Autophagy Contributes to Caspase-independent Macrophage Cell Death*

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Yue Xu1, Sung Ouk Kim2, Yilei Li3, and Jiahuai Han1,4†*1

From the 4Department of Immunology, The Scripps Research Institute, La Jolla, California 92037 and the 6Department of Microbiology and Immunology, University of Western Ontario, London, Ontario N6G 2B4, Canada

Macrophage cell death plays a role in many physiological and pathophysiological conditions. Previous work has shown that macrophages can undergo caspase-independent cell death, and this process is associated with Nur77 induction, which is involved in inducing chromatin condensation and DNA fragmentation. Here we show that autophagy is a cytosolic event that controls caspase-independent macrophage cell death. Autophagy was induced in macrophages treated with lipopolysaccharides (LPSs) and the pan-caspase inhibitor benzylxoycarbonyl-Val-Ala-Asp (Z-VAD), and the inhibition of autophagy by either chemical inhibitors or by the RNA interference knockdown of TRIF/interleukin-1 receptor-domain-containing adaptor inducing IFN-β (TRIF)–deficient mice, we found that TRIF and RIP1 function upstream of ROS production in LPS + Z-VAD-treated macrophages and both are involved in caspase-independent macrophage cell death. We further determined that the formation of autophagic bodies in macrophages occurs downstream of PARP activation, and PARP activation occurs downstream of ROS production. Using macrophages in which receptor-interacting protein 1 (RIP1) was knocked down by small interfering RNA, and macrophages isolated from Toll/interleukin-1 receptor-domain-containing adaptor inducing IFN-β (TRIF)-deficient mice, we found that TRIF and RIP1 function upstream of ROS production in LPS + Z-VAD-treated macrophages. We also found that Z-VAD inhibits LPS-induced RIP1 cleavage, which may contribute to ROS over-production in macrophages. This paper reveals that TRIF, RIP1, and ROS production, as well as PARP activation, are involved in inducing autophagy, which contributes to caspase-independent macrophage cell death.

Macrophages are essential for the host defense system. Phagocytosis of microorganisms and the secretion of proinflammatory mediators by activated macrophages are part of the response to infection of the host (1). The importance of macrophages in this defense system is evident by the fact that the virulence of some bacteria is due to their ability to overcome the protective responses of the host by triggering the death of activated macrophages (2–4). However, prolonged activation of macrophages is also believed to be dangerous because it can cause extensive local tissue damage if uncontrolled (5–7). The death of activated macrophages could also be beneficial in controlling the level of inflammation.

The viability of activated macrophages is controlled by both death and survival pathways. Many virulent strains of bacteria kill macrophages by blocking survival pathways. For example, the suppression of the mitogen-activated protein kinase (MAPK) and NF-κB pathways occurs by means of the ubiquitin-like protein protease YopJ from Yersinia pseudotuberculosis and by direct cleavage of MAPKK by anthrax toxin from Bacillus anthracis, both of which are implicated as mechanisms used by bacteria to trigger macrophage cell death (2, 3). Macrophage activation by inflammatory stimuli also activates death and survival pathways. Perhaps due to the balance of these pathways, single stimulating agents such as bacterial lipopolysaccharides (LPSs) or tumor necrosis factor (TNF) do not cause a significant change in cell viability in macrophages in vitro. The activation of the survival pathway is evident, as the LPS-activated macrophages are more resistant to the death induced by other stimulating agents such as TNF. Cross-talk between LPS and TNF signaling has been found in macrophages (8). Macrophage activation contributes to macrophage death if the cell encounters a second stimulus that impairs the survival pathway. This has been shown by a number of in vitro experiments, which found that co-stimulation with LPS and IFN-γ or stimulation with LPS in the presence of protein synthesis inhibitor cycloheximide causes macrophage cell death (9–13).

Macrophage death can be mediated through multiple pathways. Caspase activity increases in macrophages when acti-

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2 The abbreviations used are: MAPK, mitogen-activated protein kinase; LPS, lipopolysaccharide; TNF, tumor necrosis factor; IFN, interferon; Z, benzylxoycarbonyl; ERK, extracellular signal-regulated kinase; MEF2, myocyte-specific enhancer binding factor 2; ROS, reactive oxygen species; PAR, poly(ADP-ribose); PARP, PAR polymerase; AIF, apoptosis-inducing factor; RIP1, receptor-interacting protein 1; siRNA, small interfering RNA; BHA, butylated hydroxyanisole; MNG, N-methyl-N′-nitro-N-nitrosoguanidine; DPQ, 3,4-dihydroxy-5-[4-(1-piperidinyl)-butoxy]-1(2H)-isouquinolone; DHIQ, 1,5-dihydroxyisoquinoline; PI, propidium iodide; AO, acridine orange; 3-MA, 3-methyladenine; WM, wortmannin; CM-H2DCFDA, 5- and 6-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester; GFP, green fluorescent protein; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; VAC, volume of acidic compartments; TRIF, Toll/interleukin-1 receptor-domain-containing adaptor inducing IFN-β; DQ, diquat; PN, peroxynitrite; FACS, fluorescence-activated cell sorter.

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† To whom correspondence should be addressed: Dept. of Immunology, The Scripps Research Institute, 10550 North Torrey Pines, La Jolla, CA 92037. E-mail: jhan@scripps.edu.
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...vated by LPS or other stimulating agents (14, 15). Caspase activation may play a dual role in macrophage death since the inhibition of caspase by the pan-caspase inhibitor benzoxy carbonyl-Val-Ala-Asp (Z-VAD) blocks some apoptotic events, such as DNA fragmentation, but enhances cell death overall (14, 16–21). It is known that the death of activated macrophages can be executed by both caspase-dependent and caspase-independent mechanisms. The caspase-dependent death pathway in macrophages regulates cytochrome c release from mitochondria and DNA fragmentation (22). Nur77 contributes to caspase-independent macrophage death since LPS + Z-VAD-induced cell death is reduced significantly in Nur77-deficient macrophages (16). The extracellular signal-regulated kinase (ERK) pathway (which is activated by LPS) and the activity of myocyte-specific enhancer binding factor 2 (MEF2) (which is up-regulated by Z-VAD) are both required for Nur77 induction in LPS + Z-VAD-treated macrophages (16). The generation of superoxide is believed to play a role in the death of activated macrophages (14). A study showed that STAT1 knock-out macrophages exhibit decreased superoxide production and were resistant to LPS + Z-VAD treatment (14). The influence of p38 MAPK was implied in STAT1 activity and so was its participation in caspase-independent macrophage cell death. How STAT1 regulates superoxide production is largely unknown.

Although caspase-independent cell death is not well studied in comparison with classic caspase-dependent apoptosis, several mechanisms have been suggested. Reactive oxygen species (ROS) production is required for caspase-independent cell death in many different cell types (14, 23–25). The poly(ADP-ribose) (PAR) polymerase-1 (PARP-1) hyperactivation-mediated depletion of cellular NAD+ and its precursor ATP is responsible for DNA damage-reagent-induced caspase-independent cell death (26–28). It has been reported that PARP-1 hyperactivation prompts mitochondria dysfunction, which in turn releases apoptosis-inducing factor (AIF) from the mitochondria to the nucleus (29, 30). The involvement of c-Jun N-terminal kinase has recently been demonstrated in caspase-independent cell death by several groups of investigators (31–33). Receptor-interacting protein 1 (RIP1) and TNF receptor-associated factor 2 (TRAF2) are known to play important roles in cellular responses to TNF and TNF family members, and both were shown to be required for TNF-induced caspase-independent cell death (24, 34). Autophagic cell death, also called type 2 programmed cell death, is characterized by an accumulation of autophagic vacuoles in the cytoplasm (35, 36). It is also a cytoprotective mechanism in response to nutrient depletion. Several phylogenetically conserved genes (Atg genes) are required for autophagy (37, 38). Small interfering RNA (siRNA) specific for Atg genes can inhibit the autophagic cell death of L929 cells induced by Z-VAD (39) and the autophagic cell death of Bax−/− Bak−/− mouse embryonic fibroblasts treated with etoposide (20). In addition, autophagic cell death seems to be a predominant mechanism for controlling cell viability in the absence of apoptosis.

To better understand the mechanisms of caspase-independent cell death in macrophages, here we examined the death pathway of macrophages triggered by co-stimulation with LPS and Z-VAD. We found that autophagy contributes to the execution of cell death in activated macrophages. We show that the following signal pathway participates in the death of activated macrophages: TRIF → RIP1 → ROS → PARP → autophagy.

**EXPERIMENTAL PROCEDURES**

Reagents and Plasmids—Antibodies against PAR, RIP1, and beclin-1 were purchased from Pharmingen. Z-VAD and butylated hydroxyanisole (BHA) were purchased from Calbiochem. N-Methyl-N’-nitro-nitrosoguanidine (MNNG), 3,4-dihydro-5-[4-(s1-piperidinyl)-butoxy]-1(2H)-isoquinolinone (DPQ), 1,5-dihydroxyisoquinoline (DHIQ), propidium iodide (PI), acridine orange (AO), 3-methyladenine (3-MA), and wortmannin (WM) were obtained from Sigma. CM-H2DCFDA was purchased from Molecular Probes (San Diego, CA). The green fluorescent protein (GFP)-LC3 expression vector was kindly provided by Dr. T. Yoshimori (National Institute for Basic Biology, Okazaki, Japan).

Cell Culture and Treatments—The murine monocyte/macrophage cell line, RAW264.7, was cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Resident peritoneal macrophages were obtained from mice by normal saline lavage. Mice received intraperitoneal injections of 3% thioglycollate 4 days before the preparation of peritoneal macrophages. Peritoneal macrophages were harvested by the lavage of the peritoneal cavity with 5 ml of normal saline. The stock solution of LPS was 100 μg/ml in PBS and the stock solution of Z-VAD was 50 mM in Me2SO. As the control of Z-VAD treatment, the same volume of Me2SO was added into cell cultural medium. The Z-VAD dose curves were done by diluting Z-VAD with Me2SO—induced cell death is reduced significantly in

**Cell Viability Assay**—The integrity of the plasma membrane was assessed by determining the ability of cells to exclude PI. Cells were trypsinized, collected by centrifugation, washed once with PBS, and resuspended in PBS containing 1 μg/ml PI. The levels of PI incorporation were quantified by flow cytometry on a FACScan flow cytometer. Cell size was evaluated by forward-angle light scattering. PI-negative cells of normal size were considered to be live cells.

**MTT Assay**—MTT assays were performed in a 96-well plate according to manufacturer’s instruction (Sigma). Briefly, after treatment, MTT was added to each well at a final concentration of 250 μg/ml. After 1–2 h, the medium was removed, and the cells were dissolved in MTT solubilization solution (Sigma). Absorbance at 590 nm (A590) was determined for each well using a microplate reader (Bio-Rad). After subtracting the background absorbance, the A590 of the cells treated with drugs was divided by that of the untreated cells to obtain the percentage of viable cells.

**Transmission Electron Microscopy**—Cells were grown in 35-mm Petri dishes, fixed for 30 min on ice in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) plus 1 mM CaCl2, and were washed in 0.1 M cacodylate buffer. After treatment with 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 h at room temperature, and further washing in 0.1 M sodium cacodylate buffer, each sample was incubated in 0.5% tannic acid...
Acid in 0.05 M cacodylate for 30 min, rinsed in 1% Na$_2$SO$_4$ in 0.1 M cacodylate buffer. The cells were dehydrated in 30% ethanol and en bloc stained in 1% uranyl acetate in 50% ethanol for 15 min, followed by further dehydration in 50%, 70%, 90%, and two changes of 100% ethanol. Because the cells were grown in regular plastic Petri dishes, they were then cleared in 2-hydroxypropylmethylacrylate two times for 15 min each and were embedded in LX-112 (Ladd, Burlington, VT). After polymerization at 60% for 24 h, the plastic Petri dish was broken away, and the thin resin disk with cells was cut up into small pieces that were attached to blank blocks with SuperGlue. Thin sections were cut using a diamond knife, mounted on copper slot grids, stained with uranyl acetate and lead citrate, and examined on a Philips CM-100 electron microscope. Specific images were recorded photographically using Kodak SO-163 film.

Immunochemistry—Immunostaining for PAR was performed on cells grown on glass cover slips and fixed in cold 4% paraformaldehyde and permeabilized by 0.1% Triton X-100 for 5 min at room temperature. Monoclonal antibodies for PAR were used at a 1:200 dilution. For detecting acidic compartments, treated cells were stained with 1 μg/ml AO for 5 min. Samples were examined under a fluorescence microscope. When GFP-LC3 was used as an autophagosome marker, RAW264.7 cells were transfected with the GFP-LC3 expression vector using Lipofectamine (Invitrogen) according to the manufacturer’s instructions. After overnight culture, cells were treated with various chemicals, fixed with 4% paraformaldehyde, and examined under a fluorescence microscope.

Detection of ROS Accumulation—Cells in 6-well plates were cultured in a phenol red-free medium and treated with LPS and/or Z-VAD for the indicated time periods. CM-H$_2$DCFDA (20 μM) was added 30 min before collecting cells. The stained cells were analyzed with a flow cytometer, and the data were processed with the CellQuest program.

Measurement of the Relative Value of Acidic Compartments—Cells in various stages were harvested and then incubated at 37°C for 15 min with 1 μg/ml AO. Cells were then washed, pelleted, and resuspended in 1 ml of PBS for the measurement of fluorescence derived from the aggregated AO in acidic compartments. The healthy cells (with intact lysosomes) were gated, and the mean value of the fluorescence intensity was used as the relative value of acidic compartments.

Western Blot Analysis— Cells were collected and lysed in a lysis buffer (20 mM Tris (pH 7), 0.5% Nonidet P-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 20 mM β-glycerol phosphate, and 1 mM sodium vanadate). Fifty micrograms of the cell lysate from each sample were fractionated by SDS-PAGE and analyzed by Western blotting. ECL was used in the detection process according to the manufacturer’s instruction.

Results

Autophagy Is Involved in Macrophage Death Triggered by Coincubation of LPS and Z-VAD—Because LPS-activated macrophages undergo caspase-independent cell death in the presence of Z-VAD (14, 16), we decided to determine whether autophagy plays a role in this death process. We first determined the dose of Z-VAD required for the death of LPS-treated macrophages. The viability of peritoneal macrophages from C57BL/6j mice or RAW264.7 cells was measured after the cells were treated with LPS (100 ng/ml) and different concentrations of Z-VAD for 24 h. As shown in Fig. 1A, 10 μM Z-VAD is sufficient to cause maximal death of LPS-treated primary macrophages and 50 μM is required for that in RAW264.7 cells. Since we needed to use plasmid transfection in many of our experiments, and primary macrophages are very difficult to transfect, we used macrophage cell line RAW 264.7, which closely resembles the primary macrophages in regards to caspase-independent cell death (14, 16) except for its higher Z-VAD dose requirement. Fig. 1B shows the time course of LPS + Z-VAD-induced cell death in RAW264.7 cells measured by PI exclusion and MTT assay. To determine whether autophagy was induced in RAW264.7 macrophages, we first examined whether LPS + Z-VAD treatment increases the volume of the acidic compartments, which is one of the characteristics of autophagy. AO is an acidic probe and was used to stain acidic compartments in cells. Treatment with Z-VAD or LPS alone had no significant effect on the volume of the acidic compartments in RAW264.7 cells, while LPS + Z-VAD treatment increased AO staining (Fig. 1C). When AO intensity was used to compare the total volume of acidic compartments (VAC), we found a significant VAC increase in LPS + Z-VAD-treated cells by FACS analysis (Fig. 1D). The increase of VAC suggests that autophagy is induced in LPS + Z-VAD-treated RAW264.7 cells. Autophagy formation can be visualized with an electron microscope, which is by far the most confirmative analysis for autophagy. As shown in Fig. 1E, autophagy vacuoles were readily detected in LPS + Z-VAD-treated RAW264.7 cells. LC3, a mammalian homologue of Apg8p, is recruited to the autophagosome membrane during autophagy (40). The GFP-tagged LC3 has been used as a marker for the induction of autophagy (40). To evaluate the role of autophagy in LPS + Z-VAD-treated RAW264.7 cells, we transiently expressed GFP-LC3 and analyzed GFP-LC3-expressing cells before and after LPS + Z-VAD stimulation. Similar to previously reports which found that the induction of autophagy can be monitored by an increase of GFP-LC3-containing punctate structures (autophagic vacuoles) (40), our treatment of LPS + Z-VAD resulted in increased punctate staining of GFP-LC3 in macrophages (Fig. 1F).

The formation of autophagic bodies is supported by the observation that the autophagy inhibitor 3-MA inhibited the formation of these punctate stainings (Fig. 1F).

To determine whether the autophagy seen in LPS + Z-VAD-treated macrophages is involved in cell death, we used 3-MA and WM to inhibit autophagy in LPS + Z-VAD-stimulated RAW264.7 cells and primary macrophages. As shown in Fig. 1, G and H, 3-MA and WM significantly reduced the cell death. Beclin-1, the mouse homologue of yeast ATG6, is a key autophagy gene. An siRNA knockdown of beclin-1 has been used to inhibit autophagic cell death (39). To confirm that autophagy indeed contributes to LPS + Z-VAD-induced macrophage death, we used siRNA to knockdown beclin-1 in RAW264.7 cells (Fig. 1J) and determine whether autophagy is required for LPS + Z-VAD-induced cell death. Reduction of beclin-1 (Fig. 1J) resulted in a resistance to LPS + Z-VAD-induced macrophage death in RAW264.7 cells (Fig. 1J).
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**FIGURE 1.** Autophagy is involved in caspase-independent macrophage cell death. A, peritoneal macrophages isolated from C57BL/6J mice and RAW264.7 macrophages were treated with LPS (100 ng/ml) and different dose of Z-VAD as indicated. The cell viability was determined by PI exclusion. B, RAW264.7 were treated with LPS (100 ng/ml), Z-VAD (50 μM), or both, for different periods of time. The cell viability was determined by AO intensity using FACS. C, cells were treated for 12 h with or without Z-VAD, LPS, or both and then stained with AO and analyzed under a microscope. The results shown are representative photomicrographs (fluorescence). D, cells treated, as in C, were collected and incubated with AO for 15 min. The relative values of the total VAC were determined by AO intensity using FACS. E, non-treated cells (control) or cells treated with LPS + Z-VAD for 12 h were fixed as described in the experimental procedures and were analyzed using a transmission electron microscope. The asterisks denote autophagic vacuoles. Scale bars, 1 μm. F, the cells that had been transfected with the GFP-LC3 expression plasmid for 24 h were pretreated with or without 3-MA (10 mM) for 1 h and then treated with or without LPS, Z-VAD, or LPS + Z-VAD for 12 h. The cells were analyzed under a microscope. The representative photomicrographs (fluorescence) are shown. G, RAW264.7 cells were pretreated with 3-MA, WM (0.1 μg/ml), or Me2SO (control) for 1 h and then treated with LPS + Z-VAD for 18 h. The cell viability was measured by PI exclusion. H, the same as in G except that peritoneal macrophages from C57BL/6J mice were used. I, cells were transfected with siRNA targeting beclin or GFP (control). Beclin protein levels were analyzed by Western blot analysis 48 h after transfection. J, the cells that had been transfected with siRNA targeting beclin or GFP for 48 h were treated with LPS + Z-VAD for 18 h. Cell viability was measured by PI exclusion.

PARP Activation Is Involved in the Induction of Autophagy in LPS + Z-VAD-treated Macrophages—Since PARP activation is implicated to play a role in caspase-independent cell death (26–28), we decided to investigate whether LPS + Z-VAD treatment could induce PARP activation by detecting PAR formation. We treated RAW264.7 cells with LPS, Z-VAD, or LPS + Z-VAD and then checked PARP activity. LPS or Z-VAD alone did not induce PAR formation, whereas LPS + Z-VAD caused a significant increase in PAR staining (Fig. 2A). The increase in PAR staining was PARP-dependent, since the PARP inhibitor DHIQ significantly inhibited the LPS + Z-VAD-induced formation of PAR (Fig. 2A). Because a small amount of PARP was cleaved by caspase-3 in LPS-treated macrophages (data not shown) (8), it is possible that the overactivation of PARP in LPS + Z-VAD-treated macrophages is due to the inhibition of caspase-3 by Z-VAD. However, treatment of LPS-activated macrophages with caspase-3 inhibitor cannot cause PARP overactivation and cell death (data not shown) (16). Therefore, PARP hyperactivation in LPS + Z-VAD-treated macrophages requires more than preventing PARP cleavage.

To determine whether PARP activation is involved in LPS + Z-VAD-induced macrophage death, we used the PARP inhibitors DHIQ and DPQ to inhibit PARP activity in LPS + Z-VAD-stimulated cells. As shown in Fig. 2B, both DHIQ and DPQ reduced cell death in LPS + Z-VAD-treated RAW264.7 cells. Therefore, the activation of PARP contributes to LPS + Z-VAD-induced cell death.

One of the consequences of PARP activation is intracellular ATP depletion, which could be related to autophagy induction by LPS + Z-VAD treatment. Therefore, we examined whether the inhibition of PARP had an effect on LPS + Z-VAD-induced autophagy. As shown in Fig. 2C, the inhibition of PARP reduced the translocation of GFP-LC3 to the autophagosome in LPS + Z-VAD-treated cells, suggesting that PARP activation is involved in autophagy. Similarly, PARP inhibitors inhibited the LPS + Z-VAD-induced acidic compartment volume increase (Fig. 2D). Therefore, PARP activation appears to be upstream of autophagy in LPS + Z-VAD-treated macrophages.

ROS Is Upstream of PARP Activation and Is Required for Autophagy and Cell Death in LPS + Z-VAD-treated Macrophages—ROS plays an essential role in caspase-independent cell death in many different cell systems (14, 23–25). To see whether ROS in RAW264.7 cells is increased in our experimental system, we stained the cells with CM-H2DCFDA, a cell-permeable fluorescence dye that reacts to a broad spectrum of ROS. As shown in Fig. 3A, LPS treatment alone induced an increase in ROS produc-
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that the inhibition of this cleavage promotes caspase-independent cell death (43). RIP1−/− mouse embryonic fibroblast cells are resistant to caspase-independent cell death (24). Since RIP1 has been successfully knocked down by siRNA (39), we used the same strategy to knockdown RIP1 in RAW264.7 cells (Fig. 4A). As shown in Fig. 4B, knockdown of RIP1 reduced cell death in LPS + Z-VAD-treated RAW264.7 cells, indicating that RIP1 participates in LPS + Z-VAD-induced cell death. We analyzed cleavage of RIP1 in RAW264.7 cells treated with LPS or LPS + Z-VAD and found that Z-VAD treatment inhibited RIP1 cleavage (Fig. 4C). To determine the relationship between RIP1 and ROS production, we measured ROS induction by LPS + Z-VAD in RIP1 normal and RIP1 knockdown cells. siRNA knockdown of RIP1 inhibited LPS + Z-VAD-induced ROS production (Fig. 4D). Therefore, RIP1 is upstream of ROS production in LPS + Z-VAD-treated RAW264.7 cells.

Activation of RIP1 by Toll-like receptor 4 has recently been shown to be mediated through the Toll-like receptor adaptor TRIF (44, 45). Therefore, we decided to test whether TRIF is required for cell death by using primary murine macrophages deficient for functional TRIF (46). As shown in Fig. 4E, macrophages harboring nonfunctional TRIF (TRIF−/−) were significantly less sensitive to LPS + Z-VAD.

Exogenous ROS and NO Trigger Autophagic Cell Death by Activating PARP in Macrophages—Since ROS is a mediator of LPS + Z-VAD-induced autophagic cell death in macrophages, we wanted to determine whether exogenous ROS or other free radicals, such as NO, can also cause autophagic cell death. Diquat (DQ) is a bipyridyl herbicide that uses molecular oxygen to produce superoxide anions and was used here to induce oxidative stress in cultured cells (47). Peroxynitrite (PN) is an NO producer in culture medium and was used here to treat macrophages. Both DQ and PN dose-dependently induced death in RAW264.7 cells (Figs. 5A and 6A). DQ and PN both activate PARP, as determined by PAR staining (Figs. 5B and 6B), suggesting that DQ- and PN-induced cell death is associated with PARP activation. To determine whether autophagy was activated by DQ and PN, we transiently expressed GFP-LC3, as described earlier, and monitored autophagic body formation by viewing the location of GFP-LC3. As shown in Figs. 5C and 6C, DQ and PN induced an increase in autophagic bodies. The involvement of autophagy formation in DQ and PN-induced death by using primary murine macrophages deficient for functional TRIF (46). As shown in Fig. 4E, macrophages harboring nonfunctional TRIF (TRIF−/−) were significantly less sensitive to LPS + Z-VAD.

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Macrophage death was confirmed by using the autophagy inhibitor 3-MA or WM (Figs. 5D and 6D).

We also determined whether hyperactivation of PARP alone is sufficient to trigger macrophage death. MNNG is a DNA alkylating agent used to activate PARP-1 in studying PARP-1-mediated cell death (28). MNNG treatment-induced cell death in RAW264.7 cells (Fig. 7A), suggesting that PARP activation is sufficient to cause cell death in macrophages. Pretreatment with the autophagy inhibitors 3-MA or WM attenuated cell death, suggesting that autophagy is downstream of PARP activation in MNNG-induced cell death. BHA had no effect on cell death, demonstrating that in macrophages ROS does not function downstream of PRAP in PARP activation-induced cell death (Fig. 7A). Consistent with the role of autophagy in PARP-mediated macrophage cell death, we found MNNG-induced autophagic bodies (Fig. 7B) and an increase in the volume of acidic compartments (Fig. 7, C and D).

**DISCUSSION**

There is now accumulating evidence indicating that caspase-independent cell death pathways are important safeguard mechanisms to protect organisms against unwanted and potentially harmful cells (48, 49). Although caspase activation has been viewed as synonymous with programmed cell death, it is known that caspases can also contribute to processes that do not culminate in cell demise (50–53). Moreover, the inhibition of caspases may even have cytoprotective effects in some cell systems, including activated macrophages and neutrophils (14, 16, 19, 21, 54). Previous studies have shown that the pan-caspase inhibitor Z-VAD increases MEF2 transcription factor activity by preventing the proteolytic cleavage and degradation of MEF2 proteins in macrophages and have shown that MEF2-dependent expression of Nur77 is involved in macrophage cell death (16). Here we show that autophagy is a major executing mechanism of caspase-independent macrophage death. In the model system in which macrophages are activated by LPS in the presence of caspase inhibitors, the inhibition of caspase-mediated cleavage of RIP1 may be responsible for the significant increase of ROS production in LPS-treated macrophages. The ROS overproduction appears through an unknown mechanism to trigger PARP activation, and the PARP activation contributes to the induction of autophagy in macrophages. Autophagy may cooperate with other cell death pathways, such as Nur77 and STAT1, to ultimately cause cell death. Based on the information available and the data described here, the death pathways in LPS + Z-VAD-treated macrophages are proposed in Fig. 8.

A role of RIP1 in autophagic cell death has been shown in L929 fibroblast cells (39). The requirement of RIP1 in TNF-induced ROS production and caspase-independent cell death in mouse embryonic fibroblast cells has also been demonstrated (24). We show here that RIP1 is upstream of ROS production and ROS production is upstream of autophagy in macrophages. To date, no study has reported that ROS production initiates autophagy. We show in this study that ROS-mediated autophagy requires PARP activation. It should be mentioned that it is not clear why 2–3-fold increases of ROS cause macrophage death. Since what we measured is the total intracellular ROS level, it is possible that there is a dramatic change of ROS in certain subcellular locations, and the imminent cytotoxicity can only be caused...
by a specific subcellular location of ROS formation. Exogenously introduced free radicals (either ROS or NO) in macrophages can cause cell death in a PARP-dependent manner. A possible mechanism is that free radicals induce DNA strand breaks which lead to overactivation of PARP, causing an excessive use of energetic substrates such as NAD(H) and ATP. The ATP depletion in overly PARP-activated macrophages is likely to be a trigger of autophagy.

Cleavage of PARP-1 by caspases at the DEVD site is a universal phenomenon during apoptosis (55). It is postulated that PARP-1 cleavage occurs in cells undergoing apoptosis to inactivate their ability to repair DNA to preserve energy pools. It was proposed that the inhibition of PARP-1 cleavage by caspase

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FIGURE 8. A summary of the pathways involved in caspase-independent cell death of macrophages. LPS activates many intracellular pathways, including the TRIF/RIP1, ERK, and STAT pathways. Inhibition of caspases by Z-VAD prevents MEK2 cleavage, which cooperates with the ERK pathway to up-regulate Nur77 expression. Z-VAD also inhibits the cleavage of RIP1 and results in enhanced activation of the RIP1 pathway. Z-VAD may also affect the STAT pathway. The data presented in this study shows that the TRIF/RIP1 pathway triggers ROS overproduction, leading to PARP overactivation, which in turn causes autophagic cell death in macrophages.

results in ATP depletion and the conversion of apoptosis to necrosis (28). This notion was supported by reports of cell lines expressing a caspase-resistant PARP-1, by mutating the DEVD$_{214}$ site, which displayed increased necrosis (caspase-independent cell death) after treatment with TNF (56). However, the inhibition of PARP-1 cleavage by the specific inhibitor of caspase-3 is insufficient to cause cell death in LPS-stimulated macrophages (16). It is possible that the cleavage of more than one protein must be inhibited to trigger macrophage death. It is also possible that the inhibition of an unknown cysteine protease(s) by Z-VAD plays a role in triggering macrophage cell death. PARP-1 is known to be involved in various cellular processes, such as DNA repair and transcription, so the in vivo role of PARP-1 in macrophage death could be very complicated. Since both PRAP-1 knock-out mice and non-cleavable PARP-1 knockin mice are resistant to endotoxic shock, it is possible that the caspase cleavage products of PARP-1 are important in regulating inflammatory response in vivo (42, 57).

A current theory of PARP-1 hyperactivation-mediated cell death is that PARP-1 initiates a nuclear signal that propagates to the mitochondria and triggers the release of AIF (29). AIF then shuttles from the mitochondria to the nucleus and induces peripheral chromatin condensation, large scale fragmentation of DNA, and ultimately cytotoxicity. We observed positive TUNEL (terminal nick-end label) staining and condensed nuclei in cells that underwent LPS + Z-VAD-induced caspase-independent macrophage death (16). We found that in addition to nuclear changes, cytosolic events like autophagy also play an important role in the LPS + Z-VAD-induced macrophage death. Since there is no evidence so far suggesting AIF has any role in autophagy, it is possible that the PARP-1 → AIF → nuclei theme and PARP-1-mediated autophagy are independent pathways, both contributing to caspase-independent macrophage death.

The important role of autophagy in macrophage death may be due to the nature of macrophages, as they are highly capable of generating and utilizing acidic compartments. The acidic compartments appear not only to participate in the killing of infectious microbial pathogens but also appear to be involved in the death process of macrophages.

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