Proteasomes Control Caspase-1 Activation in Anthrax Lethal Toxin-mediated Cell Killing

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Activation of caspase-1 through the inflammasome protein Nalp1b controls anthrax lethal toxin (LT)-induced necrosis in murine macrophages. In this study we analyzed physiological changes controlled by caspase-1 in LT-treated murine macrophages. The caspase-1 inhibitor Boc-D-cmk blocked caspase-1 activity and membrane impairment in LT-treated cells. To determine the relationship between caspase-1 activation and membrane integrity, we added Boc-D-cmk to J774A.1 macrophages at different time points following LT exposure. Remarkably, Boc-D-cmk rescued LT-treated macrophages, even when added at the peak of caspase-1 activation. Late addition of the caspase-1 inhibitor reversed the losses of plasma membrane integrity and metabolic activity in these cells. Similar results were obtained with the proteasome inhibitor MG132, one of the most potent inhibitors of LT toxicity. LT-treated macrophages displaying evidence of membrane impairment recovered upon the addition of MG132, mirroring the Boc-D-cmk response. Strikingly, late addition of proteasome inhibitors also abrogated caspase-1 activity in LT-treated macrophages. Pro teaseal control of caspase-1 activity and membrane impairment, however, was restricted to LT-induced cytolysis, because proteasome inhibitors did not block caspase-1 activation and cell death triggered by lipopolysaccharide and nigericin. Our findings indicate that proteasome inhibitors do not target caspase-1 directly but instead control an upstream event in LT-treated macrophages leading to caspase-1 activation. Taken together, caspase-1-mediated necrosis appears to be tightly controlled and differentially regulated by proteasomes depending on the source of caspase-1 induction.

The spore-forming bacterium Bacillus anthracis is the causative agent of anthrax disease (1–3). It secretes lethal toxin (LT),4 which alone can induce many anthrax disease symptoms when injected into mice (1, 4). LT is composed of two protein subunits: protective antigen (PA) and lethal factor (LF). The zinc-dependent metalloprotease LF specifically cleaves MAPKKs in mammalian cells, thereby disrupting three MAPK signaling pathways (5–10). Although LT uptake and subsequent MAPK cleavage occur in all mammalian cells, LT is lethal to just a few targets, including antigen-presenting cells (11–17). Therefore, MAPKK cleavage is insufficient for LT killing and is possibly not even required for this process (17).

In the mouse model, the susceptibility of antigen-presenting cells to LT killing is strain-dependent. For example, BALB/c-derived macrophages and dendritic cells undergo rapid cytoly sis following LT exposure, whereas those from C57BL/6 mice are resistant to rapid LT killing (12–14, 18–21). The strain-specific sensitivity to LT is controlled by a dominant isoform of the inflammasome protein Nalp1b (22). Nalp1b belongs to the nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) family of intracellular surveillance receptors. Upon recognition of pathogen-associated molecules, NLR proteins recruit downstream factors, resulting in the formation of the inflamma some complex (23–25). This complex includes caspase-1, a well characterized inflammatory caspase, which is activated through oligomerization (26). Activated caspase-1 then goes on to process the constitutively expressed cytokines pro-interleukin (IL)-1β and IL-18, generating an inflammatory response (27).

Caspase-1 activity is central to LT killing of BALB/c-derived macrophages. Cells harboring the dominant isoform of Nalp1b but deficient in caspase-1 are resistant to the toxin (22). Furthermore, we recently found that specific caspase-1 inhibition by small peptides efficiently blocks LT-mediated necrosis in murine macrophages (21).

Caspase-1 has also been implicated in mediating killing of immune cells infected with several microbial pathogens. Infection of macrophages by Salmonella enterica (28), Salmonella typhimurium (29, 30), Shigella flexneri (31), Legionella pneumophila (32, 33), Francisella tularensis (34), and Burkholderia pseudomallei (35) triggers cell death in a caspase-1-dependent fashion. The mechanism of cell death induction by these pathogens remains to be shown. Several reports suggest that caspase-1-mediated cell death is independent of interleukin processing and release (21, 34, 36). Therefore, caspase-1 appears to target cellular proteins other than IL-1β and IL-18 to mediate cytotoxic effects.

Here we report that caspase-1 activity correlates with impairment of the plasma membrane in macrophages undergoing LT-
induced necrosis. We found that caspase-1 inhibitors reversed membrane perturbation and restored metabolic function in LT-treated macrophages, even when added at the peak of caspase-1 activity. These results point to a remarkable capacity for repair in these cells. Caspase-1 inhibitor studies mirrored those obtained with proteasome inhibitors, which also abolished caspase-1 activity in LT-treated macrophages. However, caspase-1 is not directly controlled by the proteasome, because proteasome inhibition had no effect on caspase-1 activated by lipopolysaccharide (LPS) and nigericin. Taken together, we demonstrate differential regulation of caspase-1-mediated necrosis in murine macrophages.

**EXPERIMENTAL PROCEDURES**

**Animals, Cell Culture, and Materials**—C57BL/6 and BALB/c mice were obtained from Jackson Laboratories (Bar Harbor, MN). Murine J774A.1 and RAW264.7 macrophages (ATCC, Manassas, VA) were maintained in Dulbecco’s modified Eagle’s medium and RPMI medium, respectively, supplemented with 10% fetal bovine serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml) (Mediatech, Herndon, VA). Recombinant anthrax PA and LF (List Biological Laboratories, Campbell, CA) were reconstituted in sterile water and used at the concentrations indicated. LPS (Sigma-Aldrich) was applied to cells at a final concentration of 1 μg/ml.

**Generation of Murine Bone Marrow-derived Macrophages (BMMs)**—BMMs were prepared from C57BL/6 and BALB/c mice and maintained as described previously (21). Briefly, bone marrow cells were flushed from tibias and femurs and differentiated into BMMs by incubation for 6 days in Dulbecco’s modified Eagle’s medium supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, 2.0 mM L-glutamine, 0.05 mM 2-mercaptoethanol, 0.1 mM minimum essential medium nonessential amino acids, 10% fetal bovine serum, and 20% conditioned medium from a confluent culture of L929 fibroblasts as a source of CSF-1. After removal of nonadherent cells, macrophages were recovered by washing plates with cold PBS containing 5 mM EDTA. BMMs were routinely assayed by flow cytometry and were positive for surface markers F4/80 and CD-11b. The medium for BMM maintenance was as above but with 10% conditioned L929 medium.

**Propidium Iodide Uptake Assays**—Macrophages were seeded on 96-well plates and exposed to LT or to LPS and nigericin, in the presence or absence of 40 μM Boc-D-cmk, z-VAD-cmk, Ac-YVAD-cmk, or 10 μM MG132. At different time points post-treatment, the cells were detached in PBS containing 2.6 μM TO-PRO-3 (Molecular Probes, Carlsbad, CA). The membrane permeability of TO-PRO-3 was detected in the FL-4 channel on a FACS-Calibur running CellQuest Pro software (Becton Dickinson, Franklin Lakes, NJ).

**Cell Viability by Water-soluble Tetrazolium Assay**—Cell viability was measured by analysis of the cleavage of the water-soluble tetrazolium (WST) salt WST-1 to formazan by cellular redox enzymes (37). RAW 264.7 macrophages were cultured in 96-well plates and treated with LT in the presence or absence of 10 μM MG132 and 80 μM Boc-D-cmk. Following treatment, 10 μl of WST-1 solution (BioVision, Mountain View, CA) was added to each well, and the plates were incubated for 3 h at 37 °C. The colorimetric reading of the WST-1 signal was determined in a Wallac Victor II multi-titer plate reader at 450 nm (PerkinElmer Life Sciences).

**Western Blotting**—Macrophages were cultured in 24-well plates and treated with LT or with LPS and nigericin, in the presence or absence of 40 μM Boc-D-cmk, z-VAD-cmk, Ac-YVAD-cmk, or 10 μM MG132. For Western blotting, culture medium was removed, and the cells were lysed in the wells with radioimmune precipitation assay buffer (Boston BioProducts, Worcester, MA) supplemented with a protease inhibitor mixture (Roche Applied Science). The supernatants were cleared by centrifugation, and the sample concentration was determined by Bradford assay (Bio-Rad). The samples were then mixed with SDS buffer and denatured at 100 °C for 3 min. Equivalent amounts of protein were size-fractionated on 15-well SDS-Tris-HCl polyacrylamide gels (Bio-Rad) and transferred to polyvinylidene difluoride membranes (Amer sham Biosciences). The membranes were probed with primary anti-murine polyclonal antibodies against IL-18 (BioVision, Mountain View, CA), actin (Sigma-Aldrich), or ubiquitin (Santa Cruz Biotechnology, Santa Cruz, CA). We used a secondary horseradish peroxidase-conjugated polyclonal antibody (Santa Cruz Biotechnology). The blots were developed using ECL Plus solution (Amer sham Biosciences).

**Fluorescence Microscopy**—J774A.1 macrophages were cultured on Lab-Tek chamber slides (Nunc, Rochester, NY) and exposed to LT in the presence or absence of 9 or 40 μM Boc-D-cmk. At different time points post-treatment, the cells were washed in PBS and stained with 0.5 μM PI for 5 min before fixation in 4% paraformaldehyde. The cells were washed again in PBS and then permeabilized in ice-cold PBS containing 0.1% Triton X-100 and 40 μg/ml Hoechst 33342 nuclear counterstain (Invitrogen). The coverslips were mounted using Vectashield (Vector Laboratories, Burlingame, CA). The experiments were performed with an Olympus IX81 electronically motorized microscope (Center Valley, PA).

**RESULTS**

Caspase-1 Activity Controls Membrane Integrity in LT-treated Macrophages—Nalp1b was recently identified as the genetic factor responsible for induction of LT-induced necrosis in BALB/c macrophages (22). A component of the murine inflammasome complex, Nalp1b presumably activates caspase-1, which has been linked to LT-induced necrosis (21, 22). To explore the connection between caspase-1 activation and necrosis in LT toxicity, we challenged J774A.1 macro-
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A LT-induced inhibition blocks LT-mediated IL-18 cleavage and necrosis. A, LT-induced IL-18 processing by caspase-1 in the presence of caspase-1 inhibitors. Lysates of J774A.1 macrophages treated with LT in the presence of 40 µM Boc-D-cmk, Ac-YVAD-cmk, and z-VAD-fmk were immunoblotted using an anti-IL-18 antibody. B, LT-induced necrosis in the presence of caspase-1 inhibitors. Necrosis of J774A.1 macrophages treated with LT in the presence of 40 µM Boc-D-cmk, Ac-YVAD-cmk, and z-VAD-fmk was determined at 120 min post-LT by analyzing uptake of the membrane-impermeable fluorescent dye TO-PRO-3 by flow cytometry.

To further analyze the efficiency of caspase-1 inhibition in blocking LT toxicity, we subjected LT-treated macrophages to increasing concentrations of Boc-D-cmk. Induction of necrosis was monitored using PI uptake assays. As expected, 40 µM Boc-D-cmk completely prevented PI uptake in LT-treated J774A.1 macrophages (Fig. 2). However, we detected transient membrane impairment in LT-treated J774A.1 cells in the presence of suboptimal (9 µM) Boc-D-cmk concentrations. The PI signal in these cells peaked 120 min following Boc-D-cmk exposure, before decreasing to background levels 150 and 180 min post-Boc-D-cmk exposure (Fig. 2). Likewise, temporary membrane impairment also occurred in LT-treated BALB/c-derived RAW 264.7 macrophages in the presence of suboptimal (2 µM) Boc-D-cmk concentrations, as analyzed by PI uptake assays (see supplemental Fig. S1). In these cells, PI uptake peaked at 180 min before rapidly decreasing to background levels. As described for J774A.1 cells, 40 µM Boc-D-cmk completely prevented dye uptake in LT-treated RAW 264.7 cells (Fig. 1B and supplemental Fig. S1). Similar results were obtained when we monitored lactate dehydrogenase release in lieu of PI uptake (data not shown).

Inhibitor efficiency was also examined by PI staining using fluorescence microscopy. As in Fig. 2, J774A.1 macrophages were treated with LT and either 9 or 40 µM Boc-D-cmk. A 79% increase in PI fluorescence over background was visible in J774A.1 cells 90 min after toxin exposure, approaching 98% PI-positive cells by 120 min (Fig. 3). The suboptimal dose of 9 µM Boc-D-cmk permitted a similar increase in membrane perturbation by 90 min post-LT exposure, reaching ~87% PI-positive cells by 120 min (Fig. 3). In agreement with findings in Fig. 2, membrane impairment in the presence of 9 µM Boc-D-cmk was transient, because PI-fluorescence in these cells rapidly returned to control levels by 150 min post-LT. As expected, 40 µM Boc-D-cmk completely prevented any increase in membrane perturbation and PI uptake in LT-treated J774A.1 macrophages (Fig. 3). Taken together our results suggested that complete inhibition of caspase-1 is delayed in cells treated with suboptimal Boc-D-cmk concentrations, resulting in transient membrane impairment.

Caspase-1 Controls Membrane Integrity and Cell Viability—Our findings provide evidence that LT-treated macrophages can recover from transient membrane impairment. To further characterize the correlation between membrane repair and caspase-1 inhibition, we added the caspase-1 inhibitor Boc-D-cmk to macrophages at different time points following LT exposure. J774A.1 macrophages were incubated with 40 µM Boc-D-cmk at 0, 90, and 120 min post-LT exposure, and membrane integrity was determined by PI uptake assays. As expected, Boc-D-cmk fully inhibited lysis of J774A.1 cells when added simultaneously with LT (Fig. 4). Remarkably, macrophages showed significant recovery when Boc-D-cmk was added as late as 90–120 min after LT exposure (Fig. 4). This recovery was preceded by a transient increase in membrane perturbation following addition of Boc-D-cmk, presumably because of a delay in complete caspase-1 inhibition (Fig. 4).

Similar results were obtained in LT-treated RAW 264.7 macrophages following late addition of Boc-D-cmk (see supplemental Fig. S2), albeit with slower kinetics than observed in J774A.1 cells. Membrane integrity recovered in RAW 264.7
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Figure 3. Transient membrane perturbation occurs with suboptimal inhibition of caspase-1. Fluorescence microscopy details the extent of transient PI uptake in cells treated with a low concentration of Boc-D-cmk. Top panels, J774A.1 macrophages treated with LT were stained with PI (red) and Hoechst (blue) and photographed at 30, 90, 120, 150, and 180 min. Middle panels, J774A.1 macrophages treated with LT and 9 µM Boc-D-cmk. Bottom panels, J774A.1 macrophages treated with LT and 40 µM Boc-D-cmk.

Figure 4. Caspase-1 activity is linked to membrane impairment. Late addition of caspase inhibitor Boc-D-cmk rescues membrane integrity in LT-treated J774A.1 cells. PI uptake was assessed in J774A.1 cells treated with LT in the absence or presence of 40 µM Boc-D-cmk added at 0, 90, or 120 min post-LT exposure.

Figure 5. Caspase-1 controls cell viability. Late addition of Boc-D-cmk rescues cell metabolism. WST assays were performed on RAW 264.7 cells treated with LT in the absence or presence of 80 µM Boc-D-cmk added at 0, 120, or 150 min post-LT exposure.

Cells when 80 µM Boc-D-cmk was added as late as 120 and 150 min after LT exposure. These experiments indicate that caspase-1 inhibitors can rescue LT-treated macrophages at multiple late time points with nearly complete cell recovery.

We also monitored cell viability in LT-treated RAW 264.7 cells by WST-1 assay, which is based on the reduction of a tetrazolium compound by cellular oxidoreductases utilizing mitochondria-generated NADH as the primary reductant (37, 39). A loss of WST signal indicates impairment of cellular and mitochondrial function. The WST signal dropped precipitously in the presence of LT, indicating severe metabolic impairment. This decrease was prevented when 80 µM Boc-D-cmk was added simultaneously with LT (Fig. 5).

Similar to membrane rescue, WST activity recovered rapidly in RAW 264.7 macrophages following the addition of Boc-D-cmk at 120 and 150 min post-LT exposure (Fig. 5). These findings indicated that caspase-1 plays a role in metabolic impairment in LT killing. Taken together, our data point to the involvement of caspase-1 in the concurrent losses of cell metabolism and membrane integrity in LT-treated macrophages.

Proteasomes Control Caspase-1 Activity in LT-treated Macrophages—The recovery of LT-treated macrophages following late addition of caspase-1 inhibitors mimics earlier findings with proteasome inhibitors (20). We and others have shown that proteasome inhibitors efficiently block LT toxicity, even when added late to LT-treated macrophages or dendritic cells (12, 20, 38). To directly compare the effects of caspase-1 and proteasome inhibition on LT killing, we treated J774A.1 macrophages with 40 µM of the caspase-1 inhibitor Boc-D-cmk or 10 µM of the proteasome inhibitor MG132 at 0, 60, 90, and 120 min post-LT exposure. By 60 min, cells exposed to LT alone had already undergone significant membrane perturbation (Fig. 6). Strikingly, membrane integrity completely recovered when the caspase-1 inhibitor Boc-D-cmk was added to macrophages 60 and 90 min post-LT exposure (PI uptake was tested at 180 min; Fig. 6A). Remarkably similar results were obtained following late addition of the proteasome inhibitor MG132 (Fig. 6B). The addition of Boc-D-cmk or MG132 120 min post-LT exposure, however, resulted only in limited recovery, indicating that the majority of cells had accumulated too much damage by that time (Fig. 6).

Because both inhibitors exhibited similar kinetics and efficacy in rescuing membrane integrity in LT-treated macrophages, we tested whether proteasome inhibitors, like caspase-1 inhibitors, blocked caspase-1 activity. We added 40 µM Boc-D-cmk or 10 µM MG132 to J774A.1 macrophages at different time points following LT exposure and determined caspase-1 activity by analysis of IL-18 cleavage 180 min post-LT. Despite evidence of significant IL-18 processing 60 and 90 min into toxin treatment, no cleaved IL-18 was observed following late addition of the proteasome inhibitor MG132 (Fig. 7). Late MG132 addition efficiently blocked caspase-1 activation as well as cytolyis in LT-treated macrophages. The absence of processed IL-18 in the MG132-treated macrophages 180 min post-LT suggested that the inhibitor blocked further...
IL-18 processing but did not interfere with the release of already processed IL-18.

As expected, no IL-18 cleavage products were detected 180 min post-LT following addition of the caspase-1 inhibitor Boc-D-cmk for 60 min into toxin treatment (Fig. 7). Residual processed IL-18, however, was detected when the caspase-1 inhibitor was added 90 min post-LT exposure. Taken together, caspase-1 and proteasome inhibitors blocked IL-18 processing and LT killing even when added at the peak of caspase-1 activation. This remarkable similarity suggested that both inhibitors block caspase-1 activation in LT-treated macrophages.

To rule out the possibility that Boc-D-cmk interfered with LT toxicity by inhibiting the proteasome, we probed lysates of Boc-D-cmk- and MG132-treated macrophages for levels of ubiquitinated proteins by Western blot. Inhibition of the proteasome leads to accumulation of ubiquitinated proteins (40). Lysates prepared from Boc-D-cmk-treated macrophages were similar to untreated controls, whereas significant accumulation of ubiquitinated proteins was observed in lysates from MG132-treated macrophages (Fig. 8). These results suggested that Boc-D-cmk does not prevent LT killing by inhibiting the proteasome. Furthermore, findings by our group and others have shown that neither Boc-D-cmk nor MG132 interferes with LF uptake or MAPKK cleavage (21, 38), implying that both inhibitors block downstream events in LT killing.

LPS/Nigericin-mediated Macrophage Killing Is Controlled by Caspase-1, in a Proteasome-independent Pathway—Given the proteasomal control of LT-induced cytolysis and caspase-1 activation, we tested whether proteasomes control caspase-1 activation directly. To activate caspase-1 in an LT-independent fashion, we treated murine macrophages with LPS and nigericin. In human cells, LPS activates the inflammasome through NALP3, and the ionophore nigericin provides the necessary secondary signal (K+ efflux) for caspase-1 activation (27, 41).

We treated primary BALB/c and C57BL/6-derived BMMs with concentrations of LPS (1 μg/ml) and nigericin (12.5 μM) that alone were nontoxic to these cells (Fig. 9). When used in combination, however, LPS and nigericin were highly cytotoxic in BMMs, as assessed by PI uptake 5 h after exposure (Fig. 9). LPS/nigericin also triggered caspase-1 activation, as indicated by the presence of processed IL-18. The caspase-1 inhibitor Boc-D-cmk blocked LPS/nigericin-mediated IL-18 cleavage, as well as necrosis of BALB/c and C57BL/6 macrophages. These results are consistent with earlier findings indicating that LPS/nigericin mediates cell death via a caspase-1-dependent mechanism (27, 42).

The proteasome inhibitor MG132, however, did not block caspase-1 activation or cytopathic effects in LPS/nigericin-treated BALB/c and C57BL/6 macrophages (Fig. 9). This is con-
of plasma membrane integrity following LT exposure is not terminal and that macrophages can recover from even extended membrane impairment. Cell repair after plasma membrane disruption has been widely studied. Membrane disruption is commonplace and almost inevitable, occurring in physiological events such as tissue damage and muscle contraction (46–49) or during experimental manipulation such as microinjection (50, 51) and electroporation (52, 53). Membrane repair is necessary to retain intracellular composition, minimize loss of nonrenewable cell types, curtail release of proteases into the surrounding cell population, and mitigate inflammatory responses in the host (54–57). In these cases, cell survival depends on the ability of injured cells to reseal impaired plasma membranes. It remains to be shown how activation of caspase-1 triggers membrane perturbation in LT-treated macrophages and how inhibition of this enzyme results in recovery.

We observed that caspase-1 inhibition blocked not only membrane perturbation in LT-treated macrophages but also the loss of metabolic function as determined by WST assays. It is conceivable that the loss in WST activity is caused by caspase-1-induced damage at the plasma membrane. It is also possible that caspase-1 has multiple cellular targets, thereby controlling several events in LT-treated macrophages, possibly by the same underlying mechanism. The rescue of metabolic activity by Boc-D-cmk occurred most efficiently in RAW 264.7 macrophages, whereas the inhibitor provided only minimal protection against a loss in the WST signal in J774A.1 cells (data not shown). The reason for this cell line discrepancy may lie in the slower progression of RAW264.7 macrophages into LT-induced necrosis than J774A.1 cells, giving Boc-D-cmk more time to block caspase-1 activity.

Recent studies documented that proteasome inhibitors efficiently block LT-mediated killing of murine macrophages and dendritic cells (12, 20, 38). In this study we provide evidence that caspase-1 and proteasome inhibitors prevent LT killing in a similar fashion; both inhibitors blocked caspase-1 activity and restored cell metabolism and membrane integrity even after late addition to LT-treated macrophages. We further showed that proteasome inhibitors prevent LT killing of murine macrophages by blocking caspase-1 activation. Although caspase-1 and proteasome inhibitors exhibited similar effects on LT killing, they differed significantly in their effect on LPS/nigericin-mediated cell death. In contrast to the effect on LT-treated macrophages, proteasome inhibitors did not block caspase-1 activation or cell killing triggered by LPS/nigericin. These results are consistent with a report by Kahlenberg et al. (41), who showed that proteasome inhibitors do not interfere with
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LPS-induced caspase-1 activation in murine macrophages. The failure of proteasome inhibitors to block LPS/nigericin-mediated caspase-1 activation suggested that these inhibitors do not target caspase-1 directly. Our studies imply that proteasomes control an upstream event leading to caspase-1 activation in LT-treated macrophages. It is conceivable that a protective factor blocks caspase-1 activation mediated by LT and that proteasomal degradation of this factor is required for LT killing. One relevant analogy is caspase-3, which requires proteasomal degradation of its endogenous IAP inhibitors for activation (58, 59). Our theory is corroborated by an early report showing that proteasome inhibition could prevent proteolytic activation of caspase-1 (formerly called ICE) (60). Caspase-1 itself has several endogenous inhibitors, like COP, ICEBERG, and POP1, that appear to block caspase-1 interaction with the inflammasome (26). It is possible that the activity of these inhibitors is controlled by the proteasome. Inflammasome components assembled after LT treatment might include a protective factor(s) that is under proteasomal control.

Our findings suggested that proteasome inhibitors do not directly block caspase-1 activity in LT-treated macrophages but may instead prevent an upstream event required for caspase-1 activation. It is surprising that proteasome inhibitors reverse membrane impairment when added at the peak of caspase-1 activation, because these inhibitors do not interfere with already activated enzyme. We therefore speculate that activated caspase-1 is short-lived and rapidly secreted in LT-treated cells. Rescue from cell killing at multiple late time points suggested that continual caspase-1 activation is required for LT-mediated necrosis. The quick recovery of LT-treated macrophages following delayed MG132 or Boc-D-cmk exposure further suggested a close link between caspase-1 activity and membrane perturbation. It is unclear how membrane integrity is compromised by activated caspase-1 and how cells repair themselves following inhibition of the enzyme. It remains to be shown whether activated caspase-1 directly targets cell membranes or membrane proteins.

We found that LPS/nigericin-mediated caspase-1 activation induces cell death in both BALB/c and C57BL/6-derived macrophages. This strain-independent response to LPS/nigericin is in stark contrast to LT, which mediates strain-specific cell killing in murine macrophages. LT triggers caspase-1 induction and necrosis in BALB/c macrophages but not in C57BL/6 macrophages (21, 22). As described above, LT and LPS/nigericin differed not only in their strain-specific effect on macrophages but also in their effect on macrophages in the presence of proteasome inhibitors. In contrast to caspase-1 inhibitors, the proteasome inhibitor MG132 failed to prevent LPS/nigericin-induced toxicity and in fact exacerbated necrosis by this inducer combination or by LPS alone. Moreover, late addition of caspase-1 inhibitors, despite rescuing LT-treated macrophages, was completely ineffective in preventing LPS/nigericin-induced death (data not shown). The reason for the discrepancy between LT- and LPS/nigericin-mediated cell killing may lie in the mechanics of caspase-1 activation by these inducers. While LT triggers caspase-1 via Nalp1b, LPS/nigericin is thought to activate caspase-1 via Toll-like receptor-4 and the inflammasome protein NALP3 (42). It is therefore reasonable to assume that the significant differences in caspase-1 activation and cell death induction following LT or LPS/nigericin treatment are dependent on the signaling pathways involved. This leads us to speculate that caspase-1 activation and proteasomal control of this process is inducer-specific and dependent on the NLR recruited to the inflammasome.

In summary, our findings suggested that proteasome-controlled caspase-1 activation mediates continuous necrotic damage in LT-treated macrophages. Rescued in time, these macrophages display a remarkable capacity for cellular repair. Our data also imply the formation of unique inflammasome complexes in response to diverse sources of caspase-1 induction, resulting in differential regulation of caspase-1.

Acknowledgments—We thank Eric Boyden for critical reading of the manuscript and Michael Cammer (Analytical Imaging Facility at the Albert Einstein College of Medicine) for technical assistance with image editing.

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