Cutting Edge: Necrosis Activates the NLRP3 Inflammasome

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Cells undergoing necrosis release endogenous danger signals that possess proinflammatory potential. In this study we show that mature IL-1β and IL-18 are released by necrotic cells but not by apoptotic cells. We identify 7-bromoindirubin-3′-oxime, an indirubin oxime derivative that induces necrosis, as a potent inducer of caspase-1 activation and release of mature IL-1β and IL-18. Inflammasome activation was triggered by other necrosis-inducing treatments but was not observed in response to apoptosis-inducing stimuli. Necrosis-induced inflammasome activation was mediated by the NLRP3 and ASC molecules. Release of IL-18 and IL-1β in response to necrosis-inducing stimuli was observed in THP-1 macrophages and the MSTO-211H human mesothelioma cell line independently of LPS priming. Using the in vivo model of naphthalene-induced airway epithelial cell injury, we showed that necrosis activates the ASC inflammasome in vivo. Our study identifies a new mechanism through which necrosis generates proinflammatory molecules that contributes to the sterile inflammatory response. The Journal of Immunology, 2009, 183: 1528–1532.

Necrotic cells release endogenous molecules, such as heat shock proteins, uric acid, ATP, and DNA that alert the innate immune system of the danger associated with tissue damage and infection (1). These molecules act as endogenous “danger signals” by activating a variety of pattern recognition receptors, including TLR and Nod-like receptors (NLR). As a result, necrotic cells and several danger signals directly trigger proinflammatory responses, potentiate signaling by classical proinflammatory stimuli, and possess adjuvant capacity. Inflammation triggered by tissue damage in the absence of infection is referred to as sterile inflammatory response.

Members of the NLR family are key components of the inflammasome (2), a multiprotein cytoplasmic complex that regulates secretion and bioactivity of cytokines belonging to the IL-1 family (IL-1β, IL-18, and IL-33). The protease caspase-1 and the adaptor molecule ASC are also part of the inflammasome. The function of NLR is to detect the presence of microbial products, toxins, and danger signals in the cytoplasm leading to caspase-1 activation, a necessary step for the processing and secretion of the immature forms of IL-1β, IL-18, and IL-33. One of the best-characterized NLRs is NLRP3 (also known as cryopyrin and Nalp3). This molecule mediates inflammasome activation in response to several intracellular bacteria (3) and viruses (4, 5), danger signals such as extracellular ATP and uric acid crystals (monosodium urate) (6), and particles of different origin such as asbestos fibers (7), silica particles (8, 9), and alum crystals, as we and other groups have recently demonstrated (10–13). The differences in the structure and function of the factors reported to activate the NLRP3-inflammasome make it unlikely that they directly interact with the receptor. Rather, the emerging consensus is that NLRP3 may recognize a “messenger molecule” that is generated inside the cell in response to the insult that these agonists may cause. Recently published evidence suggests that lysosome destabilization and the protease cathepsin B are critically involved in NLRP3 activation (8).

Considering the evolutionary advantage that detection of tissue damage confers to the organism, it is conceivable that, in addition to passive release of danger signals, necrotic cells may actively generate proinflammatory molecules as result of the activation of endogenous signaling pathways. In this study we report that the NLRP3 inflammasome is activated in necrotic but not in apoptotic cells, leading to the release of IL-1β and IL-18. Our study identifies a new mechanism through which necrosis generates proinflammatory molecules that contribute to the sterile inflammatory response.

Materials and Methods

Mice

NLRP3−/−, ASC−/−, and NLRCA1−/− mice on C57BL/6 background were kindly provided by Dr. V. Dixit, Genentech, South San Francisco, CA. Age and sex-matched C57BL/6 mice were purchased from The Jackson Laboratory. All experiments using mice were approved by the University of Tennessee Health Science Center Institutional Animal Care and Use Committee (Memphis, TN).

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3 Abbreviations used in this paper: NLR, Nod-like receptor; AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride; BAL, bronchoalveolar lavage; 7BIO, 7-bromoindirubin-3′-oxime; BMM, bone marrow-derived mononuclear cell; HMGB1, high mobility group box 1; IO, indirubin oxime; LDH, lactate dehydrogenase; STS, staurosporine; Z-YVAD-FMK, N-benzoyloxycarbonyl-Tyr-Val-Ala-Asp-fluoromethyl ketone.

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Reagents

Indirubin derivatives (resuspended in DMSO at 25 mM) and the caspase-1 inhibitor N-benzoyloxycarbonyl-Tyr-Val-Ala-Asp-fluoromethyl ketone (Z-YVAD-FMK) were purchased from Axoza, Alum (Alhydrogel) was from Sigma-Aldrich. The cathepsin B inhibitor CA-074-Me was from Biomol International. All other chemicals were from Sigma-Aldrich. The following Abs were used: rabbit anti-caspase-1 (Upstate Biotechnologies), goat anti-IL-1β (R&D Systems), anti-human IL-1β 3ZD mAb (Preclinical Repository, Biological Research Branch, National Cancer Institute), and rabbit anti-HMGB1 (where HMGB1 is high mobility group box 1; Santa Cruz Biotechnology).

Human cell lines

The THP-1 macrophages and MSTO-211H lung mesothelioma human cell lines were obtained from American Type Culture Collection and grown in RPMI 1640 with 10% FCS.

PBMC and bone marrow-derived mononuclear cell (BMM) isolation

Human PBMC were isolated from leukopacks by Ficoll-Histopaque density gradient centrifugation. Mouse BM were generated by incubating bone marrow in RPMI 1640 with FCS and supplemented with recombinant murine GM-CSF (20 ng/ml) for 8 days. This procedure routinely results in 60–80% CD11c⁺ dendritic cells.

Cytokine measurements

Cytokine levels in conditioned supernatants and bronchoalveolar lavage (BAL) fluids were measured by ELISA using the following paired Abs kits: human IL-1β and human IL-18 (R&D Systems), murine IL-1β (eBioscience), and murine IL-18 (MBL Nagoya, Japan).

Statistical analysis

All data were expressed as mean ± SEM. Student’s t test was used for statistical evaluation of the results. Significance was set at p < 0.05.

Results and Discussion

Indirubin oxime (IO) derivative 7BIO activates the inflammasome

In a search for small molecules capable of activating the inflammasome, we identified 7-bromoidirubin-3’-oxime (7BIO), an IO derivative (see supplemental Fig. 1 for structure), as a potent inducer of the release of IL-1β and IL-18 from LPS-primed human THP-1 macrophages or human PBMC (Fig. 1, A and B). Surprisingly, indirubin and the other derivatives 6-bromoidirubin-3’-oxime (6BIO), IO, 5-iodo-indirubin-3’-oxime (5IO), and 7BIO-Me (an inactive compound with a methyl group on N1 of 7BIO) were poor activators of the inflammasome. 7BIO induced processing of pro-IL-1β and procaspase-1 (Fig. 1C), a biochemical demonstration of inflammasome activation. Inflammasome activation by 7BIO was inhibited by treatment with the caspase-1 inhibitor Z-YVAD-FMK and the cathepsin B inhibitor CA-074-Me, but not by cytochalasin D (Fig. 1D), which blocks inflammasome activation by alun crystals (14) and other particles (7). Necrotic cells are known to release uric acid and ATP, both of which activate the inflammasome (3, 6). Inflammasome activation in response to 7BIO was not blocked by uricase, which inhibits inflammasome activation by uric acid crystals. The ATP-hydrolyzing enzyme apyrase or oxidized ATP, which blocks inflammasome activation by extracellular ATP, did not inhibit IL-1β release in response to 7BIO (Fig. 1D). Moreover, inflammasome activation by 7BIO was still observed in BMDC derived from mice deficient in P2X₇, the cell surface receptor for extracellular ATP that is critically involved in inflammasome activation by ATP (supplemental Fig. 2). These results show that inflammasome activation by 7BIO is not mediated by the release of endogenous danger signals like monosodium urate or ATP.

Necrosis but not apoptosis activates the inflammasome

All of the indirubin derivatives tested have been demonstrated to inhibit several kinases and to induce apoptosis with the exception of 7BIO, which does not induce apoptosis or inhibit kinases but rather rapidly induces a form of caspase-independent necrosis (15–17). The unequal ability of the indirubin derivatives to induce necrosis was confirmed by measuring release in the culture supernatant of the enzyme lactate dehydrogenase (LDH) or the nuclear protein HMGB1, an endogenous danger signal that is released by necrotic, but not apoptotic, cells or during infection (18). In agreement with previous results (15–17), 7BIO was a much stronger inducer of necrosis than the other indirubin derivatives (Fig. 2A). The fact that only 7BIO, among the indirubin derivatives, induced IL-1β release suggested that inflammasome activation may be triggered in cells undergoing necrosis. To test this hypothesis, LPS-primed THP-1 cells or PBMC were rendered apoptotic or necrotic by additional treatments and inflammasome activation was tested.

To induce apoptosis, cells were treated with the kinase inhibitor staurosporine (STS) or the topoisomerase inhibitor etoposide or were UV irradiated. Cells were rendered necrotic by one cycle of freeze and thaw, by osmotic shock, or by treatment with STS in presence of oligomycin (an inhibitor of ATP synthase). It has been demonstrated that cells depleted of ATP through oligomycin treatment undergo necrosis rather than apoptosis when cotreated with STS (19),
As shown in Fig. 2B, the necrotic stimuli were strong inducers of inflammasome activation as measured by the level of IL-1β released in culture supernatants and by the processing of pro-IL-1β and procaspase-1. In contrast, inflammasome activation in apoptotic cells was much weaker. It should be noted, however, that during prolonged stimulation with apoptotic agents we observed IL-1β release as previously shown (20). This is a likely reflection of the induction of secondary necrosis of the apoptotic cells and, in fact, the release of LDH and HMGB1 was greatly increased by overnight incubation of the apoptotic cells (not shown). It is also interesting that among the indirubin derivatives, after 7BIO the induction of apoptotic cells was much weaker. It should be noted, however, that during prolonged stimulation with apoptotic agents we observed IL-1β release as previously shown (20). This is a likely reflection of the induction of secondary necrosis of the apoptotic cells and, in fact, the release of LDH and HMGB1 was greatly increased by overnight incubation of the apoptotic cells (not shown). It is also interesting that among the indirubin derivatives, after 7BIO the induction of apoptotic cells was much weaker.

**Necrosis activates the NLRP3 inflammasome**

The fact that the cathepsin B inhibitor CA-074-Me suppressed IL-1β release in response to necrotic stimuli suggested the involvement of the NLRP3 inflammasome (8). To test this hypothesis, BMM from wild-type, NLRP3−/−, ASC−/−, or NLRC4−/− mice were pretreated with LPS and then stimulated with the necrotic stimuli 7BIO, STS/oligomycin, or one freeze-thaw (F&T) cycle of freeze-thaw. As shown in Fig. 3, caspase-1 activation and secretion of mature IL-1β independently of LPS in MSTO-211H, a mesothelioma cell line that constitutionally expresses the inflammasome by 7BIO was inhibited by 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), a serine protease inhibitor that was reported to block the induction of necrosis by 7BIO (17), demonstrating that induction of necrosis is necessary for inflammasome activation by 7BIO.

**Necrosis activates the inflammasome in vivo**

To test whether necrosis can activate the inflammasome in vivo, we used the naphthalene-induced lung injury model. In this model, i.p. injection of naphthalene results in selective necrosis of the epithelial cell lining the airways. As shown in Fig. 4, the marked, secretion of IL-18, which is constitutively expressed by several cell types, was induced by 7BIO or STS/oligomycin in the absence of LPS priming in THP-1 cells (Fig. 2C). Similarly, these necrotic stimuli induced the release of mature IL-1β independently of LPS in MSTO-211H, a mesothelioma cell line that constitutionally expresses pro-IL-1β (our unpublished observation). In these cells, inflammasome activation by 7BIO was inhibited by 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), a serine protease inhibitor that was reported to block the induction of necrosis by 7BIO (17), demonstrating that induction of necrosis is necessary for inflammasome activation by 7BIO.
necrosis, however, was not dependent on inflammasome activation (as judged by LDH or HMGB1 release; data not shown), as it was induced to the same extent in wild-type or NLRP3−/− or ASC-deficient cells and was not blocked by caspase-1 inhibitors, cathepsin B inhibitors, or necrostatin, a necroptosis inhibitor (23). It is also interesting to note that other necrosis-inducing conditions such as treatment with hydrogen peroxide or paclitaxel, which induces caspase-independent necrosis, failed to induce inflammasome activation. Similarly, induction of necrotosis through TNF-α stimulation in the presence of caspase-3 and caspase-9 inhibitors (23) did not result in inflammasome activation. Finally, if necrosis was induced too rapidly by repeated cycles of freeze-thaw or excessive osmotic shock, inflammasome activation was greatly reduced. Based on these observations, we speculate that the dissolution of the cellular architecture that occurs during some specific forms of necrosis may lead to lysosome destabilization, resulting in activation of the NLRP3 inflammasome (8). Recently, it has been shown that necrotic cells release IL-1α (24), although inflammasome activation was not reported. Interestingly, in contrast to IL-1β, the biological activity of which depends on caspase-1-mediated processing, the bioactivity of IL-1α does not require processing of the precursor. Our results identified activation of the NLRP3 inflammasome during necrosis as a novel mechanism through which necrotic cells release proinflammatory molecules that contribute to the sterile inflammatory response.

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Disclosures

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References


