Cutting Edge: FAS (CD95) Mediates Noncanonical IL-1β and IL-18 Maturation via Caspase-8 in an RIP3-Independent Manner


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Fas, a TNF family receptor, is activated by the membrane protein Fas ligand expressed on various immune cells. Fas signaling triggers apoptosis and induces inflammatory cytokine production. Among the Fas-induced cytokines, the IL-1β family cytokines require proteolysis to gain biological activity. Inflammasomes, which respond to pathogens and danger signals, cleave IL-1β cytokines via caspase-1. However, the mechanisms by which Fas regulates IL-1β activation remain unresolved. In this article, we demonstrate that macrophages exposed to TLR ligands upregulate Fas, which renders them responsive to receptor engagement by Fas ligand. Fas signaling activates caspase-8 in macrophages and dendritic cells, leading to the maturation of IL-1β and IL-18 independently of inflammasomes or RIP3. Hence, Fas controls a novel noncanonical IL-1β activation pathway in myeloid cells, which could play an essential role in inflammatory processes, tumor surveillance, and control of infectious diseases. The Journal of Immunology, 2012, 189: 000–000.

As (also called CD95, APO-1, or TNFRSF6) is a membrane protein that belongs to the TNFR family. Binding of its physiological ligand, Fas ligand (FasL), to Fas causes apoptosis, a process that is thought to be critical for the control of tumor cells, infected cells, or otherwise damaged cells (1). Fas-mediated killing of immune cells is important for immune homeostasis, because mice with spontaneous mutations in Fas or FasL develop autoimmunity (2, 3), and a fraction of human patients with autoimmune lymphoproliferative syndrome carry inherited mutations in Fas (4). Similar to other death-inducing signaling receptors, Fas signaling also has nonapoptotic functions in cellular proliferation and differentiation and cytokine activation (1). However, little is known about the consequences of Fas signaling on cytokine activation for the control of tumorous, damaged, or infected cells or for the development of pathologies in autoimmune diseases. In cells of the adaptive-immune system, Fas signaling is important for the regulation of apoptosis and, therefore, is essential for the establishment of self-tolerance (5, 6).

Of importance for inflammatory conditions, Fas signaling in cells of the innate immune system, such as neutrophils, dendritic cells, or macrophages, can mediate the production and activation of proinflammatory cytokines of the IL-1β family, as well as chemokines (7–9). The proinflammatory effect of Fas signaling on innate immune cells is particularly noticeable in microbially infected cells or in cells that have been primed by stimulants, such as TLR ligands (10).

The proinflammatory IL-1β family of cytokines is controlled at the transcriptional and posttranscriptional levels and produced as biologically inactive precursors, which, upon proteolytic processing, become active cytokines. Several proteases have been implicated in the processing of IL-1β cytokines (11); among these, the best studied is caspase-1. Caspase-1 activity is controlled by inflammasomes, which are multiprotein signaling complexes that detect microbial-derived molecular signatures or endogenous danger signals (12). However, Fas-mediated IL-1β activation is caspase-1 independent; thus, it remains to be determined how Fas induces IL-1β activation (7, 8).

Several lines of evidence suggest the existence of a noncanonical IL-1β activation pathway that involves caspase-8 and receptor-...
interacting protein (RIP) kinases. In primed macrophages, activation of the TRIF-engaging TLR3 and TLR4 led to IL-1β maturation via caspase-8 in conditions in which protein synthesis was pharmacologically inhibited (13). Furthermore, experiments using antagonists of inhibitors of apoptosis proteins (IAPs) revealed IL-1β maturation via the NLRP3 inflammasome and caspase-1, as well as via a caspase-8–dependent pathway. Notably, the protein kinase RIP3, a key enzyme in the crossroads between apoptosis and necrosis, and reactive oxygen species were required for both of these pathways (14). Finally, dectin-1 can activate IL-1β via caspase-8 in a pathway that requires the inflammasome adapter molecule ASC (15).

In this article, we demonstrate that Fas-mediated IL-1β activation does not require the inflammasome components NLRP3, ASC, or caspase-1; instead, it proceeds in a process requiring the adapter molecule FADD and caspase-8. In contrast to previously described caspase-8–activation pathways, Fas-induced IL-1β maturation via caspase-8 proceeds independently of RIP3 kinase.

**Materials and Methods**

**Reagents**

Anti–IL-1β Ab was from R&D Systems, anti-ASC Ab was purchased from Santa Cruz Biotechnology, and anti–caspase-1 Ab was from eBiosciences. Nigericin and poly(deoxyadenylyl-thymidylic) acid (dATP) were from Sigma, zVAD were from Promega, and Z-IEDT-FMK, Z-DQMD-FMK, and Z-VEID-FMK were from EMD Biosciences. The IL-1β Elisa kit was from BD Biosciences. IL-18 ELISA was performed using rat anti-mouse IL-18 capture Ab (clone 74) and biotinylated rat anti-mouse IL-18 detection Ab (clone 93-10C) with recombinant murine IL-18 cytokine standard (MBL).

**Mice**

The following mice were used: Nlrp3 knock-out (KO), ASC-KO (Millenium Pharmaceuticals), caspase 1/11 double KO (R. Flavell, Yale University, New Haven, CT), caspase 8/Rip3 double KO (E. Mocarski, Emory University School of Medicine, Atlanta, GA), Rip3 KO (E. Mocarski or F. Chan, University of Massachusetts Medical School, Worcester, MA), and Flip+/Rip3+/Fadd+/+, Flip−/Rip3+/Fadd+, and Flip−/Rip3−/Fadd−/− (D.R. Green, St. Jude Children's Research Hospital, Memphis, TN). The Fas+/+ mice were backcrossed for eight generations from MRL to BALB/c. C57BL/6 mice were from The Jackson Laboratory.

**Cell stimulation and analysis**

Bone marrow–derived macrophages (BMDMs) or bone marrow–derived dendritic cells (BMDCs) were primed with 20 ng/ml ultrapure LPS or with 1 μg/ml CLO97 (InvivoGen), as indicated. Cell-free membrane-bound FasL-expressing microvesicles (mFasL) or control microvesicles from neomycin-resistant vector (Neo) transfected Neuro2a cells were prepared, as described (9). For immunoblot analysis, serum-free supernatants (SNs) were precipitated by chloroform/methanol. The caspase-8 activity assay (Promega) was performed per the manufacturer's instructions and read after 90 min. Cell death was determined by flow cytometry (LSRII; BD Biosciences) using TOPRO-3 (Invitrogen) or 7-aminoactinomycin D (BD Pharmingen) dyes.

**Results and Discussion**

**Priming of macrophages leads to Fas expression**

Fas signaling can be studied efficiently using cell-free membrane-bound mFasL prepared from Fas-expressing cells (16, 17). Using these vesicles, we showed previously that peritoneal mFasL administration induced resident peritoneal macrophages to transcribe a number of proinflammatory cytokines and chemokines. Of note, upon mFasL exposure, peritoneal macrophages produce large amounts of IL-1β and other inflammatory cytokines, prior to undergoing apoptosis. Activated IL-1β results in the subsequent recruitment of high numbers of neutrophils into the peritoneum (9). Hence, Fas signaling in peritoneal macrophages induced an inflammatory response in vivo that is very similar to that seen in response to inflammasome activators (18, 19). Inflammasomes can be activated in response to necrosis-inducing agents (20) and, because inflammasomes control IL-1β maturation, we hypothesized that they may be engaged downstream of Fas.

**BMDMs require a priming signal for the upregulation of NLRP3 and pro–IL-1β that allow inflammasome activation by danger signals (21). Although a 2-h priming step is sufficient to render BMDMs responsive to NLRP3 activators, we found that IL-1β release in response to mFasL required significantly longer priming periods (Fig. 1A, Supplemental Fig. 1A). Control microvesicles (Neo) did not stimulate IL-1β release at either time point after LPS priming. In contrast to resident peritoneal macrophages, BMDMs constitutively express only low amounts of Fas (9). Therefore, we assessed whether priming of cells could induce Fas expression on BMDMs. Indeed, TLR4 or TLR7 priming for 24 h led to increased staining of membrane Fas. Notably, the IL-1β response (Fig. 1A, 1C) correlated with the surface expression of Fas (Fig. 1B), and priming was required for Fas-dependent cell killing (Fig. 1D). In agreement with the notion that mFasL stimulation of cells is dependent on Fas expression, mFasL stimulation of primed cells isolated from Fas−/− mice failed to respond, while being responsive to NLRP3 activators (Supplemental Fig. 1B). Furthermore, BMDCs also released large amounts of IL-1β in response to mFasL. (Supplemental Fig. 1C). Together, these studies suggest that Fas signaling is sufficient to induce IL-1β release from myeloid cells, and TLR-induced priming licenses IL-1β cleavage via the regulation of Fas expression.

**FasL activates IL-1β in an ASC- and caspase-1–independent manner in primed macrophages**

Fas activation normally leads to cellular apoptosis, but it also was reported to induce necrosis (22), which was recently placed upstream of NLRP3 inflammasome activation (20). To test
whether Fas engagement by mFasL activates an inflammasome, we isolated wild-type BMDMs and compared their IL-1β response to Asc-deficient or caspase-1/11-deficient BMDMs. The AIM2 activator dsDNA (dAdT) robustly activated wild-type, but not Asc- or caspase-1/11-deficient, BMDMs (23), whereas mFasL activated BMDMs independently of NLRP3, ASC, or caspase-1 (Fig. 2A, Supplemental Fig. 1D). Additionally, and in contrast to most conditions that induce inflammasome activation, mFasL incubation of BMDMs led to a marked increase in pro–IL-1β production (Fig. 2B). This effect is consistent with the ability of Fas to activate NF-κB (24), which leads to further priming of cells. Together, these data suggested that Fas induced an inflammasome-independent IL-1β–activation pathway.

Fas activates IL-1β and IL-18 in a caspase-8- and FADD-dependent and Rip3-independent pathway

Recent work demonstrated that IL-1β cleavage downstream of the C-type lectin receptor dectin-1 in dendritic cells can proceed in a noncanonical pathway involving the activation of caspase-8 (15). Interestingly, caspase-8 activation in this setting required the inflammasome adapter molecule ASC. Furthermore, an IAP antagonist, which leads to the inhibition of XIAP and degradation of cellular inhibitor of apoptosis 1 and 2, more, an IAP antagonist, which leads to the inhibition of XIAP and degradation of cellular inhibitor of apoptosis 1 and 2. Also, genetic evidence that caspase-8 is involved in IL-1β processing (25). We first tested whether caspase-8 was efficiently activated by mFasL. Indeed, Fas activation induced caspase-8 activity in BMDMs, whereas AIM2 or NLRP3 inflammasome activators failed to do so (Fig 3A, Supplemental Fig. 2A). In addition, RIP3 single-deficient cells showed normal caspase-8 activity in response to Fas signaling (Fig. 3A), suggesting that RIP3 is dispensable for caspase-8 activity downstream of Fas. We next assessed whether caspase-8 was required for Fas signaling–mediated IL-1β maturation in BMDMs. We found that Fas signaling led to IL-1β processing in primed WT BMDMs, whereas primed caspase-8/Rip3 double-deficient BMDMs failed to cleave IL-1β (Fig. 3B). Consistently, BMDMs from caspase-8/Rip3 double-deficient mice failed to secrete IL-1β and IL-18 in response to mFasL (Fig. 3C, 3D). To assess whether RIP3 plays a role in IL-1β or IL-18 activation, we next challenged primed RIP3 single-deficient BMDMs with mFasL. In contrast to previous reports implicating RIP3 in the activation of IL-1β downstream of IAPs (14), Fas signaling mediated IL-1β and IL-18 activation independently of RIP3 (Fig. 3D–F). In line with the inflammasome-independent release of IL-1β by mFasL, we found that the secreted IL-18 was also released independently of caspase-1 or -11 in caspase-8/11 double-deficient BMDMs (Supplemental Fig. 2B). Because the adaptor protein FADD is also critical for signaling from Fas by recruiting caspase-8, we wondered whether its genetic deletion would also inhibit IL-1β maturation. Indeed, deletion of FADD also abolished IL-1β cleavage and secretion in BMDCs, and this effect was paralleled by a resistance to cell death induction via mFasL (Supplemental Fig. 2C–E). Finally, to confirm that IL-1β activation is mediated directly by caspase-8 and not by the downstream executioner caspases (caspase-3, -6, and -7), we tested a panel of inhibitors of executioner caspases.
inflammasome activation is that, once licensed, Fas signaling most likely, other factors important for Fas signaling can be signals required for IL-1\(_b\) induction of pro–IL-1\(_b\). Similar to the NLRP3 inflammasome, cytokines via caspase-1 (21). Similar to the NLRP3 inflammasome, this pathway leading to an inflammasome-independent activation produced after inhibition of caspase-8 (Supplemental Fig. 2F).

The authors have no financial conflicts of interest.

Disclosures

We thank F. Chan for providing the Rip3-KO mice.

References


**Supplemental Figure 1. IL-1β release after Fas engagement via mFasL vesicles is specific to functional Fas receptor expression and independent of NALP3 in BMDM.**

A, IL-1β ELISA of supernatants from BMDMs left untreated (none) or primed with LPS and stimulated for 0h or 2h with nigericin. B, IL-1β ELISA from supernatants of wild-type and Fas<sup>lpr/lpr</sup> BMDMs stimulated with mFasL for 6h or nigericin for 1h. C, IL-1β ELISA from supernatants of wild-type BMDCs stimulated with mFasL for 6h or nigericin for 1h. D, Immunoblot for IL-1β from supernatants of LPS primed wild-type or Nlrp3<sup>−/−</sup> BMDMs as indicated.

**Supplemental Figure 2. IL-1β processing by Fas ligation is dependent on FADD and independent of the executioner caspases downstream of caspase-8.**

A, Caspase-8 activity in cell lysates from LPS primed wild-type or Caspase-8<sup>−/−</sup>/Rip3<sup>−/−</sup> BMDMs stimulated for 6h with mFas-L or dAdT or stimulated for 1h with nigericin. B, ELISA for IL-18 of SN from LPS-primed (24h) BMDCs from wild-type (white bars) or Caspase-1/11<sup>−/−</sup> (black bars) mice stimulated as indicated for an additional 6h with mFas-L or Neo. C and D, BMDC derived from Flip<sup>+/−</sup>Rip3<sup>+/−</sup>Fadd<sup>+/−</sup>, serving as control, were compared to Flip<sup>+/−</sup>Rip3<sup>+/−</sup>Fadd<sup>+/−</sup> and Flip<sup>+/−</sup>Rip3<sup>+/−</sup>Fadd<sup>+/−</sup> and supernatants were analyzed by ELISA for IL-1β (C) and immunoblot (D). E, BMDC of the same experiment as in (C) were stained with 7AAD and analyzed by flow cytometry. Histograms depicting the % of live cells excluding 7AAD at the 6-hour timepoint after mFas-L stimulation. F, IL-1β ELISA from supernatants of wt BMDM preincubated with the 20μM of indicated caspase inhibitors for 1h prior to addition of mFasL for 6h.
Supplemental Figure 1
Supplemental Figure 2