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Cutting Edge

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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: 5508–5512.**

Fas (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-

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Abbreviations used in this article: BMDC, bone marrow–derived dendritic cell; BMDM, bone marrow–derived macrophage; CL, cell lysate; dAdT, poly(deoxyadenylic-thymidylic) acid; FasL, Fas ligand; IAP, inhibitor of apoptosis protein; KO, knockout; mFasL, Fas ligand–expressing microvesicle; Neo, control microvesicles from neomycin-resistance vector-transfected cells; RIP, receptor-interacting protein; SN, supernatant.

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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(deoxyadenylic-thymidylic) acid (dAdT) were from Sigma, zVAD was 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*-knockout (KO), ASC-KO (Millennium 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 $Fas^{lpr/lpr}$ 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-resistance 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 FasL-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 $Fas^{lpr/lpr}$ 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

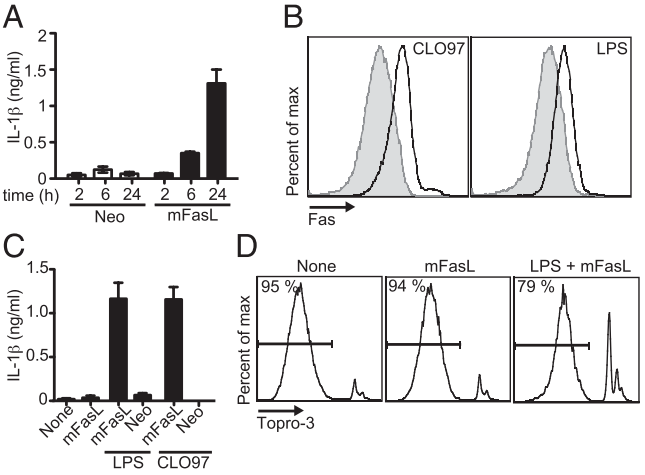

FIGURE 1. IL-1β activation by Fas is dependent on Fas receptor upregulation secondary to TLR priming in BMDMs. (**A**) IL-1β ELISA of SNs from BMDMs primed with LPS and stimulated for the indicated times with Neo or mFasL vesicles. (**B**) Graphs depicting the mean fluorescence intensity of Fas on live-gated BMDMs after 24 h of stimulation with 300 ng/ml CLO97 (open graph) or 20 ng/ml LPS (open graph) and untreated controls (shaded graph). (**C**) IL-1β ELISA of SNs from Neo- or mFasL-stimulated BMDMs, which were left untreated or primed for 24 h with LPS or CLO97. (**D**) Graphs of TOPRO-3–stained BMDMs in medium only (*left panel*), after the addition of mFasL without priming (*middle panel*), or after 24 h of priming with LPS and stimulation with mFasL for 6 h (*right panel*).

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, resulted in both NLRP3-dependent and caspase-8–dependent activation of IL-1β. Both of these pathways were dependent on the RIP3-mediated production of reactive oxygen species (14). Because Fas activation is also known to activate caspase-8, we hypothesized that Fas signaling could lead to cleavage of IL-1β via caspase-8 in primed BMDMs. Thus, we sought to establish genetic evidence that caspase-8 is involved in IL-1β maturation. *Caspase-8*–deficient mice show embryonic lethality, which is thought to be a consequence of unperturbed RIP3 activity leading to increased necrosis. However, *caspase-8/Rip3* double-deficient mice are viable and, hence, allowed the testing of BMDMs for mFasL-mediated IL-1β activation (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-1/11* 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, caspase-6, and caspase-7), we tested a panel of inhibitors of executioner

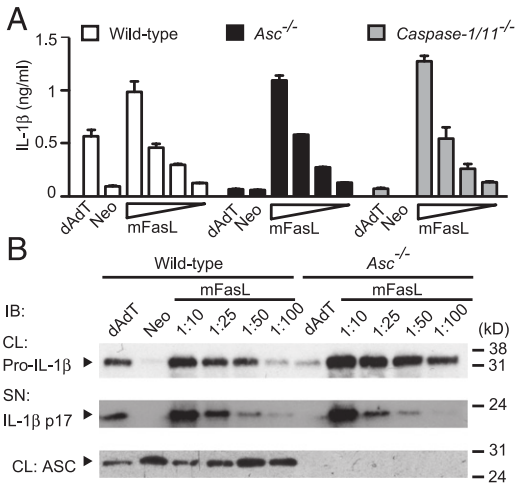

FIGURE 2. Inflammasome and caspase-1/11–independent IL-1β processing in BMDMs stimulated with mFasL. (**A**) ELISA for IL-1β of SNs from LPS-primed (24 h) BMDMs from wild-type, *ASC*$^{-/-}$, or *caspase-1/11*$^{-/-}$ mice, stimulated as indicated for an additional 6 h with decreasing concentrations of mFasL, Neo, or dAdT. (**B**) Immunoblot of SNs and cell lysates (CL) of wild-type and *ASC*$^{-/-}$ BMDMs using the same assay conditions as in (**A**).

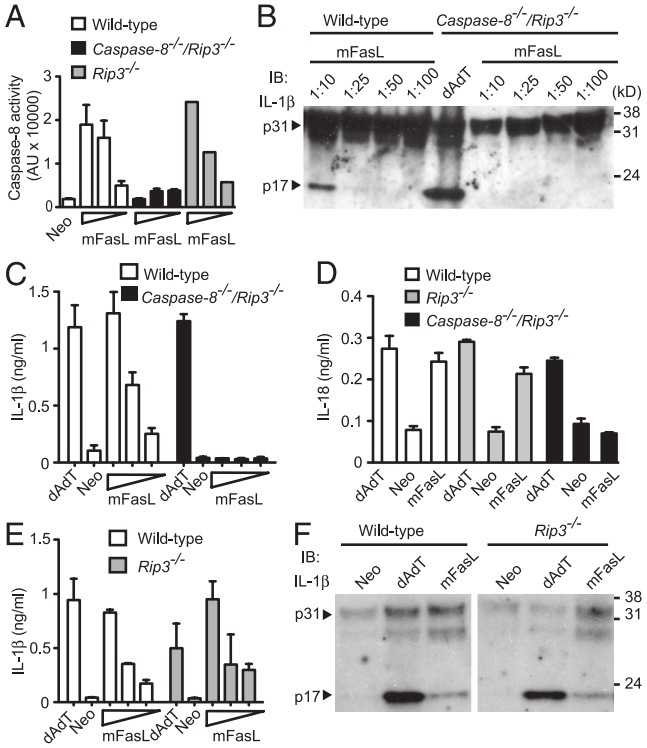

FIGURE 3. FAS-induced IL-1β and IL-18 processing is caspase-8 dependent and RIP3 independent. (**A**) Caspase-8 activity in cell lysates from wild-type, *caspase-8*$^{-/-}$*/Rip3*$^{-/-}$, or *Rip3*$^{-/-}$ BMDMs stimulated for 6 h with decreasing concentrations of mFasL. Immunoblot (**B**, **F**) or ELISA for IL-1β (**C**, **E**) and IL-18 (**D**) in SNs from wild-type, *caspase-8*$^{-/-}$*/Rip3*$^{-/-}$, or *Rip3*$^{-/-}$ BMDMs that were stimulated as indicated.

caspases and confirmed that IL-1β activation was only reduced after inhibition of caspase-8 (Supplemental Fig. 2F).

We found that Fas activation mediates a nonapoptotic pathway leading to an inflammasome-independent activation of IL-1β family cytokines. This pathway may be of great relevance for a number of processes downstream of Fas signaling. It is well established that IL-1β family members are important for antimicrobial defenses and that they play key roles during the development of adaptive immune responses (12). More recently, viruses were shown to block the activity of key innate immune pathways, such as the DNA- or RNA-sensing or inflammasome pathways (26). Thus, it is conceivable that infected cells that upregulate Fas in response to infections could induce an inflammatory response to Fas signaling by autocrine or paracrine mechanisms or via the interaction with FasL on adaptive-immune cells. A similar scenario could be relevant for infectious defense against microbes that target the Fas-mediated apoptosis pathways downstream of caspase-8 (i.e., that block components of the extrinsic apoptosis pathway) (26, 27). In such a scenario, activation of Fas and caspase-8 would still benefit the host, because it would lead to the activation of IL-1β cytokines, thus alerting other immune cells of the infection and initiating an antimicrobial response.

However, the described Fas-dependent noncanonical IL-1β activation pathway could also cause harm under conditions where endogenous danger signals excessively trigger innate immune pathways, as seen, for example, in systemic lupus. Under these situations, upregulation of Fas could predispose innate immune cells to a proinflammatory response via FasL ligation that could further potentiate autoimmune pathology (28). In dendritic cells or microglia cells, apoptosis-inducing and inflammation-inducing Fas signaling is uncoupled; therefore Fas could be of significance for the development of inflammatory conditions (29, 30). Because the activation of IL-1β cytokines can have dramatic consequences for the establishment of inflammation and immunity, it is not surprising that it is highly regulated. In the case of the NLRP3 inflammasome, transcriptionally active pattern recognition receptors or cytokine signaling receptors prime cells, leading to the induction of pro–IL-1β and NLRP3 itself. Activation of NLRP3 by a danger signal—the second signal—then leads to the proteolytic processing of IL-1β cytokines via caspase-1 (21). Similar to the NLRP3 inflammasome, Fas signaling in myeloid cells requires a licensing signal provided by pattern recognition receptors, such as Fas itself; most likely, other factors important for Fas signaling can be induced by TLR activation. However, a notable difference from inflammasome activation is that, once licensed, Fas signaling further induces pro–IL-1β and, hence, can provide the two signals required for IL-1β activation (i.e., the transcriptional induction of pro–IL-1β and the maturation of the cytokine via caspase-8). This suggests that once primed myeloid cells have upregulated Fas, they can produce large amounts of key inflammatory cytokines of the IL-1β cytokine family in response to cues received from other immune cells via Fas, which could have important implications for the control of inflammation.

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Disclosures

The authors have no financial conflicts of interest.

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