Fas Activation in Alveolar Epithelial Cells Induces KC (CXCL1) Release by a MyD88-Dependent Mechanism

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Activation of the Fas/Fas ligand (FasL) system is associated with activation of apoptotic and proinflammatory pathways that lead to the development of acute lung injury. Previous studies in chimeric mice and macrophage-depleted mice suggested that the main effector cell in Fas-mediated lung injury is not a myeloid cell, but likely an epithelial cell. The goal of this study was to determine whether epithelial cells release proinflammatory cytokines after Fas activation, and to identify the relevant pathways. Incubation of the murine alveolar epithelial cell line, MLE-12, with the Fas-activating monoclonal antibody, Jo2, resulted in release of the CXC chemokine, KC, in a dose-dependent manner. KC release was not prevented by the pan-caspase inhibitor, zVAD.fmk. Silencing of the adaptor protein, MyD88, with small interfering (si)RNA resulted in attenuation of KC release in response to Jo2. Fas activation resulted in phosphorylation of the mitogen-activated kinases extracellular signal-regulated kinase (ERK) and c-Jun-N-terminal kinase (JNK), and pharmacologic inhibition of ERK and JNK attenuated KC release in a dose-response manner. Similarly, primary human small airways epithelial cells released IL-8 in response to soluble FasL, and this was abrogated by inhibition of JNK and ERK. *In vivo* confirmatory studies showed that MyD88-null mice are protected from Fas-induced acute lung injury. In summary, we conclude that Fas induces KC release in MLE-12 cells by a mechanism requiring MyD88, mitogen-activated protein kinases, and likely activator protein-1.

Keywords: Fas; Fas ligand; KC; IL-8; MyD88

Acute lung injury (ALI) remains an important problem in critically ill patients, with a mortality rate that ranges between 26 and 40% (1, 2), and a lifetime cost-of-care-per-patient of \$99,588–\$106,821 (3). The pathogenesis of ALI remains unclear, but experimental evidence garnered from studies with animal and cellular model systems suggests that ALI is associated with activation of inflammatory, apoptotic, and oxidative injury pathways (4). However, it is unclear whether these pathogenic mechanisms are independent of each other or, instead, are linked by common activating pathways.

The Fas/Fas ligand (FasL) system, composed of the membrane surface receptor, Fas (CD95), and its cognate ligand, FasL (CD95L), has emerged as a particularly interesting pathway that can lead to both inflammation and apoptosis (5–7). The proapoptotic function of the Fas/FasL system is well described, and involves the formation of a “death-inducing signaling complex” (DISC), consisting of the intracellular domain of Fas receptor multimers and adaptor proteins, such

CLINICAL RELEVANCE

The Fas/Fas ligand system plays a role in the pathogenesis of inflammation and apoptosis during acute respiratory distress syndrome. This work clarifies the pathways associated with inflammation in epithelial cells, and shows that proinflammatory and proapoptotic pathways can be activated simultaneously.

as Fas-associated protein with death domain (FADD). The DISC rapidly recruits the cysteine protease, caspase-8, which cleaves itself and subsequently leads to proteolytic activation of the executioner caspases 3 and 7, which then activate effector proteins, such as poly ADP-ribose polymerase and caspase-activated DNase (reviewed in Ref. 8). This well studied caspase pathway results in apoptosis. A number of studies have shown that the proapoptotic function of the Fas/FasL system is active in humans with ALI/acute respiratory distress syndrome, and may be involved in the disruption of the alveolar capillary barrier by inducing apoptosis of alveolar epithelial cells (9–11).

In addition to the numerous studies examining the role of the Fas/FasL system in triggering apoptosis through the traditional caspase pathway, it has become clear that activation of the Fas/FasL system can also lead to cytokine release *in vivo* and *in vitro* (12, 13–16). Although the caspase pathway likely plays a role in ALI, other proinflammatory pathways downstream of Fas/FasL appear to be more important in the development of experimental ALI than previously thought. The activation of the Fas/FasL system in the lungs can lead to the development of neutrophilic inflammation; specifically, mice lacking functional Fas show impaired neutrophil responses to intranasal LPS (12, 17, 18). Several studies have investigated the mechanisms behind the proinflammatory function of Fas/FasL in the lung and its role *vis-a-vis* the proapoptotic function. Initial studies suggested that human and rabbit alveolar macrophages release the neutrophil chemoattractant, IL-8 (CXCL8), in response to Fas activation *in vitro* and *in vivo* (13, 17). Because of these findings, it has been proposed that the Fas/FasL system might contribute to ALI by triggering cytokine release in macrophages and apoptosis in alveolar epithelial cells (13, 17). This hypothesis was tested in a study using chimeric mice; however, contrary to the hypothesis, the mice lacking Fas in their macrophages and other myeloid cells retained the ability to release KC (the murine analog of IL-8) and recruited neutrophils to the lung after Fas activation (15). These findings were later confirmed in a second study using mice depleted of alveolar macrophages (19, 20). Thus, it appears that the macrophage is not required for the acute inflammatory response to Fas activation in the lungs, suggesting the possibility that, in addition to apoptosis, activation of Fas may be inducing cytokine release in nonmyeloid cells.

In this study, we tested the hypothesis that Fas activation triggers caspase-independent pathways that lead to release of

(Received in original form April 26, 2010 and in final form December 19, 2010)

This work was supported in part by National Institutes of Health grants HL83044 (G.M.-B.) and HL086883 (W.A.A.).

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 45, pp 650–658, 2011

Originally Published in Press as DOI: 10.1165/rcmb.2010-0153OC on January 21, 2011

Internet address: www.atsjournals.org

the murine IL-8 ortholog, KC, in alveolar epithelial cell lines. To test this hypothesis, we developed a model system based on the murine epithelial cell line, MLE-12, and determined that Fas activation leads to KC release in these cells. Interestingly, the signaling pathway was dependent on MyD88, an adaptor protein more commonly associated with inflammatory signaling triggered by the Toll family of pattern-recognition receptors (Toll-like receptors [TLR]). We then confirmed the findings with *in vivo* experiments using *Myd88*^{-/-} mice. Thus, these studies demonstrate that the Fas/FasL system induces release of proinflammatory cytokines in mice, and that MyD88 is involved in non-TLR-dependent inflammatory pathways.

MATERIALS AND METHODS

For detailed methods, please *see* the online supplement.

Animal Model

Animal studies were approved by the Animal Care Committee of the University of Washington. C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) and *Myd88*^{-/-} mice (originally provided by Shizuo Akira) received the Fas-activating monoclonal antibody (mAb), Jo2, or a control IgG, 2.5 ng/g (BD Pharmingen, San Diego, CA) intratracheally every 24 hours for a total of three instillations (21). At 7 days after the first instillation, mice were killed, the right lung was lavaged with three 0.5 ml instillations of PBS containing 0.6 mM EDTA, and the left lung was homogenized. The bronchoalveolar lavage (BAL) fluid and homogenates were processed as previously described (21–23).

Cell Studies

MLE-12 cells (no. CRL-2210; ATCC, Manassas, VA) were grown per ATCC suggestions to roughly 75% confluency. Human small airway epithelial cells (SAECs) (Lonza, Walkersville, MD) were grown per Lonza's suggestions to 100% confluency. Experiments were performed after 24-hour incubation in serum-free media. After some experiments, the supernatants were removed and stored at -80°C; alternatively, cells were retrieved for RNA extraction or protein extraction. Viability was assessed with alamarBlue (Biosource, Carlsbad, CA).

Transfection Protocols

MLE-12 cells were grown to 45% confluency, then lipofectamine transfected for 24 hours with 12.5 μ M small interfering (si)RNA targeting death-associated protein 6 (DAXX), MyD88, and receptor-interacting protein (RIP) or for 48 hours with FADD siRNA (Dharmacon, Lafayette, CO).

NF- κ B Luciferase Reporter Cells

Lentivirus was made in HEK-293T cells (ATCC no. CRL-11268) by cotransfecting a pSL9 backbone plasmid containing an NF- κ B-luciferase vector with a hygromycin-resistance gene (kindly provided by Travis Biechele, University of Washington) with the lentiviral packaging plasmids, pSL4 (16 μ g), pSL3 (4 μ g), and pSL5 (8 μ g). MLE-12 cells were grown to 50% confluency and then incubated for 48 hours with complete media containing 5% lentivirus. Stable transfectants were selected by growing the cells twice to 80% confluency in media supplemented with 300 μ g/ml hygromycin B (Invitrogen, Carlsbad, CA). Transfection was confirmed by incubation with serial doses of TNF- α .

Determination of mRNA Expression Using Real-Time PCR

Quantitative mRNA expression was performed using the following TaqMan primer-probe sets: DAXX (ID, Mm01278902_g1), MyD88 (ID, Mm01351743_g1), and RIP (ID, Mm01354381_m1) (Applied Biosystems, Carlsbad, CA). The $\Delta\Delta$ threshold cycle ($\Delta\Delta$ Ct) method was used to analyze gene amplification, using eukaryotic 18 s rRNA (ID, Hs99999901_s1; Applied Biosystems) as the reference gene, and cDNA from pooled samples as the calibrator.

Cytokine measurements

KC (CXCL1) and IL-8 were measured with specific immunoassays (R&D Systems, Minneapolis, MN). KC, granulocyte/macrophage colony-stimulating factor, monocyte chemoattractant protein 1 (MCP-1), vascular endothelial growth factor (VEGF), TNF- α , macrophage inflammatory protein 2 (MIP-2), and IL-1 β were measured simultaneously using a multiplex assay (Fluorokine; R&D Systems).

Immunoblotting

Immunoblotting was performed by SDS-PAGE electrophoresis using rabbit antibodies against human DAXX, RIP, phospho-c-Jun-N-terminal kinase (JNK) and β -actin (Cell Signaling Technology, Danvers, MA). Rabbit anti-MyD88 antibodies were obtained from ProSci (Poway, CA), and rabbit anti-FADD antibodies from Abcam (Cambridge, MA).

Statistical Analysis

Comparisons between two groups were performed with two-tailed *t* tests. Comparisons between multiple groups were performed using one-way ANOVA with Tukey's *post hoc* analysis. A *P* value of 0.05 or less was considered significant.

RESULTS

In this study, we investigated whether Fas induces the release of proinflammatory cytokines in alveolar epithelial cells using the murine cell line, MLE-12, as our *in vitro* model, and the murine ortholog of IL-8, KC, as the main outcome measurement. We chose MLE-12 cells because they do not become apoptotic in response to Fas ligation, thus avoiding confounding issues related to variation in cell number during the experiments.

Activation of Fas in Alveolar Epithelial Cells Induces Release of KC *in vitro*

First, we measured expression of the cytokines KC, granulocyte/macrophage colony-stimulating factor, MCP-1, VEGF, TNF- α , MIP-2, and IL-1 β in the supernatant of MLE-12 cells incubated for 18 hours with Jo2 mAb, 500 ng/ml, in the presence or absence of the caspase inhibitor, zVAD (100 μ M). Control cells were incubated with isotype control IgG in the presence of 0.05% DMSO, which is the diluent for zVAD. Interestingly, of all the cytokines tested, the Jo2 mAb induced expression of only the neutrophil chemoattractant, KC (62 ± 7 pg/ml in the Jo2 group, compared with 12 ± 0.4 in the IgG group). This increase in KC was not prevented by zVAD at doses of 10 or 100 μ M (60 ± 5 and 79 ± 2 pg/ml, respectively; Figure 1A). We then measured KC release in response to serial concentrations of the Fas-activating Jo2 mAb (0–1,000 ng/ml) or an isotype control IgG (1,000 ng/ml). The cells incubated with Jo2 mAb released KC in a dose-dependent manner (Figure 1B). Next, we incubated the cells with 500 ng/ml of Jo2 and measured KC at serial times (Figure 1C). The release of KC occurred between 6 and 18 hours after treatment with the Fas-activating Jo2 mAb. Thus, Jo2 induces KC release in an alveolar epithelial cell line *in vitro*.

The KC Response to Fas Activation Is Impaired by Silencing of MyD88

The canonical activating pathway downstream of the Fas receptor involves the adaptor protein FADD. FADD binds to the cytoplasmic domain of Fas via a common motif known as the death domain (DD). We tested whether the signaling pathway linking Fas activation with KC release involved FADD by using a loss-of-function approach based on siRNA silencing of mRNA translation. Transfection of FADD siRNA into MLE-12 cells for 48 hours resulted in attenuation of FADD protein, as compared with cells transfected with a control siRNA (Figure 1D). However, MLE-12 cells transfected with FADD siRNA

second lane, was used as a control for protein loading. (E) The cells transfected with siRNA showed no impairment in KC release after incubation with Jo2 mAb ($n = 3$). * $P < 0.05$ compared with the control condition, which is IgG in (A) and (B) and time zero in (C).

released similar concentrations of KC in response to Jo2 mAb, as did cells transfected with the control siRNA (Figure 1E).

In addition to FADD, other death domain-containing proteins, such as DAXX and RIP, can also bind to Fas and initiate signaling. However, silencing of DAXX or RIP with siRNA did not prevent KC release in MLE-12 cells treated with Jo2 mAb for 18 hours, although the negative result could have been due to residual protein present in the cells (Figure 2A). Another adapter protein that contains a death domain is MyD88 (24). MyD88 is better known for participating in the signaling pathway downstream of the TLR family of pattern recognition receptors; however, MyD88 is also known to interact with members of Fas signaling pathways via its death domain (25). Silencing of MyD88 with siRNA resulted in a significant—but not complete—decrease in KC release after exposure to Jo2 mAb (Figure 2A). The extent of siRNA silencing was similar for both the RNA and protein levels for all three target proteins (Figures 2B and 2C). Thus, the combined evidence from these siRNA experiments suggests that MyD88 participates in the Fas proinflammatory signaling pathway.

The KC Response to Fas Activation Requires Phosphorylation of the Mitogen-Activated Protein Kinases, Extracellular Signal-Regulated Kinase and c-Jun-N-Terminal Kinase

Mitogen-activated protein kinases (MAPKs) are known to participate in a number of proinflammatory and proapoptotic pathways, including pathways associated with Fas and MyD88.

We therefore tested whether extracellular signal-regulated kinase (ERK), c-Jun-N-terminal kinase (JNK), and p38 isoforms are phosphorylated 5–60 minutes after exposure of MLE-12 cells to Jo2 mAb. Phorbol myristate-acetate was used as positive control and an isotype mAb as negative control. Exposure to Jo2 for 60 minutes resulted in phosphorylation of ERK and JNK, and minimal phosphorylation of p38 (Figures 3A and 3B). This increase in phosphorylation was not seen at earlier time points, specifically after 5, 15, or 30 minutes of treatment with Jo2 mAb. Next, we investigated whether pharmacologic blockade of the MAPKs would decrease KC release in MLE-12 cells exposed to Jo2 for 18 hours. Inhibition of ERK with PD98059 and of JNK with SP600125 was followed by a dose-dependent inhibition of Fas-induced KC release in MLE-12 cells (Figures 3C and 3D). The p38 inhibitor, SB203580, did not impair KC release in cells treated with Jo2 (Figure 3E). Thus, the MAPKs, ERK1/2 and JNK, are involved in the KC response to Fas activation.

KC Release Is Dependent on Activation of Activator Protein-1, but Not NF-κB

The CXC cytokines, including KC, are transcriptionally regulated. Transcription factors, such as NF-κB and activator protein (AP)-1, are important regulators of KC production, and may be activated by pathways dependent on MAPK phosphorylation. Thus, we investigated NF-κB and AP-1 activity in MLE-12 cells treated with Jo2 mAb or a control mAb for 18

Figure 1. Activation of Fas in alveolar epithelial cells induces release of KC. MLE-12 cells were incubated with media supplemented with the Fas-activating monoclonal antibody (mAb) Jo2 (500 ng/ml), or a control IgG, in the presence or absence of the pan-caspase inhibitor, zVAD (10 and 100 μM). The media were collected after 18 hours incubation and the concentrations of KC, granulocyte/macrophage colony-stimulating factor (GM-CSF), MCP-1, VEGF, TNF-α, MIP-2, and IL-1β were measured in the supernatants with a multiplex assay. The only cytokine induced by FasL was KC (A) ($n = 6$). The concentrations of KC were measured by ELISA. The release of KC at 18 hours was a function of the dose of mAb Jo2 (B) ($n = 3$). MLE-12 cells were incubated with Jo2 mAb or a control mAb (500 ng/ml) and KC was measured in the media at time intervals ranging from zero to 18 hours. The mAb Jo2 resulted in KC release, which became maximal between 6 and 18 hours (C). MLE-12 cells were transfected for 48 hours with small interfering (si)RNA against FADD. A nontargeting siRNA was used as a control for transfection and siRNA processing. After transfection, cells were treated with the Fas-activating mAb, Jo2, or an isotype-control IgG (500 ng/ml) for 18 hours, after which media supernatants and protein lysates were collected for analysis. (D) Immunoblots of cell protein extracts showed silencing of FADD protein; β-actin, shown in the

Figure 2. The KC response to Fas activation requires MyD88. MLE-12 cells were transfected for 24 hours with siRNA against MyD88, DAXX, or RIP, or a nontargeting siRNA as a control for transfection and siRNA processing. After transfection, cells were treated with the Fas-activating mAb, Jo2, or an isotype-control IgG (500 ng/ml) for 18 hours, after which media supernatants and protein lysates were collected for analysis. (A) Silencing of MyD88 resulted in attenuation of KC release by Jo2 ($P < 0.05$; $n = 4$). (B) RNA expression by quantitative PCR, showing silencing of the targeted genes ($n = 3$). (C) Immunoblots of the transfected cells revealed partial silencing of the target proteins ($n = 3$).

hours in the presence or absence of MAPK inhibitors. To investigate the role of NF- κ B, MLE-12 cells were permanently transfected with an NF- κ B luciferase reporter construct. Exposure of the cells to Jo2 mAb resulted in activation of the reporter, but the activation was not prevented by pharmacologic inhibition of JNK or p38, and may have even increased by

inhibition of ERK (Figure 4A). Although pharmacologic inhibition of the MAPKs did not attenuate NF- κ B activation, it was effective in attenuating KC release (Figure 4B). To confirm that Fas activation leads to KC production by a mechanism partly independent of NF- κ B activation, we treated MLE-12 cells with Jo2 mAb, TNF- α or a control IgG in the presence of

(C), JNK (D), or p38 (E) for 18 hours showed a dose-dependent decrease in KC release associated with ERK and JNK inhibition (C and D). Treatment of MLE12 cells with an inhibitor against p38 did not show decreases in KC release (E) ($n = 3$ for all experiments shown).

BMS-345541 an inhibitor of $I\kappa\kappa$. $I\kappa\kappa$ is an enzyme complex required for NF- κ B signaling. Treatment with BMS-345541 (20 nM) had no effect on KC release after treatment with Jo2 mAb (Figure 4C). In contrast, the same dose of BMS-345541 abrogated KC release in response to TNF- α , which is known to signal via NF- κ B. These data indicate that the primary pathway linking Fas activation with KC release in epithelial cells likely involves a transcription factor other than NF- κ B. However, we have not excluded the possibility that Jo2 caused activation of NF- κ B by a pathway independent of $I\kappa\kappa$.

AP-1 is a transcription factor that can induce transcription of proinflammatory cytokines and is regulated by MAPKs. Specifi-

cally, AP-1, which is composed of homo- or heterodimers of the proteins, Jun and Fos, can be activated by phosphorylation of Jun by JNK (26). Thus, we tested whether Jo2 induces Jun phosphorylation in MLE-12 cells incubated with Jo2, using phorbol myristate-acetate, 500 nM as a positive control. We found evidence of Jun phosphorylation after 6 hours of incubation with Jo2 mAb, but not at 2 or 4 hours (Figures 4D and 4E).

FasL Induces IL-8 Expression in Human Lung Epithelial Cells

To determine whether our findings could be reproduced in primary human cells, we incubated primary human lung epithelial cells with serial concentrations of soluble FasL (sFasL)

Figure 4. KC release is dependent on activation of activator protein (AP)-1, but not NF- κ B. MLE12 cells stably transfected with an NF- κ B luciferase reporter were incubated for 18 hours with a control IgG (1,000 ng/ml) or Jo2 mAb (1,000 ng/ml) with or without serial concentrations of pharmacologic inhibitors of ERK (PD98059), JNK (SP600125), and p38 (SB203580) (A). At the end of the experiment, NF- κ B activity was measured as luminescence, and KC concentrations in the media were measured by ELISA. As compared with the control IgG, Jo2 induced an increase in NF- κ B activity, but this was not blocked by the MAPK inhibitors, and, in fact, was enhanced by PD98059. Despite the lack of NF- κ B inhibition, release of KC in response to Fas activation was attenuated by the inhibitors against ERK and JNK. ($^{\dagger}P < 0.05$ compared with IgG; $^*P < 0.05$ compared with Jo2) (B). To confirm that Fas-mediated KC release was independent of NF- κ B, MLE-12 cells were treated for 18 hours with IgG (1,000 ng/ml) or Jo2 (1,000 ng/ml) in the presence or absence of the $I\kappa\kappa$ inhibitor, BMS-345541 (20 μ M), or its vehicle, 0.4% DMSO (C). TNF- α (10 ng/ml) was used as a positive control. Blockade of NF- κ B with BMA-345541 abrogated KC production in response to TNF- α , but not Jo2. To

investigate if AP-1 was involved in Fas-mediated release of KC, MLE-12 cells were treated for 2, 4, or 6 hours with IgG (1,000 ng/ml), Jo2 (1,000 ng/ml), or PMA (500 nM, used as positive control) (D). Western blot analysis was performed for phospho-c-Jun (E). Treatment with Jo2 for 6 hours resulted in phosphorylation of c-Jun at 6 hours, suggesting possible AP-1 activation (D and E) ($n = 3$ for all experiments shown).

and measured cell death and IL-8 release. In contrast to the MLE-12 cells and in agreement to previous reports, the SAEs developed apoptosis in response to sFasL (Figures 5A and 5B). However, the SAEs simultaneously released IL-8 in a dose-dependent manner (Figure 6C).

Next, we determined whether the release of IL-8 was dependent on MAPK activation. The cells were incubated with sFasL (500 ng/ml) in the presence of the ERK inhibitor, PD98059, and the JNK inhibitor, SP600125 (Figure 6). Similar to what was seen with the MLE-12 cells, blockade of ERK with PD98059 and JNK with SP600125 abrogated IL-8 release (Figures 6A, 6C, and 6G). Inhibition of both ERK and JNK resulted in complete blockade of IL-8 release (Figure 6G). In contrast, IL-8 release was not suppressed by inhibition of p38 (Figure 6E). Despite their effect on IL-8 release, none of the inhibitors affected cell death (Figures 6B, 6D, 6F, and 6H), indicating that the proinflammatory pathway and apoptotic pathway are independent of each other, and that both are activated simultaneously.

MyD88 Is Required for Fas-Induced Injury *In Vivo*

Finally, we confirmed the biological relevance of our findings by testing whether mice with a targeted deletion of MyD88 (*Myd88*^{-/-} mice) show impaired inflammation after intratracheal administration of Jo2 mAb, as compared with a control mAb. As compared with IgG, the administration of Jo2 to wild-type mice resulted in a significant increase in lung homogenate myeloperoxidase (MPO) activity, BAL KC, BAL IgM, and lung caspase activity (Figure 7). In contrast, the administration of Jo2 did not lead to an increase in any of the parameters of lung injury in *Myd88*^{-/-} mice, as compared with mice treated with IgG. Thus, MyD88 is required for the lung response to Fas activation.

DISCUSSION

The goal of this study was to test the hypothesis that Fas activation leads to release of the proinflammatory cytokine KC in alveolar epithelial cells. The main finding was that Fas activation results in KC release by a mechanism that involves the adaptor protein, MyD88, the mitogen-activated kinases, ERK and JNK, and is largely independent of caspases.

Both Fas and FasL are present ubiquitously in the lungs (17, 27, 28). However, the outcome of Fas activation appears to be dependent on the target cell, with epithelial cells, in addition to apoptosis, releasing neutrophil chemoattractants, such as KC in mice and IL-8 in humans. The main source of bioactive sFasL in the lungs is unclear, but it may be released from macrophages upon activation, or more likely, cleaved from Fas-bearing cells by metalloproteinases, such as matrix metalloproteinase 7 (29, 30). In the setting of lung injury, the soluble form is active and capable of inducing apoptosis in human epithelial cells (9).

In the present study, we set out to determine whether Fas activation induces cytokine release in a lung epithelial cell line; specifically, we focused on release of the neutrophil chemoattractant, KC, by the epithelial cell line, MLE-12. One interesting aspect of MLE-12 cells is that they do not become apoptotic in response to Fas ligation, allowing for the study of the pathways of cytokine release without the added confounding factor of changing cell numbers due to cell death. We were able to reproduce previously reported data showing that Jo2 induces KC release in epithelial cells. This was not an immediate event; instead, the onset of the Jo2 response required several hours, suggesting activation of a transcriptional response. We set out to investigate the signaling pathway that leads to the transcriptional response.

Our first step was to investigate whether the canonical components of the DISC are involved in the pathway. Fas trimers

Figure 5. Primary human epithelial cells release IL-8 in response to soluble FasL (sFasL). Primary human small airway epithelial cells (SAECs) were incubated with serial concentrations of recombinant human sFasL and studied 18 hours later. Cell death was measured as percent of alamar Blue reduction compared with cells incubated in media only; caspase-3 activity was measured in lung homogenates; IL-8 was measured by immunoassay. sFasL caused a dose-dependent increase in cell death (A) and in caspase-3 activity (B). Simultaneously, sFasL treatment also resulted in release of IL-8 (C).

are thought to recruit the adaptor protein, FADD. We expected FADD to be required for the proinflammatory pathway, because, in other cells and tissues, FADD appears to be involved in inflammatory Fas signaling (31–33). However, partial silencing of FADD with siRNA had no effect on Fas-induced release of KC. We next tested other relevant adaptor proteins, including DAXX and RIP (34–36); however, partial silencing of these proteins did not abrogate KC release in Jo2-treated cells. Finally, we tested silencing of MyD88. MyD88 is better known as a central adaptor protein for inflammatory signaling in response to activation of pattern-recognition receptors, such as the Toll receptor family. MyD88 contains a death domain that can interact with other death domain-containing proteins, such as interleukin-1 receptor-associated kinase (IRAK) and FADD (24, 25). MyD88 has been associated with chemokine production in myeloid cells (37). Silencing of MyD88 with siRNA was associated with partial—but significant—reduction in Fas-induced KC release. The mechanisms mediating MyD88 activation in our study remain unclear, although one possibility is the direct interaction between Fas and TLRs, perhaps by means of ceramide bridges. However, the data

Figure 6. Inhibition of ERK and JNK attenuates IL-8 release in human epithelial cells exposed to FasL. Primary human SAEs were incubated in the presence or absence of sFasL (500 ng/ml) with or without serial doses of the ERK inhibitor PD98059 (A and B); the JNK inhibitor SP600125 (C and D); the p38 inhibitor SB203580 (E and F) or the ERK and JNK inhibitors combined (G and H). Inhibition of ERK and of JNK abrogated the release of IL-8 by SAEs treated with sFasL, and this effect was maximal when both inhibitors were combined (A, C, and G). Note that the inhibition of IL-8 was not associated with inhibition of cell death (B, D, and H), suggesting that the death pathway and proinflammatory pathway are independent of each other and simultaneously activated. Inhibition of p38 did not inhibit IL-8 release or cell death (E and F) ($n = 3$).

show that, in epithelial cells, the proinflammatory pathway triggered by Fas activation partially involves the adaptor protein, MyD88.

MyD88 has been associated with activation of MAPK. Thus, our next step was to determine if MAPK activity was required for the inflammatory pathway of Fas in alveolar epithelial cells. Our data show that Jo2 treatment results in phosphorylation of ERK1/2 and JNK. The role for these kinases in Jo2-induced KC release was confirmed by demonstrating that ERK and JNK pharmacologic inhibitors attenuate KC release in a dose-dependent manner. We also investigated whether the findings in the murine cell line are reproducible in primary human lung epithelial cells. In contrast to the MLE-12 cells, the human

cells develop apoptosis in response to Fas activation; however, they also released IL-8, indicating that apoptosis may not always be necessarily an anti-inflammatory event. As in the MLE-12 cells, the release of IL-8 was partly abrogated by inhibitors of ERK and JNK.

Eventually, MyD88-dependent signaling leads to cytokine production by activation of transcription factors, in particular NF- κ B or AP-1 (38). Using a reporter system, we found that Jo2 activated NF- κ B; however, the activity of NF- κ B was not entirely responsible for KC release. This conclusion is derived from the fact that inhibition of JNK blocked KC release, but not NF- κ B activity, and that blocking NF- κ B with the I κ B inhibitor did not reduce the induction of KC by Jo2. However, Jo2 did

Figure 7. Myd88 is required for Fas-induced injury *in vivo*. C57BL/6 mice and *Myd88*^{-/-} mice received three consecutive daily intratracheal instillations of mAb Jo2 or a control IgG, 2.5 μg/g, and were studied 7 days after the first installation. As compared with IgG-treated mice, wild-type (WT) mice treated with Jo2 mAb, but not *Myd88*^{-/-} mice, had increased total lung myeloperoxidase (MPO) (a marker of total lung neutrophil content); increased bronchoalveolar lavage (BAL) KC, TNF-α, and IgM (a marker of increased permeability), and increased lung caspase-3 activity (*n* = 6/group).

induce phosphorylation of c-jun, one of the major components of AP-1. Thus, the data suggest that the pathway linking Fas activation to KC production involves MyD88, JNK phosphorylation, and, likely, AP-1 activation.

Ultimately, we wanted to determine the biological relevance of the findings. To this end, we instilled Jo2 or control IgG into the lungs of wild-type mice with a targeted deletion of MyD88 and found that MyD88 is required for the inflammatory response to Fas activation, as measured by myeloperoxidase (MPO) activity. Notably, the *MyD88*^{-/-} mice showed only mild protection from permeability changes (measured by IgM concentrations in the BAL) and caspase-3 activity, suggesting that the MyD88 pathway is primarily associated with the proinflammatory function of Fas, but the proapoptotic function proceeds via the well described caspase pathways.

This study had a number of limitations. One important caveat is that the choice of a cell line resistant to Fas-mediated apoptosis may or may not be an appropriate model of the *in vivo* biology of the alveolar epithelium. However, the data were reproduced in the human epithelial cells, which do become apoptotic in response to Fas ligation. A second limitation is that silencing of proteins with siRNA is seldom complete, and it is possible that the residual protein is sufficient to induce signaling. However, our *in vitro* data suggesting that MyD88 is involved in the proinflammatory signaling of Fas was confirmed *in vivo* with the *MyD88*^{-/-} mice. Another limitation is that IκB may not completely inhibit NF-κB activity, and therefore we cannot completely exclude its role in KC release. Finally, we cannot effectively conclude that KC release was mediated by AP1 signaling in the absence of loss-of-function experiments. AP1 is notoriously difficult to inhibit because of the complexity of the transcription complex. However, the mouse data confirm the biological relevance of MyD88 for Fas-induced injury.

This study is important, because a growing body of evidence suggests that the inflammatory response of the lungs requires the Fas/FasL system, and that the main target of FasL is the lung epithelium. The data from this study suggest that the relevant pathway linking Fas with inflammation in the lungs involves MyD88, which is normally associated with signaling from pattern recognition receptors.

We conclude that Fas activation induces KC release in the alveolar epithelial cell line, MLE-12, by a mechanism dependent on MyD88 and requiring MAPK activation. The pathway is likely independent of NF-κB, and appears to require AP-1.

Author Disclosure: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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