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Different Signaling Pathways Stimulate a Disintegrin and Metalloprotease-17 (ADAM17) in Neutrophils during Apoptosis and Activation*[S]

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Background: Neutrophil ADAM17 (a disintegrin and metalloprotease-17) mediates the proteolytic release of receptors and soluble factors that regulate inflammation.
Results: ADAM17 stimulation during Fas-induced neutrophil apoptosis requires caspase-8, Bid, and mitochondrial reactive oxygen species.
Conclusion: Neutrophil ADAM17 stimulation occurs by different signal transduction pathways during activation and apoptosis.
Significance: ADAM17 activation in apoptotic neutrophils may rapidly down-regulate their pro-inflammatory activity.

ADAM17 is a membrane-associated metalloprotease that cleaves proteins from the surface of neutrophils and modulates the density of various receptors and adhesion molecules. The protease activity of ADAM17 is highly inducible and occurs upon neutrophil activation as well as apoptosis. At this time, little is known about the signal transduction pathway that promotes ADAM17 activity in neutrophils upon the induction of apoptosis. We show that caspase-8 activation, Bid cleavage, and the release of mitochondrial reactive oxygen species are sequential transduction components of the Fas signaling cascade that induces ADAM17. This is different from ADAM17 stimulation upon overt neutrophil activation, which requires MAPK p38 or ERK, but not caspases and reactive oxygen species. ADAM17 activity in apoptotic neutrophils may serve to inactivate select effector molecules that promote the pro-inflammatory activity of recruited neutrophils. For instance, TNFα receptors TNF-RI and TNF-RII are substrates of ADAM17, and we show that they are shed during apoptosis, decreasing neutrophil sensitivity to TNFα. Altogether, our findings provide significant new insights into the signal transduction pathway that stimulates ADAM17 during induced neutrophil apoptosis. ADAM17 induction during apoptosis may rapidly diminish neutrophil sensitivity to the inflammatory environment, complementing other anti-inflammatory activities by these cells during inflammation resolution.

Ectodomain shedding is a proteolytic event in which plasma membrane proteins are cleaved at an ectodomain location. This process controls the density of cell surface receptors and adhesion molecules and causes the formation of various soluble factors. The majority of shed proteins are cleaved by members of the *a d*isintegrin *a*nd *m*etalloprotease (ADAM)[3] family (1). ADAM17, originally referred to as TNFα converting enzyme or TACE (2, 3), plays a major role in ectodomain shedding and is expressed by most cells, including leukocytes (4). ADAM17 cleaves a growing list of factors expressed by leukocytes that play an important role in modulating inflammation (1, 4). For instance, ADAM17 in neutrophils cleaves the adhesion molecule L-selectin, TNFα, TNF-RI, and TNF-RII (5–7).

The levels of neutrophils at sites of inflammation are a balance between their recruitment and removal. The latter process, when occurring by programmed cell death, promotes the resolution of inflammation (8). Neutrophil apoptosis is characterized by caspase activation, phosphatidylserine externalization on the cell surface, and cell shrinkage. Apoptotic neutrophils are eventually engulfed and disposed of by macrophages (9–11). The catalytic activity of ADAM17 is also induced during neutrophil apoptosis (12–14). This is significant considering the pleiotropic activity of ADAM17 in regulating inflammation and that an immense number of senescent neutrophils in the blood and recruited neutrophils at sites of inflammation undergo apoptosis (8, 15). At this time, however, very little is known about the mechanisms that underpin ADAM17 stimulation in apoptotic neutrophils.

Our study provides significant new insights into the signal transduction process that induces ADAM17 activity during neutrophil apoptosis. ADAM17 stimulation in apoptotic neutrophils at sites of inflammation may be an early event in the inactivation of these cells by cleaving key receptors and adhe-

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[3] The abbreviations used are: ADAM, a disintegrin and metalloprotease; ROS, reactive oxygen species; fMLP, formyl-methionine- leucine-phenylalanine; NAC, *N*-acetyl-L-cysteine; rhFasL, recombinant human soluble Fas ligand; Z, benzyloxycarbonyl; fmk, fluoromethyl ketone; cmk, chloromethyl ketone.

sion molecules to suppress their reactivity to the inflammatory environment.

EXPERIMENTAL PROCEDURES

Antibodies and Other Reagents—PE-conjugated anti-L-selectin (LAM1–116) mAb was purchased from Ancell (Bayport, MN). Anti-LFA-1 (CD11a/CD18) mAb R3.1 was previously described (16). The anti-Fas IgM mAb CH-11 was purchased from Upstate (Charlottesville, VA). Fluorochrome-conjugated goat anti-mouse IgG and IgM were purchased from Jackson ImmunoResearch (West Grove, PA). Rabbit anti-caspase-3 antibody, rabbit anti-caspase-8 antibody, and rabbit anti-human ADAM17 prodomain polyclonal antibody were purchased from Cell Signaling (Beverly, MA). Goat anti-human ADAM17 polyclonal antibody, normal goat IgG, and normal rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). IRDyes 800CW- and 680-conjugated goat anti-mouse IgG, goat anti-rabbit IgG, and donkey anti-goat IgG were purchased from LI-COR (Lincoln, NE). Rotenone, rabbit anti-GAPDH antibody, *N*-formyl-methionine-leucine-phenylalanine (fMLP), phorbol 12-myristate 13-acetate, and DMSO were purchased from Sigma (St. Louis, MO). Annexin V-FITC was purchased from BD Bioscience (San Diego, CA). RPMI 1640, PBS, Hepes, and molecular grade water were purchased from Mediatech (Herndon, VA). Penicillin-streptomycin solution was purchased from Cellgro (Manassas, VA). Geneticin was purchased from Invitrogen (Carlsbad, CA). TAPI-1, a hydroxamate metalloprotease inhibitor, was purchased from Peptides International (Louisville, KY). The ADAM17 inhibitor BMS566394 (referred to as inhibitor 32 in Ref. 17) was obtained from Bristol-Myers Squibb. The ADAM10 inhibitor GI1254023X (18, 19) was kindly provided by Dr. Andreas Ludwig (Rhein-Westphalian Technical University, Aachen, Germany). Pan-caspase inhibitor I (Z-VAD-fmk), caspase-3 inhibitor IV (Ac-DMQD-cho), caspase-8 inhibitor II (Z-IETD-fmk), caspase-9 inhibitor III (Ac-LEHD-cmk), *N*-acetyl-L-cysteine (NAC), ERK pathway inhibitor U0126, and the p38 inhibitor SB203580 were purchased from Calbiochem. Recombinant human soluble Fas ligand (rhFasL) was purchased from Enzo (Plymouth Meeting, PA). Recombinant human TNFα was purchased from PeproTech Inc. (Rocky Hill, NJ).

Cell Isolation and Culture—All media, buffers, and reagents used for cell isolation and incubation were sterile and tested for endotoxin. Human peripheral blood was collected from healthy donors in sodium heparin. These procedures were performed in accordance with protocols approved by the Institutional Review Board: Human Subjects at the University of Minnesota. Neutrophils were enriched as described previously (12, 20, 21), and cell viabilities were assessed by exclusion of the vital dye Trypan Blue. All Jurkat lines used in this study were cultured in RPMI 1640 complete media supplemented with 8% heat-inactivated FBS and antibiotics (penicillin and streptomycin) at 37 °C in a humidified 5% CO_2 incubator. Bid-silenced (pSUPER-BID), Apaf-1-silenced (pSUPER-Apaf1), and vector control (pSUPER-neo) transfected Jurkat cells were generated as previously described (22, 23), and 1 mg/ml Geneticin was used for selection. Cells were maintained in an exponential growth phase for all experiments.

Cell Apoptosis and Activation—For cell treatments, cells were suspended in RPMI 1640 at the indicated concentrations and initially equilibrated to culture conditions at 37 °C in 5% CO_2. Cells were incubated with or without TAPI (50 μM), BMS566394 (3 μM), or GI1254023X (0.3 μM) for 30 min and then treated with fMLP (100 nM), phorbol 12-myristate 13-acetate (10 ng/ml), anti-Fas mAb CH-11 (500 ng/ml), rhFasL (100 ng/ml), or rotenone (5 μM) for the indicated time points. For some experiments, cells were initially incubated with a broad spectrum caspase inhibitor (Z-VAD-fmk, 5 μM), casapse-3 inhibitor (Ac-DMQD-cho, 100 μM), caspase-8 inhibitor (Z-IETD-fmk, 10 μM), caspase-9 inhibitor (Ac-LEHD-cmk, 20 μM), p38 MAPK inhibitor (SB203580, 10 μM), ERK MAPK pathway inhibitor (MEK1/2 inhibitor-U0126, 10 μM), NAC (2 mM), or DPI (10 μM) for 30 min prior to apoptosis induction or activation. ROS production was determined by a fluorometric assay using an Amplex Red kit (Molecular Probes, Eugene, OR), as previously described (24).

TNFα stimulation of apoptotic neutrophils involved the incubation of freshly isolated neutrophils with or without TAPI (50 nM) for 30 min, followed by neutrophil incubation with anti-Fas Ab CH-11 for 6 h, and then their treatment with TNFα (20 units/ml) for 3 h. IL-8 levels in cell supernatants were quantified by ELISA, as described below. For all experiments, cells were treated with the appropriate concentrations of DMSO or ethanol (carriers) during sham treatments.

Flow Cytometry—Cells were labeled with various mAbs, as described previously (12, 21). Cells were typically washed in cold PBS without Ca^{2+} and Mg^{2+} plus 5 mM sodium azide, mildly fixed with 1% paraformaldehyde in PBS to preserve antigen expression, and then analyzed on a FACSCanto instrument (BD Biosciences). Isotype-matched negative control mAbs were used to evaluate levels of nonspecific staining. For cell staining that involved Annexin V, non-fixed cells were used. Following their staining with antibodies, the cells were treated with Annexin V-FITC, as per the manufacturer's instructions, and immediately examined by flow cytometry. Human soluble L-selectin, TNF-RI, TNF-RII, and IL-8 levels in tissue culture media supernatants were evaluated by quantitative ELISA using Cytometric Bead Array kits (BD Biosciences) and flow cytometry, according to the manufacturer's instructions.

SDS-PAGE and Immunoblotting—Cells were detergent-lysed as previously described with slight modifications (24). Typically, 1×10^8 cells were washed with ice-cold PBS and lysed with 1 ml of Pierce IP lysis buffer (Thermo Scientific, Rockford, IL), as per the manufacturer's instructions. The detergent-extracted proteins were resolved by SDS-PAGE under reducing conditions and transferred to a PVDF membrane. Membranes were treated with Odyssey blocking buffer (LI-COR), as per manufacturer's instructions, for 60 min, and then incubated with primary antibody (diluted in Odyssey blocking buffer) for 60 min. After four times washing in PBS + 0.1% Tween-20 with gentle shaking, membranes were then incubated with the appropriate IRDye-conjugated secondary antibody (diluted in Odyssey blocking buffer) for 60 min. Membranes were washed as described above, and then scanned and analyzed using an Odyssey instrument (LI-COR), according to the manufacturer's instructions.

Statistical Analyses—Values are expressed as mean ± S.D. Data were analyzed using Student's *t* test and analyzed using Excel (Microsoft, Redmond, WA), and significance was concluded when p was <0.05.

RESULTS

Caspase Involvement in ADAM17 Stimulation during Fas-mediated Apoptosis—For the following experiments we assessed the proteolytic activity of ADAM17 in neutrophils by measuring the shedding of L-selectin. We have provided direct evidence that ADAM17 is the principle sheddase of L-selectin in neutrophils upon their activation and apoptosis (5, 14, 21, 24, 25). As further confirmation of these studies, the metalloprotease inhibitor TAPI and the selective ADAM17 inhibitor BMS566394 blocked L-selectin shedding by apoptotic human neutrophils at equivalent levels. ADAM10 is the most similar to ADAM17 in terms of structure and substrate overlap (2, 3, 26, 27); however, its selective inhibition with GI1254023X had no effect on L-selectin shedding (supplemental Fig. S1).

Fas is a quintessential death receptor in neutrophils (28, 29), and it is known to stimulate ectodomain shedding (12–14). Because Fas-mediated signaling events do not always involve caspases (30, 31), we initially examined their requirement in ADAM17 stimulation. When neutrophils were stimulated by Fas engagement in the presence of a broad spectrum caspase inhibitor (z-VAD-fmk), we observed significant attenuation of L-selectin shedding that was equivalent to treating apoptotic neutrophils with TAPI (Fig. 1*A*). In contrast to Fas-mediated signaling, L-selectin shedding upon overt neutrophil activation with the bacterial peptide fMLP (Fig. 1*A*) and other activators (data not shown) was not blocked by a broad spectrum caspase inhibitor.

Stimulation of the Fas death-inducing signaling complex initially results in caspase-8 activation (31). We found that the caspase-8 inhibitor z-IETD-fmk also blocked L-selectin shedding by neutrophils following Fas engagement (Fig. 1*A*). However, inclusion of an inhibitor of the downstream executioner caspase-3 (Ac-DMQD-cho) did not significantly block the shedding of L-selectin (Fig. 1*A*). We confirmed the activity of the caspase inhibitors by verifying that they blocked Fas-mediated neutrophil apoptosis, which was determined, in part, by examining their effect on the activation of caspase-3 (Fig. 1*B*).

Caspase-8 signaling in neutrophils to activate caspase-3 is amplified through the mitochondria (32–34). A similar mitochondrial amplification pathway takes place in the Jurkat hematopoietic cell line (23, 35). We have determined that ADAM17 cleaves L-selectin in these cells as well upon Fas engagement with the antibody CH-11 or by rhFasL (14). As a side note, unlike the highly sensitive Jurkat cells, rhFasL from several commercial sources had negligible effects on accelerating apoptosis or L-selectin shedding by neutrophils (data not shown), which may be due to rhFasL preparations not having the appropriate primary structure. Treatment of Jurkat cells with either a broad spectrum caspase inhibitor or a caspase-8 inhibitor, but not a caspase-3 inhibitor, blocked L-selectin shedding upon Fas stimulation (Fig. 2*A*). The caspase inhibitors blocked caspase-3 activation (data not shown) and phosphatidylserine exposure on the surface of Jurkat cells following the

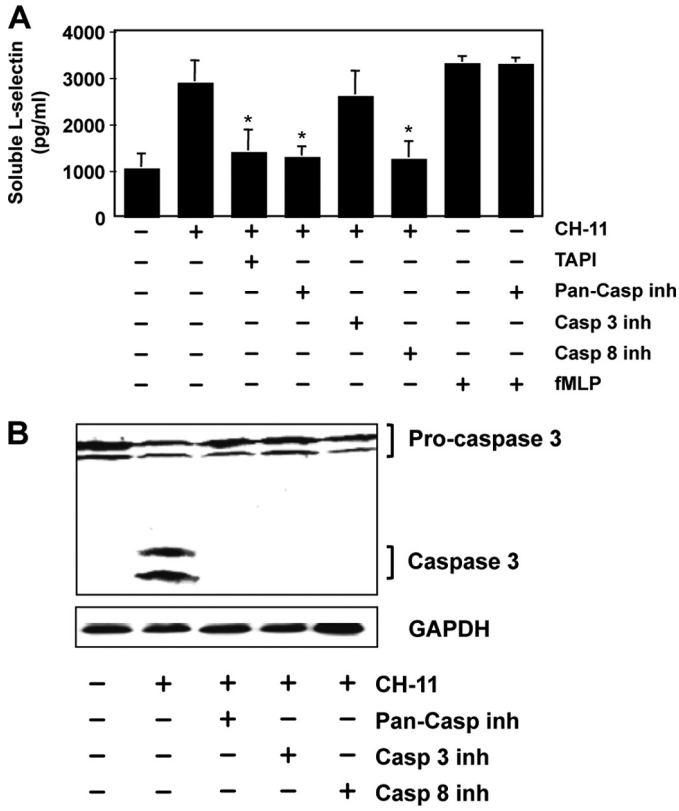

FIGURE 1. **Fas-induced ADAM17 stimulation requires caspase-8, but not caspase-3.** *A*, human peripheral blood neutrophils were either untreated or treated with the anti-Fas antibody CH-11 or fMLP for 6 h or 30 min, respectively, at 37 °C in the presence or absence of TAPI. Some neutrophils were initially incubated with a broad spectrum caspase inhibitor (z-VAD-fmk), a caspase-8 inhibitor (z-IETD-fmk), or a caspase-3 inhibitor (Ac-DMQD-cho) for 30 min at 25 °C, as indicated. Cell supernatant levels of soluble L-selectin were quantified by ELISA. Shown is the mean (± S.D.) of at least three independent experiments. *, $p < 0.05$ *versus* CH-11 treatment alone. *B*, neutrophils were treated as described above, and detergent lysates from equivalent cell numbers were subjected to reducing SDS-PAGE and immunoblotting with an anti-caspase-3 mAb. The antibody detects full-length caspase-3 (Pro-caspase 3) and the large fragment of activated caspase-3 resulting from cleavage (17/19 kDa). Data are representative of at least three independent experiments using neutrophils isolated from separate donors.

induction of apoptosis (Fig. 2*B*). To further establish the role of caspase-8 in the signaling pathway that stimulates ADAM17 during apoptosis, we used caspase-8-deficient Jurkat cells (36). These cells also demonstrated diminished L-selectin shedding upon Fas engagement when compared with control Jurkat cells (supplemental Fig. S2). Taken together, the above findings indicate that caspase-8, but not caspase-3, is required for ADAM17 stimulation in apoptotic neutrophils. In contrast to this, caspases are not required for ADAM17 stimulation upon neutrophil activation.

ADAM17 Induction during Apoptosis Requires Bid—The mitochondrial amplification pathway in neutrophils and Jurkat cells is initiated by caspase-8 cleaving the Bcl-2 homology 3 protein Bid to form the proteolytic fragment tBid (33, 37, 38). We have generated Jurkat cells expressing Bid shRNA that are resistant to Fas-induced apoptosis (23). In Fig. 3*A*, we show that these cells shed appreciably less L-selectin following Fas engagement when compared with control cells, signifying a role for Bid in the transduction pathway that activates ADAM17. To verify that differential L-selectin shedding by the Bid-silenced

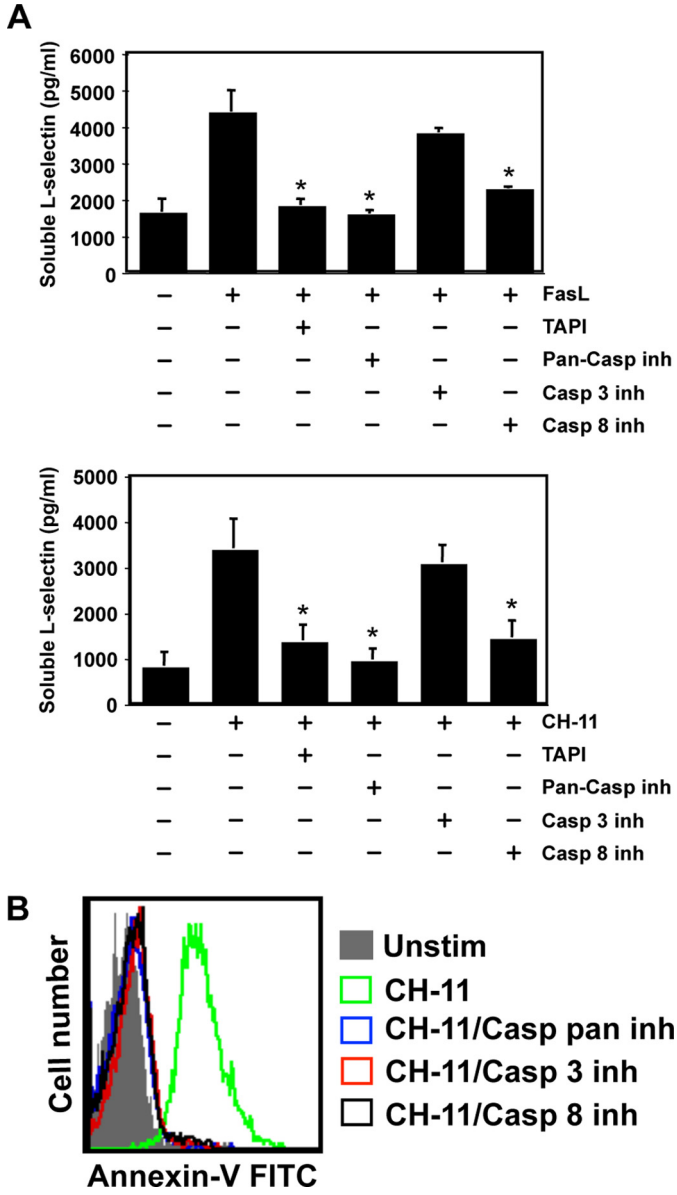

FIGURE 2. **Jurkat cells model neutrophils for ADAM17 stimulation upon Fas signaling.** *A*, Jurkat cells were either untreated or treated with the anti-Fas antibody CH-11 or rhFasL, as indicated, in the presence or absence of TAPI for 1.5 h at 37 °C. Some cells were initially incubated with the caspase inhibitors described in Fig. 1. Cell supernatant levels of soluble L-selectin were quantified by ELISA. Results are expressed as mean (± S.D.) of at least three independent experiments. *, $p < 0.05$ *versus* CH-11 or FasL treatment alone. *B*, in addition, the cell pellets were stained for Annexin V-FITC to detect cell surface phosphatidylserine exposure. The *x* axis = log 10 fluorescence. Results are representative of three independent experiments.

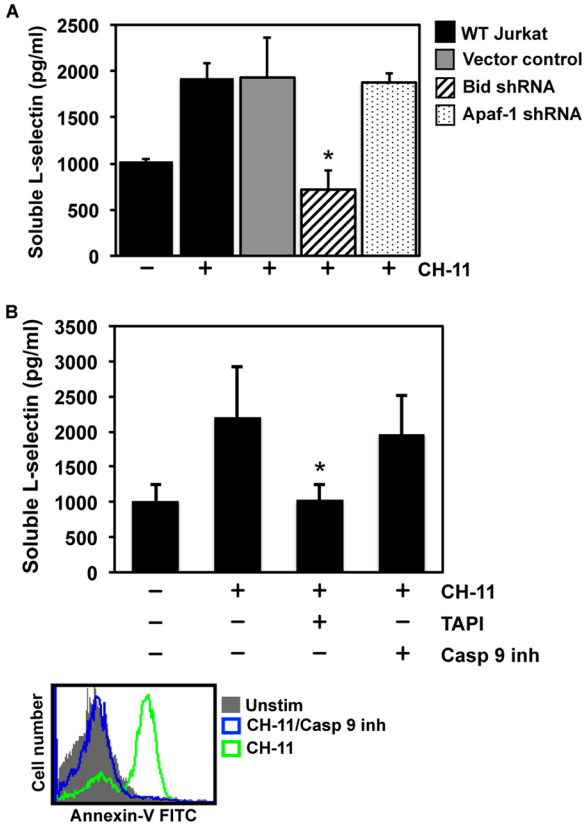

FIGURE 3. **Bid knock-down abrogates ADAM17 stimulation upon Fas engagement.** *A*, wild-type (*WT*), vector control, Bid-deficient (*Bid shRNA*), or Apaf-1-deficient (*Apaf-1 shRNA*) Jurkat cells were either untreated or treated with the anti-Fas antibody CH-11 for 1.5 h at 37 °C. Cell supernatant levels of soluble L-selectin were quantified by ELISA. Results are expressed as mean (± S.D.) of at least three independent experiments. *, $p < 0.001$ *versus* wild-type Jurkat cells treated with CH-11. *B*, Jurkat cells were either untreated or treated with the anti-Fas antibody CH-11 for 1.5 h at 37 °C in the presence or absence of TAPI. Some cells were initially incubated with a caspase-9 inhibitor (Ac-LEHD-cmk) for 30 min at 25 °C, as indicated. Cell supernatant levels of soluble L-selectin were quantified by ELISA. Shown is the mean (± S.D.) of at least three independent experiments. *, $p < 0.05$ *versus* CH-11 treatment alone. The cells were labeled with Annexin V-FITC and examined by flow cytometry. The *x* axis = log 10 fluorescence. Data are representative of at least three independent experiments.

and control Jurkat cells was not the result of differences in Fas expression, we assessed the cell lines for their surface expression levels of Fas by flow cytometry. We found that all of the Jurkat lines examined in this study expressed similar levels of Fas (supplemental Fig. S3). Moreover, all of the Jurkat lines efficiently shed their L-selectin upon activation (supplemental Fig. S3), indicating that there was not a pronounced variation in ADAM17 activity among the Jurkat lines.

Mitochondrial ROS, but Not Apoptosome Induction, Promotes ADAM17 Activity—The cleavage fragment tBid translocates to mitochondria and participates in the permeabilization of the organelle's outer membrane, promoting the release of the apoptogenic protein cytochrome *c* as well as mitochondrial ROS (33, 34). Our data indicate that Fas-mediated L-selectin shedding occurs independent of caspase-3 activation (Figs. 1 and 2), suggesting that apoptosome induction is not a component of the signaling pathway that stimulates ADAM17. Caspase-9 activation occurs in a cytochrome *c*-dependent manner involving the formation of an Apaf-1 apoptosome complex. Using Apaf-1-silenced Jurkat cells that we have described previously (22), in which apoptosome-mediated caspase-9 activation is impaired (39), we did not observe a diminishment in L-selectin shedding upon Fas engagement (Fig. 3*A*). Moreover, treating Jurkat cells and neutrophils with the caspase-9 inhibitor Ac-LEHD-cmk also did not block L-selectin shedding (data not shown and Fig. 3*B*).

We as well as others have reported that ADAM17 can be regulated by reduction-oxidation activities (14, 40, 41). Indeed, purified ADAM17 in cell-free assays is directly activated by H_2O_2 (24). Therefore, we next examined the effects of mito-

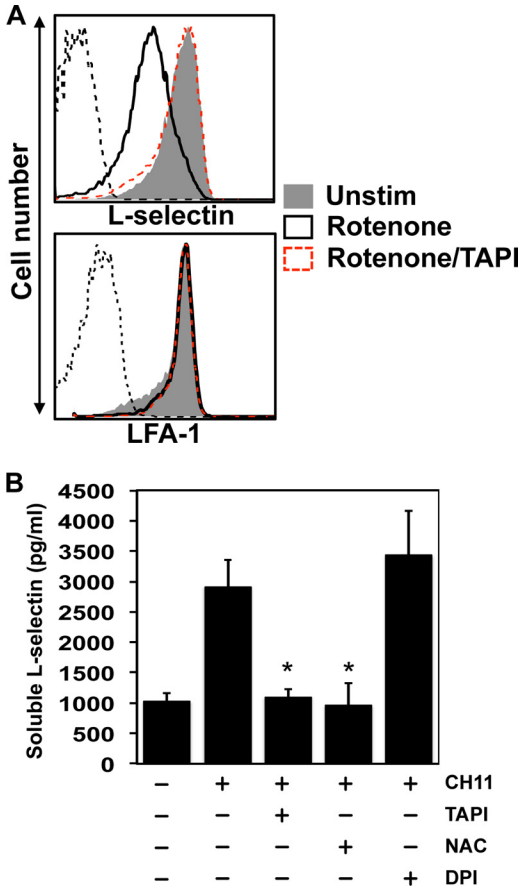

FIGURE 4. **Mitochondrial ROS in neutrophils is important for Fas-induced ADAM17 stimulation.** *A*, human peripheral blood neutrophils were either untreated or treated with rotenone for 1 h at 37 °C in the presence or absence of TAPI. Relative L-selectin or LFA-1 surface expression levels, as indicated, were determined by flow cytometry. Negative control Ab staining of untreated cells is indicated (*dashed line*). The *x* axis = log 10 fluorescence. Data are representative of at least three independent experiments using neutrophils isolated from separate donors. *B*, neutrophils were either untreated or treated with the anti-Fas antibody CH-11 for 6 h at 37 °C in the presence or absence of TAPI. Some neutrophils were initially incubated with the intracellular ROS scavenger NAC or the NADPH oxidase inhibitor DPI for 30 min at 25 °C, as indicated. Cell supernatant levels of soluble L-selectin were quantified by ELISA. Results are expressed as mean (± S.D.) of at least three independent experiments. *, $p < 0.01$ *versus* CH-11 treatment alone.

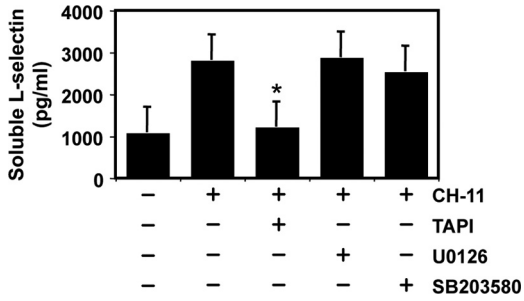

FIGURE 5. **Fas-induced L-selectin shedding occurs independent of the p38 MAPK and ERK.** Human peripheral blood neutrophils were either untreated or treated with the anti-Fas antibody CH-11 for 6 h at 37 °C in the presence or absence of TAPI. Some cells were initially incubated with a p38 inhibitor (SB203580) or an ERK pathway inhibitor (U0126) for 30 min at 25 °C, as indicated. Cell supernatant levels of soluble L-selectin were quantified by ELISA. Results are expressed as mean (± S.D.) of at least three independent experiments. *, $p < 0.05$ *versus* CH-11 treatment alone.

chondrial ROS on ADAM17 activity in apoptotic neutrophils. Inhibition of mitochondrial respiratory complex I in neutrophils by rotenone results in increased mitochondrial ROS production (42). We found that the treatment of neutrophils with rotenone alone caused a down-regulation in L-selectin surface expression, whereas the expression levels of surface proteins that do not undergo shedding (*e.g.* LFA-1) did not change (Fig. 4*A*). The treatment of Fas-stimulated neutrophils with the intracellular ROS scavenger NAC greatly diminished L-selectin shedding (Fig. 4*B*), which was observed in Jurkat cells as well (data not shown). Under certain circumstances, ROS production can alter caspase function; however, in our assays, NAC treatment did not affect caspase-8 activation following Fas engagement (data not shown). DPI is an inhibitor of NADPH oxidase-like flavoproteins, and, unlike NAC, DPI had no effect on L-selectin shedding by apoptotic neutrophils (Fig. 4*B*), which provides additional evidence for a role by ROS derived from the mitochondria and not NADPH oxidase in stimulating ADAM17 during neutrophil apoptosis. Both NAC and DPI completely blocked ROS production by activated neutrophils (supplemental Fig. S4), demonstrating their activity. We have previously reported that neutrophils either treated with DPI or obtained from patients with chronic granulomatous disease undergo efficient L-selectin shedding upon their activation (24), which was also the case for neutrophils treated with NAC (data not shown). These findings indicate that ROS derived from the mitochondria or by NADPH oxidase is not critical for the induction of L-selectin shedding upon overt neutrophil activation.

ADAM17 Stimulation during Apoptosis Is MAPK-independent—The p38 MAPK and ERK are important signaling components of ADAM17 stimulation upon the activation of various cell types, including neutrophils (43–48). Considering that kinases can be activated by caspases as well as ROS (49, 50), we next examined whether p38 and/or ERK regulates ADAM17 during neutrophil apoptosis. As expected, targeting ERK and p38 activity with the inhibitors U0126 and SB203580, respectively, blocked L-selectin shedding by neutrophils activated with fMLP or TNFα (supplemental Fig. S5). These inhibitors, however, had no effect on L-selectin shedding by Fas-stimulated neutrophils (Fig. 5). Moreover, the combined inhibition of ERK and p38 also did not block L-selectin shedding (data not shown). Similar to neutrophils, p38 and ERK were also not required for ADAM17 stimulation in Jurkat cells upon Fas engagement (supplemental Fig. S6). These data indicate that, unlike neutrophil activation, ADAM17 stimulation upon neutrophil apoptosis is not regulated by p38 MAPK and ERK.

Fas Signaling Does Not Increase Pro-protein to Mature ADAM17 Conversion—ADAM17 is synthesized as an inactive zymogen that is converted intracellularly in the late Golgi compartment to its active form upon removal of a pro-domain region by convertases (51–53). It has been proposed that ADAM17 induction during apoptosis involves the conversion of pro-ADAM17 to its mature form (13). We performed immunoblotting to quantify the levels of pro-ADAM17 and its mature form prior to and after Fas engagement. We were unable to detect the pro-form of ADAM17 in neutrophils after many attempts, and thus we did not observe a conversion of pro-ADAM17 to its mature form following Fas stimulation

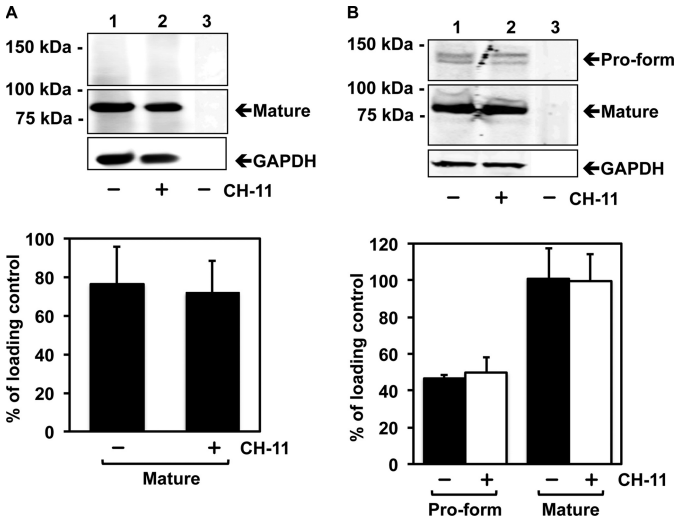

FIGURE 6. **Pro-protein to mature ADAM17 conversion is not increased during Fas-mediated apoptosis.** Human peripheral blood neutrophils (*A*) or Jurkat cells (*B*) were either untreated or treated with the anti-Fas antibody CH-11 for 6 or 1.5 h, respectively, at 37 °C, as indicated. Detergent lysates from equivalent numbers of untreated and treated cells were then subjected to reducing SDS-PAGE and immunoblotting with antibodies to mature ADAM17, the pro-domain of ADAM17, or GAPDH (loading control), as shown in *lanes 1* and *2*. Detergent lysates of untreated cells were also immunoblotted with appropriate negative control antibodies (*lane 3*). Immunoblots are representative of three independent experiments. Densitometric data are from three separate experiments (expressed as mean ± S.D.) and shown as percentage of loading control.

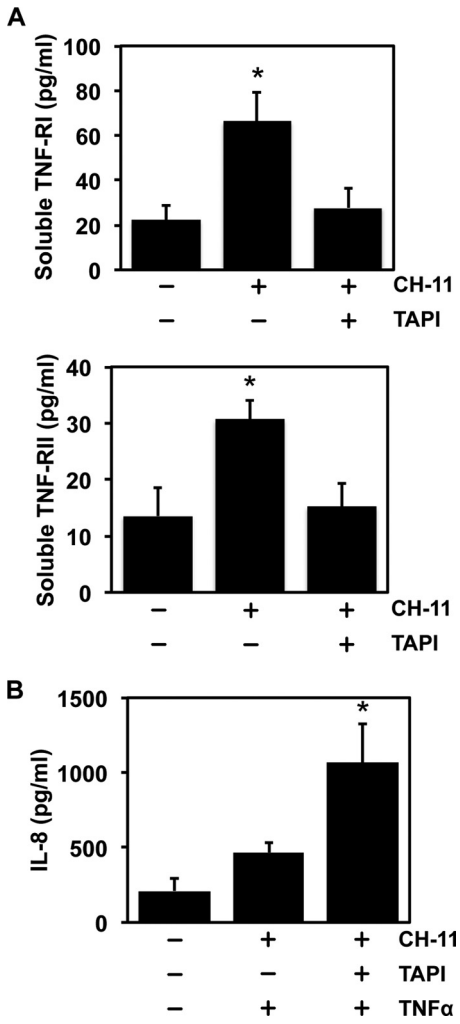

FIGURE 7. **ADAM17 activity decreases the responsiveness of apoptotic neutrophils to TNFα.** *A*, human peripheral blood neutrophils were either untreated or treated with the anti-Fas antibody CH-11 in the presence or absence of TAPI for 6 h at 37 °C. Cell supernatant levels of soluble TNF-RI and TNF-RII, as indicated, were quantified by ELISA. Shown is the mean (± S.D.) of at least three independent experiments. *, $p < 0.05$ *versus* untreated. *B*, neutrophils were either untreated or treated with the anti-Fas antibody CH-11 in the presence or absence of TAPI for 6 h, followed by TNFα stimulation for 3 additional hours at 37 °C. Cell supernatant levels of IL-8 were quantified by ELISA. Results are expressed as mean (± S.D.) of at least three independent experiments. *, $p < 0.01$ *versus* TNFα stimulation without TAPI.

(Fig. 6*A*). We did detect both the pro-form and mature ADAM17 in Jurkat cells, but we did not observe a change in their proportions in the timeframe of induced L-selectin shedding (Fig. 6*B*). Accordingly, these findings suggest that, similar to neutrophil activation (24), ADAM17 stimulation upon neutrophil apoptosis is not dependent on the processing of its pro-domain. However, we cannot formally rule out the possibility that the processed form of the ADAM17 pro-domain may non-covalently associate with mature ADAM17 and regulate its activity during neutrophil apoptosis.

ADAM17 Activity during Apoptosis Decreases Neutrophil Sensitivity to TNFα—Ectodomain shedding regulates the density of various adhesion molecules and receptors on the surface of neutrophils, and thus ADAM17 likely modulates the activity of these cells in various ways. A potential function of ADAM17 in apoptotic neutrophils at sites of inflammation may be to cleave cell surface receptors involved in activating these cells, thus rapidly reducing their sensitivity to surrounding stimuli. TNFα is a key neutrophil stimulant, and its two receptors, TNF-RI and TNF-RII, undergo ectodomain shedding by ADAM17 (6, 54, 55). We examined whether blocking ectodomain shedding by apoptotic neutrophils alters their response to TNFα. As with L-selectin, Fas stimulation increased the shedding of TNF-RI and TNF-RII by neutrophils, and this was greatly diminished by TAPI (Fig. 7*A*). In line with the latter finding is that blocking ectodomain shedding by apoptotic neutrophils increased their sensitivity TNFα, as evidenced by their enhanced production of the pro-inflammatory factor IL-8 (Fig. 7*B*). We and others have reported that metalloprotease inhibitors do not block neutrophil apoptosis (12, 13), and thus neutrophils treated with TAPI did not produce more IL-8 due to diminished levels of apoptosis. Taken together, these findings support the premise that ADAM17 activity during apoptosis decreases neutrophil sensitivity to the inflammatory environment.

DISCUSSION

Tantamount to the role of neutrophils in promoting inflammation is their role in resolving inflammation during programmed cell death (8). ADAM17 activity is induced by cell activation (2, 3, 48, 56, 57), and studies by us and others have revealed that ADAM17 can also be stimulated upon neutrophils apoptosis (12–14). At this time, very little is known about the molecular events that activate ADAM17 during neutrophil apoptosis. ADAM17 stimulation during this very widespread process may result in substantial turnover of many of its substrates. In the current study, we have elucidated a signal transduction pathway involved in ADAM17 stimulation during Fas-

induced neutrophil apoptosis that involves caspase-8, Bid, and mitochondrial ROS production.

A recurring theme of ADAM17 stimulation upon cell activation is the involvement of MAPK ERK and p38 (43–48). Consistent with this, we show that inhibitors of ERK and p38 efficiently blocked L-selectin shedding upon neutrophil activation. These inhibitors, however, did not reduce L-selectin shedding upon Fas-mediated apoptosis, indicating the involvement of different signaling components in ADAM17 stimulation upon neutrophil apoptosis. In contrast to the involvement of ERK and p38, little evidence exists that establishes a connection between MAPK JNK and ADAM17 activation. We found that JNK inhibitor VII (a selective cell-permeable peptide inhibitor based on JNK-Interacting Protein 1) had no effect on Fas-induced L-selectin shedding by neutrophils (data not shown). This is consistent with findings by others that ERK, p38, and JNK in neutrophils either are not activated or are negatively regulated upon Fas engagement (29, 58).

A broad spectrum caspase inhibitor blocked Fas-mediated induction of ADAM17 in neutrophils. This inhibitor, however, did not block ADAM17 stimulation upon neutrophil activation, further revealing the involvement of distinct signaling components in ADAM17 stimulation during neutrophil activation and apoptosis. Additional examination revealed that caspase-8, but not caspase-9 and -3, is essential for inducing ADAM17 activity during apoptosis. As with neutrophils (32, 34, 59), Fas induces a mitochondrial apoptotic pathway in Jurkat cells (23, 35), and these cells also demonstrated impaired shedding of L-selectin upon their treatment with a caspase-8 inhibitor. Jurkat cells offer the advantage of genetic manipulation, and we found that cells deficient in caspase-8 also were impaired in their stimulation of ADAM17 upon Fas engagement. A proximal downstream target of caspase-8 in the mitochondrial apoptotic pathway is Bid, a BH3-only protein member of the Bcl-2 family (37, 38). Bid undergoes activation upon cleavage by caspase-8 to form tBid, and Bid-deficient Jurkat cells also demonstrated impaired L-selectin shedding upon Fas engagement. A well characterized role of tBid is its translocation to the outer membrane of the mitochondria and induction of membrane permeabilization (34, 59). This event results in the release of the apoptogenic factors, including cytochrome *c*. Once released, cytochrome *c* binds to the cytosolic protein Apaf-1 to facilitate the formation of the apoptosome and activation of caspase-9, which then activates caspase-3 (34). We show that targeting Apaf-1 expression does not block ADAM17 stimulation. Consistent with this is that the inhibition of caspase-9 and caspase-3 also did not block ADAM17 stimulation. Our findings thus point to the involvement of other mitochondrial components stimulating ADAM17 during apoptosis.

Neutrophils have been reported to produce large amounts of ROS within their mitochondrial network, which can act as a second messenger when released into the cytoplasm (33). Mitochondrial membrane depolarization is an early event in neutrophil apoptosis, and outer membrane permeability changes induced by tBid increase the release of ROS (33). We found that the sequestration of ROS by NAC in neutrophils upon Fas engagement abrogated L-selectin shedding, implicating a role for mitochondrial ROS in the activation of ADAM17. Moreover, treatment of neutrophils with the mitochondrial respiratory complex I inhibitor rotenone, which is known to increase mitochondrial ROS production in neutrophils (42), induced L-selectin shedding. Inactivating NADPH oxidase, however, did not block ADAM17 activity in apoptotic neutrophils. Neither ROS sequestration nor NADPH oxidase inactivation blocked L-selectin shedding by fMLP-activated neutrophils (data not shown and Ref. 24). These findings thus indicate that ROS generated in the mitochondrial and not by NADPH oxidase is important for ADAM17 stimulation during neutrophil apoptosis, whereas MAPK-dependent and ROS-independent intracellular signaling appears to induce ADAM17 upon overt neutrophil activation.

The target of mitochondrial ROS involved in the induction of ADAM17 in apoptotic neutrophils remains unclear. Recent evidence demonstrates that the catalytic activity of ADAM17 can be regulated through the reduction-oxidation of a vicinal cysteine sulfhydryl motif in the extracellular region of the sheddase (24, 41). Moreover, H_2O_2 and other oxidizing agents can directly activate purified ADAM17 in cell-free assays (24, 40). In consideration of these findings, it is tempting to speculate that released mitochondrial ROS upon induced neutrophil apoptosis may activate cell surface and/or intracellular stores of mature ADAM17. Incidentally, it has been reported that the majority of ADAM17 molecules are intracellular (52).

Chalaris *et al.* have reported that ADAM17 cleaves IL-6R during neutrophil apoptosis (13). In their study, caspases were also implicated in the stimulation of ADAM17; however, in contrast to our findings, they reported that caspases-3 participates in the signaling pathway. The authors also proposed that pro-ADAM17 conversion to its mature form is important for the induction of its activity, which we did not observe. It is important to point out, however, that there are some significant differences in the experimental approaches used in the two studies. For instance, Chalaris *et al.* used non-hematopoietic cells (HepG2 cells, a human liver carcinoma cell line) to determine the role of caspases and the expression levels of ADAM17 (13). We were unable to observe pro-ADAM17 in neutrophils, but we did detect it in Jurkat cells. In the latter cells, however, there was not an appreciable conversion of the pro-protein to mature ADAM17 following Fas stimulation that occurred during the timeframe of L-selectin shedding. Chalaris *et al.* also used a caspase-3 inhibitor (Z-DEVD-fmk) in their study, which has been shown to target a broader spectrum of caspases than the caspase-3 inhibitor we used (Ac-DMQD-cho) (60). Moreover, we have recently reported that ADAM17 is not the primary sheddase of IL-6R in mouse neutrophils during lung inflammation (21), and thus the regulation of IL-6R cleavage may be convoluted.

In conclusion, we demonstrate that ADAM17 activation in neutrophils upon Fas signaling involves a transduction pathway that requires caspase-8, Bid, and mitochondrial ROS. These signaling components are different from those involved in ADAM17 stimulation during neutrophil activation, which involves p38 MAPK and ERK. It will be interesting to determine whether the same signaling cascade involved in ADAM17 stimulation upon Fas engagement is induced by other death receptors, such as TRAIL-RI, TRAIL-RII, and TNF-RI (8). However,

TNFα, for instance, is recognized by two receptors and it can induce ADAM17 activity by cell activation (supplemental Fig. S5) and apoptosis (14). Thus, isolating the death receptor signaling pathway that stimulates ADAM17 upon TNFα treatment may be challenging. An important activity by ADAM17 during apoptosis may be to initiate neutrophil inactivation by cleaving receptors and adhesion molecules normally involved in neutrophil effector activities that promote inflammation. For instance, both of the TNFα receptors are cleaved by ADAM17 in neutrophils (6, 54, 55), and we show in this study that blocking ectodomain shedding by apoptotic neutrophils increased their responsiveness to TNFα, resulting in the production of the pro-inflammatory chemokine IL-8. ADAM17 stimulation during programmed cell death may have an important role in down-regulating the inflammatory capacity of recruited neutrophils. Indeed, impaired TNFα receptor cleavage in patients with TNFα receptor-associated periodic syndrome provides an indication of the importance of ectodomain shedding in attenuating inflammation (61). In addition to neutrophil apoptosis at sites of inflammation, programmed cell death is a continual process by senescent neutrophils and other leukocytes, and thus ADAM17 may play a broad role in the homeostatic maintenance of various substrates in the blood.

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