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Article

Fas/CD95-Induced Chemokines Can Serve as "Find-Me" Signals for Apoptotic Cells

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SUMMARY

Apoptosis is commonly thought to represent an immunologically silent or even anti-inflammatory mode of cell death, resulting in cell clearance in the absence of explicit activation of the immune system. However, here we show that Fas/CD95-induced apoptosis is associated with the production of an array of cytokines and chemokines, including IL-6, IL-8, CXCL1, MCP-1, and GMCSF. Fas-induced production of MCP-1 and IL-8 promoted chemotaxis of phagocytes toward apoptotic cells, suggesting that these factors serve as "find-me" signals in this context. We also show that RIPK1 and IAPs are required for optimal production of cytokines and chemokines in response to Fas receptor stimulation. Consequently, a synthetic IAP antagonist potently suppressed Fas-dependent expression of multiple proinflammatory mediators and inhibited Fas-induced chemotaxis. Thus, in addition to provoking apoptosis, Fas receptor stimulation can trigger the secretion of chemotactic factors and other immunologically active proteins that can influence immune responsiveness toward dying cells.

INTRODUCTION

Engagement of the Fas (CD95/APO-1) receptor with its cognate ligand can result in apoptosis via a caspase-8-dependent pathway that is now well understood (Strasser et al., 2009; Lavrik and Krammer, 2012). Because the overwhelming majority of studies exploring Fas-Fas ligand (FasL) interactions have focused on cell death as the primary endpoint, this has overshadowed the role of Fas receptor engagement as a driver of other outcomes. For example, sporadic reports also suggest that Fas stimulation can result in the production of proinflammatory cytokines and chemokines (Choi et al., 2001; Park et al., 2003; Farley et al., 2006; Altemeier et al., 2007). Other reported consequences of Fas receptor engagement include activation and maturation of dendritic cells (Rescigno et al., 2000), cell motility (Barnhart et al., 2004), and tumor invasion (Kleber et al., 2008). Fas stimulation has also been implicated, counterintuitively, as a driver of tumor proliferation (Chen et al., 2010). Thus, much evidence suggests that signaling through the Fas receptor pathway can lead to endpoints other than cell death, but this aspect of Fas biology and its functional consequences are not well understood.

Fas/CD95 is a member of the TNFR superfamily, members of which play an important role in regulating cell life spans but also in cell-cell communication within the immune system (Croft et al., 2012). A key aspect of TNF function is its ability to trigger the production of numerous proinflammatory cytokines and chemokines in the context of immune reactions (Brennan and McInnes, 2008). TNF-induced cytokines and chemokines, such as IL-6, IL-8, GMCSF, CXCL1, and RANTES, can instigate and amplify immune responses through triggering the production of acute phase proteins and the recruitment of neutrophils, macrophages, and basophils to the site of inflammation, and by triggering increased production of monocytes/macrophages from bone marrow. Because the Fas receptor complex shares several constituents in common to the TNFR complex, we wondered whether Fas stimulation could also modulate inflammatory cytokine production and chemotaxis toward apoptotic cells.

Until recently, apoptotic cells were considered to be relatively immunologically inert, differing from viable cells primarily on the basis of alterations to their plasma membranes that permit recognition and engulfment by phagocytes. In sharp contrast, necrotic cells are sensed by the immune system due to the release of intracellular constituents, some of which (dubbed "alarmins" or "danger signals") have the capacity to activate macrophages and other phagocytes (Matzinger, 1994). However, recent reports suggest that apoptotic cells may also secrete soluble factors, dubbed "find-me" signals, that can promote chemotaxis of phagocytic cells toward dying cells (Gregory and Pound, 2011; Ravichandran, 2011). These factors, which include ATP/UTP (Elliott et al., 2009; Chekeni et al., 2010) and lysophosphatidylcholine (Lauber et al., 2003), represent unconventional chemotactic signals. However, it remains unclear whether conventional chemotactic factors, such as

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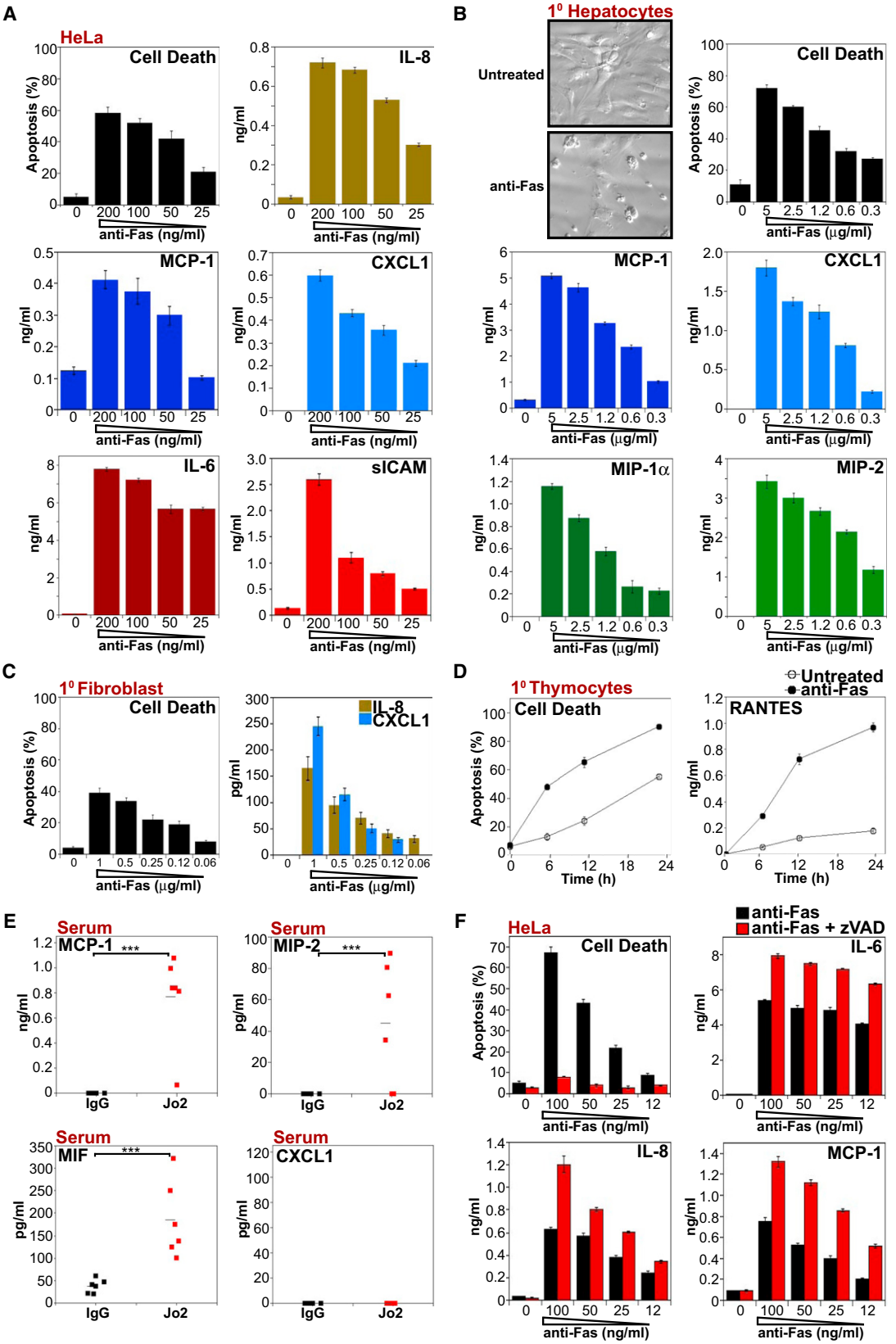

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members of the extensive family of chemotactic cytokines (chemokines), are also released from apoptotic cells. Because Fas stimulation has been linked with the production of proinflammatory cytokines and chemokines—primarily from cells of the immune system—we wondered whether nonimmunological cell types undergoing Fas-induced apoptosis also secrete conventional chemotactic factors to attract the attentions of phagocytes and coordinate removal of dying cells.

Here, we show that Fas stimulation induced the production of several proinflammatory cytokines and chemokines, some of which triggered chemotaxis of phagocytic cells toward dying cells. Importantly, although we could uncouple Fas-induced cell death from production of cytokines and chemokines, Fas-dependent apoptosis was nonetheless accompanied by robust production of several chemokines. Optimal production of Fas-induced proinflammatory factors was dependent on RIPK1 and IAPs and could be attenuated by a small molecule IAP antagonist. Thus, not all apoptotic cell deaths are immunologically silent, and certain stimuli, such as Fas receptor engagement, can actively engage the immune system through the secretion of conventional cytokines and chemokines by apoptotic cells.

RESULTS

To explore whether cells undergoing Fas/CD95-dependent apoptosis secrete proinflammatory cytokines and chemokines, we initially used HeLa cells, which are highly sensitive to the apoptosis-inducing effects of Fas receptor stimulation.

Fas Stimulation Induces Production of Multiple Cytokines and Chemokines

As shown in Figure 1A, stimulation of HeLa cells with anti-Fas IgM triggered extensive apoptosis, as expected. However, under the same conditions, HeLa cells also secreted robust amounts of IL-6, IL-8, MCP-1/CCL2, CXCL1/KC, and sICAM-1 into the culture medium (Figure 1A). Thus, in addition to provoking apoptosis, Fas receptor stimulation can also result in the concurrent production of diverse proinflammatory cytokines and chemokines. Importantly, we also observed Fas-dependent production of cytokines and chemokines during apoptosis of several additional cell types, such as primary mouse hepatocytes (Figure 1B), primary human fibroblasts (Figure 1C), primary mouse thymocytes (Figure 1D), HT-29 colon adenocarcinoma cells (see Figure S1A online), HaCaT keratinocytes (Figure S1B), and primary mouse fibroblasts (Figure S1C), demonstrating that this is a general property of Fas receptor engagement. Moreover, administration of anti-Fas antibody (Jo2) to C57BL/6 mice produced high concentrations of the chemokines MCP-1 and MIP-2 (a murine IL-8 analog), as well as MIF, in the circulation within 10 hr after antibody administration (Figure 1E).

It is important to note that production of Fas-induced cytokines/chemokines in HeLa cells failed to be attenuated through coincubation with recombinant IL-1 receptor antagonist (Figure S1D) or neutralizing antibodies against TNFα (Figure S1E), ruling out the possibility that Fas-induced cytokine/chemokine production was due to Fas-induced IL-1α/β or TNFα production acting in an autocrine or paracrine manner.

Fas-Induced Cytokine Production Can Be Uncoupled from Cell Death

One possibility was that release of cytokines and chemokines occurred secondary to Fas-induced cell death and represented a passive, as opposed to an active, phenomenon. Because caspase activation is essential for Fas-induced apoptosis, we explored whether inhibition of caspase activity suppressed cytokine release in response to Fas stimulation. While inhibition of caspase activity using the broad-spectrum caspase inhibitor zVAD-fmk efficiently blocked Fas-dependent apoptosis (Figure 1F), Fas-induced cytokine release was enhanced rather than suppressed under the same conditions (Figure 1F). We also attenuated Fas-induced apoptosis through stable overexpression of the antiapoptotic Bcl-2 family member Bcl-xL, and this also resulted in enhanced release of IL-6, IL-8, and MCP-1 (Figure S1F). Thus, Fas-dependent proinflammatory signaling can be uncoupled from caspase activity and cell death.

Apoptotic Cells Produce Cytokines and Chemokines

Although Fas-induced cytokine and chemokine secretion could be uncoupled from apoptosis, as demonstrated above, it is important to emphasize that cells undergoing Fas-induced apoptosis produced robust amounts of these inflammatory mediators (Figures 1A–1D). Indeed, the magnitude of cytokine production in response to Fas stimulation was comparable to that seen in response to TNF (Figure S2A). Moreover, immunostaining of HeLa cells undergoing Fas-induced apoptosis revealed strong upregulation of IL-6, IL-8, and MCP-1 within cells displaying clear features of apoptosis, while unstimulated cells stained very poorly for the same proteins (Figure 2A). Furthermore, coimmunostaining of Fas-stimulated cells with FLICA peptide (which detects active caspases) along with antibodies directed against IL-6, IL-8, or MCP-1 clearly demonstrated that the majority of Fas-stimulated cells were double positive for FLICA and the latter cytokines/chemokines (Figure 2B), whereas unstimulated cells were largely negative for both.

Figure 1. Fas Stimulation Induces Multiple Proinflammatory Cytokines and Chemokines in Transformed and Primary Cell Types

(A and C) HeLa cells and primary human fibroblasts were stimulated with anti-Fas IgM (clone CH-11) at the indicated concentrations. After 24 hr, apoptosis was scored by annexin V/PI staining by flow cytometry, and cytokine concentrations in the culture supernatants were determined by ELISA.

(B and D) Primary mouse hepatocytes and primary mouse thymocytes were stimulated with anti-mouse Fas (clone Jo2). After 24 hr, apoptosis and cytokine concentrations were determined as above.

(E) C57BL/6 mice (six per treatment group) were injected (i.p.) with anti-mouse Fas or isotype control IgG (5 μg). After 10 hr, mice were sacrificed, and cytokine concentrations in serum were determined by ELISA.

(F) HeLa cells were treated with anti-Fas IgM at the indicated concentrations, in the presence or absence of zVAD-fmk (25 μM), as indicated. After 24 hr, apoptosis was measured by annexin V/PI staining, and cytokine concentrations in cell culture supernatants were determined by ELISA. Results shown are representative of least three independent experiments. Error bars represent the mean ± SEM of triplicate determinations from a representative experiment. See also Figure S1.

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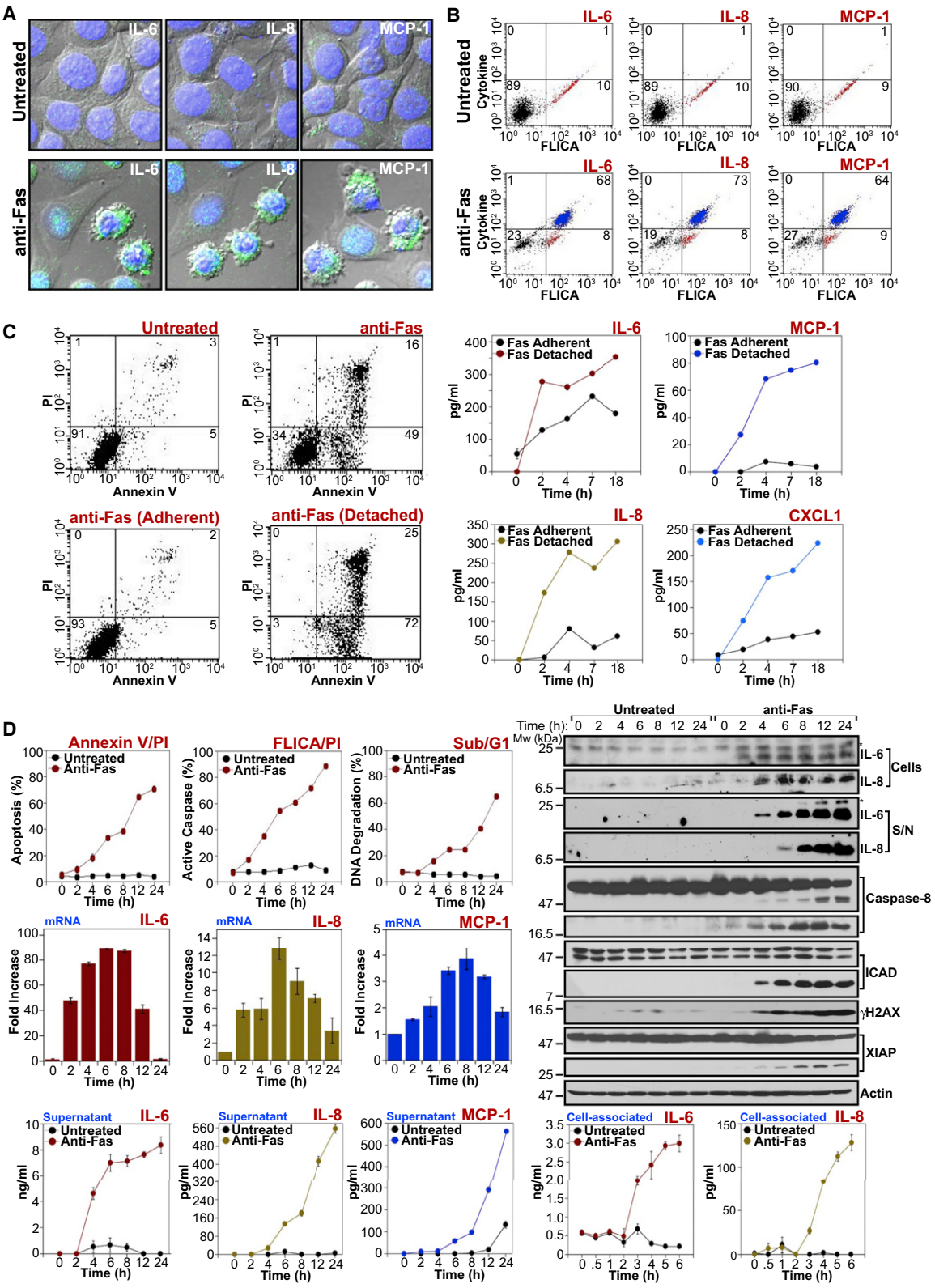

Figure 2. Fas-Induced Cytokine and Chemokine Production by Apoptotic Cells

(A) Shown are representative confocal images of HeLa cells treated with anti-Fas IgM (100 ng/ml) for 16 hr and stained for the indicated cytokines and chemokines, followed by staining of nuclei with Hoechst.

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To further explore the relative contributions of dying versus surviving Fas-stimulated cells to cytokine/chemokine production, we stimulated HeLa cells with anti-Fas antibody for 10 hr, followed by separation of detached cells (dying) from the adherent (viable) cell population by shake off. Detached versus attached cell populations were then washed, and cytokine production in both populations was followed for an additional 18 hr. As Figure 2C illustrates, the detached cell population was 97% phosphatidylserine (PS)-positive (apoptotic) and produced greater amounts of IL-6, IL-8, MCP-1, and CXCL1 during the chase period than the adherent (viable) cell population. We also conducted a similar experiment using FACS-based cell sorting to purify PS-positive apoptotic cells from the remainder of the population and again confirmed that PS-positive apoptotic cells secreted cytokines and chemokines (Figure S2B).

We also explored the relative kinetics of Fas-induced cytokine production versus DNA fragmentation, caspase activation, and other features of apoptosis. As Figure 2D shows, Fas stimulation induced robust expression of IL-6 and IL-8 mRNA within 2 hr of stimulation, well prior to widespread DNA hydrolysis (sub-G0/G1 staining) or annexin V binding, suggesting that Fas-stimulated cells transcriptionally upregulate IL-6 and IL-8 prior to the manifestation of the terminal features of apoptosis. Thus, upregulation of inflammatory mediators occurs rapidly upon Fas stimulation and is readily detectable prior to the onset of the terminal events of apoptosis. Indeed, we also found that certain cell types responded to the cytokine/chemokine-inducing effects of Fas stimulation without manifesting any features of apoptosis (Figure S2C), again underscoring the observation that these events can be separated (Figure 1F) but can also occur within the same cell.

Fas-Induced Cytokine Production Can Be Inhibited through Antagonism of IAPs

Previous studies have implicated inhibitor of apoptosis proteins (IAPs) as important regulators of TNF and Fas-induced apoptosis (Varfolomeev et al., 2007; Vince et al., 2007; Petersen et al., 2007). Thus, we asked whether IAPs were also required for optimal Fas-induced production of cytokines and chemokines. To explore this possibility, we used a bivalent small molecule IAP antagonist (BV6) that Vucic and colleagues have previously shown promotes the rapid degradation of cIAP-1 and cIAP-2 (Varfolomeev et al., 2007). BV6 greatly sensitized HeLa cells to the apoptosis-inducing effects of Fas stimulation (Figures 3A and 3B) and promoted essentially complete degradation of cIAP-1 and cIAP-2 as well as XIAP (Figure 3C).

We then explored the impact of IAP antagonism on Fas-induced cytokine production. Importantly, Fas-induced production of MCP-1, IL-8, and CXCL1 was profoundly inhibited through addition of BV6 (Figures 3D and 3E). This strongly suggests that IAPs are required for optimal Fas-induced cytokine production, consistent with previous observations that IAPs participate in signal transduction events downstream of Fas receptor ligation (Geserick et al., 2009). Because Fas-induced apoptosis was dramatically potentiated in the presence of the IAP antagonist (Figures 3A and 3B), it was possible that Fas-induced cytokine production was impaired merely as a consequence of cell death—leading to a decline in transcription/translation—rather than as a direct consequence of IAP antagonism. However, under conditions in which Fas/BV6-initiated cell death was completely suppressed by zVAD-fmk (Figures 3D and 3E), Fas-induced production of cytokines and chemokines was still inhibited in the presence of BV6 (Figures 3D and 3E).

To further separate the effects of IAP antagonism on Fas-induced apoptosis versus Fas-induced cytokine production, we screened a panel of cell types for lack of sensitization to Fas-induced death in the presence of IAP antagonist (data not shown). These experiments revealed that primary HUVECs failed to be sensitized toward the apoptosis-inducing effects of Fas through addition of BV6 (Figures S3A and S3B). This enabled us to resolve fully whether inhibition of IAPs suppressed Fas-induced cytokine production independent of sensitization toward apoptosis. As Figure S3B illustrates, BV6 inhibited Fas-induced production of IL-8, CXCL1, and GMCSF in primary HUVECs, despite failing to sensitize these cells toward Fas-induced apoptosis under the same conditions. These data again argue that IAPs are required for optimal Fas-induced proinflammatory cytokine and chemokine production and that synthetic IAP antagonists can profoundly attenuate this property of Fas receptor engagement.

To explore the identity of the IAPs required for Fas-induced cytokine production, we silenced expression of endogenous XIAP, cIAP-1, and cIAP-2 using siRNAs targeted to their respective mRNAs, followed by Fas stimulation (Figure 3F). Similar to the effects of the synthetic IAP antagonist, BV6, knockdown of cIAP-2 attenuated Fas-induced MCP-1, IL-8, and CXCL1 secretion and sensitized toward Fas-induced apoptosis (Figure 3F). Knockdown of both cIAP-1 and cIAP-2 had a greater effect

(B) HeLa cells were stimulated with anti-Fas IgM (200 ng/ml) at the indicated concentrations. After 16 hr, FAM-FLICA polycaspase reagent was added to the cells for 2 hr to facilitate detection of apoptotic caspases, and then cells were fixed, permeabilized, stained with the indicated cytokines, and analyzed by flow cytometry.

(C) HeLa cells were stimulated with anti-Fas IgM (100 ng/ml). After 10 hr, live and dying cell populations were separated by removing medium, which almost exclusively contained detached, dying cells, while the remaining adherent (live) cells were harvested separately by trypinization. Apoptosis in total or separated populations was determined by annexin V/PI staining by flow cytometry. "Adherent" and "detached" cells were subsequently replated, and cytokine concentrations in cell culture supernatants were determined by ELISA at the indicated time points.

(D) HeLa cells were stimulated with anti-Fas IgM (100 ng/ml) for the indicated times, and apoptosis was determined by annexin V/PI and FAM-FLICA/PI staining by flow cytometry, while DNA degradation was quantified by staining for Sub-G1 populations by flow cytometry. Levels of intracellular cytokines were determined by RT-PCR, or by ELISA or immunoblotting of cell lysate preparations (asterisk denotes nonspecific band). Cytokine concentrations in cell culture supernatants were determined by ELISA, while correlates of apoptosis (caspase-8, XIAP) and DNA degradation (ICAD, γH2AX) were determined by immunoblotting. Results shown are representative of least three independent experiments. Error bars represent the mean ± SEM of triplicate determinations from a representative experiment. See also Figure S2.

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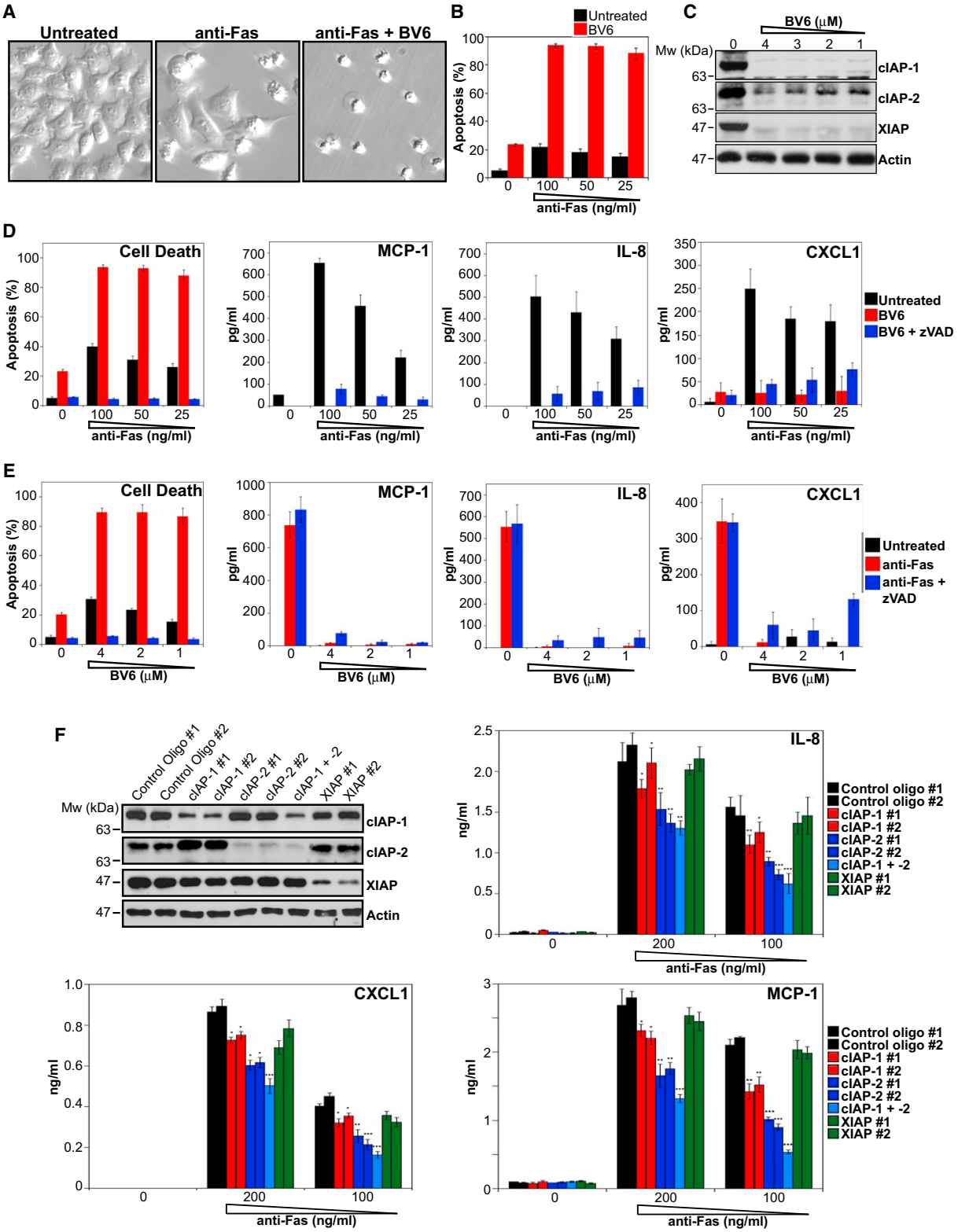

Figure 3. Neutralization of Endogenous IAPs Attenuates Fas-Induced Cytokine and Chemokine Production

(A) HeLa cells were pretreated for 2 hr with the IAP antagonist, BV6 (2 μM), followed by addition of anti-Fas (100 ng/ml). Sixteen hours later, cell cultures were visualized by phase-contrast microscopy.

(B) Cell death in cell cultures from (A) was determined by annexin V/PI staining.

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than either alone, while XIAP knockdown had little effect on Fas-dependent cytokine secretion. Taken together, these data argue that cIAP-1 and cIAP-2 are important for optimal Fas-induced cytokine production.

Fas-Induced Cytokine Production Is Regulated through RIPK1-Dependent NFκB Activation

The potent proinflammatory effects of TNFR stimulation are achieved through activation of multiple kinases including the IKK complex, p38MAPK, and MEK/ERK (Wallach et al., 1999). Indeed, we observed a similar range of kinase activation events in response to Fas stimulation, although NFκB activation was considerably delayed in response to Fas, in comparison with TNFR stimulation (Figure 4A). cIAP-1 and cIAP-2 have been implicated as key regulators of RIPK1 polyubquitination and recruitment of downstream signaling intermediates in the context of TNFR signaling (Hsu et al., 1996). Because the preceding experiments found that IAP neutralization suppressed Fas-induced cytokine production, this suggested that RIPK1 might also be important in this context. Thus, we asked whether knockdown of RIPK1 could inhibit Fas-induced cytokine production. As Figure 4B shows, silencing of RIPK1 with two different siRNAs robustly attenuated Fas-induced IL-6, MCP-1, IL-8, and CXCL1 production, suggesting that this kinase is required for the proinflammatory effects of Fas stimulation. Similarly, siRNA-mediated knockdown of the NFκB p65 subunit also profoundly attenuated Fas-induced cytokine production, whereas silencing of p38MAPK, ERK1/2, or JNK had little or no effect by comparison (Figure 4B).

Because both RIPK1 and NFκB were important for Fas-induced cytokine production, we also explored the effects of silencing RIPK1 on Fas-induced NFκB activation. As Figure 4C illustrates, Fas-induced IκB degradation and NFκB p65 nuclear translocation were considerably delayed upon RIPK1 knockdown. Similarly, neutralization of IAPs using the IAP antagonist, BV6, also considerably inhibited NFκB activation (Figure 4D) as well as MEK/ERK activation (Figure S4A), consistent with its effects on cytokine production (Figures 3D and 3E).

Consistent with a role for RIPK1 as a driver of Fas-dependent cytokine production, transient overexpression of RIPK1 was sufficient to induce apoptosis as well as robust expression and secretion of MCP-1, IL-8, and CXCL1 from HeLa cells (Figure 4E). Moreover, inhibition of RIPK1-induced apoptosis by zVAD-fmk potentiated production of these cytokines, as expected (Figure 4E). Thus, RIPK1 appears to be capable of driving the production of multiple proinflammatory factors, and this kinase is required for optimal production of cytokines and chemokines in response to Fas stimulation. To ask whether the kinase activity of RIPK1 is required for Fas-induced cytokine/chemokine production, we used a specific inhibitor of RIPK1 kinase activity (necrostatin), but as Figure 4F illustrates, necrostatin had little effect on Fas-induced cytokine production (despite inhibiting RIPK3-dependent necroptosis, see Figure S4B), suggesting that RIPK1 acts as a scaffold to promote gene expression in this context.

RIPK1 Is Found in a Soluble Complex with Procaspase-8 after Fas Stimulation

The preceding results suggested that the formation of a RIPK1 complex in response to Fas stimulation was important for promoting cytokine and chemokine production. However, only trace amounts of endogenous RIPK1 coimmunoprecipitated with the Fas receptor after stimulation, unless the polycaspase inhibitor zVAD-fmk was added (Figure 5A). Processed caspase-8, as well as FADD, was readily detected in the Fas receptor complex, as expected (Figure 5A). In contrast, immunoprecipitation of residual soluble caspase-8 (which was largely in its unprocessed form) after removal of Fas receptor-bound complexes coprecipitated considerably more RIPK1 than was found in association with the Fas receptor complex (Figure 5A). FADD was also found within this soluble caspase-8/RIPK1 complex, which was assembled within 30 min of Fas receptor engagement (Figure 5A). Once again, the addition of ZVAD-fmk greatly stabilized the association between RIPK1 and caspase-8. We also detected small amounts of endogenous cIAP-1 in association with the soluble complex under the same conditions (Figure 5A). These data are compatible with the formation of a soluble FADD/caspase-8/RIPK1 signaling complex (complex II) in response to Fas stimulation, as previously reported (Geserick et al., 2009).

To explore whether caspase-8 was required for Fas-induced cytokine production, possibly by acting as a scaffold for recruitment of RIPK1, we silenced caspase-8 expression and explored the impact of this on Fas-induced cytokine production. Although caspase activity was not required for Fas-induced cytokine production (Figure 1F), knockdown of caspase-8 robustly attenuated Fas-induced cytokine production (Figure 5B), suggesting a function of complex-associated caspase-8, but not fully cleaved heterotetrameric caspase-8, for gene induction. Knockdown of FADD had a similar inhibitory effect (Figure 5B). Collectively, these data suggest that Fas stimulation generates a soluble FADD/caspase-8/RIPK1 complex (Fas complex II),

(C) Cell cultures from (A) were analyzed by immunoblotting for endogenous IAPs.

(D) HeLa cells were pretreated for 2 hr with BV6 (2 μM) in the presence or absence of zVADfmk (25 μM), followed by stimulation with anti-Fas IgM at the indicated concentrations. After a further 24 hr, apoptosis was scored by annexin V/PI staining, while cytokine concentrations in the resulting supernatants were determined by ELISA.

(E) HeLa cells were pretreated for 2 hr with the indicated concentrations of BV6, followed by stimulation with anti-Fas IgM (100 ng/ml), in the presence or absence of zVAD-fmk (25 μM). After 24 hr, apoptosis and cytokine concentrations were determined as in (D). Error bars represent the mean ± SEM of three independent experiments.

(F) HeLa cells were transfected with either nonsilencing siRNA or siRNAs directed against cIAP-1, cIAP-2, or XIAP, as indicated. Forty-eight hours later, cells were treated with the indicated concentrations of anti-Fas IgM. After 24 hr, cell death was scored by annexin V/PI staining, cytokine concentrations in the resulting supernatants were determined by ELISA, and cell lysates were analyzed by immunoblotting for levels of endogenous proteins. Results shown are representative of least three independent experiments. Error bars represent the mean ± SEM of triplicate determinations from a representative experiment. See also Figure S3. Significance levels are ***$p < 0.01$, **$p < 0.05$, and *$p < 0.1$, measured by Student's t test.

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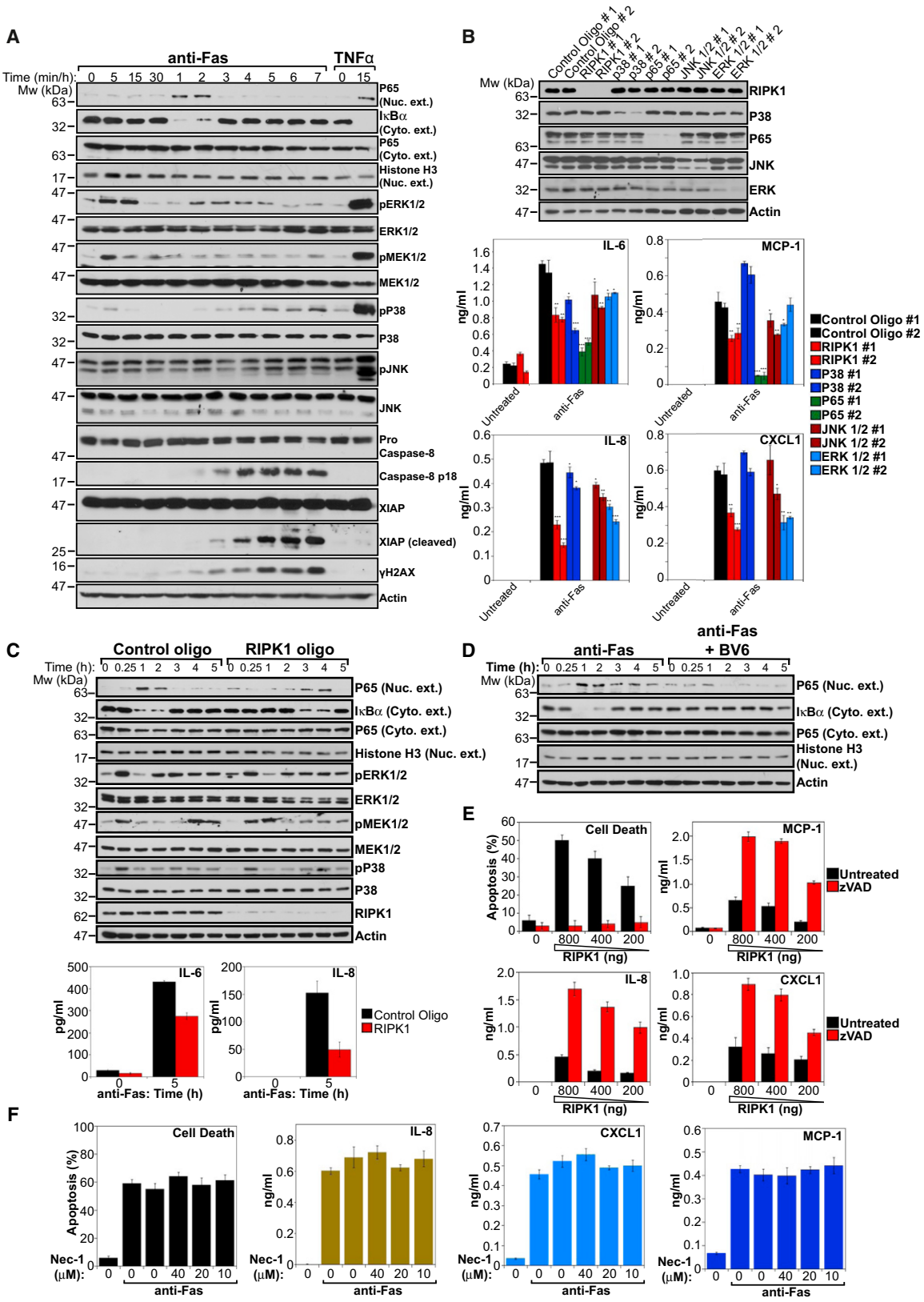

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which is important for promoting NFκB-dependent cytokine production.

We also explored whether Fas-induced cytokine production versus apoptosis could be influenced by the degree of receptor aggregation. To explore this issue, we compared soluble versus aggregated Fas ligand for its ability to induce death versus inflammatory cytokines. However, as Figure 5C shows, whereas monomeric Fas ligand failed to induce either apoptosis or cytokine production, the aggregated form of the ligand induced both.

Supernatants from Fas-Stimulated Cells Induce Phagocyte Chemotaxis

Because multiple chemokines are secreted in response to Fas receptor stimulation, this suggested that cells undergoing Fas-dependent apoptosis might actively elicit the attentions of phagocytic cells, such as neutrophils and macrophages, through production of these factors. To explore this issue, we asked whether supernatants from Fas-stimulated cells could elicit chemotaxis of the monocytic cell line, THP-1. As Figures 6A and 6B demonstrate, supernatants derived from Fas-stimulated HeLa cells were potently chemotactic for THP-1 cells. Of note, supernatants from Fas-treated HeLa cells promoted chemotaxis irrespective of whether or not the apoptotic cells were removed by centrifugation (Figures 6B–6D).

A previous report suggested that chemotaxis toward apoptotic cells, including apoptotic cells generated via Fas stimulation, may be mediated through caspase-dependent release of ATP, which is known to function as a monocyte chemoattractant (Elliott et al., 2009). To explore whether Fas-dependent release of ATP was responsible for the chemotaxis observed in our experiments, we used two different approaches. First, we used the polycaspase inhibitor, zVAD-fmk, to explore whether this blocked chemotaxis toward supernatants derived from Fas-stimulated cells. Importantly, we found that caspases were not involved in Fas-induced chemotaxis, because while zVAD-fmk protected from cell death (Figure 1F), it did not suppress the chemotactic potential of supernatants from Fas-stimulated HeLa cells (Figure 6E), nor did it suppress Fas-induced production of chemokines (Figure 1F).

Second, we directly measured ATP in supernatants from Fas-stimulated cells, but these did not contain significant amounts of this nucleotide (Figure 6F and Figure S5). We did detect modest amounts of ATP release over a time course analysis, but the kinetics of ATP release was not any faster than that of cytokine and chemokine production (Figure S5). However, it remained feasible that low concentrations of ATP or other nucleotides might still be responsible for the chemotaxis seen in response to Fas stimulation. To control for this possibility, we added the nucleotide-degrading enzyme, apyrase, to supernatants from Fas-stimulated cells and assessed the effect of this on chemotaxis. Apyrase efficiently degraded exogenously added ATP (Figure 6F), and this resulted in potent inhibition of THP-1 chemotaxis toward this nucleotide (Figure 6G). However, treatment of Fas supernatants with the same concentrations of apyrase did not reduce their chemotactic capacity (Figure 6G), suggesting that ATP or other tri- or dinucleotides were not responsible for the chemotaxis seen in response to Fas stimulation (Figure 6G).

Fas-Induced MCP-1 and IL-8 Serve as "Find-Me" Signals for Apoptotic Cells

The preceding experiments suggested that one or more Fas-induced chemokines, rather than ATP or UTP, were responsible for the chemotaxis observed. To identify the factor responsible, we used antibody-mediated depletion of specific chemokines and cytokines from Fas-stimulated HeLa cell supernatants, followed by assessment of chemotactic activity. While Fas-stimulated HeLa cells produced multiple chemokines (Figure 1A and Figure S6), depletion of MCP-1 (Figures 7A and 7B) was sufficient to block almost all THP-1 monocyte chemotaxis, while IL-8 depletion revealed a minor role for this chemokine in this context (Figure 7B). We also used primary human peripheral blood neutrophils to explore whether these cells also migrated toward MCP-1 and/or IL-8 within Fas-stimulated cell supernatants. As Figures 7C and 7D illustrate, these experiments revealed that IL-8 was essential for migration of human neutrophils toward supernatants derived from Fas-stimulated cells. We also explored whether Fas stimulation could trigger phagocyte migration in vivo by administration of anti-Fas (Jo2) antibody into C57BL/6 mice. As can be seen from Figure 7E, within 10 hr of anti-Fas administration, extensive cell death was detected in the thymus, which correlated with a dramatic increase of CD11b-positive macrophages in the same tissue.

Figure 4. RIPK1 and NF-κB Play Central Roles in Fas-Induced Cytokine Production

(A) HeLa cells were treated with anti-Fas (100 ng/ml) or TNF-α (10 ng/ml). Cell lysates were prepared at the indicated time points and analyzed by immunoblotting for the indicated proteins.

(B) HeLa cells were transfected with nonsilencing siRNA or with siRNAs directed against RIPK1, p38 MAP kinase, p65, JNK, or ERK and then 48 hr later treated with anti-Fas (100 ng/ml). After a further 24 hr, cell lysates were analyzed by immunoblotting for levels of endogenous proteins, while cytokine concentrations in the supernatants were determined by ELISA.

(C) HeLa cells were transfected with nonsilencing or RIPK1-targeted siRNAs and then 48 hr later treated with anti-Fas (100 ng/ml) followed by immunoblotting of cell lysates for the indicated proteins as in (A), while cytokine concentrations in the supernatants were determined by ELISA.

(D) HeLa cells were pretreated for 1 hr with BV6 (2 μM) and then treated with anti-Fas (100 ng/ml) followed by immunoblotting of cell lysates for the indicated proteins as in (A).

(E) HeLa cells were transfected with empty vector or the indicated concentrations of pKR.FLAG-RIPK1 in the presence or absence of zVAD-fmk (25 μM) where indicated. After 24 hr, apoptosis was scored by annexin V/PI staining, while cytokine concentrations in the resulting supernatants were determined by ELISA.

(F) HeLa cells were pretreated for 2 hr with necrostatin-1 (40 μM), then treated with anti-Fas (100 ng/ml). After a further 24 hr, cytokine concentrations in the supernatants were determined by ELISA. Results shown are representative of least three independent experiments. Error bars represent the mean ± SEM of triplicate determinations from a representative experiment. See also Figure S4.

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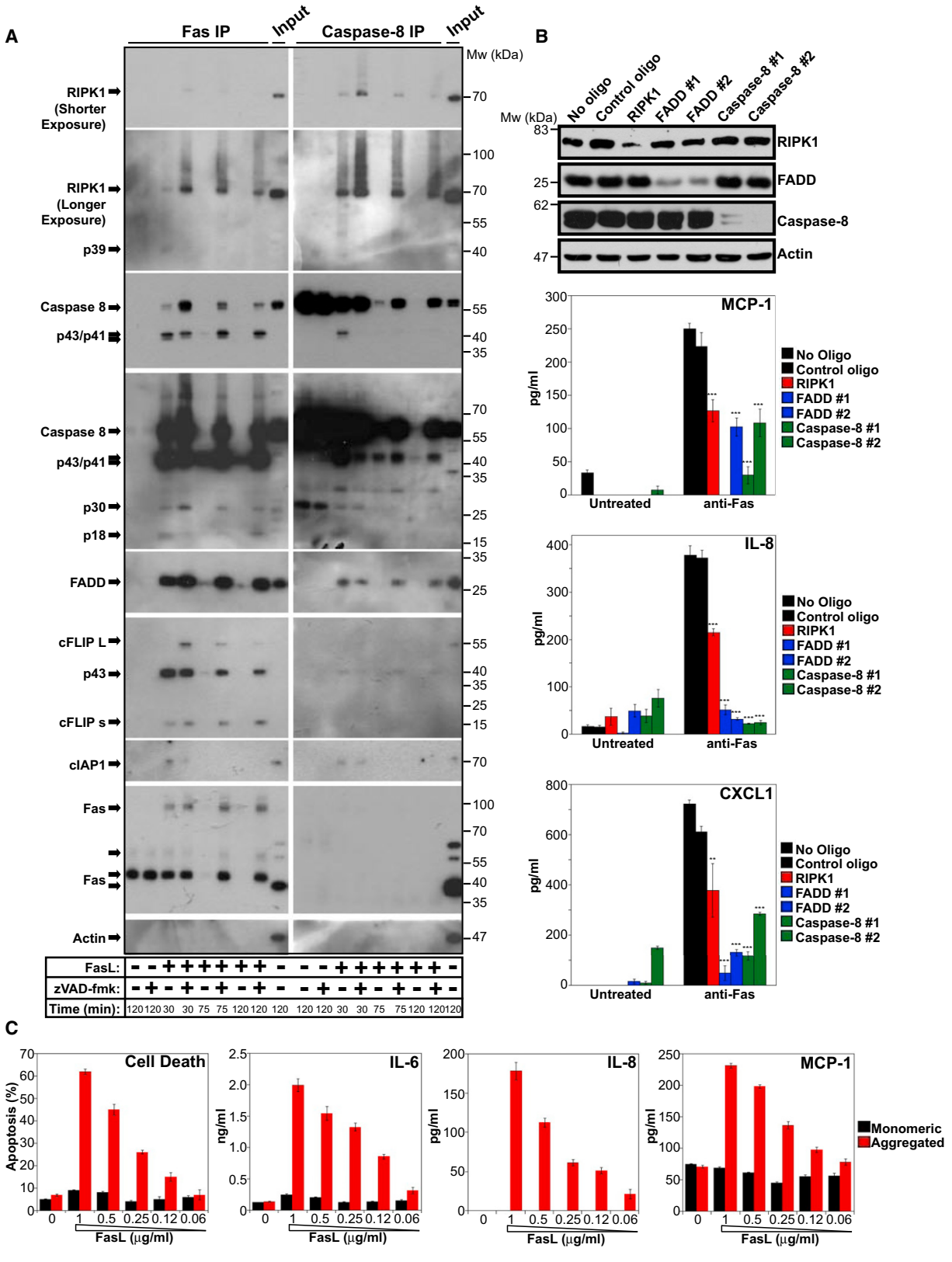

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Because Fas-induced MCP-1 and IL-8 secretion was suppressed by IAP antagonists (Figures 3D and 3E), we reasoned that inhibition of IAPs should also attenuate Fas-induced chemotaxis. As illustrated in Figure 7F, supernatants from Fas/BV6-treated HeLa cells were far less effective in promoting chemotaxis than those from cells stimulated with anti-Fas alone. Similarly, because RIPK1 was required for optimum Fas-induced MCP-1 and IL-8 production (Figure 4B), knockdown of RIPK1 prior to Fas stimulation also blunted the chemotactic potential of the resulting supernatants (Figure 7G).

We also explored whether other proapoptotic stimuli have the potential to induce the expression of cytokines and chemokines from apoptotic cells. Strikingly, etoposide and taxol both induced robust amounts of IL-8 in HeLa cells and CXCL1 (an IL-8-like chemokine) as well as MCP-1 in mouse CT26 cells (Figure S6). However, RIPK1 was not required for cytokine production in these contexts (Figure S6B).

Collectively, these results demonstrate that Fas/CD95-induced apoptosis is associated with production of proinflammatory factors, some of which (IL-8 and MCP-1) can trigger chemotaxis toward dying cells (Figure 7H). Thus, apoptotic cells can express and secrete cytokines and chemokines, which can serve as "find-me" signals for apoptotic cells in certain settings. Moreover, these data also challenge the notion that apoptosis is an "immunologically silent" mode of cell death.

DISCUSSION

Here we have shown that Fas/CD95 stimulation can activate a proinflammatory program, resulting in the expression and secretion of multiple cytokines and chemokines, that runs in parallel with the events leading to cell death. Cells dying in response to Fas stimulation produced several chemokines (MCP-1/CCL-2, IL-8, RANTES/CCL5, and CXCL1/KC), some of which promoted chemotaxis of phagocytes toward the source of these factors. Fas-induced production of cytokines was dependent on RIPK1-induced NFκB activation, which was modulated by cIAP-1 and cIAP-2. Consistent with this, a small molecule IAP antagonist attenuated the production of several Fas-induced chemokines, as well as chemotaxis toward dying cells.

Previous studies have suggested that apoptotic cells release "find-me" signals that promote chemotaxis of phagocytic cells toward the dying cell (Gregory and Pound, 2011; Ravichandran, 2011). ATP, UTP, lysophosphatiylcholine, and sphingosine-1-phosphate have all been implicated as chemotactic signals that are released during apoptosis (Lauber et al., 2003; Gude et al., 2008; Elliott et al., 2009). Indeed, Fas-induced cell death was reported to be associated with efflux of ATP and UTP via the pannexin 1 membrane channel, with these factors serving as "find-me" signals for monocytes in this context (Chekeni et al., 2010). However, we did not find evidence for the involvement of ATP in Fas-induced chemotaxis, and addition of the nucleotide hydrolyase, apyrase, failed to antagonize chemotaxis toward supernatants from Fas-stimulated cells. In contrast, depletion of MCP-1 and IL-8 potently inhibited Fas-initiated chemotaxis toward monocytic cells and neutrophils, respectively, strongly implicating these molecules as "find-me" signals in this context. Thus, conventional chemotactic cytokines, such as IL-8 (MIP-2 in the mouse) and MCP-1, may play dominant roles in guiding phagocytes to apoptotic cells in contexts in which the specific proapoptotic stimulus (such as Fas or TNF) triggers the production of these mediators.

Previous studies have also implicated Fas receptor stimulation as a trigger for the production of chemokines and cytokines from vascular smooth muscle cells, macrophages, neutrophils, and dendritic cells (Schaub et al., 2000; Rescigno et al., 2000; Shimizu et al., 2005; Guo et al., 2005). TRAIL has also been reported to induce chemokines and to promote macrophage migration in vitro, although a role for specific chemokines in this context was not demonstrated (Varfolomeev et al., 2005; Berg et al., 2009). Furthermore, transgenic expression of Fas ligand in the pancreas, with the aim of protecting these cells from immune infiltration, paradoxically induced aggressive inflammation into these organs (Kang et al., 1997; Allison et al., 1997). However, in these instances, the production of proinflammatory mediators through Fas stimulation was not observed in the context of cell death and was explored in isolation from Fas-induced apoptosis. Thus, although Fas ligand has been established to be proinflammatory in certain settings, the implications of this and its significance for the removal of apoptotic cells have not previously been appreciated or explored.

Recent studies have shown that RIPK1-mediated signaling is enhanced through cIAP-1 and cIAP-2-dependent ubiquitination of RIPK1 (Dynek et al., 2010), which promotes recruitment of the LUBAC signaling complex required for optimal RIPK1-dependent signal transduction (Haas et al., 2009). Consistent with this, we also found that interfering with IAP function, through

Figure 5. A Soluble RIPK1/FADD/Caspase-8 Complex Is Involved in Fas-Induced Cytokine Production

(A) Shown is kinetic analysis of the Fas/CD95 receptor complex (complex I, left panel) or soluble caspase-8 complex (complex II, right panel) in the presence or absence of polycaspase inhibitor zVAD-fmk. HeLa cells were preincubated for 1 hr with zVAD-fmk (10 μM) where indicated and then stimulated with an excess of Fas/CD95L-Fc for the indicated time points. Fas was then immunoprecipitated from cell lysates and the resulting complexes removed on protein G agarose. A caspase-8-containing complex (complex II) was subsequently precipitated from the Fas-depleted lysates using anti-caspase-8 antibody. Cell lysates were prepared at the indicated time points and analyzed by immunoblotting for the indicated proteins. Equal amounts of input cellular lysates were loaded to facilitate comparison of signal strength between Fas-IP and caspase-8 IPs. Results shown are representative of two independent experiments.

(B) HeLa cells were transfected with either nonsilencing siRNA or siRNAs directed against RIPK1, FADD, or caspase-8, as indicated. Forty-eight hours later, cells were treated with anti-Fas IgM (100 ng/ml) in the presence of zVADfmk (10 μM). After a further 24 hr, cell lysates were analyzed by immunoblotting for detection of endogenous proteins, while cytokine concentrations in the supernatants were determined by ELISA.

(C) His-tagged, soluble, recombinant, monomeric FasL was allowed to aggregate on tetradentate metal chelator-coated Dynabeads for 2 hr. HeLa cells were then treated with the indicated concentrations of monomeric or bead-aggregated FasL. After 48 hr, cell death was scored by annexin V/PI staining, and cytokine concentrations in the resulting supernatants were determined by ELISA. Error bars represent the mean ± SEM of triplicate determinations from a representative experiment.

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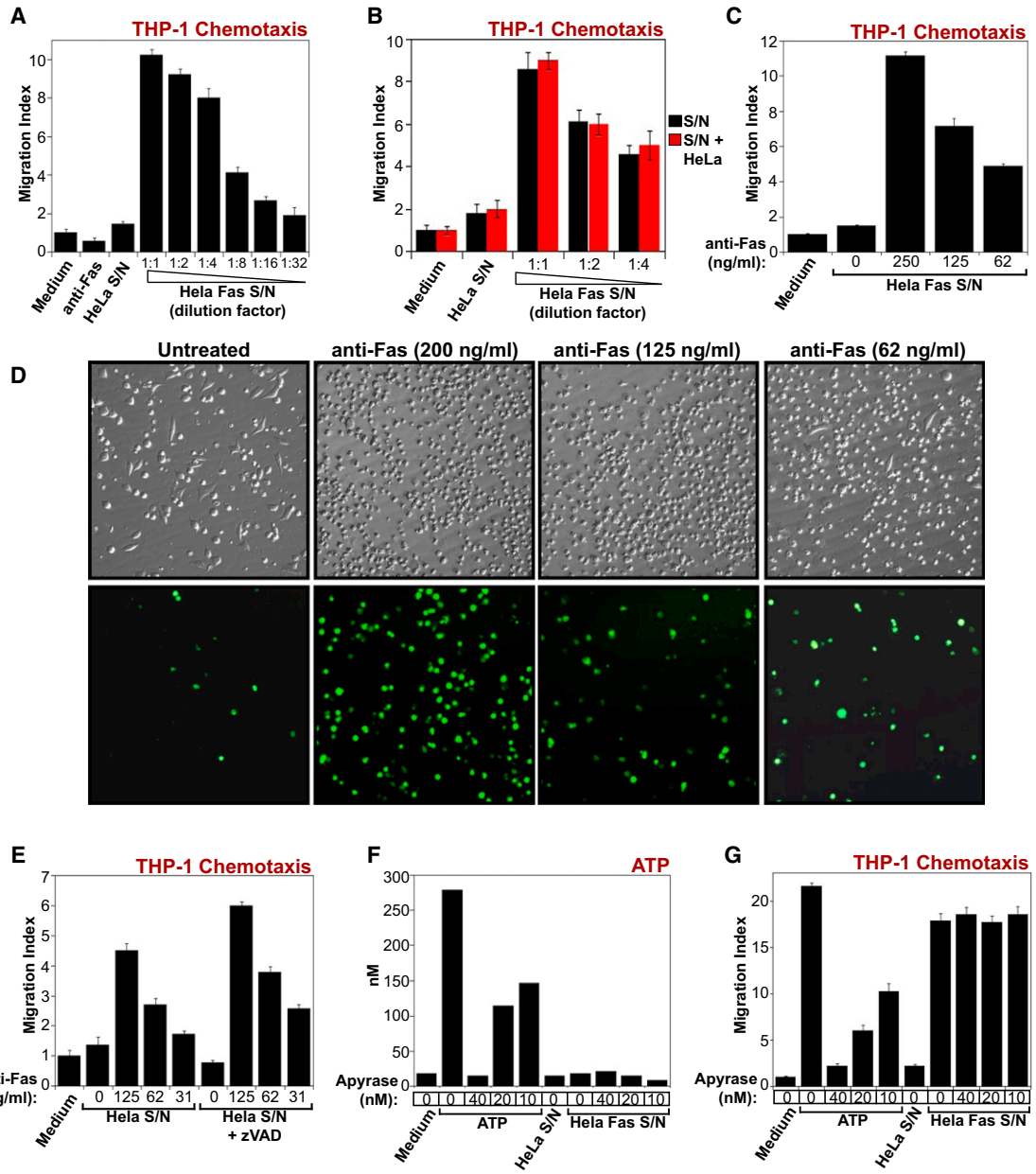

Figure 6. Fas-Induced Apoptosis Elicits Chemotaxis of Phagocytes

(A) HeLa cells either were left untreated or were treated with anti-Fas IgM (250 ng/ml) in RPMI, supplemented with 0.5% FCS. After 24 hr, culture supernatants were clarified by centrifugation, and a chemotaxis assay was performed using the indicated dilutions of supernatant (S/N) from Fas-stimulated cells. THP-1 cells were used for the chemotaxis assay, separated from the cell supernatants by 8 μM nitrocellulose filters. Cells that had migrated into the bottom wells of the chemotaxis chambers were counted after 1–2 hr and are expressed relative to the migration observed in the control wells.

(B) HeLa cells were treated as in (A), and a chemotaxis assay with THP-1 cells was performed, except that in parallel to adding supernatants alone, untreated or Fas-treated dying HeLa cells were added to the bottom wells of the chemotaxis chamber along with their respective supernatants. THP-1 cells were labeled with the fluorescent dye CFSE to facilitate detection.

(C) HeLa cells were treated with the indicated concentrations of anti-Fas IgM, and a chemotaxis assay was performed as in (B).

(D) CFSE-labeled THP-1 cells that had migrated into the bottom wells of the chemotaxis chamber from the experiment depicted in (C) were removed and photographed under UV microscopy.

(E) HeLa cells were treated with anti-Fas (250 ng/ml) for 24 hr in the presence or absence of zVAD-fmk (25 μM), followed by assessment of chemotaxis as in (A).

(F and G) (F) ATP or supernatants from Fas-treated HeLa were treated with the indicated concentrations of apyrase for 30 min; then ATP concentrations were determined by chemiluminescence, and the resulting preparations were used in a THP-1 chemotaxis assay (G). Results shown are representative of least three independent experiments. Error bars represent the mean ± SEM of triplicate cell counts from a representative experiment. See also Figure S5.

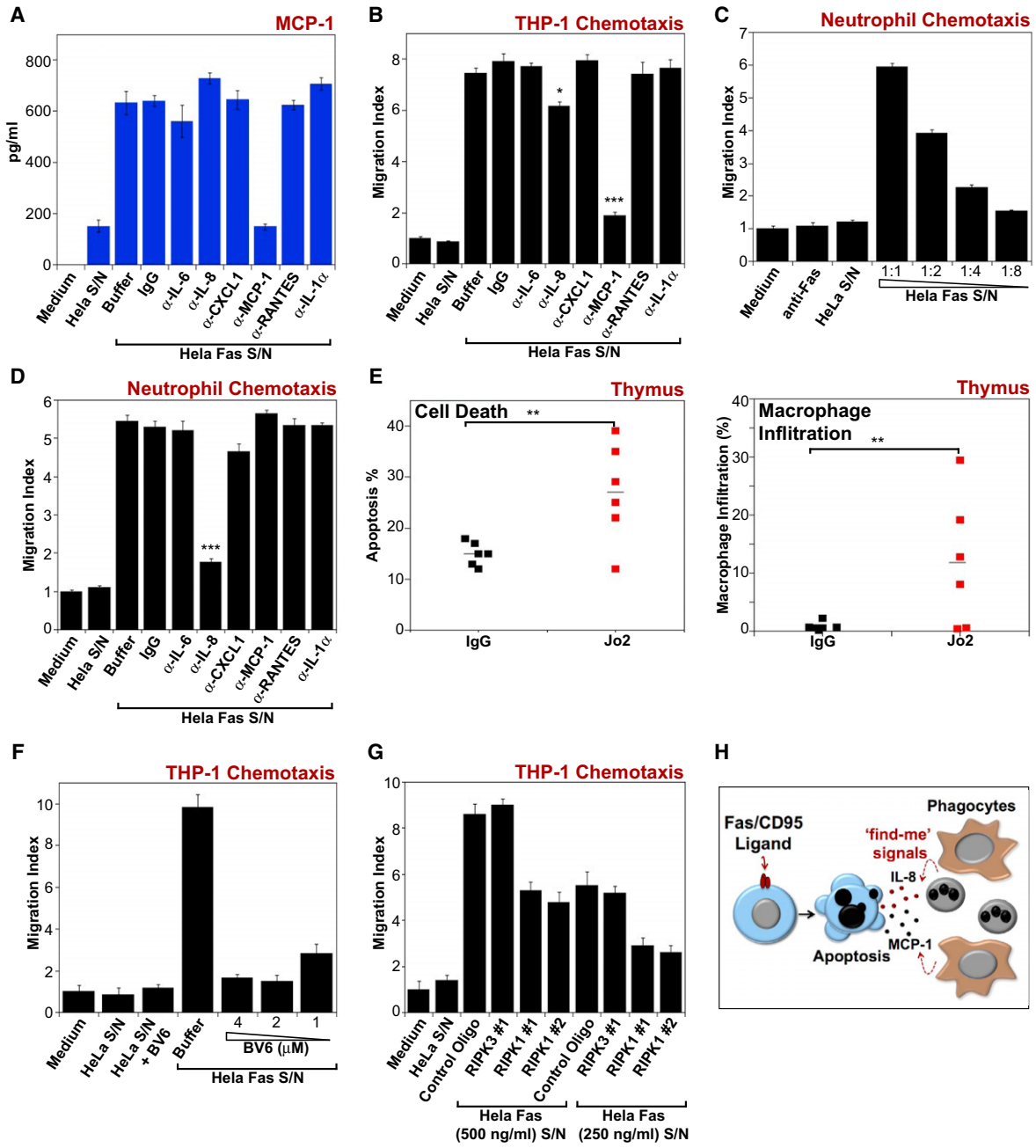

Figure 7. Fas-Induced Chemokines Promote Chemotaxis toward Apoptotic Cells

(A) HeLa cells were either unstimulated (HeLa S/N) or were treated with anti-Fas IgM (250 ng/ml) in RPMI, supplemented with 0.5% FCS. Twenty-four hours later, supernatants were immunodepleted of the indicated cytokines, and MCP-1 concentration in the culture supernatants was determined by ELISA.

(B) Supernatants from (A) were assessed for their ability to promote chemotaxis of THP-1 cells. Total migrated cells were counted after 1–2 hr and are expressed relative to chemotaxis seen in control wells.

(C) HeLa cells were treated for 24 hr with anti-Fas IgM (250 ng/ml) in HBSS, 0.5% BSA. The resulting culture supernatants were used in a chemotaxis assay using primary human peripheral blood-derived neutrophils, separated by 3 μM nitrocellulose filters.

(D) HeLa cells, in HBSS, 0.5% BSA, were treated as in (A) and then depleted for the indicated cytokines followed by a neutrophil chemotaxis assay as described above.

(E) C57BL/6 mice (six per treatment group) were injected (i.p.) with anti-mouse Fas or isotype control IgG (5 μg). After 10 hr, mice were sacrificed, and the thymus was harvested, disaggregated, and scored for cell death by annexin V/PI staining or for infiltrating myeloid cells by cell-surface staining for CD11b, F4/80, and Gr-1 by flow cytometry.

(F) HeLa cells were pretreated with the indicated concentrations of BV6 and zVAD-fmk (25 μM) for 2 hr, then treated with anti-Fas IgM (250 ng/ml) for 24 hr. A THP-1 chemotaxis assay was performed with the resulting supernatants.

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either knockdown of endogenous IAPs or use of a small molecule IAP antagonist (BV6), attenuated Fas-induced production of several proinflammatory cytokines and chemokines. Moreover, supernatants derived from Fas-stimulated cells that were treated with IAP antagonists elicited less-potent chemotaxis compared to supernatants from the same cells treated with anti-Fas alone.

Here we have shown that Fas-induced cell death is an immunologically active process and is associated with the expression and secretion of multiple immune messenger proteins from apoptotic cells. Recent studies from Kroemer and colleagues have also suggested that certain chemotherapeutic drugs, especially those belonging to the anthracyclin family, may also trigger a form of apoptosis that elicits immune activation (Obeid et al., 2007). In the latter case, anthracyclin-induced surface exposure of the ER chaperone, calreticulin, was implicated as a trigger of immune infiltration of tumors. However, precisely how calreticulin initiates an immune response to the tumor remains unclear. In future studies, it will be interesting to determine whether apoptosis induced by anthracyclins is also associated with the production of chemokines or other chemotactic factors.

In conclusion, here we report that a range of cell types dying in response to Fas receptor stimulation express and release multiple cytokines and chemokines, a subset of which attract the attentions of phagocytic cells. Thus, contrary to the classical view that apoptotic cells are immunologically inert, certain proapoptotic signals (such as Fas, TNF, and TRAIL) may instigate the production of chemokines, as well as other cytokines, that coordinate the swift removal of dying cells by acting as "find-me" signals for such cells.

EXPERIMENTAL PROCEDURES

Materials

The following antibodies were used: anti-human Fas IgM (clone, CH-11, Millipore); anti-mouse Fas IgG (clone, Jo2); anti-XIAP and anti-Bcl-xL (BD Biosciences); anti-IL-6, anti-MCP-1, anti-IL-1α, anti-RANTES, anti-cIAP-1, anti-cIAP-2, anti-IL-8, and anti-CXCL1 (R&D Systems); anti-IKKα, anti-IKBα/β, anti-phospho-IKKα/β, anti-MEK, anti-phospho-MEK, anti-ERK, anti-phospho-ERK, anti-p38, anti-phospho-p38, anti-JNK, and anti-phospho JNK (Cell Signaling); and anti-RIPK1 (Santa Cruz Biotechnology). Recombinant TNF-α was purchased from Roche. BV6 was from Genentech, and zVAD-fmk peptide was from Bachem. A/G agarose was from Santa Cruz Biotechnology. Unless otherwise indicated, all other reagents were purchased from Sigma Chemical Co. (Ireland).

Chemotaxis Assays

Chemotaxis assays were performed in Neuro Probe 10-Well Chemotaxis Chambers as per the manufacturer's instructions. Briefly, supernatants were generated from HeLa cells stimulated with anti-Fas for 24 hr in RPMI, 0.5% FCS. Supernatants (150 μl) were added to the bottom well of a chemotaxis chamber, and then 3–8 μm nitrocellulose filters were placed on top and secured by application of the top chamber, to which 250 μl of THP-1 cells or primary human peripheral blood neutrophils were added at 1–2 × 10^6/ml. Total cells entering the bottom chamber were counted after 1–2 hr. For antibody-mediated depletion of chemokines, supernatants were mock depleted with protein A/G agarose for 1 hr and then incubated with neutralizing antibodies (1–2 μg/ml) for 1 hr, followed by depletion of the antibody-protein complexes from the supernatants with A/G agarose. All cytokine and chemokine immunodepletions were confirmed by ELISA. Alternatively, HeLa cells were treated with anti-Fas (200 ng/ml) as above. After 24 hr, 2 × 10^5 cells were removed, along with their respective supernatants, to the bottom well of a chemotaxis chamber, followed by addition of CFSE-stained THP-1 monocytes to the top chamber as described above. Total migrated THP-1 cells were counted by UV microscopy.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article at http://dx.doi.org/10.1016/j.molcel.2013.01.025.

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(G) HeLa cells were transfected with nonsilencing siRNA or RIPK1-targeted siRNAs. Forty-eight hours after transfection, cells were treated with anti-Fas (250 ng/ml) for 24 hr, and then a THP-1 chemotaxis assay was performed with the resulting supernatants as described in (B). Results shown are representative of least three independent experiments. Error bars represent the mean ± SEM of triplicate cell counts from a representative experiment.

(H) Fas-induced apoptosis is associated with the production of chemokines that can act as chemotactic signals for monocytic cells and neutrophils. See also Figure S6.

Significance levels are ***$p < 0.01$, **$p < 0.05$, *$p < 0.1$, measured by Student's t test.

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