Molecular Determinants of Kinase Pathway Activation
by Apo2 Ligand/Tumor Necrosis Factor-related
Apoptosis-inducing Ligand  

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Apo2 ligand/tumor necrosis factor (TNF)2-related apoptosis-inducing ligand (Apo2L/TRLAIL) mainly activates programmed cell death through caspases. By contrast, TNF primarily induces gene transcription through the inhibitor of death through caspases. By contrast, TNF primarily induces gene homology in their extracellular, cysteine-rich domains. The intracellular signaling pathways of a primary, death-inducing signaling complex, subsequent to assembly of a primary, death-inducing signaling complex (DISC). The secondary complex retained the DISC components FADD and caspase-8, but recruited several factors involved in kinase activation by TNF, namely, RIP1, TRAF2, and NEMO/IKKγ. Secondary complex formation required Fas-associated death domain (FADD), as well as caspase-8 activity. Apo2L/TRLAIL stimulation of JNK and p38 further depended on RIP1 and TRAF2, whereas IKK activation required NEMO. Apo2L/TRLAIL induced secretion of interleukin-8 and monocyte chemoattractant protein-1, augmenting macrophage migration. Thus, Apo2L/TRLAIL and TNF organize common molecular determinants in distinct signaling complexes to stimulate similar kinase pathways. One function of kinase stimulation by Apo2L/TRLAIL may be to promote phagocytic engulfment of apoptotic cells.

Members of the tumor necrosis factor (TNF)2 superfamily regulate many physiological and pathological aspects of immune system development and function (reviewed in Refs. 1–5). The TNF ligand superfamily is defined by structural similarity and ability to recognize corresponding TNF receptor (TNFR) superfamily members, which share homology in their extracellular, cysteine-rich domains. The intracellular domains of TNFRs are devoid of enzymatic activity: The majority contain an amino acid sequence motif that mediates interaction with TNFR-associated factors (TRAFs). Conversely, a minority of TNFRs share an intracellular “death domain” motif, which interacts with adaptor molecules that contain related death domains. The signaling activities and biological functions of the death-domain-containing TNFR subgroup are diverse. A key signaling activity of Fas, which binds to Fas ligand (FasL), and of DR4 and DR5, which bind to Apo2L/TRLAIL, is apoptosis induction. On the other hand, TNFR1, which binds TNF and LT-α, primarily regulates transcription of pro-inflammatory and immunomodulatory genes and contributes to cell death signaling only in unique situations.

The apoptosis-signaling events that the death-inducing ligands FasL and Apo2L/TRLAIL trigger are relatively well defined (reviewed in Refs. 6–8). Binding of Fas, DR4, or DR5 by cognate ligand leads to conformational changes that promote interaction between the death domain of the receptor and the homologous region of the adaptor molecule Fas-associated death domain (FADD). FADD also contains a “death effector domain,” which mediates recruitment of two death effector domain-containing apical proteases: caspase-8 and caspase-10. This primary signaling complex, consisting of ligand, receptor, FADD, and apical caspase(s), is dubbed the death-inducing signaling complex (DISC) (9). Caspase-8 and -10 belong to the cysteine protease family, which plays a crucial role in programmed cell death. In living cells, caspase-8 and -10 are expressed as inactive precursors; in the DISC, these caspases become activated by oligomerization. Once activated, caspase-8 and -10 undergo proteolytic self-processing, in turn cleaving and activating downstream effector caspases, such as caspase-3, -6, and -7, which mediate the execution phase of apoptotic cell death (10, 11). In certain cell types, death receptor stimulation of caspase-8 and -10 triggers sufficient effector caspase activation to commit the cell to apoptotic death. In others, death requires further amplification of the apoptosis signal. This augmentation can be initiated by caspase-8-mediated cleavage of the proximal pro-apoptotic Bcl-2 family member Bid. The truncated form of Bid activates the distal pro-apoptotic Bcl-2 family members Bax and Bak, which trigger mitochondrial release of factors that promote activation of the apical protease caspase-9. In turn, caspase-9 stimulates further effector caspase activity, ensuring completion of the cell death program. A cellular factor called FLICE inhibitory protein (FLIP), which bears structural similarity to caspase-8 and -10 but lacks caspase activity, can inhibit death receptor-mediated apoptosis signaling (12). FLIP occurs in both short (FLIPs) and long (FLIPl) alternative mRNA splicing forms. Both forms can compete for apical caspase recruitment to the DISC, whereas FLIPs can inhibit the full processing of caspase-8 and -10.

In contrast to the apoptosis-initiating events triggered by binding of FasL and Apo2L/TRLAIL to their corresponding death receptors, TNF binding to TNFR1 leads to activation of the IKK/NF-κB, JNK, and p38 mitogen-activated protein kinase (MAPK) signaling pathways (13). Upon ligation, TNFR1 binds through its death domain to the adaptor

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The abbreviations used are: TNF, tumor necrosis factor; TNFR, TNF receptor; Ab, antibody; Apo2L, Apo2 ligand; cIAP, cellular inhibitor of apoptosis protein; DISC, death-inducing signaling complex; FADD, Fas-associated protein with death domain; FasL, Fas ligand; FBS, fetal bovine serum; GST, glutathione S-transferase; FLIP, cellular FLICE inhibitory protein; IκB, inhibitor of nuclear factor-κB; JNK, c-Jun amino-terminal kinase; NF-κB, nuclear factor-κB; RIP, Roswell Park Memorial Institute; siRNA, small interfering RNA; RIP, receptor interacting protein; TRADD, TNFR-associated death domain; TRAF, TNFR receptor-associated factor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; zVAD, benzyloxycarbonyl-Val-Ala-Asp (OMe)-fluoromethyl ketone; IKK, inhibitor of κB kinase; MAPK, mitogen-activated protein kinase; MEF, mouse embryo fibroblast; SAPK, stress-activated protein kinase; CHI, IL-8, interleukin-8; IB, immunoblot; MCP-1, monocyte chemoattractant protein-1.

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4 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–5S.
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molecule TNFR-associated death domain (TRADD). TRADD in turn binds two other adaptors: RIP1, which also contains a death domain and mediates activation of the IKK and p38 pathways, and TRAF2, which supports activation of IKK and JNK. TNFR1 activates the NF-κB pathway by inducing TRAF2-dependent formation of the signalosome complex, in which the scaffold protein NEMO/IκKγ and two kinases, IKKa and IKKB, are key catalytic components (14). IKK signalosome activation results in phosphorylation of the inhibitor of NF-κB (IκB), which in resting cells binds to NF-κB subunits and keeps them in the cytoplasm. Phosphorylation triggers proteosomal degradation of IκB, liberating NF-κB to move to the nucleus, where it can promote gene transcription. TRAF2 recruits two additional molecules, cellular inhibitor of apoptosis 1 (cIAP1) and cIAP2, to the TNFR1 complex, but the role of these cIAPs in TNFR1 signaling remains unclear.

In most cellular contexts, TNF activates the IKK, JNK, and p38 kinase pathways but not apoptotic caspase pathways. Induction of several NF-κB-dependent anti-apoptotic genes by TNF, including FLIP, cIAP1, cIAP2, XIAP, Bcl-XL, and A1, prevents apoptosis activation; however, under certain conditions, such as general inhibition of protein synthesis or specific blockade of NF-κB activation, TNF can stimulate a strong pro-apoptotic signal (reviewed in Refs. 15 and 16). Apoptosis initiation by TNF relies on the formation of a secondary intracellular signaling complex, composed of TRADD, RIP1, and TRAF2, as well as FADD and caspase-8 (17).

Although the strongest signaling activity of FasL and Apo2L/TRAIR is apoptosis induction in susceptible cells, there is accumulating evidence for the ability of these ligands to activate the IKK, JNK, and p38 pathways (reviewed in Refs. 18–23). NF-κB activation by TNF, FasL, and Apo2L/TRAIR is of particular interest, because of the anti-apoptotic activity of this transcription factor. Indeed, some tumors display a correlation between NF-κB activity and resistance to death-inducing ligands. Moreover, in some cancer cell lines, inhibition of NF-κB activity by various molecular approaches reverts resistance to cell death signaling by FasL and Apo2L/TRAIR (reviewed in Refs. 15, 24). However, the biological role and significance of kinase pathway activation in the context of apoptosis signaling by FasL and Apo2L/TRAIR are not well understood. Furthermore, in contrast to the well-defined events mediating caspase stimulation by these ligands, the molecular determinants underlying their kinase-activating function have been controversial. Published studies with FADD or caspase-8 null mice focused on apoptosis signaling and did not examine kinase pathway activation in detail (25, 26). Ectopic expression of dominant-negative FADD or caspase-8 mutants did not interfere with activation of NF-κB or JNK by TNF, FasL, or Apo2L/TRAIR (27). In contrast, detailed examination of mutant Jurkat T cell lines with deficiency in FADD or caspase-8 revealed an absolute necessity of these proteins for apoptosis as well as kinase pathway activation by death receptor ligands (28–31). More recent experiments utilizing siRNA-based gene silencing techniques have confirmed the requirement of FADD and caspase-8 for kinase pathway activation by FasL, although Apo2L/TRAIR was not investigated (32). Additional studies yielded conflicting data supporting or excluding a direct involvement of TRAF2 and RIP1 in activation of JNK and NF-κB by FasL and Apo2L/TRAIR (31–34).

Although important insights into kinase pathway activation by FasL and Apo2L/TRAIR have been achieved to date, we aimed in this present study to attain further elucidation of the molecular components and events that mediate this function, specifically as it pertains to Apo2L/TRAIR. Kinase pathway adaptors such as TRADD and RIP1 were reported to interact with the Apo2L/TRAIR receptors DR4 and DR5 in overexpression model systems (reviewed in Ref. 2). In DR4/5-transfected HEK 293 and HeLa cells, RIP1 is associated with unstimulated DR4 and DR5, increases in abundance after ligand stimulation, and undergoes caspase-dependent cleavage (35). However, studies that examined associations between endogenous cellular components did not confirm recruitment of TRADD, RIP1, or TRAF2 to the Apo2L/TRAIR DISC (22, 36–38). Here, we demonstrate the formation of a secondary signaling complex subsequent to assembly of the Apo2L/TRAIR DISC and identify several components of this complex that are crucial for kinase pathway activation. In addition, we provide evidence that kinase stimulation by Apo2L/TRAIR is associated with increased chemokine production and macrophage migration, suggesting that one role of kinase activation by this pro-apoptotic ligand may be to promote the recruitment of phagocytes to the site of apoptosis.

Materials and Methods

Cell Lines and Reagents—HT1080 human fibrosarcoma cells, A549 human lung carcinoma cells, SK-MES-1 human lung squamous carcinoma cells, human melanoma UACC62 cells, human colorectal adenocarcinoma HT-29 and HCT15 cells, and human histiocytic carcinoma HeLa cells were obtained from the ATCC. TRAF2-deficient and matched wild-type mouse embryonic fibroblasts were kindly provided by Dr. David Goeddel. Jurkat wild-type (A3 clone), FADD-deficient (E1), and caspase-8-deficient (I9.3) cells were kindly provided by Dr. John Blenis. Adherent cell lines were grown in 50:50 Dulbecco’s modified Eagle’s and FK12 medium supplemented with 10% FBS, penicillin, and streptomycin. Jurkat cell lines were grown in RPMI medium. Human recombinant soluble Apo2L/TRAIR in non-tagged or FLAG-tagged versions, FLAG-tagged FasL, and anti-DR5 (JH3) biotinylated monomeric antibodies were prepared as described (Sharp et al. (39)). Human recombinant soluble TNF was from Genentech Inc. For immunoprecipitation experiments the following Abs were used: anti-FLAG (M2, Sigma); anti-FADD (no. 06-711) and anti-TRADD (3E11, no. 05-473) from Upstate Biotechnology, NY; anti-RIP1 (G322-2, no. 551042) from BD Bioscience; anti-IκB (G151-333, no. 554286) from BD Bioscience; anti-caspase-8 (for DISC experiments, 5F7, no. IM3148) from Immunotech, France; anti-Caspase-8 (1.C12, no. 9746), anti-phospho-SAPK/JNK (G9, no. 9255), anti-SAPK/JNK (no. 9258), anti-phospho-p38 MAPK (no. 9211), anti-p38 MAPK (no. 9212), anti-phospho-1xBα (no. 9241), anti-1xBα (no. 9242) from Cell Signaling Technology, Inc.; anti-IκB-K (no. AF1846) from R&D Systems, Inc.; anti-TRAF2 (H-249, no. sc-7187) from Santa Cruz Biotechnology, Inc., CA; and anti-Actin (C4, no. 69100) from ICN Biomedicals, Inc., OH. As the secondary reagents the following horseradish peroxidase-conjugated Abs were used: anti-mouse-IgG1 (no. 559626) from BD Bioscience; anti-mouse-IgG2a and anti-mouse-IgG2b (no. 190-05) from Southern Biotechnology Associates; anti-rabbit-IgG (no. 711-056-152) from Amersham Biosciences. Recombinant c-Jun fusion protein (no. 6093) was purchased from Cell Signaling Technology, Inc.
Gene Silencing Experiments—Sequences of siRNA oligonucleotides were designed by using the Dharmacon siDesign Center (Dharmacon Research Inc., Lafayette, CO) software and synthesized at Genentech Inc. The following siRNA pairs were used for gene knockdown experiments: FADD, 5'-CCAGAGAAGGGAAACCGA and 5'-UCCGCUUCUCUCUGUGG (pair no. 4); RIP1, 5'-CAGCCUGUUGCAGCACA and 5'-UUGGACUGAAACCCGCGU (no. 16); 5'-CCAC-UAGUGUGAAGAA and UUAUCUGCACAGUUGGG (no. 70); 5'-GAGGAUGAUAACGGA and 5'-UUGGUUGUAUGGAUCA-CUCUUU (no. 71); 5'-GAAGAAGUAAUCAAGGAA and 5'-UCCG-UUGGUAACUCCUUU (no. 178); caspase-8, 5'-UGAAGAUAUA-CAAACGACUA and 5'-UUGACUGUGUAUAAUCUUA (no. 86); 5'-GUAUCCUGUUGAAGUCA and 5'-UCAGUCUCACAGUG-GAUAC (no. 87); 5'-GAUUAUAUCAUCCAACAA and 5'-UUUGA-GUGUAGUAUAAC (no. 88); TRAF2, 5'-CUGGCUCCUCUG-UCCAGA and 5'-UCUGCAAGGACUCGCAC (no. 11); 5'-GGUC-UUGGAGAUUGAAGGCA and 5'-UGCCUCUACUCCAGACCC (no. 12); JNK1, 5'-UGAUGUGCUCUAAUGUCA and 5'-UGACAU-UAGAAGAACAUAU (no. 110); 5'-UAAGCCGCAUUCAGGAA and 5'-UUCGUGGAAGUGCCGCUA (no. 111); JNK2, 5'-CCAA-CUACUUGAAGGAGA and 5'-UCUUUUGUGAAGUUUGG (no. 118). Cells were transfected as described previously (39).

Activation of JNK, p38 MAPK, and IKK/NF-κB—Cells were seeded into 10-cm dishes and in the case of gene silencing experiments were transfected as described above. Twelve hours before treatment cells were washed once with phosphate-buffered saline, and the growth media were replaced by media containing 2% heat-inactivated FBS. 8–12 h before treatment cells were washed once with phosphate-buffered saline, and the growth media were replaced by media containing 2% heat-inactivated FBS. On the next day, cells were detached from the plates with non-enzymatic solution (Sigma) and placed into 24-well dishes (0.5–2 × 10^6 cells/well) in 0.25 ml of media containing 2% heat-inactivated FBS. In the gene silencing experiments, cells were treated with 0.25 ml of media containing 200 ng/ml Apo2L/TRAIL or 30 ng/ml TNF. In other experiments cells were treated with the ligand concentrations indicated in the figure legends. Upon treatment cells were lysed with a kinase lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton, 1 μM phosphatase inhibitor mixture II (no. P-5726, Sigma)) and analyzed by SDS-PAGE followed by immunoblot analysis, using phospho-specific anti-p38, -JNK, -IκBα, or total IκBα antibodies and ECL kit (Amersham Biosciences).

In Vitro JNK1 Assay—Cells were prepared and treated similarly to the immunoblot analysis. Upon lysis, JNK1 was immunoprecipitated, and in vitro kinase reaction was performed using recombinant GST-c-Jun substrate as described before (40). 32P incorporation into GST-c-Jun was determined by SDS-PAGE analysis followed by autoradiography.

Cytotoxicity Assays—Cells (1–1.5 × 10^4 per well) were seeded into 96-well dishes in media containing 5% heat-inactivated FBS. 8–12 h later the media was changed, and cells were treated with human Apo2L/TRAIL, FasL, or TNF (cycloheximide was used in the latter case at 0.5–2 μg/ml, as indicated in the figure legends) for 18–24 h. Cell viability was measured by neutral red uptake as described (41).
of kinase activation, we analyzed the induction of mRNA encoding the NF-κB-controlled gene c-IAP2 (Fig. 1D). Apo2L/TRAIL induced ~60-fold up-regulation of c-IAP2 mRNA, as compared with induction of ~950-fold by TNF (Fig. 1D). Together, these results show that Apo2L/TRAIL is indeed capable of stimulating the JNK, p38, and IKK kinase pathways, albeit less rapidly and much less potently than TNF.

Apo2L/TRAIL Induces Formation of a Secondary Signaling Complex—Recent work by Tschopp and colleagues (17) demonstrates that, after forming a primary complex that supports kinase pathway activation, TNF stimulates a secondary signaling complex that mediates apoptotic caspase activation. We hypothesized that Apo2L/TRAIL may act in converse fashion, first inducing a DISC that activates caspases, and then a secondary complex that activates specific kinase pathways.

To examine the possibility that Apo2L/TRAIL induces assembly of a secondary signaling complex, we performed an initial immunoprecipitation of the DISC from lysates of ligand-stimulated cells through the ligand itself, and then subjected these DISC-depleted lysates to an additional immunoprecipitation, this time using antibodies to FADD, RIP1, TRAF2, or NEMO. Consistent with earlier work in non-transfected cells (36–38), within 5 min of addition to HT1080 cells, Apo2L/TRAIL stimulated the assembly of a DISC containing DR5 (DR4 is not expressed in these cells), FADD, and caspase-8, but not RIP1 (Fig. 2). Like RIP1, TRAF2 and NEMO also were absent from the DISC (data not shown). IB analysis of the second immunoprecipitation with DR5 antibody confirmed the absence of DR5, verifying complete DISC depletion (Fig. 2). FADD immunoprecipitation of DISC-depleted lysates revealed association of FADD with caspase-8 and RIP1; RIP1 immunoprecipitation indicated binding of RIP1 to FADD and caspase-8; TRAF2 immunoprecipitation suggested interaction of TRAF2 with FADD, caspase-8, and weak association with RIP1; and NEMO immunoprecipitation revealed weak NEMO interaction with FADD and stronger association with caspase-8 and RIP1 (Fig. 1).

Most of these interactions occurred within 5 min of Apo2L/TRAIL stimulation, except for TRAF2 association with FADD, which appeared a bit later, at 15 min. The interaction of RIP1 with FADD was transient, peaking at 5 min, whereas the association of RIP1 with NEMO seemed to increase over time. (Because of high background, immunoprecipitation of TRADD, or clear IB detection of TRADD, TRAF2, or NEMO were not possible.) These results indicate that Apo2L/TRAIL induces the formation of a secondary signaling complex (or complexes), which lacks DR5, but retains FADD and caspase-8, and at minimum recruits RIP1, TRAF2, and/or NEMO.

Involvement of FADD, RIP1, and TRAF2 in Kinase Pathway Activation by Apo2L/TRAIL—To explore the importance of the secondary Apo2L/TRAIL-induced complex for kinase pathway activation, we used siRNA technology to knock down individual signaling components. FADD siRNA knockdown attenuated the induction of JNK, p38 and IκB phosphorylation and of IκB degradation by Apo2L/TRAIL, but not by TNF (Fig. 3, A and B). Analysis of IκB depletion in a previously characterized mutant variant of the Jurkat T cell line that does not express FADD (29) further confirmed the importance of FADD for NF-κB activation by Apo2L/TRAIL but not by TNF (Fig. S1A). In addition, FADD siRNA knockdown inhibited Apo2L/TRAIL-, but not TNF-induced cIAP2 mRNA expression (Fig. S1B). FADD siRNA transfection protected HT1080 cells from induction of cell death by Apo2L/TRAIL or
TNF plus cycloheximide, but not by the DNA-damaging agent etoposide (Fig. 3C), verifying an effective and selective FADD knockdown.

TRADD serves as a universal adaptor for TNFR1-mediated kinase pathway activation by TNF (43). TRADD is capable of associating with TNFR1, as well as with RIP1, TRAF2, and FADD, which cannot bind directly to TNFR1. Our attempts to knock down TRADD expression in HT1080 and other cell lines by siRNA failed (we tested 16 different siRNA duplexes). Because TRADD knock-out mouse cells were not available at the time of this work, we were unable to investigate the potential involvement of this adaptor in kinase activation by Apo2L/TRAIL. We therefore turned to studying the roles of RIP1 and TRAF2 in more detail.

RIP1 is a death domain containing kinase that binds to TNFR1 in a TRADD-dependent manner and is essential for TNFR1 activation of the NF-κB and p38 MAPK pathways (44, 45). Recent work suggests that RIP1 contributes also to full JNK activation by TNF (44). There is also some evidence implicating RIP1 in kinase pathway activation by Apo2L/TRAIL or Fasl (31, 34). RIP1 siRNA knockdown in HT1080 cells did not attenuate Apo2L/TRAIL stimulation of JNK, but it substantially inhibi-
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A

B

C

D

FIGURE 4. Caspase-8 is required for effective cell death induction and activation of JNK, p38, and NF-κB by Apo2L/TRAIL. A, effect of caspase-8 knockdown on phosphorylation of JNK, p38, and IκB, and degradation of IκB induced by Apo2L/TRAIL or TNF. HT1080 cells were transfected with caspase-8-specific (C-8) or control (C) siRNA duplexes. After 48 h, the cells were treated with Apo2L/TRAIL or TNF for the indicated periods of time, lysed, and subjected to immunoblot analysis as in Fig. 3. The level of caspase-8 in cells transfected with siRNA against caspase-8 compared with control siRNA is shown at the bottom. B, effect of caspase-8 knockdown on the cytotoxic activity of Apo2L/TRAIL, TNF, or etoposide toward HT1080 cells. The graphs depict the proportion of cells, transfected with caspase-8 (black bars) or control (white bars) siRNA, remaining viable after incubation with the indicated ligands for 24 h. C, effect of zVAD on induction of JNK, p38, and IκB phosphorylation by Apo2L/TRAIL or TNF in HT1080 cells. Cells were preincubated for 1 h with zVAD (20 μM) or Me2SO vehicle control, treated with Apo2L/TRAIL or TNF, lysed, and analyzed as above. The effect of zVAD on caspase-8 activation by Apo2L/TRAIL or TNF treatment of HT1080 cells is shown in the middle panel. D, effect of zVAD on primary (right panel) and secondary (left panel) Apo2L/TRAIL signaling complexes. HT1080 cells were pretreated for 1 h with zVAD (20 μM) or a Me2SO control, then treated with Apo2L/TRAIL, and signaling complexes were analyzed as above.

JNK signaling plays an important contextual role in the control of TNF-induced cell death (16). Recent evidence indicates that JNK1 has a dominant-positive signaling function, whereas JNK2 plays an inhibitory role in apoptosis activation by TNF (48, 49). Consistently with these findings, siRNA knockdown of JNK1 in HT1080 cells diminished TNF-induced cell death, whereas knockdown of JNK2 did not (Fig. S3). In contrast, siRNA knockdown of either JNK1 or JNK2 or both did not influence Apo2L/TRAIL-induced cell death (Fig. S3). Thus, whereas...
JNK activity modulates cell death induction by TNF but not by Apo2L/TRAIL. TRAF2 seems to modulate the cytotoxicity of both ligands. Of note, knockdown of JNK1 did not reverse the sensitization of TRAF2-depleted HT1080 cells to death induction by either TNF or Apo2L/TRAIL (Fig. S3), indicating that cell death modulation by TRAF2 may be independent of and dominant over regulation by JNK activity.

Caspase-8 Activity Is Important for Effective Kinase Pathway Activation by Apo2L/TRAIL—In agreement with published data (26, 28, 38, 50, 51), siRNA knockdown of caspase-8 protected cells against death induction by Apo2L/TRAIL or TNF, but did not influence TNF-induced kinase activation (Fig. 4, A and B). However, caspase-8-depleted HT1080 cells showed markedly diminished Apo2L/TRAIL-induced phosphorylation of JNK, p38, and IκB and degradation of IκB (Fig. 4A). Although caspase-8 knockdown was less efficient in SK-MES-1 or A549 cells, these cell lines showed a similar, though less complete, attenuation or delay in kinase activation (Fig. S4). Additionally, a caspase-8-deficient Jurkat T cell line variant showed little or no IκB degradation in response to Apo2L/TRAIL, whereas wild-type Jurkat T cells showed significant IκB depletion (Fig. S1). Caspase-8 siRNA knockdown in HT1080 cells inhibited cIAP2 mRNA induction by Apo2L/TRAIL (Fig. 1B). These results generally agree with recent data implicating a structural and/or enzymatic caspase-8 requirement for effective activation of JNK and NF-κB by FasL and Apo2L/TRAIL (31). To examine further the potential importance of enzymatic caspase-8 activity for kinase stimulation by Apo2L/TRAIL, we used the pan caspase inhibitor Z-Val-Ala-Asp (OMe)-fluoromethyl ketone (zVAD) (Figs. 4C and S5). Pretreatment of HT1080 cells by zVAD blocked the activation of caspase-8 by Apo2L/TRAIL (Fig. 4C, bottom panel; depletion of pro-caspase-8 p55/p53 bands indicates stimulation). Pretreatment with zVAD substantially abrogated Apo2L/TRAIL activation of JNK and p38 while leaving TNF stimulation of these kinases intact (Figs. 4C and S5). Additionally, zVAD delayed (although it did not abolish) IκB phosphorylation in response to Apo2L/TRAIL and left TNF induction of these events unimpeded (Figs. 4C and S5).

Analysis of the effect of zVAD on Apo2L/TRAIL-induced DISC assembly showed enhanced and prolonged association between DR5 and FADD (Fig. 4D, left panels), indicating that inhibition of caspase activity by zVAD stabilized the DISC. In contrast to its effect on the DISC, zVAD delayed and attenuated formation of the secondary Apo2L/TRAIL-induced complex (Fig. 4D, right panels). These results suggest that caspase-8 activity is important for second complex formation and kinase activation by Apo2L/TRAIL. To investigate further the importance of caspase-8 activity for second complex formation, we took advantage of the fact that the caspase-related molecule FLIP<sub>L</sub> functions as an inhibitor of caspase-8 activation by Apo2L/TRAIL (39). We therefore examined the effect of siRNA knockdown of FLIP<sub>L</sub> on ligand activation of the IκK/NF-κB pathway in HT1080 cells. Consistent with earlier data, FLIP<sub>L</sub> knockdown augmented Apo2L/TRAIL induction of DISC recruitment and processing of caspase-8 in the Apo2L/TRAIL DISC (Fig. 5A), potentiating consequent cell death (data not shown). FLIP<sub>L</sub> knockdown also enhanced the induction of IκB phosphorylation and degradation by Apo2L/TRAIL, but had little effect on stimulation of these events by TNF (Fig. 5B). Furthermore, siRNA knockdown of FLIP<sub>L</sub> potentiated cIAP2 mRNA induction by Apo2L/TRAIL (Fig. 5B). These data lend further support to the notion that enzymatic caspase-8 activity (which is stimulated better in the absence of FLIP<sub>L</sub>) contributes to the formation of a secondary signaling complex that mediates kinase activation by Apo2L/TRAIL.

A Potential Biological Function for Kinase Pathway Activation by Apo2L/TRAIL—An important outcome of NF-κB activation by TNF is the induction of anti-apoptotic genes that can override the apoptosis-inducing activity of TNF, enabling the dominance of TNF pro-inflammatory function (16). We were interested in asking whether NF-κB activation by Apo2L/TRAIL affects the pro-apoptotic activity of this ligand; however, the importance of caspase-8 for Apo2L/TRAIL stimulation of kinase pathways precluded us from direct study of this question. To circumvent this limitation, we pretreated HT1080 cells with TNF to stimulate kinase pathways and subsequently examined the ability of Apo2L/TRAIL to trigger cell death. Surprisingly, TNF pretreatment provided little or no protection against induction of cell death by Apo2L/TRAIL in HT1080, A549, or HeLa cells, and if anything, it augmented Apo2L/TRAIL cytotoxicity against SK-MES-1 and HCT15 cells (Fig. 6). These results suggest that cell death induction by Apo2L/TRAIL...
TRAIL overrides the potential anti-apoptotic effects of kinase pathway activation by TNF.

Given these observations, we reasoned that the caspase-8-dependent stimulation of kinase pathways by Apo2L/TRAIL might serve to support rather than to inhibit the apoptotic program initiated by this ligand. Consistent with this notion, Apo2L/TRAIL induced significant release of the chemokines IL-8 and MCP-1 in HT1080 cells; in contrast, it had little effect on production of these chemokines in HT29 cells, which are resistant to apoptosis induction by trimeric Apo2L/TRAIL (Fig. 7A). TNF induced release of higher levels of both chemokines in both cell lines, with particularly high levels of IL-8 in HT1080 cells (Fig. 7A). To assess further the biological relevance of the chemokine levels induced by Apo2L/TRAIL, we tested the activity of conditioned media from ligand-treated cells in a Transwell cell migration assay. Conditioned media from Apo2L/TRAIL-treated HT1080 cells induced a substantial increase in the migration of human peripheral blood macrophages as compared with media from buffer-treated cells, whereas media from similarly treated HT29 cells did not alter macrophage migration (Fig. 7B). These results suggest that kinase activation by Apo2L/TRAIL increases chemokine secretion and macrophage attraction.

DISCUSSION

Earlier studies have provided evidence for the ability of the death-inducing ligands FasL and Apo2L/TRAIL to stimulate the IKK/NF-κB, JNK, and p38 MAPK pathways. However, most of the information about this activity was obtained under non-physiological conditions, namely, in the context of death receptor overexpression or cell death blockade (3, 52–54). In the present study, we used HT1080 cells as a primary model system, because these cells are only partially sensitive to Apo2L/TRAIL-induced apoptosis, and hence more amenable to physiological interrogation of kinase pathway activation. Apo2L/TRAIL treatment of HT1080 cells induced not only cell death but also activation of JNK, p38, and NF-κB, as it did in several other cell lines. Kinase pathway activation by Apo2L/TRAIL was significantly slower and weaker than stimulation by TNF, suggesting either that the molecular determinants of kinase activation by these ligands differ, or alternatively, that the two ligands use similar components, which they organize differentially in distinct signaling complexes. Consistent with the latter possibility, we found that Apo2L/TRAIL assembles not only a receptor-associated DISC, but also a secondary intracellular signaling complex (or complexes) containing molecules that are involved also in kinase pathway activation by TNF. Ligation of DR5 by Apo2L/TRAIL led to rapid recruitment of FADD and caspase-8 to the receptor, forming a DISC. The Apo2L/TRAIL DISC did not contain detectable RIP1, TRAF2, or NEMO. However, examination of secondary Apo2L/TRAIL-induced intracellular interactions by removal of the primary DISC revealed rapid organization of a complex that lacked the ligand and receptor, but contained FADD, caspase-8, RIP1, TRAF2, and NEMO. TRADD also may be involved in this secondary complex, but we were unable to verify this because of technical immunoblot and siRNA limitations. Thus, TNF and Apo2L/TRAIL appear to trigger two conversely related sets of intracellular interactions. TNF assembles a primary complex that signals kinase pathway activation and a secondary intracellular complex containing FADD and caspase-8 that under specific permissive conditions triggers apoptosis (17). Apo2L/TRAIL, on the other hand, assembles a primary complex that signals apoptosis and a secondary intracellular complex that stimulates kinase pathway activation. In each case, association of the secondary complex depends on formation of the primary complex and presumably requires dissociation of the primary complex from the receptor. The secondary complex assembled by Apo2L/TRAIL may represent multiple complexes containing overlapping and/or distinct components. The differences in
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Apo2L/TRAIL induces chemokine secretion and macrophage migration. A, induction of cell secretion of IL-8 and MCP-1 by Apo2L/TRAIL or TNF. HT1080 or HT29 cells were treated by the indicated ligand for 8 h. Conditioned media were collected, and the concentrations of IL-8 and MCP-1 were determined by cytometric bead array immunoassay as described under “Materials and Methods.” B, induction of macrophage migration by cell-conditioned media. The ability of conditioned media from cells pretreated with Apo2L/TRAIL or TNF to induce Transwell migration of human peripheral blood macrophages was analyzed as described under “Materials and Methods.” Means ± S.D. for triplicate determinations of migrating cell numbers are shown.

kinetics and strength of the associations between FADD, RIP1, and NEMO in response to Apo2L/TRAIL support the potential existence of multiple complexes, but this warrants further study.

Our functional investigation revealed that Apo2L/TRAIL requires FADD both for cell death induction and for kinase stimulation. Activation of p38 and IKK by Apo2L/TRAIL negatively regulates cell death induction. However, TNF, which strongly activates p38 and IKK signaling, did not inhibit cell death induction by Apo2L/TRAIL, suggesting that RIP1 inhibits Apo2L/TRAIL-induced apoptosis independently of its involvement in kinase activation. RIP1 may influence apoptosis indirectly, by modulating the secondary Apo2L/TRAIL signaling complex, perhaps through interaction with TRAF2 (55). Indeed, TRAF2 depletion enhanced caspase-8 activation by Apo2L/TRAIL (see below).

Apo2L/TRAIL stimulation of p38 and IKK did not require TRAF2. In contrast to p38 and IKK activity, JNK enzymatic activity was significantly reduced in TRAF2-null MEFs. Despite this reduction in JNK activity, both the basal and ligand-induced levels of JNK1 and JNK2 phosphorylation were higher in the absence of TRAF2 in all of the cell lines examined. Recent studies suggest that JNK1 promotes c-Jun phosphorylation and activation of AP-1 and ATF2, whereas JNK2 inhibits these effects by attenuating JNK1 activity and destabilizing c-Jun (48, 49). Thus, the reduction in JNK1 activity in TRAF2-deficient cells may result from dominant inhibition of phosphorylated JNK1 by phosphorylated JNK2.

TRA2 depletion surprisingly sensitized cells to death induction by Apo2L/TRAIL, FasL, as well as TNF. This finding conflicts with earlier data (obtained in overexpression studies and in the presence of high cycloheximide concentrations), which suggested that TRAF2 is not involved in modulation of apoptosis signaling by Apo2L/TRAIL and FasL (34). How might depletion of TRAF2 promote apoptosis signaling? It is well established that sustained JNK1 activation by TNF supports the ability of TNF to induce cell death (56, 57). Consistent with this, and with the inhibitory effect of JNK2 on JNK1 activity, JNK1 knockdown prevented TNF-induced cell death, whereas JNK2 knockdown potentiated this response. However, knockdown of JNK1 or JNK2 did not reverse the sensitization to TNF cytotoxicity by TRAF2 depletion, suggesting that the modulation of death signaling by TRAF2 does not involve JNK. Unlike TNF, there is no published evidence supporting a significant contribution of JNK to apoptosis induction by Apo2L/TRAIL and FasL. Indeed, siRNA knockdown of JNK1, JNK2, or both did not alter Apo2L/TRAIL-induced cell death, nor did it reverse the sensitization to this ligand by TRAF2 knockdown. Taken together, these results suggest that TRAF2 depletion causes sensitization to death ligand-induced apoptosis independently of JNK. Moreover, while JNK activation modulates TNF induction of cell death, it does not significantly impact the cytotoxic activity of Apo2L/TRAIL.

In agreement with previous studies (28), we found that Apo2L/TRAIL requires caspase-8 not only for induction of cell death, but also for kinase pathway activation. Does a mere scaffolding function or the actual enzymatic activity of caspase-8 contribute to kinase stimulation? Reconstitution of caspase-8-deficient Jurkat T cells with an enzymatically inactive caspase-8 mutant was sufficient to restore NF-κB induc-
Kinase Pathway Activation by Apo2L/TRAIL

tion by Fasl (32), suggesting a lack of requirement for caspase-8 enzymatic activity. Conversely, a catalytically inactive caspase-8 mutant blocked NF-κB induction by transiently transfected death receptors, FADD, or wild-type caspase-8 (58), supporting a requirement for enzymatic activity. We found that exposure of cells to zVAD, which blocks various caspases, including caspase-8, stabilizes the Apo2L/TRAIL DISC, and decelerates assembly of the secondary complex. Furthermore, zVAD treatment inhibited or delayed kinase pathway activation by Apo2L/TRAIL. In addition, depletion of FLIP, a molecule that inhibits recruitment and activation of caspase-8 at the DISC (39), augmented kinase activation by Apo2L/TRAIL. These results indicate that the enzymatic activity of caspase-8 is important not only for apoptosis induction but also for kinase pathway activation by Apo2L/TRAIL. These findings do not rule out the possibility that some scaffolding role of caspase-8 also may contribute to kinase pathway stimulation. Given that FADD is retained in the secondary complex, it is plausible that caspase-8 activity facilitates dissociation of FADD from the receptor, but this requires further study.

Caspase stimulation by the TNF secondary signaling complex requires inhibition of NF-κB activation by the TNF primary signaling complex (17). Our results show by contrast that caspase activation by the primary signaling complex of Apo2L/TRAIL promotes the formation of a secondary complex, which in turn leads to kinase pathway activation. One function of the secondary complex may be to curtail the level of caspase activation by Apo2L/TRAIL through an unidentified activity of TRAF2. Nonetheless, kinase pathway activation is not a dominant inhibitory determinant for, and in some cell lines it may in fact facilitate apoptosis induction by Apo2L/TRAIL.

In conclusion, our studies show that Apo2L/TRAIL induces kinase pathway activation downstream of DISC assembly and caspase-8 stimulation. Kinase pathway activation by Apo2L/TRAIL is associated with increased production of the chemokines IL-8 and MCP-1 and with enhanced macrophage migration. These results raise the possibility that, within a tissue context, Apo2L/TRAIL activates specific kinase pathways in conjunction with caspase activation to promote chemokine-supported phagocytosis of apoptotic cells (59).

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