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Bone Marrow Stroma Confers Resistance to Apo2 Ligand/TRAIL in Multiple Myeloma in Part by Regulating c-FLIP

Lia Elena Perez, Nancy Parquet, Kenneth Shain, Ramadevi Nimmanapalli, Melissa Alsina, Claudio Anasetti, and William Dalton

Apo2 ligand (Apo2L)/TRAIL induces apoptosis of cancer cells that express the specific receptors while sparing normal cells. Because the tumor microenvironment protects myeloma from chemotherapy, we investigated whether hemopoietic stroma induces resistance to Apo2L/TRAIL apoptosis in this disease. Apo2L/TRAIL-induced death was diminished in myeloma cell lines (RPMI 8226, U266, and MM1s) directly adhered to a human immortalized HS5 stroma cell line but not adhered to fibroblastic. In a Transwell assay, with myeloma in the upper well and HS5 cells in the lower well, Apo2L/TRAIL apoptosis was reduced when compared with cells exposed to medium in the lower well. Using HS5 and myeloma patients' stroma-conditioned medium, we determined that soluble factor(s) produced by stroma–myeloma interactions are responsible for a reversible Apo2L/TRAIL apoptosis resistance. Soluble factor(s) attenuated caspase-8, caspase-3, and poly(ADP-ribose) polymerase cleavage and diminished mitochondrial membrane potential changes without affecting Bcl-2 family proteins and/or Apo2L/TRAIL receptors. Soluble factor(s) increased the baseline levels of the anti-apoptotic protein c-FLIP in all cell lines tested. Inhibition of c-FLIP by means of RNA interference increased Apo2/TRAIL sensitivity in RPMI 8226 cells. Unlike direct adhesion to fibroblastic, soluble factor(s) have no impact on c-FLIP redistribution within cellular compartments. Cycloheximide restored Apo2L/TRAIL sensitivity in association with down-regulation of c-FLIP, suggesting that c-FLIP synthesis, not intracellular traffic, is essential for soluble factor(s) to regulate c-FLIP. Additionally, IL-6 conferred resistance to Apo2L/TRAIL-mediated apoptosis in association with increased c-FLIP levels. In conclusion, the immune cytotoxic effect of Apo2L/TRAIL can be restored at least in part by c-FLIP pathway inhibitors. The Journal of Immunology, 2008, 180: 1545–1555.

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2 Address correspondence and reprint requests to Dr. Lia Elena Perez, 12902 Magnolia Drive, West Clinic Building-BMT 2nd floor Blood and Marrow Transplantation Program, Tampa, FL 33612. E-mail address: lia.perez@moffitt.org
3 Abbreviations used in this paper: Apo2L, Apo2 ligand; BM, bone marrow; BMS, bone marrow stroma; CHX, cycloheximide; c-FLIP, cellular FLIP long form; CM, conditioned medium; EM-DR, environmental mediated-death resistance; EM-IR, environmental mediated-immune resistance; FN, fibronectin; HS5-GFP, HS5 cell line that expresses GFP, PARP, poly(ADP-ribose) polymerase; 7-AAD, 7-amino actinomycin-D; TW, Transwell; TW + HS5, Transwell with HS5 stromal cells; TW – HS5, Transwell without HS5 stromal cells.
improve Apo2L/TRAIL function. In this study, we investigated EM-DR in multiple myeloma because it is clear that this disease is subject to environmental influences (22), and Apo2L/TRAIL anti-myeloma activity has been confirmed in xenografted mice (7, 23). We demonstrate herein that Apo2L/TRAIL-mediated apoptosis is significantly reduced in myeloma cell lines directly attached to hemopoietic stroma in vitro (HS5 cells) but not when adhered to fibronectin (FN). Treatment of myeloma using a Transwell (TW) membrane assay or HS5 and myeloma patients’ stroma-conditioned medium has helped to elucidate that released soluble factors are responsible for a reversible Apo2L/TRAIL apoptosis resistance. Soluble factors inhibit Apo2L/TRAIL-mediated apoptosis in part by increasing baseline levels of c-FLIP. Myeloma Apo2L/TRAIL sensitivity was restored by reducing c-FLIP levels by RNA interference, demonstrating a more direct role for c-FLIP. Downregulation of c-FLIP by pretreatment with cyclohexamide (CHX) restored Apo2L/TRAIL sensitivity, indicating that protein synthesis is key in regulating c-FLIP accumulation. Unlike direct adhesion to FN, which confers resistance to Fas but not Apo2L/TRAIL apoptosis, soluble factors have no impact on c-FLIP redistribution within cellular compartments (14). IL-6 confers resistance to Apo2L/TRAIL-mediated apoptosis by increasing c-FLIP levels, among other unknown mechanisms. Our results show that interactions between the bone marrow stroma and multiple myeloma cells, via IL-6 and/or other soluble factors, promotes resistance to immune control by Apo2L/TRAIL at least in part by the anti-apoptotic factor c-FLIP.

Materials and Methods
Multiple myeloma and bone marrow stroma (BMS)
RPMI 8226 and U266 myeloma cells lines were obtained from the American Type Culture Collection. MM1s cell line was provided by Dr. S. Rosen from Northwestern University (Chicago, IL). Cell lines were maintained in RPMI 1640 medium (Mediatech-Cellgro) supplemented with 10% heat-inactivated FBS (Omega Scientific). The HS5 and the HS27a human cell lines were obtained from the AmericanType Culture Collection and maintained in RPMI 1640 medium supplemented with 10% FBS. A variant of the HS5 cell line expressing GFP (HS5-GFP) was developed in our laboratory by stably expressing enhanced GFP under hygromycin (Invitrogen Life Technologies) selection (100 μg/ml).

Human bone marrow (BM) was obtained, with Institutional Review Board approval, by aspiration from the posterior iliac crest of fully informed multiple myeloma patients. BM mononuclear cells isolated by Ficoll-Hypaque gradient purification (Amersham Biosciences) were cultured in MEM (Invitrogen Life Technologies) supplemented with 20% FBS (Invitrogen Life Technologies) and 1% penicillin with streptomycin at a concentration of 2 × 10^6 cells/ml. After 2 days, the nonadherent cells were removed and the adherent BMS
were grown to >80% confluence in the original medium. A series of six experiments, the same passage of RPMI 8226 cells was treated with Apo2L/TRAIL (10 ng/ml) for 5 h in suspension or adhered to HS5-GFP cells (left) or in a TW system (middle). To this end, myeloma cells were separated using a TW membrane from HS5-GFP cells (TW + HS5) or medium only (TW – HS5). The same passage of RPMI 8226 cells was treated with Apo2L/TRAIL (10 ng/ml) in the presence of CM derived from HS5 stroma (right). Represented is percentage specific apoptosis from six experiments with RPMI 8226 cells exposed to HS5-secreted soluble factors (black bars) or their controls (white bars).

**Compounds**

Human recombinant Apo2L/TRAIL (Alexis Biochemicals) was reconstituted in 20 mM HEPES (pH 7.4) containing 300 mM NaCl, 0.006% Tween 20, 1% sucrose, and 0.05 mM DTT and stored at −80°C before use. Apo2L/TRAIL was used at different doses (5–50 ng/ml) as specified in each experiment. Anti-Apo2L/TRAIL mAb (Alexis Biochemicals) was reconstituted in PBS (pH 7.4) and stored at −20°C before use. Recombinant human IL-6 (R&D Systems) was used from 0.1 to 10 ng/ml. Anti-human IL-6 mAb (R&D Systems) was reconstituted in PBS (pH 7.4) and stored at −80°C before use. Recombinant human IL-1R antagonist (R&D Systems) was used from 1 to 2500 ng/ml.

**In vitro tumor microenvironment models**

**Adhesion model.** HS5-GFP stromal cells were seeded to near 50% confluence and incubated overnight at 37°C in 12-well plates (3.8 cm²). The next morning stromal cells were washed three times with serum-free RPMI 1640 medium, and tumor cell lines (5 × 10⁵) were allowed to adhere for 2 h in serum-free RPMI 1640 medium (Mediatech-Cellogro). Nonadhered cells were removed and samples were cultured in 1.5 ml of medium and then exposed to vehicle control (HEPES) or Apo2L/TRAIL (see figure legends for dosage and exposure time). For a control, myeloma cells were treated in suspension. RPMI 8226 cells were also incubated on fibronectin-coated Nunc immunobeads or maintained in suspension as previously described (14).

**TW model.** RPMI 8226 myeloma (2.5 × 10⁵ cells) were cultured in the upper well and separated from the lower well containing either HS5-GFP stromal cells (TW HS5) or medium (TW HS5) by a TW insert (Costar, 0.4-μm mesh; Corning) in a 12-well plate (1.0 cm²). Control-supplemented (HEPES) medium or Apo2L/TRAIL (5–50 ng/ml) was added to the lower well.

**Apoptosis assays**

Apoptotic-induced cell death was determined by flow cytometry (FACScan and FACS-Calibur flow cytometer, BD Biosciences) after annexin V-PE (BD Pharmingen) and 7-amino actinomycin D (7-AAD; BD Pharmingen) staining as per the manufacturer’s instructions. In coculture experiments with HS5-GFP cell lines, gating on GFP-negative populations identified tumor cells. The percentage of specific cell death was calculated relative to nonspecific cell death in the controls. Apoptosis is reported as percentage of specific apoptosis: [(experimental apoptosis/ spontaneous apoptosis)/100]. The data represent analysis performed using FlowJo software (Tree Star). Student’s t test or ANOVA tests were used to determine statistical significance.

**Western blotting**

Western blotting was performed as previously described (15). Protein levels were examined with antisera specific to caspase-8, caspase-3, poly(ADP-ribose) polymerase, and Bcl-2 interacting domain (Bid) (Cell Signaling Technology); Bax, Bcl-2, and Bcl-2-x (BD Pharmingen); Bim (Calbiochem and EMD Biosciences); and c-FLIP (NF6, ALEXIS Biochemicals) visualized with ECL reagents (Amersham Biosciences). To confirm equal loading, membrane was reprobed with monoclonal β-actin Ab (Sigma-Aldrich).
Apo2L/TRAIL receptors expression in myeloma cells

Cells were placed in TW culture conditions for 5 h and then collected, washed once with ice-cold PBS, and stained with 1) PE anti-human DR4 (eBioscience), biotin anti-human DR5 (eBioscience) with PE-Cy7-conjugated streptavidin (BD Pharmingen); 2) biotin anti-human DcR1 (eBioscience) with allophycocyanin-conjugated streptavidin (BD Pharmingen); and 3) biotin anti-human DcR2 (eBioscience) with FITC-conjugated streptavidin (BD Pharmingen). In all, 10,000 events were collected and analyzed by flow cytometry using FlowJo software.

RNA interference to knockdown c-FLIP

We used a pool containing four annealed siRNA constructs targeting c-FLIP (CFLAR) (siGENOME SMARTpool reagent, Dharmacon catalog no. M-003772-06) and four independent siRNA oligonucleotides that are included in the SMARTpool used (siGENOME duplex D-003772-4-0005, D-003772-16-0005, D-003772-17-0005, D-003772-18-0005, NM_003879) (Dharmacon catalog no. MU-003772-06). RPMI 8226 cells were suspended in Nucleofector solution V (Amaxa Biosystems) as per the manufacturer’s instructions and at a final concentration of 5 × 10⁶ cells/ml. siRNA or siCONTROL NonTargeting siRNA no. 2 (Dharmacon) was added to a final concentration of 0.1 μM. Subsequently, transfection was performed using the Amaxis Nucleofector apparatus with the program A20. The cells were added to prewarmed media (5 × 10⁶ cells in 10 ml of medium) and immediately placed in a 37°C/5% CO₂ incubator for 48 h. Densitometry was performed using ImageQuant 5.2 software (GE Healthcare).

Cellular fractionation

RPMI 8226 cells (5 × 10⁶ cells) were maintained in TW/H11002 HS5 or TW/H11001 HS5 conditions for 2 h. Fractionation was performed as previously published by our laboratory (14) or using Qprotecme Cell Compartment Kit as per the manufacturer’s instructions (Qiagen catalog no. 37502).

FIGURE 3. Soluble factors mediate Apo2L/TRAIL receptors and intracellular pathway modifications. A, Apo2L/TRAIL extrinsic pathway modifications. Apo2L/TRAIL-mediated procaspase-8, procaspase-3, and PARP cleavage were determined by Western blot analysis of whole-cell lysate in TW-treated cells (cleavage products are shown by the arrows). Equal loading was determined by β-actin. B, Mitochondrial membrane potential changes. JC-1 mitochondrial membrane staining of RPMI 8226 cells treated in TW – HS5 and TW + HS5 with Apo2L/TRAIL (50 ng/ml) for 5 h. In nonapoptotic cells, JC-1 accumulates as aggregates in the mitochondria, resulting in intense red fluorescence (FL2, right upper quadrants). The loss of mitochondrial membrane potential (Δψ) is a hallmark for apoptosis where JC-1 exists in the mitochondria in monomeric form, losing red fluorescence intensity (right lower quadrant). C, Apo2L/TRAIL receptor expression. Overlay histograms of RPMI 8226 cells surface expression of Apo2L/TRAIL agonists receptors (DR4 and DR5, top plots) and Apo2L/TRAIL decoy receptors (DcR1 and DcR2, bottom plots) cultured in TW + HS5 (gray shadowed areas) and TW – HS5 (black thick lines) for 5 h. Isotype IgG control Ab is represented with dotted lines. The x-axis indicates increasing fluorescence, and the y-axis indicates events counted.
CHX treatment

RPMI 8226 cells were preincubated with 0.5 μg/ml CHX (Sigma-Aldrich) or vehicle control (DMSO) for 19 h. Cells were then washed and cultured in the TW as described with Apo2L/TRAIL (50 ng/ml) or its control (HEPES) for an additional 5 h. Cell death was determined by flow cytometry and protein levels were analyzed by Western blot as described.

Results

Adhesion of myeloma cells to HS5 stroma confers resistance to Apo2L/TRAIL-mediated apoptosis

To determine whether direct physical contact between tumor and stromal cells confers resistance to Apo2L/TRAIL, we used an in vitro adhesion assay where tumor cells were either directly attached to HS5 stromal cells or maintained in suspension. HS5 cells are a human immortalized stromal cell line that secretes multiple cytokines that support proliferation of committed hemopoietic progenitors (24, 25) and protect leukemia cells from apoptosis (26). We developed a variant of HS5-GFP that allows a better distinction between myeloma and stromal cells using flow cytometry (Fig. 1A). In a series of six experiments, myeloma cell lines were adhered to HS5-GFP for 2 h, and apoptosis mediated by recombinant human Apo2L/TRAIL was determined by annexin V/7-AAD staining analyzed by flow cytometry (Fig. 1A).

In RPMI 8226 cells, Apo2L/TRAIL-mediated apoptosis was reduced from 52.2 ± 15.8% (5 ng/ml), 74.9 ± 10.5% (10 ng/ml), and 82.2 ± 6.5% (15 ng/ml) for cells treated in suspension to 5 ± 1.2% (5 ng/ml), 12 ± 1.8% (10 ng/ml), and 22.6 ± 1.8% (15 ng/ml) for cells attached to HS5-GFP. Similar resistance to Apo2L/TRAIL-mediated apoptosis was observed in MM1s cells and U266 cells treated with Apo2L/TRAIL for 24 h (p < 0.001; Fig. 1B). Pretreatment of both RPMI 8226 cells adhered or treated in suspension with Apo2L/TRAIL-neutralizing Abs completely inhibited apoptosis, demonstrating that apoptosis observed in the presence or absence of stromal cells was Apo2L/TRAIL specific (Fig. 1C).

We have previously shown that the extracellular matrix protein FN confers resistance to FAS-mediated apoptosis (14). When RPMI 8226 were adhered to FN and treated with Apo2L/TRAIL (50 ng/ml), FN did not protect the cells from Apo2L/TRAIL-mediated apoptosis, suggesting that FN confers resistance to FAS-mediated apoptosis (14). When RPMI 8226 were adhered to HS5-GFP and treated with Apo2L/TRAIL (50 ng/ml) or its control (HEPES), cell lysates were separated with SDS-PAGE and probed with antibodies specific to c-FLIPL. Equal loading was determined with β-actin. B. c-FLIP levels after Apo2L/TRAIL treatment. c-FLIP isoforms in RPMI 8226 cells cultured in TW and HS5 and TW + HS5 after 5 h of Apo2L/TRAIL (50 ng/ml) treatment. C. Correlation with procaspase-8 cleavage. RPMI 8226 cells were cultured in TW and HS5, TW + HS5 with Apo2L/TRAIL (50 ng/ml) or with vehicle control (HEPES). A representative Western blot for procaspase-8 cleavage products is shown (arrows).

HS5 stromal cells and myeloma patients’ BMS secrete protective soluble factors that confer resistance to Apo2L/TRAIL

Soluble factors produced by myeloma cells and/or adjacent hemopoietic stromal cells have been implicated in the pathogenesis of drug resistance (12). To examine the contribution of soluble factors independent of physical cell contact, we used TW inserts that separate RPMI 8226 myeloma cells from HS5 cells (TW + HS5). As control, RPMI 8226 cells were cultured in the Transwell assay without HS5 cells (TW – HS5). In a series of six experiments, the same passage of RPMI 8226 cells was treated with Apo2L/TRAIL (10 ng/ml) either adhered to HS5 stromal cells or exposed to HS5 in the TW assay. Percentage of specific apoptosis, measured by annexin V/7-AAD staining, was reduced in these experiments as previously shown (Fig. 2A). In TW-treated cells, the percentage of specific apoptosis was decreased from 36 ± 0.7% in cells exposed to TW – HS5 to 3 ± 2.5% in cells exposed to TW + HS5 (Fig. 2A). We also tested Apo2L/TRAIL-mediated apoptosis in the presence of CM derived from HS5. To this end, HS5 stroma cells were grown to 80–90% confluence when medium was exchanged for RPMI 1640 plus 10% FBS and CM was collected following 24 h of incubation. HS5 CM was effective in preventing Apo2L/TRAIL-mediated apoptosis on RPMI 8226 cells (Fig. 2A), but to a lesser extent when compared with culture in TW + HS5, suggesting that there is a need of dynamic interaction between the cancer and stroma to sustain soluble factor production validating the TW assay (12). To gain an understanding of the significance of the role of BMS, we studied BMS isolated from patients with multiple myeloma, which may represent more closely the tumor microenvironment in vivo when compared with HS5 stromal cells. BMS from three myeloma patients were grown to >80% confluence, and at day 14 CM was collected. Soluble factors derived from BMS from three myeloma patients conferred resistance to Apo2L/TRAIL apoptosis in RPMI 8226 cells (Fig. 2B). These results demonstrate that soluble factors produced by stromal elements induce resistance to Apo2L/TRAIL-mediated apoptosis in multiple myeloma.

We next asked whether soluble factor-induced Apo2L/TRAIL apoptosis resistance is reversible. In a series of three experiments, RPMI 8226 cells were exposed to HS5 stroma in TW assay for 2 h before resuspension in medium only for 0, 20, 40, or 60 min. Within 20 min of stroma removal, Apo2L/TRAIL sensitivity was restored, implying that Apo2L/TRAIL-resistant myeloma cells could be potentially targeted (Fig. 2C).
Apo2L/TRAIL signaling pathway and receptor modifications in the EM-DR model

We next asked which Apo2L/TRAIL signaling pathway modifications related to soluble factors were responsible for the phenotype observed. To investigate the effects of soluble factors on the Apo2L/TRAIL-mediated extrinsic apoptotic pathway, RPMI 8226 cells were exposed to TW/H11001 HS5 or TW/H11002 HS5 in a TW assay. Apo2L/TRAIL was added for 5 h at 5, 10, and 15 ng/ml in the TW system. Immunoblot analysis of RPMI 8226 whole-cell lysates showed reduced cleavage of procaspase-8, effector caspase-3, and PARP in TW/H11001 HS5-treated cells, corroborating annexin V/7-AAD results (Fig. 3A).

We next assessed changes in mitochondrial membrane potential of RPMI 8226 cells in response to Apo2L/TRAIL and HS5. Mitochondria of healthy cells retain JC-1 dye, leading to formation of aggregates resulting in high red fluorescence in channel FL-2. Upon Apo2L/TRAIL activation, JC-1 leaks from the mitochondria, thereby decreasing fluorescence intensity. JC-1 staining demonstrated that Apo2L/TRAIL-induced mitochondrial permeability transition was reduced from 45 ± 5% for myeloma cells cultured in regular media to 26 ± 4% for myeloma cells exposed to TW + HS5 (p < 0.05), confirming the antiapoptotic effect of soluble factors released by HS5 stroma cells (Fig. 3B). Soluble factors produced in the TW assay had no effect on cleavage of Bcl-2 family members, including Bid, Bim, Bax, Bcl-2, and Bcl-xL (data not shown).

Resistance to Apo2L/TRAIL-mediated apoptosis has been associated with the down-regulation or mutations of agonistic receptors DR4 and/or DR5 (28, 29) and with the ectopic expression of decoy receptors DcR1 and DcR2 (30, 31). Most RPMI 8226 cells (95 ± 3%) expressed DR4 and DR5 apoptosis inducing Apo2L/TRAIL receptors, and surface expression levels were unchanged in the presence of soluble factors as determined by flow cytometry. Additionally, decoy receptors DcR1 and DcR2 were not expressed in RPMI 8226 cells (Fig. 3C).

Apo2L/TRAIL sensitivity of RPMI 8226 cells exposed to soluble factors was increased by RNA interference. RPMI 8226 cells treated for 48 h with silencing siRNA containing four duplexes targeting c-FLIP (c-FLIP siRNA) or with nonsilencing siRNA control (control siRNA) followed by culture in a TW assay with HS5 stroma (TW + HS5) or without stroma (TW − HS5). A representative Western blot showing c-FLIP down-regulation by c-FLIP siRNA is shown (left). Percentage specific apoptosis as determined by annexin V and 7-AAD staining in three independent experiments of c-FLIP siRNA or control-treated cells cultured for 5 h in a TW assay with Apo2L/TRAIL (50 ng/ml) (right). B, c-FLIP reduction by two independent duplexes. RPMI 8226 cells treated for 48 h with duplex D-003772–17-0005 (no. 17) and D-003772–18-0005 (no. 18) contained in SMART pool used in A. Representative Western blot of c-FLIP isoforms (left), apoptosis results after Apo2L/TRAIL (50 ng/ml) for 5 h (right) from three independent experiments and a representative Western blot of procaspase-8 and cleavage products as shown (bottom). Statistical analysis was done with the ANOVA test.
TRAIL receptors showed similar results (data not shown). In summary, the reduced cleavage of procaspase-8 in the face of similar levels of DR4 and DR5 receptors and the lack of expression of DcR1 and DcR2 suggest that soluble factors produced by HS5-myeloma interactions inhibit the activation of initial proteolytic events in the Apo2L/TRAIL signaling pathway.

Soluble factors are associated with increased c-FLIP protein levels

c-FLIP is a death-inducing signaling complex regulatory factor that competes with procaspase-8 for Fas-associated death domain protein, thereby inhibiting Apo2L/TRAIL-induced signaling (32, 33). When myeloma cells were exposed to TW + HS5, we observed an increase in c-FLIPL protein levels compared with cells treated in TW − HS5, suggesting that up-regulation of this antiapoptotic protein is in part involved in Apo2L/TRAIL resistance (Fig. 4A). c-FLIPL increase was more pronounced in RPMI 8226 (2.86-fold by densitometry) and in U266 (9.5-fold increase) compared with MM1s (1.34-fold increase) cells (Fig. 4A). After Apo2L/TRAIL treatment, the level of c-FLIPL was almost undetectable in RPMI 8226 cells treated in TW − HS5, whereas it was still present in TW + HS5-treated cells, suggesting that soluble factors contribute to maintain c-FLIPL levels (Fig. 4B). Decreased procaspase-8 cleavage corroborated Apo2L/TRAIL apoptosis resistance conferred by soluble factors in TW + HS5 (Fig. 4C).

c-FLIP reduction by RNA interference increases Apo2L/TRAIL sensitivity of RPMI 8226 cells exposed to soluble factors

We next evaluated whether inhibition of c-FLIP by means of RNA interference was able to overcome Apo2L/TRAIL apoptosis resistance of myeloma cells cultured with HS5 in the TW assay. In a series of three experiments, RPMI 8226 cells were treated with a c-FLIP siRNA pool containing four duplexes or nonsilencing control for 48 h. c-FLIPL expression was significantly reduced (90% down-regulation) in myeloma cells after siRNA treatment, as shown in Fig. 5A. c-FLIP siRNA-treated cells were more susceptible to Apo2L/TRAIL-mediated apoptosis, overcoming the resistance conferred by stroma cells in the TW as determined by annexin V/7-AAD staining (Fig. 5A). To exclude off-target effects, we used the siRNA oligonucleotides contained in the FLIP siRNA pool independently. Duplex D-003772-17-0005 (no. 17) and D-003772-18-0005 (no. 18) significantly diminished c-FLIP levels when compared with untreated or siRNA control-treated cells and overcame Apo2L/TRAIL resistance conferred by stromal-related soluble factors (Fig. 5B). Duplex no. 17, which was the most effective in decreasing c-FLIP levels, translated in nearly complete procaspase-8 cleavage (Fig. 5B).

Mechanisms regulating c-FLIP expression by soluble factors

We examined whether intracellular c-FLIPL mobilization within cellular compartments was responsible for increased c-FLIPL cytosolic availability. In a series of three experiments, subcellular fractionation of RPMI 8226 cells demonstrated that most c-FLIPL localizes in a membrane-associated fraction when treated in TW − HS5 (Fig. 6A, lane 4). Exposure to soluble factors (TW + HS5) did not increase c-FLIPL in the cytosolic fraction (Fig. 6A, lane 2), suggesting that soluble factors do not influence c-FLIPL localization to the cytosol where it exerts its antiapoptotic function. When cells were attached to FN, c-FLIPL levels increased significantly in the cytosolic fraction (Fig. 6A, lane 3), as we have previously shown in 8226/SH2 and U937 cell lines (14).
To assess the contribution of translational regulation, we next examined the effects of the protein synthesis inhibitor CHX that can block the expression of short-lived proteins like c-FLIP (34). In a series of three experiments, RPMI 8226 cells were pretreated with CHX (0.5 μg/ml) for 19 h before treatment in the TW assay with Apo2L/TRAIL (50 ng/ml). CHX pretreatment restored sensitivity to Apo2L/TRAIL apoptosis of RPMI 8226 cells treated in the presence of soluble factors (TW + HS5; Fig. 6B). Moreover, immunoblot analysis showed that CHX down-regulated the protein levels of c-FLIP but not those of c-IAP1, Bcl-2, and Mcl-1 proteins (Fig. 6B).

These data indicate that c-FLIP protein synthesis is important to confer Apo2L/TRAIL apoptosis resistance by soluble factors.

**IL-6 inhibits Apo2L/TRAIL-mediated apoptosis**

To determine which soluble factors are involved in Apo2L/TRAIL apoptosis resistance, we tested the effects of another immortalized stromal cell line, HS27a. Gene expression profiling of HS27a compared with HS5 stromal cells showed that IL-6 and IL-1 are within the transcripts with 10-fold lower expression in HS27a (35). In a series of three experiments, Apo2L/TRAIL-mediated apoptosis resistance induced by soluble factors. A, Apoptosis of RPMI 8226 cells treated with HS27a stroma in a TW assay. IL-6 measured by ELISA in lower well of TW inserts with HS5 or HS27a stroma at 5 h in serum free medium (left). RPMI 8226 cells cultured for 5 h with Apo2L/TRAIL (50 ng/ml) in a TW without stroma (TW − HS5) and with HS5 (TW + HS5) or HS27a (TW + HS27a) (right). Represented are means (±SD) from three experiments. Statistical analysis was performed with Student’s t test. B, IL-6 neutralization. RPMI 8226 cells treated in TW − HS5 or TW + HS5 in serum-free medium with either IL-6-neutralizing Ab (1500 ng/ml) or its IgG control Ab for 5 h when IL-6 levels were determined by ELISA (left). Percentage apoptosis of RPMI 8226 cells treated in TW − HS5 or TW + HS5 with IL-6-neutralizing Ab or IgG control and Apo2L/TRAIL (50 ng/ml) for 5 h in serum containing media (right). Statistical analysis was done with ANOVA test. C, IL-6 confers resistance to Apo2L/TRAIL-mediated apoptosis. RPMI 8226 cells cultured in suspension treated with Apo2L/TRAIL (50 ng/ml) (white bar), Apo2L/TRAIL (50 ng/ml), and IL-6 (0.001, 0.01, 0.1, 0.5, 1, and 10 ng/ml) (gray bars) or in TW + HS5 (black bar) for 5 h (left). Represented are the mean (±SD) percentages of specific apoptosis of three independent experiments. IL-6 increases c-FLIP levels (right). RPMI 8226 cells were cultured for 5 h in a TW assay with Apo2L/TRAIL (50 ng/ml) only (TW − HS5), Apo2L/TRAIL + stroma cells (TW + HS5), or Apo2L/TRAIL + IL-6 (1 ng/ml), and the respective vehicle control (HEPES or PBS). Western blot was performed with specific antisera to c-FLIP. Equal loading was determined with housekeeping β-actin protein.
was evaluated in RPMI 8226 myeloma cells in the presence of HS5 or HS27a stroma cells in a TW assay. Resistance to Apo2L/TRAIL (50 ng/ml) was conferred by HS5 but not by HS27a, suggesting that IL-6 and/or IL-1 (35) may be involved in Apo2L/TRAIL resistance (Fig. 6A).

Treatment of HS5 stromal cells cultured in a TW with a neutralizing IL-6-specific Ab significantly decreased IL-6 levels as measured by ELISA, and this treatment partially restored sensitivity to Apo2L/TRAIL, suggesting that IL-6 is one of the soluble factors involved in Apo2L/TRAIL resistance (Fig. 7B). Experiments with IL-1R antagonist either by pretreatment (1–18 h) or coadministration with Apo2L/TRAIL did not reverse Apo2L/TRAIL resistance in the HS5 TW system, suggesting that IL-1 may not be involved in this process (data not shown).

To further explore the role of IL-6, we tested increasing doses of recombinant human IL-6 (0.001–10 ng/ml) with Apo2L/TRAIL, a combination that conferred a dose-dependent resistance to Apo2L/TRAIL in RPMI 8226 cells (Fig. 7C) and increased c-FLIPL protein levels (Fig. 7C). In conclusion, IL-6 may inhibit Apo2L/TRAIL-mediated apoptosis of myeloma cells by regulating c-FLIP levels among other unknown IL-6 effects on Apo2L/TRAIL signaling (36).

Discussion

In vitro drug resistance models have provided invaluable information for the identification of mechanisms of acquired Apo2L/TRAIL resistance (11). However, one disadvantage of these models is that they do not take into consideration the influences of the stromal microenvironment on immune-mediated apoptosis. There is an increasing body of evidence that hemopoietic stromal elements and soluble factors protect hematological malignancies from chemotherapy (12, 17, 18, 37, 38), the process referred to as EM-DR. In this study, we provide evidence that the cancer-stromal interactions confer resistance to Apo2L/TRAIL, a process referred to as EM-IR.

The BM microenvironment plays a significant role in myeloma pathogenesis. Various stromal elements and growth factors mediate tumor proliferation, drug resistance, and migration to protective BM niches (39). Novel treatment approaches, including immunomodulatory agents (40, 41) and proteasome inhibitors (42), in addition to inducing apoptosis, exert an indirect effect by suppressing survival signals elaborated by the microenvironment. Apo2L/TRAIL has been implicated in immune surveillance and plays a role in myeloma apoptosis (7, 36, 43). To determine the relative contributions of the BM network on Apo2L/TRAIL immune-mediated killing, either through direct physical contact and/or by soluble factors, we cultured myeloma cell lines in microenvironment in vitro models that were previously characterized by our laboratory (16). In this study, myeloma cell lines exhibited dramatic resistance to recombinant human Apo2L/TRAIL-mediated apoptosis while directly adhering to HS5 human stroma. Treatment of myeloma cells in the TW assay and with HS5 or myeloma patients’ stroma-conditioned media demonstrate that soluble factors are mainly responsible for Apo2L/TRAIL resistance. The relative resistance to Apo2L/TRAIL observed in U266 and MM1s myeloma cells compared with RPMI 8226 cells may be related in part to decreased Apo2L/TRAIL agonistic DR4 and DR5 receptor expression (data not shown). Additionally, U266 cells are characterized by their own IL-6 production, (12) making us presume that autocrine-released IL-6 may contribute to inhibit Apo2L/TRAIL apoptosis.

To understand mechanism of resistance to Apo2L/TRAIL mediated by soluble factors, we used the TW assay, which allows testing of noncontaminated myeloma cells. c-FLIP up-regulation has been shown to be associated with chemotherapy resistance in human lymphoid (44–47) and nonhematological malignancies (48–50). c-FLIP has been implicated in escape from FAS-mediated apoptosis in murine in vivo models (51, 52). Apo2L/TRAIL-resistant myeloma cell lines have been shown to have high levels of c-FLIP (53). We have shown that c-FLIP down-regulation by RNA interference overcomes Apo2L/TRAIL resistance imposed by the HS5 stroma, a strategy that has been previously shown to be successful in overcoming apoptosis resistance in tumors with increased c-FLIP expression (47, 48). Apo2L/TRAIL-mediated apoptosis has been restored by c-FLIP down-regulation with histone deacetylase inhibitors in hepatocellular (49) and bladder cancer (50) and with 3,3′-diindolylmethane in human cancer cells (54). In our in vitro bone marrow stroma model, increased c-FLIP levels in the presence of HS5 stroma explain, in part, EM-IR to Apo2L/TRAIL, implying that c-FLIP may be a critical target for therapeutic intervention in myeloma treatment (55). Identification of therapeutic strategies to interfere with c-FLIP up-regulation and/or to target hemopoietic soluble factors may contribute to efforts to enhance the effects of c-FLIP inhibitors.

We have previously shown that in hematological malignancies (14) directly attached to FN, c-FLIP is released from a membrane-bound fraction to the cytosol to protect cells from FAS-induced apoptosis, and that protein synthesis is not responsible for c-FLIP accumulation (14). These findings demonstrate that c-FLIP mobilization alone is sufficient to confer resistance against FAS-mediated apoptosis. We herein present data showing that soluble factors do not induce c-FLIP intracellular mobilization to the cytosol, and that adhesion to FN does not confer resistance to Apo2L/TRAIL despite a significant c-FLIP increase in the cytosol, suggesting that c-FLIP mobilization alone is not adequate for Apo2L/TRAIL apoptosis resistance. CHX treatment was able to restore Apo2L/TRAIL sensitivity in tumor cells cultured with HS5 stroma, showing that c-FLIP protein synthesis induced by the BMS-secreted soluble factors is vital for resistance to Apo2L/TRAIL-mediated apoptosis. These combined findings suggest that c-FLIP mobilizes different mechanisms of action to inhibit the FAS and Apo2L/TRAIL apoptotic pathways, suggesting potential hierarchical differences between the two pathways.

IL-6 is an important survival factor in myeloma via activation of major signaling pathways (39). It has been shown to protect against dexamethasone (56–58) and FAS-mediated (37, 59) apoptosis, although its beneficial tumor survival effect is not universal because IL-6 does not protect against bortezomib (60). IL-6-neutralizing Ab was able to partially overcome Apo2L/TRAIL apoptosis resistance in the TW assay, suggesting that IL-6 may be involved in Apo2L/TRAIL resistance. A previous report showed that IL-6 combined with Apo2L/TRAIL (1000 ng/ml) resulted in no protection from Apo2L/TRAIL-mediated apoptosis (7). In our experiments, treatment of RPMI 8226 cells in suspension with Apo2L/TRAIL (50 ng/ml) and increasing doses of rh-IL-6 resulted in Apo2L/TRAIL resistance. We speculate that culture conditions with significantly higher Apo2L/TRAIL doses (1000 ng/ml) may be too toxic to elucidate the survival potential of IL-6 on myeloma cells.

IL-6 treatment was associated with c-FLIP increases, indicating that c-FLIP protein level is in part under the control of IL-6 in these cells. It has been shown that IL-6 overcomes dexamethasone-induced apoptosis via activation of the PI3K/Akt signaling pathway in myeloma (57). PI3K/Akt activity regulates c-FLIP expression in a variety of solid tumors as Akt inhibitors enhance Apo2L/TRAIL-induced apoptosis through c-FLIP reduction (61, 62). Perhaps PI3K/Akt activation induced by IL-6 in myeloma may contribute to increased c-FLIP levels, resulting in Apo2L/TRAIL-mediated apoptosis.
apoptosis resistance. Our data suggest that IL-6 mediates, in part, escape to Apo2L/TRAIL-mediated apoptosis in myeloma. It remains to be determined which other cytokines produced by BMS in vitro also contribute to Apo2L/TRAIL resistance. The results presented herein indicate that BMS may provide a site of immune privilege through anti-apoptotic signals involving soluble factors. Identification of novel key pathways involved in Apo2L/TRAIL EM-IR is crucial to develop approaches to prevent and/or overcome Apo2L/TRAIL resistance, improve immune surveillance, and to expand clinical applications of Apo2L/TRAIL.

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Disclosures
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