CD40 Ligand-Induced Carcinoma Cell Death: A Balance between Activation of TNFR-Associated Factor (TRAF) 3-Dependent Death Signals and Suppression of TRAF6-Dependent Survival Signals

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The effects of CD40 ligation in an epithelial context are complex, with the level of CD40 engagement influencing the physiological outcome. Low levels of CD40 ligation promote cell survival/proliferation, whereas high levels induce growth arrest/apoptosis. The precise form of the CD40 stimulus affects these responses with the most profound effects in carcinoma cells being induced by membrane-bound rather than recombinant soluble CD40L. However, the signaling pathways underlying these differential responses are yet to be fully characterized. We have investigated the mechanistic differences resulting from CD40 engagement by soluble and membrane-bound ligands using a novel adenovirus-delivered CD40L mutated to resist cleavage from the cell membrane in the CD40-positive EJ bladder carcinoma cell line. We have shown that membrane-bound CD40L induces apoptosis by influencing the balance between apoptotic and survival signals. Thus, membrane-bound CD40L stabilizes TNFR-associated factor 3 to induce JNK-dependent apoptosis via release of mitochondrial cytochrome c, caspase 9, and effector caspases 3/7. Further, we have shown that this process is dependent on activation of caspase 8. However, there is also a requirement for suppression of TNFR-associated factor 6-mediated PI3K/Akt-dependent survival signals for apoptosis to occur. These data provide mechanistic insights into the consequences of CD40 activation in carcinoma cells and how these might be exploited in the clinical development of CD40-targeted anticancer therapies. The Journal of Immunology, 2010, 184: 000–000.

A member of the TNFR superfamily, CD40, and its ligand (CD40L/CD154) have attracted much attention due to their central role in orchestrating immune responses and their ability to confer direct antiviral and antitumor effects (1). CD40 is expressed in normal B cells and malignant hematopoietic cells. Although CD40 ligation induces the survival and proliferation of normal B cells and of low-grade B cell malignancies, the activation of CD40 in Burkitt lymphoma cells results in growth inhibition and apoptosis (2–4).

In addition, CD40 is expressed in a number of carcinomas, including those of the ovary, liver, and bladder, although receptor expression is undetectable in normal epithelium derived from the same tissue (2). The effects of CD40 ligation in an epithelial context are complex, with the level of CD40 engagement influencing the physiological outcome. Low levels of CD40 ligation promote cell survival/proliferation, whereas high levels induce growth arrest/apoptosis (5–7). The precise form of the CD40 stimulus also affects these responses, with the most profound effects in carcinoma cells being induced by membrane-bound rather than recombinant soluble CD40L (rsCD40L) (8, 9). The signaling pathways underlying these differential responses are yet to be fully characterized, and there is controversy in the field as to the relative contribution of CD40-induced activation of the JNK/AP-1 MAPK pathway versus upregulation of membrane-bound ligands of the TNF family in CD40L-mediated carcinoma cell apoptosis (9, 10).

CD40 lacks intrinsic kinase activity and is therefore reliant on the recruitment of adapter proteins known as TNFR-associated factors (TRAFs) to mediate signal transduction. Two domains in the cytoplasmic tail of CD40 are critical for TRAF association and signal activation: a membrane-proximal region containing amino acids Glu234–Glu235, responsible for TRAF6 binding, and a Pro250–X-Glu252 motif, which is critical for interactions with TRAF2 and TRAF3 and indirect with TRAF5 (11–13). The ligand-dependent recruitment of these adapter molecules triggers the activation of multiple signaling pathways, including the JNK, ERK, and p38 MAPKs, the transcription factors NF-κB and STAT, and the PI3K cascade, which act in concert to regulate many of the pleiotropic activities of CD40 in a cell type-dependent manner (13–15). We have previously found that rsCD40L can stimulate survival-signaling pathways (including PI3K and ERK/MAPK) and induces apoptosis in carcinoma cell lines only in the presence of either the protein synthesis inhibitor cycloheximide, cytotoxic drugs, or inhibitors of the PI3K/mammalian target of rapamycin (mTOR) and/or ERK pathways (16–19). This contrasts with the effects of membrane-bound CD40L delivered by coculture of EJ cells with CD40L-expressing fibroblasts where robust apoptosis is observed in the absence of other agents (8, 9). However, the mechanisms underpinning these differential responses remain to be fully elucidated.

Thus, to investigate the mechanistic differences resulting from CD40 engagement by soluble and membrane-bound ligand, we have
explored the effects of rsCD40L and our novel adenosuvir-delivered membrane-bound CD40L in the context of CD40-positive carcinoma cell lines. An understanding of these pathways will not only provide insight into the normal function of CD40 in the epithelial cell context, but may also suggest opportunities to optimize the therapeutic exploitation of the CD40 pathway in carcinoma cells.

Materials and Methods

Maintenance of cell lines
Bladder carcinoma EJ cells, gastric carcinoma AGS cells, cervical carcinoma HeLa cells, hepatic carcinoma HepG2 cells, embryonic kidney (HEK) 293 cells, and 911 Crus were maintained in either RPMI 1640 or DMEM supplemented with 2 mM glutamine, 10% FCS.

Recombinant adenosuvir vectors and cell infection
The replication-deficient E1, E3-deleted recombinant adenosuviruses expressing either wild-type human CD40L (RAdCD40L), membrane-bound, noncleavable CD40L (RAdncCD40L), or GFP control (RAdMock) were constructed using methods as described by He et al. (20). Viruses were purified by cesium chloride banding and dialyzed against a buffer containing 10 mM Tris-HCl (pH 8.0), 2 mM MgCl2, and 5% sucrose. Virus titers were determined using the 50% tissue culture-infective dose method, based on the development of cytopathic effects in 293 cells using serial dilutions to estimate adenosuvir stock titer.

Cells were infected in 10% FCS DMEM with the appropriate multiplicity of infection (MOI) dose for 2 h at 37°C. For cell viability and caspase activation assays, infected cells were seeded in 10% FCS DMEM at 4000 cells/well in a 96-well plate or 3 × 105 cells/30-mm dish for Western blotting analysis.

Analysis of cell surface CD40 and CD40L expression
For CD40 expression, cells were plated at 5 × 103 cells/well in six-well plates for 24 h, and then washed with ice-cold PBS buffer and incubated on ice for 1 h with 100 μl of diluted mouse anti-CD40 Ab or mouse isotype Ab or left without treatment as unstimulated control. Cells were then washed three times with 100 μl ice-cold PBS followed by incubation with the anti-mouse IgG-APC Ab for 1 h. Following three washes with 100 μl ice-cold PBS, cells were fixed in 1% paraformaldehyde and analyzed by flow cytometry.

For CD40L expression, cells were infected with RAdCD40L or RAdncCD40L at 100 MOI for 24 h. A total of 3 × 105 cells were washed three times with ice-cold PBS buffer and incubated on ice for 45 min with 100 μl of diluted membrane-bound—APC Ab or mouse isotype—APC Ab conjugate or left without treatment. Cells were then washed three times with ice-cold PBS buffer, fixed in 500 μl 1% paraformaldehyde, and analyzed by flow cytometry.

TRAF mRNA expression
RNA was extracted using the RNAzol reagent (Biogenesis Ltd, Poole, U.K.), and cDNA synthesis was performed using the RETROscript RNAse reverse transcription kit (Ambion Europe, Huntingdon, U.K.) according to the manufacturer’s instructions. RT-PCR was performed using PLATINUM Taq DNA polymerase (Invitrogen, Carlsbad, CA) utilizing the human TRAF-specific primers: TRAF1: forward 5′ GTTCTAGAAACACTGGAAGCC 3′ and reverse 5′ GGAGAAGGCTGACGGTCTCT 3′; TRAF3: forward 5′ AGCCAGGTTGTCGACAGTAA3′ and reverse 5′ AAAGTAACTGCTGACTAAA3′; and TRAF6: forward 5′ ATGACCAAGCTCTTGG 3′ and reverse 5′ TCCTTTGACATGTGTGCAAA3′. The amount of cDNA template used for the RT-PCR was adjusted on the basis of amplification with primers specific for human GAPDH: forward 5′ CCTTCCCCAAATCAAGTGGGG3′ and reverse 5′ ACCACAGGTGTGCTGTA3′.

Pharmacological inhibitors
The JNK inhibitor (SP600125) was from Alexis Biochemicals (Plymouth Meeting, PA). Inhibitors of caspase-8 (z-IETD), caspase-9 (z-LEHD), pan-caspase inhibitor (z-VAD), PI3K inhibitor (LY294002), and the IKK2 inhibitor (SC-514) were all from Calbiochem (Nottingham, UK). Rapamycin was from Sigma-Aldrich (St. Louis, MO). Inhibitors were reconstituted in DMSO (Sigma-Aldrich).

RNA interference studies
Small interfering RNA (siRNA) directed to TRAF3 (SC-19510) and a control siRNA-A (SC-37007), which does not lead to specific degradation of any known cellular mRNA, were from Santa Cruz Biotechnology (Santa Cruz, CA). Cells were transfected with 50 pM of either siRNA according to the manufacturer’s instructions.

Cell viability assay
Cells were cultured in 96-well plates at 4000 cells/100 μl/well and incubated at 37°C until assessed for viability by the WST-1 assay (Roche, Basel, Switzerland). WST-1 reagent was added to the culture medium at 1:10 dilution followed by incubation at 37°C. The OD was measured by microplate ELISA reader at λ450 every 2 h up to a maximum of 6 h. The amount of the formazan formed directly correlates to the number of metabolically active cells.

Annexin V staining of apoptotic cells
Cells were infected with 100 MOI of RAdMock, RAdCD40L, or RAdncCD40L or treated with soluble CD40L (1 μg/ml) or left without treatment as a negative control for 48 h. Cells were harvested and stained with Annexin V-APC according to the manufacturer’s instruction (BD Biosciences, San Jose, CA). Cells were harvested, and the percentage of Annexin V-APC positive cells was determined within 1 × 106 cells of the population by flow cytometry.

Caspase activation assays
Cells were treated with the general caspase inhibitor zVAD or inhibitors of caspase-8 or -9 at a concentration of 30 μM or left untreated. Cells were plated in triplicate in white-walled 96-well plates at a density of 4000 cells/100 μl/well and incubated at 37°C for 36 h. The activity of caspase-3/-7, -8, and -9 were then measured using the Caspase-Glo-3/7, -8, and -9 assay kit (Promega, Madison, WI) according to the manufacturer’s instructions. Briefly, the caspase reagents were reconstituted, and 100 μl/well was added to cells and mixed for 30 s in a 96-well plate at room temperature. Cells were incubated for 1 h. The luminescence was assessed using a 96-well plate luminometer. The resulting luminescent signal is directly proportional to the amount of caspase activity present.

Mitochondrial/cytosolic fractionation
EJ cells were infected with 100 MOI of either RAdncCD40L or RAdMock for 48 h. Cells were washed twice with ice-cold PBS and then resuspended with 3 × 107 cells/ml in ice-cold lysis buffer (20 mM HEPES-KOH [pH 7.5], 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose, 1 mM DTT, 0.5 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml apronin, and 10 μg/ml trypsin inhibitor). The cell suspension was homogenized with 25 strokes of a Dounce homogenizer and centrifuged at 700 rpm for 10 min at 4°C. The pellet was discarded, and the supernatant was further centrifuged at 20,000 rpm for 15 min. The resultant pellet containing the mitochondria was resuspended in lysis buffer and the supernatant containing the cytosol retained in a fresh tube.

Measurement of mitochondrial membrane potential
EJ cells were infected with 100 MOI of RAdMock or RAdncCD40L or left without infection as a negative control. RAdncCD40L-infected cells were either treated with JNK inhibitor SP600125 (15μM), caspase-8 inhibitor (30 μM), caspase-9 inhibitor (30 μM), or left without inhibitor treatment as a control. Cells were plated into 96-well plates or a glass cover slip for 36 h. To assess the mitochondrial membrane potential, cells were stained with the 1:1 JC-1 stain according to the manufacturer’s instructions (Biotium, Hayward, CA). Red fluorescence of the mitochondria (excitation 550 nm, emission 600 nm) was measured by a 96-well plate fluorescence reader or visualized by fluorescence microscopy. The percentage of red fluorescence was considered an indicator of mitochondrial membrane potential.

Abs and immunoblotting
Phosphospecific and total stress-activated protein kinase/JNK Abs were purchased from Cell Signaling Technology (Beverly, MA). TRAF1, 2, 3, 5, and 6 Abs and JNK Ab were from Cell Signaling Technology. CD40L—APC and isotype—APC conjugates were from eBioScience. Cytochrome c and cytochrome oxidase IV (COX IV) Abs were from BD PharMingen (San Diego, CA) and Molecular Probes (Eugene, OR), respectively. For immunoblotting, 10–30 μg protein was separated by SDS-PAGE, transferred onto polyvinylidene difluoride membrane (for phospho-proteins) or Biotrace nitrocellulose membranes (Pall, Port Washington, NY) for nonphospho-proteins and subsequently blocked with 5% BSA (for phospho-proteins) or nonfat milk (for nonphospho-proteins) dissolved in TBS supplemented with 0.1% Tween 20. After three washes with TBS supplemented with 0.1% Tween 20, membranes were incubated overnight at 4°C with
Results

Adenovirus-delivered CD40L is membrane-bound and its expression induces apoptosis in CD40-positive carcinomas

In common with other TNF family ligands, CD40L is naturally expressed as a membrane-bound homotrimeric molecule that is cleaved into soluble form by a disintegrin and metalloproteinases (21). Previous studies have identified the cleavage site of Fas ligand and demonstrated that a mutant resistant to metalloproteinase cleavage is a more potent inducer of apoptosis. Through amino acid sequence alignment between FasL and CD40L, we have identified (110–116 aa, SFEMQKG) and deleted the metalloproteinase cleavage site and generated a RAd expressing this noncleavable form (RAdncCD40L) (22).

Infection of cells with RAd expressing wild-type CD40L results in ligand expression at the cell membrane as demonstrated by flow cytometry. However, RAd expressing our mutant cleavage-resistant CD40L induces significantly higher levels of ligand expression at the membrane (Fig. 1A). Unlike soluble CD40L, RAd-delivered CD40L is able to directly induce apoptosis in CD40-positive carcinoma cells (EJ, AGS) as assessed by Annexin V staining. Furthermore, significantly higher levels of apoptosis are induced by cleavage-resistant ligand (Fig. 1B, 1C). This effect is not due to interaction between CD40L and adenovirus proteins because co-treatment with rsCD40L and a control RAd did not induce apoptosis (data not shown). The requirement for CD40-CD40L interaction for apoptosis was confirmed by an absence of effect in CD40-negative HeLa cells (Fig. 1B, 1C). The presence or absence of CD40 in cell lines was determined by flow cytometry (Fig. 1C).

Thus, RAdncCD40L produces CD40L expressed on the membrane of infected cells, fails to generate soluble ligand, and demonstrates enhanced apoptosis induction compared with RAdCD40L without the requirement for protein synthesis inhibition.

RAdncCD40L induces apoptosis via caspase-3/7 activation

To determine the mechanism by which membrane-bound CD40L induces apoptosis, we examined the level of the effector caspases-3 and -7 activity within RAdncCD40L-infected cells. Thus, EJ carcinoma cells were infected with either RAdncCD40L or a control RAd or treated with rsCD40L. Cells were then treated with the broad-spectrum caspase inhibitor zVAD. Caspase-3/7 activity was significantly higher in RAdncCD40L-infected compared with the uninfected or RAdMock-infected control cells (Fig. 2A). Moreover, treatment of cells with rsCD40L did not induce caspase-3/7 activation, highlighting a major difference in the mechanism by which RAdncCD40L acts. The specificity of the assay was confirmed by the ability of the inhibitor zVAD to block caspase-3/7 activation.

RAdncCD40L-induced apoptosis requires the activation of caspase-8 and caspase-9

To examine whether RAdncCD40L-induced apoptosis requires activation of caspase-8 and/or -9, EJ cells were infected with RAdncCD40L and then either treated with caspase-8 (z-IETD) or -9 (z-LEHD) specific inhibitors. The activity of caspase-9 was robustly increased in RAdncCD40L-infected cells compared with uninfected or RAdMock-infected cells, with a more moderate elevation in caspase-8 activity (Fig. 2B, 2C).

RAdncCD40L-induced apoptosis is associated with cytochrome c release and loss of mitochondrial membrane potential

Because caspase-9 activation is associated with loss of mitochondrial membrane potential and translocation of cytochrome c from the mitochondria to cytosol, we next examined whether this is the case with RAdncCD40L infected cells. Thus, EJ cells were infected with RAdncCD40L for 48 h and then the mitochondrial and cytosolic fractions were then immunoblotted for cytochrome c and the mitochondrial marker COX IV. A significant reduction in mitochondrial cytochrome c was observed following RAdncCD40L treatment compared with RadMock-infected cells (Fig. 3A). The purity of the fractions was confirmed because COX IV was only detected in the mitochondrial fractions. Further, EJ cells were infected with RAdncCD40L in the presence or absence of inhibitors of caspases-8 or -9 and mitochondrial membrane potential was assessed by JC-1 staining. Mitochondrial-bound CD40L resulted in a significant loss of mitochondrial membrane potential (Fig. 3B). This was blocked by caspase-8 inhibitor but not caspase-9 inhibitor, suggesting caspase-8 to be upstream and caspase-9 to be downstream of the mitochondria in the CD40L apoptotic pathway (Fig. 3B).

RAdncCD40L-induced caspase-3/7 and -9 are downstream of caspase-8

To investigate whether caspase-8 or -9 is responsible for the activation of caspases-3/7 by RAdncCD40L, EJ cells were infected with RAdncCD40L and then treated with the pan-caspase inhibitor z-VAD or specific inhibitors of caspase-8 (z-IETD) or -9 (z-LEHD) and assessed for caspase-3/7 activation. The pan-caspase inhibitor z-VAD and the caspase-8 inhibitor z-IETD completely blocked caspase-3/7 activation in RAdncCD40L-infected cells. However, the caspase-9 inhibitor z-LEHD resulted in only a partial decrease in the caspase-3/7 activation, suggesting that caspase-3/7 lies downstream of caspase-8 in the cell death cascade initiated by RAdncCD40L (Fig. 2D).

The classical death receptor apoptotic pathway requires large amounts of caspase-8 recruited into the death inducing signaling complex to rapidly activate caspase-3. Alternatively, caspase-3 activation can occur after loss of mitochondrial membrane potential, resulting in mitochondria-induced amplification of apoptotic signals rather than direct caspase-8 activation (23). In this setting, it has been reported that low-level activation of caspase-8 is required to induce the cleavage of the proapoptotic Bcl family member, Bid, to enable its interaction with active mitochondrial-targeted molecules such as Bax to enable the release of cytochrome c (24). Because RAdncCD40L induces relatively greater activation of caspase-9 than caspase-8 and results in loss of mitochondrial membrane potential and cytochrome c translocation, we postulated that ncCD40L may induce apoptosis via this latter mechanism. To examine this hypothesis, EJ cells were infected with RAdncCD40L and treated with the caspase-8 (z-IETD) or -9 (z-LEHD) specific inhibitors and then assessed for caspase-9 and -8 activity, respectively. Treatment of cells with the caspase-8 inhibitor (z-IETD) inhibited RAdncCD40L-induced caspase-9 activation (Fig. 2E). In contrast, caspase-9 inhibitor (z-LEHD) had no effect on caspase-8 activation (Fig. 2F), suggesting that caspases-3/7 and -9 are downstream of caspase-8 in effecting RAdncCD40L-induced apoptosis.

RAdncCD40L but not rsCD40L constitutively induces JNK phosphorylation and inhibition of JNK signaling decreases RAdncCD40L-induced apoptosis

To investigate the contribution of JNK activation to RAdncCD40L-induced apoptosis, the JNK MAPK pathway was examined. Thus, EJ cells were infected with RAdncCD40L for 24, 48, and 72 h or treated with rsCD40L for 1, 12, and 24 h. RAdncCD40L induced sustained JNK phosphorylation even up to 72 h (Fig. 4A). However, JNK phosphorylation in response to rsCD40L was transient, being...
detectable only within 1 h of treatment (Fig. 4B). Treatment with SP600125, a specific JNK inhibitor, blocked RAdncCD40L-induced loss of mitochondrial membrane potential and cell death (Figs. 3B, 4C). The ability of SP600125 to inhibit RAdncCD40L-mediated phosphorylation of the JNK substrate, c-JUN, was confirmed (Fig. 4D).

**Membrane-bound and soluble CD40L have differential effects on TRAF regulation**

The ability of CD40 ligation to induce different signaling pathways is attributed to the differential binding of TRAF molecules to the CD40 cytoplasmic tail. To understand the difference in apoptosis induction between RAdncCD40L and rsCD40L, we examined their effect on the regulation of different TRAFs. Thus, EJ cells were infected with RAdncCD40L for 24 and 48 h or treated with soluble CD40L (1 μg/ml) or left without treatment for 48 h. Apoptotic cells were assessed by staining with Annexin V-APC and analysis by flow cytometry. Results are a mean of triplet samples ± SD. C, CD40 receptor expression: EJ, AGS, and HeLa cells were incubated with mouse anti-CD40 Ab or isotype control and analyzed by flow cytometry.

**FIGURE 1.** Adenovirus-delivered CD40L is expressed on the cell membrane and induces apoptosis in CD40-positive carcinomas. A, CD40L expression: EJ cells were infected with 100 MOI of either RAdCD40L (wild-type) or RAdncCD40L (mutated cleavage resistant) for 28 h. Harvested cells were incubated with mouse anti-CD40L–APC conjugated Ab or isotype control and then analyzed by flow cytometry. B and C, Membrane-bound CD40L induces apoptosis in CD40-positive carcinoma cells; EJ, AGS, and HeLa cells were infected with 100 MOI RAdMock, RAdCD40L, or RAdncCD40L or treated with soluble CD40L (1 μg/ml) or left without treatment for 48 h. Apoptotic cells were assessed by staining with Annexin V-APC and analysis by flow cytometry.
TRAF6 protein (Fig. 5B). However, upregulation of TRAF1 was associated with increased transcription in RAdncCD40L infected cells and, to a lesser extent, after treatment with rsCD40L. Inhibition of TRAF3 protects against CD40L-induced cell death

To examine the effect of TRAF3 inhibition on RAdncCD40L-induced apoptosis, EJ cells were transfected with TRAF3 siRNA and then infected with RAdncCD40L. Specific siRNA inhibition of TRAF3 expression was confirmed (Fig. 6A). Furthermore, TRAF3 knockdown inhibited RAdncCD40L-induced apoptosis in comparison with cells transfected with the scrambled siRNA, clearly demonstrating that TRAF3 is an up-stream adaptor involved in the induction of cell death signaling by RAdncCD40L (Fig. 6B).

Inhibition of TRAF3 expression in RAdncCD40L-infected EJ cells results in decreased JNK and increased Akt phosphorylation but no change in ERK or NF-κB

The finding that TRAF3 inhibition renders EJ cells insensitive to RAdncCD40L-induced apoptosis suggests that TRAF3 stabilization may be implicated in CD40L-induced JNK activation. To address this hypothesis, EJ cells were transfected with TRAF3 siRNA followed by infection with RAdncCD40L. Cell lysates were probed with Abs that specifically recognize the active phosphorylated forms of JNK, Akt, ERK, or IκBα. TRAF3 knockdown significantly inhibited JNK phosphorylation in RAdncCD40L-infected cells. In contrast, Akt phosphorylation was markedly increased upon TRAF3 inhibition. However, no changes were observed in ERK or IκBα phosphorylation (Fig. 6A). These results clearly suggest that TRAF3 stabilization in RAdncCD40L-infected EJ cells is required for sustained JNK activation and also inhibits Akt phosphorylation.

Inhibition of RAdncCD40L-induced TRAF3 expression restores TRAF6 expression

The ability of TRAF-binding to one domain of CD40 to stereotypically enhance or interfere with the binding of TRAFs to another domain has yet to be determined. However, because RAdncCD40L differentially regulates the expression of TRAF molecules, we postulated that TRAF6 downregulation in RAdncCD40L-infected cells could be as a result of the TRAF3 stabilization. To examine this hypothesis further, EJ cells were transfected with TRAF3 siRNA and then infected with RAdncCD40L. Inhibition of TRAF3 resulted in maintenance of the level of TRAF6 expression compared with the reduction in TRAF6 induced by RAdncCD40L in the presence of scrambled siRNA (Fig. 6B). No difference in the level of TRAF1 expression was observed (Fig. 6B). Taken together, these results suggest that TRAF3 stabilization by RAdncCD40L negatively regulates TRAF6 expression.

Inhibition of Akt, but not NF-κB signaling, in TRAF3-knockdown EJ cells restores sensitivity to RAdncCD40L-induced apoptosis and induces caspase-9 activation

We have shown above that RAdncCD40L induces increased expression of TRAF3 and that inhibition of TRAF3 in this context results in increased expression of phosphorylated Akt and reduced cell death. This suggests that Akt phosphorylation in response to CD40 ligation in RAdncCD40L-infected cells is normally impaired, or at least suboptimal, due to stabilization of TRAF3 expression. Further, this suggests a possible role for the suppression of the PI3K/Akt pathway contributing to RAdncCD40L-induced apoptosis. To test this hypothesis, we assessed whether inhibition of the PI3K/Akt pathway could restore sensitivity of EJ cells to RAdncCD40L-induced apoptosis despite knockdown of TRAF3 expression. Thus, EJ cells were
transfected with TRAF3 siRNA and then infected with RAdncCD40L. Cells were then cultured with the PI3K/Akt inhibitor LY294002. Because mTOR is one of several downstream effectors of the PI3K/Akt pathway, some samples were treated with the mTOR inhibitor, rapamycin. Inhibition of PI3K/Akt restored RAdncCD40L-induced apoptosis even despite knockdown of TRAF3 (Fig. 7A). However, only slight reduction in cell viability was observed in RAdncCD40L-infected cells treated with rapamycin. These data suggest that the PI3K/Akt pathway mediates a protection mechanism against TRAF3-dependent RAdncCD40L-induced apoptosis.

Because the PI3K/Akt pathway is implicated in antiapoptotic signaling through direct phosphorylation and inhibition of caspase-9 activation (25), we next examined the direct effect of PI3K/Akt inhibition on caspase-9 activity in RAdncCD40L-infected cells when TRAF3 was knocked down. Thus, EJ cells were transfected with TRAF3 siRNA and then infected with RAdncCD40L. Cells were then cocultured with either LY294002 or rapamycin. TRAF3 knockdown resulted in inhibition of caspase-9 activity in RAdncCD40L-infected cells consistent with our previous observation that TRAF3 knockdown reduces the degree of apoptosis induced by ncCD40L (Fig. 7B). However, inhibition of PI3K/Akt...
restored caspase-9 activity in RAdncCD40L-infected cells even when TRAF3 was inhibited. These data are consistent with our observation that blockade of PI3K/Akt restores nCD40L-induced cell death even when TRAF3 is knocked down.

The ability of LY294002 to inhibit RAdncCD40L-induced Akt phosphorylation was confirmed (Fig. 7C).

Because CD40 may also activate NF-kB, we sought to investigate whether NFκB-dependent survival signals were relevant to membrane-bound CD40L-induced apoptosis. Thus, EJ cells were treated with RAdncCD40L in the presence or absence of the IKK2 inhibitor SC-514. Membrane-bound CD40L activated the NF–κB pathway, evidenced by a reduction in the regulatory protein, IκB, and this was effectively inhibited by SC-514 (Fig. 7E). However, NFκB inhibition did not influence CD40L-induced cell death (Fig. 7D).

**Discussion**

CD40 ligation can exert either pro- and antiapoptotic effects depending on the cellular context and the nature of the ligand. Thus, soluble CD40L induces survival signals in CD40-positive carcinomas unless the protein synthesis machinery is blocked, in which case apoptotic cell death is induced (8, 10, 26, 27). Interestingly, soluble CD40L induces survival signals in CD40-positive carcinomas unless the protein synthesis machinery is blocked, in which case apoptotic cell death is induced (8, 10, 26, 27). Interestingly, membrane-bound CD40L is able to directly induce cell death without the requirement for protein synthesis inhibition.

To dissect the mechanistic differences between soluble and membrane-bound CD40L, we have engineered a RAd expressing a mutant CD40L that is resistant to proteolytic cleavage and thus expresses CD40L in a membrane-bound form. We have investigated the mechanism by which RAdncCD40L induces cell death, in particular to identify differences in the recruitment of intracellular adaptor molecules and kinase signaling pathways induced by soluble and membrane-bound CD40L. The choice of RAdncCD40L rather than RAd expressing wild-type CD40L was for the following reasons: we have found that RAdncCD40L is a more potent inducer of apoptosis than the wild-type RAdCD40L, and, through its lack of cleavage into soluble ligand, it would be more informative in allowing comparison of membrane-bound versus soluble ligand than the wild-type CD40L.
which is known to be shed into soluble CD40L by the effect of metalloproteinases.

In this study, we have shown that RAdncCD40L induces apoptosis, evidenced by caspase-3/7 activation and Annexin V staining. In agreement with previous studies, treatment of EJ cells with rsCD40L in the absence of protein synthesis inhibition did not induce caspase-3/7 activity, suggesting differential mechanisms of

![FIGURE 7](image)

**FIGURE 7.** Inhibition of Akt, but not NF-κB, signaling in the absence of TRAF3 expression in EJ cells restores RAdncCD40L-induced cell death and induces caspase-9 activation. EJ cells were transfected with TRAF3 siRNA at the concentration of 50 pM for 48 h and then infected with 50 MOI of either RAdMock (AdM) or RAdncCD40L (AdncL) or left uninfected as a control. Cells were cocultured with either 20 mM of the PI3K inhibitor LY294002 or 15 nM of the mTOR inhibitor rapamycin (Rap) or left untreated for a further 24 h. A, Cell viability was assessed by WST-1 assay. Results are a mean of triplet experiments ± SD. B, Caspase-9 activity was assessed by Caspase-Glo-9 assay. Results are a mean of triplet experiments ± SD. C, Protein lysates were probed with anti-phospho Akt and anti-β-actin as a loading control.

![FIGURE 8](image)

**FIGURE 8.** Overexpression of Akt2 results in reduction of RAdncCD40L-induced cell death in EJ cell. EJ cells were transfected with 1 μg of either empty pcDNA plasmid or pcDNA/HA-Akt2 for 36 h and then infected with 100 MOI of either RAdMock (AdM) or RAdncCD40L (AdncL) or left untransfected for a further 24 h. A, Cell viability was assessed by WST-1 assay. Results are a mean of triplet samples ± SD. B, Protein samples were probed with anti-HA to confirm expression of the HA/Akt2 fusion or with anti-β-actin as a loading control.

![FIGURE 9](image)

**FIGURE 9.** Overexpression of TRAF6 does not reduce RAdncCD40L-induced cell death in EJ cells. EJ cells were transfected with 500 ng of either empty pcDNA plasmid or pcDNA/HA-TRAF6 or left untransfected for 24 h and then infected with 100 MOI of either RAdMock (AdM) or RAdncCD40L (AdncL) or left uninfected for a further 36 h. A, Cell viability was assessed by WST-1 assay. Results are a mean of triplet samples ± SD. B, Protein samples were probed with anti-HA, TRAF6, or CD40L Abs or with anti-β-actin Ab as a loading control.
The ability of CD40 ligation to induce both survival and apoptotic signals is attributed to the ability of the CD40 receptor to differentially recruit TRAF molecules depending on the cellular context and the nature of the CD40 ligation. Differential TRAF binding induces specific receptor conformations and the degree of receptor cross-linking critically influences the outcome of signaling from the CD40 stimulus (28). In a carcinoma cell background, RAdncCD40L markedly upregulates both TRAF1 and TRAF3, with reciprocal downregulation of TRAF6. However, activation by soluble CD40L results in a more limited upregulation of TRAF1 only. The regulation of TRAF3 and -6 is a result of posttranscriptional modifications because the levels of mRNA remained unchanged. Thus, membrane-bound CD40L induces stabilization of TRAF3 and degradation of TRAF6. However, TRAF1 regulation was transcriptional, evidenced by increased mRNA in response to CD40 ligation.

In agreement with our results, it has been reported that membrane-bound CD40L but not rsCD40L upregulated TRAF3 in urothelial cancer cells but not normal urothelium (9). However, we are the first to show that there is a reciprocal downregulation of TRAF6 in response to membrane bound CD40L. Further, we show that TRAF3 may induce apoptosis via sustained induction of TRAF6 phosphorylation following CD40 ligation by nCD40L. The ability of the specific JNK inhibitor, SP600125, to decrease apoptosis further indicates that RAdncCD40L-induced cell death is JNK-dependent. Our finding that RAdncCD40L promotes cytochrome c translocation from the mitochondrial compartment to the cytosol further highlights the involvement of JNK signaling in CD40L-induced cell death. JNK activation has been reported to induce cytochrome c release through phosphorylation of c-Jun and the antiapoptotic proteins Bcl2 and BclxL (29–31). Phosphorylation of these proteins by JNK inhibits their antiapoptotic function with consequent loss of the mitochondrial membrane potential and cytochrome c release (32). The absence of an apoptotic response to JNK activation in rsCD40L-treated EJ cells appears to correlate with the transient nature of JNK activation in this setting. Indeed, it is reported that sustained but not transient activation of JNK is required for the induction of apoptosis (33).

Our finding that TRAF3 inhibition results in downregulation of JNK activation is consistent with previous reports that membrane-delivered CD40L induces JNK phosphorylation in a TRAF3-dependent manner in urothelial cancer cells (31). However, to our knowledge, this is the first study to demonstrate that inhibition of TRAF3 stabilization in RAdncCD40L-infected carcinoma cells results in upregulation of PI3K/Akt activity. Given that caspase-9 is known to be phosphorylated by Akt, resulting in suppression of its apoptotic function (25), we propose that the PI3K/Akt pathway must be functionally inactivated to allow a proapoptotic stimulus through the sustained activation of JNK. In support of this, knockdown of TRAF3 reduces RAdncCD40L-induced apoptosis and is associated with increased Akt phosphorylation. Inhibition of Akt in this context restored the apoptotic effect of nCD40L, even with only low-level JNK activation. Phosphorylated Akt can inhibit apoptosis through a variety of mechanisms, including direct inhibition of caspase-9 and via mTOR-dependent mechanisms. In this study, we have shown that the mTOR inhibitor, rapamycin, did not significantly restore cell death or caspase-3/7 activation, indicating that Akt-mediated survival signals are mTOR-independent in this setting. Thus, we propose that a key part of the mechanism of RAdncCD40L-induced cell death is through suppression of the function of the PI3K/Akt pathway. Indeed, we have shown for the first time that TRAF6, which is known to activate the PI3K/Akt pathway in an Src-dependent manner, was reciprocally downregulated as TRAF3 was stabilized in response to RAdncCD40L. Furthermore, TRAF6 expression was maintained in RAdncCD40L-infected cells when TRAF3 was inhibited by specific siRNA. However, artificial overexpression of TRAF6 could not rescue cells from RAdncCD40L-induced apoptosis where TRAF3 expression is maintained, suggesting that TRAF3 may engage the CD40 receptor in such a way as to prevent the overexpressed TRAF6 from binding and exerting its antiapoptotic activation of PI3K/Akt.

In summary, the CD40 pathway appears to provide an opportunity to muster different anticancer approaches in one therapy and offers an attractive option for clinical trials. Our preclinical research demonstrates clear advantages of adenovirus delivery of our mutant membrane-bound CD40L compared with rsCD40L in terms of directly inducing apoptosis of infected cancer cells as well as its capacity to stimulate the key components required for antitumor immune responses. In this study, we propose a model involving...
a complex interplay between pro- and antiapoptotic signals consequent upon CD40 ligation, and the context and type of ligation determine a cell’s ultimate fate (Fig. 10). It is anticipated that this knowledge will further enhance the clinical development of CD40-targeted therapies.

Disclosures
The authors have no financial conflicts of interest.

References