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A novel mechanism of CD40-induced apoptosis of carcinoma cells involving TRAF3 and JNK/AP-1 activation

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Abstract

Membrane-presented CD40 agonists can induce apoptosis in carcinoma, but not normal homologous epithelial cells, whereas soluble agonists are growth inhibitory but not proapoptotic unless protein synthesis is blocked. Here we demonstrate that membrane-presented CD40 ligand (CD154) (mCD40L), but not soluble agonists, triggers cell death in malignant human urothelial cells via a direct mechanism involving rapid upregulation of TNFR-associated factor (TRAF)3 protein, without concomitant upregulation of TRAF3 mRNA, followed by activation of the c-Jun N-terminal kinase (JNK)/activator protein-1 (AP-1) pathway and induction of the caspase-9/caspase-3-associated intrinsic apoptotic machinery. TRAF3 knockdown abrogated JNK/AP-1 activation and prevented CD40-mediated apoptosis, whereas restoration of CD40 expression in CD40-negative carcinoma cells restored apoptotic susceptibility via the TRAF3/AP-1-dependent mechanism. In normal human urothelial cells, mCD40L did not trigger apoptosis, but induced rapid downregulation of TRAF2 and 3, thereby paralleling the situation in B-lymphocytes. Thus, TRAF3 stabilization, JNK activation and caspase-9 induction define a novel pathway of CD40-mediated apoptosis in carcinoma cells.
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Abbreviations: Act1, NF-κB activator 1; AP-1, activator protein-1; CD40L, CD40 ligand (CD154); CHX, cycloheximide; ERK, extracellular signal-regulated kinase; ICAM-1, intracellular adhesion molecule 1; JNK, c-Jun N-terminal kinase; LTβR, lymphotoxin-β receptor; mAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; mCD40L, membrane-expressed CD40 ligand; MKK, Map kinase kinase; NF-κB, nuclear factor-κB; NHU, normal human urothelial; PI3-K, phosphatidylinositol triphosphate kinase; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor; TRAF, TNFR-associated factor; TRAIL, TNF-related apoptosis-inducing ligand; UCC, urothelial cell carcinoma

Introduction

CD40 is a member of the tumor necrosis factor (TNF) receptor (TNFR) superfamily, and the interaction with its cognate CD40 ligand, CD40L (CD154), is essential for the functioning of the immune system.[1] CD40 is expressed on cells of high proliferative potential of lymphoid and epithelial origins and exhibits bimodal growth-regulatory properties with distinct physiological effects.[2]

The outcome of CD40 ligation in B cells depends on differentiation stage, being mitogenic and antiapoptotic in resting cells, but growth-inhibitory in activated cells, where it can crosstalk with downstream effectors of other TNFR members.[3] In low-grade B-cell malignancies CD40 engagement contributes to cell survival and resistance to chemotherapy,[4] whereas in high-grade malignancies it causes growth arrest and apoptosis.[5]

In nonlymphoid cells, the effects of CD40 are equally pleiotropic and context-specific. Many epithelial cell types respond to CD40 by cytokine and chemokine secretion.[6,7] CD40 ligation also promotes growth of fibroblasts, angiogenesis and intrahepatic endothelial cell proliferation.[8–10] Conversely, CD40-mediated apoptosis has been documented in biliary, hepatocyte and carcinoma cells ectopically expressing CD40, where death occurs by autocrine/paracrine induction of death ligands such as TNFα, FasL and TNF-related apoptosis-inducing ligand (TRAIL).[10–14] However, apoptosis induced by soluble CD40 agonists depends on concurrent blocking of protein synthesis by cycloheximide (CHX). By contrast, membrane-presented agonists efficiently induce apoptosis in carcinoma cells without pharmacological intervention, while sparing homologous normal epithelial cells.[15,16]

In B cells and in cells engineered to coexpress CD40 and TNFR-associated factors (TRAFs), CD40 orchestrates complex signaling cascades involving kinases such as mitogen-activated protein kinase (MAPK) kinase MKK1, extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinase (JNK) and p38 stress kinase, and subsequent nuclear translocation of nuclear factor (NF)-κB and activator protein (AP)-1 transcription factor components.[17] These events are mediated by the TRAF family after recruitment and relocation to membrane lipid rafts.[17,18] TRAFs associate directly or indirectly with CD40 and convert the transduced signal into diverse functional outcomes.[19]

In B cells, CD40 induces upregulation of TRAF1 and proteasome-mediated degradation of TRAF2 and 3.[20,21]

TRAF2 has a positive role in B-cell differentiation, with opposing effects entrained by TRAF3.[22] The role of the TRAFs in epithelial cells is less clear. TRAF2 and 3 bind CD40 the most efficiently, but, despite established roles in CD40 signaling,[22] their involvement in NF-κB and JNK/activator protein-1 (AP-1) activation is more equivocal.[22,23] TRAF1 does not bind CD40 efficiently and influences the signaling pathway via association with TRAF2.[24,25]

How CD40 transmits antiproliferative or proapoptotic signals is unclear. Activation of AP-1 and subsequent induction of cell death is well documented,[26] and a direct link between CD40-mediated induction of AP-1 and apoptosis has been suggested.[10,14] Furthermore, *de novo* expression of CD40 can induce JNK activity.[17] A role for TRAF3 is implied from findings that TRAF3 specifically blocks TRAF2-mediated activation of the noncanonical NF-κB pathway following CD40 ligation[27] and TRAF3 overexpression inhibits NF-κB activator 1 (Act1) protein and thus interferes with proliferation,[28] while a dominant-negative TRAF3 mutant impairs CD40-mediated growth inhibition in malignant urothelial cells.[29] However, in HeLa cells engineered to express CD40, cell death has been attributed to TRAF6.[13]

The role of TRAFs in CD40 signaling has been mainly investigated by TRAF overexpression studies in cells ectopically expressing wild-type or mutant CD40, although this artificial setting may mask subtle interactions.[30] Furthermore, most reports have been unable to make distinctions between normal cell-specific and malignancy-associated responses to CD40 signaling in epithelial cells. To overcome these issues, we have used our *in vitro* system of normal human urothelial (NHU) cells[31,32] and their urothelial cell carcinoma (UCC) counterparts, and shown that membrane-presented, but not soluble CD40L, specifically induced apoptosis in transformed cells.[15,16] This has parallels with B-cell biology, where membrane-presented CD40L (mCD40L) exerts more profound effects than soluble agonist.[33]

Here, we have investigated the downstream effector pathways initiated following CD40 ligation in urothelial cells in order to identify components of the CD40 signaling cascade triggered by mCD40L in normal cells, and to understand the mechanism(s) of CD40-induced cell death in malignant cells.

Results

Membrane CD40L (mCD40L), but not soluble CD40 agonist, induces extensive apoptosis in malignant urothelial cells

In accordance with our previous observations,[15,16] coculture of CD40-positive UCC cells with CD40L-expressing fibroblasts resulted in at least 60–70% apoptosis of the tumor cells at 48–72 h, as determined by flow cytometry (Annexin V/PI assay) or by DNA fragmentation (JAM test). The results were identical whether the CD40L-expressing fibroblasts were used untreated or growth-arrested using mitomycin C (MMC). Control MMC-treated fibroblasts retained their integrity for at least 5 days in culture, and there was no evidence of necrotic or apoptotic cell death of either population following extended urothelial cell coculture with MMC-treated control 3T3neo fibroblasts (not shown). As demonstrated previously,[15] soluble CD40 agonist (G28-5 antibody) either alone or in the presence of 3T3neo control fibroblasts did not induce apoptosis in carcinoma cells (not shown).

CD40-mediated apoptosis does not involve induction of death ligands

Previous reports have shown that CD40-mediated apoptosis of carcinoma cells involves upregulation of death receptors and ligands, notably FasL, TRAIL and TNFα.[11–14] We used phenotypic analysis by flow cytometry and blocking antibodies to determine the involvement of such mechanisms in apoptosis induced by mCD40L. In order to more readily visualize any receptor downregulation in the coculture situation, the flow cytometer acquisition sensitivity was increased in some experiments (Figure 1a and b).

UCC cells express Fas, but not FasL (Figure 1a). Coculture with 3T3CD40L cells resulted in slight upregulation of Fas at 24 h, in agreement with previous reports,[13,14] but no detectable induction of FasL. Coculture with 3T3neo cells did not cause any change in Fas expression (Figure 1a). Although the NOK-1 monoclonal antibody (mAb) abrogated Fas-mediated killing of Jurkat cells (Supplementary Figure 1a), it had no effect on mCD40L-induced apoptosis, even at 10 μg/ml (Figure 1a), a concentration at least 10-fold higher than that used by others.[11] Identical results were obtained with the CD40$^+$ RT4 cells (Supplementary Figure 2a).

Malignant urothelial cells express TRAIL-RI and RII, but not TRAIL (Figure 1b). TRAIL-R expression remained unchanged and no *de novo* induction of TRAIL was observed following coculture with 3T3CD40L or 3T3neo cells (Figure 1b). RIK-2 mAb abrogated TRAIL-mediated apoptosis in Jurkat cells (Supplementary Figure 1b), but did not prevent killing of EJ (Figure 1b) or RT4 cells (Supplementary Figure 2b).

UCC cells also express TNF-RI and RII (Figure 1c) and respond to TNFα.[15] No differences in either TNFR or membrane TNFα expression were observed following CD40 ligation (Figure 1c). Furthermore, no TNFα was detected at 3, 6, 12, 24 and 36 h in coculture supernatants, although IL-6 secretion was seen at 3 h and peaked at 36 h (not shown), in agreement with previous studies.[13] TNFα-blocking antibody did not inhibit CD40-mediated apoptosis of EJ (Figure 1c) or RT4 cells (Supplementary Figure 2c), although it blocked TNFα-mediated intracellular adhesion molecule 1 (ICAM-1) upregulation (Supplementary Figure 1c).

Collectively, our data suggest that mCD40L-induced cell death occurs via a direct mechanism, and argue against autotropic/paratropic killing by induction of death receptors/ligands previously implicated in apoptosis induced by CD40 agonists. Thus, in UCC cells, the apoptotic pathways promoted by Fas, TRAIL-R and TNF-R are, at least in their initiation, distinct from those triggered by CD40.

Differential regulation of TRAF proteins by CD40 in normal and malignant cells

TRAF protein expression was examined by immunoblotting using human-specific antibodies. Following coculture with 3T3CD40L, NHU cells showed approximately two-fold
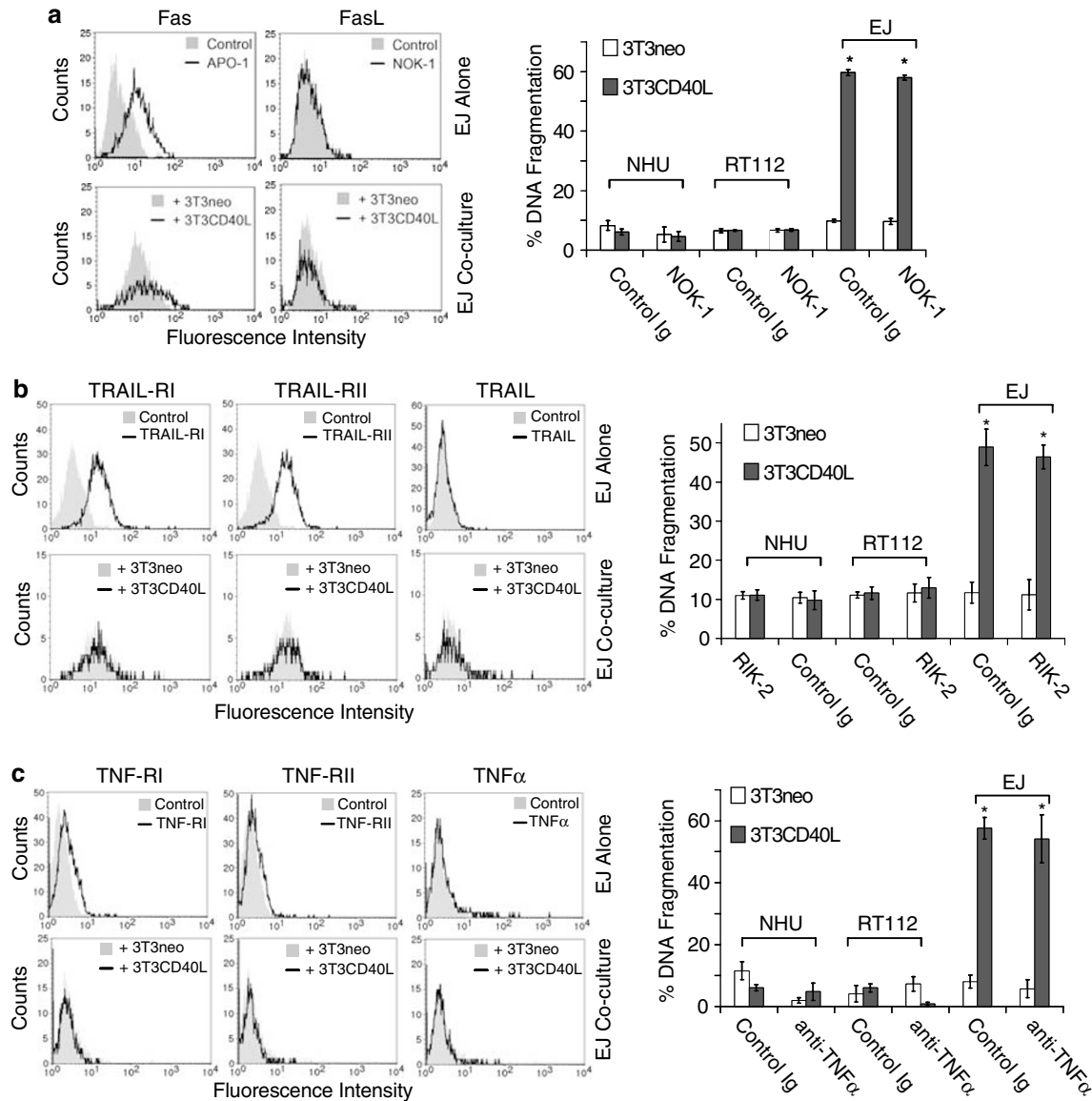
Figure 1 Autotropic/paratropic mechanisms in CD40-mediated apoptosis. Urothelial cells were cultured alone or with 3T3neo or 3T3CD40L fibroblasts. Flow cytometry was used to assess expression of death receptors/ligands on CD40-susceptible EJ cells (left panels). In some coculture experiments (**a**, **b**), acquisition sensitivity was increased to shift the negative control histograms (omitted for clarity) towards the second decade in order to permit detection of any receptor downregulation. The effect of blocking antibodies (all at 10 μg/ml) on CD40-mediated apoptosis was assessed by the JAM test of DNA fragmentation (right panels) on normal human urothelial (NHU) and transformed EJ (CD40$^+$) and RT112 (CD40$^-$) cells. Bars represent means ($\pm$ S.D.) of 12 replicates and are representative of at least three independent experiments. *$P < 0.001$ for EJ/3T3neo *versus* EJ/3T3CD40L cocultures. (**a**) The Fas/FasL System. Constitutive (top) and CD40-induced expression at 24 h (bottom) of Fas and FasL on EJ cells was assessed using APO-1 and NOK-1 mAbs, respectively (open bars), relative to negative controls (filled bars). The effect of Fas-blocking NOK-1 mAb on CD40-mediated apoptosis was assessed by the JAM test (right panel). (**b**) TRAIL and TRAIL receptors. Constitutive (top) and CD40-induced expression at 24 h (bottom) of TRAIL-RI, TRAIL-RII and TRAIL is shown (open bars) relative to negative controls (filled bars). The effect of TRAIL-blocking RIK-2 mAb on apoptosis (right panel) was assessed as above. (**c**) TNFα and TNF receptors. Constitutive (top) and CD40-induced expression at 24 h (bottom) of TNF-RI, TNF-RII and TNFα is shown (open bars) relative to negative controls (filled bars). The effect of TNF-blocking mAb on CD40-mediated apoptosis was assessed as above (right panel)

increase in TRAF1 expression after 12 h, while TRAF2 and 3 levels declined by at least two-fold after 6 h (Figure 2a). By contrast, in EJ cells, CD40 ligation resulted in moderate upregulation of TRAF2 and massive induction of TRAF1 and 3 (Figure 2a). Similar results were obtained with RT4 (not shown), whereas the CD40$^-$RT112 cells expressed virtually no TRAF1, 2 or 3 (below), and no changes in TRAF expression were seen following coculture (not shown). No

TRAF6 was detectable in NHU, EJ and RT112 cells, even after 24 h coculture (not shown). Thus, in normal urothelial cells, regulation of TRAF1–3 follows the pattern previously described for B cells stimulated by CD40 agonist,[20] whereas mCD40L causes dramatic upregulation of TRAF1 and 3 proteins in transformed cells.

As the mode of CD40L presentation critically affects the outcome of CD40 ligation,[15,33] we examined TRAF
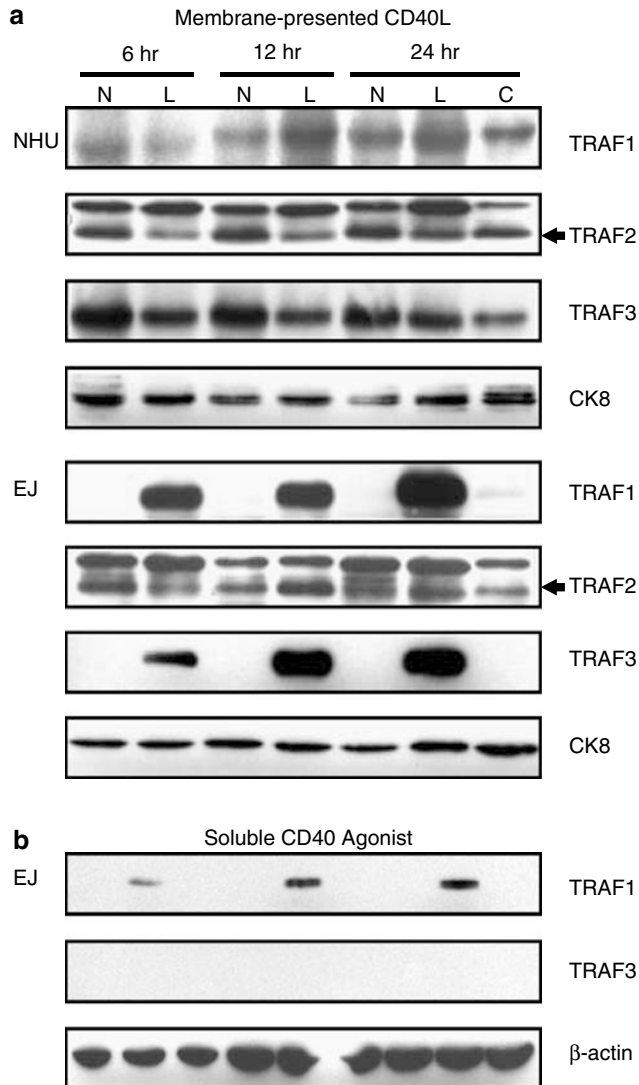
Figure 2 Expression of TRAF proteins following CD40 ligation by mCD40L *versus* agonistic anti-CD40 antibody. (**a**) NHU and EJ cells were cocultured for 6, 12 and 24 h with 3T3neo (N) and 3T3CD40L (L) cells. Cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membranes, probed with antibodies specific for human TRAFs and visualized by chemiluminescence and autofluorography. Track loading was adjusted according to reactivity with human-specific anti-CK8 mAb. Lysates from cocultures of urothelial cells with untransfected fibroblasts served as controls (C). Band intensities for TRAF1, TRAF2 (arrowed) and TRAF3 in NHU lysates were analyzed by densitometry, normalized against CK8 and expressed as ratios of 3T3CD40L *versus* 3T3neo cocultures. The results from three independent experiments were: TRAF1: 0.66 ± 0.12 (6 h), 1.78 ± 0.11 (12 h) and 1.45 ± 0.16 (24 h); TRAF2: 0.82 ± 0.09 (6 h), 0.45 ± 0.08 (12 h) and 0.72 ± 0.09 (24 h); TRAF3: 0.68 ± 0.15 (6 h), 0.41 ± 0.10 (12 h) and 0.47 ± 0.08 (24 h). (**b**) EJ cells were cultured for 6, 12 and 24 h either alone (A) or with 10 μg/ml agonistic G28-5 anti-CD40 antibody (G) and isotype-matched control mAb (C). Immunoblotting was performed as above for TRAFs 1 and 3

expression after treatment with the anti-CD40 agonistic mAb G28-5.[3] G28-5 had no effect on expression of TRAF1–3 in NHU (not shown), but caused upregulation of TRAF1 in EJ cells (Figure 2b). However, TRAF3 remained undetectable even at 24 h (Figure 2b) and TRAF2 expression was unchanged (not shown). Thus, the degree of receptor cross-linking not only determines whether the CD40 signal is proapoptotic, but also directly influences regulation of TRAF expression.

TRAF regulation by CD40 involves both transcriptional and post-transcriptional mechanisms

We performed RT-PCR to determine whether changes in mRNA accounted for TRAF protein regulation. Coculture of NHU with 3T3CD40L cells resulted in a small, but consistent, upregulation of TRAF1 mRNA within 1.5 h, whereas TRAF2 and 3 mRNA levels remained unchanged, even at 6 h (Figure 3a). CD40 ligation in EJ cells similarly resulted in no mRNA changes for TRAF2 and 3, but caused rapid induction of TRAF1 mRNA. These changes were CD40-specific, as coculture of RT112 cells with 3T3CD40L had no effect on TRAF mRNA levels (not shown). Thus, TRAF regulation in urothelial cells occurs both at the transcriptional and post-transcriptional level.

Our data suggested that TRAF3 upregulation was due to protein stabilization and previous work has suggested proteasomal degradation as a mechanism for TRAF down-regulation.[20,21] We therefore treated UCC cells with proteasome inhibitor (clasto-lactacystin) for 6 h and assessed TRAF expression by immunoblotting as above. Although limited by inhibitor toxicity at higher doses, these experiments showed that TRAF1 and 3 expression was increased in the presence of inhibitor (Figure 3b). This increase in TRAF1 and 3 expression was maintained at 24 and 48 h following treatment (not shown). Thus, TRAF1 expression appears to be regulated both at the mRNA and protein level, whereas expression of TRAF3 is regulated mainly by post-transcriptional mechanisms.

RNAi reveals an essential role for TRAF3 in CD40-mediated apoptosis

As TRAF1 and 3 proteins were induced only when CD40 was engaged by mCD40L (proapoptotic signal), we investigated TRAF involvement in CD40-mediated death by functional inactivation using RNAi. Transfection with siRNA against CD40 resulted in >50% cells losing all CD40 expression, with reduced expression in the remainder (Figure 4a). Even this incomplete loss was sufficient to virtually abrogate CD40-mediated killing in EJ (Figure 4b) and RT4 cells (not shown). Control siRNA transfection did not affect cell death in 3T3CD40L *versus* 3T3neo cocultures (Figure 4b).

The effect of functional inactivation of TRAFs by RNAi was examined in similar apoptosis assays (Figure 4c). The ability of the siRNAs designed for TRAF1 (not shown), TRAF2 and TRAF3 to knockdown protein expression was confirmed by immunoblotting (Figure 4c). Transfection with TRAF1 or TRAF2 siRNA resulted in similar decreases in CD40-mediated killing, whereas TRAF3 siRNA almost completely abrogated cell death, comparable to the effect of CD40 siRNA (Figures 4a and b). By contrast, a TRAF6
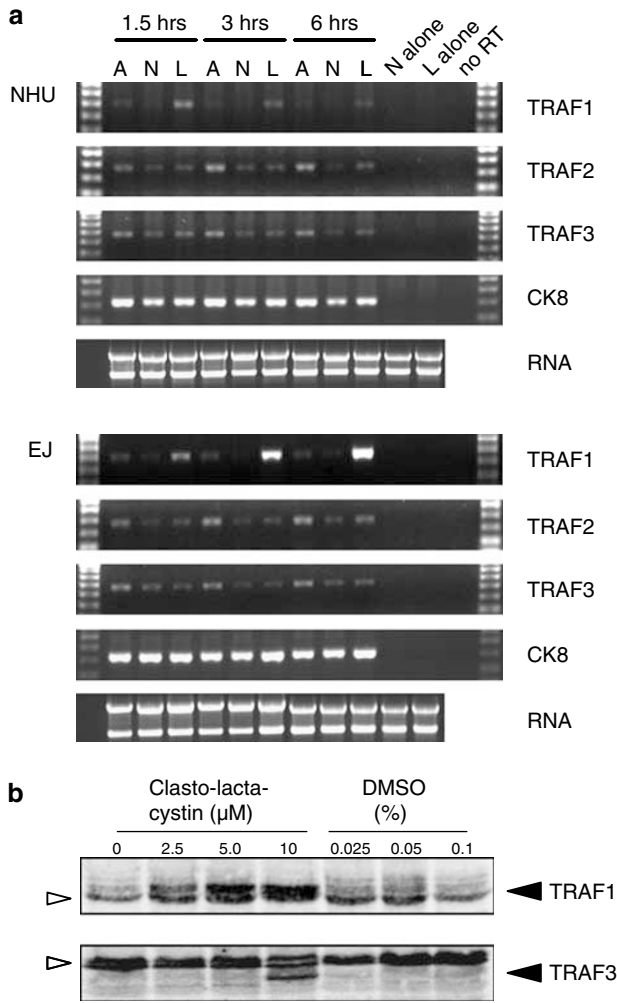
a

NHU

TRAF1

TRAF2

TRAF3

CK8

RNA

EJ

TRAF1

TRAF2

TRAF3

CK8

RNA

b

Figure 3 Mechanisms of regulation of TRAF mRNA and protein expression by mCD40L. (**a**) NHU and EJ cells were cultured alone (A) or with 3T3neo and 3T3CD40L (N and L, respectively). Following culture for 1.5, 3 and 6 h, total RNA was isolated and cDNA was prepared for PCR using human TRAF-specific primers. RNA extracts isolated from 3T3-only cultures (N alone and L alone) were also included to ensure PCR specificity for amplification of human cDNA. Lack of DNA contamination was confirmed in reactions without reverse transcriptase (no RT). PCR products were separated by 1.5% agarose gel electrophoresis and visualized with ethidium bromide. Loading of RNA samples alone (bottom panels) was included to confirm RNA integrity and quality. (**b**) EJ cells were cultured alone, treated with increasing concentrations of the proteasome inhibitor clasto-lactacystin as indicated, or cultured in the presence of equivalent concentrations of DMSO (solvent control) for 6 h. Concentrations of clasto-lactacystin above $10\,\mu M$ were toxic to EJ cells. Whole-cell lysates were prepared and immunoblotted for TRAF1 and TRAF3 as described in Figure 2b. Results are representative of three experiments. Open arrows indicate nonspecific bands

siRNA did not affect CD40-induced death (not shown), demonstrating that our findings were not due to non-specific siRNA effects. Combinations of TRAF1/TRAF2 or TRAF2/TRAF3 siRNAs were not additive or synergistic (not shown).

TRAF3 is thus essential for the induction of death by CD40, as loss of TRAF3 is functionally equivalent to loss of CD40 expression. The proapoptotic property of TRAF3 is supported by its previously reported role in soluble agonist-mediated growth inhibition of EJ cells.[29]
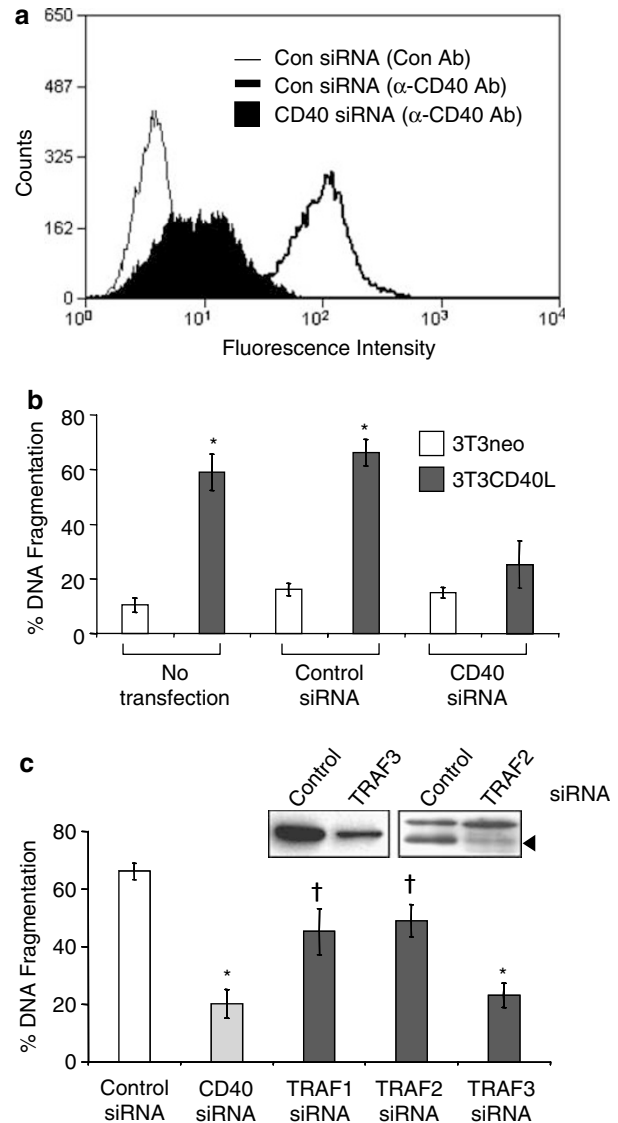
a

b

c

Figure 4 Investigation of the role of TRAF proteins in CD40-induced apoptosis by RNAi. (**a**) EJ cells were transfected with CD40 siRNA (5′-AAACAGACAC CATCTGCACCT-3′) or an irrelevant siRNA (control). Cells were harvested 12 h after transfection and analyzed for surface CD40 expression by flow cytometry. Representative histogram data from three independent experiments are shown. (**b**) At 16 h after transfection of EJ cells with control or CD40 siRNA, 3T3CD40L and 3T3neo cells were plated on top and apoptosis was assessed 48 h later by the JAM test of DNA fragmentation. No-transfection controls were included to confirm lack of transfection-related toxicity. Bars denote mean % DNA fragmentation ($\pm$ S.D.) of 16 replicates and results are representative of three experiments. *Indicates $P < 0.001$ for EJ/3T3neo *versus* EJ/3T3CD40L cocultures. (**c**) TRAF- and CD40-specific siRNAs were transfected into EJ cells as above, alongside control siRNA. The TRAF siRNAs used were: 5′-AAGCTGCGTGTGTTTGAGAAC-3′ (TRAF1), 5′-AAGATGTGTCTGCGTATC TAC-3′ (TRAF2) and 5′-AAGCAGACAGCATGAAGAGCA-3′ (TRAF3). At 16 h after transfection, 3T3s were plated on top and apoptosis was assessed 48 h later by the JAM test. Bars show mean % DNA fragmentation ($\pm$ S.D.) of 12 replicates and results are representative of three experiments. Significant differences between EJ/3T3neo *versus* EJ/3T3CD40L cocultures are indicated by *$P < 0.001$ for EJ cells pretransfected with CD40 or TRAF3 siRNA and †$P < 0.01$ for EJ cells pretransfected with TRAF1 or 2 siRNA. siRNA knockdown efficiency was confirmed by immunoblotting (insets) after transfection of EJ cells with siRNA for TRAF3 (left panel) or TRAF2 (right panel) and subsequent coculture with 3T3CD40L cells for 6 h

CD40-mediated apoptosis requires TRAF3-induced JNK phosphorylation and AP-1 activation

In B cells, CD40 ligation leads to TRAF recruitment and activation of the NF-κB and AP-1 pathways.[17] Activation of these transcription factors can promote proliferation or apoptosis in a signal- and cell type-specific manner.[26] We therefore investigated the role of components of signaling cascades leading to NF-κB and AP-1 activation using pharmacological inhibitors of JNK, MEK/ERK and p38, as well as inhibitors of NF-κB and AP-1 (Figure 5a).

CD40-mediated apoptosis following coculture of EJ cells with 3T3CD40L cells was dose-dependently abrogated

by NDGA and SP600125. By contrast, PDTC slightly, but reproducibly, enhanced cell death, supporting our previous findings.[15] Treatment with U0126 and SB202190 had no effect on CD40 killing. Similar results were obtained with RT4 cells (Supplementary Figure 3). Thus, JNK and AP-1 are essential for CD40-mediated death in UCC cells, which accords with the critical role for AP-1 reported in CD40-induced hepatocyte death.[10] The CD40 apoptotic pathway was not influenced by p38 and is not associated with ERK activity, in agreement with the well-established role for ERKs in cell proliferation and carcinogenesis, and in inhibition of apoptosis induced by death ligands such as TRAIL.[34]
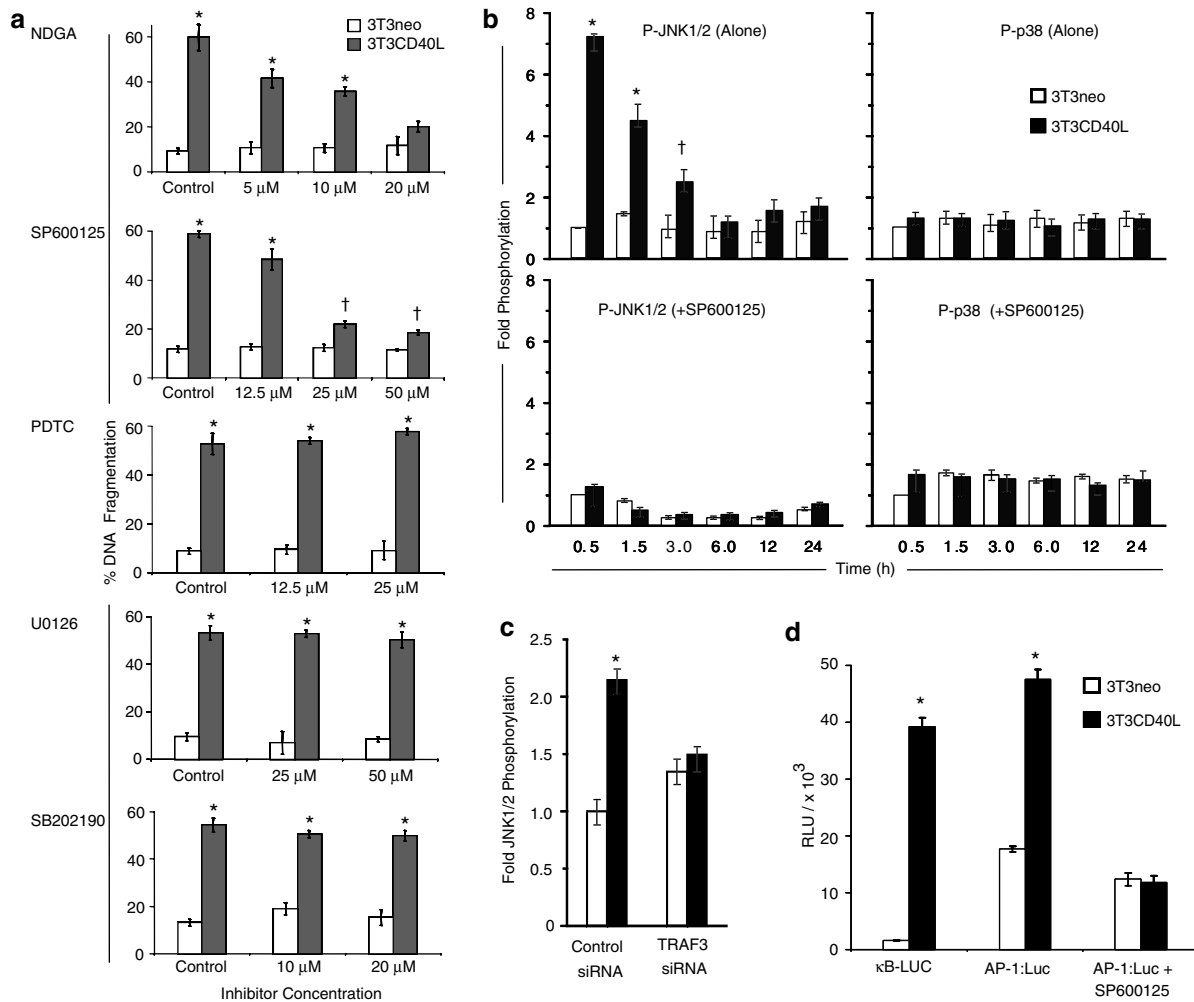

Figure 5 Intracellular signaling pathways in CD40-mediated tumor cell death. (**a**) Cocultures of EJ cells with 3T3neo and 3T3CD40L fibroblasts were performed in the presence of AP-1 inhibitor (NDGA), JNK inhibitor (SP600125), NF-κB inhibitor (PDTC), MEK/ERK inhibitor (U0126) and p38 inhibitor (SB202190). Solvent-only controls were included (control). After 48 h, apoptosis was assessed by the JAM test. Bars show means ($\pm$S.D.) % DNA fragmentation of 12 replicate wells and are representative of three experiments. *$P < 0.001$ and $^{\dagger}P < 0.01$, respectively between EJ/3T3neo *versus* EJ/3T3CD40L cocultures. (**b**) EJ cells were cocultured with 3T3neo and 3T3CD40L cells in the presence of JNK inhibitor SP600125 or DMSO alone. Cell lysates were prepared at the indicated time points and JNK1/2 and p38 phosphorylation was assessed using a total and a Phospho 3-plex Luminex kit as described in Materials and Methods. Results are expressed as mean ($\pm$S.D.) fold phosphorylation (following normalization against total protein) from triplicate samples from two independent experiments. *$P < 0.001$ and $^{\dagger}P < 0.01$, respectively, between EJ/3T3neo *versus* EJ/3T3CD40L cocultures. (**c**) EJ cells were transfected with TRAF3 siRNA before coculture with 3T3s for 1.5 h. Lysates were prepared and JNK1/2 phosphorylation was detected as above. Results are expressed as mean ($\pm$S.D.) fold phosphorylation (representative of two experiments). *$P < 0.01$ between EJ/3T3neo *versus* EJ/3T3CD40L cocultures. (**d**) EJ cells were transfected with κB-LUC or AP-1:Luc plasmids and cocultured with 3T3 cells for 16 h prior to luciferase reporter assay (see Materials and Methods). For AP-1: Luc transfections, cocultures were also performed in presence of JNK inhibitor SP600125. Results are representative of three experiments. Bars indicate relative luciferase units (RLU) (mean $\pm$ S.D.) from five replicate wells. *$P < 0.001$ in RLU between EJ/3T3neo *versus* EJ/3T3CD40L cocultures

We then examined whether TRAF3 functions as a link between CD40 engagement and induction of the JNK/AP-1 pathway. Following a multiplex bead array approach, we found that mCD40L induced rapid phosphorylation of JNK1/2, which was completely blocked by the JNK inhibitor SP600125. By contrast, soluble CD40 agonist (G28-5 antibody) did not induce JNK activation (not shown). Of note, CD40 ligation did not induce p38 phosphorylation (Figure 5b), although it did activate Akt (not shown), in agreement with the ability of CD40 to induce the phosphatidylinositol triphosphate kinase (PI3-K)/Akt pathway in epithelial cells.[35] Importantly, TRAF3 knockdown by RNAi also blocked JNK phosphorylation (Figure 5c), although addition of TRAF2 siRNA did not enhance the effect of TRAF3 siRNA alone (not shown). Finally, we performed luciferase reporter gene assays to directly assess NF-κB and AP-1 activity following CD40 ligation (Figure 5d). UCC cells did not exhibit basal NF-κB activity, but they showed basal AP-1 activity, as seen in cells cultured alone (not shown) or following coculture with 3T3neo cells (Figure 5d). Our reporter assay demonstrates that CD40 ligation induces NF-κB (in agreement with our previous findings[15]) and AP-1 activity, while induction of AP-1 was specifically abolished by the JNK inhibitor SP600125.

Collectively, our findings suggest that CD40 ligation by mCD40L, but not soluble agonists, activates JNK, which in turn mediates AP-1 induction and this signalling cascade is dependent on TRAF3.

Engineered CD40 expression in carcinoma cells confers apoptotic susceptibility via the same signaling mechanisms

We have previously shown that all UCC cells that retained CD40 expression are highly susceptible to CD40-induced apoptosis.[15] To examine whether restoration of CD40 expression restores apoptotic susceptibility, we engineered receptor expression using a retroviral vector. Transduced RT112 (RT112-CD40) cells expressed high levels of CD40 (Figure 6a) and were vulnerable to CD40-mediated apoptosis, whereas control (RT112-neo) cells remained refractory (Figure 6b). Similarly, retroviral transduction conferred apoptotic susceptibility to the highly differentiated HT1376 and the relatively anaplastic VM-CUB-1 cells (Supplementary Figure 4). In all three lines, death was elicited by mCD40L and not by soluble agonist (not shown). Thus, restoration of CD40 expression in UCC cells restored susceptibility to apoptosis, independent of the degree of cytodifferentiation.

We next investigated whether *de novo* CD40 expression renders malignant cells susceptible to death via the same TRAF3-mediated, JNK/AP-1-dependent mechanisms demonstrated above. CD40 ligation in RT112-CD40 resulted in TRAF1 protein induction and massive upregulation of TRAF3, while TRAF2 levels increased moderately (Figure 6c), reflecting the pattern of TRAF regulation in EJ cells (Figure 2a). Coculture of RT112-neo with 3T3CD40L had no effect on TRAF expression (not shown). As expected, agonistic G28-5 mAb did not cause any changes in either TRAF1 or TRAF3 expression in RT112-CD40 cells (Figure 6c) and TRAF2 levels remained unchanged (not shown). Furthermore, preliminary studies showed that a CD40 mutant truncated by 22

amino acids and thus unable to bind TRAF3 (constructed as described in Hostager and Bishop[22]) did not transmit an apoptotic signal in RT112 cells (not shown). Finally, functional blocking studies showed that the JNK and AP-1 inhibitors virtually abrogated CD40 killing, whereas NF-κB inhibitor slightly enhanced apoptosis (Figure 6d).

Therefore, our results demonstrate that *de novo* CD40 expression in malignant urothelial cells renders them highly susceptible to mCD40L-induced apoptosis and show that CD40 orchestrates the same pathways as found in CD40-intact cells to initiate and transmit its apoptotic signal.

CD40 ligation triggers apoptosis via activation of caspase-9 but not caspase-8

In order to determine whether apoptosis induced by mCD40L triggers cell death via the cell-intrinsic *versus* the cell-extrinsic pathways, we determined activation of the caspases specifically associated with each pathway. In both EJ and RT112-CD40 cells, ligation of CD40 by mCD40L resulted in activation of the initiator caspase-9 and the effector caspases 3 and 7 within 24 h (Figure 7a), with little induction of caspase-8 activity. Functional investigations were performed using biochemical caspase inhibitors. Apoptosis following coculture with 3T3CD40L cells was completely blocked by the pan-caspase inhibitor z-VAD, as well as by specific inhibitors of caspases 9 and 3 (z-LEHD and z-DQMD, respectively). By contrast, the caspase-8 inhibitor z-IETD had little effect (Figure 7b). Similar results were obtained with RT4 and RT112-CD40 cells (not shown).

Thus, CD40 ligation by mCD40L appears to trigger the caspase activation cascade associated with the intrinsic, mitochondrial pathway, an observation supported by our previous findings that mCD40L upregulates expression of Bax,[15] rather than the caspase-8-mediated extrinsic pathway reported in situations where CD40-mediated killing is by an autotropic/paratropic mechanism involving induction of death receptors/ligands.[13]

Discussion

The downstream signaling events following CD40 ligation in normal epithelial cells are largely unknown, although such pathways have been investigated extensively in carcinoma-derived cells due to the well-established ability of CD40 to induce apoptosis following CHX treatment. Yet paradoxically, only a small number of studies have addressed the exact mechanisms of CD40-induced death. Whereas studies based on ectopic overexpression of TRAFs and CD40 have contributed to our understanding of the mechanisms of TRAF action and potential intermolecular interactions, they represent an artificial scenario. Furthermore, unlike many members of the TNF family, physiological CD40 signals are provided by mCD40L, and investigations relying exclusively on soluble agonists are less relevant for the understanding of the CD40/CD40L dyad.

We have studied the effects of mCD40L in an *in vitro* epithelial model that accurately represents the situation *in vivo*. The biological relevance of our human urothelial system[32] is evidenced by the ability of cultured NHU cells to
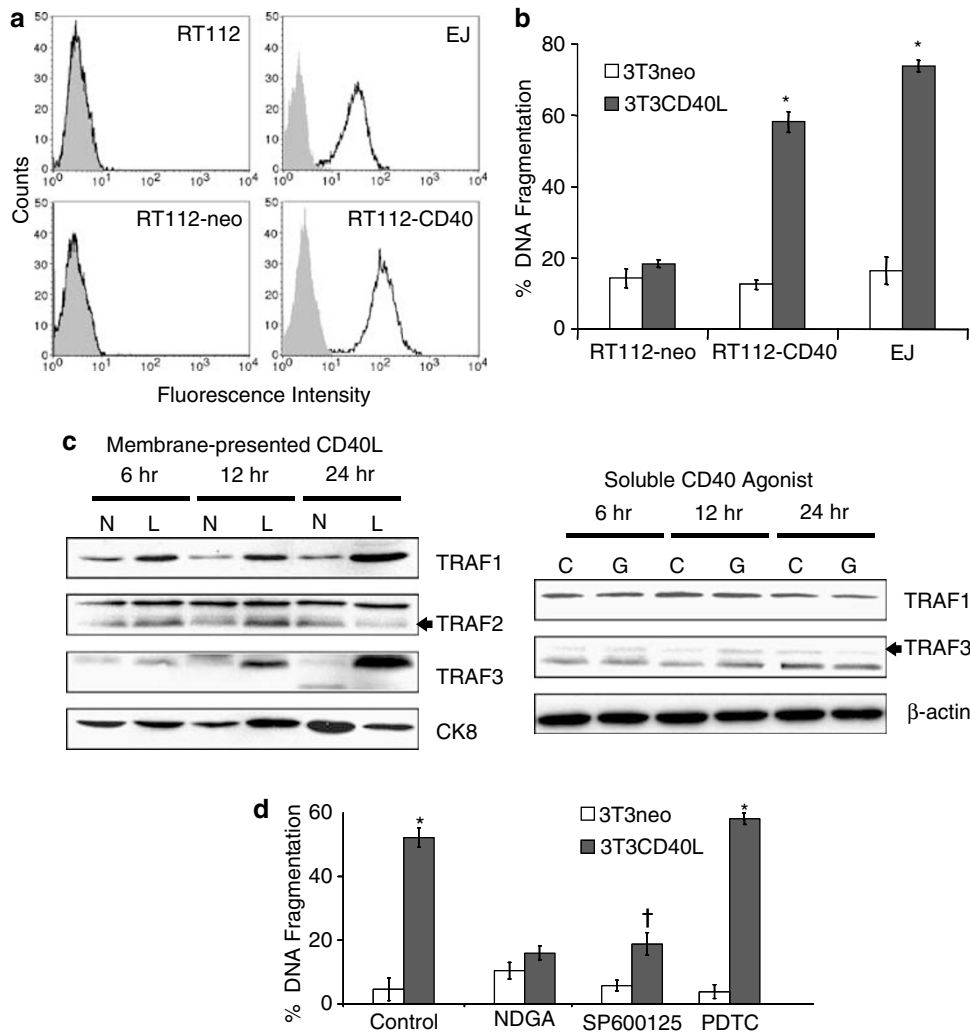
Figure 6 *De novo* CD40 expression renders CD40-negative tumor cells susceptible to CD40-mediated killing via identical apoptotic signaling mechanisms. (**a**) Stable populations of CD40-transduced RT112 (RT112-CD40) and control (RT112-neo) cells were established and CD40 expression (open histograms) was assessed by flow cytometry. RT112 and EJ cells served as negative and positive controls, respectively. Filled histograms represent antibody controls. (**b**) RT112-neo, RT112-CD40 and EJ cells pulsed with [³H]TdR were cultured alone or with 3T3 cells and apoptosis was assessed by the JAM test at 48 h (see Figure 1). Bars represent means ($\pm$ S.D.) of 16 replicate wells and are representative of three experiments. *$P < 0.001$ and $^†P < 0.01$, respectively in EJ/3T3neo *versus* EJ/3T3CD40L cocultures. (**c**) RT112-CD40 cells were cultured for 6, 12 and 24 h with 3T3neo (N) and 3T3CD40L (L) (left panel) or with 10 μg/ml G28-5 mAb (G) and isotype-matched control (C) (right panel). Immunoblotting for TRAFs 1-3 was performed as in Figure 2a. (**d**) RT112-CD40 cells were cultured alone or with 3T3 cells in the presence of AP-1 inhibitor NDGA (20 μM), JNK inhibitor SP600125 (50 μM), NF-κB inhibitor PDTC (25 μM) or DMSO alone (control) and apoptosis was assessed at 48 h by the JAM test. Bars show means ($\pm$ S.D.) of 12 replicate wells and are typical of three experiments. *$P < 0.001$ and $^†P < 0.01$, respectively, for RT112-CD40/3T3neo *versus* RT112-CD40/3T3CD40L cocultures

form histologically normal, transitional stratified urothelia when seeded onto de-epithelialized bladder stromas,[36] as well as the ability of the tumor-derived cell lines to recapitulate the grade and stage of the tumors of origins *in vitro*[37] and *in vivo*.[38] This has permitted us to make two novel observations: (a) TRAF expression patterns in normal urothelial cells following CD40 ligation parallel those of B cells,[22] and (b) CD40-induced apoptosis in transformed cells occurs via a novel mechanism that does not involve the induction of death ligands previously implicated in other cell systems.[11–14]

Critically, killing by mCD40L occurs without inhibition of protein synthesis, whereas soluble CD40 agonists entrain the apoptotic machinery only if protein synthesis is arrested, as shown by ourselves[15] and others.[11,13,29] Furthermore, the same direct killing effect can be elicited by agonistic G28-5

antibody when presented on the surface of IgFcγ (CD32)-bearing cells, but not in the presence of control fibroblasts.[15] The inability of control fibroblasts to mediate apoptosis in the presence of soluble CD40L or G28-5 antibody argues conclusively against any nonspecific effects, as suggested by others.[39]

In B cells, CD40 initiates complex signaling cascades via direct interactions with the TRAFs. Differential TRAF binding induces specific receptor conformations and the degree of receptor crosslinking critically influences the 'molecular signature' of the CD40 stimulus.[24,25] With its ability to degrade or stabilize and therefore differentially regulate the TRAFs, CD40 can provide either short-lived signals or sustained stimuli, thereby providing outcome diversity and context specificity.
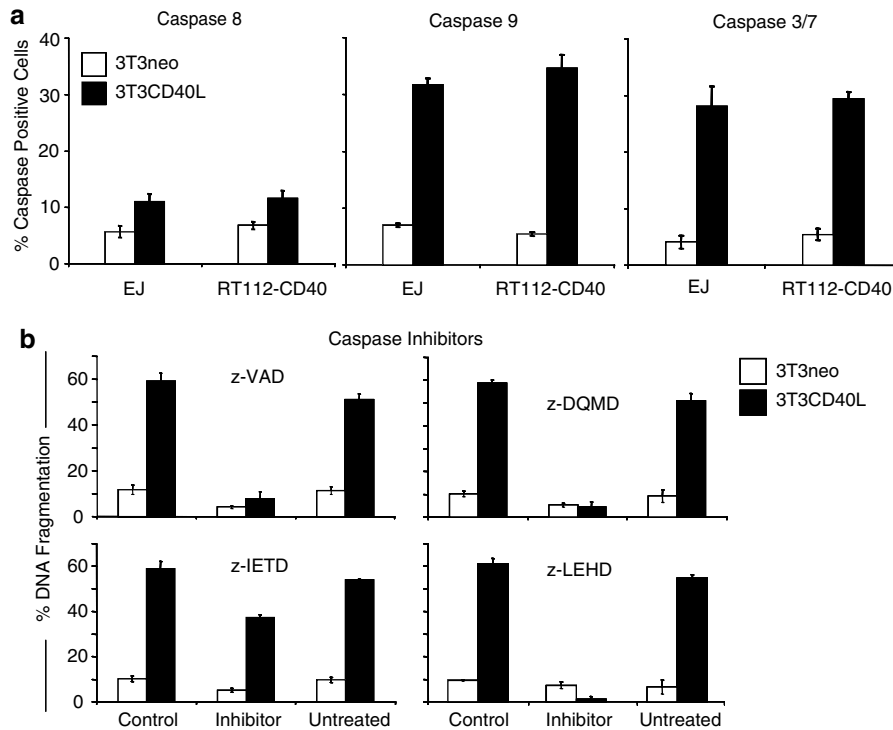
Figure 7 Caspase activation following CD40 ligation by mCD40L. (**a**) Cocultures of EJ and RT112-CD40 cells with 3T3neo or 3T3CD40L fibroblasts were performed for 24 h and induction of caspases 3/7, 8 and 9 was detected using FAM FLICA Caspase Detection Kits and flow cytometry. FAM FLICA caspase substrate fluorescence was detected in the FL-1 channel, following gating on the urothelial cells by criteria of forward/side scatter to exclude 3T3 cells from the analysis. % positive epithelial cells were calculated for five replicate samples and results are representative of four independent experiments. Bars show means ($\pm$ S.D.). (**b**) EJ cells were cultured alone or with 3T3 cells in the presence of a pan-caspase inhibitor (z-VAD) or inhibitors of caspase-3 (z-DQMD), caspase-8 (z-IETD) and caspase-9 (z-LEHD). All inhibitors were used at 100 μM, optimized for minimal toxicity by pretitration experiments. Untreated cells as well as solvent-only controls were included (control). After 48 h culture or coculture, apoptosis was assessed by the JAM test. Bars show mean ($\pm$ S.D.) % DNA fragmentation of 16 replicate wells and are representative of three experiments

CD40-mediated TRAF regulation in normal epithelial cells has not been previously reported. Here we show that TRAFs 2 and 3 are downregulated in normal urothelial cells, exhibiting a clear parallel with the situation in B cells,[21] where TRAF 2 and 3 downregulation occurs by proteasomal degradation. It is likely that the same post-transcriptional mechanism is employed by NHU cells, a hypothesis supported by our finding that downregulation of TRAF proteins induced by mCD40L was not reflected by alterations in TRAF mRNA levels.

In transformed cells, TRAF regulation by mCD40L is very different, with massive upregulation of TRAFs 1 and 3. Particularly in the case of TRAF3, we often observe upregulation as early as 2–3 h following CD40 ligation, although we could find no evidence for expression of TRAF3 splice variant isoforms (data not shown). Although induction of TRAF1 protein expression by CD40 has been documented,[40] this is the first such demonstration for TRAF3. Here, we provide evidence that upregulation of TRAF1 protein is the result of transcriptional and post-transcriptional mechanisms, whereas TRAF3 expression appears to be modulated by proteasomal degradation. We now have preliminary evidence that CD40 ligation by mCD40L induces TRAF3 colocalization, and also results in receptor downregulation, as previously reported for soluble CD40L in B cells[18] (unpublished observations). Collectively, our data imply that, under normal circumstances, TRAF3 is continually being synthesized and

proteasomally degraded, but, upon CD40 engagement by membrane-presented ligand, it is recruited to the receptor and thus escapes degradation. Protein stabilization would account for the massively increased protein expression in the absence of mRNA upregulation.

TRAF3 has previously been associated with growth inhibition[13] and inhibition of NF-κB by interaction with Act1 in carcinoma cells.[28] Our results reveal for the first time an essential role for TRAF3 in CD40-mediated tumor cell apoptosis, as TRAF3 knockdown is functionally equivalent to loss of CD40 expression. The importance of TRAF3 in apoptosis is further supported by our findings on the role of receptor crosslinking in modulating TRAF expression. Whereas the weak nonapoptotic CD40 signal provided by soluble agonist caused upregulation of TRAF1, it was not adequate to upregulate TRAF3, where only the strong proapoptotic mCD40L signal was sufficient. These findings both underline the critical role of TRAF3 and extend our previous observations that CD40 crosslinking determines functional outcome.[15]

The RNAi experiments suggest that TRAFs 1 and 2 may also influence apoptosis, albeit to a lesser extent than TRAF3. This probably reflects a role for TRAF1, rather than TRAF2, in the apoptotic pathway. TRAF1 can negatively regulate TRAF2-associated antiapoptotic signals[41,42] and, when over-expressed, can inhibit CD40-mediated NF-κB activation.[17] In *Drosophila*, TRAF1 is associated with apoptosis, whereas

TRAF2 is involved in proliferation and NF-κB activation.[43] TRAFs 1 and 3 may act independently to activate different pathways, with TRAF3 shifting the equilibrium toward apoptosis, perhaps by recruiting other signaling component(s) following relocation to CD40.

Our studies provide evidence that TRAF3 directly influences the phosphorylation status and activation of JNK, which in turn induces AP-1. We have demonstrated that JNK activation occurs rapidly following CD40 ligation, returning to normal levels by 6 h, and we have evidence of TRAF3 upregulation and relocalization to CD40 within less than 3 h by immunofluorescence microscopy (unpublished observations). Therefore, it appears that TRAF3 rapidly initiates the signaling cascade, with TRAF3 stabilization and JNK activation occurring concurrently. This suggests that further increases in TRAF3 levels following the initial TRAF3–CD40 interaction, as shown by the immunoblotting studies, do not necessarily further contribute to the apoptotic signaling, and we are currently addressing this hypothesis.

Cytoplasmic TRAF3 is a weak JNK inducer, but becomes a strong JNK/AP-1 activator upon relocation to lipid rafts,[23] possibly acting via T3JAM, a novel protein that associates with TRAF3 and facilitates its recruitment to rafts.[44] These findings imply that TRAF3 relocation juxtaposes it with signaling molecules preferentially located in rafts. Since stabilization requires extensive receptor crosslinking, TRAF3 is likely to be the determining factor that translates the CD40 signal into a proapoptotic message in malignant cells. Interestingly, TRAF3 can interact with ASK1, MKK4/7 and JNK,[17] kinases implicated in lymphotoxin-β receptor (LTβR) signaling.[19] LTβR is similar to CD40 both structurally and functionally; it has been shown to recruit TRAF3 and thus inhibit TRAF2-mediated activation of the noncanonical NF-κB pathway[27] and, most importantly, can induce cell death in malignant epithelial cells via a TRAF3-dependent mechanism.[45] It is tempting to speculate that TRAF3 interactions with kinases, such as ASK1 or MKK7, may be how it activates JNK. Although such molecular events remain unidentified, TRAF3 appears to be the crucial link between CD40 activation and JNK/AP-1 induction that subsequently triggers cell death.

Our data provide the first demonstration of a direct role for JNK and AP-1 in the CD40-mediated apoptotic pathway in urothelial cells, and are in agreement with the reported role of JNK/AP-1 in CD40 killing in other systems.[10,14] CD40 ligation in malignant cells can also induce expression of antiapoptotic proteins via PI3-K and ERK activation,[35] thereby explaining why protein synthesis blocking is necessary for apoptosis induction by soluble agonists. However, inactivation of the MEK/ERK and p38 pathways did not alter the apoptotic response to mCD40L in our study. Rather, activation of the JNK/AP-1 cascade appears to relay a powerful apoptotic signal that dominates concomitant proliferative signals such as induction of NF-κB, whose activation by CD40 we have reported.[15]

It is well documented that JNK-induced cell death often involves the mitochondrial (intrinsic) pathway, requiring the proapoptotic Bax subfamily members of the Bcl-2 family to activate caspase-9.[46,47] We have previously shown that proapoptotic CD40 ligation in UCC cells induces Bax/Bak expression and downregulates Bcl-2,[15] and we now provide direct evidence that CD40-mediated cell death is dependent on activation of caspase-9 but not caspase-8. By contrast, where CD40 causes autotropic/paratropic activation of death ligands, it triggers the extrinsic death pathway involving caspase-8.[13] Thus, mCD40L appears to trigger JNK-induced apoptosis via the intrinsic pathway.

Loss of CD40 expression is thought to confer a survival advantage to bladder cancer cells during tumor progression.[15] Here we show that *de novo* expression of CD40 in such cells renders them susceptible to elimination by mCD40L and death occurs by the same signaling mechanisms. Restoration of susceptibility to CD40 even in anaplastic tumor cells, combined with the unique ability of CD40 to spare normal cells, has implications in future approaches involving use of CD40 as a therapeutic tool against bladder cancer.

Materials and Methods

Cell culture

NHU cells were established and cultured in complete KSFM (Gibco-BRL, Paisley, UK) containing 30 ng/ml cholera toxin (Sigma Chemical Co., Poole, UK) as described.[15,16,31,32] Human UCC-derived HT1376, RT112, VM-CUB-1 (CD40$^-$) and RT4, and EJ (CD40$^+$) lines and Jurkat J6 cells were cultured in standard medium consisting of a mixture of DMEM and RPMI 1640 (Sigma) containing 5% FBS (TCS Cellworks, Bucks, UK). Retrovirally transduced cells (below) were maintained in standard medium supplemented with 1 mg/ml G418 (Invitrogen, Paisley, UK), and 3T3neo and 3T3CD40L fibroblasts[15,16] were cultured in DMEM/10% FBS containing 0.5 mg/ml G418, with omission of antibiotic for coculture experiments.

Flow cytometry

Expression of Fas, FasL and ICAM-1 was determined as described.[15] Antibodies specific to human TRAIL-RI, -RII and TRAIL were from Alexis (Alexis Corp., Nottinghamshire, UK), to TNF-RI, -RII and TNFα from R&D Systems (Abingdon, UK) and to CD40 from Serotec (Oxford, UK). PE-goat anti-mouse Ig (Fab)'2 was from Southern Biotechnology Associates (Cambridge Biosciences, Cambridge, UK). For analysis of urothelial/3T3 cell cocultures, epithelial cells were gated by the criteria of forward and side scatter. At least 3000 events were acquired per sample and analyzed on a FACSCalibur$^{\circledR}$ using CellQuest$^{\circledR}$ software (Becton Dickinson, Oxford, UK).

CD40 ligation

Urothelial cells were treated with agonistic G28-5 anti-CD40 mAb at 10 μg/ml or an isotype-matched control.[15] For coculture experiments, 3T3neo and 3T3CD40L fibroblasts were pretreated with mitomycin C (Kyowa, Tokyo, Japan) as described[15] and seeded at 10^4 or 10^5 cells/well in 96- or 24-well plates, respectively. Epithelial cells were seeded onto fibroblasts at a ratio of 0.8–0.9:1.

Assessment of apoptosis and caspase activation

Apoptosis was assessed by DNA fragmentation (JAM test) as described.[15,16] Briefly, urothelial cells were pulsed with [^{3}H]thymidine (TdR) cultured alone or with 3T3 cells in 96-well plates and harvested 48 h later onto filter mats to estimate intact DNA using a scintillation counter

(Perkin-Elmer Wallac, Bucks, UK). % DNA fragmentation was calculated as $(S - E \times 100)/S$, where S is the cpm recovered from control cultures (urothelial cells alone) and E the cpm from test cultures. In all cases, apoptosis was confirmed by flow cytometry[15] using an Annexin V-FITC/PI kit (Serotec) (not shown).

Activation of caspases 3/7, 8 and 9 was by flow cytometry detected using FAM FLICA Caspase Detection Kits according to the manufacturer's instructions (Serotec).

Functional blocking antibodies

Urothelial-3T3 cell cocultures were carried out in the presence of blocking antibodies to FasL (NOK-1), TRAIL (RIK-2) and TNFα (R&D Systems). Jurkat cells were used as a positive control for Fas- and TRAIL-mediated apoptosis and to confirm the blocking efficacy of NOK-1 and RIK-2 mAbs. The positive control for TNFα-blocking mAb was EJ cells undergoing TNFα-mediated ICAM-1 upregulation.

Luminex assays

The phosphorylation status of Akt, JNK1/2 and p38 MAPK was determined using a Total and Phospho 3-Plex kit, and secretion of TNFα, IL-6 and other cytokines was assayed using a 10-plex human cytokine kit according to the manufacturer's instructions (BioSource Europe, Belgium) on a Luminex® 100 IS System with StarStation Software (Applied Cytometry Systems, Sheffield, UK).

Pharmacological inhibitors

The AP-1 (NDGA) and NF-κB (PDTC) inhibitors were from Sigma. Inhibitors to JNK (SP600125), MEK/ERK (U0126) and p38 MAPK (SB202190) were from Alexis. Proteasome inhibitor clasto-lactacystin and inhibitors for caspase-3 (z-DQMD), caspase-8 (z-IETD), caspase-9 (z-LEHD) or pan-caspase (z-VAD) activity were all from Calbiochem (Notts, UK). Inhibitors were reconstituted in DMSO (Sigma) and solvent-only controls were included in all experiments.

RT-PCR

Total RNA was isolated from urothelial cells using an RNeasy® kit (Qiagen, Crawley, UK) and any contaminating DNA was removed with a DNA-free™ kit (Ambion, Huntingdon, UK). cDNA was prepared with a RETROscript™ First Strand Synthesis kit (Ambion). Oligonucleotides were from MWG-Biotech (Milton Keynes, UK).

Analysis of TRAF mRNA expression
PCR was performed using PLATINUM™ Taq DNA polymerase (Invitrogen) and human TRAF-specific primers: TRAF1: 5′-CAAGCTG GAGCAGAGCTTGCGCCTCAT-3′ (sense) and 5′-CTAAGTGCTGGTC TCCACAATGCACTTGAG-3′ (anti-sense); TRAF2: 5′-AAGTACCTGTG CTCCGCCTGCAGAAACGT-3′ (sense) and 5′-TGCTCCAGGTGGCGC TCCTTTTCACCAA-3′ (anti-sense); TRAF3: 5′-AAAGATGGACTCTCCT GGCGCGCTGCA-3′ (sense) and 5′-GTCTCGCAGGTCTTTCCTCAAGA CCTTTTC-3′ (anti-sense).

The amount of cDNA template used for RT-PCR was adjusted for epithelial cells on the basis of amplification with primers specific for human cytokeratin 8 (CK8): 5′-CATGAACAAGGTAGAGCTGGAGTCTCG-3′ (sense) and 5′-GTTCATCTCTGAGATCTCAGTCTTTGTG-3′ (anti-sense).

25 cycles of 94°C for 30 s, 58°C for 45 s, 72°C for 45 s were performed, followed by 72°C for 10 min. For CK8 PCR, the annealing temperature was 53°C. PCR products were separated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining, and their fidelity was confirmed by DNA sequencing (Lark Technologies, Essex, UK).

Cloning of CD40 cDNA into a retroviral vector
Full-length CD40 cDNA was amplified using oligonucleotides 5′-ACCGCTCGAGCGGCCACCATGGTTCGTCTGCCTCTGCAGTGCGTC-3′ (sense) and 5′-ACCGCTCGAGCGGTCACTGTCTCTCCTGCACTGA GATGCGACT-3′ (anti-sense). The primers were designed to incorporate flanking XhoI restriction sites (underlined) and the Kozac sequence (double-underlined) upstream of the ATG codon. PCR (94°C for 30 s, 64°C for 30 s, 72°C for 1 min, followed by 72°C for 10 min) was carried out using PLATINUM™ Pfx DNA polymerase (Invitrogen) and the product was cloned into the pLXSN retroviral vector (BD Clontech). The CD40 cDNA sequences from NHU and UCC lines were identical and a single clone (pLXSN-CD40) was used for retroviral transduction, alongside vector control (pLXSN).

Retroviral transduction

PT67 fibroblasts (BD Clontech) were plated onto 60-mm dishes (4×10^5 cells) in DMEM/10% FBS before transfection with 4 μg pLXSN or pLXSN-CD40 plasmid using LipofectAMINE™ (Invitrogen). pLXSN-CD40 and pLXSN virus-containing supernatants were collected, filtered, supplemented with 8 μg/ml polybrene (Sigma) and incubated with urothelial cells for 16 h, followed by selection in growth medium containing 1.2 mg/ml G418 for 2 weeks.

Western blotting

Whole-cell lysates were prepared,[15] separated by 12% SDS-PAGE, electroblotted to nitrocellulose membranes (Amersham Biosciences, Bucks, UK) and probed with antibodies against TRAFs 1–3 and 6 (Santa Cruz, supplied by Autogen-Bioclear, Wilts, UK). Loading was adjusted for epithelial cells according to reactivity with human-specific anti-CK8 antibody (Zymed, supplied by Invitrogen). Antibody binding was detected by enhanced chemiluminescence (Pierce, supplied by Perbio Science, Cramlington, UK) and visualized by autofluorography. The human specificity of the antibodies was confirmed by blotting against lysates of 3T3 cells cultured alone (not shown).

Luciferase reporter gene assays

The κB-LUC plasmid was kindly provided by Dr. Alan Melcher (Cancer Research UK, Leeds) and the AP-1:Luc vector was a kind gift from Dr Simon Cook (Cancer Research UK, Babraham Institute, Cambridge). Urothelial cells were plated out in 24-well plates (5×10^4/well) and transfected with 0.4 μg of luciferase plasmid and 0.1 μg of a plasmid constitutively expressing the GFP gene using Effectene™ (Qiagen). 3T3 cells (10^5/well) were then added and cocultured for 16 h. Luciferase assays were carried out using the Steady-Glo® Luciferase Assay System according to the manufacturer's instructions (Promega, Southampton, UK) on a liquid, auto-injection scintillation/luminescence counter (Perkin-Elmer Wallac). Equivalent transfection efficiencies were confirmed on the basis of GFP expression assessed by flow cytometry.

RNA interference (RNAi) studies

Small interfering RNAs (siRNAs) were synthesized with a *Silencer*™ siRNA kit (Ambion) for TRAFs 1–3, 6 and CD40, while an irrelevant (GFP-specific) siRNA served as a control. Urothelial cells were seeded (9×10^3/well) in 96-well plates before transfection with 10 nM siRNA in serum-free OptiMEM™ using OligofectAMINE™ (Invitrogen) for 4 h. Media were replenished with FBS and cells were cultured in the presence of siRNA for a further 12 h. 3T3 cells (10^4/well) were added and apoptosis was assessed 48 h later by the JAM test.

Statistics

Means and standard deviations were used as descriptive statistics. Unless otherwise stated, Student's *t*-test was used for evaluation of statistical significance.

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Supplementary Information accompanies the paper on the Cell Death and Differentiation website (http://www.nature.com/cdd)