Inhibition of PI3K and ERK MAPK-regulated protein synthesis reveals the pro-apoptotic properties of CD40 ligation in carcinoma cells.

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**Keywords**: Apoptosis, CD40, MAPK, PI3 kinase, signalling.
Abstract

CD40, a member of the TNF receptor (TNFR) superfamily is frequently expressed in carcinomas where its stimulation results in induction of apoptosis when \textit{de novo} protein synthesis is inhibited. The requirement of protein synthesis inhibition for efficient killing suggests that CD40 transduces potent survival signals capable of suppressing its pro-apoptotic effects. We have found that inhibition of CD40 signaling on the phosphatidylinositol 3-kinase (PI3K) and ERK MAPK, but not on the p38 MAPK axis disrupts this balance and sensitizes carcinoma cells to CD40-mediated cell death. The CD40-mediated PI3K and ERK activities were found to converge at the regulation of protein synthesis in carcinoma cells \textit{via} a pathway involving the activation of p90Rsk and p70S6 kinases, upstream of the translation elongation factor eEF2. In addition, CD40 ligation was found to mediate a PI3K and mTOR-dependent phosphorylation of 4E-BP1 and its subsequent dissociation from the mRNA cap binding protein eIF4E, as well as an ERK-dependent phosphorylation of eIF4E thus promoting translation initiation. Concomitantly, the anti-apoptotic protein cFLIP was found to be induced in CD40 ligand-stimulated carcinoma cells in a PI3K, ERK and mTOR-dependent manner and down-regulation of cFLIP\textsubscript{S} expression sensitized to CD40-mediated carcinoma cell death. These data underline the significance of the PI3K and ERK pathways in controlling the balance between CD40-mediated survival and death signals through the regulation of the protein synthesis machinery. Pharmacological agents which target this machinery or its upstream kinases could therefore be exploited for CD40-based tumor therapy.
Introduction

CD40, a member of the tumour necrosis factor receptor (TNFR) superfamily, is expressed on a plethora of cell types, including normal B lymphocytes, macrophages, endothelial cells and dendritic cells and this widespread expression is likely to account for its central role in the regulation of humoral immunity and host defence (1,2). The key role of the CD40-CD40L duet in orchestrating immune responses is exemplified by clinical data from patients with X-linked hyper IgM (HIGM) syndrome, a rare immune disorder caused by mutations in the CD40L gene. In these patients, thymus-dependent responses to antigens, such as immunoglobulin class switching and antibody production are impaired with consequent deficiency in germinal centre formation, recurrent infections and high frequency of carcinomas and lymphomas (3,4).

In addition to its expression in normal lymphoid cells, CD40 is also found in a variety of malignant cells, including leukemias, lymphomas and carcinomas of the ovary, nasopharynx, liver, bladder and breast (5). However, in marked contrast to the proliferative effects of CD40 ligation on normal B lymphocytes, CD40 stimulation in malignant lymphoid cells results in growth retardation both in vitro and in vivo indicating a cell type/differentiation state-dependent response to CD40 ligation (6,7). A similar growth-inhibitory effect has been noted in various carcinoma cell lines and early passage ovarian and breast tumour cells treated with a soluble trimeric form of CD40L (8,9). This treatment also promotes the endogenous production of cytotoxic ligands of the TNF family, such as FasL, TNF-α and TRAIL and results in low levels of apoptosis in carcinoma cells grown in vitro or in SCID mice as human ovarian or breast tumour xenografts (9-13).
The pro-apoptotic properties of CD40 ligation in carcinomas can be dramatically enhanced in the presence of the protein synthesis inhibitor cycloheximide (CHX) (12,14) or chemotherapeutic agents which inhibit protein synthesis (8,13). This observation suggests that CD40 transduces potent survival signals which counterbalance its apoptosis-inducing effects. In view of the potential therapeutic role of CD40L in carcinomas, we wished to probe the CD40-activated signaling pathways that mediate anti-apoptotic responses and evaluate the hypothesis that inhibition of these pathways may enhance the susceptibility of carcinoma cells to CD40L-induced cell death.

**Materials and Methods**

*Cell culture, treatments and apoptosis assays.*

EJ, HeLa/CD40 and Rat-1/CD40 cells and apoptosis assays using propidium iodide staining and UV-light microscopy have been previously described (12) (8,12). Apoptosis was also confirmed by a 7-AAD flow cytometric method (15) and a cell death ELISA (Roche), performed according to the manufacturer’s instructions. LY294002 and SB203580 were purchased from Calbiochem and used at 20µM, PD98059 (Calbiochem) used at 50µM, and rapamycin was purchased from Cell Signaling Technology and used at 10nM. Trimeric recombinant soluble CD40L (rsCD40L) was purchased from Bender MedSystems. FLIP antisense was used as previously described (16). To generate RAd-dn p85, the cDNA for dominant-negative p85 PI3 kinase was cloned into a transfer vector and a replication deficient adenovirus expressing this molecule was generated by homologous recombination with the AdEasy1 vector in BJ5183 cells. The virus produced
was expanded in HEK 293 cells and a passage 4 virus was collected, purified and used to infect cells, as previously described (12,17).

**Antibodies and Immunoblotting**

Phospho-specific antibodies were purchased from Cell Signaling Technology. RSK1 was purchased from Upstate Biotechnology and Mnk antibodies from Santa Cruz Biotechnology. The 4EBP1 antibody was kindly provided by Dr. R. Denton, University of Bristol, UK. For immunoblotting, 15-30µg protein was separated by SDS-PAGE, transferred onto PVDF membrane and blocked with 5% non-fat milk dissolved in TBS supplemented with 0.1% Tween-20 (TBS-T). Following three washes with TBS-T, membranes were incubated overnight at 4°C with primary antibody and for one hour at room temperature with the appropriate secondary antibody followed by enhanced chemiluminescence (Amersham). For cFLIP immunoblots, 80 µg protein was separated by SDS-PAGE, transferred onto Biotrace nitrocellulose membranes (Pall Gelman Laboratory, Ann Arbor, USA) and blocked with 5% non-fat milk. The NF6 antibody from Alexis Biochemicals was used for detection of cFLIP_L/S.

**In vitro kinase assays.**

For PI3 kinase assays, Rat-1/CD40 cells were seeded at 1.2 x 10^6/60mm dishes in complete media and allowed to adhere overnight. Following serum starvation for 24 hours, cells were stimulated with rsCD40L and lysed in situ with PI3K lysis buffer (20mM Tris pH 7.5, 150mM NaCl, 1mM EDTA, 1% NP40, 1mM sodium orthovanadate, 50mM NaF, 2 µg/ml leupeptin, 2 µg/ml aprotinin). 500µg of protein was pre-cleared with
Protein G-Sepharose beads (1:1 slurry) for 1hr and incubating overnight at 4°C with 4µg 4G10 anti-phosphotyrosine antibody (Upstate Biotechnology). Immunoprecipitated proteins were recovered using Protein G-Sepharose beads (1:1 slurry) and unbound protein removed by washing 4 times with PI3K lysis buffer, twice with a LiCl buffer (0.5M LiCl, 50mM Tris pH 7.5) and twice with a NaCl buffer (0.1M NaCl, 50mM Tris pH 7.5). Beads were resuspended in buffer containing 0.2mg/ml phosphatidylinositol (reconstituted in 10mM Hepes pH 7.4, 1mM EDTA) and incubated at room temperature for 10 mins. Kinase reactions were initiated by adding 40µl lipid kinase buffer (30mM Hepes pH 7.4, 30mM MgCl₂, 50µM ATP, 400µM adenosine, 10µCi ³²P γ-ATP), incubated at room temperature for 20 min and terminated with 100µl 1M HCl. To recover radiolabelled phosphatidylinositol, 200µl CHCl₃:MeOH (1:1) was added, vortex vigorously and pulse-centrifuged. 40µl of the lower organic phase was separated by thin layer chromatography in a pre-equilibrated with propan-1-ol:2M acetic acid (65:35) tank. Dried plates were quantitated by phospho-imaging. The p90Rsk and p70S6 peptide kinase assays were performed as previously describe (18), using the peptide RRRLLSSLRA which corresponds to amino-acids 231-239 of human 40S ribosomal protein S6 as a substrate (Upstate Biotechnology).

*m⁷-GTP Sepharose affinity chromatography and measurement of protein synthesis.*

HeLa/CD40 cells were stimulated with rsCD40L and lysed with buffer containing 20mM Tris pH 7.5, 100mM KCl, 0.5% Triton X-100, 0.5% NP40, 20mM β-glycerophosphate pH 7.4, 10mM NaF, 1mM EGTA, 1mM DTT, 250µM sodium orthovanadate, 1µg/ml leupeptin and 1µg/ml aprotinin. eIF4E was immunoprecipitated from 250µg total protein...
by incubating with 15µl m7GTP-Sepharose 4B beads (1:1 slurry) (Pharmacia) in a total volume of 500µl of lysis buffer. After 2hrs incubation at 4°C, the beads were washed with lysis buffer and beads were resuspended in a small volume of lysis buffer and SDS/PAGE loading buffer. Following denaturation, immunoprecipitates were run on a 15% SDS-PAGE gel and subjected to standard western blotting for eIF4E and 4EBP1. Protein synthesis was assessed as previously described (19).

**Results**

*Inhibition of CD40-mediated PI3K activation sensitises carcinoma cells to CD40L-induced apoptosis.*

To identify the CD40-activated signaling pathways responsible for counteracting its pro-apoptotic capacity, we first examined the effects of CD40 ligation on phosphatidylinositol 3-kinase (PI3K), an established survival signal. The PI3K pathway is activated in CD40L-stimulated B lymphocytes and endothelial cells (20,21) and could be involved in the protection against CD40-mediated carcinoma cell death. We confirmed that CD40 ligation engages this signaling pathway in non-lymphoid cells by performing *in vitro* kinase assays using anti-phosphotyrosine immunoprecipitates from CD40L-stimulated Rat-1/CD40 cultures and phosphatidylinositol as a substrate (Figure 1A). As a read-out for PI3K activation, the effects of CD40 ligation on the phosphorylation of Akt/PKB, an established PI3K target, were assessed by immunoblot analysis using an antibody that specifically recognises Akt phosphorylated at Ser^473^ or an antibody raised against total (phosphorylated and non-phosphorylated) Akt. The results of these experiments confirmed that CD40 ligation promotes a transient increase in the
phosphorylation of Akt in both Rat-1/CD40 and HeLa/CD40 cells. Furthermore, this effect was found to occur in a PI3K-dependent manner, as CD40L-induced Akt phosphorylation was abolished by pre-treatment with the PI3K inhibitors wortmannin or LY294002 (Figures 1B and 1C).

We then proceeded to assess whether PI3K signals are involved in the protection against CD40-mediated carcinoma cell death. To this end, serum-starved HeLa/CD40 clone 13 (cl.13) cells were pre-treated with PI3K inhibitors and then co-cultured with rsCD40L for 48 hours. The results showed that whilst rsCD40L alone induced approximately 10% cell death above background, pre-treatment with LY294002 or wortmannin significantly augmented this effect to 45% (Figure 1D and data not shown). Neither of these inhibitors affected the levels of CD40 expression in these cells (data not shown). Similar results were obtained in other HeLa/CD40 clones, as well as in Rat-1/CD40 fibroblasts but not in vector control-transfected cells. Furthermore, pre-treatment of EJ bladder carcinoma cells which naturally express CD40 (8) with 20µM LY294002 also resulted in a significant increase in CD40L-induced apoptosis, such that more than 30% of the cells were killed in the presence of both agents (Figure 1D). However, LY294002 did not enhance the cytotoxicity of the chemotherapeutic agent cis-platin and did not result in susceptibility of HeLa/CD40 cells to IGF-1 or EGF stimulation (data not shown).

To confirm that the effects of these chemical inhibitors reflect a specific phenomenon, we generated a replication-deficient recombinant adenovirus expressing a dominant-negative form of p85 (RAd-dn p85), the regulatory subunit of PI3K, for its efficient delivery to the majority of target cells. The p85 PI3K has recently been shown
to be recruited in the CD40 signaling complex (21). Infection of HeLa/CD40 cultures with RAd-dn p85 resulted in significant sensitisation to apoptosis following treatment with rsCD40L but not IGF-1 (Figure 1E and data not shown). We conclude that CD40 transduces PI3K-dependent survival signals in carcinoma cell lines and fibroblasts, capable of counteracting the apoptosis-inducing effects of CD40 ligation.

**ERK but not p38 MAPK activation counteracts CD40-transduced death signals in carcinoma cells.**

Mitogen-activated protein kinase (MAPK) signaling has been implicated in certain anti-apoptotic responses. Earlier studies suggested that CD40 stimulation does not engage the ERK MAPK pathway (22,23) but this finding has been challenged by other investigators (24-26). Taking into account the controversy surrounding the ability of CD40 to transduce ERK signals, we examined the effects of CD40 ligation on ERK phosphorylation, a surrogate for its activation. In parallel, we assessed whether CD40 also engages the p38 MAPK pathway in carcinomas. To this end, HeLa/CD40 cells were stimulated with rsCD40L for 5, 15, 30 or 60 minutes and total lysates were analysed for the phosphorylation status of the ERK isoforms p44 ERK1 and p42 ERK2 and the p38 MAPK by immunoblot. The results demonstrated a dramatic and rapid activation of ERK and a significant phosphorylation of p38 following CD40 ligation (Figures 2A & 2B).

To assess the potential involvement of ERK and p38 in the suppression of CD40-mediated cell death, serum-starved HeLa/CD40 cells were pre-treated with SB203580, a p38 inhibitor, or PD98059, an inhibitor of MEK1, the upstream kinase of ERK and then
co-cultured with rsCD40L for 48 hours. The ability of these compounds to impair CD40L-induced p38 and ERK signaling was confirmed by immunoblot analysis using antibodies specific for the phosphorylated, active forms of these kinases (Figures 2C & 2D). PD98059 or rsCD40L alone had very little effect on cell viability, however, a 2-fold increase in cell death was noted when the cells were cultured with both agents (Figure 2E). Similar results were obtained with a structurally unrelated MEK inhibitor, UO126, which further confirms the specificity of the observed phenomenon (data not shown). PD98059 also synergised with LY294002 to further potentiate CD40-mediated cell death (data not shown). Unlike the sensitising effects of PD98059, pre-treatment with SB203580 did not increase the susceptibility of HeLa/CD40 cells to CD40L-induced apoptosis (Figure 2E). We conclude that inhibition of CD40L-induced ERK but not p38 MAPK activation partly sensitises carcinoma cells to CD40-mediated apoptosis. Interestingly, activation of ERK has also been shown to override anti-Fas and TRAIL but not soluble TNF-α-mediated cell death in the same cell line (27).

*CD40 ligation promotes de novo protein synthesis in carcinoma cells that is dependent on the activation of PI3K and ERK.*

The preceding data demonstrate a critical role for the PI3K and ERK in counter-acting the apoptotic function of activated CD40. This PI3K and ERK-mediated resistance to CD40-induced cell death may depend on or occur independently (i.e. upstream) of *de novo* protein synthesis. To address this question, we treated HeLa/CD40 cells with IGF, EGF or combination of these growth factors, in the presence of rsCD40L and CHX and apoptosis was quantitated 48 hours later. IGF-1 and EGF potently activated the PI3K/Akt
and ERK pathways respectively but failed to rescue these cells from the cytotoxic effects of CD40L and CHX treatment (data not shown). This finding suggests that CD40-transduced PI3K and ERK signals promote resistance to CD40L-induced apoptosis in carcinoma cells through the regulation of de novo synthesis of some crucial anti-apoptotic protein(s).

The ability of CD40 to affect protein synthesis is however unknown. Measurements of the incorporation of $[^{35}\text{S}]$methionine into protein revealed that CD40 ligation in serum-starved HeLa/CD40 cells induces a substantial 30-40% increase in the overall rates of protein synthesis, compared to unstimulated control cells (Figure 3A). This increase was comparable to the levels of induction caused by treatment with insulin, the ‘prototypic’ inducer of de novo protein synthesis in epithelial cells (28). Whilst LY294002 and PD98059 partly affected the background levels of $[^{35}\text{S}]$methionine-incorporation, pre-treatment with these inhibitors but not SB203580 resulted in a dramatic inhibition of CD40L-mediated protein synthesis (Figure 3B). Taken together, these data suggest that CD40-activated PI3K and ERK signals regulate de novo protein synthesis, thereby promoting the production of critical survival proteins that counteract the apoptosis-inducing effects of CD40 ligation.

$CD40$ ligation promotes the activation of the ribosomal S6 kinase and the disruption of the 4E-BP1/eIF4E complex in a PI3K-dependent manner.

To confirm the ability of CD40 ligation to promote protein synthesis and probe the mechanisms by which CD40-activated PI3K and ERK signals influence this phenomenon, we first examined the effects of CD40 ligation on the phosphorylation of
p70S6k and 4E-BP1, two proteins that have been implicated in the control of translation of cytoplasmic RNAs (28,29). The activation of p70S6k results in the phosphorylation of the 40S ribosomal protein S6 that drives the translation of 5'-TOP (terminal oligopyrimidine tract) RNAs and also contributes to the phosphorylation of eEF2k, the upstream kinase of the elongation factor eEF2 (30). Phosphorylation of 4E-BP1 promotes its dissociation from eIF4E, the translation initiation factor that binds the ‘cap’ structure (7-methylguanosine triphosphate) present at the 5’ termini of mRNAs, thereby allowing ‘cap’-dependent translation.

The levels of p70S6k phosphorylation were examined by immunoblot in lysates from CD40-stimulated HeLa/CD40 cells, using an antibody that detects p70S6k phosphorylated at Thr389, a critical site for its activation (31) or an antibody that detects the protein independently of its phosphorylation status. The results showed that CD40 ligation promotes a significant increase in p70S6k phosphorylation in a time-dependent manner. Furthermore, CD40-mediated p70S6k activation was blocked by pre-treatment with LY294002, but only marginally affected by PD98059 (Figure 4A). The regulation of p70S6k is complex and involves the mammalian target of rapamycin, mTOR. Consistent with an mTOR-dependent input in p70S6k activation, CD40L-induced p70S6k phosphorylation was significantly decreased in HeLa/CD40 cells pre-treated with the mTOR specific inhibitor rapamycin (Figure 4A). The ability of CD40 to transduce PI3K and mTOR-dependent p70S6k activation was confirmed by using kinase assays in HeLa/CD40 cells stimulated with rsCD40L (Figure 4B).

To ascertain whether CD40 ligation influences 4E-BP1 phosphorylation status and function, serum-starved HeLa/CD40 cells were stimulated with rsCD40L and
analysed for 4E-BP1 phosphorylation by immunoblot, using an antibody against 4E-BP1 phosphorylated at Thr\textsuperscript{70} or an antibody that detects total protein. Furthermore, 4E-BP1/eIF4E assembly was evaluated by using m\textsuperscript{7}GTP-sepharose chromatography (30). The results showed that CD40 ligation promotes both the phosphorylation of 4E-BP1 and its dissociation from the translation initiation factor eIF4E (Figures 4C and 4D). Pre-treatment with LY294002 or rapamycin inhibited the ability of CD40 to promote the dissociation of 4E-BP1 from ‘cap’-bound eIF4E (Figure 4C). Taken together, these data provide a link between CD40-mediated PI3K activation and initiation of translation, through the modulation of p70S6k and 4E-BP1 function. Importantly, pre-treatment with the mTOR-specific inhibitor rapamycin renders HeLa/CD40 cells susceptible to CD40L-induced apoptosis (Figure 4E).

CD40 ligation promotes the phosphorylation of the serine/threonine kinase MnK1 and its downstream target eIF4E in an ERK-dependent manner.

Mnk1, a MAPK substrate, promotes the phosphorylation of eIF4E at Ser\textsuperscript{209} \textit{in vitro} and \textit{in vivo}. The effects of CD40 ligation on the phosphorylation of Mnk1 and eIF4E were assessed in HeLa/CD40 cells stimulated with rsCD40L. Immunoblot analysis was performed in total cell extracts using antibodies that specifically recognise Mnk1 and eIF4E phosphorylated at Thr\textsuperscript{197/202} and Ser\textsuperscript{209} respectively. The results showed a substantial induction of Mnk1 and eIF4E phosphorylation following CD40 ligation, which was maximal at 15 min and decreased thereafter (Figures 5A, 5B and data not shown). To identify the CD40-activated signaling pathways that are responsible for eIF4E phosphorylation, HeLa/CD40 cells were pre-treated with PD98059, SB203580 or
rapamycin and then stimulated with rsCD40L for 15 min. Protein extracts from these cells or from untreated control cultures were analysed for eIF4E phosphorylation by immunoblot. Whilst both ERK and p38 MAPKs have been shown to target Mnk1 (32), PD98059 but not SB203580 suppressed CD40L-induced eIF4E phosphorylation (Figure 5B). It is possible that p38 activation is dispensable for Mnk1 phosphorylation in CD40L-treated carcinoma cells where a rapid and more robust engagement of the ERK MAPK pathway occurs (Figure 2). Therefore, CD40 ligation promotes the phosphorylation of Mnk1 and its downstream target, eIF4E, in an ERK-dependent manner.

*CD40 ligation promotes the phosphorylation of p90Rsk and the inactivation of eEF2 in an ERK-dependent manner.*

The p90 ribosomal S6 kinase (p90Rsk) is specifically activated through phosphorylation by ERK MAPKs but not by other MAPK subfamilies and regulates the elongation of translation through the phosphorylation of eEF2k, the upstream kinase of the elongation factor eEF2 (30). CD40 ligation in HeLa/CD40 cells was found to result in a rapid and significant increase in p90Rsk phosphorylation at Ser\(^{381}\), as determined by immunoblot analysis (Figure 6A). Pre-treatment with the MEK inhibitor PD98059 abolished CD40L-induced p90Rsk phosphorylation, while LY294002 and SB203580 had no effect (Figure 6B). Kinase assays were performed in CD40-stimulated HeLa/CD40 cells to confirm that phosphorylated p90Rsk is catalytically active and that this activity depends on ERK (Figure 6C).

The activation of p90Rsk by TPA is known to result in the phosphorylation of eEF2 kinase which, in turn, inactivates the elongation factor eEF2 via an unknown
To determine whether CD40 ligation influences the phosphorylation status of eEF2, HeLa/CD40 cells were stimulated with rsCD40L and lysates were examined for expression of the phosphorylated and total eEF2 by immunoblot. Untreated cells were found to possess significant levels of the phosphorylated, inactive form of eEF2. Upon CD40 ligation, a marked and rapid de-phosphorylation of eEF2 occurred which returned to normal levels by 60 min of stimulation (Figure 6D). The kinetics of eEF2 de-phosphorylation mirrored those of p90Rsk phosphorylation. Importantly, CD40-mediated eEF2 de-phosphorylation was inhibited upon co-culture with PD98059 but not SB203580. Furthermore, eEF2 de-phosphorylation was reversed upon pre-treatment with LY294002, consistent with a PI3K-dependent, p70S6k-mediated effect on eEF2 activation (30). Therefore, CD40-activated ERK and PI3K signals converge in regulating the elongation of protein translation.

**CD40 ligation induces the expression of functional cFLIPS in a PI3K, mTOR and ERK-dependent manner.**

The anti-apoptotic protein cFLIP is induced by members of the TNF family, including CD40L (33,34) and critically depends on de novo protein synthesis to maintain its levels of expression. Thus, cFLIP is rapidly degraded following CHX treatment (16,33). cFLIP exists in various splice variants of which the long (cFLIPL) and short (cFLIPS) isoforms are expressed in cells. The short isoform is terminated by a stop codon present in exon 7 of the FLIP gene but cFLIPL does not utilize this exon (35). Multiple signaling pathways, including PI3K/Akt and ERK, have been implicated in the regulation of cFLIP expression in a stimulus and cell type-dependent manner (36). On the basis of
these data, we hypothesized that cFLIP could be a target of CD40-induced de novo protein synthesis and may play a role in counteracting CD40-transduced death signals. To address this hypothesis, we examined lysates from EJ carcinoma cells which naturally express CD40, for the expression of FLIP isoforms before and 2, 6 or 12 hours after stimulation with CD40L. In the absence of stimulus, these cells were found to possess significant levels of cFLIP\textsubscript{L} but undetectable levels of cFLIP\textsubscript{S} (Figures 7A and 7B). Treatment with CD40L induced the dramatic expression of cFLIP\textsubscript{S} and a modest up-regulation of cFLIP\textsubscript{L} while the levels of other pro- or anti-apoptotic proteins, such as Bcl-2, Bcl-x\textsubscript{L}, TRAF2, Bax and TRADD or housekeeping gene products, such as β-actin, remained essentially unaffected for a period up to 24 hours post-stimulation (Figure 7A and data not shown). The induction of both cFLIP isoforms was sensitive to CHX (Figure 7B). Interestingly, we have found that pre-treatment of these cells with LY294002, PD98059 or rapamycin, at concentrations which inhibit the effects of CD40L on protein synthesis, suppressed the ability of CD40 to induce the expression of cFLIP\textsubscript{S} but not cFLIP\textsubscript{L} (Figure 7B). This observation was reproduced in four independent experiments. None of these inhibitors affected the basal levels of cFLIP\textsubscript{L/S} expression. Moreover, the ability of dn-p85 to inhibit the CD40-mediated cFLIP\textsubscript{S} induction (Figure 7C) confirms the contribution of the PI3K pathway to this effect. As expected, CD40 ligation also stimulated the de novo production of cFLIP\textsubscript{L/S} mRNA, measured by RT-PCR, which was unaffected by rapamycin (Figure 7D). Interestingly, however, LY294002 partly reduced the inducible levels of cFLIP\textsubscript{S} but not cFLIP\textsubscript{L} mRNA (Figure 7D), suggesting that PI3K signals regulate cFLIP\textsubscript{S} expression at both transcriptional and translational level.
To determine if the *de novo* expression of this protein protects against CD40-transduced death signals, we utilized an anti-sense approach. Incubation of EJ cells with FITC-labelled cFLIP antisense oligonucleotides suppressed the endogenous levels of induced cFLIPs (Figure 8A). Flow cytometry was also performed in these cultures and confirmed the uptake of the oligo in most of the cells (Figure 8B). Apoptosis was assessed by a cell death ELISA following stimulation with rsCD40L. It was found that suppression of cFLIPs expression sensitized EJ cells to CD40-transduced death signals, whilst a non-sense oligonucleotide had no effect (Figure 8C and data not shown). As a control for these experiments, pre-treatment with LY294002 also sensitized EJ cells to CD40L-induced apoptosis as determined by the relative increase in the nucleosome enrichment factor compared to control cultures. Therefore, CD40 activation results in the *de novo* expression of functional cFLIPs, an effect mediated by PI3K, ERK and mTOR signals.

**Discussion**

CD40 conveys signals that modulate diverse cellular responses, ranging from proliferation and differentiation to growth inhibition and apoptosis, in a cell type-dependent manner. Irrespective of the precise mechanisms underlying these responses, the differential effects of CD40 ligation on normal *versus* malignant cells suggest that the expression of CD40 in transformed cells could be exploited as a novel therapeutic target. This is supported by recent *in vivo* studies and phase I clinical trials demonstrating a potent effect of CD40L administration on tumor growth (9,13,37). Apoptosis in tumor cells treated *in vitro* with CD40L can be dramatically enhanced by CHX treatment, a
phenomenon believed to be a manifestation of a regulatory circuit which facilitates a decision between life and death. Thus, the ligand-dependent activation of particular signaling pathways results in the rapid de novo synthesis of survival proteins which counteract the pro-apoptotic effects of CD40 activation.

In view of the potential therapeutic role of CD40L in carcinomas, we wished to identify the CD40-activated signaling pathways that mediate anti-apoptotic responses and evaluate the hypothesis that inhibition of these pathways may enhance the susceptibility of carcinoma cells to CD40L-induced cell death. We have found that CD40 stimulation results in the activation of PI3K, a known anti-apoptotic effector and regulator of gene expression, including transcriptional and translational control (38). Importantly, CD40-transduced PI3K signals were found to be critical in counteracting the apoptosis-inducing effects of CD40 ligation, as inhibition of PI3K sensitised carcinoma cells to CD40-mediated apoptosis. Data presented in this paper demonstrate that the activation of ERK also plays a role in counteracting the pro-apoptotic properties of CD40 ligation in HeLa cells (Figure 2; see also proposed model in Figure 9).

We then examined the possibility that CD40-transduced PI3K and ERK signals converge to the regulation of the protein synthesis machinery. This was confirmed by the demonstration that CD40 ligation in serum-starved carcinoma cells induces a substantial increase in the overall rates of protein synthesis and this induction is selectively suppressed by the PI3K inhibitor LY294002 and the MEK inhibitor PD98059. CD40 was found to affect key regulators of both the initiation, the rate-limiting step, and the elongation of translation. Thus, CD40 ligation promoted the generation of an active translation initiation complex, as evident by the dissociation of the translational repressor.
protein 4E-BP1 from eIF4E which occurred through a PI3K and mTOR-dependent manner (Figure 4). Interestingly, rapamycin sensitized HeLa/CD40 cells to CD40L-induced apoptosis. This is consistent with a role for eIF4E in controlling the expression of survival proteins to rescue transformed cells from apoptosis induced by serum withdrawal or constitutively active c-myc (39). CD40 also controls the activation of p70S6 kinase which promotes the phosphorylation of the 40S ribosomal protein S6 that drives the translation of 5’-TOP RNAs. We found that the CD40L-induced activation of p70S6 kinase depends on PI3K/mTOR and, to a lesser extent, ERK signals (Figures 4B and 9). Moreover, we demonstrated that CD40 controls key regulators of the elongation of translation, such as the elongation factor eEF2 through both the PI3K and ERK pathways (Figures 6 and 9) and inhibition of elongation by cycloheximide sensitises carcinoma cells to CD40-mediated apoptosis. Taken together, the presented data identify a novel function of CD40, namely the regulation of protein synthesis through PI3K/mTOR and ERK, the signaling pathways that counteract the pro-apoptotic properties of CD40 stimulation in carcinoma cells.

In addition to translational control, the PI3K/Akt pathway has been implicated in anti-apoptotic responses that occur independently of protein synthesis. Thus, Akt has been proposed to directly phosphorylate the pro-apoptotic effectors caspase-9 and Bad at Ser196 and Ser136 respectively, resulting in their inactivation (40,41). Theoretically, CD40-mediated PI3K signals may override CD40L-induced cell death via suppression of these effectors. Given that potent inducers of PI3K, such as IGF-1 failed to counteract CD40L and CHX-induced apoptosis (data not shown), this possibility appears remote. Furthermore, in six independent experiments we did not detect phosphorylation of Bad at
Ser\textsuperscript{136} following CD40 stimulation in HeLa cells (Davies and Eliopoulos, unpublished observations), suggesting that a threshold of PI3K activity may be required for efficient signaling on the Akt/Bad axis or that Akt may not be the principal Bad kinase. Recent findings support the latter possibility (42) and suggest that Akt promotes survival via a mechanism that is independent of Bad phosphorylation (43-45). Moreover, whilst human caspase-9 contains a putative RxRxxS\textsuperscript{196} Akt-phosphorylation motif, Ser\textsuperscript{196} is not conserved in rodent homologues. As CD40-transduced PI3K signals counteract CD40L-induced apoptosis in rodent fibroblasts (Figure 1) and the caspase-9 peptide inhibitor z-LEHD-fmk fails to rescue HeLa/CD40 cells from LY294002 and CD40L-induced apoptosis (Davies and Eliopoulos, unpublished observations), it is unlikely that phosphorylation of caspase 9 is a key mechanism of survival in our system.

Whilst we cannot exclude the possibility that CHX and/or PI3K/ERK inhibition affect the activation status or the basal levels of expression of a pre-existing protein which protects against CD40-mediated apoptosis, our observation that CD40 ligation induces the \textit{de novo} production of functional cFLIP\textsubscript{S} in carcinoma cells in a PI3K, mTOR and ERK-dependent manner (Figures 7 and 8) testifies to the contribution of protein synthesis to anti-apoptotic responses. This is consistent with recently published work demonstrating that the short but not the long spliced form of FLIP confers resistance to TNF and FasL-mediated apoptosis (46,47) and also suggests that the expression of the FLIP isoforms is regulated by different mechanisms. Many of the FLIP isoforms, including cFLIP\textsubscript{L} and cFLIP\textsubscript{S}, differ in their 5’-UTR (35) and their expression may therefore be differentially controlled by the translation initiation machinery as a result of ‘translational discrimination’ (28). In addition, the differential inclusion of intron/exon
sequences in certain FLIP isoforms may influence the stability or the secondary structure of the transcript thereby affecting the elongation step of translation. Moreover, the observation that LY294002 inhibits the induction of cFLIP at both the protein and RNA level raises the possibility that in addition to translational control, CD40-transduced PI3K signals may impinge on the regulation of FLIP pre-mRNA splicing. This hypothesis is supported by published evidence demonstrating that PI3K stimulates the activity of at least two splicing-regulatory factors, SRp40 (48) and CBC (49). Activation of the CBC is of particular interest given its ability to bind the m7G mRNA cap structure which also interacts with the translation initiation factor complex. Thus, it is possible that CD40-transduced PI3K signals regulate FLIP gene expression through the co-ordination of cap-dependent splicing and translation.

Collectively, the data presented in this paper delineate the auto-protective signaling pathways activated by CD40 ligation and provide a link between the potentiation of CD40-mediated apoptosis by PI3K and ERK inhibition and the sensitisation conferred by CHX treatment, through the PI3K and ERK-dependent modulation of protein synthesis. These findings suggest that pharmacological agents which target the protein synthesis machinery (50) or its upstream kinases could be exploited for tumor therapy involving CD40 and its ligand.

**Acknowledgments**

We are grateful to Dr R. Denton for reagents. This work was supported by a Cancer Research UK grant (SP2584) to A.G.E and L.S.Y, a Biotechnology and Biological
Sciences Research Council (BBSRC, UK) grant to M.J.O.W and by a Medical Research Council (MRC, UK) Career Development Award to A.G.E.
References


Figure Legends

Figure 1: CD40 mediated PI3K/Akt activation overrides CD40L-induced cell death.

Serum starved Rat-1/CD40 cells were stimulated with rsCD40L for the times indicated. Lysates were subjected to (A) in vitro PI3K assay and (B) immunoblot analysis using an antibody that recognises Akt phosphorylated at Ser$^{473}$ (p-Akt) or an antibody that detects Akt regardless of its phosphorylation state (Akt). To verify the PI3K-dependent activation of Akt by CD40, cells were pre-treated with wortmannin (Wm) before being stimulated for 5 mins with rsCD40L, lysed and subjected to immunoblotting as described above.

(C) HeLa/CD40 Cl.13 cells were pre-treated with LY294002 (LY) and then stimulated with rsCD40L for 15 min. IGF-1 stimulated cells (lane 4) serve as a positive control. Samples were subjected to immunoblotting using anti-phospho-Akt (Ser$^{473}$) or total-Akt antibodies.

(D) LY294002 sensitises carcinoma cells and fibroblasts to CD40L-induced apoptosis. Cells were pre-treated with LY before being co-cultured with rsCD40L for 48 hrs. The percentage of apoptotic cells (mean values ±SD) from 3 independent experiments are shown for HeLa/CD40 Cl.13 and EJ cells. Stars represent individual values from 2 independent experiments performed in HeLa/CD40 Cl.14 cells.

(E) Infection of HeLa/CD40 cl.13 cells with a recombinant adenovirus (multiplicity of infection 100) expressing dominant negative p85 (RAd-dn p85) but not with a β-galactosidase-expressing virus (RAd35) sensitises to CD40L-induced apoptosis. Mean values from 2 independent experiments are shown.
**Figure 2:** CD40 ligation activates both the ERK and p38 MAPK pathways, however inhibition of ERK selectively sensitises cells to CD40-mediated apoptosis.

(A and B) CD40 ligation induces the activation of ERK and p38 MAPK in carcinoma cells. Serum-starved HeLa/CD40 Cl.13 cells were stimulated with rsCD40L for the times indicated. Immunoblots were probed for (A) phosphorylated ERK (upper panel) and total ERK (lower panel) or (B) phosphorylated p38 (upper panel) and total p38 (lower panel).

(C) PD98059 suppresses CD40L-induced ERK activation. Cells were pre-treated with PD98059 (PD) before being stimulated with rsCD40L for 15 min. Lysates were analysed for ERK phosphorylation as described in (A).

(D) The p38 inhibitor SB203580 suppresses CD40L-induced p38 activation.

(E) PD98059 (PD) but not SB203580 (SB) sensitises carcinoma cells to CD40L-induced apoptosis. Cells were pre-treated with PD or SB and then cultured in the presence (+CD40L) or absence (-CD40L) of rsCD40L for a further 48 hrs, when apoptosis was assessed. Mean values (±SD) from 3 independent experiments are shown.

**Figure 3:** CD40 ligation promotes protein synthesis in a PI3K and ERK-dependent manner.

(A) Serum-starved HeLa/CD40 cells were stimulated with rsCD40L or IGF1 for the indicated time-points and labelled with $^{35}$S-methionine for 20 min prior to harvesting. Data are mean values from 3 independent experiments, each performed in duplicate and expressed as percentage relative to untreated control (CNTR) cultures.
(B) Serum-starved HeLa/CD40 cells were pre-treated for 30 min with kinase inhibitors, as indicated and then stimulated with rsCD40L for 60 min. Cells were labelled with $^{35}$S-methionine for 20 min prior to harvesting.

**Figure 4**: CD40 activates the p70S6K pathway and promotes the disruption of the 4E-BP1/eIF4E complex in a PI3 kinase and rapamycin-dependent manner.

(A) Serum-starved HeLa/CD40 cells were stimulated with CD40L for the times indicated (left hand panels) or pre-treated with PD98059 (PD), LY294002 (LY) or rapamycin (Rap) before being stimulated with rsCD40L for 30 min (C; control unstimulated cells) (right hand panels). Samples were then subjected to immunoblot for p70S6K phosphorylated at Thr$^{389}$ or total p70S6K1.

(B) CD40 ligation promotes the activation of p70S6k in a PI3K and mTOR-dependent manner. Cells were treated with inhibitors as in (A) and then stimulated with rsCD40L for 45 min before being analysed for endogenous p70S6 kinase activity.

(C) m$^7$GTP-Sepharose affinity chromatography demonstrating CD40-mediated dissociation of 4E-BP1 from eIF4E. Cells were stimulated with either rsCD40L or insulin as a control (left hand panels) or pre-treated with inhibitors and then stimulated with rsCD40L for 45 min (right hand panels). The eIF4E:4E-BP1 complex was blotted for 4E-BP1 (upper panels) or eIF4E (lower panels). NT; no treatment.

(D) CD40 ligation promotes the phosphorylation of 4E-BP1 at Thr$^{70}$. Data shown in (A-D) are representative of at least 3 independent experiments.

(E) The mTOR inhibitor rapamycin sensitises carcinoma cells to CD40L-induced apoptosis. Serum-starved HeLa/CD40 cl.13 cells were pre-treated with 50nM rapamycin
(Rap) and apoptosis was assessed following addition of rsCD40L as described in the materials and methods. Stars represent individual values from 2 independent experiments performed.

**Figure 5**: CD40 ligation promotes the phosphorylation of Mnk1 and its downstream effector eIF4E.

(A) Serum-starved HeLa/CD40 cells were stimulated with rsCD40L for various time-intervals, as indicated. Immunoblots of total cell lysates were probed with an antibody that recognises Mnk1 phosphorylated at Thr$^{197/202}$ or total Mnk.

(B) eIF4E, a Mnk1 target, is phosphorylated following CD40 ligation. HeLa/CD40 cells were left untreated, stimulated with rsCD40L or pre-treated with inhibitors and then stimulated with rsCD40L for 30 min. Lysates were immunoblotted for phosphorylated eIF4E (upper panel) or total eIF4E (lower panel). Data are representative of 3 independent experiments.

**Figure 6**: Regulation of p90Rsk activity and eEF2 dephosphorylation by CD40 ligation.

(A) Serum-starved HeLa/CD40 cells were stimulated with rsCD40L for the times indicated and p90Rsk phosphorylated at Ser$^{381}$ (upper panel) or total p90Rsk (lower panel) were detected by immunoblot.

(B) Cells were pre-treated with PD98059 (PD), LY294002 (LY) or SB203580 (SB) for 30 min and then co-cultured with or without CD40L for a further 15 min before analysed for phosphorylated p90Rsk.
(C) CD40 ligation promotes the activation of p90Rsk. Serum-starved HeLa/CD40 cells were treated as described in (B) and lysates were subjected to in vitro kinase assays using S6 peptide substrate, as described in the materials and methods. Incorporation of $^{32}$P was measured and activities were normalised to the background kinase activity of unstimulated (NT) lysates, which was given the arbitrary value of 1. Results represent mean values (±SD) from 3 independent experiments. Inserted panel shows a p90Rsk immunoblot of anti-p90Rsk immunoprecipitates from one representative experiment.

(D) CD40 ligation promotes the de-phosphorylation of the elongation factor eEF2 in a PI3K and ERK-dependent manner. Cells were left untreated (C; control unstimulated), stimulated with rsCD40L or pre-treated with inhibitors, as in (B) and then stimulated with rsCD40L for 15 min. Lysates were immunoblotted for eEF2 phosphorylated at Thr$^{56}$ (upper panel) or total eEF2 (lower panel).

**Figure 7:** CD40 ligation results in the de novo synthesis of cFLIP.

(A) EJ bladder carcinoma cells were stimulated with rsCD40L for 2, 6 or 12 hrs or left untreated and lysates were analysed for FLIP levels by immunoblot.

(B) EJ cells were pre-treated with 10µg/ml CHX, 20µM LY294002, 50µM PD98059 or 10nM rapamycin for 30 min, before being stimulated with 1µg/ml rsCD40L or left untreated and lysates were analysed for cFLIPS/L or β-actin expression by immunoblot.

(C) EJ cells were infected with RAd-dn p85 (multiplicity of infection 200) or a β-galactosidase-expressing control virus (RAd35) and 36 hours later were stimulated with rsCD40L as in (B). Lysates were analysed for cFLIPS/L or β-actin expression by immunoblot.
(D) EJ cells were treated with CD40L for 4 hours, in the presence or absence of inhibitors as indicated. Isolated RNA was reverse-transcribed and PCR amplified for cFLIP<sub>S/L</sub>. GAPDH serves as amplification control.

**Figure 8:** Suppression of CD40-induced cFLIP<sub>S</sub> expression sensitises carcinoma cells to CD40L-mediated apoptosis.

(A) EJ cells were incubated with 5µM FITC-labelled FLIP antisense oligonucleotide (AS) for 24 hours or left untreated (NT) before CD40 stimulation and evaluation of FLIP levels by immunoblot.

(B) Flow cytometric analysis of EJ cells following incubation with FITC-labelled FLIP antisense oligonucleotide demonstrates uptake by the majority of cells. More than 80% of the cells were found positive for FITC-labelled cFLIP antisense oligonucleotide uptake in all experiments performed. NT; no treatment.

(C) Suppression of endogenous FLIP<sub>S</sub> sensitises EJ cells to CD40L-induced apoptosis. Following incubation with antisense FLIP as described above, cells were either left untreated or stimulated with rsCD40L for 36 hours before being analysed for apoptotic content using a Cell Death ELISA (Roche). Data shown represent fold increase in nuleosome enrichment as a result of cell death, compared to control untreated cultures (NT) which have been given the arbitrary value of 1. For comparison, the effects of LY294002/CD40L combination treatment on nuleosome enrichment is also shown.

**Figure 9:** A proposed model of the signaling cascades utilised by CD40 to counteract its pro-apoptotic properties, in part through the induction of cFLIP<sub>S</sub>. On the basis of the data
presented, we propose that CD40-induced anti-apoptotic responses critically depend on the PI3K/mTOR and ERK MAPK-dependent induction of de novo protein synthesis. These signals converge in the regulation of critical components of the translation machinery, namely eIF4E, S6 and eEF2, thereby influencing both the initiation and elongation steps of translation. This is achieved through the modulation of the phosphorylation status and activity of upstream kinases and regulatory molecules, such as p90Rsk, p70S6k, Mnk1 and 4E-BP1. The sites of action of the kinase inhibitors PD98059 (PD) and LY294002 (LY) and of the mTOR inhibitor rapamycin (Rap) are also indicated.
Figure 1: Davies et al.

A.

![Graph showing P13K activity (fold increase) with bars for time points 0, 3, 5, 15, and 30 minutes. The x-axis represents time in minutes, and the y-axis represents P13K activity. The bars are labeled with Rat-1 CD40 Cl.19.]

B.

![Diagram showing Western blot results for p-Akt and Akt with conditions for CD40L, Wm, IGF-1, and LY. The legend includes conditions for 0, 5, 15, and 30 minutes.]

C.

![Diagram showing Western blot results for p-Akt with conditions for CD40L, IGF-1, and LY. The legend includes conditions for 0, 5, 15, and 30 minutes.]

D.

![Graph showing % apoptosis with conditions for NT, LY, CD40L, and CD40L + LY. The x-axis represents different conditions, and the y-axis represents % apoptosis.]

E.

![Graph showing % apoptosis with conditions for NT, CD40L, RAAd 35, and RAAd dn p85. The x-axis represents different conditions, and the y-axis represents % apoptosis.]

\[\text{Figure 1:} \text{ Davies et al.}\]

\[\text{A.} \]

![Graph showing P13K activity (fold increase) with bars for time points 0, 3, 5, 15, and 30 minutes. The x-axis represents time in minutes, and the y-axis represents P13K activity. The bars are labeled with Rat-1 CD40 Cl.19.]

\[\text{B.} \]

![Diagram showing Western blot results for p-Akt and Akt with conditions for CD40L, Wm, IGF-1, and LY. The legend includes conditions for 0, 5, 15, and 30 minutes.]

\[\text{C.} \]

![Diagram showing Western blot results for p-Akt with conditions for CD40L, IGF-1, and LY. The legend includes conditions for 0, 5, 15, and 30 minutes.]

\[\text{D.} \]

![Graph showing % apoptosis with conditions for NT, LY, CD40L, and CD40L + LY. The x-axis represents different conditions, and the y-axis represents % apoptosis.]

\[\text{E.} \]

![Graph showing % apoptosis with conditions for NT, CD40L, RAAd 35, and RAAd dn p85. The x-axis represents different conditions, and the y-axis represents % apoptosis.]

\[\text{Figure 1:} \text{ Davies et al.}\]
Figure 2: Davies et al.
Figure 3: Davies et al.
A. CD40L (min)

Time (min): 0 15 30 45

- p-p70S6K
- p70S6K

B. p70S6K activity (fold increase)

- C
- PD
- LY
- Rap

Inhibitor
Inhibitor + CD40L

C. CD40L

Time (min): 0 15 30 45 45

- 4E-BP1
- eIF4E

D. CD40L

Time (min): 0 15 30 45

- p-4E-BP1
- 4E-BP1

E. % apoptosis

- NT
- Rap
- CD40L
- Rap + CD40L

Figure 4: Davies et al.
Figure 5: Davies et al.
Figure 6: Davies et al.
Figure 7: Davies et al.
Figure 8: Davies et al.
Figure 9: Davies et al.
Inhibition of PI3K and ERK MAPK-regulated protein synthesis reveals the pro-apoptotic properties of CD40 ligation in carcinoma cells
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J. Biol. Chem. published online October 27, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M303820200

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