

Inhibition of p38 mitogen-activated protein kinase unmasks a CD30-triggered apoptotic pathway in anaplastic large cell lymphoma cells

Sergey V. Krysov, Tania F. Rowley,
and Aymen Al-Shamkhani

Cancer Sciences Division, University of Southampton School of Medicine, Southampton General Hospital, Southampton, United Kingdom

Abstract

CD30, a non-death domain-containing member of the tumor necrosis factor receptor superfamily, triggers apoptosis in anaplastic large cell lymphoma cells. The CD30 signaling pathways that lead to the induction of apoptosis are poorly defined. Here, we show that the induction of apoptosis by CD30 requires concurrent inhibition of p38 mitogen-activated protein kinase, which itself is activated by engagement of CD30 with CD30 ligand. Treatment of anaplastic large cell lymphoma cells with CD30 ligand and pharmacologic inhibitors of p38 mitogen-activated protein kinase, but not with CD30 ligand or inhibitors alone, triggered the activation of caspase-8 and the induction of apoptosis. Caspase-8 activation occurred within a few hours (2.5–4 h) after receptor triggering, was unaffected by the neutralization of ligands for the death domain-containing receptors TNFR1, Fas, DR3, DR4, or DR5, but was abolished by the expression of a dominant-negative form of the adaptor protein FADD. Importantly, we show that expression of the caspase-8 inhibitor c-FLIP_S is strongly induced by the CD30 ligand, and that this is dependent on the activation of p38 mitogen-activated protein kinase. Thus, we provide evidence that the induction of apoptosis by CD30 in anaplastic large cell lymphoma cells is normally circumvented by the activation of p38 mitogen-activated protein kinase. These findings have implications for CD30-targeted immunotherapy of anaplastic large cell lymphoma. [Mol Cancer Ther 2007;6(2):703–11]

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Requests for reprints: Aymen Al-Shamkhani, Tenovus Research Laboratory, Southampton General Hospital, Tremona Road, Southampton SO16 6YD, United Kingdom. Phone: 44-23-8079-6285; Fax: 44-23-8070-4061. E-mail: aymen@soton.ac.uk

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Introduction

CD30 is a member of the tumor necrosis factor receptor (TNFR) superfamily that is expressed on normal activated T cells, B cells, and eosinophils (1, 2). It is also a marker of the Hodgkin and Reed-Sternberg cells, the malignant cells in Hodgkin's disease, and a group of large cell lymphomas known as anaplastic large cell lymphoma (ALCL; ref. 3). Because of its association with these lymphomas and its restricted pattern of expression on normal cells, CD30 is currently being investigated as a target for monoclonal antibody-based therapy (4, 5). CD30 signaling activates nuclear factor κ B (NF- κ B; refs. 6–9) as well as the p38, c-Jun-NH₂-kinase, and extracellular signal-regulated kinase mitogen-activated protein kinase (MAPK) pathways (10, 11). Recruitment of the TNFR-associated factors directly to the cytoplasmic domain of CD30 following ligand binding is critical for the activation of downstream signaling events (7–9). Like other TNFR superfamily members, CD30 mediates diverse biological effects, ranging from the induction of cell cycle arrest and cell death, to the enhancement of proliferation and cell survival that are dependent on cell type and environmental factors (2). Thus, CD30 signaling enhanced the proliferation of antigen receptor-triggered T cells and T cell-like Hodgkin's cell lines (12), but induced cell death in T cell hybridomas (13), preactivated/memory CD8 T cells (14, 15) and ALCL cells (12, 16). In the ALCL cell line Karpas 299, CD30 triggering was shown to induce cell cycle arrest (17) and only modest levels of cell death (16). However, under conditions in which new protein synthesis was blocked, the ability of CD30 to trigger cell death in ALCL cell lines was substantially enhanced (16). Furthermore, CD30-induced cell death in ALCL cells was inhibited by the pan-caspase inhibitor zVAD-fmk, suggesting that CD30 triggers cell death by an apoptotic pathway (16). Although the mechanism of induction of cell death was not investigated in that study, the data suggest that CD30 induces *de novo* synthesis of a survival protein(s) that blocks its cell death-promoting activity.

The "classical" death receptors including Fas, TNFR1, DR3 and receptors for tumor necrosis factor-related apoptosis inducing ligand (TRAIL)-R1 (DR4), and TRAIL-R2 (DR5) contain a homologous cytoplasmic sequence termed the death domain (18). This serves to recruit cytoplasmic death domain-containing adaptor proteins, such as FADD, which can associate with procaspase-8 leading to the initiation of apoptosis (18). The cytoplasmic region of CD30 lacks the death domain, and thus, recruitment of death domain-containing adaptor proteins, such as FADD, directly to the cytoplasmic tail of CD30 is unlikely. Induction of apoptosis by non-death domain-containing TNFR superfamily members is thought to be mediated by an

indirect mechanism that involves endogenous expression of ligands for death domain-containing receptors (19, 20). Furthermore, non-death domain-containing members of the TNFR superfamily, such as TNFR2, can potentiate TNFR1-induced apoptosis (21). Signaling via TNFR2 was shown to induce c-IAP1-dependent ubiquitination and subsequent proteosomal degradation of the cellular TNFR-associated factor-2 pool leading to the shutdown of pro-survival signaling pathways and a bias of TNFR1 signaling towards the apoptotic pathway (21). This view, however, has been challenged by studies showing that TNFR2-induced apoptosis in some cells can occur in the presence of antibodies that neutralize TNF- α binding to TNFR1, suggesting that TNFR2-mediated apoptosis occurs independently of TNFR1 (22, 23). Interestingly, TNFR2-triggered apoptosis required FADD (22, 23), an adaptor protein that also mediates the apoptotic effects of death domain-containing receptors such as Fas and TNFR1 (18). In light of these studies, we sought to define the signaling pathways that lead to the induction of apoptosis by CD30, as these have not been characterized thus far.

Although CD30 engagement has been shown to stimulate p38 MAPK activity (10), its role in CD30-mediated cell death is not well established. Matsumoto and colleagues (24) suggested that p38 MAPK activation plays a proapoptotic role in eosinophils following CD30 cross-linking. However, it is noteworthy that in that study, the apoptosis of eosinophils was dependent on signaling via Fc γ receptors and seemed to require cell adhesion through β 2 integrins (24). Thus, it remains unclear if p38 MAPK activation by CD30 or other receptors determines cell death in eosinophils. In contrast, other studies have shown that pharmacologic inhibition of p38 MAPK accelerates neutrophil apoptosis and sensitizes tumor cells to Fas-induced cell death (25, 26). Here, we examine the role of p38 MAPK in CD30-triggered death of ALCL cells. We show that pharmacologic inhibition of p38 MAPK unmasks a CD30-triggered apoptotic pathway. Induction of apoptosis involved the activation of caspase-8 and was independent of endogenous expression of ligands for TNFR1, Fas, DR3, and DR4/DR5. We also show that CD30 signaling induces the expression of cellular FLICE-inhibitory protein (c-FLIP; ref. 27), and this is dependent on CD30-mediated activation of p38 MAPK. A more detailed analysis of the mechanism of induction of apoptosis revealed a critical role for the adaptor protein FADD in CD30-triggered apoptosis. These findings have implications for CD30-targeted immunotherapy of ALCL.

Materials and Methods

Cell Culture and Reagents

The human ALCL cell line Karpas 299 was obtained from the German National Resource Centre for Biological Material (DSMZ, Braunschweig, Germany) and cells were cultured in RPMI 1640 containing 10% FCS, 100 units/mL of penicillin, 100 μ g/mL of streptomycin, and 2 mmol/L of L-glutamine. The following neutralizing antibodies were

used: anti-TNF- α (mAb195; Millipore, Bedford, MA), anti-Fas ligand (mAb126; R&D Systems, Minneapolis, MN), and anti-TRAIL (mAb 375; R&D Systems). Recombinant human TNFR1-Ig and DR3-Ig proteins were from R&D Systems. Antibodies used for Western blotting were: anti-caspase-8 (3-1-9; BD PharMingen, San Diego, CA), anti-p38, anti-phospho p38 (Cell Signaling Technology, Danvers, MA), anti-actin (C11; Santa Cruz Biotechnology, Santa Cruz, CA), anti-cFLIP (NF6; Alexis Biochemicals), anti-FLAG (M2; Sigma, St. Louis, MO), and anti-CD30 (HRS-4; Abcam, Cambridge, United Kingdom). Recombinant CD70 (28) and the following antibodies were prepared in-house: anti-CD30 (BerH2; ref. 29), anti-rat CD4 (MRC OX68; ref. 30), rabbit anti-mouse Fc γ and human IgG. SB203580, PD169316, wortmannin, LY294002, SP600125, and zVAD-fmk were obtained from Calbiochem (La Jolla, CA), whereas RNase A, puromycin, and propidium iodide were purchased from Sigma.

Generation of Recombinant CD30 Ligand

A cDNA fragment corresponding to the full-length CD30 ligand (CD30L) protein (CD153) was amplified by PCR using a cDNA template prepared from Concanavalin A-activated mouse splenocytes. The following primers were used for amplification: forward primer incorporating a *Xba*I restriction site 5'-GGTCTAGACAGACTATATAAAG-CATGG-3'; reverse primer incorporating a *Bam*HI site 5'-AGGATCCTCAGTCTGAGCTACTATATAAG-3'. A cDNA fragment corresponding to the extracellular domain of CD30L (amino acid residues 72–239) was then amplified by PCR from the full-length cDNA using the following primers: forward primer incorporating a *Nsi*I restriction site and linker region 5'-AATGCATCCAAAACCTCACACAGCCCCACCGGCCCCATCCACTCCAAATA-CAACTG-3'; reverse primer incorporating an *Xba*I site 5'-GTCTAGATTCAGTCTGAGCTACTATATAAG-3'. This PCR fragment was cloned downstream of a cDNA encoding a modified human IgG₁ Fc region designed to express type II transmembrane proteins as soluble proteins (28). The human Fc-CD30L fragment was excised and then ligated into the pEE14 expression vector. CD30L was expressed in Chinese hamster ovary cells, and then purified from culture supernatant by immunoaffinity chromatography using an anti-human Fc monoclonal antibody (28). The structure and purity were then confirmed by SDS-PAGE and size exclusion chromatography.

Cell Cycle Analysis and Determination of Apoptosis

Karpas 299 cells (5×10^4) were harvested and washed in PBS and fixed in cold ethanol (70%) for 30 min at 4°C. Cells were then washed with PBS followed by treatment with RNase A (50 μ g/mL) for 1 h at 20°C. Next, propidium iodide (20 μ g/mL) was added and the cells were analyzed by flow cytometry on a FACSCalibur (Becton Dickinson, Mountain View, CA).

Transfection Procedure

Karpas 299 cells were transfected using the Nucleofector instrument and Nucleofection kit V (Amaxa, Cologne, Germany). After washing with the medium, cells (2×10^6) were resuspended in 100 μ L of Solution V containing 2 μ g

of pEF FLAGFADDDN79 puro (31). Cells were transferred into sterile cuvettes and then pulsed using the T-01 program. Immediately after that, cells were transferred into prewarmed media. The next day, cells were diluted into medium containing 0.5 $\mu\text{g}/\text{mL}$ of puromycin and transferred into 96-well plates to derive single cell clones.

Quantitative Reverse Transcriptase PCR

Karpas 299 cells (2.5×10^6) in 1 mL of the medium were incubated for 5 h with CD30L (10 $\mu\text{g}/\text{mL}$) with or without SB203580 (40 $\mu\text{mol}/\text{L}$) in the presence of zVAD-fmk (50 $\mu\text{mol}/\text{L}$). Total RNA was isolated with the RNeasy kit (Qiagen, Chatsworth, CA) and 3 μg of total RNA was used to obtain cDNA with a first-strand cDNA synthesis kit (GE Healthcare, Piscataway, NJ). As a control, reverse transcriptase was omitted from some reactions. The expression of c-FLIP_S mRNA was measured using a TaqMan Pre-Developed Assay Reagent (Applied Biosystems, Foster City, CA). Quantitative PCR (20 $\mu\text{L}/\text{reaction}$) was done using Platinum Quantitative PCR SuperMix (Invitrogen, San Diego, CA), 1 μL of TaqMan Pre-Developed Assay Reagent, 1 μL of cDNA, and 8 μL of H₂O. Amplification was done on a PTC-200 Chromo 4 instrument (Bio-Rad, Hercules, CA). The amplification protocol was 94°C for 5 min, then 35 cycles at 94°C for 30 s, 60°C for 30 s, 74°C for 1 min, and a final step at 74°C for 10 min. The assays were conducted in duplicate and the experiments were repeated at least thrice. Relative changes in c-FLIP_S mRNA levels were normalized to the levels of β 2-microglobulin mRNA and were calculated using the $2^{-\Delta\Delta C_t}$ method (32).

Measurement of TNF- α

Karpas 299 cells ($5 \times 10^5/\text{mL}$) were incubated for 24 h with or without CD30L and SB203580 in the presence of zVAD-fmk. Supernatant was collected and the amount of TNF- α measured by ELISA (Invitrogen, San Diego, CA).

Western Blot Analysis

Cells (2×10^6) were washed with PBS and then lysed in 50 μL of buffer containing 1% NP40, 10 mmol/L of Tris-HCl, 150 mmol/L of NaCl, 0.02% NaN₃ (pH 7.4), and protease inhibitors (Complete Protease Inhibitors Cocktail, Roche Diagnostics, Basel, Switzerland) for 30 min on ice. Nuclei were removed by centrifugation and the protein concentration was determined in the supernatant by the BCA protein assay (Pierce, Rockford, IL). Supernatant samples (equivalent to 40–80 μg of protein) were diluted 1:1 with protein solubilization buffer [160 mmol/L Tris-HCl, 6.4 mol/L urea, 1.6% SDS, 0.08% bromophenol blue (pH 8)] and then analyzed by SDS-PAGE. For analysis of p38 levels and phosphorylation state, 2×10^6 cells were lysed directly in protein solubilization buffer (100 μL). Samples were sonicated briefly (1 min), heated to 95°C and then 20 μL was analyzed by SDS-PAGE. Resolved proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA). Membranes were incubated with antibodies against the protein of interest and proteins visualized with horseradish peroxidase-conjugated anti-mouse antibody and the ECL Plus detection reagent (Amersham Biosciences).

Results

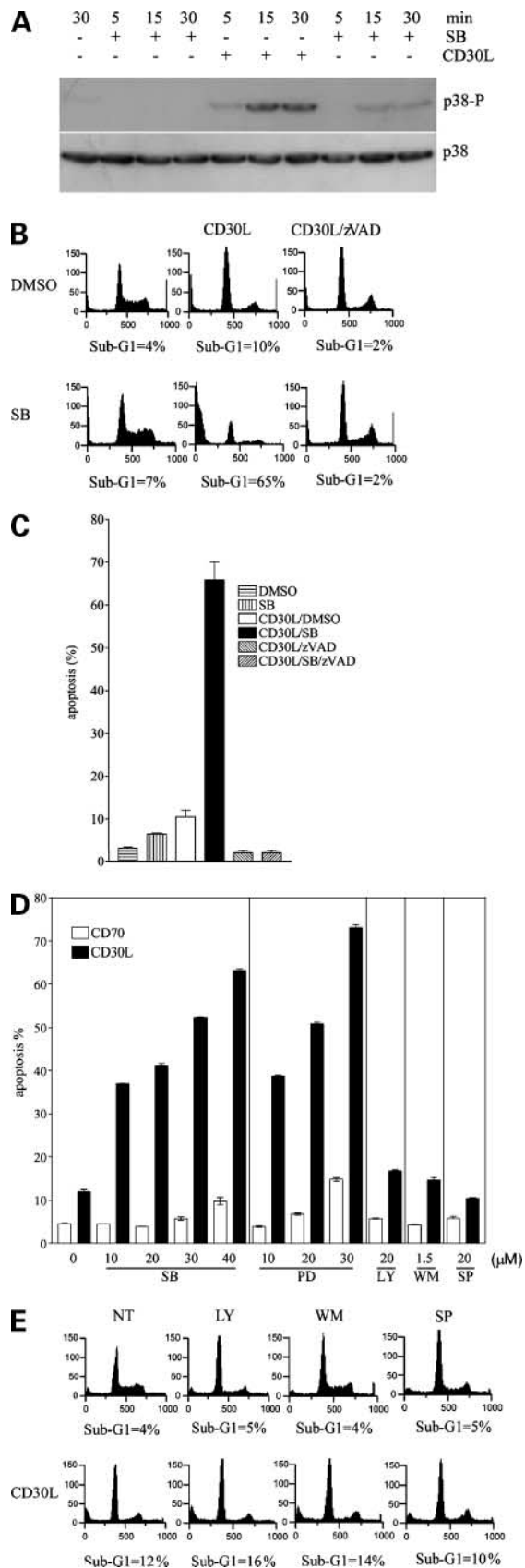
CD30L and Simultaneous Inhibition of p38 MAPK Trigger Apoptosis in ALCL Cells

Although CD30 cross-linking with antibody activates p38 MAPK (10), it is not known if this also occurs after the engagement of CD30 with its physiologic ligand, CD30L. Incubation of Karpas 299 cells with CD30L induced rapid activation p38 MAPK as assessed by the analysis of phospho-p38 MAPK levels, with maximal activation reached by 15 min (Fig. 1A). Having established that activation of p38 MAPK occurs following CD30 engagement with CD30L, we sought to investigate the role of this pathway in the regulation of CD30-triggered cell death. We used the pyridinyl imidazole compound SB203580, a well-known potent and specific inhibitor of p38 MAPK (33, 34) to investigate the role of this pathway in CD30 signaling in ALCL cells. Incubation of the ALCL cell line Karpas 299 with CD30L for 18 h significantly reduced the population of cells in S phase, indicating that CD30 signaling triggers cell cycle arrest (Fig. 1B). CD30L also caused a small increase in the proportion of cells within the sub-G₁ peak indicating that CD30L alone could trigger low levels of cell death (Fig. 1B). However, when cells were incubated with the combination of CD30L and SB203580 (CD30L/SB), there was a dramatic increase in cell death which was not observed when cells were incubated with SB203580 alone (Fig. 1B and C). The ability of SB203580 to inhibit p38 MAPK was confirmed by Western blot analysis. As shown in Fig. 1A the autophosphorylation of p38 MAPK (35) was significantly reduced by the addition of SB203580. Cell death was also observed when cells were treated with CD30L and two additional p38 MAPK inhibitors, PD169316 (Fig. 1D) and SB202190 (data not shown). Incubation of cells with soluble recombinant CD70, another member of the TNF superfamily, and SB203580 or PD169316, did not trigger cell death (Fig. 1D). Moreover, there was little, if any, increase in cell death after treatment with CD30L and inhibitors of phosphatidylinositol 3-kinase (LY294002 and wortmannin), or *c-Jun*-NH₂-kinase (SP600125) as compared with cells treated with CD30L alone (Fig. 1D and E). Cell death induced by CD30L/SB was prevented by the pan-caspase inhibitor zVAD-fmk, indicating that cell death proceeds via an apoptotic pathway (Fig. 1B and C).

To address whether caspase-8 is involved in the induction of apoptosis by CD30, we analyzed the proteolytic processing of procaspase-8 in ALCL cells. Incubation of cells with CD30L/SB, but not with either reagent alone, resulted in a reduction in the level of procaspase-8 and concurrent appearance of the cleaved caspase-8 pro-domain, both indicators of caspase-8 activation (Fig. 2A). Caspase-8 activation was barely detectable 2.5 h following the addition of CD30L/SB, but was clearly observed 4 h after stimulation (Fig. 2B). Taken together, these results show that activation of caspase-8 and efficient induction of apoptosis required CD30 engagement and concurrent inhibition of p38 MAPK.

Inhibition of p38 MAPK Prevents CD30-Mediated Up-Regulation of c-FLIP

c-FLIP is an important modulator of apoptosis that interacts with FADD and procaspase-8 (27). Two forms,



c-FLIP_L (long form) and c-FLIP_S (short form) exist as a result of alternative splicing (27). c-FLIP_L contains two death effector domains and an inactive caspase-like domain, and seems to act as an activator of procaspase-8 at physiologic levels (36). In contrast, c-FLIP_S, which contains only two death effector domains, is a bona fide inhibitor of procaspase-8 (37). Because CD30 engagement alone does not lead to the activation of caspase-8, we wondered if expression of the caspase-8 inhibitor c-FLIP_S is induced by CD30 signaling. To address the possible role of c-FLIP_S in the regulation of CD30-triggered apoptosis, we compared its expression in cells treated with CD30L, SB203580, or the combination of CD30L/SB. The pan-caspase inhibitor zVAD-fmk was included in these experiments in order to prevent cell death-associated reduction of cellular mRNA and protein. CD30 stimulation of ALCL cells triggered an ~3-fold increase in the level of c-FLIP_S mRNA (Fig. 3A). A clear increase in c-FLIP_S protein was also observed after treatment of cells with CD30L (Fig. 3B). Interestingly, inhibition of p38 MAPK with SB203580 markedly reduced the up-regulation of c-FLIP_S mRNA and protein levels by CD30L (Fig. 3A and B). These results show that the caspase-8 inhibitor c-FLIP_S is a downstream target of CD30 signaling and that its expression is highly dependent on p38 MAPK activation. Furthermore, the ability of SB203580 to inhibit c-FLIP_S up-regulation strongly correlates with its ability to sensitize ALCL cells to CD30-induced apoptosis.

CD30-Induced Apoptosis Is Independent of Endogenous Expression of Ligands for Death Domain-Containing Receptors

CD30 signaling has previously been shown to stimulate the expression of TNF- α in Karpas 299 cells (19, 38). Therefore, it is possible that CD30L/SB-triggered apoptosis could be mediated by TNF- α . Inhibition of p38 MAPK, however, abolished the ability of CD30L to trigger the production of TNF- α (Fig. 4A), suggesting that endogenous TNF- α production is unlikely to be required for CD30L/SB-induced apoptosis. To directly address the possibility that CD30-induced apoptosis was mediated through ligation of TNFR1 by endogenous TNF- α , we examined the effect of soluble TNFR1-Ig protein, a potent inhibitor of signaling

Figure 1. Induction of apoptosis by CD30L requires concurrent inhibition of p38 MAPK. **A**, analysis of p38 MAPK activation by Western blotting. Karpas 299 cells were incubated for the indicated periods of time with CD30L (10 μ g/mL) in the presence or absence of SB203580 (SB, 40 μ mol/L) and then processed as described in Materials and Methods. Phosphorylated (p38-P) and total levels of p38 MAPK (p38) were analyzed in non-treated cells (NT), or following the addition of CD30L with or without SB203580. **B**, cell cycle analysis and determination of apoptosis. Cells were incubated for 18 h with or without CD30L (10 μ g/mL) in the presence of SB203580 (SB, 40 μ mol/L) or DMSO as a control. zVAD-fmk (50 μ mol/L) was included in some experiments to examine the role of caspases in CD30-induced cell death. Cells were fixed with 70% ethanol, stained with propidium iodide to indicate the DNA content, and then analyzed by flow cytometry. Apoptotic cells were identified by their sub-G₁ DNA content. **C**, graphical representation of **B** using data obtained from four independent experiments. **D** and **E**, the effect of SB203580 (SB), PD169316 (PD), LY294002 (LY), wortmannin (WM), and SP600125 (SP) on CD30L-triggered apoptosis. Columns, mean; bars, SE.

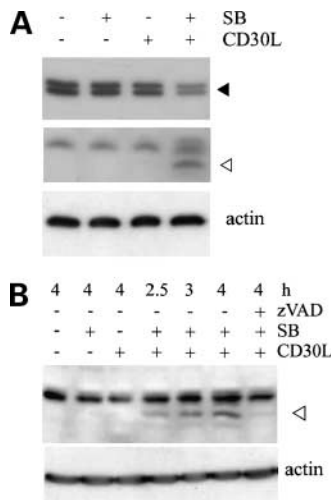


Figure 2. CD30L/CD30 interaction triggers caspase-8 activation after inhibition of p38 MAPK. Cells (2×10^6) were treated for 4 h (A), or for the indicated times with CD30L (10 $\mu\text{g}/\text{mL}$), SB203580 (SB, 40 $\mu\text{mol}/\text{L}$) or the combination of CD30L and SB203580 in the presence or absence of zVAD-fmk (50 $\mu\text{mol}/\text{L}$; B). Western blot of procaspase-8 (filled arrow), the cleaved p24 caspase-8 pro-domain (empty arrow) and actin. Actin was used to show equivalent protein loading per lane.

by membrane-anchored TNFR1 (39) and neutralizing anti-TNF- α monoclonal antibody (40), on the ability of CD30L/SB to trigger apoptosis. The addition of TNFR1-Ig or anti-TNF- α antibody to the culture media of Karpas 299 cells failed to inhibit CD30-induced apoptosis (Fig. 4B). These data strongly argue against a role for endogenous TNF- α expression in CD30L/SB-triggered apoptosis.

We also addressed if CD30L/SB-triggered apoptosis was dependent on endogenous expression of Fas ligand, TRAIL, or signaling by DR3. Cell surface expression of Fas ligand and TRAIL was either very low or absent on Karpas 299 cells and did not increase upon treatment of cells with CD30L (data not shown). Consequently, the addition of anti-Fas ligand, or anti-TRAIL neutralizing antibodies had no effect on the induction of apoptosis by the combination of CD30L/SB (Fig. 4C). Similarly, soluble DR3-Ig failed to protect cells against CD30L/SB-triggered apoptosis (Fig. 4D). Taken together, these results exclude a role for endogenous expression of ligands for TNFR1, Fas, DR3, and DR4/DR5 in CD30-mediated apoptosis.

Involvement of FADD in CD30-Induced Apoptosis

To examine the role of FADD in CD30-induced apoptosis, we generated stable ALCL cell lines that express a dominant-negative form of FADD that lacks the death effector domains (ref. 31; Fig. 5A). CD30L/SB failed to induce the activation of caspase-8 and trigger apoptosis in Karpas 299 cells expressing a dominant-negative form of FADD (Fig. 5B and C). In contrast, CD30L/SB-mediated caspase-8 activation and apoptosis occurred in wild-type cells and in Karpas 299 clones that grew under selection but lacked the expression of a dominant-negative form of FADD (Fig. 5B and C).

Induction of Cell Death by Anti-CD30 Antibodies Also Requires Inhibition of p38 MAPK

Because CD30 is currently being investigated as a target for monoclonal antibody-based immunotherapy, we compared the effects of CD30L with those of anti-CD30 antibodies. A significant induction of cell death by anti-CD30 antibodies was observed in the presence of the p38 MAPK inhibitor SB203580 (Fig. 6A). Induction of cell death by anti-CD30 antibodies was less efficient than that triggered by CD30L. However, an equivalent level of cell death to that produced by CD30L was attained upon cross-linking of anti-CD30 antibodies, suggesting that hyper cross-linking of CD30 is necessary for efficient signaling. Consistent with this notion, the ability of anti-CD30 antibodies to cause cell cycle arrest in the absence of SB203580 was also enhanced by cross-linking, although this was always lower than that observed with CD30L (Fig. 6B). Interestingly, whereas the addition of CD30L triggered clear homotypic aggregation in cell cultures, anti-CD30 antibodies had little effect on cell aggregation even after cross-linking (Fig. 6C). These data suggest that anti-CD30 antibodies do not fully mimic the activity of the physiologic ligand for CD30.

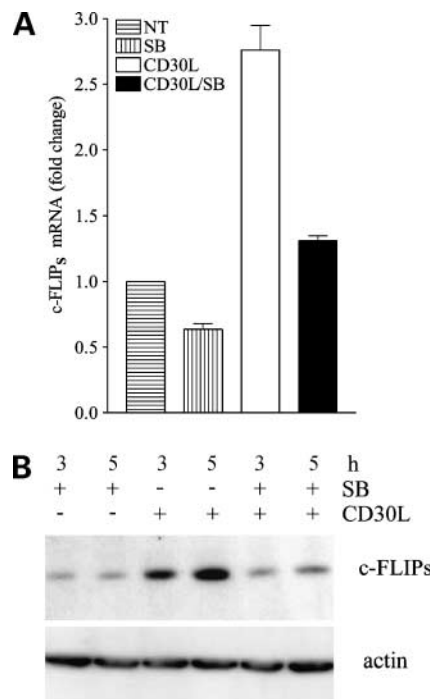


Figure 3. CD30 up-regulation of c-FLIPs is dependent on the activation of p38 MAPK. **A**, relative expression of c-FLIPs mRNA in Karpas 299 cells was determined by quantitative reverse transcriptase PCR as described in Materials and Methods. RNA was prepared from non-treated cells (NT) and cells incubated for 5 h with CD30L with or without SB203580 (SB) in the presence of zVAD-fmk. Columns, mean of three independent experiments; bars, SE. **B**, Western blot of c-FLIPs. Cells were incubated for 3 or 5 h as described in A. Actin was used to show equivalent protein loading per lane.

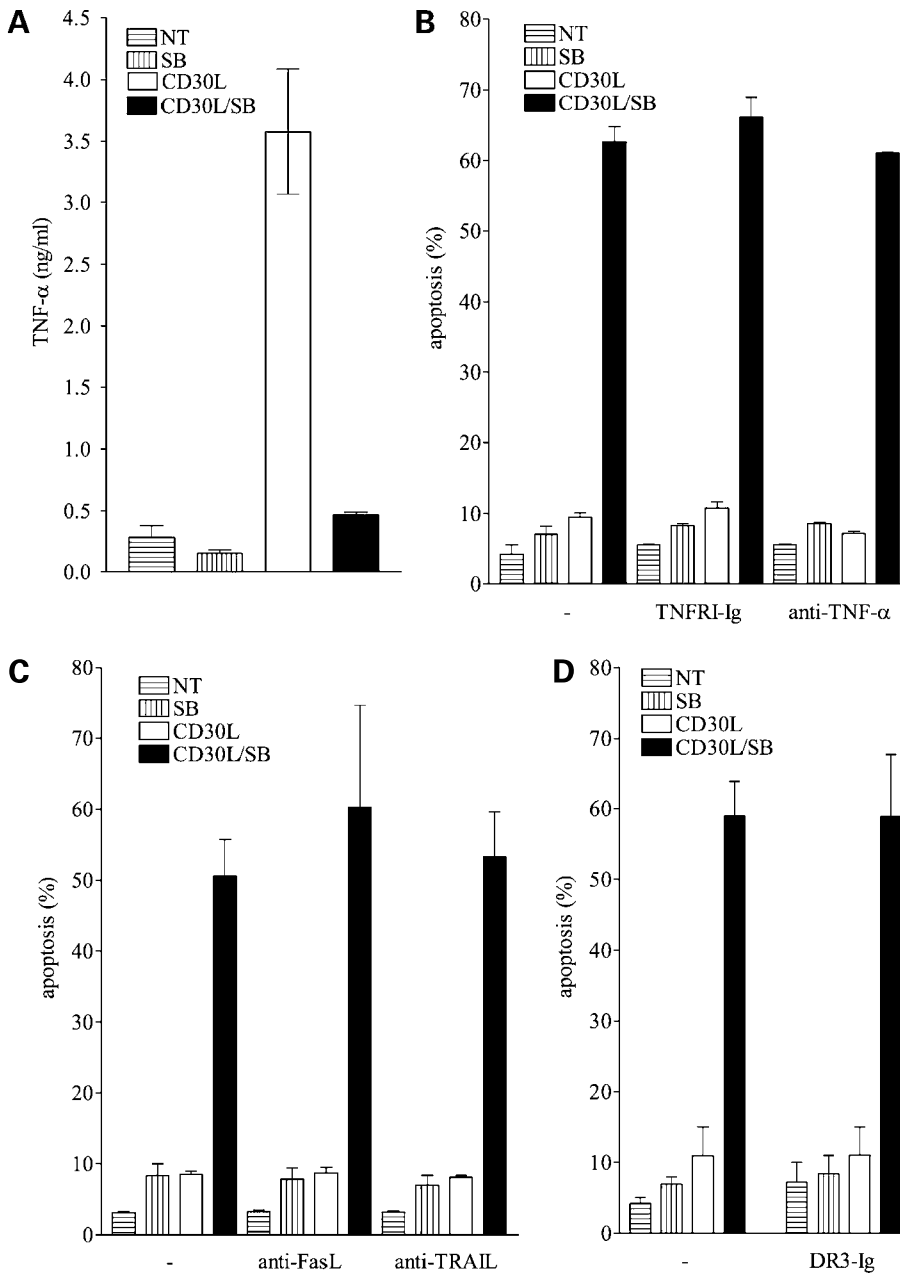


Figure 4. CD30-induced apoptosis was independent of endogenous expression of ligands for death domain-containing receptors. **A**, SB203580 inhibits CD30-mediated production of TNF- α . Cells (5×10^5) were incubated for 18 h with CD30L, SB203580 (SB), the combination of CD30L and SB203580 (CD30L/SB), or left untreated (NT). All incubations were carried out in the presence of zVAD-fmk. The concentration of TNF- α in the culture medium was measured by ELISA. Columns, mean; bars, SE ($n = 4$). **B** to **D**, induction of apoptosis by CD30L/SB was independent of endogenous expression of ligands for the death domain-containing receptors TNFR1, Fas, DR3, DR4, or DR5. Cells (5×10^4) were pretreated for 1 h with TNFR1-Ig, neutralizing antibodies against TNF- α , Fas ligand and TRAIL, or DR3-Ig. As a control, cells were pretreated with mouse anti-rat CD4 antibody or human IgG (-). All neutralizing reagents were used at a concentration of 25 μ g/mL. Cells were then incubated for 18 h with CD30L, SB203580 (SB), the combination of CD30L and SB203580 (CD30L/SB), or left untreated (NT). Cell death was analyzed by flow cytometry. Columns, mean; bars, SE ($n = 2-3$).

Discussion

We have undertaken this study in order to understand the signaling pathways that lead to cell death following engagement of CD30 by CD30L. We have made the following novel observations: (a) effective induction of apoptosis by CD30 requires concurrent inhibition of p38 MAPK, thus revealing a pro-survival role of p38 MAPK in CD30 signaling; (b) induction of apoptosis involves caspase-8 activation via the adaptor protein FADD and is independent of endogenous expression of ligands for the death domain-containing receptors TNFR1, Fas, DR3,

DR4, or DR5; and (c) CD30 triggering augments the expression of the natural caspase-8 inhibitory protein c-FLIP_s and this is dependent on CD30-mediated activation of p38 MAPK.

In a previous study, CD30 engagement by anti-CD30 antibody was shown to inhibit the ability of cells to incorporate 3 H-thymidine and this inhibition was partially overcome by the addition of a neutralizing anti-TNF- α antibody (19). Based on these observations, the authors suggested that CD30 engagement induces cell death that is, in part, dependent on endogenous expression of TNF- α .

However, by employing an assay that could differentiate between cell death and cell cycle arrest, subsequent studies showed that CD30 engagement by either anti-CD30 antibody (17) or CD30L (this study) results in cell cycle arrest with minimal levels of cell death (Fig. 1B and C). Thus, the inhibition of ³H-thymidine incorporation after treatment with anti-CD30 antibody, observed by Grell et al. (19), most likely reflects CD30-mediated cell cycle arrest rather than cell death. Using multiple reagents that neutralize TNF- α , including monoclonal antibody 195 used by Grell et al. (19), we did not observe any role for TNF- α in CD30-induced apoptosis (Fig. 4B) or the induction of cell cycle arrest (data not shown). In our study, CD30 signaling was initiated using soluble recombinant CD30L, which forms two adjacent trimeric units (41), whereas Grell and colleagues used a bivalent anti-CD30 antibody. Because the natural CD30L (CD153) is a trimer (38), we suspect that bivalent cross-linking of CD30 by antibodies does not activate the full signaling capacity of CD30 (Fig. 6). Thus, differences in

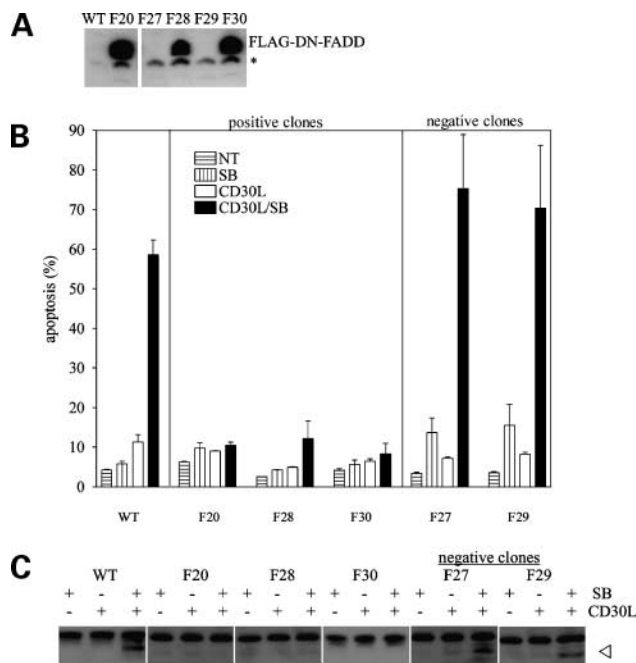


Figure 5. FADD is essential for CD30-dependent activation of caspase-8 and the induction of apoptosis. **A**, expression of dominant-negative FADD in stable Karpas 299 clones (F20, F28, and F30) detected by Western blotting using anti-FLAG monoclonal antibody. F27 and F29 are two clones that grew under selection with puromycin, but lacked the expression of dominant-negative FADD and therefore served as negative controls. *WT*, parental cell line; *, presence of a nonspecific band on the Western blot. **B**, analysis of apoptosis by flow cytometry. Clones were precultured in media without puromycin for 24 h before treatment. Cells (5×10^5) were incubated for 18 h with CD30L, SB203580 (SB), the combination of CD30L and SB203580 (CD30L/SB), or left untreated (NT). *Columns*, mean from three independent experiments; *bars*, SE. **C**, dominant-negative FADD prevents CD30L/SB activation of caspase-8. The presence of the cleaved p24 caspase-8 pro-domain (empty arrow) could be shown by Western blotting only in cells that lacked expression of dominant-negative FADD.

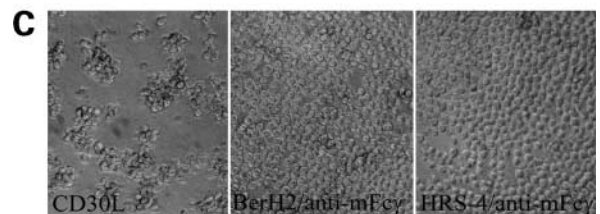
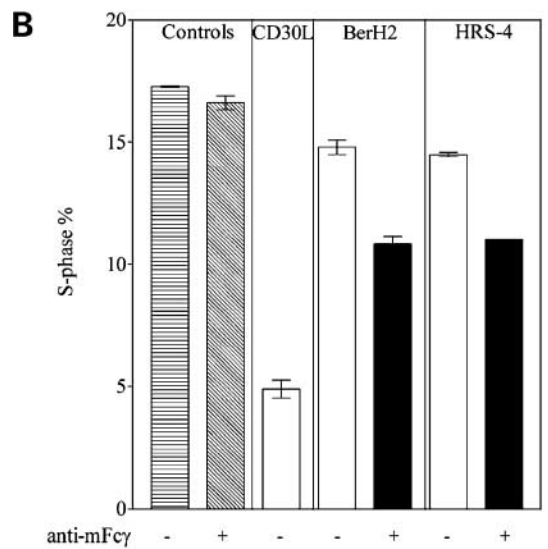
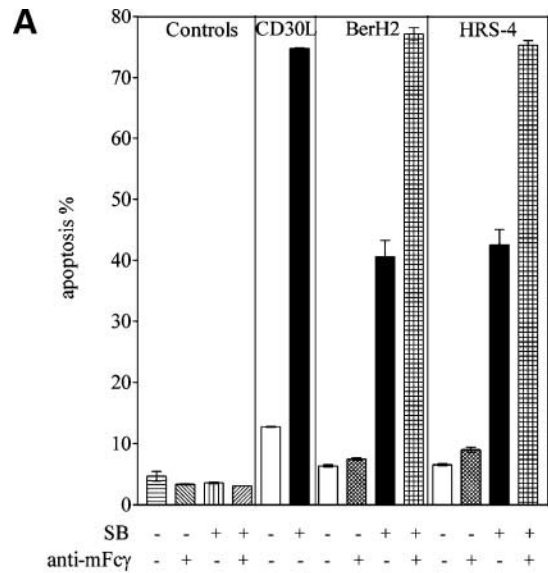


Figure 6. Induction of cell death by anti-CD30 antibodies also requires inhibition of p38 MAPK. **A**, analysis of apoptosis by flow cytometry. Cells (5×10^5) were incubated for 18 h with 10 μ g/mL of CD30L, anti-CD30 antibodies (*BerH2* and *HRS-4*) in the presence or absence of anti-mouse Fc antibody (*anti-mFc*), with or without SB203580 (SB, 40 μ mol/L). *Columns*, mean from two independent experiments; *bars*, SE. **B**, induction of cell cycle arrest as determined by flow cytometry analysis. Cells were treated as in **A**, but in the absence of SB203580. *Columns*, mean from two independent experiments; *bars*, SE. **C**, CD30L triggers homotypic aggregation of ALCL cells. Cells (5×10^5) were incubated for 18 h with 10 μ g/mL of CD30L (left panel) or anti-CD30 antibodies [*BerH2* (middle panel) and *HRS-4* (right panel)] in the presence of anti-mouse Fc antibody (*anti-mFc*). Homotypic aggregation was assessed by light microscopy.

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the signaling between antibodies and CD30L might explain why anti-CD30 antibody and not CD30L-induced cell cycle arrest shows partial dependency on endogenous expression of TNF- α .

CD30 activation of caspase-8 requires the adaptor protein FADD (Fig. 5C) and proceeds with relatively slow kinetics (Fig. 2B), similar to that observed following TNFR1 signaling (42). In contrast, caspase-8 activation by Fas is rapid, occurring within minutes of receptor engagement (43). Unlike Fas, TNFR1 is unable to recruit FADD directly to the engaged receptor at the level of the plasma membrane (so-called complex I; ref. 42). Instead, FADD-dependent activation of caspase-8 by TNFR1 occurs in a second step that takes place in the cytoplasm (complex II; ref. 42), or during formation of endocytic vesicles after internalization of the receptor (44). Because CD30 does not interact directly with FADD, it is possible that FADD-dependent activation of caspase-8 by CD30 occurs in a post-plasma membrane compartment, analogous to that described for TNFR1 (42). The relatively slow kinetics of caspase-8 activation by CD30L/SB is in agreement with this notion.

The expression of c-FLIP has been previously shown to be regulated by NF- κ B (45, 46). Thus, activation of NF- κ B by TNFR1 triggers the expression of c-FLIP leading to the inhibition of caspase-8 (42). In this study, we have shown that p38 MAPK regulates c-FLIP_S mRNA and protein levels following stimulation of ALCL cells with CD30L (Fig. 3). We observed an induction of c-FLIP_S protein expression in cells within 3 h of stimulation via CD30 (Fig. 3B), with no detectable activation of caspase-8 (Fig. 2B), suggesting that *de novo* expression of c-FLIP_S prevents CD30-triggered activation of caspase-8. The regulation of gene expression by the p38 MAPK pathway occurs through transcriptional as well as posttranscriptional mechanisms (47, 48). One possibility is that p38 MAPK might promote the expression of c-FLIP_S through the potentiation of the transcriptional activity of the p65 (RelA) subunit of NF- κ B (49), which is required for expression c-FLIP (50). However, an effect of p38 MAPK on CD30-mediated transcriptional activity of NF- κ B is unlikely because we found that CD30-mediated expression of a NF- κ B-dependent reporter gene in Karpas 299 cells is not affected by the p38 MAPK inhibitor SB203580 (data not shown). Further studies are necessary to fully understand the role of p38 MAPK in CD30-driven expression of c-FLIP_S.

In summary, we have uncovered a previously unappreciated role for p38 MAPK in the regulation of CD30-triggered apoptosis in ALCL cells. The current mainstay of therapy for ALCL is cytotoxic chemotherapy. Although this cures a proportion of patients, a significant number relapse, requiring alternative therapy (51). The findings described in this study may have implications for future development of more effective forms of therapy for ALCL.

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