CD30 Activates Both the Canonical and Alternative NF-κB Pathways in Anaplastic Large Cell Lymphoma Cells*

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The tumor necrosis factor receptor superfamily member CD30 is highly expressed on the surface of many leukemia and lymphoma cells, including Hodgkin lymphoma (HL)2 (1), anaplastic large cell lymphoma (ALCL), multiple myeloma, and adult T-cell leukemia (2–4). The fact that CD30 is present normally on only a very small fraction of activated B- and T-cells makes it an attractive target for therapeutic intervention. The physiological role of CD30 is unclear, although it has been shown to contribute to negative selection of T-cells (5–7) and has been shown to serve as a co-stimulatory molecule on T-cells (8, 9).

The tumor necrosis factor receptor superfamily can be subdivided into two groups, defined by their signaling properties (10). The group that includes CD30 (11–13) signals through recruitment of one or more of the tumor necrosis factor (TNF) receptor-associated factors (TRAFs) directly to their cytoplasmic tails (14, 15). The other group signals through an ~65-residue motif known as a death domain located in the cytoplasmic tail, which mediates proapoptotic signals through heterotypic interactions with components of the prodeath machinery. This second group also recruits adaptors via their cytoplasmic death domains that mediate interactions with the TRAFs (16). In both groups of receptors, TRAF recruitment is thought to result in activation of the nuclear factor κB (NF-κB) family of transcription factors (17).

NF-κB transcription factors are important regulators of genes whose products are necessary for the innate and adaptive immune response and for the survival and proliferation of certain cell types (reviewed by Chen and Greene (18)). Five different proteins constitute the NF-κB family, which homodimerize and/or heterodimerize to form active transcription factors with different target gene specificity. The different NF-κB factors include RelA, RelB, c-Rel, and the precursor and processed products of the NFKB1 (p105/p50) and NFKB2 (p100/p52) genes. aberrant regulation of the alternative NF-κB pathway subunit p100/p52 contributes to cancer, which has been linked to the cell cycle-regulatory function of the protein. Interestingly, a recent report demonstrated that p100/p52 is a regulator of the p21waf1 promoter in U-2 OS cells (19).

A second characteristic of ALCL, besides high surface expression of CD30, is the expression of the oncoprotein NPM-ALK, which is the product of a chromosomal translocation t(2;5)(p23;q35) that fuses nucleophosmin (NPM) with anaplastic lymphoma kinase (ALK) (20). NPM-ALK can associate with the cytoplasmic tail of CD30 (21), and a recent study
reported that the presence of NPM-ALK in ALCL cells abrogates CD30-mediated NF-κB activation (22). Alternatively, other studies that have examined CD30 signaling observed effects ranging from apoptosis to cell cycle arrest and are suggestive of NF-κB activation in ALCL cells (23–26).

In order to investigate CD30 signaling in a more physiological manner, a CD30-responsive system was established that allowed a detailed analysis of the kinetics and specific cellular responses in ALCL and HL cells. We show here that CD30 stimulation alone causes apoptosis in a fraction of ALCL cells with observed reduction in cytoplasmic TRAF2 protein levels. In surviving cells, TRAF2 was found to translocate to a detergent-insoluble fraction of the cell, and this translocation correlated with both canonical and alternative NF-κB activation as measured by cytoplasmic processing of p105 and p100, NF-κB nuclear translocation, and transactivation of NF-κB-responsive genes. Up-regulation of NF-κB-responsive genes suggested a cell cycle-progressive consequence for CD30 signaling. In contrast to CD30 ligand (CD30L/CD153), exposure of ALCL cells to TNF activated only the canonical NF-κB pathway and was a poor activator of NF-κB-responsive genes. The sustained robust activation of NF-κB following prolonged physiological CD30 stimulation resulted in cell cycle arrest, which correlated with induction of p21wafl. Surprisingly, the canonical, but not the alternative, NF-κB pathway was primarily responsible for the CD30-induced expression of p21wafl, since short interfering RNA (siRNA) targeted against RelA, but not p100/p52, resulted in a reduction of CD30-induced p21wafl. Given these results, we conclude that, regardless of the presence of NPM-ALK, CD30 signaling in ALCL results in strong NF-κB activation, directly leading to cell cycle arrest.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Cell Lines**—The coding sequence of CD30L was subcloned into pcDNA5/FRT/TO (Invitrogen) and then co-transfected with a recombinase expressing plasmid into Flp-In Chinese hamster ovary (CHO) cells (Invitrogen) according to the manufacturer’s instructions. This generated a cell line expressing CD30L and the hygromycin resistance marker to allow for selection of stable CD30L⁺ CHO cells with 500 μg/ml hygromycin B. Following selection, stable CD30L⁺ CHO cells were stained with a fluorescein isothiocyanate-conjugated anti-CD30L antibody and sorted by sterile flow cytometry. One cell was placed into each well of a 96-well plate and selected with hygromycin B. Forty-two lines, Michel and Karpas 299, have been described previously (24), and were propagated in RPMI medium (Mediatech) supplemented with 10% fetal bovine serum and 2 mM Glutamax at 37 °C, 5% CO₂.

**Physiological CD30 Stimulation**—Michel, Karpas 299, L428, and/or KM-H2 cells were resuspended in a 1:1 mixture of RPMI and F-12 nutrient media at a final concentration of 1 × 10⁶ cells/ml. One ml of the lymphoma cells was incubated for the specified time with either CHO cells (negative control) or with CD30L⁺ CHO cells that had been seeded at 0.8 × 10⁶ cells/well (0.5 × 10⁶ cells/well for cell cycle analysis) in 6-well plates the previous day. Following CD30 stimulation, the lymphoma cell lines were removed from the CHO cells with gentle pipetting and collected by centrifugation at 200 × g for 5 min. The medium was aspirated, and the cells were washed once with 1 ml of phosphate-buffered saline (PBS). The cells were centrifuged at 200 × g for 5 min, the PBS was aspirated, and the cells were resuspended according to the experimental specifications listed below.

**Propidium Iodide Exclusion and Cell Death Analysis**—CD30 on Michel, Karpas 299, L428, and KM-H2 cells was stimulated for 6 h as described above. The lymphoma cell lines were washed once with PBS and resuspended in 0.5 ml of PBS containing 2 μg/ml propidium iodide (PI). PI-positive cells were detected by flow cytometry.

Cell death analysis following CD30 stimulation with agonistic antibodies M67 and M44 was performed as described (24), except that 6-well plates were used, and each well was coated with 20 μg of either IgG1 control antibody or agonistic antibody. 1 × 10⁶ Michel or Karpas 299 cells were incubated with IgG1, M67, or M44 for 24 h followed by washing once in PBS and resuspending in 0.5 ml of PBS containing 2 μg/ml PI. PI-positive cells were detected by flow cytometry.

**TRAF2 Translocation Analysis**—CD30 on Karpas 299 and L428 cells was stimulated for 3 h as described above. The lymphoma cell lines were washed once in PBS and resuspended in Triton X-100 lysis buffer (25 mM HEPS, 100 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM PMSF, and 1 mM DTT) supplemented with additional protease inhibitors and incubated for 20 min on ice to ensure complete lysis. The soluble and insoluble Triton X-100 fractions were separated by centrifugation at 20,800 × g at 4 °C for 10 min. The soluble fractions were moved to a fresh microcentrifuge tube, and the insoluble pellets were washed once in 1 ml of Triton X-100 lysis buffer and centrifuged at 20,800 × g at 4 °C for 10 min. The Triton X-100 lysis buffer was aspirated, and the insoluble pellets were resuspended in Laemmli buffer (0.0625 M Tris-HCl, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 1 mM PMSF, and 1 mM DTT). The soluble and insoluble fractions were analyzed by immunoblotting for the presence of TRAF2 protein.

**Antibodies and Immunoblotting**—Lysates from control or CD30-stimulated lymphoma cells were prepared with radioimmune precipitation lysis buffer (PBS containing 1% Nonidet P-40, 0.5% (w/v) deoxycholic acid, 0.1% SDS, 1 mM PMSF, and 1 mM DTT) supplemented with additional protease inhibitors and incubated for 20 min on ice to ensure complete lysis unless stated otherwise. Protein samples were resolved on denaturing NuPAGE 4–12% polyacrylamide gradient gels (Invitrogen), transferred to nitrocellulose (Invitrogen), and blocked with 5%...
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powdered milk (w/v) in TBS containing 0.05–0.2% Tween 20, depending on the primary antibody used. The membranes were incubated with the specified antibodies, washed, and then incubated with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare). Peroxidase activity was detected by the enhanced chemiluminescence Western blot analysis system (GE Healthcare). Antibodies against TRAF2 (Transduction Laboratories), RelA and RelB (a kind gift from Nancy Rice), p50 and p52 (Upstate Biotechnology), p21\textsuperscript{waf1} (Transduction Laboratories), cyclin-dependent kinase 4 (CDK4; Transduction Laboratories), and \(\beta\)-actin (Sigma) were used.

Electrophoretic Mobility Shift Assay—CD30 on Karpas 299 or L428 was stimulated for 3 h as described above except that 5 \times 10^6 Karpas 299 cells were layered onto CHO and CD30L \textsuperscript{+} CHO cells previously seeded in 10-cm cell culture dishes at a density of 3.5 \times 10^5 cells/plate. The lymphoma cells were washed once with PBS, and half were used for nuclear extract preparation, whereas the remaining cells were used for total RNA isolation. All steps were performed cold. Karpas 299 cells were washed once with buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl\textsubscript{2}, 10 mM KCl, 0.1 mM PMSF, and 0.5 mM DTT) and pelleted by centrifugation at 200 \times g for 5 min. Buffer A was aspirated, and the pellet was resuspended by gentle pipetting in 30 \mu l of buffer A supplemented with 0.1% Nonidet P-40 and incubated on ice for 10 min. The nuclear pellet was isolated by centrifugation at 20,800 \times g for 10 min, resuspended in 20 \mu l of cold buffer C (20 mM HEPES, pH 7.9, 0.42 mM NaCl, 1.5 mM MgCl\textsubscript{2}, 0.2 mM EDTA, 0.1 mM PMSF, 0.5 mM DTT, and 25% glycerol), and rotated at 4 °C for 15 min. The nuclear extract was clarified by centrifugation at 20,800 \times g for 15 min, and 10 \mu l was transferred to a fresh tube and diluted with 60 \mu l of modified buffer D (20 mM HEPES, pH 7.9, 50 mM KCl, 0.2 mM EDTA, 0.1 mM PMSF, 0.5 mM DTT, and 20% glycerol), flash frozen, and stored at −80 °C.

Two complementary oligonucleotides containing NF-\(\kappa\)B consensus binding sites, \textit{kBEMSaolo}g1 (5'-TGCAGGGACT-TTCCGCAGGGACTTTC-3') and \textit{kBEMSaolo}g2 (5'-TGCTG-GAGGAATGTTCCCAAGGAAATGTCCT-3'), were annealed, and 50 ng was radiolabeled in a Klenow reaction in the presence of [\(\alpha\text{-}^{32}\text{P}\)]dCTP. The radiolabeled probe was then purified over a Sephadex column in TE containing 50 mM NaCl. To test for the presence of NF-\(\kappa\)B in the nuclear extracts, prepared as described above, 2 \mu l of nuclear extracts were incubated for 20 min at room temperature with 1 \mu g of poly(dL-dC):poly(dL-dC) in modified buffer D (minus glycerol) for a total volume of 20 \mu l. 0.2 \mu l of \(^{32}\text{P}\)-radiolabeled probe was added, and the entire reaction was separated on a nondenaturing 4% polyacrylamide gel. Autoradiography was performed overnight at −20 °C. For supershift analysis, 1–4 \mu g of antibody was incubated with the nuclear extract and poly(dL-dC):poly(dL-dC) prior to the addition of \(^{32}\text{P}\)-radiolabeled probe.

Real Time Reverse Transcription-PCR—CD30 on Karpas 299 or L428 cells was stimulated for 3 h (from 6 to 36 h for cell cycle analysis; see below) as described above. The lymphoma cells were washed with PBS followed by total RNA isolation using the RNasy minikit (Qiagen) according to the manufacturer’s instructions. 100 ng of total RNA was subjected to a reverse transcription reaction using random hexamer primers and MultiScribe\textsuperscript{TM} Reverse Transcriptase (Applied Biosystems). 1 \mu l of the resulting cDNA was analyzed with the indicated target assay using the Applied Biosystems 7500 real time PCR system. Each target assay was normalized to glyceraldehyde-3-phosphate dehydrogenase levels and performed in triplicate.

Cell Cycle Analysis—CD30 on Karpas 299 or L428 cells was stimulated for 6, 12, 24, and 36 h as described above. The lymphoma cells were washed with PBS and collected by centrifugation at 200 \times g for 5 min, and each sample was normalized to 0.5 \times 10^5 cells in 0.5 ml of PBS. The cells were fixed in 50% ethanol overnight at −20 °C. The following day, the cells were collected by centrifugation at 200 \times g, the PBS/ethanol mixture was decanted, and the cells were resuspended in 0.5 ml of PBS containing 50 \mu g/ml PI and 100 \mu g/ml RNase A. The DNA content of the cells was analyzed by flow cytometry.

RNA Interference—1 \times 10^7 Karpas 299 cells were transfected with 16 \mu g of either a control (green fluorescent protein) siRNA (sense strand AAGACCCGCGCGGAGTGAAG), siRNA targeted against p100 (sense strand AAGGCTGTTGCTGACATCCAT), or an siRNA targeted against RelA (sequence has been described (27)). The indicated siRNA was transfected using a Bio-Rad Gene Pulser II electroporator set on infinite resistance, 300 V, and 950 microfarads. Twenty-four h post-transfection, dead cells resulting from the transfection procedure were removed by centrifuging the cells at 400 \times g for 20 min on a Ficoll-Paque PLUS (GE Healthcare) density cushion. Forty-eight h post-transfection, CD30 was stimulated for 24 h on the transfected cells as described above. Following CD30 stimulation, total RNA was isolated from a fraction of the cells and subjected to real time reverse transcription-PCR. The remaining cells were divided and used for whole cell lysate preparation and Western blot analysis and cell cycle analysis.

RESULTS

Physiological CD30 Stimulation Triggers Apoptosis in ALCL Cells—Much of our understanding of CD30 function is based on the use of agonistic CD30-specific antibodies or otherwise nonphysiologic means to activate CD30 signaling cascades. Although immobilized agonistic antibodies hypothetically mimic CD30L and are a convenient tool for stimulating CD30, there are conflicting data reported for different CD30 activating antibodies. These studies suggest that different epitopes on CD30 recognized by the various agonistic antibodies may contribute to different signaling outcomes. Since CD30L is normally expressed as a membrane-bound ligand on macrophages, T-cells, and B-cells, we decided to examine CD30 signaling using a membrane-bound form of CD30L to avoid the epitope-agonistic antibody approach. To this end, a system to stimulate CD30 via its physiological ligand was established, and the subsequent role of CD30 signaling in lymphoma cells was investigated. CHO cells were chosen as a vehicle for stable expression of CD30L for two reasons. First, because CHO cells are derived from a different species, there is less likely to be contaminating stimulants, such as secreted cytokines or membrane-bound receptors/
Analysis of the stable CD30L+ CHO cells by flow cytometry and Western blot revealed that CD30L was expressed on the cell surface versus no detectable CD30L expression on stable vector control CHO cells as expected (Fig. 1B) (data not shown).

We have previously shown that stimulation of CD30 on ALCL cells with agonistic antibodies M67 and M44 resulted in a percentage of cells succumbing to death (24). Thus, to examine whether a physiological CD30 signal causes cell death, ALCL (Michel and Karpas 299) or HL (L428 and KM-H2) cell lines were layered onto control CHO cells or CD30L+ CHO cells for 6 h (Fig. 1A). Following incubation, the lymphoma cells were removed, and cell viability was evaluated by propidium iodide exclusion. As can be seen in Fig. 2A, stimulation of CD30 on both ALCL cell lines tested resulted in ~20% cell death when normalized to control cells. These results are comparable with the death observed following CD30 stimulation with M44 or M67 (Fig. 2B). Conversely, HL cell viability was barely affected upon physiological CD30 stimulation (Fig. 2A), which has previously been attributed to the constitutive prosurvival signals of NF-κB in these cells (24).

CD30 Signaling Includes TRAF2 Translocation and NF-κB Activation—Ectopic expression of constitutively active CD30 in HEK293 cells or CD30 cross-linking of ALCL and HL cells with an agonistic antibody has been reported to result in the degradation of TRAF2 (TNF receptor-associated factor 2) (24, 28), an adaptor molecule necessary for the activation of NF-κB. In order to examine whether this phenomenon occurs following CD30 stimulation with its physiological ligand, ALCL (Karpas 299) or HL (L428) cell lines were layered onto control CHO cells or CD30L+ CHO cells for 3 h. Following CD30 stimulation, the lymphoma cells were removed, and Triton X-100-soluble fractions were prepared and analyzed by Western blot. Analysis of cell lysates using an antibody directed against TRAF2 revealed that TRAF2 disappeared from the Triton X-100-soluble fractions of Karpas 299 cells as early as 1 h and was completely lost at 3 h poststimulation of CD30 (Fig. 3A) (data not shown). Loss of TRAF2 was seen to a much lesser degree in the L428 soluble fraction following CD30 stimulation (Fig. 3A). In conjunction with TRAF2 loss from the soluble fraction, TRAF2 accumulated in the Triton X-100-insoluble fraction of the cell (Fig. 3A).

Because the presence of NPM-ALK in ALCL cells has been shown to inhibit CD30-mediated NF-κB signaling by blocking the recruitment and aggregation of TRAF proteins (22), we asked whether the observed TRAF2 translocation following a CD30 response correlated with NF-κB activation, as has been shown in other signaling situations (29). In fact, the NF-κB precursors, p105 and p100, were processed in the cytoplasm of Karpas 299 cells stimulated for 3 h using our system (Fig. 3, B and C), suggesting that NF-κB was being activated following CD30 stimulation. Processing of p105 can occur co-translationally (30), which satisfies the basal necessity of NF-κB activity required by the cell. However, p105 processing has also been shown to occur post-translationally following a stimulant-dependent response (31, 32), as in the case of CD30 stimulation shown here. To our knowledge, this is the first example of CD30-induced p105 processing, and it suggests that physiological CD30 stimulation increases the cellular pool of the p50 subunit. In L428 cells, there is a loss of p105 protein following CD30 stimulation (Fig. 3B), but because of the large amount of p50 present in these cells, it is difficult to determine whether p105 is being processed to p50 as in ALCL cells or if it is being degraded, a step that leads to activation of other signaling pathways in addition to releasing NF-κB subunits for nuclear translocation (33, 34). Similar exposure of ALCL and HL cells to membrane-bound CD30L resulted in activation of the alternative NF-κB pathway, as seen by the loss of p100 and the accumulation of the active p52 subunit (Fig. 3C). It has been reported recently that CD30 stimulation, either by overexpressing CD30 or using agonistic antibodies, results in p100 processing (26, 35). The findings shown here clearly indicate that p100 processing occurs following stimulation of CD30 with its physiological ligand. Furthermore, in L428 cells that had been exposed to CD30L, p100 is processed to p52 at a higher rate than the constitutive processing already present.

To further explore the ramifications of CD30-mediated cytoplasmic processing of NF-κB, as seen in Fig. 3, the nuclear extracts of Karpas 299 cells were examined for the presence of NF-κB subunits. To measure the nuclear translocation of NF-κB subunits in control or CD30-stimulated Karpas 299 cells, an electrophoretic mobility shift assay (EMSA) was employed. Nuclear extracts isolated from Karpas 299 cells following a 3-h physiological CD30 stimulation showed a dramatic increase in the nuclear content of NF-κB as measured by the increased presence of shifted 32P-radiolabeled probe (Fig. 4A, compare lanes 1 and 2). Furthermore, when nuclear extracts prepared from similarly stimulated Karpas 299 cells were preincubated with antibodies against the various NF-κB subunits before introduction of the 32P-radiolabeled probe, it was evident by a supershifted band in the EMSA that the NF-κB
subunits RelA, p50, RelB, and p52 were all present and bound to the $^{32}$P-radiolabeled probe (Fig. 4A, lanes 3–6 and 8–11). As a control, the $^{32}$P-radiolabeled probe was incubated in the absence of nuclear extract and resulted in no shift on the EMSA (data not shown).

**CD30L Versus TNF Activation of NF-κB-responsive Genes**—As a comparison with the dramatic increase of nuclear NF-κB following CD30L exposure, Karpas 299 cells were treated with TNF, a known NF-κB-activating cytokine. Interestingly, nuclear extracts from TNF-treated Karpas 299 cells contained only nuclear RelA and p50, as indicated by the supershift of the entire NF-κB-bound probe complex when the nuclear extract was preincubated with anti-p50 (Fig. 4B, lane 6). This observation is in contrast to anti-p50 supershifted nuclear extracts isolated from Karpas 299 cells following a 3-h CD30 stimulation, where a portion of the NF-κB-probe complex remains unshifted, suggesting that TNF treatment activates the canonical NF-κB pathway but not the alternative pathway in ALCL cells (Fig. 4B, lane 8). This is further supported by the fact that there is no p52 present in nuclear extracts of TNF-treated Karpas 299 cells (Fig. 4B, compare lanes 10 and 12).

The EMSA profile of NF-κB nuclear translocation following TNF treatment as opposed to CD30L exposure of Karpas 299 cells suggested that NF-κB-responsive genes are regulated differently. To test this possibility, we first performed a low density microarray (Superarray) to identify NF-κB-responsive genes that were up-regulated in Karpas 299 cells following CD30 stimulation with CD30L (data not shown).
Nuclear extracts were then prepared and tested for the presence of NF-κB, as a strong signal for NF-κB binding activity to a 32P-radiolabeled probe, whether the extracts were isolated from control or CD30-stimulated cells, as determined by EMSA (data not shown). However, physiological CD30 stimulation with TNF, resulted in modest transcriptional up-regulation of some of the NF-κB-responsive genes tested, above the constitutive level seen in nonstimulated cells (Fig. 4D and supplemental Fig. 1B). These results suggest that CD30 signaling is responsive in HL cells, although not to the extent seen in ALCL cells. Thus, physiological CD30 stimulation activates both the canonical and alternative NF-κB pathways in ALCL cells and slightly enhances the constitutive activity of NF-κB in HL cells.

**Sustained CD30-mediated Activation of the Canonical, but Not the Alternative, NF-κB Pathway Results in Cell Cycle Arrest in ALCL Cells**—Our observation of increased transcription from the cyclin D2 locus following CD30 stimulation (Fig. 4C) suggested a cell cycle-activating signal in ALCL cells. The cyclin D proteins in particular drive the cell through the restriction point of the G1 phase (reviewed by Harper and Brooks (36)). Cyclin D proteins accomplish this by binding to CDK4, which results in a conformational change in CDK4, allowing it to become phosphorylated and activated. Because of their critical cell cycle-regulatory role, up-regulation of cyclin D proteins has been reported to play a role in tumorigenesis (37, 38). Therefore, we extended the exposure of Karpas 299 and L428 cells to the CD30L+ CHO cells for up to 36 h to test whether a CD30 response resulted in cell cycle progression changes, as suggested by the increase in cyclin D2 transcripts. Karpas 299 or L428 cells were incubated with control CHO cells or CD30L+ CHO cells for 6, 12, 24, or 36 h. At each time point, RNA was isolated from the cells and analyzed by real time reverse transcription-PCR. The 6 and 24 h time points showed that transcription of the cyclin D2 gene continued to increase over time, whereas other NF-κB-responsive genes returned to basal levels by the 24 h time point (Fig. 5A), suggesting that stimulation of CD30 with its physiological ligand might promote cell cycle progression. To test this possibility, cell cycle states during a prolonged CD30 stimulation were determined by DNA quantitation using flow cytometry.

Prolonged CD30 stimulation was found to promote a G1 and G2 cell cycle arrest, with almost complete reduction of the S phase, in Karpas 299 cells but not L428 cells (Fig. 5, B and C). The cell cycle arrest observed following prolonged CD30 stimulation was unexpected, and contrary to the predicted effect of...
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A

Fold Activation Over Control

Cyclin D2  IL-8

+ CD30L 8hr  + CD30L 24hr

Cyclin D1  TNF  RelB  p100  Bcl-xL

+ CD30L 8hr  + CD30L 24hr

B

Karpas 299 (ALCL)  L428 (HL)

6 Hour Incubation

HG1: 53%  S: 32%
HG2: 15%  Pl →

HG1: 49%  S: 34%
HG2: 17%  Pl →

HG1: 46%  S: 32%
HG2: 22%  Pl →

HG1: 44%  S: 32%
HG2: 24%  Pl →

24 Hour Incubation

HG1: 47%  S: 39%
HG2: 14%  Pl →

HG1: 66%  S: 32%
HG2: 21%  Pl →

HG1: 45%  S: 32%
HG2: 23%  Pl →

HG1: 44%  S: 32%
HG2: 21%  Pl →

Control  + CD30L

C

Karpas 299 Control  Karpas 299 CD30L

Percent Cells / Cell-Cycle Phase

G1  G2

Time (hrs)

6 12 18 24 30 36

L428 Control  L428 CD30L

Percent Cells / Cell-Cycle Phase

G1  G2

Time (hrs)

6 12 18 24 30 36
the dramatic increase in cyclin D2 transcription. These results suggested that prolonged CD30 signaling increases the expression of one or more cyclin-dependent kinase inhibitors (CDKIs). In order to determine if this was indeed the case, we analyzed the expression levels of various CDKIs using real time reverse transcription-PCR. This led to the identification of an increase in transcript levels of the CDKI p21<sup>WAF1</sup> but not in the other CDKIs tested (Fig. 6A). As shown, the detected increase in p21<sup>WAF1</sup> transcription was associated with dramatic increases in protein levels. This result supports those obtained by agonistic antibody stimulation of CD30 on ALCL cells (39). Furthermore, we found that although cyclin D2 transcript levels increase significantly in response to physiological CD30 stimulation, the protein level was only increased by ~2-fold (data not shown). We also found that CDK4 levels are not up-regulated upon CD30 stimulation (supplemental Fig. 2), suggesting that the dramatic up-regulation of p21<sup>WAF1</sup> from low basal levels is sufficient to neutralize any cyclin D2-CDK4 complexes present, resulting in cell cycle arrest.

It has recently been reported that loss of the alternative NF-κB subunit p100/p52 resulted in a dramatic increase in p21<sup>WAF1</sup> transcripts and protein levels, whereas overexpression of p52 or p100 resulted in repression or activation of the p21<sup>WAF1</sup> promoter, respectively (19). Since CD30 stimulation modulates the alternative NF-κB pathway, we wanted to test whether p100/p52 had a role in CD30-mediated p21<sup>WAF1</sup> expression. To address this question, p100/p52 protein levels were suppressed through the introduction of siRNAs targeted against p100 (siP100) or a control protein (sicontrol) into Karpas 299 cells. Following suppression of p100/p52, the Karpas 299 cells were exposed to either control CHO cells or CD30L<sup>+</sup> CHO cells for 24 h of continuous CD30 stimulation. As shown in Fig. 6, B and C, although p100/p52 was sufficiently suppressed, p21<sup>WAF1</sup> levels only increased by ~2-fold in the control-stimulated Karpas 299 cells. Furthermore, CD30-mediated p21<sup>WAF1</sup> expression was unaffected in p100/p52-suppressed Karpas 299 cells (Fig. 6, B and C). Interestingly, in Karpas 299 cells lacking p100/p52, there was a profound G<sub>1</sub> arrest (Fig. 6D, left), which is independent of p21 levels. Furthermore, these conditions resulted in a marked potentiation of apoptotic cell death, most noticeably after 24 h of incubation with CD30L<sup>+</sup> CHO cells (Fig. 6D, right) (data not shown), suggesting that p100/p52 specifically mediates a cellular prosurvival signal. Therefore, p100/p52 does not significantly affect p21<sup>WAF1</sup> levels in Karpas 299 cells, and a loss of p100/p52 results in a G<sub>1</sub> arrest, which is independent of CD30 or p21<sup>WAF1</sup> expression and synergizes CD30-induced apoptosis. This led us to test the role of the canonical NF-κB pathway in CD30-mediated cell cycle arrest using a similar approach but with siRNA targeting RelA.

RelA suppression in Karpas 299 cells had no effect on p21<sup>WAF1</sup> expression at basal status (Fig. 6, B and C). Nor did RelA suppression affect the cell cycle in control-stimulated Karpas 299 cells (Fig. 6D). However, following 24 h of CD30 stimulation, suppression of RelA blocked CD30-mediated p21<sup>WAF1</sup> expression and cell cycle arrest. These results show that the canonical NF-κB pathway mediates the induced expression of p21<sup>WAF1</sup> and the subsequent cell cycle arrest brought about by prolonged physiological CD30 stimulation.

**DISCUSSION**

In this study we show, through the use of an in vitro CD30L system, that CD30 stimulation on an NPM-ALK-containing ALCL cell line resulted in activation of both the canonical and alternative NF-κB pathways, leading to NF-κB transactivation of a number of genes. Initially, stimulation of CD30 promoted cell death in a fraction of these cells and eventually, with prolonged CD30 stimulation, resulted in a G<sub>1</sub> and G<sub>2</sub> cell cycle arrest.

An explanation for the observed apoptosis in a portion of ALCL cells following CD30 stimulation may reside in the intracellular concentration and location of the TRAF proteins, in particular TRAF2. We have previously shown that TRAF2 degradation upon CD30 stimulation sensitizes cells to apoptosis (28). In addition to this model, we show in this report that not all of the TRAF2 protein is immediately degraded, but some is directed to a detergent-insoluble cellular compartment, which has been associated with NF-κB activation in other situations (29). Thus, the observations reported herein fit our model in which the initial loss of TRAF2 following a CD30 signal may lead to cell death, while the translocation of TRAF2 coincides with the activation of NF-κB that may then “protect” the remaining cells from death. This hypothesis is further supported by the observations that suppression of NF-κB subunits synergizes CD30-induced cell death (Fig. 6).

The NF-κB activation that we observe following a physiological CD30 stimulus includes post-translational processing/degradation of p105 in ALCL and HL cells. This finding is significant on two fronts. First, p105 processing to p50 is CD30-dependent, since there was an accumulation of p50 and a loss of p105 following CD30 stimulation. Increasing the cellular pool of p50 and removing the inhibitory moiety in the C terminus of p105 may contribute to the activation of NF-κB-responsive genes. Second, the removal of p105 is necessary for the activation of the extracellular signal-regulated kinase signaling pathway (33, 34). CD30 stimulation has been reported to increase extracellular signal-regulated kinase activity in both ALCL and HL cells (40–42), suggesting that the loss of p105 that we observe may be the mechanism for CD30-mediated extracellular signal-regulated kinase activation.

We also observed the activation of the alternative pathway, involving processing of p100 to the active p52 subunit. Further-
more, we find sustained increases in cyclin D2 expression over time. It has been reported that the p52 subunit regulates expression from the cyclin D1 locus (43), and it may prove enlightening to ascertain whether up-regulation of cyclin D2 is also mediated by CD30-dependent activation of p52. Interestingly, in L428 cells (an HL cell line), physiological CD30 stimu-
lation resulted in further p100 processing, above the constitutive processing already present. The C-terminal portion of p100 contains ankyrin repeats and functions as an inhibitor of κB (IκB) moieties to inhibit the nuclear translocation of NF-κB subunits, including p52. Since L428 cells harbor a loss of function mutation in IκBα that contributes to elevated basal activity of the canonical NF-κB pathway, p100 may function as an IκB in these cells. Thus, the modest transactivation of NF-κB-responsive genes following CD30 stimulation, above the constitutive activity already present, could be explained by the CD30-mediated processing of p100 in L428 cells. In support of this, transcription of cyclin D1, a known p52 target, was induced (Fig. 4B).

CD30 stimulation on ALCL cells resulted in dramatic upregulation of NF-κB-responsive genes with an observed increase in cyclin D2 transcription and protein levels, suggesting a prosurvival/proliferative response. A CD30-mediated proliferative response is further supported by previous observations showing that the Rb protein is phosphorylated following CD30 stimulation (25, 39). Thus, the resulting cell cycle arrest of ALCL cells with prolonged physiological CD30 stimulation is in direct conflict with the indicated evidence of a CD30-mediated proliferative response. Surprisingly, the observed cell cycle arrest in ALCL cells was a direct consequence of sustained CD30-mediated RelA activation, since siRNA-mediated suppression of RelA, but not p100/p52, was found to reverse expression of the CDKI p21^{waf1}. Although p100/p52 has recently been shown to control expression of p21^{waf1} in osteosarcoma cells (19), in ALCL cells the canonical, p100/p52-independent pathway was found to play the predominant role. One explanation is that the reported modulation of the p21^{waf1} promoter by p100/p52 requires basal levels of p53 (19), and p53 in Karpas 299 cells harbors a point mutation (39), which may affect how p100/p52 regulates p21^{waf1} in ALCL cells.

Through investigation of CD30 signaling in ALCL and HL cells, these studies suggest that regardless of the presence of NPM-ALK in ALCL cells, both the canonical and alternative NF-κB pathways are activated by CD30 and that the canonical pathway plays the primary role in the induction of CD30-mediated cell cycle arrest. These findings raise the intriguing possibility that the combined use of CD30 agonists and NF-κB inhibitors may have synergistic therapeutic potential for CD30-positive lymphomas.

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