Tumorigenesis and Neoplastic Progression

CD30-Induced Signaling Is Absent in Hodgkin's Cells but Present in Anaplastic Large Cell Lymphoma Cells

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High CD30 expression in classical Hodgkin's lymphoma and anaplastic large cell lymphoma (ALCL) suggests an important pathogenic role of this cytokine receptor. To test this hypothesis, we investigated CD30 signaling in Hodgkin's and ALCL cell lines by different approaches: 1) CD30 stimulation, 2) CD30 down-regulation, and 3) a combination of both. The effects were determined at the RNA (microarray and real-time quantitative RT-PCR), protein (electrophoretic mobility shift analysis, immunoblot, and flow cytometry), and cellular/functional (proliferation and apoptosis) levels. We demonstrate that Hodgkin's cells are virtually CD30 unresponsive. Neither CD30 stimulation nor CD30 silencing of Hodgkin's cells had any significant effect. In contrast, CD30 stimulation of ALCL cells activated nuclear transcription factor- κ B (NF- κ B), induced major transcriptional changes, and decreased proliferation. These effects could be abrogated by down-regulation of CD30. Stimulation of CD30 in ALCL cells, stably transfected with a dominant-negative NF- κ B inhibitor, induced pronounced caspase activation and massive apoptosis. Our data indicate that 1) CD30 signaling is not effective in Hodgkin's cell lines but is effective in ALCL cell lines, 2) CD30 is probably not significantly involved in the pathogenesis of classical Hodgkin's lymphoma, and 3) CD30 stimulation triggers two competing effects in ALCL cells, namely activation of caspases and NF- κ B-mediated survival. These data suggest that CD30-targeted therapy in ALCL should be combined with NF- κ B inhibitors to induce effective

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CD30, a member of the tumor necrosis factor (TNF) receptor superfamily, is predominantly expressed by the tumor cells (Hodgkin's and Reed-Sternberg cells) of classical Hodgkin's lymphoma (cHL) and those of anaplastic large cell lymphoma (ALCL). In normal tissue, CD30 expression is restricted to activated T and activated B cells.¹ The consistent CD30 expression of Hodgkin's and ALCL tumor cells implies an important role in the pathogenesis of both lymphoma entities, hence it is proposed to be used as a therapeutic target.² Experiments designed to elucidate the functional impact of CD30 signaling revealed contradictory results ranging from induction of proliferation³ over cell cycle arrest^{4,5} to apoptosis.^{6–8}

CD30 signaling is transmitted by TNF receptor-associated factors (TRAFs),⁹ which are attached to the cytoplasmic tail of CD30 after receptor stimulation. In analogy to other TNF receptors, it is suggested that CD30 stimulation leads to an activation of I κ B kinases and nuclear transcription factor- κ B (NF- κ B)-inducing kinase (NIK), which phosphorylate I κ Bs and thereby activate NF- κ B.¹⁰ However, I κ B kinase and NF- κ B-inducing kinase activation is not unequivocally demonstrated for CD30 stimulation.

Two major signaling pathways lead to NF- κ B activation: the "canonical" NF- κ B1 pathway, which requires proteasomal I κ B α degradation, thereby releasing the NF- κ B subunits p50 and p65, and the "noncanonical" NF- κ B2 pathway, whereby p100 is processed to p52 and which is most commonly associated with RelB to activate gene transcription.¹¹ High constitutive NF- κ B activation is

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a characteristic feature of Hodgkin's cells,^{12,13} which is reflected by the expression of several NF- κ B-regulated targets, eg, A20, cellular inhibitor of apoptosis protein 2 (cIAP2), cellular FLICE inhibitor protein (c-FLIP), and TRAF1.^{14–16} Hodgkin's cell lines have been reported to be incapable to activate NF- κ B in response to CD40 ligation¹⁷ and demonstrated not to be susceptible to CD30-induced apoptosis,⁶ presumably because of constitutive NF- κ B activity.

Here, we report that B cell-derived Hodgkin's cells, which account for more than 98% of the cHL cases,¹⁸ are virtually CD30 unresponsive. Furthermore, down-regulation of CD30 influenced neither constitutive gene expression nor proliferation of CD30-stimulated or untreated Hodgkin's cell lines. In contrast, CD30 stimulation of ALCL cells activated the canonical and noncanonical NF- κ B pathway, induced major transcriptional changes, and decreased proliferation, which could be inhibited by CD30 silencing. However, CD30 stimulation of ALCL cells stably transfected with a dominant-negative NF- κ B inhibitor ($I\kappa$ B $\alpha\Delta$ N) induced caspase-3, caspase-8, caspase-9, and BH3 domain containing Bcl2 family member (BID) activation with the result of massive apoptosis.

Materials and Methods

Cell Lines and CD30 Stimulation

Because Hodgkin's and Reed-Sternberg cells derive from B cells,¹⁹ we used B cell-derived Hodgkin's cell lines (L1236, L428, KM-H2, and L591) for our experiments. The ALCL cell lines Karpas 299 and SU-DHL1 were obtained from the German Resource Centre for Biological Material (DSMZ, Braunschweig, Germany). The ALCL cell lines JB6 and FE-PD were generous gifts from Dr. M.E. Kadin (Harvard Medical School, Boston, MA) and Dr. K. Pulford (John Radcliff Hospital, University of Oxford, Oxford, UK), respectively. The cell lines Karpas 299, JB6, and SU-DHL1 are t(2;5) positive. Cells were maintained in RPMI 1640 (Gibco-BRL, Eggenstein, Germany), 10% (v/v) heat-inactivated fetal calf serum and 4 mmol/L glutamine at 5% CO₂, 37°C. Exponentially growing cells (1.5×10^5 /ml) were treated with CD30 ligand (100 ng/ml; R&D Systems, Wiesbaden, Germany), CD30 agonistic murine monoclonal antibody (mAb) Ki-1 (500 ng/ml; IgG₃ subclone produced in our institute), or isotype-specific control mAb (IgG₃; 500 ng/ml; FLOPC 21; Sigma, Taufkirchen, Germany) or without Ab for 2, 4, 6, 16, 48, and 72 hours. The recombinant CD30 ligand and soluble CD30 mAb (Ki-1) induced indistinguishable effects, as revealed by real-time quantitative RT-PCR (RT-RQ-PCR) analysis of CD30-stimulated ALCL cell lines (data not shown). Therefore, we performed CD30 stimulation experiments of Hodgkin's and ALCL cell lines by the use of soluble Ki-1 and used nonbinding isotype control IgG₃ for control treatments, herein after referred to as nonstimulated.

Because CD30 ligand and Ki-1 mAb induced identical effects (data not shown), we performed CD30 stimulation with Ki-1 mAb, and control treatment with IgG₃, mAb binding was verified by flow cytometric analysis. All cell

lines express wild-type CD30 (B. Hirsch and H. Dürkop, unpublished data).²⁰ The pan-caspase inhibitor Z-VAD-FMK (Calbiochem, Schwalbach, Germany) was used at 50 μ mol/L.

Generation of $I\kappa$ B $\alpha\Delta$ N Transfectants

Hodgkin's and ALCL cell lines were transfected by the nucleofector technology (AMAXA, Cologne, Germany) with the solutions and programs indicated in brackets: L1236 (V, T-01), L428 (L, X-01), KM-H2 (T, T-01), and Karpas 299 (T, V-01). Transfection conditions were optimized using a plasmid coding for green fluorescent protein (pMaxGFP; AMAXA) by flow cytometric analysis. Selection during subcloning was performed by 1.2 mg/ml G418 (Calbiochem). The expression plasmid $I\kappa$ B $\alpha\Delta$ N-pcDNA3 was kindly provided by Dr. Stephan Mathas (Charité, Berlin, Germany).

Cell Proliferation Assay

Cell proliferation was determined by the cell proliferation WST-1 reagent (Roche, Mannheim, Germany) and trypan blue dye exclusion test.

Electrophoretic Mobility Shift Analysis

Nuclear extracts of CD30-stimulated and nonstimulated cells were prepared as published previously.²¹ For binding assays, 5 μ g of nuclear extracts was incubated and processed with 8 fmol of digoxigenin-labeled NF- κ B probe: 5'-GATGGATGAGTTGAGGGGACTTTCCCTCTT-TACTG-3' according to manufacturer's instructions (Roche). To ensure specificity, a mutated NF- κ B probe (5'-GATGGATGAGTTGAGGCGACTTTCCCTCTTTACTG-3'; mutation is underlined) was used. Labeled NF- κ B oligonucleotide was added to each sample. Supershift analyses were performed with 0.4 μ g antisera of p50 (H119), p65 (F-6), c-Rel (B-6), p52 (C-19), and RelB (C-19) (Santa Cruz Biotechnology, Heidelberg, Germany).

Immunoblot Analysis and Immunocytochemistry

Cell extracts (20 μ g) were fractionated on 10% (w/v) SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Amersham, Braunschweig, Germany). Immunoblot analysis was performed by ALK1 (DakoCytomation, Hamburg, Germany), CD30 (BerH2; produced in our institute), manganese super oxide dismutase (MnSOD; Upstate Biotechnology, Lake Placid, NY), activating transcription factor 3 (ATF3) (c-19) and caspase-9 (Ab-2) (Calbiochem), $I\kappa$ B α (C-21) and p52 (C-5) (Santa Cruz Biotechnology), BID (no. 2002), caspase-3 (Asp132), caspase-8 (Asp384), and cleaved poly(ADP-ribose) polymerase (PARP) (Cell Signaling, Hamburg, Germany), β -actin (Abcam, Cambridge, UK), and horseradish peroxidase-conjugated secondary antibodies were detected by chemiluminescence (Amersham). Immunocytochemistry was performed as published previously.²²

Cytokine Analysis

Cells were treated as described above, and cytokine protein concentration of cell culture supernatants was determined by the Bio-Plex-Protein System (Bio-Rad, Munich, Germany).

Flow Cytometric Analysis

Cells were treated as described and stained by murine mAbs: CXCR4 (phycoerythrin-conjugated; R&D Systems), CD95/FAS and CD30 [fluorescein isothiocyanate (FITC)-conjugated BerH2; DakoCytomation], prostaglandin E receptor 4 (PTGER4) (Abcam), and OX40 (FITC-conjugated Ber-Act 35; generated in our institute). The polyclonal Ab CD137/4-1 BB (N-16; Santa Cruz Biotechnology) was detected by anti-goat-biotin and phycoerythrin-conjugated Streptavidin (DakoCytomation). Staining of dead/apoptotic cells was performed using the Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences Pharmingen, Wiesbaden-Nordenstadt, Germany) according to manufacturer's instructions. Labeled cells were analyzed by a FACSsort (Becton Dickinson, Heidelberg, Germany).

CD30 RNA Interference Experiments

Karpas 299 and L428 were transfected twice on subsequent days with 5 μ g of control or CD30 small-interfering RNA (siRNA) nos. 1 and 2 (Qiagen, Hilden, Germany) by nucleofection as stated above with the CD30 target sequence no. 1 (5'-ACCAATAACAAGATTGAGAAA-3') and no. 2 (5'-ATGCAAATGAGTGATGGATAA-3'). Both CD30 siRNAs were mixed 1:1 before transfection. CD30 expression was monitored by flow-cytometric and immunoblot analysis. Nucleophosmin-anaplastic lymphoma kinase (NPM-ALK) expression was analyzed by immunoblotting. siRNA-transfected cells were CD30-stimulated or IgG₃-control-treated for 16 hours. Protein and RNA analyses were performed as described above.

RNA Extraction and RT-RQ-PCR

RT-RQ-PCR was performed as published previously.¹⁵ The following pairs of oligonucleotides (forward, reverse) were used: ATF3, 5'-ACAAAATCCATGGGCAGCAT-3' and 5'-TGCTTCTTCTCTGTGACTTTGG-3'; A20, 5'-AGCTTCTCTCATGGATGTAAATGTG3' and 5'-GCATAAAGGCTGGGTGTTTAC-3'; cIAP2, 5'-GGACTCAGGTGTTGGGAATCTG-3' and 5'-CCTTTAATTCTTATCAAGTACTCACACCTT-3'; c-FLIP, 5'-CAGAGATTGGTGAGGATTTGGAT-3' and 5'-TGCCTCGGCCCATGTAA-3'; CXCR4, 5'-TTCCCTTCTGGGCAGTTGAT-3' and 5'-ACATGGACTGCCTTGCATAGG-3'; RGS1, 5'-AAGGATGTACTTTCTGCTGCTGAA-3' and 5'-CCAGTTTGGTTGGCAAGAAGTT-3'; RAD1, 5'-TTCGAGAAGGCCTCAGAACTG-3' and 5'-TGGGCACATCATCTGTTTGC-3'; PTGER4, 5'-ACGCCGCCTACTCCTACATG-3' and 5'-AGAGGACGGTGGCGAGAAT-3'; IEX-1, 5'-AACCGAACCCAGCCAAAAG-3' and 5'-ACCCTCTCAGCCATCA-

GGAT-3'; and hypoxanthine ribosyl transferase (HPRT), 5'-GAGTCCTATTGACATCGCCAGTA-3' and 5'-CTAAGCAGATGGCCACAGAACTAG-3'. HPRT was used as endogenous control and amplified in parallel. HPRT-normalized gene expression was calculated by the $2^{-\Delta\Delta CT}$ method.²³

Oligonucleotide Microarray Analysis

Cells were stimulated as described above. RNA was extracted as described previously.¹⁵ Total RNA (5 μ g) was hybridized using the Affymetrix GeneChip (U133A). The Expression Analysis System (Affymetrix, Santa Clara, CA) and a LASER confocal scanner (Agilent Technologies, Waldbronn, Germany) were used according to the manufacturers' instructions.

Statistical Analysis

Affymetrix probe intensities were normalized using a variance stabilization method.²⁴ Gene expression level were estimated by fitting an additive model using a median polish routine.²⁵ Differences in the regulation of individual genes between CD30-stimulated and nonstimulated cells were quantified by computing Euclidean distances between expression profiles of each gene in the two conditions across the three time points (4, 16, and 72 hours). For each gene, we computed the ratio of dissimilarity measures from both cell lines. It is expected to be high for genes induced/repressed in ALCL cells (numerator) but not in Hodgkin's cells (denominator). This "differential induction score" was used for ranking genes according to ALCL-specific effects of CD30 stimulation. Adding a positive constant to the denominators regularized the ratios for artificially low denominators, a procedure analogous to the fudge factor regularization of t-scores.²⁶ As fudge factor, we chose the mean of Euclidean distances. The top 250 probe sets were selected for analysis. Genes represented several times by multiple probe sets were unified according to Entrez Gene identifiers (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?DB=gene>). We searched this list for overrepresentation of GO categories using Fisher's exact test.

Results

Different Effects of CD30 Stimulation in Hodgkin's and ALCL Cells

We studied the impact of CD30 stimulation on the NF- κ B system in Hodgkin's and ALCL cell lines by analyzing proteasomal degradation of I κ B α (canonical NF- κ B activation) and processing of p100 to p52 (noncanonical NF- κ B activation) using whole-cell lysates for Western blotting. No CD30-mediated degradation of I κ B α was observed in the Hodgkin's cell lines L1236 and L591, which carry a functional I κ B α molecule (Figure 1A). The other two Hodgkin's cell lines, L428 and KM-H2, are not suitable for this analysis because their I κ B α genes carry

Figure 1. CD30 stimulation does not induce canonical and noncanonical NF- κ B activation in Hodgkin's cells but in ALCL cells. **A:** Immunoblot analysis of I κ B α after 30 minutes of CD30-stimulated or nonstimulated Hodgkin's cells (L1236, L428, KM-H2, and L591) and ALCL cells (Karpas 299, JB6, SU-DHL1, and FE-PD). The decrease of I κ B α expression (37 kDa) represents canonical NF- κ B pathway activation. **B:** Immunoblot analysis of p100 and p52 in cells after 16-hour CD30 stimulation and control treatment. The appearance of p52 denotes noncanonical NF- κ B pathway activation, ie, processing of p100 to p52. Unspecific signal is indicated. β -actin served as loading control. Experiments were repeated three times; representative results are shown.

noncoding mutations or deletions.²⁷ In contrast to Hodgkin's cells, I κ B α degradation was induced in all four ALCL cell lines investigated (Karpas 299, JB6, SU-DHL1, and FE-PD) by CD30 stimulation. CD30-induced processing of p100 to p52 was also demonstrable in all ALCL cell lines but not in the Hodgkin's cell lines (Figure 1, A and B). I κ B α degradation was analyzed after 30 minutes of CD30 stimulation whereas processing of p100 proved to be a slower process as shown by the appearance of p52 in cell lysates of 16-hour CD30-stimulated ALCL cells (Figure 1B).

Consistent with these results, electrophoretic mobility shift analysis/supershift assays did not reveal CD30-induced DNA binding of p50, p65 (canonical), or p52 (noncanonical NF- κ B pathway) in nuclear extracts of Hodgkin's cells, whereas CD30-mediated DNA binding of p50, p65, and p52 could be demonstrated in ALCL cells (Supplemental Figure S1, see <http://ajp.amjpathol.org>).

Gene Expression Analysis of CD30-Stimulated Hodgkin's and ALCL Cells

To elucidate global CD30-induced effects, we performed genome-wide gene expression profiling of CD30-stimulated and nonstimulated Hodgkin's cells (L1236) and ALCL cells (Karpas 299). For both conditions, we analyzed three time points (4, 16, and 72 hours) and computed a dissimilarity score between time series of each gene. Large dissimilarity values were considered to reflect CD30 effects. Global gene expression (22,283 probe sets) was analyzed by Wilcoxon rank sum test. There was nearly no effect in Hodgkin's cells, whereas a significant change of gene expression was induced by CD30 stimulation in ALCL cells ($P < 2.2 \times 10^{-16}$). Our complete list of CD30-regulated genes in ALCL cells can be retrieved from Supplemental Table S1 (available at <http://ajp.amjpathol.org>).

To analyze further the specific response to CD30 stimulation in ALCL cells, we developed a customized score, called "differential induction score" (for details, see Materials and Methods). Genes were ranked according to their level of CD30-dependent regulation by this score. The

top 250 probe sets of this list represent 208 genes and were considered for further exploration. One hundred, forty-nine genes from this list were induced by CD30 stimulation, whereas 59 genes were repressed (Figure 2). We categorized the 149 CD30-induced genes according to Gene Ontology (GO; <http://www.geneontology.org>) and applied Fisher's exact test, which revealed a highly significant enrichment of the following categories: 51 signal transducers

Figure 2. CD30 stimulation does not regulate gene expression in Hodgkin's cells, but it does in ALCL cells. Gene expression microarray analyses of CD30-stimulated and nonstimulated Hodgkin's cells (L1236) in comparison with ALCL cells (Karpas 299) at 4, 16, and 72 hours. The top 250 probe sets, ranked by the differential induction score, are shown. Rows represent probe sets; columns represent samples. Relative gene expression in comparison with an untreated control (medium) of Karpas 299 is displayed. CD30 stimulation strongly induced (top panel)/repressed (bottom panel) gene expression in ALCL but not in Hodgkin's cells.

Table 1. CD30 Target Gene Profiling in ALCL Cells

Diff. ind. score	Gene symbol	Probe set	Induced/repressed	NF-κB target	Diff. ind. score	Gene symbol	Probe set	Induced/repressed	NF-κB target
Receptors and ligands					Apoptosis regulation				
7.79	TNFRSF9	207536_s_at	▲	●	7.08	MnSOD	215223_s_at	▲	●
5.23	FAS	216252_x_at	▲	●	6.29	cIAP2	210538_s_at	▲	●
4.39	CHRNA1	206633_at	▲	●	6.04	FTH1	214211_at	▲	●
4.27	SLAMF1	206181_at	▲		4.38	TRAF1	205599_at	▲	●
3.39	CD5	206485_at	▲		4.19	GADD45α	203725_at	▲	●
3.35	CD59	200985_s_at	▲	●	4.79	A20	202644_s_at	▲	●
3.25	ADRB2	206170_at	▲		4.30	IER3/IEX-1	201631_s_at	▲	●
3.23	CXCR4	211919_s_at	▲	●	3.81	BCL2A1	205681_at	▲	●
3.21	TNFSF4	207426_s_at	▲		3.17	SPP1	209875_s_at	▲	
3.20	PLAUR	210845_s_at	▲	●	3.00	PDCD4	212593_s_at	▲	
3.19	CNR1	213436_at	▲		Cytokines				
3.06	EMR2	207610_s_at	▲		5.88	IL8	202859_x_at	▲	●
4.11	KLRC2	206785_s_at	▼		3.60	CCL20	205476_at	▲	●
Signaling molecules					Proteolysis				
5.16	RGS1	202988_s_at	▲		4.68	MMP12	204580_at	▲	●
3.61	RAB9A	221808_at	▲		3.30	ADAM8	205179_s_at	▲	
3.55	RGS2	202388_at	▲		3.17	CFB	202357_s_at	▲	
3.39	RRAD	204802_at	▲		3.85	GZMA	205488_at	▼	
3.37	TNIP1	207196_s_at	▲	●	Cell cycle-proliferation				
3.33	PBEF1	217738_at	▲		8.72	CCND2	200953_s_at	▲	●
3.32	SH3BP5	201811_x_at	▲		4.24	p21/WAF	202284_s_at	▲	●
3.29	SERPINB8	206034_at	▲		Cell adhesion				
3.07	LSP1	203523_at	▲		6.31	ICAM1	202637_s_at	▲	●
3.32	ANXA1	201012_at	▼		3.15	CD83	204440_at	▲	●
3.25	INPP4B	205376_at	▼		3.10	CD93	202878_s_at	▼	
3.11	TRAF3IP3	213888_s_at	▼		3.02	ITGA4	205885_s_at	▼	
Transcription factors					Miscellaneous				
4.74	HEY1	44783_s_at	▲		4.81	BTN2A2	205298_s_at	▲	
4.66	RELB	205205_at	▲	●	3.80	DRAM	218627_at	▲	
3.79	EPAS1	200878_at	▲		3.83	IBRDC3	36564_at	▲	
3.56	MAFF	36711_at	▲		3.79	EPAS1	200878_at	▲	
3.71	ATF3	202672_s_at	▲		3.71	SDC4	202071_at	▲	
3.52	IκB-α	201502_s_at	▲	●	3.69	TMOD1	203661_s_at	▲	
3.37	HIVEP1	204512_at	▲		3.63	UPK1B	210064_s_at	▲	
3.82	CENTD1	213618_at	▲		3.49	C1orf38	207571_x_at	▲	
3.51	ELL2	214446_at	▲		3.11	TRAF3IP3	213888_s_at	▼	
3.55	STAT5A	203010_at	▲	●	3.08	CA2	209301_at	▼	
3.37	NR3C2	205259_at	▼		3.08	COL4A2	211966_at	▼	
3.06	DMRT1	220493_at	▼		3.07	TMEM23	212989_at	▼	
Stress-acute phase response					3.05	AKAP2	202759_s_at	▼	
5.70	LBP	214461_at	▲	●					
3.65	SERPINA1	202833_s_at	▲	●					
3.65	S100A8	202917_s_at	▲						
3.52	HTRA2	203089_s_at	▲	●					
3.36	DUSP1	201041_s_at	▲						
4.28	AQP9	205568_at	▼	●					

Based on our microarray analysis, we configured CD30-induced (▲) and -repressed (▼) genes according to their biological/functional properties with a differential induction score (diff. ind. score) greater than 3. Quoted genes are CD30 regulated in ALCL (Karpas 299) but not in Hodgkin's cells (L1236). Targets that are reported to be NF-κB-regulated are indicated (●), referring to Refs. 10 and 29. The complete list of CD30-regulated genes in ALCL cells can be retrieved in Supplementary Table S1.

[(GO: 0004871, $P < 5 \times 10^{-8}$) eg, ATF3, STAT5A, p100, and p105], 38 immune response-related genes (GO: 0006955, $P < 2 \times 10^{-13}$), 23 apoptosis-related genes [(GO: 0006915, $P < 4 \times 10^{-7}$), eg, c-FLIP, IEX-1, and MnSOD], and 15 cytokines [(GO: 0005125, $P = 2 \times 10^{-8}$) eg, IL-8, TNF-α, and tumor necrosis factor-related apoptosis-inducing ligand]. Forty-one of the 208 top-ranking genes are NF-κB regulated. CD30-regulated genes with a differential induction score greater than 3 are listed in Table 1.

Validation of CD30 Response by RT-RQ-PCR

To validate our CD30 signaling microarray data, we performed further CD30 stimulation experiments with an expanded set of Hodgkin's cell lines (L1236, L428, KM-H2, and L591) and ALCL cell lines (Karpas 299, JB6, SU-DHL1, and FE-PD). RT-RQ-PCR was performed at 4 and 16 hours to evaluate differences of gene expression (Figure 3). For this purpose, we chose the following 10 CD30 target genes: *ATF3*, *A20*, *cIAP2*, *c-FLIP*, *CXCR4*, *RGS1*,

Figure 3. CD30 stimulation does not activate the expression of selected genes in Hodgkin's cells but induced major changes of gene expression in ALCL cells. Expression of 10 genes (ATF3, A20, cIAP2, c-FLIP, CXCR4, IEX-1, RAD1, RGS1, PTGER4, and HPRT (new CD30 targets underlined)) was analyzed by RT-RQ-PCR using 4- and 16-hour CD30-stimulated and nonstimulated Hodgkin's cells (L1236, L428, KM-H2, and L591) and ALCL cells (Karpas 299, JB6, SU-DHL1, and FE-PD). One of two experiments is shown. Relative, HPRT-normalized gene expression, measured in triplicates, is displayed.

RAD1, IEX-1, and PTGER4 (newly identified CD30 targets are underlined). The selected genes represent the major functional categories that are regulated by CD30 in ALCL, as deduced from our microarray data (Table 1). CD30 stimulation of Hodgkin's cells did not significantly

change mRNA expression of any of these genes. In contrast, CD30 stimulation of ALCL cells strongly induced the expression of all genes analyzed at 4 or 16 hours. The comparison of CD30-mediated effects in ALCL cell lines revealed that Karpas 299 cells showed the strongest CD30 response. The results confirmed the ineffectiveness of CD30 signaling in Hodgkin's cells and the capacity of ALCL cells to respond to CD30 stimulation with the up-regulation of specific target genes (Figure 3).

Effect of CD30 Stimulation on Protein Expression in Hodgkin's and ALCL Cells

To explore further the CD30-mediated changes in Hodgkin's and ALCL cells, we extended our investigations to the protein level using immunoblotting, flow cytometry, and the Bio-plex-Protein System. In line with our results at the RNA level (Table 1; Supplemental Table S1), analysis of Hodgkin's cells revealed that CD30 stimulation did not change protein expression of cytokine receptors (CXCR4, CD95/Fas, PTGER4, 4-1 BB, and OX40), cytokine secretion (IL-4, IL-8, and TNF- α), the apoptosis-related protein MnSOD, or the signal transducer ATF3. In contrast, all of the investigated proteins were up-regulated in Karpas 299 cells (ALCL) after CD30 stimulation (Figure 4, A–C). Even though a CD30-mediated induction of CXCR4, OX40, and 4-1BB expression was observed in JB6 cells, no change of CD95/FAS and PTGER4 expression occurred in this ALCL cell line.

Effect of CD30 Stimulation on the Proliferation of Hodgkin's and ALCL Cells

To determine the impact of CD30 stimulation on proliferation and apoptosis, we investigated CD30-stimulated

Figure 4. CD30 stimulation does not induce protein expression in Hodgkin's cells, but it does in ALCL cells. **A:** Flow cytometric analysis of CD30-induced CXCR4, OX40, CD95, CD137, and PTGER4 expression (novel CD30 targets underlined) in Hodgkin's cells (L1236 and L428) and ALCL cells (Karpas 299 and JB6). Data show the overlay of histograms, representing individual antibody-specific fluorescence of 16-hour CD30-stimulated (solid line/open area) and nonstimulated cells (dotted line/shaded area). One of five experiments is shown. **B:** Secretion of IL-4, IL-8, and TNF- α was determined in the supernatant of 8-, 16-, and 48-hour CD30-stimulated or nonstimulated L428 and Karpas 299 cells (measured in triplicate). SD is indicated. One of three experiments is shown. **C:** Immunoblot analysis of MnSOD, ATF3 using 16-hour CD30-stimulated and nonstimulated Hodgkin's cell lines (L1236 and L428) and ALCL cell lines (Karpas 299 and JB6). β -Actin served as loading control. Experiments were repeated three times; representative results are shown.

Figure 5. CD30 stimulation of Hodgkin's cells does not change proliferation but reduces proliferation of ALCL cells. Hodgkin's cells (L1236 and L428) and ALCL cells (Karpas 299 and JB6) were CD30-stimulated or nonstimulated for 0, 24, 48, and 72 hours, trypan blue stained, and counted. Counts of viable and dead cells are shown. Experiments were performed at least three times in triplicates, bars indicate SD.

and nonstimulated Hodgkin's cell lines (L1236 and L428) and ALCL cell lines (Karpas 299 and JB6) at three time points (24, 48, and 72 hours). CD30 stimulation had no effect on the proliferation rate of Hodgkin's cells but led to a significant reduction of proliferation of ALCL cells, which was most pronounced in Karpas 299 cells. Cell death was not induced by CD30 stimulation either in Hodgkin's or in ALCL cells as revealed by trypan blue dye exclusion test (Figure 5) and the Annexin V-FITC/propidium iodide assay (data not shown).

CD30 Silencing of Hodgkin's and ALCL Cells by RNAi

To test the concept of CD30 ligand-independent signaling in cHL,²⁸ we silenced CD30 expression in Hodgkin's cells (L428, KM-H2, and L1236) and ALCL cells (Karpas 299) by means of RNAi in two independent experiments. The application of a combination of two CD30 siRNA proved to be more effective compared with a single CD30 siRNA at a time. Down-regulation of CD30 protein expression as revealed by Western blotting and flow cytometric analysis (Figure 6, A and B) did not change the gene expression program of Hodgkin's or ALCL cells (Figure 6), indicating that there is no spontaneous or

constitutive CD30 ligand-independent signaling in these lymphoma categories. Concordant with our previous data, CD30 stimulation of CD30-silenced Hodgkin's cells did not alter their gene expression profile. In contrast, CD30 silencing of ALCL cells abrogated CD30 signaling (Figure 6).

We further analyzed the impact of CD30 expression on the proliferation of Hodgkin's cells (L428 and KM-H2) and ALCL cells (Karpas 299). Despite successful down-regulation of CD30 by CD30 siRNA transfection, no change of proliferation of Hodgkin's and ALCL cells was detected by the measurement of metabolic activity of viable cells (WST-1 reagent) after 24, 48, or 72 hours (Figure 7).

To investigate the influence of CD30 expression on the expression of NPM-ALK, we analyzed CD30-silenced NPM-ALK-positive ALCL cells (Karpas 299) by immunoblotting. There was no difference of NPM-ALK protein expression detectable in CD30-silenced and control-treated ALCL cells after 24 or 48 hours (Figure 6A). We performed three independent experiments and revealed identical results, demonstrating that NPM-ALK expression is not influenced by CD30 (Figure 6A).

The Combination of CD30 Stimulation and NF- κ B Inhibition Leads to Apoptosis in ALCL Cells

Hodgkin's cells (L1236, L428, and KM-H2) and ALCL cells (Karpas 299) were subjected to transfection of a dominant-negative inhibitor of NF- κ B ($\text{I}\kappa\text{B}\alpha\Delta\text{N}$). We did not succeed in generating stable $\text{I}\kappa\text{B}\alpha\Delta\text{N}$ transfectants of Hodgkin's cell lines, which might be explained by published data.¹² In contrast to Hodgkin's cells, ALCL cells (Karpas 299) could be successfully stably transfected with $\text{I}\kappa\text{B}\alpha\Delta\text{N}$ ($\text{I}\kappa\text{B}\alpha\Delta\text{N}$ Karpas 299) as demonstrated by expression of the truncated $\text{I}\kappa\text{B}\alpha$ protein (Figure 8, B and C). Spontaneous apoptosis of $\text{I}\kappa\text{B}\alpha\Delta\text{N}$ Karpas 299 cells was only marginally increased compared with mock-transfected Karpas 299 cells (8 versus 4% Annexin V-FITC/PI-positive cells, respectively). CD30 stimulation of $\text{I}\kappa\text{B}\alpha\Delta\text{N}$ Karpas 299 cells induced massive apoptosis when compared with nonstimulated $\text{I}\kappa\text{B}\alpha\Delta\text{N}$ Karpas 299 cells (60 versus 8%, respectively). This dramatic apoptosis-inducing effect could be abolished completely by a pan-caspase inhibitor (Z-VAD-FMK) (Figure 8A). Immunostaining of cytopins from 16-hour CD30-stimulated $\text{I}\kappa\text{B}\alpha\Delta\text{N}$ Karpas 299 cells revealed pronounced activation of caspase-3, whereas this effect was negligible in untreated Karpas 299 cells and nonstimulated cells. We further revealed CD30-mediated PARP cleavage after 16 hours and activation of caspase-8, caspase-9, and BID after 48 hours in $\text{I}\kappa\text{B}\alpha\Delta\text{N}$ Karpas 299 cells (Figure 8, B and C).

Discussion

Because the cytokine receptor CD30 is constitutively expressed in all tumor cells of cHL and ALCL, CD30 is

Figure 6. Down-regulation of CD30 does not change constitutive gene expression of Hodgkin's cells but inhibits CD30 signaling of ALCL cells. **A:** CD30 down-regulation of Hodgkin's cells (KM-H2, L1236, and L428) and ALCL cells (Karpas 299) by CD30 siRNA was verified by immunoblotting. NPM-ALK expression was analyzed in CD30 down-regulated and nonstimulated and untreated ALCL cells by immunoblotting. β -Actin served as loading control. **B:** Flow cytometric analysis of cells, analyzed in A. Data show the overlay of histograms, representing isotype IgG₃-control antibody-specific fluorescence (black dotted line), CD30 antibody-specific fluorescence of CD30 siRNA-transfected (red line), control siRNA-transfected (green line), and CD30 antibody-specific fluorescence of untreated cells (black line). One of two analyses is shown. **C:** Gene expression microarray analysis of CD30 siRNA- or control siRNA-transfected, 16-hour CD30-stimulated and nonstimulated Hodgkin's cells (KM-H2, L1236, and L428) and ALCL cells (Karpas 299). Aliquots of cells that were analyzed in A and B were used for subsequent CD30 stimulation experiments and gene expression profiling. We performed two identical experiments and analyzed the established "CD30 signaling gene set" (Figure 2). The average of fold-change (scale bar) is displayed. Rows denote probe sets. The top part depicts the 70 most CD30-induced probe sets in Karpas 299. The bottom part represents the 35 most CD30-repressed probe sets in Karpas 299. Each column represents one sample. Treatments are indicated.

suggested to be implicated in the pathogenesis of these lymphoma entities. However, unequivocal experimental proof for this assumption is still missing. To clarify the pathogenic role of CD30 in these lymphoma categories, we analyzed the effects of CD30 stimulation, CD30 silencing, and a combination of both in Hodgkin's and ALCL cells.

In this study, we showed that CD30 stimulation of B cell-derived Hodgkin's cells did not change global gene expression, constitutive NF- κ B activity, or proliferation (Figures 1, 2, and 5), which is in line with the reported failure of Hodgkin's cells to respond to CD40 ligation.¹⁷ In contrast, CD30 stimulation of ALCL cells induced pronounced changes of gene expression (Figure 2). Most of the 208 top-ranking genes (designated as "CD30 signaling gene set"), identified by our novel algorithm, represent new CD30 targets. GO analysis revealed that CD30 signaling of ALCL cells is involved in basic mechanisms of cell function, reflected by the significant induction of signal transducers, cytokines, and immune response/apoptosis-related genes. Forty-

one of the 208 top-ranking genes are reported to be NF- κ B-regulated,^{10,29} a finding that is in accordance with the activation of the canonical and noncanonical NF- κ B pathway as demonstrated by our electrophoretic mobility shift analysis (Supplemental Figure S1) and recently published data.³⁰ We confirmed CD30 unresponsiveness of B cell-derived Hodgkin's cells and high CD30 responsiveness of ALCL cells by RT-RQ-PCR analysis for nine genes in independent experiments using four Hodgkin's and four ALCL cell lines. The CD30 response of Karpas 299 cells was the highest when compared with the other ALCL cell lines analyzed (Figure 3).

The category of apoptosis-related CD30 target genes constitutes the most remarkable group. Although the apoptosis-triggering ligands tumor necrosis factor-related apoptosis-inducing ligand and TNF- α are highly expressed in Hodgkin's cells and CD30-stimulated ALCL cells (Figure 4B; Supplemental Table S1), the cells are protected from apoptosis. This is most likely due to the CD30-induced expression of the caspase-8 antagonist

Figure 7. CD30 down-regulation does not influence proliferation of Hodgkin's or ALCL cells. CD30 expression of Hodgkin's cells (L428, KM-H2, and L1236) and ALCL cells (Karpas 299) was down-regulated by CD30 siRNA. Proliferation of control siRNA (black line) and CD30 siRNA-treated cells (gray line) was measured by WST-1 assay after 24, 48, and 72 hours. Experiments were performed at least three times in triplicates; bars indicate SD.

c-FLIP (Figure 3),¹⁶ the caspase inhibitor cIAP2 (Figure 3),¹⁵ and the antioxidant enzyme MnSOD (Figure 4C),³¹ the latter of which represents a newly identified CD30 target gene. These data indicate that CD30 is able to simultaneously trigger apoptosis-inducing (eg, via tumor necrosis factor-related apoptosis-inducing ligand and TNF- α) and apoptosis-protecting mechanisms in ALCL cells.

To further resolve this paradox, we generated Karpas 299 cells (ALCL) in which NF- κ B was blocked by I κ B α Δ N, a dominant-negative NF- κ B inhibitor. Despite almost complete extinction of wild-type I κ B α , spontaneous apoptosis was increased only marginally compared with untransfected or mock-transfected Karpas 299 cells. This changed dramatically when I κ B α Δ N Karpas 299 cells were CD30-stimulated: Activation of caspase-8, caspase-3, caspase-9, Bid, and PARP cleavage resulted in massive apoptosis. This effect could be abolished completely by a pan-caspase inhibitor (Figure 8), confirming that CD30-mediated activation of caspases was responsible for the induction of massive apoptosis in I κ B α Δ N Karpas 299 cells. This approach of NF- κ B inhibition differs from pharmacological strategies^{32,33} with respect to specificity and low or no side effects. Our results are consistent with published data in which pharmacological inhibition of NF- κ B led to CD30-induced apoptosis of ALCL cells.³² Beyond that, our study demonstrated molecular aspects of this phenomenon for the first time. Unfortunately, we could not perform analogous experiments with Hodgkin's cells because stable transfection of these cells with I κ B α Δ N was not possible, most likely due to the decisive importance of NF- κ B for the survival of these cells.¹²

The observation that exclusive CD30 stimulation is unable to induce apoptosis of Hodgkin's or ALCL cells appears to be very important for CD30-targeted immu-

Figure 8. Simultaneous NF- κ B inhibition and CD30 stimulation of ALCL cells activate caspases and lead to apoptosis. Mock-transfected ALCL cells (Karpas 299) and Karpas 299 cells, stably transfected with a dominant-negative inhibitor of NF- κ B (I κ B α Δ N Karpas 299), were CD30-stimulated or nonstimulated for 16 hours (A and B) or 48 hours (C). **A:** Annexin V-FITC/PI-stained cells were analyzed by flow cytometry. **B:** Cytopins of 16-hour-treated cells were fixed and stained for active caspase-3 (top panel). Cleaved PARP was analyzed by immunoblotting. **C:** Immunoblot analysis of active caspase-8, active caspase-9, and active BID. I κ B α and I κ B α Δ N expression was analyzed in B and C; β -actin served as loading control. Three independent experiments were performed; representative results are shown.

notherapy. Whereas inhibition of NF- κ B is apparently sufficient for the induction of apoptosis in Hodgkin's cells,¹² additional CD30 stimulation is required for the induction of apoptosis in ALCL cells. Therefore, treatment of ALCL with anti-CD30 antibodies should be combined with NF- κ B inhibitors to induce highly effective CD30-mediated tumor cell killing.^{2,33} Results of a recent phase I/II study, in which cHL and ALCL patients were treated with an humanized CD30 mAb, support our conclusions. The CD30 mAb displayed limited activity as a single agent *in vivo*, ie, the effects were marginal and not convincing. Therefore, the authors suggested CD30 antibody applications *in vivo* to be used in combination with other therapies.³⁴

To verify the concept of ligand-independent CD30 signaling,²⁸ and to investigate the relevance of CD30 expression for survival and proliferation of Hodgkin's and ALCL cells, we performed CD30 knockdown experiments. De-

spite almost complete down-regulation of CD30, neither gene expression nor proliferation of Hodgkin's cells was affected by CD30 silencing (Figures 6 and 7). Hence, we do not support the concept of ligand-independent CD30 signaling of Hodgkin's cells and conclude that CD30 expression is not responsible for constitutive NF- κ B activity and NF- κ B-dependent gene expression of cultured Hodgkin's cells (Figures 2 and 6). Therefore, it is tempting to speculate that the high and consistent expression of NF- κ B dependent proteins in primary Hodgkin's and Reed-Sternberg cells, such as cyclin D2, p21/WAF, FAS, TNF- α , ATF3, STAT5A, NF- κ B2/p100, c-FLIP, cIAP2, TRAF1, CD54, and CD83,^{12,14–16,35–38} is not related to the signaling of CD30 but to constitutive NF- κ B activation. In contrast to Hodgkin's cells, CD30 silencing of ALCL cells eliminated CD30 signaling effects, as demonstrated by microarray analyses, confirming that our CD30-silencing approach was functionally effective.

Consistent with previous publications,^{4,5,7,30,32} we were able to demonstrate strong CD30 signal transduction in three NPM-ALK-positive ALCL cell lines (Karpas 299, JB6, and SU-DHL1). The extension of our approach to systemic NPM-ALK-negative ALCL cells (FE-PD) revealed a similar CD30 response compared with NPM-ALK-positive ALCL cells. This demonstrates that the fusion protein NPM-ALK has no impact on CD30 signaling. Hence, our data do not confirm the abrogation of CD30 signaling and the inhibition of constitutive NF- κ B activity by NPM-ALK. The discrepancy might be due to the artificial experimental setting applied by Horie et al,³⁹ who mainly deduced their results from NPM-ALK-transfected T-cell type Hodgkin's cells (L540) or CD30-transfected human embryonic kidney cells (HEK 293) and, most importantly, omitted CD30 stimulation experiments for the analysis of CD30 signal transduction. In this context, it is of interest that CD30 down-regulation did not influence NPM-ALK protein expression (Figure 6A), whereas NPM-ALK silencing was reported to inhibit CD30 expression.⁴⁰ These data suggest an unidirectional way of regulation, ie, from NPM-ALK to CD30, but not vice versa.

In summary, we conclude that 1) CD30 expression per se has no impact on the survival of Hodgkin's and ALCL cells, 2) in ALCL cells, CD30 stimulation releases two competing effects, namely caspase activation and simultaneous NF- κ B-mediated survival, and 3) CD30 probably does not play a significant role in the pathogenesis of cHL.

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