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CD40-ligation-mediated protection from apoptosis of a Fas-sensitive Hodgkin's-disease-derived cell line

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Abstract Modulation of Fas expression and function by CD40 ligation was investigated in the Fas-sensitive human Hodgkin's disease cell line HDLM2. The recombinant human trimeric soluble CD40L (sCD40L) protected this cell line from apoptosis induced by an agonistic Fas antibody at all concentrations tested. sCD40L also protected HDLM2 when added up to 2 h after Fas ligation. Apoptosis induced by a cell-permeable synthetic ceramide could not be prevented by sCD40L. Thus, CD40 ligation is likely to intervene in the early phases of the Fas signal transduction pathway. When CD40 ligation preceded Fas ligation, it rendered the cells refractory to Fas-induced apoptosis. sCD40L-mediated protection could not be attributed to reduction in surface Fas expression, increase in Bcl-2 levels or to increase in the levels of soluble Fas isoforms.

Key words Fas · CD40 · Apoptosis · Ceramide · Hodgkin's disease

Introduction

T-cell-dependent immunity is critically regulated by members of the tumor necrosis factor (TNF) ligand and TNF receptor superfamily [14]. These proteins are expressed on activated T cells and they control T cell interactions with other cells. Their interactions can induce pleiotropic biological responses like differentiation, proliferation, activation or even cell death [14].

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Fas is a 35-kDa transmembrane protein belonging to the TNF receptor superfamily [33]. When engaged by its specific ligand, the Fas ligand (FasL), Fas transduces a death signal by activating a complex and regulated process [10]. Mouse strains with defects either in the Fas gene (lpr or lpr^{cg} mutation) or the FasL gene (gld mutation) develop lymphadenopathy and an autoimmune disease similar to systemic lupus erythematosis in man [18, 38]. Thus, the Fas-FasL system plays a crucial role in the homeostasis of the immune system.

Activated T cells express FasL and CD40L, while all mature B cells express CD40. The CD40-CD40L interaction plays a central role in contact-dependent T-cell–B-cell collaboration [31]. In in vivo studies, interference with CD40 signalling prevents the formation of germinal centres and the generation of memory B cells [11, 43]. In vitro studies have demonstrated that ligation of CD40 on B cells results in proliferation, immunoglobulin (Ig) heavy-chain class switching and secretion of Ig in the presence of appropriate cytokines [1, 20, 30].

Signals from $CD4^+$ T cells can induce either clonal proliferation or clonal deletion of B cells. CD40L binding to CD40 on B cells leads to up-regulation of Fas, which can then be engaged by the FasL on $CD4^+$ T cells. Fas-FasL signalling brings about apoptosis of B cells that have not bound antigen via their B cell antigen receptor clonal deletion. In contrast, the concerted action of CD40L and FasL leads to proliferation and clonal expansion of B cells that have their B cell antigen receptor bound by antigens [36]. CD40L is also known to protect immature B cells and B cell lymphomas from IgM-induced apoptosis [7, 16, 45].

The neoplastic Reed-Sternberg (RS) cells in Hodgkin's disease express CD40 [13, 3] and Fas [46, 28] and are surrounded by activated $CD4^+$ T cells [35]. However, because of the paucity of the RS cells [15, 19], investigating ligand-receptor interactions in RS cells in clinical samples is difficult. The Hodgkin's-disease-derived permanent cell line HDLM2 was established from the pleural effusion of a patient with nodular sclerosis

subtype of the disease [9]. HDLM2 strongly expresses CD40 and Fas and undergoes apoptosis on Fas ligation [3, 8]. We investigated the role of CD40-CD40L interaction in modulating Fas function and expression in the Hodgkin's lymphoma cell line HDLM2.

Materials and methods

Cells and culture conditions

HDLM2 was obtained from the German collection of microorganisms and cell culture, Braunschweig, Germany. The cells were cultured in RPMI-1640 medium (Gibco, USA) supplemented with 20% inactivated fetal calf serum (FCS) (Gibco, USA) and 4 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamycin and 5 μg/ml amphotericin B. The cells were grown as suspension cultures at 37 °C in a humidified atmosphere with 5% CO_2.

Antibodies and reagents

The CD40 antibodies used were G28-5, IgG1 [6], kindly provided by Dr. E. Clarke, University of Washington, Seattle, and mAb89, IgG1, kindly provided by Dr. Banchereau, Schering Plough, France. The CD95L antibody NOK1, IgG1κ, was a gift from Dr. Yagita, Juntendo University School of Medicine. The CD95 antibody DX2, IgG1κ was a gift from Dr. Lanier, DNAX Research Institute. The recombinant human trimeric soluble CD40 ligand (sCD40L) was a gift from Dr. Armitage, Immunex Research and Development Corp., Washington. Primers FasIF, FasIIIR and GR20 were a gift from Dr. Ruberti, National Research Council, Rome, Italy. Cell-permeable synthetic C6 ceramide (*N*-hexanoyl-D-sphingosine) was from Sigma and was provided by Dr. P Sarthou, Pasteur Institute, France.

Phycoerythrin(PE)-conjugated CD95 (DX2) and CD40L (TRAP1, IgG1) and biotinylated CD95L (NOK1) were from Pharmingen, USA. The agonistic CD95 antibody CH11 (IgM) was from Immunotech, France, and the fluorescein-isothiocyanate(FITC)-conjugated bcl-2 antibody (clone 124, IgG1κ) was from Dako, Denmark. FITC- and PE-conjugated isotype control antibodies and FITC-conjugated rabbit anti-(mouse Ig) antibody were from Sigma, USA. Avidin conjugated to FITC was from Gibco, USA. Annexin V conjugated to FITC was from Kamiya Biomedical Company, USA. Glyceraldehyde-3-phosphate dehydrogenase primers were from Clontech, Calif. USA.

Phorbol myristate acetate, ionomycin, ethidium bromide, propidium iodide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Tween-20 were from Sigma, USA. Tritium-labelled thymidine ([^{3}H]dT) was from BRIT, India.

Flow cytometry

Samples containing 1×10^6 cells were washed three times in fluorescence-activated cell sorting (FACS) buffer (phosphate-buffered saline pH 7.4 containing 2% FCS and 0.02% NaN_3) and incubated with CD40 antibody at 10 μg/ml or with the CD95-PE, CD40L-PE, or CD95L-biotin antibodies or relevant-isotype-matched control antibodies at 4 °C for 40 min. This was followed by three washes in FACS buffer and incubation with either FITC-conjugated rabbit anti-(mouse Ig) for CD40 or avidin-FITC for CD95L staining for 30 minutes at 4 °C. The cells were washed three times and analysed. For analysis of Bcl-2, the protocol of Steck et al. was used [40]. Single-colour flow cytometry was performed on a FACSCALIBUR flow cytometer (Becton Dickinson Immunocytometry Systems) equipped with a 488-nm argon laser, and data were analysed using CELLQUEST software. Live cells were gated on the basis of forward and side scatter and a minimum of 10 000 events were analysed.

Apoptosis induction and detection

Samples containing 3×10^5 cells were treated with 125 ng/ml mouse IgM Fas antibody CH11, or CH11 in the presence of sCD40L at 500 ng/ml or in medium alone for 96 h in 24-well plates (Nunc, Denmark). At different intervals, i.e. 24, 48, 72 and 96 h, the cells were harvested and cellular viability was assessed by trypan blue dye exclusion. Further, CH11 and sCD40L were added to the cells at different times. Cells receiving CH11 at 0 h (i.e. at the initiation point) received sCD40L at 1, 2, 4, 8 or 12 h and vice versa. At the end of 24 h, the cells were harvested and analysed for apoptosis. In ceramide experiments, cells were treated with C6 ceramide (40 μM) alone or in the presence of sCD40L for 24 h. The cells were analysed for apoptosis by the following methods using flow cytometry.

(i) Propidium iodide staining was performed as described by Nicoletti et al. [29]. Apoptosis induction was determined by evaluating the percentage of hypodiploid nuclei (subG0-G1 peak). The light-scatter gate was drawn to exclude events with very low or high forward scatter. A gate was also drawn to select for peak rather than integral propidium iodide fluorescence that limited the analysis to single cells and excluded doublets and clumps.

(ii) Annexin V binding was used in selected experiments to detect phosphatidylserine that is preferentially expressed on the outer surface of cells undergoing apoptosis [44]. Annexin V conjugated to FITC was used according to the manufacturer's instructions.

(iii) Side- and forward-scatter analysis (scatter methodology) was also employed for detection of apoptosis. A minimum of 10 000 events were analysed.

Analysis of oligonucleosomal DNA fragments was done by extracting DNA according to the standard protocols and electrophoresis on 2% agarose gels.

[^{3}H]dT incorporation and MTT assays

HDLM2 (6×10^4 cells in 200 μl/well) were incubated with the CH11 antibody alone or with CH11 in the presence of sCD40L for the indicated times in a 96-well U-bottom plate (Nunc). The microwells received 0.5 μCi [^{3}H]dT (specific activity: 6500 mCi/mmol) 18 h before the cells were harvested on to glass-fibre filters in a Titertek cell harvester (Flow Laboratories, Norway). The samples were counted in a liquid scintillation analyser (Packard Instruments, USA). The cell viability was also assessed by colorimetric MTT assay essentially as described [41].

Reverse transcriptase/polymerase chain reaction (RT-PCR)

Total RNA was extracted using the Trizol reagent (Gibco, USA) from Jurkat cells, a T cell lymphoblastic leukemia cell line, either in the absence or presence of phorbol myristate acetate (50 ng/ml) and ionomycin (1 μM) for 5 h, or HDLM2 alone or after treatment with sCD40L for 24 h and 48 h. Two other HD-derived cell lines, L428 and KMH2, were also used. cDNA was prepared from 2 μg total RNA using the first-strand cDNA synthesis kit from Amersham, UK, and a total of 2 μl cDNA product was used in the PCR. Custom primers were from Gibco, USA. An 809-bp product was obtained for CD40L, a 610-bp product for FasL and a 983-bp product for G3PDH. The PCR conditions were as described [39, 21]. Primers FasIF, FasIIIR and GR20 were used for the analysis of soluble Fas isoforms, as described by Papoff et al. [34].

Results

Expression of CD40-CD40L and Fas-FasL in HDLM2

On flow-cytometric analysis, HDLM2 strongly expressed CD40 and Fas, but was negative for the corresponding ligands (Fig. 1). While unstimulated Jurkat cells did not express CD40L and FasL, their surface expression was induced upon stimulation with phorbol myristate acetate and ionomycin (data not shown). RT-PCR analysis in HDLM2 revealed the absence of a CD40L transcript and a very weak expression of FasL (Fig. 2). In Jurkat cells, while CD40L levels remained unchanged on stimulation, the FasL signal was enhanced on stimulation, as seen by RT-PCR. PCR reactions without the addition of cDNA served as negative controls (data not shown).

Fas ligation induces apoptosis in HDLM2

A dose-dependent inhibition of [^{3}H]dT uptake was seen upon addition of agonistic anti-Fas antibody CH11 (from 500 ng/ml to 2 ng/ml) over a period of 48 h. (data not shown). A dose of 125 ng/ml of CH11 was used in further experiments. A 24 h treatment of HDLM2 with CH11 led to the generation of oligonucleosomal DNA fragmentation, indicating induction of apoptosis on Fas ligation (Fig. 3a). We quantified apoptosis by measuring the percentage of hypodiploid events, and a significant hypodiploid shift was seen after Fas ligation (Fig. 3b). A corresponding increase in annexin V staining intensity was also observed (Fig. 3c).

Soluble CD40L protects HDLM2 from Fas-induced apoptosis

HDLM2 was incubated with medium alone, CH11 alone or CH11 with sCD40L at the initiation of the assay.

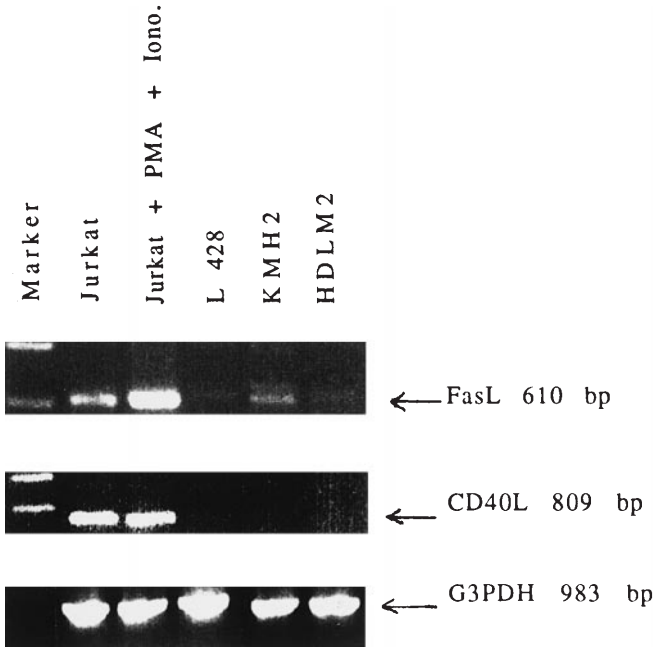


Fig. 2 Reverse transcription/polymerase chain reaction (RT-PCR) analysis of HDLM2 for CD40L (809 bp), CD95L (FasL 610 bp) and the housekeeping gene for glyceraldehyde-3-phosphate dehydrogenase (983 bp). *Jurkat + PMA + Iono* Jurkat cells stimulated with phorbol myristate acetate (50 ng/ml) and ionomycin (1 μM) for 5 h

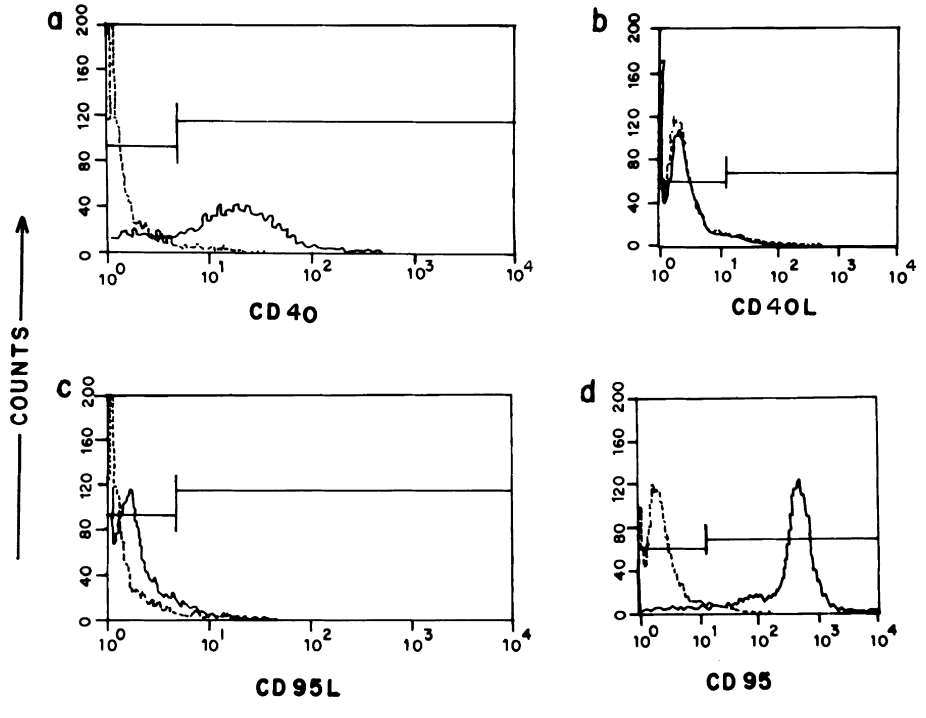


Fig. 1a–d Flow-cytometric analysis of HDLM2 for (**a**) CD40, (**b**) CD40L, (**c**) CD95L and (**d**) CD95. – – – Isotype control staining, —— staining with the respective antibodies

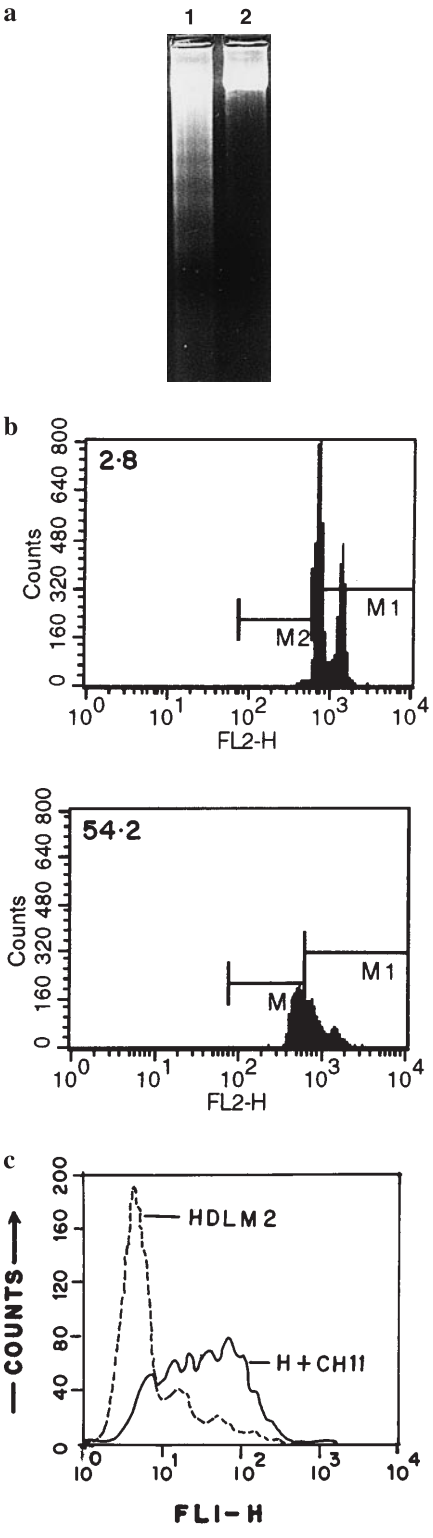


Fig. 3 **a** Internucleosomal DNA cleavage in HDLM2 after CH11 treatment (125 ng/ml) for 24 h. *1* HDLM2 treated with CH11, *2* HDLM2 alone. **b** Increase in hypodiploid (subG0–G1) events after treatment with CH11 for 48 h. *Top* cells only, *bottom* cells treated with CH11. The numbers in the left corner indicate the percentage of events in the M2 (hypodiploid) region. The results shown are representative of one of three independent experiments. **c** Annexin V staining in HDLM2 after CH11 treatment for 24 h. – – – Cells only, —— cells treated with CH11

While CH11 treatment drastically decreased the [^{3}H]dT incorporation, addition of CH11 along with sCD40L resulted in an incorporation of [^{3}H]dT that was intermediate between control and cells treated with CH11 only (Fig. 4a). This phenomenon was tested with CD40L concentrations of 62.5 ng/ml to 1 μg/ml and a concentration of 500 ng/ml was found to be optimal (data not shown). This effect of sCD40L was independent of the CH11 concentration tested (62.5–500 ng/ml) and was statistically significant ($P = 0.005$, Mann Whitney Wilcoxon test) (Fig. 4b). sCD40L alone was not mitogenic to HDLM2 at concentrations of 0.1–1 μg/ml (data not shown).

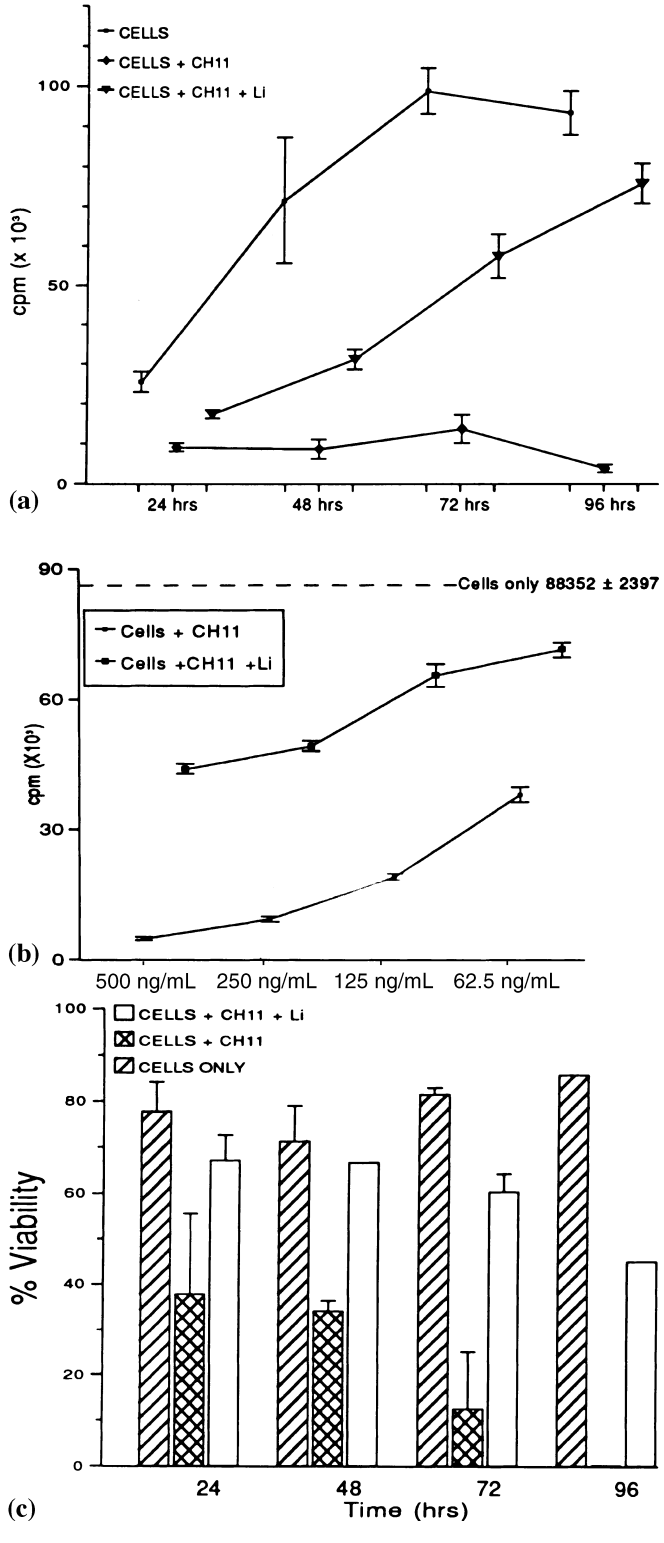
Cellular viability was assessed by trypan blue dye exclusion. With CH11 treatment, viability decreased progressively from 40% at 24 h to 0% at 96 h. In contrast, the cells incubated with CH11 along with sCD40L had a viability of 70% at 24 h and 45% at 96 h (Fig. 4c). Cell viability by the MTT assay also yielded similar results (data not shown).

Hypodiploid events quantified by flow cytometry showed an increase in the subG0–G1 region events upon a 24-h CH11 treatment (mean ± SE, 42.63 ± 4.9%) as compared to cells alone (5.44 ± 2.09%). In the presence of both CH11 and sCD40L, fewer events were seen in the subG0–G1 region (17.65 ± 6.13%). Cells treated with sCD40L alone had a cell cycle profile similar to that of the control (data not shown). Figure 5 summarizes the results obtained from three independent experiments over a period of 96 h. Annexin V staining also yielded a similar pattern, with untreated cells being predominantly negative, cells treated with CH11 showing a positive peak and cells treated with CH11 and sCD40L showing a dominant negative peak and a smaller positive peak (Fig. 6a,b).

Time kinetics of CD40L-mediated protection

sCD40L was added at different times i.e. 1, 2, 4, 8 or 12 h after Fas ligation in a 24-h assay. With an increasing time lag in the addition of sCD40L, a progressive increase in the Annexin-V-positive peak was noted. The magnitude of sCD40L-mediated protection from apoptosis was similar in wells where sCD40L was added 0, 1, or 2 h after Fas ligation. Beyond 2 h, the magnitude of the protection diminished progressively (Fig. 7).

We further studied the effect of addition of CH11 at different times following CD40 ligation. Annexin-V-positive peaks increased in magnitude with longer exposure to CH11 alone (Fig. 8, top). When CH11 was added after a time lag of 0–12 h following sCD40 ligation, it could not induce an Annexin-positive peak of a magnitude identical to that of CH11 alone for a similar exposure time (Fig. 8, bottom). The cell viability also followed a pattern similar to that of Annexin V staining in these experiments (data not shown).



◀

Fig. 4 **a** [^{3}H]dT incorporation in HDLM2 alone (●), or in HDLM2 + CH11 (◆), or in HDLM2 + CH11 + sCD40L (▼) for a period of up to 96 h. The results are expressed as means ± SE and are representative of three independent experiments. **b** CD40 ligand can protect HDLM2 at all concentrations of CH11. HDLM2 was incubated with CH11 at different concentrations (62.5–500 ng/ml) in the presence (■) or absence of sCD40L (●) for a period of 72 h. – – – Incorporation in cells only. Results are expressed as means ± SE and are representative of three independent experiments. **c** Cellular viability as assessed by trypan blue dye exclusion in HDLM2 alone (▨) or after treatment with CH11 (▩) or CH11 and sCD40L (□) for the indicated times. Results are representative of three independent experiments

sCD40L cannot protect HDLM2 from ceramide-induced apoptosis

We investigated the effect of synthetic cell-permeable C6 ceramide on HDLM2 in the presence of sCD40L. Ceramide is a downstream molecule in the Fas-induced apoptotic pathway [42]. [^{3}H]dT studies revealed that ceramide could significantly inhibit incorporation up to a concentration of 30 μM, but not below (data not shown). Cells were treated with C6 ceramide (40 μM) for a period of 24 h in the presence or absence of sCD40L. There was an increase in Annexin V staining upon C6 treatment, which remained unaltered in the presence of sCD40L. Thus, sCD40L could not protect HDLM2 from ceramide-induced apoptosis (Fig. 9a–c).

Effect of CD40 ligation on Fas receptor levels, Bcl-2 level and Fas isoforms

Fas receptor levels remained unchanged after sCD40L treatment for a period of 96 h (Table 1) indicating that CD40 ligation did not lead to any reduction in surface Fas receptor. Except for CD30, which was shed to a small extent after CD40 ligation, the levels of all other members of the TNF receptor and ligand family remained unaltered (data not shown). The level of Bcl-2,

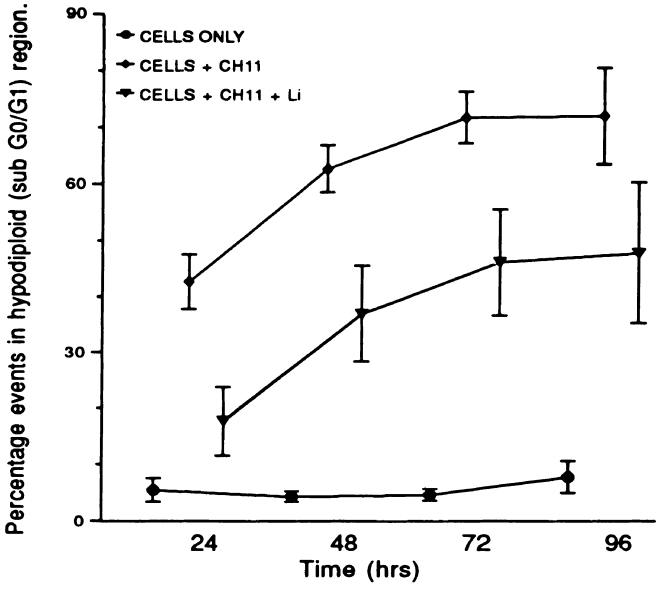


Fig. 5 Cell-cycle analysis to evaluate events in the hypodiploid region in the presence of sCD40L. The results obtained from three independent experiments over four assay times are summarized. ● Cells alone, ◆ cells + CH11, ▼ cells + CH11 + sCD40L

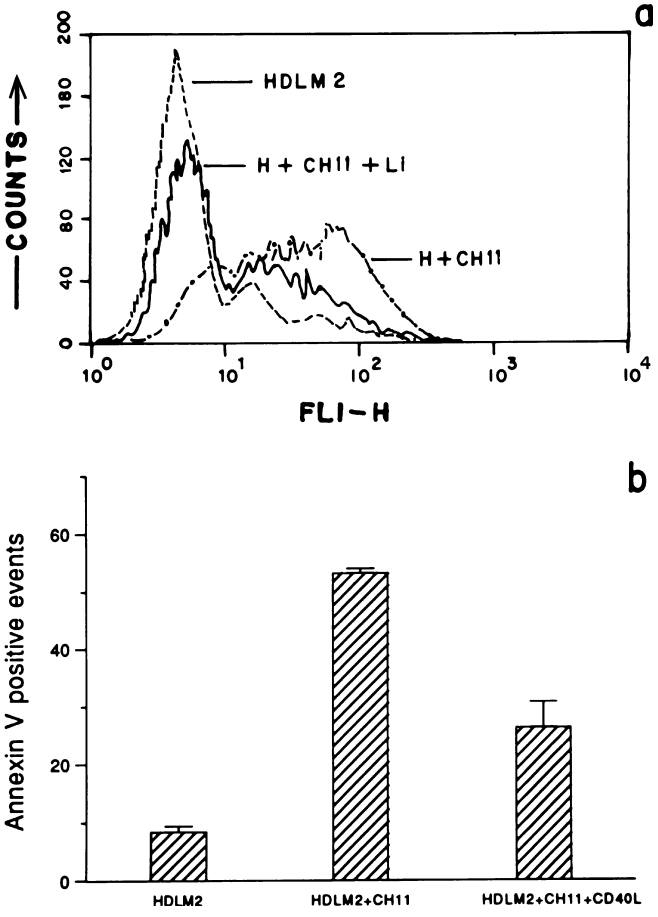


Fig. 6a,b Annexin V Fluorescein-isothiocyanate-conjugated staining pattern in HDLM2 alone or after addition of CH11 or CH11 + sCD40L for 24 h. **a** A representative experiment with a decrease in staining following CD40 ligation as compared to cells treated with CH11 alone. – – – The staining pattern in cells alone, – · – · – cells + CH11, —— staining in cells + CH11 + sCD40L. **b** Annexin-V-positive events under similar conditions. Results are representative of three independent experiments

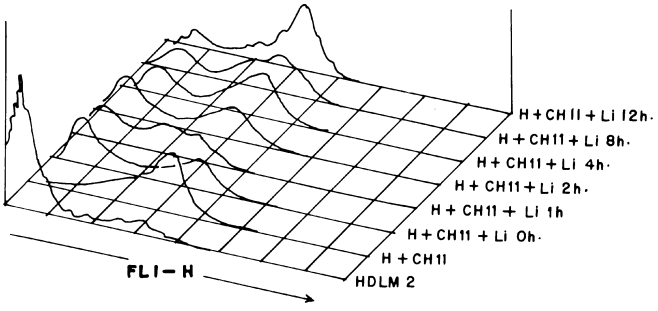


Fig. 7 Kinetics of CD40L-mediated protection from Fas-induced apoptosis: a histogram overlay of Annexin V staining when CD40L was added either at the same time as CH11 (i.e. 0 h) or at different times after Fas ligation, the total assay period being 24 h. Note that the Annexin-V-negative peak (with low fluorescence) decreases in size while the Annexin-V-positive peak (with high fluorescence) increases with increasing time lag in addition of CD40L post Fas ligation. Results are representative of two independent experiments

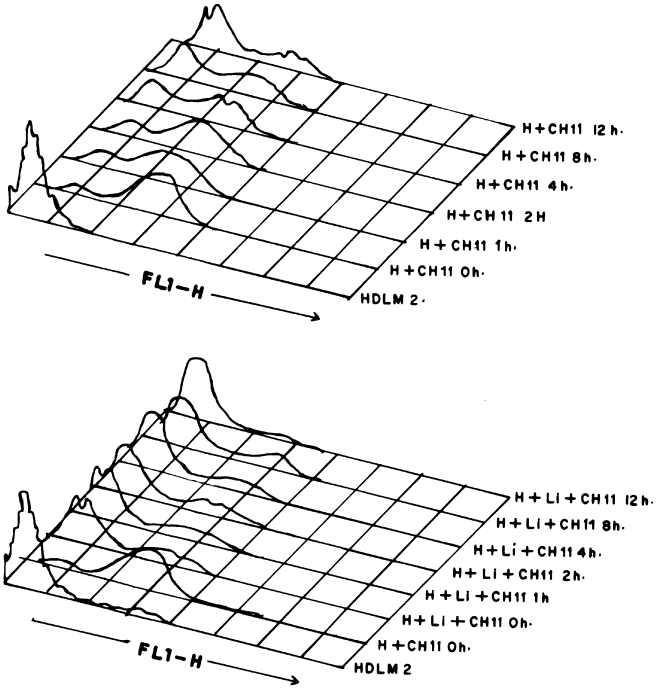


Fig. 8 Annexin V staining pattern in HDLM2 when CD40 ligation precedes Fas ligation. *Top* the staining pattern in cells treated with CH11 alone. CH11 was added at different times as indicated and cells were stained at 24 h. Note the decrease in the size of the Annexin-V-positive peak, along with an increase in the Annexin-V-negative peak with a decreased period of exposure to CH11. *Bottom* the pattern when sCD40L was added at the initiation of the assay with CH11 added at different times. Note the serial reduction in the Annexin-V-positive peak with decreasing exposure to CH11. Results are representative of two independent experiments

an anti-apoptotic protein, remained unchanged in HDLM2 following CD40 ligation (Fig. 10). MCF-7, a breast cancer cell line, and Daudi, a Burkitt lymphoma cell line, were used as positive and negative controls respectively (data not shown). Soluble Fas isoforms have been reported to protect Fas-sensitive cell lines from apoptosis. On RT-PCR analysis, we did not observe any change in types of sFas isoforms or an increase in their relative levels following 24-h and 48-h treatments with CD40L (Fig. 11a,b). PCR reactions without the addition of cDNA served as negative controls (data not shown).

Discussion

HDLM2 is a T-cell-like cell line derived from a nodular sclerosis subtype of Hodgkin's disease and is positive for CD2, but negative for CD3, CD4, CD5, CD6, CD7, CD8, and T cell receptor α/β. This cell line has recently been shown to express CD40 and Fas and to undergo apoptosis on Fas ligation. We found that CD40 ligation protected the HDLM2 cell line from Fas-induced apoptosis. Furthermore, this protective effect was irreversible and the cells remained viable for up to 96 h after

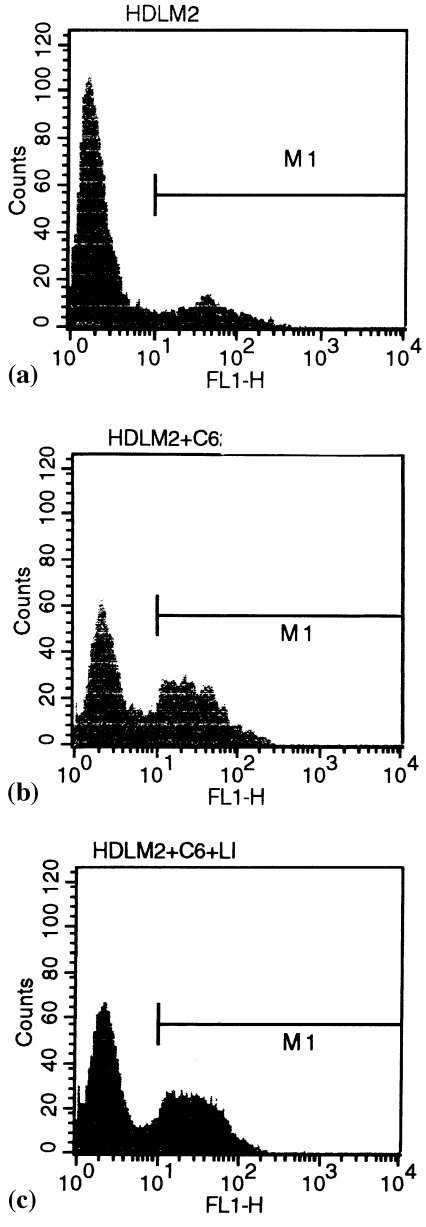


Fig. 9a–c Annexin V staining pattern in HDLM2 alone (**a**), when treated with 40 μM C6 ceramide (**b**) or when treated with ceramide in the presence of sCD40L (**c**) for 24 h. Ceramide and sCD40L were both added at the initiation of the assay. Results are representative of two independent experiments

Table 1 CD40L-mediated protection is not due to increased Fas receptor shedding. Results are expressed as the percentage of positive cells (mean ± SE) and are representative of three independent experiments

Time (h)	Cells positive for Fas (%)	
	Cells only	Cells + sCD40L
24	91.9 ± 1.0	91.4 ± 1.4
48	91.1 ± 1.7	91.1 ± 2.5
72	87.8 ± 1.0	87.9 ± 4.7
96	90 ± 1.0	89.5 ± 3.3

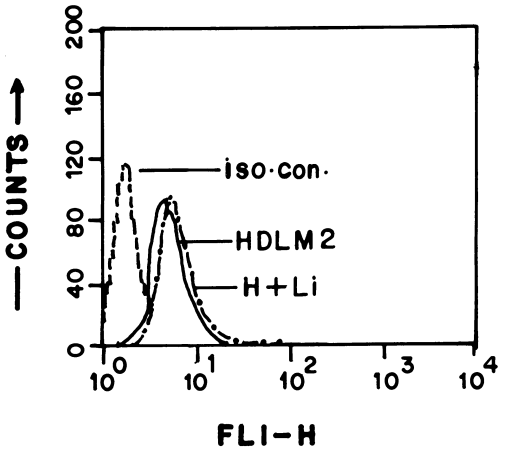


Fig. 10 CD40L does not lead to any changes in Bcl-2 level in HDLM2. – – – Isotype control staining, —— staining in cells only, – · – · – staining for cells + sCD40L

addition of CH11 and CD40L. Moreover, sCD40L could protect this cell line at all concentrations of CH11 tested. Evaluation of events in the hypodiploid region and Annexin V staining also yielded identical results.

On flow cytometric analysis, sCD40L offered a relative protection from Fas-induced apoptosis with a decrease in hypodiploid events. CD40 ligation could protect HDLM2 from Fas-induced apoptosis when added up to 2 h after Fas ligation, but not beyond. Therefore, it is likely that sCD40L intervenes at a proximal step in the Fas signal-transduction pathway. Further, when sCD40L was added before Fas ligation, there was a decrease in apoptotic events as compared to cells treated with CH11 alone. Thus, when CD40 ligation preceded Fas ligation, it could render the cells refractory to Fas-induced apoptosis.

Fas triggers two apoptosis-induction pathways, the sphingomyelin–ceramide pathway and the activation of the interleukin-1β-converting enzyme (ICE) or ICE-like protease cascade [23, 12]. It has not been determined whether these two pathways mediate cell death simultaneously or independently. Ceramide (C6) induces apoptosis in HDLM2, and sCD40L could not protect these cells from ceramide-induced apoptosis. sCD40L could therefore act before ceramide generation, implying that signals generated by CD40 ligation interfere with the early events of a Fas-mediated apoptotic pathway.

The CD40L-induced protection from Fas-mediated apoptosis could not be attributed to a reduction in the surface expression of Fas. On the other hand, CD40 ligation led to the shedding of CD30, another member of the TNF receptor superfamily. Though this has been reported earlier [13], the significance of CD40L-induced CD30 shedding in relation to protection from Fas-induced apoptosis is unclear at present. The role of the anti-apoptotic molecule Bcl-2 in preventing Fas-mediated apoptosis is also unclear [17, 24, 4, 25]. CD40 engagement produces Bcl-2 expression within germinal centres [22]. In HDLM2, sCD40L did not lead to a

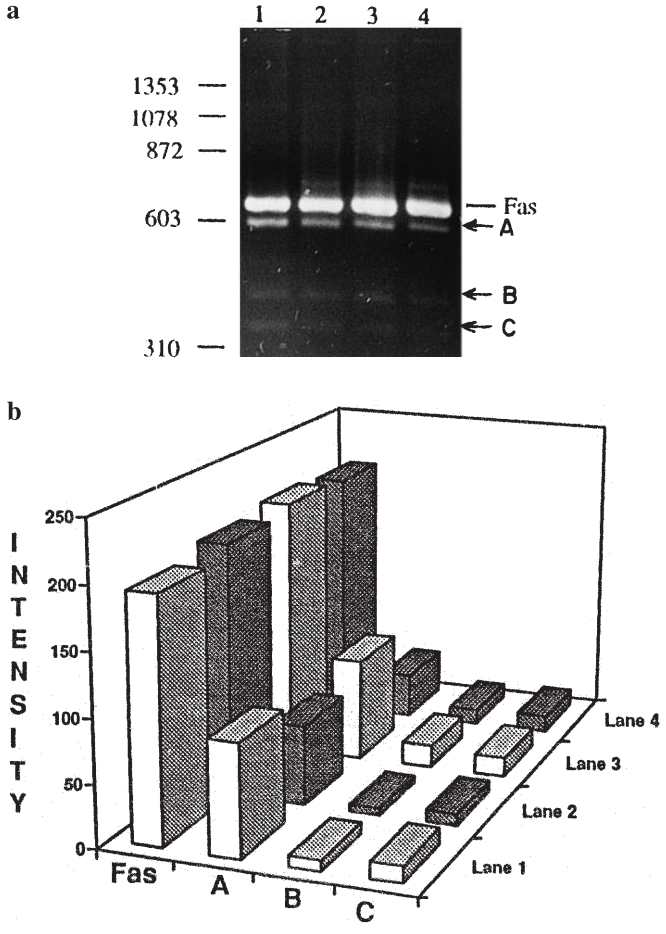


Fig. 11 a sCD40L does not lead to any qualitative changes in sFas isoforms as checked by RT-PCR using primers FasIF and GR20. An approximately 700-bp product was obtained for full-length Fas on 2% agarose gels, while the three alternatively spliced Fas isoforms (*arrows*, *A*, *B*, *C*) migrated below the full-length Fas product. Lanes: *1*, *3* HDLM2 alone at 24 h and 48 h respectively, *2*, *4* HDLM2 + sCD40L at 24 h and 48 h respectively. **b** sCD40L causes no increase in sFas isoform levels, as analysed by densitometric analysis. Densitometric analysis was performed using the UVP gel analysis system, SW2000, Cambridge. The *y*-axis represents the band intensity (in arbitrary units)

significant increase in the level of Bcl-2 following treatment for 24 h and 48 h. Activated human peripheral blood mononuclear cells and many tumour cell lines express several alternatively spliced variants in addition to the full-length Fas mRNA [34]. All the splice variants code for soluble proteins and all of them can bind to either the agonistic Fas antibody or the Fas ligand, thereby inhibiting Fas ligation. However, sCD40L could neither induce a change in the types of Fas isoforms nor increase their relative levels.

A series of murine B cell lymphomas, a subset of human Burkitt lymphomas and immature B cells from the bone marrow or neonatal spleen, are known to undergo apoptosis when their surface IgM receptors are cross-linked [5, 26, 32, 7]. CD40 ligation has been shown to provide survival signals in these B cell apoptotic models [7, 16, 45]. The Bcl-x_L protein has been found to play a major role in this system [5, 26, 45]. Bcl-x_L has also been shown to play a major role in protecting follicular centre B cells from Fas-mediated apoptosis following antigen receptor engagement [37]. It will be worthwhile to investigate Bcl-x_L levels following CD40 ligation in HDLM2.

The diagnosis of Hodgkin's disease is based on the presence of malignant RS cells embedded in an abundance of reactive cells. RS cells express CD40 and Fas and the majority of $CD4^+$ cells that rosette around the RS cells express CD40L [2, 27]. We have demonstrated FasL expression and the co-expression of both Fas and FasL on the RS cells. However, in spite of this, the RS cells do not show features of apoptosis (manuscript accepted). It is possible that the interaction between CD40 on the RS cells and the CD40L on the rosetting $CD4^+$ T cells in vivo, can make the RS cells refractory to Fas-induced apoptosis. CD40 ligation on the RS cells may thus confer a survival advantage to these cells.

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