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CD40 Ligand Protects from TRAIL-Induced Apoptosis in Follicular Lymphomas through NF-κB Activation and Up-Regulation of c-FLIP and Bcl-xL

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Follicular lymphomas (FL) are indolent non-Hodgkin lymphomas and have a relatively good prognosis with a median survival as long as 10 years. However, the majority of patients having FL will experience recurrent relapses, leading to death (1–3). The mechanisms relevant for FL B cell prolonged survival remain unclear. The anti-apoptotic Bcl-2 protein is over-expressed in most FL and results from a t(14;18) chromosomal translocation presents in 85% of the cases. However, this phenomenon cannot explain by itself the selective advantage given to tumor cells (4). Recent microarray-based gene expression profiling and immunohistochemical analyses have revealed that clinico-biologic outcome in FL patients is primarily predicted by specific molecular features of nonmalignant cells instead of tumor cells themselves (5, 6). The influence of the cellular microenvironment on the prognosis of FL probably reflects the participation of both immune and stromal cells in the biology and pathogenesis of this tumor (5, 7). This microenvironmental dependency is supported by the fact that FL B cells are very difficult to grow in vitro in the absence of stromal cells and without stimulation of the CD40 receptor, a crucial event in the interactions between B and T cells (8–10). CD40, a 48-kDa TNF superfamily transmembrane receptor, was first identified and functionally characterized on B lymphocytes (11, 12) and is involved in activation and survival of normal and malignant B cells, such as FL (8, 10). During the germinal center (GC) reaction, CD40 strongly contributes to B cell proliferation and differentiation, to somatic hypermutation and isotype switching, and to memory B cell genesis (11, 13, 14). During these processes, B cells with low-affinity Ag receptors are eliminated by apoptosis to generate a B cell repertoire with appropriate Ag specificities. Studies on GC B cells of human and mice origins lacking the functional CD95 receptor have demonstrated that this death receptor, also a member of the TNFR superfamily, is directly involved in the clonal selection of GC B cells (15, 16). Within GC, CD40 is expressed on normal B lymphocytes and interacts as a trimer with CD40 ligand (L) expressed predominantly on CD4+ activated T cells (17). CD40 activation exerts a complex modulation of B cell apoptosis: CD40 promotes GC B cell survival by protecting them against CD95-induced apoptosis (18–20), but also induces CD95 expression, thereby rendering the cells sensitive to CD95L or CD95 agonists. Cross-linking of CD40 on tonsillar B cells and B cell lines activates nuclear factor NF-κB/Rel transcription factors (21–25). This activation is required for protection against CD95-mediated apoptosis, by up-regulating cellular inhibitors of apoptosis, c-FLIP, Bcl-xL and Bfl-1/A1, or Gadd45b (20, 26, 27). It has been demonstrated that c-FLIP...
proteins serve as major antiapoptotic molecules during CD95-mediated cell death (28, 29).

Proximal signaling events engaged by DR4 and DR5, the two death receptors of TRAIL, are very similar to CD95 (30, 31). TRAIL receptors’ ligation induces activation of caspases 8 and 10, which in turn can induce the cleavage of Bid for initiation of apoptosis via the intrinsic pathway. However, some differences between CD95 and TRAIL receptor-mediated apoptosis signaling are suggested by the finding that TRAIL, in contrast to CD95L, is cytotoxic against many tumor cells but not against most normal cells (32–34) and that some CD95-resistant cell lines still exhibit sensitivity to TRAIL-mediated apoptosis (35). Moreover, ongoing preclinical and clinical trials on different tumor models are confirming the potent antitumor activity of TRAIL in vivo (36). Since FL have a slow growth profile, they may be more vulnerable to apoptotic stimuli than to cytotoxic agents targeting dividing cells. Then the use of new therapeutic molecules with proapoptotic function, like TRAIL, must be envisaged in FL therapies. Moreover, studies on TRAIL-deficient mice suggest a role of TRAIL as a tumor suppressor, where TRAIL deficiency predisposed mice to a greater number of tumors, including B cell lymphomas, making this ligand a new promising molecule in FL treatment (37). However, the cytotoxic response of neoplastic cells to proapoptotic members of the TNF superfamily can be down-regulated by the NF-κB/Rel nuclear activity (38–41). In consequence, the aim of this work was to investigate TRAIL-mediated apoptosis in primary FL B cells and different B cell lines from a GC origin in a context of a strong CD40L/CD40 costimulation to mimic one of the most important signals present in the GC microenvironment. We show with clear evidence that CD40 signaling protects tumor B cells from TRAIL-induced apoptosis and this is associated with a rapid NF-κB activation, which in turn up-regulates c-FLIP and Bcl-xL. Selective inhibition of NF-κB with appropriated drugs restores TRAIL-induced apoptosis in B cell lymphomas.

Materials and Methods

Cell samples
Human tonsils were collected from children undergoing routine tonsillectomy and primary lymph nodes were obtained from FL patients collected at diagnosis. Legal approval was obtained for this study from the institutional review board of the University Hospital of Rennes. Informed consents were provided according to the Declaration of Helsinki. Tonsils and FL lymph nodes were cut into pieces and flushed through a 21-gauge needle. Cell suspensions were cultured in RPMI 1640 (Invitrogen) supplemented with 10% FCS (Biowest) and penicillin/streptomycin. The B cell population was further analyzed by flow cytometry on CD19+CD20+ cells. More than 95% of FL B cells expressed the appropriate κ or λ chain according to the tumor monoclonal IgG.

FIGURE 1. TRAIL-induced apoptosis in primary follicular lymphoma B cells with or without a CD40L stimulation. Lymph nodes obtained from 11 FL patients and tonsils collected from 7 children undergoing routine tonsillectomy were treated or not with 100 ng/ml CD40L and/or 500 ng/ml TRAIL for 24 h, and apoptosis was further estimated by flow cytometry on CD19+CD20+ B lymphocytes with an active caspase 3 assay. A, B cell histograms representative of one of the 11 FL patients and one of the 7 human tonsils treated or not with 500 ng/ml TRAIL for 24 h are presented. The percentage of active caspase 3-positive B cells after CD40L, TRAIL, or CD40L plus TRAIL treatments of the 11 lymph nodes of FL patients (B) or the 7 children tonsils (C) was compared with untreated samples. Mean ± SD; ***, p ≤ 0.001. Asterisks express statistics relative to the control except when referenced with bars.
The peroxidase-conjugated goat anti-mouse or anti-rabbit Abs were obtained from Chemicon, and the anti-DR5, anti-DcR1, and anti-DcR2 Abs from Alexis Biochemicals, the anti-Bcl-xL inhibitor BH3I-1 from Cell Signaling, the anti-FLIP NF6, anti-NOXA, anti-DR4, anti-DR5, anti-DcR1, and anti-DcR2 Abs from Alexis Biochemicals, the anti-annexin-V was from Roche. The anti-IκB-α phosphorylation inhibitor BAY 117085 was from Calbiochem and the anti-Bad, anti-Bak, anti-Bax, anti-Bid, anti-Bcl-2, anti-Bcl-xL, and anti-PUMA were purchased from Sigma-Aldrich. FITC-conjugated anti-DR4 and anti-DR5-neutralizing Abs and then treated with 100 ng/ml TRAIL for 3 and 24 h, and annexin V-positive cells were further estimated by flow cytometry. Mean ± SD, n = 3. C, Neutralization of DR4 and/or DR5 was performed on BL2 using 0.01–10 μg/ml antagonist Abs. Residual TRAIL-mediated apoptosis was evaluated by flow cytometry and expressed as a percentage of control values (TRAIL-induced apoptosis in absence of Ab). Mean ± SD, n = 4; *, p ≤ 0.05; **, p ≤ 0.01; and ***, p ≤ 0.001. D, 16 × 10^4 BL2 cells/well were treated or not with 100 ng/ml CD40L and/or 100 ng/ml TRAIL for 3 and 24 h, and annexin V-positive cells were further estimated by flow cytometry. Mean ± SD; *, p ≤ 0.05; **, p ≤ 0.01; and ***, p ≤ 0.001. E, BL2 cells were treated or not with 100 ng/ml CD40L and/or 100 ng/ml TRAIL for 0–24 h and the percentage of active caspase 3 cells was further estimated by flow cytometry (mean ± SD, n = 3).

### Chemicals and Abs

The human recombinant soluble killer TRAIL was from Alexis Biochemicals. The human recombinant soluble CD40L trimer was a generous gift from C. Bastard (Centre Becquerel, Rouen, France).

### Apoptosis assay

Tonsils, FL lymph nodes, and B cell lines were cultured alone or with CD40L (100 ng/ml), or TRAIL (100 or 500 ng/ml), or cotreated with CD40L and TRAIL for 3 or 24 h. For B cell lines, apoptosis was analyzed using a PE-conjugated anti-active caspase 3 apoptosis kit according to the manufacturer’s instructions or a FITC-conjugated anti-annexin-V Ab. For primary tonsils and FL samples, active caspase 3 was analyzed on selectively gated CD19^+ CD20^- B cells. For NF-κB inhibition, B cell lines and primary tumor B cells were pretreated for 1 h with 75 nM or 1 μM BAY 117085, respectively, and then stimulated with 100 or 500 ng/ml TRAIL for 24 h. Active caspase 3-positive cells were quantified. Labelings were analyzed using a FACS Calibur and CellQuest Pro software (BD Biosciences).

**A fluorochrome inhibitor of caspases (FLICA) apoptosis detection kit was used to reveal active caspases. Once inside the cell, FLICA inhibitors bind covalently to active caspases; these inhibitors are cell permeable and noncytotoxic. The green fluorescent signal represents the amount of active caspases present in the cell at the time the reagent was added. After stimulation, the cell suspension was incubated with caspase 3–7, 6, 8, or 9 FLICA (AbD Serotec) for 1 h at 37°C/5% CO₂ for 1 h at 37°C/5% CO₂. After two washes, cells were directly analyzed by flow cytometry.**

#### TRAIL-receptor labeling and neutralization

B cell lines were labeled with mouse mAb directed against DR4, DR5, DcR1, DcR2, and IgG1 isotype matched as negative control. FITC-conjugated goat anti-mouse IgG1 secondary Ab was used. For TRAIL receptor inhibition, cells were pretreated for 45 min with preservative-free anti-DR4 and/or anti-DR5-neutralizing Abs and then treated with 100 ng/ml TRAIL for 24 h. FITC-annexin-V-positive cells were detected by flow cytometry.

### FIGURE 2. TRAIL-induced apoptosis in GC-derived B lymphoma cell lines with or without a CD40L stimulation.

A, 16 × 10^4 BL2, SUDHL4, RL, and VAL cells/well were treated or not with 100 ng/ml CD40L and/or 100 ng/ml TRAIL for 24 h and active caspase 3-positive cells were estimated by flow cytometry. Mean ± SD, n = 4; *, p ≤ 0.05; **, p ≤ 0.01; and ***, p ≤ 0.001. Asterisks express statistics relative to the control except when referenced with bars. B, TRAIL receptor surface expressions were evaluated in GC-derived B lymphoma cell lines by flow cytometry with anti-DR4, DR5, DcR1, and DcR2 Abs. Isotypic controls are depicted in dashed line and specific labeling in bold line. C, Neutralization of DR4 and/or DR5 was performed on BL2 using 0.01–10 μg/ml antagonist Abs. Residual TRAIL-mediated apoptosis was evaluated by flow cytometry and expressed as a percentage of control values (TRAIL-induced apoptosis in absence of Ab). Mean ± SD, n = 3; **, p ≤ 0.01 and ***, p ≤ 0.001. D, 16 × 10^4 BL2 cells/well were treated or not with 100 ng/ml CD40L and/or 100 ng/ml TRAIL for 3 and 24 h, and annexin V-positive cells were further estimated by flow cytometry. Mean ± SD; *, p ≤ 0.05; **, p ≤ 0.01; and ***, p ≤ 0.001. E, BL2 cells were treated or not with 100 ng/ml CD40L and/or 100 ng/ml TRAIL for 0–24 h and the percentage of active caspase 3 cells was further estimated by flow cytometry (mean ± SD, n = 3).
Proliferation assay

BL2 cells (32 x 10^3/well) were treated or not with CD40L, TRAIL, or a combination of both in 96-well plates. After 24 h of culture, cells were treated with 1 μCi/well tritiated thymidine ([3H]TdR; Amersham Biosciences) for the last 12 h of culture, harvested, and counted on a liquid scintillation analyzer.

For cell cycle analysis, 2.4 x 10^6 BL2 cells were treated or not with CD40L, TRAIL, or a combination of both in 75-cm² flasks. After 24 h of culture, cells were collected and fixed with 70% ethanol, then RNase A was added for 5 min. Finally, propidium iodide was admixed to the cells before flow cytometry analysis. Cell cycle distribution was determined using ModFit software (Verity Software House).

Western blot analysis

After treatments, cells were lysed in radioimmunoprecipitation assay buffer (50 mM Tris-HCl (pH 7.4), 1% Nonidet-P 40, 150 mM NaCl, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM paramethysulfonide, 1 μg/ml pepstatin, leupeptin, and aprotinin) at 4°C. After 14,000 g centrifugation for 30 min, the protein concentration was determined in the supernatant by bicinchoninic acid assay. Samples were boiled for 5 min in Laemmli buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 25% glycerol, and 0.01% bromophenol blue) containing 4.8% of 2-ME. Equal amounts of protein (30 μg) were loaded on 12–15% SDS-polyacrylamide gels and transferred to a polyvinylidene difluoride membrane (Millipore). Membranes were blocked with 5% nonfat dry milk in PBS-Tween 20 (0.1%, v/v) for 1 h and incubated at 4°C for 1 h with rabbit primary Abs. Immunoreactive proteins were visualized by chemiluminescence protocol (ECL plus; Amersham Biosciences).

NF-κB activity measurement

NF-κB activation was measured with a TransAM NF-κB family kit (Active Motif). This ELISA is based on measurements of p50-, p65-, c-Rel-, p52-, and RelB-binding activities to specific consensus DNA sequences. Nuclear extracts were purified according to the manufacturer’s instructions after 1 or 6 h of stimulation with CD40L (100 ng/ml), TRAIL (100 ng/ml), or both. Five micrograms of nuclear extracts were added per ELISA well, incubated with anti-p50, anti-p65, anti-c-Rel, anti-p52 or anti-RelB primary Abs for 1 h, washed, and then incubated with the secondary peroxidase-conjugated Ab for 1 h. After three washes, the developing solution was added for 10 min and absorbance was read at 450 nm.

Quantitative RT-PCR

RNA was extracted using a RNeasy kit (Qiagen) and cDNA was generated using Superscript II reverse transcriptase (Invitrogen). For quantitative RT-PCR, we used assay-on-demand primers and probes, and the TaqMan Universal Master Mix from Applied Biosystems. Gene expression was measured using the Applied Biosystems Prism 7900 Sequence Detection System. 18S was determined as the appropriate internal standard gene. For each sample, the cycle threshold value for the gene of interest was determined, normalized to its respective value for 18S, and compared with the value obtained for unstimulated cells.

Retrovirus production and cell transduction

The bicistronic retroviral pMIG vector containing an internal ribosome entry site upstream of the enhanced GFP gene (42) was used to introduce c-FLIPL cDNA from a pcDNA3 plasmid using BglII-SalI and BglII-XhoI.
respectively. The pMIG vector encoding Bcl-xL was purchased from Ad- 
gene (43). Retroviruses were produced using a Retro-X Universal Pack- 
ing System (BD Clontech). Briefly, GP2-293 cells were transfected us- 
ting a standard calcium phosphate technique with 10 μg H9262 g of pMIG (mock, 
encoding FLIP-L or Bcl-xL) and 5 μg H9262 g of pVSVG. Twelve hours after 
transfection, medium was removed to stimulate cells overnight with 10 μM 
sodium butyrate. Stimulated cells were then washed twice with PBS to 
remove sodium butyrate and refed with fresh medium. Viral supernatants 
were collected 24 – 48 h after, to infect SUDHL4 cells, in the presence of 
8 μg/ml polybrene for 12 h. Infection was repeated twice, then mock- 
transfected cells and cells expressing c-FLIP-L or Bcl-xL were sorted by 
flow cytometry based on GFP expression.

Statistical analyses

Statistical analyses were performed with the Student t test using GraphPad 
Prism software. The significance is shown as follows: *, p ≤ 0.05; **, p ≤ 
0.01; and ***, p ≤ 0.001.

Results

Effect of CD40 triggering on TRAIL-induced apoptosis in 
primary FL B cells

To address whether the proapoptotic TNF superfamily member 
TRAIL could be a promising therapeutic molecule in the treatment 
of FL, we first estimated its potency to induce apoptosis on pri- 
mary FL B cells (Fig. 1). After a 24-h treatment with 500 ng/ml 
TRAIL on a total cell population extracted from lymph nodes re- 
covered from patients with FL at diagnosis, we estimated by flow 
 cytometry the percentage of active caspase 3-positive cells on 
CD19+CD20+ B lymphocytes. We observed in all 11 patients 
tested that TRAIL significantly induces B cell death with a 30% 
increase of active caspase 3-positive primary FL B cells according 
to the control (Fig. 1, A and B). It is worth notice that on average 
20% of active caspase 3-positive nontreated cells were detected 
reflecting spontaneous apoptosis after 24 h of culture as already 
described, and thus indirectly confirms the role played by the 
lymph node microenvironment in FL B cell survival (44, 45). To 
evaluate a possible side effect of TRAIL, if used as an anticancer 
drug, we also tested its cytotoxicity on normal B cells from human 
 tonsils. Dose-response experiments have been 
performed on total cell populations extracted from lymph nodes 
and a higher concentration of TRAIL (500 ng/ml) was needed to

FIGURE 4. Modulation of the ap- 
optotic signaling pathway by CD40L 
in TRAIL-treated BL2 and SUDHL4 
cells. A and C, BL2 and SUDHL4 
cells were stimulated or not with 100 
g/ml CD40L and/or 100 ng/ml 
TRAIL for 3 or 24 h. Cell lysates 
were analyzed after stimulation by 
immunoblotting. β-Actin was used 
as a loading control. B, 16 × 10^6 BL2 
cells were stimulated or not with 100 
g/ml CD40L and/or 100 ng/ml 
TRAIL for 3 and 24 h and active 
caspases were evaluated with a 
FLICA detection kit by flow cytom- 
etry (mean ± SD, n = 4; **, p ≤ 
0.01).
induce strong FL B cell apoptosis as compared with the B cells lines (100 ng/ml used in the next section (data not shown). This was due to the admixture of TRAIL-sensitive and -resistant cells obtained after tissue extraction, as the CD19^+CD20^+ B lymphocyte fraction represented ~50% of the total cell population. These results confirmed the specific antitumoral activity of TRAIL. Then, we asked whether the strong survival signal provided by CD40 on B cells could interfere with a TRAIL treatment. To address this question, we treated normal and neoplastic B cells with 100 ng/ml human recombinant CD40L, alone or in cotreatment with 500 ng/ml TRAIL for 24 h. We observed that CD40L alone protected both normal and FL-derived primary B cells from spontaneous apoptosis, as already addressed but also efficiently protected tumor cells from TRAIL-induced apoptosis when used in cotreatment (Fig. 1, B and C). These results indicate that CD40 triggering, a strong signal in GC B cell differentiation, contributes to GC B cell survival but will also interfere with TRAIL-induced apoptosis in FL.

Effect of CD40 triggering on TRAIL-induced apoptosis in GC-derived B lymphoma cell lines

To characterize the molecular mechanisms which sustain CD40 protection against TRAIL-induced apoptosis, we tested different cell lines derived from GC lymphomas. We retained four of them, BL2, SUDHL4, VAL, and RL, and we analyzed for their sensitivity to TRAIL with or without a CD40L cotreatment (Fig. 2A). We consistently observed that the BL2 and SUDHL4 cell lines were highly sensitive to 100 ng/ml TRAIL with >85% of active caspase 3-positive cells, when RL was an intermediate responder and VAL was much less sensitive to the same dose of the recombinant protein after a 24-h treatment. Dose-response experiments showed that BL2 and SUDHL4 were sensitive to a lower dose of TRAIL (10 ng/ml) with 60% of active caspase 3-positive cells after a 24-h treatment (data not shown). We analyzed surface expression of DR4, DR5, DcR1, and DcR2 on the four different cell lines to appreciate whether the differences in TRAIL sensitivity were linked to receptor expression levels. As demonstrated in Fig. 2B, the level of cell surface expression of DR4 and DR5 was similar in the four different cell lines. The only differences we observed were for the decoy receptors, with DcR2 almost absent on BL2 but significantly expressed on VAL and RL. However, DcR2 was also highly expressed on the very sensitive SUDHL4 cell line. VAL was the only cell line expressing a significant amount of DcR1 on its cell surface. Using specific neutralizing Abs directed against DR4 and DR5, we analyzed the respective subpopulation, and a decrease of the S and G2-M phases reflecting apoptosis (sub-G1 phase) and a blockade of the cell cycle (Fig. 3, B and C). These last results that we also confirmed by guest on November 18, 2017 http://www.jimmunol.org/ Downloaded from

Effect of CD40 ligation on cell growth and cell cycle regulation on TRAIL-stimulated GC-derived B lymphoma cell lines

To fully appreciate the effect of TRAIL and CD40L on cell cycle regulation, we analyzed BL2 and SUDHL4 cell growth and BL2 cell cycle after a 24-h treatment with 100 ng/ml TRAIL and/or 100 ng/ml CD40L (Fig. 3). After treating BL2 separately with TRAIL, we observed that this stimulation was associated with a strong increase of the sub-G1 cell population, an increase of the G1/G0 subpopulation, and a decrease of the S and G2-M phases reflecting apoptosis (sub-G1 phase) and a blockade of the cell cycle (Fig. 3, A and B). In agreement, a strong decrease of cell growth using thymidine incorporation was obtained (Fig. 3C). BL2 treated with CD40L exhibited a slight increase of thymidine incorporation as compared with the control but with a similar repartition of cells in each phase of the cell cycle compared with the control. This difference in thymidine incorporation observed between CD40L-treated and nontreated cells was due to a slight reduction of spontaneous apoptosis detected in the sub-G1 subpopulation (Fig. 3B). When the cells were cotreated with TRAIL and CD40L, the sub-G1 cell population was considerably reduced as compared with TRAIL alone. We also noticed that cotreated BL2 cells continued to proliferate with an increase of thymidine incorporation and a recovery of the cell cycle. These last results that we also confirmed on the SUDHL4 cell line (Fig. 3C), associated with the data on apoptosis (Fig. 2), indicate that CD40 signaling not only protects...
lymphoma B cells from TRAIL-induced apoptosis but also restores the proliferation process of these cells. Modulation of the apoptotic signaling pathway by CD40L in TRAIL-treated BL2 and SUDHL4 cells

Since CD40L protects GC-derived B cell lymphomas from apoptosis and restores their progression into the cell cycle, we next analyzed the apoptotic signaling pathway engaged after CD40L and TRAIL costimulation on the BL2 and SUDHL4 cell lines. Western blot analyses were performed for most of the molecules of the apoptotic cascade (Fig. 4, A and C) and FLICA was used to evaluate caspase activation after 3 and 24 h of stimulation (Fig. 4B). We first noticed that caspase 8 cleavage induced by TRAIL was slightly decreased by a CD40L cotreatment after 3 h. This difference was significant after 24 h, with a reduction of active caspase 8 of ~40–50%. Associated with this reduction of active caspase 8, we observed an up-regulation of c-FLIP after cotreatment, also detected with CD40L alone (Fig. 4, A and C). This up-regulation was faint at 3 h but very clear after 24 h. As a consequence, Bid truncation detected after TRAIL treatment was almost completely inhibited when CD40L was added to the culture for 24 h. We also observed that receptor interacting protein was cleaved after a 24-h treatment with TRAIL as already described in other cell types (46). This cleavage was inhibited after a costimulation with CD40L. Among the other components of the apoptotic signaling pathway, the proapoptotic member Bax was up-regulated after 24 h of TRAIL treatment and a second band with a lower molecular mass of p18 appeared, corresponding to the cleavage of the p21 Bax protein. This smaller fragment, previously described by Wood et al. (47) and Cao et al. (48) as being more efficient in cell death, is generated in the late phase of apoptosis as a cleavage product of calpain (49, 50). We indeed confirmed calpain activation with the appearance of proteolysis products associated with its activation after 24 h of stimulation. CD40L cotreatment modulated calpain activation and cleavage of Bax p21 into p18. These events participated in the reduction of TRAIL-induced apoptosis by CD40L. Similarly, CD40L prevented Bad cleavage detected under TRAIL stimulation as already observed in other models (51). The up-regulation of the BH3-only member NOXA after a 24-h treatment with TRAIL was also partially inhibited by a cotreatment with CD40L. We did not detect any changes after the different stimulations for the other proapoptotic members Puma and Bak, as for the anti-apoptotic Bcl-2 protein. Conversely, the anti-apoptotic protein Bcl-xL was induced after 24 h of treatment with CD40L, cleaved with TRAIL as described by Müller et al. (52), and protected from cleavage and still up-regulated after a cotreatment.
This molecule with c-FLIP is probably another major player in the protection mediated by CD40L against TRAIL-induced apoptosis. As a consequence of the upstream modulation provided by the CD40L signaling on TRAIL-induced cell death, we observed a significant inhibition of caspase 9, 6, 7, and 3 cleavages at 24 h. Caspase activation induced by TRAIL was reduced by 40–50% after a CD40L cotreatment. This corroborates the 40% protection from apoptosis observed after a cotreatment and previously described in Fig. 2. Finally, CD40L also modulated some inhibitors of apoptosis, in particular X-linked inhibitor of apoptosis protein which is protected from degradation after TRAIL stimulation. As a final consequence of these modulations in the apoptotic pathway, CD40L reduced poly(ADP-ribose) polymerase cleavage, ultimately protecting the cells from DNA degradation.

NF-κB activation by CD40L is responsible for its protective effect against TRAIL-induced apoptosis

We observed that c-FLIP and Bcl-xL were both up-regulated after 24 h of CD40L treatment in BL2 and SUDHL4. These genes are known to be NF-κB target genes, the main signaling pathway engaged after CD40L stimulation. We then asked whether the protection mediated by CD40L was directed linked to its ability to induce NF-κB. NF-κB/Rel transcription factors are dimers of proteins (p50/p105 or NFκB1, p52/p100 or NFκB2, p65 or RelA, c-Rel and RelB) that have ~300-aa Rel regions. The NF-κB/Rel complexes are either found in cell nuclei or retained in the cytoplasm by inhibitors of the IκB (α–ε) family; these latter are proteolyzed on cell stimulation by a number of agents, allowing NF-κB/Rel dimers to reach the nucleus and control the expression of a wide range of genes. We analyzed NF-κB activation by Western blot in BL2 and SUDHL4 with an anti-IκBα Ab and by ELISA to evaluate the level of the different NF-κB subunits, p50, p65, c-Rel, p52, RelB, into the nucleus of the BL2 cells (Fig. 5). When the two cell lines were treated with CD40L with or without TRAIL, IκBα disappeared after 15 min due to its fast phosphorylation and ubiquitylation, which drive this inhibitor to its degradation by the proteasome (Fig. 5A). On ELISA, p50, constitutively expressed in BL2, p65, and c-Rel were all rapidly induced during the first hour of a CD40L treatment, reflecting their translocation into the nucleus (Fig. 5B). These three NF-κB members belong to the NF-κB1 pathway. P52 and RelB which belong to the NF-κB2 pathway were slightly induced after 1 h of stimulation (data not shown) and significantly detected in the nucleus after 6 h (Fig. 5C). These data indicate that CD40L stimulation induces very efficiently both the classical and alternative NF-κB pathways, even in cotreatment with TRAIL.

Inhibition of NF-κB signaling induced by CD40L restores TRAIL-induced apoptosis

To confirm the role played by the NF-κB signaling pathway after CD40L stimulation in the prevention of TRAIL-induced apoptosis in B lymphoma cells, we performed our treatments with or without a specific inhibitor of NF-κB signaling which prevents IκBα phosphorylation. Our results showed that the inhibition of TRAIL-induced apoptosis by CD40L on BL2 and SUDHL4 was considerably reduced when BAY 117085 was added to the culture (Fig. 6A). Similar results were obtained on primary FL B cells (Fig. 6B). We also noticed that spontaneous apoptosis was reduced when primary FL B cells were cultured with CD40L and was then restored when BAY 117085 was added into the culture medium. This indicates clearly that spontaneous apoptosis, observed when tumor B cells are removed from their microenvironment, can be partially prevented after activation of the NF-κB pathway. To confirm the role played by c-FLIP and Bcl-xL, the two NF-κB-targeted genes in the modulation of TRAIL-induced apoptosis, we first performed quantitative RT-PCR and Western blot analysis on BL2- and SUDHL4-stimulated cells treated or not with BAY 117085. As
shown in Fig. 6D, the up-regulation of c-FLIP and Bcl-xL observed by Western blot in Fig. 4A was due to the induction of both gene expressions detected by quantitative RT-PCR after CD40L stimulation alone or in cotreatment with TRAIL. This up-regulation was almost completely blocked after treatment with the NF-κB inhibitor. As a consequence, the up-regulation of c-FLIP and Bcl-xL proteins after CD40L stimulation was abrogated in the presence of BAY 117085 (Fig. 6C). To definitely address the role played by c-FLIP and Bcl-xL in the inhibition of TRAIL-induced apoptosis we have demonstrated recently that DcR2-mediated TRAIL inhibition occurs through a TRAIL-dependent interaction with DR5, leading to caspase 8 inhibition within the TRAIL death-inducing signaling complex (56). It remains unclear why SUDHL4 cells exhibit TRAIL sensitivity since they express both agonistic receptors. In this context, it is noteworthy that BL2 cells engage primarily DR4 to trigger TRAIL-induced cell death. Therefore, it remains to be determined whether the selective engagement of DR4 can be inhibited by DcR2 (54).

In this study, we have also shown that CD40L is capable of partially inhibiting the apoptotic effect of TRAIL on primary B cell lymphoma and B lymphoma cell lines as well as the activation of the cysteine protease caspases 8, 9, 6, 7, and 3. These results uncover a new mechanism of resistance to cytotoxic agents conferred by adjacent nontumoral cells expressing CD40L. This is particularly important in the context of FL, which originates from GC, where B cell selection and differentiation are tightly dependent on a CD40L stimulus provided mainly by TFH cells, a specific CXCR5highICOShigh CD4+ T cell subpopulation present within GC (57). This mechanism also prevents normal tonsil B cells and FL B cells from spontaneous apoptosis in culture and identifies this stimulation as crucial to their prolonged survival in vitro and also probably in vivo.

Modulation by CD40L of TRAIL-induced apoptosis in GC-derived B cell lymphoma is mediated by the rapid activation of the canonical NF-κB1 pathway. Proteins of the NF-κB2 pathway are also activated but after a prolonged activation as already reported in different models (58). These results are in agreement with the inhibition of 1κBζ degradation by BAY 117085, the potent NF-κB inhibitor (data not shown), which almost completely reverses the protective properties of CD40 against TRAIL-induced apoptosis. This clearly indicates the main role played by the NF-κB signaling pathway in this context. We have also evaluated by Western blot the activation of the PI3K/Akt and MAPK pathways (p44/42MAPK, JNK, and p38) after CD40L activation and have shown no involvement of these signaling pathways as opposed to previous data obtained on multiple myeloma and chronic lymphocytic leukemia B cells (59, 60). We also confirmed these results using specific inhibitor of the PI3K/Akt, MAPK, p38, and JNK pathways (data not shown).

c-FLIP and Bcl-xL genes, direct targets of NF-κB transcription factors, are both up-regulated in our study under a sustained CD40L stimulation. The anti-apoptotic molecule c-FLIP acts at the initiation phase of TRAIL-induced apoptosis. Both c-FLIP isoforms, c-FLIPshort and c-FLIPlong (61), interfere with caspase 8 activation by inhibiting the processing of procaspase 8 at the death-inducing signaling complex (28, 62), resulting in the blockade of the apoptotic cascade. CD40L-induced protection against CD95-mediated apoptosis has also been recently described by Eeva et al. (63) in a human FL cell line. These authors showed that this protection was associated with a rapid up-regulation of c-FLIP and confirmed, with our present results on FL and results on other tumors, that this inhibitory molecule is a key player in the inhibition of cell death induced by different TNF death receptor family members in human lymphoid malignancies including FL (64, 65).

In addition to c-FLIP, Bcl-xL is also involved in the antiapoptotic signaling of CD40 as a direct target gene of the NF-κB transcription factors. Recent data in solid tumors have shown that Bcl-xL was responsible for the development of acquired TRAIL resistance (66, 67). Our results demonstrate that in GC-derived B lymphoma cells, this antiapoptotic protein is also up-regulated under a CD40L stimulation. When NF-κB activation after CD40L stimulation is blocked by a specific inhibitor of 1κBζ phosphorylation, the functional regulation played by BAY 117085 is associated with a blockade of c-FLIP and Bcl-xL. The key role played by either c-FLIP or Bcl-xL in the resistance to TRAIL in B cell lymphoma after the engagement of the NF-κB signaling pathway was definitely proven after BL2 and SUDHL4 treatment with specific inhibitor of either Bcl-xL or c-FLIP and in SUDHL4-transfected cells with one of these two antiapoptotic genes (Fig. 7). These results have to be taken into account in B lymphoma cancer.
therapy, because CD40 signaling provided by TFH cells on the GC-derived B cells could completely abate a beneficial antitumor effect mediated by TRAIL through NF-κB activation. In this context, BAFF, another TNF family member also involved in normal B cell survival and B cell lymphoma proliferation (68, 69), through activation of the NF-κB pathway could also cooperate with CD40L to prevent TRAIL-induced apoptosis in FL B cells. Collectively, our results strongly suggest that microenvironmental signals are at least in part responsible for the modulation of FL survival in vitro and in vivo. Blockade of such signals may facilitate the entry of FL cells into the death pathway and might potentially provide novel approaches to alter the sensitivity of FL to therapy, because CD40 signaling provided by TFH cells on the germinal center B cells or microenvironmental factors may alleviate the differentiation and apoptosis of germinal center B cells: anti-lg down-regulates Fas expression of CD40 ligand-stimulated germinal center B cells and inhibits Fas-mediated apoptosis. J. Immunol. 157: 1006–1016.


