Rituximab-Induced Inhibition of YY1 and Bcl-x$_L$ Expression in Ramos Non-Hodgkin's Lymphoma Cell Line via Inhibition of NF-κB Activity: Role of YY1 and Bcl-x$_L$ in Fas Resistance and Chemoresistance, Respectively[1]

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Rituximab treatment of B non-Hodgkin's lymphoma (NHL) cell lines inhibits the constitutive NF-κB activity and results in the sensitization of tumor cells to both chemotherapy and Fas-induced apoptosis. Cells expressing dominant active IκB or treated with NF-κB-specific inhibitors were sensitive to both drugs and Fas agonist mAb (CH-11)-induced apoptosis. Down-regulation of Bcl-x$_L$ expression via inhibition of NF-κB activity correlated with chemosensitivity. The direct role of Bcl-x$_L$ in chemoresistance was demonstrated by the use of Bcl-x$_L$-overexpressing Ramos cells, Ramos hemagglutinin (HA)-Bcl-x, which were not sensitized by rituximab to drug-induced apoptosis. However, inhibition of Bcl-x$_L$ in Ramos HA-Bcl-x resulted in sensitization to drug-induced apoptosis. The role of Bcl-x$_L$ expression in the regulation of Fas resistance was not apparent; Ramos HA-Bcl-x cells were as sensitive as the wild type to CH-11-induced apoptosis. Several lines of evidence support the direct role of the transcription repressor yin-yang 1 (YY1) in the regulation of resistance to CH-11-induced apoptosis. Inhibition of YY1 activity by either rituximab or the NO donor DETANONOate or after transfection with YY1 small interfering RNA resulted in up-regulation of Fas expression and sensitization to CH-11-induced apoptosis. These findings suggest two mechanisms underlying the chemosensitization and immunosensitization of B-NHL cells by rituximab via inhibition of NF-κB. The regulation of chemoresistance by NF-κB is mediated via Bcl-x$_L$ expression, whereas the regulation of Fas resistance by NF-κB is mediated via YY1 expression and activity. The potential clinical significance of these findings is discussed. *The Journal of Immunology,* **2005, 175: 2174–2183.**

The majority of non-Hodgkin's lymphomas (NHL)[4] are of B cell origin, and >95% express surface CD20 (1). The chimeric mouse anti-human CD20 mAb, rituximab (Rituxan, IDEC-C2B8) (2), has significant antitumor activity and, alone or in combination with chemotherapy, has been successfully used in the treatment of patients with follicular and low grade lymphoma (3) as well as elderly patients with aggressive diffused large B cell lymphoma (4). The mechanisms of action of rituximab in vivo include Ab-dependent cell-mediated cytotoxicity, comple-

ment-dependent cytotoxicity and apoptosis (5). In vitro, we have previously reported that rituximab sensitizes B-NHL cell lines to drug-induced apoptosis in a synergistic manner (6). Additional investigation of the molecular mechanisms of synergy revealed an association with the down-regulation of Bcl-2 in AIDS-related lymphoma (ARL) (7, 8) or of Bcl-x$_L$ in non-ARL cell lines (9, 10). Additional analyses revealed that down-regulation of Bcl-x$_L$/Bcl-2 by rituximab is a result of rituximab-mediated inhibition of the p38 MAPK, NF-κB (8), and ERK1/2 signaling pathways (10). Recent findings demonstrated that rituximab up-regulates Fas expression and sensitizes B-NHL cell lines to Fas-induced apoptosis via inhibition of yin-yang 1 (YY1) (11). The transcription repressor YY1 negatively regulates surface and total Fas expression and confers resistance to Fas-induced apoptosis through binding to the silencer region of the Fas promoter (12).

The above findings demonstrate that rituximab-mediated inhibition of NF-κB and subsequent inhibition of Bcl-x$_L$ and YY1 result in both chemo- and immunosensitization. However, the roles of Bcl-x$_L$ and YY1 in the regulation of B-NHL sensitivity to drugs and Fas-induced apoptosis mediated by rituximab are not clear. Dong et al. (13) reported that NF-κB regulates Bcl-x$_L$ transcription and demonstrated that functional κBα and κBβ sites in the Bcl-x promoter are functional. Using PCR-mediated site-directed mutagenesis, mutant κBα and κBβ sites were generated in the 520-bp fragment of the Bcl-x$_L$ gene promoter. Although background levels of chloramphenicol acetyltransferase (CAT) activation were detected in Kos cells transfected with the 800- or 520-bp Bcl-x$_L$ gene promoter in CAT, little CAT activity was detected in the cells cotransfected with the Bcl-x$_L$ gene promoter CAT construct with a mutant κBα. Furthermore, it was shown that the presence of two tandem κB sites in the upstream promoter region of Bcl-x supports Bcl-x$_L$ expression (14, 15). The findings in B-NHL cell lines (10,

14) are concordant with findings showing that Bcl-x_L expression is a key determining factor in the resistance to a wide variety of chemotherapeutic drugs (16).

Rituximab is the first FDA-approved Ab for the treatment of cancer, namely B-NHL. As a single agent, it induces a response rate of 50%, which is increased to >90% when used in combination with chemotherapy (17). However, it has been reported that many patients become refractory to such therapies (18). Therefore, alternative therapeutic interventions are being sought. We hypothesized that rituximab-mediated inhibition of YY1 and Bcl-x_L, via inhibition of NF-κB activity, may be responsible in part for the regulation of tumor cell sensitivity to Fas- and drug-induced apoptosis, respectively. This hypothesis was examined, and the following were addressed. 1) Does rituximab inhibit Bcl-x_L expression and YY1 expression/activity via inhibition of NF-κB activity? 2) Does Bcl-x_L expression regulate drug- and/or Fas-induced apoptosis? 3) Does overexpression of Bcl-x_L result in resistance to drugs and/or Fas-induced apoptosis? 4) Does inhibition of YY1 by chemical inhibitors and small interfering RNA (siRNA) regulate Fas expression and sensitize the cells to Fas-induced apoptosis? The findings demonstrate that there is complementation in rituximab-mediated signaling pathways downstream of NF-κB such that the down-regulation of Bcl-x_L expression sensitizes B-NHL cells to drug-induced apoptosis, whereas the down-regulation of YY1 expression or activity regulates tumor cell sensitivity to Fas-induced apoptosis.

Materials and Methods
Cell lines and reagents

The Ramos and Raji cell lines were purchased from American Type Culture Collection. The ARL B cell line 2F7 (19) was provided by Dr. O. Martinez-Maza (Jonsson Comprehensive Cancer Center, Los Angeles, CA) and is currently available from American Type Culture Collection. Cells were grown in RPMI 1640 (Cellgro; Mediatech) in 10% heat-inactivated FBS (Invitrogen Life Technologies). Cultures were additionally supplemented with 1% bacteriofungicide solution containing 10,000 U/ml penicillin G, 10 mg/ml streptomycin, and 25 μg/ml fungizone (Cellgro).

For the generation of Ramos IκB mutant cells, the 5′-FLAG-tagged IκB mutant was fused in-frame to the 5′ end of the mutant estrogen receptor ligand-binding domain (ER), and the chimera was cloned into the *Hind*III/*Eco*RI sites of pCDNA3 to generate the pCDNA3-IκB-ER construct. For the generation of Bcl-x-overexpressing Ramos cells, the pEBB-puro-Bcl-x-hemagglutinin (HA) construct was generated by PCR cloning of human Bcl-x, which then was inserted into the *Bam*HI and *Not*I sites of pEBB-puro-HA in-frame with the 3′ influenza HA tag. The cells were pulsed using electroporation at 250 V and 975 μF and then selected and maintained in 2.5 μg/ml puromycin (Sigma-Aldrich) or in 1 mg/ml active G418 (Mediatech), respectively. These cell lines were provided by Dr. G. Cheng (University of California, Los Angeles, CA) (15). Cultures were incubated in a controlled atmosphere incubator at 37°C with saturated humidity and an atmosphere of 95% air and 5% CO$_2$ at 0.5 × 10^6 cells/ml and were subcultured every 2 days.

The Fas Ab for Western blot was purchased from DakoCytomation. The Fas agonist Ab (CH-11) and anti-Fas Ab (UB2) used for flow were purchased from MBL. Rituximab was obtained commercially, and rituximab-deleted CH$_2$ (rituximab CH2$^-$) was provided by Dr. P. Chinn (Biogen-Idec, San Diego, CA).

Flow cytometric analyses

Active caspase 3. Fas-mediated apoptosis was assessed using the anti-Fas Ab CH-11 (MBL; 5, 10, and 20 ng/ml) in an 18-h incubation assay. Active caspase 3 was analyzed by flow cytometry using a FITC-conjugated anti-active caspase-3 mAb (BD Pharmingen) as described previously (8–10). Briefly, 2 × 10^6 cells/sample were preincubated with rituximab (20 μg/ml) for 6 h and subsequently with CH-11 Ab or isotype control for 18 h. The cells were stained for intracellular active caspase 3 and analyzed by flow cytometry. As a negative control, the cells were stained with pure (isotype) IgG. Population data were acquired on a Flow EPICS XL-MCL (Coulter) with System II software, and the percentage of positive cells was recorded. ***Surface Fas expression.*** Surface Fas Ag expression was determined by flow cytometry. Cells were treated for 6 h with rituximab (20 μg/ml), IgG

(20 μg/ml) control, or medium alone. Extracellular detection was determined in cells treated with anti-Fas Ab or isotype control for 30 min. The cells were washed twice with 1× PBS, incubated with anti-mouse PE-conjugated Ab (BD Pharmingen), and analyzed by flow cytometry for percentage and mean fluorescence intensity.

Western blot analysis for protein expression

Tumor cells (2 × 10^6) were incubated at 37°C for 6 h. The cells were lysed on ice with 200 μl of ice-cold RIPA buffer (1% Nonidet P-40, 0.1% SDS, 0.5% deoxycholic acid, and complete protease inhibitor mixture tablet (Roche)). Lysates were transferred to microcentrifuge tubes, sonicated in a sonicator (model W-220F; Heat-System Ultrasonic) for 10 s, and were centrifuged at 12,000 × g at 4°C for 5 min. Protein concentrations were quantified using the Bio-Rad protein assay. Gel loading buffer (Bio-Rad) was added to the cell lysates at a 1:1 ratio. Samples were boiled for 5 min, separated on 12% SDS-polyacrylamide minigels, and transferred to nitrocellulose membrane Hybond ECL (Amersham Biosciences) in a Trans-Blot SD semidry transfer cell system (Bio-Rad). Primary Abs for Fas (DakoCytomation), anti-phospho-IKK, anti-inhibitor of κB kinase (IKK), anti-phospho-IκB (Cell Signaling Technology), anti-IκB, anti-Bcl-x_L (Santa Cruz Biotechnology), and anti-YY1 (Geneka) were used at predetermined optimal concentrations. The blots were developed by LumiGLO reagent and peroxide (Cell Signaling Technology).

Treatment of 2F7 cells with inhibitors

The specific NF-κB inhibitor Bay 11-7085, obtained from Calbiochem, was dissolved in DMSO (Sigma-Aldrich). DETANONOate was purchased from Alexis. The NF-κB inhibitor Bay 11-7085 (a specific inhibitor of IκBα phosphorylation) was added at 6 μM under the same conditions. DETANONOate inhibits YY1 function (12) and was used at 1000 μM. The inhibitor dehydroxymethylepoxyquinomicin (DHMEQ) inhibits the nuclear translocation of NF-κB (20) and was a gift from Dr. K. Umezawa (Keio University, Yokohama, Japan).

EMSA

Briefly, cells (10^6) were harvested after treatment and washed twice with ice-cold 1× PBS. After washing, the cells were lysed in 1 ml of Nonidet P-40 lysis buffer (10 mM Tris-HCl (pH 7.5), 10 mM NaCl, 3 mM MgCl$_2$, and 0.5% Nonidet P-40) on ice for 5 min. Samples were centrifuged at 300 × g at 4°C for 5 min. The pellet was washed twice in Nonidet P-40 buffer. Nuclei were then lysed in nuclear extraction buffer (20 mM HEPES (pH 7.9), 25% glycerol, 0.42 mM NaCl, 1.5 mM MgCl$_2$, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT) and sonicated for 10 s at 4°C. Both buffers contained the complete protease inhibitor mixture tablets from Roche. The protein concentration was determined using the Bio-Rad protein assay kit. The nuclear proteins were frozen at −80°C. Nuclear protein (5 μg) was mixed for 30 min at room temperature with biotin-labeled oligonucleotide NF-κB and YY1 probe using an EMSA kit (Panomics) according to the manufacturer's instructions. Ten microliters of nuclear extract was subjected to 5% PAGE for 90 min in Tris-borate EDTA buffer (Bio-Rad) and transferred to a nylon transfer membrane (Hybond-N$^+$; Amersham Biosciences) using the Trans-Blot SD semidry transfer cell system (Bio-Rad). The membranes were transferred to a UV cross-linker (FB-UVXL-1000; Fisher Scientific) for 3 min. The detection was made according to the manufacturer's instructions after the membranes were exposed on Hyperfilm ECL (Amersham Biosciences) as previously described (8–10).

Transfection with YY1 siRNA

The prostate carcinoma cell line (PC-3) was cultured in 1 ml of RPMI 1640 medium supplemented with 5% FBS. Transfections were performed using Lipofectamine 2000 CD reagent (Invitrogen Life Technologies) and the SureSilencing siRNA kit (SuperArray Bioscience) according to the manufacturers' instructions. Briefly, 3 μl of YY1 siRNA or a negative control of siRNA solution was incubated with 4 μl of the transfection reagent in serum-free RPMI 1640 medium for 25 min to facilitate complex formation. The resulting mixture was added to PC-3 cells cultured in a 24-well plate with 1 ml of medium. YY1 expression was determined by RT-PCR; Fas expression was determined by flow cytometry. To determine PC-3-induced sensitization to Fas-mediated apoptosis, 24 h after transfection the cells were treated for 18 h with CH-11, then fixed and permeabilized for anti-active caspase-3-FITC Ab staining, and analyzed by flow cytometry as described above

Isobologram analysis for synergy

To establish whether the cytotoxic effect of the combination of rituximab and CH-11 Ab was more than additive, isobolograms (21) were developed from a battery of combination at various concentrations of rituximab (10, 20, and 40 μg/ml) and anti-Fas Ab CH-11 (5, 10, and 20 ng/ml). Combinations yielding $25 \pm 5\%$ cytotoxicity were graphed as a percentage of the concentrations of single agent alone that produced the same amount of cytotoxicity as that used in combination (6–10).

Statistical analysis

All results were expressed as the mean $\pm$ SD of data obtained from three or four independent and separate experiments. The statistical significance of differences between group means was determined using one-way ANOVA to compare variance. Significant differences were considered for probabilities <5% ($p < 0.05$).

Results

Rituximab-mediated up-regulation of Fas expression and sensitization to CH-11-induced apoptosis

Treatment of Ramos, 2F7, and Raji NHL-B cell lines with rituximab sensitized the tumor cells to CH-11 (Fas agonist Ab)-induced apoptosis. The sensitization was a function of the concentration of CH-11 used (Fig. 1*A*, *top*). The sensitization achieved by rituximab was found to be synergistic, as determined by isobologram analysis (Fig. 1*A*, *bottom*). Because B cell lines also express FcRs, it was possible that rituximab-mediated sensitization to CH-11 might have resulted from cross-linking of rituximab with FcRs on the tumor cells. This possibility was shown to be unlikely by demonstrating that an engineered rituximab molecule lacking the CH2 domain (rituximab CH2$^-$) sensitized B-NHL cells to CH-11-mediated apoptosis similarly to sensitization by rituximab. The sensitization was specific, because control IgG had no effect (Fig. 1*B*).

Treatment of Ramos cells with rituximab up-regulated surface Fas expression as determined by flow cytometry. Time-kinetics analysis of treatment showed that significant up-regulation of Fas occurred as early as 6 h after rituximab treatment and returned to baseline levels at 24 h (Fig. 1*C*). The up-regulation of surface Fas expression was confirmed at the total protein level by Western blot (Fig. 1*D*).

These findings demonstrate that rituximab treatment of B-NHL cells sensitizes the cells to Fas-induced apoptosis, and synergy is achieved. Rituximab treatment also up-regulated Fas expression in these cells.

Rituximab-mediated inhibition of NF-κB activity correlates with down-regulation of Bcl-x_L expression and up-regulation of Fas expression

Treatment of Ramos cells with rituximab resulted in inhibition of NF-κB activity, as assessed by EMSA. The inhibition was significant at 3 h after treatment and was maintained for up to 24 h. The specificity was confirmed by use of the NF-κB-specific inhibitors Bay 11-7085 and DHMEQ (Fig. 2*A*). The specificity of these inhibitors has been previously established (10, 14, 20). In addition, rituximab inhibited IKK and IκB phosphorylation in a time-dependent manner beginning 3 h after treatment, and the inhibition was maintained for up to 24 h. Levels of the nonphosphorylated IKK and IκB were not affected by rituximab (Fig. 2*B*). These findings demonstrate that rituximab inhibits the NF-κB signaling pathway via inhibition of phosphorylated IKK (p-IKK) and p-IκB. Previous studies have reported that NF-κB regulates Bcl-x_L transcription (15, 22–24). Hence, treatment of Ramos cells with rituximab inhibited Bcl-x_L expression beginning at 3 h after treatment, and the inhibition was maintained for up to 24 h. The NF-κB-specific inhibitor DHMEQ (20) also inhibited Bcl-x_L expression (Fig. 2*C*).

The above findings demonstrated that rituximab inhibits the NF-κB pathway (Fig. 2*A*) and down-regulates Bcl-x_L expression

(Fig. 2*C*). Thus, we examined the direct relationship between inhibition of the NF-κB pathway and Bcl-x_L expression by rituximab. To this end, Ramos cells with a functional block in the NF-κB signaling pathway were established. The strategy involved the overexpression of a chimeric fusion protein consisting of a dominant-active IκB-α mutant (S32/36A) fused to a mutated ER (15). The IκB mutant is incapable of being phosphorylated at the critical serine residues and thus is not targeted for proteasomal degradation upon activation of IKK. The fused ER confers inducible activation of the gene of interest upon exposure to the synthetic estrogen 4-hydroxytamoxifen (4-OHT). The FLAG-IκB-mutant-ER construct was cloned into the pCDNA3 expression vector and was stably transfected into the cells. Single clones expressing the construct were isolated and used for additional analysis (15). The Ramos-IκB-ER cells were either untreated or pretreated with 4-OHT (200 nM; 8 h). Thereafter, the cell lysates were subjected to immunoblot analysis. As depicted, treatment of Ramos-IκB-ER cells with 4-OHT inhibited NF-κB activity, similar to findings observed with rituximab treatment (Fig. 3*A*), and also reduced the basal level of Bcl-x_L protein expression in these cells (Fig. 3*B*). These findings demonstrate that inhibition of NF-κB correlated with inhibition of Bcl-x_L expression. In addition, treatment of the Bcl-x_L-overexpressing Ramos cells, HA-Bcl-x, with rituximab resulted in down-regulation of endogenous Bcl-x_L, but not that driven by the overexpressing plasmid (Fig. 3*C*). Together, these findings establish a strong correlation between rituximab-induced inhibition of NF-κB activity and inhibition of Bcl-x_L expression in Ramos B-NHL cells.

We have shown above that rituximab sensitizes B-NHL cells to CH-11-induced apoptosis and also up-regulates Fas expression (Fig. 1, *C* and *D*). Because rituximab inhibits NF-κB activity (Fig. 2), the role of NF-κB in the regulation of Fas expression was examined. Treatment of Ramos IκB-ER cells with rituximab resulted in up-regulation of Fas expression, comparable to treatment of Ramos cells, as determined by flow cytometry (Fig. 4*A*) and Western blot (Fig. 4*B*). However, when Ramos IκB-ER cells were treated with 4-OHT to inhibit NF-κB activity and in the absence of rituximab, there was a much higher up-regulation of Fas expression compared with that in untreated cells as determined by both flow cytometry (Fig. 4*A*) and Western blot (Fig. 4*B*). Treatment of Ramos IκB-ER cells with the combination of rituximab and 4-OHT did not further augment Fas expression compared with the effect of treatment with either rituximab or 4-OHT alone. These findings demonstrate the regulation of Fas expression correlated with the sensitization of tumor cells to CH-11-induced apoptosis. Thus, although treatment of Ramos IκB-ER cells with rituximab sensitized the cells to CH-11-induced apoptosis, 4-OHT treatment was more efficient in sensitizing the cells to CH-11-induced apoptosis than treatment with rituximab (Fig. 4*C*). These findings demonstrate that NF-κB participates in the negative regulation of Fas expression and resistance to CH-11-induced apoptosis in Ramos cells.

Bcl-x_L expression regulates drug resistance, but fails to regulate Fas resistance

We examined the role of Bcl-x_L in regulation of the sensitivity of Ramos cells to CH-11-induced apoptosis. Treatment of Bcl-x_L-overexpressing Ramos cells (HA-Bcl-x) with rituximab resulted in significant sensitization to CH-11-induced apoptosis, comparable to that of wild-type Ramos cells. The extent of apoptosis was a function of the CH-11 mAb concentration used (Fig. 5*A*). These findings demonstrate that Bcl-x_L expression does not influence the sensitivity of Ramos cells to CH-11-induced apoptosis.

FIGURE 1. Rituximab-mediated sensitization of B-NHL cell lines to CH-11-induced apoptosis and up-regulation of Fas expression. *A*, Ramos, 2F7, and Raji B-NHL cell lines are sensitized by rituximab to CH-11-induced apoptosis, and synergy is achieved. The B-NHL cells were treated with rituximab (20 μg/ml) for 6 h, then they were treated with different concentrations of CH-11 (5, 10, and 20 ng/ml for Ramos and 2F7 and 0.1, 0.2, and 0.3 ng/ml for Raji) for 18 h. Apoptosis was determined by activation of caspase 3 as described in *Materials and Methods*. The data represent the mean ± SD from three independent experiments. ∗, $p < 0.05$; ∗∗, $p < 0.03$. The *bottom panel* shows the synergy achieved as determined by isobolograph analysis. Fractional inhibitory concentration represents the concentration from concentrations yielding a percentage of cytotoxicity alone yielding $25 \pm 5\%$. *B*, Rituximab-mediated sensitization to CH-11-induced apoptosis is independent of cross-linking of rituximab with FcR on tumor cells (20 μg/ml). Ramos cells were treated with rituximab (20 μg/ml), CH2-deleted rituximab (20 μg/ml), or control IgG (20 μg/ml) for 6 h and thereafter treated with CH-11 (10 and 20 ng/ml) for 18 h, and apoptosis was determined. ∗, $p < 0.03$; ∗∗, $p < 0.002$. *C*, Up-regulation of surface Fas expression on Ramos cells by rituximab. Ramos cells were treated for various times with rituximab (20 μg/ml), and surface Fas expression was analyzed by flow cytometry. The data represent the mean ± SD of three independent experiments performed in duplicate. ∗, $p < 0.04$. *D*, Up-regulation of Fas expression as determined by Western blot. Ramos cells were treated with rituximab (20 μg/ml) for 6 h, and total cell lysates were prepared and tested for Fas by Western blot as described in *Materials and Methods*. The *top panel* shows Fas expression, and the *bottom panel* shows densitometric analyses. There was a 6- to 7-fold increase in the level of Fas by rituximab compared with that in untreated Ramos cells.
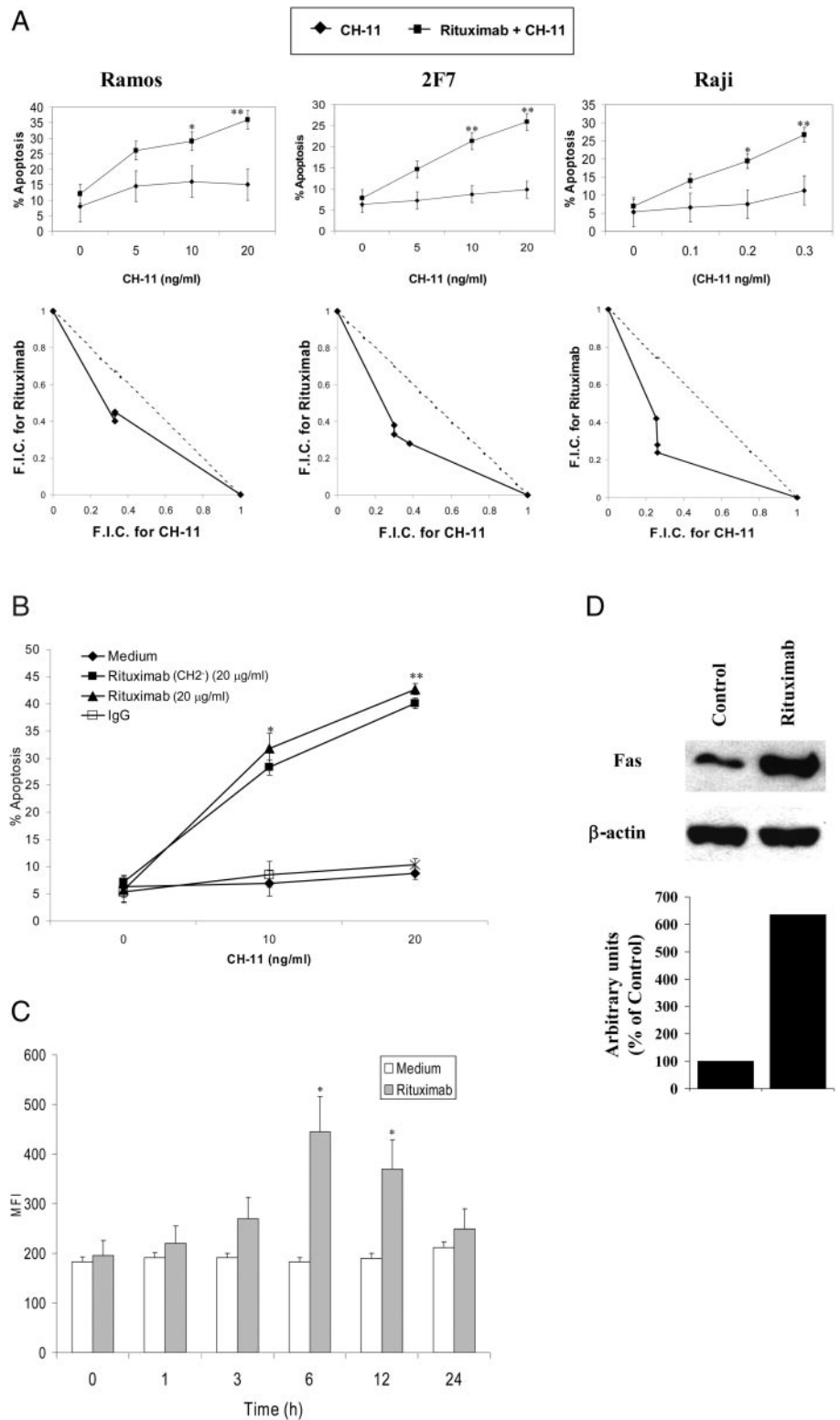

We then examined the role of Bcl-x_L in the sensitization to *cis*-platinum (CDDP)-mediated apoptosis by comparing rituximab-mediated chemosensitization in Ramos and Ramos HA-Bcl-x cells. Although treatment of Ramos cells with rituximab sensitized the cells to CDDP-mediated apoptosis, rituximab treatment of Ramos HA-Bcl-x cells, in contrast, failed to significantly sensitize to CDDP-induced apoptosis (Fig. 5*B*). Treatment of Ramos IκB-ER cells with CDDP resulted in moderate cytotoxicity; however, inhibition of NF-κB by 4-OHT and consequently by

down-regulation of Bcl-x_L sensitized the cells to CDDP-mediated apoptosis (Fig. 5*C*). These findings clearly demonstrate that Bcl-x_L expression regulates tumor cell sensitivity to drug-induced apoptosis, but not to Fas-induced apoptosis.

YY1 expression/activity regulates Fas expression and resistance to CH-11-induced apoptosis

We have previously reported that YY1 negatively regulates Fas expression through its interaction with the silencer region of the

FIGURE 2. Rituximab-mediated inhibition of NF-κB DNA-binding activity and down-regulation of p-IKK and p-IκB and Bcl-x$_L$ expression. Ramos cells were treated with rituximab (20 μg/ml) and the NF-κB inhibitors Bay11-7085 (6 μM) and DHMEQ (10 μg/ml) for 0–24 h, and nuclear lysates and total cell lysates were prepared as described in *Materials and Methods*. *A*, Inhibition of NF-κB DNA-binding activity by rituximab. This figure shows the EMSA (*top panel*) and corresponding densitometric analysis (*bottom panel*). *B*, Inhibition of p-IKK and p-IκB by rituximab. Total cell lysates were tested by Western blot for both nonphosphorylated and phosphorylated IKK and IκB. *C*, Inhibition of Bcl-x$_L$ expression by rituximab. Ramos cells were treated with rituximab (20 μg/ml) or the NF-κB inhibitor DHMEQ (10 μg/ml) for various periods of time (0–24 h), and total cell lysates were tested for Bcl-x$_L$ expression by Western blot.
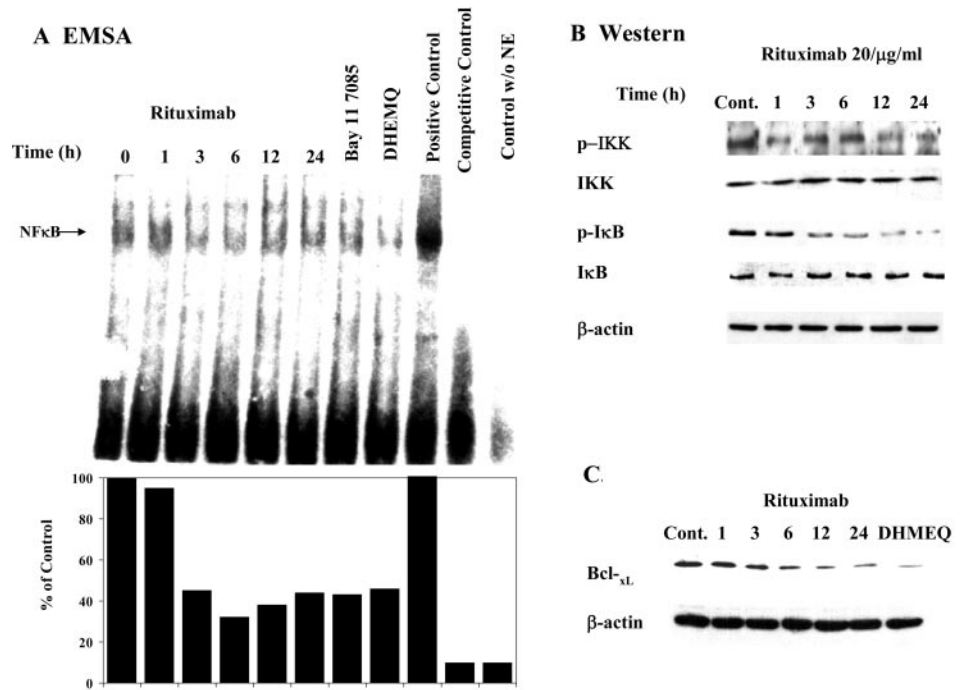
Fas promoter. Inhibition of YY1 by NO results in up-regulation of Fas expression and sensitization of ovarian carcinoma cells to Fas-induced apoptosis (12). Thus, we examined whether rituximab-induced up-regulation of Fas expression in B-NHL cells was also regulated by YY1. Treatment of Ramos cells with rituximab or specific NF-κB inhibitors (e.g., Bay 11-7085 and DHMEQ) and/or inhibition of YY1

(by the NO donor DETANONOate) resulted in significant up-regulation of surface Fas expression (Fig. 6*A*) and sensitization of Ramos cells to CH-11-induced apoptosis. The extent of apoptosis was a function of the CH-11 concentration used (Fig. 6*B*). These findings suggested that YY1 inhibition by rituximab in B-NHL cells regulates Fas expression and sensitizes the cells to Fas-induced apoptosis.
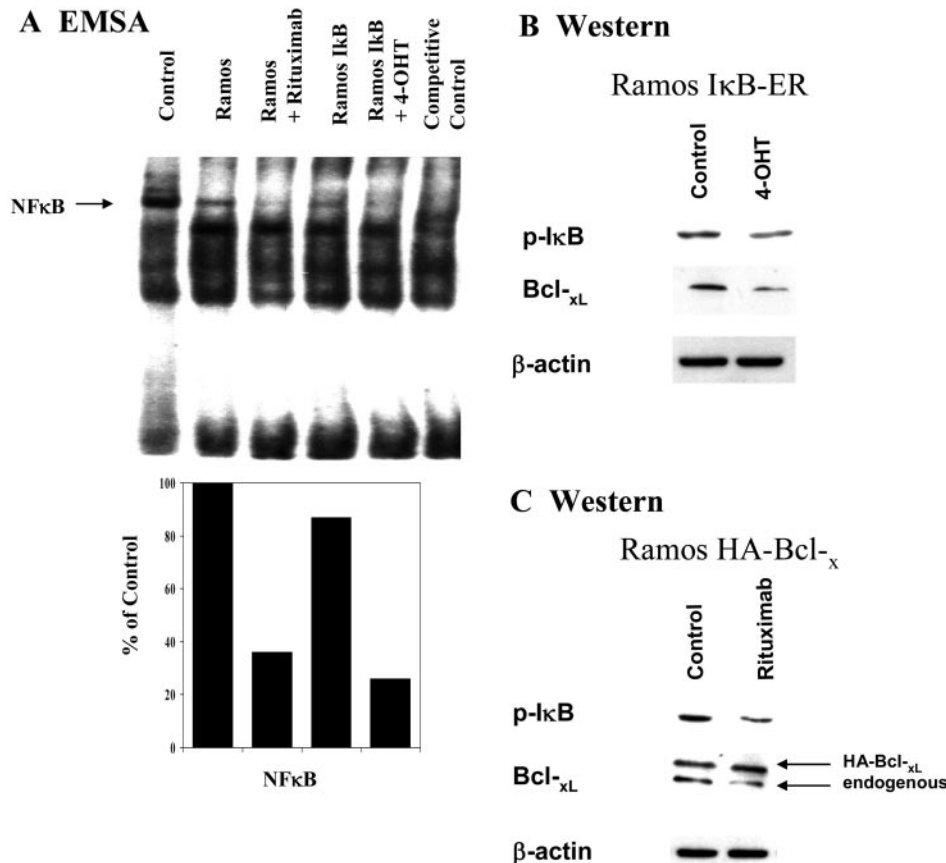

FIGURE 3. Rituximab-mediated inhibition of Bcl-x$_L$ expression via inhibition of NF-κB activity. *A*, Inhibition of NF-κB DNA-binding activity in Ramos cells by expression of dominant active IκB. Ramos cells were transfected with a dominant negative IκB under regulation by 4-OHT. Ramos-IκB cells in which IκB can be activated by 4-OHT (200 nM; for 8 h) were compared with wild-type Ramos cells for the effect of rituximab treatment on inhibition of NF-κB activity by EMSA. Densitometric analysis was also performed. *B*, Inhibition of Bcl-x$_L$ expression (Western blot) by Ramos-IκB-ER after activation of IκB by 4-OHT. Ramos-IκB-ER cells were untreated or were treated with 4-OHT (200 nM) for 8 h, and cell lysates were prepared and analyzed by Western blot for p-IκB and Bcl-x$_L$. *C*, Inhibition of only endogenous Bcl-x$_L$ expression by rituximab in Ramos-HA-Bcl-x cells. Ramos cells overexpressing Bcl-x$_L$ were treated with rituximab (20 μg/ml) for 16 h, and cell lysates were analyzed for p-IκB and Bcl-x$_L$ by Western blot. The *upper band* with higher m.w. represents the HA-Bcl-x$_L$ overexpression, and the *lower band* the endogenous Bcl-x$_L$. The data are representative of two independent experiments.
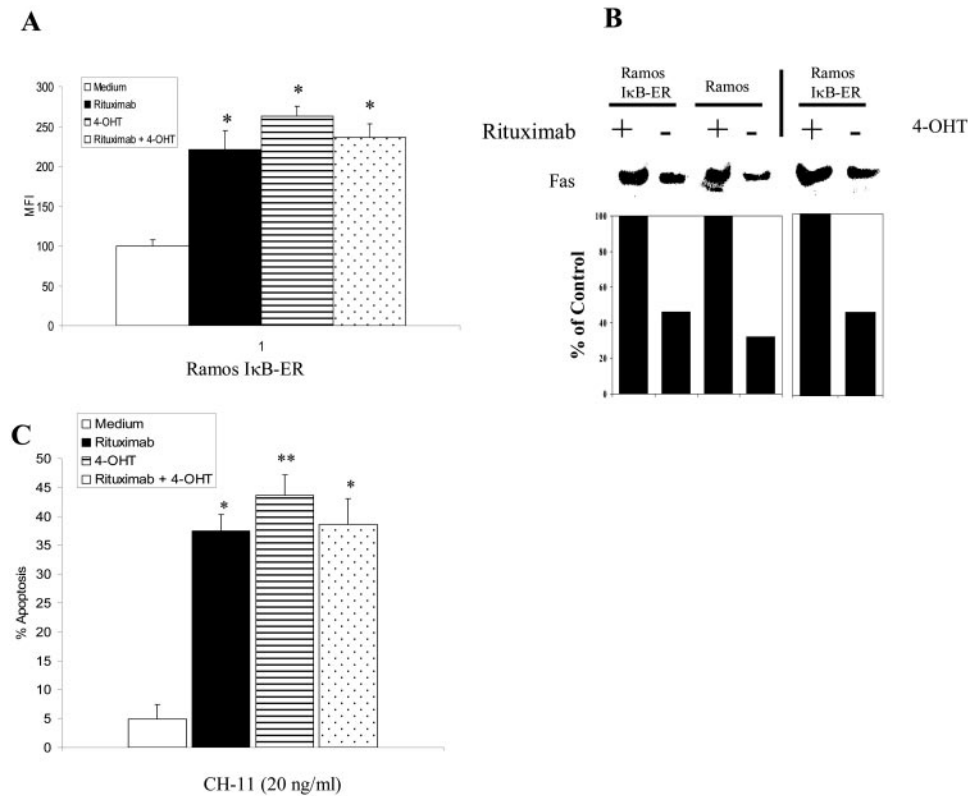
A

B

C

FIGURE 4. Rituximab-mediated up-regulation of Fas expression via inhibition of NF-κB activity. *A*, Surface Fas expression. Ramos-IκB-ER cells were treated with rituximab (20 μg/ml) in the presence or the absence of 4-OHT (200 nM) and tested for surface Fas expression by flow cytometry. *, $p < 0.05$. *B*, Fas expression by Western blot. Ramos and Ramos-IκB-ER cells were either left untreated or treated with rituximab (20 μg/ml) or 4-OHT (200 nM) for 6 h, and total cell lysates were tested for Fas by Western blot. *C*, Rituximab-mediated sensitization to CH-11-induced apoptosis via inhibition of NF-κB. Ramos-IκB-ER were treated with rituximab (20 μg/ml) in the presence or the absence of 4-OHT (200 nM) for 6 h. Thereafter, the cells were washed, fresh medium was added, and cells were incubated with 20 μg/ml CH-11 for an additional 18 h and examined for apoptosis. Results are presented as the mean $\pm$ SD of duplicate samples. *, $p < 0.05$; **, $p < 0.02$.

The direct role of YY1 in rituximab-mediated sensitization to CH-11-induced apoptosis was examined. Treatment of Ramos cells with rituximab resulted in inhibition of YY1 protein expression (Fig. 6*C*) and YY1 DNA-binding activity (Fig. 6*D*). Likewise, treatment of the cells with the NF-κB inhibitors (Bay11-7085 and DHMEQ) or the NO donor, DETANONOate, resulted in inhibition of both YY1 expression (Fig. 6*C*) and YY1 DNA-binding activity (Fig. 6*D*). These findings demonstrate that rituximab inhibits both YY1 expression and DNA-binding activity and correlated with rituximab-mediated inhibition of NF-κB activity (Fig. 4*A*). The direct role of YY1 in the negative regulation of Fas expression and sensitivity to CH-11-induced apoptosis was corroborated by the use of cells transfected with YY1 siRNA, whereby the transfectants showed significantly lower levels of YY1 expression and were significantly sensitized to CH-11-induced apoptosis, and the extent of apoptosis was a function of the CH-11 concentration used. The specificity of the siRNA was shown by transfection of the cells with a negative siRNA control, which did not show any sensitization to CH-11 (Fig. 6*E*).

The findings presented above provide clear evidence that rituximab-mediated up-regulation of Fas expression and sensitization to Fas agonist-induced apoptosis are the results of rituximab-mediated inhibition of YY1 expression and DNA-binding activity.

Discussion

The present study provides evidence demonstrating that chemoresistance and Fas resistance in B-NHL cell lines are commonly regulated by constitutive NF-κB activation. However, downstream

of NF-κB, chemoresistance and Fas resistance are differentially regulated by Bcl-x$_L$ and YY1, respectively. Rituximab-mediated inhibition of NF-κB activity resulted in both the inhibition of Bcl-x$_L$ expression and chemosensitization and the inhibition of the transcription repressor YY1 and sensitization to CH-11-induced apoptosis. These differentially regulated mechanisms for chemo- and CH-11-induced apoptosis emanated from findings making use of both biologically engineered cell lines and specific chemical inhibitors. Treatment with rituximab or specific inhibitors of NF-κB sensitized NHL cells to both drug- and CH-11-induced apoptosis. The role of Bcl-x$_L$ expression in the regulation of drug resistance, but not Fas resistance, was demonstrated by the failure of rituximab to sensitize Bcl-x$_L$-overexpressing Ramos cells (Ramos HA-Bcl-x) to drug-induced apoptosis, although these same cells were sensitized by rituximab, like the wild type, to CH-11-induced apoptosis. However, inhibition of Bcl-x$_L$ expression in Ramos-IκB-ER or by various NF-κB inhibitors sensitized the cells to CDDP-induced apoptosis. Like rituximab, inhibition of NF-κB either by specific chemical inhibitors or by 4-OHT treatment of Ramos-IκB-ER cells sensitized the cells to CH-11-induced apoptosis. The resistance to Fas-induced apoptosis was found to be regulated by the transcription repressor YY1, whereby inhibition of YY1 expression or activity by rituximab, chemicals, or YY1 siRNA resulted in up-regulation of Fas expression and sensitization to CH-11-induced apoptosis. These findings clearly establish distinct regulatory mechanisms modulated by rituximab in B-NHL cells, downstream of NF-κB for the sensitization to
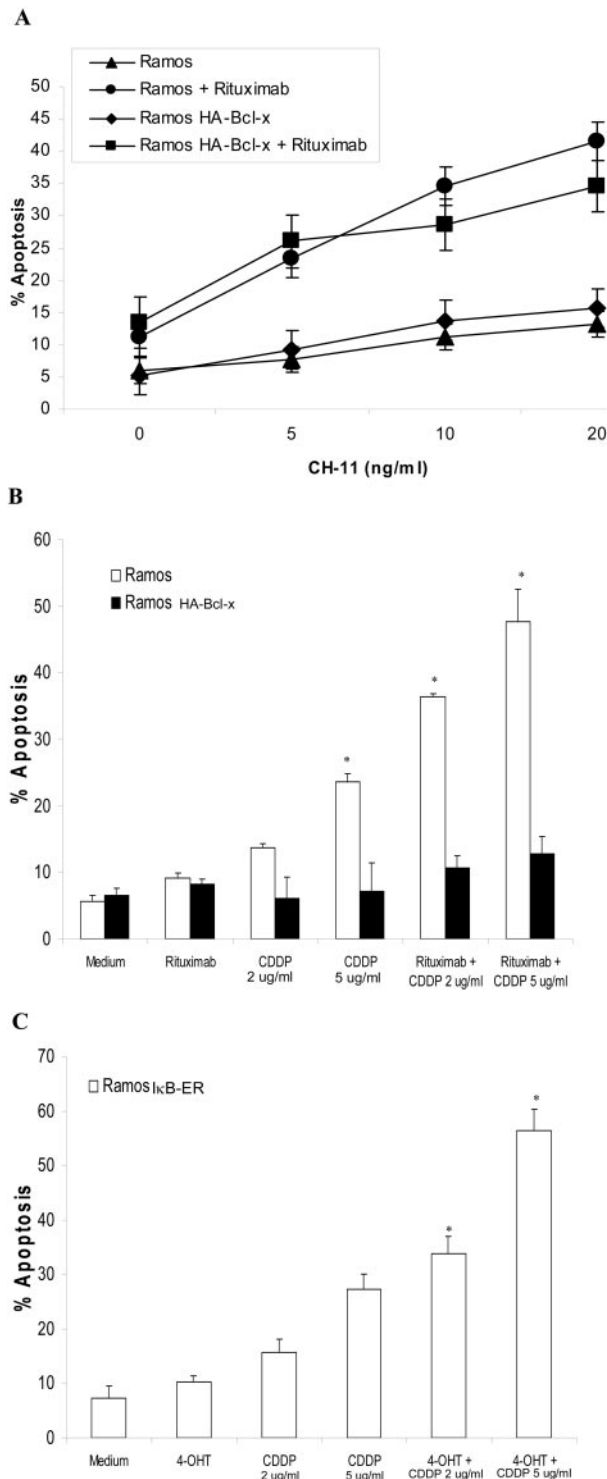
FIGURE 5. Rituximab-mediated sensitization of Ramos and Ramos-Ha-Bcl-x tumor cells to CH-11-induced apoptosis. *A*, Ramos and Ramos-HA-Bcl-x cells were treated with rituximab (20 μg/ml) for 6 h, treated with different concentrations of CH-11 for 18 h, and tested for apoptosis by caspase 3 activation as described in Fig. 4*C*. Results are presented as the mean $\pm$ SD of duplicate samples. *B*, Role of Bcl-x_L in resistance to drug-induced apoptosis. Ramos and Ramos-HA-Bcl-x cells were treated with rituximab (20 μg/ml), CDDP (2 and 5 μg/ml), and their combination for 24 h. The cells were analyzed for apoptosis by caspase 3 activation. *, $p <$ 0.05. *C*, Sensitization of Ramos cells to CDDP-induced apoptosis via inhibition of NF-κB. Ramos-IκB-ER cells were left untreated or treated with CDDP (2 and 5 μg/ml), 4-OHT (200 nM), or their combination for 18 h and tested for apoptosis. Results are presented as the mean $\pm$ SD of duplicate samples. *, $p < 0.05$

drugs and to Fas-induced apoptosis. These findings may have clinical implications and suggest that overexpression of Bcl-x_L in tumors, which are refractory to treatment with chemotherapy alone or in combination with rituximab, may still be sensitive to killing by rituximab in combination with immunotherapy.

The present findings demonstrate that rituximab inhibits NF-κB activity, as assessed by EMSA, and corroborate recent findings in our laboratory (8, 10, 14). The inhibition of NF-κB by rituximab resulted in the sensitization of B-NHL cells to both drug- and CH-11-induced apoptosis. Inhibition of NF-κB activity by various chemical inhibitors or in cells with dominant negative IκB mimicked rituximab-mediated chemo- and immunosensitization. The mechanism by which rituximab inhibits NF-κB activity is not clear. In ARL NHL cells, we have demonstrated that rituximab inhibits the p38 MAPK pathway and, consequently, NF-κB activity (8). In non-ARL NHL cells, we have demonstrated that rituximab inhibits NF-κB activity through the induction of Raf-1 kinase inhibitor protein (14), which interferes with activation of the NF-κB pathway (25).

Rituximab-mediated chemosensitization of B-NHL cell lines results from the down-regulation of Bcl-x_L expression in NHL (9, 10, 14); these findings were confirmed in this study with down-regulation of Bcl-x_L expression in Ramos cells. Visual inspection and computer analysis have revealed NF-κB binding sites on the Bcl-x promoter, and NF-κB has been shown to be responsible in part for Bcl-x_L gene expression (13, 14). In addition, activation of the NF-κB pathway by various stimuli rescues tumor cells from drug-induced apoptosis via up-regulation of Bcl-x_L expression (15, 23). Thus, Bcl-x_L is most likely a downstream target of the NF-κB pathway (23, 24, 26–28). This is supported in this study by the findings in Ramos IκB-ER, whereby NF-κB inhibition also resulted in Bcl-x_L down-regulated expression. The role of Bcl-x_L in the regulation of chemoresistance is shown in Ramos cells overexpressing Bcl-x_L, Ramos HA-Bcl-x. These cells were not significantly sensitized to CDDP-mediated apoptosis compared with the wild type. We have reported previously that rituximab sensitizes the 2F7 Burkitt lymphoma cell line to CDDP-induced apoptosis. CDDP did not modify any of the various apoptotic regulatory gene products, although it partially affected the mitochondrial membrane potential and the release of reactive oxygen species (29). Rituximab treatment of Ramos HA-Bcl-x still down-regulated the constitutive level of Bcl-x_L, but not the transduced level of Bcl-x_L, and this down-regulation was not sufficient to sensitize the cells to CDDP-induced apoptosis. We have recently demonstrated that inhibition of Bcl-x_L function by 2-methoxyantimycin-A3 sensitized Ramos HA-Bcl-x to drug-induced apoptosis (10, 14). The role of Bcl-x_L in the regulation of drug resistance was corroborated in studies using Ramos IκB-ER cells, in which inhibition of NF-κB resulted in down-regulation of Bcl-x_L expression and chemosensitization in the absence of rituximab.

In contrast to the regulation of drug resistance by Bcl-x_L expression, we demonstrate that the level of Bcl-x_L expression does not regulate the sensitivity of B-NHL cells to Fas-induced apoptosis. This idea is supported by the demonstration that Bcl-x_L-overexpressing cells were as sensitive as the parental Ramos cells to rituximab-mediated sensitization to CH-11-induced apoptosis. The regulation of Fas resistance by NF-κB was found to be the result of NF-κB-mediated regulation of the transcription repressor YY1. YY1 has been shown to negatively regulate Fas expression in ovarian and prostate carcinoma cell lines and also regulates resistance to Fas-induced apoptosis (12). The present findings in B-NHL cells showed that inhibition of YY1 resulted in the up-regulation of Fas expression and sensitization to Fas-induced apoptosis. We demonstrate that rituximab treatment of Ramos cells
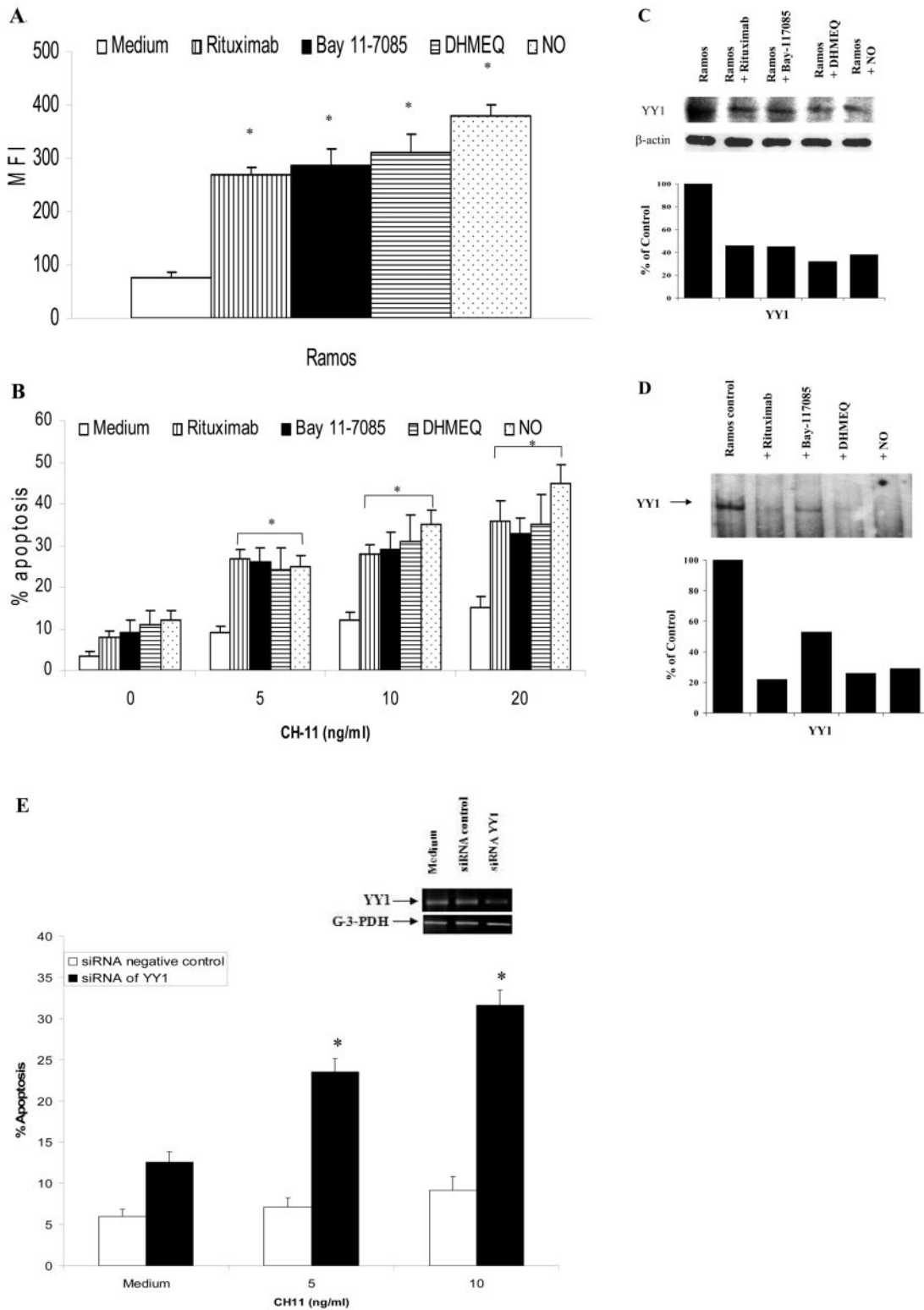
FIGURE 6. Sensitization to CH-11-induced apoptosis by inhibition of NF-κB and YY1. *A*, Surface Fas expression. Ramos cells were treated with rituximab (20 μg/ml), Bay 11-7085 (5 μM), DHMEQ (10 μg/ml), or NO donor DETANONOate (1000 μM) for 6 h, and surface Fas expression was examined by flow cytometry. ∗, $p < 0.05$. *B*, Sensitization of Ramos to CH-11-induced apoptosis by inhibition of NF-κB. Ramos cells were treated with rituximab (20 μg/ml), Bay-11-7085 (6 μM), DHMEQ (10 μg/ml), or the NO donor DETANONOate (1000 μM) for 6 h, followed by treatment with different concentrations of CH-11 (5, 10, and 20 ng/ml) for 18 h. ∗, $p < 0.01$. *C*, Inhibition of YY1 expression by rituximab. Ramos cells were left untreated or treated with rituximab (20 μg/ml), Bay-11-7085 (6 μM), DHMEQ (10 μg/ml), or the NO donor DETANONOate (1000 μM) for 18 h. The cell lysates were tested for YY1 expression by Western blot (*top panel*) and by densitometric analysis (*bottom panel*). *D*, Inhibition of YY1 DNA-binding activity by rituximab. Ramos cells were treated as described above, and nuclear lysates were analyzed for YY1-DNA-binding activity by EMSA. The data are representative of two independent experiments. *E*, Role of YY1 in the resistance to CH-11-induced apoptosis. PC3 cells (2.5×10^6) were transfected with 10 μl of Superfect and 10 μl of YY1 siRNA or control siRNA. After 48 h, the cells were treated with CH-11 (5 and 10 ng/ml), and apoptosis was measured. The inhibition of YY1 siRNA was tested by RT-PCR (*top panel*). ∗, $p < 0.03$ compared with control nontransfected cells.

FIGURE 7. A schematic diagram illustrating rituximab-mediated inhibition of NF-κB and the roles of YY1 and Bcl-x$_L$ in the regulation of Fas- and drug-induced apoptosis, respectively. Treatment of Ramos B-NHL cells with rituximab inhibited NF-κB activity and resulted in sensitization of the cells to both Fas- and drug-induced apoptosis. However, downstream of NF-κB, there was distinct, yet complementary, signaling in the regulation of Fas and drug sensitivity. YY1 inhibition by NF-κB resulted in the up-regulation of Fas and sensitization to Fas apoptosis, independent of Bcl-x$_L$ levels. However, rituximab-mediated inhibition of Bcl-x$_L$ expression was pivotal for sensitization to drug-induced apoptosis.
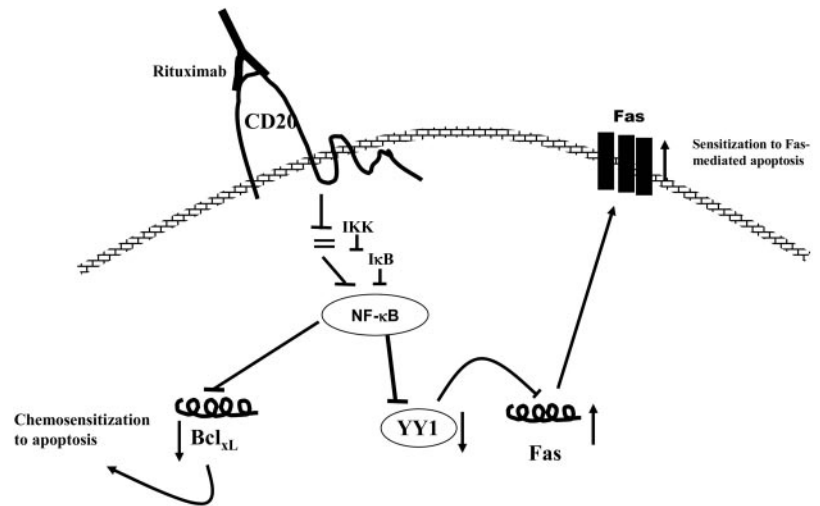
resulted in inhibition of YY1 expression and YY1 DNA-binding activity concomitant with the inhibition of NF-κB activity. The inhibition of NF-κB and YY1 activities by chemical inhibitors mimicked the inhibition produced by rituximab. The involvement of YY1 in the regulation of Fas expression and sensitivity of Ramos cells to CH-11-induced apoptosis was demonstrated by inhibition of YY1 by chemicals (such as NO or NF-κB inhibitors) or YY1 siRNA, all of which resulted in up-regulation of Fas expression and sensitization to CH-11-induced apoptosis in the absence of rituximab. These findings establish YY1 as a negative regulator of Fas expression and as a resistance factor in B-NHL cells, where its inhibition by rituximab sensitizes the cells to Fas-induced apoptosis.

Activation of NF-κB is emerging as one of the major mechanisms of tumor cell resistance to drugs (5, 22–24, 30, 31). Thus, interruption of this pathway is a target for therapeutic intervention for the treatment of tumors that has proven successful in enhancing the apoptotic effects of anticancer agents (e.g., TNF-α and carnitine palmitoyl transferase-11), resulting in tumor regression in vivo (32). Targeted suppression of Bcl-x$_L$ expression facilitated drug-induced B cell leukemia tumor regression in the SCID/NOD-Hu in vivo model (33). Thus, NF-κB and Bcl-x$_L$ inhibition augments drug-, Fas-, and TNF-α-induced apoptosis in various tumor model systems. Currently, rituximab in combination with chemotherapy improves the clinical response in patients significantly compared with treatment with either agent alone (34). However, several reports demonstrate that certain patients are not responsive to treatment by rituximab or a combination of drugs, and the underlying mechanisms of resistance are not clear.

It is possible that resistance of the tumor cells may have resulted from the overexpression of antiapoptotic gene products, such as Bcl-x$_L$, which was shown in this study in Ramos cells and other drug-induced B-NHL cells. It is possible to consider therapies aimed at reducing or inhibiting the expression of antiapoptotic gene products to reverse acquired or induced resistance. Based on our studies, this may be achieved by inhibition of NF-κB activity directly or inhibition of the antiapoptotic members of the Bcl-2 family. The underlying mechanisms of resistance of B-NHL by rituximab are not clear. There have been several reports demonstrating that resistance may be due to loss of the CD20 receptor, overexpression of complement inhibitors, inhibition of signaling for apoptosis, and inhibition of Ab-dependent cellular cytotoxicity due to the polymorphism of the FcRs (5, 35, 36). It is also possible that tumor cells overexpress antiapoptosis regulatory proteins,

such as Bcl-2 or Bcl-x$_L$ (7–11, 14, 37). The long, alternatively spliced variant of the Bcl-x gene, Bcl-x$_L$, shares a high degree of structural and functional similarity with the antiapoptotic protein Bcl-2 and exerts its protective effects mainly in the membrane of mitochondria by preventing loss of membrane potential, cytochrome c efflux upon apoptotic stimuli, and the initiation of apoptosis (38). Bcl-x$_L$ is predominantly expressed in lymphomas (39, 40) and antagonizes DNA-damaging agents, metabolic, microtubule, and topoisomerase inhibitors. Through modulation of apoptosis, Bcl-x$_L$ plays a major role in determination of the cellular response to a wide variety of apoptosis-inducing stimuli (41, 42). It is not clear whether overexpression of such molecules can also affect immune-mediated apoptosis of rituximab-treated cells. We show in this study that overexpression of Bcl-x$_L$ does not inhibit Fas-induced apoptosis in Ramos NHL cells.

The present findings demonstrate that rituximab sensitizes B-NHL cell lines to apoptotic stimuli by two different and complementary signaling pathways downstream of NF-κB. The down-regulation of Bcl-x$_L$ sensitizes to drug-induced apoptosis, and inhibition of YY1 sensitizes to Fas-induced apoptosis (see scheme in Fig. 7). Clearly, these findings establish that inhibition of NF-κB will sensitize B-NHL cells to both drugs and immunotherapy. Furthermore, although rituximab fails to chemosensitize Bcl-x$_L$-overexpressing cells, it can sensitize these cells to Fas-induced apoptosis. The present findings provide new evidence that chemoresistance in B-NHL cells after treatment with rituximab and drugs in combination may still be amenable for immunotherapeutic intervention in combination with rituximab. Our findings strongly suggest that rituximab sensitizes drug-resistant tumor cells to Fas-induced apoptosis. Therefore, it may be conceivable that treatment with rituximab in combination with activation of host immune cells to express death-inducing TNF family ligands may result in host-induced killing of the resistant tumor cells. These findings establish several targets (e.g., NF-κB, YY1, or Bcl-x$_L$) for intervention in rituximab/drug refractory cells.

Disclosures

The authors have no financial conflict of interest.

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