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# Rituximab inhibits p38 MAPK activity in 2F7 B NHL and decreases IL-10 transcription: Pivotal role of p38 MAPK in drug resistance

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**We have recently reported that Rituximab (anti-CD20) sensitizes drug-resistant 2F7 and 10C9 B Non-Hodgkin's lymphoma (NHL) cell lines to the apoptotic effects of various chemotherapeutic drugs by downregulation of IL-10 and Bcl-2 expression. The mechanism by which Rituximab induces downregulation of IL-10 was examined. We hypothesized that Rituximab may inhibit p38 MAPK activity that regulates IL-10 expression via Sp1. Treatment of 2F7 cells with Rituximab or the p38 inhibitor SB203580 inhibited the constitutive p38 MAPK activity and resulted in the inhibition of Sp1, IL-10, STAT3, and Bcl-2. Inhibition of the Src-family PTKs, Lyn, and Src-family PTKs upstream signaling molecules of the p38MAPK pathway, by PP2, a specific Src-family kinase inhibitor, resulted in the inhibition of p38MAPK and IL-10 expression. In addition to p38 MAPK, Rituximab also inhibited NF-κB activity. Inhibition of the Src PTKs, MAPK, and NF-κB activities by Rituximab or by specific chemical inhibitors sensitized the cells to CDDP-mediated apoptosis. The above signaling-mediated effects by Rituximab were observed with similar kinetics beginning at 1 h following treatment. Thus, altogether, these results demonstrate that signaling by Rituximab results in the inhibition of the p38MAPK pathway, which in turn inhibits the transcription of IL-10 via Sp1. Inhibition of the IL-10 autocrine/paracrine loop results in the inhibition of STAT3 activity and, consequently, inhibition of Bcl-2 expression and sensitization to drugs-apoptosis. Further, Rituximab-mediated signaling identifies several new intracellular targets in NHL that may be of potential therapeutic interest for the development of new drugs in the treatment of drug-refractory NHL tumor cells.**
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## Introduction

Currently, 45 000 new cases of lymphoma have been reported, which makes it the sixth leading cause of cancer death. This is in part due to the frequency of lymphoma in the AIDS population, which has increased in the past few decades (Levine, 1992). Non-Hodgkin's lymphoma (NHL) includes a large group of neoplasms of the immune system. All B cells express CD19, and approximately 50% express CD20 and some CD10 (Freedman and Nadler, 1991). The standard treatment for NHL is chemotherapy. However, many patients become refractory to chemotherapy (Foran, 2002). Recently, treatment of patients with NHL with monoclonal antibodies has provided an alternative therapeutic approach to this malignant disease.

Rituximab is a monoclonal antibody that specifically targets the CD20 antigen on the surface of normal and malignant human B cells (Reff *et al*., 1994). Its primary use has been for the treatment of follicular and low-grade NHL, but is now in wide use for an assortment of B-cell cancers and proliferative disorders (Huhn *et al*., 2001; Coiffier, 2003). CD20 is a cell surface phosphoprotein that is expressed specifically within the B-cell lineage from pre-B cells to mature B cells (Stashenko *et al*., 1980), but lost following the differentiation of B cells into plasma cells. The function of CD20 is not yet fully understood. Its role has been implicated in the differentiation, proliferation in B-lymphocyte development (Tedder and Engel, 1994) and transmembrane calcium flux (Riley and Sliwkowski, 2000). CD20 is functionally important for regulating cell cycle progression and signal transduction in B cells. CD20 is constitutively associated with lipid rafts and this association is dependent on cholesterol and on a short membrane-proximal cytoplasmic sequence (Polak *et al*., 1998).

Although the mechanism by which Rituximab mediates its effect *in vivo* is not known, previous studies have suggested that the antitumor effects are mediated by several mechanisms including antibody-dependent cellular cytotoxicity, complement-mediated cell lysis (Reff *et al*., 1994) and induction of apoptosis by CD20 crosslinking (Shan *et al*., 1998). Crosslinking of Rituximab-induced apoptosis was dependent on the activity of p38MAPK in B-cell chronic lymphocytic leukemia

(B-CLL) (Pedersen *et al*., 2002). Our laboratory has previously reported that Rituximab can reverse *in vitro* the drug-resistant phenotype of NHL tumor cell lines to a drug-sensitive phenotype (Demiden *et al*., 1997; Alas *et al*., 2001; Jazirehi *et al*., 2003). Studies with NHL B showed that Rituximab downregulates the transcription and secretion of tumor-derived IL-10 and the expression of Bcl-2 (Alas and Bonavida, 2001) or Bcl-$_{xL}$ (Jazirehi *et al*., 2003). Inhibition of IL-10/IL-10 R interaction by an autocrine/paracrine loop results in the decrease of the constitutively activated STAT3 and subsequent inhibition of Bcl-2 transcription and expression (Alas and Bonavida, 2001). The downregulation of Bcl-2 by Rituximab is responsible in large part for the sensitization of the NHL tumor cells lines to various chemotherapeutic drugs (e.g. CDDP, ADR, Vinblastine) (Alas *et al*., 2001). These findings demonstrated that IL-10 is a chemoresistant factor in NHL.

The mechanism by which IL-10 is regulated by Rituximab is not known. It has been reported that IL-10 induction is accompanied by an enhanced phosphorylation of p38/SAPK2 in the Burkitt's lymphoma cell line BL2 (Vockerodt *et al*., 2001). Other studies have shown that activation of the mitogen-activated protein kinase (MAPK) signaling pathway regulates the activation of Sp1, which in turn regulates the transcription of the hIL-10 gene (Pedersen *et al*., 2002). Therefore, we hypothesized that Rituximab downregulation of IL-10 in 2F7 NHL cells may be mediated through the p38 MAPK pathway. This study examined (1) the effect of Rituximab on the p38 MAPK signaling pathway; (2) the effect of Rituximab on Sp1 DNA-binding activity and its role in IL-10 transcription and secretion; (3) the correlation between the Rituximab effect on the p38 MAPK signaling pathway and its effect on STAT3 activity and Bcl-2 expression and (4) the role of p38 MAPK pathway in drug resistance.

## Results

### *Inhibition of IL-10 secretion from 2F7 cells by Rituximab is mediated via the p38 MAPK signaling pathway*

We have demonstrated that Rituximab downregulates the expression and secretion of IL-10 in 2F7 cells (Alas *et al*., 2001). This inhibition causes a decrease in the constitutively activated STAT3, downregulation of Bcl-2 expression and the tumor cells become sensitive to apoptotic stimuli, including the effect of various chemotherapeutic drugs (Alas and Bonavida, 2001). Previous reports showed that IL-10 regulation is via p38 MAPK activity (Ma *et al*., 2001; Vockerodt *et al*., 2001). We hypothesized, therefore, that p38 MAPK may regulate IL-10 synthesis in the 2F7 cell line. 2F7 tumor cells were treated with 10 $\mu$M of the specific inhibitor of p38 MAPK (SB203580) or with 20 $\mu$g/ml Rituximab for different time intervals (3, 6, 12, and 24 h) and IL-10 secreted into the medium was determined by ELISA. As shown in Figure 1a, treatment of 2F7 cells with either Rituximab or SB203580 significantly decreased IL-10 secretion (ranging from 50 to 95%) at 3, 6, 12, and 24 h. The inhibition of IL-10 by SB203580 correlated with that achieved by Rituximab. These results suggested that the p38 MAPK signaling cascade is involved in IL-10 transcription in the 2F7 tumor cells, and inhibition of

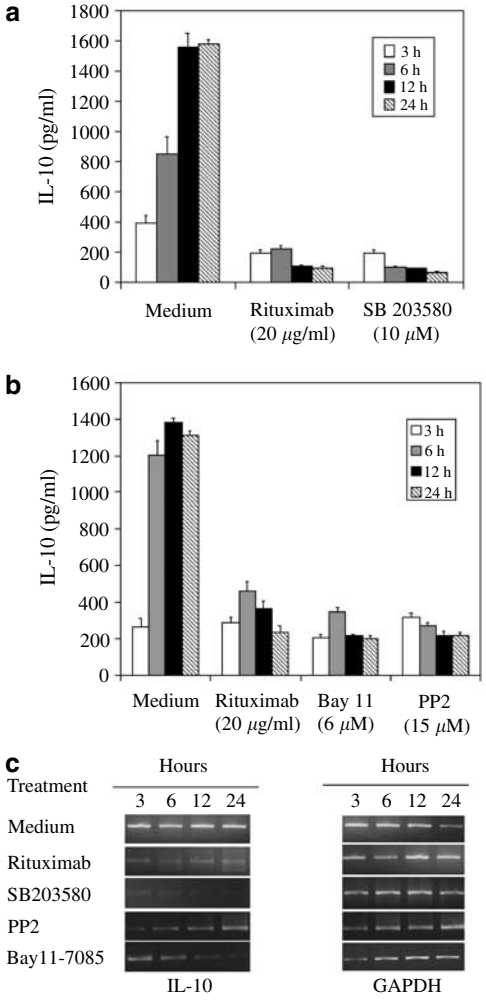



**Figure 1** Regulation of IL-10 transcription and secretion by p38 MAP kinase in 2F7 cells. The 2F7 cells were either left untreated or treated for 3, 6, 12, and 24 h at 37° with Rituximab or inhibitors. (**a**) The 2F7 cells were grown in a complete medium in the presence or absence of Rituximab (20 $\mu$g/ml) or with SB203580 (10 $\mu$M). IL-10 secretion was determined by ELISA. IL-10 secretion was significantly decreased by SB203580 at 6, 12, and 24 h ($P < 0.05$), similar to the downregulation induced by Rituximab. (**b**) The specific inhibitors of Src family kinases (PP2 15$\mu$M) and of NF-$\kappa$B (Bay 11-7085 6$\mu$M) downregulate the secretion of IL-10 in 2F7 cells. The inhibition of IL-10 secretion was statistically significant in all treated samples compared with the untreated control ($P < 0.02$). (C) The 2F7 cells were grown in a complete medium in the presence or absence of Rituximab (20 $\mu$g/ml), SB203580 (10 $\mu$M), PP2 (15 $\mu$M), or Bay 11-7085 (6 $\mu$M) for various time points ranging from 3 to 24 h. The cells were harvested and mRNA was isolated. IL-10 expression was determined by reverse transcriptase-based PCR analysis using GAPDH as an internal control for DNA loading. Transcription of IL-10 was significantly decreased by SB203580, Bay 11-7085, and PP2 at 6, 12, and 24 h, similar to the downregulation induced by Rituximab

this signaling pathway by Rituximab results in the downregulation of IL-10.

To further evaluate the involvement of the p38 MAPK signal pathway in the regulation of IL-10 secretion, 2F7 cells were treated with 15 $\mu$M of the specific inhibitor of Src family kinases PP2. The Src kinases are upstream of p38 MAPK. IL-10 secretion was inhibited by PP2 ($P<0.02$) to the same level as achieved by either SB203580 or Rituximab treatments (Figure 1b). These results strongly suggested that Rituximab inhibits the Src family kinases, which then inhibit MAPK activity and the transcriptional regulation of IL-10.

The IL-10 promoter contains many consensus-binding sites for various transcription factors such as Sp1 and NF-$\kappa$B (Kube *et al*., 1995; Eskdale *et al*., 1997; Ma *et al*., 2001). Sp1 is under the regulation of MAPK. To examine the role of NF-$\kappa$B in IL-10 transcription, 2F7 cells were treated with a specific inhibitor of NF-$\kappa$B, Bay 11-7085. IL-10 secretion was inhibited by Bay 11-7085 to the same extent achieved by either SB203580 or Rituximab treatments (Figure 1b). These findings suggest that NF-$\kappa$B regulates IL-10 expression.

The above findings that examined IL-10 production by ELISA were corroborated at the transcriptional level. Total RNA (1 $\mu$g) from 2F7 cells, untreated or treated with Rituximab (20 $\mu$g/ml), SB203580 (10 $\mu$M), PP2 (15 $\mu$M) or Bay 11-7085 (6 $\mu$M), was reverse transcribed; the resultant cDNAs were used to amplify the IL-10 transcripts. IL-10 amplicons were compared to the corresponding housekeeping gene GAPDH amplicons used as internal controls. As depicted in Figure 1c, IL-10 transcripts were approximately five-fold less in the cells treated with either Rituximab or inhibitors than in untreated cells. These findings corroborated the IL-10 secretion data determined by ELISA (Figure 1b). Altogether, the data demonstrate that Rituximab negatively signals the 2F7 cells through the Src/p38 MAPK pathway.

### *Rituximab downregulates p38 MAPK activity in 2F7 cells*

It has been reported that IL-10 induction is accompanied by an enhanced phosphorylation of p38 MAPK (Foey *et al*., 1998) and the above findings demonstrated that a highly specific inhibitor of p38 MAPK suppressed IL-10 expression. Therefore, p38 MAPK activity may be central for IL-10 gene expression. To verify whether downregulation of IL-10 expression following treatment of 2F7 cells with Rituximab is due to the inhibition of p38 MAPK activity, we analysed the phosphorylation of p38 MAPK in these cells. Using Western blot, time kinetics analyses showed that Rituximab inhibits phospho Lyn as early as 1–2 h following treatment and was maximal at 24 h (Figure 2a). Likewise, Rituximab inhibited phospho p38 MAPK with similar kinetics as for phospho Lyn (Figure 2b). We then analysed the effect of inhibitors of the p38 MAPK pathway. In addition, we analysed whether the Src family PTKs, which also contribute to IL-10 expression, are also involved in p38 MAPK activity. 2F7 cells were treated with either Rituximab (20 $\mu$g/ml) or the p38 MAPK inhibitor SB203580 (10 $\mu$M) for 3, 6, 12, and 24 h. Untreated 2F7 cells exhibited high levels of phosphorylated p38 and Rituximab inhibited p38 activity as early as 3 h (Figure 2c). Cells treated with SB203580 showed decreased levels of phosphorylated p38 MAPK as early as 3 h and complete inhibition at greater than 6 h. The specificity of SB203580-mediated effects on p38 MAPK was determined by showing that SB203580 does not inhibit JNK-1 and JNK-2 activities (Figure 2d). The inhibition of phospho Lyn by Rituximab and its role in the activation of p38 MAPK was corroborated by the use of the Src family PTK inhibitor PP2 for 24 h or with medium alone, and the cells were examined for the expression of phosphorylated p38 MAPK by Western blot. As depicted in Figure 2c, p38 MAPK activity was ablated by PP2 as early as 12 h and these findings were comparable to the cells treated with Rituximab. These findings suggested that Src family PTK activation is involved upstream of the p38 MAPK activation signaling pathway and Rituximab inhibits the Src kinases.

Recently, it has been reported that the p38 MAPK pathway coregulates with NF-$\kappa$B in the LMP1-mediated IL-6 and IL-8 genes expression (Eliopoulos *et al*., 1999), suggesting a link in this signaling pathway in the regulation of cellular gene expression. We investigated if there exists a correlation between the activity of p38 MAPK and NF-$\kappa$B in the regulation of IL-10 expression in 2F7 cells. These cells were treated with Bay 11-7085 (6 $\mu$M), a specific inhibitor of I$\kappa$B$\alpha$ phosphorylation, for 3, 6, 12, and 24 h. Bay 11-7085 decreased the levels of phospho p38 MAPK at 12 h (Figure 2c), which was correlated with the downregulation of IL-10 (Figure 1b). Inhibition of phospho p38 MAPK by Bay 11-7085 was consistent with a previous observation by Hu *et al*. (2001). In addition, the superinduction of p38 MAPK at 24 h was consistent with the findings of Hu *et al*. (2001). However, when 2F7 cells were treated with another NF-$\kappa$B inhibitor, SN50, which inhibits the translocation of NF-$\kappa$B into the nucleus (Lin *et al*., 1995), no inhibition of p38 MAPK activity was detected (Figure 2e). These findings are consistent with NF-$\kappa$B being downstream of p38 MAPK (Berghe *et al*., 1998; Carter *et al*., 1999).

### *Rituximab decreases Sp1 and NF-κB DNA-binding activities in 2F7 cells*

The Sp1 transcription factor that is activated by p38 MAPK is involved in the regulation of IL-10 gene transcription in several cell lines (Ma *et al*., 2001). We determined whether the downregulation of p38 MAPK activity mediated by Rituximab treatment decreases Sp1 DNA-binding activity in 2F7 cells. Gel-shift analysis of Sp1 activity was performed. 2F7 cells were left untreated or treated with Rituximab (20 $\mu$g/ml) for different time intervals (1–24 h) Two Sp1 bands, Sp1(A) and Sp1(B), were detected as described previously (Ma *et al*., 2001). The findings demonstrate that Rituximab decreases Sp1(A) (Figure 3a). The specificity of these two forms was demonstrated by the use of cold Sp1 oligonucleotide, which competed for both forms (data not shown). We then examined chemical inhibitors (SB203580

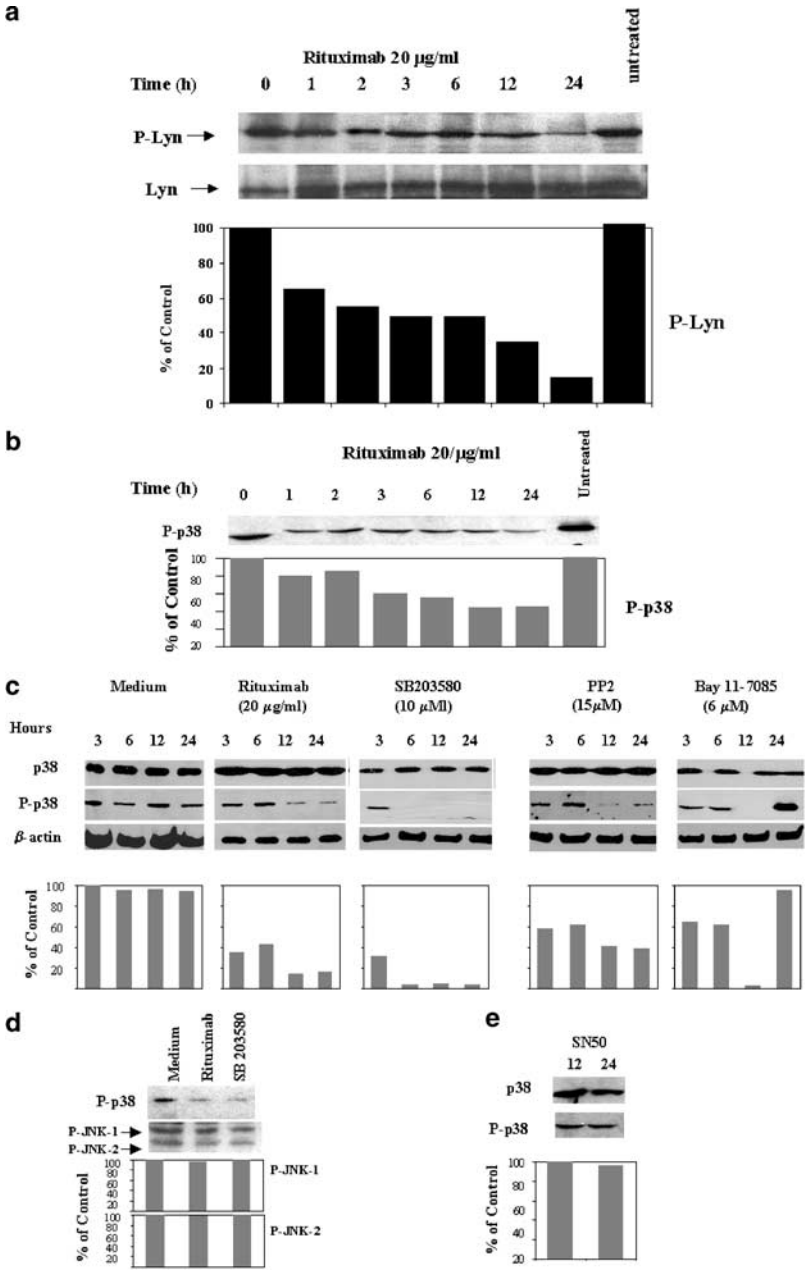
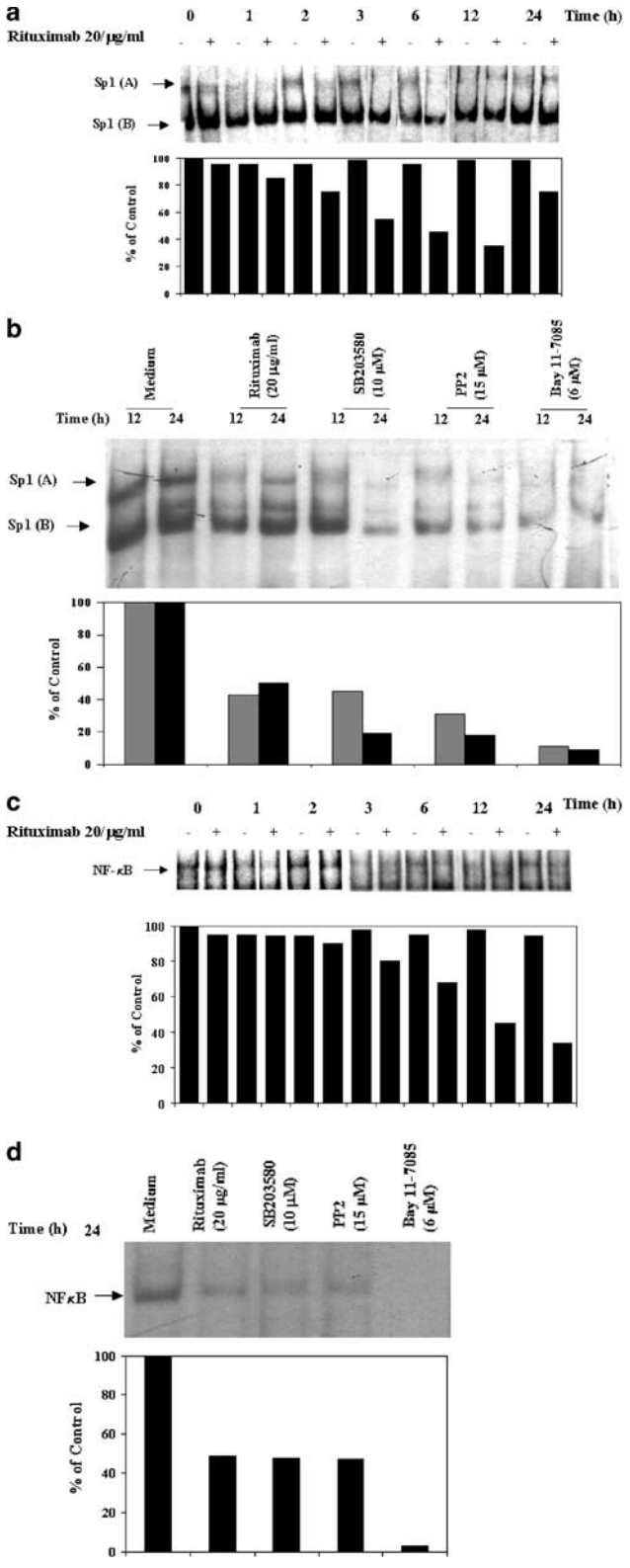



**Figure 2** Rituximab downregulates p-Lyn and p-p38 MAPK activity in 2F7 cells. 2F7 cells were cultured in 12-well plates ($10^6$/ml) for 3, 6, 12, and 24 h in the presence or absence of Rituximab or inhibitors. (**a**) 2F7 cells were cultured at different time points in the presence or absence of Rituximab (20 $\mu$g/ml) and lysates tested for p-Lyn activity. The cells showed a constitutive activity of Src kinase p-Lyn as analysed by Western blot. Treatment with Rituximab inhibited p-Lyn as early as 1 h following treatment and was maximal at 24 h. The Western blots were analysed by densitometry to show the relative levels of corresponding proteins. The data are representative of two identical experiments. (**b**) A representative experiment is shown from two independent experiments. The 2F7 cells showed a constitutive activity of p38, and treatment with Rituximab inhibited p-p38 MAPK as early as 1–2 h following treatment and was maximal at 24 h. (**c**) 2F7 cells treated with Rituximab (20 $\mu$g/ml) or inhibitors SB283580 (10 $\mu$M), PP2 (15 $\mu$M), and Bay 11-7085 (6 $\mu$M) inhibited phosphorylation of p38 MAPK. Rituximab inhibited p38 MAPK activity at $<$ 3 h, SB 283580 at 3 h, and both PP2 and Bay 11-7085 at 3 h. In all cases, inhibition was maximal at 24 h except for Bay 11-7085. Bay induced hyperphosphorylation of p38 at 24 h as reported by Hu *et al*. (2001). DMSO-treated cells did not differ from the control. The $\beta$-actin levels confirmed equal loading of protein for all treated samples. (**d**) The 2F7 cells were cultured for 24 h in the presence or absence of Rituximab (20 $\mu$g/ml) or the p38 MAPK inhibitor, SB203580 (10 $\mu$M). While SB203580 inhibited p-p38, it did not inhibit p-JNK1 and p-JNK2. (**e**) 2F7 cells were cultured in the presence of NF-$\kappa$B inhibitor, SN50, (50 $\mu$M) and, in contrast to Bay 11-7085 in (**a**), it did not inhibit the phosphorylation of p38 MAPK

10 $\mu$M, PP2 15 $\mu$M or Bay 11-7085 6 $\mu$M) for 12 and 24 h. Inhibition of Sp1 was observed with the p38 MAPK inhibitor, SB203580, Src family kinases, PP2, and the inhibitor of NF-$\kappa$B, Bay 11-7085 (Figure 3b). Rituximab also inhibited NF-$\kappa$B DNA-binding activity as early as 3 h following treatment and was maximal at

24 h (Figure 3c). Also, the various inhibitors, like Rituximab, inhibited NF-$\kappa$B DNA-binding activity (Figure 3d). These data corroborate the above findings on the role of p38 MAPK and NF-$\kappa$B signaling pathways in the regulation of IL-10 transcription.



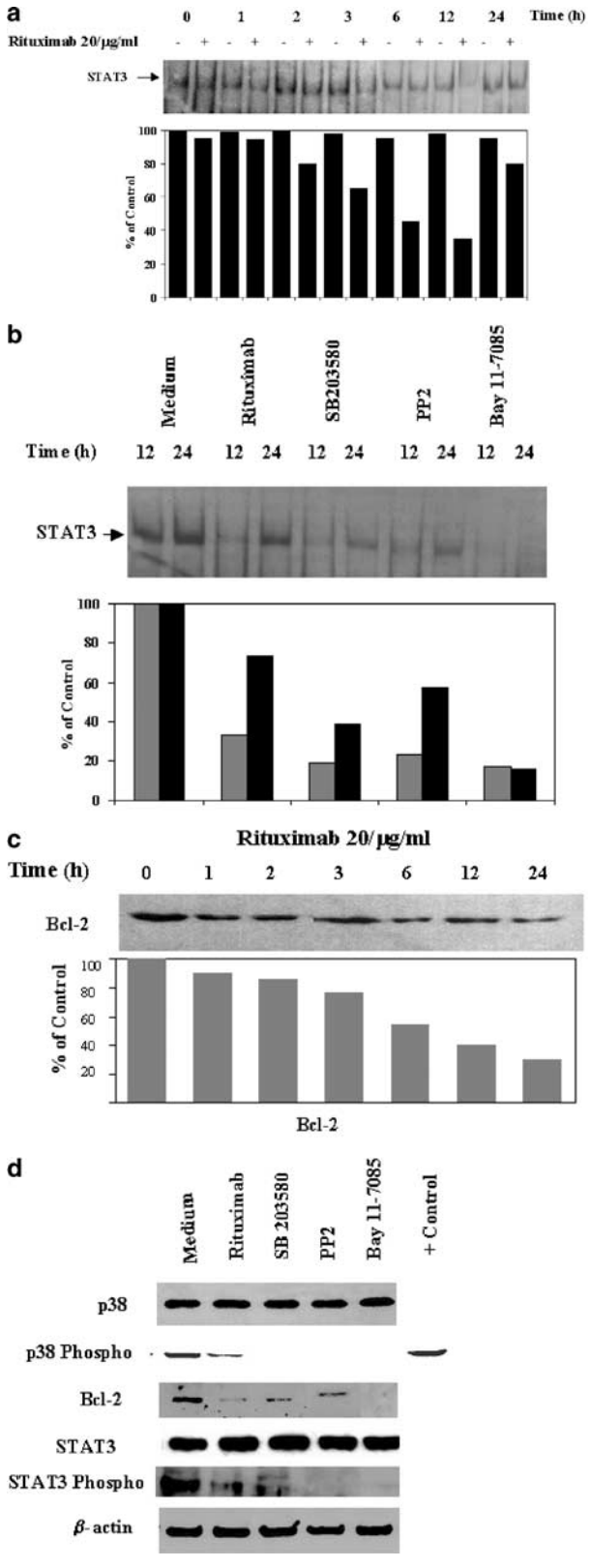

*Rituximab-mediated downregulation of p38 MAPK activity correlates with downregulation of STAT3 activity and Bcl-2 expression*

Rituximab decreases the activation of signal transducer and activator of transcription 3 (STAT3) factor, which in turn, is responsible for decreased levels of Bcl-2 in 2F7 cells (Alas and Bonavida, 2001). As shown above, Rituximab downregulates p38 MAPK (Figure 2) and Sp1 activities (Figure 3). The IL-10/IL-10 R autocrine/paracrine signal is responsible in part for the activation of STAT3. We analysed by electrophoretic mobility shift assays (EMSA) whether Rituximab-induced downregulation of p38 MAPK activity correlated with the downregulation of STAT3. 2F7 cells were left untreated or treated with Rituximab (20 $\mu$g/ml) for various time intervals and nuclear lysates were tested by EMSA. The findings demonstrate that Rituximab inhibits STAT3 DNA-binding activity as early as 2–3 h following treatment, and was maximal at 12 h (Figure 4a). We then examined the effect of the p38 MAPK inhibitor SB 203580 (10 $\mu$M), the Src kinases inhibitor PP2 (15 $\mu$M). DNA-binding of STAT3 activity was significantly inhibited by Rituximab treatment and by the different inhibitors (Figure 4b). These findings demonstrate that the downregulation of p38 MAPK, Sp1 and NF-$\kappa$B activities and IL-10 secretion mediated by Rituximab treatment correlate with inhibition of STAT3 DNA-binding activity. The Rituximab-mediated inhibition of IL-10 secretion at 24 h (Figure 1) and partial recovery of STAT3 activity at 24 h suggest that factors other than IL-10 regulate STAT3 activity (Battle and Frank, 2002; Steinman *et al*., 2003).

Previously, we reported that STAT3 activity regulates Bcl-2 expression (Alas *et al*., 2001). To demonstrate the correlation between p38 MAPK inhibition and downregulation of Bcl-2 expression induced by Rituximab, we determined by Western blot the expression of Bcl-2 in 2F7 at different time points (1–24 h). The findings

**Figure 3** Sp1- and NF-$\kappa$B DNA-binding activities are inhibited by both Rituximab and inhibitors. To analyse Sp1- and NF-$\kappa$B binding activity, EMSA were performed with a biotin-labeled probe as described in Materials and methods. (**a**) Nuclear lysates from 2F7 cells treated with Rituximab (20 $\mu$g/ml) and at different times (1–24 h) following Rituximab treatment; Sp1 DNA-binding activity was assessed. The findings demonstrate that Rituximab inhibits Sp1(A), but not Sp1(B), DNA-binding activity as early as 2 h and maximal at 24 h. DMSO had no effect on constitutive Sp1 binding. A 10-fold excess of unlabeled Sp1 probe was used as a competitor. (**b**) 2F7 cells were treated with p38 inhibitor SB 203580 (10 $\mu$M), Src kinases inhibitor PP2 (15 $\mu$M), and NF-$\kappa$B inhibitor (6 $\mu$M) for 12 or 24 h and Sp1 DNA-binding activity was determined. Clearly, the inhibitors, like Rituximab, inhibited Sp1(A) DNA-binding activity. (**c**) Nuclear lysates prepared as in (**a**) above were tested for NF-$\kappa$B DNA-binding activity. The data show that Rituximab inhibits NF-$\kappa$B activity as early as 3 h post-treatment and was maximal at 24 h. (**d**) Nuclear extracts from untreated cells or treated with Rituximab (20 $\mu$g/ml), and inhibitors (SB203580 (10 $\mu$M), PP2 (15 $\mu$M), and Bay 11-7085 (6 $\mu$M)) for 24 h were prepared and analysed for DNA-binding activity of NF-$\kappa$B by EMSA. Constitutive DNA binding of the NF-$\kappa$B was downregulated by Rituximab and all of the inhibitors

demonstrate that Rituximab downregulates Bcl-2 expression as early as 3 h following treatment and was maximal at 24 h (Figure 4c). We then examined the effect of inhibitors and correlation between phospho p38 MAPK, Bcl-2, and phospho STAT3. Cells were treated with a complete medium alone, Rituximab (20 $\mu$g/ml), SB 203580 (10 $\mu$M), PP2 (15 $\mu$M), and Bay 11-7085 (6 $\mu$M). The levels of phospho p38 MAPK, Bcl-2, and phospho STAT3 decreased following treatment with Rituximab and by the various inhibitors (Figure 4d). These findings demonstrate that there is a good correlation between the inhibition by Rituximab of STAT3 and Bcl-2 expression and the inhibition by Rituximab of the p38MAPK, Sp1, and NF-$\kappa$B-mediated IL-10 transcription.




*Downregulation of p38, NF-κB activity, and Sp1 DNA-binding activities by Rituximab and corresponding inhibitors results in the sensitization of 2F7 cells to cisplatin-mediated apoptosis*

Previous findings demonstrated that the downregulation of IL-10 expression by Rituximab sensitizes the 2F7 cells to drug-mediated apoptosis (Alas *et al*., 2001). Based on the above findings demonstrating that IL-10 expression is regulated by the Src-PTKs, p38 MAPK, and NF-$\kappa$B, we hypothesized that inhibitors of Src PTKs, p38 MAPK, and NF-$\kappa$B activities will sensitize 2F7 cells to CDDP-mediated apoptosis in the absence of Rituximab. The inhibitors used inhibited 2F7 proliferation to the same extent as Rituximab (data not shown). After 24 h of treatment with Rituximab (20 $\mu$g/ml), inhibitors (SB203580 (10 $\mu$M), PP2 (15 $\mu$M), and Bay 11-7085 (6 $\mu$M)), or CDDP (5 $\mu$g/ml), the 2F7 cells remained resistant to apoptosis. However, on treatment with a combination of Rituximab or inhibitors and CDDP, the tumor cells were significantly killed as shown by caspase 3 activation by 24 h (Figure 5). The various inhibitors did not show any killing by themselves. These findings demonstrate the role of p38MAPK and NF-$\kappa$B activities in the resistance of 2F7 to CDDP, and their inhibition by Rituximab reverses the resistance to CDDP-mediated apoptosis.

**Figure 4** Rituximab-mediated downregulation of p38 MAPK correlated with the downregulation of STAT3 activity and Bcl-2 expression. (**a**) The 2F7 cells were incubated with a complete medium or Rituximab (20 $\mu$g/ml) and STAT3 DNA-binding activity was assessed at different times (1–24 h) by EMSA. The finding demonstrates that STAT3 activity is inhibited as early as 2–3 h following treatment and was maximal at 12 h. (**b**) The inhibition of STAT3 activation induced by Rituximab (20 $\mu$g/ml) and the inhibitors SB 203580 (10 $\mu$M), PP2 (15 $\mu$M), and Bay 11-7085 (6 $\mu$M) was determined by EMSA using 2F7 nuclear extracts at 12 and 24 h. Constitutive DNA binding of the activated STAT3 was downregulated by Rituximab and inhibitors. (**c**) Western blot was used to determine Bcl-2 expression at different times after Rituximab treatment. The finding demonstrates that Bcl-2 is downregulated as early as 2–3 h following treatment as was maximal at 24 h. (**d**) Western blot was used to determine Bcl-2 expression and phosphorylation of STAT3 at 24 h. Rituximab and inhibitors all inhibited STAT3 phosphorylation as well as Bcl-2 expression and these correlated with the inhibition of p38 phosphorylation. The lysate of the control was provided by the manufacturer of the p-p38 antibody for specificity and corresponds to a lysate from anisomycin-treated C6 glioma cells

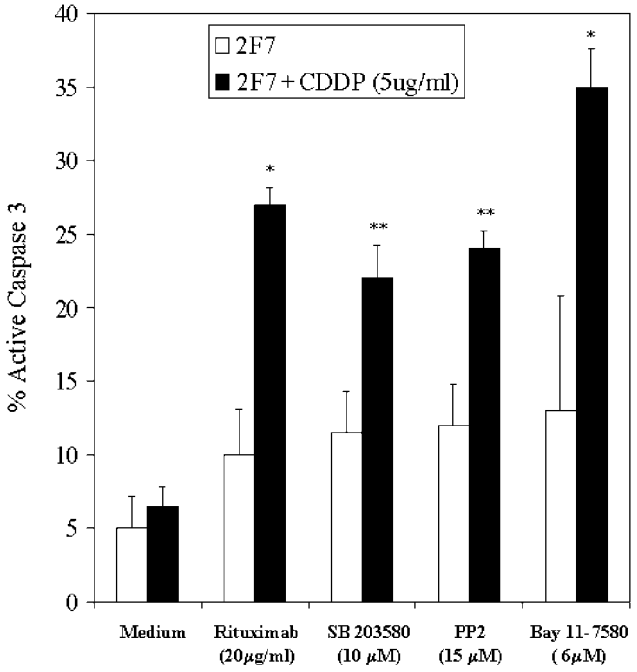



**Figure 5** Rituximab-mediated downregulation of p38 MAPK in 2F7 correlated with enhanced sensitivity to CDDP-mediated apoptosis. 2F7 cells were treated with Rituximab (20 $\mu$g/ml), inhibitors SB 203580 (10 $\mu$M), PP2 (15 $\mu$M), and Bay 11-7085 (6 $\mu$M), CDDP (5 $\mu$g/ml), or a combination of Rituximab or inhibitors with drug for 24 h. Control samples were allowed to grow in a complete medium alone. Apoptosis was determined by flow cytometry as described in Materials and methods. Both Rituximab and the inhibitors did not induce significant apoptosis. However, the enhanced cytotoxicity by a combination of Rituximab or inhibitors and CDDP was statistically significant when compared with treatment with Rituximab, inhibitors, or drug alone. This experiment is representative of three independent experiments ($^{*}P<0.01$, $^{**}P<0.02$)

## Discussion

Rituximab has been proven to be effective against low-grade NHL and against other B-cell malignancies (Thieblemont and Coiffier, 2002). However, the mechanism of Rituximab-mediated action *in vivo* is not entirely clear. It is also not clear whether Rituximab signals the B cells *in vivo* directly, and resulting in changing the phenotype of the cells for their demise. Therefore, characterization of the signaling pathways triggered by Rituximab is of considerable interest to elucidate its activity and lack of activity in patients refractory to Rituximab. Our laboratory has initiated a series of studies *in vitro* to examine the signaling pathways mediated by Rituximab in NHL B cell lines. We have reported that the downregulation of IL-10, inhibition of STAT3, and downregulation of Bcl-2 expression by Rituximab treatment in 2F7 NHL cells resulted in their sensitization to chemotherapeutic drugs. We have also shown that IL-10 is a pivotal resistant factor in these cells (Alas *et al*., 2001 and Alas and Bonavida, 2001). However, little is known of the signaling pathway involved in the regulation of IL-10 by Rituximab.

This study examined the mechanism by which Rituximab regulates IL-10 expression and presents evidence that Rituximab signals the 2F7 NHL B cells through the p38 MAPK pathway and results in the inhibition of IL-10 transcription and secretion. Rituximab inhibited the constitutive phospho Lyn and p38 MAPK activities and resulted in the inhibition of IL-10 transcription via Sp1. The role of p38MAPK in the inhibition of IL-10 was corroborated by the use of specific chemical inhibitors of the p38 MAPK pathway implicating the roles of Src kinases and NF-$\kappa$B. Rituximab-mediated inhibition of MAPK activity and IL-10 transcription correlated with the inhibition of both STAT3 activity and Bcl-2 expression and sensitization of 2F7 to CDDP-mediated apoptosis. Time kinetics analyses of the various effects induced by Rituximab establish p38 MAPK activity as a pivotal regulatory factor in the drug-resistant phenotype of 2F7. Further, the present findings identified new intracellular targets modified by Rituximab that may serve in the development of new therapeutics.

The present findings clearly demonstrate that Rituximab-induced inhibition of IL-10 expression involves the Src kinase/p38MAPK pathway. Further, we demonstrate that the treatment of 2F7 cells with Rituximab inhibited ($<2$ h) the constitutive p38 MAPK activity rapidly. These findings were corroborated by the treatment of 2F7 cells with an inhibitor of p38MAPK (SB203580), which resulted in inhibition of IL-10 transcription and expression. Constitutive activation of p38 MAP kinase has been observed in a variety of solid tumors including renal cancer, colorectal cancers, breast cancer (Miki *et al*., 1999) and, recently, it has also been observed in 50% of human primary acute myeloid leukemia (AML) cells (Lida *et al*., 1999). The mechanism by which p38 MAPK activity is constitutive in the cancer cells is not known. Our findings are consistent with the observations made in other cell lines, whereby constitutive p38 MAPK activity modulated the regulation of gene expression involved in cell-cycle progression as well as the regulation of several cytokines that included IL-1, IL-6, IL-8, IL-10, TNF-$\alpha$, and INF-$\gamma$ (Dong *et al*., 2002).

We have established the link between CD20 signaling, p38 MAPK activity, and IL-10 regulation by examining the signaling pathway upstream of p38 MAPK and downstream of p38 MAPK. A recent report by Semac *et al*. (2003) demonstrated that Rituximab inhibits the Src-kinase, Lyn in NHL Raji cells. We also demonstrate that Rituximab inhibits p-Lyn in 2F7 cells with similar kinetics as the inhibition of phospho p38 MAPK. We also examined the role of Src-family kinases by using the chemical inhibitor PP2 (Pyrazolopyrimidine), shown to be a specific inhibitor for Src-family kinases (Hanke *et al*., 1996). We demonstrate that PP2 inhibited IL-10 expression and also inactivated p38 MAPK activity and the findings were comparable to the effects induced by Rituximab treatment (Figures 1 and 2). These results established the Src-family kinase in the

Rituximab-mediated signaling pathway. Future studies are needed to examine directly the effect of Rituximab on the activity of Ras/Raf-1, MLKs, ASK1, and MEKK upstream of p38 MAPK. These kinases are involved in the activity of MKK or MEK, which activate p38 MAPK (Chang and Karin, 2001). In addition, Chang and Karin (2001) and Han *et al*. (2002) suggest that MKK3 and not MKK6 regulate p38 MAPK activity. The constitutive activation of MAP kinase observed in a variety of solid tumors and human primary AML cells is mediated by the G-protein Ras, which transduces the signal from cytokine receptors to Raf-1 resulting in the activation of the MAP kinase pathway (Lida *et al*., 1999). Our results demonstrate that inhibition of the Src family kinase by Rituximab downregulates IL-10 expression, which is also inhibited by the p38 MAPK inhibitor SB203580. Altogether, the findings suggest that the expression of IL-10 in 2F7 is mediated through the Src kinases/p38 MAPK pathway through Ras activation.

Previous analysis of NF-κB-mediated gene expression in dendritic cells revealed that p38 is required to induce some NF-$\kappa$B targets genes (Saccani *et al*., 2002). Even though there is no clear evidence that IL-10 expression is directly mediated by NF-$\kappa$B, in some experimental models, however, IL-10 regulation was observed by an indirect activity of NF-$\kappa$B (Mori and Prager, 1997). Thus, we determined if NF-$\kappa$B plays a role in the regulation of IL-10 expression in 2F7 cells by using a specific inhibitor of NF-$\kappa$B, Bay 11-7085. Specific inhibition of NF-$\kappa$B resulted in the inhibition of IL-10 expression and suggested that NF-$\kappa$B activity has a possible role in the transcriptional regulation of IL-10 gene expression in 2F7 cells. Studies in T-cell leukemia virus type 1 or in other human T-cell lines as Jurkat cells reported three sequences recognized by NF-$\kappa$B located in the 5′-regulatory region of the IL-10 gene (Mori and Prager, 1997). In these cell lines, the inhibition with antisense oligonucleotides of the p65 subunit resulted in the reduction of IL-10 gene expression and IL-10 production.

We further analysed IL-10 regulation in 2F7 cells by NF-$\kappa$B following Rituximab treatment using EMSA (Figure 3). It is worth noting that Rituximab inhibited NF-$\kappa$B activity in 2F7 cells similar to Bay 11-7085. It is possible that the downregulation of NF-$\kappa$B activity induced by Rituximab treatment resulted from the inhibition of the p38 MAPK activity and is supported by time kinetics analysis. Our findings were corroborated by examining the effect of PP2 and SB 203580 on NF-$\kappa$B activity, and show that the p38 MAPK signaling pathway regulates NF-$\kappa$B activity. On the basis of the previous and present findings, we suggest that the downregulation of NF-$\kappa$B activity is linked to p38. According to previous observations, p38 MAPK activity is regulated through the phosphorylation of IKK, an NF-$\kappa$B activator (Martin-Blanco, 2000). Although the NF-$\kappa$B inhibitor, Bay 11-7085, showed inhibition of p38 MAPK at 12-18 h, another inhibitor, SN50, which inhibits the translocation of NF-$\kappa$B into the nucleus (Lin *et al*., 1995), did not inhibit p38 MAPK (Figure 2e). Therefore, we suggest that NF-$\kappa$B is downstream of p38 MAPK. Also, previous studies determined that Ras, MEKK1, and p38 are involved in the signal pathway of activation of NF-$\kappa$B (Wang and Richmond 2001). This report demonstrated that NF-$\kappa$B activation is through the Ras/MEKK1/p38 cascade in melanocytes. Thus, we postulate that NF-$\kappa$B activity inhibited by Rituximab is mediated through a pathway that involves the p38 MAPK signaling pathway.

The p38 MAP kinase regulates the activation of Sp1, which in turn regulates the transcription of the hIL-10 gene (Ma *et al*., 2001). The molecular mechanism by which Sp1 regulates IL-10 transcription remains elusive. We investigated the regulation of the IL-10 by Sp1 in 2F7 NHL B cells following treatment with Rituximab (Figure 3). Our results demonstrate that Sp1 plays a significant role in the transcription of the IL-10 gene in B-NHL 2F7 cells. The observed induction of two distinct Sp1 DNA-protein complex bands was consistent with previous work (Ma *et al*., 2001). An alternative possibility is that Sp1 undergoes an inducible post-translational modification, which may contribute to its inducible activity (Han and Kudlow, 1997). The existence of several Sp1 coactivators has been reported, including CRSP, Rb, and hTAFII30 that acquire a post-translational modification during cell activation (Brightbill *et al*., 2000) and that, thereby, modulate the ability of Sp1 to regulate the transcription of different genes.

Since Rituximab also inhibits IL-10 autocrine/paracrine activity, we also examined if the inhibition of p38 MAPK and Src-family kinases by Rituximab results in the inhibition of STAT3 activity. Our findings were corroborated with the use of Rituximab or the inhibitors. STAT3 has been shown to be inactivated by Rituximab using EMSA and was associated with the p38 MAPK signaling pathway. We used PP2, SB 203580, and Bay 11-7085 inhibitors and compared the findings to Rituximab-mediated effects. The STAT3 activity was affected by Rituximab and by inhibitors of p38 MAPK activity (Figure 4).

Previous findings implicated IL-10 as a chemoresistance factor in 2F7 cells and showed that IL-10, via STAT3 activation, plays a role in the expression of the antiapoptotic Bcl-2 gene product (Alas *et al*., 2001; Voorzanger *et al*., 1996). Rituximab-mediated inhibition of IL-10 resulted in the downregulation of Bcl-2 and the subsequent sensitization of 2F7 cells to chemotherapeutic drugs (Alas *et al*., 2001). We expected that p38MAPK activity, which regulates IL-10 transcription, will also play a role as a chemoresistant factor and its inactivation by Rituximab will also sensitize the 2F7 cells to drug apoptosis. Indeed, our findings indicate that treatment of 2F7 with inhibitors of the Src kinase, p38 MAPK, and NF-kB, like Rituximab, sensitized the cells to CDDP-mediated apoptosis (Figure 5). Therefore, the p38 MAPK signaling pathway is directly involved in the resistance of the 2F7 cells to cytotoxic drugs.

In summary, our results clearly show for the first time the involvement of the Sp1 and NF-$\kappa$B transcription factors, and their activation via p38 MAPK, in the

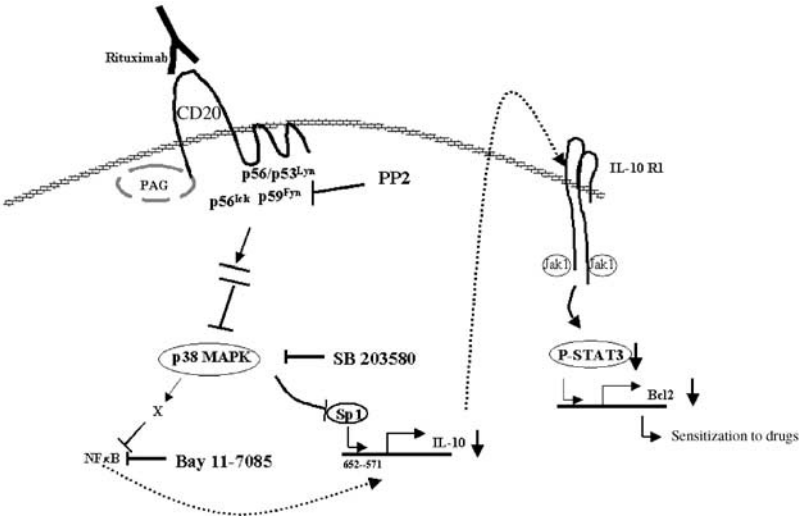



**Figure 6** A proposed model for Rituximab-regulated transcription of IL-10. Based on the findings reported in this study and the time kinetics of Rituximab-mediated effects, we propose that Rituximab inhibits the p38 MAPK signaling pathway through the inhibition of the Src family kinases. Inhibition of p38MAPK activity inhibits Sp1 and results in the downregulation of IL-10 transcription and secretion. Consequently, the inhibition of the autocrine/paracrine loop of IL-10/IL-10R signaling and partial inhibition of IL-10-mediated activation of STAT3 results in the downregulation of Bcl-2 expression. These events led to sensitization of 2F7 cells to drug-mediated apoptosis. Rituximab-mediated signaling of the MAPK pathway is corroborated by the use of specific inhibitors for Src kinases, p38 MAPK, and NF-$\kappa$B

regulation of IL-10 synthesis and secretion by Rituximab in 2F7 cells. Based on our findings with time kinetics analysis we propose that a signaling pathway mediated by Rituximab in 2F7 cells is schematically illustrated in Figure 6. This scheme shows the signaling cascade in which the inhibition of p38 MAPK and NF-$\kappa$B resulted in the inhibition of the IL-10/IL-10 receptor autocrine/paracrine loop, downregulation of STAT3 activity and Bcl-2 expression and sensitization to drug apoptosis. Rituximab also inhibits the p38 MAPK in Ramos, an EBV negative in NHL, and results in the inhibition of Bcl-$_{xL}$ and sensitization to drugs (data not shown). Therefore, modulation of the MAPK pathway has a significant potential for chemosensitization on NHL. The present findings also identify novel molecular targets for therapeutic intervention in NHL that may potentiate Rituximab's effect and/or in designing new targeted drugs in combination with Rituximab in the therapy of various lymphoproliferative disorders.

## Materials and methods

### *Cell lines and reagents*

2F7 is a CD20-positive AIDS-related lymphoma cell line that has been used in previous studies (Alas *et al*., 2001 and Alas and Bonavida, 2001). It was isolated from a single lymph node biopsy of a patient suffering from AIDS, and was kindly provided by Dr Otoniel Martinez-Maza (Jonsson Comprehensive Cancer Center, Los Angeles, CA, USA), and is also available from ATCC. The cells were grown in RPMI 1640 (Mediatech, Cellgro. Va, Ak.) supplemented with 10% heat-inactivated fetal bovine serum (Mediatech, Cellgro. Va, Ak.). All cells were cultured in 5% $CO_2$ at 37°C.

The antibody used for IL-10 capture in ELISA was purchased from PharMingen (San Diego, CA, USA). IL-10 polyclonal antibodies for the detection in ELISA were produced from rabbits in our laboratory and partially purified by ammonium sulfate as described before (Gan and Bonavida, 1999). Recombinant IL-10 was purchased from PeproTech (Rocky Hills, NJ, USA). Bcl-2 monoclonal antibody for immunoblotting was purchased from Dako (Carpinteria, CA, USA). Polyclonal antibodies against p38, antiphospho-p38, anti-STAT3, antiphospho-STAT3, Anti-Lyn and antiphospho Lyn were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). Rabbit anti-JNK 1 and 2 phospho-specific antibody were obtained from Biosource International (Camarillo, CA, USA). The chimeric anti-human CD20 monoclonal antibody, Rituximab, was provided by Genentech, Inc. (San Francisco, CA, USA).

### *Treatment of 2F7 cells with inhibitors*

The inhibitors SB203580, PP2, and Bay 11-7085 were obtained from Calbiochem (San Diego, CA, USA), SN-50 was obtained from Biomol (Plymouth Meeting, PA, USA), and the solvent, DMSO, was purchased from Sigma Chemical Co. (St Louis, MO, USA). SB203580 (Pyridinyl imidazol, a potent inhibitor of p38 and p38$\beta$MAP kinases), was added to the culture medium at a concentration of 10 $\mu$M, and the cells ($1 \times 10^6$ cells/ml) were pretreated for 1 h or incubated for 24 h at 37°C with the inhibitor or with DMSO alone. The inhibitor PP2 (pyrazolopyrimidine, specific Src-family kinase inhibitor; Hanke *et al*., 1996) was added at a concentration of 15 $\mu$M, and the cells were pretreated or incubated under the same conditions as above. The NF-$\kappa$B inhibitor Bay 11-7085 (a specific inhibitor of I$\kappa$B$\alpha$ phosphorylation) was added at 6 $\mu$M under the same conditions. SN-50 (50 $\mu$M) was used for the inhibition of NF-$\kappa$B. For the detection of phosphorylated

MAPK, the cells were cultured in 0.1% serum overnight before treatment.

*ELISA for IL-10 detection*

Supernatants from tumor cells treated with the inhibitors SB203580, PP2, Bay 11-7085, and with Rituximab (20 $\mu$g/ml) or medium alone were collected at 3, 6, 12, and 24 h post-treatment. IL-10 detection by ELISA was accomplished as described previously (Gan and Bonavida, 1999).

*RT–PCR for IL-10 transcriptional regulation*

RT–PCR was used to detect the transcriptional regulation of IL-10 in 2F7 tumor cells in the presence or absence of inhibitors (SB203580, PP2 and Bay 11-7085) and with or without Rituximab as described previously (Alas *et al*., 2001). Briefly, 2F7 cells ($2 \times 10^6$) were treated in 12-well plates (Costar, Cambridge, MA, USA) with Rituximab (20 $\mu$g/ml), with each inhibitor (SB203580, PP2 or Bay 117085) or medium alone and incubated for 6, 12, and 24 h. Total RNA was extracted from 2F7 cells at the specified time points using the single-step guanidinium thiocyanate–chloroform method with 1 $\mu$g RNA, which was reverse-transcribed to first-stranded cDNA for 1 h at 42°C using SuperScript™ II RNAase H reverse Transcriptase (Invitrogen, Life Technologies, Carlsbad, CA, USA). Amplifications of IL-10 and GAPDH transcript expression were performed with 2F7 cDNA using the hot-start technique as was described before (Garban and Bonavida, 2001). Gene-specific primers used in this study included the human IL-10 upstream 5′-CCAACAGAAGCT TCCATTCC-3′, and downstream 5′-GTTATAAAT AA-CAAGCTGGCCAC-3′ probe (Alas *et al*., 2001).

*Western blot analysis*

This was carried out as previously described (Alas and Bonavida, 2001; Alas *et al*., 2001; Jazirehi *et al*., 2003). Briefly, 2F7 cells ($1 \times 10^6$/ml) were treated in 12-well plates in the presence or absence of inhibitors (SB203580, PP2 or Bay 11-7085) or Rituximab (20 $\mu$g/ml). At specific time points (0, 3, 6, 12 and 24 h) post-treatment, the cells were pelleted and lysed on ice with cold RIPA buffer (1% NP40, 0.1% SDS, 0.5% deoxycholic acid, complete protease inhibitor cocktail tablets (Roche Diagnostic Co., Indianapolis, IN, USA), and 1 × PBS. Lysates were quantified for protein concentration using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA). An equal volume of sample buffer Bio-Rad (Bio-Rad) was added to the lysates and boiled for 10 min. The total protein lysates (20 $\mu$g) were subjected to electrophoresis on 12% polyacrylamide SDS gels. For the detection of phosphorylated p38, STAT3, Lyn, and JNK cells were pelleted and lysed on ice with the addition of sample buffer and sonicated in a SONICATOR™, Model W-220F (Heat-System Ultrasonic, Inc.), for 10 s. Proteins were transferred onto nitrocellulose membrane Hybond™ ECL™ (Amersham Pharmacia Biotech, Germany) in Trans-Blot® SD semi-dry Transfer cell System (Bio-Rad), and the membranes were probed for phosphorylated Lyn, p38, JNK, STAT3, and Bcl-2 protein using specific antibodies as per the manufacturer's instructions. Immunoblots were developed by LumiGLO® Reagent and Peroxide from Cell Signaling (Cell Signaling Technology).

*EMSA*

Briefly, cells ($10^6$) were harvested after treatment and washed twice with cold Dulbeco PBS (Cellgro). Cells were lysed in 1 ml of NP40 lysis buffer (10 mM Tris-HCl pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$ and 0.5% NP40) on ice for 5 min. Samples were centrifuged at $300 \times g$ at 4°C for 5 min. The pellet was washed twice in NP40 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 phenylmethylsulfonyl fluoride, and 0.5 mM DTT) and sonicated 10 s at 4°C. The protein concentration was determined using the Bio-Rad protein assay. The nuclear proteins were stored at −80°C until use. Both buffers contained the complete protease inhibitor cocktail tablets from Roche. Nuclear protein (5 $\mu$g) was mixed for 30 min at room temperature with the Biotin-labeled oligonucleotide probe Sp1, STAT3, and NF$\kappa$B using the EMSA Kit purchased from Panomics™ (Panomics, Inc., Redwood City, CA, USA), following the manufacturer's instructions. A measure of 10 $\mu$l was subjected to 5% polyacrylamide gel electrophoresis for 90 min in TBE buffer (Bio-Rad Laboratories), and transferred onto a Nylon transfer membrane Hybond-N+ (Amersham) using the Trans-Blot® SD semi-dry Transfer cell System (Bio-Rad). The membranes were transferred to a UV Crosslinker FB-UVXL-1000 Fisher technology (Fisher Scientific) for 3 min. The detection was carried out as per the manufacturer's instructions, after the membranes were exposed using Hyperfilm ECL (Amersham Pharmacia Biotech). The oligonucleotide sequences used for Sp1 were: 5′-ATTCGATCGGGG CGGGGCGAG-3′, for STAT3: 5′-GATCCTTCTGGGAAT TCCTAGATC-3′ and for NF-$\kappa$B: 5′-AGTTGAGGGGACTT TCCCAGGC-3′ as previously described (Sato *et al*., 1997; Harada *et al*., 1994).

*Flow cytometric analysis to determine active caspase 3*

Cells were subjected to flow cytometric analysis using an FITC-conjugated antiactive caspase-3 monoclonal antibody (BD Paharmigen, San Diego CA, USA) to determine the CDDP-mediated apoptosis of the cells treated with or without inhibitors or with Rituximab. Cells were treated with inhibitors (SB203580 10 $\mu$M, PP2 15 $\mu$M or Bay 11-7085 6 $\mu$M) Rituximab (20 $\mu$g/ml), and CDDP (5 $\mu$g/ml), or combinations of inhibitor or Rituximab with CDDP. All samples were performed in duplicate. After 24 h of incubation, the cells were stained with 5 $\mu$l of fluorescein isothiocynate-labeled active anti-Caspase 3 monoclonal antibody along with the isotype (IgG2a)-matched control antibody (Becton Dickinson). The gates were set in accordance with the gates obtained with the isotype-matched control antibody. Population data were acquired on a Flow centre EPICS$^R$ XL-MCL (Coulter, Co., Miami, FL, USA), with the System II™ Software and the percent positive cells recorded.

*Statistical analysis*

All the results were expressed as the mean ± s.d. of data obtained from three to four separate experiments. The statistical significance of differences between groups was determined using one-way ANOVA to compare variance. Significant differences were considered for those probabilities $<5\%$ ($P < 0.05$).

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