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REVIEW

Cellular and molecular signal transduction pathways modulated by rituximab (rituxan, anti-CD20 mAb) in non-Hodgkin's lymphoma: implications in chemosensitization and therapeutic intervention

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The clinical application of rituximab (chimeric mouse anti-human CD20 mAb, Rituxan, IDEC-C2B8), alone and/or combined with chemotherapy, has significantly ameliorated the treatment outcome of patients with relapsed and refractory low-grade or follicular non-Hodgkin's lymphoma (NHL). The exact *in vivo* mechanisms of action of rituximab are not fully understood, although antibody-dependent cell-mediated cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), and apoptosis have been suggested. We have proposed that modifications of the cellular signaling pathways by rituximab may be crucial for its clinical response. The B-cell restricted cell surface phosphoprotein CD20 is involved in many cellular signaling events including proliferation, activation, differentiation, and apoptosis upon crosslinking. Monomeric rituximab chemosensitizes drug-resistant NHL cells via selective downregulation of antiapoptotic factors through the type II mitochondrial apoptotic pathway. Several signaling pathways are affected by rituximab which are implicated in the underlying molecular mechanisms of chemosensitization. ARL (acquired immunodeficiency syndrome (AIDS)-related lymphoma) and non-ARL cell lines have been examined as *in vitro* model systems. In ARL, rituximab diminishes the activity of the p38MAPK signaling pathway resulting in inhibition of the interleukin (IL)-10/IL-10R autocrine/paracrine cytokine autoregulatory loop leading to the inhibition of constitutive STAT-3 activity and subsequent downregulation of Bcl-2 expression leading to chemosensitization. Rituximab upregulates Raf-1 kinase inhibitor protein (RKIP) expression in non-ARL cells. Through physical association with Raf-1 and nuclear factor κB (NF-κB)-inducing kinase (NIK), RKIP negatively regulates two major survival pathways, namely, the extracellular signal-regulated kinase1/2 (ERK1/2) and the NF-κB pathways, respectively. Downmodulation of the ERK1/2 and NF-κB pathways inhibits the transcriptional activity of AP-1 and NF-κB transcription factors, respectively, both of which lead to the downregulation of Bcl-$_{xL}$ (Bcl-2 related gene (long alternatively spliced variant of Bcl-x gene)) transcription and expression and sensitization to drug-induced apoptosis. Bcl-$_{xL}$-overexpressing cells corroborated the pivotal role of Bcl-$_{xL}$ in chemosensitization. The specificity of rituximab-mediated signaling and functional effects were corroborated by the use of specific pharmacological inhibitors. Many patients do not respond and/or relapse and the mechanisms of unresponsiveness are unknown. Rituximab-resistant B-NHL clones were generated to investigate the acquired resistance to rituximab-mediated signaling, and chemosensitization. Resistant clones display different phenotypic, genetic and functional properties compared to wild-type cells. This review summarizes the data highlighting a novel role of rituximab as a signal-inducing antibody and as a chemosensitizing agent through negative regulation of major survival pathways. Studies presented herein also reveal several intracellular targets modified by rituximab, which can be exploited for therapeutic and prognostic purposes in the treatment of patients with rituximab- and drug-refractory NHL.
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Introduction

Non-Hodgkin's lymphomas (NHL)

NHL are a heterogeneous group of malignancies that originate in the lymphoid system in which the cells usually express either B-cell or T-cell markers, or both, indicating disruption of normal development at a precursor stage. Some cases of NHL are related to immune deficiency and chronic antigenic stimulation and particularly to Epstein–Barr virus (EBV) in the context of immune deficiency; however, the exact etiology of the majority of NHL cases remains elusive (Coffey *et al*., 2003; Swerdlow, 2003). NHL accounts for about 4% of cancers in the US, ranks fifth in cancer mortality and is the leading cause of cancer-related death for people between 20 and 40 years of age. Approximately, 54 900 new cases of NHL were diagnosed in the US and 26 100 patients died of the disease

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in 2000. NHL is slightly more common in men and the median age at diagnosis is 65 years; the incidence increases with age and peaks in the 80–85 years age group (Theodossiou and Schwarzenberger, 2002; Swerdlow, 2003). The incidence of NHL has been steadily increasing for the past 50 years. The largest increases are observed within patients with aggressive lymphoma. The incidence of extranodal lymphoma has increased more rapidly than nodal form, whereas the incidence of primary central nervous system (CNS) lymphoma in the US has increased more than 10-fold between 1973 and 1992. This increase is partly due to the AIDS epidemic, although the incidence of CNS lymphoma has increased in non-AIDS populations as well. Thus, the observed increase in incidence of NHL is most likely multifactorial including chronic antigenic stimulation, reactivation of EBV infection, and severe immunodeficiency (Theodossiou and Schwarzenberger, 2002; Swerdlow, 2003).

Treatment

The major determinants for choosing the treatment options for NHL are the grade and the extent of the disease. Generally, low-grade (indolent) lymphomas are considered incurable with standard therapy when diagnosed at the advanced stages. Intermediate- and high-grade (aggressive) lymphomas are potentially curable with aggressive combination chemotherapy (Smith, 1996). The two most common histologic forms of NHL are follicular lymphoma (FL) and diffuse large B-cell lymphomas (DLBCL). FLs serve as a paradigm for the management of all indolent lymphomas. Treatment options for patients with indolent lymphomas consist of a 'watch and wait' approach, single agent alkylators, nucleoside analogues, combination chemotherapy, immunotherapy with monoclonal antibodies (mAbs), radiolabeled mAbs, or interferon (IFN). Although up to 75% of patients treated with conventional chemotherapy regimens exhibit sustained remissions, the vast majority eventually relapse. Thus, localized radiation therapy remains the treatment of choice, which is used for stages I and II disease, where 50–70% of patients will show a sustained clinical remission that lasts >5 years. Chemotherapy with alkylating agents, immunotherapy, and radioimmunotherapy are most frequently used in stages III and IV disease. Most patients with FL enjoy prolonged survival but those with stages III and IV are potentially incurable (Hiddemann, 1995; Tan and Bartlett, 2000; Theodossiou and Schwarzenberger, 2002; Coffey *et al*., 2003; Fisher, 2003).

DLBCL serves as a paradigm for the treatment and management of aggressive lymphomas. Combination chemotherapy with cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) represents the treatment of choice, and may be restricted to 3–4 cycles in patients with limited-stage disease when followed by involved field radiotherapy. Even in advanced states of the disease, long-term remission and potential cure are achieved in 30–50% of cases. Patients who fail initial management are treated with further chemotherapy. High-dose chemotherapy with stem cell rescue is effective as salvage treatment for DLBCL (Hiddemann, 1995; Smith, 1996; Tan and Bartlett, 2000; Theodossiou and Schwarzenberger, 2002; Coffey *et al*., 2003; Fisher, 2003), but relapse with the development of drug-resistance remains a major problem. In either case, intensification of therapy by myeloablative chemotherapy or combined chemoradiotherapy followed by autologous bone marrow transplantation (ABMT) or peripheral stem cell transplantation provides a promising and potentially curative prospective (Hiddemann, 1995). Eventually, most patients with low-grade (indolent) lymphoma will become refractory to treatment. Approximately 25–60% of the cases will transform to aggressive lymphoma, usually diffuse large cell type (Acker *et al*., 1983; Horning and Rosenberg, 1984).

Relapsed or refractory lymphomas

In the past 10 years, high-dose chemotherapy and autologous stem cell reconstitution have become established parts of treatment for aggressive lymphomas. CHOP continues to hold ground as first-line therapy when compared to other regimens in aggressive lymphomas. Patients with chemosensitive relapse are suitable candidates for high-dose therapy. In relapsed or refractory disease, selective compounds appear to have activity as single agents and others have shown activity in combination therapy (Hauke and Armitage 2000; Horning *et al*., 2001; Marcus 2003; Hennessy *et al*., 2004). Patients whose disease progresses after the initial administration of combination chemotherapy exhibit a poor prognosis. Retreatment with any of the first-line regimens usually does not result in sustained remission. Such patients are usually treated with one of several salvage regimens. These regimens, however, induce a response in 30–40% of patients. This unresponsiveness is due to the emergence and selective outgrowth of drug-resistant variants with multidrug-resistant (MDR) phenotype that will eventually lead to patient's demise (Hiddemann, 1995; Tan and Bartlett, 2000; Theodossiou and Schwarzenberger, 2002; Coffey *et al*., 2003; Fisher, 2003). This pattern of inevitable failure of standard therapies highlights the importance of the design of new treatment modalities. A novel strategy that has attracted much attention within the past few years is the use of Ab-mediated immunotherapy targeted against specific surface markers, alone or in combination with chemotherapy. These modalities are less systematically toxic, less myelosuppressive and more specific.

Antibody-mediated immunotherapy

Exploration of immunotherapy for the treatment of malignancies using polyclonal Ab preparations began in the 1950s. Major advances in Ab-mediated immunotherapy emerged in 1975 when techniques for producing

mAbs were developed, making it feasible to produce large quantities of identical Abs directed against specific antigens (Ags) (Köhler and Milstein, 1975). The first cancer therapeutic Abs studied were of murine, rabbit, or rat origins obtained following immunization of the animal with an antigenic preparation. Patients often generated humoral immune response against these therapeutic Abs referred to as human anti-mouse Ab (HAMA) or human anti-rabbit/rat Ab (HARA), which blocked the efficacy of the therapeutic Ab by prematurely clearing the Ab, thus, limiting the possibilities for effective antitumor response (White *et al*., 2001). The host Ab responses were mainly immune complex-related adverse events such as serum sickness and anaphylaxis. For example, HAMA rates as high as 41% have been observed in previously untreated NHL patients receiving anti-B1 Ab (Zelentez, 1999).

In addition to HARA and HAMA, murine, rabbit, and rat Abs are poorly able to recruit human effector functions such as antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), which facilitate the destruction of a tumor cell (White *et al*., 2001). To overcome obstacles inherent in the first generation Abs, hybrids composed of mouse or primate Ab regions linked with a human backbone were constructed (Reff *et al*., 1994; Zelentez, 1999). These are referred to as chimeric, humanized or primatized Abs, depending on the exact Ab structure. For instance, humanized Ab is a human Ab containing the complementarity-determining regions (CDR) of non-human origin and human constant regions. These genetically engineered Abs are potent inducers of ADCC and CDC and also have enhanced half-life which contributes to their efficacy. For instance, the half-life of the chimeric anti-CD20 Ab, rituximab, is 76 h after a single infusion and 206 h after four infusions (Newman *et al*., 1992), compared with 28 h for the murine counterpart, ibritumomab (McLaughlin *et al*., 1998). The incidence of immune responses with second-generation Abs is also significantly lower than that seen with cross-species Abs.

Examples of mAbs clinically used in the treatment of cancers are edrecolomab (Panorex®), which was approved in Europe in 1994 and trastuzumab (Herceptin®) that was approved in the US in 1998. These mAbs, in combination with chemotherapy, are effectively used in the treatment of patients with colorectal and breast cancer, respectively (White *et al*., 2001). In February 2004, the food and drug administration (FDA) approved bevacizumab, antivascular endothelial growth factor (VEGF) mAb as a first-lime therapy for metastatic colorectal cancer, which is the first therapeutic to target tumor angiogenesis (Ferrara *et al*., 2004). The anti-CD52 mAb, CAMPATH-1®, produces a combined response rate of 26% in patients with chronic lymphocytic leukemia (CLL) given extensive prior therapy (Witzig *et al*., 1999). The anti-CD20 mAb rituximab (Rituxan®, IDEC-C2B8) is the first mAb for the treatment of low-grade and follicular NHL approved by the FDA in 1997.

Targeting CD20 for therapy of NHL

CD20: structure, function, and signaling

The CD20 receptor is expressed in a lineage-specific and developmentally regulated manner (Kehrl *et al*., 1994; Tedder and Engel, 1994). It is exclusively expressed on B cells and appears during the pre-B-cell stage, but is absent during the earlier or later stages of B-cell differentiation such as pro-B cells and the Ab-secreting plasma cells. CD20 expression has not been detected on other normal tissues. The human CD20 gene is located on chromosome 11 close to the site of the t(11;14)(q13;q32) translocation that is found in a subset of B-lineage malignancies, which might explain the observed alterations in the expression of the CD20 gene occurring after t(11;14) translocation (Tedder *et al*., 1989a). Two separate groups cloned and isolated CD20 simultaneously. Tedder *et al*. (1988b) isolated CD20 from a human tonsillar B-cell specific cDNA library by differential hybridization with labeled cDNA derived from B- or T-cell mRNA. The deduced amino-acid sequence lacked an N-terminal signal sequence and contained a highly charged C-terminal domain (Tedder *et al*., 1988b) suggestive of a type III integral membrane protein. Its predicted sequence was 297 residues long and contained three hydrophobic regions, one of which spans the membrane twice (Stamenkovic and Seed, 1988). Alternatively, Einfeld *et al*. (1988) isolated CD20 from a specific cDNA clone from a lambda library using a polyclonal antiserum raised against purified CD20. It was shown that normal B cells and B-cell lines exhibit heterogeneity in the expression of CD20. These cells contain a prominent 2.6 kb mRNA and a lower level of 3.3 kb mRNA. These two mRNA species are likely derived from an alternative splicing mechanism (Einfeld *et al*., 1988; Tedder *et al*., 1988a). Southern blot analysis revealed that CD20 mRNA is transcribed from a single-copy gene and is 16 kb long composed of eight exons (Tedder *et al*., 1989b). The protein form of M_r 33 000 represents 75–80% and the M_r 35 000 represents 20–25% of total CD20. These isoforms are constitutively phosphorylated in B-cell lines and significantly phosphorylated in resting B cells upon stimulation, suggesting that CD20 may be functionally regulated by a protein kinase (Einfeld *et al*., 1988; Tedder and Schlossman, 1988).

Shortly after the cloning of human CD20, the murine counterpart was identified. The tetra-membrane spanning murine CD20 (mCD20) protein contains three hydrophobic domains and shares 73% amino-acid sequence homology with human CD20 (Tedder *et al*., 1988d). CD20 belongs to the family of four-transmembrane proteins including the β chain of the high-affinity receptor for IgE (FcεRIβ), the myeloid and lymphoid-specific protein HTm4 (Adra *et al*., 1994), and the testis specific nonhematopoietic human gene, TETM4 (Hulett *et al*., 2001). The gene encoding mCD20 is located on chromosome 19, is highly expressed in murine spleen lymphocytes, in mature B-cell lines and weakly expressed in plasmacytoma cell lines (Tedder *et al*., 1988d).

It was recently shown that CD20 gene disruption in mice has no major effect on the differentiation and function of B cells (O'Keefe *et al*., 1998), suggesting additional roles played by CD20.

Deletion analysis of the 5′ untranslated region of CD20 promoter has revealed several regulatory elements required for the cell type- and stage-specific expression of CD20. Two major positive *cis*-acting elements are localized between base pairs −290/−186 and −454/−280 and a negative regulatory element at −828/−454. The −280/−186 sequence is important for B-cell specific CD20 expression (Rieckmann *et al*., 1991). Further investigations with the 5′ deletion CD20 promoter-CAT constructs demonstrated that the cell type- and stage-specific expression of CD20 correlates with the activity of a DNA motif called BAT box. The BAT box is present in the most proximal region of the CD20 promoter between bases −214 and −201 (TTCTTCTAATTAA) and is important in the high constitutive expression of CD20 in mature B cells and the induction of CD20 in pre-B cells. BAT box-binding proteins were identified as Oct-1 and Oct-2. Oct-2 is implicated in the induction of CD20 in the pre-B cell line, PB-697 (Thevenin *et al*., 1993). *In vivo* foot printing identified two sites at −45 and −160 that were occupied by $CD20^+$ B cells. The −45 site is an E box that binds to basic helix–loop–helix zipper proteins and enhances promoter activity whereas the −160 site is a composite PU.1 and Pip-binding site, which likely accounts for both lineage and stage-specific expression of CD20 (Himmelmann *et al*., 1997).

As noted, CD20 isolated from proliferating or malignant B cells or B-cell lines is highly phosphorylated, whereas CD20 detected in nonproliferating B cells is nonphosphorylated. Crosslinking cell surface CD20 by Ab or by phorbol esters results in enhanced phosphorylation (Tedder and Schlossman, 1988). In fact, in hairy cell leukemia (HCL), CD20 phosphorylation was reduced after *in vitro* administration of IFN-α. Thus, inhibition of proliferation of HCL by IFN-α may result, at least in part, via the regulation of CD20 phosphorylation (Genot *et al*., 1991) and it can also be deduced that phosphorylation of CD20 is associated with proliferation (Tedder and Schlossman, 1988). During the initial investigations in identifying the kinase responsible for CD20 phosphorylation it was shown that PMA can induce phosphorylation and internalization of CD20 on Raji cells, both of these events were reversed by protein kinase C (PKC) inhibitor palmitoyl carnitine (Valentine *et al*., 1987). Subsequently, it was demonstrated that CD20 has multiple phophorylation sites and purified PKC can use both isoforms of CD20 as a substrate *in vitro* (Valentine *et al*., 1989). Herbimycin (tyrosine kinase inhibitor) and staurosporine (PKC inhibitor) inhibited CD20-mediated induction of c-*myc*, suggesting association of CD20 with tyrosine and serine kinases (White *et al*., 1991; Deans *et al*., 1993). It is generally believed that CD20 itself has kinase activities. Crosslinking CD20-phosphorylated phospholipase C-gamma-1 and -2 (PLC-γ-1, -2) on tyrosine residue, which correlates with increases in intracellular calcium levels (Deans *et al*., 1993). Deans *et al*. (1995), showed that CD20 is associated with the Src family tyrosine kinases ($p53/56^{lyn}$, $p56^{lck}$, and $p59^{fyn}$) and with 75–80 kDa proteins phosphorylated on tyrosine residues, the natures of which are unknown. The association of CD20 with the Src family PTK Lyn seems to be independent of cytoplasmic domains as the deletion of major portions of its cytoplasmic regions did not abolish its association with tyrosine kinases (Deans *et al*., 1995).

A new role of CD20 as a Ca^{2+} channel emerged from studies showing that transfection of various lymphoid (human T, mouse pre-B lymphoblastoid) and nonlymphoid (human K562 erythroleukemia and mouse NIH-3T3 fibroblasts) cell lines with CD20 cDNA increases transmembrane Ca^{2+} conductance (Bubien *et al*., 1993). Recently, Uchida *et al*. (2004) have provided evidence for a role for CD20 in transmembrane Ca^{2+} movement in mouse primary B cells. These data complement previous results obtained using human CD20 cDNA-transfected cell lines (O'Keefe *et al*., 1998). The authors elegantly showed that in $CD20^{-/-}$ mice, several immunological features such as B-cell development, tissue localization, signal transduction, proliferation, affinity maturation, and T-cell-dependent Ab responses are unaffected (Uchida *et al*., 2004).

Modulation of CD20 expression

A number of investigations focused on delineating the modulation of CD20 expression by cytokines. For instance, recombinant interleukin-4 (rIL-4) downregulated CD20 expression in nonstimulated and preactivated normal and leukemic B cells, while IL-1, -2, -3, -6, INF-α, INF-γ, GM-CSF, TGF-β, tumor necrosis factor (TNF)-α, and lymphotoxin had no modulatory effect on CD20 expression. rIL-4 had no effect on the steady-state CD20 mRNA and probably acts by modifying the CD20 conformation rather than by inhibiting its production or affecting its internalization (Dancescu *et al*., 1992). In contrast, in a separate study, CD20 upregulation was observed with IL-4, TNF-α, and GM-CSF treatment of samples derived from CLL patients, while other cytokines (erythropoeitin, stem cell factor, TGF-β, G-CSF, IL-1, -2, -3) did not modulate CD20 expression (Venugopal *et al*., 2000). IFN-α (500–1000 U/ml, 24–48 h) induces significant upregulation of CD20 on peripheral blood lymphocytes of patients with B-CLL with no significant increase of CD20 on normal lymphocytes (Sivaraman *et al*., 2000). Activation of lymph node T cells by mitogen and IL-2 induces CD20 expression (Murayama *et al*., 1996). IFN-γ (100–1000 U/ml, 48–72 h) augments CD20 surface expression on human myeloma cells (Treon *et al*., 2001; Jazirehi *et al*., 2002). Relatively low doses of external beam radiotherapy (10 Gy) are capable of significant and consistent increase in CD20 surface expression *in vitro* (Kunala and Macklis, 2001). Collectively, these results suggest a potential approach to combine a short pretreatment course of cytokine/radiotherapy followed by a course of chemo/immunotherapy in order to increase the efficacy of anti-CD20 therapy in B-cell malignancies.

Chimeric mouse anti-human CD20 mAb, rituximab (IDEC-C2B8, rituxan), for the treatment of NHL

CD20 is an ideal target for immunotherapy of B-cell lymphomas for the following reasons: CD20 does not circulate in the plasma as a free protein, which would block Ab binding to the B lymphoma cells (Anderson *et al*., 1984). CD20 is neither shed from the cell surface (Einfeld *et al*., 1988) nor internalized upon Ab ligation (Press *et al*., 1987). Various mAbs have been raised against CD20, which exert various effects upon CD20 ligation. Also, there exists a differential effect on B cells to anti-CD20 Abs during different stages of differentiation. For example, anti-B1a Ab inhibits B-cell progression through cell cycle (Tedder *et al*., 1988a). In contrast, peripheral blood cells did not proliferate in response to anti-CD20, but dense tonsillar B cells proliferated and appeared to be at a more activated stage compared to unresponsive B cells (Clark and Shu, 1987). Administration of anti-CD20 mAbs (IgG2a subclass) NKI-B20 and BCA-B20 mediated antilymphoma effects in a nude mouse xenograft tumor model and decreased tumor growth rate (Hooijberg *et al*., 1995). Anti-CD20 mAb B1 decreases the expression of IgM at the surface of normal human B cells and B-cell lines, suggesting a negative regulatory role of CD20 on B-cell activation through antigen receptor (Bourget *et al*., 1993). The B1-induced surface IgM downregulation was reversed by staurosporine, suggesting a role of PKC. The 1F5 anti-CD20 mAb activates resting human peripheral B cells into middle to late G1 phase of the cell cycle (Smeland *et al*., 1987; Valentine *et al*., 1987) and also rescues the germinal center (GC) B cells from apoptosis without priming for the proliferation. In contrast, 1F5 signals non-GC B cells for cell cycle progression (Holder *et al*., 1995). The murine 1F5 and B1 mAbs are not always able to efficiently recruit human immune effector functions, such as ADCC and CDC, which facilitate destruction of tumor cells. Compared to rituximab, anti-B1 mAb (tositumomab) shows lower level of activity against CDC-sensitive Daudi and Ramos cells (Cardarelli *et al*., 2002). The redistribution of CD20 into membrane lipid rafts appears to control the efficiency of anti-CD20 mAbs to mediate complement lysis of lymphoma cells (Cragg *et al*., 2003). Rituximab, unlike B1, is effective in mediating ADCC as well as CDC in the presence of human complement. Rituximab also has a longer half-life and of 459 patients treated in clinical trials with rituximab, less than 1% developed a human antichimeric antibody (HACA) titer, compared with double-digit immune responses seen with murine CAMPATH-1 and anti-B1. Also, administration of anti-B1 Ab induced HAMA rates as high as 41% in previously untreated NHL patients (White *et al*., 2001). HAMA responses have also been reported following administration of this Ab in bone marrow transplant patients. Thus, to enhance the efficacy of immunotherapy, toxins and radionuclides have been conjugated to B1 and 1F5 mAbs (Press *et al*., 1987; Press *et al*., 1993, 1995; Kaminski *et al*., 1993, 1996).

Depletion of peripheral B cells was noticed upon infusion of a chimeric mouse anti-human CD20 mAb (rituximab, Rituxan, IDEC-C2B8) of macaque cynomolgus monkeys. Rapid recovery of B cells started at 2 weeks and no other toxicities were observed in the animals. This was the first report offering the possibility of the utilization of an 'immunologically active' mAb in the treatment of B-cell lymphoma (Reff *et al*., 1994). Rituximab is a genetically engineered human/mouse chimeric anti-CD20 mAb containing murine light- and heavy-chain variable regions and human gamma 1 heavy chain and kappa light chain constant regions (IgG1κ). Rituximab was isolated from murine anti-CD20 Ab (IDEC-2B8) through immunization of BALB/c ($H\text{-}2^d$) mice with the CD20-expressing human lymphoblastoid SB cell line, and expression in Chinese hamster ovary (CHO) cells. It binds with high affinity ($\sim$8.0 nM) to CD20-expressing cells (Grillo-Lopez, 2002).

Rituximab has been an important addition to the therapeutic armamentarium against low-grade FL (Hagenbeek *et al*., 2002; Cohen *et al*., 2003; Dillman, 2003), and furthermore, its utilization alone or combined with chemotherapy has been considered as first-line therapeutic option for patients with other types of hematological malignancies (Avivi *et al*., 2003), including B-CLL (Keating *et al*., 2002; Lin *et al*., 2003; Montserrat, 2003), mantle cell lymphoma (MCL) (Hiddemann *et al*., 2003), Waldenstrom's macroglobulinemia (WM) (Ghobrial *et al*., 2003), and aggressive DLBCL (Bieker *et al*., 2003; Blum and Bartlett, 2003; Coiffier, 2003; Fisher and Shah, 2003), providing significant opportunity for improving survival of these patients. Rituximab usage has also been extended to other pathologic states often culminating in long-lasting response. These include the treatment of chronic refractory idiopathic thrombocytopenic purpura (ITP) (Risken *et al*., 2003), primary central nervous system lymphoma (PCNSL) (Pels *et al*., 2003), post-transplant lymphoproliferative disorders in lung transplant recipients (Reams *et al*., 2003), intravascular lymphoma (Weichert *et al*., 2003), and other disease states.

Rituximab exerts significant antitumor activity and induces depletion of B cells *in vivo* (Reff *et al*., 1994). The superior efficacy of CHOP + rituximab regimen compared to CHOP alone in the treatment of 399 elderly patients with DLBCL was elegantly demonstrated in a randomized trial, where the combined therapy resulted in higher rates of complete remission and survival as compared to CHOP alone (76 vs 63% and 70 vs 57%, respectively) (Coiffier *et al*., 2002). Treatment of $CD20^+$ B cells with rituximab triggers multiple cell-damaging mechanisms. Possible antitumor mechanisms involve ADCC, CDC, and the induction of apoptosis (Maloney, 2003; Smith, 2003). Rituximab can also sensitize tumor cells to the cytotoxic effects of chemotherapy (Demidem *et al*., 1997; Alas *et al*., 2001; Jazirehi *et al*., 2003; Vega *et al*., 2004). However, the contribution of these various mechanisms of action of rituximab on primary normal and malignant B cells *in vivo* needs to be defined. More detailed and comprehensive knowledge about these mechanisms and their

relative contribution in eliminating tumor cells, and hence to the clinical efficacy of the Ab, is required to optimize the treatment of NHL.

In vivo mechanisms of action of rituximab

Rituximab-mediated ADCC

ADCC mediated through ligation of the human Fc portion of rituximab to Fc receptors expressed by the accessory cells is considered as a major antitumor mechanism, thus, studies were undertaken to assess the ability of rituximab in mediating ADCC. Highly purified normal peripheral blood $CD19^+$ B cells were minimally affected by rituximab in the presence of complement. In contrast, significant reduction in the number of Daudi and B-CLL cells was observed by the addition of mononuclear ($CD56^+$ and $CD14^+$) cells. Depletion of B-CLL cells in PBMC was significantly increased in the presence of rituximab and even more pronounced following the addition of granulocyte-macrophage colony stimulating factor (GM-CSF) (Vose, 1999). Other cytokines also enhance ADCC. For instance, G-CSF primed neutrophils (PMN) were efficient effector cells in mediating rituximab-mediated killing of Ramos and Raji cells (van der Kolk *et al*., 2002). Also, combination of rituximab and low-dose IL-2 (1.2 MIU/m^2/day for 56 days s.c.) induced a response in 55% of patients with relapsed and refractory FL. Immunophenotyping of the peripheral blood of all the evaluable patients showed significant increase in the levels of circulating $CD8^+$ and $CD56^+$ lymphocytes (Friedberg *et al*., 2002). Another group showed that responses to rituximab, including ADCC and CDC, are associated with Fc receptor polymorphism, as the human Fc region of rituximab is important in mobilizing ADCC and CDC effector functions (Johnson and Glennie, 2003). Using multivariate analysis, a comparison between the expression of FcγRIIIa receptor 158V and 158F allotypes in 49 patients having received rituximab for the treatment of a previously untreated FL was made. Accordingly, the homozygosity of the FcγRIIIa-158V allotype was the single parameter associated with the clinical (response rates at 2 and 12 month post treatment) and molecular (disappearance of the Bcl-2-JH gene rearrangement in peripheral blood and bone marrow) responses (Cartron *et al*., 2002). Collectively, these results establish ADCC as a potential *in vivo* and *in vitro* antitumor mechanism of rituximab; however, the exact molecular determinants of ADCC resistance need to de defined.

Rituximab-mediated CDC

Rituximab is capable of binding to C1q (Reff *et al*., 1994), thus activating the complement cascade. C1q-binding motif on human IgG1 is comprised of D270, K322, P329, and P331 residues. Substitution of these residues to alanine significantly reduces the binding of rituximab to C1q and its ability to activate complement (Idusogie *et al*., 2000). K326 and E333 are located at the extreme ends of the C1q-binding motif and mutation to tryptophan of K326 diminishes the ability of rituximab to mediate CDC (Idusogie *et al*., 2001). Depending on their origin, lymphoma cells exhibit differential sensitivity to CDC. In a recent study, rituximab induced high CDC killing of FL cells, whereas MCL and DLBCL were moderately sensitive and small lymphocytic lymphoma (SLL) cells were almost resistant (Manches *et al*., 2003). Rituximab also efficiently kills acquired immunodeficiency syndrome (AIDS)-related lymphoma (ARL) cell lines through ADCC and CDC. Despite the high expression of CD52 on ARL cells, rituximab was superior in activating CDC compared to Alemtuzumab (anti-CD52 mAb) (Golay *et al*., 2002). Various agents have been shown to enhance CDC mediated by rituximab *in vitro*. For instance, dexamethasone enhances rituximab-mediated CDC, but has minimal and/or negative impact on ADCC (Rose *et al*., 2002). The 3E7 mAb directed against the C3b breakdown product [C3b(I)] increases C3b deposition on lymphoma cells and significantly enhances rituximab-mediated CDC in the presence of normal human serum (Kennedy *et al*., 2003).

Heterogeneity of response to rituximab therapy led to the postulation that CDC resistance may be due to the expression of complement inhibitors. Support for this idea came from studies showing that the BL and FL cells exhibit highly variable sensitivity to CDC (ranging from 100% to complete resistance). The major contributors to CDC resistance were determined to be CD46 (MCP), CD55 (DAF), and CD59 as specific antagonistic Abs against these molecules significantly augmented killing in lymphoma and multiple myeloma cell lines (Golay *et al*., 2000; Harjunapaa *et al*., 2000; Treon *et al*., 2001). Further, the levels of CD20 and complement inhibitors (CD46, CD55, CD59) were determinants in the clinical response of freshly isolated cells obtained from 33 patients with B-CLL, five with prolymphocytic leukemia (PLL), and six with MCL where functional block of CD55 and CD59 enhanced CDC (Golay *et al*., 2000). Compared to rituximab, anti-B1 mAb (tositumomab) showed lower level of activity against CDC-sensitive Daudi and Ramos cells. A negative correlation was found between CD55 and CD59 expression and sensitivity to rituximab- and tositumomab-mediated CDC (Cardarelli *et al*., 2002). In the presence of human AB serum, rituximab induced significant CDC in cells from 55 patients with B-cell lymphoproliferative disorders (B-CLL, MCL, FL, HCL). The efficiency of killing was directly related to the number of CD20 molecules per cell and preincubation with anti-CD59 increased the cytotoxic effects of rituximab (Bellosillo *et al*., 2001). Collectively, these studies support a role of complement inhibitors as a mechanism for lymphoma cells to escape CDC. Contrary to the aforementioned, the baseline expression of the Mcl-1/Bax ratio, not the expression of complement regulators (CD55, CD59), was predictive of the clinical response of a retrospective, single-agent rituximab trial on 21 CLL patients (Bannerji *et al*., 2003). Further, analysis of tumor cells

obtained from 29 rituximab-treated patients with FL showed no significant correlation between the expression of CD46, CD55, CD59, and rituximab-induced CDC (Weng and Levy, 2001). Further studies are warranted to delineate the exact role of complement inhibitors in the ability of rituximab to mediate CDC.

Lipid rafts are heterogeneous lipid microdomains, relatively enriched in sphingomyelin, glycosphingolipids, and cholesterol that spontaneously form in cell membranes as a consequence of the biophysical properties of the different lipids that comprise the membrane and serve as a platform for the signaling molecules. The binding of CD20 Abs to B cells induces rapid (as early as 15 s) redistribution of CD20 molecules (up to 95%) to low-density, detergent-insoluble lipid rafts and induces the appearance of an approximately 50 kDa tyrosine phosphorylated protein in the same compartment. This is mediated through a membrane proximal sequence in the cytoplasmic carboxyl tail of CD20 corresponding to residues 219–252 (Polyak *et al*., 1998). The redistribution of CD20 into membrane lipid rafts appears to control the efficiency of anti-CD20 mAbs to mediate complement lysis of lymphoma cells (Cragg *et al*., 2003). In contrast, no association between CD20 redistribution to membrane lipid rafts and the induction of apoptosis has been demonstrated (Claude Chan *et al*., 2003). However, the coexistence of CD20 and Src family protein kinases in lipid rafts suggests a role of CD20 in cellular signaling. Redistribution of CD20 to lipid rafts depends on the nature of Abs used and its correlation with the ability to induce apoptosis warrants further scrutiny. The ability of other anti-CD20 mAbs in mediating CDC has also been investigated. Unlike 1F5 and rituximab, B1 is ineffective in recruiting human complement. CD20 translocation to the membrane lipid rafts was observed upon crosslinking with 1F5 and rituximab, but not with B1. However, crosslinking B1 with F(ab′)2 anti-Ig enabled the Ab to redistribute CD20 into detergent-insoluble compartment which parallels the ability of the Ab to control the translocation of CD20 to membrane lipid rafts and to control the effector functions.

Some investigators consider the complement activation and cytokine accumulation culpable for the infusion-related side effects of rituximab. Considerable release of cytokines (IL-6, -8, TNF-α) and accumulation of complement activation products (C3b/c, C4b/c) were observed during the first infusion of rituximab in five patients with relapsed low-grade NHL, which might contribute to the severity of side effects of rituximab therapy (van der Kolk *et al*., 2002). Complement activation was also noticed in blood and cerebral spinal fluid in a patient with relapsing NHL with CNS involvement during the first infusion of rituximab (Harjunapaa *et al*., 2000). Conversely, the limited number of cases makes the interpretation of data difficult. Collectively, these data support an effector function of CDC in the antitumor activity of rituximab. However, the role of complement inhibitors in conferring CDC-resistance is controversial and warrants further investigation.

Rituximab-mediated induction of apoptosis

Studies were undertaken to investigate the apoptotic activity of rituximab in *in vitro* model systems. Initial studies demonstrated that rituximab is capable of inducing moderate levels of apoptosis in certain NHL cells (DHL) (Demidem *et al*., 1995). The antitumor activity of mAbs that have little or no signaling activity (e.g. anti-CD19, -CD20, -CD21, -CD 22, and Her-2) is augmented when converted into IgG–IgG homodimers. Depending on the cell surface molecules they bind to, homodimers exert antitumor activity by exerting G0/G1 arrest or induction of apoptosis (Ghetie *et al*., 1997). Thus, crosslinking rituximab became a new approach in eradicating NHL tumor cells. In a series of elegant studies it was shown that, compared to the monomeric form, homodimers [F(ab′)2] of rituximab exhibit superior antigrowth activity and induce both apoptosis and necrosis in Raji, Daudi, Ramos, DHL-4 cells irrespective of the density of CD20 or the presence of Fc receptors. Also, homodimeric rituximab sensitized these cell lines to doxorubicin and synergized with RFB4-deglycosyated ricin toxin A chain (RFB4-dg (RTA)) anti-CD22 immunotoxin (Ghetie *et al*., 2001). Monomeric rituximab induced modest apoptosis in Ramos cells that was greatly enhanced upon clustering with a secondary Ab, which accompanied tyrosine kinase activation, PLC-γ2 phosphorylation, Ca^{2+} influx, and caspase-3 activation. These events were all inhibited by PP2, a selective inhibitor of Src family kinases (Hofmeister *et al*., 2000). Another group compared the apoptotic activities of rituximab and other anti-CD20 mAbs. The results of that study reveal that anti-CD20 mAbs (1F5, anti-B1, and rituximab)) are capable of inducing modest apoptosis in various NHL cell lines. The magnitude of apoptosis induction was greater with rituximab than with the murine 1F5 and anti-B1. Crosslinking of the mAbs by secondary goat anti-mouse Ig Abs or Fc-receptor-bearing accessory cells significantly potentiated the rate of killing. PP1 (inhibitor of protein tyrosine kinases Lck and Fyn), chelators of extracellular or intracellular Ca^{2+}, and caspase inhibitors attenuated apoptosis. Also, varying the expression of Bcl-2 did not affect the magnitude of anti-B1-induced apoptosis, possibly due to the sequestering effects of other Bcl-2 family members, such as Bad (Shan *et al*., 2000). Pedersen *et al*. (2002) showed that freshly isolated B-CLL cells cultured in the presence of rituximab and a crosslinking F(ab)2 fragment results in a dose- and time-dependent induction of apoptosis independent of ADCC and CDC. Rituximab crosslinking resulted in sustained and strong phosphorylation of MAPKs (p38, JNK, ERK (extracellular signal-regulated kinase)), whereby the addition of SB203580 (p38MAPK specific inhibitor) significantly reduced the degree of apoptosis (Pedersen *et al*., 2002). Additional studies were conducted to investigate the molecular mechanism of rituximab-mediated apoptosis upon crosslinking. In BL60-2 and SU-DHL-4 cell lines, this event involved rapid upregulation of the proapoptotic Bax, as well as the activation of the extracellular-signal-regulated

kinase 1/2 (ERK1/2) and increased DNA-binding activity of activation protein 1 (AP-1) (Mathas *et al*., 2000). Also, activation of caspase-9, -3, and PARP cleavage immediately following rituximab treatment is observed in patient-derived B-CLL cells, accompanied by significant downregulation of XIAP and Mcl-1 antiapoptotic proteins (Byrd *et al*., 2002). In a separate study, CD20 crosslinking by rituximab induced apoptosis in Ramos cells via loss of mitochondrial transmembrane potential, cytochrome *c* release, and caspase activation that occurred independent of Bcl-2 levels (van der Kolk *et al*., 2002). Collectively, the data presented here demonstrate the efficacy of rituximab in killing NHL cells upon crosslinking.

Molecular determinants of rituximab-mediated modifications of signaling pathways in drug-refractory NHL cells

Rituximab-mediated signaling in AIDS-related NHL (ARL): inhibition of p38MAPK, STAT3, IL-10

The data summarized above show the *in vitro* and *in vivo* effectiveness of rituximab in eradicating NHL cells. The *in vivo* effectiveness of the combination of rituximab and drugs in the treatment of drug-resistant tumors suggests that rituximab can modify the drug-resistant phenotype by interfering with signaling pathways and augments drug-induced apoptosis. However, the molecular mechanism by which rituximab interferes with the cellular signaling pathways still remain elusive. For the first time, Alas *et al*. (2001) deciphered one potential signaling pathway modulated by rituximab in 2F7 ARL cell line (Ng *et al*., 1994), which revealed a concentration- and time-dependent downregulation of IL-10 following rituximab treatment. IL-10 is an antiapoptotic protective factor in ARL cells in response to cytotoxic drugs, which utilizes the JAK/STAT pathway mainly through the activation of STAT3. Rituximab decreases the phosphorylation and DNA-binding activity of STAT3, which correlates with a decrease in Bcl-2 expression (Alas and Bonavida, 2001; Alas *et al*., 2001).

Several studies have shown that the IL-10 autocrine/paracrine loops act as a protective factor, enhance growth progression, and assists in the pathogenesis of NHL (Benjamin *et al*., 1994; Voorzanger *et al*., 1996). Patient-derived NHL cells maintain higher viability and increase their proliferation when cultured in the presence of IL-10 (Voorzanger *et al*., 1996). Also, IL-10 production by constitutively activated $CD4^+$ T cells in mice drives the proliferation of chronically activated B cells (Yetter *et al*., 1988; Cerny *et al*., 1991; Gazzinelli *et al*., 1992). Importantly, serum levels of IL-10 are elevated in many NHL patients that correlate with poor survival rates (Blay *et al*., 1993; Sarris *et al*., 1999). These led to the examination of the involvement of IL-10 in drug-resistance where we demonstrated that IL-10 serves as a protective factor in ARLs against CDDP, etoposide, ADR, and diphtheria toxin (Demidem *et al*., 1995). We expanded these initial observations to show that tumor-derived IL-10 not only protects 2F7 cells against drug cytotoxicity but also abrogates the ability of rituximab to sensitize the cells to drugs. In our model system, IL-10 activates STAT3 and that the inhibition of endogenous IL-10 by rituximab correlates with STAT3 inactivation. Anti-IL-10 Ab and piceatannol (STAT3 inhibitor) also inactivate STAT3. These findings point to the importance of tumor-derived IL-10 in the constitutive STAT3 signaling in ARL milieu. The mechanisms by which IL-10 confers its protective effects are currently unknown, although IL-10 is a known promoter of Bcl-2 expression in hematopoietic cells (Levy and Brouet, 1994; Weber-Nordt *et al*., 1996; Cohen *et al*., 1997). Bcl-2 plays an important role in a tumor cell's ability to survive cytotoxic stimuli including UV radiation, serum starvation, and drug treatment (Reed, 1995; Domen *et al*., 1998). Owing to the ability of rituximab to regulate endogenous IL-10 levels, Bcl-2 expression upon rituximab treatment of ARL cells was analysed. The findings revealed that expression of Bcl-2 is dependent on IL-10 levels and that both Bcl-2 and IL-10 are downregulated by rituximab (Alas and Bonavida, 2001).

Rituximab-mediated inhibition of the IL-10 autoregulatory loop The studies conducted by Alas *et al*. (2000) and Alas and Bonavida (2001), however, raised a question about the signaling pathway(s) involved in the regulation of IL-10 by rituximab. The regulation of human IL-10 gene expression is not well understood, however, three transcriptional elements have been identified as IL-10 inducers in human cells. Recent work has implicated transcription factor complexes consisting of cAMP-responsive element-binding protein (CREB) 1 and activating transcription factor-1 (ATF-1) in the cAMP-dependent activation of IL-10 in monocytes (Zhu *et al*., 1996). Binding of CREB1/ATF-1 to the CRE located in the enhancer region drives IL-10 transcription. Activation of nuclear factor κB (NF-κB) activates IL-10 expression in the Hut78 T-cell lymphoma cell line and is associated with higher survival rates of tumor cells (Sanchez-Garcia and Martin-Zanca, 1997). Factors responsible for downregulation of IL-10 have not been reported. Characterization of the IL-10 promoter reveals that DNA regions upstream of the gene possess both positive and negative regulatory elements (Hewitt *et al*., 1997). Putative IL-10 repressors may serve as a means by which IL-12 suppress IL-10 expression in Th1/Th2 paracrine loops. By the same token, repressors could play a role in the downregulation of IL-10 by rituximab.

We extended our studies in an attempt to address the mechanism of regulation of IL-10 expression by rituximab in 2F7 cells by providing evidence that rituximab signals the 2F7 cells through the p38MAPK pathway culminating in the inhibition of IL-10 transcription and secretion (Vega *et al*., 2004) by rapid inhibition of the constitutive p38 MAPK activity. Constitutive activation of p38MAPK has been observed

in a variety of solid tumors including renal, colorectal, and breast cancer (Miki *et al*., 1999) as well as in 50% of human primary acute myeloid leukemia cells (Lida *et al*., 1999). These findings are consistent with the observations that constitutive p38MAPK activity regulates the expression of genes involved in cell cycle progression as well as several cytokines including IL-1, -6, -8, -10, TNF-α, and INF-γ (Dong *et al*., 2002). The mechanism of constitutive p38MAPK activity in cancer cells, although, warrants further investigation. To establish a link between CD20 signaling, p38MAPK activity, and IL-10 regulation, the signaling pathways upstream and downstream of p38MAPK were examined. A recent report reveals inhibition of the Src-kinase Lyn in Raji cells by rituximab (Semac *et al*., 2003). Likewise, rituximab inhibited p-Lyn in 2F7 cells with similar kinetics as the inhibition of p-p38MAPK. The chemical specific inhibitor of Src-family kinases pyrazolopyrimidine (PP2) (Hanke *et al*., 1996) inhibited IL-10 expression and p38MAPK activity comparable to the effects induced by rituximab, establishing a possible role of the Src-family kinase in rituximab signaling (Vega *et al*., 2004). Future studies are required to examine the direct effect of rituximab on the activity of the upstream kinases of p38MAPK such as Ras/Raf-1, MLKs, ASK1, and MEKK (Chang and Karin, 2001) and their involvement in chemosensitization.

Dendritic cells require p38MAPK for NF-κB-mediated gene expression (Saccani *et al*., 2002). IL-10 is indirectly regulated by NF-κB, although there is lack of clear evidence that IL-10 expression is directly mediated by NF-κB (Mori and Prager, 1997). The DNA-binding activity of NF-κB diminishes upon rituximab treatment, and specific inhibition of NF-κB (by Bay 11-7085) inhibits IL-10 expression, suggestive of a possible role of NF-κB in the transcriptional regulation of IL-10 in 2F7 cells (Vega *et al*., 2004). This is consistent with studies in T-cell leukemia virus type 1 or in Jurkat cells mapping three consensus κB sites in the 5′-regulatory region of the IL-10 gene where antisense p65 oligonucleotides reduced IL-10 gene expression and production (Mori and Prager, 1997). Additionally, the involvement of Ras, MEKK1, and p38 in the activation of NF-κB in melanocytes has been shown (Wang and Richmond, 2001). Thus, one can postulate that the p38MAPK pathway is involved in rituximab-mediated inhibition of NF-κB.

Rituximab-mediated inhibition of Bcl-2 expression There is little information on the interaction between IL-10-mediated signaling and known Bcl-2 regulators, such as c-Myb, WT1, AML1/ETO, BCR-ABL, CREB, Ras, and Pax proteins (Wilson *et al*., 1996; Zhu *et al*., 1996; Salomoni *et al*., 1997; Sanchez-Garcia and Martin-Zanca, 1997; Banker *et al*., 1998; Mayo *et al*., 1999). Likewise, the *bcl-2* gene promoter is poorly characterized for STAT3-binding sites and activity. Luciferase assays demonstrated that STAT3 and CT-1 increase Bcl-2 promoter activity in a pre-B-cell line (Stephanou *et al*., 2000), indicating that the promoter region contains STAT3-responsive elements. No promoter mapping was carried out in this study, however. Analysis of the Bcl-2 promoter sequence by a separate group revealed eight putative sites matching that of STAT3 consensus-binding sequence (TTNNNNNAA) (Seto *et al*., 1988). It is currently unknown which of these binding sites actively binds STAT3 or how many are critical for transcription. Nonetheless, our findings demonstrate that STAT3 inactivation by rituximab and its activation by IL-10 results in the regulation of STAT3 binding to corresponding DNA sequences and, thus, implies that STAT3 may bind to the sequences on Bcl-2 promoter and regulates Bcl-2 transcription. Although STAT3 is the major contributor of Bcl-2 expression in 2F7 cells, the involvement of intermediary factors is not excluded. Upregulation of Bcl-2 by IL-10 autocrine/paracrine loops in 2F7 cells is specific for Bcl-2, as the expression of other Bcl-2 family members (e.g. Bcl-$_{xL}$(Bcl-2 related gene (long alternatively spliced variant of Bcl-x gene))), Bax, Bad, and Bid) remained unmodified (Alas and Bonavida, 2001). STAT3 upregulates c-*myc* levels (Kiuchi *et al*., 1999); however, rituximab does not alter the expression of c-*myc* mRNA in 2F7 cells, thus, the effect of STAT3 on 2F7 cells is likely independent of c-*myc*.

Rituximab-mediated signaling in low-grade follicular NHL (non-ARL): inhibition of the NF-κB and the ERK1/2 pathways and Bcl-$_{xL}$ downregulation

The studies conducted by Alas *et al*. (2001) and Vega *et al*. (2004) demonstrate that rituximab adversely modulates signaling pathways in ARL cells. These studies were primarily performed on EBV$^+$ ARL cells, which histologically are DLBCL. This issue, however, was not addressed in non-ARL, low-grade FL lines. Consequently, we initiated a series of studies aiming to delineate the intracellular signal transduction pathways and the identification of the apoptosis-related gene product(s) regulated by monomeric rituximab. We used an EBV$^-$ low-grade FL *in vitro* model system based on the hypothesis that rituximab alters the expression profile of antiapoptotic gene products, via interruption of the major intracellular signal transduction pathways implicated in growth, survival, and apoptosis-resistance. This interruption decreases the apoptosis threshold via selective downregulation of apoptosis-associated gene products ensuing in the chemosensitization of drug-refractory NHL cells. On formulation of the above hypothesis we carried out studies showing rituximab-mediated chemosensitization of non-ARL Ramos, Daudi and Raji cells in a synergistic fashion (Jazirehi *et al*., 2003), via downregulation of Bcl-$_{xL}$.

The mechanism by which rituximab inhibits Bcl-$_{xL}$ expression is elusive. In accordance with previous reports, visual inspection and computer analysis revealed NF-κB and AP-1-bindings sites in the Bcl-x promoter region and NF-κB and AP-1, in part, regulate Bcl-$_{xL}$ gene expression (Lee *et al*., 1999; Chen C *et al*., 2000; Chen Q *et al*., 2000; Sevilla *et al*., 2001; Dixit and Mak, 2002; Ghosh and Karin, 2002; Karin and Lin,

2002). In addition, activation of the NF-κB pathway by various stimuli rescues tumor cells from drug-induced apoptosis, via upregulation of Bcl-$_{xL}$ (Lee *et al*., 1999; Chen Q *et al*., 2000). Likewise, activation of the ERK1/2 pathway by fibroblast growth factor-2 (FGF-2) rescues small-cell lung carcinoma cells from apoptosis induced by etoposide, via upregulation of Bcl-2 and Bcl-$_{xL}$ proteins (Pardo *et al*., 2002). Based on the above and since AP-1 is regulated by the ERK1/2 pathway (Karin, 1995; Lee and McCubrey, 2002), Bcl-$_{xL}$ is a downstream target of the ERK1/2 and NF-κB pathways (Lee *et al*., 1999; Chen C *et al*., 2000; Chen Q *et al*., 2000; Manna *et al*., 2000; Sevilla *et al*., 2001; Dixit and Mak, 2002; Ghosh and Karin, 2002; Karin and Lin, 2002) formulating the hypothesis of the subsequent studies that entails rituximab may inhibit the constitutive activity of the NF-κB and ERK1/2 pathways leading to inhibition of AP-1 and NF-κB transcriptional activity, hence diminishing transcription of Bcl-$_{xL}$.

Rituximab-mediated inhibition of the NF-κB pathway - Constitutive NF-κB activation has been observed in various malignancies including NHL (Fujioka *et al*., 2003) that, either via the amplification of Rel genes or through aberrant activation of the upstream regulators, contributes to pathological conditions including cancer (Dixit and Mak, 2002; Ghosh and Karin, 2002; Karin and Lin, 2002). In mammals, the NF-κB family consists of RelA (p65), RelB, c-Rel, NF-κB1 (p50 and its precursor p105), and NF-κB2 (p52 and its precursor p100) members; the most abundant form being the p65/p50 heterodimer. In normal cells, inhibitor of κB (IκB) inhibitory proteins tightly controls NF-κB activity. NF-κB activation can be induced by a plethora of extracellular stimuli resulting in phosphorylation of IκB at two conserved serines in the N-terminal regulatory region, which in IκB-α correspond to Ser$^{32/36}$. This phosphorylation step is rapidly followed by polyubiquitination and IκB degradation by the 26S proteasome allowing stable nuclear translocation of NF-κB and transcriptional activation. The multiprotein IκB kinase (IKK) complex, phosphorylated and activated by the upstream nuclear factor kappa B (NF-κB)-inducing kinase (NIK), catalyses IκB phosphorylation (Dixit and Mak, 2002; Ghosh and Karin, 2002; Karin and Lin, 2002). Significant decrease in the phosphorylation-dependent state of NIK, IKK, IκB-α as well as the DNA-binding activity of NF-κB shortly (3–6 h) post rituximab treatment in Ramos and Daudi NHL B-cells (Klein *et al*., 1968, Klein *et al*., 1975) concomitant with diminished enzymatic activity of IKK was observed suggestive of a novel function for rituximab as a negative regulator of the NF-κB pathway. However, rituximab-mediated inhibition of the NF-κB pathway was incomplete as NIK/IKK/I$\kappa\kappa$B-independent mechanisms might be implicated in the residual activity of the NF-κB signaling pathway (Fujioka *et al*., 2003). Jazirehi *et al*. (2004a) demonstrate for the first time that rituximab negatively regulates the NF-κB pathway. The NF-κB transcription factors bind to κB control elements present in the promoter region of a large number of target genes that regulate a wide variety of cellular activities including differentiation, proliferation, survival, and apoptosis (Dixit and Mak, 2002; Ghosh and Karin, 2002; Karin and Lin, 2002). Activation of the NF-κB pathway by various stimuli is, in part, responsible for the transcriptional activation and expression of antiapoptotic Bcl-2 and IAP family members, which rescue tumor cells from drug cytotoxicity (Manna *et al*., 2000; Dixit and Mak, 2002; Ghosh and Karin, 2002; Grag and Aggarwal, 2002; Karin and Lin, 2002). We have demonstrated that rituximab selectively down-regulates Bcl-$_{xL}$ expression, in part, via inhibition of the NF-κB signaling pathway (see below) (Jazirehi *et al*., 2004a).

The direct involvement of the NF-κB signaling pathway in Bcl-$_{xL}$ expression was examined by various approaches. First, using Ramos and Daudi cells expressing a super-repressor, dominant-active IκB, we established the necessity of an intact NF-κB signaling pathway for Bcl-$_{xL}$ expression. Second, promoter reporter assays showed that two tandem NF-κB-binding sites in the upstream promoter region support Bcl-$_{xL}$ expression and deletion of these sites mimicked rituximab- and Bay 11-7085-mediated effects in reducing luciferase activity. Third, the role of NF-κB in Bcl-$_{xL}$ expression was corroborated by pharmacological interruption of the NF-κB pathway using specific inhibitors (Bay 11-7085, DHMEQ, and SN50) (Lin *et al*., 1995; Pierce *et al*., 1997; Ariga *et al*., 2002; Kikuchi *et al*., 2003), which reduced Bcl-$_{xL}$ transcription. These results are of paramount importance showing that monomeric rituximab, although incapable of inducing apoptosis, can alter the dynamics of cellular signaling culminating in diminished expression of protective factors and reduced apoptosis threshold.

Rituximab-mediated inhibition of the ERK1/2 MAPK pathway In subsequent studies, we demonstrated that the mitogen activated protein kinases (MAPKs; ERK1/2, p38, JNK) are constitutively activated in Ramos and Daudi cells, with selective inhibition of ERK1/2 by rituximab (Jazirehi *et al*., 2004b). MAPKs are structurally related and are activated by similar kinase cascades, yet, they are activated by different extracellular stimuli. The ERKs are activated by Ras (via the Raf-1 and MEK1/2 kinases) in response to cytokine/growth factor stimulation, whereas the JNK and p38 kinases are activated by environmental stress through Rho family GTPases including Rac and Cdc42. Different MAPK family members also have distinct substrate specificities (Xia *et al*., 1995; Chang and Karin, 2001). Thus, differences in response to extracellular stimuli and in substrate specificity might explain selective inhibition of the ERK1/2 pathway by rituximab. We demonstrated that rituximab inhibits the ERK1/2 pathway in representative NHL cells, however, previous reports demonstrated the activation of the ERK1/2, p38, and JNK1/2 and induction of apoptosis by crosslinking rituximab (Mathas *et al*., 2000; Pedersen *et al*., 2002). Concor-

dantly, crosslinking rituximab induced robust and sustained phosphorylation of ERK1/2 and p38, accompanied with higher AP-1 DNA-binding activity and the induction of significant apoptosis suggesting that the signaling pathways utilized by monomeric vs crosslinked rituximab are different. The AP-1 transcription factor complexes, composed of Jun and Fos families, bind to specific control elements present in the promoter of genes that regulate cellular differentiation, proliferation, survival, and apoptosis (Karin, 1995). Owing to the presence of functional AP-1-binding sites in the Bcl-x promoter (Manna *et al*., 2000; Sevilla *et al*., 2001), and the fact that the ERK1/2 pathway regulates AP-1 among other transcription factors (Karin, 1995; Lee and McCubrey, 2002), Bcl-$_{xL}$ is a downstream target of the ERK1/2 pathway. Hence, we tested the hypothesis that rituximab, via inhibition of the constitutive activity of the ERK1/2 pathway, inhibits AP-1 activity and transcription of Bcl-$_{xL}$. Electrophoresis mobility shift and supershift assays revealed that AP-1 (c-Jun, c-Fos) DNA-binding activity diminishes in the presence of rituximab as early as 3–6 h post treatment, which was corroborated by the use of MEK1/2 inhibitors. Luciferase reporter assays confirmed the role of ERK1/2 pathway in Bcl-$_{xL}$ gene expression, an effect that was diminished by rituximab and ERK1/2 inhibition. Further, treatment of the cells with rituximab, or specific chemical inhibitors of Raf-1 (GW5074) (Lackey *et al*., 2000), or MEK1/2 (U0126 and PD098059) (Alessi *et al*., 1995; Dudley *et al*., 1995; Favata *et al*., 1998) decreased Bcl-$_{xL}$ gene expression at the transcriptional regulation level. Time kinetics studies revealed that rituximab-mediated inhibition of Bcl-$_{xL}$ gene expression is an early event initiating as early as 1 h post treatment and is more pronounced as a function of time. Collectively, these data denote the ability of rituximab to inhibit the ERK1/2 pathway, decrease AP-1 DNA-binding activity and Bcl-$_{xL}$ transcription (Jazirehi *et al*., 2004b). These events culminate in reduced apoptosis threshold and the cells exhibit higher drug sensitivity (see below).

Rituximab-mediated inhibition of the ERK1/2 and NF-κB signaling pathways via induction of RKIP The above studies reveal a novel function of rituximab as a negative regulator of major survival pathways, identify new targets for therapeutic intervention and provide a rationale molecular basis for the usage of rituximab and/or specific chemical inhibitors in combination with chemotherapy. However, the underlying mechanism by which rituximab interferes with the constitutive activity of these pathways is unknown. The inhibition of the ERK1/2 and NF-κB pathways might occur via several different mechanisms. It might be through the inhibition of the activity of the Src-family kinase Lyn or through disruption of cytokines (e.g. TNF-α) autoregulatory loop, respectively. Indeed, we have observed a decrease in p-Lyn (consistent with previous reports; Semac *et al*., 2003) and diminished transcription and translation of TNF-α by rituximab. Alternatively, it might be due to the modulation of Raf-1 kinase inhibitor protein (RKIP) expression that inhibits the NF-κB and ERK1/2 pathways (Yeung *et al*., 1999, 2000, 2001; Odabaei *et al*., 2004). Time kinetics studies revealed that rituximab upregulates RKIP expression and augments the association between RKIP and Raf-1 or NIK. Physical association between RKIP and Raf-1 and/or NIK will abrogate the ability of Raf-1 and NIK to phosphorylate and activate downstream molecules such as MEK1/2, ERK1/2, and IKK, IκB, respectively. *In vitro*, RKIP disrupts the interaction between Raf-1 and MEK, and that of NIK/TAK/IKK, thus, acting as a competitive inhibitor for MEK and IKK, and by suppressing the ERK1/2 and NF-κB pathways, decreases AP-1- and NF-κB-dependent gene expression (Yeung *et al*., 1999, 2000, 2001), thus, acting as a negative regulator of these pathways. Our results corroborate these findings and demonstrate that rituximab-mediated RKIP induction diminishes the phosphorylation of the components of the ERK1/2 and NF-κB pathways, reduces the DNA-binding activity of AP-1 and NF-κB and decreases Bcl-$_{xL}$ expression, all of which occur with similar kinetics (Jazirehi *et al*., 2004a). These findings provide a novel mechanism induced by rituximab that regulates cell survival and sensitizes the cells to drug-induced apoptosis through induction of RKIP and subsequent inhibition of the activity of these pathways. The cellular signaling pathways induced by rituximab in ARL and non-ARL NHL cell lines are schematically depicted in Figures 1 and 2.

Rituximab-mediated signaling is independent of its crosslinking with FcRs

The Fc portion of the IgG (e.g. rituximab) molecule recruits effector functions such as ADCC and CDC (Thommesen *et al*., 2000). Two classes of Fc receptors can be triggered for signaling. Stimulatory Fcγ receptors transduce signals through the immunoreceptor tyosine-based activation motif (ITAM) resulting in protein phosphorylation, increase in intracellular Ca^{2+}, the production of 1,4,5-triphosphate inositol and diacylglycerol mainly through the Src family kinases, activation of phospho-inositol 3-kinase (PI3-k) and phospholipase-C (PLC). Inhibitory Fcγ receptors are single-chain receptors that transduce signals through an immunoreceptor tyrosine-based inhibitory motif (ITIM) in the cytoplasmic domain and counteract the activating signals (Dearon, 1997). Depending on the cell type, various Fc receptors can be triggered for signaling. For example, the Fc-receptors Fc-γ RIII/CD16A expressed on NK cells, macrophages, and a subset of T-cells trigger ADCC. Crosslinking of CD16A on NK cells, by immune complexes or by anti-CD16 Abs, initiates a cascade of signaling events including the tyrosine phosphorylation of the ζ chain and other proteins, intracellular calcium mobilization, and activation of PLC-A2, -C, and -D (Dearon, 1997; Pan and Pei, 2003). Activation of CD16A is coupled directly or indirectly by a nonreceptor tyrosine kinase as was confirmed in

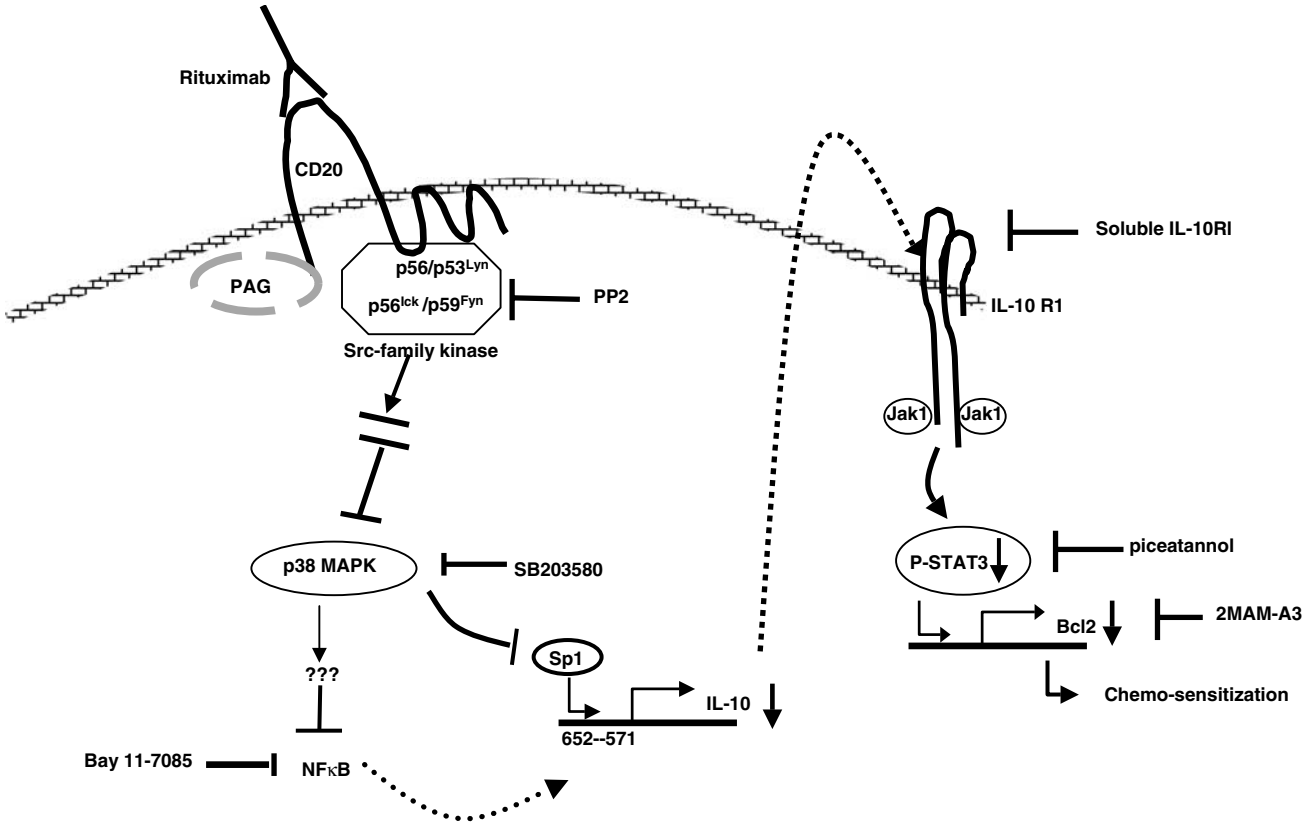

Figure 1 Proposed model for rituximab-mediated disruption of the p38MAPK pathway, regulation of IL-10 expression and chemosensitization of AIDS-related NHL (ARL) cells. Upon ligation to the B-cell-restricted marker CD20 on the surface of 2F7 ARL cells, rituximab inhibits the phosphorylation-dependent state of Lyn (Src-family tyrosine kinase), which leads to inhibition of the p38MAPK signaling pathway and diminished Sp1 transcriptional activity leading to decreased IL-10 transcription and secretion. Subsequently, the IL-10/IL-10 receptor autocrine/paracrine cytokine regulatory loop is disrupted which will inactivate STAT3. Inhibition of STAT3 transcriptional activity will result in downregulation of Bcl-2 expression and the cells become more sensitive to the apoptotic effects of chemotherapeutic drugs. Pharmacological inhibition of the Src-family kinases (e.g. PP2), p38MAPK pathway (e.g. SB203580), IL-10 loop (e.g. soluble IL-10 receptor), STAT3 activation (e.g. piceatannol) or Bcl-2 function (e.g. 2MAM-A3) mimic the effects of rituximab in sensitizing the cells to drug-induced apoptosis

studies in which the tyrosine phosphorylation of kinases including $p72^{Syc}$, $p56^{LCK}$, Shc, p36 and activation of PI3-kinase were observed (Galandrini *et al*., 1999). Also, crosslinking of FcγRIII induces robust p38MAPK phosphorylation (Hazan-Halevy *et al*., 2002).

The signaling pathways induced by rituximab may be due to the direct engagement of the CD20 receptor alone or indirectly through the crosslinking of the Fc fragment of rituximab with FcRs expressed on the tumor cells; the latter may either activate or inhibit intracellular signaling pathways. Crystallographic analysis of the FcγR III–IgG1 complex shows that amino acids in the hinge region and hinge proximal loops of CH2 are involved in binding (Canfield and Morrison, 1991). The NH2 region of the CH2 domain of human IgG1 is required for C1q and FcγRIII binding (Gergely and Sarmay, 1990; Morgan *et al*., 1995) to mediate CDC and ADCC, respectively, as a mechanism of rituximab-induced activity (Smith, 2003). In this respect, polymorphysims in the Fc receptors are determining factors in the efficacy of Ab therapy. Nonetheless, the implication of the Fc fragment in other aspects of rituximab signaling is elusive. In order to decipher the role of Fc–FcR interactions in rituximab-mediated signaling, CH2 deleted ($CH2^-$) monomeric and dimeric rituximab molecules were genetically constructed (Genentech, Inc.), and compared various aspects of signaling of the $CH2^-$ Abs with the native rituximab. As expected, the $CH2^-$ Abs were devoid of any ADCC and CDC activities. The Abs bind efficiently to the CD20 receptor on the surface of NHL B cells similar to the wild-type rituximab molecule. Further, no detectable differences between the wild-type rituximab and the $CH2^-$ forms were observed with respect to their ability to inhibit cell proliferation, induce cell aggregation, induce apoptosis upon crosslinking with secondary anti-IgG Ab, and inhibition of the p38MAPK and NF-κB pathways. In addition, the $CH2^-$ Abs are equally capable of sensitizing tumor cells to both chemotherapeutic drugs and Fas-induced apoptosis (Vega *et al*., unpublished data). These observations suggest that the various biological effects of rituximab do not require the participation or cooperation of the crosslinking of the Fc fragment of rituximab with the tumor cell's Fc receptor. The findings also imply that in situations where individuals have compromised ADCC and CDC,

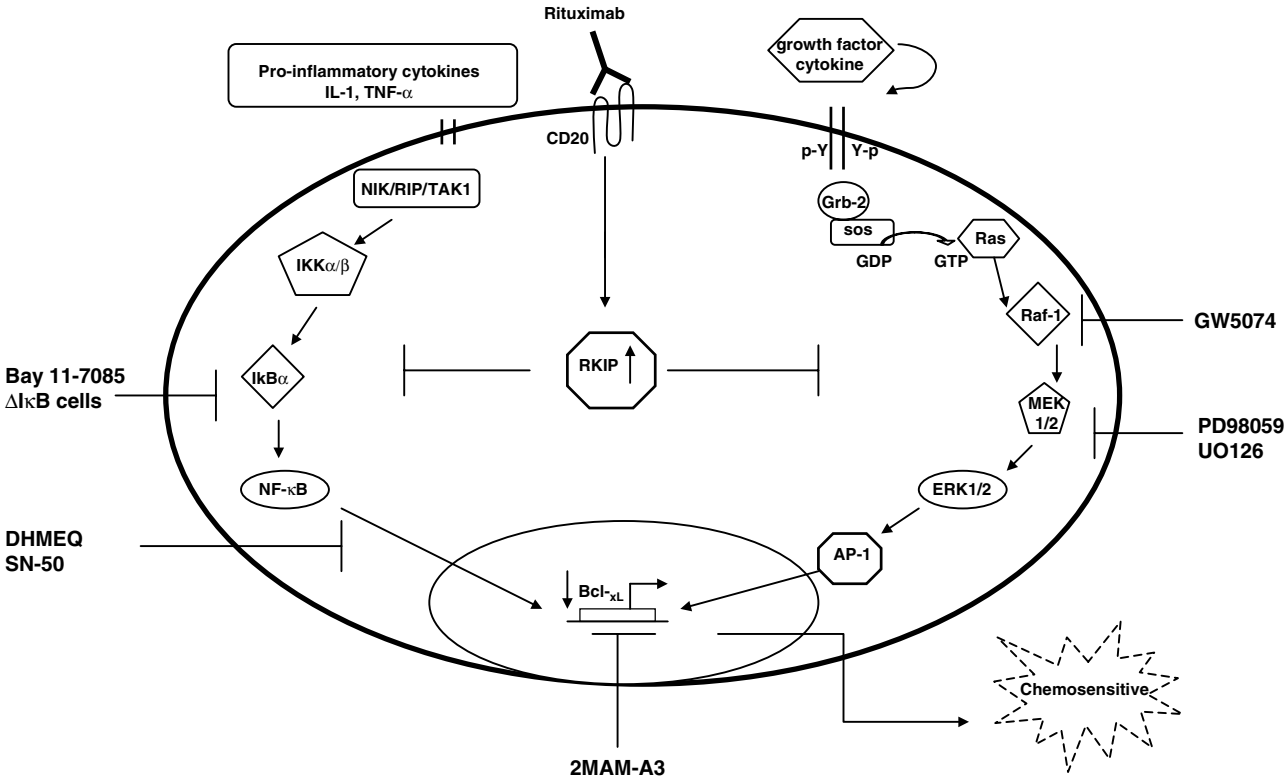

Figure 2 Proposed model of rituximab-mediated inhibition of the NF-κB and ERK1/2 signaling pathways and chemosensitization of NHL B-cells. The NF-κB and ERK1/2 signaling pathways are constitutively active in Ramos and Daudi cells and these cells express low levels of Raf-1 kinase inhibitor protein (RKIP). Rituximab, upon ligation to CD20, upregulates RKIP expression. RKIP blocks the phosphorylation and activation of endogenous NIK and Raf-1, via physical association, and renders them incapable of relaying the signal to the downstream components of their respective signaling cascade. This will in turn decrease the phospho-dependent state of the components of the NF-κB (IKK, IκB-α) and ERK1/2 (Raf-1, MEK1/2, ERK1/2) signaling pathways and inhibition of the activity of the NF-κB and ERK1/2 pathways. Subsequently, the DNA-binding ability of the transcription factors NF-κB and AP-1 is diminished culminating in decreased NF-κB- and AP-1-dependent Bcl-$_{xL}$ expression. Deactivation of the NF-κB and ERK 1/2 pathways will (**a**) decrease the proliferation rate of the NHL B cells and (**b**) diminish the levels of Bcl-$_{xL}$, which will decrease the apoptosis threshold and (**c**) will sensitize the NHL B cells to drug-induced apoptosis. Pharmacological inhibition of the NF-κB (e.g. Bay 11-7085, DHMEQ, and SN50) or the ERK1/2 pathways (e.g. PD098059, GW5074, UO126), functional block of NF-κB (e.g. IκB super-repressor cells), or functional impairment of Bcl-$_{xL}$ (e.g. 2MAM-A3) mimic the antiproliferative and chemosensitizing effects of rituximab

rituximab can still mediate chemo- and immunosensitization effects.

Monomeric rituximab as a chemosensitizing agent: synergy with drugs

As noted, monomeric rituximab is not a potent inducer of apoptosis yet the magnitude of apoptosis is significantly augmented when rituximab is used in combination with drugs. Our group was the first to report on the chemosensitizing effect of rituximab on several NHL B-cell lines prompting us to investigate the underlying mechanism of sensitization (Demidem *et al*., 1995). Our findings implicate IL-10 as a chemoresistance factor in 2F7 ARL cells and IL-10, via STAT3 activation, plays a role in the expression of the antiapoptotic Bcl-2 (Voorzanger *et al*., 1996; Alas *et al*., 2001). Our findings corroborated findings by other investigators. For instance, combination of rituximab with various drugs (bendamustine hydrochloride, cladribine (2-CdA), doxorubicin, mixoxantrone) significantly decreased the IC (30 and 50) dosages of the drugs required for the induction of apoptosis in DOHH-2, WSU-NHL, and Raji cells. This phenomenon was independent of the addition of complement and was mediated through the activation of caspases (-7 and -8) (Chow *et al*., 2002). Another study revealed that combination of *N*-(4-hydroxyphenyl) retinamide (4-HPR; known as ferentinide) and rituximab induces synergism in killing a variety of B-cell lymphoma lines (Raji, Ramos, Ramos AW, SU-DHL4) through caspase activation as the specific inhibitor of caspase activation, 2-val-ala-asp-fluromethyl ketone (z-VAD-fmk), completely blocked cell killing (Shan *et al*., 2001). Recently, it was shown that an immunotoxin comprised of rituximab coupled to type I ribosome inactivating protein (RIP) saporin-S6 significantly augments antitumor activity against $CD20^+$ NHL cells compared to individual agents and synergizes with fludarabine (Polito *et al*., 2004).

Cytokines, using the JAK/STAT signaling pathway, play a role in protecting the tumor cells against

drug-induced apoptosis. Activation of cytokine receptors induces the activation of JAKs bound to the intracellular receptor chains. In the case of IL-10R, Tyk2 activation phosphorylates JAK1. Thereafter, JAK1 phosphorylates tyrosines on the IL-10R cytoplasmic domain. Phosphorylated tyrosines bind STAT3 molecules via SH2 domains, allowing JAK1 to tyrosine phosphorylate STAT3. Upon phosphorylation, STAT3 monomers dimerize, translocate to the nucleus, and transcribe various genes. STAT3 is constitutively activated in a variety of tumor cells (Bowman *et al*., 2000). This abnormal increase in activation has implicated STAT3 as not only a factor in the onset of oncogenesis but also an antiapoptotic player in resistance to cell- and chemotherapy-induced cytotoxicity. Likewise, the ability of STAT3 to activate antiapoptosis and proliferation-associated proteins such as Bcl-$_{xL}$, Bcl-2, Mcl-1, c-*myc*, Cyclin D1, and p21^{WAF-1} is established in various model systems (Bellido *et al*., 1998; Catlett-Falcone *et al*., 1999; Kiuchi *et al*., 1999; Sinibaldi *et al*., 2000; Epling-Burnette *et al*., 2001). Compounded by evidence that STAT3, using cytokines, confers a drug-resistant phenotype in some tumors, STAT3 becomes an attractive target for intervention therapy. In our studies, the implication of STAT3 was corroborated by specific inhibition of STAT3 with piceatannol resulting in Bcl-2 downregulation and chemosensitization of the NHL B-cells in the absence of rituximab (Alas *et al*., 2001; Alas and Bonavida, 2001). The expression of the antiapoptotic Bcl-2 is implicated in the resistance of tumor cells to apoptotic stimuli. Among these are the abilities of Bcl-2 to act as an antioxidant (Hockenbery *et al*., 1993; Kane *et al*., 1993), block caspase activity (Strasser *et al*., 1994), and regulate Ca^{2+} flux (Baffy *et al*., 1993; Genestier *et al*., 1995). The commonalities between Bcl-2 function, chemotherapeutic drugs, and CD20 signaling allude to possible mechanisms involved in the reversal of drug-resistance. For instance, although Bcl-2 does not reduce the amount of DNA damage incurred by drugs, it prevents the cells from undergoing apoptosis by these drugs. Through rituximab-mediated decreases in Bcl-2 expression, the block of apoptosis by DNA-damaging drugs, such as *cis*-platinum (CDDP) and fludarabine, is circumvented. Similarly, prevention of reactive oxygen species accumulation by Bcl-2 can potentially suppress the effects of ADR, which includes the generation of free radicals. Bcl-2 prevents Ca^{2+} flux within the mitochondria and endoplasmic reticulum (Baffy *et al*., 1993) and may inhibit Ca^{2+}-dependent apoptotic pathways.

Neutralizing IL-10 has a chemosensitizing attribute, which further attests to the pivotal role of IL-10 in the chemoresistance of ARL (Alas and Bonavida, 2001). Thus, the ability of rituximab to sensitize ARL cells to functionally distinct drugs via downregulating IL-10 correlates directly with the ability of IL-10 to protect cells against a range of apoptotic stimuli. These data, however, do not preclude the involvement of IL-10-independent mechanisms in the resistance of ARL cells. Since rituximab inhibits the IL-10 autocrine/paracrine loop through inhibition of STAT3 and p38MAPK activity, it is logical to postulate that p38MAPK activation will also act as a chemoresistant factor and its inactivation will chemosensitize the 2F7 cells. We examined the consequence of p38MAPK inhibition on STAT3 activity using rituximab and specific chemical inhibitors. The DNA-binding activity as well as the phorphorylation-dependent state of STAT3 was significantly diminished in the presence of PP2, SB203580, and Bay 11-7085 (Vega *et al*., 2004) similar to the findings by rituximab (Figure 1) (Alas and Bonavida, 2001), all of which sensitized the cells to drug-induced apoptosis supporting the role of p38MAPK in the regulation of STAT3 (Vega *et al*., 2004). Altogether, these findings reveal one mechanism triggered by rituximab that interferes with multiple signaling pathways resulting in selective downregulation of antiapoptotic factors (e.g. Bcl-2, IL-10) in ARL. The findings also support the *in vivo* findings showing significant augmentation of clinical response when rituximab is used in combination with standard chemotherapeutic drugs (CHOP) in Bcl-2-expressing DLBCL patients where a response rate of 58% was achieved (Coiffier, 2003).

We have reported a reduction of Bcl-$_{xL}$ expression by rituximab and subsequent chemosensitization of drug-refractory non-ARL cell lines (Jazirehi *et al*., 2003). Our proposed molecular mechanism of this phenomenon is that on ligation to CD20, rituximab interferes with apoptotic pathways via alterations in protein expression profile. The molecular events triggered by rituximab include a selective decrease in the expression of the antiapoptotic Bcl-$_{xL}$ and the induction of the proapoptotic Apaf-1. Paclitaxel downregulates the antiapoptotic proteins Bcl-$_{xL}$ and c-IAP-1 and upregulates the expression of proapoptotic Bid and Apaf-1. Yet, these various modulatory effects by themselves were inadequate for the full induction of apoptosis, as treatment of the cells with single agents did not produce significant apoptosis. The combination treatment, via functional complementation, resulted in the formation of truncated Bid (tBid) (proapoptotic Bcl-2 family member) and the induction of apoptosis. tBid migrates to and reside in the mitochondrial outer membrane. Decreased levels of Bcl-$_{xL}$ by rituximab and paclitaxel and the presence of tBid and high levels of Bad alter the ratio of pro-/antiapoptotic Bcl-2 family members. Decrease in this ratio, which is a key determinant of cellular fate in response to cytotoxic stimuli, will assist in the formation of mitochondrial permeability transition pore (PTP) resulting in collapse in mitochondrial transmembrane potential ($\Delta\Phi m$) facilitating the unidirectional release of apoptogenic molecules such as cytochrome *c* and second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (Smac/DIABLO) into the cytosol. Smac/DIABLO will bind to and repress the inhibitory effects of IAPs. Increased levels of Apaf-1 in combination with cytochrome *c* and dATP/ATP will facilitate the assembly of the multisubunit apoptosome complex (Apaf-1/caspase-9/cytochrome *c*). Through autocatalytic processing, procaspase-9 becomes activated concurrently with decreased levels of certain IAPs, and caspase-9 will

activate caspase-3 and -7 to subsequently cleave the death substrate PARP and induce apoptosis (Jazirehi *et al*., 2003) consistent with a previous report (van der Kolk *et al*., 2002).

The long alternatively spliced variant of the Bcl-x gene, Bcl-$_{xL}$, 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 (Tudor *et al*., 2000). Bcl-$_{xL}$ is predominantly expressed in lymphomas (Xerri *et al*., 1996) and antagonizes DNA-damaging agents and metabolic, microtubule and topoisomerase inhibitors, thus, conferring an MDR phenotype. Thus, through modulation of apoptosis, Bcl-$_{xL}$ plays a major role in the determination of cellular response to apoptotic stimuli (Minn *et al*., 1995; Reed, 1995; Amundson *et al*., 2000; Tudor *et al*., 2000). These findings led us to establish the functional significance of Bcl-$_{xL}$. Using Bcl-$_{xL}$-overexpressing cells, which exhibit higher resistance against structurally and functionally distinct antineoplastic agents (paclitaxel, CDDP, ADR, VP-16, TRAIL), confirmed the protective role of Bcl-$_{xL}$ against chemotherapy-triggered apoptosis. Further involvement of Bcl-$_{xL}$ in chemoresistance (and its regulation by NF-κB) was confirmed by using cell lines stably transfected with an inducible dominant active IκB-super repressor, which upon activation contain reduced levels of Bcl-$_{xL}$ and exhibit higher drug sensitivity. Further, 2-methoxyantimycin A3 (2MAM-A3) that binds to Bcl-$_{xL}$ at the hydrophobic groove formed by the highly conserved BH1, BH2, and BH3 domains, thus, impairing the antiapoptotic ability of Bcl-$_{xL}$ without regulating its transcription or translation (Tzung *et al*., 2001), sensitized both the wild-type parental as well as the Bcl-$_{xL}$-overexpressing cells, albeit higher concentrations were required for chemosensitization of Bcl-$_{xL}$-overexpressing cells. In contrast to our findings with the 2F7 ARL cell line where Bcl-2 plays a pivotal protective role (Alas and Bonavida, 2001), in Bcl-2-expressing Daudi and Bcl-2-deficient Ramos non-ARL cells rituximab-mediated chemosensitization is independent of Bcl-2 expression concordant with previous reports (Shan *et al*., 2000; van der Kolk *et al*., 2002; Claude Chan *et al*., 2003).

Activation of NF-κB is emerging as one of the major mechanisms of tumor cell resistance to drugs, thus, interruption of this pathway is a target for therapeutic intervention (Grag and Aggarwal, 2002; Orlowski and Baldwin, 2002), which has proven successful in enhancing the apoptotic effects of anticancer agents (e.g. TNF-α, CPT-11), resulting in tumor regression *in vivo* (Wang *et al*., 1999). Bcl-$_{xL}$ is one of the downstream targets of the NF-κB pathway and is a prognostic marker in lymphoma (Xerri *et al*., 1996; Zhao *et al*., 2004). Targeted suppression of Bcl-$_{xL}$ expression facilitated drug-induced B-cell leukemia tumor regression in SCID/NOD-Hu *in vivo* model (Fennell *et al*., 2001). Rituximab selectively downregulates Bcl-$_{xL}$ expression, in part, via inhibition of the NF-κB signaling pathway (discussed above). The role of NF-κB was corroborated by pharmacological interruption of the NF-κB pathway using specific chemical inhibitors (Bay 11-7085, DHMEQ, and SN50), all of which reduced Bcl-$_{xL}$ transcription and chemosensitized the cells at levels comparable to those achieved by rituximab. Our results corroborate previous reports where NF-κB and Bcl-$_{xL}$ inhibition augmented drugs-, Fas-, and TNF-α-induced apoptosis in various tumor model systems (Lee *et al*., 1999; Wang *et al*., 1999) and support the contention that downregulation of Bcl-$_{xL}$ expression by rituximab, via inhibition of the ERK1/2 and NF-κB signaling pathways, is critical for chemosensitization (Jazirehi *et al*., 2004b).

Since the constitutive activation of the ERK1/2 signaling pathway confers a chemoresistance phenotype on tumor cells and induces their rapid proliferation (Cowley *et al*., 1994; Sivaraman *et al*., 1997; Rasouli-Nia *et al*., 1998; Watts *et al*., 1998; Fan and Chambers, 2001; Weinstein-Oppenheimer *et al*., 2001; Johnson and Lapadat, 2002; Chang *et al*., 2003), the inhibition of this pathway should theoretically confer drug sensitivity. Accordingly, interruption of this pathway is a target for therapeutic intervention for the treatment of leukemia and other tumors (Dent and Grant, 2001; Lee and McCubrey, 2002). We demonstrated that rituximab inhibits the ERK 1/2 pathway and sensitizes the cells to drug (paclitaxel, CDDP, VP-16)-induced apoptosis. The role of the ERK1/2 pathway in the drug-resistance of NHL B cells was corroborated by pharmacological interruption of the ERK1/2 pathway using specific chemical inhibitors including GW5074 (Raf-1 inhibitor), PD098059, and UO126 (MEK1/2 inhibitors), which also sensitized the cells to drug-induced apoptosis at levels comparable to those achieved by rituximab. These findings corroborate previous reports where MEK inhibition synergized with UCN-01 (Dai *et al*., 2001) and augmented the apoptotic effects of paclitaxel (MacKeigan *et al*., 2000; Yu *et al*., 2001).

Altogether, the data summarized above point to the important ability of rituximab to negatively modulate signal transduction pathways implicated in the survival of tumor cells and by altering the gene expression profile rituximab enhances the susceptibility of the tumor cells to apoptosis inducing stimuli in *in vitro* models (see Figures 1 and 2). The same principle might be operative *in vivo*. Indeed, the *in vivo* efficacy of the combination of rituximab and drugs was demonstrated. As an illustration, cytosine–guanine oligodeoxynucleotide (CpG ODN) plus anti-CD20 mAb inhibited tumor growth more efficiently than each agent alone and enhanced the efficacy of the antitumor activity of anti-CD20 mAb in a mouse lymphoma model (Warren and Weiner, 2002). The combination of rituximab and CHOP also increases the response rates, event-free survival, and overall survival of patients older than 60 years suffering from DLBCL in comparison with each agent alone (Coiffier *et al*., 2002).

Based on the discussion in the section'Rituximab-mediated inhibition of the ERK1/2 and NF-κB signaling pathways via induction of RKIP', RKIP expression may be implicated in the regulation of tumor cell's sensitivity to drugs. Our findings that RKIP is a novel

and important mediator of chemotherapy-triggered apoptosis are not exclusive to the NHL B-cells. Indeed, rapid upregulation of RKIP during drug-induced apoptosis is observed in human prostate and breast cancer cells where ectopic overexpression of RKIP chemosensitizes the cells (Chatterjee *et al*., 2004). Further, downregulation of RKIP expression confers resistance to chemotherapeutic agents by releasing its inhibitory constraint of two major survival pathways in cancer cells, namely the ERK1/2 and the NF-κB signaling pathways (Yeung *et al*., 1999, 2000, 2001; Chatterjee *et al*., 2004), suggesting that RKIP represents a novel apoptotic marker in human cancer cells (Odabaei *et al*., 2004). The mechanism by which rituximab as well as other agents regulate RKIP expression is under investigation. The role of RKIP in the regulation of cell survival and apoptosis in cancer cells may be important in clinical oncology as a novel antimetastatic function for RKIP in prostate and melanoma cancer is proposed (Fu *et al*., 2003; Schuierer *et al*., 2004), where the involvement of the ERK1/2 pathway in tumor progression and metastasis and the specific interaction between RKIP and the ERK1/2 pathway was confirmed. Notably, tumor cell invasion was abrogated only by the inhibition of the ERK1/2 pathway. Thus, the regulation of expression of RKIP in cancer cells may dictate the outcome of tumor progression and response to apoptotic stimuli.

Molecular mechanisms of rituximab resistance

Deregulation of signal transduction pathways such as the NF-κB, MAPKs, JAK/STAT, AKT/PI3 kinase, or aberrant expression of the signaling molecules can contribute to the acquired chemoresistance (Pommier *et al*., 2004; Wada and Penninger, 2004). Since chemotherapeutic drugs utilize apoptosis as a mean of exerting their cytotoxic effects, drug-resistant tumor cells develop crossresistance to apoptosis induced by structurally and functionally distinct stimuli including immunotherapy and *vice versa*. Although rituximab therapy has significantly improved the treatment outcome of NHL patients, a subset of patients does not respond or relapses after the initial treatment. Selective outgrowth of the drug-resistant cells will complicate subsequent treatment regimens and will eventually lead to patient's demise. It is therefore imperative to decipher the mechanisms of intrinsic or acquired resistance to rituximab treatment. Resistance acquired during the course of therapy that is commonly seen in various cancers appears to involve similar mechanisms of intrinsic resistance (Pommier *et al*., 2004). Thus, approaches that overcome intrinsic resistance should theoretically bypass the acquired resistance and *vice versa*. Various mechanisms have been postulated for rituximab resistance (also refer to section 'Rituximab-mediated signaling in low-grade follicular NHL (non-ARL): inhibition of the NF-κB and the ERK1/2 pathways and Bcl-$_{xL}$ downregulation' above) including transient CD20 downregulation (Foran *et al*., 2001; Pickartz *et al*., 2001; Alvaro-Naranjo *et al*., 2003; Jilani *et al*., 2003; Kennedy *et al*., 2004), loss of CD20 (Davis *et al*., 1999; Haidar *et al*., 2003), circulating CD20 (Manshouri *et al*., 2003), and expression of complement inhibitors (Treon *et al*., 2001). These issues have been elegantly discussed recently (Smith, 2003).

The above-mentioned discussions point to the efficacy of rituximab (either alone (monomeric or crosslinked) and/or in combination with drugs in eradicating tumor cells *in vitro* and *in vivo*. These mimic the responding patients. In an attempt to recapitulate the nonresponders and/or relapsed situations, we have generated rituximab refractory NHL clones by growing the cells in the presence of step-wise increasing concentrations of rituximab followed by multiple rounds of limiting dilution assay. Single cells were then propagated into clones and assayed for alterations in the signal transduction pathways compared to the parental cell lines. In these rituximab-resistant clones, rituximab is incapable of either inhibition of cellular growth or induction of apoptosis (both in monomeric and crosslinked forms). Noteworthy, rituximab has lost its chemosensitizing effect on these CD20-expressing NHL B-cell clones. Compared to the parental cells, the clones exhibited higher resistance to rituximab-mediated CDC (using human AB serum). The striking observation is the selective overexpression of Bcl-$_{xL}$ and the exhibition of higher resistance to a wide array of antineopleastic agents (Jazirehi and Bonavida, 2004a); concordant with the protective role of Bcl-$_{xL}$ (Minn *et al*., 1995; Reed, 1995; Xerri *et al*., 1996; Amundson *et al*., 2000; Tudor *et al*., 2000). These findings suggest that the selective pressure applied by prolonged rituximab treatment has coselected for tumor cells that express higher levels of antiapoptotic proteins, which have lost the capacity to undergo apoptosis in response to various apoptotic stimuli. Therefore, as NHL cells develop resistance to rituximab, they may also develop crossresistance to the cytotoxic effects of the chemotherapeutic drugs and the immune system, consistent with our observation that the rituximab-resistant clones also exhibit higher resistance to TNF-related apoptosis-inducing ligand (TRAIL) and anti-Fas Ab CH-11.

Detailed analysis of the signaling pathways revealed hyperactivation status of the components of the ERK1/2 and NF-κB signaling pathways in these cells. Unlike the parental cells, rituximab is inefficient in inhibiting these pathways in the clones. Thus, we speculated that pharmacological inhibition of these pathways could potentially avert the chemoresistant phenotype, and this prompted us to evaluate the chemosensitizing effect of specific inhibitors of the NF-κB (e.g. DHMEQ) and the ERK1/2 (e.g. PD098059) pathways as well as the recently approved proteasome inhibitor bortezomib (Velcade) (Goy and Gilles, 2004). These inhibitors efficiently sensitized the rituximab-resistant clones to structurally and functionally distinct drugs including topoisomerase II inhibitor (VP-16), DNA-damaging agents (CDDP, ADR), microtubule poisons (paclitaxel, vincristine), and TRAIL, albeit to varying degrees. The

inhibitors also reduce Bcl-$_{xL}$ levels further confirming the notion that deregulated cellular signaling culminating in overexpression of antiapoptotic gene products leads to crossresistance (Jazirehi and Bonavida, 2004a). Thus, in addition to the above-mentioned mechanisms, alterations in the signaling pathways upon continuous rituximab treatment can contribute to acquired resistance (Figure 3). Studies are underway to validate our *in vitro* findings with freshly derived rituximab-refractory tumor cells.

Implications of findings on molecular signaling by rituximab and clinical significance

The vast majority of anticancer agents eradicate tumor cells by the induction of apoptosis (Ferreria *et al*., 2002). Tumor cells, in turn, have adopted various mechanisms to resist apoptosis. Natural inhibitors of apoptosis, such as Bcl-2 (Bcl-2, Bcl-$_{xL}$, Mcl-1) and IAP (c-IPA-1, -2, XIAP, survivin) family members protect the tumor cells from the apoptotic effects of various antineoplastic agents (Ferreria *et al*., 2002) via different mechanisms. Chemotherapy resistance in NHL cells is further reinforced by the emergence of the MDR phenotype following initial chemotherapy administration due to the action of membrane-bound drug efflux pumps (Nooter and Stoter, 1996; Sandor *et al*., 1997; Filipits *et al*., 2000). Overexpression of antiapoptotic Bcl-2 family members also contributes to the MDR phenotype of NHL (Minn *et al*., 1995; Reed, 1995; Xerri *et al*., 1996; Amundson *et al*., 2000; Tudor *et al*., 2000). Hence, as NHL cells develop resistance to drugs, they may also develop crossresistance to the cytotoxic effects of the immune system. The development of crossresistance suggests that drugs and death receptors may utilize a common apoptotic pathway, and such a crossresistance phenotype cannot be solely explained by the MDR mechanism (Jazirehi *et al*., 2001; Ng and Bonavida, 2002). This is probably the main reason for the failure of MDR modulators such as retrovirus- or liposome-mediated transfer of MDR1 ribozyme or MDR reversal agents including verapamil, quinidine, and cyclosporine in the treatment of drug-resistant NHL (Tan *et al*., 2000; Kobayashi *et al*., 2001). Utilization of these agents is

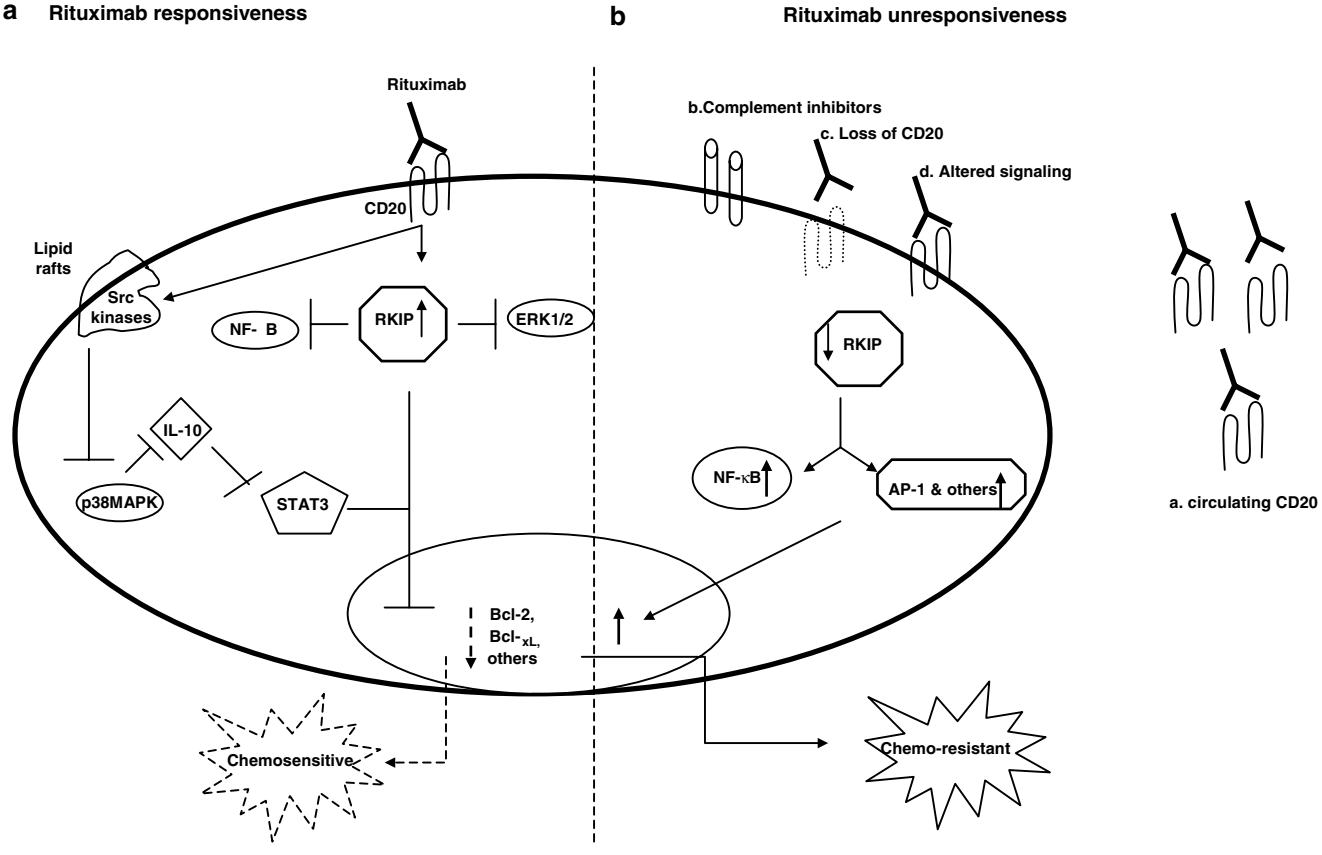

Figure 3 Proposed model of the development of rituximab resistance in NHL B cells. Various mechanisms have been proposed for the acquired or inherent resistance to rituximab. These include (**a**) the presence of circulating CD20 that could potentially bind prematurely to rituximab, (**b**) overexpression of complement inhibitors, (**c**) transient or permanent loss of CD20, and (**d**) altered cell signaling that can also contribute to rituximab resistance. For instance, in responding cells, inhibition of cellular survival pathways culminates in downregulation of antiapoptotic gene products and potentiation of chemotherapy treatment. Nonetheless, repeated rituximab exposure can result in loss of rituximab's ability to regulate molecular switches leading to constitutive hyperactivation of the survival pathways, overexpression of antiapoptotic gene products and increased apoptosis threshold (for further information, refer to the text)

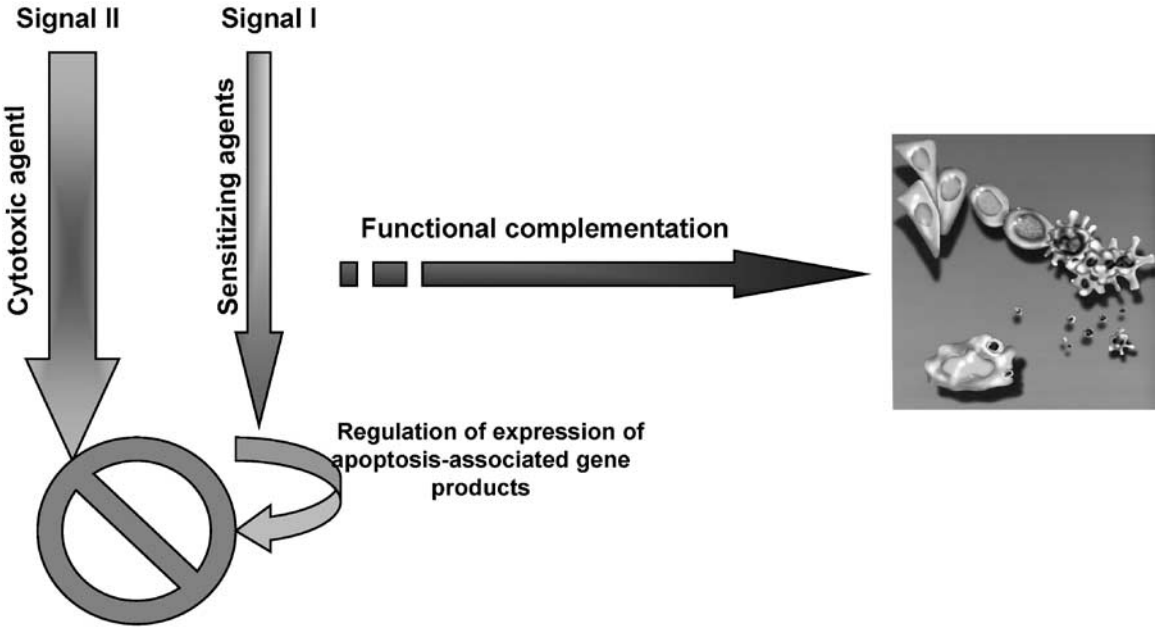

Figure 4 Proposed model of the 'functional complementation' (two signal) model. Successful execution of an apoptotic signal in drug- and/or immune-resistant tumor cells requires at least two complementary signals (functional complementation), whereby, a nontoxic sensitizing agent (e.g. cytokines, drugs, rituximab) (Signal I), via interference with the cellular signaling pathways, alters the expression profile of the apoptosis-associated molecules, removes the inhibitory block in the apoptotic pathway and facilitates the cytotoxic action of the second agent (e.g. chemotherapeutic drugs, biological response modifiers, immune system) (Signal II), thus, much lower concentrations of cytotoxic agents are required to achieve synergistic apoptosis

further limited by the presence of redundant cellular mechanisms of resistance, alterations in the pharmacokinetics of the agents, and clinical toxicities (Tan *et al*., 2000). Thus, nontoxic agents that interfere with the function of drug efflux pumps or adversely modulate the signaling pathways leading to alterations in the expression profile of apoptosis-associated gene products can be effectively used in combination with chemotherapy in the clinical treatment of drug-resistant NHL.

The other issue regarding resistance to apoptosis, such as drugs- and/or immune-mediated cytotoxicity, is often due to the inability of cells to carry out the signal transduction pathways ultimately leading to cell death (Kaufmann and Eranshaw, 2000). This may be due to insufficient expression of signaling molecules, overexpression of protective factors, or mutations in apoptotic proteins such as p53. Drugs have been shown to regulate the expression levels of anti- and proapoptotic proteins (Maldonado *et al*., 1997; Jazirehi *et al*., 2001). This phenomenon illustrates the possibility that therapeutic compounds may not directly induce cytotoxicity but nonetheless possess the ability to alter protein expression profile in a manner that would allow additional agents to induce apoptosis at much lower threshold. Thus, the direct cytotoxicity and sensitizing attributes exerted by drugs are accomplished via distinct mechanisms although some overlap may exist. The long-lasting objective of the research should determine the cellular signaling pathways regulated by sensitizing agents that avert the resistant phenotype of drug-refractory tumor cells. In this respect, the functional complementation (two signal) model is proposed (Figure 4). Accordingly, treatment of tumor cells with a nontoxic sensitizing agent (e.g. rituximab) alters the expression profile of apoptosis-associated gene products (signal I), removes the inhibitory block in the apoptotic pathway and by lowering the apoptosis threshold sensitizes the tumor cells to the cytotoxic effects of the second agent (e.g. biological response modifiers and/or chemotherapeutic drugs) (signal II) (Ng and Bonavida, 2002). Numerous studies have validated the functional complementation model and further attest to the contention that therapeutic compounds, in addition to the ability to directly induce apoptosis, are capable of altering the gene expression profile and decrease the apoptosis threshold of drug-resistant tumor cells (Alas and Bonavida, 2001; Alas *et al*., 2001; Jazirehi *et al*., 2001; Ng *et al*., 2002; Jazirehi *et al*., 2003; Jazirehi and Bonavida, 2004b; Vega *et al*., 2004), thus, overcoming the acquired or intrinsic apoptosis-resistance. In addition, delineation of the signaling pathways modulated by rituximab has revealed several intracellular targets for therapeutic intervention in the treatment of rituximab-refractory tumor cells. Further, based on the genetic profile of cancer cells and identification of the targets that control response to treatment, it is now possible to identify NHL patients for alternative therapeutic intervention to override the resistant phenotypes.

Abbreviations

ADCC, antibody-dependent cell-mediated cytotoxicity; AP-1, activator protein-1; ARL, acquired immunodeficiency syn-

drome (AIDS)-related lymphoma; Bcl-2, B-cell lymphoma protein 2; Bcl-$_{xL}$, Bcl-2 related gene (long alternatively spliced variant of Bcl-x gene); CDC, complement-dependent cytotoxicity; DHMEQ, dehydroxy methylepoxy quinomicin; DLBCL, diffuse large B-cell lymphoma; ERK1/2 MAPK, extracellular signal-regulated kinase1/2 mitogen-activated protein kinase; IKK, inhibitor of kappa B (IκB) kinase complex; IL, interleukin; JNK/SAPK, c-Jun NH_2-terminal kinase/stress-activated protein kinase; MEK1/2, mitogen-activated protein kinase kinase 1/2; 2MAM-A3, 2-methoxyantimycin-A3; NIK, nuclear factor κB (NF-κB)-inducing kinase; RKIP, Raf-1 kinase inhibitor protein; STAT, signal transducer and activator of transcription.

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