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Interleukin-21 Induces Apoptosis of Diffuse Large B Cell Lymphomas via Activation of the STAT3 - c-Myc Intracellular Signaling Pathway

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INTERLEUKIN-21 INDUCES APOPTOSIS OF DIFFUSE LARGE B CELL LYMPHOMAS VIA ACTIVATION OF THE STAT3 – C-MYC INTRACELLULAR SIGNALING PATHWAY

By

Kristopher A. Sarosiek

A DISSERTATION

Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Interleukin-21 (IL-21), a recently discovered member of the IL-2 cytokine family, has been shown to have diverse regulatory effects on B cells including the induction of antibody secretion, differentiation, or apoptosis depending on the cell milieu and activation status. However, the effects of IL-21 on B cell neoplasms such as diffuse large B cell lymphoma (DLBCL) are largely unknown. Our research uncovered the widespread expression of the IL-21 receptor (IL-21R) in B cell lymphomas including DLBCL and that IL-21 stimulation resulted in potent phosphorylation of STAT1 and 3 and weak activation of STAT5. However, our findings also showed that treatment of DLBCL cell lines with IL-21 induced cell cycle arrest and apoptosis. The cell death was caspase-dependent and evident in a majority of DLBCL cell lines. To further examine the potential therapeutic applicability of IL-21, we assessed the effects of IL-21 on primary DLBCL tumors and in vivo DLBCL xenografts in mice. In primary tumors, IL-21 induced apoptosis in five of five DLBCLs compared to two of three follicular lymphomas and two of seven chronic lymphocytic leukemias. No apoptosis or cell death was induced in normal peripheral B lymphocytes. In mice bearing DLBCL xenograft tumors, in situ
IL-21 injections induced tumor regression and dramatically extended the overall survival of mice (P<0.001).

To elucidate the mechanism of IL-21-induced cell death we analyzed the expression of apoptosis-regulating proteins and observed a strong downregulation of anti-apoptotic Bcl-2 and Bcl-XL and an upregulation of pro-apoptotic Bax post IL-21 stimulation. Subsequent experiments showed that ectopic expression of Bcl-2 or Bcl-XL was able to partially reduce cell death induced by IL-21 while Bax knockdown with siRNA blocked apoptosis completely. To gain insight into the signaling pathways shifting the expression of these proteins toward cell death we performed microarray analysis on sensitive and resistant DLBCL cell lines. The most striking difference in gene expression was observed in C-MYC which was only induced in cell lines exhibiting apoptosis upon IL-21 treatment. Previous reports have shown that c-Myc, which has been studied extensively for its oncogenic properties, can induce apoptosis via downregulation of its transcriptional targets Bcl-2 and Bcl-XL. We then showed that IL-21-induced cell death is dependent on c-Myc by utilizing specific siRNA and shRNA to block the upregulation of this transcription factor and prevent apoptosis. Since c-Myc is a bona-fide target of STAT3 we also showed that siRNA-mediated knockdown of STAT3 abrogated apoptosis by preventing c-Myc upregulation and its subsequent effects on apoptosis-regulating proteins.

Our results delineate a novel IL-21 pro-apoptotic signaling pathway and one of the first examples in which the STAT3 – c-Myc pathway, which usually promotes B cell survival and oncogenesis, can be exploited for treatment of cancer. Furthermore, our
findings demonstrate that IL-21 is a highly potent anti-DLBCL agent *in vitro* and in animal models and should be examined in clinical studies of DLBCL.
ACKNOWLEDGEMENTS

My graduate work has been a journey full of twists and turns, and certainly full of ups and downs, but it has also been an exceptionally satisfying one. As I clean my desk and move on to future challenges it would be remiss of me to not thank those people that have made this work possible. First and foremost I would like to thank Izidore Lossos, my mentor, who has been nothing short of fantastic in guiding me through experiments, presentations, and manuscripts. Every good researcher needs a strong foundation and I believe that Izidore has certainly provided me with one by continuously exhibiting a keen interest in my development as a scientist. The work in this dissertation was guided by his prolific scientific vision and experience. I would also like to thank my committee members Drs. Sean Scully, Thomas Malek, and Kerry Burnstein who were always willing to sacrifice their time to provide me with helpful suggestions and informed critiques. Finally, I appreciate Dr. John Timmerman’s attendance of my dissertation defense as an external examiner, his keen insights certainly made the final dissertation stronger.

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CHAPTER 4

Figure 4.1  A model of IL-21-induced apoptosis in DLBCL
ABBREVIATIONS

ABC – Activated B cell
ADCC – Antibody-dependent cell-mediated cytotoxicity
BCR – B cell receptor
CDC – Complement-dependent cytotoxicity
CHOP – Cyclophosphamide, Adriamycin, Prednisone, Vincristine
CLL – Chronic Lymphocytic Leukemia
CPM – Counts per minute
CSR – Class switch recombination
DLBCL – Diffuse Large B Cell Lymphoma
Dox - Doxorubicin
EL – Extra Long
FL – Follicular Lymphoma
FL – Full length
GC – germinal center
IgH – Immunoglobulin Heavy-Chain
IL – Interleukin
JAK – Janus kinase
MM – Multiple Myeloma
MZL – Marginal Zone Lymphoma
MOMP – Mitochondrial outer membrane permeabilization
NHL – Non-Hodgkin’s Lymphoma
NK – Natural Killer
NKT – Natural Killer T cells

NOD – Non-obese diabetic

PI – Propidium Iodide

RLU – Relative Luciferase Units

SCID – Severe combined immunodeficiency

shRNA – small hairpin RNA

siRNA – small interfering RNA

STAT – Signal transducer and activator of transcription

TCR – T cell receptor

YO-PRO – YO-PRO iodide
CHAPTER 1

INTRODUCTION

I. DIFFUSE LARGE B CELL LYMPHOMA

Diffuse large B cell lymphoma pathogenesis

Diffuse large B cell lymphoma (DLBCL) is the most common type of non-Hodgkin’s Lymphoma (NHL) and accounts for 30-35% of the 61,900 new cases of NHL and for more than 80% of the aggressive lymphomas diagnosed each year (Institute). It is considered to be an aggressive lymphoma due to its typically rapid growth and limited survival in the absence of effective treatment. DLBCL has a 5 year survival rate of 50-60% and presents with a high degree of heterogeneity morphologically, phenotypically, molecularly, and clinically [reviewed in (Lossos 2005; Illidge and Tolan 2008)].

DLBCL pathogenesis is a complex process in which the cell of origin, varied chromosomal translocations, and errant somatic hypermutation contribute to disease heterogeneity (Lossos 2005). DLBCLs originate from B lymphocytes at specific stages of differentiation and most frequently are composed of cells that morphologically resemble germinal center centroblasts (Friedberg and Fisher 2008).

DLBCLs frequently harbor gene translocations that can arise in normal B cells during the process of somatic hypermutation which occurs within the germinal centers. The most common translocation involves \( BCL-6 \) which is normally upregulated during the transient B cell residence in the germinal centers. The Bcl-6 protein suppresses the apoptosis that would normally be triggered by the genomic
instability that occurs during clonal expansion and immunoglobulin affinity
maturation by repressing expression of ATR, a sensor of DNA damage (Iqbal,
Greiner et al. 2007; Ranuncolo, Polo et al. 2007; Klein and Dalla-Favera 2008). This
Bcl-6 translocation, therefore, can provide DLBCL cells with resistance to apoptosis
and has also been shown to contribute to lymphomagenesis (Ranuncolo, Polo et al.
2007).

The t(14;18)(q32;q21) chromosomal translocation, juxtaposes the BCL-2 gene
with the enhancer of the immunoglobulin heavy-chain (IgH) gene leading to
overexpression of the anti-apoptotic Bcl-2 protein (Weiss, Warnke et al. 1987; Joseph
O. Jacobson 1993; Dalla-Favera, Ye et al. 1994; Huang, Sanger et al. 2002). This
BCL-2 translocation has been found in up to 85% of follicular lymphomas, a common
lymphoma that frequently progresses to DLBCL, and up to 30% of primary DLBCLs

Another common DLBCL translocation involves the IgH enhancer and the C-
MYC oncogene (Iqbal, Greiner et al. 2007; Kikuchi, Nakamura et al. 2008).
Translocation of c-Myc, a transcription factor that can drive the cell cycle and
proliferation as well as induce apoptosis, is seen in about 6% DLBCLs and is a
hallmark of Burkitt’s Lymphoma (Evan, Wyllie et al. 1992; Prendergast 1999; Boxer
and Dang 2001; Nakamura, Nakamine et al. 2002; Kevin D. Mills 2003; Dave, Fu et
al. 2006).

Additional gene mutations, such as the common TP53 gene mutation,
amplifications, and deletions such as 6q further compound variance among
malignancies. The most common translocations and other genetic lesions are included
in Figure 1.1. The heterogeneity of these genetic aberrations and of the resulting disease likely contributes to the widely ranging responses of DLBCLs to therapies and also suggests that DLBCL is actually a group of distinct aggressive B cell lymphomas instead of a single disease entity (Lossos 2005).

Figure 1.1 The most common genetic lesions found in DBLCL and their respective frequencies of occurrence (Lossos 2005).

Most malignancies, including DLBCLs, are addicted to the survival advantages afforded by genetic aberrations (Green and Evan 2002). For instance, the aforementioned translocation and resultant overexpression of \(BCL-2\) can provide DLBCLs with the ability to sequester and silence the pro-death signaling that results from not only the genomic instability, oncogene activation, and cell cycle checkpoint violations that are common in these cancer cells but also from the therapeutic agents used to treat lymphomas. In fact, it has been shown that DLBCLs are particularly sensitive to ABT-737, an agent that inhibits anti-apoptotic proteins Bcl-2, Bcl-X\(_L\), and Bcl-w which leads to the release of bound pro-apoptotic proteins and subsequent
cell death (Deng, Carlson et al. 2007). It can be assumed, therefore, that any downregulation or inhibition of these upregulated anti-apoptotic proteins could exploit this oncogene addiction and lead to cell death in DLBCL.

**DLBCL classification**

Analysis of gene expression as well as cell surface antigens can provide valuable insight into DLBCL cell of origin and by using these techniques DLBCL has been subdivided into four distinct subtypes (Alizadeh, Eisen et al. 2000; Rosenwald, Wright et al. 2002).

Germinal center (GC)-like DLBCLs have a gene expression signature that is similar to that of healthy B cells in germinal centers while the signature of activated B cell (ABC)-like DLBCLs is more similar to that of *in vitro* activated peripheral blood B cells or plasma cells (Alizadeh, Eisen et al. 2000; Rosenwald, Wright et al. 2002; Lossos 2005). Further support for the distinct origins of these subtypes of DLBCL lie in the fact that GC-like DLBCLs frequently express immunoglobulin (Ig) gene intraclonal heterogeneity which indicates the presence of ongoing immunoglobulin somatic mutations, a hallmark of germinal centers, while ABC-like DLBCLs do not (Lossos, Alizadeh et al. 2000). Taken together, these findings suggest that GC-like DLBCL originate from germinal center B cells while the cell of origin for ABC-like DLBCL is likely to be a late or post-germinal center B cell that is undergoing plasmacytic differentiation (Wright, Tan et al. 2003).

The third subtype expresses genes that are not characteristic of either the GC or ABC subtypes. Primary mediastinal lymphomas, the final subtype, are unique in
their clinical presentation and likely originate from thymic B cells and have a distinct immunophenotype and chromosomal imbalance signature while still resembling other DLBCLs morphologically (Davis, Dorfman et al. 1990; Joos, Otano-Joos et al. 1996). Shipp, et al., described another method to subdivide DLBCL into three distinct subtypes – oxidative phosphorylation, B cell receptor/proliferation, and host response – which suggests that tumor microenvironment and host inflammatory response are possibly the dominant determinants of DLBCL pathogenic characteristics (Monti, Savage et al. 2005).

**DLBCL therapy**

For 30 years the standard therapy for DLBCL had consisted of Prednisone, Cyclophosphamide, Adriamycin, and Vincristine (CHOP). Since the addition of the anti-CD20 monoclonal antibody Rituximab to standard therapy ten years ago cure rates have improved but remain at only 50-60% (Habermann, Weller et al. 2006). Stem cell transplantation following high-dose chemotherapy is able to cure another 10-20% of patients but there remains a need for well-tolerated treatments that can improve cure rates further, particularly for patients with the highest risk of treatment failure (Andreadis, Gimotty et al. 2007).

The CHOP+Rituximab (R-CHOP) therapy induces apoptosis in DLBCL via several mechanisms. Firstly, adriamycin (doxorubicin) is a potent anti-neoplastic drug used as treatment for a variety of cancers including lymphomas. Many mechanisms of adriamycin toxicity have been reported yet many have only been shown to be active at drug concentrations that exceed those achieved in human plasma after treatment
administration (Gewirtz 1999). Therefore, the two mechanisms most likely to be mediating the anti-cancer effects of this drug include DNA intercalation and subsequent inhibition of DNA strand separation and the inhibition of DNA Topoisomerase II activity which results in double-strand DNA breaks (Tewey, Rowe et al. 1984; Gewirtz 1999). Both mechanisms are able to induce apoptosis in rapidly dividing neoplastic cells.

Another component of this therapy is cyclophosphamide, an immunosuppressive and alkylating agent that induces DNA cross-linking at guanine N-7 positions and results in cell death (Ahmed and Hombal 1984). Vincristine is a vinca alkaloid that induces apoptosis by inhibiting microtubule formation in the mitotic spindle which arrests mitosis at metaphase (Cardinali, Cardinali et al. 1963; Jordan, Thrower et al. 1992). Mitotic arrest in proliferating cells activates apoptosis. Finally, prednisone is a immunosuppressive corticosteroid that inhibits the production of prostaglandins and can directly enhance apoptosis in lymphoma cells (Elias, Portlock et al. 1978).

As previously mentioned, rituximab was recently added to the standard therapy and its addition has increased overall survival by 10-12% (Habermann, Weller et al. 2006). This monoclonal anti-CD20 antibody induces apoptosis in neoplastic as well as normal CD20+ B cells by binding CD20 and inducing complement-dependent cytotoxicity, antibody-dependent cell-mediated cytotoxicity, or directly activating apoptosis (Pescovitz 2006). Over the last ten years the outcomes for patients with DLBCL have improved due to the addition of rituximab to standard therapy and the intensification of chemotherapy regimens yet with 5-year survival
rates of only 50-60% there still exists an urgent need for new therapies (Illidge and Tolan 2008).

Many cancers develop resistance to standard therapies and DLBCL is no exception. Overexpression of Bcl-2, an anti-apoptotic member of the Bcl-2 family of apoptosis-regulating proteins, can lead to resistance to chemotherapy by inhibiting the apoptotic pathways that are activated by chemotherapy and is associated with a poor prognosis (Mounier, Briere et al. 2003). Bcl-2 inhibitors, which would block the anti-apoptotic activity of this protein, are being developed as chemotherapy adjuvants that can potentially overcome this drug resistance. Cillessen, et al., recently reported that drug resistance in DLBCL can also be due to dysfunctional signaling downstream of caspase 9 which is a necessary executor of the intrinsic apoptotic pathway activated by CHOP (Cillessen, Hess et al. 2007). DLBCLs have also developed resistance to rituximab by downregulating cell surface CD20 or upregulating levels of CD55 or CD59 which block CDC killing of rituximab-labeled cells (Macor, Piovan et al. 2007; Hiraga, Tomita et al. 2009).

As further insight into the tumorigenesis and molecular characteristics of DLBCL is acquired it is likely that novel therapies will be developed and improve survival for patients with this disease.

II. INTERLEUKIN-21

Interleukin-21

Interleukin-21 (IL-21) is a recently-discovered cytokine that is produced by NKT cells and CD4+ T cells including T\textsubscript{H1}7 cells (Parrish-Novak 2000; Coquet
It is normally produced in response to T cell receptor (TCR) activation but can also be induced by calcium signaling alone in preactivated T cells (Kim, Korn et al. 2005). Innate immune signals, including mycobacterial antigen (BCG) have also been shown to induce IL-21 production, especially in NKT cells (Harada, Magara-Koyanagi et al. 2006). It is an important regulator of many immune responses such as autoimmunity, allergy, infection, and rejection of tumors (Coquet 2007; Yi, Du et al. 2009). The discovery of IL-21 included first the identification of a functional, novel class I cytokine receptor, IL-21R, and subsequent functional screening of ligand libraries isolated from activated T cells (Coquet 2007). Interleukin-21 cDNA shows extensive open reading frame homology with IL-2, IL-4, and IL-15 and belongs to the γ-common chain receptor interleukin family (Ozaki, Kikly et al. 2000). The various cytokines in the IL-2 family are responsible for such vital functions as activation of NK cells and T cells and B cell class-switch. The major functions of these cytokines are listed in Figure 1.2.
Figure 1.2 IL-21 belongs to the family of the common γ-chain binding cytokines. Shown are all members of this family, its receptors and major functions of the different cytokines in the immune system (Spolski and Leonard 2008). (you have mention mof Figure 4 in the figure that is not your figure 4)

The IL-21 gene, which lies on chromosome 4, has a 5’ regulatory region which contains three NF-AT binding sites (Kim, Korn et al. 2005). The chromosomal region that contains IL-21 also contains the genes for IL-2 and IL-15 and encompasses bands 4q26-q27 to 4q31 (Parrish-Novak 2000). The presence of these three related genes in the same region perhaps allows for coordinate expression of the cytokines as part of a “looped domain” that is similar to that seen for IL-4, IL-5, and IL-13 and makes it likely that they arose by gene duplication (Spilianakis, Lalioti et al. 2005; Coquet 2007). IL-21 was recently shown to have a vital role in the clearance of chronic infections and act as a messenger between CD4+ and CD8+ T cells thus underscoring the importance of this cytokine in regulating the immune system (Yi, Du et al. 2009).

Other cytokines such as Granulocyte macrophage-colony stimulating factor (GM-CSF), thrombopoietin, G-CSF, IL-2 and erythropoietin have been shown to be therapeutically useful in a range of inflammatory, immunological, and infectious diseases. The various ligands of the class I family of cytokine receptors, of which IL-21 is a member, regulate growth, activation, survival, and differentiation of cells in the immune and hematopoietic systems making them important candidates for research and application in the clinic (Benton 1991).
Interleukin-21 Receptor

The IL-21 receptor was discovered in 2000 by genomic and cDNA sequencing of an open reading frame that was presumed to encode a type I cytokine receptor (Ozaki, Kikly et al. 2000; Parrish-Novak 2000). The IL-21Rα chain is unique and gives the IL-21R its distinct signaling profile (Parrish-Novak 2000). The IL-21Rα gene is located on chromosome 16 in close proximity to the IL-4Rα gene, to which it is most similar in regards to domain organization. The IL-21Rα cDNA encodes a 538 amino acid receptor that contains three domains: an extracellular domain containing the highly conserved Trp-Ser-X-Trp-Ser (WSXWS) motif that binds ligand, a transmembrane domain, and a large intracellular domain that includes the Box 1 and Box 2 elements that are vital for signal transduction (Coquet 2007). NMR spectroscopy has elucidated the three-dimensional structure of the IL-21Rα as having four α helices in a typical up-up-down-down bundle (Bondensgaard, Breinholt et al. 2007). Interestingly, a segment of helix C that is involved in ligand binding is unstable which may have an effect on IL-21 signaling (Bondensgaard, Breinholt et al. 2007).

IL-21 signaling requires the IL-21Rα chain to associate with γ-common (γc) upon binding the cytokine which is a trait shared by all members of the IL-2 family which also includes IL-2, IL-4, IL-7, IL-9, and IL-15 (Habib, Senadheera et al. 2002). The γ-common chain is critical for the development and function of lymphocytes and mutations of the gene leads to X-linked, severe-combined immunodeficiency (X-SCID) (Habib, Senadheera et al. 2002).
The IL-21R was initially reported to be present on all T, B, and NK cells (Ozaki, Kikly et al. 2000; Jin, Carrio et al. 2004). B cells, which express higher levels of IL-21R than other lymphocytes, exhibit progressively higher levels of IL-21R as the cells develop from progenitors to mature B cells yet the receptor is absent in mouse memory B cells and plasma cells (Jin, Carrio et al. 2004; Good, Bryant et al. 2006). IL-21R levels have been shown to be upregulated in B cells following activation through CD40 or Toll-like receptor (TLR) (Good, Bryant et al. 2006). Similarly, receptor levels in T cells can be increased following T cell receptor stimulation.

**IL-21 signaling**

Janus tyrosine kinase 3 (Jak3) is activated upon IL-21 binding to the $\gamma$-common chain in a similar fashion as other members of the $\gamma$-common family of cytokines (Figure 1.3) (Habib, Senadheera et al. 2002). Jak1, however, is activated with IL-21 binding to the IL-21R$\alpha$ chain (Ozaki, Kikly et al. 2000). Activation of these two Jak kinases leads to phosphorylation and robust activation of Signal Transducer and Activator of Transcription 1 (STAT1) and STAT3 and weak activation of STAT4 and 5 (Ozaki, Kikly et al. 2000; Parrish-Novak 2000; Strengell, Matikainen et al. 2003). STAT3 seems to be a critical transcription factor in IL-21 signaling as mutation of STAT3 in Ba/F3 cells prevents the induction of proliferation by IL-21 that is evident in wild type cells (Zeng, Spolski et al. 2007). Data obtained with IL-21R chimeric receptors has shown that the intracellular portion of the IL-21R can transduce a weak signal as a homodimer although its natural complex includes
the γ-common chain which is required for STAT activation (Habib, Senadheera et al. 2002; Coquet 2007). IL-21 signaling can also activate PI3-kinase and RAS/MAPK pathways to modulate cell proliferation, protein translation, and survival (Brenne, Baade Ro et al. 2002; Zeng, Spolski et al. 2007).

**Figure 1.3 IL-21 – STAT3 signaling pathway.** (Spolski and Leonard 2008).

**IL-21 as a modulator of the immune system**

IL-21 is able to modulate the activity of most lymphocyte subsets (Figure 1.4). IL-21 can increase proliferation in CD4+ T cells and stimulate their differentiation
into T_{H17} cells (Parrish-Novak, Foster et al. 2002; Strengell, Sareneva et al. 2002; Suto, Nakajima et al. 2002; Wurster, Rodgers et al. 2002). IL-21 also stimulates proliferation in CD8^{+} T cells but also induces production of perforin and granzymes thus increasing their cytotoxicity (Parrish-Novak, Foster et al. 2002; Li, Bleakley et al. 2005; Zeng 2005; He, Wisner et al. 2006; Peluso, Fantini et al. 2007). These effects on T cells also require co-stimulation of the T cell receptor (TCR) or other activating cytokines such as IL-2 or IL-15. NK cells stimulated with IL-21 and other cytokines or though Fc receptors exhibit increased differentiation and maturation as well as an increase in cytotoxicity (Parrish-Novak, Foster et al. 2002; Simona Sivori 2003; Wang, Tschoi et al. 2003; Brady, Hayakawa et al. 2004; Roda, Parihar et al. 2006). IL-21 stimulation also increases ADCC of antibody coated targets by NK cells which has served as the impetus to begin clinical trials of IL-21 with rituximab for B cell malignancies. The effects of IL-21 on other lymphocyte subsets are shown in Figure 1.4.
IL-21 effects on B cells

B cells are major components of adaptive immunity and their differentiation and function need to be highly regulated. The nature of IL-21’s effects on B cells depends on the organism, specific cellular context (e.g. activation and developmental stages) and presence of costimulatory factors (Arguni, Arima et al. 2006; Brandt, Singh et al. 2007). For example, IL-21 increases growth and differentiation of murine B lymphocytes that received both B cell receptor (BCR) and T cell help mediating signals yet induces apoptosis in murine B lymphocytes post TLR stimulation which can be prevented by CD40 stimulation (Jin, Carrio et al. 2004). Research has also shown that IL-21-induced apoptosis in murine B cells occurs mainly via upregulation
of pro-apoptotic proteins such as Bim and down-regulate anti-apoptotic proteins such as Bcl-XL (Jin, Carrio et al. 2004; Herrin and Justement 2006).

Although there have been several reports of IL-21 inducing apoptosis in murine B cells, especially in the presence of strong innate signals, the effects of IL-21 on human non-neoplastic B cells have been confined to regulation of B cell activation and differentiation. Specifically, IL-21 has been shown to costimulate human B cell proliferation induced by anti-CD40 antibody, yet inhibit proliferation induced by IL-4 and BCR stimulation (Parrish-Novak 2000; Good, Bryant et al. 2006).

*In vivo* studies have shown that overexpression of IL-21 in murine B cells leads to increased numbers of isotype-switched memory cells, immature transitional cells, and plasma cells resulting in increased levels of IgG and IgM in serum (Ozaki 2004). IL-21 also contributes to the diversity of human immunoglobulin (Ig) function. Specifically, IL-21 was reported to have a central role in the differentiation of human primary B cells into plasma cells by inducing expression of AID, an enzyme which catalyzes class switch recombination (Ettinger 2005; Saito, Kitayama et al. 2008). Studies have shown that IL-21 stimulates CSR and secretion of IgG and IgA in postswitch CD40-stimulated IgM+ memory B cells (Ettinger, Sims et al. 2007).

Taken together, these findings suggest that IL-21 is a potential executor of B cell fate. IL-21 induces apoptosis in improperly-activated B cells that receive only non-specific activation of their BCR or TLR yet stimulates proliferation and differentiation of B cells that are activated by antigen and receive T cell help. As research continues into IL-21 signaling our understanding of the pleiotropic effects of IL-21 will be clearer.
**IL-21 as treatment for neoplasia**

IL-21 has been shown to have anti-tumor activity in a variety of mouse tumor models (Comes 2006). These effects are largely thought to be indirect and mediated by IL-21-induced terminal differentiation of NK cells and elicitation of protective T cell responses (Brady, Hayakawa et al. 2004). Other animal experiments showed that IL-21 is capable of enhancing anti-tumor cytotoxic T cell response and T cell memory in response to tumor re-challenge. This would suggest that use of IL-21 as an adjuvant therapy to antibody treatment may be beneficial (Smyth 2006).

In contrast to indirect immune mediating effects of IL-21 on solid tumors, IL-21 may have direct effects on IL-21R-expressing malignancies originating from B lymphocytes. Indeed, it was reported that IL-21 enhanced growth of multiple myeloma (MM) cells (Menoret, Maiga et al. 2008) yet induced mild apoptosis in chronic lymphocytic leukemia (CLL) B cells that express relatively low levels of IL-21R (de Totero, Meazza et al. 2006; Gowda, Roda et al. 2008). The apoptotic response in CLL cells was enhanced by anti-CD40 simulation which upregulated cell surface expression of IL-21R (de Totero, Meazza et al. 2006). IL-21-induced apoptosis of CLL B cells involved the activation of caspase 8 and 3, suggesting a role for the extrinsic apoptotic pathway. Interestingly, another study reported that IL-21 stimulation caused CLL cells to produce and secrete granzyme B, a potent cytotoxic protein (Jahrsdorfer, Blackwell et al. 2006). IL-21 also stimulated the cleavage and activation of Bid as well as cleavage of p27 and PARP in these cells. Finally, Hodgkin’s Lymphoma cells were shown to express the IL-21R as well as secrete IL-
21 which possibly protects the Reed-Sternberg cells from CD95-induced apoptosis (Lamprecht, Kreher et al. 2008).

**IL-21 in clinical trials**

Malignant melanoma and renal cell carcinoma were two early candidates for IL-21 treatment in clinical trials due to strong preclinical data and previous successes treating these malignancies with immunotherapy (Fyfe, Fisher et al. 1995; Negrier, Escudier et al. 1998). Two Phase I dose-escalation studies evaluated IL-21 therapy in 72 patients with either metastatic melanoma or renal cell carcinoma and demonstrated favorable pharmacodynamic and pharmacokinetic profiles and good patient tolerability with limited side effects. The observed limited toxicities consisted mainly of Grade 3 laboratory abnormalities such as neutropenia, thrombocytopenia, hyponatremia, hypophosphatemia, and increased liver transaminases (Davis, Skrumsager et al. 2007; Thompson, Curti et al. 2008). IL-21 also showed some evidence of antitumor activity in both diseases. For metastatic melanoma 1 complete response was observed, 1 partial response, 20 stable diseases, and 27 progressive diseases. In patients with renal cell carcinoma IL-21 treatment led to 4 partial responses, 13 stable diseases, and 2 progressive diseases. Overall, these studies showed that repeated cycles of IL-21 are well tolerated and encourage further study of IL-21 as a cancer therapy. Additional clinical trials are ongoing to further explore the antitumor activity of IL-21 for metastatic melanoma, renal cell carcinoma, metastatic colon cancer, and for non-Hodgkin’s lymphoma when used in conjunction with rituximab.
Pharmacologic stimulation of the immune system can lead to autoimmunity as is seen with IL-2 immunotherapy (Fyfe, Fisher et al. 1995). This is also a risk with IL-21 since the cytokine promotes cytotoxic T cell responses and $T_{H}17$ cell generation which are involved with several autoimmune disorders (Reiner 2007). Thus far IL-21 has not been shown to induce any autoimmune responses which may be due to its relatively specific role in stimulating proliferation and survival only in B cells that are appropriately responding to antigens.

The pleiotropic effects of IL-21 on B cells and lack of understanding of the cytokine’s effects on DLBCL prompted the studies in this dissertation. We hypothesize that IL-21 treatment has a pro-apoptotic effect on DLBCL. If this hypothesis is confirmed, we aim to elucidate the mechanism of IL-21-induced apoptosis in DLBCL. In this study we investigated the role of IL-21R in DLBCL, determined the effects of IL-21 on DLBCL cell lines and primary tumors, and tested the *in vivo* activity of IL-21 on DLBCL xenograft tumors in mice. Our findings elucidate a novel IL-21 signaling pathway and suggest that IL-21 may be useful as a therapeutic agent for DLBCL.
CHAPTER 2
METHODS

I. REAGENTS

Recombinant IL-21 and Biotinylated Anti-IL-21R antibody were kindly provided by Zymogenetics, Inc. (Seattle, WA). Recombinant IL-2 was purchased from R&D Systems (Minneapolis, MN). LY294002 was purchased from Sigma-Aldrich (St. Louis, MO). Biotinylated isotype control and Streptavidin-FITC used for receptor staining were purchased from BD Biosciences (San Jose, CA). Anti-Bax, Bcl-2 (50E3), c-Myc, pSTAT1 (Tyr701), STAT1, pSTAT3 (Tyr705), STAT3, pSTAT5 (Tyr694), and STAT5 antibodies were purchased from Cell Signaling Technology (Danvers, MA). Anti-GAPDH antibody was purchased from Ambion (Austin, TX). Anti-Bcl-X$_L$ (YTH-2H12) antibody was purchased from Trevigen (Gaithersburg, MD) and anti-Bim and Anti-Rat-HRP antibodies were purchased from Calbiochem (San Diego, CA). Anti-Bcl-6 (N-3), Mcl-1 (22), and Bid (FL-195) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The pan-caspase inhibitor Z-VAD-FMK, caspase-8 inhibitor Z-IETD-FMK, and caspase-9 inhibitor Z-LEHD-FMK were purchased from R&D Systems (Minneapolis, MN). Doxorubicin was purchased from Sigma-Aldrich (St. Louis, MO) and Rituximab was purchased from Genentech (San Francisco, CA).
II. DLBCL CELL LINES

The following cell lines were selected for this study: OCI-LY-3, OCI-LY-10, HBL-1 (ABC-like DLBCL); OCI-LY-19, SU-DHL-4, SU-DHL-6, VAL (GC-like DLBCL); MC116, RC-K8 (non-GC-like DLBCL); RPMI-8226, U-266 (multiple myeloma); HeLa and HEK293. MC116, RC-K8, RPMI-8226, SU-DHL-4, SU-DHL-6, U-266, and VAL cell lines were grown in RPMI 1640 medium (Mediatech, Manassas VA) supplemented with 10% fetal bovine serum (FBS) (Mediatech), 2 nM glutamine (Gibco BRL, Grand Island NY), and penicillin/streptomycin (Gibco BRL). HBL-1, OCI-LY-3, OCI-LY-10, and OCI-LY-19 cell lines were grown in Iscove’s Modified Dulbecco Medium (IMDM) (Mediatech) supplemented with 20% human plasma, 2nM glutamine, penicillin/streptomycin, and 50 µM 2-β mercaptoethanol (Gibco BRL). HeLa and HEK293 cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) (Mediatech) supplemented with 10% FBS, 2nM glutamine, and penicillin/streptomycin. The RC-K8-Resistant (RC-K8R) cell line was generated by treating IL-21-sensitive RC-K8 cells weekly with IL-21 at escalating concentrations from 10 to 100 ng/mL for a period of 10 weeks.

III. DLBCL PRIMARY TUMORS

Fresh primary tumors, obtained from routine biopsies after patients signed an informed consent approved by the Institutional Review Board, were used for preparation of viable single cell suspensions. The lymph nodes were cut steriley and forced through a metal sieve. Mononuclear cells were obtained after centrifugation of the cell suspension over Ficoll/Hypaque gradient as per the manufacturer’s
instructions (GE Healthcare, Piscataway NJ). B cell purification was performed by negative selection using a cocktail of biotinylated CD-2, CD-14, CD-16, CD-36, CD-43, and CD-235a (Glycophorin A) antibodies (Miltenyi Biotec, Auburn CA). Magnetically labeled cells were separated using an autoMACS magnetic sorter (Miltenyi Biotec). Cell viability was assessed with YO-PRO and Propidium Iodide (PI) staining. Purity was assessed by anti-CD-19 (BD Biosciences) staining and analysis on a Becton-Dickinson LSR analyzer (BD Biosciences). Samples with at least 80% viability and 95% B cell purity were cultured in RPMI 1640 medium (Fisher Scientific) supplemented with 10% FBS, 2 nM glutamine, and penicillin/streptomycin and used for subsequent experiments.

IV. CELL-SURFACE RECEPTOR STAINING

5x10^6 cells were washed with 1X phosphate-buffered saline (PBS) and resuspended in blocking buffer (Hank’s Balanced Salt Solution [Mediatech], 2% FBS, 2% Normal Goat Serum [Rockland Immunochemicals, Gilbertsville PA], 3% human AB sera) for 10 minutes. Cells were pelleted and resuspended in cold staining buffer (Hank’s Balanced Salt Solution, 2% FBS). Biotinylated Anti-IL-21R antibody or isotype control was added for 30 minutes followed by 3 washes and resuspension in cold staining buffer with Streptavidin-FITC (BD Biosciences) for 30 minutes. After three additional washes the cells were resuspended in 2% para-formaldehyde (Sigma-Aldrich, St Louis MO) for 10 minutes, washed, and resuspended in cold staining buffer. Cells were analyzed on a BD LSR Analyzer (BD Biosciences).
V. \( ^3 \)H THYMIDINE INCORPORATION

For proliferation studies, \( 10^5 \) cells/mL were incubated with or without IL-21 (100 ng/mL) for specified time periods. Concentration-corrected aliquots were transferred into a 96-well plate and incubated with \( ^3 \)H-Thymidine at a final concentration of 2\( \mu \)Ci/mL (PerkinElmer, Boston MA) for four hours. Cells were transferred onto fiberglass filters and radioactivity was measured by a TopCount-NXT scintillation counter (PerkinElmer).

VI. YO-PRO/PI CELL VIABILITY STAINING

For apoptosis studies, \( 10^5 \) cells/mL were incubated with or without IL-21 (10 or 100 ng/mL) for specified time periods, collected, washed with 1X PBS and stained with PI (Invitrogen, Carlsbad CA) and YO-PRO (Invitrogen) as per the manufacturer’s instructions. PI intercalates into DNA of dead cells that no longer have an intact plasma membrane. YO-PRO intercalates into the DNA of cells undergoing early apoptosis and exhibit small perforations in the plasma membrane. Analysis was performed on a Becton-Dickinson LSR analyzer (BD Biosciences, San Jose CA).

VII. CASPASE ACTIVATION

To assay caspase activation, cells were treated with IL-21 (100 ng/mL) for 48 hours. Caspase-Glo 3 reagent (Promega, Madison WI) was added for 30 minutes and luminescence was measured on a Luminoskan Ascent (Thermo Labsystems, Waltham MA) luminometer. Readings were normalized to total amount of protein.
VIII. WHOLE CELL EXTRACT PREPARATION AND WESTERN BLOT ANALYSIS

Whole cell extracts for Western blot analysis were prepared by lysing $5 \times 10^6$ cells in RIPA buffer (1X PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 10mM phenylmethylsulfonyl fluoride, 1µg/ml aprotinin, 100mM sodium orthovanadate) on ice for 30 minutes. Protein concentrations in cell lysates were determined using Coomassie Protein Assay Reagent (Pierce, Rockford IL) and a Genesys 10UV Spectrophotometer (Thermo Labsystems). 20 µg of whole cell lysates per experimental condition were separated by electrophoresis on 10% SDS-polyacrylamide gel, transferred to nitrocellulose membrane (BioRad Laboratories Inc., Hercules CA) and immunoblotted with specified antibodies. Supersignal West Pico chemiluminescent substrate (Thermo Scientific, Rockford IL) was added to visualize protein levels with light-sensitive film (Phenix Research, Candler NC). Film was then scanned and data were subjected to densitometric analysis using Scion Image Software (NIH). Protein levels were normalized to the corresponding loading controls and reported as ratios.

IX. TRANSFECTION OF CELL LINES

RC-K8 and MC-116 cells were transfected by Amaxa electroporation using solution L, program H-024 and solution C, program D-024, respectively, as specified by the manufacturer (Amaxa, Gaithersburg MD). Non-targeting control siRNA as well as pools of siRNAs targeting C-MYC, STAT1, STAT3, BAX, and BIM were purchased from Dhharmacon (Chicago, IL). Control shRNA and shRNA targeting C-
MYC were purchased from Origene (Rockville, MD). PCDNA3.1 empty vector, PCDNA3.1-BCL-XL, and PCDNA3.1-MCL-1 were kindly provided by Dr. Lawrence Boise (University of Miami). PCDNA3.1-BCL-2 (Addgene plasmid 8768) was purchased from Addgene (Cambridge, MA) (Yamamoto, Ichijo et al. 1999).

X. DLBCL XENOGRAFT TUMOR STUDIES

For RC-K8 xenograft tumors, six to eight-week old female NOD/SCID mice (Jackson Laboratory, Bar Harbor ME) were inoculated subcutaneously in the flank with 5x10^6 RC-K8 cells. For MC116 xenograft tumors, six to eight-week old female NOD/SCID mice were injected intravenously with Anti-Asialo GM1 antisera (Wako Chemicals, Richmond VA) 24 hours prior to subcutaneous inoculation in the flank with 5x10^6 MC116 cells. Mice were monitored daily for tumor growth. When the tumor volume reached 25 mm^2 the tumors were treated intratumorally once daily with IL-21 (10µg) or PBS for 7 consecutive days. Tumors were assessed using the 2 largest perpendicular axes (l indicates length; w, width) as measured with standard calipers. Tumor-bearing mice were assessed for weight loss and tumor size at least twice weekly. Animals were sacrificed when tumor area exceeded 100 mm^2 or after loss of more than 10% body weight in accordance with institutional guidelines. All procedures with animals were conducted in conformity with an approved institutional animal protocol.
XI. MICROARRAY HYBRIDIZATION AND ANALYSIS

OCI-LY-3, RC-K8, MC-116, OCI-LY-10 and IL-21-resistant RC-K8 cell lines were treated with IL-21 for 6 hours and harvested. RNA was isolated by TRIzol reagent (Invitrogen, Carlsbad CA) according to manufacturer’s instructions. Total RNA yield was determined spectrophotometrically.

RNA quality was assessed on the Agilent 2100 Bioanalyzer (Santa Clara, CA). 10 to 20 µg of total sample RNA or Human Reference Total RNA (Stratagene, La Jolla CA) were mixed with the 200 pMol of oligo-dT primer. After 10 min incubation at 70°C and 5 min at 4°C, 20 units of Transcriptor reverse transcriptase (Roche, Indianapolis IN), 10 nMol of each dNTP and 4 nMol of aminoallyl-dUTP (Ambion) were added, and this mix was incubated 2 hours at 42°C. After 30 min RNase treatment cDNA was purified using QIAquick PCR purification Kit (Qiagen, Valencia CA). Amount of cDNA was determined spectrophotometrically, and samples were dried on speedvac. cDNA was resuspended in the carbonate buffer (pH 9.0 – 9.3) and mixed with the Cy3- and Cy5-NHS ethers (Amersham Biosciences, Piscataway NJ). After 1 hour incubation in the dark 20 mMol of hydroxylamine was added to quench the reaction, and labeled cDNA was purified using QIAquick PCR purification Kit (Qiagen). Concentration of the labeled cDNA and labeling efficiency were determined spectrophotometrically, and labeled cDNA was hybridized to Agilent Oligo microarrays for 17 hours at 60°C according to manufacturer’s instructions.

The microarrays were scanned at 10 micron resolution using a GenePix 4000A scanner (Molecular Devices, Sunnyvale CA) and the resulting images were
analyzed with the software package GenePix Pro 5.1 (Molecular Devices). Data extracted from the images were transferred to the software package Acuity 4.0 (Molecular Devices) for normalization and statistical analysis. Each array was normalized for signal intensities across the whole array and locally, using Lowess normalization. Features for further analysis were selected according to the following quality criteria: (1) at least 90% of the pixels in the spot had intensity higher than background plus two standard deviations; (2), there were less than 2% saturated pixels in the spot; (3) signal to noise ratio (defined as ratio of the background subtracted mean pixel intensity to standard deviation of background) was 3 or above for each channel; (4) the spot diameter was between 110 and 150 micron; (5) the regression coefficient of ratios of pixel intensity was 0.6 or above. cDNA array results were filtered for absolute fold change of $\geq 2$ and genes that were modulated similarly by IL-21 across similarly-responding cell lines were identified.

**XII. STATISTICAL ANALYSIS**

To test the differences in IL-21 responses we used the 2-tailed Student t test. Mice survival curves were estimated using the product-limit method of Kaplan-Meier and were compared using the log-rank test. P-values less than 0.05 were considered statistically significant.
CHAPTER 3

RESULTS

I. IL-21 RECEPTOR STUDIES

*IL-21 Receptor is expressed on DLBCL cell lines*

Analysis of cell surface IL-21R expression in a panel of cell lines demonstrated that all tested DLBCL cell lines displayed the receptor, with the most prominent expression on RC-K8, OCI-LY-3, HBL-1, OCI-LY-10 and MC116 (Figure 3.1). Lower levels of IL-21R expression were detected in OCI-LY-19, SU-DHL-4, SU-DHL-6, and VAL DLBCL cell lines. Neither HeLa (Figure 3.1) nor 293T (not shown) cells expressed detectable levels of IL-21R while multiple myeloma cell lines expressed levels lower than DLBCL cell lines (not shown).
Figure 3.1 DLBCL cell lines express IL-21R at variable levels. DLBCL cell lines were stained for IL-21R cell surface expression as described in the Methods section. Solid histograms represent staining with biotinylated anti-IL-21R antibody and dashed histograms represent isotype control. Results shown are representative of three independent experiments.

Interleukin-21 treatment downregulates cell surface expression of IL-21R

Cytokine stimulation can lead to receptor downregulation in cells of the hematopoietic system (Parrish-Novak 2000). By measuring cell surface expression of IL-21R before and after IL-21 treatment we confirmed that receptor expression was decreased post stimulation in 3 of 4 DLBCL cell lines tested (Figure 3.2 and 3.3). The antibody used for cell surface receptor staining recognizes an epitope that does not interfere with IL-21 binding thus suggesting that the receptor is internalized in response to binding cytokine.

Figure 3.2 IL-21 treatment downregulates cell surface expression of IL-21R. OCI-LY-3 cells were stained for IL-21R cell surface expression as described in the Methods section. Dotted histograms represent staining with biotinylated anti-IL-21R antibody and solid histograms represent isotype control. Results shown are representative of three independent experiments.
Figure 3.3 IL-21 treatment downregulates cell surface expression of IL-21R. DLBCL cell lines were treated with IL-21 for the specified time periods and stained for IL-21R cell surface expression as described in the Methods section. Results shown are representative of three independent experiments.

**IL-21 stimulates phosphorylation of STAT transcription factors**

Next, we investigated the functionality of the IL-21R in the DLBCL cell lines by stimulating cells with IL-21. It has been reported that IL-21 can activate STAT1, STAT3, and to a lesser degree STAT5 in B cells (de Totero, Meazza et al. 2006; Akamatsu, Yamada et al. 2007). STAT3 phosphorylation was rapidly induced in all investigated DLBCL cell lines (Figure 3.4). OCI-LY-3 cells, which exhibit constitutive activation of STAT3, also showed increased STAT3 phosphorylation following IL-21 stimulation yet the level of induction was lower than in other analyzed cell lines. IL-21 also induced phosphorylation of STAT1 and STAT5 although to a lesser degree (Figure 3.4).
II. IL-21 EFFECTS ON DLBCL CELL PROLIFERATION AND VIABILITY

IL-21 inhibits proliferation of DLBCL cell lines

Having shown functionality of the IL-21R in DLBCL cell lines, we investigated the effects of IL-21 on DLBCL cell proliferation. Interestingly, IL-21 stimulation led to a slight increase (3-25%) in proliferation after 24 hours in approximately half of DLBCL cell lines tested by \(^3\)H Thymidine incorporation, an indicator of DNA synthesis (Figure 3.5). In contrast, IL-21 markedly inhibited cellular proliferation at 48 hours in 5 of 6 DLBCL cell lines with an average
reduction in proliferation of 73% (Figure 3.5). Finally, IL-21 induced almost complete proliferation arrest after 72 hours of treatment in all tested DLBCL cell lines (Figure 3.5).

![Figure 3.5 IL-21 inhibits DLBCL cell proliferation.](image)

**Figure 3.5 IL-21 inhibits DLBCL cell proliferation.** Cells were stimulated with IL-21 (100ng/mL) for the indicated time periods and then assayed for 3H Thymidine incorporation as described in the Methods section. Error bars represent SE. Results are representative of three independent experiments.

**IL-21 induces apoptosis in DLBCL cell lines**

We next examined whether the proliferation arrest induced by IL-21 was associated with any changes in cell survival. YO-PRO and propidium iodide (PI) staining for cell viability revealed that IL-21 treatment potently induced apoptosis and decreased cell viability in all but one (OCI-LY-3) of the examined DLBCL cell lines in a dose- and time-dependent manner (Figure 3.6, 3.7 and 3.8). The largest decreases in cell survival upon treatment with IL-21 were observed in the RC-K8, MC116 and OCI-LY-10 cell lines (Figure 3.8). IL-21 treatment did not induce apoptosis in MM cell lines which exhibit lower levels of IL-21R expression (Figure 3.8). As expected,
the viability of HeLa and 293T cell lines was also unaffected by IL-21 stimulation since these cells lack expression of IL-21R (Figure 3.8).

**Figure 3.6 IL-21 induces dose-dependent apoptosis.** Cells were stimulated with IL-21 at indicated doses for 72 hours and cell viability was assayed by YO-PRO/PI staining as described in the Methods section. Cells were considered “live” if negative for both YO-PRO and PI staining. Results are representative of three independent experiments.
Figure 3.7 IL-21 induces time-dependent apoptosis. RC-K8 cells were stimulated with IL-21 (100ng/mL) for the indicated time periods and cell viability was assayed by YO-PRO/PI staining. Results are representative of three independent experiments.
Figure 3.8 IL-21 induces apoptosis of B cell lymphomas but not control or MM cells. Cells were stimulated with IL-21 (100ng/mL) 72 hours and cell viability was assayed by YO-PRO/PI staining. Data represent means ± SE from three independent experiments.

**IL-21-induced apoptosis is additive to doxorubicin cytotoxicity**

We next examined the effects of IL-21 on cell viability when used concurrently with doxorubicin (adriamycin), a major component of the standard clinical therapy for DLBCL. Interestingly, the levels of apoptosis induced by IL-21 were comparable to or higher than those induced by doxorubicin in the RC-K8, OCI-LY-10, and MC116 cell lines (Figure 3.9). In addition, IL-21 treatment augmented the cytotoxic effects of doxorubicin in all cell lines except OCI-LY-3 which is resistant to IL-21.
Figure 3.9 Apoptosis induced by IL-21 is additive to Doxorubicin-induced cell death. Cells were treated with IL-21 (100ng/mL) and/or doxorubicin (100ng/mL) for 48 hours and cell viability was assayed by YO-PRO/PI staining. Data represent means ± SE from three independent experiments.

**IL-21 induces apoptosis of primary B cell neoplasias**

To demonstrate that the IL-21-induced cytotoxicity was not restricted to established DLBCL cell lines, we evaluated IL-21’s effects on five fresh *de novo* untreated primary DLBCL tumors. All analyzed primary tumors exhibited cell surface IL-21R expression (Figure 3.10). Notably, IL-21R was expressed at higher levels on the tumor cells compared to normal B cells obtained from non-malignant tonsil or lymph node in the same patients (Figure 3.10).
Figure 3.10 IL-21R is expressed on primary tumors and healthy B cells. Healthy B cells (Tonsil 1) and B cells isolated from primary tumors were stained for IL-21R cell surface expression as described in the Methods section. Solid histograms represent staining with biotinylated anti-IL-21R antibody and dashed histograms represent isotype control. Specimens that have matched tumor and normal tissue samples have tumors designated with a “T” and normal tissue with an “N”. Results shown are representative of three independent experiments.

IL-21 treatment induced marked apoptosis and cell death in all examined primary DLBCL tumors (Figure 3.11 and 3.12). The magnitude of the IL-21-induced DLBCL apoptosis increased over time, starting at 48 hours post treatment and reaching maximum cell death at 120 to 168 hours (Figure 3.11). The pro-apoptotic effects of IL-21 were observed in both GC-like and non-GC-like DLBCLs as determined by the Hans classifier (Hans, Weisenburger et al. 2004). Interestingly, one DLBCL tumor was obtained from a bilateral tonsillectomy and was localized only to one of the two tonsils. While IL-21 treatment induced apoptosis of 44% of
these tumor cells, no apoptosis was observed in normal B cells derived from the uninvolved tonsil from the same patient (Figure 3.12, Patient 5). In addition, IL-21 did not induce cell death of normal B cells derived from peripheral blood or non-malignant tonsils and lymph nodes from multiple other patients (Figure 3.13).

Figure 3.11 IL-21 induces time-dependent apoptosis of primary DLBCL tumor. Neoplastic B cells isolated from a representative primary tumor (Patient 4) were treated with IL-21 (100ng/mL) for the specified time period and stained with YO-PRO/PI.
Figure 3.12 IL-21 induces apoptosis of primary DLBCL tumors. Compilation of viability data for B cells isolated from five DLBCL primary tumors and one matched healthy tonsil tested by YO-PRO/PI staining after 72 hours of IL-21 treatment. Hans classifier subtypes are shown where available.
Figure 3.13 IL-21 does not affect healthy B cell viability. Flow cytometry profiles of healthy B cells isolated from a representative normal tonsil (Tonsil 1), treated for 72 hours with IL-21 (100ng/mL) and stained with YO-PRO/PI.

We also examined whether IL-21 can induce apoptosis of primary neoplastic B cells derived from other lymphoproliferative disorders. Consistent with previous reports, IL-21 induced mostly mild decreases in cell viability in 5 of 7 CLL cases (Figure 3.14). In addition, IL-21 induced apoptosis and cell death in two of the three primary follicular lymphomas which frequently transform into DLBCL in patients. IL-21 was particularly cytotoxic to neoplastic B cells isolated from one marginal zone lymphoma (Figure 3.14). Subsequent cell surface receptor level analysis of the marginal zone lymphoma showed that these cells expressed the highest IL-21R levels of any of the non-DLBCL primary tumor tested (Figure 3.10).
**Figure 3.14 IL-21 induces apoptosis of B cells from other lymphoproliferative disorders.** Compilation of viability data for B cells isolated from 7 CLL, 3 FL, and 1 MZL patient samples tested by YO-PRO/PI staining after 72 hours of IL-21 treatment.

*Level of apoptosis induced by IL-21 correlates with IL-21R expression*

Previous studies have shown that upregulation of cell surface IL-21R levels via CD40 stimulation can lead to an increase in the level of apoptosis induced in CLL (de Totero, Meazza et al. 2006). We therefore analyzed the relationship between level of apoptosis induced by IL-21 and cell surface IL-21R levels in B cell lymphomas. IL-21R expression in DLBCL cell lines was highly variable and did not correlate to level of apoptosis induced ($R^2 = 0.1767$) (data not shown). However, the level of IL-21R did correlate to extent of apoptosis induced by IL-21 when analyzing apoptotic responses in normal as well as neoplastic B cells with over 48% of the variability in response to IL-21 being determined by IL-21R levels (Figure 3.15). Nearly 57% of variability in cell viability post IL-21 treatment could be attributed to IL-21R levels if analyzing only those lymphoproliferative disorders that exhibited apoptosis post IL-21 stimulation (Figure 3.16).
Figure 3.15 Level of apoptosis induced by IL-21 in healthy B cells and B cell neoplasms correlates with IL-21R expression. Levels of apoptosis were plotted against IL-21R expression levels (% change in MFI) of all primary tumors and healthy B cells tested and a linear regression line was applied to the data. Correlation coefficient was calculated as 0.4862 with a p-value of 0.0036.

Figure 3.16 Level of apoptosis induced by IL-21 correlates with IL-21R expression in responsive lymphoproliferative diseases. Levels of apoptosis were plotted against IL-21R expression levels (% change in MFI) of all primary tumors that exhibited a decrease in cell viability after IL-21 treatment and a linear regression line was applied to the data. Correlation coefficient was calculated as 0.569 with a p-value of 0.0117.
III. IN VIVO EFFECTS OF IL-21 ON DLBCL XENOGRAFT TUMORS IN MICE

IL-21 induces regression of in vivo DLBCL xenograft tumors

The proapoptotic activity of IL-21 against DLBCL cell lines and primary tumors suggests that the cytokine could be useful for treatment of patients. To evaluate IL-21’s effects in vivo, groups of NOD/SCID mice were inoculated subcutaneously in the right flank with RC-K8 or MC116 cells and monitored for tumor development. MC116 xenograft tumor engraftment rates were aided by intravenous injection of Anti-Asialo GM antisera 24 hours prior to cell injection in order to temporarily eliminate NK cell activity (Habu, Fukui et al. 1981). After tumors reached a size of 25 mm², IL-21 (10µg) or PBS was injected in situ daily for 7 consecutive days. IL-21 treatment eradicated the RC-K8 tumor within 10 days in a majority of mice (4 of 5 mice in a representative experiment shown in Figure 3.17) while mice injected with PBS showed continued tumor growth. This effect was highly reproducible in repeated experiments and also with MC116 xenograft tumors (Figure 3.19). IL-21-induced tumor regression led to a significant improvement in overall survival compared to PBS-treated mice (P = 0.004 for RC-K8, P = 0.012 for MC116) (Figure 3.18 and 3.20).
Figure 3.17 IL-21 induces DLBCL xenograft tumor regression. 10 µL in situ injections of either PBS or IL-21 (1 mg/mL) were given to mice bearing subcutaneous RC-K8 xenograft tumors. Dashed lines represent tumor area in individual PBS-treated mice while solid lines represent individual IL-21-treated mice. Results shown are representative of two independent experiments.
Figure 3.18 IL-21 improves overall survival of mice bearing DLBCL xenograft tumors. Overall survival of mice harboring RC-K8 xenograft tumors being treated with PBS (dashed line) or IL-21 (solid line). Results shown are representative of two independent experiments.

Figure 3.19 IL-21 induces DLBCL xenograft tumor regression. 10 µL in situ injections of either PBS or IL-21 (1 mg/mL) were given to mice bearing subcutaneous MC116 xenograft tumors. Dashed lines represent tumor area in individual PBS-treated mice while solid lines represent individual IL-21-treated mice. Results shown are representative of two independent experiments.
Figure 3.20 IL-21 improves overall survival of mice bearing DLBCL xenograft tumors. Overall survival of mice harboring MC116 xenograft tumors being treated with PBS (dashed line) or IL-21 (solid line). Results shown are representative of two independent experiments.

**IV. IL-21 MECHANISM OF ACTION**

*IL-21-induced apoptosis of DLBCL cells proceeds through the intrinsic apoptotic pathway*

Previous studies reported that IL-21-induced apoptosis of CLL is associated with activation of caspase-8 leading to activation of caspases-3/7 and apoptosis (de Totero, Meazza et al. 2006). To determine which apoptotic pathways are activated by IL-21 in DLBCL, we monitored the activation of caspases-8, -9 and -3/7 during treatment. IL-21-induced apoptosis of RC-K8 DLBCL cells was associated with activation of caspases-8 and -9 as well as caspases-3/7, while no significant activation of caspases was observed in the IL-21-resistant cell line OCI-LY-3 (Figure 3.21).
Figure 3.21 IL-21 treatment activates Caspases-3/7, -8, and -9. RC-K8 and OCI-LY-3 cells were treated with IL-21 (100ng/mL) for the specified time period, lysed, and assayed for caspase activation as described in the Methods section. Relative luciferase units (RLU) readings were normalized to total protein content. Data represent means ± SE from three independent experiments.

To further elucidate the apoptotic pathway induced by IL-21, we pre-treated RC-K8 and MC116 cells with the pan-caspase inhibitor Z-VAD-FMK prior to IL-21 stimulation. Z-VAD-FMK almost completely abrogated IL-21-induced apoptosis in both cell lines (Figure 3.22). Furthermore, the selective caspase-9 inhibitor Z-LEHD-FMK also prevented IL-21-induced apoptosis in both cell lines, although this inhibitor exhibited some inherent toxicity to RC-K8 cells which has been previously reported in certain lymphoma cells (Shah, Asch et al. 2004). In contrast, the selective caspase-8 inhibitor Z-IETD-FMK did not prevent IL-21-induced apoptosis. Since caspase-9 activation can lead to downstream caspase-8 activation (Viswanath, Wu et al. 2001) these overall observations suggest that IL-21 induced apoptosis of DLBCL cells by activating the intrinsic apoptotic pathway.
Figure 3.22 IL-21-induced apoptosis is dependent on activation of caspase-3/7 and -9. RC-K8 and MC116 cells were pretreated with either DMSO or the pan-caspase inhibitor Z-VAD-FMK, the selective caspase-9 inhibitor Z-LEHD-FMK, or caspase-8 inhibitor Z-IETD-FMK at 50µM for 30 minutes and then treated with IL-21 (100ng/mL). Cell viability was assayed after 48 hours by YO-PRO/PI staining. Results shown are representative of three independent experiments.

**IL-21 modulates expression of apoptosis-regulating proteins**

We then investigated if IL-21-induced activation of the intrinsic apoptotic pathway involves changes in expression of the apoptosis-regulating proteins of the Bcl-2 family. Previous studies have shown that IL-21 may alter the expression of apoptosis-regulating proteins Bel-2, Bel-XL, Bim, and Bax in mouse lymphocytes and CLL cells (Jin, Carrio et al. 2004; Akamatsu, Yamada et al. 2007; Gowda, Roda et al.
We observed marked differences in the effects of IL-21 on some members of the Bcl-2 family between DLBCL cell lines exhibiting either apoptosis or resistance to IL-21 treatment. The expression of Bim-EL, a proapoptotic member of the Bcl-2 family, was increased at 24, 48, and 72 hours after IL-21 treatment in sensitive cell lines (RC-K8, OCI-LY-10), while its expression in IL-21-resistant OCI-LY-3 cell line was decreased (Figure 3.23). The expression levels of the antiapoptotic proteins Bcl-2 and Bcl-XL were reduced upon IL-21 treatment in the IL-21-sensitive cell lines (RC-K8, OCI-LY-10) compared to the minimal change observed in the IL-21-resistant OCI-LY-3 cell line (Figure 3.23). The protein expression of Bax, a cell death effector, increased in the IL-21-sensitive DLBCL cell lines, but not in the IL-21 resistant OCI-LY-3 cell line. Protein levels of Mcl-1, a known target of STAT3 (Ding, Yu et al. 2008), were increased in all the cell lines treated with IL-21 while no consistent changes in the expression of full-length Bid, a protein previously implicated in proapoptotic IL-21 signaling (de Totero, Meazza et al. 2006), were observed in the sensitive cell lines. Bcl-6, a known target of IL-21 (Ozaki 2004), was induced by IL-21 regardless of whether the cells were sensitive or resistant to IL-21.
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Figure 3.23 Expression levels of apoptosis-regulating proteins are altered by IL-21 treatment. (A) Cells were treated with IL-21 (100ng/mL) for the specified time period and protein expression was assayed by immunoblotting with specific antibodies. Immunoblotting for GAPDH served as a loading control. (B) Densitometric analysis of Western blots. The values in specimens at time point 0 were arbitrarily defined as 1. Data represent means ± SE from three independent experiments.

IL-21 activates the FOXO3 transcription factor

Studies by Jin, et al., showed that IL-21 is capable of inducing levels of pro-apoptotic protein Bim in mouse lymphocytes and CLL (Jin, Carrio et al. 2004; Gowda, Roda et al. 2008). Our studies of human DLBCL cell lines also showed an increase in Bim-EL, a splice variant of Bim, after IL-21 stimulation (Figure 3.23). The FOXO3 transcription factor can be activated by type I cytokines and is capable of regulating expression of Bim as well as Fas ligand (FasL) and p27 (Stahl, Dijkers et al. 2002; Tran, Brunet et al. 2003; Obexer, Geiger et al. 2006). We therefore monitored FOXO3 activation post IL-21 stimulation and observed strong dephosphorylation, and thus activation, of this transcription factor after treatment in responsive, but not resistant, cell lines (Figure 3.24). In addition to the IL-21-induced upregulation of Bim we also observed increases in levels of other transcriptional
targets of FOXO3 including p27 and FasL in responsive cell lines (Figure 3.25). These observations suggested that activation of FOXO3 and subsequent induction in Bim levels may contribute to IL-21-induced apoptosis.

**Figure 3.24 IL-21 treatment activates FOXO3 in sensitive cell lines.** (A) Cells were treated with IL-21 (100ng/mL) for the specified time period and protein expression was assayed by immunoblotting with specific antibodies. Immunoblotting for GAPDH served as a loading control. pFOXO3 is corrected for FOXO3 signal. (B) Densitometric analysis of pFOXO3 Western blots and normalized to FOXO3 signal. The values in specimens at time point 0 were arbitrarily defined as 1. Data represent means ± SE from three independent experiments.
**Figure 3.25 IL-21 treatment induces expression of FOXO3 target genes in sensitive cell lines.** (A) Cells were treated with IL-21 (100ng/mL) for the specified time period and protein expression was assayed by immunoblotting with specific antibodies. Immunoblotting for GAPDH served as a loading control. (B) Densitometric analysis of Western blots. The values in specimens at time point 0 were arbitrarily defined as 1. Data represent means ± SE from three independent experiments.

**Bim is not necessary for IL-21-induced apoptosis**

Since we observed an increase in pro-apoptotic Bim-EL expression in sensitive cell lines we tested whether this protein is necessary for IL-21-induced apoptosis. Although transfecting cells with siRNA targeting Bim prevented the induction of this protein by IL-21, it did not affect levels of apoptosis seen in treated cells (Figure 3.26). Therefore, the induction of Bim-EL by IL-21 is not a necessary part of IL-21-induced apoptosis of DLBCL.
Figure 3.26 Prevention of Bim upregulation does not affect IL-21-induced apoptosis. (A) RC-K8 cells were transfected with siRNA targeting Bim or control siRNA. 24 hours after transfection, cells were treated with IL-21 (100 ng/mL). Cellular proteins from untreated and IL-21-treated cells were resolved by SDS-PAGE and immunoblotted for Bim-EL at 24 hours post treatment. Immunoblotting for GAPDH served as a loading control. Cell viability was assayed by YO-PRO/PI staining after 48 hours of IL-21 treatment. (B) Densitometric analysis of Western blots. The values in specimens transfected with control siRNA and not treated with IL-21 were arbitrarily defined as 1. Data represent means ± SE from three independent experiments.

**IL-21-induced apoptosis is not dependent on FOXO3 activation**

FOXO3 is activated by IL-21 and is known to block cell growth and proliferation and induce cell death (Stahl, Dijkers et al. 2002; Sunters, Fernandez de Mattos et al.)
We transfected siRNA targeting FOXO3 into RC-K8 cells and although the siRNA reduced the overall levels of phosphorylated FOXO3 present in the cell by over 60%, there was no resulting change in level of apoptosis induced by IL-21 (Figure 3.27). Thus, although FOXO3 is activated by IL-21 this is likely not the major mechanism by which IL-21 induces apoptosis in DLBCL.
**Figure 3.27 Knock-down of FOXO3 does not affect IL-21-induced apoptosis.**

(A) RC-K8 cells were transfected with siRNA targeting FOXO3 or control siRNA. 72 hours after transfection, cells were treated with IL-21 (100 ng/mL). Cellular proteins from untreated and IL-21-treated cells were resolved by SDS-PAGE and immunoblotted for pFOXO3 at 24 hours post treatment. Immunoblotting for GAPDH served as a loading control. Cell viability was assayed by YO-PRO/PI staining after 48 and 72 hours of IL-21 treatment. (B) Densitometric analysis of Western blots. The values in specimens transfected with control siRNA and not treated with IL-21 were arbitrarily defined as 1. Data represent means ± SE from three independent experiments.

**IL-21-induced apoptosis of DLBCL cells is associated with c-Myc upregulation**

To further examine the mechanism of IL-21-induced apoptosis of DLBCL cells and to interrogate upstream signaling pathways that led to the observed decrease in the expression of Bcl-2 and Bcl-XL proteins, we performed gene expression profiling of untreated and IL-21-treated sensitive and resistant RC-K8 cells (the latter were generated by continuous exposure of cells to increasing concentrations of IL-21 and will be referred to as RC-K8R cells from hereon). mRNA levels of 375 genes and 402 genes were upregulated and downregulated, respectively, at least 2-fold in RC-K8 cells (Figure 3.28). A total of 763 mRNAs lost observed changes in expression upon IL-21 stimulation in RC-K8R cells (Figure 3.28). *C-MYC, GADD45b, BCL-XL, IL-16, IL-2Ra* and *SOCS3* genes were among those that exhibited the most prominent changes in mRNA expression upon IL-21 stimulation between RC-K8 and RC-K8R cell lines (Figure 3.29). Further microarray studies showed similar changes in *C-MYC* mRNA expression in additional IL-21-sensitive DLBCL cell lines (data not shown). Changes in c-Myc protein expression corresponded to gene expression data as IL-21 stimulation strongly upregulated c-Myc protein levels in the RC-K8 and MC116 cells, while no increase was observed in the RC-K8R or OCI-LY-3 cells (Figure 3.30). IL-
21 also induced c-Myc protein expression in cells from a *de novo* DLBCL primary tumor that exhibited apoptosis upon *in vitro* treatment with IL-21 (Figure 3.30, Patient 4).
Figure 3.28 Gene expression analysis of IL-21-treated DLBCL cells. RC-K8 cells were treated with IL-21 (100ng/mL) for 6 hours and total RNA was extracted as described in the Methods section. Microarray analysis was performed and listed are genes exhibiting at least a 2-fold change in expression. Upregulated genes are marked in red and downregulated genes in green. Genes that were also upregulated or downregulated by IL-21 in RC-K8R cells are marked in gray.
Figure 3.29 Microarray gene expression analysis revealed *MYC* upregulation by IL-21. Wild-type RC-K8 and IL-21-resistant RC-K8R cells were treated with IL-21 (100ng/mL) for 6 hours and RNA was collected as specified in the Methods section. Microarray gene expression analysis was performed. The 42 genes shown represent genes with the most dramatic changes between RC-K8 and RC-K8R upon IL-21 treatment.
Figure 3.30 IL-21 induces expression of Myc. (A) RC-K8, MC116, RC-K8R, OCI-LY-3 cell lines and cells from a primary DLBCL tumor from patient 4 were treated with IL-21 (100ng/mL). At 24, 48 and 78h post treatment cellular proteins were resolved by SDS-PAGE and immunoblotted for Myc. Immunoblotting for GAPDH served as a loading control. (B) Densitometric analysis of Western blots. The values in specimens at time point 0 were arbitrarily defined as 1. Data represent means ± SE from three independent experiments.

IL-21-induced apoptosis is dependent on c-Myc upregulation

Although c-Myc has been studied extensively for its oncogenic properties, it may also induce apoptosis (Pallavicini, Rosette et al. 1990; Mitchell, Ricci et al. 2000; Nilsson and Cleveland 2003). To determine whether apoptosis is dependent on c-Myc upregulation we utilized specific siRNAs (Figure 3.31 and 3.32) and shRNAs (Figure 3.33) to block the upregulation of c-Myc that is evident in the IL-21-sensitive cell lines. Prevention of the c-Myc upregulation following IL-21 treatment with either siRNA or shRNA effectively blocked IL-21-induced apoptosis of DLBCL cells while non-targeting control siRNA or shRNA did not. These observations demonstrate that IL-21-induced apoptosis is dependent on upregulation of c-Myc.
Figure 3.31 siRNA-mediated prevention of Myc upregulation prevents IL-21-induced apoptosis in RC-K8. (A) RC-K8 cells were transfected with siRNA targeting Myc or control siRNA. Twenty-four hours after transfection, cells were treated with IL-21 (100 ng/mL) for 24 hours. Cellular proteins from untreated and IL-21-treated cells were resolved by SDS-PAGE and immunoblotted for Myc or GAPDH. Cell viability was assayed by YO-PRO/PI staining after 48 hours of treatment. (B) Densitometric analysis of Western blots. The values in specimens transfected with control siRNA and not treated with IL-21 were arbitrarily defined as 1. Data represent means ± SE from three independent experiments.
Figure 3.32 siRNA-mediated prevention of Myc upregulation prevents IL-21-induced apoptosis in MC-116. (A) MC-116 cells were transfected with siRNA targeting Myc or control siRNA. Twenty four hours after transfection, cells were treated with IL-21 (100ng/mL) for 24 hours. Cellular proteins from untreated and IL-21-treated cells were resolved by SDS-PAGE and immunoblotted for Myc or GAPDH. Cell viability was assayed by YO-PRO/PI staining after 72 hours of treatment. (B) Densitometric analysis of Western blots. The values in specimens transfected with control siRNA and not treated with IL-21 were arbitrarily defined as 1. Data represent means ± SE from three independent experiments.
Figure 3.33 shRNA-mediated prevention of Myc upregulation prevents IL-21-induced apoptosis. (A) RC-K8 cells were transfected with shRNA targeting MYC or control shRNA. 24 hours after transfection, cells were treated with IL-21 (100ng/mL) for 24 hours. Cellular proteins from untreated and IL-21-treated cells were resolved by SDS-PAGE and immunoblotted for Myc or GAPDH. All immunoblots shown originated from the same membrane and identical exposure; superfluous lanes were removed. Cell viability was assayed by YO-PRO/PI staining after 48 hours of treatment. (B) Densitometric analysis of Western blots. The values in specimens transfected with control shRNA and not treated with IL-21 were arbitrarily defined as 1. Data represent means ± SE from two independent experiments.

**IL-21 modulates expression of c-Myc target proteins**

One mechanism by which c-Myc has been shown to induce apoptosis is via the upregulation of the proapoptotic protein Bax (Mitchell, Ricci et al. 2000), which was observed in the sensitive cell lines (Figure 3.23). In addition, c-Myc has been shown to suppress the expression of antiapoptotic proteins Bcl-2 and Bcl-XL (Eischen, Packham et al. 2001; Eischen, Woo et al. 2001) which also coincided with
our protein expression data (Figure 3.23). To demonstrate that the observed changes in the expression of these c-Myc target proteins may contribute to the apoptosis induced by IL-21, we transiently overexpressed Bcl-X<sub>L</sub> and/or Bcl-2 in RC-K8 cells before stimulation with IL-21. As expected, ectopic expression of either Bcl-X<sub>L</sub> or Bcl-2 partially prevented IL-21-induced apoptosis (Figure 3.34). This effect was specific since similar levels of overexpression of the antiapoptotic protein Mcl-1, which is not a known c-Myc target, did not affect apoptosis. Furthermore, knockdown of Bax, which is known to be required for c-Myc-induced apoptosis (Mitchell, Ricci et al. 2000; Eischen, Roussel et al. 2001; Dansen, Whitfield et al. 2006), completely blocked apoptosis after IL-21 stimulation (Figure 3.35).
Figure 3.34 Ectopic expression of Bcl-XL, but not Mcl-1, partially blocks IL-21-induced apoptosis. (A) RC-K8 cells were transfected with PCDNA3.1-BCL-XL, MCL-1 or empty vector. 72 hours after transfection, cells were treated with IL-21 (100 ng/mL). After 24 hours cellular proteins from untreated and IL-21-treated cells were resolved by SDS-PAGE and immunoblotted for Bcl-XL, Mcl-1. Immunoblotting for GAPDH served as a loading control. Cell viability was assayed by YO-PRO/PI staining after 48 hours of IL-21 treatment. (B) Densitometric analysis of Western blots. The values in specimens transfected with empty vector and not treated with IL-21 were arbitrarily defined as 1. Data represent means ± SE from three independent experiments.
Figure 3.35 Knock-down of Bax expression prevents IL-21-induced apoptosis. (A) RC-K8 cells were transfected with siRNA targeting Bax or control siRNA. 24 hours after transfection, cells were treated with IL-21 (100 ng/mL). Cellular proteins from untreated and IL-21-treated cells were resolved by SDS-PAGE and immunoblotted for Bax and Myc at 24 hours post treatment. Immunoblotting for GAPDH served as a loading control. Cell viability was assayed by YO-PRO/PI staining after 48 hours of IL-21 treatment. (B) Densitometric analysis of Western blots. The values in specimens transfected with control siRNA and not treated with IL-21 were arbitrarily defined as 1. Data represent means ± SE from three independent experiments.

Ectopic expression of c-Myc downregulates Bcl-X<sub>L</sub> and Bcl-2 and induces apoptosis

To confirm that c-Myc is capable of regulating Bcl-X<sub>L</sub> and Bcl-2 expression and of inducing apoptosis in DLBCL we transfected RC-K8 cells with a mammalian expression plasmid encoding C-MYC that increased c-Myc protein levels 3 to 5-fold (Figure 3.36). This upregulation of c-Myc led to downregulation of Bcl-X<sub>L</sub> and Bcl-2 thus demonstrating that the transfected protein was functional. In addition, transfection with c-Myc plasmid led to apoptosis in 11-20% of cells thus confirming that upregulation of c-Myc may induce apoptosis in DLBCL cells.
Figure 3.36 Ectopic expression of c-Myc induces apoptosis and downregulates Bcl-2 and Bcl-X\textsubscript{L}. (A) RC-K8 cells were transfected with 4.0 or 6.0 µg of pcDNA 3.1 – C-MYC or empty vector and cellular proteins were resolved by SDS-PAGE and immunoblotted for c-Myc, Bcl-XL and Bcl-2 after 48 hours. Immunoblotting for GAPDH served as a loading control. Cell viability was assayed by YO-PRO/PI staining 48 hours after transfection. (B) Densitometric analysis of Western blots. The values in specimens transfected with empty vector were arbitrarily defined as 1. Data represent means ± SE from three independent experiments.

**Activation of STAT1 is not necessary for IL-21-induced apoptosis**

The STAT1 transcription factor is largely considered to be pro-apoptotic and can induce growth arrest and cell death in DLBCL (Stephanou and Latchman 2003;
Stephanou and Latchman 2005). In addition, a previous study showed that inhibition of STAT1 can partially block IL-21-induced apoptosis in follicular lymphoma cells (Akamatsu, Yamada et al. 2007). Our analysis of STAT activity upon IL-21 stimulation showed that STAT1 is activated by the cytokine (Figure 3.4). To elucidate the role of this transcription factor in the induction of apoptosis by IL-21 we transfected cells with siRNA targeting \textit{STAT1} to knock-down its expression (Figure 3.37). Despite a significant reduction in STAT1 levels there was no change in the level of apoptosis induced by IL-21 which suggests that STAT1 activation is not necessary for IL-21-induced apoptosis.
Figure 3.37 siRNA-mediated knock-down of STAT1 does not prevent IL-21-induced apoptosis. (A) RC-K8 cells were transfected with siRNA targeting STAT1 or control siRNA. Seventy two hours after transfection, cells were treated with IL-21 (100 ng/mL). Cellular proteins from untreated and IL-21-treated cells were resolved by SDS-PAGE and immunoblotted for STAT1 at 15 minutes post treatment. Immunoblotting for GAPDH served as a loading control. Cell viability was assayed by YO-PRO/PI staining after 48 and 72 hours of treatment. (B) Densitometric analysis of Western blots. The values in specimens transfected with control siRNA and not treated with IL-21 were arbitrarily defined as 1. Data represent means ± SE from three independent experiments.
**Upregulation of c-Myc by IL-21 is mediated by STAT3 activation**

*C-MYC* is a known target of STAT3 (Bromberg, Wrzeszczynska et al. 1999; Kiuchi, Nakajima et al. 1999; Bowman, Broome et al. 2001) and STAT3 is activated by IL-21 stimulation (Figure 3.4). siRNA-mediated knock-down of STAT3 expression prevented STAT3 activation and potently blocked IL-21-induced apoptosis (Figure 3.38). Furthermore, knock-out of STAT-3 also ameliorated the IL-21-mediated upregulation of c-Myc and downregulation of Bcl-2 and Bcl-X\(_L\) (Figure 3.39). Taken together, these findings suggest that IL-21-activated STAT3 induces expression of c-Myc which subsequently modulates the expression of apoptosis-regulating proteins.
Figure 3.38 siRNA-mediated knock-down of STAT3 prevents IL-21-induced apoptosis. (A) RC-K8 cells were transfected with siRNA targeting STAT3 or control siRNA. Seventy two hours after transfection, cells were treated with IL-21 (100 ng/mL). Cellular proteins from untreated and IL-21-treated cells were resolved by SDS-PAGE and immunoblotted for p-STAT3 and STAT3 at 15 minutes post treatment. Immunoblotting for GAPDH served as a loading control. Cell viability was assayed by YO-PRO/PI staining after 48 hours of IL-21 treatment. (B) Densitometric analysis of Western blots. The values in specimens transfected with control siRNA and not treated with IL-21 were arbitrarily defined as 1. Data represent means ± SE from three independent experiments.

Figure 3.39 siRNA-mediated knock-down of STAT3 prevents IL-21-induced changes in expression of apoptosis-regulating proteins. (A) RC-K8 cells were transfected with siRNA targeting STAT3 or control siRNA. Seventy two hours after transfection, cells were treated with IL-21 (100 ng/mL). Cellular proteins from untreated and IL-21-treated cells were resolved by SDS-PAGE and immunoblotted for Myc, Bcl-XL, Bcl-2, and STAT3 at 48 hours post treatment. Immunoblotting for GAPDH served as a loading control. (B) Densitometric analysis of Western blots. The values in specimens transfected with control siRNA and not treated with IL-21 were arbitrarily defined as 1. Data represent means ± SE from three independent experiments.

**IL-2 pre-stimulation prevents IL-21-induced apoptosis**

IL-2, another member of the type I cytokine family, is an important growth factor for B cells and is secreted by activated T cells (Mingari, Gerosa et al. 1984; Nakagawa, Hirano et al. 1985; Gimmi, Freeman et al. 1991). Interestingly,
microarray gene expression analysis showed that IL-21 stimulation induces a 3 to 6-fold increase in IL-2Rα (IL-2 Receptor α) mRNA levels (Figures 3.28 and 3.29). IL-2Rα is responsible for high-fidelity binding of IL-2. To clarify any potential role of IL-2 in IL-21 signaling we pre-treated RC-K8 cells with IL-2 and then stimulated the cells with IL-21. The IL-2 pre-treatment partially blocked IL-21-induced apoptosis (Figure 3.40). This effect was specific since IL-2 stimulation did not affect apoptosis induced by immunochemotherapeutic agents doxorubicin or rituximab.

Figure 3.40 IL-2, but not IL-4, pretreatment ameliorates IL-21-induced, but not immunochemotherapy-induced, apoptosis. RC-K8 cells were pretreated with IL-2 (100U/mL) for 4 hours and then treated with IL-21 (100ng/mL), Doxorubicin (25 or 100ng/mL), or Rituximab (50µM). Cell viability was assayed by YO-PRO/PI after 48 hours of IL-21 treatment. Untreated and IL-21-treated data represent means ± SE from three independent experiments.
**PI3K inhibition partially blocks IL-21-induced apoptosis**

IL-21 has been shown to activate the PI3K pathway which is a major controller of protein translation, cell metabolism and survival (Brenne, Baade Ro et al. 2002; Zeng, Spolski et al. 2007). Cytokine-mediated phosphorylation of PI3K can lead to activation of Akt, mTOR, and S6-kinase 1 which in turn upregulate protein translation and promote cell survival. LY294002 is a potent inhibitor of this PI3K pathway. Pre-treatment of DLBCL cells with LY294002, but not vehicle, markedly blocked IL-21-induced apoptosis, likely by inhibiting the translation, but not transcription, of c-Myc (Figure 3.41 and 3.42). Indeed, protein expression analysis showed potent inhibition of IL-21-mediated c-Myc upregulation by LY294002 while microarray gene expression analysis showed that C-MYC gene transcripts were unaffected by LY294002 treatment (Figure 3.41 and 3.42).
Figure 3.41 Inhibition of PI3K partially blocks IL-21-induced apoptosis. RC-K8 cells were pretreated with 50 µM LY294002 for 1 hour and subsequently treated with IL-21 at 100ng/mL. (A) Cell viability was assayed by YO-PRO/PI staining after 72 hours of treatment. Data represent means ± SE from three independent experiments. (B) Cellular proteins from untreated and IL-21-treated cells were resolved by SDS-PAGE and immunoblotted for c-Myc after indicated time periods post treatment. Immunoblotting for GAPDH served as a loading control. (C) Densitometric analysis of Western blots. The values in specimens not treated with IL-21 were arbitrarily defined as 1. Data represent means ± SE from two independent experiments.
**Figure 3.42 Gene expression analysis of LY294002- and IL-21-treated DLBCL cells.** RC-K8 cells were pretreated with 50µM LY294002 or vehicle (DMSO) for 1 hour and subsequently treated with IL-21 (100ng/mL) for 6 hours and total RNA was extracted as described in the Methods section. Microarray analysis was performed and listed are genes exhibiting at least a 2-fold change in expression upon IL-21 stimulation. Upregulated genes are marked in red and downregulated genes in green.
CHAPTER 4

DISCUSSION

I. MODEL FOR IL-21-INDUCED APOPTOSIS IN DLBCL

**Novel IL-21 – STAT3 – c-Myc signaling pathway identified**

IL-21 is a recently discovered member of the IL-2 family of cytokines that is capable of promoting not only immunostimulatory and pro-survival effects but also cell death of certain B lymphocytes. The effects of IL-21 on B cell fate depend on their activation status and developmental stage. In addition, the effects of IL-21 differ among lymphocytes derived from humans and various strains of mice. In the present study we investigated the effects of IL-21 in human DLBCL and showed that it potently induced apoptosis of DLBCL cell lines and primary tumors and prolonged survival of mice harboring human DLBCL xenograft tumors.

Our studies have uncovered a novel IL-21 signaling pathway involving STAT-3 – c-Myc which can induce apoptosis in IL-21R-expressing DLBCLs (Figure 4.1). The proapoptotic pathway is initiated by IL-21 binding to the IL-21R and triggering Jak-mediated phosphorylation and activation of STAT3. Phosphorylated STAT3 is able to homodimerize and enter the nucleus to activate transcription of its targets including *C-MYC*. The c-Myc protein, also a transcription factor, then activates transcription of pro-apoptotic Bax and represses transcription of anti-apoptotic Bcl-2 and Bcl-XL. Decreased expression of Bcl-2 and Bcl-XL leads to a shift in the Bcl-2 rheostat within the cells and triggers mitochondrial outer membrane permeabilization, caspase activation, and apoptosis. This is a novel mechanism of IL-
21-induced apoptosis and is one of the few examples in which the STAT-3 – c-Myc pathway promotes cell death.

**Figure 4.1 A model of IL-21-induced apoptosis in DLBCL.** IL-21 binding to IL-21Rα results in Jak activation and subsequent phosphorylation and activation of STAT3. Homodimerized STAT3 then enters the nucleus and activates transcription of Myc. Myc protein promotes the transcription of Bax and suppresses Bcl-2 and Bcl-XL thus disrupting the Bcl-2 rheostat within the cells and triggering mitochondrial outer membrane permeabilization, caspase-9 activation, and apoptosis.

*Reconciliation of existing models of IL-21-induced apoptosis*

Previous studies of IL-21 activity in cancer cells showed that IL-21 treatment can induce a low level of apoptosis in CLL and that cell death was augmented by upregulating IL-21R levels via anti-CD40 pre-stimulation. IL-21-induced apoptosis of CLL B cells was associated with activation of caspases-8 and -3, upregulation of Bim, cleavage of p27, Bid and PARP and secretion of granzyme B in the presence of...
CPG-DNA (Jahrsdorfer, Blackwell et al. 2006; Gowda, Roda et al. 2008). Herein we confirmed that IL-21 treatment results in slight increases in CLL cell apoptosis. However, in contrast to the relatively mild apoptosis observed in CLL tumors that were not manipulated to increase IL-21R expression, we demonstrated that IL-21 induced pronounced apoptosis of DLBCL cell lines and de novo primary tumors that inherently express high levels of IL-21R. Intriguingly, the observed mechanism of IL-21-induced apoptosis in DLBCL cells is different from the mechanism reported in CLL cells. Of the two CLL studies, one group showed that IL-21-induced apoptosis of CLL was associated with secretion of granzyme B when cells were stimulated with IL-21 in conjunction with CpG-ODN treatment which activates TLR (Jahrsdorfer, Blackwell et al. 2006). The study did not show, however, that granzyme B secretion was responsible for apoptosis of IL-21-stimulated cells. The study reported only that CLL cells stimulated with IL-21 and CpG-ODN could gain the potential to induce apoptosis in autologous bystander CLL cells. In our studies IL-21 did not induce expression of granzyme B in DLBCL alone or when used in combination with CpG-ODN or LPS (data not shown).

The second group showed that IL-21-induced apoptosis of CLL cells was mediated by an upregulation of Bim and that siRNA-mediated knock-down of Bim levels prevented some of the IL-21-induced apoptosis (Gowda, Roda et al. 2008). However, since Bim is a pro-apoptotic member of the Bcl-2 family it would be expected that reducing its levels could decrease apoptosis from any insult nonspecifically. In our study, we show that Bim is in fact induced by IL-21 but that Bim knock-down did not prevent a significant amount of apoptosis in sensitive cell
lines (Figure 3.26). It is also possible that the differences in apoptotic pathways activated by IL-21 in different B cell neoplasms may be due to the pleiotropic effects of IL-21 seen in non-neoplastic B cells of different activation statuses and developmental stages. Clarification of those apoptotic pathways require additional studies such as the knock-out of STAT3 or c-Myc within CLL cells to determine whether these transcription factors are involved in apoptosis induced by IL-21 in these cells as they are in DLBCL.

The aforementioned and other studies in CLL and follicular lymphoma have reported that IL-21 treatment activates caspases 3/7 as well as 8 during the induction of apoptosis which would indicate that the extrinsic apoptotic pathway is activated (de Totero, Meazza et al. 2006; Akamatsu, Yamada et al. 2007). As in previous studies, we also observed activation of caspase 3/7 and 8 but also observed strong activation of caspase 9, the effector of the intrinsic apoptotic pathway. Cross-talk between the intrinsic and extrinsic apoptotic pathways is common and prompted us to use caspase inhibitors to uncover that caspase 9, and not caspase 8, was in fact necessary for IL-21-induced apoptosis. It is possible that the caspase 8 activation observed in other studies may in fact be due to caspase 9 activation and subsequent cross-activation of caspase 8 during the execution of apoptosis. Alternatively, different apoptotic pathways may be utilized by IL-21 in varying lymphoid tumors. The necessity of caspase 8 and 9 activation in these other tumor models can be clarified by utilizing specific caspase 9 inhibitors prior to IL-21 stimulation.
**STAT3 as mediator of IL-21-induced cell death**

The STAT family of transcription factors regulates diverse cellular events such as proliferation, differentiation, and cell survival (Calo, Migliavacca et al. 2003). Previous research has cast STAT1 and STAT3 in opposing roles with the former promoting cell death and the latter promoting cell survival and proliferation, especially in DLBCL (Stephanou and Latchman 2003; Stephanou and Latchman 2005; Ding, Yu et al. 2008). In the present study we demonstrated that STAT1 activation is not necessary for IL-21-induced DLBCL apoptosis, while STAT3 activation is necessary but not sufficient since STAT3 activation was observed also in the IL-21-resistant OCI-LY-3 cell line. However, the magnitude of IL-21-induced stimulation of STAT3 in the resistant cell line was lower relative to IL-21-sensitive cell lines which could account for the differences in downstream effects.

STAT3 has been previously implicated in the induction of apoptosis in limited models. There have been reports of STAT3 promoting cell death when triggering terminal differentiation and apoptosis in myeloid leukemia M1 cells or during involution of mammary glands (Minami, Inoue et al. 1996; Sutherland, Vaillant et al. 2006). Interestingly, the mechanism of apoptosis induction by STAT3 in mammary glands was shown to involve the upregulation of c-Myc (Sutherland, Vaillant et al. 2006). However, to our knowledge, this is the first example in which the STAT-3 – c-Myc pathway is employed to mediate tumor cell death by a therapeutic agent. The mechanism determining whether STAT3 activation promotes cell survival or induction of cell death is unknown. Although loss of SOCS3 has been linked to the conversion of STAT3 into a proapoptotic transcription factor (Lu, Fukuyama et al.
SOCS3 expression was induced upon IL-21 treatment in both resistant and sensitive DLBCL cell lines (data not shown) and thus could not explain the proapoptotic effects of STAT3. It is possible, however, that the strength and duration of STAT3 phosphorylation may contribute to the final phenotypic outcome of STAT3 activation and studies to address this possibility should be explored.

**c-Myc as mediator of IL-21-induced apoptosis**

We have shown that apoptosis is dependent on STAT-3-induced upregulation of c-Myc, a protein that has been studied most extensively for its oncogenic properties. It is known that c-Myc is normally expressed only transiently and at low levels in response to mitogenic signaling and is a key regulator of cellular proliferation and differentiation by controlling transcription as well as DNA replication (Shichiri, Hanson et al. 1993). Interestingly, deregulation of c-Myc has the potential to induce either tumorigenesis by stimulating proliferation or apoptosis by inducing DNA damage and suppressing Bcl-2 and Bcl-X<sub>L</sub> (Lombardi, Newcomb et al. 1987; Vaux, Cory et al. 1988; Evan, Wyllie et al. 1992). Recent studies demonstrated that the effects of c-Myc may depend on levels of its upregulation, with low level induction promoting gene transcription and cell growth while high level induction stimulating DNA damage, repression of anti-apoptotic proteins and eventually apoptosis (Prendergast 1999; Murphy, Juntila et al. 2008). In our study c-Myc levels in sensitive cell lines were strongly upregulated by IL-21 stimulation, which likely favored its pro-apoptotic effects, as was confirmed by prevention of apoptosis through abolishment of c-Myc upregulation. Resistant cells did not exhibit
an induction of c-Myc following IL-21 stimulation thus further suggesting the pro-
apoptotic effects of this transcription factor are responsible for inducing cell death in
DLBCL. As in previous studies, Bcl-2 or Bcl-XL rescue partially prevented c-Myc-
induced apoptosis, confirming the importance of their downregulation by c-Myc
during activation of the apoptotic cascade (Eischen, Packham et al. 2001; Eischen,
Woo et al. 2001; Pelengaris, Khan et al. 2002; Maclean, Keller et al. 2003).
Interestingly, the upregulation of either, or both, of these anti-apoptotic proteins did
not block IL-21-induced apoptosis in its entirety which invites conjecture that other
apoptosis-regulating proteins may be involved as well. In contrast to our finding, a
previous report linked IL-21 with downregulation, instead of upregulation, of c-Myc
in Epstein-Barr Virus (EBV)-transformed B lymphocytes (Konforte and Paige 2006).
The discrepant effects may be attributed to EBV effects on IL-21 stimulation and
require additional studies.

We also confirmed that this transcription factor is capable of promoting
apoptosis in DLBCL by ectopically expressing c-Myc which did result in induction of
apoptosis. The level of apoptosis observed, however, was not as high in these cells as
those treated with IL-21. The protein levels of c-Myc induction and Bcl-2 and Bcl-XL
downregulation via ectopic expression of C-MYC were similar to those exhibited post
IL-21 treatment, thus reducing the likelihood that the discrepancy is simply due to
differences in protein expression levels. Several possibilities may account for this
discrepancy. It is possible that the c-Myc upregulation is a vital component of IL-21-
induced apoptosis but that other proteins activated by IL-21 signaling are also
necessary. In essence, c-Myc upregulation may be necessary, but not sufficient, for
maximum induction of apoptosis by IL-21. Other proteins that could contribute to the pro-apoptotic effects of IL-21 include GADD45β and TP53BP2 which are both upregulated by IL-21 in sensitive DLBCL cell lines. GADD45β is induced by DNA damage and contributes to growth arrest and apoptosis while TP53BP interacts with p53 and induces caspase-9 activation and apoptosis (Sheikh, Hollander et al. 2000; Kobayashi, Kajino et al. 2005). The roles of these additional potential mediators of IL-21-induced apoptosis may be explored via perturbing their upregulation and can serve as foci for future studies.

**IL-2 involvement in IL-21 signaling**

Our studies demonstrated that DLBCL cell pretreatment with IL-2 was able to block IL-21-induced apoptosis. This block of apoptosis was specific since IL-2, which can promote B cell survival via Bcl-2 upregulation and proliferation, did not affect apoptosis induced by doxorubicin or rituximab. It is therefore likely that IL-2 stimulation specifically prevents some portion of the IL-21 pro-apoptotic signaling cascade. It is important to note that we observed a potent upregulation of IL-2Ra after IL-21 stimulation in DLBCL which suggests a possible IL-21-induced IL-2 dependence. We could hypothesize that IL-21, secreted by NK and helper T cells, may cause DLBCL and certain, inappropriately-activated perhaps, healthy B cells to become poised for apoptosis and rapid cell death if they fail to acquire a secondary signal from a helper T cell to validate their existence. Boise, et al., previously demonstrated the existence of stimulation-induced IL-2 dependencies in
hematopoietic systems and their report may serve as a guide for the studies needed to elucidate this signaling relationship (Boise, Minn et al. 1995).

**PI3K involvement in IL-21 signaling and induction of apoptosis**

We observed that LY294002, a potent PI3K inhibitor, prevented IL-21-induced apoptosis in DLBCL cell lines. PI3K is frequently activated in B cells due to signaling through various cytokines and growth factors and its wide-ranging effects make it an important player in nearly all cellular processes. This kinase is able to enhance protein translation via activation of Akt, mTOR, and S6K1 and its activity is required for the translation of many proteins including granzyme B and FasL (Phu, Haeryfar et al. 2001; Pardo, Buferne et al. 2003; Kelley and Efimova 2009).

Chanprasert, et al., have shown that Thrombopoietin-mediated upregulation of c-Myc is PI3K-dependent (Chanprasert, Geddis et al. 2006). It is likely, therefore, that blocking PI3K activity with LY294002, an inhibitor of PI3K, prevents the upregulation of c-Myc that is required for IL-21-induced apoptosis.

Other recent reports have demonstrated that PI3K can regulate the transcriptional activity of c-Myc by promoting the degradation of Mad1, which can suppress the transcriptional activity of c-Myc (Zhu, Blenis et al. 2008). It is also reasonable to infer, therefore, that inhibition of PI3K with LY294002 may prevent apoptosis by inhibiting the c-Myc-induced repression of BCL-2 and BCL-XL and not by blocking the translation of c-Myc.

There exists a third possibility in which activation of PI3K by IL-21 may directly lead to upregulation of c-Myc and subsequent apoptosis yet this scenario is
unlikely since there have been no reports of PI3K activation leading to an increase in C-MYC gene transcription. Further studies of PI3K involvement in pro-apoptotic signaling of IL-21 are needed to elucidate the mechanism of apoptosis prevention by LY294002 which could include studying the translation of proteins that are not products of IL-21 signaling.

II. IL-21 AS THERAPY FOR DLBCL

DLBCL resistance to IL-21-induced apoptosis

The heterogeneous nature of DLBCL with varied mechanisms of transformation likely contributes to their differing sensitivities to therapeutic interventions. We observed that despite having high levels of IL-21R the viability of the OCI-LY-3 cell line was unaffected by IL-21 treatment which contrasts with the effects of IL-21 on the remaining DLBCL cell lines and even primary tumors. We demonstrated that although IL-21 activated STAT3 in OCI-LY-3 there was no subsequent upregulation of c-Myc which is required for IL-21-induced apoptosis. Notably, while the levels of pSTAT3 achieved post IL-21 activation were similar across all DLBCL cell lines, the fold-change increase in pSTAT3 levels was markedly smaller in OCI-LY-3 due to constitutive activation of STAT3 via an IL-6 autocrine loop (Yee, Biondi et al. 1989). This might contribute to the absence of IL-21-induced upregulation of c-Myc in this cell line. Further studies to uncover the mechanism of resistance to IL-21 in OCI-LY-3 cells are needed.

Our preclinical studies demonstrated that a vast majority of DLBCL cell lines respond to IL-21 stimulation with apoptosis. However, it is known that in vitro
studies with cell lines do not necessarily mimic in vivo responses. By testing DLBCL cells isolated from de novo untreated primary tumors we observed that none of the tumors exhibited resistance to IL-21 therapy, thus making it likely that the insensitivity of OCILY-3 cells to IL-21 is the exception and not the rule. The pro-apoptotic effect of IL-21 in primary tumors is particularly encouraging as these cells are taken directly from tumors that are treated in clinical settings.

Additional mechanisms of resistance to IL-21 treatment may be uncovered as the potential of IL-21 as a therapy for DLBCL is explored. The downregulation of IL-21R post IL-21 stimulation would lead to a reduced upregulation of c-Myc and thus a reduced pro-apoptotic effect of the cytokine on DLBCL. This resistance to IL-21-induced apoptosis was evident in our own studies as chronic treatment of RC-K8 cells with increasing doses of IL-21 over a period of 10 weeks resulted in the generation of an RC-K8 cell line (designated as RC-K8R) that was wholly resistant to the apoptosis normally induced by the cytokine. Cell surface expression of IL-21R was indeed reduced in this resistant cell line although not to levels that would indicate a total absence of the receptor. Interestingly, culture of these RC-K8R cells in medium not containing IL-21 for a period of 4 weeks restored IL-21 sensitivity thus contributing to the possibility that IL-21 stimulation is affecting cell surface receptor levels, a phenomenon that we observed in our receptor expression studies. In addition, our primary B cell data suggest that high levels of IL-21R are necessary for IL-21-induced apoptosis which would make the downregulation of the receptor a particularly effective way to develop resistance.
IL-21 resistance could also arise by a block of IL-21 pro-apoptotic signaling at other points in the STAT3 – c-Myc – Bcl-2/Bcl-X₁ cascade. For instance, further upregulation of Bcl-X₁ could shift the Bcl-2 rheostat within DLBCL cells further away from apoptosis and prevent the IL-21-mediated downregulation of Bcl-X₁ from releasing sufficient pro-apoptotic factors to induce mitochondrial membrane permeabilization and caspase activation within the cell. Our studies, however, did not show evidence of any additional perturbations in this pathway leading to IL-21 resistance. Whether DLBCL tumors, especially in an in vivo setting, have the ability to evade IL-21-induced apoptosis by downregulating receptor levels or increasing expression of anti-apoptotic proteins needs to be studied in order to determine the likelihood and importance of these potential mechanisms of IL-21 resistance.

**IL-21R expression in B cells**

During our studies we uncovered differences in IL-21R expression in B cells that are neoplastic as compared to their healthy counterparts. Cell surface receptor expression analysis showed that the IL-21R is upregulated 265-360% in B cells isolated from DLBCLs as compared to those from healthy tonsils or lymph nodes in matched patients. From our data it may be inferred that upregulation of IL-21R may occur before or during B cell transformation, perhaps in order to provide a pro-survival advantage to cells. This survival advantage may be attributed to upregulation of c-Myc, a key driver of cellular growth, and activation of STAT3, a known pro-survival transcription factor. Due to low levels of IL-21 in circulation the potent STAT3 activation, strong upregulation of c-Myc, and induction of apoptosis that is
evident in our studies may not occur. We speculate that it is only when a larger, ectopically administered, dose of IL-21 stimulates DLBCLs that apoptosis is then induced due to a prohibitively strong activation of IL-21R and the STAT3 – c-Myc pathway. Interestingly, our finding also raises the possibility that DLBCL tumors may be targeted via other therapies that exploit this difference in IL-21R expression such as anti-IL-21R monoclonal antibodies which would induce apoptosis in DLBCL via a mechanism similar to rituximab. Larger-scale comparisons of IL-21R expression in DLBCLs and matched normal B cell specimens via immunohistochemistry would be required to confirm this finding and measure with more accuracy the difference in IL-21R expression.

Our preclinical studies demonstrate that IL-21 may be employed for direct targeting of DLBCL tumors that express IL-21R and may benefit patients when used as a single-agent or in conjunction with standard chemotherapies. These studies combined with further investigation of mechanisms of IL-21 resistance and facilitation of c-Myc-induced apoptosis will not only elucidate the biological functions of IL-21 but also potentially improve the outcome of DLBCL patients.
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