ABERRATIONS IN CYTOKINE SIGNALING IN LEUKEMIA:
VARIATIONS IN PHOSPHORYLATION AND
O-GLCNACYLATION

by

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A thesis submitted in conformity with the requirements
for the degree of Doctor of Philosophy
Graduate Department of Medical Biophysics
University of Toronto

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Tumor-induced immunosuppression can occur by multiple mechanisms, each posing a significant obstacle to immunotherapy. Evidence presented in this dissertation suggests that aberrant cytokine signaling, as a result of altered metabolism of Chronic Lymphocytic Leukemia (CLL) cells, confers a selective advantage for tumor survival and growth. Cells from CLL patients with aggressive disease (as indicated by high-risk cytogenetics) were found to exhibit prolongation in Interferon (IFN)-induced STAT3 phosphorylation, and increased levels of reactive oxygen species (ROS) in these cells reflected these signaling processes. Changes in the relative balance of phospho-STAT3 and phospho-STAT1 levels, in response to combinations of IL-2 + Toll-like receptor (TLR)-7 agonist + phorbol esters, as well as IFN, were associated with the immunosuppressive and immunogenic states of CLL cells. In addition, immunosuppressive leukemic cells were found to express high levels of proteins with O-linked N-acetylglucosamine (O-GlcNAc) modifications, due to increased metabolic activity through the Hexosamine Biosynthetic Pathway (HBP), which caused impaired intracellular signaling responses and affected disease progression. A conclusion of the studies presented here is that the intrinsic immunosuppressive properties of leukemic cells may be overcome by agents such as Resveratrol that target metabolic pathways of these cells.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Abstract</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ii</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>iii</td>
</tr>
<tr>
<td>List of Tables</td>
<td>v</td>
</tr>
<tr>
<td>List of Figures</td>
<td>vi</td>
</tr>
<tr>
<td>List of Publications</td>
<td>ix</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>xi</td>
</tr>
</tbody>
</table>

## CHAPTER I: General Introduction

1. **Chronic Lymphocytic Leukemia (CLL)**
   - 1.1.1 CLL staging and prognostic factors 4
   - 1.1.2 CLL therapy 7
   - 1.1.3 CLL immunotherapy 8

2. **Tumor immunogenicity**
   - 1.2.1 Weak CLL cell immunogenicity 13
   - 1.2.2 CLL cell manipulation to enhance immunogenicity 14

3. **Interferon (IFN)-α**
   - 1.3.1 IFN-α in cancer 18
   - 1.3.2 Regulation of IFN-signaling and intrinsic tumor resistance to IFN 20
   - 1.3.3 Oncogenic event: loss of tumor suppressor p53 22
   - 1.3.4 Oncogenic event: elevated ROS levels 24

4. **Glucose metabolism in tumor cells**
   - 1.4.1 Warburg effect 28
   - 1.4.2 Hexosamine Biosynthetic Pathway (HBP) and signal transduction 29
   - 1.4.3 HBP and O-GlcNAc modification in cancer 31
   - 1.4.4 Role of OGT in cancer 33
   - 1.4.5 Targeting HBP to overcome immunomodulatory resistance 33

5. **Hypothesis**

6. **Thesis objectives and organization**


CHAPTER II: Sensitization of IL-2 Signaling Through Toll-like Receptor - 7 Enhances B Lymphoma Cell Immunogenicity

2.1 Abstract 38
2.2 Introduction 39
2.3 Materials and Methods 41
2.4 Results 48
2.5 Discussion 66

CHAPTER III: Aberrant Interferon-Signaling is Associated with Aggressive Chronic Lymphocytic Leukemia

3.1 Abstract 71
3.2 Introduction 72
3.3 Materials and Methods 74
3.4 Results 83
3.5 Discussion 100

CHAPTER IV: Aberrant O-GlcNAcylation Characterizes Chronic Lymphocytic Leukemia

4.1 Abstract 106
4.2 Introduction 107
4.3 Materials and Methods 109
4.4 Results 117
4.5 Discussion 129

CHAPTER V: O-GlcNAc is a Therapeutic Target for Murine and Human Leukemia

5.1 Abstract 133
5.2 Introduction 134
5.3 Materials and Methods 136
5.4 Results 140
5.5 Discussion 153

CHAPTER VI: Discussion and Future Perspectives

6.1 ROS as a prognostic factor for CLL 156
6.2 Identifying the source of ROS 159
6.3 O-GlcNAc as a target for cancer therapy 161
6.4 Understanding Resveratrol resistance 163
6.5 Novel treatment approach 164
6.6 Concluding remarks 168

LIST OF REFERENCES 169
LIST OF TABLES

Table I. Clinical properties of CLL patients 42
Table II. Clinical properties of CLL patients 75
Table III. Clinical properties of CLL patients in whom tumor ROS levels were measured 77
Table IV. CLL patient characteristics 110
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.</td>
<td>APC:T cell interaction</td>
<td>11</td>
</tr>
<tr>
<td>Figure 2.</td>
<td>Interferon-α signaling pathway</td>
<td>17</td>
</tr>
<tr>
<td>Figure 3.</td>
<td>Hexosamine biosynthetic pathway (HBP)</td>
<td>30</td>
</tr>
<tr>
<td>Figure 4.</td>
<td>Effect of S28690 and IL-2 on proliferation of CLL cells</td>
<td>49</td>
</tr>
<tr>
<td>Figure 5.</td>
<td>Effect of S28690 and IL-2 on costimulatory molecule expression by CLL cells</td>
<td>50</td>
</tr>
<tr>
<td>Figure 6.</td>
<td>Effect of S28690 and IL-2 on signaling pathways in CLL cells</td>
<td>52</td>
</tr>
<tr>
<td>Figure 7.</td>
<td>Enhancement of IL-2 receptor expression and responses by TLR-7 activation</td>
<td>55</td>
</tr>
<tr>
<td>Figure 8.</td>
<td>Effect of S28690 and IL-2 on the ability of CLL cells to stimulate T cell proliferation</td>
<td>58</td>
</tr>
<tr>
<td>Figure 9.</td>
<td>Effect of PKC agonists on costimulatory molecule expression by CLL cells treated with S28690 and IL-2</td>
<td>59</td>
</tr>
<tr>
<td>Figure 10.</td>
<td>Effect of S28690, IL-2, and PDB on cytokine and chemokine production by CLL cells</td>
<td>61</td>
</tr>
<tr>
<td>Figure 11.</td>
<td>Effect of S28690, IL-2, and PDB on STAT1 and STAT3 activation in CLL cells</td>
<td>64</td>
</tr>
<tr>
<td>Figure 12.</td>
<td>Variations in IFN-mediated STAT3 phosphorylation and proliferation in CLL cells</td>
<td>84</td>
</tr>
<tr>
<td>Figure 13.</td>
<td>Correlation of IFN-mediated pSTAT3 duration and proliferation with cytogenetic lesions</td>
<td>87</td>
</tr>
<tr>
<td>Figure 14.</td>
<td>Increased IFN-mediated STAT3-phosphorylation and proliferation in p53^{-} B cell lines</td>
<td>89</td>
</tr>
<tr>
<td>Figure 15.</td>
<td>Increased IFN-mediated STAT3-phosphorylation and proliferation in ATM^{-} cell lines</td>
<td>91</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>----------</td>
<td>------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Figure 16.</td>
<td>Effect of phorbol esters on IFN-signaling in CLL cells</td>
<td>93</td>
</tr>
<tr>
<td>Figure 17.</td>
<td>Correlation of intracellular ROS levels with clinical parameters</td>
<td>95</td>
</tr>
<tr>
<td>Figure 18.</td>
<td>Effect of a TYK2 inhibitor on IFN-signaling and responses</td>
<td>97</td>
</tr>
<tr>
<td>Figure 19. (supplementary)</td>
<td>IFN-signaling in p53-temperature sensitive cell lines and p53−/− mice</td>
<td>102</td>
</tr>
<tr>
<td>Figure 20. (supplementary)</td>
<td>Effects of p53 and ATM inhibitors on IFN-signaling</td>
<td>103</td>
</tr>
<tr>
<td>Figure 21. (supplementary)</td>
<td>Additional examples of cell cycle analysis of IFN-treated CLL cells</td>
<td>104</td>
</tr>
<tr>
<td>Figure 22.</td>
<td>Hexosamine pathway activity in CLL cells</td>
<td>118</td>
</tr>
<tr>
<td>Figure 23.</td>
<td>Effect of immunoreceptor agonists and hexosamine pathway metabolites on O-GlcNAcylation in CLL cells</td>
<td>121</td>
</tr>
<tr>
<td>Figure 24.</td>
<td>Effect of O-GlcNAc levels on TLR-7 signaling in CLL cells</td>
<td>123</td>
</tr>
<tr>
<td>Figure 25.</td>
<td>Effect of variations of intracellular O-GlcNAcylation on responses to chemotherapeutic agents, BCR cross-linking, and phorbol esters</td>
<td>125</td>
</tr>
<tr>
<td>Figure 26.</td>
<td>Correlation of intracellular O-GlcNAcylation with clinical parameters</td>
<td>127</td>
</tr>
<tr>
<td>Figure 27. (supplementary)</td>
<td>Effect of altering O-GlcNAcylation on TLR-7-mediated JNK phosphorylation in normal B cells</td>
<td>131</td>
</tr>
<tr>
<td>Figure 28.</td>
<td>Resveratrol-mediated death of CB3 cells in vivo is associated with decreased O-GlcNAcylation and can be overcome with hexosamine pathway loading</td>
<td>141</td>
</tr>
<tr>
<td>Figure 29.</td>
<td>Effect of OGase and proteasome inhibitors on down-regulation of O-GlcNAc levels by Resveratrol (RSV)</td>
<td>143</td>
</tr>
<tr>
<td>Figure 30.</td>
<td>Clearance of CB3 cells in vivo by Resveratrol (RSV)</td>
<td>145</td>
</tr>
<tr>
<td>Figure 31.</td>
<td>Clearance of FEL cells in vivo by Resveratrol (RSV)</td>
<td>147</td>
</tr>
</tbody>
</table>
Figure 32. Modification of O-GlcNAc levels in circulating CLL cells by Resveratrol (RSV) 148

Figure 33. Effect of Glucosamine and Resveratrol (RSV) on CB3 cells in vivo 150

Figure 34. Interferon increases O-GlcNAcylation and overcomes protection by Resveratrol (RSV) in the FEL model 152

Figure 35. Oxidative stress in circulating CLL cells 158
LIST OF PUBLICATIONS


LIST OF ABBREVIATIONS

2-ME 2-mercaptoethanol
7AAD 7-amino-actinomycin D
Ab Antibody
Ag Antigen
APC Antigen presenting cell
ATM Ataxia telangiectasia mutated
BCR B cell receptor
B7-H B7 homolog
CAR Chimeric antigen receptor
CD Cluster of differentiation
CLL Chronic lymphocytic leukemia
CTL Cytotoxic T lymphocyte
CTLA-4 Cytotoxic T lymphocyte antigen-4
DC Dendritic cell
DCFH$_2$-DA 2'7'-Dichlorofluorescein diacetate
DMEM D-modified Eagle’s medium
DMSO Dimethyl sulfoxide
EBV Epstein-Barr virus
EGF Epidermal growth receptor
ER Endoplasmic reticulum
ERK Extracellular signal regulated kinase
FBS Fetal bovine serum
FCS Fetal calf serum
FFAs Free fatty acids
FISH Florescent in situ hybridization
FEL Friend Erythroleukemia
FMU/LV Friend murine leukemia virus
F6P Fructose-6-phosphate
FS Forward scatter
GAPDH Glyceraldehyde-3-phosphate dehydrogenase
GFAT Glutamine-fructose-6-phosphate amidotransferase
GlcN Glucosamine
GlcNAc N-acetylglucosamine
G6P Glucose-6-phosphate
Glc6P Glucosamine-6-phosphate
HBP Hexosamine biosynthetic pathway
HK Hexokinase
HMP Hexose-monophosphate pathway
HPLC High performance liquid chromatography
HPRT Hypoxanthine-guanine phosphoribosyltransferase
HSA Human serum albumine
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hsp</td>
<td>Heat shock protein</td>
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<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
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<td>ICOS</td>
<td>Inducible T cell co-stimulator</td>
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<td>IFN</td>
<td>Interferon</td>
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<td>IFNAR</td>
<td>Interferon-α receptor</td>
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<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin growth factor</td>
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<tr>
<td>IgVH</td>
<td>Immunoglobulin heavy-chain variable region</td>
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<tr>
<td>IkB</td>
<td>Inhibitor of NF-κB</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IS</td>
<td>Immune synapse</td>
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<tr>
<td>IR</td>
<td>Ionizing radiation</td>
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<td>ISRE</td>
<td>Interferon stimulated response elements</td>
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<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun N-terminal kinase</td>
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<tr>
<td>LDT</td>
<td>Lymphocyte doubling time</td>
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<tr>
<td>LFA-1</td>
<td>Leukocyte function-associated antigen-1</td>
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<tr>
<td>LMP</td>
<td>Low molecular mass protein</td>
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<td>LOH</td>
<td>Loss of heterozygosity</td>
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<td>LPL</td>
<td>Lipoprotein lipase</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>Mabs</td>
<td>Monoclonal antibodies</td>
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<td>MBL</td>
<td>Monoclonal B lymphocytosis</td>
</tr>
<tr>
<td>MDM2</td>
<td>Murine double minute 2</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<tr>
<td>miR</td>
<td>MicroRNA</td>
</tr>
<tr>
<td>MKK</td>
<td>MAPK kinase</td>
</tr>
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<td>MLR</td>
<td>Mixed lymphocyte reaction</td>
</tr>
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<td>m.w.</td>
<td>Molecular wight</td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetyl-cysteine</td>
</tr>
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<td>NFAT</td>
<td>Nuclear factor of activated T cells</td>
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<td>NF-κB</td>
<td>Nuclear factor-κB</td>
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<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NO</td>
<td>Nitrogen oxide</td>
</tr>
<tr>
<td>NOD/SCID (mice)</td>
<td>Non-obese diabetic/Sever combined immunodeficient (mice)</td>
</tr>
<tr>
<td>OGase</td>
<td>O-GlcNAcase or O-GlcNAc hydrolase</td>
</tr>
<tr>
<td>O-GlcNAc</td>
<td>O-linked b-N-acetylglucosamine</td>
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<td>OGT</td>
<td>O-GlcNAc transferase</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
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<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PD-1</td>
<td>Programmed death-1</td>
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<tr>
<td>PDB</td>
<td>Phorbol 12,13-dibutyrate</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
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<tr>
<td>PIAS</td>
<td>Protein inhibitor of activated STAT</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
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<tr>
<td>PPP</td>
<td>Pentose phosphate pathway</td>
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<td>PRR</td>
<td>Pattern recognition receptor</td>
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<td>Pt.</td>
<td>Patient</td>
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<td>PTK</td>
<td>Protein tyrosine kinase</td>
</tr>
<tr>
<td>PTP</td>
<td>Protein tyrosine phosphatase</td>
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<tr>
<td>RAG</td>
<td>Recombination-activating gene</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma</td>
</tr>
<tr>
<td>rHA</td>
<td>Recombinant human albumin</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>SH</td>
<td>Src homology</td>
</tr>
<tr>
<td>SHM</td>
<td>Somatic hypermutation</td>
</tr>
<tr>
<td>SOCS</td>
<td>Suppressor of cytokine signaling</td>
</tr>
<tr>
<td>SS</td>
<td>Side scatter</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TAP-1</td>
<td>Transporter associated with antigen processing-1</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
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<tr>
<td>Th</td>
<td>T helper cell</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>Tregs</td>
<td>Regulatory T cells</td>
</tr>
<tr>
<td>ts</td>
<td>Temperature sensitive</td>
</tr>
<tr>
<td>TYK</td>
<td>Tyrosine kinase</td>
</tr>
<tr>
<td>UDP</td>
<td>Uridine diphosphate</td>
</tr>
<tr>
<td>UTP</td>
<td>Uridine triphosphate</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WBC</td>
<td>White blood cell</td>
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<tr>
<td>ZAP70</td>
<td>ζ-chain-associated protein kinase 70</td>
</tr>
</tbody>
</table>
CHAPTER I:

General Introduction
It is well established that the immune system plays a key role in the prevention of cancer. By suppressing viral infections, the immune system protects against virus-induced tumors; by controlling pathogen-driven inflammatory processes, it interferes with the establishment of chronic inflammation-mediated tumorigenesis; and finally, by recognizing tumor antigens (Ags) and responding to components of cellular stress, such as reactive oxygen species (ROS), it is capable of identifying and clearing pre-cancerous and cancerous cells (known as immunosurveillance). However, genetic mutations and phenotypic changes allow for the selection and expansion of non-immunogenic tumor cell variants (known as immunoselection or immunoediting) and the active suppression of innate and adaptive immune responses (known as immunosubversion), ultimately enabling tumors to escape (Zitvogel et al., 2006).

There are number of ways by which tumors paralyze the immune system and escape, but the exact mechanisms are still under investigation. B cell leukemias, such as chronic lymphocytic leukemia (CLL), are unique in that they have the innate ability to be antigen-presenting cells (APCs). However, the inherently weak immunogenic phenotype of CLL cells inhibits any productive T cell-mediated immune response that might result in tumor clearance. In order to enhance the immunogenicity of CLL cells, understanding of cellular mechanisms that control this process is first needed.

Work presented in this dissertation will begin by identifying phospho-STAT3 as a marker of weak immunogenicity, and how STAT3 activation and dominance over phospho-STAT1 influences immunomodulator signaling in CLL cells. Furthermore, high levels of ROS and O-GlcNAc, both of which have been found to affect signaling in tumor cells, will be described as novel prognostic tools and indicators for disease progression. In the last
chapter, emphasis will be placed on how ROS and O-GlcNAc can also serve as therapeutic targets in tumor cells. First, aspects of CLL biology and mechanisms of immunomodulatory resistance in tumor cells will be introduced.

1.1 Chronic Lymphocytic Leukemia (CLL)

CLL is the most common adult leukemia in the Western world (Rozman et al., 1995), making up over 30% of all leukemias, and accounting for approximately 15000 new cases and 4500 deaths annually in the USA (Cancer Facts and Figures, 2010). Diagnosis of the disease occurs primarily in patients over the age of 40 and requires a count of over 5000 circulating CLL cells per cubic millimeter [Cheson et al., 1996]. It is now well established that genetic factors play a role in the etiology of CLL (Andritsos and Khoury, 2002), but the clinical course is remarkably heterogeneous. Biological reasons for the heterogeneity are becoming better understood. More aggressive disease (indicated by a short lymphocyte doubling time (LDT)) is associated with prognostic factors such as absence of somatic hypermutation (SHM) at the immunoglobulin (Ig) locus and increased expression of ectoenzyme CD38 (Zupo et al., 1996) or the tyrosine kinase ZAP-70 (Chen et al., 2005). Our laboratory has shown that calcium sensing by CLL cells correlates with poor outcome (Hammond et al., 2009), and cytokine signaling pathways are “re-wired” in aggressive CLL cells in order to accelerate tumor growth (Tomic et al., 2011), whereas high intracellular glycosylated protein levels in CLL cells confer a better outcome (Tomic et al., 2010).

Most circulating CLL cells do not appear to be dividing and accumulate due to decreased apoptosis and a lack of immune clearance of the malignant cells. CLL cells are B cells characterized by their abnormal co-expression of the cell surface markers CD5 (an activation marker also found on T cells) and CD19 (a B cell marker), are IgM\textsuperscript{low} and
autoreactive. These cells are thought to originate from the B1-lineage of cells presenting a number of monocytoid features (Zwiebel and Cheson, 1998), including the expression of macrophage markers such as CD11b and CD11c (Rai et al. 1975), markers often observed on CLL cells. High-sensitivity flow cytometry allows for the detection of B cells with CLL phenotype in numbers as low as 1 per 10 000 normal leukocytes [Rawston et al., 2001]. With this method CLL cells have been found in over 3% of adults with otherwise normal blood counts [Ghia et al., 2004]. This condition is termed monoclonal B lymphocytosis (MBL) and it is a relatively new diagnostic category [Rawston et al., 2008]. It is important to separate MBL from CLL because MBL does not necessarily evolve into CLL [Rawston et al., 2008].

1.1.1 CLL staging and prognostic factors

One of the most intriguing features of the disease is its clinical heterogeneity. While some patients have an indolent disease and can survive for years without requiring any treatment, others die of drug-resistant disease within a year or two of presentation. Decision to treat is guided by clinical staging, prognostic factors, symptoms and disease activity. Classical staging systems include Rai (Fitchett et al., 1987) and Binet (Dohner et al., 1997; Krober et al., 2002). The Rai system uses numbers 0 to IV to group CLL into low-, intermediate-, and high-risk categories. Generally, the higher the stage number, the more advanced the cancer. The Binet system uses letters A to C to stage CLL according to how many lymph nodes are involved and whether there is a drop in the number of red blood cells or platelets. In addition, clinical parameters such as morphology of leukemic cells and LDT (Chiorazzi et al., 2005) as well as the extent of lymphadenopathy, splenomegaly and hepatomegaly upon physical examination are included into staging the disease.
A number of prognostic markers have been identified which reflect CLL cell biology and can be used to classify CLL cells into sub-groups with differing clinical behavior. Some of these markers include absence of SHM of the Ig locus and overexpression of both ZAP-70 and CD38. These markers reflect intrinsic biological differences in CLL cells that account for different clinical behavior, and may arise from the cytogenetic changes that underlie CLL progression. Currently used method to classify CLL into 5 sub-groups on the basis of cytogenetic abnormalities is fluorescence in situ hybridization (FISH) (Dohner et al., 2000; Glassman et al., 2005). Normal cytogenetics are seen in 20-30% of patients and 40-50% of the rest have deletions at 13q14 which result in the loss of microRNAs (miR15 and miR16) (Calin et al., 2004). Another 10-20% of CLL patients have Trisomy 12 (T12) with increased expression of genes on chromosome 12, including MDM2, an inhibitor of p53 (Harris and Levine, 2005). Deletions at 11q22-q23, with loss of the ATM gene, are found in 10-20% of patients. ATM is a kinase that communicates the presence of DNA damage to p53, and is associated with DNA repair (Meek et al., 2004). In CLL, in contrast to many solid tumors, deletions at 17p13 with loss of the p53 gene are found in only 10-15% of patients. Loss of heterozygosity (LOH) is associated with inactivation by mutation of the remaining allele, and 11q- and 17p- cells have often lost ATM and p53 function, respectively. Other chromosomal aberrations occur infrequently and are not screened for routinely by fluorescence in situ hybridization (FISH). In addition, aberrant expression of genes not located on chromosomes 11, 12, 13 and 17, such as anti-apoptotic protein, bcl-2 (Schimmer et al., 2003), is likely due to the aberrant signaling caused by these cytogenetic abnormalities. One of the strongest predictors that CLL cells will exhibit aggressive clinical behavior is expression of lipoprotein
lipase (LPL), an enzyme that cleaves free fatty acids (FFAs) from triglycerides in circulating lipoproteins (Heintel et al., 2005).

Patients with normal cytogenetics or 13q abnormalities have prolonged survival (associated with SHM, low CD38, and low ZAP70 expression) and never require treatment (Kay et al., 2007). Patients with Trisomy 12, usually have a good response to initial treatment, but eventually develop drug resistance, and have a mean survival of about 7-10 years. Patients with 11q- deletions and defective ATM function have rapid disease progression and a mean survival of only approximately 5 years, while patients with 17p-deletions and absent p53 function have the worse outcome as their tumors develop drug resistance and their disease rapidly progresses resulting in a mean survival of only 2 years (Kay et al., 2007; Dohner et al., 2000).

These properties of CLL cells provide prognostic information to help guide treatment decisions but are imperfect predictors (Cramer and Hallek, 2011). For instance, serum $\beta_2$ microglobulin levels correlate positively with advanced stage of the disease and high lymphocyte count (DiGiovanni et al., 1989; Barrebi et al., 2010) however, its role as an independent prognostic marker still requires study. LDT has also been shown to be a useful dynamic prognostic marker, where patients with LDTs < 12 months have poor prognosis with a median survival of 61 months and patients with LDTs > 12 months have a long treatment-free period and survival beyond 10 years (Molina and Alberti, 1987; Montserrat et al., 1986). However, its drawbacks are that it is not available upon diagnosis and that transient rises may occur during infection or treatment with steroids. Therefore, prognostic factors capable of effectively identifying patients that will acquire aggressive disease prior to
disease progression are essential for treating those who might benefit from therapy at an early disease stage.

1.1.2 CLL therapy

Decision to start treatment is based on patient’s stage (estimated by Rai or Binet staging systems), risk status (determined by genetic profile such as the presence or absence of 17p- deletion and other prognostic factors such as SHM, ZAP-70, CD38) and overall health because of the need to tolerate the potential side effects (Hallek and Pflug, 2011).

For asymptomatic patients at an early stage (Rai 0-II or Binet A), the “watch and wait” approach (also called “active surveillance”) is applied, since it has been found that these patients do not benefit from an early start of treatment. For some of these patients (e.g. patients with normal cytogenetics) disease may develop very slowly that they may never require treatment. For others, if they become symptomatic and show a sign of worsening the treatment would then begin. At present, the question that remains unanswered is whether there is a need for early treatment for patients with high-risk cytogenetics, such as loss of 17p-, even if they do not have any symptoms. After diagnosis, all CLL patients are continuously monitored for changes in their blood counts.

Patients with advanced (Rai III-IV or Binet C stage) and symptomatic disease are placed on the treatment immediately. Among these patients, it is important to note the presence of 17p- deletion as these patients respond poorly to chemotherapy. First-line therapy drugs commonly used to treat advanced and symptomatic CLL are fludarabine (purine analogue), cyclophosphamide (alkylating agent), chlorambucil (alkylating agent) and Rituxan (anti-CD20 antibody) alone or in combination. Campath-1H (anti-CD52 antibody)
is approved for treatment of CLL patients who do not respond to fludarabine or ones that have poor prognostic features such as 17p-deletion.

Treatment decisions become more difficult for therapy-refractory patients or those with 17p-deletion. Lenalidomide (an immunomodulatory agent) has shown activity in CLL in the relapsed/refractory as well as in the untreated setting (Chanan-Khan et al., 2006; Ferrajoli et al., 2008). Other options include flavopiridol (a synthetic flavon which targets cyclin-dependent kinases and is currently used in clinical trials), and ofatumumab (fully humanized monoclonal anti-CD20 antibody). Allogeneic stem cell transplantation may only be used in younger, physically fit CLL patients with high-risk or refractory CLL. It is the only therapy that offers a potential cure for CLL, but at the expense of mortality.

1.1.3 CLL immunotherapy

Cytotoxic drugs (such as fludarabine) and monoclonal antibodies (mabs) (such as Campath-1H (Alemtuzumab) and Rituxan) alleviate symptoms of CLL, but are not curative (Hallek et al., 2010; Wierda et al., 2000). Moreover, drug resistance becomes a major problem with continued treatment. Thus, despite improved understanding of its pathogenesis (Caligaris-Cappio and Ghia, 2008) and better treatment options (Hallek et al., 2010), CLL still remains a fatal disease for many patients.

There are a number of in vivo studies that support a role for immunotherapy in the control of CLL (Ramsay and Gribben, 2008). Spontaneous remissions of CLL have been associated with increased immune activity that accompanies effective host responses against viral infections (Hillmen, 2004). In order for cancer immunotherapy to be effective, tumor-reactive T cells would have to be activated to approximately the same levels as successful anti-viral T_H1 responses (of the order of 1-10/100 CD8^+ T cells) (Hansen and Libnoch, 1978;
Shirono et al., 1995) for sufficient time (probably of the order of several months) (Dreger et al., 2003) to achieve clinically meaningful results. Recent publication by Porter et al. (2011) showing sustained remissions in three patients with advanced p53-deficient CLL who received genetically modified autologous T cells demonstrates the promise of immunotherapy as a potent and sustained method of cancer therapy. In this pilot study, patients’ T cells were genetically engineered to express an anti-CD19 chimeric antigen receptor (CAR) with intracellular domains of the CD3-zeta chain and 4-1BB protein [Porter et al., 2011]. These modified T cells were able to mount a strong anti-tumor activity and in vivo persistence of chimeric CD19 receptors produced a rapid response in patients with refractory CLL [Porter et al., 2011]. Other groups have also used CAR-expressing T cells that contained a CD28-costimulatory domain instead of 4-1BB plus the cytoplasmic portion of the CD3-zeta molecule in clinical trials [Kochenderfer et al., 2010; Brentjens et al. 2011]. The results suggest that 4-1BB moieties may be better than CD28 ones at providing enhanced proliferation and persistence of CAR-transduced T cells [Rosenberg and Kochenderfer, 2011].

Type I cellular immunity (or Th1 immunity characterized by IFN-γ production) and cytotoxic T cells (CTLs such as CD4+ and CD8+) are generally believed to be the most important for controlling tumors and cancer immunotherapy (Rosenberg, 2001). While CD8+ T cells may be the most important effectors, CD4+ T cells are essential to amplify the responses (Shedlock and Shen, 2003). Activation of CTLs occurs in the secondary lymphoid organs and requires at least two signals (summarized in Figure 1), much like APC activation. Signal 1 comes from recognition of tumor antigens by T cell receptor (TCR)/CD3 complex with its co-receptor CD4 or CD8 (CD8 in the case of CTLs) (Germain and Margulies, 1993).
CD8$^+$ cells recognize 9- or 10-mer peptide epitopes, derived from proteasomal degradation of intracellular proteins, that bind to class I MHC molecules in the endoplasmic reticulum (ER) (Germain and Margulies, 1993). CD4$^+$ T cells recognize longer peptides from membrane and internalized proteins that are degraded by lysosomes before binding to class II MHC molecules (Germain and Margulies, 1993). Internalized proteins can also enter the class I pathway by “cross-presentation” (Carbone et al., 1998; Fonteneau et al., 2003).

Proliferation and differentiation of CTL precursors into effectors requires costimulatory signals from APCs (Schwartz, 2003; Sharpe and Freeman, 2002). These second signals are provided by B7-family members (such as CD80 and CD86) and adhesion molecules (such as the Ig superfamily member CD54 and the β2-integrin, LFA1), which increase T cell binding to APCs (Figure 1). Also, second signals control survival and prevent abortive responses of CD4$^+$ and CD8$^+$ effectors (Schwartz, 2003; Sharpe and Freeman, 2002). Cytokines (such as IL-12 and IFN-α) from APCs provide additional signals that mediate Type I T cell development (Wigginton and Wiltout, 2002). Once fully stimulated by APC-T cell interaction, the CTLs proliferate and differentiate into effector cells. They are guided to the tumor sites by adhesion molecules and chemokines (such as IP-10) where they secrete cytokines like IFN-γ and mediate tumor cell killing.

The ability of viral-reactive T cells (or T$_{H}1$ cells) to clear normal B cells infected by the Epstein-Barr virus (EBV) (Nikiforow et al., 2001) suggests that T cells able to recognize cancer antigens should also be able to clear B leukemia cells, provided they are as immunogenic as B cells activated by EBV gene products. Consistent with this, allogeneic bone marrow transplantation cures cases of advanced CLL via a T cell-mediated grafted-vs-
Figure 1. APC:T cell interaction. T cell activation requires Ag-specific recognition in the context of MHC on the surface of an APC. The interaction with an APC provides the T cell with peptide-MHC ligands for the TCR (signal 1) as well as ligands (such as CD80 and CD86) for co-stimulatory receptors (such as CD28) (signal 2). T cell recognition of Ag is associated with the formation of a tight cell-cell contact with APC, termed the immunological synapse (IS). Other key molecule/ligand pairs that play part in the formation of IS are CD54/LFA1, CD83/CD83 ligand, and TNF/TNFR. The engagement of TCR and localization of proteins within IS lead to induction of specific signaling events such as regulation of glucose metabolism, cell-cycle progression, cytokine production (e.g. IL-12, TNF-α) and functional activation and differentiation of the T cell. Negative regulation of TCR signaling occurs through CTLA-4 which competes with CD28 for binding CD80/CD86.
leukemia effect (Qin et al., 1998), but the toxicity of non-specific T cell activation (graft-vs-host) and lack of donors preclude general application of this procedure (Mehta et al., 1997; Marks et al., 2002). Most leukemias express potential targets for T cells (such as the Ig idiotype) that can be formulated as vaccines to stimulate tumor-reactive T cells in vivo or ex vivo for subsequent infusion into patients (Massaia et al., 1999; Armstrong et al., 2002; Baskar et al., 2004). Vaccines based on autologous tumor cells (Schultze et al., 1997) have also been shown to produce decreases in tumor burden associated with enhanced anti-tumor T cell activity. However, the responses observed with these antibody and vaccine immunotherapeutic approaches are incomplete and temporary. Therefore, in order to improve these responses, a better understanding of cellular pathways that control tumor immunogenicity is needed.

1.2 Tumor immunogenicity

Immunotherapies (such as cancer vaccines) that promote tumor lysis by T cells have the potential to cure a number of cancers, but tumor-reactive T cells alone are not sufficient for clinical efficacy (Schreiber et al., 2010). An additional requirement is that the tumor targets be “immunogenic”, or able to activate and be killed by T cells (Schreiber et al., 2010). Unfortunately, oncogenic events and signaling pathways that promote cancer growth are associated with weak immunogenicity (Tomic et al., 2006).

Immunogenicity is a complex phenotype that includes the expression of costimulatory molecules (such as CD80, CD83, CD86 and CD40) and adhesion molecules (such as CD54 and LFA-1) (Schwartz, 2003; Sharpe and Freeman, 2002) that promote strong interactions with Ag-reactive T cells (Figure 1). Immunogenic cells also make cytokines (such as IL-2, IL-15, TNF-α, TNF-β, IFN-γ, IL-12 and IFN-α) and chemokines (such as IP-10 and IL-8)
that attract and activate Type I (or $T_{H1}$) T cells, which are associated with effective tumor clearance (Figure 1). The immunogenic phenotype is also associated with characteristic signaling properties, including strong NF-$\kappa$B and STAT1 activation (Shi et al., 2007; Tomic et al., 2006). In contrast, cell surface molecules such as CTLA-4, ICOS and PD-1 (Salih et al., 2006), cytokines such as IL-10, VEGF, and TGF-$\beta$ (Alvarez et al., 2005), and strong activation of STAT3 are associated with impaired tumor immunogenicity.

### 1.2.1 Weak CLL cell immunogenicity

Despite advances in the development of effective vaccine platforms (Weiner, 2008) and methods to generate large numbers of tumor-reactive T cells *ex vivo* (Dudley et al., 2002; Morgan et al., 2006), the clinical efficacy of these approaches (which focus mainly on activating tumor-reactive T cells) remains low (Rosenberg et al., 2004). A potential explanation for these poor results may be the intrinsically weak immunogenicity of the target tumor cells, such as CLL cells.

Consistent with the coupling of oncogenic events to weak immunogenicity (Tomic et al., 2009; Spaner et al., 2007), CLL cells are weak stimulators of T cells, despite expressing high levels of class II MHC molecules to bind to the T cell receptor and some costimulatory molecules such as CD40 (Gitelson, et al., 2003). They make immunosuppressive factors such as IL-6, IL-10, and VEGF, and generally express low levels of the costimulatory molecules CD80, CD86 and CD83. Furthermore, T cells from CLL patients with advanced disease often express CTLA-4, which downregulates T cell immune responses (Motta et al., 2005). In addition, the absence of dendrites and low phosphorylated STAT1/STAT3 ratio
(Okamura et al., 1982; Tomic et al., 2006) seen normally in CLL cells may also prevent sustained effector function of tumor-reactive T cells.

### 1.2.2 CLL cell manipulation to enhance immunogenicity

A variety of immunotherapeutic approaches have been designed and developed *in vitro* to render CLL cells more immunogenic, before they can be applied *in vivo*. Jahrsdofer *et al.* (2005) explored the impact of cytogenetic status on the immunogenicity of the CLL cells, and found that good prognosis cytogenetics (e.g. normal karyotype, 13q deletion) correlated with greater *in vitro* immunogenicity (e.g. higher basal expression of molecules involved in costimulation, adhesion and Ag presentation and induced significantly more T cell proliferation in mixed lymphocyte cultures). Using a gene therapy approach, Wendtner *et al.* (2002) found that CLL cells transduced with CD40L (a critical molecule for T cell activation that is downregulated on T cells in CLL patients (Cantwell *et al.*, 1997)) upregulated CD80 costimulatory molecules not only on infected CLL cells but also on noninfected bystander leukemic B cells, resulting in increased T cell proliferation. Litzinger *et al.*, (2009) used CLL cells that overexpress CD80, ICAM-1 and LFA-3 to demonstrate for the first time that T cells from CLL patients can acquire multiple costimulatory molecules from autologous CLL cells and can then act as APCs themselves. Given the immunodeficient characteristics of CLL cells, this work showed that enhancing the Ag-presenting function of CLL cells and T cells simultaneously could provide a distinct advantage in the effort to elicit anti-tumor immune responses. Other studies have shown that treatment of CLL cells with immunomodulatory CpG oligonucleotides (CpG ODN) (Jahrsdorfer *et al.*, 2001; Decker *et al.*, 2000) or Toll-like receptor (TLR) agonists (Shi *et al.*, 2007) also enhanced costimulatory molecules expression on CLL cells and rendered them
more sensitive to T cell-mediated killing, and apoptosis by cytotoxic agents. Additionally, treatment of CLL cells with PKC agonists, such as tumor promoting phorbol esters (phorbol 12,13-dibutyrate (PDB)), induce CLL cells to differentiate into a state of increased Ag-presentation and T cell stimulatory capability (MacDonald et al., 1994). PKC signaling is induced upon CD40 ligation, as well as upon phorbol ester treatment of normal B cells, suggesting that the PKC signaling pathway may be involved in increasing the APC capabilities of CLL cells and may be manipulated to increase immunogenicity of CLL cells for therapeutic purposes. Accordingly, addition of PKC agonist to cytotoxic agents (such as Vincristine) increased immunogenicity of CLL cells and their susceptibility to T cell-mediated killing, with implications for the design of chemo-immunotherapeutic strategies (Tomic et al., 2009).

Weak immunogenicity of CLL cells can sometimes be overcome by treatment with cytokines such as IFN-α. However, more aggressive CLL cells often exhibit aberrant signaling responses, or intrinsic resistance, to this immunomodulator. Therefore, it is essential to determine what underlies the resistance to immunomodulators in these tumors, so that IFN-based therapies may be improved. However, in order to do that, an understanding of signaling through the IFN pathway is first needed.

1.3 Interferon (IFN)-α

The IFN family includes two main classes of related cytokines: type I IFNs and type II IFN (Pestka et al., 1987; Pestka et al., 2004). There is only one type II IFN, IFN-γ, which is a single gene product that is synthesized primarily by T lymphocytes in response to foreign antigens (Pestka et al., 1987; Pestka et al., 1997; Pestka et al., 2004). In contrast, there are many type I IFNs. IFN-β is a single gene product and is produced mainly by fibroblasts
IFN-α is a multigene family and is made by many cell types, including B lymphocytes (Pestka et al., 1987; Pestka et al., 2004; Platanias et al., 2005).

Upon IFN-α binding, two receptor chains (IFNAR1 and IFNAR2) dimerize which leads to consecutive activation of receptor-associated protein tyrosine kinases (of the Janus kinase family) JAK1 and TYK2 (Figure 2). Activated JAK kinases phosphorylate tyrosine residues in the intracellular domains of these receptors (Domanski et al., 1997; Levy and Darnell, 2002). Two members of the Signal Transducer and Activator of Transcription (STAT) family, STAT1 and STAT3 then bind with their SH2 domains to these receptor docking sites (Figure 2). Subsequently, JAK1 and TYK2 phosphorylate STAT1 and STAT3 respectively on a single tyrosine residue located carboxyl-terminal to the SH2 domain (Silvennoinen et al., 1993; Darnell et al., 1994; Stark et al., 1998; Sue et al., 2000; Velichko et al., 2002). The tyrosine phosphorylation of STAT1 and STAT3 is the decisive activation event, resulting in STAT dimer formation (i.e. STAT1:STAT1 homodimer, STAT3:STAT3 homodimer as well as STAT1:STAT3 heterodimer) through mutual SH2 domain-phosphotyrosine interactions (Figure 2). The STAT dimers then translocate to the nucleus, bind to interferon stimulated response elements (ISREs) in gene promoters, and enhance the transcription of these target genes (Darnell et al., 1994; Darnell et al., 1997; Ramana et al., 2000).

STAT1 and STAT3 are STAT family members with divergent effects on immunogenicity. STAT3 regulates the expression of many genes that promote cell growth and prevent cell death. Furthermore, immunosuppressive factors (such as VEGF, IL-6 and IL-10) and factors suppressing dendritic cell maturation have been linked to high levels of phospho-STAT3 (pSTAT3) (Benkhart et al., 2000; Niu et al., 2002; Cheng et al., 2003; Wang
Figure 2. **Interferon-α signaling pathway.** Type I IFN (IFN-α) binding induces receptor tyrosine phosphorylation (P) by JAK1 and TYK2 proteins, producing a recruitment site for STAT1 and STAT3 and ultimately resulting in the formation of homo- and heterodimers of STAT1 and STAT3. Negative inhibition of the IFN pathway is accomplished by protein tyrosine phosphatases (such as CD45, SHP-1, PTP-1B) that can act at the level of the membrane-associated receptor-kinase complex, or in the nucleus (such as TC-PTP) by dephosphorylation of activated STAT dimers and recycling the latent STAT monomers to the cytoplasm. The JAKs have its own inhibitors, called SOCS proteins which directly bind to and inactivate kinases. The steady-state and signal-inducible concentrations of all the regulators determine the intensity and duration of the signal response in a cell.
et al., 2004; Nefedova et al., 2004; Yu et al., 2004; Yu et al., 2007; Humpolikova-Adamkova et al., 2009). In contrast, STAT1 (the major transducer of signals from the IFN-α and -γ receptors) (Li et al., 1996; Kaplan et al., 1996; Grimley et al., 1998, Ihle, 2001) behaves as an antagonist to STAT3. Active STAT1 (pSTAT1) has tumor suppressive properties and promotes the expression of pro-apoptotic and cell-cycle inhibitory genes, and represses cell survival and proliferation genes. In keeping with the function of STAT1 as a tumor suppressor, the loss of STAT1 signaling enhances oncogenesis (Meraz et al., 1996; Lesinski et al., 2003). STAT1 also mediates the expression of MHC and costimulatory molecules, and induces the activation of immune cells responsible for tumor clearance (Darnell et al., 1997; Tomic et al., 2006). While STAT3 expression in APCs has been associated with ineffective anti-cancer immune responses (Yu and Jove, 2004; Yu et al., 2007; Kortylewski et al., 2005), dominant STAT1 expression is associated with development of potent APCs (Tomic et al., 2006).

Taken together, a high pSTAT1/pSTAT3 ratio is associated with strong immunogenic functions. If this feature could be imposed on tumor cells, it would follow that the tumor cells should be able to better stimulate and be killed by tumor-reactive T cells.

1.3.1 IFN-α in cancer

Type I IFNs have emerged as important regulators of cancer cell growth and differentiation, affecting cellular communication and signal transduction pathways (Platanias et al., 2005; Dunn et al., 2005; Dunn et al., 2006; Smyth, 2005). Numerous studies have outlined the importance of IFN in control of cancer, both from the laboratory and clinical points of view. Some studies have shown that the endogenously produced IFN forms the basis of a tumor surveillance system that controls the development of both chemically induced and
spontaneously arising tumors (Dighe et al., 1994, Kaplan et al., 1998; Dunn et al., 2006). However, it has been shown that some cancer cells become unresponsive to IFN due to impairment in the proximal signaling events downstream of IFN receptors that lead to activation of STAT1 (Kaplan et al., 1998; Kovarik et al., 2003). In addition, studies in mice have buttressed these findings by demonstrating that STAT1-deficient mice show no overt developmental abnormalities, but display a complete lack of responsiveness to IFN (Meraz et al., 1996; Lesinski et al., 2003). These mice do not develop spontaneous tumors, but are highly susceptible to develop cancer induced by chemical carcinogens (Dunn et al., 2006), and have reduced median time onset of mammary tumors in ErbB2/neu-expressing mammary epithelium (Klover et al., 2010), suggestive of a tumor-suppressing effect of STAT1.

IFN-α is the first cytokine to find clinical application (Langenmayer et al. 1996; Grimley et al., 1998; Astsaturov et al., 2003). It has activity in several malignancies, including some leukemias and lymphomas (Foon et al., 1985; Golomb et al., 1986; Talpaz et al., 1986; Silver et al., 1999) and is also approved for treatment of high-risk melanoma patients in the adjuvant setting (Astsaturov et al., 2003; Kirkwood et al., 2000). As a single agent, IFN-α is thought to inhibit melanoma cell proliferation by directly regulating gene expression (Astsaturov et al., 2003; Lesinski et al., 2010). IFN-α may also affect antigen presentation by increasing MHC expression on both tumor cells and APCs (Lee et al., 1999). In the adjuvant setting, Astsaturov et al. (2003) demonstrated that IFN-α enhanced significantly the immunological and therapeutic effects of a cancer vaccine against the melanoma antigen. In this study, it was shown that IFN-α altered both the quantity and quality of autoreactive T cells that recognize tumor antigens, further suggesting that IFN-α may also act by recalling the responses of T cells that have been naturally activated by tumor antigens.
Despite wide evidence for the role of IFN in controlling the tumor growth, the benefit of IFN treatment is limited to some neoplasms while others are completely or partially resistant, especially advanced stage tumors. We have found that IFN-signaling is altered in leukemia cells from CLL patients with aggressive disease (Tomic et al., 2011). In these cells, the duration of IFN-mediated STAT3 activation was prolonged and the outcome switched from a tumor-suppressor phenotype involving growth arrest and enhanced immunogenicity to a phenotype involving proliferation and production of immunosuppressive cytokines, such as IL-10. Altered IFN-signaling in CLL cells was linked to increased ROS levels, which themselves are associated with more aggressive disease (Kumar et al., 2003; Szatrowski and Nathan, 1991; Behrend et al., 2003). Why might altered IFN-signaling be involved in the development of cancer? Evidence suggests that a dominant IFN-mediated STAT3 pathway negates the normal tumor-suppressor function of IFN, causing it to become a growth and immunosuppressive factor that promotes cancer development.

1.3.2 Regulation of IFN-signaling and intrinsic tumor resistance to IFN

IFN-signaling is regulated in a number of ways. The protein tyrosine phosphatases (PTPases) regulate the tyrosine kinase activities by dephosphorylating tyrosine residues involved in the catalytic functions (Mustelin et al., 2004; Shuai and Liu, 2003). Optimal activation of JAK kinases is positively regulated by phosphorylation of a critical tyrosine residue in the kinase-activating domain (Rane and Reddy, 2002). PTPases such as SHP-1 and PTP-1B have been shown to inhibit tyrosine phosphorylation of JAK kinases following their recruitment to receptor complexes (Rane and Reddy, 2002). Transmembrane PTPase CD45 also suppresses JAK kinases by directly binding and dephosphorylating them (Irie-
Sasaki et al., 2001). Another group of proteins referred to as SOCS (suppressor of cytokine signaling) play a critical role in negative regulation of cytokine signals processed by JAK kinases (Kubo et al., 2003; Yoshimura et al., 2007). For example, SOCS1 and SOCS3 are found to inhibit JAK kinase activity by directly binding to their kinase activation loop (Rottapel et al., 2004).

Downstream of IFN receptor and JAKs, yet another group of proteins negatively modulates IFN-signaling by acting on STATs. PIAS (protein inhibitor of activated STAT) family members, such as PIAS1, inhibit STAT1 activity in the nucleus (Liu et al., 1998; Shuai and Liu, 2005). The PTPase TC-PTP also exerts its effect in the nucleus where it dephosphorylates STAT1 (ten Hoeve et al., 2002).

High levels of STAT3 (relative to STAT1) can prevent STAT1 homodimer formation, by sequestering STAT1 in STAT1:STAT3 heterodimers (Jove et al., 2002). Furthermore, activated STAT3 can suppress phosphorylation of STAT1 by inducing SOCS1 (which binds to and inhibits JAK1-mediated phosphorylation of STAT1) (Rottapel et al., 2004).

Constitutive STAT3 activation is frequently detected in human cancers (Russo et al., 2003), often resulting from autocrine signaling through IL-6 and IL-10 receptors (Youinou et al., 2011; Russo et al., 2003; Dondi et al., 2001). These receptors also use JAK1 and TYK2 to communicate their signals, thus the shared use of JAK proteins leads to decreased IFN-mediated STAT1 activation (Dondi et al., 2001). Hence, tumor cells create an overall imbalance that favors STAT3 activation, through IL-6 and IL-10 signaling pathways that use the same kinases as IFN. In CLL cells, constitutive serine phosphorylation is present on both STAT1 and STAT3 (Frank et al., 1997). It was later shown by Kay et al. (2005) that these
constitutive serine-phosphoryated STAT1 and 3 molecules physically complex with VEGF receptors 1 and 2 and that this interaction may be responsible for increase in survivorship of CLL cells. Estrov et al. (2010) showed that constitutive serine-phosphorylated STAT3 can activate transcription of STAT3-regulated genes such as STAT3, Bcl2, Pim1, Bel-X<sub>L</sub>, p21 and c-myc in CLL cells. This group was also able to show that high levels of unphosphorylated STAT3 are found in CLL cells as a result of binding of serine-activated STAT3 to DNA, which then complexes with NF-κB and activates NF-κB-regulated genes such as RANTES. Remarkably, NF-κB is constitutively activated in CLL cells [Kay et al., 1992] and through coupling with cyclin D2 and c-myc [Igawa et al., 2011] may be responsible for proliferation of CLL cells in the proliferation centers.

Taken together, enhanced expression and function of PTPases or SOCS proteins, or dominant STAT3 activation in tumor cells (Tomic et al., 2011), would prevent strong IFN-signaling, more specifically IFN-mediated STAT1 effects. Unfortunately, the oncogenic events, such as loss of tumor suppressor <i>p53</i> and elevated ROS levels, that underlie tumor progression lead to precisely these conditions.

1.3.3 Oncogenic event: loss of tumor suppressor <i>p53</i>

The <i>p53</i> gene was the first tumor suppressor gene to be identified (Levine et al., 1991). Originally, <i>p53</i> was believed to be an oncogene (e.g. a cell-cycle accelerator), but genetic and functional data showed it to be a tumor suppressor (Levine et al., 1991). Unlike oncogenes (which are mutated in ways that render the gene constitutively active), for tumor suppressor genes, mutations and deletions reduce the activity of the wild-type gene product (Levine et al., 1991). Hence, it was found that the p53 protein does not function correctly in most human cancers. In about 50-55% of all human cancers, p53 is inactivated directly as a
result of mutations in the \textit{p53} gene (Hollstein \textit{et al.}, 1991). These mutations strongly select for \textit{p53} proteins that fail to bind to DNA in a sequence-specific fashion (Hollstein \textit{et al.}, 1991). In many other cancers, \textit{p53} is inactivated indirectly through binding of viral proteins (e.g. SV40 T antigen) (Levine \textit{et al.}, 1991), or as a result of alterations in genes whose products interact with \textit{p53} (i.e. MDM2) (Levine \textit{et al.}, 1991).

Normally, in a cell, \textit{p53} protein is kept at a low concentration by its relatively short half-life (about 20 minutes), which is related to ubiquitin-mediated proteolysis (Levine \textit{et al.}, 1991; Goldinger \textit{et al.}, 1994). In addition to low protein concentration, in some cells \textit{p53} exists in a latent form inactive for transcription, and must be activated by post-translational modifications, such as phosphorylation, for it to function. \textit{p53} functions as a transcription factor involved in cell cycle checkpoints, apoptosis, and genome stability (Levine, 1997). The upstream events or signals that flow to \textit{p53} are mediated by several stressful situations such as DNA damage caused by \(\gamma\)-irradiation (Meek \textit{et al.}, 2004), causing a rapid increase in the level of \textit{p53} in the cell and activation of \textit{p53} as a transcription factor. The \textit{p53} level increases because the half-life of the protein is lengthened and possibly because the rate of translational initiation of \textit{p53} mRNA in the cell is enhanced (Levine 1997; Meek \textit{et al.}, 2004). In response to ionizing radiation (IR), ATM (ataxia-telangiectasia mutated) (Savitsky \textit{et al.}, 1995; Lavin and Shiloh, 1997) protein recognizes DNA damage and communicates this via its kinase domain to \textit{p53} (Xu \textit{et al.}, 2002; Derheimer and Kastan, 2010). The subsequent change in \textit{p53} (i.e. phosphorylation) permits DNA binding to a specific DNA sequence regulating several downstream genes (such as \textit{p21} and \textit{MDM2} among others) (Meek \textit{et al.}, 2004). However, tumor cells that have acquired a mutation in the \textit{p53} gene have an impaired response to IR. For example, cells with dysfunctional \textit{p53} are unable to up-
regulate either p53 or p21 in response to γ-radiation and as a result they do not undergo cell arrest or apoptosis (Vousden and Lee, 2002). Thus p53-mediated up-regulation of p21 (p53/p21) in response to IR can be used as a test to assess the functional state of p53 pathway in tumor cells (Tomic et al., 2011).

We had found that the duration of IFN-mediated STAT3 phosphorylation was prolonged in CLL cells with deletions involving ATM and p53 (Tomic et al., 2011). Although loss of normal p53 and ATM function did not appear to directly affect IFN-signaling in CLL cells, increase in cell size and number in response to IFN resulted in a gene expression pattern and elevated ROS levels that caused IFN to be used as a growth factor rather than a tumor suppressor. Our identification of an association of altered IFN-signaling pathways with high-risk disease characteristics, such as loss of ATM and p53 function, may help explain the clinical observations that IFN-α is most effective in patients with low-risk CLL (those that have intact ATM and p53).

1.3.4 Oncogenic event: elevated ROS levels

Reactive oxygen species (ROS) are oxygen-containing chemical species, such as superoxide, hydrogen peroxide, singlet oxygen and the hydroxyl radical (Halliwell, 2007). Other reactive species exist and these include nitrogen oxides, NO and NO$_2$. Each of these possesses higher reactivity than molecular oxygen, has distinct reactive chemical properties, as well as the rate and site of production. Consequently, the biological impacts of ROS depend on the particular molecules involved and on the microenvironment (Halliwell, 2007). There are five main targets for ROS in living cells: small organic biomolecules, nucleic acid, proteins, gene activation and unsaturated fatty acids (Halliwell, 2007). The formation of ROS is prevented by an antioxidant system which consists of low molecular mass
antioxidants such as Vitamin C and glutathione and ROS-interacting enzymes such as SOD, peroxidases and catalases (Chiarugi and Buricchi, 2007).

Low levels of ROS regulate cellular signaling and play important roles in normal cell proliferation (Kamata and Hirata, 1999). In fact, ROS have a huge range of potential actions on cells that can be either anti-cancer (e.g. promoting senescence, apoptosis, necrosis, inhibiting angiogenesis) or pro-cancer (e.g. promoting uncontrollable proliferation, angiogenesis, invasiveness, metastasis and inhibiting apoptosis) (Halliwell, 2007). In a mammalian cell, ROS are constantly generated and eliminated through a variety of pathways involving mitochondria and ER. Specifically, they are by-products of mitochondrial electron chain flux as a result of aerobic metabolism, but may also be produced by cytoplasmic enzymes such as NADPH oxidase (NOX), cyclooxygenase, lipooxygenase (LOX) and NO synthases (Chiarugi and Buricchi, 2007; Benhar, et al., 2002). Also, microsomes and peroxisomes can be additional sources of ROS, mainly in stress situations such as prolonged starvation which leads to fatty acid oxidation (Chiarugi and Buricchi, 2007).

ROS production is increased in cancer cells compared to normal cells as a result of oncogenic transformation, increased metabolic activity and mitochondrial malfunction (Szatrowski and Nathan, 1991; Halliwell, 2007). Elevated ROS levels promote tumorigenesis by activating signaling pathways that control proliferation, angiogenesis and metastasis (Storz, 2005; Benhar et al., 2002). More specifically, ROS are able to oxidize both protein tyrosine phosphatases (PTPs) and protein tyrosine kinases (PTKs), leading to opposing enzymatic activity regulation (i.e. inhibition for PTPs and activation for PTKs) (Chiarugi and Buricchi, 2007). Other signaling proteins that are susceptible to oxidation by ROS are NFκB (Schrech et al., 1991), p53 (Rainwater et al., 1995), HIF (Wang et al., 1995),
and AP-1 (Okuno et al., 1993) as a result of low pK_a cysteine residues. Increased ROS production by tumor cells can also serve as an endogenous source of DNA-damaging agents that promote genetic instability and development of drug resistance (Pelico et al., 2004). ROS-induced DNA lesions and mutations are likely to provide a mechanism through which more aggressive and drug-resistant cancer cells constantly arise. However, because ROS are biochemically active and can cause severe cellular damage, the very fact that cancer cells are under increased intrinsic ROS stress may also provide a unique opportunity to kill the tumor cells based on their dependence on ROS.

We have found that aggressive CLL cells express high levels of ROS which may mediate resistance to immunomodulatory agents, such as IFN (Tomic et al., 2011). More specifically, IFN-mediated STAT3 activation was prolonged in these cells as a result of lowered phosphatase activity. Others have found that protein tyrosine phosphorylation is abnormal in CLL cells and this is evident by overactive Src kinases activity (Contri et al., 2005). Src kinase family members are positive regulators of STAT3 activation and ROS generation is involved in regulation of STAT3 and Src kinases (Xing et al., 2010). ROS-Src-STAT3 path may be another way by which immunomodulator signaling is dysregulated in CLL cells.

But where do elevated ROS levels in aggressive CLL cells originate? As survival of CLL cells may be driven by antigenic stimulation through the B-cell antigen receptor (BCR), one possibility is that engaged BCR transmits signals to mitochondria which provides energy for functional cellular responses. In fact, Palazzo et al. (2011) have shown a connection between hydrogen peroxide and signaling proteins downstream of the BCR such as phospho-Lyn, phospho-Syk, phospho-BLNK, phospho-PLCγ2 and phospho-STAT5 in CLL cells.
Even though phospho-STAT5 is not a member of the canonical BCR signaling pathway, the increase seen by Palazzo et al. in hydrogen peroxide-mediated STAT5 phosphorylation could be due to a bystander effect resulting from phosphatase inhibition with consequent increases in kinase activities for which STAT5 is a substrate.

The unique aspect of this finding in CLL cells is that elevated endogenous ROS levels in cancer cells can be exploited both as a prognostic marker as well as a therapeutic target. Recently, Lilienthal et al. (2011) have exploited the disturbed redox regulation in CLL cells by using a class of ROS catalysts to elevate already high ROS levels toward toxic levels in order to induce apoptosis. The premise for this study was to turn the “pathogenetically” beneficial ROS levels of CLL cells towards its own Achilles’ heel (Lilienthal et al., 2011).

In conclusion, ROS measurements could predict patient’s clinical course, “personalize” the treatment plan and establish ROS as a more effective target for therapy.

1.4 Glucose metabolism in tumor cells

Despite the ability to increase aspects of tumor immunogenicity in vitro, immunomodulators such as PKC agonists and IFN-α are relatively ineffective as single agents in vivo. This disparity between in vitro effects and in vivo results seems to hold for other immunomodulators, such as TLR-agonists (Spaner et al., 2008), as well as cytotoxic chemotherapies, such as Vincristine (Tomic et al., 2009).

An important distinction between in vitro experiments and clinical trials is the proliferative state of the target tumor cells. For instance, while circulating leukemia cells (such as CLL) are easily obtained from patient’s blood, targeting them contributes little to long-term clinical remissions, since the disease is maintained by proliferation centers in secondary and tertiary lymphoid tissues (Herishanu et al., 2011; Friedberg et al., 2011).
Central to proliferation is dependence on high rates of glycolysis to obtain sufficient energy for survival, and to generate substrates such as lipids and nucleotides for cell division (Bauer et al., 2004; Bauer et al., 2005; DeBerardinis et al., 2008). In fact, it has been shown that glucose usage by aggressive tumor cells in vivo modifies signal transduction and impairs responses to immunomodulators (Shi et al., 2010).

1.4.1 Warburg Effect

In the 1920’s, Otto Warburg reported an observation that rapidly proliferating tumor cells consume glucose at a surprisingly high rate compared to normal cells, and secrete most of the glucose-derived carbon as lactate rather than oxidizing it completely (Warburg, 1925; Warburg, 1956a). This phenomenon became known as “Warburg effect”. While true, this observation presented a paradox: Why do proliferating cells, which have a great need for ATP, use such a wasteful form of metabolism? Warburg proposed that tumor cells have a permanent impairment of oxidative metabolism resulting in a compensatory increase in glycolytic flux (Warburg, 1956a). However, later studies ruled out the possibility that only tumor cells use aerobic glycolysis or that the Warburg effect only develops when oxidative capacity is damaged (Wang et al., 1976; Roos and Loos, 1973). Then why does Warburg effect occur? High glycolytic rate allows cells to use glucose (which is the most abundant extracellular nutrient) to generate ATP. Even though the yield of ATP per glucose consumed is low, if the glycolytic flux is high enough, the percentage of cellular ATP produced from glycolysis can exceed that produced from oxidative phosphorylation (Guppy et al., 1993). Second, glucose degradation provides cells with intermediates needed for biosynthetic pathways, including ribose sugars for nucleotides, glycerol and citrate for lipids, nonessential amino acids, and through the oxidative pentose phosphate pathway, NADPH (DeBerardinis
et al., 2008). So the Warburg effect allows for the generation of energy and incorporation of nutrients into biomass. In fact, this phenomenon of altered metabolic state is considered to be yet another hallmark of cancer, emerging concomitantly with malignant transformation, hypoxia and mitochondrial malfunction in the majority of cancers.

To expand on this notion that tumors rely on large amounts of glucose for survival, how does the immunomodulatory resistance arise in highly glycolytic cells? The problems with PKC agonists and IFN-α signaling in cancer cells are suggestive of another clinical condition where aberrant glucose metabolism and defective signal transduction are linked. In diabetes, insulin receptor-expressing cells do not respond appropriately to insulin in the presence of hyperglycemia (Brownlee, 2001). More specifically, a minor glucose pathway, the hexosamine biosynthetic pathway (HBP), was shown to inhibit insulin responses by modifying (glycosylating) components of the insulin signaling cascade (Marshall et al., 1991).

1.4.2 Hexosamine Biosynthetic Pathway (HBP) and signal transduction

When glucose enters the cell, it is metabolized through a number of pathways. Most commonly, it is metabolized to pyruvate in the Embden-Meyerhof pathway, and then oxidized to make ATP in mitochondria, or reduced to lactate under anaerobic conditions. Glucose is also metabolized in the Hexose-Monophosphate Pathway (HMP) to Ribose-5-P, to make nucleotides for gene transcription and DNA replication by rapidly dividing cells (Bouche et al., 2004). Only 3-5% of glucose enters the hexosamine pathway (HBP) (Figure 3). HBP is a relatively minor branch of glucose metabolism (Marshall et al., 1991). In addition to glucose, glutamine and acetyl-CoA are necessary for pathway activity, suggesting that the hexosamine pathway is highly attuned to cellular metabolism. The rate-limiting
Figure 3. Hexosamine Biosynthetic Pathway (HBP). Upon entry into the cell, glucose is immediately phosphorylated by the hexokinase (HK) (step 1) to glucose-6-phosphate (G6P) to prevent diffusion out of the cell. G6P has many possible fates within the cell. If the cell needs to produce more NADPH (used for fatty acid synthesis) or nucleotides (used for DNA synthesis), G6P can enter pentose phosphate pathway (PPP). G6P can also be converted to glycogen for storage. If the cell needs energy, then G6P is first isomerized to fructose-6-phosphate (F6P) by phosphoglucoisomerase (step 2) and then targeted for glycolysis. However, the adverse effects of glucose have been attributed to its increased routing through the HBP. In the HBP, GFAT catalyzes the conversion of F6P to glucosamine-6-phosphate (Glc6P) with glutamine acting as an amino-donor. The final product of this pathway is the substrate UDP-GlcNAc, which is the donor sugar used by the enzyme OGT for O-linked glycosylation of proteins. In this reaction, a single GlcNAc moiety is enzymatically attached to the hydroxyl group of either serine or threonine residue, and removed by OGase. Glucosamine can directly activate the HBP by bypassing the reaction catalyzed by GFAT.
enzyme in this pathway is glutamine-fructose-6-phosphate amidotransferase (GFAT), whose product is glucosamine (GlcN)-6-phosphate, which is acetylated and uridinylated to form UDP-GlcNAc (which is the end product of this pathway) (Figure 3). UDP-GlcNAc is an important branch-point of HBP. This nucleotide sugar is used in the ER and Golgi to glycosylate secretory and membrane lipids and proteins (Dennis et al., 2009; Love and Hanover, 2005), which regulate signaling events at the cell surface (Lau et al., 207; Partridge et al., 2004). However, in the nucleus and cytoplasm, the enzyme O-GlcNAc-transferase (OGT) rapidly attaches UDP-GlcNAc to serine and threonine residues on substrate proteins (including metabolic enzymes, signaling molecules and transcription factors that are also kinase targets) (Hart et al., 2007; Zachara and Hart, 2004; Love and Hanover, 2005), which can be recognized by monoclonal antibodies such as RL2 and CTD110.6 (Holt et al., 1987). Analogous to the negative regulation of kinase activity by phosphatases, O-GlcNAcylation is countered by the deglycosylating enzyme, O-GlcNAcase (OGase). The dynamic modulation of serine and threonine residues by O-GlcNAcylation can potentially regulate cellular behavior in the same way as phosphorylation cascades (Hart et al., 2007; Slawson et al., 2005; Shi et al., 2010). Moreover, O-GlcNAcylation mediates resistance to physical stressors that activate similar signaling pathways as immunomodulators (Zachara, et al., 2004). The similarity of the substrates for these different post-transcriptional modifications suggests that the hexosamine pathway may negatively regulate signaling from immunologic receptors.

1.4.3 HBP and O-GlcNAc modification in cancer

HBP contributes a metabolically sensitive protein modification, O-GlcNAc, which is emerging as a key modification in cancer cells that are highly dependent on glucose for
growth. In fact, a number of tumor-associated proteins are modified by O-GlcNAc, suggesting that this glucose-sensing mechanism can directly link metabolic status to protein expression and activity. For instance, STAT5, which is involved in cell differentiation (Bromberg and Darnell, 2000; Ben-David et al., 2000), was found to be O-GlcNAc modified at Thr\textsuperscript{92} and as a result unable to interact with the transcriptional co-activator CBP to induce STAT5-dependent gene transcription (Gewinner et al., 2004). O-GlcNAc modification of tumor suppressor p53 on Ser\textsuperscript{149} decreases p53 ubiquitination/proteolysis, thus resulting in higher stability of p53 (Yang et al., 2006). The presence of O-GlcNAc modification on c-Myc at Thr\textsuperscript{58} residue was found to inhibit interaction with retinoblastoma (Rb) protein and the Rb-related protein p107, thereby interfering with transactivation of c-Myc (Gu et al., 1994). Furthermore, it has been speculated that O-GlcNAcylation of c-Myc at Thr\textsuperscript{58} competitively inhibits phosphorylation and thus suppresses proteasome-mediated degradation of c-Myc (Chou et al., 1995; Gu et al., 2010). On the other hand, a number of studies have shown that many metabolic pathway genes are targets of c-Myc (Dang et al., 2006; Zeller et al., 2006), including those involved in HBP (Li et al., 2003; Lawlor et al., 2006), suggesting that c-Myc is involved in orchestrating the changes in cell metabolism. More specifically, a study by Morrish et al. (2009) has shown that in response to serum, Myc\textsuperscript{+/+} cells exhibited an increase in global O-GlcNAcylation. We have also reported that some CLL cells express high levels of O-GlcNAcylated proteins, including p53, c-Myc, and AKT (Tomic et al., 2010). This is important because while exogenous stimulation by growth factors is needed to trigger normal cell proliferation, in tumor cells, genetic alterations favor self-sufficiency in growth signaling and/or pathways supporting nutrient capture from the microenvironment. Partridge et al. (2004) reported elevated levels of UDP-GlcNAc in the Golgi of cancer cells,
which were used in N-glycan modification of cell-surface cytokine receptors, such as EGFR and TGF-βR. In turn, this modification enabled surface retention of these cytokine receptors and contributed to tumor invasion and metastasis. The mechanisms by which O-GlcNAcylation promotes tumor progression and allows tumor cells to resist cytotoxic stresses (Zachara and Hart, 2004) are likely to be multi-factorial and include effects on signaling pathways (Shi et al., 2010) and gene expression regulated by oncogenic transcription factors such as c-Myc (Kamemura et al., 2003; Chou et al., 1995).

1.4.4 Role of OGT in cancer

O-GlcNAcylation is a process that is dynamically regulated by OGT and OGase in response to nutrient availability and extracellular signals. Mi et al. (2011) examined O-GlcNAcylation levels and the expression of OGT and OGase in human lung and colon cancer tissues and found that O-GlcNAc and OGT expression were significantly elevated in the cancer tissues compared to that in the corresponding adjacent tissue. Caldwell and colleagues (2010) also observed increased O-GlcNAcylation and elevated expression of OGT in breast cancer cells. More importantly, they found that reduction of OGT through RNA interference in breast cancer cells led to inhibition of tumor growth. In addition, pharmacological inhibition of OGT in the same cells had similar anti-growth and anti-invasion effects (Caldwell et al., 2010). We have also reported higher OGT expression in CLL cells (Tomic et al., 2010), which could account for higher O-GlcNAcylated proteins levels in CLL cells. These findings suggest that OGT is strongly linked to cancer, and may represent a novel therapeutic target.

1.4.5 Targeting HBP to overcome immunomodulatory resistance
The resistance of leukemic cells to immunomodulators (such as PKC agonists, TLR agonists and IFN-α) is an important obstacle that must be overcome for effective T cell-mediated immunotherapy. Some cancers are genetically predisposed to respond aberrantly to immunomodulators (Spaner 2004), but strong dependence on glucose to support growth is a general characteristic of aggressive cancers (Mazurek et al., 1997; Kroemer and Pouyssegur, 2008). Increased levels of glucose usage is a metabolic strategy employed by tumor cells which not only promotes the growth but also provides the energy to efficiently repair damage from cytotoxic drugs [Gatenby and Gillies, 2004] and is associated with immunosuppressive gene programs that decrease susceptibility to T cells [Tomic et al., 2010]. A conclusion of the studies in this dissertation is that the intrinsic immunosuppressive properties of CLL cells may be overcome by targeting their dependence on glucose, in particular their dependence on HBP and elevated O-GlcNAc levels. Inhibition of O-GlcNAcylation could potentially improve efficacy of cancer drugs as well as clinical efficacy of T cell-mediated immunotherapies for CLL and possibly other cancers that are dependent on HBP and O-GlcNacylation.
1.5 Hypothesis

Metabolic disregulation promotes tumor progression in part by altering cytokine signaling pathways and inhibiting immunogenicity of tumor cells.

1.6 Thesis objectives and organization

1. In an effort to increase immunogenicity of CLL cells, and in an attempt to guide the development of clinically relevant immunotherapeutic strategies for CLL, the effects of combinations of IL-2 and TLR-7 agonist on proliferation, costimulatory molecule expression, cytokine production, signaling and T cell-stimulatory capability were studied. The results of these studies are presented in Chapter II and were published in the *Journal of Immunology*.

2. While studying the effects of IL-2 and TLR-7 agonist on CLL cell immunogenicity, a phenotype was identified marked by strong STAT3 activation. However, in order to properly assess the effects of STAT3 activation on CLL cell immunogenicity, studies were undertaken to first understand how STAT3 activation and dominance over phospho-STAT1 influences IFN signaling in CLL cells. The results of these studies are presented in Chapter III and were published in the journal *Blood*.

3. Having previously found that aberrant IFN-signaling, characterized by prolonged STAT3 activation, is associated with aggressive CLL cells that express high levels of ROS, studies were undertaken to understand how cytokine responses are regulated. This could provide
insights into clinical behavior and lead to better treatment strategies for CLL. As aberrant metabolism is also a hallmark of cancer, it was found that the overactivity of the hexosamine pathway, marked by increased levels of O-GlcNAcylation, leads to impaired signaling responses to IFN and TLR agonists. The results of these studies are presented in Chapter IV and were published in the journal *Leukemia*.

4. Cancer cells were found to express high levels of proteins with O-GlcNAc modifications that interfere with normal (cytokine) signaling, and this offered a target for development of new treatment strategies. Studies were carried out to assess if agents that target this aberrant intermediary metabolism may have a role in the treatment of cancer. The findings of these studies are presented in Chapter V and are in revision for publication to journal *Blood*. 
Sensitization of IL-2 Signaling Through Toll-like Receptor-7 Enhances B Lymphoma Cell Immunogenicity

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2.1 ABSTRACT

The innate ability of B lymphoma cells to escape the effects of tumor-reactive T cells must be overcome to develop effective immunotherapies for these diseases. Because signals from both the innate and adaptive immune systems direct the acquisition of strong immunogenicity by professional antigen-presenting cells, the effects of IL-2 and the Toll-like receptor (TLR7) agonist, S28690, on the immunogenic properties of Chronic Lymphocytic Leukemia (CLL) B cells were studied. IL-2 with S28690 caused CLL cells to proliferate and increased their expression of B7-family members, production of TNF-α and IL-10, and levels of tyrosine-phosphorylated STAT-1 and STAT-3 proteins. S28690 increased CD25 expression on CLL cells and sensitized them to IL-2 signaling. However, IL-2 did not change TLR-7 expression or signaling in CLL cells. The ability to stimulate T cell proliferation required additional activation of protein kinase C, which inhibited tumor cell proliferation, “switched off” IL-10 production, and caused essentially all CLL cells (regardless of clinical stage) to acquire a CD83^{hi}CD80^{hi}CD86^{hi}CD54^{hi} surface phenotype marked by the activation of STAT-1 without STAT-3. These findings suggest that TLR-7 “licenses” human B cells to respond to cytokines of the adaptive immune system (such as IL-2) and provide a strategy to increase the immunogenicity of lymphoma cells for therapeutic purposes.
2.2 INTRODUCTION

Chronic Lymphocytic Leukemia (CLL), the most common leukemia in the western world (Cheson et al., 1996), is incurable with conventional cytotoxic chemotherapy but may be sensitive to T cell-based immunotherapies (Montserrat et al., 2005). However, despite expressing high levels of MHC molecules, at least one tumor antigen (the immunoglobulin idiotype), and sometimes even costimulatory molecules (Hammond et al., 2005; Spaner et al., 2004), CLL cells are weakly immunogenic (or unable to sustain a strong Type I immune response) and escape control by natural (Gitelson et al., 2003) or vaccine-activated (Spaner et al., 2005) tumor-reactive T cells. Consequently, the development of clinically relevant methods to increase the immunogenicity of CLL cells is an important goal.

Immunogenicity is a complex phenotype caused by signaling pathways that regulate the expression of costimulatory molecules, pro-inflammatory cytokines and chemokines, and ability to stimulate T cell proliferation and differentiation. Costimulatory molecules include CD80 and CD86, which are B7-family members that bind to CD28 on T cells and enhance their expression of anti-apoptotic and cytokine genes (Greewald et al., 2005), CD54, which stabilizes APC and T cell contacts (Hart, 1997), and CD83, a characteristic dendritic cell (DC) marker (Lechmann et al., 2002). ICOS-L and PDL-1 are additional B7 family members that regulate the behavior of activated T cells (Chen, 2004) and 4-1BB ligand (4-1BBL) is a CD28-independent costimulatory factor, primarily for CD8+ T cells (Watts, 2005).

Important transcription factors that positively regulate the immunogenic phenotype include members of the NF-kB family (Burkley et al., 1995) and the signal transducer and activator of transcription (STAT) family member, STAT-1 (Jackson et al., 2004). Other
members of the STAT family (particularly STAT-3) are negative regulators of immunogenicity, and cause production of immunosuppressive factors such as IL-10 (Cheng et al., 2003; Wang et al., 2004). Previously, we showed that both IL-2 (a cytokine mediator of adaptive immunity) (Spaner et al., 2004) and the imidazoquinoline, S28690, (a synthetic TLR-7 agonist which mediates innate immunity) (Spaner et al., 2006; Spaner et al., 2005) could enhance some aspects of CLL immunogenicity, but required additional signals (mainly from activators of protein kinase C (PKC) family members (Hammond et al., 2005)) to make CLL cells able to stimulate T cell proliferation.

The high-affinity IL-2 receptor consists of the α (CD25), β, and common γ (γc) chains (Ozaki and Leonard, 2002) and is expressed by many CLL cells (Spaner et al., 2004). Although IL-2 receptor signaling has not been well-characterized in CLL cells, IL-2 activates the mitogen activated protein kinase (MAPK) pathway that involves ERK-1/2, along with STAT-1, -3, -5a, and -5b, in most IL-2 responder cells, and the p38 phospho-relay pathway in T cells (Frank et al., 1995; Lin and Leonard, 2000). Like other TLRs (Akira and Takeda, 2004), TLR-7 activates NF-kB, p38 MAPK and the stress activated protein kinase (SAPK) pathway that involves JNK-1 and -2 (Schoenemeyer et al., 2005). Given that IL-2 and S28690 individually activate only some of the signaling pathways required for strong immunogenicity, and that highly immunogenic cells such as DCs incorporate information from both the innate and adaptive immune systems (Matzinger, 2002), the effects of combinations of IL-2 and S28690 on the immunogenicity of CLL cells were studied in this paper.
2.3 MATERIALS AND METHODS

Blood samples

Heparinized blood (30-40 ml) was collected from consenting CLL patients (diagnosed by a persistent monoclonal elevation of CD19<sup>+</sup>CD5<sup>+</sup>IgM<sup>lo</sup> lymphocytes (Cheson et al., 1996)). Patients were untreated at the time of analysis and their clinical characteristics and identification numbers are described in Table 1. Protocols were approved by the local review board.

Antibodies and Reagents

PE- or FITC-labeled CD80, CD86, CD54, CD83, CD25, 4-1BB, CD5, and CD19 antibodies (Abs) were purchased from BD Pharmingen (San Francisco, CA). PE-labeled anti-ICOS-L and PDL-1 and unlabeled CD80 and CD86 Abs for blocking experiments were obtained from eBioscience (San Diego, CA). Class I and II MHC Abs were from clones W6/32 and IVA12, respectively, obtained from the American Type Culture Collection and purified in our laboratory. Isotype control Abs for blocking experiments were obtained from BD Pharmingen. Phorbol dibutyrate (PDB) was from Sigma Chemical Co. (St. Louis, MO) and stock solutions (5 mg/ml) were made in DMSO. Clinical grade IL-2 (Chiron Corporation, San Francisco, CA) was purchased from the hospital pharmacy. S28690 and the inactive control imidazoquinoline, S26424 (Spaner et al., 2005), were from 3M Pharmaceuticals (St. Paul, MN). The powder was dissolved in AIM-V media (GibcoBRL, Grand Island, NY) (with 33% DMSO) at 1.3 mg/ml and stored in the dark at 4°C. Abs against JNK, p38, p42/p44 ERK, IκB, and the serine/threonine-phosphorylated forms of JNK, p38, ERK, and IκB were from Cell Signaling Technology, Beverly, MA. β-Actin Abs were from Sigma.
Table I: Clinical properties of CLL patients

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1. Rai stage 0=lymphocytosis; I= with adenopathy; II= with hepatosplenomegaly; III= with anemia; IV= with thrombocytopenia.
2. C=Chlorambucil, P=Prednisone, F=Fludarabine, S=Splenectomy, Rads=local radiation, R=Rituxan, CHOP=cyclophosphamide/vincristine/adriamycin/prednisone,
3. T12=Trisomy 12
ND=not done
Cell purification

CLL and T cells were isolated from fresh blood by negative selection (RosetteSep, StemCell Technologies, Vancouver, BC) as described previously (Gitelson et al., 2003).

Activation of CLL cells

Purified CLL cells (1.5x10^6 cells/ml) were cultured in serum-free AIM-V medium plus 2-mercaptoethanol (2-ME) (Sigma) (5x10^-5 M) in 6- or 24-well plates (Becton-Dickinson Labware, Franklin Lake, NJ) at 37°C in 5% CO₂ for the times indicated in the figure legends. S26424, S28690, IL-2, or PDB were used at 0.1 µg/ml, 0.1 µg/ml, 5000 U/ml, or 10 ng/ml, respectively. These concentrations were determined by the effects of the individual immunomodulators on CD80 and CD86 expression (for S28690 and IL-2) or CD83 (for PDB) (data not shown). S26424 (the control compound for S28690) did not have measurable effects on CLL cells so AIM-V medium, alone, was used as a control for most experiments.

Mixed Lymphocyte Responses (MLRs)

T cells were isolated from CLL patients and adjusted to 5x10^5 cells/ml in AIM-V medium. Activated CLL cells were washed at least four times (to remove residual immunomodulators), irradiated (2500 cGy) and suspended at 5x10^5 cells/ml (or lower concentrations) in AIM-V. Responders and stimulators were mixed in a 1:1 (vol:vol) ratio and cultured in 96-well round bottom plates (Becton Dickinson Labware) without cytokines or serum. Proliferation was measured 4-6 days later in a colorimetric assay (Gitelson et al., 2003). In some experiments, the activated CLL cells were lightly fixed (5 min) in 1% paraformaldehyde (and then washed extensively before suspension in AIM-V medium)
before being placed in the T cell cultures. It has been shown previously that APCs can present Ag even when fixed (Germain et al., 1993).

**Flow cytometry and DNA analysis**

Surface immunophenotyping was performed as described previously (Gitelson et al., 2003). For analysis of DNA content, CLL cells (~1 x 10^6) were washed and fixed in 70% ethanol at -20°C for several days at 10^6 cells/ml. The cells were then washed and resuspended in 1 ml of Ca^{2+}, Mg^{2+}-free PBS to which 0.1% Triton X-100, 0.1 mM EDTA, and 50 µg/ml RNase were added, and incubated for 1 h at 37°C (to allow the escape of low m.w. DNA through the permeabilized membranes). Cells were then washed, resuspended in staining buffer (0.1 mM EDTA, 0.1% Triton X-100, and 50 µg/ml propidium iodide; Sigma) at room temperature in the dark for 4-12 h, filtered through nylon mesh, and analyzed on a FACSCalibur flow cytometer (BD Biosciences) using CellQuest software.

**Western blots**

Proteins were extracted from activated CLL cells and immunoblotting was performed as described previously (Hammond et al., 2005), using anti-rabbit and anti-mouse IgG1 secondary Abs, as required. Blots were stripped for 15-30 min at 37°C in Restore Western Blot stripping buffer (Pierce Biotechnology Inc. Rockford, IL), washed once at room temperature, and then blocked with 10% milk for 1 h. Chemiluminescence signals were detected using Supersignal West Pico Luminal Enhancer and Stable Peroxide Solution (Pierce) and a GS-700 Imaging densitometer with Multi-Analyst software (Bio-Rad Laboratories, Hercules, CA).

**Isolation of total RNA and synthesis of cDNA**
CLL cells were activated for 2 days, harvested, and washed. Total RNA from activated CLL cells was extracted using the RNeasy kit (Qiagen, Mississauga, Ontario) according to the manufacturer's instructions. To remove contaminating genomic DNA, 10 µg of RNA were incubated with 10 U of RNase-free DNase I (Promega, Madison, WI) for 30 min at 37°C. The RNA concentration was determined in a spectrophotometer at 260 nm.

cDNA was synthesized with the Superscript First Strand Synthesis System (Invitrogen, Carlsbad, CA) in a 20 µl reaction containing 3 µg of total RNA, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM MgCl₂, 10mM dithiothreitol, 0.5 µg oligo(dT)₁₈, 0.5 mM of dATP, dGTP, dCTP, and dTTP, and 200 U of Superscript II Reverse Transcriptase. The priming oligonucleotide was annealed to total RNA by incubating the mixture at 70°C for 5 min and cooling to 4°C. Reverse transcription was conducted at 42°C for 2 h and the cDNA was stored at -20°C.

**Real-time PCR**

TLR-7, tumor necrosis factor (TNF)-α, and the housekeeping gene, HPRT, were amplified from cDNA with the following primers:

**TLR-7** forward: 5’-CTAAAGACCCAGCTGTGACCAG-3’

**TLR-7** reverse: 5’-CCAGTCCCTTTTCTCGAGACAT-3’

**TNF-α** forward, 5’-ACCTCTCTCTATACTCAGCCC-3’;

**TNF-α** reverse, 5’-AGGAGCACATGGGTGGAG-3’;

**HPRT** forward, 5’-GAGGATTTGGAAAGGGTGTT-3’;

**HPRT** reverse, 5’-ACAATAGCTCTTCAGTCTGA-3’.

PCR was performed on a DNA engine Opticon™ System (MJ Research Inc., Waltham, MA) using SYBR Green I as a double-strand DNA-specific binding dye. PCR
reactions were cycled 40 times after initial denaturation (95°C, 15 min) according to the following parameters: denaturation at 95°C for 15 sec, primer annealing at 57°C (TNF-α) or 52°C (HPRT) for 20 sec, and extension at 72°C for 20 sec. Fluorescent data were acquired during each extension phase. After each reaction, a melting curve analysis was performed by cooling the samples to 4°C and then heating them to 95°C at 0.2°C/sec. Fast loss of fluorescence is uniquely observed at the denaturing/melting temperature of the amplified DNA fragment. Standard curves were generated from serial 10-fold dilutions of DNA made with the above primers.

**Cytokine measurement**

Cytokine levels in culture supernatants (from CLL cells activated for 48 h) were determined by a multi-analyte fluorescent bead assay with a Luminex-100™ system (Luminex Corp., Austin, Texas). A kit for human IFN-γ, IL-4, IL-10 and TNF-α measurement was used, according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN). Individual cytokine concentrations were determined from standard curves using Bio-Plex 2.0™ software (BioRad, Mississauga, Ontario). The assay was linear between 3 and 15,000 pg/ml for each cytokine. Human IL-12 and TNF-α were measured with ELISA kits from Pierce, according to the manufacturer’s instructions.

**Statistical analysis**

The Student $t$ test was used to determine $p$ values for differences between sample means.
2.4 RESULTS

Effect of IL-2 and S28690 on proliferation and costimulatory molecule expression by CLL cells

As reported previously, both IL-2 (at doses above 500 U/ml) (Spaner et al., 2004) and S28690 (at doses above 0.1 µg/ml) (Spaner et al., 2006) change the shape and surface molecule expression of CLL cells. However, neither IL-2 nor S28690, alone, caused CLL cells to proliferate, as measured by counting them at the end of the culture period (Figure 4A). Note that in serum-free conditions, spontaneous CLL cell death was not observed during the course of these experiments (Hammond et al., 2005; Germain and Marguiles, 1993). In contrast, the combination of IL-2 and S28690 increased cell numbers significantly after at least 3 days of culture (Figure 4A). The net increase in CLL cells appeared to result from increased proliferation (rather than resistance to apoptosis) as more cells were found in the G2-S phase of the cell cycle (Figure 4B) without an increase in subdiploid DNA (representing apoptotic cells). Note that spontaneous CLL cell death is not usually observed in the time frame of these experiments under serum-free conditions (Hammond et al., 2005; Levesque et al., 2001).

IL-2 and S28690 individually increased the percentages of CLL cells that expressed CD80 and CD86, as well as the mean fluorescence intensity (MFI) of expression of these molecules (Figure 5). S28690 appeared to be more potent in this regard and also increased CD83 expression on CLL cells more than IL-2. As with proliferation, the effects of IL-2 and S28690 on costimulatory molecule expression were additive (Figure 5), suggesting they were mediated by different mechanisms.
Figure 4. Effect of S28690 and IL-2 on proliferation of CLL cells. (A) Purified CLL cells (1x10^6 cells/ml) from the indicated numbers of patients were cultured with combinations of S28690 (0.1 µg/ml), IL2 (5000 U/ml), or PDB (10 ng/ml) for 3-5 days. At the end of the culture period, viable cells were counted manually in a hemocytometer. The number of activated cells divided by the number of cells cultured alone was then determined for each patient sample. The average and standard errors are plotted in the graph. (B) DNA content histograms show that CLL cells (from patient 104) cultured alone, or with IL-2 or S28690 alone, were mainly in the G1-Go phase of the cell cycle while a significantly higher number were in cycle in the presence of both IL-2 and S28690. Little subdiploid DNA was apparent in these cultures. Similar results were obtained with CLL cells from four other patient samples.
Figure 5. Effect of S28690 and IL-2 on costimulatory molecule expression by CLL cells. CLL cells were isolated and cultured alone, or with IL-2, S28690, or both IL-2 and S28690, for 3-4 days. Expression of CD80, CD86, CD54, and CD83 were then determined by flow cytometry. (A) An example of one patient sample is shown. The numbers in the dot plots in the upper and lower rows are the percentages of CD80+ (sum of the right and left upper quadrants) and CD86+ (sum of the right upper and lower quadrants) CLL cells, respectively. (B) The percentage of cells expressing the different costimulatory molecules (indicated by staining intensity above the first decade of log fluorescence) (left graph) and the MFI of expression (right graph) were determined for CLL cells from the number of patients indicated in the graph legend. The average and standard error of the results for each different costimulatory molecule are shown. For CD54, only the MFI (divided by 10) is shown since essentially all CLL cells express this molecule (C). The magnitude of CD80 expression was increased especially by S28690 in combination with IL-2. The numbers over the double-headed arrows are the p values for the differences between sample means.
**Activation of signaling pathways in CLL cells by IL-2 and S28690**

The cooperation between IL-2 and S28690 in causing cell proliferation and increased costimulatory molecule expression arises presumably through interactions between their respective signaling pathways. As described in the introduction, TLR-7 activates NF-kB, p38, and JNK (Schoenemeyer et al., 2005), while IL-2 activates ERK and STAT family members, including STAT-1, 3, 5a, and 5b (Lin and Leonard, 2000). Abs against phosphorylated forms of important pathway molecules can be used to indicate the state of activation of the signaling pathways.

As shown in Figure 6A, S28690 treatment caused rapid phosphorylation of IkB, p38, and both JNK isoforms (suggesting activation of the NF-kB, p38, and SAPK pathways). IL-2 increased the phosphorylation of p42 and p44 ERK, but none of the other signaling pathways. The early signaling events that accompanied simultaneous treatment of CLL cells with IL-2 and S28690 were a composite of these effects. Thus, all the signaling pathways studied were activated, with NF-kB, p38, and JNK signaling at similar levels as cells treated with S28690 alone, and ERK signaling at the levels resulting from treatment with IL-2 alone (Figure 6A).

Unlike T cells (Lin and Leonard, 2000), IL-2 did not appear to activate JAK/STAT signaling directly in CLL cells (Figures 6B and 7C). Similarly, TLR-7 does not phosphorylate STAT proteins directly (Akira and Takeda, 2004), although autocrine production of cytokines in response to S28690 results in phosphorylation of STAT-1 and STAT-3 after several hours (Spaner et al., 2006). Consistent with an absence of early interactions between the signaling pathways, simultaneous treatment with IL-2 and S28690
Figure 6. Effect of IL-2 and S28690 on signaling pathways in CLL cells. (A) Purified CLL cells were cultured alone or treated with IL-2, S28690, or both IL-2 and S28690. After 1 h, changes in the phosphorylation status of JNK, p42/44 ERK, p38, and IkB were determined by immunoblotting with phospho-specific antibodies as described in the Materials and Methods. The blots were first probed with phospho-specific antibodies and were then stripped and probed with a pan-specific JNK, p42/p44, or p38 antibody (not shown) or a β-actin antibody as a loading control. A representative example of the results obtained with the CLL cells of patient 79 is shown. The results were similar for 4 additional patient samples. (B) CLL cells were cultured for 24 h in the presence or absence of IL-2 and/or S28690 and the levels of phosphorylated STAT-1 and STAT-3 were determined by immunoblotting. The results were similar for 6 additional samples.
did not directly phosphorylate STAT-1 or STAT-3 (data not shown). However, after 24 hours, tyrosine-phosphorylated STAT-1 and STAT-3 levels were increased (Figure 6B).

**Enhancement of IL-2 signaling in CLL cells by S28690**

Because IL-2 and S28690 appeared to have independent effects on early signaling events (Figure 6A), but increased proliferation (Figure 4), costimulatory molecule expression (Figure 5), and phosphorylated STAT-1 and STAT-3 levels (Figure 6B) after more prolonged culture periods, we considered that IL-2 might increase TLR-7 levels (and the effects of S28690 in culture) or that S28690 might increase IL-2R levels (and the effects of IL-2), or both.

Treatment with S28690 resulted in strong upregulation of CD25 on CLL cells (Figure 7A). Increases in CD25 expression were variable but did not appear to correlate with clinical staging differences. Five patients (patients 3, 4, 6, 9, 15) had stage 0 disease, two (patients 11, 17) had stage I/II disease, and six (patients 31, 32, 38, 43, 46, 47) had stage III/IV (Table I). The average and standard error of the S28690-induced changes in CD25 expression for these groups were 7.0 +/- 0.6 (n=5), 6.5 +/- 2.5 (n=2), and 8.0 +/- 2.5 (n=6), respectively. In contrast, IL-2 did not affect TLR-7 expression, at the mRNA level. Note that attempts to quantitate TLR-7 protein levels with existing commercial Abs by immunoblotting were unsuccessful, perhaps because of low expression of TLR-7. These results suggested that S28690 could enhance IL-2 signaling, but not the reverse.

To determine if the increased CD25 expression caused by pretreatment with S28690 affected IL-2 signaling, we compared changes in CD83 expression on CLL cells stimulated with a low dose of IL-2 (50 U/ml, which is 10-100 times lower than in the studies shown in Figures 4-6) before or after treatment with S28690 (Figure 7B). Only a small number of
CLL cells acquired CD83 expression when treated with low doses of IL-2, alone. However, when the cells were first exposed to S28690, the number that acquired CD83 expression in response to IL-2 was much higher (Figure 7B), even considering that CD83 expression had been increased by the preliminary culture in S28690 (Figure 5B). Similar results were seen with CD80 and CD86 expression (data not shown).

To demonstrate further that S28690 caused enhanced IL-2 signaling, early phosphorylation of STAT proteins was studied. As mentioned previously, IL-2 phosphorylates and activates STAT-5 in many IL-2 responder cells, but not CLL cells (see lanes 1 and 3, Figure 7C). When CLL cells were cultured with S28690 overnight, treatment with IL-2 now resulted in phosphorylation of STAT-5 (Figure 7C, compare lanes 5 and 6, upper panels). In contrast, using NF-kB activation to represent TLR-7 signaling, treatment overnight with IL-2 did not lead to enhanced phosphorylation of I kB in response to S28690 (Figure 7C, compare lanes 5-8 with lanes 1-4, lower panels).

Taken together with the absence of an effect on TLR-7 gene expression (Figure 7A), these results suggested that IL-2 did not sensitize CLL cells to TLR-7 signaling, whereas S28690 sensitized CLL cells to IL-2 (in part through increased IL-2R expression (Figure 7A).

Effect of PKC agonists on costimulatory function and phenotype of CLL cells treated with IL-2 and S28690

In accordance with their increased expression of costimulatory molecules, CLL cells treated with both IL-2 and S28690 were better able to support T cell proliferation (measured in MLRs) than CLL cells treated with either agent alone (Figure 8A). However, their stimulatory ability was still rather weak. Previously, we had found that the costimulatory
Figure 7. Enhancement of IL-2 receptor (IL-2R) expression and responses by TLR-7 activation. (A) CLL cells from the indicated numbers of patients were cultured for 2 days alone, with S28690, or with IL-2. CD25 expression was then determined by flow cytometry and TLR-7 mRNA expression by real-time PCR, as described in the Materials and Methods. The MFI of CD25 expression after treatment with S28690 was divided by the MFI of CD25 expression without stimulation. For TLR-7, the transcript number after treatment with IL-2 was divided by the transcript number in cells that were cultured alone. The results from the individual samples are shown, along with the average and standard error, and indicate that S28690 increased markedly the expression of CD25 but IL-2 did not change the expression of TLR-7, at the mRNA level. (B) CLL cells were cultured overnight alone or with S28690 to increase CD25 expression levels. The cells were then washed and either cultured alone or stimulated with low doses of IL-2 (50 U/ml) for 2 days. The expression of CD83 was then measured by flow cytometry. The difference in the percentage of CD83+ cells, with or without IL-2 treatment, was determined for each patient sample. The results show that S28690 increased the response of CLL cells to IL-2 in this assay, concomitant with the increase in CD25 expression. The p value is the significance of the differences between the changes in the two groups. (C) CLL cells from patient 73 were cultured for 24 h alone (lanes 1-4) or with S28690 (lanes 5-8, top panels), or IL-2 (lanes 5-8, lower panels). The cells were then washed and stimulated with IL-2 (top panels), or S28690 (bottom panels). Tyrosine-phosphorylated STAT-5 levels (representing IL-2R signaling) or serine-phosphorylated IκB levels (representing TLR-7 signaling) were then determined 0, 3, 10, 30 min later by immunoblotting, as described in the Materials and Methods. STAT-5 was phosphorylated by IL-2 only in CLL cells that had been pretreated with S28690 (compare lanes 5-8 with lanes 1-4, upper panels). In contrast, IL-2 did not augment TLR-7 signaling (compare lanes 1-4 and 5-8, bottom panels). This experiment was repeated with three other patient samples with similar results.
ability of CLL cells treated with S28690 (Spaner et al., 2006) or IL-2 alone (Spaner et al., 2004) could be enhanced significantly by concomitant stimulation with PKC agonists (such as phorbol esters, Bryostatin, or a synthetic Bryostatin analog called Picolog (Szabo et al., 2003) through a number of mechanisms, including increased expression of the DC marker, CD83. Since CLL cells treated with IL-2 and S28690 did not increase CD83 expression maximally (Figure 5B), the effect of additional treatment with PDB was then studied.

PDB decreased the numbers of CLL cells after treatment with IL-2 and S28690 (Figure 4) but caused them to become better stimulators of T cell proliferation (Figure 8A). Interestingly, CLL cells treated with both PDB and S28690 remained weak stimulators of T cell proliferation, despite increased expression of CD83. Hence, IL-2 appeared to be required for CLL cells to acquire strong T cell stimulatory ability.

Blocking experiments were conducted to determine whether the enhanced proliferation of T cells resulting from coculture with CLL cells treated with PDB, S28690, and IL-2 resulted from antigenic signaling, or was nonspecific and caused simply by high levels of cytokines in the presence of increased costimulatory molecule expression (Figure 8B). T cell proliferation was inhibited mainly by Abs against class II MHC molecules (Figure 8B, top graph), suggesting that CD4\(^+\) T cells were stimulated mainly by the activated CLL cells. Consistent with this, CD4\(^+\) T cell numbers increased more than CD8\(^+\) T cells (measured by manual counting in a hemocytometer and flow cytometry) at the end of the culture period (average numbers and standard errors with CLL cells from patients 1, 9, 23, and 104 as stimulators were: initial CD4\(^+\) cells: (90 +/- 3) x 10^4/ml. Final CD4\(^+\) cells: (213 +/- 13) x 10^4/ml. Initial CD8\(^+\) cells: (5 +/- 2) x 10^4/ml. Final CD8\(^+\) cells: (23 +/- 4) x 10^4/ml. Blocking Abs against CD80 and CD86 independently inhibited T cell proliferation.
This inhibition was stronger in the presence of both Abs (Figure 8B, bottom graph), suggesting that the two costimulatory molecules (along with class II MHC) contributed to the increased stimulatory ability of CLL cells treated with PDB, S28690, and IL-2. However, cytokine production may also play a role in the enhanced T cell stimulation. When the activated CLL cells were fixed with paraformaldehyde (PFA) (to prevent cytokine production while preserving cell membrane protein expression), the ability to stimulate T cell proliferation was inhibited strongly (Figure 8B, bottom graph, vertical bars).

Taken together, these results suggest that CLL cells treated with PDB, S28690, and IL-2 acquired properties of strong APCs and stimulated T cell proliferation through expression of Ag, costimulatory molecules (particularly CD80 and CD86), and cytokines. Note that CLL cells from patients with all clinical stages of the disease (Figure 8B) could stimulate T cell proliferation in this manner, upon activation with PDB, S28690, and IL-2.

**Costimulatory molecule expression by CLL cells treated with S28690, IL-2 and PKC agonists**

PDB, alone, caused ~90% of CLL cells to express CD83 (Figure 9B, clear bars). PDB also increased the number of CD80+ and CD86+ CLL cells (the latter more than the former), as well as the expression of 4-1BBL and PDL-1 (Figure 9C, clear bars). CD54 and ICOS-L expression were affected only marginally by PDB (Figures 9B and 9C).

Addition of IL-2 during activation of CLL cells with PDB increased mainly the number of CD80+ cells and the MFI of CD80 and CD54 expression (Figure 9B; horizontal bars). A slightly greater percentage of CD80+ cells was obtained when CLL cells were activated with both PDB and S28690 (Figure 9B; diagonal bars). The addition of IL-2 to S28690 and PDB increased strongly the expression of CD80 (compared to CD86 (Figure
Figure 8. Effect of S28690 and IL-2 on the ability of CLL cells to stimulate T cell proliferation.

(A) CLL cells were purified from individual patients and cultured alone or with IL-2, S28690, IL-2 and S28690, PDB, PDB and IL-2, PDB and S28690, or PDB, IL-2, and S28690, as described in the Materials and Methods. After 3-4 days, the cells were harvested, washed extensively, irradiated (2500 cGy) and used to stimulate T cells from CLL patients (obtained at the same time as the tumor cells and rested in culture until added to the MLRs). After 5-6 days, Alamar Blue was added and proliferation was measured in an optical density colorimetric microplate reader at wavelengths of 540 (reduced state) and 595 (oxidized state) nm. The difference between these readings was used as a measure of the number of viable cells in the culture. After subtracting the proliferation induced by non-activated CLL cell stimulators, the results from each individual experiment were used to generate the average proliferation and standard error from the number of patients indicated on the x-axis. IL-2 was required for CLL cells to acquire strong stimulatory capabilities and interacted with both PDB and S28690 to increase the stimulatory capabilities of CLL cells. (B) CLL cells from patients 104, 23, and 1 were activated with PDB, S28690, and IL-2 and used as stimulators in MLRs. Blocking class I MHC, class II MHC, CD80 and CD86 Abs (each at 20 µg/ml), as well as isotype control Abs, were added to some wells at the start of the MLR cultures. In some cases, the CLL cells were first fixed with PFA (to inhibit their ability to secrete cytokines) before use as stimulators in the MLRs. Proliferation was then measured using Alamar blue. The average of three replicate wells (after subtracting the background proliferation of the responders and stimulators) and standard error is shown for each patient. *P < 0.05; **P < 0.01.
Figure 9. Effect of PKC agonists on costimulatory molecule expression by CLL cells treated with S28690 and IL-2. (A) CLL cells from a representative patient were cultured alone (left panels) or with S28690, IL-2, and PDB for 3 days. CD80, CD83, CD54, and CD86 expression were then determined by flow cytometry. The percentages in the dot-plots refer to CD80 (sum of the right and left upper quadrants) (top panels) and to CD86 (sum of the right upper and lower quadrants) (bottom panels). (B) Summary of the flow cytometric evaluation of the percentage of CLL cells expressing CD80, CD83, CD54, and CD86 (and the MFI of expression) after culture alone, with PDB, PDB and IL-2, PDB and S28690, or PDB, IL-2 and S262690. The average and standard error of the results from the number of patients indicated in the graph legend are shown. For CD54, only the MFI (divided by 10) is shown since essentially all CLL cells express this molecule. The results indicated that PDB and S28690 caused nearly 100% of CLL cells to acquire CD80, CD86, and CD83 expression. Addition of IL-2 affected mainly the magnitude of CD80 and CD54 expression. (C) Summary of similar flow cytometric evaluation of ICOS-L, 4-1BB, and PDL-1 expression. PDB, with or without IL-2, and/or S28690 increased the expression of 4-1BB and PDL-1 but not to the same degree as CD80, CD86, CD54, and CD83. The numbers over the double-headed arrows are the $p$ values for the differences between sample means. *$P < 0.05$; **$P < 0.01$. 

A. 

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B. 

- CD80
- CD86
- CD83

C. 

- ICOSL
- 41BBL
- PDL1
as well as CD54. Remarkably, regardless of clinical stage or cytogenetic abnormalities (Table I), and in accordance with their strong T cell stimulatory ability, CLL cells treated with IL-2, S28690, and PDB acquired uniformly a CD83hiCD80hiCD86hiCD54hi cell surface phenotype (Figures 9A and 9B; horizontal bars).

**Cytokine production by CLL cells treated with S28690, IL-2, and PKC agonists**

The increased phosphorylation of STAT1 and STAT3 after 24 h in CLL cells treated with IL-2 and S28690 (Figure 6B) was consistent with autocrine production of cytokines. In addition, immunogenic cells promote activated T cell proliferation and differentiation by secreting cytokines and chemokines, as well as expressing costimulatory molecules (Pulendran, 2005). Accordingly, we measured the production of a number of cytokines and chemokines (relevant to induction of effective antitumor immunity; (Szabo et al., 2003) by CLL cells activated with various combinations of PDB, S28690, and IL-2 (Figure 10). CLL samples from 19 different patients (1, 9, 20, 23, 25, 26, 38, 67, 73, 75, 98, 99, 100, 101, 102, 103, 104, 105, 106), representing all clinical disease stages, were studied.

CLL cells treated with IL-2, alone, made very few cytokines or chemokines. As reported previously (Spaner et al., 2006), CLL cells invariably made TNF-α (Figure 10A, top left graph) and lesser amounts of IL-10 (Figure 10A, middle left graph) in response to S28690. In the presence of IL-2, S28690-induced TNF-α production was somewhat decreased. Remarkably, IL-10 production was increased significantly when CLL cells were treated with both IL-2 and S28690 (Figure 10A, middle left graph).

PKC agonists had dramatic effects on the production of these cytokines. Although a paucity of cytokines was made by CLL cells treated with PDB alone, TNF-α production was increased considerably after treatment with both PDB and S28690, and especially after PDB,
Figure 10. Effect of S28690, IL-2, and PDB on cytokine and chemokine production by CLL cells. (A) CLL cells from patients 9, 67, 73, and 104 (stage 0 disease), 1, 26, 75, 98, and 99 (stage I-II disease) and 20, 23, 25, 38, 100, 101, 102, 103, 105 and 106 (stage III-IV disease) were cultured alone or with IL-2, S28690, IL-2 and S28690, PDB, PDB and S28690, or PDB, IL-2 and S28690 for 48 h. Supernatants were then harvested for cytokine measurements as described in Materials and Methods. The results for TNF-α, IL-10, RANTES (CCL5), IL-6, GM-CSF, and IFN-γ are shown. Each line represents results for a different patient sample. Note that TNF-α production by stimulated CLL cells from patient 75 was off-scale and is not shown. RANTES levels were measured only from 13 patient samples. (B) The averages and standard errors of TNF-α (white square) and IL-10 (black square) produced by CLL cells alone, or treated with IL-2 and S28690, or PDB, IL-2 and S28690 are shown (top left). The averages and standard errors of TNF-α (top right), IL-10 (bottom left), and IL-6 (bottom right) production by stage 0 CLL cells (black square), stage I-II CLL cells (white square), stage III-IV CLL cells (diagonal lines square), and the combination of stage 0 and stage I-II CLL cells (horizontal lines square), after stimulation with S28690, IL-2, and S28690, and PDB, IL-2, and S28690, are shown. The numbers over the double-headed arrows are p values for the differences between sample means.
S28690, and IL-2 (Figure 10A, top left graph). At the same time, IL-10 production by CLL cells treated with S28690 and IL-2 was inhibited strongly by concomitant treatment with PDB. These changes in the balance of TNF-α and IL-10 production caused by PDB were highly statistically significant (Figure 10B, top left graph).

The effects of these immunomodulatory agents on the production of other chemokines and cytokines were more variable. Chemokines such as RANTES (CCL5) (Figure 10A, bottom left graph), MIP-1-α (CCL3), MIP-1-β (CCL4), and IFN-γ-inducible protein-10 (CXCL10) (data not shown) generally followed the pattern of TNF-α, and tended to be produced in greater amounts when CLL cells were treated with PDB, S28690, and IL-2, consistent with the increased ability of these cells to stimulate T cell proliferation (Figure 8). IL-6 was sometimes very high when CLL cells were treated with S28690 (with or without IL-2), but did not appear to be affected especially by concomitant stimulation with PDB (Figure 10A, top right graph). CLL cells have been reported to make Type I immune cytokine, IFN-γ (Battle and Frank, 2003). IFN-γ production was both uncommon and low in the CLL samples studied here, but tended to be increased by treatment with S28690, IL-2, and PDB (Figure 10A, bottom right graph). GM-CSF production was also infrequent, but tended to be increased more by CLL cells treated with IL-2 and S28690 (Figure 10A, middle right graph).

Although cytokine and chemokine production appeared to follow general patterns, the magnitude of production was quite variable. Such variability could be related potentially to biological differences between the tumor samples, reflected in the clinical stages of the patients. Using the Rai clinical staging system for CLL (Cheson et al., 1996), samples were grouped into stage 0 (which may never require treatment), stage I-II (which has a mean
survival of 7-10 years), and stage III-IV (which has a mean survival of <5 years) (see legend to Table I). For purposes of establishing statistical significance, stages 0.I and II (“low-risk” disease) were also compared with stages III and IV (“high-risk” disease).

On this basis, CLL cells from stage III and IV patients made significantly higher levels of immunosuppressive cytokine, IL-10, when stimulated with IL-2 and S28690 (Figure 10B, bottom left graph). Production of most other cytokines (e.g. IL-6 (Figure 10B, bottom right graph)) also tended to be higher when advanced stage CLL cells were treated with S28690 (with or without PDB or IL-2), and approached (but did not reach) statistical significance (i.e. \( p < 0.05 \)).

Interestingly, TNF-\( \alpha \) production did not seem to be affected as much by the clinical stage (Figure 10B, top right graph). Importantly, PDB was able to shut off the production of IL-10 (Figure 10B, bottom left graph), while increasing the production of TNF-\( \alpha \) (Figure 10B, top right graph), regardless of clinical stage.

**STAT-1 and STAT-3 activation in CLL cells treated with S28690, IL-2, and PKC agonists**

Because CLL cells treated with S28690, IL-2 and PDB appeared to become strong APCs, their expression of tyrosine-phosphorylated STAT-1 and -3 protein levels was measured (in view of the relationship of these signaling molecules with tumor immunogenicity (Cheng et al., 2003; Wang et al., 2004) (Figure 11). Consistent with previous results (Figure 6B), S28690 (with or without IL-2) increased activated STAT-1 and especially STAT-3 levels (lanes 3 and 7) (which was shown previously to be due to autocrine production of IL-6 and -10 (Spaner et al., 2006).
Figure 11. Effect of S28690, IL-2, and PDB on STAT-1 and STAT-3 activation in CLL cells. CLL cells were cultured for 24 h alone (lane 1), with PDB (lane 2), with S28690 (lane 3), with PDB and S28690 (lane 4), with IL-2 (lane 5), with PDB and IL-2 (lane 6), with IL-2 and S28690 (lane 7) and with PDB, IL-2, and S28690 (lane 8). Tyrosine-phosphorylated STAT-1 and STAT-3 levels were then determined by immunoblotting, as described in Materials and Methods. Similar results were obtained using three different patient samples.
PDB alone did not activate STAT-1 or STAT-3, and promoted STAT-1 activation by IL-2 (lanes 5 and 6). Remarkably, PDB inhibited strongly STAT-1 and STAT-3 activation by S28690 (lanes 3 and 4). STAT-1 activation (presumably by IL-2) was maintained in CLL cells treated with PDB, IL-2, and S28690. Therefore (in accordance with other features of strong immunogenicity), gene transcription regulated by STAT-1 appeared to be favored in CLL cells treated with IL-2, S28690, and PDB.
The results in this paper suggest that CLL cells treated with a TLR-7 agonist, IL-2, and a PKC agonist differentiate into cells that resemble DCs, with uniformly high expression of costimulatory molecules (Figure 9), and ability to stimulate T cell proliferation (Figure 8). Each of the individual immunomodulators appeared to affect specific aspects of costimulation. For example, IL-2 promoted STAT-1 activation (Figure 11). S28690 activated NF-κB, SAPK, and p38 signaling pathways (Figure 6A), and PKC agonists induced CD83 expression (Figure 9) and inhibited STAT-3 activation (Figure 11). However, all three agonists were required to cause CLL cells to undergo DC-like maturation.

IL-2 and S28690 initially provided only additive signaling to CLL cells (Figure 6), perhaps because of the locations of the respective receptors. The IL-2R is found on the plasma membrane while TLR-7 is in the endosomal compartment (Heil, 2003). However, IL-2 and S28690 signaling became more connected over time. TLR-7 activation caused increased expression of CD25, leading to enhanced signaling through the IL-2R (Figure 7). The results of these interactions between S28690 and IL-2 included activated STAT-1 and STAT-3 (Figure 6B), increased proliferation (Figure 4), increased costimulatory molecule expression (Figure 5) and increased cytokine production (Figure 10) by CLL cells.

IL-2 and S28690 had especially striking effects on IL-10 production (Figure 10A), particularly by more aggressive CLL cells from patients with advanced stage disease (Figure 10B). Although the mechanism is unclear (but probably reflects signaling aberrations caused by the cytogenetic abnormalities associated with CLL progression (Stevenson and Caligarisi-Cappio, 2004), this observation may have pathogenic implications, because IL-10 has immunosuppressive properties and promotes development of regulatory T cells that can
inhibit strong Type I responses, required for effective antitumor immunity (Spaner, 2004). It is possible that endogenous stimulation of CLL cells by IL-2 (or IL-2 family members (Spaner et al., 2004)) and endogenous TLR-7 agonists (such as oxidized guanosines (Heil et al., 2003) or single-stranded RNA (Heil et al., 2004)), produced in response to episodes of infection (both clinical and subclinical), may lead to enhanced production of IL-10 and contribute to the poor prognosis of patients with stage III and IV disease.

Despite these potent interactions between IL-2 and S28690, additional activation by PKC agonists was required to cause CLL cells to become highly immunogenic. The immunogenic importance of PKC (likely the PKCβ isozyme) has been documented previously (Hammond et al., 2005; Spaner et al., 2004; Spaner et al., 2006, Heil et al., 2003), although the mechanism is not entirely clear. PKC agonists increased CD83 expression (Figure 9), inhibited the proliferation of CLL cells treated with IL-2 and S28690 (Figure 4), “switched-off” IL-10 production (Figure 10) and significantly altered the relative amounts of phosphorylated STAT-1 and STAT-3 (Figure 11). This latter effect may be due to inhibitory phosphorylation of STAT-3-activating cytokine receptors by phorbol esters (Sengupta et al., 1998). Given the importance of STAT-3 as a negative regulator of DC and tumor cell immunogenicity (Cheng et al., 2003; Wang et al., 2004), we suggest that turning off STAT-3 activation was critical for making the CLL cells highly immunogenic.

Although the results in this paper describe a method to increase the immunogenicity of B cell tumors, they may have broader implications for human B cell immunology. The enhancement of IL-2 signaling by S28690 (a synthetic analog of the natural TLR-7 ligand, single stranded RNA (Spaner, 2004)) (Figure 7), may represent a model for the priming of an adaptive immune response to a systemic viral infection. The “one-way” nature of this
interaction (i.e. priming of IL-2 responses by S28690 but not of TLR-7 responses by IL-2) (Figure 7) may represent an immunological control mechanism to localize the effects of activated T cells (represented by IL-2) to sites of active viral infections (represented by treatment with S28690) and contrasts markedly with the ability of Type I interferons (which are cytokines of innate immunity) to increase TLR-7 expression (Heil et al., 2004). Nevertheless, B cells required concomitant stimulation with PKC agonists to become highly immunogenic. Because phorbol esters can mimic signaling through the B cell receptor (Guo et al., 2004), the requirement for simultaneous PKC signaling may represent another point of immunological control, which ensures that only antigen-activated B cells acquire strong immunogenicity in the presence of high levels of innate and adaptive immune stimulators to avoid nonspecific immune activity and the development of autoimmune diseases. We speculate further that IL-2 and TLR-7 signaling (in the absence of PKC activation) together cause strong production of IL-10 (Figure 10) as an additional mechanism to suppress nonspecific immunity.

Along with providing a potential model for understanding human tolerance and immunogenicity mechanisms, the results described in this paper may also aid in devising immunotherapeutic strategies for CLL. Despite the fact that CLL cells from different patients are heterogeneous (characterized by different cytogenetic abnormalities, mutation status of the rearranged immunoglobulin locus, or expression of CD38 and ZAP-70 (Stevenson and Caligaris-Cappio, 2004), and respond variably to S28690, phorbol esters, and IL-2 as single agents (for example, advanced stage CLL cells with high CD38 expression respond less well to IL-2 (Spaner et al., 2004) but more strongly to S28690 (Spaner et al., 2006) than early stage CLL cells with low CD38 expression), CLL cells uniformly became
highly immunogenic when treated with all three of these agents. The absence of significant heterogeneity in patient response, coupled with the ease, rapidity, and reproducibility of the method, suggest the use of imidazoquinolines, along with IL-2 and clinically relevant PKC agonists (such as Bryostatin-1 (Hammond et al., 2005)), to make DC-like CLL cells for autologous tumor vaccines in vitro, or (depending on clinical toxicity) to turn CLL cells into endogenous vaccines in vivo.
CHAPTER 3:

Aberrant Interferon-Signaling Is Associated With Aggressive Chronic Lymphocytic Leukemia

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3.1 ABSTRACT

The Type I Interferons (IFNs) normally suppress tumor growth by phosphorylating and activating the signal transducer and activator of transcription 1 (STAT1), but also briefly activate STAT3, which promotes cell growth. In Chronic Lymphocytic Leukemia (CLL) cells, the duration of IFN-mediated STAT3 phosphorylation was found to exhibit significant inter-patient variability and was prolonged in cells with high-risk features, such as 11q- and 17p- deletions involving ataxia telangiectasia mutated (ATM) and p53. This aberrant signaling pattern was associated with a paradoxical increase in cell size and number in response to IFN and similar alterations in IFN-signaling and responses were seen in cell-lines that developed in the absence of p53 or ATM. However, direct inhibition of p53 or ATM failed to cause these changes and CLL cells with aggressive clinical features were found to also express high levels of reactive oxygen species (ROS), which decrease tyrosine phosphatase activity. Prolonged IFN-mediated STAT3 phosphorylation and lowered phosphatase activity could be reversed by antioxidants. These findings suggest that increased ROS levels may corrupt IFN-signaling processes in aggressive CLL cells, causing IFN to be used as a growth factor rather than a tumor suppressor. Antioxidants or STAT3-kinase inhibitors might improve the outcome of IFN-therapy in CLL by restoring normal signaling.
3.2 INTRODUCTION

Chronic Lymphocytic Leukemia (CLL) is often benign but aggressive forms of the disease are lethal and generally incurable with conventional therapy (Caligaris-Cappio and Ghia, 2008). Like other cancers, aggressive CLL cells develop adaptive mechanisms which allow them to resist immuno- and chemotherapies (Spaner, 2004) and continue to grow despite such obstacles as hypoxia, poor vasculature, and natural host defenses including tumor-reactive T cells (Blagosklonny, 2002). A greater understanding of how CLL cells survive in these conditions is needed to improve treatment outcomes.

The Type I Interferons (IFNs) are natural tumor suppressor molecules that act by inhibiting cell proliferation and increasing the susceptibility of cancer cells to cytotoxic immune effectors (Dunn et al., 2006; Takaoka et al., 2003). Exogenous IFN also has therapeutic activity in CLL, particularly in patients with low-risk disease (Langenmayer et al., 1996). However, IFN is generally inactive in more aggressive disease, and may even accelerate tumor growth (Foon et al., 1985), suggesting that IFN-signaling may be altered in tumor cells from such patients.

Upon activation of the IFN-α/β receptor (IFNAR), signaling processes are initiated that lead to phosphorylation and activation of STAT1, via the Janus Kinase, JAK1, and a more transient phosphorylation and activation of STAT3, mainly via TYK2 (Fig.1A) (Su and David, 2000). STAT1 is a transcription factor with properties of a tumor suppressor while STAT3 is oncogenic and promotes cell growth and resistance to pro-apoptotic stimuli (Takaoka et al., 2003; Regis et al., 2008). Variations in the magnitude and timing of STAT1 and STAT3 activation (Regis et al., 2008) might then affect the responses of tumor cells to
IFN (Humpolikova-Adamkova et al., 2009). Our studies were designed to investigate IFN-signaling in leukemia cells from patients on different parts of the clinical CLL spectrum.
3.3 MATERIALS AND METHODS

Blood samples

Heparinized blood was obtained from healthy volunteers and consenting CLL patients (diagnosed by a persistent monoclonal expansion of CD19\(^+\)CD5\(^+\)IgM\(^lo\) lymphocytes (Hallek et al., 2008)). Patients were untreated for at least 3 months at the time of analysis. Protocols were approved by the Sunnybrook Ethics Review Board. Clinical characteristics of the patients are described in Tables 2 and 3.

Peripheral blood mononuclear cells (PBMCs), normal B cells, and CLL cells were isolated as previously described by negative selection with the Rosette Sep Human B cell enrichment cocktail (StemCell Technologies, Vancouver, BC) and density centrifugation with Ficoll-Paque (Amersham Pharmacia Biotech AB, Uppsala, Sweden) (Gitelson et al., 2003). This method of purification yields percentages of CD19\(^+\) and CD19\(^+\)/CD5\(^+\) cells of >98 and 96%, respectively (Gitelson et al., 2003).

Antibodies and Reagents

The 7-aminoactinomycin D (7AAD) was from Pharmingen (San Francisco, CA). Phorbol dibutyrate (PDB) was from Sigma Chemical Co. (St. Louis, MO) and stock solutions (5 mg/ml) were made in DMSO. Cycloheximide (CHX), N-acetyl-cysteine (NAC), and \(\beta\)-actin antibodies were also from Sigma. Clinical grade IFN-\(\alpha\)2b (Schering Canada Inc., Pointe-Claire, QC) was purchased from the hospital pharmacy. Go6976 (classical PKC isozyme inhibitor), KU-55933 (ATM inhibitor) (Hickson et al., 2004), AG9, and Piceatannol (TYK2 inhibitors) were from Calbiochem (San Diego, CA), and stock solutions made in DMSO. Pifithrin-\(\alpha\) (p53 inhibitor) and Nutlin-3 (p53 activator)
Table II: Clinical properties of CLL patients

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1. Corresponding patient numbers are maintained throughout.
2. Rai stage 0=lymphocytosis; 1= with adenopathy; 2= with hepatosplenomegaly; 3= with anemia; 4= with thrombocytopenia.
3. C=Chlorambucil, P=Prednisone, F=Fludarabine, S=Splenectomy, Rads=local radiation, R=Rituxan, CHOP=cyclophosphamide/vincristine/adriamycin/prednisone, FC=Fludarabine/cyclophosphamide, CVP=cyclophosphamide/vincristine/prednisone; BMT=allogeneic bone marrow transplant; Rev=revlimid
4. In the case of multiple cytogenetic lesions, the higher-risk one was used (eg. 11q- or 17p-)
NA=not available
Table III: Clinical properties of patients in whom tumor ROS levels were measured

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1. Normal range is 0.6-2.3 mg/ml; 2. Lymphocyte doubling time; 3. Splenectomy, conventional chemotherapeutic regimens, such as FC, and steroid-based regimens are each considered as a treatment. 4. MFI of DCFH staining
were from Cayman Chemical Co. (Ann Arbor, MI). Antibodies to p53, p21, STAT1, STAT3, phospho-(Tyr\textsuperscript{701})STAT1, phospho-(Tyr\textsuperscript{705})STAT3, JAK1, and TYK2 were from Cell Signaling Technology (Beverly, MA). TK6 and NH32 cells were from H. Liber and colleagues (Chuang \textit{et al.}, 1999) and BL41tsp53 cells were from N. Nagy and colleagues (Ramqvist \textit{et al.}, 1993). Recombinant murine IFN-β was synthesized as described previously (Gill \textit{et al.}, 2006). The p53-/-, ATM-/-, and control mice on the C57BL/6j background were obtained from Jackson Laboratories (Bar Harbor, Me, stock numbers 002101, 008536, and 000664, respectively) and used at 6 weeks of age.

**Cell culture and activation**

Purified CLL cells (1.5x10\textsuperscript{6} cells/ml) were cultured in serum-free AIM-V medium (GibcoBRL, Grand Island, NY) plus 2-mercaptoethanol (2-ME) (Sigma) (5x10\textsuperscript{-5} M final concentration) in 6- or 24-well plates (Becton-Dickinson Labware, Franklin Lake, NJ) for 3-4 days at 37°C in 5% CO\textsubscript{2}. TK6, NH32, and BL41 temperature sensitive (ts) p53 cell lines were cultured in RPMI 1640 media (Invitrogen) + 5% FBS (Wisent Inc.) at 37°C in 5% CO\textsubscript{2}. BL41ts53 cells were cultured at 32°C in 5% CO\textsubscript{2} to induce p53 activity. Lymphocytes from spleens and lymph nodes of wt p53, wt ATM, p53-/-, and ATM-/- mice were cultured in RPMI 1640 + 5% FBS for the times indicated in the figure legend. Human IFN-α2b and murine IFN-β were used at 1000 U/ml. In preliminary experiments this dose of IFN was found to be above the amount that caused maximal phosphorylation of STAT proteins, suggesting that plasma membrane IFNARs had been saturated. PDB was used at 100 ng/ml as before (Tomic \textit{et al.}, 2006). Signaling inhibitors (i.e. Go6976, NAC, AG9, and Piceatannol) were added at the concentrations indicated in the figure legends.
The ATM+/+ and -/- fibroblast lines, GM16666 and GM16667, were grown at 37°C in 5% CO₂ in Dulbecco’s modified Eagle medium (Invitrogen) supplemented with 10% FBS and 100 µg/ml hygromycin (Invitrogen) to maintain transgene expression.

**Cell staining**

Fibroblasts were visualized with a Diff-quick staining kit (Baxter, McGaw Park, IL), which yields a modified Romanowski stain, according to the manufacturer’s instructions.

**Mixed Lymphocyte Responses**

T cells were isolated from normal donors and adjusted to 5x10⁵ cells/ml in AIM-V medium. Activated CLL cells were washed at least 4 times (to remove residual immunomodulators), irradiated (2500 cGy) and suspended at 5x10⁵ cells/ml (or lower concentrations) in AIM-V. Responders and stimulators were then mixed in a 1:1 (vol:vol) ratio and cultured in 96-well round bottom plates (Becton Dickinson Labware) without additional cytokines or serum. Proliferation was measured 4-6 days later with a colorimetric assay (Tomic *et al.*, 2006).

**Flow cytometry**

Cell were stained with 7AAD and analyzed as described previously (Shi *et al.*, 2007). DNA content was also measured as before.¹⁷ Briefly, CLL cells (~2 x 10⁶/ml) were transferred to conical tubes, pelleted and re-suspended in 50 µl PBS/HBSS with 2% FBS, and then fixed in 1 ml of ice cold 80% ethanol. Cells were pelleted again and re-suspended in 500 µl of 0.1 mg/ml Propidium Iodide (Sigma) with 0.6% NP-60 to which 500 µl of 2 mg/ml RNAse was then added. After incubation for 30 min at room temperature (RT), the cells were filtered through nylon mesh and kept on ice in the dark before analysis on a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ) using CellQuest flow.
Western blots

Proteins were extracted from activated CLL cells, cell lines and mouse lymphocytes using RIPA buffer plus protease and phosphatase inhibitors. Immunoblotting was performed as described before (Tomic et al., 2006). Blots were stripped for 60 min at 37°C in Restore Western Blot Stripping Buffer (Pierce, Rockford, IL), washed twice in TBS-T (Tris-buffered saline plus 0.05% Tween-20) at RT, blocked with 10% milk for 1 h, and then re-probed, as required. Signals were detected with Supersignal horseradish peroxidase enhanced chemiluminescence reagent (Pierce, Rockford, IL) and blots were exposed to Kodak Biomax MR film.

Determination of relative pSTAT3 levels

The intensities of pSTAT3 and total STAT3 bands on a blot were quantified with GeneTools Analysis Software version 4.00 from Syngene Genius 2 Bioimager (Syngene, Frederick, MD, USA). To control for experimental variations that arise when comparing multiple samples on different blots, these numbers were normalized to the results obtained with 50 µg of a reference standard from Patient 92, which was always run with each gel. To further control for experimental variation, two separate gels were run. The average of the results from the two gels was called the “relative pSTAT3 level at 4h”.

Para-nitrophenyl phosphate (pNPP) assay for phosphatase activity

CLL cells were lysed in a RIPA buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.25% Na-deoxycholate, 1mM EDTA, 1mM PMSF, aprotinin and leupeptin, each at 1 mg/ml. Phosphatase activity in each lysate was assayed in 96-well plates (Becton-Dickinson Labware, Franklin Lake, NJ) in a buffer of 50 mM Tris, 100 mM NaCl and 1 mM EDTA, and 10 mM DTT (pH 7.2) using pNPP (10 mg/ml) (Sigma), which is a
chromogenic substrate for most phosphatases. The reaction yields para-nitrophenol, which becomes an intense yellow soluble product under alkaline conditions measurable at 405 nm on a spectrophotometric plate reader (Model 3550, BioRad Laboratories, Hercules, CA).

**Measurement of intracellular reactive oxygen species (ROS)**

Formation of ROS was indicated by 2’7’-Dichlorofluorescin diacetate (DCFH$_2$-DA) (Molecular Probes, Eugene, OR). Intracellular esterases cleave the acetyl groups from the molecule to produce non-fluorescent 2’7’-dichlorofluorescein (DCFH$_2$), which is trapped inside the cell. In the presence of ROS, DCFH$_2$ is oxidized to DCF, which emits fluorescence at 530 nm, after excitation at 488 nm. Activated CLL cells were incubated with 10 µM DCFH$_2$-DA at 37°C for 30 min. Samples were then washed in PBS and analyzed on a FACScaliber flow cytometer (Becton Dickinson). DCFH$_2$ oxidation was measured as “green” (FL1) fluorescence on a log scale for 10 000 events.

To measure ROS production in circulating CLL cells, the cells were first purified from the blood and then cultured in serum-free media for 2-3 days. This time was chosen on the basis of preliminary experiments showing that DCFH-staining and cell viability decreased in normal B cells after day 5 in these conditions but was relatively similar immediately after isolation or 1 and 3 days of culture.

**Cytokine measurement**

Cytokines in culture supernatants were measured in a Luminex-100™ system (Luminex Corp., Austin, Texas) as before (Tomic *et al.*, 2006). A kit for human IL-6, IL-8, IL-10, and TNF-α was used, according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN). Individual cytokine concentrations were determined from standard
curves using Bio-Plex 2.0™ software (BioRad, Mississauga, ON). The assay was linear between 30 and 1000 pg/ml for each cytokine.

**Statistical Analysis**

The Student $t$ test was used to determine $p$ values for differences between sample means.
3.4 RESULTS

Variable IFN-mediated STAT3-phosphorylation and functional outcomes in CLL cells

IFN-signaling in purified, circulating CLL cells was assessed by serial measurements of pSTAT1 and pSTAT3 proteins. Both STAT1 and STAT3 became phosphorylated within minutes of stimulation with IFN-α2b and pSTAT1 levels remained high for many hours. However, the decay of IFN-induced STAT3 phosphorylation exhibited significant inter-patient variation. For example, pSTAT3 essentially disappeared after 4 h in CLL cells from patient 31 whereas high levels persisted for up to 18 h in cells from patient 92 (Figure 12B).

This behavior of pSTAT3 appeared to be linked to defects in the p53 axis which are associated with clinically aggressive disease (Pettitt et al., 2001). In Figure 12B, the sample in which IFN-induced pSTAT3 levels were maintained had defective p53 function, in that p21 was not transcribed when the cells were radiated (Pettitt et al., 2001) (Pt. 92). In the sample where pSTAT3 disappeared within 4 h of stimulation (Pt.31), the p53 axis was intact by these criteria. Moreover, IFN induced different behaviors in CLL cells depending on the duration of STAT3-phosphorylation. If IFN-induced STAT3-phosphorylation persisted longer than 4 h (Figure 12E, bottom panels), the tumor cells became larger (Figure 12C, bottom panels), entered cell cycle (Figure 12D, bottom panels), and increased in number almost 2-fold within 4 days (Figure 12F). In contrast, cells in which IFN-mediated STAT3-phosphorylation disappeared within 4 h did not enlarge and there was no increase in DNA content (Figures 12C, 12D, 12E, top panels; 12F).
Figure 12. Variations in IFN-mediated STAT3 phosphorylation and proliferation in CLL cells. (A) Schematic diagram of IFN-signaling. (B) Primary CLL cells were treated with IFN-α2b (1000 U/ml) for the indicated times and whole cell lysates were immunoblotted with antibodies to phospho-(Tyr701)-specific STAT1 or phospho-(Tyr705)-specific STAT3, as well as total STAT1, STAT3, and β-actin, as loading controls. Cell aliquots were also exposed to ionizing radiation (2.5 Gy) and p53 and p21 levels were determined by immunoblotting 18 h later (lower panels). Tumor cells from patients 31 and 92 had intact and absent p53 axes, respectively, according to this assay. (C) Tumor cells from 2 other patients (patients 81, bottom panels and 44, top panels) were cultured for 4 days in the presence or absence of IFN. Cell size was determined by the forward scatter parameter of flow cytometry. (D) DNA content was also determined by flow cytometry at this time. The numbers on the right of the histograms represent the percentage of cells with DNA content greater than 2N and the numbers on the left represent the percentage with sub-diploid DNA content. (E) Immunoblots showing measurable pSTAT3 levels at 4 h in CLL cells from patient 81 but not patient 44. (F) Viable cells were counted in a hemocytometer after 4 days of culture. The results show that IFN induced a proliferative response when STAT3-phosphorylation persisted longer than 4 h.
Increased viable cell numbers could be caused by effects of IFN on proliferation or on survival (or both). However, because these experiments were carried out in serum-free media, little death occurred in control cells from the vast majority of patient samples during the culture period (Hammond et al., 2005). Treatment with IFN did not increase the number of apoptotic cells (indicated by the sub-diploid peak of cell cycle analysis (Spaner et al., 1998) (Figure 12D and Supplementary Figure 21) or necrotic cells (indicated by staining with the DNA dye, 7AAD (see Figure 18E for an example). We conclude that the increase in cell numbers associated with prolonged IFN-mediated STAT3 phosphorylation was due mainly to the increase (albeit small) in the number of cycling cells (Figure 12D). Accordingly, the phenotype of CLL cells which exhibited prolonged increases in pSTAT3 levels following treatment with IFN was characterized by an increase in cell size and proliferation, both of which are consistent with the known oncogene-like functions of STAT3 (Regis et al., 2008; Humpolikova-Adamkova et al., 2009).

The five sub-types of CLL are distinguished by fluorescence in situ hybridization (FISH) including those with no detectable cytogenetic lesions, trisomy 12, or deletions at 13q14, 11q22-q23 (with loss of the ataxia telangiectasia mutated (ATM) gene) and 17p13 (with loss of p53) (Dohner et al., 2000). To more fully explore the relationship of the temporal characteristics of IFN-mediated STAT3-phosphorylation with underlying cytogenetic abnormalities, pSTAT3 and total STAT3 levels were measured in 47 different CLL samples following stimulation with IFN-α2b for 4 h. This time was based on the initial analysis of the disappearance of IFN-mediated pSTAT3 proteins in individual patient samples. The amount of pSTAT3 relative to STAT3 at this time was then related to the most severe cytogenetic abnormality determined by FISH (eg. patients with both 13q- and 17p-
deletions were classed as 17p-). As shown in Figure 13A, IFN-induced pSTAT3 levels were higher in CLL cells with 11q- or 17p- deletions compared to samples with low-risk cytogenetic abnormalities and were not related to altered expression of IFN-signaling pathway components (Figure 12A and data not shown). This larger study confirmed that CLL cells which exhibited prolonged STAT3 phosphorylation (defined as a densitometric ratio of pSTAT3 to STAT3 greater than 2 after 4 h of stimulation with IFN) increased in number, whereas cells with a shorter duration of STAT3 phosphorylation (pSTAT3/STAT3 < 2 at 4h) did not show an increase (Figure 13B).

**Effect of p53 loss on IFN-mediated pSTAT3 levels in B lymphoma cells**

These results with primary CLL cells (Figures 12 and 13) suggested that adverse oncogenic lesions were associated with prolonged IFN-mediated STAT3 phosphorylation, leading to paradoxical use of IFN-α2b as a growth factor rather than a cytostatic molecule. To study this in more detail, we focused on p53 since 17p- deletions are associated with the most aggressive forms of CLL (Zenz et al., 2009). As a model, IFN-responses were studied in an Epstein-Barr Virus (EBV) transformed lymphoblastoid B cell line (LCL) with wild-type p53 (TK6) and a double p53 knockout cell line derived from TK6 using a promoterless gene targeting approach (Chuang et al., 1999). Because EBV proteins activate similar signaling pathways as inflammatory mediators encountered by CLL cells (Thorley-Lawson, 2001) in proliferation centers (Caligaris-Cappio and Ghia, 2008), we reasoned that these two cell-lines might provide insights into how signaling and responsiveness of malignant B cells to IFN are affected by loss of p53.
Figure 13. Correlation of IFN-mediated pSTAT3 duration and proliferation with cytogenetic lesions. (A) Circulating CLL cells from 47 consecutive patients presenting to the CLL clinic at Sunnybrook Odette Cancer Centre (described in Table 2) were isolated and stimulated with IFN-α2b. After 4 h, levels of pSTAT3 relative to total STAT3 were determined by immunoblotting and densitometry as described in the materials and methods. This number was then plotted as a function of the highest-risk cytogenetic abnormality. The median value for each subgroup is shown. *p<.001) (B) Cell cultures were continued for another 4 days. Viable cells were then counted in a hemocytometer and the ratio of the numbers obtained with and without IFN-α2b was plotted against the pSTAT3/pSTAT1 ratio at 4 h. Median values for the two groups are indicated. *p<.01).
The status of p53 in the cell lines was confirmed by the increased expression of p53 and p21 in irradiated TK6 cells and their absence in irradiated NH32 cells (Figure 14A, top panels) (Pettitt et al., 2001). IFN-α2b induced phosphorylation of STAT1 and STAT3 within 1 hour in both TK6 and NH32 cells. However, consistent with the findings in primary CLL cells, pSTAT3 levels remained high for more than 7 hours following stimulation with IFN-α2b in NH32 cells which lacked functional p53 while these levels decayed much more rapidly in TK6 cells (Figure 14A, middle and bottom panels). Similarly, treatment with IFN-α2b produced higher numbers of NH32 cells after 4 days of culture whereas TK6 numbers were significantly lower (Figure 14B).

Production of a number of cytokines and chemokines, including IL-6 and IL-10 which are regulated by JAK-STAT signaling modules (Shuai and Liu, 2003), was measured to determine if the altered kinetics of pSTAT3 reflected enhanced STAT3 activity. NH32 cells constitutively produced IL-10, which was greatly enhanced by IFN-α2b. Low level production of IL-6 was also increased somewhat by IFN-α2b in NH32 cells while TNF-α and IL-8 production were not affected. In contrast, IL-10 and IL-6 production by TK6 cells were not changed by IFN-α2b while IL-8 was only modestly increased (Figure 13C).

NH32 is derived from TK6 but the two cell-lines have been subjected to the stresses of tissue culture (Shay and Wright, 2007) for many generations and may have evolved different genetic and epigenetic alterations. Accordingly, an aberrant pathway other than p53 could account for prolonged IFN-mediated STAT3 phosphorylation in NH32 cells. Consistent with this possibility, IFN-signaling patterns were not different in spleen cells from p53+/+ and p53−/− mice (Supplementary Figure 19B) or at permissive
Figure 14. Increased IFN-mediated STAT3-phosphorylation and proliferation in p53/- B cell lines. (A) The p53 axes of TK6 (left panels) and NH32 cells (right panels) were assessed by measuring p53 and p21 levels 18 h after irradiation. Other cells were treated with IFN-α2b (1000 U/ml) for the indicated times and whole cell lysates were then immunoblotted with phospho-(Tyr701)-specific STAT1 or phospho-(Tyr705)-specific STAT3 antibodies. The pSTAT3 level relative to pSTAT1 was quantified by densitometry and shown below the blot. (B) TK6 and NH32 (5x10^4 cells/ml) were cultured with or without IFN-α (1000 units/ml) for 48h and then counted manually in a hemocytometer. The average and standard error of 3 separate measurements are shown. (C) Supernatants were also collected after 48 h and the concentrations of IL-6, IL-10, IL-8 and TNF-α were determined as described in the Materials and Methods.
and non-permissive temperatures for functional p53 activity in human B cells expressing a temperature-sensitive p53 mutant (Ramqvist et al., 1993) (Supplementary Figure 19A). Similarly, IFN-signaling in CLL cells was not altered by the p53 inhibitor, pifithrin (Supplementary Figure 20) (Komarov et al., 1999).

**Effect of ATM loss on IFN-mediated pSTAT3 levels**

The results in Figure 13 suggested that 11q deletions and loss of \( ATM \) were also associated with prolonged IFN-mediated phosphorylation of STAT3. Accordingly, IFN-signaling was studied in the GM16666 and GM16667 cell lines which are derived from fibroblasts of a patient with Ataxia-telangiectasia (caused by a mutant \( ATM \) gene) transfected with either an ATM expression construct (GM16667) or an empty vector (GM16666) (Ziv et al., 1997). Treatment of GM16667 cells (that express wild-type \( ATM \)) with IFN-\( \alpha \)2b caused only a brief phosphorylation of STAT3 (Figure 15A) and strongly inhibited cell growth (Figures 15B and 15C). Remarkably, STAT3 phosphorylation was prolonged in ATM-deficient GM16666 cells (Figure 15A), which grew in response to IFN (Figures 15B and C).

However, analogous to the situation with p53, IFN-signaling was similar in spleens and thymuses of ATM\(+/+\) and ATM\(-/-\) mice (Supplementary Figure 19B, right panels) and treatment of CLL cells with the ATM inhibitor, KU-55933, did not alter IFN-signaling responses (Supplementary Figure 20). Taken together, these results suggested that loss of p53 and ATM were necessary but not sufficient to corrupt IFN-signaling patterns in CLL cells.
Figure 15. Increased IFN-mediated STAT3-phosphorylation and proliferation in ATM-/- cell lines. (A) GM16667 (wild-type ATM) and GM16666 (absent ATM) cells in exponential growth phase were treated with IFN-α2b (1000 units/ml) for the indicated times and whole cell lysates were then immunoblotted with antibodies to phospho-(Tyr701)-STAT1, phospho-(Tyr705)-STAT3, total STAT1 and STAT3, or β-actin. (B) After 3 days of culture, the cells were stained and pictures were taken at a magnification of x10. Pictures were taken with a Canon PowerShot G11 Digital Camera equipped with a Carl Zeiss 426126 lens. The microscope was an Axiovert 40 C equipped with an A-plan 10x/0.25 objective, both from Zeiss. (C) The cells were also counted manually in a hemocytometer. The average and standard error of 3 separate measurements are shown and the experiment was performed twice with similar results. *p<.01; **p<.05
Effect of phorbol esters on IFN-signaling in CLL cells

The association of aberrant IFN-signaling with high-risk cytogenetic lesions that could not be reproduced by inhibitors of p53 or ATM raised the possibility that other aspects of the cellular phenotype of aggressive tumors are associated with prolonged IFN-mediated STAT3 phosphorylation. Increased ROS levels are associated with CLL cells that are sufficiently aggressive to have warranted chemotherapy (Zhou et al., 2003). Other features of high-risk CLL cells include activation of protein kinase C (PKC) (Holler et al., 2009; Barragan et al., 2002) and enhanced signaling through immunoreceptors (Soma et al., 2006). Since phorbol esters activate PKC, increase ROS and CD38 expression (Hammond et al., 2009), and promote immunoreceptor-signaling (Soma et al., 2006), exposing CLL cells to these tumor-promoters may model some of the behavioral aspects of aggressive tumor cells.

Similar to CLL cells with high-risk cytogenetic lesions, IFN-mediated STAT3 phosphorylation was prolonged by concomitant activation with phorbol esters (Figure 16A). This result was not an indirect effect of cytokines induced by IFN as it was still observed in the presence of the protein translation inhibitor, cycloheximide (Figure 16B). Phorbol esters increase ROS levels partly through classical PKC isozymes (Korchak et al., 1998) and both the antioxidant, N-acetylcysteine (NAC), and the PKC inhibitor, Go6976, shortened the duration of IFN-mediated STAT3 phosphorylation but did not otherwise affect pSTAT1 levels (Figures 16C and 16D).

Tyrosine phosphatases are inhibited by oxidation (Chiarugi et al., 2005), which could account for prolonged IFN-mediated STAT3-phosphorylation and its normalization.
Figure 16. Effect of phorbol esters on IFN-signaling in CLL cells. (A) CLL cells were cultured for 24 h with or without PDB (100 ng/ml) and/or IFN-α2b (1000 U/ml). pSTAT1, pSTAT3, and total STAT1 and STAT3 levels were then determined by immunoblotting. Immunoblots were stripped and re-probed with β-actin antibodies to assess loading. The results were similar for 21 additional samples. (B) CLL cells were cultured alone or with PDB for 48 h (to allow abatement of cytokine production caused by PDB alone), washed and then re-cultured with IFN in the presence and absence of cycloheximide (CHX) (10 µg/ml) for 6 h before determining pSTAT1 and pSTAT3 levels. Phosphorylated STAT3 levels were higher in cells treated with PDB and IFN than in cells treated with IFN alone and were not affected by the presence of CHX to block autocrine cytokine production. Similar results were obtained with 4 additional patient samples. (C, D) CLL cells were cultured alone or with PDB, IFN, or PDB and IFN with or without the classical PKC isozyme inhibitor, Go6976 (1 μM) (D), or the anti-oxidant, N-acetylcysteine (NAC) (30 μM) (C) added 1h before stimulation. Cell lysates were collected 4 h later and pSTAT1 and pSTAT3 levels determined by immunoblotting. Go6976 and NAC shortened STAT3 phosphorylation in CLL cells treated with PDB and IFN. This experiment was repeated with 8 different patient samples with similar results. (E) After 24 h of culture, global phosphatase levels were determined using pNPP as described in the materials and methods. This experiment was repeated with 8 different CLL patient samples and showed that global phosphatase activity was decreased in PDB-treated CLL cells, which could be reversed by blocking PKC and ROS. *p<0.05.
by antioxidants. Consistent with this, total phosphatase activity was diminished in phorbol ester-activated CLL cells and restored in the presence of NAC or Go6976 (Figure 16E). However, we were unable to implicate inactivation of specific tyrosine phosphatases (such as CD45 and PTP1b (Figure 12A)) that are known to regulate aspects of IFN receptor signaling (Shuai and Liu, 2003).

**ROS levels in CLL cells**

The association of prolonged IFN-mediated STAT3-phosphorylation with ROS in phorbol ester-activated CLL cells (Figure 16) suggested that higher ROS levels might be found in aggressive CLL cells which exhibit similar changes in IFN-signaling (Figure 13). Accordingly, circulating CLL cells from an additional 32 patients (Table 3) were purified and stained with DCFH after a brief period of culture in serum-free conditions. ROS levels in CLL cells were found to be much higher than in normal B cells (Figure 17A) and exhibited significant inter-patient variability. Remarkably, the pattern of DCFH staining mirrored the relative 4 h pSTAT3 levels observed in the different cytogenetic sub-types of CLL (Figures 13A and 17A). ROS levels were significantly higher in CLL cells with 11q or 17p deletions, compared to the other sub-types. Consistent with an association of increased ROS levels with more aggressive disease, DCFH-staining was also significantly higher in tumor cells with higher CD38 expression (Figure 17C). Lymphocyte doubling times (LDTs) were significantly faster (<12 months) in patients whose tumor cells had high ROS levels (Figure 17B). These patients also had a higher Rai Stage classification (Figure 17D) and were much more likely to require treatment for their disease (Figure 17E).
Figure 17. Correlation of intracellular ROS levels with clinical parameters. The mean fluorescence intensity (MFI) of DCFH staining (indicative of ROS levels) in short-term cultured CLL cells was measured as described in the materials and methods for 33 patients whose clinical features are shown in Table 3. Cytogenetic abnormalities (A), lymphocyte doubling times (LDTs) (B), percentage of CD38+ cells (C), clinical Rai stage (D), and number of treatments for symptomatic disease (E) were determined from the patients’ medical records. The average values and standard errors for the different groups are shown. Tumor cells from patients with more aggressive disease had significantly higher ROS levels, as indicated by the p-values in the respective panels.*p<.001; **p<.02. The open circles in (A) represent ROS levels in B cells from 4 normal donors, handled in the same manner as CLL cells.
Normalization of IFN-signaling and responses by TYK2 inhibition

Because prolonged IFN-mediated STAT3 phosphorylation was associated with cell growth (Figures 12D, 12F and 13B) and production of immunosuppressive cytokines (Figure 14C), a clinically relevant method to inhibit IFN-mediated phosphorylation of STAT3, while preserving STAT1 phosphorylation, might improve the therapeutic effects of IFN-α2b in high-risk CLL patients. Current models of IFNAR signaling suggest that STAT1 is phosphorylated by JAK1, whereas STAT3 is phosphorylated by TYK2 (Figure 12A) (Su and David, 2000). Accordingly the effect on IFN-responses of AG9, a tyrphostin which inhibits IL-2-stimulated TYK2 phosphorylation in T cells (Bright et al., 1999), was studied in CLL cells treated concomitantly with phorbol esters to mimic features of high-risk disease (Figure 16).

The levels of phosphorylated STAT3 normally seen after 5 hours of treatment with PDB and IFN were abrogated by AG9 in a dose-dependent manner, with maximal inhibition achieved at 300 µM (Figure 18A, compare lanes 4, 7, and 8). Consistent with direct inhibition of TYK2, early (i.e. within 10 minutes) IFN-mediated STAT3-phosphorylation was also prevented by AG9 (data not shown).

Treatment of CLL cells with AG9 decreased production of IL-6 in response to PDB and IFN, as well to IFN alone (Figure 18B, top panel) whereas IL-8 levels remained the same or were increased (Figure 18B, bottom panel). Changes in IL-10 production tended to follow the pattern of IL-6 (data not shown).

The Type I IFNs exert their normal tumor-suppressor functions in part by altering the expression of cytokines and co-stimulatory molecules in order to increase the
Figure 18. Effects of a TYK2 inhibitor on IFN-signaling and responses. (A) CLL cells were cultured for 1 h alone, or with different concentrations of AG9, before treatment with PDB (100 ng/ml), IFN-α2b (1000 U/ml), or PDB and IFN-α. After 4 h, phospho- and total STAT1 and STAT3 levels were determined by immunoblotting. STAT3 phosphorylation was abrogated by AG9, while STAT1 activation remained intact. Similar results were obtained with 5 different patient samples. (B) Concentrations of IL-6 (top panel) and IL-8 (bottom panel) were measured in culture supernatants after 48 h. IL-6 production was increased by PDB and inhibited by AG9 without significantly affecting IL-8. (C) CLL cells cultured for 3-4 days with or without PDB and/or IFN in the presence or absence of AG9 were obtained from 5 patients and used to stimulate T cells from healthy donors to proliferate as measured 5-6 days later by a colorimetric assay. Averages and standard errors from triplicate MLR cultures (after subtracting the averages from control wells containing T cells and stimulators, alone) are shown. The different symbols represent the results obtained using individual patient stimulator cells. Prevention of IFN-mediated STAT3 phosphorylation by AG9 made CLL cells, treated concomitantly with PDB and IFN, better able to stimulate T cell proliferation. (D) Percentages of viable cells that excluded 7AAD were determined by flow cytometry after 48 h. The averages and standard errors of the results from 5 different patient samples are shown. Arrow indicates a statistically significant p value of p<0.05. (E) (top panel) CLL cells from patient 304 were cultured with or without IFN and/or AG9 and cell viability after 3 days was indicated by measuring exclusion of 7-AAD by flow cytometry. The numbers in the dot-blots are the percentages of 7-AAD+ dead cells. Means of the forward scatter parameter for 10 other patient samples are summarized in the bottom graphs. ‘High-risk’ cells are defined on the basis of 11q- or 17p- cytogenetic lesions, >20% CD38+ cells, or LDTs<12 months. AG9 counters the increased cell size caused by IFN in aggressive CLL cells.
immunogenicity of tumor cells, allowing them to effectively stimulate and be killed by tumor-reactive T cells (Spaner, 2004; Dunn, 2006). The capacity of CLL cells to stimulate T cell proliferation, as measured in allogeneic mixed lymphocyte reactions (MLRs), is poor but can be improved by phorbol esters (Hammond et al., 2005). IFN did not change significantly the stimulatory abilities of PDB-activated CLL cells (Figure 18C). However, blockade of STAT3 activation by AG9 caused CLL cells treated with PDB and IFN to acquire strong stimulatory ability (Figure 18C). Similar results were obtained with another putative TYK 2 inhibitor, Piceatannol (Su and David, 2000) (data not shown).

Changing the balance of IFN-mediated gene transcription to favor STAT1 over STAT3 responses might be expected to cause cell death (Regis et al., 2008; Humpolikova-Adamkova et al., 2009). Consistent with this, significantly more cells died after 24 hours of treatment with PDB and IFN in the presence of AG9 (Figure 18D). Because phorbol ester-activated CLL cells exhibit properties associated with aggressive CLL cells (i.e. increased ROS, prolonged IFN-mediated STAT3-phosphorylation), these findings suggested that AG9 might also allow IFN to kill aggressive CLL cells directly. Accordingly, CLL cells from 3 low- and 8 high-risk patients (defined by 11q or 17p deletions, high CD38 expression, or short LDTs) were stimulated with IFN, with or without AG9, for 4 days. Cell death was then measured by uptake of the DNA dye, 7-AAD and size was measured by the forward scatter parameter of flow cytometry. The increase in size associated with prolonged IFN-mediated STAT3-phosphorylation in high-risk CLL cells (Figure 12C) was prevented by AG9 (Figure 18E, bottom right panel). As expected (Figure 12C), this IFN-mediated size increase was not seen in samples from low-risk patients (Figure 18E, bottom left panel). Unlike phorbol ester-activated cells, in only one case (Patient 304) did IFN and AG9 act in a synergistic manner to
kill cells in vitro (Figure 18E, top panel). These findings suggest that phorbol esters have additional effects on tumor cells that affect responses to IFN. However, the results also suggest that TYK2 inhibitors can counter some of the aberrant effects of IFN on high-risk CLL cells.
3.5 DISCUSSION

The study shows that IFN-signaling is altered in leukemia cells from CLL patients with aggressive clinical disease (Figure 12). The normally brief period of activation of STAT3 induced by IFN-α in normal cells becomes sustained, and the outcome switches from a tumor-suppressor phenotype involving growth arrest and enhanced immunogenicity to a phenotype involving proliferation and production of immunosuppressive cytokines, such as IL-10, consistent with the oncogenic effects of STAT3-mediated gene expression (Figures 12-16).

“Corrupted” IFN-signaling appears to be a phenotype associated with aggressive disease, intertwined with the processes of tumor progression but not linked solely to a single oncogene (Figures 13 and 15). A variety of tumorigenic pathways appear to yield this phenotype. Increased ROS levels associated with more aggressive disease (Figure 17) may lower the activity of a number of phosphatases (Figure 16) and enhance the survival (and activity) of phosphorylated proteins in general, and pSTAT3 in particular. While p53 and ATM do not appear to directly affect IFN-signaling (Supplementary Figures 19 and 20), growth of tumor cells, unrestrained by these tumor suppressors, appears to result in gene expression patterns and elevated ROS levels (Figure 17) that impart aberrant IFN-signaling (Figures 12 and 13).

Where do the elevated ROS levels in aggressive CLL cells originate? One possibility is receptors on the B cell surface that transduce signals that stimulate mitochondria to provide energy for functional cellular responses. Activated mitochondria also make ROS, which can be measured by flow cytometry with DCFH (Hammond et al., 2007). A hallmark of aggressive CLL cells is their enhanced responsiveness to environmental signals (Hammond
et al., 2009; Shi et al., 2010) and increased ROS levels in these cells may reflect these signaling processes.

It seems unlikely that corruption of IFN-signaling in aggressive tumor cells is unique to CLL (Humpolikova-Adamkova et al., 2009). Why might altered IFN-signaling be involved in the development of a cancer? Potentially, intact IFN-signaling leading to STAT1-dominated gene expression allows the immune system to initially control an evolving tumor (Dunn et al., 2006). “Re-wiring” of IFN-signaling pathways may negate the normal tumor-suppressor function of IFN, causing it to become a growth and immunosuppressive factor that promotes cancer development. Assessment of the duration of pSTAT3 persistence following stimulation with IFN-α2b in vitro might then be an independent method to assess prognosis in CLL patients (Figure 12) (Kay et al., 2007). Larger, prospective studies are needed to address this possibility.

Our identification of an association of altered IFN-signaling pathways with high-risk disease characteristics may help explain the clinical observation that IFN-α2b is most effective in patients with relatively low-risk CLL (Langenmayer et al., 1996). Our results also suggest that inhibiting the activation of STAT3 (with small molecule TYK2 inhibitors (Figure 18) or possibly anti-oxidants (Figure 16) might be a strategy to improve the therapeutic efficacy of IFN-α2b in patients with more aggressive disease.
Supplementary Figure 19. IFN-signaling in p53-temperature sensitive cell lines and p53-/- mice. (A) Burkitt Lymphoma cells expressing a temperature-sensitive p53 protein (BL41 ts p53) were treated with IFN-α2b (1000 U/ml) at the permissive (32oC) and inactive (37oC) temperature (confirmed by the presence or absence of p21 at the respective temperatures). At the indicated times, whole cell lysates were immunoblotted with phospho-(Tyr701)-specific STAT1 or phospho-(Tyr705)-specific STAT3 antibodies, and also antibodies to total STAT1, STAT3 and β-actin. (B) Unactivated spleen cells from p53+/+ and p53-/- mice (right panels) and from ATM+/+ and ATM-/- mice on the B6 background (left panels) were treated with murine IFN-β (1000 U/ml). At the indicated times, immunoblotting was carried out using antibodies to phosphorylated STAT1 and STAT3 and p53 (to confirm the presence or absence of the protein).
Supplementary Figure 20. Effects of p53 and ATM inhibitors on IFN-signaling. CLL cells were incubated with Pifithrin-α (a p53 inhibitor) or KU-55933 (an ATM inhibitor) for 30 min and then stimulated with IFN-α2b. At the indicated times, pSTAT1 and pSTAT3 levels were measured by immunoblotting. The activity of the inhibitors was confirmed by showing that p53 and p21 levels could not be increased by radiation. (bottom panels). IFN-signaling was not altered by directly inhibiting ATM or p53.
Supplementary Figure 21. Additional examples of cell cycle analysis of IFN-treated CLL cells. CLL cells from 'high-risk' patient (patient 210) and a 'low-risk' patient (patient 231, whose clinical characteristics are shown in Table 3, were treated with IFN-α2b. After 4 h, pSTAT3 levels were measured by immunoblotting and, after 4 days, DNA content was measured by flow cytometry. The results show that IFN-treatment increased the number of cells in cycle only when IFN-mediated STAT3 phosphorylation was prolonged. IFN did not significantly increase the amount of cells with sub-diploid DNA, suggesting that the increase in viable cell number was a function of increased proliferation.
CHAPTER 4:

Aberrant O-GlcNAcylation Characterizes Chronic Lymphocytic Leukemia

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4.1 ABSTRACT

O-linked N-Acetylglucosamine (O-GlcNAc) post-translational modifications originate from activity of the hexosamine pathway, and are known to affect intracellular signaling processes. As aberrant responses to microenvironmental signals are a feature of Chronic Lymphocytic Leukemia (CLL), O-GlcNAcylated protein levels were measured in primary CLL cells. In contrast to normal circulating and tonsillar B cells, CLL cells expressed high levels of O-GlcNAcylated proteins, including p53, c-myc, and Akt. O-GlcNAcylation in CLL cells increased following activation with cytokines and through Toll-like receptors (TLRs), or after loading with hexosamine pathway substrates. However, high baseline O-GlcNAc levels were associated with impaired signaling responses to TLR agonists, chemotherapeutic agents, B cell receptor (BCR) cross-linking, and mitogens. Indolent and aggressive clinical behavior of CLL cells were found to correlate with higher and lower O-GlcNAc levels, respectively. These findings suggest that intracellular O-GlcNAcylation is associated with the pathogenesis of CLL, which could potentially have therapeutic implications.
4.2 INTRODUCTION

Variations in the clinical course of CLL are related in part to the ability of the tumor cells to respond to microenvironmental signals (Hammond et al., 2009). Aggressive CLL cells, marked by unmutated rearranged heavy chain variable genes, high CD38, ZAP-70, or CD49d expression, and deletions in chromosomes 11 or 17 (Kay et al., 2007), respond more strongly to such signals than cells which exhibit indolent clinical behavior (Hammond et al., 2009). While this observation has primarily been made in studies of BCR-signaling (Ghia et al., 2008), it is also true for other signals encountered by CLL cells in the proliferation centers where circulating cells originate, including chemokines, cytokines, TLR agonists, and extracellular calcium fluctuations (Hammond et al., 2009). Understanding how such responses are regulated is important as it could provide insights into clinical behavior and lead to better treatment strategies for CLL.

Aberrant metabolism is a hallmark of cancer (Kroemer and Pouysegur, 2008) and can also affect signaling processes. For example, overactivity of a minor metabolic pathway, the hexosamine pathway, leads to impaired insulin signaling and type 2 diabetes (Vosseller et al., 2002). The rate-limiting enzyme of this pathway is Glutamine: fructose-6-phosphate amidotransferase (GFAT) (Hart et al., 2007), which ultimately makes UDP-GlcNAc (Figure 22A), a nucleotide-sugar used to glycosylate cell-surface lipids and proteins (Lau et al., 2007). UDP-GlcNAc is also employed by O-linked N-acetylglucosamine (GlcNAc) transferase (OGT) to O-GlcNAcylate serine and threonine residues on intracellular proteins, including components of kinase cascades. These modifications can be removed by the deglycosylating enzyme, O-GlcNAcase (OGase) (Hart et al., 2007). The experiments in this
study were designed to explore the possibility that the hexosamine pathway might modulate intracellular signaling in CLL cells, and affect disease pathobiology.
4.3 MATERIALS AND METHODS

Cells
Blood was obtained from consenting CLL patients (Table 4) with persistently increased clonal CD19+CD5+IgMlo cells (Kay et al., 2007) who were untreated for at least 3 months, and from normal donors, with approval from the Sunnybrook Review Board. Tonsils were obtained from the Alberta Children’s Hospital with ethics approval.

Normal B and CLL cells were isolated as before (Gitelson et al., 2003) by negative selection with the Rosette Sep Human B cell enrichment cocktail (StemCell Technologies, Vancouver BC) and density centrifugation over Ficoll-Paque (Amersham Pharmacia Biotech AB, Uppsala, Sweden). This method yields percentages of CD19+ and CD19+/CD5+ cells of >98 and >96%, respectively (Gitelson et al., 2003). Tonsillar B cells were obtained by using RosetteSep spiked with human red blood cells (Zuccolo et al., 2009). IgD+ (naive) and IgD- (germinal center and memory) cells were then isolated by incubating with biotinylated anti-IgD antibodies (Miltenyi Biotec, Cat. No. 120-094-554) for 30 min followed by washing and incubation with streptavidin-coated microbeads (New England Biolabs, Cat. No. S1420S). Cells were then washed twice and selected three times with an EasySep magnet (Stemcell technologies). IgD+ and IgD- B cells were lysed for subsequent immunoblotting. Peripheral blood mononuclear cells (PBMCs) were obtained by density centrifugation without RosetteSep.

Antibodies and reagents
Phycoerythrin- and FITC-labeled CD19, CD83 and TNF-a antibodies were purchased from Pharmingen (San Francisco, CA). N-acetylglucosamine, uridine, and phorbol dibutyrate
### Table 4: CLL patient characteristics

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1. Corresponding patient numbers are maintained throughout the article. Patients listed below the solid line were not used in the analysis of Figure 25.
2. Time since diagnosis
3. Normal serum $\beta$2M levels are less than 2.3 mg/L
4. Lymphocyte doubling times (LDTs) in months were calculated from routine blood lymphocyte counts performed at each clinic visit and are not reported if the time of follow up was insufficient.
5. A course of treatment included any of splenectomy, radiation, or standard steroid-, alkylator-, or fludarabine-based chemotherapy regimens, with or without Rituxan.
NA=not available

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(PDB) were from Sigma (St. Louis, MO). The TACE inhibitor, TAPI (Shi et al., 2007), was from Peptides International (Louisville, KY). The TLR-7 agonist, 852A (Spaner et al., 2010), was from 3M Pharmaceuticals (St. Paul, MN). The OGT inhibitor, X1, has been described previously (Gross et al., 2005). IL-2 (Novartis Pharmaceuticals Canada Inc., Dorval, Quebec), IFN-α2b (Schering Canada Inc., Pointe-Claire, Quebec), and vincristine sulfate (Faulding Canada Inc., Kirkland, Quebec) were purchased from the hospital pharmacy.

RL2 antibodies (Holt et al., 1987) were from AbCam Inc. (Cambridge, MA). Antibodies against native and phosphorylated forms of Syk, Akt, JNK, ERK, and IκB and secondary horseradish peroxidase-conjugated anti-rabbit and anti-mouse antibodies were from Cell Signaling Technology (Beverly, MA). Antibodies to p53 were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-c-myc and -β-actin were from Sigma. OGase and AL-28 antibodies that recognize OGT were raised as described previously (Wells et al., 2002; Iyer et al., 2003). Goat anti-human IgM Fc-specific antibodies were from Jackson ImmunoResearch Labs (West Grove, PA).

**Cell culture and activation**

Purified CLL cells (1.5x10⁶/ml) were cultured in AIM-V (GibcoBRL, Grand Island, NY) plus 2-mercaptoethanol (2-ME) (Sigma) (5x10⁻⁵ M) in 6- or 24-well plates (Becton-Dickinson Labware, Franklin Lake, NJ) at 37°C in 5% CO₂. To increase O-GlcNAcylation, cells were cultured overnight with N-acetylglucosamine (20 mM) and uridine (5 mM) (Grigorian et al., 2007). Short term activated B cell lines were prepared by activating normal B cells with IL-2 (100 U/ml) and Resiquimod (Cedarlane Laboratories, Burlington, ON, Canada) (0.5 mg/ml) in embryonic stem cell media (Kalla et al., 2005) (D-MEM-F12, 15% Knockout Serum Replacement™, 1% 100x Glutamax-I (all from Invitrogen, USA)), 1%
100x MEM nonessential amino acids (Wisent Inc., USA), and 2-ME). Every 5 days, the cell density was reset to 5x10^5 cells/ml and the cells were reactivated. For the experiments in Supplementary Figure 27, cells were rested for 2 days before activation with 852A, with or without overnight culture in glucosamine and uridine.

**Immunophenotyping and detection of membrane TNF-α (mTNF-α) detection**

These flow cytometric analyses were performed as described previously (Gitelson et al., 2003; Shi et al., 2007).

**Real-time-PCR**

RNA was prepared using the RNeasy mini kit (Qiagen, Germany) and cDNA was synthesized from 2 µg of RNA using Superscript II reverse transcriptase (Invitrogen, USA) according to the instructions of the manufacturer. GFAT1, GFAT2, and HPRT transcripts were amplified with the following primers: **GFAT1** forward, 5’-GCAAGCAGTTGGCACAAGG-3’; reverse, 5’-CTCCACTGCTTTTCTTCCAC-3’; **GFAT2** forward, GGA 5’-GTCCGGAGCAAATACAAT-3’; reverse, 5’-GACCCGGTGAAATGGAGAGT-3’; **HPRT** forward, 5’-GAGGATTTGGAAAGGGTGTT-3’; reverse, 5’-ACAATAGCTTCTCAGTCTGA-3’. PCR reactions were carried out in a DNA engine Opticon™ System (MJ Research Inc., Waltham, MA) and cycled 34 times after initial denaturation (95°C, 15 min) with the following parameters: denaturation at 94°C for 20 sec, annealing of primers at 58°C (GFAT1 and 2) or 52°C (HPRT) for 20 sec, and extension at 72°C for 20 sec. The abundance of GFAT 1 and GFAT 2 mRNA were evaluated by a standard amplification curve, which was set on the basis of analyzing the relationship between starting copy number and the number of cycles. Copy numbers were determined from two independent cDNA preparations for each sample. One cDNA was the target gene
while HPRT was used as the internal standard. The final result was expressed as the relative fold change of the target gene to HPRT.

**UDP-GlcNAc measurements**

Nucleotide sugars were extracted from CLL cells or normal PBMCs (with 0.3M perchloric acid, followed by 1:4 v/v of trioctylamine and 1,1,2-trichlorotrifluoroethane, on ice), separated on a Partisil SAX anion exchange HPLC column (4.6 x 250 mm), and developed in 80 mM potassium phosphate pH 2.8, 35% acetonitrile. UDP-N-acetylhexosamines were quantified by UV adsorption at 254 nm and reported relative to the absorbance of UDP-hexoses (Grigorian et al., 2007). Reference standards for elution times of UDP-GlcNAc were from Sigma.

**Immunoblotting and immunoprecipitation**

Protein extracts from freshly isolated CLL-B cells, or following different *in vitro* treatments, were prepared in RIPA buffer. For immunoprecipitation, 100 µg of total protein was incubated with RL2 antibody at 4°C overnight and then with UltraLink immobilized protein G (Pierce Chemical) for 1 h. Lysate-antibody-agarose bead mixtures were washed four times with PBS. Total protein extracts (50 µg) or immunoprecipitates (200 µg) were mixed with SDS-PAGE sample buffer (50mM Tris-HCL pH6.8, 2mM EDTA, 10% Glycerol, 2% SDS, 2% 2-ME, and 0.025% bromphenol blue), heated at 100°C for 5 min and then run in an 8% SDS polyacrylamide mini gel. Proteins were then transferred to PVDF membranes (Immobilion, Millipore, Bedford, MA). After incubation with primary Abs overnight, blots were incubated with anti-rabbit or -mouse horseradish peroxidase-conjugated secondary Abs for 1 h. To confirm that binding was specific for O-GlcNAc moieties, RL2 antibodies were pre-incubated with 500 mM N-acetylglucosamine (GlcNAc), dissolved in T-PBS, for 30 min
at room temperature before immunoblotting. Signals were detected with Supersignal horseradish peroxidase enhanced chemiluminescence reagent (Pierce, Rockford, IL) and the blots were exposed to Kodak Biomax MR film. Western blot analyses for other antibodies were performed according to the manufacturers' protocols.

**Determination of RL2 index**

The total intensity of all RL2-staining bands on a gel was quantified using the GeneTools Analysis Software version 4.00 from Syngene Genius 2 Bioimager and normalized to the intensity of β-actin staining. To control for the experimental variations involved when comparing large numbers of samples on different blots, this number was normalized again to the results obtained with 50 µg of a reference standard from a single CLL patient (patient 106), which was always run with each gel. To further control for experimental variation, 3 separate gels were run. The normalized RL2 density for the reference standard of the second and third blots was divided by the normalized RL2 density for the reference standard of the first blot and used to multiply the normalized RL2 densities of the patient samples obtained on the respective gels. The average of the results from gel 1 and the weighted results of gels 2 and 3 was called the “RL2 index”. In other words,

$$\text{RL2 index} = \frac{\sum (P106_1 \times X_n)}{(P106_n \times 3)},$$

where \(n\) is the gel number, \(X_n\) is the densitometry value of total RL2 staining relative to β-actin staining for an individual patient sample and \(P106_n\) is the densitometry value of total RL2 staining relative to β-actin staining for patient 106.

**Statistical analysis**

The Student \(t\) test was used to determine \(p\) values for differences between sample means. Best-fit lines were determined by least-squares regression.
4.4 RESULTS

*Increased levels of O-GlcNAcylated proteins in CLL cells*

As a measure of hexosamine pathway activity in CLL cells, protein extracts were immunoblotted with RL2 antibodies, which recognize O-GlcNAc moieties on proteins such as nuclear porins and transcription factors (Holt *et al.*, 1987). Examples of 5 patient samples described in Table 4 are shown (Figure 22B, top panel). The “RL2 index” was obtained by densitometry as described in the materials and methods and used to convey differences in O-GlcNAcylation between patient samples. Variations in band intensities were noted but all CLL samples (more than 80 different patients) exhibited evidence of increased O-GlcNAcylation. In marked contrast to CLL cells, normal PBMCs (of which ~20% are B cells) expressed low levels of O-GlcNAcylated proteins, as did purified circulating normal B cells and IgD⁺ naïve tonsillar B cells. Higher amounts of O-GlcNAcylated proteins were found in IgD⁻ tonsillar B cells, including both memory and germinal center cells, but not to the levels observed in CLL cells (Figure 22B).

Expression of a number of hexosamine pathway enzymes (Figure 22A) by CLL cells was determined since O-GlcNAcylation reflects activity of this pathway. Consistent with the results obtained with RL2, OGT was expressed at higher levels in CLL cells than in normal circulating or tonsillar B cells (Figure 22B). OGase was expressed by CLL cells and by IgD⁻ tonsillar B cells but was not present at very high levels in PBMCs, circulating B cells, or naïve tonsillar B cells (Figure 22B). Two GFAT isoforms have been described (Garcia-Gonzalo *et al.*, 2008). Antibodies to these proteins are not readily available but GFAT1 mRNA was expressed by CLL cells at similar levels as normal PBMCs (Figure 22C).
Figure 22. Hexosamine pathway activity in CLL cells. (A) Schema of the hexosamine pathway indicating relevant enzymes (eg. OGT, GFAT) and molecules (eg. OGT inhibitors). (B) Top: Lysates from freshly isolated normal PBMCs from 3 donors and CLL cells from 5 different patients were immunoblotted with RL2 antibodies and quantified by densitometry (RL2 index) as described in the materials and methods and shown above the blots. Bottom: Results with 4 additional CLL samples and another PBMC sample are shown. In addition, purified circulating adult B cells and IgD+ and IgD- B cells from two normal tonsils were studied. The extracts were probed with RL2 and polyclonal antibodies against OGT and OGase. Similar results were seen with 3 other tonsillar samples. (C) GFAT1 and GFAT2 isozyme expression were assessed by RT-PCR. An example of a gel is shown along with the transcript level relative to HPRT. (D) UDP-GLcNAc levels in PBMCs (n=6) and CLL cells (n=19) that had been cryopreserved immediately upon isolation were measured by HPLC. UDP-GLcNAc levels were generally higher in CLL cells than normal cells (circled). (E) Protein extracts from freshly isolated CLL cells were passed through a Protein A column containing RL2 antibodies and immunoprecipitated proteins were identified by immunoblotting with antibodies against the indicated proteins.
GFAT2 was not expressed by CLL cells but was found in PBMCs, presumably from the monocyte component (Figure 22C).

UDP-GlcNAc levels in CLL cells were measured by HPLC as OGT activity is thought to depend on the concentration of its substrates (Figure 22A) (Oki et al., 1999). CLL cells were generally found to have higher intracellular levels of UDP-GlcNAc than normal PBMCs (Figure 22D). Taken together, increased UDP-GlcNAc and OGT expression could help to account for higher O-GlcNAcylated protein levels in CLL cells.

**OGT targets in CLL cells**

More than 1000 proteins have been identified that are modified by O-GlcNAcylation, although consensus sequences are not yet available for in silico site mapping (Kreppel et al., 1999). Some of these proteins, which were identified in other systems but are relevant to CLL, include OGT itself (Kreppel et al., 1999), p53 (Zachara et al., 2006), c-myc (Yang et al., 2006), and Akt (Vosseller et al., 2002). By immunoprecipitating RL-2-binding proteins from 5 different patient samples and then immunoblotting with the specific antibodies, these proteins were also found to be O-GlcNAcylated in primary CLL cells (Figure 22E). This finding was confirmed by first immunoprecipitating with the specific antibodies and then immunoblotting with RL2 (not shown). To show that RL-2 antibodies bound O-GlcNAcylated residues, immunoblots were repeated in the presence of 0.5 M GlcNAc, as described in the materials and methods, and reactive bands disappeared (data not shown).

**Immunoreceptor-mediated increases in O-GlcNAc levels in CLL cells**

The results in Figure 22 suggested that the hexosamine pathway was activated in CLL cells. Activating signals that cause increased intracellular glucose levels classically lead to increased flux through the hexosamine pathway in non-hematopoietic cells (Hart et al.,
2007). Immunoreceptor ligands, such as antigens, cytokines, and TLR-agonists, are thought to activate CLL cells in proliferation centers (Hammond et al., 2009). Accordingly, O-GlcNAcylated protein levels were measured after stimulating CLL cells with IL-2 and a TLR-7/8 agonist (Chou et al., 1995) (Figure 23A, left panel) or Interferon (IFN)-α2b (Figure 23A, right panel), and found to increase within 1 h. Such changes could potentially be caused by increased OGT activity or decreased OGase activity (Figure 22). However, they were inhibited by X1 (Figure 23B), which was identified as an OGT inhibitor in a small molecule library screen (Gross et al., 2005) and shown previously to prevent hypoxia-induced O-GlcNAcylation in cardiomyocytes (Ngoh et al., 2008), suggesting that the immunoreceptor-mediated increases in O-GlcNAcylation in CLL cells were through OGT.

**Effect of increased O-GlcNAcylation on signaling responses in CLL cells**

Although acute stimulation with cytokines and TLR-agonists increased O-GlcNAcylated protein levels in CLL cells with relatively low baseline levels (Figure 23A), we wanted to determine the effect of high baseline O-GlcNAc levels on subsequent signaling in CLL cells. To increase O-GlcNAcylated protein levels, cells were cultured in N-acetylglucosamine (GlcNAc) and uridine (GU), which enter the hexosamine pathway downstream of GFAT (Figure 22A) and increase UDP-GlcNAc and OGT activity (Grigorian et al., 2007). To decrease O-GlcNAc levels, cells were cultured in the presence of X1 to prevent new O-GlcNAc moieties from being formed while allowing time for preexisting modifications to be removed by OGase.

In adipocytes, O-GlcNAcylation inhibits the activity of Akt by interfering with phosphorylation, particularly at Threonine 308 (Vosseller et al., 2002). To determine if Akt was regulated in a similar manner in CLL cells, fresh patient samples were cultured for 2
Figure 23. Effect of immunoreceptor agonists and hexosamine pathway metabolites on O-GlcNAcylation in CLL cells. (A) Samples from patients 26 and 111, with relatively low baseline O-GlcNAc levels, were incubated with the indicated concentrations of X1 for 30 min and then stimulated with IL2 (100 U/ml) and 852A (0.1 µg/ml) (left panel) or IFN-α2b (right panel). After 1 h, extracts were made and immunoblotted with RL2 antibodies. The intensity of RL2 staining relative to β-actin levels was determined by densitometry and shown below each lane. Similar results were obtained with 2 other samples. (B) Chemical structure of X1 (C) CLL cells from patient 46 were cultured for 2 days in AIM-V media alone, with the OGT inhibitor, X1 (50 µM), or with GlcNAc (20 mM) and Uridine (5 mM) (GU) to provide substrates to OGT. Protein extracts were then examined with RL2 and antibodies to activated AktT308. Staining intensities, relative to β-actin, were quantified by densitometry (right panels). GlcNAc and uridine increased O-GlcNAc levels and decreased AktT308 levels while X1 had the opposite effects. (D) Cell size was indicated by the forward scatter parameter of flow cytometry. Cells treated with GLcNAc and uridine were smaller while cells treated with X1 were slightly larger than cells cultured alone. Numbers in the histogram are the means of the M1 region. The graph summarizes results with 8 other CLL samples (patients: 128, 133, 171, 172, 173, 125, 26 and 174) and shows the average and standard error of the differences in cell size due to substrate loading of the hexosamine pathway. (p<0.02 for the 8 samples).
days in the presence of X1 (to decrease O-GlcNAc levels) or in GlcNAc and uridine. X1 decreased O-GlcNAc levels, as shown by RL2 staining, and resulted in higher expression of Akt$_{T308}$ (Figure 23C, middle lanes). Conversely, GlcNAc and uridine increased RL2 levels and significantly decreased the expression of phosphorylated Akt (Figure 23C, right lanes). Consistent with decreased Akt activity, CLL cells cultured in GlcNAc and uridine were smaller than cells cultured alone, as indicated by the forward scatter parameter of flow cytometry (Figure 23D). X1 treated cells were also generally larger than untreated cells.

Signaling responses through TLR-7 were indicated by measuring cell surface expression of TNF-α by flow cytometry after 4 h of stimulation (Shi et al., 2007; Ngoh et al., 2008). GlcNAc and uridine increased O-GlcNAcylated protein levels in CLL cells, as shown by immunoblotting with RL2 (Figure 24A, right panel), while TNF-α expression in response to 852A decreased (Figure 24A, compare upper and lower dot-plots). When O-GlcNAc levels were decreased by X1 (Figure 24B, right panel), TNF-α expression increased following stimulation with 852A (Figure 24B, compare upper and lower dot-plots). These findings suggested that TLR-7-signaling strength was inversely proportional to intracellular O-GlcNAc levels.

JNK and IκB are normally phosphorylated following TLR-7 activation in CLL cells (Figure 24C) (Shi et al., 2007). However, treatment with GlcNAc and uridine markedly impaired JNK phosphorylation (Figure 24C, top two panels) and altered phosphorylation kinetics of IκB (Figure 24C, third and fourth panels). Previously, a similar aberrant signaling profile was found to accompany the inability of CLL cells to make TNF-α in response to TLR-7 agonists (Shi et al., 2007). To determine if the inhibitory effect of hexosamine
Figure 24. Effect of O-GlcNAc levels on TLR-7-signaling in CLL cells. (A) CLL cells from patient 116 were cultured overnight, alone or in GlcNAc and Uridine (GU), before stimulation with the TLR-7 agonist, 852A (0.1 µg/ml). Immunoblotting of cytoplasmic extracts with RL2 antibodies confirmed the increase in O-GlcNAcylated protein levels (right panels) which was quantified (relative to β-actin levels) by densitometry (shown in the graph). Surface expression of CD83 and TNF-α (to reflecting TLR-7-signalling strength) were then measured by flow cytometry after 4 h. Numbers in the right upper quadrants are the percentages of CLL cells that express mTNF-α. (B) CLL cells from patient 164 were cultured for 48 h alone or with the OGT-inhibitor, X1 (50 µM), to decrease O-GlcNAcylation (confirmed by immunoblotting with RL2 antibodies (right panels)) before stimulation with 852A and analysis by flow cytometry. Similar increases in TNF-α production were seen with 10 other samples. (C) CLL cells from patient 80 (left panel) and patient 125 (right panel) were cultured overnight in the presence or absence of GlcNAc and Uridine (GU) and then stimulated with 852A. At the indicated times, protein extracts were collected and expression of phosphorylated and unphosphorylated JNK and IkB as well as RL2, to confirm the increased levels of O-GlcNAcylated proteins, were examined by immunoblotting. Similar results were seen with 2 other samples.
pathway loading on TLR-7-mediated JNK phosphorylation was unique to CLL cells, similar studies were performed with normal B cells isolated directly from the blood and also with short-term B cell lines, since CLL cells are considered to be the transformed counterparts of activated B cells (Cheung and Hart, 2008). In either of these states, phosphorylation of at least one JNK isoform was inhibited by over-night culture with glucosamine and uridine (Supplementary Figure 27).

JNK and NF-κB signaling pathways are also activated in CLL cells by the microtubule inhibitor, vincristine (Figure 25A) (Shaha et al., 2009). Phosphorylation of JNK in response to vincristine was also particularly impaired (Figure 25A, top two panels) when intracellular O-GlcNAc levels were increased by pre-culture with GlcNAc and uridine (Figure 25A, bottom panel).

BCR-signaling plays an important role in the pathogenesis of CLL (Shaha et al., 2009). As readouts for BCR-signaling, phospho-SykY352 and -ERK T202/Y204 levels were measured, since they usually increase following BCR-cross-linking despite the anergic features of some CLL cells (Ghia et al., 2008). In contrast to their inhibitory effects on JNK activation by TLR-agonists and microtubule inhibitors (Figures 24C and 25A), GlcNAc and uridine did not significantly affect ERK phosphorylation by BCR engagement, although background levels appeared to increase (Figure 25B, second and third panels). However, altered kinetics of Syk phosphorylation accompanied the increase in O-GlcNAcylated proteins (Figure 25B, top panel).

Taken together, these results suggested that increased O-GlcNAcylation caused by metabolic loading had marked effects on subsets of signaling pathways in CLL cells. To determine if the natural variations in baseline O-GlcNAc protein levels in CLL cells (Figure
Figure 25. Effect of variations of intracellular O-GlcNAcylation on responses to chemotherapeutic agents, BCR cross-linking, and phorbol esters. CLL cells from the indicated patients were cultured overnight in the presence or absence of GlcNAc and Uridine (GU) and treated with (A) vincristine (0.3 µg/ml) or (B) anti-human IgM antibodies (10 µg/ml). Extracts were collected at 0, 10, 30 and 60 min and expression of phosphorylated and unphosphorylated forms of JNK and IκB (for vincristine) and ERK as well as SykYY525/526 (for BCR activation), along with RL2 to confirm increased levels of O-GlcNAcylated proteins, were examined by immunoblotting. Similar results were seen with 2 other samples. (C) Freshly isolated CLL cells from patients 152, 113, 74, 149, 155, 130, 1, 5, 26, 55, 38, 154 (solid diamonds) and 3 normal donors (open circles) were stimulated with PDB (100 ng/ml). Differences in mean fluorescence intensity (MFI) of CD83 expression on CD19+ B cells, measured by flow cytometry 4 h later, compared to unstimulated cells are plotted against the RL2 indices of cytoplasmic extracts prepared at the time of flow cytometry and cryopreserved until analysis. The results suggest that CLL cells with higher baseline intracellular O-GlcNAcylated protein levels are less responsive to stimulation with PDB.
might similarly affect signaling processes, CD83 expression by freshly isolated CLL samples was measured by flow cytometry before, and 4 h after, activation with phorbol dibutyrate (PDB), an assay we have used previously to reflect protein kinase C (PKC) signaling (Hammond et al., 2009). At the same time, protein extracts were prepared and frozen to allow subsequent determination of the baseline RL2 index. CLL samples with high baseline RL2 indices proved to be less responsive to PKC agonists and vice versa (Figure 25C). In all cases, the responses of CLL cells to PDB were weaker than normal B cells, which have intrinsically very low intracellular O-GlcNAcylation (Figure 22B). Such differences in responsiveness to immunomodulators between normal B cells and CLL cells have been noted previously (Hammond et al., 2009; Klein et al., 2001).

**Correlation of O-GlcNAc levels with clinical parameters**

To determine if the variations in O-GlcNAcylated protein levels, which were associated with differential responsiveness to PDB (Figure 25C), had any clinical significance, the RL2 indices of 41 consecutive patients presenting to the CLL clinic at Sunnybrook were related to a number of clinical parameters and conventional biological prognostic factors, including CD38, cytogenetic abnormalities, lymphocyte doubling times (LDTs), and need for treatment (Kay et al., 2007) (Table 4). Note that ZAP-70 and mutation status of rearranged heavy chain variable genes are not routinely measured at our institution. An association with clinical stage was not observed, although Rai stage IV patients tended to have lower RL2 indices (Figure 26A). However, high levels of O-GlcNAcylation (i.e. RL2 index > 1) were associated with significantly lower CD38 expression by CLL cells (Figure 26B). Patients with higher RL2 indices (> 1) had much longer LDTs (36.1±5.8 months) compared to patients with RL2 indices < 1 (8.2±1.1 months) (Figure 26C). Similarly, CLL
Figure 26. Correlation of intracellular O-GlcNAcylation with clinical parameters. RL2 indices of freshly isolated CLL cells was measured for 41 patients whose clinical features are described in Table 4. Clinical Rai stage (A), CD38 expression (B), LDTs (C), number of treatments for symptomatic disease (D), and cytogenetic abnormalities (with no or 13q deletions considered ‘low-risk’ and any of trisomy 12, 11q or 17p deletions considered ‘high-risk’) (E) were plotted against the RL2 indices. The open circles in (B) and (E) represent patients that were not included in the statistical analysis. Patients with more aggressive disease had significantly lower intracellular O-GlcNAcylated protein levels, as indicated by the p values in the respective panels.
cells from patients with high-risk cytogenetic abnormalities (i.e. any of trisomy 12 or deletions of 11q22-23 or 17p13) (Figure 26E) or disease severe enough to require therapeutic intervention (Figure 26D) had significantly lower RL2 indices than patients with low-risk cytogenetics (i.e. normal chromosomes or deletions at 13q14) or disease that did not require treatment. The outlier in Figure 26E is patient 73 with a 17p deletion who has had a benign clinical course (Table 4) despite this ominous prognostic factor. Serum β2M levels, which also reflect aggressive disease, were also higher in patients with RL2 indices < 1 (3.43±.83 mg/L (n=12) compared to 1.98±0.21 mg/L (n=10) for patients with RL2 indices > 1) but this difference was not statistically significant. Taken together, these observations suggest that higher O-GlcNAc levels in CLL cells are associated with a favorable outcome while lower levels are associated with more aggressive disease.
4.5 DISCUSSION

The findings in this study suggest a role for the hexosamine pathway in the pathogenesis of CLL. Increased levels of O-GlcNAcylated proteins, including OGT, c-myc, p53, and Akt, are found in CLL cells (Figure 22), likely because of high expression of OGT and intracellular concentrations of UDP-GlcNAc (Figures 22B and 22D). Importantly, higher expression of O-GlcNAcylated proteins appears to be associated with an indolent clinical course, as evidenced by longer doubling times in vivo and absence of symptoms (Figure 26).

The reason for this apparent inverse relationship of total O-GlcNAcylated protein levels with clinical course is not clear. Given that subsets of signaling processes in CLL cells, particularly JNK-signaling (Figures 24C and 25A), appear to be inhibited when O-GlcNAc levels are increased, it is possible that CLL cells with high levels of O-GlcNAcylated proteins are less responsive to activating signals in proliferation centers (Figure 25C), resulting in a slower rate of cell division in vivo and prolonged LDTs. The mechanism by which O-GlcNAcylation is lowered in aggressive cells is also unclear. OGT and UDP-GlcNAc levels do not appear to distinguish between indolent and aggressive cells (Figure 22). Perhaps the changes reflect increased OGase activity (Slawson et al., 2001) or a failure of OGT to associate with signaling complexes in more aggressive cells (Yang et al., 2002).

This model is “OGT-centric” and ignores the potential impact of increased hexosamine activity on glycosylation of proteins and sphingolipids in the Golgi (Figure 22A). Indeed, increased flux through the hexosamine pathway has been shown to alter the formation of signaling complexes at the cell surface (Lau et al., 2007; Grigorian et al., 2007),
which could affect signal transduction in CLL cells. The relationship of Golgi pathway modification and intracellular O-GlcNAcylation in CLL requires further study.

The large increase in OGT expression in CLL cells (Figure 22B) compared to normal cells raises the possibility that the OGT gene sequence or structure may be modified as part of the leukemogenic process. OGT is found at chromosome Xq13, which is not commonly affected in CLL. Disruptions in this region have been reported but they are too infrequent (Kalla et al., 2005) to account for the essentially uniform over-expression of OGT in CLL samples (Figure 22). However, OGT is induced by cellular activation (Cheung et al., 2008) and we suggest its presence in circulating CLL cells may reflect the stimulatory events that occur in a proliferation center.

Larger, prospective studies are needed to properly address the possibility that O-GlcNAc levels may be an independent prognostic factor for high-risk CLL (Figure 26). Rather than measuring global O-GlcNAcylation, as in this paper, perhaps levels of individual O-GlcNAcylated signaling proteins, such as Akt, or possibly JNK, (Figures 23 and 24) may suffice to reveal the relevant activity of the hexosamine pathway in a patient’s tumor cells. Such assays await the development of antibodies against O-GlcNAcylated forms of these proteins. Similarly, the therapeutic possibilities of manipulating O-GlcNAc levels in CLL cells may be worthy of further exploration. For example, administration of glucosamine analogues might possibly slow proliferation of CLL cells in vivo by inhibiting their responsiveness to microenvironmental signals.
Supplementary Figure 27. Effect of altering O-GlcNAcylation on TLR-7-mediated JNK phosphorylation in normal B cells. B cells from two normal donors (A) or short-term B cell lines, prepared as described in the materials and methods (B), were cultured overnight in the presence or absence of GlcNAc and Uridine (GU) and then activated with the TLR-7 agonist, 852A. Expression of phosphorylated and total JNK expression were examined by immunoblotting 0, 30, and 60 min later and extracts were also blotted with RL2 to confirm that O-GlcNAc levels had increased.
CHAPTER 5:

O-GlcNAc is a Therapeutic Target for Murine and Human Leukemia

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5.1 ABSTRACT

Cancer cells express high levels of proteins with O-linked N-acetylglucosamine (O-GlcNAc) modifications that reflect increased metabolic flux through the hexosamine pathway. Agents that target this aberrant intermediary metabolism may have a role in the treatment of cancer. The calorie-restriction mimetic Resveratrol is purported to also have anti-cancer properties and was found to inhibit the growth of erythroleukemia in mice and transiently decrease tumor burdens in patients with Chronic Lymphocytic Leukemia (CLL). The anti-tumor activity of Resveratrol was associated with proteasomal activation and down-regulation of O-GlcNAcylated protein levels. However, the inhibitory effects of Resveratrol on leukemia growth in vivo and in vitro could be overcome by loading the hexosamine pathway with glucosamine to increase O-GlcNAcylation. Administration of IFN also increased O-GlcNAc levels and abrogated the efficacy of Resveratrol in vivo. These findings suggest that O-GlcNAcylation promotes the growth of leukemia cells, establish Resveratrol as a prototype for cancer drugs that act by down-regulating O-GlcNAcylation, and describe a mechanism for resistance to such drugs.
5.2 INTRODUCTION

Hematologic malignancies continue to kill many patients each year and new treatment strategies are needed to improve patient outcomes. The mechanisms employed by cancer cells to derive their energy may offer new therapeutic targets since indefinite proliferation cannot occur without sufficient energy even though cells can become malignant by a variety of different genetic routes (Kroemer and Pouyssegur, 2008). Many hematologic malignancies are fueled by aerobic glycolysis, or the Warburg effect (Warburg et al., 1927; Warburg, 1956), and there is intense interest in developing metabolic inhibitors as therapeutic agents (Barger and Plas, 2010).

When glucose enters any cell, a small fraction (2-5%) is directed into the hexosamine biosynthetic pathway (HBP). This minor metabolic pathway produces post-translational O-linked β-N-acetylglucosamine (O-GlcNAc) modifications of diverse nuclear and cytoplasmic proteins (Hart et al., 2007) (Figure 28A). We have found that activity of the hexosamine pathway is elevated in chronic lymphocytic leukemia (CLL) cells, producing increased levels of O-GlcNAcylated proteins which cause impaired intracellular signaling and affect disease progression (Tomic et al., 2010). Accordingly, inhibition of the hexosamine pathway and O-GlcNAcylation may be a therapeutic strategy for CLL and potentially other leukemias.

Resveratrol, a polyphenol nutraceutical derived from grapes, berries, plums and peanuts, is a prototype for anti-cancer drugs that target metabolism (Harikumar and Aggarwal, 2008). Resveratrol affects glycolysis directly by blocking glucose uptake in myelocytic tumors (Park, 2001) or indirectly by inhibiting phosphatidylinositol 3-kinase (PI-3K) in diffuse large B-cell lymphomas (Faber et al., 2006). Resveratrol has also been shown to block glucose utilization in human ovarian cancer cells (Kueck et al., 2007) and alter
metabolism in breast cancer cell lines (Jager et al., 2011). The aim of the present study was to determine if Resveratrol has activity against leukemia cells that could be associated with changes in glucose and hexosamine metabolism.
5.3 MATERIALS AND METHODS

Antibodies and Reagents

Clinical grade Interferon-α2b (IFN-α2b) (Schering Canada Inc.) was purchased from the hospital pharmacy. Resveratrol (RSV), MG132, and Lactacystin were from Calbiochem, and stock solutions made in dimethylsulfoxide (DMSO) (Sigma-Aldrich). Glucosamine, N-acetylglucosamine and Uridine were purchased from Sigma-Aldrich. Recombinant murine IFN-β was synthesized as described previously (Gill et al., 2006). 7-aminoactinomycin D (7AAD) was from Pharmingen. NButGT (OGase inhibitor) was from D. Vocadlo (Macauley and Vocadlo, 2010) and X1 (OGT inhibitor) was from S. Walker (Gross et al., 2005). BALB/c and C.B-17-scid/scid (SCID) mice were obtained from Jackson Laboratories.

Induction of erythroleukemia in vivo

The CB3 cell-line was originally derived from the spleen of an F-MuLV-infected BALB/c mouse (Shibuya and Tak, 1983). CB3 cells were maintained in DMEM (Wisent) supplemented with 10% FBS (Wisent). Leukemias were induced by by intraperitoneal (i.p.) injection of 10^6 CB3 cells into SCID mice.

To induce Friend Erythroleukemia (FEL), viral lysates were obtained from the replication-competent NB-tropic Friend Murine Leukemia Virus (F-MuLV) by repeated culturing of the fibroblastic, clone-B cell line in α-MEM (Wisent) supplemented with 10% FBS and penicillin/streptomycin at 1000U/ml (Gibco) as previously described (Shaked et al., 2005). 1 day old BALB/c neonates were injected i.p. with 100µl F-MuLV (approximately 500 focus-forming units) using a 1cc U-100 insulin syringe (Becton Dickinson) as previously described (Cervi et al., 2007; Lee et al., 2003). Age-matched non-infected littermates served as controls.

Hematocrit measurements
Tail blood was collected into heparinized hematocrit tubes (Fisher Scientific) and centrifuged at 100g for 10 min. Packed cell volumes were then measured using a hematocrit gauge.

**Tumor cell isolation**

Excised spleens from each mouse were placed in RPMI media, chopped into small pieces over a 40µm nylon cell strainer (BD Biosciences) and mashed with the plunger of a 3-ml syringe. Mononuclear cells were obtained by Ficoll (Cedarlane) gradient density centrifugation (400g, 30 min). Single-cell suspensions were washed in 1 x PBS (Wisent), and cultured in DMEM supplemented with 10% FBS in 5% CO₂.

**Flow cytometry**

Spleen cells were suspended in fluorescence-activated cell sorting (FACS) staining buffer (1 x PBS with 1% BSA) to a final concentration of 5 x 10⁶ cells/ml. Samples were blocked with an anti-Fc receptor antibody cocktail for 5 min before adding primary antibodies. Fluorescein isothiocyanate (FITC)-conjugated anti-SCA1 and anti-CD71, phycoerythrin (PE)-conjugated anti-cKIT, and allophycocyanin (APC)-conjugated anti-Ter119, anti-SCA1 and anti-cKIT antibodies (all from eBiosciences, San Diego, CA) were used at 0.5 µg/10⁶ cells. After 15 min at room temperature in the dark, cells were washed in 1 x PBS, re-suspended in FACS staining buffer, stained with 7AAD, collected in a FACSCalibur flow cytometer (Beckton Dickinson, USA) and analyzed with CellQuest Pro (Beckton Dickinson, USA) software.

**Histology**
Splenocytes were washed twice in 1 x PBS and adjusted to a concentration of 2 x 10^5 cells/ml. Cytospin preparations were stained with Wright’s stain in the Sunnybrook Clinical Hematology Laboratory.

**CLL blood samples**

Heparinized blood was obtained from 3 CLL patients (diagnosed by a persistent monoclonal expansion of CD19^+CD5^+IgM^{lo} lymphocytes) prior to and 4 weeks after consuming Resveratrol (5 g/d) purchased from a local health food store or over the internet. The patients had been previously untreated for at least 3 months. White blood cell (WBC) counts were recorded at the time of routine clinic visits. Protocols for blood collection were approved by the Sunnybrook Ethics Review Board and informed consent was obtained in compliance with the Declaration of Helsinki.

CLL cells were isolated as previously described by negative selection with the Rosette Sep Human B cell enrichment cocktail (StemCell Technologies) and density centrifugation with Ficoll-Paque (Amersham Pharmacia Biotech) (Gitelson et al., 2003). Cells were stimulated with IFN-α2b (1000 U/ml) and IFN-signaling was analyzed as previously described (Tomic et al., 2011).

**Proliferation assay**

CB3 cells were grown in the presence or absence of N-acetylglucosamine (35mM), Uridine (5mM) or Resveratrol (10 µM) in a 5% CO₂ atmosphere. After 48 h, viable cells were measured by manual counting in a hemocytometer using trypan blue exclusion and by flow cytometry using 7AAD exclusion, as previously described (Tomic et al., 2011).

**Immunoblotting**
Protein extracts from freshly isolated spleen cells, or following different *in vitro* treatments, were prepared in radioimmunoprecipitation assay (RIPA) 1 x lysis buffer (0.5% Triton X-100, 150mM NaCl, 25mM Tris/HCl buffer (pH=7.4), 0.1% SDS, 0.5% deoxycholate, 2mM EDTA, 1mM NaF, 1mM sodium orthovanadate, 1mM PMSF, and 1 µg/ml of aprotinin, leupeptin, and pepstatin). For analyses of ubiquinated proteins, freshly dissolved 10mM N-ethylmaleimide (NEM) was added to RIPA 1 x lysis buffer. Extracts were resolved by electrophoresis, and then blotted and probed with the following primary Abs: RL2 (Holt *et al.*, 1987) and Ubiquitin (Ub) P4G7 (AbCam), phospho-IκB-α (Ser32), STAT1, STAT3, phospho-STAT1 (Tyr701), and phospho-STAT3 (Tyr705) (Cell Signaling Technology). Antibodies against β-Actin (clone AC-15) (1:10,000 dilution) were from Sigma. To confirm that binding was specific for O-GlcNAc moieties, 500mM N-acetylglucosamine (Sigma-Aldrich) was added to RL2 antibodies diluted in Tween-PBS buffer, and membranes were incubated for 1 h at room temperature. Signals were detected with Supersignal horseradish peroxidase enhanced chemiluminescence reagent (Pierce). For additional signal, blots were stripped for 60 min at 37°C in Restore Western Blot Stripping Buffer (Pierce), washed twice in TBS-T (Tris-buffered saline plus 0.05% Tween-20) at room temperature, blocked with 10% milk for 1 h, and then re-probed, as required. Membranes were exposed to Kodak Biomax MR film (Sigma).

**Statistical analysis**

Survival curves were generated by using nonparametric Kaplan-Meier analysis. The Student *t* test was used to determine *p* values for differences between sample means.
5.4 RESULTS

Resveratrol-mediated killing of CB3 cells is associated with decreased O-GlcNAcylation

Initial experiments were carried out with the erythroleukemia cell line CB3 (Shibuya and Mak, 1983). Resveratrol (10 µM) inhibited the growth of CB3 cells and killed them within a couple of days (Figure 28B and 28C). Previously we found that high levels of O-GlcNAcylated proteins are present in primary leukemia cells (Tomic et al., 2010) but little information is available about how O-GlcNAc levels change in response to cytotoxic agents. Global O-GlcNAc levels in CB3 cells were found to decrease rapidly (as early as 5 and 15 min) in response to Resveratrol (Figure 28D, compare lanes 1 and 3-5) as measured by immunoblotting with RL2 antibodies that recognize O-GlcNAc moieties on proteins such as nuclear porins and transcription factors (Holt et al., 1987). We also showed previously that signaling pathways are disrupted by elevated O-GlcNAc levels, which could potentially make leukemia cells less susceptible to cytotoxic agents (Tomic et al., 2010). To determine if increased protein O-GlcNAcylation protected CB3 cells from Resveratrol, O-GlcNAc levels were raised by hexosamine pathway-loading with N-acetylglucosamine (G) and uridine (U). N-acetylglucosamine is thought to enter cells by pinocytosis while glucosamine enters through glucose transporters (Tomic et al., 2010; Grigorian et al., 2007). As shown in Figures 28B and 28C, GU-treatment protected CB3 cells from Resveratrol-induced death. The increase in intracellular O-GlcNAc levels caused by GU was confirmed by Western blot analysis (Figure 28D, lane 2). These results suggested that the cytotoxic effects of Resveratrol were associated with decreased O-GlcNAcylation and could be overcome by GU. Similar observations were made with primary CLL cells (Figure 29C, compare lanes 1 and 4 and data not shown), suggesting that the findings were not unique to CB3.
that cell viability is greatly enhanced in cultures treated with GU + RSV compared to treatment with RSV alone. The numbers in the FS vs. 7AAD dot-plots are the percentages of viable (7-AAD−) cells. (A) Schematic diagram of the Hexosamine Biosynthetic Pathway (HBP) indicating relevant enzymes (OGT and OGase), and the entry point for glucosamine. (B) CB3 cells were cultured for 48 h in DMEM + 10% FBS alone (untreated), with Resveratrol (RSV) (10 µM), or with N-acetylglucosamine (35mM) plus Uridine (5mM) (GU) to provide substrates to OGT, or with GU + RSV. Viable cells were counted by trypan blue exclusion and the averages and standard deviations of the results of 3 separate wells are shown (**P < .02). (C) Cells were analyzed for 7-AAD uptake at 48 h by flow cytometry. Representative dot-plots show that cell viability is greatly enhanced in cultures treated with GU + RSV compared to treatment with RSV alone. The numbers in the FS vs. 7AAD dot-plots are the percentages of viable (7-AAD−) cells. (D) Changes in O-GlcNAc levels in CB3 cells in response to GU and RSV are shown by immunoblotting. CB3 cells were left untreated (lane 1), treated with RSV (10 µM) for 5' (lane 3), 15' (lane 4) and 60' (lane 5), or loaded with GU (lane 2) for 60' as a positive control for changes in O-GlcNAcylation. Extracts were then made and probed with RL2 antibodies. β-actin was used as a loading control.
Down-regulation of O-GlcNAcylation by Resveratrol is associated with proteasomal activation

The O-GlcNAc moiety on proteins is mediated by O-GlcNAc transferase (OGT) and removed by OGase (Hart et al., 2007). The rapid effect of Resveratrol on total O-GlcNAc levels (Figure 28D) suggested it was not due to inhibition of OGT since our previous studies had shown that OGT-inhibitors lowered total O-GlcNAc levels only after several hours (Tomic et al., 2010). Consistent with this, the effects of Resveratrol were not mimicked by the OGT inhibitor X1 (Tomic et al., 2010; Gross et al., 2005) (Figure 29A, compare lanes 1, 4, and 5). We then tested if Resveratrol might activate OGase to cause the rapid decrease in O-GlcNAcylated protein levels. In this case, blockade of OGase should prevent O-GlcNAc levels from being lowered by Resveratrol. NButGT is a potent and specific inhibitor of OGase (Yuzwa et al., 2008) and increased total O-GlcNAc levels in CB3 cells (Figure 29A, compare lanes 1, 6, 7). However, Resveratrol still lowered O-GlcNAcylated protein levels in the presence of NButGT (Figure 29A, compare lanes 6, 8), suggesting it was not acting through OGase.

Most short-lived cytoplasmic and nuclear proteins are degraded by the 26S proteasome complex after substrate labeling with ubiquitin (Han and Kudlow, 1997) and Resveratrol has been shown to activate the proteasome in neurons (Marambaud et al., 2005). Therefore, we hypothesized that Resveratrol might promote the degradation of O-GlcNAcylated proteins. Indeed, treatment with Resveratrol resulted in decreased ubiquitination and O-GlcNAcylation in both CB3 cells (Figure 29B, compare lanes 1, 4) and primary CLL cells (Figure 29C, compare lanes 1, 4). MG132 (a general, reversible proteasome inhibitor) and Lactacystin (an irreversible 20S proteasome inhibitor) inhibited
Figure 29. Effect of OGase and proteasome inhibitors on down-regulation of O-GlcNAc levels by Resveratrol (RSV). (A) Immunoblot analysis of CB3 cells in the presence of an OGT inhibitor (X1) and an OGase inhibitor (NBuGT) at two different doses to modulate O-GlcNAc levels. CB3 cells were pre-treated with these inhibitors for 1 hour and then treated with RSV (10μM) for an additional 30 minutes. Extracts were then analyzed for O-GlcNAc levels using RL2 antibodies. Equal loading was demonstrated with anti-β-Actin antibodies. Immunoblot analyses of CB3 cells (B) and CLL cells (C) in the presence of MG132 (10μM) and Lactacystin (20μM) to stabilize ubiquitinated proteins. CB3 cells were incubated with MG132 and Lactacystin for 3 hours, and then treated with RSV (10μM) for an additional 30 minutes. Extracts were made and analyzed for O-GlcNAc levels and polyubiquitin using RL2 and polyubiquitin antibodies, respectively. Polyubiquitinated proteins are detected as a smear in a 10% gel. Detection of phospho-IκB served as a control for the efficacy of the proteasomal inhibitors. β-Actin was used as a loading control. Experiments with CB3 cells have been repeated 3 times and similar results have been observed with 5 other patient samples.
proteolytic degradation, particularly in CLL cells, as shown by measuring poly-Ubiquitin (poly-Ub) levels by immunoblotting and also by measuring pIkB as an example of a specific protein that is subject to proteasomal degradation (Marambaud et al., 2005) (Figures 29B and 29C). Importantly, the proteasome inhibitors rescued O-GlcNAc protein levels (Figures 29B and 29C, compare lanes 4, 5, 6), suggesting that Resveratrol promoted proteasomal degradation of O-GlcNAcylated proteins.

**Resveratrol inhibits murine erythroleukemia growth in vivo**

Given the ability of Resveratrol to kill CB3 cells *in vitro* (Figure 28), we wondered if it was effective *in vivo* and also associated with decreased O-GlcNAcylation. For *in vivo* studies, CB3 cells were adoptively transferred into 6 week-old SCID mice. Intraperitoneal injections of Resveratrol were begun the next day and continued every 2 days for 5 weeks. The dose of Resveratrol (50 mg/kg) was obtained from preliminary experiments which demonstrated inhibition of subsequent *in vitro* phosphorylation of signal transducer and activator of transcription 3 (STAT3) by Interferon-β (IFN-β) in spleen cells harvested 4 h after the injection of this dose (data not shown). This bioassay was based on published studies showing that a hepatic metabolite of Resveratrol, Piceatannol, inhibits the STAT3 kinase, tyrosine kinase 2 (TYK2) (Tomic et al., 2011; Su and David, 2000). Tumor-bearing mice injected with Resveratrol (CB3+RSV group) survived much longer than control mice (Figure 30A). Their spleens were also smaller (Figure 30B) and exhibited low global O-GlcNAc levels (Figure 30C). After 5 weeks of treatment, CB3+RSV mice were observed for an additional two weeks and showed no evidence of recurrent leukemia (Figure 30A).

To determine the effects of Resveratrol on more established leukemic cells *in vivo*, the Friend Erythroleukemia (FEL) model was employed (Lee et al., 2003). In this model,
Figure 30. Clearance of CB3 cells in vivo by Resveratrol (RSV). (A) Groups of uninjected (healthy controls), RSV-treated, CB3-injected (tumor hosts), CB3+DMSO-treated and CB3+RSV-treated mice were monitored on a daily basis and sacrificed according to institutional guidelines. The survival curve is shown. RSV was administered at 50mg/kg i.p. every 2 days (indicated by arrows). The experiment was terminated 2 weeks after discontinuation of RSV injections at week 5. \( P = .01 \). (B) Spleens from other mice in the groups were harvested at week 5. The averages and standard deviations of the spleen weights (\( n \geq 3 \) mice/group) are shown to indicate the tumor burdens (** \( P < .05 \)). (C) Protein extracts from splenocytes from CB3-injected and CB3+RSV-treated mice were analyzed for O-GlcNAc levels using RL2 antibodies. Each lane represents the results from a single mouse. \( \beta \)-actin was used as a loading control.
neonatal BALB/c mice develop acute erythroleukemia, marked by anemia, splenomegaly, and inflammatory cytokine production, ~4 weeks after injection with Friend Murine Erythroleukemia Virus (FMuLV) (Shaked et al., 2005). Resveratrol injections (every 2 days for 3 weeks) were commenced 3 weeks after inoculation of FMuLV. After 5 weeks of age, control mice were moribund but mice injected with Resveratrol (RSV group) experienced extended survival (Figure 31A). These mice also had significantly smaller spleens (Figure 31B) and lower splenic O-GlcNAc levels (Figure 31C) than FMuLV-inoculated mice without treatment. Erythroleukemia cells are characterized by overexpression of cKit (Usenko et al., 2009) and cKit$^+$ cell numbers were much lower in the spleens of Resveratrol-treated mice (Figure 31D, top panel). SCA-1$^+$ hematopoietic cells have been reported to inhibit leukemogenesis (Usenko et al., 2009) and were enriched in the spleens of Resveratrol-treated mice (Figure 31D, bottom panel), consistent with a low tumor burden. In summary, Resveratrol cleared tumor cells in two models of murine erythroleukemia, associated with decreased levels of O-GlcNAcylated proteins.

**Resveratrol down-regulates O-GlcNAc levels in primary CLL cells in vivo**

Based on reports of its beneficial health effects in the popular press, many cancer patients are using over-the-counter Resveratrol as an anti-cancer or anti-aging strategy (Kelkel et al., 2010). We were able to evaluate O-GlcNAc levels in circulating tumor cells of 3 CLL patients before and 4 weeks after they began taking commercial Resveratrol supplements (5 g/d). STAT3-phosphorylation in purified circulating tumor cells caused by in vitro stimulation with IFN-α2b was inhibited following ingestion of Resveratrol (Figure 32A) and served as a bioassay (see above) (Tomic et al., 2011) to suggest that sufficient levels had entered CLL cells in vivo to possibly mimic the effects observed in vitro with
Neonatal BALB/c mice were infected with F-MuLV and RSV administration began 3 weeks later (50mg/kg i.p. every 2 days). The survival curve is shown. Mice were monitored on a daily basis and killed according to institutional guidelines. The experiment was terminated after 4 weeks. **P < 0.01. (B) After 3 weeks on RSV, other mice in the group were sacrificed and spleen weights were recorded as an indicator of tumor burden (n ≥ 3 mice/group). The values represent means ± SD. **P < 0.05. (C) Spleen extracts from single mice were analyzed for O-GlcNAc levels using RL2 antibodies. β-actin was used as a loading control. (D) Spleen cells obtained at the same time were stained with anti-cKIT and anti-SCA1 antibodies and analyzed by flow cytometry. The numbers in the histograms represent percentages of positively-stained splenocytes.
Figure 32. Modification of O-GlcNAc levels in circulating CLL cells by Resveratrol (RSV). Three patients with chronic lymphocytic leukemia (CLL) ingested Resveratrol (5 g/d) over a period of 4 weeks. The patients were otherwise untreated at the time and had consented to donate blood for research purposes. (A) Purified CLL cells were treated with human IFN-α2b (1000 U/ml) for the indicated times in vitro. Extracts were made and phosphorylated STAT1 and STAT3 levels were determined by immunoblotting. The inhibition of IFN-induced STAT3-phosphorylation served as a biomarker for adequate absorption of Resveratrol. Similar results were seen for the other patients. (B) Changes in O-GlcNAc levels in each patient (Pt.) cells were determined by immunoblotting with RL2 antibodies. β-actin was used as a loading control. (C) Changes in the white blood cell (WBC) count, an indicator of tumor burden, are shown for one patient. The dotted horizontal line indicates the period of time on Resveratrol.
Resveratrol (Figure 29C). In all cases, O-GlcNAc levels in circulating tumor cells had decreased by the time of the next clinic visit 4 weeks later (Figure 32B). A decrease in the circulating white blood cell count was also observed (Figure 32C).

**Glucosamine inhibits the anti-leukemic effects of Resveratrol**

The decline in circulating CLL cells following ingestion of Resveratrol (Figure 32C) was short-lived, suggesting the existence of resistance mechanisms that may limit its effectiveness as an anti-cancer agent. A possible mechanism of resistance was suggested by the results of Figure 28 which showed that increased flux through the hexosamine pathway could increase O-GlcNAcylated protein levels and overcome Resveratrol-mediated cytotoxicity *in vitro*.

To first determine the effect of hexosamine pathway-loading on leukemic progression *in vivo*, SCID mice were injected with CB3 cells and then injected daily with glucosamine (20 mM) (Macauley and Vocadlo, 2010). Preliminary experiments indicated that high levels of O-GlcNAcylated protein levels were induced in spleens of mice injected 4 hours previously with this dose (data not shown). Splenomegaly and low hematocrits are known to correlate with murine erythroleukemia progression (Usenko *et al.*, 2009) and spleen weights were significantly higher (Figure 33A) in mice that received glucosamine injections while hematocrit values were significantly lower (Figure 33B). Percentages of cKIT<sup>+</sup>CD71<sup>+</sup>Ter119<sup>+</sup> tumor cells were also higher in spleens of CB3+glucosamine-injected mice, indicating enhanced erythroleukemogenesis (Figure 33C). O-GlcNAc levels were also elevated in the spleens of these mice (Figure 33D). These results indicated that glucosamine promoted tumor growth *in vivo* in this model, associated with increased O-GlcNAcylation.
Figure 33. Effect of Glucosamine and Resveratrol (RSV) on CB3 cells \textit{in vivo}. (A) Spleen weights from CB3-bearing mice with or without concomitant glucosamine injections (n=3 mice/group) were recorded at 3 weeks to indicate tumor burden. The values represent means +/- SD. (B) Hematocrits at the time of sacrifice were measured by tail bleeds (n = 3 mice/group) and the averages and standard deviations are shown. (C) Splenocytes were isolated from excised spleens and the percentages of cKIT$^+$CD71$^+$Ter119$^+$ cells determined by flow cytometry. The values represent means +/- SD. **P < .05. (D) Protein extracts from these cells were analyzed for O-GlcNAc levels using RL2 antibodies. \( \beta \)-actin was used as a loading control. (E) Splenocytes were stained with Wright’s stain and pictures were taken at a magnification x40 with a Canon PowerShot G11 Digital Camera equipped with a Carl Zeiss 426126 lens. The microscope was an Axiovert 40 C equipped with an A-plan 10x/0.25 objective, both from Zeiss. **P < .05.
To determine if glucosamine could overcome the effect of Resveratrol in vivo, CB3-bearing mice were treated with glucosamine and Resveratrol, alone or in combination, and sacrificed after 3 weeks. Consistent with the protective effects shown in Figure 30, spleen cells in Resveratrol-treated mice consisted mainly of normal myeloid cells as indicated by Wright’s staining (Figure 33E, top right panel). In contrast, tumor cells were mainly found in the spleens of mice injected with CB3 alone, CB3 + glucosamine, or CB3 + glucosamine + Resveratrol (Figure 33E). These results indicated that glucosamine could overcome the therapeutic effects of Resveratrol in vivo as well as in vitro (Figure 28).

**IFN up-regulates O-GlcNAcylation and inhibits the efficacy of Resveratrol**

Previously we showed that the type 1 IFNs increase O-GlcNAc levels in leukemia cells in vitro (Tomic et al., 2010). Therefore, we examined the effect of IFN on O-GlcNAcylation in vivo in the presence and absence of Resveratrol. The dose of IFN-β (1000U) was determined on the basis of preliminary experiments which indicated that STAT1 proteins in spleen cells were phosphorylated for at least 4 h following injection of this dose (not shown). The FEL model (Figure 31) was used for these studies and Resveratrol and IFN-β injections, alone or in combination, were started 3 weeks following inoculation of the viruses. Murine IFN-β (Tomic et al., 2011) did not protect mice from leukemia (Figure 34A) and reversed the protective effect of Resveratrol as measured by survival (Figure 34A) and spleen weights (Figure 34B). Acceleration of disease by IFN-β was also associated with increased splenic O-GlcNAc levels (Figure 34C). Taken together, these results are consistent with the notion that hexosamine pathway loading, either by glucosamine or IFN, is a mechanism of Resveratrol resistance.
Figure 34. Interferon increases O-GlcNAcylation and overcomes protection by Resveratrol (RSV) in the FEL model. (A) Administration of RSV (50mg/kg BW i.p. every 2 days) and IFN-β (1000U i.p everyday) were commenced at week 3 post inoculation of FMuLV and carried out until the death of the animals (n = 4 mice/group). Mice were monitored on a daily basis and killed according to institutional guidelines. The survival curve is shown. **P < .05. (B) Spleens were harvested and their weights recorded to indicate tumor burden (n = 4 mice/group). The values represent means +/- SD. ** P < .05. (C) O-GlcNAc levels in splenocytes were determined with RL2 antibodies. β-actin was used as a loading control.
5.5 DISCUSSION

The studies in this paper demonstrate that the plant polyphenol Resveratrol has therapeutic activity against murine and human leukemias and suggest that the benefits of Resveratrol are associated with down-regulation of O-GlcNAc levels in tumor cells (Figures 28-34). Proteasomal activation accounts in part for the effects of Resveratrol on O-GlcNAcylation (Figure 29) and can be overcome by increasing hexosamine pathway flux directly with glucosamine or N-acetylglucosamine (Figures 28 and 33) or indirectly with IFN (Figure 34).

High levels of O-GlcNAcylation have been shown before to characterize leukemia cells (Tomic et al., 2010) and solid tumors (Mi et al., 2011; Caldwell et al., 2010). The results presented here provide further evidence for O-GlcNAcylation as a marker of tumor progression since acceleration of disease by glucosamine or IFN was associated with increased levels of O-GlcNAcylated proteins (Figures 33 and 34) while disease control by Resveratrol was associated with lowered O-GlcNAc levels (Figures 30, 31 and 32). The mechanisms by which O-GlcNAcylation promotes tumor progression and allows tumor cells to resist cytotoxic stresses (Zachara et al., 2004) are likely to be multi-factorial and include effects on signaling pathways (Tomic et al., 2010) and gene expression regulated by oncogenic transcription factors such as c-Myc (Kamemura and Hart, 2003; Chou et al., 1995), EWS-Fli1 (Bachmaier et al., 2009) and FoxM1 (Caldwell et al., 2010). Resveratrol might then be able to act as a chemo-sensitizing agent by removing the protection associated with O-GlcNAcylation and enhancing the susceptibility of tumor cells to cytotoxic drugs. Further studies will address the issues of whether other anti-cancer agents lower O-GlcNAc levels as part of their mechanism of action and if resistance to both chemotherapy and
immunotherapeutic agents such as the type I IFNs (Tomic et al., 2011) correlate with maintenance of O-GlcNAc levels. Indeed, our observation that IFN-β overcame the protective effects of Resveratrol and accelerated leukemia progression while increasing O-GlcNAc levels (Figure 34) may offer new insights into mechanisms for failure of IFN-therapy in cancer patients (Langenmayer et al., 1999; Humpolikova-Adamkova et al., 2009).

The therapeutic effects of Resveratrol could be overcome with Glucosamine and IFN which were associated with high splenic O-GlcNAc levels (Figures 28, 29 and 34). Glucosamine may have other effects than simply increasing O-GlcNAcylation in tumor cells. For example, glycosylation of membrane proteins and the functional properties of immune effector cells could be altered by increased hexosamine pathway activity which might inhibit the rejection of adoptively-transferred cell-lines or virally-induced tumors (Grigorian et al., 2007; Lau et al., 2007). However, if increased removal of O-GlcNAcylated proteins is the mechanism by which Resveratrol lowers total O-GlcNAc levels (Figure 29), then accelerating flux through the hexosamine pathway, resulting in increased UDP-GlcNAc levels (Figure 28A) (Tomic et al., 2010) and higher OGT-mediated O-GlcNAcylation rates by mass action (Kreppel et al., 1999), could certainly account for maintenance of O-GlcNAc levels and glucosamine-mediated resistance to Resveratrol. In this case, combining Resveratrol with an OGT-inhibitor may be a rational strategy to improve the efficacy of this approach. While the first generation of OGT-inhibitors, such as X1 (Gross et al., 2005) were relatively weak and non-specific, new inhibitors based on oligosaccharide engineering are being developed (Gloster et al., 2011) and the recent solution of the crystal structure of OGT (Lazarus et al., 2011) should facilitate the design of other, more effective OGT-inhibitors.
CHAPTER 6:

Discussion and Future Perspectives
In a concerted effort to develop more effective treatments for CLL, a better understanding of its pathogenesis is needed. As a result, the major findings of this thesis include the concepts that CLL cells are driven to proliferate \textit{in vitro} and \textit{in vivo} by immunomodulators such as IL-2 and TLR-7 agonist (Chapter II), and IFN (Chapter III), which is characterized by high levels of activated STAT3 (Chapters II and III). These findings help explain previous observations, including ours, that CLL cells are relatively resistant to T cell-mediated immunotherapies \textit{in vivo}. Another major finding was the observation that CLL cells express high levels of O-GlcNAcylated proteins, as a result of overactive hexosamine pathway, which impairs signaling responses to TLR-7 agonist, IFN, chemotherapeutic agents, B cell receptor crosslinking and mitogens (Chapter IV). These findings suggest that O-GlcNAcylation is associated with the pathogenesis of CLL, which could have therapeutic implications (Chapter IV). The latter findings solidified O-GlcNAcylation as a mechanism of resistance to cancer drugs, such as IFN and Resveratrol, and suggested that the benefits of these cancer drugs are associated with down-regulation of O-GlcNAc levels in tumor cells (Chapter V). In addition, the findings of this thesis suggest that ROS (Chapter III) and O-GlcNAc (Chapters IV and V) could be developed into two independent prognostic factors for high-risk CLL. In this chapter, the importance of these findings and the direction of future research in this area will be expanded upon.

\textbf{6.1 ROS as a prognostic factor for CLL}

CLL, like most cancers, is incurable, in part because the conventional drugs that are prescribed do not kill all their targets (Caligaris-Cappio \textit{et al.}, 2008). This intrinsic drug-resistance may be intertwined with mechanisms that allow cancer cells to survive conditions in the tumor microenvironment such as tumor-reactive T cells, hypoxia and poor vasculature
(Spaner, 2004; Blagosklonny, 2002). For CLL patients, standard practice is to defer treatment until symptoms develop that are related to tumor progression (Hallek, et al., 2008). During this “watch and wait” period, cancer cells may acquire more aggressive characteristics that ensure drug resistance and a poor outcome (Stilgenbauer et al., 2007). On the other hand, treatment too early with drugs that lack curative potential may select for drug-resistant clones and shorten survival (Stilgenbauer et al., 2007). A diagnostic test that could predict the patient’s clinical course would “personalize” the treatment plan and indicate the appropriate time to intervene. If this prognostic factor is relevant to disease biology, it would also indicate a more effective target for therapy.

The findings of this thesis suggest that determining the metabolic state of patient’s tumor cells may be an ideal prognostic parameter. I have discovered that aggressive CLL cells express high levels of ROS which may mediate resistance to immunomodulatory agents, such as IFN (Tomic et al., 2011) (Chapter III). This finding came from the observation that CLL cells with aggressive clinical features such as loss of tumor suppressor p53 and faster lymphocyte doubling times had significantly higher ROS levels compared to other less aggressive CLL cells (Chapter III). This suggested that increased levels of ROS might support tumor growth. To further corroborate this, using a mass-spectrometry (MS)-based proteomics approach, we found that the largest number of proteins identified in aggressive CLL cells are involved in oxidative stress pathways and generation of ROS (Figure 35). In addition, these aggressive CLL cells with increased ROS levels, and corrupted IFN-signaling pathways caused IFN to be used as a growth factor rather than as a tumor suppressor (Tomic et al., 2011) (Chapter III). More specifically, I have found that a read-out for “corrupted” IFN-signaling in aggressive CLL cells is increased activity of tumor
Figure 35. Oxidative stress in circulating CLL cells. Pooled trypsin-digested proteins from 2 advanced stage CLL samples were fractionated by liquid chromatography interfaced with a quadrupole time-of-flight mass spectrometer. Protein pilot v2.01 was used to identify and quantify 283 proteins at the 95% confidence interval. Their accession numbers were submitted to Ingenuity pathway analysis and showed that the top pathway (indicated on the x-axis) is oxidative stress pathway (i.e. a large proportion of identified proteins are involved in oxidative pathway). Significance scores are indicated on the y-axis (negative log of p-value calculated using Fisher exact test). The line represents the number of proteins in a given pathway that meet the cutoff criteria divided by the total number of proteins that make up that pathway. Only the pathways with the highest significance scores are shown.
promoter STAT3 (Tomic et al., 2011) (Chapter III). Altogether, these findings suggest that elevated ROS levels support tumor growth and resistance to immunomodulators such as IFN by increasing activity of tumor promoter STAT3, and as a result ROS levels could serve as a novel prognostic marker as well as a therapeutic target for CLL.

**6.2 Identifying the source of ROS**

The future studies would extend and build on the findings that elevated ROS levels are a characteristic of aggressive disease, intertwined with the processes of tumor progression and resistance to treatment. Currently, there is a lot of evidence indicating that metabolic stress exerted by ROS is central to promoting cancer (Pelicano et al., 2004). Moreover, intracellular pathways implicated in tumorigenesis are intimately involved with metabolism and generation of ROS. The first goal would be to identify where the elevated ROS levels in CLL cells originate. ROS can be produced at multiple sites in a cell. One possibility is that the receptors, which send signals into the cell, stimulate mitochondria to provide energy for functional cellular responses. Mitochondria are the energy machinery of the cell and produce the largest amount of ROS (Pelicano et al., 2004; Fleury et al., 2002). Elevated mitochondrial-dependent ROS production can contribute to cancer through DNA mutation, activation of inflammatory pathways and promotion of pro-growth signaling (Pelicano et al., 2004). A hallmark of aggressive CLL cells is their enhanced responsiveness to environmental signals (Hammond et al., 2009; Tomic et al., 2010), and increased ROS levels in these cells may reflect these signaling processes.

Previous studies have shown that the main source of ROS production by mitochondria occurs via complexes I and III (Stowe and Camara, 2009). Specific inhibitors of complex I (such as diphenylene iodonium (DPI) and rotenone) as well as complex III
(such as antimycin A) can be used to demonstrate that ROS production originates in the mitochondrial electron transport chain (Massaad and Klann, 2011; Lambert et al., 2008). Additionally, Mitoquinol 10 nitrate (MitoQ), a mitochondria-targeted antioxidant, can be used to selectively block mitochondrial oxidative damage. Changes in production of superoxide can be assessed with fluorescent indicators such as dihydroehidium (DHE) and MitoSOX Red, while differences in the levels of hydrogen peroxide can be detected by Amplex Red and DCF.

Another major source of ROS is NADPH oxidase (NOX), which consists of Nox1, Nox2, Nox4, Nox5, p22phox and the small G-protein Rac1 (Lambeth, 2004; Arnold et al., 2001). NOX is activated by various growth factors, cytokines, hypoxia and high glucose (Ushio-Fukai and Nakamura, 2008; Li and Shah, 2003). Apocyanin, which prevents the assembly of NADPH oxidase subunits, and DPI have been widely used to inhibit activity of NOX (Ellis et al., 1998; Riganti et al., 2004). Generation of ROS by NADPH oxidase can be measured by lucigenin-enhanced chemiluminescence method (Block et al., 2007).

In addition to mitochondria and NADPH oxidase, endoplasmic reticulum (ER) stress can also activate inflammatory pathways and lead to accumulation of ROS inside the cell (Pelicano et al., 2004). In the ER, the major sources of ROS are cytochrome P450, NADPH-P450 reductase (NPR) and phospholipids (Premereur et al., 1986; Davydov, 2001). Treatment of cells with thapsigargin and tunicamycin (ER-stress inducers) can significantly increase ROS levels, while addition of 4-methylpyrazole (4-ME) can abrogate the effects of either activator (Kim et al., 2009). There are three key ER-membrane-associated proteins PERK, IRE1 and ATF6, which together act to relieve ER stress (Pelicaneto et al., 2004). The
changes in their expression can be correlated with changes in ROS levels, and that could support connection between ER stress and ROS accumulation.

The intracellular location of ROS can be visualized with aid of fluorescent dyes and viewed in real time *in situ* with confocal microscope. In addition, cells can be co-loaded with mitochondrion and ER-selective probes, such as MitoTracker and ER Tracker, in order to see involvement of a particular organelle in ROS generation (Halliwell and Whiteman, 2004). However, caution must be exercised to ensure that the signal from the organelle Tracker stain does not interfere with the measurement of ROS-sensitive dye.

While the studies above should solidify ROS as a prognostic marker of CLL, addition of exogenous anti-oxidants (such as N-acetyl cysteine which has ability to scavenge ROS) to IFN may improve treatments for CLL. Furthermore, assessment of the duration of STAT3 activation and persistence following IFN treatment (in the presence and absence of anti-oxidants) *in vitro* might become an independent method to assess prognosis and response to treatment in CLL patients.

However, results with oxidative stress, ROS, and antioxidants must be assessed cautiously with a clear understanding of what the methods used do or do not measure, particularly *in vivo* (Murphy *et al.*, 2011). Furthermore, in order to make the results more valid, it would be helpful to specify particular ROS involved as well as the pathways thought to be responsible for given biological effects. Likewise, it would be important to show that an antioxidant actually reacts with the particular ROS and that it can lower the concentration of that ROS, both *in vitro* and *in vivo*, in order to justify its use.

6.3 O-GLcNAc as a target for cancer therapy
Another prognostic factor that is relevant to CLL biology is O-GlcNAc, which can also be an effective target for therapy. Elevated levels of O-GlcNAc in cancer cells reflect increased metabolic flux through the hexosamine pathway. As a result, agents that target this aberrant intermediary metabolism may have a role in the treatment of cancer. A compound called Resveratrol, which is a natural product made by the immune systems of plants responding to various stressors, including microorganisms, was found to down-regulate O-GlcNAc levels in tumor cells. Initially, we became interested in this molecule because of the ability of a Resveratrol metabolite (Piceatannol) to block IFN-induced STAT3 activation. Using primary CLL cells and Friend erythroleukemia (FEL) mouse model, I was able to test IFN+Resveratrol combination both in vitro and in vivo. My studies showed that Resveratrol mediated its effects via hexosamine pathway, rather than through direct inhibition of STAT3 activation, as administration of IFN led to increase in O-GlcNAc levels and abrogated the efficacy of Resveratrol in vivo (Tomic, et al., manuscript in revision) (Chapter V). These findings established O-GlcNAc not only as a growth promoting property of cancer cells, but also as a mechanism of resistance to cancer drugs such as IFN. Therefore, the data presented in Chapter V are important as they offer new insights into mechanisms for failure of IFN-therapy in cancer patients (Langenmayer et al., 1999; Humpolikova-Adamkova et al., 2009).

The uniqueness of findings presented in Chapter V extend to include therapeutic benefits of Resveratrol, as a single agent, which are associated with down-regulation of O-GlcNAc levels in tumor cells. More specifically, results indicate that Resveratrol might be able to act as a chemo-sensitizing agent by removing the protection associated with O-GlcNAcylation and enhancing the susceptibility of tumor cells to cytotoxic drugs. However, our findings did show that the therapeutic effects of Resveratrol could be overcome with
Glucosamine and IFN which were associated with high O-GlcNAc levels (Tomic et al., manuscript in revision) (Chapter V). Thus the next step would be to look at how resistance to Resveratrol can be overcome.

6.4 Understanding Resveratrol resistance

It is not entirely surprising that Glucosamine and IFN are able to overcome the effect of Resveratrol by increasing O-GlcNAcylation in tumor cells (Tomic et al., manuscript in revision) (Chapter V). In our system, IFN serves as a growth factor for tumor cells (Chapters II and V). Others have reported that growth factor-dependent stimulation can lead to glutamine consumption, even in the absence of glucose (Wellen et al., 2010). This growth-factor-dependent nutrient uptake is essential for production of ATP and biosynthetic precursors such as protein, DNA, RNA and lipid membranes, and growth factor-stimulated uptake of glutamine could serve as a primary carbon source, in the absence of glucose, in cancer cells (Wellen et al., 2010).

It must be noted that IFN-β was used in combination with Resveratrol in FEL mouse model because IFN-α fails to activate same signaling molecules in mice as it does in human cells (e.g. IFN-α does not activate STAT4 and as a result Th1 development does not occur in mice) (Mestas and Hughes, 2004). In addition, while IFN-α and -β exert many similar biological activities including antiviral, antiproliferative and immunomodulatory, the two induce differential assembly of homo- and heteromeric STAT complexes (Rankel et al., 1998; Darnell et al., 1994), which distinguish promoter elements on the basis of distinctive binding properties (Darnell et al., 1994; Xu et al., 1996). IFN-β has also been found to have activity in cells lacking the IFN receptor-associated TYK2 and higher potency in treatment of multiple sclerosis (Rankel et al., 1998). The potential for two IFNs to differentially activate
STAT complexes or to induce other signaling events such as activation of PI3-kinase serine kinase and MAPK (Uddin et al., 1996; David et al., 1995) could result in distinctive gene activation events that potentially affect Resveratrol response.

Of equally great importance is the notion that tumor cell metabolism could regulate the fate of T cells. According to Rathmell and Michalek (2010), it may be possible to suppress T cell responses by altering specific metabolic pathways. However, if increased removal of O-GlcNAcylated proteins is the mechanism by which Resveratrol lowers total O-GlcNAc levels, then accelerated flux through the hexosamine pathway by either Glucosamine or IFN, resulting in increased UDP-GlcNAc levels (Figure 3) and higher OGT-mediated O-GlcNAcylation rates, could certainly account for maintenance of O-GlcNAc levels and resistance to Resveratrol. If this is the case, then combining Resveratrol with an OGT-inhibitor may be a rational strategy to improve the efficacy of this approach.

However, the studies above must be assessed cautiously because the biggest liability of Resveratrol as a molecule that targets O-GlcNAc is its lack of specificity. Resveratrol has been found to have other direct targets in tumor cells, some of which have their own complex and beneficial consequences, such as inhibition of NFκB (Aggarwal et al., 1996; Manna et al., 2000), activation of adenosine monophosphate protein kinase (AMPK) (Lin et al., 2010), activation of p53 (Huang et al., 1999), etc. These complications highlight the need to verify effects that have been attributed to Resveratrol using more specific methods of O-GlcNAc down-regulation in vivo.

6.5 Novel treatment approach

Given the interest in the role of O-GlcNAc, and the potential therapeutic benefits associated with manipulating O-GlcNAc levels (Chapters IV and V) (Caldwell et al., 2010;
Yuzwa *et al.*, 2008), useful chemical approaches are needed to reduce O-GlcNAc levels in tumor cells that have excessive O-GlcNAcylation levels. Currently available inhibitors such as azaserine are able to block UDP-GlcNAc synthesis but unfortunately other amidotransferases (those involved in purine and pyrimidine synthesis) are affected as well (Lyons *et al.*, 1990). Alloxan is another inhibitor that has been proposed to inhibit OGT, but it is also found to be toxic and have many other non-specific substrates (Lenzen and Panten, 1988). In the past, we too have worked with the first generation of OGT inhibitors, such as X1 (Gross *et al.*, 2005; Tomic *et al.*, 2010), which proved to be relatively weak and non-specific. Recently, Dr. Suzanne Walker’s group reported the crystal structure of OGT (Lazarus *et al.*, 2011) which should accelerate the design of other, more effective OGT inhibitors. The lack of crystal structure has been a major impediment to investigating OGT’s molecular mechanisms, understanding substrate recognition, and developing inhibitors with therapeutic value for treating disease associated with excessive O-GlcNAcylation, such as cancer (Lazarus *et al.*, 2011). Because of the interest in the biological functions of O-GlcNAc and its therapeutic benefits, there is a motivation to design a specific inhibitor that could be used effectively and with least toxicity in cancer cells.

Recently, our collaborator Dr. David J Vocadlo has developed a new OGT inhibitor, UDP-5SGlcNAc, based on oligosaccharide engineering. Dr. Vocadlo’s group synthesized a nucleotide sugar analog that is able to inhibit OGT with high potency and little toxicity both *in vitro* and *in vivo* (Gloster *et al.*, 2011). Their approach (referred to as a Trojan horse strategy) was to take advantage of the enzymes of hexosamine pathway to transform a synthetic precursor into the nucleotide sugar analog (Gloster *et al.*, 2011). They showed that UDP-5SGlcNAc does not serve as a substrate for OGT, and that it does not accumulate on
proteins. Instead, formation of UDP-5S\text{GlcNAc} using cellular machinery inhibits OGT function and leads to decreased intracellular O-GlcNAc levels, without affecting cell surface glycosylation (Gloster et al., 2011). In the future, we would like to investigate the effects of this new OGT inhibitor in combination with Resveratrol on the O-GlcNAc status of cancer cells, and if this combination could improve the effects of immunomodulators, such as IFN. Another important investigation would be to test if high O-GlcNAc levels protect tumor cells from oxidative stress and ROS, and consequently if lowering O-GlcNAc levels (by Resveratrol) would make tumor cells more susceptible to ROS-induced cell death.

In the past, FEL mouse model was used to test the effect of Resveratrol on tumor growth and progression \textit{in vivo}, and these findings were correlated with those of primary CLL cells. However, in order to properly assess the anti-CLL activity of Resveratrol alone or in combination with other inhibitors, a CLL murine model should be used. Animal models of CLL have been remarkably difficult to establish (Bertilaccio et al., 2010). The development of xenograft models has been severely limited by inefficient (Hummel et al., 1996) or short-term engraftment (Shimoni et al., 1999). In addition, recovery of CLL cells from bone marrow or peripheral blood has been very low after infusion of human primary CLL cells into immunodeficient non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice (Durig et al., 2007). The establishment of a xenograft model using CLL cell line, MEC1 (Stacchini et al., 1999), has been successful and it is useful for testing the efficacy of new therapeutic agents (Bertilaccio et al., 2010) but it must be noted that this model does not fully resemble human CLL as MEC1 cell line lacks the expression of CD5. At present, the most popular CLL model is the transgenic mouse obtained by inserting the human TCL1 gene under the control of the immunoglobulin heavy chain variable region
promoter and immunoglobulin heavy chain enhancer but a CLL-like disease develops after a long period of time (13-18 months) (Bichi et al., 2002). In order to overcome the limitations posed by these mouse models, we are working on establishing a novel CLL xenograft model using Daudi cell line that expresses CD5 (Garaud et al., 2011) in Rag2<sup>−/−</sup>γ<sub>c</sub><sup>−/−</sup> mice which have profound immunosuppression (Bertilaccio et al., 2010).

To fully understand and appreciate how Resveratrol works alone or in combination with other agents, which are able to manipulate O-GlcNAc levels, the effect on CLL microenvironment must be assessed. Herishanu et al. (2011) have elegantly shown that bone marrow and lymph node environment directly affects tumor biology of CLL cells in vivo. For example, after simultaneously purifying CLL cells from bone marrow, lymph nodes and blood and performing gene expression profiling, they observed increased BCR signaling in lymph node-derived CLL cells which was accompanied by NFκB signaling and c-Myc activation (Herishanu et al., 2011). This translated into increased tumor proliferation and rapid disease progression, suggesting that the microenvironment within the lymph node may be a key determinant with regard to disease course and outcome. We were able to mimic the CLL microenvironment in vitro using IL2+TLR7/8 agonist system (Tomic et al., 2006), however in a mouse model of CLL we should look at the effect of Resveratrol on bone marrow, lymph node and spleen environment by carefully analyzing which cell population (tumor vs. stroma and leukocytes) are showing differential O-GlcNAc signal. These results should provide a framework for an increased understanding of the pathogenesis of CLL, emphasizing the role of microenvironment and better appreciation of the role of O-GlcNAc and agents that manipulate its levels in vivo.
6.6 Concluding remarks

The altered metabolism of cancer cells confers a selective advantage for survival and proliferation in the unique tumor environment. Not only can the tumor environment select for an abnormal metabolism, but oncogene activation and tumor suppressor loss can also drive metabolic changes. Recent findings show that metabolic changes can impair presentation of tumor antigens (Herber et al., 2010), which is crucial for activation of the immune system. As a result, emphasis should be place on the development and testing of the inhibitors of metabolic enzymes such as OGT. These pharmacological inhibitors may be able to reverse tumor-induced immunosuppression and restore anti-tumor immune responses by fine-tuning the metabolism of tumor cells, and thus improve the efficacy of therapeutic vaccination and conventional chemotherapies.
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