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Femina Adatia

AUTEUR DE LA THÈSE / AUTHOR OF THESIS

M.Sc. (Biochemistry)

GRADE / DEGREE

Department of Biochemistry, Microbiology and Immunology

FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

A Role for Type I Interferons in the Resistance of Chronic Lymphocytic Leukemia to Vesicular Stomatitis Virus

TITRE DE LA THÈSE / TITLE OF THESIS

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A Role for Type I Interferons in the Resistance of Chronic Lymphocytic Leukemia to Vesicular Stomatitis Virus

Femina Adatia

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"I may not have gone where I intended to go,
but I think I have ended up where I intended to be."

- Douglas Adams (1952-2001)

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~Bismillahir rahemanir rahim~

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ABSTRACT

Vesicular Stomatitis Virus (VSV) is a naturally oncolytic agent that is exquisitely sensitive to the antiviral effects of Type I Interferons (IFN). Although 81% of human cancer cell lines in the NCI-60 panel are sensitive to VSV, Chronic Lymphocytic Leukemia (CLL), the most common adult leukemia in the West, is resistant. It is hypothesized that this resistance is due to an intact IFN pathway in CLL cells. A quantitative PCR based approach was taken to examine the IFN gene expression profile of CLL cells. It was discovered that CLL cells constitutively express IFNβ and IFNαR1 transcripts which may contribute to an inherent antiviral state in these cells. However, upon activation of CLL cells, IFN transcript levels decrease and susceptibility to VSV infection is increased. As complete sensitivity to VSV is not achieved, the contribution of other antiviral mechanisms cannot be excluded. Understanding the mechanisms of viral resistance in CLL and uncovering novel approaches of manipulating them will have future implications for VSV therapy of CLL.

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LIST OF ABBREVIATIONS

AIDS	Acquired immune deficiency syndrome
ALL	Acute lymphoblastic leukemia
biAL	Bi-phenotypic acute leukemia
AML	Acute myeloid leukemia
ATF2	Activating transcription factor 2
ATP	Adenosine triphosphate
Bp	Base pairs
CBP/p300	CREB binding protein and p300
cDNA	Complementary DNA
CLL	Chronic lymphocytic leukemia
CNS	Central nervous system
CPE	Cytopathic effect
Δ51 (d51)	Deletion of methionine 51 in matrix protein of VSV
DEPC	Diethyl pyrocarbonate
DLBCL	Diffuse large B cell lymphoma
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
dsRNA	double stranded RNA
DTT	Dithiothreitol
eIF	Eukaryotic translation initiation factor
FITC	Fluorescein isothiocyanate
G	Glycoprotein (of VSV)
GCV	Ganciclovir
GFP	Green fluorescent protein
GTP	Guanosine triphosphate
H_20	Hydrogen dioxide (water)
Hb	Hemoglobin
HER	Human epidermal growth factor receptor
HPV	Human papilloma virus
HR	Heat resistant
HSV	Herpes simplex virus
Hu	Human
IFN	Interferon
IFNα	Interferon- alpha
IFNβ	Interferon-beta
Ig	Immunoglobulin
IKKi	Inducible IKB kinase
IL	Interleukin
IMDM	Ischove's modified Dulbecco's medium
Io	Ionomycin

IRF	Interferon regulatory factor
ISG	Interferon-stimulated gene
ISGF	Interferon-stimulated gene factor
JAK	Janus kinase
L	Large polymerase protein (of VSV)
Le	Leader
LSM	Lymphocyte separation medium
M	Matrix protein (of VSV)
M51R	Substitution of methionine 51 for arginine in matrix protein of VSV
MEF	Mouse embryonic fibroblast
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
mRNA	Messenger RNA
N	Nucleocapsid protein (of VSV)
Nab	Neutralizing antibody
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NDV	Newcastle disease virus
NFKB	Nuclear factor Kappa B
NK	Natural killer (cell)
NS	Non structural
OAS	2'-5' oligoadenylate synthetase
P	Phosphoprotein (of VSV)
PBS	Phosphate buffered saline
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PFU	Plaque forming units
PHA	Phytohemagglutinin
PKR	Protein kinase R
Plt	Platelet
PMA	Phorbol 12-myristate 13-acetate
PRD	Positive regulatory domain
Pt	Patient
PWM	Pokeweed mitogen
Q-PCR	Quantitative PCR
RNA	Ribonucleic acid
RNAseL	Ribonuclease L
RNP	Ribonucleoprotein
R-PE	R-Phychoerythrin
RT	Reverse transcriptase
SEM	Standard error of the mean
siRNA	Small interfering RNA
SOCS	Suppressor of cytokine signaling
ssRNA	single stranded RNA

STAT	Signal transducer and activator of transcription
TBK	Tankyrase binding kinase
TC	Tri-colour
Te	Terminator
TK	Thymidine kinase
TLR	Toll like receptor
TNFα	Tumour necrosis factor alpha
TNTC	Too numerous to count
TYK2	Tyrosine kinase 2
U	Unit
VAK	Virus-activated kinase
V221F	Substitution of valine 221 for phenylalanine in matrix protein of VSV
VGF	Vaccinia growth factor
VSV	Vesicular stomatitis virus
WBC	White blood cell
WT	Wild type

1. INTRODUCTION

1.1 Cancer and Currently Available Treatments

On the basis of current mortality rates, one in four Canadians will die of cancer, making it the leading cause of death in the country. An estimated 149 000 Canadians will be diagnosed with some form of cancer in 2005. An increase in the number of new cases annually has been observed in Canada, likely due to the increasing and aging population. Almost half of all new cases occur in the percentage of the population that is over seventy years old [1, 2]. The link between cancer and age may lie in the time required for enough genetic abnormalities to accumulate and eventually develop into a cancer [3]. Chronic Lymphocytic Leukemia (CLL) is the most common form of leukemia in the middle-aged to elderly population in the West and is the focus of this thesis [4].

Conventional treatments for cancer fall into three broad categories: surgical resection, radiation therapy and chemotherapy, with each having certain limitations. Surgery and radiation therapy are limited to the treatment of localized tumours, radiation and chemotherapy can lead to resistant populations of cancer cells and can cause damaging side-effects to rapidly proliferating normal cells [5]. Over the last few decades however, an understanding of the molecular biology of cancer has increased tremendously; differences between normal and cancer cells have been identified and targeted at the molecular level in order to engineer more specific cancer therapeutics. For example, Herceptin (Trastuzumab) is a monoclonal antibody used specifically in the treatment of Human Epidermal Growth Factor Receptor 2 (HER2)-driven metastatic breast cancer [6].

Despite such advances at the molecular level, cancer mortality rates have decreased only marginally since the 1970s [1]. Novel treatments for cancer, such as monoclonal

antibodies, are generally much larger in size than conventional chemotherapeutic drugs, causing inefficient delivery of these drugs to solid tumours via the intravenous route [7]. Clearly, a revolutionary treatment for cancer is required which would be able to overcome the challenges that current therapies face. Oncolytic viruses may represent such a treatment.

1.2 Evolution of Oncolytic Viruses

An ideal cancer therapeutic will kill malignant cells while sparing normal cells. Oncolytic viruses are an innovative example of such a "cancer-specific killer". Naturally selected or genetically engineered replication-selective oncolytic viruses take advantage of genetic defects commonly found in cancer cells. Upon assuming a malignant transformation, cancer cells simultaneously compromise their innate anti-viral defense rendering them more susceptible to infection than normal cells that exhibit an intact viral defense. Replication-competent viruses have the added advantage of being able to spread from cell to cell within a tumour. Collectively, these properties make oncolytic viruses a promising method of specifically targeting and destroying cancer cells.

Anecdotal evidence suggests that occasional tumour regression by virus infection has been reported historically. These observations led to the deliberate treatment of animal models of cancer by viruses as early as the 1920s [8]. Discouragingly however, most viruses which were able to cure tumours were also lethal to the host. For example, in 1937, avian influenza (fowl plague) virus treatment of immune-competent mice with transplanted tumours was able to kill tumour cells, as determined through histology and by the inability of treated tumours to subsequently transplant into naïve mice. However, virus treatment also killed all mice within one week of infection. [9].

Newcastle Disease Virus (NDV) was one of the few exceptions to the rule; NDV could cure mice of lethal Ehrlich ascites tumours without killing the hosts if virus treatment

was administered within six days of tumour implantation [10]. Cured mice were also immune to challenge with the same tumour cells, suggesting that the virus may have elicited desired immune adjuvant effects against the tumour [11].

In 1969, Sinkovics and Horvath used mouse models of cancer to demonstrate the oncolytic capacity of Vesicular Stomatitis Virus (VSV), the oncolytic virus used in this study [12]. Murine lymphoma cells were infected with VSV *in vitro* and mice were subsequently challenged with these infected tumour cells. Approximately half of the mice receiving infected cells rejected the lymphoma, while all of the mice receiving uninfected cells eventually died of tumour burden [12]. In this case, the tumour cells were infected prior to implantation into mice, which is not as impressive as the occurrence of tumour regression *in vivo*. Shortly thereafter, it was shown that oncolysates, antigens isolated from Ehrlich ascites tumour cells infected with VSV *in vitro,* could immunize mice against subsequent challenge with the same cells [13]. It became apparent that oncolytic viruses exert their tumour specific killing in part, by developing a state of anti-tumour immunity.

Such promising findings in mouse models prompted several small human cancer trials. Treatments with West Nile virus, NDV, mumps and other viruses were attempted, as reviewed in Sinkovics and Horvath [8]. However, the majority of these trials were unsuccessful.

In the 1990s, two major developments occurred that motivated a renewed optimism for oncolytic virus therapy. Firstly, in 1991, Martuza *et al* brought about the age of genetically-engineered oncolytic viruses. Construction of a thymidine kinase (TK)-negative mutant of herpes simplex virus 1 was undertaken. This attenuated the virus for replication in normal neurons, but rendered it replication-competent and toxic to human brain cancer cells. This virus was capable of killing human gliomas cell lines *in vitro*. More strikingly though,

the virus also prolonged survival of athymic mice (mice lacking a normal thymus gland and an intact immune system) bearing U87 human glioma xenografts. [14].

The second major development occurred in 1994, when the first durable tumour regressions were observed through oncolytic virus treatment. Lorence *et al.* treated athymic mice bearing human neuroblastoma xenografts with Newcastle Disease Virus (NDV) and complete long-lasting tumour regression was observed in 17 of 18 mice, without any acute or chronic side-effects [15]. Similar observations were also made for mice bearing human fibrosarcoma xenografts [16]. In this case, the ability of an oncolytic virus to exert direct cytolytic effects on tumour cells was witnessed, however residual innate immunity may have still been playing a role.

These promising pre-clinical studies led to the first generation of genetically-engineered oncolytic viruses to be tested in clinical trials [17]. A mutant Adenovirus was constructed that had a deletion in its E1B gene, rendering it replication-competent in p53-deficient tumour cells. 50% of human cancers are known to have a deficiency in their p53 tumour suppressor gene [18]. TK and VGF-deleted Vaccinia virus [19], NS1-deleted Influenza A virus [20] and γ34.5-deleted HSV-1 [21] were also tested in human clinical trials. These genetically engineered attenuated viruses proved to be safe but were not as effective as desired.

The naturally occurring Reovirus and Vesicular Stomatitis Virus (VSV) are believed to be second-generation oncolytic viruses [22-24] that emerged after experience was gained from their predecessors. Although VSV had been briefly tested as a cancer therapeutic in the 1960s, its characteristic neurovirulence was a hindrance to its use at that time. However, the

recent development of neuro-attenuated oncolytic strains of VSV [25] has led to a renewed interest in its potential as a cancer therapeutic.

1.3 VSV Biology and Life Cycle

Stemming from the order *Mononegavirales* and family *Rhabdoviridae,* VSV is the prototypical virus of the genus *Vesiculovirus.* Two common serotypes of the virus are used in the lab: New Jersey and Indiana. VSV manifests clinically in a less severe form than foot-and-mouth disease, a vesicular virus infection in livestock. The virus is endemic in North America, Central America and the northern regions of South America with outbreaks commonly occurring each year. Evidence suggests that the virus propagates in insects, mostly black flies and sand flies, which can further infect animals and humans. Animals are dead-end hosts to the virus, as they can not spread the virus to others [26, 27]. VSV is not a human pathogen and virus infection of humans is rare and non-fatal. If human infection does occur, Influenza-like symptoms ensue, which quickly dissipate as the virus is completely cleared from the host [28, 29].

A VSV particle is enveloped, bullet-shaped and 180 nm long x 75 nm wide [30, 31]. Its non-segmented negative-sense RNA genome of approximately 11 kilobases encodes five mRNAs giving rise to the principal proteins. The five proteins include: Nucleocapsid (N), Phosphoprotein (P), Matrix (M), Glycoprotein (G) and Large polymerase protein (L). The viral polymerase transcribes mRNAs from the 3'end in order of appearance, stopping at the end of each transcript and re-starting inefficiently. Gene expression is regulated in this manner, with genes at the 3'end (such as the N gene for example) being transcribed to a higher copy number than genes at the 5'end (such as the L gene). A 47 nucleotide leader (Le) RNA region is found at the 3' terminus, and a 59 nucleotide trailer (Tr) sequence at the 5' terminus which are both transcribed, but not translated [26, 32].

There are two main components to the virus: a Ribonucleoprotein (RNP) core and a viral membrane. The N, P and L proteins, as well as the single strand of RNA forms the RNP. The role of the RNP is to serve as a transcriptional unit that will synthesize, cap and polyadenylate mRNAs *in vitro*. N is the most abundant of the viral proteins and it binds the RNA genome, rendering the RNA ribonuclease-resistant and allowing it to serve as a template for transcription. The role of the N protein is primarily in genome packaging. L is the RNA-dependent RNA polymerase, which requires a phosphorylated P protein to initiate transcription [32].

The second constituent of the virus is the viral membrane, which encompasses the G and M proteins, in addition to cholesterol and lipids acquired from the host cell. The G protein is a glycosylated trimer which forms the characteristic spikes in viral envelopes. Upon binding to the ubiquitously-expressed host receptor, acidification of the endosome occurs and the G-protein mediated fusion of the viral and endosome membranes is executed thereby allowing the virus particle to enter a host cell. Finally, the M protein has several important roles in directing virus assembly and budding. As well, the M protein mediates induction of virus-associated cytopathic effect (CPE) on the host cell through inhibition of host transcription, inhibition of nucleocytoplasmic transport of mRNAs and proteins, disorganization of the host cytoskeleton leading to cell rounding and detachment, and finally induction of apoptosis in the host cell [26, 32].

The rapid replication cycle of VSV allows for virus progeny to start budding from the host cell in as little as two hours post-infection [33]. The replication cycle begins with VSV adsorption to the cell membrane, which is mediated by the G protein binding to the host cell receptor. Subsequently, receptor-mediated endocytosis and pH-dependent membrane fusion occur allowing RNPs to enter the cytoplasm. Transcription of the five mRNAs then ensues,

followed by translation of the N, P, M and L proteins on free ribosomes in the cytoplasm. The G protein is translated on endoplasmic reticulum-bound ribosomes, where it is glycosylated and transported to the cell membrane [31].

Viral RNA replication commences with the synthesis of a template for the negative-sense RNA progeny viruses; essentially a full-length positive-sense copy of RNA. The RNPs and M proteins are then shuttled to the G protein-containing plasma membranes, where virion packaging and budding occur [31]. The P and M mRNAs may encode additional proteins, whose function has not yet been uncovered [34, 35].

1.4 Human Interferon Production

VSV is so well-known for its exquisite sensitivity to the antiviral effects of Type I Interferons (IFN) that the international unit of IFN activity is identified by the ability to inhibit VSV replication [reviewed in 32]. Interferon was discovered in 1957 by Drs. Issacs and Lindenmann [36] during studies on virus interference, making it the oldest known cytokine [37]. Interferon was identified as the product of influenza virus-infected chick embryo cells that could confer resistance to infection with homologous or heterologous viruses [37].

There are two types of IFN, Type I which is the focus of this study and Type II. Type I IFN consists of at least four subfamilies: IFNα of which there are 14 potential forms in humans and IFNβ, which are the two prototypical forms of Type I IFN. The final two Type I IFN family members are the lesser known IFNω and IFNT. Various cell types produce Type I IFNs. Hematopoietic cells produce IFNα, ω and varying amounts of IFNβ. In addition, IFNβ is also produced by nonhematopoietic cells. The primary functions of all Type I IFNs are to induce an anti-viral state, regulate cell growth and differentiation and to stimulate

MHC- class I antigens. IFNT is also involved in embryo implantation into the uterus. Other functions of Type I IFNs include: inhibiting cell proliferation, stimulating MHC-class II antigens to some extent, as well as activating monocytes, macrophages, NK cells, and cytotoxic T cells. Type I IFNs can also regulate immunoglobulin synthesis by B cells and have pyrogenic functions [37].

Type II IFN consists of a sole family member, IFN γ. IFN γ is synthesized by T cells, large granular lymphocytes and Natural Killer (NK) cells. The major roles for Type II IFN include: macrophage activation, induction of MHC- class I and II antigens, as well as a role in the induction of an anti-viral state [37].

In response to VSV infection, a normal cell will induce Type I IFN production (see Figure 1). During the first wave of IFN production, the transcription factor Interferon Regulatory Factor 3 (IRF-3) which is ubiquitously expressed in the cytoplasm of most cell types, is activated by the binding of pathogen-specific products such as double-stranded RNA (dsRNA) in the case of virus infection [25, 38]. DsRNA is not usually present in an uninfected cell but is present during the replication of many RNA and DNA viruses as an obligate intermediate or a side-product of infection [37]. DsRNA will bind to the toll-like receptor (TLR) 3, expressed intracellularly and on cell surface membranes, leading to the phosphorylation of serine/threonine residues in the 3' terminus (residues 385-405) of IRF-3. *In vitro* studies of human cell lines have demonstrated that the phosphorylation of IRF-3 occurs through the action of a virus-activated kinase (VAK), recently discovered to consist of two non-canoncial IKK-related kinases: IKK-E and tankyrase binding kinase I (TBK-1) [39, 40].

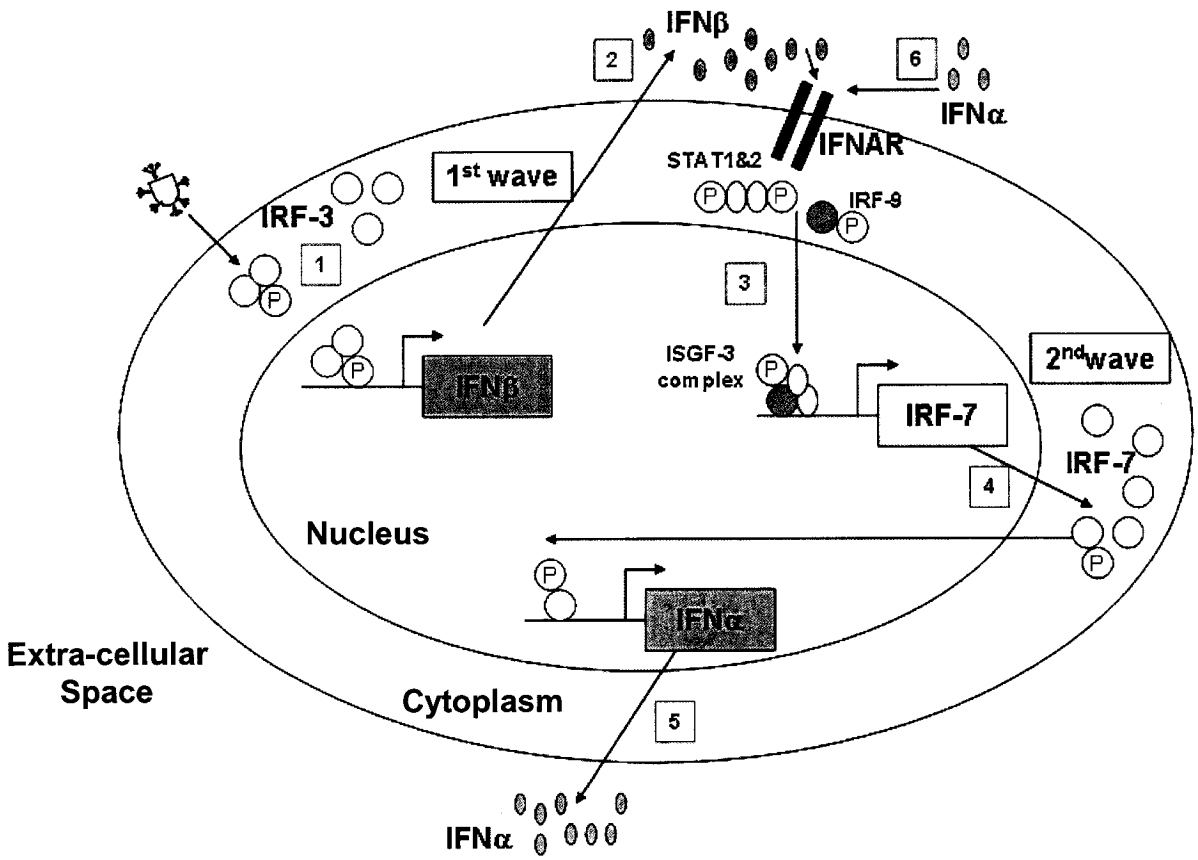
The phosphorylated IRF-3 then enters the nucleus as a homodimer and binds to the IFNβ promoter as part of a larger complex known as the enhanceosome, consisting of NF-KB, IRF-1 and ATF-2/c-Jun. The transcriptional enhancer of the IFNβ gene consists of four positive regulatory domains (PRD) I- IV. NF-KB binds to PRD II, IRF-1 and IRF-3 bind PRD I and III, while ATF2/cJun binds PRDIV. IFNβ production then occurs when all transcription factors are present on the PRDs in multiple copies and are significantly induced by a virus infection or other such stimulus [37, 40].

IFNβ is secreted and binds to the Type I IFN receptor (IFNαR, which has two components: IFNαR1 and IFNαR2). The receptor is in intimate association with two Janus tyrosine kinases (Jak1 and Tyk2) and two Signal Transducer and Activator of Transcription (Stat) molecules, Stat 1 and Stat 2. When Type I IFNs bind to their receptor, Jak1 and Tyk2 phosphorylation occurs followed by phosphorylation of the Stat 1 and 2 molecules. The latter two form a heterodimer and enter the nucleus in association with the DNA binding protein-IFN Regulatory Factor 9 (IRF-9, formerly known as p48). This heterotrimeric complex of Stat 1, Stat 2 and IRF-9, known as IFN-Stimulated Gene Factor-3 (ISGF-3), binds to the IFN Regulatory Factor 7 (IRF-7) promoter to allow for the synthesis of IRF-7 and the start of the second wave of IFN production [40, 41, as reviewed in 32].

IRF-7 is phosphorylated by the VAK and upon nuclear translocation binds to the IFNα promoter to up-regulate the transcription of IFNα and various other Interferon Stimulated Genes (ISGs). Finally, IFNα also signals through the IFNαR. The binding of IFNα to its receptor completes a positive feedback loop that allows for the development of an antiviral state in the infected cell, as well as in uninfected neighbouring cells through the

Figure 1. Waves of IFN production.

1. In the first wave following virus infection, IRF-3, which is ubiquitously expressed in the cytoplasm of all cell types, is phosphorylated by the virus-activated kinase (VAK) and is thereby activated. IRF-3 then translocates to the nucleus as a homodimer, binding the IFNβ promoter along with other members of the enhanceosome complex (ATF-2/c-Jun, IRF-1 and NF-KB).
2. IFNβ is transcriptionally up-regulated, synthesized and secreted. IFNβ then binds to the Type I IFN receptor (IFNαR) on the surface of the infected cell and neighbouring uninfected cells.
3. Binding of IFNβ to the IFNαR leads to the phosphorylation of Stat 1, Stat 2 and IRF-9. Upon nuclear translocation, these three molecules bind the IRF-7 promoter and form the ISGF-3 complex. IRF-7 is synthesized, resulting in the second wave of IFN production.
4. IRF-7 is phosphorylated by the VAK and, upon nuclear translocation, IRF-7 binds to the IFNα promoter, causing the transcriptional up-regulation of IFNα and other downstream Interferon Stimulated Genes (ISGs).
5. IFNα is synthesized and secreted.
6. Like IFNβ, IFNα also binds to and signals via the IFNαR, resulting in a positive feedback loop.


IFNβ
2
6
IFNα
IFNAR
STAT1&2
IRF-9
1st wave
IRF-3
1
3
IFNβ
ISGF-3
complex
2ndwave
IRF-7
IRF-7
4
Nucleus
IFNα
Extra-cellular
Space
Cytoplasm
5
IFNα

IFN priming effect, whereby a low dose of IFN leads to increased IFN production during a subsequent virus infection [25, reviewed in 32].

1.5 Interferon Stimulated Genes

Type I IFNs confer an antiviral state upon the cells by inducing the expression of several IFN-stimulated genes (ISGs). Amongst the most widely-studied ISGs are: 2'-5' oligoadenylate synthetase/ribonuclease L (2'-5'OAS/RNAse L), protein kinase R (PKR) and MxA. Induction of these genes leads to the development of an antiviral state by causing such downstream effects as mRNA degradation, inhibition of host cell translation, cell-cycle arrest and apoptosis [32] (see Figure 2).

In brief, IFN induces the transcription of 2'-5' OAS with dsRNA acting as a cofactor for activation. 2'-5' OAS then polymerizes Adenosine triphosphate (ATP) to produce 2'-5' linked oligoadenylates which activate the latent RNAse L and cause it to cleave single-stranded viral RNA [32].

IFN also induces the transcription of PKR which like 2'-5' OAS is activated by dsRNA. This serine/threonine kinase becomes auto-phosphorylated upon activation and then phosphorylates its targets. One such target is the translation initiation factor, eIF2α, which upon phosphorylation, causes the shutdown of protein synthesis [42]. PKR also plays various other roles, such as enhancing the activity of the NF-KB and IRF-1 transcription factors which are both part of the enhanceosome complex. It also has regulatory functions on p53 (the tumour suppressor gene), p38 MAP kinase (stress activated protein kinase) and on the JNK kinase. The functions of PKR range from antiviral and anti-tumour activities to playing a role in cell growth, cell cycle arrest, cell differentiation and apoptosis [32].

In 2000, Stojdl *et al.* portrayed a role for PKR in IFN-mediated resistance to VSV by showing that PKR -/- mouse embryonic fibroblasts (MEFs) were more susceptible to VSV infection than their wild type (wt) counterparts. The PKR-deficient mice succumbed to a lethal respiratory infection with as little as thirty virus particles given intranasally, while the wt mice could tolerate much higher doses of the virus (approximately $2x10^6$ virus particles). The latter also developed hind limb paralysis, a sign of central nervous system (CNS) pathology. However, if the wt mice were pre-treated with IFNα or β, these symptoms disappeared. In the PKR -/- mice, pre-treatment with IFNα or β did not have any effect on survival or symptoms [43].

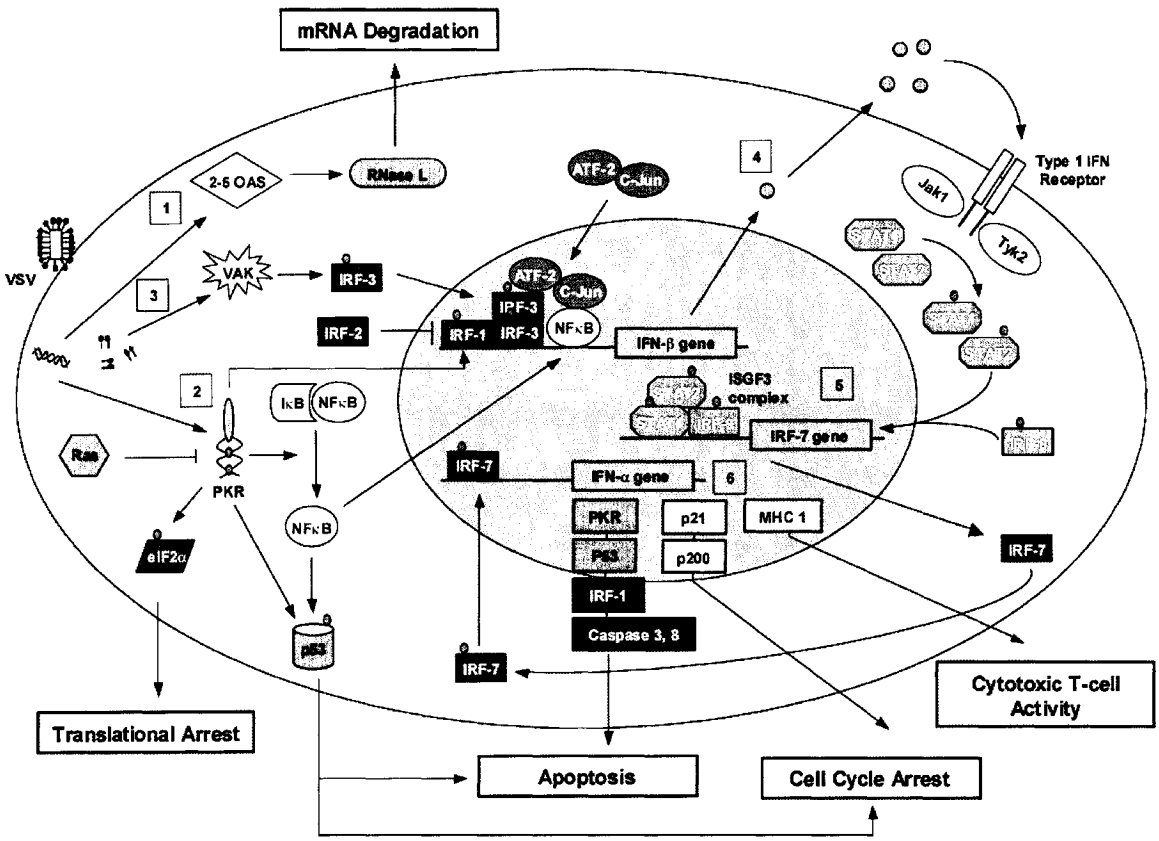
PKR also seems to be a very important antiviral protein as certain viruses have even developed methods of inhibiting the actions of PKR. To mention a few, adenovirus and reovirus sequester dsRNA so that it can not activate PKR while Herpes virus synthesizes a pseudo-substrate for PKR and poliovirus degrades PKR [32].

Finally, MxA is a GTP-binding protein, an intrinsic GTPase that exerts its antiviral actions intracellularly. It can bind cytoskeletal actin and tubulin, in addition to binding viral transcriptases. MxA is present in the cytoplasm of human cells and can inhibit the replication of VSV and orthomyxoviruses, such as influenza. In mice, the protein has two forms- Mx1 and Mx2. Mx1 is nuclear and inhibits the replication of orthomyxoviruses, while Mx2 is cytoplasmic and inhibits VSV replication [37].

IFNs can also have other effects on various pathways such as inducing caspases involved in apoptosis, or increasing cyclin-dependent kinase inhibitors to cause cell cycle arrest and finally, causing MHC I-mediated immune stimulation of cytotoxic T cells [32].

Figure 2. Host antiviral responses to VSV infection.

1. dsRNA activates 2'-5' OAS which in turn activates RNAseL, leading to the degradation of viral mRNA.
2. PKR becomes auto-phosphorylated and is activated in the presence of dsRNA. This event has several downstream effects, including: the phosphorylation of eIF2α which causes translational arrest; NF-KB and IRF-1 activation which form part of the enhanceosome complex on the IFNβ promoter; and finally, transcriptional up-regulation and activation of p53, that leads to cell cycle arrest and cellular apoptosis.
3. Viral antigens activate the virus-activated kinase (VAK), which phosphorylates IRF-3 and causes its translocation (in homodimeric form) from the cytoplasm to the nucleus. There, it forms the enhanceosome complex on the IFNβ promoter (along with NF-KB, IRF-1, ATF-2 and c-Jun) leading to the transcriptional up-regulation of IFNβ.
4. IFNβ is synthesized and secreted from the cell. IFNβ then binds to the Type I IFN receptor on its own cell surface and the surface of neighbouring cells.
5. Upon the binding of IFNβ to its receptor, Stat1 and Stat2 are phosphorylated. In conjunction with phosphorylated IRF-9, these three molecules form the ISGF3 complex on the IRF-7 promoter.
6. IRF-7 is formed and is phosphorylated by VAK, leading to its nuclear translocation. The transcriptional up-regulation of IFNα occurs followed by other downstream effects of IFNs, including the up-regulation of MHC-I that leads to cytotoxic T-cell activity.



(Viral Therapy of Human Cancers, 2005)

1.6 Interferons and Cancer

Although well-known for their antiviral properties, IFNs have also been implicated in the anti-tumour response by inducing such changes in cell physiology as cell cycle arrest, cell differentiation, apoptosis and immunomodulation [reviewed in 32]. As such, IFNs have been employed in the treatment of certain cancers including hairy cell leukemia, chronic myelogenous leukemia, cutaneous T cell lymphoma, melanoma, renal cell carcinoma and AIDS-related Kaposi's sarcoma. Unfortunately, IFN therapy proved not to be as promising as was originally anticipated. The more common malignancies such as breast, prostate and lung cancer are unable to respond to IFN therapy. As well, of the few cancers which are responsive to IFN, usually only a transient response is exhibited due to the development of IFN-resistant subclones [37, 44, reviewed in 45].

The inability to respond to IFN therapy may be due to the selective pressure that cancer cells experience during their malignant transformation to compromise portions of their IFN pathway and allow for a growth advantage over normal cells [32]. Defects in the IFN pathway may manifest through the inability of cancer cells to produce IFN subsequent to virus infection, to respond to IFN through the IFNαR or through defects in specific ISGs [32].

The lack of IFN production in cancer cells may occur through deletions on chromosome 9p, where the IFNα and IFNβ loci are located, which is known to occur in several human gliomas [46]. In cervical carcinomas, the transcription factor IRF-3 is bound by the E6 oncoprotein of human papillomavirus (HPV), thereby inhibiting the transcriptional up-regulation of IFNβ [47]. As well, IRF 1, a known tumour suppressor gene, is thought to

play a role in the normal antiviral IFN response, but is located at position 5q31, that is frequently deleted in myeloid leukemias [reviewed in 32].

Defects in the IFN response, such as in Stat signalling or in the formation of ISGF3 may also occur in cancer cells. For example, cutaneous T cell lymphoma can acquire resistance to IFN due to the presence of the Suppressor of Cytokine Signalling (SOCS-3) that inhibits Stat-1 activation. Certain human melanoma cells have also been shown to be deficient in ISGF3 components, particularly Stat-1 [reviewed in 32].

Finally, the inhibition of ISGs can also occur in cancer. In prostatic hyperplasia cells, 2'-5' OAS and IRF-9 are down-regulated in tumorigenic samples, as compared to non-tumorigenic samples [reviewed in 32]. Secondly, the murine homologue of PKR, which is encoded by the Tik gene was discovered to be catalytically inactive in a murine lymphocytic leukemia line (L1210) through the rearrangement of just one allele [48]. As well, PKR may also play a role in oncogenesis through its upstream and downstream signalling pathways.

Upstream of PKR is the Ras pathway, which is activated in most malignant cells. Upon activation, Ras induces an endogenous PKR inhibitor to down-regulate PKR activity [49, reviewed in 32]. A downstream effect of PKR is the activation of tumour suppressor gene p53. Defective p53 or Ras activation occurs in several cancers [18, reviewed in 22 and 23], linking PKR to abnormal signalling pathways occurring in most malignant cells.

Defects in the IFN pathway seem to be a recurrent theme in cancer and exploitation of this phenomenon forms the basis of the selective replication of VSV in cancer cells.

1.7 Interferon-inducing VSV mutants

Mutant forms of VSV exist either in nature or have been genetically engineered to induce IFN in order to widen the therapeutic index for these viruses. If a virus could induce IFN, it could be attenuated for replication in normal cells, but would retain its tumour-killing

properties. Wt-VSV, through the action of the M protein, is able to block host cell transcription [50]. In 1992, Black and Lyles studied the M protein gene from a mutant version of VSV- T1026R1 [51]. This version of VSV was defective in shutting down host gene expression and this property was attributed to a Methionine to Arginine mutation in the M protein at position 51 (M51R). The mutant VSV could not inhibit transcription from the cellular IFNβ promoter or from a viral promoter (SV40) thereby rendering it a good IFN inducer in contrast to wt VSV which suppressed IFN induction. Other viral or cellular factors were also likely involved in the IFN induction process.

In 2003, Obuchi *et al.* genetically engineered a VSV expressing the murine IFNβ gene and a similar form of VSV expressing the human IFNβ gene [52]. These viruses would allow for an IFN-induced antiviral effect to be mediated in normal fibroblasts, while killing malignant cells that were not receptive to IFNαβ. This phenomenon held true both *in vitro* and *in vivo* in immunocompetent metastatic lung disease models.

Finally, Stojdl *et al.* developed a series of oncolytic attenuated strains of VSV known as AV1, AV2 and AV3 [25]. AV1 was the M51R virus referred to above, AV2 had two mutations (V221F and S226R) in its M protein that differed from the wt M. Lastly, AV3 exhibited a complete deletion of methionine 51 (ΔM51), which was the virus used in this study. All three viruses could propagate in cancer cells but would not kill normal fibroblasts due to the induction of Type I IFNs.

1.8 Chronic Lymphocytic Leukemia

Chronic Lymphocytic Leukemia (CLL) is the most common adult leukemia in the West, with increasing incidence of disease after the age of 50 [53, 54]. This disorder of morphologically mature lymphocytes causes progressive accumulation of malignant cells in

the blood, bone marrow and lymphatic system. Characteristically high lymphocyte counts at or above100 x 10^9/L are present in the blood, whereas a normal count would range from 1-4 x 10^9/L [4].

Contrary to most cancers, the accumulation of CLL cells is believed to occur by a prolongation of cell survival rather than the excess proliferation of a malignant clone [55, 56]. Survival and growth signals from the micro-environment of CLL cells allow them to escape apoptosis and to proliferate. Up-regulation of anti-apoptotic genes such as BCL-2, survivin and MCL1 is observed in leukemia cells [55, 57-59]. 99% of CLL cells have been shown to be arrested in the G_0G_1 phase of the cell cycle, with a half-life of several months, as opposed to the 5-7 day half-life of normal B cells. Interestingly however, CLL cells slowly undergo apoptosis when cultured *in vitro*, suggesting that the tumour cell microenvironment contains survival factors for these cells [56].

CLL can manifest as a benign slow-developing disease or a rapidly fatal disorder [56]. The overall 5 year survival rate for CLL is about 60%, but this is dependent upon the stage of the disease at presentation. There is no clear clinical staging system for CLL but the Rai staging system and Binet classification are the most common. Therapeutic options for CLL depend on the stage of disease and come in a variety of forms from simple observation, to treatment with alkylating agents, fludarabine, radiation therapy, combination chemo-therapy or monoclonal antibodies. More aggressive treatments for advanced stages of disease like bone marrow and peripheral stem cell transplants are currently under clinical investigation for the treatment of CLL [53].

1.8.1 CLL Cell Surface Marker Expression

CLL cells can be differentiated from other hematological malignancies through the expression of specific cell surface markers used to immunophenotype the cells by flow

cytometry. As CLL is a B-cell derived disease, the cells express pan-B cell markers such as CD19, CD20 (although weakly), CD24 and CD79a. However, the cells do not express CD79b. Surface membrane immunoglobulins IgM and IgD are weakly expressed in CLL, although the cells have a characteristic clonally expanded immunoglobulin light chain expression (kappa or lambda). CD22 is weakly expressed or not present at all, FMC7 is not expressed, while CD23 and CD5 are expressed. CD5 is also found on a subset of B cells, which account for about 15% of the naïve B cells [53, 54].

An individual B cell will express either the kappa or the lambda immunoglobulin light chain. A polyclonal population of B cells will consist of a kappa to lambda ratio of approximately 2:1 [54, 55]. However, hematological malignancies represent the clonal proliferation of a single cell type that has been arrested at a particular stage of differentiation. The classic presentation of a B cell malignancy is a monoclonal B cell population with a "light chain restriction" (a single immunoglobulin light chain) [55]. For instance, an oncogenic transformation could take place in an individual B cell that expresses the kappa immunoglobulin light chain. The repeated mitotic division of the transformed cell ensues causing the over-representation of the kappa immunoglobulin light chain in the greater B cell population and making lambda expression negligible. As such, a clonally restricted cell population develops.

1.9 CLL and Type I IFNs

IFNα has been used in conjunction with certain CLL therapies in an attempt to improve their therapeutic index. When IFNα is given as a maintenance drug to fludarabine monophosphate therapy it can extend the duration of remission [60]. In addition, IFNα

priming may increase the effectiveness of CD20 monoclonal antibody (rituximab) treatment in CLL patients by up-regulating CD20 antigen expression on CLL cells [61].

However, CLL is often insensitive to the pro-apoptotic effects of IFNα which may promote survival of these cells *in vitro* [62]. A major mediator of the anti-proliferative effects of IFNα is PKR. In a study of CLL patients and the catalytic activity of their PKR enzymes by Hii *et al.* it was discovered that 21 of 28 CLL patients had an inactive full-length PKR protein likely due to the presence of a soluble inhibitor of PKR. This breakdown in PKR activity is thought to contribute to the maintenance of CLL and by extension, its inability to respond to IFNα therapy [56].

In contrast, Jurlander *et al.* demonstrated that CLL cells express functional IFNα and IFNγ receptors and, upon their activation, phosphorylation of Stat proteins occurs [63]. These findings would suggest that CLL cells have a certain portion of their IFN pathway intact and therefore should be responsive to IFN therapy.

1.10 Oncolytic Specificity of VSV

Upon acquiring the ability to proliferate uncontrollably, cancer cells concurrently compromise other cellular properties such as their antiviral defences. Most normal lymphocytes and bone marrow progenitor cells are resistant to VSV infection. Lichty *et al.* demonstrate that T cells, B cells and normal bone marrow progenitors are refractory to the virus, while neutrophils and monocytes are infected by VSV. This difference in VSV susceptibility between normal and cancer cells can be exploited allowing for leukemia cells to be purged from a culture of peripheral blood progenitor cells [45].

The NCI-60 human tumour cell panel was tested for Type I IFN defects. It was discovered that 81% (42 out of 52) of all cell lines tested harboured a defective IFN pathway,

rendering these cells susceptible to killing by wt and IFN-inducing mutants of VSV [25]. When examining VSV as a leukemolytic agent, it was found that VSV could kill 11 out of 12 human leukemia cell lines tested and 3 of 3 primary Multiple Myeloma patient samples [45]. Certain leukemias have also been shown to possess Type I IFN defects. For example, 29% of Acute Lymphoblastic Leukemia (ALL) patients have deletions in their IFNα and β genes [64].

Due to the success of VSV as a leukemolytic agent, oncolytic viruses have recently been examined as a potential therapy for CLL. Preliminary work has shown that the majority of primary CLL patient samples tested were resistant to VSV infection.

1.11 Research Objective

VSV is an effective cancer therapeutic in most cancer cells. However, it is not effective in combating CLL. The primary research objective is to further understand the mechanism(s) behind the inherent resistance of CLL to VSV infection and to determine if CLL cells can be sensitized to VSV. It is hypothesized that CLL cells exhibit an intact IFN pathway, which renders them resistant to VSV. The abrogation of this IFN pathway may allow VSV infection of CLL cells.

1.12 Experimental Approach

Leukemia cell lines, primary leukemia patient samples and normal control cells were obtained, that were either able to be infected by VSV (such as AML 3) or that were resistant to VSV infection (such as CLL). Where possible, flow cytometry was used to immunophenotype the samples. Subsequently, all cell types were either mock-infected or infected with ΔM51-GFP-VSV at a high MOI, to ensure every susceptible cell was infected with at least one virus particle. VSV infection was measured by the proportion of GFP-

expressing cells, as identified by flow cytometry. RNA was extracted from all cell types at baseline and at relevant time points post-infection. IFN bioassays were performed on cell lines that would serve as positive controls for gene expression studies. A quantitative PCR-based approach was taken to examine gene expression levels from the first and subsequent waves of IFN production in all cell types. Finally, CLL cells were activated with cellular mitogens to observe if changes in VSV sensitivity, coinciding with changes in IFN transcript levels would be observed post-activation.

2. MATERIALS AND METHODS

2.1 Cell Culture

A549 (from the National Cancer Institute's panel of 60 human tumour cell lines), HeLa (ATCC) and Vero cells (ATCC) were cultured in HyQ Dulbecco's Modified Eagle Medium (containing high glucose, L-glutamine and sodium pyruvate) (HyClone). Media was supplemented with a 3:1 mixture of donor bovine serum (Medicorp) to fetal calf serum (CanSera), to a concentration of 10%. HyQ phosphate buffered saline (PBS) (HyClone) was used to wash the cells and 0.05% HyQ Trypsin (HyClone) to make a single cell suspension. All primary suspension cells (CLL, ALL, bi-phenotypic acute leukemia cells and B cells) as well as suspension cell lines (AML 3 (kindly provided by Dr. Mark Minden, Ontario Cancer Institute) and Jurkat cells (ATCC)) were grown in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco). Media was supplemented with 10% fetal bovine serum (ICN Biomedicals). All cells were incubated at $37^{O}C$ in 5% CO_2.

2.2 Viruses

The two viral strains used in this study were the heat-resistant (HR) strain of wild-type (wt) VSV- Indiana serotype, as well as the IFN-inducing mutant of wt-VSV Indiana, also known as ΔM51-VSV (deletion of the methionine at position 51 in the matrix protein of VSV). Both of the above viruses express the green fluorescent protein (GFP) gene that was inserted between the G and L sequences of the VSV genome. GFP gene expression is driven by the viral promoter and as such, measurement of GFP expression represents a surrogate for the measurement of viral protein expression. The two viruses are described in detail in Stojdl *et al.* [25].

2.3 Virus Infections

Vesicular Stomatitis Virus (VSV) infections of suspension cells were carried out in a minimal volume of serum free media (100 μL in 15 mL polypropylene Falcon tubes) at 37°C for 45 minutes. At this point, fresh serum-containing media was added to adjust the culture concentration to approximately $1x10^6$ cells/mL and the cells were transferred to the appropriate culture flasks. Confluent adherent cell cultures were also infected in 100 μL of serum free media at 37°C for 45 minutes after which fresh serum-containing media was added to the cells. However, infections were performed in 6-well dishes or 60 mm plates. Cells were infected at various multiplicities of infection (MOI) and for different lengths of time. The proportion of virally-infected cells was identified by their green fluorescent protein (GFP) expression, as measured by flow cytometry, as done by Lichty and colleagues [45].

2.4 Plaque Assays

Cell culture supernatants were centrifuged in the Sorvall RT 6000D at 1200 rpm for 5 minutes to rid the supernatants of cell debris. The amount of VSV in cell culture supernatants was measured by plaque assays. Serial dilutions of supernatants were made in 100 μL of serum-free media and added to 60 mm plates of confluent Vero cells. After 45 minutes at 37°C, cells were overlaid with 0.5% agarose in α-mem + 10% serum (at approximately 42°C). Upon solidification of the agarose overlay, the plates were incubated overnight at 37°C and plaques were counted by visual inspection.

2.5 Patient/Donor Samples

Venous peripheral blood samples were obtained from CLL, ALL and bi-phenotypic acute leukemia patients of the Ottawa Hospital. Blood samples were also taken from healthy donors in the lab. Blood was drawn after the informed consent of patients/donors under a

protocol approved by the Ottawa Hospital Internal Review & Ethics Board (IREB). 7-15 mL of venous peripheral blood was drawn in sodium heparin coated tubes (BD Vacutainer).

2.6 Processing of Peripheral Blood Samples

7 mL of peripheral blood was layered on 3 mL of Lymphocyte Separation Medium (LSM) (ICM Biomedicals) in 15 mL polypropylene round-bottom tubes (Becton Dickinson), which were centrifuged at 1700 rpm for 20 minutes. Subsequently, the peripheral blood mononuclear cell (PBMC) layer, at the plasma-LSM interface, was isolated and re-suspended to 20 mL in PBS. Cells were pelleted by centrifugation at 1500 rpm for 5 minutes. PBMC were re-suspended in media, counted using the Bright-Line hemacytometer (Hausser Scientific) and adjusted to$1x10^6$ cells/mL in culture media. Cells were incubated at $37^{O}C$ in 5% CO_2.

2.7 Fluorescent Antibody Labeling

$1x10^6$ PBMC were placed in 15 mL polypropylene Falcon tubes (Becton Dickinson) and centrifuged at 1200 rpm for 5 minutes. Cell pellets were re-suspended in 100 μL of wash buffer (PBS+1% serum) and transferred to 5 mL polystyrene round-bottom Falcon flow cytometry tubes (Becton Dickinson). Cells were incubated with 5 μL of the appropriate anti-human antibody: CD19-tri-colour (TC) conjugate (Caltag), CD5- R-phychoerythrin (R-PE) (Caltag), CD69- fluorescein isothiocyanate (FITC) conjugate (Caltag), isotype antibody (IgG1-FITC conjugate) (Caltag) or CD19 Kappa-FITC/Lambda-R-PE (Serotec). The tubes were shaken briefly and placed on ice for 30 minutes and protected from light. Subsequently, 2mL of wash buffer was added to the cells, which were then centrifuged at 1200 rpm for 5 minutes. Cell pellets were re-suspended in 300 μL of wash buffer and flow cytometric analysis was performed.

2.8 Flow Cytometry

Adherent cells were trypsinized to be made into a single cell suspension and analyzed in their culture media. Suspension cells were also analyzed in their culture media. All flow cytometric analysis was performed on the FACScan flow cytometer (BD Biosciences) using the argon ion laser and CellQuest 3.3 software (BD Biosciences). Ten thousand events were acquired in each sample. Cells were analyzed on the forward scatter and side scatter axes. FITC emission was measured as a green signal by the FL1 detector, R-PE was measured as an orange signal by the FL2 detector, and Cy-Chrome/tri-colour was measured as a violet signal by the FL3 detector.

2.9 Fluorescence-activated Cell Sorting

PBMC from healthy donors were labeled with anti-human CD19-TC conjugate antibody (Caltag). Labeled cells were suspended in culture media and adjusted to $1x10^7$ cells/mL. Cells were then filtered through a 70 μm nylon cell strainer (BD Falcon) into 5 mL flow cytometry tubes. Fluorescence-activated cell sorting was performed on the MoFlo (DakoCytomation) at the OHRI flow cytometry facility in order to isolate a population of normal B cells (CD19-expressing cells). The CD19-expressing cells were collected in 5 mL flow cytometry tubes containing 1mL of culture media and were sub-cultured as soon as possible after sorting.

2.10 Measurement of IFN Responsiveness

HeLa cells, known to be IFN-responsive [65], were seeded at 250 000 cells per well in a 6-well dish for 24 hours. Dilutions of human IFNα (Intron a2b, Schering) and human IFNβ (Rebif, Serono) were made in DMEM + 10% serum. HeLa cells were treated with increasing doses of huIFNαβ for 18-24 hours. Cells were then infected with ΔM51-GFP-

VSV at an MOI=3 for 6 hours. Subsequently, cells were lifted off the plate through trypsinization and re-suspended in their appropriate culture media. Using 0.4% Trypan blue solution (Sigma) and a hemacytometer, trypan blue exclusion analysis was performed to measure cell viability. The proportion of virally-infected cells was identified by their GFP expression, as measured by flow cytometry. Figure 10 shows the experimental design of the IFN responsiveness bioassay.

2.11 IFN Induction in A549 Cells

$1x10^7$ A549 cells, shown to be IFNβ-producing [65], were seeded in a 150 mm dish for 24 hours. Cells were then infected at an MOI=0.1 with ΔM51-GFP-VSV for 18-24 hours. Supernatants from infected cells were collected and centrifuged to remove cell debris at 1200 rpm for 5 minutes.

2.12 Filtering of Supernatants

The following solutions were used to prime or wash the positively charged Mustang Q filters (Pall): 4 mL of 1 M sodium hydroxide (NaOH) solution, followed by 4 mL of sodium chloride (NaCl) solution and finally, 10 mL PBS. Solutions were delivered to the filter at a constant rate of 1-4mL/minute using the Bio-Rad Econo pump. Subsequently, the cell culture supernatants were passed through the filters.

2.13 Treatment of HeLa Cells

HeLa cells were treated with the filtered supernatants for 24 hours. Supernatants were removed and HeLa cells were challenged with ΔM51-GFP-VSV at an MOI=0.1 for 24 hours. The proportion of virally-infected cells was identified by their GFP expression as measured by flow cytometry and visualized by the Leica MZFLIII fluorescent microscope and camera.

2.14 IFN Neutralization

1 μL (200 neutralization units) of sheep polyclonal anti-human neutralizing antibody to IFNβ (Biosource) was incubated with 500 μL of filtered supernatant for 18-24 hours at 4°C, prior to applying the supernatants to HeLa cells. Figure 11 shows the experimental design of the IFN production bioassay.

2.15 Generation of cDNA

Total RNA was extracted from cells using the RNAeasy kit (Qiagen) as per the manufacturer's protocol. 1 μg of RNA was isolated and adjusted to 25 μL with 0.1% diethyl pyrocarbonate (DEPC)-treated H_20. cDNA was generated on the Gene Amp PCR System 9700 thermocycler. Denaturation of RNA occurred at 65°C for 5 minutes. Subsequently, samples were placed on ice for 5 minutes, while a master mix was added to the samples. The 25 μL master mix that was added to each 1 μg RNA sample consisted of: 2.5 μL 2.5 mM dNTPs (Invitrogen), 2.5 μL random primers (3μg/μL) (Invitrogen), 10 μL 5x first strand buffer (Invitrogen), 5 μL 0.1M DTT (Invitrogen), 2.5 μL Superscript II (Invitrogen) and 2.5 μL RNAse inhibitor (40U/μL) (Ambion). The PCR reaction then took place on the thermocycler at 42°C for 60 minutes followed by 70°C for 15 minutes and a cooling step to 4°C. Samples were either stored at -20°C or used immediately for Q-PCR.

2.16 Q-PCR

2.16.1 Q-PCR for IFNβ mRNA

Primers for the amplification of human IFNβ were sense 5'- CATTACCTG AAGGCCAAGGA-3' and antisense 5'-CAGCATCTGCTGGTTGAAGA-3' (Sigma Genosys). The optimized primer concentration for IFNβ was 20 pmol/μL and magnesium concentration was 4 mM. The experimental run protocol used for Q-PCR was initial

denaturation at 95 °C with a 10 minute hold, 50 cycles of amplification and quantification consisting of 95°C for a 2 second hold, 59°C with a 10 second hold and 72°C with an 8 second hold, with a single fluorescence measurement. The amplifications were followed by a melting curve analysis with one cycle from 70-95°C (with a heating rate at 0.1°C/second and a continuous fluorescence measurement) and finally a cooling step to 40°C for 10 seconds.

Q-PCR reactions were performed three independent times. β2-microglobulin served as a reference gene for the two target genes- IFNβ and IFNαR1. Signals from the target genes were normalized to the signal from β2-microglobulin.

Q-PCR was performed on Roche Lightcycler Technology (Roche Diagnostics) using the Lightcycler FastStart DNA Master Sybr Green 1 kit, as per manufacturer's instructions. Relative quantification was performed using RelQuant software. Crossing points were converted to relative quantities based on standard curves for each gene. The A549+V sample was used as a calibrator (an internal control) to normalize for differences between individual rounds of Q-PCR.

All PCR products were isolated from glass capillaries by centrifugation and subsequently underwent size-gradient gel electrophoresis on 2% agarose (UltraPure-Invitrogen) gels at 80V for 2 hours. Staining with ethidium bromide (Omnipur, 10 mg/mL) was performed to visualize DNA bands on the gel with the Epi Chem II Darkroom camera (UVP Laboratory products, BioMed Lab Supplies) and results were documented with LabWorks version 4.0 software.

2.16.2 Q-PCR for IFNαR1 mRNA

Primers for the amplification of the human IFNαR1 gene were 5'-ATCGG TGCTCCAAAACAGTC-3' and antisense 5'-GTGCTCTGGCTTTCACACAA-3' (Sigma

Genosys). Optimized primer concentration for IFNαR1 was 20 pmol/μL and magnesium concentration was 2 mM. The experimental run protocol used for Q-PCR was initial denaturation at 95 °C with a 10 minute hold, 50 cycles of amplification and quanitification consisting of 95°C for a 2 second hold, 59°C with a 10 second hold and 72°C with an 8 second hold, with a single fluorescence measurement. The amplifications were followed by a melting curve analysis with one cycle from 70-95°C (with a heating rate at 0.1°C/second and a continuous fluorescence measurement) and finally a cooling step to 40°C for 10 seconds.

Q-PCR reactions were performed three independent times using Roche Lighcycler technology. Details of the equipment and kit used as well as, relative quantification and gel electrophoresis techniques employed are provided in Section 2.16.1.

2.16.3 Q-PCR for b2-microglobulin mRNA

Primers for the amplification of β2-microglobulin were sense 5'-CGCTAC TCTCTCTTTCTGGC-3'and antisense 5'-AACTTCAATGTCGGATGGAT-3' (Sigma Genosys). Optimized primer concentration for β2-microglobulin was 30 pmol/μL and magnesium concentration was 2 mM. The experimental run protocol used for Q-PCR was initial denaturation at 95 °C with a 10 minute hold, 40 cycles of amplification and quanitification consisting of 95°C for a 1 second hold, 52°C with a 10 second hold and 72°C with a 6 second hold, with a single fluorescence measurement. The amplifications were followed by a melting curve analysis with one cycle from 61-95°C (with a heating rate at 0.1°C/second and a continuous fluorescence measurement) and finally a cooling step to 40°C for 20 seconds.

Q-PCR reactions were performed three independent times using Roche Lighcycler technology. Details of the equipment and kit used as well as, relative quantification and gel electrophoresis techniques employed are provided in Section 2.16.1.

2.17 CLL Cell Activation

CLL cells were activated for 48 hours with 25 ng/mL phorbol 12-myristate 13-acetate (PMA) and 1 μg/μL Ionomycin (Io) given concomitantly to the cells [66]. CLL cells were also treated for 48 hours with 2 μg/mL phytohemagglutinin (PHA) or 1% pokeweed mitogen (PWM) as per previously established protocols. The cell activators were donated by the laboratory of Dr. Jonathan Angel, OHRI. CLL cell activation was verified through expression of the activated leukocyte marker, CD69, by flow cytometry.

2.18 Statistical Analysis

All statistical analysis was performed with Graphpad Prism 3.0 software. Data are presented as the mean +/- standard error (SEM).

3.0 RESULTS

3.1 Clinical Patient Data

Blood samples were taken from seven patients, including five patients with Chronic Lymphocytic Leukemia (CLL), one patient with Acute Lymphoblastic Leukemia (ALL) and one bi-phenotypic acute leukemia (biAL) patient (Figure 3). Three female and four male patients were examined, who ranged from 42-81 years old. The time elapsed since diagnosis with the disease also ranged from a recent diagnosis to being diagnosed with the disease for greater than nine years. For the five patients with CLL, most of the circulating lymphocytes belonged to the malignant clone. With the acute leukemia patients, blast cells, representative of the malignant cell population, were present in both patients to varying degrees. In the case of the ALL patient, almost half of the white blood cells consisted of blast cells in contrast to the bi-phenotypic acute leukemia patient, where the majority (84%) of white blood cells were made up of blast cells. The above patient data shows a diverse population for this study.

3.2 Immunophenotyping of CLL Cells (CD19+5+Kappa+)

Immunophenotyping for characteristic CLL cell surface markers was performed on PBMC in order to verify that the majority of peripheral blood mononuclear cells (PBMC) isolated from each CLL patient blood sample belonged to the CLL clone. CLL cells generally express CD19, CD5 and a single immunoglobulin light chain isotype- Kappa or Lambda. In Figure 4, flow cytometry on PBMC from a representative CLL patient is presented. PBMC were labeled with antibodies to CD19, CD5 and CD19-Kappa/Lambda. It was observed that approximately 97% of cells in the viable cell population (gated in Region 1 or R1) express CD19 and CD5 (Figure 4D). 20% of the viable cell population also

Figure 3. Patient data tables.

In Figure **3A**, the patient (Pt)'s age, gender and time from diagnosis to obtaining the blood sample are given. The white blood cell count (WBC), lymphocyte concentration, hemoglobin concentration (Hb) and platelet count (Plt) at the time the samples were obtained for this study are presented. For the five patients with CLL, most of the circulating lymphocytes belong to the malignant clone. The lymphocyte concentration for CLL patient 3 was not available (N/A) at the time of blood draw. The blood counts and differential of the patients with acute leukemia (ALL1= acute lymphoblastic leukemia patient 1, biAL1= bi-phenotypic acute leukemia patient 1) are given in Figure **3B**. The blast cells represent the malignant cell population in acute leukemia patients which are present in both patients to varying degrees.

3A. Clinical information for leukemia patient samples

Pt #	Gender	Age	Time since diagnosis	WBC ($x10^9$/L)	Lymphocytes ($x10^9$/L)	Plt ($x10^9$/L)	Hb (g/L)
CLL1	F	80	> 2 years	87.7	80.6	215	124
CLL2	M	81	>9 years	133.9	125.9	113	134
CLL3	M	73	>9 years	185.4	N/A	81	46
CLL4	F	64	>1 year	481.1	461.9	113	48
CLL5	F	79	>5 years	45.9	39	365	119
ALL1	M	42	1 day	8.1	3.1	274	124
biAL1	M	57	1 day	220.8	13.3	58	107
Normal				3.0-10.5	1.0-4.0	125-400	130-170

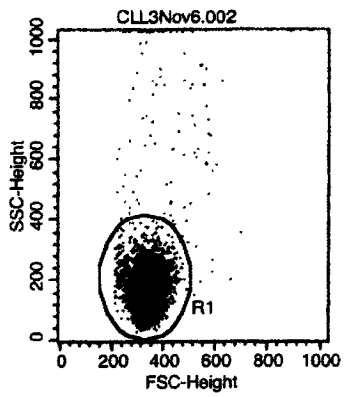
3B. White blood cell differential for acute leukemia patients

	Normal ($x10^9$/L)	Pt# ALL1 ($x10^9$/L) [% of total WBC]	Pt# biAL1 ($x10^9$/L) [% of total WBC]
WBC	3.0-10.5	8.1 [100]	220.8 [100]
Lymphocytes	1.0-4.0	3.1 [38.3]	13.3 [6.0]
Basophils	0.0-0.1	0	2.2 [1.0]
Neutrophils	2.0-7.5	1.2 [14.8]	6.6 [3.0]
Bands	<0.9	0	4.4 [2.0]
Metamyelocytes	<0.2	0	2.2 [1.0]
Myelocytes	0.0	0	0
Promyelocytes	0.0	0	6.6 [3.0]
Blasts	0.0	3.8 [46.9]	185.5 [84.0]

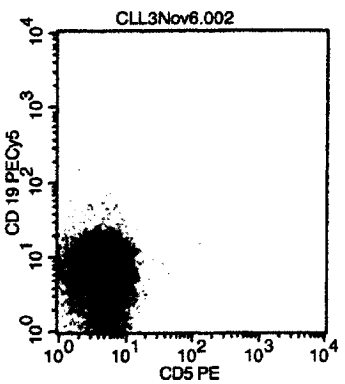
Figure 4. CLL patient cell immunophenotyping.

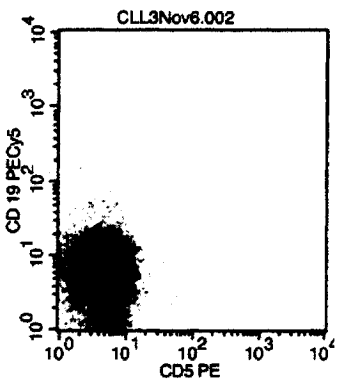
CLL cells are CD19+5+Kappa+. Representative flow cytometry data is presented from one of five CLL patients. 10, 000 cells were acquired in each case on the Becton Dickinson Flow Cytometer. Each sample consisted of $1x10^6$ PBMC incubated with the appropriate antibody. Antibodies include CD19- PE-Cy5, CD5-R-PE, or CD19 Kappa-FITC/Lambda-R-PE. In the sample **A**, R1 represents the major population of viable lymphocytes, K=Kappa, L=Lambda. Sample **B** represents the unstained control for CD19/5, which showed a similar phenotype to Sample **C**- the isotype control for CD19/5. Sample **D** is the CD19+5+ population. Sample **E** is the unstained CD19 K/L control. CD19 K/L are internal isotypes, hence these controls were not performed. Sample **F** is the CD19 K+ population.

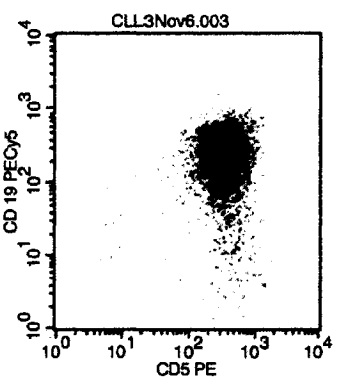
4A. Unstained control-forward/side scatter



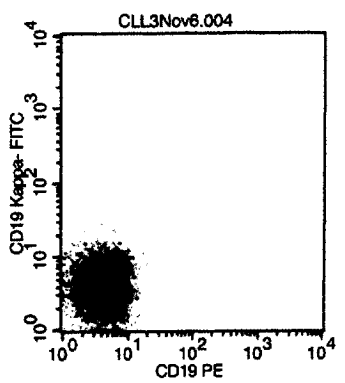
4B. Unstained control- CD19/5 **4C. Isotype control- CD19/5** **4D. CD19+5+**

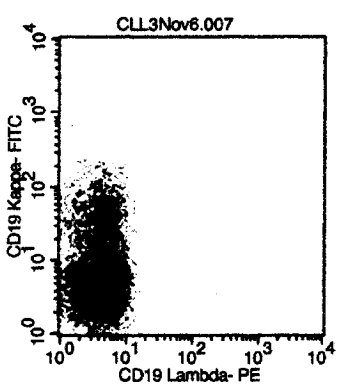






4E. Unstained control- CD19 K/L **4F. CD19 K+**





expresses surface Kappa light chains. There is an absence of Lambda light chain-expressing cells (Figure 4F). While CLL cells mainly express cytoplasmic immunoglobulins, the surface antigen expression on a proportion of the cells indicates that the isolated PBMC are almost all CLL cells.

3.3 CLL Cell Resistance to VSV

This study builds on previous laboratory observations suggesting that CLL cells are resistant to ΔM51-GFP-VSV. In this study, an additional five CLL patients were examined for sensitivity to ΔM51-GFP-VSV. In the experiments outlined below, the proportion of infected cells is measured by the percentage of GFP-expressing cells, as identified by flow cytometry. Cells were incubated with ΔM51-GFP-VSV for 24 hours at a multiplicity of infection (MOI) of three, to ensure that most cells were exposed to a viral particle. The percentage of GFP-expressing cells was minimal after a 24-hour incubation with three virus particles per cell (Figure 5A). Representative flow cytometry data is presented in Figure 5B-D. CLL cells were incubated with ΔM51-GFP-VSV at an MOI of three for six hours. The proportion of virally-infected cells was marginal. CLL cells were further exposed to ΔM51-GFP-VSV or to wild-type (wt-GFP-VSV) for a maximum of six days. The percentage of GFP-expressing cells was negligible (Appendix I). Cells were also counted in a hemocytometer following staining with trypan blue to measure cell viability (Appendix II). Correlations exist between the numbers of dead cells and the proportion of infected cells. The above flow cytometry and cell viability data suggests that primary CLL patient cells are resistant to VSV. Blood samples were taken from a patient with ALL and a patient with bi-phenotypic acute leukemia to assess if other primary leukemia cell types could be infected. PBMC were isolated and infected with ΔM51-GFP-VSV for 24 hours at an MOI of three.

Figure 5. CLL cells are resistant to VSV.

A VSV sensitivity of various primary cells and cancer cell lines. Five CLL, one ALL and one bi-phenotypic acute leukemia patient sample as well as normal primary B cells (mean of two infections is shown for B cell data), were infected with ΔM51-GFP-VSV at a multiplicity of infection (MOI) of 3 for 24 hours. Patient samples, with the exception of the bi-phenotypic acute leukemia patient sample, were resistant to virus infection, as were the normal B cells. The AML3 and Jurkat leukemia cell lines were also infected with ΔM51-GFP-VSV at an MOI of 3, for 6 hours and 48 hours respectively; both cell lines were able to be infected by VSV. The percentage of ΔM51-GFP-VSV-infected cells was assessed by the proportion of GFP-expressing cells as measured by flow cytometry.

B-D Flow cytometry representation of the resistance of CLL cells to VSV. Representative flow cytometry data from one of five CLL patients is presented. Cells were infected with ΔM51-GFP-VSV at an MOI of 3. The percentage of ΔM51-GFP-VSV-infected cells is shown in the upper right hand corner of each dot plot, as identified by the proportion of GFP-expressing cells. Plot **B** represents the forward/side scatter analysis, Plot **C** represents the uninfected control while plot **D** represents a 6 hour infection.

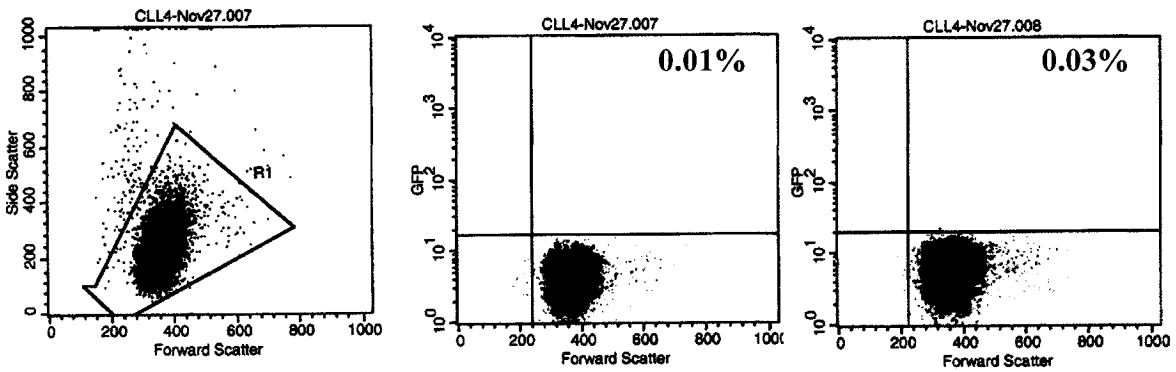
E-G Flow cytometry representation of the ability of AML 3 cells to be infected by VSV. Representative flow cytometry data from one of seven experiments is presented demonstrating that AML3 cells (a leukemia cell line used as a control for a productive virus infection) are sensitive to VSV. Cells were infected with ΔM51-GFP-VSV at an MOI of 3. The percentage of ΔM51-GFP-VSV-infected cells is shown in the upper right hand corner of each dot plot, identified by the proportion of GFP-expressing cells. Plot **E** represents the forward/side scatter analysis. Plot **F** represents the uninfected control while plot **G** represents a 6 hr infection.

5A. VSV sensitivity of various primary cells and cancer cell lines

Patient #	Percentage of ΔM51-GFP-VSV infected cells (%)
CLL1	0.02
CLL2	0.21
CLL3	0.04
CLL4	0.10
CLL5	0.65
ALL1	0.07
biA1	2.32
Normal B cells	0.23
AML 3	86.62
Jurkat	97.82

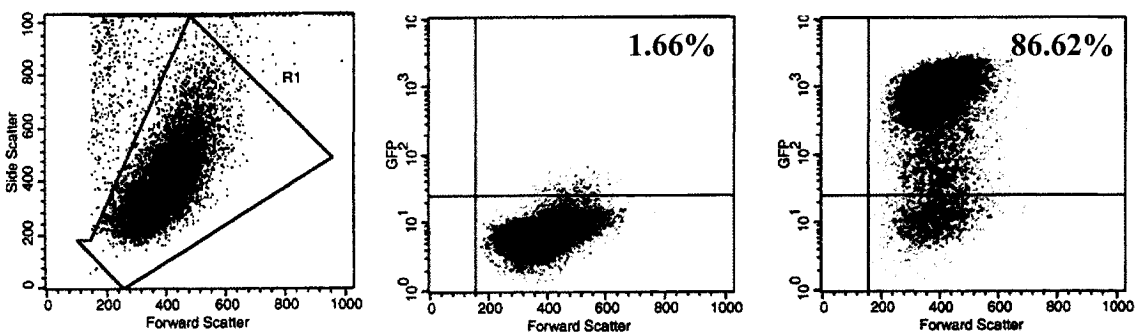
5B-D. Flow cytometry representation of the resistance of CLL cells to VSV

5B. Forward/side scatter **5C. CLL-V** **5D. CLL+V**



5E-G. Flow cytometry representation of the ability of AML 3 cells to be infected by VSV

5E. Forward/side scatter **5F. AML 3-V** **5G. AML 3+V**



Though ALL cells appear to be resistant to infection, the bi-phenotypic acute leukemia patient sample is 2.32% infected by VSV at 24 hours (Figure 5A). Cells from the latter sample were incubated for longer time points with ΔM51-GFP-VSV and with wt-VSV and a maximum of 15% infection was achieved at 24 hours post-infection with wt-VSV at an MOI of three (see Appendix III). The bi-phenotypic acute leukemia patient cells appear to be infected by both viruses at all time points. Trypan blue cell viability analysis was performed, which agreed with the percentage of infected cells measured by flow cytometry (data not shown).

Since Chronic Lymphocytic Leukemia is a B cell malignancy, the ability of VSV to infect B cells from volunteers without leukemia was of interest. This would allow assessment of the impact of the oncogenic transformation of a cell on its ability to be infected by the virus. Venous peripheral blood was obtained from ten healthy volunteers in the lab. Peripheral blood mononuclear cells were isolated and pooled. B cells were isolated by fluorescence activated cell sorting on the PBMC and infected with ΔM51-GFP-VSV for 24 hours at an MOI of three. This experiment was repeated twice in its entirety. A mean of the two independent infections showed that 0.23% of normal B cells expressed GFP following VSV infection (Figure 5A). Trypan blue cell viability analysis agreed with the proportion of infected cells (data not shown). These data suggest that the non-malignant counterparts to this disease are resistant to VSV. The resistance of normal B cells to VSV is in accordance with previous observations [45].

A human leukemia cell line, AML 3, which is readily infected by all forms of VSV [45] or Jurkat cells (a T cell leukemia which is infected by ΔM51-GFP-VSV) [45] was infected alongside the CLL cells. Infection of the AML 3 or Jurkat leukemia cell lines

represented a control for a productive virus infection. Representative flow cytometry data six hours after exposing the AML 3 cells to ΔM51-GFP-VSV at an MOI of three is presented in Figure 5E-G. At this time point, the majority of cells (86.62%) express GFP. Trypan blue analysis was done to measure cell viability and this correlated with the proportion of infected cells (data not shown). The demonstration that AML3 cells were infected by the same viral preparation as was used for the CLL samples dismisses the possibility that dead virus was being used or that the infection technique was flawed.

3.4 Testing for IFNβ Production

It was hypothesized that CLL cells had a constitutively activated IFN pathway which conferred resistance to VSV infection. In an effort to further understand CLL cells' resistance to VSV infection, components of the IFN antiviral pathway were examined at a transcriptional level by Q-PCR. Prior to performing PCR analysis however, it was important to identify positive controls for IFN production and IFN responsiveness. A549 cells served as a positive control for the induction of IFNβ following VSV infection [65, 67, 68]. A549 cells exhibit an intact proximal portion of the IFN pathway, defined by the ability to induce IFNβ production following virus infection. In our study, A549 cells did not respond to IFNαβ treatment (data not shown). HeLa cells were identified as a positive control for responsiveness to exogenous IFN treatment, as determined previously [65]. HeLa cells have an intact distal portion of the IFN pathway as represented through intact signalling following the binding of IFN to its receptor and the subsequent development of an antiviral state. The terminology proximal and distal portions of the IFN pathway will be used throughout the remainder of this thesis.

An IFN production bioassay was developed to confirm A549 cells' production of IFNβ. The experimental design for this assay is presented in Figure 6. Briefly, in order to induce IFNβ production in the cells, A549 cells were infected at a low MOI with ΔM51-GFP-VSV for 24 hours. Thereafter, supernatants from infected cells were collected, centrifuged and filtered through positively-charged Mustang Q filters to remove the negatively-charged virus particles. HeLa cells, known to be IFN-responsive [65], were treated with the filtered supernatants for 24 hours and then challenged with a low MOI of ΔM51-GFP-VSV to observe if supernatant-treated HeLa cells were protected from infection by VSV. VSV-infected cells were detected through green fluorescence by flow cytometry. To delineate that A549 cells were secreting IFNβ in response to virus infection, neutralizing antibodies to huIFNβ were pre-incubated with supernatants of infected cells before treatment of HeLa cells to observe if the protective effect of the supernatants was ablated.

To ensure that virus particles were completely removed by filtering, HeLa cells were treated with filtered and non-filtered supernatants, and green fluorescence visualized through the Leica MZFLIII fluorescent microscope and camera. Representative photographs from one of three experiments are presented in Figure 7A-B. HeLa cells were treated with non-filtered supernatant from infected A549 cells in Figure 7A. As the majority of cells are expressing GFP in this figure, it is apparent that before filtering, GFP-virus was present in the supernatant. In Figure 7B, HeLa cells were treated with filtered supernatants and no discernable GFP-expressing cells are observed, suggesting that the virus was removed by filtering. Furthermore, plaque assays were performed to measure the amount of virus in the supernatants before and after filtering (see Appendix IV). The above data suggest that virus particles were removed by Mustang Q filters and filtered supernatants did not contain virus.

Figure 6. Experimental design for IFN production bioassay.

In step **1**, $1x10^7$ A549 cells were mock-infected or infected at an MOI=0.1 with ΔM51-GFP-VSV for 24 hours. Supernatants from infected cells were collected.
In step **2**, the supernatants were centrifuged to remove cell debris and filtered through positively charged Mustang Q filters. Supernatants were applied to HeLa cells for 24 hours.
In step **3**, supernatants were removed and HeLa cells were challenged with ΔM51-GFP-VSV at an MOI=0.1 for 24 hours.
In step **4**, the proportion of GFP-expressing cells was measured by flow cytometry as a surrogate for the percentage of ΔM51-GFP-VSV-infected cells.
In step **5**, 1 μL (200 neutralization units) of neutralizing antibody to human IFNβ was incubated with a small volume of supernatant for 18-24 hours prior to applying the supernatants to HeLa cells. Steps 3 and 4 were repeated.

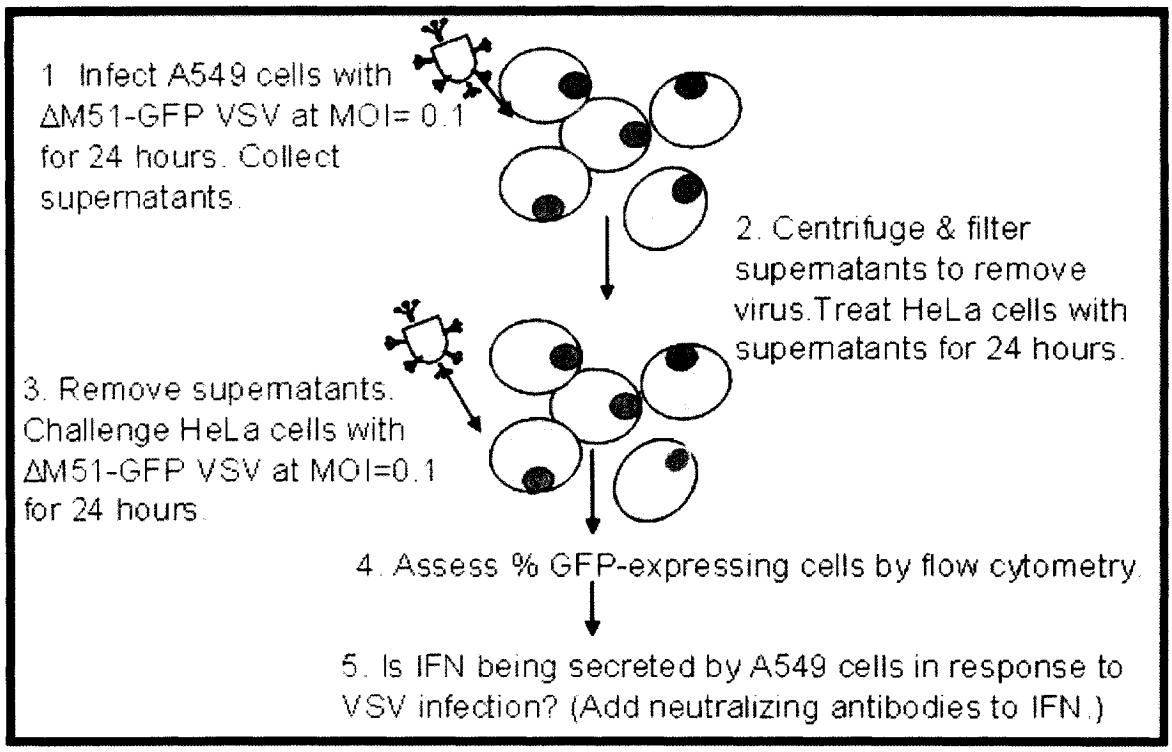

1 Infect A549 cells with ΔM51-GFP VSV at MOI= 0.1 for 24 hours. Collect supernatants.
2. Centrifuge & filter supernatants to remove virus.Treat HeLa cells with supernatants for 24 hours.
3. Remove supernatants. Challenge HeLa cells with ΔM51-GFP VSV at MOI=0.1 for 24 hours.
4. Assess % GFP-expressing cells by flow cytometry.
5. Is IFN being secreted by A549 cells in response to VSV infection? (Add neutralizing antibodies to IFN.)

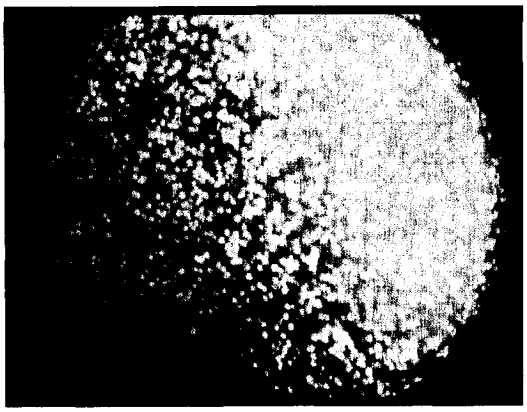
Figure 7. A549 cells secrete IFNβ in response to infection with ΔM51-GFP-VSV.

A-B Supernatants from infected A549 cells were filtered through positively charged Mustang Q filters. Filtered and non-filtered supernatants were applied to HeLa cells for 24 hours to look for the presence of GFP-expressing virus pre- and post-filtering. The Leica MZFLIII fluorescent microscope and camera were used to visualize and document GFP expression in HeLa cells. Representative data from one of three experiments are presented in this figure.

C A549 cells secrete IFNβ in response to infection with ΔM51-GFP-VSV. HeLa cells received various treatments for 24 hours: virus challenge (virus), treatment with 10U huIFNβ (IFNβ), filtered supernatants (filt. sup), non-filtered supernatants (non-filt. sup), or incubation of supernatants with neutralizing antibodies to IFNβ (Nab(IFNβ)). The percentage of VSV-infected cells was assessed by the proportion of GFP-expressing cells, as determined by flow cytometry. Data was normalized to the 'Virus' sample, which was assumed to be a 100% infection. The mean of three assays (N=3) is presented.

7A-B. Membrane filters remove virus from supernatants.

7A.

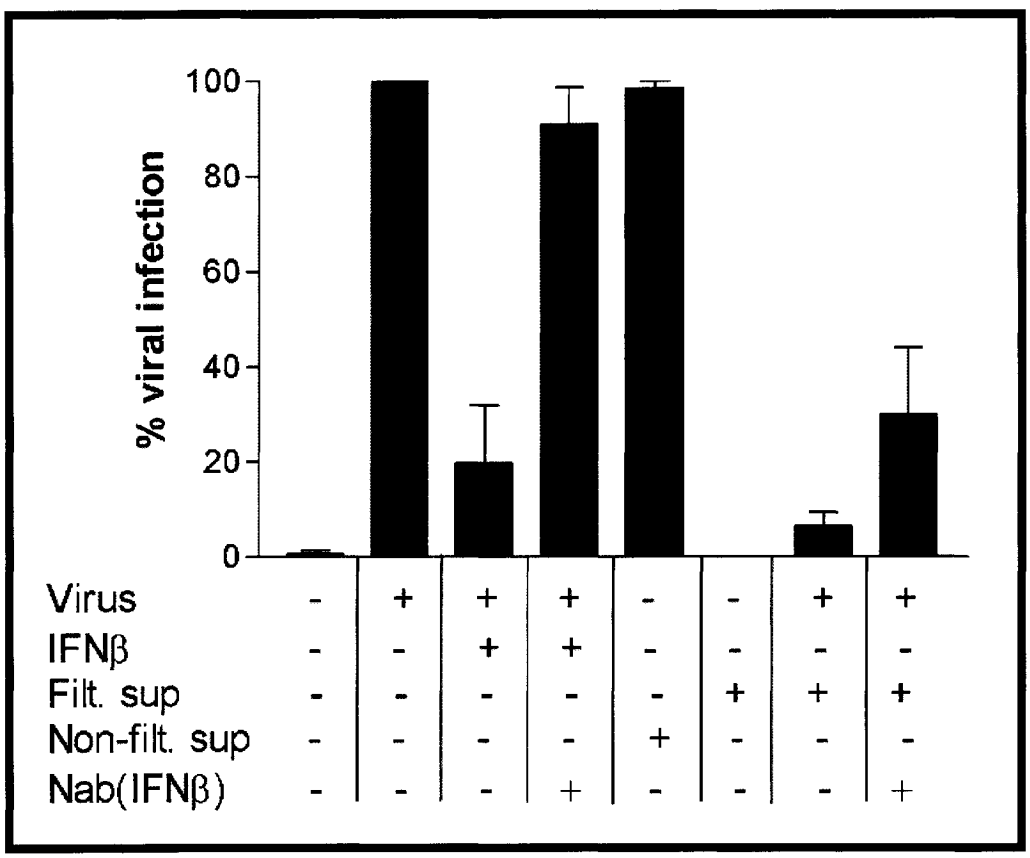


HeLa cells treated with non-filtered supernatant.

7B.

HeLa cells treated with filtered supernatant.

7C. A549 cells secrete IFNβ in response to infection with ΔM51-GFP-VSV.



The IFN production bioassay was developed to confirm if A549 cells can induce IFNβ production in response to infection with ΔM51-GFP-VSV. Figure 7C represents the mean of three assays that suggest A549 cells are capable of IFNβ production. Several controls were included in the assay such as treatment of HeLa cells with 10 U/mL of huIFNβ to demonstrate that the cells were IFN responsive, as well as treatment of HeLa cells with 10 U/mL of huIFNβ pre-incubated with 1 μL (200U) of neutralizing antibody to huIFNβ to ensure that the antibody was specific to huIFNβ. When HeLa cells were treated with 10U of IFNβ, only a small proportion (19.18%) of the cells were virally infected. Upon addition of neutralizing antibody, the percentage of virally infected cells increased to 91.16% thereby indicating that the neutralizing antibody is specific to huIFNβ. Other control treatments included an incubation of HeLa cells with non-filtered supernatant in which case every cell expressed GFP demonstrating that the virus was present in the supernatant prior to filtering. In contrast, treatment with the filtered supernatant did not yield any GFP-expressing cells, suggesting that the virus was not present in the supernatant.

When HeLa cells were treated with filtered supernatant and subsequently challenged with VSV, only a small proportion (6.54%) of the cells were infected, suggesting that the supernatant contains an antiviral substance which is able to protect HeLa cells against VSV infection. In order to demonstrate that IFNβ was specifically contributing to the protection of HeLa cells against VSV infection, filtered supernatant was incubated with 1 μL of neutralizing antibody to huIFNβ prior to treating the HeLa cells with supernatant. At this point, the proportion of GFP-expressing cells increased to 30.14%. Hence, this experiment suggests that A549 cells secrete IFNβ in response to infection with ΔM51-GFP-VSV and that IFNβ partially contributes to the protection of HeLa cells against VSV infection in this

assay. The IFN production bioassays suggest that A549 cells will serve as a good positive control for the induction of IFNβ production in response to VSV infection in the PCR studies.

3.5 CLL Cell Expression of IFNβ at the Transcriptional Level

IFNβ plays a pivotal role in the primary wave of IFN production. Hence, it was the first candidate for gene expression analysis. Relative IFNβ gene expression was determined by quantitative PCR (Q-PCR), with β2-microglobulin serving as the reference gene. Transcript levels were examined in five CLL patient samples and one ALL patient, as well as in A549, AML3, HeLa and normal B cells, before and after incubation with ΔM51-GFP-VSV. Gel electrophoresis was performed on cDNA from the Q-PCR reaction and representative gels are shown in Figure 8. As expected, constant β2-microglobulin expression was observed in all samples and its expression is absent in the Reverse transcriptase and H_20 negative controls. In the investigation of IFNβ gene expression, A549 cells were employed as a positive control for the induction of IFNβ subsequent to virus infection. Upon examination of CLL cells, the five patient samples seem to constitutively express IFNβ, which generally does not change upon virus infection, regardless of the length of infection. Normal B cells also constitutively express IFNβ at the transcriptional level which is not further enhanced subsequent to the addition of virus. Hence, constitutive IFNβ gene expression observed in CLL patient samples as well as in normal B cells seems to correlate with resistance to VSV infection. Quantification of the relative expression of IFNβ/β2-microglobulin was performed on RelQuant software and is presented in Figure 9. In A549 cells, IFNβ gene expression was increased 115 fold post infection - a highly statistically significant difference ($P<0.0001$ as measured by the unpaired T test). With CLL

Figure 8. CLL cells constitutively express IFNβ at the gene expression level which, in general, does not change subsequent to virus infection.

5 μL of cDNA from a representative Q-PCR reaction for IFNβ and for β2-microglobulin underwent gel electrophoresis. Cells from five CLL patients (C1-C5), from Acute Lymphocytic Leukemia patient 1 (ALL1) and two samples of normal B cells (B1 and B2) were infected for 24 hours with ΔM51-GFP-VSV (V) at an MOI of 3, unless indicated otherwise (in addition to the 24 hour infections, C4 was infected for 9 hours and C5 for 48 hours). The ALL-V sample was unavailable for gel electrophoresis. Reverse-transcriptase negative (RT-neg.) controls are also shown. The expected product band size for IFNβ is 178 base pairs (bp) and for β2-microglobulin, is 150 bp. The lower band observed is likely due to primer-dimer interaction as this band is also observed in the H_2O control.

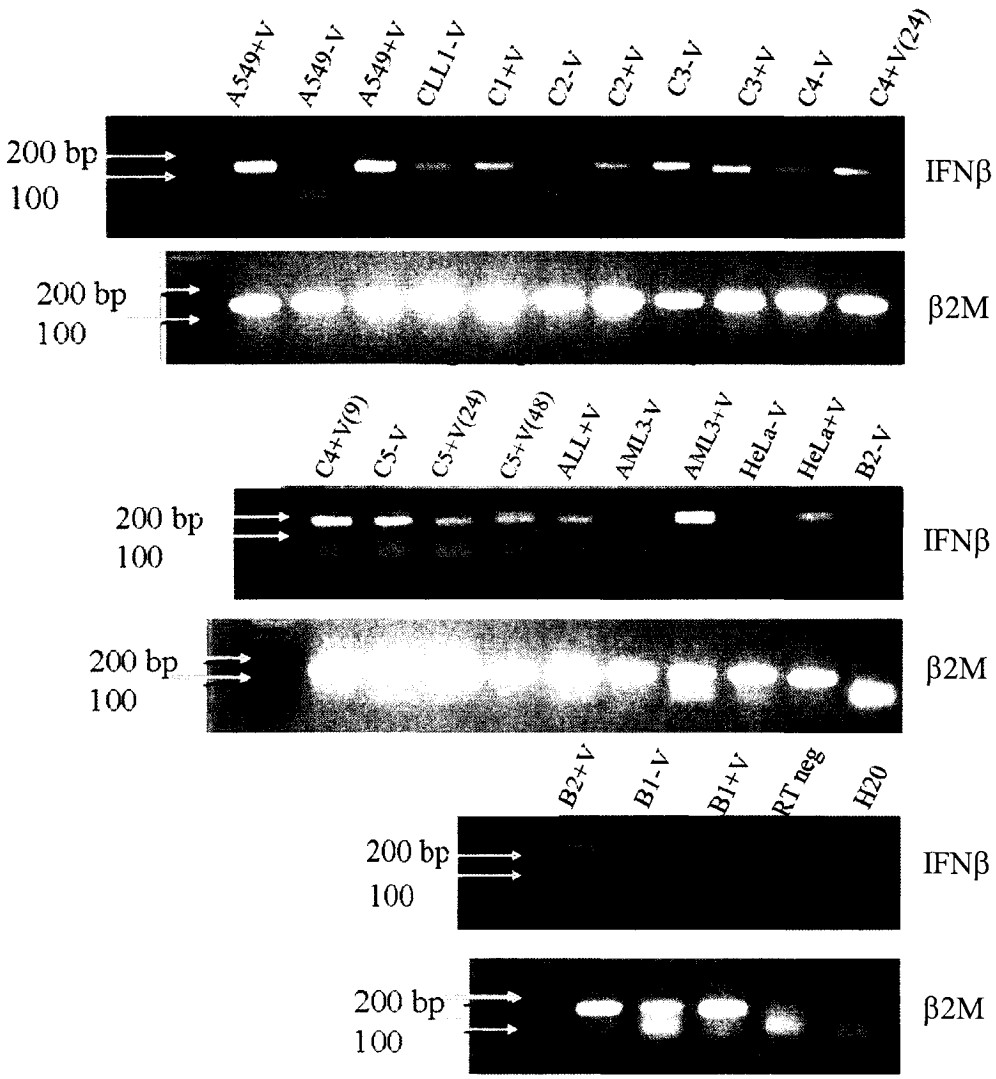
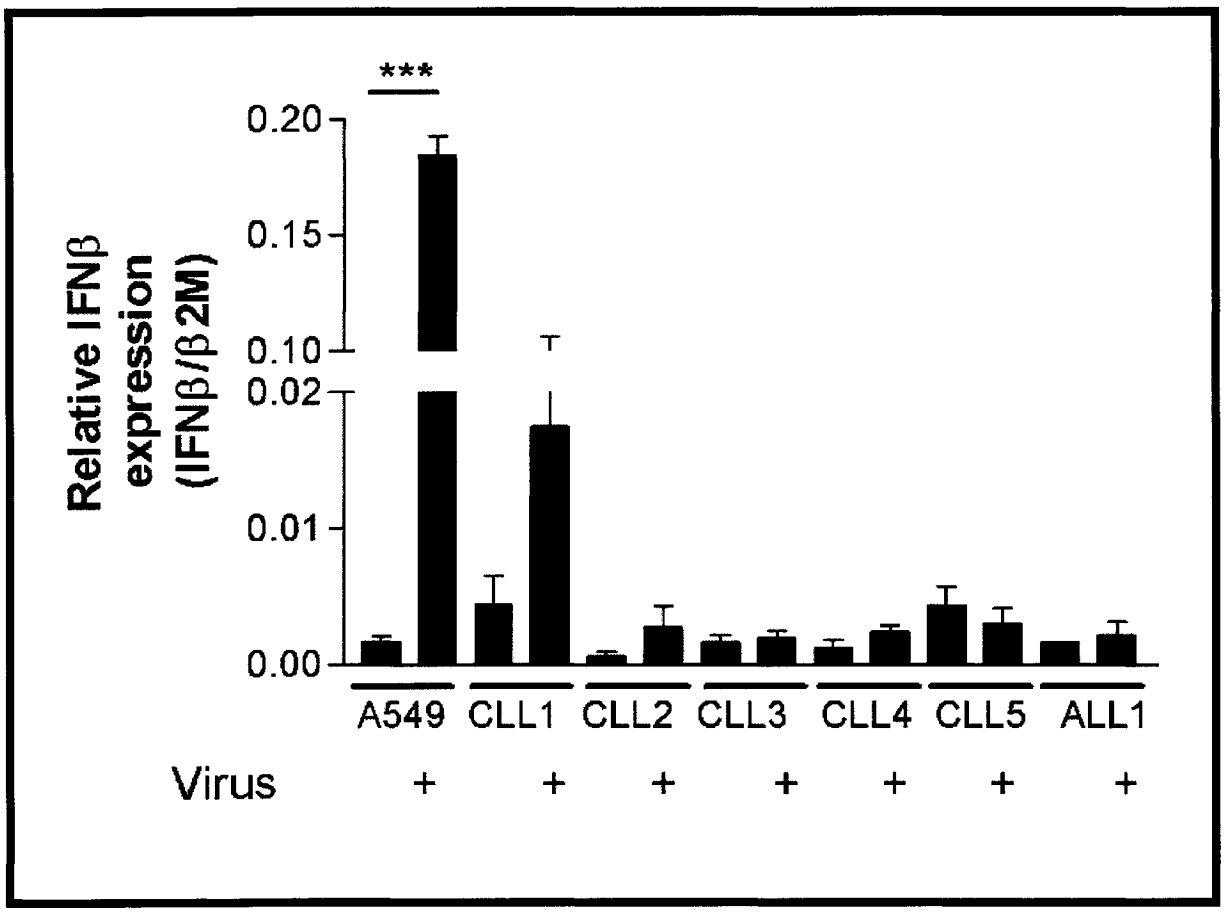

A549+V
A549-V
A549+V
CLL1-V
C1+V
C2-V
C2+V
C3-V
C3+V
C4-V
C4+V(24)
200 bp
100
IFNβ
200 bp
100
β2M
C4+V(9)
C5-V
C5+V(24)
C5+V(48)
ALL+V
AML3-V
AML3+V
HeLa-V
HeLa+V
B2-V
200 bp
100
IFNβ
200 bp
100
β2M
B2+V
B1-V
B1+V
RT neg
H20
200 bp
100
IFNβ
200 bp
100
β2M

Figure 9. Quantification of relative IFNβ gene expression levels.

Relative IFNβ gene expression was determined by Q-PCR using Lightcycler technology (Roche). RelQuant software was used to measure the relative expression of IFNβ/β2-microglobulin. The A549+V sample was also used as a calibrator (an internal control) to normalize for differences between individual rounds of Q-PCR. The mean of three experiments is presented (N=3). $P < 0.0001$ as calculated by the unpaired T test.


***
0.20
0.15
0.10
0.02
0.01
0.00
Relative IFNβ expression (IFNβ/β2M)
A549
CLL1
CLL2
CLL3
CLL4
CLL5
ALL1
Virus
+
+
+
+
+
+
+

and ALL cells, IFNβ transcript levels remained fairly constant before and after infection with the exception of CLL patient 1 where IFNβ transcript levels seem to increase after virus infection. This data quantitatively demonstrates that CLL and ALL cells constitutively express a low level of IFNβ transcripts.

3.6 Testing for IFN Responsiveness

Further distal portions of the IFN transcriptional response were also examined. Once again, a control cell line was required for PCR studies. In this case, the positive control should exhibit an intact distal portion of the IFN pathway. HeLa cells were chosen as they are known to be protected from VSV infection, by treatment with exogenous IFNαβ [65]. A bioassay was developed to confirm that HeLa cells were IFN-responsive. The experimental design for this assay is outlined in Figure 10. Briefly, HeLa cells were treated overnight with increasing doses of human IFNαβ to a maximum dose of 20 000 U/mL IFNαβ. Thereafter, cells were challenged with ΔM51-GFP-VSV at a high MOI for six hours. HeLa cells were then examined qualitatively for green fluorescence expression by flow cytometry and for virus-induced cytopathic effect. Cells were also stained with trypan blue and counted on the hemacytometer to measure cell viability. The mean of four assays is presented in Figure 11 which suggests that HeLa cells are IFN responsive. Infecting HeLa cells without pre-treatment with IFN resulted in 54.56% of the cells being infected. However, prior treatment with as little as 5 U/mL of huIFNαβ decreased the proportion of infected cells substantially to 14.88%. Pre-treatment of HeLa cells with increasing doses of IFN demonstrates a dose-dependent inhibition of cells that can support viral replication. For example when the dose

Figure 10. Experimental design for IFN responsiveness bioassay.

In step **1**, HeLa cells were treated for 18-24 hours with increasing doses of human IFNα (Intron a2b) and human IFNβ (Rebif).
In step **2**, the IFN-containing media was removed from HeLa cells and cells were infected with ΔM51-GFP-VSV at an MOI=3 for 6 hours.
In step **3**, the proportion of GFP-expressing cells was determined by flow cytometry as a surrogate for the percentage of ΔM51-GFP-VSV-infected cells.

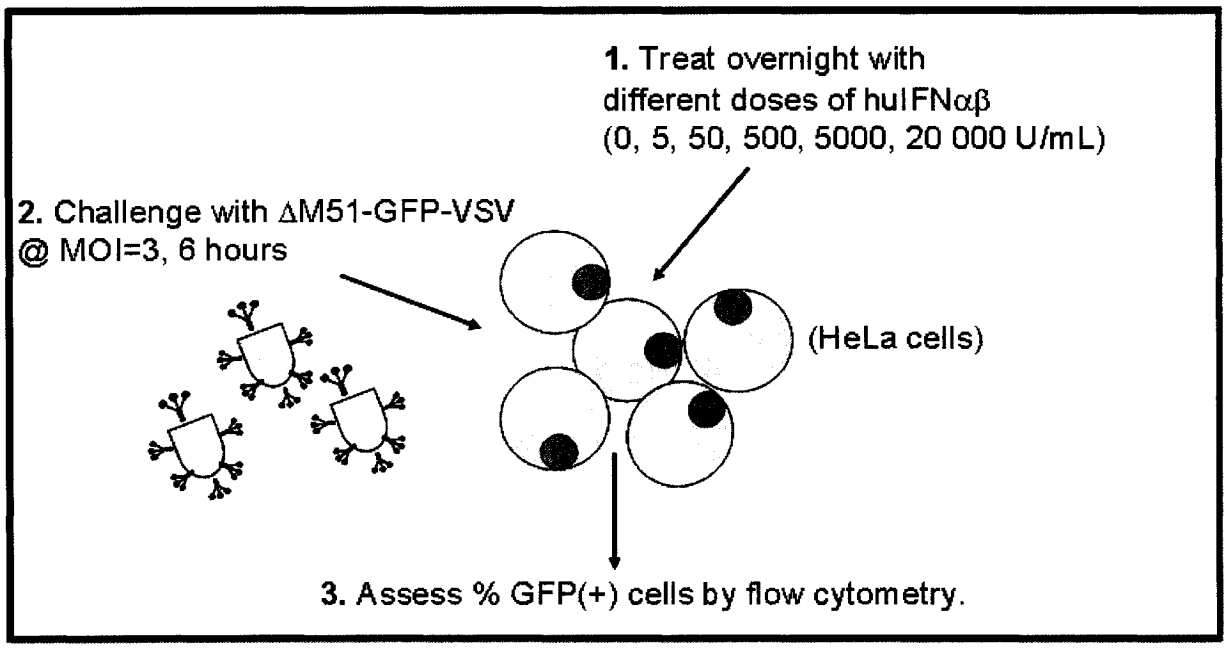
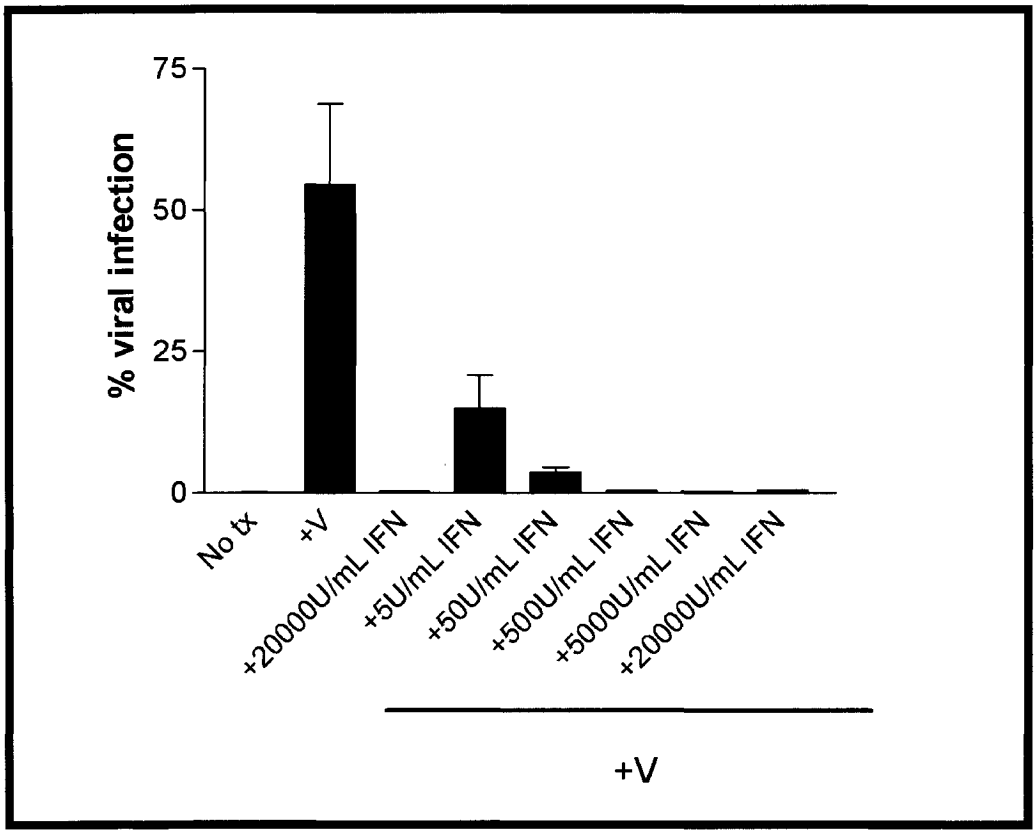

1. Treat overnight with
different doses of hulFNαβ
(0, 5, 50, 500, 5000, 20 000 U/mL)
2. Challenge with ΔM51-GFP-VSV
@ MOI=3, 6 hours
(HeLa cells)
3. Assess % GFP(+) cells by flow cytometry.

Figure 11. HeLa cells are responsive to human interferon αβ.

HeLa cells received various treatments from no treatment (No tx), challenge with ΔM51-GFP-VSV (V), huIFNαβ treatment (20 000 U/mL IFNαβ) without subsequent virus challenge, and treatment with IFNαβ in incremental doses to 20 000 U/mL followed by virus challenge. The percentage of viral infection was determined by the proportion of GFP-expressing cells by flow cytometry. The mean of four assays (N=4) is presented.


% viral infection
75
50
25
0
No tx
+V
+20000U/mL IFN
+5U/mL IFN
+50U/mL IFN
+500U/mL IFN
+5000U/mL IFN
+20000U/mL IFN
+V

is increased to 50 U/mL, only 3.65% of cells are infected. Saturation occurs at the three highest doses of IFN treatment where less than 1% of cells are virally infected. Treating the cells with human IFNαβ at 20 000 U/mL, in the absence of a viral infection, does not yield a significant cytopathic effect, as observed qualitatively. Trypan blue staining for cell viability was performed, in addition to qualitative examination of HeLa cells for virus-induced cytopathic effect. Both correlated with the proportion of infected cells (data not shown).

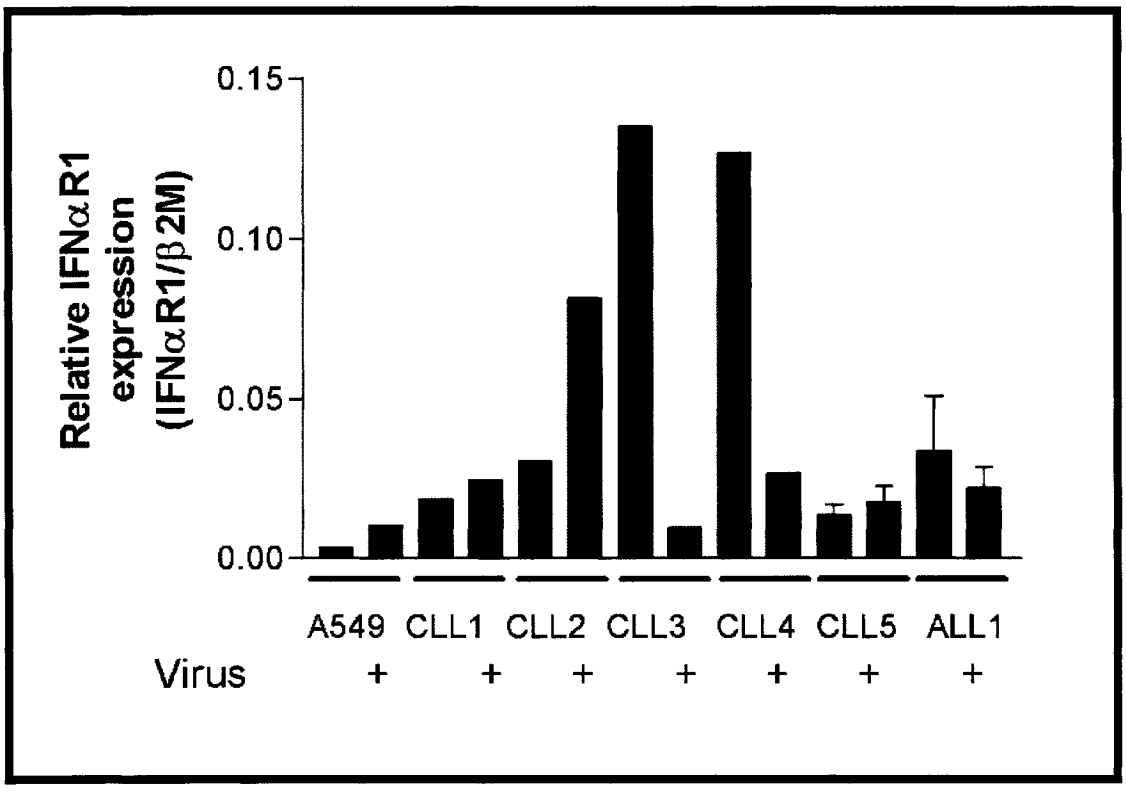
IFNα and IFNβ are able to induce equivalent antiviral states in HeLa cells (data not shown). Thus HeLa cells are able to respond to exogenous interferon and mount an appropriate antiviral response. Further, the assay is able to detect the effect of as little as 5U/mL of IFNαβ on HeLa cells. This is a demonstration of the extreme sensitivity of HeLa cells to IFN treatment.

3.7 Expression of IFNαR1 in CLL Cells at the Transcriptional Level

Based on the previous results, the distal portion of the IFN transcriptional pathway was examined. The next target gene for PCR analysis in CLL cells became IFNαR1. IFNαR1 forms part of the Type I IFN receptor complex (also composed of IFNαR2). IFNβ and IFNα bind to the same receptor to complete the signaling loop to other parts of the IFN antiviral pathway [32]. Quantification of IFNαR1 gene expression levels is demonstrated in Figure 12. Gel electrophoresis was performed on cDNA from the Q-PCR reaction (see Appendix V). Similar to IFNβ transcript levels, it was observed quantitatively and qualitatively that IFNαR1 is also constitutively expressed in all CLL and ALL samples, with the exception of CLL patients 3 and 4, where transcript levels were higher before infection. Gene expression analysis of IFNβ and IFNαR1 suggests that genes from the proximal and distal portions of the IFN pathway are constitutively expressed in CLL cells, which relates

Figure 12. Quantification of relative IFNαR1 gene expression levels.

Relative IFNαR1 gene expression was determined by Q-PCR using Lightcycler technology (Roche). RelQuant software was used to measure the relative expression of IFNαR1/β2-microglobulin. The A549+V sample served as a calibrator (an internal control) to normalize for differences between individual rounds of Q-PCR. The mean of three experiments (N=3) is presented.


Relative IFNαR1 expression (IFNαR1/β2M)
0.15
0.10
0.05
0.00
A549
CLL1
CLL2
CLL3
CLL4
CLL5
ALL1
Virus
+
+
+
+
+
+
+

to the observed resistance of these cells to VSV infection.

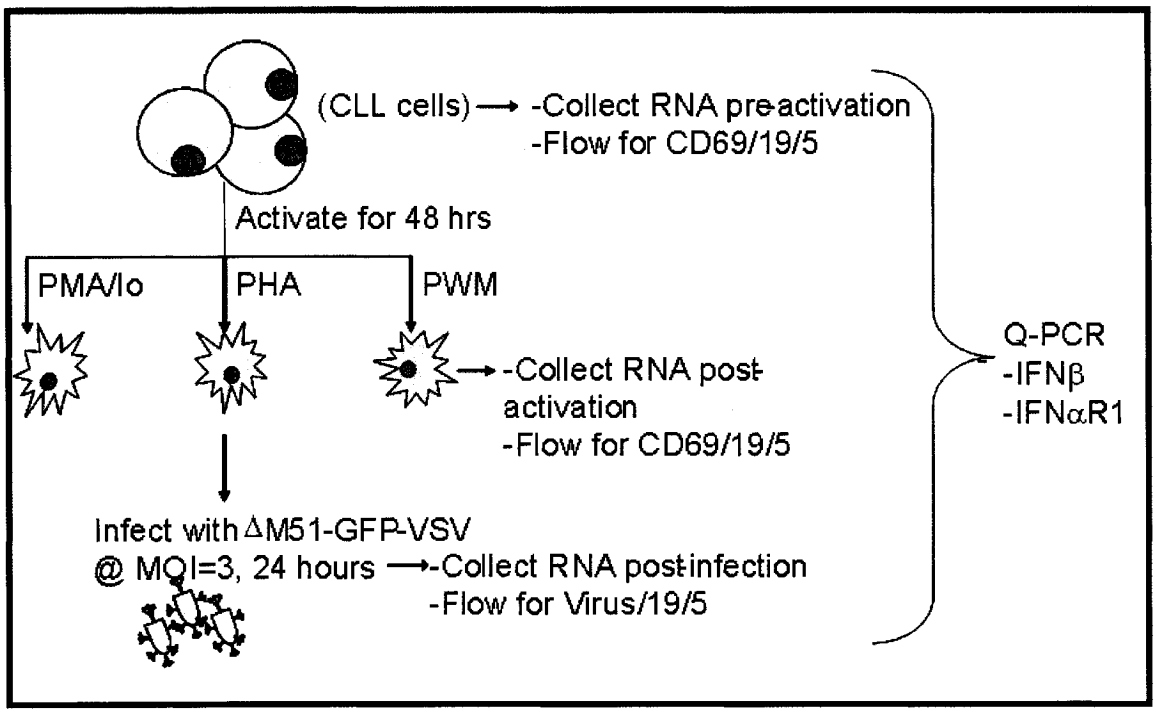
3.8 Sensitivity of Activated CLL Cells to VSV Infection

De Fanis *et al.* and Segel *et al.* have shown that CLL cells can be activated by various cell mitogens, including phorbol 12-myristate 13-acetate + ionomycin (PMA/Io), phytohemagglutinin (PHA) or pokeweed mitogen (PWM) [69, 70]. Upon activation, the cell surface marker, CD69, is induced on leukocytes. As well, other laboratory investigations have shown that activated T cells can be infected by VSV, while resting T cells are resistant [71].

In order to determine if activated CLL cells can be similarly sensitized to VSV infection, the CLL cell activation experiment outlined in Figure 13 was designed. In brief, PBMC were isolated from CLL patient 4, who was known to be resistant to VSV from previous experiments. Immunophenotyping by flow cytometry was then performed on isolated cells to ensure that the cell population consisted of resting CLL cells. Subsequently, cells were stimulated with various cell mitogens including PMA/Io, PHA or PWM. Flow cytometry was performed for CD19 and CD5 to look for potential changes in the CLL cell surface markers post-activation. Forward and side scatter analysis was also performed to look for differences in cell morphology post-activation. As well, flow cytometry for CD69 expression was done to ensure that the cells were activated. Activated and resting cells were then infected with ΔM51-GFP-VSV at an MOI of three for 24 hours. Flow cytometry was performed for CD19, CD5 and GFP-expressing virus, to examine if changes in the sensitivity of CLL cells to virus infection occurred post-activation. RNA was extracted at each time point. Finally, Q-PCR for IFNβ and IFNαR1 was carried out on all samples, to examine if changes in the IFN transcription pathway were induced through activation of CLL cells.

Figure 13. Design for CLL cell activation experiment.

Peripheral blood mononuclear cells (PBMCs) were obtained from a CLL patient who was known to be resistant to VSV. Cells were activated for 48 hours by various cell mitogens including phorbol 12-myristate 13-acetate + ionomycin (PMA/Io), phytohemagglutinin (PHA) and pokeweed mitogen (PWM). To ensure the CLL cells were activated, cells were stained with CD19-PE-Cy5, CD5-R-PE and CD69-FITC conjugate to look for CD19+5+69+ cells. Flow cytometry was also performed for CD19 and CD5 (using the antibodies mentioned above) and GFP-virus (FITC channel) to look for potential changes in sensitivity to virus infection pre- and post-activation. RNA was collected at each time point and Q-PCR was performed for IFNβ and IFNαR1.


(CLL cells)
-Collect RNA pre-activation
-Flow for CD69/19/5
Activate for 48 hrs
PMA/Io
PHA
PWM
-Collect RNA post-activation
-Flow for CD69/19/5
Infect with ΔM51-GFP-VSV
@ MOI=3, 24 hours
-Collect RNA post-infection
-Flow for Virus/19/5
Q-PCR
-IFNβ
-IFNαR1

Representative flow cytometry data is presented in Figure 14 where immunophenotyping of CLL cells pre- and post-activation with PMA/Io was performed. In the untreated control (Figure 14A-C), the majority of cells is in the viable cell population and is also fairly small and non-granular (low on the forward scatter and side scatter plots respectively). As well, they are resting cells as shown through their lack of CD69 expression. Isolated cells also express the characteristic CLL cell surface markers CD19 and CD5. In contrast, Figure 14D-F, representative of the PMA/Io treated cells, demonstrates that the cells morphology has changed with the cells larger and more granular (higher on the forward scatter and side scatter plots respectively). The CLL cells have also down-regulated their CD19 expression but have up-regulated CD5 expression. In the untreated sample, 63.76% of total cells are CD19+5+, while in the treated samples the percentage of CD19/5-expressing cells decreases to 22.94% (Figure 14). However, the cells are activated, as shown through their CD69 expression. It is important to note that CLL cells did not achieve an activated state by treatment with PHA and PWM (data not shown) and further optimization of the experimental activation procedure for these two mitogens is still required. Hence, the PHA- and PWM- treated samples acted as negative controls for activation in future experiments. Figure 14 demonstrates that CLL cells are activated by PMA/Io.

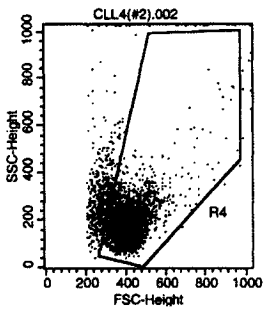
Changes in the sensitivity of CLL cells to VSV infection after activation were subsequently examined. Activated and resting cells were infected with ΔM51-GFP-VSV at a high MOI for 24 hours and flow cytometry was performed for green fluorescence-expressing cells. Representative flow cytometry data of resting and PMA/Io-activated cells and their ability to be infected by VSV are shown in Figure 15. In Figure 15A, a distinct viable cell population is observed and in Figure 15B, only 0.10% of cells are infected, which is in

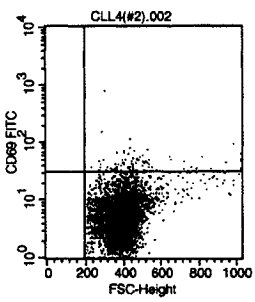
Figure 14. CLL cells are activated by PMA/Ionomcyin.

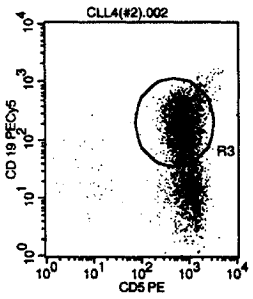
Flow cytometry data is presented where 10, 000 cells were acquired in each sample. $1x10^6$ PBMCs were incubated with the appropriate antibody. Antibodies include CD19- PE-Cy5, CD5-R-PE, or CD69-FITC. In samples presented, R4= Region 4 that represents the major population of viable cells, R3=CD19+5+ cells. Figures **A-C** represent the untreated control, while **D-F** represent the PMA/Io treated cells.

14A-C. Untreated Control

14A. Forward/side scatter **14B. CD69+ cells: 1.72%** **14C. CD19+5+: 63.76%**

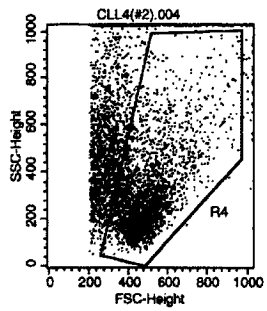


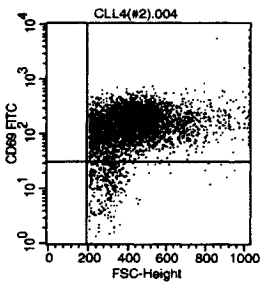


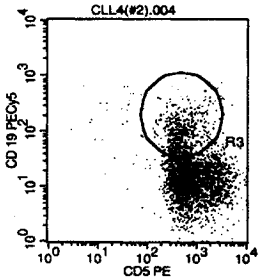


14D-F. PMA/Io Treatment

14D. Forward/side scatter **14E. CD69+ cells: 92.82%** **14F. CD19+5+: 22.94%**







agreement with previous data that this patient's cells are resistant to VSV. In Figure 15C, a distinct CLL cell population is observed (the cells express CD19 and CD5). Figure 15D demonstrates that upon activation, the cells become more granular, indicative of cytopathic effect and that there is no longer a distinct viable cell population. Also shown here is that 12.52% (Figure 15E) of the CLL cell population (Figure 15F) are infected by VSV. These observations show that CLL cells are sensitized to VSV infection after treatment with PMA/Io. PHA-treatment also caused mild sensitization to VSV infection (Appendix VI).

As mentioned earlier, upon activation, the proportion of CD5+ cells (shown in region R1) seems to increase from 0.10% to 12.84% (Figure 16A-B). These CD5+ cells (present in R1 in Figure 16B) are 99.57% activated, as shown through CD69 expression (Figure 16C). The activated CD5+ cells are also sensitized to VSV infection, with 36.29% of cells expressing GFP (Figure 16D).

3.9 Type I IFN Transcripts in Activated CLL Cells

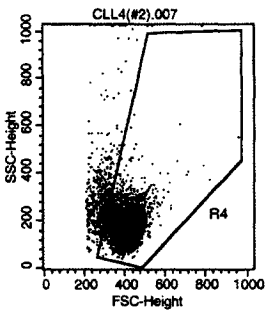
The resistance of CLL cells to VSV infection correlated with constitutive expression of IFNβ and IFNαR1 transcripts. Once activated CLL cells were able to be infected by VSV, it was hypothesized that IFN transcripts would also be decreased. To assess this hypothesis, Q-PCR was performed on CLL samples before and after activation, as well as pre- and post-virus infection. β2-microglobulin was again used as a reference gene and its expression was constant in all samples yet was absent in negative controls (shown in Appendix VII). In Figure 17A, gel electrophoresis of cDNA from a representative Q-PCR reaction for IFNβ is presented. This gel demonstrates that the IFNβ band at 178 bp is present in all CLL patient cells and in the A549 cells after virus treatment. The lower band that is observed is likely

Figure 15. Activated CLL cells are sensitive to VSV.

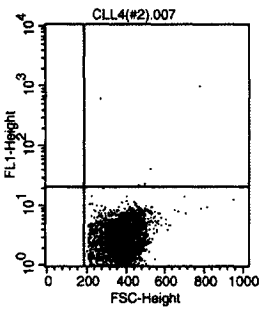
Flow cytometry data is presented where 10, 000 cells were acquired in each sample. Figures **A-C** represent the untreated cells that were infected with virus. Figures **D-F** represent the infected cells after PMA/Io treatment.

15A-C. Untreated + virus

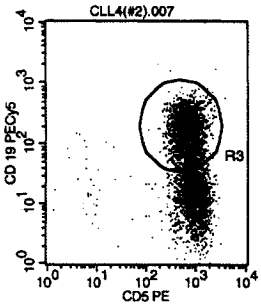
15A. Forward/side scatter



15B. GFP+ cells: 0.10%

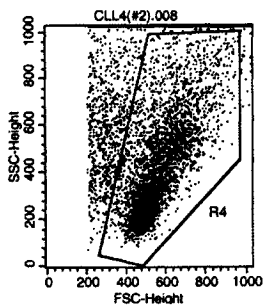


15C. CD19+5+: 57.96%

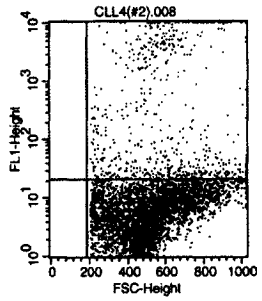


15D-F. PMA/Ionomycin treated + virus

15D. Forward/side scatter



15E. GFP+ cells: 12.52%



15F. CD19+5+: 22.48%

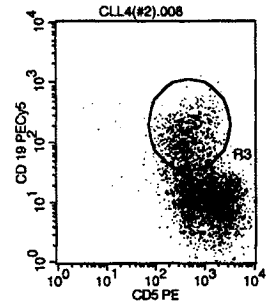
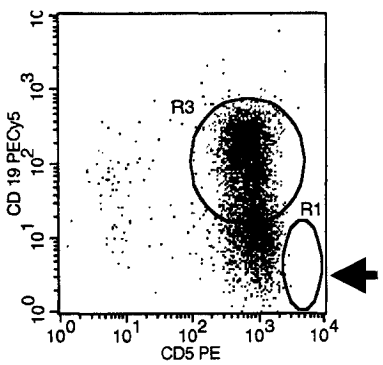


Figure 16. Activated CD5+ cells are also sensitive to VSV.

Flow cytometry data is presented where 10, 000 cells were acquired in each sample. $1x10^6$ PBMCs were incubated with the appropriate antibody. Antibodies include: CD19- PE-Cy5, CD5-R-PE, or CD69-FITC. In samples presented, R1= Region 1 that represents the CD5+ cells, R3=CD19+5+ cells. Figure **A** represents the untreated cells that were infected with virus. Figures **B-D** represent the infected cells after PMA/Io treatment.

16A. Untreated + virus

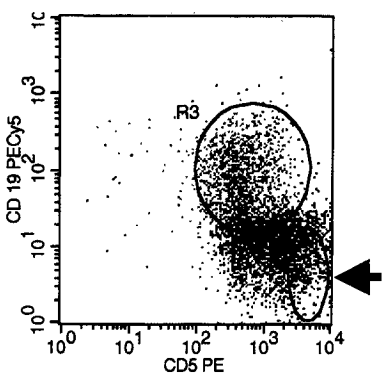
16A. Activated CD5+ cells:
0.10% (shown in R1)



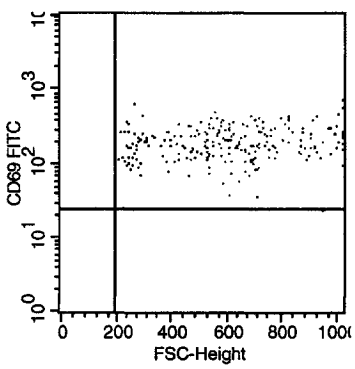
16B-D. PMA/Ionomycin treated + virus

(gated on R1 from Figure 16B)

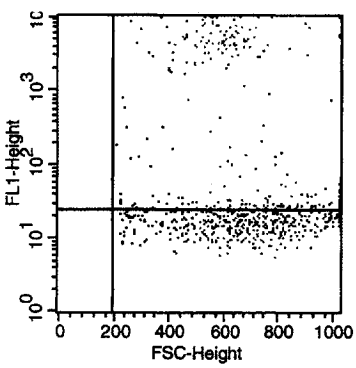
16B. Activated CD5+ cells:
12.84% (shown in R1)



16C. CD69+ cells:
99.57%



16D. GFP+ cells:
36.29%



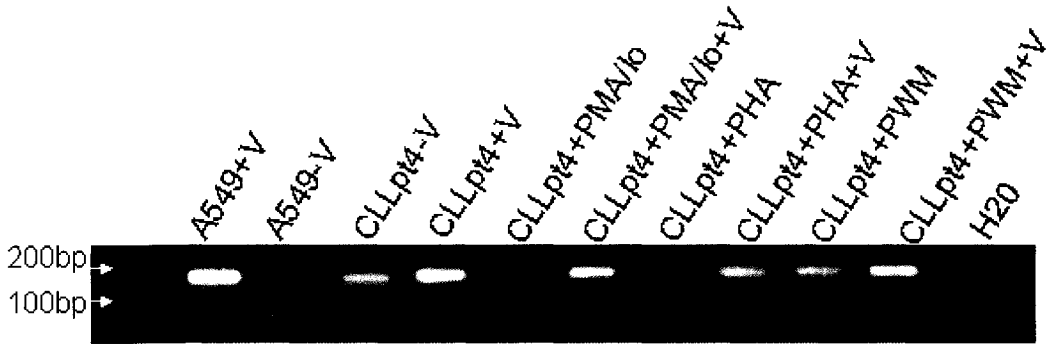
the result of primer-dimer interactions as it is also seen in the water control. In Figure 17B, quantification of IFNβ gene expression levels was performed. It was found that treating CLL patient 4 cells with PMA/Io caused IFNβ transcript levels to decrease 4.5 fold in comparison to the resting cells. This phenomenon does not occur in any of the negative control treatment cells. Also, after infection of the activated cells, transcript levels seem to return to almost their original levels. Hence, activation of CLL cells seems to cause a decrease in IFNβ gene expression levels that correlates with increased ability to be infected by VSV as compared to resting CLL cells.

In Figure 18, quantification of IFNαR1 gene expression levels in CLL patient 4 pre- and post-activation with PMA/Ionomycin and pre- and post-virus infection is shown. IFNαR1 levels appear to decrease 9 fold (a statistically significant difference where $p=0.0263$ as calculated by the Two-way ANOVA test) subsequent to PMA/Io treatment, when comparing the untreated cells versus treatment of cells with PMA/Ionomycin. After infection, the levels of IFNαR1 transcripts in untreated and treated CLL cells are quite similar. Gel electrophoresis was performed on cDNA from IFNαR1 Q-PCR reactions and a representative gel is shown in Appendix V. The results presented in Figure 17 and Figure 18 suggest that CLL cells have an increased ability to be infected by VSV after PMA/Io treatment through decreasing gene expression levels of the antiviral IFNβ and IFNαR1.

Figure 17. Activation of CLL cells with PMA/Ionomycin decreases IFNβ gene expression levels.

A 5 μL of cDNA from an IFNβ Q-PCR reaction underwent gel electrophoresis.
B Quantification of IFNβ gene expression levels in CLL patient # 4 pre- and post-activation with various cell mitogens. IFNβ levels appear to decrease subsequent to PMA/Io treatment, when comparing the uninfected CLL patient #4 versus CLL patient #4 treated with PMA/Ionomycin. Relative IFNβ gene expression was determined by Q-PCR using Lightcycler technology (Roche). RelQuant software was used to measure the relative expression of IFNβ/β2-microglobulin. The A549+V sample was also used as a calibrator (an internal control) to normalize for differences between individual rounds of Q-PCR. The mean of three experiments (N=3) is presented. P=0.0002 as calculated by the unpaired T test.

17A. IFNβ gene expression



17B. Quantification of IFNβ gene expression levels in CLL patient # 4 pre- and post-activation with various cell mitogens

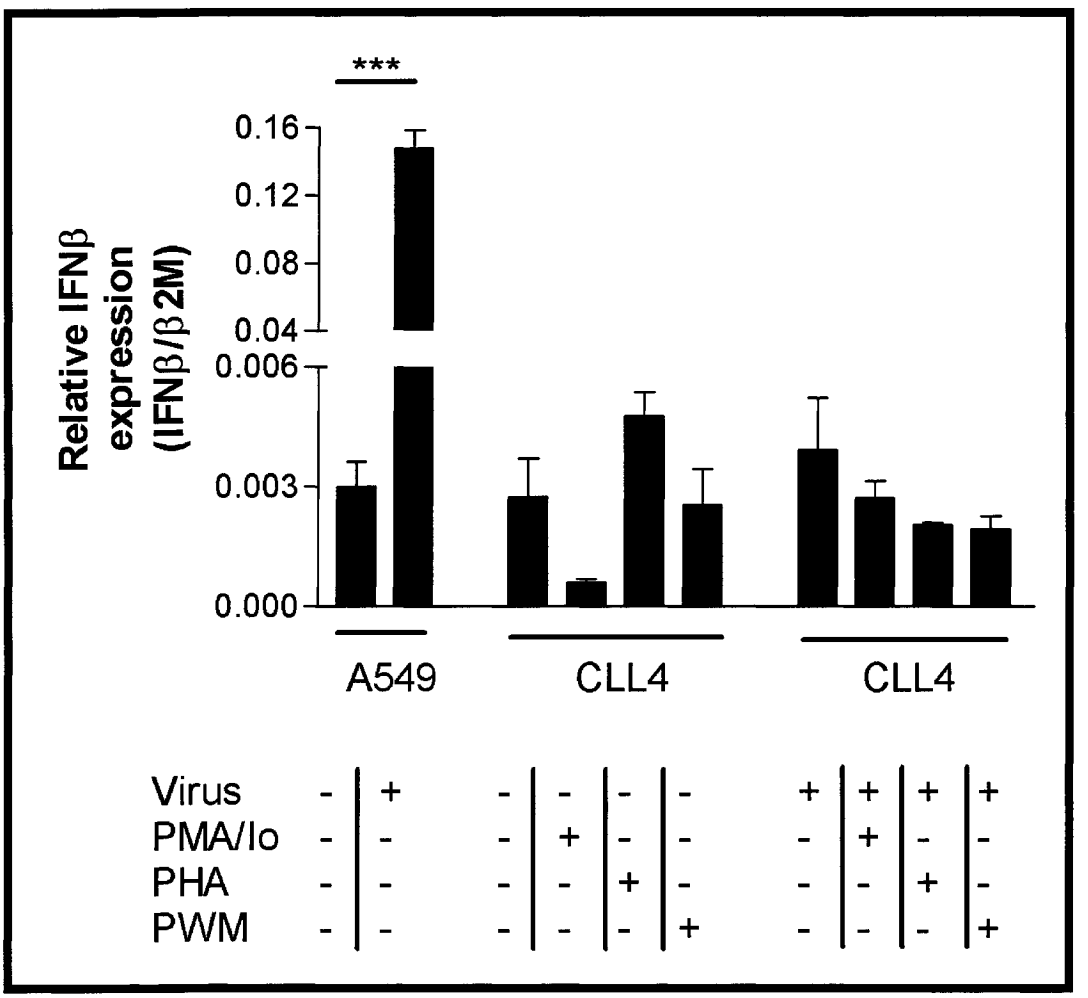
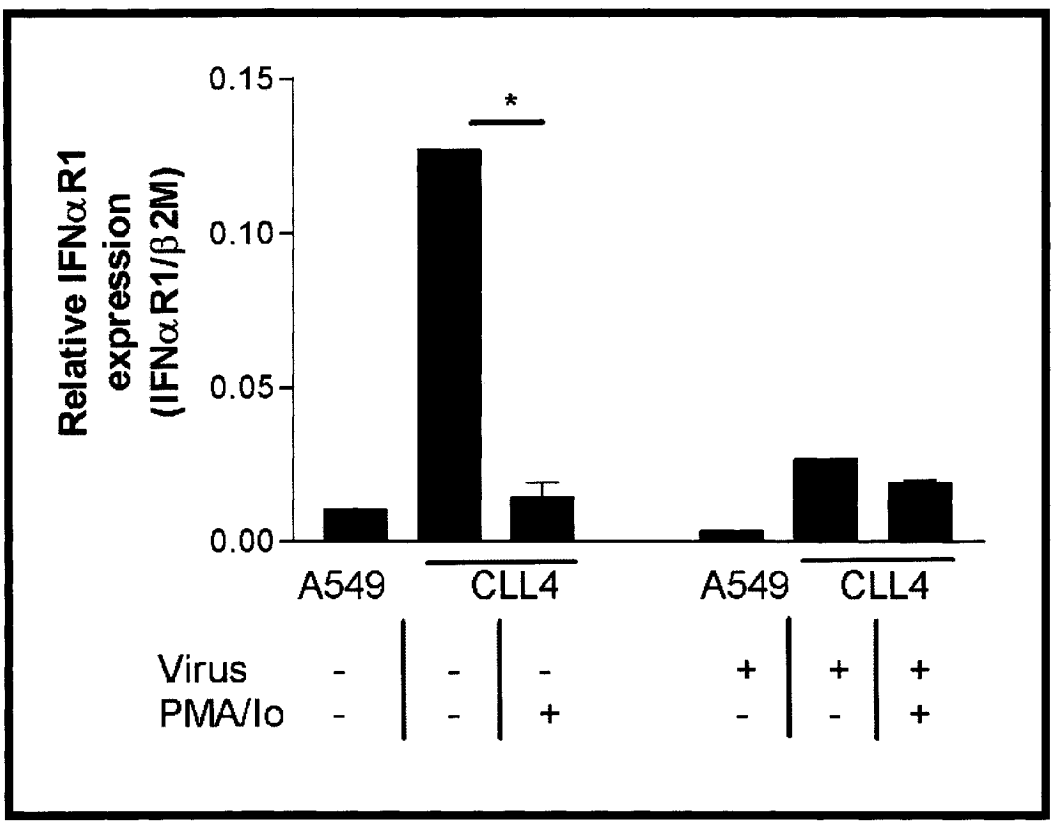


Figure 18. Activation of CLL cells with PMA/Ionomycin decreases IFNαR1 gene expression levels.

IFNαR1 levels appear to decrease subsequent to PMA/Io treatment, when comparing the uninfected CLL patient #4 uninfected versus CLL patient #4 treated with PMA/Ionomycin. Relative IFNαR1 gene expression was determined by Q-PCR using Lightcycler technology (Roche). RelQuant software was used to measure the relative expression of IFNαR1/β2-microglobulin. The A549+V sample was also used as a calibrator (an internal control) to normalize for differences between individual rounds of Q-PCR. The mean of three experiments (N=3) is presented. P=0.0263, as calculated by the Two-way ANOVA test.


Relative IFNαR1 expression (IFNαR1/β2M)
0.15
0.10
0.05
0.00
*
A549
CLL4
A549
CLL4
Virus - - - + + +
PMA/Io - - + - - +

4. DISCUSSION

4.1 Primary CLL Patient Cells are Resistant to VSV Infection

Preliminary laboratory observations suggested that CLL cells were not infected by ΔM51-GFP-VSV. This was unusual as VSV is considered an effective therapeutic in most cancer cells [25, 45]. This study aimed to better characterize this phenomenon and to determine the mechanism behind the resistance of CLL cells to VSV infection. As such, five CLL patient samples were obtained for this study and tested for sensitivity to VSV infection. A negligible proportion of cells were infected by ΔM51-GFP-VSV (Figure 5A, B-D) or wt-GFP-VSV (Appendix I).

In contrast, CLL and various other B cell malignancies are able to be efficiently infected and killed by other oncolytic viruses. Alain *et al.* have shown that 15 of 15 primary CLL patient samples tested for susceptibility to reovirus were killed by the virus. In the same study, four diffuse large B cell lymphoma lines (DLBCLs) (OCY-LY1, OCY-LY2, OCY-LY8, and OCY-LY10) also supported reovirus replication and resulted in cell death [72]. Although reovirus replication and apoptosis in host cells can be reduced through pre-treatment with IFNβ [73], reovirus preferentially acts on cancer cells with an activated Ras or Ras-signaling pathway. This is also the likely mechanism of oncolysis in CLL and DLBCLs [22, 23, reviewed in 72]. Additionally, a replication defective Herpes Simplex Virus Thymidine Kinase (HSV-TK)/ganciclovir (GCV) - mediated suicide gene therapy has shown 17.1% mean tumour killing against nine CLL patient samples [74, reviewed in 75].

VSV is however an effective therapeutic virus for the treatment of most hematological malignancies. This study demonstrates that the human leukemia cell lines AML 3 and Jurkat are readily infected by VSV as shown in Figure 5A and Figure 5E-G. Upon examination of primary patient cells, bi-phenotypic acute leukemia patient cells were

also infected by both the wild-type and the IFN-inducing forms of VSV, as seen in Figure 5A and Appendix III. Lichty *et al.* have also shown the ability of VSV to act as a leukemolytic agent, when 11 of 12 human leukemia cell lines including cell lines of myeloid and lymphoid origins were efficiently killed by VSV. In the same study, Multiple Myeloma primary samples tested were also infected by both forms of VSV [45]. Thus, the resistance of CLL cells to VSV infection is a significant observation that warrants further study.

4.2 CLL Patient Cells Constitutively Express Type I IFN Transcripts

It was hypothesized that CLL cells had a constitutively activated IFN pathway. Therefore, they were inherently resistant to VSV infection. An important characteristic of VSV is its exquisite sensitivity to IFN. An IFN-induced antiviral state in a host cell leads to inhibition of virus replication [76]. The majority of cancer cell lines (42 of 52) tested from the NCI-60 human tumour cell line panel exhibit Type I IFN defects; including six leukemia cell lines, these cell lines are infected and killed by VSV [25].

The integrity of the IFN pathway in CLL cells was examined at the transcriptional level starting with IFNβ, a member of the first wave of the human IFN cascade which is mediated by IRF 3 (see Figure 1). A549 lung carcinoma cells served as a positive control for the induction of IFNβ production subsequent to virus infection (Figure 7) based on previous studies [65, 67, 68] and confirmed by a bioassay developed in the lab (Figure 6). Relative IFNβ gene expression was determined by Q-PCR and showed that the five CLL patient samples constitutively expressed low levels of IFNβ which, in general, was not further induced through virus infection (Figure 8 and Figure 9). Hence, it appears that the proximal portion of the IFN pathway where IFNβ production ensues subsequent to virus infection, was constitutively activated to a low level in CLL cells.

The distal portion of this pathway, which begins with the binding of IFN to its receptor and ends at the development of an antiviral state, was then examined. IFNβ is secreted and binds to the Type I IFN receptor (IFNαR) which has two components, IFNαR1 and IFNαR2, to induce the synthesis of IRF 7 and cause the second wave of the human IFN cascade [40, 41, as reviewed in 32 and 77]. HeLa cervical carcinoma cells were identified as a positive control for responsiveness to exogenous IFNαβ treatment that would protect against VSV challenge, as shown by Wansley *et al.* and others [65, 68] (Figure 11). The ability of HeLa cells to respond to IFNαβ treatment was also assessed by an IFN responsiveness bioassay (Figure 10). Relative IFNαR1 gene expression showed that the distal portion of the IFN pathway was also constitutively activated in all five CLL patient samples (Figure 12).

Although the five CLL patient samples differed in progression of their disease (Figure 3), constitutive IFNβ and IFNαR1 transcript expression was a common observation in all patients. Evidence exists for the ability of CLL cells to respond to Type I IFNs and lead to cell survival *in vitro*. IFNα therapy has been shown to protect CLL cells from apoptosis-induced cell death *in vitro* [62]. Therefore, it is possible that the expression of Type I IFNs is providing resistance to cell killing by VSV infection in CLL cells.

The ALL patient sample that was examined was also shown to be resistant to VSV infection (Figure 5A) and it too displayed constitutive IFNβ and IFNαR1 transcript expression (Figure 8, Figure 9 and Figure 12). However, not all ALL patients have an intact IFN pathway. 29% of ALL patients have been shown to possess deletions in their IFNα and β genes [64]. Hence, not all ALL cells are resistant to VSV infection.

Unfortunately, gene expression analysis for the bi-phenotypic acute leukemia patient sample that was infected by VSV (Figure 5A and Appendix III) could not be conducted due to technical difficulties in the extraction of RNA from this patient sample. IFN gene expression analysis of this sample would have made an interesting comparison to the other primary patient samples which were resistant to VSV infection.

Other normal cells such as resting T cells and normal bone marrow cell progenitors are also resistant to VSV infection, while neutrophils and monocytes are infected [45]. The resistance of normal B cells to VSV that was observed in this study is in accordance with previous observations [45] (Figure 5A). One possibility for the resistance of CLL cells to VSV is that their progenitor B cells are themselves resistant to VSV and the oncogenic transformation to CLL cells did not disrupt their IFN antiviral mechanism.

This study shows a strong correlation between VSV resistant cells and constitutive IFN expression. Further experiments which specifically deplete Type I IFNs from CLL cells or other resistant cell types would be required to demonstrate a direct causal link. In one such experiment, if neutralizing antibodies to IFN$\alpha\beta$ were incubated with CLL cells prior to infection with VSV and the cells were then susceptible to VSV infection, it could be stated that Type I IFNs are responsible for the observed resistance.

In other experiments, small interfering RNAs (siRNAs) could be employed to deplete Type I IFNs or the Type I IFN receptor from CLL cells prior to infection with VSV or IFNαR -/- CLL cells could be infected with VSV. This would allow the effects of eliminating these components of the IFN pathway on the resistance of CLL cells to VSV infection to be assessed.

This study demonstrates that Type I IFN transcripts, although expressed at low levels, play a contributory role to the network of host antiviral responses to VSV (Figure 2). However, other antiviral cytokines including the Tumour Necrosis Factor (TNF) and the Interleukin-1 (IL-1) families, which are produced as a result of virus infection and have known immunomodulatory functions, could also play a role in CLL resistance to VSV infection [37].

A more complete analysis of the expression of various other genes from the IFN pathway should be conducted. The first and second waves of human IFN production are mediated by the IRF-3 and IRF-7 respectively [38]. Hence, examination of these two transcription factors would be beneficial. As well, the synthesis of IFNα and the positive feedback loop which it forms to further up-regulate the antiviral state in the infected and neighbouring cells makes IFNα a good candidate for gene expression analysis. An analysis of other ISGs such as PKR and MxA would also help to determine how the IFN pathway and its downstream signaling components in CLL cells are affected by VSV infection.

Various steps of the virus replication cycle have been shown to be inhibited by IFN-induced proteins. For instance, early replication steps such as penetration and uncoating are inhibited in SV40 and retroviruses by an unknown IFN-induced protein. Transcription of influenza, VSV and HSV RNA is inhibited by the Mx protein family and perhaps others. Translation is inhibited in picornaviruses (such as poliovirus) by the 2'-5' OAS/RNAse L mechanism. For VSV, Reovirus, adenovirus, vaccinia and influenza, translation is inhibited by PKR. In later steps of the replication cycle, such as maturation, assembly and release, retroviruses, VSV and HSV are inhibited by an unknown IFN-induced protein [37]. Hence, a

more complete analysis of IFN-induced proteins may help to define the mechanism of inhibition of VSV infection in CLL cells.

4.3 Activated CLL Cells Exhibit Increased Sensitivity to VSV Infection

Precedent exists for the activation of CLL cells through studies performed by De Fanis *et al.*, Segel *et al.* and others [69, 70, 77]. These groups have shown that CLL cells can be activated by the cell mitogens PMA/Io, PHA or PWM. However, PHA and PWM are primarily T cell activators. In addition, other laboratory investigations have demonstrated that activated T cells can be infected by VSV, while resting T cells are resistant [71].

This study demonstrates that CLL cells have been activated by PMA/Io treatment (Figure 14). Upon CLL cell activation, CD5 expression appears to increase and CD19 expression seems to decrease. This is in agreement with previously published reports of changes in CLL cell surface markers upon activation with PMA/Io [78, 79].

Of particular significance is that activated CLL cells exhibit a 20 fold increase in sensitivity to VSV infection over resting CLL cells (Figure 15). The maximum percentage of VSV infection that has been reported in resting cells is 0.65 (Figure 5A). This study shows an infection rate of 12.52% in activated cells (Figure 15). CD5+ activated cells also have an increased ability to be infected by VSV (Figure 16).

One reason for incomplete CLL cell sensitization may be that the optimal conditions for activation have not yet been achieved. These conditions include concentration of PMA/Io and length of cell incubation. Other cell activators may also provide greater sensitization to VSV infection. It is important to recognize that the cell activation experiment conducted for this work was only performed once in its entirety with CLL cells from one patient. Although very encouraging, the reproducibility of these results must be confirmed in a larger number of patient samples.

4.4 Activated CLL Cells have Down-regulated Type I IFN Transcripts

The resistance of CLL cells to VSV infection correlates with constitutive expression of IFNβ and IFNαR1 transcripts. This suggests that activated CLL cells would exhibit down-regulated IFN transcripts, thereby achieving increased sensitivity to VSV infection. IFNβ transcript levels are down-regulated 4.5 fold (Figure 17) and IFNαR1 transcripts showed a 9 fold down-regulation (Figure 18), when comparing resting and PMA/Io-activated CLL cells.

After virus infection, IFN transcript levels in activated cells return to approximately the same levels as in resting cells. This suggests that virus infection has caused re-stimulation of the IFN pathway, which may be a contributing factor to the incomplete CLL cell sensitization to VSV infection. There appears to exist an interplay between VSV infection and IFN induction. If VSV infection dominates this interplay, perhaps through a high MOI, then the proportion of infected cells may be high. On the other hand, if IFNs are given the time to be induced, perhaps through a lower MOI, then the proportion of infected cells may be lower.

Activation of cells through PMA treatment has been shown to down-regulate various ISGs. Chase *et al.* demonstrate that PMA treatment of mouse L929 fibroblasts leads to the rapid degradation of the 2'-5' OAS-dependent RNAse L [80]. Upon treatment with an inhibitor of PMA, RNAseL degradation was completely blocked. Similarly, Zhou *et al.* have shown that PMA decreased levels of auto-phosphorylated PKR in IFN-treated mouse fibroblast cells [81]. These results suggest that various genes involved in the IFN pathway can be down-regulated through treatment with PMA and perhaps should be examined.

In contrast, PMA treatment has also been shown to induce expression and activity of ISGs. A case in point: Cohen *et al.* demonstrated that PMA-induced expression and catalytic

activity of 2'-5' OAS occurs in the human myeloid leukemia cell line HL-60. Treatment with PMA led to induction of the IFN pathway [82].

4.5 A Proposed Role for Type I IFN Transcripts in Resting and Activated CLL Cells

A model of the proposed role for Type I IFNs in resting and activated CLL cells is presented in Figure 19.

Step 1: This study has shown that in resting CLL cells, IFNβ and IFNαR1 transcripts are constitutively expressed.

Step 2: This transcript expression protects the cells against VSV infection.

Step 3: CLL cells are activated with PMA/Io for 48 hours.

Step 4: The levels of Type I IFN antiviral transcripts decrease. Also upon activation, CD19 expression is decreased, while CD5 and CD69 expression increases.

Step 5: Activated CLL cells exhibit increased sensitivity to VSV infection.

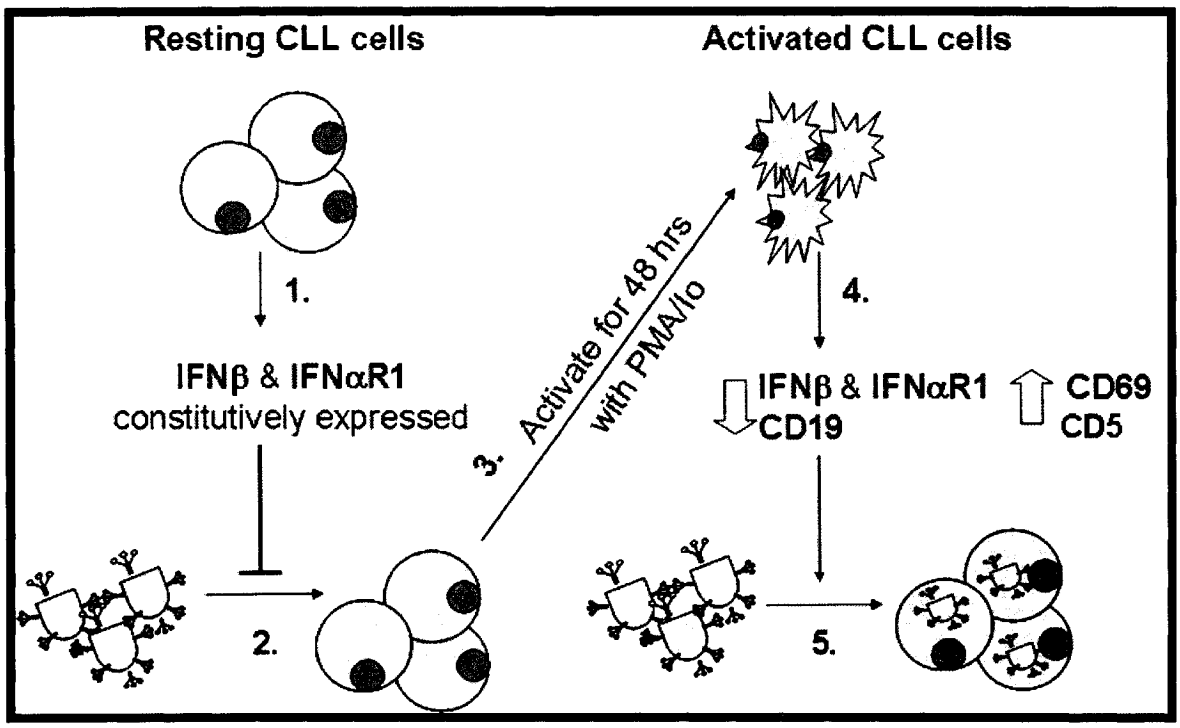
4.6 Future Directions

Preliminary work done in this study suggests that constitutive Type I IFN transcript expression in CLL cells may contribute to the inherent resistance of CLL cells to VSV. Upon activation of CLL cells, down-regulation of Type I IFN transcripts occurs in conjunction with increased susceptibility to VSV infection. This study sets the stage for many future steps in this field.

In addition to the future work presented throughout the above sections, protein levels should be examined to correlate with gene expression profiling that was performed through Q-PCR in this study. Subsequently, a cDNA microarray analysis of the differences between

Figure 19. A proposed role for the Type I IFN transcriptional response in resting and activated CLL cells.

In step **1**, the antiviral Type I IFN transcripts- IFNβ and IFNαR1, are constantly present in resting CLL cells hence blocking VSV infection from occurring in step **2**. However, upon activation of CLL cells with PMA/Ionomycin for 48 hours (in step **3**), IFNβ and IFNαR1 transcript levels decrease, as do CD19 levels, while CD5 and CD69 levels increase (in step **4**). Activated CLL cells are now able to be infected by VSV (step **5**). ⊣ represents the block in VSV infection occurring through IFN transcript expression.


Resting CLL cells
Activated CLL cells
1.
IFNβ & IFNαR1
constitutively expressed
2.
3. Activate for 48 hrs
with PMA/Io
4.
IFNβ & IFNαR1
CD19
CD69
CD5
5.

resting and activated CLL cells, before and after virus infection could be performed, to provide greater insight into other genes in the IFN pathway or other antiviral pathways that may be affected by CLL cell activation. Target candidates could then be confirmed by Q-PCR and protein level analysis.

This study has contributed to the understanding of a viral resistance pathway exhibited by CLL cells and has found a potential method of down-regulating portions of this pathway to achieve enhanced susceptibility to VSV infection. A greater understanding of the mechanisms of viral resistance in CLL and uncovering novel approaches of manipulating these resistance mechanisms may allow for the future use of VSV as a therapy for CLL.

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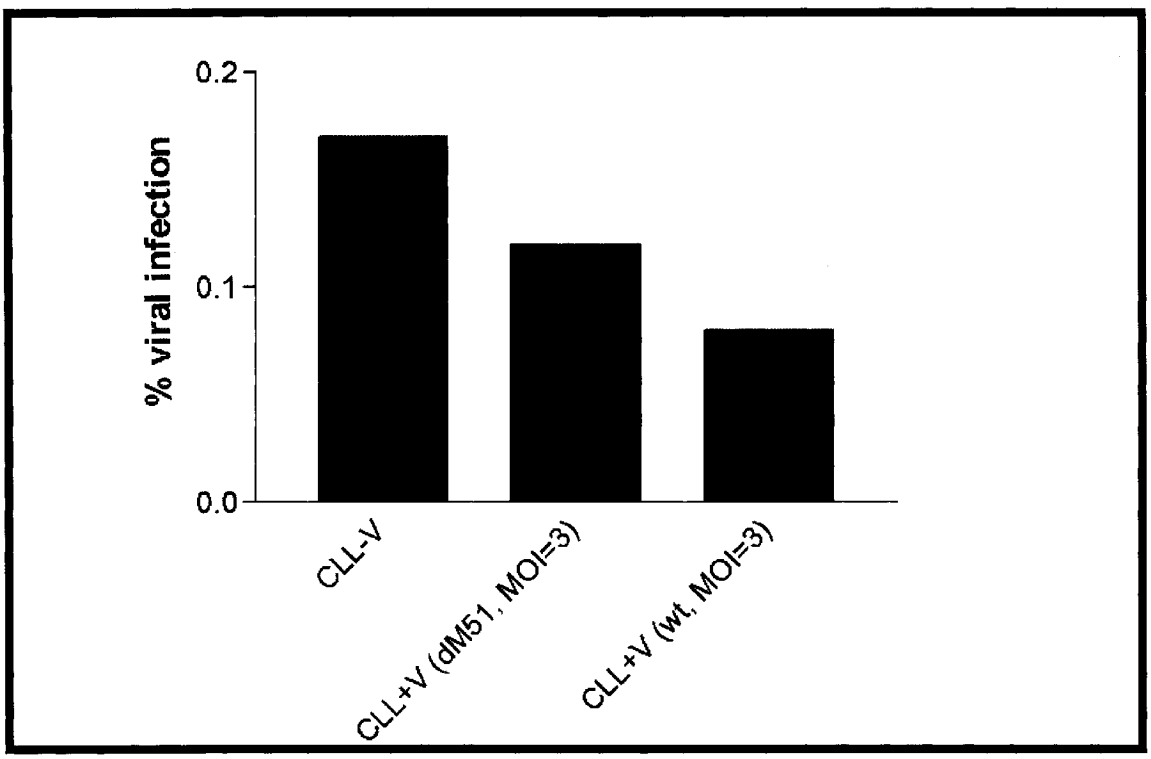
APPENDICES

Appendix I. Representative 6-day infection of CLL patient cells with ΔM51 and wt VSV.

Representative virus (V) infection from one of five CLL patient samples is presented. CLL cells were infected with ΔM51-GFP-VSV and wt-GFP-VSV at an MOI of 3 for 6 days. The percentage of VSV-infected cells was assessed by the proportion of GFP-expressing cells as measured by flow cytometry. CLL cells are resistant to infection with ΔM51- and wt-VSV.


0.2
0.1
0.0
% viral infection
CLL-V
CLL+V (dM51, MOI=3)
CLL+V (wt, MOI=3)

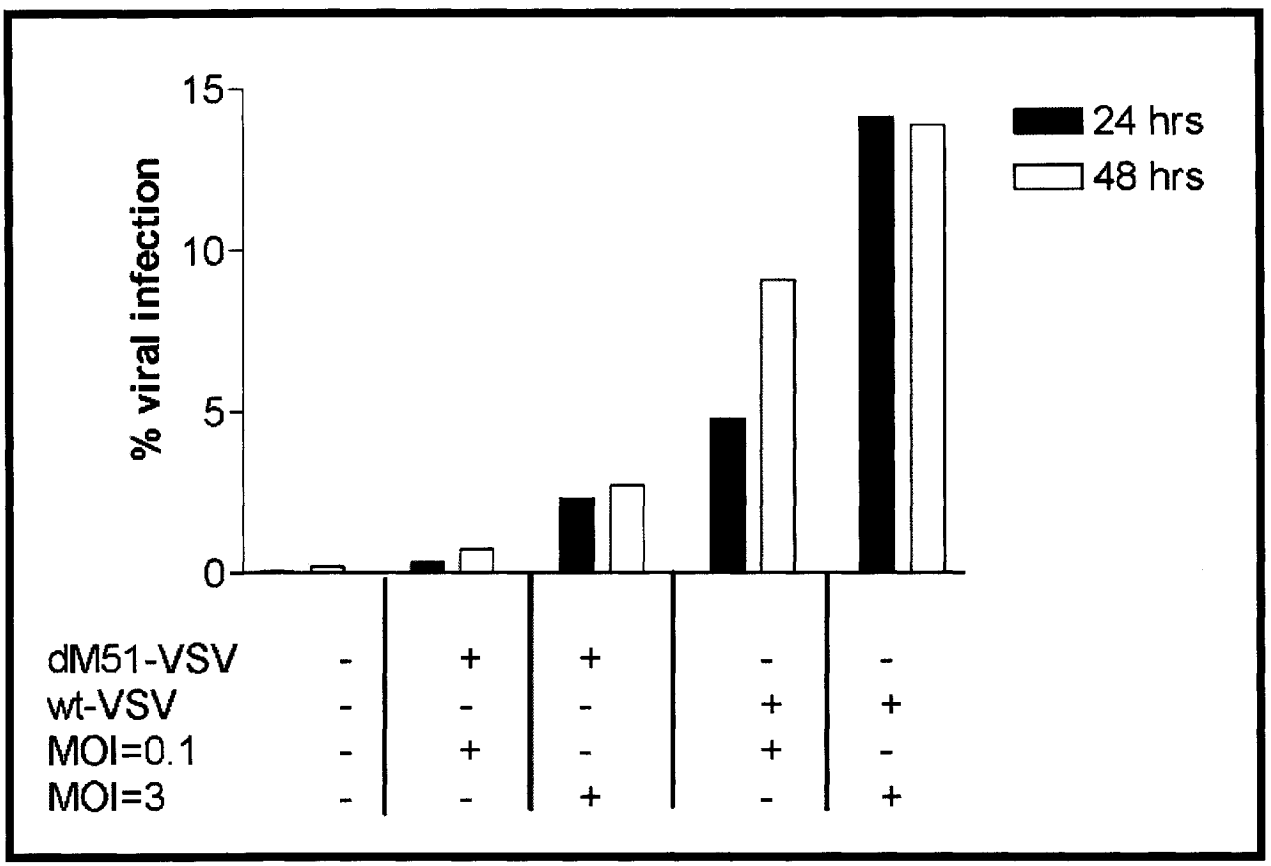
Appendix II. Trypan blue exclusion analysis from a representative CLL & Jurkat cell infection with ΔM51-GFP-VSV at an MOI=3 for 24 hours.

Representative cell viability analysis is presented from one of five CLL patient samples and control Jurkat leukemia cells. Both cell types were infected with ΔM51-GFP-VSV (V) at an MOI of 3 for 24 hours. Trypan blue staining was performed and cells were counted on a hemacytometer. CLL cells are viable after infection, whereas Jurkat cells are not.

Sample	# white (live) cells	# blue (dead) cells	% blue (dead) cells
CLL3-V	162	3	1.8
CLL3+V	154	3	1.9
Jurkat-V	17	4	19.0
Jurkat+V	20	21	51.2

Appendix III. Infection of a bi-phenotypic acute leukemia patient sample with wt and ΔM51 VSV.

Infection of a bi-phenotypic acute leukemia patient sample is presented. Cells are infected with ΔM51-GFP-VSV or wt-GFP-VSV at various MOI and time points. The percentage of VSV-infected cells was assessed by the proportion of GFP-expressing cells as measured by flow cytometry. Bi-phenotypic acute leukemia patient cells are able to be infected by VSV.

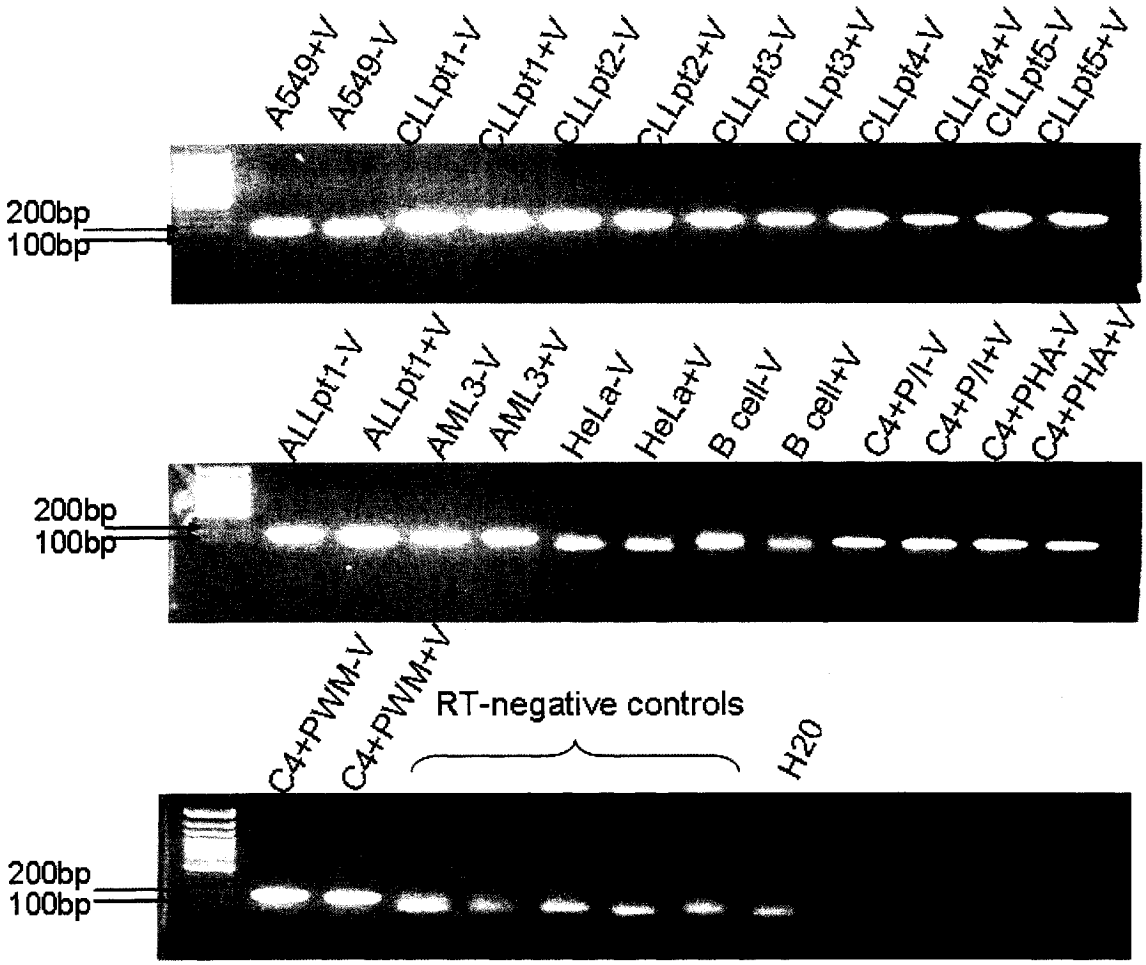

15
10
5
0
% viral infection
24 hrs
48 hrs
dM51-VSV - + + - -
wt-VSV - - - + +
MOI=0.1 - + - + -
MOI=3 - - + - +

Appendix IV. Plaque assay results from filtered and non-filtered supernatants.
Plaque assays were performed to measure the amount of virus in A549 cell infected supernatants before and after filtering with Mustang Q filters. Representative measurements are presented from one of three assays. Plaques were initially Too Numerous To Count (TNTC) in the positive control and in the non-filtered supernatant (Non-filt sup), however there was no discernable virus present in the filtered supernatant.

	# of plaques
Media alone (negative control)	0
ΔM51 infection (positive control)	TNTC
Non-filt sup undiluted	TNTC
Non-filt sup -5 dilution	136
Non-filt sup -6 dilution	31
Non-filt sup -7 dilution	0
Filtered sup undiluted	0

Appendix V. IFNαR1 expression in all patient and control cells pre- and post- virus infection and pre- and post-activation.

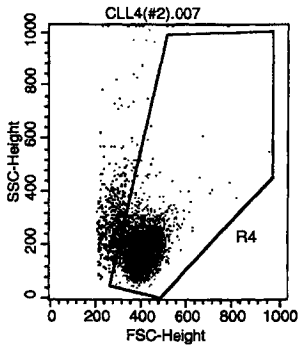
5 μL of cDNA from a representative Q-PCR reaction for IFNαR1 underwent gel electrophoresis. Cells from five CLL patients (CLL pt1-5), from Acute Lymphocytic Leukemia patient 1 (ALLpt1), A549, HeLa, AML 3 and normal B cells were infected for 24 hours with ΔM51-GFP-VSV (V) at an MOI of 3. Cells from CLL pt 4 that were activated with PMA/Ionomycin (P/I), PHA and PWM are also shown before and after infection with ΔM51-GFP-VSV (V) at an MOI of 3 for 24 hours. Reverse-transcriptase negative (RT-neg.) controls are also shown. The expected product band size for IFNαR1 is 178 base pairs (bp).


A549+V
A549-V
CLLpt1-V
CLLpt1+V
CLLpt2-V
CLLpt2+V
CLLpt3-V
CLLpt3+V
CLLpt4-V
CLLpt4+V
CLLpt5-V
CLLpt5+V
200bp
100bp
ALLpt1-V
ALLpt1+V
AML3-V
AML3+V
HeLa-V
HeLa+V
B cell-V
B cell+V
C4+P/I-V
C4+P/I+V
C4+PHA-V
C4+PHA+V
200bp
100bp
C4+PWM-V
C4+PWM+V
RT-negative controls
H20
200bp
100bp

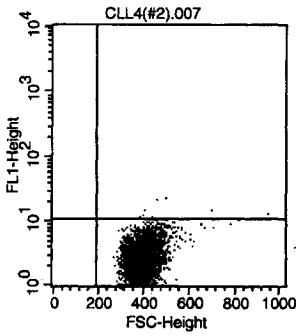
Appendix VI. VSV sensitivity of PHA-treated CLL cells.
Flow cytometry data is presented where 10, 000 cells were acquired in each sample. $1x10^6$ PBMCs were incubated with the appropriate antibody. Antibodies include: CD19- PE-Cy5 and CD5-R-PE. In samples presented, R4= Region 4 that represents the major population of viable cells, R3=CD19+5+ cells. Figures **A-C** represent the untreated cells that were infected with virus while **D-F**, represent the infected cells after PHA-treatment. CLL cells treated with PHA experience mild sensitization to VSV infection.

Untreated+V

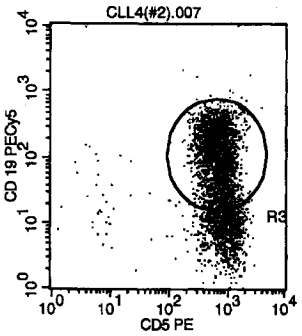
A Forward/side scatter



B %GFP+ cells: 0.20

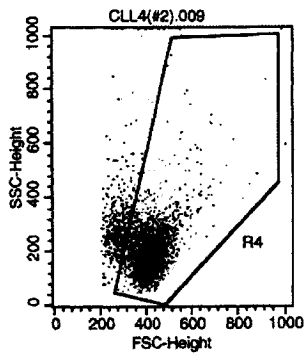


C CD19+5+ cells

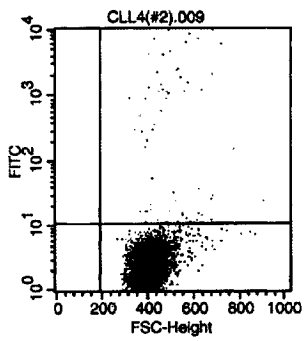


PHA-treated+V

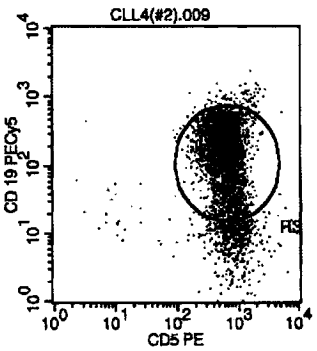
D Forward/side scatter



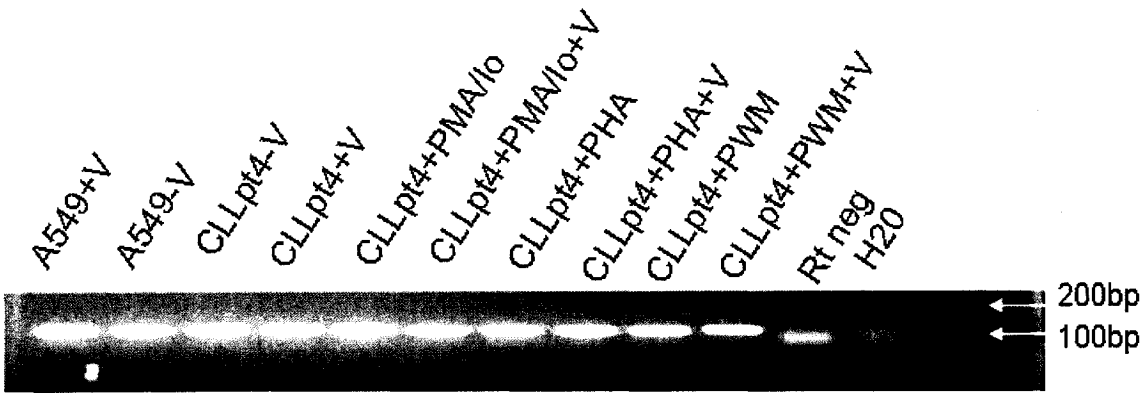
E %GFP+ cells: 1.52



F CD19+5+ cells



Appendix VII. β2-microglobulin gene expression levels in activated CLL patient 4 cells. 5 μL of cDNA from a representative Q-PCR reaction for b2-microglobulin (b2M) was analyzed by gel electrophoresis. Cells from CLL patient 4 (CLLpt4) are shown pre- and post-activation with various cell activators for 48 hours and pre- and post- infection with ΔM51-GFP-VSV (V) at an MOI of 3 for 24 hours. A549+V sample was used as an internal control (a calibrator) between experiments. Reverse-transcriptase negative (RT-neg.) and water (H_20) controls are also shown. The expected product band size for b2M is approximately150 base pairs (bp).


A549+V
A549-V
CLLpt4-V
CLLpt4+V
CLLpt4+PMA/Io
CLLpt4+PMA/Io+V
CLLpt4+PHA
CLLpt4+PHA+V
CLLpt4+PWM
CLLpt4+PWM+V
Rt neg
H20
200bp
100bp

CURRICULUM VITAE

Femina Adatia

Faculty of Medicine-Biochemistry
Human and Molecular Genetics
Ottawa Hospital Regional Cancer Centre
503 Smyth Road
Ottawa, ON, K1H 1C4
Phone: (613) 737-7700 x70353
E-mail: fadat087@uottawa.ca

Education:

Sept. 2003–Present **University of Ottawa** (Ottawa Health Research Institute), Ottawa, ON, Canada.

- Candidate for Master of Science – Human and Molecular Genetics, Cancer Research (expected date of completion: November 2005)

Sept.1998–June 2003 **McGill University**, Montreal, QC, Canada.

- Bachelor of Science – Honours Microbiology & Immunology, Minor Management

Sept.1993–June 1998 **Brookfield High School**, Ottawa, ON, Canada.

- Ontario Secondary School Diploma, graduated with Honours with Distinction
- Bilingual and Spanish Certificates obtained

Work Experience:

Sept 2003–Present Ottawa Health Research Institute, Ottawa, ON
Research Project: A Role for Type I Interferons in the Resistance of Chronic Lymphocytic Leukemia to Vesicular Stomatitis Virus

May–Aug. 2003 Merck Frosst Canada, Montreal, QC
Research Project: Examining the role of cysteine proteases in osteoporosis

May 2002–2003 Montreal Neurological Institute and Hospital, Montreal, QC
Research Project: B cell cytokine profiling in Multiple Sclerosis patients

May 2002–2003 Vice-Principal of Administration and Finance Office, McGill University, Montreal, QC
Administrative Assistant
- Communicating with senior administration
- Assisting office personnel with accounting and administrative tasks during peak times

Summers of 1997–2001 Statistics Canada, Ottawa, ON
Statistical/Administrative Assistant
- French translation of Excel tables, updating the division's web site, basic computer programming
- Mastering subject matter & training supervisors, census clerks
- Implementing system to audit the work of clerks

1996–1999 Wal-Mart Canada, Ottawa, ON
Pharmacy/Courtesy Desk Associate
- Handling the cash register, offering creative solutions to customer service problems, communicating with people of diverse needs

Volunteer Experience:

July 2005–Present **Vice-chair: Health & Wellness team, Ismaili Council,** Ottawa, ON
- Implementing programs to increase awareness in the Ottawa Ismaili community of healthy behaviour and disease prevention strategies

Sept 2004–Present **"Let's Talk Science" Partnership Program,** Ottawa, ON
- Creating and presenting interactive demonstrations on DNA forensics and biotechnology to Grade 11 Biology students and on stem cells & transgenic mice to Grade 12 Biology students
- Trying to inspire high school students in the pursuit of scientific knowledge, coordinating curriculum and presentations with teachers

Jan. 2004–Mar. 2005 **Vice-coordinator: Fire and Safety Team, Ismaili Volunteer Corps for Ottawa,** Ottawa, ON
- Educating the Ottawa Ismaili Community on disaster prevention (medical, fire or natural) via formal training sessions and simulations

Sept.2002–June 2003 **Montreal Neurological Institute and Hospital**, Montreal, QC
- Communicating with patients of all ages, lending social support and encouragement, promoting psychosocial wealth

Sept. 2001–Present **Terry Fox Foundation,** Ottawa, ON & Montreal, QC

- Organizing fundraisers in support of the foundation (BBQs, skate-a-thons)
- Participating in and volunteering at the Annual Terry Fox Run

Sept. 2000–June 2003 **Community Service Outreach Program– Red & Green Door,** Montreal, QC

- Spearheading activities performed by McGill students to assist members of the community from all age groups and marketing this project to the Ismaili Council for Quebec, recruiting volunteers to assist with activities

Sept. 2000–June 2003 **McGill University, Rotary Club**– University Chapter, Montreal, QC

- Coordinating food drives in the McGill community, serving meals at the local soup kitchen and fundraising for schools in India

Sept.–Dec. 2000 **McGill University, Project Chance**, Montreal, QC

- Providing single student mothers from McGill and Concordia with childcare assistance
- Organizing mentorship activities between children and care-givers

Sept. 1997–Present **Canadian Blood Services/Héma-Québec**, Ottawa, ON and Montreal, QC

- Organizing and ensuring successful mobile clinics in the community
- Performing rest, coffee and reception duties in the clinics

Sept. 1987–Present **Ismaili Volunteer Corps (IVC)**, Ottawa, ON and Montreal, QC

- Liaising between volunteers and community leaders, coordinating activities for volunteers (ages 8-90), implementing fun-filled activities for the Junior Volunteers (ages 8-13)

Extracurricular Activities:

Sept. 2003–Present B.E.L.I.E.F. (Becoming Educated Leaders Inspired by Exemplary Females) -Women's Development Group- Member

Sept. 2003–July 2004 I-STAR (Ismaili Students Total Achievement and Recognition Program), Ottawa, ON

- Ottawa Lead: Executing a national awards competition & networking session for high school students, motivating students to continue to excel

Sept. 2003–June 2004 "Rangeeli Sham" Talent Night (Ismaili Community Youth & Sports Board)

- V.P. External: Liaising between Ottawa, Kingston and Montreal to coordinate information delivery and performances

Sept. 2002–June 2003 Héma-Québec and the Science Undergraduate Society of McGill University

- Blood Drive Coordinator:
  -Coordinating on-campus clinics, networking with local sponsors
  -Raising awareness and promoting the importance of blood donation among students, staff and the Montreal community

Sept. 2000–June 2003 UNICEF Member- McGill University Chapter

Sept. 2000–June 2003 McGill Model United Nations Member

Sept. 2000–June 2003 ITREB (Ismaili Tariqah & Religious Education Board) Representative-McGill University chapter

- Organizing book club meetings and recruiting speakers from across the nation to deliver inspirational talks

Sept. 1999–June 2000 Montreal Ismaili Students' Association

- V.P. Social: -Organizing and implementing exciting social, fundraising and educational activities for the Montreal Ismaili University students

Sept. 1998–present McGill University & University of Ottawa Intramural Ball Hockey – Defensive player

Sept. 1994–June 1998 Brookfield High School- Student Government Representative

May 1991–Present World Partnership Walk Committee Member– Aga Khan Foundation Canada

- Being part of the organizing team and raising funds for the walk (proceeds matched by CIDA, aimed at development of Third World Countries)

Sept. 1984–Present Indian Classical and Modern Dancing

- Participating in several performances, danced in Shanghai, China during the summer of 1994 at the International Children's Art Festival

Awards/Scholarships:

2005	"Let's Talk Science" Outstanding Volunteer Award, University of Ottawa
2003	First Class Honours (Microbiology & Immunology Dept.), McGill University
2001	Ismaili Students Total Achievement and Recognition Awards (I-STAR) -Academic Achievement and Youth Leadership – Montreal, QC
2001	Golden Key International Honor Society Member – McGill University
1998-2002	J.W. McConnell Entrance Scholarship for four years – McGill University
1998	Annual Governor General's Award – Brookfield High School
1998	Annual Subject Awards OAC Chemistry, Algebra & Geometry, English, French and Spanish – Brookfield High School
1998	I-STAR Top Academic, Community Service, Student of the Year– Ottawa, ON
1998	Canadian Parents for French (CPF) Award: Top Ottawa Board of Education (OBE) bilingual program graduate
1997	OBE French Award: Second place, Public Speaking Contest
1996	CPF Award: Third place, Concours de français
1993-1998	Annual medals of merit – Renuka Sahay's Indian Dance School-Ottawa, ON

Languages:

Fluent in English, French, Gujrati, Urdu, Hindi, basic knowledge of Kachi and Spanish

Published papers/manuscripts:

Duddy M, Niino M, **Adatia F**, Hebert S, Kim HJ, Bar-Or A. A novel network of human B cell effector cytokines is implicated in autoimmunity: Dysregulation in patients with multiple sclerosis and potential as therapeutic target. (In preparation.)

Abstracts:

Adatia F, Bell JC, Atkins H. Examining host antiviral responses to Vesicular Stomatitis Virus (VSV). Annual graduate student poster day. University of Ottawa. Ottawa, ON. 2004.

Adatia F, Bell JC, Atkins H. Characterization of host antiviral responses to Vesicular Stomatitis Virus (VSV). Third international meeting on replicating oncolytic virus therapeutics. Banff, AB. 2005.

Presentations:

Adatia F, Bar-Or A, Hebert S, Duddy M. Distinct B cell cytokine profiles: potential target for therapy in Multiple Sclerosis? Honours Students Final Presentations. McGill University. Montreal. 2003.

Adatia F, Lamontagne S, Cromlish W. Examining the role of cysteine proteases in osteoporosis. Annual presentations to the Molecular Biology/Biochemistry departments. Merck Frosst Canada. Montreal. 2003.

Bar-Or A, Hebert S, **Adatia F**, Duddy M, Jalili F. Abnormal profile of B cell regulatory cytokines in MS: Potential target of therapy.

- World Congress of Immunology/FOCIS meeting, Montreal 2004.
- Platform presentation. Annual Meeting of the European Neurological Society (ENS). Barcelona, Spain. 2004
- Platform Presentation. Canadian Congress of Neurological Sciences (CCNS) Annual Scientific Meeting. Calgary, AB. 2004.
- Platform presentation. 56th Annual Meeting of the American Academy of Neurology (AAN), San Francisco. Selected for Scientific Highlights Session. 2004.

Bar-0r A., Yamashita T., Niino M., Alatab S., Jalili F., Hebert S., Ifergan I., Schreiner B., **Adatia F.** and Ghorayeb C. Prospective study of in vivo GA effects on B cell responses in patients with multiple sclerosis. Platform presentation. 57th Annual Meeting of the American Academy of Neurology (AAN), Miami. Selected for Scientific Highlights Session. 2005.

Adatia F, Bell JC, Atkins H. Determining host viral resistance mechanisms to Vesicular Stomatitis Virus (VSV).

- Annual presentations to the Cancer Research Group of the Ottawa Regional Cancer Centre. Ottawa, ON. 2004 & 2005.
- Graduate Student Seminar to the Biochemistry, Microbiology & Immunology (BMI) Department. University of Ottawa. 2005.

Adatia F, Bell JC, Atkins H. Characterization of host antiviral responses to Vesicular Stomatitis Virus (VSV). Third international meeting on replicating oncolytic virus therapeutics. Banff, AB. 2005.

Conferences:

Bionorth 2003: 10th Annual International Life Sciences Conference and Exhibition. Hosted by the Ottawa Life Sciences Council (OLSC). Ottawa, ON. 2003.

Ottawa Health Research Institute (OHRI) Research Day. Ottawa, ON. 2003, 2004

Third international meeting on replicating oncolytic virus therapeutics. Banff, AB. 2005.

Canadian oncolytic virus consortium meetings. Ottawa, ON. 2005.

CONTRIBUTION OF COLLABORATORS

Dr. Mark Minden provided the AML 3 cells.

Dr. Jonathan Angel donated the PMA/Ionomycin, PHA and PWM.

Shane Knowles and Leigh Miller provided the ΔM51-GFP-VSV and wild type-GFP-VSV.

Dr. Isabelle Bence-Bruckler and Dr. Luke Chen recruited patients for this study.

Dr. Robert El-Maraghi and Dr. James Villeneuve drew blood from healthy donors.