

**DEVELOPMENT OF VESICULOVIRUS-BASED THERAPEUTICS  
FOR ACUTE LEUKEMIA**

David Paul Conrad

This thesis is submitted to the  
Faculty of Graduate and Postdoctoral Studies  
in partial fulfillment of the requirements  
for the Doctorate in Philosophy degree in Cellular and Molecular Medicine

Department of Cellular and Molecular Medicine  
Faculty of Medicine  
University of Ottawa

# Abstract

---

Outcomes for most patients with acute leukemia remain dismal. *In-vitro*, vesiculovirus members induced rapid apoptosis of acute leukemia cells. Intravenous injection of lymphoblastic leukemia cells infected *ex-vivo* with attenuated Vesicular Stomatitis Virus or Maraba Virus followed by gamma-irradiation, controlled leukemic progression in murine recipients. Essential properties of this autologous vaccine [immunotherapy by Leukemia-Oncotropic Virus (iLOV)] and the host's immune system were characterized. iLOV durability was restricted to the leukemia used to manufacture the vaccine. At administration, virion cell-entry was required but vesiculovirus lifecycle completion was not essential. Apoptotic or necrotic leukemia cells, with/without co-injection of virus, were ineffective vaccines. Similarly ineffective were leukemia cells activated by, or injected with, Toll-like receptor agonists. Naïve recipients of adoptive splenocyte transfer from vaccine-treated immunocompetent donors were protected from leukemic challenge. Efficacy was notably diminished following matched allogeneic bone marrow transplantation; this correlated with isolated depletion of cytotoxic T-cells. iLOV was ineffective in athymic mice. Taken together, iLOV therapy relies on immediate spatiotemporal interactions between infected-dead/dying leukemia cells and the immune system; this promotes adaptive anti-tumor responses. Clinical translation could target patients in remission to control relapse.

During the above I discovered that under specific conditions, live vesiculovirus exposed to a precise window of UV fluence reproducibly generates unique “non-

replicating rhabdovirus-derived particles” (NRRPs) that maintain cell-entry and cytopathic properties. A gamut of leukemia cells, including multidrug-resistant blasts, underwent rapid NRRPs-induced apoptosis. Normal cell lines and healthy bone marrow mononuclear cells were resistant, in part through interferon-mediated signaling responses. Administering NRRPs intravenously was curative in a murine acute leukemia model, versus uniform disease progression using maximal tolerated dose of replicating virus. Serum levels of an array of immunomodulatory cytokines were significantly elevated after injection of NRRPs. iLOV prepared with NRRPs protected recipients from otherwise lethal leukemia. Intracranial administration of NRRPs proved nonlethal as opposed to neurotoxic live vesiculovirus. Following treatment, neutralizing antibodies were diminished with NRRPs compared to replicating virus. Together, NRRPs exhibit enhanced therapeutic index over replication-competent vesiculovirus. Leukemocidal activity of NRRPs is exerted through a plurality of immune-related and direct cytotoxic effects. This novel approach now extends vesiculovirus-based therapeutics into upfront treatment for acute leukemia.

## Acknowledgements

After close to a decade practicing Internal Medicine and Critical Care, more creativity was needed; I decided to pursue a research and development career within fields of longstanding interest to me – hematology, oncology and immunology. First, I thank Dr. Melissa Forgie for providing me this opportunity to re-enter academia in 2007, as a trainee in the uOttawa Hematology Residency Program. This fellowship under Dr. Forgie was immensely rewarding on several levels and provided ample research elective time to align career objectives. Next, I would like to thank Dr. Phil Wells for his support, advice and mentorship throughout my hematology fellowship and graduate school training. Despite his busy schedule, I greatly appreciated that he always found time to meet for scheduled and impromptu discussions ranging from basic and clinical research to career development and future plans. Thank you to Dr. Cory Batenchuk and Dr. Fabrice Le Boeuf for their collaboration and expertise on our various projects inside and outside the lab - another photo-finish gentleman! Thanks also goes to my friends, colleagues and professors within the various laboratories at the OHRI and uOttawa, too many to mention by name. Together you made a thoroughly gratifying graduate school experience. Thank you to members of my thesis advisory committee, Dr. Bill Cameron (also for support and mentorship), Dr. Bruce McKay and Dr. Luc Sabourin. Of course, many thanks to Drs. John Bell and Harry Atkins for introducing me to the interesting field of viral oncolytics and for providing an enriched laboratory experience – one that encourages open dialogue and exploration of scientific curiosities. As well, who knows what new therapies are spawned from the scientific discourse that occurs at Dr. Bell’s famous (infamous) house parties? Last but not least, I’d like to thank Belinda for her support and willingness to allow the dining room to disintegrate, temporarily, into a paper-strewn office.

*“The really good idea is always traceable back quite a long way, often to a not very good idea which sparked off another idea that was only slightly better, which somebody else misunderstood in such a way that they then said something which was really rather interesting.”*

John Cleese

## List of Figures

### Chapter 2. Leukemia Cell-Rhabdovirus Vaccine: Personalized Immunotherapy for Acute Lymphoblastic Leukemia

- Figure 2.1** *MG1 virus infects human and murine leukemia cells in vitro; however, it is ineffective at halting progression of established murine leukemia in vivo* ..... 57
- Figure 2.2** *MG1-iLOV eliminates otherwise lethal leukemic challenge* ..... 60
- Figure 2.3** *iLOV induces potent and durable immune-mediated leukemia-specific protection* ..... 65
- Figure 2.4** *Virus infection is critical to the induction of iLOV-mediated anti-leukemic immunity*..... 69
- Figure 2.5** *Host or leukemia cell stimulation with TLR agonists, or injection of necrotic or apoptotic leukemia cells are insufficient to produce effective anti-leukemia immunity* ..... 72
- Figure 2.6** *Pre-existing anti-viral immunity does not impair the development of anti-leukemia immunity*..... 75

### Chapter 3. Non-Replicating Rhabdovirus-Derived Particles (NRRPs) Eradicate Acute Leukemia by Direct Cytolysis and Induction of Anti-tumour Immunity

- Figure 3.1** *NRRP-mediated cytotoxicity in immortalized cells*..... 94
- Figure 3.2** *NRRP-mediated cytotoxicity in leukemic cells* ..... 97
- Figure 3.3** *NRRPs specifically target tumor cells with defects in antiviral signaling pathways*..... 101
- Figure 3.4** *Treatment of chronic myeloid leukemia (CML)-blast crisis patient samples with NRRPs* ..... 104
- Figure 3.5** *NRRPs eradicate acute leukemia by inducing immunogenic apoptosis* ... 108

### Chapter 4. General Discussion

- Figure 4.1** *Evolving treatment approach for acute leukemias* ..... 123

## Appendix Figures and Tables

<b>Appendix Figure 1</b> <i>Treatment schedules</i> .....	155
<b>Appendix Figure 2</b> <i>Cytopathic effect induced by rhabdovirus infection</i> .....	157
<b>Appendix Figure 3</b> <i>iLOV efficacy using VSVd51 virus</i> .....	159
<b>Appendix Figure 4</b> <i>Efficacy of EL4-based iLOV</i> .....	161
<b>Appendix Figure 5</b> <i>iLOV induces leukemia-specific immunity</i> .....	163
<b>Appendix Figure 6</b> <i>Activated L1210 cells</i> .....	165
<b>Appendix Figure 7</b> <i>UV-treated apoptotic and pressure-disrupted necrotic L1210 cells used for specific vaccine preparations</i> .....	167
<b>Appendix Figure 8</b> <i>NRRPs retain cell-entry capacity but exhibit disrupted genome expression</i> .....	169
<b>Appendix Figure 9</b> <i>Maraba-based NRRPs</i> .....	171
<b>Appendix Figure 10</b> <i>Fluorescent microscopy images</i> .....	173
<b>Appendix Figure 11</b> <i>Multiplex cytokine array of MG1-infected murine acute lymphoblastic leukemia cells</i> .....	175
<b>Appendix Figure 12</b> <i>Antileukemia antibody response following vaccination (x3) of iLOV and various control vaccines</i> .....	177
<b>Appendix Figure 13</b> <i>Antibody-mediated complement-dependent cytotoxicity using serum collected from mice either treated, or not, with iLOV followed by leukemic challenge</i> .....	179
<b>Appendix Table 1</b> <i>List of adapted modeling parameter estimates</i> .....	181

## **List of Abbreviations**

ABL1: Abelson murine leukemia viral oncogene homolog 1

AML: Acute myeloid leukemia

ALL: Acute lymphoblastic leukemia

A-KG: Alpha ketoglutarate

APC: Antigen presenting cell

APC-: Allophycocyanin

ATP: Adenosine triphosphate

B18R: Vaccine virus-encoded decoy receptor for Type 1 Interferons

BCR: Breakpoint cluster region

BMT: Bone marrow transplant

CART: Chimeric antigen receptor therapy

CEBPA: Enhancer Binding Protein A gene

CD: Cluster differentiation molecule

CML: Chronic myelogenous leukemia

CMV: Cytomegalovirus

CNS: Central Nervous System

CpG: Cytosine phosphate guanine

CTL: Cytotoxic T-Lymphocyte

CXCL10: C-X-C motif chemokine 10

DAMP: Danger/damage associated molecular pattern

DC: Dendritic cell

DNA: Deoxyribonucleic acid

DNMT3A: DNA cytosine 5 methyltransferase 3A

DOTL1: DOT1-like, histone H3 methyltransferase

dsDNA/RNA: Double-stranded deoxyribonucleic or ribonucleic acid

EBV: Epstein Barr Virus

EGFR: Epidermal growth factor receptor

eIF: Eukaryotic initiation factor

ER: Endoplasmic reticulum

ETV6: N-terminal remnant of ETs variant 6 gene

FLT3: Fms-like tyrosine kinase 3 gene

G: Glycoprotein of vesiculovirus

$\gamma$ -IR: Gamma irradiated/irradiation

GFP: Green fluorescent protein

GM-CSF: Granulocyte macrophage colony stimulating factor

GM38: A normal fibroblast cell line

GMP: Good manufacturing practice

GU: Guanine uracil

GvHD: Graft versus host disease

GvL: Graft versus leukemia

HA1/2: Minor histocompatibility antigens restricted to hematopoietic tissue

HER-2: Human epidermal growth factor receptor 2

HIV: Human immunodeficiency virus

HDF: Human dermal fibroblast

HDT: High dose therapy

HLA: Human leukocyte antigen

HMGB1: High mobility group B1 nuclear protein

HSCT: Hematopoietic stem cell transplantation

HSV: Herpes simplex virus

HT29: Resistant human colon cancer cell line

i20S: Immunoproteasome

ICD: Immunogenic cell death

IDH1/2: Isocitrate dehydrogenase gene 1 and 2

IFN: Interferon

IL: Interleukin family of cytokines

iLOV: Immunotherapy by leukemia-oncogenic virus

INDELS: Insertions/deletions

Intron A: Interferon alpha 2b

IRF: Interferon regulatory factor

ISG: Interferon stimulated genes

iTreg: Induced T-regulatory cell

IV: Intravenous

JAK: Janus kinase

KC: Keratinocyte chemoattractant; chemokine (C-X-C motif) ligand 1

KIT: Stem cell factor receptor

L: Large protein of vesiculovirus (polymerase)

L1210: An acute lymphocytic leukemia cell line

LAA: Leukemia associated antigen

LDLR: Low density lipoprotein receptor

LPS: Lipopolysaccharide

M: Matrix protein of vesiculovirus

MAPK: Mitogen-activated protein kinases

MAVS: Mitochondrial antiviral signaling

MDSC: Myeloid derived suppressor cells

MFI: Mean fluorescence intensity

MG1: An attenuated Maraba virus strain

mHA : Minor histocompatibility antigen

MHC : Major histocompatibility antigen

MLL: Mixed lineage gene

MOI: Multiplicity of infection

MTD: Maximum tolerated dose

mTORC1: Mechanistic target of rapamycin complex 1 (serine/threonine kinase)

MV-NIS: Measles virus encoding thyroidal sodium iodide symporter

MxA: Myxovirus resistance protein A

MyD88: Myeloid differentiation gene 88

N: Nucleoprotein of vesiculovirus

NALP3-ASC: Multiprotein complex of NLR protein NLRP3, adapter ASC and pro-caspase-1 responsible for production of IL-1 $\beta$  and IL-18

NAN: Not a Number

NDV: Newcastle Disease Virus

NF $\kappa$ B: Nuclear factor kappa-light-chain-enhancer of activated B cells

NK: Natural killer (cell)

NKG2D: Natural killer cell activating receptor encoded by *KLRK1* gene

NPM1: Nucleophosmin gene

NRRPs: Non-replicating rhabdovirus-derived particles

NUP98: Nucleoporin 98; an mRNA export factor

ODE: Ordinary differential equation

OV: Oncolytic virus

P: Phosphoprotein of vesiculovirus

PAMP: Pathogen associated molecular pattern

PBMC: Peripheral blood mononuclear cells

PBS: Phosphate-buffered saline

PD1: Programmed death 1 (receptor)

PDL1: Programmed death ligand 1

PE: Phycoerythrin

PFA: Paraformaldehyde

PFU: Plaque forming units

Ph1: Philadelphia chromosome

PI: Propidium Iodine

PKR: dsRNA-dependent protein kinase

pMHC: Peptide-loaded major histocompatibility complex

PML-RARA: Promyelocytic leukemia-retinoic acid receptor alpha

Poly I:C: Polyinosinic:polycytidylic acid; synthetic dsRNA-mimicking immunostimulant

PRR: Pathogen recognition receptor

PSI: Pounds per square inch

Rae1: Ribonucleic acid export 1; multifunctional protein involved in mRNA nuclear-cytoplasmic transport and transcription regulation

RAGE: Receptor for advanced glycosylation end products

RANTES: Regulated on activation normal T cell expressed and secreted

RAS: “Rat sarcoma” family of small GTPases; signalling proteins that activate downstream genes involved in proliferation, differentiation and survival

RdRP: RNA-dependent RNA polymerase (complex)

RIG-I: Retinoic acid inducible gene I

RNA: Ribonucleic acid

ROS: Reactive oxygen species

RUNX1: Runt related transcription factor 1

RV: Rhabdovirus

scFv: Single chain variable fragment

s.d.: Standard deviation

ssRNA: Single stranded ribonucleic acid

STAT: Signal transducer activators of transcription

TET2: Ten-eleven translocation gene

TBI: Total body irradiation

TCR: T-cell receptor

TGF- $\beta$ : Transforming growth factor beta

TK: Thymidine kinase

TKI: Tyrosine kinase inhibitor

TLR: Toll-like receptor

TNF: Tumor necrosis factor

TP53: Tumor protein p53

Treg: Regulatory T-lymphocyte

UV: Ultraviolet; UVC: Ultraviolet C

TK: Thymidine kinase

VSV: Vesicular Stomatitis Virus

VSV $\Delta$ 51: An attenuated Vesicular Stomatitis Virus strain

WT1: Wilms tumor 1 gene

$\Delta$ 51: A deletion of methionine 51 in the Matrix protein

7-AAD: 7-aminoactinomycin D; a cell viability dye

## List of Symbols

AP: Activated population

$EC_{50}$ : Half maximal effective concentration

$\gamma_c$ : Rate of cell death

$\gamma_n$ : Rate of NRRP degradation

$\gamma_{IFN}$ : Rate of IFN degradation

IP: Infected population

$K_{IFNon}$ : Rate of IFN signaling activation

$K_{IFNoff}$ : Rate of IFN signalling inactivation

$K_{IFN1}$ : IFN production rate from IP

$K_{IFN2.1}$ : IFN production rate from AP

$K_{IFN2.2}$ : IFN production rate from PP

$K_{NI}$ : Rate of NRRP internalization

$K_{NC}$ : Rate NRRP clearance from AP

N: NRRPs

PP: Protected population

UP: Uninfected population

## **Table of Contents**

<b>Abstract</b> .....	ii
<b>Acknowledgements</b> .....	iv
<b>List of Figures</b> .....	v
<b>List of Abbreviations</b> .....	vii
<b>Table of Contents</b> .....	xvii

### **Chapter 1. General Introduction**

1.1 Pathogenesis of acute leukemias .....	1
1.2 Synopsis of the molecular framework of the acute leukemias .....	4
1.3 Standard treatment for acute leukemias .....	8
1.4 The evolving treatment landscape for the acute leukemias .....	11
1.5 Oncolytic virotherapy: versatile anti-neoplastic agents .....	15
1.51 Direct and indirect tumoricidal activity .....	16
1.52 Immunobiology of tumors and virus-mediated oncotoxicity .....	21
1.53 Evasion of virotherapy by tumors cells and their microenvironment .....	22
1.54 Augmenting tumor-specificity of an oncolytic virus .....	24
1.55 The challenges of improving virus oncotoxicity .....	27
1.56 Oncolytic virus-based immunotherapy .....	29
1.6 Virotherapy in hematopoietic cancers .....	31
1.7 The vesiculovirus platform .....	34
1.8 Hypotheses .....	39
1.9 Objectives .....	40

<b>Chapter 2.0 Leukemia Cell-Rhabdovirus Vaccine: Personalized Immunotherapy for Acute Lymphoblastic Leukemia</b> .....	41
Authors' Contributions .....	42
Abstract .....	43
Translational Relevance .....	45
Introduction .....	46
Materials and Methods .....	48
Results .....	55
Discussion .....	77
<b>Chapter 3.0 Non-Replicating Rhabdovirus-Derived Particles (NRRPs) Eradicate Acute Leukemia by Direct Cytolysis and Induction of Anti-tumor Immunity</b> .....	81
Authors' Contributions .....	82
Chapter Premise and Synopsis .....	83
Abstract .....	86
Introduction .....	87
Materials and Methods .....	88
Results .....	92
Discussion .....	110
<b>Chapter 4. General Discussion</b>	
4.1 iLOV: Personalized Immunotherapy for ALL .....	113
4.2 NRRPs .....	119

<b>References</b> .....	125
<b>Appendices</b> .....	154

# Chapter 1 General Introduction

## 1.0 Pathogenesis of acute leukemia

Acute leukemia is an aggressive bone marrow-based neoplasm caused by mutation(s) that occur in a pluripotent cell or a common progenitor cell committed to either a myeloid or lymphoid lineage [1]. Most blood elements have a brief lifespan requiring brisk hematopoiesis to preserve homeostasis. Acute leukemic transformation halts this maturation process preventing replication senescence but self-renewal and clonal expansion is maintained, quickly exhausting marrow reserves leading to hematopoiesis failure. Finite resources within localized marrow niches can exert selective pressures whereby leukemic subclones evolve capable of inhabiting new territory. Ultimately marrow egress and hematogenous dissemination occurs, with potential migration into ectopic sites including the central nervous system (CNS) [2].

Several endogenous processes can spontaneously damage DNA. Chemical modification to deoxyribonucleic acid (DNA) bases constitutes the major molecular cause of mutagenesis, which can ultimately lead to leukemia. A common mechanism involves oxidative attack on nucleotides by metabolically derived reactive oxygen species (ROS) estimated to occur at least 20,000 times/cell/day [3]. ROS also create single and double-stranded DNA breaks. This can lead to leukemogenic base substitutions, small frameshift insertions/deletions (INDELs) or chromosomal rearrangements [4–6]. Indeed, among the various childhood acute leukemias are syndromes associated with germline

mutations of several genes required for efficient DNA repair, including Ataxia Telangiectasia Mutated, Bloom Syndrome, Nijmegen Breakage Syndrome, Fanconi, Breast Cancer 1 and multiple genes encoding mismatch repair proteins [7].

Exogenous sources of DNA injury associated with leukemogenesis have been identified [6]. Particles of ionizing radiation can directly break the sugar-phosphate backbone or the weak hydrogen bonds of base pairs or indirectly by the generation of free radicals and ROS, such as hydrogen peroxide. High-energy penetrating rays (gamma/X-Ray/neutron) injure bone marrow cells and an increased incidence of acute leukemia has been recorded among those proximal to nuclear bomb fallout or patients receiving radiotherapy. There is a strong causal relationship between secondary acute leukemias and previous exposure to several chemotherapeutics [1, 8, 9]. The rapid turnover of normal bone marrow progenitors make them collateral targets for these drugs, which aim to disrupt tumor cell replication through DNA damage and subsequent induction of apoptosis. Alkylating agents form covalent cross-linked bonds between functional groups (-OH, -SH, -NH) on adjacent molecules of DNA, ribonucleic acid (RNA) and/or protein. Unsuccessful repair may lead to chromosomal aberrations that promote latent acute leukemia. Partial or full loss of chromosome 5 and/or 7 has been reported in 90% of alkylator-related acute myeloid leukemia (AML)[10, 11]. The most recognized agents are perhaps the nitrogen mustard compounds (cyclophosphamide, melphalan, chlorambucil) - the clinical formulations of mustard gas, used to disrupt both allied and axis soldiers, primarily during World War I [12]. Other agents in the alkylator group include nitrosoureas and the platinum compounds. Non-covalently binding agents such as the

anthracycline toxins produced by strains of *Streptomyces* (daunorubicin, doxorubicin, idarubicin), generate ROS and intercalate between base pairs during the enzymatic cutting and unwinding of DNA transcription or synthesis [11]. Stabilized drug-topoisomerase II-DNA complexes prevent DNA synthesis and introduce double strand breaks that commonly lead to pro-leukemic translocations. Similar leukemogenicity is associated with the classic topoisomerase II inhibitors (etoposide, teniposide) [11] – formulations derived from the mandrake root, used in ancient sorcery, contemporary paganism and voodoo medicine alike. Exposure to benzene (petrochemicals), organic solvents and certain pesticides has long been associated with acute leukemia, with similar DNA interactions and abnormal karyotypes [13]. Most cases of acute leukemias are not associated with inherited predisposition or known exposure to carcinogens. However, of the known exogenous agents linked to acute leukemia perhaps cigarette smoke deserves special mention considering 2000 known carcinogens (including benzene) are transferred to the circulation upon inhalation. Significant correlations with acute leukemia have been reported in several large studies [14].

Direct viral implications in other hematopoietic malignancies are well known, such as Human T-cell lymphotropic virus (adult T-cell lymphoma), Epstein–Barr virus (Burkitt and Hodgkin lymphoma), HIV (several lymphomas); however viral etiology for human acute leukemia is not clear [1]. However it would seem appropriate that infectious agents might promote cancers of the immune system, as white blood cells are programmed to react to pathogens, some of which (lymphocytes) undergo somatic hypermutation [15]. Recent reports have shown an association between infections and

childhood leukemia [16]. Transforming growth factor-beta (TGF- $\beta$ ) released during infections normally acts to halt hematopoietic stem cell division. Childhood acute lymphoblastic leukemia (ALL) cells frequently harbor the classic *ETV6-RUNX1* t(12;21)(p13;q22) translocation, which encodes a chimeric transcription factor that represses TGF- $\beta$ -induced cell cycle regulation leading to clonal expansion without competition [17]. As a preliminary genetic lesion, this may provide the selective growth advantage to attain further genetic alterations required for outright disease.

### **1.1 Synopsis of the molecular framework of acute leukemia**

The development of modern therapeutics for leukemia should start with an understanding the molecular framework of the disease; a focused synopsis here will provide context to specific discussion points presented later. Point mutations and chromosomal alterations (i.e., fusions, amplifications, deletions) can alter or introduce neomorphic protein function [18], leading to loss of tumor suppressor or enhanced proto-oncogene activity. An original mutation produces a pre-leukemic phenotype, requiring additional genetic lesions for development of overt disease [2, 18, 19]. The self-induced genotoxicity of some initiating events may create the genomic instability necessary for the development of blatant leukemia [18, 20–22]. Precursor states are typically clinically silent particularly for ALL, although peripheral blood or marrow analyses may detect myelodysplastic or proliferative syndromes associated with progression to AML [1, 19, 23–25].

This step-wise conceptualization of tumorigenesis is not unique to acute leukemia but recently using next generation sequencing and computational genomics, the molecular framework has been further defined [26]. Pertinent aspects related to AML will be mainly discussed here as this disease has been interrogated more intensely of late compared to ALL. Several higher-than-expected recurrent molecular aberrations are implicated in the pathogenesis of AML; although on average only 13 mutations occur in genes. The majority require some cooperative activity to induce overt disease; although among them considerable mutual exclusivity is observed. These drivers most frequently involve genes encoding activated signaling, DNA-methylation, chromatin modification, nucleophosmin, transcription factors, tumour suppressors, or cohesion and spliceosome-complex proteins [26]. In *de novo* AML, coding aberrations are mostly single nucleotide variants and occasionally small INDELs occurring in karyotypically normal cells. Copy number variations (chromosomal and cryptic) are infrequent, associated mainly with unfavorable monosomy, arm length or complex chromosomal alterations. The vast majority of mutations are randomly acquired in the hematopoietic progenitor prior to the initiating event, with the founder clone typically harboring very few cooperating mutations [26].

The three most prevalent coding mutations in AML occur in the FMS-like tyrosine kinase 3 (*FLT3*), nucleophosmin (*NPM1*) and DNA (cytosine-5)-methyltransferase 3A (*DNMT3A*) genes [26]; each detected in approximately 30% of all patients and a high co-occurrence rate is observed. *FLT3* is a receptor tyrosine kinase known to regulate differentiation, proliferation and survival in hematopoietic progenitors;

internal tandem duplication of one of its two functional domains results in AML with unfavorable prognosis [27]. *NPM1<sup>mut</sup>* is associated with overexpression of homeobox transcription factor gene and miR-10a leading to increased self-renewal and enhanced myelopoiesis [28]. When exclusive expression occurs in cytogenetically normal AML, more favorable outcomes are observed [29]. *DNMT3A<sup>mut</sup>* expression is favored over the normal allele and its impaired methyltransferase activity results in pronounced under-methylation of low-density CpG regions [26]. Indeed, intergenic regulation of gene expression plays an important role in leukemic pathogenesis [30].

In approximately 20% of AML cases isocitrate dehydrogenase gene (*IDH1/2*) is mutated. IDH is a critical enzyme in the Krebs cycle normally converting isocitrate to alpha-ketoglutarate ( $\alpha$ -KG) [31–33]. Point mutations create gain-of-function activity producing 2-hydroxyglutarate instead, which appears to act epigenetically to halt myeloid differentiation and promote leukemogenesis [34]. Mutations in the ten-eleven-translocation gene *TET2*, results in loss-of-function (approximately 10% of AML) [26, 32]. TET proteins normally convert 5-methylcytosine to 5-hydroxymethylcytosine, which acts to demethylate DNA and promote gene expression. *TET2* catalytic activity is  $\alpha$ -KG dependent, which explains both the hypermethylation effect of *IDH1/2* mutants and its lack of co-occurrence with *TET2* mutations [32]. Other recurrent AML coding mutations include *RUNX1* (10%), *TP53* (8%), *CEBPA* (6%), *WT1* (4%), and *KIT* (4%) [26].

Some chromosomal alterations seem less reliant on recurrent coding mutations for leukemogenesis [26]. Highly promiscuous translocations of the histone methyltransferase *MLL* generate gain-of-function fusion proteins. This results in broad epigenetic

alterations leading to leukemic transformation [35, 36]. *PML-RARA* is the classic, albeit not sole, fusion associated with acute promyelocytic leukemia (APL). The fusion transcription factor formed by t(15;17)(q22;q21) assembles into unusual homodimers with elevated affinity for retinoic acid response elements and co-repressors with histone deacetylase activity; they also recruit DNA methyltransferases. The modified chromatin structure and promoter methylation results in pronounced transcriptional repression when target gene expression is required for continued granulocyte maturation - stalling differentiation at the promyelocyte stage [37].

In 1-2% of de novo AML (20% adult ALL) the prototypical self-sufficient Philadelphia (Ph1) chromosome is detected [38]; in this context, a mean of 5 recurrent molecular aberrations is also present [26]. Ph1 is indeed the sole lesion required for the development of chronic myeloid leukemia (CML), a rare leukemia that typically presents insidiously. Ph1 involves a reciprocal translocation of a portion of the *ABL1* gene on chromosome 9 with a segment of the *BCR* locus on chromosome 22; t(9;22)(q34;q11). *BCR-ABL* on derivative chromosome 22, encodes a tyrosine kinase with constitutive expression and gain-of-function compared to the wild type *ABL1* [39]. This single driving event in pluripotent hematopoietic stem cells favours myeloblast differentiation, leading to an accumulation of circulating granulocytes. Ph1 activity alters the apoptosis threshold, generates reactive oxygen species creating point mutations and a build-up of double stranded DNA breaks that results in further translocations through error-prone repair [20, 21]. Chronic genotoxic stress propels the development of drug resistance and

blast crisis – an aggressive form of acute leukemia typically refractory to therapy [39]. Patient-derived Ph1+ acute leukemic blasts were acquired for use in this thesis.

In contrast with AML, ALL frequently exhibits cooperating copy number alterations, [1, 40]. The molecular structure and evolution of acute leukemia can be uniquely studied in childhood ALL, where monozygotic twins have a concordance rate of only 10-15% of common (B-cell precursor) ALL. In monozygotic twins (75% of identical twins), the healthy child's lymphoid cells routinely share the priming mutation without detection of the additional clonal lesion(s) present in the affected twin - suggesting transfer of the pre-leukemic clone anastomotically [2]. Stored fraternal-twin neonatal blood spots and cord blood confirms the prenatal initiation and clonal evolution of ALL by the detection of typical PCR-amplified fusion transcripts (i.e., *ETV6-RUNX1*, *MLL-chimeras*) [41]. The genome-wide blueprint is fundamental to the acquisition and phenotype of ALL. This is exemplified by the observation that only 1% of all births with circulating *ETV6-RUNX1* clones eventually develop into the highly-treatable childhood ALL, versus 10% chance for a healthy child (with the fusion clone) whose identical twin has ALL [42].

### **1.3 Standard treatment for acute leukemia**

The basic treatment approach for acute leukemia has ostensibly not changed in over four decades [23, 43, 44]. For most patients, multidrug regimens are used to enhance the effect of therapy and circumvent drug resistance. The primary function of each chemotherapeutic is to disrupt the active cell cycling of leukemia cells and/or induce

apoptosis. Several classes of drugs are used. Pyrimidine nucleoside analogues (i.e., cytosine arabinoside) and anthracyclines (i.e., idarubicin) for AML; alkylating agents such as cyclophosphamide, vinca alkaloids (i.e., vincristine) and antimetabolites such as the folate antagonist methotrexate for ALL. Most of these agents work indiscriminately. Off-target injury harms gastrointestinal mucosa, gonadal and normal hematopoietic tissue. Depending on the leukemic subtype, additional or alternate pharmaceuticals are sometimes employed including arsenic trioxide and retinoic acid (both are highly effective at inducing normal differentiation of promyeloblasts), monoclonal antibodies targeting leukemia cell-surface molecules, L-asparaginase, purine analogues and corticosteroids [9, 45–47]. Although an initial remission is frequently attained, relapse invariably occurs usually within 6 months [43, 48]. To sustain remission durations, sequential rounds of chemotherapy are attempted. In children this can be maintained over several years, and with ALL, this is associated with ~ 90% cure rate, although several long-term adverse health effects are incurred [45, 49]. Most patients cannot tolerate such prolonged regimens; eventually some form of salvage/palliation therapy is implemented. However younger adults (i.e., < 65 years old) may be candidates for myeloablative consolidation therapy administered soon after initial remission [23, 50]. Today, this high dose therapy (HDT) typically involves administering an alkylator alongside total body irradiation with the intention to significantly reduce remaining disease. This otherwise lethal maneuver requires an immediate stem cell-enriched blood transfusion to re-establish normal hematopoiesis [51]. It was recognized over 50 years ago that HDT could not eradicate all leukemia cells [52]. However it was appreciated that rescue transfusions procured from donors also represents a form of adoptive immunotherapy [53]. The

allogeneic graft produces an immune reaction against residual leukemia cells termed graft versus leukemia (GvL), which is associated with prolonged survival. However the procedure does not involve T-cell selection and the non-specific reaction is likewise directed against normal tissue – particularly bone marrow, thymus, skin, liver and gastrointestinal, termed graft versus host disease (GvHD). Accumulated transplant data over the last half-century indicates that patients able to endure the debilitating effects of chronic GvHD have improved overall survival [54]. Nevertheless, the management of patients with acute leukemia remains controversial. The magnitude of morbidity and mortality associated with allotransplantation and the level of benefit appears to be dependent on the leukemic subtype, but conflicting studies have prevented the development of universal treatment algorithms. Modern molecular profiling is refining our management decisions [23, 51, 55–59], nevertheless leukemia immunotherapy will soon move beyond this somewhat crude form of treatment.

The immunotherapeutic contribution of human leukocyte antigen (HLA)-matched allogeneic hematopoietic transplantation depends primarily upon the interaction of reactive donor cytotoxic T-cells (CTLs) against host leukemia cells; although this seems to be more crucial in AML versus ALL [60–63]. A clonal T-cell receptor (TCR) recognizes specific antigen in the form of a peptide-loaded major histocompatibility complex I (pMHC I) displayed on recipient cells. Genetic differences between the HLA-matched donor's endogenously expressed proteins and the recipient's (i.e., allelic single nucleotide polymorphisms) can create minor histocompatibility antigens (mHAs), which represent the main peptide targets for alloreactive CTLs. [60, 64]. However, a few reports

examining relapse in recipients of haploidentical transplants (T-cell replete or delayed donor T-lymphocyte infusions) have suggested the alloreactive mHAs may not always provide sufficient anti-leukemic activity [65–67]. TCR interaction with pMHC may trigger intracellular signaling events that culminate in effector responses, whereby functionally avid CTLs release perforin, granulysin and granzymes (serine proteases), inducing apoptosis in the target leukemic cell [68–71]. Rare mHAs restricted to hematopoietic tissue (i.e., HA-1/2) or other potentially immunogenic leukemia-associated antigens (LAA) may provide more discrete GvL activity [72–74]. Nonetheless, immunoediting pressures during leukemic outgrowth promote the persistence of non-immunogenic clones. This is a result of either diminished pMHC display or expression of LAAs with low TCR affinity [75–77]. CD4<sup>+</sup> helper cells may recognize HLA II-associated LAA, when leukemic cells have low expression of class II-associated invariant chain peptide [78]. Additional processes may be minor contributors to the overall GvL reaction including sustained activation of natural killer (NK) cells and/or macrophage, cytokine-mediated tumor apoptosis and anti-tumor humoral responses [60, 69, 79, 80].

#### **1.4 The evolving treatment landscape for acute leukemia**

The incidence of AML, ALL and acute blast phase CML is approximately 4, 2 and 0.08 per 100,000 persons/year respectively [1, 39, 46, 48]. An estimated 25,000 people will be diagnosed with one of these aggressive malignancies in North America in 2014. Overall less than 20% of adults survive 5 years from diagnosis. Those amenable to HDT have approximately 30-40% long-term survival whereas the majority of patients ineligible for this procedure have a 5-year survival of less than 10% [23, 43, 81]. It is

imperative that new therapeutic modalities with greater finesse be developed that move beyond the use of early-20<sup>th</sup> century technologies of ionizing radiation and high-dose mustard gas equivalents.

Despite the longstanding interest in dissecting GvL from GvHD, no major translation to the clinic has occurred, although very promising adoptive cellular therapies are on the cusp. Reduced intensity conditioning for donor T-cell depleted transplantation followed by delayed (~ 2 months) donor lymphocyte infusions may lessen GvHD and provide a postponed GvL effect [60]. More recently it has been shown that donor effector memory CD4<sup>+</sup> T-cells can provide long-term GvL effect without GvHD; transfusions of these cells may be particularly beneficial in haploidentical or MHC II mismatched transplants, where T-cell depletion from the graft is routinely performed to mitigate catastrophic GvHD [82]. Alloreactive T-cells expanded *ex-vivo* and specifically targeting recipient tissue-restricted LAA/mHA is a promising strategy; although fraught with technical issues including pre-transfusion exhaustion [83]. Other approaches to cellular therapy involve T-cell engineering. Healthy CTLs can be modified *ex-vivo* through gene-transfer to attack leukemia associated cell-surface targets. Retroviral or plasmid delivery of genes encoding for TCRs targeting HA1/2 (for example) has shown promise. When using mHA-negative HLA-matched donors, selecting viral specific T-cells (against EBV or CMV) appears to mitigate GvHD [84]. Another engineering approach utilizes polyclonal autologous T-cells. Similar to the above, transgenic HLA-restricted TCRs can reprogram patient-derived T-cells against self-LAA targets such as the WT1 antigen [85]. Although the potential for GvHD appears to be eliminated using this approach,

endogenous-transferred TCR heterodimers may induce off-target tissue injury or lead to loss of function [86]. A more recent adoptive immunotherapeutic platform, chimeric antigen receptor T-cell (CART) therapy, involves redirecting the patient's T-cells toward *non*-MHC restricted native leukemic antigen, such as the CD19 molecule expressed on the surface of common B-cell ALL. Lentivirus delivers a synthetic gene encoding an antigen-recognition domain of an antibody (i.e., single chain variable fragment) linked to the intracellular signalling domains of CD3-zeta and co-stimulatory molecule(s). CARTs circumvent the classic tumor evasion mechanisms of MHC downregulation and impairment in immunoproteasome (i20S) processing of HLA-dependent antigen display, which could subvert the other cellular approaches described above. This is particularly pertinent in blood cancers, as i20S is predominantly expressed in hematopoietic cells. Next-generation CARTs resist peripheral immune checkpoints (described later) and have astonishingly prolonged T-cell functionality without exhaustion [87–93]. A challenge here remains finding cell-surface leukemia-specific targets that avoid depletion of normal counterparts, although this has not posed significant clinical issues thus far.

Another tactic however is to coerce the natural immune system into attacking leukemia cells. An efficient way to provoke immune cells into action is by using natural reactants - pathogens or mimicking substances (adjuvants). Innate immunocellular programs identify and vigorously react to these real or perceived microbial threats. When this alarm is linked in some manner to tumor antigen, anti-tumor immune activity may be generated [94–99]; this will be discussed in more detail in subsequent sections. White blood cells in particular have evolved an elegant pathogen recognition receptor (PRR)

system. PRRs, including toll-like receptors (TLRs), ligate a wide array of pathogen-associated molecular patterns (PAMPs). A network of PRR-mediated signal transduction pathways allows the immune cell to perform specific antimicrobial effector functions in a highly regulated fashion [100–106]. Various forms of programmed cell death may also be triggered following PRR activation - an act that may ultimately save the organism from rampant infection and/or limit the deleterious effects of extensive inflammation [100, 107, 108]. Aside from common adjuvant use, synthetic TLR agonists mimicking PAMPs can also exploit these terminal cellular responses, and as monotherapy (or combined with STAT3 silencing) have shown promise in both acute myeloid and chronic lymphoid leukemia [109–112]. These agents induce intrinsic/extrinsic apoptosis and promote the induction of anti-leukemic immunity. Interestingly, metastatic non-hematopoietic cancers may also be susceptible to TLR agonists and tumor selectivity using these agents is due to the pro-apoptotic effects of interferon (IFN) and/or impairment in NF $\kappa$ B-mediated pro-survival responses following TLR activation [113–115]. Other novel immunotherapeutic approaches are discussed within the manuscript section of the thesis.

A traditional target-based treatment strategy is to disrupt altered signal transduction pathways assumed to be directly responsible for unchecked leukemic proliferation and survival [18]. The success of tyrosine kinase inhibitors (TKI) in CML, which previously had extremely dismal outcomes, has only emboldened the hunt for targetable genomic alterations in other leukemias. Indeed, the deep sequencing of AML and ALL genomes has provided novel targets for drug development. In *FLT3*-mutated AML, multiple small molecules currently in development bind and inhibit the activated

tyrosine kinase [27]. Agents targeting KIT (stem cell growth factor receptor), the *RAS* pathways and others are in development or early phase clinical trials [9, 27, 81, 116]. However as we begin to more fully understand the underlying genetic and biomolecular underpinnings of acute leukemia, therapies are moving beyond a focused blockade of signal transduction pathways and into global or targeted epigenetic modulation [31, 32, 117]. New agents targeting the mutated IDH isoforms in AML reverse the associated methylation aberrancy and induce differentiation. In *MLL* fusion AML and ALL (~ 10% each), the hybrid protein constitutively activates the methyltransferase DOTL1; high-potency inhibitors of this protein selectively eradicate *MLL*-mutated acute leukemia. Early phase human trials are underway for both these agents [118].

There is retained optimism that one, or more likely multiple, genotype-specific small molecule-targeting agents will be able to sustain remissions. However, given the widespread dissemination and rapid kinetics of AML and ALL, multifaceted strategies incorporating potent immunotherapeutics should be explored.

## **1.5 Oncolytic virotherapy: versatile anti-neoplastic agents**

For centuries, association between spontaneous tumor regression and a temporally related bacteria or viral infection has been recognized. The “father of immunotherapy”, Dr. William Coley, treated several hundreds of patients with live and subsequently killed mixtures of *Streptococcus pyogenes* and *Serratia marcescens*. His poor documentation along with the apparent sporadic responses coincided with the advent of radiation and chemotherapy during the early 20<sup>th</sup> century [119, 120]; basic research examining this first

“immunotherapeutic” was largely dropped - but not entirely. Several large pharmaceutical corporations are presently examining the active tumor-reducing components of Coley’s toxin. This renewed interest is likely in part due to the successful development of multiple oncolytic virus (OV) platforms over the last decade. However, OVs have natural and engineered properties that may empower their efficacy beyond the capacity of Coley’s toxin.

### **1.51 Direct and indirect tumoricidal activity**

Generally, an OV is able to infect a wide range of host tissues, however upon cancer cell-entry, the obligate parasite encounters the ideal environment. Foremost, neoplastic transformation provides a rich pool of substrates (i.e., nucleosides and amino acids) used to produce the essential macromolecules and organelles that maintain an active mitotic cycle [18, 121]. This largesse conveniently provides all the elements for efficient viral replication. When tumors acquire constitutively active anti-apoptotic programs, this may also favour propagation of some viruses, particularly important for those with longer life cycles. Defects in “viral” type I IFN responses frequently arise during tumorigenesis [122, 123]. Type I IFNs are produced in most cells in response to virus infection. This family of pleiotropic cytokines induce the expression of hundreds of IFN-stimulated genes (ISGs), exerting a plurality of overlapping and interconnected effector functions that can interfere with essentially every aspect of the virus life cycle - together an antiviral state is achieved [124]. Depending on the impairment within this system, OV production may be augmented in these cells. Spread to adjacent tumor tissue occurs when viral progeny exit the host cell. Non-enveloped virions such as Adenovirus

or Reovirus do so *en masse* causing abrupt cell rupture; whereas enveloped particles such as Herpes simplex type 1, Vesicular stomatitis virus (VSV) or Newcastle disease virus, tend to consume their host's membrane during egress. Nonetheless, apoptosis is invariably triggered in susceptible tumors during the OV replication cycle and depending on the relative kinetics of both these activities, hastened cell death may limit virus production/spread [100, 125, 126].

Aside from their direct cytotoxicity, OVs trigger innate inflammatory and adaptive anti-tumor responses – dominant contributors to the overall tumor-reducing effect [127, 128]. Initially upon administration, OVs induce a similar generalized immune-provoking effect as produced by Coley's toxin. However, increased tumor-localized inflammatory responses may be realized when OV amplification occurs within inaccessible tumor masses following systemic administration [127]. Nevertheless, exposing tumor directly to certain replication-incompetent virus, or specific synthetic immunostimulatory compounds, has shown equal or superior tumor-reducing and immunomodulatory effects [94, 122, 127, 129–141]. The quality and quantity of the initial inflammatory and subsequent adaptive response is dependent on several factors including the intrinsic properties of the noxious agent(s) and the immune state of the tumor microenvironment and/or individual as a whole [142]. Optimal effector responses correlate to proper activation of an antigen-presenting cell (APC) concurrent with its tumor antigen processing and presentation to T-cells [143]. PAMPs, such as viral-derived double or single stranded RNA (ds/ssRNA), or dsDNA, ligate specific TLRs and other PRRs located on the cell surface, in the cytosol, or within endosomes. PRR-mediate

signaling cascades are initiated by the recruitment of adaptor molecules that activate downstream pathways. These involve several intermediate protein complexes, which induce the nuclear translocation of activated NF $\kappa$ B- and IFN-regulatory factors (IRFs) promoting the transcription of an array of inflammatory cytokines/chemokines and type I IFN respectively. Commonly, IFN $\beta$  is initially released to bind its cognate surface receptor on the same or adjacent cells, inducing the Janus kinase/signal transducer and activator of transcription/IFN-stimulated gene factor 3 (JAK/STAT/ISGF3) pathway leading to the transcription of potentially thousands of ISGs as well as IFN $\alpha$  [100, 101, 104, 105, 144–146]. In the context of dendritic cells (DC) or macrophage, pseudopodia and phagocytic activity is enhanced, which results in the engulfment of proximal cancer cells [102, 147, 148]. In addition, co-stimulatory molecules are upregulated and expressed on the APC's cell surface following PAMP-induced activation; ligating their cognate receptors on T-cells provides the critical second step for a regulated immune response [142, 149]. Thus, following endocytosis of a cancer cell, the pre-stimulated APC actively digests tumor antigen for loading on MHC II and cross-presentation on MHC I molecules; these complexes may be recognized by the TCR on naive T-helper and CTL respectively. So long as the amplitude of co-stimulation is sufficient, this overall engagement licenses T-cells to perform their effector duties. Although these interactions can take place in tumors, the principle innate-adaptive interface occurs in draining lymph nodes where activated tumor-sampled DCs have migrated [100, 142, 149–152].

Dead or dying tumor cells emit danger signals, damage-associated molecular patterns (DAMPs), with immunomodulatory function. Immunogenic apoptosis is characterized by cell-surface exposure of calreticulin, heat shock proteins (HSP), and phosphatidylserine. Engagement with their specific receptors induces phagocyte (including DCs) activity, tumor clearance and adaptive anti-tumor CTL responses. Suppressed CD47 expression enhances these activities [153–156]. In addition, necrotic tumor cells and viral- or cytokine-stimulated macrophage release high mobility group B1 (HMGB1) nuclear protein, which can bind (alone or in association with RNA/DNA) to an assortment of cell-surface PRRs including TLRs 2/4 and the receptor for advanced glycosylation end products (RAGE). Depending on the redox state of HMGB1, intense inflammatory reactions, DC maturation and potent adaptive anti-tumor immune responses may be elicited [157, 158]. Adenosine triphosphate (ATP) secreted during apoptosis or passively released at necrosis binds purinergic receptors recruiting monocytes. ATP receptors on DCs leads to enhanced antigen presentation and NALP3-ASC-inflammasome activation and IL-1 $\beta$  secretion, polarizing IFN $\gamma$ -secreting CD8<sup>+</sup> T-cells. However, cell-surface enzymes (CD39 and CD73) convert ATP to highly immunosuppressive adenosine, which may play a dominant role in tumor formation and metastasis [159, 160]. Several other molecules serve as DAMPs, including mitochondrial DNA, histones, F-actin, monosodium urate, ribonucleoprotein and mRNA/DNA - triggering mostly immunostimulatory activity [158]. The key to potent immunogenic cell death (ICD) is the range of DAMPs emitted and their spatiotemporal associations with each other and the dying cell. Aside from inducing HMGB1 release, OV infections can cause ICD through several other mechanisms including endoplasmic reticulum (ER)

stress and ROS generation - essential factors that induce secretory DAMPs such as calreticulin and HSPs. ICD is a major contributor to the overall efficacy of OV [153, 158, 159, 161].

Not to be discounted, the profound release of inflammatory cytokines/chemokines and Type 1 IFNs following OV administration has a number of anti-tumor effects. First many cytokines and chemokines (i.e., IL-1 $\beta$ , TNF, IL-1,-2,-6,-8, GM-CSF, IFN $\gamma$ , CXCL10, RANTES, KC) further activate and recruit both arms of the immune system [162–166]. In addition,  $\alpha/\beta$  IFNs are potent stimulants of macrophage, dendritic and NK; they also induce maturation and expansion of B- and T-lymphocytes. IFN responses include upregulation of MHC I/II complexes and antigen loading, enhancing engagement of the adaptive immune system with APCs and potentially tumor. Activated innate immune cells secrete tumor necrosis factor (TNF) and TNF-related apoptosis inducing ligand (TRAIL), which bind cognate death receptors on tumors – enlarging the kill-effect beyond OV replication zones [107, 142, 162, 167]. Indirect cytolysis is further enhanced by vascular shutdown within the tumor mass secondary to recruited neutrophils and associated ROS damage [128]. Indeed, neutrophils, plasmacytoid DCs and intact host type 1 IFN responses were required for successful Vesicular Stomatitis Virus (VSV) oncolysis, which was abolished by knockout of PRR-signaling adapter protein myeloid differentiation gene 88 (*MyD88*) or IFN $\alpha/\beta$ R, despite enhanced VSV replication within tumors [168]. Moreover, several known ISGs (most of the > 2500 ISGs have not been characterized) are involved in programmed cell death (PCD) [107]. Indeed, IFN has been

effectively used for many years as an anti-neoplastic agent in several marrow-based and lymphoid malignancies, as well as a wide range of non-hematopoietic cancers [169].

### **1.52 Immunobiology of tumors and virus-mediated oncotoxicity**

The extent of OV amplification within tumor tissue and the degree of anti-tumor immunity generated may frequently be inversely correlated. In addition to the well-known immune evasion mechanisms mentioned previously, tumors frequently upregulate the cell-surface programmed death ligand 1 (PDL1). Binding its cognate receptor (PD1) negatively regulates CTL, T-helper, B-cell and NK effector functions within the tumor microenvironment and decreases Tcell-tumor or -antigen presenting cell (APC) contact time. PDL1-PD1 engagement on tumor-resident T-regulatory cells (Tregs) enhances their proliferation and tolerizing activity [18, 170, 171]. OV-exposed tissue also induces PDL1 expression and promotes induced Treg (iTreg) as well as myeloid derived suppressor cell (MDSC) activity, which are normal counter-inflammatory responses [170, 172–174]. Further, some OVs (i.e., vesiculoviruses) actively down-regulate tumor expression of ligands for NKG2D activation receptors on NK cells [175]. OVs including vesiculoviruses have been shown to ablate viability and function of tumor-resident infected DCs, impairing antigen presentation capacity within the tumor and draining lymph node [176]. Vesiculovirus can also suppresses CD4<sup>+</sup> T-cell activity [173]. This overall tumor- and virus-induced immunosuppressive environment improves OV amplification within the tumor bed - albeit compromises the development of optimal anti-tumor immune responses and potentially outcomes [173]. On the other hand, adaptive anti-tumor immunity induced by some OV-tumor associations may actually require iTreg

activity within the microenvironment. This may be due to iTreg-dependent inhibition of MDSCs, which significantly inhibit both CTL and T-helper activities [166]. Indeed, the presence of iTregs within human tumors has been associated with favourable outcomes [177, 178]. At this time, it is impossible to predict and orchestrate the overall quality and magnitude of the immune response within individual tumors exposed to OV. Clearly the OV-tumor ecosystem is complex and only partially understood, but the multimodal nature of OVs has the potential, in some manner or another, exploit the immune status of tumors. Designing an OV that consistently provides safe yet prolonged direct oncolysis and at the same time robust anti-tumor immunity remains a challenge.

### **1.53 Evasion of virotherapy by tumor cells and their microenvironment**

Drug-induced selective pressures exerted upon tumor cells invariably leads to escape clones no longer amenable to standard therapy. This occurs through acquisition of multidrug resistance gene activity, enhanced drug efflux or target protein mutation [18, 179]. However, the metabolic abnormalities of neoplastic cells that make them ideal hosts for replicating OVs are indeed essential hallmarks of their transformed nature [18, 121, 180] – intrinsic evasion options for isolated tumor cells seem limited. Additionally, several common transforming events have been linked to impaired antiviral responses. The frequently activated *RAS/MAPK* pathway may impair eIF2 $\alpha$  phosphorylation by dsRNA protein kinase (PKR) and/or suppresses the activities of another ISG – MxA [181–183]. Other transforming events (i.e., upregulation of eIF2B $\epsilon$ , or mutations to  $\alpha, \beta, \delta$  subunits) can override PKR's translational repression [184]. Furthermore, tumor defects in type 1 IFN responses frequently denote neutral passenger (or weakly detrimental) gene

losses [162, 169, 185] - collateral vulnerabilities conveniently exploited by virotherapy. Clonal evolution leading to indirectly enhanced or directly amplified ISG activity capable of impairing OV replication appears unlikely due in part to the unsustained selective pressures from transient replication of OV within tumors. Further, virus replication is rarely dependent on the activity of a single or even several ISGs – highlighting the redundancy of the IFN network [124, 164]. When IFN deficits assist in driving tumor outgrowth (diminished MHC expression, increased apoptosis threshold or enhanced angiogenic and proliferative programs) [123, 169, 186, 187], again these losses often complement OV replication and spread.

Despite the lack of evasion options for neoplastic cells themselves, in contrast tumor beds can exhibit significant resistance to virotherapy. In marrow-based cancers, protective leukemic niches exist with the support of normal mesenchymal and endothelial cells [188]. Although considerable attention has been given to understanding the role these sanctuary sites have in sustaining leukemic “stem cells”, very little is known about this microenvironment in relation to OV resistance. However in solid tumors, it is well known that normal stromal cells and immune infiltrate with intact antimicrobial defences can swiftly shutdown the virus wavefront [189], this would be particularly problematic when the mass contains a high proportion of stroma or when resident tumor cells have IFN defects only upstream of its receptor. In addition, non-malignant cells, associated extracellular matrix and elevated hydrostatic pressure within tumors together act as physical barriers to OV spread [190]. One option to increase OV dissemination throughout a tumor mass is to integrate a foreign fusion glycoprotein into

the genome [191]; avoiding off-target cell-cell fusion remains a concern. Subverting these formidable impediments to virotherapy remains another challenging issue for the field.

#### **1.54 Augmenting tumor-specificity of an oncolytic virus**

Considerable effort has been focused on engineering OVs that possess enhanced tumor-specificity. Many ways to achieve conditional replication have been explored; only basic approaches will be discussed. Generally an OV is genetically manipulated to exploit the physiology of either tumors cells (promote replication), and/or normal cells (attenuate replication) [122, 192]. One main approach to promote replication in tumors is to remove the virus' natural regulatory sequence, and replace it with a promoter preferentially targeted in the neoplasm (or shared by its associated stromal cells). Inserting the tumor-specific response element into the endogenous viral promoter may be a more versatile approach. These techniques are not universally effective across different tumors or viral platforms [193–195]. Perhaps the ultimate barrier to effective cancer treatment, regardless of the platform, is the quiescent cancer initiating/stem cell [18, 52, 196]. So long as the molecular framework of an identified cancer initiating clone is understood, using the promoter-based targeting approach may be an effective way to drive OV replication in an otherwise resistant cancer cell [197]. An appealing approach to promote tumor replication (and simultaneously detarget normal cells) is to alter an OV's receptor-binding moiety (i.e., via scFv insert) restricting attachment to hyperexpressed tumor cell-surface molecules (i.e., HER-2) [198].

An alternative approach to enhance tumor-specificity involves attenuating viral protein production in healthy cells. Here the safety margin is increased, ultimately permitting higher, perhaps more effective OV doses to be administered. One method is to impair or remove virulence or evasion factors so long as they are dispensable in tumor cells. For example, VSVd51 has a deletion of methionine at position 51 in the viral matrix protein (M). This prevents its wildtype ability to interfere with cellular nucleoporin NUP98-Rae1 complex activity [199–201] - thus host transcription and nuclear-to-cytoplasmic mRNA transport remains unaltered. Mutating this virulence factor permits normal global gene expression and anti-viral defenses are unrestrained. Indeed, enhanced inflammatory responses are observed when VSVd51 is utilized versus the wildtype VSV [202]. However, in some tumors cells with significant IFN-defective pathways, the d51M mutation may be rather inconsequential - viral replication is minimally altered. This mutated M protein maintains operation at other cellular levels including its interaction with ubiquitin ligases (required for budding) and ability to induce cytopathic effects by altering mitochondrial action potentials or interact with cytoskeletal elements such as actin, vimentin, and tubulin, although this may be cell type dependent [200, 202–206]. Our understanding of vesiculovirus-induced IFN secretion is incomplete, as certain glycoprotein (G) mutants allow IFN to be secreted at “d51 levels” and higher, despite normal viral inhibition of cellular transcription/translation; further these mutants may exhibit increased cytotoxicity (apoptosis) than wildtype VSV in some tumor cells [207].

Another attenuated mutant (also used in my thesis work), the MG1 maraba vesiculovirus, contains a point mutation in M (L123W), altering its function by similar mechanism, while a second mutation in its glycoprotein (G) (Q242R) confers an IFN-independent attenuation [208]. Alternatively, adding the *IFN- $\beta$*  transgene into wildtype VSV to increase the production of IFN by infected cells mitigates its virulence in normal cells. Further, VSV constructs without M mutations can decrease the translation of oncogenes and enhance the degradation of their products (i.e., myeloid leukemia cell 1), relieving resistance to apoptosis and/or chemotherapy [209]. However, a major disadvantage of retaining wild-type functional M protein is that generation of tumor antigen is also diminished - skewing the immune response toward dominant viral antigens.

Nevertheless, in some cancer cells without significant impairment in the IFN system, these attenuated OV constructs may lose their direct oncolytic prowess [125, 126, 210], particularly in remote sites inaccessible to intratumoral inoculation. To regain potency in these situations a pharmacovirus approach has been utilized. Rapamycin, which inhibits mTORC1-mediated regulation of mRNA translation, has been shown to dramatically impair type I IFN responses in cancer and immune cells [211, 212]. This OV-drug combination was effective in treating malignant gliomas in pre-clinical models, without deleterious off-target effect [212]. Rapamycin abolishes the cell cycle in T-cells [213]; I speculate that it might be an attractive agent to explore in combination with VSVd51 to treat T-cell lymphomas or T-ALL (tumors amenable to rapamycin therapy),

or to eliminate residual disease in high-risk post-transplant leukemic patients already receiving this drug for chronic GvHD.

Two attenuation strategies that deserve brief mention involve inactivation/deleting a gene that drives replication in all tissues. For example, when poxvirus thymidine kinase is inactivated in the vaccinia genome, the virus will preferentially replicate in cancers typically replete with this enzyme [214]. Along the same lines, a virus may activate a signal transduction pathway to enhance replication; incapacitating this ability will not impair its replication in cancers with redundant signaling. Vaccinia growth factor gene activates the *EGFR/RAS/MAPK* pathway – deleting this gene may be inconsequential in at least 30% of cancers [125, 215]. Enhanced therapeutic index may also be attained by integrating targeting sequences for micro-RNAs differentially expressed in normal cells (+) and tumors (-) [216].

### **1.55 The challenges of improving virus oncotoxicity**

Methods to increase the oncotoxic effects of replicating OVs continue to be explored. However as OVs have multimodal effects, attempts to engineer enhancement of one component may significantly impair another. Integrating anti-angiogenic activity to starve cancer cells may reduce virus spread and/or delivery of subsequent treatments. Removing immunosuppressive genes or adding immunostimulatory genes (i.e., GM-CSF, IL-4, IL-12, IL-15, IL-23) in an attempt to enhance anti-tumor immunity may either impede virus propagation and/or further skew the immune response toward the virus [134, 217]. Adding pro-apoptotic genes to improve cytotoxicity may impede virus replication. Moreover, when OV is injected systemically, the vast majority of cells

exposed are not cancerous. Although replication in normal tissue is typically transient, engineering strategies designed for enhanced oncotoxicity may have unintended off-target effects. To minimize the problematic double-edged sword of replicating virotherapy, selecting an OV that has both rapid lifecycle kinetics *and* intrinsic immunostimulatory properties may be the most straightforward approach by allowing both OV propagation and host inflammatory responses to operate at maximal capacity. A nimble virus might rapidly form a voluminous tumor-nidus for the unfettered inflammatory response. The OV would have a small relatively uncomplicated genome capable of immediate cytosolic replication and increased dependence on the host's physiology for propagation - thus a natural proclivity for tumor cells. Several viruses indeed fit the above theoretical requirements such as Newcastle disease virus (NDV), Respiratory Enteric Orphan Virus (Reovirus), Measles and VSV; all wildtype preferentially propagate in tumor cells and variants have advanced to the clinic [154, 218–220].

One way to manipulate the tumor immune environment for enhanced direct cytotoxicity and anti-tumor immunity is through specific heterologous viral platforms. In one version, the OV administered first might temporarily induce an immunosuppressive microenvironment in the tumor, maximizing regional spread. For example a Vaccinia virus strain could be employed, which expresses genes capable of dampening the host's immune response through several known (i.e., B18R – a secreted IFN $\beta$  decoy receptor) and unknown mechanisms. Taking advantage of this milieu, a second distinct virus that has the unique characteristics described above could be administered to quickly eradicate

residual tumor while inducing the essential marked inflammatory response. This tag-team approach might also fortify the immune system's "prime-boost" response toward tumor antigen, while diverting cell and humoral anti-viral immunity. Pre-clinical work exploring this general concept has been reported [221, 222].

### **1.56 Oncolytic virus-based immunotherapy**

Several immunotherapeutic strategies for acute leukemia have involved transducing leukemia or DCs to express immunomodulatory molecules and/or LAA respectively. Several interesting vector-based approaches are outlined in the manuscript section. OVs are well positioned to extend this approach further. Reverse genetic engineering has produced virus strains encoding immunopotentiating molecules and/or tumor antigens [122, 190, 202, 217, 223, 224]. Oncolysis may be fortified through enhanced immune responses resulting from expression of these gene inserts in infected tumor and APCs alike. As discussed earlier, a wide range of cytokines and co-stimulatory molecules have been employed in this manner, although encoding a cytokine into a virus already adept at inciting broad inflammatory responses may be less than incremental. However, a well-chosen insert may have significant therapeutic effect. For example, a replication-defective adenovirus expressing the ligand for the co-stimulatory molecule CD40 normally present on APCs (Ad-CD40L) demonstrated superior therapeutic effect compared to replication-competent VSV +/- CD40L coexpression. This Ad enhancement was associated with a slightly delayed onset of tumor-specific adaptive T-cell responses, whereas fully replicative VSV induced prompt inflammatory responses and T-cell activation without tumor-antigen specificity [133, 134]. An approach to be examined in

an upcoming landmark phase-I/IIb clinical trial again employs an OV as “vector-lytic”. However, the administration of attenuated MG1 vesiculovirus expressing the ubiquitous cancer/testis antigen, melanoma antigen E (MAGE), is preceded by a priming dose of replication-defective adenovirus, also expressing the same tumor antigen. The utility of this variation on the heterologous prime-boost concept was recently reported [225] and the safety of this platform has been verified in non-human primates.

OVs have been used similar to traditional transductants for tumor antigen expression in DC-based vaccines. NK cell and tumor-specific T-cell responses have been observed with therapeutic effect in pre-clinical work [226]. However, DCs do not sustain viral protein expression and frequently undergo apoptosis once activated by infection. An improved DC-based approach employs a conditionally replicative or replication-defective OV to induce an oncolytic nidus that dramatically amplifies the potency of a tumor-lysate loaded DC vaccination series. Similar to the prime-boost modalities previously discussed, the immune system mounts a fortified adaptive anti-tumor response with minimal viral skewing; this approach is capable of eradicating metastatic disease [166].

OVs have long been used as high-potency adjuvants for the ex-vivo manufacture of whole-cell or membrane-fraction anti-tumor vaccines, with the view to eliminate minimal residual disease following initial tumor de-bulking [94, 122, 140, 141, 154, 218]. These virus-absorbed tumor cells or “oncolysates” have shown pre-clinical promise for many years. Generally these vaccines have been made from replication-defective OVs including an inactivated NDV, UV-irradiated influenza as well as replication-incompetent Vaccinia and HSV-1. These highly attenuated or inactivated viruses induce

anti-tumor immune responses primarily through DAMP-mediated ICD, augmented by the presence of viral PAMPs as previously discussed. Modest results were observed in early phase clinical trials yet none have been advanced further at this time. One recent report from our lab describes an infected-cell vaccine using VSV $\Delta$ 51-GMCSF to control a resistant murine melanoma cell line [227]; the oncolysate stimulated NK cells and DCs. Finally worth mentioning is an interesting adoptive cell therapy approach. T-cells engineered to recognize a specific immunodominant tumor antigen were infected with VSV; these cells resist apoptosis yet transfer their “protected” OV cargo to metastatic sites [228].

If the immune system is to have a significant impact on long-term survival, the ratio of tumor-specific high-avidity CTLs to neoplastic cells is likely the key determinant to controlling tumor outgrowth. Maintaining this mathematical constant over a critical time-period [229] will require rapid leukemic cyto-reduction followed by potent and sustained adaptive anti-tumor immunity. OVs may be able to contribute to both of these therapeutic essentials.

## **1.6 Virotherapy in hematopoietic cancers**

During the mid 1800’s, before and during Dr. Coley’s therapeutic exploration using bacteria, frequent case reports described regression of tumors temporally associated with an antecedent viremia resembling influenza and varicella [230]. These involved mainly patients suffering from aggressive forms of lymphoma and acute leukemias. Marked regression or remissions typically occurred in younger adults or children.

However these were transient respites – all appear to have relapsed within weeks to months. Similar reports surfaced throughout the ensuing century and several cases of measles-, EBV- or viral hepatitis-induced remission of acute leukemia, Hodgkin or Burkitt lymphomas were reported [231–233]. The very first clinical trial examining virotherapy, in 1949, involved 22 patients with Hodgkin lymphoma. The treatment involved multiple injections of infectious extracts obtained from donors with hepatitis B or picornavirus. Over half acquired hepatitis (some died on this account) and one third had improvement in symptoms [234]. The next clinical trial in OV history involved patients with acute leukemia using “glandular fever serum”. The majority of those that became infectious underwent a short-lived remission period [235]. Other agents such as flavivirus were employed during the mid-century for patients with cancer and routinely the optimal tumor responses were seen in those with acute leukemia. However, these immunocompromised patients were highly susceptible to the fatal neurotoxic effects of many of these OVs [236]. Eventually less neurotropic OVs were employed, but mainly in other cancers. Given the historic position blood cancers have played in the early development of the field, it seems incongruous that this realm of cancer has not been more thoroughly investigated in the modern era.

More recently the dsDNA Myxoma virus, segmented dsRNA Reovirus, and the ssRNA Measles virus, Coxsackievirus A21 and VSV have all been shown to selectively kill marrow-based cancers (multiple myeloma cells and/or acute myeloid leukemia) within a mixed population of peripheral blood mononuclear cells or bone marrow/progenitor cells [220, 237–244]. After virus exposure, normal stem/progenitor

cells can be engrafted into immunodeficient mice without impairment of repopulation/lineage differentiation. Myeloma/leukemia cell lines support short-lived virus replication; cell death is primarily induced through activated apoptotic pathways [220, 245]. It is envisioned that purging contaminating myeloma or leukemia cells from autologous grafts may improve remission rates, extend high dose therapy or replace allogeneic HSCT for some patients. An early report from an ongoing phase I clinical trial (NCT00450814) examining the use of cyclophosphamide and the Edmonston vaccine strain measles virus encoding thyroidal sodium iodide symporter (MV-NIS) in patients with refractory or relapsing multiple myeloma, describes a dramatic decrease in tumor burden with evidence of virus amplification (iodine-123 gamma camera imaging) within disseminated myeloma foci. Preclinical studies demonstrated that unresponsive xenografted myeloma expressing NIS after treatment with MV-NIS, regressed completely with a single dose of  $^{131}\text{I}$  [246]. Measles virus displaying scFv for CD38 detargeted normal and enhanced delivery to myeloma clones not expressing the measles cell-entry receptor CD46; enhanced therapeutic index was observed [247].

Adenovirus serotype 5, double deleted vaccinia virus (TK and vaccine growth factor genes removed) and rat parvovirus H-1PV have all been explored in marrow-based cancers or in aggressive lymphomas that frequently seed there [215, 248, 249]. However at this time, no platform outside Measles (above) and Reovirus (“Reolysin”: phase I monotherapy NCT01533194; pending NCI trial dexamethasone/carfilzomib with Reolysin) has successfully advanced to the clinic to treat hematopoietic cancer (both multiple myeloma). It is interesting to note that these promising OV's are ubiquitous

human pathogens for which essentially all individuals have acquired pre-existing immunity, either through natural infection or vaccination.

## **1.7 The vesiculovirus platform**

Vesiculoviruses belong to the family Rhabdoviridae of the order Mononegavirales. The prototypical species is the highly studied VSV, while another member of this genus – Maraba, has only recently been examined in detail. Their natural hosts are non-human mammals, frequently livestock, and their arthropod vectors (i.e., phlebotomine sandfly). Outbreaks occur seasonally on South and Central America farms and parts of the USA; the viremia is associated with cytolytic pathology with vesicular eruptions on feet, oral mucosa and teats. Humans are rarely infected - a brief flu-like syndrome may be observed. One case of fatal encephalitis has been reported [202, 242, 250].

Vesiculoviruses are bullet-shaped, on average approximately 200 x 75 nm, and enveloped with bilipid leaflets [202]. The genome is comprised of a non-segmented negative-sense ssRNA encoding 5 discrete structural genes spanning 11 kilobases: nucleoprotein (N), phosphoprotein (P), M, G and large polymerase (L). They are partitioned by transcriptional stop/start sequences and the functional RNA-dependent RNA polymerase (RdRP; L and P proteins) begins immediately at the N gene-start site and stalls at each intergenic region with the efficiency of transcription decreasing by approximately 30% at each step. This results in a quantitative gradient of mRNA

(N>P>M>G>L). Replication is facilitated by RdRP synthesis of a full-length positive-sense copy of the genome [251]. In an infected cell, P dimers are bound to the N-RNA template at regular intervals. Its flexible N-terminal arm “fly-casts” into the cytosol to capture L and position the polymerase onto the template, maintaining stability throughout transcription particularly during intergenic pauses. The RdRP complex may move along the N-RNA template by jumping between the adjacent P molecules [252]. New virion assembly occurs rapidly within 2 hours post-entry. Interestingly, this does not appear to happen stochastically, but rather in an orchestrated fashion. The ssRNA and N first associate as a condensed nucleocapsid ribbon at the tip of the bullet, which forcibly curls into larger rings that eventually inlay the trunk of the particle as a helical formation [253]. Although associated with the nucleocapsid, the exact locations within the packaged virion and the complete crystal structures of P and L remain unsolved. P however acts as a chaperone preventing nonspecific encapsidation of cellular RNA. Repeating M subunits mesh the nucleocapsid on its outer surface creating a scaffold for binding G and envelope membrane during egress [202, 252–257]. The extraviral protrusions of G facilitate cell attachment and entry. Several dozen additional host membrane-associated proteins can be “dragged” along during the budding process [255, 258]. Although host membrane is consumed in this process it is well established that replication is terminated by both mitochondrial and death receptor-mediated apoptosis [204, 205, 242, 259–261].

Vesiculoviruses are entry-pantropic, however the mechanism has been a controversial issue. Until recently vesiculovirus internalization was felt to be independent of a cell-surface receptor, facilitated through a nebulous interaction with negatively

charged host membrane lipids [258, 262]. More recently, it has been shown that the ubiquitously expressed low-density lipoprotein receptor (LDLR) serves as the major attachment gate on cancer and normal cells alike. Other members of the LDLR family also serve as less efficient ports-of-entry [263]. Interestingly, previous reports have shown that IFN-treated cells indeed secrete soluble LDLR and this significantly reduces VSV infectability [264] - a contributing factor to the IFN-sensitivity of the vesiculovirus genus. Once attached, actin- and/or dynamin-2-mediated clathrin-dependent endocytosis occurs immediately. The low pH and low calcium ion concentration within the endosome induces G-protein conformational alteration allowing virus-endosome membrane fusion and ejection of the nucleocapsid into the cytosol to begin transcription and replication [258, 262, 265]. Viral ribonucleoprotein release is not completely efficient and retained endosomal ssRNA triggers the TLR7/8 signaling cascade activating NF $\kappa$ B and prompting the brisk type I IFN release – most profoundly from infected plasmacytoid dendritic cells. Other PRR pathways contribute to the overall intracellular anti-viral response, including endosomal TLR3 and cytosolic dsRNA-sensing retinoic acid inducible gene-I (RIG-I) mitochondrial antiviral signaling (MAVS) [102, 211, 266, 267]. In normal cells, the rapid kinetics of vesiculovirus replication and M-mediated shutdown of global cellular gene expression may permit progeny formation and exit before a complement of ISG effectors proficiently abort infection [124, 181, 201]. The massive systemic release of IFN primes uninfected cells to resist vesiculovirus infection. Indeed infections are short-lived and typically self-limiting in cells with intact IFN responses.

Aside from its intrinsic predilection for tumor cell replication and ability to instigate anti-tumor immune responses (principally with non-replicating or attenuated variants), the vesiculovirus platform has several additional features that make it an attractive biotherapeutic. Replication is entirely cytosolic and cell transformation cannot occur. Furthermore, pre-existing immunity in humans does not exist, theoretically improving the initial delivery to tumor [202, 245]. Nevertheless, intrinsic neurotoxicity remains a potential concern, particularly in hyper-susceptible patients with marrow-based cancers that frequently disseminate to the CNS. Virulence is partially G-serotype dependent and encephalitis is primarily a result of the inflammatory response elicited by microglia and astrocytes, resident innate immune cells of the CNS [216, 268]. As vesiculoviruses induce intrinsic/extrinsic apoptosis and potent indirect oncolytic effects - increasingly attenuated forms are being used successfully in preclinical research. Additional mutations to the M and/or G protein can further impair inhibition of cellular gene expression, as well as virion assembly capacity [207, 269, 270]. Mutations anywhere along the genome are continually being scanned for attenuating defects that improve tumor selectivity or enhance anti-tumor responses. The intrinsic transcription gradient drop-off can be exploited by inserting genes upstream of N, which markedly perturb virus protein ratios and progeny formation, abolishing neurotoxicity in glioblastoma xenografts [269]. Full deletion of G to prevent spread altogether has been shown to eradicate injected and distant tumors [133].

It is clear that the highly versatile vesiculovirus platform imparts multimodal tumor-specific oncotoxicity. This includes the incitement of pronounced innate and

adaptive immune responses - the latter being most *tumor-focused* using attenuated or semi- or non-replicating strains. The acute leukemias have dismal outcomes for the majority, and current regimens are highly toxic. Improvements will only be realized upon the introduction of novel non-toxic cytoreductive agents followed by robust immunotherapy that is applicable to all patients. I believe cancers of the immune system may be uniquely amenable to technologies that exploit their aberrant responses to microbials or pathogen-like substances. On several levels, the continued research and development of vesiculovirus-based therapeutics for the acute leukemias merits attention.

## 1.8 Hypotheses

A. Given attenuated vesiculoviruses provoke brisk inflammatory and antiviral adaptive immune responses, administering leukemia cells infected by vesiculovirus may induce anti-leukemic immune responses capable of controlling the progression of acute leukemia.

B. Given that attenuated replication-competent vesiculovirus was unable to safely control leukemic progression and semi-replicative vesiculovirus induced apoptosis in acute leukemia cells (observed from work performed in A), bioactive non-replicating vesiculovirus may be administered intravenously safely at the doses required to control disseminated acute leukemia.

## 1.9 Objectives

A1. Confirm vesiculoviruses infect and kill a range of human and murine acute leukemias and establish a reproducible murine model of acute leukemia

A2. Develop a quality-controlled vaccine preparation method and then measure its *in-vivo* efficacy, potency and durability in the established leukemia model.

A3. Examine the individual aspects and components of the experimental vaccine and develop control vaccines based on these elements for *in-vitro* and *in-vivo* comparison analyses.

A4. Examine the host's immune responses and critical immune requirements by employing multiple *in-vitro* and *in-vivo* experimental methods ensuring reliability.

B1. Develop a reproducible method to manufacture non-replicating vesiculovirus at concentrations feasible for pre-clinical *in-vivo* experimentation.

B2. Assess the bioactivity of the non-replicating particles against a wide range of cells, including several acute leukemia lines and primary leukemic blasts from patients.

B3. Explore the utility of the non-replicating vesiculovirus particles to control acute leukemia using the established murine model of acute leukemia.

B4. Examine the immunopotentiating and anti-tumor properties of the non-replicating vesiculovirus particles using *in-vitro* and *in-vivo* methodology.

# **Chapter 2. Leukemia Cell-Rhabdovirus Vaccine: Personalized Immunotherapy for Acute Lymphoblastic Leukemia**

**Authors:** David P. Conrad<sup>1,2,3,5</sup>, Jovian Tsang<sup>1,4</sup>, Meaghan Maclean<sup>1</sup>, Jean-Simon Diallo<sup>1,2</sup>, Fabrice Le Boeuf<sup>1,2</sup>, Chantal G. Lemay<sup>1,4</sup>, Theresa J. Falls<sup>1</sup>, Kelley A. Parato<sup>1</sup>, John C. Bell<sup>1,2,4</sup>, and Harold L. Atkins<sup>1,2,5</sup>

**Authors' Affiliations:** <sup>1</sup>Ottawa Hospital Research Institute, Center for Cancer Therapeutics; Departments of <sup>2</sup>Medicine, <sup>3</sup>Cellular and Molecular Medicine, and <sup>4</sup>Biochemistry, Immunology and Microbiology, University of Ottawa; and <sup>5</sup>Ottawa Hospital Blood and Marrow Transplant Program, Ottawa, Ontario, Canada

**Correspondence:**  
Dr. Harold Atkins

**Journal:**  
Published: Clinical Cancer Research, 2013

## **Authors' Contributions**

**I wrote the manuscript and performed all experiments** except Figs. 2.1C and 2. 6B.

Conception and design: D.P. Conrad, J.C. Bell, H.L. Atkins

Development of methodology: D.P. Conrad, K.A. Parato, J.C. Bell, H.L. Atkins

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D.P. Conrad, J. Tsang, M. Maclean, T.J. Falls, J.C. Bell

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D.P. Conrad, J.-S. Diallo, C.G. Lemay, J.C. Bell, H.L. Atkins

Writing, review, and/or revision of the manuscript: D.P. Conrad, J. Tsang, F. Le Boeuf, K.A. Parato, J.C. Bell, H.L. Atkins

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D.P. Conrad, T.J. Falls

Study supervision: J.C. Bell, H.L. Atkins

## **Abstract**

**Purpose:** Acute lymphoblastic leukemia (ALL) remains incurable in most adults. It has been difficult to provide effective immunotherapy to improve outcomes for the majority of patients. Rhabdoviruses induce strong antiviral immune responses. We hypothesized that mice administered ex vivo rhabdovirus-infected ALL cells [immunotherapy by leukemia-oncotropic virus (iLOV)] would develop robust antileukemic immune responses capable of controlling ALL.

**Experimental Design:** Viral protein production, replication, and cytopathy were measured in human and murine ALL cells exposed to attenuated rhabdovirus. Survival following injection of graded amounts of ALL cells was compared between cohorts of mice administered  $\gamma$ -irradiated rhabdovirus-infected ALL cells (iLOV) or multiple control vaccines to determine key immunotherapeutic components and characteristics. Host immune requirements were assessed in immunodeficient and bone marrow–transplanted mice or by adoptive splenocyte transfer from immunized donors. Antileukemic immune memory was ascertained by second leukemic challenge in long-term survivors.

**Results:** Human and murine ALL cells were infected and killed by rhabdovirus; this produced a potent antileukemia vaccine. iLOV protected mice from otherwise lethal ALL by developing durable leukemia specific immune-mediated responses ( $P < 0.0001$ ), which required an intact CTL compartment. Preexisting antiviral immunity augmented iLOV potency. Splenocytes from iLOV-vaccinated donors protected 60% of naive recipients from ALL challenge ( $P = 0.0001$ ). Injecting leukemia cells

activated by, or concurrent with, multiple Toll-like receptor agonists could not reproduce the protective effect of iLOV. Similarly, injecting uninfected irradiated viable, apoptotic, or necrotic leukemia cells with/without concurrent rhabdovirus administration was ineffective.

**Conclusion:** Rhabdovirus-infected leukemia cells can be used to produce a vaccine that induces robust specific immunity against aggressive leukemia.

## **Translational Relevance**

Treating acute lymphoblastic leukemia (ALL) involves prolonged multi-agent radio-chemotherapy. While children are often cured, they develop significant late adverse treatment-related health effects. The majority of adult patients unfortunately succumb to the disease. Evidence derived from recipients of allogeneic hematopoietic stem cell grafts support the concept that ALL is susceptible to immune attack but treatment-related toxicity limits its broad application. Robust, yet non-toxic *leukemia-specific* immunotherapy could improve outcomes for all patients. Using several murine models of ALL, we test the immunotherapeutic potential of leukemia cells infected with engineered attenuated rhabdoviruses (iLOV). These preclinical studies demonstrate the potency and durability of the anti-leukemic immune response induced by iLOV. This is the first time an oncotropic virus has been successfully employed in clinically relevant orthotopic models using syngeneic acute leukemia cells. This novel immunotherapeutic has the potential to advance towards early-phase clinical trials for patients with ALL.

## Introduction

Acute lymphoblastic leukemia (ALL) is an aggressive hematopoietic malignancy characterized by rapid accumulation of lymphoblasts in the marrow with suppression of hematopoiesis [1]. Children are treated with prolonged multi-agent radio-chemotherapy, achieving at least 80% long-term survival [44], but this is associated with frequent late adverse effects including secondary malignancies, various chronic medical problems, and psychological and cognitive impairments [8, 49]. Although adults with ALL frequently obtain complete remission, most relapse with only a third surviving 5 years from diagnosis [44, 57]. Immunologically mediated graft versus leukemia (GvL) effects are responsible, in part, for improved outcomes in the minority of patients eligible for allogeneic hematopoietic stem cell transplantation (HSCT) [43, 47, 63, 271–274]. Unfortunately, the benefits of GvL are difficult to separate from the detrimental effects of GvHD [54]. Thus, measures that reduce the intensity or duration of chemotherapy without compromising disease control would improve the quality of life for survivors of childhood ALL, whereas more potent therapies are required for curing adult ALL. ALL is amenable to immunotherapy as shown by the effectiveness of GvL, consequently administering vaccines targeting residual leukemic cells remaining after induction chemotherapy may help achieve these goals.

Leukemia cells often harbor unique antigens with immunogenic potential. For instance, the presence of autologous CD8<sup>+</sup> and CD4<sup>+</sup> T-cell immune responses to

peptides derived from the leukemia-specific antigen NPM1<sup>mut</sup> has been shown in patients with acute myelogenous leukemia (AML) [29]. The use of adjuvants or mechanisms that upregulate cell-surface immune activation molecules, improve the intrinsic immunogenicity and therapeutic potential of leukemia cell vaccines [95, 99, 275]. Indeed, allogeneic HSCT recipients with advanced high-risk ALL and AML were shown to generate tumor specific responses and survive longer following treatment with autologous leukemia cells mixed with syngeneic skin fibroblasts expressing CD40L and interleukin (IL)-2 from adenoviral vectors [70]. Phase I/II clinical studies of idiotypic and dendritic cell-based vaccines have shown a modest survival benefit for patients with acute and chronic lymphomas [276]. Unfortunately, available immunotherapeutic technologies suffer from inefficient recognition and processing of tumor peptide(s), leading to suboptimal antitumor T-cell responses in vivo [277]. These challenges limit the potential impact of current immune-based anticancer therapies.

Neoplastic transformation is associated with defects of cellular antiviral defenses allowing selective infection of cancer cells by diverse families of attenuated viruses [122, 125, 242]. Rhabdoviruses, such as the engineered VSVd51 with deletion at methionine-51 of the matrix protein, and MG1, a recombinant Maraba virus with cooperative attenuating mutations in its matrix protein (L123W) and glycoprotein (Q242R), complement the IFN signaling defects in cancer cells. These specific mutations attenuate virulence and increase their tropism toward malignant cells [202, 278]. Vesicular stomatitis virus (VSV) can be rendered incapable of

spreading between cells by creating mutants with deletion of the viral glycoprotein gene (VSV $\Delta$ G) required for final virion assembly and egress [279]. The biology of these enveloped single-stranded negative-sense RNA viruses has been reviewed [202]. Infection of tumor cells by these oncotropic viruses initiates a chain of events causing peri-tumor inflammation, activation of NK cells, macrophage-mediated innate immune attack, as well as induction of adaptive antitumor immune responses [122, 127, 128].

Rhabdoviruses are capable of infecting leukemia cells *in vitro*, however, viral replication is quite limited in these cells [245]. This hinders their use as direct cytolytic agents for treatment of ALL. However, using immunocompetent murine models of ALL, we show that a vaccine composed of syngeneic leukemia cells infected *ex vivo* with rhabdovirus [immunotherapy by leukemia-oncotropic virus (iLOV)] generates a potent and durable antileukemia effect that is specifically directed toward the leukemia cell used to produce this vaccine.

## **Materials and Methods**

### **Reagents**

Blasticidin and Zeocin, used for VSV $\Delta$ 51G production, were purchased from Invitrogen. The Toll-like receptor (TLR) 3 agonist polyinosinic-polycytidylic acid (poly I:C) and TLR4 agonist lipopolysaccharide (LPS) were purchased from Sigma-Aldrich. Anti-mouse CD40-allophycocyanin (APC), propidium iodide (PI), 7-amino-

actinomycin D (7-AAD) viability-staining solutions, and Annexin V apoptosis detection kit APC were obtained from eBioscience. Antimouse CD19-FITC, CD3-PE, CD4-PerCP, CD8-PerCPCy5.5, biotin anti-mouse CD252 (Ox40L), and phycoerythrin (PE)–streptavidin were obtained from BD Biosciences.

### **Tumor cells**

L1210 and EL4 murine lymphoblastic cell lines, from American Type Culture Collection (ATCC), were maintained in suspension culture, Dulbecco's modified Eagle medium (DMEM)-high glucose (HyClone), with 10% fetal calf serum (FCS; CanSera) at 37<sup>0</sup>C and 5% CO<sub>2</sub>. Cells were routinely split every 2 to 4 days to maintain concentration between 0.5 to 1.0 x 10<sup>6</sup> cells/mL. The Jurkat human acute T-cell lymphoblastic leukemia cell line (from ATCC), the human acute immunoblastic B-cell line OCI-Ly-18 (kind gift of Dr. Hans Messner, Ontario Cancer Institute, Toronto, ON, Canada), and the human acute T-cell lymphoblastic cell line A301 (kind gift from Dr. Thomas Folks, AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Disease, NIH, Germantown, MD), were maintained in similar culture conditions. The Vero cell line (from ATCC) was maintained as adherent cell cultures in DMEM and 10% FCS. Vero cells were used for virus propagation, detection, or enumeration of infectious viral particles and for viral-neutralization antibody assays. T-Rex-293 cells (Invitrogen) were used for manufacturing of VSVd51ΔG virus.

## **Mice**

DBA/2, C57BL/6, athymic nude nu/nu, and B6D2F1 hybrid mice (all 6–8 weeks of age) were purchased from Charles River Laboratories and housed in a biosafety unit at the University of Ottawa (Ottawa, ON, Canada), accredited by the Canadian Council on Animal Care (CCAC). Institutional guidelines and review board for animal care (The Animal Care and Veterinary Service of the University of Ottawa) approved all animal studies.

## **Oncotropic viruses**

The rhabdoviruses, MG1 and VSVd51 were propagated in Vero cells and purified as previously described [242]. MG1-eGFP and VSVd51-eGFP, genetically engineered to express enhanced GFP (eGFP) gene, were grown and purified in similar manner. VSVd51 with deletion of the glycoprotein gene (VSVd51ΔG) was propagated in T-REx-293 cells stably transfected with pcDNA4/TO plasmid expressing VSV glycoprotein gene. VSVd51ΔG was grown by infecting this cell line with virus stock, 24 hours after glycoprotein induction with 1 mg/mL tetracycline. Supernatants were collected 24 hours after infection and virus purified. For certain experiments, 50 mL aliquots of viral preparation were exposed to UVC radiation (Spectrolinker UV Crosslinker XL-1000; Spectronics); complete inactivation was confirmed by testing for absence of cytopathic effects and infectious particles on Vero cells. Enumeration of virus particles was conducted as previously described [128]. Suspension cultures of human and murine leukemic cells were infected by

adding virus preparations directly to culture (at  $1 \times 10^6$  cells/mL), at a multiplicity of infection (MOI) of 0.1.

### **Vaccine preparations**

iLOV was prepared by infecting suspension cultures of L1210 or EL4 cells at  $1 \times 10^6$ /mL with virus at MOI of 10, and maintained at 37°C in humidified (5% CO<sub>2</sub>) incubator. Eighteen hours after infection, an aliquot of culture was analyzed by flow cytometry, assessing extent of infection (eGFP expression), concentration, and viability. The remainder was pelleted by centrifugation at 1500 RPM, media aspirated, washed once in PBS, and resuspended for final concentration of  $1 \times 10^7$  cells/mL in PBS. Vaccine preparations received 30 Gy  $\gamma$ -irradiation ( $\gamma$ -IR; HF-320; Pantak) before administration. Specific experiments used uninfected leukemia cell vaccine prepared analogously and injected alone, coinjected with MG1 (MOI 10) in a separate syringe or mixed with MG1 at MOI of 10, at room temperature 60 minutes before administration. In other experiments, virus infected L1210 cells were fixed in 1% (final) paraformaldehyde (PFA) before  $\gamma$ -IR. In specific experiments, TLR agonists replaced virus infection in the vaccine. Standard murine doses of either 150 mg/100 mL poly I:C or 17 mg/100 mL LPS were added to L1210 cells before  $\gamma$ -IR and injection. In other experiments, 150 mg/10<sup>6</sup> cells poly I:C and/or 17 mg/10<sup>6</sup> cells LPS were added to cultures of L1210 cells for 18 hours before washing and  $\gamma$ -IR. Apoptotic leukemia cell vaccine was prepared from uninfected L1210 cell cultures. Cells were pelleted by centrifugation, washed once, and resuspended in PBS at  $2 \times 10^6$ /mL. Ten milliliter of cell suspension was placed in a 150 mm x 25 mm plate and

exposed to 500 mJ/cm<sup>2</sup> UVC. Cells were then pelleted, resuspended in fresh media, and incubated at 37<sup>0</sup>C for 4 hours before preparation for use in a manner analogous to iLOV. Necrotic leukemia cell vaccine was prepared by pressure disruption (1,500 PSI) of washed uninfected L1210 cells at 1 x 10<sup>7</sup> cells/mL in a French hydraulic press (AMINCO J5-598A; Newport Scientific).

### **Virus treatment of leukemia**

DBA/2 mice received tail vein injections of 1 x 10<sup>6</sup> L1210 leukemia cells. Leukemic mice were treated with 100 mL PBS or PBS containing 1 x 10<sup>8</sup> plaque-forming units (pfu) MG1 by tail vein injection 7, 10, and 14 days later (Appendix Fig. 1A). Mice were euthanized upon development of typical signs of advanced leukemia such as hind-leg paralysis, focal tumor development, significant weight loss, and/or respiratory distress. Immunization and leukemic challenge Immunization was conducted by tail vein injection of 100 mL per mouse per dose of freshly prepared iLOV, an alternative vaccine or PBS. Vaccines were administered once weekly for 3 doses, followed 1 week later by intravenous tail vein injection of viable leukemia cells from suspension cultures. Cells were pelleted by centrifugation, media aspirated, washed once in PBS, and resuspended at 1 x 10<sup>7</sup> cells/mL in PBS. Mice received a dose of 1 x 10<sup>6</sup> cells unless otherwise specified. Mice were euthanized upon development of predetermined signs of advanced leukemia endpoints, (Appendix Fig. 1B).

### **Adoptive cell transfer**

Under sterile conditions, single-cell suspensions of splenocytes were prepared from donor spleens removed from iLOV immunized or naive DBA/2 mice using gentleMACS Dissociator (Miltenyi Biotec) according to the manufacturer's recommendations with red blood cells lysed by ammonium-chloride-potassium lysis buffer. Donor splenocytes were pooled and  $15 \times 10^7$  cells were injected intravenously via tail vein into syngeneic recipients. Splenocyte recipients received a leukemia challenge of  $1 \times 10^6$  L1210 cells, administered intravenously via tail vein, 1 week after adoptive transfer.

### **Bone marrow transplantation and vaccination**

Eight-week-old DBA/2 mice received total body irradiation (TBI) using two fractions of 450 cGy given 2 hours apart. Under aseptic conditions,  $10^6$  bone marrow cells, collected from flushed femurs of 8-week-old B6D2F1 donors, were intravenously infused into each TBI-treated recipient. On day 43, cohorts (n=5–6/group) of bone marrow transplantation (BMT) recipient DBA/2 mice and healthy B6D2F1 mice, received the first of 3 weekly iLOV vaccinations. On day 63, a leukemia challenge of  $1 \times 10^7$  L1210 cells was administered to all mice, including parallel-unimmunized cohorts (n=5/group). Additional cohorts of unimmunized TBI-treated DBA/2 and naive B6D2F1 were euthanized at day 43 for enumeration of the major lymphocyte compartments by flow cytometry.

## **Flow cytometry**

Leukemia cell infections were evaluated by flow-cytometric analysis of 10,000 cells using Quanta SC (Beckman Coulter). A 500 mL aliquot of infected cells was stained with 5 mL PI (1 mg/mL) approximately 30 minutes before data acquisition. All vaccine preparations were analyzed by flow cytometry in similar fashion to allow dose standardization and quality control of virus expression. For analysis of apoptosis and necrosis, acquisition was conducted on CyAn ADP (Beckman Coulter) using Annexin V apoptosis detection kit APC and cell viability dye 7-AAD, with minimum of 50,000 cells counted. Cell-surface expression of activation/costimulation molecules CD40 and Ox40 ligand (CD252) on L1210 cells incubated for 18 hours with either MG1 (MOI 10), LPS (17 mg/10<sup>6</sup> cells), or Poly I:C (150 mg/10<sup>6</sup> cells) was analyzed on CyAnADP (minimum of 60,000 cells acquired) and conducted in triplicate with background mean fluorescence intensity (MFI) of unstained cells under each condition subtracted from the MFI of stained cells. Single-cell suspensions of splenocytes were collected from unimmunized TBI-treated DBA/2 and naive age-matched B6D2F1 mice and total cells/spleen were measured by Trypan blue exclusion. Enumeration of B cell (CD19<sup>+</sup>), T-cell (CD3<sup>+</sup>/CD4<sup>+</sup> and CD3<sup>+</sup>/CD8<sup>+</sup>), and NK cell (NK1.1<sup>+</sup>) subpopulations was conducted on CyAn ADP, counting a minimum of 60,000 cells, conducted in technical duplicates for each biologic replicate. Data analysis was conducted with Kaluza software version 1.1 (Beckman Coulter) and Cell Lab Quanta Analysis (Beckman Coulter).

## **Statistical analysis**

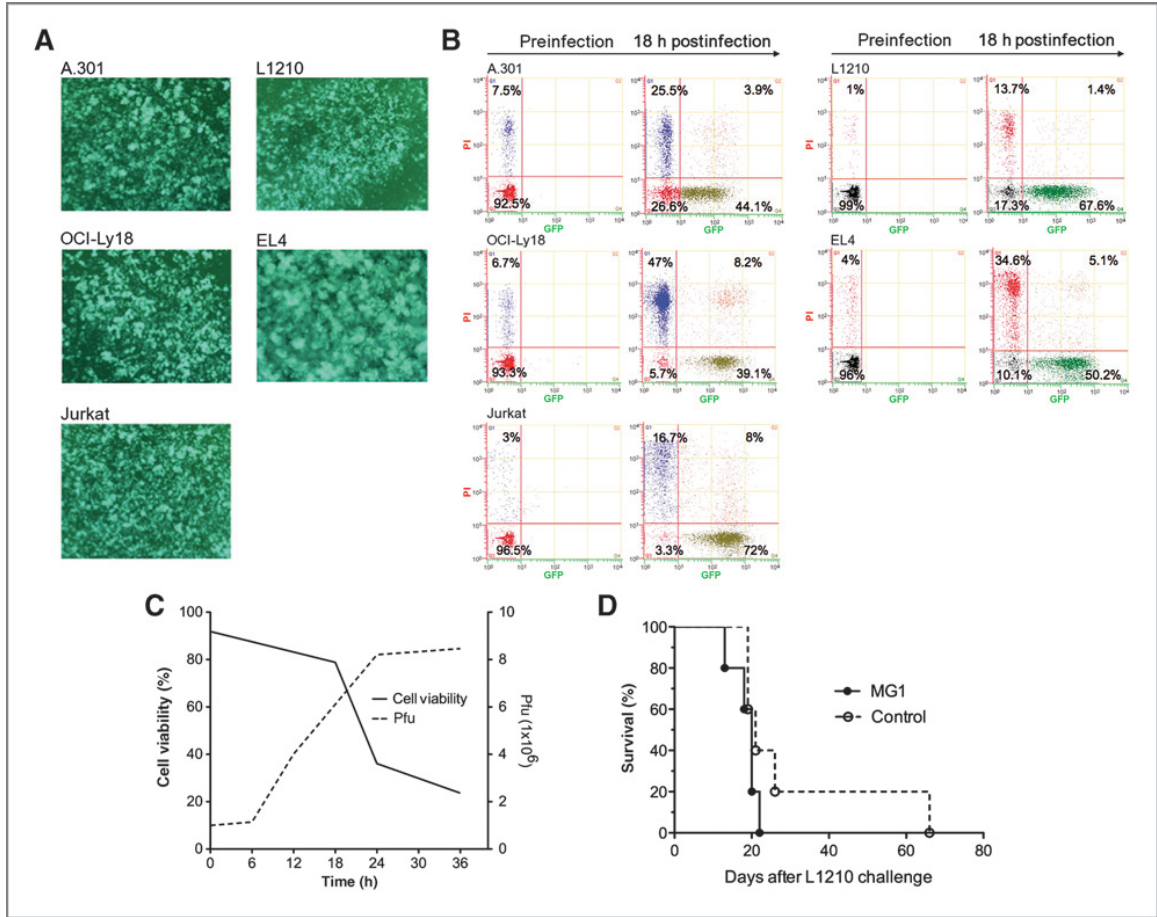
Survival curves were generated using product limit (Kaplan–Meier) method and comparisons were conducted using log-rank (Mantel–Cox) test, all P values are two-tailed. Elsewhere, data presented as mean + SEM with significance determined by Welch corrected t test. Statistical significance was determined at level of  $P < 0.05$ . Analyses were conducted using Prism 5 software (Graph-Pad Software).

## **Results**

### **MG1 infects leukemia lines in vitro but is ineffective in halting leukemia progression in vivo.**

We first wished to explore whether mice with disseminated ALL could be successfully treated by systemic delivery of live oncotropic rhabdovirus. We first established that MG1 was able to infect and kill various murine and human leukemia cell lines in vitro at low MOI (Figures 2.1A and B; Appendix Figure 2). L1210 leukemia cells show considerable permissiveness to MG1 infection and result in efficient, rapid cytolysis yet virus production is modest over 24 to 40 hours incubation (Figure 2.1C). Next, a cohort of leukemia-bearing mice was given  $1 \times 10^8$  pfu of MG1-eGFP every 3 days for 3 doses. This was unable to prevent disease progression (Figure 2.1D). Organs were recovered from all mice at endpoint. Following homogenization, an aliquot of each organ was cocultured for 15 hours on Vero cells and the monolayer was then scanned under a fluorescence microscope for presence of green fluorescing cells. Homogenates from the brain and liver of a single

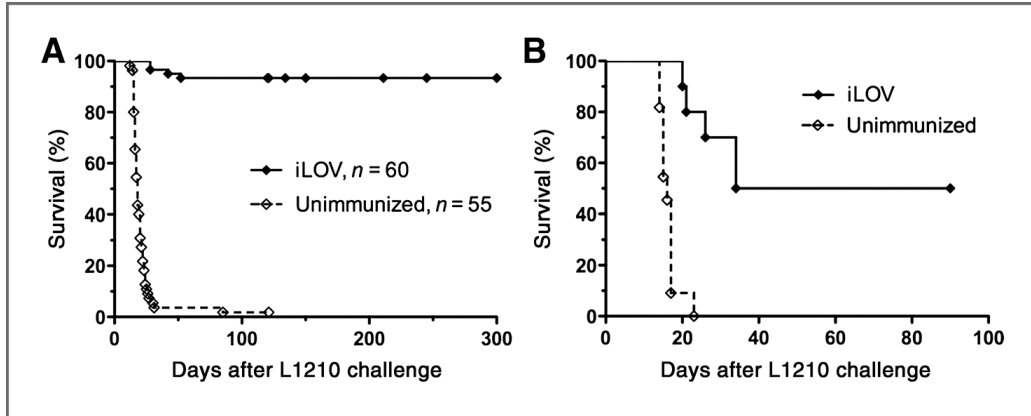
mouse, which died 3 days following the second virus injection, contained MG1 and resulted in GFP positivity on the Vero monolayer. Despite the ability of MG1 to infect and kill leukemic cells in vitro, the oncolytic effect of live MG1 was ineffective at controlling leukemia at doses large enough to cause toxicity in leukemia-bearing hosts.



**Figure 2.1 MG1 virus infects human and murine leukemia cells in vitro; however, it is ineffective at halting progression of established murine leukemia in vivo.** **A**, fluorescence microscopy (x10) showing expression of viral GFP at 18 hours after infection of MG1-GFP (MOI 0.1) in human T-cell leukemia lines A.301 and Jurkat, human B-cell leukemia OCI-Ly18, murine T-cell lymphoblasts EL4, and murine B-cell lymphoblasts L1210. **B**, flow cytometry dot plots of the same cell lines in **A**, pre- and 18 hours post-MG1 GFP infection indicating viral gene replication and protein expression by GFP. Cell viability decreases as indicated by increased PI staining. Dot plots are representative of vesiculovirus infections of cell lines conducted on several occasions. **C**, L1210 cell-viability (PI) versus virus enumeration by viral pfu over time following infection with MG1 at low MOI (0.1). **D**, survival of DBA/2 mice that received systemic MG1 virotherapy compared with untreated DBA/2 mice for the treatment of established L1210 leukemia ( $n = 5$  each group),  $P = 0.19$ .

**MG1-infected leukemia cells (iLOV) acts as a vaccine, eliminating otherwise lethal acute leukemia.**

Barriers to systemic replication-competent virotherapy for leukemia may include inadequate cytolysis at tolerable doses and rapid tumor kinetics that outpace establishment of antitumor immune responses. To address the latter issue, mice were administered 3 weekly doses of  $\gamma$ -IR virus-infected L1210 cells (MG1-iLOV). This was followed, a week later, by injection of viable L1210 cells. Mice that received MG1-iLOV showed more than 90% long-term survival following challenge with viable leukemia cells compared with untreated control mice, which reproducibly reached leukemic endpoints with median survival of 18 days (Figure 2.2A), confirming that iLOV was able to establish highly protective antitumor responses. However, when leukemic challenge was administered 1 day before the MG1-iLOV vaccination series, 100% of control mice succumbed to leukemia, whereas 50% of mice that received MG1-iLOV survived (Figure 2.2B), illustrating that iLOV is able to induce a protective effect even in the presence of early-disseminated leukemia. This incomplete protection is likely due to rapid growth of L1210 leukemia in this aggressive tumor model, which outstrips the development of antitumor responses.



**Figure 2.2 MG1-iLOV eliminates otherwise lethal leukemic challenge.** **A**, DBA/2 mice immunized with iLOV reliably (>90%) achieve long-term protection from L1210 leukemic challenge, as compared with unimmunized mice. Combined survival from 8 independent experiments ( $n = 5-15$  per group per experiment),  $P < 0.0001$ . **B**, DBA/2 mice immunized with iLOV 24 hours after high-dose L1210 administration maintain survival advantage compared with unimmunized mice ( $n = 10$  both groups),  $P < 0.0001$ .

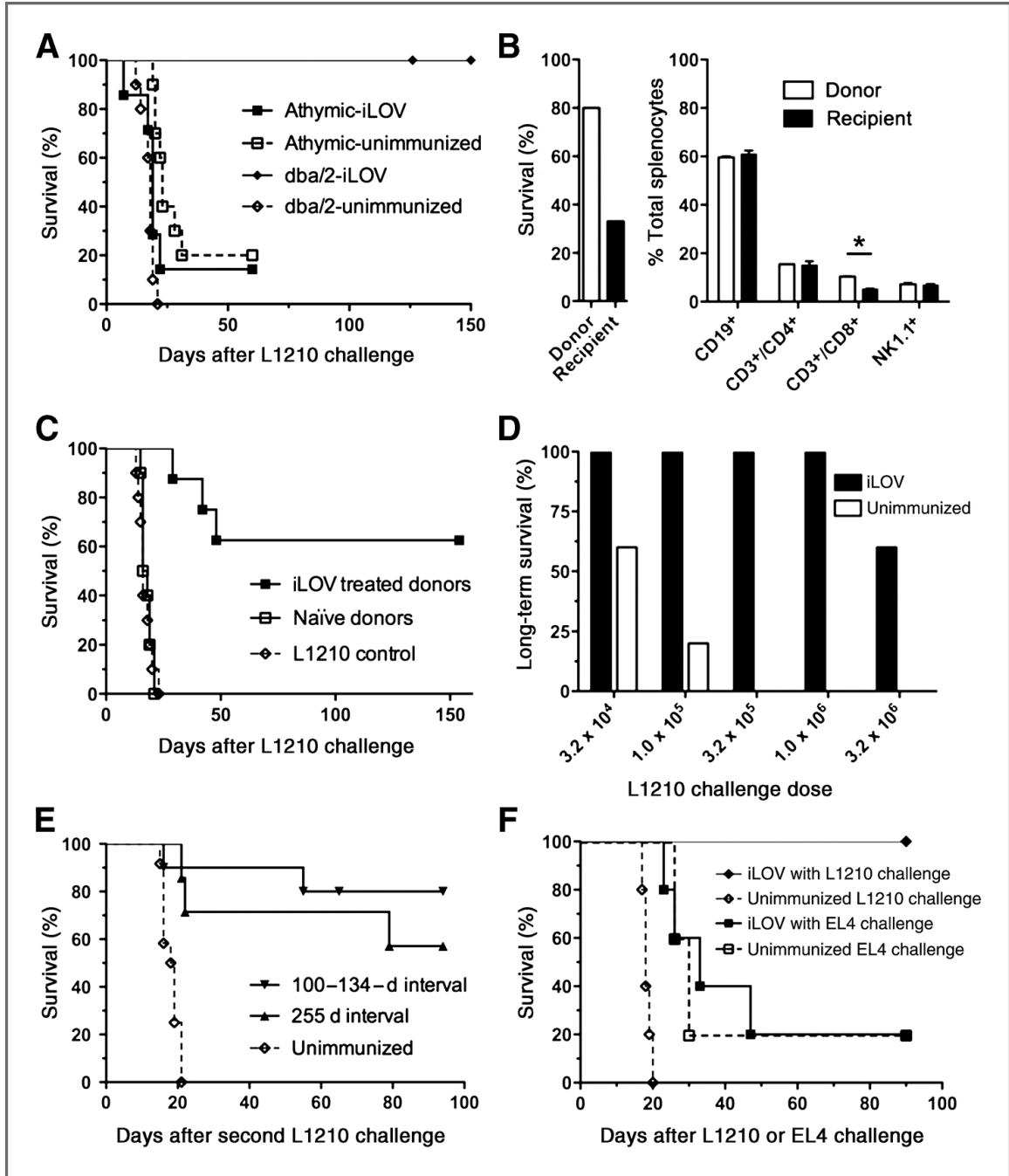
To test whether the protective effects of iLOV were mediated through development of antitumor immunity, MG1-iLOV was administered to athymic nude or immunocompetent DBA/2 mice once weekly for 3 doses. Treated and untreated nude mice died from leukemia in a median of 18 and 23 days, respectively, following injection of viable L1210 cells. In contrast, immunocompetent mice that received iLOV rejected L1210 cells (Figure 2.3A). To further examine the immune requirements of the host, a BMT model was used. After receiving myeloablative TBI, DBA/2 mice were administered  $10^6$  rescue marrow cells from B6D2F1 donors. Following a 43-day recovery, absolute numbers of lymphocyte populations were compared between unimmunized BMT recipients versus healthy donors. Although NK cells,  $CD3^+/4^+$  T-cells, and B cells were similar in number between the groups,  $CD3^+/8^+$  T-cells were significantly reduced in the BMT recipients (Figure 2.3B). This depletion of CTLs was associated with a reduction in long-term protective immunity against a leukemic challenge by approximately 40% in a parallel cohort of iLOV-vaccinated BMT recipients (Figure 2.3B). The additional control cohorts of unimmunized BMT recipients and unimmunized B6D2F1 mice all reached typical endpoints by day 28. In a separate experiment, we wished to examine the effect of adoptive splenocyte transfer from longterm iLOV-protected mice to naive recipients. Accordingly, 17 mice that received MG1-iLOV and survived between 211 to 349 days following leukemic challenge were used as splenocyte donors. Pooled donor splenocytes were administered to 8 naive DBA/2 recipients followed 7 days later by injection of viable L1210 cells. Long-term survival was observed in 63% of recipients, whereas control mice that received the same number of splenocytes from untreated donors were unable to reject leukemic challenge

(Figure 2.3C). Collectively, these observations indicate an intact thymocyte compartment mediates the antileukemic protection afforded by iLOV and CTLs are critical for optimal effect.

To examine the strength of the immune response that develops following iLOV, cohorts of unimmunized and MG1-iLOV-treated mice were challenged with increasing amounts of viable L1210 cells. The LD50 of unimmunized mice was approximately  $4.9 \times 10^4$  cells, whereas the LD50 for MG1-iLOV-vaccinated mice was estimated to be  $3.8 \times 10^6$  cells. Thus, iLOV was able to protect mice against an almost 100-fold larger inoculum of leukemia than would be spontaneously rejected by unimmunized mice (Figure 2.3D). The durability of such a response is particularly critical as the ability to prevent leukemic recurrence may wane over time. iLOV-treated mice that survived a primary leukemic challenge were administered a second L1210 leukemia challenge either 100, 134, or 255 days after initial L1210 challenge. The majority of mice were able to reject this additional leukemic challenge, but there may be a time-dependent decline in the ability to reject a late secondary leukemic challenge (Figure 2.3E).

The effectiveness of iLOV treatment was not limited to a single rhabdovirus, leukemic cell line or mouse strain. Survival following leukemic challenge was observed when animals were administered iLOV prepared using a different rhabdovirus – VSVd51, indicating that the protective effects were independent of the specific rhabdovirus (Appendix Figure 3). Similarly, mice survived an otherwise lethal challenge with EL4, a T lymphoma cell line, when MG1-iLOV prepared using

these cells was used to vaccinate syngeneic C57BL/6 mice (Appendix Figure 4). To examine the specificity of the anti-tumor protection afforded by iLOV, two cohorts of B6D2F1 hybrid mice were administered 3 weekly doses of MG1-L1210 iLOV. One cohort was subsequently challenged with viable  $1 \times 10^7$  L1210 cells while the other received  $1 \times 10^7$  EL4 cells. Mice that received the L1210-based iLOV were protected from L1210 challenge, while survival of EL4 challenged mice was identical to unimmunized mice challenged with EL4 (Figure 2.3F). The specificity of the immune response was further examined using reciprocal immunization combinations of EL4-iLOV or L1210-iLOV in cohorts of both DBA/2 and C57BL/6 mice. To control for immune recognition of leukemia cells based on the MHC disparity alone, additional cohorts of C57BL/6 and DBA/2 mice were immunized with  $\gamma$ -IR L1210 or  $\gamma$ -IR EL4 cells respectively. All groups were subsequently challenged with viable leukemia cells syngeneic to the breed. Only mice that received iLOV produced using syngeneic leukemia cells were protected (Appendix Figure 5). Together, these observations suggest that iLOV induces anti-tumor immunity functionally restricted to the specific antigenic profile of the leukemic cell used to produce iLOV rather than commonly expressed leukemic antigens.



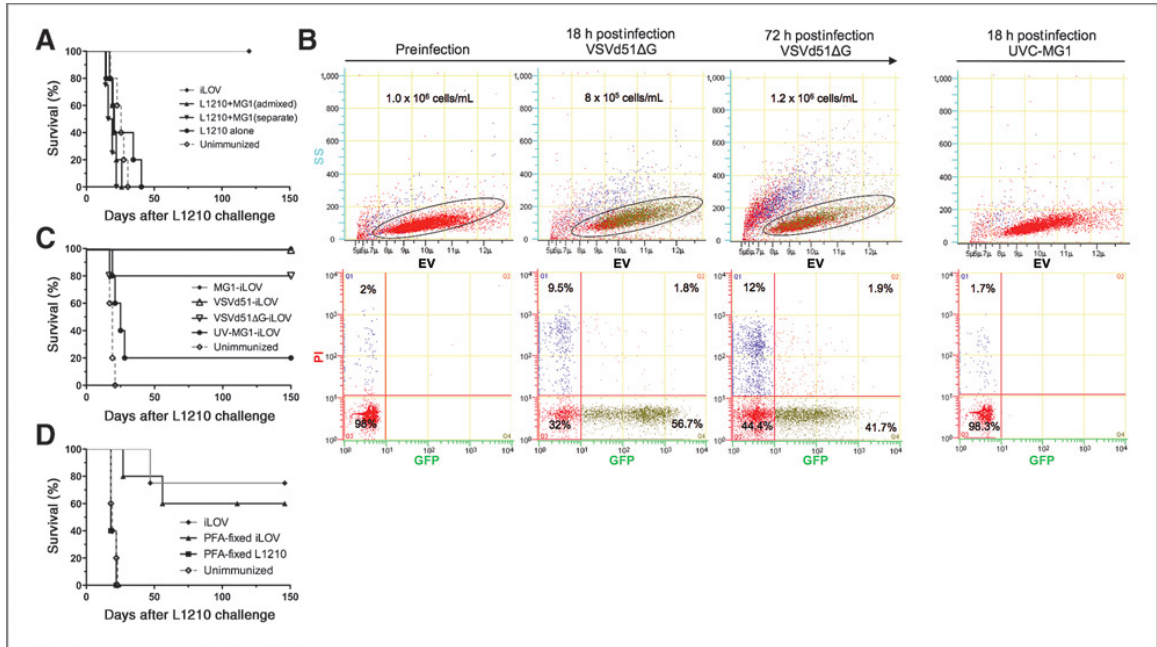
**Figure 2.3 iLOV induces potent and durable immune-mediated leukemia-specific protection.** **A**, survival following leukemic challenge for iLOV-immunized or unimmunized immunocompetent DBA/2 and immunodeficient athymic mice ( $n = 10/\text{group}$ ).  $P = 0.0002$ , iLOV-immunized DBA/2 versus iLOV-immunized athymic mice;  $P = 0.12$ , iLOV-immunized versus unimmunized athymic mice. **B**, 100 day survival following L1210 challenge ( $10^7$  cells) for iLOV-immunized DBA/2 BMT recipients ( $n = 6$ ) and immunized B6D2F1 donors ( $n = 5$ ). Lymphocyte enumeration by flow cytometry in unimmunized parallel cohorts ( $n = 2/\text{group}$ ), conducted in triplicate. \*,  $P \leq 0.007$ . **C**, survival following L1210 challenge for naïve DBA/2 recipients ( $n = 8$ ) of pooled splenocytes from iLOV-immunized DBA/2 donors surviving 211 to 349 days post-L1210 challenge versus naïve recipients ( $n = 5$ ) of pooled splenocytes from unimmunized DBA/2 donors ( $P = 0.0001$ ), and unimmunized ( $n = 10$ ) without splenocyte transfer ( $P = 0.0001$ ). **D**, long-term (125 days) survival for iLOV-immunized and unimmunized DBA/2 mice ( $n = 5$  each group) challenged with increasing doses of L1210 cells. **E**, survival following second L1210 leukemic challenge for iLOV-immunized mice surviving 100 to 134 days ( $n = 10$ ) or 255 days ( $n = 7$ ) after first leukemic challenge versus unimmunized mice ( $n = 10$ );  $P = 0.0002$  (255 day interval) and  $P < 0.0001$  (100–134–day interval) versus unimmunized. Trend observed ( $P = 0.45$ ) for time-dependent decrease in survival following late second challenge. **F**, survival of L1210-iLOV-immunized and unimmunized B6D2F1 mice ( $n = 5$  each group) following L1210 or EL4 challenge ( $10^7$  cells).  $P = 0.0133$ , iLOV-immunized L1210-challenged versus iLOV-immunized EL4 challenged mice;  $P = 0.0018$ , iLOV-immunized versus unimmunized L1210-challenged mice;  $P = 0.84$ , iLOV-immunized versus unimmunized EL4-challenged mice.

### **Virus infection is critical to induction of iLOV-mediated anti-leukemic immunity.**

We examined whether the cellular and viral components of iLOV could be individually effective at inducing protective anti-tumor immunity. Mice administered 3 doses of  $\gamma$ -IR ex-vivo MG1 infected L1210 cells (iLOV) survived subsequent administration of an otherwise lethal dose of L1210 cells, while all mice that received  $\gamma$ -IR uninfected L1210 cells prior to leukemic challenge succumbed with median survival that was not significantly different from unimmunized mice that received the same leukemic challenge dose. Furthermore, the virus must infect the cell for iLOV to be effective as 3 weekly separate co-injections of  $\gamma$ -IR L1210 cells and MG1, or the administration of 3 weekly doses of  $\gamma$ -IR L1210 cells mixed with MG1 at room temperature for 1 hour prior to injection, were unable to prevent the lethality of a subsequent L1210 leukemia challenge (Figure 2.4A).

In-vitro, leukemic cells exposed to UV-inactivated MG1-eGFP virus did not express GFP nor developed cytopathology. In contrast, L1210 cells exhibit green fluorescence following in-vitro exposure to live spread-incompetent VSVd51 $\Delta$ G-eGFP, and delayed cytolysis occurs as long as 72 hours after infection (Figure 2.4B). Immunization with iLOV produced by infection of L1210 cells with VSVd51 $\Delta$ G protected 80% of mice from the lethal effects of subsequent L1210 challenge (Figure 2.4C), indicating that iLOV is effective even if the virus is incapable of fully completing its lifecycle. Administration of iLOV produced with UV-inactivated MG1 prior to challenge with viable L1210 leukemia was unable to prevent death from fulminant leukemia in 80% of mice. iLOV preparations fixed with PFA after virus infection but

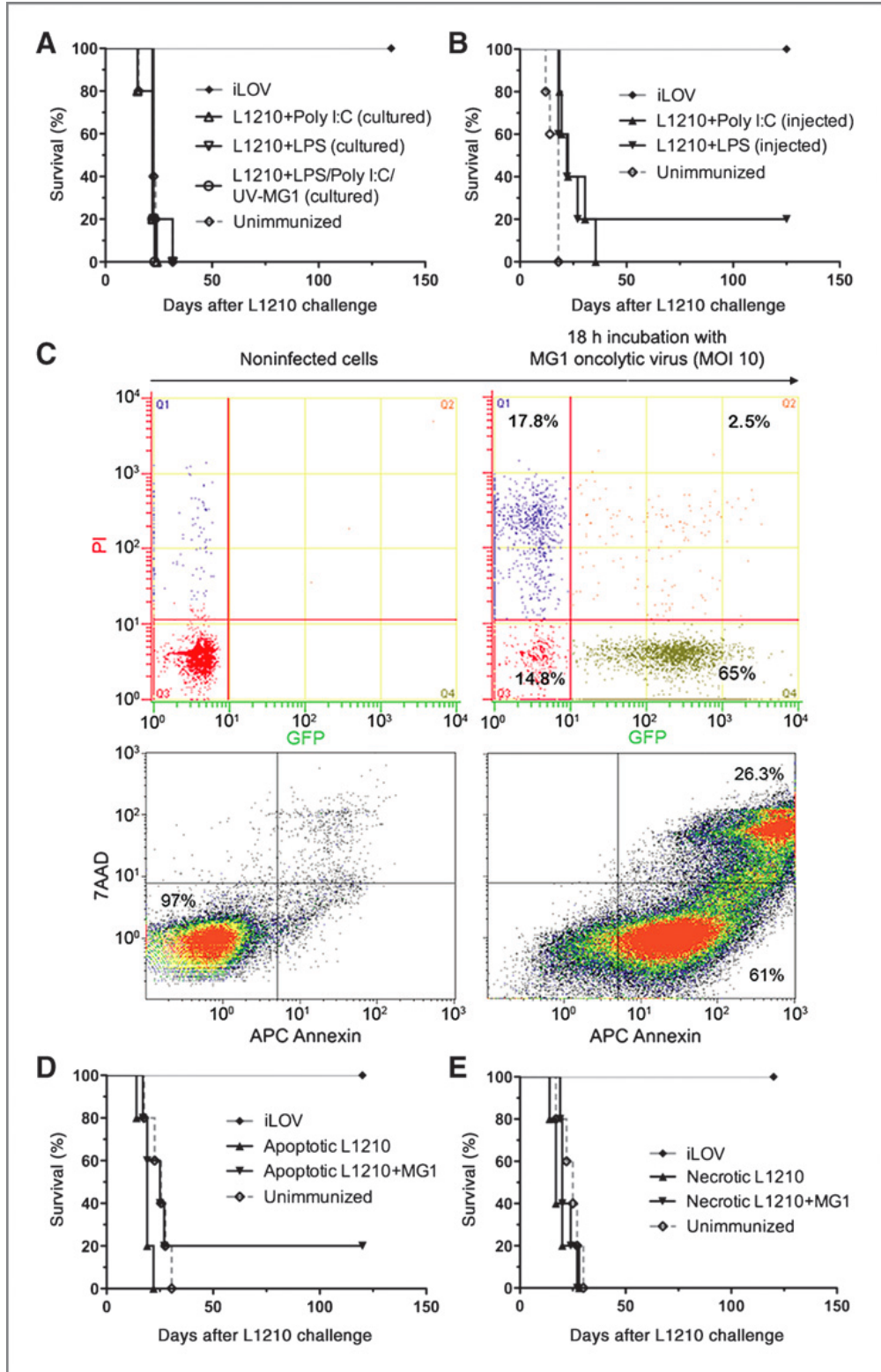
immediately prior to  $\gamma$ -IR were capable of protecting immunized mice just as effectively as freshly produced, unfixed iLOV preparations (Figure 2.4D). PFA-fixed iLOV preparations did not contain detectable viable MG1 in a standard plaque assay. Thus in this model, live rhabdovirus was used for the manufacturing of iLOV but it was not critical at the time of administration.



**Figure 2.4 Virus infection is critical to the induction of iLOV-mediated antileukemic immunity.** **A**, survival following leukemic challenge for DBA/2 mice immunized with iLOV versus L1210 cells alone, L1210 cells admixed with live MG1 for 1 hour at room temperature before injection, or unimmunized ( $n = 5$  each group), all  $P = 0.0018$ ; or versus L1210 cells coinjected simultaneously with live MG1 using a separate syringe ( $n = 5$ ),  $P = 0.0027$ . **B**, flow cytometry dot plots of infected L1210 cells. Cells incubated with UV-inactivated MG1 virus do not express GFP and exhibit negligible PI staining at 18 hours. In contrast, cells infected with attenuated VSVd51 $\Delta$ G-eGFP virus express GFP at 18 to 72 hours following infection, with viability slowly decreasing over time. Oval region indicates viable L1210 lymphoblasts. **C**, survival of DBA/2 mice following L1210 leukemic challenge. Four cohorts immunized as follows; iLOV prepared using MG1 (MG1-iLOV), VSVd51 (VSVd51-iLOV), VSVd51 $\Delta$ G (VSVd51 $\Delta$ G-iLOV), or UV inactivated MG1 (UV-MG1-iLOV;  $n = 5$  each group). The unimmunized and iLOV-immunized cohorts shown here were conducted simultaneously with experiment shown in Fig. 3F.  $P = 0.0133$ , MG1-iLOV or VSVd51-iLOV versus UV-inactivated MG1-iLOV;  $P = 0.32$ , MG1-iLOV or VSVd51-iLOV versus VSVd51 $\Delta$ G-iLOV;  $P = 0.0228$ , VSVd51 $\Delta$ G-iLOV versus unimmunized;  $P = 0.0019$ , MG1-iLOV or VSVd51-iLOV versus unimmunized. **D**, survival following leukemic challenge in DBA/2 mice immunized with PFA-fixed iLOV versus freshly prepared unfixed iLOV, PFA-fixed L1210 cells, or unimmunized ( $n = 5$  each group).  $P = 0.6649$  PFA-fixed iLOV versus iLOV;  $P = 0.0023$  PFA-fixed iLOV versus PFA-fixed L1210;  $P = 0.0017$  PFA-fixed iLOV versus unimmunized.

### **MG1 infected leukemia cells exhibit superior immunogenicity.**

The protective effects induced by injection of ex-vivo virus infected leukemia cells cannot be mimicked solely by activation of anti-viral defense pathways in leukemia cells used for immunization. L1210 cells were cultured for 18 hours with either MG1, poly I:C, LPS, or both TLR agonists and inactivated virus, prior to  $\gamma$ -IR and injection. Activation of L1210 cells by virus or TLR agonists was confirmed by measuring cell-surface expression of the B-cell immunopotentiating activation molecules CD40 and CD252 by flow cytometry (Appendix Figure 6). Mice that received 3 weekly injections of these TLR-agonist preparations succumbed to subsequent injection of L1210 cells, in contrast to mice that received MG1-iLOV (Figure 2.5A). Similarly, pulsed stimulation of host innate immunity by direct injection of either poly I:C or LPS concurrently with 3 weekly injections of  $\gamma$ -IR L1210 cells was also incapable of protecting animals from subsequent injection of viable L1210 cells (Figure 2.5B). The protective effects induced by injection of ex-vivo virus infected leukemia cells cannot be mimicked solely by the presence of apoptotic or necrotic cells that are contained in iLOV preparations (Fig. 2.5C). Apoptosis was induced in L1210 cells by UV irradiation (Appendix Fig. 7A) while parallel samples of L1210 were pressure disrupted into cellular necrosis (Appendix Figure 7B). Cohorts of mice received 3 weekly injections of either MG1-iLOV, UV-irradiated apoptotic L1210, or pressure-disrupted necrotic L1210 followed by challenge of viable L1210 leukemia. Mice that received UV-irradiated or pressure disrupted L1210 expired due to leukemia in contrast to the mice that received MG1-iLOV. Administration of 3 weekly injections of apoptotic or necrotic L1210 cells mixed with MG1 virus just prior to injection, were similarly ineffective (Figure 2.5D, E).

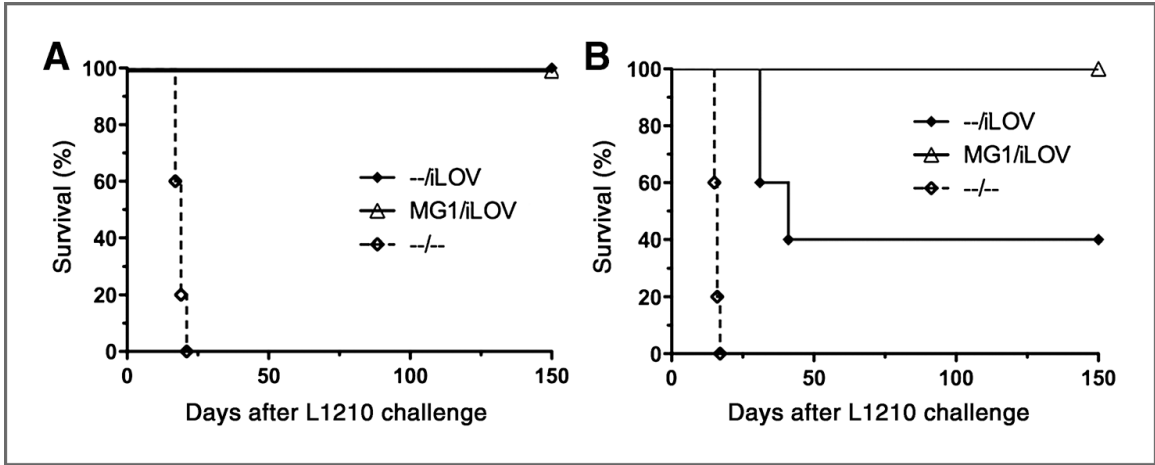


**Figure 2.5 Host or leukemia cell stimulation with TLR agonists, or injection of necrotic or apoptotic leukemia cells are insufficient to produce effective antileukemia immunity.**

**A**, survival following leukemic challenge of DBA/2 mice immunized with MG1-iLOV versus immunization with L1210 cells previously cultured for 18 hours in TLR agonists (LPS or poly I:C) or L1210 cells incubated with both TLR agonists and UV-inactivated MG1 (MOI 10;  $n = 5$  each group).  $P \leq 0.002$ , iLOV versus other groups. **B**, survival following leukemic challenge of DBA/2 mice immunized with MG1-iLOV versus immunization with L1210 cells injected with poly I:C or LPS ( $n = 5$  each group).  $P = 0.0034$ , iLOV versus unimmunized;  $P = 0.0018$ , iLOV versus poly I:C;  $P = 0.0128$ , iLOV versus LPS. **C**, flowcytometry dot plots of Annexin V (apoptotic cells) versus viability dye 7-AAD (necrotic cells). MG1-iLOV preparations include substantial proportions of apoptotic and necrotic cells at 18 hours—shown is a representative analysis. **D** and **E**, survival following leukemic challenge of DBA/2 mice immunized with iLOV versus immunization with apoptotic or necrotic L1210 cells, respectively. MG1 was either admixed (apoptotic L1210 + MG1, necrotic L1210 + MG1) or omitted (apoptotic L1210, necrotic L1210) with cells 1 hour before injection ( $n = 5$  each group). The unimmunized and iLOV immunized cohorts shown were conducted simultaneously for this experiment and the experiment shown in Fig. 4A.  $P = 0.0133$ , iLOV versus apoptotic cells . MG1;  $P = 0.002$ , iLOV versus apoptotic cells;  $P = 0.0018$ , iLOV versus necrotic cells  $\pm$  MG1 and unimmunized.

## **Pre-existing anti-viral immunity does not impair development of anti-leukemia immunity**

We wondered whether pre-existing anti-viral immunity to the rhabdovirus component would modulate iLOV efficacy as anti-viral responses have impaired the efficacy of other vector-based vaccines [280]. Accordingly, mice were injected  $10^7$  pfu MG1 by tail vein to generate anti-viral immunity. Prior to receiving MG1, mice did not manifest serum virus-neutralizing antibody while the titer of MG1 neutralizing antibody was  $\geq 1:800$  in serum of mice 10 days following administration of virus. Three doses of MG1-iLOV were administered starting 18 days after MG1 injection. The survival of MG1 immunized mice was no different than a cohort of mice that received MG1-iLOV without preceding MG1 inoculation when challenged with  $1 \times 10^6$  L1210 cells (Figure 2.6A). However, when L1210 challenge was increased 10 fold, mice immunized against MG1 prior to MG1-iLOV treatment had a significant survival advantage over mice that received MG1-iLOV treatment alone (Figure 2.6B). These results suggest iLOV efficacy is not dampened but indeed may be augmented following development of anti-viral immunity.



**Figure 2.6 Preexisting antiviral immunity does not impair the development of antileukemia immunity.** **A**, survival of iLOV-immunized DBA/2 mice, challenged with  $1 \times 10^6$  L1210 cells, previously immunized against MG1 acquiring neutralizing antibodies (MG1/iLOV) versus mice that were not preimmunized against MG1 (--/iLOV) and unimmunized (--/--;  $n = 5$  each group),  $P = 0.0019$ . **B**, survival of iLOV-immunized DBA/2 mice, challenged with  $1 \times 10^7$  L1210 cells, previously immunized against MG1 acquiring neutralizing antibodies (MG1/iLOV) versus mice that were not preimmunized against MG1 (--/iLOV) and unimmunized (--/--;  $n = 5$  each group).  $P = 0.0486$  (MG1/iLOV) versus (--/iLOV);  $P = 0.0019$  (MG1/iLOV) versus (--/--).

## Discussion

We show that live attenuated rhabdoviruses are able to infect and kill leukemia cells in-vitro but are incapable of treating mice with established systemic leukemia. An alternative approach, injecting mice with  $\gamma$ -IR virus-infected leukemia cells, or iLOV, controls leukemic progression. This effect is mediated by development of a robust adaptive anti-tumor immunity, wherein CTLs are essential for optimal efficacy. The immune response is specifically directed against the cell used to produce iLOV. It is longstanding, protecting the animal from repeat leukemic challenge more than 8 months following immunization. Both cellular and viral components of the vaccine are necessary. In this model, infection of viable leukemia cells with a transcription-competent oncotropic rhabdovirus leads to the induction of protective immune responses; however virus spread is not essential. Furthermore, the immune effects are not simply a consequence of administering  $\gamma$ -IR leukemic cells responding to noxious stimuli or due to simultaneous injection of  $\gamma$ -IR leukemic cells during nonspecific or viral provocation of the host's innate immune system. In preclinical models, iLOV appears to be an effective immunotherapy for ALL.

It has been suggested that at the interface between infected tumor cells and immune system, viral PAMPs ligate various TLRs in host dendritic cells (DCs), leading to NF $\kappa$ B or IRF3 activation, DC maturation and licensing for expansion of tumor specific T-cells [150]. However, the effectiveness of iLOV is not the result of presenting leukemia cells to an immune system stimulated by systemic administration of TLR agonists or viable oncotropic virus. TLRs have been shown to activate caspases leading

to apoptotic cell death [281] and constituents of dead or dying cells are immunomodulatory [94]. For example, phosphatidylserine externalized to the outer membrane during apoptosis activates both DCs and T-cells [282]. Necrotic cell debris provokes inflammation and innate immune system activation through pattern recognition receptor ligation of danger associated molecular patterns (DAMPs) [165, 283]. In this model, the use of UV-inactivated MG1 virus with or without concurrent activation through TLR-ligation using poly I:C and LPS is insufficient to increase the cell's recognition by the immune system. iLOV contains apoptotic and necrotic cells, however these alone or combined with live MG1 virus are insufficient to induce protective anti-leukemia immunity. These results suggest that additional pathways beyond PAMP or DAMP signaling may be activated and responsible for induction of an effective anti-leukemia immune response.

Vesiculovirus infections induce marked anti-viral immune responses. When used as priming or boosting agent, immunization with recombinant VSV expressing a tumor antigen prolonged survival and reduced tumor burden in cancer-bearing mice [217, 223]. Nonetheless, viruses encoding a single or few antigen targets induce an oligoclonal immune response. Further, the paucity of known TAAs along with their weak immunogenicity and variable expression on individual cancers [142], ultimately limits widespread applicability of this approach. Alternate approaches are being developed to create personalized anti-cancer vaccines. Recently, the mutanome, the sum total of somatic mutations in a tumor, was shown to encode multiple tumor-specific immunogenic peptides [284]. Vesiculovirus-expressed cDNA libraries, optimally derived

from xenogeneic tumor cells, have been reported to induce anti-tumor immunity in animal models, although varying degrees of autoimmunity were observed [285]. At the present time, substantial effort would be required to manufacture personalized vaccines based upon these methods.

Similar to the above methodology, our cell-based vaccine harnesses the entire library of immunologically recognized epitopes [94] in the leukemic mutatorome without the need for involved recombinant DNA or “-omic” technology. The cell-based nature of iLOV may confer additional advantages, as the vaccine would contain the extensive array of aberrant post-translational modified proteins common in tumors, which could serve to broaden the epitope library iLOV presents to the immune system, resulting in a potentially more robust immune response [286–288].

While the effectiveness of iLOV requires a virus, the neoplasia-selective replication of the attenuated virus, limited viral replication in leukemia cells, and the development of neutralizing anti-viral antibodies following first injection contribute to the inherent safety of this therapeutic. The small risk of uncontrolled viral replication can be mitigated by using viral mutants incapable of spreading, treating the preparation with paraformaldehyde, or by pre-immunizing the patient against the virus, none of which diminish effectiveness of the anti-tumor response. In contrast to other viral-based anti-cancer therapies [224, 280], pre-existing anti-viral immunity increases the potency of iLOV.

This platform represents a feasible technology to produce a safe and effective individualized immunotherapeutic for controlling disseminated leukemia. We envision

generating autologous iLOV from leukemia cells collected and stored at the time of diagnosis or relapse. Future clinical testing will determine its role in the treatment of patients with ALL.

# **Chapter 3. Non-replicating rhabdovirus-derived particles (NRRPs) eradicate acute leukemia by direct cytolysis and induction of anti-tumor immunity**

## **Authors:**

C Batenchuk<sup>1,2,3</sup>, F Le Boeuf<sup>1,3</sup>, L Stubbert<sup>1,3</sup>, T Falls<sup>1,3</sup>, HL Atkins<sup>1,3,4</sup>, JC Bell<sup>1,2,3</sup>, and DP Conrad<sup>1,3,4,5</sup>

## **Affiliations:**

<sup>1</sup> Ottawa Hospital Research Institute, Center for Cancer Therapeutics, Ottawa, Ontario, Canada. <sup>2</sup> Department of Biochemistry, Immunology and Microbiology, University of Ottawa, Ottawa, Ontario, Canada. <sup>3</sup> Department of Medicine, University of Ottawa, Ottawa, Ontario, Canada. <sup>4</sup> Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, Ontario, Canada. <sup>5</sup> Blood and Marrow Transplant Program, The Ottawa Hospital, Ottawa, Ontario, Canada.

## **Correspondence:**

Dr. David Conrad

## **Journal:**

Blood Cancer Journal (Nature Publishing Group), 2013

## **Authors' Contributions**

**I discovered the NRRP-UV fluence phenomenon during the first project (iLOV) and performed the initial leukemia cell line screenings.**

**I performed the majority of the *in-vitro/in-vivo* experiments including the following: Figure 3.2A and 3.2B, Figure 3.4 (all panels), Figure 3.5 (all panels)**

**I wrote the Abstract/Introduction/Discussion and wrote sections of and edited Results/Methods.**

Conception and design: DP Conrad, C Batenchuk, F Le Boeuf

Development of methodology: DP Conrad, C Batenchuk, F Le Boeuf, L Stubbert

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): DP Conrad, C Batenchuk, F Le Boeuf, L Stubbert, T Falls, JC Bell

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): DP Conrad, C Batenchuk, F Le Boeuf, JC Bell

Writing, review, and/or revision of the manuscript: DP Conrad, C Batenchuk, F Le Boeuf, JC Bell

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): DP Conrad, C Batenchuk, F Le Boeuf, T Falls

Study supervision: DP Conrad, HL Atkins, JC Bell

## Chapter Premise and Synopsis

Although vesiculovirus is gradually shed from the host during an infectious cycle, virus propagation in its mammalian host cell is terminated through apoptosis. The kinetics of this process is dependent on numerous factors and varies between cells. During the work performed in the iLOV project, I observed that all forms of acute leukemia cell lines undergo rapid apoptosis after vesiculovirus infection. This dampened the ability of these cells to produce virus as shown in Fig. 1C. This sensitivity to virus-induced apoptosis was exemplified by the observation that non-spreading mutants (semi-replicative VSVd51ΔG) were also efficient at killing leukemia cells. This led me to wonder to what extent vesiculovirus could be attenuated yet still trigger programmed cell death in leukemia cells. Previous published reports indicated that UV-inactivated vesiculovirus neither killed tumor cells *in-vitro* or elicited immune responses when administered *in-vivo* [133, 250, 278]. Indeed in the iLOV manuscript I showed that when a completely inactivated vesiculovirus is used to make the vaccine – the leukemia cells retain viability and the vaccine was ineffective. However I speculated that the ultra high levels of UV irradiation used during these inactivation procedures likely render the virions essentially inert, incapable of cell entry and/or severely impairing their ability to trigger PRR signalling. Therefore I first examined the effects that very small increments in UV dosage had on the virus' transgene expression, plaque forming and cell entry capacity, as well as induction of apoptosis. The purpose of this exercise was initially to determine whether I could abrogate genomic replication yet preserve cell-entry and tumoricidal activity. As previously discussed, intracellular viral-derived PAMPs elicit

marked inflammatory responses in normal white blood cells (WBC). Moreover, once activated by vesiculovirus, WBC subsets frequently undergo programmed cell death. I hypothesized that the unusually rapid induction of apoptosis by acute leukemia cells may be due to aberrant downstream effector responses to PRR ligation and this may not require extensive, if any, viral replication. Using several methodologies to inactivate virus by UVC, I observed that it was difficult to completely eliminate genome expression in these particles without abolishing cell-entry capacity (Appendix Figure 8). Using traditional UV methods to ablate genome expression and infecting leukemia cells at high MOI (50), no viral protein was detected by western blotting under 1-hour post exposure, supporting my notion that cell-entry functionality was abolished. Using my dose-adjusted UV procedure, particle cell-entry was preserved but full-length genome expression was significantly disrupted. Nonetheless, these non-replicating particles still induced apoptosis in production cell and leukemia cell lines.

It became clear that this production method was convenient to examine whether non-replicating virus-derived particles could be used effectively to control acute leukemia. Since poor virus production and spread between non-contiguous blood cancer cells limits the usefulness of a replicating format, this novel approach seemed reasonable to explore. Moreover, others and myself have found that our current VSVd51ΔG production method yields very modest titers. Mathematically, I could not foresee using these engineered constructs to examine the utility of a non-spreading virus platform. Based on the work performed in the iLOV project, the only feasible production method to supply sufficient quantities of agent needed for both *in-vivo* experimental regimens and

*in-vitro* work was by applying the above UV-based approach on high-titer stocks of wildtype vesiculovirus.

My next experiment, which eventually led to initiation of the NRRP project, used the iLOV method to ascertain whether these particles could induce ICD; indeed they could. I reasoned that since NRRPs retained immunostimulatory properties and killed acute leukemia cells directly; they might be used safely when applied at higher doses or as continuous/serial regimens. I posited that in the setting of a highly accessible tumor, a non-replicating bioactive particle was a logical way to maximize delivery required for efficacy, while maintaining safety.

As detailed in chapter 3, “*Non-replicating rhabdovirus-derived particles (NRRPs) eradicate acute leukemia by direct cytolysis and induction of antitumor immunity*”; similar to live virus, NRRPs are evolutionary refined packets containing multiple TLR-agonist that exhibit broad immunostimulatory properties and intrinsic leukemocidal activity. However NRRPs can be used safely at significantly higher doses compared to less attenuated vesiculovirus, thereby preventing disease progression as tested in a murine model of acute leukemia. Using samples acquired from patients I had seen in the clinic with multi-drug resistant acute leukemia, I observed NRRPs are tumor-selective, as leukemic blasts were purged within a mixed population of normal peripheral blood mononuclear cells. Injury to healthy human bone marrow cells is not apparent. Direct delivery of a non-replicating vesiculovirus-based therapeutic to circulating liquid tumor can be safely amplified by using dose-adjusted intravenous regimens – this represents a novel approach to the treatment of hematopoietic cancers.

## **Abstract**

Rhabdoviruses (RVs) are currently being pursued as anticancer therapeutics for various tumor types, notably leukemia. However, modest virion production and limited spread between noncontiguous circulating leukemic cells requires high-dose administration of RVs, which exceeds the maximum tolerable dose of the live virus. Furthermore, in severely immunosuppressed leukemic patients, the potential for uncontrolled live virus spread may compromise the safety of a live virus approach. We hypothesized that the barriers to oncolytic virotherapy in liquid tumors may be overcome by administration of high-dose non-replicating RVs. We have developed a method to produce unique high-titer bioactive yet non-replicating rhabdovirus-derived particles (NRRPs). This novel biopharmaceutical is multimodal possessing direct cytolytic and immunomodulatory activity against acute leukemia. We demonstrate that NRRP resistance in normal cells is mediated by intact antiviral defenses including interferon (IFN). This data was substantiated using murine models of blast crisis. The translational promise of NRRPs was demonstrated in clinical samples obtained from patients with high-burden multidrug-resistant acute myeloid leukemia. This is the first successful attempt to eradicate disseminated cancer using a non-replicating virus-derived agent, representing a paradigm shift in our understanding of oncolytic virus-based therapies and their application toward the treatment of acute leukemia.

## Introduction

Rhabdoviruses (RVs), such as vesicular stomatitis virus (VSV) and Maraba, are currently being explored as anticancer therapeutics [245, 250, 278, 289]. Through cell lysis and activation of antitumor immune responses [125, 127], live RVs are multitasking self-amplifying cytolytic agents. In tumors, viral propagation is enabled by disrupted metabolic activities [290, 291] and impaired antiviral programs [292, 293]. Typically, oncolytic viruses are genetically altered to reduce virus replication in healthy tissue, constraining viral spread to the tumor microenvironment. Although live RVs are being pursued to treat a wide variety of tumor types, their application in hematopoietic malignancies is complicated by several factors. Limited virion production and reduced spread between leukemic cells requires high-dose viral therapy to overcome these inefficiencies. However, uncontrolled live virus spread and off-target effects in normal tissue compromise the safety of this approach, particularly in immunosuppressed patients [294].

In the RV field, all oncolytic platforms developed to date utilize a replication-competent virus that spreads between tumor cells [245, 250, 278, 289]. The dogma pillar to these therapies is that virus replication is a prerequisite for treatment efficacy [126, 295, 296]. Indeed, reports describing the use of live replication/expression-competent RV as a direct virotherapy for cancer, typically compare efficacy with non-expressing virus controls where no measurable efficacy is observed [133, 250]. In these studies, the control virus is treated with a substantial dose of ultraviolet (UV) irradiation to intentionally generate an inert bioparticle. We posited that if one could devise a method

to generate non-replicating particles, which maintain both oncolytic and immunogenic properties, many of the above barriers in the treatment of hematopoietic malignancies may be overcome.

## **Materials and Methods**

### **Cells**

L1210, A20, A301, Jurkat, HL60 cell lines were from American Type Culture Collection (Manassas, VA, USA), and maintained in suspension culture, Dulbecco's modified Eagle's medium-high glucose (HyClone, Logan, UT, USA), with 10% fetal calf serum (CanSera, Etobicoke, ON, Canada), at 37°C and 5% CO<sub>2</sub>. Cells in culture were maintained at a concentration between 0.5 and 1.0×10<sup>6</sup> cells/ml. Vero cells (kidney epithelial cells extracted from an African green monkey), GM38 (normal human fibroblast cell line) and human dermal fibroblast-adherent cells were from American Type Culture Collection and propagated in same culture media used for the suspension cell lines. Normal bone marrow cells acquired from STEMCELL Technologies Inc. (Vancouver, BC, Canada) were obtained from a healthy volunteer and consented to an approved institutional review board protocol. Acquisition of human leukemic patient samples was approved by the institutional review board of the Ottawa Hospital Research Ethics Board. All patients provided written informed consent for the collection of samples and subsequent analysis.

## **Virus**

The Indiana serotype of VSV and wild-type Maraba used throughout this study were propagated in Vero cells, as previously described [242]. VSV titration was performed using serial dilutions in Dulbecco's modified Eagle's medium. Hundred microlitres of each dilution was applied to a confluent monolayer of Vero cells for 45 min. Subsequently, the plates were overlaid with 0.5% agarose in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, and the plaques were grown for 24 h. Carnoy's fixative (methanol:acetic acid in a 3:1 ratio) was then applied directly on top of the overlay for 5 min. The overlay was removed and the fixed monolayer was stained with 0.5% crystal violet for 5 min, after which the plaques were counted.

## **Viability assays**

Viability assays were done in a 96-well plate format. Cells were plated at specific density and treated with live VSV virus or non-replicating rhabdovirus-derived particles (NRRPs). After 72 h, cytotoxicity was assessed by adding 25  $\mu$ l of alamar blue reagent, (AbD Serotec, Raleigh, NC, USA).

## **Modeling**

The model used to describe NRRPs specificity against cells with defects in antiviral signaling pathways was adapted from our previous work described in Le Boeuf et al [297]. In brief, this model is represented by a subset of six ordinary differential equations describing the transition between the cell populations (UP, IP, AP and PP) depending on

the concentration of NRRPs (N) and interferon (IFN) in the environment. These equations are:

$$\frac{dUP}{dt} = -K_{NI} \times [N] \times [UP] - \left( \frac{-K_{IFN_{on}}}{1 + \left(\frac{[IFN]}{EC_{50}}\right)^2} + K_{IFN_{on}} \right) \times [UP] + K_{IFN_{off}} \times [PP],$$

$$\frac{dIP}{dt} = K_{NI} \times [N] \times [UP] - \left( \frac{-K_{IFN_{on}}}{1 + \left(\frac{[IFN]}{EC_{50}}\right)^2} + K_{IFN_{on}} \right) \times [IP] - \gamma_c \times [IP],$$

$$\frac{dAP}{dt} = \left( \frac{-K_{IFN_{on}}}{1 + \left(\frac{[IFN]}{EC_{50}}\right)^2} + K_{IFN_{on}} \right) [IP] - K_{NC} \times [AP] - \gamma_c \times [AP],$$

$$\frac{dPP}{dt} = \left( \frac{-K_{IFN_{on}}}{1 + \left(\frac{[IFN]}{EC_{50}}\right)^2} + K_{IFN_{on}} \right) [UP] + K_{NC} \times [AP] - K_{IFN_{off}} \times [PP].$$

The parameters used in the above equations represent the internalization rate ( $K_{NI}$ ), the rate of IFN-signaling activation ( $K_{IFN_{on}}$ ), the rate of IFN-signaling inactivation ( $K_{IFN_{off}}$ ), the EC50 of IFN ( $EC_{50}$ ), the rate of cell death ( $\gamma_c$ ) and the rate NRRP clearance ( $K_{NC}$ ).

The next subset of equation describes the dynamics of NRRPs (N) and IFN whereby:

$$\frac{dN}{dt} = -K_{NI} \times [N] \times [UP] - \gamma_n \times [N],$$

$$\frac{dIFN}{dt} = K_{IFN1} \times [IP] + K_{IFN2.1} \times [AP] + K_{IFN2.2} \times [PP] - \gamma_{IFN} \times IFN.$$

The parameters described in the above equations represent the rate of NRRP internalization ( $K_{NI}$ ), NRRP degradation ( $\gamma_n$ ), IFN production from IP, AP and PP ( $K_{IFN1}$ ,  $K_{IFN2.1}$  and  $K_{IFN2.2}$ , respectively) and IFN degradation ( $\gamma_{IFN}$ ).

The Monte Carlo simulation was performed by randomly varying the above parameters within a 1 log window (see Appendix Table I) surrounding physiological parameter derived from literature and experimental evidence [297]. Simulations were performed in Matlab using ODE15s imposing a non-negativity constraint. Trends described in Figure 3.3b represent the median value over 1000 simulations. The number of cells used in these simulations was  $2.5E5$ , the media volume was set at 1 ml and the particle to cell ratio was set at 100. In these simulations, defects in IFN-signaling pathways were simulated by decreasing  $K_{IFN1}$ ,  $K_{IFN2.1}$ ,  $K_{IFN2.2}$ , KVC and  $K_{IFN}$  on from 100 to 1% of their original value.

### **Flow cytometry**

Anti-CD33-PerCP Cy5.5 and Anti-CD33-PC5 antibodies obtained from eBioscience (San Diego, CA, USA) were used. Annexin-V and 7-AAD viability dye were obtained from eBioscience and performed according to manufacturer's instructions. Flow cytometry was acquired using a CyAn ADP (Beckman Coulter, Brea, CA, USA). Analysis was performed using the Kaluza software version 1.1 (Beckman Coulter).

### **Cytokine array**

Bio-Plex Pro mouse luminex-based multiplex cytokine assay (Bio-Rad, Hercules, CA, USA) was performed on mouse serum 20 h after NRRP treatment. The assay was performed according to the manufacturer's instructions.

## **Murine experiments**

For the L1210 vaccination strategy, cohorts of DBA/2 mice (Charles River, Wilmington, MA, USA) received three weekly intravenous doses of  $1 \times 10^6$   $\gamma$ -irradiated L1210 cells pretreated, or not, with NRRPs. One week following this regimen, a L1210 leukemic challenge ( $1 \times 10^6$  cells) was administered via tail vein, and survival was followed. Mice were euthanized upon development of predetermined signs of advanced leukemia end points. For the L1210 treatment strategy, DBA/2 mice were challenged with  $1 \times 10^6$  dose of L1210 blast cells. The following day, mice began a regimen of  $3 \times 10^9$  NRRPs administered intravenously for 3 consecutive days, or treated with live VSV at the maximum tolerable dose (MTD) of  $2 \times 10^6$  viruses per injection, and survival was followed. Institutional guidelines and review board for animal care (The Animal Care and Veterinary Service of the University of Ottawa) approved the above animal studies.

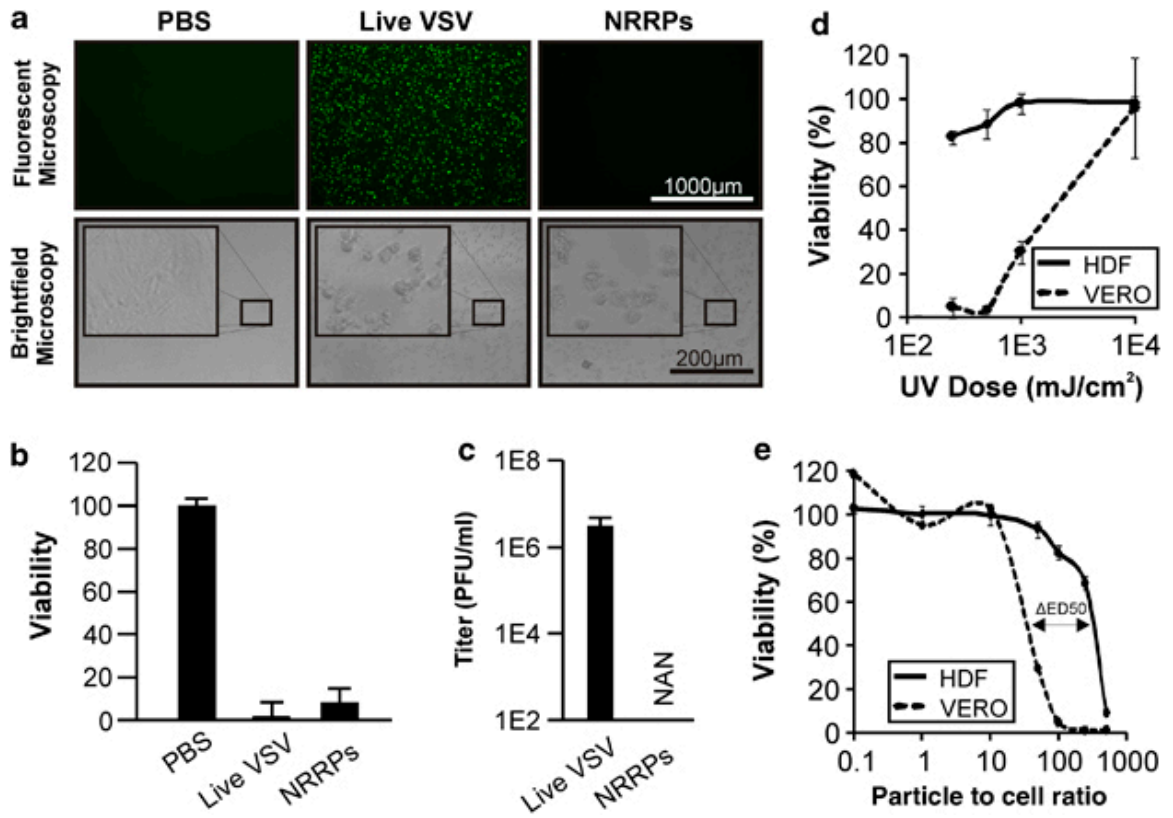
## **Results**

### **Generation of high-titer NRRPs**

We theorized that UV photonic damage of RVs could be used to generate a non-replicating particle that retained bioactivity. In the past, high-dose UV irradiation has been used to ablate the RV genome rendering a biologically inert virus [133, 250]. We hypothesized that when applied at moderated doses, UV irradiation could be used to generate a quasiparticle where replication is lost yet biological activity is maintained. To investigate this possibility, samples of purified VSV-expressing green fluorescence

protein (GFP) were exposed to a wide range of UV irradiation intensities and the biological effects of this treatment were examined. When irradiated at a low dose (250mJ/cm<sup>2</sup>), VSV-eGFP lost its expression capabilities, yet maintained potent cytotoxicity against our immortalized production cell line (Vero; Figures 3.1a and b). Titering for virus following UV treatment confirmed that the resulting particles were unable to replicate in these cells in sharp contrast with live virus infection (Figure 3.1c). This effect was equally observed when using other members of the RV family including Maraba (Appendix Figure 9).

Dose–response curves provide a better understanding of the relationship between cytotoxicity and the irradiation procedure. In these experiments, we observed that only a narrow window of UV fluence allows for the NRRP to maintain cytotoxicity against immortalized cells (Figure 3.1d), and that this effect requires a particle to cell ratio >10 (Figure 3.1e). By comparing and contrasting with normal neonatal human dermal fibroblasts (Figures 3.1d and e), it appears that cytotoxicity is tumor specific. Indeed, normal cells appear to require at least a 10 times higher dose to become sensitive to NRRP-mediated cytotoxicity.

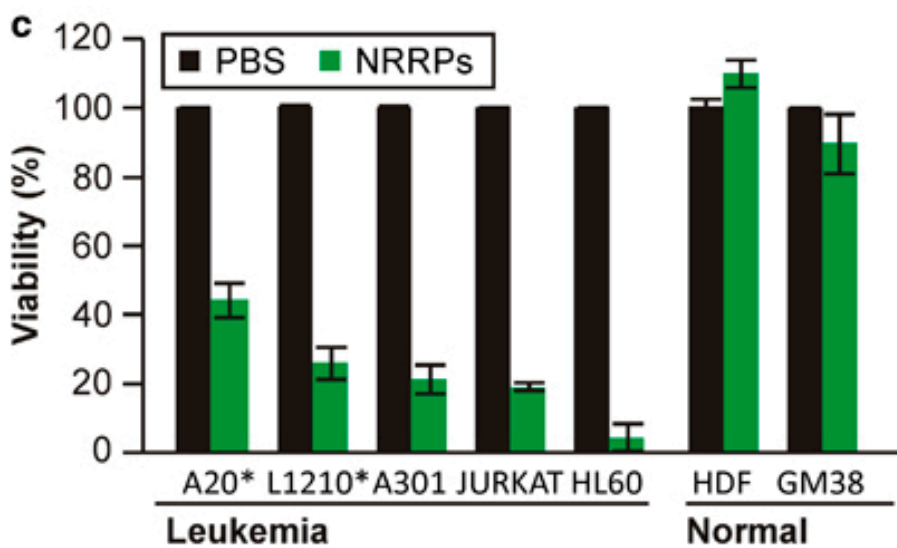
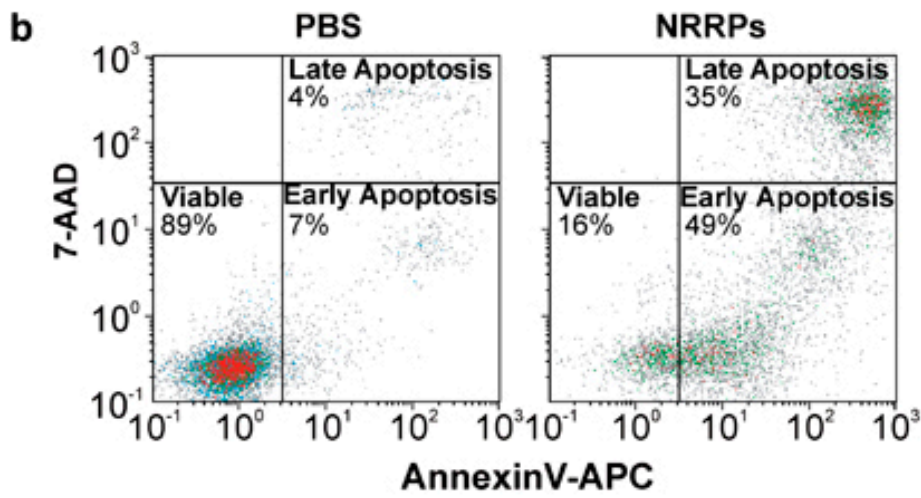
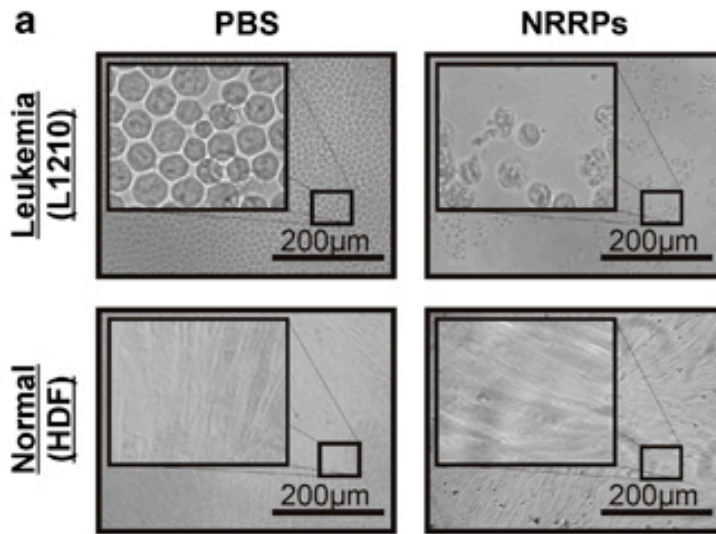


**Figure 3.1 NRRP-mediated cytotoxicity in immortalized cells.**

(a) Fluorescent and brightfield images of Vero cells treated with PBS, live VSV-GFP and NRRPs taken at 24 and 72 h after exposure, respectively. The multiplicity of infection (MOI) used in these experiments was set at 100 particles per cell. (b) Resazurin quantification of viability in Vero cells treated with PBS, live VSV-GFP or NRRPs 72 h after exposure. The MOI used in these experiments was set at 100 particles per cell. (c) Viral titers produced from the above experiments. NAN is defined as not a number, as no virions were detected. (d) Impact of UV dosage on NRRP-mediated cytotoxicity. All doses illustrated had no detectable GFP signal following UV-induced NRRP generation. This experiment used 100 particles per cell. Viability was quantified using the resazurin assay 72 h after treatment. (e) Impact of particle to cell ratio on the cytotoxicity induced by NRRPs in Vero and HDFN cells. The UV dosage of this experiment was set at 250 mJ/cm<sup>2</sup>. Viability was quantified using the resazurin assay 72 h after treatment. Error bars represent the s.d. between technical triplicate replicates.

### **NRRPs are an efficient treatment against leukemic cells in vitro**

We next examined whether acute leukemic cells were equally associated with an increased sensitivity to NRRPs. In these experiments, we first compared the cytotoxicity induced in the acute lymphoblastic L1210 cell line with that observed in normal human dermal fibroblast cells. Whereas the normal cell line was resistant to NRRPs, L1210 cells were eradicated by NRRP treatment (Figure 3.2a). The classic apoptotic phenotype was observed in leukemic cells characterized by a reduced cell diameter, a shrivelled appearance with numerous apoptotic bodies and fragmented nuclear content. To validate the cell death mechanism, we quantified the level of apoptosis in L1210 cells by flow cytometry. In these experiments, we observed potent apoptosis in NRRP-treated samples 30 h after treatment, where ~84% of population was in an early- or late-stage apoptosis (Figure 3.2b). Cytotoxicity was next quantified using a standard resazurin assay in a panel of human and murine cell lines representative of hematological malignancies and normal tissue. In these experiments, acute leukemias were highly susceptible to NRRP-mediated cell death while preserving the viability of normal cells (Figure 3.2c, Appendix Figure 10).



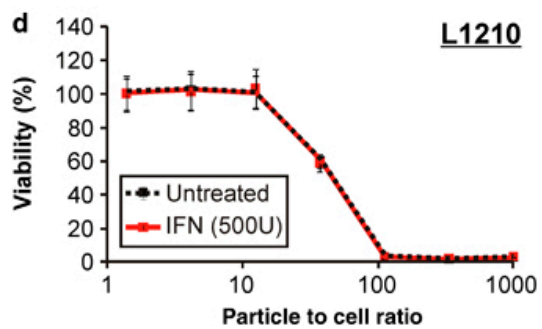
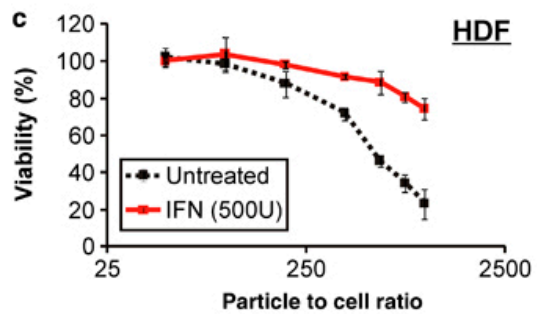
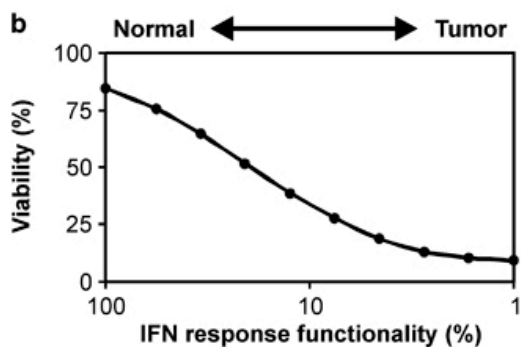
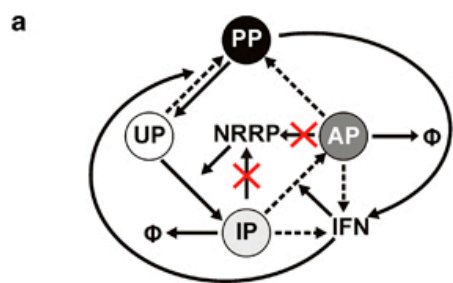
**Figure 3.2 NRRP-mediated cytotoxicity in leukemic cells.**

(a) Brightfield images of L1210 and human dermal fibroblast (HDF) cells treated with PBS or NRRPs (particle to cell ratio=100), 72 h after treatment. (b) Flow cytometry analysis of Annexin-V-APC and 7-AAD staining in L1210 cells treated with PBS or NRRPs for 30 h. (c) Resazurin quantification of viability in leukemic and normal cell lines. Murine cell lines are denoted by \*.

### **Modeling depicting NRRPs' antitumor specificity**

To investigate the mechanism by which specificity against tumor cells is achieved, we simulated the cytotoxicity induced by NRRPs in normal and tumor cells. Recently, we have developed a population-based model describing the relationship between cytotoxicity and live oncolytic virus replication dynamics in normal and tumor cells [297]. According to this model (Figure 3.3a), an infection cycle begins as the uninfected population of cells (UP) encounters virions. This allows the population to become infected, where in the context of live virus, virions and the cytokine known as IFN are released into the environment. As IFN gradually increases, the population of cells activates antiviral signaling (AP), which over time allows this population to clear the viral infection and become protected against further insult (PP). To adapt this model to NRRPs, we removed virus replication dynamics from the model, and simulated the relationship between NRRP-mediated cytotoxicity and the extent of defects in IFN-signaling pathways, a process known to occur in ~80% of cancers [250]. These defects were simulated by decreasing the rate of IFN production, the rate of activation of IFN signaling and the rate of NRRP clearance between tumor and normal cells. To ensure that this observation is systematic, a Monte Carlo simulation platform was utilized. Here, all kinetic parameters were varied within a 1 log window surrounding estimates derived from literature or experimental evidence (Appendix Table 1) [297]. Following simulation across 1000 random parameter pairings (Figure 3.3b), we observed that as the cancer cells lose their ability to signal or respond to IFN, these cells become more sensitive to NRRP-mediated cytotoxicity. To validate this observation, we investigated the impact of

IFN on NRRP-mediated cytotoxicity in normal (human dermal fibroblast) and leukemic (L1210) cells. Interestingly, although Intron A (recombinant IFN) could further increase normal cell protection against NRRP insult (Figure 3.3c), it had no detectable impact on leukemic cells (Figure 3.3d).

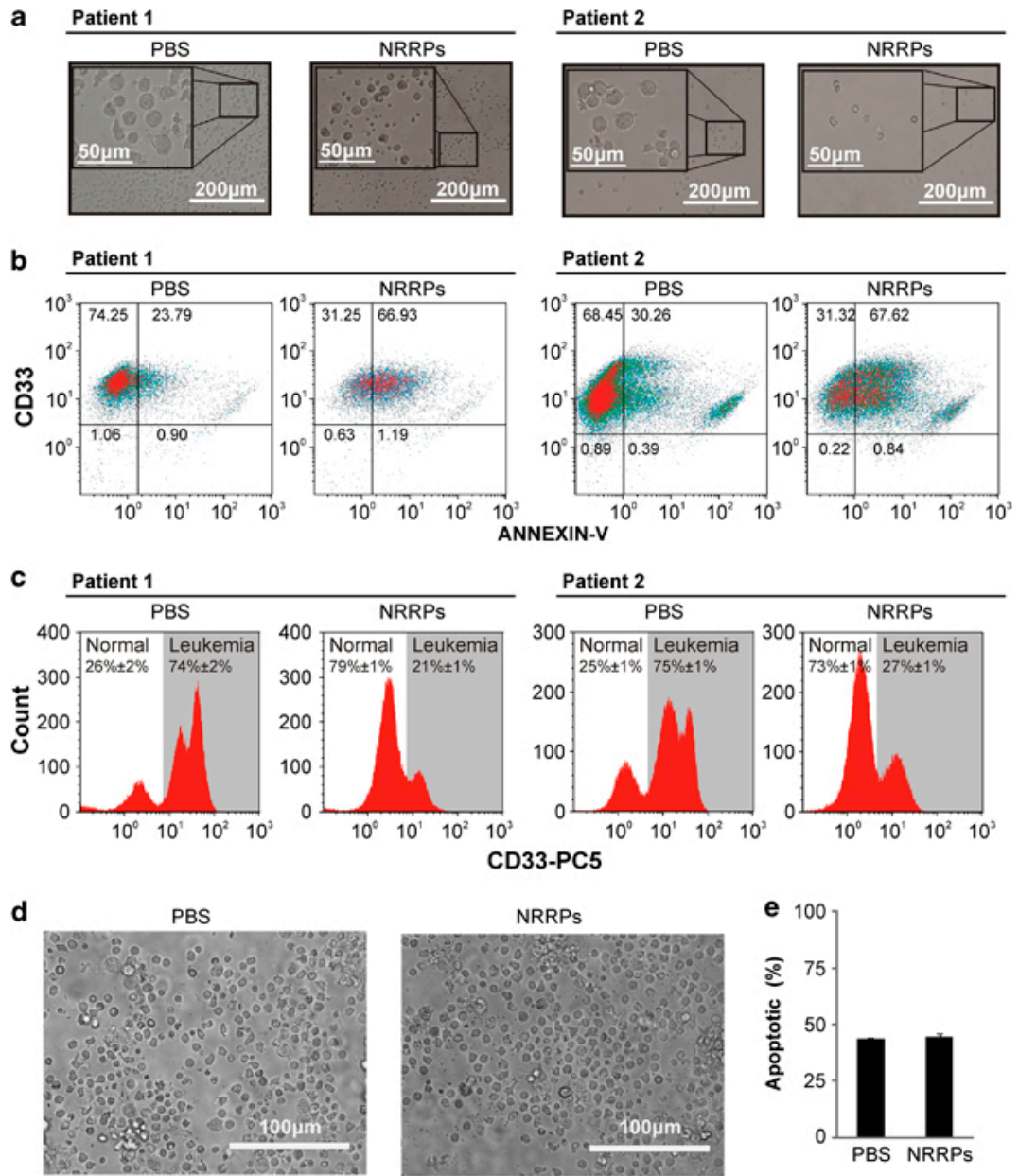


**Figure 3.3 NRRPs specifically target tumor cells with defects in antiviral signaling pathways.**

(a) Phenomenological model developed by Le Boeuf et al. (253) amended to simulate NRRPs cytotoxicity in normal cells and tumors with defects in antiviral signaling pathways. Kinetics removed from the Le Boeuf model to describe NRRP dynamics are marked in red. Hashed lines describe the IFN defects associated with tumor cells. (b) Simulated relationship between defects in the antiviral signaling pathways and viability 72 h after treatment with NRRPs. Trend represents the median value obtained over 1000 Monte Carlo simulations (see Materials and Methods). Defects in IFN-signaling pathways were simulated by decreasing the rate of IFN production, the rate of activation of IFN signaling and the rate of NRRP clearance from 100 to 1% of their original value in normal cells. (c) In vitro relationship between particle to cell ratio and viability 72 h after treatment with NRRPs in normal human dermal fibroblast (HDF) cells in the presence or absence of IFN. (d) In vitro relationship between particle to cell ratio and viability 72 h after treatment with NRRPs in leukemic L1210 cells in the presence or absence of IFN.

### **NRRP activity in AML blast crisis**

The translational potential of the NRRP platform was investigated in human clinical samples. Peripheral blood mononuclear cells were obtained from two patients with high-burden acute blast crisis. The patients had circulating blasts with a CD33<sup>+</sup> phenotype. Both had previously received extensive treatment for chronic myeloid leukemia and developed multidrug resistance. Similar to our observation in L1210 blast cells, patient samples cultured to enrich for the CD33<sup>+</sup> fraction developed obvious NRRP-induced apoptosis with classic morphology (Figure 3.4a). Indeed, the leukemic CD33<sup>+</sup> cells within this population avidly bound the apoptotic marker Annexin-V (Figure 3.4b). Use of the non-cultured patient samples was used to evaluate the specificity of this response. In both patients, the preponderant leukemic CD33<sup>+</sup> population was ablated following NRRP treatment, leaving normal cells to dominate the sample (Figure 3.4c). To ensure that NRRPs do not affect normal white blood cells, the bone marrow mononuclear cells isolated from a healthy donor were treated with phosphate-buffered saline (PBS) or NRRPs. At both early (18 h) and late (65 h) time points, NRRPs did not appear to induce apoptosis within these samples (Figures 3.4d and e).



**Figure 3.4 Treatment of chronic myeloid leukemia (CML)-blast crisis patient samples with NRRPs.**

(a) Brightfield microscopy images of two CML-blast crisis patient samples treated with PBS or NRRPs. (b) Representative flow cytometry diagram of Annexin-V and CD33 staining in two CML-blast crisis patient samples treated with PBS or NRRPs (particle to cell ratio=100) 48 h after treatment. The CD33+ blast population was enriched by long-term culture of these cells. (c) Flow cytometry of CD33 staining in two non-enriched CML-blast crisis patient samples treated with PBS or NRRPs. (d) Brightfield microscopy images of a healthy bone marrow sample treated with PBS or NRRPs for 18 h. (e) Quantification of Annexin-V staining in the healthy bone marrow sample treated with PBS or NRRPs for 65 h.

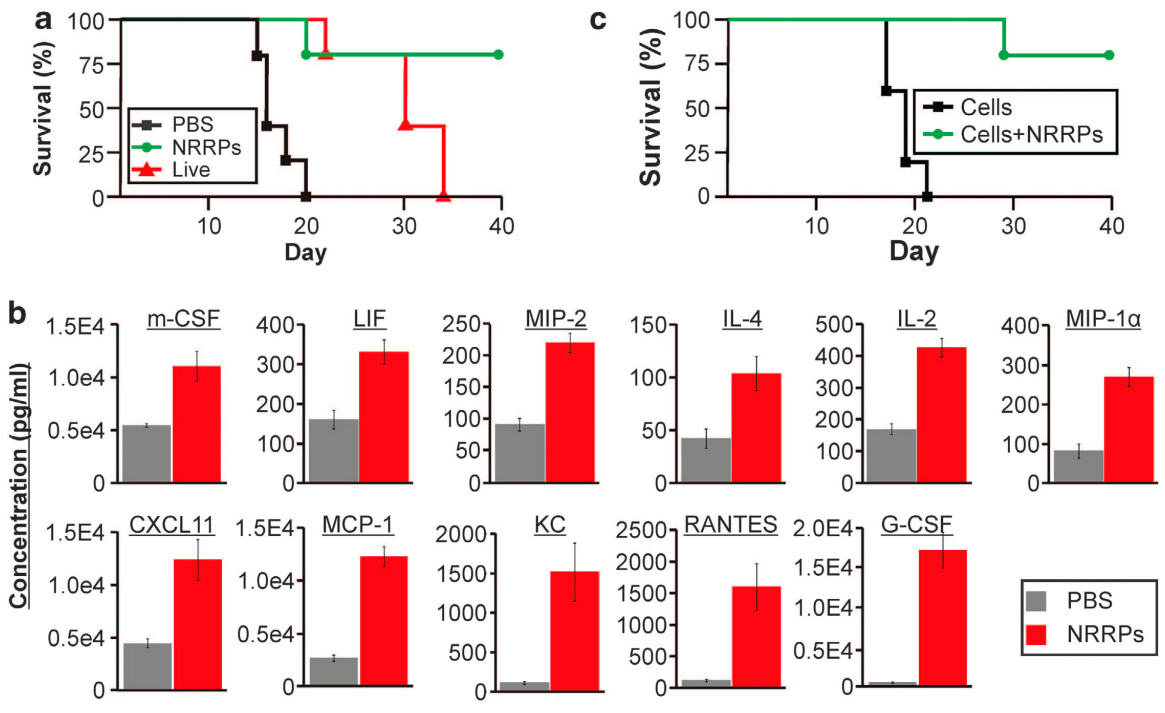
### **NRRPs antileukemic activity in vivo**

A murine model of leukemic blast crisis was used to evaluate the potential of NRRPs as a therapeutic agent. In brief, on day 1, DBA/2 mice were challenged with  $1 \times 10^6$  dose of L1210 blast cells. The following day, mice began a regimen of  $3 \times 10^9$  NRRPs administered intravenously for 3 consecutive days, and survival was monitored. In parallel, separate cohorts of mice were treated with live VSV at the MTD of  $2 \times 10^6$  viruses per injection [298] or PBS under the same treatment schedule. NRRP-treated mice achieved 80% survival up to day 40, representing a significant advantage versus those treated with PBS ( $P \leq 0.0045$ ) or live virus ( $P \leq 0.044$ ). Indeed, mice treated with PBS or live virus, all succumbed to overwhelming leukemia (Figure 3.5a). NRRPs were well tolerated and administered at the maximal feasible dose for this particular experiment, which represented a 1500 times higher dose than the MTD of live virus. Given that acute leukemia frequently disseminates to the central nervous system, and that wild-type VSV is highly neurotoxic, intracranial injections of NRRPs and live virus were performed. Although mice could tolerate the maximum production dose for intracranial injections of  $1 \times 10^8$  NRRPs, all mice rapidly succumbed to a  $1 \times 10^4$  dose of live virus.

Prompted by the superlative efficacy and differential MTD afforded by the NRRP therapy, we wondered whether the recipient's immune system is activated following NRRP administration. To answer this, the peripheral blood serum was collected from L1210 tumor-bearing mice 20 h after treatment with PBS or NRRPs (Figure 3.5b). In this analysis, we observed that multiple cytokines typically known to recruit and differentiate T cells are induced and circulating following NRRP treatment. Examples of such

immunomodulatory cytokines [299–301] significantly induced by NRRP treatment include leukemia inhibitory factor, interleukin-2, interleukin-4, monocyte chemoattractant protein-1, regulated on activation normal T cell expressed and secreted (RANTES), and macrophage inflammatory protein-1 $\alpha$  (Figure 3.5b).

To confirm immune system stimulation, in particular T-cell activation, we adopted a vaccine strategy as previously described [227, 302]. Experimentally, this platform consists of injecting apoptotic cells into immunocompetent animals and measuring protective adaptive immunity against subsequent tumor challenge. We adopted this classical experimental approach to explore whether NRRPs trigger immunogenic apoptosis [303]. Two cohorts of DBA/2 mice (syngeneic to L1210) received three weekly intravenous doses of  $1 \times 10^6$   $\gamma$ -irradiated L1210 cells pre-treated with NRRPs. Another cohort received the same number of  $\gamma$ -irradiated L1210 cells. One week following this regimen, a L1210 leukemic challenge ( $1 \times 10^6$  cells) was administered via tail vein, and survival was recorded. The cohort receiving NRRP-treated L1210 cells had 80% protection after the leukemic challenge, which was otherwise uniformly lethal in the untreated L1210-treated cohorts (Figure 3.5c). Surviving mice were kept for >150 days to ensure long-lasting protection. These results are consistent with the notion that NRRP-treated acute leukemic cells undergo immunogenic apoptosis.



**Figure 3.5 NRRPs eradicate acute leukemia by inducing immunogenic apoptosis.** (a) Survival in a murine-blast crisis treatment model. Following L1210 challenge on day 1, mice received three daily doses of NRRPs ( $3 \times 10^9$ ) or PBS, or 3 daily injections at MTD for live VSV. (b) Luminex-based quantification of cytokines induced by NRRPs in L1210-bearing mice. All cytokines illustrated are induced over twofold by NRRPs and are statistically significant (non-paired t-test  $p < 0.05$ ).  $p$  values have been corrected to account for multiple hypothesis testing (Benjamini and Hochberg method). (c) Survival in a murine model of immunogenic apoptosis. Before L1210 challenge on day 1, mice received three weekly doses of  $\gamma$ -irradiated L1210 cells preincubated or not with NRRPs.

## Discussion

This is the first successful attempt to eradicate disseminated cancer using non-replicating virus-derived particles, and represents a paradigm shift in the field of oncolytic virus-based therapeutics. Through in silico and in vitro testing, we demonstrate that NRRPs, analogous to live virus, are tumor selective, given that they exploit defects in innate immune pathways common to most tumors. However, this platform is unencumbered by the principle safety concern associated with live virus replication, that is, the potential for uncontrolled viral spread in immunocompromised patients. Indeed, the superior safety margin afforded by the NRRP platform was exemplified by the observation that high-titer intracranial NRRP administration was well tolerated by murine recipients.

The outcome for the majority of adult patients suffering from acute lymphoblastic or acute myeloid leukemia remains dismal [304, 305]. For a minority of patients, allogeneic hematopoietic stem cell transplantation (HSCT) after myeloablative conditioning is potentially curative; however, this procedure is associated with frequent adverse events and significant treatment-related mortality [304]. For many patients with chronic-phase CML, targeted tyrosine kinase inhibitor therapy offers excellent disease control [306]. However, when progression into acute blast crisis occurs, very limited therapeutic options exist owing to development of multidrug resistance and the rapid kinetics of this form of recalcitrant leukemia [39]. Clearly, new and innovative therapeutic approaches are urgently required.

Here we establish that NRRPs exhibit both direct cytolytic and potent immunogenic properties in multiple acute leukemia models. A peculiar form of programmed cell death involves the induction of adaptive immune responses against the dying cell. This process, commonly referred to as immunogenic apoptosis, is essential to the efficacy of several current chemotherapeutics [303] and is required for host defense against viral infection [307] including live RVs [308]. Our in vivo results indicate that a similar process is induced by NRRPs and is a driving factor for treatment efficacy.

More relevant are the observations that multidrug-resistant primary myeloblasts from patients with CML in acute blast crisis are forced into apoptosis and finally eradicated by NRRP treatment. In addition, non-leukemic white cells procured from healthy bone marrow were not adversely affected. This observation suggests that despite the potent tumoricidal activity of NRRPs, the leukopenia commonly observed after standard induction and consolidation chemotherapy could be avoided by using NRRP-based regimens. This would likely significantly decrease treatment-related adverse events. Further, given the preservation of normal white blood cells during leukemic cyto-reduction by NRRPs, the simultaneous induction of an effective antileukemic immune response may be attainable for the majority of patients who are not candidates for high-dose radiochemotherapy and HSCT. With the induction of immunogenic apoptosis by NRRPs, a broad array of immunomodulatory cytokines are released by the recipient's intact immune system, and this likely contributes to development of the effective adaptive antitumor immune activity—a critical component to achieving durable curative responses.

This work demonstrates a feasible biotechnology that produces high-titer NRRPs—an essential requirement for wide-scale clinical advancement. We are currently validating the MTD of NRRPs in several animal models, and are developing good laboratory practice safety measures to ensure stringent confirmation of the non-replicating nature of these bioactive particles. We expect that the pathway to approval should be less onerous than current live RV platforms under development by our laboratory and others. This promising multimodal therapeutic platform is poised for early-phase clinical trials.

## Chapter 4. General Discussion

### 4.0 iLOV: Personalized immunotherapy for ALL

Cell based vaccines have been in pre-clinical development for decades. Very few have made it to the clinic past early phase trials. This failure is multifactorial as outlined previously. On a fundamental level, most antigens expressed by tumors are likewise displayed on normal tissue and T-cells reactive against these antigens have been deleted during thymic maturation. Despite a potential array of LAAs provided by a leukemia cell-based vaccine, the effector response relies on a scarce number of endogenous T-cells that have survived the negative selection process; these cells are either anergic or possess extremely weak affinity for the mostly “self” LAAs. Proteasome processing of neo-fusion proteins from classic oncogenic translocations have a low probability of producing an immunogenic antigen that spans the breakpoint. Moreover, as outlined earlier, molecular aberrations are relatively infrequent in most cases of acute leukemia and there is a low likelihood that a coding mutation will generate an epitope that is provocative to the native immune system. Clones that do acquire immunoreactive neo-epitopes are removed by the immune system during tumor outgrowth. Diminished pMHC display by tumors and enhanced immune checkpoints augment the overall immunotolerance [64, 94, 95, 142, 149, 154, 280, 309–317].

It has been established that persistent TLR signaling is required for proper APC stimulation and reversal of Treg-mediated CTL tolerance [318]. Moreover, endosome-localized TLR signaling is the fundamental determinant for the identification of

“foreignness”. This is particularly important when ssRNA is detected, since no specific molecular motif allows the cell to distinguish its source [132, 187, 266, 319]. Indeed, TLRs sensing ssRNA are exclusively located inside endosomes and ligation of these specific receptors (i.e. endosomal TLR7/8) is associated with marked downstream activity particularly in hematopoietic cells. This includes upregulation of co-stimulatory molecules, cytokine secretion, enhanced i20S biogenesis [132, 266, 320, 321]. Indeed, ligation of TLR7 has been shown to simultaneously activate the innate and adaptive arms of the immune system [137]. I suggest that a unique feature of vesiculovirus-based therapies is that, aside from their initial stimulation of phagocytes, subsequent digestion of virus-infected tumor cells (from a vaccine such as iLOV or after upfront virotherapy) further sustains TLR signaling within the endosomal compartment of APCs. In addition, when semi- or replication-competent vesiculovirus is utilized, ongoing endosome-located TLR activation may occur through virus-induced macroautophagy. TLR7/8 activation leads to this cellular degradative pathway, which involves sequestration of internal organelles and cytosol into autophagosomes that eventually mature upon fusion with endosomes [322–324]. Thus the presence of vesiculovirus ss/ds RNA species in the cytosol of infected APCs transiently sustains the compartmentalized TLR activation. Ligation of other PRRs such as RIG-I and TLR4 (cell-surface/endosomal sensing of G protein), TLR3 (endosomal sensing of short dsRNA) as well as DAMP-mediated signaling contribute to the overall immunomodulatory effect. Indeed, the entire innate-to-adaptive immune orchestration must be tightly synchronized in order to effectuate an optimal anti-tumor response. Since most tumor antigens are at best feebly reactive, it is extremely important that all of the essential spatiotemporal elements be taken into

account when designing a novel cancer immunotherapeutic. This remains a pressing challenge in modern cancer vaccinology. Most immunostimulatory agents are not adequately associated with active APC tumor antigen processing – this prevents a properly licensed collective of tumor-targeting CTLs [143, 149, 277, 313, 325]. Vesiculovirus-based vaccine platforms may represent a convenient way to overcome these immunotherapeutic efficiency barriers.

Interestingly, when I prepared a vaccine using a high concentration of a synthetic GU-rich phosphorothioate-protected ssRNA complexed with cationic lipid (to prevent degradation and improve internalization), the leukemia cells failed to undergo apoptosis and the vaccine treatment was ineffective, using my standard experimental set-up. This most probably was due to relatively inefficient internalization (compared to VSVd51). However, TLR7/8 downstream signaling may favor NF $\kappa$ B activation [132, 187]. Although this will enhance cytokine secretion and the antigen presentation machinery, prosurvival pathways may also predominate. This highlights two caveats. First, single TLR agonists are potentially perilous agents shown to not only induce cell tumor death but also promote tumor growth and metastasis [103, 326]. Secondly, in some leukemic clones, ligating alternative or multiple PRRs (as provided by vesiculovirus-based agents) may be required for induction of apoptosis. Moreover, replicating/semi-replicating vesiculovirus strains amplify their spectrum of intracellular PRR ligands, which may improve the efficiency of the intracellular payload delivery.

Aside from the unique ability of vesiculovirus-based therapy such as iLOV to properly stimulate the healthy immune system, I observed that malignant immune cells

(B-lymphoblasts) also displayed a stimulatory phenotype upon exposure to virus, as detected by flow cytometry (Appendix Figure 6) and multiplex cytokine array (Appendix Figure 11). Several immunomodulatory cytokines were secreted from these infected leukemia cells including RANTES, IL-6 and MIP-1b. Although it is tempting to speculate that virus-infected leukemia cells with upregulated co-stimulatory molecules (“leukemic APCs”) could efficiently present tumor antigen directly to T-cells, this is tempered by my observation that LPS, and to a lesser extent poly I:C, also upregulated potent co-stimulatory molecules (Appendix Figure 6), yet were not useful as adjuvants for a leukemia cell-based vaccine in the prophylactic setting. Attempts at transducing acute leukemia cells into activated leukemic APCs for direct licensing of T-cells [327–329] has not yet been overly successful. The efficacy of iLOV is predominantly due to the synchronized activity of tumor antigen processing with DAMP/PAMP-mediated APC activation.

It is well recognized that elicitation of anti-tumor humoral responses following vaccine or chemotherapy administration is a sensitive and reliable surrogate for global anti-tumor immune responses [330–332]. I found that iLOV induced marked humoral responses (as detected by western blot) directed against the constituents of lysed tumor cells used in the vaccine preparation. However nil to minor humoral responses in comparison were elicited with control vaccines including those prepared with standard TLR agonists (Appendix Figure 12). These were confirmed humoral responses in each animal, as protein G absorption of immunoglobulin from serum prevented banding on mini-western blots. Although the level of detection was greatest against the tumor, serum

from iLOV treated animals (used as source of primary antibody) also induced some banding patterns against lysed cells obtained from several organs of syngeneic donors. Interestingly, colleague J. Tang and I also detected a level of antibody-mediated complement-dependent cytotoxicity against the same leukemia cell line used in the vaccine (Appendix Figure 13). Thus a fraction of the humoral response elicited may indeed be targeting the vaccine's leukemia cells, although at this time it is uncertain whether any are leukemia-specific antibodies. Interestingly, other data from our lab suggests normal B-lymphocytes are not depleted after the B-cell leukemia-iLOV treatment and splenic architecture/morphology is preserved.

However, it is known that generalized or tissue-restricted autoimmunity occurs after successful immunotherapy involving whole tumor cells or normal antigens overexpressed on tumors; leucopenia may be observed with certain LAA-targeting vaccines [225, 333, 334]. In unique cases, autoimmunity is likely due to the heightened baseline immunogenicity of the tissue itself (i.e., melanocytic self-antigens); indeed vitiligo can be induced by skin inflammation [335, 336]. Nevertheless, most normal tissue antigens are ostensibly non-immunogenic, thus cessation of leukemia-restricted tolerance (without autoimmunity) might be induced by a cooperative array of individually weakly immunogenic *neo*-LAAs. However optimal immunomodulation is required - such as provided by iLOV, or a VSV vector-based technology [285]. To be fair however, the model system used in this project is admittedly extremely contrived. Our highly passaged cell lines have diverged considerably from their origin, and immunogenic LAAs are undoubtedly expressed given the lack of immunoediting

pressures that are normally exerted on *in-situ* tumor outgrowth. In reality, leukemia-specific antigens have been difficult to identify, this correlates with the paucity of coding mutations in many individual acute leukemias [26].

Although cell line-iLOV administered to naïve recipients in the prophylactic setting or early-metastatic period is clearly effective, this cannot predict outcomes of residual tumor developed *in-situ*. Even in our contrived murine models, when  $\gamma$ -irradiated leukemia cells are administered just several weeks before an iLOV schedule, efficacy is markedly diminished. Moreover, antecedent therapies frequently cause immune paralysis for extended periods impairing vaccine responses. Indeed, when I administered several common blood cancer chemotherapeutics (i.e., cyclophosphamide and/or fludarabine) before iLOV therapy, this compromised the overall efficacy of the vaccine.

Although iLOV may be able to break immunotolerance on some level, the intrinsic poor immunogenicity of most *in-situ* tumors and the immunosuppressed state of patients remain significant barriers as it is with all autologous leukemia cell-based vaccines, regardless of the adjuvant used. Early phase clinical trials in non-transplant candidates might be designed to examine the ability of iLOV to control leukemic progression or prevent relapse post-induction therapy. To promote leukemia-focused immune responses iLOV should ideally be prepared with NRRPs or  $\Delta$ G strains. However, targeted small-molecules and/or other novel drugs, followed by high potency redirected T-cell therapies will likely emerge as the new acute leukemia treatment paradigm. It is uncertain how tumor cell- or DC-based vaccines could be integrated into these future regimens (Figure 4.1).

## 4.1 NRRPs:

OV therapy is often thought to rely on *virus-amplification*, which is ultimately dependent on several factors as discussed previously. These include the heterogeneity of the tumour bed, the immunosuppressive state of the tumor microenvironment and the overall functionality of the host's immune system often left impaired from previous treatments. These factors can be markedly different from patient-to-patient. Despite its theoretical and observed advantages in pre-clinical research using OV-sensitive cell lines, overt replication and spread within *ex-vivo* human tumor samples or *in-situ* tumors (clinical trials) is unpredictable and frequently observed to be a short-lived phenomenon. Intratumoral amplification is dependent on the amount first delivered to the tumor bed, which is limited primarily due to the toxic effects of active (albeit transient) replication in normal tissue. This leads to cytokine storm, organ impairment and for some viruses such as vesiculovirus – neurotoxicity/encephalitis. Fortunately, many of these potential adverse events can be quickly shutdown by the IV administration of interferon- $\alpha$ . However, in immunocompromised individuals (i.e., leukemic patients) uncontrolled replication should remain a concern. Indeed, a large pre-clinical program using VSV-IFN $\beta$  to treat multiple myeloma has been suspended. In advanced disease, meningoencephalitis occurs as a result of virus replication [337]. This adverse impact on the therapeutic index of attenuated replication-competent vesiculoviruses is a concern particularly with malignancies (such as the acute leukemias) that frequently seed to the CNS.

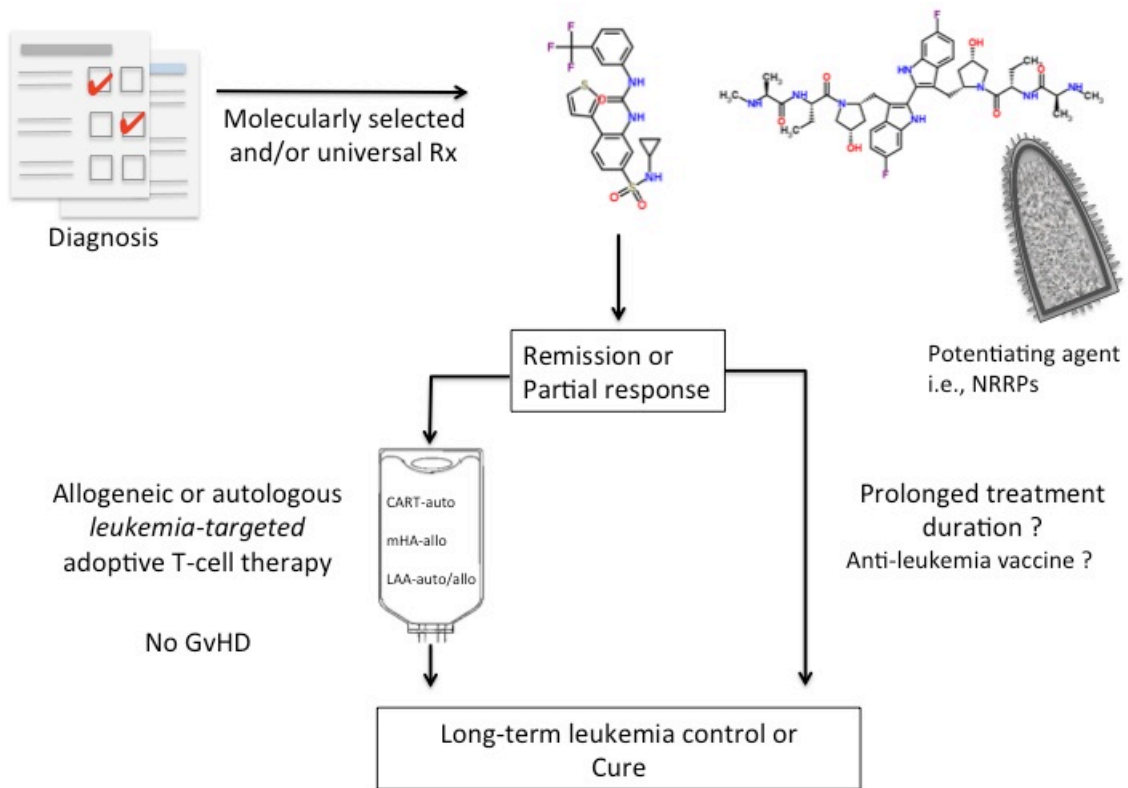
An alternative NRRP-based approach relies on deliberate *dose-amplification* to achieve its goals. This is particularly attractive when targeting circulating liquid cancers, given the immediate tumor-exposure the agent has during IV infusion. This IV-intratumoral application not only maximizes direct leukemotoxicity, but since doses can be administered at several logarithms higher than standard OV therapy, the *indirect* oncolytic effects are augmented by a more widely dispersed elicitation of the immune system - the dominant oncolytic mechanism for OVs. In addition, time lapsed videos of NRRP-treated K562 cells (Ph1 leukemic blasts) indicate that apart from apoptosis induction and the dense accumulation/dissemination of apoptotic bodies (likely logarithmically fueling phagocyte stimulation *in-vivo*), juxtacrine-mediated signaling may contribute to the expansion of death across a population of leukemia cells.

An added potential benefit exists for using a bioactive replication-defective virus for “needle-accessible” tumors. The levels of specific anti-virion antibodies were diminished considerably using this method compared to fully replicating virus. Therefore I anticipate prolonged minimally neutralized regimens may be administered - increasing total direct tumor and immune system exposure. The minimal virus protein production and the blunted anti-NRRP humoral responses suggest that the immune system will be less skewed toward anti-virion activity; a problem well known to limit the efficacy of replication competent OVs and for which several heterologous virus approaches are being explored as discussed earlier.

A potential issue in advancing this, or other virus-based platforms, involves the ability to safely infuse an effective dose of NRRPs without incurring some degree of

renal impairment from the excessive protein load. Although our *in-vitro* particle-to-cell ratios (>10) in the NRRP manuscript suggested that we would need untenable *in-vivo* doses, this of course does not take into consideration the major indirect tumoricidal activities. Ongoing optimization experiments are indicating feasible translation into human dose equivalents. Lastly, during my initial trouble-shooting of the UV irradiation procedure, I noticed that a number of technical factors required addressing to obtain reliable non-replicating bioactive particles from batch-to-batch. These included the volume of saline diluent, the dish size and the quality of the stock preparation. Using stabilized ultra-filtered GMP-grade batches during scale-up manufacturing will mitigate many of our common bench-side variables and limit the amount of UV-induced particle waste. Indeed a limitation of this approach is the high probability that a fraction of particles are rendered inert during the process. Alternatively, new methods to produce high titers of VSVd51ΔG or similar highly attenuated strains or “replicons” should be undertaken. On a per-particle basis, semi-replicating viruses (with full-genome expression) may have enhanced direct cytotoxicity, although this can likely be compensated by NRRP dose escalation. However as discussed earlier, published data is clear that increased viral mRNA translation unnecessarily diverts adaptive immune responses away from tumor antigen, impairing overall therapy. Nevertheless, vesiculovirus-resembling particles manufactured at high concentrations with preserved cell-entry functionality but minimal/defective translation capacity might represent a considerable technological advancement. These manufacturing and immunobiological issues need further investigation.

In summary, when applied to sensitive and highly accessible cancers such as AML or ALL, NRRPs possess an enhanced therapeutic index in pre-clinical investigations. This thesis provides new evidence that highly attenuated vesiculovirus-derived therapy can be safely administered at intensified doses in a fully controllable, titratable and predictable fashion. This work highlights the importance of contextualizing the development of biotherapeutics in general, particularly those with built-in versatility such as the vesiculovirus platform. Rational modifications to their basic properties and application should be attempted to address the biophysical parameters of the disease in question.



**Figure 4.1 Evolving treatment approach for the acute leukemias.** Immediate leukemic cytorreduction will be increasingly achieved through the use of novel combinatorial small molecule-based regimens with enhanced therapeutic index. Drugs are selected based on the molecular profile of the leukemia - such as the highly potent tri-fluorinated agent on the left, which targets mutated IDH protein present in 20% of AML patients. Other compounds will be employed more broadly - such as the molecule on the right, which targets inhibitors of apoptosis (IAPs). Multimodal biologics or immunostimulants such as NRRPS, attenuated OVs, cytokines or synthetics will be applied as potentiating agents. Prolonged administration may be required to achieve longstanding disease control or cure. It is uncertain whether adding an anti-leukemia vaccine will be needed, particularly if novel multi-drug regimens induce ICD. However in the near-term, therapies such as iLOV, DC-based or vector-antigen vaccines could potentially be integrated into our current standard regimens. Adoptive T-cell therapy is unquestionably the most effective immunotherapy currently advancing toward the clinic. These leukemia-directed cellular therapies are more potent than standard HSCT, do not require HDT and eliminate the toxicity of GvHD. Various forms are in development using both autologous and allogeneic T-cell procurement. As manufacturing efficiencies improve, retargeted T-cells expressing mHA/LAA-specific TCRs or CARTs will emerge as standard immunotherapeutic approaches.

## References

1. Swerdlow, S.H. et al (2008) WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues, 4th ed. 149–178.
2. Greaves M (2009) Darwin and evolutionary tales in leukemia. The Ham-Wasserman Lecture. Hematology Am Soc Hematol Educ Program 3–12. doi: 10.1182/asheducation-2009.1.3
3. Jackson AL, Loeb LA (2001) The contribution of endogenous sources of DNA damage to the multiple mutations in cancer. 477:7–21.
4. Caldecott KW (2008) Single-strand break repair and genetic disease. doi: 10.1038/nrg2380
5. Robertson a B, Klungland a, Rognes T, Leiros I (2009) DNA repair in mammalian cells: Base excision repair: the long and short of it. Cell Mol Life Sci 66:981–93. doi: 10.1007/s00018-009-8736-z
6. Goodarzi A a, Jeggo P a (2013) The repair and signaling responses to DNA double-strand breaks., 1st ed. Adv Genet 82:1–45. doi: 10.1016/B978-0-12-407676-1.00001-9
7. Stieglitz E, Loh ML (2013) Genetic predispositions to childhood leukemia. 270–290. doi: 10.1177/2040620713498161
8. Robison L (2011) Late Effects of Acute Lymphoblastic Leukemia Therapy in Patients Diagnosed at 0-20 Years of Age. ASH Educ. Progr. B.
9. Kolitz JE (2006) Current therapeutic strategies for acute myeloid leukaemia. Br J Haematol 134:555–72. doi: 10.1111/j.1365-2141.2006.06219.x
10. Bhatia S (2013) Therapy-related myelodysplasia and acute myeloid leukemia. Semin Oncol 40:666–75. doi: 10.1053/j.seminoncol.2013.09.013
11. Pedersen-Bjergaard J, Andersen MK, Andersen MT, Christiansen DH (2008) Genetics of therapy-related myelodysplasia and acute myeloid leukemia. Leukemia 22:240–8. doi: 10.1038/sj.leu.2405078
12. Oronsky BT, Reid T, Knox SJ, Scicinski JJ (2012) The Scarlet Letter of Alkylation: A Mini Review of Selective Alkylating Agents. Transl Oncol 5:226–229. doi: 10.1593/tlo.12187

13. Natelson EA (2007) Benzene-induced acute myeloid leukemia: A clinician's perspective. 826–830. doi: 10.1002/ajh
14. The L, Vi J, James K, et al. (2007) Cigarette smoking, cytogenetic abnormalities, and acute myelogenous leukemia. 1137–1140. doi: 10.1038/sj.leu.2404698
15. Kinoshita K, Honjo T (2001) Linking class-switch recombination with somatic hypermutation. *Nat Rev Mol Cell Biol* 2:493–503. doi: 10.1038/35080033
16. Ford AM, Palmi C, Bueno C, et al. (2009) The TEL-AML1 leukemia fusion gene dysregulates the TGF- $\beta$  pathway in early B lineage progenitor cells. doi: 10.1172/JCI36428DS1
17. Siegel PM, Massagué J (2003) Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer. *Nat Rev Cancer* 3:807–21. doi: 10.1038/nrc1208
18. Hanahan D, Weinberg RA (2011) Review Hallmarks of Cancer: The Next Generation. *Cell* 144:646–674. doi: 10.1016/j.cell.2011.02.013
19. Welch JS, Ley TJ, Link DC, et al. (2011) The Origin and Evolution of Mutations in Acute Myeloid Leukemia. doi: 10.1016/j.cell.2012.06.023
20. Dierov J, Sanchez P V, Burke BA, et al. (2009) ORIGINAL ARTICLE BCR / ABL induces chromosomal instability after genotoxic stress and alters the cell death threshold. 279–286. doi: 10.1038/leu.2008.308
21. Nowicki MO, Falinski R, Koptyra M, et al. (2014) BCR / ABL oncogenic kinase promotes unfaithful repair of the reactive oxygen species – dependent DNA double-strand breaks BCR / ABL oncogenic kinase promotes unfaithful repair of the reactive oxygen species – dependent DNA double-strand breaks. 3746–3753. doi: 10.1182/blood-2004-05-1941
22. Ciriello G, Miller ML, Aksoy BA, et al. (2013) Emerging landscape of oncogenic signatures across human cancers. *Nat Genet* 45:1127–1133. doi: 10.1038/ng.2762
23. Rowe JM, Tallman MS (2010) How I treat acute myeloid leukemia. *Blood* 116:3147–56. doi: 10.1182/blood-2010-05-260117
24. Kon A, Shih L-Y, Minamino M, et al. (2013) Recurrent mutations in multiple components of the cohesin complex in myeloid neoplasms. *Nat Genet* 45:1232–7. doi: 10.1038/ng.2731
25. Vassiliou GS, Cooper JL, Rad R, et al. (2011) Mutant nucleophosmin and cooperating pathways drive leukemia initiation and progression in mice. doi: 10.1038/ng.796

26. Leukemia AM (2013) Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. *N Engl J Med* 368:2059–74. doi: 10.1056/NEJMoa1301689
27. Grunwald MR, Levis MJ (2013) FLT3 inhibitors for acute myeloid leukemia: a review of their efficacy and mechanisms of resistance. *Int J Hematol* 97:683–94. doi: 10.1007/s12185-013-1334-8
28. Bryant A, Palma CA, Jayaswal V, et al. (2012) miR-10a is aberrantly overexpressed in Nucleophosmin1 mutated acute myeloid leukaemia and its suppression induces cell death. *Mol Cancer* 11:8. doi: 10.1186/1476-4598-11-8
29. Greiner J, Ono Y, Hofmann S, et al. (2012) Mutated regions of nucleophosmin 1 elicit both CD4+ and CD8+ T-cell responses in patients with acute myeloid leukemia. *Blood* 120:1282–9. doi: 10.1182/blood-2011-11-394395
30. Wu H, Coskun V, Tao J, et al. (2010) Dnmt3a-dependent nonpromoter DNA methylation facilitates transcription of neurogenic genes. *Science* 329:444–8. doi: 10.1126/science.1190485
31. Sasaki M, Knobbe CB, Munger JC, et al. (2012) IDH1(R132H) mutation increases murine haematopoietic progenitors and alters epigenetics. *Nature* 488:656–9. doi: 10.1038/nature11323
32. Wakita S, Yamaguchi H, Omori I, et al. (2012) Mutations of the epigenetics-modifying gene ( DNMT3a , TET2 , IDH1 / 2 ) at diagnosis may induce FLT3 -ITD at relapse in de novo acute myeloid leukemia. 1–9. doi: 10.1038/leu.2012.317
33. Ward PS, Patel J, Wise DR, et al. (2010) The common feature of leukemia-associated IDH1 and IDH2 mutations is a neomorphic enzyme activity converting alpha-ketoglutarate to 2-hydroxyglutarate. *Cancer Cell* 17:225–34. doi: 10.1016/j.ccr.2010.01.020
34. Wang F, Travins J, DeLaBarre B, et al. (2013) Targeted inhibition of mutant IDH2 in leukemia cells induces cellular differentiation. *Science* 340:622–6. doi: 10.1126/science.1234769
35. Biswas D, Milne TA, Basrur V, et al. (2011) Function of leukemogenic mixed lineage leukemia 1 ( MLL ) fusion proteins through distinct partner protein complexes. doi: 10.1073/pnas.1111498108
36. Okuda H, Kawaguchi M, Kanai A, et al. (2014) MLL fusion proteins link transcriptional coactivators to previously active CpG-rich promoters. 1–16. doi: 10.1093/nar/gkt1394

37. Wang Z, Chen Z (2014) Acute promyelocytic leukemia : from highly fatal to highly curable ASH 50th anniversary review Acute promyelocytic leukemia : from highly fatal to highly curable. 2505–2515. doi: 10.1182/blood-2007-07-102798
38. Soupir CP, Vergilio J, Cin PD, et al. (2007) Philadelphia Chromosome – Positive Acute Myeloid Leukemia A Rare Aggressive Leukemia With Clinicopathologic Features Distinct From Chronic Myeloid Leukemia in Myeloid Blast Crisis. 642–650. doi: 10.1309/B4NVER1AJJ84CTUU
39. Hehlmann R (2012) How I treat How I treat CML blast crisis. *Blood* 120:737–747. doi: 10.1182/blood-2012-03-380147
40. Mullighan CG, Goorha S, Radtke I, et al. (2007) Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. *Nature* 446:758–64. doi: 10.1038/nature05690
41. Wiemels JL, Cazzaniga G, Daniotti M, et al. (1999) Prenatal origin of acute lymphoblastic leukaemia in children. *Lancet* 354:1499–503.
42. Mori H, Colman SM, Xiao Z, et al. (2002) Chromosome translocations and covert leukemic clones are generated during normal fetal development. *Proc Natl Acad Sci U S A* 99:8242–7. doi: 10.1073/pnas.112218799
43. Goldstone AH, Richards SM, Lazarus HM, et al. (2008) In adults with standard-risk acute lymphoblastic leukemia, the greatest benefit is achieved from a matched sibling allogeneic transplantation in first complete remission, and an autologous transplantation is less effective than conventional consolidation/. *Blood* 111:1827–33. doi: 10.1182/blood-2007-10-116582
44. Pui C, Robison LL, Look AT (2008) Acute lymphoblastic leukaemia. *Lancet* 371:1030–1043.
45. Schafer ES, Hunger SP (2011) Optimal therapy for acute lymphoblastic leukemia in adolescents and young adults. *Nat Rev Clin Oncol* 8:417–24. doi: 10.1038/nrclinonc.2011.77
46. Gökbuget N, Hoelzer D (2002) Recent approaches in acute lymphoblastic leukemia in adults. *Rev Clin Exp Hematol* 6:114–41; discussion 200–2.
47. Fielding AK (2011) Current therapeutic strategies in adult acute lymphoblastic leukemia. *Hematol Oncol Clin North Am* 25:1255–79, viii. doi: 10.1016/j.hoc.2011.09.008

48. Oran B, Weisdorf DJ (2012) Survival for older patients with acute myeloid leukemia: a population-based study. *Haematologica* 97:1916–24. doi: 10.3324/haematol.2012.066100
49. Oeffinger, KC et al. (2006) Chronic health conditions in adult survivors of childhood cancer. *N Engl J Med* 355:1572–82.
50. Ribera J-M (2011) Advances in acute lymphoblastic leukemia in adults. *Curr Opin Oncol* 23:692–9. doi: 10.1097/CCO.0b013e32834bb854
51. Koreth J, Schlenk R, Kopecky KJ, et al. (2009) Allogeneic stem cell transplantation for acute myeloid leukemia in first complete remission: systematic review and meta-analysis of prospective clinical trials. *JAMA* 301:2349–61. doi: 10.1001/jama.2009.813
52. Weissman IL, Shizuru J a (2008) The origins of the identification and isolation of hematopoietic stem cells, and their capability to induce donor-specific transplantation tolerance and treat autoimmune diseases. *Blood* 112:3543–53. doi: 10.1182/blood-2008-08-078220
53. Mathé G, Amiel JL, Schwarzenberg L, et al. (1965) Adoptive Immunotherapy of Acute Leukemia : Experimental and Clinical Results Adoptive Immunotherapy of Acute Leukemia : Experimental eradicate. 1525–1531.
54. Socié G, Blazar BR (2009) Acute graft-versus-host disease: from the bench to the bedside. *Blood* 114:4327–36. doi: 10.1182/blood-2009-06-204669
55. Ukena SN, Grosse J, Mischak-Weissinger E, et al. (2011) Acute but not chronic graft-versus-host disease is associated with a reduction of circulating CD4(+)CD25 (high)CD127 (low/-) regulatory T cells. *Ann Hematol* 90:213–8. doi: 10.1007/s00277-010-1068-0
56. Novitzky N, Thomas V, du Toit C, McDonald A (2011) Is there a role for autologous stem cell transplantation for patients with acute myelogenous leukemia? A retrospective analysis. *Biol Blood Marrow Transplant* 17:875–84. doi: 10.1016/j.bbmt.2010.09.016
57. Fielding AK, Richards SM, Chopra R, et al. (2007) Outcome of 609 adults after relapse of acute lymphoblastic leukemia (ALL); an MRC UKALL12/ECOG 2993 study. *Blood* 109:944–50. doi: 10.1182/blood-2006-05-018192
58. Patel JP, Levine RL (2012) How do novel molecular genetic markers influence treatment decisions in acute myeloid leukemia? *Hematology Am Soc Hematol Educ Program* 2012:28–34. doi: 10.1182/asheducation-2012.1.28

59. Seibel NL (2008) Acute lymphoblastic leukemia: an historical perspective. *Hematology Am Soc Hematol Educ Program* 365. doi: 10.1182/asheducation-2008.1.365
60. Kolb HJ (2008) Graft-versus-leukemia effects of transplantation and donor lymphocytes. *Blood* 112:4371. doi: 10.1182/blood-2008-03-077974
61. Reddy P, Maeda Y, Liu C, et al. (2005) A crucial role for antigen-presenting cells and alloantigen expression in graft-versus-leukemia responses. *Nat Med* 11:1244–9. doi: 10.1038/nm1309
62. Vincent K, Roy D, Perreault C (2011) Next-generation leukemia immunotherapy. *Blood* 118:2951–2959. doi: 10.1182/blood-2011-04-350868.
63. Jansson J, Hsu Y-C, Kuzin II, et al. (2011) Acute lymphoblastic leukemia cells that survive combination chemotherapy in vivo remain sensitive to allogeneic immune effects. *Leuk Res* 35:800–7. doi: 10.1016/j.leukres.2010.10.018
64. Smits ELJ, Lee C, Hardwick N, et al. (2011) Clinical evaluation of cellular immunotherapy in acute myeloid leukaemia. *Cancer Immunol Immunother* 60:757–69. doi: 10.1007/s00262-011-1022-6
65. Kobayashi S, Kikuta A, Ito M, Sano H (2014) Loss of Mismatched HLA in Myeloid / NK Cell Precursor Acute Leukemia Relapse After T Cell-Replete Haploidentical Hematopoietic Stem Cell Transplantation. *Pediatr Blood Cancer* 2–4. doi: 10.1002/pbc
66. Vago L, Perna SK, Zanussi M, et al. (2009) Loss of mismatched HLA in leukemia after stem-cell transplantation. *N Engl J Med* 361:478–88. doi: 10.1056/NEJMoa0811036
67. Villalobos IB, Takahashi Y, Akatsuka Y, et al. (2010) Brief report Relapse of leukemia with loss of mismatched HLA resulting from uniparental disomy after haploidentical hematopoietic stem cell transplantation. *Blood* 3158–3161. doi: 10.1182/blood-2009-11-254284.The
68. Vincent K, Hardy M-P, Trofimov A, et al. (2014) Rejection of leukemic cells requires antigen-specific t cells with high functional avidity. *Biol Blood Marrow Transplant* 20:37–45. doi: 10.1016/j.bbmt.2013.10.020
69. Welniak L a, Blazar BR, Murphy WJ (2007) Immunobiology of allogeneic hematopoietic stem cell transplantation. *Annu Rev Immunol* 25:139–70. doi: 10.1146/annurev.immunol.25.022106.141606

70. Rousseau RF, Biagi E, Dutour A, et al. (2006) Immunotherapy of high-risk acute leukemia with a recipient (autologous) vaccine expressing transgenic human CD40L and IL-2 after chemotherapy and allogeneic stem cell transplantation. *Blood* 107:1332–41. doi: 10.1182/blood-2005-03-1259
71. Lord SJ, Rajotte R V, Korbitt GS, Bleackley RC (2003) Granzyme B: a natural born killer. *Immunol Rev* 193:31–8.
72. Van Loenen MM, de Boer R, Hagedoorn RS, et al. (2011) Optimization of the HA-1-specific T-cell receptor for gene therapy of hematologic malignancies. *Haematologica* 96:477–81. doi: 10.3324/haematol.2010.025916
73. Marijt W a E, Heemskerk MHM, Kloosterboer FM, et al. (2003) Hematopoiesis-restricted minor histocompatibility antigens HA-1- or HA-2-specific T cells can induce complete remissions of relapsed leukemia. *Proc Natl Acad Sci U S A* 100:2742–7. doi: 10.1073/pnas.0530192100
74. Rezvani K, Yong ASM, Savani BN, et al. (2007) Graft-versus-leukemia effects associated with detectable Wilms tumor-1 – specific T lymphocytes after allogeneic stem-cell transplantation for acute lymphoblastic leukemia. *Blood* 110:1924–1932. doi: 10.1182/blood-2007-03-076844.The
75. Vesely MD, Kershaw MH, Schreiber RD, Smyth MJ (2011) Natural innate and adaptive immunity to cancer. *Annu Rev Immunol* 29:235–71. doi: 10.1146/annurev-immunol-031210-101324
76. DuPage M, Mazumdar C, Schmidt LM, et al. (2012) Expression of tumour-specific antigens underlies cancer immunoediting. *Nature* 482:405–9. doi: 10.1038/nature10803
77. Koebel CM, Vermi W, Swann JB, et al. (2007) Adaptive immunity maintains occult cancer in an equilibrium state. *Nature* 450:903–7. doi: 10.1038/nature06309
78. Luijn MM Van, Chamuleau MED, Ossenkoppele GJ (2012) Tumor Immune Escape in AML Class II-invariant peptide chain expression. *Oncoimmunology* 1:211–213.
79. Barrett a J, Le Blanc K (2010) Immunotherapy prospects for acute myeloid leukaemia. *Clin Exp Immunol* 161:223–32. doi: 10.1111/j.1365-2249.2010.04197.x
80. Benjamin JE, Gill S, Negrin RS (2010) Biology and clinical effects of natural killer cells in allogeneic transplantation. *Curr Opin Oncol* 22:130–137. doi: 10.1097/CCO.0b013e328335a559.Biology
81. Portell C a, Advani AS (2014) Novel targeted therapies in acute lymphoblastic leukemia. *Leuk Lymphoma* 55:737–48. doi: 10.3109/10428194.2013.823493

82. Zheng H, Matte-martone C, Li H, et al. (2008) Effector memory CD4<sup>+</sup> T cells mediate graft-versus-leukemia without inducing graft-versus-host disease. *Blood* 111:2476–2484. doi: 10.1182/blood-2007-08-109678.
83. Warren EH, Fujii N, Akatsuka Y, et al. (2010) Therapy of relapsed leukemia after allogeneic hematopoietic cell transplantation with T cells specific for minor histocompatibility antigens. *Blood* 115:3869–3878. doi: 10.1182/blood-2009-10-248997.
84. Heemskerk MHM, Hoogeboom M, Hagedoorn R, et al. (2004) Reprogramming of virus-specific T cells into leukemia-reactive T cells using T cell receptor gene transfer. *J Exp Med* 199:885–94. doi: 10.1084/jem.20031110
85. Ochi T, Fujiwara H, Okamoto S, et al. (2011) Novel adoptive T-cell immunotherapy using a WT1-specific TCR vector encoding silencers for endogenous TCRs shows marked antileukemia reactivity and safety. *Blood* 118:1495–1503. doi: 10.1182/blood-2011-02-337089.
86. Ngo MC, Rooney CM, Howard JM, Heslop HE (2011) Ex vivo gene transfer for improved adoptive immunotherapy of cancer. *Hum Mol Genet* 20:R93–9. doi: 10.1093/hmg/ddr102
87. Cartellieri M, Bachmann M, Feldmann A, et al. (2010) Chimeric antigen receptor-engineered T cells for immunotherapy of cancer. *J Biomed Biotechnol* 2010:956304. doi: 10.1155/2010/956304
88. Imai C, Mihara K, Andreansky M, et al. (2004) Chimeric receptors with 4-1BB signaling capacity provoke potent cytotoxicity against acute lymphoblastic leukemia. *Leuk Off J Leuk Soc Am Leuk Res Fund, UK* 18:676–84. doi: 10.1038/sj.leu.2403302
89. Porter DL, Levine BL, Kalos M, et al. (2011) Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. *N Engl J Med* 365:725–33. doi: 10.1056/NEJMoa1103849
90. Milone MC, Fish JD, Carpenito C, et al. (2009) Chimeric receptors containing CD137 signal transduction domains mediate enhanced survival of T cells and increased antileukemic efficacy in vivo. *Mol Ther* 17:1453–64. doi: 10.1038/mt.2009.83
91. Kalos M, Levine BL, Porter DL, et al. (2011) T Cells with Chimeric Antigen Receptors Have Potent Antitumor Effects and Can Establish Memory in Patients with Advanced Leukemia. *Sci Transl Med* 3:95ra73–95ra73. doi: 10.1126/scitranslmed.3002842

92. Sadelain M, Brentjens R, Rivière I (2009) The promise and potential pitfalls of chimeric antigen receptors. *Curr Opin Immunol* 21:215–23. doi: 10.1016/j.coi.2009.02.009
93. Ngo MC, Rooney CM, Howard JM, Heslop HE (2011) Ex vivo gene transfer for improved adoptive immunotherapy of cancer. *Hum Mol Genet* 20:R93–9. doi: 10.1093/hmg/ddr102
94. Chiang CL-L, Kandalaft LE, Coukos G (2011) Adjuvants for enhancing the immunogenicity of whole tumor cell vaccines. *Int Rev Immunol* 30:150–82. doi: 10.3109/08830185.2011.572210
95. Dubensky TW, Reed SG (2010) Adjuvants for cancer vaccines. *Semin Immunol* 22:155–61. doi: 10.1016/j.smim.2010.04.007
96. Krishnan L, Sprott GD (2008) Archaeosome adjuvants : Immunological capabilities and mechanism ( s ) of action. doi: 10.1016/j.vaccine.2008.02.026
97. Pufnock JS, Cigal M, Rolczynski LS, et al. (2011) Priming CD8+ T cells with dendritic cells matured using TLR4 and TLR7/8 ligands together enhances generation of CD8+ T cells retaining CD28. *Blood* 117:6542–51. doi: 10.1182/blood-2010-11-317966
98. Vujanovic L, Szymkowski DE, Alber S, et al. (2010) Virally infected and matured human dendritic cells activate natural killer cells via cooperative activity of plasma membrane-bound TNF and IL-15. *Blood* 116:575–83. doi: 10.1182/blood-2009-08-240325
99. Wang J, Zhang W, He A, et al. (2011) Screening of novel immunostimulatory CpG ODNs and their anti-leukemic effects as immunoadjuvants of tumor vaccines in murine acute lymphoblastic leukemia. *Oncol Rep* 25:519–29. doi: 10.3892/or.2010.1093
100. Broz P, Monack DM (2013) Newly described pattern recognition receptors team up against intracellular pathogens. *Nat Rev Immunol* 13:551–65. doi: 10.1038/nri3479
101. Melchjorsen J (2013) Learning from the messengers: innate sensing of viruses and cytokine regulation of immunity - clues for treatments and vaccines. *Viruses* 5:470–527. doi: 10.3390/v5020470
102. Takeda K, Akira S (2005) Toll-like receptors in innate immunity. *Int Immunol* 17:1–14. doi: 10.1093/intimm/dxh186

103. Basith S, Manavalan B, Yoo TH, et al. (2012) Roles of toll-like receptors in cancer: a double-edged sword for defense and offense. *Arch Pharm Res* 35:1297–316. doi: 10.1007/s12272-012-0802-7
104. Bowie AG, Unterholzner L (2008) Viral evasion and subversion of pattern-recognition receptor signalling. *Nat Rev Immunol* 8:911–22. doi: 10.1038/nri2436
105. Lee MS, Kim Y (2007) Signaling Pathways Downstream of Receptors and Their Cross Talk. doi: 10.1146/annurev.biochem.76.060605.122847
106. Lopez J, Meier P (2010) To fight or die — inhibitor of apoptosis proteins at the crossroad of innate immunity and death. *Curr Opin Cell Biol* 22:872–881. doi: 10.1016/j.ceb.2010.08.025
107. Lindner DJ, Liu Y, Williams BR, et al. (2003) Apoptosis and interferons : Role of interferon-stimulated genes as mediators of apoptosis. 8:237–249.
108. Lei Y, Moore CB, Liesman RM, et al. (2009) MAVS-mediated apoptosis and its inhibition by viral proteins. *PLoS One* 4:e5466. doi: 10.1371/journal.pone.0005466
109. Hossain DMS, Dos Santos C, Zhang Q, et al. (2014) Leukemia cell-targeted STAT3 silencing and TLR9 triggering generate systemic antitumor immunity. *Blood* 123:15–25. doi: 10.1182/blood-2013-07-517987
110. Zhang Q, Hossain DMS, Nechaev S, et al. (2013) TLR9-mediated siRNA delivery for targeting of normal and malignant human hematopoietic cells in vivo. *Blood* 121:1304–15. doi: 10.1182/blood-2012-07-442590
111. Liang X, Moseman EA, Farrar M a, et al. (2010) Toll-like receptor 9 signaling by CpG-B oligodeoxynucleotides induces an apoptotic pathway in human chronic lymphocytic leukemia B cells. *Blood* 115:5041–52. doi: 10.1182/blood-2009-03-213363
112. Rezvani K, Barrett J (2014) STAT3: the “Achilles” heel for AML? *Blood* 123:1–2. doi: 10.1182/blood-2013-11-537092
113. Kim IY, Yan X, Tohme S, et al. (2012) CpG ODN, Toll like receptor (TLR)-9 agonist, inhibits metastatic colon adenocarcinoma in a murine hepatic tumor model. *J Surg Res* 174:284–90. doi: 10.1016/j.jss.2010.12.021
114. Umemura N, Zhu J, Mburu YK, et al. (2012) Defective NF-κB signaling in metastatic head and neck cancer cells leads to enhanced apoptosis by double-stranded RNA. *Cancer Res* 72:45–55. doi: 10.1158/0008-5472.CAN-11-1484

115. Sun R, Zhang Y, Lv Q, et al. (2011) Toll-like receptor 3 (TLR3) induces apoptosis via death receptors and mitochondria by up-regulating the transactivating p63 isoform alpha (TAP63alpha). *J Biol Chem* 286:15918–28. doi: 10.1074/jbc.M110.178798
116. Nepstad I, Hatfield KJ (2013) Pharmacologic targeting of the PI3K/mTOR pathway controls release of angioregulators from primary human acute myeloid leukemia cells and their neighboring stromal cells. 4:830–843.
117. Rock Y, Emma C, Abdel-wahab O, et al. (2012) Epigenetic alterations in hematopoietic malignancies. doi: 10.1007/s12185-012-1181-z
118. Dinner SN, Giles FJ, Altman JK (2014) New strategies for relapsed acute myeloid leukemia: fertile ground for translational research. 79–86. doi: 10.1097/MOH.0000000000000018
119. Hoption Cann S a, van Netten JP, van Netten C (2003) Dr William Coley and tumour regression: a place in history or in the future. *Postgrad Med J* 79:672–80.
120. McCarthy EF (2006) The toxins of William B. Coley and the treatment of bone and soft-tissue sarcomas. *Iowa Orthop J* 26:154–8.
121. Ward PS, Thompson CB (2012) Metabolic reprogramming: a cancer hallmark even warburg did not anticipate. *Cancer Cell* 21:297–308. doi: 10.1016/j.ccr.2012.02.014
122. Vähä-Koskela MJ V, Heikkilä JE, Hinkkanen AE (2007) Oncolytic viruses in cancer therapy. *Cancer Lett* 254:178–216. doi: 10.1016/j.canlet.2007.02.002
123. Katze MG, He Y, Gale M (2002) Viruses and interferon: a fight for supremacy. *Nat Rev Immunol* 2:675–87. doi: 10.1038/nri888
124. Schoggins JW, Rice CM (2011) Interferon-stimulated genes and their antiviral effector functions. *Curr Opin Virol* 1:519–25. doi: 10.1016/j.coviro.2011.10.008
125. Parato K a, Senger D, Forsyth P a J, Bell JC (2005) Recent progress in the battle between oncolytic viruses and tumours. *Nat Rev Cancer* 5:965–76. doi: 10.1038/nrc1750
126. Hernández-Alcoceba R (2011) Recent advances in oncolytic virus design. *Clin Transl Oncol* 13:229–39. doi: 10.1007/s12094-011-0647-4
127. Melcher A, Parato K, Rooney CM, Bell JC (2011) Thunder and lightning: immunotherapy and oncolytic viruses collide. *Mol Ther* 19:1008–16. doi: 10.1038/mt.2011.65

128. Breitbach CJ, Paterson JM, Lemay CG, et al. (2007) Targeted inflammation during oncolytic virus therapy severely compromises tumor blood flow. *Mol Ther* 15:1686–93. doi: 10.1038/sj.mt.6300215
129. Kurooka M, Kaneda Y (2007) Inactivated Sendai virus particles eradicate tumors by inducing immune responses through blocking regulatory T cells. *Cancer Res* 67:227–36. doi: 10.1158/0008-5472.CAN-06-1615
130. Zajac P, Oertli D, Marti W, et al. (2003) Phase I/II clinical trial of a nonreplicative vaccinia virus expressing multiple HLA-A0201-restricted tumor-associated epitopes and costimulatory molecules in metastatic melanoma patients. *Hum Gene Ther* 14:1497–510. doi: 10.1089/104303403322495016
131. Zamarin D, Holmgaard RB, Subudhi SK, et al. (2014) Localized oncolytic virotherapy overcomes systemic tumor resistance to immune checkpoint blockade immunotherapy. *Sci Transl Med* 6:226ra32. doi: 10.1126/scitranslmed.3008095
132. Sary G, Bangert C, Tauber M, et al. (2007) Tumoricidal activity of TLR7/8-activated inflammatory dendritic cells. *J Exp Med* 204:1441–51. doi: 10.1084/jem.20070021
133. Galivo F, Diaz RM, Wongthida P, et al. (2010) Single-cycle viral gene expression, rather than progressive replication and oncolysis, is required for VSV therapy of B16 melanoma. *Gene Ther* 17:158–70. doi: 10.1038/gt.2009.161
134. Galivo F, Diaz RM, Thanarajasingam U, et al. (2010) Interference of CD40L-Mediated Tumor Immunotherapy By Oncolytic Vesicular Stomatitis Virus. *Hum Gene Ther* 450:439–450.
135. Jogler C, Hoffmann D, Theegarten D, et al. (2006) Replication Properties of Human Adenovirus In Vivo and in Cultures of Primary Cells from Different Animal Species. *J Virol* 80:3549–58. doi: 10.1128/JVI.80.7.3549
136. Prestwich RJ, Ilett EJ, Errington F, et al. (2009) Immune-mediated antitumor activity of reovirus is required for therapy and is independent of direct viral oncolysis and replication. *Clin Cancer Res* 15:4374–81. doi: 10.1158/1078-0432.CCR-09-0334
137. Bourquin C, Schmidt L, Hornung V, et al. (2007) Immunostimulatory RNA oligonucleotides trigger an antigen-specific cytotoxic T-cell and IgG2a response. *Blood* 109:2953–2960. doi: 10.1182/blood-2006-07-033258.The

138. Wirth T, Zender L, Schulte B, et al. (2003) A Telomerase-dependent Conditionally Replicating Adenovirus for Selective Treatment of Cancer A Telomerase-dependent Conditionally Replicating Adenovirus for Selective Treatment of Cancer 1. *Cancer Res* 63:3181–3188.
139. Adamina M, Rosenthal R, Weber WP, et al. (2010) Intranodal immunization with a vaccinia virus encoding multiple antigenic epitopes and costimulatory molecules in metastatic melanoma. *Mol Ther* 18:651–9. doi: 10.1038/mt.2009.275
140. Wakamiya N, Wang YL, Imai H, et al. (1986) Feasibility of UV-inactivated vaccinia virus in the modification of tumor cells for augmentation of their immunogenicity. *Cancer Immunol Immunother* 23:125–9.
141. Ito T, Wang D, Maru M (1990) Antitumor Efficacy of Vaccinia Virus-modified Tumor Cell Vaccine. *Cancer Res* 50:6915–6918.
142. Mellman I, Coukos G, Dranoff G (2011) Cancer immunotherapy comes of age. *Nature* 480:480–9. doi: 10.1038/nature10673
143. Schlosser E, Mueller M, Fischer S, et al. (2008) TLR ligands and antigen need to be coencapsulated into the same biodegradable microsphere for the generation of potent cytotoxic T lymphocyte responses. *Vaccine* 26:1626–37. doi: 10.1016/j.vaccine.2008.01.030
144. De Veer MJ, Holko M, Frevel M, et al. (2001) Functional classification of interferon-stimulated genes identified using microarrays. *J Leukoc Biol* 69:912–20.
145. Chang H, Paulson M, Holko M, et al. (2004) Induction of interferon-stimulated gene expression and antiviral responses require protein deacetylase activity.
146. Richards KH, Macdonald A (2011) Putting the brakes on the anti-viral response: negative regulators of type I interferon (IFN) production. *Microbes Infect* 13:291–302. doi: 10.1016/j.micinf.2010.12.007
147. Benencia F, Courrèges MC, Fraser NW, Coukos G (2008) Herpes virus oncolytic therapy reverses tumor immune dysfunction and facilitates tumor antigen presentation. *Cancer Biol Ther* 7:1194–205.
148. Ilett EJ, Prestwich RJD, Melcher a a (2010) The evolving role of dendritic cells in cancer therapy. *Expert Opin Biol Ther* 10:369–79. doi: 10.1517/14712590903559830
149. Palucka K, Banchereau J, Mellman I (2010) Designing vaccines based on biology of human dendritic cell subsets. *Immunity* 33:464–78. doi: 10.1016/j.immuni.2010.10.007

150. Lapteva N, Aldrich M, Rollins L, et al. (2009) Attraction and activation of dendritic cells at the site of tumor elicits potent antitumor immunity. *Mol Ther* 17:1626–36. doi: 10.1038/mt.2009.111
151. Tel J, Smits EL, Anguille S, et al. (2012) Human plasmacytoid dendritic cells are equipped with antigen-presenting and tumoricidal capacities. *Blood* 120:3936–44. doi: 10.1182/blood-2012-06-435941
152. Mittelbrunn M, Martínez G, Lo M, et al. (2009) Imaging of plasmacytoid dendritic cell interactions with T cells. *Blood* 113:75–84. doi: 10.1182/blood-2008-02-139865.The
153. Obeid M, Tesniere A, Ghiringhelli F, et al. (2007) Calreticulin exposure dictates the immunogenicity of cancer cell death. *Nat Med* 13:54–61. doi: 10.1038/nm1523
154. Schirmacher V (2005) Clinical trials of antitumor vaccination with an autologous tumor cell vaccine modified by virus infection: improvement of patient survival based on improved antitumor immune memory. *Cancer Immunol Immunother* 54:587–98. doi: 10.1007/s00262-004-0602-0
155. Donnelly OG, Errington-mais F, Steele L, et al. Title page Measles virus causes immunogenic cell death in human melanoma.
156. Hannani D, Sistigu A, Kepp O, et al. (2011) Prerequisites for the antitumor vaccine-like effect of chemotherapy and radiotherapy. *Cancer J* 17:351–8. doi: 10.1097/PPO.0b013e3182325d4d
157. Sims GP, Rowe DC, Rietdijk ST, et al. (2010) HMGB1 and RAGE in inflammation and cancer. *Annu Rev Immunol* 28:367–88. doi: 10.1146/annurev.immunol.021908.132603
158. Krysko D V, Garg AD, Kaczmarek A, et al. (2012) Immunogenic cell death and DAMPs in cancer therapy. *Nat Rev Cancer* 12:860–75. doi: 10.1038/nrc3380
159. Michaud M, Sukkurwala AQ, Martins I, et al. (2012) Subversion of the chemotherapy-induced anticancer immune response by the ecto-ATPase CD39. *Oncoimmunology* 1:393–395. doi: 10.4161/onci.19070
160. Stagg J, Smyth MJ (2010) Extracellular adenosine triphosphate and adenosine in cancer. *Oncogene* 29:5346–58. doi: 10.1038/onc.2010.292
161. Galluzzi L, Kepp O, Morselli E, et al. (2010) Viral strategies for the evasion of immunogenic cell death. *J Intern Med* 267:526–42. doi: 10.1111/j.1365-2796.2010.02223.x

162. Smyth MJ (2005) Type I interferon and cancer immunoediting. *6*:646–648.
163. Schoggins JW, Wilson SJ, Panis M, et al. (2011) A diverse range of gene products are effectors of the type I interferon antiviral response. *Nature* 472:481–5. doi: 10.1038/nature09907
164. Sen GC (2007) The Interferon-Stimulated Genes : Targets of Direct Signaling by Interferons , Double-Stranded RNA , and Viruses. *CTMI* 316:233–250.
165. Strowig T, Henao-Mejia J, Elinav E, Flavell R (2012) Inflammasomes in health and disease. *Nature* 481:278–86. doi: 10.1038/nature10759
166. Woller N, Knocke S, Mundt B, et al. (2011) Virus-induced tumor inflammation facilitates effective DC cancer immunotherapy in a Treg-dependent manner in mice. doi: 10.1172/JCI45585DS1
167. Wang S, El-Deiry WS (2003) TRAIL and apoptosis induction by TNF-family death receptors. *Oncogene* 22:8628–33. doi: 10.1038/sj.onc.1207232
168. Wongthida P, Diaz RM, Galivo F, et al. (2011) VSV oncolytic virotherapy in the B16 model depends upon intact MyD88 signaling. *Mol Ther* 19:150–8. doi: 10.1038/mt.2010.225
169. Wang BX, Rahbar R, Fish EN (2011) Interferon: current status and future prospects in cancer therapy. *J Interferon Cytokine Res* 31:545–52. doi: 10.1089/jir.2010.0158
170. Pardoll DM (2012) The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer* 12:252–64. doi: 10.1038/nrc3239
171. Intlekofer AM, Thompson CB (2013) At the bench: preclinical rationale for CTLA-4 and PD-1 blockade as cancer immunotherapy. *J Leukoc Biol* 94:25–39. doi: 10.1189/jlb.1212621
172. Bilate AM, Lafaille JJ (2012) Induced CD4<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells in immune tolerance. *Annu Rev Immunol* 30:733–58. doi: 10.1146/annurev-immunol-020711-075043
173. Willmon C, Diaz RM, Wongthida P, et al. (2011) Vesicular stomatitis virus-induced immune suppressor cells generate antagonism between intratumoral oncolytic virus and cyclophosphamide. *Mol Ther* 19:140–9. doi: 10.1038/mt.2010.224
174. Oliveira V, Agua-Doce A, Duarte J, et al. (2006) Regulatory T cell maintenance of dominant tolerance: induction of tissue self-defense? *Transpl Immunol* 17:7–10. doi: 10.1016/j.trim.2006.09.028

175. Jensen H, Andresen L, Nielsen J, et al. (2011) Vesicular stomatitis virus infection promotes immune evasion by preventing NKG2D-ligand surface expression. *PLoS One* 6:e23023. doi: 10.1371/journal.pone.0023023
176. Leveille S, Goulet M-L, Lichty BD, Hiscott J (2011) Vesicular stomatitis virus oncolytic treatment interferes with tumor-associated dendritic cell functions and abrogates tumor antigen presentation. *J Virol* 85:12160–9. doi: 10.1128/JVI.05703-11
177. Yoon HH, Orrock JM, Foster NR, et al. (2012) Prognostic impact of FoxP3+ regulatory T cells in relation to CD8+ T lymphocyte density in human colon carcinomas. *PLoS One* 7:e42274. doi: 10.1371/journal.pone.0042274
178. Tzankov A, Meier C, Hirschmann P, et al. (2008) Correlation of high numbers of intratumoral FOXP3+ regulatory T cells with improved survival in germinal center-like diffuse large B-cell lymphoma, follicular lymphoma and classical Hodgkin's lymphoma. *Haematologica* 93:193–200. doi: 10.3324/haematol.11702
179. Gillet J-P, Gottsman M (2010) Mechanisms of Multidrug Resistance in Cancer. *Methods Mol Biol* 596:47–76.
180. Vander MG (2014) Understanding the Warburg Effect: doi: 10.1126/science.1160809
181. Balachandran S, Barber GN (2004) Defective translational control facilitates vesicular stomatitis virus oncolysis. *Cancer Cell* 5:51–65.
182. Farassati F, Yang a D, Lee PW (2001) Oncogenes in Ras signalling pathway dictate host-cell permissiveness to herpes simplex virus 1. *Nat Cell Biol* 3:745–50. doi: 10.1038/35087061
183. Noser J a, Mael A a, Sakuma R, et al. (2007) The RAS/Raf1/MEK/ERK signaling pathway facilitates VSV-mediated oncolysis: implication for the defective interferon response in cancer cells. *Mol Ther* 15:1531–6. doi: 10.1038/sj.mt.6300193
184. Pavitt GD, Yang W, Hinnebusch A (1997) Homologous segments in three subunits of the guanine nucleotide exchange factor eIF2B mediate translational regulation by phosphorylation of eIF2 . Homologous Segments in Three Subunits of the Guanine Nucleotide Exchange Factor eIF2B Mediate Translational. *Mol Cell Biol* 17:1298–1313.
185. Muller FL, Colla S, Aquilanti E, et al. (2012) Passenger deletions generate therapeutic vulnerabilities in cancer. *Nature* 488:337–42. doi: 10.1038/nature11331

186. O'Sullivan T, Saddawi-Konefka R, Vermi W, et al. (2012) Cancer immunoediting by the innate immune system in the absence of adaptive immunity. *J Exp Med* 209:1869–82. doi: 10.1084/jem.20112738
187. Meylan E, Tschopp J, Karin M (2006) Intracellular pattern recognition receptors in the host response. *Nature* 442:39–44. doi: 10.1038/nature04946
188. Nwajei F, Konopleva M (2013) The bone marrow microenvironment as niche retreats for hematopoietic and leukemic stem cells. *Adv Hematol* 2013:953982. doi: 10.1155/2013/953982
189. Liu Y-P, Suksanpaisan L, Steele MB, et al. (2013) Induction of antiviral genes by the tumor microenvironment confers resistance to virotherapy. *Sci Rep* 3:2375. doi: 10.1038/srep02375
190. Smith E, Breznik J, Lichty BD (2011) Strategies to enhance viral penetration of solid tumors. *Hum Gene Ther* 22:1053–60. doi: 10.1089/hum.2010.227
191. Brown CW, Stephenson KB, Hanson S, et al. (2009) The p14 FAST protein of reptilian reovirus increases vesicular stomatitis virus neuropathogenesis. *J Virol* 83:552–61. doi: 10.1128/JVI.01921-08
192. Bell JC, Garson K a, Lichty BD, Stojdl DF (2002) Oncolytic viruses: programmable tumour hunters. *Curr Gene Ther* 2:243–54.
193. Ko D, Hawkins L, Yu D-C (2005) Development of transcriptionally regulated oncolytic adenoviruses. *Oncogene* 24:7763–74. doi: 10.1038/sj.onc.1209048
194. Lopez MV, Viale DL, Cafferata EG a, et al. (2009) Tumor associated stromal cells play a critical role on the outcome of the oncolytic efficacy of conditionally replicative adenoviruses. *PLoS One* 4:e5119. doi: 10.1371/journal.pone.0005119
195. Rojas JJ, Guedan S, Searle PF, et al. (2010) Minimal RB-responsive E1A promoter modification to attain potency, selectivity, and transgene-arming capacity in oncolytic adenoviruses. *Mol Ther* 18:1960–71. doi: 10.1038/mt.2010.173
196. Reya T, Morrison SJ, Clarke MF, Weissman IL (2001) Stem cells, cancer, and cancer stem cells. *Nature* 414:105–11. doi: 10.1038/35102167
197. Mahller YY, Williams JP, Baird WH, et al. (2009) Neuroblastoma cell lines contain pluripotent tumor initiating cells that are susceptible to a targeted oncolytic virus. *PLoS One* 4:e4235. doi: 10.1371/journal.pone.0004235

198. Menotti L, Nicoletti G, Gatta V, et al. (2009) Inhibition of human tumor growth in mice by an oncolytic herpes simplex virus designed to target solely HER-2-positive cells. *Proc Natl Acad Sci U S A* 106:9039–44. doi: 10.1073/pnas.0812268106
199. Faria AMC, Levay A, Wang Y, et al. (2006) The nucleoporin Nup96 is required for proper expression of interferon-regulated proteins and functions. *Immunity* 24:295–304. doi: 10.1016/j.immuni.2006.01.014
200. Rajani KR, Pettit Kneller EL, McKenzie MO, et al. (2012) Complexes of vesicular stomatitis virus matrix protein with host Rae1 and Nup98 involved in inhibition of host transcription. *PLoS Pathog* 8:e1002929. doi: 10.1371/journal.ppat.1002929
201. Faria P a, Chakraborty P, Levay A, et al. (2005) VSV disrupts the Rae1/mrnp41 mRNA nuclear export pathway. *Mol Cell* 17:93–102. doi: 10.1016/j.molcel.2004.11.023
202. Lichty BD, Power AT, Stojdl DF, Bell JC (2004) Vesicular stomatitis virus: re-inventing the bullet. *Trends Mol Med* 10:210–6. doi: 10.1016/j.molmed.2004.03.003
203. Solon J, Gareil O, Bassereau P, Gaudin Y (2005) Membrane deformations induced by the matrix protein of vesicular stomatitis virus in a minimal system. *J Gen Virol* 86:3357–63. doi: 10.1099/vir.0.81129-0
204. Kopecky SA, Lyles DS (2003) The Cell-Rounding Activity of the Vesicular Stomatitis Virus Matrix Protein Is due to the Induction of Cell Death. *J Virol* 77:5524–5528. doi: 10.1128/JVI.77.9.5524
205. Kopecky SA, Willingham MC, Lyles DS (2001) Matrix Protein and Another Viral Component Contribute to Induction of Apoptosis in Cells Infected with Vesicular Stomatitis Virus Matrix Protein and Another Viral Component Contribute to Induction of Apoptosis in Cells Infected with Vesicular Stomatitis V. doi: 10.1128/JVI.75.24.12169
206. Lichty BD, McBride H, Hanson S, Bell JC (2006) Matrix protein of Vesicular stomatitis virus harbours a cryptic mitochondrial-targeting motif. *J Gen Virol* 87:3379–84. doi: 10.1099/vir.0.81762-0
207. Janelle V, Brassard F, Lapierre P, et al. (2011) Mutations in the glycoprotein of vesicular stomatitis virus affect cytopathogenicity: potential for oncolytic virotherapy. *J Virol* 85:6513–20. doi: 10.1128/JVI.02484-10
208. Brun J, McManus D, Lefebvre C, et al. (2010) Identification of genetically modified Maraba virus as an oncolytic rhabdovirus. *Mol Ther* 18:1440–9. doi: 10.1038/mt.2010.103

209. Schache P, Gürlevik E, Strüver N, et al. (2009) VSV virotherapy improves chemotherapy by triggering apoptosis due to proteasomal degradation of Mcl-1. *Gene Ther* 16:849–61. doi: 10.1038/gt.2009.39
210. Hastie E, Grdzelishvili VZ (2012) Vesicular stomatitis virus as a flexible platform for oncolytic virotherapy against cancer. *J Gen Virol* 93:2529–45. doi: 10.1099/vir.0.046672-0
211. Cao W, Manicassamy S, Tang H, et al. (2008) Toll-like receptor-mediated induction of type I interferon in plasmacytoid dendritic cells requires the rapamycin-sensitive PI(3)K-mTOR-p70S6K pathway. *Nat Immunol* 9:1157–64. doi: 10.1038/ni.1645
212. Alain T, Lun X, Martineau Y, et al. (2010) Vesicular stomatitis virus oncolysis is potentiated by impairing mTORC1-dependent type I IFN production. *Proc Natl Acad Sci U S A* 107:1576–81. doi: 10.1073/pnas.0912344107
213. Araki K, Ellebedy AH, Ahmed R (2011) TOR in the immune system. *Curr Opin Cell Biol* 1–9. doi: 10.1016/j.cecb.2011.08.006
214. Parato K a, Breitbach CJ, Le Boeuf F, et al. (2012) The oncolytic poxvirus JX-594 selectively replicates in and destroys cancer cells driven by genetic pathways commonly activated in cancers. *Mol Ther* 20:749–58. doi: 10.1038/mt.2011.276
215. Deng H, Tang N, Stief a E, et al. (2008) Oncolytic virotherapy for multiple myeloma using a tumour-specific double-deleted vaccinia virus. *Leuk Off J Leuk Soc Am Leuk Res Fund, UK* 22:2261–4. doi: 10.1038/leu.2008.120
216. Kelly EJ, Nace R, Barber GN, Russell SJ (2010) Attenuation of vesicular stomatitis virus encephalitis through microRNA targeting. *J Virol* 84:1550–62. doi: 10.1128/JVI.01788-09
217. Bridle BW, Stephenson KB, Boudreau JE, et al. (2010) Potentiating cancer immunotherapy using an oncolytic virus. *Mol Ther* 18:1430–9. doi: 10.1038/mt.2010.98
218. Eager RM, Nemunaitis J (2011) Clinical development directions in oncolytic viral therapy. *Cancer Gene Ther* 18:305–17. doi: 10.1038/cgt.2011.7
219. Alain T, Hirasawa K, Pon KJ, et al. (2002) Reovirus therapy of lymphoid malignancies. 100:4146–4153. doi: 10.1182/blood-2002-02-0503.Supported
220. Stief AE, McCart JA (2008) Oncolytic virotherapy for multiple myeloma. *Expert Opin Biol Ther* 8:463–73. doi: 10.1517/14712598.8.4.463

221. Le Boeuf F, Diallo J-S, McCart JA, et al. (2010) Synergistic interaction between oncolytic viruses augments tumor killing. *Mol Ther* 18:888–95. doi: 10.1038/mt.2010.44
222. Le Boeuf F, Bell JC (2010) United virus: the oncolytic tag-team against cancer! *Cytokine Growth Factor Rev* 21:205–11. doi: 10.1016/j.cytogfr.2010.02.008
223. Bridle BW, Boudreau JE, Lichty BD, et al. (2009) Vesicular stomatitis virus as a novel cancer vaccine vector to prime antitumor immunity amenable to rapid boosting with adenovirus. *Mol Ther* 17:1814–21. doi: 10.1038/mt.2009.154
224. Bell JC (2007) Oncolytic viruses: what’s next? *Curr Cancer Drug Targets* 7:127–31.
225. Pol JG, Zhang L, Bridle BW, et al. (2014) Maraba virus as a potent oncolytic vaccine vector. *Mol Ther* 22:420–9. doi: 10.1038/mt.2013.249
226. Boudreau JE, Bridle BW, Stephenson KB, et al. (2009) Recombinant vesicular stomatitis virus transduction of dendritic cells enhances their ability to prime innate and adaptive antitumor immunity. *Mol Ther* 17:1465–72. doi: 10.1038/mt.2009.95
227. Lemay CG, Rintoul JL, Kus A, et al. (2012) Harnessing oncolytic virus-mediated antitumor immunity in an infected cell vaccine. *Mol Ther* 20:1791–9. doi: 10.1038/mt.2012.128
228. Kottke T, Diaz RM, Kaluza K, et al. (2008) Use of biological therapy to enhance both virotherapy and adoptive T-cell therapy for cancer. *Mol Ther* 16:1910–8. doi: 10.1038/mt.2008.212
229. Budhu S, Loike JD, Pandolfi A, et al. (2010) CD8+ T cell concentration determines their efficiency in killing cognate antigen-expressing syngeneic mammalian cells in vitro and in mouse tissues. *J Exp Med* 207:223–35. doi: 10.1084/jem.20091279
230. Bierman H, Crile D, Dod K, et al. (1953) Remissions in leukemia of childhood following acute infectious disease: staphylococcus and streptococcus, varicella, and feline panleukopenia. *Cancer* 6:591–605.
231. Gross S (1971) MEASLES AND LEUKÆMIA. *Lancet* 297:397–398. doi: 10.1016/S0140-6736(71)92232-X
232. TAQI A (1981) REGRESSION OF HODGKIN’S DISEASE AFTER MEASLES. *Lancet* 317:1112. doi: 10.1016/S0140-6736(81)92286-8
233. Bluming A, Ziegler J (1971) REGRESSION OF BURKITT’S LYMPHOMA IN ASSOCIATION WITH MEASLES INFECTION. *Lancet* 298:105–106. doi: 10.1016/S0140-6736(71)92086-1

234. Hoster HA, Zanes RP, Haam E Von (1949) Studies in Hodgkin ' s Syndrome : IX . The Association of Viral Hepatitis and Hodgkin ' s Disease ( A Preliminary Report ). *Cancer Res* 9:473–480.
235. Taylor a W (1953) Effects of glandular fever infection in acute leukaemia. *Br Med J* 1:589–93.
236. Southam C, Moore A (1952) Clinical studies of viruses as antineoplastic agents with particular reference to Egypt 101 virus. *Cancer* 5:
237. Au GG, Lincz LF, Enno A, Shafren DR (2007) Oncolytic Coxsackievirus A21 as a novel therapy for multiple myeloma. *Br J Haematol* 137:133–41. doi: 10.1111/j.1365-2141.2007.06550.x
238. Césaire R, Oliére S, Sharif-Askari E, et al. (2006) Oncolytic activity of vesicular stomatitis virus in primary adult T-cell leukemia. *Oncogene* 25:349–58. doi: 10.1038/sj.onc.1209055
239. Parrula C, Fernandez S a, Zimmerman B, et al. (2011) Measles virotherapy in a mouse model of adult T-cell leukaemia/lymphoma. *J Gen Virol* 92:1458–66. doi: 10.1099/vir.0.028910-0
240. Peng K-W (2001) Systemic therapy of myeloma xenografts by an attenuated measles virus. *Blood* 98:2002–2007. doi: 10.1182/blood.V98.7.2002
241. Rahman MM, Madlambayan GJ, Cogle CR, McFadden G Oncolytic viral purging of leukemic hematopoietic stem and progenitor cells with Myxoma virus. *Cytokine Growth Factor Rev* 21:169–75. doi: 10.1016/j.cytogfr.2010.02.010
242. Stojdl DF, Lichty B, Knowles S, et al. (2000) Exploiting tumor-specific defects in the interferon pathway with a previously unknown oncolytic virus. *Nat Med* 6:821–5. doi: 10.1038/77558
243. Thirukkumaran CM, Luidar JM, Stewart D a, et al. (2003) Reovirus oncolysis as a novel purging strategy for autologous stem cell transplantation. *Blood* 102:377–87. doi: 10.1182/blood-2002-08-2508
244. Peng W, Russell SJ (2013) Curative one-shot systemic virotherapy in murine myeloma. *Leukemia* 26:1870–1878. doi: 10.1038/leu.2012.70.Curative
245. Lichty BD, Stojdl DF, Taylor RA, et al. (2004) Vesicular stomatitis virus: a potential therapeutic virus for the treatment of hematologic malignancy. *Hum Gene Ther* 15:821–31. doi: 10.1089/hum.2004.15.821

246. Dingli D, Peng K, Harvey ME, et al. (2004) Image-guided radiotherapy for multiple myeloma using a recombinant measles virus expressing the thyroidal sodium iodide symporter. *Blood* 103:1641–1646. doi: 10.1182/blood-2003-07-2233.Supported
247. Peng K, Donovan KA, Schneider U, et al. (2003) Oncolytic measles viruses displaying a single-chain antibody against CD38 , a myeloma cell marker. *Blood* 101:2557–2562. doi: 10.1182/blood-2002-07-2195.Supported
248. Senac JS, Doronin K, Russell SJ, et al. (2010) Infection and killing of multiple myeloma by adenoviruses. *Hum Gene Ther* 21:179–90. doi: 10.1089/hum.2009.082
249. Angelova AL, Aprahamian M, Balboni G, et al. (2009) Oncolytic rat parvovirus H-1PV, a candidate for the treatment of human lymphoma: In vitro and in vivo studies. *Mol Ther* 17:1164–72. doi: 10.1038/mt.2009.78
250. Stojdl DF, Lichty BD, tenOever BR, et al. (2003) VSV strains with defects in their ability to shutdown innate immunity are potent systemic anti-cancer agents. *Cancer Cell* 4:263–75.
251. Whelan SPJ, Wertz GW (2002) Transcription and replication initiate at separate sites on the vesicular stomatitis virus genome. *Proc Natl Acad Sci U S A* 99:9178–83. doi: 10.1073/pnas.152155599
252. Leyrat C, Schneider R, Ribeiro E a, et al. (2012) Ensemble structure of the modular and flexible full-length vesicular stomatitis virus phosphoprotein. *J Mol Biol* 423:182–97. doi: 10.1016/j.jmb.2012.07.003
253. Ge P, Tsao J, Green TJ, et al. (2014) Cryo-EM Model of the Bullet-Shaped Vesicular Stomatitis Virus. *Science* (80- ) 327:689–693. doi: 10.1126/science.1181766
254. Ribeiro E a, Favier A, Gerard FC a, et al. (2008) Solution structure of the C-terminal nucleoprotein-RNA binding domain of the vesicular stomatitis virus phosphoprotein. *J Mol Biol* 382:525–38. doi: 10.1016/j.jmb.2008.07.028
255. Thomas D, Newcomb WW, Brown JC, et al. (1985) Mass and molecular composition of vesicular stomatitis virus: a scanning transmission electron microscopy analysis. *J Virol* 54:598–607.
256. Grdzlishvili VZ, Smallwood S, Tower D, et al. (2005) A Single Amino Acid Change in the L-Polymerase Protein of Vesicular Stomatitis Virus Completely Abolishes Viral mRNA Cap Methylation A Single Amino Acid Change in the L-Polymerase Protein of Vesicular Stomatitis Virus Completely Abolishes Viral mRNA Cap M. doi: 10.1128/JVI.79.12.7327

257. Gaudier M, Gaudin Y, Knossow M (2002) Crystal structure of vesicular stomatitis virus matrix protein. *EMBO J* 21:2886–92. doi: 10.1093/emboj/cdf284
258. Albertini A a V, Baquero E, Ferlin A, Gaudin Y (2012) Molecular and cellular aspects of rhabdovirus entry. *Viruses* 4:117–39. doi: 10.3390/v4010117
259. Gaddy DF, Lyles DS (2007) Oncolytic vesicular stomatitis virus induces apoptosis via signaling through PKR, Fas, and Daxx. *J Virol* 81:2792–804. doi: 10.1128/JVI.01760-06
260. Tumilasci VF, Olié S, Nguyễn TL-A, et al. (2008) Targeting the apoptotic pathway with BCL-2 inhibitors sensitizes primary chronic lymphocytic leukemia cells to vesicular stomatitis virus-induced oncolysis. *J Virol* 82:8487–99. doi: 10.1128/JVI.00851-08
261. Gaddy DF, Lyles DS (2005) Vesicular Stomatitis Viruses Expressing Wild-Type or Mutant M Proteins Activate Apoptosis through Distinct Pathways Vesicular Stomatitis Viruses Expressing Wild-Type or Mutant M Proteins Activate Apoptosis through Distinct Pathways. doi: 10.1128/JVI.79.7.4170
262. Johannsdottir HK, Mancini R, Kartenbeck J, et al. (2009) Host cell factors and functions involved in vesicular stomatitis virus entry. *J Virol* 83:440–53. doi: 10.1128/JVI.01864-08
263. Finkelshtein D, Werman A, Novick D, et al. (2013) LDL receptor and its family members serve as the cellular receptors for vesicular stomatitis virus. *Proc Natl Acad Sci U S A* 110:7306–11. doi: 10.1073/pnas.1214441110
264. Fischer D, Tal N, Novick D, et al. (1993) An antiviral soluble form of the LDL receptor induced by interferon. *Science* (80- ) 262:250–253.
265. Cureton DK, Massol RH, Whelan SPJ, Kirchhausen T (2010) The length of vesicular stomatitis virus particles dictates a need for actin assembly during clathrin-dependent endocytosis. *PLoS Pathog* 6:e1001127. doi: 10.1371/journal.ppat.1001127
266. Lund JM, Alexopoulou L, Sato A, et al. (2004) Recognition of single-stranded RNA viruses by Toll-like receptor 7. *Proc Natl Acad Sci U S A* 101:5598–603. doi: 10.1073/pnas.0400937101
267. Rajagopal D, Paturel C, Morel Y, et al. (2010) Plasmacytoid dendritic cell – derived type I interferon is crucial for the adjuvant activity of Toll-like receptor 7 agonists. *Blood* 115:1949–1957. doi: 10.1182/blood-2009-08-238543.The

268. Chauhan VS, Furr SR, Sterka DG, et al. (2010) Vesicular stomatitis virus infects resident cells of the central nervous system and induces replication-dependent inflammatory responses. *Virology* 400:187–96. doi: 10.1016/j.virol.2010.01.025
269. Wollmann G, Rogulin V, Simon I, et al. (2010) Some attenuated variants of vesicular stomatitis virus show enhanced oncolytic activity against human glioblastoma cells relative to normal brain cells. *J Virol* 84:1563–73. doi: 10.1128/JVI.02040-09
270. Irie T, Carnero E, Okumura A, et al. (2007) Modifications of the PSAP region of the matrix protein lead to attenuation of vesicular stomatitis virus in vitro and in vivo. *J Gen Virol* 88:2559–67. doi: 10.1099/vir.0.83096-0
271. Ram R, Storb R, Sandmaier BM, et al. (2011) Non-myeloablative conditioning with allogeneic hematopoietic cell transplantation for the treatment of high-risk acute lymphoblastic leukemia. *Haematologica* 96:1113–20. doi: 10.3324/haematol.2011.040261
272. Verneris MR, Eapen M, Duerst R, et al. (2010) Reduced-intensity conditioning regimens for allogeneic transplantation in children with acute lymphoblastic leukemia. *Biol Blood Marrow Transplant* 16:1237–44. doi: 10.1016/j.bbmt.2010.03.009
273. Litzow M (2009) Evolving paradigms in the therapy of Philadelphia chromosome–negative acute lymphoblastic leukemia in adults. *ASH Educ. Progr. B*.
274. Boatsman EE, Fu CH, Song SX, Moore TB (2010) Graft-versus-leukemia effect on infant lymphoblastic leukemia relapsed after sibling hematopoietic stem cell transplantation. *J Pediatr Hematol Oncol* 32:e57–60. doi: 10.1097/MPH.0b013e3181c6beef
275. Köchling J, Prada J, Bahrami M, et al. (2008) Anti-tumor effect of DNA-based vaccination and dSLIM immunomodulatory molecules in mice with Ph<sup>+</sup> acute lymphoblastic leukaemia. *Vaccine* 26:4669–75. doi: 10.1016/j.vaccine.2008.06.094
276. Rezvanie K, de Lavallade H (2011) Vaccination Strategies in Lymphomas and Leukaemias. *Drugs* 71:1659–1674.
277. Ma W, Smith T, Bogin V, et al. (2011) Enhanced presentation of MHC class Ia, Ib and class II-restricted peptides encapsulated in biodegradable nanoparticles: a promising strategy for tumor immunotherapy. *J Transl Med* 9:34. doi: 10.1186/1479-5876-9-34

278. Brun J, McManus D, Lefebvre C, et al. (2010) Identification of genetically modified Maraba virus as an oncolytic rhabdovirus. *Mol Ther* 18:1440–9. doi: 10.1038/mt.2010.103
279. Lai F, Kazdhan N, Lichty BD (2008) Using G-deleted vesicular stomatitis virus to probe the innate anti-viral response. *J Virol Methods* 153:276–9. doi: 10.1016/j.jviromet.2008.07.001
280. Irvine KR, Chamberlain RS, Shulman EP, et al. (1997) Enhancing efficacy of recombinant anticancer vaccines with prime/boost regimens that use two different vectors. *J Natl Cancer Inst* 89:1595–601.
281. Lehner M, Bailo M, Stachel D, et al. (2007) Caspase-8 dependent apoptosis induction in malignant myeloid cells by TLR stimulation in the presence of IFN-alpha. *Leuk Res* 31:1729–35. doi: 10.1016/j.leukres.2007.05.001
282. Freeman GJ, Casasnovas JM, Umetsu DT, DeKruyff RH (2010) TIM genes: a family of cell surface phosphatidylserine receptors that regulate innate and adaptive immunity. *Immunol Rev* 235:172–89. doi: 10.1111/j.0105-2896.2010.00903.x
283. Iyer SS, Pulskens WP, Sadler JJ, et al. (2009) Necrotic cells trigger a sterile inflammatory response through the Nlrp3 inflammasome. *Proc Natl Acad Sci U S A* 106:20388–93. doi: 10.1073/pnas.0908698106
284. Castle JC, Kreiter S, Diekmann J, et al. (2012) Exploiting the mutanome for tumor vaccination. *Cancer Res* 72:1081–91. doi: 10.1158/0008-5472.CAN-11-3722
285. Pulido J, Kottke T, Thompson J, et al. (2012) Using virally expressed melanoma cDNA libraries to identify tumor-associated antigens that cure melanoma. *Nat Biotechnol* 30:337–343. doi: 10.1038/nbt.2157
286. Petersen J, Purcell AW, Rossjohn J (2009) Post-translationally modified T cell epitopes: immune recognition and immunotherapy. *J Mol Med (Berl)* 87:1045–51. doi: 10.1007/s00109-009-0526-4
287. Krueger KE, Srivastava S (2006) Posttranslational protein modifications: current implications for cancer detection, prevention, and therapeutics. *Mol Cell Proteomics* 5:1799–810. doi: 10.1074/mcp.R600009-MCP200
288. Sarkies P, Sale JE (2012) Cellular epigenetic stability and cancer. *Trends Genet* 28:118–27. doi: 10.1016/j.tig.2011.11.005
289. Willmon C, Saloura V, Fridlender C, et al. (2009) Expression of IFN- $\beta$  enhances both efficacy and safety of oncolytic vesicular stomatitis virus for therapy of mesothelioma. *Cancer Res* 69:7713–7720.

290. McCart J, Ward J, Lee J, et al. (2001) Systemic cancer therapy with a tumor-selective vaccinia virus mutant lacking thymidine kinase and vaccinia growth factor genes. *Cancer Res* 61:8751–8757.
291. Barber G (2005) VSV-tumor selective replication and protein translation. *Oncogene* 24:7710–7719.
292. Russell S, Peng K, Bell J (2012) Oncolytic virotherapy. *Nat Biotechnol* 30:658–670.
293. Naik S, Russell S (2009) Engineering oncolytic viruses to exploit tumor specific defects in innate immune signaling pathways. *Expert Opin Biol Ther* 9:1163–1176.
294. Bais S, Bartee E, Rahman MM, et al. (2012) Oncolytic virotherapy for hematological malignancies. *Adv Virol* 2012:186512. doi: 10.1155/2012/186512
295. Zeyauallah M, Patro M, Ahmad I, et al. (2012) Oncolytic viruses in the treatment of cancer: a review of current strategies. *Pathol Oncol Res* 18:771–781.
296. Wildner O (2003) Comparison of replication-selective, oncolytic viruses for the treatment of human cancers. *Curr Opin Mol Ther* 5:351–361.
297. Le Boeuf F, Batenchuk C, Vaha-Koskela M, et al. (2013) Model-based rational design of an oncolytic virus with improved therapeutic potential. *Nat Commun* 4:1974.
298. Ebert O, Harbaran S, Shinozaki K, Woo S (2005) Systemic therapy of experimental breast cancer metastases by mutant vesicular stomatitis virus in immune-competent mice. *Cancer Gene Ther* 12:350–358.
299. Dinarello C (2007) Historical insights into cytokines. *Eur J Immunol* 37:S34–S45.
300. Luther S, Cyster J (2001) Chemokines as regulators of T cell differentiation. *Nat Immunol* 2:102–107.
301. Metcalfe S (2011) LIF in the regulation of T-cell fate and as a potential therapeutic. *Genes Immunol* 12:157–168.
302. Conrad DP, Tsang J, Maclean M, et al. (2013) Leukemia Cell-Rhabdovirus Vaccine: Personalized Immunotherapy for Acute Lymphoblastic Leukemia. *Clin Cancer Res*. doi: 10.1158/1078-0432.CCR-12-3199
303. Zitvogel L, Kepp O, Sinovella L, et al. (2010) Immunogenic tumor cell death for optimal anticancer therapy: the calreticulin exposure pathway. *Clin Cancer Res* 16:3100–3104.

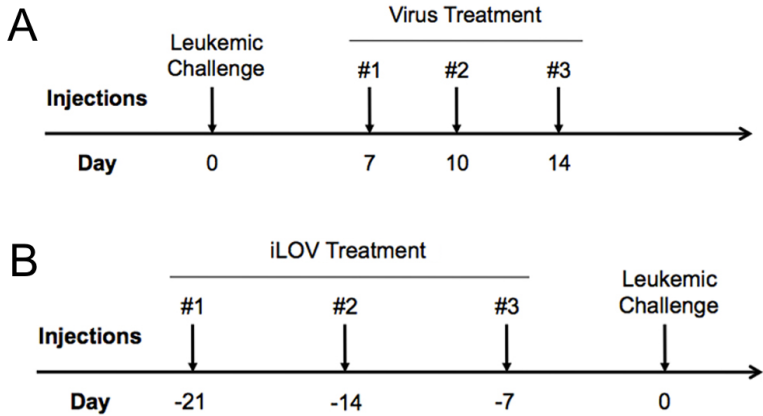
304. Finke J, Nagler L (2007) Viewpoint: what is the role of allogeneic haematopoietic cell transplantation in the era of reduced-intensity conditioning - is there still an upper age limit? A focus on myeloid neoplasia. *Leukemia* 21:1357–1362.
305. Daenen S, Van Der Holt B, Dekker A, et al. (2012) Intensive chemotherapy to improve outcome in patients with acute lymphoblastic leukemia over the age of 40: a phase II study for efficacy and feasibility by HOVON. *Leukemia* 26:1726–1729.
306. Giles F, O'Dwyer M, Swords R (2009) Class effects of tyrosine kinase inhibitors in the treatment of chronic myeloid leukemia. *Leukemia* 23:1698–1707.
307. Kepp O, Senovilla L, Galluzzi L, et al. (2009) Viral subversion of immunogenic cell death. *Cell cycle* 8:860–869.
308. Mahoney DJ, Lefebvre C, Allan K, et al. (2011) Virus-tumor interactome screen reveals ER stress response can reprogram resistant cancers for oncolytic virus-triggered caspase-2 cell death. *Cancer Cell* 20:443–56. doi: 10.1016/j.ccr.2011.09.005
309. Keilholz U, Martus P, Scheibenbogen C (2006) Immune monitoring of T-cell responses in cancer vaccine development. *Clin Cancer Res* 12:2346s–2352s. doi: 10.1158/1078-0432.CCR-05-2540
310. Beer TM, Bernstein GT, Corman JM, et al. (2011) Randomized trial of autologous cellular immunotherapy with sipuleucel-T in androgen-dependent prostate cancer. *Clin Cancer Res* 17:4558–67. doi: 10.1158/1078-0432.CCR-10-3223
311. Petricciani J, Egan W, Vicari G, et al. (2007) Potency assays for therapeutic live whole cell cancer vaccines. *Biologicals* 35:107–13. doi: 10.1016/j.biologicals.2006.05.001
312. Tong X, Wang L, Liu Y, et al. (2010) The complete preventive effect of homologous tumor vaccines--based on a 5-year experimental study in mice. *Biomed Pharmacother* 64:605–8. doi: 10.1016/j.biopha.2010.09.011
313. Comber JD, Philip R (2014) MHC class I antigen presentation and implications for developing a new generation of therapeutic vaccines. *Ther Adv vaccines* 2:77–89. doi: 10.1177/2051013614525375
314. Turnis ME, Rooney CM (2012) Enhancement of dendritic cells as vaccines for cancer. *Immunotherapy* 2:847–862. doi: 10.2217/imt.10.56.
315. Copier J, Ward S, Dalglish A (2007) Cell based cancer vaccines: regulatory and commercial development. *Vaccine* 25 Suppl 2:B35–46. doi: 10.1016/j.vaccine.2007.06.041

316. Håkansson L, Dalgleish a., Kallen KJ, Schuessler-Lenz M (2007) Vaccines: A rapidly evolving technology – Are the hurdles being addressed? *Eur J Cancer Suppl* 5:29–32. doi: 10.1016/j.ejcsup.2007.09.004
317. Le DT, Pardoll DM, Jaffee EM (2010) Cellular vaccine approaches. *Cancer J* 16:304–10. doi: 10.1097/PPO.0b013e3181eb33d7
318. Yang Y, Huang C, Huang X, Pardoll D (2004) Persistent Toll-like receptor signals are required for reversal of regulatory T cell-mediated CD8 tolerance. *Nat Immunol* 5:508–515.
319. Brencicova E, Diebold SS (2013) Nucleic acids and endosomal pattern recognition : how to tell friend from foe? *Front Cell Infect Microbiol* 3:1–14. doi: 10.3389/fcimb.2013.00037
320. Groettrup M, Kirk CJ, Basler M (2010) Proteasomes in immune cells: more than peptide producers? *Nat Rev Immunol* 10:73–8. doi: 10.1038/nri2687
321. Palmowski MJ, Gileadi U, Salio M, et al. (2006) Role of immunoproteasomes in cross-presentation. *J Immunol* 177:983–90.
322. Lee HK, Lund JM, Ramanathan B, et al. (2007) Autophagy-dependent viral recognition by plasmacytoid dendritic cells. *Science* 315:1398–401. doi: 10.1126/science.1136880
323. Chiramel AI, Brady NR, Bartenschlager R, et al. (2013) Divergent Roles of Autophagy in Virus Infection. 83–104. doi: 10.3390/cells2010083
324. Amaravadi RK (2011) Autophagy in Tumor Immunity. *Science (80- )* 334:1501–1502. doi: 10.1126/science.1216428
325. Sheng W-Y, Huang L (2011) Cancer immunotherapy and nanomedicine. *Pharm Res* 28:200–14. doi: 10.1007/s11095-010-0258-8
326. Guha M (2012) NEWS & ANALYSIS Anticancer TLR agonists on the ropes. 11:503–505.
327. Stripecke R, Levine a M, Pullarkat V, Cardoso a a (2002) Immunotherapy with acute leukemia cells modified into antigen-presenting cells: ex vivo culture and gene transfer methods. *Leuk Off J Leuk Soc Am Leuk Res Fund, UK* 16:1974–83. doi: 10.1038/sj.leu.2402701
328. Ingram W, Chan L, Guven H, et al. (2009) Human CD80/IL2 lentivirus-transduced acute myeloid leukaemia (AML) cells promote natural killer (NK) cell activation

- and cytolytic activity: implications for a phase I clinical study. *Br J Haematol* 145:749–60. doi: 10.1111/j.1365-2141.2009.07684.x
329. Smith BD, Kasamon YL, Kowalski J, et al. (2010) K562/GM-CSF immunotherapy reduces tumor burden in chronic myeloid leukemia patients with residual disease on imatinib mesylate. *Clin Cancer Res* 16:338–47. doi: 10.1158/1078-0432.CCR-09-2046
330. Fischer E, Kobold S, Kleber S, et al. (2010) Cryptic epitopes induce high-titer humoral immune response in patients with cancer. *J Immunol* 185:3095–102. doi: 10.4049/jimmunol.0902166
331. Murphy MA, O’Leary JJ, Cahill DJ (2012) Assessment of the humoral immune response to cancer. *J Proteomics* 1–7. doi: 10.1016/j.jprot.2012.01.021
332. Noguchi M, Mine T, Komatsu N, et al. (2010) Assessment of immunological biomarkers in patients with advanced cancer treated by personalized peptide vaccination. *Cancer Biol Ther* 10:1266–1279. doi: 10.4161/cbt.10.12.13448
333. Oka Y, Sugiyama H (2010) WT1 peptide vaccine, one of the most promising cancer vaccines: its present status and the future prospects. *Immunotherapy* 2:591–594.
334. Yee C, Thompson J, Roche P, et al (2000) Melanocyte destruction after antigen-specific immunotherapy for melanoma: direct evidence of t cell-mediated vitiligo. *J Exp Med* 192:1637–1644.
335. Lane C, Leitch J, Tan X, et al. (2004) Vaccination- induced autoimmune vitiligo is a consequence of secondary trauma to the skin. *Cancer Res* 64:1509–1514.
336. Bridle BW, Li J, Jiang S, et al. (2010) Immunotherapy can reject intracranial tumor cells without damaging the brain despite sharing the target antigen. *J Immunol* 184:4269–75. doi: 10.4049/jimmunol.0901447
337. Yarde DN, Naik S, Nace R a, et al. (2013) Meningeal myeloma deposits adversely impact the therapeutic index of an oncolytic VSV. *Cancer Gene Ther* 20:616–21. doi: 10.1038/cgt.2013.63

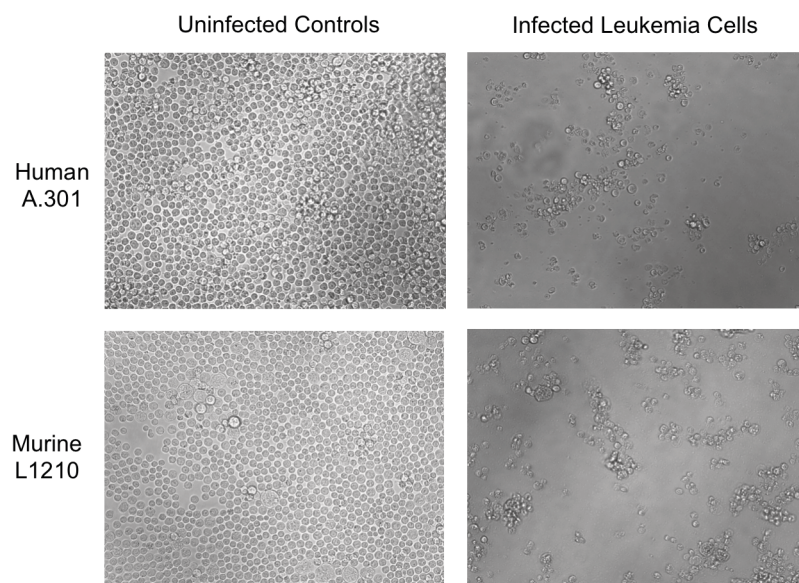
## Appendices

**Supplemental Figure 1**



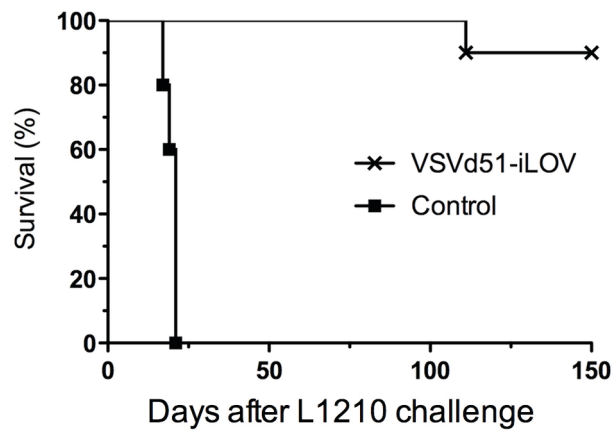
**Appendix Figure 1. Treatment schedules.** A, Schedule of administration using MG1 virotherapy for treatment of established L1210 leukemia in DBA/2 mice. B, Immunization schedule for iLOV and other vaccine preparations for use in DBA/2, C57BL/6, B6D2F1 and athymic nude mice. In certain experiments leukemic challenge preceded iLOV treatment, wherein the weekly x 3 immunization series was initiated on Day 1.

**Supplemental Figure 2**



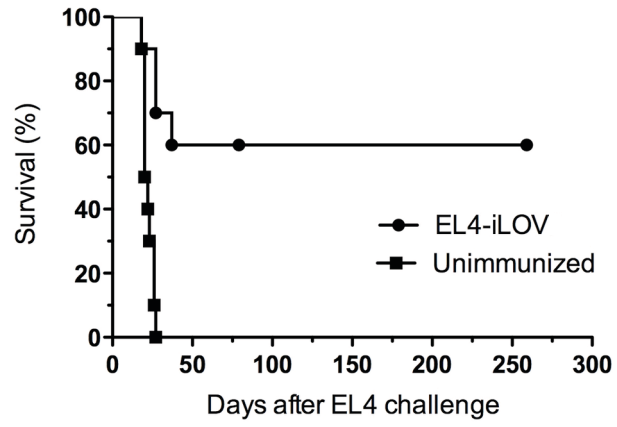
**Appendix Figure 2. Cytopathic effect induced by rhabdovirus infection.** Murine and human acute leukemia cells following infection with rhabdovirus, versus uninfected acute leukemia cells at 24 hours. Rhabdovirus-infected leukemic blasts typically display the morphological features of disrupted cell membranes, reduced cell diameter, apoptotic bodies, fragmented nuclear content and cellular debris. Shown are representative images (20X) of murine (L1210) and human (A.301) leukemia blasts.

Supplemental Figure 3



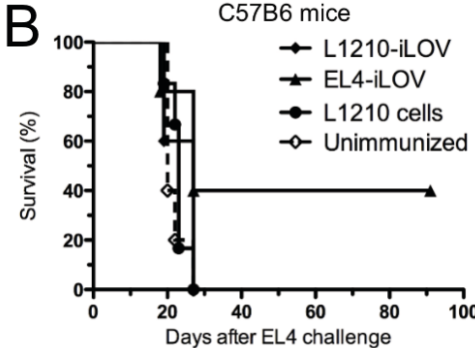
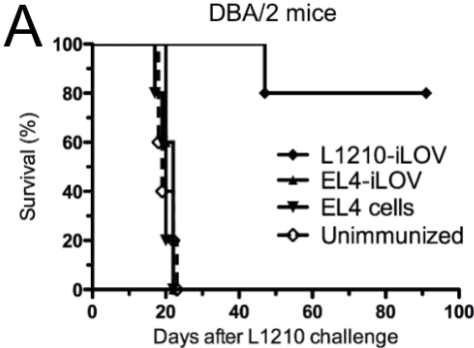
**Appendix Figure 3. iLOV efficacy using VSVd51 virus.** Survival following injection of  $1 \times 10^6$  L1210 leukemia cells as a challenge for the development of anti-leukemia immunity in naive or VSVd51-iLOV immunized DBA/2 mice ( $n = 10$  each group),  $P < 0.0001$ .

Supplemental Figure 4



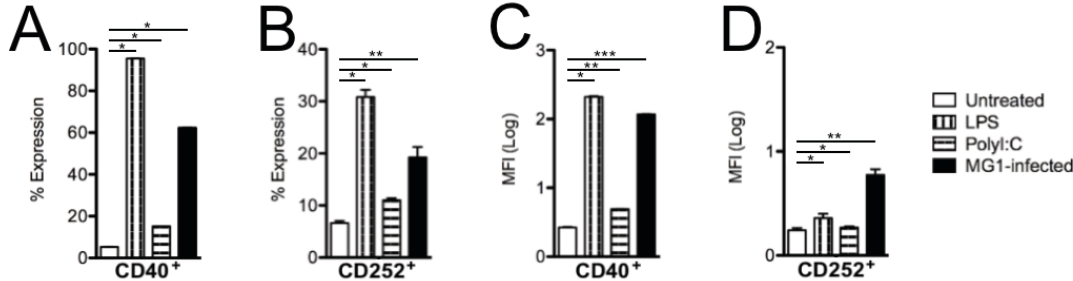
**Appendix Figure 4. Efficacy of EL4-based iLOV.** Survival following injection of  $1 \times 10^6$  EL4 leukemia cells as a challenge for the development of anti-leukemia immunity in unimmunized or EL4-based iLOV immunized C57BL/6 mice ( $n = 10$  each group),  $P = 0.0003$ .

**Supplemental Figure 5**



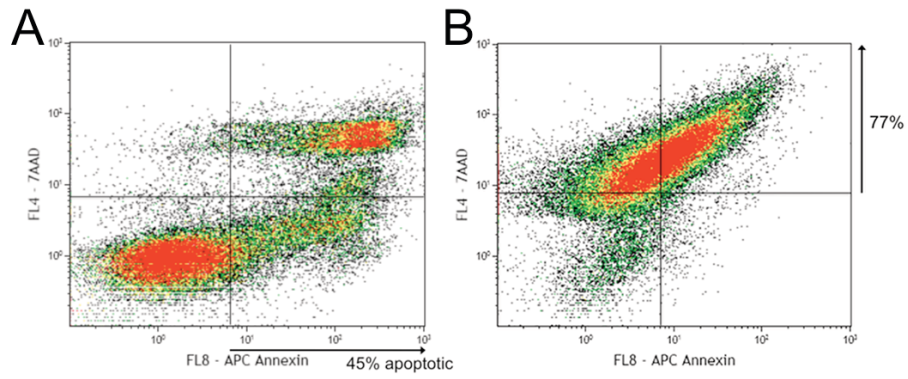
**Appendix Figure 5. iLOV induces leukemia-specific immunity.** Cohorts ( $n=5$  each group) of C57BL/6 and DBA/2 mice were immunized once weekly x 3, with either iLOV prepared with L1210 cells or EL4 cells. To control for immune recognition of leukemia cells based on the MHC disparity alone, cohorts of each breed were immunized with gamma-irradiated L1210 or EL4 cells alone. Each breed was challenged with syngeneic leukemia cells 1 week following the last immunization. DBA/2 mice were only protected by L1210-iLOV and C57BL/6 mice were only protected EL4-iLOV immunization.

Supplemental Figure 6

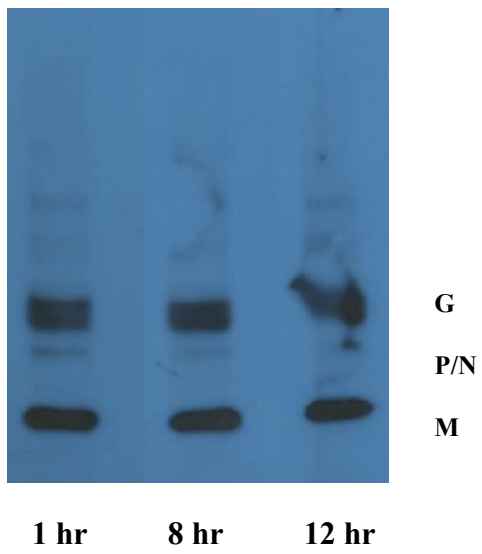
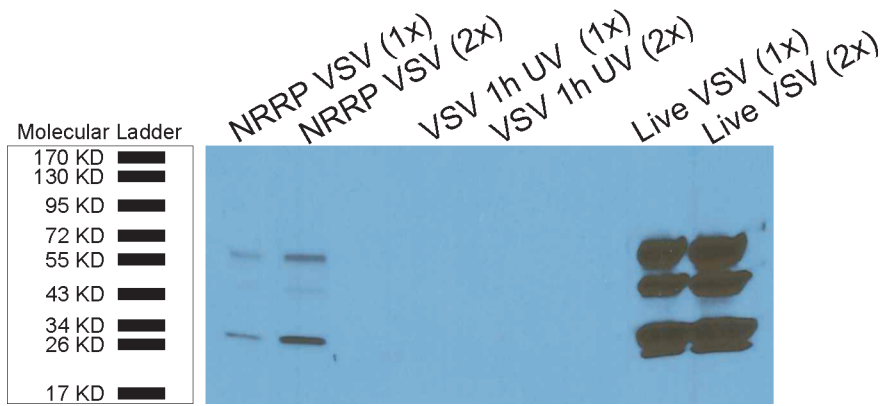


**Appendix Figure 6. Activated L1210 cells.** Expression of B-cell activation markers on L1210 cells following 18-hour incubation with TLR agonists or MG1-infection. Percentage of cells expressing A, CD40 (\*  $P < 10^{-7}$ ) and B, CD252 (\*  $P < 0.002$ , \*\*  $P < 0.02$ ). Cell-surface expression density as mean fluorescence intensity (MFI) of C, CD40 (\*  $P < 0.0007$ , \*\*  $P < 10^{-5}$ , \*\*\*  $P < 0.0002$ ) and D, CD252 (\* NS, \*\*  $P < 0.05$ ). Measurements performed in triplicate.

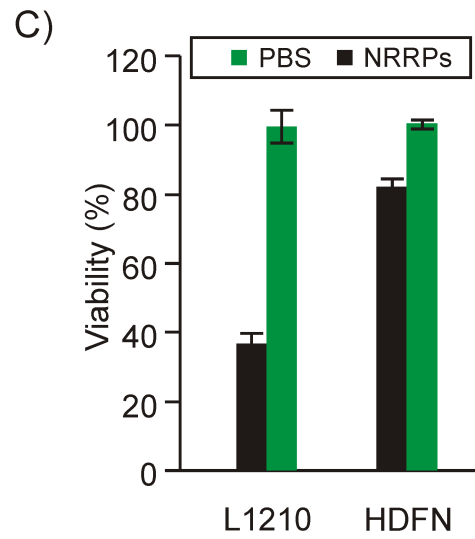
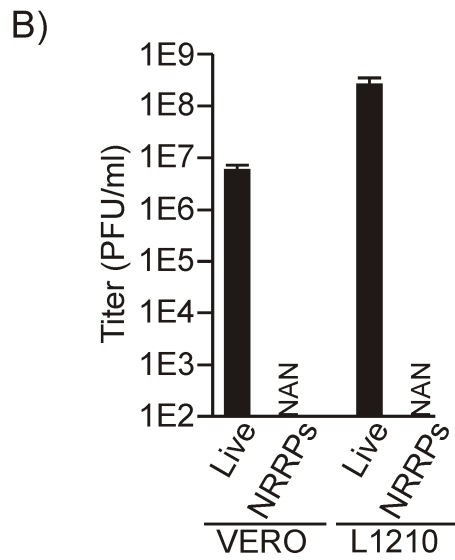
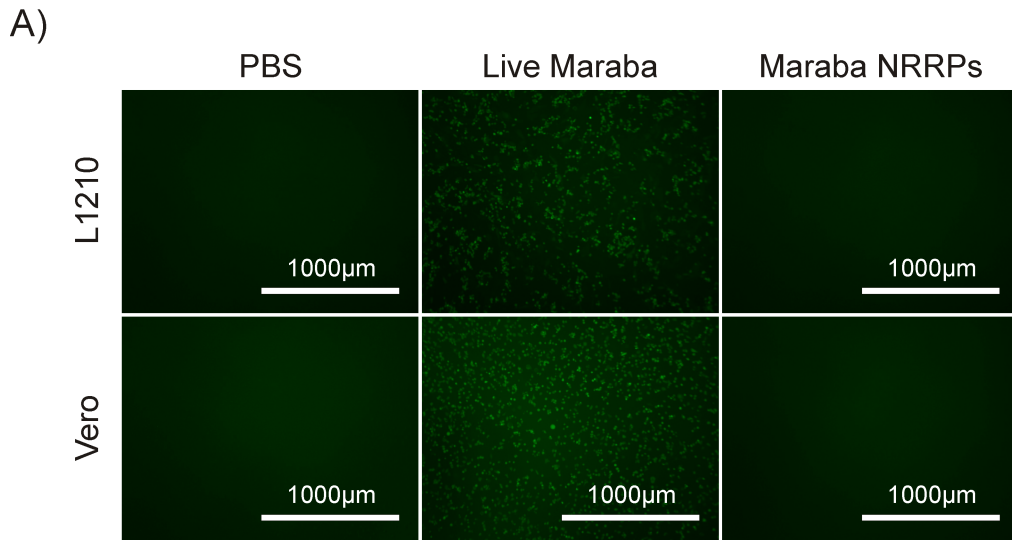
### Supplemental Figure 7



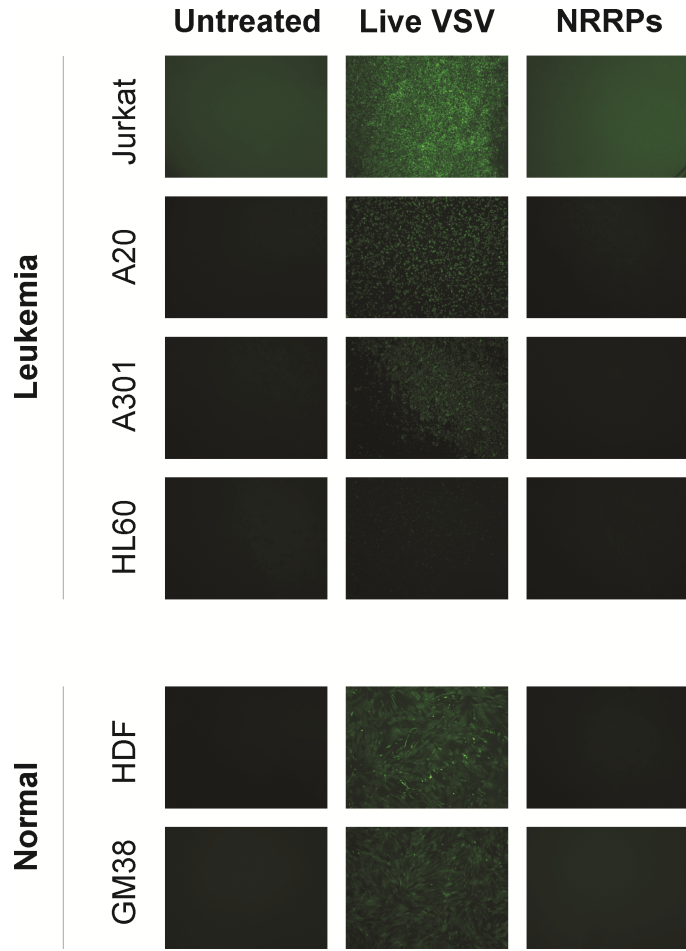
**Appendix Figure 7. UV-treated apoptotic and pressure-disrupted necrotic L1210 cells used for specific vaccine preparations.** Shown are representative flow cytometry dot plots of L1210 cells stained with Annexin V-APC versus 7-AAD cell viability dye. A, UVC-induced early apoptosis (Annexin V-APC +) and late apoptosis (Annexin V-APC+ and 7-AAD+) observed in L1210 cells 4 hours after UVC treatment. B, Pressure disruption of L1210 cells (1500 PSI) reliably produces necrosis as indicated by 7-AAD+ events.



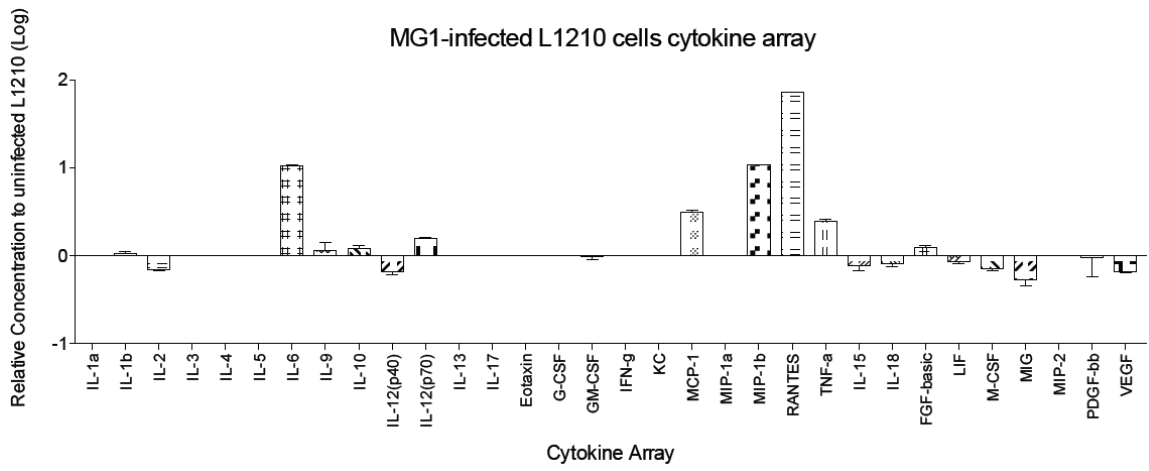
**Appendix Figure 8. NRRPs retain cell-entry capacity but exhibit disrupted genome expression.** Top: L1210 leukemia cells were treated with NRRPs, traditionally UV-inactivated VSV and live VSV, all at MOI 50. 15 hours post treatment, cells were washed 3X and then lysed for protein extraction. Electrophoresis (15/30 microgram protein) and western blotting using rabbit polyclonal anti-VSV antibodies: Strong detection of VSV protein (G, P, N, M) with live VSV, whereas significantly altered/reduced genome expression is observed in NRRP-exposed cells and absent with traditional UV-inactivated method. Bottom: Aberrant VSV genome expression pattern and non-apparent amplification in NRRP-treated leukemia cells. Complete absence of banding was observed at all times points for traditionally UV-inactivated VSV.



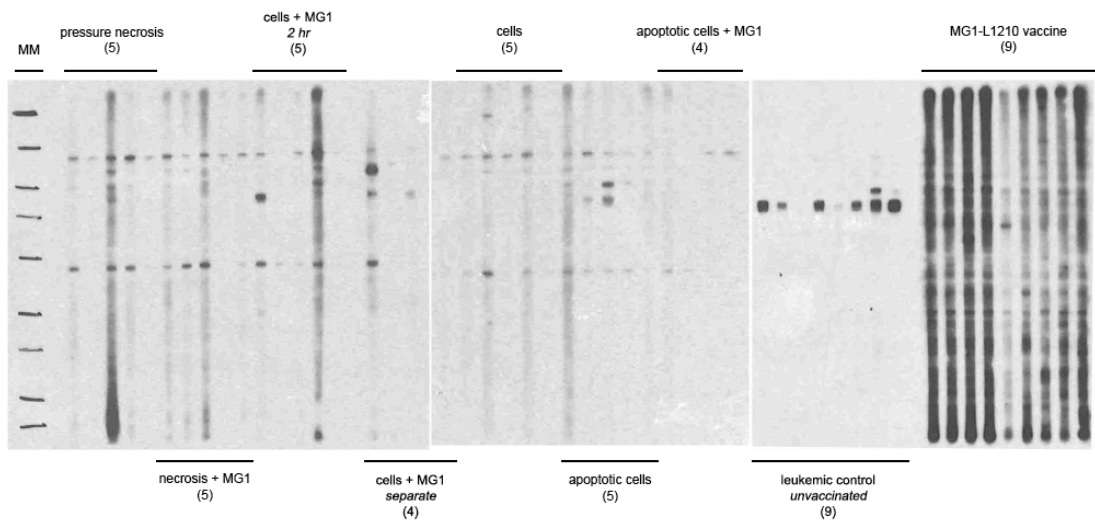
**Appendix Figure 9. Maraba-based NRRPs.** **A)** Fluorescent images of L1210 and Vero cells treated with PBS, Live Maraba-GFP, or Maraba-based NRRPs. **B)** Viral titers obtained from these immortal cell lines. **C)** Resazurin quantification of cellular viability in L1210 and HDF cell lines. Error bars represent the standard deviation between triplicate experiments.



**Appendix Figure 10. Fluorescent microscopy images** of PBS, Live VSV-GFP, or NRRP treatment in murine human Jurkat T-cell acute leukemia, murine A20 B-cell lymphoblastic leukemia, A301 T-cell lymphoblastic leukemia, and HL60 acute promyelocytic leukemia and GM38 and HDF normal cell lines.

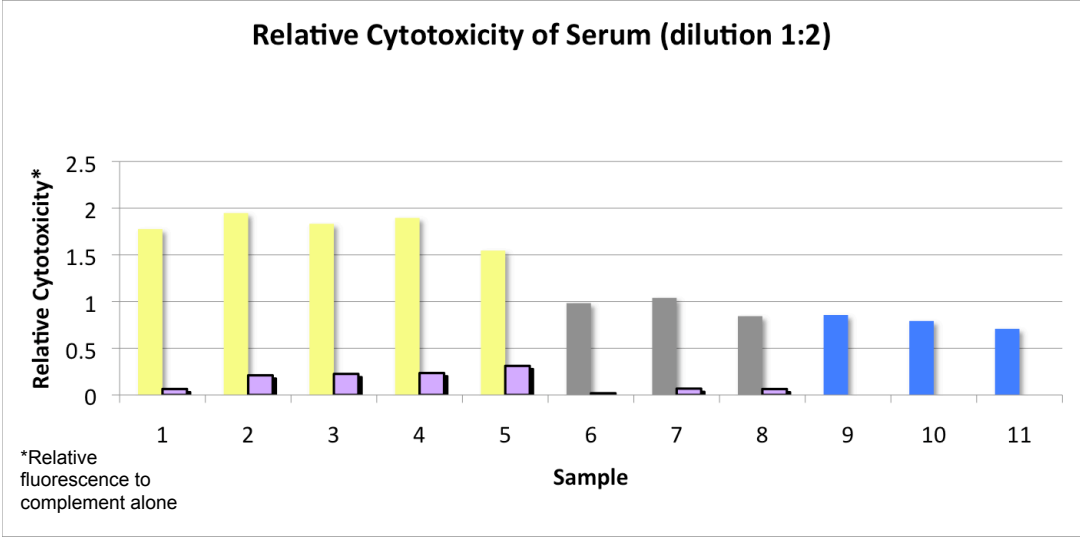


**Appendix Figure 11. Multiplex cytokine array of MG1-infected murine acute lymphoblastic leukemia cells.** Cytokine array (luminex-based) was performed on supernatant collected 18 hours after infection with MG1 vesiculovirus at MOI 10. Performed in duplicate. Relative levels depicted (compared to uninfected leukemia cells) determined against a standard curve performed at the same time.



**Appendix Figure 12. Antileukemia antibody response following vaccination (x3) of iLOV and various control vaccines.**

Representative miniwestern blots using serum from individual mice, probing L1210 cell lysate. Vaccinated types indicated. Marked increase in antibody binding to lysate protein from iLOV treated mice compared to various control groups and unvaccinated mice. Long-term survival was observed in the iLOV cohort. Control cohorts' survival were similar to untreated. For both unvaccinated, control and iLOV mice, additional biological replicates showed similar banding patterns (~N=60).



- Treated with iLOV + received leukemic challenge
- Leukemic challenge only
- Naïve mice (no vaccine, no leukemic challenge)
- Heat inactivated serum only (no complement added)

**Appendix Figure 13. Antibody-mediated complement-dependent cytotoxicity using serum collected from mice either treated, or not, with iLOV followed by leukemic challenge.** Biological replicates as shown. Relative fluorescence (L1210-targeted cytotoxicity) to complement alone.

Parameter	Range Utilized
$K_{NI}$	7.5E-5 to 7.5E-4 ( $V^{-1}h^{-1}$ )
$EC50$	0.25e-12 to 2.5e-12 (M)
$K_{IFN\ on}$	$\ln(2)/(0.2\ to\ 2.0)$ ( $h^{-1}$ )
$K_{IFN\ off}$	$\ln(2)/(5\ to\ 50)$ ( $h^{-1}$ )
$\gamma_c$	$\ln(2)/(2.5\ to\ 25)$ ( $h^{-1}$ )
$K_{NC}$	$\ln(2)/(0.25\ to\ 2.5)$ ( $h^{-1}$ )
$K_{IFN1}$	$K_{IFN2} \times 10$ to 100% (M/h)
$K_{IFN2.1}$ & $K_{IFN2.2}$	8.3e-18 to 8.3e-17 (M/cell/h) (ie 5000-50000 molecules/cell/h)
$\gamma_{IFN}$	$\ln(2)/(5\ to\ 50)$ ( $h^{-1}$ )
$\gamma_N$	$\ln(2)/(2.5\ to\ 25)$ ( $h^{-1}$ )

**Appendix Table 1. List of adapted modeling parameter estimates.**