The Role of APOBEC3 in Controlling Retroviral Spread and Zoonoses

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This thesis is submitted to the Faculty of Graduate and Postdoctoral Studies of the University of Ottawa in partial fulfillment of the requirements for the degree of Masters in Science.

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"(...) chance only favours the prepared mind."

- Louis Pasteur
ABSTRACT

APOBEC3 (A3) proteins are a family of host-encoded cytidine deaminases that protect against retroviruses and other viral intruders. Retroviruses, unlike other viruses, are able to integrate their genomic proviral DNA within hours of entering host cells. A3 proteins hinder retroviral infectivity by editing retroviral replication intermediates, as well as by inhibiting retroviral replication and integration through deamination-independent methods. These proteins thus constitute the first line of immune defense against endogenous and exogenous retroviral pathogens. The overall goal of my Master's project was to better understand the critical role A3 proteins play in restricting inter- and intra-host transmission of retroviruses. There are two specific aspects that I focused on: first, investigating the role of mouse APOBEC3 (mA3) in limiting the zoonotic transmission of murine leukemia retroviruses (MLVs) in a rural environment; second, to identify the molecular features in MLVs that confer susceptibility or resistance to deamination by mA3. For the first part of my project, we collected blood samples from dairy and production cattle from four different geographical locations across Canada. We then designed a novel PCR screening strategy targeting conserved genetic regions in MLVs and Mouse Mammary Tumor Virus (MMTV) and MMTV-like betaretroviruses. Our results indicate that 4% of animals were positive for MLV and 2% were positive for MMTV. Despite crossing the species barrier by gaining entry into bovine cells, our study also demonstrates that the bovine A3 protein is able to potently inhibit the spread of these murine retroviruses in vitro. The next question we asked was whether mA3 could also mutate and restrict murine endogenous retroviruses and thereby partake in limiting zoonotic transmission. Moloney MLV and AKV MLV are two highly homologous murine gammaretroviruses with opposite sensitivities to restriction by mA3: MoMLV is resistant to restriction and deamination
while AKV is sensitive to both. Design of MoMLV/AKV hybrid viruses enabled us to map the region of mA3 resistance to the region encoding the glyco-Gag accessory protein. Site-directed mutagenesis then allowed us to correlate the number of N-linked glycosylation sites with the level of resistance to deamination by mA3. Our results suggest that Gag glycosylation is a possible viral defence mechanism that arose to counteract the evolutionary pressure imposed by mA3. Overall, my projects show the important role A3 proteins play in intrinsic immunity, whether defending the host from foreign retroviral invaders or endogenous retroviral foes.
ACKNOWLEDGEMENTS

First and foremost, I would like to express my gratitude to my supervisor Dr. Marc-André Langlois for allowing me to conduct such rewarding and fulfilling research under his supervision. I would also like to thank him for his constant support and guidance during experimental design, troubleshooting as well as scientific writing. I believe that under his tutelage I have been able to develop into a better scientist and for this, I am truly grateful.

I would also like to thank my Thesis Advisory Committee members, Dr. Kathryn Wright and Dr. Martin Pelchat, for their support, guidance and critical input on the two projects I worked on during my Masters.

I am extending thanks to my lab mates, Kasandra Bélanger, Cindy Lam, Tyler Renner, Tara Read, Mark Campbell and past members Hail Aydin, Mathieu Savoie, Shabnam Rahimi and Olga Agah who made working in the lab such a great and enjoyable experience. I sincerely appreciate their technical assistance and advice during my time at the Langlois lab. Special thanks go to Kasandra Bélanger for being an amazing mentor and friend, as well as to Cindy Lam and Tyler Renner for being there through thick and thin.

Finally, I would like to thank my family, partner and friends for pushing and motivating me when times were tough.

This work was funded by a CIHR operating grant to Dr. Marc-André Langlois.
LIST OF CONTRIBUTIONS

Tara Read designed the MLV $gag$ and GAPDH primers for the Zoonosis Project.

Kasandra Bélanger generated the catalytically inactive hA3G/E259Q expression vector for infectivity as well as the AKV MLV CTA mutant viral construct. She also designed the SDM primers for MoMLV N113Q, N480Q and N505Q and for MoMLV's and AKV MLV's CTA.

Cindy Lam performed the 3D PCRs in Figures 15, 18, and 21-23 under my supervision and guidance.
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LIST OF ABBREVIATIONS

aa: Amino acid
AID: Activation-induced cytidine deaminase
AKV MLV: AKV murine leukemia virus
AKV-NB: AKV with Fv1 NB-tropism
APOBEC: Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like
APOBEC1: Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 1
APOBEC2: Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 2
APOBEC3: Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3
APOBEC3A: Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3A
APOBEC3B: Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3B
APOBEC3C: Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3C
APOBEC3DE: Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3DE
APOBEC3F: Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3F
APOBEC3G: Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G
APOBEC3H: Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3H
APOBEC4: Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 4
ALV: Avian Leukosis Virus
CA: Capsid
CD: Catalytic domain
CMV: Cytomegalovirus
DMEM: Dulbecco's Modified Eagle's Medium
DNA: Deoxyribonucleic acid
dsDNA: double stranded deoxyribonucleic acid
ECL: Enhanced chemiluminescence
eGFP: Enhanced green fluorescence protein
EIAV: Equine infectious anaemia virus
EMLV: Ecotropic Murine Leukemia Virus
Env: Ecotropic Murine Leukemia Virus
Env: Envelope
FrMLV: Friend murine leukemia virus
FBS: Fetal bovine serum
FIV: Feline immunodeficiency virus
Fv1: Friend virus susceptibility 1
Fv4: Friend virus susceptibility 4
Gag: Group specific antigen
gDNA: genomic DNA
gRNA: genomic RNA
HERV: Human endogenous retrovirus
HIV: Human immunodeficiency virus
HIV-1: Human immunodeficiency virus type-1
HMM: High molecular mass
HRP: Horseradish peroxidase
HTLV: Human T cell leukemia virus
IAP: Intracisternal A particle
IN: Integrase
IPTG: Isopropyl β-D-1-thiogalactopyranoside
kDa: Kilodalton
LMM: Low molecular mass
LTR: Long terminal repeat
M/Pmv: Modified Polytropic or Polytropic Murine Leukemia Virus
MA: Matrix
mA3: Mouse APOBEC3 protein
mCAT-1: Murine cationic amino acid transporter 1
MCS: Multiple Cloning Site
MERV: Murine endogenous retrovirus
MLV: Murine leukemia virus
MMTV: Mouse mammary tumour virus
MoMLV: Moloney murine leukemia virus
mRNA: Messenger ribonucleic acid
NC: Nucleocapsid
Nef: Negative regulatory factor
nm: Nanometer
ng: Nanogram
NP40: Nonidet P-40
PBS: Phosphate buffered saline
PBS-T: Phosphate buffered saline with Tween 20
PCR: Polymerase chain reaction
PERV: Porcine endogenous retrovirus
PMLV: Polytropic Murine Leukemia Virus
Pol: Polymerase
PPT: Polypurine Tract
Pro: Protease
R: Repeat region
Rem: Rev-like nuclear export protein
Rev: Regulation of virion
RNA: ribonucleic acid
Rpm: Revolutions per minute
RSV: Rous sarcoma virus
RT: Reverse transcriptase
Sag: Superantigen
SDS: Sodium dodecyl sulphate
SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SIV: Simian immunodeficiency virus
SIV: mac Simian immunodeficiency virus of macaques
SIV: agm Simian immunodeficiency virus of African green monkey
ssRNA: single stranded RNA
SU: Surface protein
TfR-1: Transferrin receptor 1
TM: Transmembrane protein
Trim5α: Tripartite motif-containing protein 5
TRIMCypA: Tripartite motif-containing protein Cyclophilin A
tRNA: Transfer ribonucleic acid
TU: Transducing unit
U: Unique region
U3: Unique 3’ region
U5: Unique 5’ region
XMLV: Xenotropic Murine Leukemia Virus
XMRV: Xenotropic murine leukemia virus-related virus
Xmv: Xenotropic murine leukemia virus
XPR1: Xenotropic and polytropic retrovirus receptor 1
Vif: Viral infectivity factor
Wt: Wild-type
μg: Microgram
μm: Micrometer
μl: Microliter
+ssRNA: positive single stranded RNA
-ssDNA: negative single stranded DNA
1.0 INTRODUCTION

1.1. Retroviruses

Retroviruses were first studied as tumour-causing agents in animals such as birds, mice and sheep in the 1930s and 1950s (reviewed in [6-9]). After the discovery of the reverse transcriptase, they were no longer known as RNA tumour viruses but rather retroviruses (reviewed in [9]). The most studied of all retroviruses is perhaps Human Immunodeficiency Virus (HIV), the causative agent for the Acquired Immunodeficiency Syndrome (AIDS). The link of HIV to AIDS and later on, the finding that this virus evolved from a zoonotic infection of Simian Immunodeficiency Virus (SIV) (reviewed in [10]) in the wild gave much perspective to the study of endogenous retroviruses, retrotransposons and retroelements, and further insight into the process of genome evolution.

Remarkably, retroviruses have also become a tool in the study of cancer. In fact, the oncogene theory of tumourigenesis arose thanks to the study of these viruses and in particular with the study of the Rous Sarcoma Virus (RSV) (reviewed in [11, 12]). The oncogene theory of tumourigenesis was based on observations of alpha- and beta-retroviruses, such as Mouse Mammary Tumour Virus (MMTV), RSV and Avian Leukosis Virus (ALV). The latter induce tumour formation because they either contain genes that directly cause cancer or genes which indirectly trigger malignant cellular growth by activating possible host oncogenic genes in a trans-activating manner (reviewed in [13]). This knowledge of tumourigenesis has opened new avenues in the field of gene therapy where retroviral vectors can now be used for treatment of malignant tumours (reviewed in [11, 14]).
Moreover, antiretroviral drug research continues to benefit from the breadth of information now available on the interaction of HIV accessory proteins and cellular factors. Indeed, the particular relationship that certain retroviruses such as HIV developed with the immune system has also helped the scientific community gain a deeper understanding of the adaptive as well as innate, and intrinsic immune systems. Novel reports of host-encoded factors such as tetherin, APOBEC3 and TRIM5α proteins in recent years would not have been possible had it not been for the thorough introspection of the retroviral life cycle and pathogenesis (reviewed in [15-17]). In turn, these discoveries paved the way for constructing other possible antiretroviral therapeutic agents. Without doubt, retroviral research is of importance to the scientific community for its contributions to many biological and chemical fields.

1.2 Taxonomy of Retroviruses

Retroviruses are single-stranded RNA (ssRNA) viruses of positive polarity, which belong to the Retroviridae family of viruses. This family is subsequently divided into two subfamilies based on differences within their genomes: Spumaretrovirinae and Orthovirinae. The former are called spuma or “foamy” viruses due to the characteristic phenotype observed in the cells they infect. The latter subfamily comprises of six categories, the first 5 of which were based on the electron-microscope appearance of the capsid structure, and are called alpha-, beta-, delta-, epsilon- and gamma-retroviruses. The sixth category, lentiviruses, are denoted as lenti or “slow” for the characteristically persistent and chronic infections they cause. These consist of the notorious HIV, SIV and Human T-cell Leukemia Virus (HTLV). Other retroviruses remain vaguely unclassified such as the Human Endogenous Retroviruses (HERVs). However, phylogenetic analysis has categorized the recently discovered HERV-W and
HERV-K as a gammaretrovirus and a betaretrovirus, respectively (Figure 1) (reviewed in [18, 19]).

Figure 1. Retroviral Family Phylogeny Tree. Figure adapted from Weiss et al. 2006. Those families which contain endogenous retroviruses are labelled with a red asterisk.
This thesis will focus on MMTV, which belongs to the betaretrovirus family, and Xenotropic, Polytropic and Ecotropic Murine Leukemia Viruses (XMLVs, PMLVs, EMLVs), which are distinct Murine Leukemia Viruses (MLVs) from the gammaretroviruses family. In particular, I will elaborate on Xenotropic Murine Leukemia virus-Related Virus (XMRV), an XMLV and Moloney Murine Leukemia Virus (MoMLV) and AKV Murine Leukemia Virus (AKV MLV), which are EMLVs.

1.3 Retrovirus Genome and Morphology

As mentioned previously, the retroviral genome consists of two copies of positive sense, single stranded RNA. Retroviral genomes range between 7-12 kb and are housed within viral capsids, also called virions, with diameters between 80-100nm. The basic blueprint of every retroviral genome consists of the following genes: \textit{gag}, \textit{pol} and \textit{env}. These are essential for the retrovirus to complete its replication cycle. The coding sequence of the virus is flanked at each extremity by Long Terminal Repeats (LTRs), which harbour the viral promoters, reverse transcription primer binding sites (PBSs), polyadenylation signal as well as transcription enhancers. In addition to the previously mentioned genes, complex retroviruses like HIV encode accessory proteins. In contrast, those that do not contain these additional proteins are denoted “simple” retroviruses, such as MoMLV and AKV MLV[9].

1.3.1 "Simple" Retroviruses: A Look at Gammaretroviruses and in Particular Murine Leukemia Viruses (MLVs)

The MLV genome consists of three essential genes: \textit{gag}, which encodes the structural proteins that surround the pair of single stranded genomic RNA; \textit{pol}, which encodes the protease that takes care of the maturation of the retroviral proteins, the reverse transcriptase (RT) polymerase that is responsible for making the double stranded DNA (dsDNA) intermediate
and the integrase (IN), which takes the dsDNA and inserts it into the host cell's genome; and finally, \textit{env}, which encodes the viral envelope glycoproteins (reviewed in [7, 9, 11]).

The MLV protein of interest in this project is the Gag polyprotein, which in turn is cleaved into four major proteins by the protease: the p15 or matrix (MA), p12, the p30 or capsid (CA) and the p10 or nucleocapsid (NC). Prior to protease processing, in the immature virus particle, these proteins are organized such that the NC protein faces the inner portion of the virion and coats the viral genomic RNA [20-22]. The CA forms a protective inner shell for the RNA, and finally the MA, which provides the outer structure under the envelope. The viral envelope is a combination of viral (Env) and cellular proteins anchored to the host’s lipid bilayer membrane that the virion takes with it as it is released from the cell during budding (reviewed in [7]).

In addition to the Gag polyprotein (Pr65) described above that is approximately 65 kDa long prior to protease processing, MLVs, unlike other retroviruses, also produce a glycosylated form of Gag that has a molecular weight of approximately 80kDa (gPr80\textsuperscript{Gag} or glyco-Gag). Translation of Pr65 mRNA begins with an AUG codon, whereas the additional N-terminal sequence of gPr80\textsuperscript{Gag}, is translated from a non-conventional CUG codon. The amino acids (a.a) immediately upstream of the CUG encode a signal peptide that sequesters the gPr80\textsuperscript{Gag} protein to the endoplasmic reticulum where glycan moieties are added to the protein. A study by Dr. Hung Fan, however, showed that mannose glycans tend to be the sugar moieties that are most commonly used to decorate the glyco-Gag protein [23]. This glycosylation is termed N-linked glycosylation because only asparagine residues that follow the N-X-S or N-X-T sequence pattern, where X is any amino acid except proline, are adorned with sugar groups.
Two examples of gammaretroviruses are the replication-competent, endogenous ecotropic AKV MLV and MoMLV retroviruses. They cause T-cell malignancies in mice. AKV MLV was originally discovered in the Akr strain of mice. In these mice, AKV MLV undergoes recombination with other non-ecotropic murine retroviruses encoded in the genome of Akr mice. These recombination events give rise to leukemogenic mink cell focus-forming viruses which are responsible for causing thymic lymphomas in Akr mice[24-39]. MoMLV and AKV MLV also share more than 95% a.a. identity in proteins like the CA p30 and NC p10. Despite the strong homology between the two viruses, they behave remarkably different in the presence of mouse APOBEC3 (mA3), as mentioned previously. Though AKV MLV is restricted and hypermutated, MoMLV is able to overcome the effects of this protein[40]. To this date, the reasons behind AKV's sensitivity and MoMLV's resistance to mA3 inhibition are yet to be resolved.

1.3.2. Retroviruses with oncogenic proteins: A look at Betaretroviruses and in particular MMTV

MMTV is a milk-borne betaretrovirus which is hormone-regulated and capable of indirectly transforming mammary epithelial cells as well as cells of the immune system. It does so by integrating in areas where major oncogenes are found, driving their activation in trans via viral LTR enhancers, and integrating as well within protein-coding sequences, modifying the type of gene product that is later expressed.

The MMTV genome contains the main structural and functional gag, pol and env genes as other retroviruses,. Interestingly, the env of MMTV has also been associated with cancer. This should come as no surprise as other betaretroviruses such as Jagsiekte Sheep Retrovirus
(JSRV) and Enzootic Nasal Tumour Virus (ENTV) have Env proteins capable of transforming the lung epithelial cells they infect (reviewed in [41]).

The genes described above are flanked by non-structural genomic regions, the LTRs, which contain promoters and primer sites for reverse transcription as well as transcriptional enhancers. However, unlike most retroviruses, the LTRs of MMTV also encode a gene, expressed through alternative splicing that produces a superantigen (Sag). This protein is responsible for the proliferation of MMTV within the lymphatic system before it spreads within mammary tissue. The LTRs also determine tissue tropism as they harbour hormone-sensitive enhancers, which stimulate virus replication in mammary tissue and regulatory elements that facilitate virus spread in T- and B-cells.

MMTV gains entry to cells via the Transferring Receptor 1 (TfR1), which is conserved in all mammals[42]. In mice, TfR1 is highly expressed in dividing mammary epithelial cells as well as activated immune cells, which is another way that MMTV may be limited to this cellular repertoire. Oddly enough, despite the fact that TfR1 is also expressed in human cells, MMTV cannot enter human cells using the human TfR1 receptor[42, 43]. Despite this, multiple studies, have reported findings of MMTV-like sequences in human malignancies and diseases, indicating that MMTV may have found another route for trans-species transmission[3, 44-47].

1.3.2.1. The Replication Cycle of Gammaretroviruses Retroviruses

MLVs, like other retroviruses, use their Env glycoproteins to interact with host cell receptors and gain entry to the cell. Once in the cell, the envelope-striped core is activated by still unknown factors that could include the presence of free deoxynucleotides in the cytoplasm.
The RT makes dsDNA from the ssRNA. This dsDNA intermediate is taken to the nucleus by the Pre-Integration Complex (PIC) (Reviewed in [16]) (Figure2).

There are three major types of MLVs: ecotropic, xenotropic and polytropic. EMLVs are endogenous MLVs, which are only capable of infecting murine cells, much like their name suggests; eco comes from the Greek “oikos”, meaning home. EMLVs infect murine cells through the mCAT-1 receptor. XMLVs, on the other hand, are “foreign” MLVs in that though endogenous to mice, cannot spread within murine cells but rather infect other species. PMLVs have the broadest range in tropism since they are able to infect both murine and other cells. Thus, PMLVs are able to use the CAT-1 receptor utilized by ecotropic MLVs in murine cells, but like XMLVs, they also use the Xenotropic and Polytropic Receptor-1 (XPR-1), which is conservatively expressed in all mammalian and avian cells[48, 49]. MLV is also thought to use actin polymerization to cluster the receptors so their Env glycoproteins may interact better with them before entering the cell (reviewed in [7, 16]).

In contrast to the rest of the retroviruses, once MLVs enter a host cell and the core particle becomes exposed, all the Gag proteins remain as part of the RTC and eventually also as part of the PIC, except perhaps for the MA. In the RTC, the RT transcribes the positive polarity RNA into a single stranded, negative sense DNA (-ssDNA). RT accomplishes this via template switching, a process that describes the 'jumping' of the enzyme between the two positive-sense ssRNAs. The way that reverse transcription is carried out ensures that genome integrity may be preserved as well as favour genome evolution. The RT of retroviruses is known to have poor fidelity. This propensity to make mistakes also assures that genome evolution is a constant
Retroviruses enter the cell via a variety of mechanisms, which usually resort on entry via a cell surface receptor and proceed to replicate their genome via the process known as reverse transcription. They then integrate their genome into the host cell chromosome. **B) Late steps in the retroviral life cycle.** After integration, the retroviral genome depends on the cellular machinery for RNA transcription and processing and for protein translation and modification. The viral proteins then assemble at the plasma membrane and bud out as immature viral particles. A mature particle is achieved after protease processing occurs outside of the cell. This cleavage changes the structure of the viral core for betaretroviruses and lentiviruses but not for gammaretroviruses, which remain spherical.
driving force behind the persistent infections of viruses such as HIV-1, whose RT makes 1 error per 1.7kb [50]. The RT also destroys the RNA with its RNaseH activity as it makes the ssDNA. Once the dsDNA is manufactured, it is ready to be shipped to the host genome where the integrase will insert it into CpG islands and other preferred hotspots[9, 51-56].

1.4. Retroviral Pathogenesis and Zoonosis

1.4.1. Pathogenesis

Retroviruses target many multicellular, vertebrate organisms, which include mammals, birds and fish, and within these species, retroviruses may also affect different cell types, organs, and tissues. For instance, although HIV-1 prefers to infect cells of the immune system, it does not shy away from the digestive system, where it can gain entry to the cells and damage them (reviewed in [57]).

Because of the diversity and range of retroviral pathogenesis, it makes sense that the disease and syndromes, retroviruses cause are also varied. Retroviruses have been known to cause anemia, arthritis, cancer, osteoporosis, malignant tumour growth, immunodeficiencies, and cognitive and neurodegenerative disorders. Interestingly, HIV-1 had already been shown to cause neurological symptoms when untreated. But recently, HERVs have been linked to diseases such as Multiple Sclerosis (MS), and even to mental illnesses such as Schizophrenia (SZ) and bipolar disorder (BP). HERV type W (HERV-W) and HERV type K (HERV-K) have both been linked to MS, SZ and BP[58-64].

Retroviruses are not airborne, with the exception of the betaretrovirus Jaasiekte Sheep Retrovirus (JSRV)[41, 65], and cannot spread through surface or skin contact like other viruses or pathogens. They rely on being as close as can be to the body in order to invade the host.
Thus, the most efficient way retroviruses manage to spread is through mucosal and blood interactions. These interactions are limited to blood or bodily fluids contact with opened wounds, sexual contact and during birth through the birth canal, though they may also be passed down genetically to offspring, a process known as vertical transmission.

The most studied retrovirus to date is HIV-1, which causes a severe immunodeficiency. Unlike the rest of the retroviruses, HIV-1, being a lentivirus, must first establish a persistent, chronic infection before leading to an immunodeficiency syndrome in the host. HIV-1 does this by attacking the cells of the immune system, which it also uses as a hiding reservoir for future infections even in the presence of antiretroviral therapy (reviewed in [9, 66]).

1.4.2 Zoonosis

Zoonosis is a process that occurs when a pathogen is transmitted from one species to another. The most famous of all zoonoses is probably that of the bubonic plague; where murine vectors carried fleas harbouring the bacterium *Yersinia pestis* that then went on to infect humans. In virology, a most common zoonotic agent is the influenza virus, which has various vertebrate hosts, and HIV-1, which was transmitted to humans from chimpanzees carrying a recombinant SIV (reviewed in [67-69]).

The scientific community was alerted once more to the potential dangers of zoonotic transmission of viruses with the discovery of XMRV. This virus was originally, and wrongfully associated with prostate cancer and chronic fatigue syndrome[70, 71]. However, studies later confirmed that XMRV actually developed as a result of recombination events between a polytropic replication-competent virus and replication-incompetent xenotropic viruses which were present endogenously in mice that underwent xenograft procedures for the generation of the prostate cancer cell line 22rv1[72, 73]. Despite the fact that XMRV is not
found in the wild, what remains is its undeniable ability to infect human tissues and cells *in vitro*, which was proven in numerous studies (reviewed in [74]). This thus poses the question of whether or not polytropic and xenotropic murine retroviruses are able to spread in humans since they can readily infect mammalian cells through the XPR-1 receptor (reviewed in [7, 75]). Interestingly, macaques infected with XMRV do not develop disease[76], though whether or not the virus is able to cause an AIDS-like syndrome remains. However, a possible reason why this virus might not cause disease could also be due to its inability to fight off host-encoded restriction factors such as the likes of the APOBEC3 proteins that inhibit the virus via the addition of deleterious mutations to its genome and the inhibition of reverse transcription and integration processes[77, 78]. Despite not causing an apparent infection, however, the insertions of XRMV into the host genome could potentially lead to cancer. Thus, it is important to determine the risk of murine retroviral zoonosis.

Notably, murine retroviral zoonosis may occur more frequently than thought. For instance, the Koala Leukemia Virus (KLV) and Gibbon Ape Leukemia Virus (GALV) are very closely related retroviruses and it is thought that GALV is actually the result of a horizontal transmission from a vector carrying KLV. Recently, speculation has also highlighted the possibility of a vector carrying a retrovirus that led to both GALV and KLV. The vector for this transmission has yet to be discovered (reviewed in [79-81]). However, phylogenetic analysis of both of these retroviruses seems to point out that the latter speculation might be true, implicating yet again mice as the potential vector of zoonotically transmitted diseases. Recently, it was discovered that south-eastern Asian mice and bats carry gammaretroviruses that closely resemble KLV (reviewed in [79-81]). In addition, mammalian retroviral zoonosis to birds has also been observed. Reticuloendotheliosis viruses that afflict many types of
poultry actually originated from recombinant mammalian endogenous retroviruses during a study of malaria in the 1930s[82].

It is important to track zoonoses because retroviruses can cause malignancies and monitoring these malignancies can help prevent them. In fact, ever since the discovery that one of the causative agents of breast cancer in mice was a filterable agent now known as MMTV, there has been an ongoing search for a possible MMTV-like retroviral culprit for some human breast cancers. The search remains ongoing. MMTV-like sequences have also been found in a number of other malignancies other than breast cancer, such as lung cancer and primary biliary cirrhosis[3, 44-47, 83-86]. The link between cancer and endogenous retroviral integration must be investigated further.

1.5 Host-encoded restriction factors are a component of intrinsic immunity

Retroviruses must be resourceful; they must know how to find their way around the cellular framework and take advantage of the cellular machinery. If this were not enough, retroviruses must also be able to fend off the host cell’s defenses. After all, they are intruders that need to be stopped. It is of no surprise then that many retroviral accessory proteins arose as a counterpoint to a cellular protection mechanism.

The first line of defense of the cell against retroviruses is, of course, prevention. Most retroviruses use the classical route of glycoprotein-receptor interaction to gain entry into a cell. If the host cell does not express this receptor or if the receptor is somehow different within species, this will confer resistance to the host to that particular retrovirus. Good examples for this are the previously mentioned ecotropic Murine Leukemia Viruses. These viruses use a cationic amino acid transporter which is expressed in all mammals. However, EMLVs are only
able to access the murine version of this transporter, the mCAT-1 receptors (reviewed in [16]), sparing humans since their CAT-1 receptor does not contain the binding site for the ecotropic glycoprotein[87]. Another type of restriction factor that works in a similar way is the Fv4 protein, encoded by the Fv4 gene. This gene produces a defective Env-like protein that assembles in the cell membrane and is picked up by budding virions, preventing the Fv4-containing virus from entering the target cell (reviewed in [16]). Fv4 was discovered in relation to gammaretrovirus Friend Leukemia Virus (FrMLV), just like the Fv1 restriction factor.

The Fv1 gene expresses a Gag protein homologous to that of the MERV-L murine endogenous retrovirus family. Initially a single amino acid residue in the CA, residue 110 was thought to be the single determinant of sensitivity to Fv1 restriction; however, others have been described[88-90]. The Fv1 gene has two alleles the Fv1\textsuperscript{a} and Fv1\textsuperscript{b}, denoted as such because they are present in NIH/swiss mice and Balb/C mice, respectively. B-tropic MLVs which do not carry the E110 CA residue are restricted by the expressed Fv1\textsuperscript{a} allele, whereas N-tropic viruses with the R110 CA residue[88, 90] are restricted by Fv1 from mice carrying the Fv1\textsuperscript{b} allele. Studies in other species showed that there were restriction factors capable of inhibiting retroviruses at the same step as Fv1, after reverse transcription and before viral genome integration. Mammalian Fv1-like restriction factors are the TRIM5\(\alpha\) and TRIMCypA protein, which target retroviruses for proteasomal degradation.

In contrast to the above restriction factors which work by interacting with the CA protein and interfering with retroviral genetic material integration into the host’s genome, the restriction factors SAMHD1 and APOBEC3 proteins, work prior to this event in the replication life cycle. SAMHD1, for instance, works during the reverse transcription replication step, limiting the pools of dNTPs needed by the RT. The APOBEC3 proteins and their mode of action is
described in better detail in section 1.5. Other restriction factors that act during different steps in the viral replication cycle are the recently discovered Tetherin, ZAP, MOV10 and TRIM28 proteins. Tetherin obstructs virion release by fastening budding virions to the cell surface. The Zinc-finger Antiviral Protein (ZAP) is an anti-retroviral protein that inhibits the accumulation of retroviral RNAs. MOV10 is able to decrease the levels of Gag expression in HIV-1 producer cells while also capable of inhibiting reverse transcription in newly-infected HIV-1 target cells (reviewed in [91-95]). Finally, TRIM28 is a protein that binds an as-of-yet unidentified DNA-binding protein that adheres to the primer binding site of MLV RNA, leading to the transcriptional silencing of transcribed MLV RNAs[96] (Figure 3).
Figure 3. Host cell restriction factors. A) Events in the early retroviral cycle blocked by natural host defenses and restriction factors. B) Late events in the retroviral life cycle inhibited by innate restriction factors.
1.5.1. APOBEC3 proteins and their antiretroviral activity

Our cells protect us from retroviral invaders using a repertoire of intrinsic restriction factors like those mentioned above. This thesis focuses on one type of restriction factor in particular, APOBEC3 and specifically mouse APOBEC3.

1.5.1.1. Cytidine Deaminases

APOBEC proteins (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide), are mammalian-encoded cytidine deaminases which use RNA or ssDNA as substrates (Figure 4). The first studied family members of the APOBEC proteins were APOBEC1 (A1) and the Activation-Induced Deaminase (AID). Present in gastrointestinal cells, A1 is capable of editing through cytosine deamination the mRNA of apolipoprotein B causing a truncation that leads to two types of mRNA with distinct roles in the metabolism of cholesterol, triglycerides and lipids. AID, on the other hand, plays a crucial function in antibody affinity maturation and isotype class switching by deamination of the ssDNA regions of genomic DNA during transcription in B cells[97]. Hypermuation induced by AID's deaminating activity leads to a diverse immunoglobulin(Ig) antibody repertoire (reviewed in [69, 98]).

Two paralogs of the APOBEC3 proteins, APOBEC2 (A2) and APOBEC4 (A4), do not seem to encode cytidine deaminase activity. Found mostly in skeletal muscle and in the heart, A2 has been hypothesized to be involved in muscle differentiation, despite murine knock outs of A2 being healthy and viable[99, 100].

A3 proteins were originally discovered during studies with the lentivirus HIV-1[101]. In efforts to find the possible role for one of HIV-1’s accessory proteins, Vif, some cells of the immune systems, namely CD4 T-cells and macrophages, were discovered to be "non-
Figure 4. Representative Schematic of the APOBEC3 Proteins Mechanism of Action using human APOBEC3G as an Example. Figure adapted from Jonsson et Andresdottir 2013[1, 2]. Human A3G gets encapsidated into the virus by binding both the retroviral RNA genome and the nucleocapsid protein. Once in the target cell, hA3G restricts the retrovirus via dependent and independent ways.
permissive" to the production of infectious HIV-1 particles. This phenotype of the HIV-1 lacking Vif (ΔVif) was narrowed to a particular cDNA named CEM15 when a subtractive screen was done in CEM and CEM-SS cells to differentiate cDNAs from HIV-1 ΔVif permissive and non-permissive cells, respectively. The CEM15 factor was later identified as human APOBEC3G (hA3G) (reviewed in [69]). Since then, attempts to understand the cytidine deaminase activity of this A3 protein have led to the discovery of 6 others in humans: A3A, A3B, A3C, A3DE, A3F, and A3H. Of note, only A3G and A3F have the most potent restriction activity against HIV-1, followed by A3H, with some of its haplotypes being more active than others[102-104]. These proteins are expressed in various human tissues. For instance, A3G is found in high amounts in the spleen and in the cells of the immune system such as CD4 T-cells, monocytes, and macrophages, as well as being observed in the tonsil, testis, ovary, brain, heart and other tissues (reviewed in [69, 105, 106]).

1.5.1.2. **Domain Architecture of the APOBEC3 Proteins**

Preceding the origin of lentiviruses such as HTLV-1 and HIV-1, the A3 gene evolved close to 30 million years ago from a common ancestor thought to be the activation-induced deaminase (AID). The A3 gene ramification also reflects an expansion of this gene through vertebrate speciation. For instance, mice only have one A3 gene in chromosome 15, artiodactyls have 2 to 3 genes (2 in pigs, 3 in sheep and cattle), cats have 4, horses encode 6 and primates possess 7. The 7 human A3 genes are located in tandem in chromosome 22[2, 69, 105, 107]. The A3 genes are characterized by their Zinc-coordinating domains which have the general amino acid sequence H-X1-E-X_{25-31}--C-X_{2-4}-C, where X can be any amino acid. Further phylogenetic analysis of the Zinc-binding domains across species proved that the domains could be
classified into three classes, where the Z domain-recognizing motifs differ from one another within the $X_{25-31}^-C-X_{2-4}^-C$ portion as well as within the entire motif. For instance, both deaminases with Z1 and Z2 domains are characterized by the SW-S/T-C-X2-4-C motif, whereas proteins with Z3 domains have the following TW-S/T-C-X2-C motif (Figure 5). Of note, these S/TWS motifs have been associated with RNA-binding capacity, with the tryptophan being of utmost importance[108, 109].

A3 proteins scan proviral (−)ssDNA for specific sequences to deaminate. For instance, human A3G predominantly deaminates the second cytosine in a 5′-CC-3′ context in the nascent transcribed minus strand cDNA, whereas mouse A3 and other human A3s, except for hA3DE, prefer 5′-TC-3′ dinucleotides over 5′-CC-3′ dinucleotides. Porcine A3 and that of other artiodactyls prioritize a 5′-GC-3′ context, which is not observed in any of the other cytidine deaminases, except for in AID, hA3DE and the feline A3Z2Z3[110-112]. Nevertheless, the N-terminal domain (NTD)Z2 domain remains catalytically inactive for hA3F and hA3G. To add to this conundrum, hA3B happens to have the same genetic architecture as hA3G, yet both domains are catalytically active. Further characterization of these domains should help elucidate a clearer picture of the domain functionality of these proteins (reviewed in [2, 112]).

The APOBEC3 proteins play a strong role in our immune system. They are able to combat retroviruses at the early stages of their infection when the innate and adaptive immune system have yet to recognize them. Therefore, it is important to continue to study the APOBEC3 proteins. Understanding their interactions with retroviruses such as HIV-1 can aid in the advancement of pharmaceutical research. And finally, researching the APOBEC3 proteins can provide more information about how retroviral zoonosis may be prevented in mammals.
Figure 5. Schematic of the Mammalian APOBEC3 genes and their respective Zinc-binding domains. Adapted from LaRue et al. (2008).
A3A  A3B  A3C  A3DE  A3F  A3G  A3H  Human

A3Z2-Z3
Mouse & Rat

A3Z1a  A3Z1b  A3Z2a-Z2b  A3Z2c-Z2d  A3Z2e  A3Z3  Horse

A3Z2-Z3
A3Z2  A3Z3
Pig

A3Z2-Z3
A3Z1  A3Z2  A3Z3
Cattle & Sheep

A3Z2b-Z3
A3Z2a  A3Z2b  A3Z2c  A3Z3
Cat
2.0 HYPOTHESIS

**Project 1: Role of APOBEC3 in Protecting Cattle Against Murine Retroviral Zoonosis**

Polytropic and xenotropic murine retroviruses are theoretically able to infect the cells of various animal species, including that of cattle. Mice and cattle cohabit in barns in rural settings which thereby fosters an opportunity for the transmission of retroviral pathogens. We hypothesize that bovine APOBEC3 restricts the infection and spread of these retroviruses.

**Project 2: N-linked glycosylation of MLV Gag is a viral defence mechanism against restriction and deamination by mA3**

Because MoMLV and AKV display different sensitivities to deamination by mA3 despite sharing over 95% sequence identity, I hypothesize that there is a genetic component making either MoMLV resistant or AKV sensitive to deamination.
3.0 OBJECTIVES

Project 1: Role of APOBEC3 in Protecting Cattle Against Murine Retroviral Zoonosis

1. To identify murine retroviral sequences in the genomic DNA of cow lymphocytes
2. Determine whether bovine APOBEC3 protects against murine retroviral zoonosis

Project 2: N-linked glycosylation of MLV Gag is a viral defence mechanism against restriction and deamination by mA3

1. To identify the genetic differences between AKV MLV and MoMLV that render MoMLV resistant to mA3
2. To characterize how glyco-Gag prevents restriction and deamination by mouse APOBEC3
4.0 MATERIALS AND METHODS

4.1 Cells and Expression Vectors

Bovine Mammary Alveolar Cells immortalized with SV-40 T-antigen (MAC-T) were a gift from Dr. Lacasse (Agriculture Canada). The MAC-T, Human Embryonic Kidney Epithelium (HEK 293T) and mouse embryonic fibroblasts NIH 3T3 cells used in this study were cultured in 75cm² flasks in 10mL of HyClone DMEM/High Glucose Medium (Thermo Fischer Scientific, Waltham, Massachusetts, USA) supplemented with decomplemented 10% Fetal Bovine Serum (FBS) (CellGro, Mediatech, Inc., Manassas, VA, USA), 100U/mL penicillin and 100μg/mL streptomycin (Multicell, Wisent Inc., Canada) and propagated at 37°C in a 5% CO₂ incubator. The FBS was decomplemented by heating at 56°C for 30min to remove any potential complement or antibodies reactive against murine retroviruses.

The pMOV-eGFP expression vector encoding replicative MoMLV has been described before[113], as well as the pAKV-NB-eGFP viral plasmid encoding replicative AKV MLV[88]. Both constructs express viral proteins and RNA under the control of a CMV promoter. Briefly, the green fluorescent protein (GFP) coding sequence was inserted in the proline-rich region of the Env gene of MoMLV, whereas a cassette containing the enhanced fluorescent protein (eGFP) fused with an internal ribosome entry site (IRES) was inserted downstream of the U3 region in AKV MLV.

The replicative chimera viruses were generated from both of these viruses, with all of them sharing a Moloney-vector backbone. Hybrid 1 MLV (pMOV-AKV Hybrid-U5) contains the AKV-NB-eGFP’s U5 region and CUG glyco-Gag initiation codon. The replicative chimera Hybrid 2 MLV contains the same genomic sequence of AKV as Hybrid 1 with the addition of the protease of AKV MLV. Replicative MLV Hybrid 3 contains the same genomic
regions as Hybrid 2 and also the pol gene up to the restriction site PmlI upstream of the Env gene of AKV (Figure 16 and 17 in the Results).

The glycosylation point mutants were generated at putative N-glycosylation sites using the QuickChange XL Site-directed Mutagenesis (SDM) Kit (Agilent Technologies, Santa Clara, California, USA) according to the manufacturer’s specifications. The following primers were used to generate the mutated CTA non-conventional start codon: FWD 5' - CACCCGGCGCAGCACCCCTAGGAGACGTCCCAGGG-3' and REV 5' - CCCTGGGACGTCTCTAGGGTGGCGGCCGGGTG-3'; the N113Q site: FWD 5' - GAGCGGATCGCTCACCACGAGTCCGTAGATGTC-3' and REV 5' - GACATCTACCCGACTGCTGGTGAAGCGATCCGCAGCTC-3'; the N113D site FWD 5' - CAGCGCATCGCGCTCCGATCAGTCCGTAGATG-3' and REV 5' - CATCTACGGACTGAGCGACGCCGATCGCAGTGTAG-3'; the N480Q site FWD 5' - CCAGGGCAAGAAACTCAAGGTGCTATGTCATTC-3' and REV 5' - GAAAGACATAGACACCTGAGTGGTTCTTGCCCTGG-3'; the MoMLV N480D site FWD 5' - CCAGGGCAAGAAACTGATGTATCTATGTCATTC-3' and REV 5' - GAATGACATAGACACATCAGATTTCCTTGCCCTGG-3'; the AKV MLV N482D site FWD 5' - CTCTAGAGATTTAAACAGAAGACGCTTGGAGATTTAG-3' and REV 5' - CTAATATCTCCTCAAGTTTTATTATTTAAGTCAGTATTGTACCGC-3'.
The XMRV VP62/pcDNA 3.1 viral vector encoding replicative XMRV was obtained from the NIH Reagents program and has been described before[114].

The plasmid encoding hA3G and eGFP has been previously described by Conticello et al., 2003[115]. To generate a FLAG-tagged A3G, the eGFP reporter gene was removed from the peGFP-C3 plasmid (Invitrogen Corp., Carlsbad, California, USA) and a FLAG sequence was inserted between the NheI and Scal restriction sites of the MCS, making a pFLAG-C3 plasmid, which was then used for cloning the coding sequence of hA3G using other restriction sites (XhoI and PstI) downstream of the FLAG. The hA3G E259Q point mutant was generated via SDM as previously mentioned.

The mA3 expression vectors and primer sequences used to generate them have been described elsewhere [40, 116]. Briefly, mA3 allelic cDNA was reverse transcribed from total RNA isolated from the spleen of different mouse strains (Reference 35), and cloned using restriction sites XhoI and KpnI of the in-house generated pFLAG-C3 plasmid’s MCS. The mA3 catalytic point mutants were created from A3[C57] ΔE5 plasmid using SDM.

**4.2 In vitro transfection and infection assays**

To produce the replicative viruses as well as the APOBEC proteins mentioned above, 2 x 10^6 cells were seeded in a 12-well plate using decomplemented media and incubated approximately 24hrs, until they reached 50-60% confluence. Before transfection, the media was replaced with 1.5mL of fresh decomplemented media. With the exception of pAKV-NB (1.2ug) and APOBEC2 (20ng), co-transfections were done with 800ng of viral plasmid and 100ng of APOBEC3 plasmid, using GeneJuice transfection agent (Novagen-EMD4 Biosciences, Darmstadt, Germany) according to manufacturer’s instructions. The HEK 293T producer cells were then cultured for 48hrs before proceeding with infection.
Twenty-four hours prior to infection, 1 x 10⁶ of NIH 3T3 target cells were seeded in a 12-well plate with decomplemented media and incubated until 50-60% confluence. Forty-eight hours post-transfection, NIH 3T3 cell media was replaced by fresh decomplemented media previously mixed with Polybrene (Sigma-Aldrich, Oakville, ON, Canada) at a concentration of 8µg/mL. The supernatant of the producer 293Ts was spun at 2000rpm in a Sorvall ST40R centrifuge (Thermo-Scientific, Mississauga, ON, Canada) for 10min before being added drop-wise to the target cells. The cells were infected at an MOI of 1. Finally, the target cells were spinoculated at 2000rpm for 1hr in the Sorvall ST40R centrifuge, and incubated for 24-, 48- and 72-hrs post-infection before being analyzed for the presence of integrated eGFP reporter gene using the Cyan ADP flow cytometer (Beckman Coulter Inc., Brea, California, USA).

4.3 Western Blot Analysis

Cells were harvested 48h post-transfection, washed with 1X phosphate-buffered saline (PBS) solution and lysed on ice for 30min with RIPA lysis buffer (50mM Tris pH 8.0, 150mM NaCl, 1% NP-40, 0.2% Sodium Dodecyl Sulphate (SDS), 0.5% Na-Deoxycholate and 1mM EDTA) supplemented with 2X complete, EDTA-free, protease inhibitor cocktail tablets (Roche, Mississauga, ON, Canada). Cell lysates were resuspended in 5X Laemmli Buffer (0.5M Tris pH 6.8, 0.2% (v/v) glycerol, 20% (w/v) SDS, 0.16% (w/v) Bromophenol blue) with 5% β-mercaptoethanol and boiled for 5min at 100°C prior to loading on gel. Samples were run on 10% SDS-PAGE acrylamide gels in 1X Running Buffer (190mM glycine, 0.1% (w/v) SDS, 25mM Tris-base) at 175V for 1 hour, and transferred to 0.45 µm PVDF membranes (Millipore, Billerica, Massachusetts, USA) at 0.35mA in 1X Novex Transfer buffer (190mM glycine, 25mM Tris-base, 20% (v/v) methanol) for 1 hour. Membranes were then blocked with 5% skim milk in 1X PBS-T (0.1% Tween 20) (Acros, New Jersey, USA) for 1hr at room
temperature or overnight at 4°C on a shaker. Blotting followed for 1 hour at room temperature or at 4°C overnight, in the case of primary antibodies only, on a shaker. The following antibodies were used for blotting the membranes, primary, monoclonal HRP-conjugated anti-FLAG M2-peroxidase (Sigma-Aldrich, St. Louis, Missouri, USA) at 1:3000 and primary polyclonal rabbit anti-β-tubulin (Abcam, Cambridge, Massachusetts, USA) at 1:20,000. All antibodies were diluted in 5% skim milk in 1X PBS-T prior to use. After blotting, membranes were washed in 1X PBS-T for 10 minutes three times on a shaker, followed by a brief wash in 1X PBS. Proteins were detected using the ECL Plus Western Blotting Detection Kit (GE Healthcare, Buckinghamshire, UK) and exposed using Clear Blue CL-XPosure imaging films (Thermo Scientific, Rockford, Illinois, USA) or Clarity Western ECL Substrate (Bio-Rad, USA) and the membranes were exposed using ImageQuant LAS 4000 software (GE Healthcare Life Sciences).

4.4 Viral Encapsidations

Prior to harvesting the virus, HEK 293T producer cells were seeded at 2 x 10^6 in 10cm dishes for 96 hours. The supernatant of these producer cells was then collected and filtered using a Millex-HV 0.45µm pore cartridge filters (EMD Millipore, Canada) to remove cell debris, and spun at 31,500 rpm for 2 hours using ultra-centrifuge L-100XP (Beckman Coulter, Mississauga, ON, Canada) with a 70Ti rotor (Beckman Coulter). The viral pellet was then resuspended in RIPA lysis buffer with 2X Roche completeEDTA-free protease inhibitor cocktail tablets (Roche) and analyzed by western blot analysis for the presence of the APOBEC proteins and viral capsid proteins. The samples were then boiled after mixing with 5X Laemmli buffer with 5% β-mercaptoethanol, and loaded on 10% SDS-PAGE acrylamide gels. Other than the anti-FLAG antibody mentioned before used to detect the encapsidated
APOBEC proteins, the antibodies used to detect the capsid protein of the pelleted virus were primary, rabbit polyclonal anti-MuLV p30CA at 1:30,000, which was a gift from Dr. Hung Fan (Department of Molecular Biology and Biochemistry and Cancer Research Institute, University of California, Irvine, California, USA), and secondary, HRP-conjugated anti-rabbit IgG at 1:10,000 (Dako, Agilent Technologies, Makham, ON, Canada). The antibodies were used and maintained as previously mentioned.

4.5 MACT Bovine APOBEC3 Proof of Concept

4.5.1 Viral Spread Assay

HEK 293T cells were seeded at 200 X 10⁴ cells in 12-well plates and transfected or co-transfected with pMOV-eGFP and/or XMRV VP62/pcDNA 3.1. After 96hrs, supernatants were collected, spun down at 2000rpm as described previously before NIH 3T3, HEK 293T and MAC-T cells, seeded at 200 x 10⁶ the day before, were infected at a MOI of 1.

4.5.2 Restriction Assay

HEK 293T and MAC-T cells were seeded at 200 x 10⁴ cells in 12-well plates and transfected or co-transfected with pMOV-eGFP or pMOV-eGFP and FLAG-C3-A3G in the case of the 293Ts only. After 72hrs, supernatants were collected and spun down as described previously and used to infect NIH 3T3 cells seeded at 200 x 10⁶ cells the day before at a MOI of 1.

For both, the viral spread and restriction assays, the infected cells were resuspended in 1X PBS-EDTA before being measured for eGFP-fluorescence 24, 48 and 72 hours by flow cytometry (Cyan ADP Flow Cytometer, Beckman Coulter) and the data was processed using Kaluza software (Beckman Coulter, Mississauga, ON, Canada).
4.5.3 Protein Expression analysis

Cells were harvested 96h after transfection, prior to infection, washed twice with 1X PBS and lysed on ice for 30min with RIPA lysis buffer (50mM Tris pH 8.0, 150mM NaCl, 1% NP-40, 0.2% SDS, 0.5% Na-Deoxycholate and 1mM EDTA) supplemented with 2X complete, EDTA-free, protease inhibitor cocktail (Roche). Lysates were resuspended in 5X Laemml Buffer and boiled prior to loading on gel. Samples were run on 10% SDS-PAGE gels and transferred to PVDF membranes. The following antibodies were used for this study: HRP-conjugated anti-FLAG and HRP-conjugated anti-β-tubulin previously described.

4.5.4 Collection and Processing of Blood Samples

Blood samples were obtained from 400 dairy and production animals in 4 cities across Canada. The samples were processed via blood fractionation by veterinarians on site. The genomic DNA was isolated using the Isolation of Genomic DNA from Buffy Coat (QIAGEN, Toronto, ON, Canada) according to manufacturer’s specifications. A clean room, where no mouse work had been previously performed was used for the processing of the samples. All surfaces were decontaminated with 5.25% Javex bleach prior to usage. Sterile filter tips were used for all experiments. The process of this project is broken down in a schematic in Figure 6.

4.6. Retrovirus screening procedure

Except for the DNA, all reagents were kept in a separate lab fridge that did not come in contact with mouse samples or the same type of murine retroviral plasmids used in our lab. For extra precaution, all mastermixes were created in another separate clean room, not previously used as a laboratory and which was decontaminated with bleach prior to being used for the first time. The DNA was added in the main lab in a separate area designated as the DNA bench, which was decontaminated with bleach prior to adding the DNA. All PCR
screens were performed using the Eppendorf Mastercycler Pro S series (Eppendorf, Hamburg, Germany).

4.6.1 Sensitivity of the PCR Screens

To determine the sensitivity of the PCR, 40ng of bovine gDNA was spiked with serial dilutions of the target DNA. The number of copies was determined using the following equation,

\[ m = n \times 1.096e^{-21} \text{g/bp} \]

where \( n \) represent the size of the target DNA and the constant \( 1.096e^{-21} \text{g/bp} \) is obtained based on the average base pair’s molecular weight of 660g/mol and Avogadro’s number. The equation outputs the mass of a single copy of the target DNA present in a PCR reaction.

The genome of the murine retroviruses and the regions targeted by our PCR assays are illustrated in Figure 2 in the Results section.

4.6.2 MLV gag PCR

The \( \text{gag} \) genes of multiple MLVs (Table S3) were aligned using Sequencher DNA Sequence Analysis software (Gene Codes Corporation, Ann Arbor, USA) and primers were designed for homologous sequences within the p30 Matrix region of the \( \text{gag} \) gene. Samples were then screened for MLV \( \text{gag} \) with a semi-nested PCR using primers Gag8-Fwd 5’-CACCAATTTGGCCAAGGTAA-3’ and Gag8-Rev 5’-GTCAGGAGGAGGTCTGGG-3’ for the 1\textsuperscript{st} round to obtain a 561bp amplicon, and the same forward primer and Gag7-Rev 5’-CTCTCCCCCTGTCTATCCTG-3’ for the 2\textsuperscript{nd} round to obtain a 436bp amplicon, using the following conditions, one cycle at 94°C for 2 min followed by 35 cycles of 94°C for 45 sec, 60°C for 30 sec or 50sec for the 1\textsuperscript{st} or 2\textsuperscript{nd} rounds respectively and 72°C for 1 min and finally, one cycle of 10 min at 72°C.
### 4.6.3 Glyco-gag GAP PCR Screen

To differentiate between XMRV and the rest of the MLVs, a nested-PCR screen targeting the glyco-Gag-coding region of the gag gene was designed to contain a 24-bp gap present in XMRV but not in most MLVs [72]. The nested PCR generated 268bp and 180bp, for the 1st and 2nd round, respectively for those MLVs lacking the 24bp, whereas XMRV-positives produced a 236bp amplicon on the 1st round and a 155bp on the 2nd round. Primers 1R-FWD GAP 5’- AACTAGATCTGTATCTGGCG-3’ and 1R-REV GAP 5’-ACGCCGCGGCGCGGTTCG-3’ were used for the first round. Primers 2R-FWD GAP 5’-CGCAGCCCTGGGAGACGT-3’ and 2R-REV GAP 5’-ACCGAAAGCAAAAAATTTCAG-3’ were used for the second round. The PCR conditions were as follows, one cycle at 94°C for 2 min followed by 35 cycles of 94°C for 45 sec, 63.2°C for 45 sec or 60.3 for 45 sec for the first and second round respectively and 72°C for 1 min, followed by one cycle of 10 min at 72°C.

### 4.6.4 Tropism Screen

The env genes of multiple MLVs (Table 6 in the Appendix) were aligned using Sequencher and three tropism screens were constructed for modified polytropic (Mpmv) and Polytropic (Pmv), Xenotropic (Xmv) and Ecotropic (Emv) MLVs. A nested PCR screen was designed for all tropism PCR assays using common forward primers pol FWD 1 5’-TGCAACTGGCACTAGGACT-3’ for the first round and pol FWD 2 5’-CTCTAAAGTAGACGGCATC-3’ for the second round in homologous areas within the integrase region of the pol gene. For the Mpmv/Pmv screen, the following reverse primers were used, M/Pmv env REV1 5’-TGATGATGATGGCTCCAGT-3’ and M/Pmv env REV2 5’-AGTCCAGTCTCTACCCAGTC-3’ for the first and second round, respectively. For the Xmv tropism screen, Xmv env REV1 5’-GAGGTATGTTGAGTAGGT-3’ was used for
the first round and Xmv env REV2 5'- CAGACACAACCAGCACTCT-3’ was used for the second round. Finally, for the Emv screen, Emv env REV1 5’- GTATGGTTGGAGTAGGTA-3’ and Emv env REV2 5’- GATTGTTGCTTACTGTGAT-3’ were used for the first and second round, respectively. The amplicon sizes for the 1st round were 1540bp, 896bp, and 1456bp and for the 2nd round, 685bp, 359bp and 1020bp for Emv, M/Pmv and Xmv, respectively.

### 4.6.5 MMTV gag PCR

A semi-nested PCR was developed using primers designed in homologous regions of the gag gene of the reference sequence of MMTV as well as recently discovered human MMTV-like tumor virus, MMTV-like betaretroviral sequences in breast, liver and lung cancers and in primary biliary cirrhosis[3, 44-47, 117]. The gag genes of multiple MMTV sequences (Table 1) were also aligned using Sequencher software. The 1st round PCR amplified a 698bp fragment that covered the matrix and capsid coding sequences of MMTV and on the second round, an inner 538bp fragment of the same region. PCR conditions were as follows, one cycle at 94°C for 2 min followed by 35 cycles of 94°C for 45 sec, 56°C for 45 sec and 72°C for 1 min 20sec, followed by one cycle of 10 min at 72°C for the first round and second round.

### 4.6.6 MMTV envPCR

The primers and PCR conditions used for this semi-nested PCR screen stem from an MMTV study by Mazzanti et al. in 2011[3] where they linked an exogenous MMTV-like env sequence to human sporadic breast carcinoma. Briefly, a nested PCR was performed to amplify a 248bp fragment of the env gene on the 1st round and a 202bp fragment on the 2nd round using the same primers and conditions as described in their paper.
4.6.7 GAPDH PCR

To confirm the presence of DNA, a PCR screen was designed to target the bovine GAPDH gene (NM_001034034.1) using forward primer 5’-GCAATTAACCTTGGCTCCTTGCT-3’ and reverse primer 5’-ATGCCAAAGTGGTTCATGGAT-3’ to produce an amplicon of 299bp. The conditions for the PCR were as follows, one cycle at 94°C for 2 min followed by 35 cycles at 94°C for 45 sec, 56°C for 45 sec and 72°C for 1 min 20 sec, followed by one cycle at 10 min at 72°C.

4.6.8 Screening for Contamination using mouse mitochondrial DNA (mtDNA) PCR

Positive gDNA samples for MLVs and MMTV were tested for the presence of mouse DNA using primers specific to Balb/C mtDNA with Lo et al’s primer set and PCR conditions[5].

4.6.9 Amplification by Differential DNA Differentiation (3D PCR)

NIH 3T3 target cells were harvested 24, 48 and 72 hours post-infection and gDNA was extracted using the Wizard Genomic DNA Purification kit (Promega, Madison, Wisconsin, USA) according to the manufacturer’s specifications. Once the gDNA was extracted, it was diluted to 5ng/µL before PCR was performed. Prior to doing 3D PCR, a first PCR was performed to amplify a 717bp region of the integrated virus’ eGFP reporter gene, using 2.5mM dNTPs, 5X Primer Star Buffer, 1UPrime Star (Takara Bio Inc., Otsu, Shiga, Japan) high fidelity polymerase, 10µM of each of the following two primers, Forward 5’-GTGAGCAAGGCGAGGAGCTGTTCA-3’ and Reverse 5’-CTTGTACAGCTCGTCCATGCCGAGA-3’ and 10ng of gDNA. All primers for this study were ordered from Sigma-Aldrich (Oakville, ON, Canada). The reaction was run in an Eppendorf Mastercycler Pro S series (Eppendorf, Hamburg, Germany) with one cycle at 98°C for 1 min, 32 cycles of 98°C for 10sec, 58°C for 5sec, and 72°C for 1 min and a final cycle at
72°C for 2min. Post completion of the PCR, the samples were run in a 1% agarose gel to confirm the right amplicon size. Samples were then diluted 1:25 using sterile commercial water and 2µL of this dilution were used for the second round, 3D PCR. The same reagents from the first round were used with the following reaction conditions, 1 cycle for 2min of a denaturation gradient from 86.5°C to 93.5°C, 32 cycles of the same denaturation gradient for 50sec, 56°C for 30 sec and 72 for 45sec, and a final cycle at 72 for 5min. Sample size of 267bp and denaturation profile were confirmed by loading the samples on a 2% agarose gel. Samples that amplified at the lowest denaturation temperature were purified, phosphorylated with T4 PNK (NEB) and cloned blunt with a 5:1 insert: vector ratio into the EcoRV site of the MCS of pBluscriptII SK+ (pBSK), dephosphorylated with Antarctic Phosphatase (NEB) using T4 DNA Ligase (NEB) with the specifications of the manufacturer. Ligations were transformed using competent DH5α cells, heat shocked at 42°C for 1min and left to grow with Luria-Bertani (LB) Broth for 1hr at 37°C before plating on LB-agar plates containing ampicillin for negative selection and X-gal with IPTG for blue-white screening. A total of 50 colonies for each amplicon cloned were grown in LB broth with 100µg/mL ampicillin for 24hrs (minipreps). These minipreps were spun down at 13,300rpm, their supernatants decanted and the pellet lysed with TENS solution (10mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0, 0.1 N NaOH, 0.5% SDS), followed by neutralization with 3M Sodium acetate pH 5.2 and centrifugation for 2 min at 13,300 rpm. The supernatants were then added to 100% ethanol kept cold at -20°C, proceeded by incubating on dry ice for 5min, and another spin at 13,300rpm for 5min. The DNA pellets were washed with 70% ethanol once and spun once again at the same speed to centrifuge any residual ethanol. Finally, the DNA was resuspended in water and

4.7 Viral Sequence Analysis

The samples were sent for sequencing to the Nanuq Sequencing Facility at McGill University and Genome Quebec Innovation Center. Sequencher software was used to process the sequencing information obtained. Sequences were analyzed for G-A mutations within the context of the human, mouse and bovine (in the case of the MACT proof of concept experiment) APOBEC3 deamination profile.

4.8 Cloning, Sequencing and Mutation Analysis of the Bovine gDNA Samples

After PCR, bands were gel extracted using the Wizard SV Gel and PCR Clean-up System kit (Promega) following manufacturer’s specifications. The purified PCR product was then cloned using the pDrive vector from the PCR Cloning Kit (Qiagen), according to manufacturer’s specifications and analyzed by sequencing and processed with Sequencher software or Nucleotide database of the National Center for Biotechnology Information (NCBI)’s BLAST sequencing software. Sequences were compared with reference sequences such as those of MoMLV, AKV MLV, XMRV and other EMLVs, PMLVs and XMLVs (Table 6 in the Appendix), and also analyzed for the presence of G-A mutations within the context of the bovine, mouse or human APOBEC3(G) deamination profile.

4.9 Statistical Analysis

All data from the infectivity and restriction assays were plotted using the standard error mean (SE) and n = 3.
5.0 RESULTS

Part I

Project 1: Role of APOBEC3 in Protecting Cattle Against Murine Retroviral Zoonosis

This part of my thesis investigates the frequency of the zoonotic transmission of murine retroviruses to cattle and the role of the bovine APOBEC3 restriction factor in preventing the spread of the infection.

5.1 Murine retroviral gag and env sequences are found in cattle

For this study, 400 production and dairy animals were selected and blood samples were acquired by veterinarians in 4 different cities across Canada (Guelph, Montreal, Charlottetown and Saskatoon) (Figure 6). The veterinarian and technicians at each respective college processed the blood samples via blood fractionation on site before the DNA was extracted from the blood fraction’s buffy coat at our premises. After the gDNA was extracted from each sample, an assay to determine if DNA was present was carried out by testing for the presence of the Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) housekeeping gene. Out of 400 samples that were originally collected and from which DNA was extracted, 325 were found to contain GAPDH. In view of the vast amount of papers citing risk of contamination, we also decided to conduct an in-house quality control assay that would rule out in-lab viral strains by targeting the eGFP gene expressed by most of our viral constructs, with the exception of pcDNA3.1/XMRV-VP62, which does not contain an eGFP reporter gene and for which a separate diagnostic assay was designed. Initially, the samples were extracted inside the hoods of our tissue culture room, which are routinely used for transfections using plasmid DNA. As a result, 49 samples were found to be laced with plasmid DNA. Thus, from an
Figure 6. Study Methodology Flow-Chart. A total of 276 production and dairy animal samples were selected from four different sites across Canada and they were processed via blood fractionation. The DNA was then extracted from the buffy coat and validated via a GAPDH screen. Once presence of DNA was confirmed, samples were screen for the gag and env genes of Murine Leukemia Viruses (MLVs) and Mouse Mammary Tumour Virus (MMTV). Finally, positive samples were screened for murine mitochondrial DNA (mtDNA) to discriminate for mouse DNA contamination and sent for sequencing to validate the presence of MLV or MMTV.
Selection of 100 Production and Dairy Animals from 4 different cities across Canada

- Guelph
- Montreal
- Charlottetown
- Saskatoon

Blood Fractionation

Buffy Coat DNA Extraction

1. Confirm for presence of DNA (GAPDH Screen)
2. Quality control screen for in-lab strains (eGFP screen)

GAPDH\(^{+}\)eGFP\(^{-}\) Samples

- MLV Screen
  - gag
  - Glycogag Screen
  - MLV\(^{env}\) Tropism Screen
- MMTV Screen
  - gag
  - env

mtDNA PCR on Positives  Sequence amplified bands
original sample size of 400 bovine gDNA samples, only 276 reliable samples remained. The samples from Guelph and Montreal were the ones initially processed in the tissue culture hoods and thus they suffered the biggest impact. Precautions were then taken as described in the Methods in order to null any future possibility of mice or plasmid DNA contamination of the remaining bovine gDNA samples. In order to determine if murine retroviruses could be zoonotically transferred to cattle, two different types of screens were performed, each with a specific PCR assay targeting MLVs and MMTV and MMTV-like viruses. Three PCR assays were designed to potentially identify MLVs and two PCR assays were established to detect MMTV-like sequences in bovine gDNA samples. See Figure 7 for a schematic showing the primer position along the targeted genomic regions of the two viruses. Sequence alignments of a vast amount of reference sequences and recently found genetic sequences from MLVs and MMTV, human mammary tumour virus, human MMTV-like mammary tumour virus, and exogenous MMTV-like viral sequences found in primary biliary cirrhosis, breast and lung cancers, retrieved from the NCBI and from Jern et al. 2007[4] were employed in the design of primers for each PCR assay. The primers were designed within highly conserved regions of the gag, env and pol genes of MLVs shown in Table 5 in the Appendix and within the gag and env genes of MMTV and MMTV-like sequences shown in Table 6 in the Appendix.
Figure 7. Primer Design Schematic for MLV and MMTV PCR Screens. A) A nested PCR strategy was designed for the XMRV-discriminatory screen (GAP) as well as for the tropism screens (env), whereas a semi-nested PCR was used to screen for the gag of MLV. B) For MMTV, semi-nested PCR strategies were used when screening for either MMTV-like gag or env sequences. The PCR screen for MMTV env was the same as used by Mazzanti et al in 2011. [1, 3] The sequences used to design the strategies in A) and B) can be seen in Tables 5 and 6 in the Appendix.
A) MLV Primer Design

B) MMTV Primer Design
5.2 The designed PCR screens can detect low copy numbers of \textit{gag} and \textit{env} viral sequences from MLVs and MMTV infected cells.

Because the integration of the murine proviruses in the bovine genome are likely to be a rare occurrence, it was important to know what copy number our PCR assays would be able to detect. In order to do this, bovine gDNA was spiked with serial dilutions of the targeted virus (MLV or MMTV), as discussed in the Methods. In the case of the MLV \textit{gag} PCR assay, the detection limit is quite sensitive since up to 1 copy of the \textit{gag} gene can be picked up in 40ng of bovine gDNA, which accounts for as much DNA as that contained within 12,500 bovine lymphocytes (Figure 8B).
Figure 8. Summary of the MLV gag Screen. A) Schematic representation of primers used to screen for the gag gene of various MLVs. The primers are outlined with half-arrows shown below the sequences. B) Sensitivity of the PCR assay: up to 1 copy can be detected. All samples, except for the water controls, contain 40ng of bovine gDNA and are spiked with no DNA to serial dilutions of pcDNA3.1/XMRV plasmid. C) Example of results from a typical screen with bovine gDNA samples from Guelph. Samples that turned out positive were also screened for murine DNA contamination. The negative control (C) denotes a sample containing only 40ng of bovine gDNA, whereas the positive control (P) consists of spiked bovine gDNA with 250pg of pcDNA3.1/XMRV plasmid. The two amplicon sizes refer to the bands expected in 1st and 2nd round PCR. "Np" stands for no plasmid (only cow DNA) and "un" stands for undiluted pcDNA3.1/XMRV DNA starting from 2.5ng.
The MLV env tropism assays are also able to detect 1 copy of ecotropic (Emv) endogenous MLVs. However, for the Polytropic and modified polytropic (M/Pmv) and xenotropic (Xmv) MLVs, the copy number detection decreases in sensitivity substantially due to the low specificity of the primers. For instance, in the case of the Pmv and Mpmv primers, env amplification is only observed when there are at least 1000 copies of the genomic DNA and in the case of the Xmv primers, the sensitivity decreases further to needing 10,000 copies of genomic DNA to pick up amplified Xmv env (Figure 9).
Figure 9. Summary of MLV tropism (env) PCR screen. A-D) Schematic representation of primers used to screen for the env gene of various Emv, M/Pmv or Xmvs (Table 6 in the Appendix). The primers are outlined with half-arrows shown below the sequences. The screen used primers for the end of the pol gene and the beginning of the env gene. The base positions are based on MoMLV Reference sequence (NC_001501.1)(B), Pmv2 (C) and Xmv8 (Pmv and Xm sequences obtained from reference [4]). (D). E-G) Sensitivity of the PCR assay: up to 1 copy can be detected in the case of Emv screen (E), 1000 copies in the case of M/Pmvs (F) and up to 10,000 copies in the case Xmvs (G). All samples, except for the water controls, contain 40ng of bovine gDNA and are spiked with no DNA to serial dilutions of pMOV-eGFP plasmid (E), and Balb/C gDNA (F,G). "Np" stands for no plasmid (only cow DNA) and "un" stands for undiluted pMOV-eGFP plasmid starting from 13pg or undiluted 0.63pg of Balb/C gDNA or 1.5ng of pcDNA-XMRV.
Early in 2011 XMRV was discovered to be a laboratory contaminant rather than a newly found feral virus. Thus, we developed an assay based on a screen by Coffin et al. [72], with the purpose of discriminating between the gag of XMRV and that of other MLVs amongst those samples positive for the presence of MLV gag in the MLV gag PCR assay. The sensitivity of this assay was high since up to 10 copies of the gag sequence can be detected in 12,500 copies of bovine gDNA (Figure 10B and C).
Figure 10. Summary of MLV glycoag gly PCR screen. A) Schematic representation of primers used to differentiate between XMRV and the rest of the MLVs. The primers are outlined with half-arrows shown below the sequences. The base positions are based on the MoMLV genome reference sequence NC_001501.1. C) Sensitivity of the PCR assay: up to 10 copies can be detected. All samples, except for the water controls, contain 40ng of bovine gDNA and are spiked with no DNA to serial dilutions of 7.4pg of pcDNA3.1/XMRV (B) or 6.7pg pMOV-eGFP plasmid (C).
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**MUMLY Plasmid**

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56
The same degree in sensitivity (10 copies) is found in both MMTV gag and env PCR assays. In this case, the type of DNA that was used to spike the bovine gDNA was gDNA extracted from a HEK 293T cell line previously infected with MMTV (Figure 11B, C).
**Figure 11. Summary of MMTV gag and env PCR screens.**

A, B) Schematic representation of primers used to screen for the *gag* (A) and *env* (B) gene of MMTV and other MMTV-like sequences. The primers are outlined with half-arrows shown below the sequences. The base positions are based on a consensus set to the MMTV genome reference sequence NC_001503.1. The *env* primers were obtained from Mazzanti et al.[3] C, D) Sensitivity of the PCR assay: up to 10 copies can be detected in both *gag* (C) and *env* (D) PCR screens. All samples, except for the water controls, contain 40ng of bovine gDNA and are spiked with no DNA to serial dilutions of 2ng of gDNA from a HEK 293T cell line infected with MMTV. "Np" stands for no plasmid and "un" stands for undiluted.
5.3 Murine Leukemia Virus *gag* and Mouse Mammary Tumour Virus *env*

Sequences are present in bovine gDNA samples

A total of 11 bovine gDNA samples, accounting for 4% of the 276 bovine gDNA samples, were found to contain MLV *gag* (Table 1). Of these MLV *gag* positives, 3 of them amplified the right size XMRV glyco-*gag* amplicon in the glyco-*gag* GAP PCR assay (Figure 10). These results were confirmed via sequencing analysis as well, where the pre-XMRV2 sequence clearly shows that XMRV is present in these samples. The pcDNA3.1/XMRV-VP62 viral construct does not encode a reporter eGFP tag. The initial quality control screen responsible for removing any possible viral plasmid contamination would not have discriminated against pcDNA3.1/XMRV-VP62-containing samples. Since XMRV is considered to be a contaminant, these samples were initially meant to be discarded from the total MLV *gag* positives but they were kept for murine mtDNA testing and future studies. Furthermore, no MLV *env* was detected and the sensitivity for this screen was very low. Six samples which constitute 2.2% of the 276 samples, displayed the correct size amplicon in the MMTV *env* PCR assay. However, despite the high sensitivity seen in the MMTV *gag* PCR assay, no MMTV *gag* was detected in the bovine gDNA samples (Figure 11C).
Table 1. **Summary of PCR Screens with Positive Results.** A total of 276 samples of bovine gDNA were assayed for the *gag* gene and of MLVs and *env* gene of all MMTV-like sequences. There were 11 (4%) samples found positive for MLV *gag* and 6 (2.2%) for MMTV *env*. 
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</tr>
<tr>
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<td>1</td>
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5.4 No conclusive positives for the presence of murine mitochondrial DNA were found amongst the bovine gDNA samples previously positive for MLV gag and MMTV env

After conducting a PCR assay on all of the MLV gag and MMTV env positives, none of the samples contained the right size amplicon for the 2nd round of the mtDNA PCR. These samples were thus kept for the next step in this study: determining whether these viruses had indeed integrated into the genome of the cows (Figure 12).
Figure 12. No definitive positive is found for the presence of murine mtDNA among samples found to be positive for MLV gag or MMTV env. A) Samples positive for MLV gag and B) samples positives for MMTV env were assayed for the presence of murine mtDNA, performed as previously shown[5]. The P stands for the presence of Balb/C gDNA only, with no bovine gDNA.
5.5 Murine Leukemia Viruses are restricted by the bovine APOBEC3

5.5.1 Murine Leukemia Viruses are able to infect Bovine Cells

After having found murine retroviral sequences in the 276 screened blood samples, we asked if murine retroviruses would in fact be capable of infecting and spreading in bovine cells cultures in vitro. We obtained a bovine mammary cell line from Dr. Lacasse of Agriculture Canada. The MAC-T cells were cultured in decomplemented media to make sure that no immune system factor such as complement proteins or neutralizing antibodies would aid the MAC-Ts in counteracting the murine retroviral infection. HEK 293T cells were used to produce MoMLV in conjunction with XMRV. Please refer to Methods for an in-depth description of the experimental setup. Given that the envelope of a retrovirus assembles at the plasma membrane, both of these gammaretroviruses would express envelope proteins at the cell surface. Since both viruses are produced in the same cells, the viruses erupting from the cells would contain a combination of MoMLV and XMRV glycoproteins (Figure 13). MoMLV is an ecotropic virus, which gains access to target cells via the murine cell receptor mCAT-1. The CAT-1 receptor present in mammalian cells is not permissive to ecotropic MLV entry. On the other hand, XMRV enters host cells through the XPR-1 receptor, constitutively expressed in many animal species, including mammals[48, 49, 75, 118]. Consequently, the XPR-1 is a functional entry receptor in both human and bovine cells. The goal of this proof of principle was to see if these gammaretroviruses co-expressing both envelopes would be able to infect human (HEK 293T) and bovine (MAC-T) cell lines. The infection was monitored via the fluorescence stemming from the eGFP reporter protein expressed by the MoMLV-encoded pMOV-eGFP viral construct.
The target murine NIH 3T3 cell line was efficiently infected as shown by a fluorescence reaching approximately 60% within the first 24hrs. However, the HEK 293T and MAC-T cell

**Figure 13. Proof of Principle Experiment.** Briefly, two viral vectors encoding MoMLV and XMRV were co-transfected into 293T cells and the produced chimeric viruses were used to infect target 293T, MACT and 3T3 cells.
lines had very different infectivity profiles from that of the NIH 3T3 positive control. Both the HEK 293T and MAC-T showed that they were indeed infected. Interestingly, the MAC-T target cells showed that the infection reached a maximum of approximately 60% at the 48hr time point. This was of special interest because cows like other animals encode APOBEC3. Since APOBEC3 is one of the first intrinsic immune factors involved in fending off retroviral infection, we were interested in studying if APOBEC3 was indeed linked to the observed plateau.

5.5.2 Murine Leukemia Viruses are restricted by the bovine APOBEC3

To perform this experiment, both HEK 293T and MAC-T cells were used for the production of viral particles. HEK 293T are known to be devoid of any APOBEC3 proteins[101]. Thus, they were used as a negative control for the production of Moloney viral particles without packaged APOBEC3 proteins. They were also used as a positive control for APOBEC3 restriction by co-transfecting them with a MoMLV and hA3G construct. See Methods for details of this experiment. The bovine cell line MAC-T was transfected only with the Moloney construct. Forty-eight hours post-transfection, the supernatants of the three sets of producer cells (HEK 293T-producing Moloney only, HEK 293T-producing both hA3G and MAC-T cells producing MoMLV) were harvested and used to infect NIH 3T3 cells (Figure 14). The infection was monitored once more through flow cytometry via the expression of integrated eGFP reporter gene from the Moloney viral construct at 24-, 48- and 72-hour time points. The results were remarkable in that only those NIH 3T3 cells infected by the supernatant of the negative control HEK 293T producer cells emitted high levels of fluorescence. Both sets of NIH 3T3s infected with supernatants from HEK 293T-producing
Figure 14. MACT cells can be infected by murine leukemia viruses but bovine APOBEC3 present in MACT cells hinders the spread of the infection. A) HEK 293T, MACT and NIH 3T3 cells were infected with supernatants from HEK 293T cells co-transfected with pMOV-eGFP and pcDNA3.1/XMRV viral constructs. B) The infectivity of MoMLV is hindered by a factor in MACT cells’ supernatant. HEK 293T cells were transfected with pMOV-eGFP or co-transfected with pMOV-eGFP and pFLAG-APOBEC3G and MACT cells were transfected with pMOV-eGFP and their supernatants were used to infect NIH 3T3 cells. Infectivity was tracked through the expression of the eGFP protein encoded in the pMOV-eGFP construct and measured via flow cytometry every 24hrs until 72hrs. C) Western of producer cells showing the expression of FLAG-APOBEC3G. D) 3D PCR mutation profiles of 3T3 mouse fibroblasts infected with Moloney MLV produced alone or in the presence of human APOBEC3G in 293T cells and with Moloney MLV produced alone in bovine MACT cells.
MoMLV and hA3G and MACT-producing only MoMLV, respectively, showed a stalled infection, only reaching 1% at the 72-hour time point in the case of the latter.

The gDNA of these NIH 3T3 cells was extracted at each of the aforementioned time points and 3D PCR was performed on the extracted gDNA at the 24hr time point. Remarkably, the gDNA from the NIH 3T3 cells infected with supernatant from MoMLV-producing MACTs shows more bands that of the negative control gDNA (NIH 3T3 cells infected with supernatant from MoMLV-producing 293Ts). To confirm these results, the lowest amplified band was cloned via TA-cloning and 24 clones, representing 24 individual sequences, were sent for sequencing. A total of 39 mutations were found amongst the 24 sequences. Furthermore, 33 of these 39 were G-A mutations (Tables 2 and 3). Closer examination revealed that these 33 mutations have a clear bovine APOBEC3 deamination footprint context, which is 5'-TGC-3' (Table 4). This is compelling evidence that the MoMLV virus is being suppressed by the strong deamination activity of the bovine APOBEC3 present in the MACT cells. These *in vitro* results provide a great example of how zoonosis is stalled in cows that interact with the feral and domesticated mice they come in contact with throughout their lives.
Table 2. Direct Sequencing Confirms G-to-A Mutations. 3D-PCR bands that amplified at the lowest temperature were column purified and cloned blunt into pBluescript. DNA clones were sequenced and analyzed for G-A mutations in the context of an APOBEC3 deamination footprint. Each sequence stems from a 281bp amplicon.
<table>
<thead>
<tr>
<th>No. of Sequences Analyzed</th>
<th>Percent of Mutated Sequences (%)</th>
<th>No. of G-A Mutations</th>
<th>Base Pairs Sequenced*</th>
<th>No. of bp in Mutated Sequences</th>
<th>Frequency of G-to-A Mutations/kb</th>
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Table 3. Extensive G-to-A mutations indicative of APOBEC3-induced cytidine deaminase activity in MLV-infected MACT cells. Out of a total of 39 sequences analyzed, 24 were mutated. Each analyzed sequence represents a single colony. The data was analyzed and collected as detailed in Table 2’s caption.
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<td><strong>C</strong></td>
<td>1</td>
<td>0</td>
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Table 4. The Footprint of Bovine APOBEC3 5’-TGC-3’ is readily observed amongst the G-to-A mutations. The preferred deamination signature of Bovine APOBEC3 is 5’-TGC-3’, further supporting a role for bovine APOBEC3 inhibiting the spread of MLV in MACT cells.
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<td>C</td>
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Results Part II

Project 2: Role of mouse APOBEC3 in the Inhibition of Murine Retroviral Infection

In this second part of my thesis, I characterize factors that render murine gammaretroviruses resistant to deamination by mouse APOBEC.

5.6 Unlike MoMLV, AKV MLV is restricted and hypermutated by mouse APOBEC3

Moloney MLV and AKV MLV are two gammaretroviruses that are 95% homologous. Yet, their response to the restriction by mA3 is markedly different. Whilst MoMLV is able to resist restriction by mouse APOBEC3, AKV MLV is severely hindered. Interestingly, the infectivity of both viruses is inhibited in the presence of mA3 in the first 24 hours post-infection. Yet, this inhibition is clearly a product of deamination-independent restriction since deamination-dead mutants of both human APOBEC3G and mouse APOBEC3 are clearly inhibiting the viruses at the same level as the wild type proteins (Figure 15A, B).

It is at the 48 hour time point that both viruses differ. Moloney is able to overcome mA3 restriction and proliferate, but AKV MLV remains repressed. Furthermore, Moloney is not hypermutated by mouse APOBEC3 whereas AKV MLV is, as can be seen by the 3D PCR results (Figure 15E and F). The protein expression levels of the APOBECs are comparable for both restriction assays. However, the two viruses possess a strong sequence homology and thus we speculate that remaining differences between the genomes of these viruses could account for the different sensitivity to restriction. For this reason, we developed chimeras between MoMLV and AKV.
Figure 15. Unlike MoMLV, AKV MLV is restricted and hypermutated by mouse APOBEC3. A,B) Virus-producing cells were harvested, lysed and assayed for the presence of FLAG-tagged APOBECs and β-tubulin. Supernatants of the same virus-producing cells were filtered and spun at ultra speed before being lysed and assayed for the encapsidated FLAG-tagged APOBECs and for the presence of viral capsid p30. C, D) Restriction Analysis of MoMLV and AKV MLV measured by the expression of eGFP in the target cells A) and B). E, F) AKV MLV is sensitive to deamination by mouse APOBEC3 but MoMLV is resistant. 3D PCR mutation profiles of MoMLV and AKV MLV’s eGFP genes after extracting gDNA of 3T3 mouse fibroblasts infected with MoMLV or AKV MLV in the presence of hA3G E259Q, hA3G wild type and mA3.
In order to determine the genomic region responsible for AKV MLV's sensitivity, regions of the AKV MLV viral construct were cloned (Figures 16 and 17) into MoMLV's viral vector, as described in the Methods. The plan was to change MoMLV from being resistant to being sensitive to mA3 deamination.
Figure 16. Schematic representation of main constructs, chimeras and glycosylation mutants. Genomic map of A) AKV-NB-eGFP and chimeras (denoted by H1 to H3); B) pMOV-eGFP; C and D) Glycosylation mutants in predicted N-linked glycosylation sites in the gag gene of MoMLV and AKV MLV. E) Leader codon mutant in which the alternative initiation codon CTG was converted to CTA to prevent expression of glyco-Gag.
Figure 17. Schematic representation of MoMLV and AKV MLV chimeras. Genomic regions of MoMLV were exchanged for those of AKV MLV as detailed in the methods. A) Hybrid 1 MLV (pMOV-AKV Hybrid-U5) contains the AKV-NB-eGFP’s U5 region and CUG glyco-Gag initiation codon. B) Hybrid 2 MLV contains the same genomic sequence of AKV as Hybrid 1 with the addition of the protease of AKV MLV. C) Replicative MLV Hybrid 3 contains the same genomic regions as Hybrid 2 and also the pol gene up to the restriction site PmlI upstream of the Env gene of AKV.
5.7 Resistance to mA3 maps to the glyco-Gag region of MoMLV

All of AKV MLV’s genomic regions, with the exception of the LTRs and the env gene, were sequentially cloned into a Moloney-based vector (pMOV-eGFP). Interestingly, only those hybrids where the AKV gag gene was swapped were mutated by mouse APOBEC3, as shown by 3D PCR (Figure 18). Interestingly, the gag genes between these two gammaretroviruses do differ slightly.

Gammaretroviruses, like AKV MLV and MoMLV, encode an additional protein called glyco-Gag or gPr80\textsubscript{Gag} that is translated from a non-conventional codon, CUG, upstream of the AUG that expresses the pr65 Gag protein, as mentioned in the introduction. The gPr80\textsubscript{Gag} harbours in its N-terminus a leader peptide that sequesters the protein to the endoplasmic reticulum where it will be glycosylated. There are three putative N-linked glycosylated residues in MoMLV’s gag gene (N113, N480 and N505). These residues are found in MoMLV’s MA (N113) and CA (N480 and N505) [9, 23]. AKV MLV and MoMLV share a strong sequence identity[40] and these residues happen to be found in AKV MLV’s MA as N113 and in its CA as N482. However, AKV MLV does not contain the third predicted N-linked glycosylation site; it is instead present as a serine (S507) in AKV MLV’s CA (Figure 19). The glyco-Gag protein continues to be of interest to the retrovirology field because its role has not conclusively been pieced together. For instance, mutants lacking this glycosylated form of the Gag are capable of replicating and remain infectious. However, they display defective morphology features of their CA and are unable to overcome restriction by the murine host factor APOBEC3[119]. In light of the result with the hybrids, point mutants were generated in the putative N-linked glycosylated sites mentioned above to
see if the individual glycosylation sites played a role in resisting mA3-dependent deamination. The point mutants generated can be seen in Figure 16.
Figure 18. Viral Spread of Hybrids and Western blots. 

A-C) Hybrids 1-3 are infectious and continue to spread in the presence of mouse APOBEC3 but remain inhibited by human A3G. G-I) Unlike Hybrid 1 (G), Hybrids 2 and 3 (H, I) have a clear mutation profile as shown by the presence of additional 3D-PCR bands compared to the negative control (E259Q). D-F) Protein expression of APOBEC and retroviral proteins in cell lysates and virions.
Figure 19. Alignment of MoMLV and AKV MLV gPr80 proteins. Using ExPaSy and NCBI the gPr80 nucleotide sequences of MoMLV and AKV MLV were translated and aligned using NCBI’s BLAST software. The arrows depict the three putative N-linked glycosylation sites, N113, N480 (MoMLV)/N482 (AKV MLV) and N505 (MoMLV)/S507 (AKV MLV).
Briefly, three single mutants N113Q, N480Q and N505Q were generated for MoMLV as well as double mutants, N113Q/N480Q, N113Q/N505Q, N480Q/N505Q and finally the triple mutant N113Q/N480Q/N505Q from the pMOV-eGFP plasmid using SDM as described in the Methods section. For AKV MLV, single mutants N113D, N482D and S507N were generated in AKV MLV as well as the double mutant N113D/N482D. Whereas N113D, N482D and the double mutants N113D/N482D were generated with the intention of removing glycosylation, the purpose of the single mutant S507N was to restore glycosylation at that site. MoMLV's double mutant N113Q/N505Q and the MoMLV's triple mutant N113Q/N480Q/N505Q will be labelled as MoMLV 2X and MoMLV 3X, respectively, for simplicity throughout this thesis.

Notably, after experiments testing the expression of the eGFP gene of these mutants via the use of flow cytometry, it became apparent that mutants at residue N480 in the case of MoMLV or residue N482 in the case of AKV MLV could not be used for future experiments. Despite the p30 levels in the supernatant of producer cells being the same, these mutants were non-infectious (Figure 20). The possible reasons for this will also be tackled in the discussion. Finally, in addition to these point mutants, another mutation was generated in both viruses which changed the non-conventional initiation codon for glyco-Gag from CTG to a CTA. These mutants are labelled as MoMLV CTA and AKV MLV CTA throughout this thesis.
Figure 20. N480 residue of the MoMLV Gag protein is functionally important for infection. A) NIH 3T3 cells were infected with the supernatant of HEK 293T cells transfected with the various mutants of MoMLV. CA p30 levels are similar for all mutants. B) Mutation of AKV MLV N482 residue also renders AKV MLV non-infectious. The AKV MLV N482D mutants were also infected as described in A).
In contrast to the hybrids, the glycosylation mutants display sensitivity to mA3 restriction up to the 48h time point. However, all MoMLV mutant viruses eventually recover at the 72h time point. The importance of the glycosylated sites that we first witnessed with the hybrids is corroborated by the 3DPCR results of the single mutants of MoMLV, which show the same 3D PCR band pattern as that of the hybrids containing the AKV MLV gag gene (Figure 21-23). Mutating just one putative N-linked glycosylation site renders the normally deamination-resistant MoMLV susceptible to mA3- mediated G-A mutations. What is even more interesting is that when more than one glycosylation site is mutated, the deamination pattern becomes more apparent. This is observed in the case of MoMLV 2X and MoMLV CTA as well as AKV N113D and AKV CTA. In the presence of mA3, these viruses sustain much more mutations compared to the wild type viruses, going from 2 bands in the case of MoMLV to 4 bands in MoMLV 2X and MoMLV CTA. Not surprisingly, the mutation burden also changes in the presence of hA3G, where more bands denoting the increase in G-A mutations are seen in less intensely glycosylated mutants like MoMLV 2X, MoMLV CTA, AKV N113D and AKV CTA. Of note, changing S to N at position 507 of AKV confers resistance against G-A mutations presumably because a putative glycosylation site is created. This data overwhelmingly supports a role for the glycosylated Gag protein in the resistance against mA3-mediated deamination.
Figure 21. MoMLV Single Glycosylation Mutants are sensitive to mA3-induced Deamination and their deamination profile is identical to that of AKV MLV. A,B) Infectivity spread of single mutants MoMLV N113Q and N505Q. C,D) Despite mutations, APOBEC proteins still encapsidate within MoMLV mutants. Protein expression of retroviral and APOBEC proteins in the lysates and virions. E, F) MoMLV N113Q and N505Q single mutants are sensitive to deamination by mA3.
Figure 22. MoMLV Double Glycosylation Mutant and the CTA mutant have an increased sensitivity to both mA3- and hA3G-Induced Deamination. A,B) Infectivity spread of MoMLV CTA and N113Q/N505Q double mutant (MoMLV 2X). C,D) Despite additional loss in putative N-linked glycosylated residues, APOBEC proteins still encapsidate within MoMLV CTA and 2X mutants. Protein expression of the retroviral and APOBEC proteins is consistent in the lysates and virions. E, F) MoMLV CTA and 2X mutants are highly sensitive to deamination by mA3 and increasingly mutated by hA3G.
Figure 23. AKV MLV Site Directed Mutagenesis Mutants have an increased sensitivity to both mA3- and hA3G-Induced Deamination but the S507N mutant with restored Asparagine shows resistance to mA3’s deamination. A-C) Protein expression of retroviral and APOBEC proteins is consistent in virions and lysates no matter the type of mutation, whether removing glycosylation (AKV MLV N113D and AKV MLV CTA) or restoring it (AKV MLV S507N). D-F) AKV MLV N113D and AKV MLV CTA mutants are highly sensitive to deamination by mA3 and hA3G as evidenced by the increased number of amplification products along the 3D-PCR gradients in contrast to the deaminase-dead negative control (E259Q), whereas S507N appears to not be mutated by mA3 (only two PCR amplicons).
6.0 DISCUSSION

Most of what we know of retroviral zoonosis stems from studies on HIV-1. However, other cases have been documented[79, 82]. Other than studies that characterized the presence of MLV-like bovine retroviruses[120] or MMTV-like betaretroviruses in horses[121], no other study has conclusively documented a zoonotic transmission from mice to cattle. A recent study attempted to find xenotropic as well as polytropic retroviruses in animal workers exposed to mice and found negative results. However, this negative result is perhaps because they strictly used XMRV-specific primers to amplify MLV sequences[122]. In our study, we designed highly sensitive screens that can detect between 1-10 copies of target MLV or MMTV DNA (Figures 8-11). With these screens, we are able to see that murine retroviral zoonosis is surprisingly common - six percent of the 276 bovine gDNA samples were positive for murine retroviral sequences (Table 1).

When we put this into perspective of the vast amount of cattle used for the production of meat and dairy in North America, a total of approximately 100 million cows and calves, six percent signifies six million cows and calves. Is it possible that all those animals are actually infected with murine retroviruses? This may not necessarily be the case. For instance, calves are usually fed whole milk from the herd. Since MMTV can be transmitted via milk-born virions, it is possible that many of the calves would be infected with the same viral source [123]. However, since the viral amplicons obtained with our screens were not found to be replicas of one another or the same as the reference viral sequences, we concluded that a germline infection of the cattle by murine retroviruses is unlikely. Rather, these viruses are probably spreading through somatic cell-to-cell infections. Perhaps the viruses are gaining entry through the gut before spreading through the lymphatic system, as is the case with MMTV. Also, cows remain
in constant contact with feral mice. Mouse feces, urine, blood and saliva are a likely source of murine retroviruses that are either ingested by the cows, or can find ways into their blood stream, in wounds for example[123].

Our *in vitro* results also provide evidence that somatic cell-to-cell spread of murine retroviruses is possible in bovine cells (Figure 14). Remarkably, this experiment also showed that it is possible for ecotropic MLVs to cross the species barrier if in the presence of an MLV capable of infecting mammalian cells. This is of interest because murine cells harbour many different types of retroviruses, including MLVs with different cell tropisms. Therefore, ecotropic MLVs are constantly produced jointly with polytropic and xenotropic endogenous retroviruses. This would mean that their envelopes would be coated with a mixture of xenotropic, polytropic and ecotropic glycoproteins, allowing these chimeric retroviruses to gain entry into foreign cells (Figure 13). Furthermore, other studies have also shown that chimeric retroviruses can be released from cells. For instance, as mentioned in the introduction, retroviral RNA is packaged in a dimerized form that occurs via complementary interactions that require the Psi packaging signal (reviewed in [9]). If a packaging signal is homologous between two different retroviruses, then dimerization is possible[124]. This phenomenon has been observed with porcine retroviruses, which have been shown to co-package with murine retroviral RNA[125].

Our study highlights how retroviruses, even those with limited tropism, may infect cells for which they do not have a genome-encoded cognate ligand. Retroviruses are formidable pathogens because of their short infection cycle. Within 4 - 6 hours after entering a host cell, the retrovirus begins replicating. After 8 hours, *env* mRNA can be detected and protein starts to accumulate at the surface of the cell and by 24hrs, maximal replication potential is reached.
[126-128]. Unfortunately, the innate and adaptive components of the immune system struggle to neutralize such fast-acting viruses when exposed to them for the first time. A potential risk to humans is that a broad tropism retrovirus enters the food chain. Nevertheless, mammalian cells have developed a set of intrinsic mechanisms to counteract the attacks of retroviruses at the early stages of their replication cycles. Such intrinsic mechanisms were mentioned in the introduction and include the actions of the APOBEC3 proteins. We were therefore interested to determine if the bovine APOBEC3 (bA3) protein could be responsible for the prevention of murine retroviral infection.

We indeed observe that bA3 is crucial in the fight against exogenous retroviral spread. The eGFP reporter gene amplified from MLV-infected NIH 3T3s was peppered with mutations that were reminiscent of bA3 as visualized by the 3D PCR data (Figure 14, Tables 2-4). Interestingly, we can clearly observe the difference in the mutation load when comparing the 3D PCR pattern of bands obtained from the NIH 3T3 gDNA sample infected with MACT supernatant and that of the hA3G positive control (Figure 14D). This result indicates that bA3 likely uses both deamination-dependent and -independent modes of restriction against MLVs. In contrast, we do not observe many G-to-A mutations typical of bA3 in the MLV gag amplified sequences from gDNA of the processed blood samples. However, the viral genome is not equally deaminated with the same intensity. For instance, the 5' end of the retroviral genome is weakly deaminated compared to the 3' end, as observed previously by our lab and we only analyzed the 5' end in our assay[129]. Nevertheless, the in vitro experiments still shed light on the very likely mechanism by which zoonotic transmission of murine retroviruses to cattle is halted. In addition, only six sequences were sent per MLV gag positives amplified from the screening assays and we only looked at a very small portion of this gene. In future
studies, after uncovering the integration sites of the retroviral sequences in this study, the genome of these viruses will also be sequenced via deep sequencing. As expected, however, we find no G-to-A mutations in the MMTV env sequences, which is consistent with results in the literature[130]. Together, the results from part 1 were meant to be a preliminary study to show that murine retroviral inter-species transmission is possible and that bA3 is capable of mutating and halting exogenous retroviral spread.

Just like the exogenous MLVs trying to spread in bovine cells, endogenous retroviruses are simply retroviruses that gained entry into germ cells and integrated into their genomes millions of years ago. Throughout the years, many of them have become inactive. It is said that 8% of the human genome is composed of retroviral DNA (reviewed in [61]). In mice, 10% of their genome is composed of endogenous retroviruses but in contrast to humans, the large portion of these are fully replicative (reviewed in [131]). A3 proteins are responsible for the regulation and control of transposable elements as well as endogenous retroviruses (reviewed in [132]).

Our study also focuses on the restriction by mouse APOBEC3 of two fully infectious mouse retroviruses, gammaretroviruses MoMLV and AKV MLV, which cause leukemia in mice. MoMLV and AKV MLV evade the effects of mA3 since they are persistent in many mouse strains and in the Akr mice, respectively. In vitro, both of these viruses display different sensitivities to mA3. Though both viruses share a high level of homology, MoMLV is not restricted or hypermutated by mA3 whereas AKV MLV is. We observe this in our study at the 48h time point (Figure 15A, B), where MoMLV is able to overcome the inhibition by mA3, but AKV MLV is not. We also observe that MoMLV does not appear to be mutated but AKV MLV does (Figure 15 E, F). These observations have also been previously reported[40]. Interestingly, however, at the 24h time point, both viruses are restricted by mA3 (Figure 15A,
B). This is likely the product of deamination-independent restriction since the deamination-dead mutants, hA3G E259Q and mA3 E73A, are able to restrict both viruses as well. This has not previously been discussed in the literature because studies have usually been conducted at the 48h infection time point[40, 133, 134].

To investigate why these viruses behaved so differently in the presence of mA3, we decided to generate chimeras that would allow us to pinpoint the genomic region that makes AKV MLV susceptible to hypermutation and restriction by mA3 (Figure 17). The hybrid's 3D PCR data clearly shows that the region responsible for AKV MLV's sensitivity to deamination by mA3 lies within the gag gene because only those hybrids containing the gag gene of AKV MLV shared the same deamination sensitivity to mA3 as AKV MLV's (Figure 15F and 18 G-I).

The gag gene of gammaretroviruses contains two different initiation codons, a conventional one that produces a 65kDa protein and a CUG, a non-conventional codon, from which a larger glycosylated 80kDa protein is produced. The latter is called glyco-Gag or gPr80Gag. Other groups have argued that gPr80Gag could be responsible for the resistance against restriction observed in the case of MoMLV[135-140]. Remarkably, after analysis of the protein sequence of MoMLV, we found that MoMLV contains three putative N-linked glycosylation sites, whereas AKV MLV only harbours two (Figure 19). Using SDM, we generated glycosylation mutants in the gag gene that aimed to either remove or restore N-linked glycosylation in MoMLV and AKV MLV, respectively. When the MoMLV mutants were tested using infection-spreading assays, they showed a decrease in infectivity after 48h, similar to AKV MLV. However, as expected, the mutants eventually grow out. In contrast, the wild type as well as the mutants do not grow out in the presence of human A3G.
The most detrimental A3-induced hypermutations in the viral genome are those that generate stop codons. Mutations caused by hA3G, for instance, are known to result in a high content of stop codons in the coding sequence. By comparison, the mA3 and bA3 mutation load results in less frequent stop codons. This is because hA3G prefers to mutate 5'-CCC-3' trinucleotides, whereas mA3 and bA3 preferentially deaminate the C in a 5'-TC-3' and 5'-GC-3' dinucleotide contexts, respectively. Thus, mA3-induced mutations do not exert the same inactivating potency than A3G mutations, which explains why though hypermutated, hybrids 2 and 3, and AKV MLV continue to spread past the 48h time point.

Like the hybrids, the single glycosylation mutations of MoMLV showed the same amplification gradient profile as AKV MLV's (Figure 15F and 21E,F). Oddly, mutating MoMLV N113 also renders the virus sensitive to mA3-induced deamination. This is bizarre because mA3 is found at the core of the virion. However, N113 is located in the N-terminus of MoMLV's MA (reviewed in [9]). The MA N-terminus is myristoylated so that it may interact with the envelope. Unless N113 were to actually point towards the inside and interact with the core like the residues in the C-terminus of MA, it is unlikely it would be in contact with core-packaged mA3 (reviewed in [7]). A recent paper has argued that the glyco-Gag is needed for core stability. By preserving a rigid core, the glyco-Gag prevents mA3 from accessing and eventually mutating the DNA in the RTC[137]. If this is the case, if N113 does not point toward the core, then a glycosylated N113 could help preserve the stability of the viral core instead. However, our study seems to point out that a completely glycosylated gPr80Gag is absolutely needed to avoid deamination. Therefore, glycosylated N113 contributes to inhibit mA3 catalytic activity.
Interestingly, despite its lower deamination toll, mA3 is still able to restrict other MLVs like FrMLV and the murine betaretrovirus MMTV in vivo. This suggests that mA3 inhibits these viruses mainly through deamination-independent methods[116, 130]. Like other MLVs, FrMLV also possesses a glycosylated Gag (reviewed in [7, 9]). Curiously, FrMLV contains the same number of putative N-linked glycosylation residues as AKV MLV but is rarely mutated by mouse APOBEC3 (Figure 24 in the Appendix). Therefore, in light of the 3D PCR results from the single glycosylation mutants and AKV MLV (Figures 15F and 21E, F), MLVs require a completely glycosylated glyco-Gag to resist deamination. Notably, though MMTV does not contain a glyco-Gag, it does however encode for two accessory proteins, Sag and Rem, which are actually glycosylated. This would suggest a novel use of the post-transcriptional machinery by murine retroviruses: to resist inhibition to deamination by the APOBEC3 proteins.

Adding weight to the importance of glycosylation, both MoMLV and AKV MLV mutants with more than one glycosylation site missing exhibited amplification at a broader denaturing temperature gradient; more bands were apparent in the case of those mutants with additional N-linked glycosylation sites mutated (Figures 22, 23). This seems to point out that when less glycosylation sites are present, the more deamination can occur. It also seems to point out that a fully glycosylated Gag protein is needed to avoid mutation by mouse APOBEC3. This is supported by the 3D PCR results obtained with the AKV MLV S507N mutants, which did not have detectable levels of mutations (Figure 23F). Curiously, the lack of glycosylation also influences the mutation load of human A3G on both MoMLV and AKV MLV. Deamination increases dramatically so that amplification is observed for the entire gradient in those mutants with fewer N-linked glycosylation sites (Figures 22E, F and 23G, H). Likely, both mA3 and
hA3G are able to bind to the capsid or p30 of MLVs, where they are either inhibited by glycosylation via steric hindrance, as previously postulated [137], or by a catalytic inhibition of these two proteins as we suggest. In addition, interaction with the glycosylated residues might bring in an unknown co-factor. This co-factor might also explain why mA3 is more inhibited than hA3G.

However, despite the overwhelming deamination-dependent phenotype observed in the 3D PCR data, it remains unclear why the MoMLV CTA and AKV MLV CTA mutants that are completely devoid of gPr80\textsuperscript{Gag} and very sensitive to deamination, are still capable of spreading in culture (Figures 15A,B, F, G, 22A, E and 23B, E). In the case of human A3G, its potent deamination activity is what renders MoMLV non-infectious. If the glyco-Gag protein protects the virus from deamination, then it would be expected that glyco-Gag-deficient mutants would succumb to mA3-induced hypermutation in a similar fashion.

Furthermore, while testing the mutants, we made an interesting discovery. Every time the putative glycosylation site at position N480 (MoMLV) or N482 (AKV MLV) is mutated, the infection of either virus is trumped (Figure 20). Residue 480 seems to be important for infection because the viruses which contain the N480Q or N480D mutation are not infectious compared to the wild-type viruses, in spite of being produced at similar levels as the wild-type viruses (Figure 20). This is regardless of the type of amino acid used to change the asparagine, at position 480 of MoMLV. The more conserved Aspartic acid was used to replace the Asparagine and the same effect was observed (Figure 20). Despite studies being done to determine important residues used in both the N- and C-termini of the MLV Gag protein[88, 141-147], this is the first time that residue 480 has been identified as a crucial and conserved residue amongst gammaretroviruses. This is likely to be the result of a possible structural
defect. For instance, Hung Fan and colleagues have shown that MLVs with defects in glyco-Gag actually have reduced infectivity and problems in budding and release from the cell \textit{in vivo} [119]. Nevertheless, we know that this impaired infectivity does not correspond with the removal of N-linked glycosylation because both MoMLV and AKV MLV's CTA mutants, which do not produce gPr80Gag, but that still contain the N480 site in the Pr65 protein, remain infectious (Figures 20 and 22). Furthermore, when MoMLV's CTA mutant contains the additional N480D residue, the virus is rendered non-infectious as well (Figure 20). Therefore, the asparagine is what is needed for the infectivity. Perhaps, the conserved asparagine residue in MoMLV and AKV MLV is important during the early entry steps in the replication cycle of gammaretroviruses by conferring stability to the capsid. Unlike HIV-1, once inside the cell, MLVs maintain their viral core consisting of p12, CA and NC up until the integration step, as mentioned in the introduction. Thus, if the structure were unstable, replication and integration would not be possible. The role of N480 will be clarified in future studies at the Langlois lab.

This study was able to pinpoint a defense mechanism against mouse APOBEC3 developed by gammaretroviruses, which have not previously been known to encode accessory proteins. This is the first time that gammaretroviruses have been shown to exploit the host’s post-translational machinery to thwart host intrinsic defenses. In this case, mouse APOBEC3 acted as a source of evolutionary pressure for gammaretroviruses like MoMLV and AKV MLV to develop countermeasures to restriction. APOBEC3-induced evolutionary pressures have been observed before in HIV and SIV, where, the Vif protein of SIV and HIV has evolved to counteract A3G [1, 148]. Thus, this study further stresses the importance of the APOBEC3 proteins in restricting endogenous retroviruses.
In contrast to its protective role, APOBEC3 deaminase activity can also have detrimental effects on the host. Of interest, HIV is a special case because it encodes the previously mentioned accessory protein, Vif, capable of targeting APOBEC3 for proteasomal degradation [115]. The presence of Vif leaves small numbers of APOBEC3 protein available, leading to sub-lethal hypermutations in HIV that instead of weakening the virus, increases fitness and promotes its spread to new cells[149]. In addition, some APOBEC proteins, A3A and A3B, have been shown to shuttle to the nucleus. Both of these proteins have now been linked to somatic C-to-T mutations, which are thought to cause cancers such as melanoma and breast cancer[106, 150, 151].

The results of the second part of this thesis suggest that N-linked glycans could serve as catalytic inhibitors of APOBEC3 deaminase activity. These could prove useful to prevent drug resistance mutations from appearing in HIV patients and as a preventive treatment for individuals at risk of certain types of cancers caused by high expression levels of A3A and A3B.
7.0 CONCLUSIONS

This thesis demonstrates the importance of A3 proteins in the restriction of both endogenous and exogenous retroviruses. The identification of murine retroviral sequences in bovine blood samples provides strong evidence that zoonotic events occur at high frequencies and need to be stopped by the host. Fortunately, we found that bovine A3 protects cattle against the spread of murine retroviral infection, much like human A3G has against most retroviruses (except HIV and HTLV). My second project explored how mouse retroviruses use the host’s post-translational machinery to protect themselves from restriction by mA3. Counteracting mA3 is so crucial for retrovirus survival that it pushed gammaretroviruses to evolve an accessory protein that potently inactivates A3 deaminase activity. In addition, my research helped identify a catalytic inhibitor of A3 deaminase activity, Glyco-gag, which could help in the development of new treatments for HIV and preventive therapy for certain types of cancers in at-risk individuals.
REFERENCES


**APPENDIX**

Table 5. **Primers used in Part 1 of this thesis.**

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*Primers obtained from Mazzanti et al. 2011.[3]*

**Primers obtained from Lo et al. 2010.[5]**
Table 6. Sequences used in Part 1 of this thesis.

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Figure 24. Alignment between the glyco-Gag coding sequences of MoMLV and FrMLV using NCBI Protein BLAST program. The putative N-linked glycosylation sites for both virus are displayed as red for MoMLV and blue for FrMLV.
CURRICULUM VITAE

MARIA CARLA ROSALES GERPE

Career Goal

- Driven towards a career in academia with focus on research and teaching in the retrovirology field

Education

Bachelor of Science (B.Sc), Honours in Biochemistry with Co-op option, Sept 2006 – May 2011
University of Ottawa – Ottawa, ON
  - Dr. Langlois Lab, University of Ottawa Co-op Placement, May 2010 – Aug 2010
  - Dr. Langlois Lab, Honours Project, Sept 2010 – April 2011

Master’s of Science (M.Sc), Microbiology and Immunology, Sept 2011 – Present (Expected: 2014)
University of Ottawa – Ottawa, ON
  - Project Title: Assessment of the Potential Zoonotic Transmission of Murine Retroviruses to Humans and Animals
    - Xenotropic and polytropic mouse retroviruses are able to cross the species-barrier and infect a broad range of animals and even humans. The aim of my project is to investigate whether mice can transmit their endogenous retroviruses to other animals through casual contact in a natural environment.
    - Supervisor: Dr. Marc-André Langlois

Awards and Scholarships

1. Canadian Society of Immunologists National Conference 4th Place Poster Award ($150) 2014
2. University of Guelph Ontario Veterinary Scholarship ($17,500) 2013 (Declined)
4. Ontario Graduate Scholarship (OGS) ($15,000) 2011 – 2012
5. University of Ottawa Excellence Scholarship ($6,866) 2011 – 2012
6. Dean’s Honour List at University of Ottawa 2011 – 2012
7. National Science and Engineering Research Council (NSERC) Undergraduate Science Research Awards (USRA) – Industry ($4,500) 2009
8. Queen Elizabeth II Aiming for the Top Scholarship ($8,750) 2006 – 2009
Publications:

Posters and Abstracts


Published Manuscripts


Manuscripts Submitted or in Preparation

1. **María Carla Rosales Gerpe**, Tyler Renner, Kasandra Bélanger, Cindy Lam, Rui Chen, Daniel Figeys and Marc-André Langlois. Glycosylation of gPr80\textsuperscript{gag} is involved in the antagonism of MLV restriction by murine APOBEC3. (To be submitted to *Journal of Virology*, July 2014).


Relevant Work

Experience

**Teaching Assistant for Biochemistry Laboratory I (BCH2333)** January - April 2011, 2012, 2013 University of Ottawa, Ottawa, ON

- Supervised 2 group sessions of about 16 students performing carbohydrate chemistry
Taught students pipetting, serial dilutions, spectrometry, column chromatography and dialysis

**Tutor**
Peer Help Center, University of Ottawa, Ottawa, ON

- Tutored Calculus I and II, Organic Chemistry I, II and III, Spectroscopy and Molecular Biology

**Research Assistant (Co-op Work Term and Summer Term)**
Faculty of Medicine, University of Ottawa, Ottawa, ON

- Analyzed restriction of mouse retroviruses like AKV-MLV, MoMLV and their chimeras, by mouse APOBEC3.
- Gained knowledge of Flow Cytometry to quantify viral infection through expression of the eGFP reporter protein and performed Western Blotting to qualify the expression of APOBEC3 protein expression.

**Research Assistant (Co-op Work Term)**
Spartan Bioscience Inc., Ottawa, ON

- Optimized assays to test the Spartan DX-12 PCR instrument for clinical diagnostics.
- Improved fluorophore detection system for the Spartan DX-12 PCR instrument and developed an application note “Fluorophores Compatible with the Spartan DX-12™ Instrument.”

**Summary of Skills**

**Technical Skills**

- Experienced in HEK 293T, NIH 3T3, L929, CEM, CEM-SS, HeLa and LNCaP cell culture
- Adept in performing DNA, RNA and protein extraction from tissue samples
- Proficient in molecular cloning techniques
- Extensive training in PCR (2&3-Step, isothermal, semi and fully-nested PCR) and genotyping
- Primer design, cloning with restriction enzymes, via TA overhangs and via Site-Directed Mutagenesis

**Interpersonal Skills**

- Fluent in Spanish and English (oral and written)
- French (fair writing and reading skills)
- Public speaking experience through Co-op work terms and seminar presentations during Master’s degree
- Able to work with a team as well as independently with effective time management skills

**Database Research and Other Software**

- BLAST, BLAT, RepeatMasker, PubMed, VectorNTI, Gene Codes Sequencher, NEBCutter, ExPASy, Kaluza and Summit 4.3 Flow Cytometry Software

**Additional Information**

**Volunteering & Clubs**

**Teacher**, Let’s Talk Science (Institution engaging youth with science) 2013-2014
**Writer**, Biochemistry, Microbiology and Immunology Graduate Students Association (BMIGSA) Bulletin 2011 – Present
Participant & Organizer, Holocaust Education Week: Dinner with Survivors 2010
Participant & Presenter, Faculty of Medicine Summer Student Program 2010
Co-chair, Social Committee for the Co-op Student Committee 2009 – 2010