Loss of Cell Surface aGal during Catarrhine Evolution: Possible Implications for the Evolution of Resistance to Viral Infections and for Oligocene Lineage Divergence

Idalia Aracely Rodriguez
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Loss of cell surface αGal during catarrhine evolution: possible implications for the evolution of resistance to viral infections and for Oligocene lineage divergence

A Dissertation Presented

by

IDALIA ARACELY RODRIGUEZ AYALA

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

FEBRUARY 2014

Anthropology
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This dissertation is dedicated to my wonderful parents, Fredy and Gladis and to my amazing sister, Gladys
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With the help of Ray as principal investigator, a version of this material has been published as “Rodriguez, I.A. and R.M. Welsh. 2013. Possible role of a cell surface carbohydrate in evolution of resistance to viral infections in Old World primates. Journal of Virology 87:8317-8326.” Additionally, under Laurie’s and Ray’s instruction, a version of this work was presented at the 81st Annual Meeting of the American Association of Physical Anthropologists (AAPA), Portland, OR, as Rodriguez, I.A., Galili, U., Godfrey, L.R. and R.M. Welsh. 2012. “The possible role of a cell surface carbohydrate in the evolution of resistance to viral infections in catarrhines”. The abstract was published online by AAPA.
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Finally, I would like to respectfully acknowledge the mice that were sacrificed as part of the production of this dissertation.
ABSTRACT

LOSS OF CELL SURFACE αGal DURING CATARRHINE EVOLUTION:
POSSIBLE IMPLICATIONS FOR THE EVOLUTION OF RESISTANCE TO
VIRAL INFECTIONS AND FOR OLIGOCENE LINEAGE DIVERGENCE

FEBRUARY 2014

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The divergence of the two superfamilies belonging to the Infraorder Catarrhini –
Cercopithecoidea (Old World monkeys) and Hominoidea (apes, including
humans) – is generally assumed to have occurred during the Oligocene, between
38 and 20 million years ago. Genetic studies indicate that this time period was
one of active genetic evolution under strong purifying selection for catarrhine
primates. This includes selective pressures on the glycoprotein
galactosyltransferase 1 (GGTA1) gene and subsequent inactivation “clocked” at
approximately 28 ma, possibly prior to the Cercopithecoidea/Hominoidea split.
The GGTA1 gene codes for an α1,3 galactosyltransferase (GT) enzyme that
synthesizes a terminal disaccharide, αgalactosyl (αGal), found on glycoproteins
and glycolipids on the surface of cells in the tissues of most mammals. Currently,
catarrhines are the only mammals studied for the terminal αGal residue that do not
express this sugar on their cell surfaces. The proposed selective advantage of this
mutation for catarrhines is the ability to produce anti-Gal antibodies, which may
be an effective immune component in neutralizing αGal-expressing pathogens, as certain helminthes, many bacteria, including those found in primate guts, and some viruses derived from GGTA1 positive species express αGal on their surfaces. However, many viruses are known to utilize host cell carbohydrates in various ways such as binding receptors or attachment proteins, making these moieties “hot spots” for selective evolution. Cell surface αGal may have predisposed ancestral catarrhines to pathogens and toxins that could utilize the terminal sugar moieties on host cells as binding sites or in other capacities during infection. I found that, in fact, the presence or absence of cell surface αGal affects the course of certain viral infections. Infections of paired cell lines with differential expression of GT showed that Sindbis viruses (SINV) preferentially replicate in αGal-positive cells, whereas herpes simplex viruses type 1 and type 2 (HSV-1 and HSV-2) preferentially grow in cells lacking αGal. In both cases, differences in infection levels resulted from the ability of the virus to successfully initiate infection. This points to a role for αGal in the early stages of viral infections. I also showed that GT knockout mice infected with HSV-2 had higher viral load and greater pathology compared to WT B6 mice that naturally express αGal. The increased susceptibility of KO mice to HSV-2 was not due to an immune component as differences in viral load and pathology were even more evident in immunocompromised mice. This clearly indicates that αGal expression in cells or animal hosts can affect the course of viral infections. I was not able to further confirm differences in susceptibility to HSV 1 and 2 using mouse backcrosses (KO x WT). Unknown genetic factors, that are independent of αGal
expression, may be introduced during the crosses that need to be further investigated. Infections of KO and WT mice with other herpes viruses did not yield definitive data and require further studies with suitable reagents. The mechanism by which GT-dependent differential susceptibility to viruses operates still remains to be deciphered. However, it is clear that susceptibility to certain viral infections is tied to the presence or absence of αGal on the surface of host cells. Overall, these results have implications for the evolution of resistance to viral infections in catarrhines. Pathogens exert great selective pressure on their hosts, and it is possible that a pathogen, able to exploit αGal, could have helped shape primate lineage evolution during the Oligocene.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS ................................................................. v</td>
</tr>
<tr>
<td>ABSTRACT ................................................................. viii</td>
</tr>
<tr>
<td>LIST OF TABLES ............................................................... xv</td>
</tr>
<tr>
<td>LIST OF FIGURES .............................................................. xvi</td>
</tr>
</tbody>
</table>

## CHAPTER

1. INTRODUCTION ................................................................. 1

1.1 Catarrhine evolution and the glycoprotein, glycosyltransferase gene 1 \((GGTA1)\) locus ................................................................. 1

1.2 Primate biogeography and biodiversity at the Eocene-Oligocene transtion ............................................................................................................. 3

1.3 \(GGTA1\) function in class Mammalia ............................................... 5

1.4 \(GGTA1\) and infectious disease ............................................................. 6

1.5 Selection for \(\Delta GGTA1\) gene during catarrhine evolution .................. 9

1.6 Summary and Chapter goals .............................................................. 12

1.6.1 Chapter goals ........................................................................... 13

1.6.2 Chapter 2: Biology of \(GGTA1\), anti-Gal and terminal carbohydrates ................................................................. 13

1.6.3 Chapter 3: Materials and methods .................................................. 14

1.6.4 Chapter 4: Differential host susceptibility to viruses, \(in vitro\) results ............................................................................................................. 14

1.6.5 Chapter 5: Differential host susceptibility to viruses, \(in vivo\) results ............................................................................................................. 15

1.6.6 Chapter 6: Summary and Conclusions .......................................... 15
2. BACKGROUND ON \textit{GGTA1}, ANTI-GAL, TERMINAL CARBOHYDRATES AND VIRUSES .................................................................17

2.1 \textit{GGTA1} gene and $\alpha$Gal .................................................................17

2.2 Anti-Gal ......................................................................................................22

2.3 Terminal carbohydrates .............................................................................25

2.4 Pathogen and host-cell glycan interactions ..............................................26

2.4.1 Alpha 2,6 sialic acid and influenza A ...............................................28

2.4.2 NeuAGc and Malaria ......................................................................29

2.5 Experimental mouse models.................................................................32

2.6 Viruses ....................................................................................................34

2.6.1 Sindbis virus (SINV) and SINV-related viruses .........................34

2.6.2 Herpes viruses ................................................................................37

3. METHODS AND MATERIALS ......................................................................40

3.1 Cell lines .................................................................................................40

3.2 Knockout mice .......................................................................................43

3.3 Mouse crosses .......................................................................................43

3.4 Enveloped viruses ................................................................................44

3.5 Time courses ..........................................................................................47

3.6 $\gamma$MHV-68 time course and PCR analysis .......................................48

3.7 Infectious center assays and fluorescent-focus assays .........................49

3.8 Blocking assays ....................................................................................51

3.9 Enzymatic removal of $\alpha$Gal from cell surfaces .................................52

3.10 Disruption of the GT gene using RNAi .............................................53

3.11 Addition of $\alpha$Gal molecules to host cell membranes using FSL Galili constructs ............................................................53
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.12</td>
<td>HSV-2 infections in adult male mice and γ-irradiation</td>
</tr>
<tr>
<td>3.12.1</td>
<td>HSV-2 infections of genetically crossed male mice</td>
</tr>
<tr>
<td>3.13</td>
<td>HSV-1 infection</td>
</tr>
<tr>
<td>3.14</td>
<td>γMHV-68 infections</td>
</tr>
<tr>
<td>3.15</td>
<td>SINV infections of adult mice and γ-irradiation</td>
</tr>
<tr>
<td>3.16</td>
<td>SINV survival studies</td>
</tr>
<tr>
<td>3.17</td>
<td>Statistical methods</td>
</tr>
<tr>
<td>4.1</td>
<td>Surface expression of αGal on experimental cell lines</td>
</tr>
<tr>
<td>4.2</td>
<td>Viral growth kinetics in the presence or absence of α1,3GT gene in the host cell</td>
</tr>
<tr>
<td>4.3</td>
<td>Viral expression of αGal does not affect host-cell susceptibility to SINV and HSV strains</td>
</tr>
<tr>
<td>4.4</td>
<td>Level of viral replication correlates with the proportion of infected cells in the population</td>
</tr>
<tr>
<td>4.5</td>
<td>Differences in host-cell susceptibility are evident early after viral infection</td>
</tr>
<tr>
<td>4.6</td>
<td>Testing restriction of SINV infection by blocking αGal sites on host cells</td>
</tr>
<tr>
<td>4.7</td>
<td>Enzymatic removal of αGal from host cells</td>
</tr>
<tr>
<td>4.8</td>
<td>Addition of αGal terminal residues to GT-negative host cells</td>
</tr>
<tr>
<td>5.1</td>
<td>GT KO mice are more susceptible than WT mice to HSV-2 infection</td>
</tr>
<tr>
<td>5.2</td>
<td>Genetic background of GT KO mice and susceptibility to HSV-2</td>
</tr>
<tr>
<td>5.3</td>
<td>WT and GT KO mice are similarly susceptible to HSV-1 infection</td>
</tr>
<tr>
<td>5.4</td>
<td>WT B6 and GT KO infections with γMHV-68</td>
</tr>
</tbody>
</table>
5.5 Adult WT and KO mice are equally permissive to SINV S.A.A.R86 infection ................................................................. 97

5.6 SINV infected WT suckling mice have accelerated symptom onset and death compared to KO counterparts ................................................................. 99

6. DISCUSSION AND CONCLUSIONS ......................................................... 105

APPENDIX: LIST OF ABBREVIATIONS ..................................................... 118

BIBLIOGRAPHY ......................................................................................... 120
<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Viruses screened during this study</td>
<td>46</td>
</tr>
<tr>
<td>2. Replication of viruses in SK human melanoma cells</td>
<td>61</td>
</tr>
<tr>
<td>3. Replication of viruses in B6 melanoma cells</td>
<td>62</td>
</tr>
<tr>
<td>4. Replication of S.A.AR86 and S.A.AR86(αgal) in BL6 cells</td>
<td>67</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Primate phylogeny on the geological time scale</td>
<td>2</td>
</tr>
<tr>
<td>2. αGal residues on a carbohydrate chain (top) and sialic acid-capped carbohydrate chain (bottom), as part of glycoproteins or glycolipids on the surface of mammalian cells</td>
<td>28</td>
</tr>
<tr>
<td>3. Expression of αGal on paired BL6 and SK cell lines</td>
<td>60</td>
</tr>
<tr>
<td>4. Growth kinetics of SINV and herpes viruses in B16 cell lines</td>
<td>63</td>
</tr>
<tr>
<td>5. Relative viral quantity in infected BL6 and BL6αGal</td>
<td>65</td>
</tr>
<tr>
<td>6. Growth kinetics of αGal expressing viruses in BL6 cell lines</td>
<td>66</td>
</tr>
<tr>
<td>7. Replication of GFP viruses in BL6 cell lines</td>
<td>70</td>
</tr>
<tr>
<td>8. Virus adsorption to BL6 and BL6αGT cell lines</td>
<td>73</td>
</tr>
<tr>
<td>9. Blocking host cell αGal sites with anti-Gal</td>
<td>75</td>
</tr>
<tr>
<td>10. αGal liposome treatment of BL6 and BL6αGal cells</td>
<td>76</td>
</tr>
<tr>
<td>11. Enzymatic treatment of BL6 and BL6αGal cells</td>
<td>77</td>
</tr>
<tr>
<td>12. Infection of enzymatically treated cells with SINV TE12-GFP</td>
<td>79</td>
</tr>
<tr>
<td>13. Assay for interferon production in the BL6 and BL6αGal cell lines</td>
<td>81</td>
</tr>
<tr>
<td>14. FSL treatment of Vero cells for SINV TE12-GFP infection</td>
<td>82</td>
</tr>
<tr>
<td>15. Infection of FSL treated Vero cells with SINV TE12-GFP</td>
<td>84</td>
</tr>
</tbody>
</table>
16. FSL treatment of Vero cells for HSV-1 GFP infection. .........................85
17. HSV-2 infections of KO and WT B6 mice ...........................................87
18. HSV-2 infections of γ-irradiated mice .............................................89
19. Expression of αGal on lymphocytes of KO and WT mice ..................90
20. HSV-2 infection of backcrossed male mice ....................................92
21. HSV-1 infection of WT, KO and backcrossed mice ......................93
22. γMHV-68 infection on KO and WT mice ..................................94
23. γMHV-68 infection on KO, WT and backcrossed mice .................96
24. S.A.A.R86 infections of WT and KO mice ..................................98
25. SINV infections of KO and WT weanling mice ............................101
26. SINV AR339 infections of KO and backcrossed mice .................103
CHAPTER 1
INTRODUCTION

1.1 Catarrhine evolution and the glycoprotein, glycosyltransferase gene 1 (GGTA1) locus

The phylogenetic split of the two superfamilies belonging to the infraorder Catarrhini – Cercopithecoidae (Old World monkeys; OWM) and Hominoidea (apes, including humans) – occurred during the Oligocene, estimated at about 29 Ma (Steiper et al., 2004; Chatterjee et al., 2009; Zalmout et al., 2010). More conservative estimates, primarily based on fossil evidence, place the OWM and ape divergence sometime between 38-20 Ma, essentially bracketing the entire Oligocene (Raaum et al., 2005; Stewart and Disotell, 1998; Steiper and Young, 2008; Perelman et al., 2011; Pozzi et al., 2011). Molecular studies indicate that the early part of the Oligocene epoch (~35.0-23.0 Ma) was a period of active genetic evolution under strong purifying selection for catarrhines (Galili et al., 1991; Koike et al., 2007). This may have included a mutation on the glycoprotein galactosyltransferase 1 (GGTA1) locus. Catarrhini are unique among mammals because a loss-of-function mutation, which has been molecularly clocked at approximately 28.1 million years ago, resulted in the inactivation of the GGTA1 gene (Figure 1) (Koike et al., 2007). In animals with an active GGTA1, the gene codes for the α1,3 galactosyltransferase (α1,3GT or GT) enzyme, which is responsible for the addition of the terminal galactosyl unit to αGal (Galα1,3Galβ1,4GlcNAc-R) carbohydrate chains found on cell surface
glycoproteins and glycolipids in the tissues of most mammals (Galili et al., 1985; Galili et al., 1987; Galili et al., 1988). Due to loss of GT expression, OWM and apes do not express the cell surface carbohydrate, $\alpha$Gal (Galili and Swanson, 1991; Koike et al., 2002). All other extant primates – Platyrhini (New World monkeys (NWM)) and Strepsirrhini (lemurs and lorises) (Galili et al., 1988; Galili and Swanson, 1991) — retain an actively expressed $\alpha$1,3GT gene and therefore have enzymatic activity that catalyzes production of the terminal galactose sugar.

Figure 1: Primate phylogeny on the geological time scale. The Hominoidea and Cercopithecoida lineages diverged during the Oligocene, some time after 28.1 Ma when the $GGTA1$ gene was inactivated. The shaded area highlights the primate lineages lacking $\alpha$Gal expression.
The significance of \textit{GGTAl} inactivation is of interest to studies in primate evolution for several reasons: (1) inactivation occurred during the Oligocene epoch, a period of ambiguity in catarrhine evolutionary history; (2) inactivation occurred in relatively few taxa in the Order Primates, but active expression is conserved across the vast majority of Class Mammalia, and of most importance for this dissertation; (3) suppression of αGal expression in catarrhines has been associated with a selective advantage against pathogenic agents.

1.2 Primate biogeography and biodiversity at the Eocene-Oligocene transition

The Grande Coupure at the Eocene-Oligocene boundary is characterized by a shift in global climate commonly referred to as “greenhouse to icehouse” (Prothero 1994; Seiffert, 2007; Pearson et al., 2008; Maravelis and Zelilidis 2012). Cool conditions and pronounced seasonality continued for most of the epoch until the onset of warmer climate approximately 25 Ma. The transition from the late Eocene into the late Oligocene is also marked by a massive turnover of marine biota as well as terrestrial fauna and flora (Prothero 1994; Kappelman et al., 2003; Pearson et al., 2008; Figueirido et al., 2012; Maravelis and Zelilidis 2012; Wade et al., 2012). Terrestrial faunal biogeography and diversity during the Oligocene epoch were likely influenced by geographic and climatic changes such as fluctuating Antarctic ice sheet formation, increased aridification and seasonality and a significant drop in global temperature and sea levels (Seiffert 2007; Maravelis and Zelilidis 2012; Wade et al., 2012). Additionally, the fall in
sea levels opened routes between previously unconnected land masses and permitted migration (Kappelman et al., 2003; Figueirido et al., 2012; Maravelis and Zelilidis 2012) that may have, in turn, affected faunal extinctions during the late Eocene, and faunal diversity into the late Oligocene (Kappelman et al., 2003; Figueirido et al., 2012).

Prior to the Eocene-Oligocene boundary, primates were widely distributed in Africa, the Arabian Peninsula, Asia and even the mid-latitudes of North America and Europe (Seiffert, 2007; Williams et al., 2010). In the latter part of the Oligocene, primates in North America disappeared, as did many stem strepsirrhine and haplorrhine lineages in Africa and Afro-Arabia (Seiffert, 2007). Cool temperatures and habitat fragmentation in northern latitudes may have restricted primate latitudinal distribution, while equatorial Africa, despite experiencing decreased plant species diversity and cooler temperatures, retained widespread tropical forest habitats suitable for primates (Seiffert, 2007). Paleontological evidence shows stem anthropoids or catarrhines (i.e., the Oligopithecidae, Parapithecoidea, Propliopithecidae) at localities in Africa, Afro-Arabia and Asia during the early Oligocene (Williams et al., 2010; Ducrocq et al., 2011) although many of these lineages were extinct by the early Miocene (Seiffert 2007). Fossil remains of stem catarrhines such as Aegyptopithecus derive from as late as 29-30 Ma, but a scarce paleontological record from the mid-Oligocene makes discerning phylogenetic relationships or divergence dates between such
stem taxa and crown catarrhines from the Oligocene-Miocene boundary very
difficult (Seiffert, 2007; Zalmout et al., 2010). Even the most informative
sediments—such as those of the Fayum depression in Egypt—lack many primate
remains (Steiper et al., 2004; Zalmout et al., 2010), resulting in a chronological
gap in the primate paleontological record.

The timing of the \textit{GGTA1} mutation can inform on the maximum bound for the
phylogenetic split of catarrhines. The mutation occurred in a basal catarrhine
prior to the divergence of cercopithecoids and hominoids. Therefore, the
phylogenetic split of these two groups must have occurred sometime after 28.1
Ma. It should be noted, however, that the timing does not indicate an exact
divergence time for the catarrhine lineages or set a minimum bound for the split.
The last common ancestor of OWMs and apes carrying the \textit{ΔGGTA1} gene may
have survived for a long period of time after the mutation was introduced into the
catarrhine lineage but prior to the divergence of cercopithecoids and hominoids.

\textbf{1.3 \textit{GGTA1} function in class Mammalia}

Loss of GT expression in catarrhines resulted in significant alteration to the
surface carbohydrate biology of these primates, but neither the physiological
function of \(\alpha\)Gal nor the metabolic consequences of inactivation of this locus in
animals with a functional GT gene is currently known. In non-catarrhine
primates, the function of \textit{GGTA1} appears to be under strong physiological
constraint, and the gene sequence has been highly conserved over evolutionary
time (Koike et al., 2007). Koike et al., (2007) suggest that inactivation of GGTA1 gene may have been detrimental to individuals and that loss of GT gene function may have been possible only after its enzymatic role had been replaced, although they do not conjecture on what replaced the enzymatic function in catarrhines.

The successful generation of experimental homozygous knockout (KO) pigs (Nottle et al., 2007) and KO mice (Thall et al., 1995) demonstrate that loss of GGTA1 activity is not lethal to an organism. A few abnormal health conditions have been observed, including early-onset cataracts in some KO mice and breeding problems in certain KO pig lineages (Koike et al., 2007). However, breeding of other KO pig lineages and KO mice (Tanemura et al., 2000) resulted in normal-sized litters that do not experience greater mortality or health defects than wild type counterparts. This suggests that basal catarrhines could have experienced loss of GT function without an elevated risk of morbidity or mortality. Alternately, the loss of αGal from tissue surfaces may have served a protective role during the evolution of catarrhines, and the benefits of losing gene function may have outweighed any negative effects.

1.4 GGTA1 and infectious disease

Infectious agents can be powerful drivers of evolutionary change. Pathogens exert great selective pressures on susceptible populations and can even be the cause of extirpation or extinction (Pederson et al., 2007; Wyatt et al., 2008). An example of the latter includes the species-level extinction of Rattus macleari after the introduction of a parasite, possibly Trypanosoma lewisi, to Christmas Island in
the Indian Ocean (Wyatt et al., 2008). The large rat population, which was immunologically naïve to the trypanosome parasite, was wiped out over a period of less than 20 years (Wyatt et al., 2008). Infectious agents can also decimate populations of susceptible hosts and have devastating consequences for these populations. Ebola virus, with up to 95% mortality rates, have reduced *Gorilla* and *Pan* populations in Africa over the past few decades (Leroy et al., 2004; Bermejo et al., 2006). In regions of the Congo, outbreaks of Ebola have resulted in *Pan troglodytes* population declines of up to 83% (Bermejo et al., 2006). This can lead to regional extirpation or bottlenecks in the population. Occasionally individuals survive due to genetic resistance (previous mutations carried in their genome) or because new mutations have rendered them less susceptible than their counterparts. This can be of adaptive significance to the entire species.

One of the most studied examples of genetic resistance in the human population is the mutation CCR5-Δ32 on the leukocyte chemokine (C-C motif) receptor 5 (CCR5). The CCR5 gene locus was under selective pressure from pathogens encountered by the human population centuries ago, namely the bacterium *Yersinia pestis* and *Variola* virus (Hütter and Ganepola, 2011). The Δ32 mutation likely provided a level of resistance against these pathogens when they were scourges in the human population, and today, individuals who possess this mutation can also benefit due to their reduced risk of acquiring human immunodeficiency virus (HIV) (Hütter and Ganepola, 2011).
It has been hypothesized that the *GGTA1* gene locus has also been under evolutionary selection due to pathogenic pressures (Galili et al., 1987; Galili et al., 1988; Galili and Swanson, 1991; LaTemple and Galili, 1999). Catarrhine primates that lack GT gene and αGal expression produce a natural antibody, anti-Gal, which recognizes the αGal disaccharide with high affinity (Galili et al., 1987) and can induce phagocytic cell and complement activity (LaTemple and Galili, 1999; Rother and Galili, 1999). Production of natural antibodies that can recognize and effectively neutralize pathogens may be beneficial for animal hosts against a variety of parasites that express the αGal domain (Galili et al., 1984; Galili et al., 1988; Avila et al., 1989; Hamadeh et al., 1992; Ramasamy and Rajakaruna, 1997; Pingel et al., 1999). Fresh sera with functional complement activity from different catarrhines can inactivate many different viruses that express αGal (Welsh et al., 1975; Welsh et al., 1976; Welsh, 1977; Rother et al., 1995; Takeuchi et al., 1997; Welsh et al., 1998; Kim et al., 2007). Sera from other mammals, including New World monkeys, rabbits, rats, mice and guinea pigs, do not lyse αGal-expressing viruses, as these species do not naturally make anti-Gal (Rother et al., 1995; Welsh et al., 1975; Welsh et al., 1976). The ability of anti-Gal to neutralize pathogenic agents via the complement system has been heavily researched, and it is suggested that loss of αGT gene activity was favored during catarrhine evolution because it allowed immune recognition of pathogens expressing αGal (Galili et al., 1987; LaTemple and Galili, 1999; Rother and Galili, 1999). As such, suppression of the GT gene would have been necessary to develop the ability to produce anti-Gal and not also stimulate an autoimmune
response (Galili et al., 1987). Under this scenario, GT-deficient individuals would have been indirectly protected from αGal-expressing pathogens by the anti-Gal activity.

1.5 Selection for ΔGGTA1 gene during catarrhine evolution

My alternative hypothesis is that positive selection for GT gene inactivation may have been favored because loss of αGal residues directly altered host susceptibility to infections. The proteins on cell surfaces are involved in interactions with the surrounding environment, and the terminal carbohydrates that decorate these proteins are prime candidates for pathogens, including viruses, which can utilize carbohydrates as binding receptors or in other capacities (Olofsson and Bergström, 2005). Historically, selective pressures from pathogens have led to major biochemical changes in carbohydrate biology of primates (Chou et al., 1998; Hayakawa et al., 2001; Gagneux et al., 2003; Varki and Varki, 2007). It is known that αGal can serve as a binding receptor for pathogens, as toxin A, produced by Clostridium difficile, binds to the αGal moiety on host cells and causes cytotoxic effects in these cells (Krivan et al., 1986; Clark et al., 1987; Teneberg et al., 1990; Phelps et al., 2003). Hamsters are highly vulnerable to severe infections by C. difficile because they express high amounts of αGal on intestinal epithelial cells, and these sites are specifically targeted by the bacterial cytotoxin (Krivan et al., 1986). Experimental infections of rabbit and rat brush border membranes and erythrocytes (Clark et al., 1987; Pothoulakis et al., 1991) have showed that αGal-capped membranes are the binding sites for C. difficile in
these species, and greater expression was associated with more targeting by the toxin (Krivan et al., 1986). Krivan et al. (1986), also found that human red blood cells with the blood group B structure, Gal\(\alpha_1,3\)Fuc\(\alpha_1,2\)Gal\(\beta_1,4\)GlcNac-R, would bind toxin A after treatment with a fucosidase. The enzymatic removal of fucose resulted in expression of Gal\(\alpha_1,3\)Gal linkages on the cell membranes. Humans can be infected with *Clostridium* spp. when intestinal or genital tract flora is disturbed (Agrawal and Garg et al., 2012), but the binding receptor in humans has not been determined. It is suggested that because humans do not naturally express \(\alpha\)Gal, \(N\)-acetyllactosamine sites bearing molecular similarity to the Gal\(\alpha_1,3\)Gal structure serve as binding sites (Krivan et al., 1986; Teneberg et al., 1990). As such, surface Gal residues are potential “hot spots” for selective evolution.

For basal catarrhines living during the Oligocene, GT expression may have predisposed them to pathogenic agents able to exploit the GT pathway or its final glycosylation product, \(\alpha\)Gal. Loss of *GGTA1* activity and of surface \(\alpha\)Gal may have increased resistance to such pathogens by GT-deficient catarrhines relative to their GT-sufficient counterparts, resulting in selection for the \(\Delta GGTA1\) gene. The pathogenic agents at work during the Oligocene epoch are unknown, and therefore it is impossible to directly study the effects of such pathogens on \(\alpha1,3\)GT positive or negative hosts. However, we can conjecture about the role of host GT activity during viral infections in the past by studying the interactions of
GT-deficient and GT-sufficient hosts with modern day viruses. Arguments can then be made regarding the potential for viral infectivity (or host resistance) in the presence or absence of $GGTA1$ gene activity.

My current hypothesis does not contradict the theory that anti-Gal production was favored in the evolution of disease resistance for catarrhines. Instead, it attempts to expand our understanding of the selection mechanism(s) that drove evolution of the $GGTA1$ gene. In fact, the inactivation of the $GGTA1$ gene, coupled with the production of anti-Gal, point to strong selective pressure for reduced expression of the $\alpha$Gal epitope on the tissues of catarrhines. It is possible that GT activity was lost due to neutral selection, through the accumulation of mutations on the gene locus that had no benefit or detrimental effect on the host. This can be easily verified, as active host expression of $GGTA1$ or $\alpha$Gal would have no effect on the course of viral infections in that host. It is also possible that certain viruses may preferentially infect hosts in the absence of GT expression. This may not necessarily contradict my working hypothesis that GT activity facilitates the course of a viral infection. Today, different viruses employ vastly different strategies to establish infections in hosts, and viruses that may have once specialized in exploiting the GT pathway may have modified their infection strategies since 28.1 Ma.
In order to demonstrate the possible adaptive significance of \( \Delta GGTA1 \) for basal catarrhines, it is necessary to show that GT or \( \alpha \text{Gal} \) expression in hosts is required—or in the least—can enhance the capacity of infection for viruses relative to non-expressing hosts. This will establish that GT or \( \alpha \text{Gal} \) expression may play a role in host susceptibility to viral infections. Additionally, it is important to provide a possible mechanism by which viruses may utilize the GT pathway or sugar. Viruses are known to utilize carbohydrate residues during entry into the cell (adsorption, penetration), or subsequently, during exit (release); as such, viruses may use surface \( \alpha \text{Gal} \) in this capacity. Until now, glycan-based interactions of pathogens and \( \alpha \text{Gal} \) positive host-cells have received only limited investigation. **This aspect of the evolution of the \( \alpha 1,3GT \) gene – the differential ability of pathogens to infect host cells through utilization of the GT pathway or sites containing \( \alpha \text{Gal} \) – is the topic of this dissertation.**

**1.6 Summary and Chapter goals**

The goal of this chapter was to provide an overview of the inactivation of the \( GGTA1 \) gene in basal catarrhines and of primate evolution during the Oligocene. Little is known about direct selective pressures underlying catarrhine evolution during this epoch; the period of catarrhine lineage evolution and the hominoid and cercopithecoid phylogenetic split. Changes in global ecology such as colder temperatures and reduced habitats likely affected primate biogeography and diversity during the late Eocene and into the Oligocene. We hypothesize that a pathogen, perhaps a virus, may have also played a role in shaping catarrhine
lineage divergence during this epoch. Inactivation of the \textit{GGTA1} gene (molecularly clocked at the midpoint of the Oligocene epoch, \( \sim 28.1 \) Ma) resulted in a major change in carbohydrate biology for catarrhines, and possibly sites for pathogens to exploit. The pressure from pathogens able to exploit the GT pathway may have been an impetus for selection of \textit{GGTA1} gene inactivation.

Understanding the possible basis for selection of the mutated gene may inform us about some of the processes that helped shape primate lineage divergence during the Oligocene.

\textbf{1.6.1 Chapter goals}

Below is a brief description of the goals of each chapter of this dissertation.

\textbf{1.6.2 Chapter 2: Biology of \textit{GGTA1}, anti-Gal and terminal carbohydrates}

In Chapter 2, I establish the evolutionary origins of \textit{GGTA1}. I review the role of anti-Gal as a protective factor in infectious diseases of primates. I also discuss the importance of terminal carbohydrates in viral infections and provide several examples of pathogen-driven modifications of carbohydrate loci during primate evolution.
1.6.3 Chapter 3: Materials and methods

*In vitro* and *in vivo* methods were utilized in order to understand the details of viral infections in GT positive and GT negative hosts. Cell lines derived from mice were used for *in vitro* experiments and a mouse model was utilized for *in vivo* work.

In Chapter 3, I describe the origins of the cell cultures and mouse lineages utilized in these experiments, as well as the significance of using a mouse model to study concepts in primate evolution. I also describe the varied experimental methods utilized in this research.

1.6.4 Chapter 4: Differential host susceptibility to viruses, *in vitro* results

Various cell lines were used to establish differential susceptibility to viral infections in the presence or absence of GT activity in the host cell. Preliminary results regarding the role of GT activity in viral infections were established in experiments using cultured cells lines because of the ease of manipulation.

In Chapter 4, I describe the results from experiments with various cell lines. I present evidence for GT-dependent differential susceptibility to viral infections, primarily with Sindbis viruses and herpes simplex viruses 1 and 2. I show that differences in infections of GT-positive and GT-negative cell lines by the different viruses are based on the ability of the viruses to initiate infection in the
host. Additionally, I show that αGal may play a role in the ability of viruses to establish infection. I also discuss experiments that were not successful or resulted in ambiguous data.

1.6.5 Chapter 5: Differential host susceptibility to viruses, in vivo results

Differences in susceptibility to viruses can be tested in vivo using a mouse model to verify that in vitro findings can translate to the organismal level.

In Chapter 5, I discuss the results of in vivo experiments. I show that the same patterns of differential infection observed in the cell lines are observed in experimental mouse lines. Clear and significant differences in susceptibility to infections are seen in GT deficient vs. GT sufficient mice with Sindbis virus infections of young mice and with herpes simplex 2 infections of adult mice. I also show the results of experiments that generated inconsistent or ambiguous data.

1.6.6 Chapter 6: Summary and Conclusions

In Chapter 6, I examine the data and interpret findings from the previous chapters. Overall, infections of paired cell lines with differential expression of α1,3GT showed that certain viruses preferentially replicated in α1,3GT-positive cells, whereas others preferentially grew in cells lacking α1,3GT. Viral growth and spread correlated with the ability of the different viruses to successfully initiate infection in the presence or absence of α1,3GT expression. Additionally, differences in gross pathology, onset of disease symptoms and mortality are correlated with the presence or absence of α1,3GT host mice. The current study
demonstrates that α1,3GT activity plays a role in the course of infections for certain viruses. Furthermore, this study has implications for the evolution of resistance to viral infections in primates.
2.1 GGTA1 gene and αGal

The genetic sequences that code for the ABO glycosyltransferases are found on
the human homolog for the GGTA1 gene. These glycosyltransferases likely form
a family that arose as a result of gene duplications (Gastinel et al., 2001). αGal is
chemically similar to the ABO blood group antigens, and the transferases
responsible for their biosynthesis share similarities in their catalytic domains
(Gastinel et al., 2001). The α-galactosyltransferase enzyme is a golgi
transmembrane protein that catalyzes the transfer of a non-reducing galactose to
an N-acetyllactosamine acceptor via the following reaction: UDP-Gal +
Galβ1,3GlcNAc-RëGalα1,3Galβ1,4GlcNAc-R. This results in Galα1,3Gal
capped glycan chains. In most mammals, including mice, rats, cows, rabbits,
hamsters, pigs, dogs and non-catarrhine primates, αGal residues are found on the
surface of nucleated cells and erythrocytes (Galili, 1993). These residues cap the
terminal ends of membrane-bound glycosphingolipids and glycoproteins (Galili et
al, 1987) and in secreted N-glycosylated glycoproteins such as thyroglobulin,
fibrinogen and IgG (Thall and Galili, 1990). With the exception of sperm cells
(Thall et al., 1995), the carbohydrate is abundantly expressed on the surface, up to
10^6 molecules per cell (Galili, 1993). Some tissue specificity in αGal expression
does exist in certain mammals. In rats, Griffonia simplicifolia B4 isoelectin (BS
IB₄), a molecule with high specificity for α-D-galactose groups, was found to bind to deeper layers in the epidermis and not at all in higher layers of the skin (Brabec et al., 1979). The quantity of αGal residues expressed on tissue is also variable by host species and by the type of the glycoconjugate. For example, bovine thyroglobulin has approximately 11 residues of αGal per molecule while *Saimiri* (squirrel monkey) expresses one residue per thyroglobulin molecule and mice express one αGal residue per every four thyroglobulin molecules (Thall and Galili, 1990).

As expected, *Macaca* (macaque) and humans do not express a terminal galactose α(1,3) linked to a penultimate galactose (Gal-Gal linkage) on thyroglobulin, or to any other issue, because they have an inactive *GGTA1* gene. In humans, only three glycoconjugates found in red blood cells (RBCs) are known to express a terminally linked α-galactosyl: CTH, P₁ and B antigen. CTH and P₁ have an α(1,4) linkage and B antigen has a fucose attached to the penultimate galactose (Galα1,3Fucα1,2). BS Lectin can bind the terminal residue of the human blood B-antigen, but anti-gal binding—which is highly specific for the Gal-Gal linkage—is blocked due to the branched fucose (Galili et al., 1984). Still, humans that express the B antigen (OB, BB or AB blood type) have reduced levels of IgG anti-Gal, possibly because there is some level of reaction between the antibody and the terminal galactose monosaccharide (McMorrow et al., 1997).
Of interest is the de novo-expression of αGal that has been observed on humans cells, including: senescent RBCs, pathologic RBCs such as those derived from thalassemic or sickle cell anemia-affected individuals (Galili et al., 1984) and cells from human mammary carcinomas (Castronovo et al., 1989). Additionally, lung carcinoma cells will sometimes secrete β-interferon containing αGal carbohydrate chains (Kagawa et al., 1988). In this way, αGal could play a role in autoimmune disease as human RBCs expressing αGal are targeted and lysed by anti-Gal mediated-complement lysis (Galili, 1993). In humans, the GGTA1 gene is not transcribed, and although mice and pigs have alternative genes that can synthesize very small quantities of αGal epitopes (Milland et al., 2006), no other human gene has been discovered that can do this. In the case of senescent RBCs expressing αGal, displaced GT-positive gut bacteria or their GTs can make their way into the circulatory system and become the source of αGal found on human RBCs (Hamadeh et al., 1996). RBCs exposed to Klebsiella bacteria and their galactosyltransferases have been shown to incorporate bacterial αGal residues on their surfaces (Hamadeh et al., 1996). Older RBCs compared to younger cells may be more likely to acquire αGal because their extended time in circulation exposes them to more lactosamine acceptor molecules that can link αGal molecules (Hamadeh et al., 1996). Aberrant expression of αGal in other human tissues could indicate that within these tissues inactivation of GGTA1 may not be absolute, or a functional form of the polypeptide can be made under certain conditions (Galili et al., 1984).
Catarrhines without a functional GT gene still have homologous sequences to \textit{GGTA1}. Two \textit{GGTA1} homologs have been found in catarrhines by using probes from cDNA sequences derived from pigs, cattle and rodents (Jozaissse et al., 1991; Strahan et al., 1992; Koike et al., 2002). The two loci evolved independently in the Haplorrhine (monkeys, apes and tarsier) lineage. All haplorrhines have an orthologue of the gene, a processed pseudogene (PPG) that does not result in production of a functional protein (Larsen, et al., 1990; Jossaize et al., 1991; Koike et al., 2007). The PPG appeared in the primate genome approximately 57.6 Ma, after the Strepsirrhine/Haplorrhine split, but likely prior to tarsier divergence from the rest of the haplorrhines. Putative grouping of the tarsier’s GT gene sequence places it with the PPG sequence (Koike et al, 2007). Catarrhines still express the PPG and also an altered \textit{GGTA1} gene referred to as an unprocessed pseudogene (UPG) (Jozaissse et al, 1991; Koike et al., 2007). The human homologues are found on chromosomes 12 (PPG) and 9 (UPG) (Jozaissse et al., 1991).

Comparisons of the human UPG with sequences from \textit{Macaca} and mouse GT genes show little homology in various portions of the regulatory region (Koike et al., 2001; Koike et al., 2002). The same is observed with comparisons of the regulatory regions (exons 1-3) of the more distantly related pig and mouse \textit{GGTA1} genes (Koike et al., 2001). Although these differences affect gene regulation in pigs and mice, the enzymatic function is identical in both species (Koike et al., 2001), indicating that conservation in the regulatory region is not
essential for gene function. In contrast, homologous genetic sequences in coding regions of the GT gene between primates, mice and bovine have been found (Larsen et al., 1990; Galili and Swanson; Jozaisse et al., 1991), indicating that the coding region is conserved. In primates, including Homo, Pongo (orangutan), Macaca and Callithrix (marmoset), homology in the gene-coding regions (exons 4-9) between apes and Callithrix was 94.7% and between Macaca and Callithrix was 92.4% (Koike et al., 2002).

Several mutations in the coding regions of the Homo, Callithrix and Pongo genes (exons 7, 8 and 9) independently result in GT gene inactivation, but two mutations that independently yield a truncated gene product are shared by all three species: (1) a deletion on exon 7, and (2) a nucleotide substitution on exon 9 (Koike et al., 2002). Nucleotide alignment of genetic sequences that included strepsirrhines, platyrrhines, cercopithecoids, and hominoids indicate the point mutation on exon 7 in the catarrhine sequences, upstream of a region specifying an enzymatic catalytic domain, was likely responsible for GGTA1 inactivation (Koike, 2007). The presence of the same mutation in cercopithecoids and hominoids, clocked at approximately 28.1 Ma, indicates that the gene was inactivated prior to an OWM-ape lineage divergence (Koike et al., 2007).

It is of interest that KO pigs and KO mice still express relatively low quantities of αGal epitopes compared to their WT counterparts as a result of iGb3S glycosyltransferase activity (Milland et al., 2006). Like α-galactosyltransferase,
this enzyme is part of the ABO-blood group family of glycosyltransferases and can catalyze the placement of a terminal Gal residue on glycoconjugates; however, in contrast to αGT enzyme activity, αGal capping via iGb3S is restricted to acceptors on iGb3 glycolipids (Milland et al., 2006). This is of general interest because αGal is the major xenoantigen that induces hyperacute rejection in organ transplants to humans that come from pigs (Phelps et al., 2003). Therefore, organs from GT KO pigs may still be immunogenic. This is of consequence to our current research because deciphering pathogen-host cell protein interactions may largely depend on absolute expression or non-expression of the protein of interest. Results must be interpreted in this context.

2.2 Anti-Gal

Catarrhines produce very high amounts of anti-gal antibodies that recognize the Galα1,3Galβ1,4GlcNAc-R epitope with high affinity (Galili et al., 1984; Galili et al., 1988). In humans, anti-Gal is most commonly found as class IgG (IgG1, IgG2a, IgG2b, IgG3 subclasses) comprising approximately 0.5-1.0% of total serum IgG (Galili et al., 1984), but it is also produced as classes IgM and IgA (LaTemple and Galili, 1998). Humans produce other naturally occurring IgG molecules in blood, including anti-blood A and B and anti-T antibodies, that similar to anti-gal, display an anti-carbohydrate specificity (Galili et al., 1984). Production of natural antibodies is attributed to constant exposure to naturally occurring antigens over evolutionary time. The constant exposure to gastrointestinal flora that expresses αGal on the cell wall, such as E. coli,
Salmonella and Shigella (Galili et al., 1988; Hamadeh et al., 1992), may stimulate the observed production of anti-Gal throughout the lifetime of humans as well (Galili et al., 1984).

Besides commensal and pathogenic gut bacteria, a variety of other pathogens also express αGal residues, including protists (Ramasamy and Rajakaruna, 1997; Pingel et al., 1999). Species of trypanosomatids (Leishmania sp. and Trypanosoma cruzi), the causative agents of leishmeniasis and Chagas disease, express lipids capped by galactosyl residues on the parasite’s surface during part of its life cycle, and infected hosts show significant increases in anti-Gal production compared to uninfected individuals (Avila et al., 1989). Some individuals also develop a class of highly specific anti-Gal antibodies to the T. cruzi trypomastigote (the infective stage) that efficiently inhibits parasite growth through complement-mediated damage (Almeida et al., 1991). The merozoites (infective stage) of Plasmodium falciparum, the most virulent malarial parasite of humans, also express αGal antigens and induce antibody-dependent complement-mediated lysis (Ramasamy and Rajakaruna, 1997; Ramasamy et al., 1999). During egress from host cells, enveloped viruses often incorporate cellular membrane components into or onto the particle; and this includes galactosyl-capped entities (Welsh, 1977; Welsh et al., 1998). Viruses that express αGal then become antigenic to GT-negative species. In this way, anti-Gal is speculated to restrict xenotransmission of viruses propagated in GT-positive species by
targeting them for complement-mediated inactivation (Welsh et al., 1975; Welsh et al., 1976; Welsh et al., 1978; Repik et al., 1994; Rother et al., 1995; Takeuchi et al., 1996; Kim et al., 2007).

Mammalian species that naturally express \textit{GGTA1} accordingly do not make anti-Gal, and are therefore tolerant to \(\alpha\)Gal-expressing viruses (Rother et al., 1995; Welsh et al., 1975; Welsh et al., 1976). It is speculated that natural anti-Gal production was an adaptive response to pathogenic infection for catarrhine primates (Galili et al., 1987; LaTemple and Galili, 1999; Rother and Galili, 1999). However, for anti-Gal expression to be a driving force behind GT gene inactivation, anti-Gal production would have had to occur concurrently with the \textit{GGTA1} loss-of-function mutations. Experimental mice that are double knockouts for the \textit{GGTA1} gene (Thall et al., 1995) do not naturally produce anti-Gal in sera and only produce low levels of IgG and IgM antibodies after initial exposure to antigenic epitopes (LaTemple and Galili, 1998). A robust antibody response, comparable to titer levels found in humans, is only achieved by multiple immunizations (LaTemple and Galili, 1998). This may indicate that upon historical loss of GT activity, anti-Gal-mediated protection was not immediate or wholly effective on primary exposures to \(\alpha\)Gal-expressing pathogens. For a period of time, prior to the natural production of anti-Gal in the absence of external antigen stimulation, catarrhines still would have been highly susceptible to pathogens that expressed \(\alpha\)Gal epitopes. As such, I suggest it is more plausible that production of natural anti-Gal occurred after inactivation of \textit{GGTA1}.
I hypothesize that loss of GT gene expression may have been a response to pathogen-mediated selection on host genotype (GGTA1) or phenotype (αGal).

Pathogens often utilize host cellular carbohydrates as binding receptors or in other capacities during the course of an infection. The terminal carbohydrates that decorate cell surfaces function in various biological capacities for the host organism. Due to their location at the outer edges of cells, carbohydrates are prime candidates for mediating interactions with other cells, other molecules, and with environmental agents. As targets to foreign agents, surface sugars are expected to be under constant evolutionary pressures and are therefore key players in organismal health and disease.

2.3 Terminal carbohydrates

Many forms of life, including bacteria, plants and animals, express carbohydrate chains on the exterior surfaces of their cells. The repertoire of glycans and the function they perform varies significantly depending on species and tissues, but the distribution of these structures in nature is indicative of their fundamental biological role. Between 1-2% of animal genes are involved in glycan modification or synthesis (Bishop and Gagneux, 2007). Relative to bacteria and plants, animals utilize a restricted set of carbohydrate units to create diverse and complex structures, generally arranged as four basic types of glycoconjugates: N-linked, O-linked, glycolipids and proteoglycans (Bishop and Gagneux, 2007). Patterns of glycan distribution can be limited to single species, be lineage-specific, be similar across distantly related species or be widely conserved.
The different distribution patterns likely resulted from various processes such as the evolution of independent lineages following phylogenetic splits or from selection pressures imposed by pathogens (Bishop and Gagneux, 2007).

**2.4 Pathogen and host-cell glycan interactions**

During the course of infections, pathogens are involved in a variety of interactions with terminal carbohydrates on host cell surfaces and have evolved numerous strategies to exploit them. Pathogens can also utilize glycosyltransferases to modify their own proteins, and this may result in increased virulence for that pathogen (Bishop and Gagneux, 2007). Pathogens have also evolved ligands (lectins) that bind host carbohydrates, and they also produce molecules that mimic host glycans and can then bind to corresponding host ligands (Bishop and Gagneux, 2007). Viruses, especially enveloped viruses, can also incorporate host cell carbohydrates or even proteins on their envelopes during egress. This can aid to promote their infection cycles and also provide reduced immunogenicity in the host (Vigerust and Shepherd, 2007). Host cell surface glycans are preferred binding sites for viruses to utilize as receptors (Olofsson and Bergström, 2005). Among these binding sites is heparan sulfate, a heavily glycosylated protein found on the surface of animal tissues. Heparan sulfate often serves as initial binding sites for various mammalian viruses, including: Foot and mouth disease virus Type O (Jackson et al., 1996), Dengue virus and yellow fever virus (Germi et al., 2002), herpes viruses (WuDunn and Spear, 1989), and Sindbis viruses (Byrnes
and Griffin, 1998). Other commonly exploited epitopes are sialic acid monosaccharides. The carbohydrate chains of sialic acids are structurally very similar to αGal glycans (Fig. 2). It is possible that αGal molecules play a role analogous to sialic acid during pathogen infection.

Sialic acids are the most commonly expressed terminal carbohydrates on cells of vertebrates (and other animal phyla) and are essential for embryonic development (Varki, 2010). Sialic acid residues are transferred to glycoconjugates by sialyltransferases. Both sialyltransferases and α-galactosyltransferases function within the trans-golgi network and link terminal residues to N-acetyllactosaminyl Galβ1,4GlcNac-R carbohydrate chains (Joziasse et al., 1991; Thall and Galili, 1990). This results in competition for linkage of the terminal unit (either sialic acid or αGal) to the common acceptor (Gorelik et al., 1995). It is of interest to consider that because α-galactosyl residues can utilize the same position on glycoconjugates as sialic acid, they may perform corresponding functions during pathogen infections, and may also be subjected to similar evolutionary pressures. During evolution, certain linkage expression patterns of sialic acid in specific vertebrate taxa underwent major genetic changes, but the same domains remained conserved for millions of years in the majority of vertebrate taxa, including closely related species (Gagneux et al., 2003). This indicates that selective pressures on these domains may be species or lineage-specific. The primate evolutionary record yields good evidence of alterations of sialic acid loci under
selection in response to pressures exerted from pathogens, notably the ST6 beta-galactosamide alpha 2,6-sialyltransferase 1 (ST6GAL1) gene and cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH) gene.

Figure 2: αGal residues on a carbohydrate chain (top) and sialic acid-capped carbohydrate chain (bottom), as part of glycoproteins or glycolipids on the surface of mammalian cells.

2.4.1 Alpha 2,6 linked sialic acid and influenza A

Influenza A and B viruses are among several viruses that can use sialic acid as an attachment receptor to gain entry into host cells (Varki and Varki, 2007), and they also encode their own sialidases (sometimes called neuraminidases) to facilitate their release from these cells (Weis et al., 1988; Susuzki et al., 2000). Influenza A viruses attach to host cell terminal sialic acid residues via the haemagglutinin (HA) glycoprotein on their surfaces; therefore, virus host range is defined by the receptor binding site of the HA they express (Vines et al., 1998; Angata and Varki, 2002). HA of avian influenza A viruses generally recognize different carbohydrate linkages (SAα2,3Gal) than human influenzas (SAα2,6Gal) do, possibly because the linkages orient the terminal carbohydrate in an energetically favorable position (Vines et al., 1998). Among primate taxa, certain influenza
viruses cause disease in humans but not in other great apes, and vice versa (Gagneux et al., 2003). This is because influenza viruses infecting the other great apes use SAα2,3Gal linkage specificity (Gagneux et al., 2003; Varki and Varki, 2007). Humans express SAα2,3Gal linkages but these sites are found lower in the airways relative to SAα2,6Gal sites, and virus may not travel that far down during host exposure (Varki and Varki, 2007). It is hypothesized that the ST6GAL1 gene, the gene responsible for the enzyme that biosynthesizes the placement of the terminal sialic acid to α2,6Gal, has undergone altered regulation in the hominin lineage (Gagneux et al., 2003), to become the predominantly expressed sialic acid linkage in the upper airways. Avian influenzas can sometimes cross the species barrier via an intermediate host—pigs—which express both SAα2,3Gal and SAα2,6Gal domains on the upper respiratory airways (Varki and Varki, 2007). The lack of SAα2,3Gal expression in human upper airways is fairly protective against direct transmission; the 1918 influenza pandemic is an example of a successful transmission event of a bird virus to the human population that could utilize both α2,3Gal and α2,6Gal linkages (Varki and Varki, 2007).

2.4.2 NeuAGc and Malaria

The vector-borne apicomplexan, Plasmodium falciparum, is the causative agent of the most virulent form of malaria in humans. P. falciparum evolved from P. reichenowi, the malarial parasite species that infects our closest living relatives, chimpanzees (Pan troglodytes and P. paniscus) (Rich et al., 2009). Pan, like other apes and most other mammals (except humans), express the terminal sialic
acid Neu5Gc, and in smaller quantities also its metabolic precursor Neu5Ac, on erythrocytes (Chou et al., 1998; Hayakawa et al., 2001). The infective stage of *Plasmodium*, the merozoite, invades host erythrocytes primarily via binding the host glycoprotein A (GYPA) receptor that is capped by sialic acid (Martin et al., 2005). *P. reichenowi* preferentially utilize the sialic acid, Neu5Gc, to cause infection in chimps, but *P. falciparum* binds Neu5Ac in humans (Orlandi et al., 1992). This is because between 2.8 and 3.2 Ma, prior to the appearance of *Homo*, an exon deletion in the cytidine monophosphate (CMP) *N*-acetylneuraminic acid hydroxylase (*CMAH*) gene responsible for conversion of Neu5Ac to Neu5Gc, rendered our ancestors unable to produce the Neu5Gc terminal domain (Chou et al., 1998; Hayakawa et al., 2001; Hayakawa et al., 2006). Only Neu5Ac, the most common sialic acid found in modern day humans, accumulates on RBCs (Hayakawa et al., 2001; Rich et al., 2009), while the Neu5Gc residue is now antigenic (Brinkman-Van der Linden et al., 2000). It is hypothesized that pressure from the ancestral *P. reichenowi* parasite may have driven selection for the mutated gene during hominin evolution that then provided natural resistance to infection (Rich et al., 2009). For some time, our ancestors would have enjoyed reduced susceptibility to malaria, but subsequently, prior to ~10,000 years ago, a population of *P. falciparum* had evolved binding specificity for Neu5Ac, and malaria, abetted by agriculture, once again became a major human pathogen (Hayakawa et al., 2001; Rich et al., 2009). In modern human populations, the sialylated GYP A domain is highly polymorphic. This suggests that even today, this site is under high selection, presumably due to continued pressure exerted
from Plasmodium (Martin et al., 2005) or numerous other pathogens that can exploit this moiety. These include various viruses such as Newcastle disease virus, Sendai virus and human parainfluenza viruses (Angata and Varki, 2002). Although the sialyltransferase responsible for addition of Neu5Ac to glycan chains is under evolutionary constraint, modification of the GYPA protein scaffolding has been an effective host strategy for pathogen evasion (Martin et al., 2005).

It is of special interest, and is perhaps not unexpected, that changes in glycosylation may exert wide-ranging effects on the organism and these effects may themselves be of evolutionary significance. Siglec-1 (sialic acid binding immunoglobulin superfamily lectin) is found in all mammals and has strong binding affinity for Neu5Ac (Brinkman-Van der Linden et al., 2000). Since the inactivation of the CMAH gene, human tissue provides abundant ligands for this lectin, and its expression is especially notable on macrophages in splenic tissue. The localization and expression of siglec-1 may provide humans an advantage against sialic acid-expressing pathogens, increasing their recognition by phagocytic cells (Brinkman-Van der Linden et al., 2000). Other studies with knockout mice for the CMAH gene show that loss of Neu5Gc expression in mice yields phenotypes that appear, to some degree, to have analogues in humans. Compared to control mice, Neu5Gc-deficient mice have age-related hearing loss and slow wound healing; these are derived traits also associated with humans after divergence from our common (CMAH gene-expressing) ancestors with
chimpanzees (Hedlund et al., 2007). Perhaps of more significance is the possibility that Neu5Gc, which is expressed in low quantities in the brain of apes and other mammals, may have restricted brain expansion in ancestral primates (Varki, 2001; Chou et al., 1998; Chou et al., 2002). The fixation of the gene, sometime by 2 Ma, coincides with a significant increase in hominin cranial capacity as evidenced by the fossil record (Chou et al., 2002). Lastly, molecular analyses of CMAH gene sequences between different human ethnic groups, Pan and Gorilla, have even been employed in assessing hominin dispersal out of Africa and in reconstructing modern human origins (Hayakawa et al., 2006).

2.5 Experimental mouse models

Cellular functions, as studied in vitro, may not always translate accurately to functional studies in a complex system. Cell lines can be very useful for testing questions about phenomena at the cellular or molecular level, but for questions that concern evolution, it is essential that in vitro findings be verified at the organismal level. Ideally, research based on a species of interest would also be tested on that animal species, as they are the test subjects. However, several small mammal species are used as models in biological research in lieu of the species of interest, which is often humans. It is neither ethical nor always feasible to experiment directly on humans. Codes of ethics in research also extend to our closest relatives, nonhuman primates, because, among other concerns such as cost and availability, many primate species are endangered.
Mice are among the most commonly used animal models in experimental research because they are less expensive, are easier to genetically manipulate and have faster reproduction times with more numerous litters compared to larger laboratory mammals (Messaoudi et al., 2011; Bähr and Wolf, 2012). These traits are especially beneficial when a great number of experiments must be completed. However, a major criticism of utilizing mice is that they are not suitable substitutes for studying the biology of other organisms (most notably humans) due to their genetic distance from humans. Additionally, inbred laboratory mice do not generally harbor the same species of pathogens as humans, and for disease research this limits the diversity of pathogens and variability in responses from host mice that can be examined (Messaoudi et al., 2011). Despite these limitations, a remarkable amount of information about fundamental cellular and molecular functions is known from using mouse models (Messaoudi et al., 2011). Because of the relative ease of genetic manipulation, mice with desired mutations often can be generated for studies of human issues (Crawley, 1999), and the mutated homologous loci result in genetic and phenotypic similarities in mice and humans (Bedell et al., 1997). Laboratory mice are even useful for studies of nonhuman primates, and serve as models for one of the most severe pathogens affecting nonhuman primates, Ebola virus (Bray et al., 1998; Bradfute et al., 2012). For my dissertation research, working with a nonhuman primate model was not possible due to cost and laboratory capabilities. Fortunately, genetically-altered mice were readily available for testing the proposed hypotheses.
2.6 Viruses

Two groups of viruses were predominantly used in this study to understand the role of GGTA1 activity and the terminal carbohydrate, αGal, in the course of viral infections: 1) Sindbis (SINV) group of viruses and 2) herpes viruses. Clearly, the viruses examined in this study would not have been the putative selection agents of 28 million years ago. As with any study focused on reconstructing the evolutionary past, it is not possible to directly evaluate the selection agents or the effect these agents may have had on primates millions of years ago, but we can make inferences based on strongly supported data. Therefore, this study should be taken as a proof of principle of what could have happened, rather than a factual account of what did occur.

2.6.1 Sindbis virus (SINV) and SINV-related viruses

The arthropod-borne alphaviruses are distributed worldwide: Old World alphaviruses include Sindbis (SINV), West Nile (WNV), Semliki forest (SFV) and Chikungunya (CHIK) viruses, and New World alphaviruses include Eastern equine encephalitis (EEE) and Western equine encephalitis (WEE) viruses (Strauss et al., 1994). The prototype alphavirus, Sindbis virus, is an enveloped, positive-sense RNA virus with a genome of 11,703 nucleotides (Strauss et al., 1994). SINV (strain AR339) was originally isolated from the Culex mosquitos in Sindbis, Egypt in 1952 (Taylor et al., 1955), but it is geographically widespread, and has been collected from infected mosquitos in Europe, Africa, Asia and Australia (Johnson, 1965; Kurkela et al., 2008). Culex and Culiseta mosquitos are
primary vectors of SINV (Taylor et al., 1955; Johnson, 1965; Kurkela et al., 2008), although ixodid (hard) tick transmission has been documented (Taylor et al., 1955; Gresikova et al., 1978). Various avian species, including game and passerine birds, serve as reservoirs for the virus (Taylor et al., 1955; Sammels et al., 1999; Kurkela et al., 2008), and it is possible that the geographical dissemination of the virus is due to the migrations of viremic birds (Sammels et al., 1999; Kurkela et al., 2008). A broad range of animal hosts, such as amphibians and mammals (including humans), are susceptible to SINV (Johnson, 1965). In humans, disease from SINV infections can range from asymptomatic to chronic, and although generally not a health threat, epidemics have occurred in Europe and Africa (Sammels et al., 1996; Kurkela et al., 2008).

In mice, SINV is neurovirulent and is generally fatal if the virus reaches the central nervous system (CNS), but infection is restricted if only extraneural tissue is involved (Johnson, 1965). The virus replicates in skin, skeletal muscle and fibroblast tissue, and then disseminates to the CNS via a viremia (Klimstra et al., 1998; Ryman et al., 2000). Lesions occur in both skeletal muscle and in the CNS, but there is no significant pathology in infected liver or abdominal organs (Taylor et al., 1955). In the brain, SINV induces apoptosis in spinal cord and brain cells (which do not regenerate), and the loss of this tissue correlates with death in mice (Lewis et al., 1996). Most strains of SINV are lethal to neonatal mice, replicating to very high levels and causing ataxia and death by five days post infection (Taylor et al., 1955; Klimstra et al., 1998). In weanling mice, the virus has a
prolonged incubation period that results in delayed onset of symptoms (Taylor et al., 1955); by approximately two weeks of age, mice have reduced viral load and no longer succumb to infection (Taylor et al., 1955; Reinarz et al., 1971; Lewis et al., 1996). In these mice, virus replication and viremia are limited primarily by a robust IFN-α/β response, which increases with age (Ryman et al., 2000).

SINV are easily adaptable to a variety of cells in vitro, and strains used in laboratory settings can be modified by their cell culture passage history (McKnight et al., 1996). For example, the virus can acquire the ability to utilize heparan sulfate as an attachment receptor when grown in BHK cells, even after one passage (Klimstra et al., 1998). SINV viruses may also become attenuated after serial passage (McKnight et al., 1996). Additionally, SINV isolates, mutants or laboratory strains may differ in their level of virulence in young and adult mice (McKnight et al., 1996). S.A.A.R86 (South African arbovirus no. 86) is a natural virus isolate of the SINV group of alphaviruses and is genetically distinct from the wild-type (WT) AR339 strain (Heise et al., 2000; Ryman et al., 2000). This isolate is neurovirulent and lethal to adult mice when inoculated intracranially (i.c.) (Russell et al., 1989; Ryman et al., 2000); this is due to a mutation in its nonstructural protein 1 (Heise et al., 2000). The TR339 strain is a laboratory-generated clone that is similar to AR339, but expresses cell-culture adaptive mutations such as heparan sulfate binding, and is more virulent to neonatal mice compared to culture-adapted strains (McKnight et al., 1996). The TE12-GFP
mutant is a recombinant virus encoding a green fluorescent protein (GFP) gene under control of a subgenomic promoter (Wang, 2008). The GFP mutant replicates to similar titers as the parental TE12 mutant (Wang, 2008). TE12-GFP was constructed from the (avirulent) Toto1101 and (virulent) NSV strains and is highly lethal to suckling mice, but not adult mice (Lustig et al., 1988).

2.6.2 Herpes viruses

The alphaherpesviruses, herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2), are large, double stranded DNA viruses that are ubiquitous human pathogens. These viruses cause primary (lytic) infections in skin and mucosal epithelia and then travel to the peripheral and central nervous system where the viruses establish latent infections in ganglia (Sodeik, 2007). Lytic infections can recur when the virus reactivates and travels back to the peripheral sites of infection (Sodeik, 2007).

Mice serve as models for infections of these human pathogens (Baker and Plotkin et al., 1978; Manickan and Rouse, 1995). The pathogenesis and neurovirulence of HSV-1 and 2 can differ, as shown in experimental mice. This is often a consequence of the route of infection. By intravaginal infections, both virus strains produce infections in the vulva and vagina, without much involvement of peripheral organs. The virus then disseminates to the CNS where infection can lead to fatal encephalitis (Renis et al., 1976). Almost any route of inoculation with HSV-2 results in apparent hepatic necrosis, but similar infections with HSV-1 seldom result in liver involvement or damage (Mogensen et al., 1974; Renis et
al., 1976). Severe infections with herpesviruses in humans (e.g., HSV-2, VZV, EBV) often involve dissemination to various organs and exhibit liver pathology (Renis et al., 1976).

The HSV-1 GFP (KOS 1.1) strain is a mutant that expresses a GFP-ICP8 fusion protein. The ICP8 protein, an early DNA-binding protein that functions in viral nucleic acid and protein localization to replication compartments, as well as regulation of late gene expression in host cells, is mutated in this recombinant virus (McNamee et al., 2000). The virus is able to effectively initiate infection, but DNA replication and late gene expression in host cells are affected, and subsequent virus production is significantly reduced compared to that of wild type HSV-1 (Gao and Knipe, 1991; McNamee et al., 2000; Taylor et al., 2003).

The murine gammaherpes virus 68 (γMHV-68) is a member of the gammaherpesviruses. The virus is closely related to the human Epstein-Barr virus (EBV; the most common agent of mononucleosis) and Kaposi’s sarcoma-associated herpesvirus. In mice, infections with γMHV-68 most commonly occur through the nasopharyngeal tissue, and the virus replicates in the lung epithelial cells (Michaud et al., 2010) where it can cause lymphoproliferative disease (Sunil-Chandra et al., 1992). During primary infection, the virus can be recovered from the lung and various peripheral tissues such as kidneys and spleen (Sunil-Chandra et al., 1992). In the spleen, the primary organ where virus persists, infection leads to enlargement of the organ (splenomegaly) (Sunil-Chandra et al.,
1992). Latent infection occurs when the virus migrates from lung and other tissues to B lymphocytes (as seen with EBV infections in humans) and macrophages (Sunil-Chandra et al., 1992; Michaud et al., 2010). Several months to a year after infection, mice can develop lymphomas or lymphoproliferative disease (Sunil-Chandra et al., 1992).
3.1 Cell lines

For *in vitro* studies, three pairs of cell lines (two mouse lines and one human line) were used to verify that findings were not unique to a single type of cell line or organism from which the cell line derived. The GT KO mouse embryonic fibroblast (MEF) cell line is derived from GT-deficient transgenic mice and therefore lacks expression of αGal. MEFs from 129 WT mice (GT-sufficient) (Jones et. al., 1996) were acquired from Dr. Stephen Jones at UMMS, as controls. Both cell lines were cultured as monolayers in 10cm petri plates because 129 WT MEFs appear to grown better in dishes than in flasks. Cultured cells were fed with Gibco Dulbecco modified Eagle’s medium (EMEM) and supplemented with 10% fetal bovine serum (FBS) and 2.5% penicillin and streptomycin and 2.5% L-glutamine. WT 129 cells produce debris after initial passages, so they were used after third passage for experiments, but after 4-5 passages, cells were no longer viable for experimentation.

A pair of modified human melanoma cell lines, the SK-N-MC cells (Biedler et al., 1973), were transduced with a retroviral vector containing a full-length porcine GT cDNA (pLGTSN) or a retroviral vector without insert (pLXSN) (Welsh, 1998). The GT-modified human cell lines were designated as SK-N-MC pLXN
(SK) (inactive GT gene) and SK-N-MC pLGTSN clone 10 (SK-GT) (active GT gene). These cell lines were grown in GIBCO Roswell Park Memorial Institute (RPMI) medium 1640 and supplemented with 10% fetal calf serum (FCS) and 2.5% penicillin and streptomycin, 2.5% L-glutamine and the selective antibiotic for the Neomycin resistance gene, G418 (500μm/mL).

The BL6 (bladder 6) cell line, a subclone of the highly invasive B16 melanoma cell line, was isolated from the bladder wall of C57BL/6J mice (Hart, 1979; Poste et al., 1980). Continuous selection for metastatic capabilities resulted in BL6 cells that spontaneously lost biosynthetic αGT activity (Gorelik et al., 1995). These melanoma cells were later transfected with the murine α1,3GT gene cDNA to create a cell line, designated BL6αGT, with similar characteristics to the parental cell line except for αGal expression on the cell surface (Gorelik et al., 1995). We used the BL6αGT (GT+) cell line as controls for BL6 (GT-) cells. Both cell lines were cultivated as monolayers in GIBCO minimum essential medium (MEM) and supplemented with 10% fetal calf serum (FCS) and 2.5% penicillin and streptomycin and 2.5% L-glutamine. The stable transfectant BL6αGT line seems to maintain the inserted gene without selection media, but the antibiotic G418 can be utilized to reduce the possibility it will lose the gene (U. Galili, pers. comm.). Cells were maintained by freezing at -80° in aliquots of approximately 1x10^6 cells/vial, in 90% fetal calf serum and 10% DMSO. Two different clones of the BL6 and BL6αGT cell lines were used throughout the course of the study, because the initial set of cells acquired began exhibiting
differences in morphology and growth rates, and BL6αGT displayed variable expression of αGal on its’ surface. The second set of cells appeared more stable. Several experiments initially completed on the first set of cells, such as time courses and adsorption assays, were repeated on the second set. Results were consistent in both sets of cell lines.

SK and SK-GT cell lines were tested for surface expression of αGal by staining with monoclonal anti-gal antibody, M86 (Enzo Life Sciences), and secondary allophycocyanin (APC)- or R-phycoerythrin (R-PE)- conjugated goat anti-mouse IgM (Invitrogen). BL6 and BL6αGT were regularly monitored for αGal expression during the course of the study with M86 and secondary APC or R-PE, or alternatively, with fluorescein isothiocyanate (FITC)-conjugated BS lectin (BSl-B₄) from Bandeiraea simplicifolia was used as an αGal binding lectin (Sigma-Aldrich). BSI has broader specificity than anti-gal, as it has binding capacity to the GalNAc and Galα1,4Gal carbohydrates, but anti-Gal is specific only to a galactose in an α(1,3) linkage to a penultimate galactose (Galili et. al, 1984). BSI can also bind to Galα1,3Fucα1,2Gal, as demonstrated by hemagglutination of erythrocytes derived from Hylobates and Pongo (Galili et al., 1987). Fluorescence was measured using a BD Biosciences LSR II flow cytometer with FACS Diva software and analyzed using FlowJo software (TreeStar Inc., Ashland, OR).
3.2 Knockout mice

GT KO mice [C57BL/6J x DBA/2J x 129sv] were generated by disruption of the α1,3GT gene by homologous recombination (Thall et al., 1995). GT KO mice were backcrossed onto the C57BL/6J background for well over ten generations in the laboratory of Dr. Uri Galili in the Department of Medicine at UMMS. Breeding pairs were kindly donated to our laboratory, and mice were bred at the UMMS animal facilities. Adult C57BL/6J mice, acquired from Jackson Laboratories, were used as control mice. C57BL/6J (B6) suckling mice were bred at the UMMS animal facilities. During the course of this study the GT KO mice generally bred well, with litter sizes that ranged between 2-10 pups, and mice only displayed occasional periods of reduced reproductive output. The mice showed no outward signs of abnormal health conditions, except a few incidents of dental malocclusion and rare cases of hydrocephaly or rectal prolapse.

3.3 Mouse crosses

Different mouse strains have variable resistance to HSV infections (Kastrukoff et al., 1985). Both 129 and DBA/2 mice are genetically more susceptible to HSV infections than B6 mice. Because B6 mice are utilized as controls to KO mice in \textit{in vivo} experiments, any underlying susceptibility that is a result of the KO genetic background would skew results. The backcrossing history of KO mice into the B6 line suggests that the disrupted \textit{GGTA1} locus should be well recombined into the B6 genotype. However, further outcrossing and backcrossing mice will provide some assurance that our loci of interest will further recombine.
into the B6 background and that possible genetic linkages from the DBA/2 and 129 backgrounds will be disassociated. Male C57BL/6J mice were mated with female GT KO to produce an F1 heterozygous generation. The heterozygotes maintain active GT expression. Backcrosses (F1xWT and F1xKO) were then created by mating the F1 generation with individuals of the parental strains. All F1xWT crosses had functional GT expression while F1xKO crosses had a 50% probability of being homozygous for the KO gene. It would be expected that the susceptibility of an F1xKO mouse that inherited two alleles of the inactive α1,3GT locus would respond in the same way as a GT KO animal, to herpesvirus infection. F1xF1 intercrossed mice were also created, but were utilized in few experiments. All mice were analyzed for GT activity by testing for surface expression of αGal. Anti-Gal or BS lectin binding of peripheral or cardiac blood cells was performed; BS lectin was more adequate for testing αGal expression in blood.

### 3.4 Enveloped viruses

The following enveloped viruses were predominantly used in these studies: Sindbis virus (SINV) HR strain (Burge and Pfefferkorn, 1966); S.A.A.R86, TR339 and TE12-GFP strains obtained from Dr. Dennis T. Brown at North Carolina State University; herpes simplex type 2 (HSV-2) strain MS (Rosenberg et al., 1972); herpes simplex type 1 (HSV-1) KOS1.1 strain (Babu et al., 1996)
obtained from Dr. David Knipe, Harvard; HSV-1 KOS 1.1 GFP (green fluorescent protein) ICP8 mutant (McNamee et al., 2000) obtained from David Knipe; mouse γ-herpes virus-68 (γMHV-68) obtained from Dr. Blake Tomkinson.

Table 1 shows additional viruses that were screened during the study. These viruses were readily available for use in the laboratory of Dr. Raymond Welsh at the UMMS. Viruses were grown in Vero cells, which do not express αGal; therefore the carbohydrate was not expressed on the viral envelope. Several SINV and herpes viruses were also grown in L929 cells for experiments where αGal expression on the viral envelope was needed. Additionally, murine cytomegalo virus (MCMV), strain Smith, was grown in GTKO cells that do not express αGal and had an initial stock titer of 3.5x10^7.
### TABLE 1: Viruses screened during this study

<table>
<thead>
<tr>
<th>Virus</th>
<th>Virus titer log_{10} PFU (mean)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vero(-αGal)(^b)</td>
</tr>
<tr>
<td>Sindbis (SINV), AR339</td>
<td>1.15x10^8</td>
</tr>
<tr>
<td>S.A.A.R86</td>
<td>5x10^6</td>
</tr>
<tr>
<td>TE12-GFP</td>
<td>5x10^7</td>
</tr>
<tr>
<td>TR339</td>
<td>3.6x10^6</td>
</tr>
<tr>
<td>SVHR</td>
<td>3.2x10^7</td>
</tr>
<tr>
<td>Herpes simplex virus type 1 (HSV-1), KOS 1.1</td>
<td>9x10^5</td>
</tr>
<tr>
<td>GFP ICP8</td>
<td>1.5x10^6</td>
</tr>
<tr>
<td>Herpes simplex virus type 2 (HSV-2), MS</td>
<td>1.24x10^6</td>
</tr>
<tr>
<td>Mouse γ-herpes virus-68 (γMHV-68)</td>
<td>1.85x10^4</td>
</tr>
<tr>
<td>Pichinde virus (PV), AN3739</td>
<td>1.4x10^7</td>
</tr>
<tr>
<td>Lymphocytic choriomeningitis virus (LCMV), Armstrong</td>
<td>1.2x10^5</td>
</tr>
<tr>
<td>Vesicular stomatitis virus (VSV), Indiana</td>
<td>1.3x10^8</td>
</tr>
<tr>
<td>Mouse hepatitis virus (MHV), A-59</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\) Viral stocks were grown in either αGal-negative Vero cells or αGal-positive L929 cells and were then titrated on Vero cell monolayers.

\(^b\) Titers shown are mean viral titers from duplicate tests.

\(^c\) – denotes that virus was not grown in that cell type.

Viruses were propagated in Vero cells (Chlorocebus sp. kidney cell line) or L929 cells (C3H mouse fibroblast cell line) and harvested at the peak of infection. Cell debris was removed from culture fluid by centrifugation. Culture fluid from virus-infected cells was titrated by plaque assay on Vero cell monolayers. The concentration of virus (multiplicity of infection or MOI) used for infections in our assays was based on titrations in Vero cells. Vero cells do not express αGal on their surfaces, and viruses propagated in Vero cells do not carry αGal.
carbohydrates on their envelopes. L929 cells express αGal on their surfaces, and viruses grown in L929 cells have αGal incorporated into their envelopes (Welsh et al, 1998). Vero and L929 cells were maintained as monolayers in MEM supplemented with 10% FCS and 2.5% penicillin and streptomycin and 2.5% L-glutamine.

### 3.5 Time courses

For time courses of viral infections, GT-positive and GT-negative cells were seeded in 6-well plastic plates and left to adhere anywhere from 4 and 24 h prior to infection. The period of time cells were left to adhere to plates did not affect the results of the time course infections. Before adding virus, media were removed and replaced with 1mL of complete MEM. Virus was diluted to an MOI from .01 to 1.0 in MEM, added to the cell monolayers, and left for adsorption periods of 60 minutes for time courses. Culture fluid with unabsorbed virus was removed, and the cells were washed twice and re-fed with 2mL MEM. Culture fluid from infected cells was collected up to 72 hours post infection (h pi) and titrated by plaque assay on Vero cell monolayers. Monolayers infected with GFP viruses were observed using a Nikon Eclipse fluorescence microscope, and the images were analyzed with NIS-Elements Advanced Research imaging software; alternatively, cells were harvested by dispersion with trypsin and analyzed by FACs analysis at each time point.
3.6 γMHV-68 time course and PCR analysis

Infections with γMHV-68 were completed on BL6 and BL6αGal cells, and virus was quantified by PCR. Cells were seeded in 6-well plastic plates and left to adhere for 24 h prior to infection. Before adding virus, media was removed and replaced with 1mL of complete MEM. Virus was diluted to an MOI of 1.0 in media, added to the cell monolayers, and left for adsorption periods of 60 minutes. Culture fluid with unabsorbed virus was removed, and cells were washed twice and re-fed with 2mL MEM. Cells were harvested at 24, 48 or 62 h pi, counted, and then prepped for PCR analysis using the CFX detection system (BioRad Laboratories, Hercules, CA). The two amplification primers for the viral ORF49 were as follows: forward primer, 5’-CCTGGCCATGTTACATACTC-3’; reverse primer, 5’-GGAACATAATCCATAAGGGT-3’ (Lee et al., 2007). No calibration standard was readily available for use, so quantification of viral copy number (absolute quantification) was not possible. Instead, samples were normalized using a reference gene, the housekeeping gene β-actin, found in host cells, to control for relative differences in spleen size. DNA synthesis was monitored using the nucleic acid stain SYBR Green I. Samples of γMHV-68 from culture fluid and water were used as positive and negative controls, respectively. PCR conditions were as follows: 95°C for 10 mins denaturation, and 95°C for 30 s, 62°C for 30 s and 72°C for 30 s, for 37 cycles. Samples were tested in duplicate. The quantification cycle or Cq (the number of cycles where the fluorescence increases above background) mean for the two values and for the values of the housekeeping gene for each sample, were imported into the
QIAGEN RT² profiler PCR array data analysis, version 3.5 website
(SABiosciences, a QIAGEN company)
(http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php), to obtain fold change calculations. The sample with the lowest Cq mean was used to set the baseline from which fold change calculations were derived. The measurement unit “fold change” refers to a fold-difference in quantity of DNA of any sample relative to the sample used as a baseline.

3.7 Infectious center assays and fluorescent-focus assays

Three different protocols were used for infectious center assays (ICA), and the different techniques did not appear to influence final results. BL6 and BL6αGal cells, at a concentration of 2x10⁵ to 3x10⁵ cells/well, were placed in tubes in 1 mL MEM. Another 1 mL of diluted virus, either HSV-2 or SINV AR339, at an MOI of 1.0, was then added the tube. The cell-virus suspension was left to incubate for 30, 60 or 90 minutes at 37° C, and tubes were shaken every 15 minutes. At the end of each incubation point, virus was washed off twice by centrifugation with 3 mL media each time. At the end of each incubation period, cells were counted and then plated over a Vero cell monolayer in serial dilution (analogous to a plaque assay).

Otherwise, BL6 and BL6αGal cells, at a concentration 3x10⁵ cells/well, were plated in 12-well plates and left to adhere from 4 to 24 h. Media were removed and virus was added in 500 µl at an MOI of 1.0 and left to incubate with cells for
30, 60 or 90 minutes at 37° C. Plates were shaken every 15 minutes. At the end of each incubation period, cells were harvested by dispersion with trypsin and washed twice by centrifugation. Cells were then counted and overlaid on Vero cell monolayers in a serial dilution.

Alternately, BL6 and BL6αGal cells, at a concentration 3x10^5 cells/well, were dispersed with trypsin and added to 96-well plates in 100 μl MEM. Virus was then added to the wells in 100 μl and left to incubate for 30, 60 or 90 minutes at 37° C. Wells were gently pipetted every 15 minutes. At the end of each incubation period, virus was washed off three times with 300 μl by centrifugation. Cells were then counted and overlaid on Vero cell monolayers in a serial dilution.

For fluorescent focus-assays, BL6 and BL6αGal cells, at a concentration of 2x10^5 to 3x10^5 cells/well, were seeded in 6-well plastic plates and left to adhere from 4 to 24 h prior to infection. At time of infection, media were removed and replaced with 1mL of complete MEM. Either TE12-GFP or HSV-1GFP virus was diluted to an MOI from 0.01 to 1.0 in MEM, added to the cell monolayers, and left for adsorption periods from 15 to 180 minutes for adsorption assays at 37° C. Culture fluid with unabsorbed virus was removed, and cells were washed twice by pipetting and were re-fed with 2mL MEM. The infection was left to run from 7 to 48 h. At the end of each time point, infected monolayers with GFP viruses were
observed using a Nikon Eclipse fluorescence microscope, and the images were analyzed with NIS-Elements Advanced Research imaging software; alternatively, cells were harvested by dispersion with trypsin and analyzed by FACs analysis.

3.8 Blocking assays

For blocking using the anti-Gal antibody, M86, $5 \times 10^5$ cells/well were seeded in 6-well plates. Cells were left to adhere to plates from 5 to 24 h in 2 mL of MEM. Media were removed and antibody was then added at different concentrations, from 1:5 to 1:100, in 1 mL of MEM. Plates were left in the 37°C incubator for 60 minutes and then SINV S.A.A.R86 or HSV-2, at an MOI of 0.1, in 100 μl MEM, was added to cells. Cells were harvested using trypsin and washed by centrifugation, and then were plated over Vero monolayers.

For blocking with liposomes (acquired from Dr. Uri Galili at the UMMS), $2 \times 10^5$ plaque-forming units (PFU) of SINV TE12-GFP virus in 50 μl media were added to a solution of 1 μl or 2 μl of αGal-liposomes or control liposomes in a 50 μl suspension (total volume of 100 μl). The virus-liposome mixtures were incubated at 4°C for 1 h. The mixture was then added to $2 \times 10^5$ BL6 or BL6αGal cells suspended in 100 μl of MEM. Cells were washed twice after a 2 h incubation period by centrifugation and plated in 6-well plates. The infection was left for 9 h and cells were harvested and analyzed by FACs. A second set of cells was left plated for examination at a later time point.
3.9 Enzymatic removal of αGal from cell surfaces

To remove surface αGal from BL6αGal cells, 2x10^5 cells/well were added to 96-well plates or to 2 mL tubes, in 100 μl of a phosphate buffered saline (PBS) solution. BL6 were treated in parallel as controls. The PBS solution was adjusted to pH ranging from 6.0 to 7.0. The enzyme is most efficient at pH 6.0, but cells do not fare well for too long. At pH 7.0, the enzyme is less efficient and cells (although seemingly more healthy in their environment) must be treated for an extended period of time. The pH levels of 6.2 and 6.4 worked best for this assay. The α-galactosidase from green coffee bean (Sigma-Aldrich) was added to cells at varying concentrations, from 1:4 (25 μl) to 1:25 (4 μl), in a total of 100 μl PBS solution. The enzyme is specific to αGal and cleaves off the terminal disaccharide from glycoproteins and glycolipids on the cell surface. Cells were incubated with enzyme for 60 min at 25°C or room temperature and shaken every 15 minutes. Cells were then washed twice by centrifugation. One set of cells was tested directly after treatment for αGal expression using anti-Gal and analyzed by FACs. SINV TE12-GFP virus was diluted to an MOI of 1.0 and added to the second set of cells in 96-well plates or 2 mL tubes in 100 μl. Cells and virus were incubated for 60 min and then were washed twice by centrifugation. Cells were then seeded in 6-well plates and the infection was left from 6 to10 h. Cells were then harvested and fixed for FACs analysis.
3.10 Disruption of the GT gene using RNAi

Four siRNA clones were purchased from QIAGEN (FlexiTube GeneSolution GS14594 for Ggta1), each targeting a different region of the gene. siRNA clones were tested individually, or in combination, to observe if any single one or combination was especially effective in disrupting enzyme production. BL6αGal cells were seeded in 12-well plates at concentrations of $2 \times 10^4$ cells/well to $6 \times 10^4$ cells/well. Each clone was tested at two concentrations, 5nM (0.3 μl) or 10nM (0.6 μl) mixed with 4 μl transfection reagent in a total of 100 μl incomplete MEM (no FCS or antibiotics). The mixture was slightly vortexed and let stand for approximately 10 minutes for the reaction to occur. Next, 100 μl of each clone mixture was added to freshly seeded cells (within 10 mins of seeding) in 500 μl MEM. The mixture was added drop-style and distributed thoroughly throughout each well. Cells were gently rocked and incubated in a 37° C incubator. Cells were analyzed at various time points, from 20 to 66 h pi. As a control, fluorescently labeled Cy3-siRNA was utilized to ensure the BL6αGal cells were permissive of siRNA transfection.

3.11 Addition of αGal molecules to host cell membranes using FSL Galili constructs

Vero cells, which do not express αGal, were used in these experiments as the TE12-GFP virus replicates very well in this cell line. Cells at a concentration of $2 \times 10^5$ cells/well were added to 96-well plates, or 2 mL tubes, in 100 μl of neutral PBS. FSL-Galili(tri) and control FSL-Biotin (Sigma-Aldrich) constructs are
composed of three fragments: a lipid, a spacer molecule and a functional component. For these experiments, an αGal disaccharide or a Biotin molecule, used as a control, constituted the functional component of the construct. These constructs insert themselves into cell membranes via the lipid segment of the construct and remain there for the lifetime of the cell. Either FSL-Galili(tri) or FSL-Biotin at various concentrations, from 1:4 (25 μl) to 1:100 (1 μl), were added to Vero cells in 100 μl PBS. The mixture was incubated from 60 -120 min and then washed twice with PBS. One set of samples was used immediately after FSL treatment, to test for αGal expression by anti-Gal binding and for Biotin expression by fluorescently labeled streptavidin. SINV TE12-GFP virus at an MOI of 1.0 in 100 μl of lipid-free media (incomplete MEM) was added to the second set of samples, and samples were incubated for 60 min in the 37° incubator. The virus was then washed off twice by centrifugation, and 6-well plates were seeded with cells. Cells were harvested between 6-10 h pi and fixed for FACs analysis.

3.12 HSV-2 infections in adult male mice and γ-irradiation

Male GT KO and C57BL/6J (WT B6) mice from 8 to 19 weeks of age were used in various experiments where no γ-irradiation was administered. Mice were inoculated intraperitoneally (i.p.) with 3.75x10⁵ PFU or 5x10⁵ PFU of HSV-2. Infected mice were examined daily for physiological or behavioral changes and sacrificed by cervical dislocation at 3, 4 and 5 days post infection. Fat pad, spleen and liver were harvested and examined for signs of pathology, such as increase in
organ size or the formation of lesions. Virus from organ suspensions was titrated by plaque assay on Vero cell monolayers. For *in vivo* HSV-2 infections with γ-irradiated animals, male GT KO and WT B6 mice between the ages of 8 and 14 weeks received 9-11Gy. Immediately after irradiation, mice were inoculated i.p. with 3.75x10⁴ PFU of HSV-2. Infected mice were observed and sacrificed as described above at days 2, 3, 4, 5 and 6. Organs were harvested, examined, and titrated as described previously.

3.12.1 HSV-2 infections of genetically crossed male mice

For irradiation experiments, WT- crossed (F₁ x WT) and KO- crossed (F₁ x KO) male mice, aged approximately 12 weeks, were exposed to 10Gy of γ-irradiation, and immediately after, were inoculated i.p. with 8.5x10⁵ PFU of virus. Infected mice were examined daily for physiological or behavioral changes and sacrificed by cervical dislocation at day 6 pi. Livers were examined for pathology such as lesions, and both liver and brain were homogenized for titration of virus. The phenotype (αGal expresser or non-expresser) of genetically crossed mice was not known; therefore, blood was taken at time of harvest via heart punctures, to analyze for αGal expression of lymphocytes using BSI.

3.13 HSV-1 infections

For HSV-1 experiments, male and female GT KO, WT B6 and crossed mice (F₁, F₁ x KO) of 4 weeks of age were used. Mice were inoculated intranasally (i.n.) with 1.8x10⁴ PFU in 20 μl. Infected mice were sacrificed by cervical dislocation
at days 1, 2 and 4 pi. Lungs were harvested and virus from organ suspensions was titrated by plaque assay on Vero cell monolayers. Blood was also taken by performing heart punctures, and utilized to test for αGal expression by BS lectin binding and analyzed by FACs.

3.14 γMHV-68 infections

Male and female KO, WT, F₁ and F₁xKO mice from 6-12 weeks of age were used in various γMHV-68 experiments. Mice were inoculated i.p. with 3.5x10³ PFU or 5.5x10³ PFU of HSV-2. Because a productive infection lasts until about 10 dpi (Sunil-Chandra et al., 1992), animals were sacrificed by inhalation of CO₂ at days 4, 5, 7, 9 and 11 pi to observe the course of the infection. Spleens were harvested and weighed at time of sacrifice and then prepped for molecular analysis. Viral genomes were quantified with real-time PCR as described for in vitro experiments.

3.15 SINV infections of adult mice and γ-irradiation

For SINV infections, male and female GT KO and WT B6 mice aged from 8 to 9 weeks were inoculated i.p. with 10⁵ PFU of SINV S.A.AR86 in 200 μl MEM. Blood, spleen, fat pads, or brain was harvested at days 1, 2, 3 and 5 for titration of virus. Additionally, 8-week old mice were γ-irradiated with 10Gy and inoculated with the same dose of virus. Blood, spleen, liver and fat pad were harvested at day 6 pi.
3.16 SINV survival studies

GT KO and C57BL/6J suckling mice were infected via different routes and doses of different SINV strains as follows: 1) 8-day old mice were inoculated intracranially (i.c.) with 200 PFU AR339 strain; 2) 13-day old mice were inoculated subcutaneously (s.c.) with 1.68x10^3 PFU. Different inoculation routes and ages were used to determine susceptibility to infection. Pregnant females were monitored for day of birth, but because births from GT KO and WT B6 pups were not synchronized to the day, infections of mice were staggered. Entire litters (ranging from 3-8 pups) were infected when pups came of age, excluding runts. Two to three litters per mouse strain were used for each experiment at each dose, SINV strain, and age of infection. Mice were weighed daily and monitored for symptoms of disease, including paralysis, lethargy and other possible signs of neurological involvement such as spasms and shaking. Additionally, WT, KO, F_1xKO and F_1xWT suckling mice aged 8 days were inoculated i.c. with 200 PFU or 1.1x10^6 PFU of S.A.AR86 virus. Brains were harvested at day 1 and day 2 pi for titration of virus.

3.17 Statistical methods

Survival data were analyzed by Log-rank (Mantel-Cox) test and Gehan-Breslow-Wilcoxon survival test using GraphPad inStat software. Analyses of individual HSV-2 in vivo experiments were done using GraphPad inStat Student’s t tests and chi-squares, and analyses of pooled experiments were completed using Minitab
Statistical software ANOVA mixed models with random assumption, factoring
dose, age, and experimental trial differences. Significance was set at $P \leq 0.05$; *
indicates a $P$ of $\leq 0.05$, ** a $P$ of $\leq 0.005$. 
4.1 Surface expression of $\alpha$Gal on experimental cell lines

Experiments were performed using paired cell lines. In each pair, one line actively expressed the GT gene and the other did not. Figure 3a shows that the BL6 cell line did not react with anti-Gal (left panel), or bind BS lectin (right panel), which also reacts with $\alpha$Gal. The population of bright BL6 cells stained with BSl (right shoulder of peak) may be due to the broader binding specificity of BSl compared to anti-Gal. The BL6$\alpha$GT line bound both anti-gal and BSl.

Figure 3b shows that the SK cell line did not react with anti-Gal, but SK-GT cells bound it. The fluorescence peak from anti-Gal-stained SK-GT cell lines is broad, indicating that there is variation in the quantity of $\alpha$Gal residues expressed on the surface of individual cells within the GT-positive population. These data confirm cell surface expression of $\alpha$Gal on BL6$\alpha$GT and SK-GT at the time of these experiments. This is an important issue, as continued passage of the cells can result in decreased expression over time.
Figure 3: Expression of αGal on paired BL6 and SK cell lines. Cells were stained with anti-Gal (IgM) or BS lectin (BSl-B₄), both of which bind αGal. The light grey peak represents αGal-negative cells and the black peak represents that of αGal-positive cells. BSl can bind αGal monomers, while anti-Gal requires multiple sites for binding. MFI is mean fluorescence intensity of entire cell population. (A) BL6 and BL6αGT cell lines stained with anti-Gal or BSL. (B) SK and SK-GT stained with anti-Gal.

4.2 Viral growth kinetics in the presence or absence of α1,3GT gene in the host cell

If GT activity is important in the course of viral infections, then viral replication should be contingent on whether or not host cells express GT. Previously, preliminary work in the laboratory of Dr. Raymond Welsh had demonstrated differential growth kinetics with Sindbis AR339, HSV-1 and HSV-2 in the paired GT-modified human melanoma cell lines, the SK-N-MC pLXN (SK) (inactive αGT gene) and SK-N-MC clone 10 (SK-GT) (active αGT gene). I also verified GT-dependent differential susceptibility of SK and SK-GT cells to these viruses during the course of this study. Sindbis viruses preferentially grew in GT-positive cells whereas HSV-2 grew better in GT-negative cells (Table 2). Although the SK and SK-GT cell lines had been similarly maintained and passaged, they had slightly different morphologies and growth kinetics. SK cells consistently
established a uniform monolayer whereas SK-GT cells appeared to lose contact inhibition after multiple passages. Because of this, it was important to verify these findings in other paired cell lines.

TABLE 2: Replication of viruses in SK human melanoma cells

<table>
<thead>
<tr>
<th>Virus (h pi)</th>
<th>Virus titer log_{10}PFU (mean ± SD)^b</th>
<th>SK (-αGal)</th>
<th>SK-GT (+αGal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-2</td>
<td></td>
<td>3.5</td>
<td>4.5</td>
</tr>
<tr>
<td>SINV (AR339)</td>
<td></td>
<td>7.3</td>
<td>6.0</td>
</tr>
<tr>
<td>SINV TE12-GFP</td>
<td></td>
<td>6.2</td>
<td>5.5</td>
</tr>
</tbody>
</table>

*aHSV and SINV viruses grown in either GT-positive or GT-negative SK cells were titrated on Vero cell monolayers. These results were completed with single samples, but are representative of between 2-4 experiments.

b Titers shown were peak titers for that time course. MOI =1.0. Bolded numbers indicate the comparably higher titer.

The majority of the research for this dissertation (including all *in vitro* experiments that follow below) was completed using the paired B6 mouse-derived melanoma cell lines, BL6 (GT-negative) and BL6αGT (GT-positive). In culture, these cells were the most robust and were also the best matched in terms of growth and phenotype. Various SINV strains, herpes viruses and other enveloped viruses were screened on these cells to observe differences in susceptibility. Table 3 shows representative data from infections on BL6 and BL6αGT indicating that several of the viruses screened (VSV, LCMV, PV, MHV, MCMV) grew equally well (as measured by titers from culture fluid) in both BL6 and BL6αGT lines. Any variations in the growth of these viruses in the two cell lines that did occur, such as with MCMV, were inconsistent.
TABLE 3: Replication of viruses in B6 melanoma cells

<table>
<thead>
<tr>
<th>Virus (h pi)</th>
<th>Virus titer log$_{10}$PFU (mean ± SD)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BL6 (-αGal)</td>
</tr>
<tr>
<td>VSV</td>
<td>(24)</td>
</tr>
<tr>
<td>PV</td>
<td>(48)</td>
</tr>
<tr>
<td>LCMV</td>
<td>(48)</td>
</tr>
<tr>
<td>MHV</td>
<td>(48)</td>
</tr>
</tbody>
</table>

$^a$ Viruses grown in either BL6 or BL6αGT cells were titrated on Vero cell monolayers. Experiments were completed in duplicate and are representative of between 2-4 tests.

$^b$ Titers shown were peak titers for that time course. MOI for VSV and PV was 1.0 and 0.1 for LCMV and MHV.

$^c$ - denotes no virus growth.

Differences in replication between cell lines were consistently observed for all tested SINV strains (AR339, S.A.AR86, TR339, SVHR and TE12-GFP), HSV-1, HSV-1 GFP and HSV-2. Notably, the SINV strains grew often to 10-20 fold higher titers in the presence of αGT, whereas the HSV strains grew often to 100-fold higher titers in the absence of αGT (Figure 4). SINV growth differences between cell lines were observable early in the time course—after a single round of viral replication (Figure 4b). SINV-infected BL6αGT monolayers also exhibited more cytopathic effects (CPE) than BL6 cells. BL6 cell monolayers, in contrast, were much more susceptible to HSV-1 and HSV-2 infections (Figure 4c) and exhibited greater CPE. A third herpes virus, murine gammaherpes virus 68 (γMHV-68), also replicated better in GT-negative cells (Figure 4c).
Figure 4: Growth kinetics of SINV and herpes viruses in B16 cell lines. Results are representative of >5 experiments for HSV and SINV and of 2 tests for γMHV-68. Virus was diluted to an MOI of 1.0 in MEM, added in 100 μl (SINV strains) or 400μl (herpes viruses) to the BL6 and BL6αGT monolayers, and left for an adsorption period of 60 minutes. (A) and (C) During time courses, culture fluid from cell monolayers were collected every 24 h for 72 h pi (SINV AR339 and γMHV-68) or 96 h pi (HSV 1 and 2). (B) For 12 h replication experiments, cell monolayers were infected with SINV viruses at an MOI of 0.1 and culture fluid was collected, starting at 12 h pi.

To further test differential replication of γMHV-68 in the two cell types, time courses were completed using the BL6 and BL6αGal paired cell lines. At various time points post infection, cell populations were harvested and DNA from cell populations was analyzed by real-time PCR. Absolute quantification of viral
copy number was not possible because no calibration standard was used. Instead, relative quantity of DNA was standardized using a housekeeping gene, as discussed in Methods and Materials. For each experiment, the sample with the lowest quantity of viral DNA was set as a baseline to compare the rest of the samples to. The unit of measurement, “fold change”, refers to a fold difference in quantity of DNA of each sample to the quantity of the baseline sample. At 24 h pi, the quantity of viral DNA in GT-negative cells was three times that of GT-positive cells, and this difference was increased to almost 13-fold by 68 h pi (Figure 5, left graph). It is of interest that there was the same relative amount of viral DNA present at 24 h and at 68 h pi in the infected BL6αGal cell population. In a separate experiment, at 48 h pi, there was approximately 1.8 times more viral DNA in the population of BL6 cells relative to the BL6αGal cells (Figure 5, right graph). The relatively greater quantity of DNA found in GT-negative cells compared to GT-positive cells correlates with higher viral titers found in culture fluid from infected BL6 and BL6αGal cell populations.
Figure 5: Relative viral quantity in infected BL6 and BL6αGal. Results are representative of two separate experiments completed in duplicate. Time courses were completed as previously described and infected cells were harvested for analysis at 24 and 68 h pi, or at 48 h pi. Virus was used at an MOI of 1.0. Viral DNA was quantified using real-time PCR.

In the systems studied here, related viruses behaved similarly during infections of each cell type. Viral preference for expression or non-expression of αGT in host cells appears to be a general characteristic of certain viruses, and also appears to be virus-dependent but not viral-strain dependent.

4.3 Viral expression of αGal does not affect host-cell susceptibility to SINV and HSV strains

Enveloped viruses incorporate host-cell carbohydrates onto their envelopes during egress from cells, and this in turn can affect interactions with subsequent host cells. Various enveloped viruses, including SINV, acquire αGal after passages in mouse fibroblast L929 (αGT-positive) cells (Repik et al., 1994; Welsh et al., 1998). To determine whether viral infectivity on our two cell lines was affected by the presence of αGal on the virion, time course assays were completed on BL6
and BL6αGT cell lines with HSV-1 and 2 and with several SINV strains that had been propagated in L929 cells (designated SINVαgal, HSV-1αgal and HSV-2αgal). These SINVαGal virus strains still preferentially replicated in cells positive for αGT gene expression (Figure 6a), while HSV-1αgal and HSV-2αgal both preferentially grew in αGT-negative cells (Figure 6b).

Figure 6: Growth kinetics of αGal expressing viruses in BL6 cell lines. Results are representative of 2 separate experiments. SINV and herpes viruses were propagated in αGal-positive L929 cells to produce virions expressing αGal. Time courses were completed as previously described. SINV(αgal) was used at an MOI of 0.1 and or HSV(αgal) at an MOI of 0.01. Culture fluid was harvested every 12 h for 48 hpi (SINV(αgal)) or every 24 h for 72 h pi (HSV(αgal)).
Further, the αGal-expressing SINV S.A.AR86 virus replicated to similar levels to non-αGal expressing virus when used at the same MOI (Table 4). These results indicate that host-cell susceptibility to various SINV strains and to HSV 1 and 2 is affected by host-cell differences in αGT gene expression but not by variable αGal expression on the viruses.

**TABLE 4: Replication of S.A.AR86 and S.A.AR86(αgal) in BL6 cells**

<table>
<thead>
<tr>
<th>Virus (24 h pi)</th>
<th>Virus titer log$_{10}$PFU(mean ± SD)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BL6 (−)</td>
</tr>
<tr>
<td>SINV S.A.AR86</td>
<td>5.5 ± .10</td>
</tr>
<tr>
<td>SINV S.A.AR86(αgal)</td>
<td>5.7 ± .10</td>
</tr>
</tbody>
</table>

$^a$BL6 and BL6αGT were infected with SINV S.A.AR86 expressing or not expressing αGal on its envelope at an MOI of 0.1. Experiment was done in duplicate. Results are representative of a single experiment, and titers are consistent with results from other time courses with S.A.AR86 at similar MOI.  

$^b$ The titers shown are peak titers during a 48 h time course. Higher titers are bolded.

4.4 Level of viral replication correlates with the proportion of infected cells in the population

Susceptibility to viral infections that is dependent on αGT-expression should be a general characteristic of the entire cell population. High viral titers as measured from culture fluid, however, could indicate two different phenomena: 1) many infected cells producing moderate levels of virus or 2) a subpopulation of infected cells producing high amounts of virus. In order to investigate whether the amounts of virus in culture fluid correlated with the fraction of infected cells in the population, we used SINV and HSV-1 recombinants that express a green
fluorescent protein (SINV TE12-GFP and HSV-1 GFP ICP8 mutant) during replication, and we performed time course assays on BL6 and BL6αGT cells. HSV-1 GFP has reduced replication potential due to an ICP8 gene mutation that interferes with late gene expression, but not with its ability to initiate infection; therefore, differences in virus yields between cell types can still indicate a difference in cell susceptibility to infection. Further, GFP is expressed as a delayed early gene product, and so fluorescence in host cells is a useful measure of a first round of infection. Infected cells were directly measured via FACs, and virus from culture fluid was quantified by plaque assay. As seen with wild type strains, SINV TE12-GFP replicated better in BL6αGT cells (Figure 7a), whereas HSV-1 GFP replicated better in BL6 cells (Figure 7b). Under fluorescence microscopy, GFP SINV-infected BL6αGT cell monolayers had relatively large areas of fluorescing cells compared to SINV-infected αGT-negative cells (Figure 7c). Conversely, HSV-1-infected αGT-positive cell monolayers exhibited relatively small foci of fluorescing cells compared to HSV-1-infected BL6 cells (Figure 7d). Time course studies using FACs analysis showed that a substantially greater fraction of SINV-infected GT-positive cells expressed GFP (Figure 7e), compared to a small subpopulation of BL6 cells, even after multiple rounds of replication. In contrast, a much greater proportion of HSV-1-infected BL6 cells expressed GFP, and only a small fraction of αGT-positive cells were GFP positive for the duration of the time course (Figure 7f). Comparable mean fluorescent intensity (MFI) levels of GFP were observed across both cell lines during SINV and HSV infections (Figures 7g and 7h), suggesting that once cells are
productively infected, virus is synthesized at similar levels in both cell lines.

These data suggest that differences in viral replication between host cell types are due to differential success in virus establishing a productive infection.
Figure 7: Replication of GFP viruses in BL6 cell lines. Time courses on BL6 and BL6αGT cells were performed as previously described. Results with SINV GFP are representative of >5 experiments and for 3 experiments with HSV GFP. SINV TE12-GFP was used at an MOI of 1 and HSV-1 GFP at an MOI of .1. (A) Culture fluid was collected from SINV-infected monolayers for titration every 6 h for 26 h pi or (B) from HSV-infected monolayers every 12 h for 48 h pi. (C) and (D) Infected monolayers were observed under a fluorescence microscope to visually monitor infection and (E) and (F) cells were harvested for FACs analysis. (G) and (H) Mean fluorescence intensity of GFP expressing cells was measured via FACs Diva Software.
4.5 Differences in host-cell susceptibility are evident early after viral infection

Using GFP viruses, the success of an initial infection in host cells can be measured by FACs analysis, as infected cells will fluoresce. Cells were incubated with either SIN GFP or HSV-1 GFP for varying time periods, and infected cells were harvested prior to completion of a first round of replication—thus measuring initial infection levels—and analyzed via FACs. In HSV-1 GFP assays longer adsorption time resulted in a small increase of infected BL6 cells, but the infection on BL6αGT monolayers was almost negligible (Figure 8a). In SINV GFP assays, longer adsorption time resulted in higher fractions of αGT-positive infected cells, but not of αGT-negative cells; infection levels in BL6 cell populations did not increase with adsorption times longer than 120 minutes (Figure 8b). Additionally, it is of interest that following multiple rounds of replication (Figure 8c) the proportion of GFP SINV-infected cells in the BL6 population declined. This was not a result of lysed cell monolayers, as at 36 h pi the BL6 cells appeared more intact than BL6αGT cell monolayers (Figure 8d) and only focal areas exhibited cytopathic effects; the majority of the monolayer simply appeared to resist infection. This could suggest heterogenicity of the BL6 cell population with respect to permissivity to SINV infection, whereby some cells are permissive to infection and others are not. This could also indicate that viral infectivity declines after a first round of replication, a possible consequence of defective interfering (DI) particles. Along with infectious particles, Sindbis virus can generate DI particles that disrupt the replication cycle (Shenk and
Stollar, 1973). However, low MOIs were often used for SINV infection assays to reduce the possibility of this occurrence. Additionally, reduced infectivity was not a result of multiple rounds of infection, but was noticeable during the initial infection. This could suggest that DI particles are present in the viral stock, yet interfererence did not appear to be an issue for infections on Vero or BL6αGT cells. In either case, the mechanism by which infection is limited in BL6 cells appears to occur early in virus-host cell interactions.
Figure 8: Virus adsorption to BL6 and BL6αGT cell lines. Results are representative of >5 experiments with SINV GFP and 3 for HSV GFP. Virus was diluted to an MOI of 1.0 (SINV TE12-GFP) or MOI of 0.1 (GFP HSV-1) in MEM, and added in 100 μl to the BL6 and BL6αGT monolayers for varying periods of time. Culture fluid with unabsorbed virus was removed after each incubation end point, and cells were washed twice with media and re-fed with 2 mL MEM. At harvest, cells were prepared for FACS analysis. (A) GFP HSV-1 was incubated with cells for 30, 60 or 90 minutes and monolayers were harvested at 18 h pi, prior to one round of replication. (B) GFP SINV was incubated with cells between 15 to 180 minutes and cells were harvested between 7 to 10 h pi, prior to one round of replication. (C) GFP SINV was incubated with cells for 60, 120 or 180 minutes and cells were harvested at 35 h pi, after multiple rounds of infection. (D) GFP SINV infected cell monolayers approximately 36 h pi under light microscopy.

4.6 Testing restriction of SINV infection by blocking αGal sites on host cells

Fluorescence focus-assays indicate that differences in viral growth correlate with the successful infection of a host cell. Host-cell glycans are commonly utilized by viruses during the entry process; as such, the α-galactosyltransferase product αGal could play a role in facilitating or restricting infection. To explore the possible
role of αGal in viral entry, αGal sites were blocked using the anti-Gal antibody (ab), M86. BL6 and BL6αGal cell monolayers were treated with the M86 ab at varying dilutions and were then inoculated with the SINV S.A.AR86 strain, which has shown preference for preferentially replicating in αGal positive cells. In cell cultures without ab treatment, SINV preferentially replicates in cells expressing αGal (Figure 9). For BL6 cell populations, the relative fraction of infected cells does not change whether it is untreated or treated with M86. In contrast, the fraction of infected BL6αGal cells treated with 1:10 dilution of anti-Gal is slightly reduced (Figure 9, left graph). In a second experiment (Figure 9, right graph), the fraction of BL6αGal cells treated with 1:50 dilution of M86 drops significantly compared to the untreated, SINV-infected cells. When the concentration of anti-Gal treatment is reduced, the fraction of infected BL6αGal cells increases, but is still lower relative to the fraction of untreated, SINV-infected cells (Figure 9, right graph). These data must be interpreted with caution, however, for two reasons: 1) the standard deviation for experimental values completed in duplicate is large, which indicates a large margin of error and 2) additional experiments to confirm these observations were not successful. In several experiments, the antibody agglutinated cells in monolayers, or in suspension, during the incubation period and experiments were terminated. Overall, although some results may indicate that blocking αGal sites restricts SINV infection (at least with the S.A.AR86 strain), the data are not sufficiently reliable to confirm this phenomenon.
Figure 9: Blocking host cell αGal sites with anti-Gal. Anti-Gal antibody was used to bind to αGal sites on BL6 and BL6αGal monolayers. Antibody was added to cell monolayer at various dilutions and left for 60 min, then washed off. Virus was then added at an MOI of 0.1 and left for 60 min. Cells were removed by dispersion with trypsin and plated over Vero monolayers in serial dilution.

Additional methods to block glycan-virus interactions were attempted, including using free αGal in solution acquired from Dr. Uri Galili at the UMMS. The αGal solution was incubated with SINV AR339 and then the αGal-virus solution was added to BL6 and BL6αGal monolayers. The rationale was that if SINV binds αGal residues on host cells, then it may also bind free αGal molecules in solution, which would then prevent binding to the host cell. Unfortunately, the cell monolayers were damaged by the treatment, so this strategy was not effective.

A third blocking method was the use of liposomes that contained αGal epitopes in the membrane and control liposomes derived from KO pig red blood cells (both acquired from Dr. Uri Galili). Liposomes were incubated with virus, and the
liposome-virus solution was added to BL6 and BL6αGal monolayers. The function of αGal liposomes in these experiments was analogues to free αGal in solution described above. However, the cells reacted poorly to the treatment (Figure 10). Similarly to anti-Gal treatment, the liposomes tended to agglutinate the cells in culture. At 9 h pi, cells from some wells were harvested and fixed for FACs analysis, and cells from other wells were left for later examination. After 24 h post-treatment, the cell monolayers began to recover and returned to their normal morphology. Despite this, due to the unhealthy morphology of cells directly after treatment, experiments were terminated.

![Figure 10: αGal liposome treatment of BL6 and BL6αGal cells. Monolayers at 2 h post-treatment with the liposome.](image)

4.7 Enzymatic removal of αGal from host cells

Results from blocking experiments were inconclusive due to problems with replicating results or with reagents that were toxic to cells. However, the role of αGal in viral infections could still be examined by removal of the sugar from the surface of host cells. BL6 and BL6αGal cells were treated with the green bean α-galactosidase enzyme that cleaves the terminal Gal residue on the cell’s surface.
Under the right conditions (discussed in methods), the enzyme was very efficient at removing surface αGal from cells without damaging cells (Figure 11), and αGal residues were not detected on BL6αGal cells at normal levels by 6 h post treatment. The unusually high MFI for the negligible αGal-positive population in the BL6 sample was possibly due to non-specific binding.

Figure 11: Enzymatic treatment of BL6 and BL6αGal cells. Cells were treated with 5 μl of enzyme in 100 μl of pbs at pH 6.2 for 45 minutes. Cells were stained for αGal expression using the anti-gal ab M86. MFI is mean fluorescence intensity of entire cell population.
Once the removal of αGal residues was confirmed via FACs analysis, cells were infected with SINV TE12-GFP. Infections were left between 6-10 h, and infected cells were harvested prior to a first round of replication—to thus measuring initial infection levels—and analyzed via FACs. As expected, a greater proportion of the BL6αGal cell population was infected with SINV compared to BL6 cells (Figure 12a, left column). Green bean α-galactosidase is most efficient at low pH (6.0-7.0). To control for any effects of the harsh conditions on cells, infections were performed in both normal cell culture media and also in low pH solution. Incubation of cells in either low pH solution or MEM did not appear to affect susceptibility to infection for either cell type (Figure 12a, right column). Removal of αGal from BL6αGal cells increased the proportion of cells infected by SINV TE12-GFP (Figure 12b, top panel). In various experiments, infection levels ranged between 2 to 6-fold lower in treated cells compared to untreated cells. Surprisingly, treatment with α-galactosidase also resulted in reduced levels of infection for BL6 cells compared to untreated cells (Figure 12b, bottom panel). The reduced infection observed in BL6 cells could indicate that α-galactosidase also interacts with another component on the cells that is involved in susceptibility to SIN infection. Overall, treatment of cells with the galactosidase did not identify a role for αGal in viral infections. More work is needed to understand the dynamics of αGal cleavage in BL6 cells and SINV infection.
Figure 12: Infection of enzymatically treated cells with SINV TE12-GFP. Bl6 and Bl6αGal cells were treated with 5 μl of α-galactosidase in 100 μl of PBS at pH 6.2 for 45 minutes. Cells were then infected with virus at an MOI of 1.0 and infection ran for 7 h. As a control, cells were infected in MEM media and also in low pH solution. Cells were harvested by dispersion using trypsin and analyzed by FACs. MFI is mean fluorescence intensity of GFP-positive cells.

These experiments also showed that the MFI of infected BL6 cells is considerably higher than infected BL6αGal cells (Figure 12b). This phenomenon was not observed in experiments completed with the first lot of virus propagated in Vero cells, but was noticed after several passages (approximately 3) in Vero cells where viral stocks were grown. MFI is used as a relative measure of viral replication within a cell; brighter cells have more viral replication and thus express greater amounts of GFP. For BL6 cells, that could suggest that although few cells become infected in the population, the virus replicates very well once
inside the cell. The relatively lower viral output, measured from culture fluid, could result from the initial low-level infection in the population or could indicate that viral egress is restricted in the infected cell subpopulation.

It was also possible that the two cell types had different IFN-induced antiviral activity, and therefore responded to viral infection differently. Sindbis virus is sensitive to antiviral effects of IFN α/β. The virus replicated well in the BL6 cells, but infection levels were low compared to BL6αGal. If BL6 cells produced greater amounts of IFN early during infection, it could have protected the rest of the population from becoming infected, and thus explain the greater proportion of infected BL6αGal cells. An inhibition assay for interferon was performed in order to measure IFN in culture fluid of SINV TE12-GFP infected BL6 and BL6αGal cells. In some assays, both cell lines had comparable levels of IFN (Figure 13, left graph) but in other assays, the BL6αGal cell population produced more IFN than the BL6 population (Figure 13, right graph). It is possible that higher levels of viral replication in the BL6αGal cell population induced higher levels of interferon production. It is clear, however, that the lower proportion of infected cells in the BL6 population was not a result of a relatively more robust IFN response.
Figure 13: Assay for interferon production in the BL6 and BL6αGal cell lines. Cells were infected with SINV TE12-GFP and culture fluid was harvested from infected cells from 6 to 36 h pi. Virus in samples of culture fluid was then inactivated under UV light to be used in the VSV protection assay.

4.8 Addition of αGal terminal residues to GT-negative host cells

Attempts to understand the role of αGal in viral-host interactions by blocking or removal of the surface sugar or knockdown of GT activity were not successful. An alternative method was to add the carbohydrate molecule to GT-deficient host cells and examine its effects on viral infections. The FSL (function, spacer, lipid) construct system was a good candidate for these experiments because the constructs are composed of three parts: a functional terminal end, a spacer molecule and a lipid molecule. The lipid portion can insert itself into membranes (be it cellular or viral) and the functional portion is exposed in the extracellular space. The functional unit of the FSL-Galili(tri) construct contains the Galα1,3Galβ1,4GalCNAc molecule. The BL6 cell line seemed a good candidate for these experiments, but these cells were not very receptive to the FSL constructs. A relatively high quantity of the reagent was necessary to produce
only a small proportion of αGal-expressing cells in the population. Instead, Vero cells that derive from *Chlorocebus* sp. and do not have a functional GT gene, were utilized. Cells were treated with the FSL-Galili(tri) to express αGal residues on the surface. Figure 14a shows that a vast majority of Vero cells was made to express αGal, although at relatively low levels. Treated and untreated Vero cells were then used to examine viral-host cell glycan interactions as both cell types were identical, but for the expression of terminal αGal. Because the addition of an exogenous molecule to the host cell might have unforeseen effects that can skew findings, a control FSL construct was used: the FSL-Biotin, which has a Biotin molecule as the functional portion of the construct. The control construct also integrates itself well into the cell membrane (Figure 14b).

![Figure 14: FSL treatment of Vero cells for SINV TE12-GFP infection.](image)

Cells were incubated with either FSL Galili(tri) or control FSL-Biotin at a concentration of 1:4 for 60 min and then infected with virus at an MOI of 1.0. At 2.5 h post treatment, cells were tested for expression of αGal or Biotin using FACs. (A) Cells treated with FSL-Galili(tri) were tested for successful insertion using anti-Gal, M86 and (B) Cells treated with FSL-Biotin were tested for successful insertion using fluorescently labeled streptavidin. MFI is mean fluorescence intensity of entire cell population.
Vero cells treated with both constructs were infected with the GFP SINV TE12 strain and cells were harvested prior to a second round of replication to look at the initial infection cycle. Compared to untreated Vero cells infected with the virus (Figure 15a), the proportion of FSL-Galili(tri) treated cells that became infected increased slightly, and the proportion of FSL-Biotin treated cells that became infected dropped slightly (Figure 15b). Strangely, when the concentration of FSL constructs is reduced (resulting in fewer constructs inserted into the host cell membrane), the infection level of FSL-Galili(tri) treated cells is slightly reduced, but infection level of FSL-Biotin treated cells is increased (Figure 15c). Other experiments demonstrated this same phenomenon. Cells treated with higher concentrations of FSL-Galili(tri) resulted in more significant increases in TE12-GFP infection; but cell treatment with low concentrations of FSL-Biotin also resulted in an increase in TE12-GFP infected cells. These data suggest that the constructs do have a moderate effect in viral-host cell interaction; however, it is not specific to αGal residues.
Figure 15: Infection of FSL-treated Vero cells with SINV TE12-GFP. Cells were incubated with either FSL Galili(tri) or control FSL-Biotin and were infected with TE12-GFP at an MOI of 1.0. (A) Control: virus infected cells without FSL treatment. (B) Cells treated with FSL-Galili(tri) or FSL-Biotin at a concentration of 1:4 and infected with virus. (C) Cells treated FSL-Galili(tri) or FSL-Biotin at a concentration of 1:10 and infected with virus.

To examine if FSL-constructs affected the course of other viral infections, Vero cells treated with the constructs were infected with HSV-1 GFP. Due to low quantity of available reagent, cells were treated with a more dilute mixture, and accordingly, a decrease in αGal expression on treated cells was observed (Figure 16a). Infected cells were harvested prior to a second round of replication in order to observe an initial infection cycle. No difference was observed in the level of infection between treated and untreated Vero cells, although it is possible results were skewed by reduced αGal expression on treated cells (Figure 16b). However, repeats of this experiment resulted in a greater proportion of cells incorporating
αGal molecules, but differences in infection levels between treated and untreated cells were still not observed. Overall, addition of αGal to GT-deficient host cells did not identify a role for αGal in viral infections.

![Figure 16: FSL treatment of Vero cells for HSV-1 GFP infection.](image)

In vitro experiments showed that the presence or absence of the GT gene in a host cell plays an important role in the course of viral infections. The viral kinetics of various herpes viruses and Sindbis virus strains seem to be dependent on the state of GT expression in the host. Herpes viruses preferentially replicate and infect GT-negative host cells, and SINV strains have greater infectivity in GT-positive cells. Host cell susceptibility to infection appears to be determined by an event early in virus-cell interaction. Enzymatic removal of αGal from cell surfaces may indicate that the presence of αGal plays a role in facilitating infection by SINV; however, the mechanism by which susceptibility is affected could not be established.
5.1 T KO mice are more susceptible than WT mice to HSV-2 infections

The role of the GT gene or αGal expression must be relevant in vivo in order to have implications for primate evolution. To test whether our in vitro data could be corroborated in vivo, we used C57BL/6J (WT B6) mice that naturally express the GT gene and GT knockout mice (GT KO) that lack it. Mice aged 8 to 19 weeks were inoculated by the intraperitoneal route with different doses of HSV-2. From i.p. inoculations, HSV-2, which is not highly pathogenic in B6 mice, spreads via blood to visceral organs. Spleen, liver and visceral fat pads proximal to the injection site were harvested at various days post infection to quantify virus. At day 3 pi, viral loads from splenic suspensions were often higher in KO mice compared to the WT, but this was not always statistically significant. However, virus accumulated significantly more in livers of GT KO mice compared to livers of age-matched WT B6 mice, regardless of initial inoculum. Virus was detected in a greater proportion of KO mice (83.4%) compared to WT mice (45.5%) ($\chi^2 = 5.9$, df = 1, $P < 0.02$), and macroscopic hepatic lesions commonly associated with HSV-2 infections following i.p. inoculation (Mogesen et al., 1974; Mogesen, 1977) were more frequently observed in KO mice (27.7%) than in WT mice (5.5%). Independent experiments did not always show statistically significant differences in liver viral load between the two mouse types, but analysis of variance (ANOVA) of titers from pooled experiments demonstrated significant
differences (F=8.36; df=1; \( p=.007 \)). Additionally, by day 4 pi, virus was detected in a significantly greater proportion of livers from KO mice (88.2%) than WT mice (35.3%) (\( x^2 = 10.09, df = 1, P < 0.002 \)). Viral load was also significantly higher in KO mice compared to WT mice, irrespective of age of mouse or viral dose administered (Figure 17a and b), and a greater proportion of KO mice exhibited hepatic necrosis (29.4%) compared to WT mice (11.8%). By day 5 post-infection, KO and WT mice had largely cleared the infection from all organs tested. These data show differences in resistance to HSV-2 between KO and WT mice, reflected in the amount of virus isolated from liver and from the level of hepatic necrosis observed.

Figure 17: HSV-2 infections of KO and WT B6 mice. Mice were inoculated i.p. with HSV-2. Organs for titration (PFU/organ) from mice were harvested at days 3, 4 and 5. In figure, wo = weeks old. (A) Mice aged 8-weeks of age were inoculated with 5x10^5 PFU in 400\( \mu l \) media or (B) of 9, 11 or 19 weeks of age were inoculated with 3.75x10^5 PFU in 300\( \mu l \) media.
The GT KO and WT B6 mouse lines, although genetically similar, could present differences in their immune response to infection. To ensure that observed differences in susceptibility to HSV-2 were not an artifact of immune system differences between the two mouse lines, mice were given a high dose of gamma-irradiation directly prior to infection. Replication of HSV-2 was considerably enhanced in the spleen and livers of these immunocompromised KO mice but only slightly improved in WT mice. At day 2, the virus was barely detectable in spleens of WT mice, but KO mice had mean viral titers up to 80-fold higher (Figure 18, left panel). By day 5 post-infection, all KO mice exhibited numerous large focal necrotic lesions and elevated viral load in livers (Figure 18, right panel). In contrast, only a few WT mice presented small hepatic lesions and relatively lower (or undetectable) viral titers. Thus, the presence of GT confers a level of resistance to HSV-2 infection in mice.
Figure 18: HSV-2 infections of γ-irradiated mice. Mice were aged 8 to 14 weeks were inoculated with 3.75x10⁵ PFU of HSV-2 in 300 μl media. Organs for titration (PFU/organ) were harvested at days 2,3,4,5 or 6. In figure, wo = weeks old.

5.2 Genetic background of GT KO mice and susceptibility to HSV-2

GT KO mice were created in a DBA/2, 129Sv and B6 background. Despite being backcrossed into the C57BL/6J background over multiple generations, it is important to ensure that any genotypic differences between KO and WT mice do not influence response to HSV-2 infection. In preliminary tests to determine this, WT, KO and various mouse crosses (F₁, F₁xWT, F₁xF₁, F₁xF₀) of varying ages were γ-irradiated with 10Gy and immediately infected with HSV-2. αGal expression was tested from peripheral blood samples using anti-Gal antibody. By BS lectin binding, expression of αGal in the cell population of GT KO mice is close to zero, while in WT B6 mice, it can range between 80-95% (Figure 19). It should be noted that in the heterogeneous population derived from blood of WT B6 mice, there was no distinct separation between the population of expressers
and of non-expressers. As in other tissues, αGal-expression likely occurs across a spectrum whereby different individual cells express different quantities of αGal on the surface.

![Figure 19: Expression of αGal on lymphocytes of KO and WT mice. Cells were stained with BS lectin (BSl-B4), which binds αGal monomers. (Left panel) Cell populations derived from KO mice express practically no αGal on their surface, while (right panel) the majority of cells in the population derived from WT mice express different quantities of surface αGal. MFI is mean fluorescence intensity of entire cell population.]

To analyze the role of the GT KO genetic background in its susceptibility to HSV-2, age-matched F1 male mice that had been backcrossed with parental strains (F1xWT or F1xKO) were γ-irradiated with 10Gy and then infected i.p. with HSV-2. Blood was collected and utilized for testing αGal expression with BS lectin. Additional antibodies were included in fluorescence analysis to locate a homogenous population of lymphocytes, and limit factors that could influence the analysis (e.g., different cell types expressing different levels of αGal). The congeneric marker, Thy 1.2, revealed that there were very few lymphocytes in peripheral blood preps, potentially because gamma radiation had eliminated much
of the population. Using the small Thy 1.2 subpopulation, individuals with very low αGal expression vs. high αGal expression were differentiated (Figure 20a). At day 6 pi, there were no statistically significant differences in mean viral titers from liver or brain suspensions of αGal-negative and αGal-positive individuals (Figure 20b). The three αGal-negative mice with the highest viral load exhibited macroscopic hepatic lesions. One αGal-positive mouse had light spotting on the liver but the corresponding viral titer was below the limit of detection. Additional experiments with crossed mice were completed with a modified protocol, whereby blood was taken a week prior to infections to determine αGal status of the animal, as gamma-irradiation depleted the immune cell population used for αGal detection. Unfortunately, in three separate experiments, KO and WT mice did not yield sufficiently high viral titers for proper analysis, even after viral inoculum was increased. Although these results trend in the direction of previous findings which showed that KO mice developed greater pathology compared to WT mice, the observed differences in this experiment were not statistically significant. Overall, these do not clarify whether the GT KO background plays a role in its increased susceptibility to HSV-2 infections.
5.3 WT and GT KO mice are similarly susceptible to HSV-1 infections

*In vitro*, herpes simplex type 1 exhibited preferential growth in cells that lacked GT activity. WT B6 mice are highly resistant to HSV-1 infection via the i.p. route, but are susceptible if infected intranasally (i.n.) and quickly develop pneumonia (Adler et al., 1997). In order to verify if *in vitro* findings translate to an *in vivo* system, males aged 4 weeks, were anesthetized and infected i.n. with 1.8x10⁴ PFU. Numerous crossed mice were readily available for testing and were used in these experiments. BS lectin was used to test for αGal expression in peripheral lymphocytes. At day 1 and day 2 pi, there was no difference in mean viral titer from suspensions of whole lung between αGal-negative and αGal-positive individuals (Figure 21). By day 4 pi, both groups of mice appeared to be clearing the virus from lungs. These results suggest KO mice do not have increased susceptibility to HSV-1 infections via the intra-nasal route, compared to their αGal-expressing counterparts.
Figure 21: HSV-1 infections of WT, KO and crossed male mice. Mice, aged 4 weeks, were inoculated i.n. with 1.8x10⁴ PFU in 20 μl. Lungs were harvested at days 1, 2 and 4. Day 1 results are representative of pooled data from two separate experiments performed under the same parameters using KO, F₁ and F₁xKO mice. Day two experiments were conducted on F₁xKO mice. Day 4 experiments were conducted on KO and WT. Blood was taken at time of sacrifice to verify αGal expression.

5.4 WT B6 and GT KO infections with γMHV-68

Similar to the herpes viruses tested previously, the gammaherpes virus, γMHV-68 replicated to higher levels in cells negative for GT expression. In order to test for possible GT-dependent differences in susceptibility to infection in vivo, GT KO and WT B6 male mice aged 8-9 weeks, were inoculated i.p. with virus, and spleens were harvested at day 7 pi. Titers from splenic suspensions (PFU/organ) were too low, or under the limit of detection, to be analyzed by plaque assay. However, in mice, after virus is cleared from circulation (acute infection), γMHV-68 establishes a latent infection in B cells of the spleen that can result in splenomegaly (Sunil-Chandra et al., 1992). By macroscopic examination, relatively larger spleens were observed from GT KO mice compared to spleens from WT mice at 7 days pi (Figure 22).
Figure 22: γMHV-68 infection on KO and WT mice. Mice aged 9 weeks were inoculated i.p. with 5.5x10³ PFU. Spleens were harvested at day 7 for viral titration. Using the Adobe Photoshop CS6 Rule Tool, a scale was calibrated using the standard provided by the program, and an arbitrary unit (a.u.) of measurement was constant throughout the scale. Spleen length according to this scale is written below each organ. The majority of spleens harvested from GT KO mice exhibited increased size relative to the spleen from an uninfected control. All spleens harvested from WT B6 were comparable in size, or smaller, to spleens from the uninfected mouse.

γMHV-68-infected GT-negative mice exhibited splenomegaly to a greater degree than GT-positive mice. In order to quantify infection, real-time PCR analysis was used to detect viral DNA present in infected GT-positive and GT-negative mice. Mice aged 6-12 weeks were inoculated i.p. with γMHV-68 and spleens were harvested at various days pi. As with in vitro experiments, absolute quantification of viral copy number was not possible because no calibration standard was readily available. Relative quantity of DNA was measured using an endogenous control; for each experiment, the sample with the lowest quantity of viral DNA was used as a standard to compare with the rest of the samples. The unit of measurement, “fold change”, refers to a fold difference in quantity of DNA to the standard sample. At 5 days pi, all GT-negative mice exhibited similar levels of viral DNA,
but a few GT-positive mice had 100’s to 1000’s-fold more DNA per gram in splenic samples relative to the standard (Figure 23a). By day 7 pi, the majority of mice from both groups expressed levels of viral DNA close to the standard. At day 9 pi, the weight of individual spleens was also collected to see if size correlated with the level of viral DNA expression. The average weight of a healthy mouse spleen is between 80-140 mg. Gal-negative mice had spleen weights ranging from 130-340 mg, while Gal-positive individuals had spleen weights ranging from 90-140mg (Figure 23b). PCR results confirmed that the larger spleens derived from Gal-negative mice. These spleens also contained relatively higher amounts of viral DNA per gram compared to spleens from GT-positive mice. By day 11, both mouse types expressed relatively similar levels of viral DNA (Figure 23c). Weights of spleens from GT-negative mice ranged from 120-270 mg and for GT-positive mice, 100-390 mg. Size of spleen again correlates with the relative amount of viral DNA present in the organ. Data are inconclusive for GT-dependent differential susceptibility to γMHV-68 infection. At day 7 and 9 pi, GT-deficient mice have higher concentration of γMHV-68 virus in spleens, and greater splenomegaly, compared to GT-sufficient mice. But this is likely not true at day 5 and day 11 pi. Working in relative quantities for viral load makes it difficult to fully understand kinetics differences of γMHV-68 infections in the two mouse strains. More work with a calibration standard would greatly elucidate the dynamics of viral infection between the two mouse types.
Figure 23: γMHV-68 infection on KO, WT and backcrossed mice. Mice aged between 6-12 weeks were inoculated i.p. with 3.5x10^3 PFU (Day 5, 9 and 11 pi) or 5.5x10^3 PFU (Day 7). Spleen was harvested at various days pi for real-time PCR analysis. (A) Spleens from 12-wk old mice harvested at day 5 pi, and spleens from 9 week old mice harvested at day 7 pi (B) Spleens from 11 week old mice were harvested and weighed at day 9 pi (C) Spleens from 6 week old mice were harvested and weighed at day 11 pi.
5.5 Adult WT and KO mice are equally permissive to SINV S.A.A.R86 infection

Findings from cell culture experiments indicated that SIN viruses preferentially replicated in cells with an active GT gene, and it was important to confirm these findings in an animal system. Most strains of SINV are avirulent in adult and suckling mice. After approximately 2 weeks of age, mice can control infections via an IFNα/β response, but for neonates that lack a functional IFN response (up to 14 days of age), most SINV strains induce a severe, lethal viral encephalitis (Reinarz et al., 1971; Ryman et al., 2000). Adult and suckling mice, however, are susceptible to the neuroadapted S.A.A.R86 strain (Russell et al., 1989; Heise et al., 2000; Ryman et al., 2000). To test for differences in susceptibility of KO and WT mice to S.A.AR86, 8 to 9 week old male mice were inoculated i.p. with the virus. From the i.p. route, the virus infects skin, muscle, and various visceral organs and disseminates to the CNS via a viremia. Mice were sacrificed by cervical dislocation, and blood, spleen, visceral fat pads or brain were harvested from day 1 to 5 pi. Additionally, a group of mice was γ-irradiated to examine the response of immunocompromised KO and WT mice to infection. Organs from irradiated mice were harvested at day 6 pi and titrated for virus. At days 1 and 2 pi, mean viral titers from organ suspensions of blood, spleen and fat pad were comparable between KO and WT mice (Figure 24a and 24b). At day 3, more virus was found in the spleens of WT mice than KO mice, but the difference was not statistically significant (Figure 24c). By day 5 pi, the virus was detected in the brains of both KO and WT mice at comparable levels (Figure 24d). In
irradiated mice, at day 6 pi, no statistically significant difference in viral growth in blood, spleen (Figure 24e), liver and fat pad (not shown) was observed between GT-positive and GT-negative mice. These results indicate that adult WT and KO mice are comparably susceptible to the virulent SINV S.A.AR86 strain.

Figure 24: S.A.A.R86 infections of WT and KO mice. Mice aged 8-9 weeks were inoculated i.p. with $10^5$ PFU in 200 μl. Blood, spleen, fat pads, or brain was harvested at days 1, 2, 3 and 5. A second group of 8 week old mice was γ-irradiated with 10Gy and inoculated with the same dose of virus. Blood, spleen, liver and fat pad were harvested at day 6 pi. (A) Day 1 results are representative of pooled data from two separate experiments performed under the same parameters (B) Day 2 results from blood, spleen and fat pad are also from pooled data from two separate experiments (C) Only spleens were harvested for titration at day 3 pi (D) Only brain was harvested at day 5 pi for titration (F) Titers from blood and spleen of γ-irradiated mice.
5.6 SINV infected WT suckling mice have accelerated symptom onset and death compared to KO counterparts

In adult mice, no differences were observed in the response to SINV infection by GT-sufficient or GT-deficient mice. However, very young mice are susceptible to most SINV strains and could provide clues about host-specific differences in response to infection. In order to determine this, GT KO and B6 mice aged 6 to 20 days were inoculated intracranially, because the virus is neurotropic, or subcutaneously, to mimic the natural route of vector-borne infection. For these experiments I used the less virulent AR339 strain and the more virulent S.A.AR86 strain. I tested a variety of ages for infection and found that mice infected at day 6 were not cared for by their mothers and that mice infected at day 20 were resistant to the lethal effect of either strain. Infections of mice from 8 to 14 days of age resulted in 100% mortality, regardless of the presence or absence of αGT. However, significant differences were observed in mean survival period prior to death and onset of pathological symptoms. Intracranial infections of 8-day old mice with the more virulent SINV S.A.AR86 resulted in a rapid onset of spasmodic behavior and lethargy. All WT mice died by day 3 and a few KO mice survived until day 4, but this difference was not statistically significant. Figure 26 shows an experiment where 8-day old WT mice infected i.c. with SINV AR339 had a shorter mean survival time than KO mice (3.0 vs. 4.0 days). WT mice exhibited spasms and trembling, unsteady locomotion as early as 24 h after inoculation and succumbed to infection beginning at 2 days post infection (Figure 25). In contrast, KO mice experienced morbidity around day 2 and 3 and began
dying at day 4. Thirteen day-old KO mice inoculated subcutaneously with S.A.AR86 had a longer mean survival time compared to WT mice (5.0 vs. 4.0 days) and experienced weight loss later than WT mice (day 3 vs. day 2). Shaking and hind limb paralysis were observed by day 2 in WT mice and on day 4 in GT KOs. At fourteen days of age, both KO and WT mice began dying on day 4 pi and survived until day 6 pi, and showed no significant differences in survival times and symptomology. These data reflect the same pattern of susceptibility to SINV seen with cultured cells. Altogether, these data show that GT-sufficient suckling mice have increased sensitivity to the lethal effects of SINV compared to GT KO counterparts. GT KO mice eventually succumb to infection, but the onset of pathological symptoms and death are significantly delayed.
Figure 25: SINV infections of KO and WT weanling mice. A) Eight-day old mice were inoculated intra-cranially with approximately 200 pfu SINV HR strain in 10 μl of media (2 litters KO n=15, 2 litters WT n=13) or (B) 13-day old mice were inoculated subcutaneously with approximately 1.68x10³ PFU of SINV S.A.AR86 in 30μl (3 litters KO n=17, 2 litters WT n=10).

To determine if differences in survival time and disease progression in GT-sufficient and GT-deficient suckling mice correlate with differences in viral replication in the brain, eight-day old mice were infected i.c. with either a high
dose \((1.16 \times 10^6)\) or low dose \((200 \text{ PFU})\) of SINV AR339. With a high dose of virus, the effects of an abundant infection can be observed, as a high number of cells should be infected. With a low dose, the ability of a virus to replicate within the host can be observed, as few cells should be initially infected. The F_{1}\times B_{6} generation of mice was used in lieu of WT to represent a GT-sufficient host because these mice were readily available and age matched to the KOs. This group was given the high dose. For the low dose experiment, age-matched GT-sufficient and GT-deficient counterparts were not available, but a litter of F_{1}\times K_{O} mice was. Each mouse had a 50\% chance of either expressing or not expressing the GT gene; a mix of GT-expressing and GT non-expressing individuals in the group was likely. These mice were used to compare viral replication in the brain from a lower dose of virus. For high-dose and low-dose experiments, brains from KO and WT mice were harvested at 24 h pi and titrated for virus. At 24 h pi, titers from brain suspensions of all three types of mice reached levels of up to \(6 \times 10^8\) PFU/mL, regardless of the initial inoculum, or possible genotype of the mouse (Figure 26). These data suggest that viral replication is not curtailed in GT-deficient mice relative to GT-sufficient mice. Delayed onset of disease and death in GT KO mice does not appear to be correlated with inhibition of viral replication in the brains of these mice.
Figure 26: SINV AR339 infections of KO and backcrossed mice. Eight-day old GT KO and genetically crossed (F1xB6) mice were given a high dose of virus, $1.1 \times 10^8$ PFU. F1xKO were inoculated with a lower dose of 200 PFU. Brains were harvested 24 h pi.

In vivo experiments have demonstrated that KO mice for the GT gene are more susceptible to HSV-2 infection compared to GT-sufficient mice. This parallels the results from in vitro studies, which show that GT expression in a host cell decreases susceptibility to herpes virus infection. Results from experiments using genetically crossed mice to verify the greater susceptibility of αGal-negative mice to HSV 1 and 2 were not as conclusive, and it is possible that a biological factor in the KO mice affects their response to infection, prior to being crossed with WT B6 mice. However, results from these experiments are difficult to interpret because the true level αGal expression in individual mice was difficult to assess. Results from experiments with γMHV-68 to verify greater susceptibility in KO mice to other herpes viruses were also not consistent with in vitro findings. More work with this virus is neccessary to verify results. For Sindbis virus infections,
suckling mice that express the \textit{GGTA1} gene experience faster onset of pathological symptoms and death compared to GT-deficient mice. This also parallels in vitro data where SINV shows greater infectivity in GT-positive hosts. Overall, \textit{in vivo} data support my hypothesis that host susceptibility to different viral infections is dependent on the expression or non-expression of \textit{GGTA1} in the host.
The present study provides the first investigation on the infection dynamics of viruses in the presence and absence of the α1,3-galactosyltransferase (GGTA1) gene. I have shown that the loss of GT expression rendered cells more resistant to several viruses belonging to the Sindbis group but more sensitive to various herpes viruses, regardless of αGal expression or non-expression on the infecting viruses. Additionally, differences in efficiency of infection were seen with SINV and herpes viruses, but once productively infected, host cells generally expressed similar amounts of viral protein as indicated by GFP expression. This points to a role for GT activity or a GT product early during the course of an infection. At the organismic level, I have shown that SINV strains are more virulent to GT-expressing young mice compared to GT-deficient suckling mice. In contrast, adult WT mice infected with HSV-2 exhibited less pathology and lower viral replication compared to KO mice. These findings demonstrate that the presence of the GT enzyme in a host can alter their susceptibility to viral infection. This study also provides insight on the evolution of disease resistance in catarrhine primates. The significance of a mutation that occurred approximately 28 Ma, that inactivated GGTA1, and modified the carbohydrate repertoire of the ancestors of catarrhine primates, is not fully understood. Here, I provided evidence that this mutation had the capacity to significantly alter host resistance to viral infections, and I speculate that this change in resistance to infections may have provided a
selective advantage to individuals harboring the mutation. In this study, SINV grew to 10-20 fold higher in GT-positive cells and caused more rapid death in GT-positive suckling mice. Selection may have acted on the GT locus, directly altering host cell susceptibility to virulent infection. However, the mechanism by which GT-dependent differential susceptibility to viruses operates still remains to be deciphered. It is important to once again note that the viruses examined in this study would not have been the putative selection agents of 28 million years ago. While it may not be possible to identify the pathogenic agent that exerted selective pressure on the GT locus, it is possible to conjecture that loss of GT expression rendered ancestral catarrhines more resistant to a virus or other pathogens with the ability of modern day SINV strains to favorably replicate in GT-positive hosts.

It is more difficult to explain how a mutation that enhanced susceptibility to some viruses, as was observed with contemporary strains of HSV, could also be advantageous to the host. It is very possible that HSV evolved specificity for cells that did not express surface αGal after the mutation became fixed in the population. For instance, selective pressure from *Plasmodium reichenowi* (Martin et al., 2005) likely led to loss of expression of the terminal sialic acid, Neu5Ac, a product of the *CMAH* gene, in the ancestral hominin lineage around 3 Ma (Orlandi et al., 1992; Chou et al., 1998; Hayakawa et al., 2001: Hayakawa et al., 2006). The inactivation of the *CMAH* gene presumably rendered humans less susceptible to malarial infections for some time until the *Plasmodium* parasite
evolved the ability to exploit the mutated site once again (Hayakawa et al., 2001; Rich et al., 2009). Today, that domain is still under strong selective pressure from *Plasmodium* and other pathogens (Martin et al., 2005), clearly indicating that host-pathogen relationships are not static. In a similar manner, inactivation of *GGTA1* may have provided ancestors of catarrhines with temporary relief from a virulent pathogen that exerted pressure on that locus, but this would not have eliminated the ability of other (even related) pathogens to exploit hosts expressing Δ*GGTA1*. It is also of interest that herpes virus infections can sometimes benefit the chronically infected host. Recent work with mice has shown that herpes virus latency can induce a state of heightened NK cell activity that results in swifter action against new infections, and certain human herpesvirus infections may have similar properties (White et al., 2012). Furthermore, the periodic activation of latent or persistent viruses can induce chronic IFN-γ secretion and macrophage activation that can provide cross protection against acute bacterial infections (Barton et al., 2007).

The greater susceptibility of GT KO mice to HSV-2 compared to WT B6 mice was evident, but the greater susceptibility of crossed mice that expressed low levels of αGal compared to mice that expressed high levels of αGal, was much less clear. Overall, GT-negative mice presented slightly higher viral titers and greater pathology than GT-positive mice, but the data were not as definitive as that obtained from infections with the parental strains of mice. Additionally, infections of crossed mice with HSV-1 did not show differences in response to
virus between αGal expressors and non- or low expressors. This could indicate that the susceptibility of GT KO mice may be due to a factor that is present in the mice prior to crossing with the WT, or alternately, increased resistance of crossed mice may be due to a factor that is present in the WT mice they are crossed with. In either scenario, the response would be independent of the GGTA1 gene or αGal. It is important to note however, that interpreting results of experiments using the genetically crossed mice was made difficult because assessing the level of αGal expression in individual mice was problematic. The expression of αGal was measured by BS1 binding to Thy 1.2 cells derived from blood of gamma-irradiated mice, but the small fraction of remaning Thy 1.2 cells post-irradiation may not be an accurate representation of αGal expression in the individual mice. More precise tests to assess genotype-phenotype associations, such as genetic tests, in addition to antibody binding tests of blood lymphocytes, would be highly useful in these experiments. In this way, a better understanding of the correlation between homozygotes to an HSV response could be measured, in conjunction with a response to virus by heterozygotes that express varying levels of αGal on their cell surfaces.

In cell culture, HSV-1 preferentially grew in cells that were GT-negative and this was not corroborated in vivo. Wild type and KO mice showed no significant differences in HSV-1 titers from lung suspensions at various days post infection. αGal sites are found on all tissues (except sperm cells) of the body in GT-sufficient species (Thall et al., 1995), but the level of expression on different
tissues can vary, as can the level of expression by individual. Thus, the level of expression on the specific cell the virus infects may determine susceptibility of the entire host. HSV-1 infections were performed intranasally, and αGal expression is abundant in the vomeronasal organ (Koike et al., 2007). Variable expression of αGal at the site of inoculation may not explain the comparable levels of HSV-1 viral replication between WT and KO mice as measured from lung suspensions. However, it is possible that route of infection and organs accessible for viral dissemination may influence the outcome of infection. For example, i.p. inoculation of HSV-1 often results in subclinical infections barely detectable in visceral organs (Renis et al., 1976), but i.n. inoculation causes obvious disease in the lung. It is possible that GT expression plays no role in HSV-1 infections in vivo, but it is important that other routes of infection (e.g., intracranial, corneal) that result in measurable infection, and the level of αGal expression in infected tissues, be tested in order to verify this possibility.

Results from in vivo infections with γMHV-68 are not easy to interpret because of repeatability issues with these assays. Titers derived from mice of the same genotype were highly variable, whereby some animals had quantities of virus many-fold higher than their littermates, and littermates would be expected to react similarly to infection. A possibility is that the immune system interacts with the virus and influences the outcome of γMHV-68 infection in individual mice. In addition, each experiment was completed once and therefore must be repeated with the same parameters to verify results. Precise quantification of viral DNA
would also benefit these experiments. Relative quantification of viral DNA was informative about differences in infection levels between individuals, but it was difficult to assess whether a relatively low amount of viral DNA indicated viral clearance or was simply the lowest measured quantity of very high levels of replication. Two experiments did show that the majority of KO mice exhibited splenomegaly while WT mice presented spleens that were comparable in size to that of uninfected control mice. This could indicate that more B cells are infected and accumulated in the spleens of KO mice compared to the WT, but additional experiments are necessary to clarify whether this observed difference is real.

Fatality in suckling mice is associated with neuronal damage (Johnson, 1965; Tucker et al., 1993). Interestingly, WT suckling mice succumbed to SINV infection and experienced disease significantly earlier than their KO counterparts, despite having similar viral titers in brain tissue. One explanation for this is that viral replication within cells of both mouse types is comparable, but dissemination of the virus—and thus amount of tissue damage—is restricted. Infections in cell culture showed that the susceptible GT-negative population was limited to a small subpopulation, and if this phenomenon translates in vivo, viral spread in brain tissue of KO mice may be slowed by the absence of GT expression. As in cell culture, viral replication is not impeded in the mouse brains, but the extent of neuron involvement may be sufficiently limited to delay onset of disease symptoms and death. Histology work of SIN GFP infected suckling mouse brains
would elucidate this point. Additionally, titration of other tissues from these mice would be informative regarding dissemination of the virus, and how extra-neural tissue involvement affects virulence.

The possible mechanism by which expression of the GT gene alters the course of viral infections is by inhibiting the ability of a virus to initiate infection. I hypothesized that αGal is involved in viral-host interactions during entry because αGal is a known active binding receptor to other pathogens, such as *Clostridium difficile*. However, results from experiments to support my hypothesis have not been conclusive. Fluorescence focus assays showed that GT-negative cells appeared to resist infection by SINV viruses, and even after long periods of incubation with virus and after several rounds of viral replication, a large fraction of cells remained uninfected. The BL6 cell population likely represents a heterogeneous group in terms of their permissiveness to SINV infection that includes high- and low-permissive clones. Previous work (Kim et al., 1993) with the MA-104 cell line, derived from monkey kidney cells, showed that upon infection with porcine reproductive and respiratory syndrome (PRRS) virus, cells produced low viral titers and monolayers showed focal cytopathic effects. The parental MA-104 cell line yielded two subpopulations with different susceptibilities to PRRS infection, high- and low-permissive cell clones (Kim et al., 1993). Infections on a different cell line, the PK15 porcine cell line, with the porcine circovirus type 2 (PCV2), demonstrated that only about 20% of the population was susceptible to infection (Zhu et al., 2007). High- and low-
permissive clones were also demonstrated in the porcine cell lines. In SINV infection assays, BL6 cells showed similar characteristics to the parental MA-104 and PK15 cell lines. SINV-infected BL6 cells produced relatively low titers that appeared to derive from a fraction of the cell population, and monolayers exhibited focal cytopathic effects. In contrast, SINV-infected BL6αGal cells yielded relatively high titers and monolayers were uniformly infected and lysed by virus infection. If BL6 cells produce clones with differences in their susceptibility to SINV, it is important to identify the factor that permits propagation in permissive cells. Perhaps results from experiments with α-galactosidase can provide an explanation. It can be argued that herpes virus infections on the BL6αGal cells may also exhibit a similar phenomenon as described above. However, assays with HSV-1 GFP showed that infection on BL6αGal cells was almost negligible, indicating that, at least with HSV-1 GFP, the cells in the population were comparably non-permissive to infection.

Enzymatic treatment of BL6 cells reduced infectivity of αGal-positive cells, possibly due to the loss of surface αGal. However, this enzyme also further reduced the low level infectivity in αGal-negative cells that do not have a functional GT gene. BL6 cells may still express low levels of terminal Galα1,3Gal residues via an additional glycosyltransferase, the iGb3S enzyme (Taylor et al., 2003; Milland et al., 2006). iGb3S enzyme activity has been observed in rats (Taylor et al., 2003) and in GT KO mice and pigs (Milland et al., 2006). Our cell lines were consistently monitored for expression of αGal using
anti-Gal and BSl, but αGal was not detected in BL6 cells. This may be because as shown in KO mice, detection by BS lectin or anti-Gal is poor as the αGal disaccharide expressed via iGb3S synthesis is restricted to glycolipids. The sites in KO mice are recognized by monoclonal antibodies produced from GGTA-/mouse splenocytes fused to NS-1 hybridoma cells (Milland et al., 2006). BL6 cells could potentially express barely detectable levels of iGb3S derived αGal residues, the presence (and removal) of which may have some effect on infection in these cells. Alternatively, BL6 cells may express terminal galactose monosaccharides in variable amounts that may not be detectable by anti-Gal or BSI binding but are removed by enzymatic treatment. The α-galactosidase enzyme has high specificity for terminal galactose substrates, which means the enzyme removes the terminal galactose from proteins that express the Galα1,3Gal structure, but it will also remove αGal residues from the surface of human red blood cells with the group B structure, Galα1,3Fuc (Zhu et al., 1996). It is possible that while SINV may have greater affinity with the Gal-Gal linkage, resulting in a more productive infection of BL6αGal cells, the virus may also have some affinity for αGal monosaccharides. SINV may infect BL6 cells that express certain levels of terminal galactose residues, the removal of which affects infectivity. These observations should be further investigated.

The involvement of αGal in the course of viral infections is thus an avenue that warrants further investigation. Cellular glycans and host glycosylation pathways play an important role in viral infections (Reitter and Desrosiers, 1998; Varki and
Varki, 2007; Vigerust and Sheperd, 2007), and in this way the distribution of host-cell carbohydrates may even determine host range and tissue tropism (Schenider-Schaulies, 2000; Suzuki et al., 2000). SIN viruses have widespread host repertoires and utilize the ubiquitously expressed protein NRAMP2 for binding and entry into host cells (Rose et al., 2011; Stiles et al., 2011). It is unlikely that αGal would be a required receptor utilized in entry for SINV strains because loss of αGT expression limits, but does not completely inhibit viral infection. Further, insect cells such as those of Aedes mosquitoes, which SINV infects, lack terminal glycosylation from galactosyltransferases and sialyltransferases (Butters et al., 1981). The most common terminal glycan expressed on SINV virions propagated in Aedes-derived cells is mannose (Hsieh et al., 1984). On mammalian cells, αGal may instead participate in attachment or binding of SINV in a process analogous to how the virus may utilize heparan sulfate (Byrnes and Griffin, 1998); these receptors may promote initial adherence to host cells until higher affinity receptors are engaged (Schneider-Schaulies, 2000). Loss of heparan sulfate on host cells reduces SINV infection, but does not abrogate it. HSV-1 and 2 entry into cells is a multi-step process initiated by viral glycoproteins binding to cellular heparan sulfate proteoglycans (WuDunn and Spear, 1989; Shieh et al., 1992; Shukla et al., 1999; Spear and Longnecker, 2003) followed by binding to entry receptors such as the herpesvirus entry mediator (HVEM) protein or nectin-1 and nectin-2 and includes other virion-cellular protein interactions (Whitbeck et al., 1997; Shukla et al., 1999; Spear and Longnecker, 2003). Active expression of the αGT gene highly impairs infection
of both herpes virus strains. Experimental blocking of viral glycoprotein interactions with cognate cellular proteins disrupted the entry processes and inhibited infection of HSV-1 (Whitbeck et al., 1997; Gianni et al., 2010). It is conceivable that the presence of αGal on the cellular surface could somehow alter the interactions of binding or entry receptors and result in reduced infection efficiency for these herpesviruses.

Pathogens are major drivers of evolution, but conservation work has shown it is often the case that synergistic causes are responsible for reducing the number of individuals in populations to the brink of extinction (Brook et al., 2008). As such, climate change can influence ecosystems and animal biodiversity via altering pathogen or host biological processes (Harvell et al., 2002). Recent studies on global climate change and infectious disease (Reiter, 2001; Harvell et al., 2002; Lafferty, 2009; Mills et al., 2010) have focused on the effects of warming climates on disease, likely due to the present-day warming phase, but it is conceivable that measurable climate change of any sort could affect the disease ecology of populations experiencing these changes. The Oligocene was a period of vast climatic and geological change that also coincides with a great chronological gap in the primate paleontological record (Seiffert, 2007). While the particular ecological conditions that basal catarrhines experienced in the Oligocene are not known, the world underwent climatic changes that affected animal habitats and the distribution of life on the planet. In general, the fossil record shows a vast turnover of faunal species found at the Eocene-Oligocene
boundary and those present at the Oligocene-Miocene boundary (Seiffert, 2007; Kappelman et al., 2003). For catarrhines, the scarcity of fossils that derive from the Oligocene makes it difficult to discern phylogenetic relationships between ancestral and descendant taxa, and to even estimate conclusive divergence dates for OWM and apes (Raaum et al., 2005; Steiper and Young, 2008; Perelman et al., 2011). Numerous factors may have affected Oligocene primate evolution and lineage divergence. \textit{GGTA1} inactivation around 28 Ma can inform us on timing of phylogenetic divergences; the work presented in this dissertation can inform us on one possible catalyst for lineage divergence in ancestral catarrhines.

Pathogens with wide host-specificity can cause declines across sympatric populations and lineage divergence can result when less resistant populations within a species are culled (Van Blerkom, 2003). In cases where genetic diversity is useful, polymorphism may be selected for in the population, such as with the major histocompatibility group of genes; however, specific alleles are favored when selection is directional (Van Blerkom, 2003). In the case of the \textit{GGTA1} gene, the mutated gene (\textit{AGGTA1}) may have been favored if \textit{GGTA1} or its gene product could be exploited by a virulent pathogen. Any factor that increases resistance to a pathogen or decreases infectivity of that pathogen is enough to increase fitness for an individual (Antonovics et al., 2013). If the hypotheses tested during this research are correct, the loss of \(\alpha_{1,3}\)GT expression may have given \(\alpha\)Gal-negative catarrhines an advantage over \(\alpha\)Gal-positive catarrhines in controlling pathogens that could exploit the \(\alpha_{1,3}\)GT pathway without also stimulating an autoimmune response. Additionally, the production of anti-Gal
antibodies would have further protected GT-negative species from zoonotic transmission. Viruses can shape the evolutionary trajectory of the organisms they infect, as well those that the infected organisms are in contact with. Experimental evidence on the significance of $\alpha_1,3GT$ activity and $\alpha$Gal expression in viral disease such as that presented here may shed light on the processes that helped shape catarrhine evolution during the Oligocene epoch.
## APPENDIX

### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>αGal</td>
<td>α1,3galactosyl</td>
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<tr>
<td>α1,3GT or GT</td>
<td>α1,3galactosyltransferase</td>
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<tr>
<td>ΔGGTA1</td>
<td>mutated glycoprotein, glycosyltransferase gene 1</td>
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<tr>
<td>γMHV-68</td>
<td>murine gammaherpes virus 68</td>
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<tr>
<td>ab</td>
<td>antibody</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>APC</td>
<td>allophycocyanin</td>
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<tr>
<td>BS1-B4 or BS1</td>
<td>lectin from <em>Bandeiraea simplicifolia</em></td>
</tr>
<tr>
<td>CCR5</td>
<td>leukocyte chemokine (C-C motif) receptor 5</td>
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<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>CHIK</td>
<td>Chikungunya virus</td>
</tr>
<tr>
<td>CMAH</td>
<td>cytidine monophosphate-N-acetylneuraminic acid hydroxylase</td>
</tr>
<tr>
<td>CMP</td>
<td>cytidine monophosphate</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
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<tr>
<td>d pi</td>
<td>days post infection</td>
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<tr>
<td>DI particle</td>
<td>defective interfering particle</td>
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<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
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<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>FCS</td>
<td>fetal calf serum</td>
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<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<td>FSL</td>
<td>functional spacer lipid construct</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>GGTA1</td>
<td>glycoprotein, glycosyltransferase gene 1</td>
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<tr>
<td>GT K0 or K0</td>
<td><em>GGTA1</em> knock out</td>
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<tr>
<td>Gy</td>
<td>gray unit</td>
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<tr>
<td>GYP A</td>
<td>glycoprotein A</td>
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<tr>
<td>h pi</td>
<td>hours post infection</td>
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<tr>
<td>HA</td>
<td>haemagglutinin</td>
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<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
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<tr>
<td>HSV-1</td>
<td>herpes simplex virus type 1</td>
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<tr>
<td>HSV-2</td>
<td>herpes simplex virus type 2</td>
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<tr>
<td>i.c.</td>
<td>intracranial</td>
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<tr>
<td>i.n.</td>
<td>intranasal</td>
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<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
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<tr>
<td>ICA</td>
<td>infectious center assay</td>
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<tr>
<td>IFN</td>
<td>interferon</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>LCMV</td>
<td>lymphocytic choriomeningitis virus</td>
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MCMV  murine cytomegalovirus
MEF  mouse embryonic fibroblast
MEM  minimal essential medium
MFI  mean fluorescence intensity
MHV  mouse hepatitis virus
mL  milliliter
MOI  multiplicity of infection
NWM  New World Monkey
NK  natural killer cell
OWM  Old World Monkey
PBS  phosphate buffered saline
PCR  polymerase chain reaction
PFU  plaque forming units
pi  post infection
PPG  processed pseudogene
PV  Pichinde virus
R-PE  R-phycoerythrin
RBC  red blood cell
RNAi  interfering RNA
s.c.  subcutaneous
SINV  Sindbis virus
SFV  Semliki forest virus
ST6GAL1  ST6 beta-galactosamide alpha 2,6-sialyltransferase 1
UMMS  University of Massachusetts Medical School
UPG  unprocessed pseudogene
VSV  vesicular stomatitis virus
VZV  varicella-zoster virus
WEE  Western equine encephalitis virus
WNV  West Nile virus
WT  wild type
WT B6  wild type C57BL/6J
BIBLIOGRAPHY


