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Investigating the Role of B Cells in Limiting the Efficacy of Oncolytic Virus Therapy

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Abstract

B cells limit the efficacy of oncolytic virus therapy, both through their role as antibody producers. After VSV infection, neutralizing antibodies are detectable in mouse serum four days post-infection, persisting for many weeks. Pre-existing immunity to VSV precludes viral replication in tumours and is caused by the action of anti-VSV antibodies, and not other immune compartments. I hypothesize that B cells are important in limiting the efficacy of oncolytic virus treatment, and that a lack of B cells would correlate with improved therapeutic outcome. To better study the role of B cells, I have chosen to study VSV infection and oncolytic activity in a B cell-deficient mouse model, muMT. My data indicate that while the absence of B cells correlates with the ability to deliver multiple doses of virus to the tumour and improve efficacy, under the current treatment protocol, this does not correlate with significantly improved survival.

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List of Abbreviations

ADCC	Antibody-dependent cell cytotoxicity
APC	Antigen presenting cell
BCDM	B cell-deficient mice
BCR	B cell receptor
Breg	Regulatory B cell
CAR	Coxsackie and Adenovirus receptor
CEV	Cell-associated enveloped virus
CTL	Cytotoxic T Lymphocyte
DC	Dendritic cell
DMEM	Dulbecco's Modified Eagle Medium
dsRNA	Double-stranded RNA
EAE	Experimental autoimmune encephalitis
EEV	Extracellular enveloped virus
eIF2α	Eukaryotic translation initiation factor 2α
ELISA	Enzyme-linked immunosorbent assay
F	Fusion protein
FCS	Fetal calf serum
fLuc	Firefly luciferase
GC	Germinal center
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage colony-stimulating factor
H	Hemagglutinin protein
H&E	Hematoxylin and Eosin
HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitor
HDI	High-dose IFNα
HSG	Heparan sulphate glycosaminoglycans
HSV-1	Herpes Vimplex Virus-1
i.n.	Intranasally
i.p.	Intraperitoneally
i.t.	Intratumorally
i.v.	Intravenously
IC	Immune complex
ICOS	Inducible costimulator protein
IEV	Intracellular enveloped virus
IFN	Interferon
IFP	Interstitial fluid pressure
IHC	Immunohistochemistry
IMV	Intracellular mature virus
iNOS	Inducible nitric oxide synthase
IRF-2	IFN response factor-2
ISG	IFN-stimulated genes
ISRE	IFN-sensitive response elements
JAK	*Janus* family of tyrosine kinase

LIGHT	Lymphotoxin-like inducible protein that competes with glycoprotein D for binding herpesvirus entry mediator on T cells
MAC	Membrane attack complex
MOI	Multiplicity of infection
MV	Measles Virus
MZ	Marginal zone
NDV	Newcastle Disease Virus
NK cells	Natural killer cells
NO	Nitric oxide
OV	Oncolytic virus
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell
Pfu	Plaque-forming unit
PI-3K	Phosphatidylinositol-3 kinase
PKR	RNA-dependent protein kinase
polyI:C	polyinosinic:polycytidilic acid
RA	Rheumatoid arthritis
RCA	Regulators of complement activation
RFP	Red fluorescent protein
ROS	Reactive oxygen species
Rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SFV	Semliki Forest Virus
SHP-1	Src homology 2 domain-containing protein tyrosine phosphatase 1
SLE	Systemic lupus erythematosus
ssRNA	Single-stranded RNA
STAT	Signal transducer and activator of transcription
T2-MZP	Transitional 2-Marginal Zone Precursor
TBS-T	Tris-buffered saline with Tween-20
TCR	T cell receptor
TI	T cell-independent
TIL	Tumour-infiltrating lymphocyte
TK	Thymidine kinase
TRAIL	TNF-related apoptosis-inducing ligand
Treg	Regulatory T cell
VSV	Vesicular Stomatitis Virus
VV	Vaccinia Virus
vvDD	Vaccinia virus double-deleted

1. Introduction

1.1 Oncolytic Viruses

Oncolytic viruses (OVs) are viruses that are able to preferentially infect and destroy cancer cells over their normal counterparts. There are a number of diverse OVs currently being evaluated, including adenovirus, reovirus, herpes simplex virus 1 (HSV-1), Newcastle disease virus (NDV), measles (MV), vaccinia virus (VV) and vesicular stomatitis virus (VSV). Although these all come from different families and have divergent characteristics, they are all able to cause tumour-specific death by hijacking cellular machinery and causing apoptosis or necrosis. There are three ways in which viruses can be targeted to infect tumours; the first is their ability to exploit defects in cellular machinery that promote tumour growth, specifically defects in the interferon (IFN) pathway. Second, many of these viruses have been engineered to be dependent on signaling pathways that are constitutively activated in cancer cells. For example, a vaccinia virus lacking both its thymidine kinase (TK) and vaccinia growth factor (VGF) genes was generated by McCart et al. (2001), (double-deleted VV, vvDD). While this mutant lacked the CNS-tropism of other VVs, its ability to infect and destroy tumours was not compromised. Third, cellular entry can be restricted based on the expression of tumour-specific antigens, allowing viruses to only infect cancer cells. Entry of viruses into cells can occur via specific receptors. For example, MV expresses the hemagglutinin protein (H) which is responsible for binding

the complement regulatory protein CD46, while the fusion protein (F) mediates the fusion of viral and cell membranes, facilitating entry (Schneider et al., 2000). In the case of adenovirus type 5, cell entry is mediated in one of three ways: a) the knob domain binds Coxsackie and Adenovirus Receptor (CAR), b) the penton base binds αvβ3 and αvβ5 integrins and c) fibre protein binds heparan sulphate glycosaminoglycans (HSG) – all of which are expressed on a large number of cells, making adenovirus quite unspecific in its binding capacity (Green et al., 2004). Other viruses including VV (Chung et al., 1998) and HSV-1 (Spear et al., 1992) are also known to bind HSG. The surface receptor bound by the VSV glycoprotein G is still unidentified, though the virus is known to enter cells via endocytosis, with membrane fusion being mediated by VSV G at low pH (Roche et al., 2007). A number of OVs have shown efficacy in both pre-clinical and clinical studies (reviewed in Parato et al., 2005). Pre-clinical studies in mice have been completed with VSV, while VV JX-594 is currently in Phase I/II clinical trials for treatment of melanoma, and the H101-E1B-deleted adenovirus vector has been tested in Phase III trials in combination with cisplatin therapy for head and neck carcinoma (Xia et al., 2004).

1.1.1 VSV

VSV is a rhabdovirus with a negative, single-stranded RNA (ssRNA) genome encoding five proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and long polymerase protein (L)

(Lichty et al., 2004). These are expressed sequentially and in a graduated fashion as the polymerase stutters at each intergenic region, resulting in more nucleoprotein than phosphoprotein, which is produced in more abundance than matrix protein, and so on. The matrix protein plays a crucial role in viral assembly and budding, as well as circumventing host innate immunity. This occurs by blocking host transcriptional programs and nuclear export of mRNA (including IFNβ transcripts expressed upon viral infection). These two functions are responsible for preventing the initiation of the innate antiviral response. VSV M blocks nuclear transport by interacting with the nucleoporin Nup98, which is an IFN-inducible protein. Nup98 is an IFN-responsive gene and its expression is enhanced upon pretreatment of VSV-infected cells with IFN, thus limiting the ability of VSV M to inhibit nuclear transport (Lichty et al., 2004). The genome of VSV can accommodate foreign genes so recombinant viruses are relatively simple to engineer. Recombinants have been created that encode immunomodulatory cytokines such as IFNβ (Obuchi et al., 2003) and IL-4 (Fernandez et al., 2001) as well as receptor targeted recombinants expressing CD4 and Sindbis glycoproteins (summarized in Lichty et al., 2004).

1.1.2 Attenuated VSV mutant

Many viruses, including wild-type VSV, have evolved the ability to block the IFN response, delaying its onset. In the case of VSV, this resistance is mediated by the matrix protein (Lichty et al., 2004). Mutations in VSV M,

such as a deletion at methionine 51 (VSVΔ51) abrogate its ability to interact with Nup98 and block the export of IFNβ transcripts, thus making the virus extremely sensitive to IFN (Stojdl et al., 2003). VSVΔ51 has an excellent safety profile; whereas wild-type VSV kills athymic nude mice in the absence of exogenous IFN, immune compromised mice are asymptomatic upon multiple infections with VSVΔ51 (Stojdl et al., 2003). Replication of VSVΔ51 in normal cells is completely abrogated, and it is attenuated in tumour cells that are IFN-responsive (such as RT4 human bladder carcinoma and PC3 human prostate cancer); pre-treatment of these cells with exogenous IFN renders them completely resistant to virus infection (Hadaschik et al., 2008 and Nguyen et al., 2008). Of concern is the possibility of outgrowth of a reversion mutant that is no longer sensitive to IFN, however, the deletion of methionine 51 of protein M makes it unlikely that this will occur, and complementary mutations that restore IFN-resistance to VSV have not been found (Stojdl et al., 2003), making VSVΔ51 a much safer therapeutic.

1.1.3 Oncolytic potential of VSV

The efficacy of VSV as an oncolytic has been demonstrated in a variety of tumour types. Stojdl et al. (2003) showed that VSVΔ51 could retard tumour growth when injected intratumorally (i.t.), intraperitoneally (i.p.), intranasally (i.n.) and intravenously (i.v.) in immune deficient and immune competent mice, with durable cures observable in a large proportion of

animals. VSV treatment was also able to prolong survival of lung tumour-bearing mice (seeded with CT26 tumours) when they were treated with six doses of virus i.n. over the span of two weeks (Stojdl et al., 2003). An IFNβ-expressing VSV mutant also showed efficacy against Renca renal cell carcinoma upon multiple i.t. administrations of virus and significantly prolonged survival in a metastatic model of mammary tumours (Obuchi et al., 2003). Hadaschik et al. (2008) recently demonstrated that intravesical VSV treatment of an orthotopic model of bladder cancer (KU-7 tumour cells of human origin) resulted in reduced tumour burden in both VSVΔ51 and VSV wild-type treated mice. VSV has been shown to be extremely selective; in co-cultures of normal fibroblasts and cancer cells, VSV will only infect cancer cells, and retains its oncolytic activity in the presence of IFN (Stojdl et al., 2000). In addition to directly causing tumour destruction, VSV may also be able to improve the anti-tumoural T cell response by recruiting antigen-presenting cells (APCs) and activating them in an otherwise non-immunogenic tumour. These APCs may have already sampled tumour antigen and become activated by the danger signals elicited by VSV infection so that upon migration back to secondary lymphoid organs, tumour-specific T cells may also become activated and in turn infiltrate and attack tumours. There may be evidence of this, as Stojdl et al (2003) have shown better efficacy in an immune competent mouse model (Balb/c) where VSV treatment caused complete tumour regression and durable cures, compared to immune compromised

athymic nudes where VSV treatment was tumour-static at best (Stojdl et al, 2000), indicating that T cells are necessary in VSV's anti-tumoural activity. However, the authors did not treat the same tumour cell line in both cases, so the nature of the tumour must also be taken into account, as some tumours may be more resistant to VSV oncolysis than others.

1.2 Barriers to effective OV dissemination and efficacy

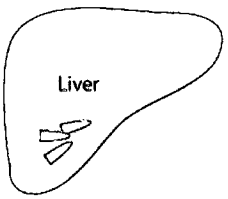
There are multiple barriers to OV infection (Figure 1). These include scavenging by the liver (Green et al., 2004, Breitbach et al., 2007), non-specific binding to erythrocytes, lymphocytes and monocytes (Lyons et al., 2006), high interstitial fluid pressure (IFP) within the tumour (Boucher et al., 1990 and Rutz, 1999), inflammation-dependent vascular shut-down (Breitbach et al., 2007) and naturally, anti-viral immunity (discussed in section 1.3). Green et al. (2004) found that they could decrease scavenging by the liver and enhance dissemination of adenovirus by coating the virus with polymers, preventing the non-specific interaction of the virus with hepatocytes and minimizing phagocytosis by Kupffer cells. Similarly, high titers of VSV were found in the livers of mice immediately after systemic infection (5 minutes) followed by lower titers in the spleens, and very little virus detectable in tumours (Breitbach et al., 2007).

Lyons et al. (2006) determined the extent of non-specific binding of adenovirus to blood cells and found that over 90% of virus was associated

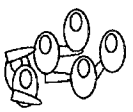
with human blood (but not murine) and that this interaction prevented the virus from infecting epithelial cells. In addition, the mere presence of blood severely diminished infection, though the virus was not bound to the cells (Lyons et al., 2006).

The importance of high tumour IFP has been recognized for many years. It has been shown to minimize the efficacy of many therapeutics including monoclonal antibodies by generating outward flux of fluid from the tumour to the periphery, or by reducing the force required for extravasation into the tumour environment (Boucher et al., 1990). IFP is uniformly elevated throughout the tumour but drops dramatically outside and is thought to be driven by systemic blood pressure – death of test animals corresponded with a quick drop in the IFP of tumours as observed by Boucher et al. (1990). In addition, Rutz (1999) hypothesizes that enhanced tumour IFP is the result of osmotic changes caused by the presence of intermediary metabolites, such as lactate and carbon dioxide. Solid tumours are known to exist in hypoxic conditions, favouring anaerobic respiration which results in the conversion of glucose into two lactate molecules, thus leading to increased osmotic pressure within tumours and increased IFP (Rutz, 1999).

Non-specific binding



Uptake by the liver



Binding to blood cells

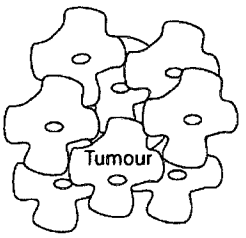
Anti-viral immunity

Anti-viral cytokines

Neutralization by antibodies

Inactivation by complement

Hostile tumour microenvironment



High interstitial fluid pressure
Hypoxia
Low pH

Figure 1: Barriers to oncolytic virus therapy. Oncolytic viruses are removed from the circulation through uptake into the liver and non-specific binding to blood cells. They can also become inactivated by mediators of antiviral immunity, including anti-viral cytokines, neutralizing antibodies and complement. Furthermore, the tumour microenvironment often precludes efficient virus entry due to high interstitial pressure, hypoxia and low pH.

Loss-of-perfusion corresponding to VSV and VV infection has recently been demonstrated by Breitbach et al. (2007). When mice bearing CT26 tumours were treated systemically with VSV (or VV), the authors observed a lack of perfusion to the centre of the tumour with virus infection restricted to the rim, though the centre of the tumour showed markers of apoptosis (active caspase 3). This vascular shut-down is attributed to neutrophils which infiltrate the infected tumour, become activated and lodged within the tumour vasculature, and initiate a cytokine storm (Breitbach et al., 2007). This cytokine storm can potentially block virus from spreading further and replicating efficiently within the tumour, limiting the efficacy of treatment.

Antiviral immunity is discussed in detail in section 1.3; however it is important to discuss the complement cascade in the context of limiting OV therapy. Complement is part of the innate immune system and can be divided into two pathways: classical and alternative. The classical pathway is initiated when the complement protein C1q recognizes mannose-binding lectin on the surface of pathogens, while the alternative pathway is the default process which is initiated upon pathogen encounter, unless it is otherwise down-regulated (Vanderplasschen et al., 1998). Activation of complement results in the activation and cleavage of C3, opsonization and deposition of C3 subunits on target cells. This is followed by C5 cleavage and assembly of the membrane attack complex (MAC) which is responsible for disrupting the cell membrane (Janeway et al., 2001). The

activation of this cascade is controlled by regulators of complement activation (RCAs). These act by inhibiting the induction of and accelerating the degradation of C3 convertases and other downstream enzymes, preventing the formation of MACs (Vanderplasschen et al., 1998). Thus complement is an important factor in limiting the spread of oncolytic viruses.

Interestingly, many enveloped viruses have evolved methods of complement resistance. For instance, the glycoprotein C of HSV-1 mimics RCAs and induces dissociation of C3 while HIV and VV both incorporate host RCAs into their membrane as they bud from cells, with VV also secreting VV complement control protein (VCP) which restricts complement activation (Vanderplasschen et al., 1998). It has been reported that VV infection of tumour cells makes them more susceptible to complement-mediated lysis (Okada et al., 1987). This was attributed to a loss of RCAs on host cell membrane, not due to global translational down-regulation during viral infection, rather due to the fact that they are being incorporated into viral envelopes (Baranyi et al., 1994). Thus, although complement severely impairs viral infection and spread, it can also enhance the effect of oncolytic viruses by lysing infected tumour cells.

1.3 Antiviral immunity

1.3.1 Antiviral IFN response

IFNs are a group of pro-inflammatory cytokines that are produced in response to viral infections, as well as a variety of other stimuli. There are two families of IFNs, type I (IFNα and IFNβ, induced in response to virus infection) and type II (IFNγ, produced in response to mitogenic or antigenic stimuli) (Samuel, 2001). IFNα/β are produced by leukocytes and fibroblasts respectively, while IFNγ is produced only by a subset of cells, including cytotoxic T lymphocytes (CTLs), CD4 Th1 cells, macrophages and natural killer (NK) cells. IFN stimulation results in growth arrest, induction of an antiviral state (such as inducible nitric oxide synthase (iNOS) expression), modulation of Th1 vs. Th2 immunity and stimulates antigen-specific cytotoxic immunity (Samuel, 2001). Importantly, IFNs play a role in anti-tumour immunity, and many cancer cells have evolved to be able to resist IFN signaling, allowing them to escape the pro-apoptotic and anti-proliferative effects of IFN. However, this resistance comes at the cost of compromised antiviral defense and increased susceptibility to virus infection. IFNα/β is the major antiviral cytokine produced by cells in response to an infection, and can induce immunoregulatory functions that affect innate and adaptive immunity.

1.3.1.1 *IFNα/β*

Upon viral infection, NF-κB is released from its inhibitory complex in the cytoplasm and translocates to the nucleus where it mediates the expression of IFNα/β. IFNα/β can also prime its own production via a positive feedback loop (Malmgaard, 2004). Essentially any nucleated cell can produce IFN if stimulated properly, and differential expression of IFNα and β depends on cell type, transcription factors and type of stimulus. However, the natural IFNα-producing cells are plasmacytoid dendritic cells (pDCs) (Samuel, 2001) and IFNα/β has also been shown to promote its own expression by acting as a survival factor for DCs (Dalod et al., 2002).

Interaction of viral factors with intra- or extracellular receptors initiates a signaling cascade that results in the translocation of several transcription factors to the nucleus where transcription of IFNβ and IFNα4 is initiated. Among these receptors are Toll-like receptors (TLRs) that are found both on the cell surface and also on intracellular compartments and are activated by a variety of viral factors, such as CpG islands, double stranded RNA (dsRNA) and ssRNA (Malmgaard, 2004). Signaling by these receptors is mediated by four different adaptor proteins, and results in the expression of IFNα/β (Malmgaard, 2004). IFNα/β binding to the IFNα/β receptor (IFNAR) results in positive feedback regulation and production of non-IFNα4 subtypes, as well as inducing the expression of IFN-stimulated genes (Samuel, 2001). IFNα/β signaling proceeds through

the JAK-STAT pathway , where signal transducer and activator of transcription (STAT) proteins that are normally in an inactive state are phosphorylated by *Janus* family of tyrosine kinase (JAK) and translocate to the nucleus, where they bind to IFN-sensitive response elements (ISRE) in IFN-inducible genes, mediating their expression (Samuel, 2001).

The antiviral effects of IFN are the result of the activity of a number of IFN-inducible genes. These include RNA-dependent protein kinase (PKR) and RNA-specific adenosine deaminase (ADAR1). PKR is induced by IFN expression and is activated by ssRNA and dsRNA. Upon activation, PKR phosphorylates the initiation factor eIF2α, resulting in inhibition of mRNA translation. On the other hand, posttranscriptional modification of RNA by ADAR1, which mediates adenosine-to-inosine conversions in both viral RNA and cellular transcripts, destabilizes dsRNA helices and disrupts protein expression (Samuel, 2001).

1.3.1.2 *IFNγ*

IFNγ is induced as part of a secondary wave of cytokines and not as a direct result of viral infection. The expression of this cytokine is stimulated by antigen presenting cells (APCs) or by other pro-inflammatory cytokines. $CD4^+$ and $CD8^+$ T cells produce IFNγ upon stimulation by APCs; binding of the T cell receptor (TCR) by antigens presented in the context of MHC molecules on the surface of APCs results in IFNγ expression and requires the involvement of the transcription factor

NFAT (Malmgaard, 2004). However, APCs may also activate T cells and NK cells by producing cytokines including IL-12, IFNα/β and TNFα, which also support the development of a Th1 response and cell-mediated immunity. IFNγ expression results in the up-regulation of MHC expression on the surface of cells, possibly targeting infected cells to cytotoxic T cell responses, as well as having anti-angiogenic and pro-apoptotic properties (Parato et al., 2005).

1.3.2 Cell-mediated immunity

Cell-mediated immunity is induced in response to virus infection and is responsible for the destruction of infected cells. APCs sampling the environment uptake viral antigens to present to $CD8^+$ T cells, but APCs can also be activated by antigen-specific $CD4^+$ T cells that are primed by encountering viral antigen in the presence of co-stimulatory signaling. $CD4^+$ T cell help to APCs has been shown to yield better $CD8^+$ CTLs as well as improving recall of memory cells upon re-stimulation of $CD8^+$ T cells with viral antigen. Recently, persistence of antigen weeks after the resolution of VSV infection has been demonstrated and is thought to contribute to the maintenance of a memory pool (Turner et al., 2007). There are three phases of the $CD8^+$ T cell response to acute viral infection: expansion, contraction and the establishment of a virus-specific memory pool (Wherry and Ahmed, 2004). During the expansion phase, activated $CD8^+$ T cells proliferate to a great extent and differentiate into effector cells which can produce cytokines (IFNγ and TNFα), as well as expressing

cytotoxic granule proteins such as granzyme B and perforin (Wherry and Ahmed). These activated cells are cytolytic and gain the ability to enter non-lymphoid organs to mediate their antiviral activities.

1.3.3 Anti-VSV antibody response

Neutralizing antibodies are produced as early as four days post VSV infection and persist for several weeks (Power et al., 2006). These antibodies prevent subsequent infection of tumour-bearing mice with VSV and correlate with reduced efficacy. VSV-specific antibodies can go on to activate phagocytic cells via their Fc receptors, triggering antibody-dependent cell cytotoxicity (ADCC), as well as sequestering virus in the form of immune complexes. The antibody response is initiated when DCs carrying viral antigen or infected with virus migrate to lymphoid organs and express viral antigen on surface MHC molecules or release small amounts of virus into the milieu. After encounter with VSV, long-lived germinal centers (GCs) containing proliferating B cells are induced, and a rapid T cell-independent (TI) IgM response is initiated (Ludewig, 2000). Only a small fraction of B cell receptors (BCR) need be cross-linked with viral antigen before a robust antibody response is initiated (Bachmann and Zinkernagel, 1997). Although the induction of the neutralizing IgM response to VSV occurs in a TI fashion, $CD4^+$ T cell help is required for isotype switching from IgM to the higher affinity, longer lasting IgG subclass and the establishment of VSV-specific GCs (Ludewig, 2000). However, when presented in the context of MHC molecules on the surface

of infected cells, poorly organized VSV G (the main target for neutralizing antibodies) is a weak TI antigen and induction of a sufficient antibody response requires T cell help (Ludewig, 2000). Interestingly, Turner et al. (2007) have demonstrated the presence of naturally-occurring anti-VSV IgM antibodies which are not the result of pre-exposure but which enhance the expression of IFNα in response to VSV infection. VSV:IgG immune complexes interact with TLR7 in an endosomal-dependent fashion, inducing IFNα production and bridging humoral with innate immunity.

The neutralizing antibody response is not only a barrier to therapeutic efficacy of VSV, but also for a variety of other oncolytics, such as HSV-1 (Coukos et al., 1999), measles virus (Iankov et al., 2007), and myxoma virus (Stanford et al., 2007). In order to overcome the antiviral effects of neutralizing antibodies several strategies have been utilized. One of these is to inject virus intratumorally thus bypassing exposure to circulating antibody or immune cells. However, this method does not address the need for systemic delivery of virus to treat disseminated tumours and alternatives such as masking viral antigens (adenovirus) or delivering virus within carrier cells (VSV, HSV-1) have been used. Masking of adenovirus antigens with multivalent co-polymers of poly N-(2-hydroxypropyl) methacrylamide not only protects this virus from neutralization by antibodies, but also limits the extent of virus binding in the liver and increases its bioavailability (Green et al., 2004),

and although carrier-cell technology shows promise as a proof of concept, much work remains to be done in order to find a cell line that yields optimal levels of virus and disseminates systemically (Guo et al., 2008 and Power and Bell, 2008). Alternatively, ablation of the immune response using cyclophosphamide has been shown to decrease neutralizing antibody titers to HSV-1 (Wakimoto et al., 2004), but this also limits the contribution of anti-tumour immunity. In fact, a number of viruses such as VSV and HSV-1 have been shown to have better efficacy in the presence of T cells, indicating that anti-tumour immunity concurrent with the oncolytic activity of viruses is necessary to achieve therapeutic efficacy. Thus, the optimal course of action is to specifically eliminate the antiviral effects of B cells, while maintaining an intact anti-tumoural response.

Although VSV is exquisitely sensitive to neutralization by antibodies, VV is quite resistant. VV has evolved in a manner that allows it to evade anti-viral antibodies. The virus exists in four infectious forms: intracellular mature virus (IMV), intracellular enveloped virus (IEV), cell-associated enveloped virus (CEV) and extracellular enveloped virus (EEV) (Law and Smith, 2001). While IMV, IEV and CEV are susceptible to antibody-mediated neutralization, EEV is protected and is shed into the circulation, making it responsible for maintaining a persistent infection. It is able to resist antibody neutralization far better than IMV, probably due to its cell-derived outer membrane which effectively shields the virus against antibodies (Law and Smith, 2001). The outer membrane is very

fragile and breaks easily during virus purification so the extent of neutralization by antibodies may depend on the level of damage to the membrane and on the accessibility of the immunogenic IMV particle inside. Law and Smith (2001) showed that low titers of anti-VV neutralizing antibodies may have actually improved infection through antibody-dependent enhancement (ADE). As evidence to the resistance of VV to pre-existing immunity, patients enrolled in clinical trials with VV JX-594 who have been vaccinated against smallpox are still susceptible to infection with repeated clinical doses of the virus. This was demonstrated by Park et al. (2008) who reported that even patients with high levels of pre-existing anti-VV antibodies responded well to treatment. Although the immune system plays a role in limiting oncolytic virus infection, this immunity may be advantageous as the presence of virus in tumours may actually induce anti-tumoural immunity, significantly improving therapeutic outcome.

1.4. Anti-tumour immunity

1.4.1 NK cells, DCs, $CD4^+$ and $CD8^+$ T cells come together

Anti-tumour immunity is mediated by DCs, $CD4^+$ T cells, $CD8^+$ T cells and NK cells. Collectively, these cells recognize the presence of tumours and mediate their rejection to varying degrees of success. Tumour clearance involves initial recognition of stressed cells by NK cells and

their subsequent activation (Chan and Houusseau, 2007). NK cells can be activated in response to both the presence of stress-related ligands on cells via the NKG2D receptor which binds MICA on tumour cells (Kim et al, 2006), and also the absence of MHC I molecules on tumour cells (Chan and Housseau, 2007). Upon activation, NK cells have multiple roles including priming $CD4^+$ and $CD8^+$ T cells and triggering and sustaining DC activity (via cell contacts and through the release of TNFα and IFNγ), as well as direct cytotoxicity. NK cell cytotoxicity is mediated by direct cell-cell contact and is the result of two different pathways: a) granule exocytosis involving the release of granzymes and perforin, and b) the release of pro-apoptotic molecules FasL and TNF-related apoptosis-inducing ligand (TRAIL), where the end result of each pathway is the activation of caspase pathways and target cell death (Chan and Housseau, 2007). IFNγ induces differentiation of CTLs, activates macrophages and NK cells, inhibits angiogenesis and may enhance ADCC (Shah et al., 2005).

DCs are crucial for the initiation of anti-tumour immunity as they are continuously sampling antigen and can activate T cells present in secondary lymphoid tissues (Ochsenbein et al., 1999). DC subsets can have different effects – pDCs produce IFNα in response to a viral infection, but they can also activate DCs involved in cross-priming of T cells (Chan and Housseau, 2007). However, some pDCs recruited by tumours have suppressive activities and result in tolerization of T cells and

other DCs (Ochsenbein et al., 1999). The degree of DC activation and maturation is important as non-activated or immature DCs are poorly cytotoxic and their interaction with T cells can be tolerogenic (Chan and Housseau, 2007). DC interaction with tumours must be accompanied by pro-inflammatory signals in order for them to subsequently activate a tumour-specific T cell response. Tumour-associated antigen (TAA) presentation by DCs to T cells can occur in three different ways: a) immature DCs engulf tumour cell debris and apoptotic bodies generated by NK cells, migrate to secondary lymphoid organs and activate tumour-specific T cells, b) DCs are activated at the tumour by the activity of IFNγ released by NK cells or by IFNα released by damaged tissue, migrate to lymphoid organs and activate T cells or c) DCs mediate their own activation and produce their own activating cytokines in the absence of NK cell help, after which time they migrate to lymphoid organs to activate T cells (Chan and Housseau, 2007). TRAIL-dependent cytotoxic action of DCs is enhanced by the presence of IFNα and IFNγ (Chan and Housseau, 2007), thus DCs can directly cause cell death and also mature into immunostimulatory APCs.

Anti-tumour activity of $CD4^+$ and $CD8^+$ T cells is dependent upon their activation by DCs in secondary lymphoid organs; however $CD4^+$ T cells can also prime cytotoxic $CD8^+$ T cells via cross-presentation to APCs (Pardoll and Topian, 1998). T cell activation is dependent upon stimulation of the T cell receptor with tumour antigen presented in the

context of MHC molecules on the surface of APCs, and the presence of co-stimulatory signals, as the absence of these signals results in T cell tolerance. Direct interaction of activated T cells with tumours is possible - most tumours are positive for MHC I but not MHC II molecules, allowing cytotoxic $CD8^+$ T cells to bind tumour cells and mediate their function in a similar way to NK cells (Paradoll and Topalian, 1998). However, direct binding of naive $CD8^+$ T cells to antigen presented on tumour cells has been shown to be tolerogenic as this interaction is usually accompanied by a lack of activating signals (Chan and Housseau, 2007). Thus, $CD4^+$ T cells and APCs are crucial to the induction and maintenance of a viable anti-tumour response through the proper activation of CTLs. The interaction between $CD4^+$ T cells and APCs facilitates communication between cells that are specific to MHC I- and MHC II-restricted epitopes, resulting in a broader anti-tumour response (Pardoll and Topian, 1998).

In addition to priming $CD8^+$ T cells, $CD4^+$ T cells can also activate NK cells and macrophages, causing the latter to produce nitric oxide which results in the recruitment and activation of eosinophils and inducing a wide-spread anti-tumour response. Pardoll and Topian (1998) propose three possible roles for $CD4^+$ T cells in the induction and maintenance of an anti-tumour response: a) they are needed to prime CTLs through the proper activation of APCs, b) they maintain CTL activity by producing cytokines such as IL-2, and c) they are needed to facilitate an immune response against MHC II-negative tumours through the recruitment of

other effector cells including NK cells, macrophages and eosinophils. $CD4^+$ T cells have also been shown to inhibit angiogenesis in an IFNγ-dependent manner by acting on non-hematopoietic cells in the tumour environment (Pardoll and Topian, 1998). However, it is important to note that $CD4^+$ T cells can differentiate into regulatory $CD4^+CD25^+$ T cells and result in the inhibition of anti-tumour immunity (Sutmuller et al., 2001). In fact, Sutmuller et al. (2001) observed that depletion of $CD4^+CD25^+$ T cells enhanced CTL-mediated anti-tumour immunity, and proposed that the role of Tregs was to keep "educated" CTLs under control, preventing autoimmunity.

Work in our lab has shown that the efficacy of VSV is enhanced in immune-competent mice versus athymic nudes. Tumour-bearing Balb/c mice can be cured of their tumours with multiple doses of virus whereas nude mice cannot (K Parato and C Lemay, personal communication). This implies that T cells are important and necessary for VSV-mediated tumour destruction, probably because VSV infection induces inflammation, immune cell recruitment and activation, resulting in a robust anti-tumoural immune response. The role of VSV in the induction of a viable anti-tumour response is important especially since the majority of tumours have developed several means of immune evasion. These mechanisms include loss of tumour antigen, alteration of MHC I expression, defective death receptor signaling, production of immunosuppressive cytokines (e.g. IL-10 and TGFβ which promote a Th2 response), lack of co-stimulation of

immune cells and the induction of immunosuppressive regulatory T cells (Tregs) (Kim et al, 2006). As well, tumour cells are able to secrete factors such as soluble MICA, Fas and MHC I molecules to act as decoys, evading cytolysis, apoptosis and inhibiting proper activation of immune cells (Kim et al., 2006). In addition, tumour cells can attack immune cells by up-regulating surface FasL expression, resulting in apoptosis of CTLs (Ochsenbein et al., 1999), as well as up-regulating TCRζ resulting in T cell apoptosis (Kim et al., 2006). Paradoxically, anti-tumoral immunity can be a double-edged sword; while CTLs can target emerging tumours, it also provides selective pressure which results in the evolution of immune edited tumours (Chan and Housseau, 2007). Inflammation caused by leukocytes in tumours can also cause tumour progression in several types of cancers including those of hepatic, gastrointestinal and gynecological origins (Kim et al., 2006). For example, reactive oxygen species (ROS), nitrogen products, prostaglandins and pro-inflammatory cytokines all promote tumour growth by inducing mutations that result in resistance to apoptosis and angiogenesis, as well as inhibiting T cell proliferation and the production of Th1 cytokines (Kim et al., 2006). However, Yamshchikov et al., (2005) described the case of a melanoma patient whose tumour displayed ongoing immune escape but whose T cells also adapted and were able to target the emerging tumour, indicating that although tumours are capable of mutating in response to an anti-tumour

response, the T cell repertoire is also able to evolve and control tumour growth.

VSV infection of tumours may be able to cause sufficient inflammation to counteract the immunosuppressive nature of tumours. Although most tumour types are deficient in their IFN response, neighboring normal cells may be able to provide the danger signal that can activate infiltrating APCs and lymphocytes, causing them to be cytotoxic. It is also possible that VSV infection recruits APCs such as pDCs, causing them to secrete IFNα, which has been shown to activate tumour-associated DCs and T cells (Kim et al., 2006). Activated immune cells can in turn stimulate IFNγ production and Th1 cytokines, overcoming the immunosuppressive tumour environment. In addition to causing generalized inflammation, VSV can be armed with cytokines that recruit and activate lymphocytes such as IL-12, lymphotoxin-like inducible protein that competes with glycoprotein D for binding herpesvirus entry mediator on T cells (LIGHT) (Shaikh et al., 2001), or granulocyte macrophage colony-stimulating factor (GM-CSF).

Rationale

There are many barriers to oncolytic viral therapy, the most important of which is the presence of neutralizing antibodies. B cells strongly inhibit VSV infection through the production of high affinity, long-lived neutralizing antibodies which limit dissemination of the virus and prevent multiple infections, thus retarding the efficacy of treatment. The use of B cell-deficient mice will allow us to clearly discern the role of B cells in limiting the efficacy of OVs.

Hypothesis

B cells are a major barrier to efficacy of VSV. The absence of B cells will allow for better virus infection of tumours which will ultimately lead to enhanced efficacy.

Aim

The aim of this study was to investigate the efficacy of VSV oncolysis in a B cell-deficient mouse (BCDM) model.

Objectives

1. Characterize VSV infection in B cell-deficient mice as compared to wild-type mice.

2. Determine if antibodies are the only barrier to multiple infections with VSV in virus-educated mice.

3a. Determine if the absence of antibodies correlates with enhanced efficacy with VSV.

3b. Is efficacy reversed in the presence of antibodies?

4. Determine if improved efficacy correlates with better survival.

2. Materials and Methods

2.1 Mice

6-8 week old female C57Bl/6 mice were obtained from Jackson Laboratories (Bar Harbour, Maine, USA) and housed according to Canadian Council on Animal Care rules and regulations. Food and water were supplied *ad libitum.*

B cell-deficient mice (muMT, C57Bl/6 background) were obtained from Jackson Laboratories and housed in a pathogen-free environment. Breeding pairs were used to establish a colony from which both male and female mice were used at 6-8 weeks of age.

Mice lacking the PKR gene ($PKR^{-/-}$) were created in our lab by Ninan Abraham (Abraham et al., 1999). Mice were bred and housed in a pathogen-free environment. Food and water were supplied *ad libitum.*

2.2 Genotyping

Genotype of mice was confirmed using PCR of genomic DNA extracted from tail snips. Forward and reverse primers were 5'-CCGTCTAGCTTGAGCTATTAGG -3' and 5'-GAAGAGGACGATGAAGGTGG-3' respectively. The mutant allele results in a 1500bp product, while the wild-type allele results in a 210bp product. PCR reactions were prepared as follows: 5-20ng DNA, 1X PE Buffer II (10mM Tris-HCl pH 8.3, 50 mM KCl), 4mM MgCl2, 0.2mM dNTP, 0.5μM forward and reverse primers, 10U/μL Taq Pol (Invitrogen, Carlsbad, California, USA), 1.15% total volume 60% sucrose/50mM Cresol Red sodium salt (Sigma-Aldrich, St Louis, Missouri, USA) and ddH_2O to a total volume of 12μL per sample. Fragments were resolved on a 1% agarose gel.

2.3 Flow cytometry

Peripheral blood samples were obtained via the saphenous vein of C57BL/6 and muMT mice. 50uL of blood was incubated at a dilution of 1/10 with anti-mouse CD45-PE-Cy5 (Cedarlane, Burlington, Ontario, Canada), or CD22-FITC (Cedarlane) and CD19-PE-Cy5 (Cedarlane) for 1 hour at 4° C. Samples were then incubated with 500μL 1X RBC Lysis Buffer (BioLegend, San Diego, California, USA) in de-ionized water for a maximum of 6 minutes at 4°C, or until solution was clear. Lysis buffer was neutralized by the addition of 4.5mL phosphate-buffered saline (PBS)

(Hyclone) at room temperature. Samples were centrifuged at 1500 rpm for 5 minutes and washed with PBS three times before being re-suspended in 500μL PBS for flow cytometric analysis. All samples were gated for the $CD45^+$ lymphocyte population on the Beckmann Coulter EPICS XL flow cytometer (Beckman Coulter, Fullerton, California, USA).

2.4 Cell Culture

Vero and B16 murine melanoma cells were grown in Dulbecco's Modified Eagle Media (DMEM) (Hyclone, South Logan, Utah, USA) supplemented with 10% fetal calf serum (FCS) (GibcoBRL, Carlsbad, California, USA), and maintained at 37°C and 5% CO_2. B16F10-LacZ (courtesy of Dr Ann Chambers, University of Western Ontario) cells were grown in alpha-MEM (Hyclone) supplemented with 10% FCS and maintained at 37°C and 5% CO_2.

2.5 Viruses

The AV3 recombinant strain of VSV (Indiana strain) containing an attenuating deletion of methionine 51 of the matrix protein and a transgene encoding green fluorescent protein (GFP), red florescent protein (RFP) or firefly luciferase (VSVΔ51-GFP, -RFP, -fLuc) was propagated on Vero cells (American Type Culture Collection, Manassas, Virginia, USA). Virions were purified from cell culture supernatants passed through a 0.22 μm Steritop filter (Millipore) and centrifuged at 14 000 rpm for 90

minutes. Viral pellets were re-suspended in PBS and aliquots stored at -80°C.

JX-594 is a vaccinia virus (Western Reserve) expressing GM-CSF that was developed by Jennerex Therapeutics and is currently being tested in clinical trials.

2.6 VSV plaque assay

Serial dilutions of VSV were prepared in serum-free media to a final volume of 1mL. 100uL of diluted virus was applied to Vero monolayers and the infection allowed to proceed for 1 hour at 37°C and 5% CO_2. 3mL of agarose overlay (1:1 1% agarose in ddH_2O:2X DMEM+20% FCS at 42°C) were then added to each well. When the overlay had solidified, plates were incubated overnight at 37°C and 5% CO_2 and plaques counted the following day.

2.7 Plasma neutralizing antibody assay

Peripheral blood samples were collected at different time points after VSV-wt and VSVΔ51 infection of muMT and C57Bl/6 mice. Two-fold serial dilutions of plasma were incubated with $2x10^3$ pfu VSVΔ51-GFP in a total volume of 100μL serum-free media for 1 hour at 37°C and then applied to Vero monolayers in a 96-well plate for 1 hour at 37°C. 100μL DMEM supplemented with 10% serum was then added to each well and plates were incubated for 24 hour at 37°C and 5% CO_2. Wells were examined for GFP expression and cytopathic effects. The highest dilution

at which plasma prevented VSV infection was taken as the neutralizing antibody titer.

2.8 Western blot detection of VSV-reactive antibodies

Western blot analysis was used to determine the presence of VSV-reactive antibodies in mice. Plasma samples from mice infected with VSV ($5x10^8$ plaque forming units (pfu), intravenously (i.v.) in C57Bl/6, $PKR^{-/-}$ and muMT) were used to probe for VSV reactivity at Day 14 post-immunization. VSV was suspended in 4% SDS sample buffer, and samples were analyzed by SDS-PAGE electrophoresis on a 12% acrylamide gel and transferred to a nitrocellulose membrane. After blocking for 1 hour with 5% skim milk in Tris-buffered saline (TBS-T; 150mM NaCl, 10mM Tris-HCl pH 7.5, 0.1% Tween-20), the membrane was placed in a miniblotter apparatus (Immunetics, Boston, Massachusetts, USA). Mouse plasma samples were diluted in 5% milk in TBS-T at a dilution of 1:100 and were applied to the membrane for 1 hour at room temperature, followed by three five-minute washes with TBS-T. The membrane was incubated with an HRP-conjugated secondary antibody for 45 minutes at room temperature. Following three five-minute washes in TBS-T, the membrane was incubated with a chemiluminescent substrate (Pierce ECL Western Blotting Substrate, Thermo Fisher) for five minutes and exposed to X-ray film. Films were developed on the SRX-101A processor (Konica Minolta, Japan).

2.9 Mouse Interferon-α ELISA

Plasma samples were collected prior to, and at 6 and 24 hours after VSV treatment via saphenous vein bleeds. Samples and controls were prepared according to manufacturer's instructions (Mouse Interferon-α ELISA kit, R&D Systems, Minneapolis, Minnesota, USA). Absorbance was read at 450nm on an MRX plate reader (Dynex Technologies, Chantilly, Virginia, USA).

2.10 Survival of muMT and C57Bl/6 mice after VSV-wt and VSVΔ51 infection

C57Bl/6 and muMT mice received $5x10^8$ pfu VSV-wt, VSVΔ51 or PBS i.v. and were monitored for any signs of VSV-induced illness. This included weight loss, lethargy, piloerection and in the case of wild type VSV infection, hind limb paralysis. At the time of loss-of-wellness endpoint, mice were euthanized and the brains removed and titered for the presence of VSV.

2.11 Ability of VSV to infect tumours of pre-immunized C57Bl/6 and muMT mice

Mice were infected with $5x10^8$ pfu VSVΔ51-RFP i.v. and after 6 weeks had B16 tumour cells implanted bilaterally ($3x10^5$, subcutaneously). Upon development of palpable tumour masses (Day 11), mice received $5x10^8$ pfu VSVΔ51-GFP and were euthanized two days later. At the time of

euthanasia, tumours were removed and one tumour was snap-frozen in dry ice for virus titering and the other fixed in 10% formalin for paraffin embedding, sectioning and Hematoxylin and Eosin (H&E) staining. Titered tumours were visualized by fluorescent microscopy for the presence or absence of GFP-positive plaques.

2.12 Detecting VSV infection in tissues

Tumors and organs were removed from euthanized mice and homogenized in 1mL of PBS. VSV infection of tissues was determined by plaque assay (described in section 2.5).

2.13 Luciferase assay

The lungs of tumour-bearing and tumour-free C57Bl/6 and muMT mice treated with five doses of $5x10^8$ pfu VSVΔ51 and a final sixth dose of $5x10^8$ pfu VSVΔ51-fluc i.v. were assayed for luciferase expression. Mice were euthanized and the lungs removed, snap-frozen and stored at -80°C until the time of processing. At the time of processing, lungs were thawed and four lobes used to assay luciferase activity. Tissues were then homogenized in 1mL PBS and half the volume used for downstream processing. Homogenates were lysed and processed according to the manufacturer's instructions (Dual-luciferase reporter assay system, Promega, Madison, Wisconsin, USA). Luminescence was assayed within 5 minutes of adding the substrate. All samples were read in triplicate on a

Lumat LB 9507 luminometer (EG&G Berthold, Gaithersburg, Maryland, USA).

2.14 B16F10-LacZ metastatic lung tumour model

Mice received $3x10^5$ B16F10-LacZ cells i.v. at Day 0. They were then treated with six doses of either PBS, $5x10^8$ pfu VSVΔ51 or $1x10^7$ pfu JX-594 i.v. in a two-week span. Mice were euthanized on Day 14 post-tumour injection, lungs were harvested and stained for LacZ (described in section 2.15) and the number of tumours on each lobe quantified. The efficacy of each virus in diminishing tumour burden in both C57Bl/6 and muMT mice was determined relative to untreated controls. Peripheral blood from PBS and VSV treated mice was sampled and the neutralizing antibody titers determined as explained in sections 2.3 and 2.7).

2.14a *Metastatic lung model and survival*

C57Bl/6 and muMT mice received B16F10-LacZ cells and were treated with PBS, VSV or JX-594 as described in section 2.14. Mice were euthanized when they displayed signs of disease that resulted in loss-of-wellness including weight loss, labored respiration and fatigue. muMT mice were also euthanized upon development of tumour masses at various locations including the face and back. Survival was marked as the length of time between the last treatment and death of the animal.

2.14b *Metastatic lung model after passive serum transfer*

C57Bl/6 mice were immunized with $5x10^8$ pfu VSVΔ51 i.v. and left to develop antibodies for four-to-five weeks and some animals remained unimmunized as a source of naïve plasma. Mice were terminally bled under anesthetic and plasma stored at 4°C until use. muMT mice received 250uL of plasma (naïve or immune) i.p. Mice were given B16F10-LacZ cells i.v. and treated as described in section 2.14.

2.14c *Metastatic lung model in pre-immunized mice*

Mice (C57Bl/6 and muMT) were immunized with $5x10^8$ pfu VSVΔ51 or PBS i.v. Four to five weeks later, mice received B16F10-LacZ cells i.v. and were treated as described in section 14.

2.15 LacZ staining of B16F10-LacZ lung tumours

Lungs were washed in 0.1M phosphate buffer (0.1M sodium phosphate monobasic, 0.1M sodium phosphate dibasic, pH 7.3) upon removal from the mice and stored in phosphate buffer on ice until the next step. Lungs were then placed in fixative solution (5mM EGTA pH 7.3, 2mM $MgCl_2$, 0.02% gluteraldehyde in 0.1M phosphate buffer) for 15-20 minutes at room temperature. Lungs were then washed twice with wash buffer (2mM $MgCl_2$, 0.01% deoxycholate, 0.02% Nonidet-P40 in 0.1M phosphate buffer) for 10 minutes per wash at room temperature. Staining was carried out by incubating lungs in X-gal staining solution (1mg/mL X-gal stock [in dimethylformamide], 5mM potassium ferrocyanide, 5mM potassium

ferricyanide in wash buffer) overnight at 37°C. Lungs were transferred to wash buffer and stored at 4°C overnight for staining to intensify and then transferred to 10% buffered formalin for counting and long-term storage.

3. Results

3.1 Genotyping

All muMT mice were confirmed to be homozygous B cell knock-outs as indicated by the presence of the 1500bp fragment that includes the neomycin resistance cassette which disrupts the membrane spanning domain of the B cell receptor (Kitamura et al., 1991). The DNA of C57Bl/6 yielded as expected, a 210bp fragment corresponding to a wild-type genotype (Figure 2).

3.2 B cell flow cytometry of peripheral blood

Two markers of mature B cells, CD19 and CD22, were used to determine the B cell content in the peripheral blood of both muMT and C57Bl/6 mice. Gating on the $CD45^+$ population (lymphocytes), it was noted that the blood of muMT is wholly devoid of mature B cells whereas that of C57Bl/6 mice produced the normal proportion of circulating B cells (Figure 3)

3.3 Antibody response

Plasma from muMT, C57Bl/6 and $PKR^{-/-}$ mice immunized with VSV was examined for the presence or absence of VSV-reactive antibodies by Western blot analysis. $PKR^{-/-}$ mice are known to have a diminished antiviral immune response and were used as a tool to compare the antibody response of normal C57Bl/6 and that of immune compromised mice, including muMT and $PKR^{-/-}$ mice. The results confirm that plasma of muMT mice completely lacks anti-VSV antibody, whereas both C57Bl/6 and $PKR^{-/-}$ have robust levels of anti-VSV antibodies (Figure 4).

3.4 Anti-VSV neutralizing antibody response

As further confirmation of the lack of VSV-reactive antibodies in muMT mice, peripheral blood from mice immunized with $5x10^8$ pfu VSV-wt and VSVΔ51 i.v. was sampled at Day 0, 4, 11, 18, 25 and 30. muMT mice that received VSV-wt reached endpoint between Day 9 and 11 and so were excluded from the later time points. A neutralizing antibody assay showed increasing titers of anti-VSV antibodies in C57Bl/6 mice treated with VSV, but that there were no anti-VSV neutralizing antibodies in the plasma of any of the muMT mice (Figure 5).

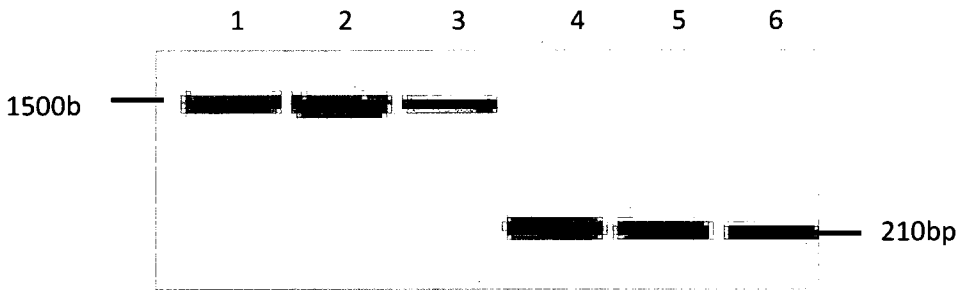
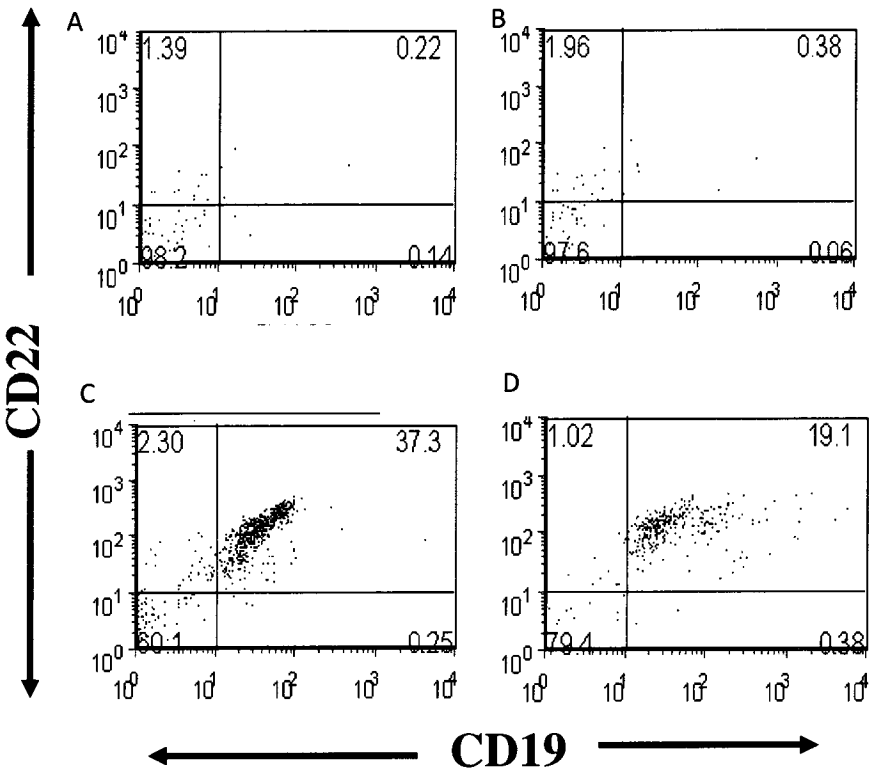

1
2
3
4
5
6
1500b
210bp

Figure 2: Homozygous null mutation for *muMT* gene in founder B cell deficient mice. PCR of genomic DNA shows the presence of neomycin insertion cassette within Exon 1 of the *muMT* gene corresponding to 1500bp in all parental muMT mice (lanes 1-3). C57Bl/6 mice (lanes 4-6) have the wild-type Exon 1 corresponding to 210bp.


A
1.39
0.22
98.2
0.14
B
1.96
0.38
97.6
0.06
C
2.30
37.3
60.1
0.25
D
1.02
19.1
79.4
0.38
CD22
CD19

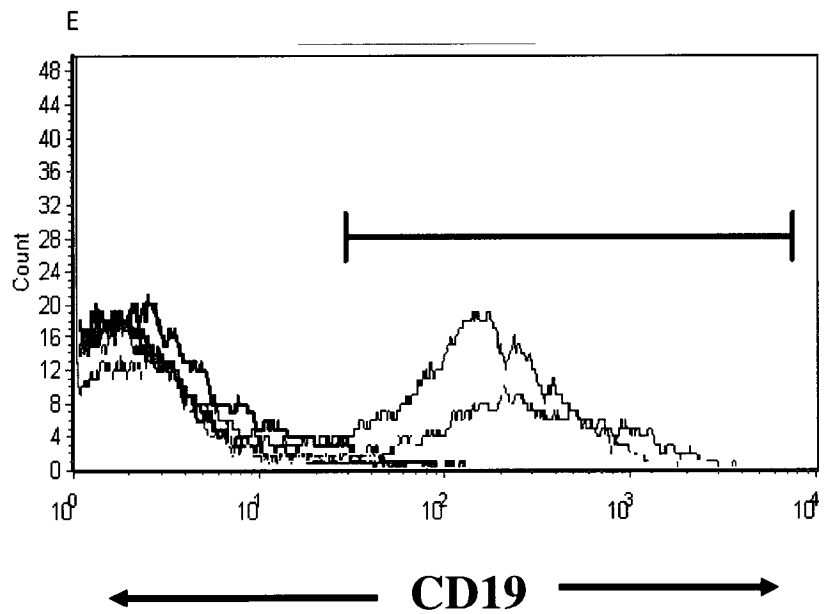

E
Count
CD19

Figure 3: muMT mice lack mature circulating B cells. Double-staining of two mature B cell markers, CD22 (FL1) and CD19 (FL4), on lymphocytes isolated from two different peripheral blood samples of muMT mice (panels A and B) and two different C57Bl/6 mice (panels C and D). All samples are gated on a $CD45^+$ population (not shown). Panel E shows histograms of the same samples where black, blue and red lines correspond to double staining of muMT lymphocytes, and green, purple and yellow lines correspond to double staining of C57Bl/6 lymphocytes (samples from three different mice are shown).

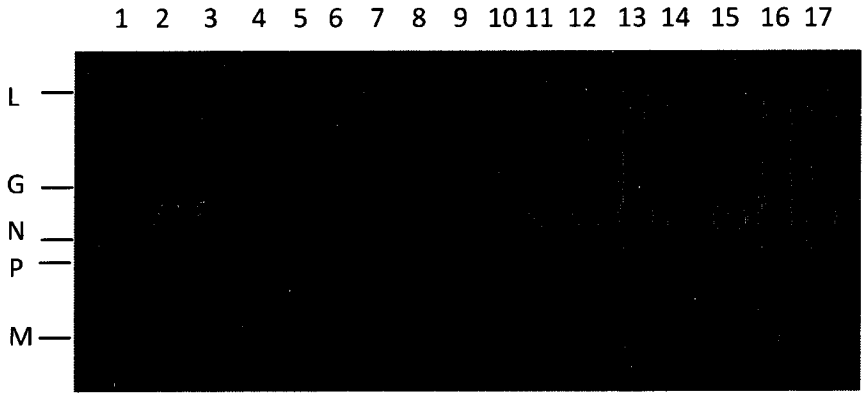

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
L
G
N
P
M

Figure 4: VSV-immunized muMT mice lack antibodies. Plasma isolated from peripheral blood of VSV-immunized ($5x10^8$ pfu, i.v.) C57Bl/6 (lane 3), muMT (lanes 4-10) and $PKR^{-/-}$ (lanes 11-17) mice was used to probe for VSV by Western blot. Lane 1 is a naïve plasmacontrol and in lane 2 a monoclonal mouse anti-VSV-G antibody was used. n=3.

3.5 Comparing plasma IFNα levels in response to VSV infection in muMT and C57Bl/6 mice

C57Bl/6 and muMT mice received $5x10^8$ pfu VSVΔ51 i.v. and blood was sampled at 0, 6 and 24 hours, and again 14 days later to examine the ability of muMT mice to produce IFNα in response to VSV infection. At 6 hours, plasma IFNα levels in C57Bl/6 mice reached an average of 733.3pg/mL, while those of muMT mice were at an average of 264.2pg/mL. By 24 hours, IFNα levels had dropped to 117.4pg/mL in C57Bl/6 and were undetectable in the plasma of muMT mice (Figure 6). Neither group of mice had any detectable plasma levels of IFNα at 14 days post-VSV infection (data not shown). It is possible that levels of IFNα in muMT mice peaks after the 6 hour time point, but this has not been validated.

3.6 Survival of VSV challenge

Upon challenge of mice with VSV-wt, only muMT mice showed any signs of CNS disease (including symptoms of hind limb or general paralysis, anorexia, fatigue, lack of mobility) and reached the loss-of-wellness endpoint between days 9 and 11 post-infection, whereas C57Bl/6 mice showed no signs of disease (Figure 7). All mice treated with VSVΔ51 survived disease-free for >7 months. Upon reaching endpoint, the brains of muMT mice treated with VSV-wt were removed and frozen for VSV titering. At 30 days post-infection, mice from each treatment group were

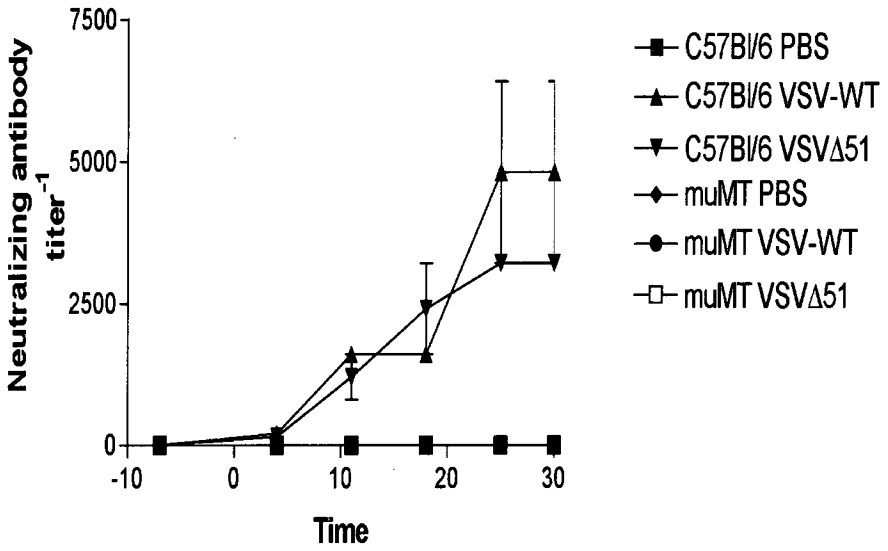

7500
5000
2500
0
-10
0
10
20
30
Time
Neutralizing antibody titer-1
C57Bl/6 PBS
C57Bl/6 VSV-WT
C57Bl/6 VSVΔ51
muMT PBS
muMT VSV-WT
muMT VSVΔ51

Figure 5: Anti-VSV neutralizing antibodies are present in VSV-immunized C57B/6 mice but not in muMT mice. Plasma isolated from peripheral blood samples at days -7, 4, 7, 11, 18, 25 and 30 post-VSV infection ($5x10^8$ pfu, i.v.) was analyzed for the presence of anti-VSV antibodies by neutralizing antibody assays. Means +/- SEM are shown. n=3.

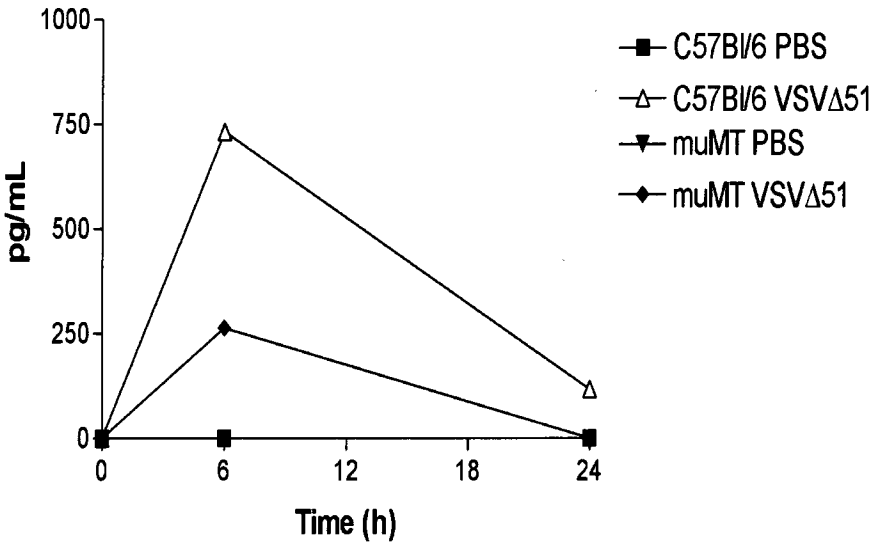

1000
750
500
250
0
pg/mL
0
6
12
18
24
Time (h)
C57Bl/6 PBS
C57Bl/6 VSVΔ51
muMT PBS
muMT VSVΔ51

Figure 6: Plasma IFNα levels are higher in VSV-immunized C57Bl/6 mice than in muMT mice. Plasma isolated at 0, 6 and 24 hours post-VSVΔ51 infection ($5x10^8$ pfu, i.v.) was analyzed for IFNα levels by ELISA. Means are shown. n=3

euthanized and their brains removed for titering as well. Only the brains of muMT mice that died of VSV-wt infection contained any infectious viral particles, to the order of $4x10^4$ pfu/mL or $1x10^2$ pfu/mg (Figure 8).

3.7 Improved infection of tumours with VSV in pre-immunized mice lacking B cells

Pre-immunity of mice to VSV has been shown to be a limiting factor in the ability of secondary doses of virus to infect tumours. For this reason, I sought to determine if the B cells (and therefore antibodies) were the cause of this limitation. C57Bl/6 and muMT mice were both immunized with a dose of VSVΔ51-RFP to distinguish it from the second challenge with VSVΔ51-GFP. All tumours removed from muMT mice (naïve and pre-immunized) contained VSVΔ51-GFP but not VSVΔ51-RFP, whereas only the tumours of naïve C57Bl/6 contained any virus. VSVΔ51-GFP titers were $7.9x10^2$ pfu/mg and $9.23x10^1$ pfu/mg for pre-immunized and naïve muMT mice, respectively, and 1.6x10 pfu/mg for naïve C57Bl/6 mice. No virus was detectable in the tumours of pre-immunized C57Bl/6 mice (Figure 9). These results indicate that B cells, and in turn antibodies, are responsible for limiting virus infection of tumours after prior exposure to the virus. H&E staining of subcutaneous tumours showed no difference in the amount of dead tissue in any of the treatment groups, although tumours of muMT mice contained more "bloody" areas containing intact red blood cells than those of C57Bl/6 mice (Figure 10).

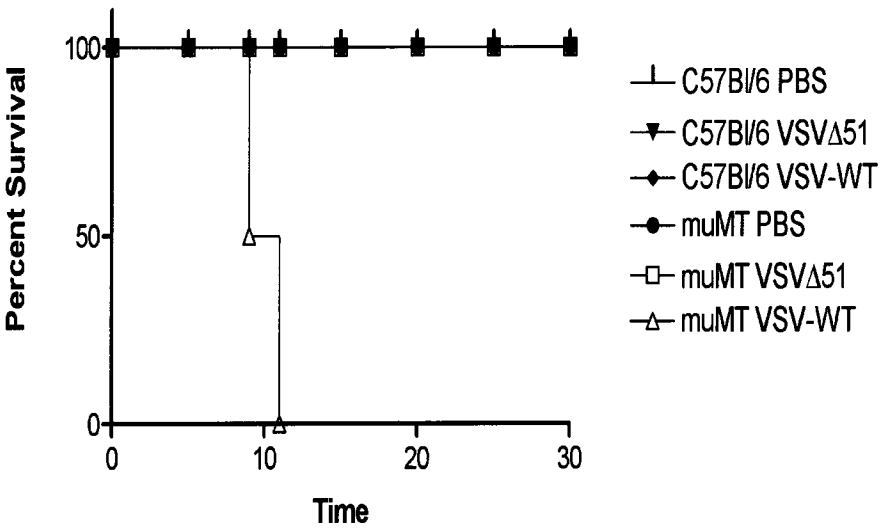

Percent Survival
100
50
0
0
10
20
30
Time
C57Bl/6 PBS
C57Bl/6 VSVΔ51
C57Bl/6 VSV-WT
muMT PBS
muMT VSVΔ51
muMT VSV-WT

Figure 7: B cells are required to protect against lethal VSV-WT but not VSVΔ51 challenge. muMT mice infected with VSV-WT ($5x10^8$ pfu i.v.) reached endpoint due to CNS disease at 9 and 11 days post-infection. n=2.

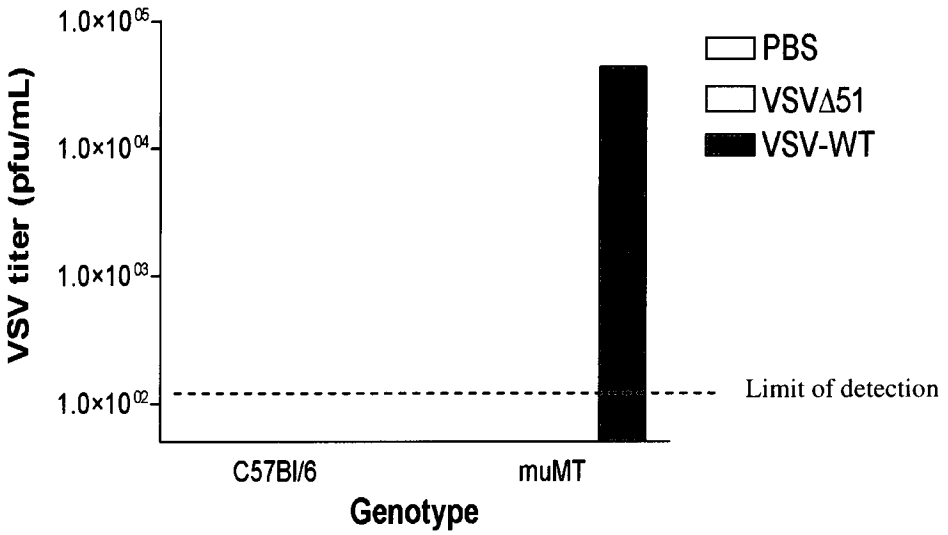

1.0×10^05
1.0×10^04
1.0×10^03
1.0×10^02
VSV titer (pfu/mL)
PBS
VSVΔ51
VSV-WT
Limit of detection
C57Bl/6
muMT
Genotype

Figure 8: VSV-WT is detectable in the brains of muMT mice. Upon reaching loss-of-wellness endpoint, muMT mice were euthanized and their brains removed for VSV titering (day 9 and 11). At Day 30, two mice each of the surviving treatment groups (PBS and VSVΔ51 ($5x10^8$ pfu), C57Bl/6 and muMT) were euthanized and their brains removed for VSV titering. n=2.

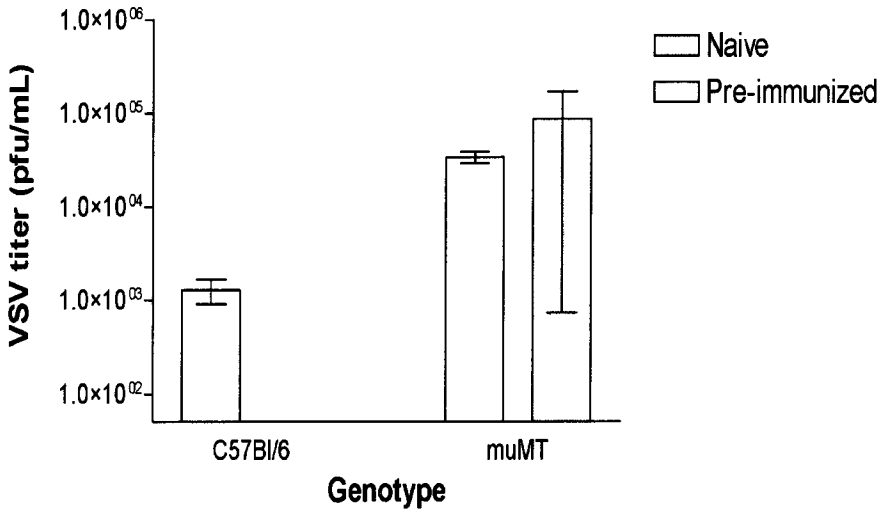

1.0×10^06
1.0×10^05
1.0×10^04
1.0×10^03
1.0×10^02
VSV titer (pfu/mL)
C57Bl/6
muMT
Genotype
Naive
Pre-immunized

Figure 9: Improved infection of tumours with VSV in pre-immunized mice lacking B cells. C57Bl/6 and muMT were immunized with a single i.v. dose of VSVΔ51-RFP ($5x10^8$ pfu), or left as PBS controls. After six weeks, mice were seeded with $3x10^5$ B16 cells subcutaneously. Upon development of palpable tumours (~11 days), mice were challenged with a single i.v. dose of VSVΔ51-GFP ($5x10^8$ pfu), euthanized 48 hours later and their tumours excised for titering. Means +/- SEM are shown. n=5.

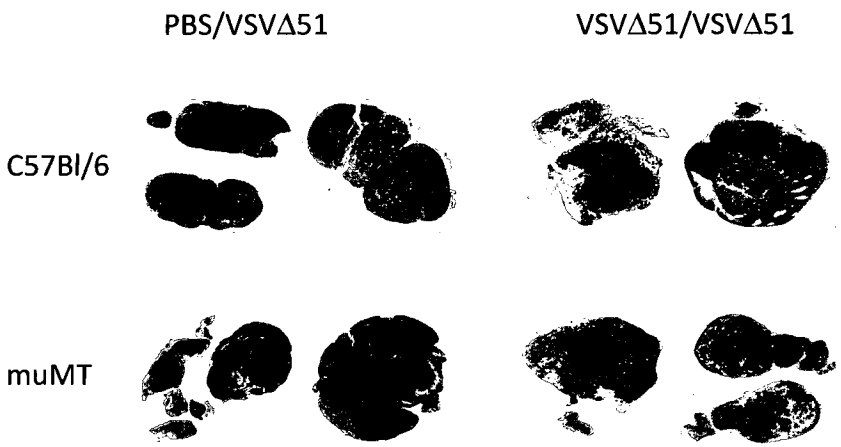

PBS/VSVΔ51
VSVΔ51/VSVΔ51
C57Bl/6
muMT

Figure 10: H&E staining of subcutaneous tumours in naïve and pre-immunized C57Bl/6 and muMT mice. Subcutaneous tumours of naïve (PBS/VSVΔ51), or pre-immunized (VSVΔ51/VSVΔ51) C57Bl/6 and muMT mice were formalin-fixed and paraffin-embedded. Sections were then stained with H&E and the proportion of necrotic or apoptotic tissue in each group compared. n=5.

3.8 Comparing the efficacy of VSV and JX-594 treatment of B16F10-LacZ lung tumours in C57Bl/6 and muMT mice

I next sought to determine whether the presence or absence of B cells correlated with improved efficacy of virus treatment in a metastatic model of lung cancer. This model allows the determination the efficacy of treatment based on the number of B16F10-LacZ tumours in the lungs of treated animals. This study showed that there was no significant difference in the number of lung metastasis in mice treated with JX-594, but that there was a significant decrease in the number of lung tumours in muMT mice treated with VSVΔ51 (Figure 11). The absence of B cells does not improve efficacy of JX-594 treatment in this model, but does correlate with much increased efficacy of VSV treatment. Neutralizing antibody titers in VSV-treated C57Bl/6 mice reached an average of 1200 units by Day 7, but were undetected in untreated C57Bl/6 and in all muMT mice (data not shown).

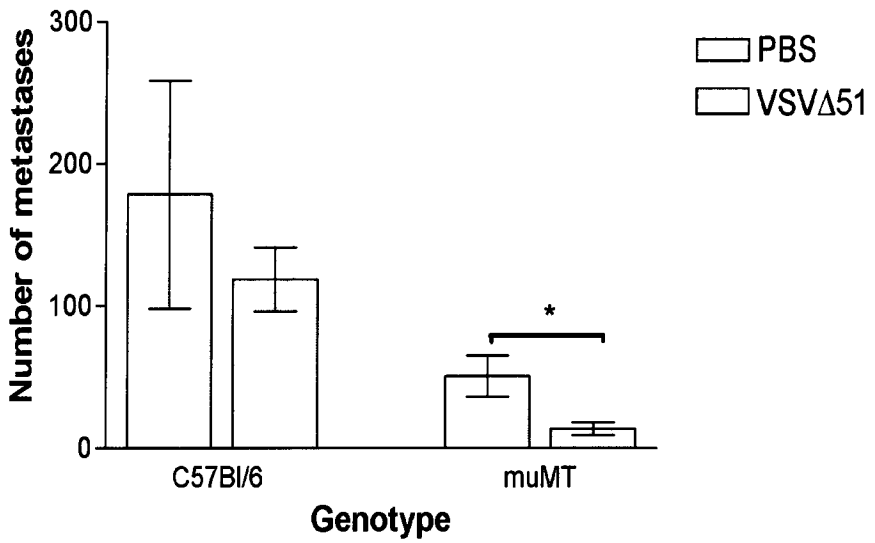
Concurrent with this experiment, we sought to determine if the last dose of virus was in fact successfully infecting tumours. To this end, three mice each of tumour-bearing C57Bl/6 and muMT mice were given five doses of VSVΔ51, and the sixth dose was VSVΔ51-luc. Lungs of these mice were assayed for luciferase expression and activity normalized against luciferase activity in the lungs of similarly treated, tumour-free C57Bl/6. Our results show that there is no significant difference between the extent of luciferase activity in the tumour-bearing lungs of C57Bl/6

and muMT mice, but that the average luciferase activity in the lungs of muMT mice was higher than that of C57Bl/6 mice (Figure 12).

3.9 Pre-existing immunity does not significantly alter the efficacy of VSVΔ51 treatment

In order to establish a causative role for B cells in limiting VSV's oncolytic activity, we studied the effect of restoring anti-VSV immunity on efficacy of treatment. This was investigated in two ways: C57Bl/6 and muMT were immunized with VSVΔ51 prior to tumour injections and virus treatment, and alternately, muMT mice received passive transfer of immune plasma from donor C57Bl/6 mice. Results show that naïve and pre-immunized C57Bl/6 mice both had insignificant reduction in the number of tumours upon virus treatment as would be expected from the presence of antibodies. However, of note, none of the muMT mice (naïve, pre-immunized or passively immunized) showed a significant reduction in tumour burden upon virus treatment (Figure 13). These results are surprising as previous observations showed more significant reduction in the number of tumours in treated animals. This may be attributed to a more even tumour growth rate and distribution in previous experiments that resulted in more statistical significance that may not have actually been due to the direct oncolytic activity of VSV. It is important to note that there were no detectable levels of anti-VSV neutralizing antibodies in

A


300
200
100
0
Number of metastases
C57Bl/6
muMT
Genotype
*
PBS
VSVΔ51

B

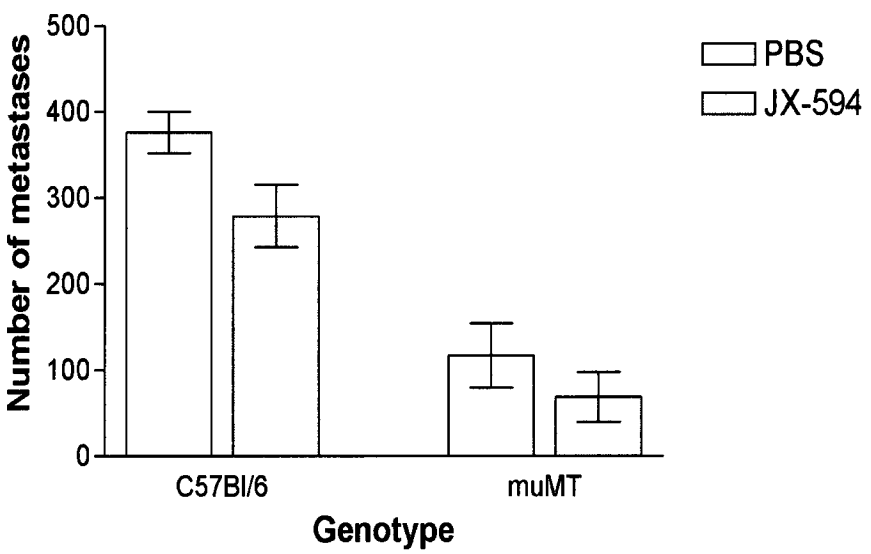
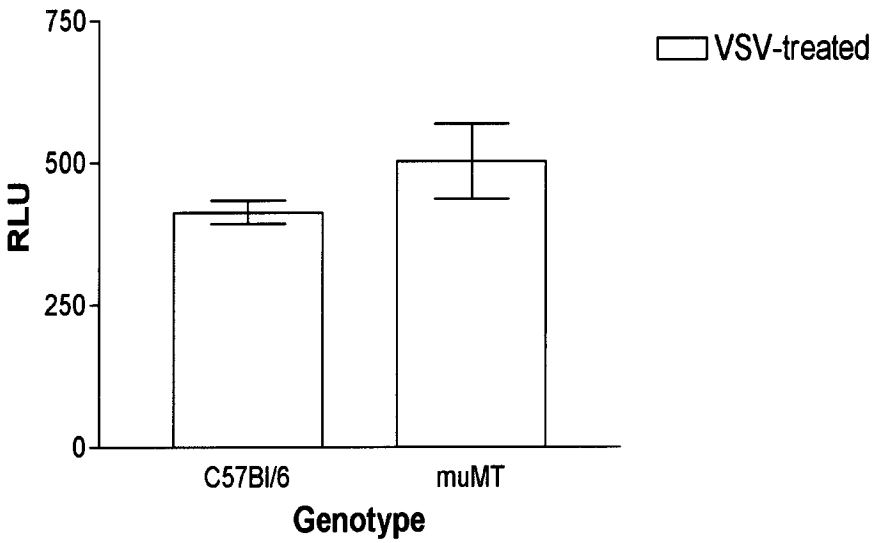

500
400
300
200
100
0
Number of metastases
C57Bl/6
muMT
Genotype
PBS
JX-594

Figure 11: B cells limit the efficacy of VSVΔ51 treatments of B16F10-LacZ tumours, but have no significant effect on the efficacy of JX-594 treatment. C57Bl/6 and muMT mice were seeded with $3x10^5$ B16F10-LacZ cells i.v. and treated with six i.v. doses of virus ($5x10^8$ pfu VSVΔ51, panel A, or $1x10^7$ pfu JX-594, panel B) over a two week period. * denotes $P<0.01$. Means +/- SEM are shown. n=5.

A


750
500
250
0
RLU
VSV-treated
C57Bl/6
muMT
Genotype

B

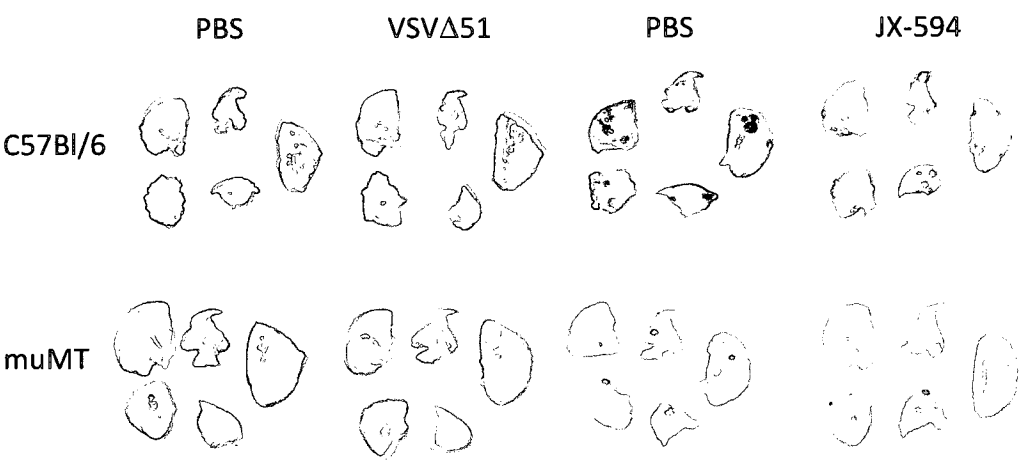

PBS
VSVΔ51
PBS
JX-594
C57Bl/6
muMT

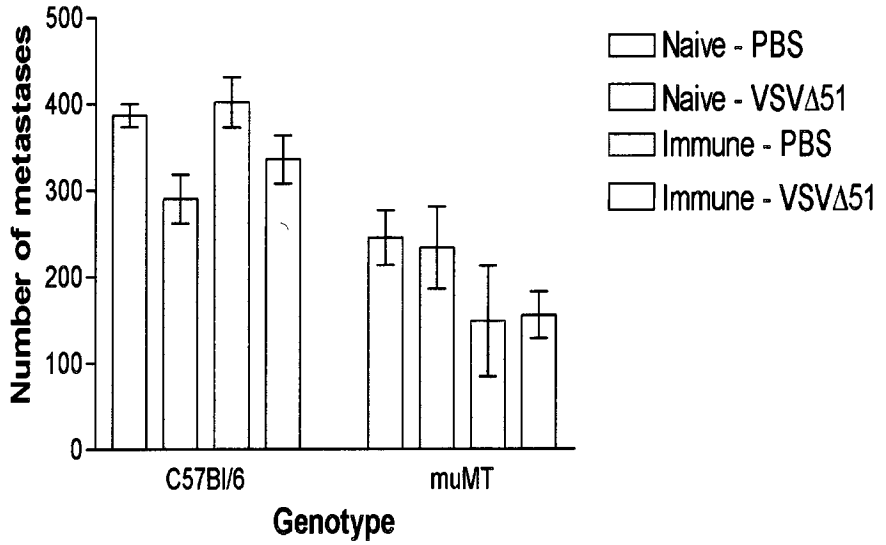
Figure 12: Luciferase activity in the tumour-bearing lungs of C57Bl/6 and muMT mice treated with VSVΔ51-fLuc does not differ significantly in the presence or absence of B cells. Tumour-bearing C57Bl/6 and muMT mice were each treated with five i.v. doses of VSVΔ51 ($5x10^8$ pfu), followed by one i.v. dose of VSVΔ51-fLuc ($5x10^8$ pfu). Mice were euthanized the next day and the lungs assayed for luciferase activity (panel A). Means +/- SEM are shown. Pictures representative of the tumour-bearing lungs of C57Bl/6 and muMT mice (panel B). All mice received $3x10^5$ B16F10-LacZ cells followed by six treatments of either PBS, $5x10^8$ pfu VSVΔ51 i.v. or $1x10^7$ pfu JX-594 i.v. over two weeks. Mice were euthanized ad Day 14 and lungs stained for LacZ expression. n=3.

the plasma of muMT mice (data not shown) that received immune plasma transfer so the lack of effect on the efficacy of virus treatment can be attributed to a lack of immunity in this case.

3.10 Ability of VSV to improve survival in a B16F10-LacZ model of lung cancer in C57Bl/6 and muMT mice

Whether or not virus treatment correlated with enhanced survival of treated animals was investigated next. Lung tumour-bearing mice were treated as described previously, with PBS, VSVΔ51 or JX-594. Survival time was calculated as the time between the last injection and the loss-of-wellness endpoint. Survival times are summarized in Figure 14. Virus treatment of each group extended survival times but this did not reach statistical significance, although there was a trend for increased survival in muMT mice relative to C57Bl/6, and for virus-treated mice versus untreated controls. VSVΔ51 treatment of muMT mice resulted in the most extended survival time of 24 days. On average, muMT had improved survival over C57Bl/6 mice (both treated and untreated).

A


500
400
300
200
100
0
Number of metastases
C57Bl/6
muMT
Genotype
Naive - PBS
Naive - VSVΔ51
Immune - PBS
Immune - VSVΔ51

B

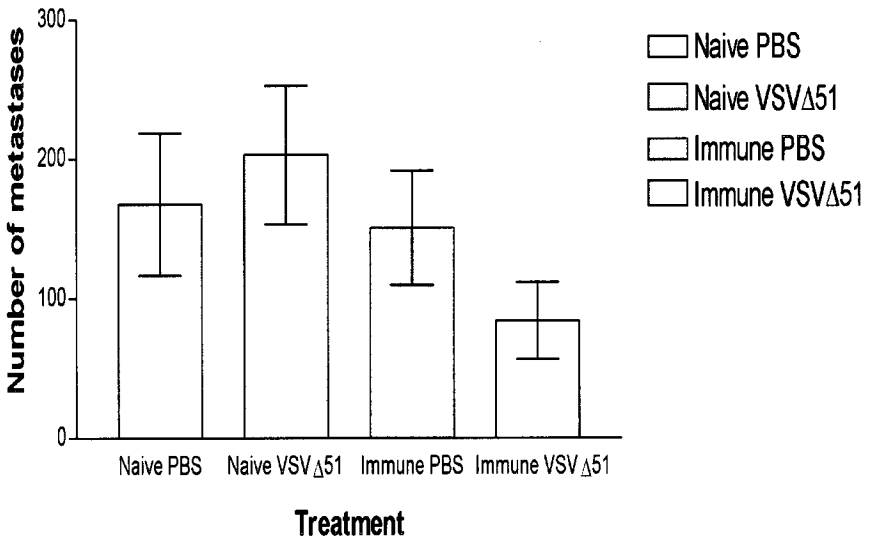

300
200
100
0
Number of metastases
Naive PBS
Naive VSVΔ51
Immune PBS
Immune VSVΔ51
Treatment
Naive PBS
Naive VSVΔ51
Immune PBS
Immune VSVΔ51

Figure 13: Pre-existing immunity to VSVΔ51 does not affect the efficacy of treatment. C57Bl/6 and muMT were immunized with $5x10^8$ pfu i.v. VSVΔ51 four weeks prior to being seeded with $3x10^5$ B16F10-LacZ tumours i.v. and treated with six doses of virus over a two-week span (panel A). Alternatively, muMT mice received passive transfer of immune plasma (panel B) prior to tumour seeding and treatment with VSVΔ51. Means +/- SEM are shown.

4. Discussion

Neutralizing antibodies have been shown to be a limiting factor in the efficacy of many oncolytic viruses, including Semliki Forest Virus (SFV) (Ketola et al., 2008), MV (Iankov et al., 2006) and VSV (Power et al., 2007). VSV infection has been shown to induce a robust anti-VSV neutralizing antibody response, initiated at Day 4 post-infection, with titers increasing and remaining at a high level for up to two months (Power et al., 2007). These neutralizing antibodies were shown in the same study by Power et al. (2007) to prevent VSV infections. Neither the tumours of immunized mice, nor in mice receiving a passive transfer of serum from immunized mice, show any virus infection. However, virus is able to infect the tumours of mice receiving adoptive transfer of T cells from immunized mice, clearly demonstrating that it is the antibody-mediated arm of adaptive immunity that is responsible for attenuating the efficacy of oncolytic virus treatment.

Stojdl et al. (2003) have shown that it is possible to cure mice of subcutaneous CT26 colon carcinoma tumours upon treatment with multiple doses of virus, delivered systemically. Although the second and third doses are known not to directly infect tumours (Power et al., 2007), the hypothesis is that the virus is able to elicit a "danger signal" that recruits immune cells to the tumour and facilitates the initiation of a T cell-mediated anti-tumour immune response.

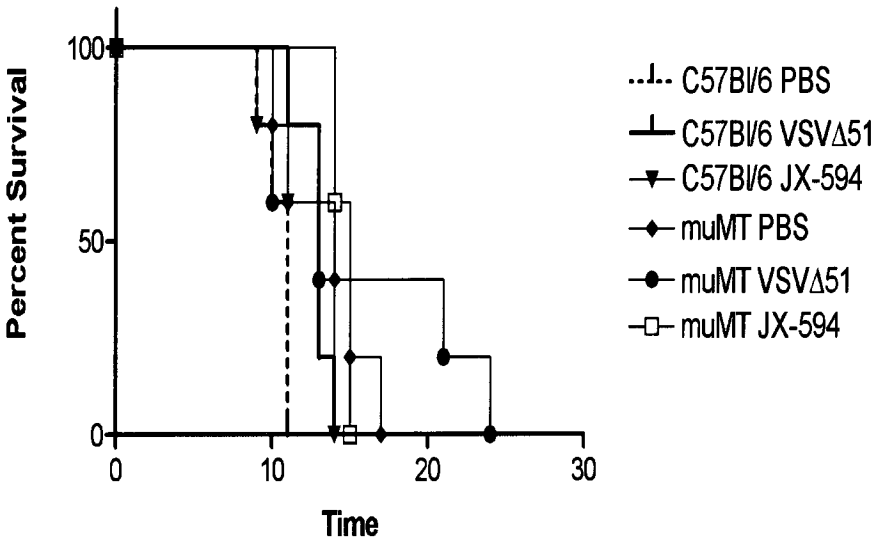

Percent Survival
100
50
0
0
10
20
30
Time
C57Bl/6 PBS
C57Bl/6 VSVΔ51
C57Bl/6 JX-594
muMT PBS
muMT VSVΔ51
muMT JX-594

Figure 14: Virus treatment did not significantly alter the survival of tumour-bearing mice. Tumour-bearing C57Bl/6 and muMT mice were treated with six doses of PBS, $5x10^8$ pfu VSVΔ51 i.v. or $1x10^7$ pfu JX-594 i.v. over a two-week period and then monitored for loss-of-wellness endpoint.

It is not possible to cure nude mice of subcutaneous CT26 tumours, indicating that T cells play an important role in anti-tumour immunity, and that this immune response is required for oncolytic virus efficacy.

This work focuses on the importance of B cells in limiting the efficacy of OV therapy. This work sought to determine the relevance of antibodies as a primary barrier to OV delivery and whether or not the presence of B cells was the primary factor in diminishing the therapeutic effects of OVs.

4.1 muMT mice and VSV

Whether the absence of B cells and anti-VSV antibodies alone is sufficient to improve efficacy of OV therapy was the question to be addressed using muMT mice. The B cells of muMT mice are unable to progress past the pre-B cell stage due to disrupted expression of the B cell receptor (Kitamura et al., 1991). Functional assays ensured a complete lack of VSV-reactive antibodies, as expected. Importantly, the lack of B cells in these mice did not compromise their ability to resolve VSVΔ51 infection. Analyzing the IFN-expression profile of muMT mice versus C57Bl/6 mice post-VSV infection showed that muMT mice produced less IFNα than their wild-type counterparts.

The difference in the levels of plasma IFNα following VSVΔ51 infection can be attributed to a disruption in the immune profile of muMT

mice. Studies characterizing muMT mice have shown three-to-four fold fewer DCs (the primary cell secretors of IFN in response to VSV infection), $CD4^+$ and $CD8^+$ T cells in their spleens, as well as impaired splenic T zone development (Ngo et al., 2001). Shah et al. (2005) also reported abnormal cytokine expression profiles in these mice where levels of Th1 cytokines were significantly higher than Th2 cytokines, while some have reported that splenic cells from tumour-bearing muMT mice released higher levels of IFNγ when challenged with irradiated tumour cells than did wild-type mice (Inoue et al., 2006 and Shah et al., 2005).

Despite the presence of lower levels of IFNα in response to VSVΔ51 infection, muMT mice were protected by the remaining innate immune compartment, as well as T cells. However, infection with wild-type virus is vastly different. Infection with wild-type VSV has been shown to cause CNS disease and death in immune competent Balb/C and immune compromised $PKR^{-/-}$ mice deficient in their IFN response (Stojdl et al., 2003). It has also been demonstrated that B cells are crucial to preventing CNS disease and limiting the neuropathology of VSV infection, where the absence of B cells resulted in 100% mortality, reversible upon passive transfer of immune serum (Thomsen et al., 1997). $CD4^+$ T cells are also important for protection against lethal VSV-wt infection and play a role in long-term survival and providing cognate help to B cells, whereas antibodies are crucial for controlling the acute phase of infection (Andersen et al., 1999). The studies above demonstrate the

importance of the adaptive immune response, whereas the data presented here supports the role of IFN in limiting VSV infection. The lack of B cells was inconsequential when VSV lacked the ability to block IFN production (VSVΔ51), indicating that innate immunity and T cells can be sufficient to control virus infection.

4.1.1 The role of antibodies in limiting OV infection

Although protective in terms of virus infection, the presence of neutralizing antibodies is a barrier to effective treatment of tumours with VSVΔ51. Our group has shown previously that the tumours of pre-immunized mice cannot be infected with a second dose of VSVΔ51 and implicated antibodies rather than T cells in this effect (Power et al., 2007). Confirming these findings was our work in muMT mice. Whether or not mice were immunized did not affect the level of infection with VSV, showing that antibodies are responsible for preventing VSV from infecting tumours of immunized mice. In the absence of B cells, it is possible to deliver as many doses of virus as is necessary to achieve a cure. Mice could be treated an unlimited number of times as there is no observed toxicity associated with VSVΔ51 infection.

Other viruses have been shown to be differentially susceptible to neutralization by antibodies; such is the case with the vaccinia virus JX-594. JX-594 has been shown to replicate in the presence of high antibody titers (Liu et al., 2008) and its enveloped infectious form is resistant to

neutralization by antibodies, as well as complement-mediated lysis. VSV however, is very sensitive to the presence of antibodies and is efficiently neutralized. This difference was apparent when comparing the efficacy of each virus treatment in the presence or absence of B cells. While C57Bl/6 mice treated with either VSVΔ51 or JX-594 showed a marginal reduction in tumour burden, muMT mice had greatly reduced numbers of metastases when treated with VSVΔ51, and a slight improvement when treated with JX-594. These results confirm the expectation that lack of antibodies contributes greatly to the ability of VSV to reduce tumour load, but less so for JX-594. The degree of tumour reduction noted in C57Bl/6 mice can be attributed to the activity of early doses of VSV that are able to reach the tumours and infect them prior to the induction of the antibody response.

4.2 VSV and inflammation

These data suggest that although virus may not be able to infect tumours of immunized mice, there may still be some degree of inflammation that results in increased IFN levels, most importantly IFNγ. IFN has been shown to have a negative role on the growth of both immunogenic and non-immunogenic tumours and mediates effects such as increasing levels of inducible nitric oxide synthase (iNOS) by macrophages, as well as having anti-angiogenic and anti-proliferative effects. Resistance to IFN signaling is a mechanism of immune evasion and is often the result of

disrupted signaling between the IFNγ receptor (IFNGR) and the nucleus (Yim et al., 1999). However, B16 tumours are IFN-responsive. This is important as B16 tumours have been shown to up-regulate MHC I expression upon exposure to IFNγ, as well as up-regulating Fas/FasL expression, targeting cells for CTL-mediated destruction (although to date no group has shown an efficient anti-B16 CTL response). IFNγ signaling in B16 cells has also been shown to cause apoptosis (Böhm et al., 1998). So although VSVΔ51 is not able to directly infect tumours in immunized mice, the inflammation caused by VSV may be enough to retard tumour growth to a certain extent, though not robust enough to clear tumours completely.

The other possibility is that the expression of LacZ (which has long been known to be a highly immunogenic reporter protein (Mælandsmo et al., 2004)) in the B16 cells used makes them more immunogenic and likely contributes towards inducing an anti-tumour immune response, though not specifically targeted to B16 cells. Although VSV infection will cause inflammation and recruitment of immune cells, this does not seem to be sufficient to reduce the growth of B16 tumours, as I have yet to observe any efficacy using this tumour model, unlike the frequent cures that are achievable when treating subcutaneous CT26 tumours. A reason for this could be the IFN-responsive nature of these cells. Although B16 tumours have low levels of the negative regulator of the IFN response, IFN response factor-2 (IRF-2), IFN signaling still

mediates its effects on these cells by decreasing cell proliferation *in vitro* and *in vivo* (Bart et al., 1980 and Kakuta et al., 2002) and increasing MHC I expression (Böhm et al., 1998). However, it is possible that causing more virus to infect tumours will improve immune cell recruitment and overcome their immune suppressive nature by altering the balance of regulatory versus effector cells. The IFN-responsive nature of B16 melanomas means that these cells are able to establish an anti-viral state, limiting the ability of VSV to replicate within the tumours.

4.3 The contribution of B cells to efficacy of VSV – are antibodies the only problem?

VSVΔ51 treatments of B16F10-LacZ lung tumour-bearing mice resulted in a significantly lower number of metastases in muMT mice, an observation attributed to the lack of antibodies. However, an alternate explanation could be that due to the decreased take-rate of tumours in muMT mice (discussed in section 4.5.4), any decrease in tumour burden can be statistically significant, even if the contribution of VSV is minimal. The IFN-responsiveness of B16 tumours is also of concern. Although there are no neutralizing antibodies, the IFN response in B16 tumours is still probably capable of attenuating VSV infection to some degree, having an effect on VSV's oncolytic ability. In fact, our group has observed that VSVΔ51 infects B16 tumours quite poorly compared to other tumours

(e.g. CT26 colon carcinoma), which is not surprising given that VSVΔ51 is extremely sensitive to the activity of IFN, and B16 tumours represent a hostile environment in which the virus cannot replicate very well. This may be apparent based on the Firefly luciferase activity in lungs of muMT versus C57Bl/6 mice. When tumour-bearing muMT and C57Bl/6 mice were given five doses of VSVΔ51 followed by Firefly luciferase-expressing VSVΔ51, an assay of luciferase activity in the lungs of these mice showed only a slight increase relative to C57Bl/6 mice. Low luciferase activity was expected in the lungs of C57Bl/6 mice as these animals would have been immunized to VSV and the last doses of virus would not have reached the tumours. However, the insignificant increase of luciferase activity in the lungs of muMT mice can be attributed to an environment that even in the absence of antibodies does not support robust VSV infection. Alternatively, a lower overall tumour burden in muMT probably contributed to lower levels of virus in the lungs.

4.3.1 Immunity to VSV does not alter efficacy of VSV treatment in B16 lung tumours

In an effort to further distinguish the role of B cells in limiting the efficacy of oncolytic virus therapy, we studied the consequence of pre-existing anti-VSV immunity on the efficacy of treatment. While naïve C57Bl/6 mice showed a small decrease in tumour burden when treated with VSVΔ51, surprisingly, so did pre-immunized mice. The lack of efficacy of

VSVΔ51 treatments in muMT mice was also unexpected, given that mice should not have been affected by previous infections with the virus. Tumour take-rate in this subset of muMT mice was higher than in previous experiments where the tumour load was low (even in untreated animals), so this lack of efficacy may be a function of the difficulty in treating B16 tumours that do not support robust VSV infection, even in the absence of antibodies. muMT mice that were the recipients of either immune or naïve plasma also showed no difference in efficacy. This could be due to insufficient antibody titers in transferred plasma (undetectable by neutralizing antibody assay) that were not able to control VSV infection. muMT mice do have the necessary effector cells that can respond to the presence of antibodies, and there have been studies published that demonstrate the ability of these mice to respond to antibody normally (Thomsen et al., 1997). Other reasons for these observed results may very well be more trivial, such as poor virus stocks or inadvertently selecting for more aggressive cells by prolonged cell culture.

4.3.2 VSV-treated tumour-bearing BCDM show a trend towards enhanced survival

The data showed a trend towards enhanced survival corresponding to reduced tumour burden. All muMT mice (which had lower numbers of lung tumours), survived well past C57Bl/6 mice. In both types of mice, VSVΔ51 treatment prolonged survival most, corresponding with the observation that it was better at reducing tumour burden than JX-594. The

majority of treated muMT mice reached endpoint due to symptoms not related to lung cancer, rather due to the growth of tumour masses at sites distant from the lungs. It is still possible for metastasis to occur when the growth of lung tumours seemed to be checked, and VSVΔ51 was not able to control the growth of these tumuors. Although virus treatment can slow down tumour growth, under the current protocol, I am unable to achieve a cure. Due to the aggressive nature of the tumours and the limited amount of virus replication in these tumours, a complete cure may not be achievable with VSVΔ51 alone, but survival can be significantly increased by continuing to treat animals with VSVΔ51 well after the usual two-week period. The antibody-free status of muMT mice makes this approach feasible as there is no longer an immune component barring repeated virus infection of tumours.

4.4 The role of B cells in anti-tumour immunity – regulatory B cells and their effects

B cells are known to play pathogenic roles in autoimmune diseases such as rheumatoid arthritis (RA) and systemic erythematosus lupus (SLE), however, there is a subset of B cells that play an important role in suppressing autoimmunity and promoting tumourigenesis. Regulatory B cells (Bregs) are B cells that develop in inflammatory environments and function to suppress autoimmunity (Qin et al., 1998, Shah et al., 2005,

Inoue et al., 2006 and Mauri and Ehrenstein, 2007). In fact, in murine models of ulcerative colitis and experimental autoimmune encephalitis (EAE), B cell-deficient mice (BCDM) were less able to combat the onset of disease, implicating B cells in prevention of inflammation and autoimmunity in these models (Mauri and Ehrenstein, 2007). Bregs are known to originate from the Transitional 2-Marginal Zone Precursor (T2-MZP) pool of B cells and the B1 subset of B cells in the peritoneal cavity and marginal zone (MZ) B cells are also significant sources of IL-10 (Mauri and Ehrenstein, 2007). However, there are still discrepancies in the reported phenotypes of these cells, possibly due to the existence of different subsets (Mauri and Ehrenstein, 2007). Breg function is dependent on inflammation and activation via CD40/CD40L interactions (Inoue et al., 2006), possibly concurrent with recognition of self-antigen (Mizoguchi and Bhan, 2006). Once developed, Bregs mediate their effects in three ways: a) through the release of inhibitory cytokines IL-10 and TGFβ, b) through the production of antibodies, c) antigen presentation to $CD4^+$ T cells, causing them to differentiate into Tregs (Mizoguchi and Bhan, 2006).

4.4.1 Breg-secreted cytokines

IL-10 and TGFβ are inhibitory cytokines that are produced by a variety of cells, including tumour cells, Bregs and Tregs. IL-10 production by Bregs regulates the Th1/Th2 balance, favouring the induction of a humoral Th2 response, found to be non-protective in cancer vaccination studies (Qin et

al., 1998). In fact, Perricone et al. (2004) reported better efficacy in BCDM after vaccination with melanoma antigens when compared to C57Bl/5 mice. Th1 and Th2 responses are mutually exclusive – the induction of one prevents the activation of the other, so when B cells initiate a Th2 response, the cell-mediated Th1 response is completely silenced (Shah et al., 2005). IL-10 down-regulates pro-inflammatory networks initiated by macrophages DCs and down-regulates surface expression of MHC I molecules (Inoue et al., 2006). It has also been shown to inhibit IFNγ production by NK cells and $CD8^+$ T cells increase the expression of Treg marker Foxp3 on $CD4^+$ T cells (Mizoguchi and Bhan, 2006). As well as IL-10, a subset of Bregs can produce TGFβ in response to LPS stimulation. These TGFβ-producing Bregs are involved in the induction of oral tolerance, as well as causing apoptosis of effector T cells (Mizoguchi and Bhan, 2006).

4.4.2 The role of antibodies in limiting anti-tumour immunity

Anti-self antibodies are produced by Bregs and are responsible for binding to and enhancing the clearance of apoptotic bodies that express pathogenic self-antigen (Mizoguchi and Bhan, 2006). Unlike antibodies that promote inflammation, these antibodies prevent inflammation by clearing cell debris which would normally activate macrophages and DCs, resulting in down-regulation of pro-inflammatory networks. It is also possible for antibodies to suppress APCs through ligation of the inhibitory Fc receptor FcγRIIb, which is very highly expressed on the surface of DCs

(Mizoguchi and Bhan, 2006). Thus, the presence of these antibodies anergizes cells that would normally be reactive to the antigen with which the antibody is complexed. While this is beneficial in the prevention of autoimmunity under normal circumstances, the presence of these antibodies can abrogate the establishment of an anti-tumour response by APCs that may have sampled and become activated by tumour cells. Thus, as well as promoting tumour growth by enhancing angiogenesis and growth, the presence of antibodies also correlates with preventing the recognition of tumour antigens. Another hypothesis is that the presence of tumour-reactive antibodies aid in immune evasion by shielding tumour antigens that would normally be recognized by APCs (Tan and Coussens, 2007).

4.4.3 B cell Antigen-presentation to T cells induces their differentiation into Tregs

Most tumours are MHC II negative, so $CD4^+$ T cells require APCs for their priming. These APCs can be macrophages, DCs, or B cells. B cells have been shown to be the most abundant APCs, and antigen presentation by B cells to $CD4^+$ T cells is known to favour the induction of a Th2 response (Shah et al., 2005). As well, antigen presentation by B cells has been shown to induce differentiation of $CD4^+$ T cells into Tregs (Mauri and Ehrenstein, 2007). The effects of Bregs are summarized in Figure 15.

4.4.4 Bregs dampen anti-tumour CTL responses

BCDM have been used to determine the role of B cells in anti-tumour immunity (Qin et al., 1998, Perricone et al., 2004 and Shah et al., 2005). Qin et al. (1998) examined the efficacy of pre-immunizing mice with irradiated tumours prior to live cell challenge and found that BCDM were better able to reject the challenge than wild-type mice. They also reported a better CTL response in BCDM compared with wild-type mice and enhanced tumour-rejection in $CD4^+$-depleted mice, implicating $CD4^+$ T cells in the prevention of anti-tumour immunity (Qin et al., 1998). Perricone et al. (2004) also observed enhanced efficacy of tumour vaccination in the absence of B cells. Immunizing mice with adenoviruses expressing melanoma antigens, the authors noted improved survival and tumour rejection in BCDM, four-fold increased proportion of IFNγ-producing T cells in BCDM spleens, and implicated NK cells in the induction of a robust, tumour-specific CTL response (Perricone et al., 2004). Th1 vs. Th2 responses were studied by Shah et al. (2005), who showed increased rejection of primary tumours by BCDM, without any treatment. The authors studied three different tumours – EL4 leukemia, MC38 colon cancer and B16F10 melanoma cells and found that EL4 and MC38 cells were both spontaneously rejected by BCDM, while B16F10 cells grew at a slower rate than they do in C57Bl/6 mice. Immunohistochemistry (IHC) of excised MC38 and B16F10 tumours showed a large number of $CD4^+$ and $CD8^+$ infiltrating T cells, which in the

case of MC38 (but not B16F10) tumours correlated with a better CTL response (Shah et al., 2005). This was attributed to a more immunosuppressive environment in B16F10 tumours – in fact, tumour cells were shown to secrete low levels of IL-10 (30pg/mL) which prohibited the induction of a robust NK and CTL response (Shah et al., 2005). Supernatants of BCDM splenocyte:tumour co-cultures yielded higher levels of Th1 cytokines, such as IFNγ and IL-12 and also demonstrated enhanced CTL activity when compared with C57Bl/6 splenocyte:tumour co-cultures. Importantly, reconstitution of BCDM with sorted B cells abrogated tumour-rejection lowered IFNγ expression and dampened the CTL response of these mice. Possible evidence of the enhanced anti-tumour capacity of BCDM was seen in our experiments. When mice were seeded with B16F10 tumours, muMT mice consistently developed fewer lung metastases in comparison with C57Bl/6 mice (Figure 13b), even if they remained untreated. This lowered tumour-take rate could not have been due to haplotype mismatch as muMT mice are certified by Jackson Laboratories to have the MHC haplotype H2b, as do B16F10 cells. Interestingly, subcutaneous B16 tumours grew similarly in muMT and C57Bl/6 mice (despite conflicting reports by Shah et al., 2005), implying that the enhanced anti-tumour CTL response in muMT mice was still not robust enough to combat bolus injection of cells, but it was sufficient to retard the growth of cells in the lungs.

4.5 Limitations associated with B16 melanoma tumours

There are several factors that contribute to the aggressiveness of B16 cells and the inherent difficulty of treating these tumours. These include an immunosuppressive nature which

precludes the establishment of an anti-tumour response, as well as the ability of B16 cells to resist robust VSV infection.

4.5.1 B16 tumours are immunosuppressive

Some tumours are more capable of eliciting an immune response than others. In general, most tumours practice some degree of immune evasion such as down-regulating the expression of surface proteins and resistance to IFN signaling. B16 tumours do not elicit an anti-tumour response, possibly due to their poor immunogenicity – however, MC38 colon cancers are also poorly immunogenic but they can elicit a robust CTL response *in vivo* (Shah et al., 2005). There seems to be an actively immunosuppressive component to B16 tumours that is preventing the maturation of T cells into effector cells. Shah et al (2005) showed large numbers of tumour infiltrating lymphocytes (TILs) ($CD4^+$ and $CD8^+$ T cells) in the tumours of B cell-deficient mice, but this did not correspond to a strong CTL response. In fact, when Böhm et al studied T-cell mediated rejection of B16 melanomas, they did so with B16 cells expressing viral antigens, as the wild-type cells themselves elicit a very

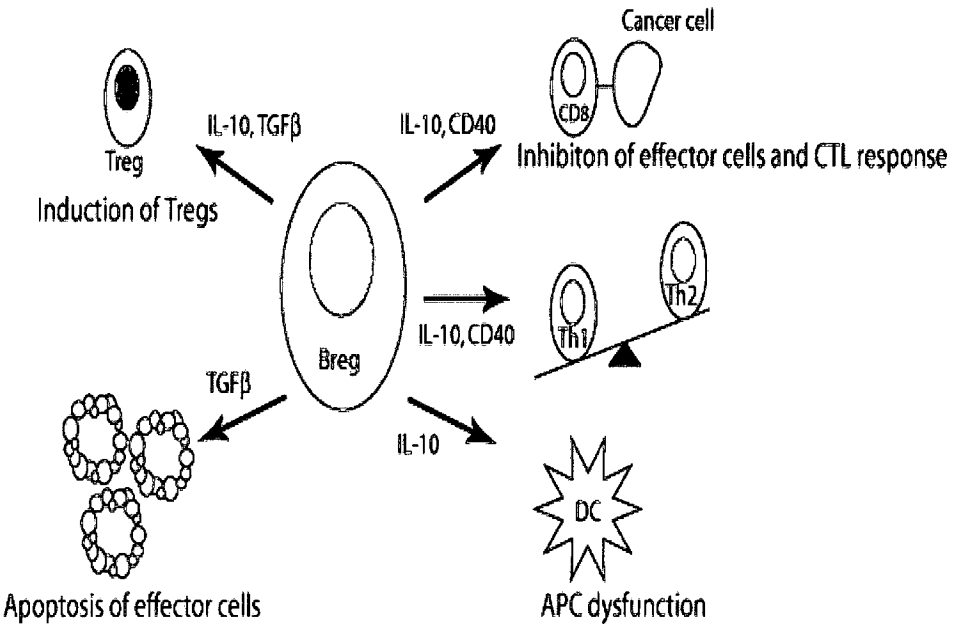

Cancer cell
CD8
Treg
IL-10, TGFβ
IL-10, CD40
Inhibiton of effector cells and CTL response
Induction of Tregs
Breg
IL-10, CD40
Th1
Th2
TGFβ
IL-10
DC
Apoptosis of effector cells
APC dysfunction

Figure 15: Bregs suppress the induction of an effective anti-tumour response and favours the development of a non-protective Th2 response. B regs mediate their effects in a CD40/CD40L-dependent fashion. Secretion of IL-10 and TGF-β by Bregs causes $CD8^+$ T cell silencing, APC dysfunction and apoptosis of effector cells, while it induces the development of $CD4^+$ Tregs and favours a non-protective Th2 response. This results in a dampened anti-tumour CTL response and tumour progression. Adapted from Mauri and Ehrenstein (2007).

poor immune response (Böhm et al., 1998). A possible explanation for the seemingly immunosuppressive nature of B16s may be the ability of these cells to secrete low levels of IL10. These levels were low but detectable (Shah et al., 2005) and may very well contribute to the poor CTL response.

4.5.1.1 *The role of IL10 in immune suppression*

IL10 secreted by macrophages and DCs plays a role in the differentiation of naïve T cells into regulatory T cells in the periphery (Mills, 2004) and Jarnicki et al. (2006) have shown that IL10 secretion by tumour infiltrating T cells has an effect on the induction of regulatory T cells. More recently, IL-10 has been implicating in tolerizing T cells by inhibiting CD28/ICOS co-stimulatory signaling (Taylor et al, 2007 and Taylor et al., 2008). These effects are mediated by the activation of the Src homology 2 domain-containing protein tyrosine phosphatase 1 (SHP-1), resulting in the de-phosphorylation of CD28, inducible co-stumilatory protein (ICOS) and CD2 and preventing phosphatidylinositol-3 kinase (PI-3K) from binding (Taylor et al., 2007 and Taylor et al., 2008). Signaling through the T cell receptor in the absence of co-stimulation results in T cell anergy, so although tumour-infiltrating T cells may recognize tumour-associated antigens, they are anergized by IL-10. It is then possible that even low levels of IL10 produced by B16 tumours are causing substantial immune suppression.

4.5.2 B16 tumours are IFN-responsive

B16 tumours are difficult to treat with oncolytic viruses because of their ability to respond to IFN. Studies by Bart et al. implicated IFNα in the inhibition of B16 growth almost 40 years ago, using polyinosinic:polycytidilic acid (polyI:C) to treat tumour-bearing C57Bl/6 mice. Later, Kakuta et al. (2002) showed that the occurrence of B16 metastasis to the lungs and liver was increased in $IFN\gamma^{-/-}$ mice but not in $IFN\gamma R^{-/-}$ mice, indicating that at least in the metastatic model, the direct activity of IFNγ on the cells is more important than the recruitment of effectors such as NK cells (Kakuta et al., 2002). Translating these studies to human disease, high-dose IFNα (HDI) is now the standard therapy for malignant melanomas in North America, although Europe has yet to follow suit, and the benefit of HDI for high-risk melanoma patients is still being debated (Kefford, 2003). Important for us is improving the efficacy of oncolytic virus treatment for this and other tumour types. We have shown that a lack of B cells corresponds to better virus infection of tumours and now the role of IFN in limiting efficacy needs to be addressed.

4.6 Possible mechanisms of overcoming IFN-responsiveness in B16 tumours

We have several options available to us in order to improve the efficacy of VSVΔ51 treatment by modulating the IFN response in tumours. One option is combination therapy with histone deacetylase inhibitors (HDACis) and VSVΔ51, while another is pre-treatment of tumour-bearing mice with JX-594, followed by VSVΔ51 (Figure 16).

4.6.1 HDACis and IFN

Histone deacteylase inhibitors (HDACis) are a class of molecules that inhibit the activity of histone deacetylases (HDACs), resulting in hyper-acetylation of chromatin, increased transcriptional activation of chromatin and disrupting gene expression (Schmudde et al., 2008). However, there is evidence that while HDACis can induce gene expression, they can also cause repression of an equal number of genes. This is thought to be due to the activity of HDACis on non-histone proteins, including transcription factors that are differentially acetylated, thus, hyper-acetylation disrupts the function of these proteins (Adcock, 2007). HDACis have been shown to induce cell cycle arrest, differentiation or apoptosis in cancer cells and most importantly, they hinder the transcriptional activation of IFN-stimulated genes (ISGs) (Otsuki et al., 2008), however, the effects of HDACis on inflammatory gene expression vary depending on cell type and the nature of the stimulus (Adcock, 2007). In tumour cells, HDACis

have been shown to improve the efficacy of ionizing radiation, chemotherapy (Schmudde et al., 2008) and oncolytic viruses (Nguyen et al., 2008 and Otsuki et al., 2008). In addition, HDACis have been shown to target tumour cells for NK cell-mediated destruction by upregulating the expression of the NK cell ligand MICA/MICB on the surface of tumour cells (Schmudde et al., 2008).

4.6.1.1 *HDACi and OV therapy*

In addition to altering the transcriptional profile of cells, HDACis can upregulate the transcriptional activity of HSV genes, resulting in increased cytotoxicity of the virus in human glioma cells, as well as improving efficacy in a xenograft model (Otsuki et al., 2008). Thus, the role of HDACis was two-fold in this study. First, HDACis caused apoptosis in a tumour-specific manner, and second, they enhanced the oncolytic activity of HSV. Nguyen et al. (2008) also showed a strong synergistic relationship between HDACis and VSV. HDACis were showing to sensitize refractory tumours to virus infection and increased the replication of VSV in a tumour-specific manor. Importantly, the presence of HDACis counteracted the anti-viral effects of exogenous IFNα and was able to restore virus infection in pre-treated cells (Nguyen et al., 2008). The ability of HDACis to augment efficacy of VSV treatment in refractory tumours was shown to be dependent upon their effect on the IFN response. The activation of the IFN cascade was studied in human prostate cells (PC3) which showed that cells treated with HDACi had much lower levels

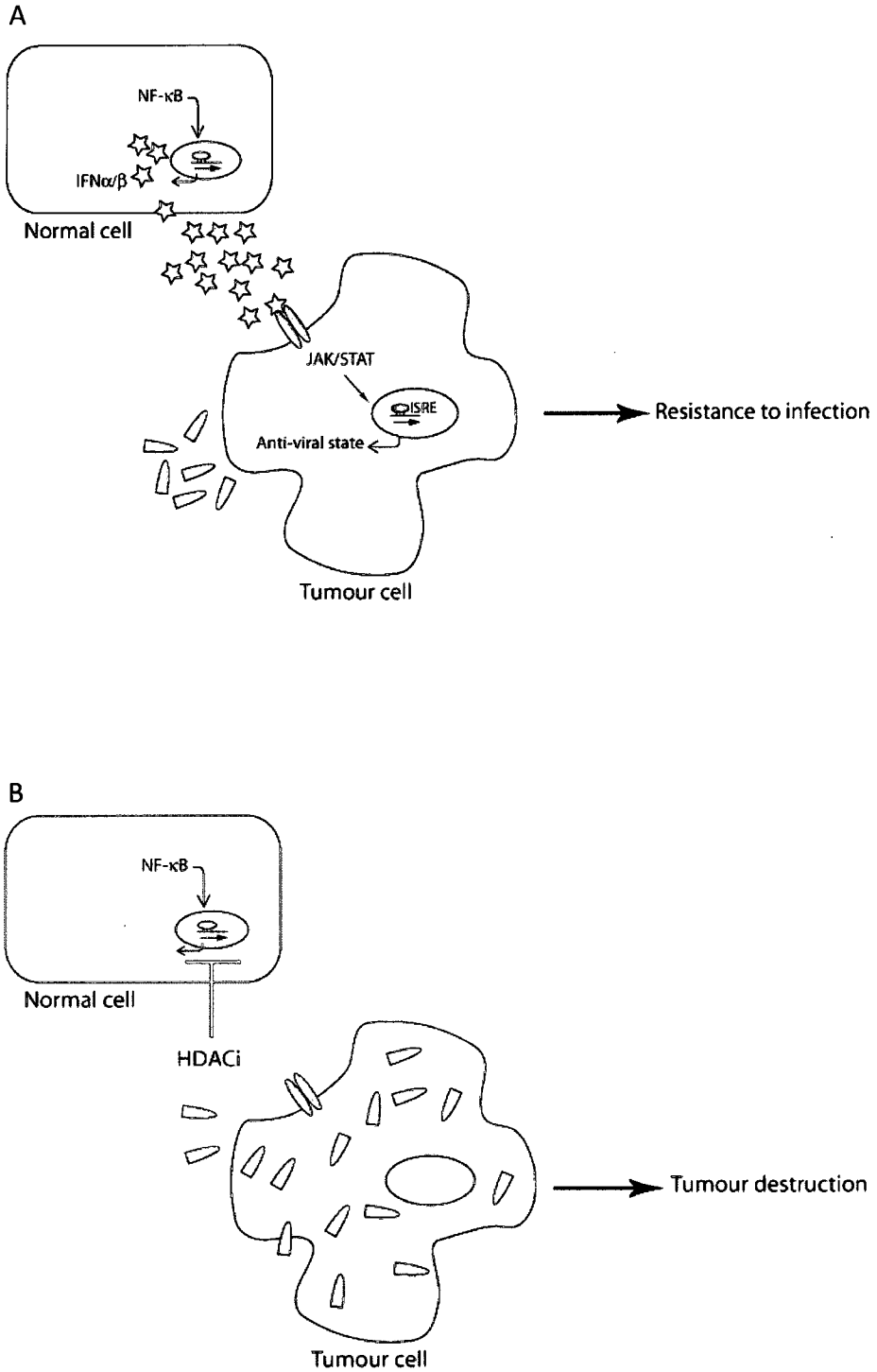
of IFNβ mRNA than did untreated cells (Nguyen et al., 2008). Dual treatment with HDACi and VSV was also shown to have a greater inhibitory effect on tumour growth in a xenograft model.

The antitumor effects of HDACis and their synergistic effect with VSV make this an attractive approach to improving efficacy in our B16 lung tumour model. However, due to the effects of HDACi on altering the IFN response, it will be important to determine if co-treatment will result in virus-related toxicity. This is especially important since the influence of HDACis on the immune system is still unknown, with some studies implicating HDACis in an enhanced immune response and others in a dampened immune response. Data from our lab has shown that pre-treating mice with an HDACi prior to VSV infection significantly impairs neutralizing antibody production – either through mediating B cell dysfunction or apoptosis (Appendix I, JSDiallo and C Lemay, personal communication). Interestingly, a recent study showed the ability of the HDACi depsipeptide FK228 (isolated from *Chromobacterium violaceum*) to cause Fas/FasL-dependent apoptosis in B16 cells, as well as increased expression of the tumour-specific antigen gp100/pmel17 (Murakami et al., 2008). This study also showed an improved CTL response to B16 tumour cells, but this was shown when irradiated B16 cells were incubated with T cells that were stimulated with Pmel17. The immunosuppressive environment of B16 tumours makes it unlikely that TIL will be sufficiently activated, even in the presence of highly expressed tumour

antigen, so the effect of HDACis on the CTL response may not be as dramatic as indicated in this study. However, the role of HDACis in dampening the IFN response in these cells will be very important in improving viral replication and efficacy.

4.6.2 Vaccinia virus and IFN

Another approach to inhibiting the IFN response and improving efficacy is to combine treatment with both JX-594 and VSV as therapeutics. JX-594 is an attractive therapeutic not only because of its proven efficacy in clinical trials, but also because of its ability to circumvent the IFN response. Poxviruses have several highly effective strategies to avoid the effects of IFN. Among these is the vaccinia virus protein E3L which binds dsRNA preventing it from activating PKR, a key activator of the IFN response upon viral infection; while K3Lbinds PKR and blocks the phosphorylation and inactivation of host eukaryotic translation initiation factor 2α (eIF2α) (Symonds et al., 1995). As well, B18R is a soluble cytokine receptor that binds type I IFNs (IFNα/β) with broad species specificity. B18R blocks the binding of IFN to its cell surface receptor, both in infected cells and in those adjacent to them, thus preventing the establishment of an antiviral state (Kirn et al., 2007). Deletion of B18R has been shown to reduce the virulence of vaccinia virus in Balb/C mice (Symons et al., 1995) and can be used as a means of targeting the virus specifically towards IFN-deficient tumour cells.


A
NF-κB
IFNα/β
Normal cell
JAK/STAT
ISRE
Anti-viral state
Resistance to infection
Tumour cell
B
NF-κB
Normal cell
HDACi
Tumour destruction
Tumour cell

C

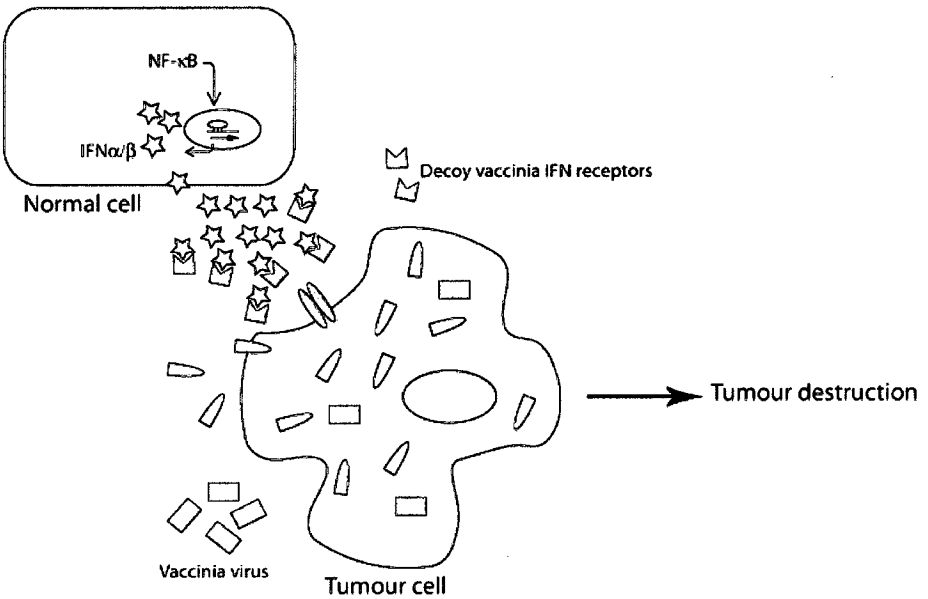

NF-κB
IFNα/β
Normal cell
Decoy vaccinia IFN receptors
Tumour destruction
Vaccinia virus
Tumour cell

Figure 16: Possible methods of overcoming the immunosuppressive and virus-restrictive nature of B16 tumours. B16 tumours are IFN-responsive, making them more resistant to VSVΔ51 infection than other tumour types (panel A). When virus infection is detected by normal cells, IFN is produced which protects B16 tumours against VSVΔ51 infection. However, the use of HDAC inhibitors (panel B) can prevent IFN expression by normal cells thus making B16 cells more susceptible to infection. Alternatively, the vaccinia virus JX-594 (panel C) can block the IFN response by secreting decoy IFN receptors, preventing the cytokine from binding its proper receptor on tumour cells and establishing an antiviral state.

4.6.2.1 *Synergism between VV and VSV*

The ability of vaccinia virus to resist the establishment of an antiviral response can be exploited by VSV. In fact, our group has shown that pre-treatment of VSV-restrictive cancer cells with JX-594 can sensitize these cells to VSV infection (Appendix II, F LeBoeuf, manuscript in preparation). This effect can be attributed to the anti-inflammatory activity of B18R where the virus is able to enter cells, replicate and express its arrays of immune modulating genes before VSV infection. This would be an excellent strategy for treatment of B16 tumours. Unlike HDACis, the activity of JX-594 is restricted to the tumour environment and does not influence the activity of T cells, rather it can make the tumours more hospitable to VSV infection and virus-mediated destruction. JX-594 can also contribute to better tumour killing due to its own oncolytic capacity and its ability to evade host immunity.

5. Concluding remarks

This report demonstrates some of the difficulties associated with oncolytic virus therapy. The majority of studies with oncolytic viruses identify antibodies as the main hurdle on the way to better treatment efficacy, but while B cells play a very large role in limiting virus delivery, we must also take into consideration the nature of the tumour and its microenvironment. The absence of B cells in this model allowed multiple doses of virus to infect tumours, but it did not confer improved efficacy. This could be a feature of the aggressive nature of B16 melanomas, or it could indicate that there are more significant barriers to optimal virus efficacy than previously anticipated. These barriers include IFN responsiveness of some types of tumours (including B16 melanomas) which limits virus infection. Both proposed mechanisms of by-passing IFN-responsiveness of tumours (HDACi and VSV combination therapy or vvDD/VSV co-infections) are subjects that are under investigation in our lab. Potentially, treatment of tumours with HDACis will prevent the establishment of an IFN response (by limiting access to the cell's genome), thus stopping the induction of IFN-responsive genes in tumour cells and enhancing virus infection. Alternatively, VV expresses soluble IFN decoy receptors that can prevent tumour cells from responding to IFN, allowing for better infection. Our group and others have shown these methods to be feasible - Ngyuen et al. (2008) demonstrate that the HDACi MS-275 successfully sensitizes tumours to VSV infection, while work by F LeBoeuf has elegantly

demonstrated a synergistic relationship between VV and VSV, where pre-infecting VSV-restrictive cells with VV paves the way for robust VSV infection. Further experiments using the above-mentioned HDACis and VV/VSV co-infections in the context of B-cell deficiency may yield much better efficacy than was observed in these experiments. However, it is important to proceed with caution so as not to severely compromise the remaining components of the immune system of muMT mice. HDACi will globally suppress IFN expression and may affect other immune cell functions as well, while VV can counteract the effects of both type I and type II IFNs, potentially compromising mouse defenses, such that they cannot sustain VSVΔ51 infection. There are also many studies calling for B cell depletion therapies to be used in the clinic in order to take advantage of the enhanced CTL response in the absence of Bregs. However, it would be interesting to examine the feasibility of this approach by depleting tumour-bearing mice of their B cells (using anti-mouse B cell monoclonal antibodies) and analyzing the effects of this on the induction of a CTL response against an established tumour. While B cells may not be the only barrier to effective OV therapy, their absence certainly paves the way towards enhanced efficacy.

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7. Contributions of Collaborators

Jean-Simon Diallo and Chantal Lemay carried out and provided the data for the effect of HDACis on anti-VSV antibodies.

Fabrice LeBoeuf carried out and provided the data for vvDD/VSV co-infections

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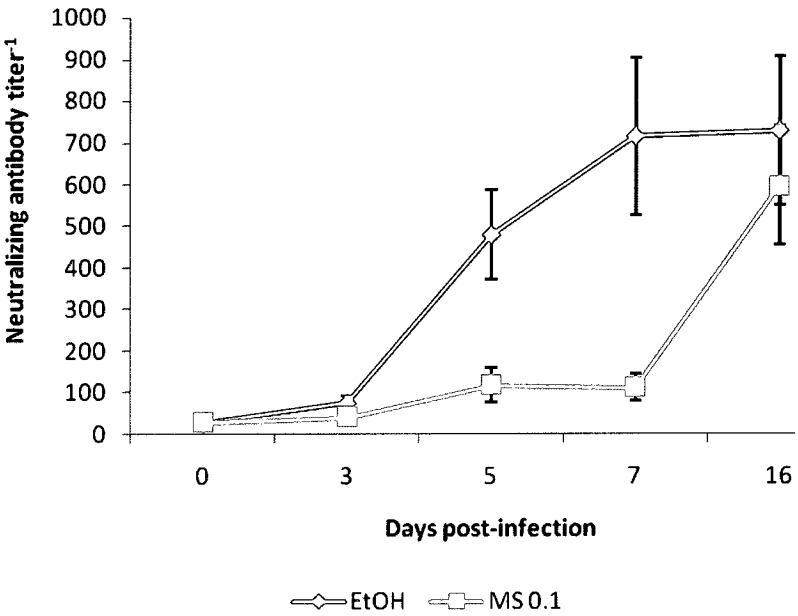
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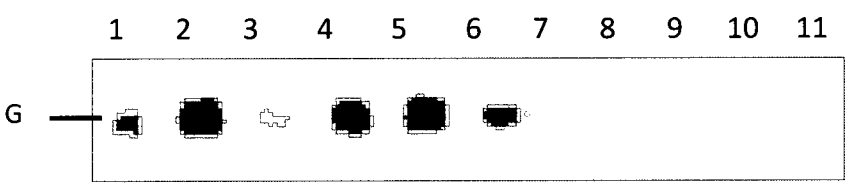
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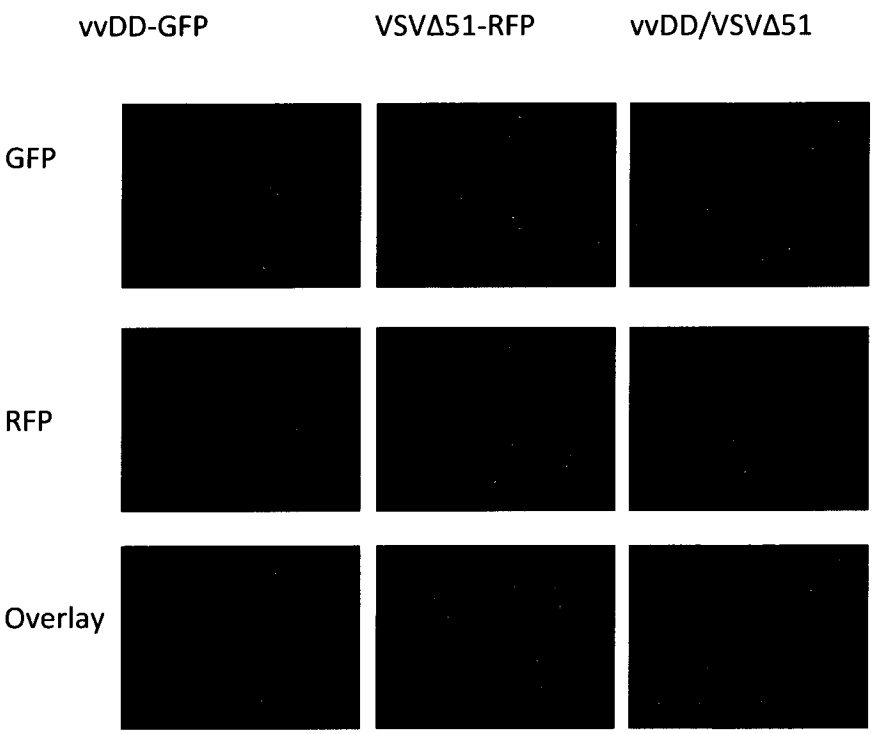
8. Appendices

Appendix I: HDACi suppress the induction of an anti-VSV neutralizing antibody response. Balb/C mice were treated with daily, i.p. doses of 0.1M MS-275 or ethanol (vehicle) for one week. Plasma samples were collected by saphenous vein bleeds and analyzed for anti-VSV antibodies by neutralizing antibody assay (panel A) at Day 0, 1, 3, 5, 7 and 16d post-VSV infection. Panel B shows the presence of anti-VSV antibodies by Western blot, 7 days post-VSV infection. Lane 1 is a positive control from a pre-immunized Balb/c mouse, lanes 2-6 are plasma samples of ethanol-treated mice and lanes 7-11 are plasma samples of mice treated with 0.1M MS-275. These data were provided by JS Diallo and C Lemay.


vvDD-GFP
VSVΔ51-RFP
vvDD/VSVΔ51
GFP
RFP
Overlay

Appendix II: Vaccinia virus increases VSVΔ51 infection/replication in SKOV3 ovarian cell line. SKOV3 cells were infected with vvDD-GFP at a multiplicity of infection (MOI) of 0.1 or with VSVΔ51-RFP at an MOI of 0.1, or both. During co-treatments, vvDD-GFP was added to cells and allowed to infect for 2 hours and then the cells incubated with fresh media for 4 hours. VSVΔ51-RFP was then added to the cells and allowed to infect for 45 minutes. All pictures are taken at 44 hours post-infection. This experiment was designed and executed by F LeBoeuf, who has also kindly provided these images.

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PERSONAL DETAILS

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EDUCATION

September 2006 – April 2009	**University of Ottawa, Ottawa, Ontario** **Department of Biochemistry, Microbiology and Immunology** **MSc Biochemistry, Human Molecular Genomics** **Title of Thesis: Investigating the role of B cells in limiting the efficacy of oncolytic virus therapy** Completed departmental seminars, thesis research terms and two graduate-level courses – Advanced Topics in Gene Expression and Protein Synthesis (January 2007 – April 2007) and Advanced Topics in Cancer Biology (January 2008 – April 2008).
September 2002 – April 2006	**University of Ottawa, Ottawa, Ontario** **BSc Hons. Biopharmaceutical Science** Graduated *Cum Laude*
September 1998 – June 2002	**St. Patrick's High School, Ottawa, Ontario** Ontario High School Diploma – OAC Honour Roll Student

RELEVANT COURSES

Biochemistry
Bioinformatics
Probability and Statistics
Biology of Cancer
Virology
Immunology
Gene and Protein Expression
Organic Chemistry
Physical Chemistry
Physics
Microbiology

Non-Science Electives:
Bioethics
Essay writing

Genetics
Genomics
Pharmacology
Molecular Biology
Mammalian
Physiology

History of
Criminological
Thought
Contemporary
Religious Movements

EDUCATIONAL PROGRAMS AND PROFESSIONAL EXPERIENCE

**September 2006-
April 2009**

**University of Ottawa, Ottawa, Ontario
Department of Biochemistry,
Microbiology and Immunology
MSc Thesis Research - Biochemistry**
Supervisor: Dr John C. Bell

Studied the interaction between oncolytic viruses and the adaptive immune response, namely the role of B cells in hindering virus infection of tumours

**May 2007 –
September 2007**

**Ottawa Health Research Institute /
Cancer Research Group
Ottawa, Ontario
Summer Student**
Supervisor: Dr John C. Bell

Optimising the production of Vaccinia virus in cell culture conditions, as well as characterising the biodistribution of the virus in mice.

**September 2005-
April 2006**

**University of Ottawa, Ottawa, Ontario
Department of Biochemistry,
Microbiology and Immunology
Undergraduate Independent Study
Thesis**
Supervisor: Dr John C. Bell
Investigating methods of improving the efficacy of oncolytic virus treatment for potential clinical use.

**August
2005 –
September
2005**

**Ottawa Health Research Institute /
Cancer Research Group
Ottawa, Ontario
Summer Student**
Supervisor: Dr John C. Bell

Recombinant protein production and characterization.

**June 2004 –
July 2005**

**Appletree Medical Group, Ottawa,
Ontario
Medical Assistant**
Supervisor: Dr Thom Tyson

Patient triage, assisting doctors during procedures (suture removal, dressing changes etc.) following up with patients regarding test results and future appointments, clerical and administrative duties, administering allergy injections, influenza vaccines and tuberculosis skin tests, as well as general housekeeping duties.

FURTHER INFORMATION AND AWARDS

I have attended the Fourth International Conference on Oncolytic Viruses (2007, Carefree, Arizona) at which I submitted an abstract and presented a poster regarding my work with oncolytic viruses.

I have also attended and presented a poster at the Ottawa Hospital Research Institute Research Day (2008, Ottawa, Ontario).
I have given a poster presentation and departmental seminar (2007 and 2008 respectively, Biochemistry, Microbiology and Immunology Day, University of Ottawa), as well as acting as a student evaluator in the same time period.

I was awarded an Entry Scholarship from the University of Ottawa in the amount of $10 000 (2002).

I was awarded an Ontario Scholar certificate for maintaining an above-80% average throughout the graduating year (2002).

PERSONAL SKILLS

- Self-motivated, hard-working and reliable
- Self-directed learning
- Work well independently and in a team setting
- Strong interpersonal skills developed through working with patients and colleagues
- Ability to teach and communicate with others outside the field
- Able and willing to learn very quickly and adapt to any environment
- Detail-oriented and take a great deal of pride in my work.
- Excellent writing and presentation skills
- Critical appraisal of scientific publications
- Grant-writing experience
- Statistical data manipulation
- Experience with diverse scientific techniques, animal manipulation and basic physiology

POSITIONS OF RESPONSIBILITY

- Supervised two undergraduate students during their Independent Study project and provided guidance during their term (September 2007 - April 2008). Introduced students to their projects and taught them new techniques and helped them develop analytical and problem-solving skills.
- Edited other people's written work including several Honours and Masters Thesis reports (2006 - 2008).
- Tutored students in Math and English at the high school level (September 2001 – February 2002).
- Worked as a teacher's assistant (Summer 1998) teaching children at the Kindergarten level.

INTERESTS AND ACTIVITIES

I enjoy travelling and have visited a number of countries in Europe, Asia and Africa. I am interested in different cultures, world politics and history and am an avid reader. I also enjoy music and play the flute at a Grade 6 level. I enjoy go-karting, skating and being outdoors.

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