Combination strategies in the development of oncolytic virus cancer therapy

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by

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Abstract

The ability of certain viruses to exploit cancer cell abnormalities for their own replication represents a remarkable opportunity in the development of cancer therapy. Although oncolytic viruses such as Vesicular Stomatitis virus (VSV) possess a variety of intrinsic properties that can be exploited to this end, important aspects of their nature are not optimized for this purpose and may benefit from refinement. Therefore, strategies to enhance VSV's direct or immune-mediated tumor cell killing have been designed and tested in the course of the studies presented in this thesis.

First, VSV was engineered to express the CD::UPRT suicide enzyme that allowed the conversion of a non-toxic, systemically delivered drug into a cytotoxic chemotherapeutic compound only at the tumor site. This strategy demonstrated a synergistic enhancement of tumor cell killing, with lysis of non-infected cancer cells contributing to increased efficacy in tumor cells partially resistant to VSV. In addition, combination treatment was optimized *in vivo* by taking into consideration the kinetics of virus replication at the tumor and the bioavailability of the non-toxic pro-drug. Results not only demonstrated enhanced therapeutic effects on tumor-bearing mice but also highlighted important characteristics of *in vivo* VSV replication kinetics. A second strategy combined VSV with an immunomodulatory approach in an attempt to boost VSV-induced anti-tumor adaptive immune response. Using Flt3L growth factor to promote dendritic cell population augmentation, antigen presentation capacity was highly enhanced concomitantly with VSV oncolysis. Although improving therapeutic outcome, the strategy did not improve anti-tumor adaptive immune response. The approach uncovered an unexpected aspect of the immune response: VSV treatment was found to profoundly affect the viability of immune cells and dendritic cells at the tumor and to block their migration to the draining lymphoid organs. Consequently, tumor antigen presentation was abolished.

The absence of tumor antigen presentation following VSV treatment is a mechanistic explanation for the limited ability of VSV to induce a tumor-specific adaptive immune response. Altogether, the strategies developed in the course of this work enhanced VSV's oncolytic properties and greatly advanced our general understanding of VSV anticancer therapy.

Résumé

Certains virus possèdent la capacité d'exploiter les défauts métaboliques des cellules cancéreuses pour leur propre réplication. Ces virus, nommés virus oncolytiques, représentent une remarquable opportunité pour le développement de thérapies contre le cancer. Malgré cette prédisposition, certaines caractéristiques des virus oncolytiques ne sont pas optimales pour cette fonction et pourraient être améliorées. Dans cette optique, des stratégies visant à augmenter l'oncolyse induite par le virus Vesicular Stomatitis (VSV) ont été développées et testées chez la souris au cours de ce doctorat.

Dans un premier temps, VSV a été modifié génétiquement afin qu'il exprime l'enzyme suicide CD ::UPRT lui permettant de réaliser la conversion d'un composé non-toxique administré de façon systémique en composé cytotoxique uniquement au site de la tumeur. La stratégie a permis de démontrer une augmentation synergique de la lyse des cellules cancéreuses ainsi que l'induction de la mort de cellules cancéreuses non infectées et partiellement résistantes au VSV. De plus, la combinaison *in vivo* a été optimisée afin de tenir compte de la cinétique de réplication du virus à la tumeur ainsi que de la biodisponibilité de la drogue. Les résultats ont permis non seulement d'obtenir une amélioration de l'effet thérapeutique mais également de souligner d'importantes caractéristiques de la réplication virale *in vivo*. Dans une seconde stratégie, VSV a été combiné avec une approche d'immunomodulation ayant pour but d'engendrer une réponse immunitaire acquise spécifique à la tumeur. En employant le facteur de croissance Flt3L qui favorise la prolifération et la différentiation des cellules dendritiques, la capacité de présentation d'antigènes a été grandement renforcée simultanément à l'oncolyse induite par VSV. En dépit du fait que la combinaison n'a que partiellement amélioré l'effet thérapeutique, elle a révélé un aspect inattendu de la réponse immunitaire engendrée par VSV. Les résultats ont démontré que VSV affecte grandement la viabilité des cellules immunitaires et des cellules dendritiques à la tumeur, qu'il bloque leur migration aux organes lymphatiques et que, par conséquent, la présentation d'antigènes tumoraux est abolie.

La démonstration de l'absence de présentation d'antigènes tumoraux suivant le traitement oncolytique de VSV représente un important concept expliquant la piètre capacité de VSV en ce qui a trait à l'induction d'une réponse immunitaire aquise spécifique à la tumeur. En conclusion, les stratégies développées aux cours de ces travaux ont permis d'améliorer les propriétés oncolytiques de VSV ainsi que de grandement contribuer à la compréhension de la thérapie anti-cancer de VSV.

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Preface

In accordance with the "Guidelines for Thesis Preparation", this thesis is presented in a Manuscript-based format. A general introduction precedes the results chapter which is divided into tree sections:

1- Manuscript I

Leveille S, Samuel S, Goulet ML, Hiscott J. **Enhancing VSV oncolytic activity with an improved cytosine deaminase suicide gene strategy.** Cancer Gene Ther. 2011 Jun;18(6):435-43.

2- Manuscript II

Leveille S, Goulet ML, Lichty BD, Hiscott J. **VSV oncolytic treatment interferes with tumor associated dendritic cell function and abrogates tumor antigen presentation.** J. Virol. 2011 in revision.

3- Other VSV combinations

Contributions of the authors

Original idea from SL.

Manuscript I: SL conceived, designed, performed the experiments and wrote the manuscript. SS performed experiments, MLG revised the manuscript and JH conceived experiments and wrote the manuscript.

Manuscript II and other VSV combinations: SL and MLG conceived, designed, performed the experiments and wrote the manuscript. BDL revised the manuscript and JH conceived experiments and wrote the manuscript.

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Nguyên TL, Abdelbary H, Arguello M, Breitbach C, Leveille S, Diallo JS, Yasmeen A, Bismar TA, Kirn D, Falls T, Snoulten VE, Vanderhyden BC, Werier J, Atkins H, Vähä-Koskela MJ, Stojdl DF, Bell JC, Hiscott J. **Chemical targeting of the innate antiviral response by histone deacetylase inhibitors renders refractory cancers sensitive to viral oncolysis.** Proc Natl Acad Sci U S A. 2008 Sep 30;105(39):14981-6.

Holm GH, Zurney J, Tumilasci V, Leveille S, Danthi P, Hiscott J, Sherry B, Dermody TS. **Retinoic acid-inducible gene-I and interferon-beta promoter stimulator-1 augment proapoptotic responses following mammalian reovirus infection via interferon regulatory factor-3**. J Biol Chem. 2007 Jul 27;282(30):21953-61

Publications from Master's degree:

Léveillé S, Caza M, Johnson JR, Clabots C, Sabri M, Dozois CM. **Iha from an Escherichia coli urinary tract infection outbreak clonal group A strain is expressed in vivo in the mouse urinary tract and functions as a catecholate siderophore receptor**. Infect Immun. 2006 Jun;74(6):3427-36

Sabri M, Léveillé S, Dozois CM.A **SitABCD homologue from an avian pathogenic Escherichia coli strain mediates transport of iron and manganese and resistance to hydrogen peroxide.** Microbiology. 2006 Mar;152(Pt 3):745-58.

Lymberopoulos MH, Houle S, Daigle F, Léveillé S, Brée A, Moulin-Schouleur M, Johnson JR, Dozois CM. **Characterization of Stg fimbriae from an avian pathogenic Escherichia coli O78:K80 strain and assessment of their contribution to colonization of the chicken respiratory tract.** J Bacteriol. 2006 Sep;188(18):6449-59.

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OTHER VSV COMBINATIONS

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List of abbreviations

5-FC	5-Fluorocytosine
5-FU	5-Fluorouracil
Ab	Antibody
APC	Antigen presenting cell
ATF2/c-Jun	Activating Transcription Factor 2
B7.1	Cluster of differentiation 80
B7.2	Cluster of differentiation 86
Bcl-2	B-cell lymphoma 2
BCR	B cell receptor
CARD	Caspase recruitment domains
CCL	Chemokine (C-C motif) ligands
CCR	C-C chemokine receptors
CD	Clusters of differentiation
CD::UPRT	Fusion enzyme of *E. coli* cytosine deaminase (CD) and uracil phosphoribosyltransferase (UPRT)
CD40L	Cluster of differentiation 40 ligand
cDC	Conventional DC
CI	Combination index
CPA	Cyclophosphamide
DC	Dendritic cells
DNA	Deoxyribonucleic acid
dsRNA	Double stranded RNA
E1A and B	Adenovirus protein
EC50	Half maximal effective concentration
ED50	Effective doses in 50%
EGF	Epidermal growth factor
eIF2α	Eukaryotic translation initiation factor 2A
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum

FACS	Fluorescence Activated Cell Sorting
FAS-L	Fas death receptor ligand
FDA	US Food and Drug Administration
FOXP3	Forkhead box P3 transcriptional regulator
GFP	Green fluorescent protein
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
HDAC	Histone deacetylase
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
hNIS	Human sodium iodine symporter
HSV	Herpes Simplex virus
ICP	Infected cell proteins, HSV proteins
IFN	Interferons
IKKε	Inducible IκB kinase
IL	Interleukins
IP	Intraperitoneal
IPAF	NLR family CARD domain-containing protein 4
IRF	Interferon regulatory factors
ISG	IFN-stimulated genes
ISVP	Intermediate subviral particles
IT	Intratumoral
IV	Intravenous
JAK/STAT	Janus Kinase/ Signal Transducer and Activator of Transcription
kb	Kilobase
LD50	Lethal Dose, 50%
LPG2	Laboratory of genetics and physiology 2: RNA sensor
LPS	Lipopolylsaccharide
LUC	Luciferase
MAVS	Mitochondrial antiviral signaling adaptor
MDA5	Melanoma differentiation-associated gene 5
MDSC	Myeloid-derived suppressor cells

MHC	Major histocompatibility complex
MOI	Multiplicity of infection
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium
NALP3	NACHT, LRR and PYD domains-containing protein 3
NCI60	National Cancer Institute 60: a set of 60 cancer cell lines
NF-κB	Nuclear factor kappa B
NK	Natural killer
NKG2D	Natural killer group 2, member D receptor
NLR	NOD-like receptors
NOD1 and 2	Nucleotide-binding oligomerization domain-containing protein
nt	Nucleotides
Nup98	Nuclear pore component 98
OV	Oncolytic viruses
OVA	Chicken ovalbumin
OX40L	Cluster of differentiation 134 ligand
PAF	Platelet-activating factor
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
pDC	Plasmacytoid DC
PFU	Plaque-forming unit
PI	Propidium iodide
PKR	Double-stranded-RNA-dependent protein kinase
PRRs	Pattern recognition receptors
Rae1	RNA export 1
Ras	Small GTPase
Rbz	Ribozyme
RIG-I	Retinoic acid-inducible gene I
RLR	RIG-I-like receptors
RNA	Ribonucleic acid

ROS	Reactive oxygen species
rVSV	Recombinant VSV
SE	Standard error
SEM	Standard error of the mean
SS	Stop/start sequence
ssRNA	Single stranded RNA
TAP	Transporter associated with antigen presentation
TBK1	TANK-Binding kinase 1
TFIID	Transcription factor II D
TGFβ	Transforming growth factor beta
Th	T helper cells
TK	Thymidine kinase
TLR	Toll-like receptors
Treg	Regulatory T cell
VEGF	Vascular endothelial growth factor
VGF	Vaccinia growth factor
VSV	Vesicular Stomatitis virus
VV	Vaccinia virus

CHAPTER 1

INTRODUCTION

1.1 Oncolytic viruses

Viruses can been described as "organisms at the edge of life" based on their dependence on living cells for survival (Rybicki, 1990). As a consequence of this obligatory interaction, both entities – the host and the virus – have evolved mechanisms that respond to one another. The hosts have developed refined systems, such as the immune system, to counteract infection, while viruses display a multitude of impressive mechanisms to bypass these systems and take control of the host. In this endless war, viruses are confined to a very precise “ecological niche” where the host antiviral response is unable to control the infection until an evolutionary change occurs to upset the balance. Viral evolutionary changes can allow access to new niches of infection, as illustrated by the recent rise of new Influenza virus strains or by the evolution of Human immunodeficiency virus (HIV) from non-human primates. Host changes such as dysregulation or partial suppression of host immune surveillance can also give rise to opportunistic viral infections. For example, immunosuppressed HIV patients or post-transplant patients using immunosuppressive drugs are more prone to opportunistic infections.

Cancer represents another undeniable case of cellular function dysregulation. The tropism of viruses for cancer cells has a long history and was observed even before they were first identified, when they were still classified as “filterable agents”. Case reports of cancer patients experiencing temporary remission following natural viral infections can be found as early as the mid-1800s (Kelly and Russell, 2007). One of the most cited examples is the case of a 42 year-old woman with chronic myelogenus leukemia who experienced a dramatic decrease in leukocyte count and remission after a presumed influenza infection. (Dock, 1904). The case was reported in 1904, before influenza was established to be a viral infection and before the virus was isolated in 1933 (Wilson Smith, 1933). Oncological benefits were also reported following vaccination, as demonstrated in the case of a woman suffering from cervical carcinoma who responded to repeated rabies vaccinations in 1912 (Liu *et al.*, 2007). More recent clinical reports describing cancer regression concomitant with viral infection are abundant. For example,

in the 1970-80s, beneficial outcomes resulting from natural infection with Measles virus were reported in leukemia (Pasquinucci, 1971), Hodgkin's disease (Taqi *et al.*, 1981) and Burkitt's lymphoma (Bluming and Ziegler, 1971). The observation that natural virus infection can counteract tumor progression under particular circumstances led to the idea that viruses can be used to treat cancer and created a completely novel research field for the development of therapeutic approaches in cancer therapy. Viruses taking advantage of genetic abnormalities and altered signaling pathways in cancer cells for their replication were termed oncolytic viruses (OVs). OVs are capable of infecting and killing tumor cells, while normal cells, which have an intact antiviral and cycling state, are refractory to the infection.

1.1.1 Desirable properties of OVs

OVs represent a heterogeneous group of viruses possessing diversified intrinsic properties that make them attractive tools for the development of experimental therapeutic approaches. Several characteristics are common to all of these viruses, while others represent advantages harbored by specific viruses. Communal characteristics include: (1) cancer specificity, as OVs should be able to efficiently target cancer cells while sparing normal cells; (2) the ability to target a wide range of tumor cells and cancer types; (3) sensitivity to the host antiviral response, which allows the patient to control the "infection"; (4) non-pathogenic in humans, to minimize side effects; and (5) genetically stable, to avoid any viral changes that could modify tropism. Apart from these essential characteristics, many desirable properties can also be found in different OVs, such as: (6) rapidity of replication cycle, resulting in more rounds of replication and thus enhancing the spread of the virus before immune surveillance interferes with viral propagation; (7) the absence of pre-existing neutralizing antibodies in the population that could limit viral spread to the tumor; (8) the ability to produce and purify the virus to high titers as a commercial asset; (9) profound knowledge of their basic virology at a molecular and infectious level; (10) the ease of genetic manipulation and the amount of genetic material that can be modified, a feature that can be exploited in order to generate novel

recombinant viruses combining intrinsic oncolytic properties with a cancer-specific gene delivery system, and finally (11) the susceptibility to anti-viral drugs as a safety feature.

1.1.2 Molecular basis for OVs' cancer specificity

Transformed cells are characterized by the accumulation of mutational events that alter multiple cellular metabolic pathways. Cancer cells have been defined by six specific essential alterations in cell physiology: (1) self-sufficiency in growth signals; (2) insensitivity to growth-inhibiting (antigrowth) signals; (3) evasion of programmed cell death (apoptosis); (4) limitless replicative potential; (5) sustained angiogenesis, and (6) tissue invasion and metastasis (Hanahan and Weinberg, 2000). Therefore, new metabolic phenotypes allow for the generation of sufficient energy for constitutive proliferation and evade the checkpoint controls that would normally block uncontrolled proliferation (Cairns *et al.*, 2011). These changes largely contribute to the viral susceptibility of cancerous cells; OVs infecting these cells therefore do not face the problem of preventing the cell from shutting down its protein synthetic machinery or entering prematurely into apoptosis (Naik and Russell, 2009). In addition, signals leading to cell death or proliferation are intimately related to antiviral and interferon (IFN) responses such as the Janus Kinase/ Signal Transducer and Activator of Transcription (JAK/STAT) pathway, whose activation trigger is largely responsible for antiviral state amplification and also controls cell survival and proliferation (Constantinescu *et al.*, 2008). Other examples of the relation between cell cycle regulation, apoptosis and antiviral response includes the relationship between tumor suppressor and cell cycle regulator protein p53 and type I IFN proteins IFNα/β (Takaoka *et al.*, 2003), and the dual role of interferon regulatory factor 3 (IRF3) transcription factor in regulating both IFN and the antiviral state as well as apoptosis (Solis *et al.*, 2006). Therefore, although distinct at some point, cancer mutations that interfere with innate immune signaling and mutations that prevent apoptosis or suppression of protein synthesis are closely related and both contribute to making cancer cells susceptible to OVs.

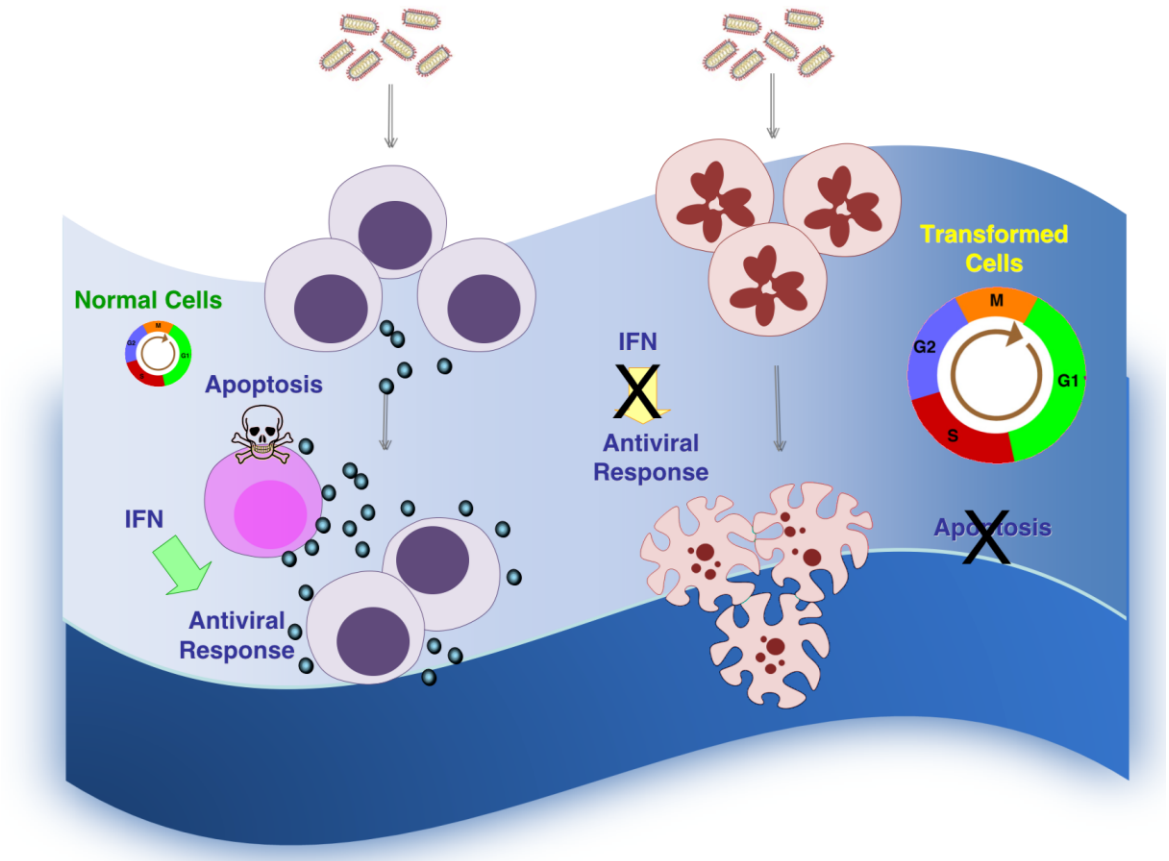

Figure 1. Schematic representation of the difference between normal cells and cancer cells that dictate OV tropism. Normal cells, have a regulated cell cycle and growth rate, produce and respond to IFN by inducing antiviral state or apoptosis and are therefore resistant to OV infection. Cancer cells are continuously cycling and possess an unregulated growth rate, associated with the lack of response to IFN or the induction of apoptosis, which renders them susceptible to OVs.

1.1.3 OV diversity

OVs are defined as replicative competent viruses as opposed to replicative defective viruses, which are only used as viral gene vehicles. The OV family can be separated into two distinct subgroups: (1) wild-type viruses or naturally occurring OVs, and (2) viruses with deleted genes. The first subgroup is composed of viruses that naturally replicate in cancer cells, while the second includes viruses that have been modified to improve their cancer specificity. Modifications are designed to delete viral genes that are essential for replication in normal cells but are dispensable in cancer cells.

The OVs that are most commonly used and studied are presented in the following sections with a focus on current hypotheses and knowledge of their tumor selectivity mechanisms. This list is not exhaustive, and other important OVs include Measles virus, Newcastle disease virus, etc. One prototypical OV is Vesicular Stomatitis virus (VSV); this virus is the main focus of the work presented here, and will be described in detail in section 1.2.

1.1.3.1 Reovirus

Reoviruses - the family name *Reoviridae* originates from the acronym "respiratory enteric orphan virus" - are characterized by non-enveloped icosahedral capsids and segmented double stranded RNA (dsRNA) genomes. Reovirus is widely distributed and can infect many types of mammals, including humans. Human infection is non-pathogenic and usually not associated with symptoms. The virus is found naturally in sewage and the water supply, which explains its mode of transmission and its high exposure prevalence in the population (Schiff *et al.*, 2007). The reovirus group contains three serotypes and the prototype strain "type 3 Dearing" (T3D), which was isolated from the intestinal track of an apparently healthy individual, is the strain most widely used for OV purposes (Van Den Wollenberg *et al.*, 2009). Because no modifications of the wild-type isolate were necessary, Reovirus is considered a naturally occurring OV.

Reovirus' preference for transformed cells probably involves many components. The most accepted mechanism is mediated through the Ras signaling pathway. Ras protein family members are involved in the regulation of a variety of cellular functions including cell growth, differentiation and cell survival (Shmulevitz *et al.*, 2005). Considering the central role of Ras signaling, it is not surprising that its constant activation found in many cancer cells sensitizes these cells to Reovirus replication. The interferon-inducible dsRNA-dependent protein kinase (PKR) was identified as one of the main Ras targets involved in Reovirus cancer specificity (Shmulevitz *et al.*, 2005; Strong *et al.*, 1998). PKR is well known to regulate translation in response to stress and its low expression level can be upregulated in response to IFN. PKR recognizes dsRNA, dimerizes upon activation and then phosphorylates the eIF2α translation initiation factor, thus preventing the loading of Met-tRNA and the formation of the initiation complex, to block translation of viral and cellular proteins (Williams, 1999). The current hypothesis is that in untransformed cells, PKR activation following Reovirus RNA recognition blocks translation and abrogates the infection cycle, while in transformed cells the presence of highly active Ras prevents the phosphorylation of PKR and permits viral protein translation (Shmulevitz *et al.*, 2005; Strong *et al.*, 1998; Van Den Wollenberg *et al.*, 2009).

Bearing in mind the complexity of Ras downstream elements it is likely that the functional consequences of Ras signaling sensitizing to Reovirus are multiple. Another recently reported aspect critical in determining cells' sensitivity to Reovirus is viral uncoating. As a consequence of its capsid structure, the Reovirus replication cycle requires uncoating before the first viral transcription can occur. Capsid proteolysis is an essential step that can either be performed in the endosome vacuole following virus endocytosis or can be mediated by a variety of extracellular proteases that generate intermediate subviral particles (ISVP) (Schiff *et al.*, 2007). Upregulation of cellular proteases such as cathepsin B and L have been reported in cancer cells. *In vitro* protease treatment of Reovirus virions - experimentally generated ISVP - significantly increased their ability to infect parent cells, and therefore tumor cells' uncoating capacities were

proposed to contribute to Reovirus susceptibility (Alain *et al.*, 2007; Marcato *et al.*, 2007). This is supported by experiments demonstrating that *in vitro* Reovirus-resistant cell lines are, in the context of an increased protease presence of the tumor microenvironment, sensitive to Reovirus oncolysis *in vivo* (Alain *et al.*, 2007; Lemay *et al.*, 2007). Both Reovirus uncoating events and Ras-mediated PKR inactivation likely contribute to Reovirus natural cancer cell selectivity, although other factors are most likely involved as well.

1.1.3.2 Vaccinia virus

Vaccinia virus (VV) is renowned for its crucial role in perhaps the most successful vaccination campaign in history, the eradication of smallpox disease in 1979 (Ellner, 1998). VV is part of the orthopoxvirus family, which also includes Cowpox virus and the smallpox-causing agent Variola virus; they all share more than 90% homology. As a consequence of its extensive use in vaccines, VV has been widely propagated for over 200 years and probably no longer exists in a natural host (Moss, 2007). The long history of VV use in humans is an important asset for an OV as proof of safety. VV has a large dsDNA genome of approximately 190 kilobase (kb) that encodes for over 200 proteins. Due to selective pressure from the innate immune response, VV encodes various proteins that target almost every aspect of the host antiviral response, including inhibition of complement activation, binding to type I and II IFN or IL-18, RNA binding proteins that prevent the activation of PKR, proteins involved in the activation of the cell cycle or the blocking of apoptosis and others (Moss, 2007). Because VV has evolved many gene products to manipulate different pathways that are already altered during malignant evolution of tumor cells, such viral genes are dispensable for the replication of VV in these cells. The fact that cancer cells are already in an optimal state for VV replication provides an opportunity to create VV strains that are dependent on this cellular status.

VV strains with specific viral immunomodulatory gene deletions have been created and greatly reduce the ability of the virus to replicate in normal cells. Two

important examples of deletions found in VV OV strains involve the viral TK and the vaccinia growth factor (VGF) (Kirn and Thorne, 2009). TKs are cellular and viral enzymes involved in the purine synthesis pathway. Their key function is to produce deoxythymidine, which is essential for DNA synthesis (Kauffman and Kelly, 1991). Cellular TKs are only expressed in a transient manner in normal cells, and therefore VV has evolved to express its own TK in order to fulfill its replication needs in nucleotides when infecting normal cells. In contrast, cancer cells constitutively express TK, and consequently a sufficient amount of deoxyribonucleotides are available for virus DNA replication and the VV TK is unnecessary (Buller *et al.*, 1985; Hengstschlager *et al.*, 1994). Thus, a virus defective in the TK is able to replicate preferentially in cycling cells.

VGF is an early viral protein secreted from vaccinia-infected cells that stimulates the proliferation of any adjacent quiescent cells. VGF is a close homologue of the epidermal growth factor (EGF), a secreted cellular growth factor that plays an important role in the regulation of cell growth and proliferation (Buller *et al.*, 1988). VGF significantly impacts the ability of VV to spread within normal complex tissues while, as for TK, it is dispensable for replication in actively proliferating host cells such as tumor cells (Kirn and Thorne, 2009). Other deletions in the immunomodulatory genes of VV to improve OV properties include the B18R gene, which prevents the virus from sequestering secreted IFN (Kirn *et al.*, 2007), or antiapotosis genes SPI-1 and 2 (Guo *et al.*, 2005). VV bearing multiple deletions of these virus-evading genes are demonstrated to have an enhanced safety profile, tumor selectivity and oncolytic effects, as illustrated by the VVdd strain harboring both TK and VGF deletions (McCart *et al.*, 2001).

1.1.3.3 Herpes virus

Herpes simplex viruses (HSV) are large enveloped DNA viruses with a genome of about 150 kb that encodes a least 84 proteins. There are two main types of human HSV: type 1 (HSV-1) and type 2 (HSV-2). HSV-1 is commonly known as the causal agent of cold sores, while HSV-2 is associated with sexually transmitted genital herpes. HSV has two unique biological properties that influence human disease: neurovirulence and latency. HSV has the ability to invade and replicate in the central nervous system, which can result in a severe neurologic disease known as HSV encephalitis. This neurotropism contributes to the establishment of latency, providing a reservoir for virus recurrence. Reemergence occurs following diverse external factors including immune suppression, where the virus in a nerve cell becomes active and is transported via the nerve's axon to the skin, where lesions may occur (Roizman *et al.*, 2007; Shen and Nemunaitis, 2006). Despite its neurotropism, HSV-1 possesses many of the previously mentioned characteristics desirable for an OV, including a large genome allowing the deletion/replacement of up to 30 kb, no integration step even if its replication occurs in the nucleus, and the availability of a number of antiherpetic drugs (Varghese and Rabkin, 2002). As for VV, and especially considering its neurovirulence, HSV-1 OV strains were engineered to inactivate or delete viral genes that are essential for viral replication in normal cells but dispensable in tumor cells.

Important gene deletions that render HSV-1 a safe OV include ICP34.5, the major determinant of HSV-1 neurovirulence which allows for the bypass of PKR translation shutdown antiviral action. The ICP34.5 gene is present in two copies of the HSV-1 genome and the deletion of both genes correlates with avirulence in animal models while maintaining oncolytic properties (Chambers *et al.*, 1995; Randazzo *et al.*, 1995). A second virulence factor that can be deleted in HSV-1 is the viral TK enzyme, on the same basis as its deletion in VV (Martuza *et al.*, 1991). ICP6, which is involved in the viral ribonucleotide reductase required for efficient viral DNA replication, can also be deleted without compromising the OV properties of HSV-1 (Mineta *et al.*, 1994). A last example of a commonly deleted gene is ICP47, a viral protein responsible for inhibiting the

transporter associated with antigen presentation (TAP), thus blocking major histocompatibility complex (MHC) class I antigen presentation. As most of the HSV-1 strain carrying only one mutation often retained residual toxicity in normal cells, strains carrying multiple alterations were created to increase safety without compromising cancer therapeutic efficacy (Mineta *et al.*, 1995; Todo *et al.*, 2001).

1.1.3.4 Adenovirus

Adenoviruses are non-enveloped viruses with a linear, dsDNA genome of approximately 36 kb. Adenoviruses have been isolated in vertebrates from fish to humans and are highly diversified, including 51 serotypes in the human family alone. The different adenoviruses cause a wide variety of common and sporadic infections; most infections of the upper respiratory tract are associated with mild or no symptoms but can also lead to more severe diseases (Wold and Horwitz, 2007). Most OV adenoviruses are derived from serotype 5 of species C (Yamamoto and Curiel, 2010). Non-replicative Adenoviruses have been widely used as gene delivery tools over the past two decades and strategies to generate replicative competent Adenovirus OVs have been developed based on two approaches: mutations in critical viral genes that are compensated by tumor cell defect, or cancer-specific control of essential viral genes via cancer-specific promoters. The major viral genes involved in these replication specificity approaches are early genes in the E1 region implicated in the control of the innate antiviral immunity (Green and Seymour, 2002; Naik and Russell, 2009).

E1A encodes two transcripts and functions to stimulate S phase entry, which trans-activates both viral and cellular genes that are critical for a productive viral infection (Wold and Horwitz, 2007). Considering its crucial role in viral replication by increasing the rate of viral transcription, the E1A coding region has been placed under the regulation of different cancer-specific promoters (Kurihara *et al.*, 2000; Rodriguez *et al.*, 1997). This strategy restricts E1A coding region expression in a cancer cell based on the aberrant overexpression of particular genes in different forms of cancers, for example

genes that are normally exclusively expressed during early embryonic development for which the expression reoccurs during the malignancy process. The use of strategies employing cancer-specific promoters driving the expression of important OV genes is not exclusive to Adenovirus and has been employed with other DNA viruses such as HSV (Chung *et al.*, 1999; Kasuya *et al.*, 2004). In addition to this approach, E1A deletions have also been reported and generated more attenuated OV strains (Heise *et al.*, 2000).

The second region is E1B, a region that encodes two proteins shown to interfere with apoptosis. E1B-55K binds p53, a transcription factor that activates genes leading to cell-cycle arrest or apoptosis, and delays cell lysis during viral replication. E1B-19K binds to pro-apoptotic B-cell lymphoma 2 (BCL-2) family members (apoptosis regulator proteins), preventing them from co-oligomerizing, forming pores in the mitochondrial membrane and inducing apoptosis (Wold and Horwitz, 2007). Blocking the apoptotic process is an essential step in the infection of normal cells but is not required in cancer cells. Therefore, deletion in the E1B region generates an Adenovirus that selectively infects tumor cells. One of the more renowned examples is the ONYX-015 Adenovirus strain, which was the first engineered replication-selective virus used in humans (Liu *et al.*, 2007). ONYX-015 contains a deletion in the E1B region and has been proposed to preferentially replicate in p53 mutant cells (Bischoff *et al.*, 1996). Their are currently debates regarding the mechanism of ONYX-015 tumor cell selectivity (Goodrum and Ornelles, 1998; Rothmann *et al.*, 1998), thus highlighting the fact that most OV tumor selectivity mechanisms described are only a partial answer to the phenomena and should not be taken as individual dogma but rather as interconnected factors that together permit the use of specific wild-type or engineered viruses for cancer treatment.

1.1.4 OV therapeutic efficacy

The development of efficient cell cultures for viral propagation and tumor rodent models in the early 1950s led to the first trials of OVs as a cancer treatment. In these early trials, non-engineered viruses were generally attenuated through serial passage *in vitro*. These first case reports brought proof of safety and signaled the efficacy of this emerging cancer therapy approach (Kelly and Russell, 2007; Liu *et al.*, 2007). Today, the number of OV clinical trials has expanded and many have recently entered phase III. As a result of the financial needs and commercial potential of the field, a number of biotechnology companies have now invested in this venture (Bell, 2010; Rowan, 2010). The first replicative competent OV was approved in 2005 for head and neck cancer treatment in China: H101, an Adenovirus similar to the ONYX-015 strain carrying an additional deletion in the E3 region (Garber, 2006). Safety issues are of the utmost importance in these trials, and results tend to demonstrate very limited toxicity with mild to inexistent side effects even at the highest doses tested thus far. In addition, very encouraging results concerning anti-tumor therapeutic effects are also recorded (Breitbach *et al.*, 2010; Liu *et al.*, 2007).

As an example, OncoVEX$^{GM\text{-}CSF}$, an HSV-1 virus containing a double ICP34.5 and ICP47 deletion in addition to the expression of Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF), has recently demonstrated clinical efficacy in melanoma patients. In a phase II trial, 50 patients having failed conventional and experimental prior therapies received the OV treatment as a stand-alone therapy. An overall positive therapeutic response was observed for 28% of these patients, including 10 patients experiencing complete response (Senzer *et al.*, 2009). For additional examples and information, **Table 1** presents an overview of the current stage of the most advanced OV clinical trials. Clinical trials are not exclusively concentrated on these three major OVs seeking US Food and Drug Administration (FDA) approval but also include many other viruses, such as Adenovirus, Measles virus, Newcastle disease virus and others, although these trials are much less advanced (Liu *et al.*, 2007). Of note, even though abundant literature supports the efficacy of VSV as a powerful OV, the virus has not yet entered

clinical trial studies. Patent issues are responsible for this slow start. Nevertheless, safety issues were addressed in non-human primates with positive outcomes and Good Manufacturing Practice has recently been published for VSV (Ausubel *et al.*, 2011; Jenks *et al.*, 2010). The publication of these two studies, in addition to the characterization of Maraba virus, a closely related Rhabdovirus, as an OV may reflect its development in the near future (Brun *et al.*, 2010).

The multi-faceted nature of cancer renders it unlikely that the use of a single agent will constitute a complete treatment strategy. Despite promising pre-clinical and clinical case results, OVs do not make exception in this regard. The full therapeutic potential of OVs will probably be best exploited through combination with other anticancer agents (Nguyen *et al.*, 2010). Efforts in pre-clinical approaches and clinical trials are therefore integrating combination strategies of chemotherapeutic compounds with the OV approach. Examples of such combination therapies are depicted in **Table 1**, in section 1.6.2 of the introduction chapter and are further discussed in Manuscript I presented in chapter 2.

Virus	Strain	Characteristics	Company	Target cancer	Phase
Herpes Virus	OncoVEX^{GM-CSF}	• ICP34.5 (2X) and ICP47 deletion • Expresses GM-CSF	BioVex, Massachusetts and UK	Metastatic melanoma	III
				Head and neck cancer	III
Reovirus	Reolysin	Naturally occurring OV	Oncolytics Biotech, Calgary	Head and neck cancer (in combination with carboplatin and paclitaxel)	III
				Ovarian, peritoneal or fallopian tube carcinoma	I/II
				Malignant gliomas	I/II
				Metastatic pancreatic adenocarcinoma (in combination with gemcitabine)	II
				Non-small-cell lung cancer (in combination with paclitaxel and carboplatin)	II
				Head and neck cancer (in combination with paclitaxel and carboplatin)	II
				Metastatic melanoma (as a single agent or in combination with paclitaxel and carboplatin)	II
				Squamous cell carcinoma; lung	II
Vaccinia	JX-594	• TK deletion • Expresses β-Galactosidase and GM-CSF	Jennerex, San Francisco and Ottawa	Hepatocellular carcinoma (as a single agent or in combination with sorafenib)	II
				Metastatic colorectal cancer	II

Table 1. Principal OVs in advanced clinical trial stages. Adapted with permission from (Rowan, 2010).

1.2 Vesicular Stomatitis virus

VSV is a member of the *Rhabdoviridae* family, which can be found in plants, invertebrates and vertebrates. The family consists of more than 185 different viruses and includes the well-known Rabies virus. Rhabdoviruses are enveloped bullet-shaped viruses containing a single-stranded nonsegmented negative-sense RNA genome (Lyles and Rupprecht, 2007).

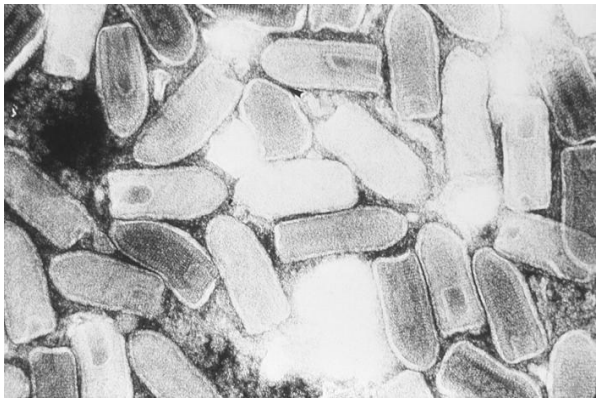

Figure 2. Electron microscopy picture showing the bullet form of VSV virions. Virions are approximately 180 nm long and 80 nm in diameter (Schnell *et al.*, 1996a) (Image source: Centers for Disease Control and Prevention, Atlanta, GA).

VSV is mainly found in horses, cattle, swine, mosquitoes and sandflies. Different strains of VSV have been isolated from all over the world and have been responsible for several outbreaks in cattle in the US, Canada and South America. The virus produces an acute disease in cattle, characterized by fever and vesicles as well as ulceration in the mucosa of the oral cavity (Lichty *et al.*, 2004; Lyles and Rupprecht, 2007). Transmission between infected animals has been proposed to be mediated via insect vectors (Mead *et al.*, 2000). Laboratory infection of mice with wild-type strains often lead to neurotoxicity. Human infection is rare and remains mainly asymptomatic or causes mild flu-like symptoms. In regions endemic for VSV, individuals seropositive for VSV antibodies are common and can reach high percentages of the population (Lichty *et al.*, 2004; Strauss and Strauss, 2008).

1.2.3 Molecular virology of VSV

1.2.3.1 VSV proteins and general structure

VSV possesses a genome of approximately 11 kb that encodes for five proteins. The nucleoprotein N forms a complex with the RNA genome to generate the ribonucleocapsid. In this complex each N protein covers nine base pairs of the RNA genome, therefore approximately 1200 copies are required to cover the entire genome (Thomas *et al.*, 1985). This structure protects the virus from cellular nucleases because the genome is never found as a naked RNA. The phosphoprotein P and the large polymerase protein L form the viral RNA-dependent RNA polymerase. P is responsible for binding the L polymerase to the N protein-RNA template and L is responsible for enzymatic activity. In the virion, the nucleocapsid is associated with approximately 466 copies of P and 50 copies of L (Lyles and Rupprecht, 2007). The matrix protein M is present in approximately 1800 copies per virion and appears to serve as a link between the nucleocapsid and the envelope by interacting with both components (Thomas *et al.*, 1985). The M protein also gives the bullet shape structure of the virion (Lyles *et al.*, 1996; Newcomb and Brown, 1981). In addition, the M protein plays a crucial role in controlling VSV replication and pathogenesis and has previously been qualified as the "brain" of the virus (Lichty *et al.*, 2004). The virus envelope consists of a lipid bilayer derived from the host cell membrane during assembly and budding. The envelope is covered with approximately 300 to 400 spikelike trimers of the glycoprotein G for virus attachment and penetration (Lyles and Rupprecht, 2007; Whitt *et al.*, 1991).

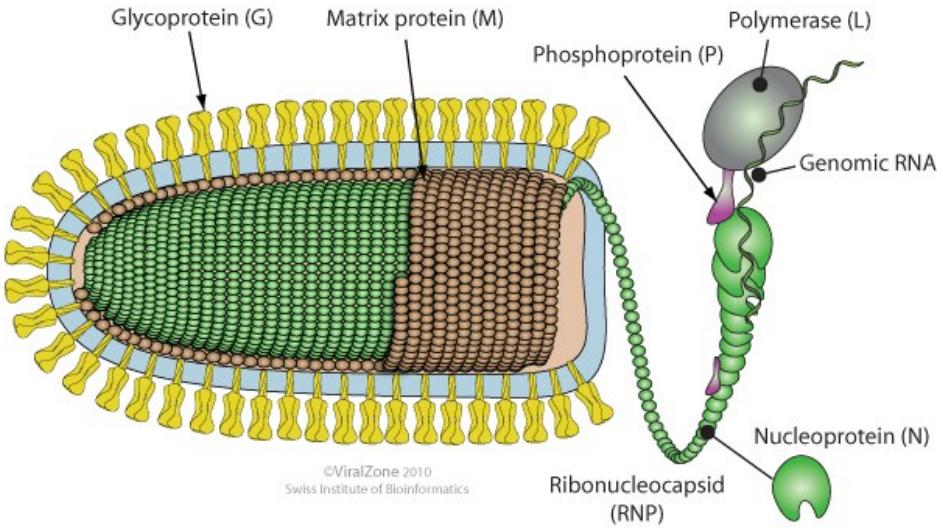

Figure 3. Illustration of VSV virion and the different viral proteins. The viral ssRNA- genome is covered with the N protein to form the ribonucleocapsid. The P and L proteins form the functional viral RNA polymerase, while the M protein links the ribonucleocapsid with the membrane containing the G trimer viral receptor. Reproduced with permission from (Hulo *et al.*, 2011).

1.2.3.2 VSV genome

The VSV RNA genome is composed of a leader region at the 3' end, five open reading frames and a trailer region at the 5' end. The genome is transcribed by the viral RNA-dependent RNA polymerase complex into five mRNA and a 47 nucleotide (nt) leader RNA of unknown function. The leader and trailer regions serve as promoters for transcription and replication of genomes and replication-intermediate antigenomes, and as signals for encapsidation following replication (Wertz *et al.*, 1994). Each intergenic region contains a highly conserved sequence serving as a stop and start signal for the polymerase (see **Figure 4**) (Rose, 1980). The genome is transcribed in a sequential event starting at the 3' leader region, which acts as a promoter, and moving to the 5' end (Barr *et al.*, 2002). Because the polymerase fails to re-initiate transcription for the subsequent mRNA around 30% of the time, viral gene mRNA expression is much more abundant in

the genes closer to the 3' end and decreases by about 30% at each junction (Iverson and Rose, 1981). This gradient concentration of mRNA results in the same decrease in protein abundance. Of note, the G-L junction generates a much higher level of polymerase re-initiation failure and consequently a much lower expression level of L protein (Ball *et al.*, 1999). Following elongation, the viral polymerase encounters a U-trap that results in the poly-adenylation of the mRNA with the addition of approximately 200 adenosine (Barr and Wertz, 2001). The viral polymerase is also responsible for the mRNA capping reaction with a 5' methylated guanoside triphosphate (Grdzelishvili *et al.*, 2005).

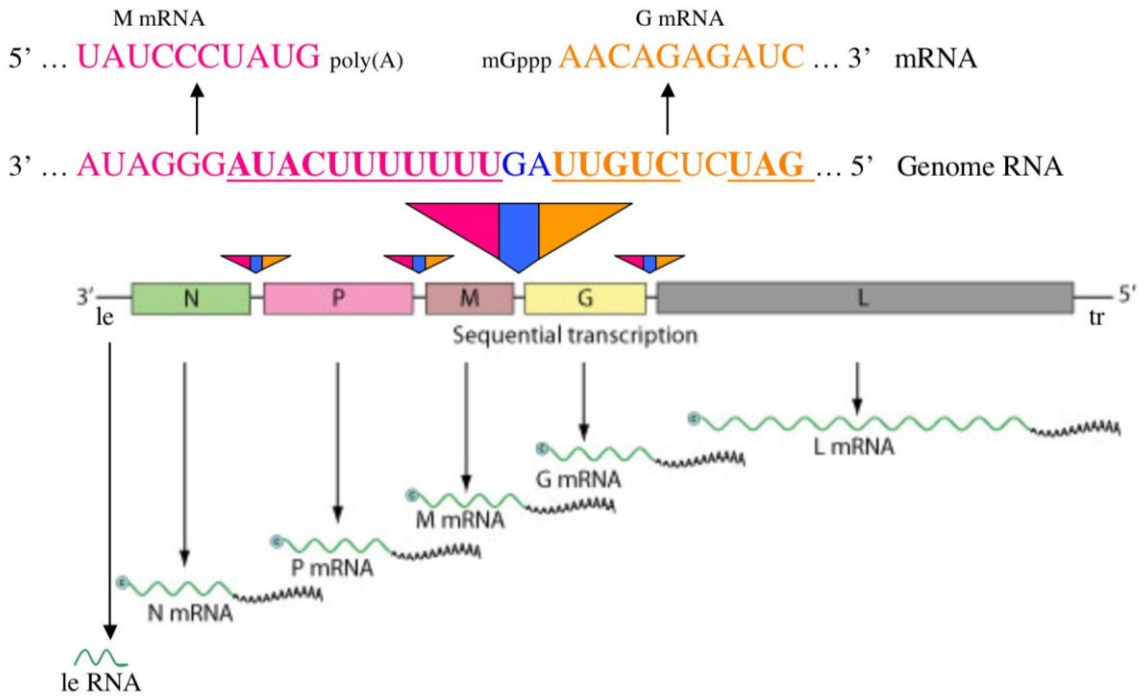

Figure 4. Schematic representation of VSV genome. The genome's different regions, gene orders and transcripts are illustrated. Each intergenic region is composed of a highly conserved ending sequence (underlined in pink), two variable nucleotides (in blue) and a second highly conserved sequence serving as the beginning of the next transcript (underlined in orange). le: leader, tr: trailer. Figure adapted with permission from (Hulo *et al.*, 2011) and (Strauss and Strauss, 2008).

1.2.3.3 VSV replication cycle

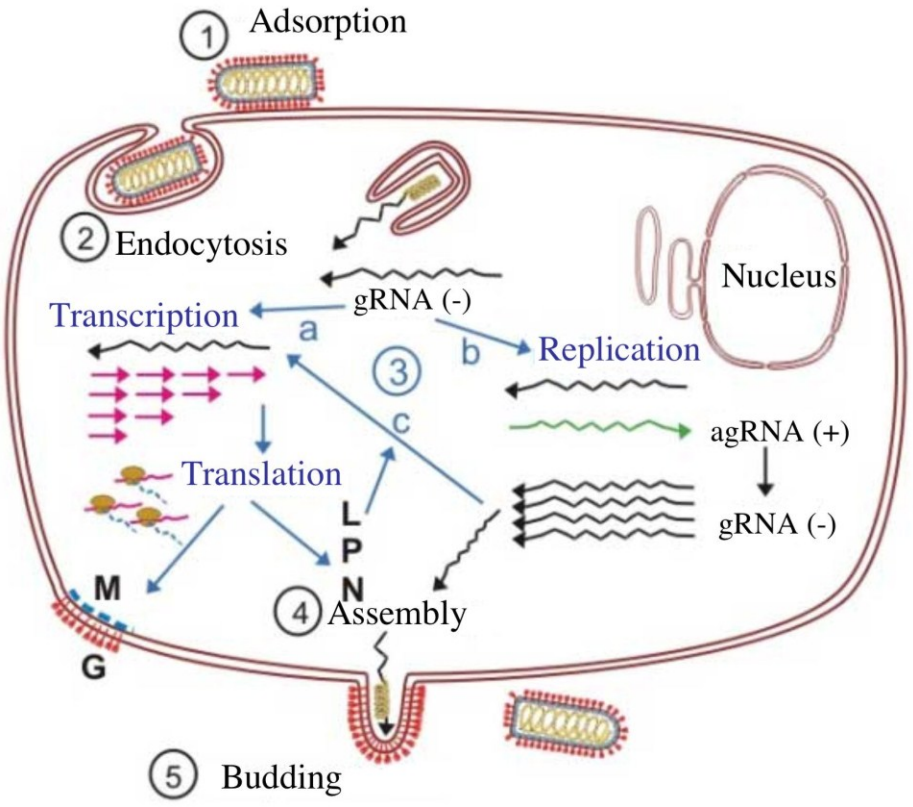

Figure 5. Overview of VSV replication cycle. Illustration of the attachment at the cell surface (1), penetration by envelope fusion with endosomal membrane (2), release of the nucleocapsid containing the genomic RNA (gRNA) into the cytoplasm, transcription of the genome (3a) and viral protein synthesis, replication of the genome to produce antigenome (agRNA) (3b), which serves as a template for the generation of progeny genome and for a second wave of transcription (3c) followed by assembly of new virions (4) and budding from the host plasma membrane (5). Adapted with permission from (Descartes, 2011).

The VSV replication cycle is an acute event that normally reaches maximal virion release within 8-10 hours of infection, decreasing towards the end of the cycle and with induction of apoptosis occurring around 16-20 hours post infection (Lyles and Rupprecht, 2007). The main features are illustrated in **Figure 5** and are presented in more detail below.

Binding at the host cell surface and fusing of viral and cellular membranes are two events mediated by the G protein. The VSV-G receptor has not been clearly identified; phosphatidylserines have been proposed to serve for viral attachment, but this description has not reached a consensus (Coil and Miller, 2004; Schlegel *et al.*, 1983). It is generally accepted that nonspecific electrostatic and hydrophobic interaction with negatively charged lipids mediate attachment of VSV to the host cell (Carneiro *et al.*, 2002; Lyles and Rupprecht, 2007). In either case, the apparent absence of a specific marker or the ubiquitous expression of phosphatidylserine enables VSV to infect virtually all animal cells (Lichty *et al.*, 2004). This extensive tropism represents a major advantage for its use as an OV, enabling the targeting of a variety of cancer types. As an example of VSV-G broad tropism application, the Human immunodeficiency virus (HIV) research field routinely uses VSV-G to generate pseudotyped-HIV - HIV particles coated with VSV-G trimers - to permit the *in vitro* infection of a variety of cell types without requiring the typical HIV receptor and co-receptor (Akkina *et al.*, 1996; Cronin *et al.*, 2005).

Following attachment, the G protein mediates the penetration of the virion into the cell via a clathrin-dependent endocytosis mechanism. Clathrin is a cellular protein that plays a major role in the endocytosis process by promoting the formation of coated vesicles. The VSV-containing vesicle will subsequently loose its clathrin coat and become an early endosome, which will then fuse with late endosomes and lysosomes. During this process the virion is progressively exposed to a lower pH, which induces the G protein to mediate the fusion of the viral envelope with the endosomal membrane, and consequently the release of the nucleocapsid into the cytoplasm (Matlin *et al.*, 1982)(Durrer *et al.*, 1995). Alternatively, the virion content may be released into the

internal vesicle and require further fusion with the late endosome membrane (Le Blanc *et al.*, 2005).

Once in the cytoplasm, the nucleocapsid is believed to spontaneously dissociate from the M protein that appears to be a soluble protein (Rigaut *et al.*, 1991). The RNA-dependent RNA polymerase is then free to initiate the primary transcription of the viral mRNA from the encapsidated RNA. As mentioned above, the two subunits of the RNA polymerase each have a distinct role, with P binding the L polymerase to the N protein-RNA template and L performing the enzymatic activities. The RNA polymerase performing transcription is composed of four P proteins and one L protein and appears to incorporate an N protein in the complex when performing genome replication (Gupta *et al.*, 2003). As described earlier, the stop/start transcription mechanism of VSV results in a gradient of the different mRNAs that are subsequently translated by the host cellular machinery. The accumulation of viral proteins, especially the N protein, dictates the transcription versus replication mode of the viral RNA polymerase. The replication process is dependent on the presence of newly synthesized N protein to encapsidate the new genomes (Patton *et al.*, 1984). The encapsidation of the newly synthesized RNA also appears to involve the P protein (Peluso, 1988) and sanctions the polymerase to ignore the stop/start sequences and synthesize a full complementary genome (antigenome). Encapsidated antigenome then serves as the template for negative-sense genome replication. As an outcome of sequence differences between the leader and trailer regions, more genomes than antigenomes are synthesized (Finke and Conzelmann, 1997). The accumulation of sense genomes following this step generates a second wave of viral transcription. Viral assembly then takes place concomitantly with the transcription amplification.

Following production, viral proteins are redirected to different cell compartments in order to perform their functions. As previously mentioned, the N protein is found in the cytoplasm associated with the RNA genome along with the P and L polymerase proteins. On the other hand, the G protein is constantly bound to the membrane through its synthesis with ribosomes bound to the endoplasmic reticulum

(ER), chaperone folding and trimer association at the ER, posttranslational modification at the Golgi and final "host destination", the plasma membrane (Bergmann *et al.*, 1981; Doms *et al.*, 1988; Katz and Lodish, 1979). The M protein is synthesized as a soluble protein found in the cytosol of infected cells, and is to a smaller extent associated with the membrane (Knipe *et al.*, 1977; Ohno and Ohtake, 1987). At the time of virion assembly, the M protein binds the nucleocapsid to the host plasma membrane containing the G trimers and condenses the nucleocapsid into a tightly coiled complex (Flood and Lyles, 1999; Odenwald *et al.*, 1986). By interacting with host proteins involved in the multivesicular bodies/endosome machinery, the M protein also mediates the budding of the complex from the plasma membrane (Harty *et al.*, 2001). The subsequent lysis of the host cell ends the cycle and potentially releases up to 100 000 virions/cells, although a high percentage of these virions are defective particles (Barber, 2005).

1.2.3.4 Molecular and cellular basis of VSV pathogenesis

The infection of a host cell by VSV is a multi-faceted event: on the one hand, the cell's survival mechanism has evolved to protect itself and its neighbors, while on the other hand the virus attempts to block these reactions, and moreover redirects all cellular machinery for its own purpose. Host cells defend themselves by sensing the infection and triggering signalization and subsequent induction of an antiviral state (described in more detail in section 1.3). Host cell protection mainly involves the induction of proinflammatory cytokines and antiviral genes such as type 1 IFN. VSV is very sensitive to the IFN antiviral response and has evolved different mechanisms to counteract the cell by blocking host gene expression. VSV inhibits both host transcription and translation almost completely within the first 4-6 hours of infection (Ahmed *et al.*, 2003). This strategy also ensures that the host protein translation machinery is available for viral replication.

The viral protein responsible for this viral evasion mechanism is the M protein. The mechanism can be separated into three aspects: inhibition of host mRNA transcription, transport from the nucleus to the cytoplasm and translation. The M protein

was demonstrated to inhibit transcription from the host RNA polymerases I, II and III (Ahmed and Lyles, 1998). More precisely, in the case of RNA polymerase II, M has been shown to inhibit the general host transcription TFIID factor, resulting in the inhibition of transcription (Yuan *et al.*, 2001; Yuan *et al.*, 1998). Secondly, direct evidence of interaction between the M protein and RNA export 1 (Rae1), a protein involved in nuclear-cytoplasmic mRNA transport, was demonstrated (Faria *et al.*, 2005). Rae1 is known to interact with the nuclear pore component 98 (Nup98), and therefore interference by VSV with these cells' regulatory proteins results in the blocking of host mRNA transport (Enninga *et al.*, 2002). Lastly, VSV blocks host mRNA translation while pursuing the translation of the viral mRNA. Blocking of host mRNA translation appears to be mediated by the inactivation of eukaryotic initiation factors, resulting in the abrogate formation of the mRNA-ribosome complex and absence of translation (Connor and Lyles, 2002; Connor and Lyles, 2005). Although the mechanism of viral mRNA preferential translation over the host mRNA has yet to be elucidated, major aspects have been identified. Viral mRNA preferential translation is conferred from the viral genome and is not related to cis-acting elements such as internal ribosome entry sites; this is supported by the observation that recombinant viruses have their transgene expressed at a similar level to the native viral genes (Schnell *et al.*, 1996b; Whitlow *et al.*, 2006). The timing of the mRNA appearance during infection appears to be crucial, such that newly synthesized mRNA are translated in favor of the host mRNA synthesized prior to infection (Whitlow *et al.*, 2008). In summary, host gene expression inhibition by VSV represents an important evasion mechanism mediated by the M protein.

Another aspect of pathogenesis mediated by the M protein is the induction of apoptosis (Kopecky *et al.*, 2001). Apoptosis is generally accepted as a host defense mechanism to limit viral spread and is often blocked or controlled by viruses. VSV is a potent inducer of apoptosis and the absence of blocking strategies is most likely due to the rapidity of its replication cycle, which is often complete before the cell has a chance to undergo apoptosis (Lyles and Rupprecht, 2007). VSV-induced apoptosis has been shown to employ both intrinsic mitochondrial pathways and extrinsic death receptor pathways (Cary *et al.*, 2011; Gaddy and Lyles, 2005; Pearce and Lyles, 2009).

1.2.3.5 VSV MΔ51

Naturally occurring attenuated strains of VSV have been isolated in laboratories for more than 40 years (Flamand, 1970). One of the most characterized strains of VSV is an M protein mutant. First isolated as a thermo-resistant strain in 1972 (Farmilo and Stanners, 1972), the virus has since been characterized for its reduced cytopathogenicity and inhibition of protein synthesis capacities (Stanners *et al.*, 1977). The phenotypic attribute was then mapped to a single amino acid substitution from a Methionine to an Arginine at position 51 of the M protein (VSV M51R) (Desforges *et al.*, 2001). Stojdl *et al* were the first to use the strain for OV purposes in 2003. In order to eliminate any chance of reversion, a strain containing a complete deletion of the Methionine 51 has been created and is termed VSV MΔ51 (Stojdl *et al.*, 2003).

VSV MΔ51 is deficient in its ability to inhibit both host RNA/protein synthesis and nucleocytoplasmic RNA transport. Therefore, VSV MΔ51 allows host gene expression and the subsequent establishment of an antiviral state (Ahmed *et al.*, 2003). As a consequence, VSV MΔ51 induces much more IFN than its wild-type counterpart. The production of IFN - also referred to as cytokine cloud - induces an antiviral state in non-infected non-cancerous cells, thus protecting them from infection (Lichty *et al.*, 2004). VSV MΔ51 demonstrates a profound diminution of neuropathogenicity observed in mice; for example, mice injected intravenously can tolerate 80 times more VSV MΔ51 than wild-type VSV, and the LD50 (Lethal Dose, 50%) of VSV MΔ51 is 10 000 times greater than that of the wild-type strain (Stojdl *et al.*, 2003). Considering the multiple functions of the M protein, the difference between the two strains is not restricted to this IFN aspect. For example, VSVs expressing wild-type or mutant M protein have been shown to activate apoptosis through distinct pathways (Gaddy and Lyles, 2005). The increased safety profile and therapeutic index conferred to VSV MΔ51 does not compromise its oncolytic properties and renders the strain even more potent as an OV (see **Table 2**).

1.2.4 VSV as an OV

VSV possesses many of the key characteristics necessary for an OV. First, it efficiently targets a wide variety of cancer types, as shown by the high percentage of sensitive cells in a panel of 60 cancer cell lines from the National Cancer Institute (NCI60) (see **Table 2a**). The virus is non-pathogenic for humans and preexisting immunization is rare in the industrialized countries, thus minimizing the possibility of inactivation of a VSV-based OV treatment. Due to its strictly cytoplasmic replication cycle, there is no chance of host cell genome altercation. Although the polymerase of RNA viruses has an error rate of approximately 1/10 000, hence theoretically resulting in around one base pair substitution per VSV genome replication (Drake and Holland, 1999), this virus is considered to be genetically stable when compared with other viruses known for their high mutation rate and taking into consideration the absence of recombination or rearrangement possibilities. For example, the HIV reverse transcriptase is known to have an average error rate per nucleotide incorporated of 1/1700 (Roberts *et al.*, 1988). The basic virology of the virus is very well documented, and the ease of producing high viral titer stocks also contributes to the value of VSV as an OV candidate (Barber, 2005; Lichty *et al.*, 2004). In addition to these characteristics, VSV has proven its oncolytic efficiency against established tumors in a number of preclinical animal models (Balachandran *et al*., 2001; Ebert *et al*., 2005; Ebert *et al*., 2003; Shinozaki *et al*., 2005b; Stojdl *et al*., 2000; Stojdl *et al*., 2003).

	a. Sensitivity to infection		**b.** IFN defect
	VSV	VSV M51R	
Leukemia	67% (4/6)	nd	100% (6/6)
Non-small-cell lung carcinoma	78% (7/9)	60% (3/5)	71% (5/7)
Colon carcinoma	86% (6/7)	100% (5/5)	100% (7/7)
Central nervous system	80% (4/5)	50% (1/2)	75% (3/4)
Melanoma	75% (6/8)	100% (2/2)	85% (6/7)
Ovarian carcinoma	100% (6/6)	67% (2/3)	67% (4/6)
Renal carcinoma	88% (7/8)	100% (3/3)	75% (6/8)
Prostate	100% (2/2)	100% (2/2)	100% (2/2)
Breast	83% (5/6)	75% (3/4)	60% (3/5)
All cell lines tested	82% (47/57)	80% (21/26)	81% (42/52)

Table 2. VSV is highly lytic to the members of the NCI60 panel of cancer cell lines. **a.** Percent of NCI60 panel cell lines by tumor type that denoted susceptibility greater than or equal to EC50 at 1 MOI of VSV wild-type or VSV M51R for a period of 48h. **b.** Percent of cell lines that were unresponsive to IFN protective treatment prior to VSV infection. Adapted with permission from (Stojdl *et al.*, 2003).

1.2.4.1 VSV cancer cell tropism dogma

One of the major advantages of using VSV as an OV is its sensitivity to the IFN anti-viral response. In addition to its safety component, allowing human hosts to efficiently control infection, this hypersensitivity is the basis for the main dogma of VSV cancer cell selectivity. Due to its acute lytic replication cycle, VSV is a potent inducer of IFN. Normal tissue and normal cells are responsive to IFN and induce an anti-viral state that protects them from VSV infection. However, many transformed cells harbor defects in the signaling leading to IFN production and are unresponsive to IFN. This is not surprising, considering the relationship between the innate IFN signaling and cell cycle / apoptosis regulation pathways (Constantinescu *et al.*, 2008). The main accepted dogma is that VSV exploits this tumor cell deficiency and selectively replicates and lyses cancer cells, while normal cells are left unaffected due to the protective effect of IFN and anti-viral response. This is supported by strong evidence showing that IFN pretreatment of primary cells in cultures completely abrogates VSV replication while tumor cell lines are less affected (Balachandran and Barber, 2000; Stojdl *et al.*, 2000). The broad application of this explanation is illustrated in **Table 2b,** which shows that about 80% of the NCI60 cancer cell panel are unresponsive to IFN pretreatment. Further supporting the concept that normal tissues are protected by IFN-mediated responses is evidence demonstrating that mice lacking intact IFN mechanisms such as STAT1 or S6K1/2-deficient mice are more susceptible to VSV infection than their wild-type counterparts (Alain *et al.*, 2010; Meraz *et al.*, 1996). In addition, the use of drugs dampening the IFN response in conjunction with VSV oncolysis treatment permits enhanced viral replication at the tumor site and improves survival of tumor-bearing animals (Alain *et al.*, 2010; Nguyen *et al.*, 2008).

Aside from the IFN system, other key cellular pathways found to be defective in transformed cells may also cooperatively contribute to VSV preferential oncolysis. Balachandran *et al.* have argued that PKR and defects in translational control of cancer cells contribute, along with IFN signaling, to VSV onco-selectivity (Balachandran and Barber, 2004). In addition, Oliere *et al.* have demonstrated that VSV replication in

primary T lymphocytes relies on cell cycle transition (Oliere *et al.*, 2008). Translation control, cell cycle, apoptosis and the IFN pathways all share a certain degree of connection and are most often found mutated in transformed cells, and may all contribute to rendering cancer cells ideal for VSV replication.

1.2.5 Engineering VSV

The development of viral genome manipulation and the advances in reverse genetic techniques have significantly benefited our understanding of basic virology and virus behavior. The first viruses to be genetically manipulated were the DNA viruses, rapidly followed by the positive-strand RNA viruses (Pekosz *et al.*, 1999). For these viruses, the simple transfection of cDNA (for DNA viruses) or full-length genomic RNA (for the RNA+ viruses) into host cells was shown to be sufficient to start an infection. (Goff and Berg, 1976; Racaniello and Baltimore, 1981). The case of negative-strand RNA virus differed in many aspects and posed a greater challenge to the development of a reverse genetic system. Firstly, negative-sense genomic RNA is not infectious by itself following transfection and must be encapsidated - covered with N proteins - in order to be transcribed by the viral polymerase. Secondly, only the viral polymerase can transcribe the genome. Third, the sequence at the extremity of the genome (or antigenome) needed to be precisely cut in order to be recognized by the viral polymerase (Palese *et al.*, 1996; Rose, 1996).

The system developed to rescue infectious VSV from plasmid transfection incorporates these important aspects, which permit it to overcome the restrictions affecting negative-sense RNA virus. The technique is based on the transfection of 3 plasmids encoding for the P and L genes in order to generate the viral RNA polymerase, and for the N gene to allow the encapsidation of the newly synthetized viral RNA template along with a plasmid coding for the antigenome sequence (see **Figure 6**). The antigenome coding plasmid is used rather than a sense genome coding plasmid because the antigenome is encapsidated much more efficiently than the sense genome. It was

hypothesized that the viral mRNA encoding for the N, P and L proteins, resulting from the cotransfection with the genome encoding plasmid, would interfere with the sense genome encapsidation process because of complementarity annealing. On the contrary, these mRNAs are not complementary to the antigenome and permit the encapsidation process by the N protein, resulting in an antigenome nucleocapsid that can serve as a template for the viral polymerase.

The fully cytoplasmic replication cycle of VSV dictated the choice of bacteriophage T7 RNA polymerase to generate viral antigenomes from the transfected plasmid. Another important point of this first template is the precise end required for polymerase recognition. To meet this obligation, the hepatitis delta virus ribozyme sequence was inserted at the 3' end of the antigenome transcription unit. The ribozyme's precise cleaving of the "antigenome RNA" end combined with its efficient encapsidation generates a template that can be recognized by the viral RNA polymerase. This precisely cut encapsidated antigenome template is now capable of initiating viral replication of the sense genome, followed by the initiation of the infection cycle (Lawson *et al.*, 1995; Palese *et al.*, 1996; Roberts and Rose, 1998; Rose, 1996; Whelan *et al.*, 1995).

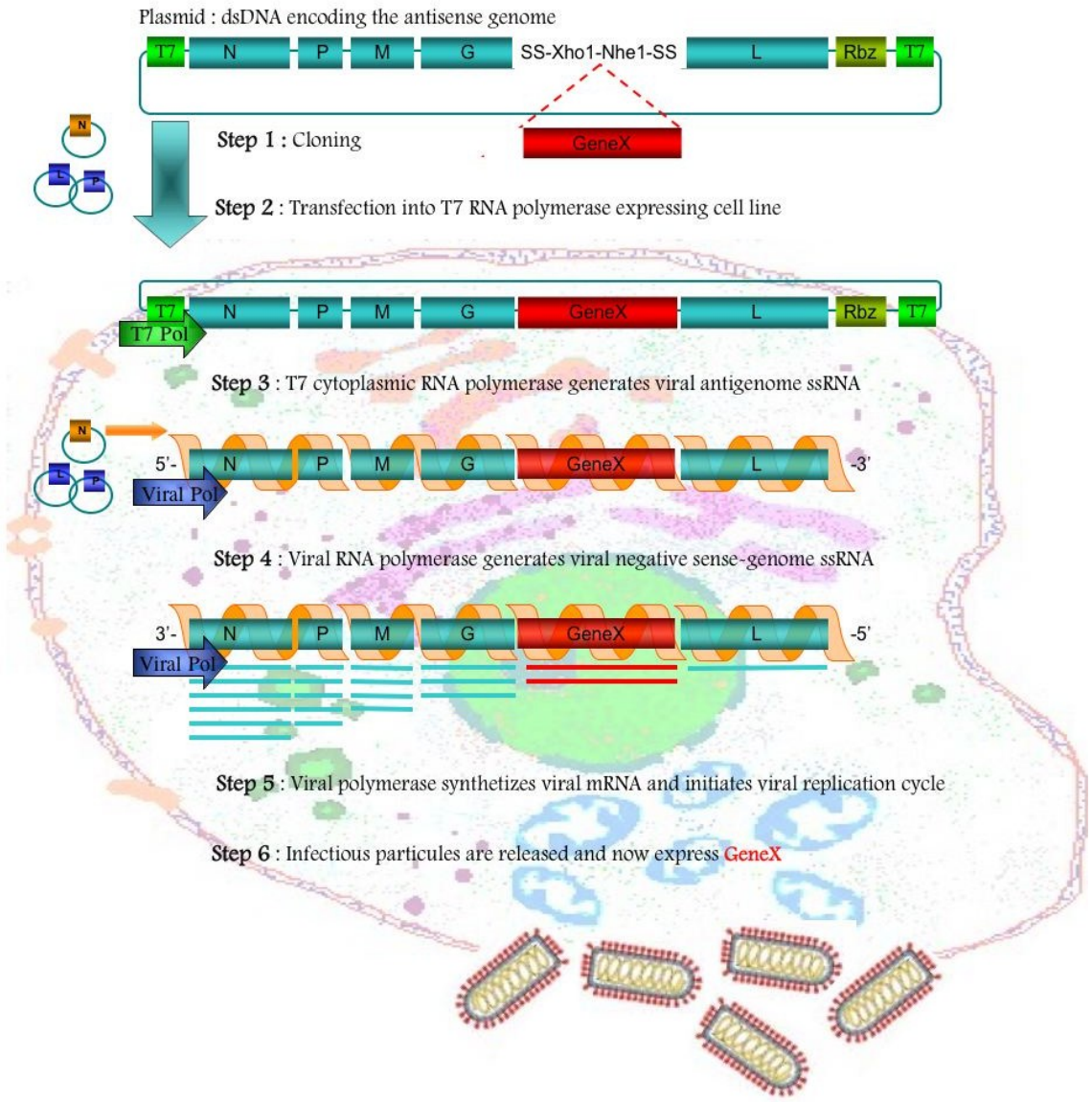
Plasmid : dsDNA encoding the antisense genome
T7
N
P
M
G
SS-Xho1-Nhe1-SS
L
Rbz
T7
GeneX
Step 1 : Cloning
Step 2 : Transfection into T7 RNA polymerase expressing cell line
T7 Pol
Step 3 : T7 cytoplasmic RNA polymerase generates viral antigenome ssRNA
5'-
-3'
Viral Pol
Step 4 : Viral RNA polymerase generates viral negative sense-genome ssRNA
3'-
-5'
Step 5 : Viral polymerase synthetizes viral mRNA and initiates viral replication cycle
Step 6 : Infectious particules are released and now express GeneX

Figure 6. Schematic representation of VSV recombinant virus rescue system. Step 1 is to clone the gene of interest between two viral polymerase stop/start sequences (SS; see Figure 4 for more details) in one of the intergenic viral regions; most of the rVSV are generated by inserting the foreign gene between the G and L genes. The flanking regions of the antigenome are composed of the T7 promoter and ending sequences (T7), as well as the hepatitis delta virus ribozyme (Rbz). **Step 2** consists of transfecting the viral genome plasmid along with the three plasmids encoding for the N, P and L genes of the virus in a cell line expressing the T7 bacteriophage RNA polymerase. For this transfection process, the plasmid ratio is crucial to allowing the proper balance between antigenome production, viral polymerase complex and N protein presence. The T7 polymerase was initially introduced into the transfected cell using a Vaccinia virus expressing the enzyme, but is now more commonly directly expressed by a stable cell line, thus avoiding a purification step from Vaccinia virus contamination following rescue. During **Step 3**, the T7 RNA polymerase synthesizes the antigenome transcript, which is encapsidated into a nucleocapsid by the presence of the N protein and is properly cleaved at its 3' end by the action of the ribozyme. In **Step 4**, the N and L viral polymerase complex recognizes the trailer region of the antigenome and performs a first replication to generate the sense genome, which is also encapsidated. At **Step 5**, viral polymerase transcribes the sense genome into viral mRNA and initiates the replication of the virus containing an external coding sequence in its genome. The release of the new infectious particles in **Step 6** constitutes a successful recovery of rVSV.

Although the insertion of an additional gene transcript into the genome could potentially reduce the efficiency of virus replication due to increased viral polymerase transcription attenuation caused by additional stop/start sequences, as well as due to the extended genome size, incorporation of a single foreign gene usually does not notably reduce virus yields (Fernandez *et al.*, 2002; Obuchi *et al.*, 2003). As the size of the virion depends on the size of the genome, incorporation of more genomic material simply increases the size of the bullet (Schnell *et al.*, 1996a). In addition, for the vast majority of recombinant VSVs (rVSVs) the foreign gene is inserted between the G and L coding sequences, a region shown to be more prone to transcription attenuation, therefore limiting the impact of reduced transcription on the subsequent viral genes (Ball *et al.*, 1999). In the absence of selective pressure rVSVs are generally stable and do not easily lose expression of their transgene despite the relatively high error rate of the RNA polymerase. For example, an rVSV encoding the T cell surface marker CD4 lost its expression only after 26 passages, while a second rVSV engineered to express the Measles virus fusion F protein, which apparently affects virus replication efficacy, rapidly lost its expression of the foreign insert (Quinones-Kochs *et al.*, 2001; Schnell *et al.*, 1996a).

Since the introduction of the rescue system for VSV in 1995, many rVSVs have been created and utilized in order to understand basic VSV virology and in vaccine development (Lichty *et al.*, 2004). For example, the phenotypical consequences of rearranging the order of the three central genes of the VSV genome were studied using a combination of six different rVSVs (Ball *et al.*, 1999); alternatively, an rVSV was generated as a vaccine platform against Ebola virus by encoding the Zaire Ebola virus glycoprotein and demonstrated encouraging results when tested in rhesus macaques (Geisbert *et al.*, 2008). In the context of VSV use for cancer treatment, the availability and relative ease of creating rVSV allows for the generation of dual-approach strategies combining the oncolysis properties of VSV with its use as a cancer-specific gene delivery system. Several rVSVs have been generated over the years to improve oncolytic efficacy or cancer cell specificity. These rVSVs will be discussed in section 1.6.3 and **Table 4**.

1.3 The antiviral immune response

Vertebrates' defense mechanisms have evolved to recognize and neutralize pathogens via two main branches: the innate and the adaptive immune response. The innate system is considered the first line of defense and occurs in the earlier stages of infection, as opposed to the adaptive response which takes place at a later stage and increases the specificity of the response. The innate response is permanently present and is not the result of previous exposure to microorganisms. Although very different, the two systems pursue the same goal and are interconnected.

1.3.1 Sensing of pathogens and activating the innate response

The early recognition of intruding pathogens represents the first crucial role of the innate immune system. Pathogens such as bacteria, viruses, fungi and parasites display conserved signature elements that are absent from the host constitution. These self-distinguishable elements are termed Pathogen-associated molecular patterns (PAMPs) and can be recognized by the host. PAMPs are essential components of the invading microorganism such as lipopolylsaccharide (LPS), dsRNA, unmethylated nucleic acids, flagellin, peptidoglycan and many others. PAMPs' sensor molecules are known as Pattern recognition receptors (PRRs) and act as a molecular switch to trigger the immune response following contact with one of the PAMPs. PRRs are widely expressed by a variety of cell types and include members that have evolved to recognize a specific molecular signature. The PAMPs' specificity and the PRRs' different locations in the cell allow their classification into three main families: the Toll-like receptors (TLR), the NOD-like receptors (NLR) and the Retinoic acid-inducible gene I (RIG-I)-like receptors (RLR) (Akira and Takeda, 2004; Ishii *et al.*, 2008; Nakhaei *et al.*, 2009). **Table 3** shows the diversity of PRRs, the variety of microorganisms recognized by PRRs, their cell localization and PAMP specificity.

Family	Receptor (PRRs)	Microbial signature (PAMPs)	Cell localization	Recognized Microorganism
Toll-like receptors	TLR 1	Triacyl lipopeptides	Plasma membrane	Bacteria and mycobacteria
	TLR 2	Lipoprotein Lipopeptide Peptidoglycan others	Plasma membrane	Gram positive bacteria Mycobacteria Fungi
	TLR 3	dsRNA	Endosome	Viruses
	TLR 4	LPS Taxol Fusion protein others	Plasma membrane	Gram negative bacteria Plants Viruses
	TLR 5	Flagelline	Plasma membrane	Bacteria
	TLR 6	Diacyl lipopeptides Lipoeichoic acid Zymosan	Plasma membrane	Mycoplasma Gram positive bacteria Fungi
	TLR 7	ssRNA	Endosome	Viruses
	TLR 8	ssRNA	Endosome	Viruses
	TLR 9	CpG-containing DNA	Endosome	Bacteria and viruses
	TLR 10	N.D.	N.D.	N.D.
	TLR 11	N.D.	N.D.	Uropathogenic bacteria
RIG-I-like receptors	RIG-I	short dsRNA	Cytoplasm	Viruses
	MDA5	long dsRNA	Cytoplasm	Viruses
	LGP2	RNA	Cytoplasm	Viruses
NOD-like receptors	NALP3	RNA Perotoxin dsRNA non-methylated DNA others	Cytoplasm	Bacteria and viruses
	NAIP5	Flagellin	Cytoplasm	Bacteria
	IPAF	Flagellin	Cytoplasm	Bacteria
	NOD1	Peptidoglycan	Cytoplasm	Bacteria
	NOD2	Peptidoglycan	Cytoplasm	Bacteria

Table 3. Host innate immune receptors of microbial pathogen-associated molecular patterns. From (Ishii *et al.*, 2008; Nakhaei *et al.*, 2009) and (Akira and Takeda, 2004)

Following pathogen recognition PRRs signal through various adaptor molecules, resulting in the activation of downstream kinases that in turn lead to the stimulation of latent transcription factors such as interferon regulatory factors (IRFs) or nuclear factor kappa B (NF-κB). Activation of these transcription factors - mainly through phosphorylation - triggers their nuclear translocation and subsequent DNA binding, resulting in the initiation of a series of transcriptional events that lead to the upregulation of diverse cytokines and soluble mediators, which then launch an immune response.

One of the central secreted mediators of antiviral response are the interferons (IFNs). IFNs are rapidly secreted proteins from most nucleated cells following PRR activation. IFNs are subdivided into three main groups: type I - IFNα and IFNβ, type II - IFNγ, and type III - IFNλ (Pestka *et al.*, 2004). Secreted type I IFNs alert surrounding cells, induce antiviral state and exert many immunomodulating functions on both the innate and adaptive responses.

Type I IFN induction is characterized by a first wave of transcription stimulation, followed by second wave of amplification and activation of hundreds of genes encoding for diverse functions related to the anti-microbial response. As a more precise example, RIG-I or MDA5 recognition of viral RNA induces their conformational change, which allows the interaction motifs "Caspase recruitment domains" (CARD) regions to bind downstream signaling molecules. The adaptor molecule that provides a link between RIG-I activation and downstream activation events is the mitochondrial antiviral signaling adaptor (MAVS). MAVS acts as a platform for subsequent signaling and leads, among other things, to the activation of the two kinases TBK1 and IKK, which in turn phosphorylate interferon regulatory factor 3 (IRF3). IRF3 is a constitutively expressed transcription factor that resides in the cytoplasm in unstimulated cells; upon phosphorylation it forms a homodimer, translocates into the nucleus, and binds to DNA to regulate expression of IFN and other IFN-stimulated genes (ISG). Of note, the first wave production of IFNβ is not solely mediated by IRF3 but also involves the cooperative binding of two other transcription factors to the positive regulatory domain of the IFNβ promoter: NF-κB and the activating transcription factor 2 (ATF2)/c-Jun. This

initial secretion of IFNβ acts in an autocrine and paracrine fashion to activate the Janus Kinase (JAK)-Signal Transducer and Activator of Transcription (STAT) pathway via the type I IFN receptor to induce IRF7 expression and other ISGs. This positive feedback loop mechanism allows the phosphorylation of IRF7, its homodimerization or heterodimerization with IRF3, translocation into the nucleus and amplification of type I IFN induction through the production of IFN and the transactivation of hundreds of other ISGs involved in the antiviral response (Kawai and Akira, 2008; Nakhaei *et al.*, 2009; Randall and Goodbourn, 2008). The IFNs, cytokines and other ISG products establish an antiviral state in neighboring cells but also attract various immune cells of the innate and adaptive branches to modulate their subsequent activation.

1.3.1.1 Innate immune cells and the antiviral response

Innate immune cells are the first cells attracted to the site of infection and their prime functions are to phagocytose and eliminate foreign pathogens as well as to secrete a variety of cytokines and chemokines that act in an autocrine and paracrine manner. Leukocytes linked to the innate system can be divided into four main branches: the granulocytes, the mast cells, the monocytes/macrophages and the Natural killer (NK) cells (Janeway, 2001).

Granulocytes can be characterized by the presence of granules in their cytoplasm and include three cell populations: Neutrophils, Eosinophils and Basophils. All granulocytes are blood-circulating cells that migrate to the site of infection or inflammation following chemoattractant gradient (Janeway, 2001). Neutrophils are the most abundant type of white blood cell and are one of the first inflammatory cells to migrate towards the site of inflammation. They are attracted to the site of inflammation by the increasing gradient of various chemokines produced by endothelium cells of the inflammed microvessels, such as Platelet-activating factor (PAF), C5a complement component, interleukine-8 (IL-8) and leukotirene B4 (Witko-Sarsat *et al.*, 2000). Following their arrival at the infection site, their primary role is to phagocytose and

eliminate infectious agents as well as infected or damaged cells. In addition to their antimicrobial effects, many of the granule proteins secreted by the neutrophils directly or indirectly mobilize immune cells (Chertov *et al.*, 2000). Neutrophils have been shown to directly phagocytose viruses (West *et al.*, 1987) and to impact virus replication *in vivo* (Tate *et al.*, 2011; Tumpey *et al.*, 1996). Eosinophils and Basophils are much less abundant than Neutrophils. Eosinophils are thought to play a defensive role against pathogens, while Basophils have been associated, along with Mast cells, with histamine release and allergic reactions (Janeway, 2001).

The second population of phagocytic cells are the monocytes/macrophages. Monocytes are the circulating precursors to the most mature and tissue-specific macrophages. Monocytes are attracted to the site of inflammation in a chemotaxis process similar to the one that takes place for neutrophils (Imhof and Aurrand-Lions, 2004). Phagocytosis of osponized cells occurs via the Fc and complement receptors, additionally macrophages possess a mannose receptor that recognizes mannose and fructose directly on the surface of pathogens. Macrophages are also capable of apoptotic cell phagocytosis via diverse receptors, including class A and B scavenger receptors or vitronectin receptors (Aderem and Underhill, 1999). In addition to its role in eliminating damaged cells and pathogens, macrophage phagocytosis is followed by digestion of microbes and microbial antigen presentation to lymphocytes, thus also serving as an antigen-presenting cell (APC). The digestion process occurs through phagosome-lysosome fusion, where enzymes and toxic peroxides digest the pathogen (Aderem and Underhill, 1999).

The last important group of innate immune cells are the NK cells. NK cells are cytotoxic cells circulating in the blood. They are considered innate immune cells because they can directly induce the death of tumor cells and virus-infected cells in the absence of other costimulatory signals or previous specific immunization. NK cells are equipped with an array of receptors that can either stimulate or inhibit NK cell reactivity (Lanier, 2008). Several NK cell-activating receptors have the capacity to detect self-molecules induced in conditions of cellular stress (Raulet and Guerra, 2009). For example, the

receptor natural killer group 2, member D (NKG2D) interacts with various ligands that are normally expressed at low levels in tissues but are overexpressed upon cellular distress such as DNA damage or viral infection (Bauer *et al.*, 1999; Pende *et al.*, 2006). In addition, some of these receptors can recognize viral-induced changes on the surface of infected cells. For example, NK cells can detect the lack of major histocompatibility complex class I (MHC-I), a situation that can occur as a viral evasion mechanism or cellular transformation (Karre *et al.*, 1986). NK cell cytotoxicity can also occur via the Fc receptor and antibody cell opsonization. Following activation, NK cells release cytoplasmic granules containing perforin and granzyme proteases. Perforin forms pores in the cell membrane, creating channels that allow osmotic cell lysis or allow the granzymes to enter and induce apoptosis. Additionally, NK cells are recognized as major producers of cytokines such as IFN-γ (Vivier *et al.*, 2011). Although mainly characterized as innate immune cells, recent evidence demonstrates the presence of memory functions in the NK cell population, therefore suggesting that they harbor characteristics attributed to the adaptive immune branch (Sun *et al.*, 2011).

1.3.2 Dendritic cells: linking innate and adaptive immunity

The adaptive immune system is characterized as an acquired, long-term protective and pathogen-specific response. The activation and differentiation of highly specialized effector lymphoid cells form the basis of this system and are dependent on three major aspects: the presence of a specific antigen, its presentation by antigen-presenting cells (APCs), and the delivery of costimulation signals also mediated by APCs. APC functions can be performed by three main types of immune cells: macrophages, B cells and dendritic cells (DCs). DCs are generally recognized as the most efficient cells performing APC functions.

1.3.2.1 DC diversity

DCs are often referred to as sentinels of the immune system, providing a link between the pathogen and the adaptive immunity. DCs represent a diverse subset of cells that can be divided into two main categories: the conventional DC (cDC) and the plasmacytoid DC (pDC). Although they share common characteristics, these two subsets perform distinct functions; cDCs are professional antigen-presenting cells, while pDCs are professional IFN-producing cells (Masson *et al.*, 2008). cDCs are also a heterogeneous group and can be subdivided into three main subsets: migratory DCs, lymphoid organ-resident DCs and monocyte-derived DCs. A certain number of phenotypic differences between human and mice DCs exist, but the main subset characteristics and subgroup divisions apply (Miloud *et al.*, 2010).

Migratory DCs can be found in almost all organs and lymph nodes and can also be subdivided into two main groups: interstitial DCs and Langerhans cells. Langerhans cells are mainly found in the epidermis, while interstitial DCs are found throughout the body (Naik, 2008). The classical paradigm of migratory cDC function implies that DCs are present in peripheral tissues, sampling and presenting the environment on their MHC molecules. Although these DCs are very efficient at capturing, processing and presenting antigens, they express very low levels of costimulatory molecules and are termed immature DCs (Villadangos and Schnorrer, 2007). Immature DCs express a variety of PRRs and inflammatory signal receptors such as TLRs, cytokines and chemokine receptors. Upon activation of these receptors, DCs initiate migration towards secondary lymphoid organs and develop a mature phenotype. DC trafficking is controlled by the expression of chemokine receptors – for example, immature DCs express high levels of CCR6, the receptor for the pro-inflammatory chemokine CCL20, thereby allowing them to migrate towards the site of inflammation (Merad *et al.*, 2004). Upon maturation, DCs downregulate their response to the peripheral inflammatory chemokine and upregulate the expression of chemokine receptors such as CCR7, which redirects migration towards the lymph nodes in response to chemokines like CCL-19 and CCL-21 (Dieu *et al.*, 1998; Sallusto *et al.*, 1998). Mature DCs are also characterized by the upregulation of their

MHC expression levels, as well as many costimulatory molecules such as CD40, CD86 and CD80, allowing them to deliver the first and second signals required for T cell activation - antigen and costimulation (Villadangos and Schnorrer, 2007). The "mature" designation refers to DCs' ability to induce naïve T cell proliferation, although this phenotype can also be associated with tolerance functions (Steinman and Nussenzweig, 2002). Antigen uptake, maturation and migration processes are rapid events that occur within hours (Granucci *et al.*, 1999). Once in the lymphoid organs, cDCs come into contact with naïve T cells and induce their antigen-specific differentiation and activation (Masson *et al.*, 2008).

The second cDC subset, lymphoid-organ-resident DCs, also called resident DCs, are DCs that spend their lifetime in one lymphoid organ (Miloud *et al.*, 2010). Lymphoid-organ-resident DCs remain in an immature state unless they encounter pathogen products or inflammatory signals *in situ* (Villadangos and Schnorrer, 2007). These DCs can be further subdivided into 3 main groups based on their expression of CD4 and CD8 surface markers: the CD4+, the CD8+, or the CD4-CD8- (double negative) subsets. Of these subsets, the CD8 DCs have been the most intensively studied and have been associated with multiple functions, such as T cell-priming APC function in response to infection and maintaining tolerance to self-antigens (Naik, 2008; Shortman and Heath, 2010).

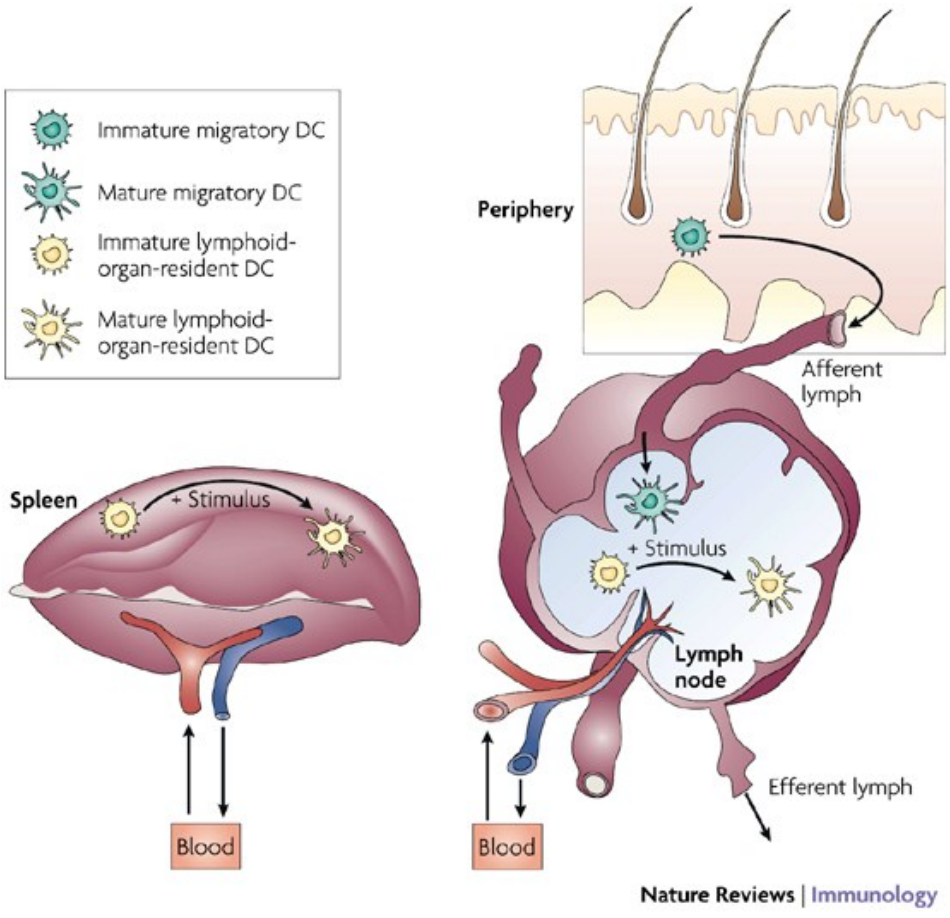

Figure 7. Schematic representation of the DC state and cycle. Migratory DCs are sampling antigens at the periphery, acquiring maturation phenotype and migrating by the lymph. In contrast, Lymphoid-organ-resident DCs acquire both signals directly in the lymphoid organs. Reproduced with permission from (Villadangos and Schnorrer, 2007).

The third type of cDC is termed the monocyte-derived DC. These are less characterized and are also known as inflammatory DCs. Monocytes are known to be blood-circulating precursors of macrophages and DCs (Gordon and Taylor, 2005). During certain infections, monocytes appear to accumulate at the site of inflammation and differentiate into this type of DC. Monocyte-derived DCs therefore boost the normal rate of DC differentiation to accommodate a more urgent need (Masson *et al.*, 2008; Villadangos and Schnorrer, 2007). In addition, human monocytes are used to generate DCs *in vitro* in order to fulfill research needs, and are also proposed to be used to generate DC-based immune therapies (Garderet *et al.*, 2001).

The second main type of DC is the pDC. They represent a very distinct group with diverging characteristics and functions. pDCs are found in most tissues and blood circulation and were first identified as type-1-IFN-producing cells (Siegal *et al.*, 1999). Although they share common characteristics with cDCs, they appear to weakly stimulate naïve T cells and their main function resides in the production of pro-inflammatory cytokines upon sensing of viral infection (Barchet *et al.*, 2005). As an illustration of the magnitude of pDC IFN production efficacy, pDCs have been shown to dedicate 60% of newly induced transcriptome to type 1 IFN genes upon virus activation (Ito *et al.*, 2006). One major physiological distinction between cDCs and pDCs is the selective expression of TLRs. Unlike cDCs, pDCs do not express TLR 1, 2, 4, 3, 5 and 8 but selectively express TLR 7 and 9, and are therefore specialized in sensing nucleic acids (Barchet *et al.*, 2005). Secondly, pDCs have been found to constitutively express IRF-7, an important transcription factor that is inducible in most cell types following IFNβ production in order to induce IFNα secretion and amplify the IFN-induced response (Coccia *et al.*, 2004). These two main characteristics are thought to explain the rapidity and efficacy of IFNα production by pDCs.

1.3.2.2 DC-presenting antigen

Two types of antigens are presented by the MHC molecules of DCs: endogenous antigens (present within APCs) and exogenous antigens (engulfed from the surrounding environment). DCs constitutively present peptides derived from their own components on MHC class I and II molecules (Villadangos and Schnorrer, 2007). Antigens derived from peptides found in the cytosol are presented via the classical MHC-I pathway. In this pathway, endogenous antigen peptides originate in the host cell or from the presence of viral proteins in the cytosol of infected DCs. Endogenous proteins can also be presented onto the MHC-II molecules, membrane proteins or proteins originating from autophagy – a catabolic process aimed at maintaining a balance between cellular products that induce the degradation of a cell's own components by the lysosomal machinery (Mizushima and Klionsky, 2007) – through the classical MHC-II pathway (see **Figure 8**). Exogenous antigens are known to be presented principally via the MHC-II pathway; following endocytosis of exogenous antigens, pathogens and apoptotic debris are degraded and directed to the MHC-II presentation machinery. Alternatively, exogenous antigens can be presented on MHC-I via a mechanism known as cross-presentation. In this process, endocytosed antigens are redirected to proteasomal degradation and MHC-I loading. CD8+ DCs have been shown to be the most efficient at uptaking exogenous material and performing cross-presentation (den Haan *et al.*, 2000; Pooley *et al.*, 2001; Shortman and Heath, 2010). A third process allowing antigen presentation on MHC-I is referred to as cross-dressing. This mechanism implies the transfer of a preformed peptide-MHC complex from the surface of an infected cell to the DC without the need for further processing. Recent data in support of this model have been accumulated both *in vitro* and *in vivo* (Dolan *et al.*, 2006; Qu *et al.*, 2009; Wakim and Bevan, 2011). Lastly, BMDCs were also shown to be able to present peptides on empty MHC-II molecules through an extracellular process (Santambrogio et al., 1999).

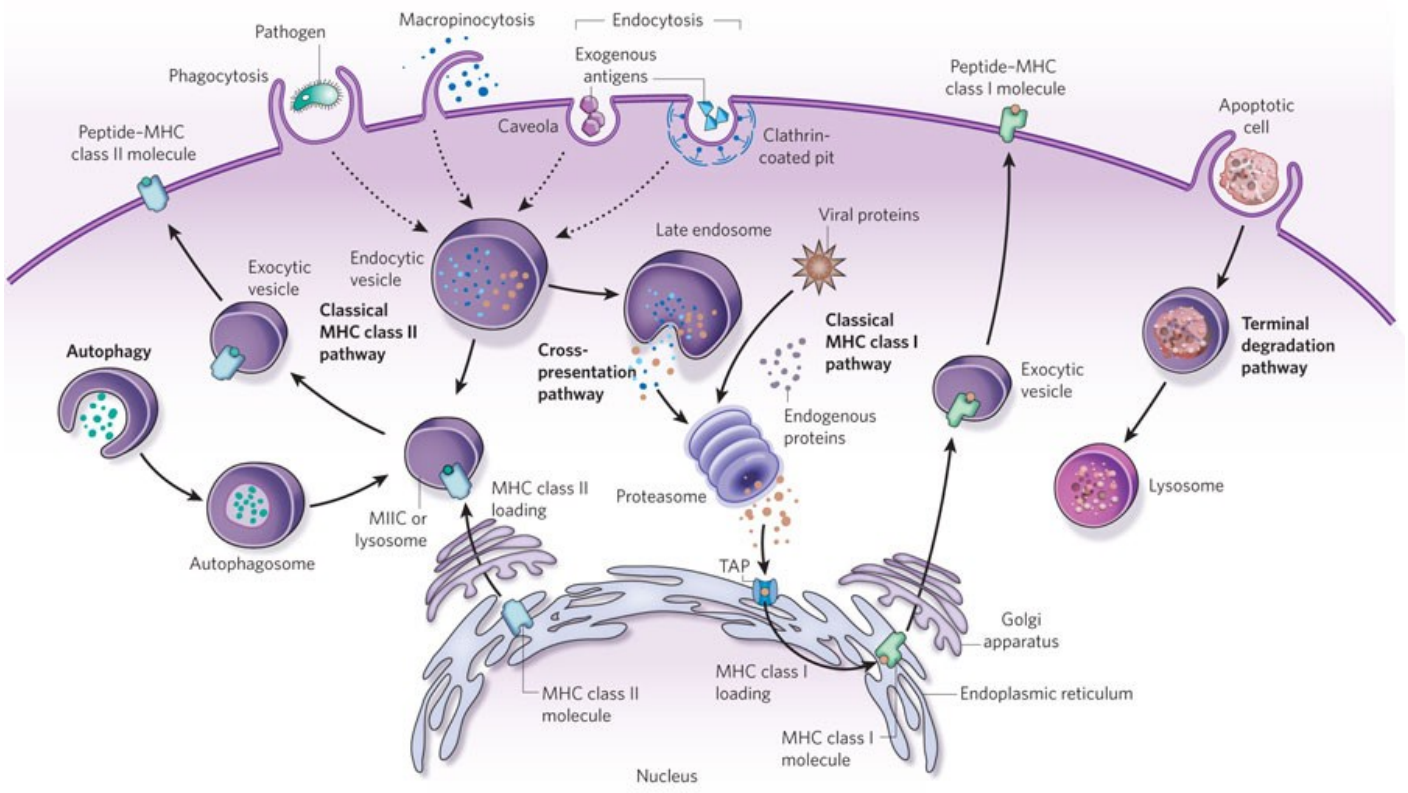

Figure 8. Illustration of the principal antigen presentation pathways of DCs. Endogenous antigens are presented on MHC-I through the classical MHC-I pathway, while exogenous antigens are presented through the classical MHC-II or on MHC-I through the cross-presentation pathway. Reproduced with permission from (Hubbell *et al.*, 2009).

1.3.2.3 DC signaling to T cells

Following pathogen encounter, processing and presenting an antigen on its MHC molecules and maturation, DCs will prime T cell activation and proliferation via three signals. Upon DC-T cell contact in the lymphoid organ, T cell antigen recognition occurs through the interaction of the T cell receptor (TCR) and co-receptor (CD4 or CD8) with the peptide loaded on the MHC molecule; MHC-I is bound by CD8 T cells, while MHC-II is bound by CD4 T cells. This signal - referred to as signal 1 - depends on the molecular identity of the pathogen and is responsible for the antigen specificity of the T cell response. The second signal arises from the co-stimulatory molecules that are upregulated upon APC maturation (for example CD80 and CD86); these co-stimulatory molecules interact with the CD28 receptor on the T cell and induce the clonal expansion of the naïve T cells into effector and memory T cells. CD40 is another costimulatory molecule upregulated following DC maturation. Contact with its T cell ligand CD40L acts in both directions to further induce DC maturation and to synergize with other costimulatory molecules to activate tumor-reactive T cells (Chatzigeorgiou et al., 2009). In the absence of co-stimulation – i.e. the T cell encountering an immature DC – T cell anergy is induced (Hugues, 2010; Janeway, 2001). Signal 3 refers to the cytokine mediators secreted by the DC during the T cell-DC contact event. This signal has a profound impact and will modulate the nature of the T cell response induced (Kalinski *et al.*, 1999; Kapsenberg, 2003). Different cDC subsets are known to prime distinctive T cell responses: the CD8+ DCs were shown to activate naïve CD8 T cells, whereas non-CD8 DCs were shown to be responsible for the activation of naïve CD4 T cells (Dudziak *et al.*, 2007; Masson *et al.*, 2008).

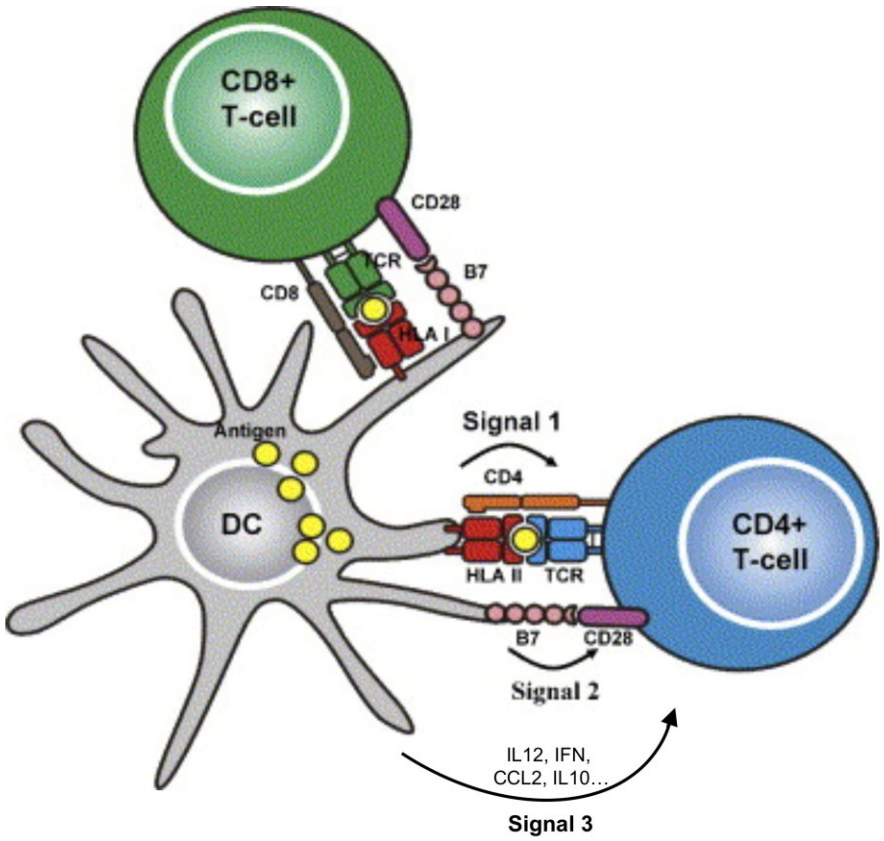

Figure 9. DC activation of naïve CD8 or CD4 T cells. DCs activate T cells through three signals: the CD4/8-MHC interaction, the B7-CD28 family costimulation signal and cytokine release during the DC-T cell synapse. HLA stands for Human leukocyte antigen, the name given to MHC in humans; the B7 molecules on the DC stand for the co-stimulatory molecules B7.1 (CD80) or B7.2 (CD86). Adapted with permission from (Berntsen *et al.*, 2006).

1.3.3 The Adaptive immune response to viruses

1.3.3.1 T cell response to viruses

T cells are found circulating in the blood stream and throughout the immune organs in different states: naïve, effector and memory T cells. Generally T cells are subdivided into two main groups: cytotoxic CD8 T cells and CD4 T helper cells.

CD8 T cells have a predominant role in controlling viral infection; upon activation, they undergo massive expansion and acquire the capacity to kill the infected antigen-bearing cell by releasing granzymes and perforin or by direct induction of apoptosis through the Fas death receptor ligand (FAS-L) (Hassin *et al.*, 2011; Parish and Kaech, 2009). DCs and other phagocytes provide signal 3, which permits the development of optimal response. This signal is composed of pro-inflammatory cytokines, mainly IL-12, but also type 1 IFN (Cui *et al.*, 2009; Williams and Bevan, 2007). The maximal number of effector cytotoxic CD8 T cells generally occurs around 7-8 days post infection (Williams and Bevan, 2007). After completion of the CD8 T cell immune response, most of the effector cells undergo apoptosis and only a small subset persist as a memory T cell population that confers long-term protection against re-infection (Joshi and Kaech, 2008; Parish and Kaech, 2009).

The CD4 T cell subset appears to be much more diversified and includes many different subsets and classifications. T helper cells exert their functions indirectly by secreting cytokines and chemokines that activate other immune cells. Historically the T helper cell subset was divided into two main branches, the Th1 and the Th2 cells, however other distinctions have now been made and more specialized CD4 T cells have been characterized.

In the Th1/Th2 paradigm, Th1 mediates immune responses against intracellular pathogens (cellular immunity), while Th2 mediates host defense against extracellular parasites (humoral immunity) (Zhu and Paul, 2008). Th1 cytokine production's signature is characterized by IFNγ, lymphotoxin α and IL-2 (Zhu *et al.*, 2010). IFNγ exerts its biological effects by increasing the microbicidal activity of macrophages (Suzuki *et al.*, 1988), while IL-2 assists in the proliferation of cytotoxic CD8 T cells and the generation of both CD8 and CD4 T memory cells (Williams *et al.*, 2006; Zhu and Paul, 2008). Th2 cells are the principal producers of IL-4, IL-5, IL-9 and IL-14, and exert their regulatory functions mainly on B cells (Zhu *et al.*, 2010). For example, IL-4 is known to induce antibody isotype switching in B cells (Kopf *et al.*, 1993).

Th17 is another subset of CD4 T cells recently discovered, and is involved in the immune response against bacteria and fungi (Weaver *et al.*, 2006). The cytokine signature produced by this subset includes IL-17A, IL-17F, IL-21 and Il-22 (Zhu *et al.*, 2010).

As one of the main functions of CD4 T cells is to orchestrate the adaptive immune response, controlling and shutting down the response is as critical as promoting proper induction. Regulatory T cells (Tregs) play an essential role in maintaining self-tolerance as well as in the regulation of the immune response. Tregs are generated from two sources: naïve CD4 T cells (as for Th1, Th2 and Th17 subsets) or directly from the thymus. Tregs are known to secrete anti-inflammatory cytokines such as IL-10, TGFβ and IL-35. One key characteristic of Tregs is the expression of the FOXP3 transcription factor, which appears to play a crucial role in their differentiation (although the expression level is different depending on the origin of the Treg) (Campbell and Koch, 2011; Sakaguchi *et al.*, 2010).

Naïve CD4 T cell polarization into the different subsets described above is driven by signal 3 produced by the DC during the DC-T cell contact-priming event. The nature of that signal is dictated by the pathogen recognition receptor that has been triggered during infection (Kapsenberg, 2003). For example, DC signaling through TLR 4

following LPS contact strongly induces the production of IL-12 in order to polarize the response towards Th1 (Langenkamp *et al.*, 2000). Other Th1-cell-polarizing factors include other IL-12 family members and type 1 IFN. Th2-cell-polarizing factors include CCL2 and OX40L and can also be induced *in vitro* by IL-4 and IL-2 combination, while Treg-cell-polarizing factors are known to be IL-10 and TGFβ (Kapsenberg, 2003; Zhu and Paul, 2008). Th17 polarization appears to be more complex and requires a combination of IL-6, IL-21 and IL-23 (Weaver *et al.*, 2006; Zhu and Paul, 2008).

Overall, CD8 as well as CD4 T cell functions are mainly dictated by the DC (or other APC) signals produced at the time of the priming and highlights once again the critical role of this important immune cell type.

1.3.3.2 B cell response to viruses

B cells are typically known to directly recognize free soluble antigens found in circulation to induce massive antibody production. Recent evidence also suggests that B cells could recognize the antigen on the surface of other APCs such as DCs (Batista and Harwood, 2009). Antibodies are high affinity molecules that bind directly to a specific antigen and function to directly neutralize pathogen attachment to host cells, to promote phagocytosis of the pathogen by macrophages and other immune cells through a process known as opsonization, or to activate the complement cascade for the destruction of infected cells (Baumgarth *et al.*, 2008; Janeway, 2001). Following detection of their specific antigen through the B cell receptor (BCR), B cells internalize it and process it for presentation through the MHC-II molecule. This event allows them to perform an APC function (although at a much less efficient level than DCs), and to receive the co-stimulation signal from a Th2 cell required for most antigens for their complete activation and antibody production (Rodriguez-Pinto, 2005). The B cells and CD4 T cells in this immunological synapse are specific to the same antigen and interact through their respective MHC-TCR molecules, as well as through the costimulation receptor-ligand CD40-CD40L, leading to the secretion of cytokines by the T cell (such as IL-4) and the

complete activation of the B cell (Baumgarth *et al.*, 2008; Janeway, 2001). There is also an activation mechanism that is independent of CD4 T cell signals for a certain type of antigen that can directly deliver both signals (Vos *et al.*, 2000). During a typical virus infection, these events induce the clonal proliferation of B cells, which ultimately results in the massive production of antibodies within days of the initial infection. Antiviral antibodies can be directed against a virion's external protein such as the viral receptor, or against a functional viral protein released by dying cells; the first group of antibodies will possess the capacity to interfere with viral entry into new host cells and are termed neutralizing antibodies, whereas the second type are unable to perform this function (Hangartner *et al.*, 2006). Additionally, different classes of antibodies exist and are in part characterized by their serum half-life, specialized effector functions or tissue access properties (Hangartner *et al.*, 2006; Janeway, 2001).

1.3.4 Immune response to VSV

The acute pro-inflammatory nature of VSV infection robustly triggers both the innate and the adaptive immune response. VSV generates a massive IFN response early after infection, and this response mainly restricts the infection to the central nervous system (Lyles and Rupprecht, 2007). The extreme susceptibility of STAT1 knockout mice to VSV infection is a striking example of the importance of the IFN response to VSV infection (Muller *et al.*, 1994). The PRRs responsible for launching the IFN response following VSV infection are TLR7 and RIG-I (Kato *et al.*, 2006; Lund *et al.*, 2004; Yoneyama *et al.*, 2004). As pDCs constitutively express TLR7 and are the main IFN-producing cells, it is not surprising that they have been found to be the major mediator in IFN response following VSV infection (Barchet *et al.*, 2002; Waibler *et al.*, 2007). In addition, a possible involvement of TLR4 in IFN induction by VSV has been reported in cDCs and macrophages; VSV G protein was shown to trigger a pathway downstream of TLR4 that is different from LPS (Georgel *et al.*, 2007; Schabbauer *et al.*, 2008).

The adaptive immune response against VSV depends on both B and T cells. Mice deficient for B cells are extremely susceptible to VSV and die from challenge in about 9 days, highlighting the early function of the B cells in VSV infection. In contrast, T cell-deficient mice die from infection approximately 30 days post challenge, indicating the long-term function of T cells in controlling VSV infection (Thomsen *et al.*, 1997). The B cell-mediated antibody response is a very early event that takes place within the first few days following infection (see **Figure 10**) (Bachmann *et al.*, 1995; Ochsenbein *et al.*, 2000). The main epitope recognized by VSV antibodies is against the receptor G protein and therefore allows for the neutralization of virions (Lefrancois and Lyles, 1982). Although less pathogenic and more rapidly cured, mice infected with the VSV MΔ51 mutant strain produced comparable amounts of neutralizing antibodies to their wild-type counterpart (Ahmed *et al.*, 2008).

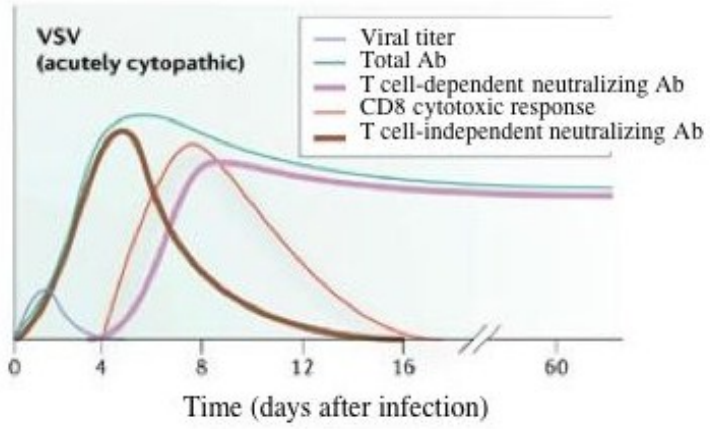

Figure 10. Adaptive immune response triggered by VSV infection. The virus engenders a rapid first wave of short-lived neutralizing antibodies (Abs) that do not require the CD4 T cell co-stimulation signal, followed by a second wave of long-lived antibodies for which the Th2 co-stimulation signal is required (Bachmann *et al.*, 1995). Total number of Abs are therefore elevated as early as day 4 and include neutralizing Abs as well as Abs specific for other viral components that do not possess the ability to prevent the virus from entering the cell. Total cytotoxic T cell response against the virus peaks around day 8. Adapted with permission from (Hangartner *et al.*, 2006).

VSV has been shown to polarize the CD4 T cell to both Th1 and Th2 lineage (Ciavarra *et al.*, 2005; Lyles and Rupprecht, 2007). CD4 T cell functions are critical to promoting the B cell-mediated antibody and CD8 cytotoxic responses. Th cells have been shown to play an important role in antibody isotype switching in response to VSV (Lefrancois, 1984; Thomsen *et al.*, 1997) and clearance of the virus (Maloy *et al.*, 2000). As required for their specific function on the B cell, the CD4 T cell's major epitope appears to be from the G protein (Burkhart *et al.*, 1994). In addition, VSV generates the proliferation of VSV-specific CD8 T cells that becomes maximal around day 6 following infection (Andreasen *et al.*, 2000). In the case of this cytotoxic response, the major epitope has been identified to be the most abundant protein present in the virus, the N protein (Puddington *et al.*, 1986). Although CD4 T cells have been demonstrated to be essential for the long-term survival of VSV-infected mice, the CD8 response appears to be dispensable (Leist *et al.*, 1987; Thomsen *et al.*, 1997).

The generation of these adaptive effector cells requires the presentation of the virus by APCs. The crucial role of DCs in this task in the context of CD4, CD8 and B cell antibody responses has been demonstrated through multiple *in vivo* DC depletion assays (Ciavarra *et al.*, 2000; Ciavarra *et al.*, 2006; Ciavarra *et al.*, 2005; Ludewig *et al.*, 2000; Zammit *et al.*, 2005). VSV-infected DCs have been proposed to be responsible for the transportation of the virus to lymphoid organs where the response can be initiated. In this model, the response may be primed through two mechanisms: directly through the infected DC, or indirectly through the release of the virus to other lymphoid organ APCs (Ludewig *et al.*, 2000). In addition, viremia may also contribute to the distribution of the virus through the lymphoid organs.

1.4 Cancer and the immune system

The immune system forms a dynamic network that protects against foreign pathogens, while simultaneously maintaining homeostasis and tolerance towards self-antigens. Based on this central role, the immune system has long been hypothesized to prevent cancer development by eliminating cancerous cells and therefore serving as an immunosurveillance system (Schreiber *et al.*, 2011). In support of this hypothesis is the observation that immunocompromised animals were more susceptible to tumor formation than immunocompetent ones (Kaplan *et al.*, 1998; Shankaran *et al.*, 2001). More recent studies led to the revision of this model when it was discovered that tumors formed in immunocompromised animals were more immunogenic than the ones formed in immunocompetent mice (Dunn *et al.*, 2002; Shankaran *et al.*, 2001). This observation led to the notion that the immune system may also shape tumor immunogenicity, and thus a new model was proposed (Vesely *et al.*, 2011). The immunoediting model stipulates that the immune system plays a critical role in three steps of the malignancy process: 1) eliminating developing tumors (in a similar manner to the immunesurveillance model); 2) equilibrating and maintaining in dormancy the rare tumor cell variants that may have survived elimination; during this step the immunogenicity of the tumor would be greatly modulated by the immune system, and 3) the escape phase, where tumor cells have acquired reduced immune recognition and are able to progress into visible tumors (Schreiber *et al.*, 2011; Vesely *et al.*, 2011).

The immune system is not only associated with the surveillance and modulating aspects of cancer formation, but also in certain circumstances to its promotion. For example, chronic inflammation has been shown to potentiate cancer development at almost all stages (Grivennikov *et al.*, 2010). For this reason, tumor infiltration with innate immune cells normally associated with inflammation such as neutrophils and macrophages has been associated with poor prognostics (Allavena *et al.*, 2008; de Visser *et al.*, 2006).

In the course of malignancy development, tumor cells acquire characteristics that allow them to evade immune surveillance and create an immunosuppressive microenvironment. For example, tumor cells have been shown to have reduced MHC-I expression, which may also serve to prevent their recognition by immune cells (Hicklin *et al.*, 1999). Additionally, tumor cells have been shown to express immunosuppressive factors such as Transforming growth factor β (TGFβ), vascular endothelial growth factor (VEGF) and IL-10. These immunosuppressive cytokines exert their functions by recruiting regulatory immune cells like Tregs and myeloid-derived suppressor cells (MDSC), or by abrogating the function of immune cells that would contribute to tumor recognition and elimination such as DCs, CD8 cytotoxic T, and NK cells (de Visser *et al.*, 2006; Igney and Krammer, 2002; Schreiber *et al.*, 2011).

DC function at the tumor site appears to favor tolerance (Vesely *et al.*, 2011). Tumor DCs are often found in low numbers and are associated with immature resting phenotype (Miloud *et al.*, 2010). TGFβ has been shown to directly inhibit DC activation (Kao *et al.*, 2003; Laouar *et al.*, 2008), while targeting VEGF and IL-10 in cancer immunotherapy strategies improved DC function and efficacy (Chen *et al.*, 2007; Gabrilovich *et al.*, 1999; Kim *et al.*, 2011). In addition, tumor cells have been shown to secrete sterol metabolites that suppress the expression of CCR7 on DCs, thereby disrupting DCs' ability to migrate to the lymph nodes (Villablanca *et al.*, 2010). A recent study also demonstrated that secretion of an unknown tumor-derived factor induces increased uptake of extracellular lipids in DCs and reduced their antigen-processing capacities (Herber *et al.*, 2010). Therefore, tumor conditions favor the inhibition of DCs as well as other immune effector cells.

1.5 OV treatment and the immune system

The immune system has long been speculated to have a negative impact on OV therapies for cancer treatment, based on its potent ability to limit viral replication and spread within a tumor. This concept is derived from the thought that OVs' efficacy in killing tumor cells relies solely on viral replication. As a matter of fact, most of the first *in vivo* experiments demonstrating OV efficacy in treated engrafted tumors were performed in immunodeficient animals (Grote *et al.*, 2001; Stojdl *et al.*, 2000). It is now known that other factors, including the immune system, contribute to tumor reduction at the onset of OV treatment. In fact, OVs are now considered by many as a "danger signal" that might help to counteract the immunosuppressive microenvironment of the tumor (Naik *et al.*, 2011; Prestwich *et al.*, 2009a). For instance, OV replication and localization at the tumor site represents a non-negligible presence of PAMPs capable of triggering different PRRs. As previously mentioned, activation of these receptors directly results in the secretion of inflammatory cytokines and represents a profound modification of the cytokine profile existing at the tumor site. For example, *in vitro* infection with Reovirus has been shown to lead to the release of a variety of cytokines such as IL-6 and RANTES, while decreasing the expression of immunosuppressive cytokines such as IL-10 (Errington *et al.*, 2008b; Gujar *et al.*, 2011). The presence of the virus and these virally induced changes launch an antiviral immune response that inevitably exerts collateral effects on the tumor burden and potentially the anti-tumor immune response.

A number of studies have now highlighted the role of the immune response in the positive outcome of OV tumor treatment. However, the innate vs adaptive nature of this immune response is not easily distinguishable and requires careful analysis. In addition, the diversity of OVs and animal models used to analyze such responses also append variability.

1.5.1 The innate immune response during VSV and other OV therapies

The innate response is the first alarm of the immune system, and from a viral point of view the smaller the alarm is, the more freely replication can occur. Many groups have developed strategies to silence or attenuate the innate immune response in order to allow increased viral replication at the tumor site with the hope of increasing tumor destruction. For example, treatment with cyclophosphamide (CPA), an agent used for chemotherapeutic and immunosuppresive purposes, combined with HSV or adenovirus-based therapy was shown to improve viral replication at the tumor as well as the overall efficacy of the treatment (Fulci *et al.*, 2006; Lamfers *et al.*, 2006; Thomas *et al.*, 2008). Rapamycin is another compound that possesses immunosuppressive characteristics; this agent targets the important mammalian cell growth-regulating kinase mTOR and is used to prevent transplant rejection. Combination of Rapamycin with many OVs such as VSV, Myxoma virus and HSV have also been shown to favor viral replication and global tumor control effectiveness (Alain *et al.*, 2010; Lun *et al.*, 2010; Lun *et al.*, 2009). Type 1 IFN also represents a key target in circumventing the negative effect of the innate immune response. Histone deacetylase (HDAC) inhibitors, through their profound impact on the modulation of chromatin topology and the regulation of gene transcription, have been shown to prevent IFN-stimulated gene expression (Chang *et al.*, 2004). These drugs have long been used as mood stabilizers and anti-epileptics and are now being considered for their application in cancer and inflammatory diseases. The interference of HDAC inhibitors with the IFN response has been shown to permit the increased viral replication of many OVs such as VSV, Vaccinia and HSV, and to allow a synergistic effect on tumor cell killing (Mactavish *et al.*, 2010; Nguyen *et al.*, 2008; Nguyen *et al.*, 2010; Otsuki *et al.*, 2008).

With the exception of these dampering strategies, the immune response is something that OV treatment cannot avoid. Therefore, the positive impacts of the innate immune response in OV treatment, such as direct enhancement of tumor cell killing and/or subsequent anti-tumor shaping of the response, must be sought out. One of the predominant illustrations of the direct effect of the innate immune response on tumor

burden is neutrophil-induced vascular shutdown. As a consequence of the strong proinflammatory release of IFN and other cytokines following VSV or Vaccinia virus OV treatment, neutrophils have been shown to massively influx the tumor and block the tumor vasculature. The blocking of tumor vascularization induces a transient tumor hypoxia that results in the killing of non-infected tumor cells (Breitbach *et al.*, 2011; Breitbach *et al.*, 2007). This is a key mechanism demonstrating that tumor cell killing does not rely solely on direct virus cell killing following infection. In addition to this specific role, neutrophil infiltration has also been associated with enhanced OV outcomes with HSV (Wakimoto *et al.*, 2003). Another example of the direct influence of the innate immune system on the tumor is that of NK-mediated tumor cell killing. NK cells have been shown to be crucial for VSV-mediated oncolysis in the B16OVA tumor mouse model. In this particular example, expression of the IL-28 receptor by B16 cells rendered these tumor cells susceptible to IL-28-mediated NK ligand upregulation and subsequent recognition by the NK cells (Wongthida *et al.*, 2010b). To the opposite end, VSV was engineered to express a protein from human cytomegalovirus known to downregulate the NK cell-activating ligand CD155. This rVSV demonstrated reduced antiviral response, increased viral replication and improved animal survival in Buffalo rats harboring orthotopic hepatocellular carcinoma (Altomonte *et al.*, 2009). The discrepancy between the positive and negative impacts of NK cells on overall therapy may be related to differences in animal tumor models. In support of the positive impact of NK cells on OV-mediated therapies, other viruses have been shown to enhance or to be dependent on NK cell-mediated antitumor response (Bhat *et al.*, 2011; Granot *et al.*, 2011). Furthermore, indirect NK activation by DCs following OV treatment have been proposed in many OV-based therapies, including VSV and Reovirus (Boudreau *et al.*, 2009; Boudreau *et al.*, 2011; Prestwich *et al.*, 2009b).

The crucial role of the innate immune system in OV tumor treatment efficacy has been demonstrated in knockout mice as well as in selected cell subset antibody-mediated depletion studies. In the particular case of VSV, in MyD88 (a central adaptor molecule downstream of many PRRs required for functional IFN signaling) knockout mice VSV's oncolytic properties were shown to be completely abolished (Wongthida *et al.*, 2010a).

The B16OVA mice model used in this study has been shown to have a limited level of viral infection since only one round of replication – through the use of VSV lacking the G (receptor) gene (VSVΔG) – displayed the same therapeutic effect as the fully replicative competent virus, and therefore further highlights the crucial role of mechanisms other than direct viral replication for oncolysis (Galivo *et al.*, 2009). In addition to the MyD88 -/- mice, the therapeutic benefits of VSV were also completely absent in mice lacking expression of the IFNα/β receptor (VSVΔG had to be used in this assay since VSV infection is lethal in mice lacking this receptor) (Stojdl *et al.*, 2003; Wongthida *et al.*, 2010a). Furthermore the importance of innate immune cells, in particular the NK cells, in the therapeutic benefits of VSV and Reovirus have been demonstrated by significantly reducing the therapeutic effects in animals treated with NK cell depletion antibody (Diaz *et al.*, 2007; Prestwich *et al.*, 2009a; Wongthida *et al.*, 2010b).

In summary, the innate immune system acts on multiple facets of the treatment: it is involved in the cancer cell-selective tropism of OVs; its action mediates viral clearance and may reduce viral replication and virus-directed tumor cell death; it is implicated in the killing of tumor cells via mechanisms independent of infection; and lastly it directs the adaptive immune response.

1.5.2 The Adaptive immune response in VSV and other OV therapies

The action of OVs on the tumor and the triggering of the innate immune response at the site of oncolysis undoubtedly results in the initiation of an adaptive immune response against the virus. Virally directed antibodies and CD8-specific cytotoxic T cells can be detected shortly after treatment with most OVs (Bridle *et al.*, 2009; Jenks *et al.*, 2010; Sobol *et al.*, 2010). The question remaining is whether this immune activation can lead to bystander triggering of a tumor-specific adaptive response. The answer to this question remains elusive and may vary from one OV to another and perhaps also from one animal tumor model to another.

The tumor cell lysing effect of OV treatment presumably releases a mass of tumor antigens in a milieu that has now changed from immunosuppressive to highly inflamed. These conditions might represent favorable circumstances for initiating a tumor-specific response. The initiation of such a response mainly depends on APCs, and DCs are one of the most potent APCs. The effects of OV treatment on DC state and function has been investigated for several different OVs, but there does not seem to be a consensus on the actual effect of OV therapy on DCs.

Filtered medium from Reovirus-infected tumor cells induced the maturation of DCs and enhanced their ability to prime the activation of NK cells (Errington *et al.*, 2008a; Errington *et al.*, 2008b). The activation potential of Reovirus on DCs has also been observed directly *in vitro* and during *in vivo* tumor treatment experiments (Gujar *et al.*, 2010). However, mouse bone marrow DCs have been shown to support Reovirus infection and to undergo virally induced cell death, data that diverges from that obtained for human DCs (Errington *et al.*, 2008a; Ilett *et al.*, 2009).

VSV has also been shown to infect and induce DC maturation *in vitro* (Ahmed *et al.*, 2003; Boudreau *et al.*, 2009). The viability of VSV-infected DCs also present conflicting results. Ahmed *et al.* first published that GM-CSF mouse bone marrow-derived DCs infected by wild-type VSV were killed by the infection, unlike infection

using the MΔ51 mutant (Ahmed et al., 2003; Boudreau et al., 2009). Both Reovirus and VSV-infected DCs have been successfully used in OV cell carrier strategies, an approach based on the delivery of OVs using injection of infected cells as a treatment in order to overcome the serum / antibody neutralizing effect on naked virions (Boudreau *et al.*, 2009; Ilett *et al.*, 2009).

Other OVs such as wild-type Measles virus have been shown to infect human DCs and impair their function (Grosjean *et al.*, 1997). Wild-type Vaccinia virus also impairs DC function, while a mutant strain demonstrated stimulatory effects (Greiner *et al.*, 2006). HSV apparently has a positive impact on DC priming abilities (Benencia *et al.*, 2008). Altogether these divergent observations concerning the effects of OVs on DCs do not support the notion of that OVs universally promote the initiation of the adaptive anti-tumor response.

One of the most convincing observations supporting the idea that OVs generate a tumor-specific immune response is that animals cured of their tumors following OV treatment were resistant to a second engraftment challenge of the same tumor cell line (Fernandez *et al.*, 2002; Kirn *et al.*, 2007; Nakao *et al.*, 2007). Although very persuasive, these data were obtained in artificial engraftments of tumor cell line models that are potentially highly immunogenic on their own and for which a response sufficient to block the establishment of new tumors, but insufficient to control the initial mass, may occur. Assessing the anti-tumor CD8 cytotoxic response represents an important aspect in determining whether the adaptive immune response is enhanced following OV treatment or remains at a level similar to that of untreated tumor-bearing animals. While tumor CD8 infiltration following OV treatment has been observed for many OVs (Benencia *et al.*, 2008; Diaz *et al.*, 2007; Thirukkumaran *et al.*, 2010), this does not necessarily correlate with the enhancement of tumor-specific T cells; CD8 T cells may only be directed towards controlling the “invading” OV.

Reports of CD8 response specificity following OV treatment are highly variable, depending on the OV and the tumor model used. In the case of VSV, a strong response against the virus is observed independently of the tumor model used. Tumor-specific CD8 T cells following VSV treatment have been reported (Diaz *et al.*, 2007); however, subsequent studies from the same group and others presented abundant data suggesting that the tumor-specific adaptive immune response induced by VSV is inexistent or weak (Bridle *et al.*, 2009; Bridle *et al.*, 2010b; Galivo *et al.*; Willmon *et al.*, 2009b; Wongthida *et al.*, 2011). In order to explain the dominant virus response as opposed to the tumor response, Bridle *et al.* have suggested that the very immunogenic nature of VSV may overshadow any response against the tumor (Bridle *et al.*, 2010b). Other OVs such as HSV were shown to induce both virus-specific as well as tumor antigen-specific CD8 response (Li *et al.*, 2007; Sobol *et al.*, 2010; Toda *et al.*, 1999). Reovirus has also been found to promote anti-tumor specific CD8 response (Gujar *et al.*, 2010; Prestwich *et al.*, 2008). However, in all these cases oncolytic-induced anti-tumor immunity is often of low magnitude (Bridle *et al.*, 2010a).

Regardless of CD8 T cell response specificity, the contribution of this adaptive immune cell subset in OV-mediated therapeutic effects has been demonstrated to be essential. Tumor treatment efficacy by VSV, Reovirus and HSV is highly impaired when CD8 cells are depleted (Diaz *et al.*, 2007; Galivo *et al.*, 2010; Prestwich *et al.*, 2009a; Sobol *et al.*, 2010). Although the first logical explanation is to conclude that OV oncolysis leads to anti-tumor cytotoxic CD8 response, which is required in order to achieve the full potential of OV treatment, the reality may be more complex. Considering that depleting CD8 T cells results in decreased therapeutic benefits regardless of tumor-specific CD8 T cell detection, non-specific T cell bystander effects are likely implicated. As a matter of fact, Sobol *et al* recently reported that antiviral CD8 cytotoxic T cells were central to HSV-mediated oncolysis (Sobol *et al.*, 2010). Furthermore, VSV was proposed to induce a general non-specific T cell activation that could assist in tumor cell killing (Galivo *et al.*, 2010). Anti-CD8 antibody depletion could also deplete other cell types expressing the CD8 molecule such as NKT cells and/or DCs, and therefore CD8 depletion may have a much broader impact than simply on the CD8 T cell population.

Proof that OVs engender a tumor-specific CD8 response directly contributing to the overall therapeutic outcome of the treatment remains elusive and may reflect the indirect aspect of this “assistance”.

The direct priming of tumor-specific T cell response following OV treatment may suffer from tolerization induced by the tumor microenvironment. To illustrate this reality, it has been shown that tumor-specific T cells following HSV treatment are reduced in tumor antigen tolerized animals, compared to wild-type animals bearing tumors derived from the exact same cell line (Sobol *et al.*, 2010). The low frequency of tumor-specific CD8 precursors has been hypothesized to be at least partly responsible for the low magnitude of tumor-specific response. Two different strategies were recently designed in an attempt to circumvent this weakness when using VSV oncolytic treatment. Bridle *et al.* developed a vaccination strategy using two viruses expressing the same endogenous tumor antigen. A first dose of adenovirus served to increase the pool of specific CD8 T cells. VSV oncolysis then enhanced the response and allowed increased therapeutic effect. This strategy was shown to promote the generation of an important tumor-specific CD8 response and to diminish the response directed against VSV (Bridle *et al.*, 2009; Bridle *et al.*, 2010b). Similarly, Whongtinda *et al* recently used a combination of tumor-specific T cell adoptive transfer and rVSV expressing the same antigen in order to generate functional tumor-specific CD8 T-mediated response. The use of a wild-type VSV (i.e. VSV not expressing the tumor antigen) combined with the same adoptive transfer failed to provide such CD8 T cell response (Wongthida *et al.*, 2011). The major limitation in the application of these two strategies resides in the need to boost CD8 T cell pools and generate an rVSV specific to a known tumor antigen; achieving a similar response to an unknown tumor antigen released from oncolysis remains an open project.

1.6 VSV combined treatment strategies

1.6.1 OV limitations

OVs are extremely efficient anti-cancer agents, but as for all cancer treatment strategies to date they are facing a certain number of limitations. OVs face multiple obstacles at all stages of strategy treatment, from delivery to tumor cell susceptibility for the initial round of replication, to the spread and subsequent tumor cells' reach. Although intratumoral delivery of the virus bypasses many of the blood-associated obstructions such as the neutralizing antibodies, complement and cell absorption (Willmon *et al.*, 2009a), OVs still face important barriers impeding its penetration and spreading into the tumor mass (Nguyen *et al.*, 2009). The first challenge is the susceptibility of the tumor cell to the specific OV, based on the pathway defect characteristics of the tumor cell as discussed at the beginning of this chapter. In addition, there are a number of physical barriers within the tumor mass that limit viral spread such as the extracellular matrix of proteoglycans, the density of the tumor cells and high interstitial pressure (Smith *et al.*, 2011). Furthermore, there is the problem of the dual impact of the immune system. Although initiating collateral tumor cell death and under certain circumstances also benefitting the adaptive response, the immune system also plays a predominant role in limiting viral replication and spread. Many of these obstacles limit OV-induced tumor cell killing and strategies to overcome these barriers and improve the benefits of OV treatment have been designed and tested over the last decade.

1.6.2 VSV in combination with other therapeutic agents

The combination of VSV with chemotherapeutic drugs or immune system-modulating agents achieved a very encouraging enhancement of VSV's therapeutic effects in number of preclinical animal models. As mentioned in section 1.5.1, combination treatment using drugs that silence the antiviral innate immune response such as HDAC inhibitors or Rapamycin allowed for increased viral replication, resulting in better treatment outcomes (Alain et al., 2010; Nguyen et al., 2008). In contrast to these combinations designed to impair IFN response, Shinozaki *et al.* combined VSV with a prophylactic injection of IFNα to reduce the neurotoxicity of wild-type VSV. Their results demonstrated enhanced VSV safety without compromising treatment efficacy in a tumor-bearing rat model (Shinozaki *et al*., 2005a). Other strategies combined VSV with drugs aimed at restoring the altered pathways of cancer cells to permit virally-induced apoptosis. This approach was shown to be efficient in certain types of cancer cells typically known to overexpress anti-apoptotic proteins. For example, B-cell chronic lymphocytic leukemia cells are known to highly express BCL2 and have an impaired apoptosis pathway; combining VSV with BCL2 inhibitors thus rendered these cells susceptible to VSV-induced apoptosis (Samuel *et al*., 2010; Tumilasci *et al*., 2008). Other approaches were designed to obtain synergistic killing effects on tumor cells using drug and OV combinations or multiple OV treatment regimens (Le Boeuf *et al*., 2010; Schache *et al*., 2009). To overcome the serum-neutralizing effect of naked virions when injecting OV intravenously, the cell carrier strategy was developed. In this approach cancer cells or immune cells are infected *in vitro* with an OV and are then injected into the animals. Using this approach, the virus is protected from the blood environment and was shown to reach the tumor more efficiently. Cancer cells, T cells and DCs have been successfully used as cell carriers for VSV (Boudreau et al., 2009; Power et al., 2007; Qiao et al., 2008a; Qiao et al., 2008b). To summarize, **Table 4** presents an overview of the different VSV and chemotherapeutic agent combinations developed to date.

Combined agent	Strategy	Aim	References
HDAC inhibitors	Modulating cancer cell gene expression	Enhancing virus replication	(Nguyen *et al.*, 2008)
BCL2 inhibitors	Restoring apoptosis susceptibility	Allowing virus apoptosis induction	(Tumilasci *et al.*, 2008) (Samuel *et al.*, 2010)
Doxorubicin	Synergistic induction of apoptosis	Enhancing cytotoxic effect	(Schache *et al.*, 2009)
Rapamycin	Impairing mTOR-dependent type 1 IFN production	Enhancing virus replication	(Alain *et al.*, 2010)
Vaccinia virus	Multiple OV targeting	Enhancing cytotoxic effect	(Le Boeuf *et al*., 2010)
Tumor specific T cell	Cell carrier	Avoiding serum neutralization Improving delivery	(Qiao *et al.*, 2008b)
T cell	Cell carrier	Avoiding serum neutralization Improving delivery	(Qiao *et al.*, 2008a)
DC	Cell carrier	Avoiding serum neutralization Improving delivery	(Boudreau *et al.*, 2009)
Tumor cell	Cell carrier	Avoiding serum neutralization Improving delivery	(Power *et al.*, 2007)
IFNα	Interferon α	Increasing safety	(Shinozaki *et al*., 2005a)

Table 4. VSV oncolytic combination therapies.

1.6.3 rVSV combination strategies

The direct engineering of VSV to express selected therapeutic genes represents an even more efficient combination strategy. VSV can be modified to express genes that enhance its overall toxicity, as in the case of VSV engineered to express the Newcastle disease virus fusion protein (Ebert *et al.*, 2004). Increased tumor cell killing and the targeting of non-infected tumor cells can also be achieved via insertion of suicide gene cassettes into the VSV genome. By expressing these genes the viruses acquire the ability to convert non-toxic compounds, which can be delivered systemically, into highly toxic chemotherapeutic drugs at the tumor site. This very attractive approach has been adopted for VSV expressing the HSV TK enzyme, *E. coli* CD::UPRT fusion enzyme or the human iodine symporter gene, and have all demonstrated enhanced therapeutic benefits (Fernandez et al., 2002; Goel et al., 2007; Porosnicu et al., 2003). In other approaches, VSV was designed to increase its tumor cell specificity. Bergman *et al.* developed a system where the G protein of VSV was replaced with a fusion antibody receptor specific to the Her2/neu cell marker. This cell marker is known to be overexpressed in a high percentage of breast cancers, and therefore this rVSV is specifically targeted to breast cancer (Bergman *et al.*, 2003). Edge *et al*. also increased the tumor specificity of VSV by incorporating a microRNA targeting strategy into the virus. MicroRNA are small noncoding RNA that direct the translation repression of host mRNA, and their expression is known to be downregulated in a number of cancers. Incorporating these microRNA complement sequences into VSV mRNA was shown to enhance its safety while retaining its full therapeutic potential (Edge *et al.*, 2008). VSV was also engineered to express IFN-β, with the same objective of increasing the safety profile of the virus. This virus demonstrated attenuated *in vivo* side effects while retaining its full oncolytic properties (Jenks et al., 2010; Obuchi et al., 2003). The potential of this specific rVSV to enhance the adaptive anti-tumor immune response was also analyzed, and was found to have no impact (Willmon *et al.*, 2009b). Many other rVSVs were designed to modulate the innate and adaptive immune response by expressing various chemokines such as IL-4, IL-12 or IL-23 (Fernandez et al., 2002; Miller et al., 2009; Shin et al., 2007). Although these viruses were more efficient at inducing tumor growth delay in mouse tumor models, these

studies lacked a rigorous analysis of the immune impact of these cytokine expressions. VSV was also engineered to express the growth factor GM-CSF; even though the report focused on the attenuation phenotype of the rVSV, the authors reported an increased CD11b+ (referred to as macrophages) population in the lung following intranasal immunization (Ramsburg *et al.*, 2005). Various groups also generated VSV to directly express a tumor antigen, with the direct objective of enhancing the anti-tumor specific adaptive immune response, (Bridle et al., 2009; Bridle et al., 2010b; Diaz et al., 2007; Wongthida et al., 2011). These viruses were shown to successfully induce an adaptive immune response to their respective antigen; however, maximal therapeutic efficacy was only obtained through their combination with adoptive T cell transfer or a vaccination approach (Bridle et al., 2009; Bridle et al., 2010b; Wongthida et al., 2011). To summarize, **Table 5** presents a global review of recent literature on cancer treatment strategies that involved engineered VSV.

On a final note, the work presented in this thesis incorporates novel aspects to this combination field, aspiring to enhance and modulate the oncolytic properties of VSV.

rVSV	Strategy	Aim	References
VSV-NIS	Iodine symporter suicide gene system	Improving non-infected tumor cell killing and oncolysis imaging	(Goel *et al.*, 2007)
VSV-CD::UPRT	Cytosine deaminase suicide gene system	Improving non-infected tumor cell killing	(Porosnicu *et al.*, 2003)
VSV-TK	Thymidine kinase suicide gene system	Improving non-infected tumor cell killing	(Fernandez *et al.*, 2002)
VSV-NDV/F	Newcastle disease virus fusion protein	Enhancing cytotoxic effect	(Ebert *et al.*, 2004)
VSV-gG	HSV-1 glycoprotein G; chemokine binding protein	Enhancing virus replication through impaired NK and NKT cell tumor attraction	(Altomonte *et al.*, 2008)
VSV-Her2/neu	Sindbis-SCA-erbb2 receptor instead of VSV-G	Retargeting VSV to Her2/neu overexpressing cancer	(Bergman *et al.*, 2004)
VSV-IFNβ	Interferon β	Enhancing safety and innate immune response	(Obuchi *et al.*, 2003) (Willmon *et al.*, 2009b) (Jenks *et al.*, 2010)
VSV-Il4	IL-4 to promotes Th2 differentiation bias	Modulating adaptive immune response	(Fernandez *et al.*, 2002)
VSV-GM-CSF	Growth factor	Reducing pathogenicity and enhancing immune response	(Ramsburg *et al.*, 2005)
VSV-IL12	Il-12 proinflammatory cytokine, NK and T cell activation	Modulating adaptive immune response	(Shin *et al.*, 2007)
VSV-IL23	IL-23 proinflammatory cytokine	Modulating adaptive immune response	(Miller *et al.*, 2009)

VSV-OVA	Ovalbumin tumor antigen	Improving tumor-specific adaptive immune response	(Diaz *et al.*, 2007)
VSV-hDCT	hDCT tumor antigen	Improving tumor-specific adaptive immune response (in a vaccine strategy)	(Bridle *et al.*, 2009) (Bridle *et al.*, 2010b)
VSV-hgp100	Hgp100 tumor antigen	Improving tumor-specific adaptive immune response (in an adoptive transfer strategy)	(Wongthida *et al.*, 2011)
VSV-ASEL	cDNA librairy	Improving tumor-specific adaptive immune response	(Kottke *et al*., 2011)
VSV-CD40L	CD40 ligand	Enhancing general T cell activation	(Galivo *et al.*, 2010)
VSV-let7	Let7 microRNA complementary sequence	Increasing safety	(Edge *et al.*, 2008)
VSV-SV5-F	SV5 Fusion protein of paramyxovirus instead of VSV-G	Increasing safety	(Chang *et al.*, 2010)

Table 5. rVSVs implicated in oncolytic viral therapy.

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CHAPTER 2

RESULTS

Rationale and specific aims

Through years of evolution VSV has developed as an optimal organism at performing its main duty, that of replication. Taking advantage of this virus's important characteristics for cancer therapy is a very promising approach, however some viral properties are suboptimal for the redirected duty of this virus. The objective of this thesis is to improve VSV's oncolytic characteristics by engineering the virus to express genes that could potentiate tumor cell killing or modulate the immune response.

Accordingly, specific aims were to:

1- **Enhance VSV's local induction of apoptosis at the tumor site by incorporating a suicide gene system.**

2- **Improve VSV's induction of the tumor-specific adaptive immune response by increasing the tumor antigen presentation capacities simultaneously with VSV oncolysis.**

3- **Use the developed approaches as tools to better characterize the multiple aspects of VSV oncolytic treatment and to improve the design of current and novel VSV-based strategies.**

The recombinant VSVs involved in this study

To achieve these aims, four recombinant VSVs were created and are presented in the three sections of this result chapter.

Name	Representation	Section
VSV-C		Manuscript 1
VSV-F		Manuscript 2
VSV-C/F		Other combinations
VSV-20		Other combinations

Figure 1. Schematic representation of the four rVSVs involved in this study. VSV-C encodes the CD::UPRT fusion enzyme used in a suicide gene strategy to improve VSV-induced apoptosis. VSV-F encodes the Flt3L growth factor, promoting the augmentation of the DC population. VSV-C/F was engineered to encode both C and F factors. VSV-20 encodes a chemokine that is known to recruit immature DCs. All viruses were created using the VSV MΔ51 mutant background.

Manuscript I

Enhancing VSV oncolytic activity with an improved cytosine deaminase suicide gene strategy

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Running title: Improving VSV-CD::UPRT suicide gene combination

Keywords: VSV, oncolysis, suicide gene, 5FC, combination therapy, synergism

Preamble for Manuscript I

VSV is very efficient at infecting and inducing apoptosis of cancer cells *in vitro*; however, *in vivo* reality imposes a more challenging situation, as presented in the introduction chapter. In the case of a solid tumor, it is unlikely that all tumor cells will be infected, and therefore the uninfected proportion of tumor cells could potentially evade treatment and cause cancer regrowth. The VSV-based suicide gene strategy presented in this section aims at targeting the uninfected cancer cells and inducing their apoptosis. The CD::UPRT / 5FC system is one of the more promising and efficient suicide gene approaches. VSV-C allows the expression of the CD::UPRT enzyme only where replication occurs, and thus the enzymatic conversion of a systemically delivered non-toxic drug into a highly active chemotherapeutic agent occurs only at the tumor site. Infected cancer cells producing the enzyme and releasing the active form of the drug generates collateral induction of cancer cell killing in uninfected cancer cells. As this strategy is aimed at increasing *in vivo* cancer cell killing, the next section emphasizes the fine-tuning of the different components of the *in vivo* treatment to reach maximal tumor killing benefits.

Abstract

Oncolytic viruses (OVs) are promising therapeutic agents for cancer treatment, with recent studies emphasizing the combined use of chemotherapeutic compounds and prodrug suicide gene strategies to improve OV efficacy. In the present study, the synergistic activity of recombinant VSV-MΔ51 virus expressing the CD::UPRT suicide gene and 5FC prodrug was investigated in triggering tumor cell oncolysis. In a panel of VSV sensitive and resistant cells - prostate PC3, breast MCF7 and TSA, B-lymphoma Karpas and melanoma B16-F10 - the combination treatment increased killing of non-infected bystander cells *in vitro* via the release of 5FC toxic derivatives. In addition, we showed a synergistic effect on cancer cell killing with VSV-MΔ51 and the active form of the drug 5FU. Furthermore, by monitoring VSV replication at the tumor site and maximizing 5FC bioavailability, we optimized the treatment regimen and improved survival of animals bearing TSA mammary adenocarcinoma. Altogether, this study emphasizes the potency of the VSV-CD::UPRT and 5FC combination and demonstrates the necessity of optimizing each step of a multi-component therapy in order to design efficient treatment.

Introduction

Targeted cancer therapy using oncolytic viruses (OVs) is an experimental therapeutic approach that is now supported by promising pre-clinical and clinical advances [1, 2]. OVs exploit genetic abnormalities and altered signaling pathways in tumor cells to achieve selective virus replication and tumor cell lysis [3]. Vesicular Stomatitis Virus (VSV) is a negative single stranded RNA virus that has served as an important prototype OV. VSV is exquisitely sensitive to type 1 interferon-mediated antiviral responses in untransformed cells, and consequently its selective onco-tropism is attributed to the innate antiviral response suppression in tumor cells [4-7]. Although successful tumor eradication and tumor growth delay have been reported in animal models following treatment with OVs, several cancer models remain partially or completely resistant to viral oncolysis. Barriers to effective tumor oncolysis include intrinsic resistance of tumor cells to infection, limited tumor cell death induced by direct viral replication and inefficient viral spreading within the tumor mass [8].

In order to overcome these barriers to oncolysis, experimental strategies are now combining OVs with different cytotoxic agents to generate a synergistic effect between the OV and the chemotherapeutic compound that augment tumor cell killing. VSV has been used in combination with chemotherapeutic agents such as histone deacetylase inhibitors, BCL-2 inhibitors, rapamycin, doxorubicin and other compounds to enhance therapeutic activity [9-12]. Limitations arising from the use of chemotherapeutic agents include non-selective toxicity in healthy tissues and development of drug resistance. One way to circumvent these restrictions and to further utilize OV combinations is to incorporate a suicide gene strategy that allows specific gene transduction of tumor cells with non-mammalian enzymes that convert innocuous prodrugs into highly toxic chemotherapeutic compounds within the tumor [13].

VSV represents an excellent choice as a vector for suicide gene transduction because of selective cancer cell tropism and the relative ease of foreign gene insertion [4, 5, 14]. VSV engineered to express suicide enzymes allow the conversion of non-toxic prodrug into a

toxic form only at the site of viral replication, thus generating specific bystander killing at the tumor site. Recombinant VSV carrying the Herpes virus TK enzyme has been shown to phosphorylate the nontoxic prodrug ganciclovir, facilitating its DNA integration and subsequent local toxicity [13, 15]. A similar strategy was employed with VSV expressing the human sodium iodine symporter (hNIS) that resulted in the accumulation of radioactive iodine at the tumor site [16]. Both of these recombinant VSV strategies were shown to delay tumor growth in murine models, however there action is restricted to cells that are directly infected by VSV.

One of the most potent suicide gene strategies involves local expression of the fusion enzyme CD::UPRT and the systemic delivery of the non-toxic 5-Fluorocytosine (5FC). CD::UPRT enzyme is the fusion of *E. coli* cytosine deaminase (CD) and uracil phosphoribosyltransferase (UPRT); CD catalyzes the deamination of the non-toxic 5FC into the commonly used chemotherapeutic 5-Fluorouracil (5FU). 5FU undergoes further enzymatic conversion by mammalian enzymes to form toxic derivatives that incorporate into DNA and RNA, and inhibit thymidylate synthetase enzymatic activity. The UPRT enzyme bypasses some rate-limiting mammalian enzymatic steps in the conversion of 5FU and therefore increases its toxicity. The high solubility of 5FU promotes a strong bystander effect on neighboring tumor cells that are not actively infected, which confers an important advantage to this combination compared to other suicide gene strategies [13, 17]. The CD::UPRT-5FC system is thus a powerful tool for cancer therapy, including oncolytic experimental strategies [18-22].

In addition to these suicide gene approaches, VSV variants that possess increased oncolytic potential compared to wild-type VSV have been characterized. The methionine 51 deletion in the matrix gene (VSV-MΔ51) is the best-characterized and probably most potent variant. [6] Because of the altered M protein, VSV-MΔ51 no longer blocks the nuclear export of host RNA encoding antiviral proteins, including multiple species of interferons (IFN), and thus triggers a stronger IFN response in normal tissues that inhibits VSV infection, whereas VSV replication in IFN-defective tumor cells is not altered [4, 6, 23, 24]. In the present study we merged the attributes of VSV-MΔ51 with the CD::UPRT/5FC

suicide gene system and further improved the previously reported VSV - CD::UPRT strategy[22]. Recombinant VSV-MΔ51 engineered to express CD::UPRT in combination with 5FC increased cancer cell killing *in vitro* in VSV resistant cell lines and in a viral dissemination blocking model. In addition, a synergism between VSV oncolysis and 5FU chemotherapeutic killing was observed. Furthermore, to improve the *in vivo* efficacy of the combination we coordinated the kinetics of VSV replication within the tumor with 5FC bioavailability, and obtained stronger therapeutic effect in a syngeneic tumor model.

Results

Generation and characterization of VSV expressing CD::UPRT enzyme. VSV-MΔ51 was genetically modified to express the *E. coli* fusion enzyme CD::UPRT (VSV-C) by inserting the gene cassette between the G and L coding regions of the VSV-MΔ51 genome vector (Figure 1a). To confirm that the insertion did not affect viral replication, the growth rate of the resulting recombinant VSV was determined and was similar to VSV-MΔ51 without gene insertion (Figure 1b). To assure the expression and functionality of the CD::UPRT fusion enzyme, a spectrophotometric enzymatic assay was used to measure 5FC prodrug conversion into 5FU and downstream products. VSV-C infected cell lysates contained functional enzyme that allowed degradation of 5FC into its active 5FU cytotoxic derivatives, while uninfected or VSV-MΔ51 infected cell lysates did not show degradation of 5FC (Figure 1c).

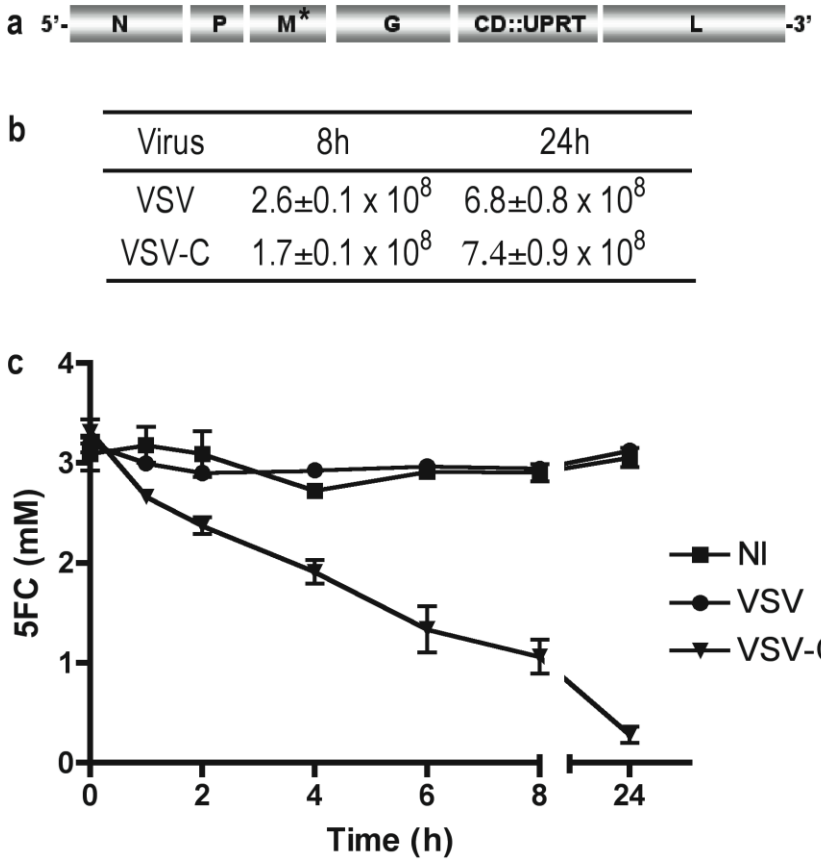

Figure 1. VSV-C generation and enzyme expression. (a) Representative schematic of VSV-C genome with the *E. coli* fusion enzyme CD::UPRT cassette inserted between the G and L genes. **(b)** Viral titers expressed in PFU/ml in the supernatant of MCF7 cells 8h and 24h post-infection (0.1 MOI). **(c)** 5FC conversion by VSV-C CD::UPRT expression; MCF7 cell lysates 24h post infection (0.1 MOI) were resuspended in PBS containing 3mM of 5FC to monitor 5FU conversion by spectrophotometry. *: Methionine 51 deletion. Error bars represent SE of triplicate.

VSV-C/5FC combination results in bystander cell killing *in vitro*. Having confirmed functional expression of the transgene, we next examined whether the combination approach would increase bystander cell killing. In VSV-sensitive cell lines, viral lysis occurs rapidly and death of all cells is normally achieved within 48h. In these cells, killing induced exclusively by 5FU and its cytotoxic derivatives cannot be measured directly *in vitro* and the two events – direct VSV cell lysis and cytotoxicity of 5FU following 5FC conversion – must be separated. Events were initially separated in time as previously reported [22]. VSV-sensitive MCF7 cells were infected with VSV-C for 24h in the presence of 5FC. Conversion of 5FC into 5FU and downstream products reached 39% and 90 % at 24h and 48h, respectively (data not shown). Supernatants were collected, heat-inactivated to destroy the virus, diluted and added to freshly plated MCF7 cells for 48h before cell viability was monitored. The toxic effect of 5FU derivatives that had been converted during VSV-C replication was observed with supernatants diluted as high as 1 in 250. Supernatants from non-infected cells supplemented with 5FC did not result in cell killing, confirming that 5FC does not cause cytotoxicity and is not spontaneously converted. In addition, cell death by any remaining infectious virus after heat inactivation was not a contributing factor, since infected supernatants did not affect cell viability in the absence of 5FC (Figure 2a).

An additional experimental model in which infected cells were separated from non-infected cells was developed to mimic a tumor environment where only a portion of the cells are actively infected by VSV. Murine TSA mammary adenocarcinoma cells were plated in trans-well chambers separated by a 0.02um membrane that allowed free diffusion of drugs while blocking passage of virions. On one side of the chamber, cells were infected with VSV-C in the presence of 5FC. At 48h, the cell population infected with VSV-C in the presence of 5FC reduced the viability of the population on the other side of the trans-well to 28%, whereas the absence of either VSV-C or 5FC did not reduce cell viability of the second population (Figure 2b). Thus, in a tumor environment, cancer cells actively infected with VSV-C in the presence of 5FC are able to drive cell death of non-infected surrounding tumor cells by the diffuse chemotherapeutic action of 5FU.

VSV-C/5FC increases the spectrum of cancer cell killing. To further demonstrate the increased therapeutic potential of the combination, the efficacy of the VSV-C/5FC combination was examined in cancer cell lines that are resistant to VSV oncolysis and restrict virus replication, even at high MOI. In these cell lines, VSV does not lead to massive cell lysis and therefore 5FU mediated cell killing could be directly measured. Karpas - human B-lymphoma, B16-F10 - mouse melanoma, and PC3 - human prostate cancer cell lines were infected with VSV-C in the presence of 5FC. In all three cell lines, despite the limited number of infected cells, the conversion of 5FC to 5FU and its derivatives significantly decreased cell viability compared to VSV treatment alone - 72 to 36%, 73 to 41% and 50 to 27%, respectively (Figure 2c). Thus the VSV-C/5FC prodrug combination expands the spectrum of tumor cells that can be targeted using VSV by promoting efficient cell killing through 5FC toxic derivative release.

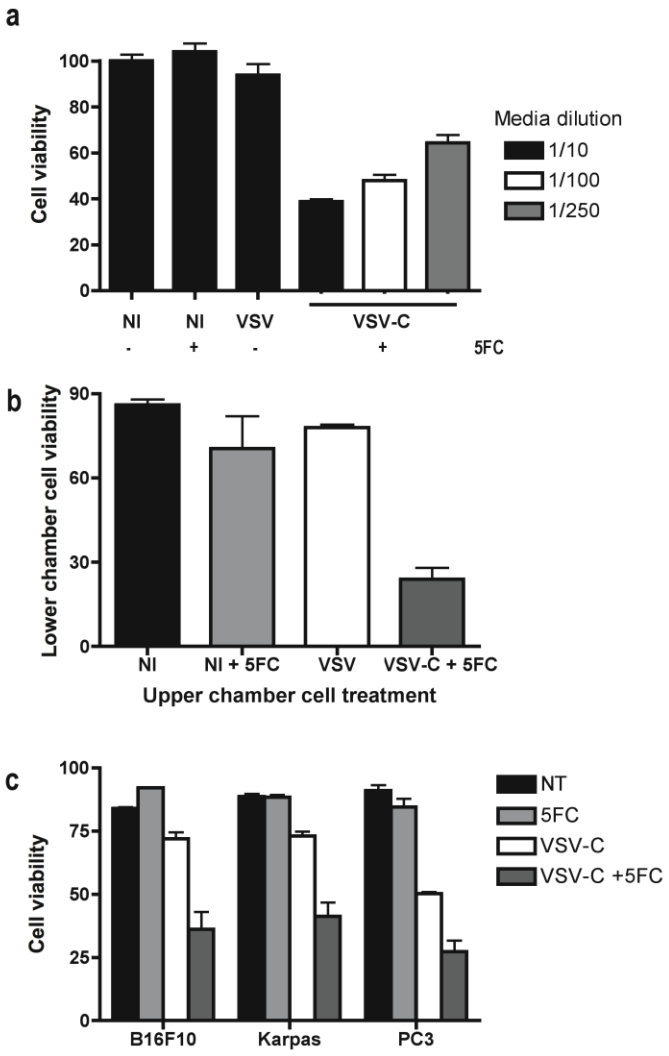

Figure 2. VSV-C/5FC combination results in bystander cell death of non-infected cells. (a) Supernatants from MCF7 infected cells 24h post-infection (0.1 MOI; with or without 5FC) were heat inactivated and fresh cells were incubated with different dilutions of supernatants. Cell viability was monitored after 48h by MTT assay. **(b)** TSA cells were seeded (with or without 5FC) in two compartments separated by a 0.02um membrane; the upper chamber cells were infected at an MOI of 0.1 with or without 5FC and viability of cells in the lower chamber were monitored 48h later by AnnexinV/PI FACS analysis. **(c)** B16-F10, Karpas-422 and PC3 cells were infected with VSV-C at 10, 1 and 0.1 MOI, respectively (with or without 5FC). Cell viability was monitored 96h later by AnnexinV/PI FACS analysis. Error bars represent SE of triplicate.

VSV and 5FU have a synergistic effect on cancer cell killing. Combination treatment leads to the simultaneous presence of VSV and 5FU within the tumor and therefore potentially affects two aspects of the treatment: cancer cell killing and viral replication. To determine the effect of the simultaneous presence of the two active agents on cancer cell killing, Karpas and PC3 cell lines (both VSV resistant), were incubated with 5FU and VSV-MΔ51 (harboring no gene insertion). As expected, VSV-MΔ51 treatment alone modestly reduced Karpas and PC3 cell viability to 70% and 57%, respectively, while 5FU treatment alone reduced survival to 48% and 38%, respectively. The combined treatment of VSV-MΔ51 and 5FU resulted in a dramatic reduction in tumor cell viability, with only 29% of Karpas and 24% of PC3 cells remaining alive (Figure 3a). Cell viability results were used to calculate the combination index (CI) based on the method of Chou and Talalay [25] and in both cell lines, the combination of 5FU and VSV-MΔ51 resulted in CI values that revealed significant synergism (Figure 3b). These data further highlight the advantage of the VSV-C/5FC combination since the active form of the drug - which is only present at the site of the tumor - can synergize with VSV to enhance tumor cell killing.

Secondly, the effect of 5FU on VSV replication was examined. At sub-effective 5FU concentration, part of the cells survive 5FU treatment and the ability of VSV to replicate in these cells was evaluated. Murine TSA cells were pretreated with a sub-effective concentration of 5FU for 24h and then infected with VSV-GFP; living cells were discriminated from apoptotic cells by Annexin V/PI staining and expression of GFP in living cells was used as a marker of viral replication. At 1 MOI, VSV infected TSA cells at a similar rate, regardless of the presence of 5FU, whereas at 0.1 and 0.01 MOI, 5FU treatment delayed the onset of VSV replication, although VSV infection levels were restored by 24h (Figure 3c). Thus in a tumor environment, VSV replication may be slowed down but should not be blocked by the presence of 5FU.

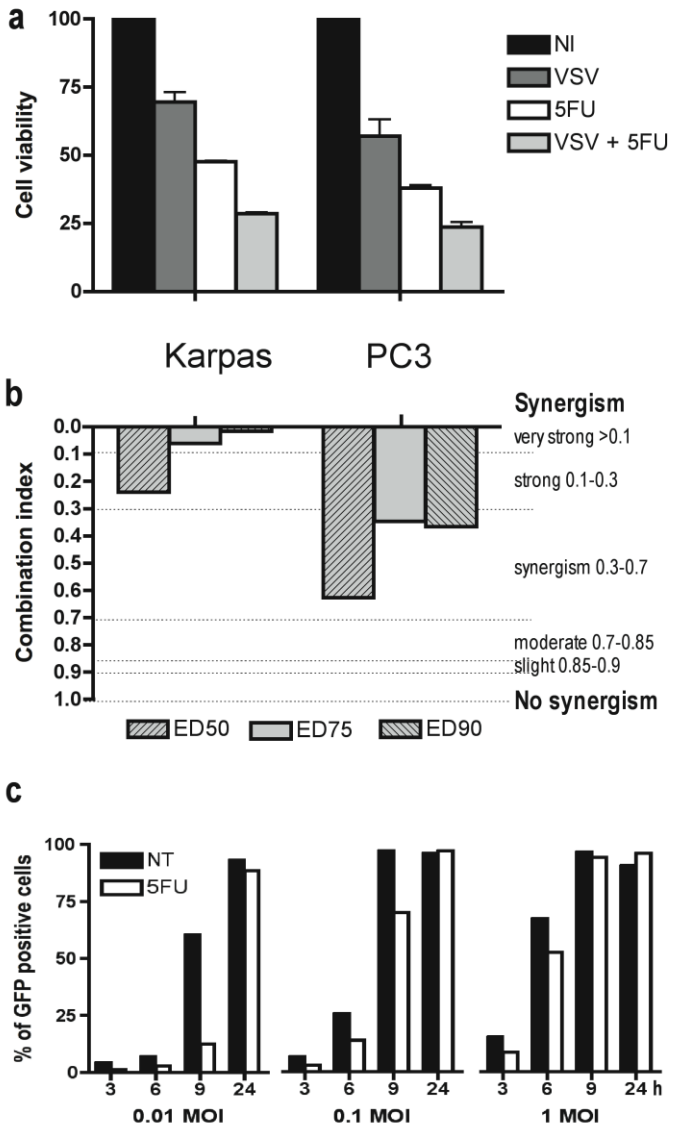

Figure 3. VSV and 5FU synergistically increase cell killing without inhibiting VSV replication. Karpas and PC3 cells were infected with VSV at 10 and 0.1 MOI, respectively (with or without 5FU); **(a)** cell viability was monitored 48h later by MTT assay and **(b)** combination index were calculated according to the method of Chou and Talalay and reported for 3 different effective doses **(c)** VSV replication in the presence of 5FU; TSA cells were pre-incubated with sub-toxic concentration of 5FU for 24h and infected with VSV-GFP at different MOIs (5FU was kept in the media). The percentage of live GFP-positive infected cells was monitored over a period of 24h by FACS. Error bars represent SE of three independent experiments.

Adapting the prodrug strategy to VSV-MΔ51 *in vivo*. The *in vitro* efficacy of the VSV-C/5FC combination prompted us to examine the parameters for *in vivo* treatment. VSV is an acutely replicating oncolytic virus with a short replication cycle; it is rapidly cleared by the immune system and consequently its presence at the tumor is transient [26, 27]. In order to correlate the expression of the prodrug-converting enzyme with 5FC availability at the tumor site, VSV was monitored in two tumor models. Subcutaneous TSA mammary adenocarcinomas and EG7 T cell lymphoma were treated with two intra-tumoral doses of a VSV-MΔ51 expressing luciferase on day 0 and 3. In the TSA model, high virus titers were detected in the tumor homogenates during the first three days after injection but decreased thereafter until titers were below detection by day 6 (Figure 4a). Although viral replication was higher in the EG7 model compared to the TSA model, viral titers also drastically decreased at 6 days (Figure 4b). Monitoring luciferase activity from the tumor homogenates also offered a relative measure of the time frame of CD::UPRT expression, since the luciferase gene was inserted into VSV genome at the same position as CD::UPRT. The relative light unit generated by luciferase expression was high during the first few days after inoculation but faded rapidly, although a second injection extended enzyme expression for an additional day in both tumor models (Figure 4b and d). Altogether these data indicate that VSV replication and gene expression at the tumor was high for a period of 3-4 days, but essentially disappeared by day 6. Thus in the VSV-C/5FC system, 5FC conversion would occur for ~96h after VSV injection.

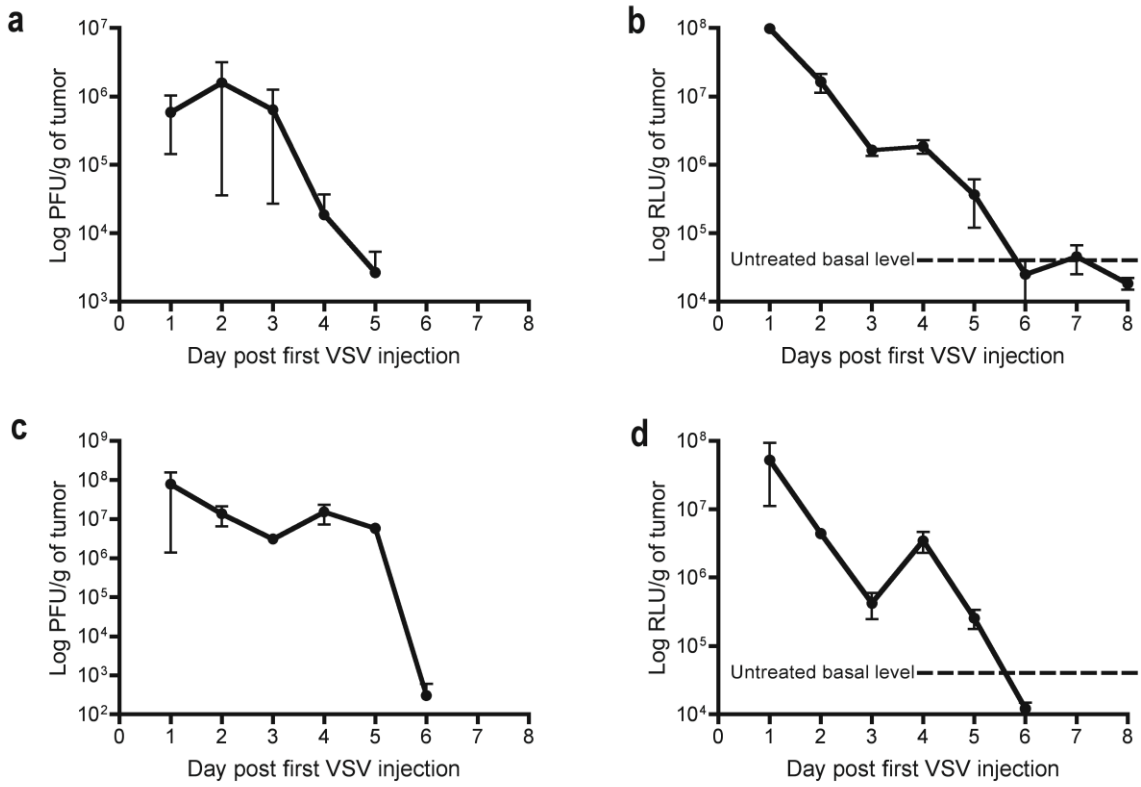

Figure 4. VSV presence and enzyme expression at the tumor is a highly acute event. Syngenic TSA mammary adenocarcinoma **(a, b)** or EG7 T cell lymphoma **(c, d)** tumors were established subcutaneously; 7 days later when tumors were palpable, $2x10^7$ PFU of VSV-Luc was injected intra-tumorally on day 0 and 3. Two mice were sacrificed daily over 8 days and tumors were collected to determine **(a, c)** viral titers and **(b, d)** Luciferase activity.

Adapting the VSV-C/5FC strategy to 5FC bioavailability *in vivo*. In previous reports, 5FC was administered to mice at 500mg/kg/day intra-peritoneally for 10 days [19-22]. Meanwhile, other studies demonstrated that the half-life of 5FC was approximately 40 minutes and that 5FC freely diffused into tissue [28-30], suggesting that after 160 minutes (i.e. 4 half-life), only 6% of the initial 5FC dose would be present at the tumor. Therefore, an adapted treatment regimen was designed to maximize the bioavailability of CD::UPRT enzyme at the tumor (Figure 5a). To test the toxicity of the modified regimen, mice were injected with 500mg/kg every 160 minutes: 4 times per day for 4 days. No sign of toxicity was observed and animals did not suffer any weight loss compared to control animals treated with PBS (Figure 5b).

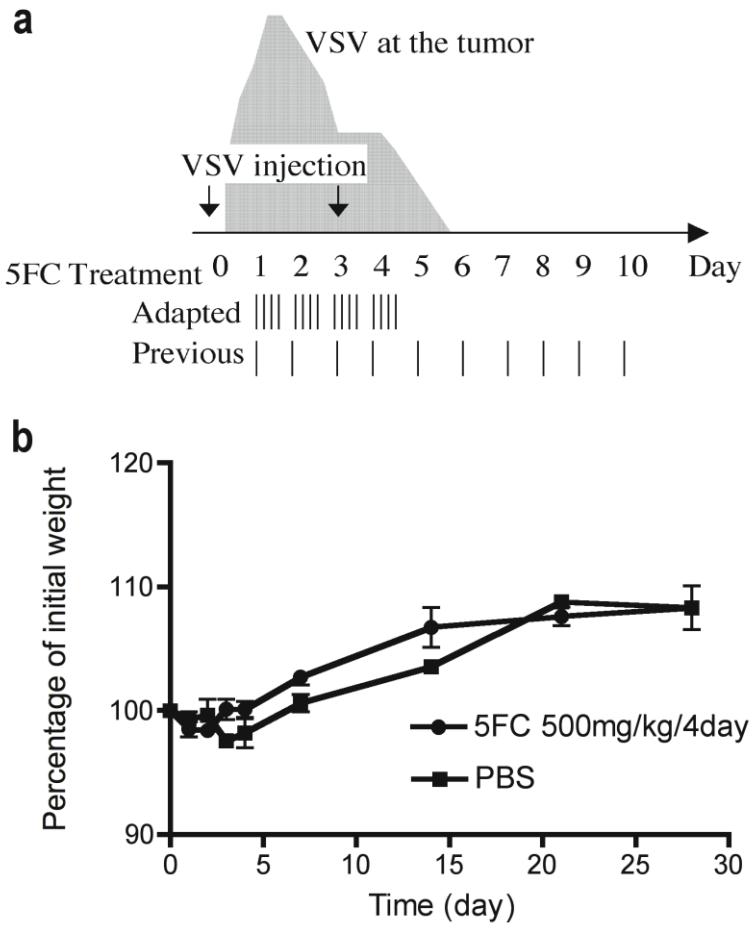

Figure 5. Adapting the 5FC dosing regimen to the presence of VSV at the tumor. (a) Schematic representation of the VSV/5FC-prodrug treatment schedule. **(b)** Balb/c mice received a 5FC dosing regimen of 500mg/kg every 160 minutes 4 times/day for a period of 4 days and weight was monitored (n=4). Error bars represent SE.

The adapted VSV-C/5FC regimen improved therapeutic effect. The efficacy of the adapted VSV-C/5FC regimen was tested *in vivo* in TSA tumor bearing mice. The adapted combined treatment delayed the onset of tumor growth compared to VSV-MΔ51 treatment alone (P value of 0.002) (Figure 6a). Furthermore, survival improved compared to VSV-MΔ51 treatment alone (P value of 0.0006), with 3 of 10 animals completely tumor free for >60 days post-treatment (Figure 6b). On the other hand, in animals inoculated with VSV-C and 500mg/kg/day of 5FC for 10 days, no significant delay in tumor growth and/or survival was observed compared to animals treated with VSV-MΔ51 alone (data not shown). Altogether, these data demonstrate that increasing the frequency of 5FC administration to coordinate bioavailability of 5FC with acute VSV replication significantly improved the therapeutic effect of VSV-MΔ51.

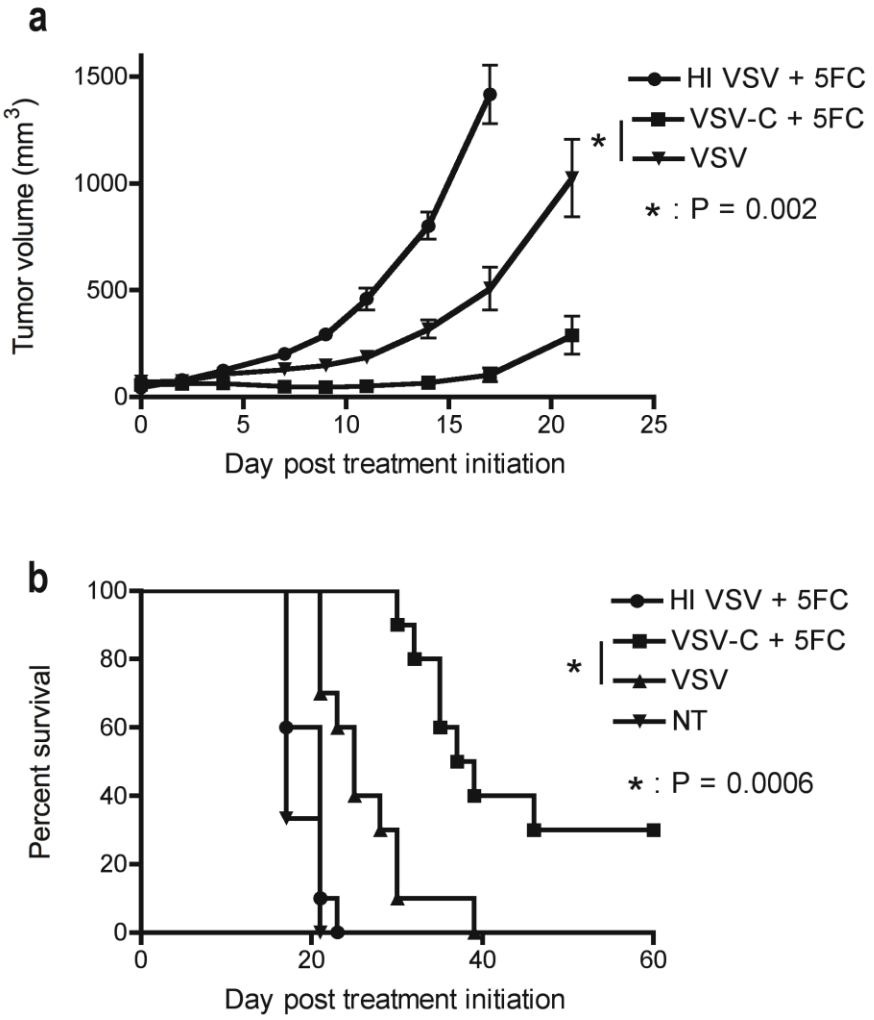

Figure 6. VSV-C and the adapted 5FC dosing regimen increase animal survival. TSA mammary adenocarcinoma tumors were established by subcutaneous inoculation of $3x10^5$ cells; 7 days later when tumors were palpable, $2x10^7$ PFU of VSV-C, VSV or heat-inactivated VSV were injected intra-tumorally on day 0 and 3. 5FC was administered by intra-peritoneal injections corresponding to 500mg/kg every 160 minutes 4times/day for a period of 4 days **(a)** Tumor volume was monitored and **(b)** animal survival was recorded (n = 10). Error bars represent the SE.

Discussion

The combination of CD::UPRT suicide gene expression and 5FC prodrug conversion – together with tumor specific VSV oncolysis – represents a powerful experimental therapeutic approach, characterized by a dual tumor cell killing effect. In the present study, we incorporated a potent oncolytic VSV variant (VSV-MΔ51) with the enzymatic activity of CD::UPRT that converts 5FC into the toxic 5FU. Building upon the previous report of VSV expressing this powerful suicide gene, we further demonstrated the potential of this strategy by exploring new approaches *in vitro* and more importantly by refining the therapy *in vivo*. By monitoring VSV replication at the tumor site and correlating results with the bioavailability of 5FC, the modified treatment regimen improved survival of animals bearing TSA mammary adenocarcinoma subcutaneous tumors.

In vitro data demonstrated that a few infected cancer cells through the release of 5FU and other toxic derivatives synergistically increased cell killing of non-infected cells. Furthermore, the combination targeted tumor cells that are normally refractory to VSV oncolysis, thus potentially increasing the spectrum of cancer cells susceptible to VSV treatment. Given that higher tumor cell killing is achieved from fewer infected cells, it appear that tumor cell killing induced by the combination treatment *in vivo* would not be limited by direct virus replication, inefficient spread of virus or intrinsic resistance to oncolysis, therefore reinforcing the capacity of this approach to overcome several limitations to OV-mediated oncolysis [8].

Combination strategies using chemotherapeutic compounds and OVs simultaneously are designed to target and increase cancer cell killing, in part by modulating the antiviral state of the tumor cell, or by enhancing the capacity to stimulate apoptosis and/or cell cycle arrest [9-12]. Histone deacetylase inhibitors or rapamycin have demonstrated enhanced effects on oncolysis, in part through their capacity to increase OV replication, by dampening the innate antiviral response [9, 11]. Increased therapeutic effect has also been achieved under circumstances when viral replication and spreading are not enhanced. [10]

Tumor cells encountering 5FU at sub-toxic concentrations are altered metabolically by the drug[31]; such cellular changes could potentially affect OV permissiveness. The previous study[22] had addressed this question by pre-treating cells with 5FU for 24h, removing the drug at the time of infection and monitoring viral GFP expression. No change in VSV replication was reported, although a 25% reduction in GFP positive cells was observed at 12h and a ~7-fold reduction in viral titer 24h following infection at 0.1MOI. We addressed this concern differently by using continuous 5FU treatment at concentrations corresponding to the ED50 over a period of 48h - which is necessary to allow for 5FU cytotoxicity [31] - and by analyzing the susceptibility of the surviving cells to VSV. Although infection kinetics was slowed at low MOI, VSV was able to infect viable cells, indicating that 5FU did not affect viral infection. While our data clearly demonstrated a synergistic effect on cancer cell killing between 5FU and VSV, the VSV-C/5FC combination and subsequent conversion to 5FU did not increase virus replication in cancer cells *in vitro*. Therefore, the beneficial effect of the VSV-C and 5FC combination *in vivo* resides in bystander killing of surrounding non-infected cells by 5FU and other toxic derivatives.

One of the important challenges of this study was to refine the *in vivo* VSV-C/5FC strategy to improve therapeutic outcome. The 5FC administration was modified to coincide with the peak of VSV replication within the tumor. Using two different subcutaneous syngenic tumor models - TSA, a VSV resistant model where virus treatment causes only tumor growth delay and EG7, a VSV sensitive model where VSV oncolysis cures the majority of the mice (S.L. unpublished data) - we demonstrated the transient nature of VSV kinetics at the tumor following two intra-tumoral injections, consistent with other studies in CT26 and B16OVA models [26, 27]. The acute pattern of VSV replication prompted us to re-evaluate the timing of 5FC administration, in order to maximize CD::UPRT enzyme expression with 5FC bioavailability. While previous studies with CD::UPRT suicide gene approaches reported a typical 5FC dosing of 500mg/kg/day for 10 days [18-22], transient VSV replication and gene expression within the tumor indicated that this regimen was not optimal for a VSV-based therapy. Furthermore, studies evaluating 5FC bioavailability and half-life in serum and tissue highlighted a

multiple-dose-a-day regimen [28-30]. Although increasing the administration frequency raises concerns about toxicity, 5FC has been used experimentally as an anti-fungal agent at doses up to 200mg/kg every 6h for 7 days without signs of toxicity [32]. The current regimen of 500mg/kg four times a day for four days did not reveal any signs of toxicity and permitted a correlation between maximal 5FC bioavailability and high level expression of CD::UPRT at the tumor. The modified VSV/5FC prodrug combination inhibited tumor growth better than either treatment alone, and increased animal survival, relative to the previously published combination approach in the TSA syngenic tumor model [22]. These experiments emphasized the advantages of analyzing each step of a multi-component therapy in order to optimize the treatment schedule and achieve maximal therapeutic benefit. In conclusion, this study further demonstrates the potential of an OV-suicide gene strategy for cancer therapy and highlights the necessity to analyze different kinetic aspects in order to design the most effective treatment.

Acknowledgments

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Material and Methods

Cell lines and viral production. MCF7 human breast cancer, B16-F10 mouse melanoma and Vero cell lines were cultured as recommended by ATCC. TSA mammary adenocarcinoma cells were a kind gift of Dr. Barber (University of Miami, Miami, FL) and were grown as previously described [33]. Karpas-422 cells were purchased from the German Collection of Microorganisms and Cell Cultures and were grown in RPMI 1640 medium with 10% FBS. EG7 mouse T cell lymphoma were a kind gift of Dr. Galipeau (McGill University, Montreal, Canada) and were grown in RPMI 1640 medium with 10% FBS with 0.5mg/ml of G418. Virus stocks were grown in Vero cells, concentrated from cell-free supernatants by centrifugation and titrated by standard plaque assay as previously described [10, 34].

Viruses and construction of recombinant VSV. To create VSV-C, the CD::UPRT *E. coli* fusion enzyme was amplified from a derivative plasmid (kindly provided by Dr. Bernard Massie, NRC Biotechnology Research Institute, Montreal, QC) containing CodA::upp gene (Invivogen, San Diego, CA) using 5'-AAGGACTCGAGCCATGGTGTCGAATAACGCTTT-3' and 5'-ATTTCTCTAGACTTATTTCGTACCAAAGATTTTGTC-3'. Amplified product was digested with Xho1/Xba1 and ligated into the Xho1/Nhe1 unique site between the G and L viral genes of the VSV genome expressing vector harboring the Methionine 51 deletion in the M coding sequence [6]. Infectious recombinant VSV was recovered as described previously [14]. VSV-GFP and VSV-Luc are recombinant VSV-MΔ51 containing GFP or GFP::Firefly Luciferase gene insertion between the G and L viral genes and were kindly provided by Dr. John Bell (Ottawa Cancer Centre, Ottawa, ON).

CD::UPRT enzymatic assay. 5-Fluorocytosine (Sigma, St-Louis, MO) conversion into 5FU and derivatives was measured by spectrophotometry and calculations were performed as previously described [35]. 5FC conversion was measured using cell lysate from MCF7 cells infected for 24h with 0.1 MOI in PBS containing 3mM 5FC,

alternatively conversion was measure directly in 5FC supplemented media of infected cells.

Measurement of bystander killing by CD::UPRT/5FC. VSV-sensitive MCF7 cells were infected at 0.1 MOI, with or without 3mM 5FC into the culture media for 24h or 48h. CD::UPRT enzymatic activity was measured and supernatants were heat inactivated at 65˚C for 20 min. MCF7 cells were plated in 96 well plates and various dilutions of infected supernatant were then added. Cell viability was monitored 48h later by 3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium (MTT) dye absorbance according to the manufacturer's instructions (Chemicon, Billerica, MA). In the second experimental set up, TSA cells were seeded into 6 well plates; 0.02µM Anapore membrane cell culture insert (NUNC, Rochester, NY) was then placed in the wells and TSA cells were also seeded in the top chamber. Medium was supplemented or not with 3mM 5FC and the upper chamber cell population was infected with VSV-C at 0.1MOI. At 48h, viability in the lower chamber was monitored by flow cytometry on a FACS Calibur (Becton-Dickinson) after staining with propidium iodide and AnnexinV-APC (BD biosciences, Franklin Lakes, NJ). FACS analysis was performed with FCS Express version 3 (De Novo Software, Los Angeles, CA). For VSV resistant cell lines (B16-F10, Karpas-422 and PC3), cells were infected with VSV-C at 10 MOI for Karpas-422 and B16-F10 and 0.1 MOI for PC3. For some conditions 3mM of 5FC was added and cell viability was monitored 96h later by flow cytometry using AnnexinV/PI staining.

VSV and 5FU combination and synergism. Karpas-422 and PC3 cells were treated with 375uM of 5FU (Sigma, St-Louis, MO) and infected with VSV-MΔ51 (harboring no insertion) at 10 and 0.1 MOI, respectively. Cell viability was assessed by MTT assay 48h later and data were used to determine combination index (CI) using Calcusyn program (Biosoft, GB, United Kingdom). Similar CI were obtained when cell viability was monitor by AnnexinV/PI staining (data not shown). To evaluate VSV replication in the presence of 5FU, TSA cells were pretreated with 0.5uM 5FU for 24h, infected with 0.01, 0.1 or 1 MOI of VSV-GFP in presence of 5FU. The percentage of infected cells (GFP positive) and live cells (AnnexinV/PI negative) were discriminated by flow cytometry.

In vivo tumor model and treatment. TSA mammary adenocarcinoma cells ($3x10^5$) or EG7 T cell lymphoma ($3x10^6$)were subcutaneously injected into the flank of 8 weeks female Balb/c or C57Bl/6 mice respectively; 7 days post inoculation, tumors were palpable and treatment was started. To monitor the presence of VSV at the tumor, two intra-tumoral injection of VSV-Luc ($2x10^7$ PFU) were administered on day 0 and day 3. Two mice were sacrificed every day for 8 days and tumors were weighed and snap frozen. Tumors were homogenized in 500ul of PBS using a Polytron PT1200 homogenizer (Kinematica inc, Bohemia, NY) and virus titration was performed. To measure Luciferase activity, tumor homogenate were frozen and thawed twice before they were used for luciferase reporter assays (Promega Corporation, Madison, WI) according to the manufacturer's instructions using a GLIOMAX 20/20 luminometer (Promega Corporation). To evaluate the toxicity of the adapted 5FC-dosing schedule, 5FC was diluted in PBS and Balb/c mice received intraperitoneal injections of 500mg/kg every 160 minutes 4 times per day over a period of 4 days, control mice were injected with PBS (n=4). For tumor volume and survival experiments, TSA tumors were established as described above and mice were treated with two dose of $2x10^7$ PFU of VSV-C, VSV or heat-inactivated VSV intra-tumorally on day 0 and 3. Mice injected with VSV-C or HI-VSV received I.P. injection of 5FC corresponding to 500mg/kg every 160 minutes 4times/day over a period of 4 days. Tumor size was measured using a caliper and tumor volume was calculated using the formula: length x $width^2/2$. Mice were sacrificed when tumor volume reached $1500mm^3$. Unpaired T test and LogRank statistical analyses were performed on growth curve and Kaplan-Meier survival graph using Prism 4 (GraphPad, San Diego, CA).

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Manuscript II

VSV oncolytic treatment interferes with tumor associated dendritic cell function and abrogates tumor antigen presentation

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Running title: VSV DC infection abrogates tumor antigen presentation

Preamble for Manuscript II

VSV oncolysis is a very acute event, and while combining it with a suicide gene system improves tumor cell killing, it remains a very transient therapy. Induction of an effective adaptive immune response against the tumor in conjunction with VSV oncolytic treatment would allow for a long-term therapy. A rapid cell killing burst in the tumor caused by VSV oncolysis followed by a long-lasting tumor-specific immune response could potentially generate an effective treatment leading to complete tumor clearance. The work presented in this section was aimed at creating favorable conditions in which VSV could promote an anti-tumor adaptive immune response. As presented in the introduction chapter, DCs represent one of the central immune cells that initiate the adaptive immune response. Knowing that VSV induces an inflammatory milieu that could represent a favorable environment for DC tumor antigen uptake and activation, strategies to increase the DC population concomitant with VSV oncolysis were developed and tested. Characterization of important aspects of the immune response engendered at the tumor site following VSV injection is a key part of this study.

Abstract

Oncolytic virotherapy is a promising biological approach to cancer treatment that contributes to tumor eradication via immune and non-immune mediated mechanisms. One of the remaining challenges for these experimental therapies is the necessity to develop durable adaptive immune response against the tumor. Vesicular stomatitis virus (VSV) is a prototypical oncolytic virus (OV) that exemplifies the multiple mechanisms of oncolysis, including direct cell lysis, cellular hypoxia resulting from the shutdown of tumor vasculature, and inflammatory cytokine release. Despite these properties, the generation of sustained anti-tumor immunity is observed only when VSV is engineered to express a tumor antigen directly. In the present study, we sought to increase the number of tumor associated dendritic cells (DC) *in vivo* and tumor antigen presentation by combining VSV treatment with recombinant Fms-like tyrosine kinase 3 ligand (rFlt3L) - a growth factor promoting the differentiation and proliferation of DC. The combination of VSV oncolysis and rFLt3L improved animal survival in two different tumor models - VSV-resistant B16 melanoma and VSV-sensitive E.G7 T lymphoma; however increased survival was independent of the adaptive CD8 T cell response. Tumor associated DC were actively infected by VSV *in vivo*, which reduced their viability and prevented their migration to the draining lymph nodes to prime a tumor-specific CD8 T cell response. These results demonstrate that VSV interferes with tumor DC function and blocks tumor antigen presentation.

Introduction

Cancer therapy using oncolytic viruses (OV) has achieved remarkable therapeutic effects in numerous preclinical tumor models and clinical trials (4, 30). Of the different OV currently evaluated for efficacy, Vesicular stomatitis virus (VSV) has emerged as a prototypical OV based on properties such as cancer cell tropism, cell lysis efficacy and sensitivity to host antiviral responses (3, 24, 33). Tumor regression induced by VSV oncolysis is a complex event that is not limited to direct cell killing by virus infection; cellular hypoxia resulting from the shutdown of tumor vasculature also cooperates to reduce tumor burden (7, 8). Moreover, the innate immune response and accompanying inflammatory cytokine release contributes to the therapeutic effect observed in various murine models (18, 28, 36).

VSV oncolytic therapy has also been proposed to induce a tumor-specific adaptive immune response because infection and concomitant cell lysis expose tumor antigens within a pro-inflammatory milieu. Early studies demonstrated the presence of tumor-specific CD8 T cells following VSV treatment and a reduction of the therapeutic effect after CD8 T cell depletion (15). However, subsequent studies indicated that tumor-specific CD8 T cells were either not detected in the tumor, spleen or draining lymph nodes following VSV treatment (35) or were detected at low levels that were not statistically significant (9, 10, 17, 37). Tumor regression in CD8 T cell depletion experiments was suggested to be the result of non-specific CD8 T cell activation induced by VSV rather than a tumor-specific response (17, 35). Furthermore, VSV treatment did not lead to significant IFNγ secretion in tumor-specific CD8 T cells, even when tumor specific T cells were adoptively transferred (15, 37). In fact, anti-tumor immunity following VSV oncolytic treatment has been successfully generated only when VSV was engineered to directly express a tumor antigen (9, 10, 15, 21, 37). Altogether, these studies argue that effector T cell functions remain intact during VSV oncolysis, but indicate that antigen presentation may be a limiting step in the initiation of a tumor-specific adaptive immune response.

Dendritic cells (DC) are the most potent antigen presenting cells and represent the main cell subset capable of cross-presentation of tumor antigens in association with MHC class I molecules. Several immunotherapy strategies have targeted DC to break tumor tolerance and prime tumor immune responses (19, 27, 32); however in the context of oncolytic virotherapy, studies on the interaction of VSV and DC remain limited. VSV has been shown to induce the maturation of bone marrow-derived dendritic cells (BMDC) *in vitro* and infected BMDC were successfully used as cell carriers for VSV oncolytic therapy (1, 2, 5). However, the effect of VSV oncolytic treatment on DC function *in vivo* has not been studied in detail.

We hypothesized that robust tumor antigen presentation may be the missing link required to mount an anti-tumor adaptive immune response. To boost the antigen presentation capacity during VSV oncolysis *in vivo*, the number of tumor associated DC was increased by using recombinant Fms-like tyrosine kinase 3 ligand (rFlt3L) - a growth factor promoting the differentiation and proliferation of DC (26). In the present study, we demonstrate that the combination of VSV oncolysis and rFLt3L improved animal survival in two different tumor models - VSV-resistant B16 melanoma and VSV-sensitive E.G7 T lymphoma. Although rFlt3L treatment did increase tumor antigen presentation, VSV abrogated this effect by infecting tumor DC, resulting in the failure of DC to migrate to draining lymph nodes to prime a tumor-specific CD8 T cell immune response.

Results

Increasing the number of dendritic cells using rFlt3L improves animal survival. A strategy was designed to combine VSV and rFlt3L to enhance tumor antigen presentation during VSV oncolysis. The combination approach was evaluated *in vivo* in two different subcutaneous tumor models expressing ovalbumin (OVA) as a model tumor antigen: the B16 melanoma model is relatively resistant to VSV oncolysis and high intratumoral doses of virus ($2x10^8$ PFU) are required to inhibit tumor growth (Thesis Supplemental Figure 1 (p.163) and ref. (18)); in contrast, the E.G7 T lymphoma model is sensitive to VSV and tumors are cured by VSV at ~ $1x10^7$ PFU (Thesis Supplemental Figure 1). As previously reported (26), daily administration of rFlt3L increased DC number in the blood and lymphoid organs at 9-10 days following treatment; moreover, DC infiltrated the tumor mass with similar kinetics, resulting in a 10-fold increase in tumor DC at day 9 after treatment (**Fig. 1a**). To optimize the presentation of tumor antigens, rFlt3L injections were overlapped with VSV infections so that the peak number of tumor DC coincided with maximal tumor cell lysis and antigen release, which occurs 24-48h following the initial injection of VSV (18, 23) (**Fig 1b**). While treatment of animals with rFlt3L alone had no effect, the combination of rFlt3L with VSV treatment significantly improved animal survival (**Fig. 1c**). Because of the sensitivity of E.G7 to VSV, the efficacy of the combination *in vivo* was evaluated in a distant, non-treated E.G7 tumor on the opposite flank, such that animal survival was dictated by a therapeutic immune response in the distant tumor. VSV as a single treatment led to a minor delay in the growth of the distant tumor early after treatment (**Fig. 1d**). Similarly, rFlt3L treatment improved animal survival, indicating that the E.G7 tumor model was partially sensitive to the effects of rFlt3L (**Fig. 1d**). Nevertheless, the combination of VSV with rFlt3L significantly improved animal survival and completely cured approximately 30% of animals (**Fig. 1d**). Therefore, augmenting the number of DC prior to VSV treatment statistically improved animal survival in two different tumor models.

As a second strategy to augment DC, VSV was engineered to express Flt3L directly. Virally expressed Flt3L was detected in the serum of treated animals by ELISA (data not shown). In the B16 model, recombinant VSV-Flt3L did not provide a survival advantage compared to VSV **(Fig. 1e)**, whereas using the same approach as above, VSV-Flt3L significantly improved survival in the E.G7 model **(Fig. 1f)**. rFlt3L has been shown to augment circulating DC in humans and mice after eight to ten days of continuous treatment (26); given that VSV is detected at the tumor for approximately 5 days (18, 23)**,** VSV-Flt3L expression may be not sustained for a sufficient time to reproduce the survival advantage observed with the rFlt3L.

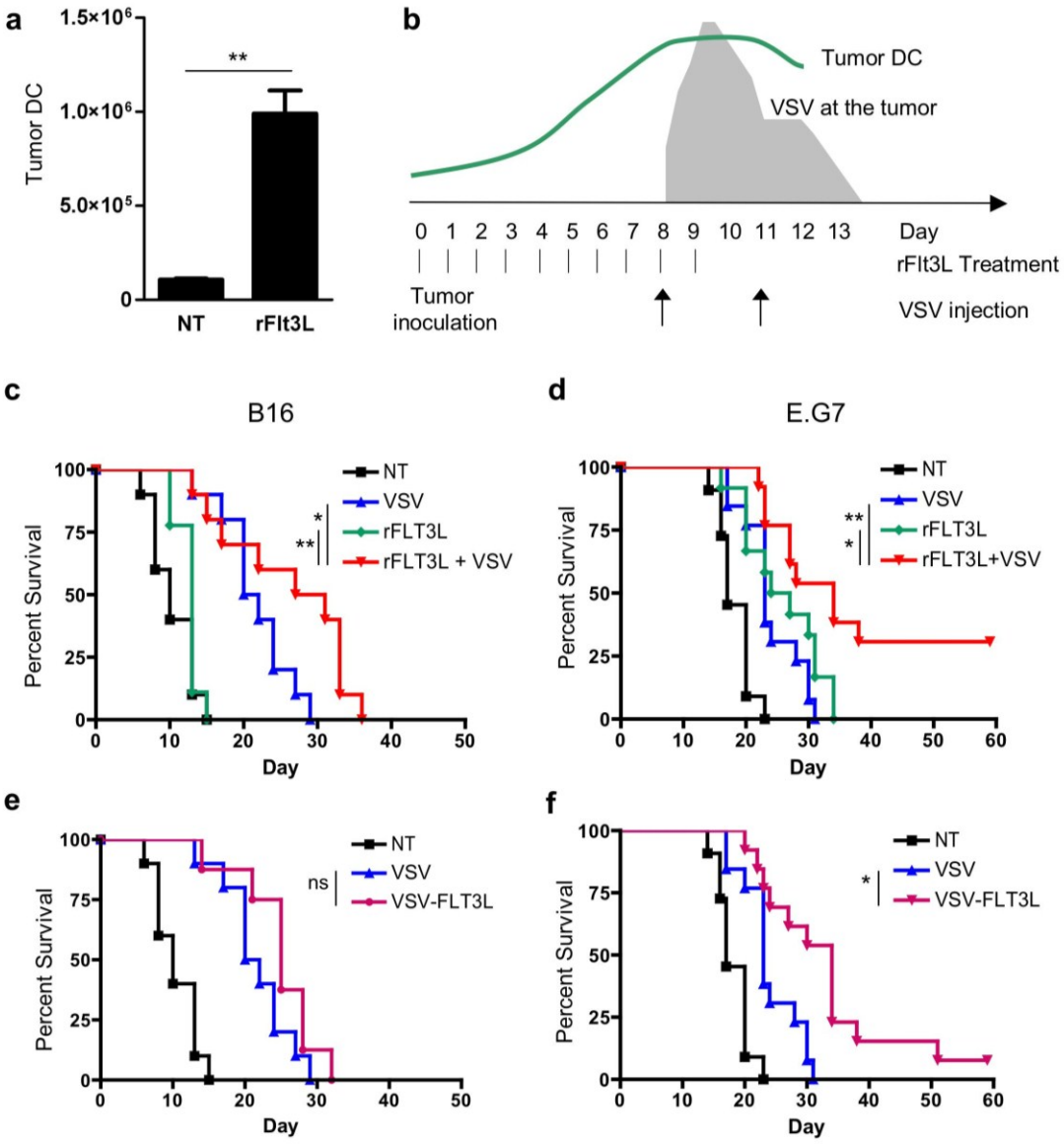
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Figure 1. The VSV-rFtl3L combination improved animal survival. (a) B16 tumor DC were quantified by flow cytometry after 9 days of rFlt3L treatment and the data are presented as CD11c+ cell per 100mg of tumor. **(b)** Schematic representation of the different treatment regimens; rFlt3L was administrated daily for 10d starting 8d before the first dose of VSV. Animals received two additional IT VSV injections 3 days apart. **(c,e)** B16 or **(d,f)** E.G7 tumors bearing mice were treated with either rFlt3L, VSV, rFlt3L and VSV, VSV-FLT3L or non treated (NT) and survival was monitored ($2X10^8$ PFU for the B16, n=10 and $2X10^7$ PFU in the right flank tumor only for EG7, n=13). In the E.G7 model, a tumor was engrafted on each flank of the mice, one tumor was infected with VSV and the opposite tumor was untreated and monitored for an immune mediated increase in survival. * $P< 0.05$, ** $P< 0.005$, ns: not significant

The efficacy of VSV and rFlt3L combination is independent of the adaptive CD8 T cell response. The combination of VSV oncolysis and rFlt3L was intended to increase tumor antigen presentation and favor a tumor-specific adaptive immune response. Therefore, the specificity of CD8 T cells for tumor or viral antigen was monitored in tumor draining lymph nodes 10 days after VSV injection, by re-stimulating lymphocytes with either an OVA or a VSV peptide, followed by IFNγ quantification by flow cytometry. In both the B16 and the E.G7 tumor models, VSV treatment induced a strong anti-viral response (**Figs. 2a, b**). However, as previously reported (10, 35), VSV alone did not generate a significant CD8 T cell response against SIINFEKL, when compared to the anti-viral response (**Fig. 2a, b**) or to the vaccination with mature bone-marrow derived dendritic cells (BMDC) pulsed with SIINFEKL peptide (**Fig. 2c**). Following combination treatment, the proportion of IFNγ-producing CD8 T cells specific for the SIINFEKL peptide was not increased (**Figs. 2a, b**), a result that was further confirmed via SIINFEKL-tetramer staining (data not shown). Thus, the improved survival rate observed with the combination therapy cannot be attributed to the generation of a tumor-specific CD8 T cell response.

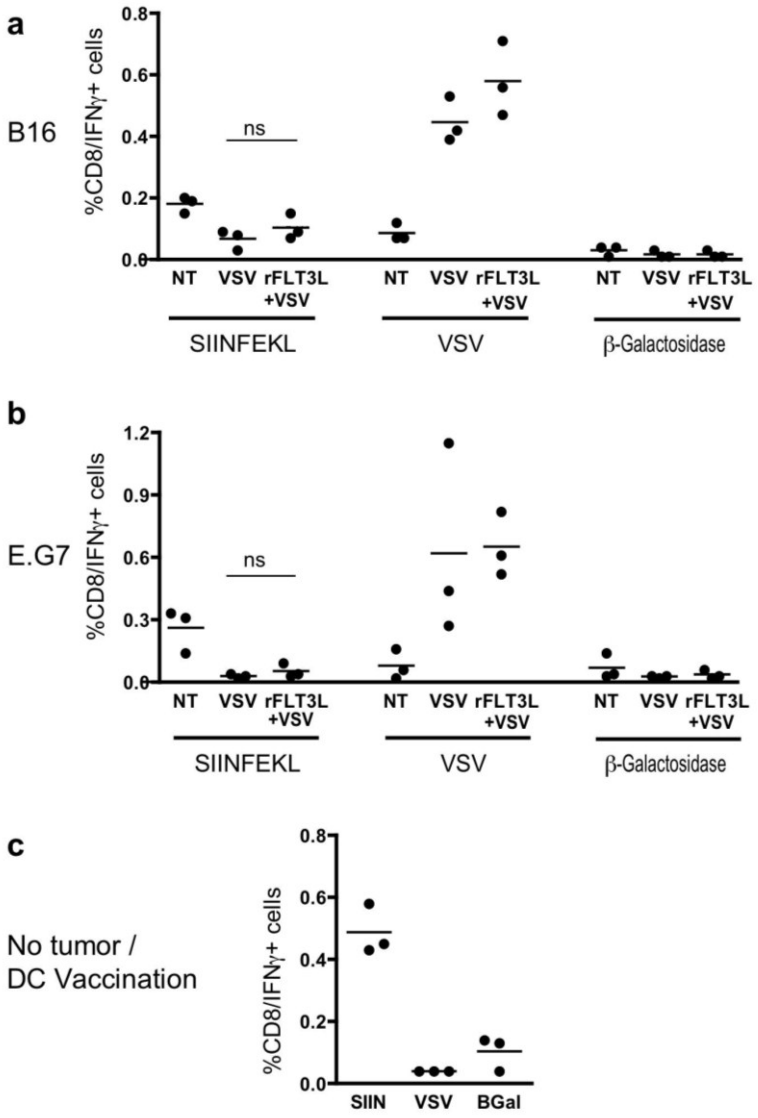


Figure 2. The VSV-rFtl3L combination did not improve tumor specific CD8 T cell response. (a) B16 or **(b)** E.G7 tumors were injected in one flank with VSV; at 10d following the first dose of VSV, draining lymph nodes were harvested and CD8+/CD11c- T lymphocytes specific for tumor SIINFEKL or VSV N peptides were monitored using *ex vivo* peptide restimulation followed by IFNγ intracellular staining. **(c)** As a positive control, mice were injected i.p. with bone marrow derived dendritic cells, pulsed with SIINFEKL and analyzed in parallel 10d following vaccination. β-Galactosidase peptide was used as a negative control. ns: not significant.

VSV infection abrogates tumor antigen presentation. To investigate why augmenting DC did not favor an adaptive immune response, the effect of rFlt3L treatment and VSV oncolysis on tumor antigen presentation was analyzed *in vivo*. OT-1 CD8 T cells specific for SIINFEKL were adoptively transferred to tumor-bearing animals, and their proliferation in response to antigen presentation was traced through CFSE dilution in the tumor draining lymph nodes. In both tumor models, OVA antigen was constitutively presented in untreated animals (**Figs. 3a,b Non-treated**); however, the absence of an inflammatory stimulus likely prevented the generation of a functional adaptive CD8 T cell response (**Figs. 2a,b**). rFlt3L treatment further increased OT-1 T cell proliferation (**Figs. 3a,b rFlt3L**), indicating that the increase in DC number improved tumor antigen presentation. Surprisingly, after VSV treatment, the proliferation of OT-1 T cells was completely arrested in both the B16 and the E.G7 tumor models (**Figs. 3a,b VSV and rFlt3L+VSV**). To rule out the possibility that OT-1 T cells had migrated from the lymph nodes to perform effector functions at the tumor site, the tumor was also analyzed. A small number of proliferating OT-1 T cells were detected in the tumor of untreated animals; however no OT1 T cells were detected in tumors that had received VSV treatment (**Fig. 3c**). Thus, VSV treatment abrogated tumor antigen presentation *in vivo* and augmenting DC using rFlt3L was not sufficient to overcome this block.

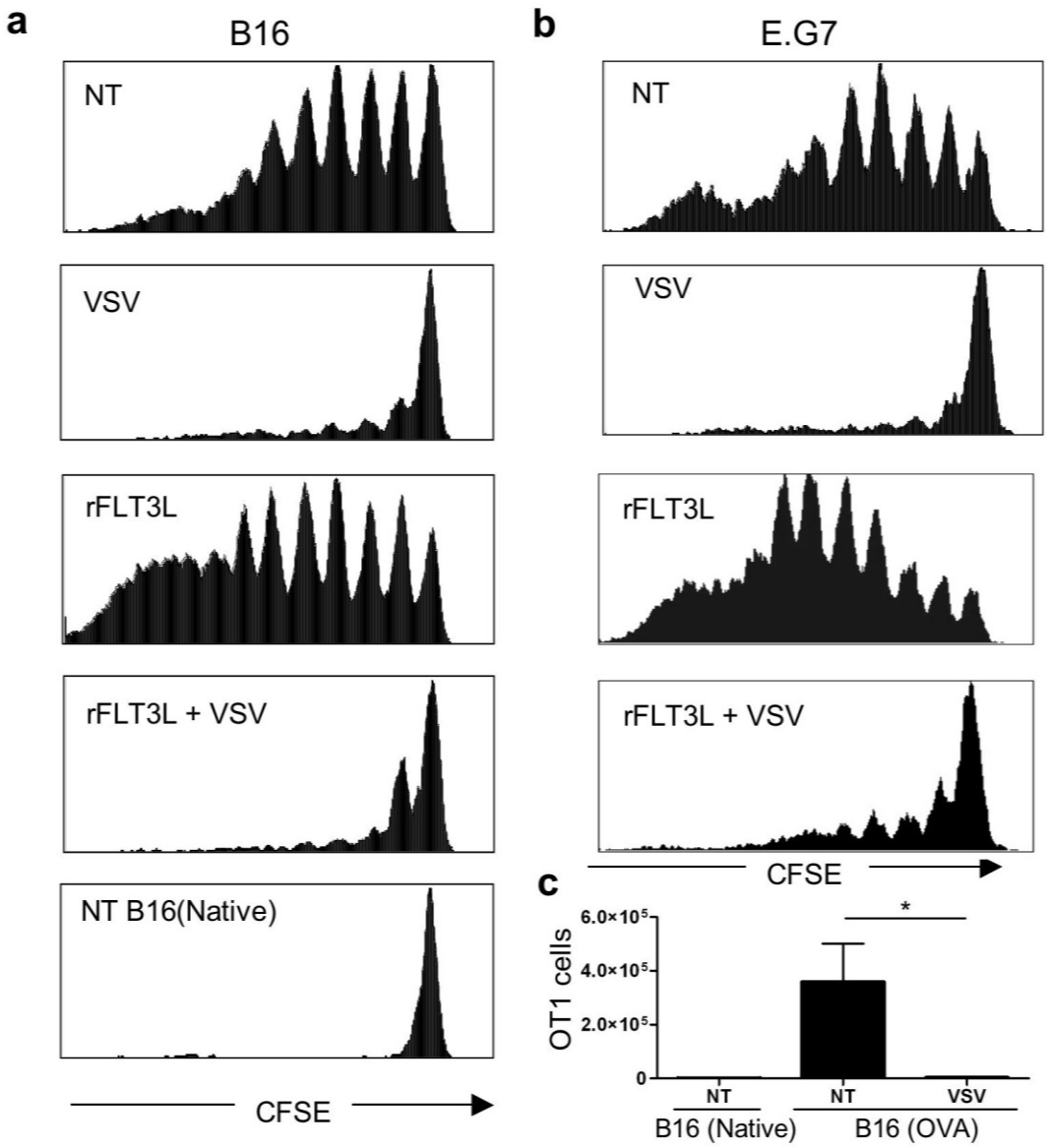
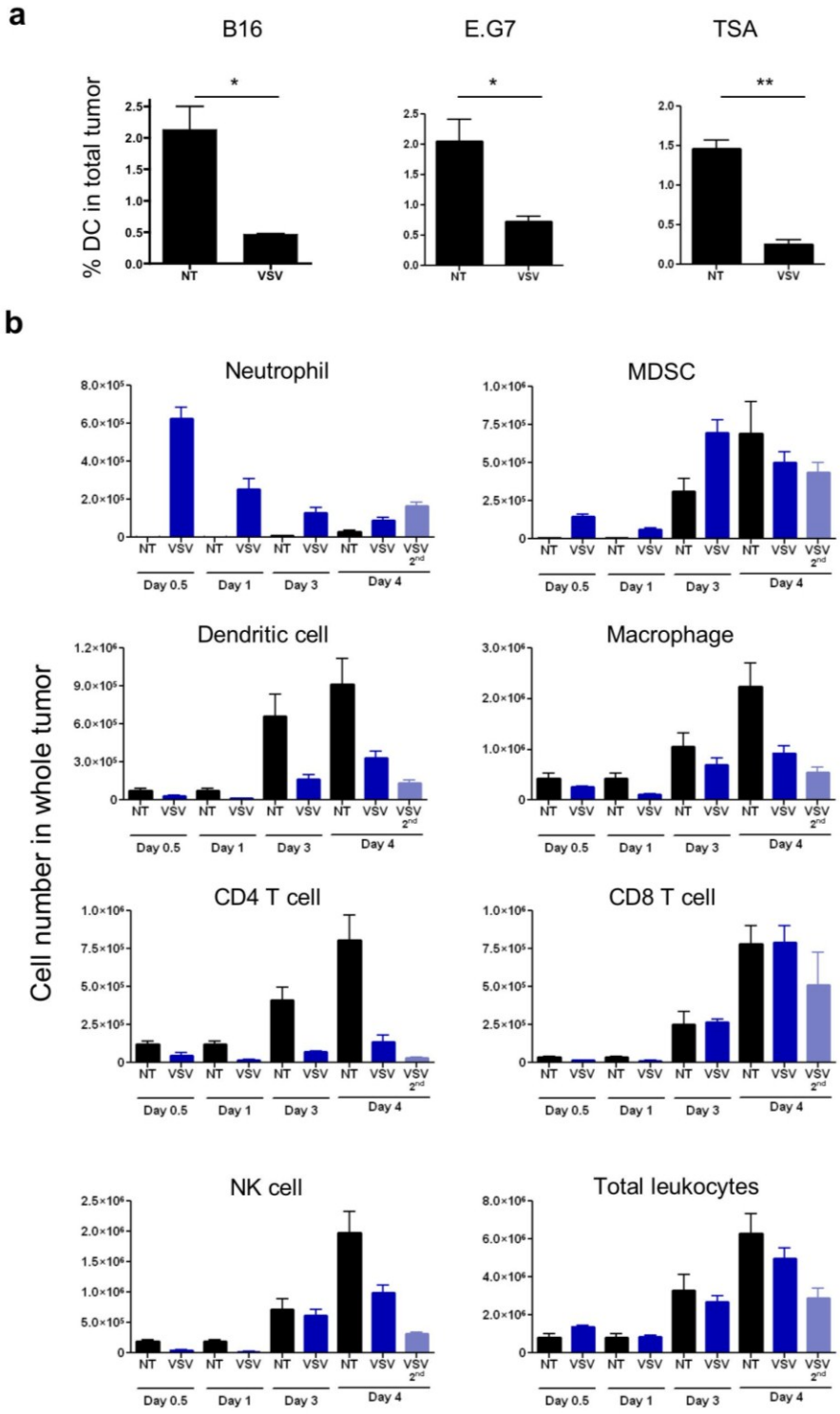


Figure 3. VSV infection abrogates antigen presentation by DC. (a) B16(OVA), B16(Native) or **(b)** E.G7 tumor bearing mice (Thy1.1) were injected i.v. with CFSE-labeled OT1 CD8 T cells (Thy1.2) 24h after the first VSV injection. At 6 days after adoptive transfer, tumor draining lymph nodes were collected and CFSE dilution in Thy1.2+ cells was monitored by FACS. **(c)** Tumors from animals in a were analyzed by FACS to identify effector Thy1.2+ OT-1 T cells. Error bars represent SEM (n=3). * P< 0.05

VSV treatment reduces the number of tumor associated dendritic cells. Given that VSV treatment abrogated tumor antigen presentation, we next examined the fate of DC during VSV oncolysis. Flow cytometry analysis revealed that VSV intratumoral injection rapidly decreased the number of tumor DC after treatment (**Fig. 4a**), rather than recruiting more DC to the site of inflammation. Moreover, the loss of tumor DC was consistent in three tumor models, syngenic to different murine genetic backgrounds.

To expand on the effect of VSV treatment, the immune cell populations infiltrating B16 tumors were analyzed. An extensive but transient infiltration of neutrophils was observed in the tumor shortly after VSV treatment (**Fig. 4b**) - a cell type that has been implicated in tumor vasculature shutdown and tumor cell hypoxia (8). Myeloid derived suppressor cells (MDSC) were also detected within the tumor, although the kinetics of their infiltration was slower than neutrophils (**Fig. 4b**). In contrast, other populations of leukocytes analyzed - DC, macrophages, NK cells, CD4 T cells and CD8 T cells - significantly decreased as early as 12h after VSV injection and remained low for several days. Further confirming this observation, reduced infiltration was reproduced 24h after the second injection of VSV (Day 4 - VSV 2^{nd}), compared to animals that had received only one injection (Day 4 - VSV). The loss of immune cells following VSV treatment was not reflected in the total number of leukocytes present at the tumor because substantial numbers of infiltrating neutrophils compensated for the loss (**Fig. 4b**). Thus, VSV treatment had a profound impact on tumor infiltrating immune cells, resulting in the recruitment of only neutrophils and MDSC to the tumor and the loss of DC and other leukocyte populations analyzed.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CD8 T cell
NK cell
Total leukocytes
Cell number in whole tumor
Day 0.5
Day 1
Day 3
Day 4
VSV 2nd

Figure 4. Tumor DC and tumor infiltrating lymphocytes decrease following VSV treatment. (a) B16, E.G7 or TSA tumors were treated with VSV and the proportion of CD11c+ DC in the tumor cell homogenate was evaluated by FACS 24h after injection (n=3). **(b)** B16 tumors were treated with VSV and collected at time points - 12h, 24h, 72h and 96h after the first VSV injection, as well as 24h after the second VSV injection. Cells were stained with different panels of antibodies and enumerated by flow cytometry using counting beads. Data are presented as absolute cell number in whole tumor to account for the neutrophil infiltration that would bias relative proportions. Error bars represent SEM (n=4). * $P< 0.05$, ** $P< 0.005$

VSV infects tumor DC and decreases their survival. To examine the possibility that tumor DC were infected by VSV and eliminated from the tumor *in vivo,* VSV-GFP was injected into the B16 tumors and GFP expression was monitored by flow cytometry. Given that viral infection and cell death induces auto-fluorescence, viral GFP fuorecence was compared to an infection using VSV that did not express GFP. The shift in GFP fluorescence intensity confirmed that tumor DC were infected by VSV *in vivo* following treatment (**Fig. 5a**); ~12% of dendritic cells were infected by VSV, compared to ~3% of tumor cells (**Fig. 5b**). Similar results were observed in the E.G7 tumor model (data not shown).

Next, the impact of VSV treatment on tumor DC viability was assessed *in vivo* by flow cytometry using Annexin V and propidium iodide (PI) discrimination of apoptotic/dead cells. Following VSV treatment, the percentage of recovered live tumor DC decreased from ~80% in untreated tumors to ~50% in VSV-treated tumors (**Fig. 5c**). As additional support, the viability of BMDC was evaluated *in vitro* following VSV infection. Flow cytometry analysis using annexin V/ PI (**Fig. 5d**) and direct cell count using trypan blue exclusion (**Fig.5e**) demonstrated that BMDC died following VSV infection, dependent on the multiplicity of infection (MOI) and elapsed time. Thus, VSV infected and killed tumor DC *in vivo,* as well as BMDC *in vitro*.

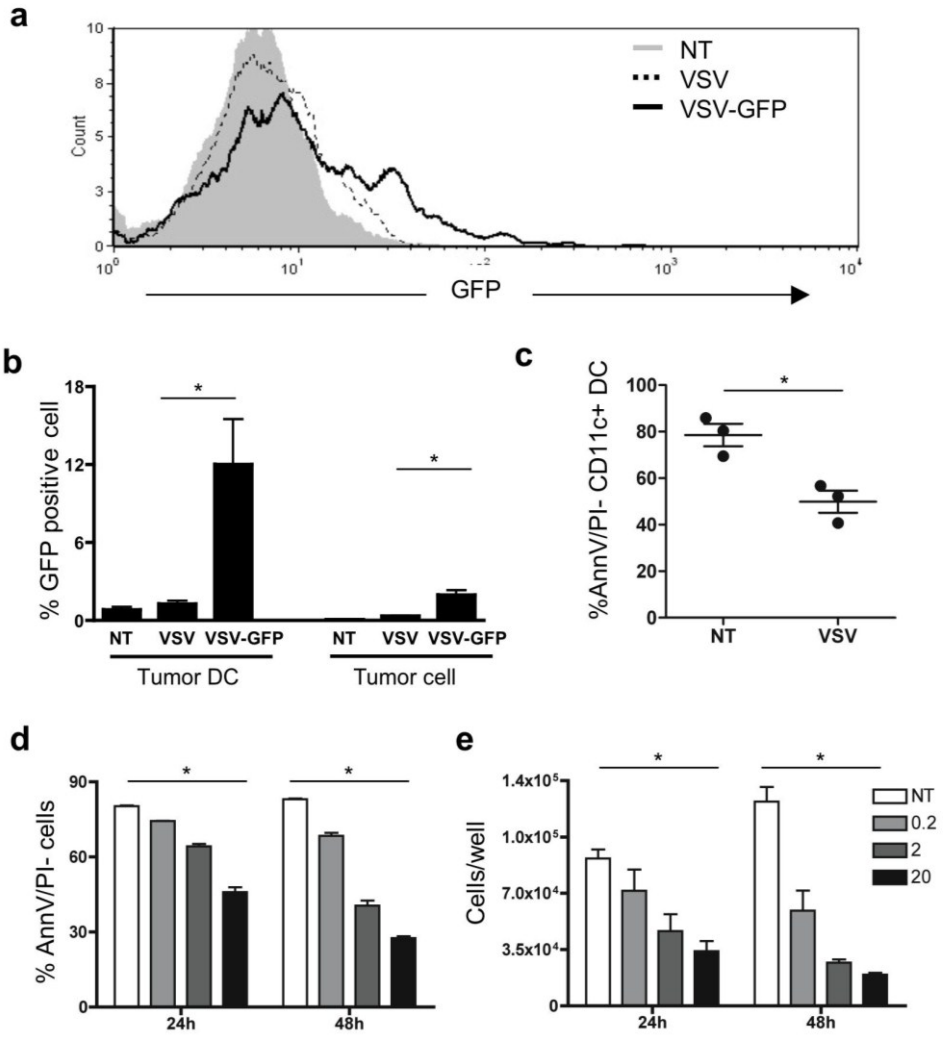

Figure 5. VSV infection of tumor homing dendritic cells reduced viability. (a, b) B16 tumors were either left untreated or injected with VSV or VSV-GFP. **(a)** GFP expression level in CD11c+ tumor DC was analyzed by FACS 10h post-injection. **(b)** Percentage of tumor DC or tumor cell expressing GFP was also analyzed (n=3). wtVSV without GFP was used as negative control to account for auto-fluorescence induced upon virus infection. **(c)** Viability of CD11c+ tumor DC was assessed in B16 tumors 10h post VSV injection by AnnexinV/PI staining (n=3). **(d, e)** BMDC were infected with VSV-GFP at 0.2, 2 or 20 MOI and cell viability was monitored 24h and 48h later by **(d)** AnnexinV/PI staining or **(e)** live cell count using trypan blue (n=3). Error bars represent SEM. * P< 0.05

Loss of functions in tumor DC. Although a portion of the tumor associated DC were infected and killed following VSV treatment, the loss of DC from the tumor may also result from their migration to the draining lymph nodes. Concomitant with the loss of DC at the tumor, VSV treatment caused an accumulation of leukocytes in the tumor draining lymph nodes (**Fig. 6a**). The large number of cells recruited to the draining lymph nodes suggested that immune cell migration from the tumor was not the only source of increased lymph node cellularity. Indeed, total blood leukocyte counts drastically decreased upon VSV treatment (**Fig. 6a**). Moreover, intratumoral VSV treatment induced a systemic inflammation since contralateral lymph nodes were also inflamed, albeit to a lesser extent than tumor draining lymph nodes (**Fig. 6a**).

To determine whether tumor DC migrated to the draining lymph nodes, BMDC were matured using LPS, labeled with CFSE and injected intratumorally 4h before or after VSV injection; traceable cells from the draining lymph nodes were then quantified by flow cytometry. Maturation of BMDC is known to upregulate the CCR7 receptor and induce homing to the lymph nodes (29) and as expected, mature BMDC from untreated tumors migrated to the draining lymph nodes (**Fig. 6b**). However, a 10-fold decrease in migrating cells was observed following VSV infection, indicating that VSV treatment drastically diminished the migration process. Moreover, DC adoptively transferred after VSV injection migrated less efficiently to the draining lymph nodes compared to DC allowed to migrate for 4h prior to VSV injection (**Fig. 6b**).

Using a more physiological approach, tumor infiltrating lymphocytes were isolated by Ficoll gradient from tumors growing in Thy1.1 mice and re-injected intratumorally to Thy1.2 mice in physiological numbers. A small number of Thy1.1 tumor lymphocytes spontaneously migrated from the tumor to the draining lymph nodes following transfer, and consistent with **Fig. 4**, VSV treatment significantly reduced the number of Thy1.1 cells in the tumor (**Fig. 6c**). However, this decrease was not associated with the migration of Thy1.1 cells from the tumor to the draining lymph nodes **(Fig. 6c)**. Thus, VSV treatment is pro-inflammatory, as demonstrated by the recruitment of immune cells to

lymphoid organs; even so, tumor DC failed to migrate to the tumor draining lymph nodes.

Finally, the effect of inefficient tumor DC migration on tumor antigen presentation was next assessed. DC were isolated from tumor draining lymph nodes and presentation of tumor antigen was evaluated by the ability of DC to induce the proliferation of CFSE-labeled OT1 T cells following co-culture. DC isolated from untreated animals induced the proliferation of OT1 T cells following co-incubation (**Fig. 6d**), while VSV treatment arrested OT-1 proliferation. MDSC induced upon VSV treatment were shown previously to interfere with priming of the adaptive immune response (34). To ascertain that a low frequency of MDSC in purified DC preparations did not interfere with OT-1 proliferation, neutralizing antibodies against TGF-β and IL-10 were supplemented during culture and failed to restore OT1 proliferation (data not shown). Therefore, VSV oncolytic treatment prevented efficient presentation of tumor antigen necessary to initiate an adaptive immune response against the tumor.

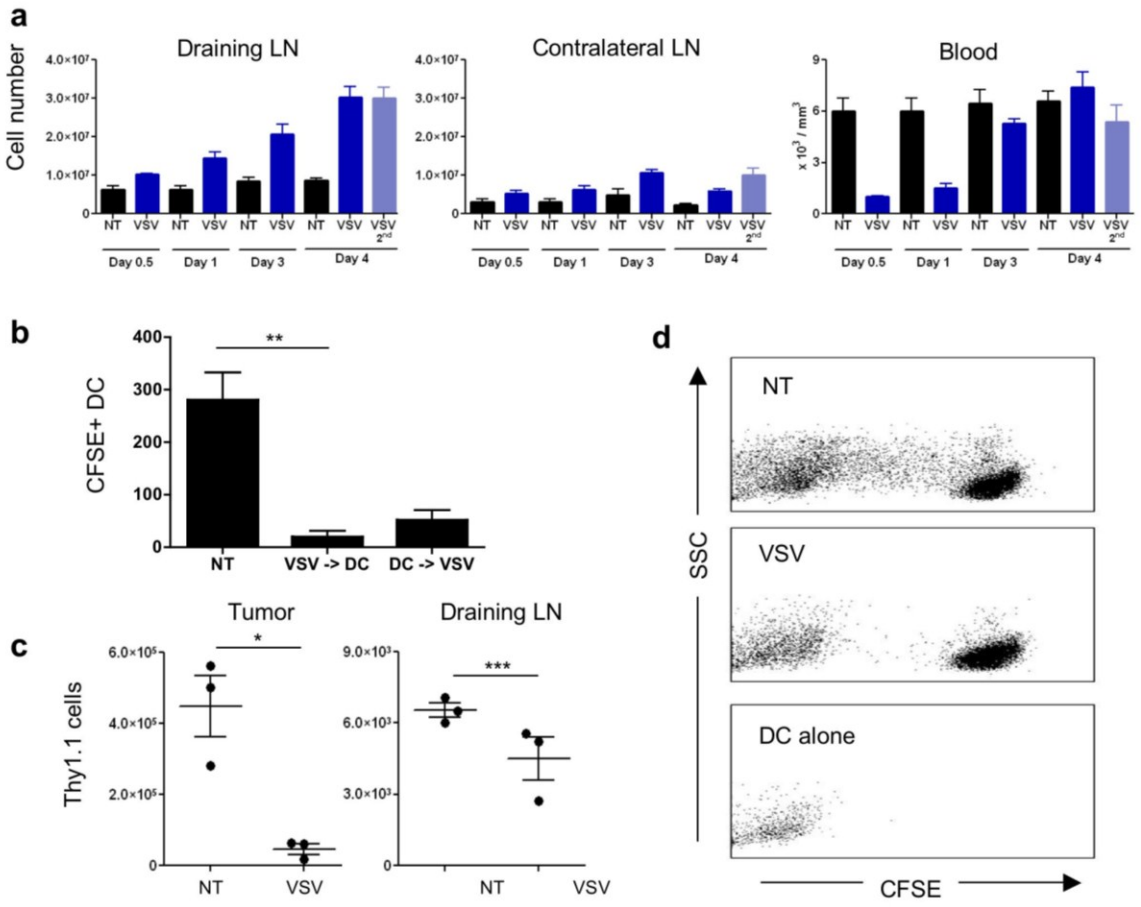

Figure 6. Tumor DC fail to migrate to the draining lymph nodes after VSV infection. (a) Total leukocyte counts from tumor draining lymph nodes, contralateral lymph nodes and peripheral blood from B16 tumor-bearing animals at different times following treatment (n=4). **(b)** LPS-activated BMDC were labeled with CFSE and injected into B16 tumors 4h before or after VSV IT injection. At 40h later CD11c/CFSE+ DC were measured in draining lymph nodes by FACS. Error bars represent SEM (n=4). **(c)** B16 tumor lymphocytes were isolated by Ficoll gradient from Thy1.1 mice and adoptively transferred by IT injection into identical B16 tumors in Thy1.2 mice; VSV was injected 4h after adoptive transfer. Tumors and draining lymph nodes were collected 40h later and Thy1.1+ cells were monitored by FACS. **(d)** DC were isolated from B16 tumor draining lymph nodes 24h post VSV treatment and cocultured with CFSE-labeled OT1 T cells and CFSE dilution in CD8+ cells was assess by FACS. Fluorescence of DC is presented as a reference for fluorescence from non-OT1 cells. * P< 0.05, ** P< 0.005, ns: not significant.

Discussion

Tumor oncolysis driven by VSV exemplifies the complex mechanisms associated with tumor regression, where oncolysis is related to direct cell killing, cellular hypoxia resulting from the shutdown of tumor vasculature, and inflammatory cytokine release. One of the remaining challenges in the development of OV therapies for cancer is the necessity to develop a sustained, durable adaptive immune response against the tumor. Although VSV oncolytic therapy has been proposed to induce a tumor-specific adaptive immune response, a number of studies indicated that the generation of sustained anti-tumor immunity was observed only when VSV was engineered to express a tumor antigen directly (9, 10, 15, 21, 37).

In the present study, we sought to increase the number of tumor associated dendritic cells *in vivo* with the goal to boost tumor antigen presentation by tumor associated DC and bypass the necessity for viral expression of tumor antigens. The combination of VSV oncolysis and rFLt3L improved animal survival in two different tumor models - VSV-resistant B16 melanoma and VSV-sensitive E.G7 T lymphoma; however increased survival was independent of the adaptive CD8 T cell response. rFlt3L treatment also increased tumor antigen presentation, but VSV oncolysis abrogated this effect by inducing the rapid disappearance of tumor associated DC. VSV treatment led to the infection and killing of tumor DC *in vivo*, thus preventing their migration to the lymphoid organs to initiate an antigen-specific immune response. Our results showing that inhibition of antigen presentation to tumor specific CD8 T cells differs from an earlier report describing the proliferation of OT1 T cells in the tumor draining lymph nodes following VSV-GFP treatment (15); however, a recent study demonstrated that the percentage of adoptively transferred OT1 T cells in the tumor draining lymph nodes was actually lower following VSV-GFP treatment compared to control animals (37). Consistent with the latter observation, VSV oncolysis did not activate OT1 T cells and did not lead to tumor infiltration by OT1 T cells (**Fig. 3** and (15, 37)), thus supporting the observation that VSV interferes with tumor antigen presentation.

Dendritic cells are the most efficient antigen presenting cells and function as the link between innate and adaptive immunity. The immunological paradigm is that DC capture antigens, while inflammatory signals trigger their maturation and migration to the draining lymph nodes to initiate an antigen-specific immune response (19, 32). As previously described *in vitro* for BMDC (1, 2, 5), we also observed that VSV infection induced the maturation of tumor DC *in vivo* through the up-regulation of different co-stimulatory molecules (Thesis Supplemental Figure 2 (p.164)). Hence, VSV has the capacity to convert immature tolerogenic DC into mature cells capable of priming T cells. The present results revealed that VSV infection blocked tumor antigen presentation, even when the number of DC was increased by prior treatment with rFlt3L.

Although VSV interfered with tumor dendritic cell function and prevented a tumor-specific adaptive immune response, a strong anti-viral response was mounted following VSV treatment. DC have been shown to play a crucial role in priming anti-VSV immune responses (11-13, 25). Recently, the dissemination of highly immunogenic viral particles to the tumor draining lymph nodes shortly after intra-tumoral injection was reported (37). Infection of resident lymph node DC by recombinant VSV expressing OVA was proposed to be responsible for the OVA-specific immune response (37), thus implying that the anti-viral response depends on lymph node DC, rather than tumor DC.

Variability in the percentage of infected and apoptotic DC was observed in the *in vivo* assays and we suspect that the extent of DC killing may be underestimated, given the rapid clearance of apoptotic cells, the loss of dead cells during sample processing, and reduced membrane integrity upon cell death. The ability of VSV to infect and kill DC *in vivo* was also tested in an *in vitro* setting, where we showed that BMDC were also infected and killed by VSV. Previous studies demonstrated that BMDC were infected by VSV *in vitro* but that cell viability was not affected (1, 5). This discrepancy can be explained by the flow cytometry analysis that selected a live cell population based on light scatter characteristics or CD11c expression (**Fig. S1** (p.162)). The total cell count following infection further confirmed that BMDC were killed following VSV infection. Factors other than direct viral infection may also contribute to tumor DC cell death. For

example, deficiency in blood flow resulting from tumor vascular shutdown could induce tumor DC death by hypoxia, as reported for non-infected tumor cells (8). This blockage of vascularization could also confine cells to a microenvironment that favors prolonged exposure to virus and thus increases cell susceptibility to infection.

The combination of VSV and rFLT3L treatment improved survival of the animals through a mechanism independent of the CD8 T cell response. Flt3L - in addition to increasing the number of conventional myeloid DC - acts as a growth factor for plasmacytoid DC and NK cells (26, 31). Increasing the number of plasmacytoid DC, which are a major interferon (IFN)-producing cell type, would likely enhance the local production of type I IFN upon VSV infection, thus contributing to essential role in the therapeutic effect of VSV treatment (36). Furthermore, the expansion of NK cells is suggestive of an enhanced NK cell-mediated tumor cytotoxicity and crosstalk with abundant DC (6, 14, 16). Thus, although designed to improve the anti-tumor adaptive immune response, the combination of Flt3L with VSV may further benefit the innate immune response against the tumor.

In conclusion, the results presented here describe a mechanism that explains the limited capacity of VSV to trigger a tumor-specific adaptive immune response. Integrating these findings into the rational design of new VSV-based cancer immunotherapy will be a major step toward complementing the acute oncolytic properties of VSV with long-lasting tumor immunity.

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Material and Methods

Cells. B16 expressing ovalbumin - referred to as B16 in the text - were a kind gift from Dr. RG Vile (Mayo Clinic, Rochester, MN) and were grown in DMEM, 10% FBS, 5mg/mL G418. E.G7 were provided by Dr. J Galipeau (McGill University, Montreal, Canada) and were grown in RPMI; 10% FBS; 0.5mg/mL G418. TSA mammary adenocarcinoma were provided by Dr. G Barber (University of Miami, Miami, FL) and grown in RPMI; 10% FBS. B16-F1 (referred to as B16 Native) were obtained from ATCC (Manassas, VA) and cultured as recommended. BMDC were differentiated as previously described (20) with 10ng/mL of mouse GM-CSF and IL-4 (R&D Systems, Minneapolis, MN) for 6 days and were typically >85% CD11c+. Where indicated, 1ug/mL of LPS (Sigma, St-Louis, MO) and/or 5ug/mL SIINFEKL peptide was added for the last 24h.

Viruses and construction of recombinant VSV. All VSV harbor the methionine 51 deletion in the matrix protein coding sequence (33). The soluble form of human Flt3L gene was amplified from pUMVC3-hFlex (Aldevron, Fargo, ND) and cloned between the G and L genes. Infectious recombinant VSV were recovered as previously described (22) and replicated as efficiently as parental VSV. VSV-GFP was kindly provided by Dr. J Bell (Ottawa Health Research Inst.). Virus stocks were grown in Vero cells, concentrated from cell-free supernatants by centrifugation and titrated by standard plaque assay.

Tumor models and VSV treatment. C57Bl/6 (Thy1.2) and Balb/c mice were purchased from Charles River (Wilmington, MA); C57Bl/6 (Thy1.1) and OT1 (C57Bl/6; Thy1.2) from Jackson Laboratory (Bar Harbor, MA). E.G7($3x10^6$), B16($1x10^6$) or TSA($3x10^5$) cells were injected subcutaneously (s.c) into the flank of 8-10 weeks old syngenic female; 7 days post-inoculation, two intratumoral injections of VSV were given on Day 0 and 3 ($2x10^7$ PFU for E.G7 and TSA; $2x10^8$ PFU for B16). rFlt3L, kindly provided by Celldex therapeutics (Phillipsburg, NJ) was administered s.c. in the nape of the neck (10ug/day) for 10 days starting 8 days before the first VSV injection (26). Tumor volumes were

calculated using the formula: length x width^2/2 and mice were sacrificed when tumor volumes reached 2000mm^3. All animal experimentations were approved by the McGill University Animal Care Committee.

In vivo assays and flow cytometry analysis. Blood leukocyte counts were obtained using Vet ABC hematology analyzer (SCIL, Gurnee, IL). Tumor draining lymph nodes refer to both inguinal and axilliary lymph nodes. Cell suspensions were prepared by meshing through a 70um nylon cell strainer (BD Falcon). Total counts were obtained using Z2 counter (Beckman Coulter, Brea, Ca) and multiplied by the proportion obtained by flow cytometry to obtain absolute counts. B16 tumors were weighted, meshed through a 100um nylon cell strainer (BD Falcon) and resuspended in 20% w/v to stain comparable number of cells for flow cytometry. Absolute number of tumor cell populations was determined using SPHERO™ AccuCount Fluorescent beads (Spherotech, Lake Forest, IL) as per manufacturer's instructions. Briefly, cells were treated with Fc Block (BD Bioscience), incubated with antibodies, washed once, resuspended in 1 mL, and 50uL of counting beads was added and vortexed just prior to acquisition. Populations in Fig. 4 were gated as follow: total leukocytes CD45+; neutrophil CD45+/CD11b+/Gr1+/F4/80-; MDSC CD45+/CD11b+/Gr1+F4/80+; macrophage CD45+/F4/80+/Gr1-; DC CD45+/CD11c+/NK1.1+; CD4 T cells CD45+/CD3+/CD4+/CD8-; CD8 T cell CD45+/CD3+/CD8+/CD4-;. NK cells CD45+/CD11c-/NK1.1+. B cells (CD45R+) were not significantly represented in the tumor and pDC could not be reliably analyzed. All antibodies were purchased from eBioscience (San Diego, CA) unless indicated otherwise. Samples were acquired on a FACScalibur (BD bioscience) and analyzed with FCS Express 3 (De Novo Software, Los Angeles, CA).

In vitro peptide restimulation. Cells (2x10^6) were incubated with 5ug/mL of peptide and 2ug/mL of CD28 antibody (BD bioscience) for 5h. GolgiPlug (BD biosciences) was added after 1h and IFNγ (BD Bioscience) intracellular staining was performed using the BD Cytofix/Cytoperm kit as per manufacturer's instructions. SIINFEKL (OVA), RGYVYQSL (VSV N) and DAPIYTNV (Irrelevant: β-Galactosidase) peptides were produced by the Sheldon Biotechnology Center (McGill University, Montreal, Canada).

For positive control of OVA specific response, $2.5x10^6$ LPS-matured BMDC pulsed with SIINFEKL were injected intraperitonally.

OT1 proliferation assays. CD8 OT1 T cells (Thy1.2) were isolated using a CD8 T cell enrichment kit (Stemcell, Vancouver, BC, Canada) and labeled with 5μM CFSE. For *in vivo* proliferation, $3x10^6$ OT1 were injected i.v. to C57Bl/6 (Thy1.1) mice 24h after the first dose of VSV. CFSE dilution was analyzed by FACS 6 days later. For *in vitro* proliferation, draining lymph nodes DC were isolated from C57Bl/6 (Thy1.2) mice 24h following VSV treatment using a CD11c positive selection kit (Stemcell) and incubated with OT1 T cells at a 2:1 ratio for 3 days.

In vivo migration assays. For DC migration, LPS-matured BMDC (>85% CD11c+) were labeled with 5μM CFSE (Invitrogen, Carlsbad, CA) and $1.5x10^6$ cells were injected intratumorally in B16 tumors. Upregulation of CCR7 by LPS was confirmed by FACS. Tumor lymphocytes were isolated by Ficoll gradient (GE Healthcare, United Kingdom) from 7 days-old B16 tumors growing in C57Bl/6 (Thy1.1) mice and re-injected intratumorally into C57Bl/6 (Thy1.2) mice bearing 7 days-old B16 tumors. Cells isolated from a certain number of tumors were re-injected into the same number of tumors. Cell migration was evaluated 40h following treatment.

DC infection and viability. The analysis was performed 10h following VSV injection prior to DC loss from the tumor. B16 tumors were stained with anti-CD45 and CD11c antibody and GFP was analyzed by FACS. DC were analyzed as CD45+/$CD11c^{Hi}$ and tumors as CD45-. For *in vivo* tumor DC viability, B16 tumors were gently dispersed by pipetting and stained with CD11c, Annexin V and PI for FACS analysis. For *in vitro* infectivity, BMDC were infected with VSV in a small volume of media without FBS for 1h; cells were then incubated in complete media containing 10ng/mL GM-CSF and IL-4. Cell viability was assessed using AnnexinV (BD) and propidium iodide (PI) (Sigma-Aldrich, St. Louis, MO) by FACS or by direct count using Trypan blue.

Statistical analysis. Unpaired t-test and LogRank statistical analyses were performed using Prism 4 (GraphPad, San Diego, CA).

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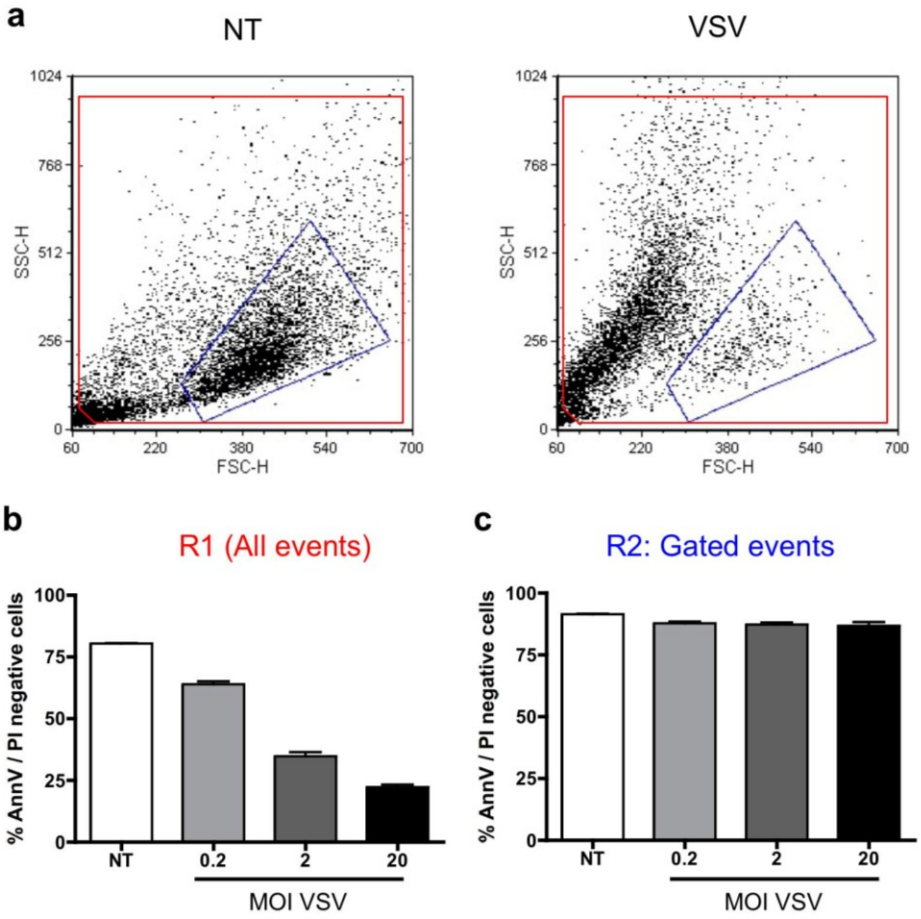
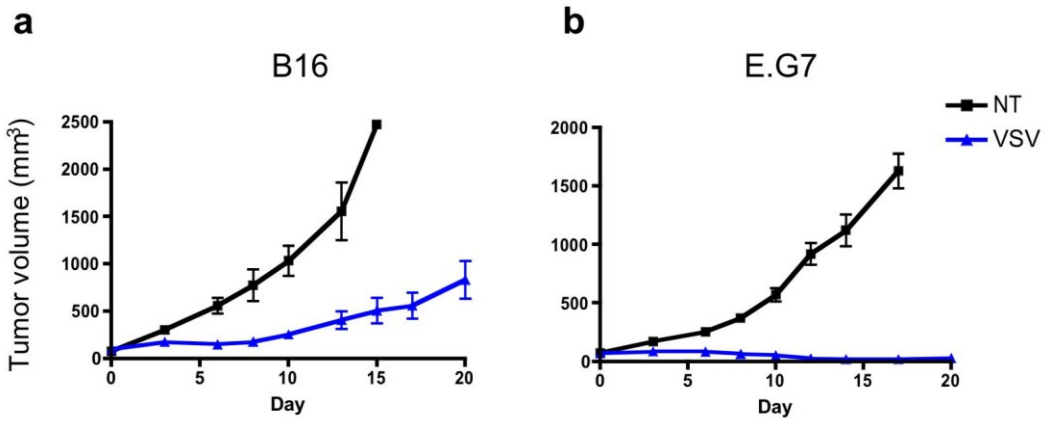
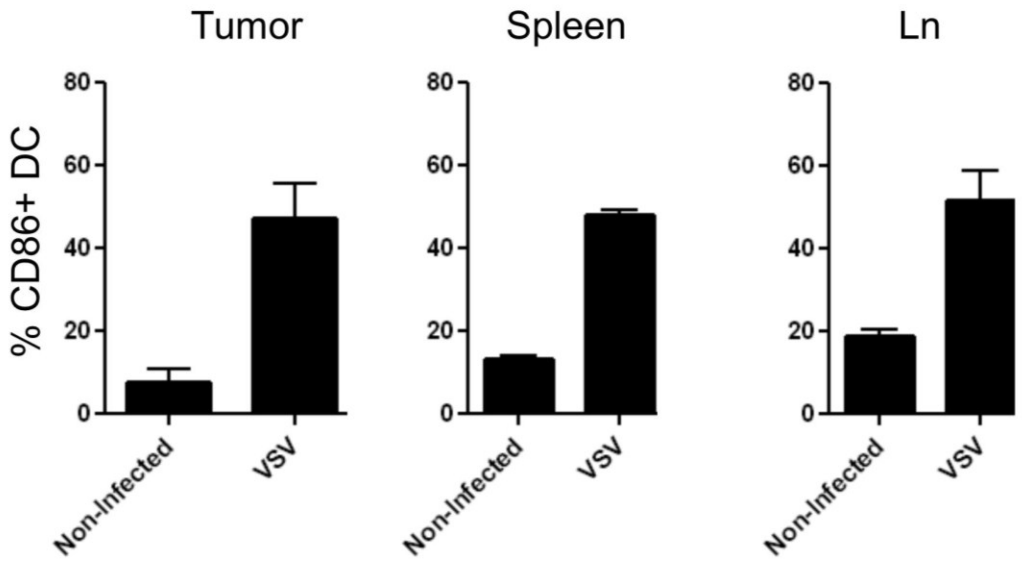


Figure S1. BMDC are killed by VSV infection. (a) SSC/FCS dot plot of BMDC 48h post VSV infection at 20 MOI or left non-infected. **(b, c)** AnnexinV/PI cell viability discrimination of BMDC 48h post VSV infection using all acquired events (b, presented in this study) or only the gated events as previously reported (c).



Thesis supplemental Figure 1. VSV cures E.G7 tumors and delays B16 growth rate. **(a)** B16 or **(b)** E.G7 tumors were VSV treated with two IT injections 3 days apart ($2X10^8$ or $2X10^7$ PFU for each model, respectively) and tumor volume was monitored (n=15).



Thesis supplemental Figure 2. VSV induces DC maturation *in vivo*. E.G7 tumors were VSV treated and tumor, spleen and lymph node (Ln) DC were analyzed for the upregulation of CD86 costimulatory molecule (n=3). Similar results were also obtained for CD40 costimulatory molecule.

Other VSV combinations

The initial designs of the global Ph.D. project incorporated the two approaches presented in Manuscripts I and II - increasing non-infected tumor cell killing as presented in Manuscript I and enhancing tumor antigen presentation as presented in Manuscript II - into a single multi-component treatment strategy. The rationale was that a VSV-C suicide gene system would enhance tumor cell killing and consequently the amount of tumor antigen released, and that VSV-F would increase the number of infiltrating DCs capable of capturing and processing these tumor antigens. VSV signals through PRRs and pro-inflammatory cytokines released from the infection would induce DC maturation and migration to the draining lymph nodes to subsequently initiate an anti-tumor adaptive immune response. In light of the results presented in Manuscript II, it is obvious that the second part of this initial hypothesis was inadequate. Nethertheless, several "non-retained" strategies that contributed to our understanding and scientific approach were developed in the course of this study.

VSV treatment combining CD::UPRT and FLT3L

To combine these various aspects into one rVSV, VSV-C/F was engineered to express both CD::UPRT and FLT3L. Both coding sequences were added to the genome as separate transcripts. Because this engineered virus contained two additional stop/start sequences for the viral RNA polymerase, as well as a significant amount of additional coding sequences, VSV-C/F had a slower growth rate and was therefore attenuated when compared to the non-engineered virus or a virus containing only one gene insertion. Although expressing both agents in fully active forms, VSV-C/F's slower replication represented a disadvantage, when compared to the single factor-expressing VSV-C and VSV-F. Therefore, subsequent experiments combining these two improvements were performed using a combination of both "single" rVSVs: VSV-C and VSV-F.

As shown in Manuscript I, the VSV-C strategy presented a therapeutic advantage; however, data presented in Manuscript II demonstrated the limited impact of the Flt3L and VSV combination and the almost complete absence of efficacy with VSV-F (Manuscript II, **Figure 1e,f**). Previous studies using recombinant Flt3L injection to increase the DC population were performed as a continuous treatment for approximately 10 days (Maraskovsky *et al*., 1996), or using adenovirus-expressing vectors demonstrating release of growth factor for up to two weeks (Bernt *et al*., 2005). Data presented in Manuscript I highlights VSV's very short–lived replication at the tumor site. Considering these timeline aspects, the poor efficacy of VSV-F may result from the short-lived expression of the growth factor. In fact, serum Flt3L was detected for up to 4 days after VSV-F, and to a lesser extent in VSV-C/F treatment, but was completely absent by day 6 (**Figure 1**).

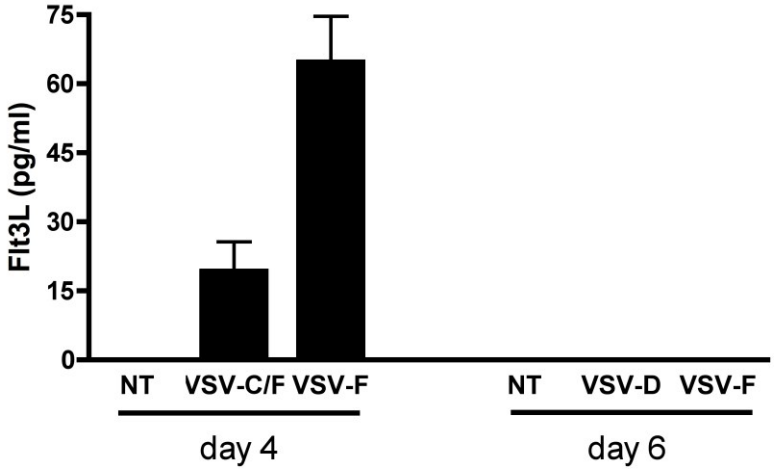


Figure 1. Serum concentration of Flt3L following VSV IT treatment. TSA tumors were treated with two IT doses of $2x10^7$ PFU of either VSV-C/F or VSV-F on days 0 and 3, and Flt3L serum concentration was assessed by ELISA at 4 and 6 days following the first dose of VSV.

A second consideration in employing VSV-F is the time required for Flt3L treatment to engender DC augmentation. Previous studies demonstrated that DC enhancement peaks around day 10 following the initial administration of Flt3L, results we were able to confirm (Maraskovsky *et al*., 1996)(Bernt *et al*., 2005). Considering this timeline issue and the fact that VSV tumor cell killing and antigen release most likely occurs at the early peak of replication, the use of VSV-F would probably not result in the overlap of these two events. Therefore, to coincide the significant increase in the number of tumor-infiltrating DCs with VSV oncolysis and tumor antigen release, recombinant Flt3L (rFlt3L) was administered to animals in combination with VSV oncolytic treatment instead of using VSV-F (Manuscript II, **Figure1a**).

VSV expressing CCL20

During the course of the study presented in Manuscript II, we hypothesized that attracting more DCs to the tumor site might help to resolve part of the problem of the absence of tumor DCs following VSV treatment. Thus, VSV was engineered to express CCL20, a chemokine known to attract immature DCs (antigen capture is more efficient before maturation occurs). In addition, combining increasing DC population through rFlt3L treatment with DC attraction using a chemokine expressed by VSV, represented a novel and exciting strategy to test. CCL20 is implicated in normal homeostatic as well as inflammatory trafficking of some leukocytes (Schutyser *et al*., 2000; Schutyser *et al*., 2003). CCR6 is the receptor for this chemokine and is largely expressed by immature DCs (Baba *et al*., 1997; Schutyser *et al*., 2003). Although CCL20 has also been associated with tolerance and regulatory functions (Comerford *et al*., 2010), its use in cancer therapy approaches has been shown to attract DCs and to favor tumor regression (Furumoto *et al*., 2004). In some of these approaches, a PAMP signal was required to induce the maturation of the DCs and probably also to inhibit regulatory function, a function that could easily be completed by VSV.

VSV-CCL20 expressed the chemokine and was effective in attracting BMDC as well as splenocytes in a chemotaxis assay *in vitro* (**Figure 2**). Supernatant from VSV-CCL20-infected tumor cell lines attracted more DCs and splenocytes than supernatant from VSV-infected cells. Supernatant from VSV infected tumor cells also contained attracting factors because more migration was observed than in non-infected supernatant. Therefore, the strategy appeared very promising *in vitro*.

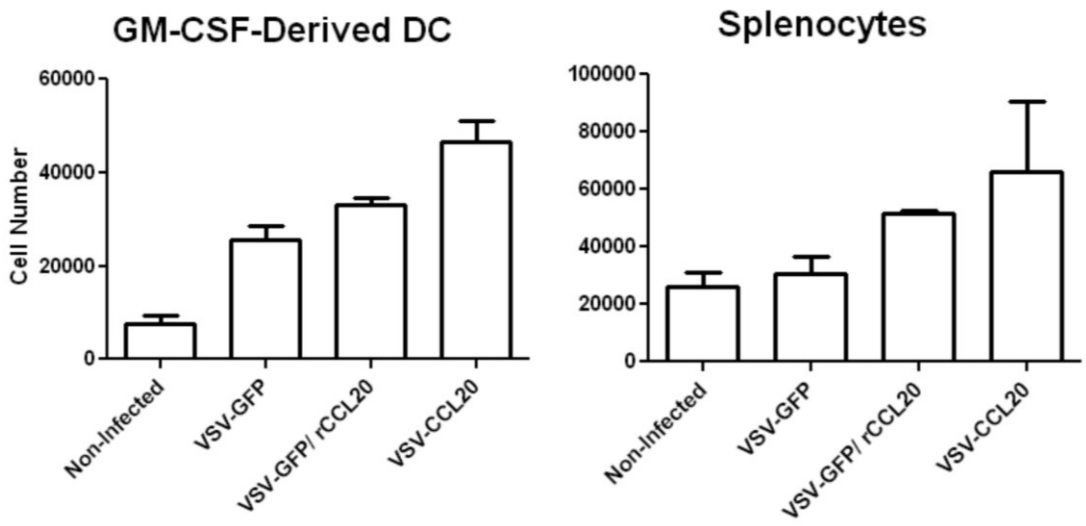


Figure 2. VSV-CCL20 is functional and demonstrated chemo-attracting properties *in vitro*. Supernatant from VSV-CCL20-infected cell lines induced migration of bone marrow-derived DCs or splenocytes through a 5μm pore membrane in a chemotaxis assay. TSA cells were infected with 1 MOI of VSV-GFP or VSV-CCL20 for 24h; supernatant was placed in the lower chamber of a chemotaxis assay plate and bone marrow-derived DCs and splenocytes ($5x10^4$ and $1x10^5$ cells respectively) were seeded on top of the membrane. Following 2h of incubation, cells that crossed the membrane were counted. Recombinant CCL20 at 300ng/ml was used as a control.

In vivo experiments using VSV-CCL20 revealed that chemokine expression by VSV was unable to enhance DC presence at the tumor following treatment (**Figure 3a**). Consequently, VSV-CCL20 did not demonstrate enhanced therapeutic effects on tumor-bearing mouse survival when compared to VSV expressing an irrelevant transgene (**Figure 3b**). Experiments in a B16 or EG7 tumor model using VSV-CCL20 displayed similar results when compared to the TSA model (data not shown). The observation that the virally-expressed chemokine was able to promote DC attraction *in vitro* but not *in vivo* contributed to the characterization of the negative impact of VSV on tumor antigen presentation. Any potential benefits derived from the increased presence of the chemokine in attracting DCs appear to be overshadowed by the killing and migratory blocking outcomes of VSV. In addition, the vascular shutdown of the tumor also likely interferes with DCs that would be attracted to the tumor site during oncolysis. Altogether, these results further support the data presented in Manuscript II.

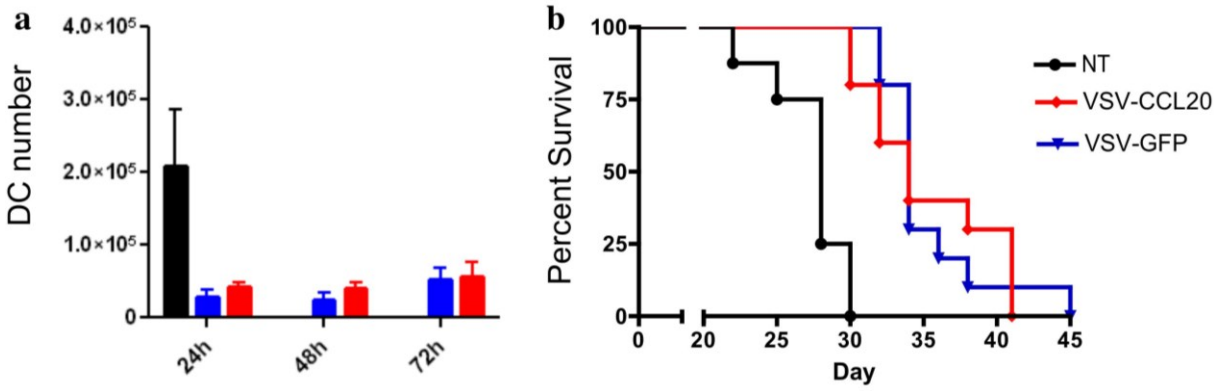


Figure 3. VSV-CCL20 does not confer advantages *in vivo*. a. VSV-CCL20 does not increase tumor DCs following intratumoral injection. TSA tumors were injected with $2x10^7$ PFU of VSV-CCL20 (red) or VSV (blue) and CD11c+ tumor DCs were numbered by FACS at 24, 48 and 72h post-VSV treatment. **b.** VSV-CCL20 does not improve animal survival when compared to VSV-GFP treatment. TSA tumor-bearing animals were treated with two intratumoral doses of VSV 3 days apart, tumor volume was monitored and animals were sacrificed when the tumor reached $1500mm^3$ (n= 10).

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CHAPTER 3

DISCUSSION

Cancer is a broad, diverse and complex problem that requires highly innovative treatment strategies. OVs represent a major tool in the development of cancer therapy. OVs not only engender direct antitumor effects and side effects like most anti-cancer treatments, but they also generate a reaction from the host. Multiple facets of oncovirotherapy are still poorly characterized, and this fact contributes to the limitations of the approach. Strategies developed in the course of this doctoral study greatly contributed to our knowledge of VSV as an oncolytic virus. The improved characterization of VSV's presence at the tumor site allowed us to adapt the treatment and enhance the therapeutic benefits of the suicide gene strategy presented in Manuscript I. The combination therapy presented in Manuscript II allowed for the characterization of the immune cell state at the tumor following VSV treatment. Although this combination permitted a certain level of enhanced therapeutic benefit, it did not transduce into the generation of an effective tumor-specific adaptive immune response. Therefore, even though only one strategy directly reached its initial goal, both approaches revealed key properties of VSV oncolysis that were previously unknown or undercharacterized.

Optimizing Oncolytic virus combination therapies

Cancer therapy needs to consider multiple aspects in order to provide efficient and adapted treatment. Combination treatments are likely to be more efficient than single treatment due to the multiple angles of attack. These multiple component therapies must be well designed and require careful analysis in order to avoid redundant toxicity and permit maximal benefit from all therapeutic agents. Therefore, each aspect of the treatment, such as the half-life of the compounds, their bioavailability and toxic side effects, must be well characterized. These important concepts also need to be carefully applied to the design of OV combination therapy.

Combining OVs with other anticancer agents represents an excellent opportunity to enhance the therapeutic effects of OVs and/or to project additional effects onto the cancer target. As each virus possesses unique properties and replication characteristics, the full potential of a particular combination can only be achieved by adapting each aspect of the strategy to the features specific to the OV in question. The data presented in manuscript I presents a clear example of this fine-tuning approach based on a detailed characterization of each component, which allowed us to obtain maximal benefits from a combination. In similar CD::UPRT suicide gene approaches, such as the CD::UPRT Adenovirus expressing vector (Koyama *et al*., 2000; Liu and Deisseroth, 2006) or CD::UPRT stably transfected tumor cells (Khatri *et al*., 2006), the 5FC treatment consisted of one injection per day for a period of 7-14 days. The basic application of this low dosage over a long period of time was not adapted to the oncolytic characteristics intrinsic to VSV and therefore did not achieve the best possible results. Using VSV vectors, the enzyme is expressed at the tumor for a very transient period of time. This VSV characteristic may first appear as a disadvantage, but the period of time for which the enzyme is expressed may not be the only criteria involved in judging the efficiency of VSV or comparing it to other OVs in a suicide gene system. For example, even if an adenoviral vector allows the expression of the transgene for a much longer period of time (Bernt *et al*., 2005), the site of viral replication may be less specific (i.e. Adenovirus is known to be trapped and expressed in the liver (Bernt *et al*., 2003)). In addition, tumor cells may be more susceptible to one OV than to another. Furthermore, a robust expression in a very acute event, as in the case of VSV, might be more effective than a more sustained expression at a much lower level. In all cases, direct comparison is difficult, and it is possible that a combination of different OVs with the same suicide gene might represent an even better approach.

The search for an optimal treatment specific to a particular OV and the adaptation of the different aspects of the approaches has also been pursued in other combination strategies. Recombinant OVs expressing the iodine symporter gene, which permits the accumulation of radioactive iodine at the tumor site, is a good example. Measles virus

and VSV recombinant viruses expressing this particular suicide gene system were developed by the same group, and the analyses of the approach presented in their two reports also demonstrated that OVs' specific characteristics must be taken into account in the application of combined therapies. In the case of Measles virus expressing the symporter gene, a maximal radioactive iodine uptake on day 3-6 was demonstrated (Penheiter *et al*., 2010), while a single injection of radioactive iodine 24h post-OV injection was performed in a strategy using the rVSV engineered to express the same symporter (Goel *et al*., 2007). One major advantage of this iodine symporter approach is the possibility of visualizing iodine uptake using a radio-imager, therefore allowing to follow not only the presence of the virus but also to evaluate the pertinence of pursuing the injection of radioactive material. This advantage would allow for a patient-based adaptation of the treatment (Penheiter *et al*., 2010).

Other cases in which the combination strategy required optimization include the concomitant use of HDAC inhibitors and OVs. HDAC inhibitors have been demonstrated to enhance VSV, HSV and Vaccinia's therapeutic effects mainly through an increase of viral replication due to the dampering of the innate immune response (Mactavish *et al*., 2010; Nguyen *et al*., 2008; Otsuki *et al*., 2008). In most of these studies, efficient enhanced anti-tumor effects were observed when the animals or cells were pretreated with the HDAC inhibitor to block IFN release and other innate immune modulators before virus infection. Similarly, the combination of OVs with rapamycin, also employed to reduce the innate immune mechanisms and allow enhanced viral replication, was also adapted to include a pretreatement approach (Alain *et al*., 2010). In contrast, combination of heat shock protein inhibitors and Measles virus demonstrated optimal synergsims when the inhibitors were added after infection. The authors observed an increase in syncytia formation that did not correlate with an increase in viral replication and hypothesized that this effect was caused by changes in the actin cytoskeleton (Liu *et al*., 2008). Therefore these examples illustrate that the design of a combination approach must take into account not only the OV's viral characteristics but also the desired impact on virus oncolysis.

VSV and the immune system combination

OV treatment engenders massive immune reactions at the tumor site and therefore represents an automatic combination between the OV and the immune system. In Manuscript II, we demonstrated that VSV oncotherapy interferes with tumor antigen presentation by infecting tumor dendritic cells and preventing their migration to the draining lymph nodes, thus defining the mechanism underlying VSV's limited ability to promote tumor-specific CD8 T cell response. Although this characteristic may be specific to VSV, some aspects may also be relevant to other OVs. In Manuscript II, one of the factors suspected to contribute to DC killing and blockage of migration is the tumor vascular shutdown, a mechanism that is not restricted to VSV but also applies to other OVs such as Vaccinia virus (Breitbach *et al*., 2007). Moreover, Breitbach *et al*. have described the mechanism as part of an innate host confinement response that would decrease blood flow and prevent the spread of pathogens during normal infection, therefore relating the concept to broad virus infections (Breitbach *et al*., 2011). In addition, while some OVs have been proposed to enhance DC properties, many of them, such as Measles virus, HSV and Vaccinia virus, have been shown to negatively affect DC functions (Errington *et al*., 2008; Greiner *et al*., 2006; Grosjean *et al*., 1997; Ilett *et al*., 2009). Furthermore, the infection of DCs by non-OV viruses such as Cowpox virus and Respiratory syncytial virus have also been shown to impair their function as a viral evasion mechanism (Cunningham *et al*., 2010; Hansen *et al*., 2011). Thus, this description of the killing effect and migration blockage of VSV on tumor DCs is coherent with other negative impacts on DCs described for both OV and non-OV viruses.

Our data clearly describes the absence of antigen presentation from tumor DCs in the days following VSV treatment where maximal oncolysis and tumor antigen release occur. Although the strategy presented was based on tumor DCs, following the hypothesis that they would be the most suited to capturing and presenting tumor antigen released from oncolysis, it is possible that VSV oncolytic treatment enhances tumor antigen release, which could directly reach the lymphoid organs. Considering this eventuality, lymphoid-organ DCs could possibly promote the anti-tumor specific adaptive

response even if tumor DCs do not arrive at the draining lymph nodes. This possibility was not directly discussed in Manuscript II and will be addressed here.

The systemic immune consequences of VSV oncolysis

Data from Manuscript II also demonstrates that VSV oncolytic treatment has an impact beyond the tumor microenvironment and the surrounding tissues. Results showing the movement of the total leukocyte population from the blood to the lymphoid organs demonstrate that a systemic inflammatory reaction is engendered. This reaction is likely the result of a systemic release of IFN and other pro-inflammatory cytokines. As a matter of fact, a high concentration of IFN in the serum can be observed following IP or IV VSV inoculation (Barchet *et al*., 2002; Schattner *et al*., 1983). Furthermore, systemic blood transient lymphopenia induced by VSV has previously been reported following VSV IP inoculation and was shown to be mediated by serum IFN (Schattner *et al*., 1983). This global host defense mechanism not only helps to protect normal tissues from infection and dictates the movement of the immune cells, but also exerts a profound effect on the their activation state.

Analysis of DC maturation *in vivo* following VSV oncolytic treatment revealed that DCs undergo general maturation. Our results demonstrated that the small amount of remaining tumor-infected DCs displayed upregulation of costimulatory molecules, as it has been previously reported for BMDCs infected with VSV (Ahmed *et al*., 2006; Boudreau *et al*., 2009). In addition to these infected DCs, lymph node DCs and spleen DCs, which are presumably not infected, also displayed a global upregulation of their costimulatory molecules (see **Manuscript II, Thesis Supplementary Figure 2**). The systemic maturation of DCs *in vivo* has also been previously reported following non-viral PAMP injection (Sporri and Reis e Sousa, 2005). Furthermore, IFN has been demonstrated to induce the maturation of BMDCs and monocyte-derived DCs *in vitro* by many groups (Dauer *et al*., 2006; Della Bella *et al*., 2004; Paquette *et al*., 1998; Santini *et al*., 2000). Therefore, the secretion of large amounts of IFN and the systemic

inflammatory response following VSV treatment likely favors a general systemic maturation of DCs *in vivo*.

Although several groups demonstrated the maturation of DCs following contact with IFN, this does not necessarily correlate with enhanced antigen presentation and T cell priming capacities. Many reports also suggest that IFN-mediated maturation of *in vitro*-generated DCs abrogates their T cell priming capacities (Dauer *et al*., 2003; McRae *et al*., 2000). These DCs failed to produce IL-12 (third signal for T cell activation), even when highly expressing costimulatory molecules (Joffre *et al*., 2009; Sporri and Reis e Sousa, 2005). Furthermore, even when IFN-maturated DCs were able to induce CD8 T cell proliferation, these CD8 T cells were unable to produce IFNγ (Joffre *et al*., 2009). Thus in the context of oncolytic virotherapy, although the systemic DC maturation induced by VSV *in vivo* appears at first to potentially benefit overall antigen presentation, it may in fact have a negative impact. It could be hypothesized that general non-functional maturation of lymphoid-organ DCs may occur during VSV oncolysis and may contribute to the impaired antigen capacities reported in Manuscript II. In comparison, systemic activation of DCs following TLR ligand injection or malaria infection has previously been demonstrated to impair DC cross-presentation capacities, but not the endogenous MHC-I processing pathway (Wilson *et al*., 2006). As cross-presentation of tumor antigen by DCs was the main objective of the Flt3L and VSV combination therapy, the systemic activation of lymphoid-organ DCs following VSV oncolysis likely also affected this crucial function in our treatment strategy.

VSV affecting DC functions

In manuscript II, we and others proposed that the antiviral response and the successful anti-OVA response achieved through the use of a VSV-OVA relies on the infection of lymphoid-organ DCs. Whether infected DCs possess the capacity to directly prime a T cell response or whether the virally expressed antigen must be presented by an uninfected DC before the infected DC dies is a question that requires further investigation. One possibility is that infected DCs release the virus or virally expressed

tumor antigen at the lymphoid organs and non-infected DCs subsequently capture these antigens and initiate a response through the cross-presentation process. A second possibility would implicate other APC subsets such as B cells or macrophages to serve as intermediaries to present antigens. A third possibility would be the direct transfer of MHC molecules; recent reports have addressed the possibility of an exchange of preloaded antigen MHC molecules from one DC to another. The process has been observed *in vitro* as well as *in vivo* and has been termed cross-dressing (Dolan *et al*., 2006; Qu *et al*., 2009; Wakim and Bevan, 2011). Therefore, it is possible that an infected DC presenting VSV antigen (or the VSV-expressed antigen) on its MHC-I molecule could exchange preformed peptide-MHC complex with an uninfected DC. Recent reports investigating cross-dressed DCs' capacity to prime CD8 T cells *in vivo* have addressed this question using VSV-OVA and OT1 naïve or memory CD8 T cells. In a non-OV context of VSV intranasal inoculation, cross-dressed DCs were able to prime memory but not naïve OT1 cells (Wakim and Bevan, 2011). Although the experimental model differs from VSV tumor oncolysis, this observation does not argue for the role of the cross-dressing pathway in the initiation of anti-viral or virally-expressed antigen adaptive immunity.

Regardless of whether infected lymphoid-organ DCs are able to directly prime T cells or in fact require uninfected DCs to perform indirect priming, it remains to be determined whether they are still capable of capturing, processing and presenting exogenous antigens through the cross-presentation pathway. Preliminary experiments in the course of this Ph.D. study using BMDC and whole OVA as source of exogenous antigens were performed *in vitro* to address this issue. Different readouts assessing cross-presentation during VSV infection resulted in conflicting data and did not permit us to determine whether these intrinsic DC capacities were affected (data not presented in the thesis). However, it is reasonable to presume that infected DCs would suffer from impaired cross-presentation ability based on two observations: first, that VSV-infected DCs undergo a maturation process known to diminish the endocytosis and antigen capture aptitude of the DC (Villadangos and Schnorrer, 2007), and secondly, that most infected DCs undergo apoptosis, which makes it unlikely that they could continue

performing any metabolic function. Supporting the notion of diminished cross-presentation abilities is the fact that anti-tumor immunity following VSV oncolytic treatment has been successfully generated only when VSV was engineered to directly express a tumor antigen, therefore allowing direct antigen presentation via the classical MHC-I pathway and bypassing the step of antigen capture and cross-presentation (Bridle *et al*., 2009; Bridle *et al*., 2010; Kottke *et al*., 2011; Wongthida *et al*., 2011).

VSV and tumor immune tolerance

To avoid auto-immunity, the immune system constantly eliminates auto-reactive immune precursor cells (Janeway, 2001). Considering the fact that a tumor originates from small modifications of a host cell, immune cell precursors directed against the modified tumor cell are likely to have already been eliminated (Prestwich *et al*., 2008). Furthermore, the tumor immunosuppressive environment and the immunoediting of the tumor (Vesely *et al.*, 2011) likely contributes to the tolerization of the immune system towards the tumor. The number of immune cells susceptible to developing into anti-tumor effector cells is very low, and preexisting tolerance does not hold any advantage for immune-based treatment. Therefore, it is possible that even if lymphoid-organ DCs would capture and present a tumor antigen following VSV treatment, the amount of CD8 T cells specific to this antigen would be very limited and that no effective response would be mounted. Recently, two strategies were developed to circumvent the limited amount of anti-tumor CD8 precursors prior to the use of rVSV expressing a tumor antigen. In the first strategy, a pre-vaccination approach using an adenovirus vector encoding a tumor antigen was designed, while in the second a co-treatment with adoptively transferred tumor-specific T cells was performed (Bridle *et al*., 2009; Bridle *et al*., 2010; Wongthida *et al*., 2011). The main limitation of these approaches rests in the generation of a response specific to a known tumor antigen, as opposed to the combination strategy of Flt3L and VSV, which was developed to increase the immune response to any tumor antigen released through oncolysis.

In a recent publication, Kottke *et al.* engineered VSV to express a cDNA library derived from normal host tissue (or from tumor cells) and demonstrated a strong CD8 response against selected epitopes from both libraries. Although this strategy appears to generate and activate autoimmunity, its anti-tumor therapeutic effect was predominant (Kottke *et al*., 2011). The authors argued that the *in vivo* selection of tumor-specific T cells is probably due to the greater amount of T cell precursors to tumor antigens when compared to self-non-altered antigens. This strategy generates a tumor response against multiple unknown but tissue-specific antigens without requiring endogenous uptake and presentation by DCs. Because the cDNA libraries used to generate the immune response were not unique to cancer, this successful induction of an anti-tumor immune response carries the risk of autoimmune manifestations. Therefore, it represents an interesting approach that, even if requiring the virus to directly express the antigen, allows to bypass the necessity of tumor DCs or lymphoid-organ DCs to capture and cross-present tumor antigens in order to launch an anti-tumor immune response.

In summary, we observed the systemic maturation of lymphoid-organ DCs following VSV oncolytic treatment and hypothesized that it might interfere with cross-presentation capacities. In addition, we clearly established in Manuscript II that tumor DCs are infected and killed by VSV and are unable to reach the lymph nodes. Altogether, both tumor DCs and lymphoid-organ DCs fail to present tumor antigen and to prime tumor-specific CD8 T cells following VSV oncolytic treatment. In support of this conclusion we demonstrated the absence of OT-1 T cell proliferation *in vivo* following VSV treatment (**Manuscript II, Figure 3**). We believe that direct infection of lymphoid organ DCs may allow to bypass the antigen capture and cross-presentation pathways and therefore to explain the successful generation of the anti-viral response or response against a virally-expressed tumor antigen.

Concluding remarks

In conclusion, the application of oncolytic viruses as novel agents in combination cancer therapy should be based on a thorough understanding of each aspect of the involved treatment. Characterization of the chemotherapeutic aspect, viral specificity and host immune response are all necessary in order to obtain the full therapeutic potential of these combined agents. **Figure 1** summarizes the therapies and findings described in this thesis. The CD::UPRT suicide adapted treatment permitted increased tumor cell killing and improved therapeutic outcome, while the VSV and Flt3L combination revealed that tumor DCs are killed by VSV and are unable to leave the tumor to induce an adaptive immune response at the lymph node. The field of oncolytic viruses in cancer therapy will certainly benefit from the knowledge uncovered during the work performed in the course of this PhD.

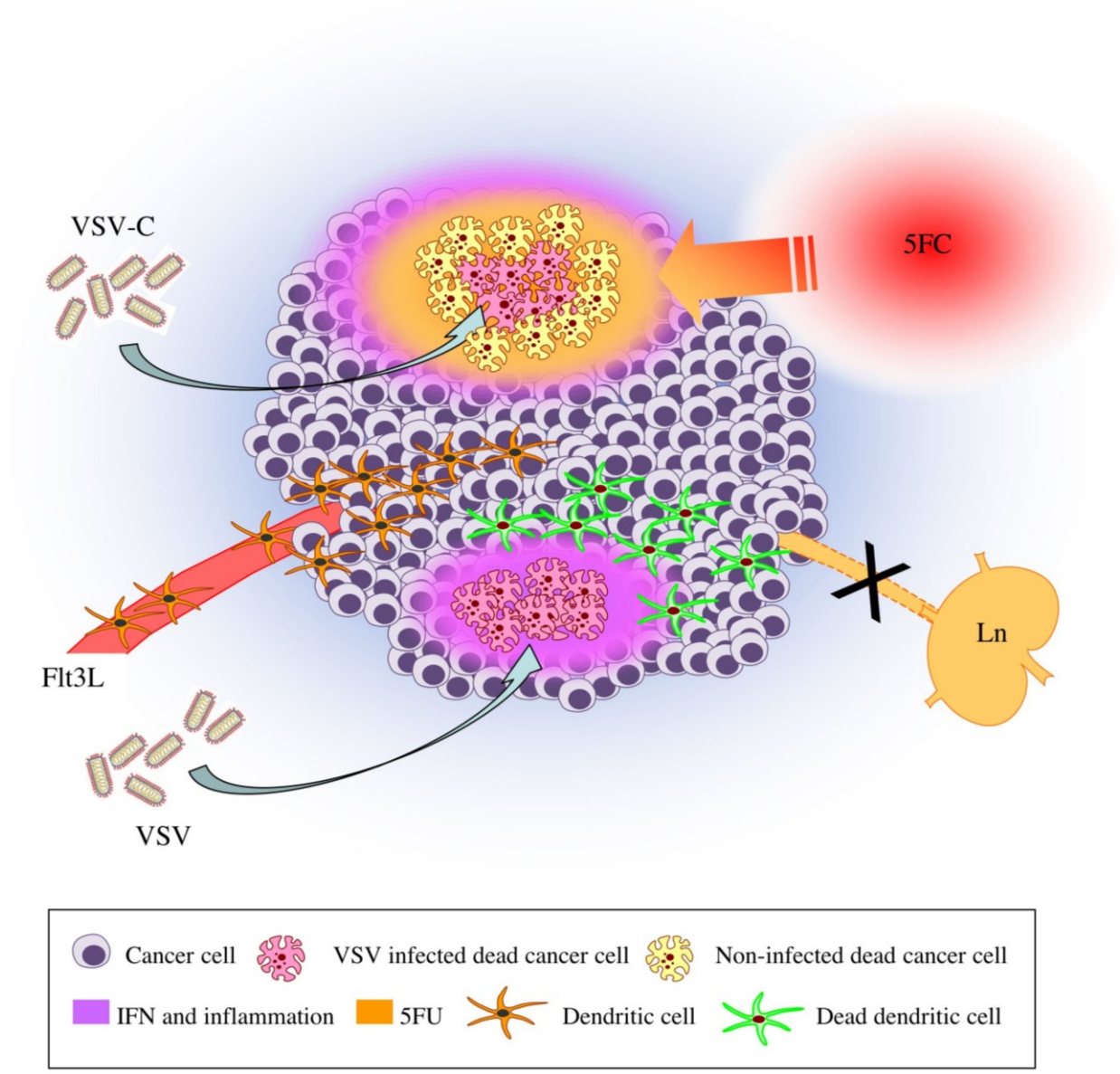

Figure 1. Summary of the findings presented in this thesis. VSV expressing the CD::UPRT suicide gene, in combination with a systemic treatment of non-toxic 5FC adapted to the short period of VSV gene expression at the tumor, allows the conversion of the prodrug into toxic 5FU at the tumor and enhances tumor cell killing.

Flt3L systemic treatment increases the number of DCs infiltrating the tumor, but VSV infects and kills tumor DCs, therefore abrogating their migration to the draining lymph nodes (Ln) and subsequent priming of the anti-tumor CD8 T cell adaptive immune response.

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CHAPTER 4

CONTRIBUTION TO ORIGINAL KNOWLEDGE

The contributions to original knowledge emerging from the work presented in this thesis are:

1. The characterization of the transient VSV cycle at the tumor following intra-tumoral injection.

2. The adaptation of the VSV-C/5FC combination treatment by integrating VSV *in vivo* oncolytic properties with the bioavailability of 5FC to reach maximal therapeutic benefit.

3. The demonstration that VSV intra-tumoral oncolytic treatment induces a systemic inflammatory response provoking the general maturation of DCs.

4. The demonstration that VSV infects tumor DCs and abrogates their migration to the draining lymph node, therefore explaining the limited ability of VSV to launch an anti-tumor adaptive immune response.

5. The description of VSV's effects on the tumor microenvironment, which implies that future VSV-based immunotherapy strategies should rely on sources of APCs other than tumor DCs.

Altogether, the results presented in this thesis have contributed to a more thorough understanding of VSV's *in vivo* oncolysis characteristics and host immune responses. Integrating these findings into new VSV-based cancer therapies will certainly provide an opportunity for complementing the acute oncolytic properties of VSV with increased bystander tumor cell killing or long-lasting tumor immunity.