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INVESTIGATING ACUTE EFFECTS OF ONCOLYTIC VIRUS INFECTION OF TUMOURS

By
Caroline Breitbach

A thesis submitted in partial fulfillment of the
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ABSTRACT

Oncolytic viruses (OVs) are selected or designed to eliminate malignancies by direct infection and lysis of cancer cells. In contrast to this concept of direct tumour lysis by viral infection, a significant portion of the *in vivo* tumour killing activity of two OVs, vesicular stomatitis virus (VSV) and vaccinia virus is caused by indirect killing of *uninfected* tumour cells. Limited virus infection rapidly triggers a loss of tumour perfusion within the tumour core. Tumour cells within the tumour rim remain viable and are associated with intact vasculature. Transcript profiling of tumours demonstrates a pro-inflammatory gene signature with significant induction of neutrophil chemo-attractants. Depletion of neutrophils in animals prior to VSV administration eliminates uninfected tumour cell apoptosis and permits more extensive replication and spreading of the virus throughout the tumour. Tumour targeted inflammation triggers extensive intravascular coagulation which is responsible for the loss of perfusion as inhibition of coagulation results in maintenance of tumour perfusion in the context of VSV therapy. Interestingly, the remaining viable tumour rim does not expand in tumours implanted into immune competent mice. Conversely, a massive expansion of the viable tumour rim ensues following treatment of tumour bearing nude mice that possess a defective adaptive T cell response. These results suggest that outgrowth of viable tumour cells from the tumour rim following OV therapy is inhibited by an adaptive immune response triggered to target tumour cells. Taken all together, these results

indicate that targeted recruitment of neutrophils triggers coagulation within infected tumour beds and enhances the killing of malignant cells. Activation of inflammatory cells may be used for enhancing the effectiveness of oncolytic virus therapeutics as well as other anticancer agents that may similarly trigger inflammation. Understanding mechanism and consequences of inflammation dependent tumour cell death is important as loss of tumour perfusion has now also been measured in patients treated with vaccinia virus.

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LIST OF ABREVIATIONS

OVs: oncolytic viruses

VSV: vesicular stomatitis virus

DNA: deoxyribonucleic acid

RNA: ribonucleic acid

ER: estrogen receptor

CTLs: cytotoxic T lymphocytes

EGFR: epidermal growth factor receptor

VEGF: vascular endothelial growth factor

GM-CSF: granulocyte-macrophage colony stimulating factor

VDAs: Vascular disrupting agents

CA-4-P: combretastatin A-4 3-*O*-phosphate

DMXAA: 5,6-dimethylxanthenone-4-acetic acid

TNF: tumour necrosis factor

PDT: Photodynamic therapy

ROS: reactive oxygen species

HSV: herpes simplex virus

HER2: human epidermal growth factor receptor 2

MMP: matrix metalloprotease

TRAIL: tumour necrosis factor–related apoptosis-inducing ligand

VSV-M: VSV matrix protein

VSV-N : VSV nucleocapsid protein

VSV-P : VSV phosphoprotein

VSV-L: VSV large

VSV-G : VSV glycoprotein

IFN: interferon

PKR: RNA-dependent protein kinase

eIF2α: eukaryotic translation initiation factor 2 alpha

WT: wild-type

ISGs: interferon stimulated genes

MHCI: major histocompatibility complex I

IMV: intracellular mature virions

CEV: cell-associated enveloped virus

EEV: extracellular enveloped virion

TK: thymidine kinase

VGF: vaccinia growth factor

PAMPs: pathogen associated molecular patterns

LPS: lipopolysaccharide

dsRNA: double stranded RNA

PRRs: pattern recognition receptors

TLR: Toll-like receptor

RIG-I: retinoic acid-inducible gene I

NALP3: NACHT-, leucine-rich-repeat- and pyrin-domain-containing protein 3

HMGB1: high-mobility group box 1 protein

RAGE: advanced glycation end-product-specific receptor

COX: cyclooxygenase

IL: interleukin

MPO: myeloperoxidase

TGF- β: transforming growth factor beta

NFκB: nuclear factor kappa-light-chain-enhancer of activated B cells

ssRNA: single stranded RNA

NSAIDs: non-steroidal anti-inflammatory drugs

HPV: human papilloma virus

TAMs: tumour associated macrophages

bFGF: basic fibroblast growth factor

TF: tissue factor

TFPI: tissue factor pathway inhibitor

tPA: tissue plasminogen activator

uPA: urokinase type plasminogen activator

PAI: plasminogen activator inhibitor

ECM: extracellular matrix

TIMPs: tissue inhibitors of matrix metalloproteases

shRNA: short hairpin RNA

NK cell: natural killer cell

HCC: hepatocellular carcinoma

CPA: cyclophosphamide

MIG: monocyte chemoattractant protein

NDV: Newcastle Disease Virus

HDAC: histone deacetylase

MTD: maximum tolerated dose

CT: computed tomography

MBV: mixed bacterial vaccines

DAMPs: damage associated molecular patterns

MIP-2: macrophage-inflammatory protein-2

G-CSF: granulocyte colony-stimulating factor

GFP: green fluorescent protein

TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP nick end label

UV: ultraviolet

nm: nanometer

μm: micrometer

h: hour

PBS: phosphate buffered saline

mAb: monoclonal abntibody

PET: positron emission tomography

BrdU: bromodeoxyuridine

I/R injury: ischemia/reperfusion injury

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CHAPTER 1: INTRODUCTION

1.1 Cancer

1.1.1 Overview

Cancer is defined as an abnormal growth of cells that has the ability to proliferate uncontrollably and capability to invade and spread (or metastasize). Cancer is not one disease, but a group of over 100 diseases which are characterized by their tissue and cell of origin. According to Canadian Cancer Society statistics, an estimated 166,400 new cancer cases will be diagnosed in 2008 and 73,800 deaths will occur due to cancer in Canada in this year. Furthermore, based on current incidence rates, approximately 40% of women and 45% of men will be diagnosed with cancer over the course of their lifetime. Approximately 1 in 4 Canadians will die from the disease (1).

1.1.2 Conventional cancer therapy

Currently, the most successful form of cancer therapy is the treatment of local disease by *surgical resection. Radiation therapy* comprises another form of loco-regional anticancer treatment (2). *Chemotherapy* and novel *biological therapies* can be administered systemically and are used to control metastatic disease in which cancer cells have left the tissue/organ of origin and have spread to other sites within the body (3, 4).

1.1.2.1 Cancer Surgery

Cancer surgery is the oldest form of anticancer treatment. However, in the mid-1800s, surgery often was not successful in removing the primary tumour and the procedure could even

result in death. These outcomes were due to complications such as insufficient respiration during surgery (resulting in a hurried surgical procedure) or patients acquiring bacterial infections following treatment. With the discovery of antibiotics and mechanisms to avoid airway obstruction, surgery has become an effective option to remove tumours at the primary site. However, metastatic disease is difficult to treat with surgery alone. In the treatment of various cancers, including ovarian, esophageal, gastric and lung cancers, surgery is used along with radiation- or chemotherapy to improve survival of patients with metastatic disease (5-8).

1.1.2.2 Radiation therapy

Radiation therapy is a component of disease management for about half of patients diagnosed with cancer. The advantages of this treatment modality is that it can be applied locally, avoiding the deleterious side effects associated with systemic administration of chemotherapeutics and that it can be used to treat local tumours that do not qualify for surgical resection (2). Radiation therapy uses ionizing radiation to create highly reactive radicals in a well-defined area of tissue. These activated molecules will then in turn cause DNA damage. Once a cell goes on to divide, its damaged DNA will not be able to be copied properly and the cell will die (termed reproductive death). Since cancer cells divide more rapidly than most normal cells, malignant cells are more sensitive to damage induced by radiation than normal cells (2). Tumours developing hypoxic regions due to inefficient blood supply represent the major problem in the field of radiation therapy. As oxygen is needed for the generation of free radicals following exposure to ionizing radiation, hypoxic cells are approximately 3 times more resistant to radiation therapy than euoxic cells (9). Therefore, much research has been focused on combining radiation therapy with compounds that sensitize hypoxic cells to radiation or compounds that are cytotoxic to hypoxic cells (10).

1.1.2.3 Chemotherapy

Currently, most metastatic disease is treated with systemic chemotherapy. The principle of chemotherapy is that certain chemical reactions occur more frequently in malignant cells and can be disrupted in order to preferentially target tumours. In particular, most chemotherapeutics target DNA or the process of DNA synthesis as cancer cells generally possess a higher rate of DNA synthesis than normal cells. However, one caveat is that some types of normal cells may possess a very high rate of DNA replication and cell division, such as bone marrow elements, gastrointestinal epithelium and hair follicles. Damage to these normal dividing cells is the cause of moderate to severe side effects upon systemic treatment with chemotherapeutics. Since chemotherapeutics are targeted to cancers due to *quantitative* differences rather than *qualitative* ones, the therapeutic index (i.e. the ratio of toxic dose to effective dose) of many chemotherapeutics is low (3).

Different classes of chemotherapeutics exist, based on their mechanism of action as well as their origin. Briefly, *alkylating agents* (e.g. melphalan, cyclophosphamide and busulphan) are highly reactive agents that substitute alkyl groups for hydrogen atoms. These substitutions can cross-link DNA, create kinks and produce DNA strand breaks, interfering with DNA replication and RNA transcription. *Antibiotics* are natural products isolated from soil fungus *Streptomyces.* These agents intercalate within DNA strands, distorting the molecule, disrupting DNA or RNA synthesis. Some antibiotics also inhibit topoisomerases, enzymes involved in unwinding coils within DNA during DNA replication. Examples of antitumour antibiotics include doxorubicin, mitoxantrone, bleomycin and mitomycin C. Other chemotherapeutics are classified as *plant derivatives* which include vincristine, paclitaxel and topotecan. These agents were found in plants and disrupt microtubule function (inhibiting proper mitotic spindle formation) and inhibit

topoisomerase II and I activity, respectively. *Antimetabolites*, which include methotrexate, 5-fluorouracil, gemcitabine and hydroxyurea, are structural analogues of normal cell metabolites. They cause disruption of cellular processes by substituting for a metabolite (whose improper incorporation results in disruption of molecule function) or by competing for active or regulatory sites within enzymes, therefore disrupting the enzyme's ability to function normally. Finally, *hormonal agents* (e.g. tamoxifen, dexamethasone and prednisone) have long been used as anticancer therapy in cancers derived from tissues that respond to endocrine signals (11). For instance, the majority of breast cancers express estrogen receptor (ER) and require estrogen receptor activity for growth. When bound to estrogen, ER induces expression of a variety of genes involved in tumour growth promotion while downregulating cell cycle inhibitors (12). Therefore, treatment with tamoxifen which recruits corepressors when complexed with ER, inhibits the expression of ER induced genes (13).

1.1.3 Recent additions to the anticancer arsenal

1.1.3.1 Biological cancer therapies

Most recent additions to the anticancer arsenal are known as *biological response modifiers* that modify the interaction between the tumour and the host. Their purpose can be to reduce tumour growth or overcome side-effects of conventional anticancer therapies (4). Briefly, *immunotherapy* is a biological therapy in which immune cells (either from the host or adoptively transferred from another person) are activated to reject the cancers, based on peptides (termed antigens) uniquely expressed by tumour cells. Immunotherapy generally aims to activate cytotoxic T lymphocytes (CTLs) that specifically recognize identified tumour antigens. This therapy has met with some success in patients with melanoma and kidney cancer (14).

Antibodies are proteins produced by B cells that specifically recognize epitopes on proteins and have been developed as a therapeutic platform for recently emerging anticancer therapies. Antibody binding to ligands or receptors can *abrogate signal transduction.* For instance, antibodies have been generated to target the oncogenic epidermal growth factor receptor (EGFR) (15). EGFR activation triggers signaling cascades that promote cell survival and proliferation (16). Inhibition of EGFR dependent signaling results in the induction of apoptosis (17). Cetuximab, an antibody that binds EGFR, was shown to prolong survival in patients with metastatic colorectal cancer (18) and head & neck cancer (19) when administered in combination with chemotherapy. These regimes have now been approved for clinical use. Antibodies have also been developed as *antiangiogenic agents*, blocking formation of new blood vessels, a process critical for tumour growth and invasion (11). Antibodies against vascular endothelial growth factor (VEGF), a critical mediator of angiogenesis that is produced by tumour cells or normal supporting cells (see below), have now been approved for clinical use. Bevacizumab is recommended in combination with chemotherapy for the treatment of colorectal, lung and breast cancer (20-22).

The major class of biological response modifiers that is used to overcome side-effects from conventional chemotherapeutic treatment is *growth factors.* These mediators are normally produced endogenously and act on the bone marrow to trigger production of immune cells. As an example, granulocyte-macrophage colony stimulating factor (GM-CSF) is used in conjunction with conventional chemotherapy in order to boost immune cell populations that have been depleted by cytotoxic drugs (reviewed in (23)). GM-CSF has also recently been used to boost anti-tumour immune responses in the context of anticancer immunotherapy (24).

1.1.3.2 Vascular targeting therapies

Vascular disrupting agents (VDAs) disrupt endothelial cell integrity and function, resulting in a loss of tumour perfusion. Combretastatins (e.g. combretastatin A-4 3-*O*-phosphate (CA-4-P) and Oxi4503) are tubulin binding agents that trigger microtubule depolymerization (25). The cytoskeletal effects of 5,6-dimethylxanthenone-4-acetic acid (DMXAA) are confined to the actin cytoskeleton, though it is thought that a portion of the antivascular effects of DMXAA are attributed to cytokine induction, in particular tumour necrosis factor (TNF) (25). Combretastatins induce endothelial shape changes resulting in increased vascular permeability, leakage of plasma proteins and an increase in interstitial fluid pressure which can trigger a decrease in vessel diameter and increased hematocrit – all contributing to a reduction in tumour blood flow. TNF decreases endothelial cell adhesion by disrupting integrin binding (26) (see discussion) which will also lead to increased vessel permeability and the subsequent events that lead to a disruption in tumour perfusion. Tumour vasculature is erratic and abnormally formed (see below). It is thought that these different properties of tumour vasculature make it more susceptible to the damaging effects of VDAs (i.e. small changes in the vessel vascular permeability following VDA administration may have catastrophic effects in the tumour).

Photodynamic therapy (PDT) involves the systemic infusion of non-toxic photosensitizers and application of visible light in the area to be targeted. Photosensitizers are activated by light and act as catalysts that convert oxygen to reactive oxygen species (ROS). These reactive species damage nucleic acids and proteins in surrounding cells, in particular the tumour vasculature, resulting in a disruption of tumour blood flow (27, 28). Both VDAs and PDT induce considerable tumour necrosis within a short time after treatment and are currently being investigated as a platform for combination with radiation- or chemotherapy (29, 30).

1.1.4 The need for better anticancer therapies

Though our knowledge of genetic differences between cancers and normal cells has expanded greatly over the last 15 years, relatively few targeted therapeutics that have been developed have shown sufficient survival benefit to patients to have been approved clinically. Novel therapeutics now applied clinically result in improved survival of only small cohorts of patients and have not led to significant improvements in how cancer is treated overall. This is because researchers are not dealing with a single disease, but hundreds of different diseases that have undergone different genetic changes. Recently, investigators have been able to generate the sequence of the same tumour (e.g. glioblastoma) derived from different patients and found different mutational profiles even in this one type of cancer (31, 32). We must find therapies that target a fundamental difference that sets tumours apart from normal cells.

1.2 Oncolytic viruses

1.2.1 Using replicating viruses to treat cancer

Oncolytic viruses (OVs) may represent a promising novel cancer therapy (33). OVs are replication competent viruses that preferentially infect cancer cells. Rather than discriminate between normal and malignant cells based on proliferation rate (which is the property that conventional chemotherapy and radiation therapy use to 'discriminate' normal and malignant cells), OV replication in cancer cells is dependent on fundamental differences between tumours and normal tissue (e.g. a block in pro-apoptotic pathways). This will likely increase the therapeutic index as OVs seek out *qualitative* differences versus *quantitative* differences between transformed and untransformed cells.

1.2.1.1 History

Even in the early 1900s there was evidence from case reports suggesting that virus infection coincided with spontaneous tumour regressions (34-36). Early clinical trials using sera or tissue extracts from infected individuals with viral hepatitis in patients with Hodgkin's disease resulted in 7 patients out of 22 responding, though improvements were short lived (37). Southam and Moore went on to pioneer the field of oncolytic virus therapy by studying the use of various viruses in preclinical tumour models as well as in humans. Tumour regression was demonstrated for the first time in rodent models (38). Southam went on to study the effects of human pathogens in causing cancer regression in patients. Though some patients responded to therapy, many suffered severe side effects. In a study that would be considered seriously ethically flawed today, Southam and Moore went on to study the efficiency of virus replication in human tumour cell lines in patients. Tumour cells were inoculated into the forearm of patients and virus replication and antibody production was monitored. Unfortunately, tumour regressions were not robust in patients and some of the transplanted cells grew out to from tumours and even metastasized (39). Any diminished efficacy in humans (as compared to rodent models) is likely attributable to the anti-virus immune response. Despite these discouraging results, a trial in Japan led by Asada showed that administration of mumps virus to patients with various types of tumours resulted in appreciable responses in 90% of patients (40). However, using non-attenuated human pathogens as a common accepted cancer therapy did not seem reasonable at that time.

1.2.1.2 Oncolytic viruses today

The field of oncolytic virus therapy has experienced a major resurgence in the 1990s thanks to an increase in understanding of the pathogenesis of these viruses. With the characterization of genetic defects in tumours, as well as our capability of genetically engineering

viral genomes, we are now able to rationally design viruses to target specific tumour types. Though some OVs are animal pathogens that naturally replicate better in cancer cells in humans (e.g. VSV, see below), most OVs are human pathogens that are engineered to target tumours. Strategies to target tumours include *(1)* restricting virus entry to tumour cells, *(2)* targeting transcription or translation of viral genes to cancer cells or *(3)* attenuating viruses by exploiting genetic defects within cancer cells (e.g. vaccinia, see below) (reviewed in (41) and (42)).

Virus entry can be restricted to cancers by targeting a receptor that is upregulated on tumours, therefore targeting viruses to exclusively replicate in tumours. For instance, herpes simplex virus (HSV) infection has been targeted to the human epidermal growth factor receptor 2 (HER2), a receptor overexpressed in approximately 1/3 of mammary tumours and some ovarian tumours, using the single-chain antibody targeting HER2 as a ligand (43). Beyond using a receptor/ligand interaction to target virus entry to tumour cells, a property of the tumour microenvironment has been capitalized on to target OV infection to tumours. Cancers are known to secrete high amounts of proteases and reovirus entry has been shown to be dependent on cleavage by cathepsins B and L. Expression of these proteases in cancer cells determined their susceptibility to the OV (44), though reovirus replication in cancer cells has also been shown to be dependent on intracellular Ras signaling (45). Also, a measles virus with an engineered fusion protein that must be activated by matrix metalloprotease (MMP), an enzyme often overexpressed in cancers, was able to infect an MMP expressing tumour. Inoculation of this virus into the brain of mice did not result in infection or death, in contrast to the wild type virus (46).

A popular approach of targeting human viruses to tumours has been to restrict viral transcription or translation to transformed cells. Adenovirus was one of the first viruses to be engineered to target tumours with this method by deleting adenoviral E1B, a protein shown to

bind to and destroy the p53 tumour suppressor protein. Adenovirus requires entry into the S phase of the cell cycle and DNA synthesis for its own propagation. Presence of p53 restricts entry into the cell cycle and E1B has been shown to bind to and destroy the p53 tumour suppressor protein. Therefore, an E1B deleted oncolytic adenovirus was thought to be targeted to tumours with mutated p53 (47). However, it has since been demonstrated that tumour selectivity of this virus is attributed to differences in late mRNA export between normal and cancerous cells. In the absence of E1B, viral mRNA was only able to be exported to the cytoplasm for subsequent translation in transformed cells (48). An alternative strategy to target adenovirus replication to tumours is to place promoters active in cancer cells to drive expression of genes critical for virus replication (e.g. telomerase promoter ahead of *E1* gene (49)).

Viruses that normally infect humans have been engineered to be attenuated in normal cells while allowing for continued replication in malignant cells. Deletion of ICP34.5 in herpes virus diminishes neurovirulence while maintaining infectibility of transformed cells (50). The tumour specificity of many OVs is due to genetic defects within tumour cells that render them more susceptible to virus infection. For instance, one mechanism for a multicellular organism to protect itself from viruses is for infected cells to undergo apoptosis, or programmed cell death. The pro-apoptotic machinery in cancer cells is frequently mutated in order to generate a proliferative advantage therefore making many cancer cells more susceptible to infection by viruses. Both VSV and vaccinia virus target tumours based on genetic defects in tumour cells, either defective innate immunity (VSV) or overexpression of oncogenic signaling proteins (vaccinia virus) (see below).

OVs have also been engineered to produce *transgenes* that enhance virus potency. Prodrug converting enzymes such as thymidine kinase and/or cytosine deaminase are thus produced by

infected cells within the tumour and convert systemically administered prodrugs to active cytotoxic agents only within the tumour microenvironment (51-53). Inserting pro-apoptotic genes such as tumour necrosis factor–related apoptosis-inducing ligand (TRAIL) into the virus genome is also an attractive strategy to improve the potency of OVs (54).

OVs have also been used as an *immunotherapy* platform. In some preclinical models, evidence is emerging that OV infection of tumours induces an adaptive immune response that contributes to tumour rejection. Following treatment with VSV, mice were shown to have activated T cells that recognize both virus and tumour associated antigens (55). Similarly, tumour infection by vaccinia virus primed an antitumour immune response (56). OVs expressing mediators that stimulate the immune system, such as GM-CSF have been engineered into HSV and vaccinia virus (57, 58). Immune stimulation, in addition to the lytic effect of direct virus infection was shown to contribute to tumour rejection. Despite generation of tumour reactive T cells, current immunotherapies are failing as T cells are not sufficiently activated within the tumour microenvironment. It is thought that 'danger signals' within the tumour microenvironment may activate cytotoxic T cells to reject the tumour (59). OV infection of tumours may well represent the danger signal that ultimately allows for the immune destruction of tumours. Therefore, OV therapy may also be a good platform to combine with existing immunotherapies (60).

OVs introduced here as well as others are being intensively investigated in preclinical models. New strategies to better target OVs to cancer as well as reduce OV toxicity are continuously emerging. Also, further insights into the sometimes unexpected mechanisms of tumour destruction are emerging from experiments in rodent models. Indeed, as discussed previously, using the host to reject the tumour in addition to damage induced by direct viral

infection and lysis of tumour cells likely represents a promising approach to generating cancer cures. Both vesicular stomatitis virus as well as vaccinia virus are being studied extensively in preclinical models. Excitingly, some OVs (including vaccinia virus) are also being tested in clinical trials. A partial summary of OVs currently being investigated in clinical trials is listed in Table 1.1. H101, an E1B deleted adenovirus (in combination with chemotherapy) has been approved clinically in China for the treatment of late stage nasopharyngeal cancer.

1.2.2 Vesicular Stomatitis Virus

1.2.2.1 Virus particle and life cycle

Vesicular Stomatitis Virus (VSV) is a bullet-shaped, enveloped virus 100 nm in diameter and belongs to the *Vesiculovirus* genus, family *Rhabdoviridae*. VSV has a negative sense, single stranded 11.2 kb RNA genome which encodes only 5 genes. The VSV nucleocapid consists of the matrix protein (VSV-M), while the RNA genome is associated with the viral nucleocapsid protein (VSV-N) as well as phosphoprotein (VSV-P) and Large (VSV-L) which comprise the RNA-dependent RNA polymerase (61). The nucleocapsid particle is encompassed by the envelope, a cell-derived lipid membrane containing viral glycoprotein (VSV-G) that is acquired by the virus particle during the budding process. Briefly, the virus life cycle consists of VSV-G binding an unknown, ubiquitous cell surface receptor and viral endocytosis via clathrin-coated pits (62). pH dependent fusion of the viral envelope with the endosomal membrane is mediated by VSV-G (63). Transcription of the viral genome is performed by the viral polymerase and newly made genomes are encapsidated in VSV-N. The VSV-G is synthesized in the endoplasmic reticulum and processed by the Golgi before being shuttled to microdomains within the cell's plasma membrane where budding takes place (64).

Table 1.1 *Oncolytic viruses tested in clinical trials.* Partial list, adapted from Liu et al (65) and Liu et al (66).

Virus	*Genetic target in cancers*	*Genetic modification*	*Transgene*	*Latest phase trial*
Non-engineered viruses				
Newcastle disease virus	Unknown	none	none	II
Reovirus	Defects in PKR/interferon pathway	none	none	II
Mumps	Unknown	none	none	I-II
Engineered, non-armed viruses				
Adenovirus dl1520 (ONYX-015)	p53 pathway defects, late RNA transport defects	E1B-55K(-), E3B(-)	none	III
H101	p53 pathway defects, late RNA transport defects	E1B-55K(-), E3(-)	none	Approved in China
HSV1716	Defects in PKR/interferon pathways	ICP34.5(-)	none	III
Engineered, armed viruses				
Vaccinia JX-594	Cellular thymidine kinase expression E2F pathway driven	Thymidine Kinase (-)	GM-CSF	II
Adenovirus Ad5-CD/Tkrep	p53 pathway defects, late RNA transport defects	E1B-55K(-), E3B(-)	CD/TK (prodrug activation)	II
HSV Oncovex-GM-CSF	Defects in PKR/interferon pathways	ICP34.5(-), ICP47(-), Us11 upregulation	GM-CSF	II
Measles MV-CEA	Tumour-selective binding through CD46	None	CEA (serum monitoring)	I-II

1.2.2.2 Interferon-inducing VSV-M mutants as oncolytic virus candidates

Though VSV-M is a critical structural protein for the formation of the nucleocapsid particle and is also important for virus budding (67), VSV-M has also been shown to manipulate critical cellular processes, improving the ability of the virus to infect and spread. VSV-M binds the nuclear pore complex, inhibiting mRNA transport from the nucleus to the cytoplasm, thereby inhibiting translation of host mRNAs (68). Since VSV replication is restricted to the cytoplasm, viral mRNA can still be transcribed.

VSV is exquisitively sensitive to the antiviral effects of the host interferon response and virus replication is shut down upon production of type I interferons (IFN-α/β). A critical interferon stimulated gene (ISG) that inhibits VSV replication is the dsRNA dependent serine/threonine protein kinase PKR (69). An important substrate of PKR is the eukaryotic translation initiation factor eIF2α (70). Upon phosphorylation of eIF2α, translation of (viral) proteins is shut down, allowing for the host to respond to virus infection by mounting an innate (IFN) and subsequently an adaptive (neutralizing antibody) immune response (69). Wild-type (WT) VSV inhibits production of IFN by blocking mRNA export and therefore translation of the IFN transcripts. Three VSV mutants (AV1 (M51R), AV2 (V221F and S226R) and AV3 (MΔ51)) were generated whose VSV-M was not able to bind the nuclear pore complex and therefore were unable to block shuttling of mRNA (71). These mutants are termed interferon-inducing as the cells are able to respond to virus infection by production of interferon. Importantly, 80% human cancer cell lines of the NCI60 panel were not able to produce or respond to interferon allowing for interferon-inducing VSV mutants to continue to replicate and spread in these transformed cells although unable to do so in normal cells (71). Though WT VSV was also able to replicate in transformed cells, this virus caused neurotoxicity *in vivo* (71). It is thought that many cancer cells

possess defects in the interferon response as interferon stimulated genes (ISGs) are unfavourable for the transformation process. For instance, ISGs have been shown to promote apoptosis (72-74). Also, major histocompatibility complex I (MHCI) is an ISG that may present tumour associated antigens to the immune system, resulting in rejection of the transformed cell (75). Mutant VSV infection of normal cells results in interferon production and a rapid shut-down of virus replication. Thus, these VSV mutants have increased selectivity for tumours with increased therapeutic index (76).

Over the course of the last number of years, VSV is intensively being investigated as a possible oncolytic agent. VSV was capable of infecting a wide array of cancer cell lines derived from lung, ovary, prostate, colon, breast, brain and skin (71, 77). *In vivo* tumour eradication was demonstrated by a number of groups in diverse tumour models, including human xenografts as well as immune competent syngeneic models (78-81).

1.2.3 Vaccinia Virus

1.2.3.1 Virus particle and life cycle

Vaccinia virus is a member of the *Poxviridae* family, genus *Orthopoxviridae.* The approximately 191 kb genome encodes 263 proteins of 65 or more amino acids (82, 83). The large (350 nm diameter) viral particle consists of the core structure, containing the viral DNA as well as enzymes involved in viral mRNA expression which is coated by lipoprotein membranes (84). Vaccinia binds ubiquitous heparin sulfate and chondroitin sulfate on the cell surface (85, 86) and membrane fusion ensues to allow the viral core structure to gain access to the cytoplasm. Early viral gene expression is rapidly induced. Proteins encoded by early transcripts are involved in uncoating the DNA genome and are also transcription factors for the intermediate genes. The latter encode for late protein transactivators that trigger synthesis of structural proteins as well as

early transcription factors that are incorporated into newly formed virus particles (87). These particles, termed intracellular mature virions (IMV) can be released as infectious particles upon cell lysis. Alternatively, the IMV can acquire a membrane in the Golgi, subsequently fusing with the cell's plasma membrane and forming the cell-associated enveloped virus (CEV). Upon release of CEV, the extracellular enveloped virion (EEV) is generated. EEV and IMV are the infectious virus particles formed during vaccinia infection (88).

1.2.3.2 Development as an oncolytic

Vaccinia virus was initially administered to humans many years ago in the successful campaign to vaccinate against and eradicate smallpox, though its species of origin is unclear (89). Though smallpox was successfully eradicated in the 1970s, vaccinia virus is continuously being investigated as a vaccination platform (84), in gene therapy (90) as well as immunotherapy (91) and more recently, also as an oncolytic agent (92). Characteristics that make vaccinia virus an attractive oncolytic are *(1)* its rapid life cycle and efficient spread from cell to cell, *(2)* its ability to incorporate large amounts of foreign DNA (25 kb), *(3)* its strong promoters drive foreign gene expression at high levels and *(4)* its wide host range and ability to infect almost any cell (93).

Vaccinia viruses are naturally targeted to cancers. Upon intravenous infusion of virus, most viral particles are delivered to tumours followed by the ovary with significantly less delivery to other normal tissues (94). It is thought that virus is preferentially delivered to tumours as vaccinia requires leaky vasculature in order to enter tissues. Both tumours and ovaries have been shown to possess leaky vasculature, likely due to high expression of vascular VEGF in both sites (95). Furthermore, vaccinia viruses can be genetically engineered to preferentially replicate in tumours. One strategy has been to delete the viral thymidine kinase (TK) gene as cancer cells possess higher TK activity to ensure high nucleotide pools in order to support the high rate of cell

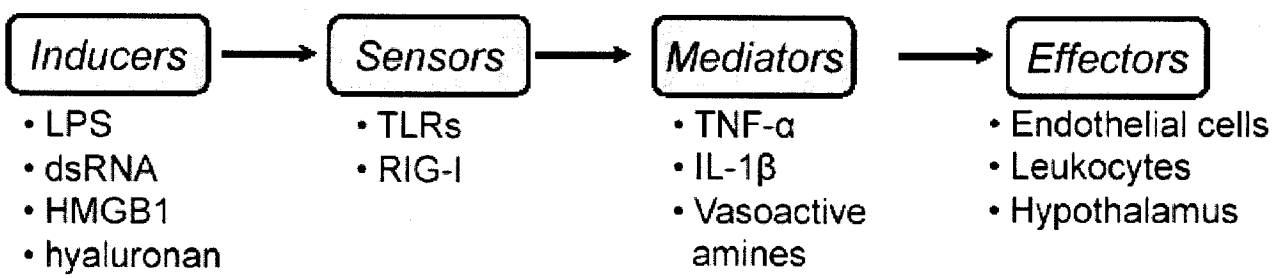
proliferation (96). Vaccinia virus with a deleted TK gene preferentially replicates in malignant cells (97). In addition, as viruses generally prefer to infect rapidly dividing cells, wild type vaccinia virus has the ability to trigger cell proliferation through production of vaccinia growth factor (VGF). VGF binds EGFR, a receptor commonly overexpressed or mutated in cancers, initiating Ras/MAPK/ERK signaling. As activation of this pathway is necessary for viral replication (98), TK–VGF double-deleted vaccinia virus demonstrates enhanced anti-tumour efficacy (due to increased tumour selectivity) while triggering less toxicity (99).

1.3 Inflammation

1.3.1 Introduction

Inflammation is a process that has evolved to return injured tissue back to homeostasis. The inflammatory response aims to stem the spread of infection, eradicate invading pathogens, remove dead tissue and aid in wound healing by coordinating delivery of leukocytes and blood components to the site of injury (100, 101). Cells of the inflammatory system include granulocytes (neutrophils, eosinophils and basophils), monocytes, macrophages and mast cells. The inflammatory process involves production of mediators of inflammation that induce vasodilation, increase vascular permeability, activate and recruit other immune cells and kill invading pathogens (reviewed in (102)). Medzhitov described the inflammatory 'pathway' that starts with *inducers* (exogenous or endogenous), which trigger activation of *sensors* which results in production of *mediators* that modulate *effectors* that respond to infection or tissue injury and return the tissue to homeostasis (103). See Figure 1.1 for overview.

Figure 1.1 *Overview of the inflammatory pathway.* Exogenous (e.g. LPS, dsRNA) or endogenous (HMGB1, hyaluronan) inducers trigger sensors on cells involved in inflammation. Pro-inflammatory mediators are then released and act on effectors to respond to injury, limit infection and ultimately restore tissue homeostasis. (Adapted from Medzhitov 2008 (103).


Inducers
• LPS
• dsRNA
• HMGB1
• hyaluronan
Sensors
• TLRs
• RIG-I
Mediators
• TNF-α
• IL-1β
• Vasoactive amines
Effectors
• Endothelial cells
• Leukocytes
• Hypothalamus

1.3.2 Inducers and sensors of inflammation

Inducers of inflammation are components of invading pathogens or aberrantly exposed endogenous tissue that signal distress (i.e. infection or injury). *Exogenous inducers* are pathogen associated molecular patterns (PAMPs) on bacteria or viruses, products exclusively produced by these invading organisms. Examples include lipopolysaccharide (LPS), components of the bacterial cell wall, or double stranded RNA (dsRNA), exclusively associated with some viral genomes or produced during the virus replication cycle. PAMPs are sensed by pattern recognition receptors (PRRs) expressed on the plasma membrane, endosomal membrane or in the cytoplasm. PRRs are therefore *sensors* of infection and include members of the Toll-like receptor (TLR) family as well as cytoplasmic retinoic acid-inducible gene I (RIG-I). Other inducers of inflammation associated with invading pathogens can be virulence factors. Typically the effect of virulence factors on tissue is detected e.g. the effect of pore-forming toxins produced by some bacteria are detected by the NALP3 (NACHT-, leucine-rich-repeat- and pyrin-domain-containing protein 3) inflammasome (sensor) (104).

Endogenous inducers have been less well characterized but are generally endogenous molecules exposed during tissue injury upon disruption of homeostasis. For example, HMGB1 (high-mobility group box 1 protein) is an endogenous inducer exposed during tissue injury. HMGB1 is recognized by RAGE (advanced glycation end-product-specific receptor) which cooperates with TLRs to induce inflammation (105). Also, components of the extracellular matrix can act as inducers of inflammation. Hyaluronate is normally present in long polymers. Upon damage to tissues, low-molecular-weight fragments are formed that are recognized by TLR4 (106).

1.3.2 Mediators and effectors of inflammation

Mediators of inflammation are released following activation of sensors and act to change the functionality of damaged tissue. Mediators may be present as precursors in plasma, or can be produced or released by leukocytes or other cells present in the affected tissue. Mediators of inflammation are divided in seven subgroups based on biochemical properties: vasoactive amines, vasoactive peptides, fragments of complement components, lipid mediators, cytokines, chemokines and proteolytic enzymes.

Early in the inflammatory response, chemical mediators that affect vasculature are released. *Vasoactive amines* include histamine and serotonin which are stored in granules within mast cells and platelets respectively, allowing for their rapid release at the site of injury. Vasoactive amines induce vasodilation which results in increased blood flow at the site of injury. Also, vasoactive amines increase vascular permeability allowing for the leakage of fluid and plasma proteins into the extravascular space. Increased fluid is meant to dilute bacterial toxins. *Vasoactive peptides*, e.g. the kinin and fibrinolytic systems are formed following proteolytic cleavage and also cause vasodilation and increased vascular permeability. Bradykinin, like vasoactive amines induce gap formation between endothelial cells to promote increased vascular permeability. The fibrinolytic system will be discussed in more detail later in the context of coagulation. However, fibrin degradation products can also induce vasodilation further promoting inflammatory influx. The *complement system* is a series of plasma and cell membrane proteins that can be activated by pathogen invasion and mediates vascular responses (e.g. by inducing release of histamine), recruits leukocytes and opsonizes invading pathogens which targets them for uptake and digestion by phagocytic cells as well as directly damaging target cells. *Lipid mediators* of inflammation include eicosanoids (prostaglandins and leukotrienes). These mediators are produced from arachidonic

acid by cyclooxygenase enzymes (COX-1 and COX-2) or lipoxygenases. Lipid mediators affect vascular tone and can recruit and activate inflammatory cells (107). Inflammatory *cytokines* including tumour necrosis factor (TNF), interleukin-1 (IL-1) and IL-6 are produced mainly by macrophages and mast cells. These cytokines have pleiotropic effects, increasing vascular permeability, activating local endothelium as well as infiltrating immune cells. Finally, *chemokines* or *chemo*attractant cyto*kines* bind chemokine receptors on immune cells and trigger their recruitment. Chemokines are divided into four groups (C, CC, CXC, CX_3C), based on the spacing between cysteines in the N-terminal region of the protein. Chemokines bind chemokine receptors, seven-transmembrane-domain G-coupled proteins whose nomenclature matches that of their respective ligands (e.g. CXCR). In some cases, multiple chemokines can bind one receptor, though generally ligands must belong to the same family. Chemokine receptors are expressed by different cells, though expression is most predominant on leukocytes. Indeed, e.g. macrophages and neutrophils will express different types of chemokine receptors in order to be able to respond to different chemokine stimuli during inflammation (108).

Effectors of inflammation represent the cells or tissues that mediators act upon to restore homeostasis. These can have local effects, e.g. formation of exudate at the site of infection or systemic effects, acting on the neuroendocrine system to regulate homeostasis in general.

It is difficult to briefly outline the inflammatory process as there exists much cross-talk and redundancy between mediators of the inflammatory cascade. The inflammatory response has likely evolved this way in order to be able to rapidly respond to a diverse array of insults and potential threats. Importantly, the pro-inflammatory reaction also triggers a negative feedback loop in which the inflammatory process is dampened to avoid systemic induction of inflammation (i.e. septic shock) as well as to promote wound healing (see below).

1.3.4 Cells of the inflammatory system

The main functions of different inflammatory cell populations will be outlined briefly. *Neutrophils* are the most abundant leukocytes circulating in blood and rapidly respond to infection and injury. A slower rate or complete stagnation of blood flow upon leakage of fluid and plasma proteins results in the margination of neutrophils (neutrophils migrate along the vessel wall). This allows for binding of neutrophils to adhesion molecules (e.g. E-selectin) upregulated on activated endothelial cells. Neutrophils extravasate, leaving the blood stream through gaps between endothelial cells and gaining access to extravascular space. Complement components and degradation products act as chemoattractants, forming a gradient along which neutrophils migrate to arrive at the site of infection. Neutrophils go on to phagocytose opsonized pathogens. A respiratory burst ensues in the phagosome, resulting in production of oxygen radicals that damage the ingested pathogen. Neutrophilic myeloperoxidase (MPO) converts ROS (e.g. hydrogen peroxide) to HOCl, a highly reactive molecule that is able to kill the ingested pathogen. Proteases (e.g. elastase) also damage the phagocytosed pathogen (e.g. by attacking the cell wall of bacteria) (109).

Eosinophils and *basophils* are rare granulocytes. Eosinophils possess similar mediators to neutrophils and are involved in the control of parasitic infection. This cell type is more long-lived than neutrophils, being able to persist for weeks in tissue. Basophils possess similar vasoactive agents as mast cells.

Circulating *monocytes* are also recruited to the site of infection. These cells are more long lived and circulate in smaller numbers than neutrophils yet expand significantly during infection. Monocytes extravasate into the affected tissue where they differentiate into macrophages. Tissue

resident *macrophages* are activated by bacterial or viral products as well as interferon-γ (IFN-γ). Macrophages are phagocytic cells that ingest bacteria but can also remove dead tissue at the site of injury. Activated macrophages produce large amounts of pro-inflammatory cytokines as well as chemokines that recruit other immune cells to the site of infection or tissue injury. Tissue resident macrophages are important in the promotion of inflammation resolution and wound healing. Macrophages cease to produce pro-inflammatory eicosanoids and secrete lipoxins that trigger the recruitment of monocytes, phagocytic cells that remove the cell debris (110). Monocytes also produce resolvins and protectins as well as transforming growth factor β (TGF-β), important mediators in inflammation resolution and tissue repair (110, 111).

1.3.3 Inflammation triggered by virus infection

Mammalian cells have numerous systems that sense invading bacteria or viruses. Upon recognition of virus infection, cells generally respond to virus infection by signaling neighbouring cells to activate their antiviral defenses while infected cells often undergo cell cycle arrest or apoptosis in order to prevent production of virus particles that would be able to infect other cells of the body. The innate response to virus infection also stimulates the adaptive immune response in an attempt to clear the virus from the host.

Viral RNA is detected by sensors, including TLRs and RIG-I (112, 113). Receptor ligation results in downstream signaling that triggers production of type I interferons and subsequent induction of ISGs that are involved in antiviral activities such as cell cycle arrest, apoptosis and immune stimulation. TLR signaling also results in the nuclear translocation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) transcription factor which upregulates the

transcription of genes involved in inflammation, including cytokines and chemokines that activate and recruit diverse immune cell populations (see Figure 1.2 for overview).

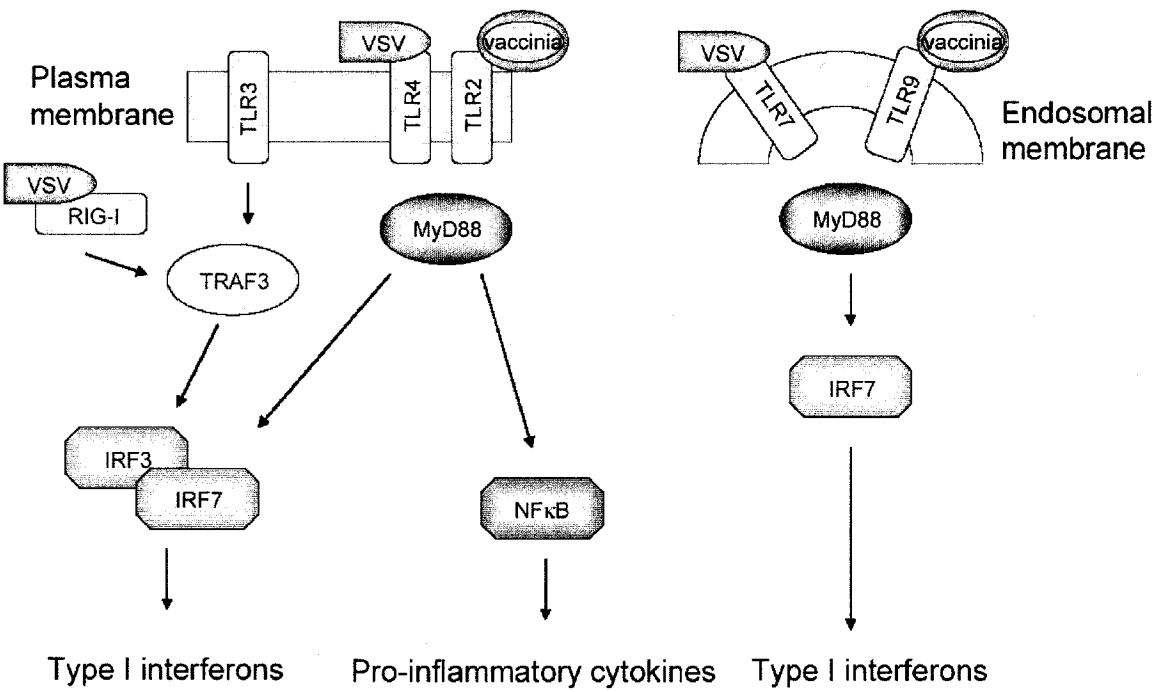
VSV single stranded RNA (ssRNA) is recognized in the endosomal compartment by TLR7. Both TLR7$^{-/-}$ mice as well as MyD88$^{-/-}$ mice (that lack a critical downstream TLR signaling molecule) possess a defective antiviral response following VSV challenge, in particular responding with decreased production of IFN-α (114, 115). VSV-G is recognized by TLR4 on the plasma membrane (116). Cytosolic RNA sensor RIG-I has also been shown to recognize VSV RNA though the involvement of RIG-I in the induction of interferon following VSV challenge has not yet been assessed (117).

Vaccinia virus is detected by TLR2 (118) and TLR9 (119) though both studies demonstrate that vaccinia virus infection is also recognized by a currently unknown TLR-independent pathway. Cytokine induction was dependent on TLR signaling, while IFN-β induction was TLR-independent (118). Interestingly, both TLR-dependent and –independent pathways were required to activate both innate and adaptive immunity *in vivo*. Vaccinia virus is a much larger, more complex virus and like other poxviruses, vaccinia has acquired numerous mechanisms to inhibit function of various players of the inflammatory cascade. Vaccinia virus encodes proteins that inhibit TLR signaling (e.g. A52R) (120) as well as bind and inhibit cytokines (e.g. B8R) (121), chemokines (122) or complement (123) (reviewed in (124-126)).

1.3.4 Acute versus chronic inflammation

The *acute inflammatory response* has evolved as a rapid response to infection, limiting damage to a restricted area within the host and subsequently triggering tissue repair. When dysregulated, systemic induction of the inflammatory response may result in septic shock. This represents the

Figure 1.2 *Overview of innate immune recognition of vesicular stomatitis virus and vaccinia virus.* Simplified diagram illustrating key receptors and adaptor molecules that sense and respond to vesicular stomatitis virus (VSV) and vaccinia virus infection. Recognition of virus infection leads to downstream production of type I interferons and pro-inflammatory cytokines. (Adapted from Kawai and Akira 2006 (113) and Trichnieri and Sher 2007 (112)).


Plasma membrane
TLR3
VSV
TLR4
vaccinia
TLR2
VSV
RIG-I
TRAF3
MyD88
IRF3
IRF7
NFκB
Type I interferons
Pro-inflammatory cytokines
VSV
TLR7
vaccinia
TLR9
Endosomal membrane
MyD88
IRF7
Type I interferons

pathological counterpart of the physiological role of inflammation. Though the acute inflammatory response to infection has been characterized in detail, the *chronic inflammatory state* has not been as well characterized. Chronic inflammation constitutes a pathological state characterized by a prolonged, dysregulated, maladaptive inflammatory reaction and is beginning to emerge as a major pathological factor in a wide range of diseases such as cancer (discussed in next section), arthritis, autoimmune diseases as well as type 2 diabetes and cardiovascular diseases. Medzhitov has recently proposed that parainflammation is a state associated with stressed or malfunctioning cells in which tissue associated macrophages help a tissue adapt to the noxious conditions in order to restore tissue function. Medzhitov suggests that parainflammation represents the continuum between homeostasis and acute inflammation and, when dyrsegulated, might be responsible for chronic inflammatory states associated with human diseases such as type 2 diabetes and atherosclerosis (103).

1.3.5 Inflammation in cancer

1.3.5.1 Epidemiological evidence

Cancer growth and progression was initially thought to be dependent on the ability of cancer cells to divide and invade normal tissues. However, over the past decade it has become apparent that untransformed cells associated with tumours often play a critical role in tumour progression. In particular, chronic presence of inflammatory cells in tumours is now known to promote tumour progression (127) and inflammation has been linked to up to 15-20% of all cancer deaths (128). Chronic administration of drugs that dampen inflammation such as non-steroidal anti-inflammatory drugs (NSAIDs) reduce the risk of developing certain cancers and decrease mortality that results from these diseases (129-131). Inflammatory diseases such as inflammatory bowel disease increase the risk of developing (colon) cancer (132). Chronic viral

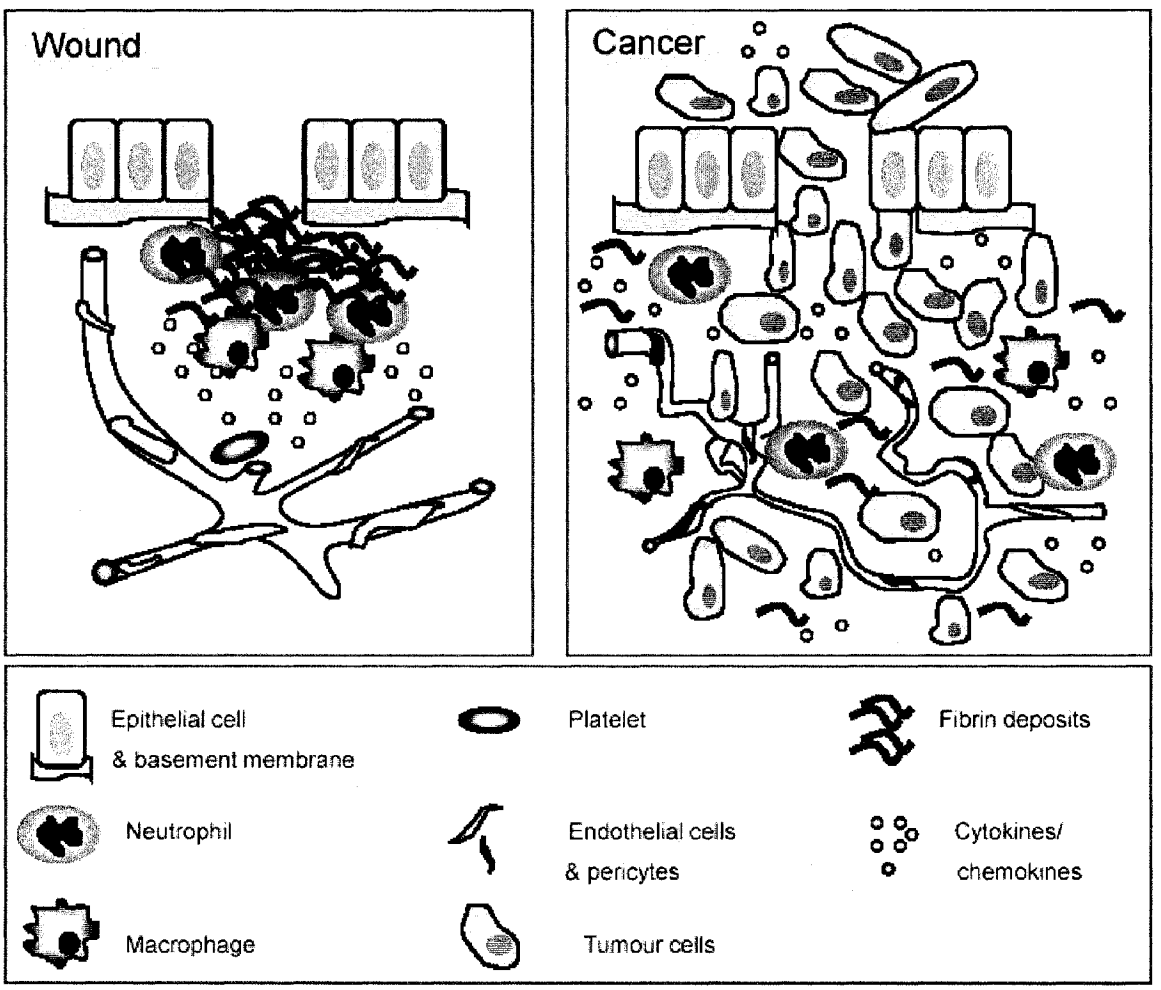
infections such as human papilloma virus (HPV) infection of the cervix and hepatitis viruses of the liver have been implicated in the onset of cervical and liver cancers respectively (133). Interestingly, hepatitis B virus vaccination programs resulted in decreased incidence of hepatocellular carcinoma in children (134).

1.3.5.2 Mechanisms of tumour promotion

Cancer has been described as 'wounds that do not heal'. Indeed, many cancer types are associated with smouldering inflammation that is required for enhanced tumour growth or tumour progression (e.g. by promoting angiogenesis and metastasis). See Figure 1.3 for comparison of inflammation in the context of a wound versus cancer.

Several extensive reviews have focused on mechanisms of tumour promotion by a chronic inflammatory reaction (135-139), an overview of which will be presented here. During chronic inflammation free radicals (e.g. ROS) are generated that can react with DNA (140). This introduces mutations that may result in *enhanced tumour growth.* Furthermore, upregulated COX-2 in tumour-associated immune cells is thought to increase tumour cell survival through mechanisms that have mainly been unidentified but may involve prostaglandin E2 receptor EP2 subtype (141). Also, mice deficient in TNF were resistant to skin carcinogenesis (142). Furthermore, in mice prone to developing hepatocellular carcinoma, NFκB (a transcription factor known as the 'master switch' of inflammation) enhanced survival of transformed cells and promoted conversion to malignancy (132). MyD88, a critical signaling adapter molecule of most TLRs has also been shown to have a pro-tumour role in certain circumstances. Mice lacking this adaptor protein were less prone to developing intestinal and liver cancers (143, 144).

Figure 1.3 *Comparison of inflammation in wound healing and cancer.* (a) Upon disruption of the endothelium and basement membrane (wound formation), blood components are first exposed to the subendothelial matrix. Platelets rapidly adhere and spread along the damaged surface. Fibrin deposits form to prevent loss of blood. Neutrophils and monocytes/macrophages are recruited to the damaged site by chemokines and are activated by cytokines. These cells prevent the spread of tissue damage and ultimately promote wound healing. Vessels are formed in a hierarchical structure and are composed of endothelial cells and covered by pericytes. (b) Cancers are less organized, with an unclear border between the epithelial layer and the basal mesenchymal layer. Cytokines and chemokines are aberrantly expressed by tumour cells and result in constant recruitment and activation of neutrophils and monocytes/macrophages. Infiltrating inflammatory cells promote tumour growth and stimulate angiogenesis within the tumour microenvironment. Rapidly proliferating endothelial cells form aberrantly shaped vessels that are not adequately covered by pericytes. The tumour microenvironment is associated with increased fibrin deposition. (Adapted from Coussens and Werb 2002 (136)).


Wound
Cancer
Epithelial cell
& basement membrane
Platelet
Fibrin deposits
Neutrophil
Endothelial cells
& pericytes
Cytokines/
chemokines
Macrophage
Tumour cells

More commonly, tumours depend on the inflammatory response to mature and thrive. As tumour cells continue to divide, hypoxic areas begin to form in the core of the tumour mass as oxygen is only able to diffuse though 1mm of tissue (145). Hypoxic cells produce stress signals that recruit bone-marrow derived myeloid cells, cells that differentiate into tumour associated macrophages (TAMs) (146). TAMs produce and secrete growth factors, cytokines, chemokines and tissue remodeling enzymes to support *angiogenesis* (147-149). In particular, MMPs degrade extracellular matrix, allowing for the diffusion of endothelial activators such as VEGF or basic fibroblast growth factor (bFGF) (150). These growth factors promote endothelial cell proliferation and migration towards hypoxic areas of tumours, which will lead to vessel formation and perfusion of hypoxic regions (151, 152). The angiogenic process is critical for continued tumour growth.

Inflammatory cells and inflammatory mediators have also been shown to be critical for *metastasis*, the spread of tumour cells from the primary site to distant sites within the body. Mice deficient in macrophages were not able to generate lung metastases in a breast cancer model (153), suggesting that TAMs act as "obligate partners of tumour-cell migration, invasion and metastasis" (154). Malignant cells express chemokine receptors that affect cell motility, invasiveness and survival (155). Cytokines such as TNF, IL-1β and IL-6 can increase the capability of malignant cells to invade tissues (135), likely through the induction of chemokine receptors (e.g. CXCR4) on cancer cells (156).

1.4 Coagulation

1.4.1 Hemostasis and thrombosis

The hemostatic system maintains fluid blood flow during physiologic conditions but is able to react rapidly to prevent blood loss in the case of injury. The mediators of hemostasis and thrombosis are endothelial cells (long, elongated cells that form the vessel wall), platelets (small circulating blood cells) and the coagulation system (a cascade of plasma proteins involved in generation of blood clots). The fibrinolytic system is activated during blood clot formation to stem the spread of the clots as well as dissolve blood clots and aid in wound healing (157).

Endothelial cells form a barrier between hemostatic blood components on the luminal surface and reactive substances on the basal surface (e.g. adhesive extracellular matrix components such as laminin and fibronectin). Beyond forming a physical barrier, endothelial cells maintain blood fluidity by producing inhibitors of platelet aggregation (prostacyclin) and coagulation (thrombomodulin) and modulators of fibrinolysis such as tissue plasminogen activator, urokinase and plasminogen activator inhibitor (to be discussed later). The interaction between endothelial cells also dictates vascular permeability and mediators such as nitric oxide produced by endothelial cells modulate vascular tone (vasodilation versus vasoconstriction) (158).

Platelets are produced from megakaryocytes which are large, multi-nucleated cells in the bone marrow (159). They adhere to exposed basement membrane upon injury to endothelium, spread along the injured surface, aggregate and form a plug (160). The plasma membrane of clotted platelets also becomes a surface for the activation of the clotting system. Subsequent fibrin deposition is critical in forming a stable blood clot. Platelets also produce mediators that manipulate vascular function and hemostasis, e.g. clotting factors, histamine, extracellular matrix components and growth factors (e.g. VEGF, FGF). These molecules are stored in granules and

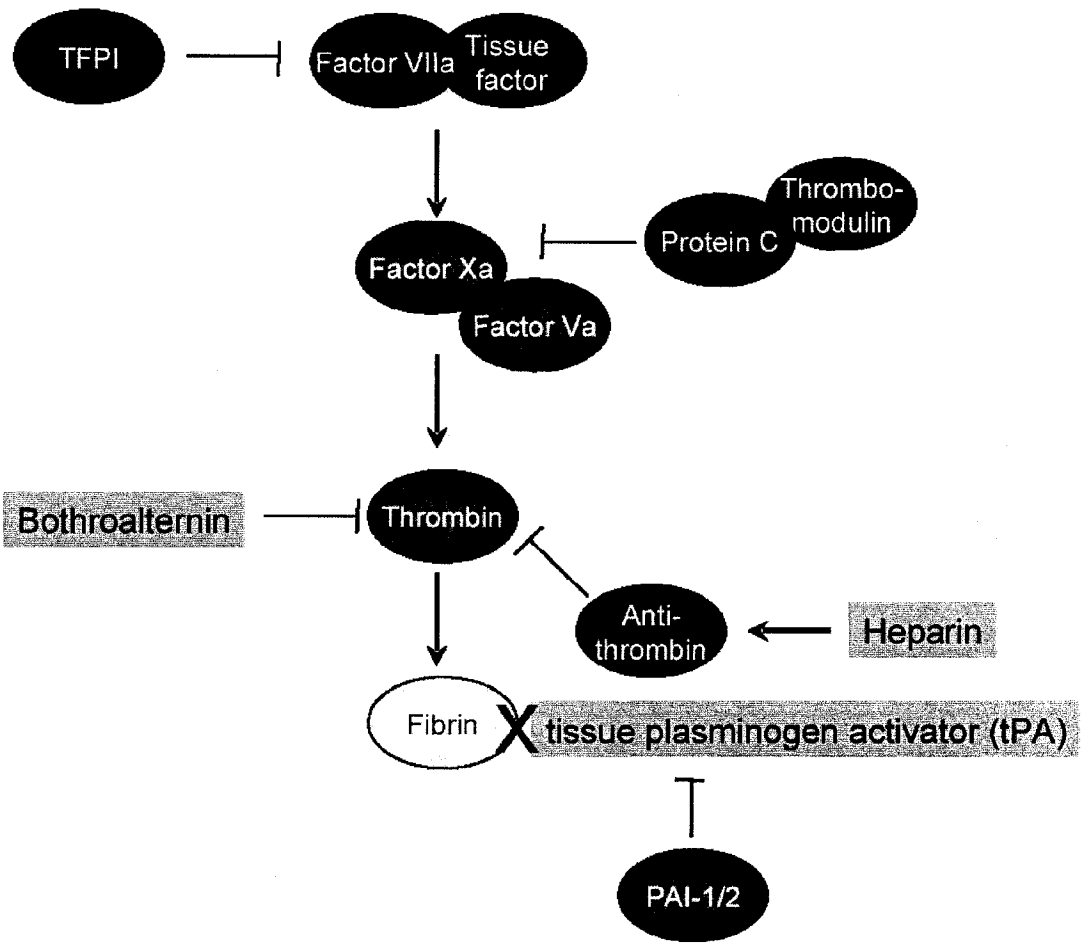
can be quickly released upon platelet activation, allowing platelets to rapidly respond to endothelial damage, initiate clot formation and ultimately be involved in clot resolution and wound healing (161).

The *coagulation system* consists of two pathways, the intrinsic and extrinsic coagulation cascade that converge to trigger blood clot formation. The extrinsic cascade will be briefly described here and is outlined in Figure 1.4. The primary inducer of the coagulation cascade is tissue factor (TF), a transmembrane glycoprotein induced on activated endothelial cells and monocytes. Exposure of TF to blood components triggers a series of reactions in which zymogen (enzyme in its inactive form) serine proteases are activated and go on to activate downstream mediators in the cascade. Coagulation factors are numbered with Roman numerals and the suffix 'a' indicates the factor has been activated. TF forms a complex with Factor VIIa, present in small amounts in circulating blood (162, 163). TF-VIIa then goes on to activate factor X. Xa, along with cofactor Va, converts prothrombin to thrombin (164). Thrombin then cleaves fibrinogen into fibrin monomers that polymerize and form the fibrin clot (165).

1.4.1.4 Inhibition of coagulation and the fibrinolytic system

Clotting must be tightly regulated in order to limit the size of the initial clot and avoid systemic spreading of blood clot formation. Therefore, activation of clotting factors simultaneously activates a negative feedback loop *(inhibition of coagulation).* Activated thrombin is bound by thrombomodulin on endothelial cells which then inactivates factors V and VIII through the action of protein C and protein S. Tissue factor pathway inhibitor (TFPI) binds factor Xa and inhibits the TF-factor VIIa complex. Soluble activated coagulation factors such as thrombin or factor Xa are quickly inactivated by the action of antithrombin III (165). Antithrombin III, the endogenous inhibitor of coagulation is activated by heparin, a blood thinner commonly used

Figure 1.4 *The coagulation cascade, inhibition of coagulation & fibrinolysis.* Tissue factor (TF) is upregulated on endothelial cells and monocytes and activates coagulation factor VII (resulting in its conversion to factor VIIa). Factor VIIa converts factor X to Xa which activates thrombin. Thrombin activation results in the generation of fibrin, which forms the clot. Clot formation is tightly controlled. Thrombomodulin is present on endothelial cells and inhibits factor Va through the action of protein C and protein S. TFPI binds factor Xa and inhibits TF-factor VIIa complex. Soluble activated clotting factors are rapidly inhibited by antithrombin, which is activated by heparin. Bothroalternin is a direct thrombin inhibitor isolated from snake venom. Fibrin clots are disrupted through the action of 'clot-busting enzymes' such as tissue plasminogen activator (tPA). tPA is inhibited by inhibitors of fibrinolysis such as PAI-1 and -2. TFPI: tissue factor pathway inhibitor. PAI-1: Plasminogen activator inhibitor 1. (Adapted from Mackman 2008 (166))


TFPI
Factor VIIa
Tissue factor
Thrombo-modulin
Protein C
Factor Xa
Factor Va
Bothroalternin
Thrombin
Anti-thrombin
Heparin
Fibrin
tissue plasminogen activator (tPA)
PAI-1/2

clinically (167). *Fibrinolytic mediators* are also circulating in blood as zymogens. Plasminogen is converted to its active form plasmin by plasminogen activators (e.g. tissue plasminogen activator (tPA), urokinase type plasminogen activator (uPA)) produced by activated endothelial cells. Initially, endothelial cells and platelets associated with plugs produce higher levels of plasminogen activator inhibitors (PAI-1 or -2) to favour blood clot formation. However, in a well timed sequence, tPA and uPA are produced in sufficient quantities to overcome inhibition by PAI and mediate clot dissolution (165).

1.4.2 Links between inflammation and coagulation

Pro-inflammatory cytokines manipulate expression and function of proteins involved in the coagulation cascade as well as critical inhibitors of clotting. Indeed, pro-inflammatory mediators have been shown to be critical in pathology due to microthrombus formation (168). TNF-α or IL-1β produced by activated monocytes trigger TF production on endothelial cells leading to activation of the extrinsic coagulation cascade (169). Activation of endothelial cells then results in the adhesion of monocytes and cytokines also induce TF on the monocyte cell surface (170). Conversely, anti-inflammatory cytokines such as TGF-β and IL-10 have been shown to decrease TF expression on endothelial cells and monocytes (171, 172). Pro-inflammatory cytokines also downregulate various inhibitors of coagulation. Cytokines can manipulate the thrombomodulin-protein C-protein S pathway. For instance, TNF-α was shown to decrease levels of thrombomodulin by inhibiting its transcription (173) and by increasing its rate of internalization (174). Pro-inflammatory cytokines may further skew towards thrombosis by inhibiting fibrinolysis. Cytokines such as TNF-α have been shown to induce inhibitors of fibrinolysis (e.g. PAI-1) *in vitro* (175-177). *In vivo*, pro-inflammatory cytokines have been shown to first induce mediators of fibrinolysis (e.g. tPA and uPA) but subsequently rapidly respond by inducing inhibitors of

fibrinolysis thereby decreasing plasminogen activator activity in plasma (178). Inflammatory mediators can also induce endothelial cell damage, exposing the procoagulant subendothelial matrix (179). An overview of the links between inflammation and coagulation is shown in Figure 1.5.

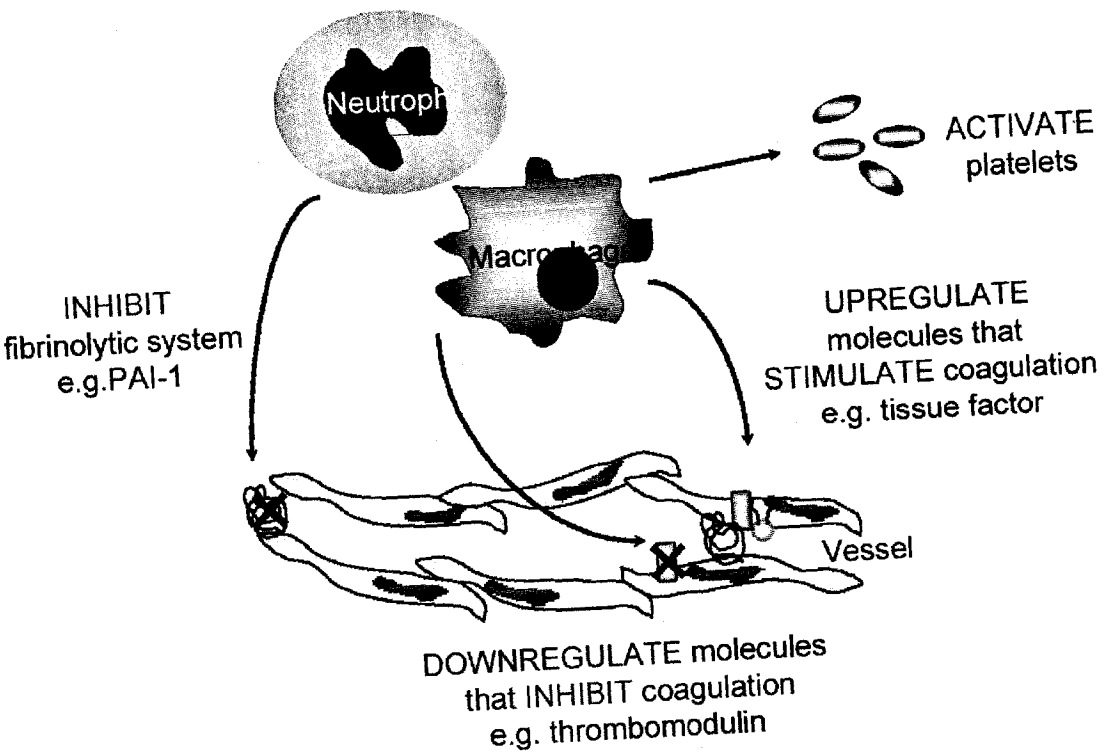
1.4.3 Coagulation in cancer

1.4.3.1 The hypercoagulable state in cancer

The relationship between increased disseminated thrombosis and cancer was first recognized by Trousseau in 1865 (180). Indeed, venous thromboembolisms carry with them an increased risk of cancer diagnosis and roughly 1 in 2 patients with cancer (up to 90% of patients with metastases) present with abnormal coagulation characteristics (181-183). The increased risk of thrombosis during malignancy is attributable to the fact that many tumour cells produce procoagulant factors that skew the hemostatic balance towards coagulation and also produce factors that activate normal host cells to promote clotting (184). Some of these factors are briefly discussed here.

Malignant cells express high levels of procoagulant molecules such as TF and cancer procoagulant (thought to be a cysteine protease produced by cancer cells that directly activates factor X, independently of factor VII). Many cancer cells produce products that degrade anti-coagulant molecules such as antithombin and protein C and S. Malignant cells produce tPA, uPA as well as PAI-1 and -2, thereby disrupting the balance in the fibrinolytic system. As discussed previously, cancers are often associated with a chronic inflammatory response. High expression of pro-inflammatory cytokines (e.g. TNF-α and IL-1β) activates the tumour endothelium (generating a procoagulant surface) and results in recruitment and activation of inflammatory cells that are known to promote coagulation (reviewed in (185)). Cytokine production by tumours also results

Figure 1.5 *Links between inflammation and coagulation.*


Neutroph
ACTIVATE
platelets
INHIBIT
fibrinolytic system
e.g.PAI-1
UPREGULATE
molecules that
STIMULATE coagulation
e.g. tissue factor
Vessel
DOWNREGULATE molecules
that INHIBIT coagulation
e.g. thrombomodulin

in increased production (e.g. by IL-6) and activation (by production of ADP or cathepsin like cysteine protease) of platelets.

The tumour microenvironment itself is also conducive to blood clot formation. Vessels associated with the tumour are aberrantly shaped and do not have a defined hierarchy. Tumour vasculature is defined by different properties and structure than vasculature associated with normal tissue. Tumour vessels possess a larger vessel diameter than normal vessels (186). They also display an irregular branching pattern, lacking the regular vessel organization of arterioles, capillaries and venules (187). Endothelial cells are rapidly proliferating and are abnormally shaped, resulting in the formation of gaps between cells and vessel leakiness (188). Endothelial cells are often not associated with pericytes and basement membrane is structurally abnormal (189). These features result in vasculature that is highly permeable to macromolecules, increasing interstitial fluid pressure within tumours (190, 191). These vessels with irregular shape and structure are prone to blood clot formation (192). Certain types of cancer therapies may further promote blood clot formation by inducing procoagulant molecules while decreasing anticoagulant molecules (193-195). Furthermore, cell lysis due to administration of cytotoxic drugs or radiation therapy can release procoagulants and cytokines that further promote blood clot formation (196, 197).

1.4.3.2 Coagulation factors in angiogenesis and metastasis

Clotting factors, in particular TF and thrombin have been shown to be important for the process of angiogenesis (198). In addition to VEGF expression (known to be a critical mediator of angiogenesis), TF expression also correlates with microvascular density in a variety of tumours (199-202). TF has been shown to increase VEGF expression via coagulation dependent and -independent mechanisms (184). As previously mentioned, blood products, including fibrinogen, leak into the extravascular space of tumours. Fibrinogen is then converted to fibrin as clotting

factors are abundant in tumours. These fibrin deposits form a network on which endothelial cells as well as tumour cells can migrate (192). Migrating endothelial cells have been shown to bind fibrin (203) and fibrin is thought to inhibit endothelial cell apoptosis by stabilization of integrin mRNA (204).

Platelets and clotting factors are important for metastasis (205). Depleting platelets or inhibiting coagulation by TF inhibition or administration of warfarin results in decreased formation of metastasis in various animal models (206-209). Platelets are thought to protect circulating tumour cells from host defense mechanisms and are also thought to help tumour cells attach to downstream endothelium and gain access to distant tissues by disrupting the endothelial cell integrity and increasing vessel permeabilization (210-213).

RATIONALE

Oncolytic viruses (OVs) are promising novel anticancer therapeutics. Based on *in vitro* data, different types of OVs are able to specifically infect and replicate in a variety of cancer cell types. However, dynamics between OVs and tumour cells are drastically altered *in vivo*. Tumours are associated with inflammatory cells as these cells have been shown to enhance tumour growth when chronically activated within the malignancy. Tumour perfusion is not reliable due to erratic and tortuous tumour vasculature that is more prone to coagulation. In order to successfully translate this promising novel class of cancer therapeutic to the clinic, the interaction of oncolytic virus infection in tumours and the acute host response to virus infection within tumours must be understood. Indeed, oncolytic virus infection has the potential to trigger an inflammatory

response that affects intravascular coagulation within tumours. This reaction may affect therapeutic dosing and ultimately the therapeutic outcome of oncolytic virus therapy.

HYPOTHESIS

Oncolytic virus infection of tumours triggers acute inflammation that affects the tumour microenvironment by altering blood flow.

AIMS

1.) Study the effect of oncolytic virus infection of tumours on the tumour microenvironment by assessing tumour cell apoptosis and proliferation, tumour blood flow and vessel distribution

2.) Identify genes involved in the host response to oncolytic virus infection *in vivo*

3.) Determine the role of neutrophils in the response to oncolytic virus infection

4.) Determine the effect of the coagulation cascade in the response to oncolytic virus infection

CHAPTER 2: TARGETED INFLAMMATION DURING ONCOLYTIC VIRUS THERAPY SEVERELY COMPROMISES TUMOUR BLOOD FLOW

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Running title: Viral Therapy Compromises Tumour Blood Flow

Keywords: oncolytic virus, vesicular stomatitis virus, vaccinia virus, chemokines, inflammation, neutrophils, blood flow

Contribution of Authors: The manuscript was written by CJ Breitbach, aided by JC Bell. All figures were generated by CJ Breitbach, except for Fig 1 and Supplementary Figures 3 and 4. Figure 1 was prepared by a previous Master's student of JC Bell, JM Paterson with the help of her summer student, A McGuire. CG Lemay validated microarray results as presented in Supplementary Figure 3. TJ Falls performed blood neutrophil counts presented in Supplementary Figure 4. M Daneshmand provided help with analysis of histological tumour sections. Kelly Speth and David Kirn contributed personal communication indicating clinical relevance to results presented in this paper. JA McCart provided vaccinia virus strain used in this manuscript. DF Stojdl provided In Vivo Imaging System used to generate Figure 6. KA Parato, H Atkins and JC Bell provided continued guidance over the course of this work.

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Abstract

Oncolytic viruses (OVs) are selected or designed to eliminate malignancies by direct infection and lysis of cancer cells. In contrast to this concept of direct tumour lysis by viral infection, we observed that a significant portion of the *in vivo* tumour killing activity of two OVs, vesicular stomatitis virus (VSV) and vaccinia virus is caused by indirect killing of *uninfected* tumour cells. Shortly after administering the oncolytic virus we observed limited virus infection, coincident with a loss of blood flow to the interior of the tumour that correlated with induction of apoptosis in tumour cells. Transcript profiling of tumours showed that virus infection resulted in a dramatic transcriptional activation of pro-inflammatory genes including the neutrophil chemoattractants CXCL1 and CXCL5. Immunohistochemical examination of infected tumours revealed infiltration by neutrophils correlating with chemokine induction. Depletion of neutrophils in animals prior to VSV administration eliminated uninfected tumour cell apoptosis and permitted more extensive replication and spreading of the virus throughout the tumour. Taken all together, these results indicate that targeted recruitment of neutrophils to infected tumour beds enhances the killing of malignant cells. We propose that activation of inflammatory cells can be used for enhancing the effectiveness of oncolytic virus therapeutics, and that this approach should influence the planning of therapeutic doses.

Introduction

Oncolytic viruses (OVs) have been engineered or selected to exploit genetic defects in tumour cells.[1–4] Unlike conventional gene therapy which involves using viral vectors for targeted gene delivery to diseased tissues, OVs are fully replication-competent and are expected to actively spread through cancerous tissue, killing tumour cells in their path by direct lysis. Indeed, *in vitro* models have clearly demonstrated that this occurs, and that therapeutic windows of greater than 1,000-fold between normal cells and tumour cells are common.[5,6] *In vivo*, the situation is far more complex, because there are many barriers to virus delivery and replication that can compromise therapeutic efficacy. Often, much of a systemically administered viral therapeutic is scavenged by normal cells of the liver,[7] or neutralized in the blood by antibodies[8] or complement[9,10] or through non-productive binding to white blood cells.[11] The effective dose of an OV that reaches a tumour is significantly less than the administered dose and multiplicities of infection *in vivo* are much lower than commonly used *in vitro*.[12] Furthermore, the replication and spreading of the virus within tumours is likely to be compromised by the restrictive properties of solid tumour architecture and micro-environment (*e.g.*, extracellular matrix, hypoxia, high interstitial tumour pressure, and low pH).[13,14] The first few days of OV infection and amplification within the tumour are of critical importance, considering that both innate and adaptive immune systems eventually curtail the spreading of the virus. Indeed, despite the excellent *in vitro* activity of a variety of OVs, systemic delivery in early phase I human trials has resulted in very limited anti-tumour activity.[15] We set out to investigate some of the factors that could limit or enhance OV therapy *in vivo*, with an eye to identifying key areas of possible intervention for improving therapeutic outcomes. Using two different OVs (vesicular stomatitis virus (VSV) and vaccinia virus) and a combination of imaging approaches, we found that only a small amount of systemically delivered virus reaches tumour

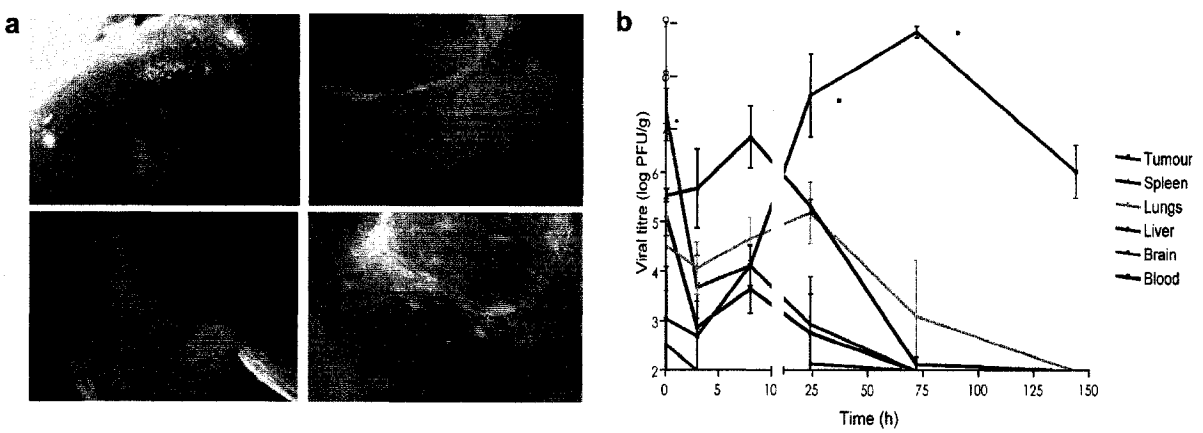
sites. Despite poor virus delivery to the tumours, we found that a significant portion of cells within OV-treated tumours were triggered to undergo apoptosis. Importantly, virus infection of a small proportion of tumour cells resulted in indirect killing of uninfected tumour cells. We discovered that viral infection triggered a loss of blood flow to the interior of the tumour, causing induction of massive cellular apoptosis in tumour cells while leaving normal tissues unaffected, and that the absence of vascular perfusion within infected tumours was induced by the recruitment of neutrophils to the tumour bed. The depletion of neutrophils from animals prior to OV administration eliminated apoptosis of uninfected tumour cells and permitted a more extensive replication and spreading of the virus throughout the tumour. Our results reveal a previously unappreciated and unexpected interplay between OVs, tumours, and inflammatory response.

Results

Extensive replication of VSV in subcutaneous tumours despite poor virus delivery

In order to gain a better understanding of the mechanisms of action of the therapeutic viral agents, we used a combination of imaging strategies to assess viral replication *in vivo*. Initially, BALB/c mice with subcutaneous CT-26 colon cancer tumours were intravenously infected with a recombinant version of VSV that expresses green fluorescent protein (GFP). Fluorescence microscopy of tumours excised 24 hours after infection showed limited VSV infection of CT-26 cells (Figure 2.1a), thereby suggesting that only a small amount of the virus had leaked out of the tortuous network of blood vessels at the rim of the tumour.

Figure 2.1. *Systemically administered VSV is poorly delivered to subcutaneous tumour but is rapidly, extensively and selectively amplified in tumour cells surrounding tumour neovasculature.* (a) CT-26 subcutaneous tumour bearing BALB/c mice were treated intravenously with VSV expressing GFP and euthanized 24 h later. Dissecting fluorescence microscopy was used to perform in situ imaging of tumour. Overlay of bright field and green fluorescence. (b) CT-26 subcutaneous tumour bearing BALB/c mice were intravenously infected with VSV. Pairs of mice were sacrificed and perfused with PBS at timepoints between 5 minutes, and 144 hours. Organs were frozen and virus titers were subsequently determined by plaque assay. Data are represented as a mean +/- standard error. (c) CT-26 tumour bearing BALB/c mice were treated with VSV expressing GFP intravenously and euthanized 48 h later. Sections were stained for apoptosis by TUNEL assay and VSV localization is detected by immunofluorescence. Hoechst stain and overlay are also shown (20x).


a
b
Viral titre (log PFU/g)
Time (h)
Tumour
Spleen
Lungs
Liver
Brain
Blood
c
Hoechst
VSV
TUNEL
Overlay

Given that large amounts of systemically administered OV resulted in only limited infection of tumours, we investigated to what extent systemically administered oncolytic virus reached the tumour bed, and also studied the kinetics of virus replication in normal and tumour tissues. Mice with subcutaneous CT-26 tumours were treated intravenously with 10^9 plaque forming units of VSV (AV1) and pairs of mice were sacrificed at time points between 5 minutes and 6 days. Homogenized tissues were titered to quantify virus delivery and replication within various organs. Immediately following virus administration, the highest virus titer was found in the liver (7.2 ± 0.5 $\log_{10}$ plaque forming units/g), followed by the spleen (5.5 ± 0.13), whereas virus titer in the tumour was much lower (3.0 ± 1.1). Strikingly, at 24 hours after injection, titers in the tumours had increased by over 4 logs, while liver titers had decreased by the same amount (Figure 2.1b). This suggests that the attenuated form of VSV is able to productively infect only the tumour, while much of the injected virus is rapidly cleared by the liver (likely due to mechanical deposition as well as uptake in Kupffer cells[16,17]). This observation is consistent with the results of our imaging study that showed that only a small amount of virus is delivered to tumours, and that it replicates at the tumour rim.

Replication of VSV in the tumour rim induces extensive apoptosis in the uninfected tumour core

The finding relating to poor OV delivery and replication within tumours was in sharp contrast to our original expectations, given that CT-26 cells are exquisitely vulnerable to VSV *in vitro*[5] and that VSV is very effective therapeutically against localized and disseminated CT-26 tumours.[5] We therefore re-confirmed the virus distribution as seen by GFP imaging, by carrying out immunofluorescence analysis of thin sections of tumours, using antibodies directed against VSV proteins. In addition, we detected terminal deoxynucleotidyl transferase-mediated dUTP nick

end label (TUNEL) staining, a marker of apoptosis, as we would expect to see significant cell killing in CT-26 tumours that respond to OV therapy. As suggested by the experiments described earlier, infection was, in general, sparse and confined to the outer edge of the tumour (Figure 2.1c). In contrast, we found extensive apoptosis throughout much of the tumour, even in areas not infected with virus (Figure 2.1c). This observation was confirmed by immunohistochemical detection of VSV and active caspase 3, and the results demonstrated poor virus distribution with extensive apoptosis of uninfected cells at both 24 and 48 hours after infusion (Figure 2.2a). These results suggest that a major portion of the *in vivo* tumour killing activity of VSV is not by direct viral infection but, rather, by an apoptotic signal associated with neighboring virus infection. Importantly, active replicating virus was found to be a pre-requisite for the induction of apoptosis in non-infected tumour cells, as verified by the fact that this phenomenon was not observed following the administration of ultraviolet (UV)-inactivated virus (Figure 2.2b). In addition, this indirect apoptotic effect is confined to tumour cells and was not detected in adjacent normal tissue (Figure2.2a).

OV infection triggers a loss of tumour perfusion in different models of tumour and virus

We reasoned that the massive cell death resulting from a very restricted viral infection could be caused by an acute loss of blood perfusion within the tumour. In order to visualize perfusion, 100 nm fluorescent beads (microspheres), were injected intravenously into VSV-treated mice with CT-26 tumours. The mice were then sacrificed after 5 minutes. Microspheres remain in the vascular system and are distributed throughout perfused regions of the normal organs and the tumour[18] and can be visualized microscopically. We confirmed the presence of microspheres within blood vessels by co-injecting Hoechst dye #33242.[19,20] Indeed, Hoechst-stained endothelial cells are found surrounding the luminally contained fluorescent beads (Figure 2.3a). In tumours

Figure 2.2 *VSV infection triggers uninfected tumour cell killing and loss of tumour perfusion in subcutaneous CT-26 tumours.* (a) BALB/c mice bearing CT-26 tumours were treated intravenously with VSV expressing GFP and sacrificed at the indicated timepoints. Representative scans of serial sections of tumours treated as indicated and stained by immunohistochemistry for VSV and active caspase 3 to visualize areas of virus infection and apoptosis respectively. Prior to sacrifice, mice were perfused with fluorescent microspheres for 5 minutes. Tumour sections were prepared and analyzed for fluorescence using a microarray scanner. Tumours excised from VSV treated mice show no fluorescence in the tumour core (large light areas), indicating that tumour tissue in VSV treated mice is not perfused ($\overline{V}$). In contrast, adjacent normal tissue remains well perfused (+). 10 tumours were examined and representative tumours are shown. (b) BALB/c mice bearing CT-26 tumours were treated intravenously with UV inactivated VSV particles and sacrificed 24h later. Fluorescent microspheres are distributed evenly throughout the tumour as evidenced by uniform fluorescence (black dots).

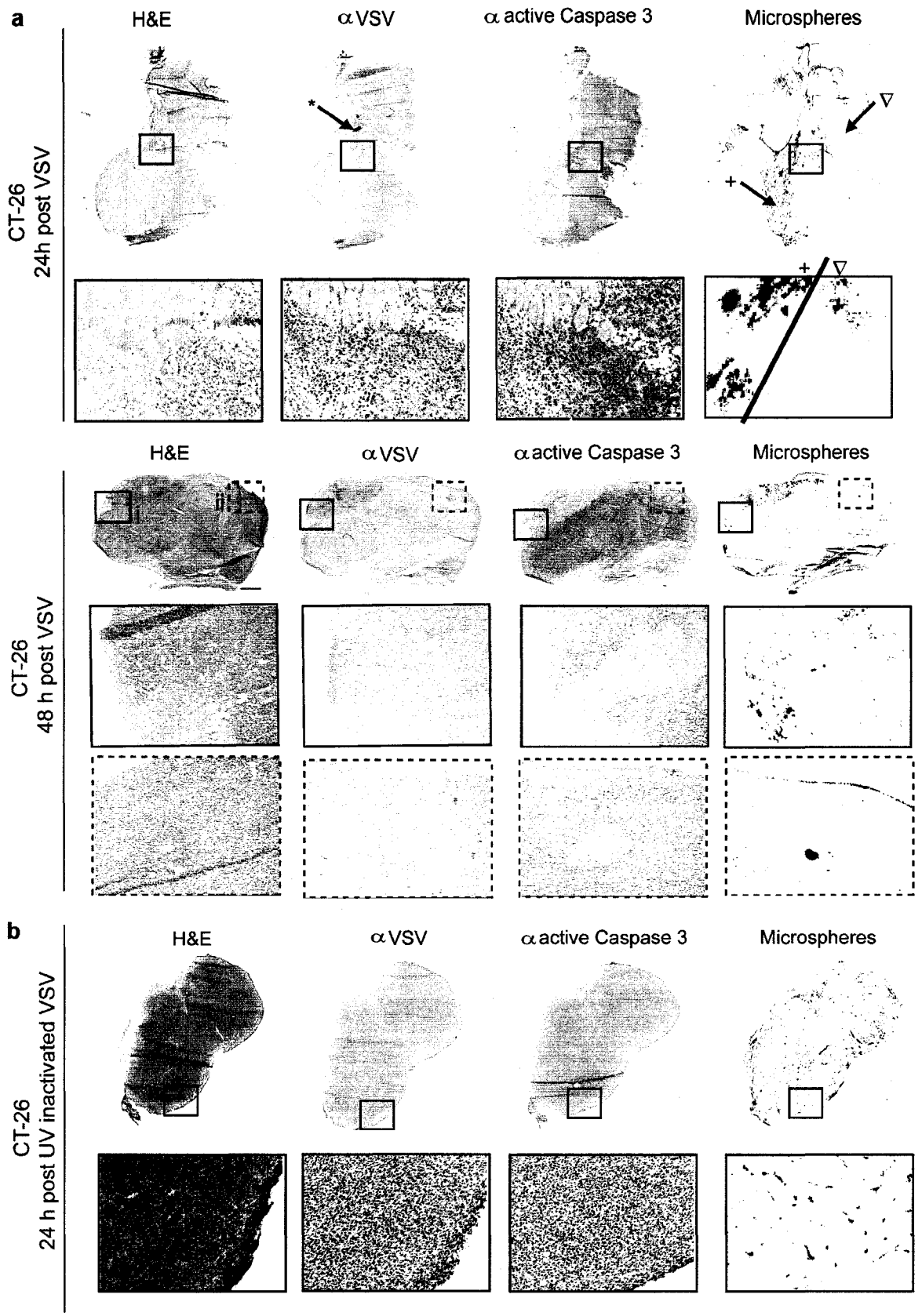
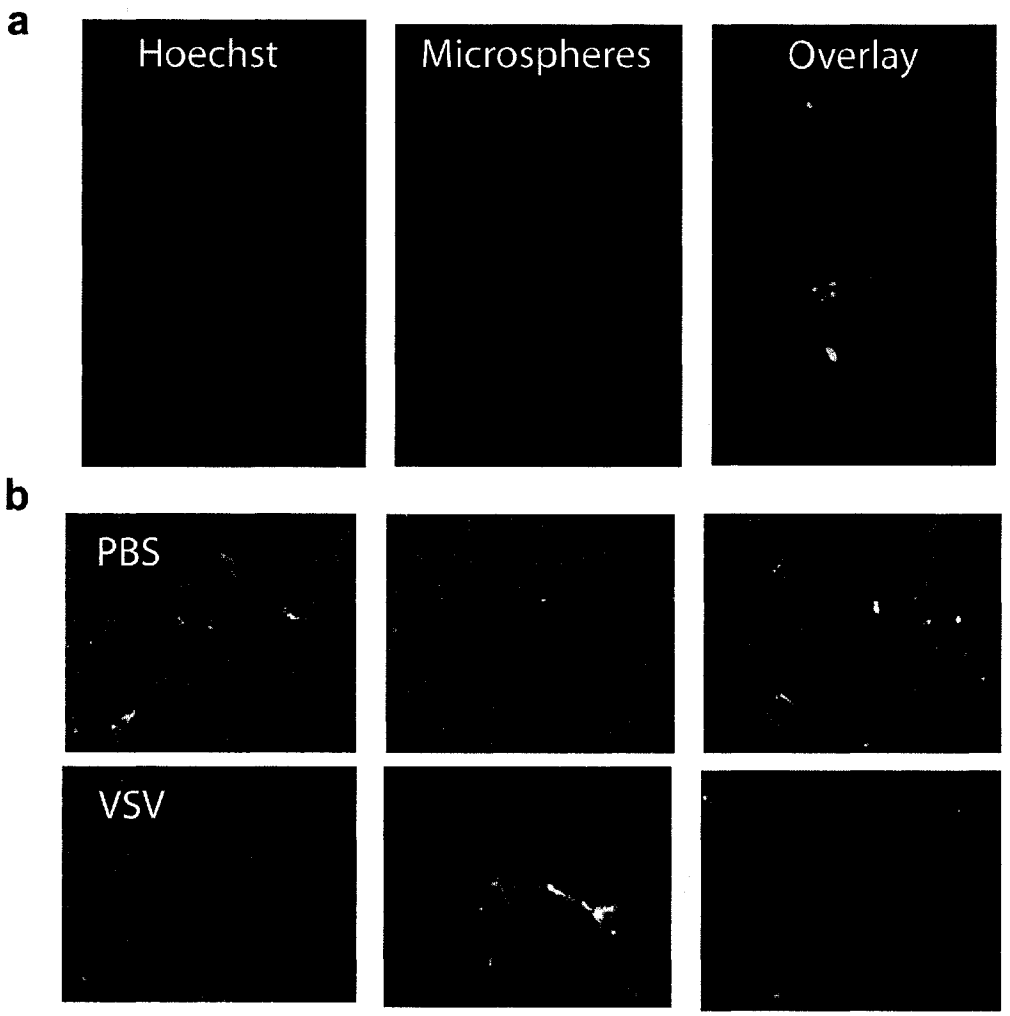

a
H&E
αVSV
α active Caspase 3
Microspheres
CT-26
24h post VSV
H&E
αVSV
α active Caspase 3
Microspheres
CT-26
48 h post VSV
b
H&E
αVSV
α active Caspase 3
Microspheres
CT-26
24 h post UV inactivated VSV

Figure 2.3 *Absence of intra-tumour blood flow is confirmed with in vivo Hoechst staining of endothelial cells.* (a) Mice with CT-26 tumours were perfused with Hoechst #33342 stain and fluorescent microspheres for 5 minutes prior to being sacrificed. Sections were visualized with a fluorescence microscope. Pictures captured at 40x. (b) Mice with CT-26 tumours were treated intravenously with vesicular stomatitis virus (VSV) or phosphate-buffered saline (PBS) and were perfused with Hoechst #33342 stain and fluorescent microspheres for 5 minutes, 24 hours after infection-. Sections were visualized under a fluorescence microscope and representative pictures are shown (20x).


a
Hoechst
Microspheres
Overlay
b
PBS
VSV

treated with phosphate-buffered saline (PBS), a relatively uniform distribution of fluorescent beads and Hoechst staining is observed (Figure 2.3b). In contrast, 24 hours after infection with VSV tumour perfusion is markedly affected, as measured by both microsphere distribution and Hoechst staining (Figure 2.3b). The fact that apoptotic staining occurred simultaneously with the loss of tumour perfusion is quite striking (Figure 2.2a). As predicted, in adjacent normal tissues in which we observed no secondary apoptosis, microspheres were found uniformly distributed (Figure 2.2a).

The killing of uninfected tumour cells and the loss of tumor perfusion was not unique to the murine CT-26 colon cancer model. This phenomenon was observed also when nude mice implanted with the human SW620 tumor cell line were systemically treated with VSV (Figure 2.4 and Supplementary Figure S1). Furthermore, upon administration of an oncolytic version of vaccinia virus[21] to mice with CT-26 tumours, we observed a similar loss of blood flow in the core of the tumour (Figure 2.4 and Supplementary Figure S2). However, in the case of vaccinia (in contrast to the results with VSV), killing of uninfected cells was not initiated until approximately 120 hours after infection, consistent with the slower replication cycle of this virus.

Pro-inflammatory molecular signature in VSV treated tumours

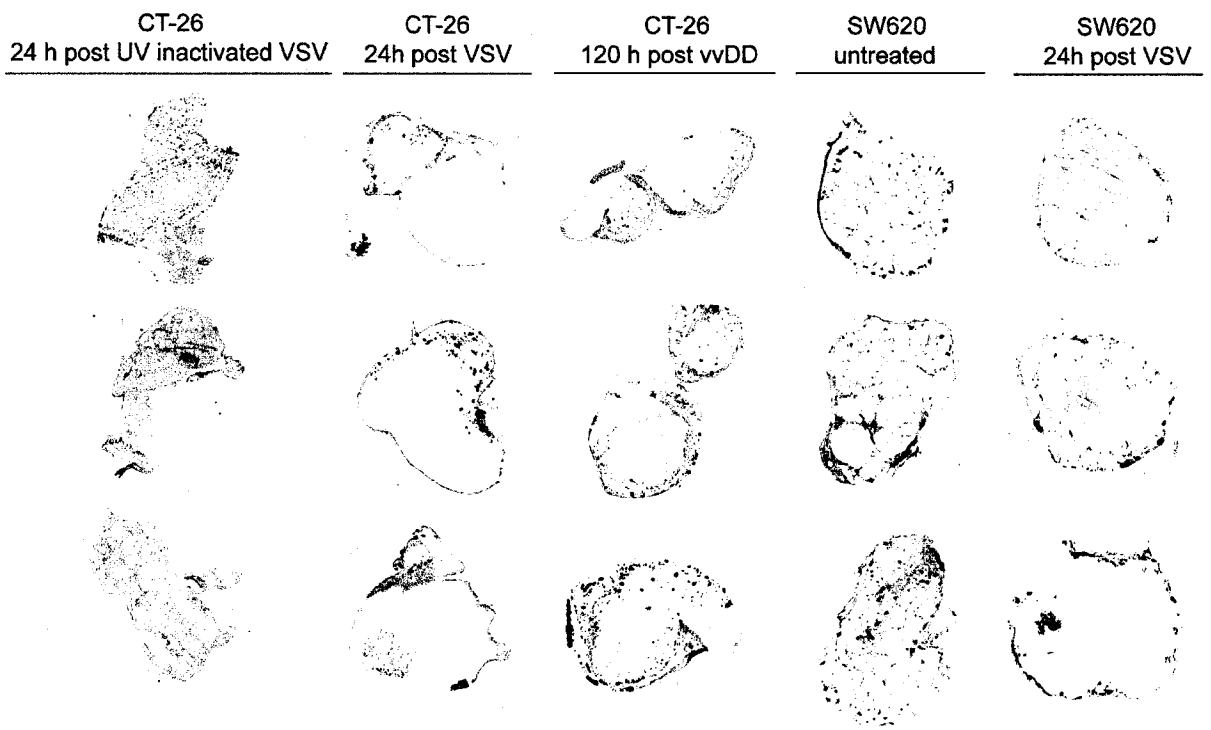
In order to elucidate the molecular events that cause the acute loss of blood flow within the tumour, we carried out transcript profiling of tumours treated with either VSV or VSV inactivated by UV (Table 2.1). Most genes that are up-regulated in response to VSV therapy are nuclear factor-κB-responsive, as would be expected from a natural infection.[22] Using a quantitative real-time polymerase chain reaction approach (on 5 additional tumours in each group) we confirmed that a number of the genes are reproducibly up-regulated during OV therapy (see

Table 2.1 *Genes upregulated in CT-26 tumour following 24 h infection with VSV as compared to genes expressed in tumour treated with UV inactivated VSV.*

Category & probe set	Fold increase[a]	Description
chemotaxis		
NM008176	127	chemokine (C-X-C motif) ligand 1
NM009114	86	S100 calcium binding protein A9 (calgranulin B)
NM013653	38	chemokine (C-C motif) ligand 5
BC024392	37	chemokine (C-X-C motif) ligand 5
NM013650	29	S100 calcium binding protein A8 (calgranulin A)
NM017466	21	chemokine (C-C motif) receptor-like 2
NM009140	14	chemokine (C-X-C motif) ligand 2
NM011333	14	chemokine (C-C motif) ligand 2
NM011337	11	chemokine (C-C motif) ligand 3
NM021274	9	chemokine (C-X-C motif) ligand 10
NM013654	4	chemokine (C-C motif) ligand 7
NM019494	4	chemokine (C-X-C motif) ligand 11
Inflammation		
BC020275	624[b]	Neutrophil gelatinase associated lipocalin 1
NM010510	294	interferon beta, fibroblast
NM031168	52	interleukin 6
NP034099	40[b]	colony stimulating factor (granulocyte-macrophage)
NM011198	35	prostaglandin-endoperoxide synthetase 2 (COX-2)
AB185896	18	Tumor necrosis factor alpha
NM009778	12	complement component 3

[a] Values taken from microarray experiment, Q RT-PCR analysis shown in Figure S3
[b] Signals for these genes were absent in UV inactivated sample. Fold upregulation was calculated using an arbitrary signal value of 600 for the UV inactivated sample.

Figure 2.4 *Uninfected tumour cell killing occurs with vaccinia virus and in a xenograft model of human cancer.* CT-26 tumour bearing BALB/c mice were treated with UV inactivated VSV particles, perfused with fluorescent microspheres 24 h post injection and sacrificed 5 min later. Fluorescence microspheres are distributed evenly though out the tumour, evidenced by uniform fluorescence (black dots). CT-26 tumour bearing BALB/c mice were treated intravenously with VSV expressing GFP and tumours analyzed for perfusion 24 h later. CT-26 tumour bearing BALB/c mice were treated intraperitoneally with vaccinia virus and perfused with fluorescent microspheres 120 h post infection. Three tumours were tested. CD1 nude mice bearing subcutaneous SW620 human colon carcinoma tumours were treated with VSV expressing GFP intravenously and perfused with microspheres 24 h post infection. Five tumours were tested and representative tumours are shown.

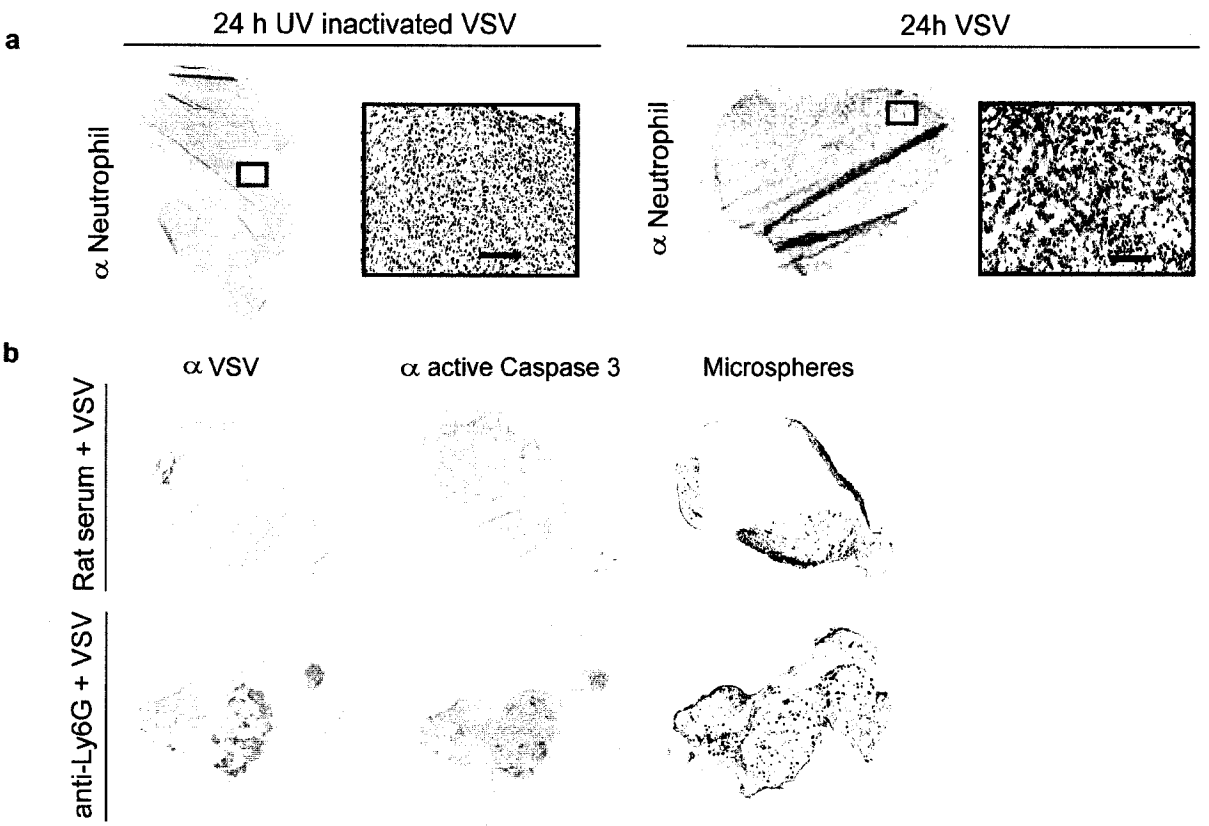

CT-26
24 h post UV inactivated VSV
CT-26
24h post VSV
CT-26
120 h post vvDD
SW620
untreated
SW620
24h post VSV

Supplementary Figure S3). A particularly interesting finding was that the transcripts encoding the neutrophil chemo-attractants CXCL1 and CXCL5 were increased 127- and 38-fold respectively, thereby suggesting that recruitment of inflammatory cells could be a key event in the process leading to loss of intra-tumour blood flow (Table 2.1). Quantitative real-time polymerase chain reaction experiments showed that VSV infection of CT-26 cells *in vitro* can also result in up-regulation of pro-inflammatory genes (*e.g.*, CXCL1) (data not shown).

Neutrophil depletion abrogates vascular shutdown and allows for more persistent virus infection in tumours

Neutrophils are known to be one of the first cell types recruited to the sites of infections,[23] and have been implicated in causing substantial tissue damage following exposure to double-stranded RNA[24] or after ischemia/reperfusion.[24,25] Indeed, immunohistochemical analysis of VSV-treated CT-26 tumours demonstrated neutrophil infiltration after virus infection (Figure 2.5a). Others have shown that activated neutrophils can inhibit blood flow in some pathological situations.[26,27] From mice with tumours, we therefore selectively depleted this cell type by injecting rat mAb RB6-8C5 (refs. 28,29) intraperitoneally 24 hours prior to initiating OV therapy. In these experiments we were able to demonstrate that the RB6-8C5 antibody effectively depleted neutrophils (see Supplementary Figure S4). In animals that had a normal complement of neutrophils, OV therapy resulted in loss of tumour perfusion and massive induction of apoptosis (Figures 2.2a and 2.5b). By contrast, in neutrophil- depleted animals blood flow in the tumour was not significantly interrupted, the killing of uninfected cells did not take place, and the replication and spread of the virus within the tumour were enhanced (Figure 2.5b). Also, in neutrophil-depleted animals, the active caspase 3 staining (signaling apoptosis) in the tumours seemed to be restricted to areas undergoing active viral infection, whereas in neutrophil-replete animals, the

Figure 2.5 *Uninfected tumour cell killing requires presence of neutrophils.* (a) BALB/c mice bearing subcutaneous CT-26 tumours were treated intravenously with UV inactivated VSV particles or VSV expressing GFP and euthanized 24 h later. Representative scans of tumours treated as indicated and stained for neutrophils (NIMP-R14). Scale bars, 100 μM. (b) BALB/c mice bearing subcutaneous CT-26 tumours were pretreated with 100 μl 50:50 rat serum:PBS or 7.5 μg RB6 8C5 antibody intraperitoneally 24 h prior to intravenous treatment with VSV expressing GFP. 24 h following virus treatment, mice were perfused with fluorescent microspheres for 5 minutes and euthanized. Five mice were tested per group and representative images are shown.


a
24 h UV inactivated VSV
24h VSV
α Neutrophil
α Neutrophil
b
α VSV
α active Caspase 3
Microspheres
Rat serum + VSV
anti-Ly6G + VSV

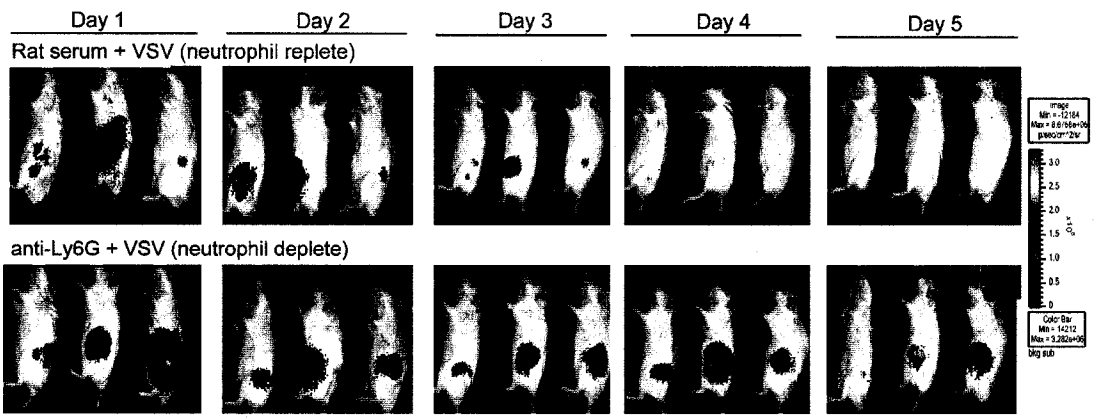
staining was found in areas ahead of the infection, suggesting that killing of uninfected tumour cells was in progress (compare Figures 2.2a and 2.5b). The absence of blood flow (triggered by neutrophil infiltration) is likely to restrict the spread of virus within the tumour, and emerging viruses would remain confined to the perfused tumour rim where "fertile ground" for virus replication would be quickly exhausted. These results suggest that neutrophil depletion prior to therapy may lead to a greater number of tumour cells becoming infected with virus.

Next, mice with tumours were infected with VSV engineered to express firefly luciferase upon infection, and we carried out imaging over time using the IVIS imaging system (Xenogen, Alameda, CA). Mice with tumours were given intraperitoneal injections of RB6-8C5 antibody every other day (starting 1 day prior to VSV infection) and were imaged every day for the following 5 days. Mice that received normal rat serum showed VSV replication until day 3 after infection, while mice that were depleted of neutrophils demonstrated tumour-specific viral replication until at least 5 days after infection (Figure 2.6). Together, these results demonstrate that OV infection within tumours triggers the release of cytokines that attract neutrophils, leading to localized acute ischemia on account of the loss of perfusion, and resulting in tumour cell death.

Discussion

It is widely believed that oncolytic viruses are effective therapeutics due to their ability to directly infect and kill tumour cells. There is accumulating evidence that an adaptive anti-tumour immune response triggered by virus infection contributes to *in vivo* efficacy. We show here that targeted activation of localized inflammation is yet a third component involved in tumour killing.

Figure 2.6 *Neutrophil depletion allows for more persistent VSV replication.* BALB/c mice bearing subcutaneous CT-26 tumours were pretreated with 100 µl 50:50 rat serum:PBS or 100 µg RB6 8C5 antibody intraperitoneally 24 h prior to intravenous treatment with VSV expressing a GFP luciferase fusion protein (Day 0). Neutrophil depletion was ensured by subsequent injection of 100 µg anti-Ly6G antibody on Day 1, Day 3 and Day 5. Mice were imaged using the IVIS200 system (Xenogen Corp., Alameda, CA) starting 24 h post infection (Day 1) and every following day for 5 days.


Day 1
Day 2
Day 3
Day 4
Day 5
Rat serum + VSV (neutrophil replete)
anti-Ly6G + VSV (neutrophil deplete)
3.0
2.5
2.0
1.5
1.0
0.5
0

Indeed, within 24 hours of VSV administration *in vivo*, viral replication can be severely restricted to the tumour periphery yet causes massive cell death in uninfected cells. In order to generate a better understanding of the tumour environment following oncolytic virus infection, transcriptional profiling was performed and revealed a pro-inflammatory gene signature. Since OVs are engineered to selectively infect tumour tissues they direct an inflammatory response to the tumour bed and initiate a cascade of events that culminate in tumour destruction. The impressive tumour killing effect is the result of "a perfect storm" within the malignancy requiring the co-incident replication of the OV, secretion of pro-inflammatory cytokines and recruitment of inflammatory cells to the tumour microenvironment.

We clearly show that neutrophils play a critical role in the shutdown of tumor blood flow. During natural infections, neutrophils, are the first line of defense against invading pathogens and are often responsible for collateral tissue damage following exposure to dsRNA[24] or hypoxia[25] likely through the production of reactive oxygen species [30,31], secretion of proteases[32] or impairment of micro-vascular perfusion[26,27]. Like other immune cells, neutrophils must make their way through the vast network of vasculature including the smallest capillaries and in their normal state can negotiate through microvasculature by distorting their shape. However, during inflammatory reactions activated neutrophils adopt a "rigid" phenotype which can result in clogging of small capillaries[33]. As tumour vasculature possesses inherently different properties than normal vessels[34], tortuous capillaries within the tumour may be more likely to act as a sink for activated neutrophils. Co-incident with the accumulation of neutrophils in the tumour, we observed a decrease in the number of neutrophils in the peripheral blood of treated mice. Although it is not unusual to see a transient loss of neutrophils from peripheral blood following infection[35-37], it is reasonable to suggest that in these animals, it is in part due to the sequestration

of neutrophils to tumour beds. Parenthetically, in an ongoing phase I trial, patients treated with oncolytic vaccinia virus experienced a similar transient decrease in neutrophil counts in their peripheral blood (D. Kirn, Jennerex Biotherapeutics, personal communication, 1 November 2006) but it remains to be seen if this is due to recruitment to the tumour.

The results obtained from mouse models reported here are consistent with the observation that adoptive transfer of tumour reactive CD8 T cells triggers granulocyte infiltration and hypoxia within tumours[38]. This would suggest that the described mechanism of tumour cell death may not be specific to oncolytic virus therapeutics but is applicable to all therapies that involve immune activation and rapid apoptosis in solid tumours. Interestingly, a clinical study of an oncolytic adenovirus demonstrated by CT scan that virus infection triggering extensive necrosis in a liver metastasis of a colon adenocarcinoma[39]. In addition PET scan images show large areas that are metabolically inactive[40], consistent with the idea that virus infection is triggering inhibition of blood flow to tumours. In addition, another clinical trial studying HSV-1 also reported extensive necrosis in an OV treated tumour, demonstrating similar histology to the OV treated tumours from mouse models in this report[41].

We have found that *targeted* inflammation to tumour beds leads to additional loss of cells in the tumour probably through acute oxygen deprivation caused by a rapid loss of tumour perfusion. While absence of intratumour blood flow and uninfected tumour cell killing may provide a significant therapeutic advantage, these phenomena may also limit virus spread and persistence within the tumour. The concept that immune cells have deleterious effects on OV therapy is consistent with reports from others that chemotherapeutic agents can enhance viral therapy by eliminating both the adaptive and innate immune responses[42]. However, generalized immune suppression poses risks to the patient and would prevent the generation of anti-tumour

immune responses noted by our group and others during OV therapy[5,43]. Grote et al[44] correlated enhanced tumour regression following treatment of a granulocyte/macrophage colony stimulating factor (GM-CSF) expressing measles virus with enhanced neutrophil recruitment to tumours. This study, however, attributed neutrophil mediated tumour regression due to expression of the transgene, and not OV replication. The results presented here suggest that inflammatory immune cells can be beneficial for OV therapy suggesting that more refined approaches to manipulating individual cell populations will be required to maximize oncolytic virus therapeutic regimes. Perhaps neutrophil activity could be down-regulated to promote initial viral replication in the tumour to be followed by neutrophil enhancement to capitalize on the ensuing tumour cell death secondary to absence of blood flow to tumours. In addition, measurement of tumour blood flow during treatment may provide a window to observe tumour response to OVs. This might influence the dose and timing of OV administration in a multiple dosing regime. Overall, we describe mechanisms by which inflammatory cells can be manipulated to cause enhanced and targeted tumour cell death. By investigating early events following OV infection in tumours, we have discovered a novel, targeted mechanism of tumour destruction, requiring the correct combination of virus infection, tumour microenvironment and inflammation. It is clear that a better understanding of the direct and indirect mediators of OV tumour lysis will allow us to fine tune therapeutic protocols to achieve the best possible outcome for patients.

Materials & Methods

Viruses. The Indiana serotype of VSV was used throughout this study and was propagated in Vero cells (American Type Culture Collection). AV1 VSV is a naturally occurring interferon inducing mutant of VSV[5] while Δ51 VSV expressing GFP[5] and GFP-firefly luciferase fusion[45] are recombinant interferon inducing mutants of the HR strain of wild-type VSV Indiana. TP3[46], herein referred to as AV2, was inactivated by UV light. Doubled deleted vaccinia virus expressing GFP[21] was also propagated in Vero cells. Virions were purified from cell culture supernatants by passage through a 0.2 μm Steritop filter (Millipore, Billerica, MA) and centrifugation at 30 000 x g before resuspension in phosphate-buffered saline (PBS) (HyClone, Logan, UT).

Cell lines. CT-26 (murine colon adenocarcinoma) and SW620 (human colon carcinoma) derived cells were purchased from American Type Culture Collection and cultured in HyQ Dulbecco's Modified Eagle Medium (High glucose) (HyClone) supplemented with 10% fetal calf serrum (CanSera, Etobicoke, Ontario, Canada).

Tumour models. Female 6-8 week old BALB/c mice were obtained from Charles River Laboratories (Wilmington, MA). Syngeneic subcutaneous tumours were established by injection of 3 x 10^5 cells in 100 μl PBS (CT-26) or 1 x 10^6 cells in 100 μl PBS (SW620) in the left and right hind flanks. When tumours reached a palpable size, mice were treated with VSV by tail vein injection and vaccinia virus intraperitoneally. Mice were sacrificed at the indicated timepoints by cervical dislocation and tumours were frozen in Shandon Cryomatrix freezing medium (ThermoElectron Corporation, Waltham, MA) on dry ice. Five or 10 μM sections were cut using a Microm HM500 OM cryostat. For fluorescence imaging of intact tumors, mice were euthanized

by halothane overdose. Skin was removed, and the tumour was visualized in a dissecting fluorescent microscope (Leica MZFLIII) with a standard GFP filter set. Pictures were captured with a Nikon Coolpix 100 digital camera. Fluorescent and light images were overlayed in Adobe Photoshop 7.0. Exact magnifications cannot be provided because this microscope has a continuous gradient of magnifications. All experiments were conducted with the approval of the University of Ottawa Animal Care and Veterinary Service.

Titration of VSV from mouse tissues. Tissues were removed at the indicated timepoints, weighed and homogenized in 1 ml of PBS using a homogenizer (Kinematica AG-PCU-11). Serial dilutions of tissue preparations were performed in serum free media, applied to confluent Vero cells for 45 min. Subsequently, the plates were overlayed with 0.5% agarose in media and the plaques were grown overnight. Plaques were counted by visual inspection (between 50-200 plaques/plate).

Immunohistochemistry, Immunofluorescence and TUNEL staining. Immunohistochemistry was performed using the Vectastain ABC kit for rabbit primary antibodies (Vector Labs, Burlingame, CA), according to instructions provided. Primary antibodies were employed as follows: VSV (gift of Earl Brown), active caspase 3 (BD Pharmingen, Rockville, MD) and anti-neutrophil NIMP-R14 (Abcam, Cambridge, MA). Horseradish peroxidase (HRP) activity was visualized with a Diaminobenzene-HRP kit (KPL Biosciences). Nuclei were counterstained in hematoxylin. For assessment of cell morphology, sections were stained with hematoxylin and eosin according to standard protocols. Whole tumour images were obtained with an Epson Perfection 2450 Photo Scanner while magnifications were captured using a Xeiss Axiophot HBO 50 microscope. Bound anti-VSV antibody was detected with a Cy3 conjugated donkey anti-rabbit antibody (Jackson Immunoresearch Laboratories, West Grove, PA). TUNEL

staining was carried out according to manufacturer's instructions (In Situ Cell Death Detection Kit - FITC, Roche, Mississauga, Ontario, Canada). Nuclei were counterstained with Hoechst #33242 (2.5 μg/ml). VSV and TUNEL staining was visualized in a Zeiss Axiocam HRM Inverted fluorescent microscope and analyzed using Axiovision 4.0 software. Negative control sections were used to set exposures, which were kept constant throughout. Neutrophil staining was captured using Aperio ScanScope (Axiovision Technologies) and analyzed using Aperio ImageScope software.

Analysis of tumour perfusion. Mice were injected intravenously with 100 μl of a 50% solution of 100 nm diameter orange fluorescent microspheres (Molecular Probes, Burlington, Ontario, Canada). Five minutes later, animals were sacrificed and tumours immediately snap frozen as previously described. Tumour perfusion was analyzed by visualizing fluorescent microspheres in the vasculature of 10 μm unfixed frozen sections using a ScanArray Express microarray scanner with a standard Cy3 laser (Packard Bioscience). For *in vivo* Hoechst staining, mice were injected with 200 μl 10 mg/kg Hoechst #33242 with 0.25 x microspheres and sacrificed by cervical dislocation after 5 min. Tumours were immediately snap frozen as previously described and 6 μM sections prepared and visualized using a Zeiss Axiocam HRM Inverted fluorescent microscope and analyzed using Axiovision 4.0 software.

Microarray. BALB/c mice bearing subcutaneous CT-26 tumours were treated with 5 x 10^8 pfu Δ51 VSV GFP or UV inactivated VSV control and tumours were harvested 24 hours later for total RNA extraction. Total RNA was isolated using the Qiagen RNeasy kit (as per manufacturer's instructions; Qiagen, Mississauga, Ontario, Canada) followed by sodium acetate/ethanol precipitation to concentrate each sample. Twenty micrograms of each RNA

sample was processed according to manufacturer's standard protocol (Affymetrix) and hybridized to an Affymetrix Mouse430_2 chip. Signals were normalized to the UV inactivated VSV control sample on a gene per gene basis. Signals below 600 were considered absent and fold changes below 2 were considered insignificant. Data were analyzed using Genespring software (SiliconGenetics).

Reverse transcription and Q-PCR. CAT RNA was made by *in vitro* transcription with the RiboMAX™ Large Scale RNA Production Systems (Promega, Madison, WI) using the pCAT plasmid as template. Total tumour or cell derived RNA was reverse transcribed (1 or 2 μg RNA) using Superscript II reverse transcriptase (Invitrogen, Mississauga, Ontario, Canada) with a spike of 5 ng of CAT RNA, an exogenous control used to quantitate and normalize for reverse transcription (RT) efficiency. Quantitative real-time PCR (Q-RTPCR) was performed in triplicate on all samples on the Roche LightCycler rapid thermal .cycler system (according to the manufacturer's instructions) and using the FastStart DNA Master SYBR Green I kit (Roche Diagnostics, Laval, Canada). Standard curves were initially generated by standard dilutions and used to find absolute values for each reaction. All values obtained were normalized to CAT values to normalize the RT efficiency. Primers were designed with Primer3 software. Primers used for each gene are as listed in Supplementary Materials and Methods.

In vivo neutrophil depletion. Mice were injected intraperitoneally with 150 μg purified RB6 8C5 rat monoclonal antibody, clone RB6-8C5[29] (BD Pharmingen, Rockville, MD) in order to systemically deplete granulocytes. 150 μl non-immune rat serum was utilized as a negative control. 24 h later, mice were treated intravenously with 5 x 10^8 pfu Δ51 VSV GFP, perfused with fluorescent microspheres 24 h later and sacrificed by cervical dislocation and tissues collected and

stained as described above. For *in vivo* imaging, mice VSV GFP-luciferase expressing mice were anesthetized under 3% isofluorane (Baxter Corp., Deerfield, IL), injected intraperitoneally with 2 mg luciferin (Sigma, Oakville, Ontario, Canada) and imaged using IVIS 200 Imaging System (Xenogen Corp, Alameda, CA). Data acquisition analysis was performed using Living Image v2.5 software. All images were captured under identical exposure, aperture and pixel binning settings and bioluminescence is plotted on identical color scales.

Statistical analysis. All statistical analysis was performed using Graphpad Prism 3.0 software. Data are represented as a mean +/- standard error. Virus quantifications from plaque assays were log transformed before statistical analysis and plotting.

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Supplementary Material

Figure S1 *VSV causes uninfected tumour cell killing and vascular shutdown in human SW620 colon carcinoma xenograft.* CD1 nude mice bearing SW620 tumours were left untreated or treated intravenously with VSV and sacrificed 24 h later. Representative scans of serial sections of tumours treated as indicated and stained by immunohistochemistry for VSV and active caspase 3 to visualize areas of virus infection and apoptosis respectively. Prior to sacrifice, mice were perfused with fluorescent microspheres for 5 minutes. Tumour sections were prepared and analyzed for fluorescence using a microarray scanner.

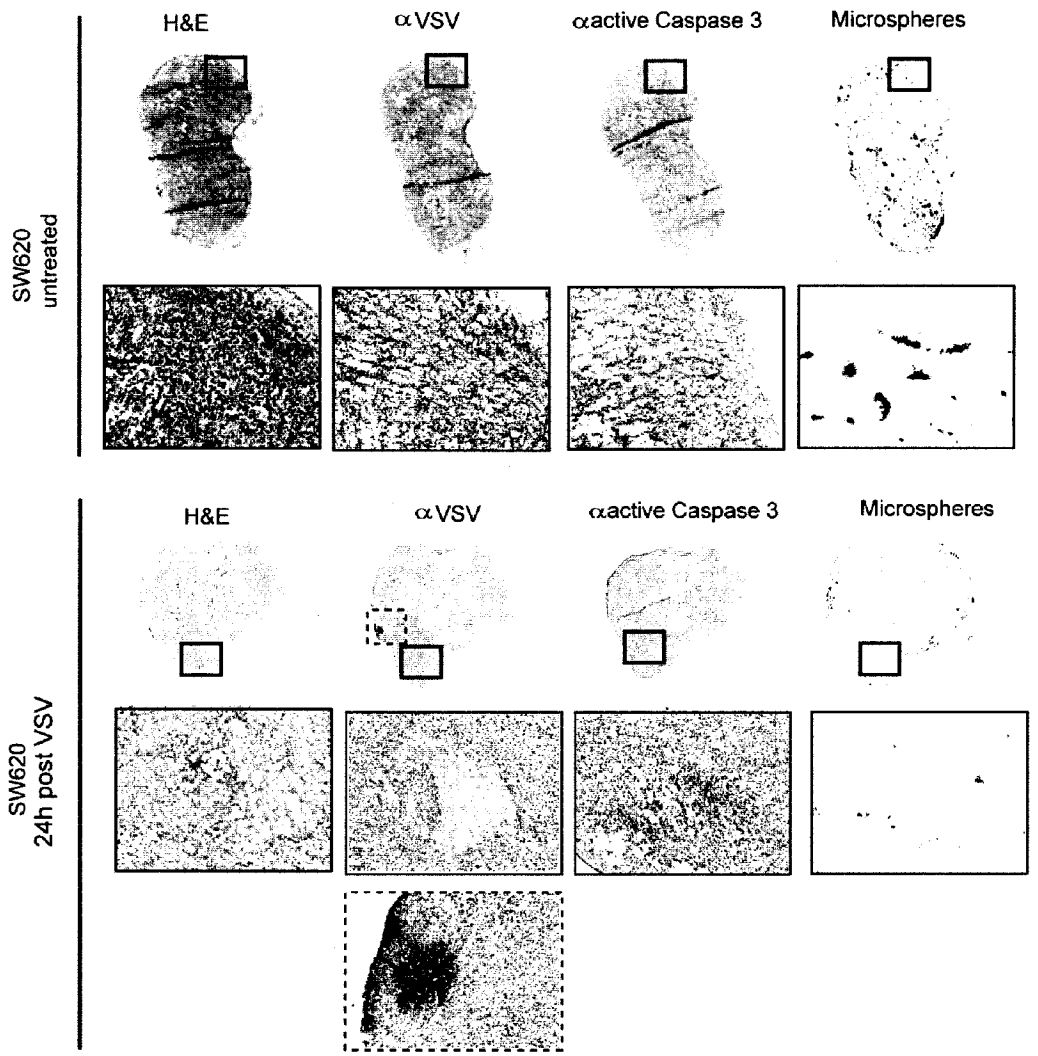

H&E
αVSV
αactive Caspase 3
Microspheres
SW620
untreated
H&E
αVSV
αactive Caspase 3
Microspheres
SW620
24h post VSV

Figure S2 *Vaccinia virus causes vascular shutdown in CT-26 colon adenocarcinoma tumours.* BALB/c mice bearing subcutaneous CT-26 tumours were left untreated or treated with vaccinia virus intraperitoneally. Representative scans of serial sections of tumours treated as indicated and stained by immunohistochemistry for vaccinia virus antigen. Prior to sacrifice, mice were perfused with fluorescent microspheres for 5 minutes. Tumour sections were prepared and analyzed for fluorescence using a microarray scanner.

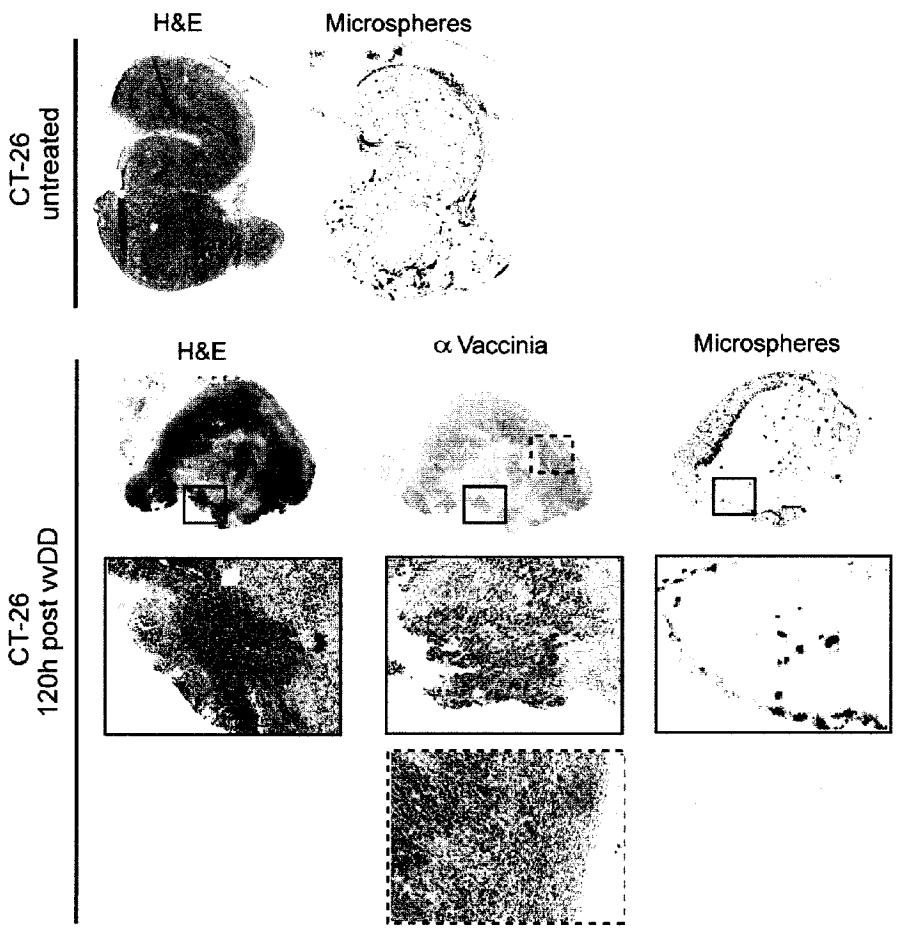

H&E
Microspheres
CT-26
untreated
H&E
α Vaccinia
Microspheres
CT-26
120h post vvDD

Figure S3 *VSV infection causes upregulation of pro-inflammatory genes in CT-26 tumours.* BALB/c mice bearing CT-26 tumours were treated intravenously with UV inactivated VSV particles or VSV expressing GFP and sacrificed 24 h later. Quantitative RT-PCR was performed on RNA extracted from the tumour analyzed by microarray (Microarray sample) as well as 5 additional VSV treated tumours (Tumours 1-5). mRNA expression levels were compared to the normalized UV inactivated VSV treated tumour used in the microarray analysis and fold upregulation is indicated.

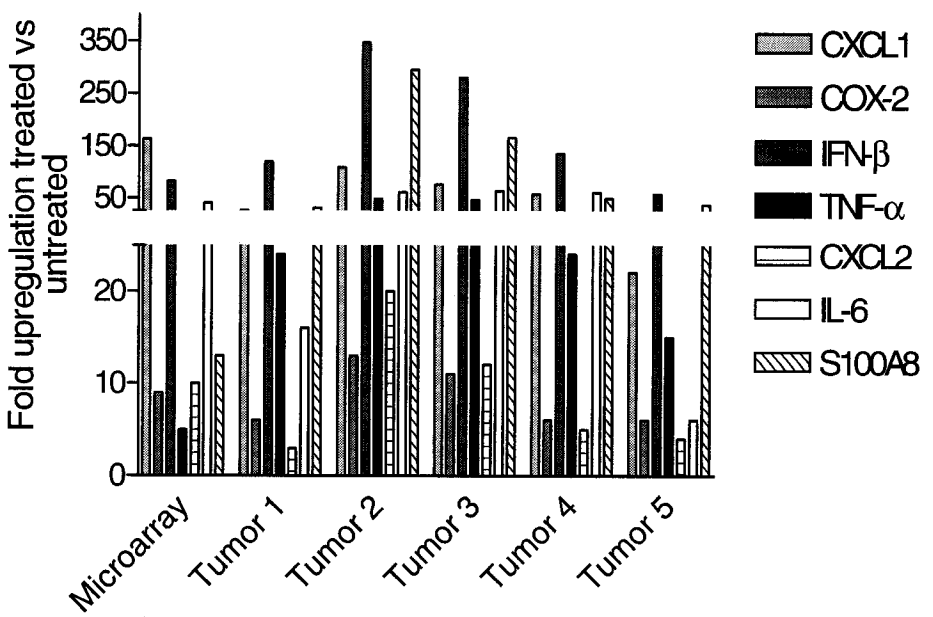
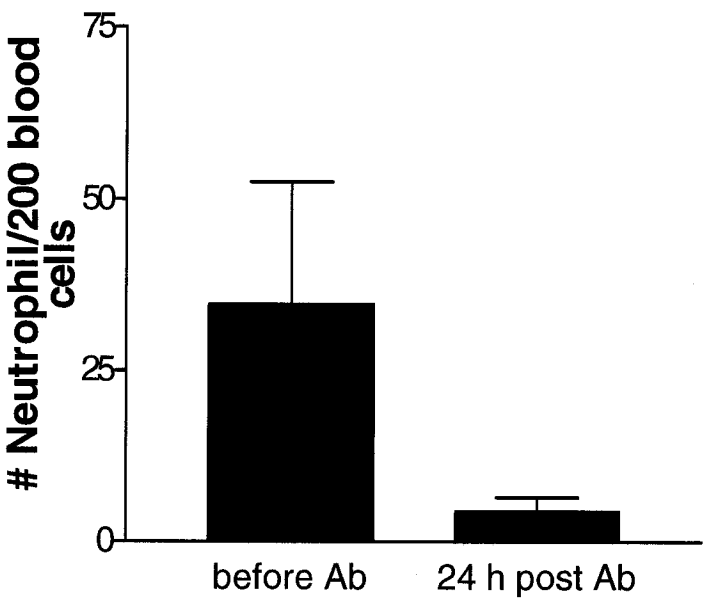

Fold upregulation treated vs untreated
350
250
150
50
20
10
0
Microarray
Tumor 1
Tumor 2
Tumor 3
Tumor 4
Tumor 5
CXCL1
COX-2
IFN-β
TNF-α
CXCL2
IL-6
S100A8

Figure S4 *RB6 8C5 anti-neutrophil antibody treatment reduces peripheral neutrophil counts.* BALB/c mice were injected intraperioneally with 125 ug RB6 8C5 antibody and peripheral blood counts were performed before and 24 h after treatment. Number of neutrophils shown per 200 blood cells.


Neutrophil/200 blood cells
75
50
25
0
before Ab
24 h post Ab

Supplementary Materials and Methods

Table S.2 Primer sequences for quantitative RT-PCR used in this study

Gene	Left Primer	Right Primer
COX-2	5'-tcctcctggaacatggactc-3'	5'-ccccaaagatagcatctgga-3'
complement component 3	5'-ctgtgtgggtggatgtgaag-3'	5'-gtcagaagccagcgttcac-3'
CXCL1	5'-gctgggattcacctcaagaa-3'	5'-gtcagaagccagcgttcac-3'
CXCL2	5'-tccagagcttgagtgtgacg-3'	5'-aggcacatcaggtacgatcc-3'
IL-6	5'-aacgatgatgcacttgcaga-3'	5'-ggaaattggggtaggaagga-3'
IFN-β	5'-ataagcagctccagctccaa-3'	5'-ctgtctgctggtggagttca-3'
S100A9	5'- tcatcgacaccttccatcaa-3'	5'- tcaactttgccatcagcatc-3'
S100A8	5'-atgccgtctgaactggagaa-3'	5'- tggctgtctttgtgagatgc-3'
CXCL5	5'-gccctacggtggaagtcata-3'	5'-gggacaatggtttccctttt-3'
NGAL-1	5'-atggtgaaaccaaggagctg-3'	5'-gctctctggcaacaggaaag-3'
GM-CSF	5'-tattcgagcagggtctacgg-3'	5'- tcattacgcaggcacaaaag-3'
TNF-α	5'-ccacatctccctccagaaaa-3'	5'-agggtctgggccatagaact-3'
CAT	5'-gcgtgttacggtgaaaacct-3'	5'-gcgtgttacggtgaaaacct-3'

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CHAPTER 3: TARGETING TUMOURS ON MULTIPLE FRONTS USING ONCOLYTIC VIRUSES

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Contributions of authors: The manuscript was written by CJ Breitbach. N De Silva generated 3D model of virus infected tumour (Figure3. 1) with software developed by U Aladl and A Fenster. L Evgin helped generate the perfusion model of an untreated control tumour. N De Silva also performed the BrdU pulse experiment (Figure 3.7). TJ Falls performed mouse treatments. M Daneshmand provided help with analysis of histological tumour sections. David Kirn contributed clinical results presented in Figure 9. KA Parato, MM Stanford, H Atkins and JC Bell provided continued guidance over the course of this work.

In preparation

Abstract

Oncolytic viruses (OVs) are currently being developed as a novel anticancer therapy. OVs are replication competent viruses that specifically infect and lyse cancer cells. Vesicular stomatitis virus (VSV) naturally targets tumours as most tumour cells possess a defective innate antiviral response. VSV has the capability of rapidly infecting and spreading in a variety of tumour cell lines *in vitro*. In contrast, 3D rendering of images detecting VSV infection in tumours *in vivo* following intravenous infusion of virus reveals only limited infection of tumour cells. Limited infection is likely attributed to a massive loss of perfusion in the tumour core, also rendered in 3 dimensions. Neutrophils have previously been shown to be critical for the loss of tumour perfusion in the context of virus therapy. Here we report formation of extensive coagulation in tumour vasculature following VSV infection. Furthermore, inhibition of coagulation using heparin or bothroalternin results in maintenance of tumour perfusion upon virus treatment suggesting that coagulation is required for the reduction of tumour perfusion in the context of VSV therapy. Interestingly, decreased coagulation was detected in tumours taken from neutrophil depleted animals. This observation suggests that inflammation triggers coagulation which in turn results in a reduction of tumour perfusion. Consistent with the observation of maintenance of perfusion within the tumour rim, viable vessels are also exclusively associated with the tumour rim. In addition, cell proliferation is limited to areas within the perfused tumour rim though tumour cells are not able to expand in tumours implanted into immune competent mice. Conversely, a massive expansion of the viable tumour rim ensues following virus treatment of tumour bearing nude mice that possess a defective adaptive T cell response. These results suggest that outgrowth of viable tumour cells following OV therapy is inhibited by an adaptive immune

response triggered to target tumour cells. Finally, a reduction of tumour perfusion has been reported in patients treated with oncolytic vaccinia virus. Here we present results from patient positron emission tomography (PET) imaging that demonstrate metabolic activity associated with the tumour rim following vaccinia virus treatment. Furthermore, we demonstrate that coagulation was also detected in a tumour associated vessel (and not vessels associated with normal tissue) from the same patient. Importantly, both observations are consistent with results from pre-clinical studies and suggest that in both animal models and patients, oncolytic viruses target tumours on multiple fronts.

Introduction

Oncolytic viruses (OVs) exploit genetic defects in cancer cells and specifically replicate in and lyse tumour cells [1-3]. A mutant form of vesicular stomatitis virus (VSV) that is unable to shut down host gene expression targets tumours with a defective innate antiviral response [4]. We have previously characterized and reported that an acute, targeted inflammatory response within tumours is triggered by OV infection [5]. Mice bearing tumours treated systemically with diverse OVs mount an inflammatory response which triggers a reduction in tumour perfusion. Therefore, virus infection of tumours induces extensive apoptosis of uninfected tumour cells due to the reduction of tumour blood flow. We have previously identified neutrophils as critical mediators of the shutdown in tumour blood flow. We now demonstrate that inflammation triggers coagulation within tumour vasculature and coagulation is also critical for loss of tumour perfusion. Further characterization of the tumour microenvironment shortly following OV therapy revealed that inflammation mediated damage to tumours is restricted to the tumour core. Interestingly,

outgrowth of the viable tumour rim is stagnated in immune competent animals while the viable rim rapidly expands in mice lacking functional thymus derived T cells. These results suggest that virus infection and inflammation mediated damage to tumours may trigger an adaptive anti-tumour immune response that subsequently inhibits outgrowth of viable tumour cells. All together, these data demonstrate that oncolytic viruses cause tumour cell death via multiple mechanisms: *(1)* lysing of virus infected cells, *(2)* triggering tumour targeted inflammation and coagulation resulting in death of uninfected tumour cells early after infection and *(3)* inducing an adaptive anti-tumour T cell response that controls viable rim outgrowth.

Identification of mediators critical for the anti-tumour inflammatory response is essential, given that a reduction of tumour perfusion associated with increased pro-inflammatory cytokine production in response to oncolytic virus therapy has now also been observed in patients with hepatocellular carcinoma [6]. Furthermore, we report here the detection of high metabolic activity within the tumour rim as well as intravascular coagulation within tumour associated vasculature in a tumour from a patient treated with oncolytic vaccinia virus. These results suggest that OV infection of patient tumours may similarly be prone to the acute effects of OV infection characterized here in murine tumour models.

Results

3D rendering of images of tumour perfusion and virus infection reveals isolated areas of virus infection and a large reduction in tumour perfusion

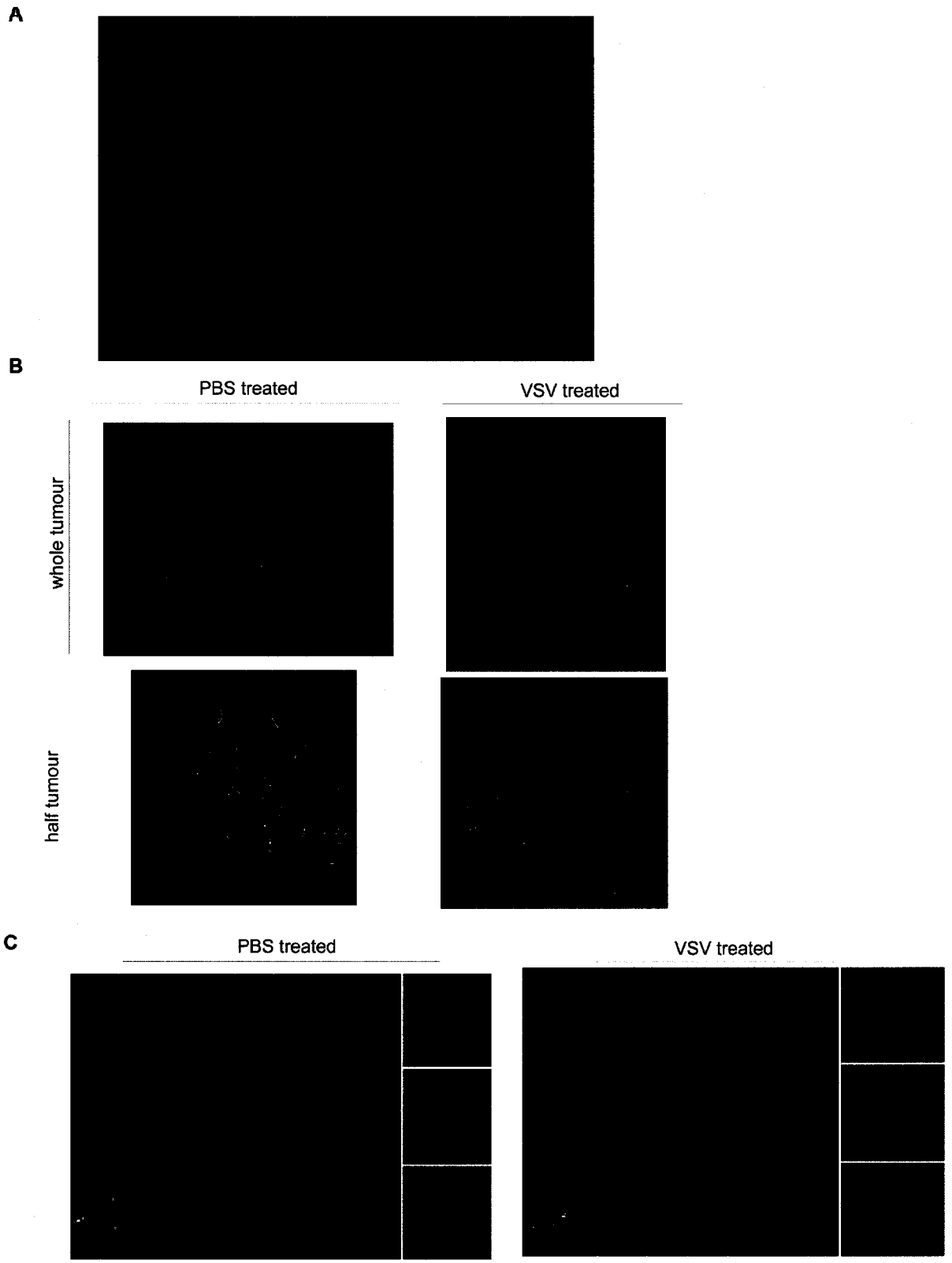
Analysis of virus infection and intratumoural blood flow of 2D representative sections published previously [5] formed the basis of 3 dimensional rendering of these parameters. We

reported that VSV infection of subcutaneous CT-26 colon adenocarcinoma tumours in syngeneic BALB/c mice results in a loss of tumour perfusion as visualized by the distribution of intravenously infused fluorescent microspheres. We now demonstrate that intravenously infused virus mainly infects numerous areas restricted to the tumour rim at 24 h post infection (Figure 3.1a). One large area of virus infection is likely the site of efficient virus spread. Importantly, 3 dimensional rendering of tumour perfusion validates initial observations that the tumour core is devoid of blood flow. This is especially evident when viewing the 3D representation of a cross section of the tumour (Figure 3.1b). Similar rendering of perfusion images of an untreated tumour reveals that basal CT-26 tumour perfusion is quite uniform (Figure 3.1b) and provides a reference for the reduction in perfusion triggered by virus infection. In another representation, cross-sections of the untreated and treated tumour in all 3 planes demonstrate a complete loss of perfusion in the tumour core of the treated tumour with uniform perfusion in the untreated control (Figure 3.1c). Three dimensional rendering of virus infection and tumour perfusion facilitates our understanding of early effects of virus infection on the tumour microenvironment. Furthermore, these models validate our initial and future analyses of two dimensional representations of tumours.

Inhibition of blood clotting results in sustained intratumour perfusion

Though we have previously found neutrophils to be critical in triggering a loss of tumour perfusion [5], we went on to investigate what downstream mediators may be activated by these inflammatory cells. It is well known that a pro-inflammatory environment can promote blood clot formation [7,8] suggesting a mechanism that could lead to the observed virus induced shutdown of tumour blood supply. Indeed, fibrin staining [9,10] demonstrates that VSV infection of CT-26 tumours triggers extensive blood clot formation within 24 h of infection (Figure 3.2a) and areas

Figure 3.1 *VSV infection and tumour perfusion are restricted to the tumour surface.* (a) A 3D tumour model was reconstructed from 2D digital images of serial histological sections. A CT-26 tumour-bearing BALB/c mouse was treated with VSV for 24 hours and perfused with fluorescent microspheres. Serial sections from the excised tumour were scanned for fluorescent microspheres in order to visualize perfusion and VSV infection was detected by immunohistochemistry. Viewing the tumour in *HTK Histology Toolkit* reveals that infection (red) and perfusion (blue) are limited to the tumour surface. Areas of infection are localized to one region of the tumour surface. 3D models of a VSV treated tumour and a PBS treated tumour were similarly constructed. (b) 3D models of tumour perfusion of a VSV treated tumour and a PBS treated tumour were reconstructed from images of serial histological sections. Viewing perfusion of whole and half 3D models reveals that the surface of both VSV and PBS treated tumours is perfused. Half models show that the tumour core of the VSV treated tumour is dramatically less perfused when compared to the PBS treated tumour. **(c)** Using *HTK Histology Toolkit*, scanning through the VSV treated tumour along x, y, and z axes consistently shows perfusion that is present in the tumour rim but absent from the core. In contrast, visualization of the PBS treated tumour shows perfusion throughout the tumour.


A
B
PBS treated
VSV treated
whole tumour
half tumour
C
PBS treated
VSV treated

of hemorrhage (as evidenced by extensive fibrin staining outside the vessels indicated leakage of blood products into extravascular space). In contrast, untreated control tumours contain unclogged vessels with no detectable fibrin deposits (Figure 3.2a). To test whether the presence of fibrin clots was responsible for the loss of blood flow in OV infected tumours, we attempted to inhibit clot formation during therapy using heparin or bothroalternin, a thrombin inhibitor isolated from snake venom [11]. In mice continuously treated with either thrombin inhibitor in combination with VSV, we found an even distribution of microspheres throughout the tumour (Figure 3.2b) suggesting that formation of micro-clots within tumour vasculature is responsible for the loss of blood flow we observed during OV therapy. In contrast, in mice treated with VSV in conjunction with tissue plasminogen activator (tPA) we observed maintenance of blocked blood flow in the tumour core (Figure 3.2b). Since tPA, a so-called "clot busting enzyme" is unable to prevent the loss of blood flow to the tumour, we conclude that the enzyme is unable to reverse clot formation once initiated in the tumour microvasculature. These experiments demonstrate that inflammation triggered by OV infection within tumours activates blood clot formation in tumour microvasculature resulting in a loss of tumour perfusion, an effect that can be reversed by treatment with thrombin inhibitors.

Blood clot formation is limited to tumours in OV treated mice

As an impressive induction in intravascular blood clot formation was observed in tumours of VSV treated mice, we went on to confirm that blood clot formation was limited to tumour tissues. We analyzed brain, lung, heart and tumour-adjacent skeletal muscle tissue and observed no increase in blood clot formation in these normal tissues taken from treated mice when compared to untreated controls (Figure 3.3a, quantification Figure 3.3 b).

Figure 3.2 *Oncolytic virus infection triggers coagulation within tumour vasculature and inhibition of coagulation results in sustained perfusion of OV treated tumours.* (a) BALB/c mice with CT-26 tumours were treated intravenously with VSV expressing green fluorescent protein (GFP) or PBS and sacrificed 24 h later. Sections were stained with antibody that detects fibrindeposits. Scale bars: 500 μm left images, 200 μm right images. (b) BALB/c mice with CT-26 tumours were treated intravenously with PBS or VSV as well as tissue plasminogen activator (tPA), bothroalternin or heparin intraperitoneally. Prior to being sacrificed, mice were perfused with fluorescent microspheres for 5 minutes. Tumour sections were prepared and analyzed for fluorescence using a microarray scanner. Black spots indicate perfused areas. Tumours excised from mice treated with PBS are uniformly perfused. Tumours taken from mice treated with VSV or VSV + tPA demonstrate a loss of tumour perfusion. In contrast, mice treated with thrombin inhibitors bothroalternin or heparin + VSV demonstrate uniform perfusion.

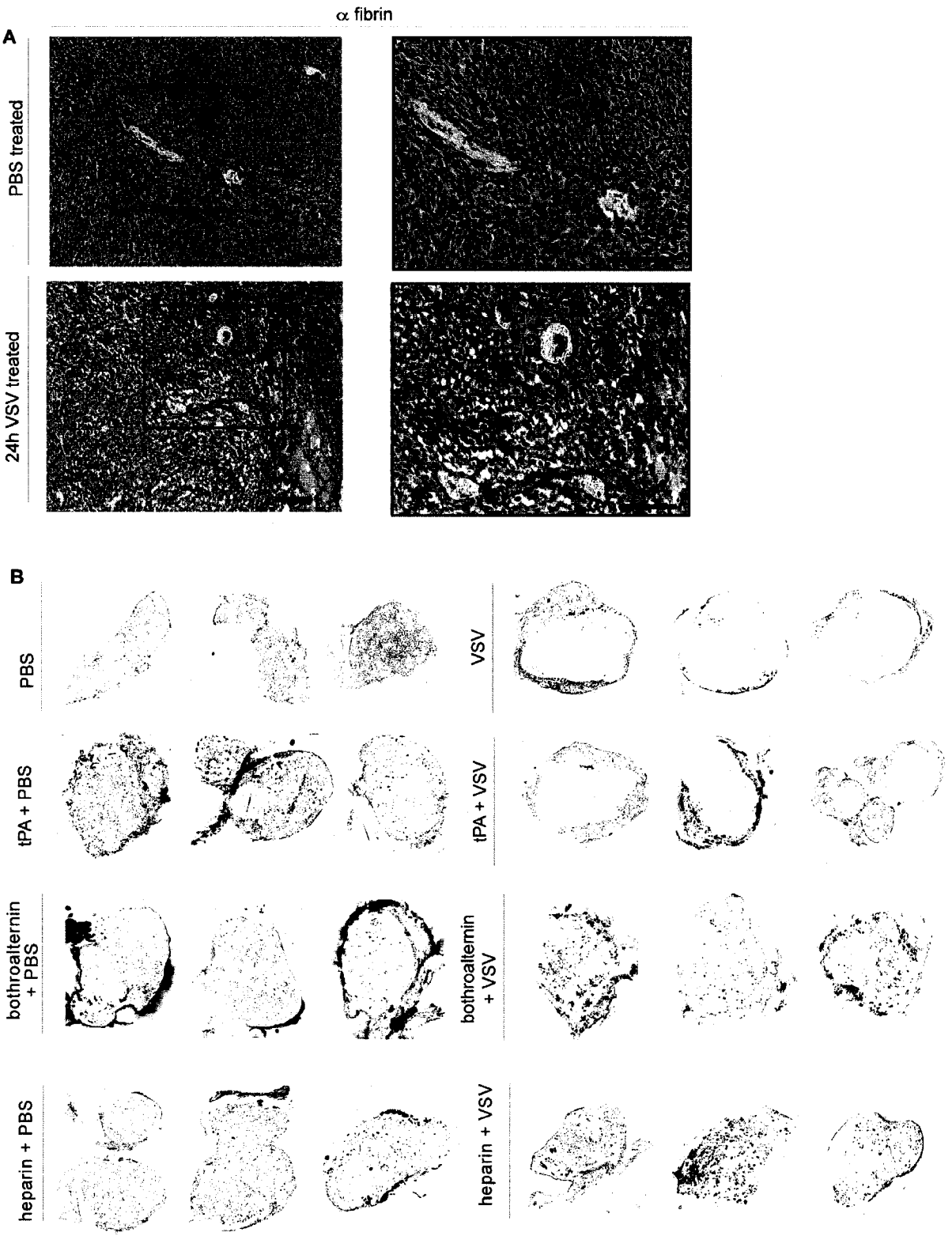
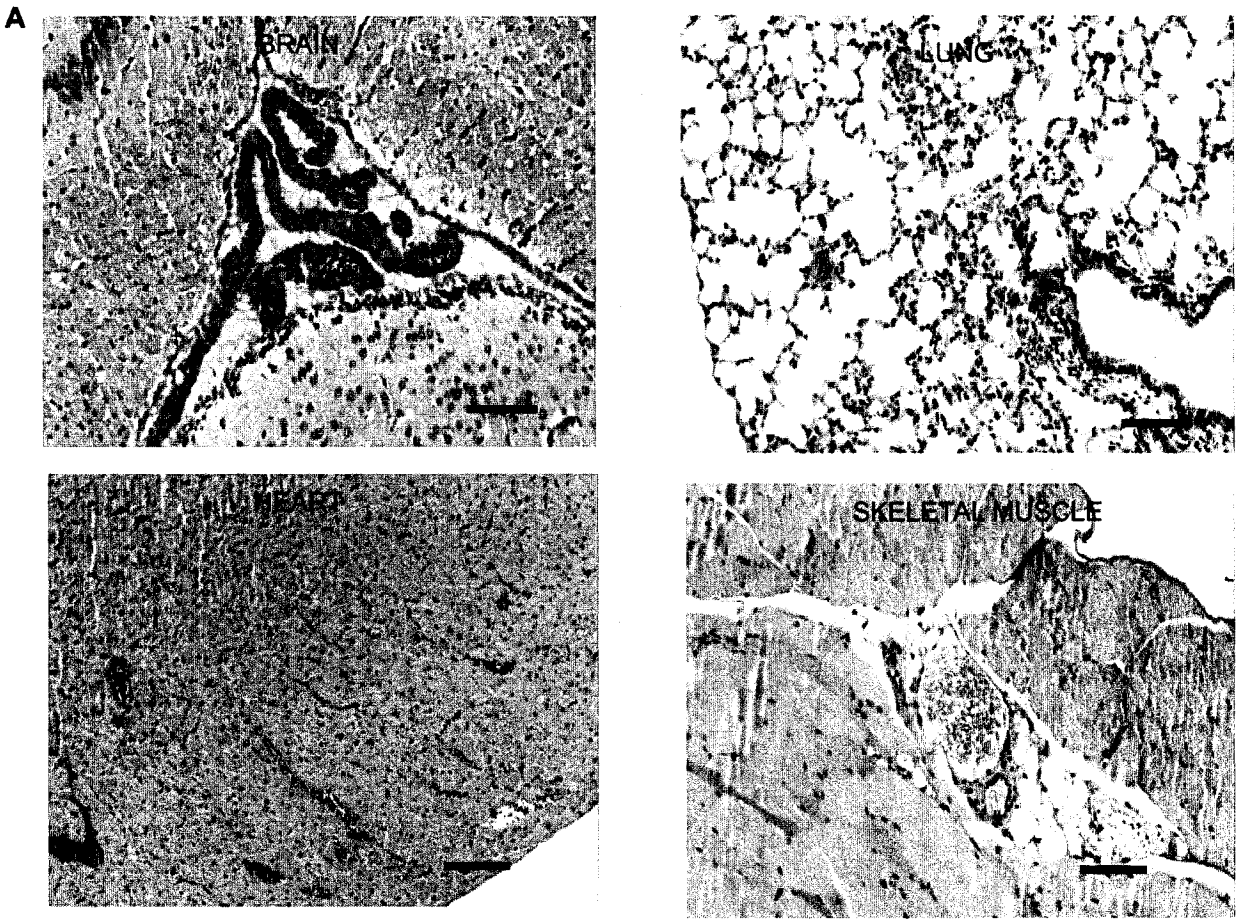

α fibrin
A
PBS treated
24h VSV treated
B
PBS
VSV
tPA + PBS
tPA + VSV
bothroalternin + PBS
bothroaltemin + VSV
heparin + PBS
heparin + VSV

Figure 3.3 *Vessels associated with normal tissues do not exhibit increased coagulation.* (a) Brain, lung, heart and tumour-adjacent skeletal muscle tissue were excised from tumour bearing BALB/c mice that were treated intravenously with VSV. Hematoxylin & Eosin stained sections of vessels associated with normal tissues are shown. Scale bars: 100 μm (b) % clotted vessels were quantified in tumour, brain, lung, heart and tumour-adjacent skeletal muscle tissue from untreated mice and mice treated intravenously with VSV.


A
BRAIN
LUNG
HEART
SKELETAL MUSCLE
B
% clotted vessels
100
75
50
25
0
untreated
treated
tumour
brain
lung
heart
skeletal muscle

Intratumour coagulation is dependent on neutrophils

We have demonstrated previously that depletion of neutrophils abrogates loss of tumour perfusion following oncolytic virus infection [5]. Given the observation that virus infection triggers coagulation in tumour associated vessels, and that coagulation can be triggered by inflammation during 'natural' pathological situations [12], CT-26 tumours taken from neutrophil depleted mice treated with VSV were tested for the presence of fibrin clots. Interestingly, a reduction of blood clot formation was observed in tumours taken from neutrophil depleted VSV treated mice which suggests that neutrophils are required for induction of coagulation in tumours treated with oncolytic viruses (Figure 3.4). This underlines the importance of an inflammatory reaction in triggering coagulation and loss of tumour perfusion.

Distribution of tumour vasculature changes upon virus treatment

Next we wanted to study the consequence of drastic changes in tumour perfusion upon virus treatment on the distribution of tumour vasculature. Tumours removed from mice treated with control, replication incompetent UV inactivated VSV were compared to tumours taken from mice treated with VSV for 15h (a timepoint at which we observe loss of tumour perfusion, data not shown). Overall fewer vessels could be detected in virus treated tumours. Also, a change in the distribution of vessels was observed with control UV inactivated VSV treated tumours exhibiting uniformly distributed vessels while tumours taken from replication competent VSV treated mice mainly contained vessels in the tumour rim(Figure 3.5). This is consistent with the observations from microsphere infusion experiments in which perfusion is consistently associated with the tumour rim.

Figure 3.4 *Decreased clot formation in tumours from neutrophil depleted mice.* BALB/c mice bearing CT-26 tumours were pre-treated with anti-GR-1 antibody or 50:50 rat serum:phosphate-buffered saline intraperitoneally 24 h prior to intravenous treatment with VSV. Twenty-four h after virus treatment, mice were euthanized and tumours embedded for sectioning. Sections were stained to detect fibrin deposits. Decreased fibrin deposition is detected in tumours taken from neutrophil depleted mice. Scale bars:100 μm.

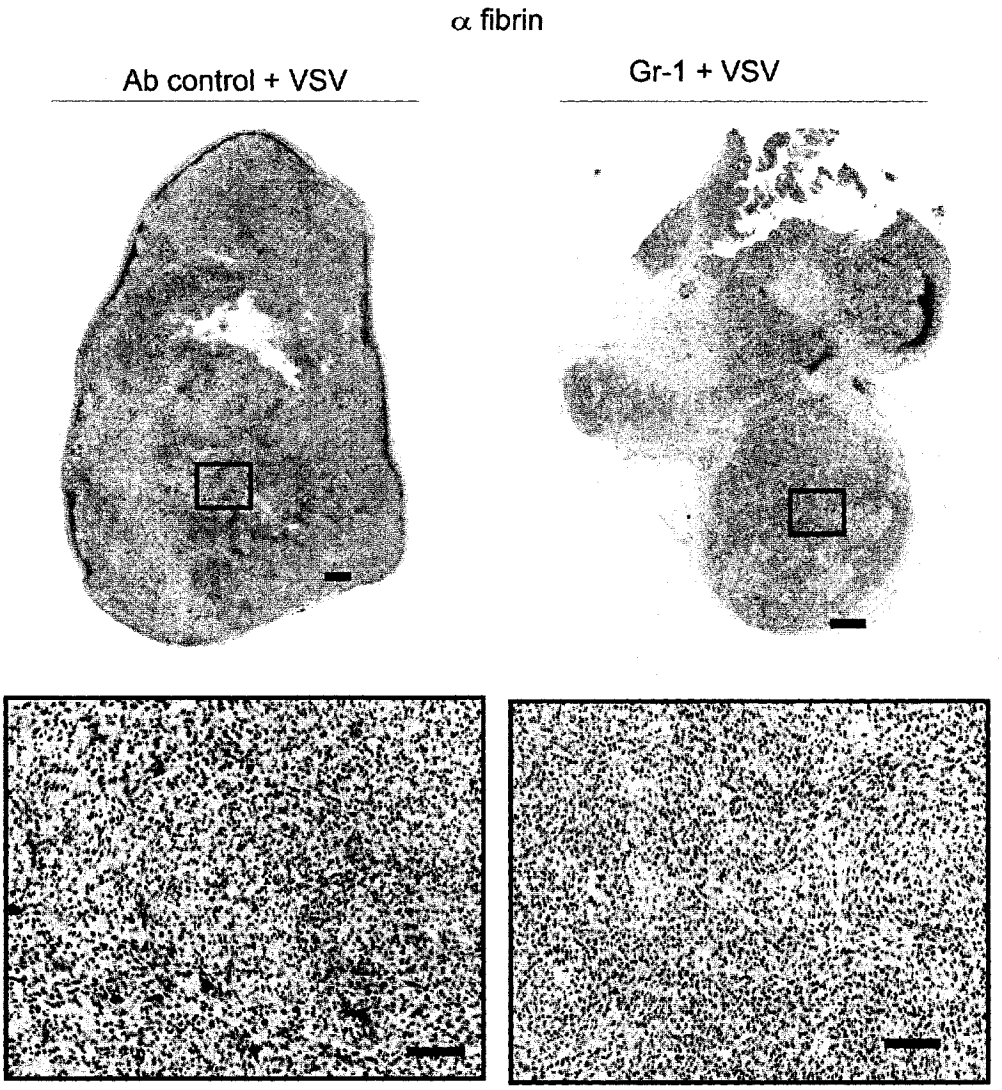
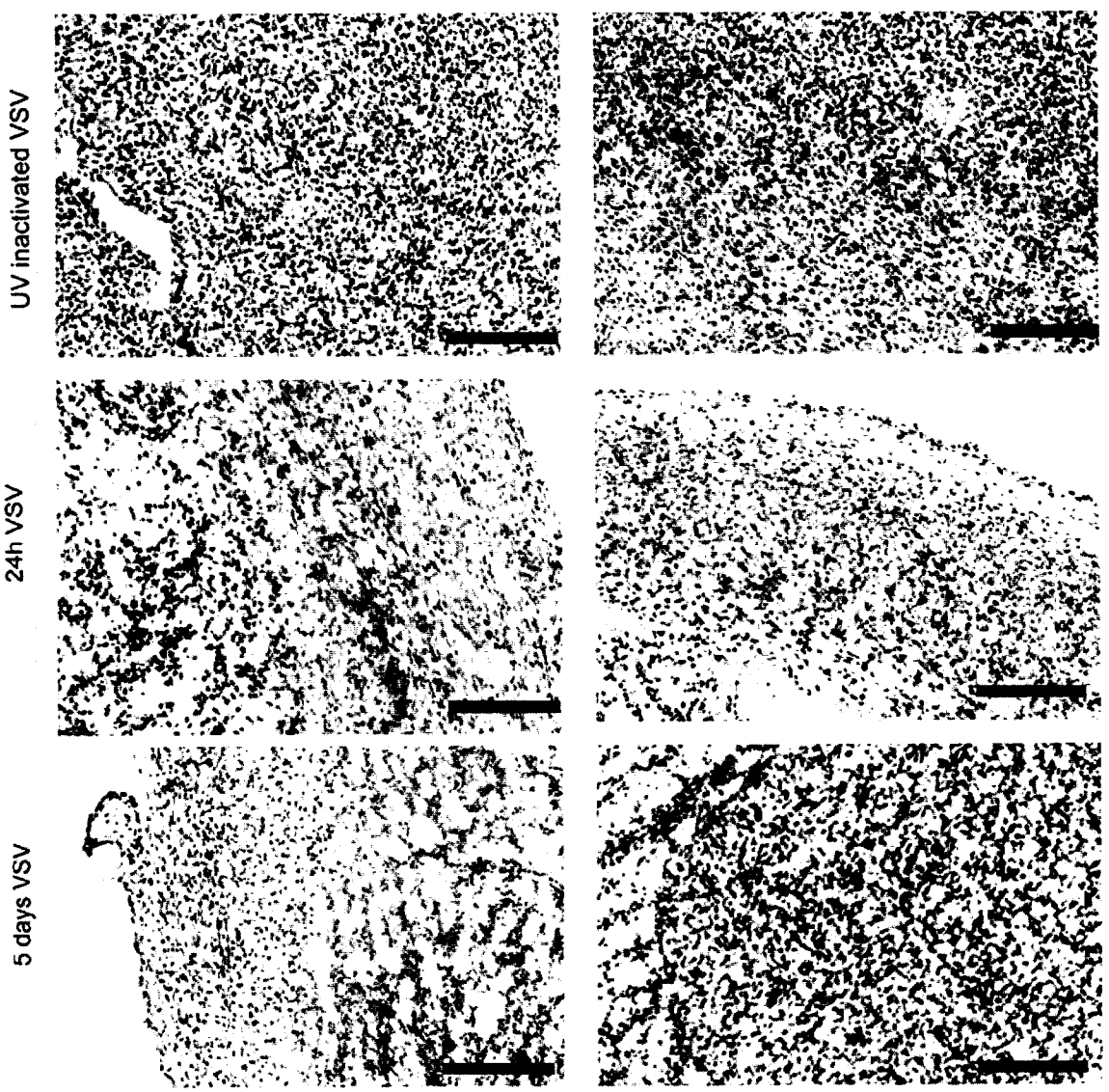

α fibrin
Ab control + VSV
Gr-1 + VSV

Figure 3.5 *Vessels are restricted to tumour rim following VSV treatment.* BALB/c mice bearing CT-26 tumours were treated intravenously with UV inactivated VSV, or VSV intravenously. Mice were euthanized at the indicated timepoints. Tumours were frozen for sectioning. Tumour sections were stained with an antibody detecting CD31, an endothelial cell marker. Endothelial cells are uniformly distributed in control tumours but are restricted to the tumour edge following VSV treatment. Scale bars: 200 μm. Image contrast was adjusted to 50 in all images using Adobe Photoshop Version 8.


UV inactivated VSV
24h VSV
5 days VSV

Viable tumour rim persists following virus treatment

As we have reported previously [5], virus infection triggers apoptosis of the tumour core along with a reduction of tumour perfusion. Given that intact tumour vasculature is associated with tumour cells in the outer tumour rim, we went on to investigate whether the tumour cells in the rim also remain viable. Hematoxylin & eosin staining of VSV treated tumours reveal an intact tumour rim with unclotted vessels (Figure 3.6). There exists a clear delineation between the tumour rim and the apoptotic tumour core at 24 h post infection. Indeed, cells within the tumour core are undergoing apoptosis as evidenced by condensed nuclei (and as has previously been demonstrated with immunohistochemical detection of active caspase 3 [5]). Furthermore, red blood cells have lost their definition and color, a property red blood cells acquire when associated with blood clots.

Immunohistochemical staining detecting Ki67, a marker of proliferating cells, confirmed that many cells within the tumour rim remain viable (Figure 3.6). A comparison of tumour cell replication between different tumours (e.g. virus treated versus untreated) is not ideal, as it is well established that tumour cells do not proliferate at the same rate throughout the tumour. Therefore, mice were pulsed with BrdU which is incorporated into the DNA of replicating cells at the time of infusion. At 24 h post BrdU treatment mice were challenged with VSV and euthanized the following day (Figure 3.7a). BrdU staining of untreated tumours correlates with Ki67 staining which marks cells proliferating at the time of mouse euthanasia. However, in tumours taken from mice treated with virus, BrdU staining is uniform throughout the entire tumour, while Ki67 staining is only detected in the tumour rim, as described above. Small areas of virus staining were also detected within the tumour rim (Figure 3.7b). This experiment therefore demonstrates that the distribution of tumour cell proliferation changes drastically over the course of virus treatment. In addition, this observation confirms that the reduction in cell proliferation,

Figure 3.6 *Cell proliferation is restricted to the tumour rim at early and late timepoints following VSV treatment.* BALB/c mice bearing CT-26 tumours were treated intravenously with VSV. Mice were euthanized at the indicated timepoints and tumours embedded for sectioning. Serial sections were stained by hematoxylin & eosin stain as well as immunohistochemical detection of Ki67, a marker of cell proliferation. At 24h, there is a clear delineation between viable tumour tissue in the tumour rim versus apoptotic cells within the tumour core. By 22 days post infection, extensive necrosis is evident within the tumour core however viable tumour cells persist within the tumour rim. Scale bars: 500 μm left images, 200 μm right images. Image contrast was adjusted to 50 in all Ki67 stained images using Adobe Photoshop Version 8.

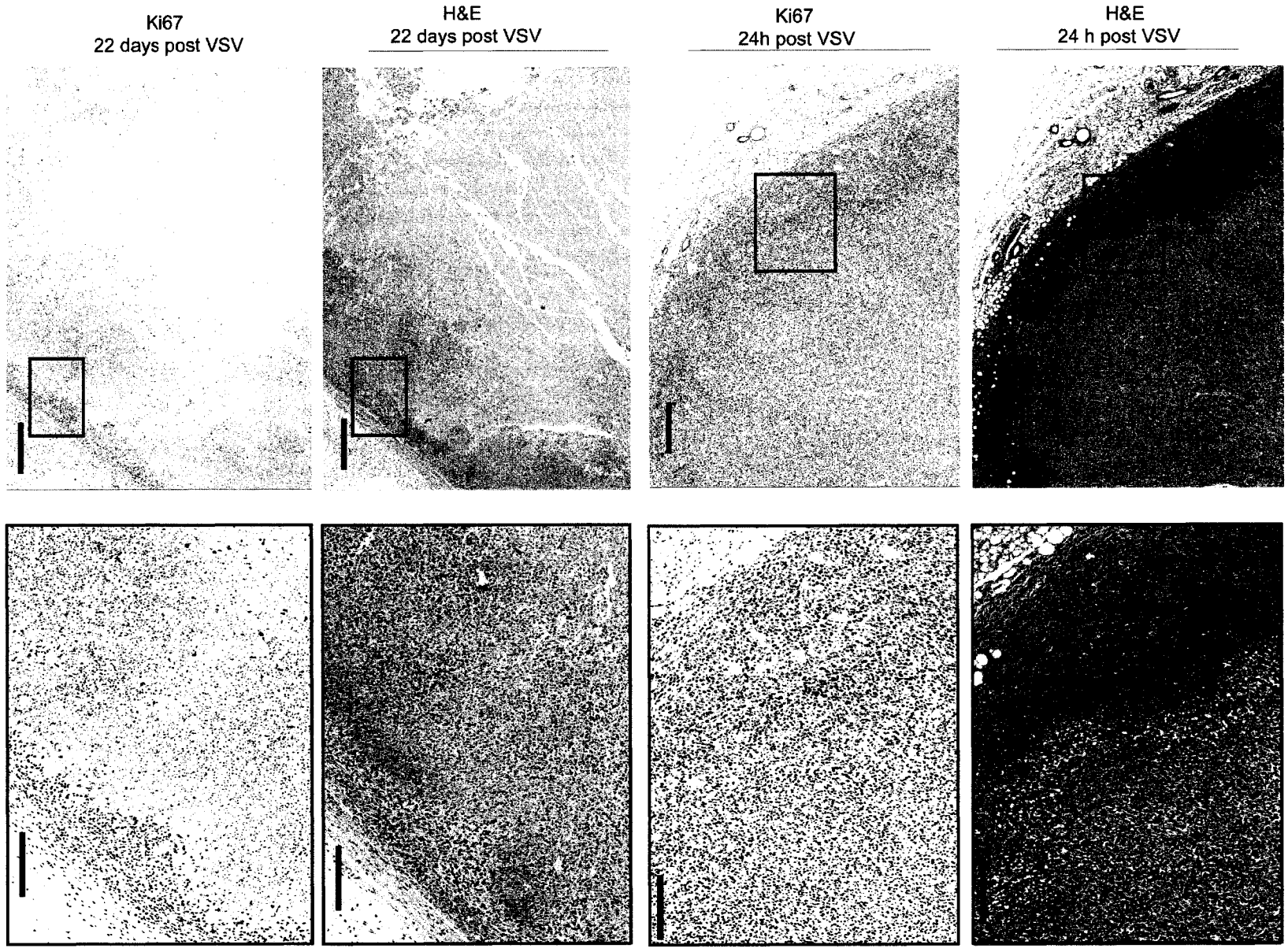
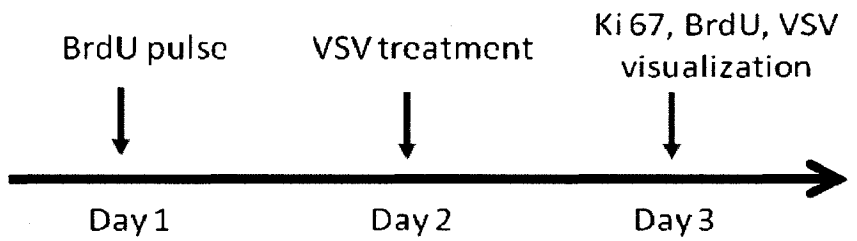
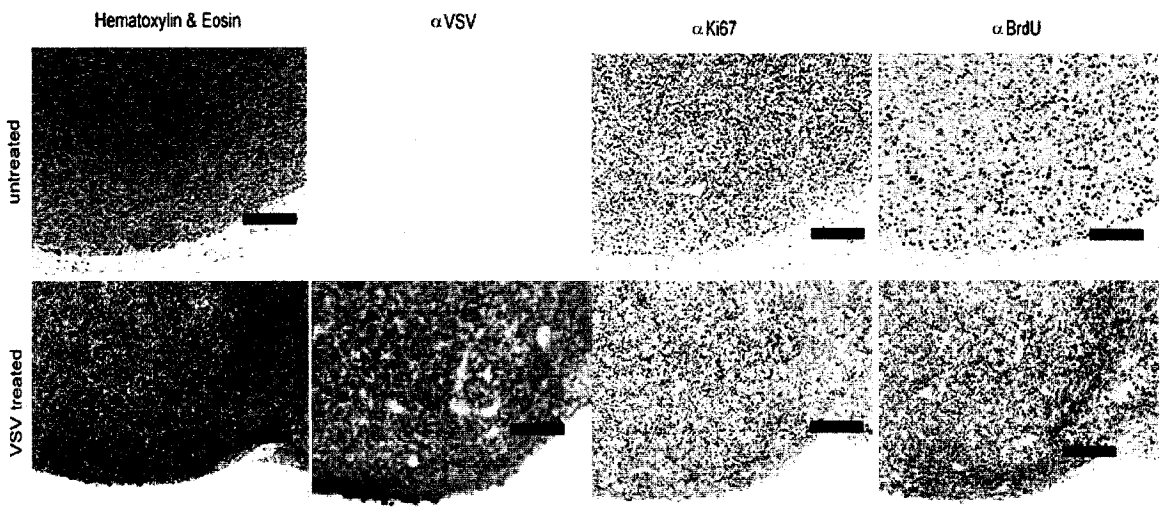

Ki67
22 days post VSV
H&E
22 days post VSV
Ki67
24h post VSV
H&E
24 h post VSV

Figure 3.7 *BrdU pulse experiments validate change in the distribution of cell proliferation over the course of VSV treatment.* (a) Experimental setup. BALB/c mice bearing subcutaneous CT-26 tumours were pulsed with BrdU 24 h prior, or 22 h after, receiving VSV intravenously (or left untreated). 24 h following virus challenge, mice were euthanized and tumours were embedded for sectioning. (b) Tumour sections were stained for immunohistochemical analysis to detect VSV (virus infection), Ki67 (cell proliferation) and BrdU (proliferating cells that incorporated the dye). Serial sections were stained with hematoxylin & eosin to visualize tissue architecture. Scale bars: 200 μm. Image contrast was adjusted to 50 in all Ki67 stained images using Adobe Photoshop Version 8.

A


BrdU pulse
VSV treatment
Ki 67, BrdU, VSV visualization
Day 1
Day 2
Day 3

B


Hematoxylin & Eosin
αVSV
αKi67
αBrdU
untreated
VSV treated

though triggered by virus infection of tumours, is largely a result of uninfected tumour cell death triggered by tumour targeted inflammation and coagulation.

Though virus infection is observed mainly within areas in the tumour rim (Figure.3.1a) it is apparent that the virus is not able to infect all viable tumour cells. This observation would lead to the prediction that tumour bearing mice would not be cured with one dose of VSV treatment. However, a cure rate of 60% is observed in this model (data not shown) which led to the investigation of tumour histology at late timepoints post infection. Analysis of tumours 22 days post virus infection surprisingly revealed a thin remaining viable rim of tumour cells (Figure 3.6) as reported in [13] and [5], virus replication persists for 3 to 4 days prior to the onset of a neutralizing antibody response, suggesting that virus infection is not limiting outgrowth of the tumour rim.

The adaptive anti-tumour immune response controls viable rim outgrowth

As virus infection is not controlling tumour cell replication at late timepoints and since we have observed poor efficacy in a nude (immune deficient) mouse model of the same CT-26 tumour (unpublished data), we investigated tumour histology at early (1 day) and late (22 day) timepoints post virus infection. Acute treatment of CT-26 bearing nude mice with VSV results in a loss of tumour perfusion (similar to results obtained with human SW620 tumours implanted subcutaneously in nude mice as reported previously [5]). Therefore, tumour histology one day post VSV treatment is similar to that observed in the immune competent model (comparing Figure 3.6 to Figure 3.8). However, tumours taken from nude mice at the late timepoint are large (note change in scale) with an expanded viable rim. This observation suggests that an anti-tumour T cell response is triggered in immune competent mice and controls outgrowth of the viable tumour rim. Conversely, nude mice do not produce thymus-derived T cells and are therefore unable to control tumour outgrowth following oncolytic virus therapy.

Figure 3.8 *Viable tumour rim expands in immune competent mice following VSV treatment.* CD1 nude mice bearing CT-26 tumours were treated intravenously with VSV. Mice were euthanized at the indicated timepoints and tumours embedded for sectioning. Serial sections were stained by hematoxylin & eosin stain as well as immunohistochemical detection of Ki67, a marker of cell proliferation. At 24h, there is a clear delineation between viable tumour tissue in the tumour rim vs apoptotic cells within the tumour core. By 22 days post infection, extensive necrosis is evident within the tumour core and viable tumour cells have expanded to form a large tumour rim (note change in scale). Scale bars: 200 μm, 500 μm left images (Ki67 22 days left bottom). Image contrast was adjusted to 50 in all Ki67 stained images using Adobe Photoshop Version 8.

H&E
24 h post VSV
Ki67
24h post VSV
H&E
22 days post VSV
Ki67
22 days post VSV

Viable tumour rim and tumour restricted coagulation detected in patient following treatment with vaccinia virus

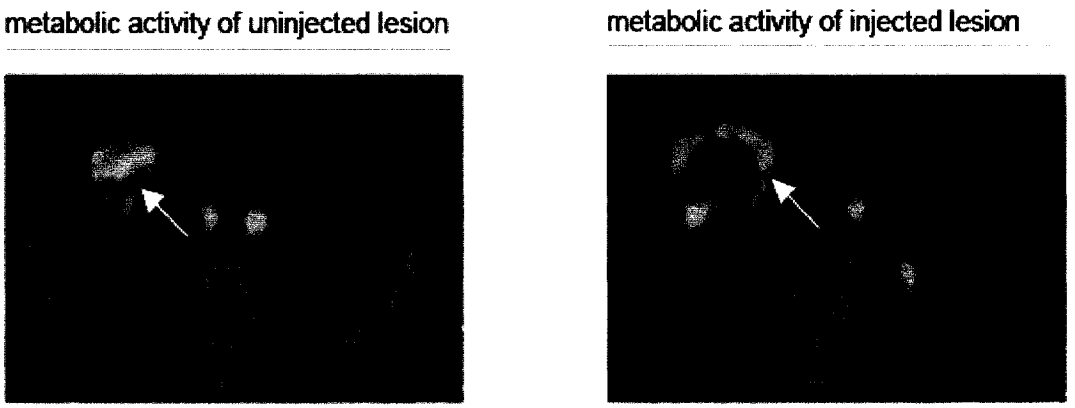
An oncolytic version of vaccinia virus has previously been shown to trigger loss of tumour perfusion in preclinical models, similar to VSV [5]. In addition, acute effects of vaccinia virus infection of a liver mass (hepatocellular carcinoma) were investigated in patients enrolled in a phase I clinical trial. Vaccinia virus was shown to similarly cause a loss of tumour perfusion in patients [6]. Functional positron emission tomography (PET) scanning post treatment in a patient with metastatic esophageal cancer indicates that metabolic activity is restricted to the tumour rim following vaccinia virus therapy, consistent with the high rate of tumour cell proliferation observed in preclinical models presented above. A control uninjected tumour from the same patient demonstrates more uniform metabolic activity (Figure 3.9a). Finally, we obtained autopsy specimens from the same patient. Blood clot formation was exclusively detected within tumour vasculature (Figure 3.9b). Vessels associated with adjacent normal tissue remain unclotted. Histological analysis confirmed that the clot was formed pre-mortem. This observation is consistent with our findings from preclinical models that demonstrate blood clot formation within tumour associated vasculature. This observation, combined with the finding that vaccinia virus replication results in a significant decrease of tumour perfusion indicates that OVs may also cause tumour regression via multiple mechanisms (including targeted inflammation and coagulation) in patients.

Discussion

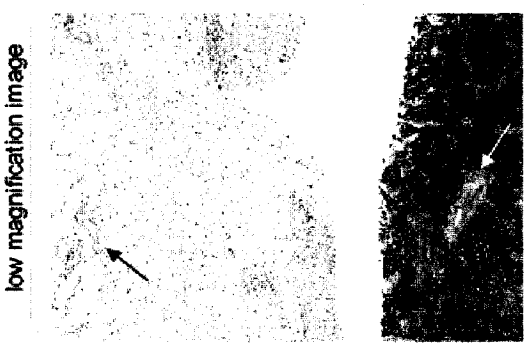
The impact of the acute host response to OVs has previously been investigated by our group as well as others and is reviewed in [14,15]. Though tumour targeted inflammation can result in

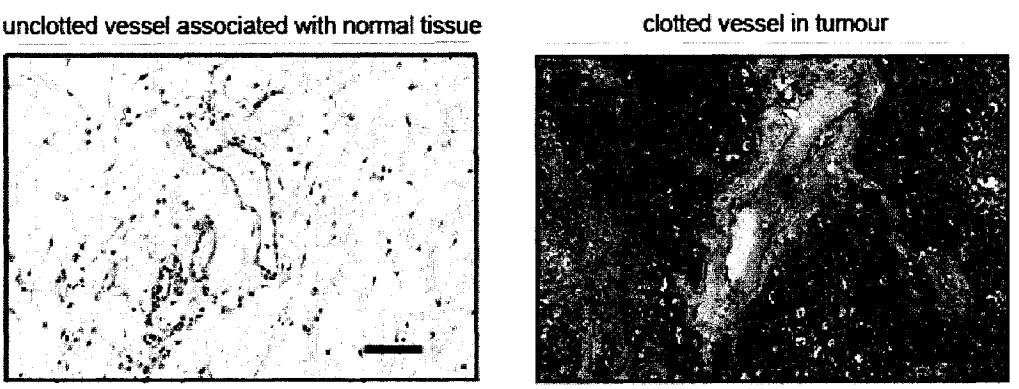
Figure 3.9 *Decreased metabolic activity and coagulation detected in injected liver lesion from patient with metastatic esophageal cancer treated with oncolytic vaccinia virus.* (a) Patient was treated with 3.3 x 10^8 pfu vaccinia virus into liver metastasis of esophageal cancer on days 1 and 15. Patient was given additional injection of 3.3 x 10^8 pfu into neck lesion. Positron emission tomography (PET) image was acquired 30 days after the first virus injection. Ring of metabolic activity remains in injected tumour while metabolic activity in uninjected control tumour (note different plane) remains evenly distributed. (b) Coagulation in autopsy specimen taken from neck lesion of same patient. Clotted vessel detected in tumour associated tissue while vessel associated with adjacent normal tissue remains unclotted. Scale bars 200 μm.

A


metabolic activity of uninjected lesion
metabolic activity of injected lesion

B


low magnification image

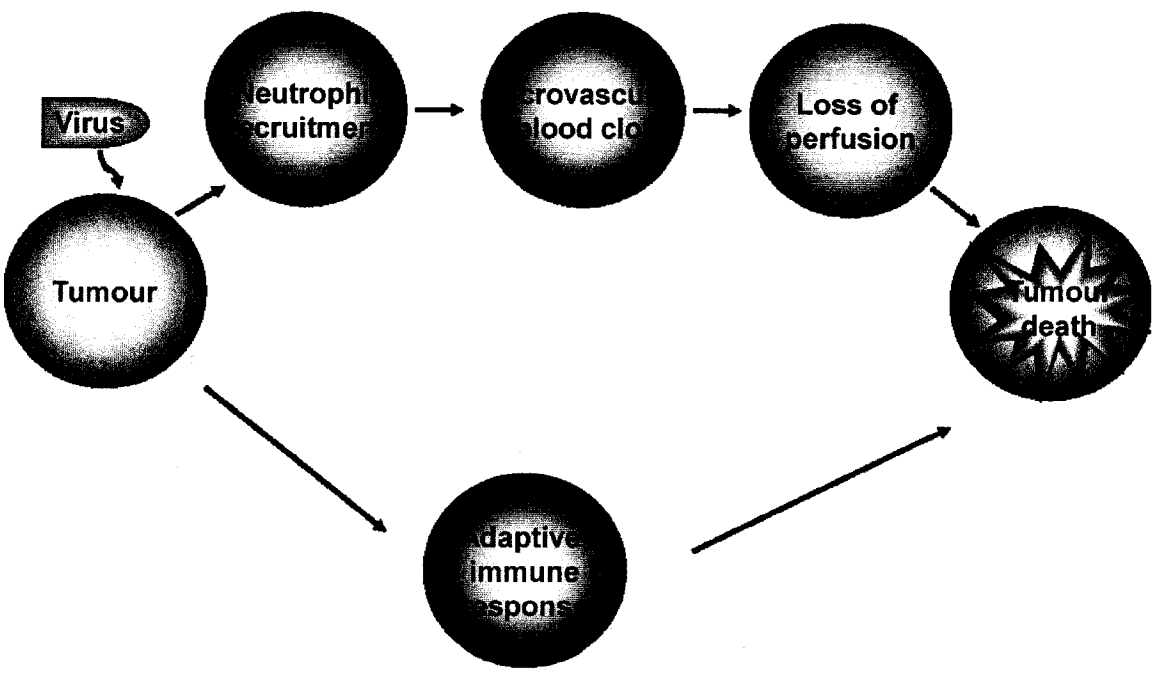

unclotted vessel associated with normal tissue
clotted vessel in tumour

significant cell death of uninfected tumour cells it may also limit the ability of the virus to persist in tumours. Indeed, neutrophil depletion allowed for more robust virus replication in tumours when compared to neutrophil replete controls [5]. Furthermore, numerous reports now indicate that general immunosuppression can result in increased virus titers within tumours. This is in part attributed to decreased clearance of virus by cells of the monocytic lineage [16-18]. Increased VSV replication was also detected upon inhibition of NK and NKT cells *in vivo* [19,20]. However, there are disadvantages to immunosuppression, including increased toxicity due to ablation of a neutralizing antibody response [21] as well as possibly inhibiting the beneficial anti-tumour immune response triggered by OV infection of tumours. Indeed, there exists increasing evidence that VSV infection of tumours can induce an adaptive anti-tumour immune response [22]. We have demonstrated here that virus infection within tumours and the ensuing inflammatory response trigger an adaptive immune response that controls outgrowth of viable tumour cells within the tumour rim. The exact delineation of the adaptive immune response in the context of VSV therapy of CT-26 tumours is currently being investigated and is beyond the scope of this study. All together, these data demonstrate that oncolytic viruses may unexpectedly target tumours in 3 different ways: *(1)* lysing of virus infected cells, *(2)* triggering tumour targeted inflammation and coagulation resulting in death of uninfected tumour cells early after infection and *(3)* inducing an adaptive anti-tumour T cell response that controls viable rim outgrowth and is essential to ultimately cure mice (see model Figure 3.10). Though chronic, smouldering inflammation has been linked to the progression of various cancers [23-26], evidence is beginning to emerge that the inflammatory response can aid tumour regression in the context of anticancer therapies. Indeed, the concept of using the acute host response to target tumours was explored as early as the 1800s when William Coley injected bacterial products into patients with advanced stage cancers [27,28]. Though the approach was even controversial at the time, his treatments resulted in tumour regression in certain types of cancers

including sarcomas, lymphomas, breast and ovarian cancers [29]. With accumulating evidence that inflammation may be a double-edged sword in the context of cancer [30,31], it has become important to understand under what circumstances the acute host response to infection and tissue injury can be harnessed to target tumours.

We have used oncolytic viruses (OVs) to probe the acute host response to anticancer therapy. Conceptually, virus infection is likely to stimulate an exaggerated host response to infection as the host has developed a rapid response system to contain and shut down virus infection. However, with the continuous identification of greater numbers of endogenous danger signals with the capability of triggering inflammation [32-34], conventional anticancer therapies that induce tissue damage within tumours may have the capability of triggering a similar host response. Indeed, a similar pattern of cell death (destruction of the tumour core with maintenance of a viable rim) has long been recognized following treatment with vascular disrupting agents (VDAs) [35]. Though evidence of the contribution of inflammation to tumour regression is only beginning to emerge in the context of VDA treatment, tumour regression following photodynamic therapy (PDT) has been attributed to the acute inflammatory response triggered by initial tissue damage [36]. Interestingly, inhibition of coagulation also reduced PDT efficacy [36]. Though an exaggerated inflammatory response has the capability of causing damage to normal tissues (e.g. ischemia/reperfusion injury [37-39]), acute inflammation causes greater damage to tumour associated vasculature rather than normal vasculature in the context of OV, VDA and PDT therapies. The tumour targeted effect is likely attributed to the erratic structure and composition of tumour vasculature that renders it more prone to coagulation [40-44]. The difference in susceptibility of normal versus tumour associated vessels may be exploited to improve the tumour targeted cytotoxicity of

Figure 3.10 *Model of oncolytic virus mediated tumour killing.* Virus infection of tumours triggers an inflammatory response and subsequent microvascular blood clot formation which results in a loss of tumour perfusion and uninfected tumour cell death. Virus infection also triggers an adaptive anti-tumour immune response which controls outgrowth of viable tumour cells.


Virus
Tumour
Loss of
perfusion
death

diverse cancer therapeutics. Therefore, further study of the effect of inflammation on tumour regression in response to diverse anticancer agents is warranted.

Importantly, loss of tumour perfusion following OV therapy has now also been documented in patients. Vaccinia virus infection of hepatocellular carcinoma resulted in a 43% decrease in tumour perfusion at 6 days post treatment [6]. Similar to the acute effects of VSV infection of tumours, vaccinia virus was also shown to disrupt tumour perfusion in preclinical models [5]. Here we report decreased metabolic activity associated with the tumour core as well as the presence of blood clots exclusively within tumour associated vasculature in a patient tumour following treatment with vaccinia virus. These observations are consistent with acute effects of OV infection of murine tumours. Also, preliminary data out of clinical trials testing oncolytic adenovirus or herpes simplex virus (HSV) indicate decreased metabolic activity and increased necrosis of tumours which are also consistent with the effects observed in murine tumour models [45,46]. The study presented here contributes to our knowledge of the mechanism of inflammation induced tumour cell death. With increased evidence that inflammation may cause damage to tumours through disruption of tumour perfusion in patients undergoing OV therapy as well as evidence that inflammation can cause damage to tumours following treatment with diverse anticancer agents, this study has major clinical implications possibly beyond the field of oncolytic virus therapy.

Materials & Methods

Viruses. The Indiana serotype of VSV was used throughout this study and was propagated in Vero cells (American Type Culture Collection). AV1 VSV is a naturally occurring interferon inducing mutant of VSV [4] while Δ51 VSV expressing GFP [4] is a recombinant interferon inducing mutant of the HR strain of wild-type VSV Indiana. Virions were purified from cell culture supernatants by passage through a 0.2 μM Steritop filter (Millipore, Billerica, MA) and centrifugation at 30 000 x g before resuspension in phosphate-buffered saline (PBS) (HyClone, Logan, UT). A modified Vaccinia virus (JX-594) was used to treat patient with hepatocellular carcinoma. Genetic modifications are deletion of thymidine kinase gene and insertion of human granulocyte-colony stimulating factor (GM-CSF) [48].

Cell lines. CT-26 (murine colon adenocarcinoma) derived cells were purchased from American Type Culture Collection and cultured in HyQ Dulbecco's Modified Eagle Medium (High glucose) (HyClone) supplemented with 10% fetal calf serum (CanSera, Etobicoke, Ontario, Canada).

Tumour models. Female 6-8 week old BALB/c mice were obtained from Charles River Laboratories (Wilmington, MA). Syngeneic subcutaneous tumours were established by injection of 3 x 10^5 cells in 100 μl PBS (CT-26) in the left and right hind flanks. When tumours reached a palpable size, mice were treated with VSV by tail vein injection. Mice were euthanized at the indicated timepoints by cervical dislocation and one portion of tumours were frozen in Shandon Cryomatrix freezing medium (ThermoElectron Corporation, Waltham, MA) on dry ice. Five or 10 μM sections were cut using a Microm HM500 OM cryostat. Another portion of tumours was placed in 10% formalin and embedded in paraffin. For some experiments, brain, lung, heart and

tumour adjacent skeletal muscle tissue were also collected and paraffin embedded for histological analysis. Mice were not perfused prior to organ collection. All experiments were conducted with the approval of the University of Ottawa Animal Care and Veterinary Service.

Histological and Immunohistochemical analysis. Immunohistochemistry detecting fibrinogen and its cleaved form fibrin (1:500, DAKO, Glostrup, Denmark) was performed on paraffin-embedded sections using the Vectastain ABC kit for rabbit primary antibodies (Vector Labs, Burlingame, CA), according to instructions provided. The antigen retrieval step was omitted. For Ki67 detection, paraffin embedded sections were boiled in 10 mM citrate buffer, pH 6. Primary anti-Ki67 antibody (1:25 dilution, DAKO, Glostrup, Denmark) was applied overnight and detected using anti-rat antibody detection system (DAKO, Glostrup, Denmark). BrdU was detected using the Vectastain ABC kit for rat primary antibodies (Vector Labs, Burlingame, CA), on paraffin embedded sections treated with 4N HCl. Primary anti-BrdU (1:100 dilution, Abcam, Cambridge, USA) was applied for an hour. Horseradish peroxidase (HRP) activity was visualized with a Diaminobenzene-HRP kit (KPL Biosciences). Nuclei were counterstained in hematoxylin. For assessment of cell morphology, sections were stained with hematoxylin and eosin (H&E) according to standard protocols. Staining was digitized using Aperio ScanScope (Axiovision Technologies) and analyzed using Aperio ImageScope software. Blood clots in normal tissues were quantified on H&E stained sections. Thirty vessels were counted per section.

Analysis of tumour perfusion. Mice were injected intravenously with 100 µl of a 50% solution of 100 nm diameter orange fluorescent microspheres (Molecular Probes, Burlington, Ontario, Canada). Five minutes later, animals were euthanized and tumours immediately snap frozen as previously described. Tumour perfusion was analyzed by visualizing fluorescent

microspheres in the vasculature of 10 μm unfixed frozen sections using a ScanArray Express microarray scanner with a standard Cy3 laser (Packard Bioscience).

***In vivo* neutrophil depletion.** Mice were injected intraperitoneally with 150 μg purified RB6 8C5 rat monoclonal antibody, clone RB6-8C5 [49] (BD Pharmingen, Rockville, MD) in order to systemically depleteneutrophils. An aliquot of 150 μl non-immune rat serum was utilized as a negative control. Twent four hsubsequent to virus infection, mice were treated intravenously with 5 x 10^8 pfu Δ51 VSV GFP, perfused with fluorescent microspheres 24 h later and sacrificed by cervical dislocation and tissues collected and stained as described above.

Manipulation of blood clot formation. Tumour bearing mice were treated with VSV or PBS i.v. and 200 U/kg heparin (dalteparin sodium injection (Fragmin), Pfizer, New York, NY), i.p. 4 times over the course of 24 h starting at the time of VSV injection and ending at 22 h when the mouse was euthanized. Bothroalternin (Cedarlane, Burlington, ON) or tissue plasminogen activator (tPA) (Activase rt-PA, Roche, Basel, Switzerland) were injected at 20 U/kg and 4 mg/kg respectively i.p. 1 h prior to intravenous VSV or PBS infusion and another 3 times over the course of 21 h prior to euthanizing the mice. In all experiments, mice were injected intravenously with fluorescent microspheres prior to sacrifice in order to visualize tumour perfusion.

BrdU pulse. Tumour bearing mice were treated with 100mg/kg 5'-Bromo-2'-Deoxyuridine (BrdU) (Sigma, Oakville, ON) i.p. 24 h prior, or 22 h post, intravenous injection of VSV or PBS. Twenty four h following virus challenge, mice were euthanized and tumours removed, paraffin embedded and analyzed by immunohistochemistry as described above.

3D Modeling of Tumour Vascularity During Oncolytic Virus Therapy. CT-26 tumour bearing mice were treated with VSV or PBS and euthanized 24 hours later. Microspheres were injected as described above and the tumour was collected and frozen. The tumour was cut

into 1085 6μm tissue sections and mounted on slides. Every fifth section was scanned for microspheres and immunohistochemically stained for VSV as described above. The sections were stained for VSV using the Autostainer Plus (DakoCytomation). HTK Histology Toolkit software (Robarts Imaging Institute, University of Western Ontario) was used to convert 2D images into 3D models. Volume reconstruction was completed using alignment and segmentation contouring algorithms which oriented each tissue section on top of one another. Each tissue section image, once oriented, was then converted from 2D pixels into 3D voxels. These 3D stacks were then rendered to generate the reconstructed tumour. 2D images of microspheres were used to generate a model of perfusion, while 2D images of VSV staining were used to generate a model of infection. An overlay model was generated from superimposed images of microspheres and VSV. Regions of infection were highlighted in red to aid in visualization (Adobe Photoshop CS2).

Statistical analysis. All statistical analysis was performed using Graphpad Prism 3.0 software. Data are represented as a mean +/- standard deviation.

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CHAPTER 4: DISCUSSION

4.1 The tumour microenvironment and acute response to oncolytic virus infection: friend or foe?

Though much is known about the interaction of viruses and the host in pathological situations, 'artificial' intravenous or intratumoural administration of OVs for the treatment of cancer likely does not mimic normal exposure to virus pathogens. Furthermore, the nature of the tumour microenvironment is different from normal tissues. Tumour perfusion is not reliable due to erratic and tortuous tumour vasculature (240). Tumours are associated with inflammatory cells (as discussed in introduction) as these cells have been shown to enhance tumour growth when chronically activated within the malignancy. This 'unnatural' environment within tumours distorts the interaction between viruses and tumour tissue. Therefore, much is to be learned about the effect of the tumour microenvironment and the associated innate immune response on the ability of the virus to be delivered and efficiently spread throughout the tumour.

Intravenous infusion of VSV results in poor delivery of virus to the tumour (215). This virus amplifies within the tumour microenvironment. Systemically, the host responds rapidly by producing neutralizing antibodies to VSV-G, thereby inhibiting virus spread at approximately 4 days post infection of naïve mice (222). Virus infection of tumours also triggers an inflammatory response within the tumour microenvironment. Likely due to differences between normal and tumour vasculature (e.g. the erratic structure and composition), tumour vasculature is more prone to damaging effects of inflammation and resultant coagulation than normal vasculature. This

observation may have positive or negative ramifications on the efficaciousness of OV therapy. The following paragraphs initially discuss detrimental effects of tumour microenvironment and the acute response to OV infection. Subsequently, aspects of tumour vasculature and host response to OV infection that may benefit therapy will be discussed.

4.1.1 Detrimental effects

In this first section, innate barriers to oncolytic viruses will be outlined, along with strategies to overcome these barriers (some of which are discussed in (223)).

4.1.1.1 Inefficient virus delivery to tumours

Many oncolytic viruses are attenuated human pathogens (42). Therefore, humans may produce circulating neutralizing antibodies to these viruses following exposure or vaccination which would result in rapid neutralization and clearance of the virus. For example, HSV-1 is rapidly attacked by complement induced antibody binding in human serum (245). Treatment with cobra venom factor, which depletes complement, was shown to enhance virus delivery to gliomas implanted in the brain of athymic rats (246). Even if the host has previously not been exposed to the OV, intravenous injection of viruses induces a rapid and robust antibody response, preventing efficient delivery of subsequent virus doses (222, 247).

Cloaking of certain viruses with *multivalent hydrophilic polymers* prevents antibody binding to the virus particle, allows for longer circulation time of the virus in the blood and therefore enhanced delivery to the tumour (248-250). This strategy has been successful for unenveloped viruses (adenovirus), though other groups have had some trouble adapting this approach to enveloped viruses, e.g. VSV (AT Power, unpublished results). Therefore, an alternative approach has been to pre-infect cancer cells with virus *ex vivo* and infuse these infected *'carrier cells'*

intravenously. VSV was efficiently delivered to disseminated lung tumours in previously immunized mice (in the presence of neutralizing antibody) (222). This approach also resulted in improved delivery of herpes virus (251), parvovirus (252) and measles virus (253).

4.1.1.2 Physical barriers within the tumour

The tumour does not consist exclusively of tumour cells. Rather, a significant proportion of the tumour mass consists of normal cells supporting tumour growth, e.g. tumour resident macrophages, fibroblasts and endothelial cells (192, 240). Normal cells are resistant to virus infection, therefore constituting a barrier to virus spread. Also, fibrous extracellular matrix (ECM) can be a physical barrier to virus spread (reviewed in (214)). For example, tumour dissemination of myxoma virus was inhibited by extracellular matrix protein deposition (247). ECM also restricted adenovirus access to breast cancer metastases (254).

A number of strategies have been employed to disrupt physical aspects of the tumour microenvironment in order to improve virus spread. Tumour cells expressing matrix metalloproteases (MMP-1 and -8), enzymes that degrade components of ECM respond better to HSV treatment than controls (255). Increased spread was attributed to depletion of tumour-sulfated glycosaminoglycans, which increased the hydraulic conductivity of tumours. Co-injection of collagenase, a bacterial enzyme that degrades collagen, a major component of ECM, resulted in higher HSV titers and more uniform virus distribution in the tumour (256). Co-injection of hyaluronidase, an enzyme that degrades hyaluronan, a high molecular weight nonsulfated glycosaminoglycan polymer that is a constituent of the ECM, with oncolytic adenovirus improved antitumour activity and overall survival in tumour bearing mice (257). An adenovirus expressing relaxin, another cell matrix degrading enzyme was able to grow to increased virus titers within tumours and resulted in increased survival of mice (258). Recently, Nagano *et al* demonstrated that

pre-treatment with a chemotherapeutic that induces apoptosis of tumour cells can increase virus spread throughout tumours due to formation of gaps between cancer cells that allow for better migration of HSV (259).

Another strategy has been to target the tumour vasculature and the angiogenic process. Though inducing collagen degradation may improve virus spread throughout the tumour, other groups have suggested that inhibiting MMPs and matrix degradation may slow down the angiogenic process. OVs producing tissue inhibitors of matrix metalloproteases (TIMPs) were cloned with the hope of limiting angiogenesis in OV treated tumours. Indeed, treatment of human xenografts in mice with herpes virus expressing TIMP-3 resulted in delayed tumour growth and reduced tumour vascular density (260). VEGF mRNA levels have been controlled by an adenovirus expressing a VEGF promoter-targeted transcriptional repressor (261) or a VEGF targeted short hairpin RNA (shRNA) (262) which resulted in reduction in tumour vessel density. Expression of endostatin, a potent anti-angiogenic protein, dominant-negative fibroblast growth factor or platelet factor 4 by OVs are other strategies to inhibit angiogenesis in the context of OV therapy (263-266). Rather than target angiogenesis to enhance anti-tumour effect, Libertini *et al* successfully combined adenovirus with bevacizumab (Avastin) in order to achieve better delivery and dissemination of virus (267). The Jain laboratory has now robustly shown that rather than decreasing tumour perfusion, treatment with antiangiogenic therapy may 'normalize' tumour vasculature to allow for improved delivery of large therapeutics (268). When combined with bevacizumab, adenovirus was able to grow more uniformly in tumours. Also, bevacizumab alone did not induce appreciable tumour regression, suggesting that its therapeutic benefit is due to enhancing oncolytic potency of the OV (267). Co-administration of oncolytic HSV-1 with the angiostatic cRGD peptide that antagonizes intergrin signaling resulted in decreased vascular

permeability and increased virus titers in a rat glioma model (227). The same group also demonstrated increased vessel density in gliomas that regrow after HSV-1 treatment. CYR61 gene expression was increased in tumours following OV treatment and inhibition of CYR61-induced integrin activation suppressed the pro-angiogenic effect of OV therapy (269). Aghi *et al* demonstrated that co-administration of thrombospondin, an anti-angiogenic protein also inhibited HSV-induced angiogenesis (270).

Clearly, investigators have taken diverse - even contradictory - approaches to enhancing the potency of OVs by manipulating the tumour microenvironment and tumour vasculature. For instance, one group found it to be beneficial to enhance matrix degradation to improve virus spread while another inhibited matrix degradation to prevent the angiogenic process in the context of OV therapy. Data presented in Chapter 2 suggests that OV infection itself can induce a loss of tumour perfusion and data in Chapter 3 demonstrates a significant loss of tumour vasculature shortly following OV infection of colon adenocarcinoma tumours in mice. Therefore, the host response to OVs can cause significant changes to normal cells within the tumour, without the virus having to express transgenes designed to manipulate the tumour microenvironment.

4.1.1.3 Tumour infiltrating innate immune cells as barriers of OV infection and spread

The innate immune response to oncolytic virus infection has been shown to inhibit spread and persistence of viruses within tumours. In the study using systemic infusion of VSV to treat mice with subcutaneous CT-26 colon adenocarcinoma tumours discussed in Chapter 2, *neutrophils* inhibited the persistent infection of tumours (virus infection in tumours was observed at day 5 in neutrophil depleted mice, compared to day 3 in neutrophil replete controls) (215). This is the first report of neutrophils inhibiting OV persistence in tumours. Other immune cells, in particular tissue resident *macrophages*, have been implicated in virus clearance and therefore inhibition of

efficient virus spread throughout tumours. Systemic depletion of macrophages using clodronate liposomes, lipid vesicles that deliver a toxic payload to phagocytic cells, resulted in decreased macrophages in herpes virus treated tumours and a 5-fold increase in virus titers (226). Similarly, depletion of microglia, brain resident phagocytes from *ex vivo* cultured brain slices resulted in a 10-fold increase in herpes virus titer in brain tumours (226). Shashkova *et al* undertook liver resident macrophage (Kupffer cell) depletion by pre-treatment with adenovirus and warfarin (271). Systemic adenovirus infusion was shown to deplete Kupffer cells but was associated with toxicity due to hepatocyte transduction (272). Co-treatment with the anti-coagulant warfarin detargeted infection of hepatocytes and exclusively resulted in Kupffer cell depletion. Upon subsequent treatment with oncolytic adenovirus, increased transduction of tumours was detected. This experimental protocol has its flaws though as dosing of mice with multiple doses of the same virus may affect innate and adaptive anti-virus immunity. Besides tumour infiltrating macrophages, other innate immune cell types have also been implicated in restricting virus spread throughout tumours. Two VSV vectors expressing proteins that inhibit *natural killer cell* (NK cell) function and recruitment (herpes virus-1 glycoprotein G or cytomegalovirus protein UL141) were able to grow to increased virus titers in tumours in an orthotopic hepatocellular carcinoma (HCC) rat model. The decreased inflammatory response resulted in increased necrosis due to better virus spread and also resulted in enhanced survival of the animals (228, 229). Expression of these transgenes also resulted in decreased T cell infiltration to tumours. UL141 did not significantly affect neutrophil or macrophage infiltration to tumours, though there was a trend towards decreased macrophage recruitment (229).

General immunosuppression has been investigated by many groups as a mechanism of increasing virus titers within tumours. Cyclophosphamide (CPA), whose metabolite can damage

DNA, has been used clinically as an anticancer agent as well as an immunosuppressant. Due to this dual activity, CPA represents a rational combination therapeutic. Fulci *et al* demonstrated that CPA enhances HSV replication and oncolysis in a rat glioma model by reducing infiltration of tumour-associated phagocytic cells (225). Peripheral blood mononuclear cells produce anti-viral cytokines, such as type I IFNs, IFN-γ, TNF-α, IL-15 and IL-18 that inhibit virus proliferation in tumours (273). Furthermore, a high dose of CPA was shown to ablate development of neutralizing antibodies to reovirus, resulting in replication in normal tissue and severe toxicity. Careful administration of lower doses of CPA resulted in increased intratumour virus titers with few side-effects (230). This study highlights the importance of cautious administration of immune suppressants with replicating cancer therapeutics. Though able to enhance virus replication in tumours (a beneficial effect of immunosuppression), this strategy may also result in systemic spread of the virus (a potential significant detrimental side-effect). Subsequent strategies aim to provide a local immunosuppressive effect within the tumour microenvironment, e.g. by engineering an OV that is able to locally produce the drug. The CYP2B1 enzyme has been cloned into herpes virus to allow for local activation of the immunosuppressant (274).

Data from macrophage depletion experiments as well as general immunosuppression suggest that inhibition of phagocyte activity promotes OV infection and spread throughout tumoursMany of the published studies on the effects of macrophages on oncolytic virus spread have been reported in glioma models. The brain is an immune privileged site that may respond to OV infection differently than infection of a tumour e.g. in the hind flank of an animal. It would be interesting to study acute changes in perfusion in these models in order to be able to correlate this data with results presented in this thesis. In the experiments reported in Chapter 2, neutrophils are likely inhibiting spread of virus due to apoptosis of uninfected cells in the tumour core (triggered

by the loss of tumour perfusion). Therefore, virus is unable to spread and persist in tumours because apoptotic cells are no longer fertile ground that can support virus infection. Perhaps macrophages would similarly impede virus persistence in this tumour model as this cell population has been shown to be the major source of pro-inflammatory cytokine production following infection or tissue injury (275). Depletion of macrophages in the context of VSV therapy in the CT-26 colon adenocarcinoma tumour may therefore also result in abrogation of loss of tumour perfusion, no apoptosis of uninfected cells and better virus spread.

4.1.2 Beneficial effects

4.1.2.1 Tumour targeted inflammation can cause tumour cell death: lessons from preclinical models

The majority of the work presented in this thesis suggests that tumour targeted inflammation associated with virus infection enhances tumour cell death. Though extensive literature cites the tumour microenvironment and innate immune response as a barrier to virus infection, other publications also report beneficial anti-tumour effects of the host response to virus infection.

Injection of HSV in a syngeneic model of ovarian carcinoma in mice resulted in induction of IFN-γ, monocyte chemoattractant protein (MIG) and IP-10 cytokines and recruitment of NK cells and $CD8^+$ T cells which is thought to contribute to tumour rejection (276). Pro-inflammatory cytokines and chemokines induced by reovirus infection of melanoma cells caused bystander toxicity to virus-resistant tumour cells *in vitro* and activated dendritic cells (277). Though OVs have been engineered to express immunomodulatory genes that aim to enhance the adaptive anti-tumour immune response, e.g. IL-4 (278), IL-12 (279-283) or GM-CSF (284), enhanced tumour regression due to these modified viruses has been partially attributed to activation of the

inflammatory response. For instance, tumour rejection following administration of measles virus expressing GM-CSF is attributed to enhanced neutrophil infiltration to tumours (285). GM-CSF produced by a recombinant Newcastle Disease Virus (NDV) was shown to activate monocytes and dendritic cells *in vitro* (286).

Combination therapy with histone deacetylase (HDAC) inhibitors is a promising new approach to sensitizing resistant cancer cells to OV therapy. Treatment with HDAC inhibitors with VSV resulted in decreased interferon production as compared to treatment with VSV alone. HDAC inhibitor treatment resulted in increased infection of resistant cancer cell lines *in vitro*, as well as human tumour specimens *ex vivo* and human tumour xenografts in immunocompromised mice (287). Interestingly, enhanced infection of tumours in the presence of HDAC inhibitors resulted in a reduction of tumour perfusion, similar to that reported in Chapter 2 in tumour models that are susceptible to VSV infection. This suggests that tumours may be sensitized to vascular disruption if virus infection can be sufficiently enhanced thereby likely triggering more inflammation. Concurrent treatment of trichostatin A (an HDAC inhibitor) with HSV resulted in decreased production of VEGF. Therefore, authors suggest that HDAC inhibitors may also possess an antiangiogenic effect when combined with OV infection (288).

4.1.2.2 Tumour targeted inflammation can cause tumour cell death: lessons from clinical trials

Oncolytic viruses are quickly entering clinical trials (66). Though limited efficacy was demonstrated in early trials, rational combination therapies as well as better oncolytic agents are now being tested with more promising results. For instance, vaccinia virus JX-594 (thymidine kinase deleted, expressing GM-CSF) has recently been tested in a small cohort of patients with advanced stage HCC (289). Treatment with GM-CSF expressing vaccinia virus increased circulating neutrophil counts in four out of six patients receiving virus at the maximum tolerated

dose (MTD) (290). Importantly, based on the results presented in Chapter 2, investigators sought to monitor tumour perfusion over the course of virus treatment and patients underwent perfusion computed tomography (CT) scanning at baseline and day 6. A significant 43% decrease in tumour perfusion was measured (289). This result validates the observations from pre-clinical models of syngeneic and xenograft tumours in mice. Furthermore, increased serum levels of pro-inflammatory cytokines were measured, including GM-CSF, TNF-α, IFN-γ, IL-4 and IL-6. In particular, TNF-α and IFN-γ have been shown to have anti-vascular effects (26). Also, GM-CSF production by infected tumour cells led to an increase of up to tenfold in white blood cells, including neutrophils, eosinophils and monocytes. These results are being followed up in an ongoing phase II trial testing JX-594 in patients with HCC.

Furthermore, clinical studies with other oncolytic vectors, such as adenovirus and HSV, demonstrated that tumours in patients undergoing viral therapy showed decreased metabolic activity (as measured by CT scan) and increased tumour necrosis (by histological analysis) (241, 242). These results are consistent with observations made in the preclinical tumour models presented in this thesis.

Oncolytic virus infection of tumours triggers a host response that is evolutionarily designed to inhibit spread of the invading virus. Conceptually, this will have detrimental effects on virus infection and spread within tumours. However, the increasing evidence from experiments using oncolytic viruses in preclinical tumour models as well as clinical trials presented here suggests that tumour targeted inflammation may contribute to tumour regression. Since there are indications from clinical trials that inflammation may trigger a loss of tumour perfusion and coagulation within tumour associated vessels, it remains important to understand how virus

infection of tumours is triggering this detrimental response. Furthermore, if this response is only triggered under certain circumstances in a subset of tumour models, it would be interesting to elucidate reasons for this discrepancy.

4.2 The acute response to other anticancer agents

4.2.1 Going back in history: Coley's toxin

Over the course of the initial studies testing VSV infection of tumours *in vivo*, we expected to observe efficient virus spread throughout tumour tissue, similar to the efficient eradiation of tumour cells observed *in vitro*. Limited infection and associated disruption of tumour perfusion was unexpected. However, upon review of the literature, these observed effects of OV infection of tumours are no longer as surprising. In the 1800s William B. Coley noticed that patients developing infections post surgery were more likely to go into remission than those that did not experience infections. Capitalizing on this observation, Coley prepared Coley's toxin, or 'mixed bacterial vaccines' (MBV), containing heat killed bacteria that were used to deliberately induce fevers in patients (231, 232). A safer approach than exposure to live bacteria, treatment with MBV increased survival in patients with sarcomas, lymphomas, breast and ovarian cancers (233). Endotoxin (or LPS) was later isolated and shown to be a component of the bacterial cell wall that is a very potent stimulator of inflammation. LPS administration resulted in the rapid induction of central hemorrhagic necrosis and a shutdown of blood flow to tumours (291, 292). In the 1970s an endogenous serum factor induced upon LPS injection was shown to be responsible for tumour cell death (293, 294). This factor was evidently termed 'tumour necrosis factor' and has now been demonstrated to cause vascular disruption along with IFN-γ by disrupting endothelial cell adhesion by inhibiting integrin αVβ3 integrin signaling (26). *Tumour necrosis factor* is only

recommended for few indications clinically, e.g. local drug administration by isolated limb perfusion to treat melanoma (295). Systemic treatment with TNF induces vasoplegia, dysfunction of vasculature that leads to a "septic shock like syndrome" and potential multiorgan failure (296). As virus infection can induce these same serum factors (215), it follows that OVs may induce a reduction in tumour perfusion by a similar mechanism.

4.2.2 Parallels with current cancer therapeutics

More recently, much attention has been paid to understanding how chronic, smouldering inflammation is implicated as a mediator of tumour progression (discussed in the 'inflammation & cancer' section of the introduction). However, there also exists more recent evidence (in addition to that presented in this thesis) that the acute inflammatory response to other anticancer agents may be similar to that observed in our OV models (see Appendix 2). These agents include photodynamic therapy and vascular disrupting agents, p53 gene therapy and adaptive antitumour immune therapies, among others. Interestingly, the pattern of tumour necrosis following treatment with these diverse anticancer agents in different tumour models is strikingly similar (Figure 4.1). These therapies will be briefly discussed here and insight into the mechanism of inflammation-mediated tumour damage gained from experiments with these other therapeutics will be presented.

4.2.2.1 Photodynamic therapy

Photodynamic therapy (PDT) involves systemic infusion of a photosensitizer that is activated upon exposure to visible light (27). This results in the production of reactive oxygen species (ROS) that go on to damage tumour and endothelial cells in the area exposed to light. Rather than this initial insult mediating the major cytotoxic effect, the ensuing inflammatory

Figure 4.1 *Examples of tumour histology following treatment with diverse anticancer agents.* Pattern of viable tumour rim and necrotic tumour core observed following treatment with different anticancer therapies. Images taken from (298-301).

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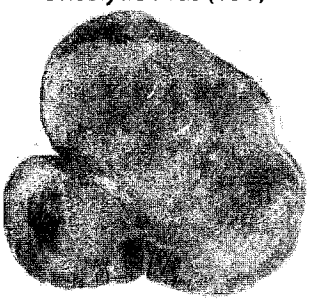
Oncolytic virus (VSV)

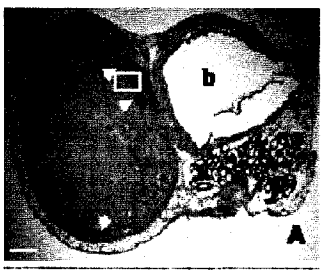


p53 gene therapy

Vascular disrupting agent (ZD6126)

Metronomic chemotherapy
(cyclophosphamide & thalidomide)

Photodynamic therapy (verteporfin)



response that is activated upon cell damage has been shown to be critical for tumour reduction and eradication upon PDT (236, 297). Recently, a number of damage associated molecular patterns (DAMPs) have been identified. DAMPs are formed or exposed upon damage to endogenous tissue and are able to stimulate inflammation through activation of TLR signaling, similar to the signaling cascade induced upon detection of PAMPs associated with invading bacteria and viruses. Triggering of TLR signaling results in production of pro-inflammatory lipid mediators such as arachidonic acid metabolites as well as chemokines, cytokines and adhesion molecules that recruit and activate various immune cell types. In particular, production of macrophage-inflammatory protein-2 (MIP-2, the murine equivalent of IL-8) and E-selectin were shown to result in neutrophil recruitment to the local site that had been damaged following PDT (302). Importantly, neutrophil depletion experiments demonstrated that neutrophils were required for tumour regression following PDT (303). Consistent with this result, combining PDT with granulocyte colony-stimulating factor (G-CSF) which increases neutrophil counts results in improved efficacy of PDT (304). Regressing tumours treated with both agents demonstrated increased neutrophil infiltration and apoptosis. The pro-inflammatory tumour microenvironment results in the generation of a procoagulant surface on tumour-associated endothelium (as discussed in the introduction). Platelet aggregation, vasoconstriction, vessel occlusion and finally microvascular collapse ensue (236). Activation of the coagulation cascade has been shown to contribute to PDT efficacy and administration of the anticoagulant warfarin resulted in decreased efficacy (236). It follows that PDT has also been shown to disrupt blood flow dynamics within tumours (305).

4.2.2.2 Vascular disrupting agents

Vascular disrupting agents (VDAs), e.g. CA-4-P, Oxi4503 and ZD6126 target existing tumour vasculature by preventing polymerization of tubulin resulting in the disruption of cytoskeletal structure and changes in the shape of endothelial cells (25). These events lead to endothelial cell monolayer barrier dysfunction and loss of blood flow to tumours. Loss of tumour blood flow is rapidly followed by induction of central tumour necrosis (25). This has been demonstrated in numerous pre-clinical models (299, 306-308) as well as in patients (309). There is some evidence that sustained induction of tumour necrosis is due to an inflammatory reaction that responds to the initial tissue damage. Expression of E-selectin was induced on endothelial cells exposed to CA-4-P *in vitro* and endothelial cells became adhesive to neutrophils (310). Increased MPO activity and neutrophil infiltration to tumours following treatment with VDA have been recorded (311, 312). However, the direct involvement of neutrophils in tumour regression following administration of VDAs has not yet been tested. Furthermore, the induction of microvascular thrombosis following treatment with VDAs has been recorded in diverse pre-clinical models (299, 313-316). Damage to vasculature is known to create a pro-thrombotic surface, inducing platelet aggregation and thrombosis.

4.2.2.3 Adaptive immune therapies

Cancer immunotherapy involves stimulating immune cells to specifically recognize and attack tumour cells. Adaptive immune therapies either activate endogenous tumour reactive CTLs or alternatively involve the adoptive transfer of CTLs that target tumour cells. This is an attractive anticancer strategy as *(1)* activation of endogenous T cells generates immunological memory that is able to be reactivated upon tumour relapse and *(2)* tumour reactive CTLs recognize 'altered self' antigens aberrantly expressed on transformed cells and release their toxic payload specifically to

these target cells. However, evidence is beginning to emerge that activated CTLs may be inducing tumour regression not solely due to specific recognition of tumour cells as CTL killing – the classic method of measuring T cell activation – only sometimes correlates with the extent of tumour regression *in vivo* (317, 318). Various mediators produced by tumour reactive T cells can cause damage to tumours, including perforin/granzyme B, IFN-γ, Fas-ligand or TNF-α. Interferon-γ and TNF-α are cytokines known to cause vascular disruption. Release of IFN-γ into the tumour microenvironment may therefore cause disruption of tumour vasculature and tumour cell death independently of the specific killing capabilities of CTLs. Indeed, infusion of either $CD8^+$ or $CD4^+$ T cells into tumour bearing mice can result in decreased vascular density and damage to the tumour core (319, 320). Furthermore, Blohm *et al* reported decreased tumour vascularity and accumulation of granulocytes in tumours of mice infused with cytotoxic T cells (321). The effect of products of activated CTLs on tumour vasculature indeed may be underappreciated, thereby warranting further study.

4.2.2.3 Reintroduction of p53

p53 is a tumour suppressor mutated in over 50% of human cancers. *p53 gene therapy* has recently been proposed as a viable anticancer strategy. Indeed, reintroduction of p53 in various tumours in mice resulted in tumour regression (298, 322). However, p53 gene therapy triggered senescence in liver cancer and sarcoma. Investigators were therefore surprised to observe tumour regression *in vivo*. The mechanism of tumour regression was found to be the activation of innate immune cells due to production of pro-inflammatory cytokines by senescent cells. This triggered innate immune cell infiltration into tumours. Depletion of various innate immune cell populations, including neutrophils, abrogated tumour regression. Interestingly, histological analysis of a sarcoma following p53 gene therapy revealed central tumour necrosis and a viable tumour rim

(Figure 4.1), consistent with the pattern of cell death following OV infection presented in this thesis.

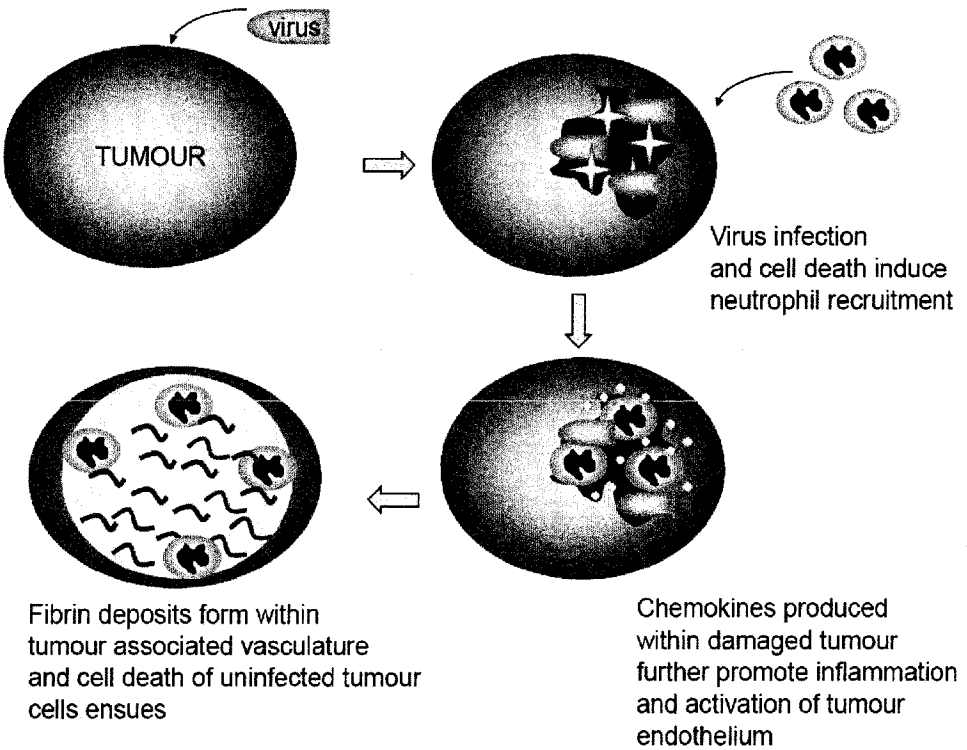
Together, evidence from *in vivo* studies of photodynamic therapy, vascular disrupting agents, adaptive immune therapies and p53 gene therapy would suggest that inflammation can unexpectedly contribute to tumour regression. Given that oncolytic viruses are also likely to trigger inflammation *in vivo*, it is not as surprising that OV infection of tumours may trigger a similar host response that may contribute to tumour regression. Lessons regarding the mechanism of inflammation mediated tumour regression may be learned from studies of these diverse anticancer agents, in particular PDT. Fundamentally, it is important to recognize that these seemingly diverse approaches to treating cancer may induce tumour cell death and tumour regression in a similar fashion *in vivo*.

4.3 Future directions

4.3.1 Investigate other players in inflammation mediated tumour damage.

Outlined here is the impact of the acute host response to oncolytic virus infection of tumours in mouse models of cancer. Granulocytes can restrict the ability of viruses to spread and persist in tumours but are also able to cause significant cell death of uninfected tumour cells by triggering intravascular coagulation and disrupting tumour blood flow (see Figure 4.2 for summary). Importantly, a reduction in tumour blood flow has now also been recorded in patients undergoing vaccinia virus therapy for hepatocellular carcinoma, validating results from the pre-clinical models (289). A deeper understanding of the mechanism and other critical mediators of the acute tumour targeted inflammatory response is warranted upon the observation that the phenomenon also occurs clinically. For instance, the importance of macrophages, eosinophils and

Figure 4.2. *Overview of acute effects of oncolytic virus infection of tumours.*


virus
TUMOUR
Virus infection
and cell death induce
neutrophil recruitment
Chemokines produced
within damaged tumour
further promote inflammation
and activation of tumour
endothelium
Fibrin deposits form within
tumour associated vasculature
and cell death of uninfected tumour
cells ensues

mast cells in the context of this reaction has not yet been elucidated. As these innate inflammatory cells produce pro-inflammatory mediators possibly involved in the disruption of tumour vasculature and induction of coagulation, individually depleting these immune cell populations may shed further light onto the mechanism of the tumour targeted inflammatory reaction. Furthermore, generating a better understanding of the triggers of inflammation in the context of OV therapy is warranted. In particular, which PAMPs or DAMPs are exposed over the course of OV therapy that trigger inflammation? Is inflammation exclusively triggered by patterns associated with the virus or is damage to tumour cells or other cells within the tumour microenvironment sufficient to induce tumour targeted inflammation?

4.3.2 Learn from research into mechanisms of ischemia/reperfusion injury

Beyond the field of cancer therapeutics, lessons may be learned from knowledge of mediators of pathology in other diseases, such as ischemia/reperfusion (I/R) injury acquired during myocardial infarction or stroke (237, 238, 323). Transient hypoxia results in activation of local endothelium and aberrant activation of neutrophils upon reperfusion (reviewed in (239, 324)). Critical mediators leading to the activation of neutrophils in this setting have been elucidated. Though the goal in the context of I/R injury is to dampen these effects, we may want to promote these locally within the tumour environment in the context of cancer therapy.

4.3.3 Understand the 'tumour rim phenomenon'

Another interesting avenue of research to pursue is to understand why tumour vasculature seems more prone to inflammation-induced coagulation than vessels associated with normal tissue. In particular, vessels within the tumour rim are often also not associated with blood clots in response to OV therapy or VDAs (25). Though it is assumed that this is due to the fact that vessels within the tumour rim resemble normal vasculature, this has not been proven. Possibly

simply proximity to normal tissue is required for protection from coagulation. If the mechanism of coagulation within the tumour core was elucidated, further studies could investigate whether vessels in the tumour rim could be sensitized, therefore possibly targeting the entire tumour for inflammation-mediated destruction.

4.3.4 Study implications for other anticancer therapies

Beyond implications for the field of oncolytic virus therapy, these studies may shed light on the mechanism of tumour destruction in response to other diverse anticancer agents. As has been discussed earlier, different, unrelated anticancer agents may cause tumour regression by stimulating acute inflammation. Furthermore, there exists rationale that other common drugs used to treat cancer may also trigger tumour targeted inflammation. Indeed, anticancer drugs, by definition, damage tumour cells and therefore may expose DAMPs that activate the host's inflammatory response. This acute host response to anticancer treatment may go unnoticed as investigators assume that tumour regression is due mainly to direct killing of cancer cells by the drug. Though this was our assumption upon OV treatment, a system was in place in which cells dying due to drug (i.e. cells directly infected with virus) versus cells dying due to a secondary insult (i.e. disruption of tumour perfusion) could easily be identified. Though the impact of primary versus secondary insults may not be as simple to probe with other anticancer agents, the contribution of inflammation to tumour regression can be investigated by systematic depletion of immune cell populations.

4.3.5 Harness inflammation to benefit anticancer therapy?

Even in the context of oncolytic virus therapy, the impact of innate inflammatory cells on the ability of virus to cause tumour regression remains ambiguous. Numerous studies have now demonstrated that general immunosuppression may be a mechanism by which to decrease virus

clearance and thereby improve efficacy of OV therapeutics (225, 273, 274). However, there remain some safety concerns of applying replicating virus therapeutics in combination with immune suppressants (230). Also, several studies have now demonstrated that virus infection of tumours may trigger an adaptive anti-tumour immune response (325). Therefore, though dampening the immune response to the virus may be beneficial, immunosuppression may also affect the ability of the host immune system to reject the tumour. With the development of clinically applicable drugs that specifically target certain innate immune pathways (e.g. TLR signaling cascade) (326) we may be able to tailor the acute host response to allow for virus spread initially (by dampening the inflammatory response with a TLR inhibitor) but subsequently augment the acute host response to virus infection (by augmenting innate immune signaling) thereby causing inflammatory damage to tumours and signal for the adaptive immune response to reject the tumour.

Results presented in this thesis demonstrate the important distinction between the mechanism of tumour regression *in vitro* versus *in vivo*. Acute inflammation triggers apoptosis of uninfected tumour cells in the context of oncolytic virus therapy by inducing coagulation and resultant loss of tumour perfusion. The involvement of chronic inflammation in the promotion of tumour progression has now been definitively established. The results presented in this thesis do not contradict this dogma. Rather, they suggest that an acute inflammatory response, rapidly responding to a danger signal arising from a tumour following therapy has a different effect on the tumour microenvironment as compared to a chronic, dysregulated inflammatory response. Indeed, in physiological settings, acute inflammation constitutes a critical rapid response to invasion by pathogens. Conversely, chronic inflammation is currently only associated with pathological situations – it is uncontrolled and can cause significant damage to normal tissues. Therefore, just as there exists a significant difference between acute and chronic inflammation associated with

normal tissues, it could be argued that acute and chronic inflammation may possess opposite effects in the tumour microenvironment. As inflammation has now been implicated in the tumour damage following diverse anticancer agents, we must continue to *(1)* understand the mechanism of inflammation mediated tumour destruction – why tumours are more sensitive to acute inflammation than normal tissue *(2)* investigate whether inflammation contributes to tumour regression following conventional anticancer therapies *(3)* continue to study the phenomenon in patients and *(4)* determine whether acute inflammation can indeed be harnessed to improve efficacy of anticancer therapeutics.

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APPENDICES

Appendix I. Innate immunity, tumor microenvironment and oncolytic virus therapy: Friends or foes?

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Innate immunity, tumor microenvironment and oncolytic virus therapy: Friends or foes?

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Abstract

The field of oncolytic virus (OV) therapy is an innovative and evolving science, taking advantage of the ability of select viruses to preferentially infect and kill human tumor cells. However, the contribution of the tumor microenvironment, and particularly the induced innate immune responses to both the tumor and the virus, has been shown to be a major player in the success of OV therapies. Innate immunity and inflammation in particular can have opposing effects: they can augment OV therapy though enhancing tumor destruction yet can also act to recognize and clear the invading virus to significantly hinder viral dissemination through the tumor tissues. This review focuses on the emerging knowledge considering how inflammation and innate immunity impinge on current OV candidates, to either facilitate or hinder virotherapy. We also discuss novel approaches that modulate or harness the innate immune system to specifically enhance OV mediated tumor destruction.

Innate Immunity and Cancer: the double-edged sword

Cancer has been described as a 'wounds that do not heal' [1], indicating the pivotal role of the innate immune system and activated inflammatory responses in the development and maintenance of tumors. Over the past 15 years, there has been an explosion of research into the role of inflammation in the promotion of malignancy. Here, we would like to argue that the consequences of activated innate inflammatory cells in cancer are more ambiguous, particularly in the context of emerging therapies with oncolytic viruses (OVs).

The innate immune system and inflammatory response constitute the immediate and first-line responses to assault from pathogens and trauma. Cells of the innate immune system, including dendritic cells (DCs), natural killer (NK) cells, macrophages, neutrophils, basophils, eosinophils and mast cells have evolved to rapidly recognize pathogens and various signs of tissue distress. These cells respond to danger signals triggered by pathogens by secreting proteins (e.g. cytokines, chemokines, proteases) and soluble mediators (e.g. reactive oxygen species (ROS), histamines) which function to directly kill pathogens, activate other inflammatory cells, induce tissue remodeling and repair as well as begin the mobilization of cells of the adaptive immune system. However, the same mediators that may be beneficial in responding to invading pathogens may also be "highjacked" by tumors to create a micro-environment that is instead more conducive for tumor progression and invasion [2]. For

example, factors produced by tumor associated macrophages (TAMs) have been shown to induce tissue remodeling and angiogenesis though the secretion of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF), matrix metalloproteinases (MMPs), cathepsins and interleukin 8 (IL-8) [3]. Tumor associated immune cells have also been shown to produce a suite of immunosuppressive factors (e.g. transforming growth factor beta (TGF-β), IL-10) that dampen adaptive anti-tumor responses.

Beyond the role of pro-inflammatory mediators in promoting cancer progression, evidence from mouse knockout studies has revealed that an intact innate immune system is required for full-blown tumorigenesis in a number of cancer models. For instance, tumor necrosis factor (TNF) knockout mice were found to be more resistant to skin carcinogenesis [4]. NF-κB, the 'master switch' of many inflammatory cascades, was shown to enhance survival of transformed cells, which promoted conversion to malignancy in *Mdr-2* knockout mice that spontaneously develop hepatocellular carcinoma [5]. Mice lacking MyD88, a key adaptor molecule in the signaling cascade triggered by recognition of pathogen associated molecular patterns (PAMPs) by toll like receptors (TLRs) are less prone to develop intestinal and liver cancers [6,7]. Furthermore, chronic infections which are thought to trigger chronic inflammation have been implicated in the generation of up to 15% of human cancers (e.g. human papilloma virus (HPV), *Helicobacter pylori* and hepatitis C virus in cervical, gastric and liver cancer, respectively) [8].

However, the innate immunity within a tumor does not exclusively have deleterious effects by just promoting tumor progession [9]. Xue et al reported that reactivating the tumor suppressor p53 in a murine model of liver carcinoma can induce complete regression of tumors [10]. Rather than inducing apoptosis, p53 reactivation instead includes cellular senescence in tumor cells, resulting in upregulation of pro-inflammatory cytokines. Infiltration of newly activated innate inflammatory cells subsequently contributed to tumor clearance *in vivo*. Furthermore, recent progress in the understanding of how innate immunity is regulated with the identification of host proteins, including toll like receptors (TLRs), retinoic acid inducible gene I (RIG-I) and multi drug resistance 5 (MDR-5) is shifting our view of the role of innate immunity in tumor immunology. It has now been proposed that the manipulation of innate immune signaling to create a pro-inflammatory environment within tumors (e.g. with adjuvant or cytokine therapy) may be critical for the success of cancer immunotherapy [11].

Beyond adjuvant therapy for tumor vaccines, innate immune signaling has recently been implicated in increased efficacy following conventional chemo- or radiation therapy. Apetoh et al report that endogenous danger signals elaborated from dying tumor cells activate dendritic cells, which present tumor antigens more efficiently to tumor reactive CTLs [12]. This report includes an analysis of clinical data in which patients with breast cancer carrying a loss-of-function allele in TLR4 relapse more quickly than women with functional TLR4, strongly implicating innate immune signaling with disease control. With a greater appreciation of the role of innate immunity in the anticancer effects of current

drug treatments, it is entirely warranted to further study the effects of manipulating the innate immune response to improve other modalities of cancer therapy.

To a large extent, the role of the innate immune system in cancer immunotherapy has been understudied in deference to a presumed primary role of the adaptive anti-tumor immune response (see corresponding review by Parato et al, in this issue). Extensive studies have explored the use of viruses in gene therapy strategies for cancer [13,14]. Targeting the adaptive immune response to tumors, essentially "vaccinating" against tumor antigens, has been a popular approach as a directed cancer therapy. To date, a multitude of mechanisms to deliver and present tumor-associated antigens have been investigated, including recombinant peptide vaccines, DNA vaccines, as well as epitope delivery with recombinant viruses or antigen-loaded dendritic cells. However, such cancer vaccines tested thus far in clinical trials have largely reported poor response rates [11]. Indeed, even studies in mouse models of cancer have largely been unsuccessful in triggering regression in established, solid, vascularized tumors [11]. Since classic anti-viral vaccines are not used to treat established viral infections, it is perhaps not surprising that anti-tumor vaccines need to be administered before or shortly following tumor challenge in many animal models. Generally, in order for a tumor vaccine to result in tumor regression, three criteria need to be achieved: (1) the generation of a large frequency of tumor-reactive T cells with a high avidity, (2) the trafficking of tumor-reactive T cells to the tumor site and (3) the activation of T cells needs to be

achieved and sustained *within the tumor*. Though the first two criteria can frequently be achieved upon vaccination against tumor antigens, the activation of tumor-infiltrating T cells once within tumors is often absent or suboptimal because the tumor cells have themselves evolved to hide from the immune cell recognition. As previously discussed, tumor-associated immune cells often secrete immunosuppressive cytokines like IL-10 and TGF-β to dampen adaptive anti-tumor immunity. In addition, T regulatory cells have been shown to suppress the proliferation and effector function of cytotoxic T lymphocytes (CTLs). Thus, the suppression of what should be robust anti-tumor adaptive immunity is likely being hindered by what is effectively a supervening pro-tumor innate immune response.

Despite the frequent lack of success in targeting adaptive immune cells to destroy tumors, there has been much less research in mobilizing host innate immune leukocytes against tumors. Although the concept of more efficiently targeting innate immune cells to respond to tumor beds may initially be less attractive than triggering adaptive anti-tumor immunity (e.g. because the innate immune response does not generate long lasting memory), it has long been recognized that innate inflammatory cells can, when appropriately activated, induce significant clearance of tumors. We argue that the host innate immune system may present a significant barrier to cancer therapies, particularly OV therapy. Instead, we posit there exists a significant gap in our knowledge in the interaction between tumor microenvironment, the innate responses induced in situ, and viruses. Although the 'natural' interaction between host and virus has

been often well studied in the context of a pathogenic infection, how this interaction may be altered within a tumor micro-environment remains largely unknown. Indeed, whether a vigorous inflammatory response to an OV is even beneficial to the therapy is unexplored, and could very likely be variable for the different OV candidates and the targeted tumors. This review will focus on some of the current knowledge with regards to OV interactions with the host innate immune system in the presence and absence of tumor tissues, and give perspective on the fundamental questions that still need to be addressed.

The innate immune system and OV: friend and foe?

OV therapy takes advantage of the preferential replication of select viruses in tumor tissue. As pathogens, all viruses likely need to subvert triggering the 'danger signals' that lead to activation of an inflammatory reaction and clearance by innate immunity. Some OVs have evolved to produce immunomodulatory proteins that manipulate both innate and adaptive immune responses and this strategy is particularly dramatic for oncolytic viruses with larger genomes like herpesviruses, poxviruses and certain RNA viruses [15,16]. However, most clinical OV candidates derived from human viruses have been both lab-adapted through continuous culture as well as attenuated, often through selective gene deletion to hopefully achieve both increased tumor selectiveness and safety in patients. In general, gene-deleted viruses allow for more preferential replication in the mutated signaling cascades of cancer cells. For example, a current oncolytic version of adenovirus has a deletion in the E1B gene to allow for replication in cells where the p53 is non-functional, although

other cellular factors may be involved [17,18]. Both herpes simplex virus and vaccinia virus have had their thymidine kinase genes ablated to selectively restrict these viruses to more rapidly cycling tumor cells [19,20]. By contrast, other OVs, whose primary host is not of human origin, express immunomodulatory proteins that vary in their capacity to mediate viral escape of human innate immunity.

In addition to the genetic selectivity, many OV take advantage of aberrant interferon (IFN) responses within many tumor types. Viruses such as VSV [21], reovirus [22], and myxoma virus [23] are naturally sensitive to IFN, thus their replication is selective to tumor cells where this pathway is abrogated. However, other viruses such as measles virus, have been genetically modified so that they elude IFN responses, thus facilitating spread throughout tumors where the IFN responses are intact [24].

Whereas the interaction of individual viruses and their host has been studied intensively, the virus-host interaction dynamics within the tumor milieu is not well studied. In particular, the mechanism by which innate immune cells can either enhance or inhibit OV tumor clearance effectiveness remains elusive. OVs encounter 'conventional' host innate immune responses during delivery of virus, yet the dose and administration route of the virus (often intravenous or intratumoral) almost always differs from what occurs during a natural viral infection. The majority of studies into the immune response to OVs in tumor-bearing hosts have focused on the ability of the virus to induce anti-tumor immunity. However, the anti-viral immune responses elicited after OV treatment

of tumors often occur during the acute phase of viral replication, when the major host antiviral response will be the early innate pathways.

Efficient delivery of virus into tumor beds, particularly at metastatic sites, has been a major barrier to successful OV therapy in humans. Direct injection of virus into a primary tumor is still the most effective delivery method, and has resulted in the most successful anti-tumor effects thus far in animal models, yet many clinical cancers are not easily accessible for this type of administration. However, the alternative systemic (often intravenous) route of administration represents a significant hurdle. In the case of human OV candidates, such as reovirus, adenovirus and herpes simplex virus, most members of the human population have neutralizing antibodies to the virus through previous natural exposures to related family members of the candidate virus. For example, the activated complement cascade induced antibody binding to HSV-1 in the blood after systemic administration of virus was a major barrier to delivery of HSV-1 to tumors [25,26]. Whereas primary clinical isolates of wild type HSV-1 have multiple pathways to avoid complement activation, common laboratory strains have largely lost their ability to survive incubation with serum containing complement [26,27], making them much more susceptible to clearance from the blood. Furthermore, systemic administration of OVs, even in the absence of pre-existing anti-viral immunity, triggers a robust antibody response, thus limiting the ability to give subsequent doses [28,29].

In addition to issues with systemic administration, intra-tumoral administration itself often does not result in complete tumor regression. In both

animal studies and subsequent clinical studies, even after repeated doses, an incomplete infection and subsequent tumor escape is a common and persistent issue with many OV candidates [30-34]. The tumor microenvironment may represent a significant passive barrier through its unique physiology (reviewed in [35]), yet there is also an additional active barrier set up within the tumor tissue after the initial wave of infection and destruction of tumor cells. Host cell fibroblasts, that are often refractory to infection by OV, produce extracellular matrix that can both absorb virus particles, as well as provide a physical barrier to restrict further viral penetration [29,36].

In addition, direct viral neutralization by the host acquired immune system after OV administration has been reported after intra-tumoral injection. Although this is an area that warrants significant further study, it has been shown in two separate models of glioma that a majority of oncolytic virus is rapidly cleared from the tumor and this clearance is associated with infiltration of innate immune cells of the monocytic lineage [37,38]. This was also demonstrated in both an immunocompetent rat model using HSV-1 [37] as well as in a nude mouse model using an oncolytic adenovirus [38]. It would be important to know how phagocytic clearance in other OV models contributes to the viral clearance.

Despite reports that innate immune cells can inhibit virus spread and therefore act to restrict effective OV therapy, a recent report suggests that certain activated innate immune cells and inflammation can enhance killing of tumor cells. A rapid loss of blood flow to tumors is induced by both VSV and vaccinia virus infection of syngeneic and xenograft models of colon tumors [39] and this

vascular shutdown is associated with granulocyte infiltration of tumors and extensive apoptosis of cells in the tumor core. These results suggest that, even beyond direct virus infection and adaptive anti-tumor immunity, targeted inflammation to tumors represents an additional synergistic mediator of OV therapy induced cell death. However, though loss of blood flow to tumors is associated with increased cell death in the tumor tissue, the phenomenon may also reduce further virus spread and persistence in tumors. Neutrophil depletion of tumor-bearing mice resulted in prolonged VSV replication in colon tumors [39]. Preliminary results from clinical trials with oncolytic adenovirus and HSV indicate decreased metabolic activity (as measured by computed tomography scans) and increased necrosis (by histological analysis), respectively, consistent with observations made in murine tumor models [40,41]. In an alternative approach, some OV platforms have added immunomodulatory genes to enhance OV therapy. Many of these strategies are geared towards facilitating an adaptive anti-tumor immune response, e.g. insertion of interleukin 4 (IL-4) [42], IL-12 [43-45] or granulocyte-macrophage colony stimulating factor (GM-CSF) [41,46]. However, these approaches also manipulate the innate response to virus. For instance, the magnitude of neutrophil infiltration to human lymphoid tumors following administration of measles virus expressing GM-CSF correlated with tumor regression [47].

Harnessing the innate immune system: Building a better OV through teamwork

Although the innate immune system may hinder OV effectiveness there are different adjunct therapies have been tested to modulate the innate immune system in OV therapy. Novel combination therapies have been adopted to result in both better delivery and effectiveness of OVs by modulating how viruses activate the innate immune system.

As the neutralization of virus by the blood and hepatic system represents the most significant barrier to systemic administration, several methods to deliver viruses without this extensive early clearance have been developed. This clearance hurdle also exists in the administration for viral vectors in gene therapy, and the proposal of coating virus particles with shielding polymers have emerged from this field. For example, adenoviruses have been coated with multivalent hydrophillic polymers which shield the virus from neutralizing antibodies present in serum of most people [48]. These coated viruses have also been shown to have a higher circulation time in blood [49], and can passively accumulate within tumors and without large scale clearance, and therefore represent one way by which large scale neutralization can be minimized [50]. Although this technology has not yet been attempted with OV, it represents an easy and feasible manner in which to modify virus preparations to reduce the early systemic effects of pre-existing immunity in hosts.

Another method with which to deliver OVs and minimize detection by the immune system is known as the 'trojan horse' or carrier cell method (reviewed in [51]). This approach seeks to 'hide' the virus within migratory tumor-targeting cells that can then traffic to tumor sites and deliver virus shielded from

neutralizing antibodies, and reducing typical danger signals that trigger an immediate innate response. This method of virus delivery has shown great promise for OV therapy with herpes virus [52], parvovirus [53] and VSV [28], and in each case, an increase in delivery to tumors as well as a decrease in the immune response to the virus was demonstrated. However, in these types of therapies, specific delivery of the virus-laden cells to tumor tissue after systemic administration has yet to be achieved yet will represent a further fine tuning of safe delivery methods that will hopefully increase OV effectiveness by dampening innate (& adaptive) immune responses to the virus.

In addition to subversion of the innate immune system through viral stealth mechanisms, pharmacologic attenuation of the innate immune responses have also resulted in increased OV efficiency in tumor killing. For example, cobra venom factor (CVF) has been used to deplete complement facilitated HSV OV infection of brain tumors [54]. However, this alone was not sufficient to allow for effective tumor destruction, indicating that other innate mechanisms also contributed significantly to virus clearance. However, when cyclophosphamide, a cancer therapeutic drug that also inhibits innate immune responses, was used in combination with CVF, there was a significant increase in oncolytic capacity of this virus [54]. It has been demonstrated that this drug can allow for effective OV responses following a lower dose of herpes virus [55], and has since been demonstrated to increase the oncolytic capacity of other OV, such as HSV-2 [56] and adenovirus [38]. In addition, other immunosuppressive drugs such as rapamycin [57-59] has been shown to increase the oncolytic capacity of OVs

such as adenovirus and myxoma virus. In another approach, administration of an angiostatic cRGD peptide resulted in reduced vascular permeability and leukocyte infiltration in response to HSV administration [60]. This combination therapy resulted in increased viral titers in tumor tissue and an increase in median survival over OV therapy alone. In a recent report, clondrate liposomes were used to deplete phagocytic cells prior to oncolytic HSV administration, and this treatment resulted in a 5 fold increase in viral titres [61], further indicating the drastic anti-viral effects that can be exacted by these innate immune cells, and how their modulation can enhance OV effectiveness.

Finally, novel therapies have been adapted to combat physical barriers created through extracellular matrix deposition by tumors infected with OV. In a human xenograft model, co-injection of oncolytic HSV and bacterial collegenase resulted in higher viral titres, and more complete dissemination of the virus throughout the tumor tissue [36]. Furthermore, Mok et al recently reported that mice bearing human soft tissue sarcomas overexpressing matrix metalloproteinases-1 and -8 (MMP-1 and MMP-8) responded better to HSV treatment than controls [62]. The enhancement in survival was attributed to a depletion of tumor-sulfated glycosaminoglycans, resulting in increased hydraulic conductivity of tumors, allowing for enhanced delivery and spread of virus. An adenoviral vector has also been designed to express the cell matrix degrading protein relaxin and this engineered oncolytic virus had significantly better tumor penetration and increased viral titres and increased survival of mice [63]. These

studies represent the innovative ways in which the barriers of OV effectiveness presented by the innate immune system can be strategically managed.

Conclusions

As more candidate OVs move from preclinical study to clinical trials, we are learning that although usually safe, many OVs have exhibited disappointing anti-cancer effectiveness in human subjects. Understandably, in the interest of safety most trials have preferentially tested viruses with compromised capacity to escape the innate immune system, and are cleared before potentially therapeutic effects within the tumor can be fully realized. As we better understand the importance of the innate immune system against the virus and the critical role of the unique microenvironment of the tumor tissues, we should be in a better position to design an OV therapy regime that is both able to more effectively deliver virus into tumor tissue and avoid being prematurely recognized and cleared. Conversely, targeting some inflammatory cells to tumors has been shown to enhance OV mediated killing of tumors. The challenge is to better understand which innate pathways are beneficial to OV therapy and which ones oppose it. To do this, a systematic examination of the relative contribution of different cell populations of the innate immune system in the context of individual OV candidate administration will need to be undertaken.

Our appreciation of the critical role of innate immune cells within the tumor microenvironment, as well of methods of manipulating this dynamic interaction, has been expanding rapidly. Furthermore, some progress has been made in

strategically harnessing innate immunity and inflammation in the context of OV therapy. As innate immune cells can have both deleterious and supportive effects in the context of OV therapy, its manipulation will likely have to be designed for each OV and tumor context. This will require are more detailed understanding of the unique immunomodulatory characteristics of each OV candidate, as well as a closer examination of the evolving paradigm of tumor tissues that are supported by an active inflammatory state.

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Appendix II. Acute targeted inflammation: A behind the scenes tumour killer!

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Acute Targeted Inflammation: A Behind the Scenes Tumour Killer!

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Introduction

The inflammatory reaction is a component of the innate, non-specific immune response that has evolved to recognize signs of infection or tissue damage. The inflammatory response is meant to prevent damage spread, contain disrupted homeostasis, remove dead/damaged tissue, promote local healing and restore tissue function. This acute aggressive response to tissue damage has long been recognized to cause particularly significant injury to tumours. Even in the 1800s spontaneous remissions were documented in cancer patients that developed fevers. William Coley noticed that patients developing infections post surgery were more likely to go into remission. Capitalizing on this observation, Coley prepared 'mixed bacterial vaccines' (MBV) containing heat killed bacteria that were used to deliberately induce fevers in patients. A safer approach than exposure to live bacteria, treatment with MBV also increased survival in patients with sarcomas, lymphomas, breast and ovarian cancers (1). Later studies revealed that components of bacteria, including endotoxin and exotoxins, were sufficient to induce an inflammatory response in the tumour. Examination of tumour histology following endotoxin administration revealed the rapid induction of central hemorrhagic necrosis and a shutdown of blood flow to tumours (2, 3). In the 1970s, Carswell *et al* demonstrated that endotoxin induces an endogenous serum factor that triggers loss of blood flow and tumour necrosis (Tumour necrosis factor, TNF) (4, 5). It was therefore recognized that a host inflammatory mediator induced damage to tumours rather than the bacterial products. Though systemic administration of recombinant TNF to patients resulted in serious side effects, though isolated limb perfusion of TNF is now used in some clinical applications (6).

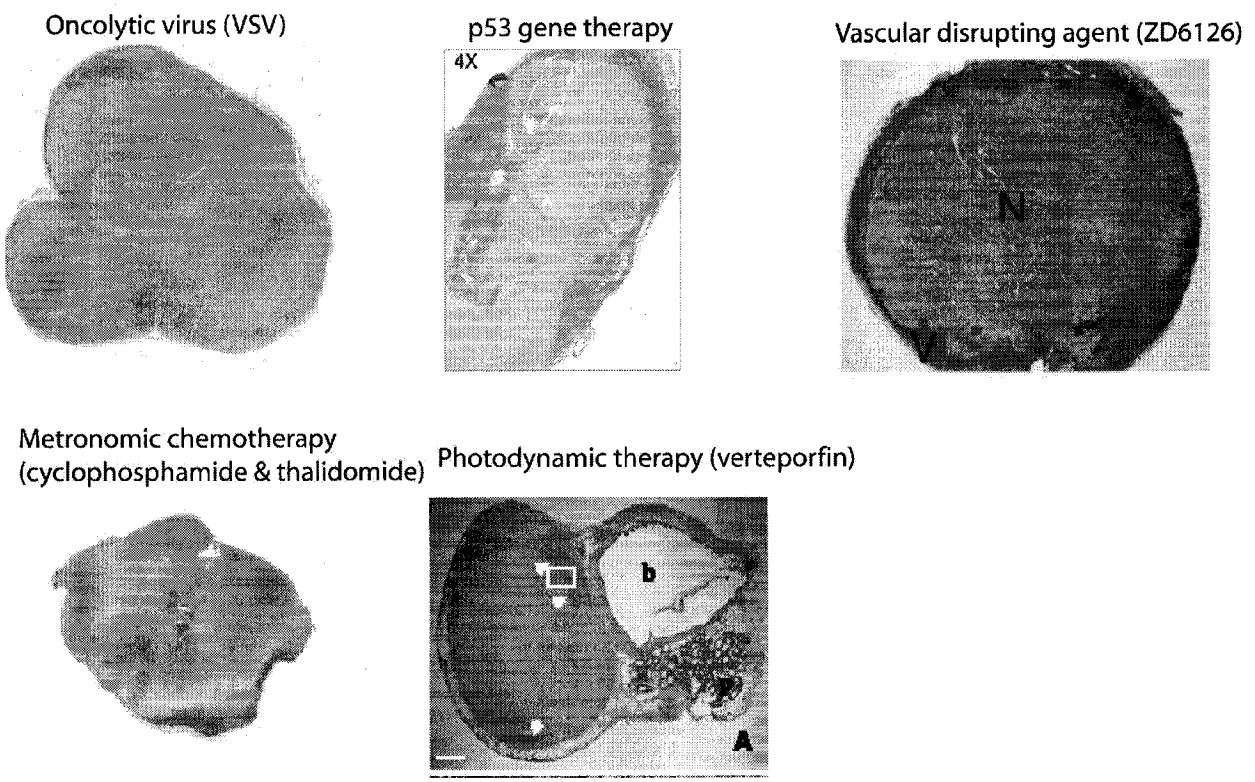
These early observations lead to the hypothesis that manipulation of the tumour microenvironment could result in the generation of anti-tumour adaptive immunity which has lead to the vast field of immune therapy, the goal being to "vaccinate" against tumor antigens. However, cancer vaccines tested thus far in clinical trials have largely reported poor response rates (7). Indeed, even studies in mouse models of cancer have largely been unsuccessful in triggering regression in established, solid, vascularized tumors (7). Since classic anti-microbial vaccines are not used to treat established infections, it is perhaps not surprising that anti-tumor vaccines need to be administered before or shortly following tumor challenge in many animal models.

Despite the poor efficacy of targeting adaptive immune cells to tumours, this has been the primary focus of investigation in the field of immune anticancer therapies. However, increasing recent evidence indicates that innate immune cells can play an important role in cancer therapy. Beyond adjuvant therapy for tumour vaccines, innate immune signaling has been implicated in tumour destruction following conventional chemo- or radiation therapy. Though targeting innate inflammatory cells to tumours has been proposed before (6, 8-11), we review more recent evidence here that diverse anticancer agents induce a tumour targeted inflammatory reaction that can cause significant damage to tumours, largely through effects on the tumour vasculature.

Differences in mechanism of induction of tumour cell death *in vitro* versus *in vivo*: experience from investigations into oncolytic virus therapy

A number of years ago, our group identified an oncolytic virus (Vesicular stomatitis virus) as a potential novel cancer therapy. Indeed, the virus was able to infect a number of cancer cells well *in vitro*, while sparing normal cells. We therefore went on to study the efficacy of the virus in murine tumour models and were able to cure mice of their disease. We assumed that this effect was the result of direct killing of infected cells because we knew that our virus was cytolytic in the target tumour cells *in vitro*. However, the response to the virus *in vivo* was not what we had predicted from our *in vitro* models. Upon histological investigation of treated tumours at short timepoints post infection, we discovered large areas of apoptosis and necrosis in the absence of direct virus infection (12). Upon further investigation, we found that the damage to central areas of the tumour (**Figure 1**) was also associated with a loss of blood flow within the tumour core. We had failed to recognize the considerable differences that exist between cultured cells and solid tumours. The tumour microenvironment includes non-malignant cells, such as endothelial cells, pericytes, lymphatic cells, fibroblasts and innate immune cells, including tumour associated macrophages (TAMs) that impact drug response. Though most literature focuses on deleterious effects of tumour environment on therapy, e.g. poor drug delivery to tumours (13), we observed enhanced tumour cell death as a consequence of virus infection within tumours. We realized that we needed to consider how the uninfected constituents of the tumour and the immune system might respond to the death of virally infected tumour cells. Interestingly, an inflammatory reaction was necessary to trigger the shutdown of blood flow, as depletion of neutrophils in the context of OV therapy resulted in the maintenance of uniform blood flow to tumours. In particular, neutrophil depletion resulted in decreased apoptosis of uninfected tumour cells as well as uniform tumour perfusion. Neutrophils are capable of secreting proteases, and other toxic products that can damage tumour tissue and associated vasculature. Though uninfected tumour cell killing was reported with different OVs in both syngeneic and xenograft tumour models, the most compelling evidence is now emerging from clinical trials with vaccinia virus,

Figure 1. *Examples of tumour histology following treatment with diverse anticancer agents.* Pattern of viable tumour rim and necrotic tumour core observed following treatment with different anticancer therapies. Images taken from (14-17).



where 43% loss of blood flow was measured in a patient with hepatocellular carcinoma following treatment with vaccinia virus (18).

In the setting of OV therapy we have detected fibrin deposition in tumour microvasculature and have shown that, similar to neutrophil depletion, inhibition of coagulation by heparin administration prevents vascular shutdown and tumour destruction following viral infection (unpublished results).

Targeted inflammation may contribute to therapy upon administration of diverse agents

Photodynamic therapy

Upon characterizing the capacity of inflammation to cause significant targeted damage to tumours, we expected this effect to solely be a byproduct of virus infection in tumours. However, the contribution of an inflammatory response in tumours following a seemingly unrelated anticancer treatment - photodynamic therapy (PDT) – has been well characterized (19-21). Indeed, the tumour microenvironment following PDT and OV therapy are similar (**Figure 1**). Tumour vessels are targeted by reactive oxygen species, which results in inflammation, vessel collapse and cell death in the tumour core (17, 22-24).

Photodynamic therapy triggers oxidative damage to tumours by exposing a photosensitizer to light and molecular oxygen. Upon activation by light, photosensitizers produce reactive oxygen species (ROS), that damage tumour cells and associated vasculature, which can impact blood flow as early as 1 h post treatment (22). Upon reperfusion and reintroduction of oxygen, sudden ROS generation induces oxidative stress which results in endothelial activation and formation of pro-inflammatory mediators. As a consequence of these events, leukocytes adhere to endothelium at the damaged site and induce further vascular damage by production of oxidizing species, predominantly by neutrophilic myeloperoxidase (MPO) (25-28).

Initial damage induced by the therapy results in production of damage associated molecular patterns (DAMPs, e.g. Heat Shock Proteins, degradation products of cellular membranes, intracellular molecules in necrotic tissue, extracellular matrix fragments) and leads to Toll like receptor (TLR) stimulation, production of pro-inflammatory cytokines and further immune cell recruitment. In addition, hypoxic endothelial cells and tumour resident immune cells produce of pro-inflammatory molecules (e.g. arachidonic acid metabolites) and expression of pro-inflammatory genes and adhesion molecules such as P-selectin, ICAM and VCAM (29) resulting in the recruitment and activation of innate immune cells. In particular, an influx of neutrophils to tumours was detected in response to PDT due to a transient and local increase in the expression of the chemokine MIP2 (murine equivalent of IL-8) and an increase in expression of E-selectin (30). Importantly, neutrophil depletion abrogated tumour growth retardation in a rat

rhabdomyosarcoma model, indicating that the inflammatory response following PDT was responsible for tumour regression (19). Neutrophil infiltration was preceded by increased expression of interleukin 1 β (IL-1). Inhibition of GCSF (granulocyte colony-stimulating factor), which decreases neutrophil counts, reduced the efficacy of PDT.

Complement activation has also been shown to promote damage to tumours following PDT. Blocking complement activation reduced cure rates following treatment with PDT in a murine tumour model (31). Vascular damage can be triggered by the assembly of complement lytic membrane attack complexes. In addition, complement fragments C3a and C5a are induced following PDT and induce neutrophil accumulation in tumours further exacerbating the increased vascular permeability, microvascular hemorrhage and inflammatory response within tumours (32). Downstream consequences of pro-inflammatory cytokine secretion, complement activation and neutrophil influx are the conversion of the endothelium from a non-adhesive barrier to a pro-coagulant surface. Platelet aggregation, vasoconstriction, vessel occlusion and finally microvascular collapse ensue. Activation of the coagulation cascade has been shown to increase PDT efficacy and administration of the anticoagulant warfarin resulted in decreased efficacy (31).

Vascular disrupting agents

Upon recognition of similarities in tumour histology following oncolytic virus therapy and PDT (**Figure 1**), we realized that tumours following treatment with vascular disrupting agents also present a similar pattern of cell death. Vascular disrupting agents (VDAs) e.g. Disodium combretastatin A-4 3-O-phosphate (CA-4-P, Oxi4503 and ZD6126) target existing tumour vasculature by preventing polymerization of tubulin resulting in the disruption of cytoskeletal structure and changes in the shape of endothelial cells. These events lead to endothelial cell monolayer barrier dysfunction and loss of blood flow to tumours. Central tumour necrosis and loss of blood flow have been documented with an array of imaging methods in a variety of murine and rat tumour models (15, 33-35) and is now also being reported in clinical trials (36). It is thought however that the host response to endothelial damage contributes to a sustained loss of blood flow to tumours. *In vitro* studies have demonstrated that HUVECs treated with VDA upregulate adhesion molecules such as E-selectin, becoming an adherent surface to neutrophils (37). Furthermore, increased myeloperoxidase activity was detected in VDA treated tumours *in vivo* (38), and detection of neutrophil infiltration to tumours has been demonstrated in a number of *in vivo* studies (39). Further evidence for the involvement of neutrophils in tumour damage following VDA treatment stems from a study in which it was shown that nitric oxide (NO) production by tumours can contribute to vessel protection. Nitric oxide synthase (NOS) inhibition decreased MPO activity in tumours (38) suggesting that the protective effects of NO may result from its inhibition of neutrophil activity (39). Though the contribution of neutrophils to VDA therapy

has not been directly tested, it is thought that these inflammatory cells contribute to the tumour cytotoxic activity of VDAs (40). Furthermore, microvascular thrombosis involving platelet activation has also been reported as a consequence of VDA treatment, likely triggered by damage to tumour microvessels, (15, 41-44). Platelet activation was observed following treatment with cationic liposomes containing paclitaxel, in which this chemotherapeutic is preferentially delivered to tumour associated vasculature (45). Similarly, coagulative necrosis of tumour tissue was reported upon Doxil (STEALTH liposomal doxorubicin) treatment alone or in combination with low-dose tumour necrosis factor α (46). Again in another VDA modality (antibody targeting toxic moieties to tumour vasculature), widespread thrombosis and hemorrhage within tumours was observed (47). More recently, Ran *et al* demonstrated that an antibody directed against anionic phospholipids primarily exposed on the surface of tumour associated blood vessels resulted in vascular damage, binding of monocytes to endothelium & infiltration of macrophages to orthotopic breast tumours in mice (48).

Another class of VDAs, which include 5,6-dimethylxanthenone-4-acetic acid (DMXAA) possess a number of antivascular actions, in particular the induction of cytokines that disrupt vascular integrity. Numerous studies have demonstrated the contribution of tumour necrosis factor α (49, 50) as well as interferon β (51) to the induction of tumour necrosis following treatment with DMXAA. TNFα is a pleiotropic cytokine primarily produced by macrophages/monocytes (6). TNF has been shown to increase vascular permeability and trigger the activation of endothelial cells, resulting in the recruitment of and activation of immune cells (52, 53). These observations suggest the importance of innate immune cells in the induction of vascular disruption and subsequent damage to tumours.

Thus far, few studies measure the potential role of an inflammatory reaction in sustaining tumour vascular shutdown. Investigators simply monitored tumour blood flow following administration of VDAs and have not suspected that inflammation was a major cause of vascular damage. Given the evidence presented above, a case can be made that the ultimate mechanism of tumour destruction following VDA treatment is similar to the tumour-targeted inflammation observed following treatment with OVs and PDT.

Adaptive immune therapies

Cancer immunotherapy involves stimulating immune cells to specifically recognize and attack tumour cells. However, it has been recognized that CTL killing – the classic method of measuring T cell activation – only sometimes correlates with the extent of tumour regression *in vivo* (54, 55). Various mediators produced by tumour reactive T cells can cause damage to tumours, including perforin/granzyme B, interferon γ (IFN γ), Fas-ligand or tumour necrosis factor α (TNF α). Classically, T cells are thought to be able to specifically deliver their toxic payload to their target cell. However, in many tumour models, the

production of IFN γ by T cells targets tumour vasculature, resulting in significant damage to surrounding tumour cells. Indeed, infusion of either $CD8^+$ or $CD4^+$ T cells into tumour bearing mice can result in decreased vascular density and damage to the tumour core (56, 57). Furthermore, Blohm *et al* reported decreased tumour vascularity and accumulation of granulocytes in tumours of mice infused with cytotoxic T cells (58).

Interleukin-2 (IL-2) treatment is among the few immunotherapies approved for clinical use. Several reports suggest that inflammation and destruction of tumour vasculature contribute to tumour regression following IL-2 treatment. Di Carlo *et al* found that rejection of tumour cells expressing IL-2 was mediated primarily by macrophages and granulocytes (59). In addition, damaged microvessels were associated with these tumours. In another study, intratumoural administration of IL-2 was shown to enhance $CD8^+$ T cells that target both tumour cells and tumour-associated vasculature (60).

p53 gene therapy

Beyond the effect of targeted inflammation on tumour regression in response to a variety of cancer therapeutics, innate immune cells were required for tumour regression following reintroduction of p53 in some tumour models. In particular, p53 reintroduction induced senescence in liver tumours and sarcomas in mice and was sufficient to induce tumour regression (14, 61). However, based on *in vitro* observations, tumours would be expected to stop growing but not regress as senescent cells persist in culture. It was shown that cells undergoing senescence upregulated pro-inflammatory cytokines that resulted in recruitment of innate immune cells to the tumour. Upon depletion of a number of innate immune cell types, tumours did not regress and a lower cure rate was recorded. Interestingly, the effect extended beyond macrophages and dendritic cells – whose activity and activation status is critical in generating an adaptive anti-tumour immune response, as granulocytes were also critical for tumour regression upon reintroduction of p53. Interestingly, tumour histology of a sarcoma following reintroduction of p53 demonstrates central necrosis with a viable rim, consistent with histology observed following e.g. treatment with OVs (**Figure 1**). We would predict that based on these studies the re-introduction of p53 in these tumours induced an acute inflammatory reaction, possibly through a stress response, leading to vascular shutdown and central tumour necrosis.

Further diverse anticancer therapies

The frequency of inflammation implicated in the loss of blood flow to tumours may be underestimated as many groups do not investigate tumour histology shortly following treatment. Disease progression is monitored and the mechanism of tumour regression is assumed to be the same as the mechanism of cell death observed *in vitro*. Just as we assumed that we knew HOW we were destroying tumours (and were happy to see an effect) many others may be missing the actual mechanism of destruction. For instance, anti-angiogenic

approaches may trigger acute inflammation. Gene transfer of tissue inhibitor of metalloproteinases-1 (TIMP-1) resulted in formation of large necrotic areas associated with many inflammatory cells (62). Furthermore, individual reports of tumour histology following administration of such diverse agents as chemoablation therapy (63), cryosurgery (64), ultrasound therapy (65), hyperthermia (66), therapeutic antibodies (67-69), small molecule angiogenesis inhibitors (70, 71), and continuous low-dose metronomic chemotherapy (16), reveal formation of a large central necrotic fraction reminiscent of that seen upon onset of tumour-targeted inflammation. Furthermore, some conventional chemotherapeutics such as doxorubicin, cyclophosphamide and melphalan have been shown to activate signaling pathways that lead to the induction of pro-inflammatory cytokines (72-74). This may result in induction of mediators responsible to inflammatory damage to tumours directly or to tumour vascultature. However, the impact of inflammation on tumour regression following these treatments is not known.

The deleterious sterile inflammatory response: lessons from other diseases

The contribution of inflammation to destruction of tumour cells is likely unappreciated. In contrast, a similar process has been implicated in the insult of normal tissues in the context of ischemia/reperfusion injury as well as chronic lung diseases. I/R injury is responsible for significant tissue damage in different pathological conditions such as myocardial infarction, pulmonary and haemorrhagic stroke, acute kidney and liver failure and organ transplant rejection (75-77). Similar to the effect of neutrophil depletion in the context of OV and PDT therapies, much less tissue damage is induced in response to an ischemic insult in the absence of neutrophils (reviewed in (78, 79). Furthermore, other molecular mediators produced by the host in response to the initial damage induced by PDT are similar to those initiated during normal tissue ischemia/reperfusion injury. Upon vessel occlusion and onset of ischemia in normal tissues, xanthine dehydrogenase is converted into xanthine oxidase (XO), which generates reactive oxygen species (ROS) (80). PDT was shown to induce XO (81). As mentioned previously, ROS generation results in endothelial cell activation, leukocyte adhesion and vascular barrier dysfunction. These events give rise to a sustained loss of blood flow, termed the 'no-reflow phenomenon', when blood flow to an ischemic organ is not fully restored (82), resulting in substantial tissue damage. This evidence suggests that mediators responsible for enhanced damage to tumours following anticancer therapies may be similar to those causing the exaggerated deleterious response to an ischemic insult. An important question that needs to be addressed now is why tumour vasculature (particularly the vasculature in the tumour core) seems more prone to damage induced by inflammation than vasculature associated with normal tissue. As well we need to determine whether or not the mechanisms that protect the viable rim in each case are related and whether we can impact on these processes to

extend tumour damage (possibly by taking an opposite tack to that taken to rescue more tissue following other ischemic events).

The (inflammatory) stress response & resistance to chemotherapy

A cell responds to an insult by changing its redox state, resulting in activation of an acute inflammatory response. This host reaction can then contribute to the onset of the cellular stress response (83). Studies have found increasing evidence of induction of cellular stress in tumours leading to increased resistance to chemotherapy. Multiple mechanisms have been proposed and will be discussed here. Firstly, stressed cells may arrest in the G1 phase of the cell cycle, reducing susceptibility to anticancer drugs that have been designed to target rapidly dividing cells (84). Pro-inflammatory cytokines have been shown to increase expression and activity of multidrug transporters (MDRs) in tumours (85). There is also some evidence that the level of chemotherapeutic drug targets is reduced upon induction of cellular stress. For instance, it has been reported that expression of topoisomerase II is decreased during stress conditions (84). Furthermore, enhanced heat shock protein expression has been associated with resistance to chemo- or radiation therapy in breast cancer and leukemia (86). However, upon recognition of the onset of a cellular stress response in tumours, rational drug combinations may be chosen to target stressed cells. For instance, Hsp inhibitors have been shown to sensitize cells to chemotherapeutics (87-89). In addition, cisplatin has been shown to be an effective treatment for stressed cells (84). These observations suggest that induction of a stress response due to acute inflammation of tumours may result in resistance to chemotherapy, though this may be overcome through rational drug combinations.

Interplay of targeted inflammation and generation of adaptive anti-tumour immune response

If it is true that acute inflammatory responses induced by a variety of cancer therapeutics can lead to a similar, transient vascular shutdown event then what happens to this mass of dead tumour tissue? We believe that there is a possibility that this has the potential to generate an immunogenic form of cell death where this mass of dead and dying tumour tissue will be phagocytosed and presented to the acquired immune system. Indeed, following PDT, OV therapy or even TNF administration in mouse models several groups have reported the induction of an anti-tumour immune response (90-98). Tumour targeted inflammation is thought to induce increased capillary leakage (99, 100) and expression of adhesion molecules (101, 102), which is likely facilitating entry and function of effector T cells. The exact mechanisms underlying this induction are not fully elucidated but it is generally thought that rapid killing of tumour cells results in increased antigen uptake by antigen presenting cells that go on to stimulate specific T cells.

There is a growing realization that certain methods of killing tumour cells may be more immunogenic than others due to the production of particular danger or eat-me signals by the dying tumour cells (103, 104). Apetoh *et al* report that endogenous danger signals exposed by dying tumour cells following treatment of seemingly non-immunogenic therapies (chemo- or radiation therapy) activate dendritic cells, which present tumour antigens to tumour reactive CTLs (105). The report includes an analysis of clinical data in which patients with breast cancer carrying a loss-of-function allele in TLR4 relapse more quickly than women with functional TLR4, strongly implicating innate immune signaling with disease control. This result suggests that innate cells also have the potential to contribute indirectly to anticancer therapies through subsequent acquired immune responses.

It is not entirely novel to suggest that innate inflammatory responses are important in inducing or regulating acquired immunity (as recently reviewed here (106)). We do believe, however, that the role of acute inflammation in mediating significant tumour destruction, possibly through loss of tumour perfusion, and the subsequent importance of removal of dead or dying tumour cells has been overlooked in many therapeutic models.

Implications for further investigations

Study the contribution of inflammatory cells to tumour response

Much research has been focused on the deleterious effects of the tumour microenvironment on the ability of the drug to function. However, investigators are likely underestimating potential benefits of non-malignant cells within tumours. Different cancer therapies seem able to induce initial tumour injury due to lesions on endothelium and tumour cells which creates local trauma, triggering a response meant to prevent the spread of tissue damage, contain disrupted homeostasis, remove dead/damaged tissue, promote local healing and restore tissue function: the definition of an inflammatory response. This response can cause damage to tumour cells not exposed to the primary insult. We would therefore like to highlight that though chronic inflammation has been implicated in the progression of malignancy, acute, targeted inflammation can cause significant damage to tumours specifically while leaving normal tissue unscathed. Indeed, evidence is beginning to emerge that tumour regression can be dependent on innate immune cells in response to diverse anticancer agents – even those that would not traditionally be viewed as immunostimulatory (e.g. radiation- or chemotherapy).

In the development of novel cancer therapies, researchers initially test their compound of choice in tissue culture. Upon proving a therapeutic window *in vitro*, compounds are then tested in animal models of disease. Few reports

include histological findings investigating which cells within tumours are dying after treatment and if, for instance, tumour regression is acutely associated with immune cell infiltration. In the field of oncolytic virus therapy, we have the advantage of being able to visualize virus infection in histological sections of tumours, allowing us to identify which cells are dying due to the *primary* insult (a direct effect of virus infection and cell killing) versus the *secondary* insult (the ensuing inflammatory response). Though identifying target cells of other anticancer therapies is not as simple, we encourage investigators using various cancer therapies to investigate the role played by acute inflammation in tumour destruction by depleting inflammatory cell populations in over the course of treatment.

The inflammatory response can be manipulated in the context of anticancer therapies

The benefit of understanding the contribution of inflammation to tumour regression includes the fact that we now possess tools to manipulate the inflammatory response. With increasing knowledge of how the innate immune response is regulated, e.g. with the characterization of the TLR signaling cascade and identification of pathogen well as endogenous damage associated molecular patterns (PAMPs and DAMPs) that are TLR ligands we now have the ability to up- or downregulate this signaling in order to steer the immune response. Manipulation of innate immune signaling is being tested as a strategy to improve adaptive anti-tumour therapies (7). Importantly, a diverse array of anticancer therapies will induce tumour damage (by definition) and exposure of DAMPs.

The fact that this pattern of tissue destruction and preservation closely resembles that seen in ischemia/reperfusion injury in the settings of myocardial infarction and stroke makes us wonder if those in the cancer therapeutics field may not be able to learn something from their colleagues who are studying these pathologies. In the setting of stroke the goal has been to save the viable rim (termed penumbra) (107) and to minimize tissue damage but cancer researchers may be able to reverse these strategies to enhance tumour destruction following inflammation-induced vascular shutdown.

The inflammatory response: impact on combination therapy

Targeting inflammation to the tumour microenvironment can result in destruction of up to 95% of the tumour within hours of drug administration. Importantly, in the context of the therapies investigated, inflammation has minimal effects on normal tissue. In addition to the normal tissue being spared, it is consistently observed that the tumour rim remains viable. Though seemingly a detriment, the viable, oxygenated rim provides fertile ground for combination therapies especially as the effect is more beneficial for large tumours, which are normally more resistant to standard therapies. Indeed, there has been some

success with combining vascular disrupting agents and conventional chemotherapy, anti-angiogenic therapy or radiation (108-110). Though conceptually a loss of blood flow to tumours would impede drug delivery, studies have shown that this change in the tumour microenvironment actually results in enhanced drug retention in tumours (111-113). Furthermore, time dependant release of a VDA first followed by a cytotoxic agent from nanoparticles resulted in enhanced drug retention and therapeutic index (114). Evidently, experiments must be carefully laid out to determine the best sequence, dose and timing of combination therapies as new agents will likely be introduced clinically in combination with current anticancer therapies.

Conclusions

We review here some evidence that inflammation is capable of causing targeted damage to tumours by affecting tumour blood flow. Interestingly, therapies with different primary modes of action, including generating reactive photoproducts in the context of photodynamic therapy, or tumour cell infection by oncolytic virus, a major cause of tumour cell death is the ensuing inflammatory response that compromises tumour blood flow. Even seemingly unrelated interventions such as the reintroduction of p53, which is now being considered as a novel therapeutic approach (115) have demonstrated a crucial role of inflammation in tumour regression. Furthermore, inflammation may also play an important role in the context of treatment with vascular disrupting agents. Is this effect truly limited to these therapies? We argue that they are probably not. If an inflammatory response is triggered by exposure of danger signals by dying cells within a tumour, a diverse range of insults may trigger this response. We would like to encourage a closer examination of the role played by innate immunity and inflammation in the destruction of tumours by diverse means. We often use mice with defects in acquired immunity in cancer models but rarely are mice with defective or suppressed innate immunity included to examine the role played by this often ignored portion of the immune system. Therefore, communication should be fostered among scientists involved in diverse areas of cancer research as they may be studying common mechanisms of tumour destruction. Furthermore, literature from seemingly unrelated fields such as ischemic diseases may be relevant to cancer researchers. Though diverse anticancer agents possess different mechanisms of targeting cancer, we should recognize that the reaction to these diverse agents *in vivo* can be quite similar. Importantly, inflammation and disruption of blood flow to tumours have now also been recorded in patients underscoring potential clinical implications of targeted tumour inflammation.

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Appendix III. Chemical targeting of the innate antiviral response by histone deacetylase inhibitors renders refractory cancers sensitive to viral oncolysis.

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Contribution of Authors: This publication demonstrates that histone deacetylase (HDAC) inhibitors can sensitize otherwise resistant cancer cells to oncolytic viruses while still sparing normal tissue. Most of the work was performed by TL Nguyen and Hesham Abdelbary, under the supervision of JC Bell and J Hiscott. As a part of their *in vivo* studies, we tested the effect of HDAC inhibitors and oncolytic viruses on the effect of tumour perfusion (CJ Breitbach contribution).

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Chemical targeting of the innate antiviral response by histone deacetylase inhibitors renders refractory cancers sensitive to viral oncolysis

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Intratumoral innate immunity can play a significant role in blocking the effective therapeutic spread of a number of oncolytic viruses (OVs). Histone deacetylase inhibitors (HDIs) are known to influence epigenetic modifications of chromatin and can blunt the cellular antiviral response. We reasoned that pretreatment of tumors with HDIs could enhance the replication and spread of OVs within malignancies. Here, we show that HDIs markedly enhance the spread of *vesicular stomatitis virus* (*VSV*) in a variety of cancer cells *in vitro*, in primary tumor tissue explants and in multiple animal models. This increased oncolytic activity correlated with a dampening of cellular IFN responses and augmentation of virus-induced apoptosis. These results illustrate the general utility of HDIs as chemical switches to regulate cellular innate antiviral responses and to provide controlled growth of therapeutic viruses within malignancies. HDIs could have a profoundly positive impact on the clinical implementation of OV therapeutics.

HDAC inhibitor | oncolytic virus | refractory tumors | combination therapy

Oncolytic virotherapy is an innovative alternative to conventional cancer therapies based on the concept that it is possible to select or engineer viruses to preferentially replicate in and kill tumor cells (1–5). A variety of strategies are being developed to restrict oncolytic virus (OV) growth to malignancies (4), but one common cellular characteristic that likely plays a role in the selectivity of a spectrum of OVs is an acquired, tumor specific defect in cellular innate antiviral responses (6). As an example, tumors often develop a diminished response to the antiviral IFN cytokines, perhaps because of strong selective pressure to avoid immune surveillance (6). Although aberrations in the cellular antiviral response occur frequently in tumors, the magnitude of the defect is quite variable and can be a barrier to effective OV spread through a malignancy (7–9). Indeed more potent OVs are being developed that express viral gene products to combat cellular innate immune responses (10, 11); however, this genetic approach may ultimately limit the safety of the therapeutic. We reasoned that combining a viral therapeutic with a compound that reversibly compromises host antiviral genetic programs could provide a means to enhance OV growth in tumor cells. Histone deacetylase inhibitors (HDIs) are small molecules currently in clinical development that have demonstrated potent anti-tumor activity but are also known to prevent the transcriptional activation of antiviral genes after IFN stimulation or virus infection (12–19). Here, we demonstrate that a variety of HDIs markedly enhance OV killing of tumor cells *in vitro* and *in vivo* but do not increase OV growth in normal tissues. HDIs, as tumor specific viral sensitizers, have the potential to significantly increase the spectrum of malignancies amenable to OV therapy.

Results

HDI Treatment Sensitizes PC3 Prostate Cancer Cells to VSV-Mediated Oncolysis. *Vesicular stomatitis virus* (*VSV*) is a prototypical *rhabdovirus* that grows poorly in normal tissues but replicates efficiently in cells lacking an intact IFN response (20), a property that prompted the development of *VSV* as an oncolytic agent for tumor cells with acquired defects in IFN signaling. We have shown that approximately 75% of tumor cell lines tested lack a normal IFN response (8); however, the extent of this defect is variable and, at low virus concentrations, IFN production may be sufficient to blunt *VSV* spread (21, 8, 9). As an example, the androgen independent prostate cancer cell line PC3 is partially responsive to IFN and at low multiplicities of infection, is refractory to *VSV* infection (21) (Fig. 1*A*). Because HDIs are known to interfere with the ability of cell lines to mount an IFN response, we examined the possibility that pretreatment of PC3 cells with the HDIs would sensitize them to *VSV* infection and subsequent virus-induced apoptosis. For these experiments, we used an attenuated strain of *VSV* encoding the *GFP* gene (*VSV*-Δ51-GFP) and 2 distinct HDI—MS-275 and SAHA (Vorinostat)—which have shown promising anti-cancer activity in preclinical (MS-275) and clinical (SAHA/Vorinostat) trials (22–31). Both HDIs dramatically increased *VSV* replication in PC3 cells as early as 24 h after infection, at which time robust *GFP* expression was detected by fluorescence microscopy and FACS analysis. Increased *GFP* expression correlated well with virus production from HDI treated cells [Fig. 1 *A* and *B* and supporting information (SI) Fig. S1*A*] and by 96 h, enhanced induction of apoptosis was observed in cells treated with HDI plus *VSV*, compared with cells treated with virus or HDI alone (Fig. 1*A* and *C*).

Addition of the caspase inhibitor z-VAD-fmk abrogated induction of apoptosis by *VSV* alone or in combination with MS-275 or SAHA, demonstrating that induction of cell death was caspase-dependent (Fig. 2*A*). Changes in mitochondrial membrane potential (JC-1 staining) were used as a measure of activation of the intrinsic apoptotic pathway (Fig. 2*B*). The combination of *VSV* and MS-275 or SAHA increased the number of cells exhibiting mitochondrial membrane depolarization to 72% and 59%, respectively, compared with *VSV* (30%), MS-275 (12%) or SAHA (21%) alone. Finally, the activation of apical and effector caspases was investigated by immunoblot analysis (Fig. 2*C*). Although *VSV* proteins

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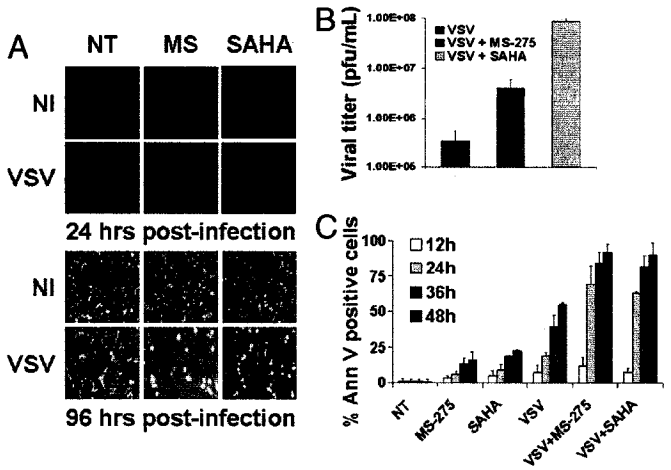

Fig. 1. HDIs enhance *VSV* oncolysis in partially resistant PC3 cancer cell line. PC3 cells were either not treated (NT) or pretreated with MS-275 or SAHA for 24 h, then infected or not (NI) with *VSV*-Δ51-GFP. (*A*) Viral replication was assessed by fluorescent microscopy for GFP expression after infection with *VSV* at 10^{-4} MOI. Phase-contrast microscopy at 96 h demonstrated massive cell death in combination-treated cells. (*B*) Viral titers as determined by standard plaque assay after *VSV* infection at 0.1 MOI. (*C*) Induction of apoptosis was established by Annexin-V staining after *VSV* infection at 0.1 MOI.

were clearly expressed in the presence of HDAC inhibitors, the level of active caspase 8 was not affected, illustrating that HDIs did not affect the extrinsic apoptotic pathway. In contrast, activation of caspase 9 and downstream effector caspase 3 was observed after infection in the presence of MS-275 or SAHA (compared with *VSV* alone, Fig. 2*C*), indicating that combination treatment impacted at the level of the mitochondrial apoptotic pathway. These results reveal that HDIs act at 2 levels by increasing virus replication and spread, and affecting the intrinsic apoptotic pathway. The striking increase in oncolysis suggested the possibility that our virus pharmacophore and the HDIs interacted in a synergistic fashion. To test this idea, *in vitro* cytotoxicity of PC3 cells was assessed at varying

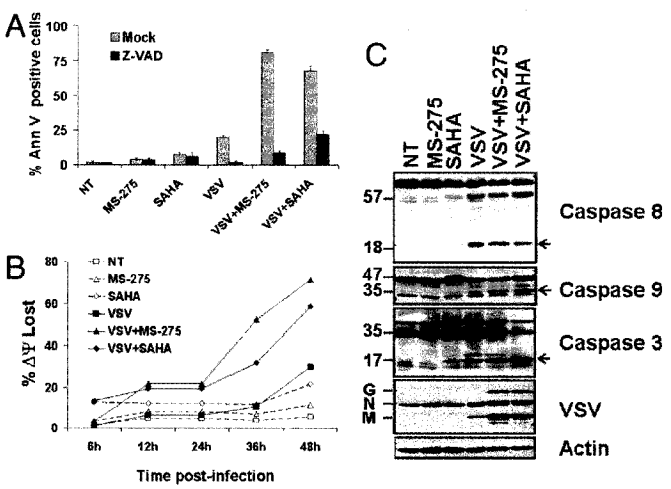

Fig. 2. *VSV* plus HDI combination treatment synergistically increases apoptosis in PC3 cells. PC3 cells were either not treated (NT) or pretreated with MS-275 or SAHA for 24 h then infected with *VSV* at 0.1 MOI. (*A*) Induction of apoptosis was measured by Annexin V staining in the presence or absence of the pan-caspase inhibitor Z-VADfmk. (*B*) Mitochondrial membrane-depolarization was assessed by JC-1 staining. (*C*) Caspase 8, 3, and 9 cleavage was determined 48 h after *VSV* infection in the presence or absence of HDIs by immunoblot of whole cell lysates. Cleaved caspase forms are indicated by open arrows.

concentrations of *VSV* and HDI in a fixed ratio design. Combination indices (CI) (10) were then used to qualify the interaction between *VSV* and MS-275 or SAHA. It is generally considered that CI values <0.7 indicate bona fide synergy. Both HDIs interacted with *VSV* in a highly synergistic fashion (CI < 0.4 at ED_{50}). In addition, the decreasing CI values obtained with increasing cellular fractions affected (CI < 0.12 at ED90) indicate that the synergistic interaction between *VSV* and HDIs may be clinically relevant (Fig. S1*B*).

HDIs Enhance *VSV* Replication in PC3 Cells by Dampening the IFN Response. To gain an understanding of how MS-275 and SAHA enhanced *VSV* infection, virus activation of the IFN cascade in PC3 cells was examined in the presence or absence of HDIs. *VSV* infection of PC3 cells induced the expression of several gene products from the IFN cascade, including RIG-I, IFN alpha and beta, IRF-7, ISG56 and MxA, an IFN-inducible GTPase with direct involvement in the inhibition of *VSV* replication (32, 33) (Fig. 3 *A–C*, and Fig. S2*A*). HDI treatment led to the blunting of the cellular IFN response and robust virus protein production (Fig. 3*C*). RT-PCR revealed that infected PC3 cells treated with HDIs expressed less IFN-β mRNA at 12 h and essentially undetectable levels at 24 h after infection, in contrast to the infected untreated cultures (Fig. 3*B*). Similarly, the levels of MxA and IRF-7 mRNA were inhibited in HDI treated PC3 cells (Fig. 3*B*). However, HDIs did not impact on the IFN signaling cascade upstream of IRF-3. Indeed, virus-induced IRF-3 phosphorylation/degradation was easily detected in samples pretreated with HDIs but not in samples infected with *VSV* alone (Fig. 3*C*, first row). This absence of IRF-3 activation by *VSV* alone is in agreement with previous studies demonstrating that at low MOI, IRF-3 phosphorylation/degradation was not detected, yet was sufficient to induce expression of downstream ISGs that inhibited virus multiplication (34, 35). Treatment of PC3 cells with HDIs in the absence of virus infection did not affect IFN levels or IFN-inducible gene expression (data not shown).

Many tumor cells maintain a partial response to IFN that is sufficient to interfere with oncolytic virus spread in cultures (21). A collection of cancer cell lines with this phenotype was infected with *VSV*-Δ51-GFP in the presence or absence of IFNα (Fig. 3*D*) and GFP expression was used as a measure of virus infection and spread. As predicted, virus infection was severely impaired by addition of IFNα to the culture media, but this protective effect was reversed by pretreatment of cells with MS-275 or SAHA (Fig. 3*D* and Fig. S3). These data support the notion that HDIs act to inhibit expression of both IFN and IFN-inducible genes. The potentiation of virus spread was not restricted to oncolytic *VSV*, as we found that both *vaccinia virus* (36) and *Semliki Forest virus* also rapidly spread through tumor cell cultures exposed to HDIs (Fig. S2 *B* and *C*).

HDIs Specifically Enhance *VSV* Spread in Primary Human Tumor Specimens. To determine whether HDI enhancement of *VSV* replication and tumor cell killing was effectively translated to primary human samples, malignant and adjacent normal prostate cell cultures were established from radical prostatectomy samples. Dissociated cultures infected with *VSV*-Δ51-GFP in the presence or absence of HDIs were analyzed for virally expressed GFP and Annexin-V staining by flow cytometry (Fig. 4*A*). No evidence of *VSV* replication or virus-induced apoptosis was observed in either normal or tumor cultures in the absence of HDIs. However, prostate cancer cells became GFP positive and were ultimately killed after *VSV* infection in the presence of either MS-275 or SAHA (Fig. 4*A*, *upper panels*). In contrast, normal prostate cells from the same patient remained refractory to *VSV* infection, even when exposed to HDIs (Fig. 4*A*, *lower panels*). In both normal and tumor tissue, the efficacy of HDI treatment was confirmed by monitoring acetylation of histone H3 by immunoblot in PC3 cells and normal PBMCs (Fig. S4*A*).

Next, the ability of HDIs to stimulate virus growth in intact primary tumor samples was evaluated in human explants obtained

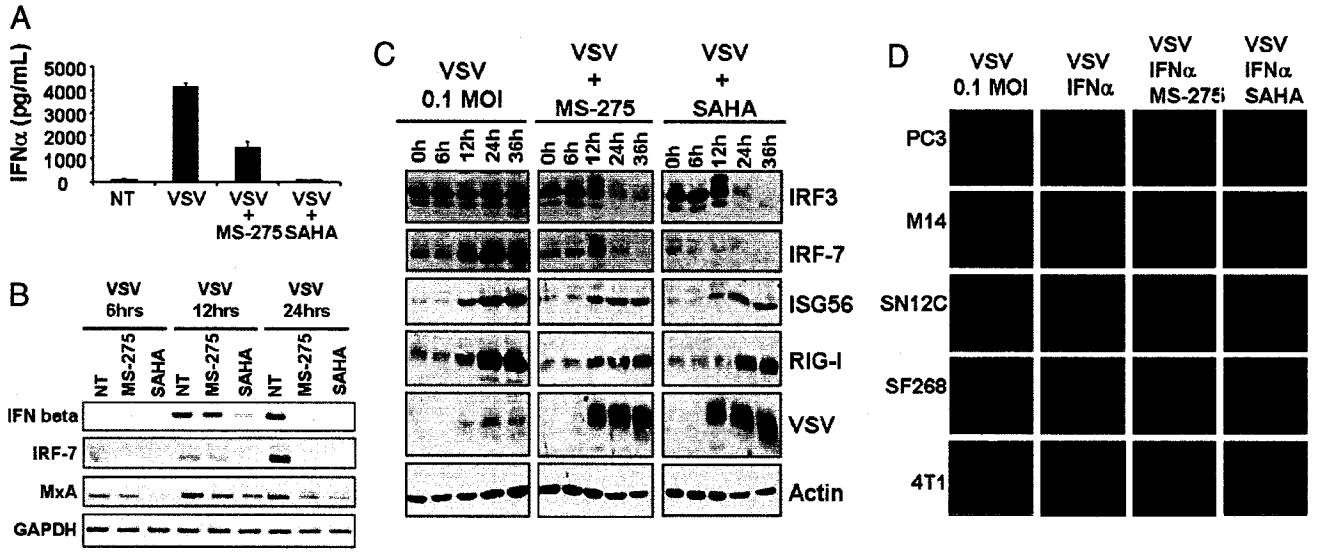

Fig. 3. HDIs augments *VSV* replication through inhibition of the IFN antiviral response. PC3 cells were either not treated (NT) or pretreated with MS-275 or SAHA for 24 h then infected with *VSV* at 0.1 MOI. (*A*) IFN-α levels in supernatants were assayed by ELISA at 24 h after infection. (*B*) Induction of antiviral genes *IFN-beta, IRF-7* and *Mxa* was assessed at different times after infection by RT-PCR. NT denotes non-HDI treated cells (*C*) IFN-stimulated antiviral genes were analyzed by immunoblot at different times after *VSV* alone or *VSV* plus HDI treatment. (*D*) Different cell lines were pretreated with HDIs for 7 h and then infected with *VSV*-Δ51-GFP at 0.1 MOI in the presence or absence of recombinant IFNα. GFP expression was monitored 24 h after *VSV* inoculation.

from patients undergoing surgical resection. Tissue slices were incubated with *VSV* in the presence or absence of the drug; similar to the dispersed primary cultures, HDIs specifically enhanced *VSV* replication and spread only in primary tumor explants but not in normal tissue slices. For example, in both ovarian cancer and sarcoma samples, intense GFP fluorescence and virus replication were detected in the combination treated samples (Fig. 4*B* and Fig. S4*B*), whereas slices of normal colon, muscle, and lung tissue remained refractory to virus infection even in the presence of HDIs. Furthermore, PBMCs isolated from healthy donors (Fig. S4*C*) remained resistant to *VSV* infection and killing at high MOI even with HDI pretreatment. Taken together, these results demonstrate the ability of HDIs to specifically enhance virus replication in primary tumor tissues but not in normal human samples.

***In vivo*, Systemic Coadministration of MS-275 with *VSV* Augments Viral Oncolytic Activity Strictly at the Tumor Site.** Five different *in vivo* cancer models were used to investigate the safety and anti-tumor efficacy profiles of HDI plus *VSV* combination therapy. These models included mice bearing PC3 (prostate), M14 (melanoma), HT29 (colon), 4T1 (breast), and SW620 (colon) s.c. tumors, and a spontaneous bilateral transgenic ovarian cancer model. MS-275 was chosen for the treatment of all models because of the noticeable enhancing effects demonstrated *in vitro* (Fig. S5 *A* and *B*), and the safety profile demonstrated in multiple phase I clinical trials (24, 37, 38). In all cases, the drug was administered i.p. (IP), while an assortment of virus delivery routes was tested. For these experiments, *VSV*-Δ51 was engineered to express firefly luciferase (*VSV*-Δ51-Luc) so that virus replication could be monitored *in vivo* using an In Vivo Imaging System (IVIS). In initial studies, mice bearing s.c. PC3, M14 or HT29 tumors were treated by direct intratumoral (IT) injection, along with daily drug (or vehicle) administration (Fig. S5*C*). In each tumor model, MS-275 therapy was able to promote *VSV* replication *in vivo*, as measured by firefly luciferase activity and by immunohistochemical (IHC) staining for *VSV*

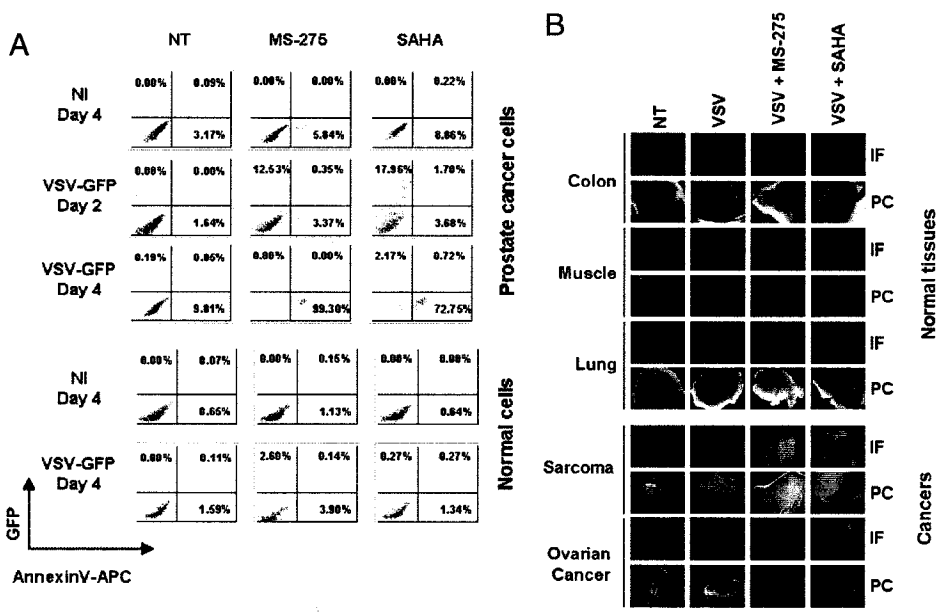

Fig. 4. HDI pretreatment enhances *VSV* oncolytic activity in primary tumor specimens. (*A*) *Ex-vivo* primary cancer or normal prostate cells were subjected to 24 h HDI pretreatment followed by *VSV*-Δ51GFP infection (5 MOI). *VSV* replication (GFP, *y* axis) and apoptosis induction (AnnexinV-APC staining, x axis) were determined at 2 and 4 days after infection by FACS. NT denotes non-HDI treated cells, NI denotes non-*VSV*-infected samples. (*B*) Human *ex-vivo* cancer or normal tissue specimens were inoculated with *VSV*-Δ51-GFP in the absence or presence of HDI pretreatment for 7 h. GFP expression was monitored 48 h after viral inoculation by fluorescence microscopy (IF). Phase contrast (PC) images of tissue samples are shown. NT denotes non-HDI treated, non-*VSV*-infected cells.

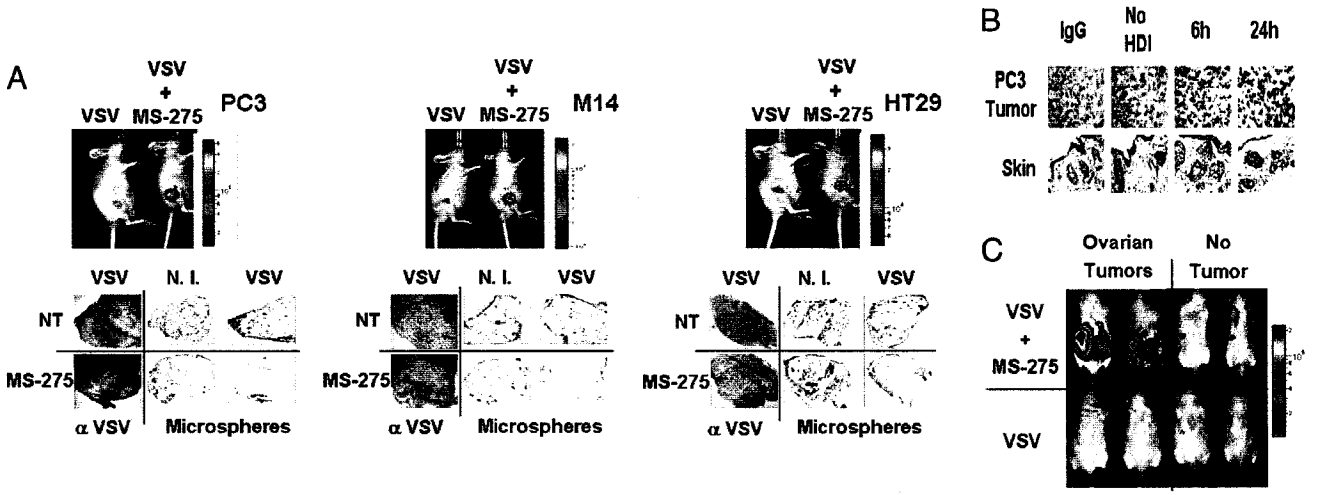

Fig. 5. HDI treatment specifically enhances *VSV*-Δ51-Luc replication at the tumor site. (*A*) PC3, M14, and HT29 s.c. xenograft tumor models were established in nude mice and treated with a single intratumoral *VSV*-Δ51-Luc injection (1×10^6 pfu) alone or in combination with MS-275 IP every 24 h. Viral replication at the tumor site was imaged using the IVIS system. Fluorescent orange microspheres were perfused to outline the tumor microvasculature (Microspheres). Frozen tumor sections were stained with anti-*VSV* antisera (α-*VSV*). NT = non-HDI treated, N.I. = non-*VSV*-infected. (*B*) Acetylation of histone H3 proteins was assessed in PC3 tumors by IHC at 6 h and 24 h after single IP delivery of MS-275. Skin sections were used as normal controls. (*C*) Transgenic mice bearing bilateral ovarian tumors were administered a single dose of *VSV* IP (1×10^8 pfu) alone or in combination with MS-275 IP. Viral replication in live animals was assessed 48 h after viral infection by IVIS imaging.

antigens (Fig. 5*A*). Hyperacetylation was furthermore confirmed in the PC3 tumors by immunohistochemistry at 6 and 24 h after treatment (Fig. 5*B*). Notably in these and subsequent experiments, the luciferase signal was restricted to the tumor mass (Figs. 5 and 6 and Fig. S6*A*) and infection of normal tissues in any of the mice receiving combination therapy was not detected (data not shown). Recently, we demonstrated that OV infection of tumors initiates a rapid and profound loss of blood flow to the tumor that can be measured by decreased uptake of intravenously administered fluorescent microspheres (39). This "vascular shutdown" phenomenon was clearly replicated in perfusion studies performed after coadministration of *VSV* and MS-275 in these 3 models (Fig. 5*A*).

We next tested a spontaneous transgenic tumor model of ovarian cancer with the combination of MS-275 and *VSV*, both delivered by intraperitoneal injection. In Fig. 5*C*, IVIS imaging demonstrated that *VSV* replication was augmented by HDI treatment and restricted to the bilateral tumors arising on the ovaries of these mice. In both tumor bearing and tumor free mice treated with *VSV* alone, low level luciferase activity was occasionally observed in the spleen but this activity was transient and was not significantly enhanced by HDI treatment.

The 4T1 breast cancer model is highly metastatic when implanted s.c. and is refractory to *VSV* therapy (40) (Fig. 6*A* and Fig. S6*A*). In mice infused IV with *VSV* alone, a weak signal emanating from infected metastatic nodules was observed between 24 and 48 h but ultimately *VSV* was cleared (Fig. S6*A*) and treatment had no effect on disease progression. However, when combined with HDI therapy, virus replication was evident both in the primary s.c. lesion, and at multiple metastatic sites; replication persisted past 80 h and had a dramatic impact on tumor size (Figs. 6*A* and Fig. S6*A*). A more marked effect on tumor size was observed in the SW620 model of colon cancer, where i.v. *VSV* infection of s.c. SW620 tumors concomitant with daily MS-275 therapy resulted in a steady increase in luminescence at the tumor site and virtually no tumor growth in the combination treatment group (Fig. 6*B*).

Not only did MS-275 show tumor specific enhancement of *VSV* replication, but the HDI also behaved as a regulatory chemical switch that can be used to control virus replication within the tumor (Fig. 6*C*). This unique characteristic of the combination therapy was demonstrated in the SW620 model, where MS-275 therapy was halted on day 4 and the luciferase signal was lost from the tumor by day 7. Reinitiation of HDI therapy at day 7.5 resulted in a reemergence of the luciferase signal at day 9 and continued regression of the tumor (Fig. 6*C* and Fig. S6*B*). This observation demonstrates that *VSV* replication closely correlated with the pharmacokinetics of the HDI used and the combination strategy

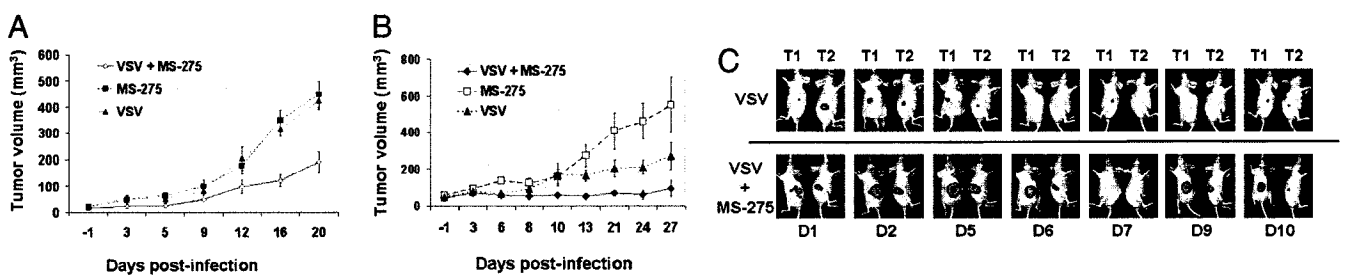

Fig. 6. HDI plus *VSV* combination treatment augments tumor-specific viral replication and significantly reduces tumor size. (*A*) Immunocompetent BALB/c mice bearing s.c. 4T1 tumors were treated with *VSV* (1×10^8 pfu), alone or in combination with MS-275 IP. The efficacy of MS-275, *VSV* and *VSV*/MS-275 combination treatment in reducing tumor growth was assessed by tumor volume measurement over time. The average tumor size and standard error for each treatment group was calculated. (*B*) and (*C*) CD1 nude mice bearing SW620 tumors in hind flanks were treated with a single i.v. *VSV* injection (1×10^7 pfu) alone or in combination with MS-275. The efficacy of MS-275, *VSV* and *VSV*/MS-275 combination treatment in reducing tumor growth was assessed by tumor volume measurement over time (*B*). Virus replication at the tumor site was revealed in live animals by IVIS imaging (*C*). Time course of treatments are schematically presented in Figs. S5 and S6.

requires effective protein hyperacetylation within the tumor to reach maximal effective oncolytic activity.

Discussion

Oncolytic viruses are often engineered to exploit tumor specific genetic deficiencies or constitutively activated signaling pathways (1–5). Regardless of the mechanism of selective targeting, all oncolytic viruses must also overcome the arsenal of antiviral programs that the individual cell has at its disposal to resist infection and/or the spread of viruses. One strategy to generate a more potent OV is to arm the virus with genes that express products to circumvent or blunt the innate antiviral response of the individual cell (10, 11). A different approach is to engineer viruses that are unable to resist cellular antiviral programs (8, 41), thus increasing their safety profile because they cannot replicate in normal tissues but retain the ability to grow in malignant tissues. During the evolution of malignancies, genetic abnormalities accumulate that provide cancer cells with growth and survival advantages but at the same time compromise the ability of individual tumor cells to mount a robust antiviral response (8, 32, 42–44). The inability of a tumor cell to secrete or respond to IFN may facilitate tumor escape from immune surveillance (44). Although defects in cellular innate immunity are commonly found in tumor cells, the extent of the defect is quite variable (8, 21, 40). Because histone deacetylases have been implicated in modulating the IFN response in cell lines (12–19, 45), we hypothesized that HDIs could complement OVs and facilitate the infection and killing of tumors that had an impaired antiviral response. Our study design used relatively low mulitiplicities of infection (MOI) *in vitro*, to closely approximate the situation *in vivo*; furthermore we expected the effects of HDIs on virus spread to be most pronounced at low MOI. An unanticipated finding from this study was the effect of HDIs on the apoptotic program of infected cells; the intrinsic mitochondrial pathway appears to be preferentially targeted by the combination of HDIs and *VSV*, as demonstrated by an increase in mitochondrial depolarization and cleavage of caspases 9 and 3 in combination treated cells. The effect on cell death was synergistic and increased at higher effective doses of combined treatment, supporting a clinically relevant interaction between HDIs and *VSV*. In addition to a direct virus-mediated induction of tumor cell death, oncolysis may also be mediated by indirect mechanisms triggering apoptosis through the release of inflammatory cytokines and mediators from infected and dying tumor cells. *In vivo*, the effective dose of an OV that reaches a tumor is further compromised by the limitations imposed by tumor architecture and microenvironment. However, despite poor virus delivery, a significant portion of cells within OV-treated tumors undergoes apoptosis. We recently demonstrated that loss of blood flow to the interior of the tumor causes massive cellular apoptosis. Although the mechanisms remain to be defined, the absence of vascular perfusion within infected tumors was induced by neutrophil recruitment to the tumor bed (39). These current studies reinforce the involvement of "vascular shutdown" as an important mechanism of tumor cell killing, and further illustrate that the combination of OV and HDI augments vascular shutdown in the tumor bed.

Importantly, the combination of *VSV* and HDIs enhances tumor killing but does not significantly increase *VSV* infection of normal human or mouse tissues. Although the exact basis for this exquisite selectivity remains to be determined, the results are consistent with the idea that HDIs blunt the cellular IFN response in tumor cells. In combination treated PC3 cells, key antiviral genes such as *IRF7*, *ISG56* and *MXA* were expressed at low levels and were poorly inducible by virus infection. Our results and those of others are consistent with the effects of HDIs occurring at the level of gene transcription (12–19, 45), but clearly HDAC activity is found in other cellular compartments besides the nucleus and has been implicated in the cellular response to a variety of stresses (46, 47). Although we favor a key role for HDIs in dampening IFN activity in tumor cells, the existence of additional stress responses affected by HDI that impact on virus replication cannot be excluded. Furthermore, different classes of HDIs may have distinct effects depending on the tumor or the oncolytic virus, in part because HDIs target different classes of HDACs (24, 27, 28).

A potential clinical advantage of this combination therapy approach is highlighted by the observation that continuous systemic administration of HDI is required to maintain robust virus replication within the tumor; it may thus be possible to regulate the magnitude of OV therapy by withdrawing or applying HDI. This strategy could be particularly advantageous when the OV therapeutic harbors a gene that facilitates imaging of the infection, or if the malignancy is located in an area (e.g., brain) where tumor swelling—as a consequence of virus infection—could lead to unwanted complications. HDI induced sensitization of tumor cells to viral oncolysis was not restricted to *VSV*, because both *Semliki Forest virus* and *vaccinia virus* also displayed increased oncolytic activity in the presence of HDIs. This observation is immediately relevant because vaccinia virus has entered into clinical trials and the effects of HDIs on the magnitude of oncolysis may be applicable to a wide spectrum of OVs under development. In conclusion, the diverse and safe applicability of HDI plus oncolytic virus combination therapy should facilitate rapid translation toward clinical application. Investigations are under way to delineate how HDIs may modulate transcriptional programs in tumor cells and how the host adaptive immune response may further enhance the antitumor effects of oncolytic viruses.

Methods

Viruses. *VSV*-Δ51 expressing GFP and GFP-firefly luciferase fusion are recombinant derivatives of *VSV*-Δ51, a naturally occurring IFN-inducing mutant of *VSV* Indiana serotype (8). Viruses were propagated and purified as described (7, 8) in Vero cells (American Type Culture Collection).

Primary *ex-Vivo* Prostate Cancer Cell Cultures. Radical prostatectomy specimens and their adjacent normal tissues (as histologically defined by a pathologist) were washed immediately in cold, sterile PBS (PBS). After removing excess, damaged epithelium and stromal tissue, specimens were cut into small pieces and incubated for 10 min at 37 °C in 0.05% trypsin/0.53 mM EDTA (Wisent). Surface epithelium was mechanically separated to dissociate cells into a single cell suspension. Prostate epithelial cells were grown in KSF medium supplemented with 5 mg/100 ml of bovine pituitary extract (BPE) (Gibco/BRL).

Flow Cytometry. After staining with AnnexinV-APC (BD biosciences) or JC-1 stain (Invitrogen Canada Inc.) as per manufacturer's instructions, cells were subjected to flow cytometry analysis (10^4 events/measurement) on a FACS Calibur (Becton-Dickinson) and analyzed with FCS Express V3 software (8, 48, 49).

***In Vivo* Tumor Models.** All mice used were obtained from Charles River Laboratories. HT29 and M14 xenograft models ($n = 2$) were established in the hind flanks of 6–8-week-old female; nu/nu mice PC3 xenograft models ($n = 2$) were established in male nu/nu mice. After tumors became palpable, the double treated group received MS-275 i.p. at a concentration of 23 mg/kg/day. Four hours after administering the second HDI dose, all mice were injected intratumorally with 5×10^6 pfu of *VSV*-Luc. The double treated and MS-275 treated groups continued to receive 23 mg/kg of MS-275 i.p. every 24 h until killed (Fig. S5C). The ovarian transgenic tumor model tgMISIIRTAg564 (Garson *et al.*, manuscript in preparation) is based on the transgenic model from Connolly *et al.* (50). At approx. 13 weeks of age, mice were administered MS-275 IP every 12h at 6 mg/kg, while *VSV* was administered IP at 1×10^8 pfu 4h after the initial HDI dose. Viral replication in live animals was assessed 48h after viral infection by IVIS imaging.

Tumor growth analysis was carried out in 2 models: human colon carcinoma SW620 xenografts in nude mice and 4T1 syngeneic breast carcinoma model in immuncompetent animals, all groups with $n = 4–5$. When tumors were palpable, MS-275 was administered i.p. at a concentration of 7 mg/kg every 12 h for 10 consecutive doses. *VSV*-Luc (1×10^7 pfu) was administered intravenously 4 h after the second MS-275 dose. This treatment cycle was repeated once more 72 h after the last MS-275 dose. Tumor sizes were measured every 3–4 days using an electronic caliper. The average tumor size from each treatment group was calculated for each time point and standard error was calculated to determine statistical significance.

IVIS Imaging. Mice were injected i.p. with D-luciferin (200 ml at 10 mg/ml in PBS, Molecular Imaging Products Company), anesthesized <3% isofluorane (Baxter Corp.) and imaged with the In Vivo Imaging System 200 Series (Xenogen Corporation). Data acquistion and analysis was performed using Living Image v2.5 software.

Analysis of Tumor Perfusion. Mice were injected intravenously with orange fluorescent microspheres and killed as described in ref. 39. Tumor perfusion was analyzed by visualizing fluorescent microspheres in the vasculature of 10 μm unfixed frozen sections using a ScanArray Express microarray scanner with a standard Cy3 laser (Packard Bioscience).

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Appendix IV. Efficacy of systemically administered mutant Vesicular Stomatitis Virus combined with radiation for nasopharyngeal carcinoma is associated with EBV latency.

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Contribution of Authors: This manuscript investigates whether Vesicular Stomatitis Virus (VSV) may be a novel therapy for nasopharyngeal carcinoma (alone or in combination with radiation treatment). The authors describe a number of mechanisms by which virus infection is triggering tumour damage. Among these is an observed reduction in tumour associated blood vessels. One mechanism by which VSV may damage and destroy tumour associated vessels is by direct infection. This result is CJ Breitbach's contribution to the manuscript.

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Efficacy of Systemically Administered Mutant Vesicular Stomatitis Virus (VSVΔ51) Combined with Radiation for Nasopharyngeal Carcinoma

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Abstract

Purpose: Nasopharyngeal carcinoma (NPC) is a malignancy of the head and neck region that is associated with EBV latency. Curative treatments for NPC achieve modest survival rates, underscoring a need to develop novel therapies. We evaluated the therapeutic potential of a mutant vesicular stomatitis virus (VSVΔ51) as single treatment modality or in combination with ionizing radiation (RT) in NPC.

Experimental Design: MTS assay was used to assess cell viability *in vitro*; apoptosis was measured using propidium iodide staining and caspase activation. *In vivo* experiments were conducted using tumor-bearing nude mice with or without local RT (4 Gy). Apoptosis was assessed in excised tumor sections with terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling staining.

Results: Our data showed that NPC cells are exquisitely sensitive to VSVΔ51 oncolysis, which correlated with the presence of EBV. Efficacy of VSVΔ51 against NPC cells was further augmented when combined with RT. A single systemic injection of VSVΔ51 achieved 50% survival in treated mice, which increased to 83% when combined with local tumor RT. In addition to induction of apoptosis, an antiangiogenic effect of VSVΔ51 was observed *in vivo*, suggesting a novel tumoricidal mechanism for VSVΔ51. This virus also prevented growth of NPC sphere-forming cells *in vitro*, showing potential utility in targeting NPC-initiating cells.

Conclusions: Our data represent the first report showing that EBV-positive NPC cells are exquisitely sensitive to VSVΔ51 oncolysis and documenting the successful utilization of this combinatorial regimen as a novel curative therapeutic strategy for NPC.

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Head and neck cancer is the fifth most common malignancy worldwide, affecting ~946,745 individuals annually (1). Almost one-fifth of the total global cancer burden is associated with bacterial or viral infections (2); among these, nasopharyngeal carcinoma (NPC) represents a unique head and neck cancer, which is almost always associated with the EBV (3). The EBV episome is maintained in a latent phase within NPC cells, expressing a restricted set of genes including EBNA1, LMP1, LMP2A, and LMP2B (4), along with the presence of EBV-encoded RNA (5). Standard therapy for NPC patients comprise ionizing radiation (RT) only for early disease, with the addition of chemotherapy for patients with advanced disease. Using conformal or intensity-modulated RT, excellent local control can be attained; however, 43% of patients will still develop distant metastases within 2 years (6). This modest clinical outcome underscores the necessity to develop novel therapies for NPC, which has been the research focus of our group for the past several years (7–9). Our previous treatment approaches (viral and molecular) showed promising results yet were unable to achieve complete eradication of established nasopharyngeal tumors.

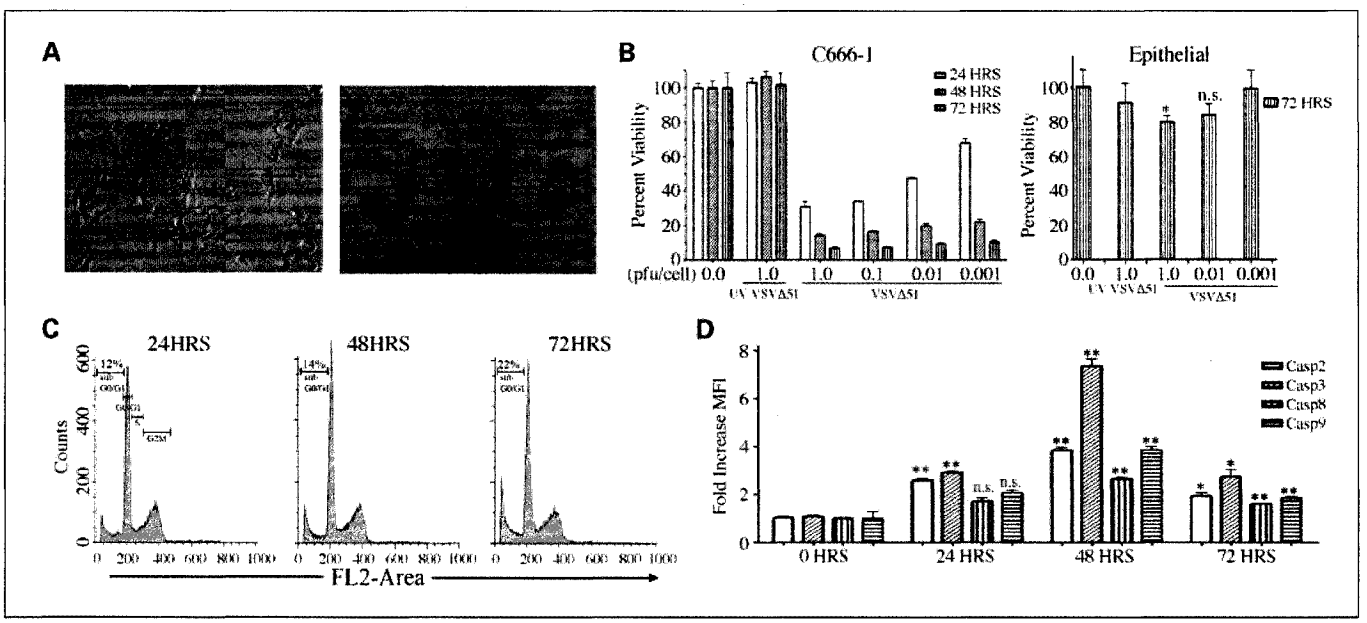

Fig. 1. VSVΔ51 infects C666-1 cells and induces apoptosis. *A,* C666-1 cells were infected with VSVΔ51-GFP (1 pfu/cell); 48 h later, mock (*left*) or VSV-infected (*right*) cells were visualized under light microscopy. *B,* MTS assay for C666-1 cells (*left*) or normal human oral epithelial cells (*right*) viability at the indicated time points following VSVΔ51 infection. Mean ± SD from experiments conducted in triplicate. *C,* representative experiment of propidium iodide staining of C666-1 cells infected with VSVΔ51-GFP (1 pfu/cell) at 24, 48, and 72 h. *D,* fold increase of caspase activation in C666-1 cells following VSVΔ51-GFP infection (1 pfu/cell) at 24, 48, and 72 h compared with mock-infected cells for each time point. Mean ± SE from at least two independent experiments conducted in triplicate. *, $P < 0.05$; **, $P < 0.005$.

Recently, mutant VSV strains (AV1 and AV2), which lack the ability to shut down the host innate immunity, have been described (10). Toxicity experiments showed that the AV1 mutant was ~80-fold less toxic than the wild-type VSV while maintaining similar antitumor efficacy, showing a therapeutic window for these mutant VSV strains. In the current report, the therapeutic potential of using a mutant (VSVΔ51) virus for EBV-positive NPC was investigated either alone or in combination with RT. When VSVΔ51 was combined with RT, an additive antitumor effect was observed both *in vitro* and *in vivo*. In addition, an antiangiogenic effect of VSVΔ51 was also apparent, suggesting a novel tumoricidal mechanism for VSVΔ51. These results show a novel therapeutic approach for NPC, by combining VSVΔ51 with RT, which appears to be particularly effective in EBV-positive disease.

Materials and Methods

Cell lines and reagents. C666-1, CNE-1, CNE1-EBV, HONE1, and HK-1 NPC lines were described previously (11–13). C15 and C17 nasopharyngeal xenograft tumors were maintained *in vivo* (14). Human oral epithelial cells were obtained from Celprogen and were cultured according to company recommendations. Other cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 100 mg/L penicillin, and 100 mg/L streptomycin (RPMI-10) at 37°C, 5% CO_2. The VSVΔ51-green fluorescent protein (GFP) virus has been described previously (10).

Cell infection. Twenty-thousand cells were seeded in 100 μL RPMI-10 in a 96-well plate. Twenty-four hours later, the medium was removed, and VSVΔ51-GFP [10-0.0001 plaque-forming units (pfu)/cell] was added in 20 μL α-MEM, with the plates maintained at 37°C for 60 min to allow virus attachment before the addition of 80 μL normal growth medium (RPMI-10). UV inactivation was done by exposing the virus for 30 min at 10 cm distance under UV light.

Cell viability and measurement of apoptosis. Cell viability was assessed using the MTS assay according to the manufacturer's recommended protocol (Promega). To measure the fraction of cells in the sub-G_0-G_1 phase of the cell cycle, C666-1 cells were infected with VSVΔ51-GFP at 1 pfu/cell, and at indicated time points, cells were harvested and fixed in 70% ethanol for 1 h on ice. Cells were washed once before resuspending in 500 μL fluorescence-activated cell sorting buffer (PBS/0.5% bovine serum albumin) supplemented with 40 μg/mL RNase A (Sigma) and 50 μg/mL propidium iodide. Cells were incubated at room temperature for 30 min in the dark before being analyzed in BD FACSCalibur using FL-2A and FL-2W channels. Cell debris was gated out before the analysis. CaspGlow kit (Biovision) was used to measure caspase activity in virally infected C666-1 cells.

NPC sphere culture. C15 and C17 NPC xenografts were propagated in severe combined immunodeficient mice. The tumor was excised on reaching 1 cm in diameter, cut into small pieces, minced with a scalpel, and then digested with 200 units/mL collagenase type I (Worthington Biochemical) in HBSS. The mixture was incubated for 1.5 h at 37°C, 5% CO_2 followed by repeated pipetting using a 10 mL pipette every 15 to 20 min. Cells were then filtered through a 40 μm cell strainer (BD Biosciences). Cells infected with VSVΔ51 or with UV-inactivated virus were seeded in T75 flask at 1×10^6 per 20 mL RPMI-10 and then incubated at 37°C, 5% CO_2. The number of subsequent NPC spheres (NPCS) was counted on day 21.

Animal experiments. CD-1 nude male mice (5-6 weeks old) were purchased from Charles River Laboratories; all experiments were conducted in accordance with the guidelines of the Animal Care Committee, University Health Network. For tumor formation assays, 1×10^6 C666-1 cells were infected at 5 pfu/cell in α-MEM for 1 h, spun down, and then resuspended in 100 μL PBS before s.c. implantation in CD-1 nude mice. Tumor volume was measured using the formula: $V = LWW / 2$, where L and W represent tumor length and width, respectively. For therapeutic experiments, 3×10^6 C666-1 cells were injected either s.c. on the upper hind flank or i.m. into the left leg. When tumor volume reached 100 mm^3 (for the sc model) or when tumor plus leg diameter reached 8.5 to 9.5 mm (for the i.m. model), treatment was initiated. Mice were treated as indicated by injecting the appropriate dose of virus in 100 μL PBS i.v. via tail vein. For local radiation treatment, animals were immobilized in a lucite box with the

tumor-bearing leg exposed to 100 kV at a dose rate of 10 Gy/min (Gemini Vertical X-ray Beam, Picker Industrial).

Microdistribution studies. CD-1 nude mice bearing C666-1 tumors were injected i.v. with 5×10^8 VSVΔ51-GFP in 100 μL PBS. At 24, 96, or 144 h postinjection, Hoechst 33342 (600 μg in 100 μL PBS) was injected i.v. for visualization of active vasculature (15); the mice were sacrificed 1 min later. Tumors were immediately excised, frozen in OCT compound (Bayer) and then stored at -80°C. Serial sections (5 μm thickness) were cut through each tumor at three levels, 500 μm apart.

The slides were scanned using a fluorescence microscope to visualize GFP expression and Hoechst 33342 perfusion and were then stained with rat anti-mouse CD31 antibody (BD PharMingen) at 1:500 dilution followed by secondary incubation with anti-rat biotinylated antibody and tertiary incubation with FITC-conjugated streptavidin to fluorescently visualize total tumor vasculature. The slides were then stained with terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling to assess for apoptosis or necrosis. All staining and cryosectioning procedures were done by the Pathology Research Services, University Health Network.

Microscopy. Unstained and FITC anti-CD31-stained slides were imaged at ×10 magnification using an Olympus BX50 tiling fluorescence stereomicroscope (FITC/GFP: λ_{ex} = 482 nm, λ_{em} = 535 nm, 500 ms exposure; Hoechst 33342: λ_{ex} = 360 nm, λ_{em} = 460 nm, 100 ms exposure). Images were captured using ImagePro 5.1 (Media Cybernetics). Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling slides were scanned at ×20 magnification using an Aperio ScanScope CS automated tiling bright-field microscope (Aperio). Fluorescence microscopy images were imported into ImageJ (NIH),[12] false colored, and merged into a composite image, where the red, green, and blue channels corresponded to Hoechst 33342 (active vasculature), GFP (VSV infection), and FITC anti-CD31 (total vasculature), respectively. For the quantification of VSV replication and tumor vascular functionality, four square regions (~100 × 100 μm) were taken per slide for each time point, and pixel intensity values for all three channels (green, red, and blue) were calculated as described before (16).

Statistical analysis. Statistical analyses and graphing were done using Microsoft Excel 2003 and Graphpad Prism 4.0 software.

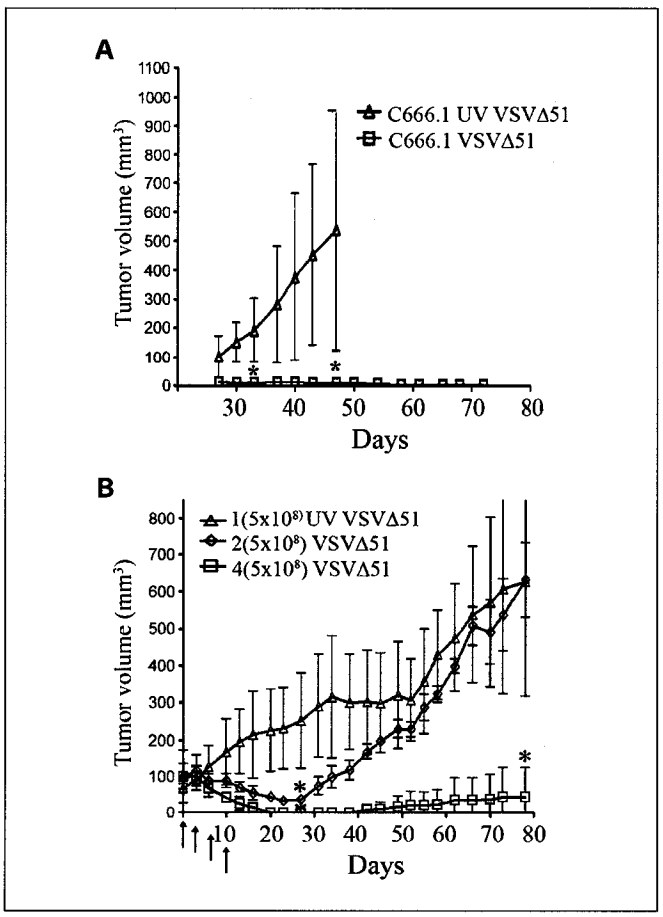

Fig. 2. VSVΔ51 is effective against NPC *in vivo*. *A*, C666-1 cells were infected with UV-inactivated virus or with VSVΔ51 (5 pfu/cell) before implanting into CD1 nude mice. Each group comprised three mice. *B*, C666-1-bearing CD1 nude mice were treated i.v. with two injections (days 0 and 3) or four injections (days 0, 3, 7, and 10) of 5×10^8 VSVΔ51. Control mice received 5×10^8 UV-VSVΔ51 on day 0, and tumor volume was measured over time. Mean ± SD (n = 3 mice in each group). *, $P < 0.05$. *Arrow*, day of virus injections.

Results

***NPC cells are sensitive to VSVΔ51 infection* in vitro.** To evaluate if NPC cells are sensitive to VSVΔ51 infection, EBV-positive NPC C666-1 cells were infected with VSVΔ51-GFP and then examined morphologically. Figure 1A (*left*) shows the appearance of normal C666-1 cells; in contrast, VSVΔ51-infected cells were rounded and detached from the plate (Fig. 1A, *right*), consistent with the appearance of virally infected cells (17). VSVΔ51 was then examined as a single agent, showing significant toxicity in C666-1 cells in a dose- and time-dependent manner (Fig. 1B). By 72 h, even at 0.001 pfu/cell, 89.6% cytotoxicity was shown (Fig. 1B, *left*). In contrast, VSVΔ51 had no significant toxicity against normal human oral epithelial cells at 0.01 pfu/cell (Fig. 1B, *right*). Similarly, the virus exhibited no significant toxicity against the GM05757 normal human fibroblast line (data not shown).

VSVΔ51-GFP kills NPC cells by disrupting the cell membrane and inducing apoptosis. To elucidate the mode of cell death of NPC cells following VSVΔ51 infection, C666-1 cells were infected with VSVΔ51-GFP and at 24, 48, and 72 h postinfection and were stained with propidium iodide to assess membrane integrity. A time-dependent increase in the population of cells with damaged cell membranes was observed, reaching 45% at 72 h postinfection (data not shown). To assess if VSVΔ51 induced apoptosis, cell cycle analysis was done on VSVΔ51-infected C666-1 cells. Again, a time-dependent increase in the sub-G_0-G_1 population was observed, reaching 22% at 72 h postinfection (Fig. 1C). This increase in apoptosis was associated with activation of caspase-2, caspase-3, caspase-8, and caspase-9 (Fig. 1D), with maximal activation observed for the effector caspase-3 (7.3-fold) at 48 h postinfection.

Systemic administration of VSVΔ51 is effective against established nasopharyngeal xenograft tumors. To evaluate whether VSVΔ51 infection can prevent tumor formation *in vivo*, C666-1 cells were infected with VSVΔ51 at 5 pfu/cell before s.c. implantation into CD-1 nude mice. VSVΔ51-infected C666-1 cells failed to form tumors when monitored for >70 days, whereas cells treated with UV-inactivated VSVΔ51 formed tumors as early as 20 days postimplantation (Fig. 2A).

VSVΔ51 was then administered systemically to evaluate its therapeutic potential in mice bearing C666-1 tumors. Two injections of 5×10^8 VSVΔ51-GFP significantly reduced tumor growth compared with mice that received UV-inactivated virus (Fig. 2B). Four injections of VSVΔ51-GFP reduced tumor size below the detection limit as early as 25 days post-treatment;

[12] http://rsb.info.nih.gov/ij/

however, all tumors in this group eventually recurred. Treating tumor-bearing mice with six injections of 5×10^8 UV-inactivated virus had no significant effect on tumor growth (data not shown).

RT in combination with VSVΔ51 is effective against NPC in vitro. Given the data in Figs. 1 and 2, RT was then added to evaluate efficacy of this combined approach. Surprisingly, RT-treated cells were less permissive to virus replication when compared with nonirradiated cells at 24 h post-RT *in vitro* (Supplementary Fig. S1A). This was transient, however; at 48 h, RT plus VSVΔ51-treated cells produced a similar viral titer compared with nonirradiated cells. Lower viral titer was again observed at 72 h likely attributable to the significant cytotoxicity with the combination treatment. RT plus VSVΔ51 killed >99.0% of C666-1 cells compared with 89.0% killing for the VSVΔ51 alone group on day 6 post-treatment ($P = 0.005$; Fig. 3A). Cell cycle analysis showed a slight increase in the percentage of apoptotic cells (sub-G_0-G_1) in the RT plus VSVΔ51 (30.6%) group compared with VSVΔ51 only (28.0%) at 72 h (Supplementary Fig. S1B). As expected, RT alone induced G_2-M arrest, which was significantly reduced when RT-treated cells were infected with VSVΔ51.

Efficacy of RT combined with VSVΔ51 on established nasopharyngeal xenograft tumors. To determine whether combining RT with VSVΔ51 was effective against established xenograft tumors, C666-1 tumors were generated and then randomized to six treatment groups. RT (4 Gy) alone caused significant reduction in tumor growth, which was less effective than single i.v. injection of VSVΔ51 (5×10^8 pfu; Fig. 3B). Three different schedules of RT plus VSVΔ51 were evaluated: simultaneously or 48 h apart, with either RT preceding VSVΔ51, or vice versa. The most effective combination was the simultaneous administration of RT with VSVΔ51, achieving almost complete tumor eradication, followed for 42 days. With longer follow-up time, and plotted as a function of survival, these data showed significant survival advantage for the group of mice treated with the simultaneous administration of RT plus VSVΔ51 ($P = 0.0005$), with 83% (5 of 6) mice surviving beyond 100 days (Fig. 3C).

No evidence of in vivo selection of VSV-resistant NPC cells following systemic administration of VSVΔ51. It has always been unclear whether tumors that regrow have acquired *in vivo* resistance to VSVΔ51 or if they have never been exposed to the virus. This issue was addressed by isolating tumor recurrences,

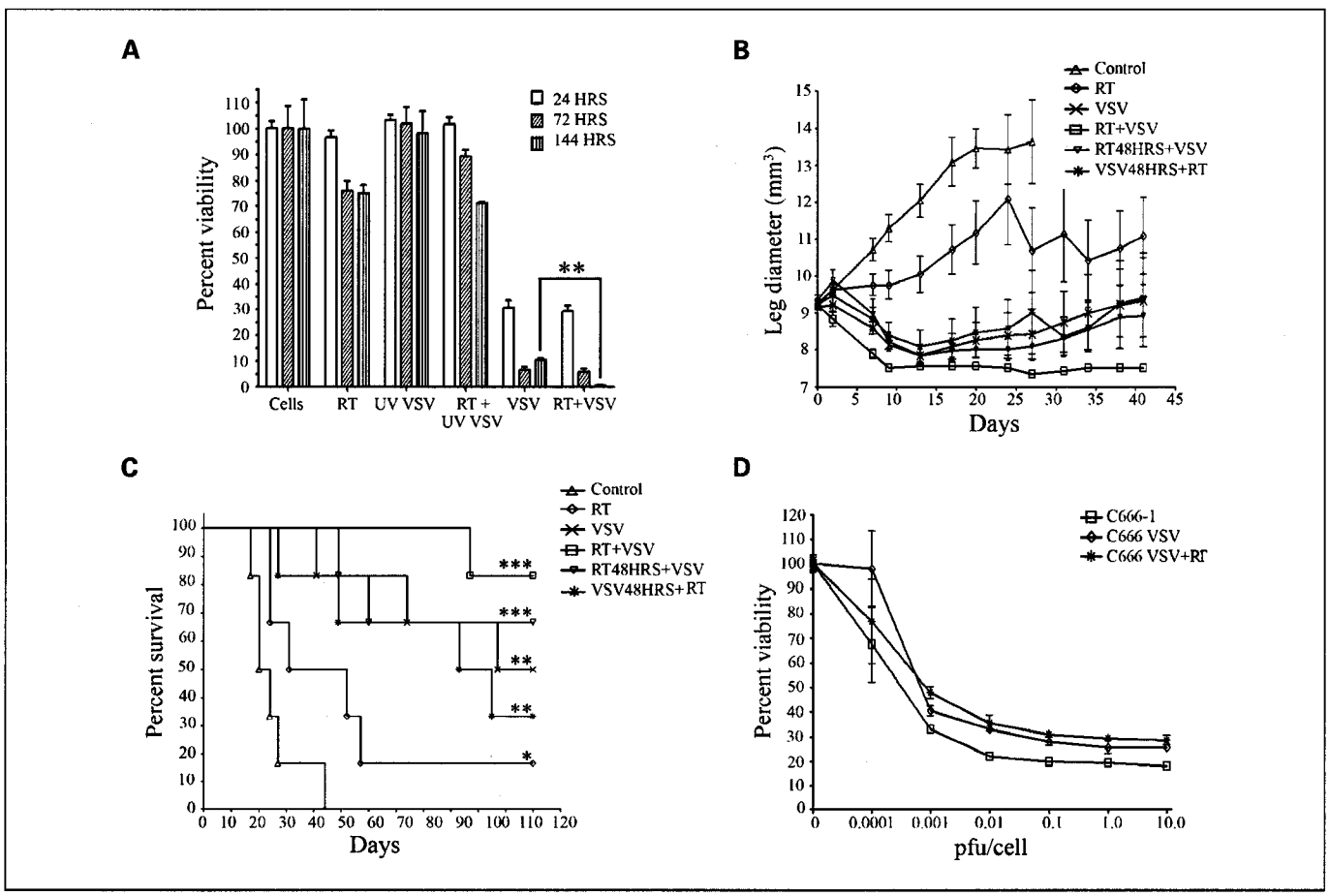

Fig. 3. VSVΔ51 in combination with RT is effective against NPC *in vitro* and *in vivo*. *A*, viability of C666-1 was measured using the MTS assay following VSVΔ51 (1 pfu/cell) or RT (6 Gy) plus VSVΔ51 at the indicated time points. Mean ± SE from at least two independent experiments conducted in triplicate. **, $P < 0.005$. *B*, C666-1 tumors were established i.m. in the left leg of CD1 nude mice to allow for local RT delivery. Mice were then randomized to six groups: (*a*) no treatment, (*b*) RT (4 Gy) alone, (*c*) VSV (5×10^8 pfu i.v.) alone, (*d*) RT plus VSV simultaneously, (*e*) RT preceding VSV by 48 h, and (*f*) VSV preceding RT by 48 h. Leg plus tumor diameter was monitored over time. Mean ± SE ($n = 6$ mice in each group). *C*, mice in *B* were monitored for survival for up to 120 days. All *P* values were calculated in comparison with the control group (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$). *D*, sensitivity of the parental C666-1 cells compared with C666-1 cells reestablished from tumor recurrences after VSVΔ51 administration or VSVΔ51 plus RT measured using the MTS assay. Mean ± SE ($n = 6$).

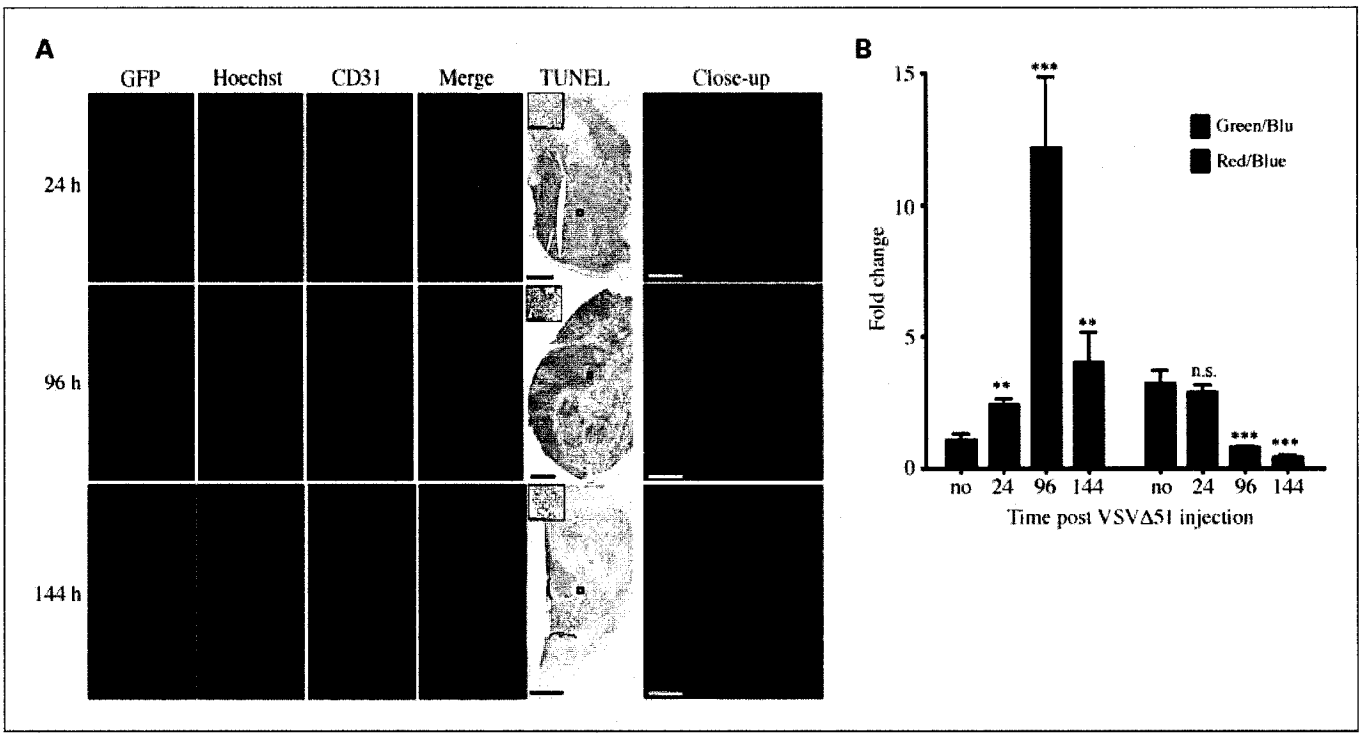

Fig. 4. Representative examples of VSVΔ51 tumor microdistribution in C666-1 xenograft tumors. *A*, viral distribution (GFP) was assessed at three time points (24, 96, and 144 h after infection) in relation to active vasculature (Hoechst, *red*), total vasculature (CD31, *blue*), and necrotic or apoptotic tissues (terminal deoxynucleotidyl transferase – mediated dUTP nick end labeling). The merged field consisted of superimposed VSV, Hoechst, and CD31 images; the close-up images (×100) are high-resolution representations of GFP distribution in relation to local blood vessels. Bars on the wide-field images (*cyan* and *black*), 1 mm. Bars on the terminal deoxynucleotidyl transferase – mediated dUTP nick end labeling close-up images, 125 μm. Bars on the close-up merged images (*white*), 200 μm. *B*, quantification of virus replication and tumor vascular functionality at the indicated time points was done by calculating the ratios of GFP/CD31 (*green/blue*) and Hoechst/CD31 (*red/blue*), respectively. Mean ± SD ($n = 4$). *no*, regions of no virus replication at 24 h. **, $P < 0.005$; ***, $P < 0.0005$.

following VSVΔ51 or RT plus VSVΔ51, and then reexposing to VSVΔ51 *in vitro*, which showed similar sensitivity compared with parental cells, indicating that tumors recurred likely due to inadequate exposure to VSVΔ51 *in vivo* (Fig. 3D).

VSVΔ51 replicates within C666-1 tumors and affects the tumor vasculature. The precise mechanism by which VSVΔ51 causes tumoricidal effects *in vivo* is still not fully elucidated. Hence, C666-1 tumor-bearing mice were treated with a single i.v. injection of VSVΔ51-GFP (5×10^8 pfu), and at 24, 96, and 144 h postinjection, the mice were injected i.v. with Hoechst 33342 (stains functional blood vessels) and sacrificed 1 min later. As early as 24 h postinjection, multiple foci of viral replication were observed (Fig. 4A, *green*), which colocalized with areas containing active blood vessels (*red*). CD31 staining was done to examine for total (functional and nonfunctional) blood vessels (*blue*). At 96 h postinjection, a marked increase in viral replication is clearly observed, accompanied by significant loss in staining for functional blood vessels (*red*) in regions of viral replication (Fig. 4A and B). Most of the tumor area still retained CD31 staining, suggesting that tumor vessel functionality (*red*) might have been destroyed, secondary to viral replication. At 144 h postinjection, there was a significant increase in terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling staining (indicating apoptosis or necrosis), with minimal Hoechst 33342 uptake, indicating extensive tumor cell death.

***VSV kills NPCS-forming cells* in vitro.** C15 and C17 NPC xenografts can only be propagated in mice (14). When these nasopharyngeal tumors are cultured *in vitro*, the tumor-associated murine fibroblasts form a monolayer within a few days. After 3 to 4 weeks, NPC cells start to proliferate on top of the fibroblasts, forming a three-dimensional spheres (Fig. 5A), which we denoted as NPCS. Each NPCS likely originated from a single clonogen. To evaluate whether VSVΔ51 could prevent sphere formation *in vitro*, C15 and C17 xenografts were digested with collagenase, infected with VSVΔ51 or UV-inactivated virus, and seeded in RPMI-10. Three weeks later, the number of NPCS in each sample was counted. VSVΔ51 infection completely prevented NPCS formation *in vitro* for both NPC models (Fig. 5B); VSVΔ51 was also capable of destroying established C17 NPC within 72 h postinfection (Fig. 5C).

Discussion

We present here the first report documenting the successful utilization of the RNA oncolytic virus (VSVΔ51) in combination with RT for the treatment of NPC. This combinatorial strategy was extremely effective, causing complete regression of established nasopharyngeal xenograft tumors in >80% of treated mice, which appears superior to even our previous conditionally replicating adenovirus (18). Firstly, adenoviruses could only be administered using the intratumoral route because systemic administration result in significant liver expression and hepatotoxicity (19). In contrast, systemically administered VSVΔ51-GFP result in no GFP expression in either

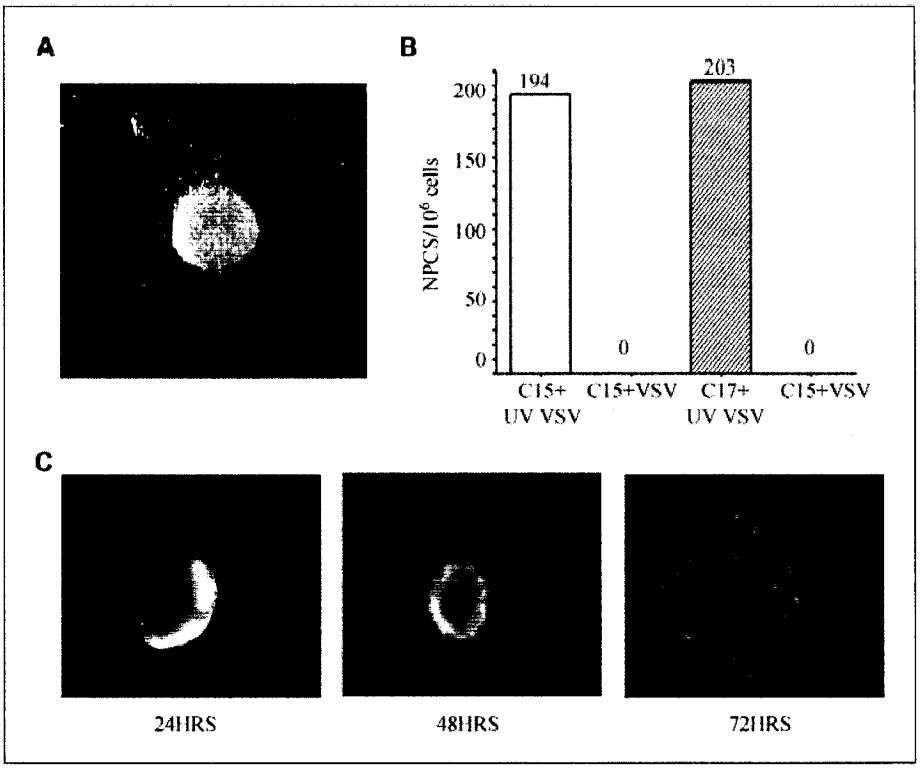

Fig. 5. VSVΔ51 is effective against NPCS *in vitro*. *A*, normal appearance of C17 NPCS in culture (day 21). *B*, number of C15 or C17 NPCS/10^6 cells forming after infection with either UV-inactivated virus (0.1 pfu/cell) or VSVΔ51-GFP (0.1 pfu/cell) on day 21 in culture. *C*, C17 NPCS infected with VSVΔ51-GFP (2×10^4 pfu) visualized using fluorescence microscopy at 24, 48, and 72 h postinfection.

the liver or the spleen of treated mice (data not shown), supporting the clinical utility of systemic administration of VSVΔ51 for NPC patients.

Secondly, a significant immune response is observed with even intratumoral adenoviral therapy, which again limits its tumoricidal efficacy. Certainly, repeated VSV would not be feasible in an immune-competent host, but with the combinatorial approach of local tumor RT with a single injection of VSVΔ51, significant tumor regression was achieved (Fig. 3B and C), thereby potentially bypassing the issue of VSV immunogenicity.

An important mode of cytotoxicity and tumoricidal effects of VSV appears to be apoptosis. In VSVΔ51-infected cells, activation of caspase-2, caspase-3, caspase-8, and caspase-9 were observed as early as 24 h postinfection, which is similar to that previously reported for murine fibroblasts following infection with another mutant (M51R-M) VSV (20). The interaction between RT and VSV is not well understood. RT can induce G_2-M arrest (21), but when VSVΔ51 infection follows RT, this G_2-M arrest was significantly reduced (Supplementary Fig. S1B), which might possibly relate to down-regulation of p53 function in VSVΔ51-infected cells (22, 23).

Yet another novel observation in this report relates to the exquisite sensitivity of NPC cells to VSVΔ51 as a function of EBV status (Supplementary Fig. S2). One possible explanation for this EBV-associated sensitivity to VSVΔ51 infection might relate to data generated from our own group showing induction of EBV lytic genes (*BZLF1* and *BRLF1*) when NPC cells are exposed to DNA damage via RT or chemotherapy (24). We have also observed an induction of these EBV lytic genes in NPC cells during VSVΔ51 infection (Supplementary Fig. S3), which might thereby enhance VSVΔ51 toxicity against NPC cells. The possible involvement of different EBV latent genes in this process remains to be investigated.

The third novel observation in this report relates to the apparent tumor vascular effects of VSVΔ51 infection *in vivo*. There is a clear time-dependent increase in viral replication following systemic administration of VSVΔ51 into NPC-bearing mice accompanied by tumor apoptosis or necrosis (Fig. 4A and B). Thus far, these data are consistent with other reports of apoptosis in glioblastoma xenograft tumors following intratumoral administration of wild-type VSV (25). Interestingly however, when we examined for active tumor vasculature (Hoechst 33342), there was an apparent time-dependent reduction in functional tumor blood vessels, suggesting that VSV could be targeting the tumor vasculature as another mechanism by which VSVΔ51 damages tumors. In fact, active viral replication was observed in tumor vascular endothelial cells as early as 24 h post-VSV injection (Supplementary Fig. S4); hence, these data, combined with the vascular functionality data (in Fig. 4), affirm a direct antivascular effect of this virus *in vivo*. Breitbach et al. have recently reported that tumor vessels are infiltrated by neutrophils following systemic administration of VSV, which would also impair vascular functionality (26). Concordantly, we also observed increased infiltration of nasopharyngeal tumors with neutrophils over time, suggesting additional tumoricidal effect of this virus against nasopharyngeal tumors *in vivo*.

There is increasing evidence of the existence of cancer stem cells (CSC) in human leukemias and solid tumors (27–29). The clonality of NPC and the presence of EBV genome in every NPC cell suggest that there might be a subpopulation of initiating or CSC in this malignancy. We have been able to culture NPC cells *in vitro*, derived originally from NPC

xenograft, to form three-dimensional colonies growing on top of a monolayer of murine fibroblasts. By definition, these NPCS have originated from a single NPC clonogen. Our data showed that VSVΔ51 is capable of infecting and destroying such NPCS *in vitro* and also preventing such spheres from forming, suggesting that VSVΔ51 could also target NPC CSC. It was shown recently that the interaction between vascular endothelial cells and CSC in brain tumors is crucial for the maintenance of "stemness" of such CSC (30). The use of antivascular agents was able to disrupt this interaction, thus ablating CSC from those xenografts. Because VSVΔ51 can also exert antivascular effects on NPC xenografts, this might therefore provide an alternate therapeutic option to inhibit NPC-initiating cells from self-renewal.

In conclusion, this is the first report of the exquisite efficacy of systemically administered VSVΔ51 in combination with RT for EBV-positive NPC. There are likely multiple mechanisms mediating this tumoricidal effect, including direct oncolysis, apoptosis, and antiangiogenesis. We propose that future NPC therapy should evaluate VSVΔ51 in the context of its ability to cure NPC, by eradicating the subpopulation of nasopharyngeal CSC, which could be responsible for recurrences and subsequent deaths of our NPC patients.

Disclosure of Potential Conflicts of Interest

J. Bell, financial interest in Jennerex Biotherapeutics. The other authors declare no potential conflicts of interest.

Acknowledgments

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Appendix V. Translational control of the innate immune response through IRF-7.

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Contribution of Authors: CJ Breitbach performed microarray analyses comparing transcriptionally and translationally upregulated genes in eIF4EBP wildtype and knockout cells. Knockout cells were resistant to infection by a wide variety of viruses, including VSV. The microarray study identified a number of genes involved in the interferon response to be upregulated in knockout cells (including IRF7). These findings helped identify IRF7 to be a critical mediator of the innate immune response (that was translationally upregulated).

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ARTICLES

Translational control of the innate immune response through IRF-7

Rodney Colina[1]*, Mauro Costa-Mattioli[1]*, Ryan J. O. Dowling[1], Maritza Jaramillo[1], Lee-Hwa Tai[2], Caroline J. Breitbach[3], Yvan Martineau[1], Ola Larsson[1], Liwei Rong[1], Yuri V. Svitkin[1], Andrew P. Makrigiannis[2], John C. Bell[3] & Nahum Sonenberg[1]

Transcriptional activation of cytokines, such as type-I interferons (interferon (IFN)-α and IFN-β), constitutes the first line of antiviral defence. Here we show that translational control is critical for induction of type-I IFN production. In mouse embryonic fibroblasts lacking the translational repressors 4E-BP1 and 4E-BP2, the threshold for eliciting type-I IFN production is lowered. Consequently, replication of encephalomyocarditis virus, vesicular stomatitis virus, influenza virus and Sindbis virus is markedly suppressed. Furthermore, mice with both *4E-BP1* and *4E-BP2* genes (also known as *Eif4ebp1* and *Eif4ebp2*, respectively) knocked out are resistant to vesicular stomatitis virus infection, and this correlates with an enhanced type-I IFN production in plasmacytoid dendritic cells and the expression of IFN-regulated genes in the lungs. The enhanced type-I IFN response in *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ double knockout mouse embryonic fibroblasts is caused by upregulation of interferon regulatory factor 7 (*Irf7*) messenger RNA translation. These findings highlight the role of 4E-BPs as negative regulators of type-I IFN production, via translational repression of *Irf7* mRNA.

Virus infection activates a subset of genes encoding cytokines and other antiviral proteins that trigger first the innate immune response and subsequently the adaptive immune response. Type-I interferons (IFN-α and IFN-β) are widely expressed cytokines that constitute the first line of defence against virus infections[1–4]. Although transcriptional control of IFN gene expression has a major role in the activation of the innate immune response, it is not known whether translational control is important for this process. Translational control of gene expression provides the cell with a rapid response to external and internal triggers or cues, without invoking the slower nuclear pathways for mRNA synthesis and transport. In eukaryotes, translational control mostly occurs at the rate-limiting initiation step, during which the small 40S ribosomal subunit is recruited to the mRNA[5]. Ribosome recruitment is facilitated by the 5′-cap structure (m^7GpppN, where N is any nucleotide) present on all nuclear transcribed eukaryotic mRNAs[6]. The cap structure is recognized by eukaryotic initiation factor 4F (eIF4F)[7], which consists of eIF4E, the cap-binding subunit[8], eIF4A, a bidirectional RNA helicase[9], and eIF4GI or eIF4GII, scaffolding proteins that bind directly to eIF4E and eIF4A and bridge the mRNA to the ribosome through interaction with eIF3 (ref. 10).

eIF4F complex assembly is inhibited by the 4E-BP translational repressors[7]. Mammals contain three highly related 4E-BPs that compete with eIF4G for a shared binding site on the convex dorsal surface of eIF4E[11,12]. mTOR-mediated phosphorylation of 4E-BP1 (the best characterized 4E-BP) stimulates translation by dissociating 4E-BPs from eIF4E. Hypophosphorylated 4E-BP1 binds to eIF4E with high affinity, whereas increased phosphorylation decreases its affinity for eIF4E[13].

Because viruses have evolved elaborate strategies to usurp the host translation machinery[14], we wished to study the effect of 4E-BPs on virus infections. To this end, we used mouse embryonic fibroblasts (MEFs) and mice deficient in 4E-BP1 and 4E-BP2.

Virus infection is suppressed in *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ MEFs

To study the role of 4E-BPs in the cell's response to virus infections *ex vivo*, MEFs derived from *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ double knockout and wild-type mice were used[15]. The *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ double knockout MEFs lack all three 4E-BPs, because 4E-BP3 is not expressed in MEFs[16]. MEFs were infected with vesicular stomatitis virus (VSV) at a multiplicity of infection (MOI) of 0.5 plaque-forming units (PFU) per cell and viral protein synthesis was analysed by pulse labelling with [^{35}S]methionine at various times after infection. In wild-type MEFs, synthesis of VSV proteins was first detected at 4 h after infection (Fig. 1a). Notably, in *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ MEFs, no viral proteins were detected at this, or even later, time points (Fig. 1a). Western blot analysis over the time course of infection demonstrated a robust expression of VSV proteins in wild-type MEFs, but not in *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ MEFs (Fig. 1b). A VSV-induced cytopathic effect at 10 h after infection was observed only in wild-type MEFs (Fig. 1c). The lack of 4E-BPs resulted in reduced (~700-fold) virus titres (Fig. 1d). These data demonstrate that removing 4E-BPs abrogates VSV propagation. At a higher MOI (5 PFU per cell), the difference in the kinetics of VSV replication in wild-type and *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ MEFs was less pronounced. In wild-type MEFs, VSV proteins were first detected as early as 2 h after infection, as compared to 4 h after infection in *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ MEFs (Supplementary Fig. 1A). The VSV-induced shut off of host translation in wild-type MEFs occurred at 5 h after infection, whereas in *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ MEFs, the reduction of cellular translation was barely detectable at 6 h after infection. In agreement with these data, the lack of 4E-BPs resulted in a decrease in infectious virus production (Supplementary Fig. 1B).

To confirm that the VSV-resistant phenotype observed in the *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ MEFs is due to the absence of 4E-BPs, and not to some unintended effect of the gene-targeting manipulation, we introduced 4E-BP1 together with 4E-BP2 into *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$

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MEFs. Expression of 4E-BP1 and 4E-BP2, but not empty vector, restored the susceptibility of the *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ MEFs to VSV infection (Supplementary Fig. 2). These data demonstrate that the virus-resistant phenotype of *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ MEFs is directly associated with the lack of 4E-BPs.

Next, we investigated whether *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ MEFs are resistant to infection by other viruses. Wild-type and *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ MEFs were infected with positive and negative strand RNA viruses, such as Sindbis virus (alphavirus, positive strand), encephalomyocarditis virus (EMCV; picornavirus, positive strand) and influenza virus (orthomyxovirus, negative strand). Similar to VSV (rhabdovirus, negative strand), protein synthesis of Sindbis virus (Supplementary Fig. 3A), EMCV (Supplementary Fig. 3B) and influenza virus (Supplementary Fig. 3C) was severely impaired in *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$, as compared to wild-type, MEFs. Accordingly, a marked reduction (>3,000-fold) in virus titres was observed for all three viruses in *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ MEFs (Supplementary Fig. 3D). Taken together, these data demonstrate that the lack of 4E-BPs markedly impairs the replication of a broad spectrum of viruses.

Upregulation of type-I IFN response in *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ MEFs

How do the 4E-BPs affect the replication of disparate viruses? A simple explanation would be that the threshold for eliciting type-I IFN production is lowered in MEFs lacking 4E-BPs. To test this hypothesis, wild-type and *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ MEFs were treated with poly(I:C), a synthetic double-stranded (ds)RNA that is a potent inducer of type-I IFN. The production of IFN was determined by assessing the inhibition of the VSV-induced cytopathic effect. Six hours after treatment with poly(I:C), culture medium from wild-type and *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ MEFs was collected and added to wild-type MEFs (Fig. 2a). After overnight incubation, MEFs were infected with VSV (MOI of 0.1 PFU per cell) and virus yield was determined by a plaque assay. Culture medium from wild-type MEFs treated with a low concentration of poly(I:C) (0.1 μg ml^{-1}) failed to protect cells from VSV infection (Fig. 2b). In contrast, no cytopathic effect was observed and virus production was significantly reduced (~200-fold) in wild-type MEFs incubated with the culture medium from *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ MEFs. Consistent with the notion that the enhanced resistance of the *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ MEFs to virus infection is due to increased type-I IFN production, incubation with a neutralizing antibody against IFN-β rescued the VSV-resistant phenotype (Supplementary Fig. 4A).

Expression of IFN-α (*Ifna*) and IFN-β (*Ifnb*) mRNAs was more responsive to treatment with either 0.1 μg ml^{-1} (Fig. 2c) or 1 μg ml^{-1} (Supplementary Fig. 4B) of poly(I:C) in *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ MEFs compared with wild-type MEFs, as determined by reverse transcriptase polymerase chain reaction (RT–PCR). Moreover, the induction of *Ifna* and *Ifnb* mRNA synthesis after VSV infection was greater in *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ than in wild-type MEFs (Supplementary Fig. 4C). Consistent with these data, poly(I:C) treatment of *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ MEFs, but not wild-type MEFs, elicited a robust production of IFN-α (Fig. 2d and Supplementary Fig. 5C) and IFN-β (Supplementary Fig. 5A, B), as determined by enzyme-linked immunosorbent assay (ELISA). Collectively, these data show that the lack of 4E-BPs results in enhanced type-I IFN production.

Double knockout mice are protected against VSV infection

To determine whether the virus-resistant phenotype of *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ MEFs is recapitulated *in vivo*, wild-type and *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ mice were infected intranasally with VSV (5×10^7 PFU). We chose VSV because its replication is exquisitely sensitive to inhibition by type-1 IFN[17]. By day 6 after infection, 80% of

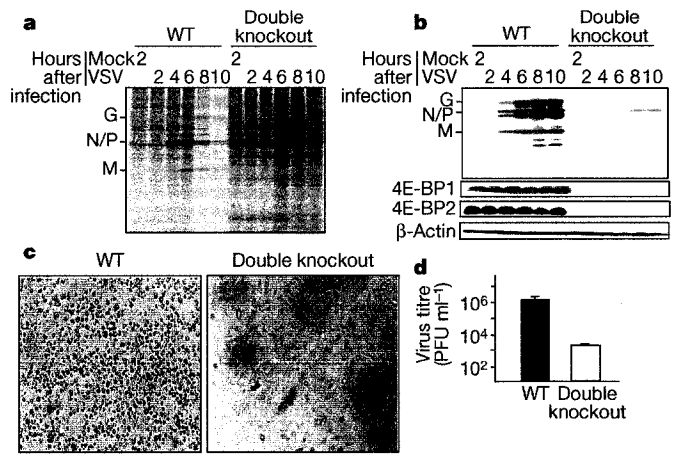

Figure 1 | Lack of 4E-BPs renders MEFs refractory to VSV replication. Wild-type and *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ MEFs were mock-infected or infected with VSV at an MOI of 0.5 PFU per cell. **a**, MEFs were incubated with [^{35}S]methionine for 30 min at the indicated times after infection. Proteins were subjected to SDS–PAGE (15%). An autoradiogram of the dried gel is shown. Viral proteins are indicated on the left. G, glycoprotein; M, matrix protein; N/P, nucleocapsid protein/phosphoprotein. **b**, Western blotting analysis using antibodies against VSV proteins, 4E-BP1, 4E-BP2 and β-actin. **c**, **d**, Cytopathic effect (**c**) and virus yield (**d**) at 10 h after infection in wild-type and *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ MEFs (mean ± s.d. of four experiments).

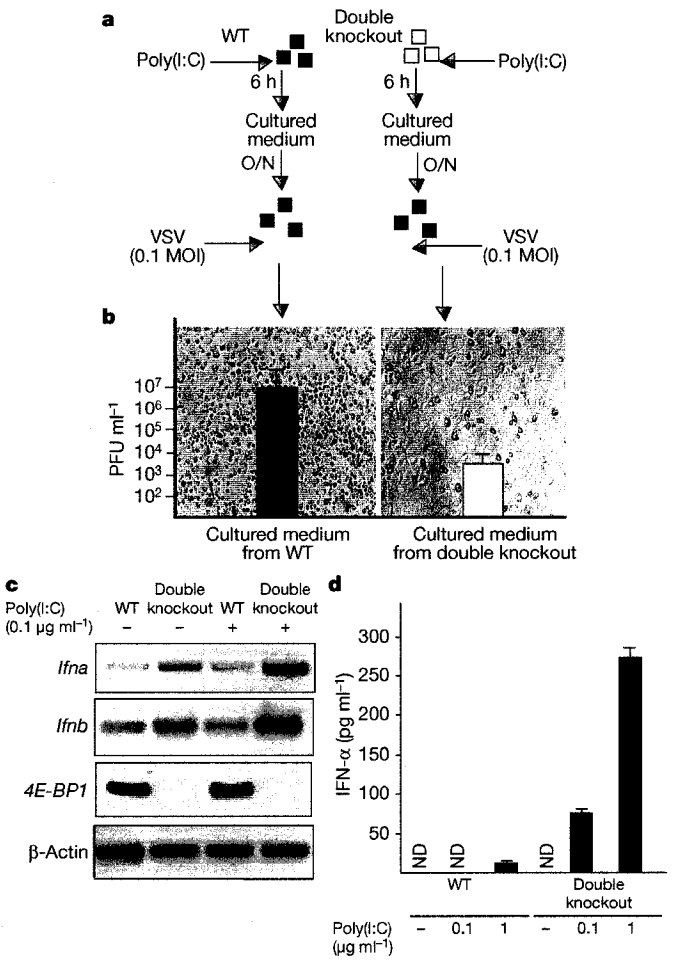

Figure 2 | Enhanced production of type-I IFN in *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ MEFs. **a**, Diagram of experimental protocol. Wild-type (filled squares) and *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ (open squares) MEFs were treated with poly(I:C) (0.1 μg ml^{-1}) for 6 h and medium was collected. Wild-type MEFs were incubated overnight (O/N) with the cultured medium from wild-type and *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ MEFs and then infected with VSV. **b**, Cytopathic effect and virus titres at 24 h after infection. **c**, MEFs were treated with poly(I:C) for 6 h and the induction of *Ifna* and *Ifnb* mRNAs was determined by RT–PCR. **d**, MEFs were treated with poly(I:C) for 6 h and the production of IFN-β was determined by ELISA (mean ± s.d. of three experiments). ND, not detected.

VSV-infected *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ mice survived, in comparison to 20% of the wild-type mice (Fig. 3a). Furthermore, VSV-infected *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ mice, in contrast to wild-type mice, failed to exhibit severe respiratory distress. In a second experiment, mice were infected intranasally with VSV (10^5 PFU) and killed 5 days after infection. In lungs from *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ mice, virus load was reduced (~100-fold; Fig. 3b). The expression of both *Ifna* and *Ifnb* mRNAs, as assayed by RT–PCR, was significantly increased (~3-fold) already by 2 days after infection, as compared to wild-type mice (Fig. 3c). In addition, the serum of VSV-infected *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ mice contained increased IFN-α levels, as compared to the serum of VSV-infected wild-type mice (Supplementary Fig. 6). Thus, mice lacking 4E-BP1 and 4E-BP2 are resistant to VSV infection and produce more type-I IFN, as compared to wild-type mice.

Plasmacytoid dendritic cells are the main producers of systemic type-I IFN in response to virus infection[18]. To determine whether plasmacytoid dendritic cells from *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ mice contribute to the virus-resistant phenotype, splenic plasmacytoid dendritic cells were incubated with VSV (MOI of 1 and 10 PFU per cell) for 6 h. Plasmacytoid dendritic cells from *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ mice generated significantly more (>7-fold) IFN-α, as compared to plasmacytoid dendritic cells from wild-type littermates (Fig. 3d). Similarly, plasmacytoid dendritic cells from *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ mice, which were co-cultured with synthetic CpG-oligodeoxynucleotides (CpG-ODN), produced more IFN-α (>4-fold) than plasmacytoid dendritic cells from wild-type littermates (Fig. 3e). Thus, the virus-resistant phenotype of *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ mice correlates with the ability of their plasmacytoid dendritic cells to produce increased amounts of IFN-α in response to virus infection.

Translational control of *Irf7* mRNA by 4E-BPs

To determine the molecular mechanism by which type-I IFN production is enhanced in *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ mice, we used gene-expression microarrays. A number of genes in the IFN pathway were upregulated in uninfected *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ double knockout MEFs as compared to wild-type MEFs (Supplementary Fig. 7A; for a complete list of genes see Supplementary Fig. 7C). Furthermore, a number of genes involved in inflammation and the immune response were also upregulated (Supplementary Fig. 7A).

Because 4E-BPs are translational inhibitors, they are predicted to repress the translation of a subset of mRNAs that are critical for type-I IFN production. To identify these mRNAs, polysomal RNA from wild-type and double knockout MEFs was analysed by gene-expression microarrays. We identified mRNAs with low or no induction at the mRNA level (<1.5-fold), but with a robust induction of translation (>4-fold) (Supplementary Fig. 7B). Notably, *Irf7* mRNA, which encodes the master regulator of the type-I IFN response[19], was the highest ranked gene in this analysis as its expression was increased ~12-fold in *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ MEFs as compared with wild-type MEFs (Supplementary Fig. 7B).

To validate these results, we studied the recruitment of ribosomes to *Irf7* mRNA using an RT–PCR assay that tracked the polysomal distribution of mRNAs in extracts from wild-type and *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ MEFs along a sucrose density gradient (Fig. 4a). *Irf7* mRNA was mainly associated with light polysomes in wild-type MEFs (Fig. 4b, left panel), consistent with its inefficient translation initiation. β-Actin mRNA, by contrast, was distributed mainly in heavy polysomes, as expected for an mRNA that is translated efficiently (Fig. 4c). In agreement with the derepression of *Irf7* mRNA translation in *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ MEFs, the distribution of *Irf7*

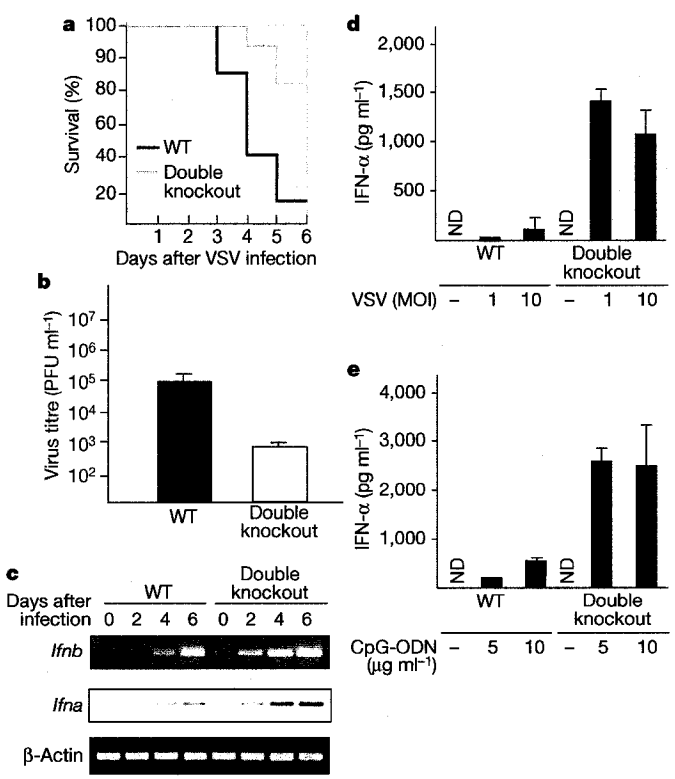

Figure 3 | *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ mice are resistant to VSV infection. **a**, Mice ($n = 10$) were intransally infected with VSV (5×10^7 PFU) and their survival was plotted as a Kaplan–Meier curve. **b**, Lungs from VSV-infected (10^5 PFU) mice ($n = 3$) were dissected 5 days after infection and virus yield was determined by plaque assay (mean ± s.d.). **c**, Expression of *Ifna* and *Ifnb* genes was determined by RT–PCR in lungs ($n = 3$). **d**, **e**, Splenic plasmacytoid dendritic cells were isolated and cultured for 6 h with VSV (**d**) or incubated overnight in the presence of CpG-ODN (**e**). Secreted IFN-α was measured by ELISA. ND, not detected.

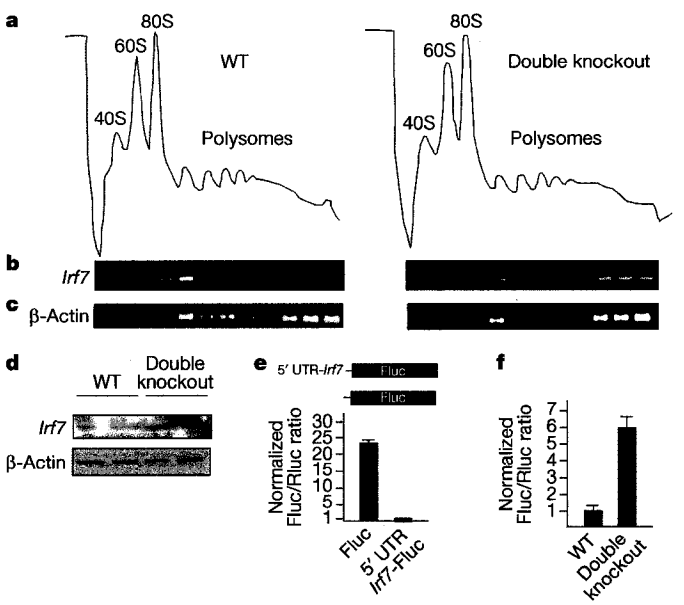

Figure 4 | 4E-BPs inhibit translation of *Irf7* mRNA. **a**, Polysome profiles of wild-type (left) and *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ (right) MEFs. **b**, **c**, RT–PCR of *Irf7* (**b**) and β-actin (**c**) mRNAs. **d**, Western blot of IRF-7 and β-actin. **e**, Translation of 5′ UTR-*Irf7*-Fluc mRNA (Fluc, top) relative to Fluc mRNA. A *Renilla* luciferase (Rluc) reporter vector was co-transfected with both reporters as a transfection control. Fluc was normalized against Rluc. Values for the Fluc reporter were $\sim 7 \times 10^4$ RLU (relative light units) and $\sim 3 \times 10^3$ RLU for the 5′ UTR-*Irf7*-Fluc reporter. Rluc values were $\sim 1 \times 10^6$ RLU. **f**, Ratio of expression of 5′ UTR-*Irf7*-Fluc/Rluc. The 5′ UTR-*Irf7*-Fluc and Rluc reporters were co-transfected. Fluc activity was normalized against Rluc activity. The Fluc value for wild-type MEFs was set as 1. For wild-type MEFs, Fluc ranged between 1×10^3 and 6×10^3 RLU and Rluc between 1.2×10^6 and 3.4×10^6. For *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ MEFs, Fluc ranged between 3.5×10^3 and 2.4×10^4 RLU and Rluc between 9×10^5 and 1.5×10^6 RLU.

mRNA was significantly shifted to heavier gradient fractions (Fig. 4b, right panel). Accordingly, in the absence of 4E-BP1 and 4E-BP2, IRF-7 protein amounts were increased (>4-fold), as determined by western blotting (Fig. 4d). These data support a role for the 4E-BPs in the repression of translation of *Irf7* mRNA.

Changes in the 4E-BPs/eIF4E ratio do not alter general translation, but rather affect the translation of a subset of mRNAs that harbour a structured 5′ untranslated region (5′ UTR)[7,20]. Notably, the *Irf7* mRNA has a highly structured 5′ UTR that is evolutionarily conserved (data not shown). To examine directly the role of the 5′ UTR in the regulation of *Irf7* mRNA translation, we generated a plasmid vector in which the SV40 promoter drives the expression of the 5′ UTR of *Irf7* mRNA fused to a firefly luciferase (Fluc) reporter (5′ UTR-*Irf7*-Fluc; Fig. 4e). As expected, because of the 5′ UTR secondary structure, the expression of 5′ UTR-*Irf7*-Fluc in wild-type MEFs was reduced relative to the control (lacking the 5′ UTR) Fluc reporter. A *Renilla* luciferase (Rluc) reporter plasmid was used as a transfection control (Fig. 4e). Next, wild-type and double knockout MEFs were co-transfected with 5′ UTR-*Irf7*-Fluc and an Rluc reporter plasmid. Consistent with the repression of *Irf7* mRNA translation by 4E-BPs, the normalized Fluc/Rluc ratio was significantly increased (~6-fold) in *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ MEFs as compared with wild-type MEFs (Fig. 4f). Therefore, we conclude that the *Irf7* mRNA 5′ UTR has a critical role in its translational repression by 4E-BPs.

To determine whether the virus-resistant phenotype and the enhanced type-I IFN production in *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ MEFs are due to increased IRF-7 expression, we reduced the IRF-7 level in *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ MEFs using a short hairpin (sh)RNA against *Irf7* mRNA. shRNA against *Irf7* mRNA, but not a control shRNA, restored the sensitivity of these cells to VSV infection and blocked type-I IFN production (Fig. 5). These data provide genetic evidence that the enhanced type-I IFN response in *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ MEFs is caused by upregulation of IRF-7 expression.

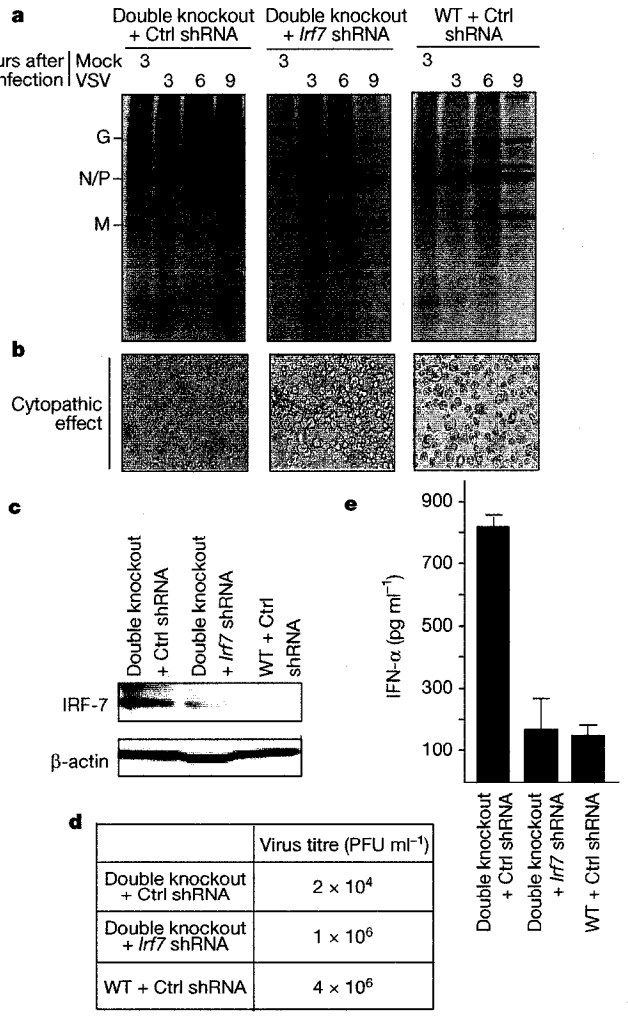

Figure 5 | Reduction of IRF-7 in *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ MEFs renders the cells susceptible to VSV infection and blocks type-I IFN production. a, *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ MEFs were first transfected with a control (Ctrl) shRNA or an shRNA against *Irf7* and then infected with VSV (MOI of 1 PFU per cell) and incubated with [^{35}S]methionine. Proteins were analysed by SDS–PAGE (15%). Viral proteins are indicated on the left. **b**, Cytopathic effect was visualized at 9 h after infection. **c**, Western blot analysis using antibodies against IRF-7 and β-actin. **d**, Virus yield was determined at 9 h after infection. **e**, IFN-α was determined 9 h after infection by ELISA (mean ± s.d. of three experiments).

Discussion

Virus infection results in the induction of type-I IFN, which confers an antiviral state on the host cell. New expression of IFN-related genes is required for the activation of the innate immune response[21,22]. The balance between activators and repressors of type-I IFN response is critical for this process. Until now, studies described only positive regulators of the innate immune response. For instance, *Stat1* and *Stat2* knockout mice[23–25] and RNase L (*Rnasel*) knockout mice[26] exhibit increased sensitivity to virus infections because of impaired IFN response. Knockout mice for two virus cytoplasmic sensors, retinoic-acid-inducible gene I (RIG-I) and melanoma differentiation associated gene 5 (MDA5), display a similar phenotype[27,28]. Our results provide new insight into the molecular mechanism of the innate immune response by providing strong evidence for a repressive translational mechanism that impedes type-I IFN production. We show that MEFs lacking 4E-BPs are largely refractory to infection by a variety of viruses (Fig. 1 and Supplementary Fig. 3). The virus-resistant phenotype is due to an increased type-I IFN production, as determined by inhibition of VSV replication, as well as RT–PCR, microarray and ELISA analyses (Fig. 2 and Supplementary Figs 4 and 5). In *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ double knockout mice VSV replication was suppressed. The VSV-resistant phenotype correlates with enhanced production of IFN-α in plasmacytoid dendritic cells (Fig. 3). These data indicate a causative role of the enhanced expression of type-I IFN in plasmacytoid dendritic cells in the resistance of *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ mice to VSV infection.

It is thought that plasmacytoid dendritic cells produce large amounts of type-I IFN as a consequence of a high constitutive expression of IRF-7 (ref. 18). Consistent with the control of *Irf7* mRNA translation by 4E-BPs, we demonstrated that the level of 4E-BP1 and 4E-BP2 in plasmacytoid dendritic cells is significantly lower as compared to MEFs (Supplementary Fig. 8). Thus, the low levels of 4E-BPs could explain the constitutive expression of IRF-7 in plasmacytoid dendritic cells.

Other lines of evidence support the idea that downstream targets of mTOR such as 4E-BPs, S6K1/2 or PRAS40 have an important role in repression of IFN production. First, *4E-BP1*$^{-/-}$ MEFs primed with mouse IFN exhibit enhanced expression of IFN-related genes[29]. Second, activation of toll-like receptor 3 (TLR3), which is a major mediator of the cellular response to virus infection through dsRNA, activates phosphatidylinositol-3-OH kinase (PI(3)K), an upstream regulator of 4E-BPs[30]. Interestingly, PI(3)K-γ and PI(3)K-δ knockout mice exhibit a defect in innate immunity[31]. In addition, pharmacological and genetic inhibition of PI(3)K blocks the induction of IFN-stimulated genes by dsRNA[32]. Third, similar to the phenotype of the *4E-BP1*$^{-/-}$ cells, the expression of type-I IFN genes was enhanced in MEFs lacking the mTOR negative regulator, tuberous sclerosis complex 2, when primed with mouse IFN[29].

How do 4E-BPs regulate type-I IFN production? Our model (Supplementary Fig. 9) is based on the translational control of *Irf7* mRNA by the 4E-BPs. *Irf7* mRNA translation is derepressed in *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ MEFs (Fig. 4) owing to increased amounts of the eIF4F complex. Because of its highly structured 5′ UTR, the translation of *Irf7* mRNA would be extremely sensitive to changes in the

amounts of eIF4F. In agreement with this model, translation of a reporter mRNA harbouring the 5′ UTR of *Irf7* mRNA is enhanced in *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ MEFs. An increase in IRF-7 expression triggers type-I IFN production, which subsequently evokes a transcriptionally dependent positive feedback regulation of IRF-7 signalling[33,34]. Consistent with this, we showed that the expression of IFN-α and IFN-β is enhanced (Fig. 2 and Supplementary Figs 4 and 5) and IRF-7 levels are upregulated in uninfected *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ MEFs (Fig. 4 and Supplementary Fig. 7B). Enhanced type-I IFN production in *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ MEFs seems to be highly dependent on IRF-7, as decreasing IRF-7 levels by shRNA rendered cells susceptible to virus infection and blocked type-I IFN production (Fig. 5). Accordingly, *Irf7*$^{-/-}$ mice exhibit increased susceptibility to virus infection both *in vivo* and *ex vivo* as well as impaired induction of type-I IFN[19]; that is, a phenotype opposite to that of the *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ mice. The activation of the IRF-7-mediated type-I IFN response induces the expression of RIG-I and MDA5, which triggers the induction of NF-κB, IRF-3 and IRF-7 that co-operate in the production of the antiviral type-I IFN response. As expected from this model, we found enhanced expression of RIG-I and MDA5 proteins in MEFs lacking 4E-BPs (Supplementary Fig. 10).

Our data provide biochemical, genetic and biological evidence that 4E-BPs constitute a critical step in the activation of the innate immune response. These findings raise the intriguing possibility that regulators of translation might serve as therapeutic targets to boost the innate immune response against virus infection.

METHODS SUMMARY

Mice, cell culture and viruses. *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ double knockout mice have been described previously[15]. MEFs derived from wild-type and *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ mice were immortalized by sequential passaging[35]. Splenic plasmacytoid dendritic cells were isolated using anti-mPDCA1 magnetic beads. *Ex vivo* virus infection and metabolic labelling were performed as described[36]. Virus titres were determined by a plaque assay[37,38]. *In vivo* virus experiments were performed as described[38].

Polysome profiling. Polysomes were prepared and analysed as described[39]. *Irf7* and β-actin mRNAs were amplified by RT–PCR reactions, which were optimized to measure the exponential phase on the amplification curve.

Microarray analysis. Total or polysomal RNA was isolated from MEFs using Trizol and hybridized to an Affymetrix Mouse430_2 chip. To identify genes upregulated in *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ MEFs, total RNA samples were analysed using normalization that reduced the range for the fold change (>1.5-fold used as threshold). Translationally upregulated genes were identified by selecting genes whose regulation in total RNA samples was low (<1.5-fold), but their abundance on polysomes was >4-fold.

ELISA. MEFs were transfected with poly(I:C) using the FuGENE 6 transfection reagent according to the manufacturer's protocol (Roche). Murine IFN-α and IFN-β production was detected by ELISA according to the manufacturer's procedure (PBL Biomedical Laboratories).

Plasmid construction, transfection and luciferase assay. The 5′ UTR of mouse *Irf7* mRNA was amplified and cloned into the pGL3 firefly luciferase (Fluc) reporter vector (Promega). MEFs were co-transfected with 5′ UTR-*Irf7*-Fluc and *Renilla* luciferase (Rluc; Promega) as described[36]. Cell extracts were prepared in passive lysis buffer and assayed for Rluc and Fluc activity using a dual-luciferase reporter assay system (Promega). Fluc activity was normalized against Rluc activity, which was used as a transfection control.

Rescue experiments. pBABE-4E-BP1, pBABE-4E-BP2 and empty vector were transfected into phoenix-293-T packaging cells and virus-containing medium was used to infect *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ MEFs. *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ MEFs were transfected with PLKO.1-puro-Ctrl-shRNA or PLKO.1-puro-*Irf7*-shRNA as described[36]. Transfected MEFs were selected with puromycin for 1 week.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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METHODS

Mice and cell culture. *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ mice have been described previously[15]. MEFs derived from wild-type and *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ mice were immortalized by sequential passaging[35]. All experiments were performed at least three times and repeated with independently derived MEFs. Splenic plasmacytoid dendritic cells were isolated using anti-mPDCA1 (murine plasmacytoid dendritic cell antigen-1) magnetic beads according to the manufacturer's instructions (Miltenyi Biotech). The IFN neutralization experiment was performed using a monoclonal antibody against mouse IFN-β according to the manufacturer's procedure (PBL Biomedical Laboratories).

Viruses. The Indiana serotype of VSV was previously described[40]. Influenza virus A/HK/1/68-MA20 was provided by E. G. Brown, EMCV K-2 by V. Agol, and Sindbis virus by J. Berlanga. EMCV, Sindbis virus and VSV were propagated in BHK21 cells. *Ex vivo* virus infection and metabolic labelling were performed as described[36]. Virus titres were determined by a standard plaque assay method[37,38]. For *in vivo* experiments, mice were infected intranasally with VSV and killed 5 days after infection. Lungs were aseptically removed and snap-frozen in liquid nitrogen. Specimens were homogenized in 3 ml of PBS on ice, and titres were determined in BHK21 cells[38].

RT–PCR and RNA extractions. Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed (RT) with Superscript III reverse transcriptase (Invitrogen) for 1 h at 50 °C using oligodT. One microlitre of RT template was incubated with specific primers (described below) and with Taq Polymerase (Fermentas) according to the supplier's instructions. The number of PCR cycles ranged from 23 to 34 depending on the linearity of the reaction. PCR primers included (5′ to 3′): IFN-α sense (CCTTCCACAGGATCACTGTGTACCT), IFN-α antisense (TTCTGCTCTGACCACCTCCC); IFN-β sense (CACAGCCCTCTCCATCAACT), IFN-β antisense (TCCCACGTCAATCTTTCCTC); IRF-7 sense (ATGATGGTCACATCCAGGAACCCA), IRF-7 antisense (TCAGGTCTGCAGTACAGCCACAT); β-actin sense (GGACTCCT ATGTGGGTGACGAGG), β-actin antisense (GGGAGAGCATAGCCCTCGTAGAT). PCR reactions were optimized to measure the exponential phase on the amplification curve.

Polysome profiling. MEFs were washed twice with cold PBS containing 100 μg ml^{-1} cycloheximide, suspended in lysis buffer (5 mM Tris-HCl, pH 7.5, 2.5 mM $MgCl_2$, 1.5 mM KCl, 100 μg ml^{-1} cycloheximide, 2 mM dithiothreitol, 0.5% Triton X-100 and 0.5% sodium deoxycholate), and centrifuged for 2 min at 14,000*g* (Eppendorf centrifuge). The supernatant was loaded onto a 10–50% sucrose gradient prepared in 20 mM HEPES-KOH, pH 7.6, 100 mM KCl and 5 mM $MgCl_2$ and centrifuged at 35,000 r.p.m. for 2 h at 4 °C in an SW40 rotor. Fractions were collected by piercing the tube with a Brandel tube piercer, passing 60% sucrose through the bottom of the tube, followed by monitoring the absorbance using an ISCO UA-6 UV Detector. RNA was isolated from individual fractions using Trizol reagent (Invitrogen).

Microarray analysis. Total RNA or polysomal RNA was isolated from wild-type and *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ MEFs using Trizol. RNA samples were purified using the Qiagen RNeasy kit (according to the manufacturer's instructions; Qiagen) followed by sodium acetate/ethanol precipitation. Twenty micrograms of each RNA sample were processed according to the manufacturer's protocol (Affymetrix) and hybridized to an Affymetrix Mouse430_2 chip. Primary image analysis of the arrays was performed using the Genechip 3.2 software package (Affymetrix). The data were normalized using Robust Multichip Averaging (RMA)[41] using updated probe set definitions 'REFSEQ_8'[42,43]. RMA leads to a reduced dynamic range of obtained fold changes and hence a moderate threshold was used. To identify genes upregulated in *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ MEFs, total RNA samples were analysed using normalization that reduced the range for the fold change (>1.5-fold used as threshold).

Translationally upregulated genes were identified by selecting genes whose regulation in total RNA samples was low (<1.5-fold), but their abundance on polysomes was >4-fold (by calculating the ratio between the translational microarray and the total gene expression microarray and then comparing between *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ and wild-type MEFs). Genes that were related to inflammation or IFN responses were identified manually using information from the Gene Ontology Consortium[44].

Western blot analysis. MEFs were homogenized in buffer A (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM dithiothreitol, protease inhibitors cocktail (Roche), 20 mM β-glycerophosphate, 0.25 mM Na_3VO_4, 10 mM NaF, 10 nM okadaic acid, 1 mM PMSF) and incubated for 30 min at 4 °C. Cell debris was removed by centrifugation at 10,000*g* (Eppendorf centrifuge) for 10 min at 4 °C and total protein content was determined using a Bio-Rad assay. Laemmli sample buffer was added to the supernatant, which was then subjected to SDS–PAGE (15%). Proteins were transferred onto a nitrocellulose membrane, which was blocked for 2 h at room temperature with 5% skim milk in PBS containing 0.2% Tween 20 (PBS-T) and washed twice with PBS-T. The membrane was incubated overnight at 4 °C with primary antibodies followed by three 10-min washes in PBS-T and further incubated with peroxidase-coupled secondary antibody for 30 min at room temperature, and washed three times. Detection of peroxidase-coupled secondary antibody was performed with ECL (GE-Healthcare).

RIG-I and MDA5 antibodies were provided by H. Kato. IRF-7 antibody was purchased from Santa Cruz Biotechnology.

ELISA. MEFs were transfected with poly(I:C) using FuGENE 6 transfection reagent (Roche) according to the manufacturer's protocol. Cultured medium was recovered at 3 h and 6 h after transfection. Murine IFN-α and IFN-β production was detected in the cultured medium by ELISA according to the manufacturer's procedure (PBL Biomedical Laboratories).

Plasmid construction, transfection and luciferase assay. A 411-bp DNA corresponding to the 5′ UTR of mouse *Irf7* mRNA was amplified by PCR from MEF genomic DNA. HindIII and NcoI restriction sites were added to the 5′ and 3′ ends, respectively. Using the same restriction sites, the *Irf7* 5′ UTR was cloned into the pGL3 firefly luciferase (Fluc) reporter vector (Promega). MEFs were co-transfected with 500 ng of 5′ UTR-*Irf7*-Fluc and 100 ng of *Renilla* luciferase (Rluc; Promega) vector, which is under the control of the CMV promoter, in 24-well plates using Lipofectamine 2000 as described[36]. Cell extracts were prepared in passive lysis buffer (Promega) 20 h after transfection and assayed for Rluc and Fluc activity in a Lumat LB9507 bioluminometer (EG&G Bertold) using a dual-luciferase reporter assay system (Promega), according to the manufacturer's instructions. Fluc activity was normalized against Rluc activity, which was used as a transfection control.

Rescue experiments. pBABE-4E-BP1, pBABE-4E-BP2 and empty vector constructs were transfected into phoenix-293-T packaging cells. After 48 h, virus-containing medium was filtered, collected and used to infect *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ MEFs in the presence of 5 mg ml^{-1} of polybrene (Sigma-Aldrich). Cells were re-infected the next day and supplemented with puromycin (2 μg ml^{-1}, Sigma-Aldrich) for selection for five days.

shRNA against *Irf7*. *4E-BP1*$^{-/-}$ *4E-BP2*$^{-/-}$ MEFs were transfected with PLKO.1-puro-Ctrl-shRNA (CAACAAGATGAAGAGCACCAA; Origene) or PLKO.1-puro-*Irf7*-shRNA (GTCACCACACTACACCATCTA; Origene) as described[36]. Transfected MEFs were selected with puromycin for 1 week.

40. Stojdl, D. F. *et al.* Exploiting tumor-specific defects in the interferon pathway with a previously unknown oncolytic virus. *Nature Med.* **6**, 821–825 (2000).
41. Irizarry, R. A. *et al.* Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Res.* **31**, e15 (2003).
42. Dai, M. *et al.* Evolving gene/transcript definitions significantly alter the interpretation of GeneChip data. *Nucleic Acids Res.* **33**, e175 (2005).
43. Sandberg, R. & Larsson, O. Improved precision and accuracy for microarrays using updated probe set definitions. *BMC Bioinformatics* **8**, 48 (2007).
44. Gene Ontology Consortium.. The Gene Ontology (GO) project in 2006. *Nucleic Acids Res.* **34**, D322–D326 (2006).

CURRICULUM VITAE

EDUCATION

Ph.D. of Science, Biochemistry, Microbiology & Immunology 2004-present
Laboratory of Dr. John Bell, University of Ottawa, Ottawa, ON
Title: Investigating acute effects of oncolytic virus infection of tumours

University exchange, Business Courses 2003-2004
University of Vienna, Vienna, Austria

Bachelor of Science, Honour's, Microbiology & Immunology 2000 - 2003
McGill University, Montreal, QC
Title: Structure of antibiotic resistance enzyme aminoglyocoside acetyltransferase Ii
Dean's List
Golden Key Honours Society
(GPA: 3.89)

Diploma of Collegial Studies (DEC), Health Sciences 1998 - 2000
Marianopolis College, Montreal, QC
Dean's List

High School Graduation Diploma (DES) 1992 - 1998
German School Alexander von Humboldt, Montreal
Equivalent German certificate received

PUBLICATIONS

Breitbach C.J., De Silva N., Falls T., Aladl U., Evgin L., Parato K., Stanford M.,Fenster A., Kirn, D., Atkins H., Bell J.C. Attacking tumours on multiple fronts using oncolytic viruses (in preparation).

Breitbach C.J., Lichty B.D., Bell J.C. Tumour targeted inflammation: The behind the scenes tumour killer! (in preparation)

Nguyen T.L., Abdelbary H., Arguello M., **Breitbach C.J.**, Leveille S., Diallo J., Yasmeen A., Bismar T., Kirn D., Falls T., Snoulton V., Vanderhyden B., Werier J., Atkins H., Hiscott J., Bell J.C. Chemical targeting of the innate antiviral response by histone deacetylase inhibitors renders refractory cancers sensitive to viral oncolysis. *PNAS*. 2008. 105(39):14981-6.

Alajez, N.M., Mocanu J.D., Shi W., Chia M.C., Hui A.B.Y., **Breitbach C.J.**, Knowles S., Busson P., Takada K., Lo K., Bell J.C., O'Sullivan B., Gullane P., Liu F. Efficacy of systemically

administered mutant Vesicular Stomatitis Virus combined with radiation for nasopharyngeal carcinoma is associated with EBV latency. *Clinical Cancer Research.* 2008. 14(15):4891-7.

Colina R., Costa-Mattioli M., Dowling R.J.O., Tai L., Jaramillo M., **Breitbach C.J.,** Martineau Y., Larsson O., Rong L., Svitkin, Y.V., Makrigiannis A.P., Bell J.C., Sonenberg N. Translational control of the innate immune response via IRF-7. *Nature.* 2008. 20;452(7185):323-8.

Stanford M.M., **Breitbach C.J.,** Bell, J.C and McFadden G. Innate immunity, tumor microenvironment and Oncolytic virus therapy: Friends or foes? *Current Opinion in Molecular Therapeutics.* 2008. 10(1):32-7.

Breitbach C.J., Paterson J.M., Lemay C.G., Falls T.J., McGuire A., Parato K.A., Stojdl D.F., Daneshmand M., Speth K., Kirn D., McCart J.A., Atkins H., Bell J.C. Targeted inflammation during oncolytic virus therapy severely compromises tumor blood flow. *Mol Ther.* 2007. 15(9):1686-93.

Burk DL, Xiong B, **Breitbach C**, Berghuis AM. Structures of aminoglycoside acetyltransferase AAC(6')-Ii in a novel crystal form: structural and normal-mode analyses. *Acta Crystallogr D Biol Crystallogr.* 2005. 61(Pt 9):1273-9.

PATENTS

Breitbach, C and Bell J. Patent Cooperation Treaty, under revision. "Staged modulation of Neutrophil Infiltration in Oncolytic Therapy"

PRESENTATIONS

Tumour targeted inflammation and coagulation following oncolytic virus therapy compromises tumour blood flow and triggers apoptosis of uninfected tumours cells. Caroline J. Breitbach, Harold Atkins, John C. Bell

- Poster presentation at the Annual Meeting of the American Association for Cancer Research. April 2008.
- Poster presentation at the 4th Canadian Gene Therapy and Vaccines Symposium. May 2008.

Targeted inflammation during oncolytic virus therapy severely compromises tumor blood flow. Caroline J. Breitbach, Harold Atkins, John C. Bell.

- Ottawa Health Research Institute Research Day. November 2007 (poster presentation, award, outstanding poster presentation)
- International conference on vascular targeted therapies in oncology. October 2007. Mandelieu, France
- University of Ottawa, Biochemistry, Microbiology & Immunology Research Day. May 2007. (poster presentation, award, outstanding poster presentation)
- The Fourth International Meeting on Replicating Oncolytic Virus Therapeutics. March 2007, Scottsdale, AZ, USA (travel award)

Investigation of the oncolytic activity of Vesicular Stomatitis Virus in murine cancer models. Caroline J. Breitbach, Harold Atkins, John C. Bell.

- University of Ottawa, Biochemistry, Microbiology & Immunology Research Day. February 2006. (oral presentation)
- The Third International Meeting on Replicating Oncolytic Virus Therapeutics. March 2005, Banff, AB, Canada
- University of Ottawa, Biochemistry, Microbiology & Immunology Research Day. May 2005. (poster presentation, award, outstanding poster presentation)

Oncolytic viruses: Novel replicating virus therapeutics.
- Invited presentation at the Ottawa Health Research Institute (OHRI) research day. November 2005.

AWARDS

Ron Worton Researcher in Training Award 2008. $2,000
Award recognizes graduate student or postdoctoral fellow at the Ottawa Health Research Institute (OHRI) that has demonstrated outstanding effort in their research and is deemed highly likely to succeed in future research endeavors.

Natural Sciences and Engineering Council of Canada (NSERC) Canada Graduate Scholarship-doctoral (CGS D) 2006- 2008. $35,000 per annum.

NSERC Canada Graduate Scholarship-master's (CGS M) 2004- 2006. $17,500 per annum.

University of Ottawa Entrance Award 2004-2008. $6,000 per annum.

NSERC Industrial Undergraduate Student Research Award (USRA) Summer 2003. Merck Frosst Inc. $4,500 plus company contribution

NSERC Undergraduate Student Research Award (USRA) Summer 2002. $4,500 plus university contribution

LANGUAGES

English, German and French (spoken and written), Spanish (basic)

WORK EXPERIENCE

Supervision of students in a basic research laboratory
Naomi de Silva: Biopharmaceuticals (Genomics) Undergraduate, Summer Student & Honour's Research Project. University of Ottawa
Chantal Lemay: Biopharmaceuticals (Genomics) Undergraduate, Honour's Research Project. University of Ottawa

Summer Student Intern, Summer 2004, **Merck Frosst Canada Ltd.** Kirkland, QC
Laboratory of Dr. Kathryn Skorey

English native speaker, Fall 2003, **Vienna Business School**, Vienna, Austria

Summer Student Internship, Summer 2003, **Merck Frosst Canada Ltd.,** Kirkland, QC
Laboratory of Dr. Kathryn Skorey

Honours Research Project, Fall 2002-May 2003, **McGill University**, Montreal, QC
Laboratory of Dr. Albert Berghuis

Laboratory Assistant, Summer 2002, **McGill University**, Montreal, QC
Laboratory of Dr. Albert Berghuis

Laboratory Assistant, Summer 2001, **McGill University**, Montreal, QC
Laboratory of Dr. Hervé Le Moual

Accounting Clerk, Summer 2000, **Telav Inc.**, Montreal, QC

Junior Accounting Clerk, Summer 1999, **Telav Inc.**, Montreal, QC

MEMBERSHIPS & ACTIVITIES

Trainee Member, Canadian Oncolytic Virus Consortium (2004-present).
Member of group of researchers that are funded through a large multi-user grant from the Terry Fox Foundation examining oncolytic viruses through collaborative research. Headed by Dr. John Bell, University of Ottawa.

Let's Talk Science (2004-present).
Performed interactive science-related activities (including DNA forensics, The 5 Senses, Human Body Activities, Fingerprint analysis) with elementary and high school students.

Speaker for the Canadian Cancer Society, Ottawa Regional Cancer Foundation (2005-present).
Invited presentations to diverse audiences to inform the public about the value of research (and importance of continued research funding) using oncolytic virus research as an example of a promising novel cancer therapeutic

Clinical Observership (2007-present).
Observed Dr. Harold Atkins, haematologist, in multiple myeloma & multiple sclerosis clinics as well as on haematology ward.

Science Travels (Fall 2006).
Was sponsored to travel to northern Ontario for one week to perform science-related activities (including DNA forensics, Chemistry Magic Show, HIV & AIDS) with students in rural communities.