ENGINEERING NOVEL TNFα-ARMED ONCOLYTIC VIRUSES FOR COMBINATION IMMUNOTHERAPY WITH SMAC MIMETICS

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ABSTRACT

Small molecular Inhibitor of Apoptosis (IAP) antagonists, known as Smac mimetic compounds (SMCs), are a novel class of anti-cancer drugs currently undergoing clinical trials. SMCs were designed to mimic the function of the pro-apoptotic protein, Smac, which directly depletes cells of cIAP1 and cIAP2, and consequently renders tumour cells sensitive to death in the presence of proinflammatory ligands such as TNFα. The Korneluk lab recently reported that SMCs synergize with the attenuated oncolytic virus Vesicular stomatitis virus (VSVΔ51) by eliciting an enhanced immune response in mice, such that the combined therapy is vastly superior to stand-alone therapies. To improve on this SMC-mediated synergistic response, I generated variants of TNFα-armed VSVΔ51. Due to high ectopic expression of TNFα in infected cells, a five times lower viral dose of TNFα-armed VSVΔ51 combined with SMC treatment was sufficient to improve the survival rate as compared to SMC and VSVΔ51 co-therapy. This improved synergistic response is attributed to a bystander effect whereby the spread of TNFα from infected cells leads to the death of neighbouring, uninfected cells in the presence of a SMC. In addition, the double treatment induced vasculature collapse in solid tumours, revealing another mechanism by which cytokine-armed VSVΔ51 in combination with a SMC can induce cancer cell death. This approach demonstrates great potential for engineered oncolytic virus and SMCs as a new combination immunotherapy for cancer treatment.

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β-ME	β-mercaptoethanol
A549	Human lung alveolar cell line
ACK	Ammonium-chloride-potassium
BHK-21	Baby hamster kidney cell line
BIR	Baculovirus IAP repeat
BSA	Bovine serum albumin
CARD	Caspase recruitment domain
CD31	Vasculature endothelial cell marker
cIAP 1/2	Cellular inhibitor of apoptosis 1 and 2
CPE	Cytopathic effects
CT-26	Mouse colorectal carcinoma cell line
DAMPS	Danger-associated molecular patterns
DIABLO	Direct IAP binding protein with low PI
DMSO	Dimethyl sulfoxide
DR5	Death receptor 5
ELISA	Enzyme-linked immunosorbent assay
EMT6	Mouse mammary carcinoma cell line
EMT6-Fluc	EMT6 cell line tagged with Fluc-expressing gene
FADD	Fas-associated protein with death domain
FCS	Fetal calf serum
Fluc	Firefly luciferace

G	Glycoprotein-coding gene
GFP	Green fluorescent protein
GM-CSF	Granulocyte-monocyte colony stimulating factor
GM38	Normal human fibroblast cell line
hIFNβ	Human interferon beta
hTNFα	Human TNFα
IAP	Inhibitor of apoptosis
IFN	Interferon
IFNβ	Interferon beta
IFNγ	Interferon gamma
IgG	Immunoglobin G
IκB	Inhibitor of κB
IKK	Complex containing IKKα and IKKβ
IKKα/β	Inhibitor of NF-κB kinase subunit alpha/beta
IL	Interleukin
IP	Intraperitoneal
IT	Intratumoural
IV	Intravenous
IVIS	In vitro imaging system
KO	Knock out
L	Polymerase-coding gene
M	Matrix-coding gene
mAb	Mouse-specific antibody

mIFNβ	Mouse interferon beta
mTNFα	Mouse TNFα
N	Nucleoprotein-coding gene
ND	Not detected
NEMO	NF-κB essential modulator
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIK	NF-κB inducing kinase
NIS	Sodium iodide symporter
NT	Non-targeting
OV	Oncolytic virus
P	Phosphoprotein-coding gene
PAMPS	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PFU	Plaque forming units
PRR	Pattern recognition receptors
rhTNFα	Recombinant human TNFα
RING	Really interesting new gene
RIP	Receptor interacting protein
RIPK1	RIP kinase
RLR	RIG-I-like receptor
siRNA	Silencing RNA
Smac	Second mitochondrial activator of caspases
SMC	Smac mimetic compound

SNB75	Human glioblastoma cell line
$TCID_{50}$	50% tissue culture infective dose
TLR	Toll-like receptors
TNF	Tumour necrosis factor
TNFα	Tumour necrosis factor alpha
TNFR	Tumour necrosis factor receptor
TNF-R1	Tumour necrosis factor receptor 1
TRADD	TNFR associated death domain
TRAF 2/3	TNFR associated factors 2/3
TRAIL	TNF-related apoptosis-inducing ligand
TWEAK	TNF-related weak inducer of apoptosis
Vero	Monkey kidney epithelial cell line
VSV	Vesicular stomatitis virus (wild-type)
VSVΔ51	Vesicular stomatitis virus that contains a deletion in the 51^{st} amino acid of the M protein
VSVΔ51-Fluc	VSVΔ51 that contains the Fluc-coding sequence
VSVΔ51-GFP	VSVΔ51 that contains GFP-coding gene inserted between G and L protein-coding genes
VSVΔ51-SA:TNFα	VSVΔ51 that contains serum albumin signal peptide sequence upstream TNFα -coding gene inserted between G and L protein-coding genes
VSVΔ51-TNFα	VSVΔ51 that contains full length TNFα -coding gene inserted between G and L protein-coding genes
XIAP	X-linked inhibitor of apoptosis

1.0 INTRODUCTION

1.1 The Inhibitors of Apoptosis

The Inhibitors of Apoptosis (IAPs) are a common target of cancer therapeutics due to their ability to suppress programmed cell death. In addition, the IAPs are key regulators of differentiation, proliferation, and signal transductions of immune and inflammatory pathways (1, 2). Before a discussion on therapeutics that target the IAPs can be described, an understanding of IAP structures, functionalities and involvement in the survival pathways needs to be detailed.

1.1.1 IAP structure and function

The IAPs were originally discovered in the baculoviral genes of the *Cydia pomonella* granulosis virus and the *Orgyia pseudosugata* nuclear polyhedrosis virus. These gene products were known to prevent apoptosis in infected host cells in order to allow for viral propagation (3, 4). Since their discovery, numerous homologues have been found in a range of different species.

The mammalian family of IAPs are characterized by the presence of one or three 70-80 amino acid Baculovirus IAP Repeat (BIR) domains. Of particular importance are the cellular IAP proteins 1 and 2 (cIAP 1/2) and X-linked IAP (XIAP). In addition to three BIR domains, cIAP1/2 and XIAP each contain a zinc finger carboxy-terminal Really Interesting New Gene (RING) domain (Figure 1-1). While the BIR repeats allow for the IAPs to inhibit both initiator and effector caspases, the RING domains harbour E3 ubiquitin ligase properties that allow IAPs to directly regulate auto- or trans-ubiquination and protein degradation (5-7). The addition of ubiquitin chains results in either proteasomal degradation or signal transduction. Typically, a K48 ubiquitin linkage destines the protein to the proteasome, while polyubiquitination at K63 promotes signal transduction (8, 9). Unique to cIAP 1 and 2 is the Caspase Recruitment Domain

(CARD) motif, which has been speculated to play a role in protein-protein interactions with other CARD containing constituents (10).

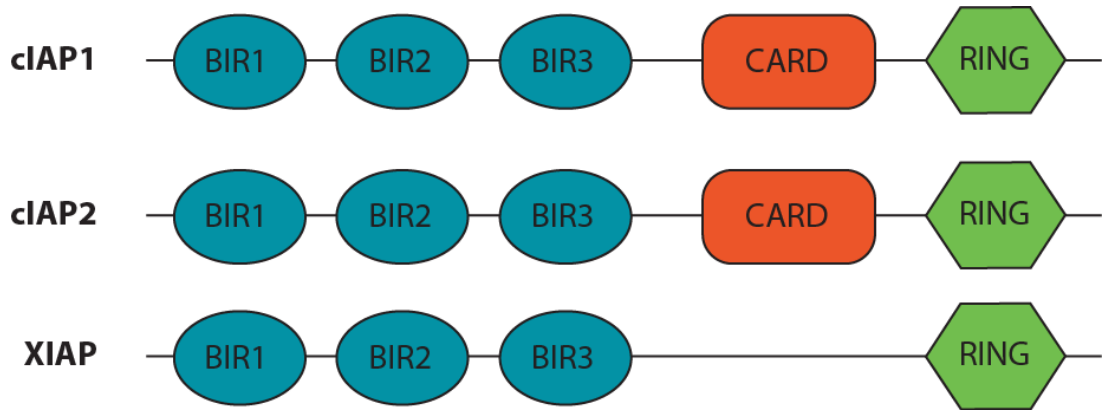

Figure 1-1. Domain structures of three mammalian IAP family members. These IAPs are characterized by the inclusion of at least one BIR domain, where cIAP 1/2 and XIAP contain three BIR domains. The BIR domains are responsible for the targeting and inhibition of initiator and effector caspases. The CARD motifs, which are unique to cIAP 1/2, have been speculated to participate in interactions with other CARD-containing proteins. The RING motifs contain E3 ubiquitin ligase activity which allows for the regulation of auto and trans-ubiquitination protein degradation *(*Modified from *LaCasse et al., Oncogene, 2008).*

IAPs function by inhibiting caspase activity, and modulate the classical nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway to promote cancer cell proliferation. The mechanism of IAP inhibition of apoptosis was first discovered by Deveraux *et al.* (11). It was determined that XIAP directly inhibits initiator and effector caspase proteins within every programmed cell death cascade, and cIAP1 and 2 primarily regulate apoptosis through the extrinsic tumour necrosis factor (TNF) receptor family and NF-κB signalling pathway (12) (Figure 1-2). Both the extrinsic and intrinsic apoptotic pathways converge on the signalling of the effector caspases -3 and -7. The effector caspase-7 appears to be inhibited simply by steric hindrance due to complexes formed with the XIAP BIR2 domain and its linker region between the BIR1 domain. This blocks the subtrate entry site of caspase-7, whereas capsase-3 is inhibited exclusively by the linker region (13, 14). Similarly, the BIR3 domain of

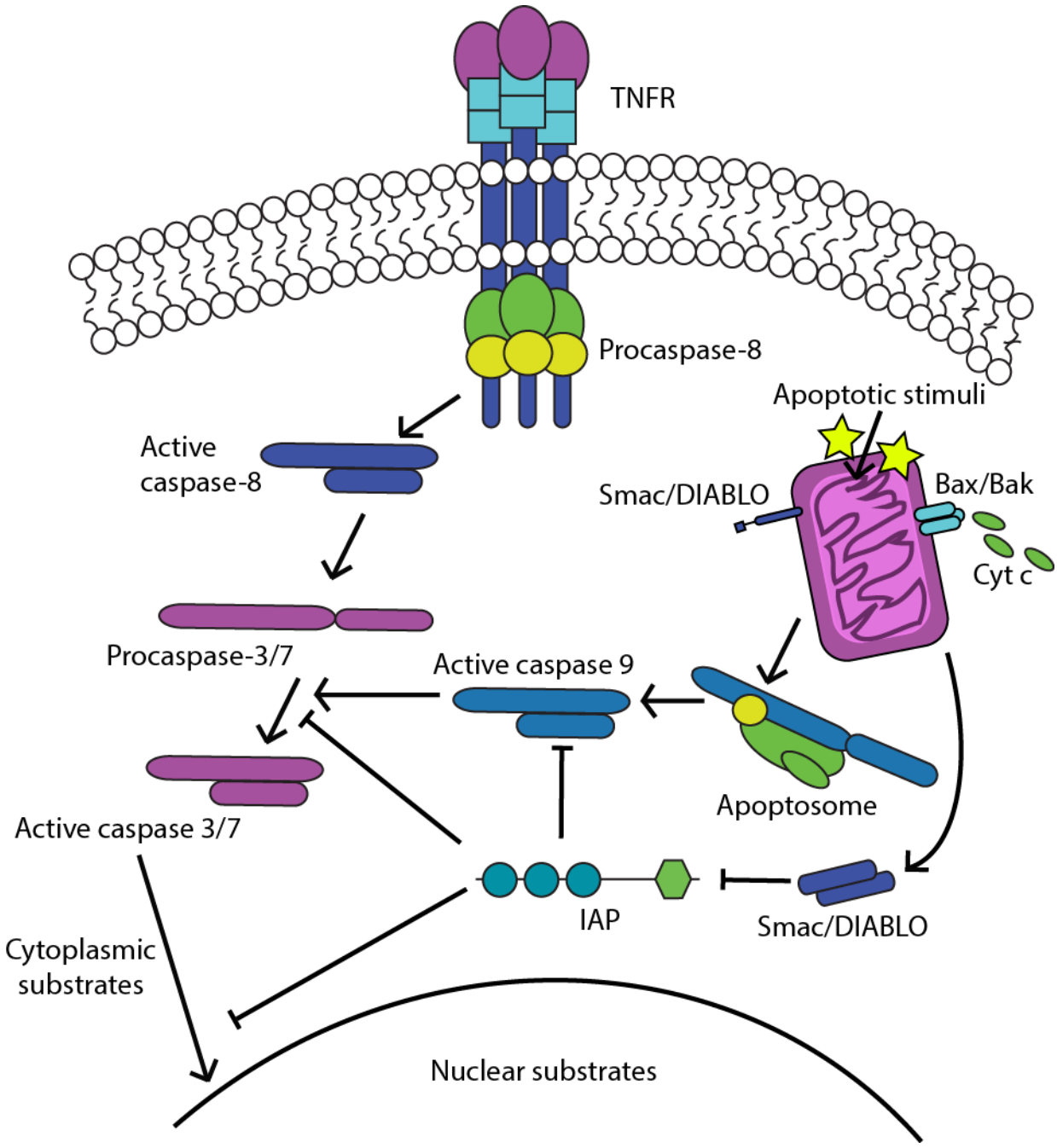

Figure 1-2. IAPs effectively inhibit the extrinsic and intrinsic apoptotic pathways. In the extrinsic apoptotic pathway, the binding of death ligands Fas or TNFα to their respective receptors activate the initiator caspase-8 which promotes initial cleavage of the effector caspase-3. The IAPs inhibit downstream caspase processing into its mature, active form and thereby control apoptosis. Apoptotic stimuli such as cell stress induces the release of cytochrome c and Smac from the outer mitochondrial membrane through the intrinsic apoptotic pathway. This activates caspase-9 through the formation of the apoptosome, which is then inhibited by the IAPs, specifically by XIAP. *(*Modified from *Hunter et al., Apoptosis, 2007).*

XIAP has been shown to stabilize caspase-9 in an inactive state, by preventing its autocatalytic activity (15).

Although they are not direct inhibitors of caspases, cIAP1 and 2 have been shown to inhibit the initiator and effector caspases by indirect means through the classical NF-κB pathway (16). The question of cIAP 1 and 2 being direct inhibitors of caspases has been difficult to prove and is still under debate.

1.1.2 cIAP modulation of the classical NF-κB pathway

The NF-κB pathway is known as a pleiotropic signalling pathway which plays a role in an array of biological processes including innate and adaptive immunity, as well as in cell proliferation and survival (12, 17-19). As mentioned above, the cIAPs are responsible for modulating the classical NF-κB pathway, also known as the canonical pathway. This pathway depends on the presence of the death ligand, TNFα, in which cIAP 1 and 2 positively regulate the classical NF-κB pathway to promote inflammation (Figure 1-3). In fact, in the absence of the cIAPs, cells become sensitized to TNFα-mediated apoptosis (20, 21). Mechanistically, when TNFα binds the TNFα receptor (TNF-R1), this signals the recruitment of the TNFR associated death domain (TRADD) and the receptor interacting protein (RIP). TRADD signals the TNFR associated factors 2/3 (TRAF 2/3), an adaptor that recruits the cIAPs to the receptor site. This complex formation allows for the polyubiquitination of RIP kinase (RIPK1) at the K63 site, which is mediated by the RING domain E3 ligase of cIAP1 and 2. Studies have shown that the recruitment of TRAF 2/3 is essential for the bridging of the gap between the cIAPs and RIP1 to occur, in order for RIP1 to be ubiquitinated by cIAP1 and 2 (12). This activates the recruitment of the IKK complex containing the inhibitor of NF-κB kinase subunit alpha (IKKα), inhibitor of NF-κB kinase subunit beta (IKKβ) and NF-κB essential modulator (NEMO). The activated IKK

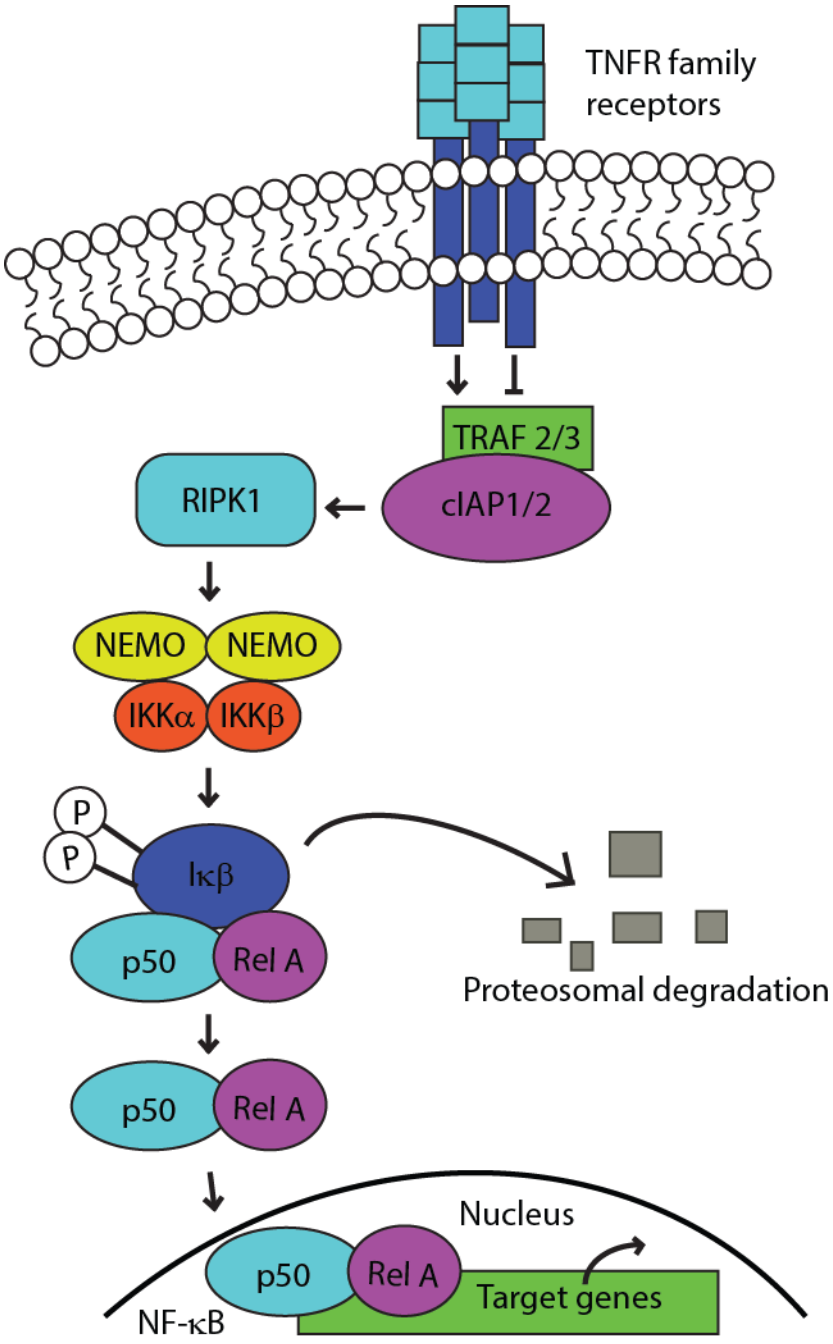

Figure 1-3. cIAP1 and cIAP2 regulation of the NF-κB pathway. In the classical NF-κB pathway, the binding of TNFα to its receptor, TNFR, signals the complexing of TRAF2/3 at the receptor which recruits cIAP 1 and 2. This formation allows for cIAP 1 and 2 to polyubiquitinate RIPK1, which activates the formation of the IKK complex composed of IKKα, IKKβ and NEMO. The IKK complex then phosphorylates IκB leading to its proteasomal degradation, allowing the transcription factors, p50 and Rel A (p65), to freely translocate from the cytoplasm in to the nucleus in order to initiate the transcription of target genes. *(*Modified from *LaCasse et al., Oncogene, 2008).*

complex phosphorylates the inhibitor of κB (IκB), leading to its degradation, allowing for the NF-κB dimers, p50 and RelA (p65), to translocate from the cytoplasm into the nucleus for transcriptional activation of target genes. This process essentially promotes cell proliferation through the functionality of cIAP 1 and 2, and is dependent on the presence of TNFα.

1.1.3 cIAP modulation of the alternative NF-κB pathway

While the cIAPs are positive modulators of the classical NF-κB pathway, they also negatively regulate the alternative, or non-canonical, NF-κB pathway (22). In contrast to their function in the classical pathway, the cIAPs inhibit the expression of the alternative pathway via K48-mediated ubiquitination and proteosomal degradation of the NF-κB inducing kinase (NIK), leaving it dormant until activation by ligation at various TNFRs. For example, the alternative pathway is activated in lymphoid cells upon binding of CD40-L, the B-cell activation factor, or the TNF-related weak inducer of apoptosis (TWEAK). Upon stimulation, NIK accumulates until there is a sufficient amount to induce downstream phosphorylation events for activation of the target genes. Taken together, the cIAPs play a central role in the regulation of both the classical and alternative NF-κB pathways in normal cells as well as in cancer cells.

Although both NF-κB pathways are activated by two very distinct mechanisms, there is cross-talk that exists between the two (23). In fact, studies have shown that co-stimulation of TNF-R2, which is restricted to lymphoid cells, with TNF-R1 allows for cIAP1 to sensitize cells to TNFα-mediated apoptosis through TNF-R1. The fact that the cIAPs are able to both negatively and positively regulate the NF-κB pathways indicates that they have both pro- and anti-survival properties depending on the cell type and conditions. In fact, there is some speculation that expression of the alternative pathway balances the classical pathway in normal cell homeostasis, whereas in the case of inflammatory disorders and cancer, the balance is shifted

toward an increase in activity of the classical pathway. With this in mind, the work discussed in this thesis focuses primarily on the TNFα-dependent classical NF-κB pathway.

1.1.4 Intrinsic IAP antagonism

Homeostasis is critical for normal cell function, and there exists other proteins responsible for repressing the roles of the IAPs in order to induce apoptosis when necessary. Intrinsic antagonists of the IAPs exist to help maintain a balance between cell survival and cell death. The second mitochondrial-derived activator of caspases (Smac), also known as the Direct IAP Binding Protein with Low PI (DIABLO), is an endogenously expressed IAP antagonist that is released from the mitochondria following the induction of the intrinsic mitochondrial stress pathway (Figure 1-2).

Initiation of Smac occurs following the proteolytic cleavage of the 53-55 amino acid leader signal sequence into an AVPI-containing amino polypeptide. Smac then resides in the mitochondria until further signalling events occur. Upon the induction of apoptotic stress such as chemotherapeutic agents, irradiation, oxidative stress, or ER stress, Smac and cytochrome c are released into the cytoplasm via the same Bax- or Bak-formed membrane pores (24). The kinetics at which this occurs are similar between the two proteins; however, it has been speculated that the signalling events differ between Smac and cytochrome c, and that Smac efflux is a caspase-catalyzed event (24, 25). It is also possible that Smac may require further permeability due to increased mitochondrial damage (16).

Smac is known as a general IAP inhibitor, and has been shown to bind XIAP, cIAP1 and cIAP2 (26-31). Smac forms a highly stable protein dimer, which is critical for its function, and the AVPI terminus recognizes and binds the BIR2 and BIR3 domains of XIAP (32-34). By binding the BIR domains of the IAPs, Smac is able to inhibit the IAP-caspase interactions

through steric hindrance, repressing ubiquitin ligase activities of the IAPs, allowing caspases to be freely activated in order to promote apoptosis (32).

1.1.5 IAPs and cancer

A higher expression of IAPs has been found in numerous cancer cell lines in which the homeostasis between cell survival and cell death is no longer in check (35-38). In fact, IAP knock out (KO) models in mice have exhibited increased sensitivity of certain cell types to different death stimuli (39-42) (Table 1-1). Similarly, loss-of-function mutations in different disorders have highlighted the importance of the roles of IAPs in cell homeostasis (43-46) (Table 1-1). There also exist many known cases of increased IAP expression and decreased IAP antagonist expression in human tumours (Table 1-2). The upregulation of IAPs in cancer cases have shown increased resistance to chemotherapy and radiation therapy (16). Hence, the targeting of IAPs proves to be a promising avenue for cancer therapeutics. With the exploitation of IAPs, mechanisms of reversing the uncontrolled cell proliferation in various cancers can be established.

1.2 Smac mimetics

Commonly practiced cancer treatments such as chemotherapy and radiation therapy kill cancer cells via apoptosis, however, cancer cells often develop resistance to these treatments due to the upregulation of IAPs. Hence, the use of alternative therapeutics which potently and safely induce apoptosis in cancer cells is needed. Understanding the structures of IAPs and their function in inflammatory disorders has allowed for their exploitation and for the development of innovative therapeutics. IAP-targeted approaches have proved to be highly efficacious for cancer treatment, with numerous forms advancing through clinical trials. Herein the IAP antagonists,

Smac mimetic compounds (SMCs) will be discussed as they are one of the most clinically advanced approaches used for cancer treatment.

Table 1-1. Summary of IAP knock outs and natural mutations in mammals leading to death or disease *(*Modified from *Hunter et al., Apoptosis, 2007)*

Gene	KO or natural disease	Phenotype/mutation*
cIAP1	Complete ablation of cIAP1 in mice	No obvious sensitization to proapoptotic stimuli, presumably due to the compensation by cIAP2, indicating that cIAP2 is a ubiquitin ligase target of cIAP1.
cIAP2	Complete ablation of cIAP2 in mice	Macrophages die upon LPS-induced sepsis. T cells die when treated with glucocorticoids, even in the presence of survival factors.
XIAP	Complete ablation of XIAP in mice	Initially no phenotype detected due to compensation of cIAP1 and 2. Delayed lobuloalveolar development in mammary gland. Sensitive to death induced by cytochrome c alone, unlike wild-type cells. Lack of XIAP and survivin complexes also lead to increased incidence of cell death.
cIAP2	MALT Lymphoma	Translocation of cIAP2 that fuses with MALT1 leads to the development of lymphoma. Continues immune stimulation leads to lymphoma development in mice.
XIAP	X-linked lymphoproliferative disorder (XLP)	Mutations in XIAP leading to loss of expression were found in XLP cases. An increased incidence of apoptosis of lymphocytes was enhanced in XIAP-deficient patients. Additionally, a lower number of natural killer cells was found in XLP patients. XIAP has been shown to be a potent regulator of lymphocyte homeostasis.

* See references in *(Hunter et al., Apoptosis, 2007)*

1.2.1 Design and function of SMCs

Structural analysis of how Smac binds to the BIR2 and BIR3 domains of XIAP, primarily through the AVPI amino acid sequence, initially shed light on how to mimic the function of endogenous Smac by designing Smac peptides as cancer therapeutics. Smac peptides that consisted of 4-8 amino acids effectively targeted XIAP and cIAP1 in MCF-7 breast cancer cells,

and enhanced the induction of apoptosis and long term anti-proliferative effects of a range of chemotherapeutics (47, 48). The use of these agonists also showed efficacy when combined with other cancer therapeutics such as Apo2L and TNF- related apoptosis inducing ligand (TRAIL)

Table 1-2. Cases of IAP overexpression and IAP antagonist downregulation in human tumours *(*Modified from *Dubrez, Berthelet & Glorian, OncoTargets and Therapy, 2013)*

IAP or IAP antagonist	Tumours*
XIAP overexpression	Acute myeloid leukemia, bladder carcinoma , breast carcinoma, cervical carcinoma, colorectal cancer, ovarian cancer, prostate carcinoma, thyroid carcinoma
cIAP1 and cIAP2 overexpression (amplicon 11q21-22)	Cervical cancer, medulloblastoma, non-small and small cell lung cancer, pancreatic cancer, oral squamous cell carcinoma, hepatocarcinoma
Smac downregulation	Acute myeloid leukemia, bladder carcinoma, breast carcinoma, cervical carcinoma, chronic lymphocytic leukemia, colorectal carcinoma, lung cancer, rectal adenocarcinoma

* See references in *(Dubrez, Berthelet & Glorian, OncoTargets and Therapy, 2013).*

(48, 49). However, because the Smac peptides are only able to bind to the BIR3 domain of XIAP, they are susceptible to proteolytic degradation (50). This led to the discovery of small molecule Smac mimetics, which inhibit the IAPs at a higher affinity than Smac peptides. While Smac peptides consist of 4-8 amino acids, the small molecule Smac mimetics, or SMCs, were created by replacing several amino acids while maintaining the essential interactions required to bind to BIR2 and BIR3 of XIAP (51).

SMCs are now a promising small molecular therapeutic drug and are currently undergoing clinical trials for cancer treatment (52, 53). SMCs function by binding and degrading the IAPs (Figure 1-4). Hyperactive activation of the NF-κB pathway promotes inflammation and tumorigenesis while repressing anti-tumour immunity (54), however, the antagonism of the

cIAPs by SMC treatment results in conversion of the NF-κB-mediated survival signal into a pro-death response (55). Mechanistically, SMCs induce the auto-ubiquitination of cIAP1 and 2, which re-sensitizes the cells to TNFα-induced death through the classical NF-κB pathway. Coincidentally, this allows for the accumulation of NIK, which in turn activates the alternative NF-κB pathway (23). As the functions of cIAP1 and 2 are redundant, and one can compensate for the absence of the other (12, 18), both need to be degraded in order for the treatment to be effective. In the absence of the cIAPs, the secretion of TNFα from a subset of cancer cells induces the activation of caspase-8, inducing extrinsic apoptosis in an autocrine fashion (12, 18, 56). TNFα secretion upon IAP antagonism may also engage paracrine TNFR signalling, in adjacent tissues (57). Theoretically, SMCs require additional stimulus such as chemotherapy to induce apoptosis, however, numerous cancer cell lines are sensitive to SMC-alone treatment, which could be attributed to the autocrine TNFα loop (23).

1.2.2 SMCs as effective cancer therapeutics

There are a number of different SMCs that have been designed for cancer treatment, and continue to advance through clinical trials (Table 1-3). Preclinical studies have demonstrated the ability of SMCs to inhibit tumour growth in multiple solid tumours (58-60), acute lymphoblastic leukemia (60), and multiple myeloma (61), among other types. The compounds are well tolerated by animals with no signs of toxicity against normal lymphocytes and bone marrow stromal cells or normal mammary epithelial cells (61, 62).

Bivalent SMCs containing two AVPI motifs, such as AEG40826 and TL-32711 (Table1-3) have proved to exhibit higher potency over monovalent SMCs, as they are able to block both the BIR2 and BIR3 motifs to better liberate caspase activation (50). Bivalent SMCs can also

induce the ligation of BIR2 to BIR3 within an IAP, or between two IAPs, promoting the incidence of auto- or trans-ubiquitination and protein degradation.

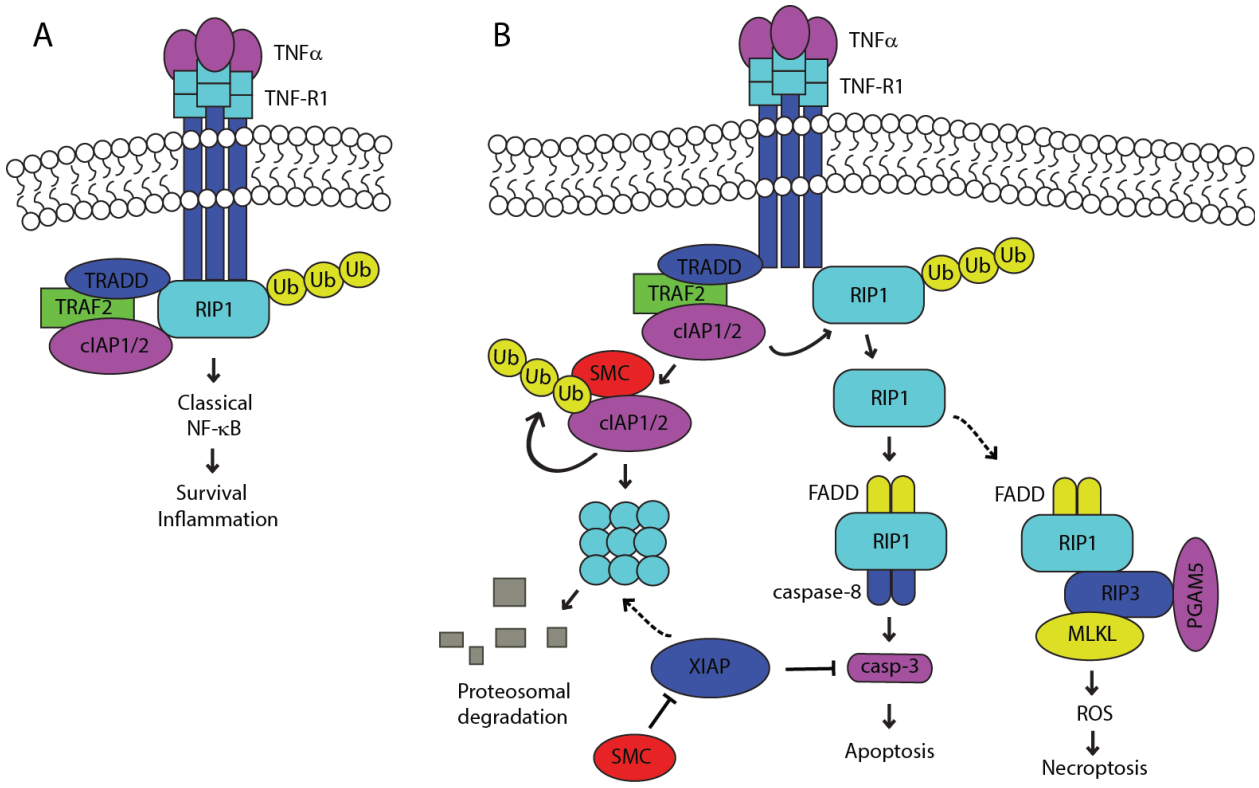

Figure 1-4 SMCs antagonize the IAPs to induce apoptosis in the presence of TNFα. (A) In the case of cancer in which the IAPs are hyperactive, RIP1 is constantly being polyubiquitinated by cIAP1 and 2 following its recruitment to the TNFα receptor via TRAF2 and TRADD complex formation as described in Figure 1-3. This promotes survival of the cancer cells through positive regulation of the classical NF-κB pathway. **(B)** In the presence of SMCs, the cIAPs are targeted for degradation through the induction of auto ubiquitination. This allows free RIP1 to form the death inducing complex consisting of RIP1, FADD and caspase-8. Upon activation of the death inducing complex, the effector caspase-3, which is free from the inhibition of XIAP, is cleaved in order to promote apoptosis. This addition of SMC to abolish the function of the cIAPs essentially switches the pro-survival response into a death response in the presence of TNFα, which induces apoptosis in an autocrine fashion.

Table 1-3. SMCs undergoing clinical trials (Modified from LaCasse et al., Oncogene, 2008; Dubrez, Berthelet & Glorian, OncoTargets and Therapy, 2013)

Compound	Developmental Stage	Company
AEG40826/HGS1029	Phase I	Aegera
LBW424	Preclinical	Novartis
Compound C	Phase I	Genentech
SM-164, SM-122	Preclinical	Ascenta
GT-T, compound A	Preclinical	Tetralogic
LCL161	Phase I/II	Novartis
TL-32711	Phase I/II	Tetralogic

Although bivalent SMCs are able to induce dimerization of the BIR2 and BIR3 domains of the cIAPs, this interaction does not appear to be necessary for SMC-induced ubiquitination and degradation, as monovalent SMCs are equally effective at similar concentrations (22). LCL161, a monovalent SMC from Novartis (Figure 1-5), is currently one of the most clinically advanced compounds, and can be delivered orally, unlike bivalent SMCs which are delivered by injection (63). Due to the degradation of the cIAPs and NF-κB activation, the use of LCL161 results in the upregulation of cytokines and chemokines, making the SMC particularly immunomodulatory (64-66). The proinflammatory response elicited by SMCs such as LCL161 could also activate the adaptive antitumour immune response in different cancers (67).

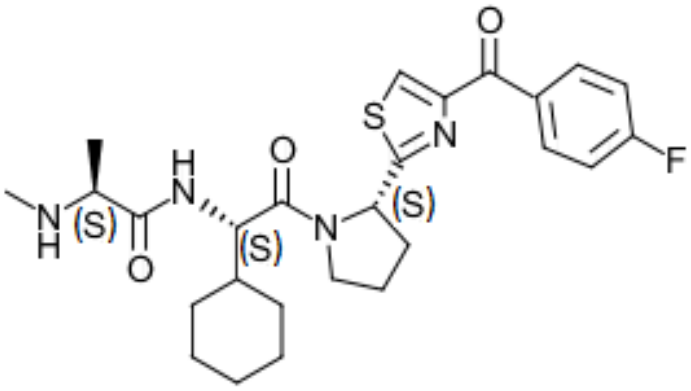

Figure 1-5. Structure of the monovalent SMC, LCL161, from Novartis *(*Modified from *Dubrez, Berthelet & Glorian, OncoTargets and Therapy, 2013).*

1.2.3 SMCs synergize with innate immune stimulants

While the function of SMCs depends on the presence of inflammatory cytokines such as TNFα for maximal efficiency, studies have also shown that these compounds are able to synergize with innate immune response stimulants (68, 69). The use of combination immunotherapies that potentiate SMC treatment poses a very promising future, however, methods to safely and effectively provide an exogenous source of cytokines to boost the antitumour effect in cancer patients still need to be developed. In essence, combination immunotherapies with SMCs may be more efficacious than single-agent approaches, and are translatable to clinical testing (70).

1.3 Cancer Immunotherapy

As different cancers have become resistant to some forms of monotherapies, the development of combination approaches that elicit stronger anti-tumour immune responses have become increasingly important. The use of cancer immunotherapies with SMCs can act as a multi-faceted approach that could potentially avoid the development of resistance in cancer. Previously it has been shown that SMCs can synergize with inflammatory cytokines and

adjuvants such as oncolytic viruses, which stimulate the host's innate immune response (68). Immunotherapeutic approaches have also been used to target developing neovasculature of tumours, another mechanism of inducing cancer cell death.

1.3.1 Oncolytic viruses

Oncolytic viruses (OVs), which have gone through phase I-III clinical evaluation, have shown great promise in cancer immunotherapy (71, 72). OVs are an attractive therapeutic tool due to their preferential infection of cancer cells, easily manipulated genome and immunogenicity. In fact, more than 1,000 patients have been treated with OVs, demonstrating acceptable safety and tolerability with flu-like symptoms being the most common adverse events (72-74). These tools first entered clinical testing during the 1950s, and were originally intended for tumour debulking through viral oncolysis. It was later discovered that infection with OVs engaged the host's immune system to help fight off malignancies through the induction of anti-tumour immune responses (75, 76). Due to its high tropism, VSV can rapidly infect and spread through tumours. This effectively "de-cloaks" the tumours, making them visible to infiltrating immune cells. In addition, some OVs have demonstrated the ability to debulk tumours through acute vasculature disruption (77, 78). The use of OVs is a valuable approach to cancer immunotherapy due to their ability to target tumour beds, overcome the ability of cancers to evade the immune response, and re-engage the host's immune system to eliminate malignancies (79).

The *Vesicular stomatitis* virus (VSV) is a commonly studied OV. VSV is a member of the "bullet-shaped" Rhabdoviridae family that contains a negative sense RNA genome which encodes the viral glycoprotein (G), matrix protein (M), phosphoprotein (P), polymerase (L), and nucleoprotein (N). Attachment of the G protein to host receptors such as LDLR allows for the

virus to be endocytosed into the host cell, leading to sequential transcription of viral mRNAs in the cytoplasm, followed by replication once enough N protein is available to encapsidate the RNA. The P protein is associated with the L polymerase and allows the polymerase to bind to the ribonucleocapsid in order for replication to occur (80). The M protein has the ability to block the host cell's antiviral response by inhibiting the host RNA polymerase activity and interferon beta (IFNβ) mRNA export out of the nucleus through an association with the interferon (IFN)-inducible cellular gene product (81). This allows VSV to evade a host's antiviral response, and replicate quite rapidly.

In order to obtain a therapeutical benefit of using VSV, an attenuated version was created in 2003 through a deletion in of the 51st amino acid in the M protein (VSVΔ51), preventing its ability to antagonize the host's IFNβ -mediated antiviral immune response (81). Thus, due to the defects in tumour IFN pathways, VSVΔ51 is able to preferentially target and infect cancer cells, while leaving normal cells unharmed. Furthermore, the release of IFNβ from infected and neighboring cells induces a storm of proinflammatory cytokines, activating immune effector cells and triggering an anti-tumour immune response (79). This leads to the rationale that VSVΔ51, among other OVs, could be engineered to express certain cytokines in order to enhance this effect (79). These aspects, including its highly studied genome and easily manipulated cloning sites, make VSVΔ51 a useful tool for cancer therapeutics.

The development of genetically modified VSV technologies is a relatively new field, and has shown significant efficacy in different cancers since OVs were first engineered in the 1990s (82). Recombinant VSV that consecutively express IFNβ have been shown to induce cancer cell death in multiple models including mesothelioma, squamous cell, head and neck, endometrial and canine cancer (83-89) (Table 1-4). Similarly, VSV that express interleukin (IL)-15 and IL-12

have demonstrated regression in colon adenocarcinoma and squamous cell carcinoma,

Table 1-4. VSV constructs expressing immunostimulatory transgenes

Transgene	Tumours	Therapeutic Effects
GM-CSF	Murine melanoma	Rapid infiltration of IFNγ-producing T and NK cells. Tumour control (90)
IFNβ	Squamous cell carcinoma (head and neck), endometrial cancer, mesothelioma	Tumour growth delay and prolonged survival (89), tumour regression and increased survival (83), enhanced T cell response (86), tumour lysis of multiple cell lines (87),
IFNβ-NIS	Murine melanoma	Complete tumour debulking within 72h, activation of anti-myeloma T cells (85)
IFNγ	Murine mammary adenocarcinoma	Activation of dendritic cells and secretion of proinflammatory cytokines, slowed tumour growth minimized lung tumours, and prolonged survivial (91)
IL-12	Squamous cell carcinoma	Reduction in tumour volume, increased survival (92)
IL-15	Murine colorectal cancer	Enhanced anti-tumoural T cell responses and enhanced survival (93)

respectively, resulting in the upregulation of anti-tumour immunity and increased survival of treated mice (92, 93). The use of an IFN gamma (IFNγ) transgene expressed from a VSV vector successfully drove a more profound secretion of proinflammatory cytokines and enhanced therapeutic activity compared to the parental virus (91). Other studies have looked at the use of VSV expressing fusion proteins and tumour antigens to prolong survival in syngeneic animal models (90, 94, 95). All treatments performed were well tolerated in the animal models, with the most severe effects being flu-like, similarly to what has previously been seen in clinical trials with OVs.

More clinical studies will need to be performed in order to determine the safest doses and modes of viral delivery to avoid toxicity while maintaining efficacy in treatments. In addition, engineering attenuated, yet potent OVs can help balance the relationship between OVs and the immune system, producing a happy medium between the two. Since OVs are able to locally induce inflammation, facilitate drug delivery, and induce tumour lysis, they can form potent partnerships with other cancer therapeutics. This opens the doors to a number of combination immunotherapies, and the use of immune modulators that synergize with OVs continue to be explored.

1.3.2 TNFα as a therapeutic

TNFα was initially discovered in 1975 to have the ability to induce necrosis in the tumours of bacillus Calmette-Guérin-primed and endotoxin treated mice (96). Since its discovery and subsequent cloning into recombinant human TNFα (rhTNFα), TNFα was proposed to be an attractive therapeutic given its anti-tumour activity and ability to damage the neovasculature of developing tumours (97, 98). In fact, *in vitro* and *in vivo* data illustrated the high specificity of TNFα treatment and activity against a number of murine tumour types as well as colorectal cancer, lung cancer and human xenografts (99, 100). Preclinical studies also demonstrated synergy of TNFα with a number of chemotherapeutics and cytokines. This led to a wide range of phase I and phase II clinical studies using rhTNFα. However, the administration of exogenous TNFα for cancer treatment during clinical trials was found to cause systemic toxicity with limited efficacy, diluting the initial promise of its use as a cancer therapeutic (101, 102). It is important to note that in a majority of these clinical trials, TNFα was administered as an intravenous bolus injection or infusion (97). Additionally, it was speculated that the doses of TNFα administered were denser compared to other phase II clinical studies, which may have

contributed to the increases systemic toxicity. Efforts have since then been focused on the design of vectors that can deliver the cytokine locally in order to circumvent the unwanted toxic effects of TNFα. The use of TNFα has also improved the penetration of chemotherapeutics into cancer tissues, showing potential in increasing drug delivery as an indirect method of treating cancer (97).

New, indirect methods of administering TNFα to tumour sites have been developed, and have proved to induce little to no toxicity. The use of TNFα in conjunction with polyethylene glycol or asparagine-glycine-arginine on the N-terminus has improved the specificity while decreasing the immunogenicity of TNFα therapy for cancer (103, 104). Another study practiced the use of recombinant self-seeding cancer cells that expressed TNFα at the site of growing tumours (105). This effectively reduced the systemic spread of TNFα; in fact, extremely low concentrations successfully induced apoptosis in primary tumours as well as in the CD31-expressing cells of developing neovasculature. It was speculated that the damage of a single endothelial cell could theoretically induce the vasculature shutdown of large areas of the tumour. Interestingly, lentivirus TNFα-Tumstatin expressed by stem cells has been shown to inhibit prostate cancer growth (106). As an alternative approach to introducing exogenous TNFα to cancer cells, the use of IAP antagonists has been shown to induce the release of TNFα from cells via the NF-κB pathway, killing tumours via disruption of the tumour vasculature (57).

A number of studies have explored the use of oncolytic viruses as tools for delivering TNFα locally to cancer cells while leaving normal cells unharmed. An adenovirus vector capable of expressing high concentrations of human TNFα in infected tumour cells demonstrated high efficacy and immunogenicity, yielding cures in syngeneic melanoma models (107). No significant levels of TNFα were detected in the blood of treated mice, showing that the

administration remained local, attributing to low toxicity. Similarly an adeno-associated virus bacteriophage that expressed TNFα combined with LCL161 was significantly effective at treating melanoma compared to single agent treatment (108). The synergism of the SMC and TNFα can be attributed to the fact that TNFα is able to increase the permeability of vasculature, allowing for easier delivery of LCL161 to the tumour tissues (109, 110). In addition, because the virus was designed to target tumour-associated vasculature, there was no sustained secretion of TNFα in normal cells, and the treatment was tolerated well in the animal models. It is important to note, however, that TNFα has been found to have different toxicities in different species, an aspect that can be overcome by improving the selectivity of TNFα secretion.

Although initially avoided due to its toxicity, these recent studies have demonstrated that the successful local delivery of TNFα can be used to treat cancer with less adverse effects, and that the combination of TNFα with commonly practiced radiation therapy, chemotherapies and SMCs can be potentially more efficacious.

1.4 Rationale and hypothesis

Previously, the Korneluk lab has shown that SMCs synergize with different innate immune stimuli including VSVΔ51, and this effect is dependent on TNFα, TRAIL and IFNβ secretion (68). The use of synergistic treatments that preferentially target carcinomas demonstrates a huge advantage over chemotherapies and radiation therapies (111-113). Dual immunotherapy approaches that utilize small-molecule antagonists of IAPs in combination with innate immune stimuli have shown high efficacy in animal models, and can overcome the limitations of single agent therapies (70). In addition, the ability of immunotherapies to activate the host's adaptive immune response helps to overcome the issue of resistance, greatly reducing the limitations of this therapeutic route.

1.4.1 Hypothesis

Given the observed synergy of SMC and inflammatory cytokines such as TNFα, TRAIL and IFNβ, I sought to generate a VSVΔ51 vector capable of secreting TNFα in infected cells. I hypothesized that a more localized and concentrated release of TNFα could improve the efficacy of SMC treatment and bystander cell killing. Together, SMCs and engineered OVs could be a new, promising combination immunotherapy for cancer treatment.

1.4.2 Objectives

The first objective of this project was to successfully generate and characterize TNFα-armed VSVΔ51 that are able to infect, replicate and express TNFα that can synergize with the SMC, LCL161. In addition, I sought to confirm that the ability of the recombinant viruses to induce an innate antiviral response is not impaired in the presence and absence of SMC. After ensuring that the new constructs were functioning properly, I then determined whether the local secretion of TNFα from cancer cells can potentiate SMC treatment and bystander killing. The second objective was to determine whether this approach would be effective in treating cancer *in vivo*. Thirdly, the occurrence of tumour vasculature collapse was studied as another potential mechanism for inducing apoptosis in developing tumours. Together these studies demonstrate the potential of SMC and TNFα combination immunotherapy for cancer treatment.

2.0 MATERIALS AND METHODS

2.1 Reagents

The SMC, LCL161, was kindly provided by Novartis (Cambridge, MA, United States).

2.2 Antibodies

2.2.1 Western blot antibodies

Death receptor 5 (DR5) (#3696, Cell Signalling Technology, Danvers, MA) and TNF-R1 (#3736, Cell Signalling Technology, Danvers, MA, United States).

2.2.2 Immunohistochemistry antibodies

α-cleaved Caspase-3 (#9661, Cell Signalling Technology, Danvers, MA, United States) and CD31 (ab28364, Abcam, Toronto, Canada).

2.3 Cell culture

2.3.1 Cancer cells

Cancer cell lines were grown at 37°C and 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 1% penicillin streptomycin, 1% glutamine and 1% nonessential amino acids (NEAA) (Invitrogen Life Technologies, Burlington, ON, Canada). Mouse mammary carcinoma (EMT6) cells were obtained from ATCC. Human glioblastoma (SNB75) cells were provided by Dr. David Stojdl (Children's Hospital of Eastern Ontario Research Institute, Ottawa, ON, Canada). CT-26 cells were provided by Dr. Jean-Simone Diallo (Ottawa Hospital Research Institute, Ottawa, ON, Canada).

2.3.2 Splenocytes

Spleens were isolated from 8 week old female BALB/c mice, homogenized with surgical scissors and then passed through a 70µm nylon mesh. Red blood cells were removed from spleen homogenate using 1ml of ammonium-chloride-potassium (ACK) lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.2-7.4) for 2 min. Isolated splenocytes were then cultured in Roswell Park Memorial Institute (RPM1)-1640 media supplemented with 10% FCS, 10uM beta-mercaptoethanol (β-ME), 1 % penicillin streptomycin and 1% NEAA (Invitrogen), and maintained at 37°C and 5% CO_2.

2.4 Enzyme-linked immunosorbent assays (ELISAs)

2.4.1 From tissue culture

Cell culture supernatants were collected from 96-well plates containing EMT6, monkey kidney epithelial (Vero) or SNB75 cells treated with vehicle (0.05% DMSO) or 5 µM SMC and 0.01-1 MOI of VSVΔ51-GFP, VSVΔ51-TNFα or VSVΔ51-SA:TNFα for 16h. Cell debris was removed by centrifugation and levels of human TNFα (hTNFα) were measured using the TNFα DuoSet ELISA kit (R&D Systems, Minneapolis, MN, Unites States), mIFNβ with the Verikine™ Mouse Interferon Beta ELISA Kit (PBL Interferon Source, Piscataway, NJ, United States) or for hIFNβ with the Verikine™ Human Interferon Beta ELISA Kit (PBL Interferon Source). Supernatant from EMT6 and SNB75 cells was diluted 10X before hTNFα ELISAs were performed in order to obtain detectable results. Supernatant from Vero cells was diluted 100X before detection of hTNFα.

2.4.2 From animal serum

For ELISAs on mouse serum, whole blood was collected from 4-5 week old BALB/c mice infected with 1 x 10^8 plaque forming units (PFU)/ml virus, clotted for 1h, and then spun for 10

min at 4900 rpm. Human and mouse TNFα levels were measured using TNFα Quantikine high sensitivity assay kit (R&D Systems). An n = 3 of biological replicates was used to determine statistical analysis (mean, s.d.).

2.5 Recombinant viruses

The Indiana serotype of VSVΔ51 was used in these studies. The VSVΔ51 backbone was kindly provided by Dr. Fabrice LeBoeuf and Dr. John Bell (Ottawa Hospital Research Institute).VSVΔ51-GFP is a recombinant derivative of VSVΔ51 expressing jelly fish green fluorescent protein. VSVΔ51-Fluc is a recombinant derivative of VSVΔ51 expressing firefly luciferase.

2.5.1 VSVΔ51-TNFα

RNA was extracted from VMM3 DNA using an RNeasy Mini Kit (Qiagen, Toronto, ON, Canada). cDNA was generated using a Superscript III reverse transcriptase reaction (Invitrogen). The sequence coding for the full-length human TNFα (hTNFα) was then amplified from the cDNA using a reverse transcriptase polymerase chain reaction (RT-PCR) with Platinum Pfx (Invitrogen). The forward primer consisted of a 5' overhang, Xho1 restriction site, followed by the first 21 nucleotides of the hTNFα-coding sequence (5'-GCGCTCGAGATGAGCACTGAA AGCATGATC -3'). The reverse primer consisted of a 5' overhang, the Nhe1 restriction site, and the last 21 nucleotides of the hTNFα-coding sequence (5'GCGGCTAGCTCACAGGGCAATGA TCCCAAA-3'). The PCR product was purified using a QIAquick PCR purification kit (Qiagen).

The amplicon and parental plasmid were double digested with the Xho1 and Nhe1 restriction enzymes (Invitrogen) for 2h at 37ºC. An extra PCR purification step was performed to remove any excess salts and reagents. The amplicon was inserted into the VSVΔ51 backbone using T4 DNA Ligase (Invitrogen) at 4ºC overnight. The ligated recombinant plasmids were

transformed into TOP10 cells (*E. coli*) (Invitrogen) using heat shock at 42ºC for 45 seconds. The transformation was then incubated in lysogeny broth (LB) at 37ºC overnight. Aliquots of the transformed cells were spread onto LB-ampicillin agar plates in order to screen for positive clones. Positive colonies were picked for further incubation followed by plasmid extraction using a QIAprep Spin Miniprep kit (Qiagen). Plasmids were sent to the Sick Kids TCAG DNA facility in Toronto for sequencing in order to confirm the positive clones.

Human lung alveolar (A549) cells were used to transfect the recombinant plasmid. $3x10^5$ cells were incubated overnight in a 6-well plate and then were infected with 10 MOI of the T7 vaccinia virus. The transfection mixture containing the recombinant plasmid, VSV N, P and L protein-coding plasmids and Lipofectamine 2000 (Invitrogen) in OptiMEM (Invitrogen) was added to the infected cells and incubated overnight. Cell supernatant was replenished then collected 24h later, passed through a 0.2µm filter, and then added to $6x10^5$ Vero cells in a 6-well plate. Once cytopathic effects (CPE) were observed, the virus-containing media was collected, filtered, and then applied to new Vero cells.

A series of plaque purifications were performed. A serial dilution of the viral rescue was added to fresh Vero cells, then incubated in 200µl of warm DMEM for 1hr. An agarose overlay was placed over each well and then visualized for plaque formation after 24h. The largest plaques were selected using a p1000 pipette tip, the plugs vortexed in warm media, and then applied to another round of purification two more times.

For the final viral expansion and purification, a 10cm plate of Vero cell was infected with 0.01 MOI of the third plaque purification rescue and incubated until CPE was observed 24h later. Once titred by plaque assay, the virus was further expanded in 50-15cm plates at 0.1 MOI. 24h later supernatants were collected, pelleted at 10,000 rpm for 1.5 h and then purified by

ultracentrifugation at 36,000 rpm for 1.5h using a 22-32% Optiprep gradient (Sigma-Aldrich, Oakville, ON, Canada).

2.5.2 VSVΔ51-SA:TNFα

The same procedure used to generate VSVΔ51-TNFα was used for VSVΔ51-SA:TNFα, with two exceptions: SNB75 cDNA was used for the RT-PCR, and the forward primer consisted of a 5' overhang, the Xho1 restriction site, the Kozak sequence, the human serum albumin signal peptide sequence, and the first 21 nucleotides of the soluble form of the hTNFα-coding sequence (5'-GCGCTCGAGGCCGCCACCATGAAGTGGGTAACCTTTATTTCCCTTCTTTTTCTCT TTAGCTCGGCTTATTCCGTCAGATCATCTTCTCGAACC -3').

2.6 Viral growth curves

EMT6 and SNB75 cells seeded in 96-well plates were treated with vehicle or 5 μM SMC and infected with 0.1-1 MOI of virus for 48h with aliquots collected at each indicated time point. Serially diluted supernatant was added to naïve cells (EMT6 supernatant to EMT6 cell, SNB75 supernatant to baby hamster kidney (BHK-21) cells), and incubated up to 3 days. The cells were then fixed with a 3:1 mixture of methanol:acetic acid and then dyed with 0.05% crystal violet in 25% methanol. Plates were scored for live and dead wells in order to calculate the 50% tissue culture infective dose ($TCID_{50}$) for each time point in order to generate the viral growth curves.

2.7 Cell viability assays

EMT6, normal human fibroblasts (GM38) and SNB75 cells were seeded in 96-well plates, treated with vehicle or 5 μM SMC and infected increasing MOI of virus for 48h. Cell viability was measured using Alamar Blue (Resazurin sodium salt (Sigma-Aldrich)) with data normalized to vehicle treatment. In order to measure the rate of cell death, 1 μM Cas 3/7 DEVD dye (Invitrogen) was added to treated cells and captured over 24h using the IncuCyte Zoom

(EssenBioScience, Ann Arbor,MI, United States). Enumeration of fluorescence signals was processed using the integrated object counting algorithm within the IncuCyte Zoom software. An $n = 9$ was used to determine statistical measurements (mean, s.d.).

2.8 Virus spreading assays

6-well plates were seeded with EMT6 and mouse colorectal cancer (CT-26) cells and incubated overnight until confluent. Cells were overlaid with 1% agarose in DMEM containing vehicle or 5 μM SMC and inoculated in the middle of each well with 500 PFU of VSVΔ51-GFP, VSVΔ51-TNFα or VSVΔ51-SA:TNFα. 48h later, agarose layers were removed with 3:1 mixture of methanol:acetic acid, and cytotoxicity was assessed following staining with 0.05% crystal violet in 25% methanol.

2.9 Conditioned media

2.9.1 Generated from cancer cells

EMT6 and SNB75 cells were grown in 6-cm plates and then infected with 0.1-1 MOI of virus for 24h. Cell supernatant was subjected to UV light for 1h and then frozen at -80ºC. Conditioned media was added to naïve EMT6 or SNB75 cells in a 4-fold serial dilution in the presence of vehicle or 5 μM SMC for 48h. Cell viability was measured using Alamar Blue (Sigma-Aldrich). An $n = 3$ of biological replicates was used to determine statistical analysis (mean, s.d.).

2.9.2 Generated from splenocytes

Splenocytes were grown in 6-well plates and then infected with 0.1-1 MOI of virus for 24h. Cell supernatant was subjected to UV light for 1h and then frozen at -80ºC. Conditioned media was added to naïve EMT6 cells in a 2-fold serial dilution in the presence of vehicle or 5

μM SMC for 48h. Cell viability was measured using Alamar Blue (Sigma-Aldrich). An $n = 3$ of biological replicates was used to determine statistical analysis (mean, s.d.).

2.10 TNF-R1 and DR5 knockdown

EMT6 and SNB75 cells were transfected with non-targeting (NT), TNF-R1 and/or DR5 siRNA (Silencer Select or Ambion *In Vivo* siRNA, Thermo Fisher Scientific, Waltham, MA, United States) for 48h in 96-well plates. EMT6 cells were subjected to 50μg/ml of TNFα neutralizing antibody (Clone: XT3.11, BioXCell, West Lebanon, NH, United States) to remove endogenous TNFα prior to treatment. Following treatment with vehicle or 5 μM SMC cells were infected with 1 MOI of virus, and cell viability was assessed by Alamar Blue (Sigma-Aldrich) 24h and 48h later. Knockdown of TNF-R1 and DR5 was determined by Western blot analysis.

2.11 TNFα antibody neutralization

EMT6 were grown in 96-well plates and 24h later were subjected to 50μg/ml of mouse IgG control (Clone: HRPN, BioXCell) or TNFα neutralizing antibody (Clone: XT3.11, BioXCell). Cells were treated with vehicle, 5μM SMC and/or 1 MOI virus after 1h. Cell viability was assessed 24h post treatment using Alamar Blue (Sigma-Aldrich).

2.12 Western blot analysis

In order to confirm the knockdown of TNF-R1 and DR5, treated cells grown in 6-well plates were scraped over ice in phosphate-buffered saline (PBS), pelleted by centrifugation at 1200 rpm, and lysed in radioimmunoprecipitation assay (RIPA) buffer containing a protease inhibitor cocktail (Roche, Mississauga, Canada). Equal amounts of soluble protein were separated on polyacrylamide gels followed by transfer to nitrocellulose membranes.

2.13 Survival studies

To establish syngeneic, refractory breast cancer animal models, $1x10^5$ EMT6 cells tagged with firefly luciferase (EMT6-Fluc) were implanted into the mammary fat pads of 4-5 week old, female BALB/c mice. Subcutaneous CT-26 tumours were established by injecting mice with $3x10^5$ cells in the right hind flank. Tumours were grown until palpable (~100 mm^3), about 1 week. Mice were then treated twice weekly with $1x10^8$ PFU/ml of virus (i.t.) early morning followed by 50 mg/kg SMC late afternoon (gavage). Tumours and weights were measured three times weekly, and the tumour volume was calculated using the formula: $(\pi)(W)^2(L)/4$, where W = tumor width and L = tumor length. Animals were euthanized by anaesthesia and cervical dislocation when tumor burden exceeded 2,000 mm^3. All animal studies were performed under approval of the University of Ottawa's Animal Care and Veterinarian Services protocols. An in-vitro imaging system (IVIS) was used to measure the growing EMT6-Fluc tumours based on bioluminescence following intraperitoneal (i.p.) injection of 200µl luciferin.

2.14 Tumour vasculature shutdown

EMT6-bearing mice were treated with 50mg/kg SMC and $1x10^8$ PFU/ ml VSVΔ51-TNFα 15 days post-implantation. 24h later mice were injected with 100 ul (i.v.) of a 50:50 solution of 100nm diameter orange fluorescent microspheres in PBS (Molecular Probes, Burlington, Canada), then after 5 minutes mice were euthanized by cervical dislocation without anesthesia. Anesthesia was omitted in order to avoid fluctuations in the delivery of the microspheres between each subject. Tumours were harvested immediately, cut in half and snap frozen in optimal cutting temperature (OCT) compound in isopentane over liquid nitrogen. Tumour halves were protected from light and cut into 10µm sections to visualize the vasculature

using a DNA Microarray Scanner (Agilent Technologies, Seattle, WA) and red laser. The second half of the tumours were embedded in 4% PFA for immunohistochemistry.

2.15 Immunohistochemistry

2.15.1 α-cleaved Caspase-3

Excised tumors were fixed in 4% PFA, embedded in a 1:1 mixture of OCT compound and 30% sucrose, and sectioned on a cryostat at 12 μm. Sections were incubated in a 1:500 dilution of primary antibody, followed by secondary incubation using the Vectastain system (Vector Laboratories, Burlingame, CA).

2.15.2 CD31

Excised tumors were flash frozen in OCT compound as described previously, fixed in 75% acetone 25% ethanol for 5 min at 4ºC, and sectioned on a cryostat at 10 μm. Sections were incubated in a 1:50 dilution of primary antibody, followed by secondary incubation using the Vectastain system (Vector Laboratories, Burlingame, CA).

2.16 Statistical analysis

Statistical analysis for quantification of microspheres/area and cleaved Caspase-3 was performed using non-parametric ANOVA (Sigma plot). Log-rank with Holm-Sidak multiple comparison was performed for Kaplan-Meier curves depicting mouse survival (Sigma Plot).

3.0 RESULTS

3.1 Characterization of TNFα-expressing VSV constructs

There are a number of different OVs that are currently undergoing clinical trials (72). To overcome the issue of toxicity, OVs have been engineered to elicit higher specificity and efficacy in killing numerous cancers while inducing only mild adverse effects in the host (71-74). In addition, OVs have been discovered to establish anti-tumour immunity (79). The attenuated VSVΔ51 has shown promising results in different cancer cell lines in terms of increased survival and immunogenicity (79). The VSVΔ51 genome has been engineered not only to attenuate pathogenic properties, but also to allow for easier alteration and expression of foreign transgenes. Previously, we have demonstrated that VSVΔ51 synergizes with LCL161 to induce more potent responses in treated cells (68). This effect is dependent on the presence of inflammatory cytokines such as TRAIL and TNFα, which attributes to the potentiation of SMC treatment. TNFα itself as a therapeutic has been scrutinized due to its systemic toxicity. However, recent studies have shown that the local delivery of TNFα using recombinant OVs can overcome this challenge (107, 108).

In pursuance of enhancing SMC treatment, I sought to generate recombinant VSVΔ51 that could effectively express human TNFα (hTNFα). Importantly, hTNFα is recognizable by the mouse TNFR, allowing its efficacy to be treated in murine tumour models and cell lines (114). The secretion of TNFα from infected cells could induce a more localized and potent response to SMC treatment, preferentially killing cancer cells while normal cells are left unharmed.

3.1.1 Generation of TNFα-armed constructs

The VSVΔ51-TNFα construct was initially generated by inserting the full length hTNFα-coding sequence between the G and L-coding sequences of the Indiana serotype VSVΔ51

backbone. To improve upon the issue of the TNFα remaining membrane bound, we proposed that the inclusion of a constitutively secreted signal peptide would allow TNFα to be secreted more efficiently from infected cells. I thus generated a second construct with the anchoring domain of TNFα replaced by the human serum albumin signal peptide sequence (VSVΔ51-SA:TNFα), a highly expressed sequence that has commonly been used for protein expression vectors (115). Preceding this signal peptide sequence, a Kozak sequence was included to enhance the expression of the inserted hTNFα gene. Rudimentary plaque assays and RT-PCR confirmed successful expression of the recombinant constructs at high viral titres (data not shown). VSVΔ51-GFP was included in these studies for comparison reasons, due to its transgene being located in the same cloning region and it being similar in length as the VSVΔ51-TNFα constructs. A schematic representation of the viruses is shown in Figure 3-1a.

3.1.2 VSV constructs effectively express TNFα

To ensure that the constructs could express TNFα, I performed a series of ELISAs on the supernatant of monkey kidney epithelial (Vero) cells infected with VSVΔ51-TNFα and VSVΔ51-SA:TNFα (Figure 3-1b). Vero cells do not elicit an antiviral, IFN response and are therefore highly permissible to infection. In fact, Vero cells do not respond to SMC and TNFα combination treatment as they lack TNF-R1 (Figure 3-1c). Therefore, infection of Vero cells with a virus allows for its optimal amplification, and tests the ability of the virus to replicate in the absence of an antiviral response. First attempts at observing the expression of virally derived TNFα from infected cells could not be quantified as the levels were too high to measure by the ELISA kit. To address this issue, I diluted supernatants extracted from infected Vero cells 100X before performing another high sensitivity ELISA. Just over 1µg

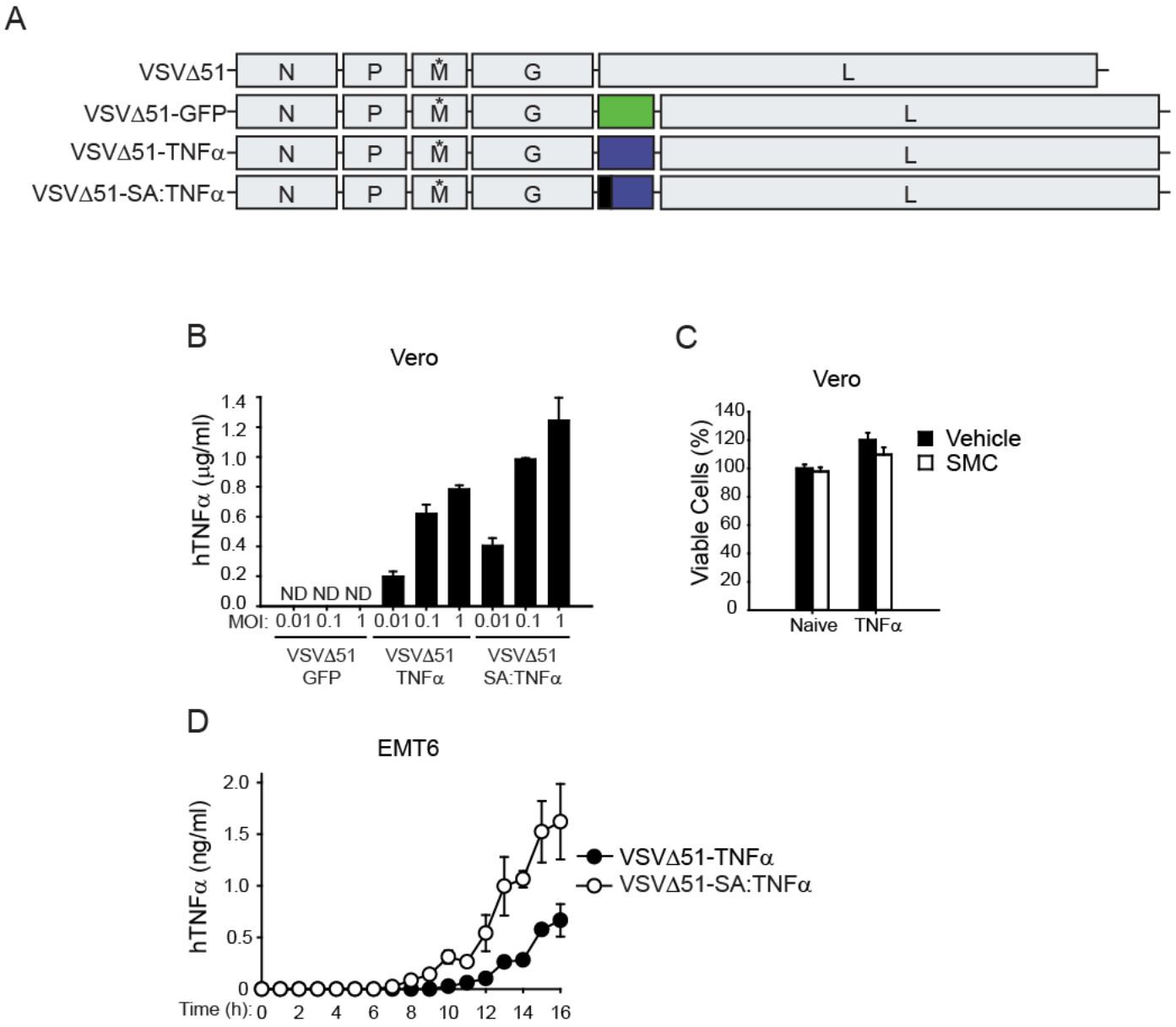

Figure 3-1. TNFα-armed VSV produce elevated levels of TNFα in infected cells. (A) Schematic representation of the VSVΔ51-GFP, VSVΔ51-TNFα and VSVΔ51-SA:TNFα constructs. Colour scheme: Green represents the GFP-coding gene, blue represents the hTNFα-coding gene, black represents the serum albumin signal peptide sequence. (*) denotes the deletion of the 51st amino acid in the M protein. **(B)** Expression of hTNFα. Cell culture supernatant extracted from Vero cells treated with vehicle and increasing MOI of virus for 16h were processed for the presence of hTNFα (mean + SD, n = 3). (ND) denotes non-detected protein. **(C)** Alamar blue assays of Vero cells treated with vehicle or 5 μM SMC and 10μg/ml hTNFα. **(D)** Time course assessment of hTNFα secreted from infected EMT6 cells. Cell culture supernatant extracted from EMT6 cells, treated with 5 μM SMC and 1 MOI of virus for 16h were processed for the presence of hTNFα (mean + SD, n = 3).

of hTNFα was detected in Vero cells infected with VSVΔ51-TNFα and VSVΔ51-SA:TNFα (Figure 3-1b). Higher levels of hTNFα was detected from cells infected with VSVΔ51-SA:TNFα compared to VSVΔ51-TNFα . As expected, no hTNFα was detected in the supernatant of cells infected with the control virus, VSVΔ51-GFP.

A time course ELISA performed on infected mouse mammary carcinoma (EMT6) cells demonstrated that VSVΔ51-SA:TNFα expresses hTNFα at a faster rate than VSVΔ51-TNFα (Figure 3-1d). EMT6 cells are more resistant to viral infection than Vero cells as they elicit an antiviral response. Hence, the levels of virally derived hTNFα were detected in nanograms.

3.1.3 TNFα is spread systemically and cleared over time

Based on these observations, I then asked if virally derived TNFα could be spread systemically in mice, and safely cleared by the host's system. This could possibly enhance the therapeutic effect of the treatment, given that endogenous TNFα is delivered long enough to potentiate the SMC-mediated killing, and then is effectively cleared to avoid toxicity.

An ELISA performed on serum isolated from the whole blood of infected BALB/c mice confirmed that TNFα is spread systemically and is gradually cleared out over time (Figure 3-2a). With the removal of the attenuated virus by the innate immune response, the release of TNFα is not sustained, allowing the host the chance to recover. In fact, during *in vivo* studies performed with VSVΔ51, the virus needed to be re-administered every two days for the treatment to be effective (68). In addition to this, we found that the levels of endogenous mouse TNFα (mTNFα) are also slightly elevated in mice infected with VSVΔ51-TNFα and VSVΔ51-SA:TNFα compared to mice infected with VSVΔ51-GFP (Figure 3-2b). The elevated levels of endogenous TNFα found in the serum from VSVΔ51-GFP-infected mice could be attributed to an elevated IFNβ-mediated antiviral response inducing the release of endogenous TNFα.

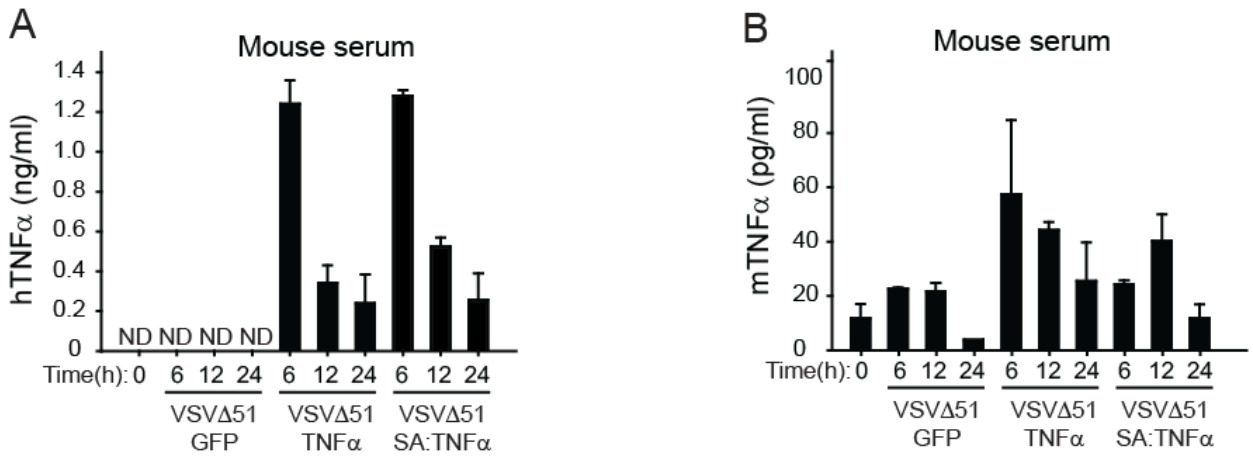

Figure 3-2. Virally derived TNFα is spread systemically in mice. (A) Systemic delivery of hTNFα in mice was assessed using a Quantikine high sensitivity ELISA kit on serum isolated from whole mouse blood following treatment with 1 x 10^8 pfu virus (i.v.) for 6, 12 and 24h (mean + SE, n = 3). (ND) denotes non-detected protein. **(B)** Similarly to A, systemic delivery of endogeous mTNFα in mice was assessed using a Quantikine high sensitivity ELISA kit on serum isolated from whole mouse blood following treatment with 1 x 10^8 pfu virus (i.v.) for 6, 12 and 24h (mean + SE, n = 3).

Taken together this demonstrates that the viruses effectively express TNFα, and the inclusion of the secretory signal peptide successfully promotes the shuttling of more TNFα out of infected cells into the supernatant. Additionally, an abundant amount of virally derived TNFα can be spread through a host systemically, and is effectively cleared over a short period of time.

From this data we concluded that the recombinant TNFα-armed viruses were functioning based on their designed intent, to express and secrete TNFα. To further confirm their efficacy, I next aimed to determine whether the viral kinetics, TNFα expression and antiviral response elicited by the recombinants are modified by SMC treatment.

3.2 Recombinant viruses are functional with SMC treatment

Stimulation of the host's innate immune response occurs through the detection of pathogen-associated molecular patterns (PAMPs) or cellular damage-associated molecular patterns (DAMPs) by the pattern recognition receptors (PRRs). Upon infection of a mammalian cell by an RNA virus, the PRRs trigger a series of signalling pathways including those regulated by RIG-I-like receptors (RLRs) and toll-like receptors (TLRs) sparking the release of an array of inflammatory cytokines and chemokines, and warning the neighboring cells of the viral infection (116). This initiates the recruitment of immune cells in order to fight off the impeding infection. Due to the fact that the cIAPs interact with the NF-κB pathway, there has been some speculation that the use of SMCs can diminish the efficacy and specificity of the combination treatment due to the alterations of innate immune cell signalling (117). Essentially, a diminished IFNβ-mediated antiviral response would remove the protection of normal cells from the attenuated OV. Hence, before the efficacy of recombinant OV and SMC co-treatment could be assessed, I next had to determine that, in addition to the successful expression of the human transgenes, that the

viral kinetics and viral growth of the constructs are comparable to the control virus, and that an antiviral response could still be elicited in the presence and absence of the SMC.

3.2.1 Viruses express TNFα in the presence of SMC

In order to confirm that the recombinant viruses could still functionally express TNFα with SMC treatment, ELISAs were performed on the supernatants of treated Vero, EMT6, and human glioblastoma (SNB75) cells (Figure 3-3a). I used the SMC, LCL161, for these studies as it is currently the most clinically advanced, can be administered to animals orally, and exhibits additional immunomodulatory effects (60, 118, 119). As expected, combination treatment had no effect on the Vero cells, as they are insensitive to TNFα-mediated death, therefore did not affect the level of expression of TNFα compared to vehicle treatment. Moreover, hTNFα production was observed in the supernatant extracted from treated EMT6 cells, demonstrating that the detected protein is virally derived. Similarly to what was seen in Figure 3-1d, EMT6 cells infected with VSVΔ51-SA:TNFα expressed higher amounts of hTNFα compared to those infected with VSVΔ51-TNFα. ELISAs performed on supernatant extracted from treated SNB75 cells also showed that the TNFα-armed viruses still produce TNFα in the presence of SMC, with higher levels being detected from the cells infected with the soluble TNFα-expressing virus . However, relatively lower levels of TNFα were detected in combination treatments of the EMT6 and SNB75 cells due to a higher incidence of death over time (Figure 3-3b). With less cells being present following treatment, there is less material to express and detect the virally-derived TNFα.

3.2.2 SMC treatment does not perturb the antiviral response

Previous reports claim that the cIAPs are involved in innate immune cell signalling (117, 120). Hence, I wanted to confirm that SMC treatment does not perturb the ability of cells to elicit an antiviral response, as we have shown previously (68). This response is crucial for the

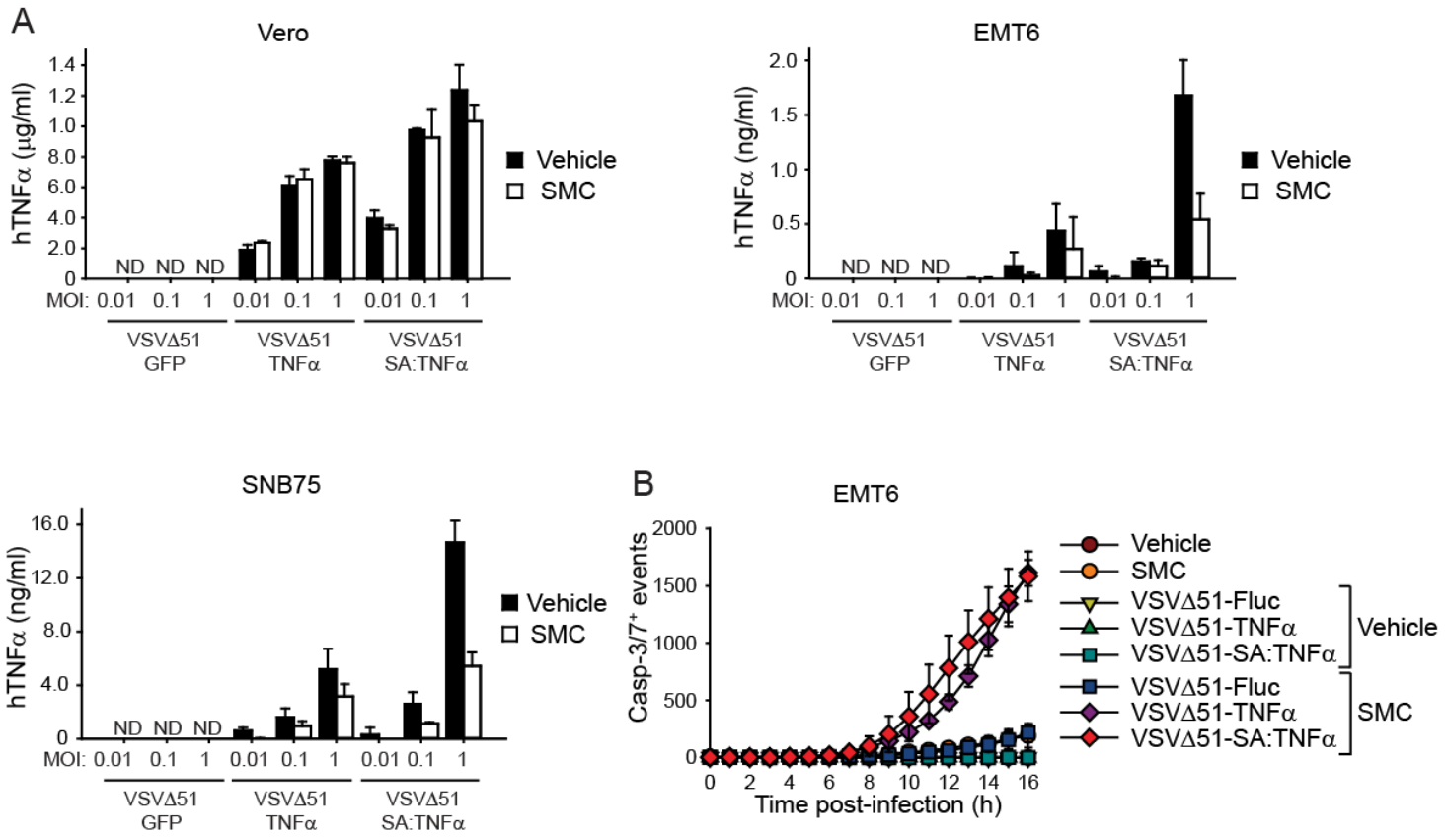

Figure 3-3. VSV transgenic TNFα expression is not affected by SMC treatment. (A) Cell culture supernatant extracted from Vero, EMT6 and SNB75 cells treated with vehicle or 5 µM SMC and increasing MOI of virus for 16h were processed for the presence of hTNFα (mean + SD, n = 3). (ND) denotes non-detected protein. **(B)** Quantification of Caspase -3/7-DEVD-FITC signals. EMT6 cells were treated with vehicle or 5 µM SMC and 1 MOI of VSVΔ51-Fluc, VSV51-TNα, or VSVΔ51-SA:TNFα. Images were collected for 16h using IncucyteZoom (mean + SD, n = 3).

specificity of the treatment, as normal cells are protected from the attenuated OV due to the IFNβ-mediated antiviral response. Using ELISAs, I measured the production of endogenous mouse and human IFNβ (mIFNβ and hIFNβ) from treated EMT6 and SNB75 cells, respectively, and found that both the recombinants VSVΔ51-TNFα and VSVΔ51-SA:TNFα still elicit an antiviral response in the presence of SMC (Figure 3-4). As mentioned previously, the detected protein levels in cells treated with SMC are lower due to increased cell death in doubly treated samples. Interestingly, there was an increase in the level of endogenous IFNβ detected from cells infected with the TNFα-armed viruses compared to the control virus. This could be attributed to the high secretion of TNFα that may be inducing the additional release of IFNβ, or to a stronger innate immune response induced by the TNFα-expressing constructs.

3.2.3 Viral kinetics are unaffected by SMC treatment

I next aimed to confirm that the TNFα-armed VSV constructs still replicate and infect cells comparably to the control virus, VSVΔ51-GFP, in the presence and absence of SMC. Viral growth curves calculated based on 50% tissue culture infective dose ($TCID_{50}$) confirmed that the viral kinetics of both the VSVΔ51-TNFα and VSVΔ51-SA:TNFα in infected EMT6 and SNB75 cells are not affected by SMC treatment (Figure 3-5). This finding correlates with what has been previously shown with cells infected with VSVΔ51 (68), demonstrating the viruses' ability to replicate and spread normally. More specifically, the viral kinetics measured at 0.01 MOI confirms that the viruses are able to replicate effectively following infection (Figure 3-5a), whereas the viral kinetics measured at 1 MOI demonstrates the ability of the viruses to infect and kill (Figure 3-5c). At 0.1 MOI, a measure of both the replication and infectivity of the viruses was observed (Figure 3-5b). The lower viral growth in EMT6 cells infected with 0.01 MOI of virus could be due to a higher IFN secretion. $TCID_{50}$ was used to titre the viruses in these

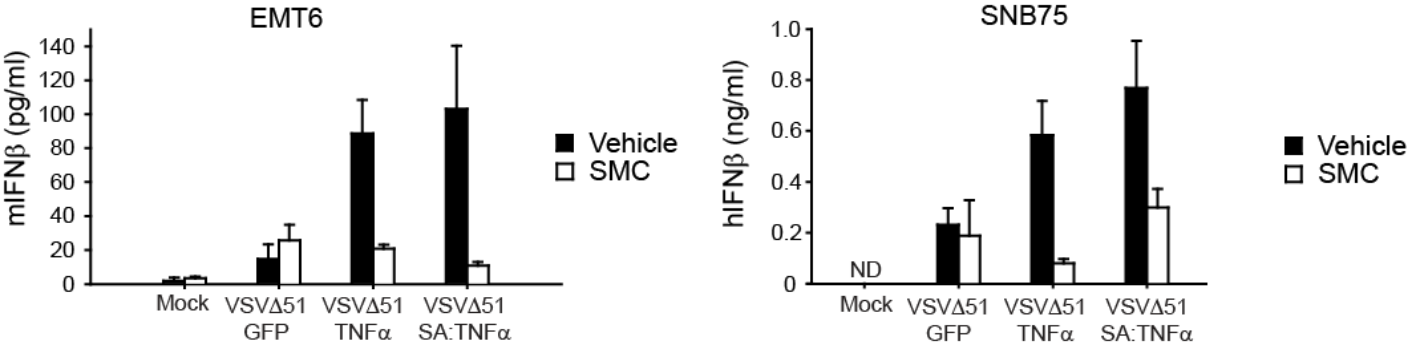

Figure 3-4. SMC treatment does not alter the antiviral response of cancer cells. Expression of type 1 IFNβ. Cell culture supernatant extracted from EMT6 and SNB75 cells treated with vehicle or 5 μM SMC and 1 MOI of virus for 16h were processed for the presence of endogenous IFNβ (mean + SD, n = 3).

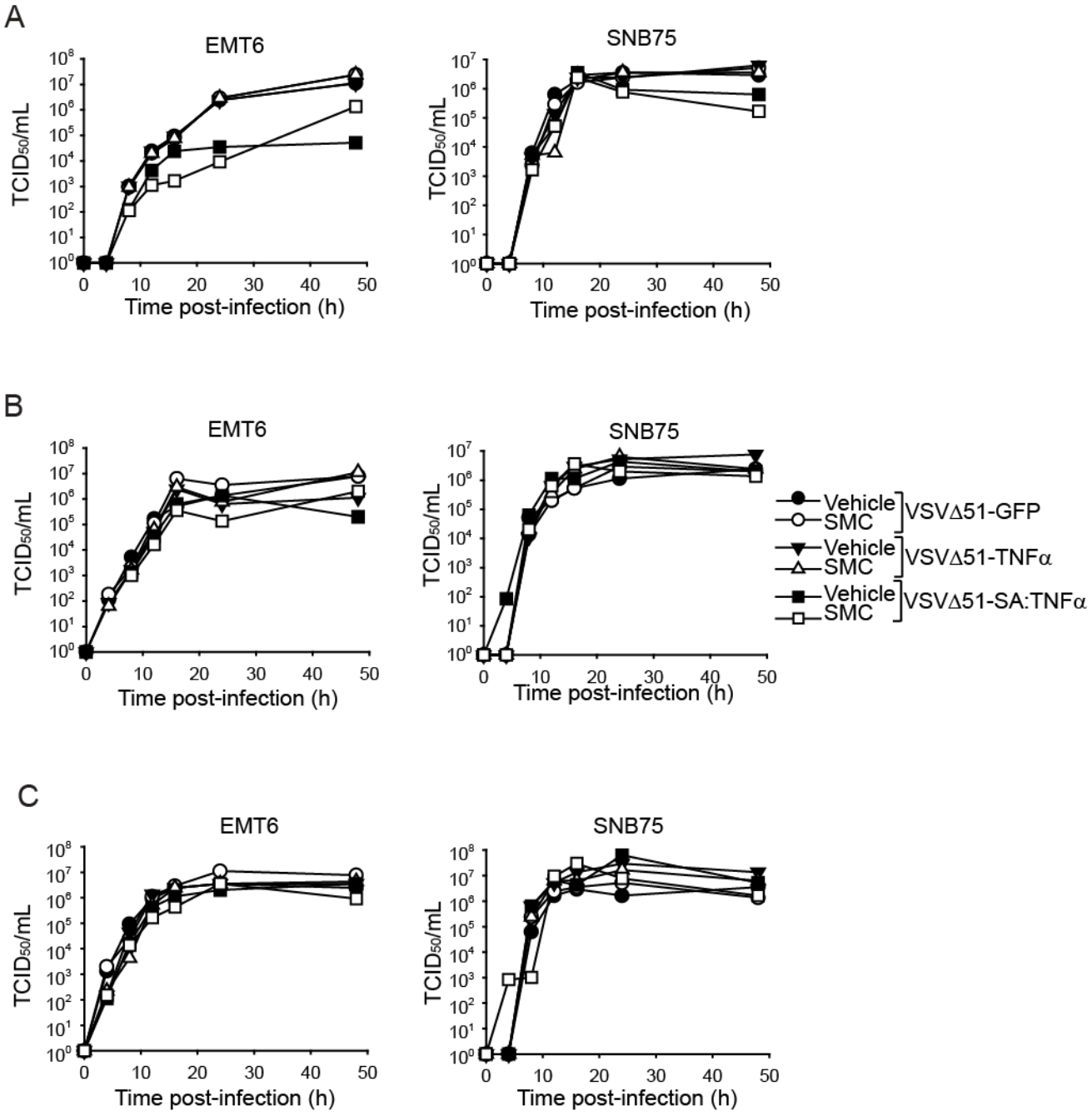

Figure 3-5. VSV transgenic constructs and SMC treatment does not alter viral kinetics. Virus growth curves. Vero and BHK-21 cells were subjected to supernatant from EMT6 and SNB75 cells, respectively, which had been treated with vehicle or 5 μM SMC and infected with **(A)** 0.01 MOI, **(B)** 0.1 MOI, or **(C)** 1 MOI of virus. Plates were stained with crystal violet and scored for living and dead cells in order to calculate TCID50/mL titres (mean + SD, n = 3). TCID50 is defined as 50% tissue culture infective dose.

experiments since this method allows a higher throughput with less materials wasted.

All in all, this data suggests that both the generated constructs' growth and functionalities are not impeded by the presence of SMC or the transgenes they express. I have shown that LCL161 does not inhibit the IFNβ-mediated antiviral response of cells, or the ability of the viruses to infect and replicate. The comparable viral kinetics are also indirect evidence that SMC treatment does not affect the antiviral response. Furthermore, these experiments confirm that the inclusion of the human transgene in the viruses does not negatively affect their efficacy. Hence, the combination treatment should effectively induce cancer cell death while normal cells are left unaffected, maintaining the high specificity and efficacy of this approach.

3.3 TNFα-armed VSV are superior to VSVΔ51-GFP *in vitro*

After successfully characterizing the new viral constructs and confirming their functionality with SMC, I sought to determine if the viral delivery of TNFα could improve the efficacy of SMC treatment *in vitro.* I hypothesized that a higher concentration of TNFα would enhance the efficacy of SMC treatment. In previous work we have studied the synergy between a number of SMCs and OVs, and determined that LCL161 exhibits the highest amount of synergy with VSVΔ51 (68). Herein, I sought to improve this synergy with the TNFα-expressing recombinant viruses.

3.3.1 TNFα-expressing constructs potentiate SMC-mediated cell death

To verify whether TNFα-armed VSV enhanced cancer cell death in the presence of SMCs, I performed Alamar Blue viability assays on treated SNB75 and EMT6 cells and showed that both VSVΔ51-TNFα and VSVΔ51-SA:TNFα are superior to VSVΔ51-GFP in inducing SMC-mediated cell killing (Figure 3-6). Approximately 1 MOI of the TNFα- expressing constructs displayed the same level of synergy with SMC as 10 ng/mL exogenous TNFα,

resulting in 20-40% viable cells 24h post-treatment (Figure 3-6a). The efficacy of both TNFα-expressing viruses appeared to be comparable after 24h; however, 48h post treatment, VSVΔ51-TNFα and VSVΔ51-SA:TNFα enhanced the SMC-mediated killing by one log compared to co-treatment with VSVΔ51-GFP (Figure 3-6b). In contrast to these results, normal human fibroblasts (GM38) are left unaffected by the treatment, confirming the specificity of the treatment toward cancer cells.

3.3.2 Viral TNFα expression enhances rate of killing

The superiority of VSVΔ51-TNFα and VSVΔ51-SA:TNFα in comparison to the control virus was also demonstrated by qualitative and quantitative Caspase -3/7-DEVD-FITC counts in co-treated EMT6 and SNB75 cells (Figure 3-7). Phase-contrast and fluorescent micrographs were acquired every hour on an IncucyteZoom apparatus to track morphology and enumerate the number of Caspase -3/7-DEVD-FITC positive cells. VSVΔ51-Fluc was used as a control for these studies due to the fact that the fluorescence of VSVΔ51-GFP would interfere with the measurement of the positive caspase-3 signals. The TNFα constructs showed an increased rate of cell killing compared to the control. The rate of cell killing by VSVΔ51-TNFα and VSVΔ51-SA:TNFα appeared to plateau after 24h, whereas cells treated with VSVΔ51-Fluc were just starting to be killed after 24h. In fact, the incidence of cell death began 8h earlier than cells treated with the control virus (Figure 3-7a). Micrographs demonstrated that while SNB75 cells were ultimately left unaffected by treatment with VSVΔ51-Fluc, the TNFα-expressing constructs induced a significant amount of cell death (Figure 3-7b). To our surprise, there was cell death observed in SNB75 cells treated with SMC alone, a phenomenom that we do not yet fully understand being that this cell line is usually hard to kill with SMC alone.

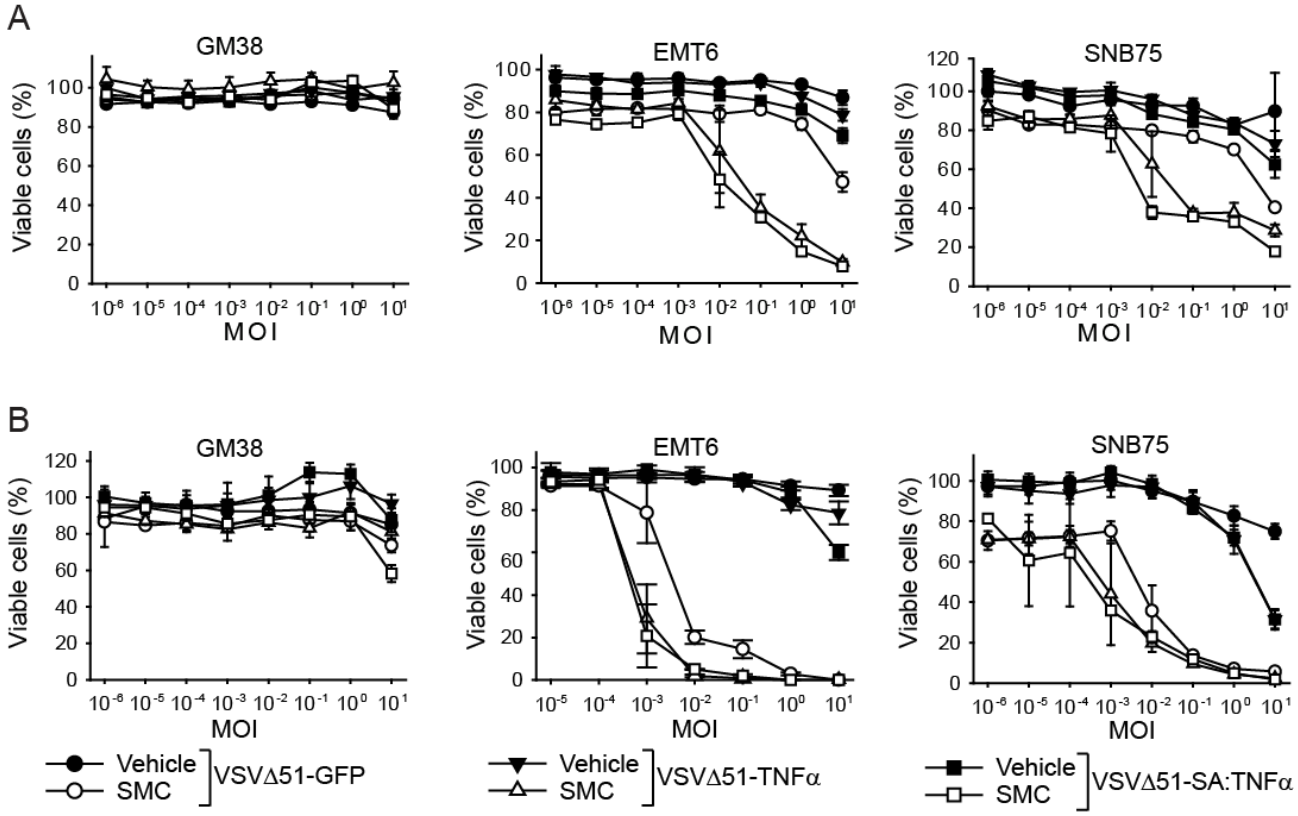

Figure 3-6. VSVΔ51-TNFα and VSVΔ51-SA:TNFα constructs are superior to VSVΔ51-GFP in potentiating SMC treatment. Alamar blue assays of EMT6, SNB75 and GM38 cells treated with vehicle or 5 μM SMC and increasing MOI of VSVΔ51-GFP, VSVΔ51-TNFα or VSVΔ51-SA:TNFα for **(A)** 24h and **(B)** 48 h (mean + SD, n = 3).

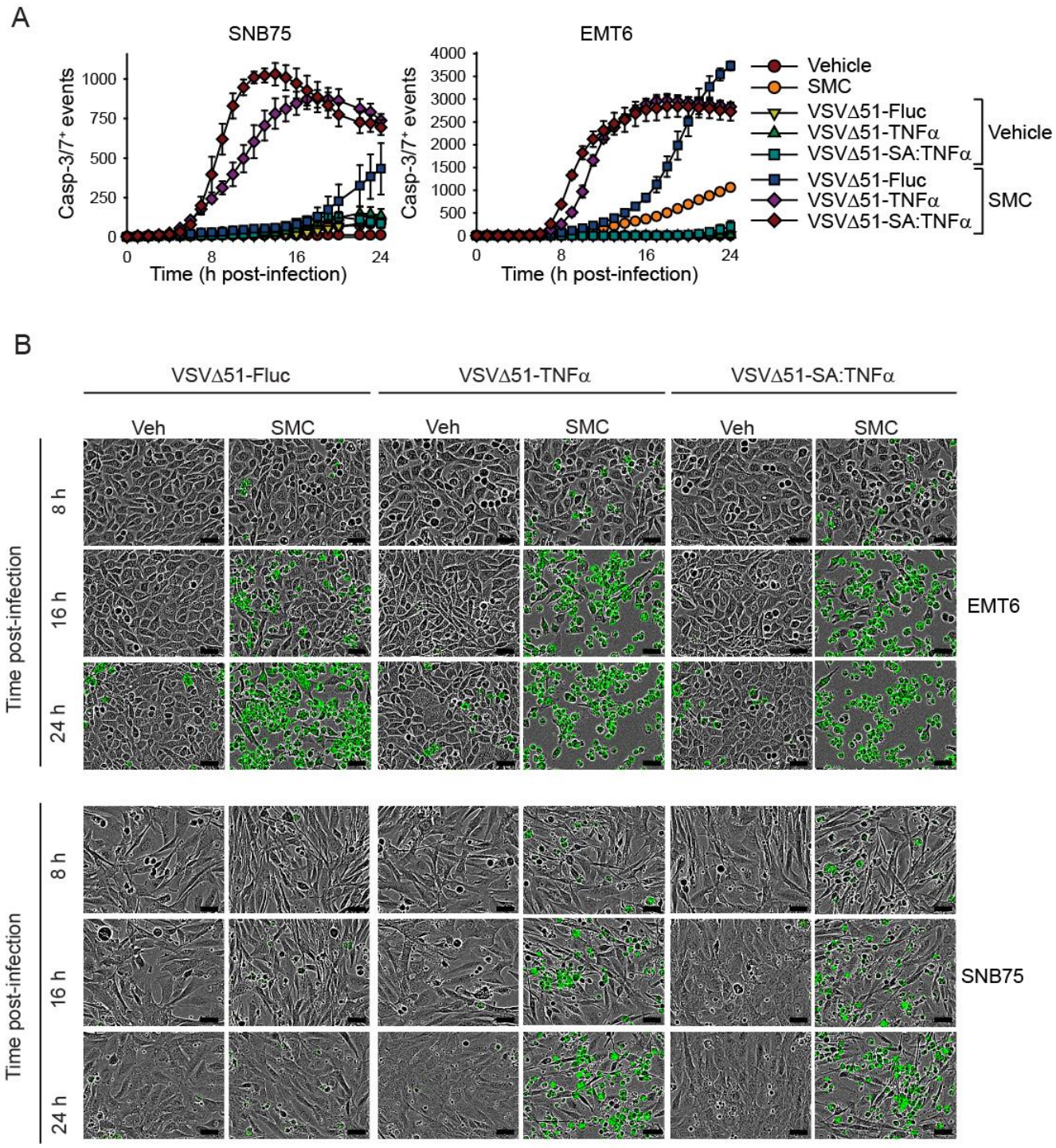

Figure 3-7. TNFα expressing constructs induce cell death at a faster rate than VSVΔ51-Fluc with SMC treatment.(A) Quantification of Caspase -3/7-DEVD-FITC signals from EMT6 and SNB75 cells treated with vehicle or 5 μM SMC and 1 MOI of VSVΔ51-Fluc, VSVΔ51-TNFα, or VSVΔ51-SA:TNFα (mean + SD, n = 3). **(B)** Caspase -3/7-DEVD-FITC staining micrographs of EMT6 and SNB75 cells treated with vehicle or 5 μM SMC and 1 MOI of VSVΔ51-Fluc, VSVΔ51-TNFα, or VSVΔ51-SA:TNFα. Images were collected using IncucyteZoom. Scale bars, 50 μm.

Collectively this data illustrates that the TNFα-armed viruses greatly improve oncolytic rhabdovirus combination treatment with SMCs, specifically in tumour cells, and do so at a faster rate of killing.

3.4 Combination treatment enhances the bystander cell killing effect

Before exploring the efficacy of the treatment *in vivo* to assess whether it can be translated to clinical applications, I wanted to investigate whether the combination treatment improved the bystander killing effect. With the abundant amount of TNFα being expressed and secreted, I hypothesized that the SMC treatment would not only be efficacious in killing infected cells, but also in killing neighboring cancer cells that are exposed to TNFα and other death-inducing factors.

3.4.1 Treatment induces spread of death from point of infection

In addition to the high levels of TNFα that are secreted from infected cells, the viral infection can induce a "cytokine storm" and the release of a plethora of soluble factors that also contribute to SMC-mediated cell death (71). To explore the effects of bystander cell killing, a cytotoxicity assay was performed on EMT6 and human colorectal cancer (CT-26) cell lines overlaid with agarose that functioned to slow viral spread. This prevents cell death induced by viral infection, and allows for the observation of cell death induced solely by the spread of soluble proteins. Following treatment, I observed a drastic spread of cell death outside the zone of infection (Figure 3-8a), indicating that the viruses are promoting the release of soluble factors that aid in SMC-mediated killing of neighboring cells. Again, the TNFα-armed viruses prove to have a stronger response in treated cells than those infected with VSVΔ51-GFP. These are striking results given that the untagged CT-26 and EMT6 are typically aggressive, refractory cell lines that have proved difficult to kill with OV or SMC treatment alone.

3.4.2 Viral infection promotes the release of soluble, death-inducing factors

To compliment the observation of increased bystander cell killing, I performed conditioned media assays to confirm that there are soluble factors secreted by infected cells that contribute to the spread of cancer cell death. Cells were first infected with VSVΔ51-GFP, VSVΔ51-TNFα or VSVΔ51-SA:TNFα, and then the supernatant was UV inactivated and re-applied to naïve cells in the presence of SMC. Virus inactivation was confirmed through visualization under a fluorescent microscope and/or through plaque assays (data not shown). Conditioned media from infected EMT6 and SNB75 cells and splenocytes isolated from BALB/c mice added to naïve EMT6 and SNB75 cells showed a strong dose-dependent increase in cell killing (Figure 3-8b and c). Cells exposed to the conditioned media from cells infected with VSVΔ51-TNFα and VSVΔ51-SA:TNFα elicited a drastic increase in killing in the presence of SMC compared to VSVΔ51-GFP. Unexpectedly, there was less cell death observed with conditioned media isolated from splenocytes, which consist of a variety of immune cells, notably, lymphocytes, macrophages and dendritic cells. This can be attributed to the fact that splenocytes are less permissible to viral infection, and thus, less death-inducing soluble factors were present in the conditioned media to contribute to cell death.

3.4.3 TNFα signalling is required for efficacy of combination treatment

There are a number of soluble factors that have been speculated to induce cell death, such as TNFα, TNFα-induced cytokines and chemokines, Cytochrome C, endogenous Smac and defective viral particles. In recent studies we have shown that SMCs sensitize cancer cells to caspase-8-dependent apoptosis through TNFα and TRAIL-signalling, which is coincidentally triggered by RNA viral infection upon IFNβ secretion (68, 69). In order to determine whether secreted TNFα is indeed one of the factors participating in the bystander cell killing, I assessed

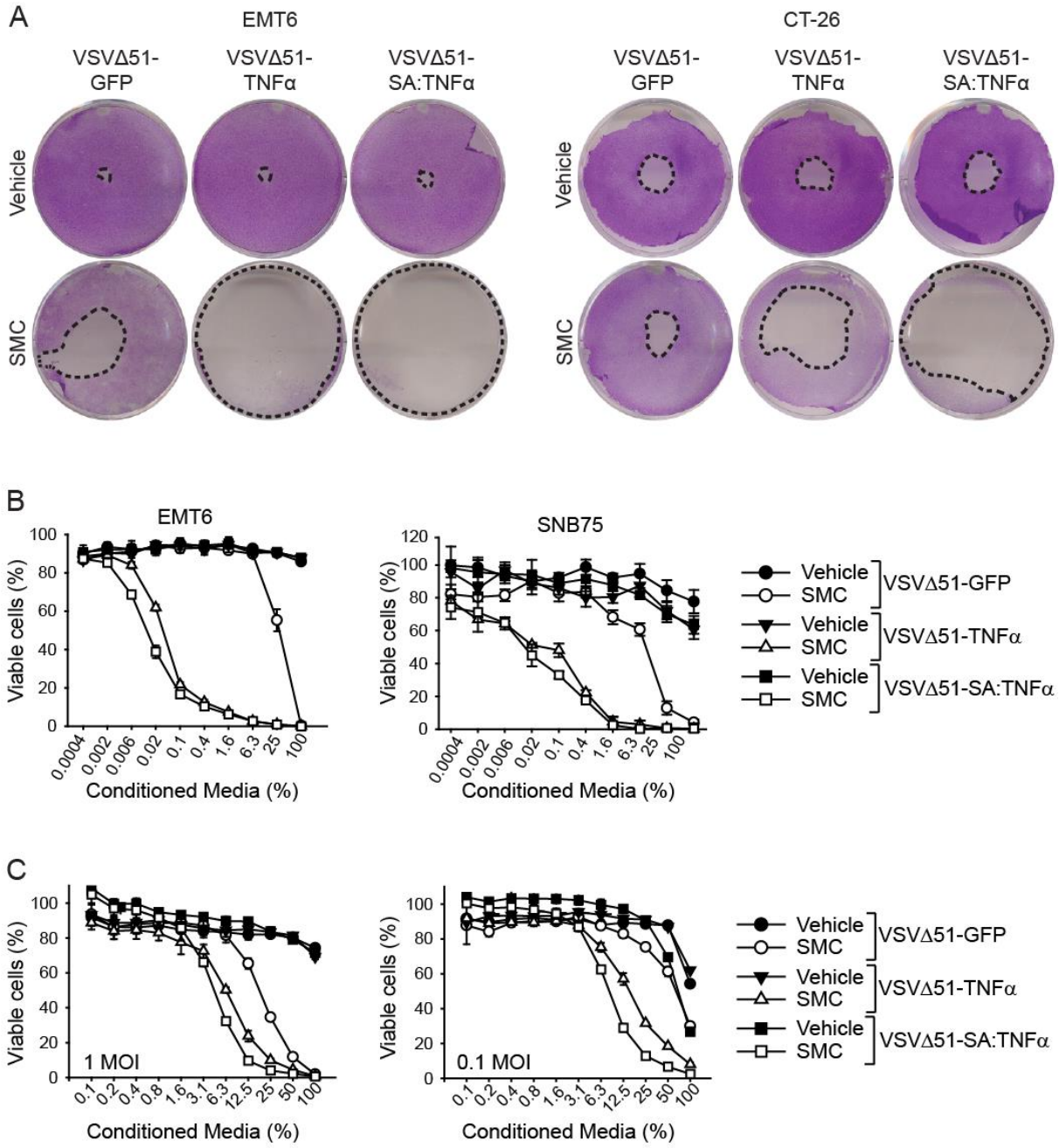

Figure 3-8. Viral infection induces the release of soluble factors that induce bystander cell death. **(A)** Virus spreading assay. Confluent monolayers of EMT6 and CT-26 cell lines were overlaid with agarose media containing 5 μM SMC and inoculated with 500 PFU of virus in the middle of the well. 2 days post-treatment, cytotoxicity was assessed by crystal violet staining (mean + SD). **(B)** Conditioned media assays. EMT6 and SNB75 cells were infected with VSVΔ51-GFP, VSVΔ51-TNFα or VSVΔ51-SA:TNFα at 1 MOI for 24 h. Cell supernatants were exposed to virus-inactivating UV light and then the supernatant was applied at indicated dilutions to new EMT6 or SNB75 cells in the presence of vehicle or 5 μM SMC. Alamar blue viability assays were performed 48 h after treatment (mean + SD, n = 3). **(C)** Conditioned media from splenocyte cultures. Splenocytes collected from BALB/c mice were used to generate conditioned media and added to EMT6 cells as described in B at indicated MOIs of virus (mean + SD, n = 3).

the effects of treatment following RNAi knockdown of TNF-R1 and/or death receptor 5 (DR5) in SNB75 cells. Cell viability assays showed that in the absence of TNF-R1 and DR5, the function of VSVΔ51-GFP in killing is removed, demonstrating that TNFα and TRAIL are indispensable for bystander cell death induced by the combination treatment (Figure 3-9a). Surprisingly, VSVΔ51-TNFα and VSVΔ51-SA:TNFα potency is only partially diminished following knockdown of TNF-R1 and DR5, suggesting that there are components other than TNFα and TRAIL signalling contributing to cell death (Figure 3-9a). TNF-R1 and DR5 knockdown was confirmed by western blot analysis (Figure 3-9b).

To demonstrate that TNFα is still required for SMC-mediated cell death, EMT6 cells were depleted of endogenous TNFα using neutralizing antibodies, and then treated to observe whether cell death still occurred. As expected, cells treated with VSVΔ51-GFP and SMC had high viability following removal of TNFα, whereas cells treated with the TNFα-secreting viruses and SMC still died in the presence of SMC (Figure 3-9c). This illustrates that the secretion of virally derived TNFα synergizes with SMC to induce death, even in the absence of endogenous TNFα.

Collectively, this data confirms that TNFα-armed VSVΔ51 infection leads to the release of soluble factors, including TNFα, that potently induce bystander cell death in neighboring, uninfected cancer cells treated with SMC. Given the promising results collected *in vitro*, I next moved on to *in vivo* survival studies to explore whether the TNFα-armed viruses could enhance the efficacy of SMC treatment in animal models of cancer.

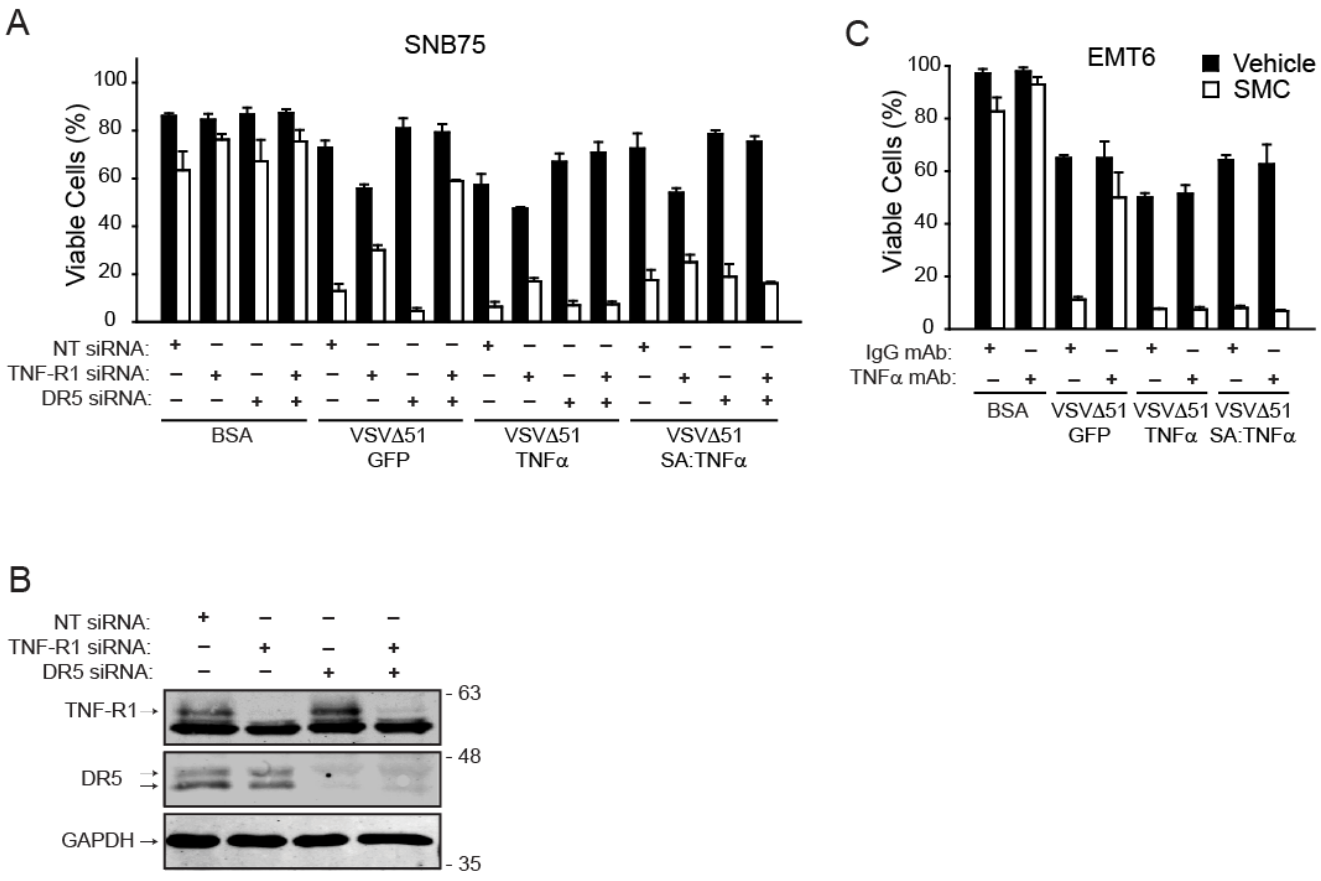

Figure 3-9. TNFα signalling plays a major role in combination treatment. (A) Knockdown of TNF-R1 and DR5. SNB75 cells were reverse transfected with TNF-R1 and/or DR5 silencing RNA for 48h. Cells were then treated with vehicle or 5μM SMC. Cell viability was assessed 48h post treatment using Alamar Blue (mean + SD, n = 3). **(B)** Knockdown was confirmed by Western blot analysis . **(C)** Antibody neutralization of endogenous TNFα. EMT6 cells were pretreated with 50μg/ml of TNFα neutralizing antibody for 1 h and subsequently treated with 5 μM SMC and 1 MOI of virus.

3.5 VSVΔ51-TNFα and VSVΔ51-SA:TNFα potently synergize with SMC *in vivo*

Previously we have shown that the OV and SMC co-treatment is a safe and efficacious approach to treat cancer in animal models (68). Preliminary safety and pharmacodynamics experiments showed that a dose of 50 mg/kg LCL161 delivered orally (gavage) and 5 x 10^8 PFU/ml of virus was well tolerated in mice with only mild adverse effects. Survival studies performed on EMT6 models co-treated with VSVΔ51 and LCL161 yielded durable cures, proving to be a successful treatment approach (Figure 3-10) (68). Given the superiority of the TNFα construct compared to VSVΔ51-GFP, I hypothesized that combination treatment with SMC and VSVΔ51-TNFα/VSVΔ51-SA:TNFα would improve the survivability of tumour bearing mice compared to those previously treated with VSVΔ51.

3.5.1 Combination immunotherapy with recombinant OV and SMC is well tolerated in mice

Preliminary tolerance tests showed that the recombinant virus could be administered to the mice at a dose of 1 x 10^8 PFU/ml intratumourally (i.t.) with 50mg/kg of SMC gavage as body weights of treated mice soon returned to baseline levels (data not shown). Once tumours were palpable (~100 mm^3) we began treating the mice twice weekly with vehicle or SMC and virus. Multiple treatments were required as the attenuated virus is cleared from the animals' system over time. Mice appeared to exhibit piloerection and ~5% transient weight loss after infection, however returned back to normal after 48h (data not shown).

3.5.2 SMC synergizes with TNFα-armed viruses, leading to durable cures

To evaluate the efficacy of SMC and TNFα-armed virus co-therapy *in vivo*, survival studies were performed on both the established CT-26 and firefly luciferase-tagged EMT6 (EMT6-Fluc) models in mice (Figure 3-11). SMC alone slowed tumour growth and prolonged survival; however, virus alone had little to no efficacy, and mice had to be euthanized after 20-30 days

(Figures 3-11a and b). Strikingly, combination treatment of SMC and VSVΔ51-SA:TNFα yielded 10% and 70% durable cures in the CT-26 and EMT6-Fluc models, respectively (Figures 3-11a and b). Additionally, EMT6-bearing mice treated with SMC and VSVΔ51-TNFα showed a 30% cure rate (Figure 3-11b). The EMT6-Fluc tumour burden was visualized using an in vivo imaging system (IVIS), as shown by three representative models from each treatment group in Figure 3-11c. Mice that were re-challenged with tumour cells did not show any tumour growth (Beug *et al*., unpublished data), confirming the long-term effects of the combination treatment. This treatment proved to be almost twice as effective as treatment with SMC and VSVΔ51, with five times less virus administered to the animals.

These results support my initial hypothesis that by inducing an enhanced release of TNFα in cancer cells, the efficacy of SMC and oncolytic virus cotherapies can be greatly improved. Hence, a stronger response to SMC treatment can be achieved with a higher induction of TNFα release, and can help treat highly aggressive cancers. The striking cure rates found in the animal models suggest a promising future for potentiating SMC treatment with TNFα-armed oncolytic viruses, and could be translatable to clinical studies.

3.6 Combination treatment targets tumour neovasculature

Previous studies have shown that VSV is capable of inducing vasculature collapse via clot formation and endothelial cell damage in CT-26 tumours (77). Additionally, the administration of exogenous TNFα has been shown to be toxic to the neovasculature of growing tumours (57, 107, 108). The use of TNFα-expressing OV with SMC to induce vasculature collapse could be another mechanism of effectively promoting cancer cell death. I proposed to explore whether VSVΔ51-TNFα can inhibit blood flow in infected EMT6 tumours, in the presence and absence of SMC.

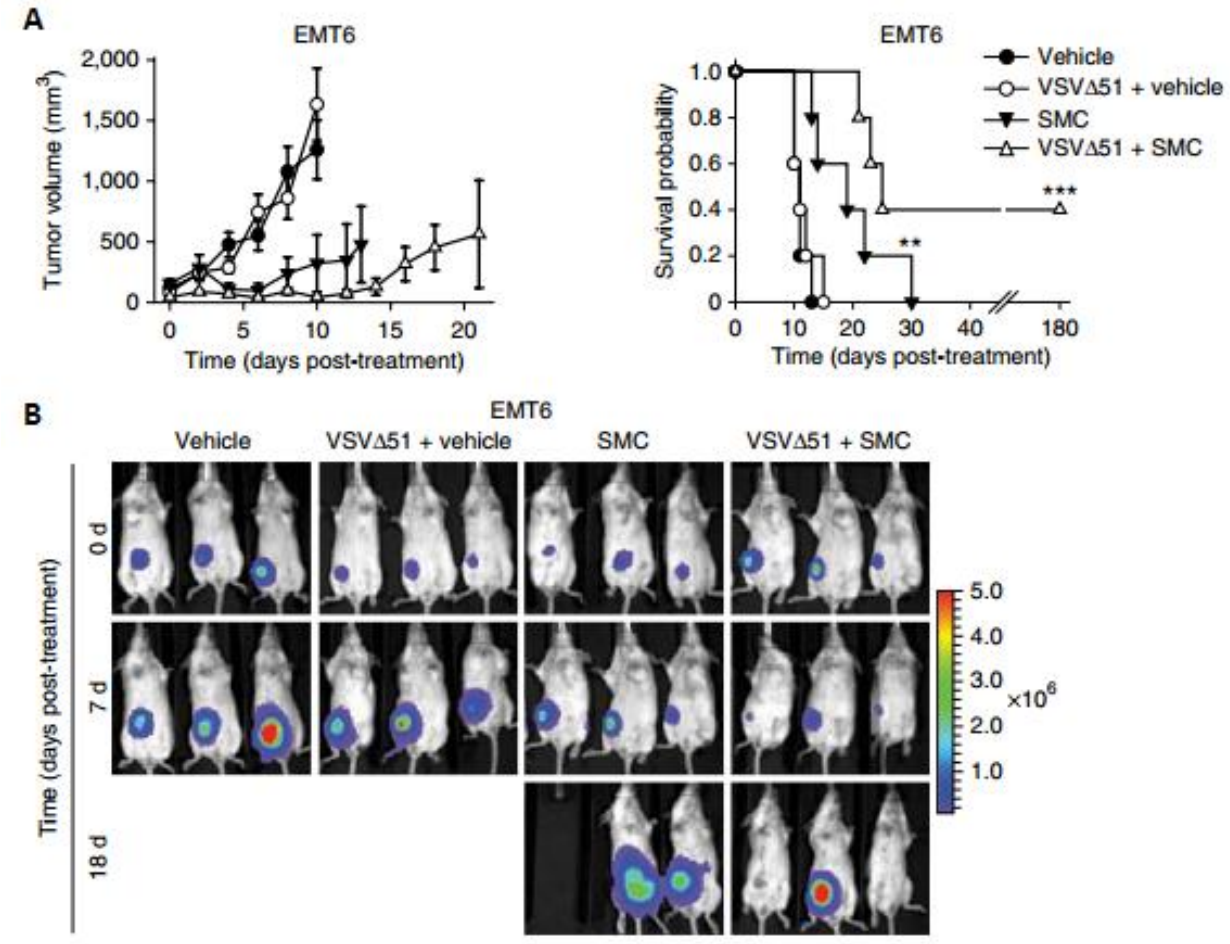

Figure 3-10. Combination VSVΔ51 and SMC treatment is efficacious *in vivo*. (A) EMT6 mouse model survival and growth rates. Mice bearing ~100mm^3 EMT6-Fluc tumors were treated with vehicle or 50 mg/kg of SMC (gavage) and 5 x 10^8 PFU/mL of VSVΔ51 (i.v.). Left panel depicts tumour growth, right panel represents the Kaplan-Meier curve depicting mouse survival. n = 5 per group. Log-rank with Holm-Sidak multiple comparison: *, $P < 0.01$, ***, $P < 0.001$ **(B)** IVIS images of representative EMT6-Fluc-bearing mice described in **A**. Scale: p/sec/cm2/sr *(Beug et al., Nature Biotechnology, 2014).*

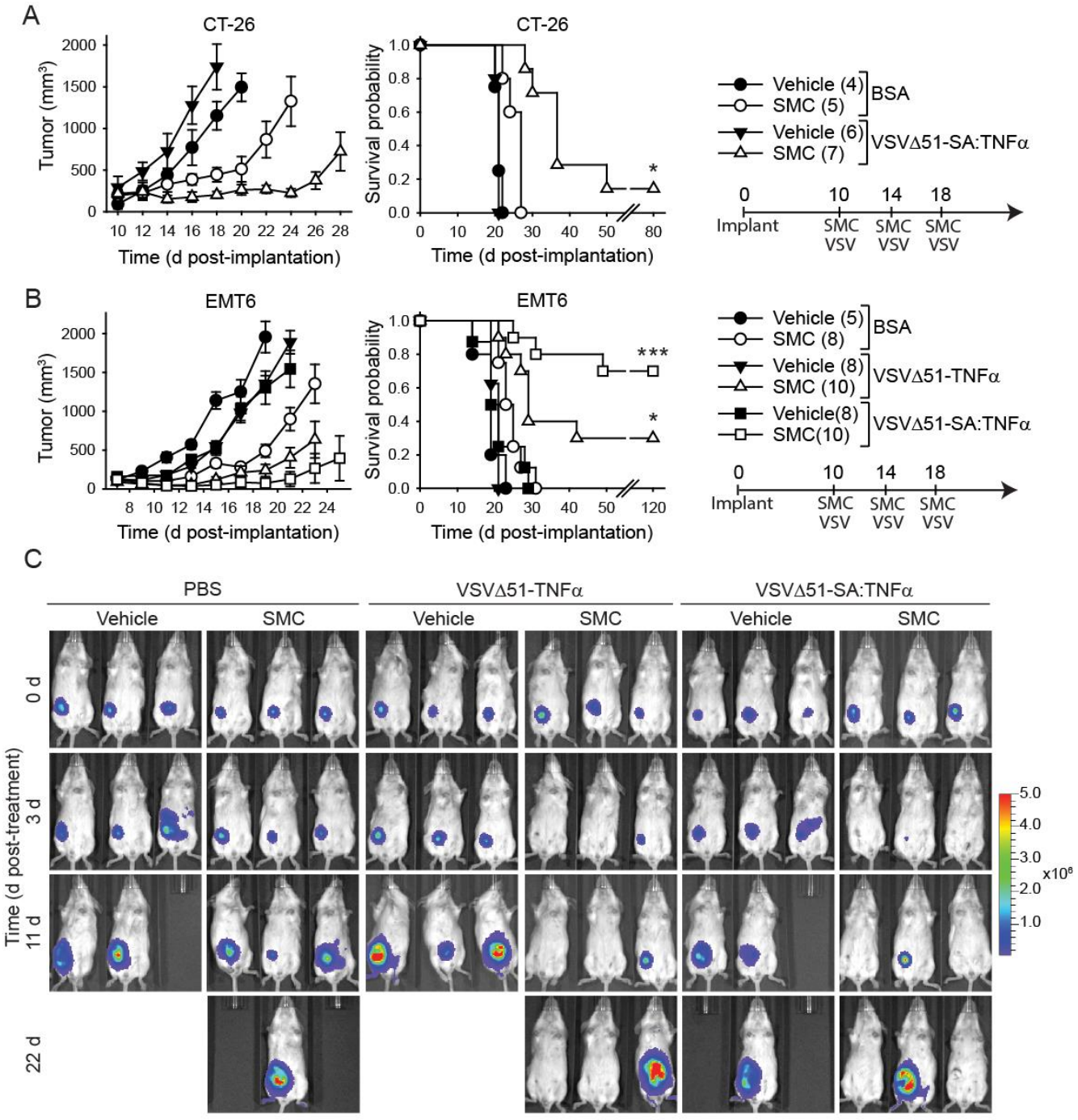

Figure 3-11. VSVΔ51-TNFα and VSVΔ51-SA:TNFα potently synergize with SMC in vivo (A) CT-26 model survival and growth rates. Mice bearing ~100mm^3 CT-26 tumors were treated with vehicle or 50 mg/kg of SMC (gavage) and 1 x 10^8 pfu/mL of VSVΔ51-SA:TNFα (i.t.). Left panel depicts tumour growth, right panel represents the Kaplan-Meier curve depicting mouse survival. Log-rank with Holm-Sidak multiple comparison: *, $P < 0.05$. **(B)** EMT6 model survival and growth rates. Mice bearing ~100mm^3 EMT6-Fluc tumors were treated with vehicle or 50 mg/kg of SMC (gavage) and 1 x 10^8 pfu/mL of VSVΔ51-TNFα and VSVΔ51-SA:TNFα (i.t.). Left panel depicts tumour growth, right panel represents the Kaplan-Meier curve depicting mouse survival. Log-rank with Holm-Sidak multiple comparison: *, $P < 0.05$, ***, $P < 0.001$. Number of mice per treatment group is displayed in brackets. **(C)** IVIS images of representative EMT6-Fluc-bearing mice described in B. Scale: p/sec/cm2/sr.

3.6.1 VSVΔ51-TNFα and SMC treatment induces tumour vasculature collapse

To determine whether treatment could successfully disrupt the tumour vasculature, EMT6 tumour-bearing BALB/c mice were injected with fluorescent microspheres into the tail vein that is used to observe the blood flow through the tumours. A lack of bead perfusion indicates tumour vascular collapse (Figure 3-12a). Surprisingly, while the single treatment of VSVΔ51-TNFα was not very efficacious, SMC alone reduced perfusion through the tumours and almost complete tumour vasculature collapse was observed with the double treatment. The degree of vasculature shutdown was quantified using ImageJ based on the presence of microspheres that had perfused through the tumour area (Figure 3-12b). Additional tumour sections are shown in Figure 3-13.

3.6.2 Double treatment enhances caspase-3 mediated death of neovasculature

To confirm that the occurrence of tumour collapse could be correlated with the induction of apoptosis in targeted neovasculature, immunohistochemical analysis of tumour sections stained for cleaved caspase-3 was performed (Figure 3-12c). In parallel to the results obtained with the perfusion experiments, the untreated tumour sections showed no incidence of activated caspase-3. Tumours treated with virus alone showed some staining for cleaved caspase-3, more so in the SMC alone treated samples, and an abundant amount of staining was found in doubly treated tumours. The positive caspase-3 pixels were quantified using ImageJ (Figure 3-12d). These results go hand-in-hand with the bead perfusion patterns observed in Figure 3-12a, suggesting that the disruption of the neovasculature is induced via apoptosis. To confirm that the endothelial cells of the vasculature are targeted by this treatment, I next looked at the staining patterns of CD31 positive cells following treatment.

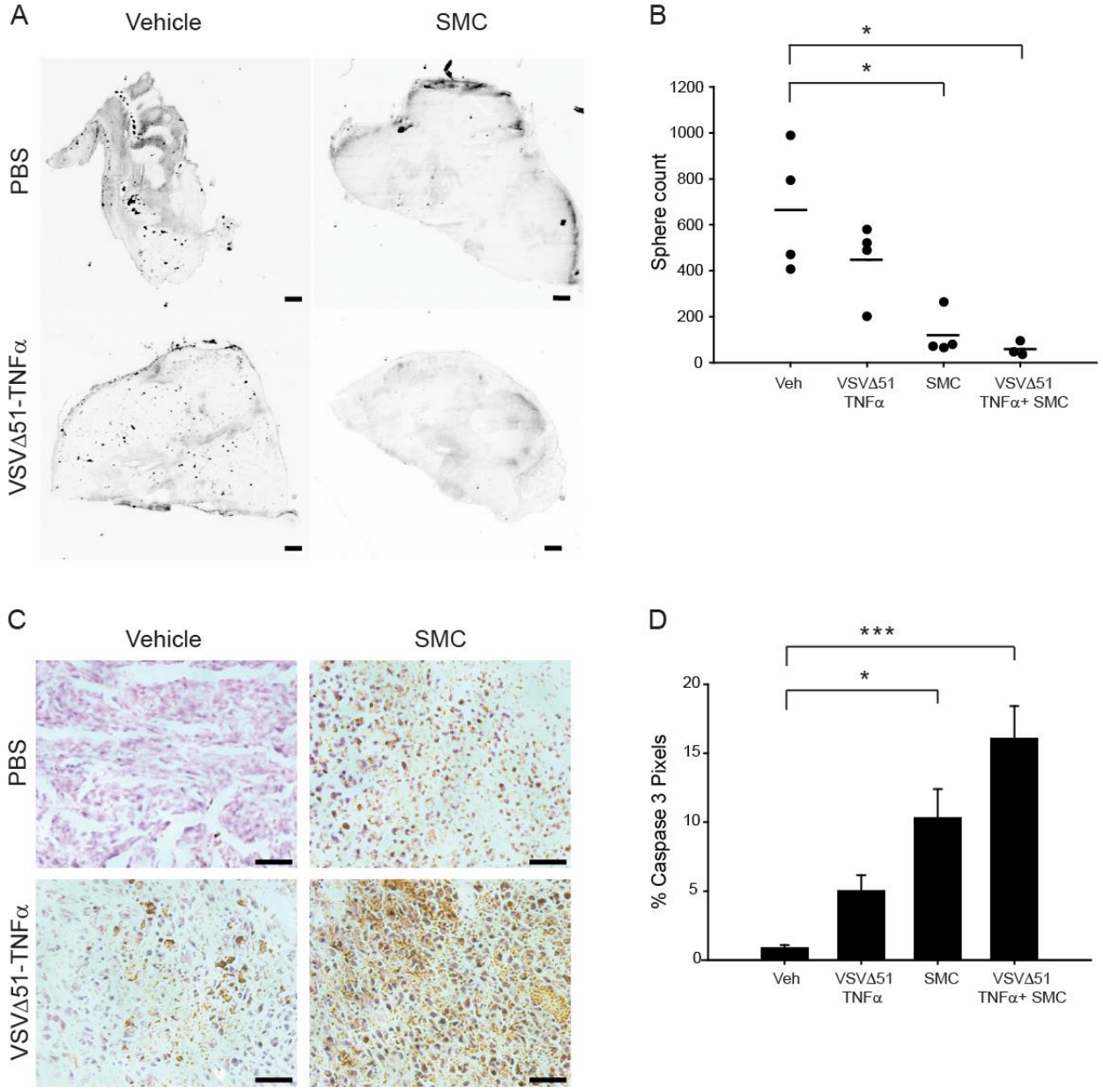

Figure 3-12. Combination treatment of SMC and TNFα-armed VSV induces vasculature shutdown and tumour cell death. (A) Analysis of perfusion through tumour vasculature. EMT6-bearing mice were treated with 50 mg/kg of SMC (gavage) and 1 x 10^8 pfu/mL of VSVΔ51-TNFα (i.t.) for 24h, then were injected with fluorescent microspheres (i.v.) and then euthanized 5 minutes later. Degree of vasculature shutdown was assessed by presence of microspheres (black dots) using the Agilent Technologies DNA microarray scanner. Scale bars, 500µm. **(B)** Quantification of perfused microspheres described in A (mean + SD, n = 4). One way ANOVA: *, $P < 0.05$. **(C)** Immunohistochemistry of tumour sections from A stained for cleaved caspase-3. Apoptotic cells are marked by the DAB (brown) reagent and a counterstain was performed using hematoxylin. Scale bars, 50µm. **(D)** Quantification of activated caspase-3 from C. One way ANOVA: *, $P < 0.05$, ***, $P < 0.001$.

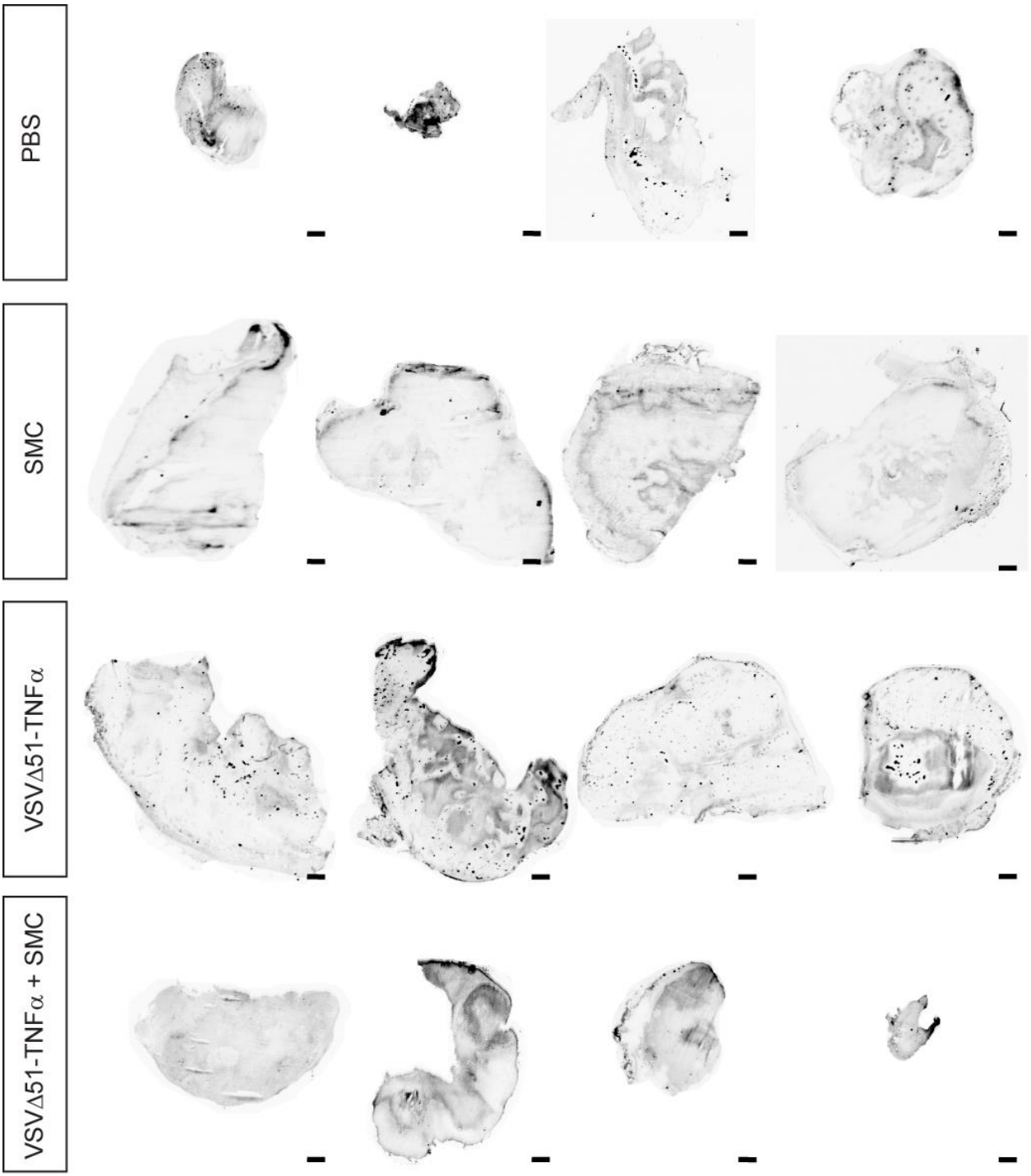

Figure 3-13. Combination treatment induces vasculature shutdown. Analysis of perfusion through tumour vasculature. EMT6-bearing mice were treated with 50 mg/kg of SMC (gavage) and 1 x 10^8 pfu/mL of VSVΔ51-TNFα (i.t.) for 24h, then were injected with fluorescent microspheres (i.v.) and then euthanized 5 minutes later. Degree of vasculature shutdown was assessed by presence of microspheres (black dots) using the Agilent Technologies DNA microarray scanner. Scale bars, 500µm.

3.6.3 VSV constructs target endothelial walls of neovasculature

To determine whether the endothelial cells of the tumour neovasculature were being targeted with SMC and VSV treatment, I stained the tumour sections for the endothelial cell marker, CD31 (Figure 3-14). Untreated and single treatment with VSV showed that the vasculature was still intact within the tumour, whereas SMC and combination treatment showed the endothelial cells near the perimeter of the tumour. This coincides with the occurrence of vasculature collapse observed in the SMC and doubly treated tumours (Figure 3-12a and 3-13).

Together these experiments demonstrate the ability of VSVΔ51-TNFα and SMC combination treatment to target the tumour neovasculature and deprive metastatic cells of oxygen required to survive, eventually leading to their death. This added insight will aid in further development and optimization of combination therapies.

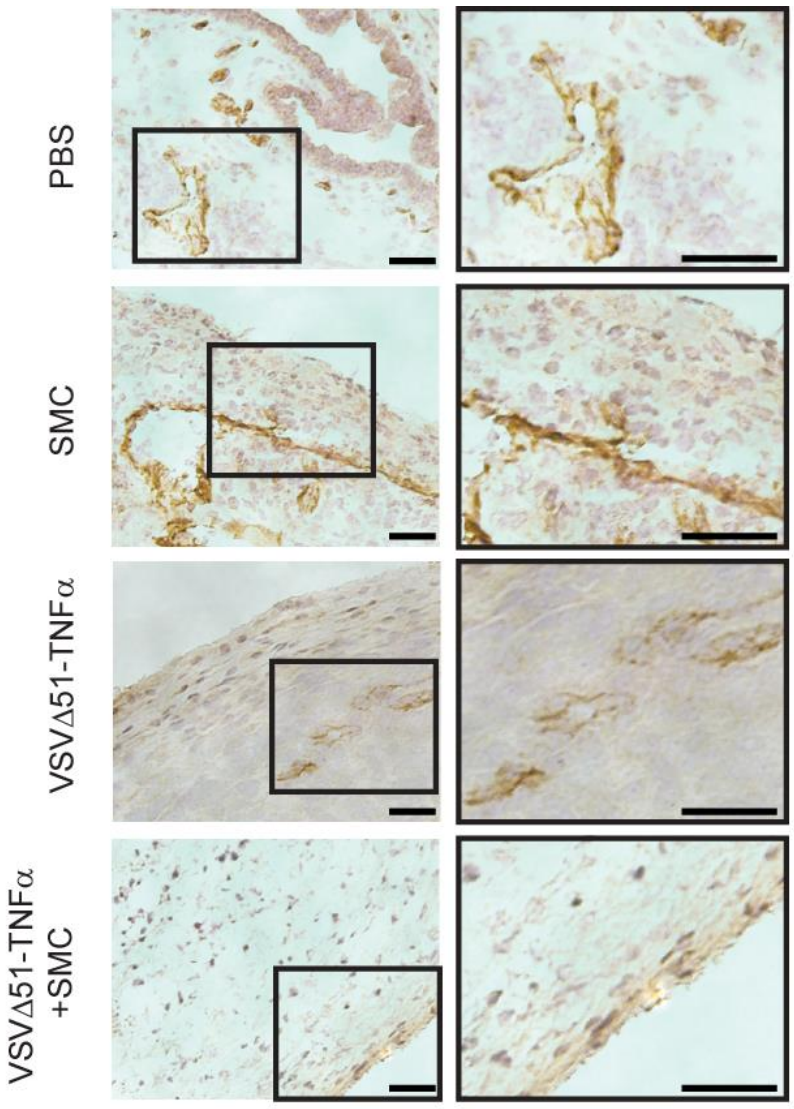

Figure 3-14. Combination treatment disrupts endothelial walls of developing neovasculature.
EMT6-bearing mice were treated with 50 mg/kg of SMC (gavage) and 1 x 10^8 pfu/mL of VSVΔ51-TNFα (i.t.) for 24h. Tumour sections were fixed and then stained with the CD31 endothelial cell marker. Endothelial cells are marked by the DAB (brown) reagent and a counterstain was performed using hematoxylin. Scale bars, 50µm.

4.0 DISCUSSION

4.1 The promise of IAP antagonists

IAP antagonists, such as SMCs, have demonstrated significant therapeutic benefits in preclinical tumour models and have therefore proved to be a very efficacious approach used to target and inhibit cancer (58-60). Given that the IAPs are upregulated in inflammatory disorders, and that cell viability is shifted to a pro-survival state, IAP antagonists have been designed to target and inhibit the IAPs in order to reinstate cell homeostasis. Here, I have shown that the therapeutic anti-cancer effects of the SMC LCL161 are enhanced when combined with an OV that expresses human TNFα.

4.1.1 Targeting the IAPs

The IAPs promote cell survival through the modulation of the classical NF-κB pathway and through the inhibition of caspase activity. In fact, in the absence of the IAPs, cancer cells are sensitized to cell death mediated by TNFα signalling (20, 21). This has sparked the exploration of methods that aim to repress IAP expression in order to promote cell death for the treatment of cancer.

Elucidating the function of the intrinsic inhibitor of the IAPs, Smac, has been crucial for understanding the function of the IAPs. Upon apoptotic stimuli, Smac is released from the mitochondria and then forms a stable protein dimer with the BIR motifs of the IAPs. This inhibits IAP-caspase interactions through steric hindrance, allowing for the caspases to freely promote apoptosis (51). Small molecular inhibitors of IAPs (SMCs) have mimicked this property and have proved to be highly efficacious in potentiating immunotherapies for cancer.

4.1.2 SMCs as potent IAP antagonists

One of the earliest attempts to antagonize the IAPs involved the assessment of Smac peptides, which were designed based on the AVPI motif of the Smac protein. The use of Smac peptides were found to increase the anti-cancer efficacy of chemotherapeutics (47, 48). However, Smac peptides were prone to degradation in these applications. To improve upon the specificity of this form of treatment, small molecule SMCs were designed to mimic the ability of the Smac protein to bind both the BIR2 and BIR3 motifs of the IAPs. This binding can promote the autoubiquitination of the cIAPs, switching the pro-survival response to a death response in cancer cells in the presence of TNFα. SMCs are currently undergoing clinical trials, and show promise given that the approach has the potential to be more specific and less toxic than radiation and chemotherapies (52, 53).

Previous studies have demonstrated that SMCs synergize with pro-inflammatory cytokines such as TNFα and TRAIL, thereby showing that the loss of the IAPs allows for cancer cells to respond to death-ligand signalling (68). Additionally, SMCs such as LCL161 are particularly immunomodulatory, and their performance is improved when combined with different innate immune stimuli including the mimetics of microbial RNA and DNA, poly(I:C) and CpG, and OVs (68).

4.2 The promise of oncolytic virus immunotherapy

Combination immunotherapies are advancing rapidly, effectively replacing less efficacious monotherapies (121). Recently, more studies have shifted to focus on immunogenic treatments that function by exploiting the host immune response, in order to work against drug-resistant cancers. Following their discovery in the 1950s, OVs have demonstrated intensifying anti-tumour activities, and have shown tolerable toxicities in treated patients (72). OVs have

since been engineered to elicit increased specificity and efficacy, in order to target cancer cells while reducing adverse effects to normal cells. The ability to manipulate OVs to express immune-stimulating proteins, induce immunogenic tumour cell death and selectively target tumour beds has opened new possibilities to enhance the anti-tumour immune response.

4.2.1 Engineering OVs as therapeutic tools for cancer treatment

The stimulation of a host's immune system can induce a robust T-cell response, thereby boosting anti-tumour immunity and inhibiting metastasis (79). Hence, given the promising possibilities, the development of combination immunotherapies with OVs continues to be explored in order to exploit this effect.

The use of VSV- or adenoviral- mediated secretion of GM-CSF for treatment of malignancies has shown promise in mouse models and phase I clinical trials, respectively (90, 122). Multiple efforts have been carried out to improve the potency of VSV using constructs that express inflammatory cytokines such as IFNβ and IL-15; however, these agents have proven to be limited in their efficacy as monotherapies (83-85, 89, 93). Preclinical studies have verified the safety and efficacy of IFNβ-expressing VSV in squamous cell and mesothelioma cell lines (86, 87, 123). Similarly, intratumoural injections of VSV-IFNβ have proven to be safe in toxicology studies performed in rats and rhesus monkeys (88). Moreover, systemically administered VSV-IFNβ prolonged survival in mice bearing orthotopic myeloma (124). To our knowledge, there is no previously reported data on VSVs engineered to secret TNFα. However, one recent study demonstrated that TNFα-armed adenovirus can elicit a strong immunogenic response and mediate apoptosis in tumours (107). Similarly, it has been shown that TNFα-expressing adeno-associated virus bacteriophage synergizes with LCL161, yielding prolonged survival in

melanoma models (108). New studies are moving toward combination treatments that aim to be more effective than single-agent therapies.

4.2.2 OV and SMC combination therapy for cancer

We have shown that VSVΔ51, in comparison to other OVs, is most efficacious in combination with SMC, in part because of its ability to induce a more robust release of cytokines (68). Other tested OVs including the herpes simplex virus, vaccinia and wild-type VSV, have been shown to be less efficacious, a property possibly due to the increased ability of these viruses to suppress innate immune signalling. The fact that VSVΔ51 is unable to antagonize a host's IFNβ-mediated antiviral response may give it an advantage over the latter three viruses, as it is not only able to infect cancer cells, but can also activate an immune response in adjacent cells following infection. These features further contribute to the efficacy of the treatment through potentiating SMC-mediated cell death. In addition, VSVΔ51 has been shown to target and infect a large range of cell types, including different types of cancer cells (125). Given that we have successfully treated cancer models with the VSVΔ51 and SMC combination treatment (which is dependent on the presence of TNFα or TRAIL) I hypothesized that VSVΔ51 expressing TNFα could further enhance SMC treatment. As discussed previously, LCL161 was selected for these studies, as it is immunomodulatory in itself, and can be delivered orally, making it an accessible and efficacious small molecular therapeutic that can be used safely in combination with VSVΔ51 constructs.

4.3 TNFα-armed VSV improve specificity and efficacy of SMC treatment

Few examples of TNFα-expressing OVs have been reported likely because this inflammatory cytokine has demonstrated considerable toxicity in cancer patients (101, 102). However, this negative effect could possibly be overcome by the use of more direct methods to

deliver endogenous TNFα to the tumour bed. Here I have shown that VSVΔ51 that secretes TNFα potently synergizes with SMC, leading to safe, durable cures in refractory murine tumour models (Figure 3-11). But whether or not this effect is due to the localized delivery of TNFα remains to be established. Regardless, these findings demonstrate a new and potent approach to enhancing the efficacy of SMC therapeutics for cancer treatment.

4.3.1 TNFα-armed VSV synergize with SMC

I successfully generated two constructs, VSVΔ51-TNFα and VSVΔ51-SA:TNFα, of which the latter expresses high protein levels of TNFα in the supernatant from infected cells (Figure 3-1 and 3-2). The use of SMC in combination with the recombinant viruses had no negative effects on the viral kinetics, antiviral response, or expression of the human transgenes (Figure 3-3, 3-4 and 3-5), confirming that the combination could be used to its full therapeutic potential. Initial *in vitro* experiments demonstrated that the TNFα-expressing constructs are superior to VSVΔ51-GFP in potentiating SMC-mediated cell death (Figure 3-6 and 3-7). Virus alone was not particularly effective, however, there was a noticeable increase in cell death overtime following infection with VSVΔ51-SA:TNFα (Figure 3-7). In addition, the release of death-inducing factors, including TNFα, upon viral infection is greatly enhanced, given that a larger spread of bystander cell death was observed in doubly treated refractory cancer cell lines (Figure 3-8). This is an exciting discovery that suggests that the treatment is not limited to the cancer cells infected by virus, but, in fact, the infection of one cell could potentially induce the killing of a larger area of the tumour bed. Interestingly, unlike the infection of cells subjected to TNF-R1 and DR5 knockdown with VSVΔ51-GFP, cells infected with the TNFα-armed constructs still showed synergy with SMC (Figure 3-9a). This suggests that the recombinant TNFα-expressing viruses are triggering additional methods of killing through signalling cascades

that are independent of TNFα expression. This unexpected result may pose as an advantage, as the recombinant viruses are triggering alternative methods of cancer cell death that could be explored in further detail. There is a possibility that the viral infection, for example, is also promoting cell death via necroptosis through TRAIL-mediated signalling pathways induced by pathophysiological processes (126).

The combination of SMC with VSVΔ51-SA:TNFα proved to have the highest therapeutic outcome as 70% durable cures were observed in treated animal models (Figure 3-11). Other studies have shown that mice treated with a TNFα-armed oncolytic adenovirus (performed as a stand-alone treatment) yielded a 40% cure rate (107). Similarly, an approach that involved the administration of an adeno-associated virus bacteriophage-tumour TNFα with the SMC, LCL161, which had effectively cured half of the treated models (108). Another related study focused on the self-seeding of TNFα-releasing cancer cells, which successfully increased tumour regression (105). Finally, given that the VSVΔ51-TNFα and VSVΔ51-SA:TNFα constructs are effectively producing high levels of TNFα, the double treatment with SMC is almost twice as effective as what we have previously shown, with five times less virus being administered (Figure 3-10) (68). Together this data confirms that the combination immunotherapy of TNFα-armed VSV with SMC is a highly effective treatment, and has shown promising results.

4.3.2 Overcoming the toxicity of TNFα

Although TNFα is known for its anti-cancer properties, its use as a therapeutic has mostly been avoided due to its systemic toxicity. It has since been determined that the local delivery of TNFα to cancer cells can overcome this toxicity, using vectors such as OVs. From these studies I was able to determine that the release of TNFα does not remain in the host, rather, the cytokine is effectively removed from the system hours post-treatment (Figure 3-2). This indicates that the

viruses are releasing bursts of TNFα long enough to induce SMC-mediated cell death and, in a self-regulating manner, the virus particles are cleared, or at least neutralized, by the induced host immune response. The virus is able to replicate and express TNFα in order to enhance SMC treatment, then over time the viral attenuation allows for it to be removed, giving the host a chance to recover. This approach is broadly useful in that it is able to selectively infect cancer cells, elicit a highly concentrated release of TNFα, and enhance SMC-mediated killing of infected and bystander cells. Due to the circulation of diffusible TNFα, cancer cells do not necessarily need to be infected by virus to be killed (Figure 3-8). The combination approach also proved to be well tolerated in mice, leading to durable cures (Figure 3-11) and was found to be resistant to the re-challenge of cancer cells (Beug *et al*., unpublished data). This demonstrates the treatment's ability to induce long-term anti-tumour immunity of the host.

While the use of an immunostimulatory OV is necessary for innate immune activation, using a replicating virus for the treatment of cancer could also pose a threat to a patient's health. In a recent phase I clinical trial, a patient passed away from liver and kidney failure induced by viremia and a cytokine storm (consisting primarily of IFNβ and TNFα) following treatment with the dose of $1.8x10^7$ PFU/ml VSV-hIFNβ (127, 128). The study claimed that the viremia ensued following rapid replication of the virus after direct injection into a large tumour mass in the liver. Although the viral expression of IFNβ was intended as a safeguard, the levels of IFNβ produced following the growth of the virus in the tumours was too detrimental to the patient, as it also induced the release of high levels of TNFα, leading to an inadvertent toxic and lethal effect. While the attenuated VSVΔ51-TNFα constructs used in these studies are intended to primarily infect cancer cells, with the expression of TNFα only occurring over the course of a few hours, there are extra safeguards that can be established during a clinical trial. Namely, TNFα

neutralizing or TNF-R1 blocking antibodies clinically used for autoimmune disease therapy such as rheumatoid arthritis, inflammatory bowel disease and psoriasis could help alleviate TNFα toxicities when treating a patient if the need arises (129). All in all, the specificity and efficacy of this technique poses a therapeutic benefit that could potentially tackle issues of drug-resistance and cancer cell evasion of the host immune response, while doing so in a safe and efficient manner.

4.4 Shutting down the tumour vasculature

Other mechanisms of inducing cancer cell death have been explored. Given that angiogenesis is dependent on the delivery of oxygen to the growing tumour through its blood supply, studies have focused on targeting the development of neovasculature. Hence, the induction of tumour vascular endothelial cell death, or tumour vasculature collapse, poses another avenue for inhibiting tumour growth.

The use of IAP antagonists has been shown to sensitize tumour endothelial cells to TNFR-mediated killing. Interestingly, other studies have shown the ability of engineered OVs to target tumour vasculature (77). Furthermore, given the higher expression of TNFR in cancer cells, the neovasculature of developing tumours is expected to be more responsive to TNFα-mediated killing compared to normal blood vessels (130). Based on these properties, I hypothesized that, in addition to the combination treatment inducing cytotoxicity in tumour cells, the viral delivery of TNFα following IAP antagonism could inhibit tumour growth through disruption of the tumour vasculature.

4.4.1 Targeting the tumour vasculature

Previous work has shown that tumour vasculature collapse can be induced by IAP antagonism with a SMC via TNFα-mediated endothelial cell apoptosis (57). Interestingly, I

found that EMT6 tumours treated with SMC alone induced significant tumour vasculature collapse (Figure 3-12 and 3-13). This can be attributed to the possibility that LCL161 in itself can induce the release of TNFα, and that systemic TNFα levels can concentrate in the tumour microenvironment (57). Unexpectedly, single treatment with VSVΔ51-TNFα induced a lesser degree of death on its own, indicating that the antagonism of the IAPs using the addition of SMC is necessary for the cells to be sensitized to TNFα induced death. This is contrary to a study that claimed that VSVΔ51 alone has the ability to induce vasculature shutdown in CT-26 tumours (77). Nonetheless, combination treatment of VSVΔ51-TNFα and SMC did induce a higher degree of vasculature collapse compared to SMC alone, illustrating that the viral delivery of TNFα does enhance the SMC-dependent killing of the neovasculature (Figure 3-12 and 3-13). The synergism of VSVΔ51-TNFα with SMC can also be attributed to the fact that TNFα has been shown to increase the vasculature permeability of microvascular endothelial cells, which could potentially enhance the penetration of LCL161 to the tumour tissue (110).

The inhibition of tumour growth can also be explained by the IAP antagonization specifically targeting the tumour blood supply (57). Not only does TNFα have the ability to induce apoptosis through TNF-R1 signalling, but it can also induce the secretion of blood clotting and adhesion proteins in vascular endothelium, resulting in vascular necrosis at the tumour tissue (130). Therefore, the induction of tumour vasculature collapse can also be attributed to the high sensitivity of tumour vasculature endothelial cells to TNFα upon IAP antagonism. In fact, it is possible that the targeting and killing of a single endothelial cell can induce the death of a larger surrounding area of the tumour (105). Consistent with this concept, I observed that the CD31 positive-stained vascular endothelial cells were limited to the periphery

of the tumour mass, implying that the vasculature inside the tumour was no longer intact (Figure 3-14).

4.4.2 The significance of vasculature collapse

From these studies I have shown that TNFα and SMC treatment induces the apoptosis of tumour tissues, and can disrupt the tumour vascular endothelium. This effect could potentially decrease the occurrence of metastasis by targeting the tumour at its source, thereby inducing cell death over large sections of the tumour. Taken together, the combination treatment effectively induces vasculature shutdown, a mechanism that can be exploited for the design of dual therapies that can be applied in future clinical trials.

4.5 The drawbacks of this TNFα-armed OV/SMC combination approach

While the experiments of this project pose some interesting and promising discoveries, there are still some limitations that need to be addressed. One such concern is that the single treatment with the TNFα-armed viruses or with SMC did not yield durable cures in the animal models (Figure 3-11). Despite the fact that SMC treatment alone induces a significant degree of vasculature collapse in treated tumours (Figure 3-12 and 3-13), this mechanism alone is not sufficient enough to induce sustained tumour regression (Figure 3-11). This suggests that the combination of the two agents is necessary in order for the treatment to be completely effective.

Given that TNFα has displayed different levels of species-specific toxicities, studies performed with hTNFα expressed in mice could underestimate the potential toxicities in humans (131). However, I have shown that that the systemic spread of virally derived hTNFα from the attenuated constructs is not sustained (Figure 3-2). Further studies would need to be performed to investigate this effect in tumour bearing mice. In addition, the local injection of the TNFα-armed viruses to the tumour bed may limit the systemic spread of the virus and reduce toxicity.

The use of OVs for cancer therapy continues to be optimized, in order to circumvent possible toxicities. The generation of novel engineered OVs that preferentially infect cancer cells have been developed in order to overcome this concern, yielding some promising results. Although there are concerns over the toxicity of OVs as therapeutic tools, their use as vectors for the local delivery of TNFα to cancer cells is still proof that SMC efficacy can be potentiated in combination with immune stimulants.

4.6 Conclusions

The findings presented here clearly demonstrate that, in combination with SMC treatment, TNFα-armed VSVΔ51 are superior to VSVΔ51 and VSVΔ51-GFP in the killing of cancer cells. Five times less virus was used to achieve an improved cure responses when compared to *in vivo* experiments performed with VSVΔ51 and SMC (68). In addition, I have shown that VSVΔ51-TNFα and SMC combination treatment targets tumour vasculature.

In summary, I report the successful generation of novel, TNFα-secreting viruses, VSVΔ51- and VSVΔ51-SA:TNFα, which have proven to be superior to VSVΔ51 at inducing SMC- mediated cancer cell death. Both TNFα viruses were shown to have similar potency *in vitro*; however, the inclusion of the secretory sequence in VSVΔ51-SA:TNFα induced a stronger response to SMC treatment *in vivo* compared to VSVΔ51-TNFα. This approach was found to be safe and efficacious in aggressive murine tumour models. Although the attenuated VSVΔ51 is less likely to induce uncontrolled viremia, its infection and lysis of cancer cells releases tumour antigens and DAMPs that trigger the host immune response, leading to tumour eradication (72). In addition, the disruption of the tumour vasculature and the deterioration of the tumour bed can further sensitize cancer to apoptosis. Together, this data demonstrates that the therapeutic

efficacy of SMC is greatly enhanced when used in combination with the endogenous delivery of TNFα to the tumour bed.

Future work could focus on elucidating which immune cell populations play a role in the combination SMC and VSVΔ51-TNFα therapy. We have previously documented that the depletion of macrophages by clodrosome (clodronate liposome) treatment completely abrogated SMC and cytokine co-therapy (Beauregard *et al*., unpublished data). Further studies are necessary to understand the contribution of macrophages, and other immune cells, in the mechanism of this combination approach to treat cancer. Furthermore, it has been speculated that SMC treatment could lead to the activation of the adaptive immune response following the killing of tumour cells and recruitment of innate immune cells (120).

The use of check-point inhibitors such as anti PD-L1/PD-1 blockades could also be studied in combination with OV and SMC therapy to assess whether this can enhance the anti-tumour T-cell response. In fact, the use of oncolytic reovirus in combination with anti PD-1 blockade has proved to prolong survival of mice compared to the single-agent therapy approaches (132). The immune activation of the OV combined with the PD-1 blockade successfully enhanced the T-cell-dependent anti-tumour response, while improving the antiviral response. This could further potentiate the SMC and OV combination immunotherapy through promoting the adaptive anti-tumour immune response.

With the promise of SMCs and growing advantages of OVs as therapeutic agents, a more efficacious and selective approach to targeting cancer can be achieved (see model depicted in Figure 4-1). Preclinical studies indicate that the combinatorial use of SMCs with immune stimulants may be more efficacious than single agent approaches (70). Given the results

produced in my thesis project, I believe that SMC and TNFα-armed OVs could potentially be used as a new, highly efficacious form of combination therapy for cancer treatment.

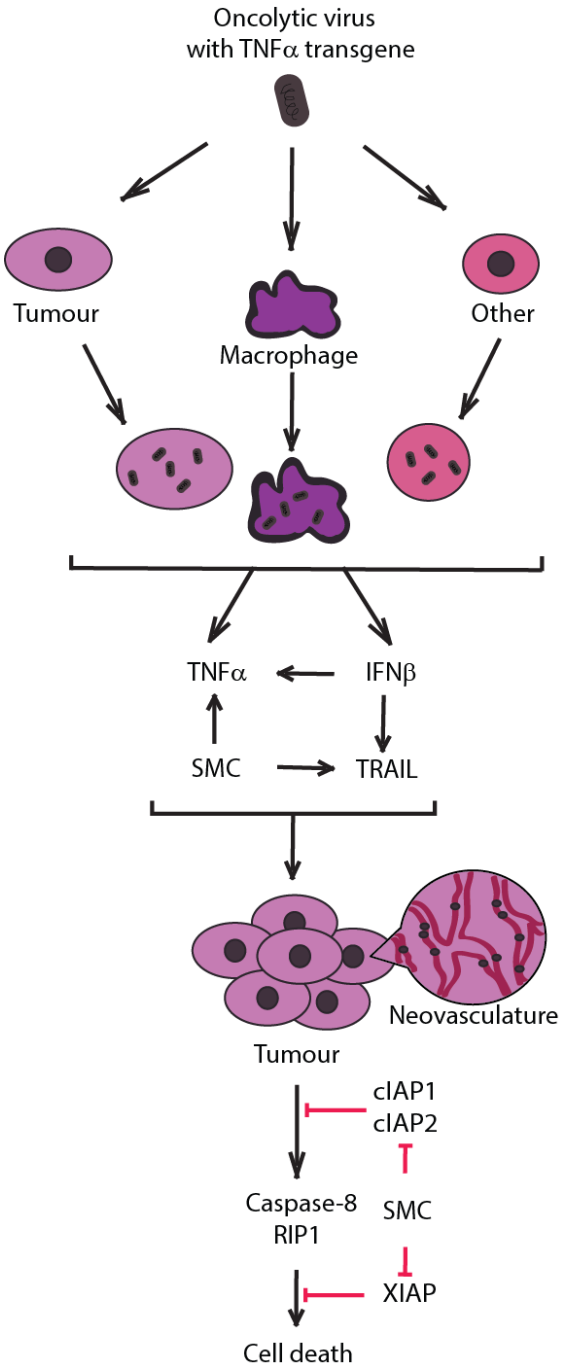

Figure 4-1. Schematic representation of SMC and oncolytic virus combination therapy. Following infection of a cell with an OV that encodes the hTNFα transgene, the over-expression of hTNFα combined with the IFN-mediated antiviral response induces the indirect release of a range of endogenous inflammatory cytokines, including mTNFα and TRAIL. This response synergizes with SMC to induce a dual mechanism of tumour killing through caspase-8-mediated cancer cell death with IAP antagonism (as shown in Figure 1-4) and neovasculature collapse. The overall efficacy of OV and SMC combination immunotherapy is dependent on the presence of the proinflammatory cytokines. (Modified from *Beug, LaCasse & Korneluk, OncoImmunology, 2014*).

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