TYPE I INTERFERON-MEDIATED KILLING OF CANCER CELLS WITH IAP-TARGETED COMBINATION IMMUNOTHERAPY

Caroline Beauregard

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Department of Biochemistry, Microbiology and Immunology
Faculty of Medicine
University of Ottawa

ABSTRACT

SMAC mimetic compounds (SMCs) are small molecule antagonists of the Inhibitor of Apoptosis (IAP) family of proteins. Binding of SMCs to the IAPs results in the sensitization of cancer cells to apoptosis in the presence of death ligands, such as tumour necrosis factor alpha (TNFα). I hypothesize that type I interferon (IFN) stimulation in cancer cells and in immune cells leads to the production of TNFα, which can then synergize with SMCs to kill cancer cells. The combined treatment of SMC and IFNα induces tumour regression in mice, and this effect is completely abrogated upon treatment with TNFα-neutralizing antibody. The synergistic effects are mediated by tumour cells and by contribution of immune cells, particularly macrophages and dendritic cells, as the systemic depletion of phagocytic innate immune cells results in an increase in tumour volume following combination treatment. The characterization of immune cell contribution will aid in the translation of the SMC combination therapy into clinical applications for the treatment of cancer.

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To Patrick, who has never been anything but supportive, patient and kind, thank you. To my parents, who, in the end, really made this all possible – I can't say thank you enough.

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LIST OF ABBREVIATIONS

2-ME	β-mercaptoethanol
ACK	Ammonium-chloride-potassium
APC	Antigen-presenting cell
BIR	Baculovirus IAP repeat
BMDM	Bone marrow-derived macrophage
BSA	Bovine serum albumin
cIAP1/2	Cellular inhibitor of apoptosis-1/2
CARD	Caspase recruitment domain
CD40	Cluster of differentiation 40
CD40L	Cluster of differentiation 40 ligand
CpG-ODN	CpG-containing deoxyoligonucleotide
CSF-1R	Colony stimulating factor 1 receptor
DC	Dendritic cell
DIABLO	Diablo homolog
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
FADD	Fas-associated protein with death domain
FBS	Fetal bovine serum
Fluc	Firefly luciferase
G	Glycoprotein
HOIL-1	Heme-oxidized IRP2 ubiquitin ligase 1
HOIP	HOIL-1 interacting protein
IAP	Inhibitors of apoptosis

IFN	Interferon
IFNAR	Interferon alpha/beta receptor
IKKα/β	Inhibitor of nuclear factor kappa-B alpha/beta
IKKB	Inhibitor of nuclear factor kappa-B kinase subunit B
IL	Interleukin
ILP2	IAP-like protein 2
IP	Intraperitoneal
IPS-1	Interferon-beta promoter stimulator 1
IRF	Interferon regulatory factor
ISG	Interferon-stimulated gene
ISGF3	Interferon-stimulated gene factor 3
ISRE	Interferon-stimulated response elements
IT	Intratumoural
IV	Intravenous
JAK1	Janus kinase 1
LLC	Lewis lung carcinoma
LPS	Lipopolysaccharide
LUBAC	Linear ubiquitin chain assembly complex
M	Matrix
MAVS	Mitochondrial antiviral signalling protein
M-CSF	macrophage colony stimulating factor
MDA5	Melanoma differentiation-associated gene 5
MEF	Mouse embryonic fibroblast
MLKL	Mixed lineage kinase domain-like protein

MyD88	Myeloid differentiation primary response gene 88
NAP-1	Nucleosome assembly protein-1
NEAS	Non-essential amino acids
NEMO	NF-κB essential modulator
NF-κB	Nuclear factor kappa B
NIAP	Neuronal apoptosis inhibitory protein
NIK	NF-κB inducing kinase
NIS	Sodium iodide symporter
NK	Natural killer
NT	Non-targeting
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PFU	Plaque-forming units
Poly(I:C)	Polyinosinic:polycytidylic acid
PRR	Pattern recognition receptor
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RelA/B	V-rel reticuloendotheliosis viral oncogene homolog A/B
RIG-1	Retinoic acid-inducible gene 1
RING	Really interesting new gene
RIP	Receptor-interacting protein
RLR	RIG-1-like receptor
RMPI	Roswell Park Memorial Institute
Sharpin	Shank-associated RH domain-interacting protein
siRNA	Small interfering RNA

SMAC	Second mitochondrial activator of caspases
SMC	Smac mimetic compound
STAT	Signal transducer and activator of transcription
TAB2/3	TGF-beta activated kinase 1/MAP3K7 binding protein 2/3
TAK1	Transforming growth factor-beta-activated kinase 1
TANK	TRAF family member-associated NF-kappa-B activator
TBK1	TANK-binding kinase 1
TBS-T	Tris-phosphate buffered saline with tween
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
TNFα	Tumour necrosis factor alpha
TNF-R1	Tumour necrosis factor receptor 1
TRADD	TNF receptor-1-associated death domain
TRAF	TNF receptor-associated factor
TRAIL	TNF-related apoptosis-inducing ligand
TRIF	TIR-domain-containing adapter-inducing interferon-beta
TYK2	Tyrosine kinase 2
UBA	Ubiquitin-associated domain
VSV	Vesicular stomatitis virus
WT	Wild-type
XIAP	X-linked inhibitor of apoptosis

1: INTRODUCTION

1.1: Introduction to the Inhibitors of Apoptosis (IAPs)

The Inhibitors of Apoptosis (IAP) family of proteins is defined by the presence of the baculovirus IAP repeat (BIR) domain. The IAPs are involved in many cellular processes including division, differentiation, proliferation, apoptosis and signal transduction (1). Three members of the family are of particular importance as established oncogenes: cellular IAP (cIAP) 1 and 2 and X-linked IAP (XIAP) (Figure 1). Other members of the IAP family exist and have more specialized roles in the cell. Survivin and BRUCE are involved primarily in mitosis while neuronal apoptosis inhibitory protein (NAIP) is involved in neuroprotection and inflammation. Livin has a role in development and IAP-like protein 2 (ILP2) might be involved in spermatogenesis.

The IAPs have been shown to have important, albeit complex roles in the progression of cancer. Mutations in IAP genes have been found to be implicated in certain types of cancer. For example, gene amplification of cIAP1/2 can lead to certain forms of carcinoma due to the enhanced inhibition of apoptosis that accompanies overexpression of these genes (2, 3). Similar effects can be seen with high expression of XIAP (4). The IAPs are promising targets for cancer therapies and the downregulation of these proteins may promote apoptosis of certain forms of cancer.

1.2: The IAPs and cell death

One of the most important roles of these proteins is their regulation of caspase activity. The BIR domains of the IAPs can directly or indirectly inhibit caspases. XIAP has been shown to directly bind caspase-3, -7 and -9 (5–7). Binding of the effector caspase-9 to the BIR3 domain of XIAP inhibits dimerization of caspase-9, thereby preventing processing

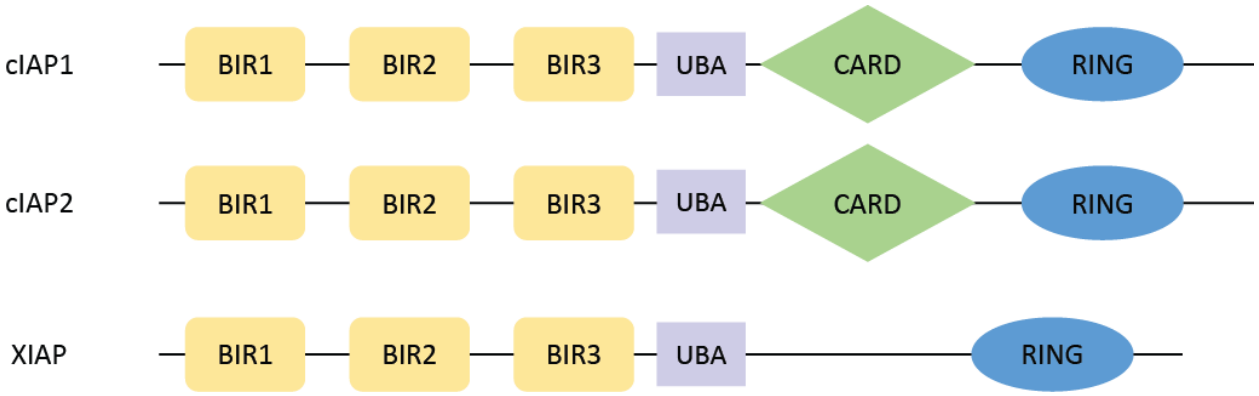


Figure 1. The Inhibitors of Apoptosis. Shown are cellular IAP1 and IAP2 (cIAP1/2) and X-linked IAP (XIAP), although there exist eight mammalian IAPs. The family of proteins is defined by the presence of the baculovirus IAP repeat (BIR) domains, which are involved in the regulation of caspases. cIAP1, cIAP2 and XIAP possess a Really Interesting New Gene (RING) domain responsible for the E3 ubiquitin ligase activity. They also possess an ubiquitin associated domain (UBA), responsible for binding of ubiquitin chains. cIAP1/2 have a caspase recruitment domain (CARD) which suppresses E3 ubiquitin ligase activity.

of the inactive procaspase-3 into its active form (8). Alternatively, binding of caspase-3 by the BIR2 domain of XIAP blocks the active site on caspase-3 so it cannot cleave its substrates. Thus, XIAP effectively blocks the caspase cascade at both the initiator and effector stages. It is generally accepted that cIAP1 and cIAP2 antagonize the activity of caspases and prevent apoptosis. However, the process by which this occurs remains unclear (1). cIAP1 and cIAP2 do bind caspase-7 and -9 via their BIR2 and BIR3 domains, but do not directly inhibit caspase activity as a result of this binding (9). It is hypothesized that cIAP1/2 may ubiquitinate caspases or inhibit their activity by a more indirect method.

The cIAPs and XIAP possess E3 ubiquitin ligase activity via their Really interesting new gene (RING) domains (10, 11). This activity is responsible for auto- or trans-ubiquitination of the IAPs and other proteins. Studies have shown that cIAP1 is capable of ubiquitinating cIAP2 but not vice versa (12). Ubiquitination via a K48 linkage generally leads to proteasomal degradation of the target protein (13), whereas ubiquitination via a K63 linkage is important for signal transduction through various pathways (14–16). The IAPs are capable of both K48- and K63-mediated ubiquitination. The E3 ubiquitin ligase activity is also linked to the anti-apoptotic effects of the IAPs. The IAPs are capable of ubiquitinating caspases, leading to the degradation of caspases and preventing apoptosis (17, 18). Conversely, auto-ubiquitination and subsequent degradation of the IAPs may promote apoptosis of certain cells (19). The balance of these two roles likely determines the fate of the cell (20).

1.3: The role of the cIAPs in Nuclear Factor κB (NF-κB) signalling

The IAPs are also key mediators of important signalling pathways. Of particular interest is the role of cIAP1 and cIAP2 in NF-κB signalling (21–23). This pathway is involved in many cellular processes including, but not restricted to, the apoptotic response

(24, 25), stress signals (26), proliferation (27), immunity (28) and inflammation (29, 30). The NF-κB pathway is comprised of two arms, the classical (canonical) and alternative (non-canonical) branches (Figure 2). The classical pathway is primarily activated by binding of tumour necrosis factor alpha (TNFα) to its receptor, tumour necrosis factor receptor 1 (TNF-R1). The alternative pathway is activated by other TNF superfamily members such as cluster of differentiation 40 ligand (CD40L) binding to its cognate receptor, CD40. cIAP1/2 are involved in the regulation of both branches, acting as critical regulators of ligand-induced signal transduction. The cIAPs are redundant in this role and one can compensate for the other (23, 31).

The cIAPs are positive regulators of the classical pathway. The cIAPs are recruited to TNF-R1 via the adaptor proteins TNF receptor-associated factor 2 (TRAF2) and TNF-receptor-1-associated death domain (TRADD) (Figure 2a). The receptor-interacting protein 1 (RIP1) is also recruited to the complex via TRADD. Upon binding of TNFα to TNF-R1, cIAP1/2 ubiquitinates RIP1 via a K63 linkage, leading to recruitment of TGFβ activated kinase 1/MAP3K7 binding protein 2 (TAB2) and transforming growth factor-beta-activated kinase 1 (TAK1). Linear ubiquitin chain assembly complex (LUBAC, consisting of consisting of heme-oxidized IRP2 ubiquitin ligase 1 [HOIL-1], HOIL-1 interacting protein [HOIP] and Shank-associated RH domain-interacting protein [Sharpin]), recruited to cIAP1/2 via an ubiquitin chain on cIAP1/2, is also capable of ubiquitinating RIP1. Inhibitor of nuclear factor kappa-B kinase subunit B (IKKB) is phosphorylated by TAK1, which leads to activation of the IKK complex that consists of IKKα, IKKβ and NF-κB essential modulator (NEMO). The IKK complex phosphorylates IκB, which is in complex with p50 and v-rel reticuloendotheliosis viral oncogene homolog A (RelA). IκB is then degraded by

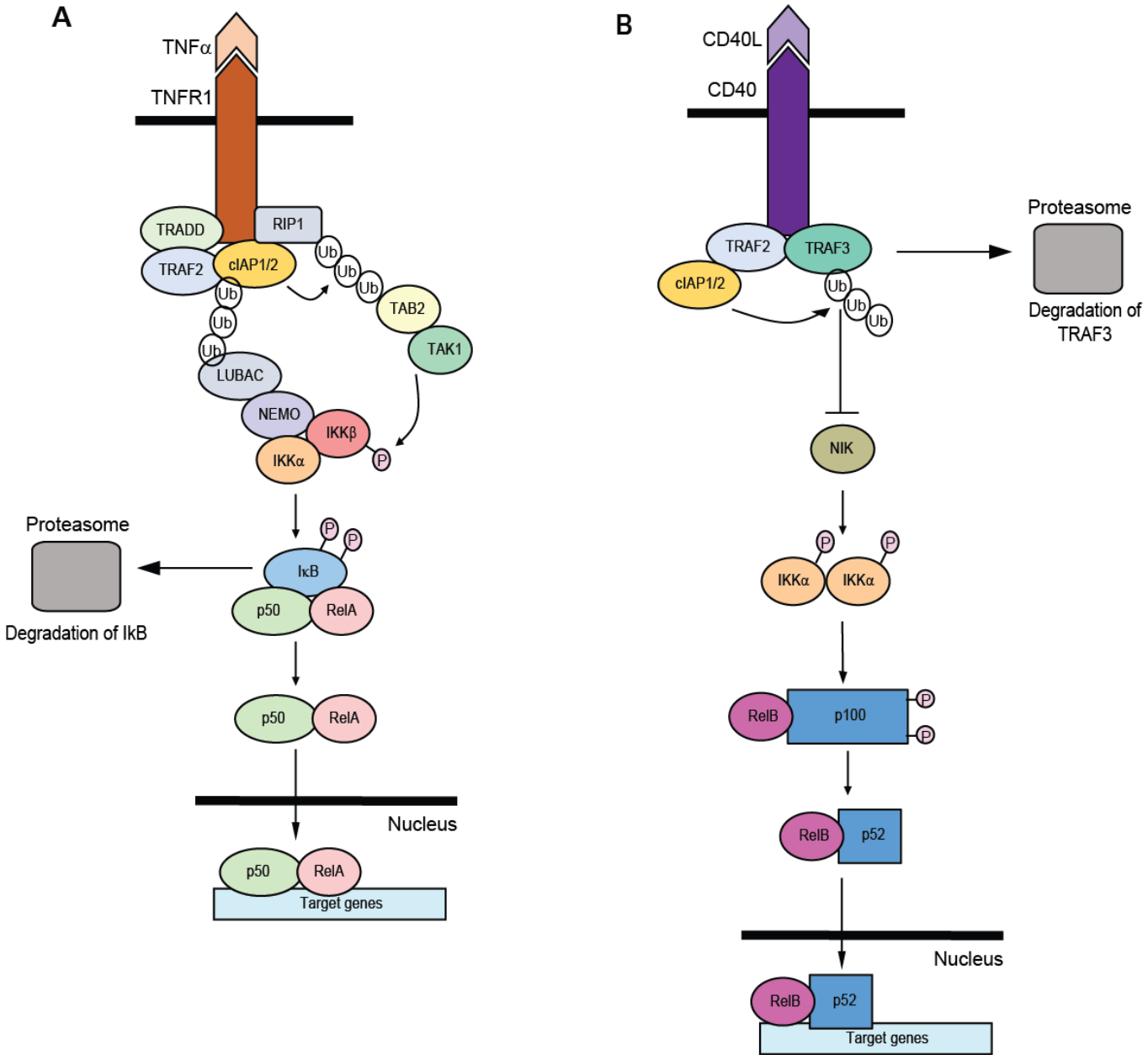

A
TNFα
TNFR1
TRADD
RIP1
TRAF2
cIAP1/2
Ub
TAB2
TAK1
LUBAC
NEMO
IKKβ
IKKα
P
Proteasome
Degradation of IkB
IκB
p50
RelA
Nucleus
Target genes
B
CD40L
CD40
TRAF2
TRAF3
cIAP1/2
Proteasome
Degradation of TRAF3
NIK
IKKα
RelB
p100
p52

Figure 2. IAP control of NF-κB signalling. (A) The classical (canonical) NF-κB pathway is activated when TNFα binds to its receptor, TNF-R1. cIAP1 and cIAP1 are recruited to TNF-R1 via the adaptor protein TRAF2 and TRADD. RIP1 is recruited via TRADD. cIAP1/2 ubiquitinate RIP1 via a K63 linkage, which leads to recruitment of TAB2 and TAK1. LUBAC, recruited to cIAP1/2 via an ubiquitin chain, is also capable of ubiquitinating RIP1. IKKB is phosphorylated by TAK1, which leads to activation of the IKK complex, consisting of IKKα, IKKβ and NEMO. The IKK complex phosphorylates IκB, which is in complex with p50 and RelA. IκB is then degraded by the proteasome which leaves p50 and RelA free to translocate to the nucleus as a heterodimer and promote transcription of target genes. **(B)** The alternative (noncanonical) NF-κB pathway is normally supressed by the degradation of NIK by TRAF2, TRAF3, cIAP1 and cIAP2. Upon binding of TNF superfamily ligands, such as CD40L, to their receptors, the cIAPs ubiquitinate TRAF3 which leads to the degradation of TRAF3 by the proteasome. Loss of TRAF3 allows stabilization of NIK which, together with IKKα, phosphorylates p100. This protein is found in an inactive complex with RelB. Phosphorylated p100 is processed into the active p52. The RelB/p52 complex then translocates to the nucleus and leads to transcription of target genes. TRAF - TNF receptor-associated factor, TRADD - TNF-receptor-1-associated death domain, RIP1 - Receptor-interacting protein-1, TAB2 - TGF-beta activated kinase 1/MAP3K7 binding protein 2, TAK1 - transforming growth factor-beta-activated kinase 1, LUBAC - Linear ubiquitin chain assembly complex (consisting of heme-oxidized IRP2 ubiquitin ligase 1 [HOIL-1], HOIL-1 interacting protein [HOIP] and Shank-associated RH domain-interacting protein [Sharpin]), IKKB - Inhibitor of nuclear factor kappa-B subunit B, NEMO - NF-κB essential modulator, RelA - v-rel reticuloendotheliosis viral oncogene homolog A, NIK - NF-κB inducing kinase, CD40L – cluster of differentiation 40 ligand, RelB - v-rel avian reticuloendotheliosis viral oncogene homolog B.

the proteasome which leaves p50 and RelA free to translocate to the nucleus as a heterodimer and promote transcription of target genes (31).

Under normal conditions, the cIAPs suppress the alternative NF-κB pathway through ubiquitination of NF-κB-inducing kinase (NIK) via the adaptor proteins TRAF2 and TRAF3. Upon activation by binding of TNF superfamily ligands such as CD40L to its receptor, cIAP1/2 ubiquitinate TRAF3, leading to its degradation by the proteasome (Figure 2b). Loss of TRAF3 allows stabilization of NIK, which, together with IKKα, phosphorylates p100. Under unstimulated conditions, p100 is found in an inactive complex with v-rel avian reticuloendotheliosis viral oncogene homolog B (RelB). Phosphorylated p100 is processed into active p52, and the RelB/p52 heterodimer then translocates to the nucleus and leads to transcription of target genes (31). As with the classical arm, the target genes of the alternative NF-κB pathway are involved in many cellular processes such as B cell maturation and dendritic cell (DC) activation (32).

1.4: The IAPs in innate immunity

One important role of NF-κB signalling is the modulation of immunity in innate immune cells such as macrophages, DCs and natural killer (NK) cells, and in adaptive immune cells such as T cells and B cells (33–37). Of particular interest is the ability of cIAP1, cIAP2 and XIAP to regulate inflammatory cytokine production in response to NF-κB activation in innate immune cells (38, 39). Innate immune cells are capable of sensing pathogen-associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs) (40, 41). The first family of PRRs discovered, the Toll-like receptors (TLRs), is involved in the recognition of many types of PAMPs. Other families of PRRs have also been discovered, including the retinoic acid gene-1 (RIG-1)-like receptors (RLRs) (42). Stimulation of any of

these groups of receptors can lead to activation of innate immune responses and the production of proinflammatory cytokines via IAP-mediated NF-κB activation.

The TLR family of receptors is defined by the presence of a Toll/interleukin (IL)-1 receptor (TIR) domain and is comprised of 10-13 proteins, depending on the species. Humans possess TLRs 1-10 whereas mice possess TLRs 1-9 and TLRs 11-13. Each TLR recognizes a specific ligand (Table 1) (41). TLR signalling can be mediated by two adaptor proteins: myeloid differentiation primary response gene 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-beta (TRIF). Binding of a ligand to its specific TLR recruits MyD88 and/or TRIF to the receptor. Both MyD88 and TRIF can activate cIAP1/2-mediated ubiquitination of TRAF6. Ubiquitination of TRAF6 recruits TGF-beta activated kinase 1/MAP3K7 binding protein 2/3 (TAB2/3) and TAK1, which phosphorylate the IKK complex (consisting of IKKβ, IKKα and NEMO). This activates NF-κB via proteasomal degradation of IκB, leaving p50 and RelA free to translocate to the nucleus and transcribe target genes (31). TLR signalling can also activate type I interferon (IFN) production via TRIF, which activates TANK-binding kinase 1 (TBK1) to lead to phosphorylation of interferon regulatory factor (IRF) 3 and IRF7 (Figure 3) (43, 44). In this manner, the IAPs control NF-κB signalling in innate immune cells.

1.5: IAP antagonism

1.5.1: SMAC-mimetic compounds (SMCs)

The IAPs are antagonized by the Second mitochondrial activator of caspases (SMAC; also known as Diablo homolog [DIABLO]). SMAC is released from the mitochondria into the cytosol following a strong apoptosis stimulus. Upon its release from the mitochondria, SMAC binds the BIR2 and BIR3 domains of XIAP and thereby prevents the ability of XIAP to directly inhibit initiator and effector caspases (45, 46). SMAC also binds to the BIR3

Table 1. Subcellular localization and ligands of relevant toll-like receptors (TLRs).

Toll-like receptor	Ligand	Subcellular location
TLR3	Double-stranded RNA (e.g. poly(I:C), some viruses)	Endoplasmic reticulum, endosome, phagolysosome
TLR4	Lipopolysaccharide (LPS)	Plasma membrane
TLR7	Single-stranded RNA (e.g. some viruses, bacteria)	Endoplasmic reticulum, endosome, phagolysosome
TLR9	CpG-ODN, DNA	Endoplasmic reticulum, endosome, phagolysosome

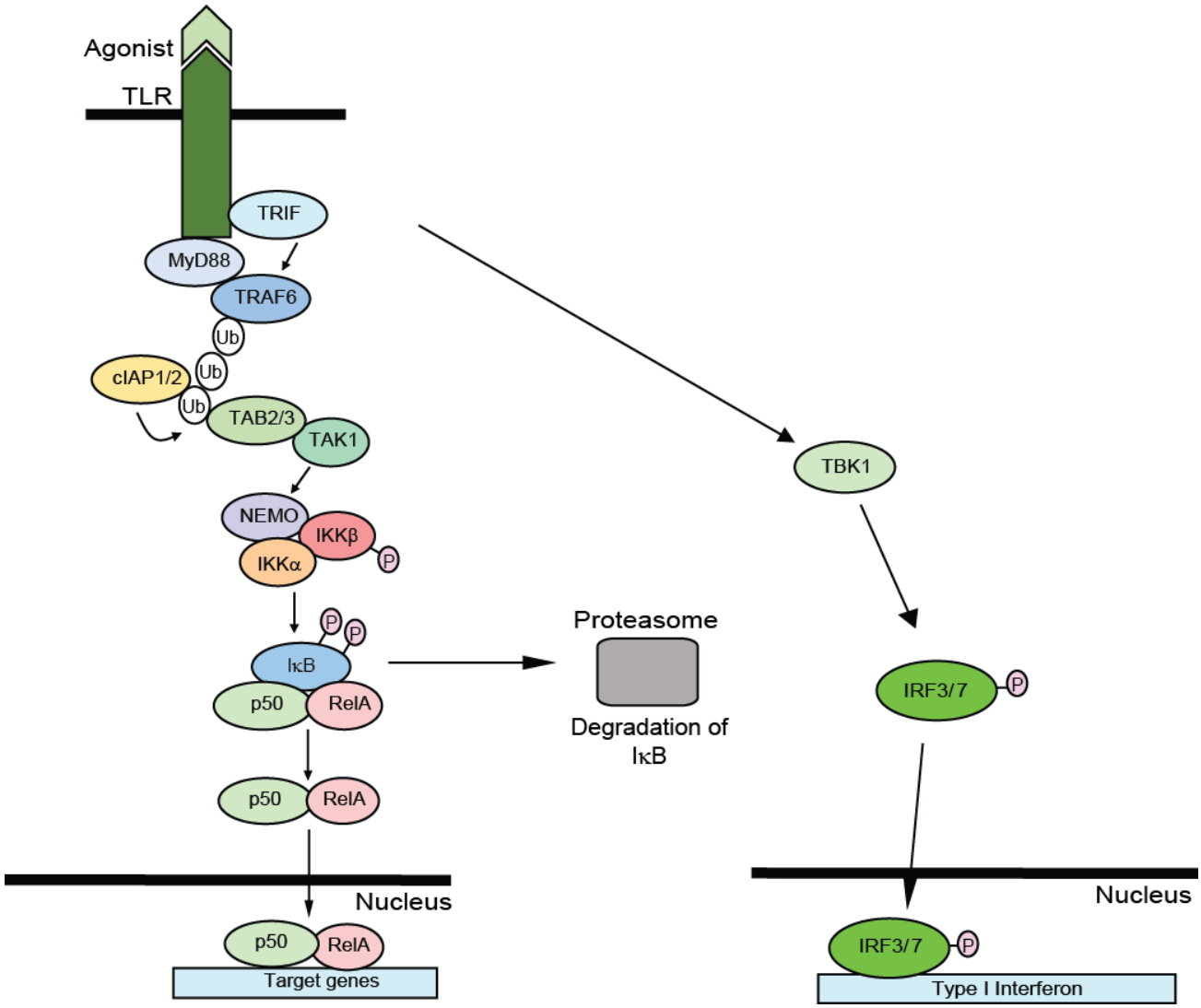
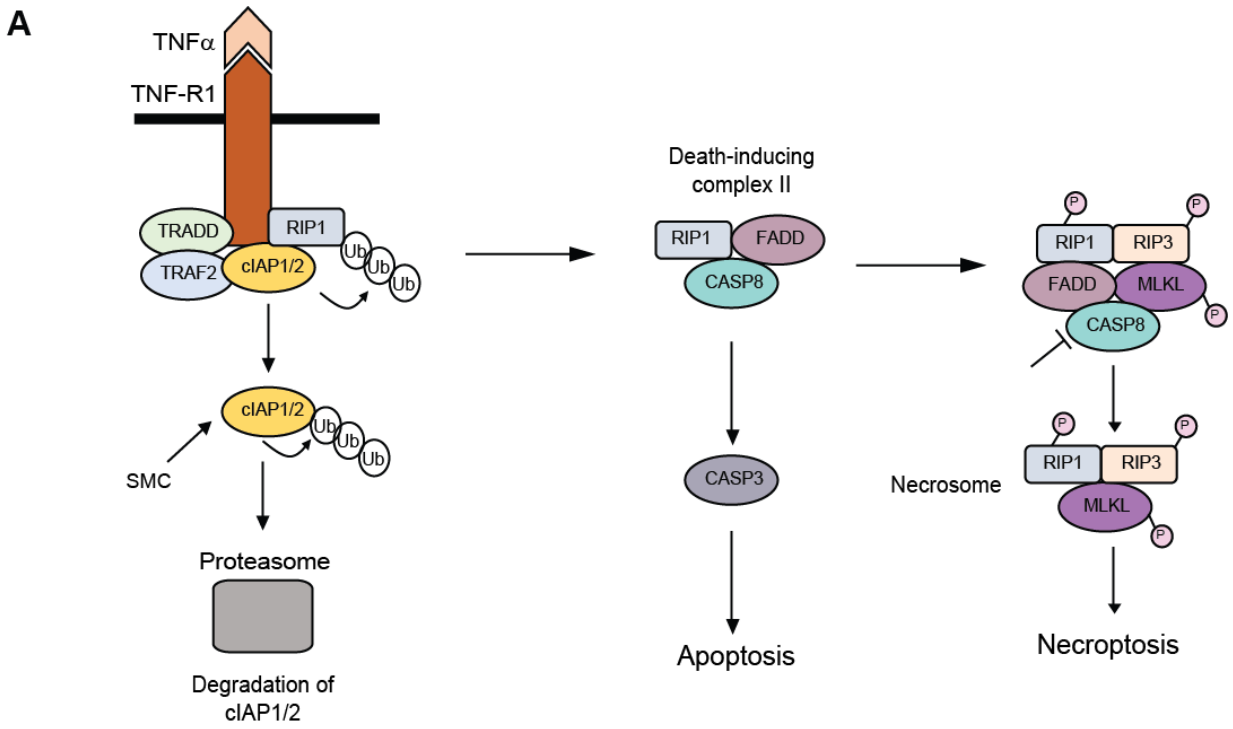


Figure 3. IAP involvement in innate immunity. Agonist binding to its appropriate TLR recruits MyD88 to the receptor, which then activates downstream signalling involving cIAP1/2. cIAP1 and cIAP2 ubiquitinate TRAF6 which recruits TAB2/3 and TAK1. TAB2/3 and TAK1 phosphorylate IKKβ, found in complex with IKKα and NEMO. This activates NF-κB via proteasomal degradation of IκB, leaving p50 and RelA free to translocate to the nucleus and transcribe target genes. Depending on the TLR being activated, TRIF can activate TRAF6 independently of MyD88 and lead to NF-κB signalling. TLR signalling also activates the type I IFN production via TRIF, which activates TBK1 to lead to phosphorylation of IRF3 or IRF7. TLR - toll-like receptor, MyD88 - myeloid differentiation primary response gene 88, TRAF6 - TNF receptor-associated factor 6, TAB2/3 - TGF-beta activated kinase 1/MAP3K7 binding protein 2/3, TAK1 - transforming growth factor-beta-activated kinase 1, IKK - inhibitor of nuclear factor kappa-B kinase, NEMO - NF-κB essential modulator, IFN - interferon, TRIF - TIR-domain-containing adapter-inducing interferon-beta, TBK1 - TANK-binding kinase 1, IRF - interferon-regulatory factor.

domain of cIAP1/2 and promotes auto-ubiquitination of these proteins (47). Conversely, the IAPs are capable of ubiquitinating SMAC, leading to its degradation (48). The balance of IAP and SMAC degradation, mediated by other proteins such as the IAP members Survivin (in the case of XIAP) or Livin (in the case of cIAP1/2), contributes to the outcome of the cell: the release of SMAC from the mitochondria does not guarantee that the cell will undergo apoptosis (49).

In attempts to specifically target the IAPs, a class of small molecule drugs have been designed to mimic the activity of SMAC. Named SMAC-mimetic compounds (SMCs), these drugs can inhibit XIAP and lead to the degradation of cIAP1/2 by binding to their BIR3 domains (50). Importantly, SMCs cannot be ubiquitinated by cIAP1/2 and these drugs therefore avoid proteasomal degradation. SMCs have been designed to fit two structural categories: monovalent and bivalent. Monovalent SMCs bind one BIR domain of the IAPs, while bivalent SMCs possess two functional binding motifs and can effectively inhibit both the BIR2 and BIR3 domains of XIAP, rendering bivalent SMCs more effective for downregulation of XIAP (50). Several SMCs are currently in clinical trials for the treatment of human cancers (50–53). The goal of SMC therapy is to inhibit the activity of the IAPs and allow the progression of apoptotic pathways in cancer cells. SMC monotherapy has been effective against certain forms of cancer and is well-tolerated in late-stage cancers but has had lacklustre results overall (50).

In the presence of SMCs, cIAP1/2 auto-ubiquitinate and are degraded (54). As a result of this loss, RIP1 is no longer ubiquitinated and is freed from the TNF-R1 complex to form the death inducing complex II along with Fas-associated protein with death domain (FADD) and caspase-8 (55). This complex activates caspase-3 and ultimately leads to apoptosis (Figure 4a). In this way, the IAPs act as a molecular switch that can change TNFα-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TNFα
TNF-R1
TRADD
RIP1
TRAF2
cIAP1/2
Ub
Ub
Ub
cIAP1/2
Ub
Ub
Ub
SMC
Proteasome
Degradation of
cIAP1/2
Death-inducing
complex II
RIP1
FADD
CASP8
CASP3
Apoptosis
RIP1
RIP3
FADD
MLKL
CASP8
P
Necrosome
RIP1
RIP3
MLKL
Necroptosis

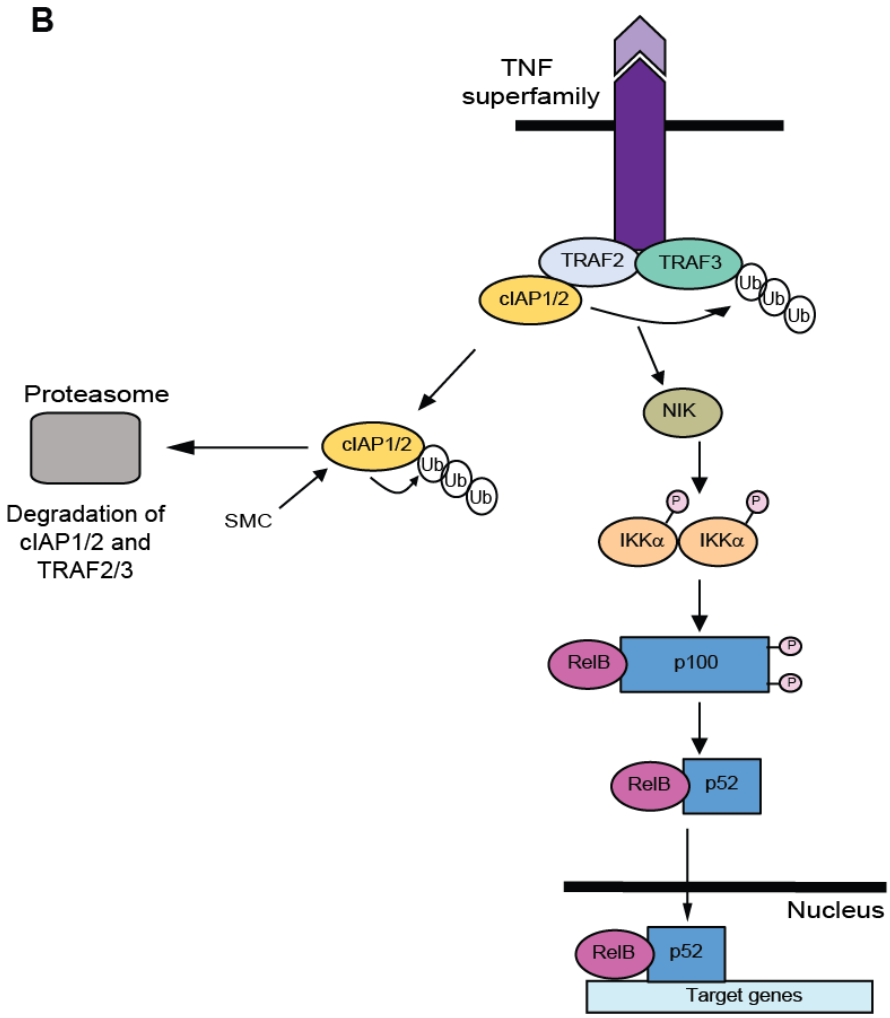

B
TNF
superfamily
TRAF2
TRAF3
cIAP1/2
Ub
Ub
Ub
Proteasome
Degradation of
cIAP1/2 and
TRAF2/3
cIAP1/2
Ub
Ub
Ub
SMC
NIK
IKKα
IKKα
P
RelB
p100
P
RelB
p52
Nucleus
RelB
p52
Target genes

Figure 4. The effects of SMAC-mimetic compounds (SMCs) on IAP-mediated NF-κB signalling. (A) In the presence of SMCs, cIAP1/2 auto-ubiquitinate and are degraded. Thus, with TNFα stimulation, RIP1 is no longer ubiquitinated and is freed from the TNF-R1 complex to form the death inducing complex II which includes FADD and caspase-8, resulting in apoptosis of the cell via activation of caspase-3. If caspase-8 is not present or is antagonized, RIP1 can form a complex with RIP3 and MLKL called the necrosome. Phosphorylation of MLKL and RIP3 promotes their translocation to the plasma membrane which induces necroptosis. **(B)** SMCs activate the alternative NF-κB pathway by promoting the degradation of cIAP1/2 and TRAF2/3. This leads to stabilization of NIK which allows processing of p100 to p52 and translocation of p52 and RelB to the nucleus. FADD - Fas-associated protein with death domain, MLKL - mixed lineage kinase domain-like protein.

mediated classical NF-κB prosurvival signalling to a pro-death signal. Importantly, apoptosis is not the only death pathway that can be activated with SMC treatment. Recent work has shown that if caspase-8 is not present or its function is impaired, RIP1 can form a complex with RIP3 and mixed lineage kinase domain-like protein (MLKL). Together, these three proteins form a complex what is known as the necrosome, which leads to necroptosis. Necroptosis is a regulated form of necrosis and ultimately leads to cell death (Figure 4a) (56). In this way, cell death can still be achieved even if there are inactivating mutations in caspase-8-mediated signalling, which can occur in some cancer types (57, 58). The exquisite control of the IAPs over life and death makes enables SMC therapy to be a promising anti-cancer option.

The presence of SMCs also promotes the activation of the alternative NF-κB pathway. SMC-mediated degradation of cIAP1/2 allows for the stabilization of NIK, thereby inducing the activation of the alternative pathway (Figure 4b) (59). It is thought that the activation of this branch of NF-κB may allow transcription of more inflammatory cytokines that can then synergize with SMCs, as discussed in the following section.

1.5.2: SMCs and inflammatory cytokines

For maximal SMC efficacy, inflammatory cytokines that activate the classical NF-κB pathway must be present (60, 61). The presence of TNFα, for example, will promote the formation of the death-inducing complex II when cIAP1/2 are degraded (Figure 4a). Some cancers produce sufficient TNFα via an autocrine loop and will respond to SMC treatment alone. However, the majority of cancers require the administration of exogenous cytokines to die in the presence of SMCs (62). Unfortunately, treatment of patients with recombinant TNFα, for example, is not a viable therapeutic option because it can lead to serious systemic toxicity. It was then reasoned that production of cytokines by the patient's own immune

system can be a safer method of elevating the levels of these cytokines. Thus, SMC combination immunotherapy was designed to create a short, tolerable burst of cytokine release which can synergize with SMCs to kill cancer cells. This method has been shown to be effective both *in vitro* for many cancer cell lines and *in vivo* for EMT6 mammary carcinoma (60).

1.6: Innate immune stimulants

1.6.1: Oncolytic viruses

It was shown in the early 20th century that patients infected with certain viruses occasionally saw spontaneous tumour regression (63). Revisiting this idea has sparked investigation in the field of oncolytic viruses, which are viruses that specifically infect and replicate in cancer cells while sparing normal cells. Oncolytic viruses target cancer cells by recognizing cell surface receptors and changes in gene expression that arise as a result of the mutated cancer cell development. Importantly, some types of cancer cells do not mount a functional antiviral response by type I interferon (discussed in section 1.6.2), which renders them particularly sensitive to oncolytic virus infection. Recent research has been conducted to engineer oncolytic viruses that possess certain characteristics such as higher oncolytic activity, greater immunostimulatory effects, or the expression of transgenes (64, 65). Several oncolytic viruses are in clinical trials for the treatment of various cancers but results have been inconsistent (66, 67).

Derivatives of vesicular stomatitis virus (VSV), a member of the Rhabdoviridae family, have been shown to be an effective as oncolytic viruses in a large number of preclinical studies. One detrimental characteristic of VSV is the ability for the viral matrix (M) protein to inhibit mRNA trafficking from the nucleus to the cytosol, thus shutting down host protein synthesis (68–71). This blockage prevents the expression of host antiviral genes

which, when lost, allows the virus to replicate and spread unhindered. Rationally designed derivatives of VSV have been generated to address this issue. For example, VSVΔ51, has a deletion at amino acid 51 in the M protein, compromising the functionality of the M protein while still allowing for the formation of new virions. This deletion allows for re-expression of host antiviral proteins and inflammatory cytokines such as type I IFNs and TNFα, respectively (64). Production of these proteins increases the safety of VSVΔ51 compared to wild-type (WT) VSV by allowing the recruitment of the host immune system to the site of infection. Other derivatives of VSV aim to reintroduce type I IFNs to the site of infection by inserting the desired type I IFN gene into the viral genome, which will then be produced in the host cells upon expression viral proteins. One example of such a virus is VSV-IFNβ. Release of virus-derived IFNβ has the same effects as host-derived IFNβ and allows mounting of an immune response to control viral spread, as discussed in the next section (65, 72). Importantly, the recruitment of immune cells to the site of infection (i.e. the tumour) encourages specific anti-tumour immune responses.

1.6.2: Type I interferons (IFNs)

Type I IFNs are a family of several proteins defined by their ability to bind to the type I IFN receptor, interferon alpha-beta receptor (IFNAR). This heterodimeric receptor consists of two chains: IFNAR1 and IFNAR2. The two well-studied type I IFNs are IFNα and IFNβ. There are 13 human and 14 mouse isoforms of IFNα, each encoded by a specific gene, whereas there is only one IFNβ gene and protein product (73). The other type I IFNs (κ, ε, ω, δ, τ, ς) are more species-specific and generally have more constrained functions, such as immunity of the skin or immunity of the reproductive tract (74). Each of these proteins binds to IFNAR with differing affinities and by doing so can induce individual cell- and protein-specific responses (75). IFNβ binds to IFNAR with the greatest affinity (76).

Type I IFNs are the primary antiviral proteins. They are expressed by cells upon infection with a virus and are released from infected cells to promote an antiviral state in neighbouring cells. This antiviral response is achieved by many cellular mechanisms activated by type I IFN signalling. For example, inhibition of viral transcription and translation prevents formation of functional virus molecules (73). Additionally, altered membrane functionality can prevent entry of viral molecules in uninfected neighbouring cells (73). In this way, type I IFNs control viral spread.

Type I IFNs are not only involved in the antiviral response; these IFNs can be released in response to engagement of other PRRs such as TLRs and RLRs (77). In this context, type I IFNs function as immune modulators and are important in both innate and adaptive immunity. They promote antigen presentation in innate immune cells such as macrophages and DCs. The release of other cytokines, such as TNF-related apoptosis-inducing ligand (TRAIL), IL-12 and IL15 from these cells can also be regulated by type I IFNs (78). Additionally, IFNα is an important regulator of NK cell activity, with some isoforms of IFNα leading to activation of these cells, while other isoforms have no effect (79). Type I IFNs also activate the adaptive immune system and promote T-cell memory by encouraging specific T-cell responses following antigen presentation by innate immune cells (80).

Engagement of IFNAR by type I IFN leads to dimerization of IFNAR1 and IFNAR2, then recruitment of Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2), which in turn phosphorylate the receptor. Phosphorylation of IFNAR leads to recruitment of signal transducer and activator of transcription (STAT) 1 and 2 to the receptor. STAT1/2 are activated via phosphorylation by JAK1 and can then dimerize in specific complexes depending on the signal being transduced (i.e. depending on which type I IFN has bound

IFNAR as described above) (75). For activation of the antiviral response, STAT1 and STAT2 dimerize and recruit IFN-regulatory factor 9 (IRF9). Together, these three proteins form the IFN-stimulated gene factor 3 (ISGF3) complex which then translocates to the nucleus and binds to IFN-stimulated response elements (ISREs), thereby promoting transcription of interferon-stimulated genes (ISGs). STAT1 can also form a homodimer and translocate to the nucleus to promote transcription of genes involved in the inflammatory response (Figure 5) (73).

Several recombinant type I IFNs are approved for the treatment of some forms of cancer. Recombinant type I IFNs are also in clinical trials for the treatment of cancer in combination with other agents. The efficacy of these treatments has been inconsistent but is often linked to T-cell infiltration into the tumour. Type I IFN production from antigen presenting cells (APCs) can be induced by the tumour itself, which increases T cell responses. It has also been shown that type I IFN signalling is required for radiation and immunotherapies to be effective (81). Therefore, there is a clear necessity for type I IFN at the tumour site to promote tumour regression in many cases.

1.6.3: CpG-containing oligodeoxynucleotides (CpG-ODNs)

CpG-containing oligodeoxynucleotides (CpG-ODNs) are short synthetic molecules that mimic bacterial DNA. CpG-ODNs contain unmethylated CpG islands, which are common in the bacterial genome but less so in the mammalian genome. These molecules activate innate immune cells by binding to TLR9 and leading to NF-κB activation as shown in Figure 3 (82).

There are three classes of CpG-ODNs: A, B and C. Class A CpG-ODNs promote the secretion of IFNα and the maturation of DCs. Class B CpG-ODNs promote the secretion of TNFα, stimulate B cells and promote the maturation of monocytes. Class C CpG-ODNs

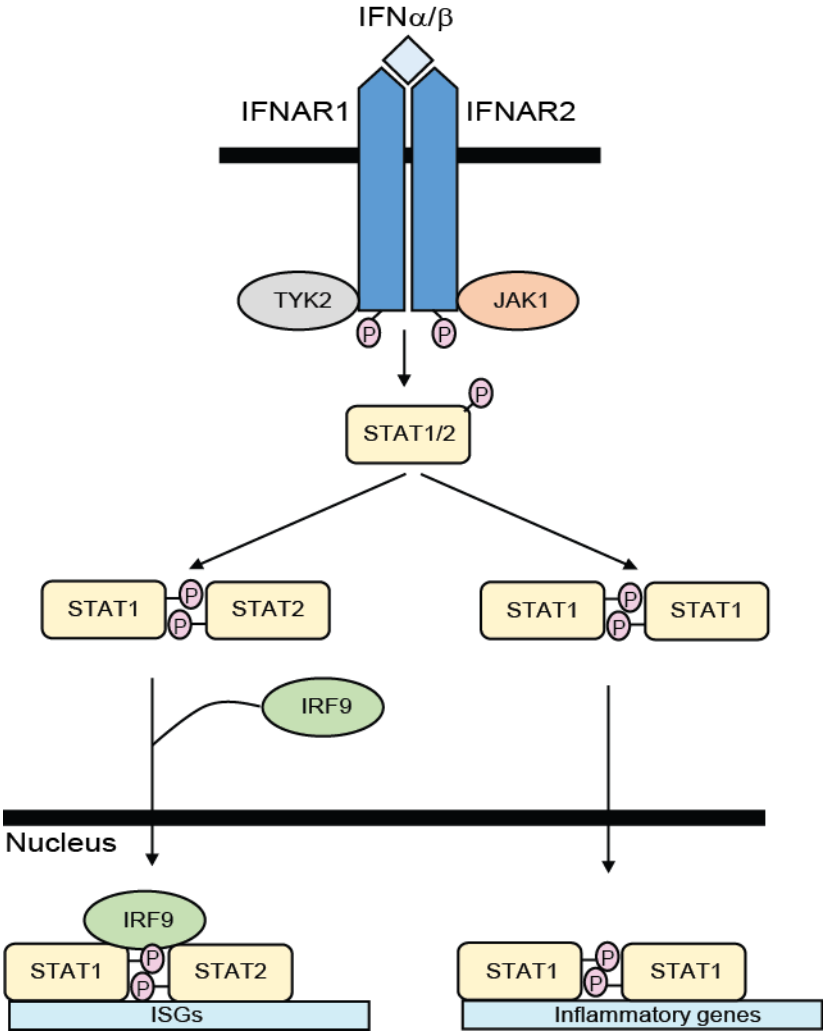


Figure 5. Type I interferon (IFN) signalling pathway. Engagement of the IFNAR by type I IFN leads to activation of JAK1 and TYK2, which phosphorylate the receptor complex. Phosphorylation of IFNAR leads to recruitment of STAT1 and STAT2 to the receptor. STAT1/2 are activated via phosphorylation by JAK1 and can then dimerize. For the antiviral response to be activated, STAT1/2 form heterodimers and recruit IRF9 to form the ISGF3 complex which then translocates to the nucleus and binds to ISREs to promote transcription of ISGs. STAT1 can also form a homodimer and translocate to the nucleus to promote transcription of genes involved in the inflammatory response. IFNAR - type I IFN alpha/beta receptor, JAK1 - Janus kinase 1, TYK2 - tyrosine kinase 2, STAT - signal transducer and activator of transcription, IRF - interferon-regulatory factor, ISGF3 - interferon-stimulated gene factor 3, ISRE - interferon-stimulated response elements, ISG - interferon-stimulated genes.

possess the characteristics of both classes A and B, and promote the secretion of IFNα and stimulate B cells (82).

CpG-ODNs are often used as vaccine adjuvants and have also been used in clinical trials as adjuvants in anti-cancer vaccines. They were tested as standalone anti-tumour agents. CpG-ODNs were shown to be well tolerated and elicit innate immune responses but did not show significant decreases in tumour burden (83).

1.6.4: Polyinosinic:polycytidylic acid (poly(I:C))

Another synthetic immunostimulant that can stimulate the production of cytokines is polyinosinic:polycytidylic acid (poly(I:C)). Poly(I:C) is a synthetic molecule that mimics the structure of double-stranded RNA (84). As such, it is often used as a substitute for virus infections in immune studies. Poly(I:C) can be used as an effective agent to sensitize cancer cells to apoptosis and to produce cytokines (85).

Poly(I:C) binds TLR3, which signals through both MyD88 and TRIF (Figure 3). Activation of these pathways leads to transcription of inflammatory cytokines and apoptosis of activated cancer cells as well as to the production of type I IFNs. However, intracellular poly(I:C) is also capable of signalling via the cytosolic sensors RIG-I and melanoma differentiation-associated gene 5 (MDA5). Activation of RIG-I or MDA5 recruits interferon-beta promoter stimulator 1 (IPS-1; also known as mitochondrial antiviral signalling protein [MAVS]), which activates nucleosome assembly protein-1 (NAP1) and TRAF3. These will then activate IRF3/7 via the TBK1, TRAF family member-associated NF-κB activator (TANK) and IKK complex to promote transcription of type I IFNs (Figure 6a). Activation of this pathway also promotes greater TLR3 expression which can then enhance the pro-apoptotic signalling in cancer cells. Importantly, there is cross-talk between the two

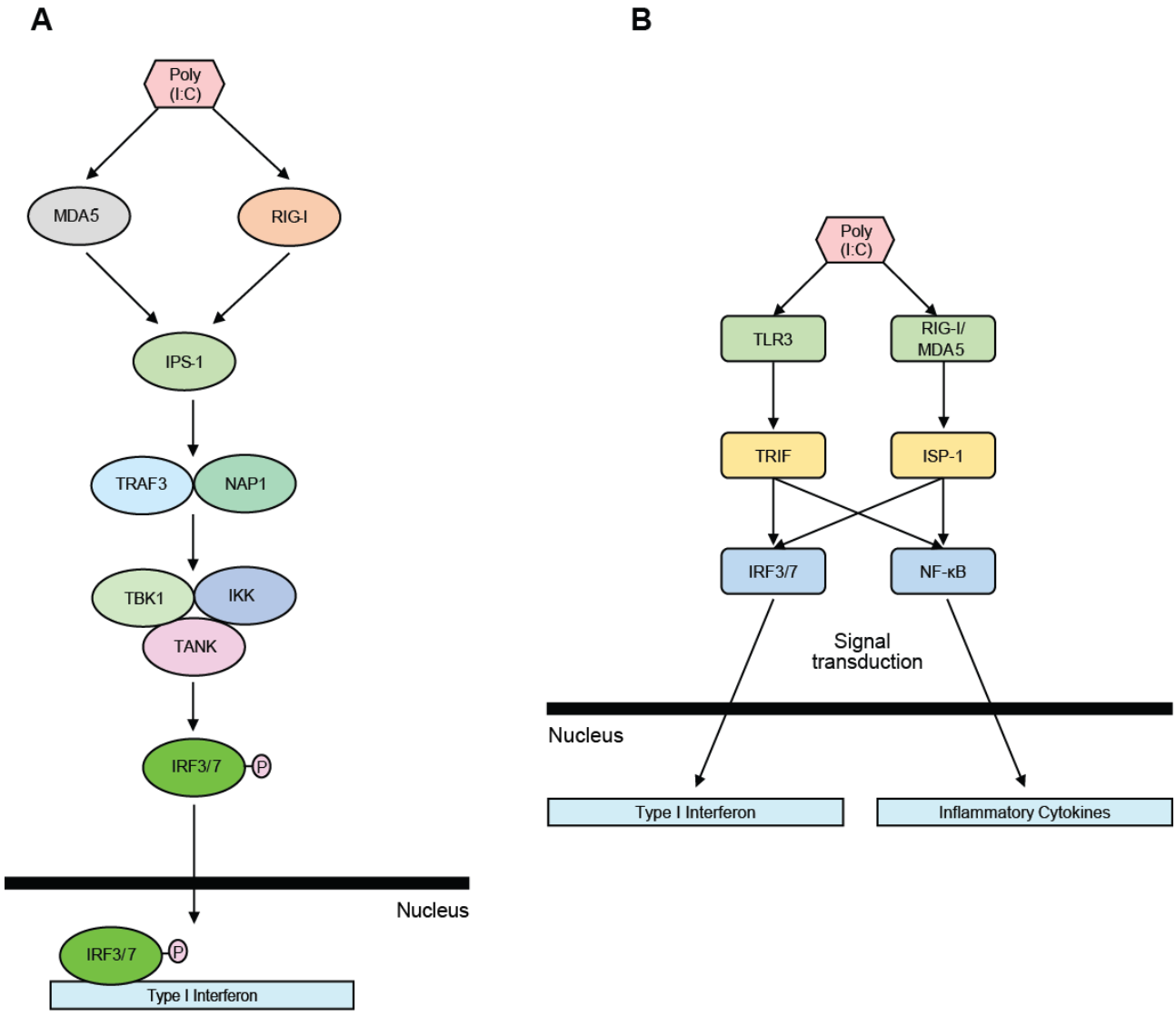


Figure 6. Poly(I:C)-induced signalling pathways. (A) Activation of RIG-I or MDA5 recruits IPS-1 (also known as MAVS), which activates NAP1 and TRAF3. These activate the TBK1, TANK and IKK complex to induce phosphorylation of IRF3/7 by TBK1. IRF3/7 will then translocate to the nucleus and promote transcription of IFNβ. It is not yet clear how IPS-1, TRAF3 and NAP1 are involved in the activation of the TBK1, TANK and IKK complex. **(B)** Cross-talk between the two poly(I:C) pathways allows for the production of both type I IFNs and inflammatory cytokines from one stimulus. TLR3 binds extracellular poly(I:C) while RIG-1/MDA5 bind cytosolic poly(I:C). Cytosolic poly(I:C) is generally achieved via transfection in vitro, but some can be taken up by immune cells following topical treatment. RIG-I - retinoic acid inducible gene 1, MDA5 - melanoma differentiation-associated protein 5, IPS-1 - interferon-beta promoter stimulator 1, MAVS - mitochondrial antiviral-signalling protein, NAP1 - nucleosome assembly protein 1, TRAF3 - TNF receptor-associated factor 3, IRF - interferon-regulatory factor, TANK - TRAF family member associated NF-κB activator, TBK1 - TANK-binding kinase, IKK - IκB kinase.

poly(I:C) pathways, which allows for the production of both type I interferon and inflammatory cytokines from the same stimulus (Figure 6b) (85–87).

Poly(I:C) has been used as a vaccine adjuvant and was tested in a phase II clinical trial for cancer. As a standalone therapy, poly(I:C) does not have very potent anti-tumour effects. It is now being considered as a combination therapy to enhance the effects of chemotherapy (88).

1.7: Rationale and hypothesis

1.7.1: Combination immunotherapy

We have shown that the combination of a monovalent SMC, LCL-161, and innate immune stimuli effectively synergize and lead to death of cancer cells *in vitro* and *in vivo*. In particular, VSVΔ51, poly(I:C) and CpG-ODN 2216 (Class A) have been proven effective in combination with LCL-161 to kill EMT6 mammary carcinoma cells in a syngeneic tumour model (BALB/c mice). Notably, significant and potent efficacy of VSVΔ51 and SMC combination therapy is dependent on TNFα signalling (60).

1.7.2: Innate immune cell involvement

The effectiveness of the above combination immunotherapy can be seen as soon as three days post-treatment. This observation suggests that the anti-cancer effects are mediated by the innate immune system rather than a specific adaptive immune response against the tumour. We hypothesized that cytokine-producing cells such as macrophages can contribute to the efficacy of treatment. This cytokine release can synergize with LCL-161 to induce cancer cell death. Other innate immune populations, such as DCs and monocytes, may also contribute to these effects.

1.7.3: Hypothesis

VSVΔ51, poly(I:C) and CpG-ODN 2216 are inducers of type I IFNs. We hypothesize that type I IFNs are necessary and sufficient mediators of TNFα production. Therefore, type I IFNs can also be used as cytokine-inducing agents to kill cancer cells in combination with SMCs. In addition to the direct effects of type I IFN on SMC-treated tumor cells, macrophages may also be responsible for the production of TNFα in response to type I IFN treatment.

1.8: Objectives

There are two objectives to this research project. The first objective is to confirm that type I IFN is necessary and sufficient to mediate SMC combination therapy efficacy. By doing so, I will determine if type I IFN is the primary mediator of TNFα production. It is also important to determine whether the source of type I IFN affects the efficacy of the treatment. Specifically, I will investigate the impact of type I IFN that is produced "endogenously" through the use of innate immune stimulants compared to that administered "exogenously" by using recombinant IFN. The second objective of this project is to determine the role of immune cells in the response to innate immune stimuli and for the production of inflammatory cytokines, specifically TNFα. By understanding the mechanism of action of SMC combination therapy, we can add insight to the use of this treatment regimen in human clinical trials. Importantly, this combination approach can be easily translated to the clinic by combining SMCs with drugs currently being used in the clinic or as approved anti-cancer therapies.

2: MATERIALS AND METHODS

2.1: Reagents

The SMC LCL-161 was provided to us by Novartis (Cambridge, MA, USA). Dr. John Bell (Ottawa Hospital Research Institute, Ottawa, ON, Canada) provided the WT VSV, VSVΔ51, VSV-mIFNβ-NIS, VSV-mIFNβ and VSVΔG viruses. Recombinant mouse TNFα (410-MT) was obtained from R&D Systems (Burlington, ON, Canada). Recombinant mouse IFNβ, human IFNβ and universal IFNα were obtained from PBL Assay Science (Pistacaway, NJ, USA). Recombinant human IFNα B/D was kindly provided by Dr. Peter Staeheli (University of Freiberg, Freiberg, Germany). Dimethyl sulfoxide (DMSO) was used as the vehicle control in all *in vitro* experiments.

2.2: Antibodies

2.2.1: Western antibodies

Primary antibodies: TNF-R1 (Abcam, 19139), TRAF2 (Cell Signalling, 4172), TRAF3 (Santa Cruz, G-6), TRAF6 (Santa Cruz, H-274), IRF9 (Santa Cruz, H-143), STAT1 (Cell Signalling, 9712), β-tubulin (Developmental Studies Hybridoma Bank, E7). Secondary antibodies: AlexaFluor 680 (Molecular Probes) and IRDye800 (Rockland).

2.2.2: Flow cytometry antibodies

All flow cytometry antibodies were obtained from Biolegend (San Diego, CA, USA) unless otherwise indicated. CD11b-APC (M1/70), CD11c-FITC (N418), F4/80-PE/Cy7 (BM8), F4/80-PE/Cy5 (eBioscience, BM8), Gr1-FITC (BD Biosciences, RB6-8C5), Ly6C-PerCP/Cy5.5 (HK1.4), Ly6G-PerCP/Cy5.5 (1A8), NK1.1-FITC (PK136), CD3-APC/Cy7 (17A2), CD19-PE (6D5), TNFα-APC (MP6-XT22), streptavidin-BV421, biotinylated anti-IFNβ (PBL Assay Science).

2.3: Cell culture

2.3.1: Cell lines

All cell lines were maintained at 37°C and 5% CO_2. All cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin/streptomycin, glutamine and 1% non-essential amino acids (NEAA) (Invitrogen, Burlington, ON Canada). EMT6 cells were obtained from ATCC. SNB75 cells were provided by Dr. David Stojdl (Children's Hospital of Eastern Ontario Research Institute, Ottawa, ON, Canada). Lewis lung carcinoma (LLC) cells were provided by Dr. Nadine Wiper-Bergeron (University of Ottawa, Ottawa, ON, Canada).

2.3.2: Splenocytes

Spleens were removed, minced with scissors and passed through a 70 μm mesh. Red blood cells were lysed by resuspending the whole spleen in 1 mL ammonium-chloride-potassium (ACK) lysis buffer for 2 min (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.2-7.4). ACK buffer reaction was neutralized using media. Splenocytes were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% heat-inactivated FBS, penicillin/streptomycin, 1% NEAA (Invitrogen), and 10 μM β-mercaptoethanol (2-ME) (Sigma). Splenocytes were maintained at 37°C and 5% CO_2.

2.3.3: Bone marrow-derived macrophages (BMDMs)

Tibiae, femurs, humeri and ischia were removed intact from mice. Ends of bones were cut and bones were flushed with R8 medium (RPMI-1640 medium supplemented with 8% FBS, 55 μM 2-ME and 5 μg/mL gentamycin [Life Technologies]). 10-15 million bone marrow cells were plated in R8 medium on non-tissue culture-treated Petri dishes containing 5 ng/mL recombinant macrophage colony stimulating factor (M-CSF; R&D Systems). Bone marrow cells were left to differentiate for 7 days without changing media. BMDMs were

harvested by removing the media and scraping the cells in phosphate buffered saline (PBS). BMDMs were cultured in R8 medium and maintained at 37°C and 5% CO_2.

2.4: Protein

2.4.1: Cell protein extraction

Cells were collected by scraping in cold PBS followed by centrifugation. Cell pellets were frozen at -80°C until required. Samples were thawed on ice and lysed in cold RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM sodium fluoride, 1 mM vanadate, 1% Nonidet P-40, 0.25% Na-deoxycholate, leupeptin, aprotinin and PMSF) for 15 minutes. Samples were centrifuged for 15 minutes, $1.4x10^4$xG, at 4°C. Protein quantification was performed using the Bio-Rad Protein Assay (Mississauga, ON, Canada).

2.4.2: Tissue protein extraction

Tumours were collected from mice, frozen in liquid nitrogen and stored at -80°C. Tumours were homogenized on ice using a benchtop homogenizer (Pro Scientific, Oxford, CT, USA) in extraction buffer (20 mM HEPES-KOH, pH 7.4, 150 mM NaCl, 10% glycerol, 1 mM $MgCl_2$ and EDTA protease inhibitor cocktail [Roche]). Samples were centrifuged for 10min, 1000xG at 4°C to remove large debris. Supernatant was transferred to a new tube and centrifuged for 30min, $2x10^4$xG at 4°C. Supernatant was transferred and the protein concentration was determined using the Bio-Rad Protein Assay.

2.4.3: Western immunoblotting

Protein samples were allowed to thaw on ice. 20 μg of protein per sample was diluted in loading buffer containing 2-ME and boiled for 5 minutes. Samples were separated on polyacrylamide gels (10%) for 1.5h at 130V. Proteins were transferred to a 0.45 μm nitrocellulose membrane by wet transfer for 1.5h at 400 mA. Membranes were blocked for 30-60 minutes using 1:1 solution of PBS and Odyssey Blocking Buffer (LI-COR

Biosciences, Lincoln, NE, USA). Primary antibodies were diluted in blocking buffer and incubated overnight at room temperature on a shaker. The primary antibody was removed and the membrane was washed 3 times in tris-phosphate buffered saline with tween (TBS-T) for 10 minutes. Secondary antibodies were diluted in blocking buffer and incubated for 2h at room temperature. The membranes were washed as previously described. Membranes were scanned and analyzed using the Odyssey Infrared Imaging System (LI-COR Biosciences).

2.4.4: Enzyme-linked immunosorbent assay (ELISA)

Cell culture debris was removed by centrifugation. To detect TNFα in cell culture supernatant, the Mouse or Human TNFα DuoSet ELISA Kits (R&D Systems) were used. Human TRAIL DuoSet ELISA Kit (R&D Systems) was used to detect TRAIL from cell culture supernatant. To detect TNFα from tissue homogenates and serum, the Mouse TNFα Quantikine ELISA Kit (R&D Systems) was used. The Verikine Mouse IFNβ ELISA Kit (PBL Assay Science) was used to detect IFNβ from all samples. All ELISA kits were used according to manufacturer instructions.

2.5: Animal experiments

Mice were housed in the University of Ottawa Animal Care and Veterinary Services facility in accordance with the Institutional Animal Care and Use Guidelines. Animals were given free access to food and water. All animal experiments were performed according to approved protocols. C57BL/6 and BALB/c animals were obtained from Charles River Laboratories (Montreal, QC, Canada). Type I interferon receptor (IFNAR1) knockout mice were very kindly provided by Dr. Subash Sad (University of Ottawa, Ottawa, ON, Canada).

2.5.1: Tumour implantation and endpoints

$1x10^5$ EMT6 cells expressing Firefly luciferase (EMT6-Fluc), in 50 µl of PBS, were implanted into the lower right mammary fat pat of 4-5 week old female BALB/c mice. $3x10^5$

LLC cells, in 100 µl of PBS, were injected subcutaneously into the right flank of 4-5 week old female C57BL/6 mice. Tumours were grown for 7 days post implantation and were palpable (~100 mm^3) prior to treatment. Mice were euthanized when one of the following endpoints was reached: the tumour volume reached 2000 mm^3, the tumour was measured with calipers as 15 mm x 15 mm or when the tumour impaired the normal activities of the mouse. Tumour volume was calculated as: $(\pi)(W)2(L)/4$, where W = tumor width and L = tumor length.

2.5.2: In vivo *treatments*

Timelines in Chapter 3 indicate the specific administration of various agents. Vehicle (30% 0.1 M HCl, 70% 0.1 M NaOAc pH 4.63) or 50 mg/kg LCL-161 were administered orally. Per round of treatments, IFNα B/D was administered intratumourally (IT) as one daily 2 µg dose or intraperitoneally (IP) as two 1 µg doses on consecutive days. One 1 µg dose of IFNβ was administered IT or IP per round of treatments. 0.5 mg of TNFα-neutralizing (XT3.11) or isotype control IgG antibodies (HRPN; Bio X Cell, West Lebanon, NH, USA) were administered IP. Control liposomes or 0.625 mg Clodrosome (Encapsula Nanosciences, Brentwood, TN, USA) were administered intravenously (IV).

2.5.3: Measurement of disease progression

Mice were weighed daily during treatment and every 2-3 days once treatments ceased. Tumours were measured manually with calipers every 2-3 days. Luminescent EMT6-Fluc tumours were visualized using a Xenogen2000 IVIS CCD-camera system (Caliper Life Sciences, Hopkinton, MA, USA) following IP injection of 4 mg luciferin (Gold Biotechnology, Olivette, MO, USA).

2.6: *In Vitro* experiments

2.6.1: Viability assays

EMT6 cells were seeded at a density of $1x10^4$ cells/well in a 96-well plate and incubated overnight. Cells were treated with vehicle or 5 μM SMC, infected with the indicated MOI of VSVΔ51, WT VSV, VSV-mIFNβ-NIS or VSV-mIFNβ or treated with the indicated concentrations of universal IFNα, IFNα B/D or IFNβ for 24h or 48h. Media was removed and replaced with 1% Alamar Blue (Resazurin sodium salt; Sigma) in media. Data were for all Alamar Blue assays were normalized to vehicle-treated cells.

LLC cells were seeded at a density of $7.5x10^3$ cells/well in a 96-well plate and incubated overnight. Cells were treated with 1000 U/mL IFNβ or 10 ng/mL TNFα with vehicle or 5 μM SMC for 48h. Viability was determined by Alamar Blue.

2.6.2: Conditioned media transfers

EMT6 cells were seeded at a density of $1x10^4$ cells/well in a 96-well plate and incubated overnight. Cells were treated with VSV-mIFNβ-NIS at a MOI of 3 for 24h. The virus was inactivated by UV for 1h. Supernatant was transferred to new EMT6 cells (seeded at a density of $1x10^4$ cells/well in a 96-well plate and adhered overnight) in serial 1:2 dilutions with vehicle or 5 μM SMC for 48h. Cell viability was assessed by Alamar Blue.

BMDMs from C57BL/6 or IFNAR KO mice were isolated as described above. $1.5x10^6$ macrophages/well in a 6 well plate were cultured and treated with 5 μM SMC, 1 μg/mL CpG-ODN 2216, 1 mg/mL poly(I:C), 1 MOI VSVΔG or 10 ng/mL LPS for 24h. LLC cells were seeded at a density of $7.5x10^3$ cells/well in R8 medium in a 96-well plate and incubated overnight. BMDM-conditioned media was transferred to LLC cells in serial 1:2 dilutions and incubated with vehicle or 5 μM SMC for 24h. LLC cell viability was determined by Alamar Blue. For the ELISA in parallel to the conditioned media, $5x10^4$ macrophages/well were seeded in a 96-well plate and treated with the same concentrations as for the conditioned media.

2.6.3: TNFα-neutralizing antibody

EMT6 cells were seeded at a density of $1x10^4$ cells/well in a 96-well plate and incubated overnight. Cells were pre-treated for 2h with isotype control IgG antibody or TNFα-neutralizing antibody (Bio X Cell). Cells were treated with BSA or 250 U/mL IFNβ and vehicle or 5 μM SMC for 48h. Cell viability was determined by Alamar Blue.

2.6.4: Small interfering RNA (siRNA) transfections

EMT6 cells were seeded at a density of $1x10^4$ cells/well in a 96-well plate or at a density of $3x10^5$ cells/well in a 6-well dish. Cells were reverse transfected for 48h with a final siRNA concentration of 10 nM. Reverse transfections were performed in DMEM with Opti-MEM and RNAiMax transfection reagent (Invitrogen). Non-targeting siRNA (Dharmacon, Lafayette, CO, USA) or siRNA for TNF-R1 (Dharmacon, ON-TARGET plus SMARTpool) were used. Cells were treated with BSA or 250 U/mL IFNβ and vehicle or 5μM SMC for 48h. 96-well plates were used for Alamar Blue viability assay following treatment and 6-well dishes were used to collect cells for Western immunoblotting.

SNB75 cells were seeded at a density of $3x10^5$ cells/well in a 6-well dish. Cells were reverse transfected for 48h with a final siRNA concentration of 10 nM as described above. Non-targeting siRNA or siRNA for TRAF2, TRAF3, TRAF6, STAT1 or IRF9 (Dharmacon, ON-TARGET plus SMARTpool) were used. Cells were treated with 1000 U/mL IFNβ, 1000 U/mL universal IFNα, 1 μg/mL poly(I:C) or 10 ng/mL LPS for 24h and supernatant was collected. Supernatant was concentrated using Amicon Ultra-4 Centrifugal Filters (EMD Millipore, Billerica, Massachusetts) and ELISAs for TNFα or TRAIL (R&D Systems) were performed using the concentrated supernatant. Cells were also harvested for Western immunoblotting.

2.6.5: Tumour-produced TNFα

EMT6-Fluc tumours were implanted (see section 2.5.1) and grown in BALB/c mice for 2 weeks. For *in vitro* treatment, tumours were dissociated by agitating in 1 mg/mL collagenase IV in RPMI-1640 media (as described for splenocyte culture) for 45 minutes at 37°C, then by passing through a 70 μm filter. ACK buffer was used to lyse red blood cells. $5x10^5$ tumour or EMT6-Fluc cells were incubated overnight in RPMI-1640 media then treated with 100 ng/mL IFNα B/D. Supernatant was collected and concentrated using Amicon Ultra-4 Centrifugation filters. The amount of TNFα was determined by ELISA. For *in vivo* treatment, mice were treated orally with vehicle or 50 mg/kg SMC, IT with 2 μg IFNα B/D for 6, 12, 16 or 24h or IP with 1 μg for 6h. Tumours were harvested and processed according to section 2.4.2. The amount of TNFα in the tumour was determined by ELISA.

2.6.6: Clodrosome-depleted splenocytes

BALB/c mice were injected intravenously with PBS, control liposomes or 0.625 mg Clodrosome (Encapsula Nanosciences). Spleens were removed 24h later and cultured as described above. Spleens were treated with 1 MOI of VSVΔ51 or VSV-mIFNβ-NIS, 1 μg/mL poly(I:C), 1000 U/mL IFNβ or 10 ng/mL LPS. The amount of TNFα (R&D Systems) or IFNβ (PBL Assay Science) in the supernatant was determined by ELISA.

2.7: Flow cytometry experiments

Appropriate unstained and single stained controls were incorporated into all experiments. Data from all flow cytometry experiments were analyzed using Kaluza (Beckman Coulter, Pasadena, CA, USA).

2.7.1: Splenocytes treated with VSVΔ51

Whole spleens were enriched for CD11b using the EasySep Mouse CD11b Positive Selection Kit (Stemcell, Vancouver, BC, Canada). $CD11b^-$ cells were enriched for CD49b

using the EasySep Mouse CD49b Positive Selection Kit (Stemcell). $CD11b^+$ cells were stained with F4/80-PE/Cy5 and Gr1-FITC then sorted using the MoFlo Astrios Sorter (Beckman Coulter). Sorted populations were treated with 1 MOI of VSVΔ51 for 24h. Conditioned media was transferred to EMT6 cells seeded at a density of $1x10^4$ cells/well with vehicle or 5 μM SMC for 24h. EMT6 cell viability was determined by Alamar Blue.

2.7.2: Macrophage depletion using Clodrosome

BALB/c mice bearing 2-week tumours (see section 2.5.1 for tumour implantation) were treated intravenously with control liposomes or 0.625 mg of Clodrosome (Encapsula Nanosciences) for 24 or 48h. Spleens, blood, lungs and tumours were removed and dissociated. Spleens and tumours were dissociated by mincing and passing the tissues through 70 μm mesh filters. Blood was obtained by cardiac puncture. Red blood cell lysis was performed using ACK lysis buffer, where whole blood was resuspended twice in 1 mL ACK lysis buffer for 2 minutes. Lungs were dissociated using 640 U/mL collagenase IV in RPMI-1640 media for 30 minutes at 37°C then passed through 70 μm mesh filter. Macrophage depletion in tissues was determined using CD11b-APC and F4/80-PE/Cy7 antibodies. Samples were analyzed using the Cyan ADP 9 Analyzer (Beckman Coulter).

2.7.3: Poly(I:C)-induced TNFα and IFNβ production

Whole spleens were enriched for CD11b using the EasySep Mouse CD11b Positive Selection Kit (Stemcell). Equivalent numbers of $CD11b^+$ and $CD11b^-$ cells were treated with PBS or 1 μg/mL poly(I:C) for 12h. GolgiPlug (BD Biosciences) was added 2h following poly(I:C) stimulation, for a total time in culture of 10h. Cells were stained with surface markers CD11c-FITC, F4/80-PE/Cy7, Ly6C-PerCP/Cy5.5, Ly6G-PerCP/Cy5.5, NK1.1-FITC, CD3-APC/Cy7, CD19-PE then permeabilized using the Cytofix/Cytoperm kit (BD Biosciences) according to manufacturer instructions. Permeabilized cells were stained for

intracellular cytokines using TNFα-APC and biotinylated anti-IFNβ/streptavidin-BV421. Samples were analyzed using the Cyan ADP 9 Analyzer (Beckman Coulter).

2.8: Statistical analysis

Sigmaplot 11.0 (Systat Software) was used to analyze data. Comparison of Kaplan-Meier survival plots was conducted by log-rank analysis then Holm-Sidak multiple comparisons were used to determine the statistical significance of survival experiments.

3: RESULTS

3.1: Vesicular stomatitis virus (VSV) synergizes with LCL-161

3.1.1: Derivatives of VSV are effective in vitro

Many oncolytic viruses are being designed for the use as specific anti-cancer treatments. There are some limitations to the use of oncolytic viruses as standalone therapies. Notably, the risk of viremia in the case of uncontrolled oncolytic viral replication poses a safety hazard (89). In human patients most oncolytic viruses have only been shown to induce modest tumour regression (66, 67). To our benefit, we have found that effective agents for synergy with SMCs do not necessarily need to lead to extensive killing of cancer cells when used as a monotherapy. However, when combined with a SMC, these synergizing agents demonstrate the ability to induce apoptosis in cancer cells at relatively low concentrations. Accordingly, we prefer to use oncolytic viruses as immune stimulants which can be paired with SMCs to reduce the risk of viremia and to create a therapy that is more effective than each treatment alone. It was previously shown that VSVΔ51 effectively synergizes with LCL-161, a monovalent SMC, to kill cancer cells *in vitro* and *in vivo* (60) (Figure 7a).

I was interested in investigating the use of other derivatives of VSV, specifically those expressing the type I interferon, IFNβ, because variants of this particular virus are undergoing clinical evaluations in patients (89). Two variants were tested for synergy with SMCs: one expressing mouse IFNβ (VSV-mIFNβ) and the other expressing mIFNβ and the sodium iodide symporter (NIS) gene. The NIS transgene can be used for *in vivo* tracking of virus spread by positron emission tomography (PET) scan but does not affect its infectivity or ability to replicate (90). Henceforth this latter virus will be referred to as VSV-mIFNβ-NIS. These transgenes are expressed upon infection of a host cell and appropriation of the

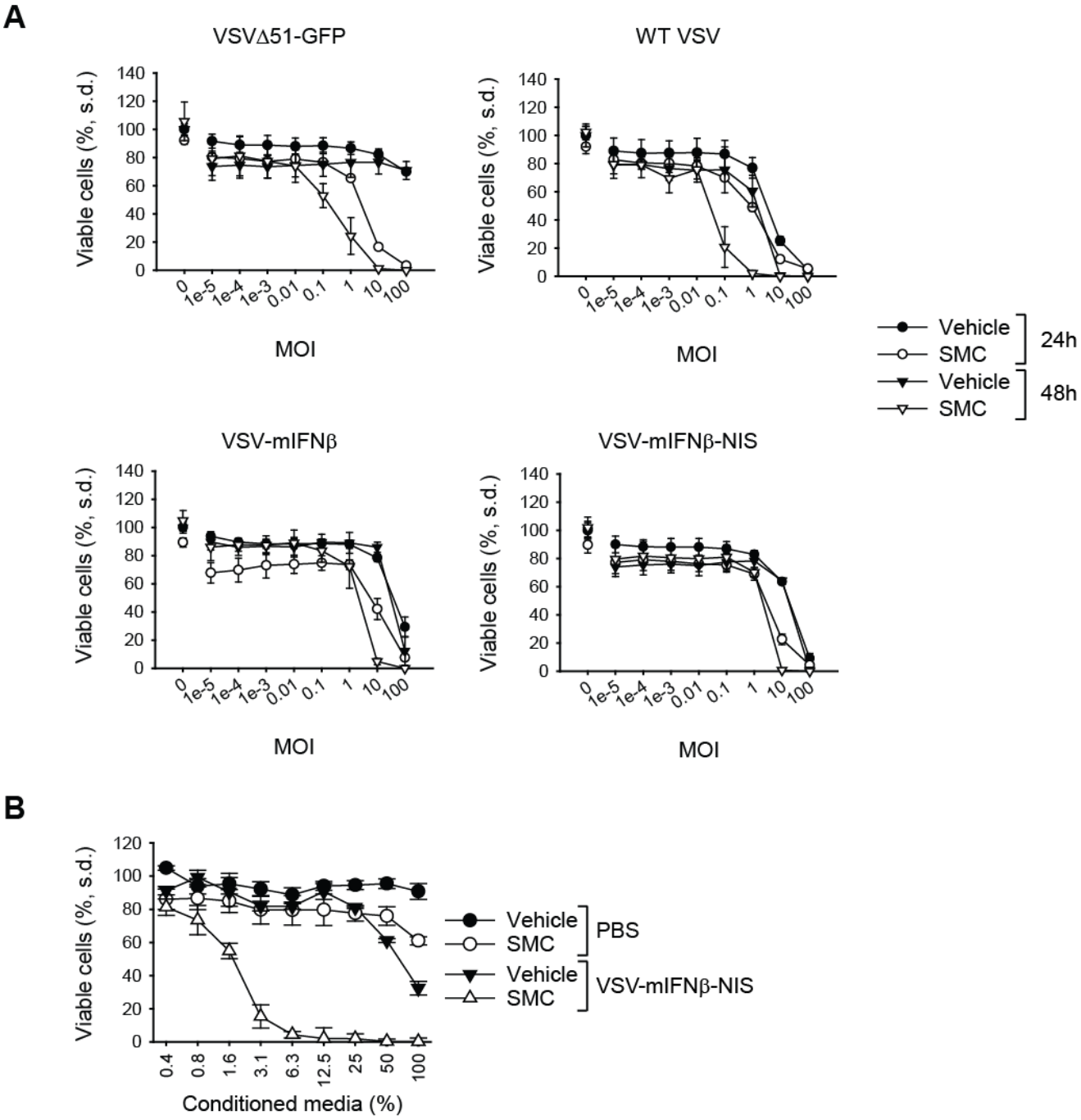


Figure 7. VSV derivative viruses synergize with SMCs in vitro. (A) EMT6 cells were treated with various MOI of the indicated virus and with vehicle or 5 μM SMC for 24h or 48h. **(B)** Conditioned media was generated from EMT6 cells treated with PBS or 3 MOI of VSV-mIFNβ-NIS for 24h. Conditioned media was exposed to UV light for 1h to inactivate any residual virus. Supernatant was transferred to new EMT6 cells with vehicle or 5 μM SMC for 48h. All panels: Viability was determined by Alamar Blue.

host protein synthesis machinery. *In vivo*, these viruses reintroduce IFNβ to the site of infection and, as a result, control viral spread and increase the recruitment of immune cells.

Both VSV-mIFNβ and VSV-mIFNβ-NIS synergized with LCL-161 in EMT6 cells, with an increase of cell death at a log lower dose in combination treatment compared to virus alone (Figure 7a). These two viruses also showed increased oncolytic activity than VSVΔ51. Because VSV-mIFNβ and VSV-mIFNβ-NIS are made using the wild-type (WT) VSV backbone, WT VSV was used as a comparison for efficacy of the VSV-mIFNβ viruses. WT-VSV displays greater lytic activity than the other viruses but also demonstrated synergy with LCL-161 at a later time point (48 hours) (Figure 7a), although this may be the result of direct lysis of the EMT6 cells rather than cytokine release from infected but intact cells. This should be confirmed through the use of cytokine-neutralizing antibodies.

It was previously shown that most of the cancer cells were killed via cytokine production from only a few infected cells following treatment with VSVΔ51 and LCL-161 (60). To mimic this scenario *in vitro* and determine if VSV-mIFNβ-NIS is also capable of killing cancer cells via a bystander effect, a conditioned media assay was performed using this virus. VSV-mIFNβ-NIS demonstrates a very high production of cytokines from EMT6 cells that can then synergize with SMCs to kill new EMT6 cells in culture, as shown by the decreased viability of these cancer cells at concentrations of conditioned media as low as 1.6% (Figure 7b). The death of these cells is not a result of virus-induced lysis because the virus was inactivated by UV-irradiation prior to the transfer of supernatant to new cells.

3.1.2: VSV derivatives are effective in vivo

It was previously demonstrated that VSVΔ51 effectively synergizes with LCL-161 to induce tumour regression in an EMT6-Fluc (Firefly luciferase) mammary carcinoma syngeneic mouse model. It was also demonstrated that this effect is dependent on TNFα

signalling (60). Because type I IFN is the primary antiviral molecule and was likely to be released in large amount following treatment with a virus, I hypothesized that the type I IFN is the main mediator of SMC efficacy by leading to production of TNFα and other inflammatory cytokines. I then investigated whether the expression of virus-derived IFNβ lead to the production of TNFα. BALB/c mice bearing syngeneic EMT6-Fluc tumours were treated intratumourally (IT) with PBS, $1x10^8$ PFU of VSV-mIFNβ-NIS or WT VSV, in the presence or absence of LCL-161 (Figure 8a). WT VSV was used as a comparison because VSV-mIFNβ-NIS was generated on a WT VSV backbone, whereas VSVΔ51 has been modified by deleting the 51^{st} amino acid on the matrix protein; as a consequence, IFN release induced by each of these viruses uses a different mechanism (64). Mice treated with the combination therapy of either virus showed decreased tumour volume (Figure 8b, left panel) and statistically significant increase in survival (Figure 8b, right panel) compared to untreated or single-treated groups. WT VSV combination therapy demonstrated a 50% increase in survival and VSV-mIFNβ-NIS combination therapy showed a 35% increase in survival. Survival between these two groups was very similar until one mouse treated with VSV-mIFNβ-NIS combination therapy displayed latent tumour regrowth.

3.2: Recombinant type I interferon synergizes with LCL-161

3.2.1: Various type I IFNs synergize in vitro

Because both VSV-mIFNβ and VSVΔ51 enable cells to mount a primary antiviral response via the production of type I IFN and because both these viruses show synergy with LCL-161, I hypothesized that recombinant type I IFNs can synergize with LCL-161. The effects of three recombinant type I IFNs were assessed in the presence or absence of LCL-161 on EMT6 cells in culture: (1) universal IFNα which is a hybrid of human IFNα A and human IFNα D, (2) IFNα B/D which is a hybrid of human IFNα B and human IFNα D (91)

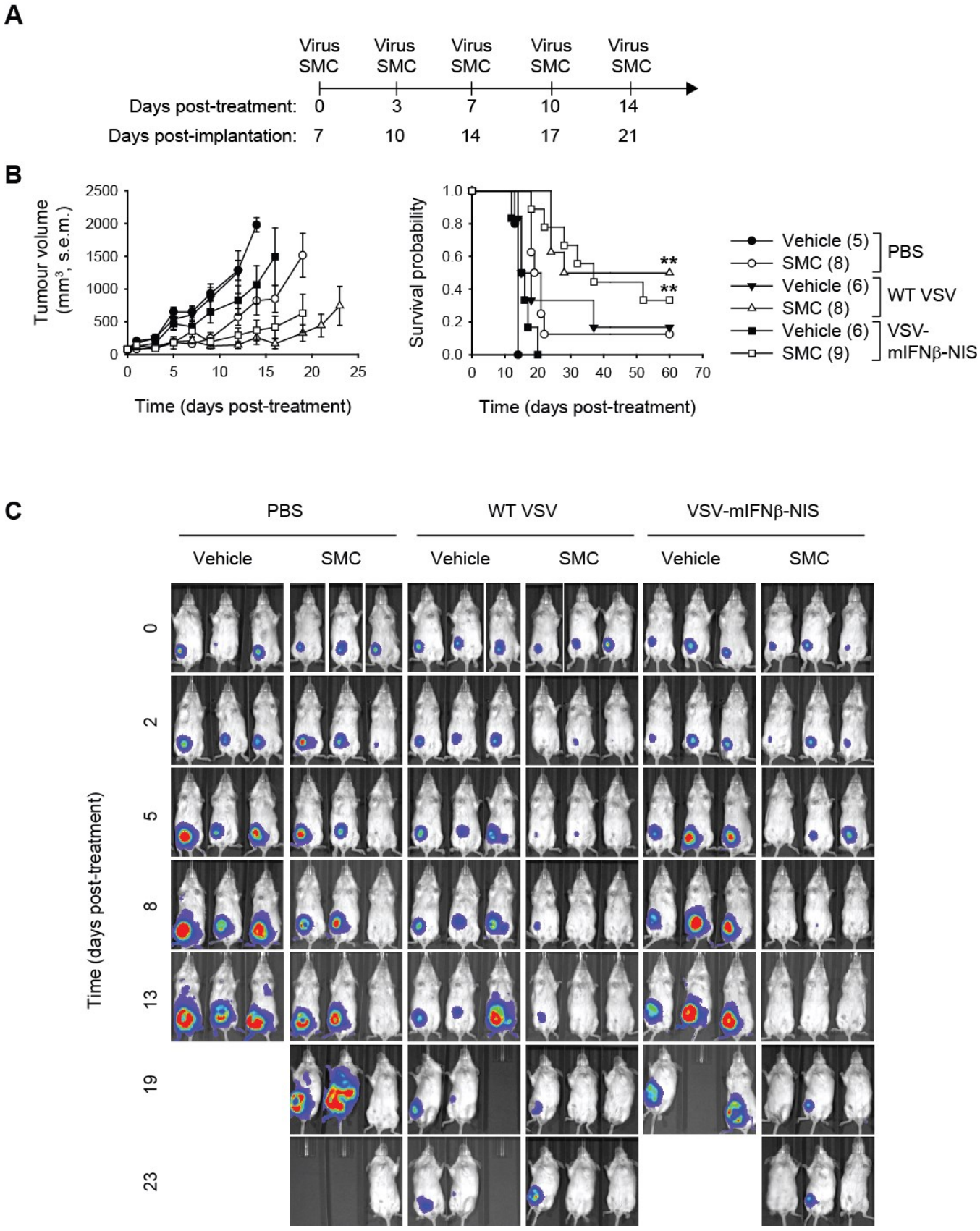

A
Virus SMC
Virus SMC
Virus SMC
Virus SMC
Virus SMC
Days post-treatment: 0 3 7 10 14
Days post-implantation: 7 10 14 17 21
B
Tumour volume (mm3, s.e.m.)
Time (days post-treatment)
Survival probability
Time (days post-treatment)
**
**
Vehicle (5)
SMC (8)
PBS
Vehicle (6)
SMC (8)
WT VSV
Vehicle (6)
SMC (9)
VSV-mIFNβ-NIS
C
PBS
WT VSV
VSV-mIFNβ-NIS
Vehicle
SMC
Vehicle
SMC
Vehicle
SMC
Time (days post-treatment)
0
2
5
8
13
19
23

Figure 8. VSV synergizes with LCL-161 *in vivo*. (A) BALB/c mice bearing EMT6-Fluc tumours were treated intratumourally (IT) with PBS, $1x10^8$ PFU of WT VSV or VSV-IFNβ-NIS and orally with vehicle or 50 mg/kg of LCL-161 (SMC) according to the timeline. **(B)** Left panel: Tumour growth of mice from (A), measured with calipers. Right panel: Kaplan Meier survival curve of mice from (A). Log-rank with Holm-Sidak multiple comparisons. **, $p < 0.01$. The number of mice per treatment group is indicated in brackets. **(C)** Representative luminescent IVIS images.

and (3) mouse IFNβ. The human hybrid IFNα proteins are cross-reactive to mouse cells. Universal IFNα is commercially available and has been shown to elicit type I IFN responses in mouse cells (92, 93). IFNα B/D has been shown to bind to the mouse type I IFN receptor (IFNAR), induce type I IFN signal transduction pathways and effectively control viral infections in mice (94–96). To date, IFNα B/D has not been studied as an anti-cancer agent or immune stimulant.

All three types of IFN were effective to induce death of EMT6 cells when combined with LCL-161 (Figure 9). Recombinant mouse IFNβ (bottom panel) and universal IFNα (top panel) showed similar levels of efficacy, with a 50% reduction in cancer cell viability at a concentration of 500 U/mL and near complete death of EMT6 cells at concentration of 1000 U/mL. On the other hand, it was shown that IFNα B/D only induces a small synergistic effect with LCL-161 after 48 hours treatment, with a 30% reduction in viability of EMT6 cells at a concentration of 100 ng/mL (middle panel).

3.2.2: IFNα B/D synergizes with LCL-161 to kill EMT6 tumours

Because human IFNα B/D has been shown to be effective at controlling viral infections in a mouse model (94–96) by inducing type I IFN responses, we investigated the efficacy of this agent as an immune stimulant in SMC combination therapy for treatment of the EMT6-Fluc syngeneic mammary carcinoma model. This protein was provided in a clinical-grade formulation and the results with which can be easily translated into clinic for human use. Mice were treated twice with one 2 μg dose IT or two 1 μg doses intraperitoneally (IP) of BSA or IFNα B/D, and orally with vehicle or 50 mg/kg of LCL-161 (Figure 10a). Mice were injected with BSA both IT and IP to determine if disruption of the

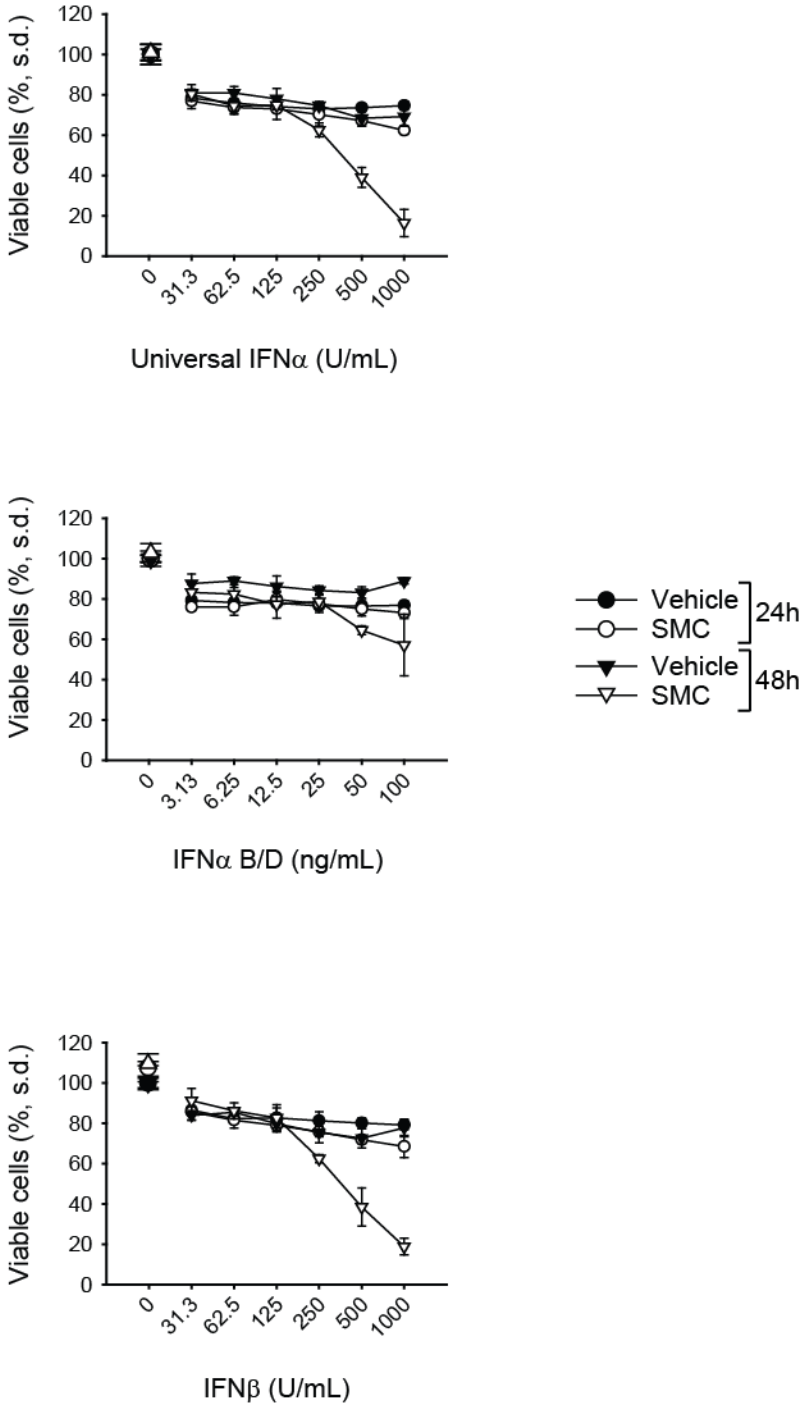
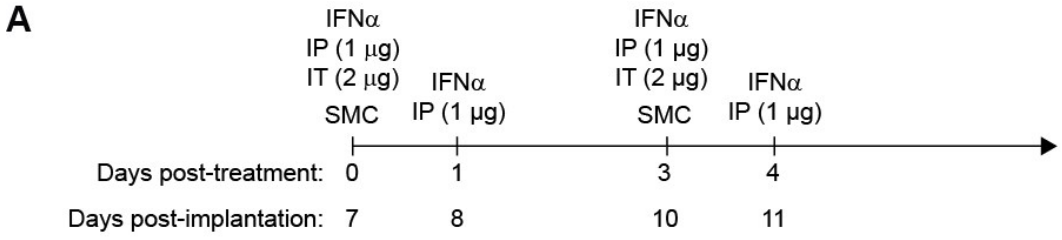
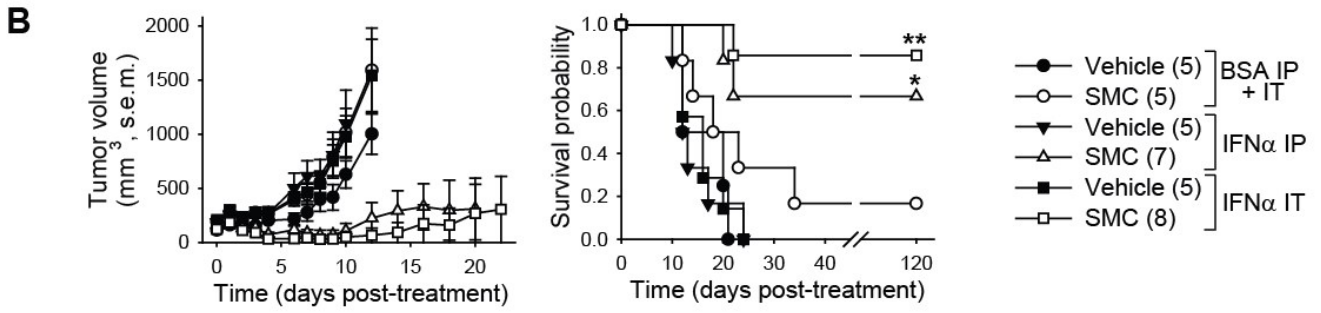


Figure 9. Recombinant type I IFNs synergize with SMCs in vitro. EMT6 cells were treated with various concentrations of the indicated recombinant type I IFN with vehicle or 5 μM SMC for 24h or 48h. Viability was determined by Alamar Blue.


A
IFNα
IP (1 μg)
IT (2 μg)
SMC
IFNα
IP (1 μg)
IFNα
IP (1 μg)
IT (2 μg)
SMC
IFNα
IP (1 μg)
Days post-treatment: 0 1 3 4
Days post-implantation: 7 8 10 11


B
Tumor volume (mm3, s.e.m.)
2000
1500
1000
500
0
0 5 10 15 20
Time (days post-treatment)
Survival probability
1.0
0.8
0.6
0.4
0.2
0.0
**
*
0 10 20 30 40 120
Time (days post-treatment)
Vehicle (5)
SMC (5)
BSA IP + IT
Vehicle (5)
SMC (7)
IFNα IP
Vehicle (5)
SMC (8)
IFNα IT

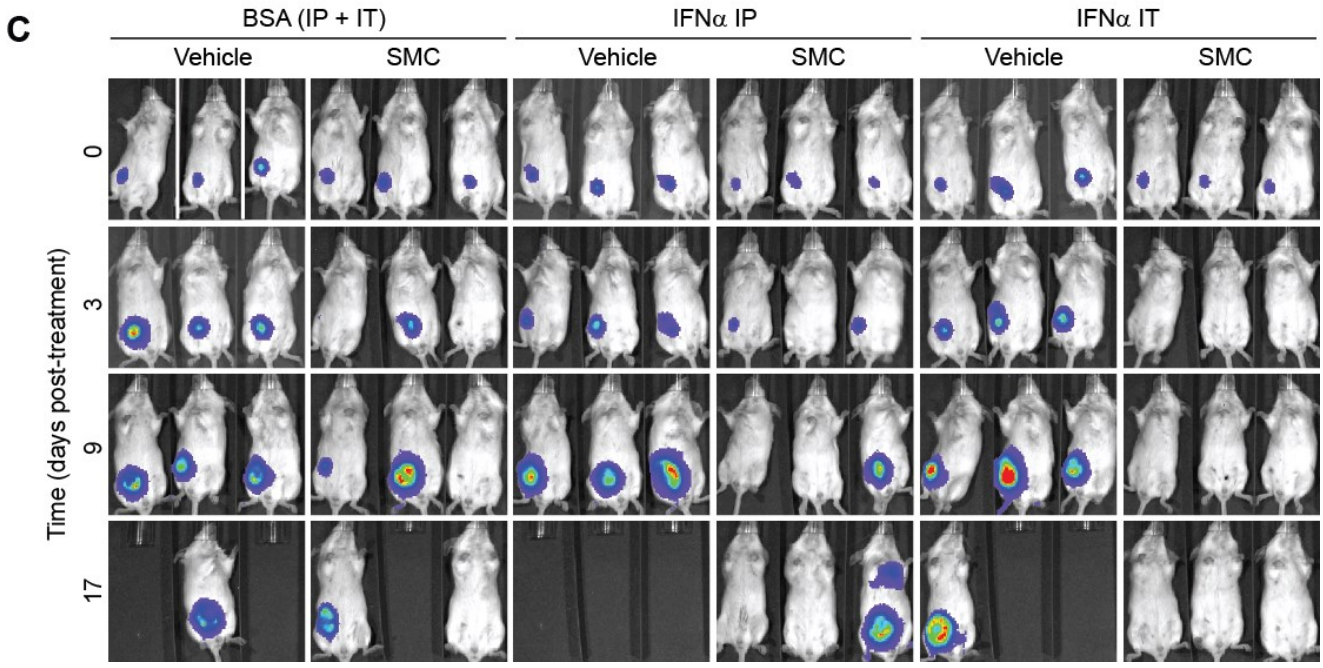

C
BSA (IP + IT)
IFNα IP
IFNα IT
Vehicle
SMC
Vehicle
SMC
Vehicle
SMC
Time (days post-treatment)
0
3
9
17

Figure 10. SMCs synergize with recombinant type I IFN to cause mammary tumour regression. (A) BALB/c mice bearing EMT6-Fluc tumours were treated with BSA, intratumourally (IT) with one 2 μg dose or intraperitoneally (IP) with two 1 μg doses of IFNα B/D, and orally with vehicle or 50 mg/kg of LCL-161 (SMC) according to the timeline. **(B)** Left panel: Tumour growth of mice from (A), measured with calipers. Right panel: Kaplan-Meier curve depicting mouse survival. Log-rank with Holm-Sidak multiple comparisons. *, $p < 0.05$; **, $p < 0.01$. The number of mice per treatment group is indicated in brackets. **(C)** Representative luminescent IVIS images.

tumour microenvironment affected tumour growth. Groups that received combination therapy showed decreased tumour volume (Figure 10b, left panel) and statistically significant increased survival (Figure 10b, right panel) compared to controls. Remarkably, durable cures were seen in in 85% and in 70% of mice treated IT and IP, respectively, with IFNα B/D and LCL-161. No mice were cured with IFNα B/D alone. Notably, the curative effects of the combination treatment were rapid, as a loss of measurable Fluc activity was evident by three days post-treatment.

3.2.3: IFNα B/D has no effect upon LLC tumours

It is important to determine if IFNα B/D is also effective in other mouse models. Accordingly, C57BL/6 mice bearing Lewis lung carcinoma (LLC) syngeneic tumours were treated with LCL-161 and IFNα B/D (Figure 11b). Notably, LLC cells die only in response to the combination of TNFα and LCL-161 in culture and other cytokines such as type I IFN does not synergize with SMC (Figure 11a). Therefore, this model presents an ideal opportunity to determine if IFNα B/D treatment leads to the death of cancer cells via the production of TNFα *in vivo*. There was no decrease in tumour burden (Figure 11c, left panel) or increase in survival (Figure 11c, right panel) in groups treated with IFNα B/D and SMC compared to the controls. We have seen that the LLC syngeneic tumour model does not respond well to treatment (data not published). We have not determined the cause of the lack of efficacy but it may be related to the inherent differences between the C57BL/6 and BALB/c immune systems. BALB/c mice produce higher levels of TNFα and have higher counts of macrophages in the spleen compared to C57BL/6 mice (97). It is also possible that LLC cells undergo a phenotype shift once implanted *in vivo* and become resistant to TNFα and SMC treatment.

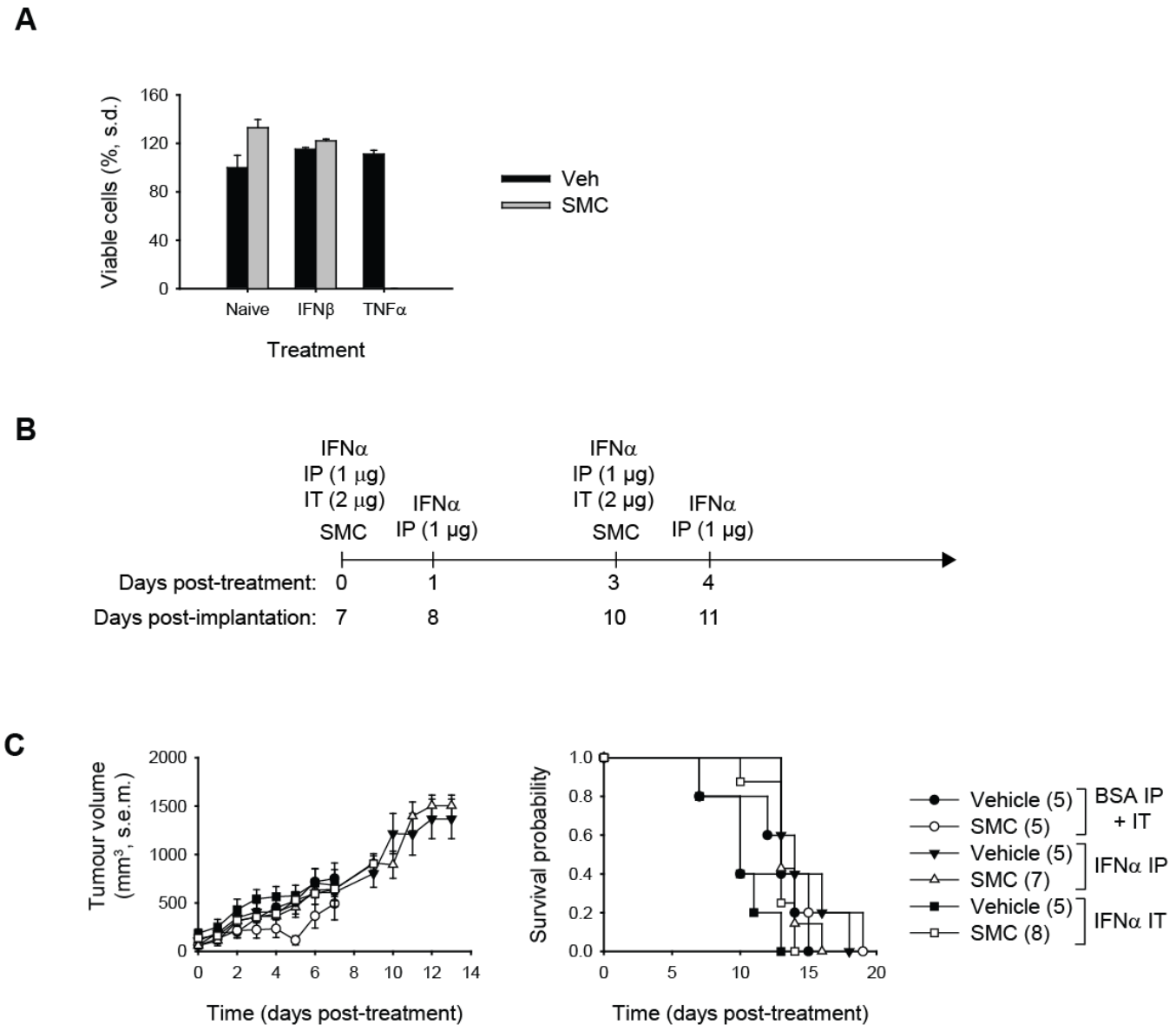


Figure 11. Lewis lung carcinoma (LLC) cells do not respond to type I IFN treatment. **(A)** LLC cells were treated with 1000 U/mL IFNβ or 10 ng/mL TNFα with vehicle or 5 μM SMC for 48h. Viability was determined by Alamar Blue. **(B)** C57BL/6 mice bearing LLC tumours were treated with BSA, intratumourally (IT) with one 2 μg dose or intraperitoneally (IP) with two 1 μg doses of IFNα B/D, and orally with vehicle or 50 mg/kg of LCL-161 (SMC) according to the timeline. **(C)** Left panel: Tumour volume of mice in (A), measured with calipers. Right panel: Kaplan-Meier curve depicting mouse survival. Log-rank with Holm-Sidak multiple comparisons. The number of animals per treatment group is indicated in brackets.

3.2.4: Mouse IFNβ does not synergize with LCL-161 to kill EMT6 tumours

IFNβ is the most potent of the type I IFN family, as it has the highest binding capacity to the type I IFN receptor and leads to the highest activity of the ISGF3 complex (76). Hence, to determine whether the ability of IFNβ to induce expression of type I IFN target genes corresponds to the ability to provide better synergy with LCL-161 *in vivo* compared to IFNα B/D, we administered mIFNβ to BALB/c mice bearing EMT6-Fluc tumours. The dosing schedule and amount was reduced to 1 μg of mIFNβ per treatment either IT or IP, due to limitations in available material (Figure 12a). Overall, no decrease in tumour burden (Figure 12b, left panel) or a statistically significant increase in survival (Figure 12b, right panel) was evident in these mice. However, it is uncertain whether the lack of efficacy is due to the difference in activity of recombinant mIFNβ compared to IFNα B/D, or whether it is due to the difference in the amount of drug administered.

3.3: The efficacy of IFNα B/D and LCL-161 combination therapy is dependent on TNFα signalling

EMT6 cells can be killed by treatment with type I IFNs and SMCs. This can be attributed to two possible mechanisms: 1) Type I IFNs synergize directly with SMCs to induce death in these cells or 2) Type I IFN treatment leads to the production of other cytokines that can then synergize with the SMC. Based on previous reports, I hypothesized that SMC-mediated death of cancer cells is due to the IFN-mediated production of TNFα (60). To determine how TNFα signalling affects the outcome of type I IFN and LCL-161 combination therapy *in vitro*, the TNFα receptor, TNF-R1, was downregulated using siRNA in EMT6 cells. These cells were then treated with IFNβ and LCL-161 for 48 hours. Cells in which TNF-R1 had been knocked down showed an 80% rescue in viability compared to cells transfected with non-targeting (NT) siRNA (Figure 13a). In parallel, EMT6 cells were pre-

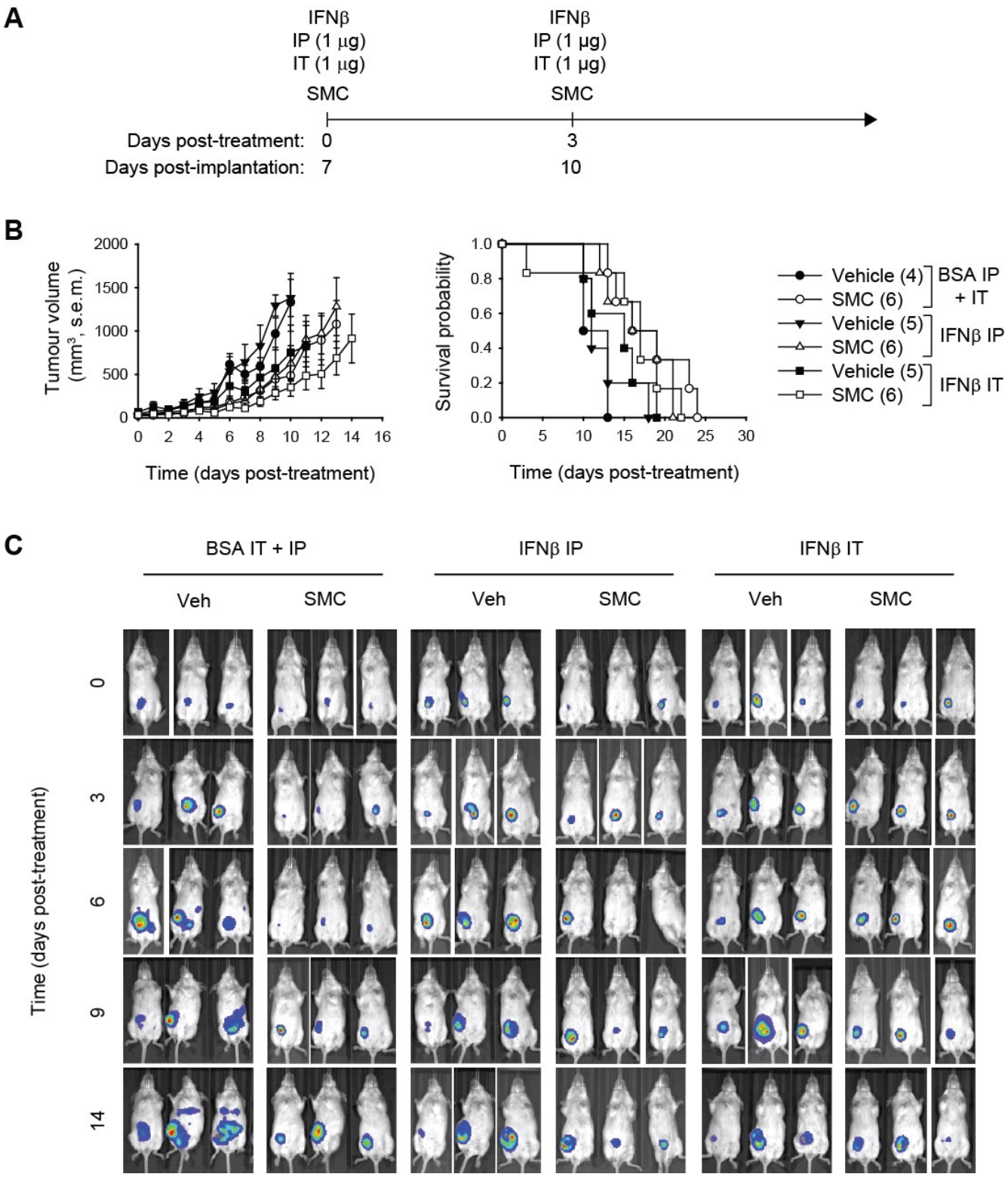

A
IFNβ
IP (1 μg)
IT (1 μg)
SMC
IFNβ
IP (1 μg)
IT (1 μg)
SMC
Days post-treatment: 0 3
Days post-implantation: 7 10
B
Tumour volume (mm³, s.e.m.)
Time (days post-treatment)
Survival probability
Time (days post-treatment)
Vehicle (4)
SMC (6)
BSA IP + IT
Vehicle (5)
SMC (6)
IFNβ IP
Vehicle (5)
SMC (6)
IFNβ IT
C
BSA IT + IP
IFNβ IP
IFNβ IT
Veh
SMC
Time (days post-treatment)
0
3
6
9
14

Figure 12. IFNβ has little effect on tumor cell growth in vivo. (A) BALB/c mice bearing EMT6-Fluc tumours were treated with BSA, 1 μg IFNβ intraperitoneally (IP) or 1 μg IFNβ intratumourally (IT) and orally with vehicle or 50 mg/kg LCL-161 (SMC) according to the timeline. **(B)** Left panel: Tumour growth of mice from (A), measured with calipers. Right panel: Kaplan Meier survival curve of mice from (A). Log-rank with Holm-Sidak multiple comparisons. The number of mice per group is indicated in brackets. **(C)** Representative luminescent IVIS images.

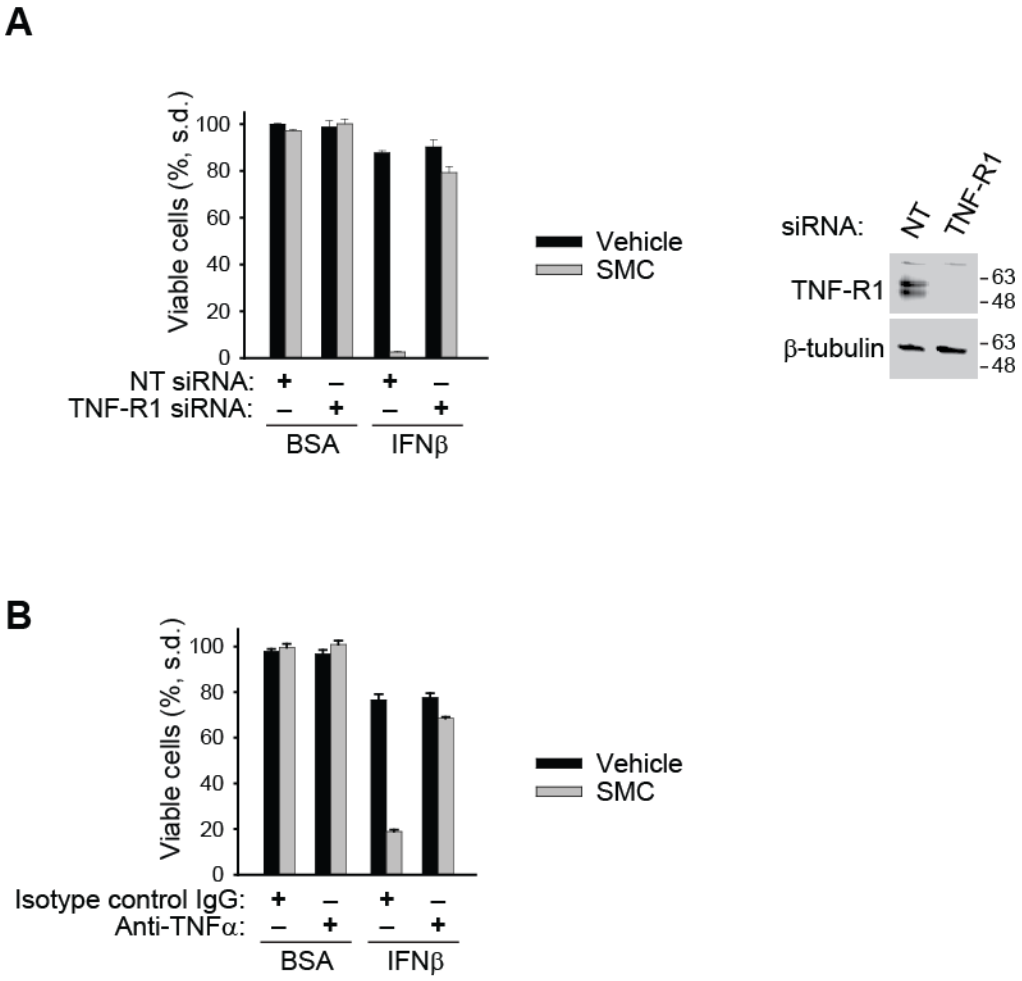


Figure 13. TNFα signalling is required for IFNβ-mediated cell death. (A) EMT6 cells were transfected for 48h with non-targeting (NT) or TNFα-receptor (TNF-R1) siRNA, then treated with 250 U/mL of IFNβ and 5 μM LCL-161 (SMC) for 48h. Western blot to confirm TNF-R1 knockdown was performed. **(B)** EMT6 cells were pre-treated with TNFα-neutralizing or isotype control IgG antibodies for 2h, then treated with BSA or 250 U/mL IFNβ and vehicle or 5 μM SMC for 48h. All panels: Viability assessed by Alamar Blue. Figure published in Beug, S. *et al*., *Nature Biotechnology* (2014).

treated with a TNFα-neutralizing antibody, then treated with IFNβ and LCL-161 for 48h. Cells treated with the TNFα-neutralizing antibody displayed a 75% rescue in viability compared to cells treated with an isotype control antibody (Figure 13b). Together, these results demonstrate that the anti-cancer effects seen with IFNβ and SMC treatment is dependent on the presence of intact TNFα signalling.

The requirement for TNFα signalling following type I IFN treatment is simple to demonstrate *in vitro*. However, it is important to determine if this phenomenon is also applicable *in vivo*. Because the combination of SMC and IFNα B/D was efficacious *in vivo*, IFNα B/D was used to determine the role of TNFα in response to type I IFN treatment. BALB/c mice bearing EMT6-Fluc tumours were treated with combinations of with IFNα B/D and LCL-161 and with either 0.5 mg of a TNFα-neutralizing antibody or isotype control antibody (Figure 14a). As expected, 60% of mice treated IT and 30% of mice treated IP with IFNα B/D, SMC and the control antibody showed an decreased in tumour size (Figure 14b, left panel) and a statistically significant increase in survival (Figure 14b, right panel) compared to controls. The synergistic effects were abrogated when the mice were treated with the TNFα-neutralizing antibody, as shown by a reduction in survival to 20% for both IT and IP treated groups (Figure 16b, right panel). These results demonstrate that type I IFN and SMC combination treatment *in vivo* is largely dependent on TNFα signalling. The difference between IT- and IP-treated groups suggests that TNFα released from the tumour is the main mediator of SMC combination treatment efficacy.

3.4: Contribution of the tumour for TNFα production

3.4.1: Tumours produce TNFα

As EMT6 cells are capable of producing cytokines that can synergize with SMCs following stimulation with oncolytic viruses, recombinant proteins and synthetic nucleic

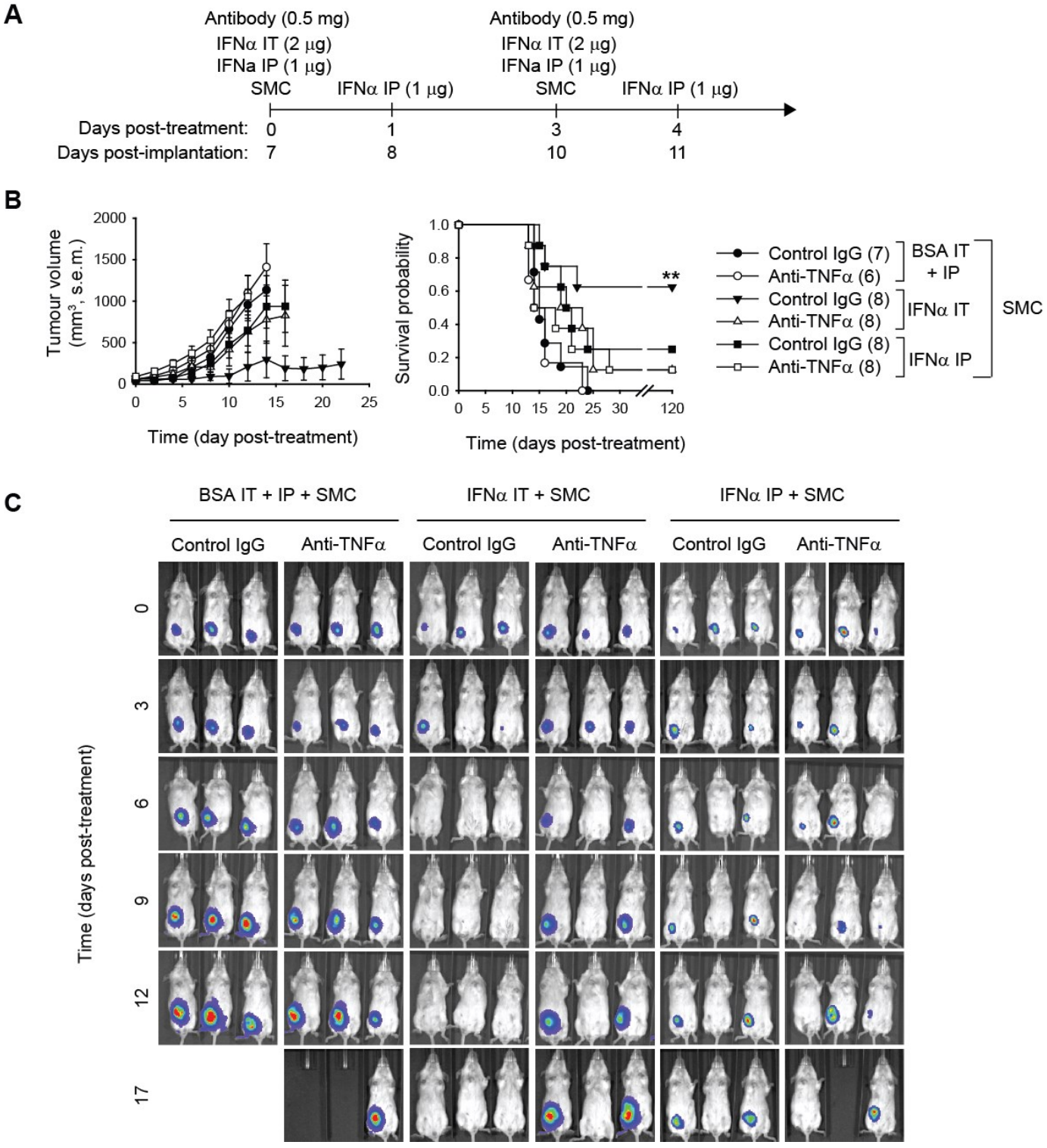

A
Antibody (0.5 mg)
IFNα IT (2 μg)
IFNa IP (1 μg)
SMC
IFNα IP (1 μg)
Antibody (0.5 mg)
IFNα IT (2 μg)
IFNa IP (1 μg)
SMC
IFNα IP (1 μg)
Days post-treatment: 0 1 3 4
Days post-implantation: 7 8 10 11
B
Tumour volume (mm3, s.e.m.)
Time (day post-treatment)
Survival probability
**
Time (days post-treatment)
Control IgG (7)
Anti-TNFα (6)
BSA IT + IP
Control IgG (8)
Anti-TNFα (8)
IFNα IT
Control IgG (8)
Anti-TNFα (8)
IFNα IP
SMC
C
BSA IT + IP + SMC
IFNα IT + SMC
IFNα IP + SMC
Control IgG
Anti-TNFα
Time (days post-treatment)

Figure 14. Efficacy of IFNα B/D and SMC combination therapy is dependent on TNFα. **(A)** BALB/c mice bearing EMT6-Fluc tumours were treated intraperitoneally (IP) with 0.5 mg anti-TNFα or control IgG antibodies, intratumourally (IT) with one 2 μg dose of IFNα B/D or intraperitoneally with two 1 μg doses of IFNα B/D, and orally with 50 mg/kg LCL-161 (SMC) as indicated on the timeline. **(B)** Left panel: Tumour volume of mice from (A), measured with calipers. Right panel: Kaplan-Meier survival curve of mice from (A). Log-rank with Holm-Sidak multiple comparisons. **, $p < 0.01$ compared to control. The number of animals per treatment group is indicated in brackets. **(C)** Representative luminescent IVIS images.

acids, it was important to determine the contribution of the tumour to cytokine production *in vivo*. To measure the amount of TNFα produced in the tumour, two methods were employed. The first approach was the *in vitro* treatment of a dissociated tumour and the second method was the *in vivo* treatment of a tumour-bearing mouse. EMT6-Fluc tumours were grown in BALB/c mice for two weeks, then removed or treated. TNFα production was then determined by ELISA. In comparison to cultured EMT6-Fluc cells, dissociated EMT6-Fluc cells from tumours produced elevated basal levels of TNFα *in vitro*. However, there was no increase in the production of TNFα following *in vitro* treatment with 100 ng/mL of IFNα B/D (Figure 15a). The lack of TNFα production may be the result of the lacklustre stimulatory activity of IFNα B/D treatment *in vitro*. In parallel, mice bearing EMT6-Fluc tumours were treated IT with 2 μg of IFNα B/D. At the designated time points, tumours were harvested and flash frozen in liquid nitrogen. Tumours were then homogenized and the protein extracted. Two mice were used per time point. Similar to the *in vitro* stimulation, there was no increase in TNFα production following *in vivo* stimulation with IFNα B/D over the course of treatment (Figure 15b).

3.4.2: LCL-161 affects TNFα production from tumours

Due to the unexpected result that there was no difference in TNFα production from the tumour, I hypothesized that SMC treatment may enhance TNFα production following stimulation. Notably, I have observed that intratumoural stimulation can lead to systemic responses (Figures 8, 10, 14) and an increase in TNFα from cancer cells treated with LCL-161 and VSVΔ51 or IFNβ (60). Therefore, I assessed for the amount of TNFα in serum as well as the tumour following either IT or IP administration of IFNα B/D in combination with LCL-161. Interestingly, the amount of TNFα in the serum increased following IT or IP treatment with IFNα B/D (Figure 15c). Perhaps more importantly, this response was only

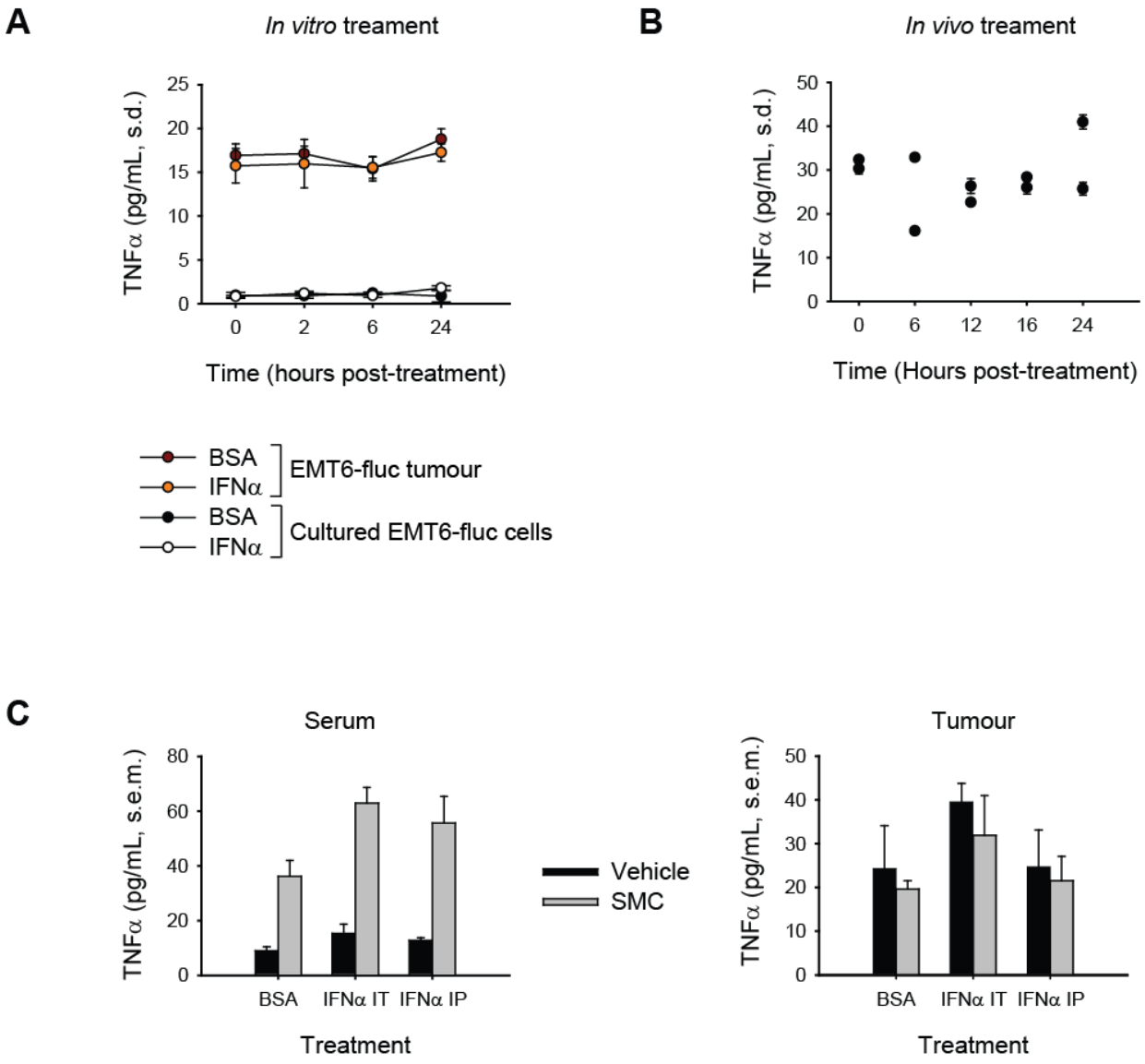

A
In vitro treament
TNFα (pg/mL, s.d.)
25
20
15
10
5
0
0
2
6
24
Time (hours post-treatment)
BSA
IFNα
EMT6-fluc tumour
BSA
IFNα
Cultured EMT6-fluc cells
B
In vivo treament
TNFα (pg/mL, s.d.)
50
40
30
20
10
0
0
6
12
16
24
Time (Hours post-treatment)
C
Serum
TNFα (pg/mL, s.e.m.)
80
60
40
20
0
BSA
IFNα IT
IFNα IP
Treatment
Vehicle
SMC
Tumour
TNFα (pg/mL, s.e.m.)
50
40
30
20
10
0
BSA
IFNα IT
IFNα IP
Treatment

Figure 15. Tumours produce TNFα in response to treatment. (A) EMT6-Fluc tumours were grown for 2 weeks in BALB/c mice. Tumours were removed, dissociated with collagenase IV and allowed to adhere overnight. Cultured tumours were treated with 100 ng/mL of IFNα B/D for 24h. EMT6-Fluc cells (not from tumours) were cultured in parallel and treated in the same manner. Supernatant was harvested from both cell types at the indicated times and concentrated by centrifugation. The amount of TNFα was determined by ELISA. **(B)** EMT6-Fluc tumours were grown for 2 weeks in BALB/c mice. Mice were treated intratumourally with 2 μg of IFNα B/D for 24h. Tumours were harvested according to the time course then flash frozen in liquid nitrogen. Tumours were homogenized and the protein extracted. An ELISA for TNFα was performed on the homogenates. **(C)** EMT6-Fluc tumours were grown for 2 weeks in BALB/c mice. Mice were treated with BSA, intratumourally (IT) with 2 μg IFNα B/D or intraperitoneally (IP) with 1 μg of IFNα B/D and orally with vehicle or 50 mg/kg LCL-161 (SMC) for 6h. Serum and tumours were harvested. Tumours were homogenized as above. An ELISA for TNFα was performed using the serum and tumour homogenates. Serum and tumours were taken from the same mice; three biological replicates were used.

seen when LCL-161 was also administered. There was very little increase in serum TNFα in vehicle-treated mice (Figure 15c, left panel).

The production of TNFα from the tumour is less well-defined. There is a mild increase in TNFα from IT treated mice. However, this increase of TNF seems to be blunted when with the inclusion of LCL-161. Mice treated IP with IFN did not show much increase in tumour TNFα in either vehicle or SMC groups (Figure 15c, right panel). Importantly, due to the variation that occurs *in vivo*, greater than three mice per group is necessary to determine the significance of these trends.

3.5: Immune cell involvement

As total tumour regression can be seen as soon as three days following treatment (Figures 8, 10, 14, 19), I hypothesized that innate immune cells are responsible for the production of cytokines that can synergize with SMCs. This short time span is insufficient for a robust, specific adaptive response to be mounted against the tumour cells (98). Preliminary data suggest that, of immune cell populations isolated from splenocytes, macrophages potentially contribute the greatest degree of cytokine production compared to the other profiled cell types in response to infection with VSVΔ51 *in vitro* (Figure 16). However, some cell types, such as DCs and monocytes, were not investigated but might also play a role and require further study.

3.5.1: Depletion of phagocytic cells using Clodrosome

A method to determine if innate immune cells such as macrophages and DCs are involved in the response to certain immunostimulants is to treat mice with Clodrosome (liposomal clodronate). Administration of Clodrosome depletes phagocytic cells (primarily macrophages, but also DCs). I then decided to assess subsequent *in vitro* responses via the culture of splenocytes, or *in vivo* responses following treatment with combination therapy.

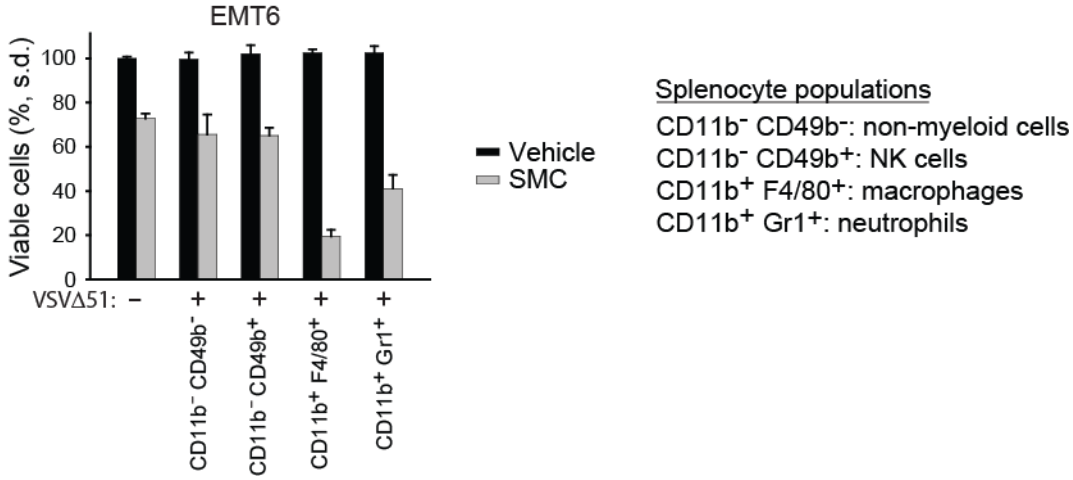


Figure 16. Innate immune cells produce cytokines that can synergize with SMCs following stimulation with VSVΔ51. A CD11b positive selection kit was used to enrich for $CD11b^+$ cells from whole spleens. $CD11b^-$ cells were enriched for CD49b. Myeloid subpopulations were stained and sorted from enriched populations by FACS, then were infected *in vitro* with 1 MOI of VSVΔ51 for 24h. Supernatant was transferred to EMT6 cells with vehicle or 5 μM LCL-161 (SMC) for 24h. Viability of EMT6 cells was determined by Alamar Blue. Figure published in Beug, S. *et al.*, *Nature Biotechnology* (2014).

First, I wanted to confirm whether treatment with Clodrosome effectively depleted macrophages from various tissues. BALB/c mice bearing EMT6-Fluc tumours were treated intravenously (IV) with control liposomes or Clodrosome. Spleen, blood, tumour and lung were assessed for the presence of $CD11b^{+}$ $F4/80^{+}$ macrophages 24 or 48 hours after administration. Notably, only the spleen showed depletion of macrophages, while the tumour, blood and lungs remained unaffected (Figure 17). In the spleen, there was a reduction in $CD11b^{+}$ $F4/80^{high}$ cells and, interestingly, there was also an increase in the $CD11b^{+}$ $F4/80^{-}$ population. This suggests that there are other myeloid cell populations unaffected by the treatment, and they may attempt to compensate for the loss of macrophages. Lungs and tumour were assessed to determine if Clodrosome was capable of affecting tissue-resident macrophages following IV administration. No difference in tissue-specific macrophages was detected. These results show that to target tissue-specific macrophages, such as tumour associated macrophages, different routes of Clodrosome administration are required.

3.5.2: Phagocytic cells produce TNFα in response to immunostimulants

The production of inflammatory cytokines such as TNFα is required for synergy with SMCs. Because it has been shown that splenic macrophages produce TNFα in response to infection with VSVΔ51 (Figure 16), I determined whether this effect can be abrogated upon depletion of these splenic macrophages. Mice were treated with PBS, control liposomes and Clodrosome. At 24h following IV administration, splenocytes were treated with various immune stimulants for 24h and the cell culture supernatant were processed for ELISA to detect IFNβ and TNFα. IFNβ production was increased following treatment with VSVΔ51 and VSV-mIFNβ-NIS, but not following poly(I:C) or LPS treatment (Figure 18a). In all cases, TNFα production was increased in untreated and control liposome-treated splenocytes

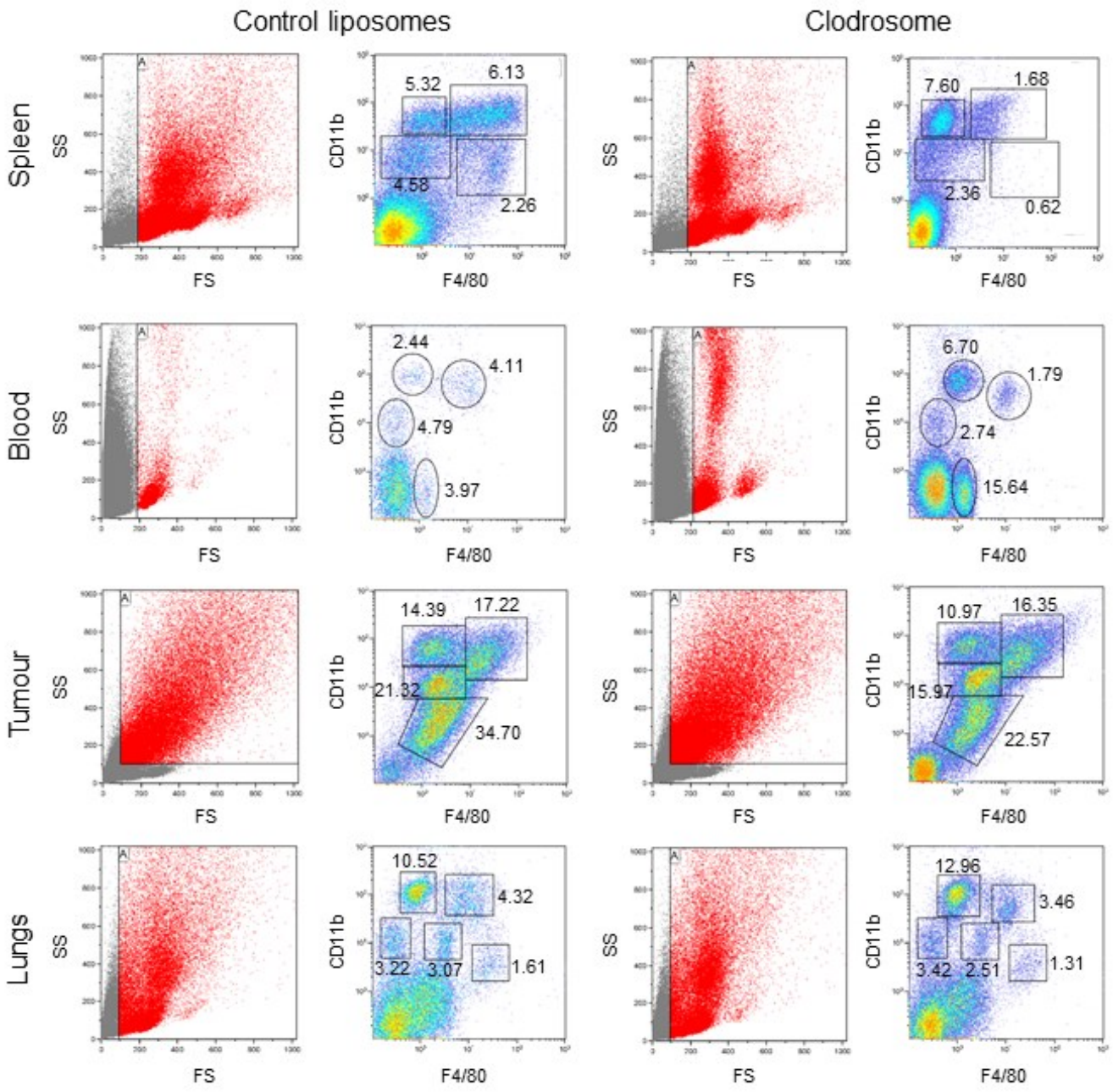


Figure 17. Depletion of macrophages using Clodrosome. BALB/c mice bearing 2-week EMT6-Fluc tumours were injected intravenously with control liposomes or 0.625 mg of Clodrosome for 48h. Spleen and tumour were harvested and dissociated to a single cell suspension using 70 µm mesh. Lungs were dissociated using 640 U/mL collagenase IV then passed through 70 µm mesh. Blood was collected into EDTA-containing tubes and red blood cells were lysed using ammonium-chloride-potassium (ACK) buffer. Tissues were stained with CD11b and F4/80 antibodies then analyzed by flow cytometry. Population percentages are indicated beside each gate.

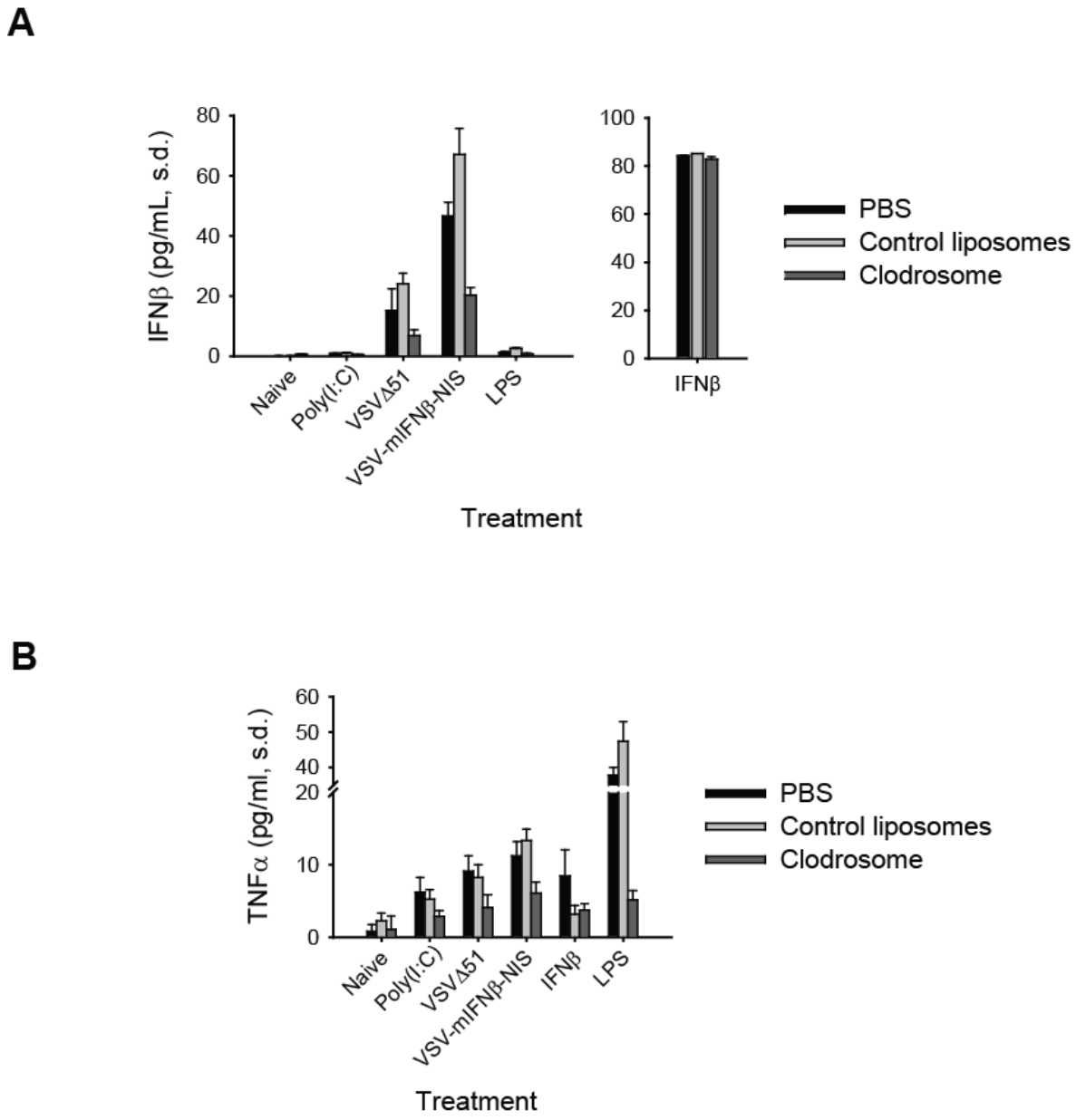


Figure 18. Clodrosome treatment reduces TNFα production in vitro. Mice were treated with PBS, control liposomes or 0.625 mg Clodrosome for 24h. Spleens were removed and splenocytes were cultured and treated for 24h with 1 MOI of VSVΔ51 or VSV-mIFNβ-NIS, 1 μg/mL poly(I:C), 1000 U/mL IFNβ or 10 ng/mL LPS. **(A)** Supernatant was processed and used for detection of IFNβ via ELISA. The signal following IFNβ treatment is solely the result of exogenous IFNβ binding to the capture antibody. **(B)** Supernatant was processed and used for the detection of TNFα via ELISA.

following stimulation. This effect was abolished in the Clodrosome-treated splenocytes (Figure 18b), which demonstrates that phagocytic cells such as macrophages and perhaps DCs are responsible for the production of the majority of TNFα following short-term stimulation.

3.5.3: Immune cell involvement in type I IFN and SMC combination therapy

There is complex interplay among immune cell populations *in vivo*. To determine whether phagocytic cells were also responsible for cytokine production in response to type I IFN and SMC combination therapy, BALB/c mice bearing EMT6-Fluc tumours were treated with Clodrosome to deplete phagocytic cells and then subsequently treated with IFNα B/D and SMC (Figure 19a). In mice that were treated with IFNα B/D IT, there was no difference in tumour growth (Figure 19b, top panel) or survival (Figure 19b, bottom panel) between control liposome and Clodrosome-treated mice. There was a cure rate of 20% in both of these groups. On the other hand, mice treated with Clodrosome and IFNα B/D IP showed an increase in tumour growth rate compared to mice treated with control liposomes (Figure 19b, top panel). There were no mice cured in the Clodrosome treated group, while 20% of the control liposome treated group showed durable cures (Figure 19b, bottom panel). These findings demonstrate that there is a role for systemic phagocytic cells such as macrophages for the production of cytokines in combination immunotherapy for the treatment of cancer.

3.6: Type I IFN signalling in TNFα production using immune stimulants

3.6.1: Immune cells responsible for type I IFN and TNFα production

Because of the complex interplay of immune responses following treatment with an immune stimulant, I was interested in determining the role of each immune cell population in the production of both type I IFNs and TNFα. Due to the limited response obtained when cells are treated with IFNα B/D *in vitro*, I chose poly(I:C) as a potent stimulant of cytokine

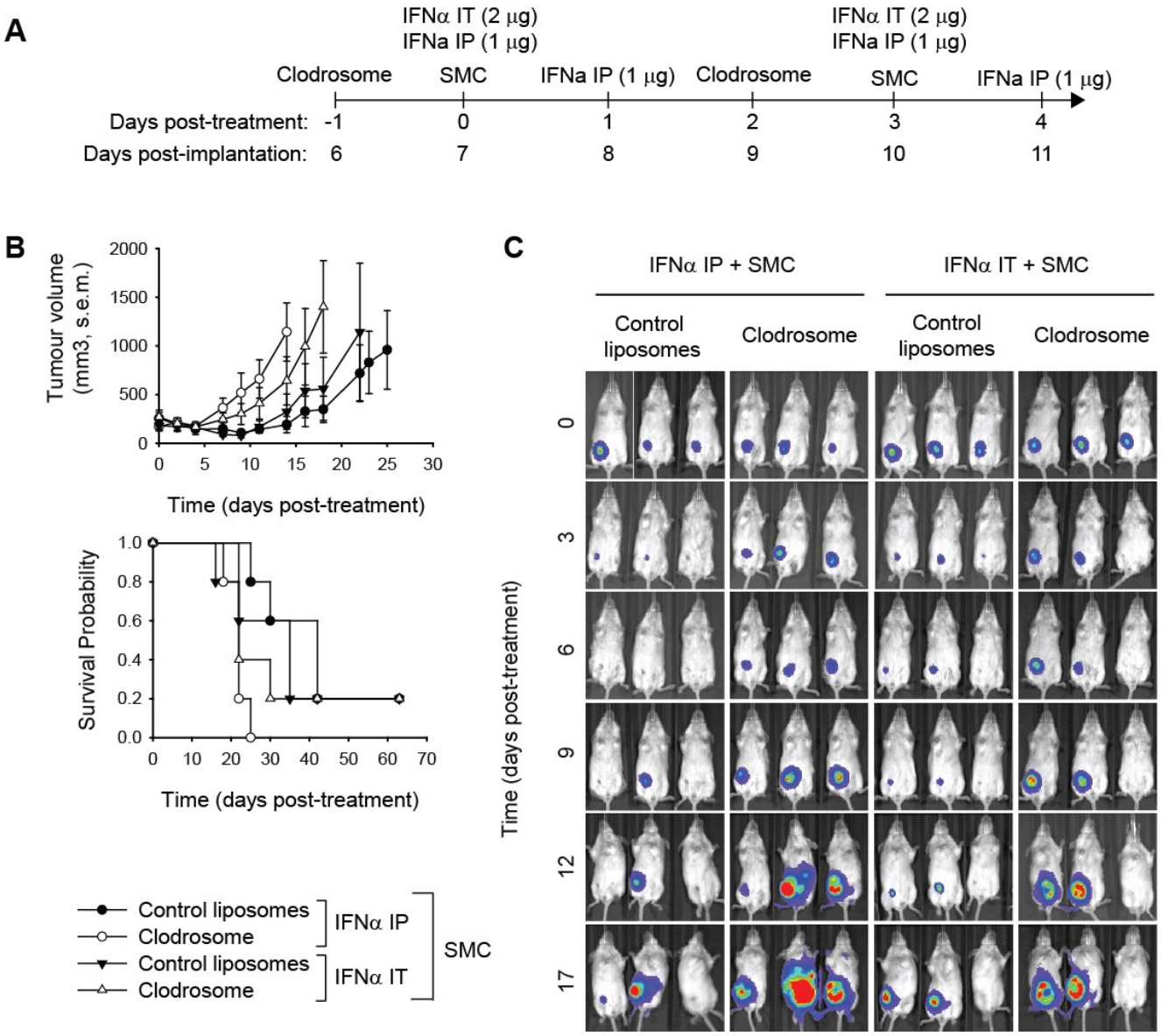


Figure 19. Phagocytic cells contribute to the production of cytokines in response to IFNα B/D. (A) BALB/c bearing EMT6-Fluc tumours were treated intravenously with control liposomes or 0.625 mg Clodrosome, intratumourally (IT) with one 2 μg dose of IFNα B/D or intraperitoneally (IP) with two 1 μg doses of IFNα B/D, and orally with 50 mg/kg SMC according to the timeline. **(B)** Top panel: Tumour volume of mice from (A), measured by calipers. Bottom panel: Kaplan-Meier survival curve of mice from (A). Log-rank with Holm-Sidak multiple comparisons. n=5 mice per treatment group. **(C)** Representative luminescent IVIS images.

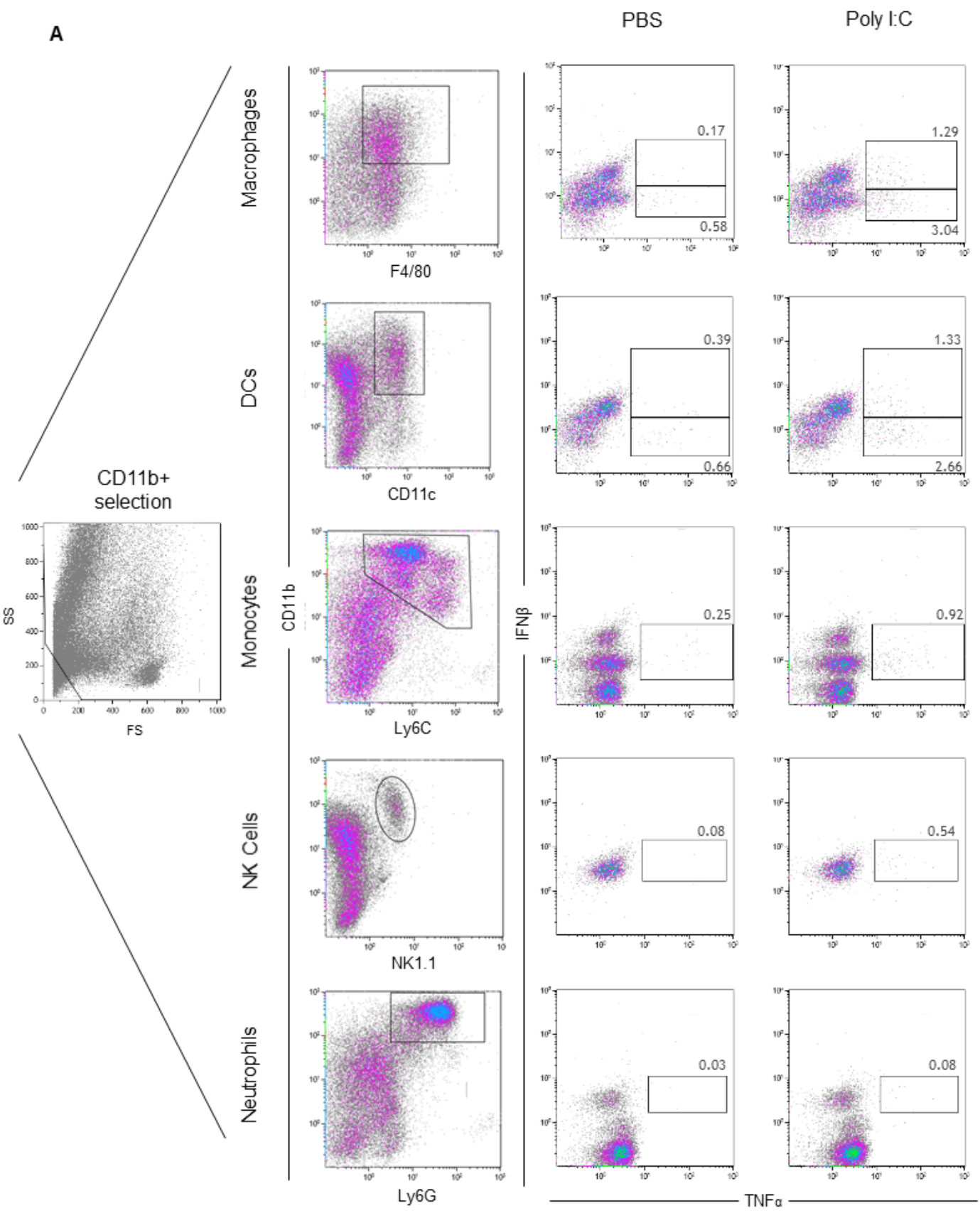
production. Poly(I:C) treatment has been shown to induce type I IFN and TNFα production via different cellular pathways and is therefore a good stimulant to assess global cellular responses (85, 86) (Figure 6). To assess type I IFN and TNFα production in immune cells, splenocytes stimulated *in vitro* with poly(I:C) and analyzed by flow cytometry.

All innate immune cell populations show some degree of basal cytokine expression in culture with GolgiPlug (Brefeldin A). However, macrophages ($CD11b^+$ $F4/80^+$) and DCs ($CD11b^+$ $CD11c^+$) displayed an increase in $TNF\alpha^+$ cells, as well as $IFN\beta^+$ $TNF\alpha^+$ cells (Figure 20a). This suggests that macrophages and DCs are capable of producing IFNβ in response to poly(I:C) treatment and that these same cells produce TNFα. Macrophages and DCs also demonstrate IFNβ-independent production of TNFα, which is not unexpected given the modes of stimulation of poly(I:C) (85). Monocytes ($CD11b^+$ $Ly6C^+$) were shown to produce a mild increase in TNFα following treatment, although there were fewer $IFN\beta^+$ $TNF\alpha^+$ cells (Figure 20a). NK cells ($CD11b^+$ $NK1.1^+$) showed a modest increase in $IFN\beta^+$ $TNF\alpha^+$ positive cells following treatment (Figure 20a). Neutrophils ($CD11b^+$ $Ly6G^+$) did not demonstrate an increase in either cytokine following treatment with poly(I:C) (Figure 20a).

The general adaptive immune cell populations were also analyzed by staining for the production of IFNβ and TNFα in response to poly(I:C). No response was expected due to the short timeline of treatment and lack of activation by antigen-presenting cells. Unsurprisingly, there was a minute increase in IFNβ or TNFα production from either T cells ($CD11b^-$ $CD3^+$) or B cells ($CD11b^-$ $CD19^+$) (Figure 20b).

3.6.2: Cytokine production is both type I IFN-dependent and -independent

We have shown that type I IFNs and other type I IFN-inducing agents can be used to produce cytokines that would synergize with SMCs to induce death of cancer cells (Figures


A
PBS
Poly I:C
Macrophages
DCs
Monocytes
NK Cells
Neutrophils
CD11b+
selection
SS
FS
CD11b
F4/80
CD11c
Ly6C
NK1.1
Ly6G
IFNβ
TNFα
0.17
1.29
0.58
3.04
0.39
1.33
0.66
2.66
0.25
0.92
0.08
0.54
0.03
0.08

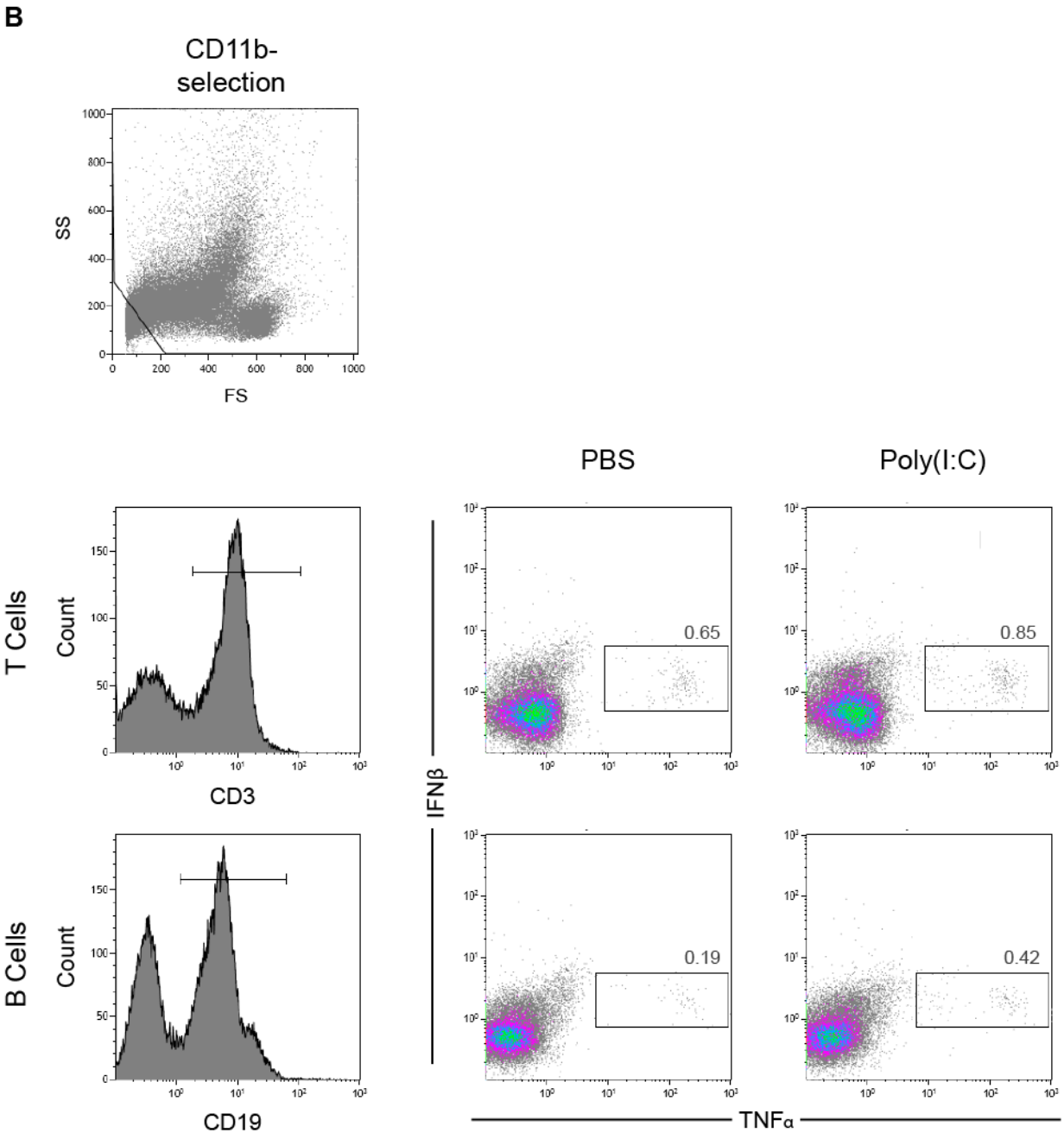


Figure 20. Innate immune cells produce TNFα and IFNβ following stimulation with poly(I:C). All panels: Cells were treated for 12h with PBS or 1 μg/mL poly(I:C). GolgiPlug (Brefeldin A) was added 2h following poly(I:C) for a total of 10h in culture. Cells were stained intracellularly for TNFα and IFNβ. Analysis was performed by flow cytometry. **(A)** $CD11b^+$ cells were isolated from whole spleens using a positive selection kit. Cells were stained for F4/80, CD11c, Ly6C, NK1.1 and Ly6G to determine innate immune populations. **(B)** $CD11b^-$ cells were kept following CD11b selection and stained for CD3 and CD19.

8, 10). (60) However, it remains unknown whether this synergy is dependent on intact type I IFN signalling pathways. A conditioned media experiment was performed using several immune stimulants that are known to induce type I IFN production from immune cells (86, 99). As primary macrophages have been shown to produce cytokines in response to immune stimulants, and because they are easily isolated from a mouse, bone marrow-derived macrophages (BMDMs) were used to generate the conditioned media.

BMDMs from WT or IFNAR1 receptor knock out (IFNAR KO) mice were stimulated with SMC, CpG-ODN 2216, poly(I:C), VSVΔG or LPS. The conditioned media was transferred to LLC cells and treated vehicle or LCL-161. LLC cells only die in the presence of TNFα and SMC (Figure 11), so this assay can be used as an indirect measure of TNFα production.

Interestingly, the degree of LLC death depended on the type of immune stimulant. Conditioned media from untreated BMDMs and those treated with LCL-161 did not show any increase in LLC death, which implies that no cytokines were produced by BMDMs (Figure 21a). SMCs have been shown to induce death of macrophages in a TNF-R1-dependent manner, so LCL-161 was used to determine if SMC treatment leads to the release of stress signals that can stimulate cytokine production (100). Conditioned media from BMDMs treated with CpG-ODN 2216, which belongs to CpG class A and therefore induces production of IFNα, only showed LLC death from supernatant from WT BMDMs. Poly(I:C), which can lead to production of both TNFα and type I IFN by different signalling pathways (85), lead to LLC death from both WT BMDM and IFNAR KO BMDM conditioned media. Instead of VSVΔ51, a form of VSVΔ51 that is restricted to one round of infection and replication (VSVΔ51ΔG) was used because a non-replicating virus was required to treat the IFNAR KO BMDMs, as they are unable to control virus spread. Treatment with

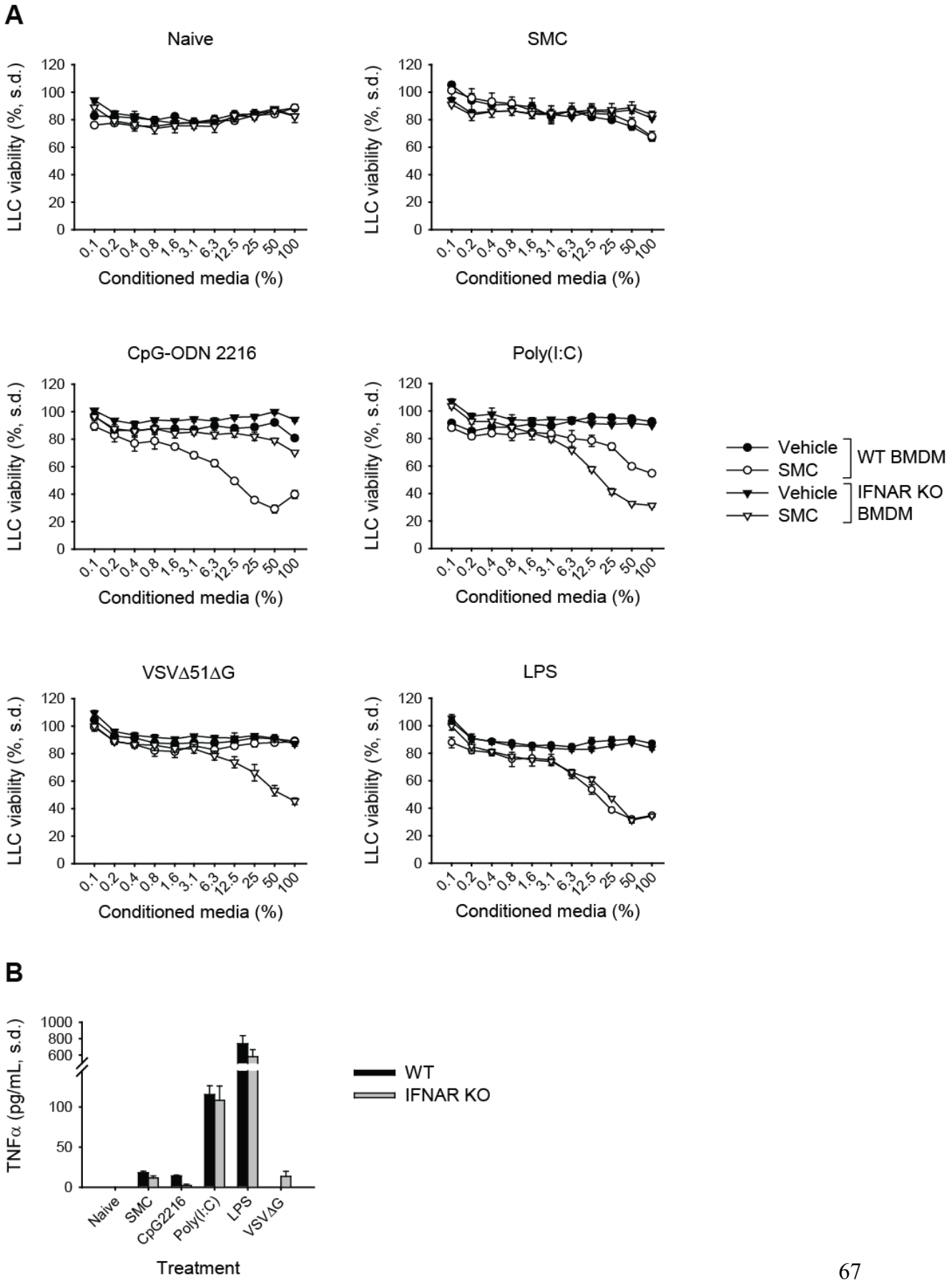

A
Naive
SMC
CpG-ODN 2216
Poly(I:C)
VSVΔ51ΔG
LPS
LLC viability (%, s.d.)
Conditioned media (%)
0.1 0.2 0.4 0.8 1.6 3.1 6.3 12.5 25 50 100
Vehicle
SMC
Vehicle
SMC
WT BMDM
IFNAR KO BMDM
B
TNFα (pg/mL, s.d.)
1000
800
600
100
50
0
Naive
SMC
CpG2216
Poly(I:C)
LPS
VSVΔG
Treatment
WT
IFNAR KO

Figure 21. Cytokine production is both type I IFN-dependent and -independent. (A) Bone marrow-derived macrophages (BMDMS) were generated from wild type (WT) or IFNAR knockout (IFNAR KO) mice. BMDMs were stimulated for 24h with 5 μM SMC, 1 μg/mL CpG-ODN 2216, 1 μg/mL poly(I:C), 1 MOI VSVΔ51ΔG or 10 ng/mL LPS, as shown above each panel. Conditioned media was transferred to Lewis lung carcinoma (LLC) cells for 24h with vehicle or 5 μM SMC and the viability was assessed by Alamar Blue. **(B)** BMDMs from WT or IFNAR KO mice were stimulated with the agents as described in (A). The supernatant was processed and the amount of TNFα was determined by ELISA.

VSVΔ51ΔG at a MOI of 1 resulted in the death of LLC cells only in IFNAR KO BMDMs, which is likely the result of the infected BMDMs unable to control viral infection. Finally, LPS-treated BMDMs showed no difference in cytokine production as LPS is known to generate TNFα independently of type I IFN signalling pathways (101).

An ELISA for TNFα was performed in parallel to the conditioned media experiments. It was shown that a large amount of TNFα (as seen from LPS-treated splenocytes) does not guarantee a greater decrease in cancer cell viability (Figure 21b). This suggests that there is a threshold of TNFα required to kill a fixed number of cancer cells when combined with LCL-161. A possible explanation for this discrepancy is that the media for the ELISA was generated in a different format than the conditioned media, as much more media was required for the conditioned media experiment.

3.7: Mechanistic insights into the production of TNFα by type I IFN

It is likely that there is cross-talk between type I IFN and TNFα signalling pathways because both of these pathways are involved in the immune response to many stimulants such as oncolytic viruses and TLR agonists (60, 102). To determine if common factors between these two pathways are involved in the production of TNFα in response to type I IFN stimulation, particular genes involved in NF-κB and type I IFN signalling, specifically the TRAFs, were transiently knocked down using siRNA in SNB75 human glioblastoma cells. The cells were then treated with IFNβ and vehicle or SMC for 16h and ELISAs were performed for TNFα or TRAIL, another cytokine capable of synergizing with SMCs (103). TRAIL is also a well-defined interferon-stimulated gene (104)

Untreated cells showed no measureable amount of the TNF or TRAIL and are not shown. On the other hand, the addition of LCL-161 enhanced the production of TNFα in IFNβ-treated cells. Knockdown of the obligate factors for type I IFN signalling,

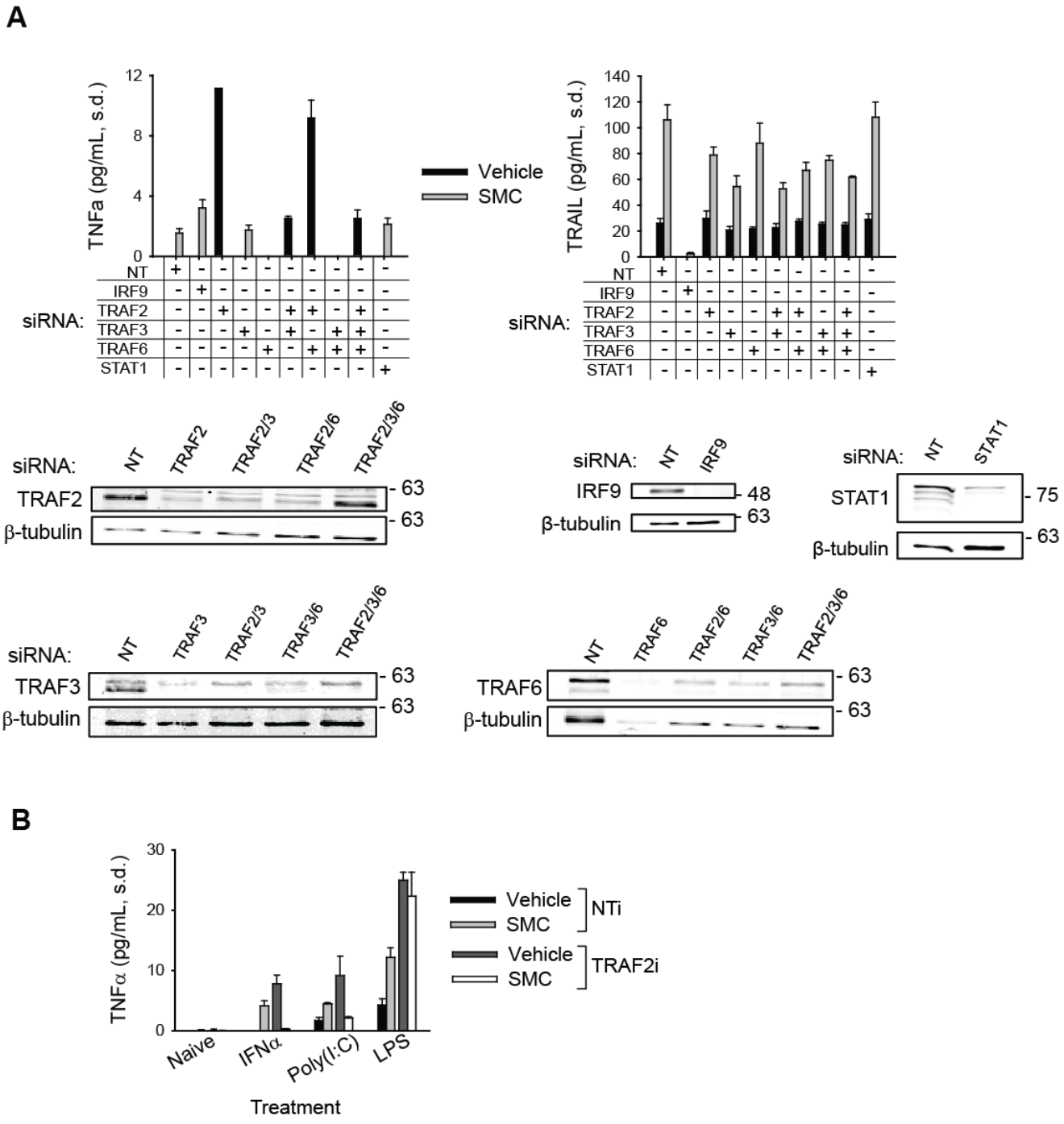


Figure 22. TRAF2 mediates cross-talk between type I IFN and NF-κB pathways. (A) SNB75 cells were transfected for 48h with non-targeting (NT), IRF9, TRAF2, TRAF3, TRAF6 or STAT1 siRNA then treated with 1000 U/mL IFNβ and vehicle or 5 μM SMC for 16h. The amounts of TNFα and TRAIL were determined by ELISA. Western blots to confirm knockdowns were performed. **(B)** SNB75 cells were transfected with non-targeting (NTi) or TRAF2 (TRAF2i) siRNA for 48h then treated with 1000 U/mL universal IFNα, 1 μg/mL poly(I:C) or 10 ng/mL LPS for 16h. An ELISA for TNFα was performed using the supernatant.

IRF9 or STAT1, had little effect on TNFα production (Figure 22a, left panel). While downregulation of TRAF3 had little effect on TNFα production, the knockdown of TRAF2 lead to a large increase in TNFα production, but only in the *absence* of LCL-161. On the other hand, knock down of TRAF6 completely abolished any response to IFNβ. Knockdown of TRAF2, TRAF3, TRAF6 or STAT1 did not affect TRAIL production (Figure 22a, right panel). However, knockdown of IRF9 abolished TRAIL production following IFNβ treatment. As expected, LCL-161 treatment enhanced the production of TRAIL in all cases (60).

Following this, I was interested to determine if the increase in TNFα following the downregulation of TRAF2 was a global response upon treatment with other immune stimulants. As expected, untreated cells showed no detectable levels of TNFα (Figure 22b). SNB75 cells treated with IFNα and poly(I:C) followed a similar trend to IFNβ-treated cells: there was an increase in TNFα production following knockdown of TRAF2 and stimulation, but only in the absence of LCL-161. This pattern was not seen in LPS, although knockdown of TRAF2 did promote an overall increase in TNFα production (Figure 22b). Together, these results demonstrate that TRAF2 is a mediator of type I IFN-induced TNFα production, but that its role is dependent on the presence of the IAPs.

4: DISCUSSION

The role of type I IFN in SMC-mediated anti-tumour efficacy is complex. I have shown that type I IFN treatment does lead to the production of TNFα, and that the TNFα can then synergize with a SMC to induce cancer cell death (Figures 10, 13, 14, 15). However, type I IFN signalling is not the only requirement for synergy with a SMC and certain IFN-inducing agents can lead to cancer cell death independently (Figure 21). Additionally, treatment with certain oncolytic viruses such as WT VSV can lead to synergy with a SMC and tumour regression without type I IFN production (Figures 7, 8). Importantly, the host immune system plays a very important role in the efficacy of SMC combination therapy, in both type I IFN-dependent and –independent systems (Figures 16, 18, 19, 20). The molecular mechanism by which type I IFN signalling mediates TNFα production remains to be elucidated, but preliminary data suggest that TRAF2 is involved in IAP-mediated TNFα production (Figure 22). Many agents can be used to synergize with SMCs to kill cancer cells (60), but those that induce type I IFN signalling are excellent candidates for translation into human therapies.

4.1: Oncolytic viruses as SMC combination immunotherapy stimulants

Oncolytic viruses are seen as a promising avenue for cancer treatment. However, their efficacy as standalone treatments remains low (66, 67). I believe that the combination of oncolytic viruses with SMCs provides a more robust anti-cancer therapy due to the mechanism of action of SMC combination therapy. The virus is not required to infect every cancer cell. Indeed, virus exposure may only be needed to stimulate cancer cells and the immune system to produce anti-viral and proinflammatory cytokines that can then synergize with SMCs. Together, these characteristics show that oncolytic viruses may be excellent agents to promote production of cytokines for use in SMC combination immunotherapy.

On the other hand, not all oncolytic viruses are equally effective as immune stimulants. I discovered that WT VSV and certain derivatives designed using the wild type backbone, such as VSV-mIFNβ-NIS, possess greater lytic ability than VSVΔ51 (Figure 7). While these oncolytic viruses can effectively synergize with SMCs, their lytic activity may affect the use of these viruses in combination therapy due to the possibility of tumour lysis syndrome (89). Consequently, certain viruses can be perceived to be unsafe in SMC combination therapy, particularly because SMC treatment can enhance cytokine production after treatment with oncolytic viruses (Figure 15, 22) (60). Despite the efficacy of WT VSV combination with SMC *in vivo*, the use of a highly pathogenic virus poses too many risks to patient safety. Indeed, I have found that the therapeutic window of a genetically modified virus is greatly improved in combination with SMC therapy. Based on these results, the most effective oncolytic virus for the combination therapy would be one that has low lytic ability, such as VSVΔ51, and that also carries a type I IFN transgene to introduce even more of this protein into the tumour. Alternatively, VSVΔ51 armed with a TNFα transgene may prove to be the most effective agent for combination with a SMC.

We hypothesized that virus-induced type I IFN is the primary mediator of inflammatory cytokine production (60). My results show that this is not always the case. For example, WT VSV shows excellent synergy with LCL-161 *in vivo* (Figure 8). WT VSV shuts down the host type I IFN response by preventing trafficking of type I IFN mRNA from the nucleus to the cytosol. By proxy, the export of other mRNAs such as interferon-stimulated genes and proinflammatory cytokines, such as TNFα, would also be inhibited (70). Without the discrete release of both type I IFN and TNFα, WT VSV and SMC combination therapy should not be effective. However, this approach does lead to durable cures in mice (Figure 8), which suggests that other mechanisms can viably contribute to

SMC efficacy. Tumour cell lysis may promote infiltration of cytokine-producing immune cells independently of type I IFN release. Tumour cell death induced by WT VSV may not be apoptotic but instead may be a more immunogenic form of cell death such as necrosis (105). Additionally, the presence of SMCs has been shown to enhance T cell responses against the tumour (106), which may be a contributing factor to WT VSV-mediated tumour regression. Therefore, type I IFN signalling is not a requirement for effective SMC combination therapy in all systems.

4.2: Type I IFNs as SMC combination immunotherapy stimulants

The use of non-viral stimulants as agents to synergize with SMCs holds much promise as an effective anti-cancer therapy. Recombinant proteins such as type I IFNs may represent a safer option compared to the use of replicating viruses because this avoids the possibility of viremia. Our data shows that a recombinant human hybrid interferon alpha, IFNα B/D, effectively synergizes with LCL-161 to lead to durable cures in BALB/c mice bearing EMT6-Fluc mammary carcinoma tumours in a fashion that is dependent on TNFα signalling (Figures 10, 14). These findings demonstrate that type I IFN signalling does promote the production of inflammatory cytokines (107).

4.2.1: The role of TNFα in type I IFN signalling

TNFα is a very important cytokine in the pathogenesis and progression of cancer. There is conflicting data suggesting that this ubiquitous cytokine can both promote tumour growth and lead to cancer cell death (108). However, it is likely that TNFα will only lead to cancer cell death in the presence of a SMC. It has been shown that inclusion of a TNFα-neutralizing antibody in mice treated with VSVΔ51 and SMC abrogates the curative effects of the combination therapy (60). This same result was seen when tumour-bearing mice were

treated with IFNα B/D, a SMC and a TNFα-neutralizing antibody (Figure 14). As such, this finding is consistent with the hypothesis that type I IFN signalling leads to the production of TNFα, which can then synergize with the SMC to induce tumour cell death.

The method of IFNα B/D administration affects the efficacy of the TNFα-neutralizing antibody. When IFNα B/D was administered intratumourally (IT) in combination with a TNFα-neutralizing antibody, there was a statistically significant reduction in efficacy than when treated with an isotype control antibody. When IFNα B/D was administered intraperitoneally (IP), there was only a 10% reduction in efficacy with the TNFα-neutralizing antibody (Figure 14). This suggests that the local production of TNFα within the tumour is the primary mediator of tumour death when LCL-161 is present. Considering that there was only a 25% cure rate in mice treated IP with IFNα B/D, SMC, and the isotype control antibody, it is unlikely that there will be a statistically significant difference in survival when using the TNFα-neutralizing antibody. Regardless, the relatively low cure rate in IP-treated mice suggests that systemic responses are less important to TNFα production when using IFNα B/D as an immunostimulant. Overall, I would conclude that TNFα release from the tumour is critical to the efficacy of IFNα B/D and SMC combination treatment. Importantly, TNFα production from immune cells following treatment with IFNα B/D is likely an important factor in the efficacy of SMC treatment. The immunomodulatory effects and TNFα production following IFNα B/D treatment may be greatly affected by the route of administration. Immune cell involvement in this system will be discussed in greater detail in section 4.3.

4.2.2: The requirement of SMCs to promote cytokine production

In vitro data show that the addition of a SMC to an immune stimulant promotes an increase in cytokine production compared to the stimulant alone (Figures 15, 22) (60). The

reason for this is not yet clear. The use of a SMC invariably results in the activation of the alternative NF-κB pathway by the stabilization of NIK (Figure 4b), which could subsequently lead to the production of TNFα. This possibility is further corroborated by *in vivo* data showing an increase in serum TNFα following stimulation with IFNα B/D and LCL-161 (Figure 15c, left panel). However, the potential decrease of TNFα production in the tumour itself following stimulation with IFNα B/D and LCL-161 shows that the tumour microenvironment is more complex than simple systems. If the tumour is consistently producing TNFα, as tumours are often described as "addicted" to NF-κB signalling, (109, 110), the addition of a SMC may actually dampen this constitutive activation of the classical NF-κB by inhibiting signalling via auto-ubiquitination of cIAP1/2. Even with activation of the alternative branch, the overall TNFα levels within the tumour may be reduced. The potential decrease of TNFα in the tumour remains to be confirmed and would require the use of a larger number of mice per treatment group. It would also be informative to determine the levels of TNFα mRNA in tumour cells by quantitative reverse transcription PCR (qRT-PCR). Additionally, it possible that TNFα in the tumour is membrane-associated rather than soluble as would be found in serum. And, finally, it is also important to confirm that tumour-produced TNFα is indeed being accurately measured by ELISA.

4.3: Immune cell involvement

4.3.1: The contribution of phagocytic innate immune cells

Innate immune cells are also responsible for the production of cytokines which can synergize with SMCs both *in vitro* and *in vivo* (Figures 16, 18, 19, 20, 21). The use of Clodrosome provides interesting insight into the immune cell involvement of type I IFN and SMC combination immunotherapy, as it depletes phagocytic innate immune cells. The effects of Clodrosome treatment vary with the route of administration of IFNα B/D. When

IFNα B/D was given IT, there was little difference in the tumour burden of animals treated with Clodrosome or control liposomes. On the other hand, mice treated IP with IFNα B/D demonstrated a rapid increase in tumour growth when treated with Clodrosome compared to control liposomes (Figure 19). A simple explanation for this difference is that Clodrosome is incapable of crossing the vascular endothelial layer for engulfment by tumour-associated phagocytic cells. This suggests that systemic innate immune cells contribute to the control of tumour growth but that similar results could potentially be seen if tumour associated immune cells could be depleted.

This result is in rather striking contrast to the results seen from the use of the TNFα-neutralizing antibody, which demonstrated that the tumoural response to treatment was more important than the systemic contribution to the production of cytokines (Figure 14). It is important to note that the TNFα-neutralizing antibody will effectively inhibit TNFα from both host and tumour cells. Contrastingly, Clodrosome has no effect on the cancer cells themselves, so the tumour can still produce TNFα following IT stimulation. Therefore, mice treated with IP IFNα B/D will demonstrate a greater increase in tumour growth following Clodrosome treatment because there is little TNFα being produced in the tumour. Taken together, these results show that there is contribution of cytokine production from both tumour-associated and systemic immune cells, as well as the cancer cells themselves.

Due to the broad-acting nature of Clodrosome, it is impossible to claim that one specific phagocytic immune cell type is responsible for the production of all cytokines released following treatment with IFNα B/D and SMC *in vivo*. However, macrophages and DCs likely play a major role since these cells are primarily affected by Clodrosome. A more specific method such as the use of cell-neutralizing antibodies would provide better insight into the roles of each cell type. For instance, to provide evidence that macrophages are

essential to the production of cytokines following IFNα B/D and SMC treatment, one could use the anti-colony stimulating factor 1 receptor (CSF-1R) antibody to deplete tumour-associated macrophages (111). Furthermore, because of the immensely complex interplay of the immune system, characterizing the cells wholly responsible for cytokine production in SMC combination therapy is difficult. Even if one innate immune cell population, such as macrophages, is the main contributor of cytokine production, the involvement of many other cell types is likely. For example, another innate immune cell population may not directly produce cytokines but could recruit more macrophages through the release of chemokines and lead to an overall increase of cytokine production.

Innate immune cells mediate the majority of cytokine production following treatment with an immunostimulant. However, in order to create durable cures there must also be involvement of the adaptive immune system. An important future line of investigation would be to re-challenge the cured mice with EMT6-Fluc cells in both the original site of implantation and in other breast fat pads to demonstrate a global anti-tumour immunity in the mouse. The addition of another cancer cell line such as 4T1 breast cancer (BALB/c background) in this experiment could show the specificity of the adaptive immune responses against the original tumour.

4.3.2: Identification of TNFα-producing cells

Using an *in vitro* model of cytokine production, I demonstrated that all innate immune cells produce a basal level of IFNβ and/or TNFα. Following a 12h treatment with poly(I:C), I observed that macrophages have a small but noticeable increase in cells expressing high levels of IFNβ and TNFα. This finding is not surprising, as macrophages are known to be robust producers of cytokines (112). Although it is uncertain whether such a small difference can account for the effects of combination therapy seen *in vivo*, it

nevertheless suggests that macrophages are capable of producing high levels of TNFα which would then synergize with a SMC.

Other innate immune cell populations, such as DCs, are also capable of producing TNFα and IFNβ in response to poly(I:C) treatment. While DCs are generally considered primarily responsible for antigen presentation and T cell activation, studies have shown that these cells are capable of producing TNFα in response to infections as part of the innate immune response (113, 114). Monocytes are also capable of producing a small amount of TNFα following poly(I:C) stimulation. It has been shown that $Ly6C^+$ monocytes are involved in the inflammatory response (115). NK cells and neutrophils do not directly produce TNFα, but NK cells, as their name suggests, perform more direct killing of infected or cancerous cells through the release of perforin and granzymes (116). Neutrophils are involved in inflammation primarily through phagocytosis of foreign particles and the release of antimicrobial granules and are generally recruited by to the site of infection by TNFα released from macrophages (117). The cooperation of all of these cell types is important to the overall response to treatment *in vivo* but this data shows that macrophages and DCs are the primary producers of TNFα following poly(I:C) treatment.

Importantly, subtypes of each immune cell population exist, and using one broad cell surface marker may not be the best way to understand the relative contribution of each population. For example, macrophages are often divided along a spectrum, denoted M1-M2, where M1 macrophages promote inflammation and M2 macrophages are considered to reduce inflammatory responses and promote healing, although designation of defined subsets remains difficult (118). The overall contribution of macrophages would then be a balance of these phenotypes, which cannot be differentiated using only one surface marker (i.e. F4/80). This relationship becomes even more complex in the tumour microenvironment, which,

while generally accepted as being inflammatory, also contains anti-inflammatory resident populations (119, 120). Characterization of specific tumour immune cell populations will provide greater insight into the role of anti-cancer immune responses following SMC combination therapy.

4.4: TRAF2 as a mediator of type I IFN-stimulated NF-κB induction

There is evidence that type I IFN signalling leads to activation of NF-κB signalling complexes but the precise mechanism of these responses remains to be determined. My work suggests that TRAF2 has a role in type I IFN-mediated NF-κB activation, although there is very little evidence showing that TRAF2 is involved in the regulation of type I IFN signalling pathways. One group has demonstrated that TRAF2 can be recruited to the type I IFN receptor (IFNAR) following type I IFN binding in Daudi cells, but this has not been confirmed in other cell lines (121). This same group found that TRAF2 is important for the activation of the alternative NF-κB pathway following stimulation with type I IFN in mouse embryonic fibroblasts (MEFs), but that the classical pathway remains inactive. In contrast, my results show that the loss of TRAF2 promotes the production of TNFα following stimulation with type I IFN, which demonstrates that TRAF2 is a negative regulator of TNFα production. Perhaps most importantly, this negative regulation is wholly dependent on the presence of the IAPs. When the IAPs are lost through the treatment with a SMC, TRAF2 siRNA-mediated TNFα production is abrogated (Figure 22). There are two possible explanations for this result: 1) cIAP1/2 and TRAF2 can be found in complex at IFNAR and are involved in signal transduction following type I IFN treatment; or 2) some unknown intermediary proteins are activated by IFNAR which then activate cIAP1/2 and TRAF2 complexes that are found coupled to other receptors in the cell. Much more work must be done to determine if either of these scenarios is true. One particular experiment of interest

would be to determine if cIAP1/2 can be pulled down with IFNAR in a co-immunoprecipitation experiment, and whether this interaction is dependent on TRAF2. Most importantly, this work must be repeated in other cell lines to determine if it is a universal finding or if it is peculiar to the mutation in SNB75 cancer cells.

4.5: Pitfalls of this research

The majority of this project focuses on the use of one cell line, specifically the EMT6 mammary carcinoma. This line, while useful for the purpose of SMC combination immunotherapy, is not necessarily representative of all cancers. Indeed, IFNα B/D and LCL-161 combination treatment did not cure Lewis lung carcinoma (LLC) tumours (Figure 11). Other cell lines must be used to determine if this treatment is a viable therapeutic option for many forms of cancer because each line has its own irregularities which may affect its susceptibility to SMC-mediated killing. For example, certain cancer cell lines have been shown to have high expression of c-FLIP, which acts as a resistance factor to SMC-mediated cell death (62). It may be important to determine other genetic factors that affect the efficacy of SMC combination therapy before this treatment can be used in human trials. Additionally, the use of primary human tumour tissues in preclinical stages will enhance our understanding of the usefulness of SMC combination immunotherapy.

Although my studies demonstrate that IFNα B/D shows promise as an immunostimulant in SMC combination immunotherapy, all of these investigations have been done with mouse models of cancer. While translation into human patients will be easier with the use of a human recombinant protein, this inherently poses some difficulties in determining doses and predicting responses in humans. Recombinant IFN protein may actually show higher activity in humans due to the possibility of a higher affinity for the type I IFN receptor. This may lead to increased cytokine production but uncontrolled cytokine

release is an undesirable reaction following stimulation. Clinical studies will greatly help to clarify these issues of efficacy.

4.6: Conclusions

We hypothesized that type I IFN signalling is required to produce TNFα that can then synergize with SMCs. I determined that there are other factors at play in the production of inflammatory cytokines and that it is unlikely that type I IFN signalling is the only requirement for cytokine production. The use of a recombinant type I IFN protein in combination with a SMC shows that type I IFN signalling is sufficient to cause the death of cancer cells *in vivo* and *in vitro*. However, the use of WT VSV as an effective stimulant for *in vivo* combination therapy demonstrates that type I IFN signalling is not necessary to lead to the killing of cancer cells, but that cytokine release and immune cell recruitment from lysed cells may also be a contributing factor in this case (Figure 23). Certain stimulants may show better results with certain forms of cancer or with a particular immune profile of a given cancer patient. The power of SMC combination immunotherapy lies in the possibility of pairing it with many other agents, including recombinant type I IFNs and type I IFN-inducing compounds.

We suggest that SMC-combination immunotherapy is a safe and effective treatment option for cancer. Many different immunostimulants can effectively be used to create a potent cytokine storm that can synergize with a SMC. The use of clinically-approved immunostimulants or vaccine adjuvants could expedite the process of investigating this therapy in humans. Immune stimulants that can activate several different cellular signalling pathways leading to cytokine production may be the most effective agents to use in combination with a SMC to treat and eradicate cancer in patients.

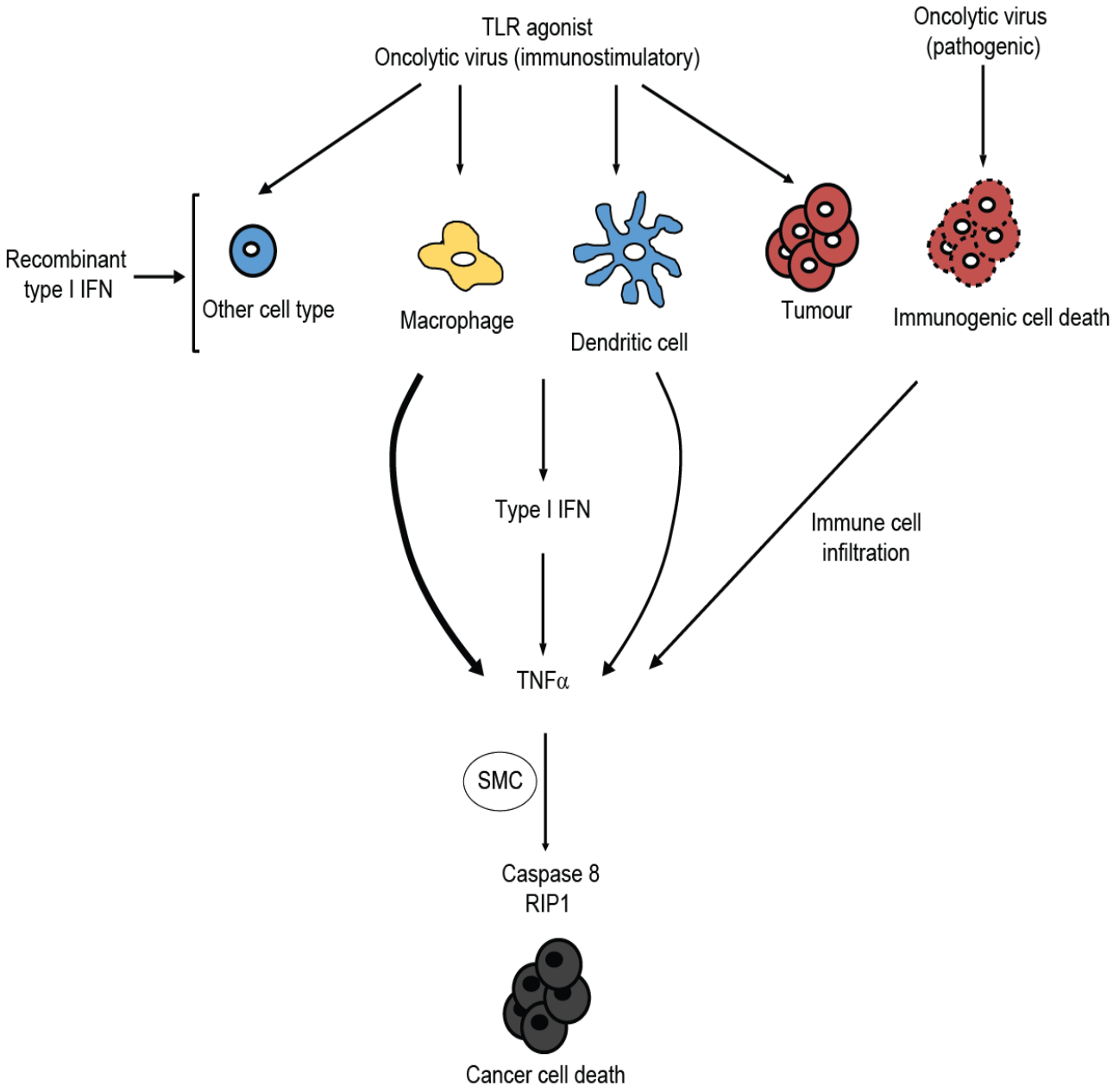


Figure 23. Type I IFN is sufficient but not necessary to the production of TNFα. The use of type I IFN-producing immunostimulants leads to the expression of TNFα, which can then synergize with SMCs to induce apoptosis of cancer cells. Many cell types can respond to stimulation, including innate immune cells, particularly macrophages and DCs, tumour cells and other host cells. Recombinant type I IFN can also be used to induce TNFα production. The use of pathogenic, replicating viruses that inhibit type I IFN production also contribute to cytokine production through tumour lysis and immune cell infiltration. Figure adapted from Beug, S. *et al.*, OncoImmunology (2014).

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