IMMUNOLOGICAL EFFECTS OF ONCOLYTIC VIROTHERAPY IN THE CONTEXT OF A PRECLINICAL MODEL OF MELANOMA

By

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ABSTRACT

Immunological effects of oncolytic virotherapy in the context of a preclinical model of melanoma

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Conventional therapies have demonstrated little to no extension in overall survival in the context of metastatic melanomas. As a result there is growing interest in the use of novel immunotherapies. Oncolytic viruses (OVs) that are genetically engineered to express tumour antigens offer the unique combination of direct oncolytic activity and induction of tumour-specific immune responses. Interactions between OVs and the immune system are complex and further complicated by the immunosuppressive tumour microenvironment. We identified mechanisms that allow OVs to mediate their oncolytic effects, despite pre-existing immunity against a virus-encoded transgene, investigated how OVs modulate the immunological landscape within a tumour, as well as the effects of metformin supplementation on melanogenesis and the immunogenicity of melanoma cells. The goal of this thesis is to explore the interactions between the tumour, immune system and OVs in a pre-clinical model of melanoma, ultimately to support the development of effective cancer biotherapies.

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Wishing you all the very best,

- Amanda

Declaration of work performed

I declare that Wing Ka Amanda AuYeung performed all the work; with the exceptions listed below, under the supervision of Dr. Byram Bridle, and an advisory committee composed of Dr. Geoffrey Wood, Dr. James Petrik and Dr. Paul Woods.

1. Tail-vein injections, retro-orbital blood collection, tissue perfusion and spleen and tumour sample collections were performed by Dr. Byram Bridle and Dr. Khalil Karimi.
2. Robert C. Mould, Jacob van Vloten and Mahi Azizi aided in processing of various tissues and staining for flow cytometry.
3. Immunohistochemistry embedding, sectioning and staining was performed by Dr. James Petrik.

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Abbreviation	*Explanation*
AMPK	5' adenosine monophosphate-activated protein kinase
ANOVA	one-way analysis of variance
cAMP	cyclic adenosine monophosphate
CREB	cAMP-responsive element binding protein
DAMP	danger-associated molecular patterns
DC	dendritic cell
DCF-DA	2',7' - dichlorofluorescin diacetate
DCT	dopachrome tautomerase
DHI	5,6-dihydroxyindole
DHICA	indole 5,6-quinone 2 carboxylic acid
DNA	deoxyribonucleic acid
DOPA	3,4-dihydroxyphenylalanine
FOXP3	forkhead box P3
GMCSF	granulocyte-macrophage colony stimulating factor
HPI	hours post-infection
IFN	interferon
MAA	melanoma-associated antigen
MDSC	myeloid-derived suppressor cells
MITF	microphthalmia-associated transcription factor
MOI	multiplicity of infection
NDV	Newcastle disease virus
NK	natural killer (cells)
OrfV	Orf virus
OV	oncolytic virus
PBS	phosphate buffered saline
PD-1	programmed cell death protein 1
PD-L1/2	programmed cell death ligand 1 and 2
PKA	protein kinase A
PMNC	peripheral blood mononuclear cells
RNA	ribonucleic acid
ROS	reactive oxygen species
SEM	standard error of mean
TAA	tumour associated antigen
TCM	T central memory
TEM	T effector memory
TIDC	Tumour-infiltrating dendritic cells
TIL	Tumour-infiltrating lymphocytes (Chapter 1) / tumour-infiltrating leukocytes (Chapter 2)
TLR	toll like receptor
TREG	regulatory T cells
TRP2	tyrosinase related protein 2
TYR	tyrosinase
VSV	vesicular stomatitis virus

CHAPTER ONE:

REVIEW OF LITERATURE

1. Melanomas

1.1. Human Melanomas

1.1.1. Background

Cancers are the leading cause of mortalities in Canada, accounting for almost 30% of all deaths annually[1]. Skin cancers are the most common types with approximately 80,000 cases diagnosed in Canada every year[1]. There are two main classifications of skin cancers, designated by their cellular origin: non-melanomas and melanomas. Non-melanoma skin cancers typically arise from the basal and squamous cells of the epidermis, while melanomas arise from the melanocytes, which are responsible for the production of melanin[2]. Melanocytes originate from the neural crest, from which they migrate to a range of other tissues including the epidermis, uvea, meninges and ectodermal mucosa, where they perform their pigment producing function. Melanin is an endogenous pigment that protects the skin from ultraviolet (UV) radiation[2]. UV radiation is considered to be the most significant environmental factor leading to development of melanomas[2].

Melanomas have been increasing in incidence worldwide over the past several decades[3–5], with recent annual increases of approximately 2.3% among men and 2.9% among women reported from 2001 to 2010[1]. This increase is thought to be attributed to changes in lifestyle and social behaviour, including increased popularity for outdoor activities and tanning, which lead to

increased exposure to UV radiation. Melanomas are most common in patients between the ages of 55-64 and affect more men than women. Melanomas may be the rarest forms of skin cancers but they are the ones that are most likely to form metastases. In fact, melanomas only account for about 4% of cases of skin cancers, however, they are responsible for approximately 80% of skin cancer-associated deaths[6], causing approximately 50,000 deaths annually worldwide[7,8]. An estimated 1.8% in men and 1.1% in women of all annual cancer deaths in Canada are due to metastatic melanomas[1].

1.1.2. Prognosis and Standard of Care

In early stages of the disease, melanomas are considered curable by surgical resection, with a survival rate of 98.3% for localized cases, decreasing substantially to 63% for regional melanomas, where the disease has spread to local lymph nodes. Metastatic melanomas (in which the cancer has spread to distant parts of the body) are the most aggressive. Metastatic melanomas have an extremely poor prognosis, with an approximate10-15% five year survival rate, even with ongoing treatments[4,9].

Treatment of melanomas typically consists of surgical resection of the primary tumour, followed by adjuvant therapy. Unfortunately, traditional therapies, including radiation and chemotherapy, have largely failed to effectively treat metastatic melanomas, demonstrating little to no extension in overall survival, and as a result, interest has grown dramatically in the development of novel, targeted biotherapies[4,5].

1.2. Mouse Models of Melanomas

Animal models have played an important part in the development of novel therapeutics as well as in studies of biological mechanisms. The most widely used pre-clinical models for cancer therapy are murine models, because of their inexpensiveness, availability, ease of manipulation, well characterized genetic backgrounds, as well as the availability of analytical reagents[10,11]. Despite the many differences between mice and humans, the use of murine models allow researchers to examine key principles and to study molecular pathways that lead to melanomas *in vivo*[11]. There are many different murine models used in research, and can be broadly categorized into 3 types: xenograft, syngeneic and genetically engineered models (refer to Table 1)[10,12,13]. Xenograft melanoma models involve engrafting human melanoma cells into immune-deficient mice[10]. Xenograft models can be further divided into cell line-derived xenograft models (CDX) and patient-derived xenograft models (PDX)[14–16]. Syngeneic melanoma models involve transplantation of melanoma cells into recipients of the same inbred strain of mice[10]. These syngeneic models can have functionally intact immune systems[10]. Genetically engineered melanoma models use transgenic mice with modified gene expression, which are beneficial for elucidating mechanisms of melanoma development[10]. Genetic modifications leading to the development of cancers include activating oncogenes or inactivating tumour suppressor genes [17]. Each model provides unique and valuable insights into the development, and progression of melanomas as well as genetic predispositions, potential risk factors, and responses to therapies, all of which ultimately help to better understand the disease. Inevitably, each model has its limitations, along with its advantages, and choosing which model to use depends on the specific scientific questions being addressed.

MODEL		ADVANTAGES	LIMITATIONS	EXAMPLES
Cell line-derived xenograft (CDX)	Standard human cell lines in immunodeficient mice	Study growth behaviour and response to therapy of human melanoma cells *in vivo*. Can study metastasis Higher engraftment rates than PDX	Immunodeficiency *In vitro* culturing of the cells over time results in loss of genetic diversity found in human tumours	WM164 in SCID/SCID mice[18]
Patient-derived xenograft (PDX)	Primary human tumour tissue in immunodeficient mice	More aligned with human cancers, often parallels human outcomes Intact tumour tissue transferred to recipient mice, therefore preserved tumour architecture	Immunodeficiency Difficulty collecting samples, and achieving sufficient engraftment rates Technically challenging	Primary melanoma cells in NOD/SCID mice[19]
Syngeneic	Genetically identical donor and recipients	Study melanoma behaviour and metastasis Intact immune system Study immunotherapies	Differences between human and murine melanomas (i.e. adhesion proteins, growth factor production, enzymes and mechanisms for invasion into host tissues) Not genetically diverse	B16F10 in C57Bl/6 mice[20] Harding-Passey cells in BALB/c x DBA/2F1 mice[21–23] Cloudman S91 cells in DBA/2 mice[23]
Genetically Engineered	Modified gene expression (i.e. knock-in and -outs resulting in loss or gain of function, inducible genes)	Investigate effects of genetic alterations in initiation, progression and metastasis Most complete representation of cancer development	Costly, labour-intensive and difficult to obtain desired genotypes	CDKN2A models[24] RAS models[24]

Table 1: Mouse models of melanomas[10,12–16,25,26]

2. Cancer Immunotherapy

2.1. The Role of the Immune System in Cancer

The role of the immune system in controlling cancer has been a heavily debated topic for over a century[27,28]. For a long time, many thought that tumour cells could not be recognized by the immune system. Paul Ehrlich was perhaps the first to propose that cancer would be a much more common disease in long-lived organisms if it wasn't for the immune system[27,29,30]. However, this prediction was difficult to validate due to the limited understanding of the immune system up until recent decades. Now, with improved experimental models, it is recognized that the immune system does have the ability to detect and even exert some control over the development and progression of cancer.

2.2. Cancer Immunoediting

Immune system-tumour interactions occur through a complex process called "immunoediting". Initially described as the immunosurveillance hypothesis by Burnet and Thomas, the immune system was believed to have a sentinel function in the host[27]. Burnet and Thomas proposed that the immune system was capable of recognizing and eliminating transformed cells. Fundamental to proving this hypothesis was the discovery of tumour-specific antigens. In order for the immune system to specifically recognize tumours, the cancerous cells must express antigens to allow them to be differentiated from normal cells[27]. The finding that mice immunized with chemically-induced tumours could be protected against subsequent re-challenge, provided proof that tumour antigens existed, thus supporting the immunosurveillance hypothesis[27].

Since knowledge in the field of immunology has grown, the interaction between a tumour and the immune system was revealed to be a more complicated and dynamic process than once thought[29]. Not only does the immune system play a protective role, but it also helps to shape the developing tumour, in some instances promoting aggressive tumour growth by selecting for variants that are less immunogenic[27,31]. This complex process is now termed cancer immunoediting[29]. There are three distinct phases characterized in the immunoediting process, including: elimination, equilibrium and escape[30,32]. The elimination phase is marked by the success of immunosurveillence, where the cytotoxic cells of the immune system detect the presence of the cancerous cells and destroy them before clinical disease can develop. The second phase is equilibrium, marked by tumour dormancy. The third phase, coined the escape phase, is when the tumour has evaded or developed some resistance to the immune system, resulting in uncontrollable growth. This transition from equilibrium to escape is recognized as a hallmark of cancer[33]. Major cellular changes have to occur before the tumour is capable of completing the transition from the equilibrium to escape phase.

2.3. Immunosuppressive Tumour Microenvironments

In order to suppress anti-tumour immune responses, aggressive tumours often hijack negative regulatory feedback loops, which are necessary for maintaining homeostasis of the immune system and preventing excessive immune activation[34,35]. In addition to playing a vital role in promoting tumour growth and dissemination, the tumour microenvironment also facilitates a highly immunosuppressive environment, helping the cancer to evade anti-tumoural immunity[36]. The tumour can suppress the immune system through a variety of mechanisms including: reducing the presentation of target antigens and/or co-stimulatory molecules,

increasing expression of inhibitory ligands for immune receptors, producing immunosuppressive cytokines and/or by activating immunosuppressive cell populations[30].

Some tumours are known to increase expression of ligands that bind to inhibitory receptors on T cells, such as programmed cell death protein ligands 1 and 2 (PD-L1, PD-L2), which bind to their receptor, the programmed cell death protein 1 (PD-1) on activated T cells[34]. Additionally, these ligands can also be expressed by the surrounding stromal cells in the tumour microenvironment. Interactions between PD-1 and its ligands result in inhibition of the effector cells, and dampen the overall anti-tumour response. Tumour cells can also down-regulate the expression of MHC class I and other stimulatory ligands, while upregulating inhibitory signals[37].

Tumour and tumour-stromal cells are also capable of secreting various cytokines and chemokines, such as TGF-β and IL-10, which inhibit immune responses[34,38–40]. Studies have shown that IL-10 reduces the production of inflammatory cytokines, impedes antigen-presenting cell function and interferes with T and B cell responses[34]. Furthermore, tumour cells secrete other cytokines and chemokines, such as CCL22, CCL2, CXCL1, which recruit and activate inhibitory cell populations such as regulatory T cells, myeloid-derived suppressor cells (MDSCs), and tumour-associated macrophages, all of which potently suppress T cells[34,38,40].

2.4. Tumour-Infiltrating Leukocytes

The key to success for immunotherapies involves overcoming the resistance established by local tumour immunosuppression, to unleash the capacity of the immune system to eradicate cancerous cells[41,42]. Nowadays, many different types of therapies are being developed to elicit and to augment the magnitude of tumour-specific immune responses.

Although T lymphocytes are often the focus of cancer immunotherapies, they are not the only leukocytes present in tumours. B cells, neutrophils, eosinophils, natural killer (NK) cells, dendritic cells (DCs), macrophages and MDSCs are several among many subsets that can also be found in the tumour. Presently, the prognostic value of some of these different immunological populations are unclear[43]. While infiltration of $CD8^+$ T lymphocytes is generally associated with a more favourable outcome, other cell types, such as macrophages may be associated with both positive and negative outcomes, often depending on the context[44]. For most solid tumours, increased immune infiltrates appear to be beneficial, however, a couple studies have shown the presence of neutrophils in renal cell carcinomas to be associated with negative outcomes[45,46].

For melanomas, one study found that a higher density of immunological cells in the tumour was associated with improved survival[47]. In particular, higher densities of $CD8^+$ T cells correlated best with patient survival[47]. Nevertheless, some melanomas with high densities of leukocytes also did continue to progress. When considering the prognostic value of any given subset, it may be important to assess the functionality and quality of the cells in addition to the quantity and type of infiltrates. Furthermore, the spatial distribution of these leukocyte subsets may add yet another layer of complexity to the immunological landscape of tumour microenvironments[47].

2.4.1. $CD8^+$ T lymphocytes

T cells are central players in acquired immunity[48]. The $CD8^+$ T cell compartment, in particular, plays an essential role in defense against pathogens such as viruses and intracellular bacteria, as well as various malignancies[49]. The primary effector cells in anti-tumoural responses are the cytotoxic $CD8^+$ T cells. The presence of tumour-infiltrating lymphocytes (TILs) have

shown to be a favourable prognostic marker in several types of cancers[50,51], including: metastatic melanomas[52], ovarian[53,54], colorectal [55,56] and breast cancers[57]. Furthermore, studies have shown that adoptive T cell transfer can result in regression of various tumours, including melanomas[58], colon cancers, head and neck squamous cell carcinomas[59], and renal cell carcinomas[48]. Therefore, it stands to reason that augmenting TIL numbers is beneficial for treating cancers.

However, infiltrating T lymphocytes are known to adopt an exhausted or dysfunctional phenotype in cancers due to the immunosuppressive effect of the tumour microenvironment. Studies have shown that tumour-infiltrating T cells express higher levels of inhibitory receptors. Tumour-specific T cells isolated from metastatic tissue in patients with melanoma, displayed an exhausted phenotype, with increased expression of inhibitory receptors, CTLA4 and LAG3, in contrast to circulating T cells which displayed an effector cell profile[60]. Tumour-infiltrating $CD8^+$ lymphocytes have also been demonstrated to have higher expression of PD-1, which was also correlated with decreased production of IFN-γ, compared to blood-derived lymphocytes from the same patient[61]. Tumour-infiltrating leukocytes may be dominant players in the anti-tumour response, but there are obstacles they must overcome in the tumour microenvironment to mediate effective killing of tumour cells.

2.4.2. $CD4^+$ T lymphocytes

There are different subtypes of $CD4^+$ lymphocytes: Th1, Th2 and $FoxP3^+$ regulatory T cells (Tregs). Roles for $CD4^+$ T cells include: secretion of effector cytokines, recruitment of other leukocytes, such as eosinophils and macrophages, as well as providing help for the priming of $CD8^+$ effector T cells and generation of $CD8^+$ memory T cells.

The role of $CD4^+$ lymphocytes in anti-tumour responses is unclear, obscured by contradicting evidence from several studies. One study showed that vaccinated $CD4^{-/-}$ mice were unable to mount successful anti-tumour responses against B16 tumours, in contrast to wild-type and even $CD8^{-/-}$ mice[62]. Both Th1 and Th2 cytokines were shown to be important to induce robust anti-tumoural responses[62]. Th1 help is important for $CD8^+$ lymphocytes, and eosinophils are believed to be the effector cells downstream of Th2 help provided by $CD4^+$ T cells[62]. However, in another study, memory $CD8^+$ T cell responses against melanomas could still be generated in the absence of CD 4^+ T cells [63]. Therefore, it is unclear to what extent $CD4^+$ T cells are involved in anti-tumour responses.

Additionally, there is a distinct subset of $CD4^+$ lymphocytes responsible for regulating immune responses. These regulatory T cells are essential for maintaining peripheral tolerance, and are critical in preventing autoimmune diseases and in limiting chronic inflammation[64]. In cancers, however, Tregs are believed to facilitate evasion of the immune system by the tumour, as they have shown to suppress anti-tumoural responses. Studies have shown that increased frequencies of Tregs present in the tumour have been correlated with poorer prognoses[64]. Hence, the role of $CD4^+$ lymphocytes likely depends on the specific subset in question.

2.4.3. B lymphocytes

While T cells usually hold the spotlight with regards to TILs, there is emerging evidence suggesting that infiltration of B cells and plasma cells may also have prognostic potential for patient outcomes. B cells are found in relatively low numbers in melanomas compared to T cells[65,66]. However, increased B cell density has been shown to be favourable in several cancer types, including: gastric, breast, colorectal cancers and melanomas[47,67–69]. In adenocarcinoma of

the esophagogastric junction, increased B cell infiltration correlated with prolonged survival[67]. In this study, plasma cell infiltration was an even stronger indicator of improved survival[67]. Similarly, dense B cell infiltration was associated with improved patient outcomes in colorectal cancers[68]. In melanomas, increased tumour-infiltrating B cells correlated with longer survival[47]. Despite these studies, the role of B cells and plasma cells in cancers are still poorly understood. B cells are known to elicit humoral immune responses, but they can also do much more than that, including: recognizing antigens, regulating antigen processing and presentation as well as modulating T cell and innate immune responses[70]. B cells can both positively and negatively regulate immune responses and have been shown to play both pro- and anti-tumorigenic roles[68]. It has been suggested that the ambiguous role of B cells may be due to the existence of functionally distinct subsets[70]. B cells with a more regulatory phenotype may directly or indirectly suppress T cell functions. On the other hand, tumour-infiltrating B cells may also serve as antigen presenting cells locally, and contribute to the survival and proliferation of T cells in the tumour microenvironment[68,70]. B cells can also produce cytokines that promote organization of local tertiary lymphoid structures[69], which may be important in coordinating anti-tumour immune responses.

2.4.4. NK cells

The innate immune system also plays an important role in the anti-tumour immune response. NK cells make up 5-20% of blood-derived lymphocytes[71,72]. NK cells are innate lymphoid cells that can kill target cells without prior immunization or MHC restriction, setting them apart from other lymphocytes[73]. NK cells have been shown to recognize and kill melanoma cells *in vitro*[74]. NK1.1 is a marker that is expressed on NK cells of C57BL/6 mice. Studies in

which NK1.1-specific antibodies were used to deplete NK cells showed a protective effect of this subset against murine melanomas *in vivo*[75]. NK cells also play a role in preventing metastasis *in vivo*[76]. The presence of tumour-infiltrating NK cells was also shown to be a positive prognostic marker in other cancers such as colorectal carcinoma and squamous cell lung cancer[70,71].

Recognition and lysis of tumour cells by NK cells involve receptor-ligand interactions[79]. Activation of NK cells depends on the integration of multiple activating and inhibitory signals[73]. Tumour cells will down-regulate MHC class I as a mechanism to avoid $CD8^+$ T lymphocytes, but this also makes them more susceptible to NK cytotoxicity, since MHC class I molecules bind inhibitory receptors on NK cells to inhibit NK activation[73]. On the other hand, interactions between NK cells and tumour cells can also lead to selection of cancer cells with a "protective" phenotype, such as losing expression of adhesion molecules or ligands for activating receptors[73,79]. These tumour cells can undergo changes in expression of surface molecules, rendering them resistant to NK cells[79].

2.4.5. Dendritic cells

Dendritic cells were first described in the early 19th century by Paul Langerhans, but the name was given by Ralph Steinman and Zanvil Cohn in 1973. DCs are considered to be professional antigen-presenting cells, as they are capable of presenting antigens along with all of the necessary signals: co-stimulatory molecules and cytokines, for T cell activation and differentiation[80]. DCs are not only the initiators of immune responses, they also shape and modulate the ensuing response, via secretion of cytokines[80,81]. DCs are able to capture and process antigens, and they can present them on both MHC class I and II molecules[81]. Then they migrate to secondary lymphoid organs, such as the spleen and lymph nodes where they can

engage T cells[81,82]. The ability of DCs to induce robust immune responses depends on their maturation status. While mature DCs can readily prime T cells, immature DCs are inefficient at initiating an immune response. Tumour-infiltrating DCs (TIDCs) have been shown to adopt an immature or paralyzed phenotype, with low expression of co-stimulatory molecules, high expression of regulatory molecules and impaired antigen presentation; rendering them more immunosuppressive[80,83].

Due to this dual role of DCs, increased infiltration of DCs into tumours has been associated with both positive and negative prognoses in different types of cancers[80]. One study showed that TIDCs were, in fact, capable of processing antigens and migrating to the local lymph nodes to activate T cells[82]. In another study, however, DCs were shown instead to be potent suppressors of T cell responses[84]. Harimoto *et al.* also provided evidence that TIDC-mediated cross-presentation of antigens was impaired, which led to the induction of tolerance rather than activation of T cells[83]. Furthermore, depletion of DCs in murine ovarian cancer has also been shown to enhance anti-tumour immune responses[85]. It is also worth noting that different DC subpopulations have been shown to infiltrate different mouse melanomas[82]. Perhaps these contradicting prognostic impacts of TIDCs are related to the different phenotypes or relative proportion of the different phenotypes of TIDCs.

2.4.6. Neutrophils

Neutrophils make up 50-70% of all circulating leukocytes in humans[86]. Neutrophils are classically recognized as the first responders to inflammation. While inflammation is important in the initiation of immune responses, inflammation is also involved in tumourigenesis. The role of neutrophils is currently under much debate, as there is evidence of both pro-tumorigenic and

anti-tumorigenic functions from these cells. For instance, increased neutrophil infiltration has been shown to be correlated with higher grades of gliomas and tumour progression[87]. In this study, scientists found that co-culturing glioma stem cells with neutrophil progenitor cells, or even just conditioned media from the neutrophil progenitor cells enhanced the growth of the glioma cells[87]. Depletion of circulating neutrophils has also shown to reduce the metastatic burden in a liver metastasis model[88]. On the other hand, some studies show that neutrophils are crucial players in immunotherapies. Neutrophils were found to be mandatory for monoclonal antibody anti-gp75 immunotherapy in a mouse melanoma model[89]. Furthermore, this study also showed that anti-Gr1-mediated depletion of neutrophils abolished the therapeutic effects of Trastuzumab immunotherapy in a murine model of breast cancer[89]. This complex picture has led researchers to believe that there may be more than one subset of neutrophils. In fact, there is emerging evidence suggesting that it may be more appropriate to further differentiate neutrophils into N1 and N2 subtypes, similar to macrophages[90]. But similar to macrophages, neutrophils may actually exist as a spectrum of phenotypes, rather than two clear-cut subtypes. However, this distinction has only been shown in mouse models, and so it is unclear if human neutrophils behave in the same way.

2.4.7. Eosinophils

Eosinophils are classically known for their role in regulation of allergic and autoimmune responses as well as host defense against parasites[91]. They are also believed to play a role in homeostasis and tissue repair[91]. However the role of eosinophils in cancer is unclear. While they have been suggested to have tumourcidal potential, it has not been formally shown. However, depletion of eosinophils resulted in impeded tumour rejection in mice, suggesting that

eosinophils do in fact play a role in the anti-tumour response[91]. Eosinophils are responsible for secreting chemoattractants, which recruit T cells to tumours[91]. Eosinophils are also implicated in the regulation of T cell activation, proliferation, and cytokine secretion[92]. Furthermore, eosinophils may play a role in normalization of tumour vasculature. Depletion of eosinophils was also shown to impair infiltration of $CD45^+$ leukocytes[91]. Therefore, rather than mediating direct anti-tumour effects, eosinophils may be important accessory cells in the anti-tumour response.. Nevertheless, the prognostic value of eosinophils remains inconclusive. While eosinophila is associated with improved prognosis in several types of solid tumours, including: colon tumours, oral squamous cell carcinomas, bladder, and prostate cancers[92,93], increased accumulation of eosinophils is associated with poor outcomes in cervical cancers and Hodgkin's lymphoma[93].

2.4.8. Myeloid-derived suppressor cells

Myeloid-derived suppressor cells are a population of phenotypically heterogeneous cells of myeloid origin that have not fully matured and have immunosuppressive functions[94]. There are generally two major subsets of MDSCs: monocytic MDSCs and granulocytic MDSCs. MDSCs have shown to suppress T cell responses, and modulate cytokine production[95]. MDSCs also play a role in promoting tumour angiogenesis, tumour cell invasion and metastasis[95]. Others have also demonstrated a relationship between MDSCs and chronic inflammation, which is known to be associated with the onset and progression of melanomas[96]. MDSCs can exert their immunosuppressive functions through multiple mechanisms that are dependent on cell-to-cell contact and oxidative metabolism[94,97]. Inhibiting ROS reduces the suppressive potential of MDSCs[94,97]. MDSCs can also mediate immunosuppression via depletion of arginine and induction of Tregs. Low numbers of MDSCs in tumours are associated with longer survival in

patients with melanomas[98]. Similarly, increased numbers of MDSCs in pancreatic, esophageal and gastric tumours were shown to be a strong independent prognostic factor of poorer survival[99].

3. Cancer Vaccines

3.1. Vaccine Platforms

Several decades of research have been invested into developing cancer vaccines that are capable of increasing the number of tumour-specific T cells to treat and provide protection against recurrence of cancers and metastatic disease in patients[100,101]. Classically, vaccines are administered in prophylactic settings, in order to prevent infectious diseases[102,103]. Some cancer vaccines are used in a similar manner, such as Gardasil® (Merck & Co., Inc.) and Cervarix® (GlaxoSmithKline), which are used to prevent infections with human papillomavirus (HPV), an essential step in the pathogenesis of many cervical cancers[104]. Other cancer vaccines are used therapeutically; administered after the onset of disease[102]. Therapeutic immunization in the setting of established, chronic disease has generally achieved less success than prophylactic immunization in preventing infectious diseases, due to a number of factors. Firstly, immune responses to acute and chronic disease are different. Humoral immunity is important for controlling acute infections, while control of chronic diseases relies more heavily on cellular immunity[103]. Secondly, in the context of therapeutic immunization, the immune system is being alerted to a chronic disease context in which immunoregulatory pathways have been developed to limit the immune response[103]. Thirdly, the burden of disease in a chronic context may

outweigh the immune response, thus limiting the therapeutic impact of the immune response induced by the vaccination[103]. Additionally, in the context of cancers, most tumour-associated antigens are expressed by both malignant and normal cells[41], which means the majority of cancer vaccines are designed to generate immune responses against self rather than foreign antigens[105]. Therefore, cancer vaccines must overcome immunological tolerance to self to induce an immune response against the cancer cells, while sparing the normal cells.

Currently there are a multitude of vaccine platforms that are being explored for the delivery of these tumour-associated antigens. With melanomas, vaccine platforms can be categorized into two major groups: cell-based and non-cell based[42]. Cell-based vaccines include: non-modified whole cell vaccines, genetically modified tumour vaccines, and DC-based vaccines; while non-cell based vaccines include: peptide-based, viral vectors, RNA and DNA vaccines[42,100]. Cancer vaccines in general have shown to be well tolerated, with minimal to no adverse events. Many of these have shown some promising efficacy in pre-clinical studies; for instance, lentiviral vectors encoding melanoma-associated antigens were able to induce potent antitumour immunity, which prevented and inhibited the growth of B16F10 tumours in mice[106]. Other studies also showed that DNA-based vaccines targeting gp100 and DCT induced T cell-dependent immune responses that inhibited tumour growth[107,108]. Other melanoma vaccines are currently being examined in clinical trials, such as the Melanoma GVAX, a lethally irradiated whole cell vaccine, that was genetically engineered to secrete granulocyte-macrophage colony stimulating factor (GMCSF) [109]. This vaccine was administered in an adjuvant setting to patients who had received surgery but were at a high risk for recurrent disease, and was found to be safe and well tolerated by the patients[109]. In veterinary medicine, there is currently a USDA-approved vaccine available for treating stage II and III canine oral melanomas. The Oncept vaccine

(Merial) is a DNA vaccine against tyrosinase, a protein involved in the synthesis of melanin[110]. It is often used post-surgery to prevent the development of metastases. The therapeutic benefit of this vaccine is controversial, due to a lack of randomized control trials used to test its efficacy. Recent retrospective studies have not shown significant increases in progression-free or median survival following treatment with the Oncept vaccine[110,111]. However, as retrospective studies are limited by low statistical power, due to small numbers of replicates and group heterogeneity, controlled studies are needed to more accurately assess whether this vaccine is able to prolong survival in dogs[111]. Though, in theory, cancer vaccines represent a promising avenue to achieve durable systemic immune responses against tumours, there is still a long way to go to develop safe and efficacious vaccination strategies capable of significantly prolonging overall survival in patients.

3.1.1. Viral vectors

Recently, recombinant viral vectors have gained interest among vaccine researchers, as they provide an effective method for delivering heterologous antigens *in vivo*, representing a promising platform for novel vaccines[112,113]. They are being recognized for their capacity to infect cells, and to express transgenes that would be released into the surrounding millieu, providing a source of target antigens for inducing both humoral and cellular immunity[112,113]. Viruses also have intrinsic adjuvant properties, as they naturally express a wide range of pathogen- and danger-associated molecular patterns[112]. Viral vectors represent useful and efficient platforms for vaccine development in human and veterinary research and are also suitable for large, industrial-scale production under good manufacturing protocol conditions[114].

The main concerns with viral vaccines are with regards to safety. But there are now many different methods established to attenuate virulent strains of viruses for therapeutic use, especially with emerging technology that allows for targeted gene deletions. Modification or deletion of important proteins that facilitate the cellular entry of the virus[113], as well as altering the expression of crucial structural proteins, can limit the replicative potential of viruses, and enhance their safety in clinical applications[112,113]. Other concerns regarding the use viral vaccines include pre-existing viral vector-specific immunity, which can limit their effectiveness[112], and optimization of vector design to drive robust responses against encoded transgenes rather than vector-specific responses.

Other considerations in selecting optimal viral platforms for immunization include tissue tropism, immunogenicity of the virus - especially in the context of a vaccine, as well as the potential for large-scale production.

3.1.2. Prime-boost immunization strategy

One strategy to induce potent cell-mediated immunity is the use of multiple vaccinations[115]. Homologous boosting, which involves boosting with the same agent repeatedly, is effective for inducing humoral immunity, but is relatively inefficient at inducing robust secondary cellular responses, due to prior immunity to the vaccine vector[115]. An approach to circumvent this problem, is sequential administration of vaccines using different delivery vectors[115]. This is known as heterologous boosting, and has shown to be effective at inducing robust responses from both $CD4^+$ and $CD8^+$ cells, as well as T cell memory[20,115]. This prime-boost regimen has been used to combat a variety of pathogens, including: M. tuberculosis, human immunodeficiency virus, Ebola virus, Hepatitis C virus, Herpes Simplex Virus and

Human Papillomavirus[116]. The first proof-of-concept heterologous prime-boost vaccination was used to treat malaria in the 1900s, and incorporated the use of a DNA primary vaccine, followed by a recombinant vaccinia virus[112]. This heterologous prime-boost regimen generated responses that were of much higher magnitude than that of either vaccine alone, demonstrating a synergistic effect of the combination[48]. Although current vaccines for melanomas have demonstrated limited efficacy, prime-boost immunization nonetheless represents a strategy that could be incorporated to enhance weak immune responses.

Prime-boost vaccination is effective because while primary vaccines induce effector cells that display robust cytotoxicity, these effector cells are limited in their capacity to proliferate[49]. This being said, primary vaccines also induce memory T cells, and subsequent administration of booster vaccines stimulate these memory cells, which proliferate rapidly upon antigen encounter, and then convert into effector cells, to eradicate the targets[49]. One study demonstrated that a prime-boost regimen involving two different viral vectors, expressing an identical transgene, could effectively generate tumour-specific immune responses, leading to reduced tumour burdens in mice[117]. Another study using a B16F10 mouse model demonstrated, that a prime-boost strategy with intravenous delivery of an oncolytic virus booster vaccine, could generate anti-tumour immune responses of greater magnitude, and could do so at the peak of the primary response[49]. The rapid induction (with a shortened prime-boost interval) of a higher magnitude T cell response was revealed to be due, at least in part, to the route of administration[49]. The intravenous delivery of the OV allowed the virus to travel straight to the B cell follicles of the spleen where it infects B cells, which subsequently pass the OV-encoded TAAs to follicular DCs for presentation to local central memory $CD8^+$ T cells[49]. This unique interaction identified between the splenic B cells and DCs allowed for engagement of the central memory T cells even

during an ongoing effector phase, bypassing the negative regulation that would normally occur due to elimination of antigen laden DCs by activated effector T cells[49]. This novel strategy allowed the expansion of $CD8^+$ T cells to be maximized in a short period of time, resulting in a robust anti-tumour immune response[49].

3.2. Oncolytic Viruses

Oncolytic viruses have the ability to selectively target and destroy cancerous cells[118]. Some OVs naturally target cancer cells, because they require certain receptors for cell entry, which are overexpressed on the surface of malignant cells[119]. Other OVs preferentially target cancerous cells due to altered signalling pathways, which make these cells ideal environments for the virus to replicate[120]. These defects often confer growth and survival advantages to the cancerous cells, but they also leave them highly susceptible to infection with viruses[119,121]. Viruses like reovirus and vaccinia have been shown to exhibit a preference for infecting cells with overactive Ras signalling[119]. Vesicular stomatitis virus (VSV) demonstrates a preference for infecting cells with defective IFN signalling[122,123]; a defect that is found in 50% of human melanomas[122].

Besides mediating oncolysis, the tumour selectivity of OVs allows them to be ideal "in situ cancer vaccines"[120]. OVs not only kill tumour cells, their presence also causes local inflammation within the tumour microenvironment[124–126]. The current paradigm is that this intra-tumoural inflammatory response induced by the virus subsequently recruits and activates a multitude of immunological cells[124,125]. Infection of cancer cells with OVs has shown to cause the release of various cytokines and chemokines that are involved in the attraction and activation of various leukocytes. For instance, infection with VSV induces the production of IFN-α[127].

Neutrophil chemoattractants have also shown to be increased in response to infection with VSV[126]. While neutrophils are classically the first responders to inflammation, they are also believed to play a role in shutting down blood flow to tumours[126]. Reovirus also induces production of a variety of pro-inflammatory cytokines, including: RANTES, IL8, MIP-1a, MIP-1b and IFN-b, which were shown to activate NK cells, DCs and cytotoxic T lymphocytes[128]. Further, viral infection results in the release of DAMPs and heat-shock proteins that potentiate immune responses[124].

As a therapeutic, OVs are also well-tolerated compared to conventional chemotherapies and radiation therapies[129]. These viruses can also be manipulated with relative ease for attenuated pathogenicity, and enhanced immunogenicity[119]. Some can be engineered to express tumour-associated antigens in order to drive robust tumour-specific immune responses. Other OVs even induce immunogenic cell death, presenting a natural repertoire of tumour-associated antigens and danger signals[120].

3.2.1. Adenoviruses

Adenoviruses are double-stranded DNA viruses with a genome of approximately 34-43 kb[130], and are currently the most widely used viral vector platform for vaccine design[131]. They have demonstrated immune-mediated efficacy and safety in numerous clinical applications[114], and a large body of evidence suggests that adenoviruses are able to prime and boost T and B cell responses remarkably well, conferring protection for a variety of pathogens[42,116].

Originally, adenoviruses showed promise for gene replacement therapy, due to their ability to be easily modified, their safety profile, broad tissue tropism and ability to drive robust expression of transgenes[131]. Unfortunately, their clinical efficacy was short-lived due to robust

immune responses against the virus and transgene products[131]. Being highly immunogenic, along with its other favourable attributes quickly made adenoviruses attractive platforms for vaccines[131,132].

Other advantages of adenoviruses include: a well characterized genome, with the potential capacity for integrating transgenes over 6kb in length, allowing the delivery of multiple or large antigens[133]. Recombinant adenoviruses have also been used for gene therapy at a wide range of doses with minimal side effects[133]. In addition, studies suggested that adenoviruses transfer genes effectively to antigen-presenting cells *in vivo*, thus promoting rapid and robust transgene-specific humoral and cellular immunity[132,133]. Adenoviruses appear to be especially effective at inducing $CD8^+$ T cell responses[131]. Adenoviruses can also be grown to high titres easily, and are relatively thermostable[133]. Lastly, the extensive clinical history with adenoviral vector therapies has made the process to scale up and purify adenoviral-based vectors a much more standardized and efficient practice than compared to other viral vectors[131].

Although adenoviruses represent nearly ideal vaccine vectors, they are not without limitations. One major hurdle of using adenoviruses as vaccine vectors is pre-existing immunity in the human population[130,134,135]. Natural adenoviral infection has a worldwide distribution[135]. Unfortunately, human subjects, unlike experimental animals, which are kept in controlled environments, can be pre-exposed to adenoviruses, resulting in antibody titres, which rapidly neutralize adenovirus-based vaccines[130]. Neutralizing antibodies reduce the uptake of adenovirus vectors by cells, thus reducing expression of the desired transgenes, ultimately dampening the resulting immune response[130]. Some methods to circumvent this problem include, using a higher dosage of the vaccine to overwhelm antibody-mediated neutralization, or the use of alternative adenovirus serotypes that are less prevalent, or the use of adenoviruses from species other than

humans[130,134]. There are currently 51 different known human serotypes of the virus[134]. However, cross-reactive anti-vector immune responses between serotypes are possible, and may limit the effectiveness of this alternative approach.

3.2.2. Vesicular Stomatitis Virus (Rhabdobviruses)

VSV is a rhabdovirus with a single-stranded, negative-sense RNA genome that can infect both insects and livestock[136,137]. VSV is classified in the *Rhabdoviridae* family, which are bullet or cone-shaped viruses, which are typically 100-430 nm in length and 45-100 nm in diameter[136]. VSV carries a compact 11 kb genome, which allows it to synthesize subgenomic mRNAs for its 5 distinct proteins[121,137]. Recombinant VSV genomes can accommodate over 4.5 kb of foreign RNA, which can be expressed at high levels[137]. It is relatively easy to engineer VSV vectors to express transgenes for vaccine use[137].

Naturally occurring infections of humans are rare, but have been reported in individuals exposed to infected livestock and in researchers exposed to laboratory environments[137]. Most infections with VSV are asymptomatic in humans, although there have been cases of mild flu-like symptoms reported previously[137]. The occurrence of antibodies against VSV in the human population is also very low[121,137]. The fact that VSV is a RNA virus also makes it safe, as there is no known risk of its integration into host genomes[121]. In addition, VSV can be produced to very high titers, which is crucial for therapeutic use[137]. These are all important considerations for the potential of VSV as a tumour vaccine candidate.

Furthermore, VSV is highly sensitive to interferon-mediated anti-viral mechanisms, and therefore, does not efficiently replicate in normal cells[121,138]. Tumour cells often have defective IFN signalling, and as a result, are more susceptible to infection and subsequent killing by

VSV[138]. However, the efficacy of VSV may be diminished in a tumour where surrounding stromal cells care capable of producing interferons. VSV also has an extensive tissue tropism and a short replicative cycle, allowing VSV to rapidly propagate and spread within tumours[123,137]. The ability of VSV to replicate rapidly in a wide range of tissues, while being exquisitely sensitive to anti-viral responses, and therefore highly attenuated in normal tissues, makes VSV an excellent choice for oncolytic virotherapy.

3.2.3. Viral-induced lymphopenia

Characteristic of many viral infections is a transient reduction in lymphocyte counts in blood, a phenomenon known as lymphopenia[139,140]. This occurs during acute infections and has been shown to be mediated by type I interferons[139]. Lymphopenia was observed as early as three hours post-injection of VSV and lymphocyte numbers returned to normal after six days[140]. This lymphopenic effect has been shown to extend to the tumour as well[141].

There is evidence that virus-induced lymphopenia is at least partially mediated by type I interferons, as the lymphopenic effect was less prominent in IFNAR$^{-/-}$ mice[139]. Peritoneal injections of IFNs also resulted in lymphopenia[140]. IFNα/β play important roles in induction of immune responses. Type I IFNs have been shown to activate NK cells, be involved in DC maturation and survival, as well as act as the third signal for lymphocytic activation[142]. Macrophages, NK cells, DCs, and fibroblasts can produce type I interferons in response to viral infections[142]. Furthermore, lymphopenia was observed in mice (both IFNAR$^{-/-}$ and WT) with adoptively transferred WT leukocytes, whereas the phenomenon was abrogated when the transferred cells were from IFNAR$^{-/-}$ mice, into WT and IFNAR$^{-/-}$ recipients. These observations

suggest that the lymphopenia was not a result of stimulating the endothelium, nor other surrounding factors, but a direct effect upon the lymphocytes themselves[139].

Due to the rapid, and dramatic depletion of lymphocytes, Shattner *et al.* suggested it was unlikely that the effect was due to cytopathic effects of VSV[140]. Using CFSE-labelled splenocytes transferred from a syngenic mouse, Kamphuis *et al.* demonstrated that, while the number of CFSE-labelled cells was also reduced during the leukopenia, these labelled cells would eventually return[139]. However, contrary to these observations, Leveille et al. showed that VSV was able to infect and kill DCs both in vivo and in vitro[141]. Additionally, there was no evidence of changes in the bone marrow, suggesting the disappearance of lymphocytes was not due to reduced production from the bone marrow[140]. There was also no evidence of trapping of lymphocytes in the liver[140].

Rather than lymphocytic destruction, homing of lymphocytes to secondary lymphoid organs (SLOs) was alternately proposed as an explanation for the decline of these cells in the blood[140]. It is known that lymphocytes continuously home to secondary lymphoid organs as they circulate through lymphatic and blood vessels[139]. The trafficking and homing of lymphocytes is a complicated process that involves a variety of selectins, integrins and chemokines etc. However, while B cells appear to accumulate in the spleen, there is no evidence that T cells do so as well[139]. Furthermore, Leveille *et al.* also show that VSV actually decreased the number of intratumourally injected CFSE-labelled DCs to the draining lymph node[141]. Similarly, VSV treatment reduced the number of $Thy1.1^{+}$ lymphocytes, adoptively transferred intratumourally to $Thy1.2^{+}$ mice, that travelled to the draining lymph node[141].

Another hypothesized mechanism that hasn't been explored is the adherence of leukocytes to the endothelium of blood vessels. Under inflammatory conditions, chemokines

direct leukocytes to the site of damaged tissue, through a process called extravasation[143]. Endothelial cells upregulate expression of selectins, which capture the leukocytes to initiate the rolling of these cells along the endothelial wall[143,144]. This leads to activation of leukocyte integrins which bind to adhesion molecules such as ICAM1 and VCAM1, on endothelial cells for a firm adhesion, prior to extravasation of the leukocytes into the damaged or infected tissue[143]. This process is important for the recruitment of leukocytes, and has been suggested as a mechanism for the lymphopenia that occurs with viral infections.

It is unclear whether all leukocytes are affected by viral infection in the same manner. In one study, it was found that administration of VSV resulted in a decrease in intratumoural DC numbers 24 hours later, consistent across three different tumour types (with different murine backgrounds)[141]. Similarly, tumour-infiltrating T cells and NK cells demonstrated a decrease, detected as early as 12 hours after administration of the virus, but neutrophils found in the tumour demonstrated an acute increase[141]. The different populations also seemed to return at different rates, some having increased beyond the number in control mice by four days. In another study, tumours were analyzed on day 10 with VSV injections on day 1, 3 and 5[145]. The results in this study demonstrated an increase in $CD8^+$ T cells, but a decrease in MDSCs and no significant changes in NK and DC numbers[145]. Since the tumours were analyzed at different times, these results do not appear to be contradictory. However, both studies show that distinct leukocyte subsets are affected differently by VSV. The mechanisms behind this phenomenon as well as effects on specific immunolgical subpopulations are still unclear and warrant further investigation.

4. Melanogenesis

4.1. The melanogenesis pathway

Melanin is the pigment responsible for skin colour. It is produced in specialized organelles called melanosomes that are found in melanocytes[146]. Melanin also provides defense against UV radiation-induced skin damage[146,147]. Exposure to UV radiation results in the generation of reactive oxygen species (ROS), and also induces the biosynthesis of melanin[146,148].

Melanogenesis is the process by which tyrosine is converted into melanin (**Fig. 1**). Melanogenesis involves a series of reactions catalyzed by three essential enzymes: tyrosinase, tyrosinase-related-proteins 1 and dopachrome tautomerase (TRP1 and DCT)[146,147]. This process also involves a number of transcription factors and diverse signalling pathways. Tyrosinase is responsible for two rate-limiting reactions of the melanogenesis pathway: the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and the oxidation of DOPA to dopaquinone. DCT is responsible for rearranging dopachrome to 5,6-dihydroxyindole (DHI) or to indole 5,6-quinone 2 carboxylic acid (DHICA). The transcription factor that regulates the expression of these three key enzymes is MITF. In fact, increased melanogenesis due to UV exposure is believed to occur via the upregulation of tyrosinase[147]. This being said, melanogenesis has been shown to be modulated by a variety of pathways, such as the cyclic adenosine monophosphate and protein kinase A pathway, and mitogen activated protein kinase signalling.

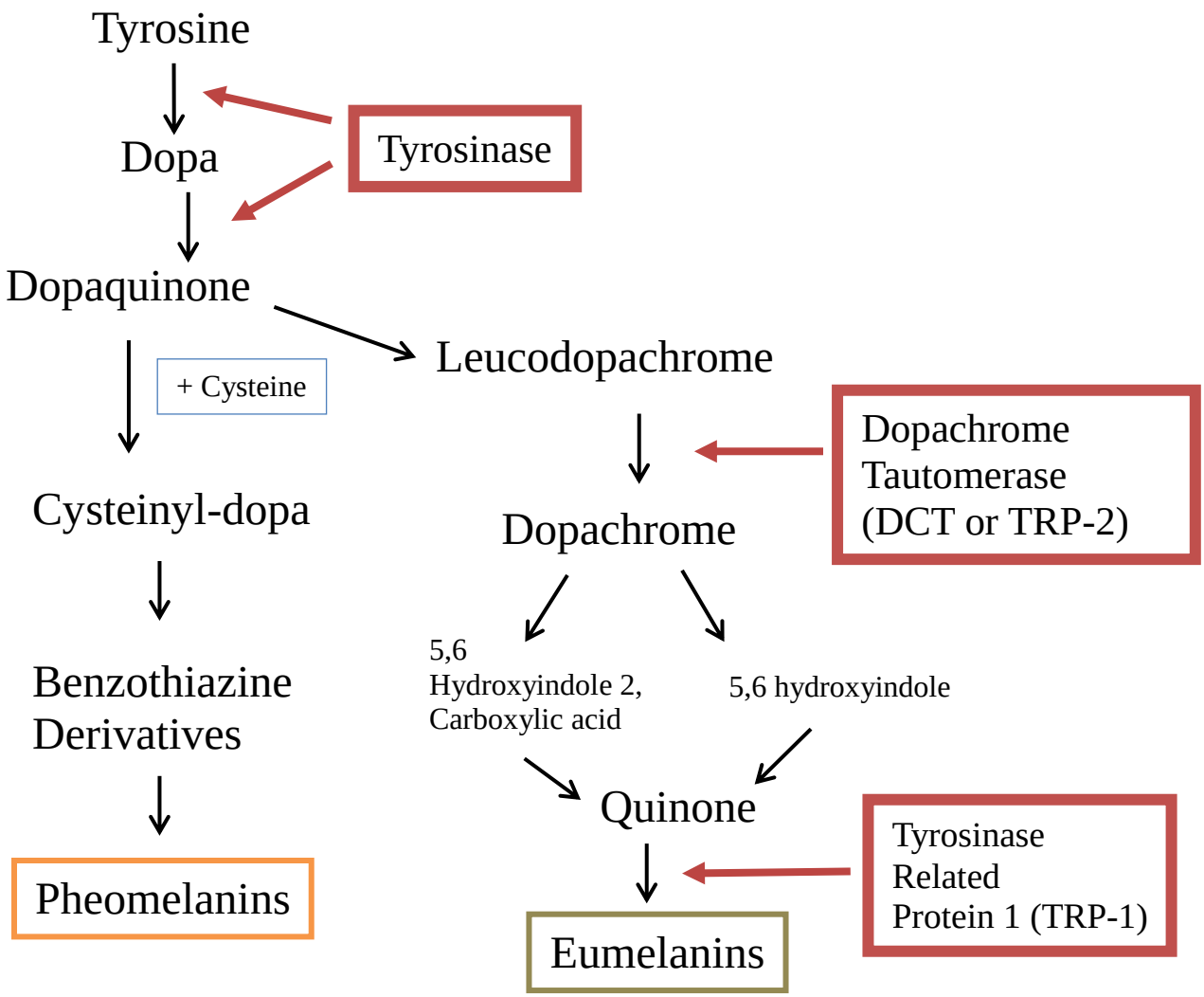

Figure 1: Melanin synthesis pathway

4.1.1. Reactive oxygen species and melanogenesis

Reactive oxygen species are important for cell survival and integrity[149]. ROS are produced by mitochondria and peroxisomes during normal metabolic processes[148]. However, high concentrations of ROS can be deleterious to proteins and enzymes, and it can disrupt

signalling pathways. Excessive oxidative stress can disrupt homeostasis of melanocytes, resulting in cell death or leading to malignant transformation[148]. Melanocyes in particular are sensitive to the presence of ROS[149]. High levels of ROS have been shown to inhibit tyrosinase, yet on the other hand, lower concentrations of ROS can upregulate and activate tyrosinase as well as other important enzymes in the pathway.

In general, the relationship between ROS and melanin is not well understood. There are conflicting reports regarding melanin playing a pro or antioxidative role within melanocytes. On one hand, studies have shown that melanogenesis generates ROS as a byproduct[150,151], yet other studies have demonstrated a protective role of melanin against oxidative damage. In one study, increases in cellular peroxide production by human melanoma cells in response to UVA irradiation was not found to be correlated with pigmentation of the cells[152]. However, increases in production of mitochondrial superoxides were reduced in highly pigmented human melanoma cells compared to non-pigmented and poorly pigmented human melanoma cells, suggesting that melanin may play a protective role against mitochondrial superoxide production[152]. Furthermore, the presence of melanin in these cells also conferred protection against mitochondrial DNA damage[152].

4.2. Metformin

Metformin is a member of the biguanide family, and is currently the most widely used oral anti-diabetic drug for Type 2 diabetes mellitus[153–155]. Metformin is highly favoured for its effectiveness, low cost and safety profile[154]. Adverse events include mild gastrointestinal symptoms that are often transient[154].

Studies have shown an association between diabetes and increased risk for pancreatic, hepatocellular, endometrial and bladder cancers[154]. On the flip side, a number of studies have also shown a correlation with metformin treatment and a decreased risk of developing certain types of cancers, as well as improved outcomes for metformin-treated diabetic cancer patients[153,154]. Additionally, metformin has also shown some potential as an anti-cancer therapeutic. In several studies, treatment with metformin has demonstrated a reduction in *in vitro* cell growth and proliferation in several different cancer types, including: hepatocellular carcinoma, colon cancer cells, glioma and melanoma cells [153,155–157]. Another study also showed that metformin negatively impacted the viability of melanoma cells, and did so through both AMPK-dependent, and -independent pathways[155]. This same study further demonstrated in vivo, that metformin inhibited tumour growth in mice[155].

The mechanisms behind the suggested anti-cancer effects of metformin are unclear. Metformin appears to affect a number of different signalling pathways. For instance, Metformin exerts effects on LKB1 - a protein involved in the AMPK/mTOR pathway, which ultimately leads to reduced protein synthesis and cell proliferation[153,155]. Metformin also disrupts complex I of the mitochondrial respiratory chain, which leads to changes in the production of ROS[154,157]. Furthermore, metformin also has effects on glucose homeostasis, as it reduces hepatic gluconeogenesis and increases uptake of glucose by skeletal muscle [155].

The effects of metformin on the immune system have yet to be explored in depth. One study showed that metformin protects $CD8^+$ TILs from apoptosis, and favours generating effector memory T cells, which circulate in the blood, versus central memory T cells cells, which home to secondary lymphoid organs[158].

5. Summary

Overall, melanomas represent a type of cancer for which conventional therapies have failed to yield effective results in advanced stages. Immunotherapies have emerged as promising alternatives in the last several decades. Viral vaccines in particular have continued to demonstrate exciting efficacy in pre-clinical settings, generating robust immune responses against melanoma-associated antigens. Furthermore the use of genetically engineered OVs presents the unique opportunity to combine direct oncolysis with induction of targeted immune responses against tumours. But despite this, these agents have largely failed to impress in clinical settings. Clearly, there still remains room for improvements of these immunotherapies. Answers likely lie behind a cloud of complex interactions between the tumour, immune system and viruses. The goal of this thesis is to explore some of these complicated relationships; to highlight the immunosuppression exerted by the tumour over various leukocyte subsets, to investigate how administration of OVs modulates the immunological landscape within a tumour, and to evaluate the potential effects of drugs that modulate melanogenesis on the immunogenicity of melanomas. Greater understanding of the tumour-immune system-virus network will provide a clearer way forward in the development of efficacious immunotherapies for melanomas.

CHAPTER TWO:

MECHANISMS THAT ALLOW ONCOLYTIC VIRAL REPLICATION DESPITE PRE-EXISTING IMMUNITY AGAINST A VIRUS-ENCODED ANTIGEN

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ABSTRACT

Oncolytic viruses (OVs) selectively target and destroy cancerous cells, while leaving normal cells unharmed. In addition to directly reducing tumour burden, OVs engineered as vaccine vectors can boost tumour-specific immune responses. Having confirmed that OVs are safer when used as booster vaccines, a mystery remained: How could OVs replicate and mediate oncolysis in hosts with pre-existing T cells specific for a tumour antigen that was also encoded by the

virus? We hypothesized two possible mechanisms to explain this phenomenon: (1) Acute virus-induced lymphopenia that transiently reduced transgene-specific tumour-infiltrating lymphocytes (TILs), and/or (2) The immunosuppressive tumour microenvironment rendered TILs dysfunctional and inefficient at clearance of viruses. We used an intradermal B16F10 melanoma model in which mice were primed with an adenovirus expressing the melanoma antigen dopachrome tautomerase (DCT) and boosted with vesicular stomatitis virus also encoding DCT. Our results show that the numbers of DCT-specific T cells inside tumours were reduced at six hours post-boost, with a preferential reduction of those with a multi-functional phenotype. Assessments of transgene-specific $CD8^+$ TILs revealed impaired degranulation, reduced production of multiple cytokines, and lower T cell receptor avidity suggesting they were functionally impaired compared to blood- and spleen-derived T cells. Analysis of *in vivo* cytotoxicity also revealed reduced killing of target cells in the tumour. Our results suggest both mechanisms are involved in allowing virus-mediated oncolysis despite pre-existing virus-specific immunity. Although seemingly counterintuitive, this research identifies induction of OV-specific immunity as a strategy to enhance the safety of oncolytic virotherapy while retaining direct oncolytic activity.

INTRODUCTION

Melanomas have been increasing in incidence worldwide over the past several decades[4,5,159]. Melanomas are cancers that originate from melanocytes, which are the cells responsible for producing the pigments that protect against UV radiation[2]. Although melanomas

only account for 4% of all cases of skin cancer, they account for approximately 80% of skin cancer-related deaths[9]. Metastatic melanomas in particular have very poor prognoses, as conventional therapies have not been successful in improving overall survival in this stage of the disease[4,9]. As a result, much current research is focused on the development of novel biotherapies with the potential to effectively treat cancers like melanomas.

Oncolytic virotherapy, in particular, has gained recent interest due to its ability to selectively target and destroy cancerous cells[118]. In addition to direct oncolysis, which is transient in nature, oncolytic viruses (OVs) can be used as *in situ* vaccines, or can be engineered to express tumour-associated antigens (TAAs) to elicit robust and long-lasting immunity against cancerous cells[120].

Oncolytic virus-vectored vaccines are likely best utilized as secondary vaccines in the context of heterologous prime-boost strategies for increased treatment efficacy. In previous studies, a heterologous vaccine strategy with a replication deficient adenoviral vaccine encoding a TAA followed by an oncolytic vesicular stomatitis virus (VSV) encoding the same TAA demonstrated efficacy in inducing robust melanoma-specific $CD8^+$ T cell responses[20]. This approach not only increased the safety of the OV that was used, it also directed the immune response towards the TAAs rather than the viral antigens[124]. More recently, it was discovered that intravenous administration of the OV led to infection of follicular B cells and subsequent antigen presentation by neighbouring DCs, resulting in rapid and robust induction of secondary responses[49]. This method of viral administration allowed secondary responses to be generated even when boosting at the peak of the primary response[49]. This novel finding shortens the classic

prime-boost interval substantially, which may be an added benefit for treating cancers, since it also reduces the time that cancerous cells can continue to proliferate rapidly. Since the OV is being administered at the peak of the primary response, there is an existing pool of TAA-specific effector T cells present. This is a time at which the immune system is expected to be most efficient at recognizing and eliminating sources of the TAA. Since the TAA is also expressed by the OV, a virus-specific response is also expected. In spite of this, it appears that OV replication within the tumour is only minimally attenuated by the immune system, evidenced by increasing viral titers found in the tumour.

In this present study, we seek to elucidate the mechanisms behind this phenomenon, in order to explain how the virus is able to replicate in a host despite pre-existing immunity against a viral-encoded antigen. There are two mechanisms we hypothesize are involved, which include: (1) acute virus-induced lymphopenia, which transiently removes TAA-specific TILs from the tumour microenvironment, and (2) the highly immunosuppressive tumour microenvironment which renders T cells dysfunctional and unable to effectively clear the infection. Lymphopenia is characteristic of acute viral infections and can be detected as soon as three hours post-infection[140]. We hypothesized that this phenomenon could also be occurring in the tumour. The reduced quantity of effector cells coupled with a dysfunctional phenotype of the remaining effector cells could provide a short window of opportunity for the virus to mediate oncolysis in a relatively unimpeded fashion.

MATERIALS AND METHODS

Mice. Eight to ten-week-old (at study initiation) female C57BL/6 (H-2^b) and Balb/c (H-2^d) mice (Charles River Laboratories, Wilmington, MA) were housed in a specific pathogen-free animal isolation facility at the University of Guelph. All animal experimentation was approved by the University of Guelph's Animal Care Committee and complied with the Canadian Council on Animal Care guidelines.

Virus-vectored vaccines. An E1/E3-deleted, replication-deficient human serotype 5 adenovirus (Ad) engineered to encode the full-length gene for hDCT (Ad-hDCT) has been described previously and was used as a primary cancer vaccine[160]. Ad-hDCT was propagated in HEK293 cells (ATCC, Manassas VA) and purified on a cesium chloride gradient. Titers for Ad-hDCT were determined using a adenovirus hexon protein-specific antibody (abcam; Cambridge, UK; Cat#: ab8249) to stain infected HEK293 cells. A recombinant VSV with a methionine deleted at position 51 of the matrix protein that had been engineered to express hDCT (VSVΔm51-hDCT) was propagated in Vero cells. VSVΔm51-hDCT has been described previously and has been used as a booster vaccine in the past[20,138]. VSVΔm51-hDCT was purified by ultracentrifugation on a sucrose gradient followed by dialysis in phosphate-buffered saline (PBS) and titers were determined by plaque assays, using Vero cells. Anesthetized mice were immunized by intramuscular injection of $1x10^8$ plaque-forming units (pfu) of Ad-hDCT in 100µL of PBS (50µL/hamstring), followed by injection of $1x10^9$ pfu of VSVΔm51-hDCT in 200µL of PBS into a tail vein.

In vivo *tumour models.* To establish melanomas, C57BL/6 mice received intradermal injections of $2.5x10^5$ B16F10 cells in 30 µL of PBS. The cells were maintained in Dulbecco's modified eagle medium (Fisher Scientific; Hampton, NH; Cat #: SH3002201) and 10% heat-inactivated bovine calf serum, and washed twice using PBS prior to injections. Anesthetized mice were immunized with Ad-hDCT four days post-tumour challenge, and received VSVΔm51-hDCT 13 days post-tumour challenge. Tissues were harvested 6 hours post-VSV treatment. (Refer to **Suppl. Fig. 1a**).

Flow cytometry reagents. The following monoclonal Abs were used in flow cytometry assays: anti-CD16/CD32 (clone 93; ThermoFisher Scientific; Waltham MA; Cat#14-0161-86), anti-CD3 BV421(clone 1 45-2C11; BD Horizon; Cat# 562600), anti-CD8 BV510 (clone 53-6.7; BD Horizon; Cat# 563068) and anti-CD4 FITC (clone RM4-4; ThermoFisher Scientific; Waltham MA; Cat# 11-0043-85) for detecting surface markers, Fixable Viability Dye-efluor780 (ThermoFisher Scientific; Waltham MA; Cat# 65-0865-18) to exclude dead cells, anti-IFNγ APC (clone XMG1.2; ThermoFisher Scientific; Waltham MA; Cat# 17-7311-82), anti-TNFα PE (clone MP6-XT22; ThermoFisher Scientific; Waltham MA; Cat# 12-7321-82) for intracellular cytokine staining and CD107a PE Cy7 (clone eBio1D4B; ThermoFisher Scientific; Waltham MA; Cat #46-1071-82) for detecting degranulation. Violet Proliferation Dye (BD Horizon; Cambirdge, UK; Cat #: 562158) was used to label donor target splenocytes for *in vivo* cytotoxicity analyses.

Preparation of T cells. Blood was collected from the periorbital sinus and red blood cells were osmotically lysed using ACK (ammonium-chloride-potassium) lysing buffer. For TIL

preparations, anesthetized mice received transcardial perfusions with PBS prior to excision of intradermal melanomas to minimize potential contamination with blood-borne lymphocytes. Tumours were then weighed, minced and dissociated using gentleMacs dissociator (Miltenyi Biotec; Germany; Cat#: 130-093-235). Following dissociation, cells were filtered through a strainer with a 70 µm pore size and washed twice. Spleens were dissected and pressed between the frosted ends of glass microscope slides to make single-cell suspensions. Red blood cells were lysed using ACK lysing buffer.

Re-stimulation with peptides and staining of intracellular cytokines. Mononuclear cells from blood and single-cell suspensions from spleens and tumours were stimulated with 1 µg/mL of the immunodominant peptide from DCT that binds to H-2K^{b} ($DCT_{180\text{-}188}$, SVYDFFVWL; shared by human and murine DCT) in the presence of brefeldin A (1 µg/mL, added after 1 hour of incubation; ThermoFisher Scientific; Waltham MA; Cat#: 00-4506-51). Anti-CD107a was also added at this time, which binds to CD107a expressed on the inside of granules, which are exposed temporarily as the T cells undergo degranulation. After 5 hours of total incubation time, cells were treated with anti-CD16/CD32 to block Fc receptors and surface markers were fluorescently labelled by the addition of Abs. Cells were then permeabilized and fixed with intracellular fixation buffer (ThermoFisher Scientific; Waltham, MA; Cat#: 88-8824-00) and stained for intracellular cytokines. Data were acquired using a FACS Canto II flow cytometer with FACSDiva 8.0.1 software (BD Pharmingen) and analyzed with FlowJo version 10.1 software (FlowJo LLC, Ashland, OR). Lymphocytes were gated based on size and complexity of the cells, doublets were then excluded, dead cells were subsequently excluded. T cells were

gated based on CD3 and CD8 expression, and $DCT_{180-188}$-specific cells were identified by IFN-γ^+ staining. (Refer to **Suppl. Fig. 1b**).

Avidity assay. Mononuclear cells from blood and single-cell suspensions from spleens and tumours were stimulated with various concentrations of peptides (starting at 0.1 µg/mL with logarithmic serial dilutions) in the presence of brefeldin A (1 µg/mL, added after 1 hour of incubation) (eBioscience; Cat# 00-4506-51). After 5 hours of total incubation time, cells were stained and data acquired and analyzed as described above.

Tetramer staining. Single cell suspensions (from blood, spleen and tumour samples) were stained with H-$2K^b$/SVYDFFVWL-PE tetramer (1:300), as well as anti-CD3, anti-CD4, and anti-CD8 for 20 minutes at 4^oC. The cells were then stained with fixable viability dye efluor780. Data were acquired and analyzed as described above.

In vivo *Cytotoxicity Assay.* Syngeneic spleens were harvested from donor mice, and processed into single-cell suspensions. Red blood cells were lysed osmotically using ACK lysing buffer. Cells were plated into 100 mm non-coated plates for four hours to allow adherent cells to attach. Suspended cells were collected gently and split into two groups. These cells were pulsed with either –$OVA_{257-264}$ (SIINFENKL; irrelevant negative control) or $DCT_{180-188}$ (SVYFFVWL; target) peptides at 10 µg/mL for 1 hour at 37°C. OVA- and DCT-pulsed cells were stained with 0.2 and 1 µM of Violet Proliferation Dye (VPD) (BD Horizon; Cambridge, UK; Cat #: 562158), respectively. OVA- and DCT-pulsed cells were then combined at equal numbers. Because intravenous administration resulted in inadequate numbers of target cells trafficking to tumours,

half of the cells were administered to mice intratumourally in a volume of 100 µL, and the other half were injected intravenously in a volume of 200 µL. Tumours, blood and spleens were harvested 13 hours later and VPD-labelled cells were quantified in these three tissues.

Statistical analyses. GraphPad Prism version 7 for Windows (GraphPad software, San Diego, CA) was used for all graphing and statistical analyses. Survival curves were estimated by the Kaplan-Meier method, and differences between groups were investigated using the log-rank test. Differences in $CD8^+$ T cell numbers and IFN-γ^+ $CD8^+$ T cell numbers in response to stimulation were analyzed by an unpaired *t*-test or ordinary one-way analysis of variance (ANOVA) with multiple comparisons using Tukey's multiple comparisons test. *In vivo* cytotoxicity ratios were also analyzed using ordinary one-way ANOVA with Tukey's multiple comparisons test. All reported *p* values were two-sided and considered significant at $p \leq 0.05$. Error bars indicate standard error throughout.

RESULTS

Inducing a primary response against an OV-encoded TAA made the OV safer. Previous studies have shown that an OV expressing an identical TAA as a primary adenovirus vaccine is capable of boosting tumour-specific T cells to massive numbers[20]. Heterologous prime-boost vaccination strategies are often employed to avoid neutralizing antibodies against the primary vaccine vector[161]. However, since the transgene expressed by both of our vaccines was identical, we expected that the primary T cell response induced by Ad-hDCT would recognize and

eliminate VSVΔm51-hDCT-infected cells, and that this would, therefore, improve the safety profile of the OV. **Fig. 1** shows survival data obtained from C57BL/6 (**panel a**) and BALB/c (**panel b**) mice, immunized with a primary adenovirus vaccine that either lacked a transgene (BHG) or that expressed an identical transgene to the subsequent OV (SIINFEKL or hDCT). When the transgenes encoded by the two viruses were different, the OV challenge was lethal to 50% of the mice. In both strains, 100% of mice that were pre-vaccinated with an Ad expressing the same transgene as the OV were protected against OV-mediated toxicity. Therefore, vaccination with Ad can generate protection against an OV-vectored booster vaccine, if the two carry the same transgene. However, if an Ad-immunized host develops immunity against a subsequent VSV booster vaccine, how is it possible that the VSV is still able to replicate and mediate oncolysis?

Boosting with replicating virus induced transient lymphopenia, which was evident in the transgene-specific subset. Lymphopenia is often observed in the blood in response to early viral infections[139]. We hypothesized that this phenomenon might extend to the tumour microenvironment, resulting in a transient reduction in the number of effector T cells capable of recognizing and eliminating an OV. We therefore quantified the total number of viable lymphocytes, to determine if lymphopenia occurred in response to administration of VSV in Ad-vaccinated, tumour-bearing hosts, and whether this occurred in tissues other than the blood, such as in tumours and spleens (**Fig. 2a**). B16F10 melanoma bearing C57BL/6 mice were vaccinated intramuscularly with Ad-hDCT 4 days post-tumour challenge. The mice were boosted 9 days later with VSVΔm51-hDCT intravenously, prior to harvesting tissues the following day (refer to **Suppl. Fig. 1a** for experimental timeline). Flow cytometry data was obtained using the gating

strategy shown in **Suppl. Fig. 1b**. There were significant reductions in the numbers of lymphocytes in response to intravenous infusion of VSV in tumours and blood. Next we assessed whether this lymphopenic effect affected the $DCT_{180-188}$-specific $CD3^+$ $CD8^+$ T lymphocyte population (**Fig. 2b**). Indeed, significant reductions in numbers of transgene-specific cytotoxic T lymphocytes in response to administration of VSV were observed in all three tissues. Notably, this reduction appeared to be especially prominent in the $CD3^{lo}CD8^{lo}$ population, which represents an activated T cell phenotype in C57BL/6 mice (**Fig. 2c**). We compared the ratio of $CD3^{lo}$ $CD8^{lo}$ to $CD3^{hi}$ $CD8^{hi}$ lymphocytes in the blood of mice treated with Ad-hDCT only to the mice treated with Ad-hDCT and VSV-hDCT, and demonstrated a significant preferential reduction in the $CD3^{lo}$ $CD8^{lo}$ lymphocyte population (**Fig. 2d**).

***Transgene-specific T cells were functionally impaired* ex vivo.** In addition to the lymphopenic effect, we suspected that effector T cells were also rendered dysfunctional due to the highly immunosuppressive tumour microenvironment. Therefore, we evaluated the *ex vivo* function of $DCT_{180-188}$-specific T cells from the tumours of melanoma-bearing mice that had been vaccinated with Ad-hDCT (**Fig. 3**). The production of multiple cytokines and relative amount of degranulation tend to correlate with the degree of activation of T cells[162,163]. We compared the percentage of DCT-specific cells that produced multiple cytokines (i.e. both TNF-α^+ and IFN-γ^+) as well as the percentage of DCT-specific cells that underwent degranulation ($CD107a^+$ IFN-γ^+) in the three tissues (tumour, blood and spleen) in response to re-stimulation with peptides. It was found that the percentage of DCT-specific cells that produced multiple cytokines (**Fig. 3a**), as well as those that degranulated (**Fig. 3b**), was reduced in tumours compared to blood and spleens. **Fig. 3c** shows dot plots from a representative mouse to demonstrate the reduction in

TNF-α^+ IFN-γ^+ and CD107a$^+$ IFN-γ^+ T cells from the tumour compared to those derived from the spleen. In addition, the relative quantities of IFN-γ, TNF-α, and CD107a were assessed by measuring mean fluorescence intensities, and found to be significantly lower in T cells derived from tumours compared to blood and spleens (**Fig. 3d**). Collectively these data suggest that the functions of tumour-infiltrating T cells were impaired compared to blood and spleen-derived T cells.

Transgene-specific T cells had TCRs of lower avidity. While optimizing our experimental protocols, we noticed a discrepancy between DCT-specific tumour-infiltrating T cells identified by IFN-γ^+ production in response to re-stimulation with peptides and those identified via tetramer staining (**Suppl. Fig. 2**). We suspected that this difference could be attributed to tumour-derived T cells having TCRs of lower avidity than those in the blood and spleens. To assess the avidity of the DCT-specific T cells isolated from various tissues, they were re-stimulated with a broad range of concentrations of the $DCT_{180\text{-}188}$ peptide to generate dose-response curves (**Fig. 4**). The percentage of maximal response at a peptide concentration of $1x10^{-3}$ ug/ml was significantly different between tumour-infiltrating and blood-derived CD8+ T cells, as well as tumour-infiltrating and spleen-derived CD8+ T cells. Further the EC_{50} values were found to be 3 times higher for tumour-derived T cells compared to blood and spleen-derived T cells. In other words, T cells isolated from tumours required a much higher concentration of peptide in order to generate 50% of the maximal response in that tissue, compared to the blood and spleen, suggesting the TCRs of T cells in the melanomas were of relatively low avidity. Nevertheless, thus far in the study, we had only demonstrated tumour-infiltrating T cell dysfunction using *ex vivo* functional assays, and were unsure if this dysfunction

was sufficient to influence the *in vivo* clearance of cells expressing target antigens inside the tumour microenvironment.

***Transgene specific T cells were unable to efficiently kill DCT targets* in vivo.** Motivated by the evidence of tumour-infiltrating T cell dysfunction accumulated from *ex vivo* assays, we optimized an *in vivo* cytotoxicity assay to assess the ability of tumour-resident $CD8^+$ T cells to kill target cells *in vivo* (**Fig. 5**). In this assay, we transferred differentially labeled syngenic splenocytes that had been pulsed with OVA- or DCT-derived immunodominant peptides (SIINFEKL (SIIN) and SVYDFFVWL (SVY), respectively) into mice that had been vaccinated with Ad-hDCT to compare the cytolytic potential of T cells in various tissues (tumours, blood and spleens). As expected, the mean SIIN:SVY ratio of unvaccinated mice in all three tissues was approximately one, indicating that there was no DCT-specific cytolysis (**Fig. 6**). The increased SIIN:SVY ratios in vaccinated mice suggested that there was DCT-specific killing, and this ratio was significantly lower in tumours compared to the blood and spleens. We also determined the percentage of specific lysis in the three tissues. It was found that the percentage of specific lysis was dramatically reduced in tumours compared to the blood and spleens. The sum total of our *ex vivo* and *in vivo* results suggest that antigen-specific T cells in tumours were functionally impaired compared to those in the blood and spleens, and could explain, at least in part, the inefficient clearance of an OV within the tumour microenvironment despite pre-existing immunity against an OV-encoded transgene.

DISCUSSION

Oncolytic viruses have gained a lot of interest in the field of cancer immunotherapy because they are capable of direct lysis of cancer cells as well as simultaneously inducing innate and adaptive immune responses[164]. By engineering OVs to express TAAs, the immune responses can be directed against cancerous cells. However, the anti-tumour response elicited against the TAA may be limited due to the response elicited against the highly immunogenic viral backbone proteins[165]. However, OVs incorporated as booster vaccines in prime-boost vaccination regimens have demonstrated efficacy in eliciting robust secondary anti-tumour responses[20,160]. Furthermore, we have shown that administration of an OV following a primary Ad vaccine expressing an identical transgene, can improve the safety profile of the OV vector, as shown in **Fig. 1**. We speculate that this protection was due to clearance of off-target infections by the host immune system. Indeed, our *in vivo* cytotoxicity assay demonstrated efficient killing of cells expressing the same antigen as the OV booster vaccine in extra-tumoural tissues such as the blood and spleens of mice that had received a primary vaccine.

Having confirmed that mice vaccinated with a primary Ad vaccine are protected against unwanted toxic side-effects resulting from subsequent vaccination with a very high dose of an OV expressing an identical transgene, it was surprising that OV replication was only minimally attenuated in the tumour of vaccinated mice, especially when the OV was administered at the peak of the primary effector response[20]. We hypothesized that either a deficiency in the quantity or quality of transgene-specific cytotoxic T cells in the tumour at the time of administration of the OV could explain the inefficient clearance of the virus.

We considered the possibility of the virus altering the quantity of T cells in the tumour. Interestingly, lymphopenia is often observed in the blood during early viral infections, and is even used as a diagnostic indicator[139,140] We hypothesized that this lymphopenic effect may not be restricted to the blood, but was actually also occurring in other tissues, including tumours. In this study, we demonstrated that there was a reduction in the number of DCT-specific cytotoxic T lymphocytes in tumours within 6 hours post-administration of VSV-hDCT. To the best of our knowledge, this represents the first report that acute virus-induced lymphopenia extends to tumour microenvironments. Previous studies looking at VSV-induced lymphopenia in the blood identified the phenomenon to be transient, with recovery of lymphocyte numbers beginning within 48 hours of exposure to the virus, and returning to baseline after six days)[140]. The phenomenon has also been shown to be dependent on type I interferons[139,140]. It is believed that the transient disappearance of these lymphocytes is due to a combination of reasons, including: redistribution to various tissues, virus-mediated killing, and/or cytokine-induced apoptosis[166].

However, in other previous studies, lymphocyte accumulation was not detected in any particular organ[139,140]. Furthermore, studies with PTX-inhibited adoptively transferred lymphocytes, which are unable to enter lymph nodes nor splenic white pulp, still demonstrated lymphopenia in response to treatment with poly (I:C)[139]. Additionally, in our current study, we also demonstrated a lymphopenic effect in the spleen. Therefore it is questionable whether all of the cells are simply homing to lymphoid organs. Previous experiments with labelled, adoptively transferred cells also showed that these cells returned to circulation after 48 hours, suggesting that their transient loss in blood was not due to cellular death followed by replenishment from the bone marrow (unpublished data). All in all, it appears more likely that VSV-induced

lymphopenia may be due to a transient trafficking phenomenon involving distribution to anatomical locations in the body other than the blood and spleen.

Lymphocyte migration is a highly organized and complicated process, orchestrated by chemokines[167]. Cellular trafficking involves physical cellular changes, including expression of various chemokine receptors and adhesion molecules that allow cells to migrate from one location to another. One theory suggests that the 'missing' lymphocytes are not actually removed from the blood, but rather adhere to the endothelial wall, and, therefore, are not collected when blood samples are drawn[139]. Adhesion molecules are up-regulated upon activation of lymphocytes, possibly facilitating this process, which is known as margination[168,169]. Previous studies have shown that activated lymphocytes can adhere to tumour endothelium; however they found no adhesion in normal vessels[168]. This is particularly interesting because we performed transcardiac perfusions before harvesting tumours to minimize their contamination with blood and, therefore, blood-borne antigen-specific T cells. It is possible that some of the tumour-specific T cells that we detected shortly after administration of VSV were not actually in the tumour parenchyma but, instead, adhered to the endothelial lining of tumour-associated blood vessels. If true, this would suggest that the transient virus-induced lymphopenia that we demonstrated in the tumour might slightly underestimate the effect.

Intriguingly, closer analysis of the blood samples in our study also revealed that there was a preferential removal of cytotoxic T lymphocytes with a $CD3^{lo}CD8^{lo}$ phenotype. Previous studies have also shown that after activation, T cells will down-regulate expression of surface TCR-CD3 complexes[170,171]. This is believed to play a role in dampening the response[171]. These

TCRs are internalized and degraded within lysosomal compartments[170–172]. Other studies have also reported that the $CD8^{lo}$ T cell population represented activated effector T cells, resulting possibly from chronic and/or repeated exposure to antigens[173]. These cells also displayed increased cytotoxicity[173]. Other studies have shown that this down-regulation correlates with the strength of the antigenic stimulation[174] and is Zap70-dependent[171]. The preferential OV-induced loss of $CD3^{lo}CD8^{lo}$ cytotoxic T lymphocytes, identified in this current study, raises the suspicion that the cells activated in response to the OV are the ones preferentially involved in virus-induced leukopenia.

Immunosuppression exerted by the tumour microenvironment has been documented extensively in literature. To evade detection and killing by the immune system, cancers utilize a variety of immunosuppressive and evasive mechanisms[33,34,37,39,40]. We suspected that this tumour-induced immunosuppression might also play a role in preventing efficient clearance of OVs from tumours, despite pre-existing transgene-specific immunity. In this current study, we demonstrated that intratumoural T cells produced less cytokines, underwent less degranulation, had TCRs of lower avidity, and were less efficient at killing target cells *in vivo.* All of these data taken together strongly suggests that tumour-infiltrating T cells were impaired compared to spleen- and blood-derived T cells, which matches what has been previously shown in literature. This being said, we recognize that the *in vivo* cytotoxicity assay has a few limitations. These assays are frequently done *ex vivo*, when known numbers of effector cells are mixed with a known number of target cells. Unfortunately this is not the case in an *in vivo* situation. However, the results from this assay must be considered in the context of the other results, which together, paint a clear picture of tumour-infiltrating T cell dysfunction.

This present study identifies two independent mechanisms that facilitate transient oncolytic activity in the tumour microenvironment to be retained when using an OV-vectored booster vaccine despite previous vaccination against a virus-encoded antigen. We demonstrated that virus-induced lymphopenia acutely removes activated DCT-specific $CD8^+$ cytotoxic T lymphocytes from tumours and that the relatively few remaining tumour-infiltrating T cells are dysfunctional. Simultaneously, at least some extratumoural tissues, such as the spleen, retain normal T cell numbers and these T cells remain functional, thereby providing the resources to clear off-target infections. Although initially counterintuitive, this study identifies mechanisms that argue in favour of inducing OV-specific immunity as a strategy to enhance the safety of oncolytic virotherapy without overly compromising direct oncolytic activity. We are applying this concept in the context of oncolytic booster vaccines, in which primary transgene-specific immune responses are required. However, one could envision a scenario in which pre-vaccination could be used against any OV-encoded protein, including imaging markers such as enhanced green fluorescent protein, etc., as a way to increase their safety profile. Future directions of this research could include determining if OV-induced lymphopenia in tumours can be mediated by viruses other than VSV and whether the phenomenon extends to other leukocyte subsets.

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FIGURES

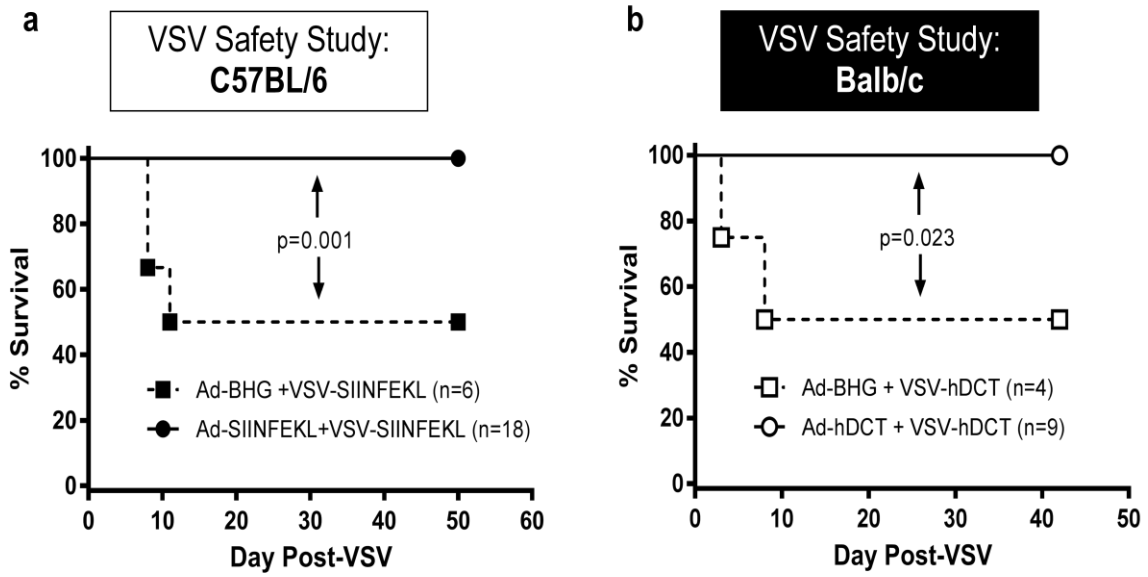


Figure 1: Inducing a primary immune response against an OV-encoded TAA made the OV safer. Two strains of mice vaccinated with an Ad-vector expressing the same transgene as the subsequent OV (VSV; $3x10^9$ pfu i.v.) had enhanced survival, demonstrating the improved safety generated by the prime-boost strategy. Mice that reached endpoint was due to the development of hind-limb paralysis. **(a)** C57BL/6 mice treated i.m. with $1x10^8$ pfu of Ad-BHG (n=6) or Ad-SIINFEKL (n=18). Both groups were subsequently treated i.v. with VSV-SIINFEKL. Survival was significantly enhanced in the group primed and boosted with the Ad-vector and OV expressing SIINFEKL (p=0.001). **(b)** Balb/c mice treated i.m. with $1x10^8$ pfu of Ad-BHG (n=4) or Ad-hDCT (n=9). Both groups were subsequently treated with VSV-hDCT. Survival was significantly enhanced in the group primed and boosted with the Ad-vector and OV both expressing DCT (p=0.023). Kaplan-Meier survival plots shown with log-rank test used for statistical analysis. Ad, adenovirus; OV, oncolytic virus; hDCT, human dopachrome tautomerase.

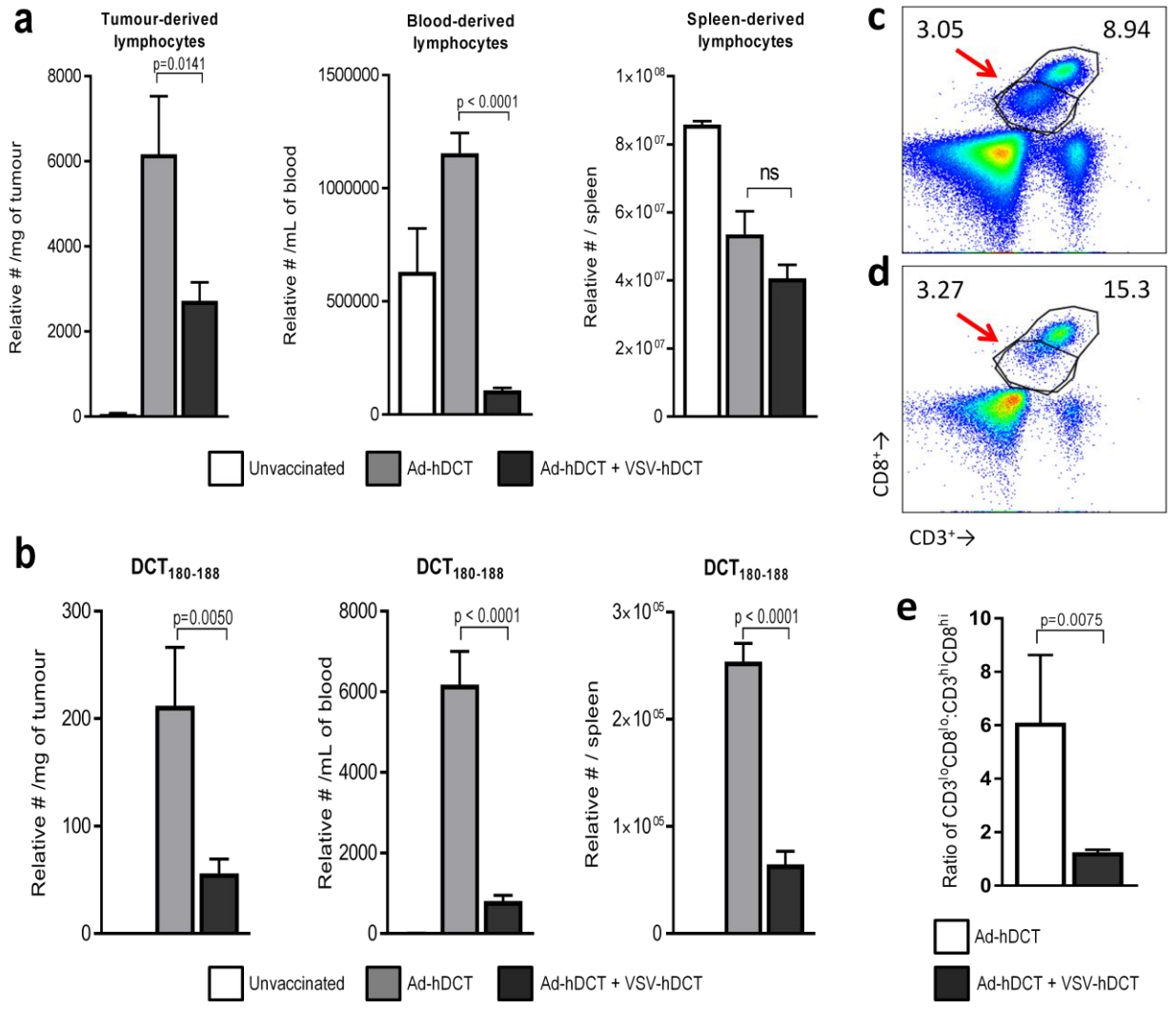


Figure 2: Boosting with a replicating virus induced transient lymphopenia, which was evident in the transgene-specific T cell subset. Melanoma bearing C57BL/6 mice were unvaccinated (n=4), vaccinated i.m. with $1x10^8$ pfu of Ad-hDCT on d4 only (n≥6), or vaccinated with Ad-hDCT on d4 + $1x10^9$ pfu of VSV-hDCT i.v (n≥8) on d13. On d14, tumours, blood and spleens were harvested and the quantity of viable lymphocytes and hDCT-specific T cells were assessed via flow cytometric analysis after *ex vivo* re-stimulation with the immunodominant epitope $DCT_{180-188}$ for 5 hours in the presence of Brefeldin A, followed by surface and

intracellular cytokine staining. The relative numbers of (a) viable lymphocytes and (b) DCT-specific $CD3^{+}CD8^{+}$ T cells isolated from tumours, blood and spleens were quantified; data was pooled from three experiments. (c) Dot plots from blood of representative mice treated with Ad-hDCT only and with (**d**) Ad-hDCT + VSV-hDCT. (**e**) The ratio of $CD3^{lo}CD8^{lo}$:$CD3^{hi}CD8^{hi}$ T cells in the blood of Ad-hDCT (n=4) or Ad-hDCT + VSV-hDCT (n=10) -treated mice. Means and standard errors are shown in all graphs; unpaired *t*-tests were used to compare differences between Ad-treated and Ad+VSV-treated mice. Ad, adenovirus; hDCT, human dopachrome tautomerase; VSV, vesicular stomatitis virus; OV, oncolytic virus.

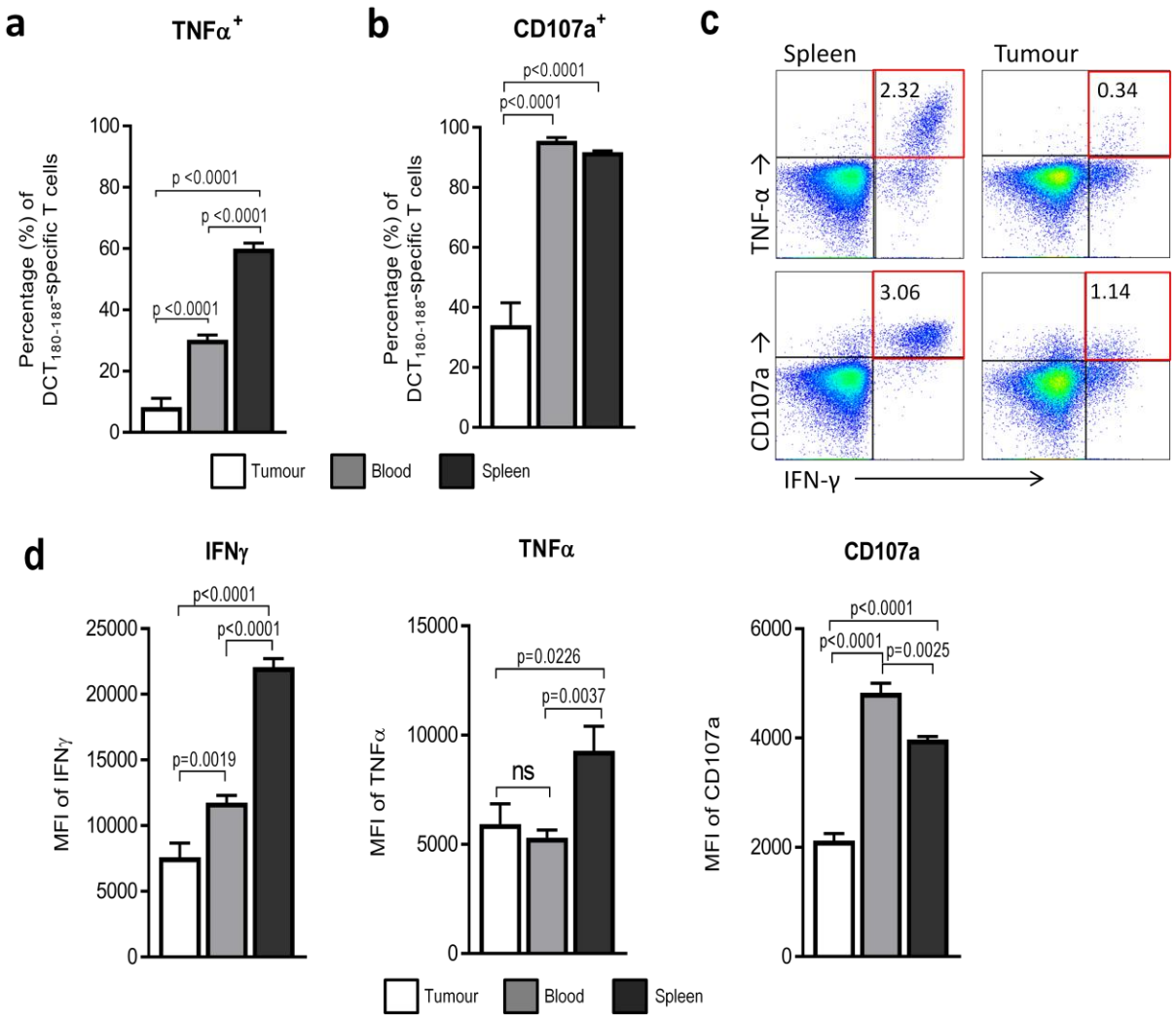
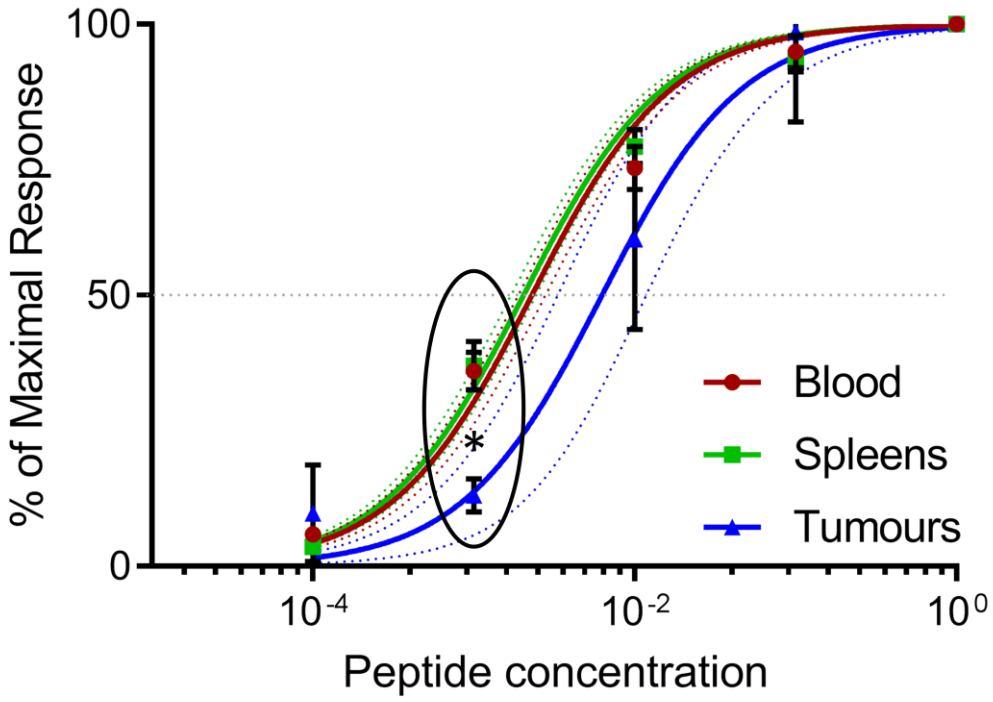


Figure 3: Transgene-specific tumour-infiltrating T cells were functionally impaired *ex vivo*. B16F10 melanoma-bearing C57BL/6 mice were vaccinated with Ad-hDCT 4 days after tumour challenge. 10 days later, tumours (n=7), blood (n=10) and spleens (n=6) were harvested and *ex vivo* function was assessed via intracellular cytokine production and degranulation assays following $DCT_{180-188}$ peptide restimulation; data pooled from 3 experiments. (**a**) Percentages of hDCT-specific lymphocytes that produced multiple cytokines were compared across tissues of origin (tumours, blood and spleens). A significant reduction was observed in the tumour compared to blood and spleen samples; p<0.0001. (**b**) Percentages of hDCT-specific

lymphocytes that degranulated (IFN-γ^+CD107a$^+$ cells) were compared across tissues of origin (tumours, blood and spleens). A significant reduction was observed in the tumour compared to blood and spleen samples; $p<0.0001$. (**c**) Dot plots from the spleen and tumour of a representative Ad-hDCT treated mouse to demonstrate difference in cytokine production and degranulation. (**d**) Significant reductions were also observed in the mean fluorescence intensities of IFN-γ, TNF-α and CD107a staining, of hDCT-specific T cells found in the tumour compared to blood and spleen. Means and standard errors are shown for all graphs; one way analysis of variance with multiple comparisons used to compare differences between tissues. Ad, adenovirus; hDCT, human dopachrome tautomerase.



		Blood	Spleens	Tumours
EC50	Value	0.0023	0.0020	0.0063
	SEM	0.0003	0.0002	0.0025

Figure 4: Transgene-specific tumour-infiltrating T cells had TCRs of lower avidity. B16F10 melanoma bearing C57BL/6 mice were vaccinated with Ad-hDCT 4 days after tumour challenge (n=12). 10 days later, tumours, blood and spleens were harvested and assessed via FACs analysis. Relative TCR avidity was formally assessed between the three tissues by EC_{50} assay. Lymphocytes isolated from the three tissues were re-stimulated with serially log-diluted doses of $DCT_{180\text{-}188}$ peptides for 5 hours in the presence of Brefeldin A, followed by surface and intracellular cytokine staining, to generate a dose response curve. Means with standard error are shown. The EC_{50} of lymphocytes derived from the three tissues were determined using this

curve, to compare the avidity of TCRs from tumour-infiltrating, blood-derived and spleen-derived T cells. Significant differences in avidity were found at a peptide concentration of 10^{-3} ug/ml, between blood vs. tumour (p=0.0306), and spleens vs. tumours (p=0.0226); two way analysis of variance with Tukey's multiple comparisons test used to compare differences between tissues. Ad, adenovirus; hDCT, human dopachrome tautomerase; EC_{50}, effective dose to elicit 50% of maximal response; TCR, T cell receptor.

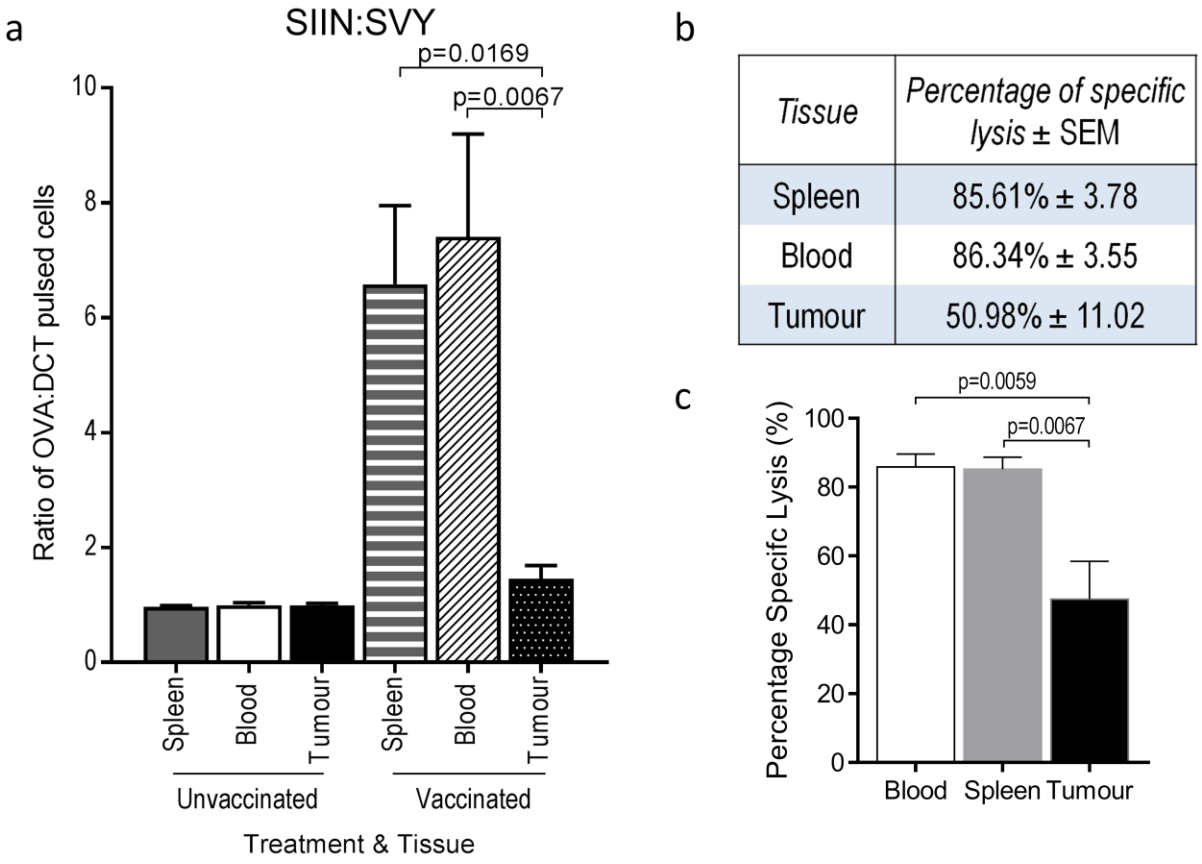
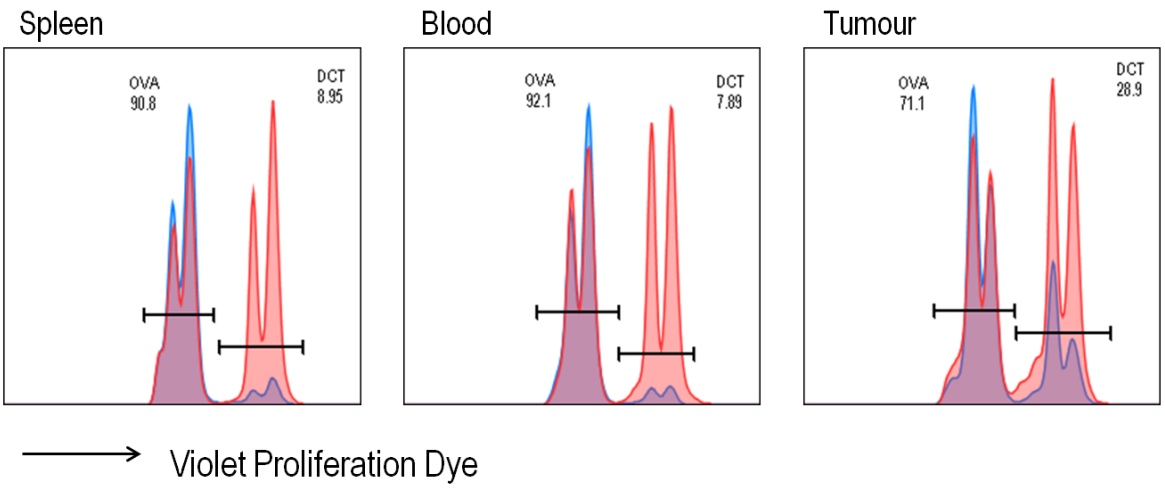


Figure 5: Transgene-specific tumour-infiltrating T cells are unable to efficiently kill DCT targets *in vivo*. (**a**) *In vivo* cytolytic ability of T cells found in the spleen, blood and tumour were compared. B16F10 melanoma bearing C57BL/6 mice were either left unvaccinated (n≥3) or vaccinated with Ad-hDCT (n≥7), 10 days prior to receiving a 1:1 mixture of hDCT and OVA-pulsed splenocytes labelled with 1µM and 0.2µM of VPD, respectively, as targets. Tissues (tumours, blood and spleens) were harvested 13 hours following, to detect and quantify the VPD labelled cells via FACs analysis. Ratio of OVA:DCT-pulsed cells recovered from spleens, blood and tumours were assessed. Means and standard errors are shown; data pooled from two experiments; one way analysis of variance with Tukey's multiple comparisons test used to

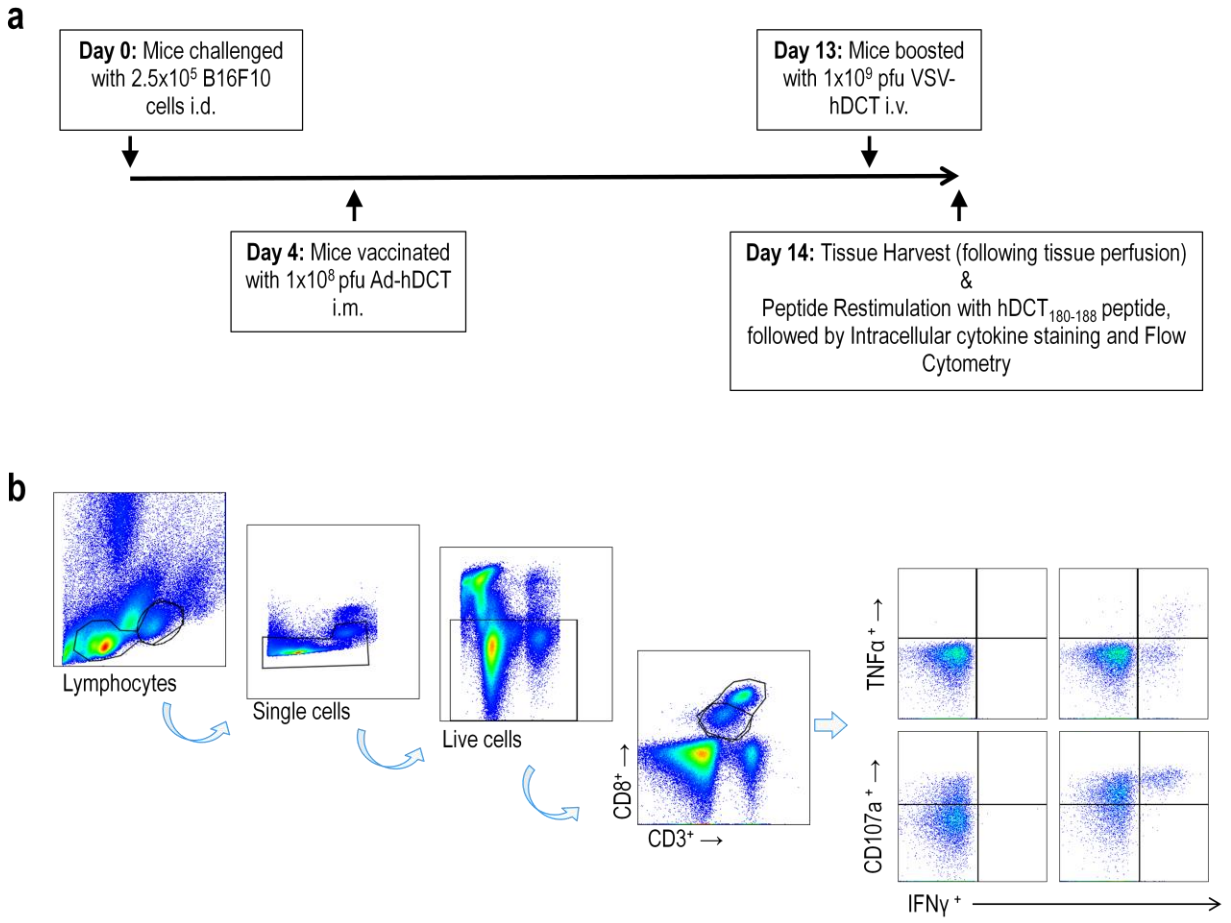
compare differences between tissues. (**b**) Percentages of specific lysis were calculated for each tissue using the following formula: $100 - [\frac{\left(\frac{VPDhigh}{VPDlow}\right)vaccinated}{\left(\frac{VPDhigh}{VPDlow}\right)unvaccinated\ avergae}] \times 100$. (**c**) Percentages of specific lysis were graphed, means and standard error are shown; one way analysis of variance with Tukey's multiple comparisons test used to compare differences between tissues. Ad, adenovirus; hDCT, human dopachrome tautomerase; OVA, ovalbumin; VPD, violet proliferation dye.



		Spleen		*Blood*		*Tumour*	
		SIIN (%)	*SVY (%)*	*SIIN (%)*	*SVY (%)*	*SIIN (%)*	*SVY (%)*
	Unvaccinatted	48.9	51.0	47.2	52.3	46.0	53.9
	Ad-hDCT	90.8	8.95	92.1	7.89	71.1	28.9

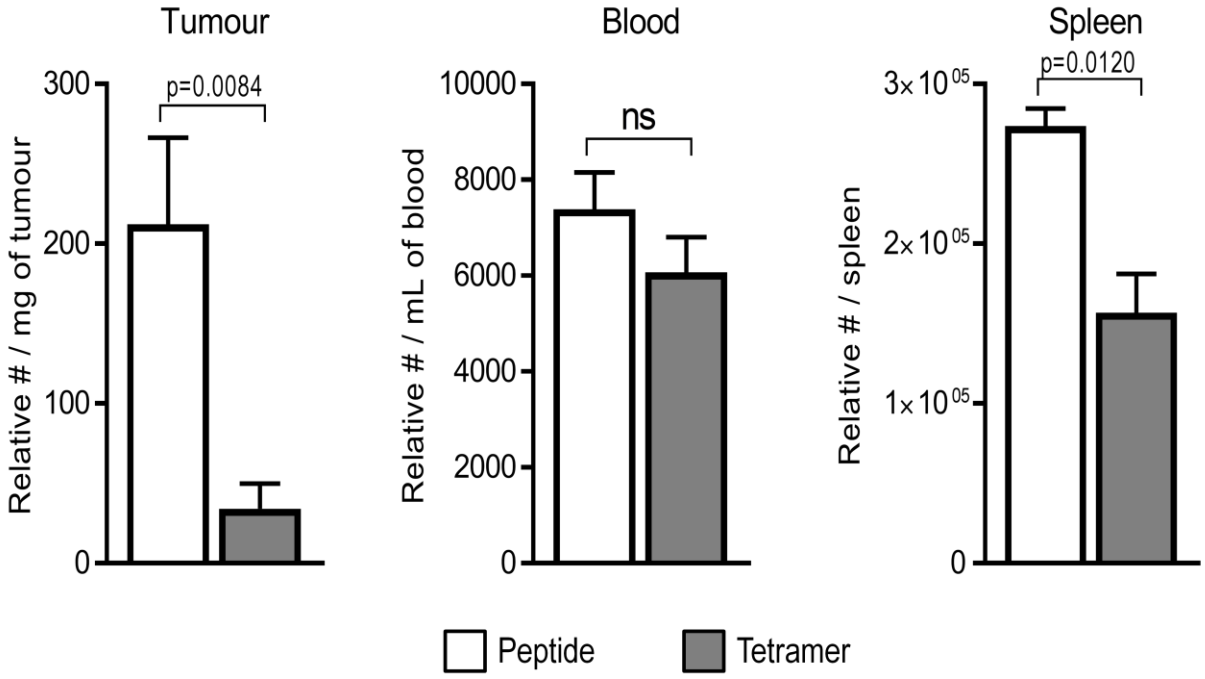
Figure 6: Transgene-specific tumour-infiltrating T cells are unable to efficiently kill DCT targets *in vivo*. In vivo cytolytic ability of T cells found in the spleen, blood and tumour were compared. B16F10 melanoma bearing C57BL/6 mice were either left unvaccinated or vaccinated with Ad-hDCT, 10 days prior to receiving a 1:1 mixture of hDCT and OVA-pulsed splenocytes labelled with 1µM and 0.2µM of VPD, respectively, as targets. Tissues (tumours, blood and spleens) were harvested 13 hours following, to detect and quantify the VPD labelled cells via FACs analysis. Ratio of OVA:DCT-pulsed cells recovered was assessed to determine *in vivo* cytolytic ability of T cells from the spleen, blood and tumour. Representative histograms of one unvaccinated and one vaccinated are shown; to demonstrate difference in cytolysis of labelled cells in the three tissues. Ad, adenovirus; hDCT, human dopachrome tautomerase; OVA, ovalbumin; VPD, violet proliferation dye.

SUPPLEMENTARY FIGURES



Supplementary Figure 1. Experimental time line and Representative dot plots demonstrating the gating strategy applied to flow cytometry data. **(a)** Timeline for experiments **(b)** An example of the gating strategy used during flow cytometry analysis of blood-derived leukocytes taken from a C57BL/6 mouse that received $2.5x10^5$ B16F10 cells intradermally, and was vaccinated with Ad-hDCT ($1x10^8$ pfu; intramuscularly) four days post-tumour challenge. Lymphocytes were gated based on forward and side scatter-area (FSC-A vs. SSC-A), doublets were excluded, dead cells were excluded using a fixable viability dye that stains dead cells, $CD8^+$ cytotoxic T

lymphocytes were gated based on $CD3^+$ and $CD8^+$ staining, DCT-specific cells were identified by staining for intracellular $IFN\gamma^+$ following re-stimulation with the immunodominant epitope $DCT_{180\text{-}188}$. Degranulation was assessed by staining for surface-expressed CD107a, and multi-cytokine-producing cells were assessed by staining for intracellular TNFα. An unstimulated control sample is shown for comparison.



Supplementary Figure 2: Transgene-specific tumour-infiltrating T cells have TCRs of lower avidity. B16F10 melanoma bearing C57BL/6 mice (n=7) were vaccinated with Ad-hDCT 4 days after tumour challenge. 10 days later, tumours, blood and spleens were harvested and assessed via FACs analysis. DCT-specific T cells were identified using two methods: $DCT_{180\text{-}188}$ peptide re-stimulation and tetramer staining. Significant differences in DCT-specific T cell numbers identified by the two methods were observed in the tumour and spleen. Means and standard errors are shown; unpaired t-test used to compare differences between tissues. *p≤0.05, **p ≤0.01. Ad, adenovirus; hDCT, human dopachrome tautomerase; TCR, T cell receptor.

CHAPTER THREE:

VIRUS-INDUCED LEUKOPENIA: CHALLENGING THE CELL TRAFFICKING PARADIGM DURING ONCOLYTIC VIROTHERAPY

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RUNNING TITLE: Effect of OV-induced leukopenia in tumours

KEYWORDS: Oncolytic virus, leukopenia, immune infiltration, cell trafficking, inflammation

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ABSTRACT

The current paradigm is that oncolytic viruses (OVs) induce pro-inflammatory cytokines and chemokines that recruit and activate tumour-infiltrating leukocytes. However, our previous data suggested that vesicular stomatitis virus-induced lymphopenia acutely decreased melanoma-infiltrating lymphocytes. In the current study, our goal was to determine if this phenomenon

extended to other leukocyte subsets, other OVs and a different type of cancer. We quantified tumour-infiltrating leukocytes in intradermal B16-F10 melanomas, blood and spleens of mice treated intravenously with $1x10^9$ pfu of vesicular stomatitis virus (VSV; Δm51 mutant). The number of $CD45^+$ tumour-infiltrating leukocytes significantly decreased 24 hours post-infection (hpi). There was a profound loss of B cells and plasma cells, as well as a reduction of myeloid-derived suppressor cells (MDSCs), dendritic cells (DCs) and $CD4^+$ T cells, except those with a regulatory phenotype (Tregs). The number of tumour-infiltrating leukocytes returned to baseline by 72 hpi, at which time intratumoural $CD8^+$ T cell and NK cell numbers had increased above baseline. Interestingly, B cells and plasma cells remained at low numbers in tumours at 72 hpi. We extended our study to include Newcastle disease virus and Orf virus. Both OVs induced similar changes as the VSV. Treatment with VSV in an EMT6 orthotopic model of mammary carcinoma in BALB/c mice did not cause significant decreases in numbers of tumour-infiltrating leukocytes at 24 hpi. Nevertheless, in contrast to the current paradigm, we show that many leukocyte subsets temporarily leave B16F10 tumours following treatment with OVs. Immunohistochemical staining suggests that one mechanism may be a result of increased leukocytic margination to the endothelium of blood vessels. Understanding how OVs modulate trafficking of TILs can provide strategies to alter the intratumoural cytokine/chemokine milieu to optimize immunological cell profiles.

INTRODUCTION

Oncolytic viruses (OVs) are able to selectively target and destroy cancerous cells[175]. In addition to this ability, the presence of OVs within a tumour is believed to act as a provocative

'danger signal,' alerting and activating the immune system to eliminate a perceived threat[176]. Furthermore, OVs can be engineered to express tumour-associated antigens (TAA) to direct this response against the tumour. Other supplemental therapies are being explored to further augment these tumour-specific immune responses. In essence, it is believed that OV infection of tumour cells induces local inflammation, which subsequently recruits various populations of leukocytes into the tumour microenvironment[125,176].

Vesicular stomatitis virus (VSV) has emerged as a promising candidate for oncolytic virotherapy due to its rapid replication kinetics, inherent tumour specificity, its potential to elicit robust immune responses, and the lack of pre-existing immunity in humans[175]. VSV is a negative strand RNA virus of the *Rhabdoviridae* family[175,177]. The selectivity of VSV is due to its susceptibility to the anti-viral effects of interferons, which prevent efficient replication in normal cells[177]. However, cancer cells tend to be susceptible to infection with and destruction by VSV due to inherent defects in the interferon signalling pathway [177]. We previously showed that VSV caused a transient intratumoural lymphopenia during acute infection (Chapter II). This apparent contradiction to the current OV paradigm highlights the need for further investigation into how OVs modulate leukocyte subpopulations within tumours.

Other viruses that have shown potential as candidates for oncolytic virotherapy include Newcastle disease virus (NDV) and Orf virus (OrfV). NDV is a type of avian paramyxovirus that causes respiratory disease in birds, but only causes mild flu-like symptoms in humans[178]. NDV is a single-stranded, negative-sense RNA virus that replicates in the cytoplasm of infected cells[179,180]. The oncolytic potential of NDV has been demonstrated in several types of cancers, including: neuroblastoma[181], fibrosarcoma[182], melanoma[183,184], as well as head and neck cancers[178].

OrfV is a member of the *Parapoxvirus* genus and causes acute dermal infections in goats and sheep[185]. OrfV is a double-stranded DNA virus[185]. A previous study demonstrated the ability of OrfV to reduce tumour burden in B16F10-lacZ and CT26-lacZ lung metastatic mouse models, as well as a human lung adenocarcinoma xenograft model[186]. This study also implicated a crucial role for NK cells in the efficacy achieved by OrfV in reducing tumour burden[186]. Furthermore, OrfV is an attractive platform for oncolytic virotherapy, due to the limited exposure the human population has had to OrfV, as well as the ability of the virus to re-infect animals repeatedly[185,186].

Infiltration of immunological cells into solid tumours has been documented in many studies, but the prognostic value of some specific subsets remain controversial[43]. Tumour-infiltrating leukocytes are part of the complex tumour microenvironment, which also contains many other cell types, including fibroblasts and endothelial cells, as well as a multitude of other growth factors, cytokines and chemokines, all of which play some sort of role in the development and progression of the cancer[187]. Many of these factors can also play a role in activating or suppressing the immune system.

$CD8^+$ T lymphocytes are considered to be the main effector cells in anti-tumour responses, and have also shown to be associated with improved patient outcomes[47]. The role of effector $CD4^+$ T lymphocytes on the other hand, is more uncertain. $FoxP3^+$ regulatory T cells are a subpopulation of $CD4^+$ T lymphocytes that have an immuno-regulatory role[64]. This subset is believed to suppress anti-tumour responses, and has been shown to be correlated with negative outcomes. B cells and plasma cells have been associated with improved prognoses in various types of cancers[47,67–69]. Immune subsets of the innate arm of the immune system have also been shown to infiltrate tumours. NK cells can recognize and kill melanoma cells *in vitro*[74] and studies

using anti-NK1.1 to deplete NK cells showed a protective effect of this subset against murine melanomas *in vivo*[75]. Neutrophils are classically known to be the primary responders to inflammation, and are suspected to be involved in the development of tumours, as well as vasculature shut-down[126,188]. Myeloid-derived suppressor cells are cells of myeloid origin that have a suppressive role in the anti-tumour response[95]. MDSCs are associated with tumour progression, and invasion[95]. Dendritic cells are professional antigen presenting cells that are critical for initiation of immune responses; however, tumour-infiltrating DCs (TIDCs) have been shown to adopt an immature or paralyzed phenotype, with low expression of co-stimulatory molecules, high expression of regulatory molecules and impaired antigen presentation; rendering them more immunosuppressive[80,83].

Since treatment with OVs is believed to recruit and activate leukocytes, it is important to consider the impact of current oncolytic virotherapy on individual leukocyte subsets. Modifications to OV therapy could then be made to specifically recruit favourable cell subsets, while removing or preventing the infiltration of other unwanted cell types, thus generating optimal immunological profiles within tumours. The goal of this study was to provide an overview of how VSV acutely modulates the immunological landscape of tumour microenvironments. Our data suggest that at 24 hours post-infection, many leukocyte subsets were reduced in tumours of mice treated with VSV.

MATERIALS AND METHODS

Mice. 8-10 weeks old (at study initiation) female C57BL/6 (H-2^b) and BALB/c (H-2^d) mice (Charles River Laboratories, Wilmington, MA) were housed in a specific pathogen-free facility at the University of Guelph. All animal experimentation was approved by the University of Guelph's Animal Care Committee, and complied with the Canadian Council on Animal Care guidelines.

Viral vaccines. The VSV used in *in vivo* experiments was a recombinant VSVΔm51 expressing SIINFEKL. VSVΔm51-SIINFEKL has been described previously[189]. VSV-WT-eGFP was used in *in vitro* experiments and has been described previously[138]. Both VSVs were grown on Vero cells and titers were determined by plaque assay on Vero cells. NDV-F3aa-GFP has been described previously[190]. NDV-F3aa-GFP plasmids (a kind gift from Peter Palese, Mount Sinai) were rescued using recombinant MVA virus expressing T7 RNA polymerase and grown in eggs[191]. The virus was first filtered using a Supracap ™ 50 Depth Filter Capsules (Pall, Canada), followed by purification and concentration by tangential flow filtration using a Centramate™ LV holder (Pall, Canada) and a 100 kDa Omega Screen channel-cassette per the manufacturer's protocol. Next, NDV underwent a sucrose gradient centrifugation using a SW41 rotor at 27,000 rpm for 3 hrs to remove any remaining chicken host proteins, where the virus was collected between the 40-50% sucrose band. Finally, virus was dialyzed in PBS using a 10kDa Slide-A-Lyzer dialysis cassette (Thermo Fisher Scientific, Canada) to remove any remaining sucrose. Virus was titrated in DF-1 cells by the tissue culture 50% infective dose TCID50 method. OrfV-NZ2[186] was propagated in OA3.T cells (ATCC; Manassas, VA; Cat#: CRL-2755), purified via

sucrose cushion purification and titrated in OA3.T sheep testis cells using a standard plaque assay.

In vivo *tumour models.* To establish melanoma tumours, C57BL/6 mice received intradermal injections of $2.5x10^5$ B16F10 cells in 30 µL of phosphate buffered saline (PBS). The B16F10 cells were maintained in DMEM and 10% heat-inactivated bovine calf serum, and washed twice using PBS prior to injections. BALB/c mice had $1x10^5$ EMT6 mammary carcinoma cells in 30 µL of PBS injected into the fourth (inguinal) mammary fat pad. The EMT6 cells were maintained in Dulbecco's modified eagle medium (Fisher Scientific; Hampton, NH; Cat #: SH3002201) with 10% heat-inactivated bovine calf serum, and washed twice with PBS prior to injections. Anesthetized mice received i.v. injections of $1x10^9$ pfu of VSV, $1x10^8$ pfu of NDV or $5x10^7$ pfu of ORFV, which represented maximum tolerable doses for each OV, in 200µL of PBS 13 days post-tumour challenge (refer to **Suppl. Fig 1** for experimental timeline).

Table 1: Staining panels used to identify various immunological cell subsets.

Panel	Antibody	Clone	Company	Cat#	Immunological subset phenotypes
NK	anti-CD16/CD32	93	ThermoFisher Scientific; Waltham, MA;	14-016186	Pan leukocyte marker: CD45.2+; NK cells: CD3-NK1.1+; CD8 Cytotoxic T cells: CD3+CD8+;
	anti-CD45.2 PE-Cy7	104	BD Pharmingen; San Jose, CA;	560696	
	anti-CD3 BV421	1 45-2C11	BD Horizon; Cambridge, UK;	562600	
	anti-CD8 BV510	53-6.7	BD Horizon; Cambridge, UK;	563068	
	anti-NK1.1 APC	PK136	BD Pharmingen; San Jose, CA;	550627	
	anti-CD69 FITC	H1.2F3	BD Pharmingen; San Jose, CA;	557392	
	7-Aminoactinomycin D (7AAD)		ThermoFisher Scientific; Waltham, MA;	00-6993-50	
B cell	anti-CD16/CD32	93	ThermoFisher Scientific; Waltham, MA;	14-016186	Pan leukocyte marker: CD45.2+; B cells: CD3-CD19+; Plasma cells: CD3-CD38+CD138+; CD8 Cytotoxic T cells: CD3+CD8+;
	anti-CD45.2 PE-Cy7	104	BD Pharmingen; San Jose, CA;	560696	
	anti-CD19 FITC	1D3	BD Pharmingen; San Jose, CA;	553785	
	anti-CD38 AlexaFluor647	90	BD Pharmingen; San Jose, CA;	562769	
	anti-CD138 PE	281-2	BD Pharmingen; San Jose, CA;	553714	
	anti-CD3 BV421	1 45-2C11	BD Horizon; Cambridge, UK;	562600	
	anti-CD8 BV510	53-6.7	BD Horizon; Cambridge, UK;	563068	
	7-Aminoactinomycin D (7AAD)		ThermoFisher Scientific; Waltham, MA;	00-6993-50	
T cell	anti-CD16/CD32	93	ThermoFisher Scientific; Waltham, MA;	14-016186	Pan leukocyte marker: CD45.2+; Regulatory T cells: CD3+CD4+FoxP3+; CD4 Helper T cells: CD3+CD4+FoxP3-; CD8 Cytotoxic T cells: CD3+CD8+;
	anti-CD45.2 PE-Cy7	104	BD Pharmingen; San Jose, CA;	560696	
	anti-CD3 BV421	1 45-2C11	BD Horizon; Cambridge, UK;	562600	
	anti-CD8 BV510	53-6.7	BD Horizon; Cambridge, UK;	563068	
	anti-CD4 FITC	RM4-4	ThermoFisher Scientific; Waltham, MA;	11-0043-85	
	anti-FOXP3 PE	MF23	BD Pharmingen; San Jose, CA;	560414	
	anti-CD25 APC	PC61.5	ThermoFisher Scientific; Waltham, MA;	17-0251-82	
	anti-CD279 (PD-1) PerCP efluor710	J43	ThermoFisher Scientific; Waltham, MA;	46-9985-82	
	efluor780 Fixable Viability Dye		ThermoFisher Scientific; Waltham, MA;	65-0865-14	
Myeloid	anti-CD16/CD32	93	ThermoFisher Scientific; Waltham, MA;	14-016186	Neutrophils: CD11b+CD11clowLy6G+; Eosinophils: CD11b+F4/80midSiglecF+; Dendritic cells (DC): CD11bdim-brightCD11c+MHCII+ Myeloid derived suppressor cells (MDSC): CD11b+F4/80midSiglecF-Ly6G-Ly6C+SSChi;
	anti-CD11b eFluor 450	M1/70	ThermoFisher Scientific; Waltham, MA;	48-0112-82	
	anti-CD11c PE-Cy7	N418	ThermoFisher Scientific; Waltham, MA;	25-0114-82	
	anti-Ly6C APC-Cy7	AL-21	BD Pharmingen; San Jose, CA;	560596	
	anti-Ly6G PE	1A8	BD Pharmingen; San Jose, CA;	551461	
	anti-F4/80 FITC	BM8	ThermoFisher Scientific; Waltham, MA;	11-4801-85	
	anti-CD170 (Siglec F) PerCP efluor710	1RNM44N	ThermoFisher Scientific; Waltham, MA;	46-1702-82	
	anti-MHC II APC	M5/114.15.2	ThermoFisher Scientific; Waltham, MA;	47-5321-82	
	efluor506 Fixable Viability Dye		ThermoFisher Scientific; Waltham, MA;	65-0866-14	

Leukocyte preparation. For blood mononuclear cell collections, blood was collected from the periorbital sinus and red blood cells were lysed osmotically using ACK (ammonium-chloride-potassium) lysing buffer. For tumour-infiltrating leukocyte preparations, intradermal tumours were perfused with PBS, excised, weighed, minced, dissociated using a gentleMacs dissociator (Miltenyi Biotec; Germany; Cat#: 130-093-235). Following dissociation, cells were filtered through a 70 µm strainer and washed. Red blood cells were lysed using ACK lysing buffer, then samples were washed twice. For splenocyte preparations, spleens were dissected and pressed between glass slides to release cells into single cell suspension. Red blood cells were lysed using ACK lysing buffer.

Surface marker and intracellular staining. Mononuclear cells from blood, splenocytes and tumour were treated with anti-CD16/CD32 and surface markers fluorescently labelled by the addition of Abs. For identification of regulatory T cells, cells were then permeabilized and fixed with intracellular fixation buffer (ThermoFisher Scientific; Waltham, MA; Cat#: 00-5523-00) (1 part concentrate with 3 parts diluent) and stained for FoxP3. Data were acquired using a FACS Canto II flow cytometer with FACSDiva 8.0.1 software (BD Pharmingen; San Jose, CA) and analyzed with FlowJo version 10.1 software (FlowJo LLC, Ashland, OR). Doublets and dead cells were excluded, and then various immunological cell subsets were identified using surface and intracellular markers (refer to **Table 1** for list of antibodies used in various staining panels, and **Suppl. Fig. 2-5** for dot plots demonstrating gating strategies).

Immunohistochemical staining. Lungs were harvested from EMT-6 mammary carcinoma-bearing BALB/c mice and fixed overnight with 10% buffered formalin (Fisher Scientific;

Hampton, NH; Cat#:SF100-4). The lungs were washed with 70% ethanol, embedded in paraffin wax and 5 μm thick sections were cut on a rotary microtome and mounted on glass slides (Superfrost Plus, Fisher Scientific). Sections were rehydrated in decreasing concentrations of ethanol and endogenous peroxidase activity was quenched with 3% (vol/vol) hydrogen peroxide. Antigen retrieval was performed with citrate at 90°C and sections were blocked with 3% bovine serum albumin with 0.01% sodium azide. Tissues were incubated with anti-smooth muscle actin overnight at 4°C, followed by incubation with a biotinylated-specific secondary antibody at room temperature for 2 hours. Sections were counterstained in Carazzi's Hematoxylin, dehydrated, and mounted on glass coverslips using Permount. Images were captured with an upright Olympus brightfield microscope using integrated morphometry software (Metamorph, Burlingame, CA). All images were captured at 400x magnification.

Statistical analyses. GraphPad Prism version 7 for Windows (GraphPad software, San Diego, CA) was used for all graphing and statistical analyses. Differences in immune cell numbers and expression of functional markers were analyzed by an unpaired *t*-test or ordinary one-way analysis of variance with multiple comparisons. All reported *p* values were two-sided and were considered significant at $p \leq 0.05$. Error bars indicate standard error throughout.

RESULTS

Tumour-infiltrating leukocytes decreased following treatment with VSV. We previously demonstrated that VSV transiently reduced melanoma (DCT)-specific lymphocytes in the blood,

spleens and tumours of melanoma-bearing mice. Here, we sought to investigate whether this phenomenon was restricted to activated transgene-specific lymphocytes, or whether this reduction occurred non-discriminately to other leukocytes as well. To determine if other immune subsets underwent similar changes, we chose to assess a wide variety of immunological cell subsets, including: $CD45.2^+$ leukocytes, $CD8^+$ and $CD4^+$ T lymphocytes, B lymphocytes, plasma cells, NK cells, neutrophils, eosinophils, myeloid-derived suppressor cells (MDSCs) and dendritic cells (DCs). Flow cytometry analysis revealed that intravenous delivery of VSV resulted in a rapid (within 24 hours) decrease in the number of cells within all of these subsets in the blood (**Fig. 1a)**. By 72 hours after treatment with the virus, these subset numbers, with the exception of neutrophils and eosinophils, had returned to or surpassed the baseline values of untreated mice. In the spleens of VSV-treated mice, there was a significant decrease in the numbers of $CD45^+$ leukocytes, plasma cells, NK cells, neutrophils, eosinophils, MDSCs and DCs at 24 hours post-treatment, and all these subsets returned to or surpassed the baseline values at 72 hours. However, no significant changes in the numbers of spleen-derived $CD8^+$ or $CD4^+$ T cells, or B cells were found.

Tumour-infiltrating leukocytes appeared to behave differently compared to those in the blood and spleen. In the tumour, there was a profound loss of B cells and plasma cells that did not return to baseline numbers despite the increased numbers in circulation. There was also an acute reduction of $CD45^+$ leukocytes, $CD4^+$ T cells, MDSCs and DCs at 24 hours after administration of the virus. Analysis of various subsets of DCs demonstrated an overall significant loss of DCs at 24 hours, indiscriminate of the phenotype identified based on CD11b and CD11c expression (**Fig. 1b**). Differences in tumour-infiltrating $CD8^+$ T cells, NK cells, neutrophils and eosinophils were not found to be significant.

Tumour-infiltrating leukocytes decreased following treatment with NDV and OrfV. Next, we wondered if this transient intratumoural leukopenia was a response exclusive to treatment with VSV. Flow cytometry analysis revealed that intravenous infusions of melanoma-bearing mice with NDV (**Fig. 2**) and OrfV (**Fig. 3**) induced similar effects. With the exception of neutrophils in NDV-treated mice, there was a significant reduction in all subsets in the blood at 24 hours post-treatment with both NDV and OrfV. However, in NDV-treated hosts, only blood-derived NK cells, MDSCs and DCs had returned or surpassed baseline values by 72 hours. Similarly, in OrfV-treated mice, only blood-derived NK cells, eosinophils, MDSCs and DCs had returned to (or surpassed) the baseline values by 72 hours.

Further, with the exception of B cells and neutrophils in NDV-treated mice, as well as neutrophils in OrfV-treated mice, there was also a significant reduction in all subsets in the spleens of mice 24 hours after administration of viruses. These subsets, in both NDV- and OrfV-treated mice, except for $CD8^+$ and $CD4^+$ T cells, returned to or surpassed baseline values by 72 hours. There were also significant decreases in tumour-infiltrating $CD4^+$ T cells, B cells, plasma cells and NK cells of NDV-treated mice, as well as MDSCs of OrfV-treated mice at 24 hours following treatment. Otherwise, the numbers of tumour-infiltrating leukocytes were not found to be significantly different from untreated mice at 24 hours post-treatment with viruses.

Intratumoural NK cells were no longer activated 72 hours post-treatment with OVs and tumour-infiltrating $CD8^+$ T lymphocytes had increased expression of PD-1. Flow cytometry analysis revealed a dramatic increase in the number of activated NK cells circulating in the blood at 72 hours after infusion of OVs, however it did not translate to an increase in numbers of activated NK cells in tumours, except for NDV (**Fig 4**). Furthermore, despite an increase in the

overall numbers of activated NK cells present in the blood of OV-treated mice, the proportion of total NK cells that were $CD69^+$, which is an early activation marker, was found to have decreased in the tumours of VSV- and OrfV-treated mice by 72 hours post-treatment.

Flow cytometry analysis also revealed that expression of PD-1on $CD8^+$ T lymphocyte increased as their numbers returned to baseline values, in the blood and tumours of VSV-treated mice (**Fig. 5**). Additionally, our data shows substantially higher PD-1 expression on tumour-infiltrating $CD8^+$ T cells compared to blood-derived $CD8^+$ T cells, even without treatment with VSV.

VSV-induced leukopenia was not restricted to B16F10 melanomas in C57BL/6 mice; however, a reduction of intratumoural subsets did not occur in the EMT6 mammary carcinoma model in BALB/c mice. Although B16F10 melanomas are one of the most immunogenic tumours, leukocytic infiltration has also been reported in other tumour types, such as breast cancers[192]. Therefore we wondered if the reduction of leukocytes observed in B16F10 tumours would also occur in other cancer models, and in different strains of mice. Hence, we also investigated the effect of VSV administered intravenously on leukocyte subsets in EMT6 mammary carcinomas in BALB/c mice. Flow cytometry analysis revealed that while the blood and spleen-derived leukocytes underwent an acute reduction in numbers at 24 hours post-treatment with VSV, most intratumoural leukocyte subsets did not (**Fig. 6**). As shown in **Fig. 6**, there was an acute loss in all blood-derived leukocytes, with only $CD4^+$ T cells, plasma cells, eosinophils and DCs having returned to baseline by 72 hours post-treatment. $CD45^+$ leukocytes, $CD8^+$ T cells, B cells, NK cells, neutrophils, and MDSCs had increased in numbers from 48 to 72 hours post-treatment, but were still lower than untreated controls. Similarly, all subsets of spleen-derived leukocytes

underwent an acute decrease in numbers at 24 hours, but had returned or surpassed baseline values by 72 hours post-treatment. Tumour-infiltrating $CD45^+$ leukocytes, B cells, plasma cells, and NK cells demonstrated a significant increase at 24 hours post-VSV treatment compared to untreated controls. $CD4^+$ and $CD8^+$ T cells, neutrophils, eosinophils, MDSCs and DCs did not. However, all tumour-infiltrating subsets had returned to baseline or lower at 48 hours post-treatment.

Treatment with VSV caused acute margination of leukocytes. We showed that leukopenia can occur within 24 hours of infusion of an OV, but the underlying mechanisms were still unclear. We postulated that activated leukocytes might adhere to the endothelium of blood vessels. To investigate whether treatment with VSV increased margination of leukocytes in blood vessels, immunohistochemical staining was performed on lungs from EMT-6 tumour-bearing mice treated with VSV 24 hours prior to euthanasia. Immunohistochemical staining of smooth muscle actin in a lung section showed the presence of leukocytes in close proximity to the endothelium of arterioles in mice 24 hours after treatment with VSV (**Fig. 7**). In contrast, the lumena of the arterioles appeared to contain exclusively erythrocytes.

DISCUSSION

In the current study, we demonstrated that treatment with OVs can induce acute intratumoural leukopenia, in contrast to the current paradigm of oncolytic virotherapy. The current paradigm regarding OVs is that they infect tumour cells, subsequently causing an

inflammatory response within tumours, resulting in recruitment of leukocytes[125,176]. However, we found that at acute time points after treatment with OVs, this was not what was happening. Instead, we found that administration of OVs induced intratumoural leukopenia, which was apparent among $CD8^+$ T cells, $CD4^+$ T cells, B cells, plasma cells, NK cells, neutrophils, eosinophils, MDSCs and DCs. Interestingly, this phenomenon was induced by all three OVs that were evaluated (VSV, NDV and OrfV) in melanoma (B16F10)-bearing C57BL/6 mice. In contrast, however, tumour-infiltrating leukocytes did not undergo this reduction in the EMT6 model in BALB/c mice.

A transient reduction of blood-derived lymphocyte counts is characteristic of acute viral infections[139,140]. The reduction could be observed as early as three hours post-infection, and was shown to return to normal after six days[140]. Additionally, VSV-induced lymphopenia was shown to be mediated by type I interferons[139]. In the present study, we found similar changes in the blood and spleens of mice treated with VSV, NDV and OrfV. However, changes in tumour-infiltrating leukocyte numbers were not as consistent between the three viruses. It is unclear why there were differences between the viruses, but perhaps they could be attributed to inherent differences in the viruses; for instance their ability to infect and/or replicate in tumours, or in the interferon responses they induce. Cellular responses to VSV are initiated by pattern recognition receptors, such as toll-like receptors 3, 4, and 7, which induce expression of type I interferons after sensing VSV-derived proteins or genomes[193,194]. NDV has been shown to induce production of IFNα/β in infected cells[195,196], through RIG-I-mediated stimulation[197,198].

C57BL/6 and BALB/c mice differ in their immune responses against infections and inflammatory diseases[199,200]. Differences observed in the EMT6 model in the present study might be due to such differences in the two genetic backgrounds. For instance, BALB/c macrophages

have shown to produce less interferon-β compared to C57BL/6 mice in response to ligation of TLR 4[201]. There were also higher amounts of pro-inflammatory cytokines produced by macrophages from BALB/c mice, and higher expression of IL-10 by C57BL/6 macrophages[201]. Further investigation into the role of type I interferons in regulating immune responses may help to unveil its role in mediating virus-induced leukopenia.

The presence of intratumoural NK and T cells is associated with improved patient outcomes in various types of cancers[78,202,203]. However, another factor to consider, which may be of greater importance, is the functional quality of these cells. In this current study, we showed that NK cells in all three tissues were activated by 24 hours after treatment with OVs. Activated NK cells are capable of killing tumour cells *in vitro*[74]. However, we show that at 24 hours post-treatment, NK cell numbers remained unchanged in the tumours of VSV- and OrfV-treated mice, or transiently decreased in tumours of NDV-treated mice. We suspect that these activated NK cells either trafficked out of tumour and/or were prevented from infiltrating the tumour due to OV-mediated vascular shutdown. We hypothesize that vascular normalization may be a method to enhance the recruitment of circulating acutely activated NK cells into OV-treated tumours.

Many previous studies have shown, as we have here, that expression of PD-1 is increased in the tumour microenvironment. Expression of PD-1 is an indicator of T cell exhaustion. Exhausted $CD8^+$ T cells in tumours express a higher level of inhibitory surface receptors and a lower level of IFN-γ compared to extra-tumoural T cells[60,61]. Further, treatment with an OV appeared to increase expression of PD-1 on $CD8^+$ T cells after 72 hours, which is expected since PD-1 is involved in the negative feedback mechanism for shutting down immune responses. However, this is counterproductive for the desired anti-tumour response. In addition, tumour

cells have been shown to up-regulate the ligand for this receptor, PDL-1, and the binding of PDL-1 to PD-1 can result in potent suppression of the anti-tumour response. Combining checkpoint inhibition therapy with oncolytic virotherapy may be a strategy to augment the efficacy of effector cells in the tumour microenvironment.

Due to the rapid disappearance and subsequent return of leukocytic subsets, we suspect that cells were disappearing temporarily due to a trafficking phenomenon. One previously hypothesized mechanism for virus-induced leukopenia was transient adherence of leukocytes to the endothelial wall of blood vessels[139]. In this current study, we showed by immunohistochemical staining that this may indeed be the case. Leukocytes in lungs appeared to be in close proximity to the endothelium of arterioles of mice 24 hours post-treatment with VSV, suggesting that the virus caused acute margination. Margination or adherence to the endothelium is an important step in the process of transendothelial migration, which is a critical process for the recruitment of leukocytes to inflamed tissues[144]. This process of rolling along and migration across the endothelium is mediated by adhesion molecules on both endothelial and immune cells[144]. Several studies have shown increased expression of adhesion molecules on endothelial cells during viral infections. Treatment of human endothelial cells with a HIV tat protein or TNF increased E-selectin expression within five hours[204]. Infection with respiratory syncytial virus also increased I-CAM expression on human endothelial cells within as little as 24 hours, but in this study, concomitant expression of selectins on leukocytes were not found to be increased[205]. Furthermore, infection of endothelial cells with RSV resulted in increased adhesion and transmigration of neutrophils[205]. Adhesion molecules can also be expressed on leukocytes themselves. For instance, L-selectin on leukocytes is critical for homing of leukocytes to virus-infected tissues, and goes through cycles of high and low expression following viral infection[206].

However, information regarding selectin expression on leukocytes at acute time points post-viral infection is limited.

Although transient adherence to the endothelium of blood vessels could explain the sudden and dramatic loss of leukocytes in blood and tumours, this mechanism may not be the only explanation for the leukopenia observed in virus-treated mice. Virus-induced leukopenia could be the result of multiple mechanisms affecting different immunological subsets in the host. Further research is required to elucidate the processes involved in regulating this acute and transient margination.

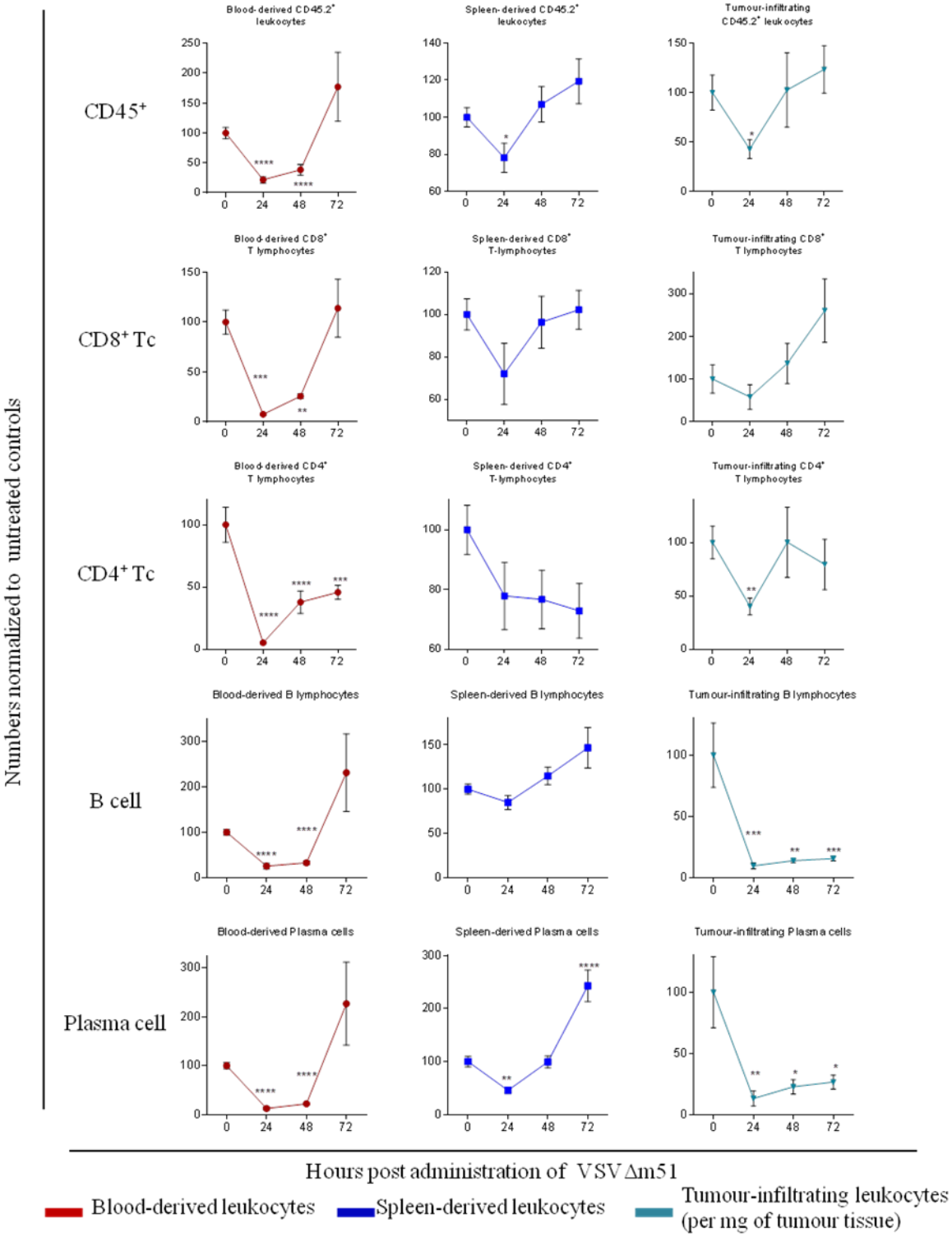
One particularly interesting finding was the observation that B cells did not return after 24 hours to the tumour despite increases in circulating and spleen-derived B cells. In a previous study, follicular B cells in the spleen were shown to be susceptible to infection with VSV[49]. Virus-mediated killing of B cells could potentially explain the lack of, or at least delayed return in numbers. Often overshadowed by the T cells in the lymphocyte compartment, B cells have not been extensively studied for their role in the anti-tumour response. However, several studies show that infiltration of B cells into solid tumours is associated with favourable patient outcomes[47,67–69]. It is speculated that B cells participate in forming/organizing tertiary lymphoid structures in the tumour microenvironment, which may be beneficial in orchestrating and coordinating the overall anti-tumour immune response[207]. As such, it may be important to consider how treatment with OVs is modulating this specific subset, and further, how we can optimize OV therapy to recruit and retain more B cells in the tumour microenvironment.

In conclusion, the impact of OV therapy on the immunological millieu of the tumour microenvironment is much more complex than what the current paradigm suggests. Future consideration should be given to the complex nature of leukocyte trafficking into and out of tumours following administration of an OV, with the goal of promoting favourable immunological profiles. The results of this current study provide the rationale to dissect the cytokine/chemokine milieu induced by OVs for the purpose of designing cytokine/chemokine-expressing OVs that can alter numbers and ratios of specific leukocyte subsets at acute time points.

ACKNOWLEDGEMENTS

We thank Drs. Brian Lichty (McMaster University), Peter Palese (Mount Sinai School of Medicine, USA) and John Bell (Ottawa Hospital Research Institute), who provided the VSV, NDV and OrfV vector systems, respectively; and Campus Animal Facilities (University of Guelph) for animal care services.

FIGURES


Blood-derived CD45.2+ leukocytes
Spleen-derived CD45.2+ leukocytes
Tumour-infiltrating CD45.2+ leukocytes
CD45+
Blood-derived CD8+ T lymphocytes
Spleen-derived CD8+ T-lymphocytes
Tumour-infiltrating CD8+ T lymphocytes
CD8+ Tc
Blood-derived CD4+ T lymphocytes
Spleen-derived CD4+ T-lymphocytes
Tumour-infiltrating CD4+ T lymphocytes
CD4+ Tc
Blood-derived B lymphocytes
Spleen-derived B lymphocytes
Tumour-infiltrating B lymphocytes
B cell
Blood-derived Plasma cells
Spleen-derived Plasma cells
Tumour-infiltrating Plasma cells
Plasma cell
Numbers normalized to untreated controls
Hours post administration of VSVΔm51
Blood-derived leukocytes
Spleen-derived leukocytes
Tumour-infiltrating leukocytes (per mg of tumour tissue)

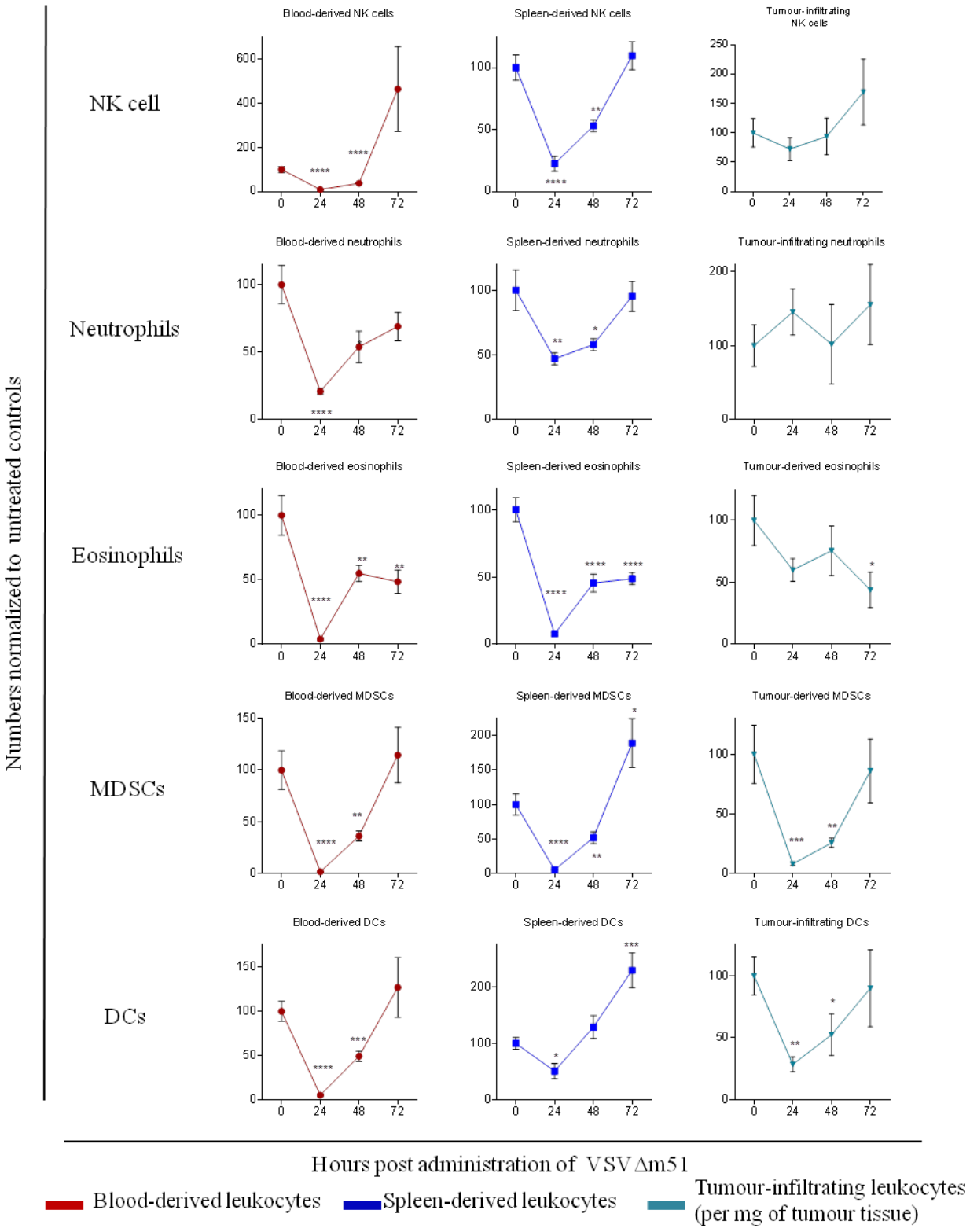

Numbers normalized to untreated controls
NK cell
Neutrophils
Eosinophils
MDSCs
DCs
Blood-derived NK cells
Spleen-derived NK cells
Tumour-infiltrating NK cells
Blood-derived neutrophils
Spleen-derived neutrophils
Tumour-infiltrating neutrophils
Blood-derived eosinophils
Spleen-derived eosinophils
Tumour-derived eosinophils
Blood-derived MDSCs
Spleen-derived MDSCs
Tumour-derived MDSCs
Blood-derived DCs
Spleen-derived DCs
Tumour-infiltrating DCs
Hours post administration of VSVΔm51
Blood-derived leukocytes
Spleen-derived leukocytes
Tumour-infiltrating leukocytes (per mg of tumour tissue)

Figure 1a: Tumour-infiltrating leukocytes decreased following treatment with VSV. Acute changes in various immunological cell subsets in blood, spleens and tumours, in response to intravenous treatment with VSV. B16F10 melanoma-bearing C57BL/6 hosts (n=8) were treated intravenously with VSVΔm51-SIINFEKL ($1x10^9$ pfu) 13 days post-tumour challenge. Tissues were harvested 24, 48 and 72 hours post-virus administration, and various immunological subsets were identified via FACs analysis. Numbers were expressed as percentages normalized to untreated controls. All graphs show means ± standard error. Results were analyzed by one-way analysis of variance with Tukey's multiple comparisons test. Significant differences compared to zero hours (prior to administration of OV) are indicated (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$).

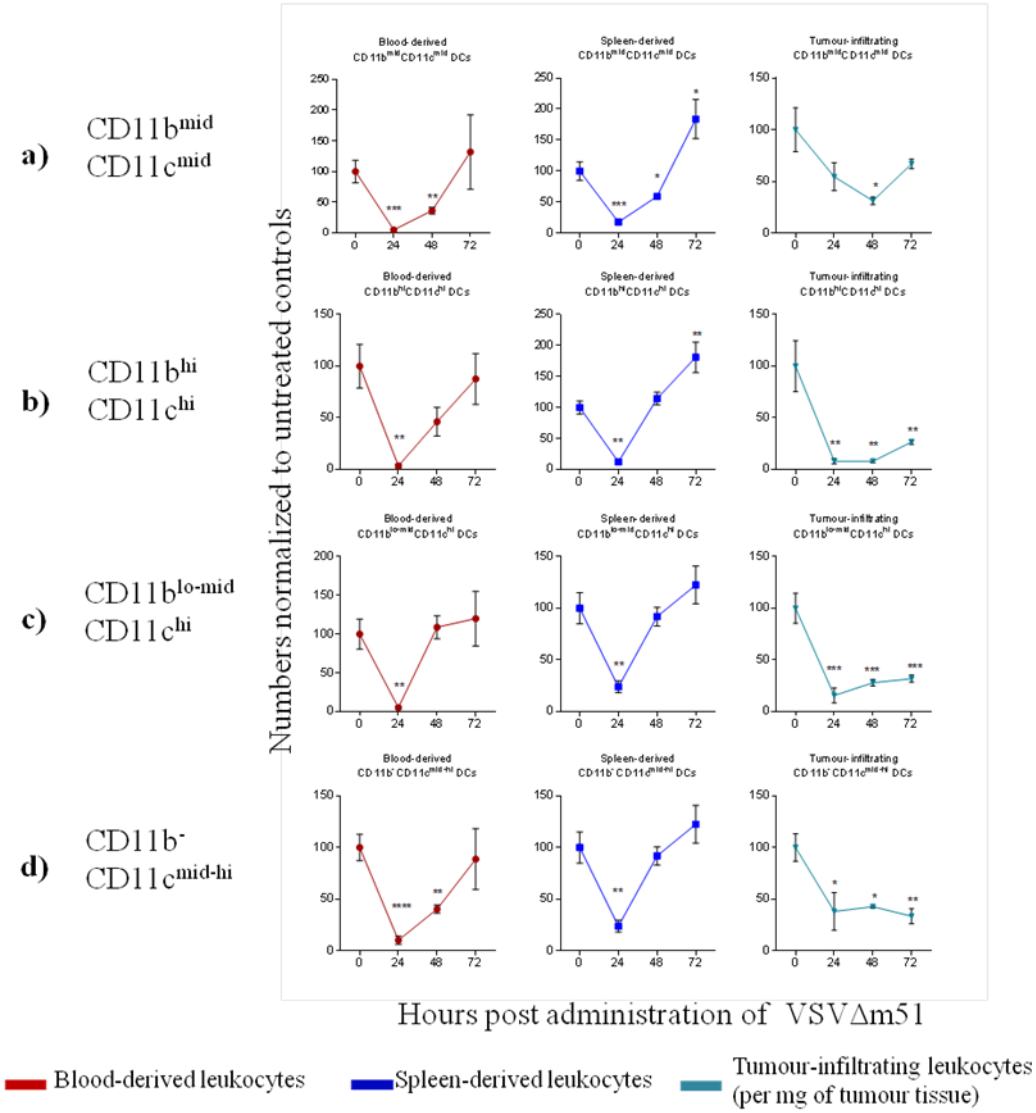
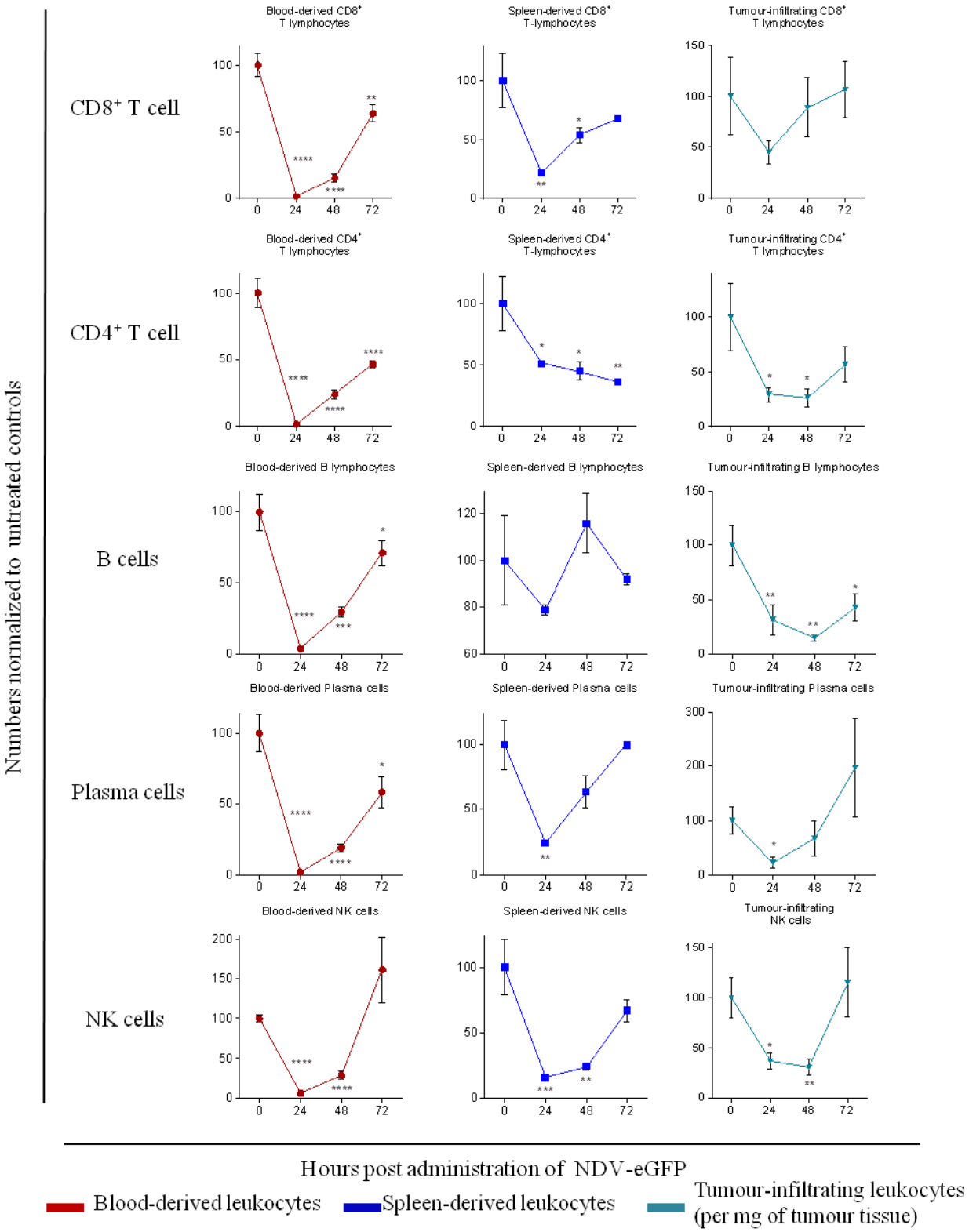


Figure 1b: Different tumour-infiltrating DC subsets also decreased following VSV treatment. Acute changes in DC subsets, (differentiated based on CD11b and CD11c expression) in blood, spleens and tumours, in response to intravenous treatment with VSV were assessed. B16F10 melanoma-bearing C57BL/6 hosts (n=8) were treated intravenously with VSVΔm51-SIINFEKL ($1x10^9$ pfu) 13 days post-tumour challenge. Blood, spleens and tumours were harvested 24, 48 and 72 hours later. Percentages were normalized to untreated controls. All graphs show means ± standard error. Results were analyzed by one-way analysis of variance

with Tukey's multiple comparisons test. Significant differences compared to zero hours (prior to administration of OV) are indicated (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).


Numbers normalized to untreated controls
CD8+ T cell
CD4+ T cell
B cells
Plasma cells
NK cells
Blood-derived CD8+ T lymphocytes
Spleen-derived CD8+ T-lymphocytes
Tumour-infiltrating CD8+ T lymphocytes
Blood-derived CD4+ T lymphocytes
Spleen-derived CD4+ T-lymphocytes
Tumour-infiltrating CD4+ T lymphocytes
Blood-derived B lymphocytes
Spleen-derived B lymphocytes
Tumour-infiltrating B lymphocytes
Blood-derived Plasma cells
Spleen-derived Plasma cells
Tumour-infiltrating Plasma cells
Blood-derived NK cells
Spleen-derived NK cells
Tumour-infiltrating NK cells
Hours post administration of NDV-eGFP
Blood-derived leukocytes
Spleen-derived leukocytes
Tumour-infiltrating leukocytes (per mg of tumour tissue)

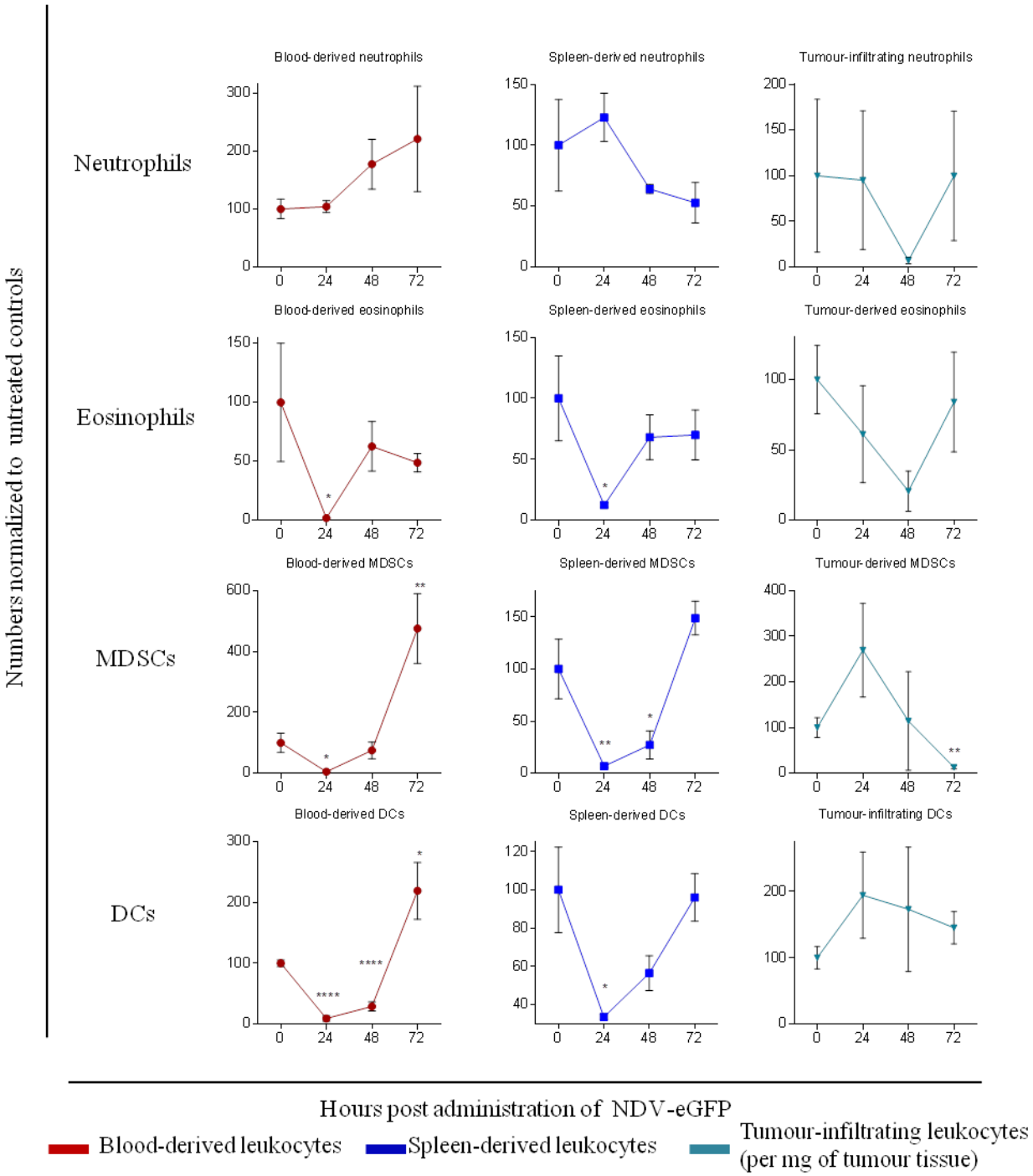
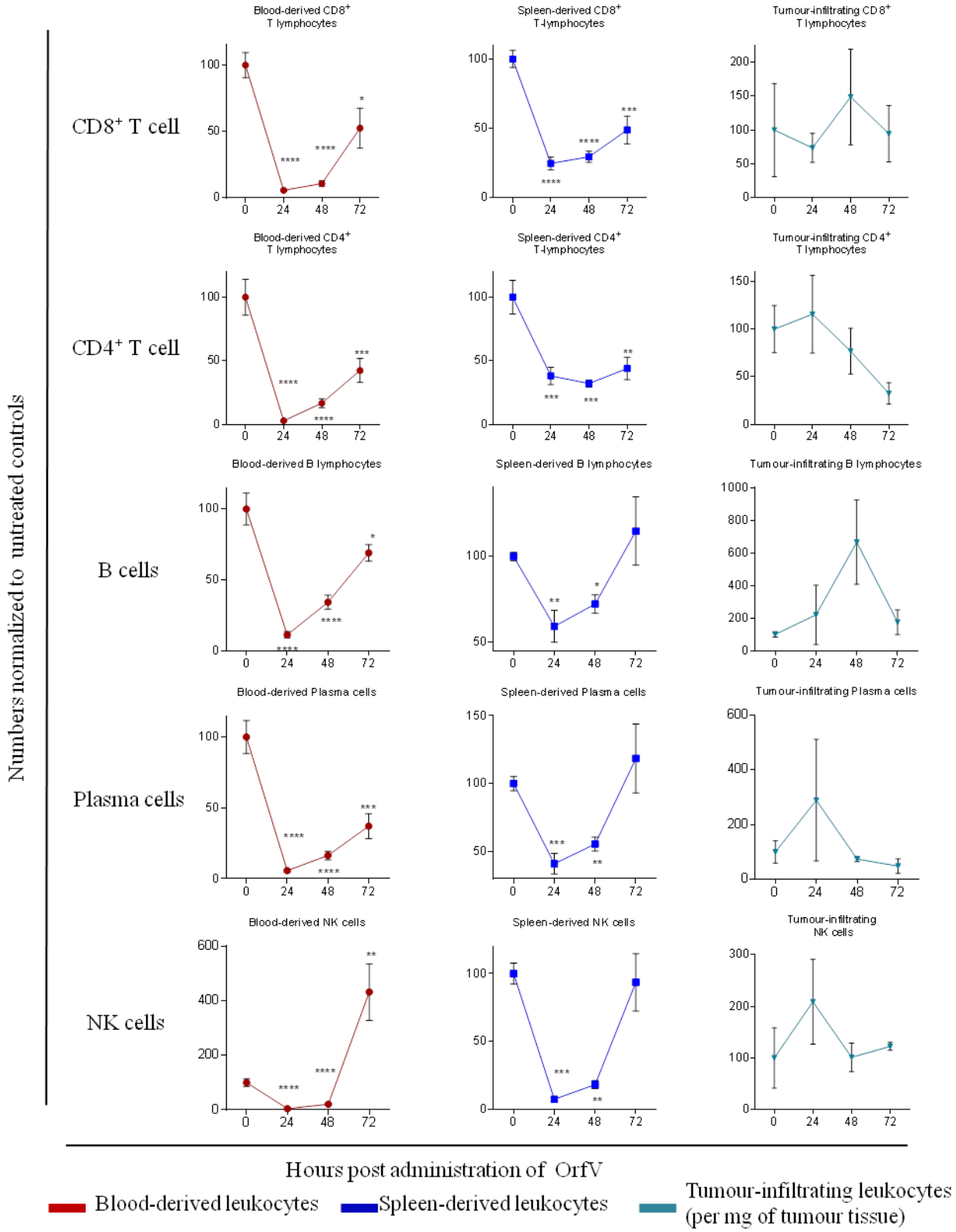

Numbers normalized to untreated controls
Neutrophils
Eosinophils
MDSCs
DCs
Blood-derived neutrophils
Spleen-derived neutrophils
Tumour-infiltrating neutrophils
Blood-derived eosinophils
Spleen-derived eosinophils
Tumour-derived eosinophils
Blood-derived MDSCs
Spleen-derived MDSCs
Tumour-derived MDSCs
Blood-derived DCs
Spleen-derived DCs
Tumour-infiltrating DCs
Hours post administration of NDV-eGFP
Blood-derived leukocytes
Spleen-derived leukocytes
Tumour-infiltrating leukocytes (per mg of tumour tissue)

Figure 2: Tumour-infiltrating leukocytes also decreased following treatment with NDV.

Acute changes in various immunological cell subsets in blood, spleens and tumours, in response to intravenous treatment with NDV. B16F10 melanoma-bearing C57BL/6 hosts (n=4) were treated intravenously with NDV-F3aa-eGFP ($1x10^8$ pfu) 13 days post-tumour challenge. Tissues were harvested 24, 48 and 72 hours post-virus administration, and various immunological subsets were identified via FACs analysis. Numbers were expressed as percentages normalized to untreated controls. All graphs show means ± standard error. Results were analyzed by one-way analysis of variance with Tukey's multiple comparisons test. Significant differences compared to zero hours (prior to administration of OV) are indicated (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$).


Numbers normalized to untreated controls
CD8+ T cell
CD4+ T cell
B cells
Plasma cells
NK cells
Blood-derived CD8+ T lymphocytes
Spleen-derived CD8+ T-lymphocytes
Tumour-infiltrating CD8+ T lymphocytes
Blood-derived CD4+ T lymphocytes
Spleen-derived CD4+ T-lymphocytes
Tumour-infiltrating CD4+ T lymphocytes
Blood-derived B lymphocytes
Spleen-derived B lymphocytes
Tumour-infiltrating B lymphocytes
Blood-derived Plasma cells
Spleen-derived Plasma cells
Tumour-infiltrating Plasma cells
Blood-derived NK cells
Spleen-derived NK cells
Tumour-infiltrating NK cells
Hours post administration of OrfV
Blood-derived leukocytes
Spleen-derived leukocytes
Tumour-infiltrating leukocytes (per mg of tumour tissue)

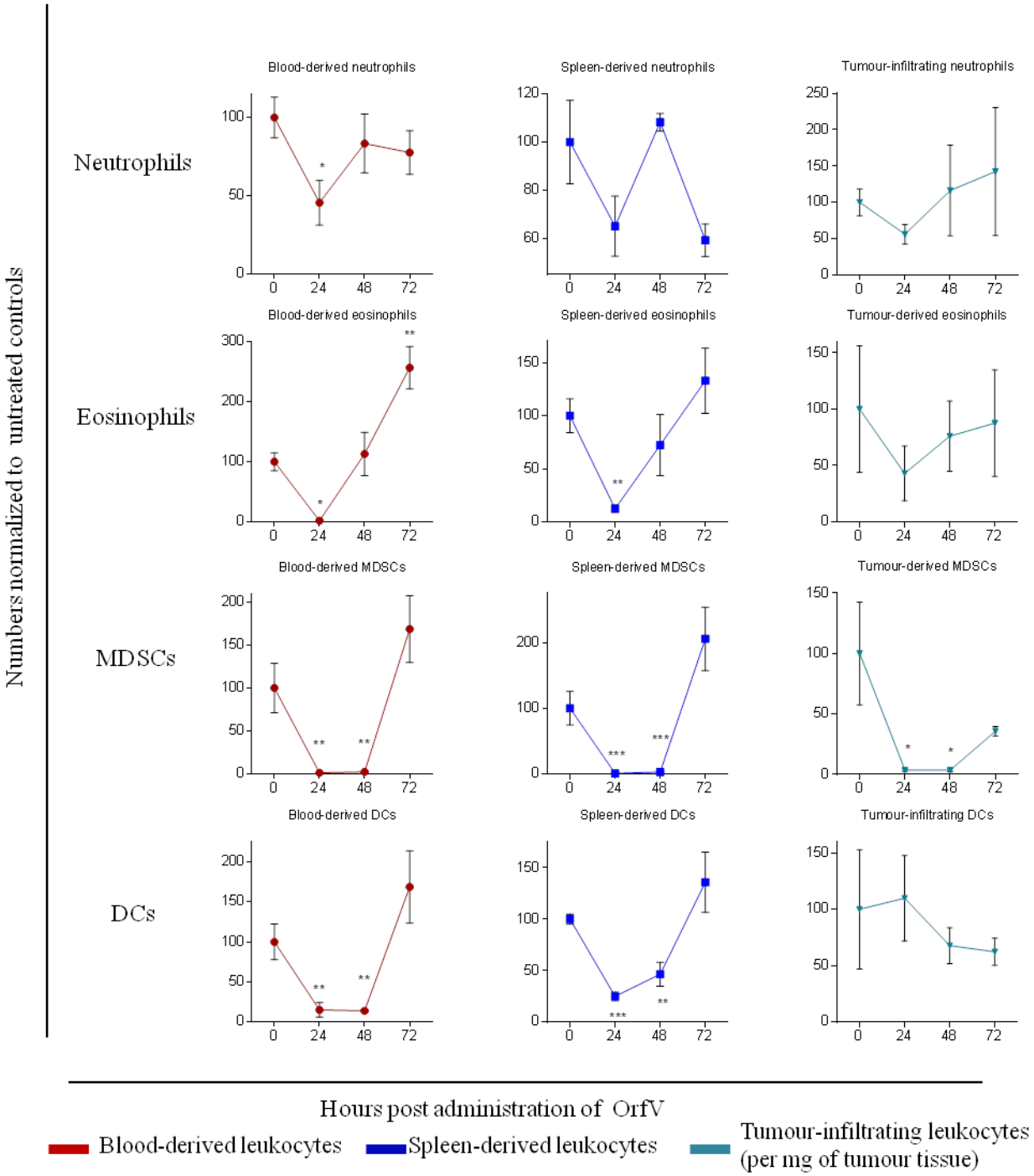

Numbers normalized to untreated controls
Neutrophils
Eosinophils
MDSCs
DCs
Blood-derived neutrophils
Spleen-derived neutrophils
Tumour-infiltrating neutrophils
Blood-derived eosinophils
Spleen-derived eosinophils
Tumour-derived eosinophils
Blood-derived MDSCs
Spleen-derived MDSCs
Tumour-derived MDSCs
Blood-derived DCs
Spleen-derived DCs
Tumour-infiltrating DCs
Hours post administration of OrfV
Blood-derived leukocytes
Spleen-derived leukocytes
Tumour-infiltrating leukocytes (per mg of tumour tissue)

Figure 3: Tumour-infiltrating leukocytes decreased following treatment with OrfV. Acute changes in various immunological cell subsets in blood, spleens and tumours, in response to intravenous treatment with OrfV. B16F10 melanoma-bearing C57BL/6 hosts (n=4) were treated intravenously with OrfV ($3x10^7$ pfu) 13 days post-tumour challenge. Tissues were harvested 24, 48 and 72 hours post-virus administration, and various immunological subsets were identified via FACs analysis. Numbers were expressed as percentages normalized to untreated controls. All graphs show means ± standard error. Results were analyzed by one-way analysis of variance with Tukey's multiple comparisons test. Significant differences compared to zero hours (prior to administration of OV) are indicated (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$).

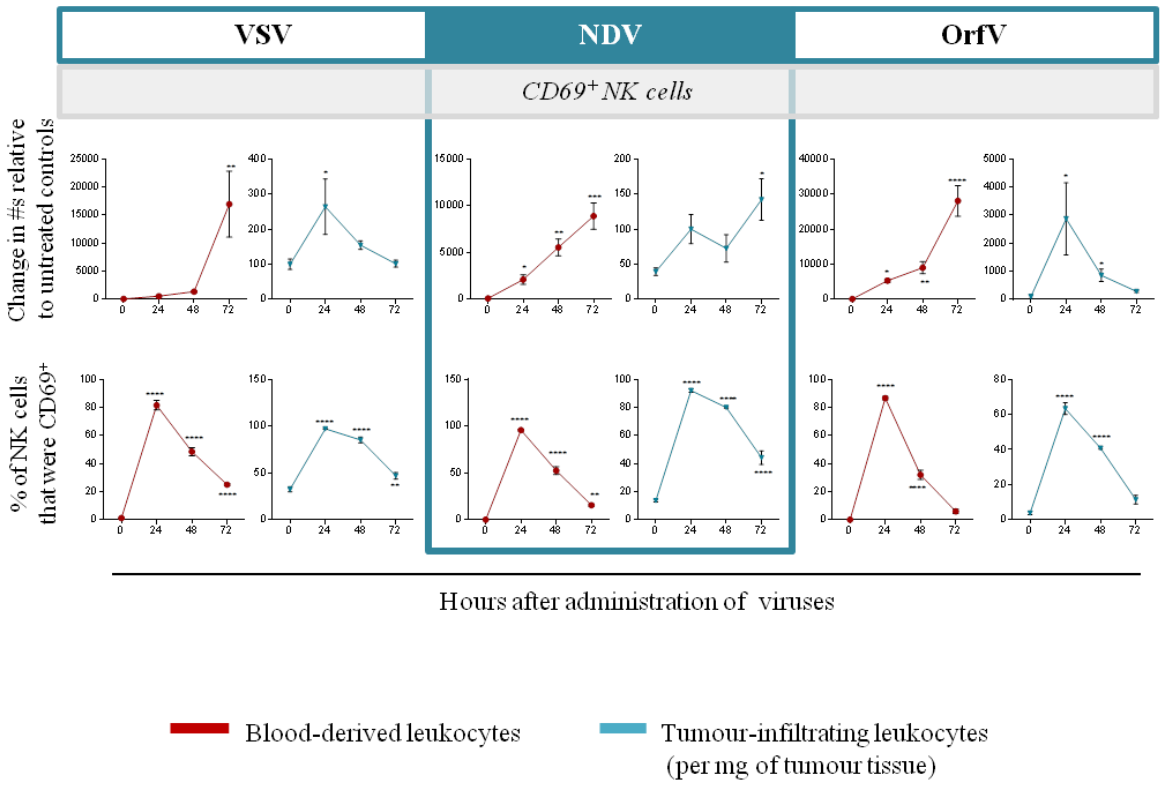


Figure 4: Intratumoural NK cells were no longer activated at 72 hours post-treatment with OVs. Acute changes in NK cell numbers and their expression of the early activation marker CD69 in blood and tumours after intravenous administration of OVs (VSVΔm51-SIINFEKL: $1x10^9$ pfu; NDV-F3aa-eGFP: $1x10^8$ pfu; OrfV: $3x10^7$ pfu) ($n \geq 4$). B16F10 melanoma bearing C57BL/6 mice were treated intravenously with OVs 13 days post-tumour challenge and tissues (blood and tumours) were harvested 24, 48 and 72 hours later. Percentages were normalized to untreated controls; means ± standard error are shown. Data were analyzed by one-way analysis of variance with Tukey's multiple comparisons test. Significant differences compared to zero hours (prior to administration of OV) are indicated (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$).

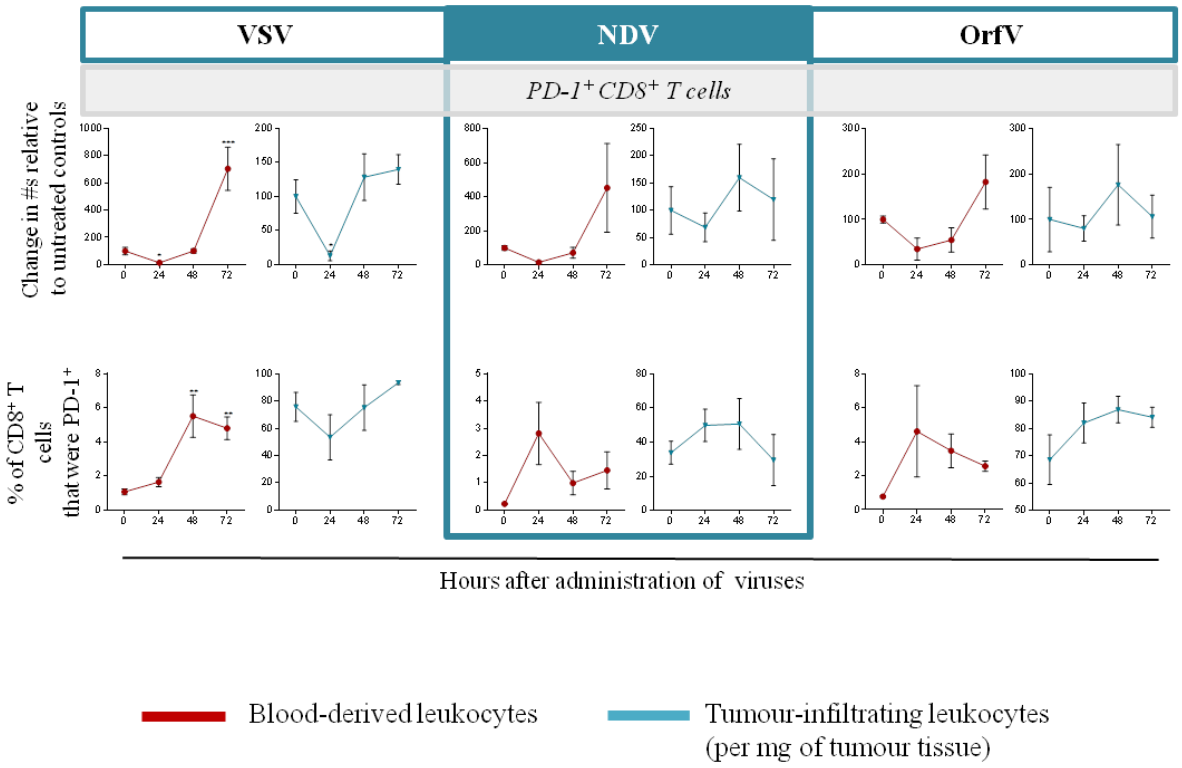
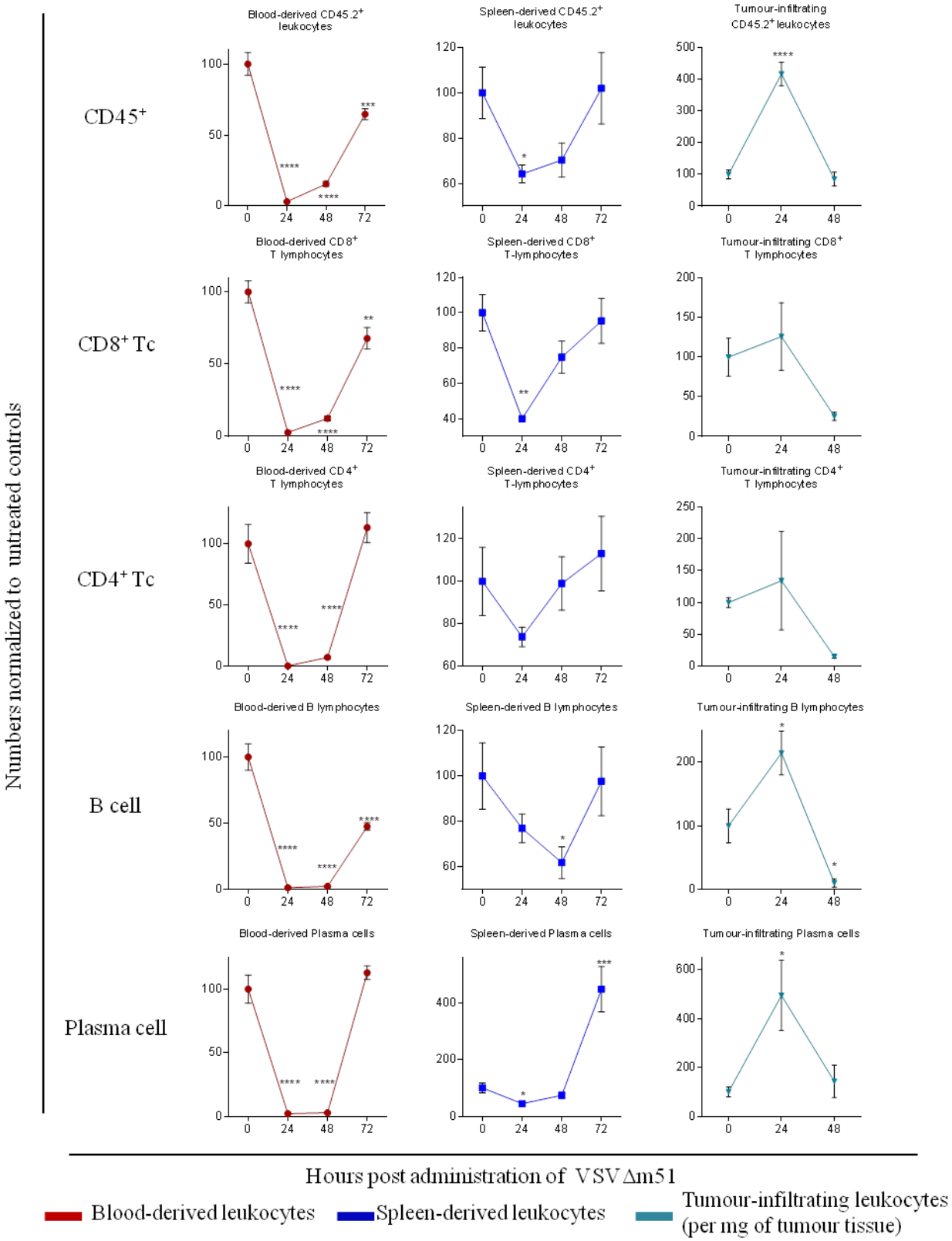


Figure 5: Tumour-infiltrating CD8$^+$ T lymphocytes had increased expression of PD-1. Acute changes in the number of CD8$^+$ T cells and their expression of programmed death receptor-1 (PD-1) in blood and tumours after intravenous administration of OVs (VSVΔm51-SIINFEKL: 1x10^9 pfu; NDV-F3aa-eGFP: 1x10^8 pfu; OrfV: 3x10^7 pfu) (n≥4). B16F10 melanoma bearing C57BL/6 mice were treated intravenously with OVs 13 days post-tumour challenge and tissues were harvested 24, 48 and 72 hours later. Percentages were normalized to untreated controls; means ± standard error are shown. Data were analyzed by one-way analysis of variance with Tukey's multiple comparisons test. Significant differences compared to zero hours (prior to administration of OV) are indicated (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).


Numbers normalized to untreated controls
CD45+
CD8+ Tc
CD4+ Tc
B cell
Plasma cell
Blood-derived CD45.2+ leukocytes
Spleen-derived CD45.2+ leukocytes
Tumour-infiltrating CD45.2+ leukocytes
Blood-derived CD8+ T lymphocytes
Spleen-derived CD8+ T-lymphocytes
Tumour-infiltrating CD8+ T lymphocytes
Blood-derived CD4+ T lymphocytes
Spleen-derived CD4+ T-lymphocytes
Tumour-infiltrating CD4+ T lymphocytes
Blood-derived B lymphocytes
Spleen-derived B lymphocytes
Tumour-infiltrating B lymphocytes
Blood-derived Plasma cells
Spleen-derived Plasma cells
Tumour-infiltrating Plasma cells
Hours post administration of VSVΔm51
Blood-derived leukocytes
Spleen-derived leukocytes
Tumour-infiltrating leukocytes (per mg of tumour tissue)

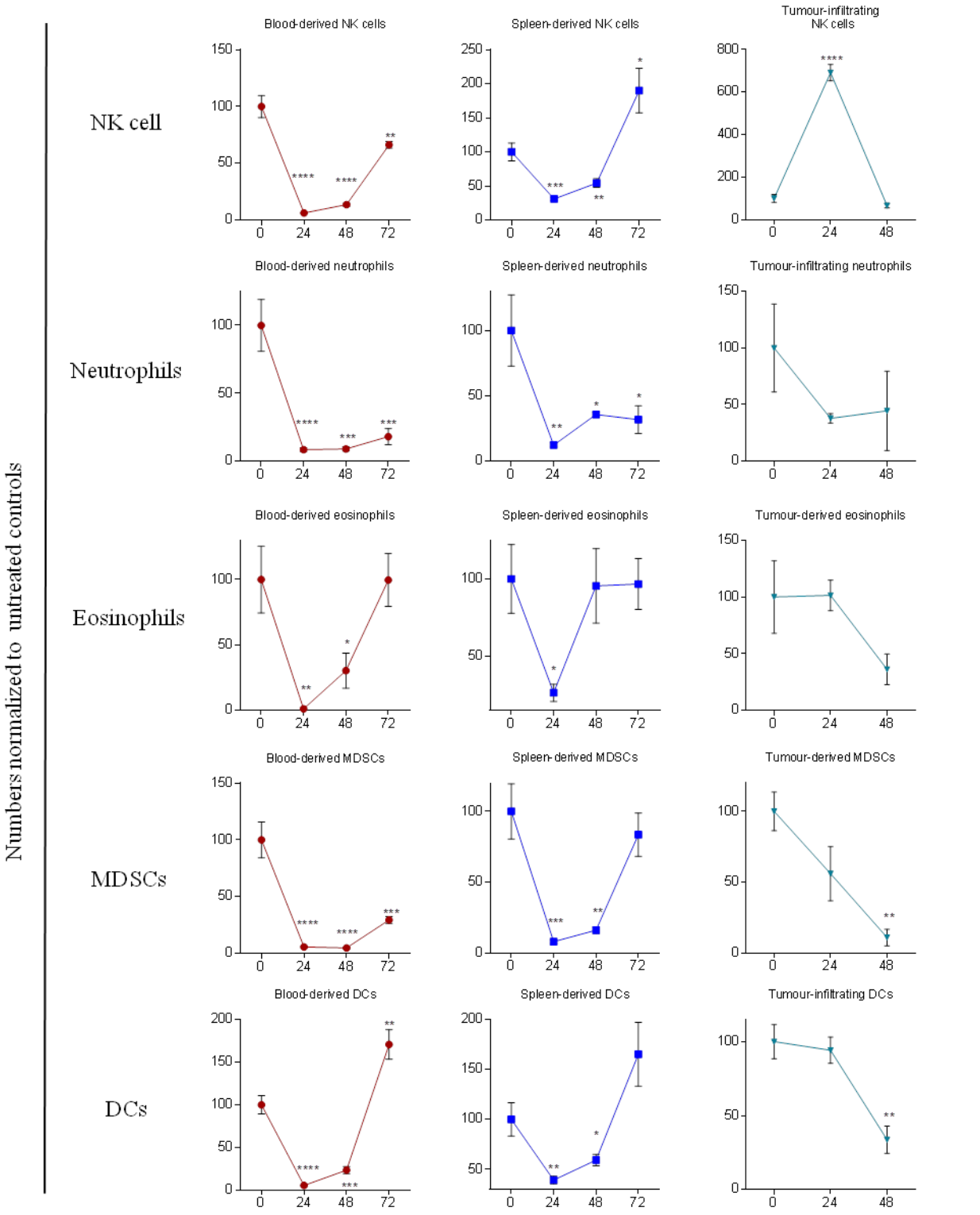

Numbers normalized to untreated controls
NK cell
Neutrophils
Eosinophils
MDSCs
DCs
Blood-derived NK cells
Spleen-derived NK cells
Tumour-infiltrating NK cells
Blood-derived neutrophils
Spleen-derived neutrophils
Tumour-infiltrating neutrophils
Blood-derived eosinophils
Spleen-derived eosinophils
Tumour-derived eosinophils
Blood-derived MDSCs
Spleen-derived MDSCs
Tumour-derived MDSCs
Blood-derived DCs
Spleen-derived DCs
Tumour-infiltrating DCs
Hours post administration of VSVΔm51
Blood-derived leukocytes
Spleen-derived leukocytes
Tumour-infiltrating leukocytes (per mg of tumour tissue)

Figure 6: VSV-induced leukopenia was not restricted to B16-F10 melanoma in C57Bl/6 mice, however reduction of intratumoural subsets did not occur in the EMT6 model in BALB/c mice. Acute changes in various immunological cell subsets in blood, spleens and tumours, in response to intravenous treatment with VSV. EMT6 mammary carcinoma-bearing BALB/c hosts (n=4) were treated intravenously with VSVΔm51-SIINFEKL ($1x10^9$ pfu) 13 days post-tumour challenge. Tissues were harvested 24, 48 and 72 hours post-virus administration, and various immunological subsets were identified via FACs analysis. Numbers were expressed as percentages normalized to untreated controls. All graphs show means ± standard error. Results were analyzed by one-way analysis of variance with Tukey's multiple comparisons test. Significant differences compared to zero hours (prior to administration of OV) are indicated (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$).

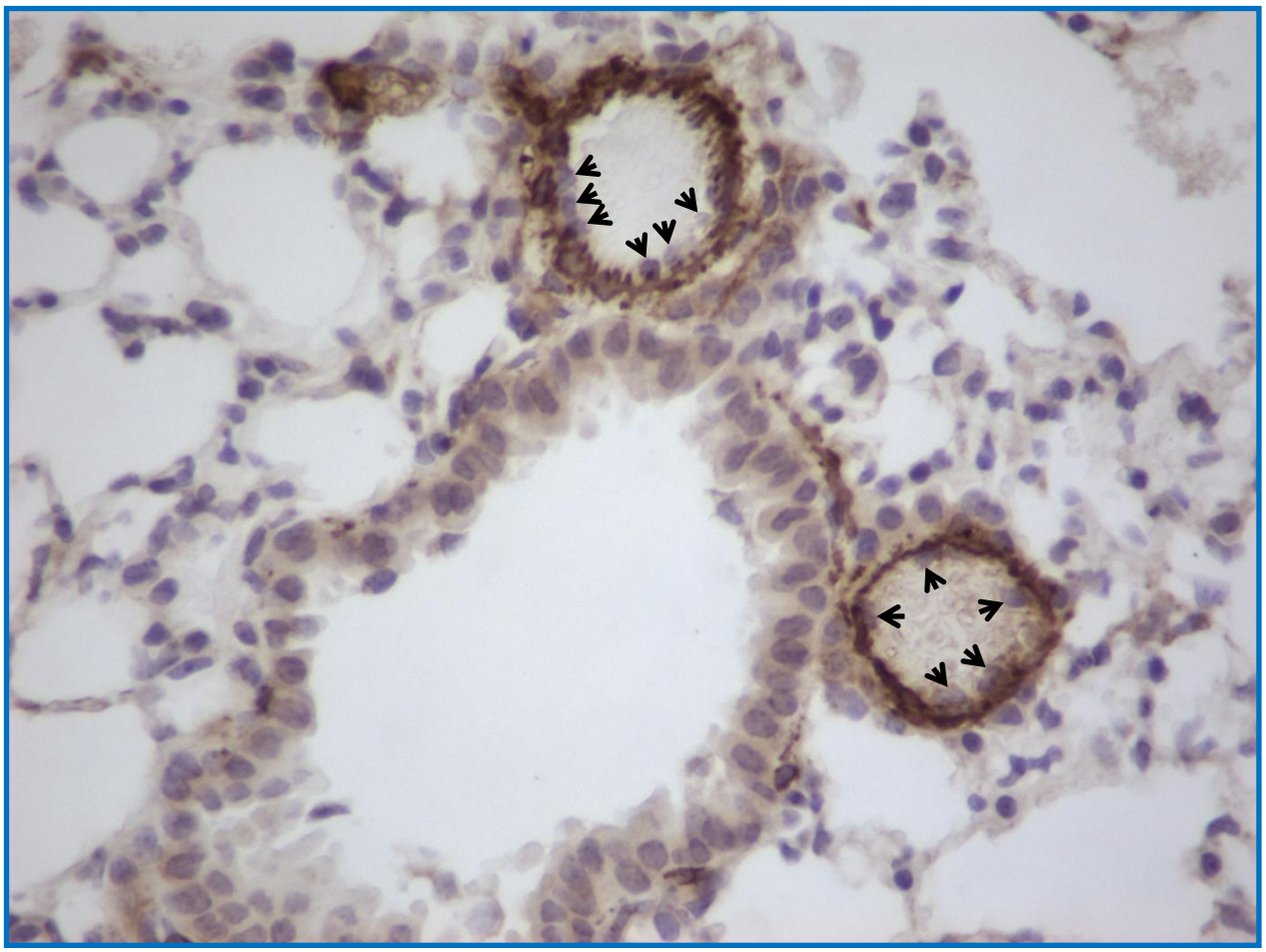
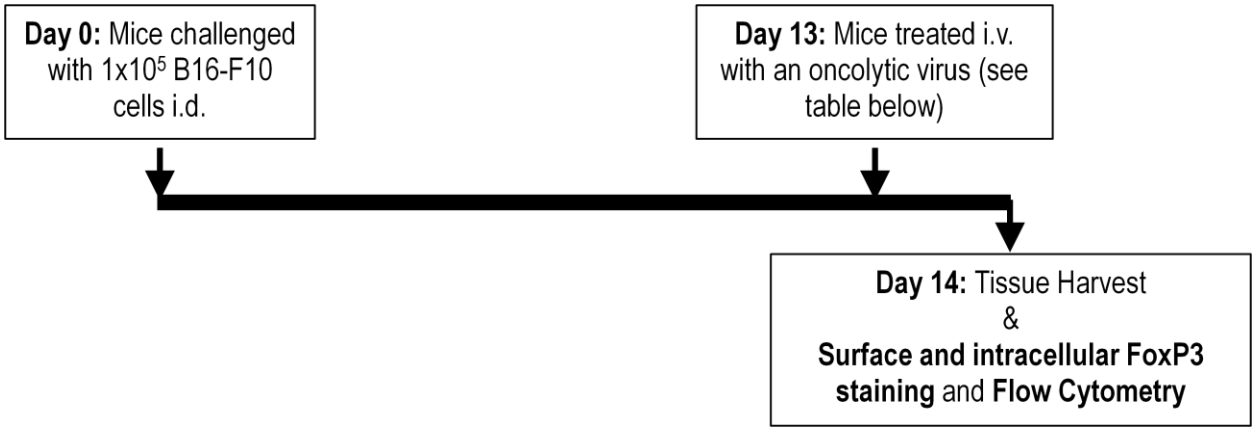
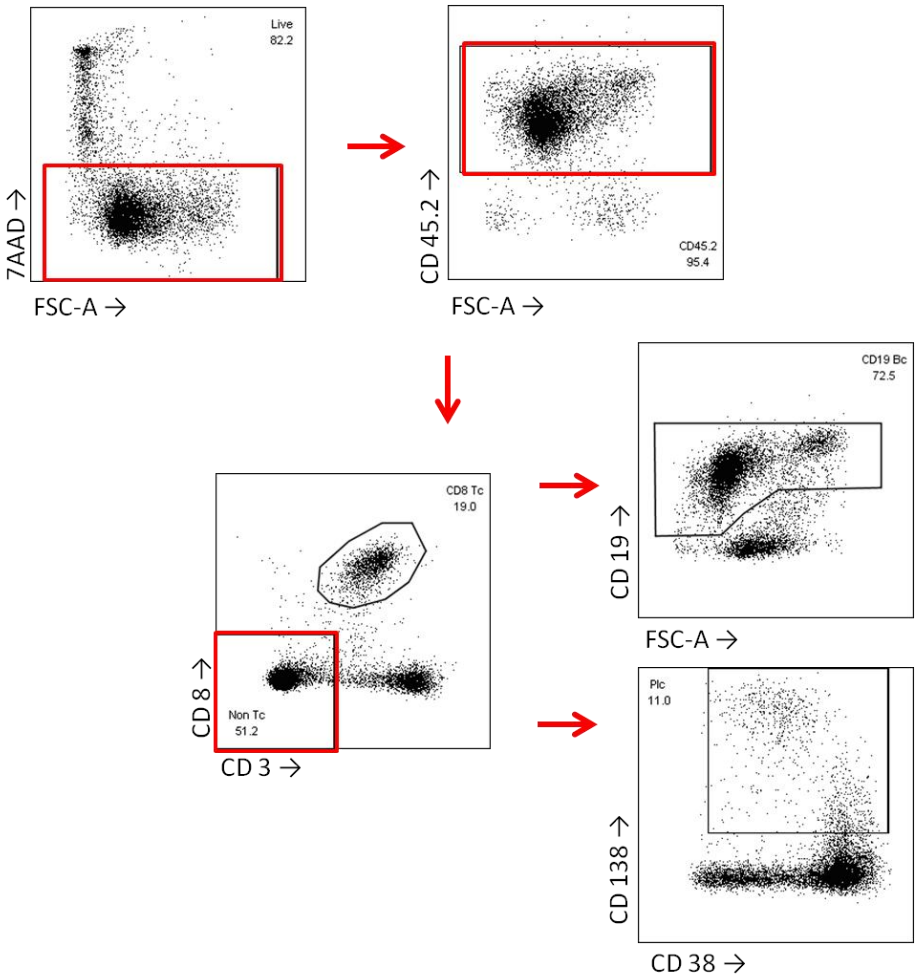


Figure 7: Treatment with VSV caused acute margination of leukocytes. Immunohistochemical staining of smooth muscle actin (brown) in a lung section from an EMT-6 mammary carcinoma bearing BALB/c mouse that received intravenous treatment with VSVΔm51-SIINFEKL ($1x10^9$ pfu), 24 hours prior to euthanasia. Leukocytes are indicated by the black arrows. All images were captured at 400x magnification.

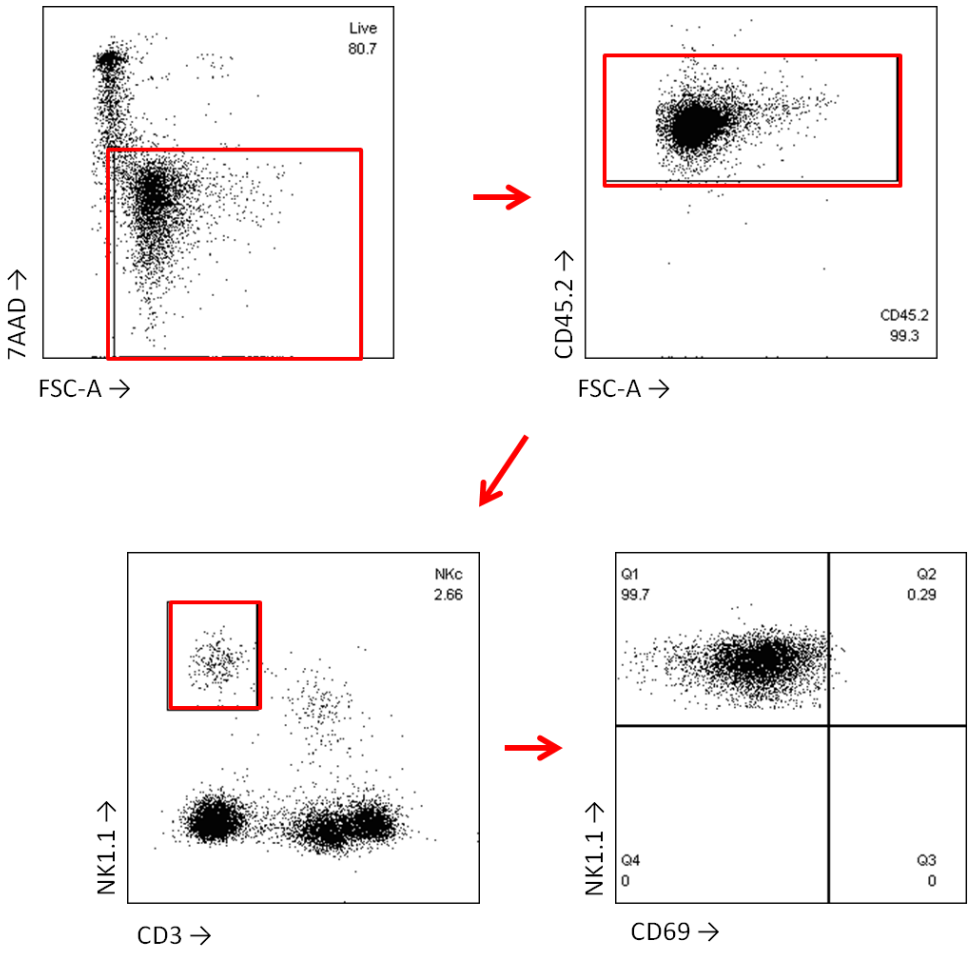
SUPPLEMENTARY FIGURES



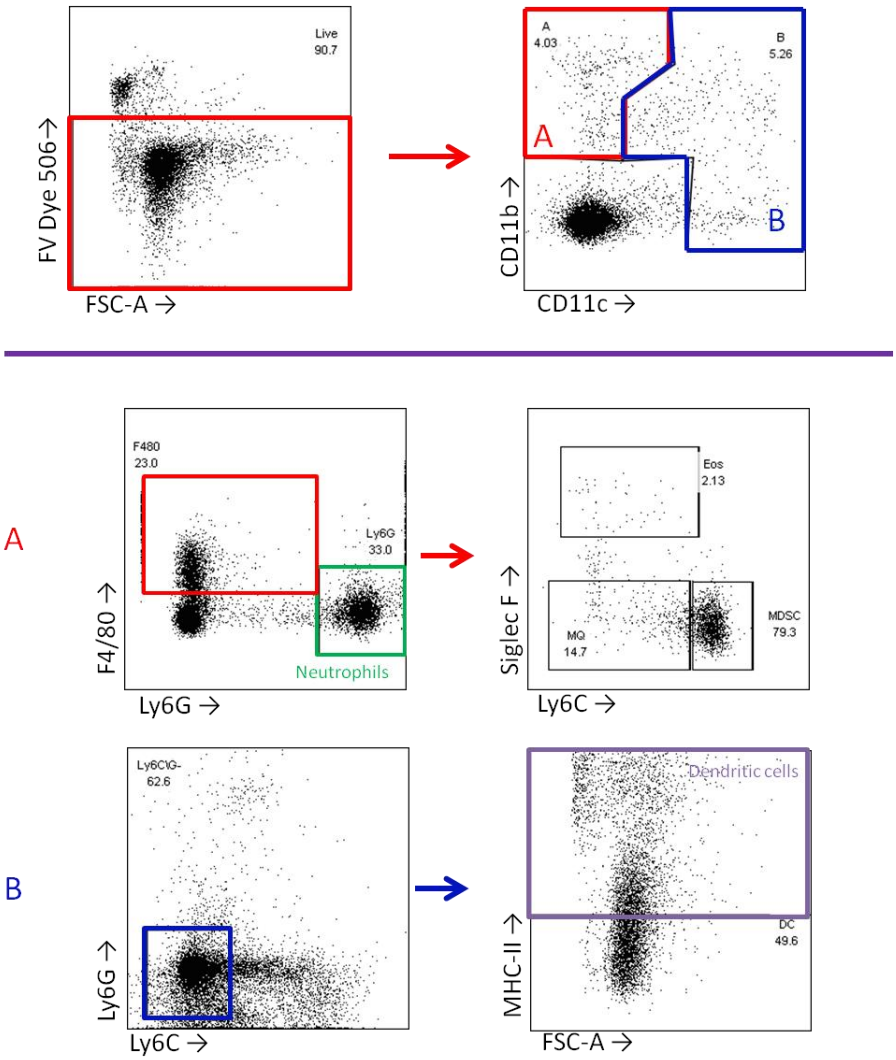
Supplementary Figure 1: Timeline of experiments.



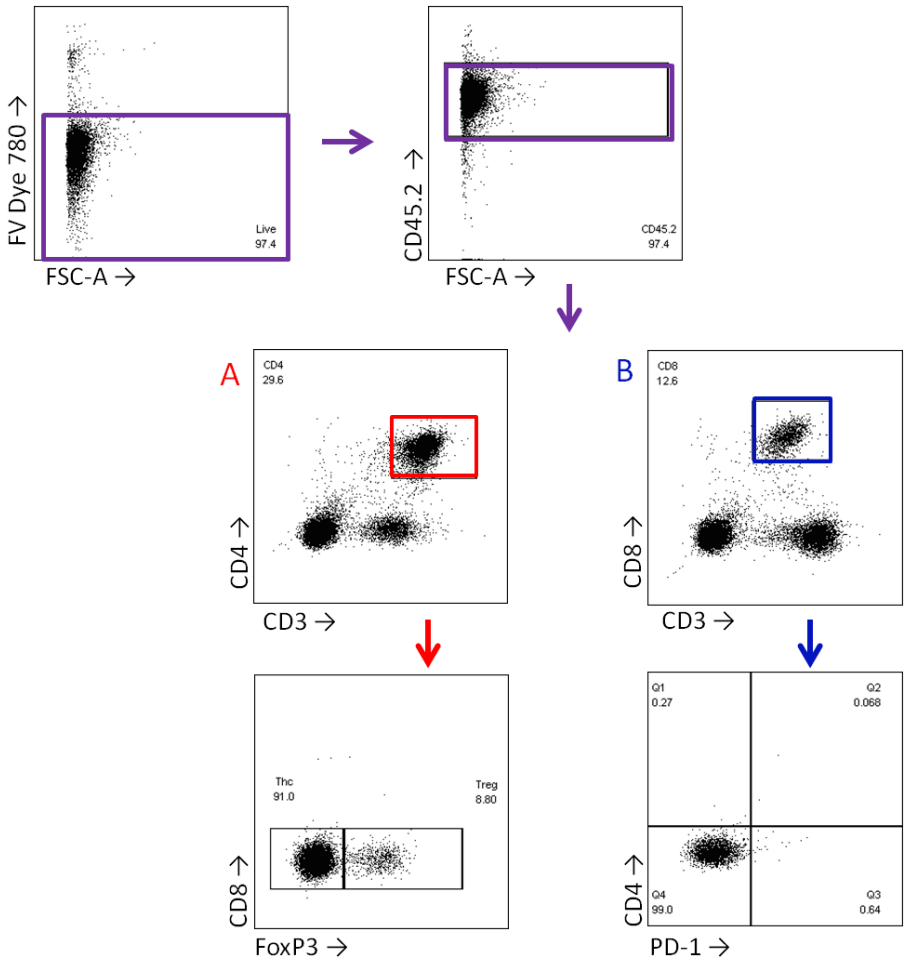
Supplementary Figure 2a: Representative dot plots from the spleen of an untreated mouse demonstrating the gating strategy used for $CD45^+$ leukocytes, B cells, Plasma cells and $CD8^+$ T cells. Spleens were processed, and red blood cells were lysed prior to counting total splenocytes. Leukocytes were first gated based on forward and side-scatter area (FSC-A vs. SSC-A). Doublets were excluded, followed by dead cells, using a fixable viability dye. Leukocytes were then identified by CD45.2 expression. CD8 T cells were identified based on positive CD3 and CD8 staining. B cells were identified by negative CD3 staining and positive CD19 staining. Plasma cells were identified by negative CD3 staining, positive CD138 and mid to high CD38 staining.



Supplementary Figure 2b: Representative dot plots from the spleen of an untreated mouse demonstrating the gating strategy used for NK cells and CD69 expression. Spleens were processed, and red blood cells were lysed prior to counting total splenocytes. Leukocytes were first gated based on forward and side-scatter area (FSC-A vs. SSC-A). Doublets were excluded, followed by dead cells, using 7AAD staining. NK cells were identified by negative CD3 staining and positive NK1.1 staining. CD69 expression was then assessed.



Supplementary Figure 2c: Representative dot plots from the spleen of an untreated mouse demonstrating the gating strategy used for neutrophils, eosinophils, myeloid derived suppressor cells (MDSC) and dendritic cells (DC). Spleens were processed, and red blood cells were lysed prior to counting total splenocytes. Leukocytes were first gated based on forward and side-scatter area (FSC-A vs. SSC-A). Doublets were excluded, followed by dead cells, using a fixable viability dye. Subsets were further identified by staining of various surface markers. (Neutrophils: $CD11b^+$ $F4/80^-$ $Ly6G^+$; Eosinophils: $CD11b^+$ $F4/80^+$ $SiglecF^+$; MDSCs: $CD11b^+$ $F4/80^+$ $Ly6C^+$; DCs:$CD11b^+$ $CD11c^+$).



Supplementary Figure 2d: Representative dot plots from the spleen of an untreated mouse demonstrating the gating strategy used for $CD4^+$ and $CD8^+$ T cells. Spleens were processed, and red blood cells were lysed prior to counting total splenocytes. Leukocytes were first gated based on forward and side-scatter area (FSC-A vs. SSC-A). Doublets were excluded, followed by dead cells, using a fixable viability dye. CD8 T cells were identified by positive CD3 and CD8 staining. CD4 T cells were identified by positive CD3 and CD4 staining. Regulatory and helper T cells were further identified by FoxP3 staining.

CHAPTER FOUR:

MODULATION OF MELANOGENESIS AND IMMUNOGENECITY OF MELANOMAS BYMETFORMIN

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ABSTRACT

Melanomas are relatively immunogenic cancers derived from melanocytes that produce the pigment melanin, which gives skin, fur and hair their colour. Darker skin is associated with a reduced incidence of melanomas. Spontaneous immune responses in patients are often directed against melanoma antigens that include enzymes involved in melanogenesis. Amelanotic tumours are difficult to treat and have reduced immunogenicity. Biochemists have shown that reactive oxygen species (ROS) promote melanin synthesis. There are many factors that can alter levels of ROS in melanomas, including activation of macrophages, hypoxia and treatments such

as photodynamic therapy. Murine B16F10 melanoma cells were cultured with or without metformin, which reduced melanogenesis as assessed by microscopy and absorbance readings. Treatment with metformin also reduced cellular ROS. Cells grown with or without metformin were implanted intradermally into mice. Melanomas derived from metformin-containing cultures had a higher rate of engraftment and grew significantly faster. We suspect that a reduction in ROS abrogated the immunogenicity of the cells. We predict that metformin-treated cells will express lower levels of melanoma antigens such as tyrosinase and dopachrome tautomerase. We propose that incorporation of treatments that promote intratumoural ROS may potentiate melanoma immunotherapies and that use of antioxidants may be counterproductive.

INTRODUCTION

Melanomas have been increasing in incidence worldwide over the past several decades[4,5,159]. Melanomas are cancers that originate from the melanocytes, the cells responsible for producing the pigments that protect against UV radiation[2]. Although melanomas only account for 4% of all cases of skin cancer, they account for approximately 80% of skin cancer-related deaths[9]. Interestingly, the incidence of skin cancers in darker skin remains relatively low[208–210]. However, when skin cancer does occur in patients of darker skin complexions, patients usually present with an advanced stage, and therefore face a worse prognosis[210,211].

Metformin is of the biguanide family, and is currently the most widely used oral anti-diabetic drug for Type 2 diabetes mellitus[153–155]. Metformin is highly favoured for its effectiveness, low cost and safety profile[154]. Adverse events include mild gastrointestinal

symptoms that are often transient[154]. Studies have shown an association between diabetes and increased risk for pancreatic, hepatocellular, endometrial and bladder cancers[154]. On the flip side, a number of studies have also shown a correlation with metformin treatment and a decreased risk of developing certain types of cancers, as well as improved outcomes for metformin-treated diabetic cancer patients[153,154]. Additionally, metformin has also shown some potential as an anti-cancer therapeutic. In several studies, treatment with metformin has demonstrated a reduction in *in vitro* cell growth and proliferation in several different cancer types, including: hepatocellular carcinoma, colon cancer cells, glioma and melanoma cells[153,155–157].

The mechanisms behind these anti-cancer effects are unclear, as metformin appears to affect a number of different signalling pathways. For instance, metformin exerts effects on LKB1, a protein involved in the AMPK/mTOR pathway, which ultimately leads to reduced protein synthesis and cell proliferation[153,155]. Metformin also reduces hepatic gluconeogenesis and increases uptake of glucose by skeletal muscle[154]. Metformin also disrupts complex I of the mitochondrial respiratory chain, which leads to changes in the production of reactive oxygen species[154,157].

Reactive oxygen species (ROS) is an important component for cell survival and integrity[149]. However, high concentrations of ROS can be deleterious to proteins and enzymes, and it can disrupt signalling pathways. Melanocytes, in particular, are sensitive to the presence of ROS[149]. High levels of ROS inhibit tyrosinase, yet on the other hand, lower concentrations of ROS can upregulate and activate tyrosinase.

Tyrosinase, tyrosinase-related protein (TRP)-1 and TRP-2 are the three main enzymes involved in the melanin synthesis pathway, and are found in all melanocytes[146,147]. Tyrosinase is

responsible for two reactions of the melanogenesis pathway: the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and the oxidation of DOPA to dopaquinone. TRP2 is also known as dopachrome tautomerase (DCT) and is responsible for rearranging dopachrome to 5,6-dihydroxyindole (DHI) or to indole 5,6-quinone 2 carboxylic acid (DHICA).

The use of cancer vaccines has shown that it is possible to elicit immune responses against these antigens. For example, a current USDA-approved DNA-based vaccine for canine melanomas is ONCEPT™, which is a bacterial plasmid DNA vaccine, encoding human tyrosinase[212]. It is currently used in dogs with stage I and II melanomas, to support surgery and/or radiation therapy[212]. Thus, we suspect a reduction in expression of these enzymes in response to a reduction in intracellular ROS may also affect the immunogenicity of melanoma cells. The goal of this present study was to investigate the effects of culturing B16F10 melanoma cells with metformin, which may reveal therapeutic strategies to improve tumours as targets for immunotherapies.

MATERIALS AND METHODS

Mice. 8-10 weeks old (at study initiation) female C57BL/6 (H-2^b) (Charles River Laboratories, Wilmington, MA) were housed in a specific pathogen-free facility at the University of Guelph. All animal experimentation was approved by the University of Guelph's Animal Care Committee, and complied with the Canadian Council on Animal Care guidelines.

Metformin hydrochloride. Purchased from Sigma-Aldrich (St. Louis, MO; Product#: PHR1084-500MG).

Quantifying melanin production. B16F10 cells were seeded in a 96-well plate in phenol red-free media and left to culture for several days, until the media changed to a dark brown colour due to the deposition of melanin. Absorbance readings were taken at 405 nm (a wavelength previously used to measure melanin[147,213]) to quantify any differences in melanin production between cells with and without treatment with metformin.

Tyrosinase activity assay. B16F10 cells cultured with and without metformin were lysed using 1% Triton-X, after washing twice with PBS. A Bradford assay was performed so that protein concentrations could be adjusted by adding more lysis buffer. Lysates were then plated in a 96-well plate, and 10 mM of 3,4-Dihydroxy-L-phenylalanine (L-Dopa) was added (Sigma-Aldrich; St. Louis, MO; D9628). The plate was incubated at 37°C for 15 minutes, and then absorbance readings were taken at 405 nm to detect differences in melanin production.

ROS quantification assay. 2',7'-Dichlorofluorescin (DCF-DA) (Sigma-Aldrich; St. Louis, MO; Cat#: D6883) is a fluorogenic dye that measures ROS. After entering the cell, DCFDA is oxidized by ROS into DCF, which is a highly fluorescent compound. Melanoma cells cultured with or without metformin were washed twice with PBS and then incubated with DCF-DA for 30 minutes in PBS. Then cells were harvested using a rubber scraper, and assessed for fluorescence by flow cytometry (FACS Canto II with FACSDiva 8.0.1 software; BD

Biosciences; San Jose, CA). Data were analyzed with FlowJo version 10.1 software (FlowJo LLC, Ashland, OR).

Western Blotting. Lysates were obtained from B16F10 cells treated with increasing concentrations of metformin for 72 hours before being harvested using a rubber scraper. The cells were washed twice using PBS, and lysed by RIPA buffer containing a cocktail of protease inhibitors. Concentrations of proteins were determined by a Bradford assay. Sample buffer was added to lysates, and boiled for five minutes before running through a 10% SDS-PAGE gel for 1.5 hours at 120 V. Proteins were transferred from the gel to a PVDF membrane (VWR; Radnor, PA; Cat#: CA28148-752) for 1 hour at 100 V. The membrane was placed in blocking buffer for 1 hour on a shaker, then left overnight in primary antibodies (anti-tyrosinase (Abcam; Cambridge, UK; Cat#: AB74073) and anti-TRP-2 (aka. DCT) (Abcam; Cambridge, UK; Cat#: AB79673)), which were diluted 1:1,000 in blocking buffer (5% skim milk powder in PBST (PBS + 0.0005% tween)). The membrane was washed three times in PBST, for 15 minutes each time, then left in horseradish peroxidase-conjugated secondary anti-mouse or anti-rabbit IgG antibodies (Fisher Scientific; Hamptom, NH; G21040 and G21234 respectively), diluted 1:2,000 in blocking buffer. This was followed by three washes with PBST, for 10 minutes each. ECL substrate was added to the membrane and a ChemiDoc XRS (BioRad; Hercules, CA) with Image Lab software (BioRad; Hercules, CA) was used to take an image of the membrane.

In vivo *tumour models.* To establish melanomas, C57BL/6 mice received intradermal injections of $2.5x10^5$ B16F10 cells in 30 µL of phosphate buffered saline (PBS). The cells were maintained in Dulbecco's modified eagle medium (DMEM) (Fisher Scientific; Hampton, NH; Cat #:

SH3002201) with 10% heat-inactivated bovine calf serum with or without 2.5 mM of metformin. The cells were washed twice with PBS prior to injections. Tumour growth was monitored closely over time. Length, width and height measurements were taken to calculate tumour volumes using the following formula: $3.14 \times \frac{(L \; x \; W \; x \; H)}{6}$. Mice were considered to be at endpoint if there was ulceration, or if tumours reached ≥10 mm in two dimensions, or ≥15 mm in one dimension. For T cell depletion experiments, mice were given 500 µg of anti-CD8a antibodies (clone 2.43; BioXCell; West Lebanon, NH; Cat#: BE006) or Rat IgG2b Isotype control (clone LTF2; BioXCell; West Lebanon, NH; Cat#: BE0090) intraperitoneally, 24 hours prior to tumour challenge.

Cell viability assay. Rezasurin is a nonfluorescent reagent that is reduced to highly fluorescent resorufin in metabolically active cells, which correlates with viability[214]. B16F10 cells were seeded in a 96-well plate, with or without metformin added. The plate was incubated at 37°C for 72 hours, before a resazurin sodium salt solution (Sigma-Aldrich; St. Louis, MO; Cat#: R7017) was added to the plate at a final concentration of 0.5mg/mL and fluorescence was measured four hours later (excitation wavelength=535/25 nm, emission wavelength= 590/35 nm).

Cell proliferation assay. B16F10 cells were washed twice with PBS, then resuspended in 1 mL of PBS. Violet proliferation dye (BD Horizon; Cambirdge, UK; Cat #: 562158) was added to the 1 mL of cells in PBS for a final concentration of 1 µM. Cells were incubated at 37°C for 10 minutes. PBS was added to the cells prior to centrifugation at 500xg for 5 minutes to remove the excess dye. Cells were washed again in PBS, resuspended in DMEM supplemented with 10%

BCS, plated into flasks and treated with various concentrations of metformin for 24, 48, 72, 96 or 144 hours before flow cytometric analysis.

Statistical analyses. GraphPad Prism version 7 for Windows (GraphPad software, San Diego, CA) was used for all graphing and statistical analyses. Survival curves were estimated by the Kaplan-Meier method, and differences between groups were investigated using the log-rank test. Means with standard errors are shown in all other graphs. All reported *p* values were two-sided and considered significant at $p \leq 0.05$.

RESULTS

Treatment with metformin reduced melanogenesis. Melanocytes are the pigment-producing cells of the body. Melanomas are cancers of the melanocytes. Hence, B16F10 cells produce a lot of melanin *in vitro,* often turning the media to a very dark brown colour. However, when metformin was added into the culture medium, we observed a reduction in the production of melanin. We formally tested this hypothesis, and found that meformin reduced melanin synthesis in the B16F10 cells in a dose-dependent manner (**Fig. 1**). Absorbance readings showed that melanin production was inversely correlated with the concentration of metformin in the culturing media (**Fig. 1b**).

Treatment with metformin reduced the production of reactive oxygen species. Metformin is known to disrupt complex I of the mitochondrial respiratory chain, which leads to changes in the

production of ROS[154,157]. We investigated whether treatment with metformin changed the quantity of intracellular ROS (**Fig 2**). A DCF-DA assay was used to assess the levels of intracellular ROS. Treatment of B16F10 cells with metformin decreased the production of intracellular ROS. We suspected that the reduction of melanin production could be linked to this reduction of intracellular ROS.

Treatment with metformin reduced the expression and activity of enzymes associated with melanogenesis. In addition to reducing the production of melanin, skin whitening reagents are often also found to reduce expression of melanogenesis-associated enzymes. Therefore, we suspected that metformin may also induce similar effects. Western blots show that treatment with metformin reduced the expression of TRP-2, in a dose-dependant manner (**Fig 3a**). Although expression of tyrosinase (primary enzyme involved in the synthesis of melanin) was found to be unaffected (**Fig 3a**), tyrosinase activity was decreased in cells treated with metformin (**Fig 3b**). These results indicate that metformin treatment reduces expression and activity of melanogenesis-associated enzymes.

Pre-treatment of melanoma cells with metformin resulted in tumours that were less immunogenic. Due to its ability to reduce melanogenesis and the expression of associated enzymes, which are known to be melanoma-associated antigens, we wondered if metformin might influence the immunogenicity of B16F10 cells *in vivo*. Hence, we challenged mice with cells cultured with and without the addition of metformin at a concentration of 2.5 mM (**Fig 4**). Our results showed that cells treated with metformin engrafted in C57BL/6 mice quicker and resulted in shorter overall survival ($p=0.0075$).

Metformin did not reduce viability nor proliferative capacity of B16F10 cells. We wondered if the difference in tumour growth was due to innate differences in cell viability or proliferation. Therefore, we used a resazurin assay to assess cell viability through direct measurement of cell metabolism (**Fig 5**). Increasing concentrations of metformin did not affect cell viability; most notably, the concentration the cells were cultured in for the tumour challenge studies did not affect cell viability (**Fig 5a**). We also assessed cell proliferation using the resazurin assay as well as a violet prolieration dye assay (**Fig 5b&c**), and found that metformin did not increase cell proliferation. Hence, differences observed *in vivo* were unlikely to be due to inherent kinetics of cell growth and proliferation.

DISCUSSION

We examined the effects of metformin on B16F10 cells. Our results demonstrated that the addition of metformin in the culturing medium reduced the production of melanin in B16F10 cells. Since there are lower incidences of skin cancers associated with darker skin, we wondered if increased production of pigment was also associated with increased immunogenicity of the melanocytes.

Previous studies have shown that metformin disrupts complex I of the mitochondrial respiratory chain, which alters the production of ROS[154,157]. High levels of ROS have shown to inhibit tyrosinase; yet on the other hand, lower concentrations of ROS can upregulate and

activate tyrosinase as well as other important enzymes in the melanin synthesis pathway. One study found that increasing H_2O_2 increased tyrosinase activity up until a concentration of $3x10^{-4}$ M, after which, H_2O_2 had an inhibitory effect on tyrosinase[215]. As we did not measure the exact concentrations of ROS, we cannot be sure if the levels in our study were above or below this threshold.

However the effects of ROS on melanogenesis remain elusive. Results from one study showed that the presence of superoxide anion radical increased the oxidation of L-tyrosine to dopachrome 40-fold[216]. However, in the same study, hydrogen peroxide was shown to be a reversible inhibitor of tyrosinase[216]. In the current study, we suspect that the reduction of ROS by metformin is associated with the reduction in melanin production.

Interestingly, treatment of B16F10 cells with metformin led to a decrease in the expression of TRP-2, and also a reduction in TYR activity. A previous study looking at the effects of metformin also assessed expression of tyrosinase, DCT (TRP-2), TRP-1, MITF as well as proteins involved in the AMPK pathway, in melanoma cells[217]. The researchers found that meformin decreased expression of all three melanogenesis-associated enzymes as well as microphthalmia-associated transcription factor (MITF), which regulates the expression of these enzymes[217]. Additionally, this effect was found to be AMPK-independent[217]. Instead, it was demonstrated that metformin decreased the production of cAMP[217]. Intracellular cAMP activates protein kinase A (PKA) which phosphorylates cAMP-responsive element binding protein (CREB), which regulates MITF expression[218,219].

Surprisingly, our results did not suggest that metformin was toxic to the B16F10 cells. A previous study demonstrated a dose-dependent deleterious effect of metformin on human melanoma cell lines, when administered at a dose in the range of 1-10mM, and treated for a total of 72 hours[155]. *In vivo* data from this study suggested that metformin also attenuated the ability of the melanoma cells to develop tumours[155]. Furthermore, other studies have shown anti-proliferative effects of metformin on hepatocellular, colon, glioma, prostate, ovarian, and endometrial cancer cell lines[153,156,220–223]. We also treated B16F10 cells within the dose range of 1-10mM *in vitro*, and our results did not show any toxicity with metformin treatment. Metformin has also been shown to induce B16 melanoma cell cycle arrest and apoptosis *in vitro*, and also reduced growth of melanomas *in vivo*[157]. These anti-melanoma effects were found to be AMPK-independent[157]. Furthermore another study also demonstrated an anti-proliferative effect of metformin in glioma cells, and similarly, this effect was not replicated with a direct AMPK activator, suggesting it was AMPK-independent[224].

One type of melanoma that was found to be resistant to the anti-cancer effects of metformin, was BRAF mutant melanomas[225]. In fact, treatment with metformin accelerated the growth of melanomas with BRAF mutations, a process which was found to be driven by VEGF-A[225]. In the present study, we challenged mice with B16F10 cells that had been cultured with and without the addition of metformin. Our results suggested that metformin-treated cells resulted in earlier tumour engraftment and lower overall survival in C57BL/6 mice. However, analysis of the B16 mutanome did not reveal mutations in BRAF, in contrast to human melanomas, where at least 60% express BRAF mutations[226].

In conclusion, we have shown an anti-melanogenic effect of metformin on melanoma cells, which we found to be a result of reduced expression and activity of melanogenesis-associated enzymes. When injected intradermally into mice, the metformin-treated cells demonstrated earlier engraftment and resulted in reduced survival. We suspect that levels of intracellular ROS are involved in this phenomenon, but their exact role is unclear. Further examination is required to ascertain the full effects of metformin on melanomas.

ACKNOWLEDGEMENTS

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FIGURES

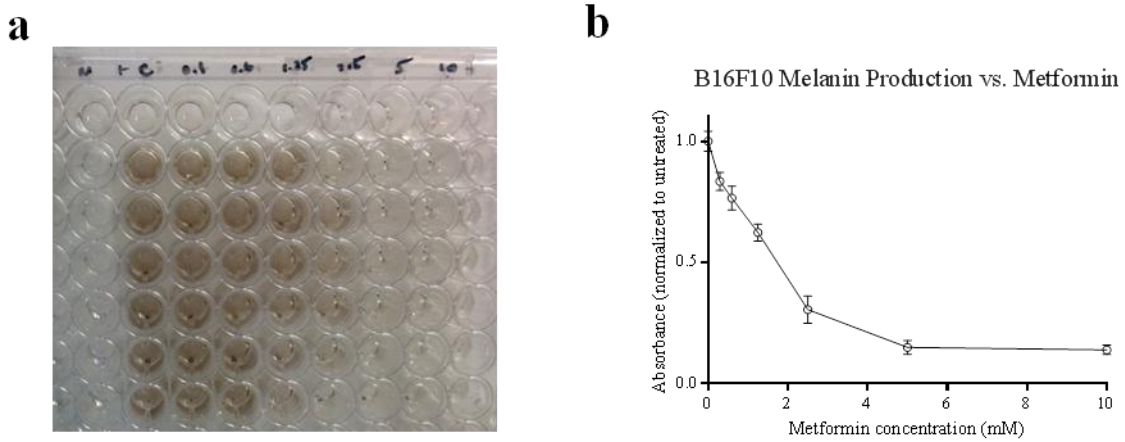

Figure 1: Treatment with metformin reduced melanogenesis. $5x10^3$ B16F10 cells were seeded prior to *in vitro* treatment with Metformin, at a range of concentrations including: 0 mM, 0.3 mM, 0.6 mM, 1.25 mM, 2.5 mM, 5 mM and 10 mM (negative control) for 96 hours. (**a**) Image of plate of B16F10 cells treated with Metformin with the highest concentration on the right, and the negative control on the left. (**b**) Absorbance values were taken to quantify the effect of Metformin treatment on the production of melanin.

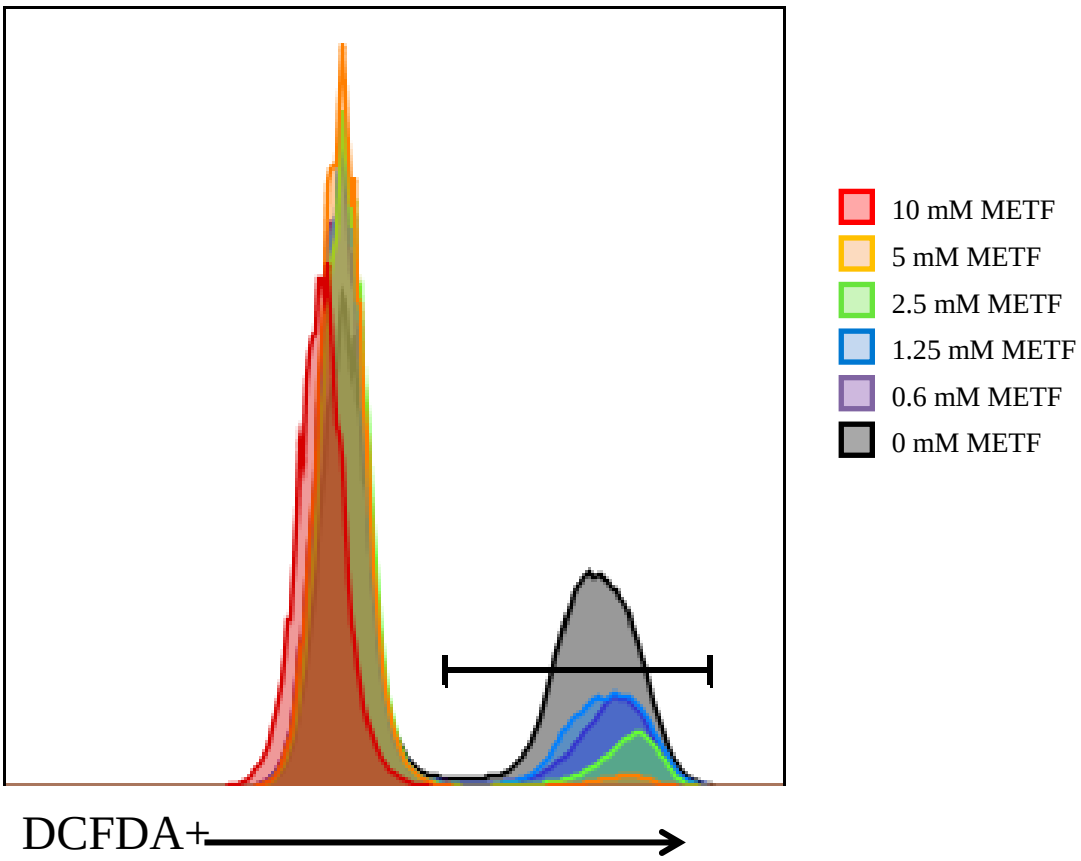

Figure 2: Treatment with metformin reduced the production of reactive oxygen species. Intracellular reactive oxygen species (ROS) was quantified using an DCFDA assay. DCFDA is oxidized to fluorescent DCF upon exposure to intracellular ROS. $2.5x10^5$ B16F10 cells were seeded into 6 well plates prior to the addition of metformin. Cells were washed 48 hours later, and DCFDA was added for 30 minutes at 37°C. Cells were then washed again, and analyzed using the FACs CANTO II for fluorescent DCF.

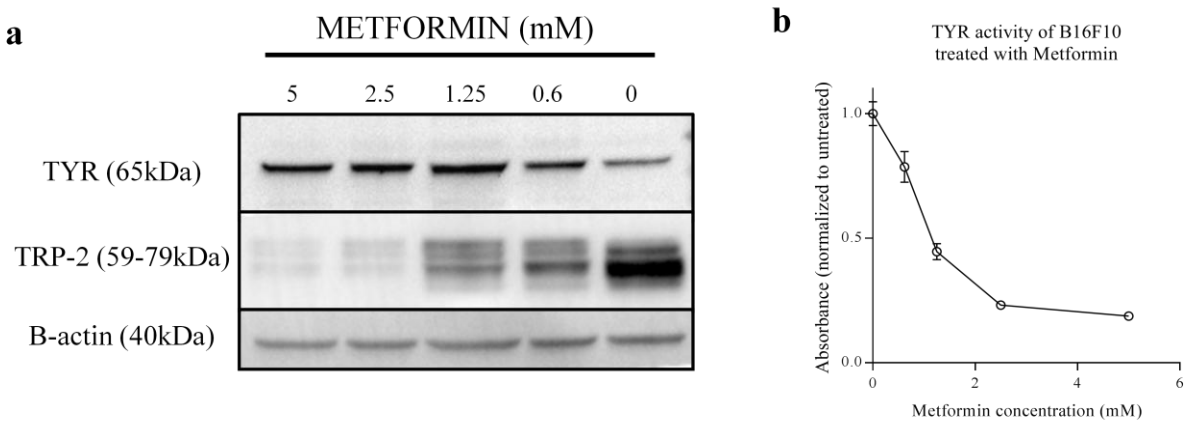

Figure 3: Treatment with meformin reduced the expression and activity of enzymes associated with melanogenesis. B16F10 cells were treated with metformin at a range of concentrations for 96 hours prior to harvesting the lysates. Lysates from the cells were then either processed for Western blot analysis or prepared for a tyrosinase activity assay. (**a**) Western blot of tyrosinase and TRP-2 (DCT) expression in B16F10 cell cultures, with B-actin as a control. (**b**) L-dopa was added to cell lysates for 30 minutes before obtaining absorbance readings on plate reader at a wavelength of 405nm, to assess activity of tyrosinase.

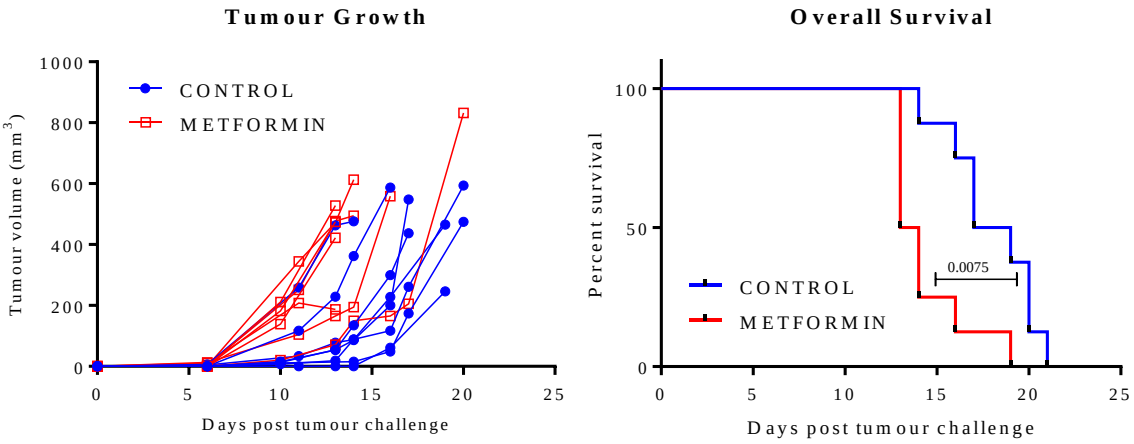

Figure 4: Pre-treatment with metformin resulted in tumours that were less immunogenic. C57BL/6 mice were challenged intradermally with $2.5x10^5$ B16F10 cells cultured with and without the supplementation of Metformin in the culture medium. (**a**) Tumour growth of mice treated with B16F10 cells cultured with and without metformin was monitored regularly. Volumes of tumours were calculated using the following formula: $3.14 \times \frac{(L\ x\ W\ x\ H)}{6}$. Volumes shown on graph were recorded for each individual mouse. (**b**) Survival of mice treated with B16F10 cells cultured with and without metformin was assessed. Mice challenged with B16F10 cells cultured with metformin demonstrated a significant reduction in overall survival. Mice were considered at end point if the tumours reached 10 mm in two dimensions or 15 mm in a single dimension. Kaplein-Meier survival plot shown with statistical significance assessed using the log-rank test (p=0.0075).

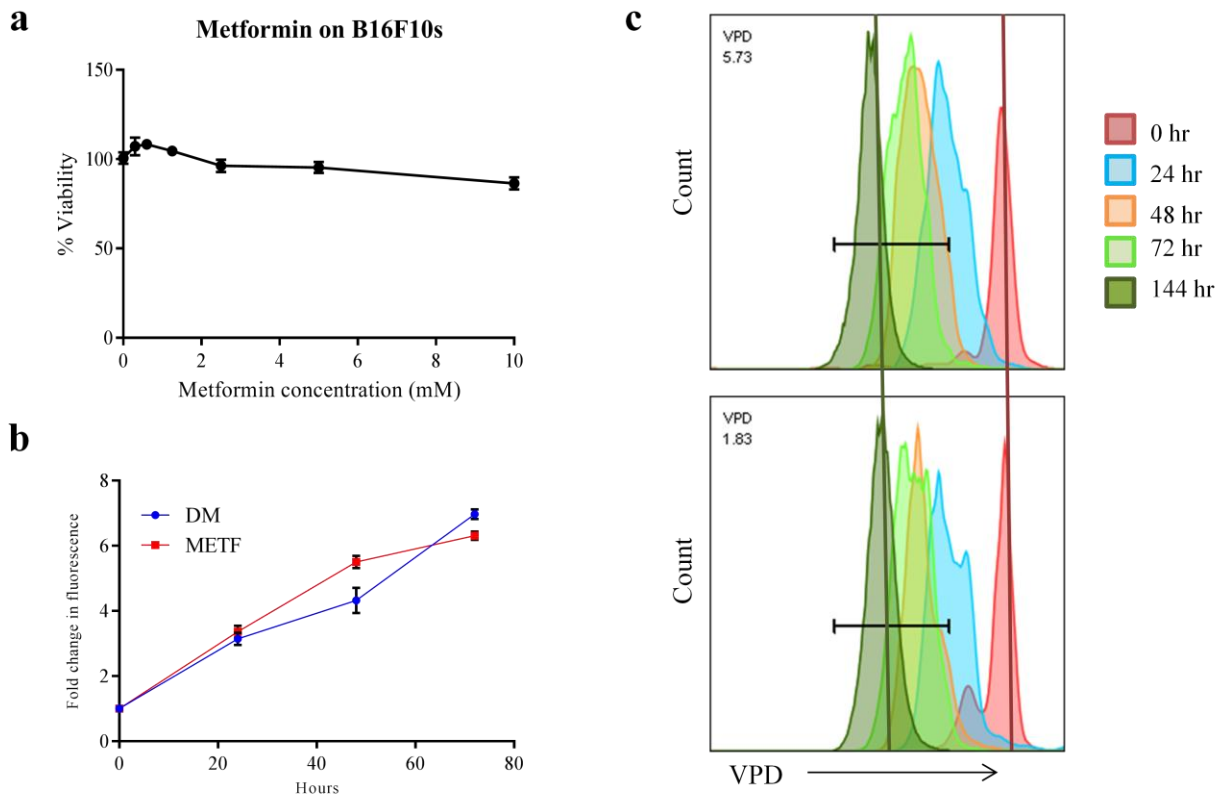

Figure 5: Metformin did not reduce viability nor proliferative capacity of B16F10 cells. (**a**) Rezasurin assay was used to compare the viability of B16F10 cells treated with increasing concentrations of metformin. (**b**) Rezasurin assay was used to compare the viability of B16F10 cells treated with and without metformin (5mM) over time. (**c**) Violet proliferation dye was incorporated into B16F10 cells cultured with (bottom) and without (top) metformin to compare proliferation of the cells over time.

CHAPTER FIVE: GENERAL SUMMARY AND DISCUSSION

The overall objective of this thesis was to investigate the complex interactions between the immune system, tumour microenvironment and oncolytic viruses in the context of a pre-clinical model of melanoma. We first sought to identify mechanisms which allowed oncolytic viruses to replicate in hosts pre-vaccinated against a virus encoded transgene. Next, we investigated how oncolytic viruses modulate the immunological landscape of tumours during acute infection. Lastly, in a somewhat separate study, we investigated the effects of metformin-induced reductions in melanin synthesis on the immunogenicity of melanomas. Ultimately, the goal is to use the knowledge gained from these studies to improve oncolytic virotherapy and immunotherapy regimens to induce more robust and longer lasting anti-tumour immune responses.

Brief summaries of individual research chapters

(1) In our first study (chapter 2), we identified two mechanisms that allow oncolytic viruses to replicate in melanomas of hosts with pre-existing immunity against a virus-encoded antigen. Our results show that an acute virus-induced leukopenia transiently removes transgene-specific TILs from the tumour microenvironment, Secondly, our results also suggest that the highly immunosuppressive microenvironment of the tumour renders the remaining tumour-resident lymphocytes dysfunctional and therefore unable to efficiently kill virus-infected cells. Together, these mechanisms allow a short window of unimpeded viral replication.

(2) Since we discovered, for the first time, that virus-induced lymphopenia extends to the tumour microenvironment, we decided to see if other intratumoural leukocyte subsets might be similarly modulated. Therefore, in our second study (chapter 3) we quantified various populations of leukocytes including: $CD8^+$ and $CD4^+$ T cells, B cells, plasma cells, NK cells, neutrophils, eosinophils, MDSCs and DCs and found that all of these populations acutely decreased in blood following treatment with an oncolytic virus. However, not all of these populations were reduced in melanomas at acute time points post-infection. We demonstrated that at least some of these cells may be temporarily adhering to the endothelial wall of blood vessels and were, therefore, undetectable at these acute time points post-infection.

(3) Lastly, in the third study (chapter 4), we investigated the effects of metformin on B16F10 cells because we observed marked differences in the production of melanin by melanoma cells cultured with and without the addition of metformin. We suspected that this was due to the ability of metformin to alter ROS production in the cells. We hypothesized that this may also lead to a reduction in the immunogenicity of the cells, and therefore produce tumours that were more aggressive and more difficult to treat. We found that melanomas derived from cells pre-treated with metformin engrafted earlier and resulted in poorer overall survival in mice than those cultured without metformin.

Future directions

One barrier to efficient systemic delivery of oncolytic viruses is the immune response elicited by viruses. Antibodies in circulation have the potential to neutralize the virus. While

immunosuppressive treatments could act to increase viral spread and subsequent tumour debulking, it also diminishes the anti-tumour immune response. On the other hand, enhancing the immune response could prematurely terminate the direct debulking effect of the virus. Our present research identifies a unique window of opportunity for tumour debulking prior to eliciting an anti-viral/tumour immune response, due to the complicated interactions between the virus, immune system and tumour. Altering one or more of these components could lead to changes in favour of either effect. Future optimization of oncolytic virotherapy in the context of cancer will require greater understanding and insight into these complex interactions as well as their underlying mechanisms.

Although oncolytic virotherapy holds a lot of promise, there is still room for improvement. As mentioned previously, there exist barriers to efficient viral delivery. In order for viruses to mediate their oncolytic effects, they have to be able to enter tumours. Increased penetration of tumours by viruses would enhance oncolysis, as well as improve *in situ* vaccination effects.

One problem is that tumour vasculature is often leaky[227], and while that has been exploited to enhance delivery of therapeutic macromolecules in the past, the build-up of interstitial fluid pressure prevents efficient diffusion of viruses into tumours[228]. Altering the permeability of tumour-associated blood vessels could enhance the delivery of viruses. One study showed that increasing the leakiness of tumour vascular with VEGF actually promoted oncolytic Sindbis virus delivery[229]. Others have shown that administration of TNFα improved oncolytic adenovirus delivery by inducing endothelial permeability which subsequently increases adenoviral extravasation[228].

Others are exploring the use of various delivery vehicles to promote the access of viruses to tumours. For example, some researchers are investigating the use of cells as carriers for viruses. This method protects the virus from neutralization in circulation, and specific cell types have shown preferential tropism for certain tissues in preclinical studies. Cells that have been tested include: mesenchymal cells[230,231], macrophages[232], tumour cells[233], T cells[234] and dendritic cells[235,236].

As technology used to manufacture the viruses continues to improve, higher viral yields will also be possible. Increased viral delivery could also be achieved by administering a higher dose. However, toxicity of the virus could also be a potential risk. Vaccination strategies such as the prime-boost immunization discussed in chapter 2, could have the potential to increase maximum tolerable doses, which means it would be safer to use higher doses when treating cancers.

Moving beyond the oncolytic effect of viruses, another cutting-edge area of research surrounding oncolytic virotherapy involves overcoming various obstacles to eliciting robust anti-tumour immune responses. One aspect involves improving infiltration of leukocytes into tumours. Previous studies have shown efficacy using viruses engineered to express immunostimulatory cytokines or chemokines, in order to stimulate effective immune responses against tumours. Some examples that have shown some success in pre-clinical studies include: chemokine ligand 5 (CCL-5)[237], interferon-β[238], granulocyte macrophage colony-stimulating factor (GM-CSF)[239,240], IL-12[241], and IL-24[242]. These chemokines and cytokines were expressed by various viruses, and demonstrated success in potentiating the immune response, in the context a variety of cancers.

Not only is increased infiltration important, but it is also critical to improve function and/or quality of the tumour-infiltrating leukocytes. The tumour microenvironment is known to be highly immunosuppressive, and our data supports this notion. As a result it is also important to overcome this barrier of immunosuppression in order to elicit anti-tumour immune responses of greater magnitude and efficacy. In the last two decades, we have seen the rise of antibody-mediated immune checkpoint blockade therapy, demonstrating exciting pre-clinical and clinical trial efficacy in improving outcomes for cancer patients[243]. The anti-CTLA4 inhibitor Ipilimumab, and the anti-PD-1 antibodies Pembrolizumab and Nivolumab have been approved by the FDA for the treatment of melanomas. The success of these immune checkpoint inhibitors demonstrates the need to overcome the immunesuppression exerted by the tumour in order to elicit effective immune responses. It also makes them great candidates for combination therapies with agents, such as oncolytic viruses, that have the capacity to stimulate cancer-specific immune responses. Preclinical studies testing combinations of immune checkpoint blockade and oncolytic viruses have demonstrated potential to reduce tumour size and extend overall survival[243].

Chimeric antigen receptor (CAR)-T cell therapy, is another method to improve the affinity of T cell receptors for tumour antigens, subsequently enhancing the specificity and strength of the immune response. CARs are genetically engineered to combine a TAA-specific domain with T cell activation domains[244]. This therapy involves extracting a patient's T cells, genetically modifying them to express CARs and then expanding them *ex vivo*, before being infused back into the patient. A previous study demonstrated increased overall survival in mice with the combination of adoptively transferred CAR-T cells and an oncolytic adenovirus

expressing RANTES and IL-15, which facilitated the migration and survival of CAR-T cells in tumours[245].

There are still many other ideas that are currently being developed, to enhance tumour-specific immune responses. For example, there is some work being done on depletion of specific regulatory immune subsets, which often dampen the immune response, as well as various immunization strategies that can help to increase the magnitude of responses.

Furthermore, as raised in chapter 4, there is also the possibility of altering the malignant cells themselves in order to improve immunogenicity of the tumour cells, making them better targets for the immune system. In fact, similar to this train of thought, there are therapies currently being explored to induce immunogenic cell death of tumour cells, in order to provide a repertoire of natural danger signals and tumour-derived antigens for eliciting immune responses.

In conclusion, this thesis explored the immunological effects of oncolytic virotherapy in melanoma-bearing mice. The mechanisms identified in this study provided insights to the complex nature of the interactions between oncolytic viruses, the immune system and the tumour microenvironment. Oncolytic virotherapy holds promise as an immunotherapeutic agent against tumours, and moving forward, can be used in combination with other therapeutic modalities to combat cancers. Ongoing advances in the fields of tumour biology, immunology and virology have helped and continue to aid in overcoming many barriers preventing effective cancer treatments. But there is still much more to be done. The future of oncolytic virotherapy holds hope for further improvement to current treatment protocols, and the development of superior combinations that can be translated into veterinary and human medicine.

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