LOCAL IL-15-IMMUNOTHERAPY TO TREAT MELANOMA

by

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ABSTRACT

Intratumoural (i.t.) IL-2 is an effective treatment for cutaneous, metastatic melanoma. However, treating patients with high metastatic burden can be very challenging. Another common γ chain cytokine, IL-15, facilitates the expansion and maintenance of antigen (Ag)-experienced $CD8^+$ T cells and endows naïve $CD8^+$ T cells with cytotoxic capability and surface molecules that enhance their ability to traffic to secondary lymphoid organs (SLOs) for primary immune activation. We asked whether i.t. IL-15 might increase antitumour immunity by increasing Ag-specific $CD8^+$ T cell numbers. To test this, we used the murine bilateral B16F10 melanoma model, high-dimensional single cell immune profiling, and *in vitro* co-culture assays. We show that i.t. IL-15 is equal to if not better than IL-2 at controlling the growth of treated B16F10 lesions; and that IL-15 has notable regulatory effects on distant non-treated tumours; orchestrates an influx of Ag-experienced $CD8^+$ T cells to treated tumours and SLOs; enhances the functional quality/reactivity of systemic $CD8^+$ T cells; while beneficially having limited influence on regulatory T cell numbers.

LIST OF ABBREVIATONS USED

Ag	Antigen
AICD	Activation induced cell death
ANOVA	Analysis of variance
APC	Antigen presenting cell
AQP9	Aquaporin 9
ATP	Adenosine triphosphate
BATF	Basic leucine zipper transcriptional factor ATF-like 3
BCL	B cell lymphoma
Blimp-1	B lymphocyte-induced maturation protein-1
BLT	Bone marrow/liver/thymus
BRAF	B-rapidly accelerated fibrosarcoma
CRT	Calreticulin
CCL	Chemokine (C-C) ligand
CCR	Chemokine receptor
CD	Cluster of differentiation
CDK	Cyclin-dependent kinase
CDKN	Cyclin-dependent kinase inhibitor
CL	Contralateral
CNS	Central nervous system
CSF-1	Colony-stimulating factor 1
CTLA-4	Cytotoxic T-lymphocyte-associated antigen 4
DAMP	Damage-associated molecular pattern

DC	Dendritic cell
Dsg2	Desmoglein 2
Eomes	Eomesodermin
ERK	Extracellular signal-regulated kinase
ESL-1	E-selectin ligand-1
ET-1	Endothelin-1
FAO	Fatty acid oxidation
Foxp3	Forkhead-box protein 3
GM-CSF	Granulocyte-macrophage colony stimulating factor
G-MDSC	Granulocytic myeloid-derived suppressor cell
HD	High-dose
HGF	Hepatocyte growth factor
HLA	Human leukocyte antigen
HMGB1	High mobility group protein B1
HSV-1	Herpes simplex virus type-1
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IL	Interleukin
irAE	Immune-related adverse effect
i.v.	Intravenous
i.t.	Intratumoural
JAK	Janus kinase
K_d	Dissociation constant

MAPK	Mitogen-activated protein kinase
Mc1-1	Myeloid cell leukemia sequence 1
MC1R	Melanocortin 1 receptor
MITF	Microphthalmia-associated transcription factor
MPS	Mononuclear phagocyte system
NK	Natural killer cell
OV	Oncolytic virus
Par-1	Protease activated receptor 1
PBS	Phosphate-buffered saline
PD-1	Programmed cell death protein 1
PDGF	Platelet-derived growth factor
PFS	Progression free survival
PI3K	Phosphoinositide 3-kinase
PSGL-1	P-selectin glycoprotein ligand-1
Ras	Rat sarcoma
RBC	Red blood cell
ROS	Reactive oxygen species
s.c.	Subcutaneous
SF	Scatter factor
SHP2	Src homology 2 domain-containing tyrosine phosphatase 2
SLO	Secondary lymphoid organ
STAT	Signal transducer and activator of transcription
TAM	Tumour-associated macrophage

T_{CM}	Central memory T cell
TCR	T cell receptor
T_{EFF}	Effector T cell
T_{EM}	Effector memory T cell
Tg	Transgenic
TGF	Transforming growth factor
T_H	T helper cell
T_{REG}	Regulatory T cell
TNF	Tumour necrosis factor
T-VEC	Talimogene laherparepvec
UV	Ultraviolet
WT	Wild-type
α-MSH	Alpha-melanocyte stimulating hormone

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CHAPTER 1. INTRODUCTION

1.1. Cancer of melanocytes

The term *cancer* describes a collection of diseases that are often classified based on origin and molecular or phenotypic signatures. Cancer cells are altered self cells that have escaped normal growth-regulating mechanisms. Melanoma is a cancer of melanin-producing melanocytes, cells of neuroectodermal origin that reside throughout the body (e.g. skin, iris, mucosa). The significance of cutaneous melanoma (hereafter, melanoma) in humans is of utmost importance, as it accounts for more than 132 000 new cases each calendar year, with 48 000 deaths (~1200 Canadians) (CCS 2016; WHO 2017). The incidence of melanoma is rising faster than any other solid tumour. Incidence is highest among residents of Australia and New Zealand (~60 cases/100 000 inhabitants) and lowest in pigmented populations from Africa and Asia at one case per 100 000 (Erdmann et *al*. 2013). Men have an unexplained higher incidence and mortality rate compared to women at all tumour-stages (Joosse et *al*. 2013; Siegel et *al*. 2016). Areas of risk for this demographic include white populations with fair-skin, a history of excessive ultraviolet (UV)-radiation exposure, age, and immunosuppression/immune dysregulation from medications or other diseases such as hepatitis or human papilloma virus. Other risk factors may include living near the equator and reduced ozone layer (WHO 2017).

1.2. Establishment of melanoma

Skin homeostasis involves sequential differentiation of keratinocytes to replace tissue turnover, beginning as basal epidermal stem cells and culminating in the upper epidermal layer, fully-differentiated. Dendrites extending from melanocytes populating the

epidermal-dermal junction coordinately associate with keratinocytes (Golan et *al*. 2015). The main function of melanocytes is to produce and distribute melanin to keratinocytes. Melanocytes produce two types of melanin, a UV absorbent form (eumelanin) and a photounstable form (pheomelanin). Eumelanin protects the nuclei of keratinocytes and the underlying tissues by physically shielding and scattering UV-light and by quenching toxic reactive oxygen species (ROS) and metals (Bustamante et *al*. 1993; Zecca et *al*. 2002). Melanin production is largely dictated by undifferentiated keratinocytes. Disruption in adhesion between these cells by downregulation of cadherins (e.g. E-cadherin) (Table 1), along with other UV-independent oncogenic events play major roles in malignant transformation of melanocytes. However, sun (UV) exposure remains the main etiological factor for melanoma. Stromal elements such as fibroblasts and vascular endothelial cells together with a myriad of immune cells support melanomagenesis and aid in its progression (see section 1.4.1.).

1.2.1. Molecular drivers of melanomagenesis

Discovery of driver mutations in melanomagenesis has proven difficult due to an abundance of passenger mutations caused largely by UV-radiation, although age and chemotherapeutics are also contributors (Alexandrov et *al*. 2013). Driver mutations endow tumour cells with a fitness advantage, passenger mutations do not. The base mutation rate of melanomas is greater than all other solid tumours (Pleasance et *al*. 2010). Forecasts by Lawrence et *al*. (2014) predict that over 5000 tumour samples with 12.9 mutations per megabase are required to catalogue putative cancer genes mutated in ≥2%

of patients. Despite these challenges, major advances in understanding the genetic and environmental influences of melanomagenesis have been made.

The Rat sarcoma (Ras)-mitogen-activated protein kinase (MAPK) pathway relays external growth-promoting signals to the nucleus by transmembrane receptor tyrosine kinases (Figure 1) (Lito et *al*. 2013). Ras proteins are guanosine nucleotide-bound GTPases that function as a signalling hub within cells. Over 20% of melanomas harbour mutations in Ras genes (*HRAS*, *NRAS*, and *KRAS*) (Shi et *al*. 2014; Wagle et *al*. 2014). Activated Ras causes membrane recruitment and activation of Raf (ARAF, BRAF, and/or CRAF), the first MAPK kinase-kinase of the pathway. The B-rapidly accelerated fibrosarcoma (BRAF) gene is mutated in ~50% of melanomas, with alterations mainly occurring at residue V600E (Shi et *al*. 2014). Raf phosphorylates and activates the MAPK kinase MEK1 and/or MEK2, which initiates the extracellular signal-regulated kinase (ERK) cascade, ultimately allowing cells to carry out normal cellular processes such as proliferation and differentiation (Figure 1). Aberrations affecting MEKs are also crucial in driving melanomagenesis (Rizos et *al*. 2014). The discovery of abnormal Ras-MAPK pathway signalling in melanomas was instrumental in kick-starting a targeted drug discovery effort that continues today (see section 1.3.1. for targeted therapies). It also provided compelling evidence that melanoma is not exclusively UV-driven, as most mutations in this pathway are not attributable to direct UV-damage (Hodis et *al*. 2012).

Germline alterations in melanocortin 1 receptor gene (*MC1R*) underpin the fair-skin and red-hair phenotype. MC1R is a G_s protein-coupled receptor with seven transmembrane

domains on melanocytes that signals through the cyclic AMP pathway, upregulating microphthalmia-associated transcription factor (MITF) in response to alpha-melanocyte stimulating hormone (α-MSH), a product of UV-exposure (Chakraborty et *al*. 1995; Raimondi et *al*. 2008). MITF is the master regulator of the melanocyte pigmentation-program. Select variants in *MC1R* (e.g. E318K) promote the production of pheomelanin, the photolabile, pro-oxidant pigment overrepresented in white populations (Panzella et *al*. 2014; Yokoyama et *al*. 2011). *MC1R* mutations are linked to increased melanoma risk independent of UV-radiation (Mitra et *al*. 2012). Amplification of MITF is observed in 4-21% of melanomas (Garraway et *al*. 2005; Hodis et *al*. 2012). Mutations in the cell-cycle control genes, cyclin-dependent kinase inhibitor 2A (*CDKN2A*) and cyclin-dependent kinase 4 (*CDK4*) are also common in melanoma-prone families and can cooperate with MAPK defects in the genesis of melanoma (Shain et *al*. 2015; Zuo et *al*. 1996).

UV-damage accounts for over 46% of melanoma-driving genetic changes (Hodis et *al*. 2012). Most detrimental is UVB-radiation, which causes cytosine to thymidine transitions at the 3′ base of pyrimidine, comprising ~70% of nucleotide substitutions in melanomas. Among the attested driver mutations, the tumour-suppressor gene, *TRP53*, possesses the highest number of these transitions, although also prevalent in *p16INK4a*, *p14ARF*, and *PTEN* genes (Hodis et *al*. 2012). UVA-light indirectly causes another major mutational signature marked by guanine to thymidine transversions mediated by ROS generated by endogenous photosensitisers (e.g. pheomelanin) (Figure 2). A comprehensive list of known UVA-induced driver mutations is published in the landmark

study by Hodis et *al*. (2012). Mutagenesis by way of infrared radiation and visible light has also become well appreciated in recent years, as both induce ROS production (Figure 2).

1.3. Approved strategies for treatment of melanoma

Given the nature of melanoma as an aggressive metastatic cancer, early treatment measures of localised early-staged disease understandably include surgery. Relatedly, complete lymphadenectomy of melanoma-positive sentinel lymph nodes remains general practice, despite high false positive rates and evidence that it may not improve survival (Morton et *al*. 2014). However, surgery is generally not curative for patients with metastatic melanoma, only palliative. In cases of bulk localised disease, surgery is often combined with isolated limb perfusions or infusions of chemotherapy or immunotherapy, which is technically challenging, invasive, and toxic (Deroose et *al*. 2015). Standard treatment options for patients with melanoma prior to 2011 was limited to systemic administration of Hydroxyurea, Dacarbazine, or interleukin (IL)-2. The two alkylating agents yield objective response rates of 20% and 5-6%, respectively, while IL-2 achieves rates of 16-17% (Atkins et *al*. 2000; Cassileth and Hyman 1967; Chapman et *al*. 2011; Hauschild et *al*. 2012). IL-2 was the staple for melanoma treatment after its approval in 1998, as it achieved complete response rates of 6-7% with 60-80% of these responders remaining disease free for 10-15 years (Atkins et *al*. 1999; Rosenberg et *al*. 1994). However, since 2011, eight new field-revolutionising therapeutic agents were approved by Health Canada, shifting the metastatic melanoma landscape from palliative management to disease control for most patients (see sections 1.3.1., 1.3.2., and 1.5.)

1.3.1. Small-molecule MAPK inhibitors

The discovery that nearly half of all melanomas harbour *BRAF* mutations fueled the development of *BRAF* inhibitors (Davis et *al*. 2002). The first-in-class agent receiving approval in 2011 was Vemurafenib, which targets melanomas harbouring active somatic $BRAF^{V600E}$-mutations. Approved shortly thereafter was Dabrafenib (2013), another *BRAF* inhibitor for treatment of melanomas with $BRAF^{V600}$-mutations in amino acid residues V600E, V600D, and V600K. The overall objective response rates of both tablets in $BRAF^{V600}$-mutant populations (per RECIST criteria) hover around 50%, with progression-free survival (PFS) at 5.3 and 5.1 months, respectively (McArthur et *al*. 2014; Hauschild et *al*. 2012). MEK inhibition is highly effective for treatment of patients with non-V600-mutant or wild-type (WT) *BRAF* melanomas. Approved in 2013, Trametinib is the only MEK1 and MEK2 inhibitor available for monotherapy. In the phase III METRIC trial by Flaherty et *al*. (2012), 22% of patients with these forms of melanomas achieved objective responses after treatment with oral Trametinib. The other MEK inhibitor, Cobimetinib, was approved in 2015 for combination therapy with Vemurafenib. This combo yields objective response rates of 87% and a median PFS of 13.7 months (Ribas et *al*. 2014). Despite mounting evidence on the short-term efficacy of small-molecule inhibitors, particularly when used in combination, nearly all patients relapse within 9 months after treatment cessation due to acquired resistance (Carlino et *al*. 2016; Flaherty et *al*. 2012; personal communication with Carman Giacomantonio, Dalhousie University). Considering this success, there are a multitude of ongoing trials

testing the combination of MAPK inhibitors with immunotherapies in an effort to increase complete response rates (e.g. NCT02263508 and NCT01940809).

1.3.2. Oncolytic virotherapy

Oncolytic viruses (OVs) selectively infect and lyse cancer cells without harming normal cells. OVs have specific cellular tropisms that govern which cells are preferentially infected (Coffey et *al*. 1998; Wang et *al*. 2006). The genetically modified herpes simplex virus type-1 (HSV-1), Talimogene laherparepvec (T-VEC), has deletions in two nonessential viral genes: *ICP34.5* and *ICP47*. Removal of the herpes virus neurovirulence factor gene, *ICP34.5*, attenuates neuropathogenicity and improves tumour-selective replication (Chou et *al*. 1990). Deletion of *ICP47* prevents its interference with peptide processing and presentation on HLA-molecules, which otherwise helps WT HSV-1 evade immune-detection. Addition of the human granulocyte-macrophage colony stimulating factor (GM-CSF) gene (two copies) also aids in the recruitment and maturation of dendritic cells (DCs) and macrophages (Liu et *al*. 2003). The lytic life cycle of T-VEC, along with its ability to stimulate antitumour immune responses led to its approval for the treatment of unresectable advanced-stage melanoma in 2015. Despite approval, the efficacy of T-VEC has been called into question. In the phase III study by Andtbacka et *al*. (2015), intralesional T-VEC significantly increased response rates (16.3%) compared with subcutaneous (s.c.) GM-CSF treated controls (2.1%), but failed to achieve statistically significant differences in overall survival (23.3 versus 18.9 months). The pitfalls of T-VEC may be attributed to induction of antiviral immune responses, which dampen antitumour responses. However,

given the low toxicity profile of T-VEC, T-VEC could be combined with immunotherapies. Indeed, preliminary reports have described acceptable adverse effects rates (19%) in patients with late-stage melanoma treated with T-VEC and Pembrolizumab (programmed cell death protein 1 (PD)-1 inhibitor) (Long et *al*. 2016). The efficacy rates are not yet available for this study.

1.4. $CD8^+$ T cell responses to melanoma within the human host

While tumour heterogeneity presents the field of oncogenomics with challenges, it is highly beneficial in the context of tumour immunotherapy (Lawrence et *al*. 2014). High mutational and neoantigen load significantly correlate with clinical benefit from T cell-based cancer immunotherapies, providing evidence for T cell recognition of melanoma-associated immunogens (Van Allen et *al*. 2015). However, the effectiveness of T cell responses against tumours is dependent on initial innate responses.

1.4.1. Initiation and regulation of $CD8^+$ T cell responses

Early responses by the human host to melanomas is marked by increased recruitment of antigen (Ag)-presenting cells (APCs) such as macrophages, neutrophils, and DCs to tumours by cytokines and nucleotides. UV-injury altering adhesion molecule and cytokine expression of skin-resident cells like keratinocytes may initiate initial immune infiltration (Ansel et *al*. 1990; Pastore et *al*. 2006). Macrophages traffic to tumours in response to cytokine colony-stimulating factor 1 (CSF-1), chemokine (C-C motif) ligand (CCL) 2 and CCL5 (Azenshtein et *al*. 2002; Harlin et *al*. 2009; Lin et *al*. 2001). Upon entering tumours, tumour-associated macrophages (TAMs) are typically tumouricidal

(M1), particularly if influenced by interferon (IFN)-γ or tumour necrosis factor (TNF)-α (Dalton et *al*. 1993; Noy and Pollard 2014; Yarilina et *al*. 2008). M1 TAMs can recognise and uptake damage-associated molecular patterns (DAMPs) from stressed, injured, or dying cells for Ag presentation to naïve T cells. Among the most immunogenic DAMPs are calreticulin (CRT), uric acid, adenosine triphosphate (ATP), and high mobility group protein B1 (HMGB1), which are often induced by UV-exposure with or without cell death (Garg et *al*. 2012; Scaffidi et *al*. 2002). However, TAMs are readily educated to become pro-tumourigenic (M2) after exposure to high concentrations of IL-4, IL-10, IL-13, glucocorticoid hormones, or transforming growth factor (TGF)-β, for example, from cells such as cancer-associated fibroblasts embedded within established immune-escaped melanomas (Ding et *al*. 1993; Gocheva et *al*. 2010; Kalluri 2016). M2 TAMs highly express PD-1 and secrete TGF-β that can negate antitumour T cell responses (Chen et *al*. 2003; Gordon et *al*. 2017). In addition to increasing angiogenesis, $TIE2^+$ M2 TAMs facilitate tumour cell entry around blood vessels and extravasation into circulation (De Palma et *al*. 2005). Moreover, CCL17 and CCL22 secretion by M2 TAMs recruits immunosuppressive regulatory T cells (T_{REG}) that further promote melanomagenesis (Curiel et *al*. 2004; Fujimura et *al*. 2015; Joshi et *al*. 2015). Overall, TAMs correlate with poor clinical outcomes in patients with melanomas (Torisu et *al*. 2000).

Likewise, neutrophil (e.g. $CD66b^+$ cells) infiltration of melanomas is also a predictor of poor prognosis (Jensen et *al*. 2012). This is due in part to the recent revelation that highly suppressive granulocytic myeloid-derived suppressor cells (G-MDSCs) are likely

immature neutrophils (Sagiv et *al*. 2015). In any case, after migrating toward chemokine gradients of CXCL5, CXCL6, and CXCL8, mature neutrophils (like TAMs) display plasticity and are easily polarised from Ag-presenting N1 neutrophils toward the protumoural (N2) phenotype (Dawes et *al*. 2011; Jovic et *al*. 2016; Seidal et *al*. 2007). TGF-β-induced N2 neutrophils secrete high amounts of gelatinase B (MMP9), a matrix metalloproteinase that plays a crucial role in tumour angiogenesis and metastasis (Deryugina et *al*. 2014; Fridlender et *al*. 2009). N2 neutrophils also support tumour growth through secretion of ROS and reactive nitrogen species, which promotes T cell apoptosis (Cemerski et *al*. 2002; Brito et *al*. 1999). Moreover, N2 neutrophil release of arginase 1 depletes tumour microenvironments of critical T cell nutrients such as L-arginine, required for anabolic functions, that further hinder T cell responses against melanomas (Rotondo et *al*. 2009; Rodriguez et *al*. 2004). Scientists also debate whether neutrophils produce IL-10 in response to tumours (Davey et *al*. 2011). IL-10 may slow down migration of DCs and Langerhans cells to secondary lymphoid organs (SLOs) to initiate primary T cell responses (Wang et *al*. 1999; Zhang et *al*. 2009). DCs expressing DNGR-1 (CLEC9a) and XCR1 are particularly effective at cross-presenting exogenous Ags to $CD8^+$ T cells, an important measure in priming the adaptive arm (Poulin et *al*. 2012).

By suppressing innate immune cell function, tumours are effectively regulating T cell activation (signals 1-3, see next section). Improperly activated T cells adopt dysfunctional states marked by loss of proliferative capacity, cytotoxicity, cytokine production, and maintenance of inhibitory receptors (reviewed in Wherry and Kurachi

2015). Melanomas can also directly render $CD8^+$ T cells hyporesponsive with multiple inhibitory receptors by secretion of indoleamine 2,3-dioxygenase (IDO) (Holmgaard et *al*. 2015; Mellor et *al*. 2002). Moreover, melanomas are typically highly acidic with toxic fatty acids, a harsh environment that can further promote the deterioration of T cell function (Böhme et *al*. 2016).

1.4.2. $CD8^+$ T cell primary responses

APCs containing melanoma-associated Ags migrate to SLOs to present Ag bound to human leukocyte Ag (HLA) molecules (signal 1), and provide co-stimulation (signal 2) and cytokines (signal 3) to complementary T cells to initiate primary responses. Co-stimulation fine-tunes T cell receptor (TCR) signalling through positive signals from molecules such as CD28, CD278, CD40, and CD27 (Coyle et *al*. 2000; Munroe and Bishop 2007; Song et *al*. 2012; Yokosuka et *al*. 2008). Co-stimulatory signalling augments cytokine and growth factor production, and expression of anti-apoptosis proteins such as B cell lymphoma (BCL)-2 and BCL-XL. However, these positive-signals are counter-balanced by negative-signals from inhibitory-receptors such as cytotoxic T-lymphocyte-associated Ag 4 (CTLA-4), and PD-1, designed to protect hosts from autoimmunity (Nishimura et *al*. 1999; Takahashi et *al*. 2000; discussed further in section 1.5.). Similarly, pro-inflammatory cytokines such as IL-12/-21, and type I/II IFNs influence the functionality and fate of T cells, even at low concentrations (10^{-10} to 10^{-15} M) (Badovinac et *al*. 2000; Curtsinger et *al*. 1999; 2005). However, it is the collective input of all three signals that dictate the magnitude and quality of $CD8^+$ T cell responses to melanoma.

Appropriate activation of Ag-specific $CD8^+$ T cells leads to their clonal expansion and differentiation into a functionally heterogeneous pool of short-lived effector T cells (T_{EFF}) and memory precursor cells – collectively regarded as Ag-experienced cells – identified by high-expression of the hyaluronan receptor CD44 (Joshi et *al*. 2007; Kaech and Ahmed 2001; Peske et *al*. 2015; Xin et *al*. 2016). Several models have been proposed to explain $CD8^+$ T cell differentiation into effector and memory populations but the transition remains controversial (discussed in Buchholz et *al*. 2016). Regardless, the differential program of T cells is clearly dictated by numerous signals including successive encounter with Ag-bearing APCs and cytokines/growth factors (signal 3) (Curtsinger et *al*. 2003; Joshi et *al*. 2007; Masopust et *al*. 2006; Schluns et *al*. 2002). T cells span this continuum until reaching the terminally-differentiated T_{EFF} stage. T_{EFF} cells express low surface levels of the lymph node-homing molecules CD62L (L-selectin) and CCR7, and contain high amounts of the transcription repressors T-bet, Eomesodermin (Eomes), and B lymphocyte-induced maturation protein-1 (Blimp-1); where T-bet and Eomes drive the production of cytotoxic granules and cytokines, and Blimp-1 represses the transcriptional program of immunological memory (e.g. *Id3*) (Intlekofer et *al*. 2008; Rutishauser et *al*. 2009; Yang et *al*. 2011). Among the purported memory T cells, effector memory (T_{EM}) and central memory (T_{CM}) subtypes are the best characterised. T_{EM} cells are defined based on high migration potential ($CD62L^{lo}$, $CCR7^{lo}$), high effector molecule production, and low proliferative capacity. In contrast, T_{CM} cells readily traffic to SLOs ($CD62L^{hi}$, $CCR7^{hi}$), are less cytotoxic, and have high proliferative potential (Table 2) (Masopust et *al*. 2001; Sallusto et *al*. 1999). Both

memory subsets also extracellularly express IL-7R α-chains ($CD127^{hi}$), necessary for acquiring important IL-7-survival-signals (Kaech et *al*. 2003; Schluns et *al*. 2000).

Initial trafficking of Ag-specific T cells to melanomas is largely guided by CCR10, which binds to CCL27, a product of keratinocytes after exposure to UVB-light (Homey et *al*. 2000; Reiss et *al*. 2001). CCR4 and CCR8 also participate in T cell homing to melanomas, particularly the dermis (Harlin et *al*. 2009; Reiss et *al*. 2001; Schaerli et *al*. 2004). Melanoma localisation is mediated by a series of steps that include tethering and rolling along vessel walls, activation and arrest, and migration and diapedesis (Schön et *al*. 2003). Core 2 O-glycans such as P-selectin glycoprotein ligand-1 (PSGL-1), and E-selectin ligand-1 (ESL-1) are of prime importance for initial transient adhesive interactions with selectins (Nolz and Harty 2014; Schön et *al*. 2003). Ag-experienced T cells strongly express core 2 O-glycans, allowing for enhanced localisation to inflamed tissues (e.g. many tumours) compared to naïve T cells. TCR-ligation induces PSGL-1 and ESL-1 expression on T_{EFF} cells, whereas IL-15 promotes such expression on memory T cells by driving *Gcnt1* expression (Nolz and Harty 2014). Upon melanoma infiltration, Ag-experienced $CD8^+$ T cells lyse tumour cells by way of two main mechanisms: the Fas/Fas-ligand apoptotic pathway and perforin and granzyme (Kägi et *al*. 1994). Perforin forms transmembrane channels in tumour cells, allowing granzyme to pass-through and induce apoptosis (Heusel et *al*. 1994). T cells can also secrete IFN-γ involved in HLA upregulation and Ag presentation by tumours which may potentiate T cell responses, as transformed melanocytes are known to express low levels of co-stimulatory signals (Freedman et *al*. 2000; Kalbasi et *al*. 2010). If tumours are cleared, most T_{EFF} cells

succumb to apoptosis while memory T cells live to provide rapid tumour immunity for the lifetime of hosts.

1.5. Immunotherapy of melanoma

Even though the first cancer immunotherapy was delivered more than a century ago, by Dr. William Coley (Coley's Toxins), research surprisingly remained dormant until the recent clinical success of anti-CTLA-4 blockade (Coley 1984). This perhaps reflects the competing financial incentives that were associated with advocating the radiation and chemotherapy movement. Even Coley's mentor, James Ewing, refuted Coley's results while receiving large financial gifts from supporters of radiation (McCarthy 2006).

CTLA-4 is expressed by activated T cells and constitutively by T_{REG} cells (Takahashi et *al*. 2000). Its cognate ligands CD80 and CD86 on APCs are shared with CD28, though CTLA-4 has a higher binding-affinity which often limits CD28 of ligand-engagement (Krummel and Allison 1995). Moreover, CTLA-4 can trans-endocytose these stimulatory ligands, further prohibiting their interaction with CD28 (Qureschi et *al*. 2011). Upon stimulation, CTLA-4 may also send inhibitory signals to the nucleus that primarily counteract kinase signals induced by TCRs, although the mechanism remains unclear (Walker and Sansom 2015). CTLA-4 ligations can also signal APCs to release immunosuppressive IDO (Fallarino et *al*. 2003). Inhibiting CTLA-4 by antibody results in enhanced activation of T cells by depleting CTLA-4-mediated immunoinhibitory activity by T_{REG} cells (Peggs et *al*. 2009). Clinically, intravenous (i.v.) delivery of low-dose anti-CTLA-4 (Ipilimumab) has achieved objective response rates of 11-15%, a

median PFS of ~3 months, and remarkable survival benefit (Hodi et *al*. 2010; Robert et *al*. 2011). In a retrospective study by Schadendorf and colleagues (2015), 22% of 1861 patients remained alive at 3 years post treatment. However, 80-95% of patients endure immune-related adverse events (irAE) – which resemble autoimmune disease – with over a third of these patients experiencing grade 3-4 irAEs (Weber et *al*. 2009). The grade refers to the severity of irAEs, with grade one being mild and five being death. There is currently no consensus on how to accurately standardise and report iAEs in clinical trials; most guidelines are a modified version of the NCI 2009 guidelines and tailored to the specific immunotherapy. Overall, anti-CTLA-4-therapy is an effective treatment for melanoma, though new strategies must be devised to reduce toxicities (e.g. regimen change).

After Ipilimumab achieved medical promise, considerable scientific effort went into understanding the biology underlying other regulatory pathways (immune checkpoints). One highly evaluated pathway is that of PD-1. The PD-1 immunohibitory receptor is expressed by activated T cells, B cells and several myeloid cells (Chemnitz et *al*. 2004; Petrovas et *al*. 2006). PD-1 has two known ligands, PD-L1 and PD-L2, and both are surface expressed – particularly after exposure to interferons – on a range of immune cells (e.g. T and B cells) and nonimmune cells including those of the heart, lungs, and skin (Kim et *al*. 2005; Ritprajak et *al*. 2010; Rodig et *al*. 2003). Widespread expression may serve as a key measure in controlling peripheral T cell tolerance (Keir et *al*. 2006). Upon engagement, PD-1 molecules transiently associate with Src homology 2 domain-containing tyrosine phosphatase 2 (SHP2) phosphatases to primarily dephosphorylate

CD28, but also TCR-signalling molecules to inhibit proper T cell activation (Hui et *al*. 2017; Yokosuka et *al*. 2012). Importantly, melanomas have co-evolved with their hosts to exploit the PD-1-inhibitory pathway, by expressing both PD-1 and PD-1 ligands, for evasion of T cell attack and to enhance their own proliferation (Kleffel et *al*. 2015). For treatment of advanced-stage melanomas, the approved anti-PD-1 antibody, Nivolumab, achieves objective response rates of 31-40% and 1-year and 2-year survival rates of 62-72.9% and 43%, respectively (Robert et *al*. 2015; Topalian et *al*. 2014). The other approved PD-1 antibody, Pembrolizumab, shows objective response rates of 26-51% (Hamid et *al*. 2013). Despite these excellent results, over three quarters of patients treated with PD-1-blockade experience serious irAEs with 11.7-22% suffering from grade 3-4 toxicities of the gastrointestinal tract and. Expectedly, combination therapy with Ipilimumab and Nivolumab improves objective response rates (61%) and survival rates – 73% at 1 year and 64% at 2 years – compared to Nivolumab monotherapy, but at the cost of higher irAEs (Hodi et *al*. 2016). Indeed, treatment-related grade 3-4 irAEs are observed in 54% of patients receiving this combination therapy. Another shortcoming of PD-1-therapy, like targeted therapies, is that melanomas can develop resistance. For example, melanomas can accrue loss-of-function mutations in *JAK1*/*JAK2* that downregulate PD-L1 expression and IFN signalling, shifting their immune-evasion tactics from inhibiting T cell function to preventing T cell encounter by reducing IFN-mediated Ag presentation (Böhm et *al*. 1998; Zaretsky et *al*. 2016).

1.6. Systemic versus local delivery of therapeutics

Historically, anticancer therapeutics have been delivered systemically, often causing off-target, dose-limiting adverse effects. Another major problem with most systemically delivered systems is ensuring medicine localisation within solid tumours. Agents with a mass of less than 60 kDa are particularly susceptible to renal clearance and excretion (Schenk et *al*. 2008). Medicines can also be degraded by low pH or oxygenation upon entry into tumour microenvironments or prevented from entry by other physiological factors such as high interstitial fluid pressure (Böhme et *al*. 2016; Curti et *al*. 1993). If able to penetrate tumour microspace, therapeutics must frequently best other barriers such as physical obstruction from fibrous tumour stroma, or cell membrane barriers/intracellular machinery if medicines exert their therapeutic effects on nuclear or cytoplasmic targets (Peetla et *al*. 2010; Zhitomirsky and Assaraf 2014-). New bioconjugates that combine medicines (e.g. small molecule chemicals), carrier molecules (e.g. polymers, lipids, peptides, proteins), and chemical linkers, obviate many of these problems by improving disease specific targeting, medicinal half-life, and toxicity profiles; while decreasing agent clearance and systemic exposure (Li 2017). However, the route of therapeutic administration remains an important factor in determining the effectiveness of most therapeutics.

Medicines in tablet form are typically preferred by patients, due to their ready-to-use and non-invasive nature, with pre-determined and measured doses and defined dosing times that reduce hospitalisation (Bandari et *al*. 2014). However, oral delivery of cancer therapy (i.e. protein/peptides) has lagged other administration routes for numerous reasons. First, the gastrointestinal (GI) tract is highly acidic and hosts high proteolytic

activity, which readily compromises the integrity of therapeutics. Second, the low permeability and high expression and activity of ATP-binding cassette drug (ABC) transporters and solute carrier (SLC) transporters in intestinal membranes hinders agent transport into circulation and contributes to high inter-individual variability in pharmacokinetics (Oostendorp et *al*. 2009). Although bioconjugation improves the bioavailability of tablets, this mode of delivery is challenging for many treatments that depend on agent localisation into tumour microenvironments, as medicines must first pass through the GI tract and circulation. Moreover, oral delivery of anticancer therapeutics may detrimentally increase the responsibility of uninformed patients and pharmacists for management of complex dosing regimens and monitoring of toxicities (Abbott et *al*. 2014).

I.v. delivery of anticancer agents is arguably the most common delivery route, as agents are less likely to interact with enzymes (e.g. lysozyme), chemicals (e.g. NaCl), and bacteria occupying mucosal surfaces. Moreover, i.v. delivered therapeutics are now often constructed to avoid blood protein opsonisation, which can lead to agent clearance by cells of the mononuclear phagocyte system (MPS) such as activated monocytes/macrophages and DCs. These constructs are typically neutrally charged, hydrophilic, and similar in hydrodynamic size to serum proteins to minimise electrostatic contact points and collision kinetics (Smith and Nie 2008).

Intraperitoneal (i.p.) delivery of therapeutics can be a means for either systemic or local disease targeting. The distribution patterns of i.p. administered agents is dependent on

charge, dimension, and mass. Anionic agents with low molecular weight (less than 20 kDa) can efficiently cross the peritoneal membrane and the lymphatic duct openings (stomata) on the diaphragm (Flessner et *al*. 1985; Tsai et *al*. 2007). In contrast, these orifices (of micrometer diameters in mice) serve as bottlenecks for cationic medicines of large dimension, which increases agent retention within the abdomen (Dadashzadeh et *al*. 2010; Tsai et *al*. 2007). However, this mode of delivery is typically only clinically efficacious for therapy of peritoneal metastases, or gastrointestinal or other gastric cancers of microscopic size (Feldman et *al*. 2003; Markman et *al*. 1991). Moreover, i.p. delivery of anticancer agents has clear pharmacodynamic and pharmacokinetic disadvantages for therapy of other cancers (non-peritoneal) compared to other routes of administration such as i.v. or intratumoural (i.t.). Agents administered into the peritoneal are likely exposed to more biofluids (e.g. peritoneal fluid, blood, plasma, serum) of different compositions compared to i.v. or i.t., making medicine construction a complex task if the goal is systemic dispersal. Other potential issues involving i.p. administration include agent uptake by peritoneal phagocytic cells, medicine aggregation, catheter obstruction, chemical peritonitis, infection, and ascitic fluid buildup (Dakwar et *al*. 2014; Ikehara et *al*. 2006; Lesnock et *al*. 2010).

I.t. administered therapy offers a myriad of benefits and challenges compared to systemic therapy. First, local delivery allows for increased therapeutic indices, as agents can be administered at high concentrations without heightened systemic toxicities. Because local delivery ensures high medicine localisation, local therapies typically require less agent overall compared to systemic therapies, making treatment less costly. By using

bioconjugates, medicines are even more constrained to the confines of tumours and less likely to escape past leaky tumour vasculature into circulation (Kwong et *al*. 2013; Lammers et *al*. 2011). Moreover, some i.t. delivered therapeutics evoke antitumour immune-responses that have abscopal effects (e.g. T-VEC) (Andtbacka et *al*. 2015). Importantly, the effectiveness of some therapeutics such as IL-2 is greatly increased when delivered i.t. (see section 1.7.1.3.). However, tissue-injury due to needle-injections can potentially be a source of immunosuppressive inflammation, as observed in core-needle biopsies (Mathenge et *al*. 2014). In theory, such injections could also increase the likelihood of metastasis and infection, although not proven in this context.

1.7. Common γ chain receptor cytokines

Interleukins 2, 4, 7, 9, 15, and 21, and thymic stromal lymphopoietin (TSLP) identified in mice and humans constitute the common γ-chain receptor family. All seven of these four-α-helix bundle cytokines signal through a receptor-complex comprising of the γ-chain and one or two additional chains (Rochman et *al*. 2009). Therefore, it is not surprising that all γ-chain cytokines activate signal transducer and activator of transcription (STAT) 5 molecules and promote the survival and proliferation of multiple T cell subsets (Rochman et *al*. 2009). The receptors of these cytokines are mainly expressed by immune cells, though low level expression on tissues such as lung endothelial cells and fibroblasts may serve to protect hosts from bacteria-induced cytokine storms (Gruss et *al*. 1996; Krieg et *al*. 2010). The outcomes of γ-chain-signalling are cell type-specific and to an extent, unique from cell-to-cell, as the

functional status of target cells and the strength and duration of signalling are determinants of cell activation and function.

1.7.1. IL-2

Monomeric IL-2 is produced predominantly by $CD4^+$ T cells in SLOs upon appropriate TCR engagement and activation. However, $CD8^+$ T cells, natural killer (NK) cells, NKT cells, activated DCs, and mast cells may also produce IL-2 in lesser amounts (Granucci et *al*. 2001; Hershko et *al*. 2011; Setoguchi et *al*. 2005; Yui et *al*. 2004). The IL-2 receptor (IL-2R) is composed of three subunits: IL-2Rα (CD25); IL-2/15Rβ (CD122); and the common γ-chain (CD132). These receptor chains may combine in numerous forms, each encompassing unique affinity for IL-2 and signalling capability. Following ligation of IL-2 with its low-affinity α-chain (dissociation constant, K_d, of 10^{-8} M), IL-2 undergoes conformational changes that increase its affinity for IL-2/15Rβ-chains (Takeshita et *al*. 1992). Dimerisation of these chains upregulates γ-chains, which leads to the formation of high-affinity receptors (IL-2Rαβγ) (K_d of 10^{-11} M). The α-chain also serves as a carrier for IL-2, as it can *trans* present IL-2 to cells expressing intermediate-affinity receptors (IL-2Rβγ) (K_d of 10^{-9} M) (Takeshita et *al*. 1992; Wuest et *al*. 2011). Assembly of this complex activates intracellularly associated Janus kinases (JAKs) (JAK1 via IL-2/15Rβ-chains and JAK3 via γ-chains), which phosphorylate and activate STAT5A and STAT5B (hereafter together denoted as STAT5), the phosphoinositide 3-kinase (PI3K)-Akt pathway, and the Ras-MAPK pathway to mainly mediate life and death signals (Figure 3) (Liu et *al*. 1997).

1.7.1.1. IL-2-mediated STAT5-signalling

STAT5 plays a crucial role in regulating the transcription of *CD25*, *Foxp3* and *FasL* in T cells, particularly $CD4^+$. Relatedly, STAT5-signalling antagonises $CD4^+$ T cell commitment toward the T helper (T_H) 17 lineage (Laurence et *al*. 2007). The Foxp3 transcription factor is required for the development of natural T_{REG} cells and the regulatory function of differentiated T_{REG} cells; for example, *Foxp3* enhances CTLA-4 expression (Hori et *al*. 2003). T_{REG} cells depend on paracrine IL-2-sources, as *Foxp3* directly represses *Il2* and hence, IL-2 production (Wu et *al*. 2006). Such dependency may account for their constitutive expression of IL-2Rαβγ, which may also serve as a regulatory mechanism to sequester free IL-2 from resting antitumour lymphocytes such as $CD8^+$ T cells and NK cells expressing IL-2Rβγ (Pandiyan et *al*. 2007). The high-affinity IL-2R is rapidly upregulated by $CD8^+$ T cells after TCR stimulation (Kim et *al*. 2001). IL-2-mediated signalling through STAT5 also promotes transcription of *FasL*, which enhances T cell apoptosis through the Fas death receptor pathway in a process known as activation-induced cell death (AICD) (Refaeli et *al*. 1998). By increasing *FasL*, hosts can maintain immune homeostasis by eliminating unwanted cells after Ag-clearance (Van Parijs and Abbas 1998). However, this is highly detrimental for cancer immunotherapies, as tumour-reactive T cells may be programmed for death prior to establishment of immunity.

1.7.1.2. IL-2 influence on $CD8^+$ T cell differentiation

Substantial evidence supports the role of IL-2-mediated-signalling, particularly through STAT5, in the acquisition of cytolytic machinery and gain of immunological memory

(Williams et *al*. 2006; Xin et *al*. 2016; Yang et *al*. 2011). Specifically, repeated-exposure to potent doses of IL-2 has been shown by Pipkin et *al*. (2010) to be key in generating T_{EFF} cells, due in part by sustained Blimp-1 expression. Conversely, brief, potent doses selectively drive memory T cell formation (Kaila et *al*. 2010). Although manageable in controlled conditions and simple in theory, this delicate balance is nearly impossible to control within the clinical environment.

1.7.1.3. Intratumoural IL-2-immunotherapy for management of melanoma

Systemic administration of IL-2 for management of melanomas requires high-dose (HD) boluses to obtain clinical benefit, as IL-2 has a low-molecular weight (15.4 kDa) and is readily cleared by the renal system. Predictably, this induces preferential and sustained T_{REG} cell expansion and causes dose-limiting severe irAEs that include vascular leakage with hypotension, impaired renal function, and CNS toxicity (Sim et *al*. 2014). This limits the availability to patients and widespread application, as HD IL-2 must be delivered at specialised centers with experienced oncologists and nurses specifically trained in management of IL-2 patients (Dutcher et *al*. 2014). To improve the safety profile, Canadian oncologists are delivering IL-2 i.t., which enhances objective response rates (82%) while maintaining high complete responses (51%) compared to systemic administration (Temple-Oberle et *al*. 2014). These data and its cost-effectiveness led to its universal coverage in Canada in 2015 for the treatment of melanoma (pCODR 2015). However, the presence of multiple metastatic lesions creates a laborious challenge for applying local therapies, particularly for IL-2 as it appears to have a predominantly localised efficacy.

1.7.2. IL-15

Cells of the MPS are the primary producers of IL-15. IL-15 mainly signals through heterotrimeric receptors made of unique high-affinity α-chains (CD215) (K_d of 10^{-11} M), and IL-2/15Rβ- and γ-chains. IL-15 binds to IL-15Rα within the endoplasmic reticulum/Golgi compartments of MPS cells prior to being surface expressed (Mortier et *al*. 2008). IL-15Rα presents IL-15 *in trans* to responding cells such as $CD8^+$ T cells expressing intermediate-affinity receptors (IL-15Rβγ) (K_d of 10^{-9} M) to initiate intracellular signalling pathways, JAK-signalling and STAT5 phosphorylation in the case of $CD8^+$ T cells. MPS cells can express these membrane-bound complexes for several days by recycling them through endosomal vesicles (Dubois et *al*. 2002). IL-15 can also signal *in cis* to cells expressing IL-15Rα or IL-15Rβγ, though less likely given the nature of IL-15 as a dimeric complex (Bergamaschi et *al*. 2012; Rowley et *al*. 2009).

1.7.2.1. IL-15 influence on $CD8^+$ T cells

Regardless of presentation mode, the signalling events generated by IL-15 are nearly identical to IL-2 (Figure 3) (Ring et *al*. 2012). Of the IL-2 and -15-responsive proteins in F15R-Kit T cells (39 proteins in total), all but two have equivalent quantitative increases in phosphotyrosine sites after exposure to IL-2 or IL-15 (Arneja et *al*. 2014). The two outliers, STAM2 and VPS18, increase phosphorylation ~2-fold when signalling events are triggered by IL-2 relative to IL-15. These proteins are involved in endosomal trafficking of proteins (Endo et *al*. 2000; Peng et *al*. 2012). Nevertheless, at saturating doses, the gene-expression profiles of $CD8^+$ T cells exposed to these interleukins are very

similar (Ring et *al*. 2012). Disparate functional outcomes are mainly attributed to cytokine doses, and the increased stabilisation of IL-15 for IL-2/15Rβ after it first binds to IL-15Rα compared to IL-2 after it binds to IL-2Rα (Ring et *al*. 2012). Thus, the spatiotemporal and expression levels of their α-chains is crucially important for the regulation of $CD8^+$ T cell processes.

IL-15 plays a central role in the survival of Ag-experienced T cells and the development and homeostasis of memory T cells, especially in the bone marrow (Becker et *al*. 2005). Indeed, T cells from transgenic (Tg) mice that overexpress secreted IL-15 do not undergo AICD as efficiently as T cells from WT mice; a phenomenon that can be restored by culturing T cells isolated from IL-15-Tg mice with anti-IL-15 antibodies (Marks-Konczalik et *al*. 2000). IL-15 prevents T cell death by stabilising the anti-apoptotic protein, myeloid cell leukemia sequence 1 (Mcl-1), and through inhibition of Fas-induced apoptosis (Mueller et *al*. 2003; Shenoy et *al*. 2014). Defining features of memory T cells are their rapid recall responses and longevity. Both actions are metabolically demanding and may require high mitochondrial mass and spare respiratory capacity compared to naïve and effector T cells, a topic still under high investigation (Gubser et *al*. 2013; Phan et *al*. 2016). IL-15 enhances mitochondrial biogenesis in memory T cells by promoting fatty acid oxidation (FAO) and synthesis by increasing rate-limiting components of FAO such as carnitine palmitoyl transferase 1a (CPT1a); and by increasing intracellular glycerol levels through upregulation of glycerol channels including aquaporin 9 (AQP9) (Cui et *al*. 2015; van der Windt et *al*. 2013). Consistent with this, IL-15-knockout mice

infected with vesicular stomatitis virus have a deficit in their ability to produce and maintain T_{EM} and T_{CM} cells (Schluns et *al*. 2002).

Interestingly, IL-15 can imprint Ag-inexperienced (naïve) $CD8^+$ T cells with surface molecules that include prototypical NK cell markers and adhesion molecules used for localisation to inflamed tissues and entry into SLOs such as CXCR3 and CD44 (Al-Banna et *al*. 2014; Hidalgo et *al*. 2007; Sosinowski et *al*. 2013; White et *al*. 2017). Imprinted $CD8^+$ T cells can kill target cells expressing non-classical MHC class I or class I-like molecules such as MHC class I polypeptide-related sequence A (MICA) or MICB in an NK group 2, member D (NKG2D)-dependent fashion prior to primary activation through TCRs (Chu et *al*. 2013).

1.7.2.2. Clinical experience with IL-15

To my knowledge, there are only two trials using IL-15 in melanoma patients, one completed and one ongoing. In the completed phase one dose-escalation study, eleven patients with melanoma received i.v. bolus infusions of IL-15 for twelve consecutive days (Conlon et *al*. 2015). Expectedly, high toxicities were observed, but vascular leakage was minor. Notably, lymphocytes immediately exited circulation upon IL-15 administration, a pattern observed at each time point (i.e. 10-50 min, 4 h, 24 h, 72 h). This efflux pattern was most apparent within the T_{EM} population. IL-15 had a half-life of about 2.4 ± 0.25 hours for all evaluated doses. Preliminary results from the ongoing phase one study on s.c. IL-15 and melanoma highlight improved tolerability compared to

i.v. delivery and profound increases in circulating NK cells and significant increases in $CD8^+$ T cells (NCT01727076) (Morishima et *al*. 2015).

1.8. Animal models for melanoma

The genetic proximity to humans makes chimpanzees (*Pan troglodytes*) an attractive model for evaluating the efficacy of immunotherapies; however, in addition to ethical concerns, Canada is not equipped to conduct primate research. In fact, chimpanzees have never been used for scientific purposes in Canada before or since the creation of the Canadian Council on Animal Care in Science in 1968 (CCAC representative on CBC News 2017). Although there is no ban on primate research in Canada there are also no housing facilities, and constructing a primate center would be highly costly, tens of millions of dollars. Unfortunately, this extraordinary animal is currently listed as endangered, according to the World Wildlife Fund, which effectively ends even the slightest notion of chimpanzee use for research or any other purpose for that matter, except for conservation (WWF 2017).

Humanised mice (*Mus musculus*) represent perhaps the most feasible surrogate for investigating human immune therapy of melanomas. The popular bone marrow/liver/thymus (BLT) model is constructed by engrafting human $CD34^+$ hematopoietic stem cells, and liver and thymus tissues, into immunodeficient NOD-*scid* $IL2Rg^{null}$ (NSG) mice (Lan et *al*. 2006). After 12-16 weeks, all lineages of hematopoietic cells develop, along with a remarkable mucosal immune system. At this stage, mice receive patient-derived melanoma xenografts and are monitored for tumour establishment

before treatment of immunotherapy. Despite having no adaptive immunity and a depleted innate immune arm, these mice eventually develop a graft-versus-host-like syndrome, which limits the time frame for experimentation (Greenblatt et *al*. 2012). Once such issues are resolved, and more patient samples become accessible, humanised mice will likely become the most efficacious model for studying all human diseases.

Considering the profound impact of UV-exposure for melanomagenesis in man, it is important to recapitulate such influence within all preclinical studies involving melanoma. The genetically engineered HMGB1 model utilises hepatocyte growth factor (HGF)-CDK4(R24C) mice and 7,12-dimethylbenz(a)anthracene (DMBA) to induce epigenetic events that contribute to melanoma formation, and UV-radiation to encourage metastasis (Bald et *al*. 2014). HGF is overexpressed to promote melanocyte proliferation by interacting with tyrosine kinase c-Met receptors; and *CDK4* is mutated to impair cell cycle regulation by inactivating p16INK4a. Mechanistically, UV-radiation promotes metastasis by stressing epidermal keratinocytes, which drives HMGB1 translocation from their nucleus into extracellular spaces; eventually, HMGB1 triggers a toll-like receptor (TLR)-4-mediated neutrophilic inflammatory response that includes secretion of TNF, which shifts transformed melanocytes toward a migratory profile (Bald et *al*. 2014). This model mimics the stepwise malignant transformation of melanocytes seen within humans, but DMBA is immunosuppressive and its ability to induce somatic mutations may tolerise T cells to novel antigens (Landsberg et *al*. 2010). For these reasons, possibly among others, these autochthonous melanomas are immune cell-poor and therefore not traditionally used for immunotherapeutic evaluations (Bald et *al*. 2014;

Landsberg et *al*. 2010). Even after adoptive transfer of tumour-specific $CD8^+$ T cells, mice are cured temporarily, but later succumb to the disease (Landsberg et *al*. 2010). Overall, this model demonstrates the need for incorporating UV-radiation into experimentation, as it has profound influence on metastasis.

The transplantable B16F10 murine cell model for melanoma is typically used as the benchmark for evaluating the effectiveness of cancer immunotherapies due to its poor immunogenicity and low MHC expression (Castle et *al*. 2012). This cell subline, and B16F1, were derived from the mother line (B16F0), which was generated by chemical induction on a mouse of the C57BL/6 (H-2b) genetic background in 1954. These sublines were selected for based on their ability to form lung nodules following i.v. injection; where the former has high potential, and the latter has low metastatic potential. Indeed, upon s.c. engraftment, B16F10 cells form palpable tumours in ~5-10 days with lymphatic and lung metastatic tendencies (Ya et *al*. 2015). Like the human disease, B16F10 melanomas typically have inactivating mutations in *CDKN2A*, causing low expression of p19ARF (p14ARF in humans) and p16INK4a, deleterious mutations in *PTEN*, *TRP53*, among others, while also expressing antigens that are common to most melanomas (e.g. MART1, Trp1, Trp2, gp100) and targetable by tetramers and antibodies (Castle et *al*. 2012). Moreover, B16F10 tumours are known to express HMGB1 in areas of low oxygen (Huber et *al*. 2016). On the other hand, these melanomas have few activating mutations in *BRAF*, *c-Kit, KRAS*, and *NRAS*, limiting its utility for understanding melanoma pathophysiology. Another problem with this cell line is its high mutability over time. Reports underscore significant differences in B16F10 cells between

and among laboratories that include variant and pigmentation levels, antigen expression, tumourigenic and metastatic potential, and recognition by cytotoxic T lymphocytes (Castle et *al*. 2012; Melnikova et *al*. 2004; Ya et *al*. 2015). Despite these concerns, this model is probably the most frequently used model in oncoimmunology, and the model used in this thesis (Castle et *al*. 2012).

1.9. Rationale and hypotheses

Despite recent advances in the management of melanoma, most available therapeutics are highly toxic and the clinical benefits are often temporary. With melanoma remaining a global threat to human health, new therapies are urgently needed. IL-15 enhances innate and adaptive immune responses against invaders and diseases. Namely, IL-15 drives the expansion and survival of Ag-experienced $CD8^+$ T cells, while imprinting naïve $CD8^+$ T cells with cytotoxic capability and surface molecules that promote their localisation to areas of inflammation and SLOs for primary immune activation. Further to this, preliminary clinical results using s.c. IL-15 suggest that i.t. IL-15 will be well-tolerated in patients with melanoma. This evidence positions IL-15 as an excellent candidate for i.t. immunotherapy of melanoma. We set out to comprehensively assess the role of IL-15 in controlling the growth of treated and non-treated distant tumours compared to clinically approved IL-2. We hypothesised that IL-15 might enhance systemic antitumour immunity by increasing the number of Ag-experienced $CD8^+$ T cells.

CHAPTER 2. MATERIALS AND METHODS

2.1. Cells

B16F10 cells purchased from the American Type Culture Collection (Manassas, VA) were maintained at 37°C in 5% CO_2 concentration in Dulbecco's modified Eagle's medium, supplemented with 10% (vol/vol) fetal bovine serum (FBS) and 1x penicillin-streptomycin, all purchased from Life Technologies (Gaithersburg, MD). Cells never reached full-confluency and were passaged every 3 days at a ratio of 1:10 using standard cell culture procedures. Primary T cells were cultured in Roswell Park Memorial Institute medium containing 10% (vol/vol) FBS.

2.2. Mice

Female C57BL/6 mice purchased from Charles River Laboratory (Montreal, QC) were aged between 6-10 weeks before tumour induction. All animal work was conducted under the approval of the Ethics Committee at Dalhousie University (Halifax, NS) in accordance with the guidelines of the Canadian Council on Animal Care. The animal holding rooms were on a 12-hour light-dark cycle and food and water were provided *ad libitum*.

2.3. Interleukins

Recombinant murine interleukins used in the experiments outlined herein were derived from *Escherichia coli* bacterium; More specifically, from the BL21(DE3) strain for IL-2 and a derivative of W3110, a K12 strain, for IL-15. Compared to human and murine IL-2 and -15, the recombinant *E. coli*-derived form possesses methionine at the *N*-terminal and

is not glycosylated (Kurys et *al*. 2000; Liang et al. 1985). The purity of IL-2 for our lot was > 95% as measured by reducing and non-reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). IL-15 purification also involved SDS-PAGE in addition to high-performance liquid chromatography (HPLC), which may explain its higher purity (≥ 98%). However, endotoxin levels for both cytokines were less than 0.1 EU/μg, determined by the kinetic limulus amebocyte lysate (LAL) method. The specific activity of these cytokines was 9.1 x 10^5 U/mg and 2 x 10^5 U/mg, respectively, calculated by evaluating their ability to drive the proliferation of IL-2-dependent murine T cells called CTLL-2.

2.4. Tumour inoculation and treatments

An inoculum of 10^5 viable B16F10 cells in 100 μL of phosphate-buffered saline (PBS) were injected s.c. into the flanks of anesthetised C57BL/6 mice of equal age and allowed to establish for 10-13 days. Viability was assessed by trypan blue staining. Tumour area was calculated using the formula: length x width^2 x $\pi/6$. Mice with palpable right flank tumours were randomised into treatment groups and treated with 100 μL i.t. injections of IL-15 (3750 U) or IL-2 (30 000 U), both reconstituted in sterile water, or PBS. Treatments were separated by two days and mice were sacrificed one day after the third treatment. The dose of IL-15 was eight times less than IL-2 to balance their intratumoural persistence, as the half-life of IL-2 is in the range of minutes (Donohue and Rosenberg 1983). IL-15 was purchased from PeproTech (Rocky Hill, NJ) and IL-2 from Shenandoah Biotechnology (Warwick, PA). Body weight and clinical signs of mice were measured daily as an indicator of adverse effects.

2.5. Isolation of immune cells

For tissue extraction, euthanised mice were dorsally pinned (using 30 gauge needles) on expanded polystyrene covered in plastic wrap, with their legs stretched outright. Briefly, skin from the abdomen was pinched with forceps and a midline incision was made along the epithelial layer, from the inner thighs to the neck using surgical scissors. For internal tissue collection, both skin sections were retracted and pinned, avoiding disturbance of tumours and inner skin layers. The draining ipsilateral lymph nodes of tumours were collected first, followed by splenectomy and extraction of tumours. Each tissue was placed in separate 6 cm tissue-culture plates containing PBS (2 mL). Single cell suspensions of leukocyte infiltrates were achieved by mechanical-disruption of tissue with scalpels and syringe plungers (1 mL) and filtration (40 μm). Infiltrates of tumours were further isolated by Ficoll density gradient centrifugation (GE Healthcare, Montreal, QC). Red blood cells (RBCs) within suspensions were lysed with ammonium chloride prior to use in co-culture experiments or staining with antibodies.

2.6. Flow cytometry

Fluorescent-labeled antibodies to CD16/CD32 (2.4G2), CD127 (SB/199), CD62L (MEL-14), CD3e (145-2C11), CD8a (53.6.7), CD44 (IM7), CD4 (GK1.5), and IFN-γ (XMG1.2) were purchased from BD Biosciences (San Jose, CA). Antibodies to Foxp3 (150D) and CD25 (PC61) were purchased from BioLegend (San Diego, CA). All staining was performed in BD Horizon Brilliant Stain Buffer to prevent fluorescent dye interactions (BD Biosciences). Viability was assessed by BD Horizon™ Fixable Viability Stain

(FVS700) and Fc interactions were blocked using CD16/32. Cells were stained using titrated antibodies to identify T cell populations (Table 2) and surface-stained samples were fixed using Fixation Buffer (554655) from BD Biosciences. Intracellular staining of Foxp3 and IFN-γ was completed according the Foxp3 kit protocol (eBiosciences, San Diego, CA). Cells were stored at 4°C in the dark until flow cytometry quantification using a FACSCalibur cytometer or a LSRFortessa cytometer (BD Biosciences). Compensation was performed with compensation beads (552845) (BD Biosciences) prepared on the day of data acquisition. Fluorescence minus one controls were used as required. Data were analysed in batch using FCS Express (DeNovo Software, Los Angeles, CA).

2.7. IFN-γ assay

Bilateral tumour-bearing C57BL/6 mice were treated as outlined previously and $CD8^+$ T cells were isolated from RBC-lysed single-cell splenic suspensions upon animal sacrifice by negative selection (> 95% purity determined by flow cytometry, see Figure 4) (Miltenyi Biotech Gmbh, Bergisch Gladbach, NRW). During isolation, T cells were suspended in buffer containing PBS, 0.5% bovine serum albumin, and 2 mM EDTA. Selected T cells were cultured in 12-well plates alone, with IL-2 (200 U/mL), or co-cultured with B16F10 cells seeded previously (12 h prior) at a 1:1 ratio. T cells were cultured for 24 h and Brefeldin A (10 μg/mL) (Sigma Aldrich) was added for the final 6 h, followed by extracellular staining for Ag-experienced $CD8^+$ T cells and intracellular staining for IFN-γ. Brefeldin A blocks intracellular transport and secretion of secretory proteins.

2.8. Cell proliferation assay

The effects of interleukins on B16F10 cell proliferation was determined using the CellTiter 96® Aqueous Non-radioactive Cell Proliferation Assay (Promega, Madison, WI) according to the manufacturer's protocol. After B16F10 cells (5000/well) were treated for 24 h, MTS solution was added to each well and 96-well plates were covered in foil for 3 h at 37°C in 5% CO_2. The absorbance at 490 nm was recorded using a Bio-Tek microplate autoreader (Bio-Tek Instruments, Inc., Winooski, VT).

2.9. Statistical analysis

Statistical methods were not used to predetermine required sample sizes to achieve significance, though sample sizes were selected based on estimates from pilot experiments. Experiments were not performed in a blinded fashion. All statistical analyses were performed using one-way ANOVA with a Bonferroni *post hoc* test using GraphPad Prism software. Variance between groups were similar based on Bartlett's test. The p values were represented as follows: $*p \leq 0.05$, $**p \leq 0.01$, and $***p \leq 0.001$.

CHAPTER 3. RESULTS

3.1. Intratumoural IL-15-immunotherapy controls B16F10-melanoma growth *in vivo*

To explore the potential of IL-15 to eliminate established lesions, syngeneic C57BL/6 mice were s.c. inoculated with B16F10-melanoma cells on both hind flanks and i.t. treated with IL-15, IL-2, or PBS upon development of palpable tumours. Treatments were delivered at 3-day intervals for a total of three treatments; mice were sacrificed one day after the final treatment (Figure 5A). Mouse tumours receiving IL-15-therapy showed significantly smaller tumours and hampered growth of distant non-treated tumours compared to controls (Figure 5B and 5C). In keeping with our clinical observations, high-bolus IL-2 also hampered local tumour growth (Figure 4A), but had little influence on distant tumour burden (Figure 5C). All doses of IL-15 and IL-2 appeared well-tolerated by the mice, as measured by clinical observations including weight loss (Figure 5D).

3.2. Interleukins 2 and 15 do not inhibit cell proliferation

Because interleukin-receptors are not only expressed by immune cells, but also by human and murine melanomas including B16F10, we sought to determine the direct effect of interleukins on B16F10 cells (He et *al*. 2004; Palomares et *al*. 1997; McMillan et *al*. 1995). Consistent with the literature, IL-2 significantly enhanced cell proliferation at doses 300 and 500 U/mL (Figure 6A), whereas IL-15 had minor to no influence (Figure 6B) (Palomares et *al*. 1997; Song et *al*. 2016). Hence, the growth control observed *in vivo* is not attributable to direct interleukin-B16F10 interaction.

3.3. Local IL-15 enhances antigen-experienced CD8$^+$ T cell infiltration into B16F10 tumours and secondary lymphoid organs

IL-15 promotes the expansion, survival, and surface expression of core 2 O-glycans on Ag-experienced CD8$^+$ T cells. Ag-specific cells are defined by high expression of CD44 (Figure 7A). Thus, we hypothesised that i.t. IL-15-therapy would increase systemic numbers of Ag-experienced CD8$^+$ T cells. Indeed, IL-15 significantly elevated the number of Ag-specific CD8$^+$ T cells within treated B16F10 tumours (Figure 7B) and contralateral (CL) tumour draining lymph nodes (Figure 7E) relative to PBS-treated control mice; and within the spleens (Figure 7F) of both PBS- and IL-2-controls. Spleens were also significantly occupied by IL-15-programmed Ag-experienced CD4$^+$ T cells relative to PBS (Figure 7B). Surprisingly, the number of naïve and Ag-specific T cells within the non-treated tumours (Figure 7C) and the draining nodes of the treated tumours (Figure 7D) were not dissimilar. Overall, numerical increases in Ag-specific CD8$^+$ T cells corresponds with lower tumour burden, linking T cells with improved antitumour efficacy.

3.4. IL-15-programmed antigen-specific CD8$^+$ T cells readily produce IFN-γ after re-exposure to tumour-associated antigens

Given that IL-15 can imprint naïve T cells with Ag-experienced phenotypes, independent of Ag exposure, we next evaluated the overall reactivity of IL-15-programmed Ag-experienced CD8$^+$ T cells to B16F10 Ag and their level of functionality via measurement of IFN-γ production. To do this, bilateral tumour bearing mice were treated as described

previously and splenic Ag-specific $CD8^+$ T cells were sorted by negative selection, identified as depicted in Figure 8A, and co-cultured with B16F10 cells (1:1 ratio) or stimulated with IL-2 or not (Figure 8B). Collected T cells were identified as illustrated in Figure 7A from both IL-15- and IL-2-treated mice had enhanced basal activation compared to those from PBS-control (Figure 8C). Importantly, more IL-15-programmed T cells produced IFN-γ after exposure to B16F10 Ag compared to T cells cultured alone or from IL-2- or PBS-treated mice, suggesting that IL-15-treatment may increase the overall $CD8^+$ T cell response to tumour-associated Ag (Figure 8C). T cells from IL-2-treated mice were also highly reactive to tumour Ag, but less-so than IL-15-exposed T cells (Figure 8C). Less Ag-specific T cells from PBS-treated mice were synthesising IFN-γ after re-exposure to B16F10 cells compared to those cultured alone; and *in vitro* stimulation with IL-2 did little to reinvigorate these nonresponsive T cells (Figure 8C). In contrast, IL-2 stimulation significantly enhanced the number of IFN- γ synthesising T cells collected from IL-15- and IL-2-treated mice relative to basal and co-culture levels (Figure 8C). These data suggest that IL-15 orchestrates a greater overall T cell response to melanomas by numerical and functional enhancement of T cells.

3.5. Intratumoural IL-15-immunotherapy does not expand regulatory T cell populations

T_{REG} cells typically require specific Ag-activation to initiate their suppressive activity, but can blindly inhibit or mask the antitumour mechanisms of tumour-specific $CD8^+$ T cells (Szymczak-Workman et *al*. 2009; Thornton and Shevach 2000). Motived by the increased localisation of Ag-specific $CD4^+$ T cells in treated tumours and spleens after i.t.

IL-15-therapy (Figure 7B and 7F), we next analysed the effect of IL-15-therapy on the tissue homing or residence of T_{REG} cells to B16F10 tumours and spleens (see Figure 9A for gating strategy). Notably, IL-15 did not upregulate T_{REG} cell accumulation within the tumours (Figure 9B and 9C) or in the spleens (Figure 9D). However, i.t. IL-2-therapy significantly expanded T_{REG} cells found within treated tumours (Figure 9B). This expansion was mirrored within distant tissues compared to PBS-treatment (Figure 9C and 9D). Thus, i.t. IL-15, unlike IL-2, does not influence T_{REG} cell numbers within this preclinical B16F10 melanoma model.

3.6. IL-15-programmed antigen-specific $CD8^+$ T cells are predominately effector and effector memory T cells

IL-15 is crucial for mature $CD8^+$ T cell acquisition of immunological memory. Thus, $CD8^+$ Ag-experienced T cells were further analysed for expression of CD62L (L-selectin) and CD127 (IL-7Rα). Figure 10A highlights the strategy for identification of effector memory T cells ($CD127^{hi}CD62L^{lo}$) (T_{EM}), central memory T cells ($CD127^{hi}CD62L^{hi}$) (T_{CM}), and effector T cells ($CD127^{lo}CD62L^{lo}$) (T_{EFF}) after gating for Ag-experienced T cells as outlined in Figure 7A. Local IL-15-therapy significantly elevated T_{EFF} numbers within treated tumours (Figure 10B) and T_{EM} and T_{EFF} numbers within CL tumour draining lymph nodes and spleens (Figure 10E and 10F).

CHAPTER 4. DISCUSSION

4.1. Summary of major findings

T cells are crucially important in tumour clearance and development of immunity. While high-bolus systemic IL-2-immunotherapy promotes both processes, dose-limiting toxicity prevents its utilisation in this way (Sim et *al*. 2014). To counteract this, clinicians are delivering IL-2 locally which alleviates most adverse effects. However, targeting systemic disease has become increasingly difficult (Unpublished clinical data; Temple-Oberle et *al*. 2014). We demonstrate herein that i.t. IL-15-therapy potentiates control of local tumour growth while having notable regulatory effects on distant non-treated tumours and limiting collateral toxicity via immune modulation. We report that i.t. IL-15-drives systemic expansion of Ag-experienced $CD8^+$ T cells, as demonstrated within treated tumours and SLOs, and link distant tumour control to the heightened reactivity and functionality of splenic IL-15-programmed $CD8^+$ T cells to tumour Ag. Moreover, our data indicate that local IL-15-therapy does not influence T_{REG} cell numbers within tumours and spleens compared to IL-2. Furthermore, the IL-15-programmed $CD8^+$ T cells within lymphoid organs were mostly of the T_{EM} and T_{EFF} phenotypes, whereas the T_{EFF} population occupied the treated tumours.

Paramount to cytokine immunotherapy is the short half-life of cytokines and the serious pulmonary and hepatic toxicities, particularly for IL-2-based-therapies (Conlon et *al*. 2015; Lotze et *al*. 1985; Sportès et *al*. 2010). However, both complications can be avoided by i.t. delivery (Temple-Oberle et *al*. 2014). Song et *al*. 2016 recently demonstrated that i.t. IL-15 inhibited local B16F10-melanoma growth. However,

treatment was initiated prior to establishment of palpable tumours, limiting its translational relevancy. We proved that i.t. IL-15 slightly improved control of treated B16F10 tumours and had marked influence on distant non-treated tumours over that of IL-2 and PBS-controls. The dosing of IL-15 appeared safe and well-tolerated by all mice, which continued to gain weight throughout treatment.

The question of what cells control tumours remains. We show that i.t. IL-15 directs Ag-experienced $CD8^+$ T cells to treated tumours and SLOs. Immune responses are initiated within SLOs due to the intricate supportive architecture that brings mature APCs in contact with T cells (Aoshi et *al*. 2008; Stoll et *al*. 2002). Because IL-15 upregulates core 2 O-glycans on memory T cells and SLO-associated ligands on naïve T cells, it is likely that IL-15 facilitates primary and secondary T cell activation within SLOs (Al-Banna et *al*. 2014; Nolz and Harty 2014; Sosinowski et *al*. 2013; White et *al*. 2017). In relation to this, IL-15 also enhances DC maturation, and mature DCs are better equipped to process and present Ag, migrate to SLOs, and provide co-stimulation to T cells relative to immature DCs (Mellman 2013; Mohamadzadeh et *al*. 2001; Pulendran et *al*. 2004). Factors such as these may account for the increased number of Ag-experienced $CD8^+$ T cells within IL-15-treated mice. Another more simple explanation is that IL-15 may be driving T cell proliferation at a rate greater than that of IL-2, particularly within treated sites (Arneja et *al*. 2014). Alternatively, IL-15 could also be preventing AICD of T cells by upregulating anti-apoptosis proteins, causing a net increase in T cell numbers (Shenoy et *al*. 2014). Overall, it is highly likely that several IL-15-linked mechanisms act in concert to increase the number of $CD8^+$ T cells.

We next show that i.t. IL-15 increases the frequency of splenic Ag-experienced $CD8^+$ T cells that can readily produce IFN-γ upon re-exposure to tumour Ag relative to T cells derived from PBS-treated or IL-2-treated mice. IFN-γ upregulates HLA molecules (MHC in mice) and promotes Ag presentation by tumour cells, making it critical for T cell-mediated tumour clearance (Böhm et *al*. 1998). IFN-γ is released after TCR-engagement, suggesting that IL-15-treated mice have more T cells specific for tumours (Adachi and Davis 2011). Moreover, these cells were highly capable of IFN-γ production in response to IL-2 stimulation *in vitro*, further highlighting their functional status. Dysfunctional T cells hierarchically lose the ability to proliferate and then synthesise cytokines such as IL-2, TNF-α, and IFN-γ (Wherry et *al*. 2003). It is possible that IL-15-imprinted $CD8^+$ T cells are releasing IFN-γ and hence skewing our interpretation of the data. However, this is highly unlikely to occur without proper APC-activation and within the unsupportive co-culture conditions (Lee et *al*. 2013). Moreover, these cells produce little or no IFN-γ after *in vitro* stimulation with IL-2 (Haluszczak et *al*. 2009). Another possibility to consider is that IL-15 could be rescuing T cells from immunosuppressive effects produced by B16F10 cells (e.g. TGF-β), enabling them to maintain their functionality, a role not played by IL-2 (Benahmed et *al*. 2007; Gorelik and Flavell 2001; Perna et *al*. 2013). Together, these findings illustrate that IL-15 not only increases $CD8^+$ T cells numbers, but also their overall reactivity/function to B16F10 tumours, which may explain the control of tumour burden.

Within the $CD4^+$ T cell population, we saw a numerical increase in Ag-experienced T cells in treated tumours and distant lymphoid organs from IL-15-treated mice. $CD4^+$ T_{REG} cells are highly immunosuppressive following TCR-ligation (Thornton and Shevach 2000). Upon further evaluation, IL-15-treatment had no effect on T_{REG} cell numbers within lesions and spleens compared to IL-2, suggesting that the $CD4^+$ T cell population consisted primarily of T_H cells. IL-15 does not endow $CD4^+$ T cells with Ag-experienced phenotypes (Purton et *al*. 2007). In the case of Ag-experienced $CD8^+$ T cells, IL-15-treatment resulted in recruitment of T_{EFF} cells to treated tumours and T_{EFF} and T_{EM} cells to SLOs. Given that T_{EFF} cells are instrumental for protecting peripheral sites from infections (e.g. vaccinia virus and HSV) and that T_{EM} cells can be rapidly recruited to inflamed tissues, it is reasonable to associate the distant non-treated tumour control to their antitumour activity (Jiang et *al*. 2012; Mackay et *al*. 2012).

One intriguing observation made from this study is the striking disparity in $CD8^+$ and $CD4^+$ T cells within distant non-treated tumours compared to treated tumours, especially in the PBS-treated mice. It could be argued that i.t. treatment initiates a pro-inflammatory wound healing response that includes recruitment of N2 neutrophils and M2 macrophages (Eming et *al*. 2014). Indeed, we show in our recent work on core needle biopsies that needle incisions significantly increase immature neutrophil/G-MDSC localisation within tumours and concurrently decrease $CD8^+$ T cell numbers (Mathenge et *al*. 2014). Consistent with this observation, in this study we observe slight increases in T_{REG} cell numbers within PBS-treated tumours compared to non-treated tumours.

I.t. IL-2-therapy was effective in impeding the growth of treated B16F10 tumours without significantly changing the levels of $CD8^+$ T cells compared to control mice receiving i.t. injections of PBS. IL-2 may have very transient yet profound effects on the tumouricidal activities of these T cells, as IL-2-programmed $CD8^+$ T cells were functional *in vitro*. IL-2 could also promote other T cell-independent immune responses that could retard tumour growth. IL-2 is known to enhance the antitumour activities and development of NK cells, though not to the extent of IL-15 (Kennedy et *al*. 2000; Kündig et *al*. 1993). Thus, it is unlikely that tumour growth was explicitly stalled due to IL-2-mediated NK cell enhancement. The vascular tree and endothelial lining of microvessels of most fast-growing tumours is typically poorly developed. The addition of IL-2 may further promote vascular leakage, resulting in a cascade of oedema, necrosis and apoptosis, and ultimately high antigen release that could stimulate immune responses. In any case, more work is required to elucidate the role of IL-2 in slowing the growth of B16F10 tumours.

4.2. Clinical implications

Here, we affirm previous works on the ability of IL-2 to promote B16F10 proliferation (Palomares et *al*. 1997). These data indicate that IL-2 may directly aid disease progression. We also show that i.t. IL-15 can give rise to systemic Ag-specific $CD8^+$ T cell responses that control systemic B16F10 tumours more effectively than i.t. IL-2-therapy. What are the consequences of these observations? Primarily, they suggest that clinicians may be able to target distant disease by treating few local lesions with i.t. IL-15-therapy. This is of central importance, as metastases and disease recurrence are the foremost cause of mortality by melanomas. This therapeutic strategy should also reduce

patient-physician time, and improve patient outcomes and quality of life by limiting melanoma-resistance and collateral toxicity, two common pitfalls associated with current approved biologics (Carlino et *al*. 2016; Sim et *al*. 2014; Zaretsky et *al*. 2016).

It is difficult to predict whether our results may become more pronounced if treatment persisted for a longer period. One caveat of our model is the short experimental window for i.t. IL-15-monotherapy after establishment of palpable tumours due to the metastatic tendencies of B16F10 cells. The less aggressive subline, B16F1, may represent a better model for evaluating the long-term effects of i.t. IL-15-therapy (Danciu et *al*. 2015). Perhaps the longest immunologic evaluation on locally administered IL-15 is from Sneller and colleagues (2011). This group delivered s.c. IL-15 twice-weekly for two weeks to healthy rhesus macaques (*Macaca mulatta*) and report a 10-fold expansion of circulating T_{EM} cells. However, it is not possible to hypothesise how these data may translate within the human host without some knowledge on how these cells were effected within the periphery. Another disadvantage of using B16F10 cells is that they do not possess oncogenic BRAF mutations, the most common driver mutation observed clinically.

If taken literally, we predict that i.t. IL-15 may particularly benefit a subset of patients with sun-induced or sun-exposed melanomas. UV-light initiates the isomerisation of 7-dehyrocholesterol into vitamin D3 within human skin (Holick et *al*. 1980). After additional enzymatic activity by hydrooxylases, vitamin D3 is converted into its active form, $1,25(OH)_2D3$, a potent inducer of CCR10 on activated T cells (Sigmundsdottir et

al. 2007). In these cases, UV-radiation may synergise with IL-15 to potentiate T cell recruitment to sun-exposed melanomas. However, $1,25(OH)_2D3$ is also a known suppressor of T cell proliferation and Ag presentation by DCs (Karmali et *al*. 1991; Penna and Adorini 2000). Thus, we may perform preclinical experiments to evaluate the efficacy of IL-15 plus vitamin D3 (both i.t.) within our established bilateral B16F10 model.

4.3. Proposed future directions of this work

Here, we report that IL-15-programmed $CD8^+$ T cells are correlated with B16F10-tumour control. However, to confirm this notion antibody depletion studies are necessary. Another remaining question is the mechanism behind how IL-15 orchestrates unrivaled T cell responses to tumour Ag. We propose that IL-15 may rescue T cells from immunosuppression, as observed previously, or by promoting primary and secondary T cell responses. To address the latter proposition, we will first investigate the spectrum of B16F10-directed $CD8^+$ T cell responses, the product of increased T cell priming. To do this, our B16F10 cells will be evaluated for surface expression of gp100, Trp1, and Trp2. These Ags are mainly B16F10-specific and are targetable by tetramers, fluorescent molecules that bind to specific TCRs. By adding these tetramers to our high-dimensional flow cytometry panel, we can better determine whether IL-15 enhances the range of T cell responses. On that note, to consolidate our *in vitro* co-culture findings on T cell cytotoxicity, we also add anti-CD107a to our panel – a protein expressed on cytotoxic granules – for measurement of T cell degranulation (Betts and Koup 2004).

Because IL-15 is known to enhance DC maturation and Ag presentation, we will also evaluate the effect of IL-15 on cross presentation by DCs in SLOs. By using GFP-expressing B16F10 tumours we can effectively compare the Ag-uptake of mature CD8-α^+ DCs and $CD103^+$ DCs exposed to IL-15. These two DC subtypes are particularly effective at cross-presenting exogenous Ags to $CD8^+$ T cells (Broz et *al*. 2014; Hildner et *al*. 2008). Development of these DCs is dependent on the transcription factor basic leucine zipper transcriptional factor ATF-like 3 (Batf3) (Hildner et *al*. 2008). By using *Batf3*$^{-/-}$ mice, we can further demonstrate the influence of IL-15-exposed DCs on priming broad T cell responses. By taking this orthogonal approach to evaluate both arms of the immune response we can confidently determine how IL-15 promotes robust antitumour T cell responses.

IL-15 is also known to endow naïve $CD8^+$ T cells with NK-like cytotoxic functions and molecules that enable SLO trafficking. In the future, it will be important to determine the contribution of IL-15-programmed Ag-inexperienced $CD8^+$ T cells within our model. To answer this question, Nur77-GFP mice may be used, as these mice express GFP after TCR-engagement; a critical tool to definitively separate the antitumour activity of Ag-inexperienced from activated Ag-experienced $CD8^+$ T cells (Moran et *al*. 2011).

Before extending these findings within humanised mice, we will further validate the translational relevancy of our findings by using *BRAF*^{CA} *PTEN*^{loxP} *Tyr::CreER*^{T2} mice. This model represents key pathophysiological aspects of the human disease. By painting tamoxifen on the ears of these mice, one can induce the expression of oncogenic *BRAF*,

which causes deletion of tumour suppressor gene *PTEN* in melanocytes and formation of melanoma within 21 to 28 days (Dankort et *al*. 2009).

Since advanced tumours deploy numerous means to escape T cell attack, combination therapies are more likely to provide T cells with the required support necessary for tumour eradication. Since IL-15 upregulates PD-1 on Ag-specific $CD8^+$ T cells and triggers secretion of IFN-γ – another potent inducer of PD-1 on T cells and PD-L1/L2 on melanoma cells – the first combination therapy we will explore is i.t. IL-15 plus low-dose anti-PD1 (Blank et *al*. 2004; Garcia-Diaz et *al*. 2017; Kinter et *al*. 2008; Taube et *al*. 2012). Another possible synergistic combination may be i.t. IL-15 plus MEK inhibitors, as recent work has shown that IL-15 can protect T cells from the deleterious effects of MEK agents (Allegrezza et *al*. 2016).

IL-15 may naturally exist as a heterodimeric complex in circulation, coupled to IL-15Rα (Bergamaschi et *al*. 2012). This form has a much increased half-life and greater ability to stimulate immune cells compared to monomer IL-15 (e.g. T cell proliferation and IFN-γ production) (Chertova et *al*. 2013). Importantly, this complex is superior to IL-15 at attenuating the growth of s.c. B16F10 tumours when delivered i.p (Stoklasek et *al*. 2006). Moreover, an ongoing trial investigating the efficacy of a modified complex – mutant IL-15 (IL-15N72D) complexed with IL-15RαSu/IgG Fc fusion protein – for i.v. therapy of relapsed hematologic malignancies has yielded promising toxicity profiles, and favourable immune cell activation and proliferation (Miller et *al*. 2015). It must be noted that high serum levels of IL-15 are associated with neurotoxicity, as observed in trials

evaluating ischemic brain injuries (Li et *al*. 2017). Luckily preclinical data suggest that s.c. delivery decreases peak serum concentrations (Liu et *al*. 2017). Nonetheless, moving forward we will carefully evaluate the potential of the IL-15/IL-15Rα complex for i.t. therapy of melanomas.

4.4. Conclusions

This study provides novel evidence that i.t. IL-15 is equal to if not better than IL-2 at controlling the growth of treated B16F10 tumours and attenuating the growth of non-treated lesions. In line with our hypothesis, we show that i.t. IL-15 promotes the localisation of Ag-experienced $CD8^+$ T cells to treated tumours and SLOs of mostly of the T_{EFF} and T_{EM} phenotypes. Moreover, we demonstrate that IL-15 enhances the functional quality/reactivity of splenic Ag-experienced $CD8^+$ T cells to B16F10 immunogens, while beneficially causing limited overt irAEs and influence on T_{REG} numbers. As we begin to elucidate the mechanisms underlining these heightened IL-15-governed antitumour $CD8^+$ T cell responses, we can begin to concentrate our efforts on understanding how IL-15 programs other immune cells such as NK cells.

Table 1. Changes in melanocyte-keratinocyte association implicated in melanoma.

Alteration	Cause	Putative role in melanoma	Putative mechanism	References
E- and P-cadherin switch to N-cadherin	ET-1, HGF/SF, PDGF	Metastasis	Twist and Snail transcription factors	Jamal and Schneider 2002; Li et *al*. 2001; Poser et *al*. 2001
↑ Adhesion molecules (e.g. MCAM, L1-CAM)	ET-1, cytokines	Metastasis, angiogenesis	PAR-1 signalling	Lei et *al*. 2015; Shih et *al*. 1994; Thies et *al*. 2002
↑ Dsg2	Unknown	Vasculogenic mimicry	Serine protease inhibitors	Tan et *al*. 2016
↑ Integrins (e.g. $\alpha_v\beta_3$, $\alpha_2\beta_1$)	TGF-β	Metastasis	Bind to ligands on bone	Seguin et *al*. 2015; Siret et *al*. 2015

↑, increased expression; ↓, decreased expression; ET-1, endothelin-1; HGF/SF, hepatocyte growth factor/scatter factor; PDGF, platelet-derived growth factor; Par-1, protease activated receptor 1; Dsg2, desmoglein 2; TGF-β, transforming growth factor- β

Table 2. Established classification scheme of murine T cell populations

Subset	Phenotype
Naïve T cells	$CD3^{+}CD44^{lo}CD4^{+}$/$CD3^{+}CD44^{lo}CD8^{+}$
Antigen (Ag)-experienced T cells	$CD3^{+}CD44^{hi}CD4^{+}$/$CD3^{+}CD44^{hi}CD8^{+}$
Effector T cells (T_{EFF})	$CD3^{+}CD44^{hi}CD8^{+}CD127^{lo}CD62L^{lo}$
Effector memory T cells (T_{EM})	$CD3^{+}CD44^{hi}CD8^{+}CD127^{hi}CD62L^{lo}$
Central memory T cells (T_{CM})	$CD3^{+}CD44^{hi}CD8^{+}CD127^{hi}CD62L^{hi}$
Regulatory T cells (T_{REG})	$CD3^{+}CD4^{+}CD25^{+}Foxp3^{+}$

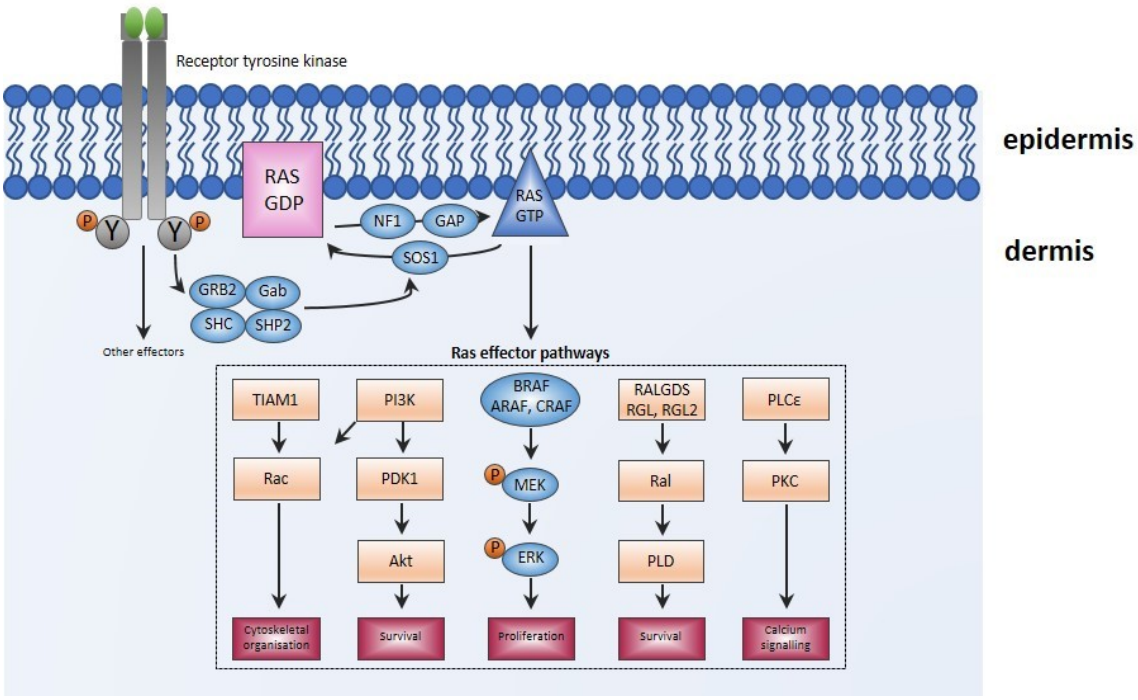


Figure 1. The Ras-MAPK pathway. Activation of Rat sarcoma (Ras) is initiated after binding of growth factors to receptor tyrosine kinases. Adaptor proteins such as growth-factor-receptor bound protein 2 (GRB2), GRB2-associated binding (Gab), and SRC-homology/collagen (SHC) recruit SRC homology 2 (SHP2) and son of sevenless 1 (SOS1); SOS1 is involved in exchanging guanosine diphosphate (GDP) for guanosine triphosphate (GTP) to initiate downstream Ras effector pathways such as proliferation and survival. NF1, neurofibromin; GAP, GTPas-activating proteins; BRAF, B-rapidly accelerated fibrosarcoma; MEK, mitogen-activated protein kinase (MAPK) kinase; ERK, extracellular signal-regulated kinase; RALGDS, Ral guanine nucleotide dissociation stimulator; Ral, Ras-related protein; PLD, phospholipase D; PLCε, phospholipase C epsilon; PKC, protein kinase C; PI3K, phosphoinositide 3-kinase; PDK1, phosphoinositide-dependent kinase 1; Akt, protein kinase B; TIAM1, T cell lymphoma invasion and metastasis 1. Redrawn and modified after Schubbert et *al*. 2007.

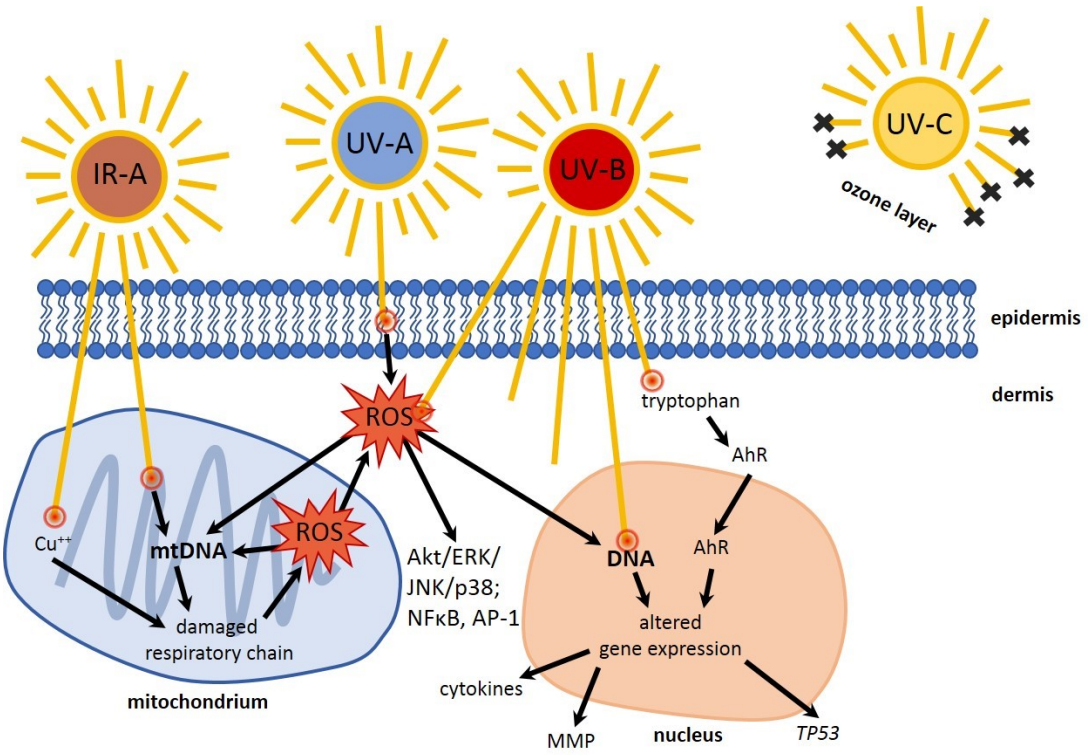


Figure 2. Light induced skin damage. Copper atoms (Cu++) within mitochondria are the major chromophores of infrared (IR)-A light (wavelength (λ) = 770-1400 nm) and are a source of damage to respiratory chains. IR-A can also directly cause mutagenesis of DNA. Ultraviolet (UV)-A radiation (λ = 320-400 nm) may harm epidermal lipid rafts to force reactive oxygen species (ROS) production. ROS alter DNA and have various stimulatory and inhibitory roles in many signalling pathways. The chromophores for UV-B light (λ = 290-320 nm) include nuclear DNA and cytoplasmic tryptophan. UV-B light-induced tryptophan metabolites can drive aryl hydrocarbon receptor (AhR) activity such as dysregulating gene expression. UV-C radiation (λ = 200-280 nm) is most damaging to skin, but is absorbed by intact ozone layer before reaching Earth's surface. mtDNA, mitochondrial deoxyribonucleic acid; Akt, protein kinase B; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; NFκβ, nuclear factor κβ; AP-1, activator protein 1; MMP, matrix metalloproteinase; TP53, tumor protein 53. Redrawn and modified after Wölfle et al. 2013.

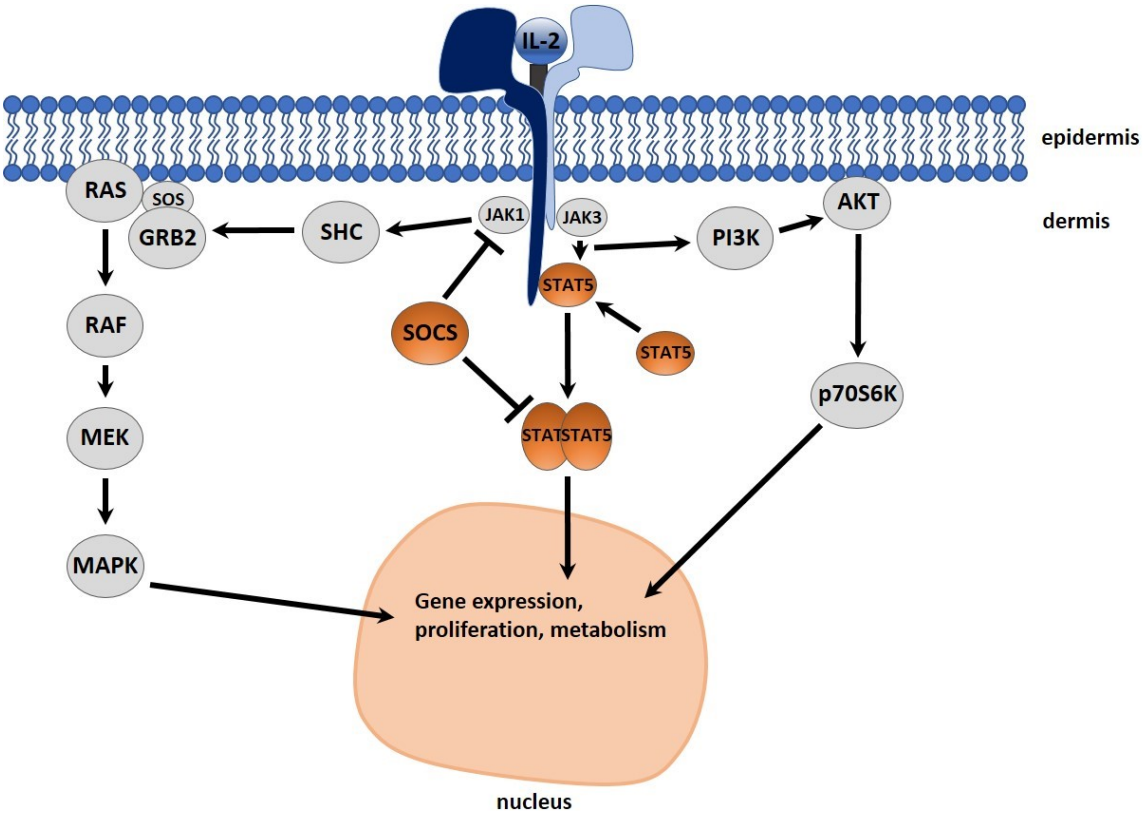


Figure 3. IL-2/-15 signalling pathway. Illustrated are the signalling events that are initiated after interleukin (IL)-2 or IL-15 ligate with the IL-2/-15 receptor β chain and the common γ-chain. JAK, Janus kinase; SHC, SRC-homology/collagen; GRB2, growth-factor-receptor bound protein 2; SOS, son of sevenless; RAS, rat sarcoma; RAF, serine/threonine-protein kinase; MEK, MAP/ERK kinase; MAPK, mitogen-activated protein kinase; SOCS, suppressor of cytokine signalling; STAT, signal transducer and activator of transcription; PI3K, phosphoinositide 3-kinase; AKT, protein kinase B; p70S6K, p70 ribosomal protein S6 kinase. Redrawn and modified after Arneja et *al*. 2014.

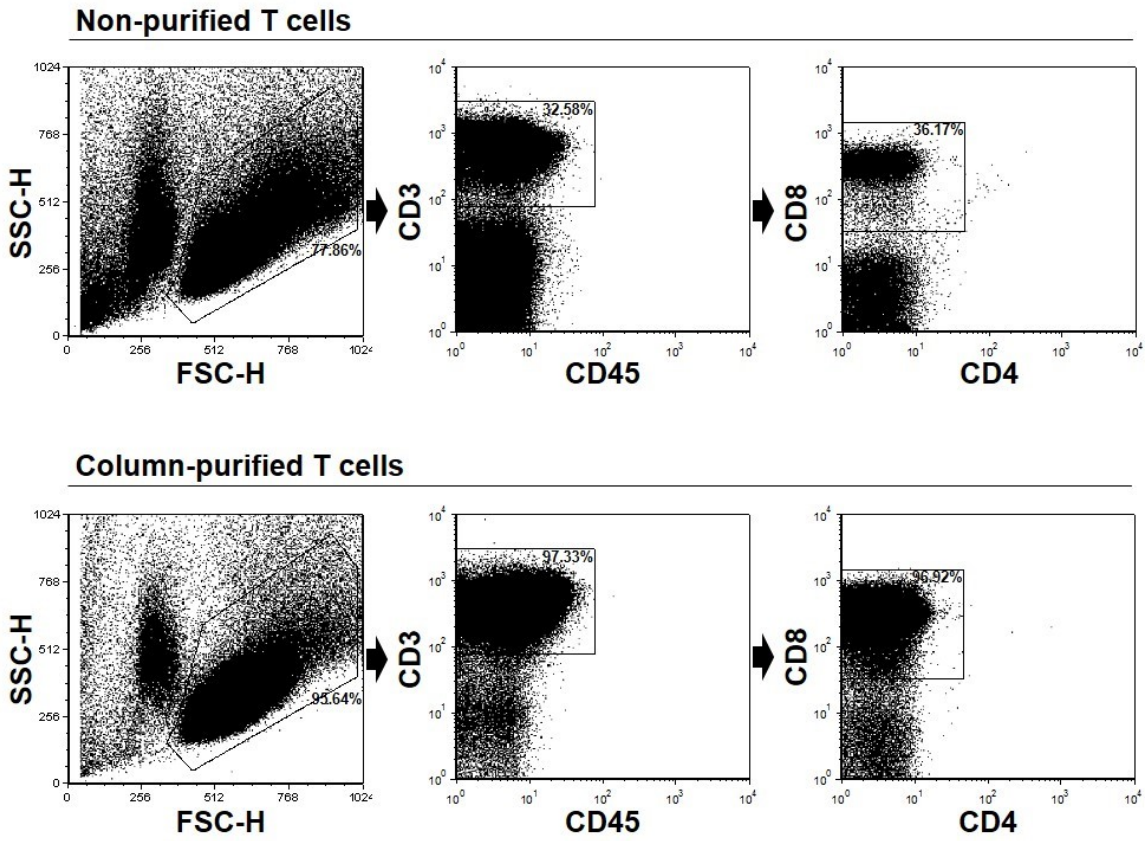
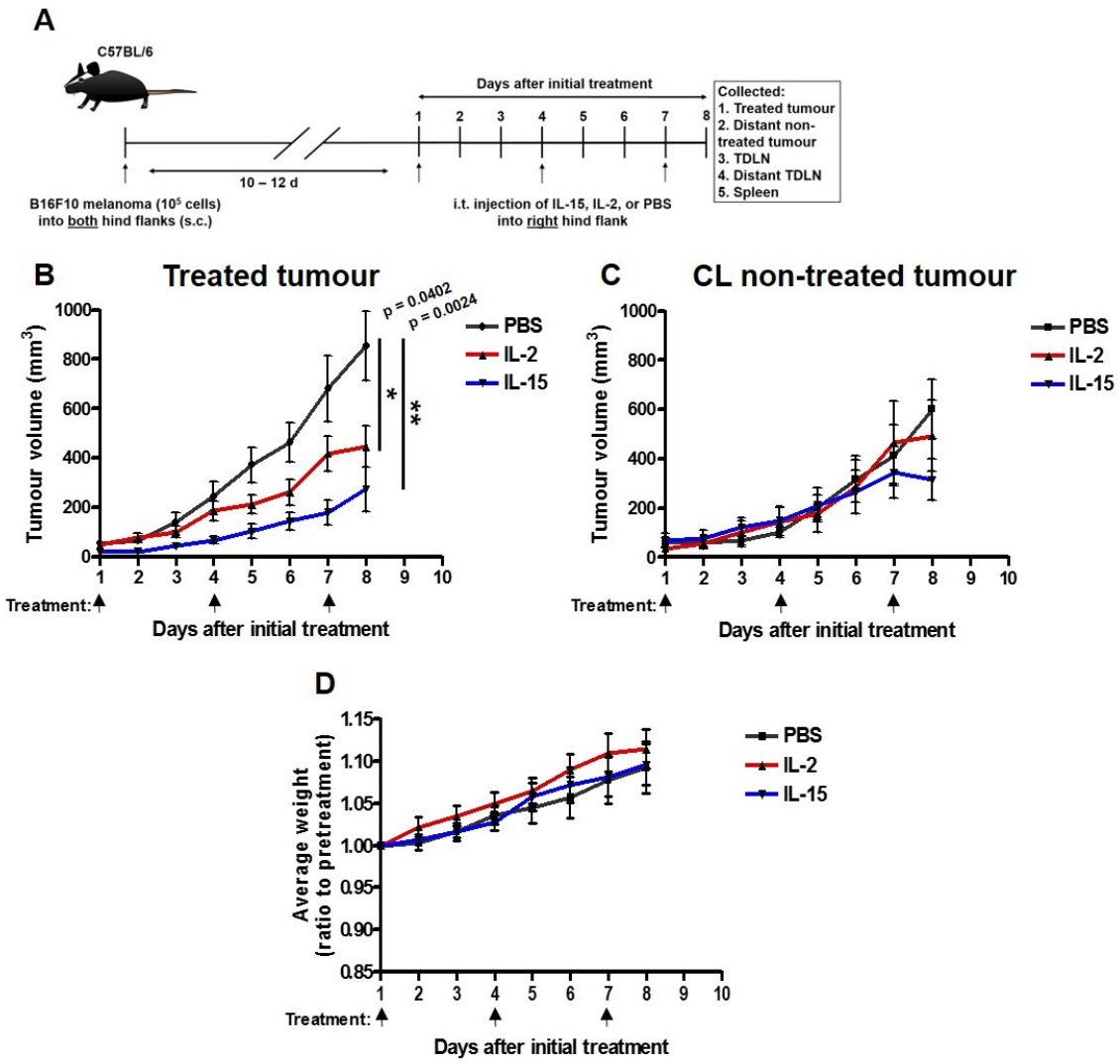


Figure 4. Example of $CD8^+$ T cell enrichment from bulk splenocytes from a healthy untreated mouse. Red blood cells were lysed with ammonium chloride and $CD8^+$ T cells were negatively selected from splenocytes by column purification and visualised by flow cytometry. Shown are relative percentages of $CD8^+$ T cells among $CD3^+CD45^+$ cells from (**A**) non-purified (**B**) and purified samples.

Figure 5. Intratumoural IL-15-immunotherapy controls treated tumour growth. (**A** – **D**) C57BL/6 mice bearing subcutaneous (s.c.) B16F10 tumours on both hind flanks were treated with PBS, IL-15 (3750 U), or IL-2 (30 000 U). Treatment was delivered intratumourally (i.t.) to right flanks after the establishment of palpable tumours. Mice received treatment once every three days, for a total of three treatments, and were sacrificed one day after the last treatment. (**B**) Treated-tumour and (**C**) contralateral (CL) non-treated tumour growth was evaluated over the course of therapy. (**D**) Mouse weight was also monitored as an indicator of animal health and systemic toxicity. Data represent cumulative results from two independent experiments summarised as mean ± SEM ($n \geq 10$). Statistical analyses were performed using one-way ANOVA with a Bonferroni *post hoc* test. Asterisks represent p values as follows: $*p \leq 0.05$ and $***p \leq 0.001$. Arrows indicate treatment time points. TDLN; tumour draining lymph node.


A
C57BL/6
Days after initial treatment
1 2 3 4 5 6 7 8
Collected:
1. Treated tumour
2. Distant non-treated tumour
3. TDLN
4. Distant TDLN
5. Spleen
10 – 12 d
B16F10 melanoma (10^5 cells) into both hind flanks (s.c.)
i.t. injection of IL-15, IL-2, or PBS into right hind flank
B
Treated tumour
p = 0.0402
p = 0.0024
*
**
PBS
IL-2
IL-15
Tumour volume (mm^3)
Treatment:
Days after initial treatment
C
CL non-treated tumour
PBS
IL-2
IL-15
Tumour volume (mm^3)
Treatment:
Days after initial treatment
D
Average weight (ratio to pretreatment)
PBS
IL-2
IL-15
Treatment:
Days after initial treatment

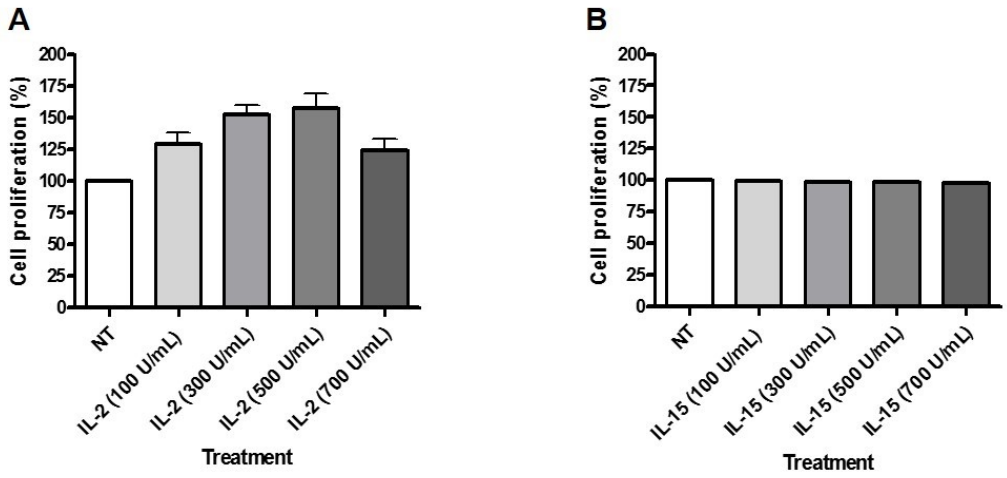
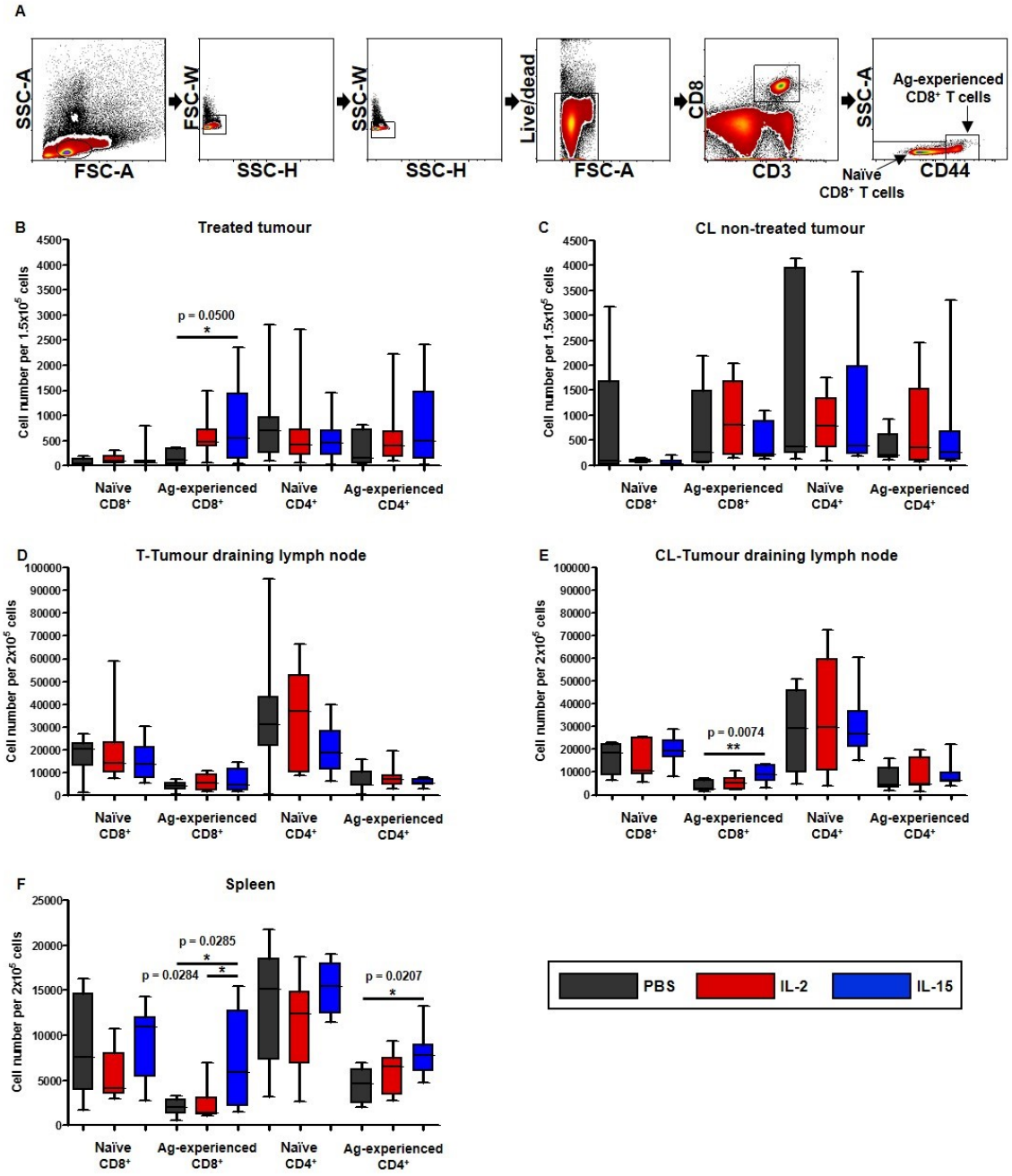


Figure 6. B16F10 cell proliferation is not hindered by IL-2 or IL-15. B16F10 cells treated with various doses of (**A**) IL-2 or (**B**) IL-15 for 24 h. Cell proliferation was quantified by formazan release ($n = 1$). Error bars represent replicates.

Figure 7. Intratumoural IL-15 induces antigen-experienced $CD8^+$ T cell accumulation within treated B16F10 tumours and secondary lymphoid organs. C57BL/6 mice with bilateral flank B16F10 tumours were treated with interleukins as outlined in **Figure 2**. Representative gating strategy (**A**) for identification of naive ($CD3^+CD44^{lo}$) and antigen (Ag)-experienced ($CD3^+CD44^{hi}$) $CD8^+$ T cells and $CD4^+$ T cells. Shown are box plots of the medians (horizontal line), interquartile ranges (box), and minimum/maximum values (whiskers) of naive/Ag-experienced $CD8^+$ T cells and $CD4^+$ T cells within (**B**) treated (T) tumours; (**C**) contralateral (CL) non-treated tumours; (**D**) tumour draining lymph nodes (TDLNs); (**E**) CL TDLNs; and (**F**) spleens. Pooled data from two independent experiments (n = 5-12/group). p-values were calculated by one-way ANOVA with Bonferroni post *hoc* test and are represented as follows: $*p \leq 0.05$ and $**p \leq 0.01$.


A
SSC-A
FSC-A
FSC-W
SSC-H
SSC-W
SSC-H
Live/dead
FSC-A
CD8
CD3
SSC-A
CD44
Ag-experienced CD8⁺ T cells
Naïve CD8⁺ T cells
B
Treated tumour
Cell number per 1.5x10⁶ cells
p = 0.0500
*
C
CL non-treated tumour
Cell number per 1.5x10⁶ cells
D
T-Tumour draining lymph node
Cell number per 2x10⁶ cells
E
CL-Tumour draining lymph node
Cell number per 2x10⁶ cells
p = 0.0074
**
F
Spleen
Cell number per 2x10⁶ cells
p = 0.0285
*
p = 0.0284
*
p = 0.0207
*
Naïve CD8⁺
Ag-experienced CD8⁺
Naïve CD4⁺
Ag-experienced CD4⁺
PBS
IL-2
IL-15

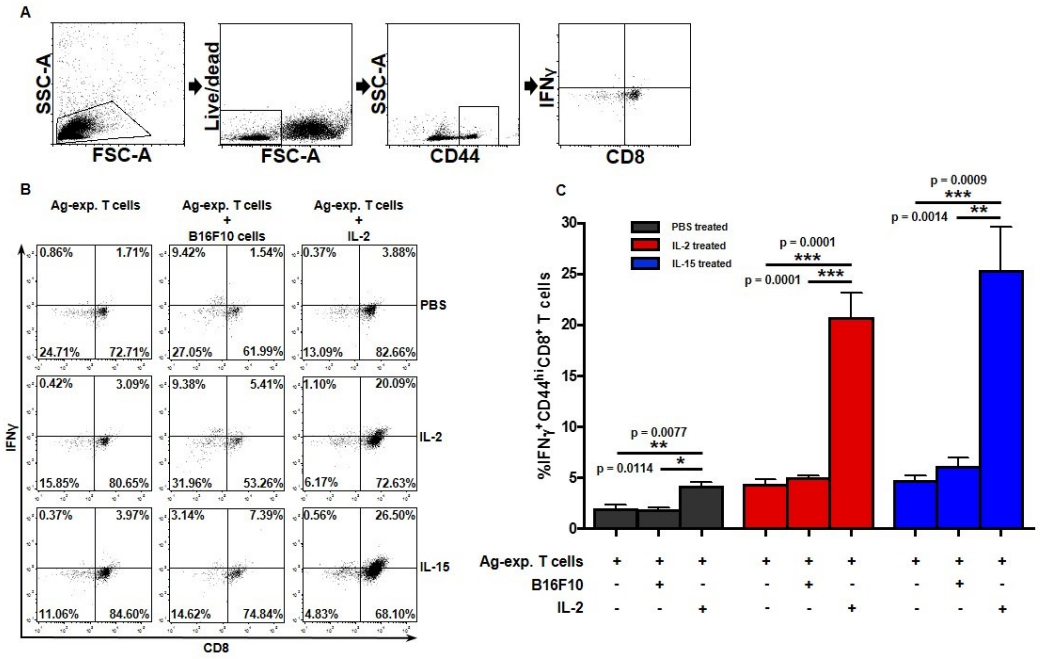


Figure 8. Peripheral IL-15-programmed antigen-specific $CD8^+$ T cells readily produce IFN-γ upon secondary challenge with B16F10. Mice were treated as per the established regimen in **Figure 2** and splenic $CD8^+$ T cells were sorted by negative selection and cultured *in vitro* alone, with B16F10, or IL-2. (**A**) Representative dot plots and (**B**) percentage of antigen (Ag)-experienced $CD8^+$ T cells producing IFN-γ following indicated co-culture conditions. $n = 4$/group. Mean ± SEM is shown. $*p \leq 0.05$, $**p \leq 0.01$, and $***p \leq 0.001$ by one-way ANOVA with Bonferroni post *hoc* test.

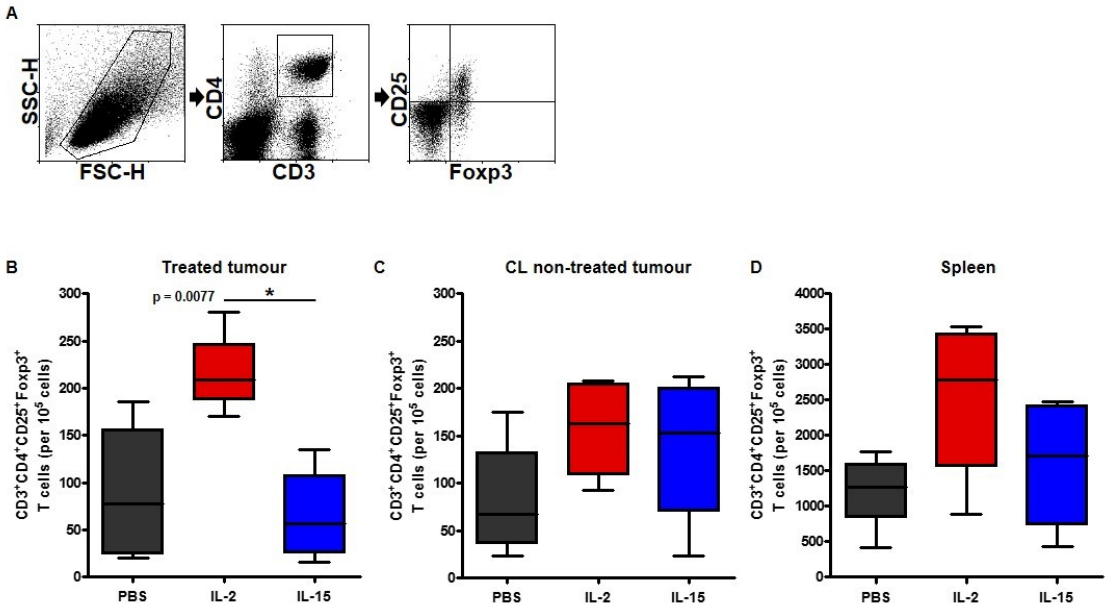
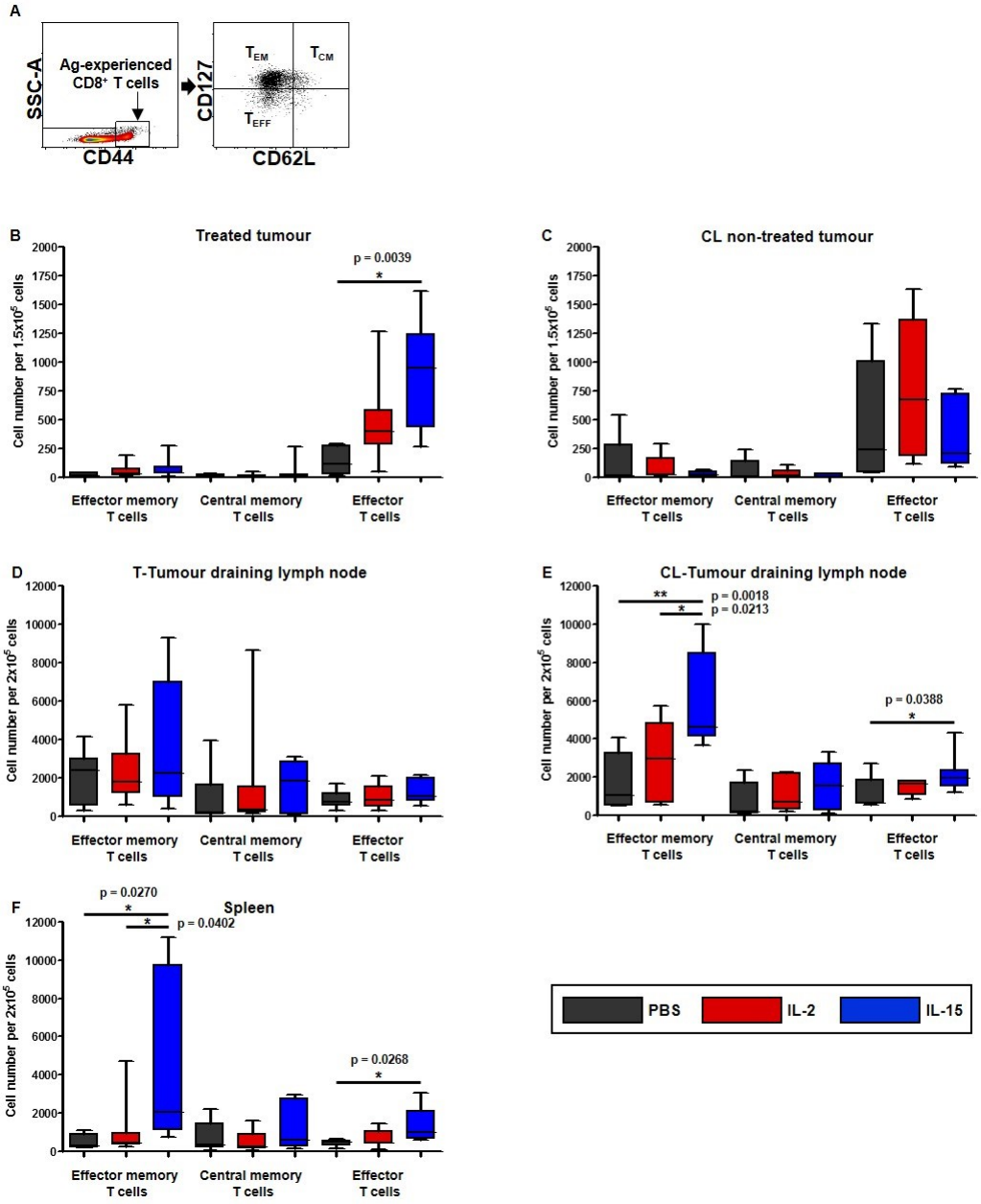


Figure 9. IL-15 does not induce systemic expansion of regulatory T cells. C57BL/6 mice with hind flank B16F10 tumours were treated with PBS, IL-2, or IL-15 as described previously (**Figure 2**). (**A**) $CD3^+CD4^+CD25^+Foxp3^+$ regulatory T cells were identified as shown within (**A**) treated tumours; (**B**) contralateral (CL) non-treated tumours; and (**C**) spleens via flow cytometry and are depicted by box and whisker plots. Plots show median (horizontal line), interquartile ranges (box), and minimum and maximum values (whiskers). n = 4-5/group. p value was calculated using one-way ANOVA with Bonferroni post *hoc* test; $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$.

Figure 10. Local IL-15-therapy influences effector memory T cell and effector T cell localisation. C57BL/6 mice with established hind flank tumours (B16F10) were treated as in **Figure 2**. (**A**) Gating strategy for identification of effector memory T cells (T_{EM}), central memory T cells (T_{CM}), and effector T cells (T_{EFF}) based on CD127 and CD62L after gating for Ag-experienced T cells outlined in **Figure 7a**. (**B**) Representative dot plots of these populations and the abbreviations used to describe each subset. Box and whisker plots depicting T_{EFF}, $T_{CM,}$ and T_{EFF} cell numbers within (**B**) treated (T) tumours; (**C**) contralateral (CL) non-treated tumours; (**D**) tumour draining lymph nodes (TDLNs); (**E**) CL TDLNs; and (**F**) spleens. The median is represented by the horizontal line, the interquartile range by the box, and the range by the whiskers. Data from two independent experiments (n = 5-12/group). Statistical analysis was performed with one-way ANOVA with Bonferroni post *hoc* test; $*p \leq 0.05$, $**p \leq 0.01$, and $***p \leq 0.001$.


A
SSC-A
Ag-experienced CD8+ T cells
CD44
CD127
CD62L
TEM
TCM
TEFF
B
Treated tumour
Cell number per 1.5x10^6 cells
p = 0.0039
*
Effector memory T cells
Central memory T cells
Effector T cells
C
CL non-treated tumour
Cell number per 1.5x10^6 cells
Effector memory T cells
Central memory T cells
Effector T cells
D
T-Tumour draining lymph node
Cell number per 2x10^6 cells
Effector memory T cells
Central memory T cells
Effector T cells
E
CL-Tumour draining lymph node
Cell number per 2x10^6 cells
**
p = 0.0018
*
p = 0.0213
p = 0.0388
*
Effector memory T cells
Central memory T cells
Effector T cells
F
Spleen
Cell number per 2x10^6 cells
p = 0.0270
*
*
p = 0.0402
p = 0.0268
*
Effector memory T cells
Central memory T cells
Effector T cells
PBS
IL-2
IL-15

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