

RATIONALLY DESIGNING STRATEGIES TO AUGMENT CHECKPOINT THERAPY IN CANCER

by

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ABSTRACT

Cancer immunotherapy, such as immune checkpoint modulation (ICM), has revolutionised the treatment of cancer in recent decades. Unfortunately, only a small fraction of patients respond to the treatments, and they are associated with several immune-mediated toxicities. This project is divided into two research chapters, both aiming to rationally design a combination ICM therapy for the treatment of cancer.

Research chapter 1 focuses on designing a novel treatment for hepatocellular carcinoma (HCC), which is a type of primary liver cancer and is in the top 3 causes of cancer related deaths owing to most patients being diagnosed at advanced stage of the disease. This first research chapter uses reovirus, an oncolytic virus, to prime the tumour microenvironment for subsequent ICM therapy to treat HCC with a specific focus on the immune-mediated mechanism of oncolytic reovirus.

Research chapter 2 focuses on rationally combining antibody-mediated programmed cell death ligand 1 (PD-L1) blockade with other ICM therapy and utilises an immunogenic mouse cancer model. This chapter has a key focus on T-cell tolerance, utilising T-cell receptor (TCR) reporter mice to explore the dynamics of orphan nuclear receptor subfamily 4 (Nr4a) expression following *in vitro* and *in vivo* TCR stimulation of Tg4 transgenic mice with agonist tyrosine at position 4 [4Y]-Myelin Basic Protein (MBP) peptide. The data in research chapter 2 using these novel reporter mice reveals that anti-PD-L1 blockade leads to increased susceptibility to peptide rechallenge, lowering the T-cell activation threshold. Furthermore, anti-PD-L1 blockade in combination with OX40 agonism further boosts T-cell activation in this adaptive tolerance model. Finally, it is shown that this combination of anti-PD-L1 blockade with OX40 agonism can lead to enhanced anti-tumour effects.

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To my best friends, Alex, Charlotte, and Ruby. Thank you for your constant love, support, and friendship throughout my journey as a PhD student and beyond. Thank you for not letting me give up and helping me see that I could do this. And to my family, thank you for constant support over the past few years to help me get to the finish line.

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STATEMENT OF CIRCUMSTANCES

Covid-19 impacted the undertaking of this research project in several ways. This research project began under the supervision of Dr Adel Samson at the University of Leeds in October 2019. The first year and a half of the project were significantly disrupted due to not having access to the lab for a prolonged period during government mandated lockdowns. Due to unforeseen circumstances at Leeds, namely fire regulations, this limited access was retained beyond Covid-19 lockdowns. To achieve the most from the project, and to improve mental health, a relocation was sought to work with Dr David Bending at the University of Birmingham whose facilities were up and running again. This relocation has resulted in two uniquely distinct research chapters (1 and 2), with an overarching theme of enhancing cancer immunotherapy through combinatorial strategies. These circumstances have unfortunately meant that some aspects of research chapter 1 could not be repeated due to time limitations during the first year of the project.

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1 INTRODUCTION

1.1 Immune System

Split into two arms, innate and adaptive, the immune system is vital for recognising and eliminating a variety of pathogens to protect organisms from diseases. The innate immune system (see section 1.1.1) provides the first line of defence by eliciting a rapid, non-specific, response to any pathogen. The adaptive immune system (section 1.1.2) elicits a slower, antigen-specific, response.

1.1.1 Innate immune system

The innate immune system begins with a physical barrier, the skin and mucous membranes, protecting against pathogen entry. In the case this barrier is breached, innate immune cells are activated to elicit a non-specific response against the pathogen (Kaur and Secord, 2021). To elicit a generalised response, innate immune cells can detect and respond to a vast range of common structural and functional features that are associated with different pathogens. This is mediated by pattern recognition receptors (PRRs), e.g. toll-like receptors, which are expressed on the surface of innate immune cells such as macrophages and dendritic cells (DCs). PRRs can detect pathogen-associated molecular patterns (PAMPs), including cell-wall components of bacteria and fungi (e.g. lipopolysaccharides) and viral nucleic acids (Thompson *et al.*, 2011). The detection of PAMPs by PRRs initiates an innate immune response against the pathogen. Furthermore, antigen processing followed by subsequent surface presentation by professional antigen-presenting cells (APCs) of the innate immune system, e.g. DCs, leads to activation of adaptive immune responses (see section 1.1.2 and 1.1.3).

In addition to PRRs, another method used for detecting foreign bodies is through missing-self recognition, an approach used by natural killer (NK) cells to distinguish between normal and infected cells (Cruz-Munoz *et al.*, 2019). NK cells express inhibitory receptors (e.g. killer Ig-like receptors; KIR) which detect major histocompatibility complex class I molecules (MHC I) on the surface of target cells to avoid killing uninfected 'self' cells (Shifrin *et al.*, 2014). Viral-infected cells reduce their surface expression of MHC I and are thus detected by NK cells as infected cells and are subjected to NK cell-mediated lysis via release of granules containing granzymes. The innate immune system uses these various forms of recognition to elicit non-specific responses against foreign antigens, in addition to recruiting (e.g. via the release of IFN- γ) and activating (e.g. via antigen presentation) the adaptive immune system for the mounting of an antigen-specific response and subsequent memory.

1.1.2 Adaptive immune system

The adaptive immune system provides the second line of defence to pathogens, incorporating T and B lymphocytes to mount an antigen-specific immune response (Bonilla and Oettgen, 2010). Adaptive immunity is specific to antigens presented on the surface of APCs and is associated with rapid clonal expansion of antigen-specific lymphocytes. Contrary to the innate immune system, effects are slower but are long-lasting and sustained by the induction of memory T-cells. Functional adaptive immune responses are initiated by APCs, such as DCs, which present antigens on their cell surface to stimulate antigen-specific T and B cells. DCs express high levels of MHC molecules, both class I and class II, leading to the activation of naïve CD8 and CD4 T-cells, respectively (see section 1.2.3).

1.1.2.1 CD4+ T-cells

CD4+ T-cells, also known as helper T-cells, produce cytokines to mediate immune responses. CD4+ T-cells can be further characterised by their capacity to produce different cytokines and subsequently promote differing types of immune responses. Type 1 helper (Th1) cells are characterised by their production of interleukin 2 (IL-2), interferon- γ (IFN γ) and granulocyte-macrophage colony-stimulating factor (GM-CSF), whilst type 2 helper (Th2) cells are characterised by production of IL-4, IL-5, B-cell-stimulating factor (BSF-1) and mast-cell growth factor (MCGF) (Mosmann *et al.*, 1986, Mosmann and Coffman, 1989 and Paul and Seder, 1994). Th1 and Th2 CD4+ T-cells were first characterised, with subsequent characterisation of type 17 helper (Th17) cells, characterised by their expression of IL-17 (Park *et al.*, 2005). Th1 cells are critical against intracellular pathogens, e.g. viruses, and activate macrophages via their production of IFN γ . Th2 cells mount responses against extracellular parasites whilst Th17 cells are essential against extracellular bacteria and fungi. In addition, regulatory T (Treg) cells, important for mediating immune tolerance, are known to express CD25 and FOXP3 constitutively. In addition, Type 1 regulatory (Tr1) T-cells have anti-inflammatory roles mediated by their production of IL-10 (Chihara *et al.*, 2016). Tr1 cells importantly contribute to immunological tolerance and help prevent uncontrolled inflammation (Freeborn *et al.*, 2022). Several other types of CD4+ T-cells have been characterised, though are beyond the scope of this text. These different CD4+ T-cells all differentiate from naïve CD4+ T-cells when they are activated through T-cell receptor (TCR) signalling events in addition to cytokine-mediated signals. For example, IL-12, produced by DCs and macrophages, induces Th1 differentiation (Hsieh *et al.*, 1993). IL-2 is capable of inducing Th2 differentiation, whilst TGF- β induces Th17 differentiation (Zhu *et al.*, 2003 and Bettelli *et al.*, 2006). As the main role of CD4+ T-

cells is to produce cytokines; the different CD4⁺ T-cell populations characterised above are associated with different functions, depending on their cytokine production, as outlined.

1.1.2.2 CD8⁺ T-cells

CD8⁺ T-cells, also known as cytotoxic T-cells, elicit target-cell killing via secretion of cytotoxic granules leading to subsequent delivery of pro-apoptotic molecules to target cells (St Paul and Ohashi, 2020). CD8⁺ T-cells recognise antigenic peptide bound to MHC I molecules on the surface of APCs, upon which, in the presence of co-stimulatory signalling with CD28, they become activated and elicit cytotoxic effects. These effects comprise cytokine secretion, including TNF- α and IFN- γ , which have both anti-tumour and anti-viral effects (Koh *et al.*, 2023). CD8⁺ T-cells also release cytotoxic granules, including perforin and granzymes. In addition to the release of cytokines and cytotoxic granules, CD8⁺ T-cells can also induce target cell killing via FasL, expressed on the surface of activated CD8⁺ T-cells, binding with Fas on the surface of the target cell. CD8⁺ T-cells are therefore critical in responding to viruses and bacteria, in addition to playing a crucial role in tumour surveillance (described in further detail in section 1.2.1).

1.1.3 Antigen processing and presentation

As described briefly in section 1.1.2, antigen presentation via MHC molecules on the surface of APCs, such as DCs, is critical for the activation of an adaptive immune response (Doherty and Zinkernagel, 1975). Antigens are specific peptide sequences that are presented on MHC I molecules to CD8⁺ T-cells, and on MHC II molecules to

CD4⁺ T-cells (Neefjes *et al.*, 2011). Structurally, MHC I molecules are polypeptides composed of a single polymorphic heavy chain that associates with soluble β 2-microglobulin whereas MHC II molecules are composed of two polypeptide chains, α and β (Wieczorek *et al.*, 2017). The polymorphic nature of the MHC allows for the recognition of many different antigens. MHC I is expressed by all nucleated cells and present endogenous antigens, leading to CD8⁺ T-cell activation where 'non-self' antigens are presented (Neefjes *et al.*, 2011). MHC II is primarily found on professional APCs and presents exogenous antigens to CD4⁺ T-cells. Additionally, exogenous antigens can also be presented via MHC I, resulting in the activation of CD8⁺ T-cells, in a process known as cross-presentation (Bevan, 2006). This form of antigen presentation is crucial in response against virally infected cells that are not professional APCs and therefore do not express MHC II. In addition to cross-presentation, autophagy is the process of endogenous antigens being presented via MHC II.

Before antigens can be presented, antigen processing must first occur (Kotsias *et al.*, 2019). Antigen processing differs between exogenous and endogenous, and between MHC I and II. For MHC I, endogenous proteins are cleaved via the proteases and the proteasome in the cytosol and are then transported to the endoplasmic reticulum (ER) via the transporter associated with antigen processing (TAP) for further processing (Song *et al.*, 1996). Here, they are assembled with MHC I and, if bound with high affinity, are subsequently transported to the cell surface for presentation via the Golgi apparatus. For MHC II, exogenous antigens are internalised via endocytosis where they are enclosed within endosomes which activate proteases for degradation (Roche *et al.*, 2015). MHC II molecules are transported into the endocytic vesicles where they bind the antigen and subsequently travel to the cell surface for presentation.

TCRs are able to recognise a wide range of antigens due to their diverse antigen-binding fragments (Mariuzza *et al.*, 2020). These portions are known as variable (V) regions, and exons coding this region are known as variable (V), diversity (D) or joining (J) (Honjo *et al.*, 1981). It is the recombination of these three regions that is critical to produce antigen receptor diversity. Due to V(D)J recombination during thymic development, T-cells express a wide range of TCRs, each with unique specificity. This diversity allows for the recognition of a wide range of antigens in a specific nature. The TCR itself is a membrane bound protein that usually consists of highly variable α and β chains that are expressed as part of the TCR complex (Mariuzza *et al.*, 2020). The α chain is involved in V and J recombination owing to V and J genes in the α gene locus, with the β chain involved in V(D)J recombination owing to D genes in the β gene locus. This process is regulated by the recombinant activating genes (RAG) 1 and 2. On rare occasions, variable γ and δ chains are expressed instead of α and β chains, these are referred to as $\gamma\delta$ T-cells (Morath and Schamel, 2020). The cluster of differentiation 3 (CD3) molecule is also part of the TCR complex, though this is invariable (San Jose *et al.*, 1998). CD3 is a protein complex comprised of CD3 $\gamma\epsilon$ -CD3 $\delta\epsilon$ -CD3 $\zeta\zeta$, this is known as a signalling hexamer (Dong *et al.*, 2019).

1.1.4 T-cell activation

As described, antigen-specific T-cell activation is dependent on TCR recognition of antigen bound to MHC molecules on the surface of APCs, including DCs and macrophages (Sharma and Allison, 2015). As illustrated in figure 1.1, full T-cell activation only occurs if TCR engagement with an MHC-antigen complex occurs alongside a second co-stimulatory signal, in the presence of a third 'danger' signal, e.g. heat shock proteins (Prestwich *et al.*, 2008). The second stimulus is formed by

binding of CD27/CD28 on the T-cell to CD80/CD86 on an APC. In the context of cancer, full T-cell activation is critical to produce an antigen-specific anti-tumour response; this is associated with an enhancement in the expression of cell surface activation markers, e.g. the early activation marker CD69 (Simms and Ellis.,1996) and release of inflammatory cytokines, e.g. IFN- γ .

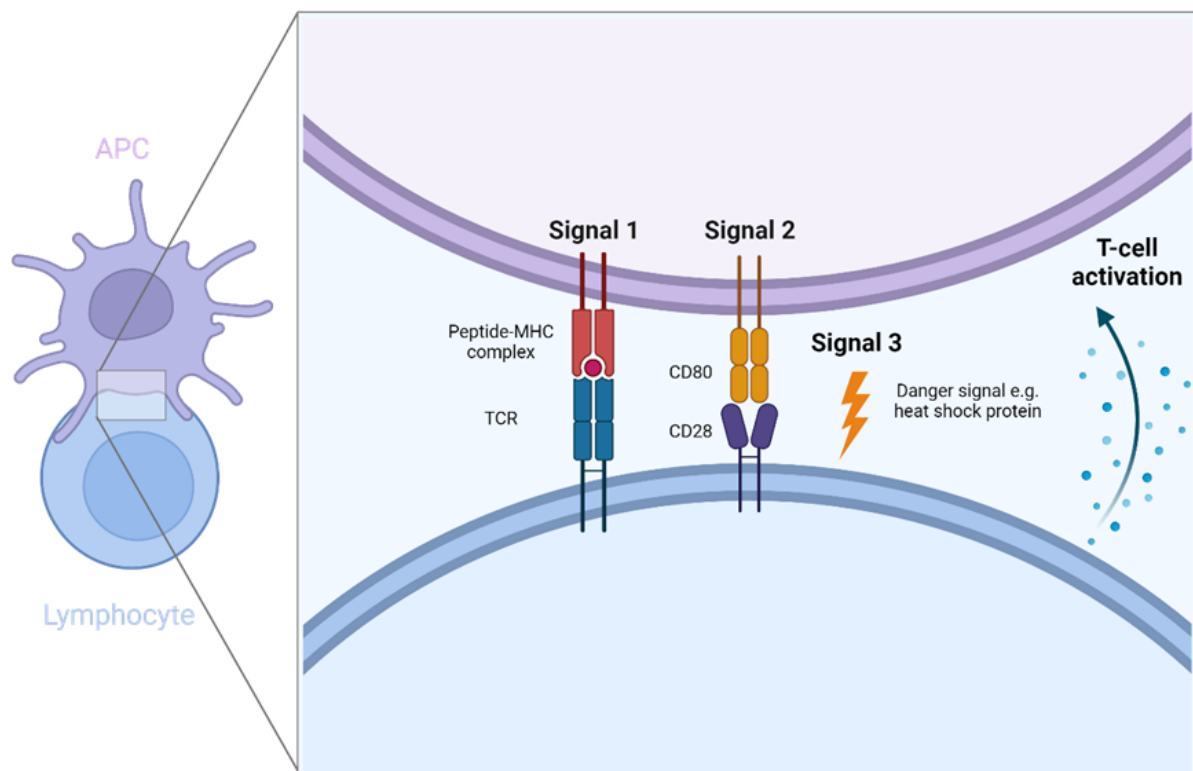


Figure 1.1. Illustration of T-cell activation via TCR ligation. TCR on the surface of T-cells, in this case CD4⁺ T-cells, recognise peptide-MHC complexes, in this case MHC II, on the surface of professional APCs. This TCR ligation provides signal 1. Signal 2 is provided by the binding of co-receptor, CD28 to its ligand, CD80. If this binding occurs during a third, danger signal, then full T-cell activation occurs. Made in Biorender.com.

In addition to TCR recognition and co-stimulatory signals, co-inhibitory signals are also critical in regulating the control of T-cell activation and proliferation. Upon the activation of T-cells, inhibitory pathways are induced that can terminate T-cell responses. This mechanism of negative regulation ensures control when reacting to a foreign antigen, only allowing a T-cell response to persist for the necessary period (Topalian *et al.*, 2015). Examples of these negative regulatory pathways that are commonly studied include the cytotoxic T lymphocyte antigen-4 (CTLA-4) and programmed cell death 1 (PD-1) molecules (Sharma and Allison, 2015). Both receptors are upregulated on the surface of T-cells following T-cell activation. The binding of these receptors to their corresponding ligands situated on APCs, CD80/CD86 and programmed cell death ligand 1 (PD-L1), respectively, inhibits expansion and activation of T-cells, thereby controlling the immune response (Smith-Garvin and Koretzky., 2009). It has also been reported that PD-L1, the known ligand of PD-1, is able to bind CD80, a known CTLA-4 ligand (Zhao *et al.*, 2019). PD-L1 and CD80 are able to heterodimerize in cis formation and this binding, in turn, prevents the binding of PD-L1 and CD80 with their respective binding partners, PD-1 and CTLA-4 (Zhao *et al.*, 2019).

1.2 Immune system and cancer

Though not included in the initial six hallmarks of cancer in 2000, the importance of the crosstalk between cancer cells and immune cells was incorporated into the emerging hallmarks of cancer in 2011 (Hannahan and Weinberg, 2000, Hannahan and Weinberg, 2011 and Hannahan, 2022). One key aspect of the immune system and cancer includes preventing cancer development through immunosurveillance, now suggested as the elimination phase of immunoediting (section 1.2.1). Once tumorigenesis has occurred, the immune system has an important role in controlling the tumour where possible. Increased expression of tumour associated antigens

(TAAs) and the release of DAMPs and PAMPs (section 1.2.2) aids the immune system in mounting immune responses against the tumour, but immune evasion is still a key characteristic of cancer (Hanahan and Weinberg, 2011).

1.2.1 Immunoediting

Previously coined immunosurveillance, the more refined theory of immunoediting consists of three main phases; elimination, equilibrium and escape (Figure 1.2; Dunn *et al.*, 2002 and Dunn *et al.*, 2004). The first phase, elimination, most likely resembles the theory of immunosurveillance and consists of the destruction of transformed cells by innate and adaptive mechanisms including IFN- γ , perforin and recognition molecules including NKG2D (Mittal *et al.*, 2014). Here, the immune system outcompetes the cancer cells. In some cases, tumour cells may survive this phase of immune-mediated killing and the second phase of immunoediting is entered; equilibrium. At this point, the immune system and the tumour is balanced, with effective immune control holding the tumour dormant. Through a variety of mechanisms, including reduced immune recognition and increased survival, tumour cells can escape this immune-control and the third phase of immunoediting is entered; escape. Here, the balance favours cancer and tumour progression occurs. In addition to reduced immune recognition (e.g. loss of MHC I) and increased survival (e.g. increased expression of anti-apoptotic molecule Bcl2), upregulation of immunoregulatory molecules such as PD-1/PD-L1 and LAG-3 is associated with tumour-cell escape (Mittal *et al.*, 2014).

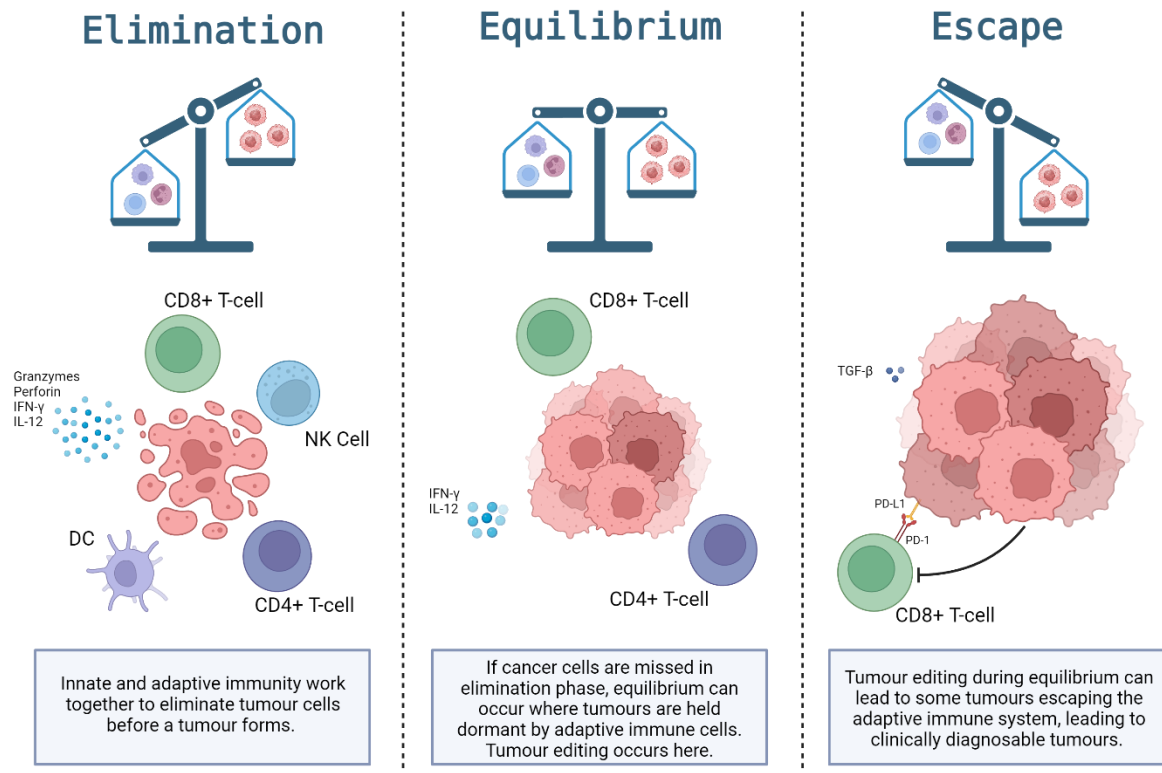


Figure 1.2. Illustration of immunoediting. There are three phases to immunoediting: elimination, equilibrium and escape. In elimination phase, the innate and adaptive immune systems work together to eliminate transformed cells before a tumour forms. Some cells are missed during this phase, and can replicate. This can lead to equilibrium, where the tumour is controlled via the adaptive immune system and is in a dormant state. During this phase, immunoediting occurs, where transformed cells can further mutate to begin to evade the immune system. This enters the escape phase, where a clinically detectable tumour is formed that has characteristics of immune evasion, including the upregulation of co-inhibitory ligands, e.g. PD-L1. Adapted concept from Dunn *et al.* (2004). Made in Biorender.com.

1.2.2 DAMPs/PAMPs

PAMPs or DAMPs are released by cells in times of stress or upon cell death and subsequently induce inflammation (Tang *et al.*, 2012 and Ahmed and Tait, 2020). The term DAMPs incorporates a number of immune mediators including heat shock proteins, ATP and calreticulin that are usually retained within the cell under normal conditions and are released in response to cell stress or cell death. Their release potently induces an immune response via DAMP receptors. DAMPs were initially

thought to be released from necrotic cell death, though immunogenic cell death (ICD) following immunotherapy treatments like oncolytic viruses are also known to induce the release of DAMPs. The release of DAMPs is associated with anti-tumour responses but also DAMP-induced inflammation which, in the absence of an effective anti-tumour immune response, can lead to tumour promotion (Vaes *et al.*, 2021).

1.2.3 Tumour-infiltrating lymphocytes

Immune cells within the TME are important for tumour progression/control, with tumour associated immune cells having potentially tumour-antagonizing or tumour-promoting roles. Tumour-antagonizing immune cells primarily incorporate effector T-cells, including CD4⁺ and CD8⁺ T-cells, NK cells, DCs and macrophages (Lei *et al.*, 2020). CD8⁺ T-cells are known for their cytotoxic activity against cancer cells due to MHC I recognition on their cell surface. In addition, CD8⁺ T-cells are activated via antigen presentation on DCs, thus inducing their cytotoxic activity. The activity of CD4⁺ T-cells is less direct, but they are known to directly help CD8⁺ T-cell activation via CD40L binding in addition to promoting the proliferation of CD8⁺ T-cells via IL-2 production (Lei *et al.*, 2020). Similarly to CD8⁺ T-cells, NK cells elicit cytotoxic effects against tumour cells via the release of perforin and granzymes. In addition, they are known to secrete pro-inflammatory cytokines to promote anti-tumour immune effects. Tumour associated macrophages, particularly M1-type, are important when considering tumour microenvironments and play critical roles in mediating cytotoxicity against tumour cells (Pan *et al.*, 2020).

On the other hand, tumour-promoting immune cells, including Tregs and tumour-associated neutrophils, enhance tumorigenesis (Tanaka and Sakaguchi, 2019). Tregs,

characterised as CD4⁺ FoxP3⁺ T-cells, have a critical role in maintaining immune homeostasis and tolerance (Freeborn *et al.*, 2022). FoxP3 is critical for their regulation of suppressive signalling. Their suppressive effects, whilst beneficial for preventing over-reactive immune responses in auto-immunity, has negative effects within the TME due to limiting effector T-cell functions against cancer cells (Lei *et al.*, 2020). An emerging form of cancer treatment is immunotherapy, acting via harnessing immune cells within or outside the TME to elicit anti-tumour effects. In addition to Tregs, some subsets of tumour-associated neutrophils are linked tumour promotion due to their expression of the inhibitory ligand, PD-L1 (Yan *et al.*, 2022).

1.3 Immune checkpoint receptors and ligands

Immune checkpoint receptors are surface receptors on immune cells that can have co-stimulatory or co-inhibitory effects when bound to their corresponding ligands (Figure 1.3.). Cancer cells are known to inhibit effector T-cells in the TME by upregulating inhibitory ligands, such as PD-L1, that bind to co-inhibitory checkpoints, e.g. PD-1, on immune cell surfaces (Zhang *et al.*, 2020). This interaction can lead to effector T-cells entering exhausted phenotypes, ultimately leading to T-cell dysfunction and tipping the balance of cancer/immunity in favour of tumour progression. In addition to PD-1/PD-L1 or PD-L2, other examples of co-inhibitory receptor/ligand pathways include CTLA-4/CD80 or CD86, LAG-3/MHC molecules and TIM-3/galectin-9 (Qin *et al.*, 2019). The binding of these receptors on the surface of T-cells, to their corresponding ligands on the surface of APCs or tumour cells, elicits inhibitory effects on T-cell activation. In contrast, the binding of costimulatory receptors, such as OX40 (also known as tumour necrosis factor receptor superfamily member 4, or TNFRSF4, and CD134), to their corresponding ligands, such as OX40L (also known as TNFSF4 and CD252), has

costimulatory effects (Lu, 2021). Other costimulatory receptor/ligand pathways include ICOS/ICOSL, CD27/CD70 and GITR/GITRL. Within the tumour microenvironment, checkpoint ligands are expressed on the surface of both tumour cells and immune cells. For example, PD-L1 is often induced on the surface of multiple types of immune cells by IFN γ . The cell types include, but are likely not limited to, monocytes, macrophages and neutrophils (Moon et al., 2021).

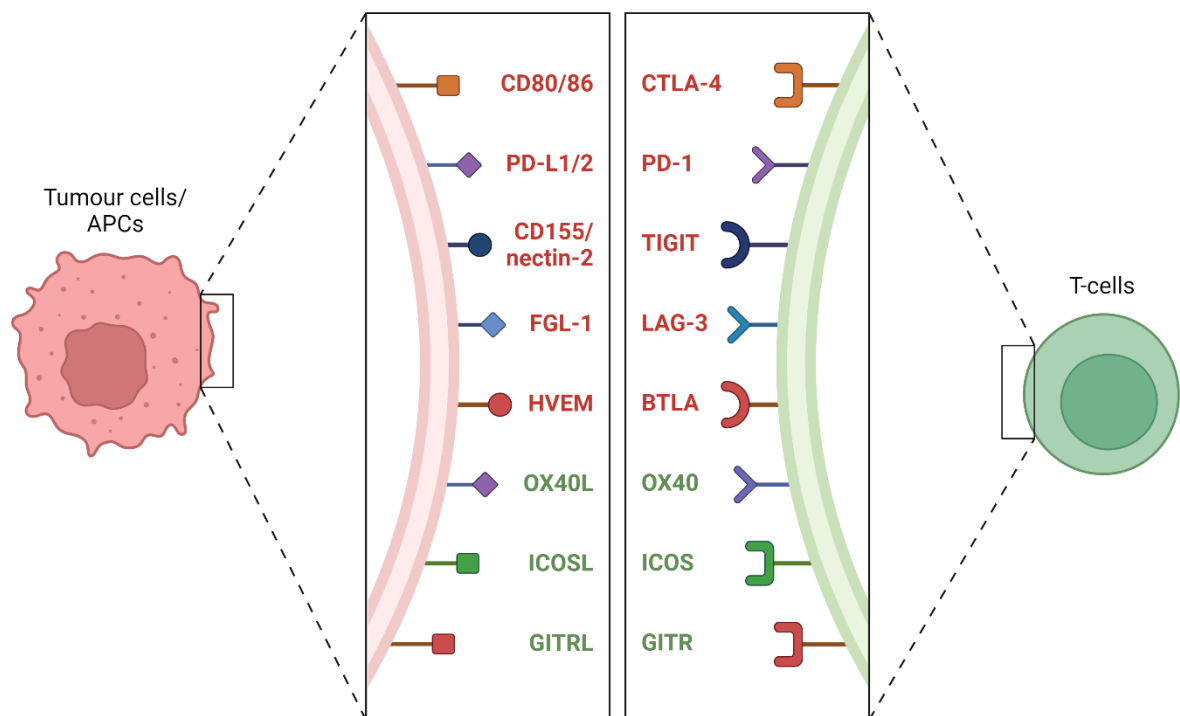


Figure 1.3. Illustrative example of co-inhibitory and co-stimulatory checkpoint pathways. Co-inhibitory (red) and co-stimulatory (green) receptors on the surface of T-cells (right) bind to their corresponding ligands on the surface of APCs or tumour cells (left). Made in Biorender.com.

Immune responses are a balance between inhibitory and stimulatory signals, for example, when stimulatory signals outweigh inhibitory signals in the presence of TCR-ligation, an effective immune response is mounted.

1.4 T-cell exhaustion

Cancer is associated with T-cell exhaustion, a state of T-cell dysfunction that is defined by poor effector functions and loss of self-renewal capacity (Chow *et al.*, 2022). Immune cell exhaustion is commonly observed in response to prolonged antigen exposure and is characterised by a progressive loss of function, e.g. CD8⁺ T-cells losing their ability to kill target cells during chronic viral infection (Wherry, 2011). T-cell exhaustion is often associated with increased expression of co-inhibitory receptors (see section 1.3). Whilst phenotypically similar to anergy, exhaustion occurs over a longer period and is progressive over time as opposed to anergy which is observed to be induced rapidly post-stimulation (Schietinger and Greenberg, 2013). T-cell anergy has been shown to be driven by impaired antigen presentation, reduced costimulation and the expression of immunomodulatory molecules (Rodrigues *et al.*, 2014). T-cell exhaustion on the other hand is typically driven by antigen persistence and chronic activation (Rodrigues *et al.*, 2014). One key mechanism of action of ICM therapy is to reinvigorate T-cells towards activation and to reverse the cancer-related exhausted phenotype.

It is important to note that whilst earlier studies have highlighted shared features between dysfunctional T-cells in cancer, specifically CD8⁺ T-cells, these cells have subsequently been shown to be distinct from a state of exhaustion (Thommen and Schumacher, 2018, Ahmadzadeh *et al.*, 2009 and Baitsch *et al.*, 2011). A population of progenitor exhausted CD8⁺ T-cells were found to share dysfunctional exhaustion features but were able to respond to PD-1 blockade (Miller *et al.*, 2019).

1.5 Immune Checkpoint Modulation

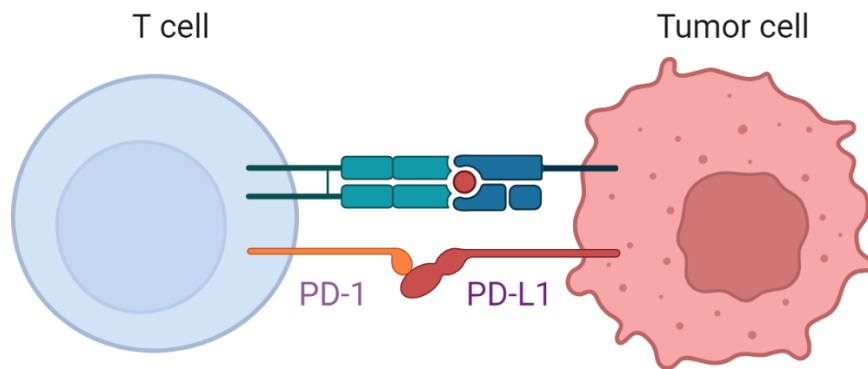
Cancer development is associated with immune suppression that hinders anti-tumour responses and tumour clearance (Smith-Garvin and Koretzky, 2009). Cancer can escape the immune system by inhibiting the recognition of tumour associated antigen by T-cells and also by triggering the dysfunction of cytotoxic T-cells (Pardoll., 2012). In the tumour microenvironment, T-cells are incessantly exposed to antigen stimulation, which is associated with enhanced expression of checkpoint molecules, like B and T-cell lymphocyte attenuator (BTLA) and T-cell immunoglobulin and mucin-domain containing-3 (TIM-3). Increased expression of inhibitory checkpoint molecules can lead to downregulation of T-cell activity. This is further exploited by an amplified expression of inhibitory ligands, like PD-L1, on tumour cells (Rotman *et al.*, 2020). These regulatory molecules have, likewise, been shown to have an increased expression on cancer cells within tumour microenvironments. These findings, amongst others, have provided the foundation for immune checkpoint inhibitors and their use as a therapeutic mediator for cancer treatment (Rotman *et al.*, 2020).

1.5.1 Antibody-mediated checkpoint blockade

To date, immune checkpoint antibodies primarily target pathways responsible for regulating T-cell activation (Pardoll., 2012). Checkpoint blockade of inhibitory immune receptor-ligand interactions boosts a generalised immune response, which, in turn, can enhance the natural anti-tumour immunity that is downregulated by tumours (Pardoll., 2012). Both the PD-1 receptor and its ligand, PD-L1, have EMA approved inhibitors for use in metastatic cancers, including melanoma and renal cancers (Topalian *et al.*, 2015). Antibody-mediated PD-1/PD-L1 pathway blockade works by preventing the

binding of PD-1 with its ligand PD-L1 (Figure 1.4). This prevents the inhibitory effects of the PD-1/PD-L1 pathway from suppressing T-cell responses through their engagement, thus enhancing T-cell responses around/towards the tumour.

Co-inhibitory receptor/ligand interaction prevents T-cell activation



Antibody-mediated PD-1 blockade permits T cell activation

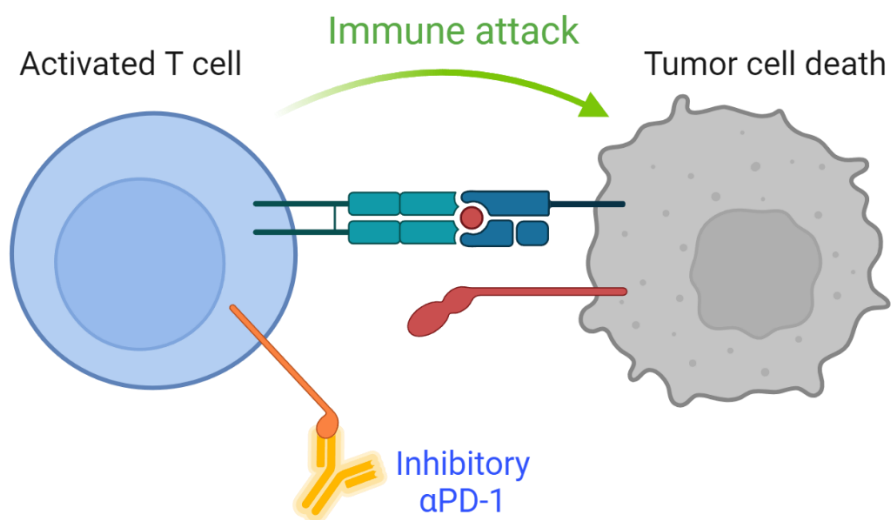


Figure 1.4. Antibody-mediated PD-1 blockade induces T-cell activation. The binding of co-inhibitory receptor, PD-1, on the surface of T-cells to its ligand on the surface of tumour cells leads to T-cell suppression (Top). Inhibition of PD-1/PD-L1 binding with therapeutic PD-1 antagonistic antibodies leads to T-cell activation by preventing this co-inhibitory signalling pathway. Made in Biorender.com.

It is widely accepted that PD-1, upon activation by its ligand, PD-L1, suppresses T-cell activation. However, there are conflicting theories behind the exact mechanism of action of PD-1, with TCR and CD28 both being potential targets for PD-1 function (Hui *et al.*, 2017 and Mizuno *et al.*, 2019). Hui *et al.* (2017) demonstrated that the CD28 co-receptor is preferentially targeted CD28 over the TCR for PD-1/Shp2-mediated dephosphorylation. Conversely, Mizuno *et al.* (2019) have recently published that PD-1 primarily targets the TCR as its mechanism of action. Their work demonstrated that PD-1 was sufficiently able to inhibit T cell activity (through cytokine production) in the absence of CD28, thus suggesting PD-1 primarily acts via another target. Their work demonstrated that T-cell activation involving ICOS co-stimulation was able to be inhibited by PD-1 at the same extent as T-cell activation involving CD28 co-stimulation (Mizuno *et al.*, 2019). This particular paper therefore suggests that the TCR is the most likely target in PD-1 function, owing to both co-stimulatory receptors relying on TCR signalling.

In addition to PD-1/PD-L1 pathway blockade, antibody-mediated CTLA-4 and LAG-3 inhibitors have been approved for clinical use by the FDA (Table 1.1). CTLA-4 and LAG-3 are both inhibitory receptors expressed by exhausted T-cells in cancer (Jiang *et al.*, 2015). CTLA-4 belongs to the CD28 immunoglobulin family and binds CD80 and CD86. CTLA-4 is known to be a tight regulator of T-cell homeostasis and in self-tolerance (Van Collie *et al.*, 2020). LAG-3 is a CD4 homolog and has roles in T-cell dysfunction, chronic viral infections and tumour responses (Aggarwal *et al.*, 2023). Antibody-mediated blockade of CTLA-4 and LAG-3 has been shown to restore T-cell function (Leach *et al.*, 1996 and Workman *et al.*, 2002).

Name	Target	Initial Cancer Type	Approval	References
Ipilimumab	CTLA-4	Advanced melanoma	2011	Hodi <i>et al.</i> , 2010
Pembrolizumab	PD-1	Unresectable or metastatic melanoma	2014	Robert <i>et al.</i> , 2014
Nivolumab	PD-1	Unresectable or metastatic melanoma	2014	Brahmer <i>et al.</i> , 2012
Atezolizumab	PD-L1	Locally advanced or metastatic urothelial carcinoma	2016	Balar <i>et al.</i> , 2017
Avelumab	PD-L1	Metastatic Merkel cell carcinoma	2017	Kaufman <i>et al.</i> , 2018
Durvalumab	PD-L1	Locally advanced or metastatic urothelial carcinoma	2017	Powles <i>et al.</i> , 2017
Cemipilimab	PD-1	Metastatic or locally advanced cutaneous squamous cell carcinoma	2018	Migden <i>et al.</i> , 2018
Dostarlimab	PD-1	Advanced endometrial cancer or mismatch repair deficient cancer	2021	Goulden <i>et al.</i> , 2023
Nivolumab and Relatlimab	PD-1 and LAG-3	Unresectable or metastatic melanoma	2022	Tawbi <i>et al.</i> , 2022

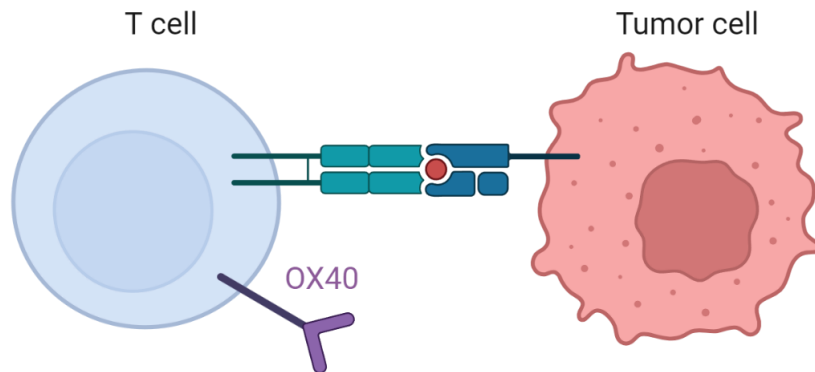
Table 1.1. FDA approved immune checkpoint inhibitors and their initial cancer targets. Nine checkpoint inhibitors have been approved by the FDA for clinical use against a range of cancers, as outlined here. Information taken from Sun *et al.* (2023).

Outlined in Table 1.1 are the initial cancer types, though multiple subsequent trials have led to subsequent approval of these drugs both as monotherapies for other cancer types, and in combination with other immunotherapies and/or other therapies. For example, clinical trials involving ipilimumab have led for its approval in combination with nivolumab for treating patients with advanced renal cell carcinoma and metastatic colorectal cancer.

1.5.2 Antibody-mediated checkpoint agonism

Whilst tumour cells upregulate their expression of co-inhibitory ligands in order to dampen the anti-tumour immune response in the TME, tumour cells are also shown to downregulate their expression of ligands for co-stimulatory molecules. In contrast to checkpoint inhibitors, that block the binding of receptors to their corresponding ligand counterparts, checkpoint agonists functionally bind to their receptor targets, initiating a co-stimulatory signal and enhancing T-cell activation (Figure 1.5).

Reduced expression of co-stimulatory ligands prevents T-cell activation



Antibody-mediated OX40 agonism permits T cell activation

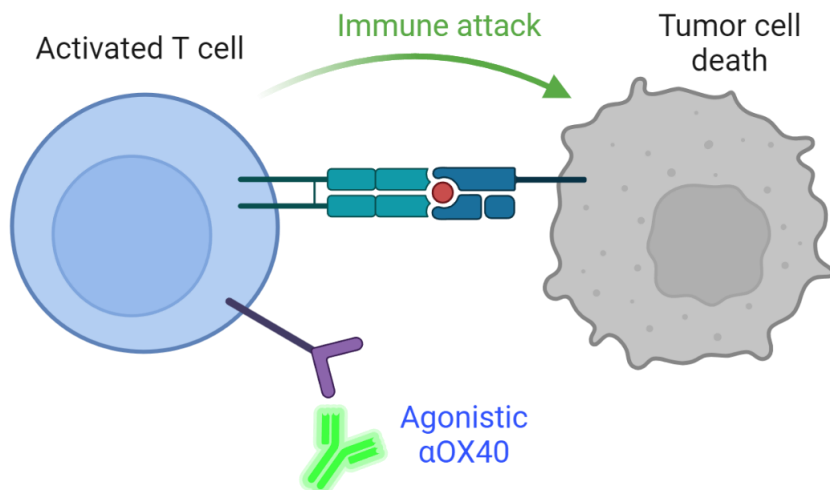


Figure 1.5. Antibody-mediated OX40 agonism induces T-cell activation. The downregulation of co-stimulatory ligands, e.g. OX40L, on the surface of tumour cells prevents receptor/ligand binding and prevents co-stimulatory signalling and T-cell activation (Top). The functional binding of co-stimulatory receptor, OX40, on the surface of T-cells by an OX40 agonistic antibody leads to a co-stimulatory signal and T-cell activation (Bottom). Made in Biorender.com.

Whilst a range of pre-clinical data suggests the use of co-stimulatory agonists, including OX40, there are currently no FDA approved checkpoint agonists suitable for clinical use. That being said, a number of trials are ongoing or have recently been completed (Davis *et al.*, 2022 and Desai *et al.*, 2023). Agonistic OX40 has been shown to elicit anti-tumour efficacy in a few ways, for example, engagement of OX40 has been shown to elicit anti-tumour effects through depletion of intratumoral Tregs (Bulliard *et al.*, 2014). In addition, agonistic OX40 has been shown to increase tumour infiltrating CD8+ T-cells (Weixler *et al.*, 2015). Results from a first-in-human phase I/II study of INCAGN01949, an OX40 agonist, showed no safety concerns but unfortunately also showed limited tumour responses in post-therapy biopsies of solid tumours (Davis *et al.*, 2022). A phase I study of a different OX40 agonist, BGB-A445, also demonstrated that the drug was well tolerated but this time also demonstrated preliminary antitumour effects (Desai *et al.*, 2023). These results are promising, but grade 3 or higher treatment-emergent adverse events were reported in 41 % of patients for BGB-A445 and 47.1 % reported for INCAGN01949.

1.5.3 Hot or cold tumour microenvironment

Whilst ICM therapies have been approved to treat a number of cancers over recent years, it is crucial to note that response rates are variable. The terms ‘hot’ and ‘cold’ are regularly utilised to describe the immunogenicity of a TME, with hot tumours responding well to clinical ICM and cold tumours often responding poorly. Several characteristics are involved in these descriptions, including a TME with increased TILs, PD-L1 overexpression and pre-existing anti-tumour immune responses for hot tumours. Cold tumours on the other hand, have opposing features, with reduced TILs,

limited PD-L1 expression and no pre-existing anti-tumour immune responses (Wang *et al.*, 2023).

For both hot and cold TMEs, therapeutic intervention with ICM aims to harness the immune system to promote anti-tumour immune effects. Due to cold tumours being associated with limited therapeutic efficacy compared with hot tumours, conversion of cold tumours to hot tumours would be advantageous to augment ICM efficacy. One example of this is radiotherapy. Radiotherapy can enhance the efficacy of antibody-mediated PD-1 blockade by promoting T-cell infiltration within the tumour, subsequently inducing immunogenic cell death (ICD) and leading to increased anti-tumour immune responses compared with PD-L1 blockade alone (Deng *et al.*, 2014). A second example of this is oncolytic virus therapy, which elicits ICD and primes the TME for subsequent ICM (Samson *et al.*, 2018). Oncolytic virus therapy, explained in further detail in research chapter 1, has been a promising treatment for cancer since the observation that some cancers regressed if infected with specific viruses (Kelly and Russell, 2007). This observation was a catalyst into a depth of research into cancer-selective viruses that specifically lyse cancer cells whilst sparing non-tumour cells. Since then, the FDA approval of T-VEC, a herpes simplex virus (HSV)-based oncolytic virus has led to a number of other viruses being evaluated (Andtbacka *et al.*, 2015).

Recently, whole-exome and transcriptome meta-analysis of over 1000 patients treated with immune checkpoint blockade revealed mechanisms of sensitisation to therapy (Litchfield *et al.*, 2021). The analysis included data across seven tumour types, including malignant melanoma and colorectal cancer where patients were treated with anti-CTLA-4, anti-PD-1 or anti-PD-L1. Of note, *CXCL9* expression was the greatest

response predictor (Litchfield *et al.*, 2021). Furthermore, there are several links between the tumour microenvironment and response to ICM therapy (Petitprez *et al.*, 2020).

1.5.4 Associated ICM-induced toxicities

ICM can lead to potentially life threatening immune-related adverse events, these are different to the toxicities associated with standard chemotherapy as they are immune-related, and are usually treated with corticosteroids. The most affected organs in the body by immune-related adverse events are the skin, the endocrine glands, liver and gastrointestinal system (Gumusay *et al.*, 2022 and Conroy and Naidoo, 2022). These events are believed to be derived from the mechanism of action of ICM, with their function being prolonged activation of adaptive immunity. Studies have proposed that these adverse effects are mediated through a combination of factors, including cytokines, autoantibodies and autoreactive T-cells. These adverse events are an important consideration when thinking about designing ICM therapy options.

When considering antibody-mediated therapies, such as CTLA-4 and PD-1 blockade which are among the most studied inhibitory pathways and have rapidly become standard of care for a number of cancer types, it is important to consider these adverse events. For example, a phase III trial with combinatorial nivolumab (anti-PD-1) and ipilimumab (anti-CTLA-4) for the treatment of melanoma showed an objective response rate of 57.6 %, which was greater than either monotherapy (Larkin *et al.*, 2015). This seems promising, but 55 % of patients suffered from adverse events at grade 3 or higher, unfortunately meaning that a number of patients likely suffered severe side effects, whilst receiving very limited benefit from the treatment.

1.5.5 Increasing efficacy of ICM therapies

Despite recent advances in immunotherapy, single-agent ICM therapy has shown limited clinical efficacy (Sharma *et al.*, 2021). The future of immunotherapy is thought to now lie with combination therapy, including combination antibody-mediated treatments, combination of ICM with standard chemotherapy/radiotherapy, and potential combination of antibody-mediated treatments with other ICM treatments like oncolytic viruses. The first FDA approved combination immunotherapy was for BRAF^{WT} metastatic melanoma to be treated by a combination of Nivolumab (anti-PD-1) and Ipilimumab (anti-CTLA-4). In addition to combining immune checkpoint blockades, in 2018, the FDA approved the combination of Durvalumab (anti-PD-L1) with chemoradiation for the treatment of Stage III non-small cell lung cancer. Chemoradiation refers to the combination of chemotherapy and radiation and has become an established treatment for solid tumours (Rallis *et al.*, 2021). Combining chemoradiation with ICM therapy has the potential to synergise and augment anti-tumour activity (Schirrmacher *et al.*, 2019). In addition, combination treatment with vascular endothelial growth factor (VEGF) inhibitors (e.g. Bevacizumab) have also been approved, for example the treatment of first-line unresectable hepatocellular carcinoma (HCC) with the combination of Atezolizumab (anti-PD-L1) with Bevacizumab (anti-VEGF) was approved by the FDA in 2020. Anti-VEGF drugs interfere with VEGF, which is a protein responsible for stimulating the growth of new blood vessels. Anti-VEGF treatments like Bevacizumab therefore prevent angiogenesis, which when in combination with other ICM therapies like anti-PD-L1 can lead to enhanced anti-tumour efficacy by potentially increasing CD8⁺ T-cell infiltration (Ishikura *et al.*, 2022). Though not currently approved, combinations of antibody-mediated immunotherapy with oncolytic viruses are also of particular interest since the

FDA approval of single-agent T-VEC in 2015 for the treatment of metastatic melanoma. Subsequent evidence suggests that treatment with oncolytic viruses can prime the tumour microenvironment for subsequent antibody-mediated therapy (Samson *et al.*, 2018).

1.6 Experimental models of cancer

Effective and reliable pre-clinical models of cancer are crucial for the exploration of novel therapies, including the recent advances in ICM. There are several models widely accepted, including *in vitro* systems (e.g. cancer cell lines) and *in vivo* models (e.g. genetically engineered mice). Each models have their benefits and limitations, as discussed in sections 1.6.1 and 1.6.2.

1.6.1 *In vitro* cell culture

The most widely used *in vitro* system for cancer research is the two-dimensional (2D) monolayer cancer cell line. Several benefits of the cancer cell line include ease of use, cost effectiveness, limited cellular heterogeneity and high proliferation rates (Sajjad *et al.*, 2021). In addition to these benefits, high throughput screening of different therapeutics can occur utilising cancer cell lines, and often several cell lines exist for once cancer type, allowing for independent validation of therapies in different cell line models. The limitation of cancer cell lines alone, particularly when considering ICM therapies, include limited cell-cell interactions and poor modelling of a TME. For example, lack of stromal components including ECM and associated immune cells.

One recent advancement for *in vitro* cell culture to circumnavigate these issues are organoids (Kretzchmar, 2020). Organoids are three-dimensional (3D) structures that are derived from pluripotent, tissue-resident stem cells or from tumour cells (Zhao *et*

al., 2022). Patient-derived organoids can be generated from normal or cancer tissue, with an ability to match patient samples for increased power. Organoids more closely mimic an organ or tumour than standard 2D cultures due to the presence of extracellular matrix (ECM).

1.6.2 *In vivo* models

The most widely used *in vivo* system in pre-clinical cancer research is the genetically engineered mouse model. Sharing up to 99 % of the human genome, mice are a widely accepted *in vivo* model for several diseases, including cancer. As with any type of model, *in vivo* models have both advantages and disadvantages. For example, compared with standard *in vitro* methodologies, *in vivo* models tend to be more costly and more time consuming, with tumour models typically lasting several weeks. That being said, there are several benefits to using an in-tact organism for experimental research, including increased reliability for observing biological effects that include considerations often not explored *in vitro*, e.g. drug excretion/metabolism. One potential limitation to *in vivo* work is the strict regulations that oversee all animal work, the obtaining of which may be time consuming. It is also worth noting that drug candidates are required to undergo animal testing in order to evaluate toxicity, metabolism and efficacy prior to testing in clinical trials. Thus, whilst *in vitro* work is crucial to exploring potential treatment options in a cost- and time-effective manner, there is no current replacement for pre-clinical *in vivo* work.

When studying T-cell responses to ICM therapies, TCR reporter systems are a crucial tool. Elliot *et al.* (2021) lay the foundation for using adaptive tolerance models to

explore mechanisms of ICM therapies, a model chosen for use in this research project and a concept further explored in Research Chapter 2.

1.7 Areas of unmet need

Whilst the field of immunotherapy has revolutionised the treatment of cancer in the past decades, current treatments still have limited efficacy and are associated with several immune-mediated adverse events. Research is therefore needed to develop specific combination therapies, looking carefully at their effects on key immune components, especially T-cells. It is vital that these combinations are rationally sought, with considerations in both timing and expression of ICM targets. This project is divided into uniquely specific chapters, the first chapter was conducted at the University of Leeds and aims to combine oncolytic reovirus treatment with subsequent antibody-mediated ICM for the treatment of hepatocellular carcinoma. The second chapter, conducted at the University of Birmingham, aims to seek a rational antibody-mediated ICM therapy for combination with α PD-L1 blockade utilising an immunogenic cancer model.

2 MATERIALS AND METHODS

2.1 *In vitro*

2.1.1 Tissue Culture

Tissue culture was performed using aseptic methodologies in a class II biological safety cabinet with laminar flow (Nuaire or SafeFAST). Virkon (ThermoFisher Scientific) at 2 % (w/v) was used to clean the cabinet and 70 % (v/v) ethanol (Sigma-Aldrich) was used to maintain sterility of equipment. All plasticware were purchased in sterile packaging from Corning Costar unless otherwise stated. Centrifugations for all tissue culture methodologies were performed at 400 g for 5 minutes in an Eppendorf 5810 or Thermofisher Megafuge ST plus centrifuge (acceleration 9, brake 9), unless otherwise stated. Cells were maintained in a Sanyo incubator at 37 °C with 5 % CO₂. Cell counts were performed by trypan blue (Sigma-Aldrich) exclusion method using a Nikon Eclipse TS100 or Motic AE 2000 microscope and a standard haemocytometer (Marienfield) or Fast-Read® 102 (BioSigma). Culture media for tissue culture and freezing media for long-term storage of both cell lines and primary cells are described in Table 2.1.

Cell Type	Medium Composition
JHH-4 Culture Medium (Human HCC cell line)	Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10 % (v/v) foetal calf serum (FCS; heat-inactivated at 56 °C for 30 minutes). Both Sigma-Aldrich. (Complete DMEM)
1MEA Culture Medium (Murine HCC cell line)	Rosswell Park Memorial Institute 1640 (RPMI-1640; Sigma-Aldrich) supplemented with 10 % (v/v) heat-inactivated FCS. (Complete RPMI)
Hep53.4 Culture Medium (Murine HCC cell line)	Complete DMEM
MC38 Culture Medium (Murine colorectal carcinoma cell line)	Complete RPMI
PBMC Culture Medium	Complete RPMI
Primary Hepatocyte Culture Medium	Complete RPMI
Cell line and PBMC Freezing Medium	FCS supplemented with 10 % (v/v) dimethylsulfoxide (DMSO) (FM1)
Primary Hepatocyte Freezing Medium	RPMI-1640 supplemented with 50 % (v/v) human serum (SeraLab) and 10 % (v/v) DMSO (FM2)

Table 2.1. Media compositions for culture and freezing of primary cells and cell lines. Details of both culture and freezing media for primary cells and cell lines used throughout.

2.1.2 Human Cell Lines

One human HCC cancer cell line (JHH-4, Japanese Collection of Research Bioresources Cell Bank; Hasumura *et al.*, 1988) was utilised as described throughout. Cells were allowed to reach 70-80 % confluency, washed with phosphate buffered saline (PBS; Sigma-Aldrich), coated with PBS-EDTA (Sigma-Aldrich) for 5 minutes at 37 °C and passaged as appropriate using Trypsin-EDTA (Sigma-Aldrich).

2.1.3 Murine Cell Lines

Two murine HCC cancer cell lines (Hep-53.4, C57BL/6 derived and 1MEA, Balb/c derived, Cytion) and one colorectal cancer cell line (MC38, C57BL/6 derived, Kerafast) were utilised as described throughout. Cells were allowed to reach 70-80 % confluency, washed with Dulbecco's phosphate-buffered saline (dPBS; Gibco) and passaged as appropriate using 1x Trypsin-EDTA (Gibco).

2.1.4 Isolation and Culture of hPBMCs

Leucocyte cones as a source of blood were obtained from healthy donors from the National Health Service Blood and Transplant unit (Seacroft Hospital, Leeds); donors gave full informed consent. Density gradient centrifugation was performed on diluted blood to isolate PBMCs as previously described (Alter *et al.*, 2004) and cells were cryopreserved in FM1 until use. Thawed PBMCs were seeded at 2×10^6 cells/ml in complete PBMC RPMI for use as described.

2.1.5 Isolation and Culture of Human Primary Hepatocytes

Ex vivo normal tissues were obtained from patients undergoing routine planned cancer-related surgery at St. James' University Hospital, Leeds, UK. Written and informed consent was obtained in accordance with local institutional ethics review and approval. Patient information and consent form version 4.1 dated 22 Feb 2016. HRA number 06/Q1206/106.

Livers were minced with scalpels and mechanically pressed through 70 μ M cell strainers, washed with 10 ml 10 % FBS RPMI (v/v) and centrifuged at 400 g for 5 minutes. Red blood cell (RBC) lysis (eBioscience™) was then performed on ice for 3

minutes according to manufacturer's instructions and quenched with 10 % FBS RPMI (v/v). Cells were centrifuged at 400 g for 5 minutes, washed once, and resuspended in an appropriate volume of FM2 for storage at -80 °C until use.

2.1.6 Virus Stock and Treatments

Reovirus Type 3 Dearing Strain (Oncolytics Biotech Inc.) was stored at a stock concentration of 2×10^9 plaque forming unit (pfu)/ml. Virus stocks were stored in cryovials at -80 °C for long term storage or 4 °C for shorter periods. Treatment conditions were 1 or 4 pfu/cell for time periods indicated throughout for *in vitro*, and 1×10^7 pfu for *in vivo*.

2.1.7 Generating Conditioned Medium and Treatment of PBMCs

PBMCs were treated with 4 pfu/cell reovirus for 48 hours and is defined as reovirus conditioned medium (RCM) throughout, or with PBS as a negative control to generate conditioned medium (CM). Samples were harvested, centrifuged at 400 g for 5 minutes and cell-free supernatants were collected and passed through a Viresolve™ Optiscale™ 25 capsule filter (Millipore) in order to remove any virus before use. RCM and CM were stored at -20 °C until required and were used to treat PBMCs at 5 % (v/v) in complete RPMI.

2.1.8 Transwell Assays

PBMCs were co-cultured with either non-malignant primary hepatocytes or JHH-4 cells for up to 72 hours using 0.4 µm transwell plates (Corning Costar). Cultures were treated with either 1 pfu/cell reovirus or PBS and analysed by flow cytometry for a panel of checkpoint receptors over a period of 72 hours. The pore size of the transwell

plates allowed the virus and virus-induced cytokines to pass through from the hepatocytes/cell line to the PBMCs but did not allow cellular transfer between compartments.

2.2 *In vivo*

2.2.1 Ethical Statement

All animal experiments were approved by the local animal welfare and ethical review body (AWERB) and authorised under the authority of Home Office licenses P0CFA732A, P18A892E0A, PP3965017 and PP9984349. Animals were housed in pathogen-free conditions.

2.2.2 Mouse Strains

Several mouse strains were utilised throughout this thesis. Details of the mice strains used can be found in Table 2.2 and strains are utilised as specified throughout.

Name	Modification	Obtained	Key references
C57BL/6	None	Charles river laboratories	
BALB/c	None	Charles river laboratories	
Tg4 Tiger	Tg4 TCR transgenic (specific for MBP peptide), crossed with IL-10-IRES-GFP knock in reporter mice	Wraith Lab	Burton <i>et al.</i> , 2014 and Liu <i>et al.</i> , 1995
Nur77-Tempo	BAC with Nr4a1 modified to express FT Fast (Timer) from the first coding exon	Bending Lab	Elliot <i>et al.</i> , 2022
Nr4a3-Tocky	BAC containing Nr4a3 gene modified to swap first coding exon for FT Fast (Timer) protein	Ono Lab, via MTA	Bending <i>et al.</i> , 2018 and Jennings <i>et al.</i> , 2020
Nr4a3-Tocky Tg4 Tiger (IL-10-IRES-GFP)	Nr4a3-Tocky crossed with Tg4 Tiger	Bending Lab	Jennings <i>et al.</i> , 2020 and Elliot <i>et al.</i> , 2021

Table 2.2. Mouse strains and their associated genetic modifications utilised throughout this study. List of WT and genetically modified mice strains utilised experimentally throughout this study, along with their associated genetic modifications where appropriate. Further details can be found in the key references.

2.2.3 Syngeneic Subcutaneous Tumour Models

Three murine cell lines, described in section 2.1.3 were implanted subcutaneously (cell densities are described throughout and prepared in 100 μ L PBS unless otherwise stated) into the right flank of syngeneic mice for tumour growth studies evaluated over 7-25 days as individually defined.

2.2.4 Intravenous Reovirus Treatment *in vivo*

Oncolytic reovirus, as described in section 2.1.6, was injected intravenously at 1×10^7 pfu at specified time points into 1MEA-BALB/c or Hep53.4-C57BL/6 syngeneic subcutaneous tumour models.

2.2.5 Intraperitoneal Checkpoint Modulation *in vivo*

ICM was achieved *in vivo* through I.P injection (200 μ L) of antagonistic (PD-L1) or agonistic (OX40 and ICOS) mABs at defined doses (see individual figure legends) diluted in PBS. Details of the antibodies and appropriate isotype controls utilised are defined in table 2.3.

Antigen	Antagonistic or agonistic	Clone	Species	Isotype	Obtained
PD-L1	Antagonistic	MIH5	Rat	IgG2a	Provided from Prof. Anne Cooke, University of Cambridge but held under MTA with Miyuki Azuma, Japan
OX40	Agonistic	OX-86	Rat	IgG1	Ultra Leaf Purified, Biolegend
ICOS	Agonistic	C398.4A	Armenian hamster	IgG	InVivo mAb™
Isotype control	N/A	MAC221	Rat	IgG1	Kind gift from Prof. Anne Cooke, University of Cambridge
Isotype control	N/A	MAC219	Rat	IgG2a	Kind gift from Prof. Anne Cooke, University of Cambridge
Isotype control	N/A	Polyclonal	Armenian hamster	IgG	InVivo mAb™

Table 2.3. Details of agonistic and antagonistic antibodies used for checkpoint modulation *in vivo*. Details of the antigens targeted, alongside the clones, species and isotypes of the therapeutic antibodies utilised for *in vivo* modulation of checkpoints.

2.2.6 Subcutaneous Peptide Treatment

Tg4 transgenic mice react to specific acetylated N-terminal region of murine myelin basic protein (MBP) and displays high affinity for MHC due to substitution of the native lysine (K) at position 4 for tyrosine (4Y-[MBP]) peptide. This peptide substitutes tyrosine (Y) at position 4 in place of the native lysine binds to MHC class II with much higher affinity than the native lysine (Fairchild *et al.*, 1993). Initially, mice were given S.C. doses of 80 µg (strong) or 8 µg (moderate) 4Y-[MBP]. This method was refined to use a weight normalised dose to further reduce variation: doses delivered were 4 mg/kg (strong) and 0.4 mg/kg (moderate).

2.2.7 Schedule 1

The humane killing of mice through dislocation of the neck was performed in accordance with schedule 1 of the Animals (Scientific Procedures) Act 1986.

2.2.8 Tissue Processing

Tissues were harvested as appropriate and collected in 10 % FBS RPMI (v/v) on ice until tissue processing.

2.2.8.1 Lymph Nodes

Lymph nodes (LNs) were mechanically pressed through 70 µm cell strainers (Griener BioOne) and washed with 3 ml PBS (Gibco), centrifuged at 400 g for 5 minutes and resuspended in an appropriate volume of FACS buffer for immunophenotyping as described in section 2.5.

2.2.8.2 Spleens for ex vivo analysis

Spleens for *ex vivo* analysis were mechanically pressed through 70 µM cell strainers, washed with 10 ml 10 % FBS RPMI (v/v) and centrifuged at 400 g for 5 minutes. Red blood cell (RBC) lysis (eBioscience™) was then performed on ice for 3 minutes according to manufacturer's instructions and quenched with 10 % FBS RPMI (v/v). Cells were centrifuged at 400 g for 5 minutes, washed once, and resuspended in an appropriate volume of FACS buffer for immunophenotyping as described in section 2.5.

2.2.8.3 Spleens for In vitro Culture

Spleens for *in vitro* culture were processed in sterile conditions and were enzymatically digested in digestion buffer (RPMI 1 % FBS (v/v), 1 mg/ml collagenase D (Sigma-Aldrich), 0.1 mg/ml DNase I (Sigma-Aldrich)) at 37 °C for 30 minutes prior to RBC lysis as previously described in section 2.2.8.2. Cells were washed, counted utilising trypan blue exclusion (Gibco) and resuspended in complete RPMI at 1×10^7 cells/ml for *in vitro* culture.

2.2.8.4 Tumours

Tumours were transferred to digestion buffer (RPMI 1 % FBS (v/v), 1 mg/ml collagenase D (Sigma-Aldrich), 0.1 mg/ml DNase I (Sigma-Aldrich)) and minced to small pieces with scissors before being enzymatically digested at 37 °C for 30 minutes then gravitationally passed through a 70 µM cell strainer (Greiner Bio-One) and washed twice with 10 % FCS RPMI (v/v) before being resuspended in an appropriate volume of FACS buffer for immunophenotyping as described in section 2.5.

2.3 *In vivo*/*In vitro* Hybrid Immunisation Platform

The *in vivo*/*in vitro* hybrid immunisation platform is a primary S.C. immunisation of 4mg/kg 4Y-[MBP] *in vivo* (see section 2.2.6) for 24 hours, followed by enzymatic digestion of spleens for *in vitro* culture (see section 2.2.8.3). A secondary treatment of 4Y-[MBP] is given *in vitro* $\pm$ ICM treatments where appropriate. Doses are defined throughout.

2.4 Immunofluorescence

Cells were cultured in a 6-well plate (Corning costar) as a monolayer before being washed twice with PBS. Cells were then fixed in 4 % PFA/PBS (v/v) for 15 minutes before being washed twice with PBS and permeabilised in 0.2 % Triton X-100/PBS (v/v) for 5 minutes. Cells were washed twice in PBS before being stained with anti-reovirus type 3 sigma 3 protein primary antibody (Creative Diagnostics) 1:250 in 10 % FCS/PBS (v/v) at RT for 2 hours. Cells were washed twice in PBS and stained with anti-mouse IgG2a secondary antibody FITC (Creative Diagnostics) 1:1000 in 10 % FCS/PBS (v/v) at RT for 1 hour in the dark. Cells were washed twice with PBS and imaged on a microscope (Nikon Exlipse).

2.5 Immunophenotyping

2.5.1 Multi-colour Flow Cytometry (Research Chapter 1)

After culture under conditions defined throughout, PBMCs or tissue-specific single cell suspensions were harvested, washed in PBS and prepared for fluorescence-activated cell sorting (FACS) analysis. Cells were first stained with viability dye (BD Biosciences) at 1 in 1000 (v/v) PBS at 4 °C for 10 minutes. Cells were pelleted and resuspended in

a pre-prepared antibody master mix (Tables 2.4 and 2.5) in FACS buffer (PBS supplemented with 1 % (v/v) FCS and 0.1 % (w/v) sodium azide (Sigma-Aldrich)), allowing 100 μ L per sample. Samples were incubated at 4 °C for 30 minutes in the dark before being washed in 200 μ L FACS buffer x 3 and resuspended in 150 μ L paraformaldehyde (PFA; Sigma-Aldrich) to fix the cells. Samples were stored at 4 °C until acquisition on a Cytotflex LX and analysed using CytExpert software (both Beckman Coulter). Cells were analysed for the effects of different treatment conditions on cell surface expression of immune checkpoint receptors within the CD4⁺ T cell, CD8⁺ T cell and NK cell immune subpopulations. Data were analysed to measure the frequency of expression of a cellular marker (% positive expression). Fluorescence minus one (FMO) controls were used to aid interpretation of data and to set correct gating locations.

Panel	Marker	Fluorophore	Manufacturer	Clone	Dilution
1 and 2	CD3	FITC	BD Horizon	HIT3a	1 in 100
	CD56	PE-Cy7	BD Horizon	B159	1 in 50
	CD8	AF700	BD Horizon	RPA-T8	1 in 100
	CD4	APC-H7	BD Horizon	RPA-T4	1 in 100
	Viability Dye	BV510	BD Horizon	N/A	1 in 1000
1	PD-1	PE	BD Horizon	MIH4	1 in 5
	PD-L1	PE-CF594	BD Horizon	MIH1	1 in 20
	OX40	BV421	BD Horizon	L106	1 in 100
	GITR	BV605	Biolegend	108-17	1 in 50
	ICOS	BV650	BD Horizon	DX29	1 in 50
	BTLA	BUV395	BD Horizon	J168-540	1 in 20
2	CD25	BB700	BD Horizon	M-A251	1 in 20
	CD137	PE	Biolegend	4B4-1	1 in 10
	LAG-3	BV650	Biolgend	11C3C65	1 in 50
	TIGIT	BV421	BD Horizon	741182	1 in 100
	TIM-3	BV785	BD Horizon	7D3	1 in 50

Table 2.4. Flow cytometry panels for hPBMCs. Two flow cytometry panels were utilised for the staining of hPBMCs post culture/treatments as described throughout research chapter 1. Markers, fluorophores, manufacturer and their dilution (v/v in FACS Buffer) are recorded here.

Antibody	Fluorophore	Manufacturer	Clone	Dilution
CD3	FITC	Biolegend	17A2	1 in 100
LAG-3	PerCP-Cy5.5	Biolegend	C9B7W	1 in 50
CD137	PE	Biolegend	17B5	1 in 100
PD-L1	PE-Dazzle 594	Biolegend	10F.9G2	1 in 50
GITR	PE-Cy7	Biolegend	DTA-1	1 in 50
Viability stain	BV510	BD Horizon	N/A	1 in 1000
PD-1	BV605	Biolegend	29F.1A12	1 in 50
CD69	BV650	Biolegend	H1.2F3	1 in 50
TIM-3	PE-Cy7	Biolegend	B8.2C12	1 in 50
CD8a	BUV395	BD Horizon	53-6.7	1 in 100
OX40	APC	Biolegend	OX-86	1 in 100
ICOS	AF700	Biolegend	C398.4A	1 in 50
CD4	APC-Cy7	Biolegend	GK1.5	1 in 50

Table 2.5. Flow cytometry panels for isolated murine immune cells. One flow cytometry panel was utilised for the staining of murine immune cells post tissue processing as described throughout research chapter 1. Markers, fluorophores, manufacturer, and their dilution (v/v in FACS Buffer) are recorded here.

2.5.2 Multi-colour Flow Cytometry (Research Chapter 2)

Multi-colour flow cytometry staining for research chapter 2 was performed as in section 2.5.1 with the following amendments.

Fixable live dead eFluor780 (Invitrogen™ eBioscience™) was included in the antibody master mix, alongside the antibody panel. FACS buffer (PBS supplemented with 2 % FBS, v/v) was used to create an antibody master mix at 25 µL per sample. Cells were resuspended in FACS buffer and remained unfixed, with acquisition on the Fortessa (BD) occurring immediately. Cells were analysed on FlowJo (BD) for the effects of different treatment conditions on cell surface expression of immune checkpoint receptors within the CD4⁺ T cell and in some cases, CD8⁺ T cell immune subpopulations. Data were analysed to measure the geometric mean fluorescence intensity (GeoMean) or frequency of expression of a cellular marker (% positive expression) where appropriate. Single stain controls were used appropriately for setting up compensation.

2.5.3 Intracellular Staining

Surface staining for extracellular markers was performed prior to intracellular staining. Intracellular staining was then performed using the eBioscience™ intracellular fix & perm kit according to manufacturer's instructions. In brief, cells were fixed at 4 °C for 30 minutes, stained at 4 °C for 20 minutes and resuspended in a final volume of 300 µL and stored at 4 °C until acquisition.

2.5.4 Spectral Cytometry

Samples for spectral cytometry were prepared as in section 2.5.2, with the following amendments. Antibody master mixes (Tables 2.6 and 2.7) were made in Brilliant Stain Buffer (BD) to mitigate non-specific interactions between polymer dyes. Samples were run on the ID7000 spectral cell analyzer (Sony Biotechnology) and unmixed using Sony ID7000 analysis software. Single stain controls and FMOs were utilised for spectral unmixing and setting analysis gates. FCS files were then imported into FlowJo for subsequent analysis as in section 2.5.2.

Single colour references for FT Blue, FT Red and eGFP were previously generated by Dr David Bending. Briefly, CD8⁺ T-cells were purified from Nr4a3-Tocky mice and a wild type mouse (for normalisation of signal) by immunomagnetic negative selection (BioLegend) and then stimulated for 2 hrs with PMA and Ionomycin (both Sigma) to induce de novo expression of the Timer blue protein. The WT mouse was used to normalise the background signal and identify Blue⁺ signal in the CD8⁺ T-cells from Nr4a3-Tocky mice. Given low levels of Timer Red, the positive signal for FT Blue was set by gating on FTBlue+FTRed^{low} cells. To generate FT Red single colour reference, CD4⁺ T-cells were cultured at 37 °C 5 % CO₂ with 20 µg/mL anti-MHC II (clone M5/114, BioLegend) antibodies to prevent any CD4⁺ TCR signalling. The following day, cells were acquired on the Spectral Cytometer. To identify positives for FT Red, gates were set on FTBlue^{neg}FTRed^{bright} cells. To generate eGFP single colour reference, splenocytes from Tcf7-GFP mice (Jax 030909) were used and normalised to wildtype splenocytes.

Single colour references for single fluorophores, which are robust, were generated by staining UltraComp eBeads (Invitrogen™ eBioscience™) with 1 µL of antibody in the dark at 4 °C for 10 minutes and acquired on the Spectral Cytometer. Single colour references for these fluorophores were generated and stored for multiple uses. Single colour references for other antibodies, specifically tandem fluorophores which are liable to breakdown, were prepared on the day by staining UltraComp eBeads with 1 µL of antibody in the dark at 4 °C for 10 minutes. Samples were acquired on the Spectral Cytometer and single colour references were generated for use in that experiment. These single colour references, either stored or collected in that experiment, were used to mathematically unmix each colour from another in the multicolour samples.

One other key feature of spectral cytometry is the ability to find autofluorescent populations using the novel autofluorescence (AF) Finder tool and subsequently remove them from experimental samples. AF is a feature of all cell types and can be a source of high background which in turn can hide low fluorescent signals. In depth details of using the AF tool are outlined by Wanner *et al.* (2022).

Marker	Fluorophore	Manufacturer	Clone	Dilution
CD4	BUV737	BD Horizon	GK1.5	1 in 200
TCRvb8.1/8.2	BUV395	BD Horizon	MR5-2	1 in 200
FT Blue	Timer Reporter	N/A	N/A	N/A
FT Red	Timer Reporter	N/A	N/A	N/A
IL-10	eGFP Reporter	N/A	N/A	N/A
TIGIT	APC	Biolegend	4D4/m	1 in 200
PD-1	BV605	Biolegend	29F.1A12	1 in 200
PD-L1	BV711	Biolegend	10F.9G2	1 in 200
LAG-3	BV785	Biolegend	C9B7W	1 in 200
OX40	Pe-Cy7	Biolegend	OX86	1 in 200
GITR	PerCP-Cy5.5	Biolegend	DTA-1	1 in 200
ICOS	AF700	Biolegend	C398.4A	1 in 200
CCR6	PE	Biolegend	29-2L17	1 in 200
CD25	BV510	Biolegend	PC61	1 in 200
Viability Dye	ef780	Invitrogen	N/A	1 in 5000

Table 2.6. Spectral Panel 1 for Research Chapter 2. Two spectral panels were utilised for the staining of murine immune cells post tissue processing as described throughout research chapter 2 for high parameter data analysis. Markers, fluorophores, manufacturer and their dilution (v/v in FACS Buffer) are recorded here for spectral panel 1.

Marker	Fluorophore	Manufacturer	Clone	Dilution
CD45	BUV395	BD Horizon	30-F11	1 in 200
CD4	BUV737	BD Horizon	GK1.5	1 in 200
CCR6	PE-Fire640	Biolegend	29-2L17	1 in 200
CD25	BV510	Biolegend	PC61	1 in 200
PD-1	BV605	Biolegend	29F.1A12	1 in 200
PD-L1	BV711	Biolegend	10F.9G2	1 in 200
LAG-3	BV785	Biolegend	C9B7W	1 in 200
OX40	Pe-Cy7	Biolegend	OX86	1 in 200
ICOS	AF700	Biolegend	C398.4A	1 in 200
Viability Dye	ef780	Invitrogen	N/A	1 in 5000
TCRb	FITC	Biolegend	H57-597	1 in 200
TIGIT	PE-Dazzle 594	Biolegend	1G9	1 in 200
GITR	BV650	BD Horizon	DTA-1	1 in 200

Table 2.7. Spectral Panel 2 for Research Chapter 2. Two spectral panels were utilised for the staining of murine immune cells post tissue processing as described throughout research chapter 2 for high parameter data analysis. Markers, fluorophores, manufacturer, and their dilution (v/v in FACS Buffer) are recorded here for spectral panel 2.

2.6 Coding for Heatmap

Heatmaps were generated in R (version 4.2.2) using the package gplots and the function heatmap.2. A csv file containing the MFI values in the samples was used as an input data file and a heatmap output in PDF format using the following code (generated by Dr David Bending):

```
>hm=read.csv("mfi.csv", header=T)
>hm=as.matrix(hm)
>pdf("pd1allhm.pdf", height=8, width=6)
>heatmap.2(hm3, scale="row", col=bluered(100), density.info="none",
trace="none", colsep=c(4,9,14), sepcolor="none", cexRow=0.4)
>dev.off()
```

2.7 Statistical Analysis

Statistical analysis (Table 2.8) was performed on GraphPad prism as appropriate. Sample sizes were not pre-determined. Summary data is shown as mean $\pm$ SEM for parametric testing and as median with interquartile range for non-parametric – this is indicated in the figure legends throughout. Significance ($P \leq 0.05$) is shown as either raw P values or using the following key: $P > 0.05$ (), $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***) and $P \leq 0.0001$ (****). Details of which method of reporting is included in each figure legend. Each experimental unit represents one donor, either mouse or human as described throughout. Paired analysis was used when variables were tested on the same subject (i.e. the same donor was used throughout the conditions). Unpaired analysis was used for the inverse (i.e. the subject was not matched across the variables).

Mann-Whitney U tests were chosen to compare two groups where the data is unpaired and the assumption of normal distribution was not met. Repeated measure (RM) one-way ANOVA were used to compare three or more groups where data was paired. Ordinary one-way ANOVA was used to compare three or more groups where data was unpaired. 2way ANOVA was used to compare two independent variables (i.e. antibody-mediated checkpoint modulation and 4Y-[MBP] treatment). For 2way ANOVA, Dunnett's or Sidak's multiple comparisons tests were used. Dunnett's was used when row means were compared, whilst Sidak's was used for independent comparisons. 3way ANOVA was used in cases of three separate variables (i.e. cell type, reovirus treatment and time post-treatment). For 3way ANOVA, Tukey's multiple comparisons testing was used to compare the means of all possible paired datasets.

NON-PARAMETRIC	PARAMETRIC
Mann-Whitney U test	2way ANOVA with Dunnett's multiple comparisons test
	3way ANOVA with Tukey's multiple comparisons test
	2way ANOVA with Sidak's multiple comparisons test
	RM one-way ANOVA with multiple comparisons
	Ordinary one-way ANOVA with multiple comparisons

Table 2.8. Non-parametric and parametric statistical tests utilised throughout. Non-parametric or parametric analyses were chosen as appropriate and indicated throughout.

2.8 Software

CytExpert was used for flow cytometric analysis of data obtained in Research Chapter 1 whilst FlowJo was used for flow cytometric analysis of data obtained in Research Chapter 2. Sony ID7000 Analysis software was utilised for spectral unmixing and initial analysis of data obtained through spectral cytometry in research chapter 2 before final analysis was completed in FlowJo. GraphPad Prism 9.4.1 was utilised for statistical analysis and data presentation throughout both research chapters. Biorender.com was used to generate illustrative figures as described throughout under a paid student plan. R (version 4.2.2) was used to generate the heatmap as in section 2.6.

3 RESEARCH CHAPTER 1: MODULATION OF IMMUNE ENVIRONMENT BY ONCOLYTIC REOVIRUS IN HCC

This research chapter, conducted primarily under the supervision of Dr Adel Samson at the University of Leeds, seeks to evaluate the immune-mediated effects of oncolytic reovirus in HCC, a top three cause of cancer related deaths. In a recent study, oncolytic reovirus has been shown to prime the tumour microenvironment in brain cancer for subsequent checkpoint blockade (Samson *et al.*, 2018). Recent advances in immunotherapy for the treatment of HCC have been promising, but treatments are still associated with significant treatment-related adverse events and not all patients respond to treatment at all. This area of unmet need requires exploration into novel combination therapies in order to increase treatment efficacy, with a consideration of adverse events. This chapter aims to address this gap in knowledge by exploring the immune-mediated effects of oncolytic reovirus in HCC, looking specifically at expression levels of co-stimulatory and co-inhibitory receptors post-treatment in order to seek effective combination strategies.

3.1 Introduction

3.1.1 Liver structure and function

As the largest organ in the body, the liver plays a vital role in macronutrient metabolism and toxic substance clearance, as well as immune system support (Trefts *et al.*, 2017). One main role of the liver is glucose metabolism, with the liver being able to store, synthesise, metabolise and release glucose as necessary. In addition to glucose metabolism, the liver can metabolise lipids and cholesterol, therefore being critical for

digestive absorption. Due to its role in protein and amino acid metabolism, the liver is responsible for the majority of proteins which are secreted in the blood.

Vasculature of the liver is unique, with three main networks playing a part. The hepatic artery and portal vein, which bring blood into the liver, run in parallel. The hepatic artery is responsible for bringing oxygenated blood into the liver, whilst deoxygenated blood is brought in by the portal vein. These two blood sources meet in the sinusoids, which form liver lobules. These lobules, the smallest functional unit of the liver, sit alongside the hepatic veins which are the outlet source for taking the blood to the vena cava inferior.

3.1.2 Immune tolerance in the liver

The liver is an important frontline immune defence and contains the largest population of phagocytic cells in the body, making the organ ideally prepared to capture and clear pathogens entering the tissue (Kubes and Jenne, 2018). The portal vein transports a number of harmless foreign molecules, e.g. food antigens and gut-derived pathogens, into the liver through portal blood, therefore the liver is immune tolerant. However, the liver is able to mount a rapid immune response under the right conditions.

Liver resident macrophages, known as Kupffer cells, are the largest population of macrophages in the body and contribute to the liver natural immune tolerance (Kubes and Jenne., 2018). Kupffer cells are intravascular and fill the sinusoidal lumens, scanning for foreign material in the blood. The majority of the time, Kupffer cells recognise, capture, and remove bacteria, e.g. *Escherichia coli*, from the bloodstream, resulting in pathogen killing. Some encapsulated bacteria, e.g. *Streptococcus*

pneumonia are able to evade the liver, but are subsequently recognised and destroyed by splenic macrophages (Kubes and Jenne., 2018). In addition to Kupffer cells, the liver contains the largest population of NK cells in the body, playing an important role in screening for infection (Kubes and Jenne., 2018). As described in detail in section 1.1.1, NK cells screen for the absence of antigen-presenting molecules such as MHC I through their expression of activating (e.g. natural cytotoxicity receptors and NKG2D) and inhibitory (e.g. killer cell immunoglobulin-like receptor and NKG2A) receptors. Failure to engage an inhibitory receptor with its ligand due to the target cell downregulating the ligands, leads to NK cell activation and target cell killing. NK cell activity is crucial for eliminating viral pathogens within the liver, often arriving via the gut.

In addition to Kupffer and NK cells, liver sinusoidal endothelial cells (LSECs) are liver resident cells which function as APCs, with an ability to present endogenous antigens to CD8+ T-cells but also to cross-present exogenous antigens (McConnell *et al.*, 2023). LSECs are therefore able to activate both CD4+ and CD8+ T-cells to blood-derived and gut-derived antigens. Of note, enhanced expression of inhibitory molecules in the liver (e.g. PD-L1) suppresses the immune response to gut-derived pathogens (Zheng and Tian, 2019). This same expression of inhibitory molecules also affects the ability to respond to systemic therapies, e.g. immunomodulatory compounds. Antigen presentation by LSECs, in addition to the expression of inhibitory molecules, aids T-cell tolerance. Furthermore, despite their primary role of metabolism, hepatocytes can act as APCs in the liver under certain conditions due to their expression of MHC I (Kubes and Jenne., 2018). In addition, under inflammatory conditions, MHC II expression can be induced on the surface of hepatocytes. Hepatocytes therefore

directly support T-cell activation and immunity against intracellular pathogens, e.g. viral infections.

This range of immune cells in the liver provides a balance of immune tolerance and immune activation that is critical to the liver's normal function. Uncontrolled immune activation in the absence of infection can lead to liver damage, whilst poor immune responses can lead to chronic infection and cancer (Kubes and Jenne., 2018).

3.1.3 Liver damage

Liver fibrosis, arising from liver disorders and diseases which disrupt liver regeneration, is characterised by an excessive accumulation of connective tissue components (Iredale, 2007 and Roehlen *et al.*, 2020). This disrupts liver homeostasis, often changing the physiological structure of the liver and in turn, liver function. Continuous liver injury can lead to cirrhosis, an irreversible condition requiring transplantation, and can be fatal.

In general cases of tissue injury, dead cells release DAMPS; including pro-inflammatory molecules which initiate inflammatory responses (Yoshizawa *et al.*, 2022). This inflammation leads to the subsequent recruitment and polarisation of macrophages which in turn release profibrotic mediators, including TGF- β (Wight and Potter-Perigo, 2011 and Yoshizawa *et al.*, 2022). These profibrotic mediators go on to activate a number of signalling pathways associated with fibroblast activation, thus producing extracellular matrix (ECM) proteins. Excessive ECM protein production can lead to a change in the tissue structure and, ultimately, function. This process forms a

feedback loop, which leads to the progression of fibrosis in tissues, ultimately leading to cirrhosis if left untreated (Wight and Potter-Perigo, 2011).

In the liver, fibrosis is generally promoted via liver resident macrophages (Kupffer cells) and infiltrating monocyte-derived macrophages that were recruited from circulating blood via the production of TGF- β alongside other profibrotic mediators (Yoshizawa *et al.*, 2022). This leads to the secretion of ECM by hepatic myofibroblasts, derived from activated hepatic stellate cells (HSCs). Again, this causes a feedback loop which can cause fibrotic tissue to become cirrhotic if left untreated.

3.1.4 Hepatocellular carcinoma

In 2020, 905,700 people were diagnosed with liver cancer, with this number predicted to rise by >55 % by 2040 (Rumgay *et al.*, 2022). Hepatocellular carcinoma (HCC), a type of liver cancer, is a top three highest cause of cancer-related deaths worldwide (McGlynn *et al.*, 2021). In just 20 years, from 1997 to 2016, both incidence and deaths from HCC trebled in the United Kingdom (UK) (Yang *et al.*, 2019). Most HCC patients are diagnosed at an advanced stage of the disease, often due to lack of symptoms early on, with early stages of HCC being characterised by dysplastic lesions in the liver (West *et al.*, 2017). HCC generally arises from an abnormal background liver, damaged by clearly defined aetiological factors such as hepatitis B virus (HBV) or hepatitis C virus (HCV) infection (Yang *et al.*, 2019). Approximately 90 % of HCC cases develop in patients with a background of cirrhosis, therefore liver health is an important factor when considering novel HCC treatment advances, as this substantially contributes to cancer-specific survival (Garrido and Djouder, 2021). Advanced stage of disease means therapy options are restricted as patients are not suitable for

potentially curative treatments such as local ablation, resection or liver transplantation. With this in mind, novel and effective therapeutic treatment options are greatly required for this cancer of unmet need.

3.1.5 Disease staging for hepatocellular carcinoma

Disease staging systems are critical within oncology to guide clinical assessments and select therapeutic options. The Barcelona-clinic liver cancer (BCLC) staging classification is a validated staging system used for HCC that links disease stage to clinical outcomes and, hence, a preferred treatment approach. There are five stages to the BCLC staging criteria: very early (0), early (A), intermediate (B), advanced (C) and end-stage (D) (Pons *et al.*, 2005 and Tellapuri *et al.*, 2018). Importantly, the functional standing of the background liver is included in the BCLC staging system, as an important determining factor of HCC survival and therapeutic options, alongside the performance status of the patient. Typically, stage 0 can be treated with liver resection or curative treatments, whilst stage A can be treated with liver transplantation or curative treatments. Stage B is classically treated with locoregional therapies including radiofrequency ablation (RFA) and transarterial chemoembolization (TACE), whilst advanced stage C is treated with systemic therapies like multi-kinase inhibitors and stage D with best supportive care (Tellapuri *et al.*, 2018 and Han *et al.*, 2015).

3.1.6 Multi-kinase inhibitors for hepatocellular carcinoma

Kinases are enzymes that are responsible for catalysing the transfer of a phosphate group from adenosine triphosphate (ATP) to a substrate molecule. Until recently in the UK, the only first-line treatments for advanced incurable HCC were sorafenib and lenvatinib, with the potential for treatment with regorafenib as a second-line therapy

post-sorafenib (Shannon *et al.*, 2022). These treatments are multi-kinase inhibitors that act against many protein kinases but are preferentially targeted against vascular endothelial growth factor (VEGF) receptor tyrosine kinases which are involved in the growth of new tumour blood vessels (Shannon *et al.*, 2022). The approval of sorafenib by the European Medicines Agency (EMA) in 2007 followed results from the sorafenib HCC assessment randomised protocol (SHARP; NCT00105443) trial (Llovet *et al.*, 2008) and the Asia-Pacific trial (NCT00432752) (Cheng *et al.*, 2009) which showed an increased overall median survival, albeit less than three months in both cases. Lenvatinib, the second approved first-line treatment for advanced HCC, was approved by the EMA in 2015 following the REFLECT trial (NCT01761266) (Kudo *et al.*, 2018) which showed an overall survival of 13.6 months for lenvatinib compared to 12.3 months for sorafenib, ultimately meeting the criteria for non-inferiority in comparison to sorafenib.

Regarding second-line treatments, regorafenib was granted approval by the EMA in 2013 as a second-line therapy for patients with advanced HCC who had tolerated and benefited from prior sorafenib treatment. This approval followed results from the RESORCE trial (NCT01774344) (Bruix *et al.*, 2017) where regorafenib added a further 2.8 months median overall survival following first-line sorafenib treatment.

3.1.7 ICM for HCC

Unfortunately, the last decade of clinical trials testing the use of various receptor tyrosine kinase inhibitors for the treatment of advanced HCC has brought underwhelming results in the field. However, one recent advancement in HCC research is the use of immunotherapies. These therapies are showing promising

results in the treatment of many cancer types in addition to advanced HCC. Of note, the recent IMbrave150 study (NCT03434379) (Finn *et al.*, 2020) is a randomised phase III study combining atezolizumab and bevacizumab (336 patients), PD-L1 and VEGF-A inhibitors, respectively, compared to sorafenib treatment (165 patients). Preliminary results were very promising, with a hazard ratio for death with atezolizumab-bevacizumab when compared with sorafenib of 0.58. Overall survival at 12 months was 67.2 % with atezolizumab-bevacizumab and only 54.6 % with sorafenib. Median progression-free survival was 6.8 months for atezolizumab-bevacizumab and only 4.3 months with sorafenib. This combination has been approved for use by the U.S. Food and Drug Association (FDA) and in the European Medicines Agency (EMA) for accelerated use. Second-line therapies following this combination immunotherapy are now needed.

In addition to the approval of atezolizumab and bevacizumab, durvalumab (PD-L1 blockade) and tremelimumab (CTLA-4) have also been recently approved for first-line therapy of HCC (Abou-Alfa *et al.*, 2022). This approval followed a global, open-label, phase 3 trial in which most patients enrolled presented with unresectable HCC and had no prior experience with systemic treatments (HIMALAYA; Abou-Alfa *et al.*, 2022). Three treatment arms were incorporated into the trial; tremelimumab plus durvalumab (STRIDE), durvalumab alone, or sorafenib. The primary objective was overall survival for STRIDE versus sorafenib. The HIMALAYA study totalled 1171 patients (STRIDE, n=393; durvalumab, n=389 and sorafenib, n=389). The median OS (STRIDE, 16.43 months; durvalumab, 16.56 months and sorafenib, 13.77 months) showed STRIDE to be more effective than sorafenib. Though promising, and approved by the FDA, this dual checkpoint blockade of PD-L1 and CTLA-4 only marginally extended median OS

compared to sorafenib (around 3 months) and is associated with significant toxicity. Around half (50.5 %) of patients in the STRIDE treatment group had grade 3/4 treatment-emergent adverse events, like that of sorafenib (52.4 %).

Although several promising advances have been made in the treatment of HCC in recent years, treatment of advanced-HCC is still limited by poor survival times and associated with significant toxicities (e.g. autoimmune-mediated hepatitis), therefore new treatment advances should be sought. Immunotherapeutic treatments for HCC have been promising, but overall response rates are still much slower than other cancer types, including melanoma. The recent approval of ICM therapies for HCC are promising, and a better understanding of the immune system in HCC and a rationally designed combination therapy of ICM treatments could be invaluable in the field when exploring new combination therapies.

3.1.8 Immune microenvironment in HCC

Immune evasion is a key feature of the initiation and development of HCC with the progression of disease being supported by an increase in immunosuppressive cell populations, defective antigen presentation and the amplified expression of inhibitory checkpoint co-receptor/ligand interactions (Yarchoan *et al.*, 2019). Immunosuppressive cell components of HCC include myeloid-derived suppressor cells (MDSCs), tumour-associated macrophages and regulatory T-cells (Tregs) (Lu *et al.*, 2019).

Unlike the background liver where T-cell priming frequently takes place in the liver itself, the main sites of T-cell priming in HCC are the tumour draining lymph nodes (TDLNs) (Shuang *et al.*, 2017). Conventional dendritic cells (DCs) are the most highly

specialised APC and play a critical role in the initiation and direction of T-cell responses. In a steady state, there is continuous migration of antigen-loaded conventional DCs from the HCC tumour through the lymphatic system towards the TDLNs. In the TDLNs, the conventional DCs present their loaded antigens, acquired in their tissue of origin, to the T-cells; this process is accelerated in response to inflammatory signals. During the migration of conventional DCs to the TDLNs from the non-lymphoid tissue, they complete their maturation and acquire potent T-cell stimulatory capacity – this is done through the upregulation of MHC molecules and co-stimulatory molecules such as CD83 and CD86 (Ormandy *et al.*, 2006 and Aerts-Toegaert *et al.*, 2007).

The role of the liver, with its ability to efficiently control immune responses to dietary antigens, microbial agents and environmental stimuli, generates a uniquely tolerogenic immune environment within the liver (Chen *et al.*, 2023). PD-L1+ T-cells play a critical role in maintaining this tolerant environment, interacting with PD-1 to limit T-cell activation in the liver (Tiegs and Lohse, 2010). In addition to the PD-L1/PD-1 inhibitory axis, many co-inhibitory and co-stimulatory checkpoint receptors and ligands are expressed within the liver, making ICM suitable therapies to explore for the treatment of HCC.

One crucial factor to note is the differences in immune microenvironments in HBV-driven and non-viral HCC. Studies have shown that HBV-driven HCC patients have circulating lymphocytes with exhausted features including elevated levels of inhibitory receptors such as TIGIT and TIM3 (Sun *et al.*, 2023). Perhaps unsurprisingly, HBV-

driven HCC patients also have the best overall response rates to checkpoint modulation in clinical trials (Liu *et al.*, 2021).

3.1.9 Oncolytic virus therapy

Oncolytic viruses (OVs) are able to selectively infect and destroy cancer cells whilst leaving non-malignant cells unharmed. OVs come from a range of viral families, of both DNA and RNA viruses (Rahman and McFadden, 2021). Examples of oncolytic DNA viruses include adenoviruses, herpes simplex virus and vaccinia virus. Oncolytic RNA viruses include Coxsackie virus, Vesicular stomatitis virus and reovirus.

OVs can be naturally occurring, with inherent cancer selective properties, such as oncolytic reovirus, or they can be genetically modified to be cancer targeting, like Pexa-vec. Pexa-vec is a Wyeth strain vaccinia virus genetically modified to have an inactive thymidine kinase gene (Breitbach *et al.*, 2015). Thymidine kinase is crucial for viral replication, and whilst non-malignant cells tightly control the enzyme, cancer cells have an abundance, leading to the cancer selective properties of the genetically modified OV, Pexa-vec.

OVs, either natural or genetically modified, act via two main mechanisms. The first being the direct lysis of tumour cells and the second being the induction of an anti-tumour immune-mediated response. The first OV (talimogene laherparepvec; T-VEC) was approved for use by the FDA in 2015 for the treatment of melanoma (Ott and Hodi., 2016). T-VEC is a herpes simplex virus (HSV) type 1, genetically modified to contain granulocyte macrophage colony-stimulating factor (GM-CSF). Whilst, the

recent progress of OV is extensively reviewed by Rahman and McFadden (2021), the list of approved oncolytic viruses is shown in Table 3.1.

Name	Virus Type	Approval	Cancer Type
Rigvir (ECHO-7)	Picornavirus	2004, Latvia	Melanoma
Oncorine (H101)	Adenovirus serotype 5	2005, China	Head and neck cancer
T-VEC (Imlygic)	HSV-1	2015, USA & Europe	Metastatic melanoma
DELYTACT (G47Δ)	HSV-1	2021, Japan	Malignant glioma or any primary brain cancer

Table 3.1. Globally approved oncolytic viruses. Oncolytic viruses that have been approved for clinical use across the world, their year and country of approval and the cancer type.

Currently, despite four OV being approved for clinical use, only T-VEC is approved for use by the FDA/EMA (Table 3.1). A number of pre-clinical and clinical studies are ongoing to evaluate the effectiveness of multiple OV in different cancer types, including the use of oncolytic reovirus.

3.1.10 Oncolytic reovirus

Reovirus (respiratory enteric orphan virus) is one of many oncolytic viruses being trialled and developed for cancer therapies (Phillips *et al.*, 2018). There are three serotypes of reovirus which circulate in humans, serotypes 1-3 (T1-3), with most of the

population being infected with reovirus during childhood (Phillips *et al.*, 2018 and Sahin *et al.*, 2013). Reovirus typically presents as a subclinical disease and is therefore rarely detected. This characteristic, in addition to being rapidly cleared by healthy cells, makes the virus an ideal candidate for therapeutic development. Importantly, this safety profile is maintained in patients who are receiving immunosuppressive therapy, which many cancer patients will already be on. Although reovirus T3 Dearing (T3D) strain has been demonstrated as safe in patients with a variety of cancers, unfortunately early clinical trials using reovirus monotherapy did not mirror the promise that was generated from pre-clinical studies (Galanis *et al.*, 2012 and Vidal *et al.*, 2008). Nonetheless, reovirus does hold promise in combination with complementary immunotherapies, including ICMs, as shown in recent studies (Rajani *et al.*, 2016, Mostafa *et al.*, 2018 and Samson *et al.*, 2018).

3.1.11 Oncolytic reovirus for HCC

Samson and colleagues demonstrated that oncolytic reovirus was able to efficiently replicate in several HCC cells lines, in addition to showing cytotoxic effects via apoptosis in these lines (Samson *et al.*, 2018). In addition, in the same hands, oncolytic reovirus was shown to elicit both anti-tumour and anti-viral properties in multiple cancers associated with oncogenic viral infections, including HCC (Samson *et al.*, 2018). Independently, it has been demonstrated that SB431542, a TGF- β inhibitor, is able to inhibit reovirus-mediated lysis of HCC cell lines in a TGF- β -independent manner (Ishigami *et al.*, 2021). Furthermore, it has been shown that reovirus is able to replicate in and destroy cancer cells derived from HCC biopsies, further supporting oncolytic reovirus as a potential therapeutic agent for HCC (Kazemi *et al.*, 2021).

3.1.12 Oncolytic reovirus combination therapy

Despite impressive preclinical observations with oncolytic reovirus as a single-agent therapy, clinical observations did not meet expectations. To circumnavigate this, oncolytic reovirus has been combined with a range of anticancer treatments, including chemotherapy and radiotherapy (Clements *et al.*, 2014). Combination of oncolytic reovirus with regularly used chemotherapy drugs, e.g. cisplatin, demonstrated synergistic effects both *in vitro* and *in vivo* (Roulstone *et al.*, 2013, Pandha *et al.*, 2009 and Heinemann *et al.*, 2011). In general, combination of oncolytic reovirus with chemotherapy or radiotherapy has shown more promise than reovirus monotherapy, both pre-clinically and clinically. In addition to these combination therapies, and with the field of ICM therapy rapidly advancing, oncolytic reovirus has been shown to prime the tumour microenvironment for subsequent checkpoint blockade in brain cancer (Samson *et al.*, 2018). Furthermore, in a B16 melanoma model, combination of oncolytic reovirus with anti-PD-1 blockade has been demonstrated to control tumour growth through innate and adaptive immune responses (Rajani *et al.*, 2016).

These observations, combined with the earlier observations that reovirus targets HCC and ICM has had promising effects in HCC, suggest that oncolytic reovirus in combination with ICM could be a potential treatment for HCC. The aim of this chapter is to rationally explore a reovirus-based combination therapy for the treatment of HCC. Specific attention is to be paid to the expression of co-stimulatory and co-stimulatory checkpoint receptors on the surface of immune cells in response to indirect reovirus treatment.

3.2 Results

3.2.1 PD-L1 expression increases on immune cells in response to treatment with reovirus conditioned medium

Oncolytic reovirus acts via two main mechanisms, firstly, the preferential direct lysis of cancer cells and secondly, the subsequent activation of the immune system to elicit anti-tumour effects (Prestwich *et al.*, 2008 and Prestwich *et al.*, 2009). The nature of this chapter is to focus on the second mechanism of action, looking at reovirus-mediated immune cell activation. Oncolytic reovirus has been shown to sensitise tumours to subsequent ICM therapies (Samson *et al.*, 2018). Choosing which co-stimulatory or co-inhibitory pathway to modulate in combination with oncolytic reovirus treatment requires exploration of their expression. A panel of co-stimulatory (ICOS, GITR, CD137) and co-inhibitory (PD-L1, PD-1, BTLA, LAG-3 and TIGIT) receptors were explored in response to indirect reovirus treatment. These markers were chosen as they have are promising targets in novel ICM therapeutics, either pre-clinically or clinically (Darvin *et al.*, 2018, Knee *et al.*, 2016, Claus *et al.*, 2023, Sordo-Bahamonde *et al.*, 2023 and Ziogas *et al.*, 2023). In addition, CD25 expression was explored as a late marker of cellular activation due to its observed prominent upregulation on T-cells within 24 hours of stimulation of the TCR/CD3 complex (Reddy *et al.*, 2004).

In vitro analyses were used to explore the temporal changes of co-stimulatory and co-inhibitory checkpoint receptors in response to indirect reovirus treatment. Conditioned medium from PBMCs $\pm$ reovirus were generated as described in section 2.1.7; where RCM and CM were viresolve™ filtered to remove any residual reovirus, thus ensuring that effects were mediated only by soluble factors produced by reovirus-treated immune cells. PBMCs were cultured with either RCM or CM up to 96 hrs. Surface

expression of co-stimulatory and co-inhibitory receptors were then analysed on NK, CD4⁺ and CD8⁺ T-cells (Figures. 3.1-3.3).

Within NK cells, PD-L1 was the only marker to significantly increase response to RCM compared to CM (Figure 3.1). Though significant at 72 hrs, the increased expression of PD-L1 was observed from 8 hrs, peaking at 48-72 hrs, and beginning to decline by 96 hrs. Although not significant, trends were also observed for ICOS, PD-1, CD25 and LAG-3 with increased expression visible post-RCM treatment compared with CM. Other markers, GITR, BTLA, CD137 and TIGIT appeared unaffected by the treatment. It is worth noting that although some markers do not differentiate between treatments, their expression levels change over time, e.g. TIGIT, which increases over time in response to tissue culture alone.

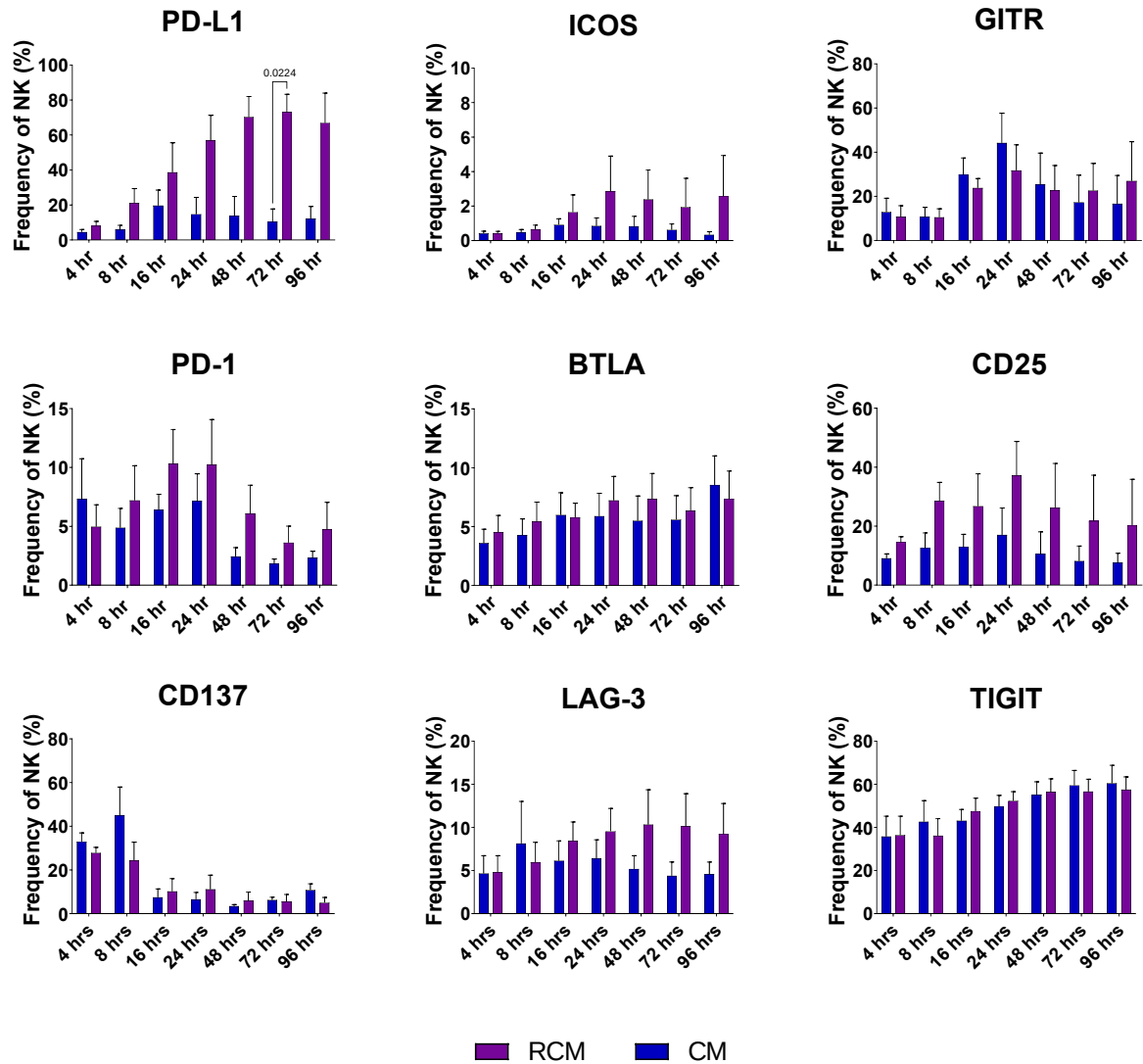


Figure 3.1. Reovirus conditioned medium induces PD-L1 expression on the surface of NK cells. PBMC-derived reovirus conditioned medium (RCM) and conditioned medium (CM) were generated as in section 2.1.7. Mixed PBMCs were cultured with 5 % RCM or CM from 4-96 hrs and analysed via flow cytometry for positive expression of co-stimulatory and co-inhibitory receptors on the surface of CD56+CD3- NK cells. Summary graphs representative of two independent experiments and show mean $\pm$ SEM (n=4). Statistical results show results of a 2way ANOVA with Dunnett's multiple comparisons test $p \leq 0.05$.

Similarly to NK cells, PD-L1 expression is significantly greater on those CD8⁺ T-cells exposed to RCM compared with CM at 48-72 hrs (Figure 3.2). ICOS and LAG-3 both show a trend of increased expression in response to RCM compared to CM, though this is not statistically significant. Differences are also observed within expression of PD-1 and CD25 to a much lesser extent, with GITR, BTLA, CD137 and TIGIT showing no differences.

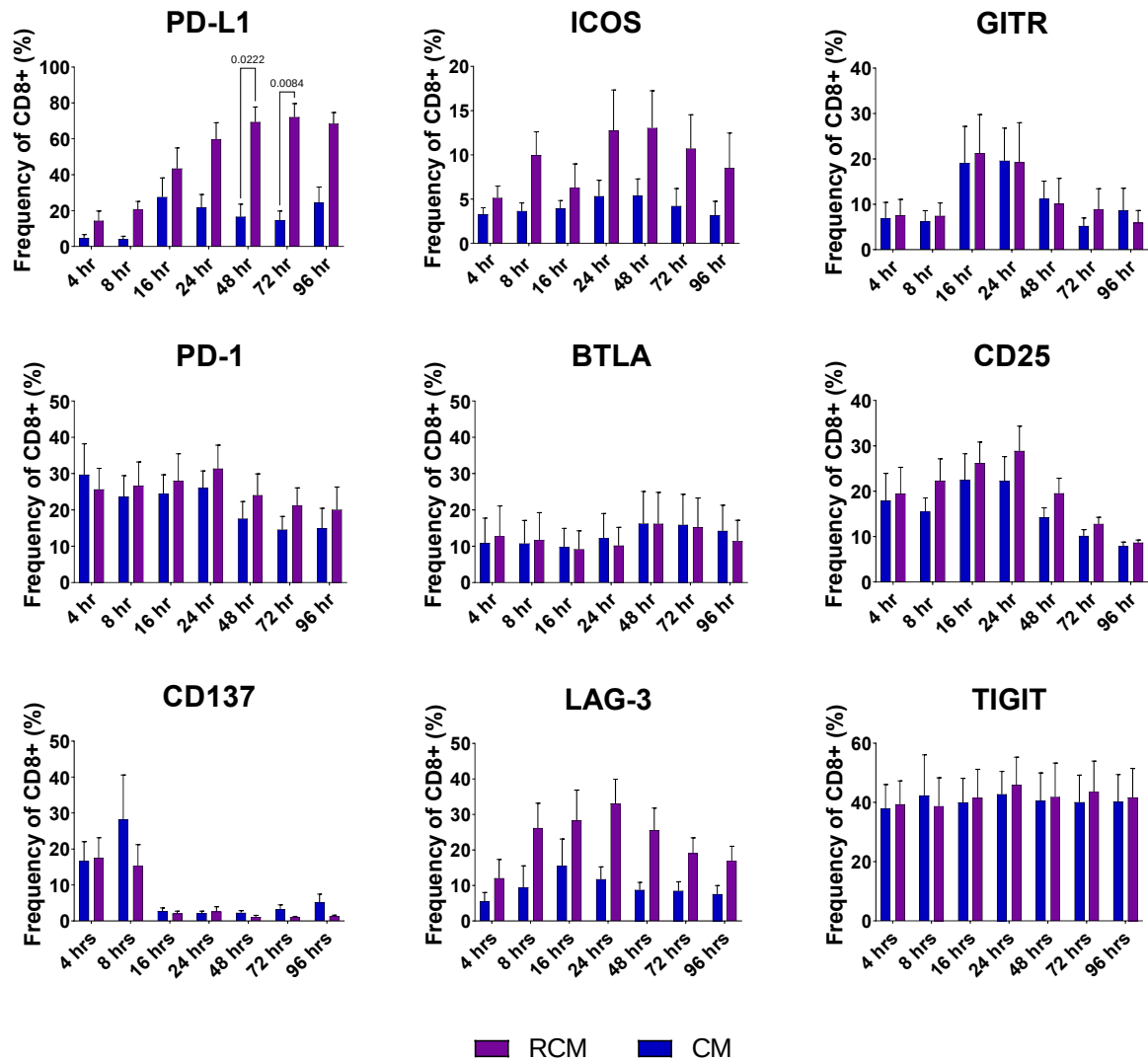


Figure 3.2. Reovirus conditioned medium induces PD-L1 expression on the surface of CD8+ T-cells. PBMC-derived reovirus conditioned medium (RCM) and conditioned medium (CM) were generated as in section 2.1.7. Mixed PBMCs were cultured with 5 % RCM or CM from 4-96 hrs and analysed via flow cytometry for positive expression of co-stimulatory and co-inhibitory receptors on the surface of CD3+CD8+ T-cells. Summary graphs representative of two independent experiments and show mean $\pm$ SEM (n=4). Statistical results show results of a 2way ANOVA with Dunnett's multiple comparisons test $p \leq 0.05$.

For CD4+ T-cells, PD-L1 is again the only marker significantly higher on those CD4+ T-cells treated with RCM compared to CM, with these differences observed between 24 and 96 hrs (Figure 3.3). ICOS, PD-1 and LAG-3 all show trends towards increased expression post-RCM treatment compared to those treated with CM, but effect sizes were too small to reach statistical significance with the sample sizes. GITR, BTLA, CD25, CD137 and TIGIT all show no differences.

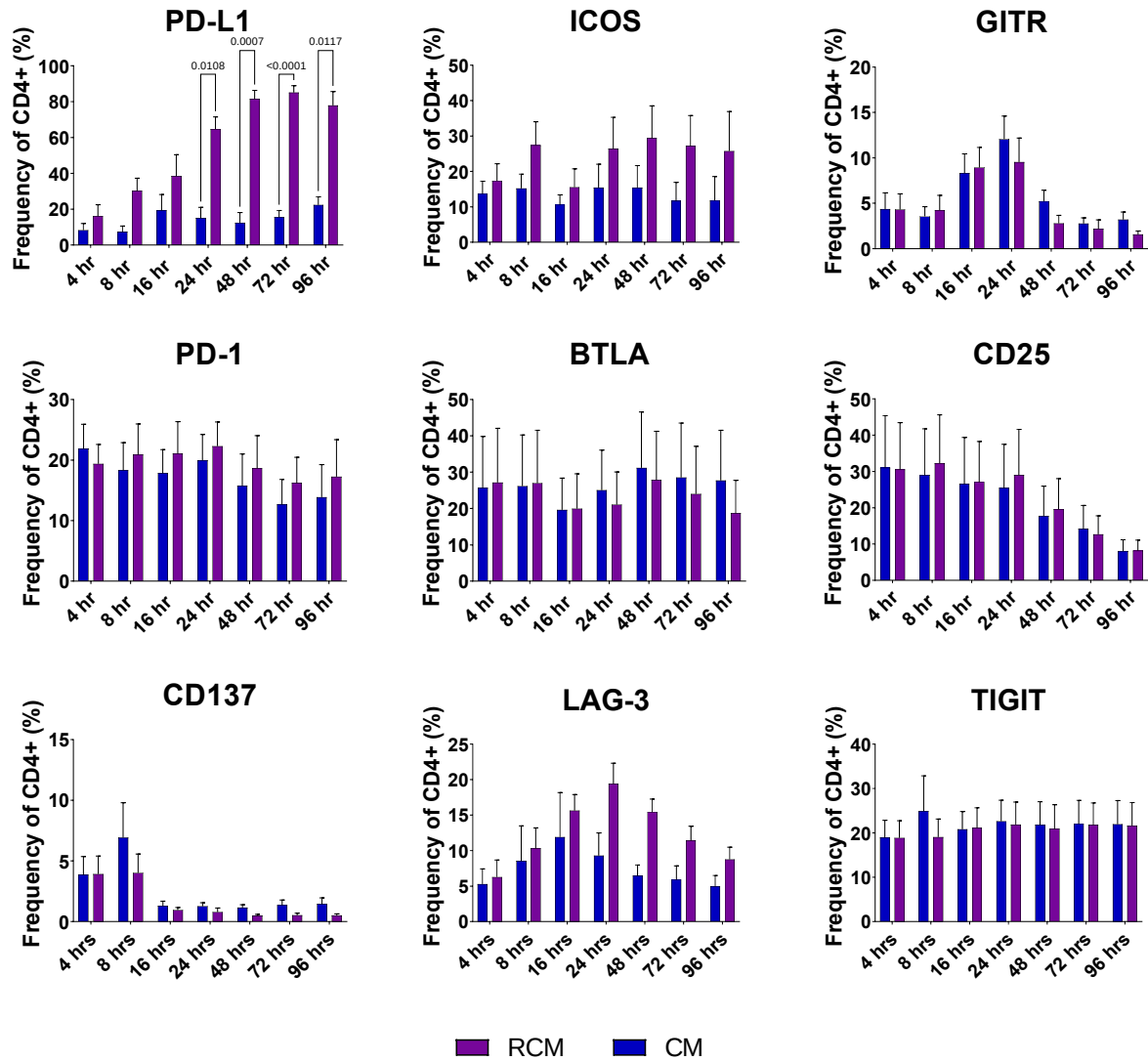


Figure 3.3. Reovirus conditioned medium induces PD-L1 expression on the surface of CD4+ T-cells. PBMC-derived reovirus conditioned medium (RCM) and conditioned medium (CM) were generated as in section 2.1.7. Mixed PBMCs were cultured with 5 % RCM or CM from 4-96 hrs and analysed via flow cytometry for positive expression of co-stimulatory and co-inhibitory receptors on the surface of CD3+CD4+ T-cells. Summary graphs representative of two independent experiments and show mean $\pm$ SEM (n=4). Statistical results show results of a 2way ANOVA with Dunnett's multiple comparisons test $p \leq 0.05$.

One key observation from these experiments is that the panel of co-stimulatory and co-inhibitory receptors studied have varied expression time post-RCM treatment, and that careful consideration should be taken when designing treatment schedules. For example, PD-L1 expression post-RCM treatment peaks at 48-72 hrs on all cell types explored and still has high expression at 96 hrs, but LAG-3 is observed to peak at 24 hrs on CD8+ and CD4+ T-cells, with the frequency of cells expressing the marker declining by 48 hrs (Figures 3.1-3.3).

3.2.2 Oncolytic reovirus preferentially induces PD-L1 and LAG-3 on the surface of immune cells cultured with cancer cells

Initial findings in section 3.2.1 demonstrated that RCM can induce the expression of co-stimulatory and co-inhibitory markers on the surface of immune cell populations. However, to better model the indirect effects of reovirus within the tumour microenvironment, a transwell co-culture system was utilised (Figure 3.4). Here, isolated primary PBMCs were co-cultured in transwell plates with either primary background hepatocytes or JHH-4 cancer cells, a human HCC cell line. Transwell plates allowed the PBMCs and background hepatocytes/JHH-4 cancer cell line to be co-cultured in the same environment without direct cell-cell contact, thus focusing on indirect responses. Temporal analysis of co-stimulatory (OX40, ICOS, GITR) and co-inhibitory (PD-L1, PD-1, LAG-3, TIM-3, TIGIT and BTLA) receptors on the surface of NK, CD4+ and CD8+ T-cells were investigated (Figures 3.5-3.7, respectively).

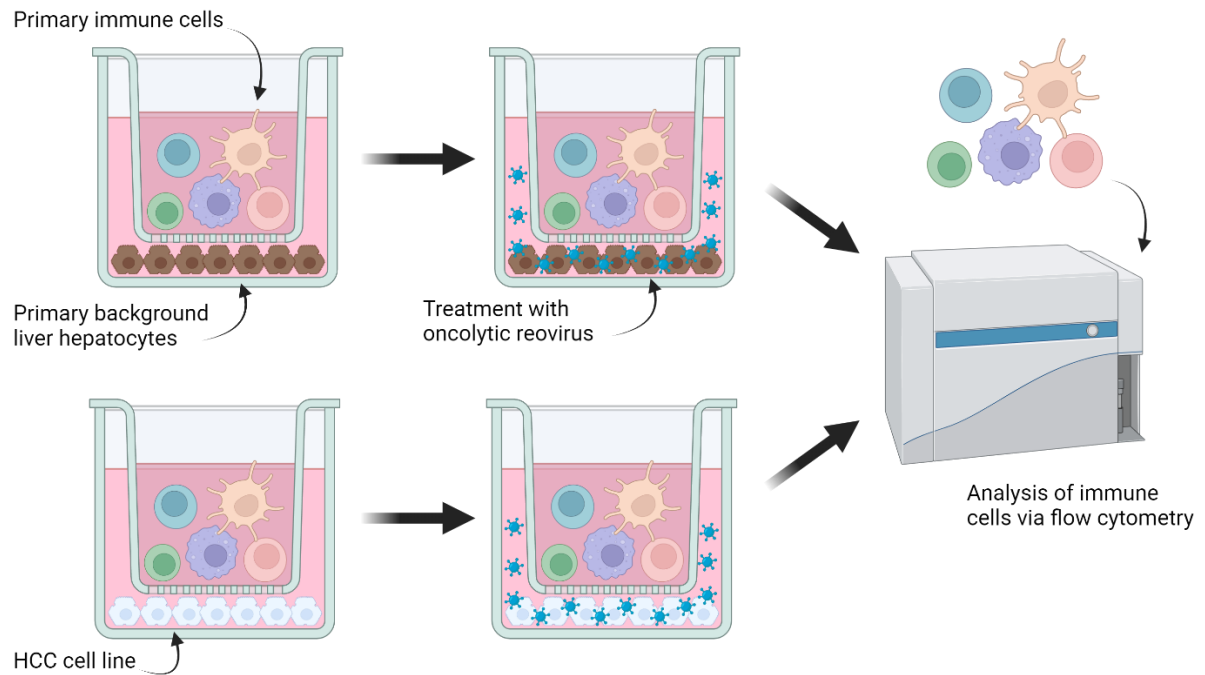


Figure 3.4. Schematic overview of transwell co-culture experimental design. Primary PBMCs were co-cultured with either primary background liver hepatocytes or JHH-4 cells, a HCC cell line. Co-cultures were then treated with either 1 pfu/cell oncolytic reovirus or PBS before flow cytometric analysis of NK, CD4+ and CD8+ T-cells for surface expression of co-stimulatory and co-inhibitory immune checkpoint receptors.

Looking at NK cells, PD-L1 expression increases on the surface of NK cells that were cultured with JHH-4 cancer cells and treated with reovirus by 72 hrs (Figure 3.5). One reason for this could be oncolytic reovirus preferentially targeting the JHH-4 cancer cells and the subsequent release of cytokines has increased surface expression of the inhibitory molecule. Similar to PD-L1, though not significant, there is a trend of increased expression of LAG-3 in response to reovirus treatment in the JHH-4 co-cultured cells (Figure 3.5). Expression of OX40, PD-1, TIGIT and BTLA are all increased on the surface of NK cells that were co-cultured with JHH-4 cancer cells compared to those co-cultured with primary hepatocytes, suggesting they may be upregulated within the tumour microenvironment, but are not affected by oncolytic reovirus treatment.

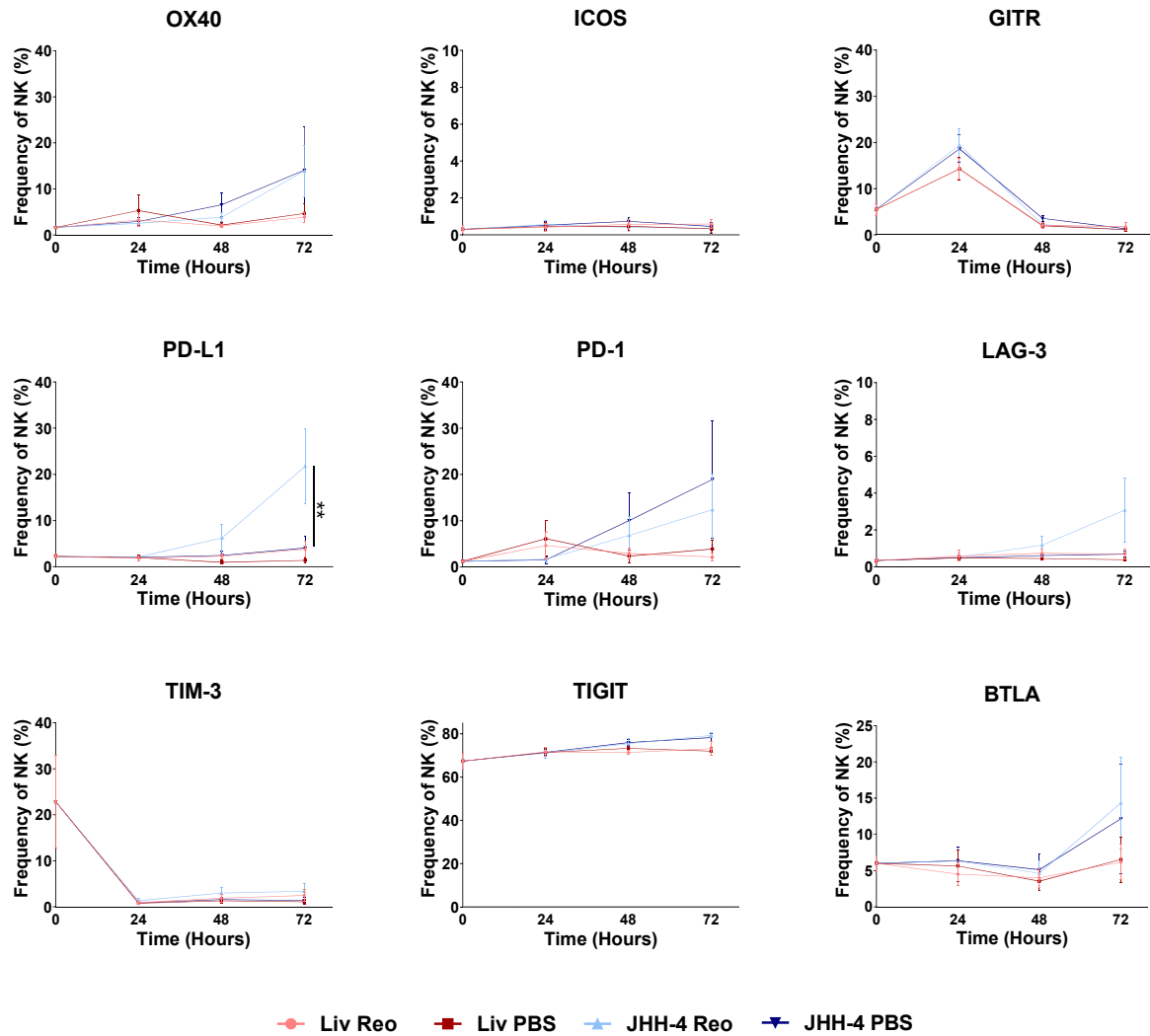


Figure 3.5. Indirect oncolytic reovirus treatment selectively induces PD-L1 expression on NK cells co-cultured with HCC cell line. Transwell co-culture experiments were conducted as in Figure 3.4, where JHH-4 (Blue) represents a human HCC cell line and Liv (Red) represents primary background human hepatocytes. Co-cultures were treated with either PBS (Dark) or 1 pfu/cell oncolytic reovirus (Pale) and in Figure 3.4. CD56+CD3- NK cells were analysed via flow cytometry for positive expression of co-stimulatory and co-inhibitory receptors from 0-72 hrs. Summary graphs representative of two independent experiments and show mean $\pm$ SEM (n=4). Statistical results show results of a 3way ANOVA with Tukey's multiple comparisons test at 72hrs. $P > 0.05$ (), $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***) and $P \leq 0.0001$ (****).

On the surface of CD4⁺ T-cells, similarly to NK cells, expression of PD-L1 and LAG-3 is increased in response to reovirus treatment in the JHH-4 cancer cell co-culture system and not in the background hepatocyte co-cultures at 72 hrs (Figure 3.6). PD-L1 increases on the JHH-4 co-cultured CD4⁺ T-cells at around 72 hrs, though later time-points may reveal a greater difference between the other treatment conditions. Compared to 0-24 hrs, LAG-3 is trending increased in all conditions at 48 hrs, with expression remaining significantly higher on the JHH-4 co-cultured CD4⁺ T-cells at 72 hrs compared to the other treatment conditions where expression has returned to baseline.

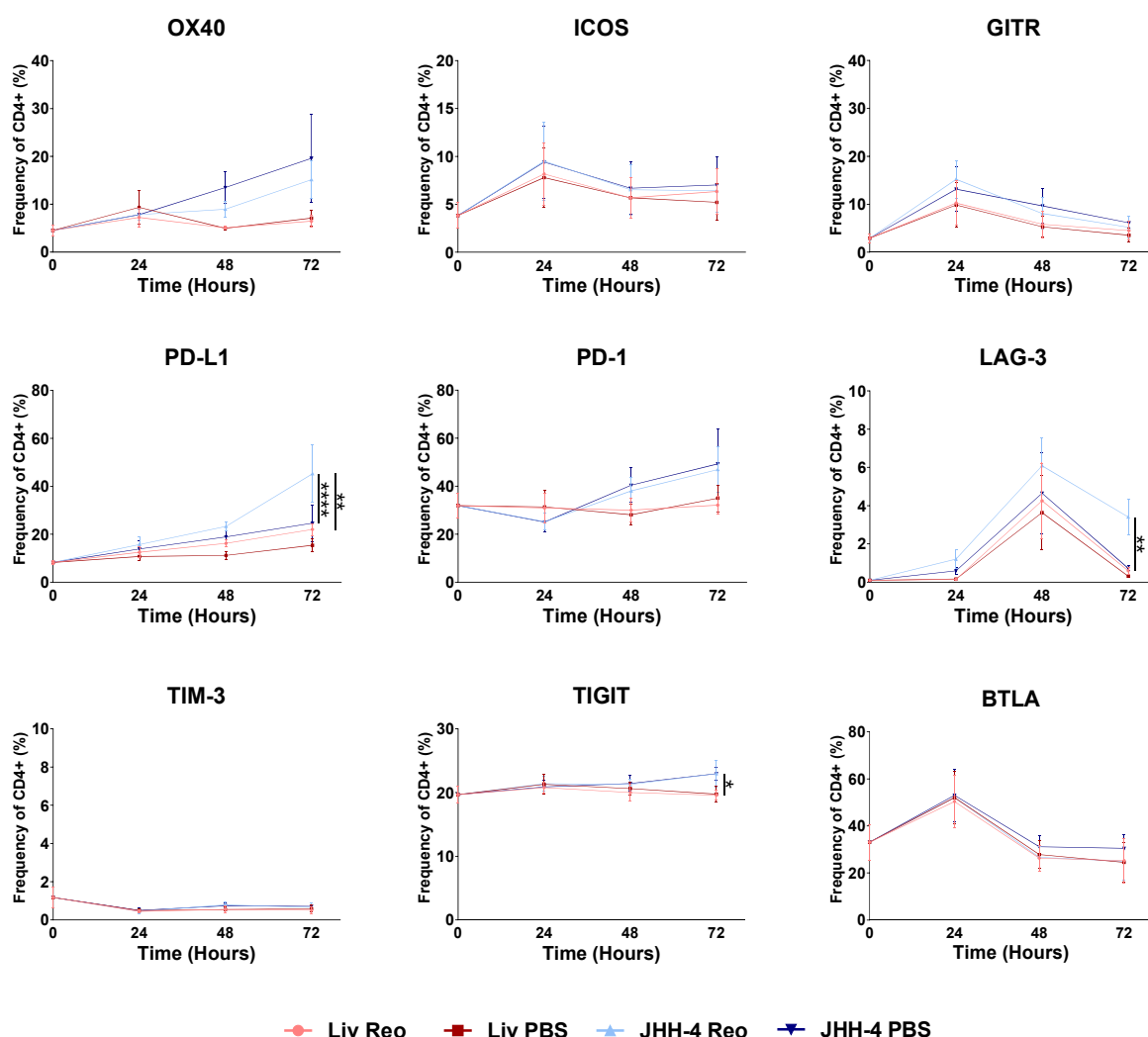


Figure 3.6. Indirect oncolytic reovirus treatment selectively induces PD-L1 and LAG-3 expression on CD3+CD4+ T-cells co-cultured with HCC cell line. Transwell co-culture experiments were conducted as in Figure 3.4, where JHH-4 (Blue) represents a human HCC cell line and Liv (Red) represents primary background human hepatocytes. Co-cultures were treated with either PBS (Dark) or 1 pfu/cell oncolytic reovirus (Pale) and in Figure 3.4. CD3+CD4+ T-cells were analysed via flow cytometry for positive expression of co-stimulatory and co-inhibitory receptors from 0-72 hrs. Summary graphs representative of two independent experiments and show mean $\pm$ SEM (n=4). Statistical results show results of a 3way ANOVA with Tukey's multiple comparisons test at 72hrs. $P > 0.05$ (), $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***) and $P \leq 0.0001$ (****).

Looking at CD8⁺ T-cells, LAG-3 is the only marker explored to significantly increase differentially in the JHH-4 co-cultured cells treated with reovirus (Figure 3.7). There is a strong trend between 24-72 hrs, with a significant difference being observed at 72 hrs.

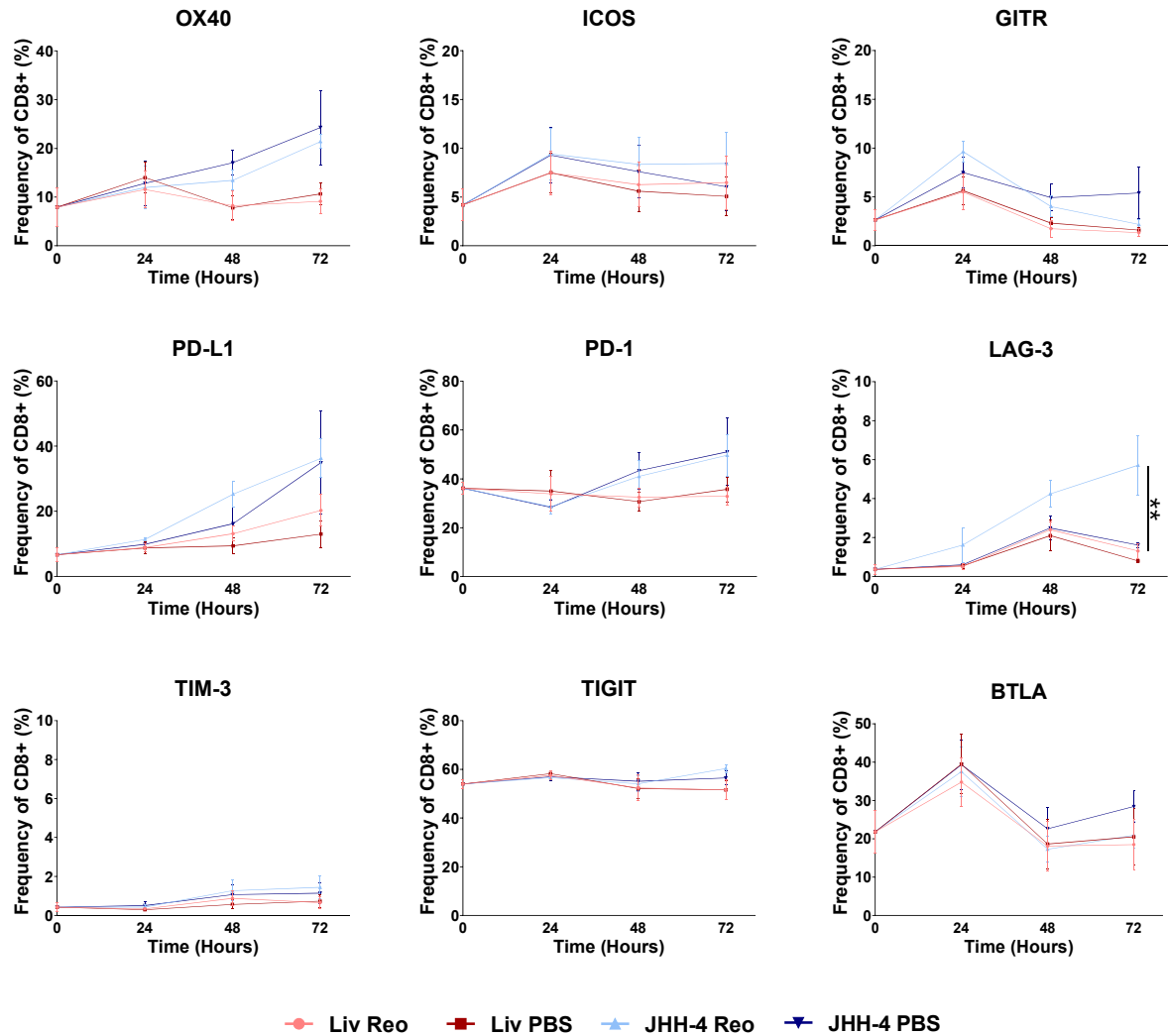


Figure 3.7. Indirect oncolytic reovirus treatment selectively induces LAG-3 expression on CD3+CD8+ T-cells co-cultured with HCC cell line. Transwell co-culture experiments were conducted as in Figure 3.4, where JHH-4 (Blue) represents a human HCC cell line and Liv (Red) represents primary background human hepatocytes. Co-cultures were treated with either PBS (Dark) or 1 pfu/cell oncolytic reovirus (Pale) and in Figure 3.4. CD3+CD8+ T-cells were analysed via flow cytometry for positive expression of co-stimulatory and co-inhibitory receptors from 0-72 hrs. Summary graphs representative of two independent experiments and show mean $\pm$ SEM (n=4). Statistical results show results of a 3way ANOVA with Tukey's multiple comparisons test at 72hrs. $P > 0.05$ (), $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***) and $P \leq 0.0001$ (****).

As LAG-3 and PD-L1 are co-inhibitory markers, their increased expression on JHH-4 co-cultured immune cells in response to reovirus treatment would likely inhibit T-cell activation when binding to their receptor/ligand counterparts. Together, these results suggest that PD-L1 and LAG-3 may be suitable targets for combination ICM treatment with oncolytic reovirus, and timing of this regimen should be explored. These preliminary data suggest that PD-L1 and LAG-3 are differentially expressed on the surface of immune cells 72 hrs post-reovirus treatment, and in most cases, an upward trend of expression suggests further time-points should also be explored. One consideration to note is that the increased expression of LAG-3, whilst demonstrating a rise, is restricted to around 4-6 % of the cell population evaluated (Figures 3.5-3.7). It would be important to explore the biological impact of this increased expression, to evaluate the effectiveness/importance of an ICM combination with LAG-3 blockade.

3.2.3 Oncolytic reovirus successfully enters Hep53.4 HCC cancer cell line

To better understand the immune mediated effects of oncolytic reovirus in the treatment of HCC, a syngeneic subcutaneous tumour model was to be used. The HCC cell line, Hep53.4, was implanted in C57JBL/6 mice and optimised for tumour growth experiments $\pm$ reovirus. The nature of this experimental design meant it would be vital that reovirus is able to enter the tumour cell line. Thus, Hep53.4 cells, were cultured for 24 hrs $\pm$ oncolytic reovirus (1 pfu/cell) and stained via IF for reovirus $\sigma 3$, a reovirus outer capsid protein (Figure 3.8). It was observed that reovirus successfully entered Hep53.4 cells, making the cell line a suitable choice for reovirus-based murine tumour models.

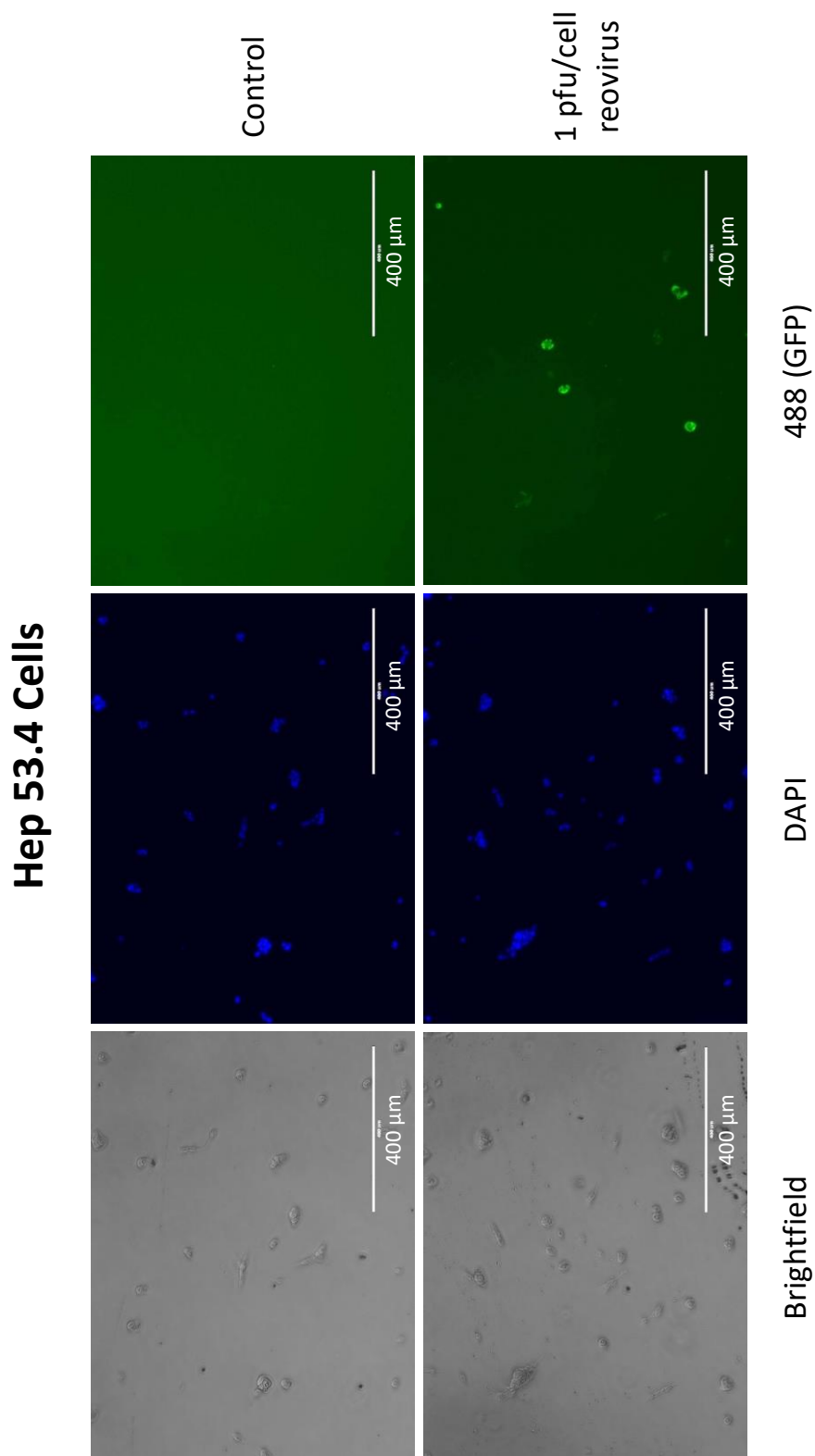


Figure 3.8. Immunofluorescence reveals oncolytic reovirus successfully enters Hep53.4 murine HCC cell line. Hep53.4 cells were treated with PBS (control) or 1 pfu/cell oncolytic reovirus for 24 hours before being stained for reovirus type 3 sigma 3 protein primary antibody, followed by FITC-conjugated IgG2a secondary antibody. Cells were imaged to reveal reovirus successfully enters Hep53.4 cells.

3.2.4 Hep53.4 HCC cell line grows palpable tumours in a subcutaneous model

Primary investigations into the Hep53.4-C57Bl/6 subcutaneous tumour model looked at tumour growth kinetics with differing cell implantation densities. As there was no literature around this cell line as a subcutaneous model, three implantation densities (1×10^5 , 1×10^6 and 1×10^7) based on other subcutaneous models were evaluated for tumour diameter (length) and volume $[(\text{width}^2 \times \text{length})/2]$ over 26 days. Looking at both mean tumour diameter (Figure 3.9A) and mean tumour volume (Figure 3.9D), 1×10^5 cell density was not sufficient to grow palpable tumours. Meanwhile, 1×10^6 and 1×10^7 both grew tumours of a similar diameter (Figure 3.9A-C). Looking at volume however, 1×10^7 cell implantation formed tumours with a larger volume than 1×10^6 , but not too large as to reach a primary end point earlier than day 26. As this was a sufficient timescale for the experimental design, and the mice showed no side effects of tumour implantation beyond the scope of the license, this cell implantation density was chosen for future experimental use. It is important to note that tumours derived from cell implantation densities of 1×10^6 and 1×10^7 appeared to self-plateau at around day 12.

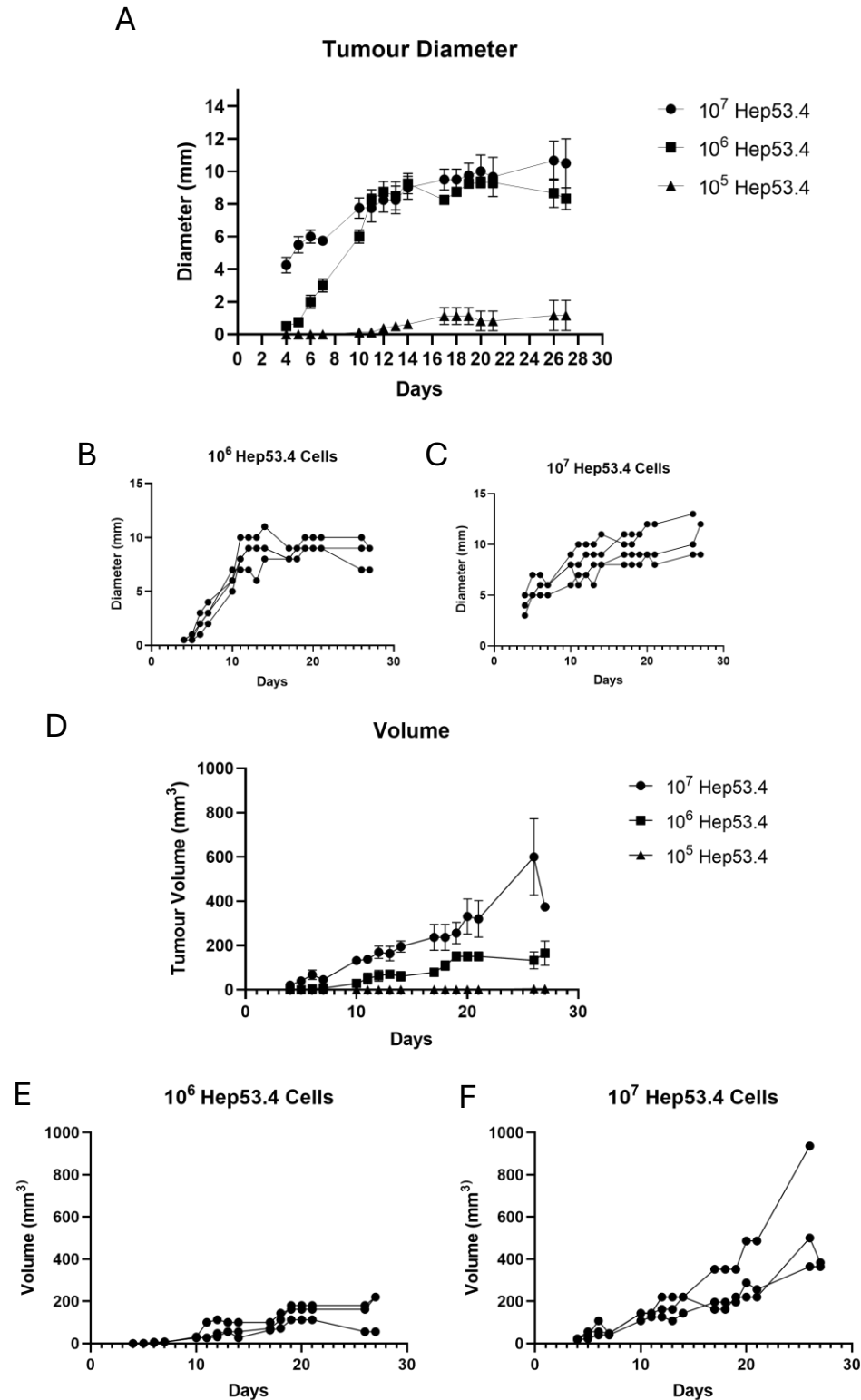


Figure 3.9. Subcutaneous implantation of 1×10^7 Hep53.4 cell line into C57JBL/6 mice successfully grows palpable tumours. Three cell densities of Hep53.4 cells (10^5 , 10^6 and 10^7 ; 200 μL PBS) were implanted subcutaneously into the right flank of C57JBL/6 mice. Tumour growth was monitored as **A-C** diameter (mm) and **D-F** volume (mm^3) over 26 days. Summary graphs A and D show mean $\pm$ SEM ($n=4$). B, C, E and F show individual mice ($n=4$).

3.2.5 Oncolytic reovirus does not show anti-tumour activity in Hep53.4-C57Bl/6 model

Following the optimisation of a Hep53.4-C57JBl/6 subcutaneous model in section 3.2.4, a cell implantation density of 1×10^7 was taken forward to evaluate the effects of oncolytic reovirus on tumour growth were evaluated. Hep53.4 tumour-bearing mice were treated with either PBS or 1×10^7 pfu reovirus, chosen due to being a murine dose comparable with human dose in clinical trials. Interestingly, despite oncolytic reovirus being able to successfully enter Hep53.4 cells *in vitro* (Figure 3.8), systemic I.V treatment with oncolytic reovirus shows no anti-tumour activity in the Hep53.4-C57JBl/6 subcutaneous tumour model compared to PBS control 7 days post-treatment when looking at tumour diameter (Figure 3.10). It would therefore be of interest to explore the effects of oncolytic reovirus on co-stimulatory and co-inhibitory expression in this model, to rationally select targets for future combination strategies.

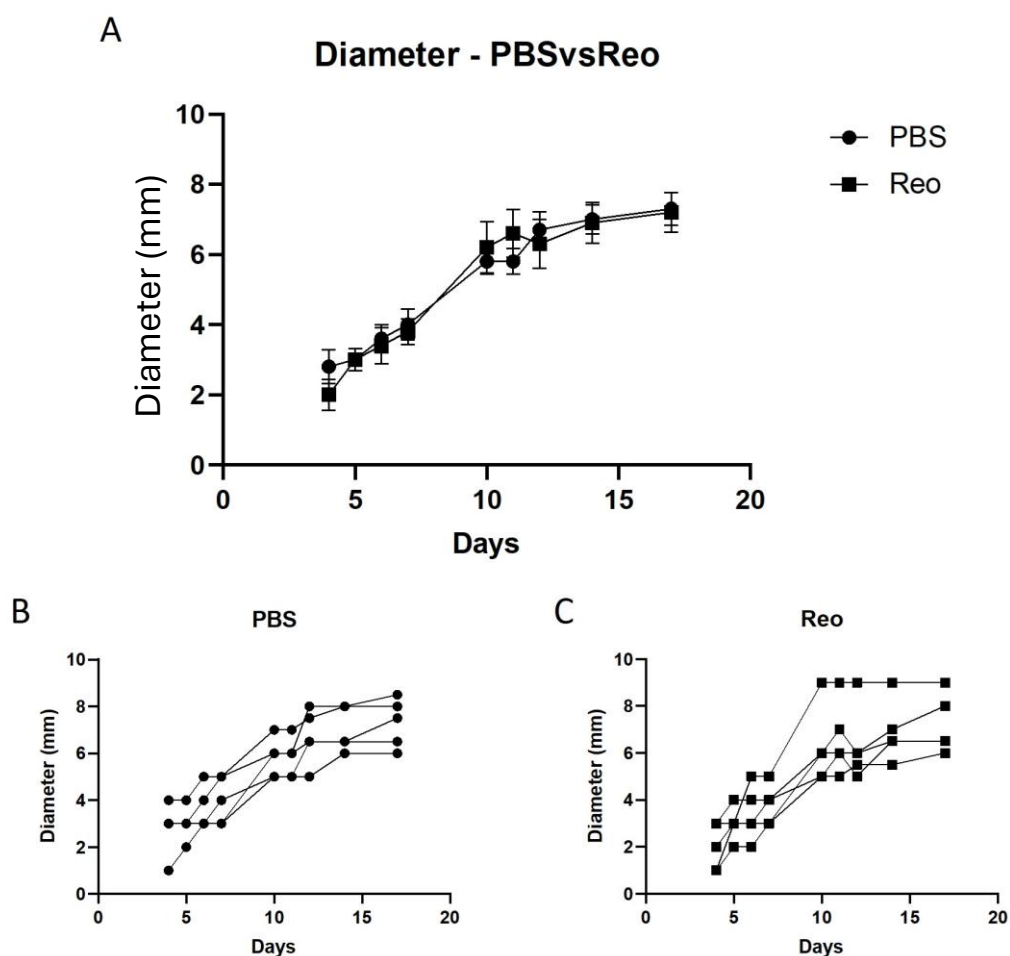


Figure 3.10. Single-agent I.V reovirus treatment *in vivo* shows poor anti-tumour efficacy. C57JBL/6 mice (n=5) were subcutaneously implanted with 1×10^7 Hep53.4 cells and treated with PBS or 1×10^7 pfu reovirus and tumour diameter (mm) was monitored over 17 days. Summary graph (A) shows mean $\pm$ SEM (n=5). B and C show individual mice (n=5).

3.2.6 Oncolytic reovirus treatment induces expression of co-stimulatory and co-inhibitory receptors on the surface of immune cells in the Hep53.4-C57Bl/6 tumour model

Following the surprising observation that systemic i.v treatment with oncolytic reovirus does not induce anti-tumour effects in the Hep53.4-C57Bl/6 model, an exploration of reovirus-induced immunity was undertaken. In the Hep53.4-C57Bl/6 subcutaneous tumour model, tissues were harvested on days 1, 4 and 7 post-reovirus treatment and CD4⁺ and CD8⁺ T-cells were explored for their expression of co-stimulatory and co-inhibitory receptors commonly associated with ICM (Figure 3.11). Unfortunately, low yields of lymphocytes were obtained from the tumours. Tissues studied therefore included the liver as an exploration into the effects of reovirus in background liver, and TDLNs as a proxy of the tumour due to low numbers of CD4⁺ and CD8⁺ T-cells retrieved from the tumour samples.

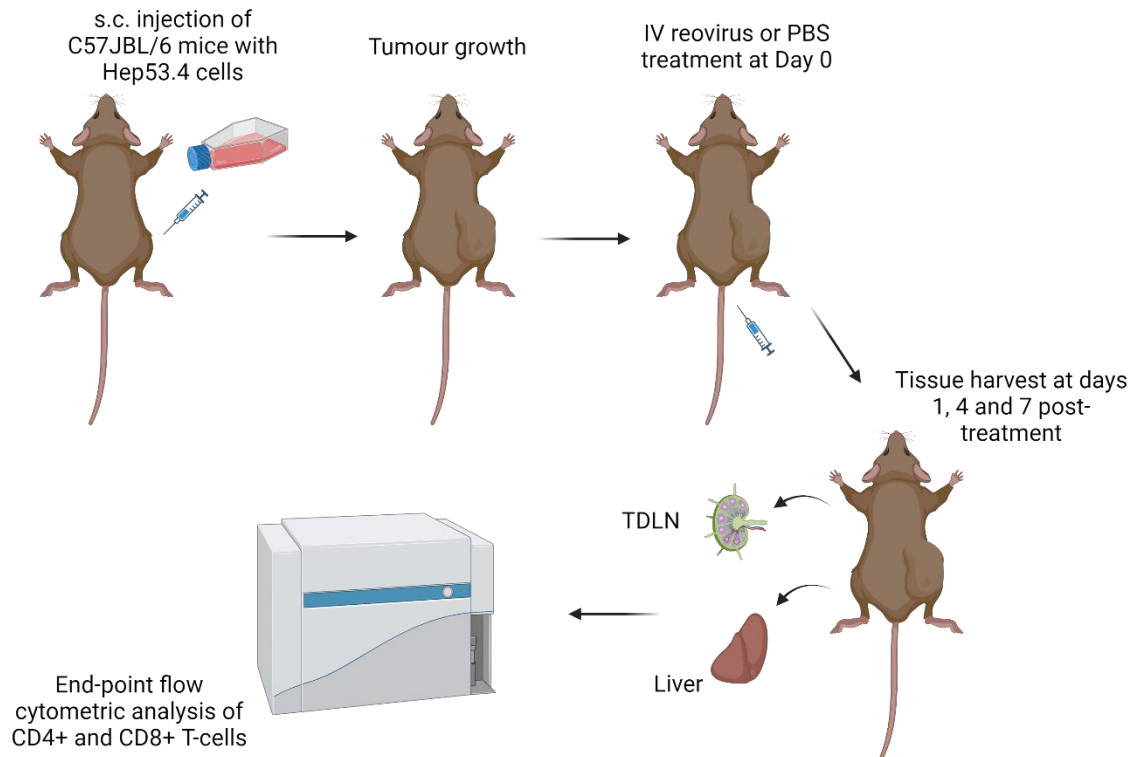


Figure 3.11. Schematic overview of subcutaneous Hep53.4-C57JBL/6 model to evaluate effects of oncolytic reovirus treatment. C57JBL/6 mice were injected subcutaneously with 1×10^7 Hep53.4 cells and tumour growth was monitored. Mice were treated i.v. with 1×10^7 pfu reovirus or PBS at Day 0 (Day 10 post-implantation). Tumour draining lymph nodes (TDLNs) and Livers were harvested at 1, 4 and 7 days post-treatment. Tissues were processed to single cell suspensions and analysed via flow cytometry for expression of co-stimulatory and co-inhibitory molecules on the surface of CD4+ and CD8+ T-cells.

A range of co-stimulatory (CD137, GITR, OX40 and ICOS) and co-inhibitory (LAG-3, PD-L1, PD-1 and TIM-3), alongside CD69 as an early marker of activation, were explored on the surface of CD4+ and CD8+ T-cells. To determine whether CD4+ and CD8+ T-cells were positive for marker expression, gates were set utilising FMOs and cells entering the gates were deemed positive for the marker in question. An example of these gates for PD-1 expression on the surface of CD8+ T-cells within the TDLN is shown in Figure 3.12. Unfortunately, PD-L1 was unable to be evaluated in TDLN due to poor quality staining. Due to time limitations, a repeat experiment was not possible.

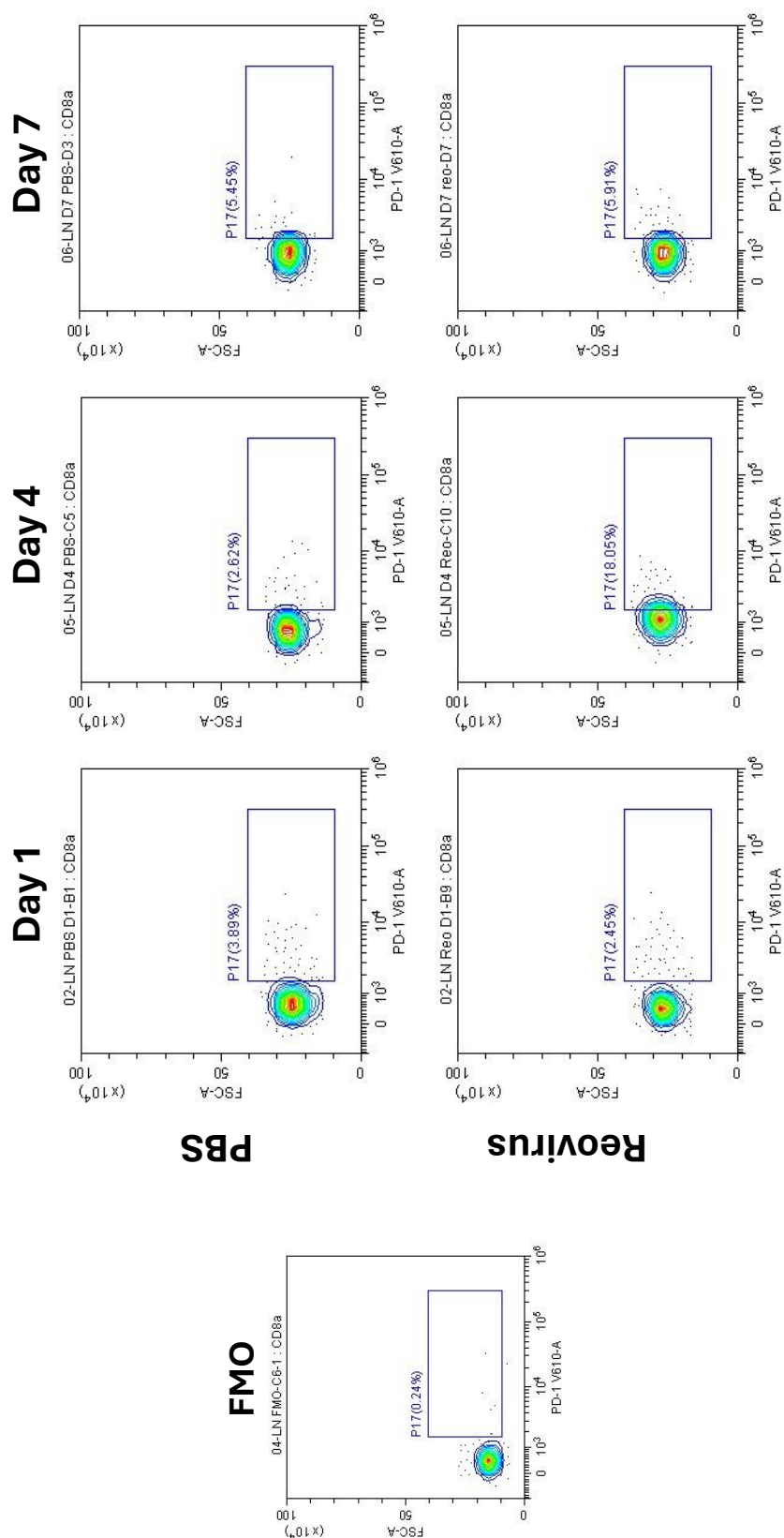
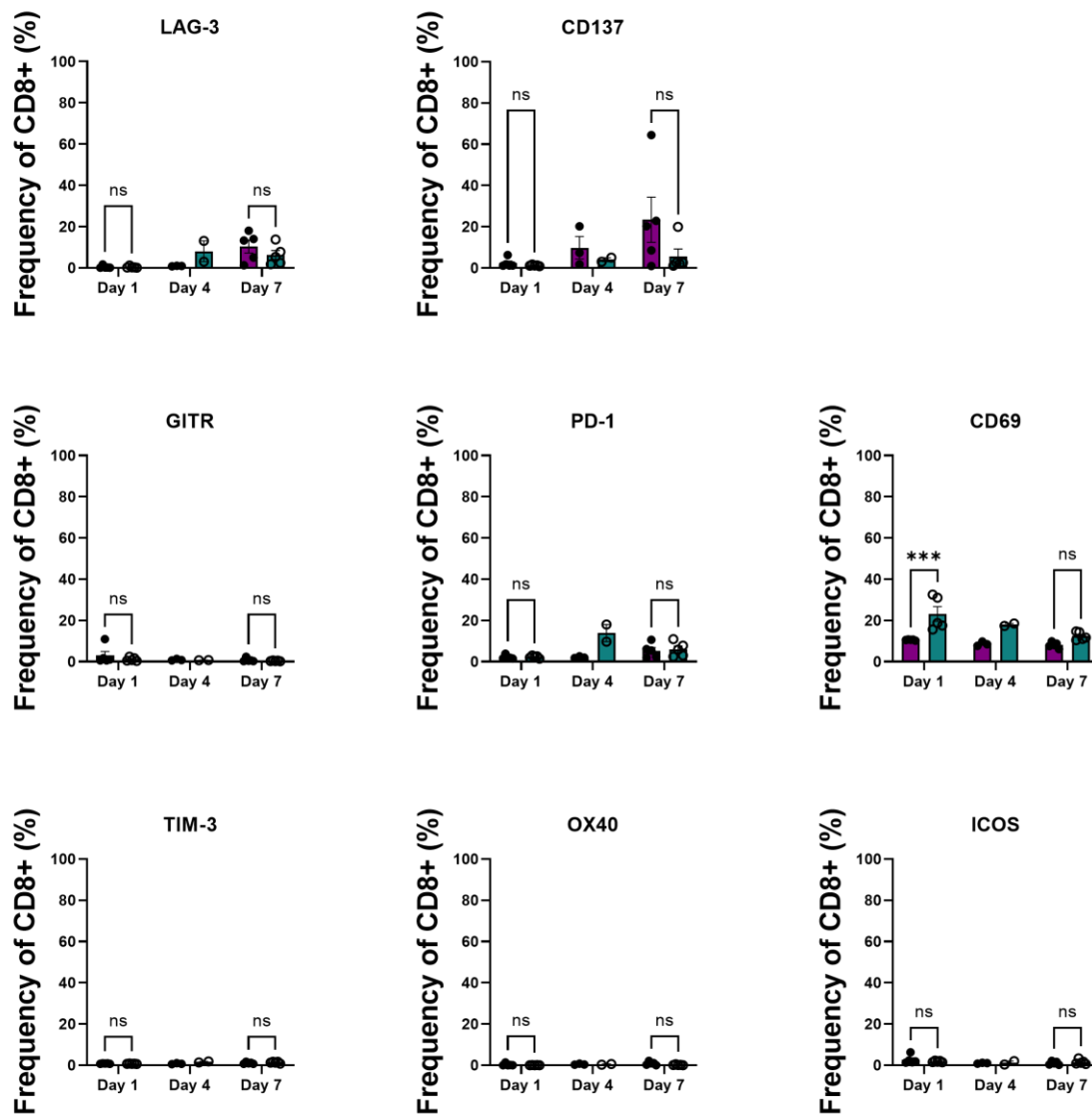


Figure 3.12. Gating strategy for positive marker expression. Gates were set to define CD3+CD8+ T-cells positive for expression of PD-1 utilising FMO. Example of TDLN. Frequency of parent (%) were taken.

The TDLNs are the primary sites of development of anti-tumour immunity due to presentation of tumour-related antigens to activate CD4⁺ and CD8⁺ T-cells (Koukourakis and Giatromanolaki, 2022). In the absence of tumour infiltrating lymphocyte yields, CD4⁺ and CD8⁺ T-cells in the TDLNs were utilised as a proxy to the tumour. The early activation marker CD69 is significantly upregulated on CD8⁺ and CD4⁺ T-cells in the TDLN (Figures 3.13 and 3.14). Within the TDLN, the frequency of CD69⁺ CD8⁺ T-cells remains significantly different at day 4, suggesting that cells are either activated for longer, or are undergoing persistent signalling with the TDLNs. This finding would be consistent with the notion that tumour-associated antigens are presented to T-cells by DCs in TDLNs (Koukourakis and Giatromanolaki, 2022). Of course, it is also worth noting here that CD69 can also be upregulated by the presence of IFNs, another potential cause of prolonged CD69 expression.

In addition, the frequency of PD-1⁺ CD8⁺ T-cells were observed to be significantly increased in reovirus-treated mice compared to the control mice at day 4 (Figure 3.13). Tim-3 showed the same trend as PD-1 albeit at a much lower level (1.6 %). No other markers were observed to significantly change on the surface of CD4⁺ or CD8⁺ T-cells in response to reovirus treatment (Figures 3.13 and 3.14).



- PBS
- Reovirus

Figure 3.13. Systemic reovirus treatment *in vivo* induces expression of PD-1 and CD69 on the surface of CD8+ T-cells in TDLN in Hep53.4-C57JBL/6 model. C57JBL/6 mice were implanted subcutaneously with 1×10^7 Hep53.4 cells and treated at day 0 with 1×10^7 pfu i.v. oncolytic reovirus. TDLN were harvested at days 1, 4 and 7 post-treatment and CD3+CD8+ T-cells were analysed for positive surface expression of co-stimulatory and co-inhibitory molecules. Summary graphs show mean $\pm$ SEM (PBS treatment, n=5 days 1 and 7, n=3 day 4; Reovirus treatment, n=5 days 1 and 7, n=2 day 4). Statistical results show results of a 2way ANOVA with Sidak's multiple comparisons test $p \leq 0.05$.

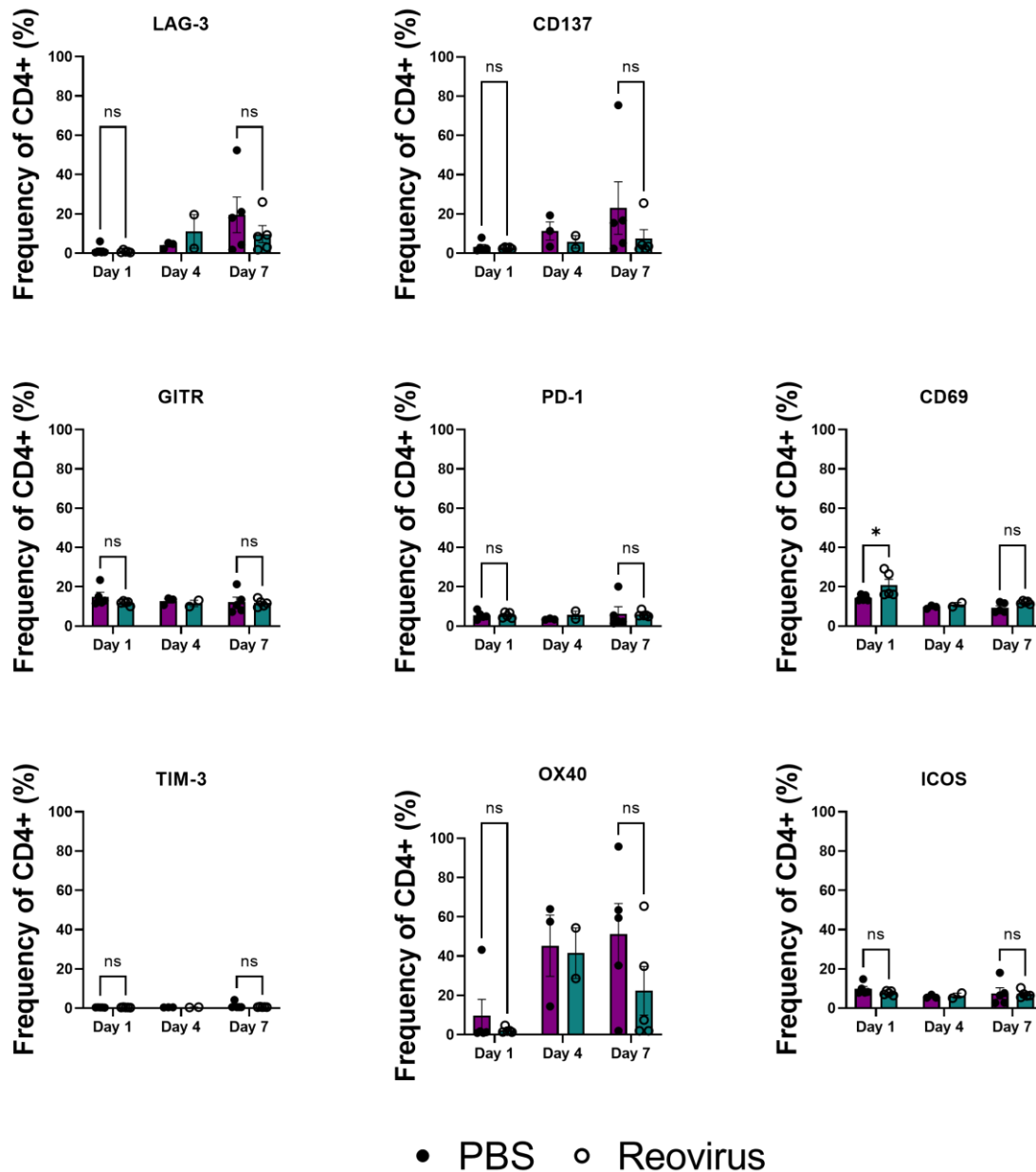


Figure 3.14. Systemic reovirus treatment *in vivo* induces expression of CD69 on the surface of CD4⁺ T-cells in TDLN in Hep53.4-C57JBL/6 model. C57JBL/6 mice were implanted subcutaneously with 1×10^7 Hep53.4 cells and treated at day 0 with 1×10^7 pfu i.v. oncolytic reovirus. TDLN were harvested at days 1, 4 and 7 post-treatment and CD3⁺CD4⁺ T-cells were analysed for positive surface expression of co-stimulatory and co-inhibitory molecules. Summary graphs show mean $\pm$ SEM (PBS treatment, n=5 days 1 and 7, n=3 day 4; Reovirus treatment, n=5 days 1 and 7, n=2 day 4). Statistical results show results of a 2way ANOVA with Sidak's multiple comparisons test $p \leq 0.05$.

To explore the immune-mediated effects of oncolytic reovirus on the background liver, intrahepatic CD4⁺ and CD8⁺ T-cell populations were explored for their expression of co-stimulatory and co-inhibitory receptors. As immune-mediated autoimmune hepatitis is a common adverse event with ICM therapies, the effect that treatments have on background liver is important to consider to potentially prevent toxicities in future ICM therapy combinations. The rationale would be to find a checkpoint receptor that is differentially induced via reovirus within the tumour, but not in the background liver, thus allowing for a more directed therapeutic approach to spare further hepatic damage.

Looking at intrahepatic CD8⁺ T-cells, CD69 was observed to increase at day 1 in response to reovirus treatment and not in the control (Figure 3.15), consistent with previous tissues studied (Figures 3.13 and 3.14). Within intrahepatic CD4⁺ T-cells, CD69 expression is not greater at day 1 for reovirus-treated mice compared to the control, but rather a significant difference is observed at day 4 post-treatments (Figure 3.16).

PD-L1 expression is also significantly induced on the surface of CD8⁺ and CD4⁺ T-cells in the liver in response to reovirus-treatment (Figures 3.15 and 3.16). This profound difference would therefore suggest that whilst PD-L1 blockade may be suggested as a suitable target for combination with reovirus *in vitro* (sections 3.2.1 and 3.2.2), blockade of PD-L1 in combination with oncolytic reovirus *in vivo* may lead to uncontrolled T-cell activation with the background liver, potentially leading to further hepatic damage and immune-mediated toxicities such as autoimmune hepatitis. Evaluating the expression of PD-1, the receptor for PD-L1, in the surrounding tissue

environment would be of importance to understand how reovirus as a monotherapy could potentially be eliciting unwanted side effects.

CD137, PD-1, TIM-3, OX40 and ICOS, all markers which have previously not showed much response to reovirus treatment, are all upregulated on the surface of reovirus-treated intrahepatic CD8⁺ T-cells 7 days post-treatment (Figure 3.15). Similarly, CD137, PD-1, TIM-3 and ICOS are all upregulated on the surface of intrahepatic CD4⁺ T-cells in response to reovirus treatment at day 7 post-treatment (Figure 3.16). OX40, whilst significantly upregulated on the surface of CD4⁺ T-cells by day 7, does show an increased trend. The importance of these markers being upregulated on the surface of intrahepatic CD4⁺ and CD8⁺ T-cells in response to reovirus treatment could be linked to the immune-mediated toxicities associated with single-agent oncolytic reovirus therapy.

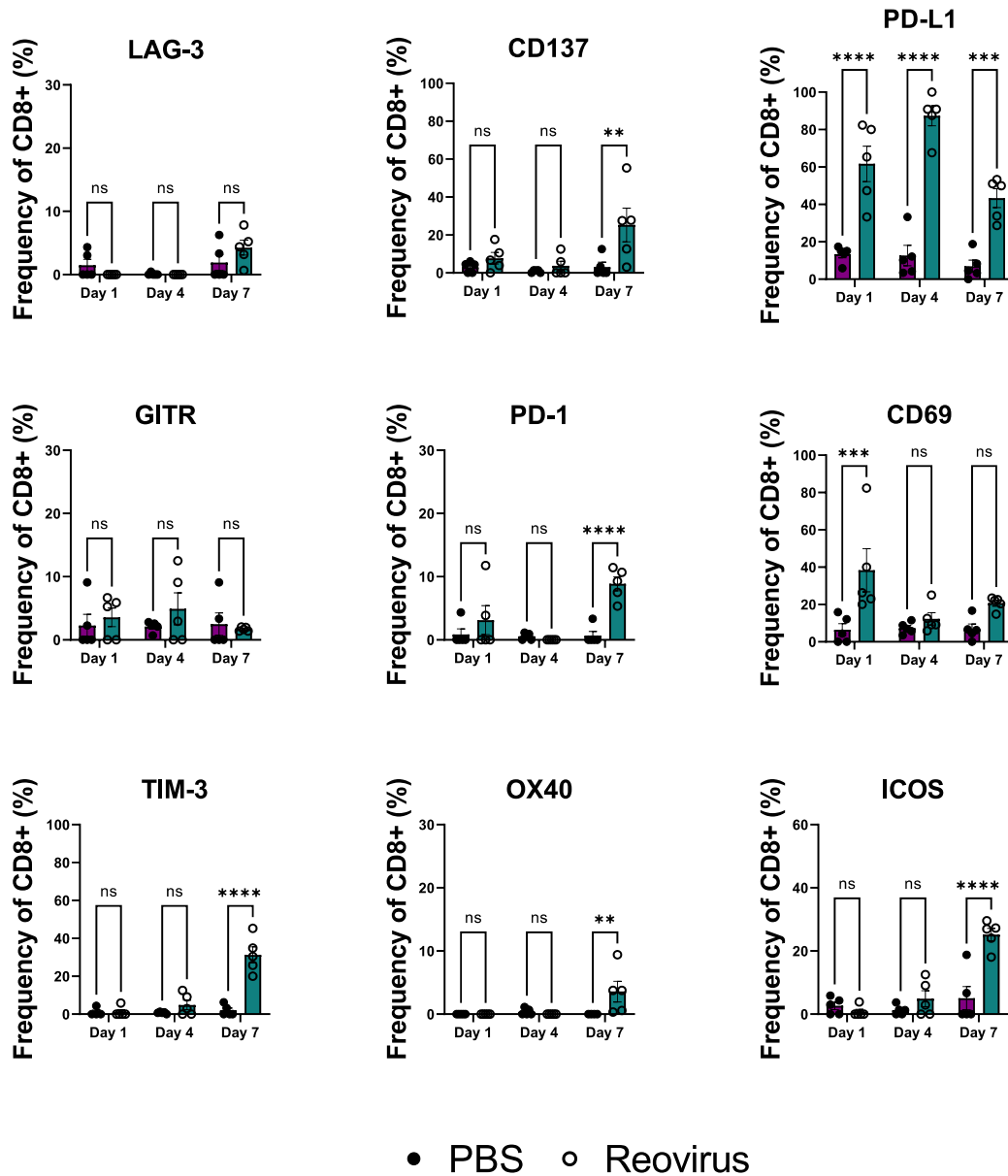


Figure 3.15. Systemic reovirus treatment *in vivo* induces expression of several co-stimulatory and co-inhibitory molecules on the surface of CD8+ T-cells in livers in Hep53.4-C57JBL/6 model. C57JBL/6 mice were implanted subcutaneously with 1×10^7 Hep53.4 cells and treated at day 0 with 1×10^7 pfu i.v. oncolytic reovirus. Livers were harvested at days 1, 4 and 7 post-treatment and CD3+CD8+ T-cells were analysed for positive surface expression of co-stimulatory and co-inhibitory molecules. Summary graphs show mean $\pm$ SEM (n=5). Statistical results show results of a 2way ANOVA with Sidak's multiple comparisons test $p \leq 0.05$.

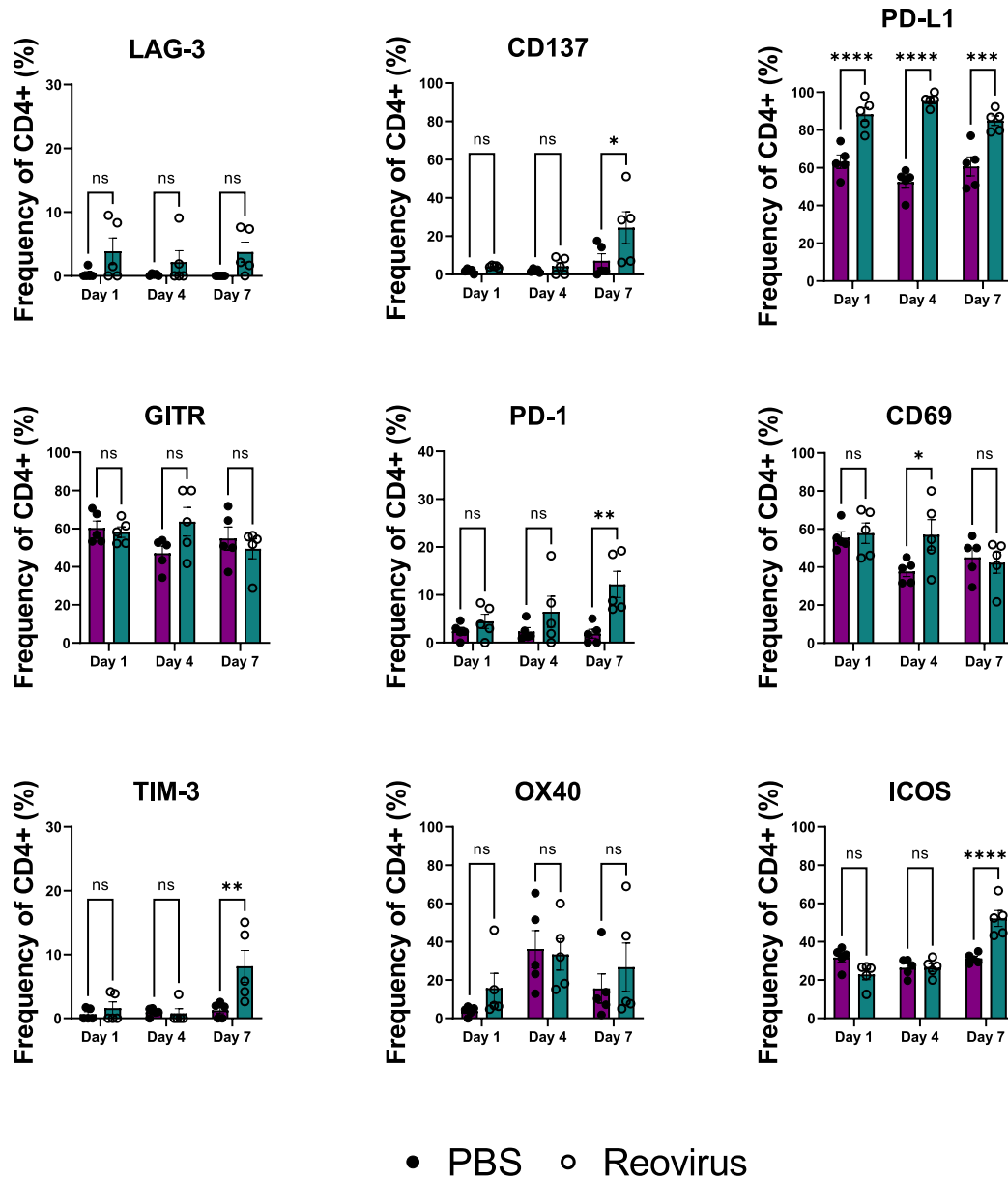


Figure 3.16. Systemic reovirus treatment *in vivo* induces expression of several co-stimulatory and co-inhibitory molecules on the surface of CD4+ T-cells in livers in Hep53.4-C57JBL/6 model. C57JBL/6 mice were implanted subcutaneously with 1×10^7 Hep53.4 cells and treated at day 0 with 1×10^7 pfu i.v. oncolytic reovirus. Livers were harvested at days 1, 4 and 7 post-treatment and CD3+CD4+ T-cells were analysed for positive surface expression of co-stimulatory and co-inhibitory molecules. Summary graphs show mean $\pm$ SEM (n=5). Statistical results show results of a 2way ANOVA with Sidak's multiple comparisons test $p \leq 0.05$.

3.2.7 Oncolytic reovirus treatment induces expression of co-stimulatory and co-inhibitory receptors on the surface of immune cells in the 1MEA-Balbc tumour model

To further validate the findings obtained in the Hep53.4-C57Bl/6 tumour model (section 3.2.6), a syngeneic subcutaneous 1MEA-Balb/c tumour model, previously used in the Samson lab to show that reovirus acts via immune-mediated mechanisms as opposed to oncolytic, was utilised to explore the effects of reovirus treatment on CD4⁺ and CD8⁺ T-cells (Samson *et al.*, 2018). This model has previously been optimised and shown to respond to oncolytic reovirus therapy and therefore was utilised in experimental design as previously optimised (Figure 3.17).

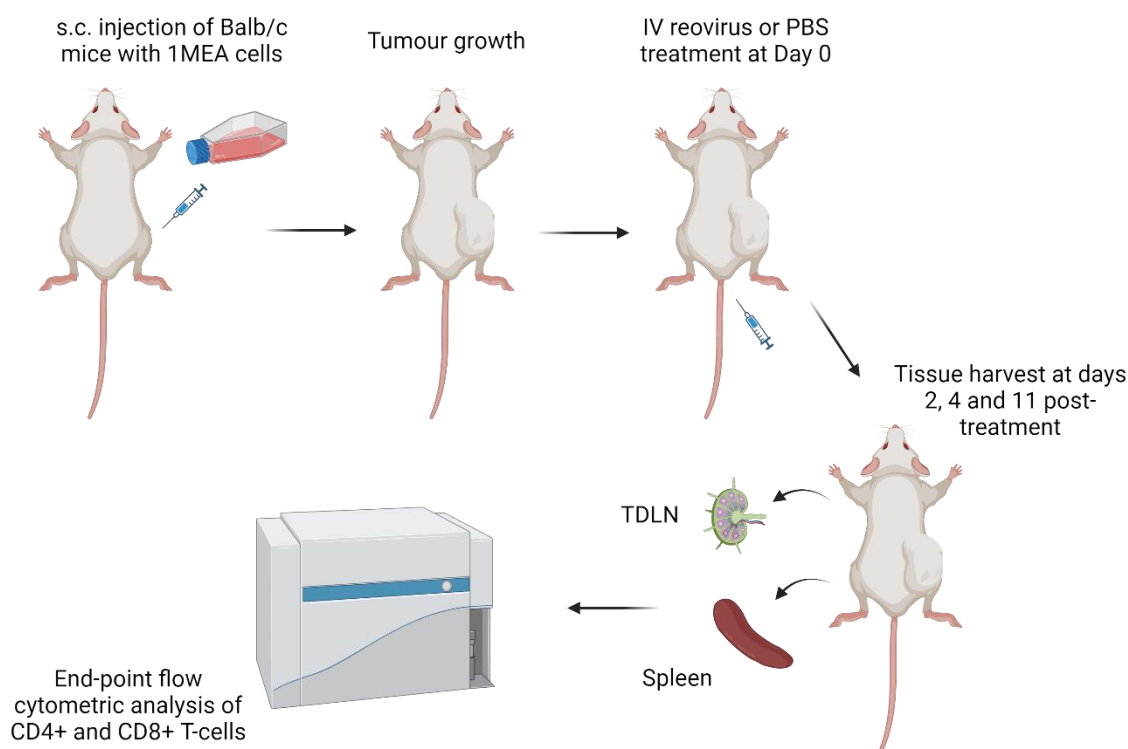


Figure 3.17. Schematic overview of subcutaneous 1MEA-Balb/c model to evaluate effects of oncolytic reovirus treatment. Balb/c mice were injected subcutaneously with 1×10^7 1MEA cells and tumour growth was monitored. Mice were treated i.v. with 1×10^7 pfu reovirus or PBS at Day 0 (Day 21 post-implantation). Tumour draining lymph nodes (TDLNs) and spleens were harvested at 2, 4 and 11 days post-treatment. Tissues were processed to single cell suspensions and analysed via flow cytometry for expression of co-stimulatory and co-inhibitory molecules on the surface of CD4+ and CD8+ T-cells.

This experimental design looked at CD4+ and CD8+ T-cells within the spleen and TDLN (Figures 3.19-3.22). Low yields of lymphocytes were obtained from the tumours and the livers; therefore in depth analysis by flow cytometry was not possible in this experiment. Unfortunately, a repeat experiment was not possible due to timing and therefore only analysable data from this experiment is included and discussed here.

As in section 3.2.6, a collection of co-stimulatory (CD137, GITR, OX40 and ICOS) and co-inhibitory (LAG-3, PD-L1, PD-1 and TIM-3), alongside CD69, were explored on the surface of CD4⁺ and CD8⁺ T-cells. Marker⁺ (%) gates were set utilising FMOs and cells within the gates were deemed positive for the marker in question. An example of these gates for PD-L1 expression on the surface of CD8⁺ T-cells within the spleen is shown in Figure 3.18. Differentially to section 3.2.6, this experimental design evaluated the expression of markers at days 2, 4 and 11 post-treatments, instead of days 1, 4 and 7 post-treatments as previous. Though an experimental design where all time points were evaluated would be ideal, the number of mice needed per group/timepoint made this unachievable for these investigations. Therefore, this secondary evaluation of receptor expression evaluated later timepoints that previously explored.

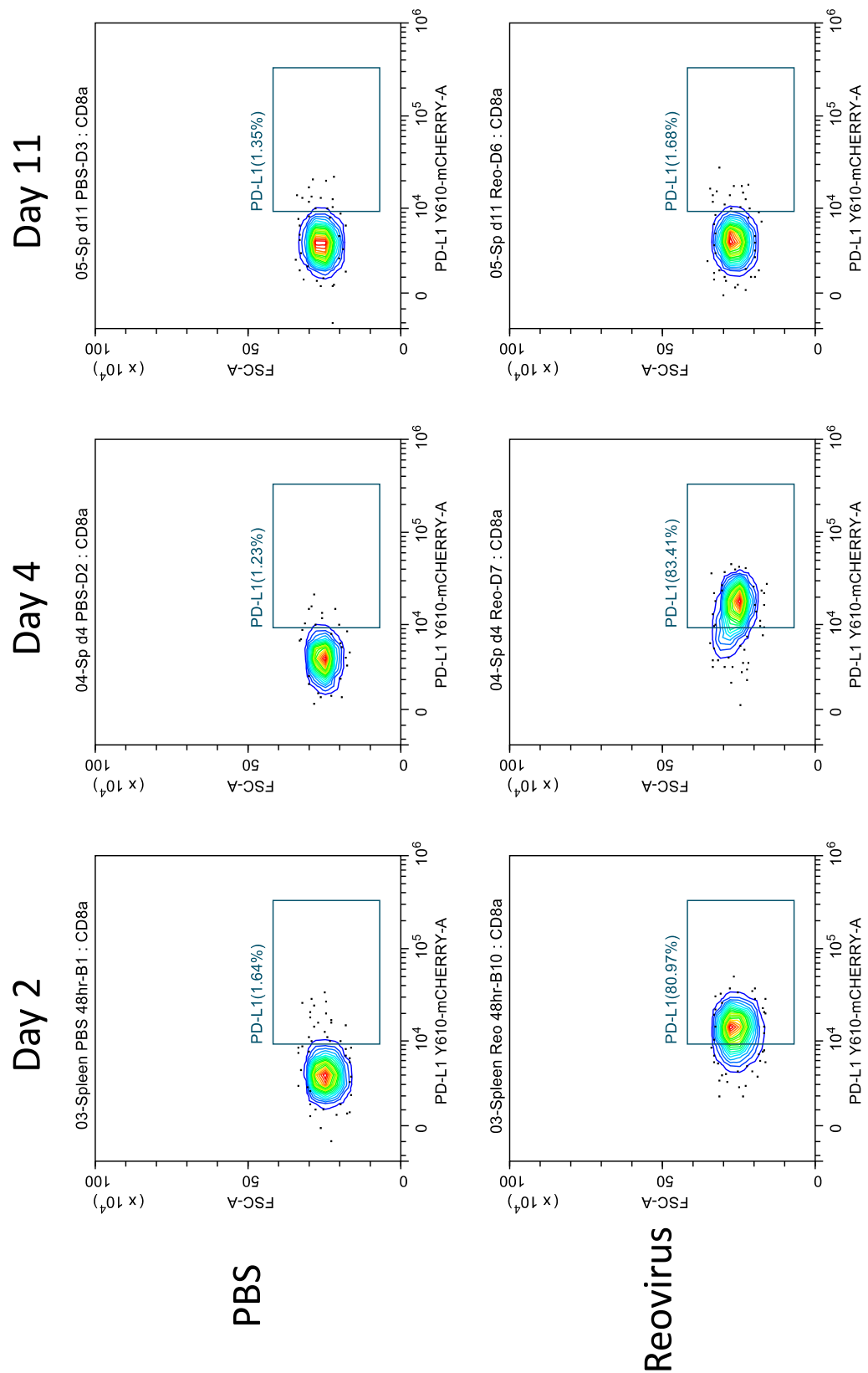


Figure 3.18. Example gating strategy for positive marker expression. Gates were set to define CD3+CD8+ T-cells positive for expression of PD-L1 utilising FMOs. Example of splenocytes. Frequency of parent (%) were taken.

In the spleen, the frequency of CD69⁺ CD8⁺ T-cells is significantly greater in reovirus-treated mice compared to the control group at day 1 (Figure 3.19). This is consistent with the notion that CD69 is an early activation marker.

Splenic PD-L1⁺ CD8⁺ T-cells are significantly greater on days 2 and 4 post-reovirus treatment compared to the control but have returned to a level comparable to the control by day 11. Interestingly, the proportion of splenic CD8⁺ T-cells positive for CD137 and PD-1 are significantly greater by day 4, and on days 2 and 4 for OX40 expression.

CD137 was observed to be significantly increased in reovirus-treated mice compared to the control 4 days post-treatment, though there is a large margin of error for the reovirus-treated mice and further repeats would reveal the validity of this significance. Also increased 4 days post-treatment in the reovirus-treated group compared to the control are LAG-3 and PD-1, although frequencies of these populations are relatively low at 16.02 % and 2.35 %, respectively. Splenic populations of OX40⁺ CD8⁺ T-cells are also significantly increased in response to reovirus treatment at days 2 and 4, with day 11 being comparable to control. ICOS expression appears later, with splenic populations of ICOS⁺ PD-L1⁺ T-cells significantly higher post-treatment with reovirus at days 4 and 11, but not at day 2 post-treatment. No changes were observed for GITR or TIM-3 on CD8⁺ T-cells in the spleen.

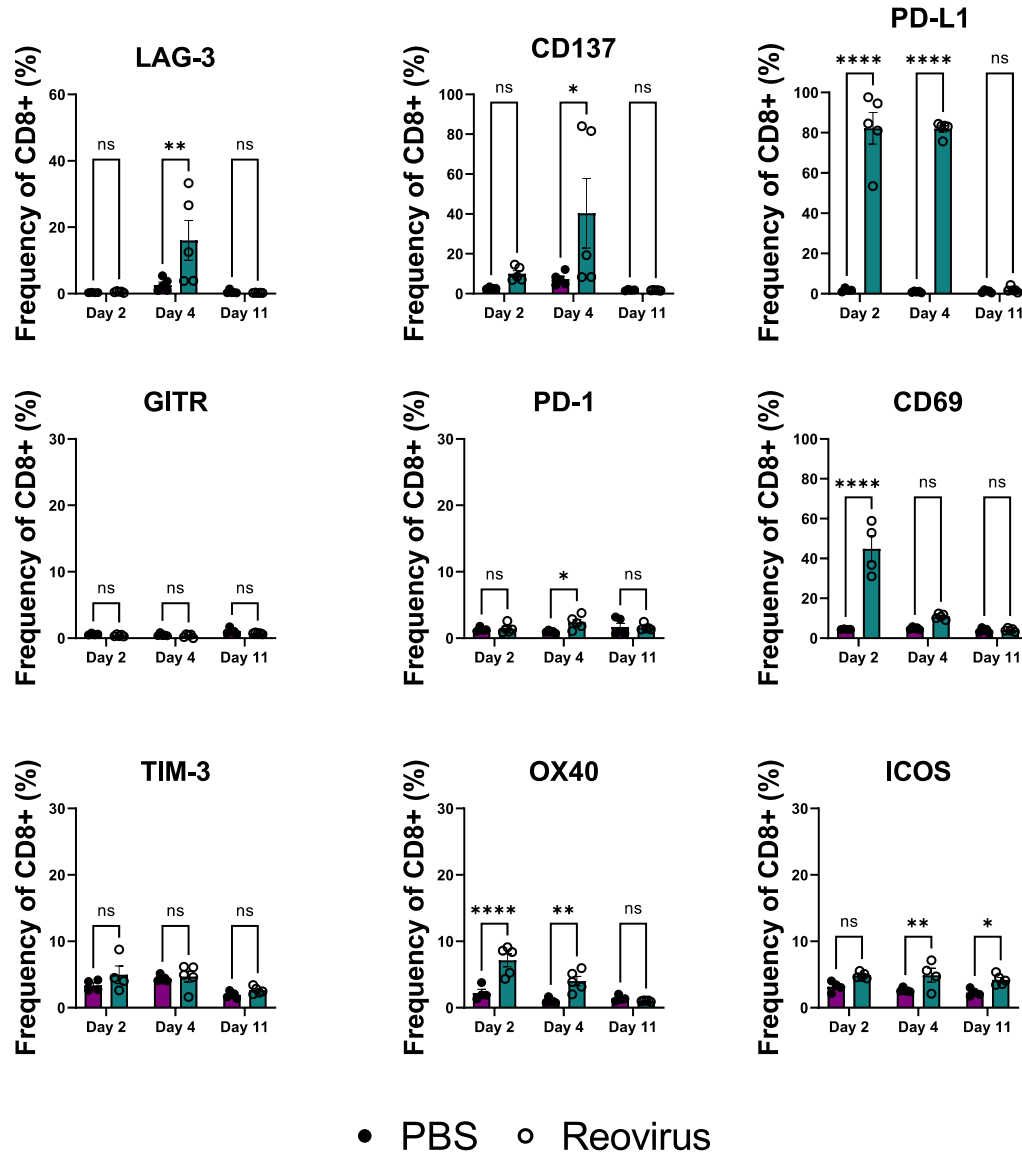


Figure 3.19. Systemic reovirus treatment *in vivo* induces expression of several co-stimulatory and co-inhibitory molecules on the surface of CD8+ T-cells in spleens in 1MEA-Balb/c model. Balb/c mice were implanted subcutaneously with 1×10^7 1MEA cells and treated at day 0 with 1×10^7 pfu i.v. oncolytic reovirus. Spleens were harvested at days 2, 4 and 11 post-treatment and CD3+CD8+ T-cells were analysed for positive surface expression of co-stimulatory and co-inhibitory molecules. Summary graphs show mean $\pm$ SEM (n=5). Statistical results show results of a 2way ANOVA with Sidak's multiple comparisons test $p \leq 0.05$.

The frequency of splenic CD69⁺ CD4⁺ T-cells is greater in reovirus-treated mice compared to the control at day 2, again, consistent with CD69 being an early marker for T-cell activation (Figure 3.20). Frequencies of splenic PD-L1⁺ CD4⁺ T-cells are significantly greater at days 2 and 4 post-treatment with reovirus compared to the control group, but comparable to the control by day 11.

Frequencies of PD-1⁺ CD4⁺ T-cells in the spleen are greatly increased in response to reovirus treatment by day 4 post-treatment and remain increased at day 11. Like on CD8⁺ T-cells, frequency of CD37⁺ CD4⁺ T-cells is significantly greater at day 4 post-reovirus treatment compared to the control, but the margin of error is large and further repeats would be needed to explore this. Similarly, the OX40⁺ CD4⁺ T-cells also shows a high margin of error, therefore also need further repeats. Frequency of Tim-3⁺ CD4⁺ T-cells are also elevated in response to day reovirus treatment in this model, though mean frequency is very low (1.24 %). The fraction of CD4⁺ T-cells positive for OX40 is also significantly increased by day 4 post-reovirus treatment compared to the control.

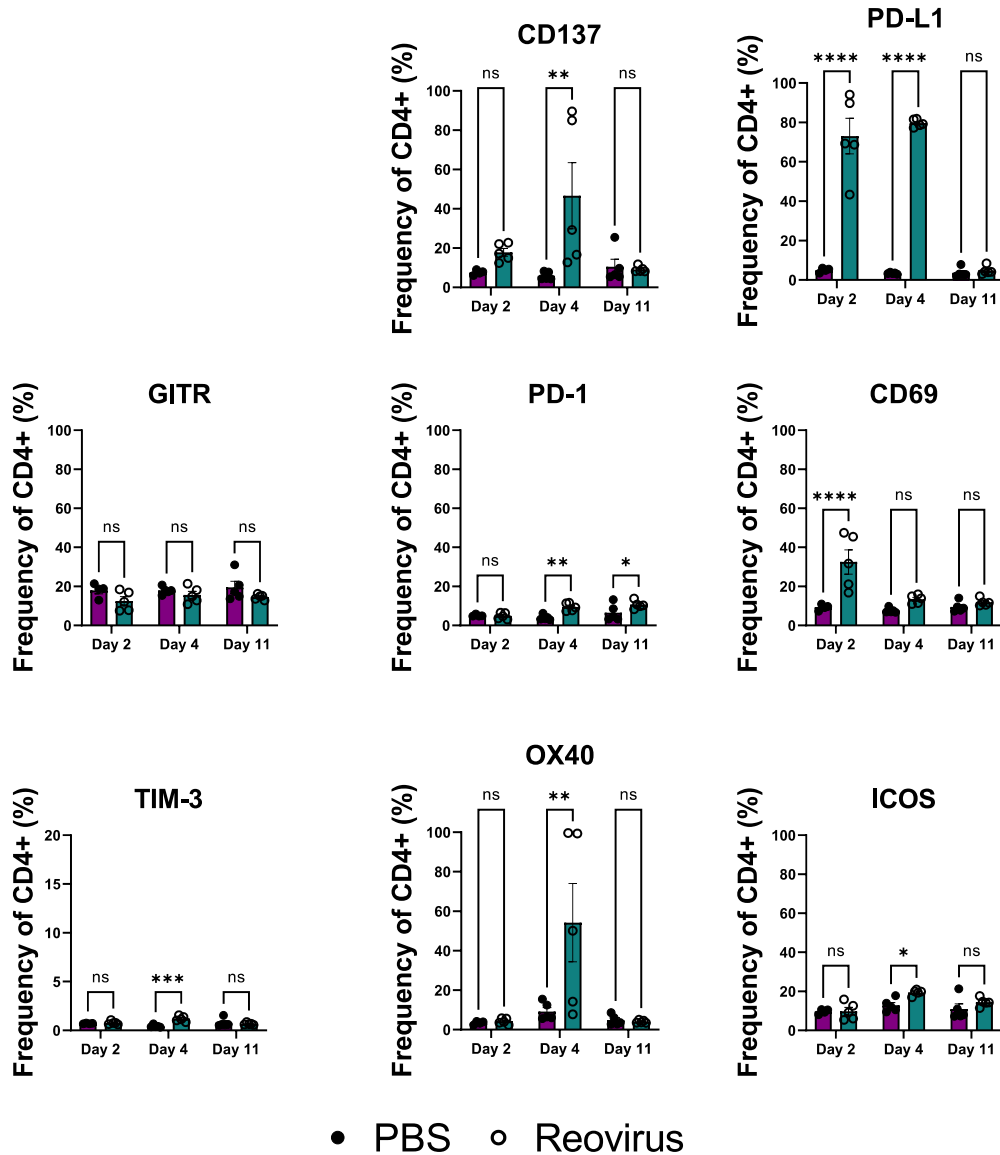


Figure 3.20. Systemic reovirus treatment *in vivo* induces expression of several co-stimulatory and co-inhibitory molecules on the surface of CD4+ T-cells in spleens in 1MEA-Balb/c model. Balb/c mice were implanted subcutaneously with 1×10^7 1MEA cells and treated at day 0 with 1×10^7 pfu i.v. oncolytic reovirus. Spleens were harvested at days 2, 4 and 11 post-treatment and CD3+CD4+ T-cells were analysed for positive surface expression of co-stimulatory and co-inhibitory molecules. LAG-3 surface staining is excluded due to staining quality. Summary graphs show mean $\pm$ SEM (n=5). Statistical results show results of a 2way ANOVA with Sidak's multiple comparisons test $p \leq 0.05$.

As a proxy for the tumour in this model, TDLNs were evaluated as in section 3.2.6. Here, for both CD4⁺ and CD8⁺ T-cell populations, CD69⁺ cells were significantly upregulated in response to reovirus treatment on day 2 post-treatment (Figures 3.21 and 3.22). This finding is consistent with previous findings. In addition, consistent with previous results, the frequencies of CD4⁺ and CD8⁺ T-cells positive for PD-L1 are significantly greater in response to reovirus treatment compared to control groups on days 2 and 4, but returning to comparable to control by day 11.

Specifically in CD8⁺ T-cells in the TDLNs, PD-1⁺ cells are significantly greater on day 4 in response to reovirus treatment compared to the control (Figure 3.21). Whilst specifically on day 2 post-treatment, reovirus-treated mice are associated with lesser frequencies of GITR⁺ CD4⁺ T-cells than the control group (Figure 3.22).

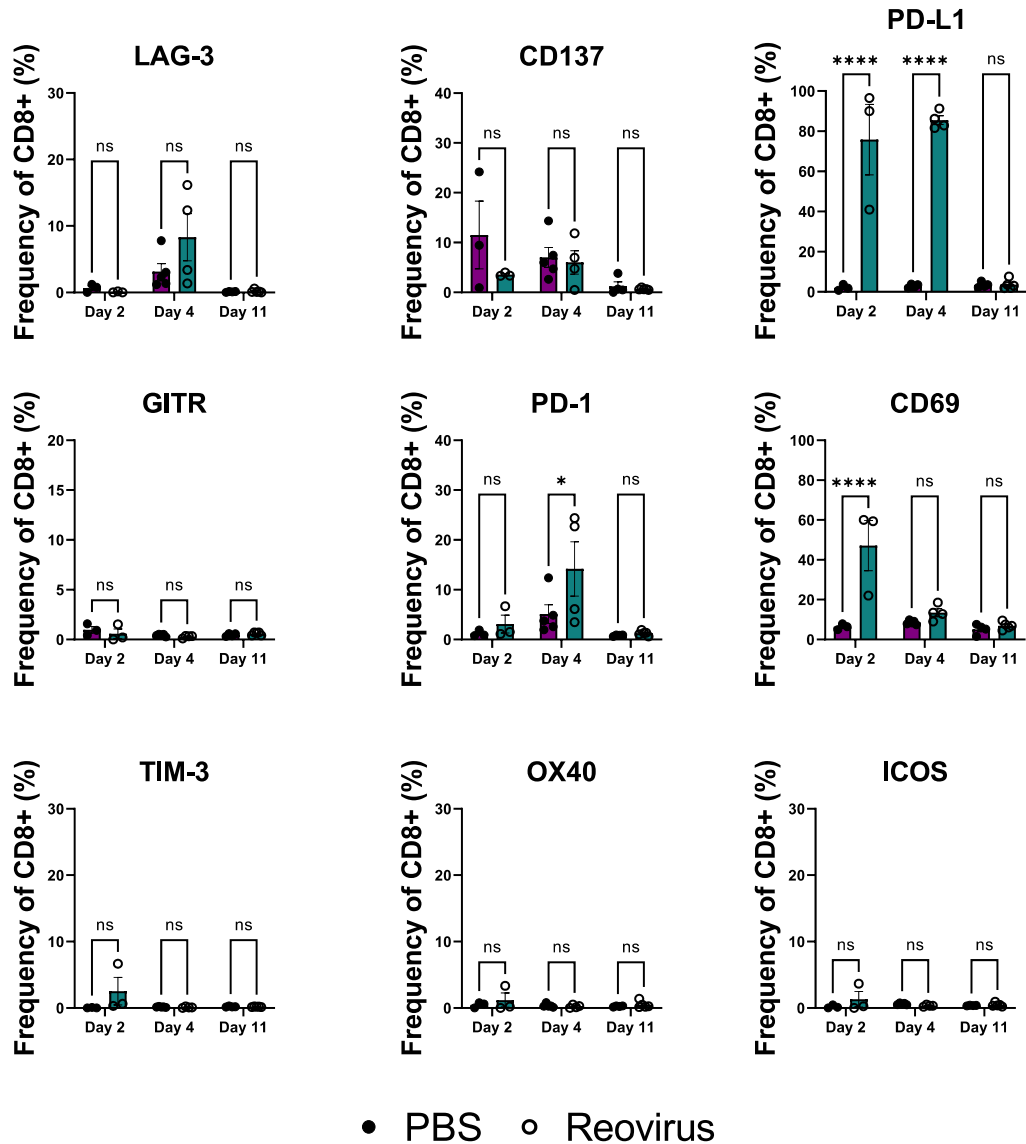
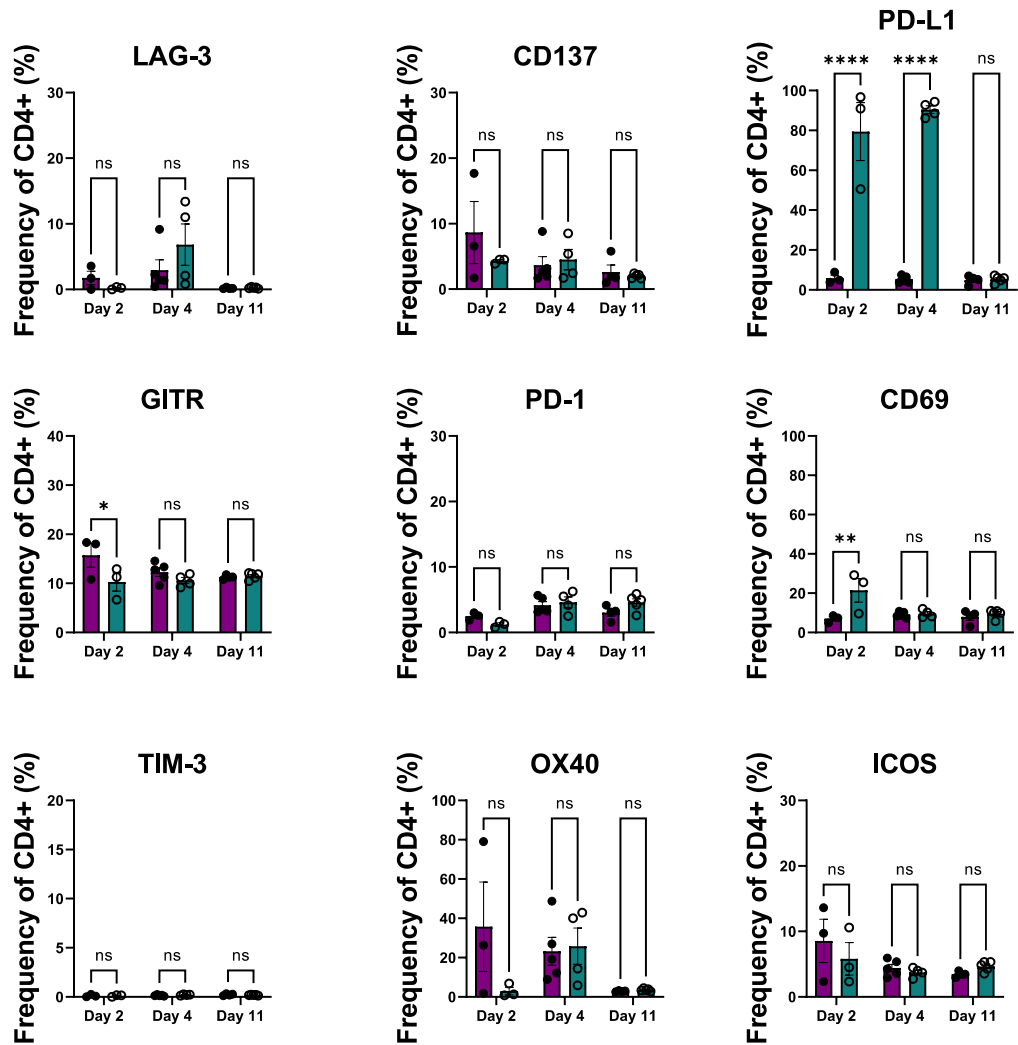


Figure 3.21. Systemic reovirus treatment *in vivo* induces expression of PD-L1 on the surface of CD8+ T-cells in TDLN in 1MEA-Balb/c model. Balb/c mice were implanted subcutaneously with 1×10^7 1MEA cells and treated at day 0 with 1×10^7 pfu i.v. oncolytic reovirus. TDLNs were harvested at days 2, 4 and 11 post-treatment and CD3+CD8+ T-cells were analysed for positive surface expression of co-stimulatory and co-inhibitory molecules. Summary graphs show mean $\pm$ SEM (n=5). Statistical results show results of a 2way ANOVA with Sidak's multiple comparisons test $p \leq 0.05$.



• PBS ◦ Reovirus

Figure 3.22. Systemic reovirus treatment *in vivo* induces expression of PD-L1 on the surface of CD4+ T-cells in TDLN in 1MEA-Balb/c model. Balb/c mice were implanted subcutaneously with 1×10^7 1MEA cells and treated at day 0 with 1×10^7 pfu i.v. oncolytic reovirus. TDLNs were harvested at days 2, 4 and 11 post-treatment and CD3+CD8+ T-cells were analysed for positive surface expression of co-stimulatory and co-inhibitory molecules. Summary graphs show mean $\pm$ SEM (n=5). Statistical results show results of a 2way ANOVA with Sidak's multiple comparisons test $p \leq 0.05$.

In brief, these data consistently suggest that oncolytic reovirus induces the expression of PD-L1 on the surface of CD4⁺ and CD8⁺ T-cells at days 2 and 4 post-treatment. In the balb/c model, this finding is true for both the spleen, and the TDLN. In addition, CD69 expression is also consistently induced by oncolytic reovirus by day 2 post-treatment. Furthermore, the upregulation of CD137, ICOS and PD-1 on splenic CD4⁺ and CD8⁺ T-cells may reflect the generation of activated antigen-specific lymphocytes within the spleen.

3.3 Discussion

There are several limitations to this chapter that should first be discussed. It is important to note the *in vitro* studies in section 3.2.1 utilise RCM and CM generated from primary PBMCs cultured $\pm$ reovirus. This treatment has led to the secretion of soluble factors, which when utilised to treat primary PBMCs, leads to an observed increased expression of co-stimulatory (ICOS) and co-inhibitory receptors (PD-L1, PD-1 and LAG-3) on different immune cell populations. The aim of this experiment was to evaluate the effects of the soluble factors released on PBMCs, as opposed to the direct lytic effects of oncolytic reovirus. As oncolytic reovirus has natural cancer-selective properties, and to bring the context of this experiment towards cancer, it would also be fitting to evaluate the effects of RCM and CM generated from treating tumour and background liver hepatocytes $\pm$ reovirus, particularly as reovirus-treated hepatocytes have been known to secrete IFN- β , which would in turn upregulate CD69 and PD-L1 (Samson *et al.*, 2018). In addition, out of the context of oncolytic viruses, it is well characterised that reovirus infection induces an IFN-based anti-viral immune response (Abad and Danthi, 2020). Conversely, it has recently been observed that the sigma 3 protein of reovirus is able to limit the induction of necroptosis by preventing excessive production of interferon in response to infection (Roebke *et al.*, 2020). Therefore, the RCM and CM generated for use in these experiments could be characterised for their levels of soluble factors, especially type I IFNs, in order to further understand what could be driving the observed changes to surface receptors.

The *in vitro* co-culture experiments undertaken in section 3.2.2 better mimic the tumour microenvironment of HCC whilst still looking at the indirect immune effects of reovirus, allowing for co-culture of primary isolated PBMCs with a HCC cell line or primary

hepatocytes, without allowing cell-cell contact. This model could be further enhanced by using matched patient samples, i.e autologous isolated PBMCs and background liver or tumour, as these samples are obtained from the patient during liver resection. Furthermore, it is important to note here that whilst it is the hepatocytes that are treated with reovirus in this experimental design, viral particles may pass through the transwell membrane due to size restrictions of available transwell products and therefore may elicit potential direct effects as opposed to purely indirect effects. It would be of interest to evaluate this in further detail by checking for viral RNA in the supernatant around the PBMCs. Another interesting evaluation that could have been performed in this experiment would have been to explore the cell viability of background hepatocytes to JHH-4 cells in response to oncolytic reovirus treatment, though the cancer selective nature of oncolytic reovirus is well reported (Kazemi *et al.*, 2021). It would also have been particularly interesting to evaluate the effects of oncolytic reovirus in a model that allows cell-cell contact, focussing on target cell killing assays, in particularly with NK and CD8+ T-cells. One novel approach to *in vitro* studies is the use of organoids to better mimic a tumour environment. It would be interesting to develop organoids using match patient samples (i.e. background liver and tumour from the same patient). These organoids could then be used to screen potential reovirus-based combination therapies.

Finally, whilst the *in vivo* models utilised in sections 3.2.6 and 3.2.7 better model HCC, they have limitations regarding tissue processing. These experiments generated extremely low yields from tumours, and in the case of the 1MEA-Balb/c model, liver samples. For the tumours, these yields could likely be improved by acquiring samples on the flow cytometer in a larger volume, and at a slower flow rate, a protocol

subsequently optimised following the transfer to the Bending Lab and used for research chapter 2. For the livers, performing a lymphoprep on the liver samples to isolate intrahepatic lymphocytes from hepatic cells in addition to acquiring samples at a slower flow rate would likely be advantageous. Repetition of these experiments would be ideal to generate a complete data set, although covid-19 circumstances and the relocation to Birmingham did not allow time for this.

Furthermore, regarding the *in vivo* work in sections 3.2.6, despite demonstrating that reovirus can enter the Hep53.4 cell line, *in vivo* treatment of Hep53.4 tumour-bearing C57JBL/6 mice produced underwhelming results regarding anti-tumour efficacy. It would be important to evaluate that systemic treatment of oncolytic reovirus does in fact lead to reovirus entering the Hep53.4 tumour *in vivo*. It is possible, when considering a self-plateau observed in tumour growth kinetics, combined with poor TILs obtained from harvested tumours, that this tumour model has poor vasculature. This could mean that systemic treatment of reovirus does not enter the tumour and therefore cannot elicit anti-tumour effects. Whilst reovirus-induced changes were observed in the TDLN, a lack of tumour data means differences cannot be evaluated within the TME. Immunohistochemistry of tumour sections for reovirus sigma 3 protein would ascertain whether reovirus has entered the tumour.

These limitations aside, the findings in this chapter demonstrate the effects of oncolytic reovirus on the surface expression of co-stimulatory and co-inhibitory receptors on the surface of immune cells in the context of HCC. Utilising both *in vitro* and *in vivo* models of HCC, it was observed that single-agent therapy induces expression of these checkpoint molecules on the surface of immune cells, and that these markers are

expressed differentially over time. Combination of a reovirus-based double-agent therapy would therefore require meticulous evaluation and a better understanding of checkpoint molecule signalling pathways to obtain increased efficacy with reduced associated toxicities.

Rationale for oncolytic reovirus for HCC stems largely from the Griffin and Samson labs, where they previously demonstrated, using both *in vitro* and *in vivo* preclinical models, that reovirus has both anti-viral and antitumour properties for HCV/HCC via type I IFNs (Samson *et al.*, 2018). They observed reovirus-induced innate immune responses within primary liver tissue which concurrently led to effective tumour killing and suppression of HCV replication. This unique anti-viral and anti-tumour effect observed by reovirus treatment has advantages in the treatment of oncogenic virus-driven tumours, like often is the case with HCC. That being said, single-agent immunotherapies have shown less efficacy than combination therapies in a range of cancers, therefore a rationally designed combination therapy should be sought (Rajani *et al.*, 2016 and Mostafa *et al.*, 2018).

One important aspect to consider when looking at co-inhibitory and co-stimulatory receptors on T-cells is the balance of activation. An up-regulation of costimulatory receptors, such as ICOS, OX40 and CD137 could tip the balance of T-cells towards activation post-reovirus treatment *in vitro*. Opposingly, an upregulation of PD-1 and Tim-3, co-inhibitory receptors, would favour suppression of T-cell activation. Further exploration into the expression of the receptor/ligand pairs of these markers would be necessary to explore whether upregulation of one part of the pair could in fact lead to increased binding. For example, an upregulation of OX40, a co-stimulatory receptor,

would not lead to biological effects unless bound to its ligand, OX40-L (Willoughby *et al.*, 2017). The only marker which was found to be significantly increased within *in vitro* tumour studies but not in the background liver was LAG-3, but the *in vitro* results of sections 3.2.1 and 3.2.2 did not translate to *in vivo* in TDLNs. These results may reflect an early type I IFN response, which can upregulate PD-1 and CD69 expression (Sumida *et al.*, 2022). In addition, the upregulation of PD-1 and ICOS, which are both NFAT targets likely driven by TCR signalling, suggests the presence of an antigen-driven response through the TCR (Boussiotis *et al.*, 2014 and Tan *et al.*, 2006).

This chapter sought to explore which ICM should be rationally combined with oncolytic reovirus by looking at reovirus-induced expression of a range of co-stimulatory and co-inhibitory molecules on the surface of immune cell populations. LAG-3, an inhibitory checkpoint receptor with a similar structure to the CD4 co-receptor (Lui and Davis, 2018), has been observed elevated on CD4+ and CD8+ T-cells and NK cells within this chapter. Expression of LAG-3 is activation dependent and is therefore associated with activated immune cells (Ruffo *et al.*, 2019). Blocking LAG-3 is associated with a prolonged T-cell response and several *in vivo* approaches to LAG-3 blockade have reportedly revealed promising anti-tumour effects to LAG-3 dual blockade, e.g. with anti-PD-1, though LAG-3 single-agent therapy has shown limited effects (Lichtenegger *et al.*, 2018 and Pühr *et al.*, 2019). The data presented here suggest that oncolytic reovirus followed by subsequent LAG-3 blockade may be worth exploring, although it is important to note the relatively low frequencies of LAG-3+ cells observed throughout.

PD-L1, a co-inhibitory checkpoint ligand, shows more profound differences in response to reovirus treatment through this chapter. Blocking PD-L1 has shown recent promise

in the treatment of HCC in phase III studies, culminating in the FDA approval of PD-L1 inhibitors (atezolizumab and durvalumab, see section 3.1.7; Finn *et al.*, 2020 and Abou-Alfa *et al.*, 2022). Results in this chapter demonstrate a reovirus-induced increase in PD-L1 expression in almost all models/cell types evaluated, thus, it would seem fitting to further explore the combination of reovirus treatment followed by PD-L1 blockade. This treatment regimen could see oncolytic reovirus priming the tumour microenvironment for subsequent PD-L1 checkpoint blockade in a similar fashion to that observed in a recent window of opportunity study by Samson and colleagues in brain tumours (Samson *et al.*, 2018). The reovirus-based induction of PD-L1 expression observed in this chapter was also observed *in vivo* in the liver, it would therefore be important to evaluate whether such a combination would lead to further hepatic damage to the background liver, leading to immune-mediated adverse events. The unique biology of the liver being immune tolerant, in combination with a common background of liver cirrhosis and fibrosis, makes immune-mediated adverse events in HCC a potentially different challenge to other cancers (Cui *et al.*, 2020). A recent meta-analysis of a global adverse drug reaction database revealed hepatitis to be among the most frequent fatal immune-related adverse events in response to PD-1/PD-L1 blockade for any cancer type (Wang *et al.*, 2019). It would therefore be crucial to evaluate the safety and efficacy of any ICM treatment for HCC in a model of cirrhosis/fibrosis.

In order to trial oncolytic reovirus with antibody-mediated blockade of PD-L1, with a specific focus on safety and efficacy, a liver fibrosis model could be utilised. Murine models of liver fibrosis can be induced by repeat i.p. injections of tetrachloromethane (CCL₄), simulating the progression of drug-induced liver fibrosis (Dutta-Moscato *et al.*,

2014). Using such a model to trial potential HCC therapies would allow for exploration into potentially unwarranted effects in a fibrotic background liver environment, thus allowing for the safety of the potential treatment to be evaluated in a truer HCC context.

It is important to consider that PD-L1 and CD69, both observed to increase in response to reovirus treatment, are considered T-cell activation markers in response to antigen (Cibrian and Sanchez-Madrid, 2017 and Elliot *et al.*, 2021). Conversely, PD-L1 and CD69 can also both be driven by JAK/STAT signalling (Gonzalez-Alvaro *et al.*, 2009 and Garcia-Diaz *et al.*, 2017). Using melanoma cell lines, surface expression of PD-L1 was evaluated in response to IFNs, with IFN γ driving the strongest expression, followed by IFN β then IFN α (Garcia-Diaz *et al.*, 2017). This chapter reveals an increased expression of PD-L1 in response to reovirus, but it is not possible to distinguish whether this upregulation is due to bystander activation or increased STAT1 signals from antigen recognition. In depth analysis of reovirus treatment utilising TCR reporters would be beneficial to distinguish here (Elliot *et al.*, 2022). As previously mentioned, PD-1 and ICOS are upregulated at later time points than CD69 expression. As PD-1 and ICOS are known to be associated with strong TCR activation, this later response could likely be antigen driven (Elliot *et al.*, 2021). Using TCR reporters here would elucidate this further.

It is also worth noting that the *in vitro* studies explored the indirect effects of reovirus on CD4 $^{+}$ and CD8 $^{+}$ T-cells, in addition to NK cells, whilst *in vivo* studies focused on T-cells. T-cells were chosen as a focus for *in vivo* evaluation due to their known importance in tumour immunotherapy, in addition to their increased expression of similar markers to that of NK cells *in vitro*. It would be interesting to evaluate NK cells

in greater detail than the *in vitro* studies performed in this chapter, either through NK cell killing assays against HCC target cell lines $\pm$ reovirus, or through exploring NK cell surface expression of co-stimulatory and co-inhibitory molecules in response to oncolytic reovirus treatment *in vivo*.

Overall, this chapter has demonstrated the use of oncolytic reovirus in priming CD4+ and CD8+ T-cells for subsequent checkpoint therapy, particularly antibody-mediated PD-L1 blockade. Further models and repeats would be necessary to fully interpret this dataset, but the findings presented are consistent with a reovirus-based immunotherapy that would potentially also benefit from subsequent PD-L1 blockade.

4 RESEARCH CHAPTER 2: RATIONALLY DESIGNING IMMUNE CHECKPOINT COMBINATION THERAPIES THAT AUGMENT α PD-L1 RESPONSES

This research chapter, undertaken under the primary supervision of Dr David Bending at the University of Birmingham, seeks to use an adaptive tolerance model to understand how to identify combinations of immunotherapy. PD-1/PD-L1 pathway modulation has revolutionised the treatment of cancer in recent decades, though importantly, not all patients respond to therapy. This chapter aims to rationally seek an antibody-mediated PD-L1 blockade-based combination therapy with a specific focus on CD4⁺ T-cell activation using an adaptive tolerance model previously developed (Elliot *et al.*, 2021). Specific focus is paid to the surface expression of co-stimulatory and co-inhibitory receptors in response to PD-L1 blockade. Effective combination therapies are explored for their anti-tumour activity in an immunogenic subcutaneous cancer model.

4. 1 Introduction

4.1.1 T-cell activation

T-cell receptor engagement, covered in more depth in section 1.1.4, is critical for T-cell activation. In brief, antigen-specific activation is dependent on TCR recognition of antigen bound to MHC molecules on the surface of APCs, such as DCs (Sharma and Allison, 2015). Full T-cell activation, which is TCR engagement with an MHC-antigen complex in addition to a second co-stimulatory signal and a third 'danger' signal, is critical for a T-cell to mount a full response against a target cell.

4.1.2 T-cell receptor signalling

TCR activation via MHC-antigen complexes as described in sections 1.1.4 and 4.1.1 initiates several signalling cascades that determine cell fate via regulation of cytokine production, cell survival, proliferation and differentiation. Proximal and distal TCR signalling are integrated via various adaptor proteins that facilitate protein-protein interactions and ultimately aid in the formation of multiprotein complexes, initiating necessary signalling cascades for effective T-cell biology (Shah *et al.*, 2021).

Initially, engagement of the TCR with MHC-antigen complex leads to proximal signalling whereby lymphocyte protein tyrosine kinase (LCK) phosphorylates immunoreceptor tyrosine-based activation motifs (ITAMs) located on the TCR/CD3 complex. Zeta-chain associated protein kinase (Zap-70) is then recruited to the TCR/CD3 complex where it is activated, subsequently promoting the recruitment and phosphorylation of downstream proteins (Ngoenham *et al.*, 2018). Proximal signalling is followed by the activation of several distal signalling cascades, including the calcineurin-NFAT, protein kinase C (PKC)/NF- κ B and MAPK/Erk pathways.

4.1.2.1 Calcineurin-NFAT pathway

Membrane bound phosphatidylinositol 4,5-bisphosphate (PIP₂) is hydrolysed by activated phospholipase C γ 1 (PLC γ 1) into diacylglycerol (DAG) and inositol-3-phosphate (IP₃). DAG and IP₃ are essential secondary messenger molecules that are responsible for activating several distal signalling pathways critical for TCR activation (Oh-hora *et al.*, 2009). DAG can activate PKC (described in section 4.1.2.2), whilst IP₃ is responsible for activating the calcineurin-NFAT pathway. IP₃ binds to the calcium-permeable ion channel receptors on the endoplasmic reticulum (ER), in turn releasing

the ER calcium stores in the cytoplasm. This depletion of intracellular calcium levels triggers an influx of extracellular calcium into T-cells which activates calcineurin, a protein phosphatase (Meng *et al.*, 2020). Calcineurin dephosphorylates NFAT, causing it to translocate to the nucleus. Nuclear NFAT binds AP-1 transcription factors JUN/FOS which are derived from the MAPK/Erk pathway described in section 4.1.2.3. This NFAT-mediated transcriptional complex induces the expression of various genes, including IL-2 among other effector molecules, that are responsible for T-cell activation (Chow *et al.*, 1999).

Interestingly, Martinez *et al.* (2015), look closely at the role of NFAT in T-cell exhaustion via the regulation of inhibitory receptors and signalling molecules which are able to subdue TCR signalling. Martinez *et al.* (2015) generated an engineered version of NFAT which is constitutively active and cannot bind AP-1, and thus elicits no effector response. They found that the engineered NFAT reduced TCR responses and induced CD8⁺ T-cell exhaustion *in vivo*, contributing to their findings that an exhausted phenotype is generated in the absence of NFAT/AP-1 binding. Coupled with this observation, NFAT-deficient CD8⁺ T-cells were seen to have diminished expression of inhibitory surface receptors commonly associated with exhaustion (PD-1, LAG-3 and TIM3). NFAT therefore drives a number of co-inhibitory receptors involved in these investigations, in addition to driving other markers such as Nr4a3 that can be used to study T-cell activation (Jennings *et al.*, 2020).

4.1.2.2 PKC/NF- κ B pathway

PKC is a family of ten kinases that have significant roles in biology. The family of proteins are divided into three subfamilies: classical, novel, and atypical. DAG and

calcium ions regulate (see section 4.1.2.1) regulate the activation of classical PKC isoforms, whilst novel PKC isoforms are regulated by DAG. Activation of atypical PKC isoforms however is independent of DAG and calcium ions. NF- κ B is an important transcription factor that plays crucial roles in regulating genes involved in immune responses. TCR-mediated T-cell activation involves the canonical and non-canonical NF- κ B pathways. PKC θ , a novel PKC isoform, is activated following TCR activation, triggering the complex of multi-protein complexes of adaptor proteins in the cytoplasm. This multi-protein subunit leads to the prevention of NF- κ B inhibition and its subsequent translocation to the nucleus where it regulates gene expression – leading to T cell activation.

4.1.2.3 MAPK/Erk pathway

DAG from PIP₂ (see section 4.1.2.2) induces the activation RAS guanyl nucleotide-releasing protein (RASGRP1) and mediates its recruitment to the plasma membrane. RASGRP1 is responsible for activation of RAS, a small G protein, in T-cells. Once activated, RAS binds to GTP and initiates the RAS-MAPK cascade, culminating in the phosphorylation and activation of ERK1/2 which control T cell development, differentiation and TCR-induced signal strength.

4.1.3 Nr4a family

The Nr4a family of orphan nuclear receptors consists of three members: Nur77 (Nr4a1), Nr4a2 (Nurr1) and Nr4a3 (Nor1). These receptors are ligand independent and are constitutively active, with Nur77 and Nr4a3 being rapidly upregulated in T-cells following TCR engagement. This ligand independent property, coupled with their TCR-specific nature, makes them a more accurate marker of TCR-mediated T-cell activation

than CD69, which can be upregulated by non-TCR stimulation, including cytokines (Bending and Zikherman, 2023).

Nr4a receptors have valuable functions in T-cell biology, with Nur77 driving apoptosis (Woronicz *et al.*, 1994) and modulating Treg differentiation (Fassett *et al.*, 2012). Nr4a2 is known to bind *Foxp3* regulatory elements and regulates the differentiation of CD4+ T-cells (Sekiya *et al.*, 2011 and Sekiya *et al.*, 2013). Additionally, expression of Nr4a3 is a marker of Treg differentiation (Sekiya *et al.*, 2013). Whilst their presence/function is of known importance in T-cell biology, their signalling pathways remained unclear until recently when Nr4a3-Tocky and Nur77-GFP mice were utilised to elucidate the differential signalling pathways driving Nr4a receptor expression (Jennings *et al.*, 2020). It was revealed that whilst *Nr4a1-3* were all Src family kinase dependent, only *Nr4a2* and *Nr4a3* were NFAT/calcineurin dependent - Nur77 was observed to be NFAT redundant (Jennings *et al.*, 2020). Additionally, it was observed that ERK signalling is required for optimal Nr4a family expression, consistent with NFAT highly co-operating with AP-1, which is dependent on the MAPK/Erk pathway, to activate crucial genes in response to TCR activation (Jennings *et al.*, 2020).

4.1.4 Critical *in vivo* tools to study T-cell activation and tolerance

4.1.4.1 *Nr4a3-Tocky*

Nr4a3-Tocky reporter mice, developed in the Ono lab, are a pivotal tool for studying temporal dynamics of TCR-mediated T-cell activation *in vivo* (Bending *et al.*, 2018). The reporter uses the fluorescent timer (FT) protein, which initially exists as an unstable blue protein before maturing to a stable red protein (Figure 4.1). The FT reporter protein, developed by Subach *et al.* (2009), is an mCherry-derived monomeric

variant. Three variants were created by Subach *et al.* (2009), fast-FT, medium-FT and slow-FT that present fast, medium and slow maturation rates.

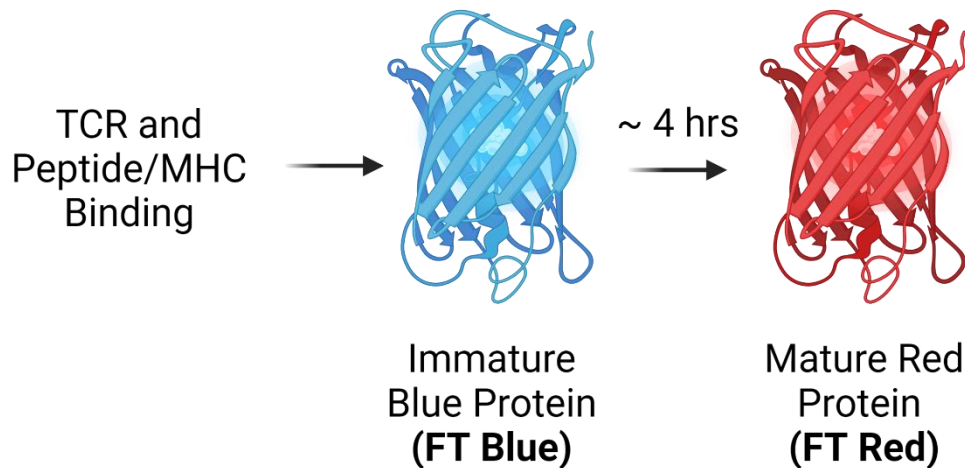


Figure 4.1. Fluorescent Timer (FT) protein maturation. FT protein is expressed after TCR and peptide/MHC binding as an immature blue form (FT Blue). After approximately 4 hours, the immature protein matures to a more stable red protein (FT Red).

In this model, fast-FT reports *Nr4a3*, which is an early activated gene downstream of TCR signalling and has a maturation half-life of ~ 4 hrs (Bending *et al.*, 2018). The development of the model consisted of identifying *Nr4a3* as the gene that was most highly associated with α CD3-mediated T-cell activation (which mimics TCR activation). *Nr4a3* was observed to rapidly increase upon α CD3 stimulation. Bending *et al.* (2018) generated Nr4a3-Tocky using a bacterial artificial chromosome (BAC) containing the *Nr4a3* gene and its regulatory elements which was modified to express FT protein expression under the control of the *Nr4a3* promoter and regulatory elements.

This chapter focuses on the use of Tg4 Nr4a3-Tocky and Tg4 Nur77-Tempo mice (see section 4.1.4.2) to explore CD4⁺ T-cell responses to specific MBP peptide. Tg4 mice

express an MHC-class II-restricted TCR specific for myelin basic protein (MBP). This was achieved using rearranged V α 4/V β 8.2 TCR chains generated from T-cell hybridoma cDNA raised against the acetylated N-terminal peptide of murine MBP. Tg4 reporter mice are therefore a useful tool to study immunological tolerance to self-antigen and antigen-specific immunotherapy. The utility of the Tg4 Nr4a3-Tocky system in investigating immunotherapy responses was revealed by demonstrating that this model could reveal the mechanisms of action for anti-PD-1 immunotherapy (Elliot *et al.*, 2021).

4.1.4.2 Nur77-Tempo

Nr4a1 (henceforth referred to as Nur77) is also specifically regulated by antigen receptor signalling, making it also suitable to use as a distal TCR reporter. Recent work in the Bending group using Nur77-GFP mice suggested that Nur77 may in fact be more sensitive to distal TCR signals compared to Nr4a3 (Jennings *et al.*, 2020). As such, Tg4 Nur77-Timer-rapidly-expressed-in-lymphocytes (Tempo) mice were generated where FT (Figure 4.1.) was expressed under the regulation of elements of the gene encoding Nur77.

Tg4 Nur77-Tempo mice were observed to be sensitive to low-strength TCR signals, with an observed brightness graded in response to TCR signal strength. In essence, Nur77-Tempo mice combine the temporal dynamics from the Tocky innovation with the increased sensitivity of Nur77 to lower TCR signal strengths (Elliot *et al.*, 2023).

4.1.5 PD-1/PD-L1 pathway

Programmed death protein 1 (PD-1) and its ligand programmed death-ligand 1 (PD-L1) are crucial immune checkpoint proteins that aid negative regulation of T-cell immune responses. PD-1 was initially observed in 1992 for its role in apoptosis (Ishida *et al.*, 1992), with PD-L1, a B7 homolog, being observed to inhibit T-cell responses followed in 1999 (Dong *et al.*, 1999). The functional binding of PD-1 to PD-L1 was observed in 2000 by Freeman *et al.* PD-1 is expressed at low levels on naïve T-cells, being upregulated following stimulation.

Conflicting theories on the mechanism of action of PD-1/PD-L1 blockade exist, with early studies suggesting a direct effect of PD-1 blockade on TCR signalling via the dephosphorylation of proximal TCR signalling molecules (Fife *et al.*, 2009 and Honda *et al.*, 2014). In contrast, recent studies have shown that the CD28 pathway is targeted via PD-1 inhibition as opposed to TCR signalling (Hui *et al.*, 2017 and Kamphorst *et al.*, 2017). Recent work by Chan *et al.* (2023), aimed to address the gap in mechanistic understanding of how inhibitory receptors affect diverse signalling pathways. To do this, Chan *et al.* (2023), developed the fluorescent intracellular live multiplex signal transduction activity reporter (FILMSTAR) system to explore critical downstream signalling events in human Jurkat T-cells. Chan *et al.* (2023) were able to separate which signalling pathways were dependent only on the TCR compared to those dependent on the CD28 receptor, and which required both. Chan *et al.* (2023) observed that PD-1/PD-L1 binding suppressed ERK activation in primary CD8⁺ T-cells, with their findings concluding that PD-1/PD-L1 binding primarily targets TCR as opposed to CD28 to inhibit T-cell activation, though their data does not conclude that CD28 expression is not required.

4.1.6 OX40/OX40L pathway

OX40, a co-stimulatory type 1 transmembrane glycoprotein, is constitutively expressed by regulatory T-cells and upregulated following activation on effector T cells (Godfrey *et al.*, 1994). Interaction of OX40 with its ligand, OX40-L, induces a signalling cascade which induces transcriptional changes to modulate immune responses, including increased T-cell proliferation (Mascarelli *et al.*, 2021). OX40 has been observed to induce the expression of anti-apoptotic proteins and offsets the inhibition of immune cells, whilst also directly stimulating effector T-cells, increasing expansion and function (Rogers *et al.*, 2001). OX40/OX4-L binding has been observed to activate NFAT (Lv *et al.*, 2019) and NF- κ B (Lubrano di Ricco *et al.*, 2020) pathways, though its exact mechanism remains unknown.

4.1.7 ICOS/ICOSL pathway

Inducible T-cell costimulator (ICOS), is a costimulatory receptor lowly expressed on naïve T-cells but rapidly induced on the surface of T-cells in response to TCR-mediated activation (Hutloff *et al.*, 1999). Structurally and functionally like CD28, ICOS has been observed to aid TCR activation in a similar manner to CD28 (Hutloff *et al.*, 1999).

4.1.8 PD-1/PD-L1 in combination with OX40 or ICOS agonism

Recent research in the Bending lab looked to help unpin how antigen and immune checkpoint engagement regulate T-cell function. Elliot *et al.* (2021), looked at how antigen abundance regulates immune checkpoint expression and recalibrates T-cell activation thresholds; with specific focus on how PD-1 blockade lowers the threshold of T-cell activation. Utilising Tg4 Nr4a3-Tocky mice, as in this chapter, it was observed

at a single cell level that antigen (4Y-[MBP]) dose drives binary activation responses but at whole population levels, activation is associated with a graded response.

Elliot *et al.* (2021) immunised mice with 4Y-[MBP] (0.8-80 µg), where PD-1 was observed to be tightly coupled to T-cell activation, as opposed to LAG3, TIGIT and CTLA-4 which were all observed to be dependent on the immunising dose of 4Y-[MBP] and not strongly coupled to T-cell activation. Elliot *et al.* (2021) evaluated the hypothesis that T-cells that had arrested TCR signalling in response to weak TCR signalling (lower doses of 4Y-[MBP]), would be more sensitive to re-stimulation with peptide than T-cells initially activated with strong TCR signalling (higher doses of 4Y-[MBP]). Their findings were congruent with this hypothesis, indicating that T-cell activation thresholds are temporarily recalibrated by the initial TCR signalling event *in vivo*.

Elliot *et al.* (2021) went on to manipulate this activation threshold through PD-1 antibody-mediated checkpoint blockade, observing that αPD-1 blockade increased the responder population of T-cells, supporting the idea that PD-1 lowers the activation threshold of T-cells *in vivo*. This increased population of responding T-cells was associated with qualitative changes, including increased expression of protein levels of OX40 and *Icos* transcriptional levels (Elliot *et al.*, 2021). It is this αPD-1 blockade-mediated modulation of T-cell activation thresholds, combined with an increased expression of the co-stimulatory receptor OX40 and/or ICOS on CD4⁺ T-cells, that lead to the notion of PD-1/PD-L1 pathway blockade in combination with agonistic OX40 or ICOS treatment explored in this chapter. Recent papers have explored the combination of OX40 agonism enhancing PD-L1 blockade in tumour efficacy studies,

with van der Sluis *et al.* (2023) observing PD-L1 blockade combined with OX40 agonism being effective at eradicating developing tumours. Their work primarily focuses on CD8⁺ T-cells, whereas this chapter is mainly focused on CD4⁺ T-cells.

Tumour-reactive CD4⁺ T-cells have recently been observed to co-express ICOS and PD-1 in head and neck squamous cell carcinoma (Duhon *et al.*, 2022). This, combined with the recent study by Yadavilli *et al.* (2023) evaluating the effects of ICOS agonism with PD-1 blockade, provided rationale for exploring PD-1/PD-L1 modulation in combination with ICOS agonism in this chapter. Yadavilli *et al.* (2023) showed that ICOS antibody-mediated agonism activated primary human CD4⁺ and CD8⁺ T-cells, in addition to showing that ICOS agonism as a monotherapy was able to elicit antitumour activity in a syngeneic breast cancer model. This observed anti-tumour effects was improved with the addition of PD-1 blockade and their results supported the use of ICOS agonism in combination with PD-1/PD-L1 modulation in their model.

4.2 Results

4.2.1 Nr4a3-Tocky and Nur77-Tempo reporter mice both identify TCR activated CD4+ populations via FT protein expression

Nr4a3-Tocky reporter mice are an innovative tool to study TCR-mediated Nr4a3 expression using Fluorescent Timer (FT) protein (Bending *et al.*, 2018). It has previously been reported by the Bending group that Nur77 may be more sensitive to distal TCR signalling events compared to Nr4a3 (Jennings *et al.*, 2020). To assess this, Nur77-Tempo FT reporter mice were generated in the Bending lab (Elliot *et al.*, 2023). This model expresses FT under the regulation of Nur77. Whilst the complete validation of the Nur77-Tempo model is shown comprehensively in Elliot *et al.* (2023), the effects of α CD3-mediated CD4+ and CD8+ T-cell activation are important to show here.

To compare FT responder populations from both models with respect to signal duration, splenocytes from Nr4a3-Tocky and Nur77-Tempo mice were cultured *in vitro* with 5 μ g/ml α CD3 for 0-4 hrs as indicated (Figure 4.1A-D). Both CD4+ and CD8+ T-cells show an increased background expression of FT Red+ in Nur77 Tempo at T0 compared to Nr4a3-Tocky (Figure 4.1A,B), indicating that Nur77-Tempo captures higher levels of *in vivo* tonic signalling (Elliot *et al.*, 2021). When looking at the levels of FT Blue+ expression via geometric mean, Nur77-Tempo showed increased expression at T120 and T240 (mins) compared with N4a3-Tocky in both CD4+ and CD8+ T-cells (Figure 4.1C), indicating that the Nur77-Tempo model provides a brighter readout for FT Blue+. However, when looking at the proportion of responder cells in CD4+ and CD8+ T-cells, defined by FT Blue+ (sum Q1 + Q2), there is little difference

between the two models (Figure 4.1D), making them both suitable models for identifying activated T cells.

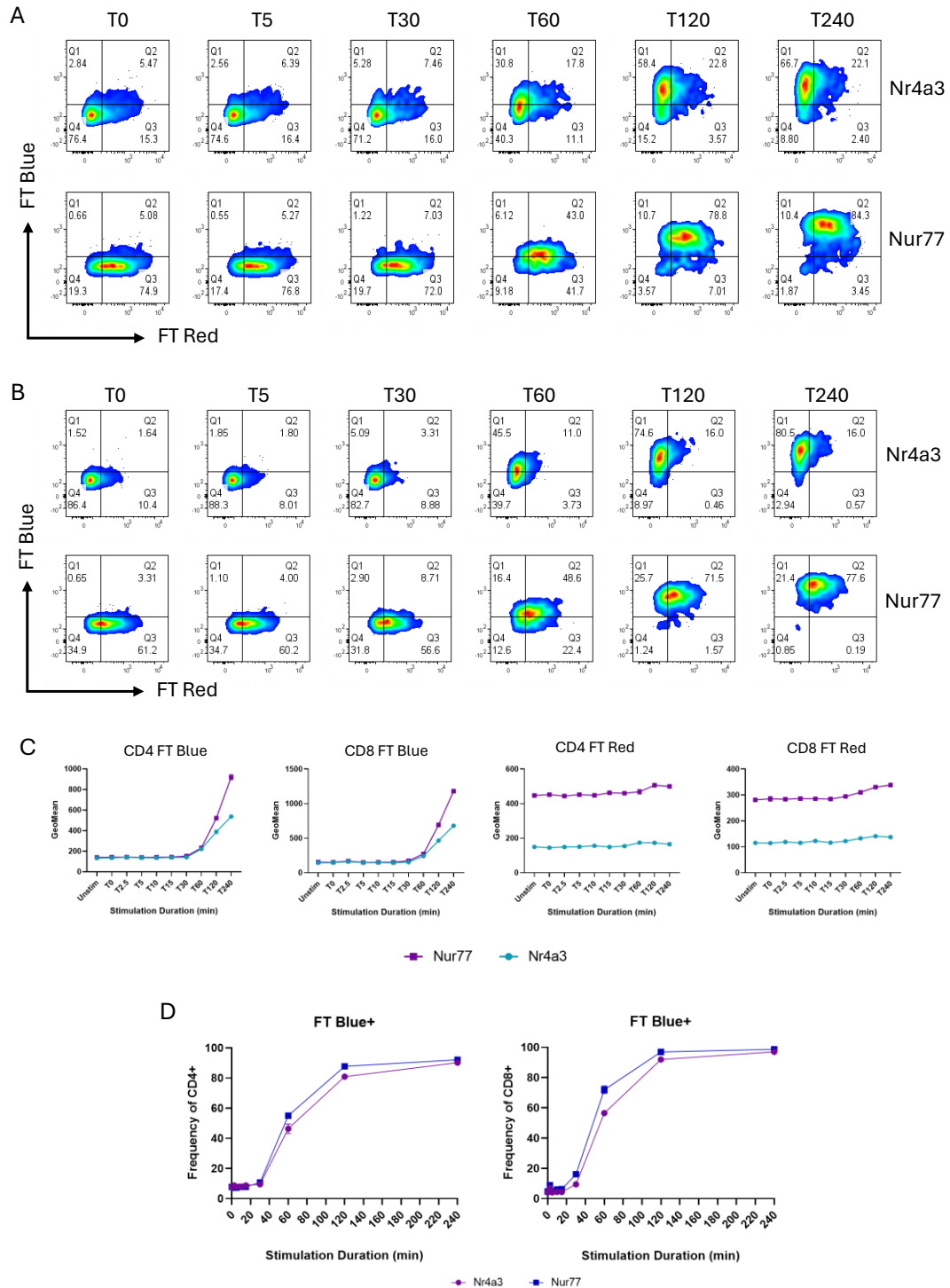


Figure 4.1. Nr4a3-Tocky and Nur77-Tempo mice capture responder populations over four hours utilising FT expression after α CD3 stimulation *in vitro*. Splenocytes from Nr4a3-Tocky and Nur77-Tempo mice were individually harvested and cultured *in vitro* with 5 μ M/ml α CD3 over 4 hours as indicated (A-D). **A and B.** CD4+ (A) and CD8+ (B) T-cells were analysed for FT Blue/Red expression, with responders characterised as FT Blue+ (sum Q1 + Q2). Flow plots are representative of one mouse for Nr4a3-Tocky and Nur77-Tempo. **C.** Summary plots of geometric mean of FT Blue/Red expression in CD4+ (A) and CD8+ (B) T-cells. Data representative of one experiment (n=3) and shows mean $\pm$ SEM. **D.** Summary plots of the frequency of CD4+ (A) and CD8+ (B) T-cells defined as FT Blue+ (sum Q1 + Q2). Data representative of one experiment (n=3) and shows mean $\pm$ SEM.

To further compare the two models, the effects of signal strength on CD4⁺ and CD8⁺ T-cells *in vitro* were analysed. Splenocytes from Nr4a3-Tocky and Nur77-Tempo mice were cultured *in vitro* for four hours with 0-1 µg αCD3 as indicated (Figure 4.2A-C). Agreeing with previous findings (Figure 4.1), unstimulated Nur77-Tempo CD4⁺ and CD8⁺ T-cells showed increased expression of FT Red⁺ compared to Nr4a3-Tocky (Figure 4.2A,B). Thus, suggesting Nur77-Tempo is a more sensitive readout for tonic signalling *in vivo* prior to *in vitro* culture. When looking at the proportion of CD4⁺ and CD8⁺ T-cells defined as FT Blue⁺ (sum Q1 + Q2), Nur77-Tempo splenocytes have a higher proportion than Nr4a3-Tocky, though this effect is more predominant at lower signal strengths (Figure 4.2C). At higher signal strengths (0.2-1 µg αCD3), whilst Nur77-Tempo T-cells are observed to retain the high FT Red⁺ expression from *in vivo* tonic signalling, FT Blue⁺ expression is like that of Nr4a3-Tocky (Figure 4.2A-C). These findings are consistent with previous findings (Figure 4.1) and together, corroborate the finding that both models are appropriate tools to define a population of 'responders'. Notably, in response to stimulation both reporters can readily identify recently activated T cells, as such, both models are utilised throughout the course of this investigation and are indicated as appropriate.

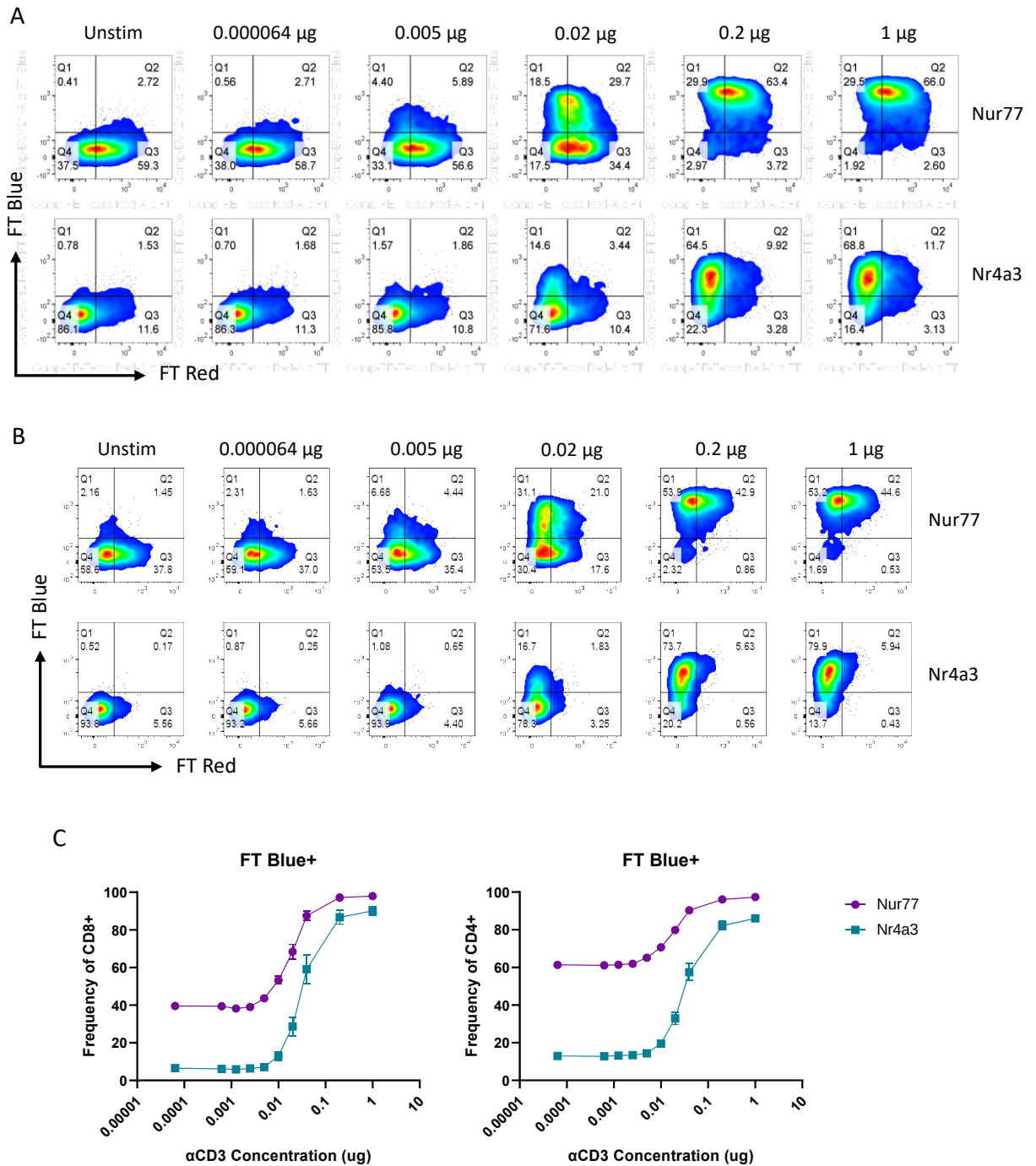


Figure 4.2. Responder populations in Nr4a3-Tocky and Nur77-Tempo models increase in a dose-dependent manner after α CD3 stimulation *in vitro*. Splenocytes from Nr4a3-Tocky and Nur77-Tempo mice were individually harvested and cultured *in vitro* with α CD3 (0-1 μ g as indicated; A-C) for 4 hours. **A and B.** CD4+ (A) and CD8+ (B) T-cells were analysed for FT Blue/Red expression, with responders characterised as FT Blue+ (sum Q1 + Q2). Flow plots are representative of one mouse for Nr4a3-Tocky and Nur77-Tempo. **C.** Summary plots of the frequency of CD4+ (A) and CD8+ (B) T-cells defined as FT Blue+ (sum Q1 + Q2). Data representative of one experiment (n=3) and shows mean $\pm$ SEM.

4.2.2 Immunisation with moderate (8 µg) vs high (80 µg) doses of 4Y-[MBP] results in qualitative differences in T cell responses

Tg4 TCR transgenic mice, generated in the Wraith lab, are a useful tool to study immunological tolerance to the myelin basic protein (MBP) self-antigen (Liu *et al*, 1995). Subsequent work has indicated that lysine at residue 4 in WT MBP controls the binding of the peptide to MHC class II, and single base substitutions of this amino acid can alter the affinity of the peptide (Fairchild *et al*, 1993). It was found that tyrosine at residue 4 of MBP (hereby known as 4Y-[MBP]) greatly increased the stability of class II binding compared to WT peptide which exhibited transient binding and resulted in less detectable functional complexes than 4Y-[MBP]. This enhanced MHC II binding ability, in combination with the high solubility of 4Y-[MBP] peptide, means that *in vivo* study of different strengths of TCR signalling can be performed (Elliot *et al*, 2021). Previous study with Tg4 Nr4a3-Tocky mice showed that weak vs strong TCR signalling drove differential expression of immune checkpoints and resulted in a recalibration of T cell activation thresholds.

To validate these past findings in Tg4 Nur77-Tempo model, the effects of moderate (8 µg) vs high (80 µg) doses of 4Y-[MBP], Tg4 Nur77-Tempo mice were injected subcutaneously with 4Y-[MBP] before splenocytes were harvested and analysed via flow cytometry 24 hrs later (Figure 4.3A). CD4⁺ T-cells were then analysed for FT Blue/Red expression (Figure 4.3B) to define a responder population (Q2). The defined responder population was then quantified and summarised (Figure 4.3C) to illustrate that there is a slight trending increase in the proportion of CD4⁺ T-cells that have responded to 4Y-[MBP] immunisation, though this is not significant.

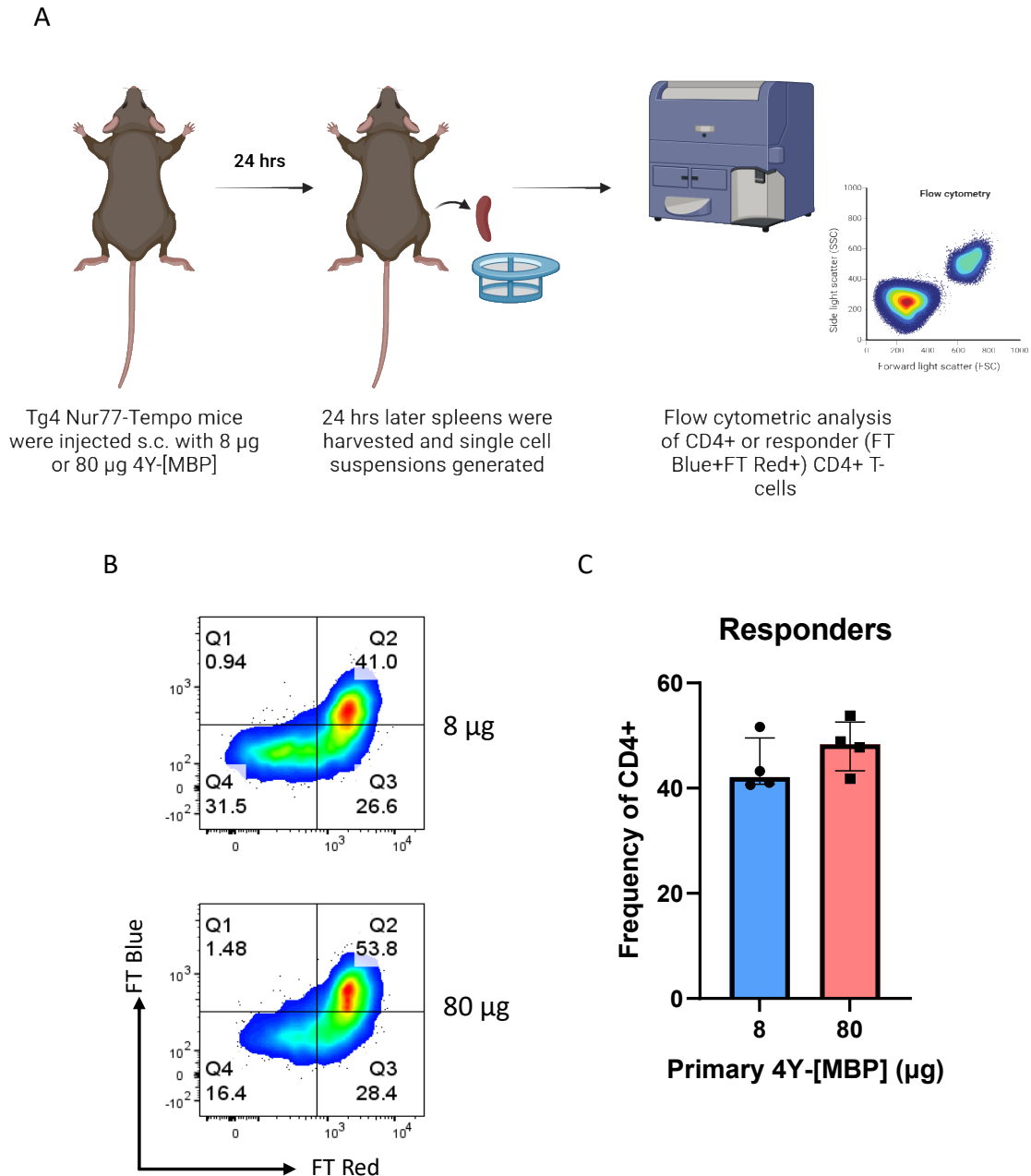


Figure 4.3. Primary immunisation of Tg4 Nur77-Tempo mice *in vivo* with moderate or high doses of 4Y-[MBP] activate CD4⁺ T-cells. Tg4 Nur77-Tempo mice were injected s.c with 8 µg (moderate) or 80 µg (high) doses of 4Y-[MBP] and splenocytes were harvested 24 hrs later (A-C). **A.** Schematic overview of experimental design. **B.** CD4⁺ T-cells were analysed for FT Blue/Red expression, with responders characterised as FT Blue⁺ FT Red⁺ (Q2). Flow plots are representative of one mouse for moderate and high doses. **C.** Summary plots of the frequency of CD4⁺ T-cells defined as responders (Q2). Data representative of one experiment (n=4) and shows median with interquartile range. Mann-Whitney test showing significance over $P \leq 0.05$.

In addition to studying the quantitative changes (i.e., frequency of responding cells) that occur post moderate (8 µg) or high (80 µg) 4Y-[MBP]-induced TCR-mediated T-cell activation (Figure 4.3), qualitative changes can also be evaluated. Co-stimulatory (e.g. OX40) and co-inhibitory (eg. PD-L1) receptor expression is often associated with T-cell activation and/or exhaustion. CD4⁺ T-cells from Figure 4.3 were evaluated for immune checkpoint expression commonly associated with favourable T-cell immune responses and/or associated with ICM therapies. In addition, IL-10 expression was explored due to its link with strong TCR signalling. IL-10, ICOS, OX40 and LAG-3 were all enhanced more predominantly in response to strong TCR-mediated activation compared with moderate (Figure 4.4A,B). The PD-1/PD-L1 pathway showed no significant difference between the two treatment doses (Figure 4.4B), consistent with the notion that PD-1/PD-L1 are early activation markers often induced on the surface of CD4⁺ and CD8⁺ T cells by antigen-specific and non-specific T-cell stimulation (Hong *et al.*, 2013). This finding is consistent with that of Elliot *et al.* (2021) whereby PD-1 was tightly coupled with T-cell activation, but LAG-3 was responsive to the immunising dose of peptide.

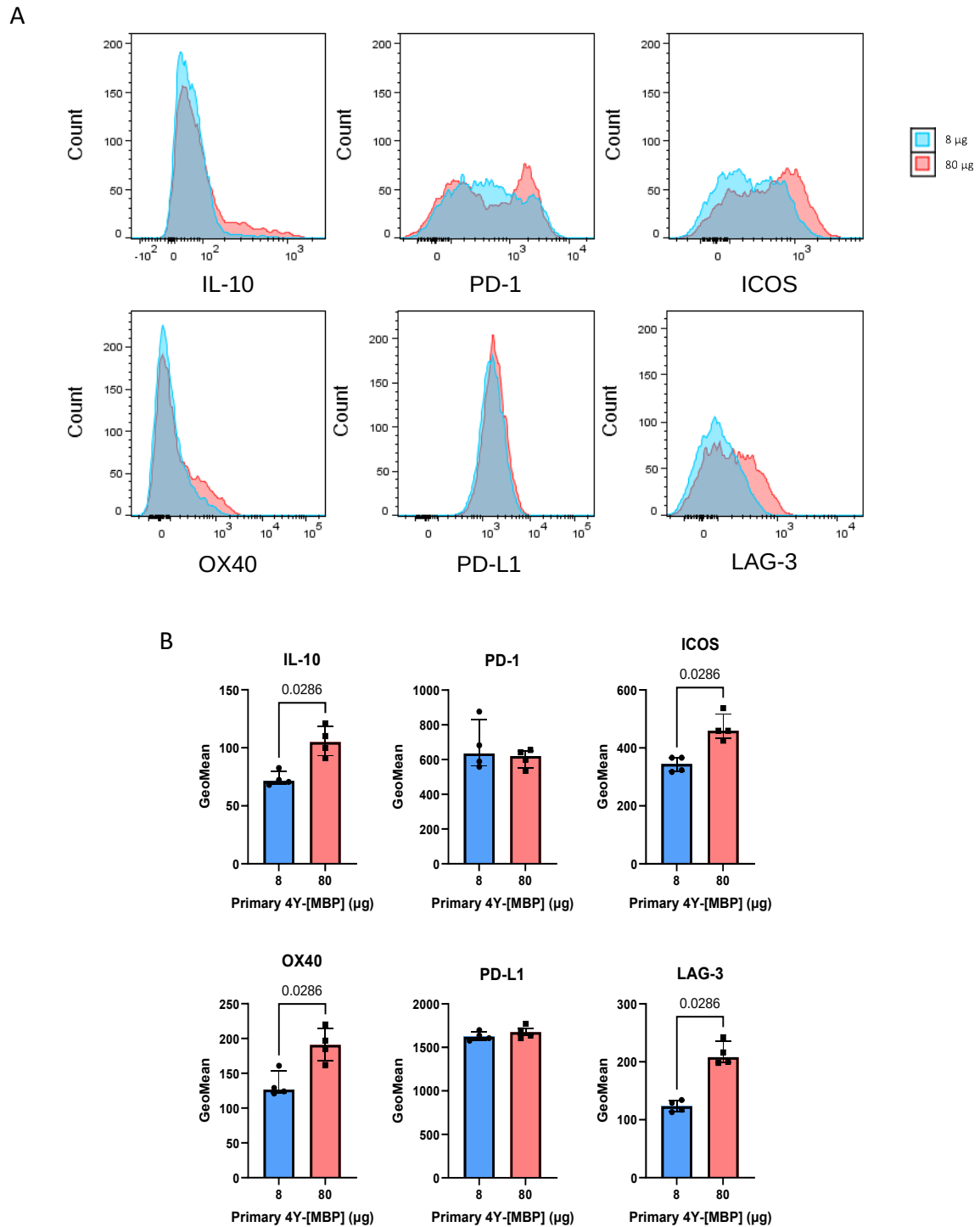


Figure 4.4. Primary immunisation of Tg4 Nur77-Tempo mice *in vivo* with moderate or high doses of 4Y-[MBP] differentially induce quantitative changes on CD4+ T-cells. Tg4 Nur77-Tempo mice were injected s.c. with 8 µg (moderate) or 80 µg (high) doses of 4Y-[MBP] and splenocytes were harvested 24 hrs later (A and B). **A.** Flow cytometric histograms of immune checkpoint expression on CD4+ T-cells (as in Figure 4.4, B and C) in response to moderate and high 4Y-[MBP] immunisation *in vivo*. Plots are representative of one mouse for moderate and high doses. **B.** Summary plots of the geometric mean for each immune checkpoint marker and IL-10 depicted in A. Data representative of one experiment (n=4) and shows median with interquartile range. Mann-Whitney test showing significance of $P \leq 0.05$.

The unique nature of the FT model allows for detailed quantitative evaluation of CD4+ T-cells which are likely to be currently receiving a TCR signal *in vivo*. This is due to the half-life of FT blue being short, around 4 hrs (Bending *et al.*, 2018). This means analysis can be refined by gating only on T-cells that show very recent TCR signalling (Blue+), a history of TCR signalling (Red+), or both (termed responder cells; Blue+Red+). Figure 4.5 illustrates those CD4+ T-cells from Figure 4.4 which were defined as responders (Q2) in Figure 4.3. The geometric mean of each marker analysed is greater on the responder populations (Figure 4.5A,B) compared to the bulk CD4+ T-cells (Figure 4.4B), though the trend of increased expression observed with a high dose of 4Y-[MBP] compared to moderate is still present, reaching significance for ICOS, OX40 and LAG-3 (Figure 4.5B). This confirms that changes in markers is not due to differential proportions in responding T-cells but reflects T-cell intrinsic changes in response to different TCR signal strengths.

Due to the upregulation of several immune checkpoints, coupled with the findings of Elliot *et al.* (2021) in the Nr4a3-Tocky model that shows α PD-1 treatment lowers the activation threshold of T-cell activation, the effects of PD-1/PD-L1 pathway blockade was to be explored in this model.

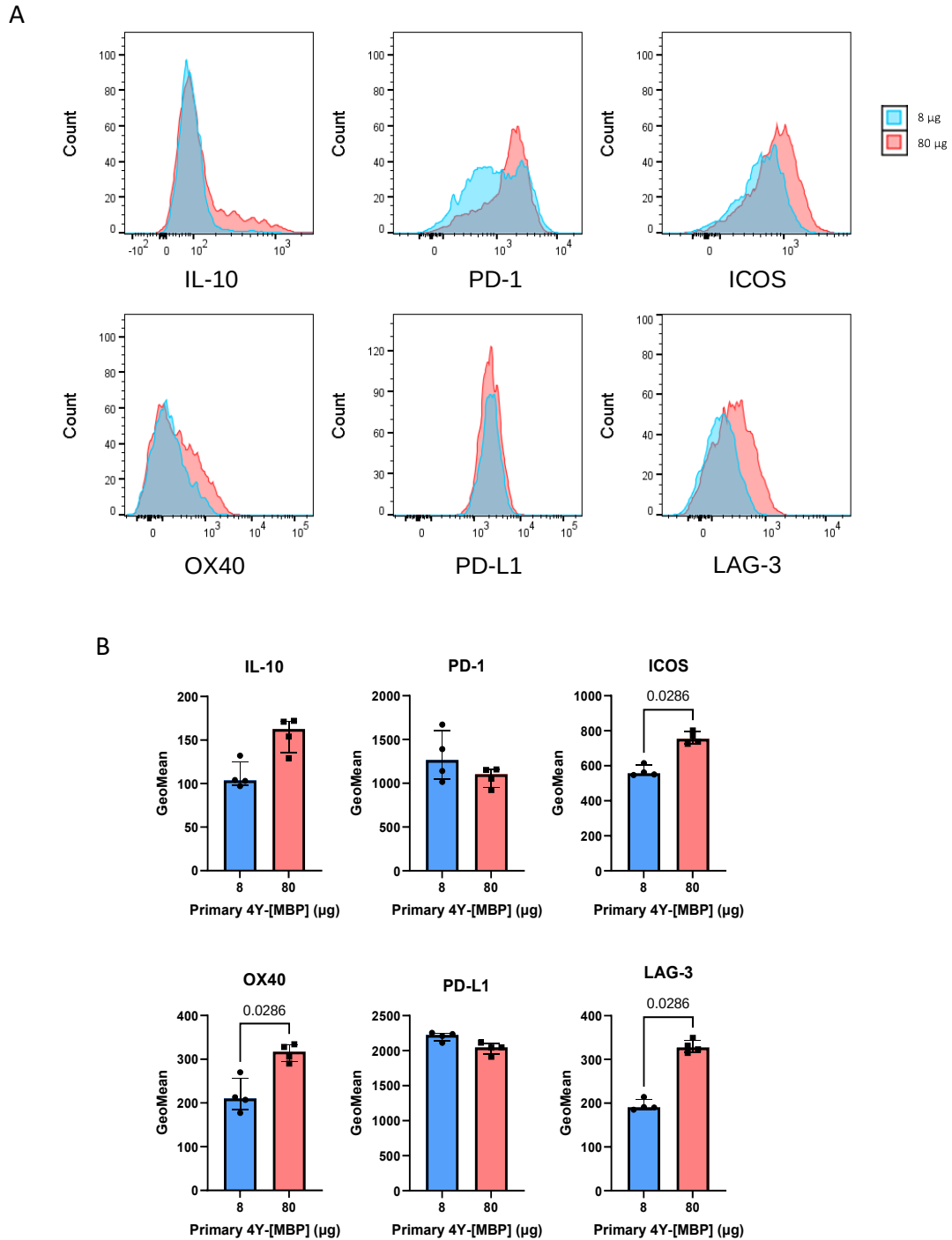


Figure 4.5. Primary immunisation of Tg4 Nur77-Tempo mice *in vivo* with moderate or high doses of 4Y-[MBP] differentially induce quantitative changes on responder (FT Blue+ FT Red+) CD4+ T-cells. Tg4 Nur77-Tempo mice were injected s.c. with 8 µg (moderate) or 80 µg (high) doses of 4Y-[MBP] and splenocytes were harvested 24 hrs later (A and B). **A.** Flow cytometric histograms of immune checkpoint expression on FT Blue+ FT Red+ (Q2) responder CD4+ T-cells (as in Figure 4.4, B and C) in response to moderate and high 4Y-[MBP] immunisation *in vivo*. Plots are representative of one mouse for moderate and high doses. **B.** Summary plots of the geometric mean for each immune checkpoint marker depicted in A. Data representative of one experiment (n=4) and shows median with interquartile range. Mann-Whitney test showing significance over $P \leq 0.05$.

4.2.3 α PD-L1 blockade in a high (4 mg/kg) and moderate (0.4 mg/kg) 4Y-[MBP] recalibration model induces expression of OX40 on the surface of FT Blue+ FT Red+ CD4+ T-cells

As Nr4a3-Tocky and Nur77-Tempo are crossed onto a Tg4 background, the high affinity 4Y-[MBP] peptide, combined with the FT reporter mice, can be utilised to study TCR responses in an adaptive tolerance model. This model, developed to look at recalibrated T-cell activation, was a critical model used in Elliot *et al.* (2021). In this model, T-cells with arrested TCR signalling (FT Blue- Red+) would move up to Q2 (Responders; FT Blue+ Red+) in response to peptide re-challenge. PD-L1, a co-inhibitory receptor constitutively expressed on T-cells, is further up-regulated upon T-cell activation (Freeman *et al.*, 2006). The negative regulatory properties of PD-1/PD-L1, in combination with its increased expression associated with T-cell activation, make the PD-1/PD-L1 pathway a suitable therapeutic target for augmenting T-cell responses in disease, including cancer.

Elliot *et al.* (2021) observed that the re-emergence of a responder population was enhanced by α PD-1 treatment. In addition to an increased responder population, Elliot *et al.* (2021) observed an increased expression of OX40. Following this publication, recent data emerged that suggested α PD-1 blockade in mice can lead to unwanted target cell depletion (Polesso *et al.*, 2021). However, antibody-mediated PD-L1 blockade has been observed to reverse the inhibitory effects of PD-1/PD-L1 binding both *in vitro* and *in vivo*, including an observed increase in T-cell cytolytic activity and decreased tumour growth kinetics (Iwai *et al.*, 2002). To circumnavigate potential target cell depletion observed with α PD-1, the effects of α PD-L1 were explored in this model to evaluate the ability of α PD-L1 to recalibrate T-cell activation thresholds in addition to measuring qualitative changes observed in response to the treatment *in vivo*. The

vast array of promising research into α PD-L1 blockade both pre-clinically and clinically over the past two decades, combined with observed high levels of PD-1 and PD L1 in 4Y-[MBP] immunised mice (Figures 4.4 and 4.5), make α PD-L1 a suitable choice of ICM therapy to explore enhancing in this model. To do this, the adaptive tolerance model was utilised, whereby Nur77-Tempo mice were immunised s.c. with 4 mg/kg [4Y]-MBP before being treated 24 hrs later with α PD-L1 or IgG (Figure 4.6A). Thirty minutes later, allowing PD-L1 blockade to take place, mice were re-challenged with a ten-fold lower dose of [4Y]-MBP (0.4 mg/kg). Four hours later, splenocytes were analysed via flow cytometry for their CD4⁺ responder properties, in addition to CD4⁺ surface expression of checkpoint markers.

In order to confirm that α PD-L1 blockade had been effective, CD4⁺ and CD4⁺ Blue⁺ Red⁺ T-cells were counterstained for PD-L1, demonstrating effective blockade of the inhibitory ligand (Figure 4.6B,C). Following this, CD4⁺ T-cells were evaluated for qualitative changes in responder populations, with α PD-L1 blockade showing 37.4 % responders compared to the 29.9 % observed in the isotype-treated mice (Figure 4.6D,E). This was coupled with an observed increase in FT Blue geometric mean, though not reaching significance (Figure 4.6E). Together, these data show that effective α PD-L1 blockade as a single-agent therapy can increase the proportion of CD4⁺ T-cells that respond to peptide re-challenge *in vivo*.

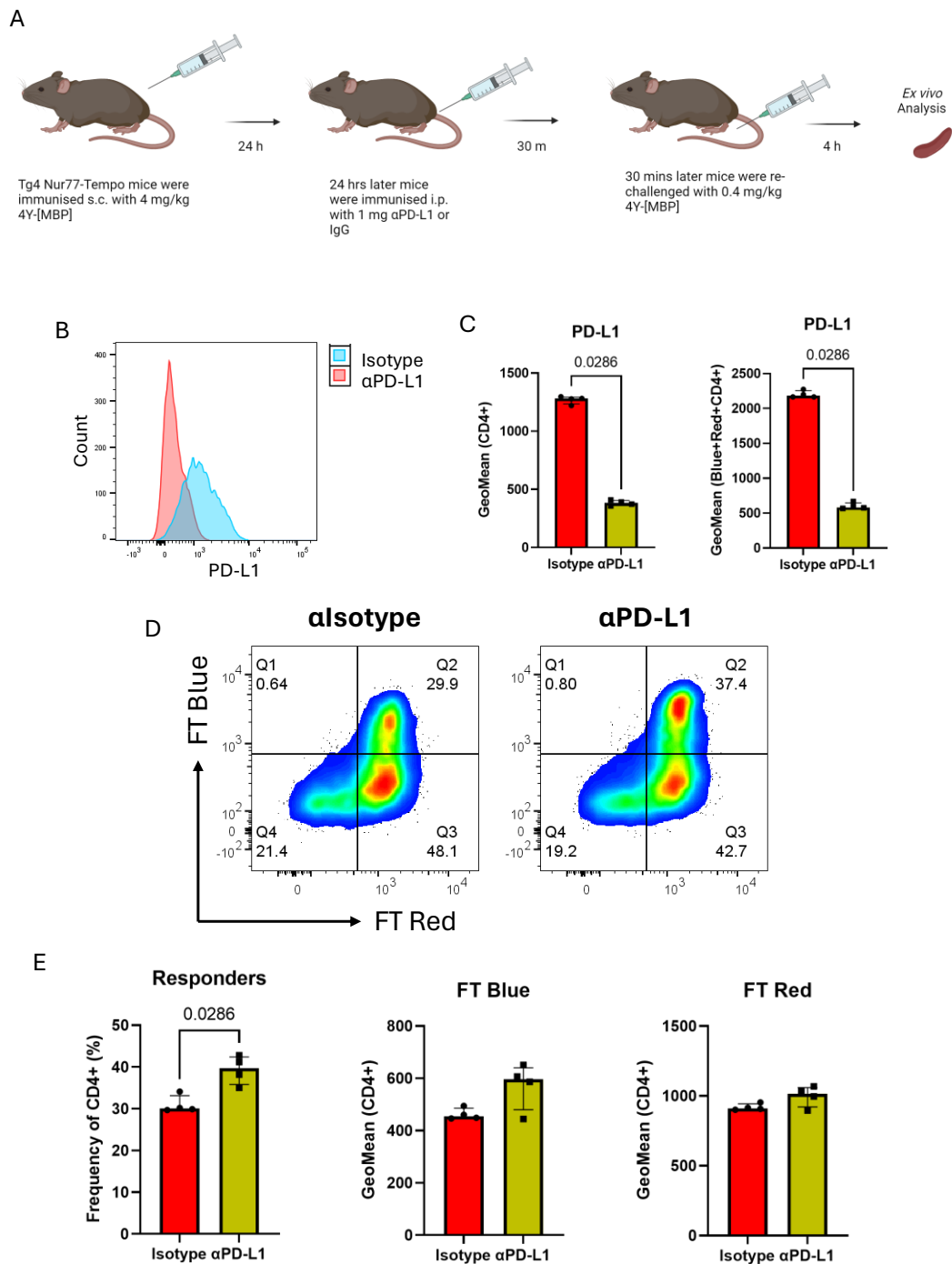


Figure 4.6. α PD-L1 blockade in Tg4 Nur77-Tempo mice *in vivo* in a 4Y-[MBP] adaptive tolerance model induce quantitative changes to responder (FT Blue+ FT Red+) CD4+ T-cells. Tg4 Nur77-Tempo mice were injected s.c. with 4 mg/kg 4Y-[MBP], followed by 1 mg α PD-L1 or IgG i.p. 24 hrs later. Mice were then re-challenged 30 mins later with 0.4 mg/kg 4Y-[MBP] s.c. for 4 hrs before splenocytes were harvested and analysed via flow cytometry. **A.** Experimental design schematic. **B.** Flow cytometric histogram of successful PD-L1 blockade on CD4+ T-cells. **C.** Summary plots of B for CD4+ T-cells and Blue+Red+ CD4+ T-cells. **D.** Flow cytometric plots of FT Blue vs Red expression showing responding Blue+Red+ CD4+ T-cells in Q2. **E.** Summary plots of D showing responding CD4+ T-cells (%) and geometric mean of FT Blue and FT Red on CD4+ T-cells. Data representative of one experiment (n=4) and shows median with interquartile range. Mann-Whitney test showing significance over $P \leq 0.05$.

Whilst this is promising, antibody-mediated PD-L1 blockade alone is likely not sufficient to elicit a favourable anti-tumour immune response *in vivo* or clinically in the majority of patients, with many pre-clinical and clinical trials focusing on combination treatments with other antibody-mediated co-stimulatory and co-inhibitory ICM therapies. It stands to reason that a rationally designed combination therapy should be sought and evaluated. To do this, surface expression of a range of co-stimulatory (OX40, ICOS and GITR) and co-inhibitory (LAG-3, TIGIT and PD-1) markers was explored in response to α PD-L1 single-agent therapy (Figures 4.7 and 4.8). ICOS, GITR and LAG-3 all had an observed slight increased expression in α PD-L1-treated mice compared to those treated with isotype on CD4⁺ T-cells, though this result was not significant (Figure 4.7A,B). One likely reason for this could be the early end-point of the experiment, with 4 hours not being long enough for these markers to be upregulated. Significant increased expression was observed in OX40, TIGIT and PD-1 expression on both CD4⁺ and Blue⁺ Red⁺ CD4⁺ T-cells (Figures 4.7 and 4.8).

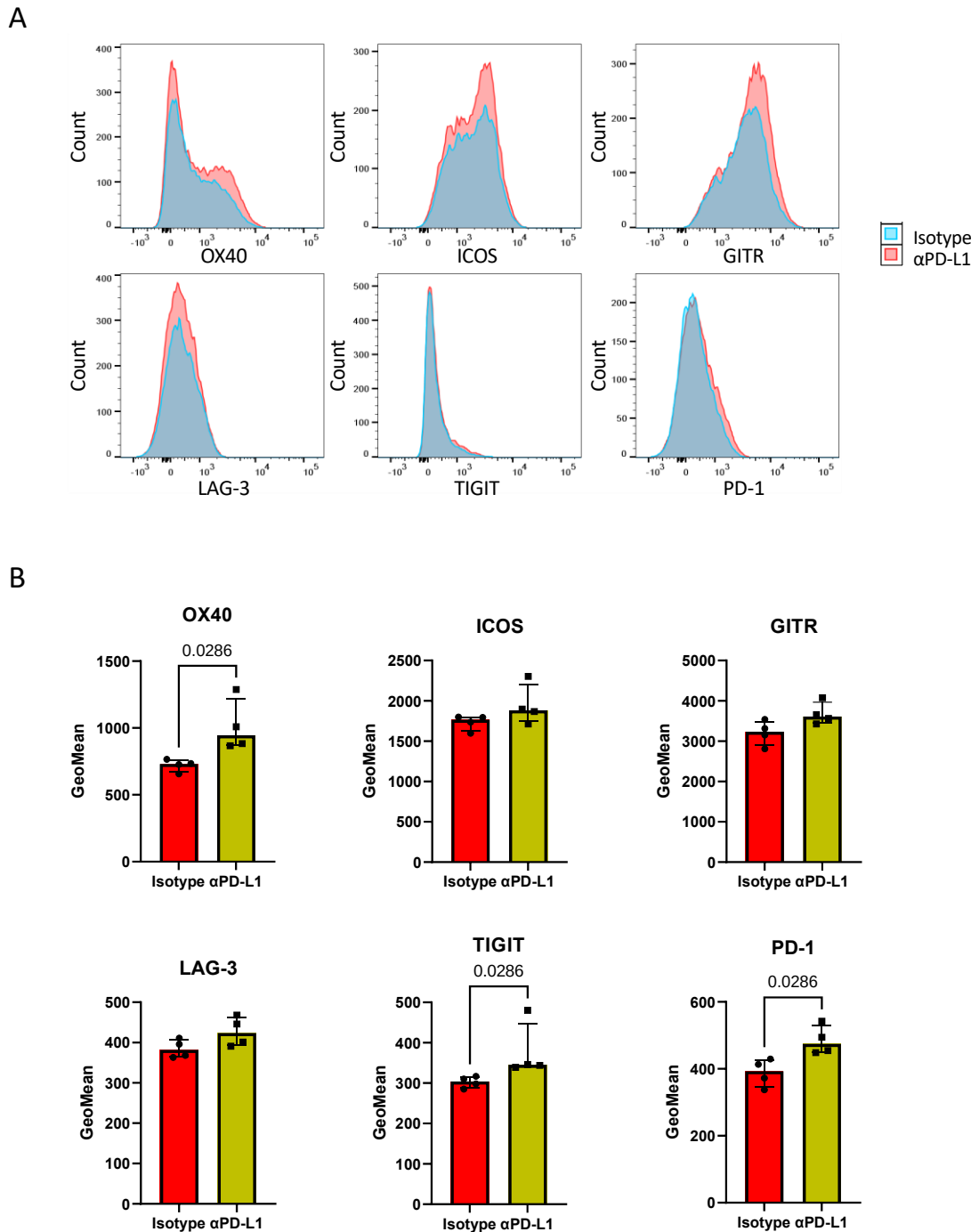


Figure 4.7. α PD-L1 blockade in Tg4 Nur77-Tempo mice *in vivo* in a 4Y-[MBP] adaptive tolerance model induces OX40 expression on the surface of CD4⁺ T-cells. Tg4 Nur77-Tempo mice were injected s.c with 4 mg/kg 4Y-[MBP], followed by 1 mg α PD-L1 or IgG i.p 24 hrs later. Mice were then re-challenged 30 mins later with 0.4 mg/kg 4Y-[MBP] s.c for 4 hrs before splenocytes were harvested and analysed via flow cytometry. **A.** Flow cytometric histograms of co-stimulatory and co-inhibitory receptors analysed on the surface of CD4⁺ T-cells from α PD-L1-treated and IgG-treated mice. **B.** Summary plots of A. Data representative of one experiment (n=4) and shows median with interquartile range. Mann-Whitney test showing significance over $P \leq 0.05$.

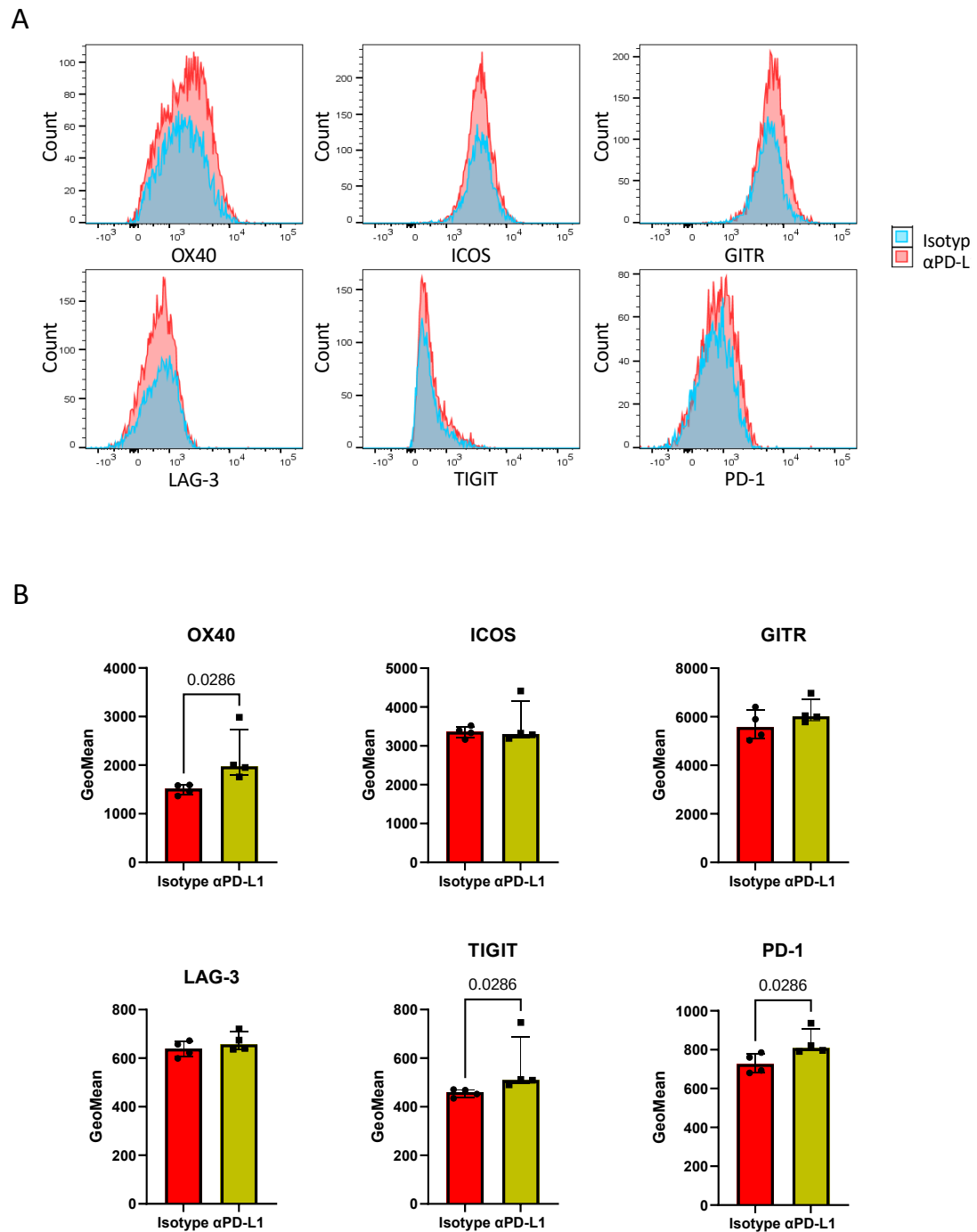


Figure 4.8. α PD-L1 blockade in Tg4 Nur77-Tempo mice *in vivo* in a 4Y-[MBP] adaptive tolerance model induces OX40 expression on the surface of responder (FT Blue+ FT Red+) CD4+ T-cells. Tg4 Nur77-Tempo mice were injected s.c with 4 mg/kg 4Y-[MBP], followed by 1 mg α PD-L1 or IgG i.p 24 hrs later. Mice were then re-challenged 30 mins later with 0.4 mg/kg 4Y-[MBP] s.c for 4 hrs before splenocytes were harvested and analysed via flow cytometry. **A.** Flow cytometric histograms of co-stimulatory and co-inhibitory receptors analysed on the surface of responder FT Blue+ Red+ CD4+ T-cells from α PD-L1-treated and IgG-treated mice. **B.** Summary plots of A. Data representative of one experiment (n=4) and shows median with interquartile range. Mann-Whitney test showing significance over $P \leq 0.05$.

The co-stimulatory nature of OX40, combined with the increased surface expression following α PD-L1 treatment *in vivo*, consistent with the findings in Elliot *et al.* (2021), led to the exploration of α PD-L1 blockade with antibody-mediated OX40 agonism (α OX40).

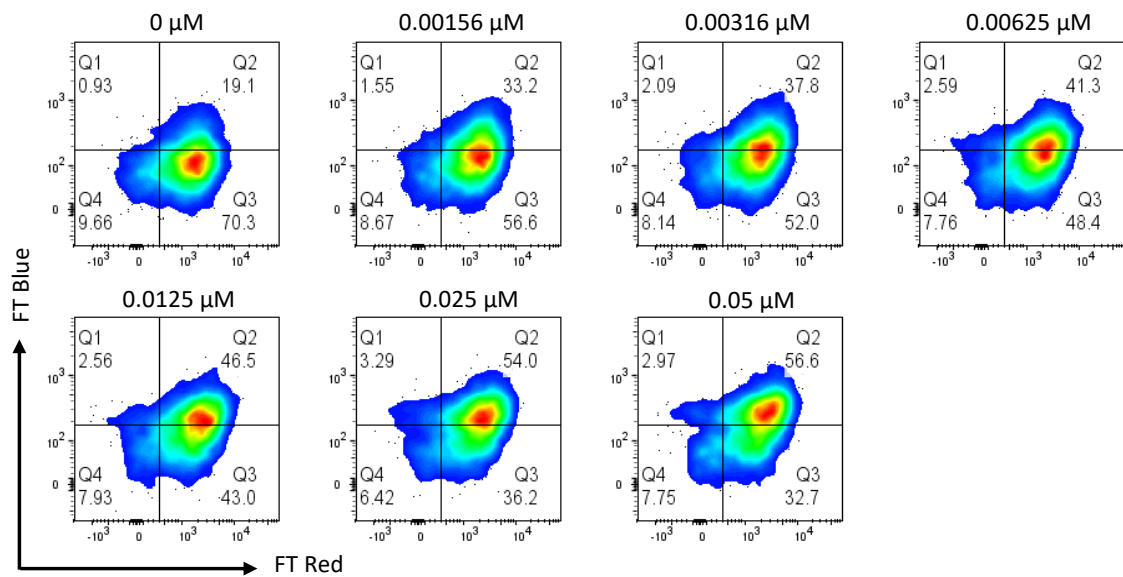
4.2.4 Development of an *in vivo/in vitro* hybrid model to explore the effects of immune checkpoint modulation in response to different TCR signal strengths

Whilst the adaptive tolerance model is an excellent model to study the effects of ICM therapy on modifying T-cell activation thresholds and associated checkpoint expression, the model is constrained by the number of mice needed per experiment, to test different combinations and how they may be modulated by different TCR signal strengths. To address these issues, a novel *in vivo/in vitro* hybrid model was developed. Here, splenocytes from an adaptively tolerised mouse can be subjected to several ICM combinations *in vitro* allowing for paired analysis of obtained data in addition to high throughput screening of many conditions per mouse.

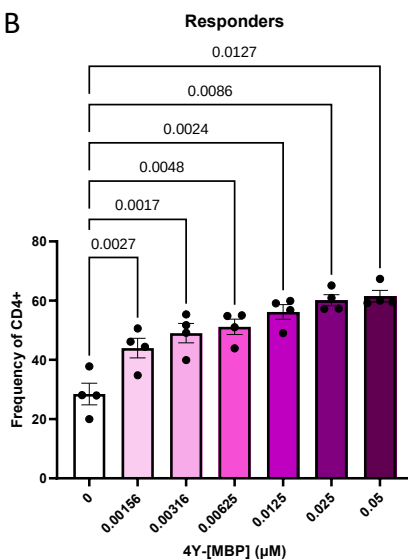
The *in vivo/in vitro* hybrid platform has two main aspects, a primary immunisation of 4Y-[MBP] *in vivo*, and a secondary treatment with a range of 4Y-[MBP] doses $\pm$ ICM combinations *in vitro*. To first explore the effects of 4Y-[MBP] in the model, Tg4 N4a3-Tocky mice were immunised subcutaneously *in vivo* with 4 mg/kg primary dose of peptide before splenocytes were harvested 24 hrs later for *in vitro* culture. Here, spleens were enzymatically digested as described in section 2.2.8.3, in order to preserve APCs for subsequent *in vitro* culture. Splenocytes were cultured for 4 hrs with a range of secondary doses of 4Y-[MBP] (0-0.05 μ M as indicated, Figure 4.10). CD4+

T-cells were then assessed for FT Blue/Red expression (Figure 4.9A) and responders were characterised as FT Blue+ (sum Q1 + Q2). This fraction of CD4 + T cells were known to respond to the secondary, *in vitro*, dose of 4Y-[MBP]. As the secondary dose of peptide increases, the fraction of responding CD4+ T cells increases in a dose-responsive manner (Figure 4.9A,B). The same trend is observed with FT Blue geometric mean (Figure 4.9C), corroborating the finding that CD4+ T-cells are capable of secondary activation, when there is enough antigen present to elicit an immune response.

A



B



C

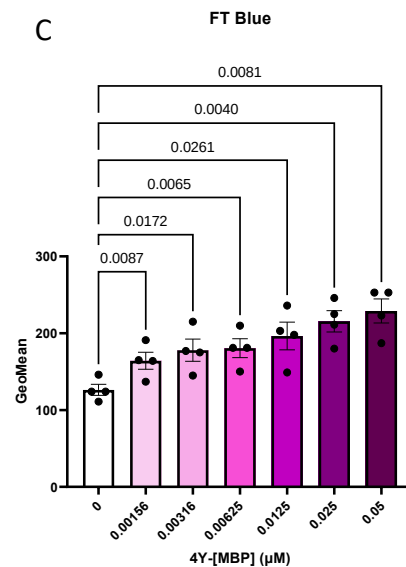


Figure 4.10. *In vivo* *in vitro* hybrid platform reveals dose-dependent qualitative changes in FT Blue+ responder CD4+ populations. Tg4 Nr4a3-Tocky mice were injected s.c. with a primary dose of 4 mg/kg 4Y-[MBP] and spleens were harvested 24 hrs later and enzymatically digested for culture *in vitro* with secondary 4Y-[MBP] (0-0.05 μM as indicated, A.C). **A.** CD4+ T-cells were analysed for FT Blue/Red expression and responders defined as FT Blue+ (sum Q1 + Q2). Plots are representative of one mouse for each dose. **B.** Summary plot for FT Blue+ responder populations as a frequency of CD4+ T-cells in response to secondary 4Y-[MBP] treatment. **C.** Summary plot for the geometric mean of FT Blue expression in CD4+ T-cells in response to secondary 4Y-[MBP] treatment. Data representative of one experiment (n=4) and shows mean ± SEM (B and C). RM one-way ANOVA with multiple comparisons showing significance (P≤0.05) (B and C).

The nature of the Tg4 Nra3-Tocky model allows the division of cells into responders (FT Blue+) and non-responders (FT Blue-). When comparing the geometric mean of co-stimulatory (OX40, ICOS, GITR) and co inhibitory (LAG-3, TIGIT, PD-1 and PD I.1) markers on the surface of FT Blue+ CD4+ and FT Blue- CD4+ T-cells, it is observed that non-responder T-cells do not show an increasing trend in marker expression, opposed to on the responder populations which increase in expression with increased secondary dose of 4Y-[MBP] (Figure 4.10). This confirms that the markers studied in this system reflect responses to TCR driven events and not changes in response to indirect effects through other pathways (e.g. cytokines) which has been a notable limitation for studies using CD69 as an activation marker.

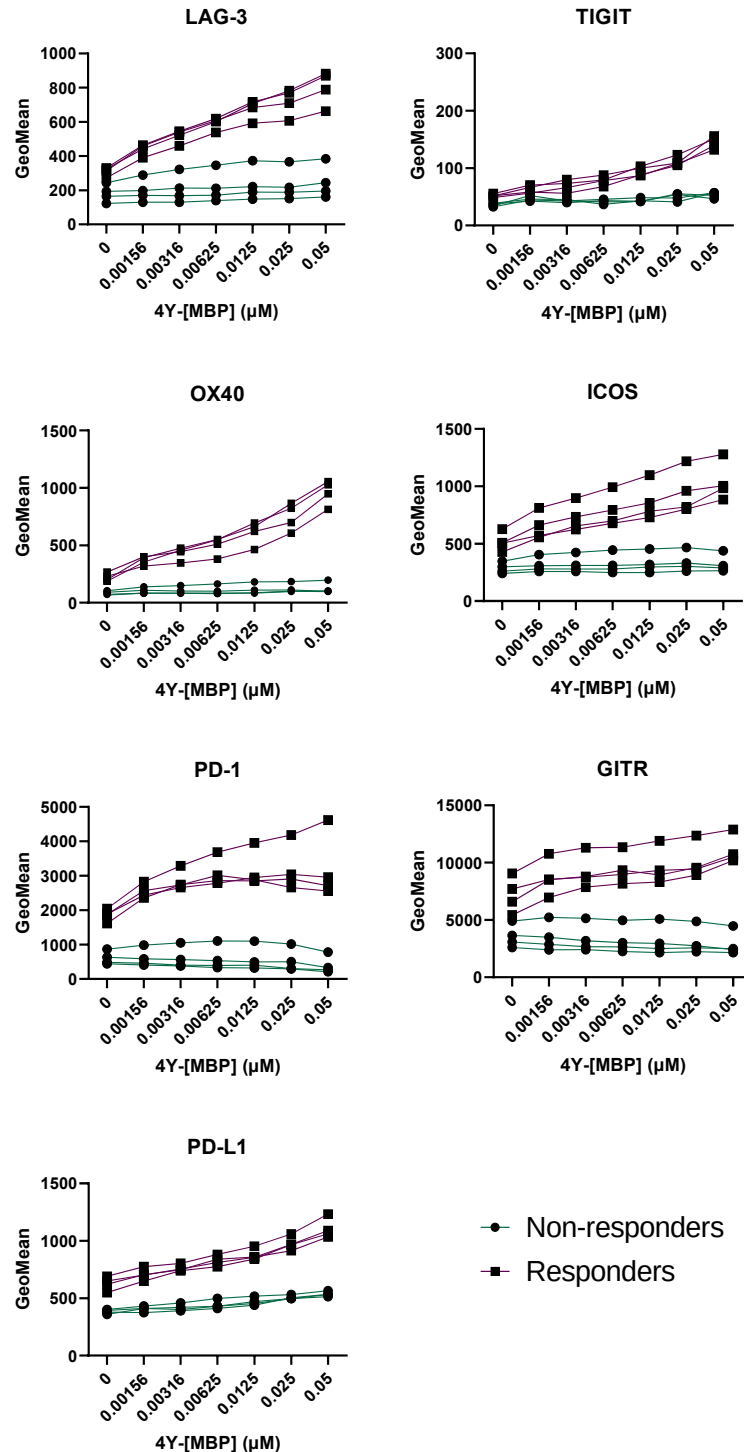


Figure 4.10. *In vivolin vitro* hybrid platform reveals dose-dependent quantitative changes in immune checkpoint expression selectively on FT Blue+ responder CD4+ populations. Tg4 Nr4a3-Tocky mice were injected s.c. with a primary dose of 4 mg/kg 4Y-[MBP] and spleens were harvested 24 hrs later and enzymatically digested for culture *in vitro* with secondary 4Y-[MBP] (0-005 μ M as indicated). CD4+ T-cells were analysed for FT Blue+ (sum Q1 + Q2) 'responders' and FT Blue- (sum Q3 + Q4) 'non-responders' populations. Summary plots illustrate the geometric mean of immune checkpoint expression on the surface of responders and non-responders in response to secondary 4Y-[MBP] *in vitro* (n=4).

A further look at responder (FT Blue+) CD4+ T-cells shows a significant increase in the geometric mean of all explored checkpoint marker surface expression in a 4Y-[MBP] dose-responsive manner (Figure 4.11A,B). This finding is consistent with co-stimulatory and co-inhibitory markers being commonly associated with increased T-cell activation. Whilst increased expression of co stimulatory markers would assist T-cell activation, an increased expression of co-inhibitory markers such as PD-1/PD-L1 poses as a potential limiting step for T-cell activation. As shown *in vivo*, PD-1/PD-L1 pathway blockade has potential to augment T cell activation by blocking the inhibitory signals associated with reducing T-cell activity and lowering the activation threshold of TCR-mediated T-cell activation (Figure 4.6). Of note, PD-1 expression plateaus at 0.0125 μ M 4Y-[MBP] treatment whilst other markers continue to rise with increased dose of peptide stimulation.

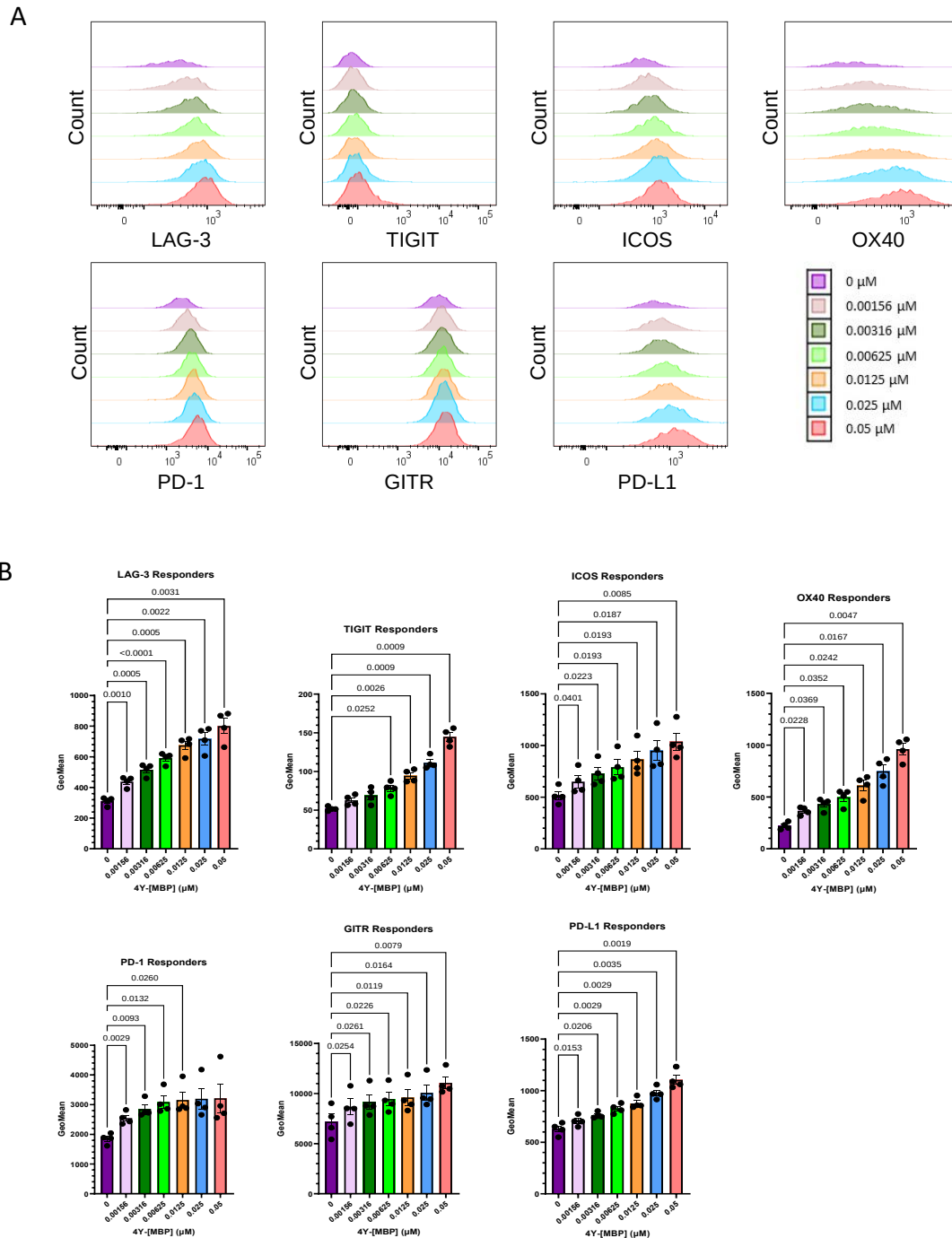


Figure 4.11. *In vivolin vitro* hybrid platform reveals dose-dependent quantitative changes in immune checkpoint expression on FT Blue+ responder CD4+ populations. Tg4 Nr4a3-Tocky mice were injected s.c., with a primary dose of 4 mg/kg 4Y-[MBP] and spleens were harvested 24 hrs later and enzymatically digested for culture *in vitro* with secondary 4Y-[MBP] (0-0.05 μ M as indicated, A and B). **A.** Flow cytometric histograms of immune checkpoint expression on the surface of FT Blue+ responder CD4+ populations in response to secondary 4Y-[MBP]. Plots are representative of one mouse for each dose. **B.** Summary plots illustrate the geometric mean of immune checkpoint expression on the surface of FT Blue+ responder populations in response to secondary 4Y-[MBP] *in vitro*. Data representative of one experiment (n=4) and shows mean $\pm$ SEM. RM one-way ANOVA with multiple comparisons showing significance ($P \leq 0.05$).

4.2.5 ICM shows quantitative and qualitative effects in a 4Y-[MBP]-induced dose-responsive and ICM-induced manner

The aim of this chapter was to explore PD-1/PD-L1 pathway modulations in combination with co-stimulatory agonism, in particular OX40 and ICOS. The *in vivo/in vitro* hybrid platform was utilised to explore the effects of a range of combination therapies on TCR-mediated T-cell activation (Figure 4.12A,B). Increased secondary dose of 4Y-[MBP] was associated with increased proportions of CD4⁺ T-cells being defined as responder cells (Figure 4.12B), consistent with previous findings (Figure 4.9). Though there was an observed increase in responder populations from ~40 % in 0 μ M to ~60 % in 0.04 μ M, there were limited effects observed between treatment conditions for the proportion of cells defined as responders (Figure 4.13A-D). It is likely that *in vivo* immunisation produces greater effects than are observed in the *in vivo/in vitro* hybrid platform due to more effective antigen presentation. In addition, during *in vitro* stimulation, it is assumed that peptide levels are constant, whilst *in vivo*, the soluble peptide will exhibit different pharmacodynamic behaviour due to excretion processes from the system.

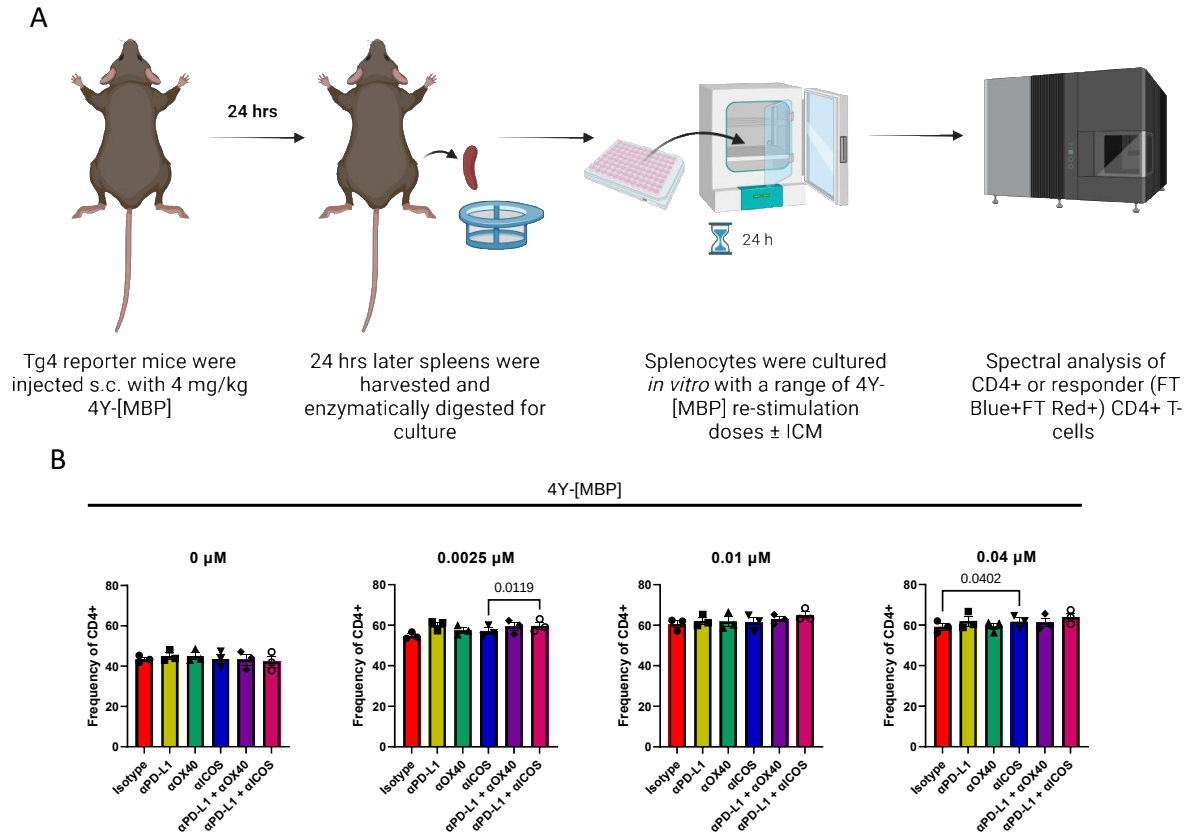


Figure 4.12. *In vivo/in vitro* hybrid platform reveals dose-dependent responses to 4Y-[MBP] treatment and limited treatment-dependent effects. Tg4 Nr4a3-Tocky mice were injected s.c. with a primary dose of 4 mg/kg 4Y-[MBP] and spleens were harvested 24 hrs later and enzymatically digested for culture *in vitro* with secondary 4Y-[MBP] (0-0.04 μ M as indicated, A and B). **A.** Schematic diagram to illustrate experimental protocol. **B.** Summary plots illustrate the frequency of CD4+ T-cells defined as responder cells (defined gate, Figure 4.14). Data representative of one experiment (n=3) and shows mean $\pm$ SEM. RM one-way ANOVA with multiple comparisons showing significance ($P \leq 0.05$).

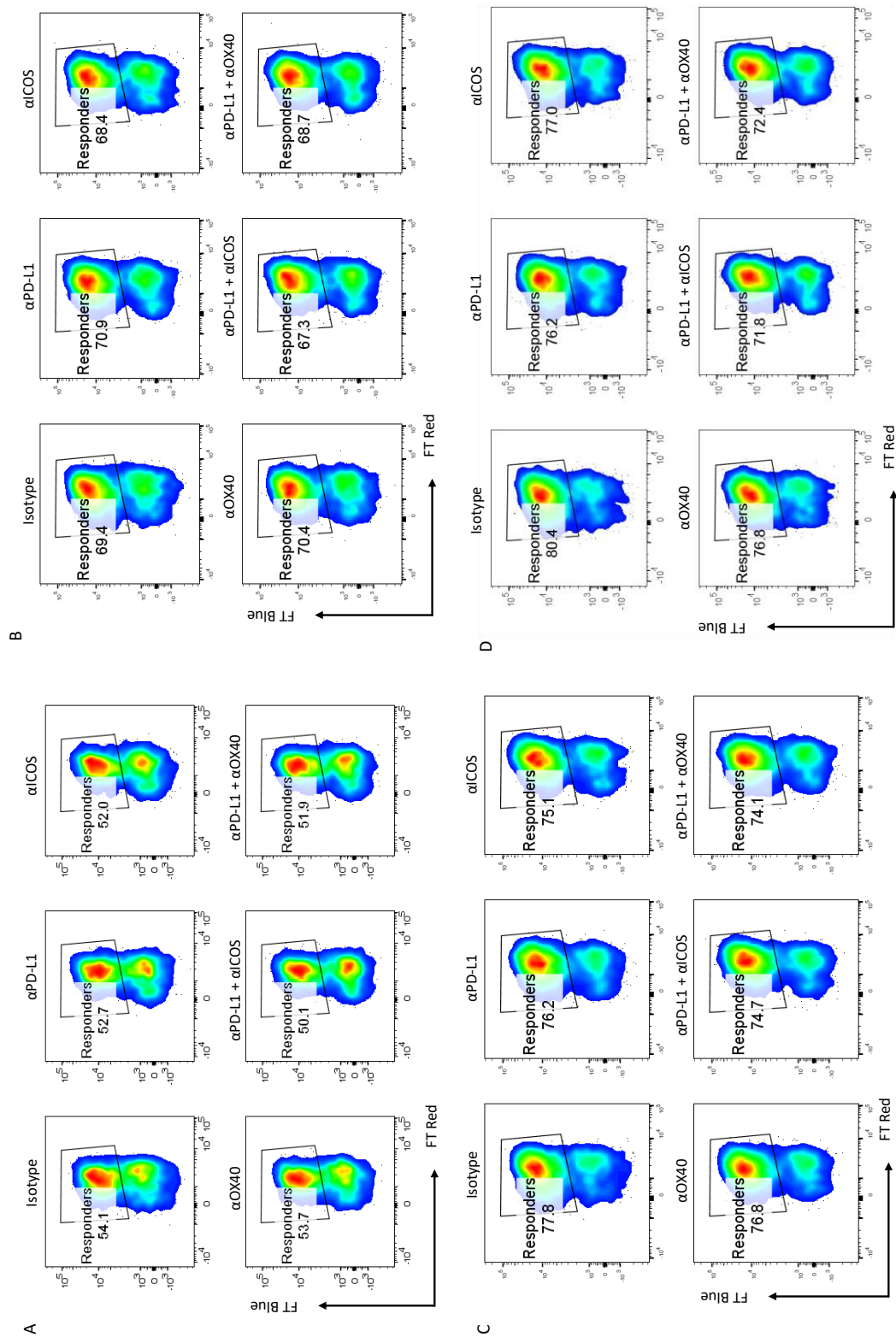


Figure 4.13. *In vivo* *in vitro* hybrid platform reveals dose dependent responses to 4Y-[MBP] treatment and limited treatment-dependent effects. Tg4 Nr4a3-Tocky mice were injected s.c. with a primary dose of 4 mg/kg 4Y-[MBP] and spleens were harvested 24 hrs later and enzymatically digested for culture *in vitro* with secondary 4Y-[MBP] at 0 μ M (**A**), 0.0025 μ M (**B**), 0.01 μ M (**C**) and 0.04 μ M (**D**). Data is representative of one mouse for all treatment conditions.

Alongside FT Blue/Red expression, CD4⁺ T-cells were evaluated for their expression of co-stimulatory and co-inhibitory checkpoint receptors. Summary data of quantified marker expression was explored for CD4⁺ (Figure 4.14) and responder CD4⁺ (Figure 4.15) T-cells. Due to using antibody-mediated agonists for ICOS and OX40 which were previously identified as strong markers of activation for T-cells, proxy markers were used as a readout, including CD25, PD-1 and GITR. Consistent with previous findings, an observed increase in geometric mean for CD25, PD-1 and GITR is observed between the secondary doses of 4Y-[MBP] 0-0.04 μ M (Figures 4.14 and 4.15).

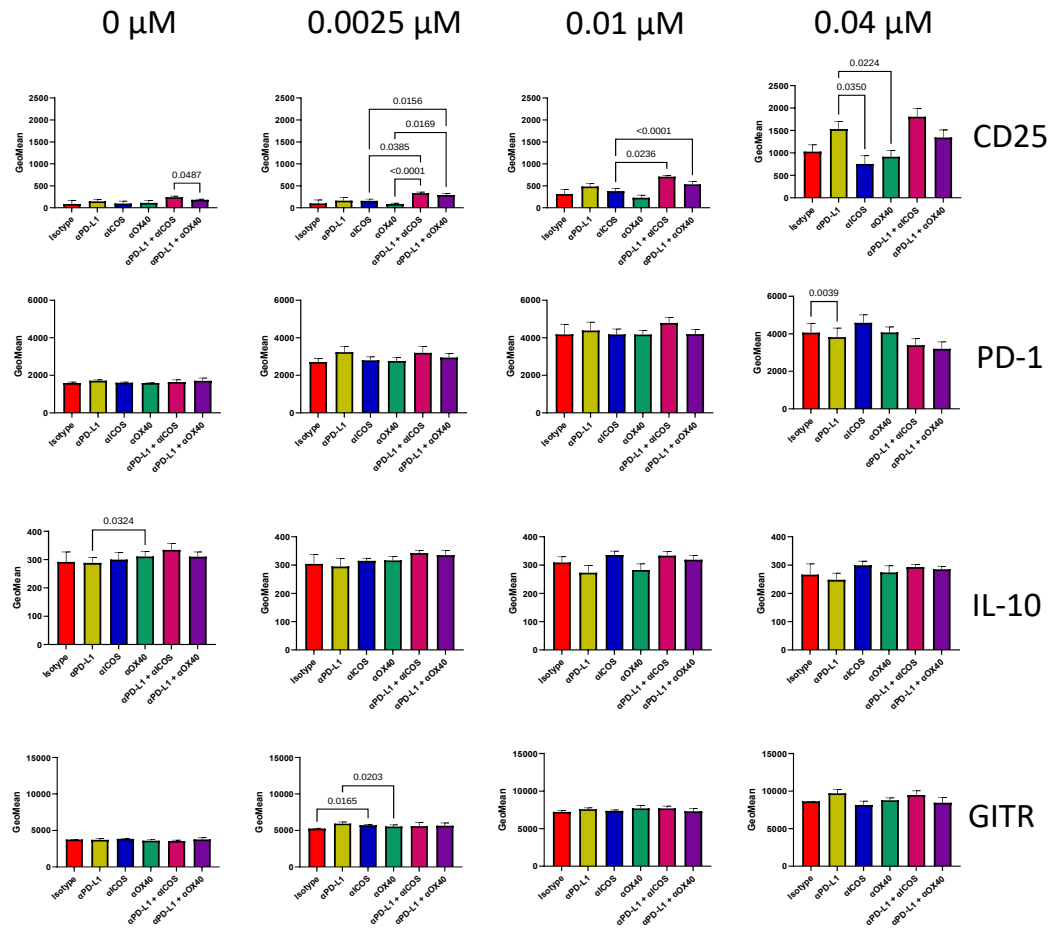


Figure 4.14. *In vivolin vitro* hybrid platform reveals dose-dependent increased expression of checkpoint markers but limited effects between treatments on CD4+ T-cells. Tg4 Nr4a3-Tocky mice were injected s.c. with a primary dose of 4 mg/kg 4Y-[MBP] and spleens were harvested 24 hrs later and enzymatically digested for culture *in vitro* with secondary 0-0.04 μ M 4Y-[MBP] as indicated. Geometric mean of CD25, GITR, PD-1 and IL-10 on CD4+ T-cells were quantified and summarised. Data is representative of one experiment (n=3) and shows mean $\pm$ SEM. RM one-way ANOVA with multiple comparisons showing significance of $P \leq 0.05$.

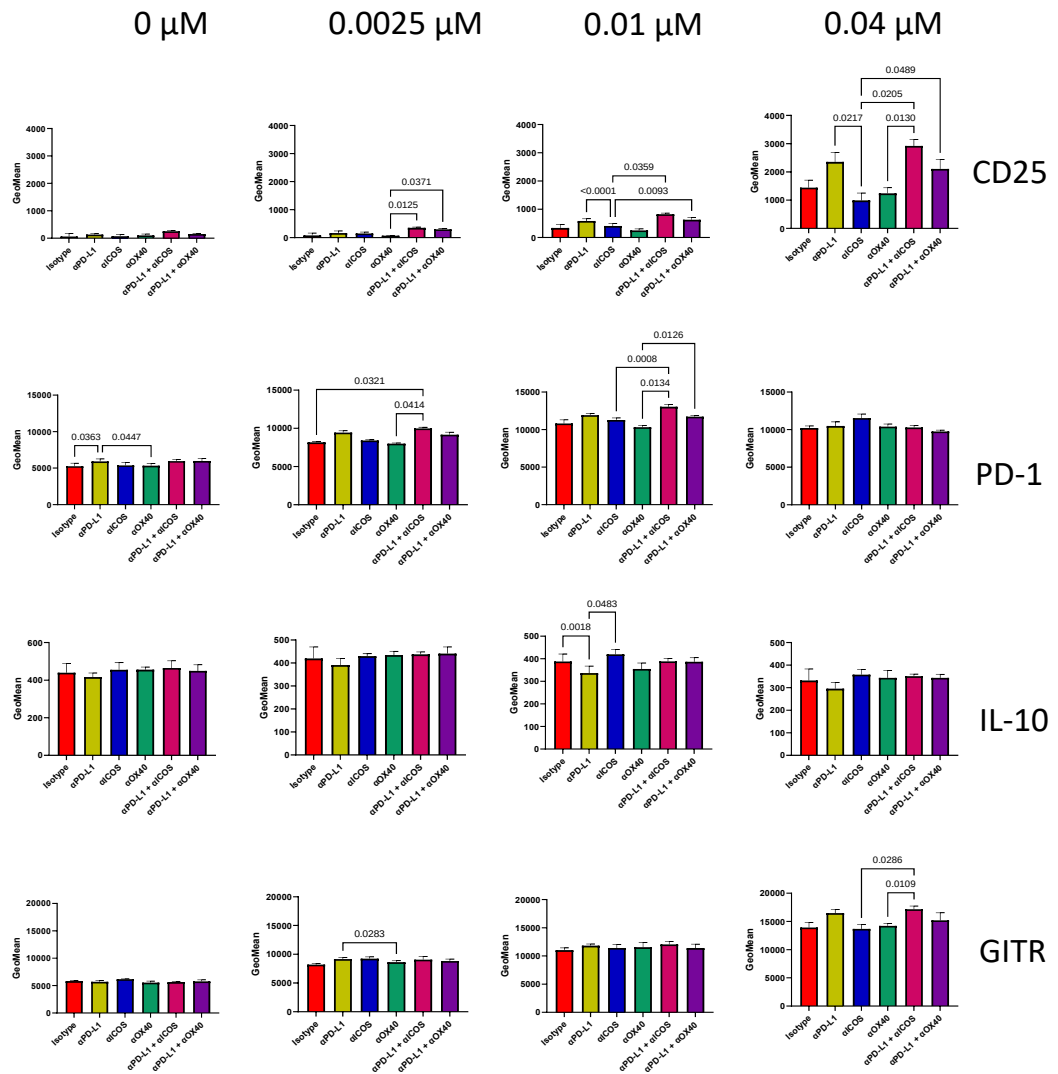


Figure 4.15. *In vivo/in vitro* hybrid platform reveals dose-dependent increased expression of checkpoint markers but limited effects between treatments on responder CD4⁺ T-cells. Tg4 Nr4a3-Tocky mice were injected s.c. with a primary dose of 4 mg/kg 4Y-[MBP] and spleens were harvested 24 hrs later and enzymatically digested for culture *in vitro* with secondary 0-0.04 μ M 4Y-[MBP] as indicated. Geometric mean of CD25, GITR, PD-1, CCR6 and IL-10 on responder CD4⁺ T-cells were quantified and summarised. Data is representative of one experiment (n=3) and shows mean $\pm$ SEM. RM one-way ANOVA with multiple comparisons showing significance of $P \leq 0.05$.

Unfortunately, individual marker summary data shows very limited meaningful differences between the isotype control and treatments within the *in vivo/in vitro* system. Largely, this could be down to the two key readouts of TCR activation, ICOS and OX40, being modulated in this experimental design and proxy readouts are therefore being used. In order to explore this data set in more detail, Uniform Manifold Approximation and Projections (UMAPs) were generated to reveal shared mechanisms of increased activation between the different treatment conditions (Figures 4.16 and 4.17).

Population 'One' is associated with CD4+ T-cells that have responded to the primary *in vivo* 4Y-[MBP] immunisation but not the secondary *in vitro* 4Y-[MBP] dose, as evidenced by increased FT Red expression but decreased FT Blue expression (Figures 4.17.1 and 4.17.2). Populations 'Two' and 'Three' both show populations of CD4+ T-cells that have responded to the primary *in vivo* 4Y-[MBP] immunisation and the secondary *in vitro* 4Y-[MBP] dose, as evidenced by presence of FT Red expression and increased FT Blue expression (Figures 4.17.1 and 4.17.2). Though populations Two and Three are both associated with responder cells, population Three is associated with elevated expression of PD-1, CD25, IL-10 and GITR, suggesting that this population is associated with a markedly stronger response to secondary 4Y-[MBP] compared to population Two (Figures 4.17.3 - 4.17.6).

CD25, commonly associated with regulatory and memory T-cell subsets where it is constitutively expressed, is a prominent marker of activation on T-cells, upregulated within 24 hours of stimulation of the TCR/CD3 complex (Reddy *et al.*, 2004). IL-10, an anti-inflammatory cytokine produced by a variety of cell types including regulatory T-

cells, has also been observed to be associated with activated T-cells (Trinchieri, 2007). GITR, a costimulatory member of the TNF receptor superfamily, is expressed at low levels on unstimulated T-cells and is upregulated upon activation (Muriglan *et al.*, 2004). Additionally, GITR has recently been observed to be elevated in tandem with Nr4a3-Tocky FT Blue expression at 4 hours with α PD-1 treatment *in vivo* (Elliot *et al.*, 2021). Expression of GITR is also associated with regulatory T-cells, having been linked with their expansion and activation (Ronchetti *et al.*, 2015). Therefore, the increased expression of CD25, IL-10 and GITR in population Three could be associated either with a stronger response, or with a regulatory T-cell population (Figure 4.16).

The proportion of CD4⁺ T-cells in population Three increases in a dose-responsive manner for all treatments, with an observed increase in the isotype from 4.43 % in 0 μ M to 31.1 % in 0.04 μ M secondary 4Y-[MBP] treatment (Figure 4.16). Between treatment conditions, at 0.04 μ M secondary 4Y-[MBP] which shows the increased proportion of responder CD4⁺ T-cells, PD-L1 is a promising monotherapy with 36.4 % in population Three compared to the 31.1 % in the isotype control (Figure 4.16). OX40 monotherapy is the most promising monotherapy at 43.6 % and ICOS is the least promising at 30.7 %, showing similar trends to the isotype control. Looking specifically at combination therapies, PD-L1 + ICOS is shows no effect compared to the isotype control at 31.9 %. Additionally, it appears that ICOS in combination with PD-L1 potentially hinders the effectiveness of PD-L1. PD-L1 + OX40 however is a promising combination therapy at 40.1 %, though like OX40 as a monotherapy, combination therapies are of importance to trial as monotherapies have produced limited effects

clinically. PD-L1 + OX40 combination therapy was taken forward to trial *in vivo*, where more pronounced effects have been observed previously, for further exploration.

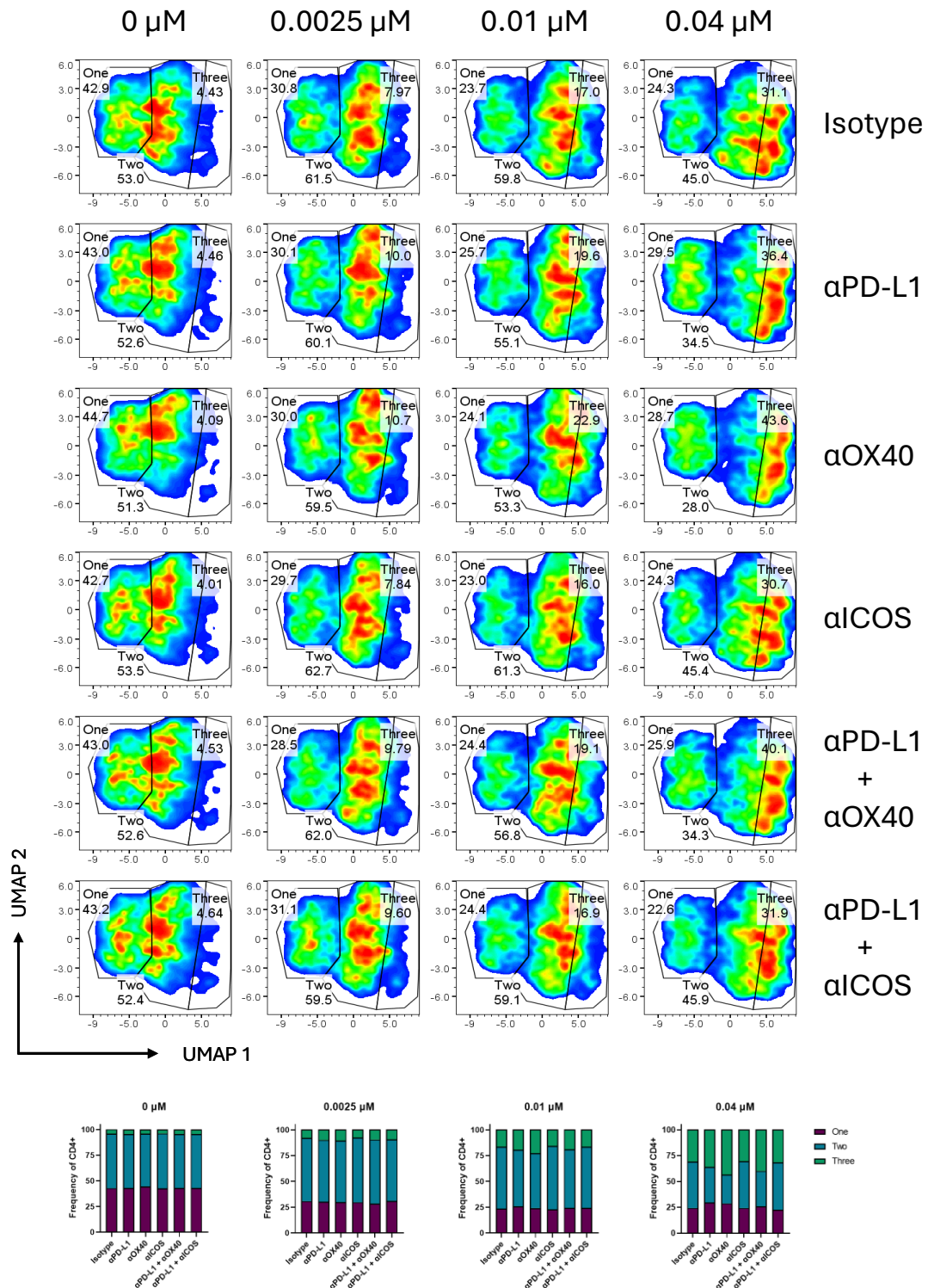


Figure 4.16. *In vivo/in vitro* hybrid platform high parameter analysis reveals shared mechanisms of dose-dependent responses to 4Y-[MBP] treatment and treatment-dependent effects. Tg4 Nr4a3-Tocky mice were injected s.c. with a primary dose of 4 mg/kg 4Y-[MBP] and spleens were harvested 24 hrs later and enzymatically digested for culture *in vitro* with secondary 0-0.04 μM 4Y-[MBP] as indicated. UMAPs were generated with data for all co-inhibitory and co-stimulatory checkpoint markers evaluated (CD25, PD-1, CCR6 and GITR) on the surface of CD4⁺ T cells along with FT Blue/Red and IL-10 reporter expression. Data is combined (n=3) to represent each treatment condition.

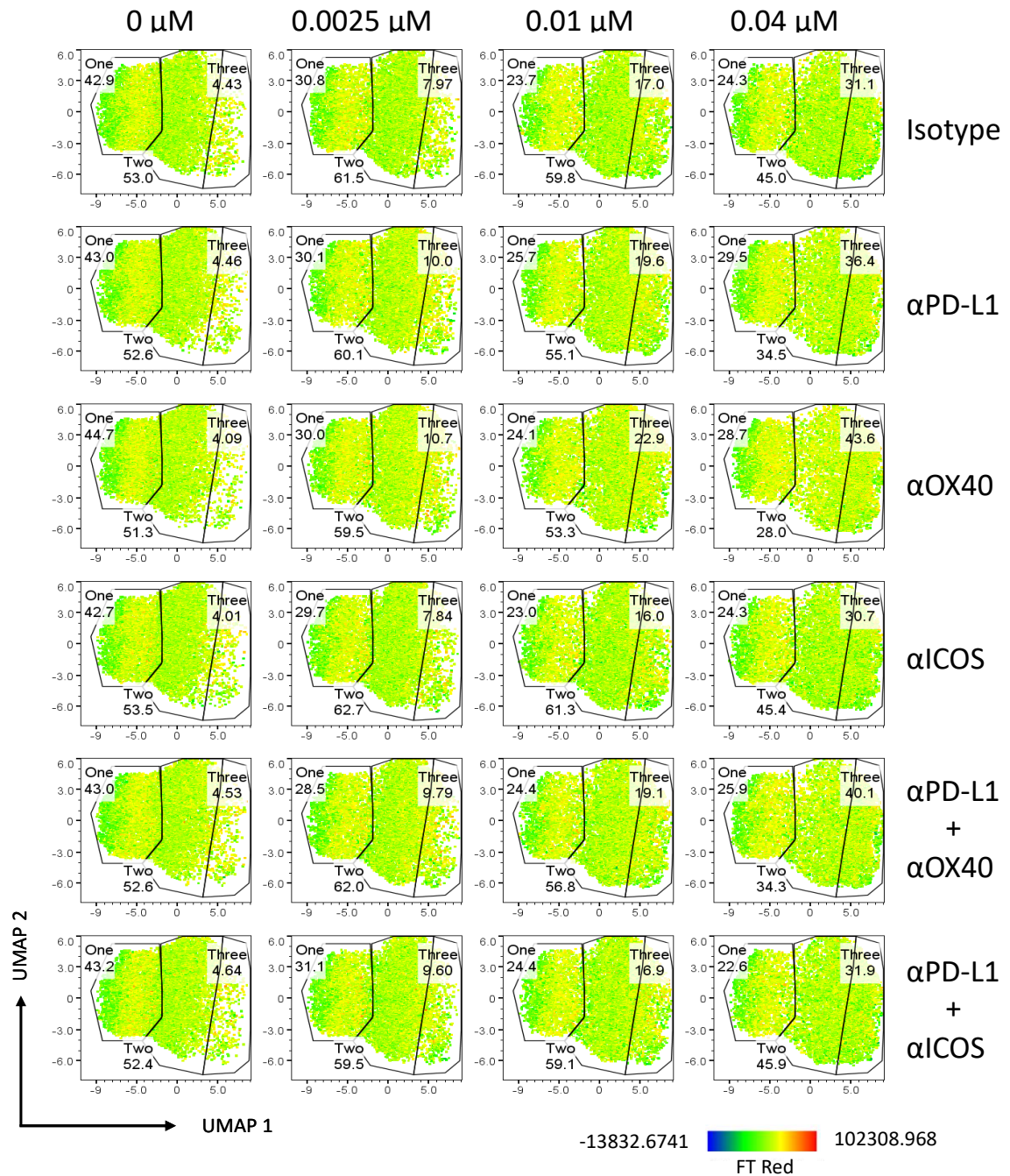


Figure 4.17.1. *In vivo* hybrid platform high parameter analysis reveals shared mechanisms of dose-dependent responses to 4Y-[MBP] treatment and treatment-dependent effects (FT Red expression overlay). Tg4 Nr4a3-Tocky mice were injected s.c. with a primary dose of 4 mg/kg 4Y-[MBP] and spleens were harvested 24 hrs later and enzymatically digested for culture *in vitro* with secondary 0-0.04 μM 4Y-[MBP] as indicated. FT Red expression heatmap was overlaid onto UMAPs generated in Figure 4.16. Data is combined (n=3) to represent each treatment condition.

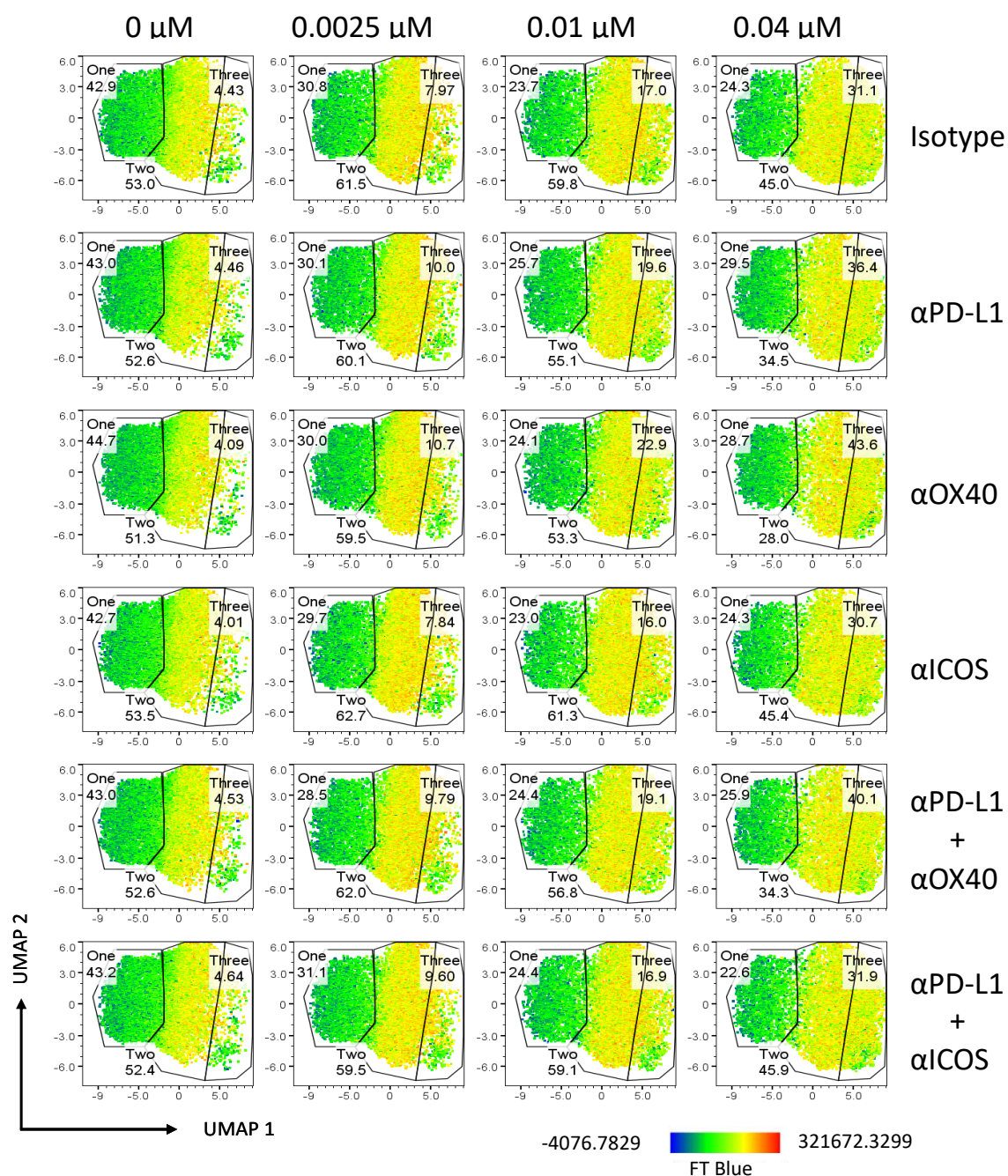


Figure 4.17.2. *In vivo* hybrid platform high parameter analysis reveals shared mechanisms of dose-dependent responses to 4Y-[MBP] treatment and treatment-dependent effects (FT Blue expression overlay). Tg4 Nr4a3-Tocky mice were injected s.c. with a primary dose of 4 mg/kg 4Y-[MBP] and spleens were harvested 24 hrs later and enzymatically digested for culture *in vitro* with secondary 0-0.04 μM 4Y-[MBP] as indicated. FT Blue expression heatmap was overlaid onto UMAPs generated in Figure 4.16. Data is combined (n=3) to represent each treatment condition.

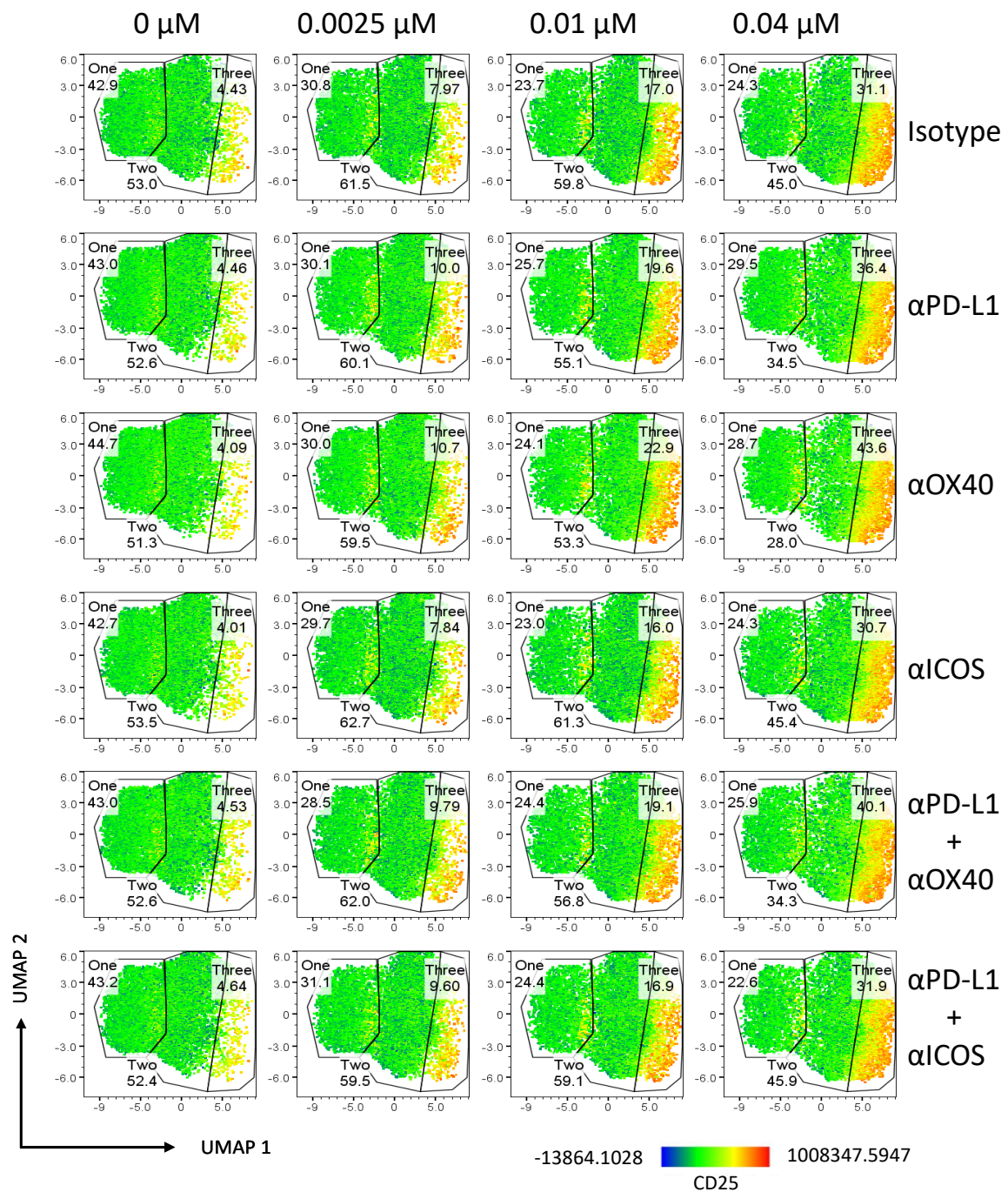


Figure 4.17.3. *In vivo* hybrid platform high parameter analysis reveals shared mechanisms of dose-dependent responses to 4Y-[MBP] treatment and treatment-dependent effects (CD25 expression overlay). Tg4 Nr4a3-Tocky mice were injected s.c. with a primary dose of 4 mg/kg 4Y-[MBP] and spleens were harvested 24 hrs later and enzymatically digested for culture *in vitro* with secondary 0-0.04 μM 4Y-[MBP] as indicated. CD25 expression heatmap was overlayed onto UMAPs generated in Figure 4.16. Data is combined (n=3) to represent each treatment condition.

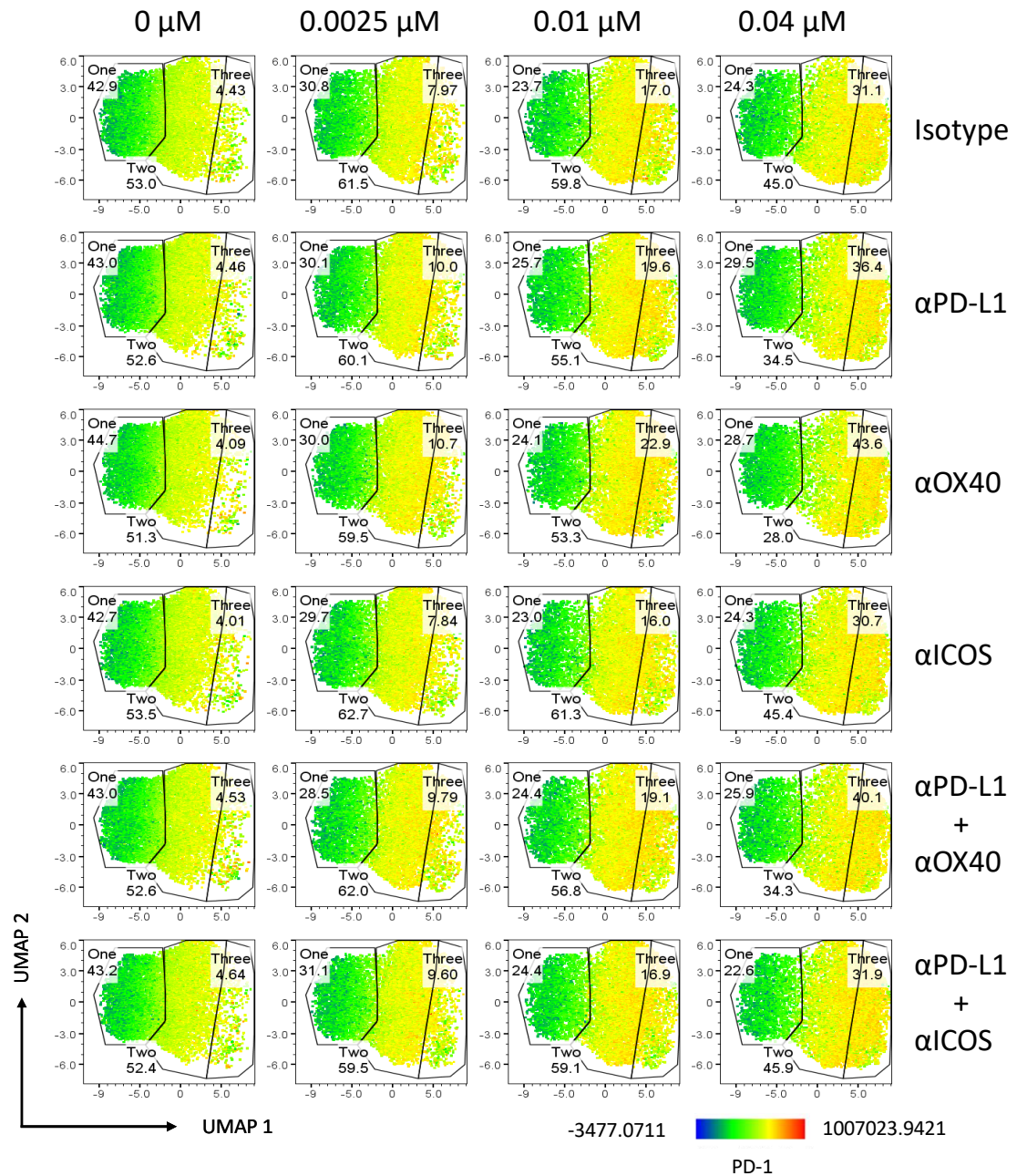


Figure 4.17.4. *In vivo/in vitro* hybrid platform high parameter analysis reveals shared mechanisms of dose-dependent responses to 4Y-[MBP] treatment and treatment-dependent effects (PD-1 expression overlay). Tg4 Nr4a3-Tocky mice were injected s.c. with a primary dose of 4 mg/kg 4Y-[MBP] and spleens were harvested 24 hrs later and enzymatically digested for culture *in vitro* with secondary 0-0.04 μM 4Y-[MBP] as indicated. PD-1 expression heatmap was overlaid onto UMAPs generated in Figure 4.16. Data is combined ($n=3$) to represent each treatment condition.

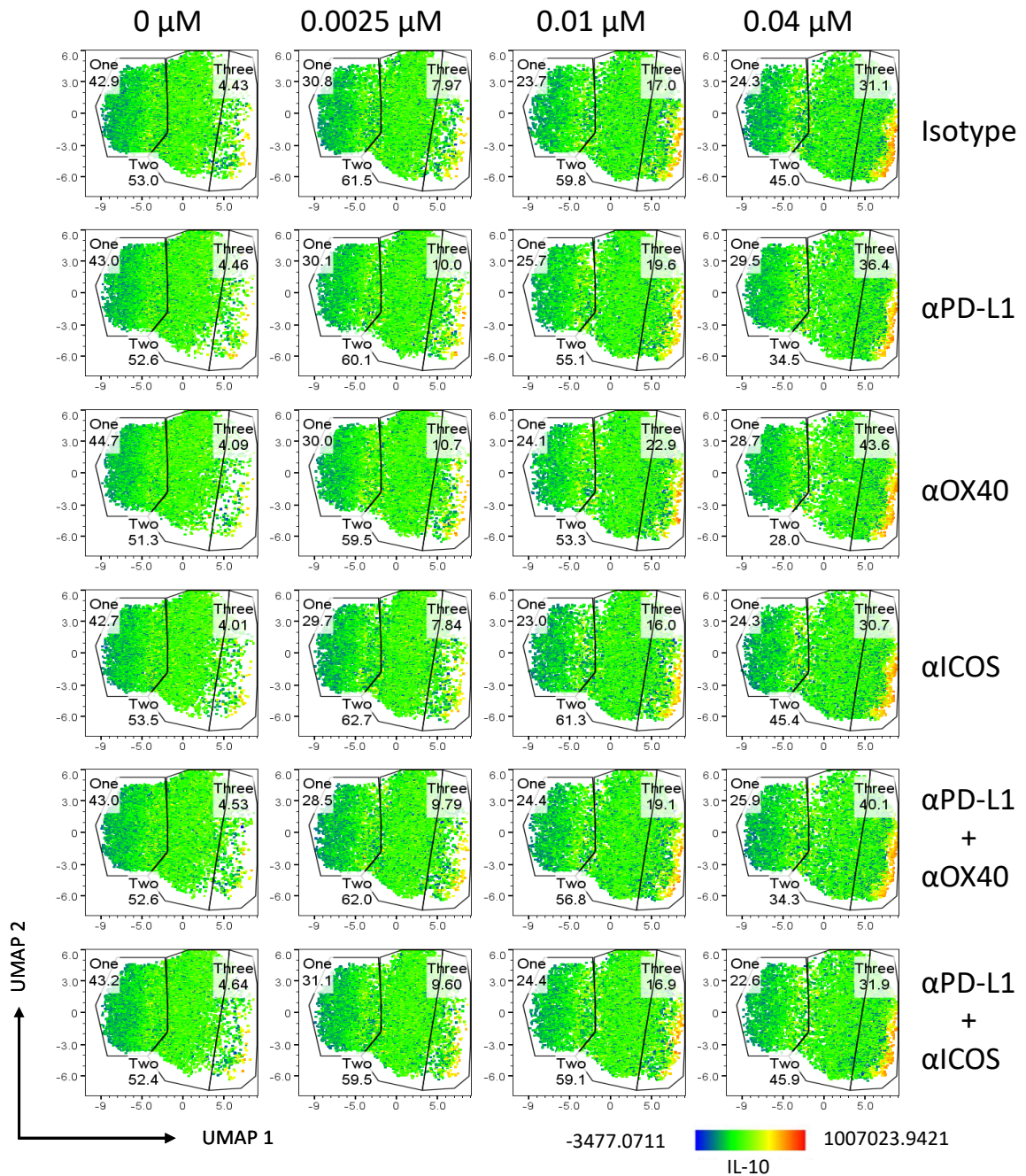


Figure 4.17.5. *In vivo* hybrid platform high parameter analysis reveals shared mechanisms of dose-dependent responses to 4Y-[MBP] treatment and treatment-dependent effects (IL-10 expression overlay). Tg4 Nr4a3-Tocky mice were injected s.c. with a primary dose of 4 mg/kg 4Y-[MBP] and spleens were harvested 24 hrs later and enzymatically digested for culture *in vitro* with secondary 0-0.04 μM 4Y-[MBP] as indicated. IL-10 expression heatmap was overlayed onto UMAPs generated in Figure 4.16. Data is combined (n=3) to represent each treatment condition.

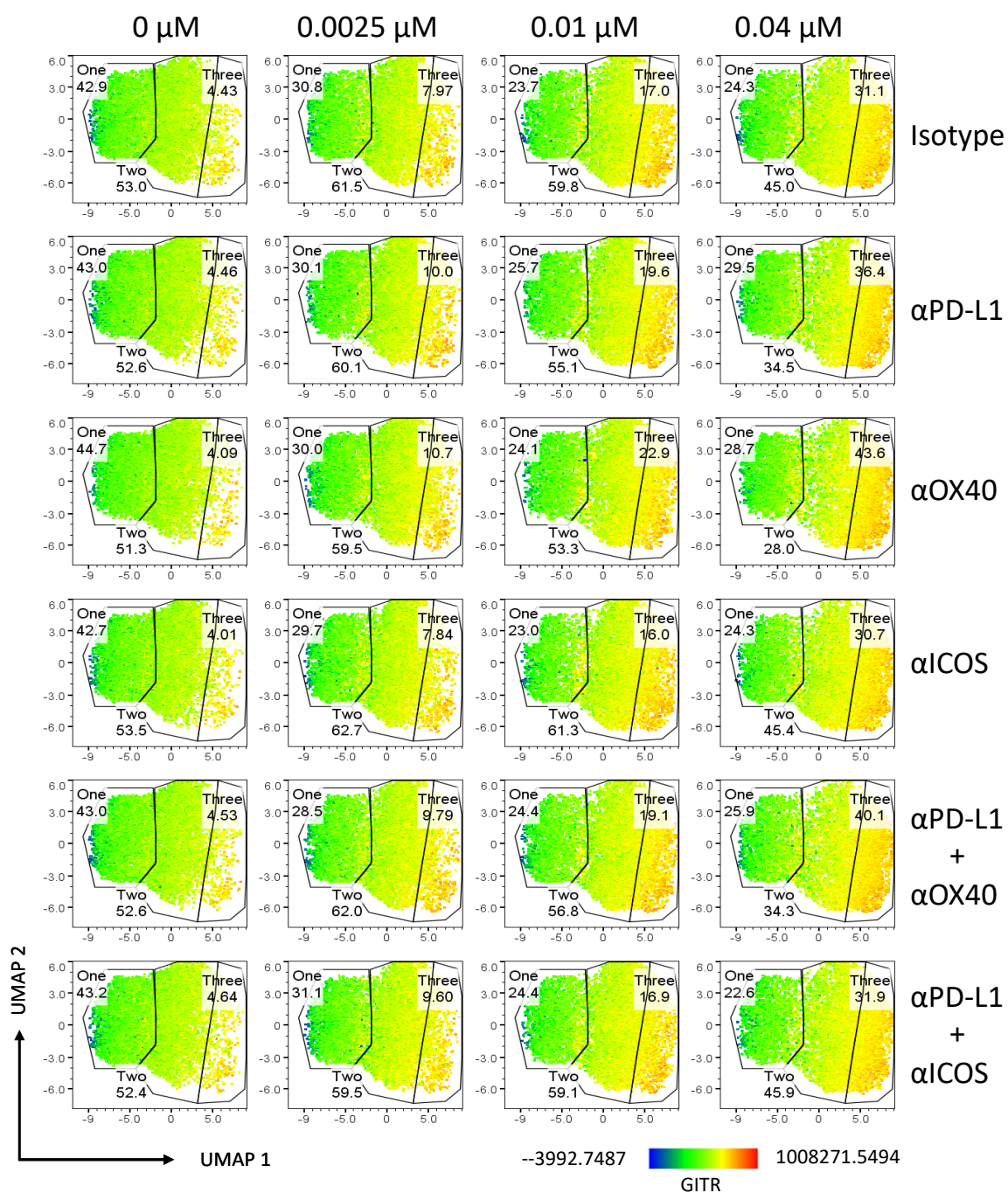


Figure 4.17.6. *In vivo/in vitro* hybrid platform high parameter analysis reveals shared mechanisms of dose-dependent responses to 4Y-[MBP] treatment and treatment-dependent effects (GTR expression overlay). Tg4 Nr4a3-Tocky mice were injected s.c. with a primary dose of 4 mg/kg 4Y-[MBP] and spleens were harvested 24 hrs later and enzymatically digested for culture *in vitro* with secondary 0-0.04 μM 4Y-[MBP] as indicated. GTR expression heatmap was overlaid onto UMAPs generated in Figure 4.16. Data is combined ($n=3$) to represent each treatment condition.

4.2.6 Immunisation of α PD-L1 + α OX40 boosts CD4⁺ responding T-cell populations *in vivo*

In vitro models likely do not capture the dynamic interactions of immune cells *in vivo* and the fluctuating levels of peptide. Given that PD-L1 in combination with agonistic OX40 showed more promise than PD-L1 and agonistic ICOS, this combination was taken forward for *in vivo* analysis in the accelerated adaptive tolerance model (Elliot *et al.*, 2021; section 4.2.3). Tg4 Nr4a3-Tocky mice were immunised with 4 mg/kg primary dose of 4Y-[MBP]. 24 hours later, mice were treated with IgG, α PD-L1, α OX40 or a combination of α PD-L1 + α OX40 before being rechallenged 30 mins later with a ten-fold lower dose of 4Y-[MBP] (0.4 mg/kg) as illustrated in Figure 4.18A. The 30 minute gap allows the circulation of blocking antibodies before re-immunisation with peptide.

In order to maximise the capture of phenotypic differences, the cells were analysed by spectral flow cytometry. α PD-L1 and α OX40 monotherapies both showed promising results, with average responder populations (Q2; Figure 4.18B) increasing from 6.5 % for the isotype, to 14.3 % and 14.4 % for α PD-L1 and α OX40 monotherapies, respectively. Combination therapy is the most promising, with an observed average Q2 responder population of 23.2 %. Summary data (Figure 4.18C) shows significant differences are observed between the isotype control and all treatment conditions evaluated, with the combination treatment showing the most striking differences. Interestingly, there is no observed difference between α PD-L1 and α OX40 monotherapies (Figure 4.18C), but there was a clear *in vivo* additive effect for combination treatment over individual monotherapies for the proportion of responder T cells.

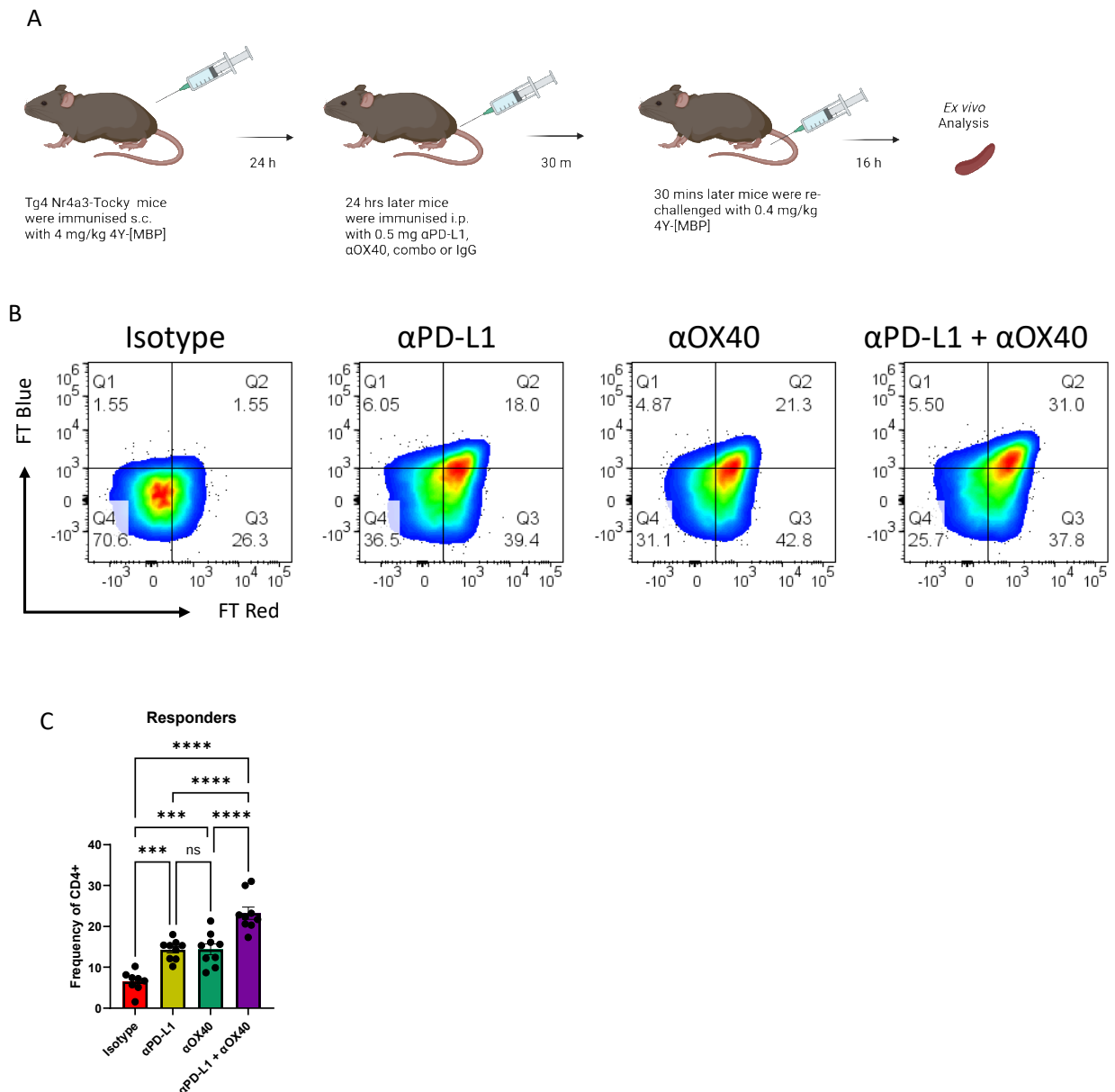


Figure 4.18. *In vivo* αPD-L1 blockade in combination with agonistic OX40 treatment induces a favourable population of responder CD4⁺ T-cells. Tg4 Nr4a3-Tocky mice were immunised s.c. with a primary dose of 4 mg/kg 4Y-[MBP]. 24 hours later, mice were immunised i.p. with isotype control, 0.5 mg αPD-L1, 0.25 mg αOX40 or a combination of αPD-L1 and αOX40 as indicated. 30 minutes later mice were re-challenged with 0.4 mg/kg secondary 4Y-[MBP] before ex vivo analysis 16 hours later. **A.** Schematic overview of experimental design. **B.** Flow cytometric plots of CD4⁺ FT Blue FT Red expression with responders defined as FT Blue⁺ FT Red⁺ (Q2). **C.** Summary of responder CD4⁺ populations. Data pooled from two independent experiments (Isotype, n=8; PD-L1, n=9; OX40, n=9 and PD-L1+OX40, n=10 cumulative). Summary data shows mean ± SEM. Ordinary one-way ANOVA with multiple comparisons showing significance of P≤0.05.

Combination therapy drove a quantitative increase in responding T cells. Next, the qualitative changes in the responding T cells were evaluated. To further evaluate the responder populations observed to increase in response to ICM, geometric means of co-stimulatory and co-inhibitory markers were evaluated on the surface of CD4⁺ (Figures 4.19A and 4.20A) and Q2 responder CD4⁺ (Figures 4.19B and 4.20B) T-cells. Expression of ICOS was observed to increase on both CD4⁺ and Q2 responder CD4⁺ T-cells in response to α PD-L1 monotherapy, or α PD-L1 + α OX40 combination therapy, but not in response to α OX40 alone. Thus, suggesting the two combination treatments act via differential signalling pathways, despite their similar trend in responder populations observed in Figure 4.18. In a similar manner to ICOS expression, PD-1 expression was also significantly increased on the surface of CD4⁺ and Q2 responder CD4⁺ T-cells in response to α PD-L1 monotherapy or α PD-L1 + α OX40 combination therapy, but not in response to α OX40 monotherapy. This finding further corroborates the potential differential mechanisms of action between the two treatment pathways, highlighting that ICOS and PD-1 are both sensitive to PD-L1 blockade. This finding could reflect increased activation of the NFAT pathway observed with α PD-L1 treatment.

TIGIT expression on CD4⁺ T-cells is only significantly elevated in response to combination treatment with α PD-L1 + α OX40, however, on responder CD4⁺ T-cells, α PD-L1 monotherapy also induces significant increased expression. This finding suggests that TIGIT has potential to be driven by PD-L1 in responder CD4⁺ T-cells, but additive effects of the combination treatment are observed at a bulk CD4⁺ population level.

CD25 expression is significantly increased on the surface of CD4⁺ and responder CD4⁺ T-cells in response to combination treatment but is not significant not in response to either monotherapy, suggesting that CD25 is not driven by PD-L1 blockade or OX40 agonist but rather an additive effect is observed when both pathways are targeted. Unpublished data from the Bending lab at the time these experiments were performed had suggested that CCR6 is sensitive to α PD-L1 blockade *in vivo*, therefore its expression was characterised in response to α PD-L1 + α OX40 here. CCR6 shows an increased expression for α PD-L1 and α OX40 monotherapies, with a greater significant increase in response to combination therapy. Whilst GITR expression is only significantly increased in response to α PD-L1 + α OX40 combination treatment, there is an observed trend of increase for both monotherapies. The lack of significance for these treatments is likely explained by the differential geometric mean observed between the two combined independent experiments. The same is likely said for LAG-3 expression, where no treatments were seen to be significantly different from the isotype control, despite an observed increased trend between all treatments and the isotype control, with the combination treatment.

In brief, the results in this section indicate that PD-1 and ICOS expression are likely regulated by PD-L1 signalling, whilst GITR, CD25 and CCR6 are likely sensitive to both PD-L1 and OX40 signalling pathways.

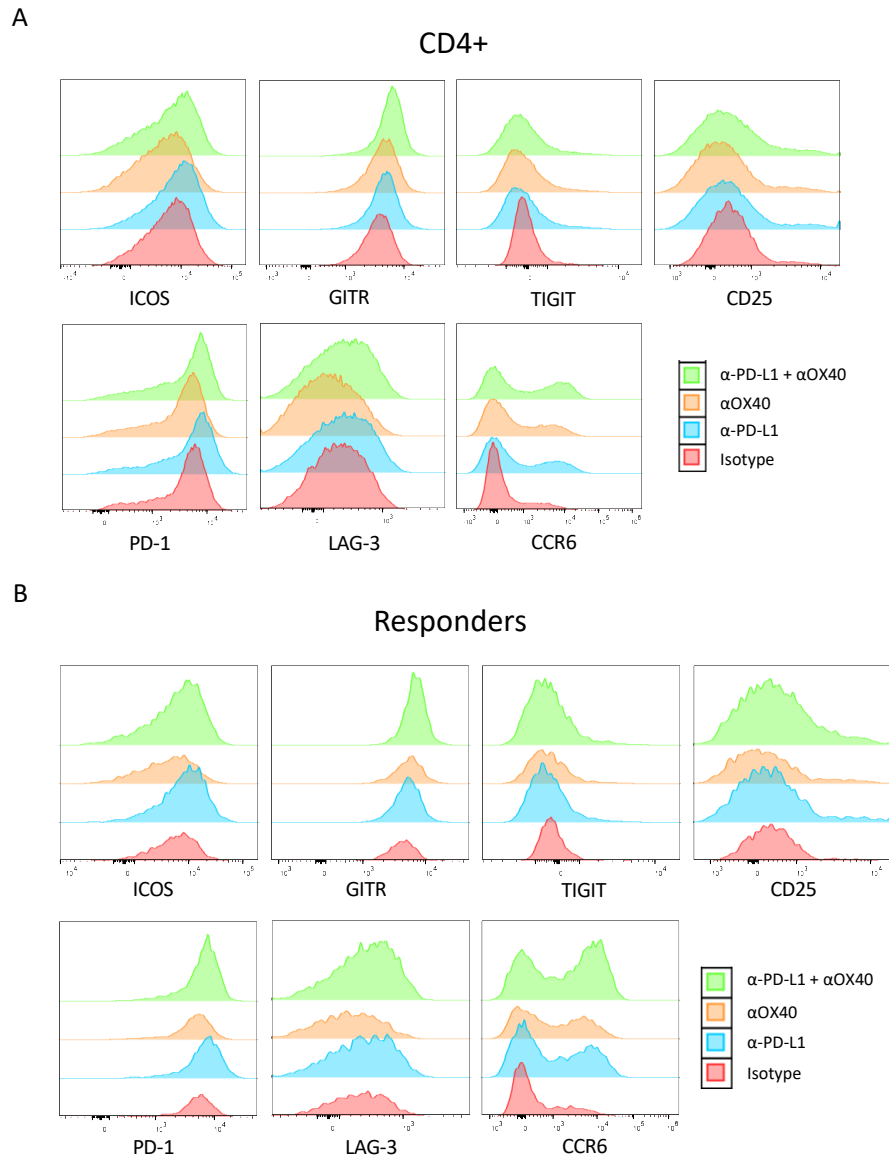


Figure 4.19. *In vivo* αPD-L1 blockade in combination with agonistic OX40 treatment induces expression of co-inhibitory and co-stimulatory receptors on the surface of CD4+ T-cells. Tg4 Nr4a3-Tocky mice were immunised with 4Y-[MBP] ± ICM modulations as in Figure 4.18A. **A.** Flow cytometric histograms of co-stimulatory and co-inhibitory receptor expression on the surface of CD4+ populations. **B.** Flow cytometric histograms of co-stimulatory and co-inhibitory receptor expression on the surface of FT Blue+ responder CD4+ populations from Figure 4.19. Data representative of one mouse for each marker.

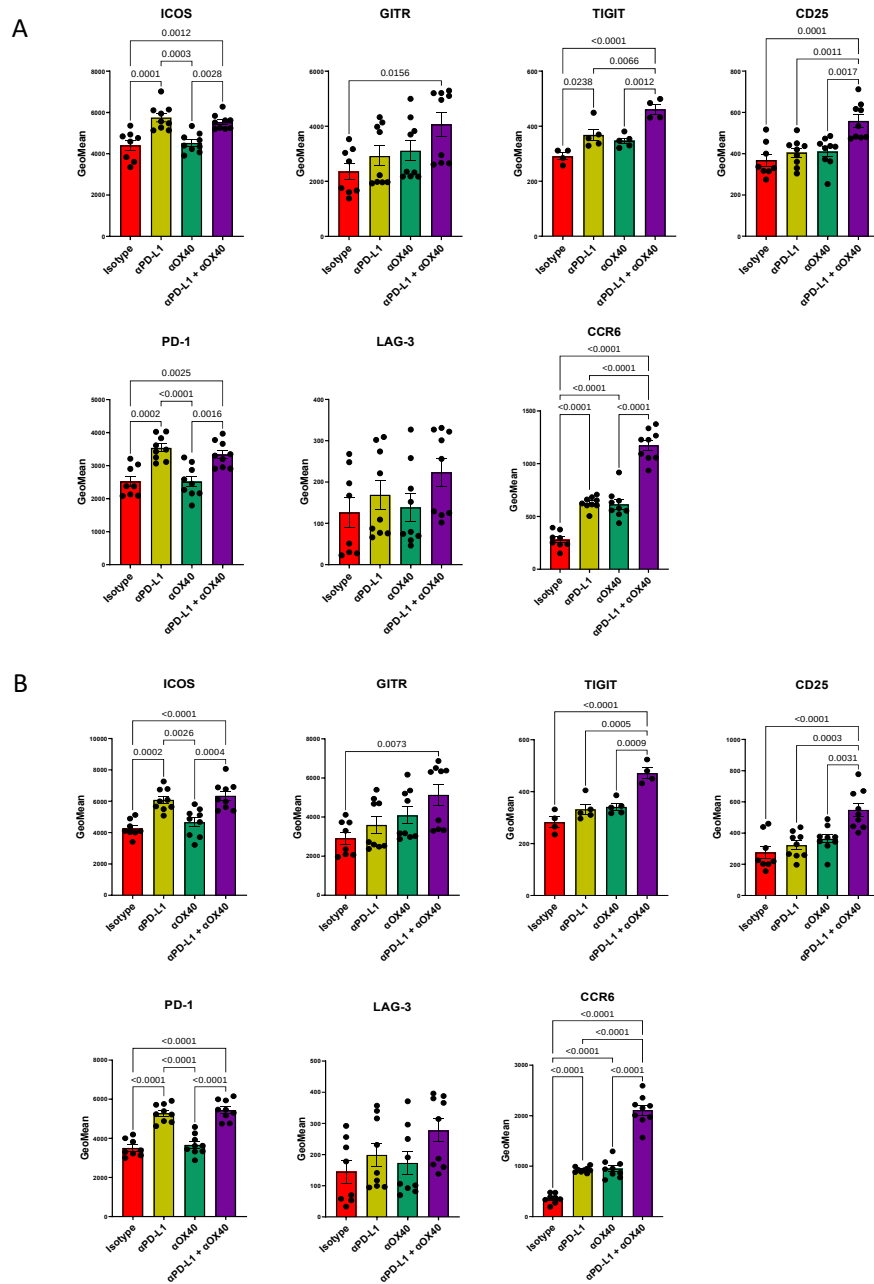


Figure 4.20. *In vivo* α PD-L1 blockade in combination with agonistic OX40 treatment induces expression of co-inhibitory and co-stimulatory receptors on the surface of CD4⁺ T-cells. Tg4 Nr4a3-Tocky mice were immunised with 4Y-[MBP] $\pm$ ICM modulations as in Figure 4.18A. **A.** Summary of co-stimulatory and co-inhibitory receptor expression on the surface of CD4⁺ populations. **B.** Summary of co-stimulatory and co-inhibitory receptor expression on the surface of FT Blue+ FT Red+ responder CD4⁺ populations from Figure 4.19. Data pooled from two independent experiments (Isotype, n=8; PD-L1, n=9; OX40, n=9 and PD-L1+OX40, n=10 cumulative). Summary data shows mean $\pm$ SEM. Ordinary one-way ANOVA with multiple comparisons showing significance of $P \leq 0.05$.

Again, to reduce the dimensionality of the dataset, UMAPs were generated from the panel of co-inhibitory and co-stimulatory receptors studied, alongside their expression for FT Blue and FT Red (Figures 4.21 – 4.23). In these UMAPs, population One is associated with CD4⁺ T-cells that have responded to the primary and secondary doses of 4Y-[MBP], as evidenced by the increased expression of several markers (CCR6, PD-1, CD25, TIGIT, GITR and ICOS; Figures 4.22 and 4.23). Population Two is likely the population of non-responders, with low observed expression of all markers evaluated. Population Three is likely the population of CD4⁺ T-cells that have responded to the primary immunisation of 4Y-[MBP], but not the secondary dose, evidence by expression of the early activation marker PD-1, but not CCR6. Population Four has observed high expression of ICOS, CD25 and GITR, but has low expression of PD-1. There is potential that population four is associated with Type 1 regulatory T-cells (as it contained IL10⁺ T cells, data not shown), but further phenotypic characterisation would be necessary to confirm this in the future.

Between treatments, population One significantly increases for both α PD-L1 (38.5 %) and α OX40 (36.4 %) monotherapies compared with the isotype control (19.3 %), but the most prominent difference is observed between the isotype control and α PD-L1 + α OX40 (46.8 %) combination therapy (Figures 4.21B and 4.21D). As population One is associated with increased expression of activation markers, it follows that α PD-L1 + α OX40 combination therapy induces a favourable T-cell response compared to the isotype control. This increased responder population (One), is also coupled with a decreased proportion of cells which have not responded to the peptide re-challenge (Two). This observed decrease, from 44.9 % in the isotype to 25.4 % in the combination treatment, agrees with the previous notion that α PD-L1 + α OX40 combination

treatment reduces the activation threshold of TCR-mediated activation and invigorates an increased proportion of T-cell response.

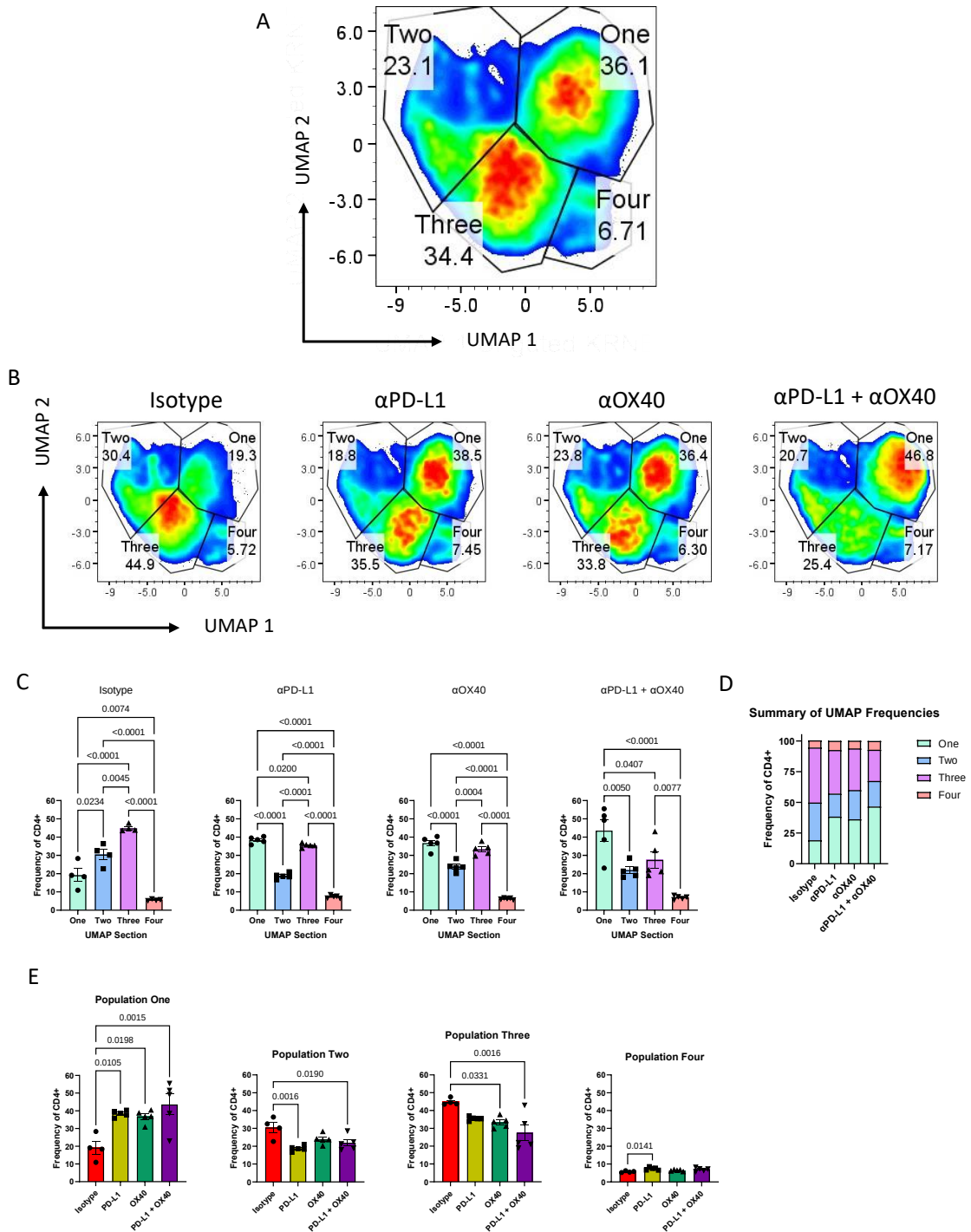


Figure 4.21. *In vivo* αPD-L1 blockade in combination with agonistic OX40 treatment increases proportions of a favourable responder population. Tg4 Nr4a3-Tocky mice were immunised with 4Y-[MBP] ± ICM modulations as in Figure 4.18A. **A.** UMAPs were generated with data for all co-inhibitory and co-stimulatory checkpoint markers evaluated (ICOS, GITR, TIGIT, CD25, PD-1, LAG-3 and CCR6) on the surface of CD4⁺ T cells along with FT Blue/Red expression. Data is representative of one experiment combined (Isotype, n=4; PD-L1, n=5; OX40, n=4 and PD-L1+OX40, n=5) to represent all treatment conditions. **B.** UMAPs were combined to represent each treatment condition in A. **C.** Summary data of quantified populations One-Four from individual treatment UMAPs combined in B and shows mean ± SEM. Ordinary one-way ANOVA with multiple comparisons showing significance of P≤0.05.

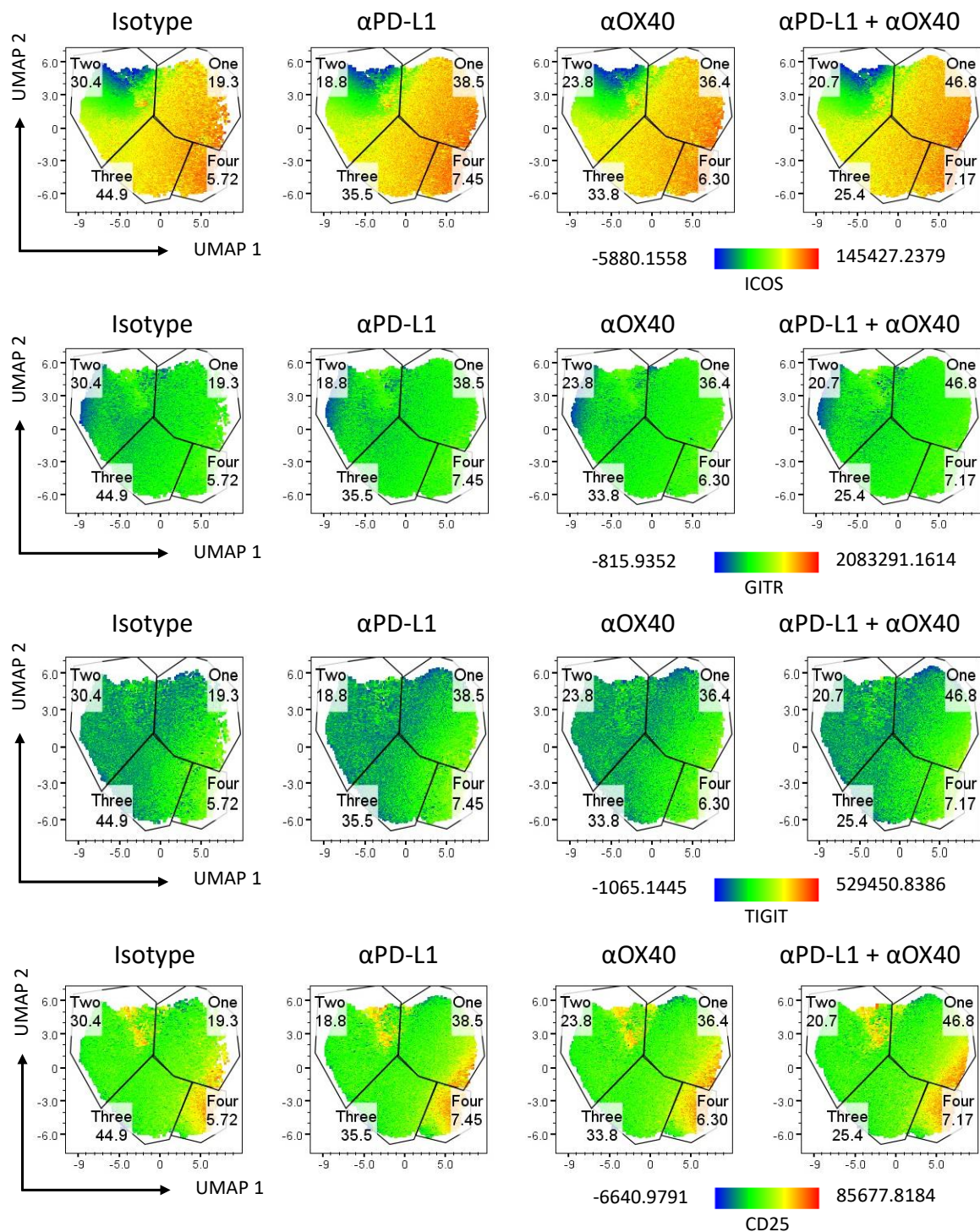


Figure 4.22. *In vivo* α PD-L1 blockade in combination with agonistic OX40 treatment increases proportions of a favourable responder population. Tg4 Nr4a3-Tocky mice were immunised with 4Y-[MBP] $\pm$ ICM modulations as in Figure 4.18A. **A.** UMAPs were generated with data for all co-inhibitory and co-stimulatory checkpoint markers evaluated (ICOS, GITR, TIGIT, CD25, PD-1, LAG-3 and CCR6) on the surface of CD4⁺ T cells along with FT Blue/Red expression. Data is representative of one experiment combined (Isotype, n=4; PD-L1, n=5; OX40, n=4 and PD-L1+OX40, n=5) to represent all treatment conditions as indicated. ICOS, GITR, TIGIT and CD25 expression heatmaps overlaid.

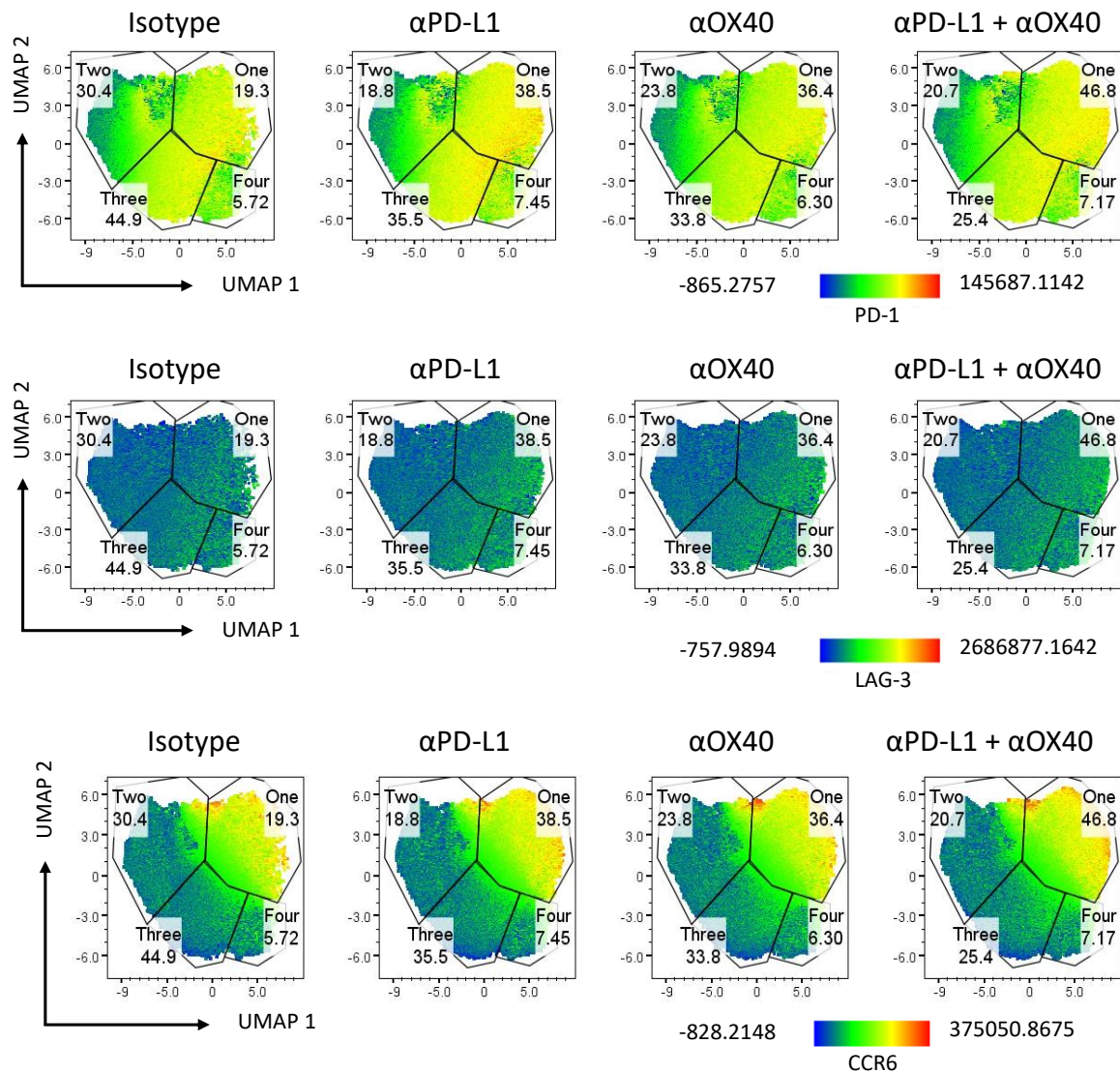


Figure 4.23. *In vivo* α PD-L1 blockade in combination with agonistic OX40 treatment increases proportions of a favourable responder population. Tg4 Nr4a3-Tocky mice were immunised with 4Y-[MBP] $\pm$ ICM modulations as in Figure 4.18A. **A.** UMAPs were generated with data for all co-inhibitory and co-stimulatory checkpoint markers evaluated (ICOS, GITR, TIGIT, CD25, PD-1, LAG-3 and CCR6) on the surface of CD4⁺ T cells along with FT Blue/Red expression. Data is representative of one experiment combined (Isotype, n=4; PD-L1, n=5; OX40, n=5 and PD-L1+OX40, n=4) to represent all treatment conditions as indicated. PD-1, LAG-3 and CCR6 expression heatmaps overlayed.

Row Z-scores were utilised to scale the collected data and a heatmap was generated to visualise the clusters of markers with trends in expression between the four treatment groups as indicated (Figure 4.24). Upregulated (Red) and downregulated (Blue) markers are illustrated and clusters form for each treatment group, thus highlighting shared response profiles. Interestingly, ICOS and PD-1 expression appears to be driven by α PD-L1 monotherapy, with α OX40 alone having relatively low expression for the two markers. α OX40 monotherapy on the other hand, slightly elevates FT expression, GITR and CD25; which are not upregulated by α PD-L1 alone. These findings further highlight the different mechanisms of action between the two monotherapies. Combination therapy of α PD-L1 + α OX40 greatly increases the expression of markers evaluated, to a much greater extent than either monotherapy. Thus, suggesting that the combination therapy produces additive effects for some markers (TIGIT, CD25, LAG3, CCR6, GITR, Nr4a3) but no further effect for others (PD-1, ICOS).

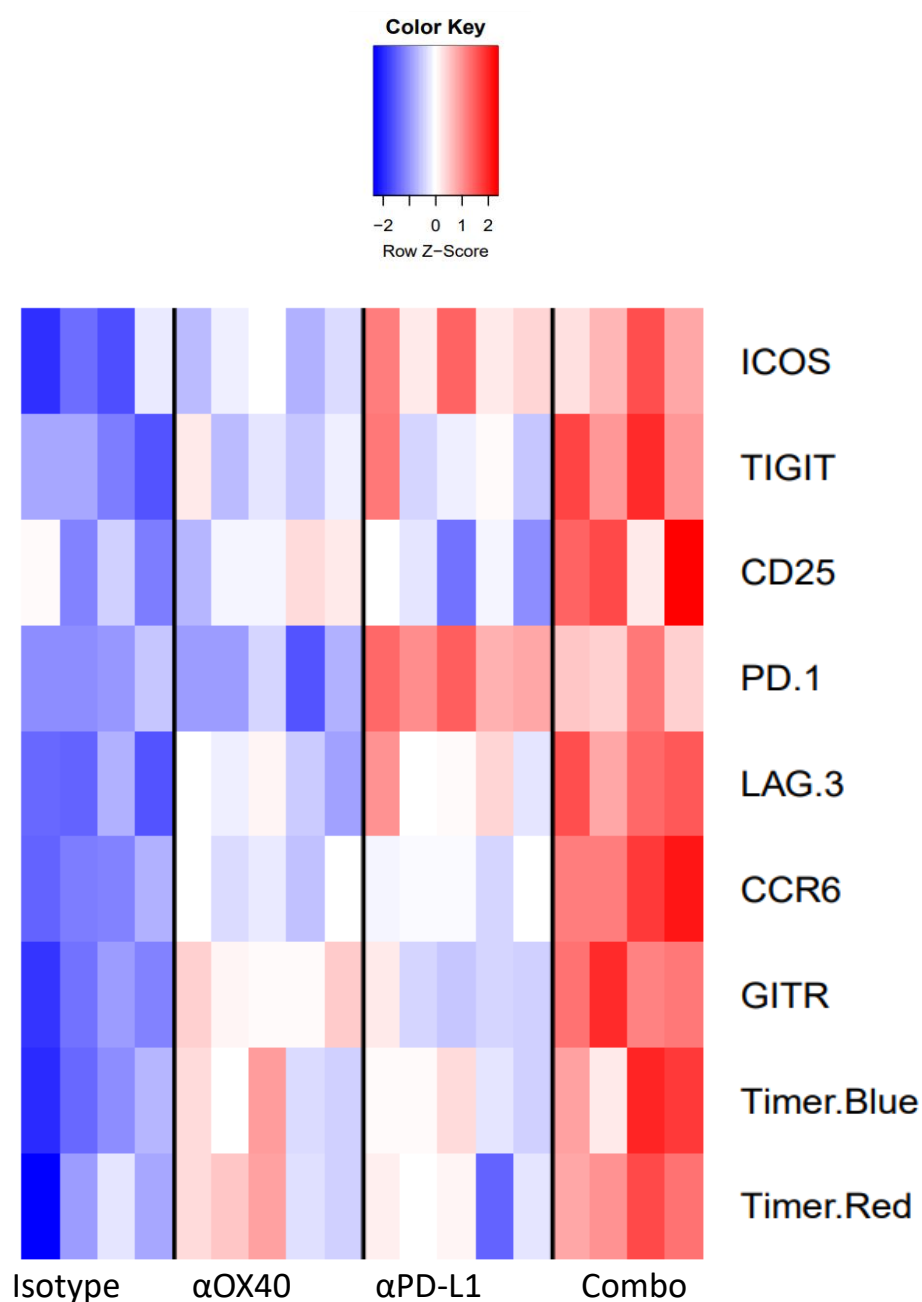


Figure 4.24. *In vivo* αPD-L1 blockade in combination with agonistic OX40 treatment increases expression of co-inhibitory and co-stimulatory markers on the surface of CD4⁺ T-cells. Tg4 Nr4a3-Tocky mice were immunised with 4Y-[MBP] ± ICM modulations as in Figure 4.18A. Geometric mean of co-stimulatory and co-inhibitory markers were analysed and a heatmap for each mouse plotted. Data generated from one of two independent experiments (Isotype, n=4; PD-L1, n=5; OX40, n=5 and PD-L1+OX40, n=4).

4.2.7 *In vivo* αPD-L1 + αOX40 induces anti-tumour effects in MC38-C57BL/6 syngeneic model

These findings showed that αPD-L1 + αOX40 combination therapy induced a population of CD4⁺ T-cells that were able to respond more favourably to peptide immunisation. To evaluate the effectiveness of this combination therapy in the treatment of cancer, a syngeneic subcutaneous MC38-C57BL/6 model was utilised (Figure 4.25A). C57BL/6 mice were implanted subcutaneously with 250k MC38 cells as described in Figure 4.25A. Mice were injected i.p. with IgG or ICM combinations on days 7, 9 and 11 and tumours were allowed to grow until protocol endpoints (12mm diameter, ulceration or day 25). In addition to evaluating the effects of αOX40 in combination with αPD-L1, αICOS was also explored *in vivo* due to previous differences observed *in vitro* compared with *in vivo*.

When looking at the mean tumour volumes (Figure 4.25B), αPD-L1 + αOX40 combination therapy is a promising treatment in this tumour model, compared to monotherapies – a finding congruent with other studies (Braeckel-Budimir *et al.*, 2021 and van der Sluis *et al.*, 2023). αPD-L1 is the most promising monotherapy, though αOX40 alone also reduced tumour volume compared to the isotype. Interestingly, αICOS monotherapy has a greater mean tumour volume compared to the isotype control, whilst the combination of αICOS with αPD-L1 is suggested to reverse the effects observed in αPD-L1 monotherapy, a finding consistent with the previous *in vitro* studies. This is reflected in the survival curves, with αICOS monotherapy and αPD-L1 + αICOS combination both having a survival rate of 10 % (Figures 4.25C and 4.26).

The Kaplan-Meier survival curves show that α PD-L1 monotherapy conveys 50 % survival compared with isotype at 20 % (Figure 4.25C and 4.26). Despite α PD-L1 and α OX40 monotherapies having similar response rates in previous findings, here, α OX40 monotherapy has 20 % survival, the same as the isotype control and much less than α PD-L1 monotherapy. The combination of α PD-L1 + α OX40 has a survival rate of 80 %, the best of all treatments explored.

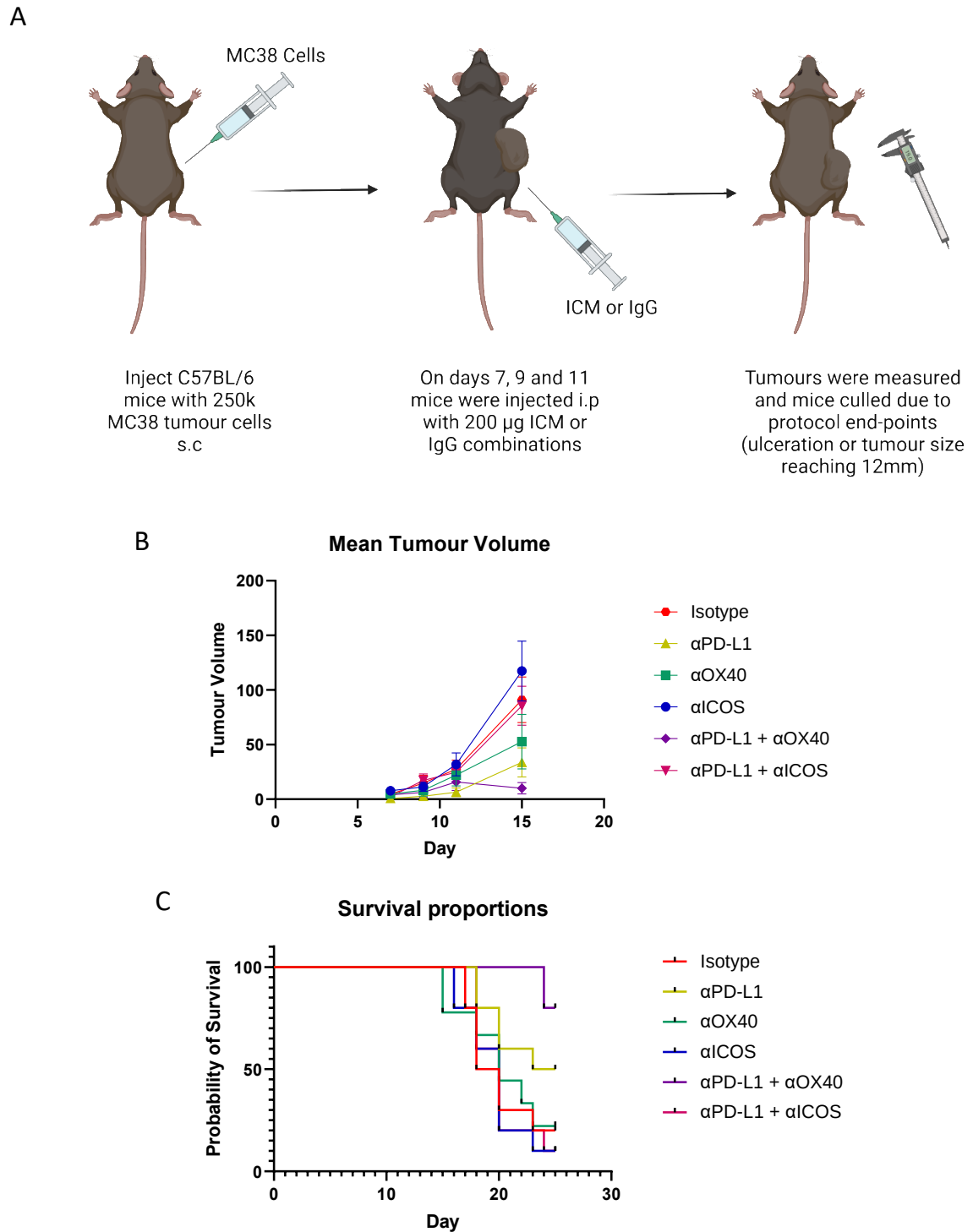


Figure 4.25. *In vivo* α PD-L1 blockade in combination with agonistic OX40 treatment shows favourable survival in syngeneic MC38-subcutaneous tumour model. C57BL/6 mice were implanted s.c. with 250k MC38 tumour cells. Mice were injected i.p. on days 7, 9 and 11 with Isotype controls or ICM treatments as indicated. Tumour measurements were recorded and culled according to human protocol endpoints (12mm diameter or ulceration). **A.** Schematic overview of experimental protocol. **B.** Mean tumour volume (mm^3) up to day 15. **C.** Kaplan-Meier survival curve. Data is representative of one experiment ($n=10$ per group). Summary data in B is mean $\pm$ SEM and profiles data up to day 15 before any mice were sacrificed for reaching their humane end points.

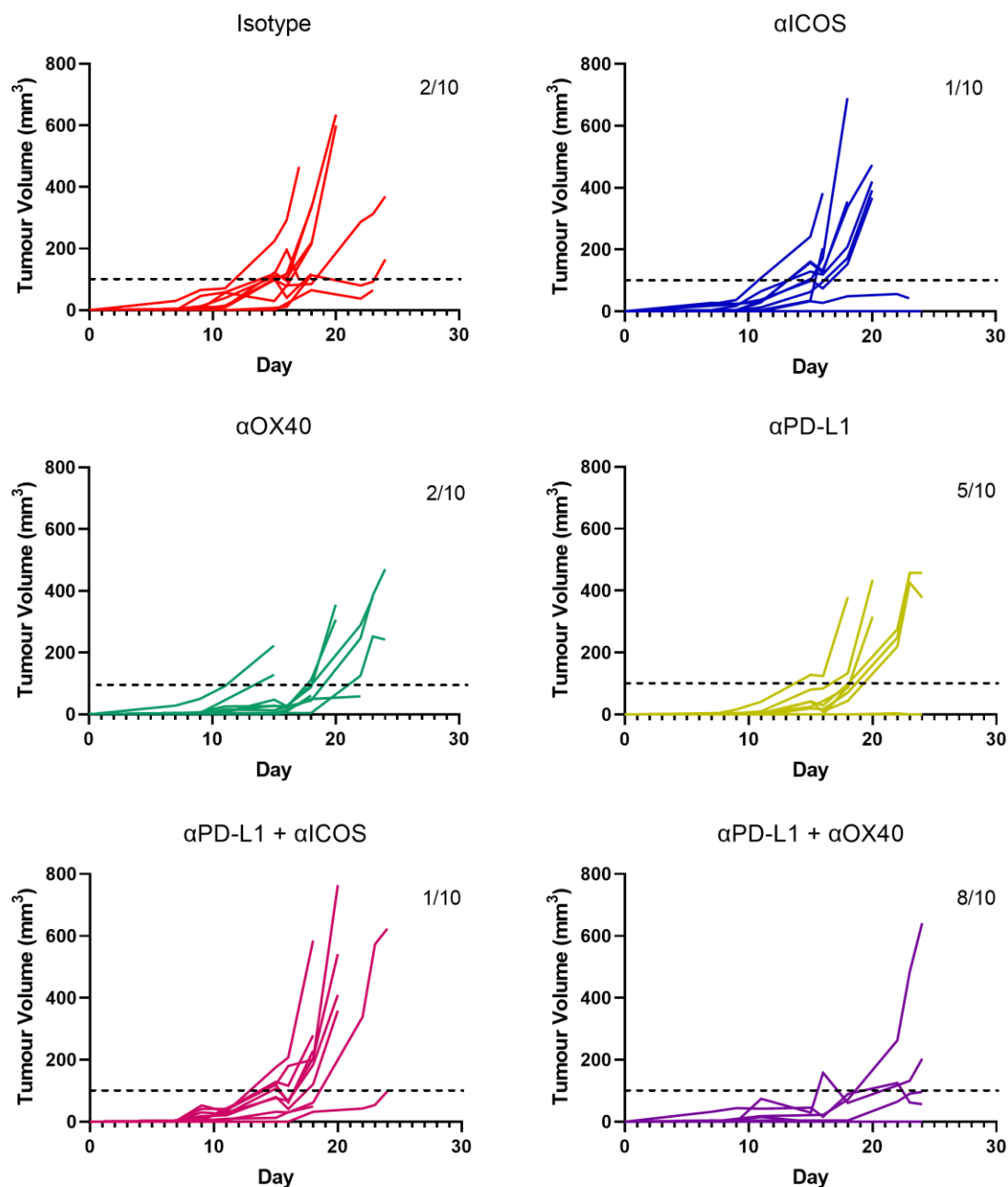


Figure 4.26. *In vivo* αPD-L1 blockade in combination with agonistic OX40 treatment shows favourable survival in syngeneic MC38-subcutaneous tumour model. C57BL/6 mice were implanted s.c. with 250k MC38 tumour cells and subjected to ICM treatments as in Figure 4.25A. **A.** Individual tumour volume until humane endpoint reached. Data is representative of one experiment (n=10 per group). Proportion of survivors indicated throughout (n/10). Dotted line represents a tumour size of 100 mm³ and visually demonstrates the smaller tumours.

4.2.8 α PD-L1 + α OX40 combination therapy increases CD45⁺ CD4⁺ T-cells in tumours

To further evaluate the immunological mechanisms behind the observed anti-tumour effects of α PD-L1 + α OX40 combination therapy, the same syngeneic subcutaneous MC38-C57BL/6 tumour model with IgG or ICM treatments at days 7,9 or 11 was utilised, with immunophenotyping employed on day 14 by spectral cytometry (Figure 4.27A). There was a noticeable increase in the fraction of CD45⁺ TCR β ⁺ CD4⁺ T-cells within the tumour in response to the combination therapy (12.6 %), compared to the isotype control (2.97 %) or either monotherapy (Figure 4.27B,C).

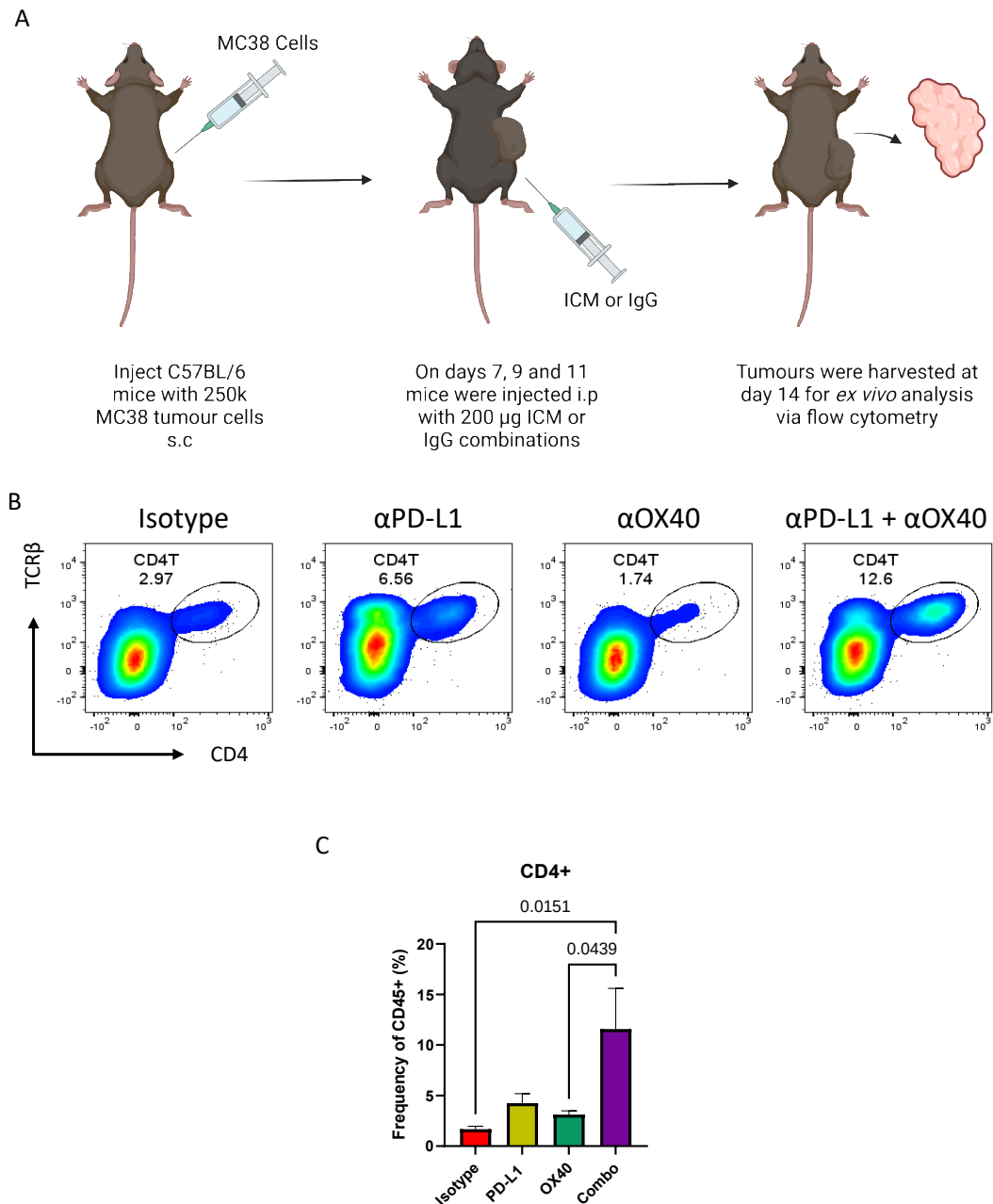


Figure 4.27. *In vivo* α PD-L1 blockade in combination with agonistic OX40 treatment shows increases fraction of CD4+ T-cells. C57BL/6 mice were implanted s.c. with 250k MC38 tumour cells. Mice were injected i.p. on days 7, 9 and 11 with Isotype controls or ICM treatments as indicated. *Ex vivo* analysis of tumours took place at day 15. **A.** Schematic overview of experimental protocol. **B.** Flow plots of TCR β /CD4 expression to define CD4+ T-cell populations. Data representative of one mouse for each treatment condition. **C.** Summary data of quantified CD4+ population in B. Data representative of one experiment (Isotype, n=7; PD-L1, n=6; OX40, n=7 and PD-L1 + OX40, n=7). Summary data is mean $\pm$ SEM. Ordinary one-way ANOVA with multiple comparisons showing significance of $P \leq 0.05$.

Due to the large number of mice required for this experiment and to minimise variation, pure C57/BL6 mice were utilised. Due to the lack of FT expression in these mice, in addition to the use of OX40 agonistic antibodies which prevent efficient staining of OX40 expression, surrogate markers of T-cell activation were used. To further evaluate these CD4⁺ T-cells, GITR and CD25 expression was explored on CD45⁺ cells and CD45⁺ CD4⁺ T-cells (Figure 4.28). GITR and CD25 were chosen as surrogate markers of T-cell activation as their expression were observed to increase in the tolerance model in response to TCR stimulation (section 4.2.6). GITR and CD25 expression was characterised as high and intermediate, with α PD-L1 + α OX40 combination treatment significantly decreasing the expression of both GITR and CD25 on CD45⁺ CD4⁺ T-cells, evidenced through a decrease in the GITR^{hi} and CD25^{hi} (proxy markers for Foxp3⁺ Treg) populations and an increase in both GITR^{intermediate} and CD25^{intermediate} (Figure 4.28).

ICOS and PD-1 populations were also explored, though no significant differences were observed compared to isotype controls for either high or intermediate populations on CD45⁺ CD4⁺ T-cells (Figure 4.29). Significant differences were observed on CD45⁺ cells however, with an increase in ICOS^{intermediate}, PD-1^{high} and PD-1^{intermediate} observed.

In brief, combination therapy was largely associated with an expansion of CD4⁺ GITR^{intermediate} CD25^{intermediate} populations with a reduction of CD4⁺ GITR^{high} CD25^{high} cells.

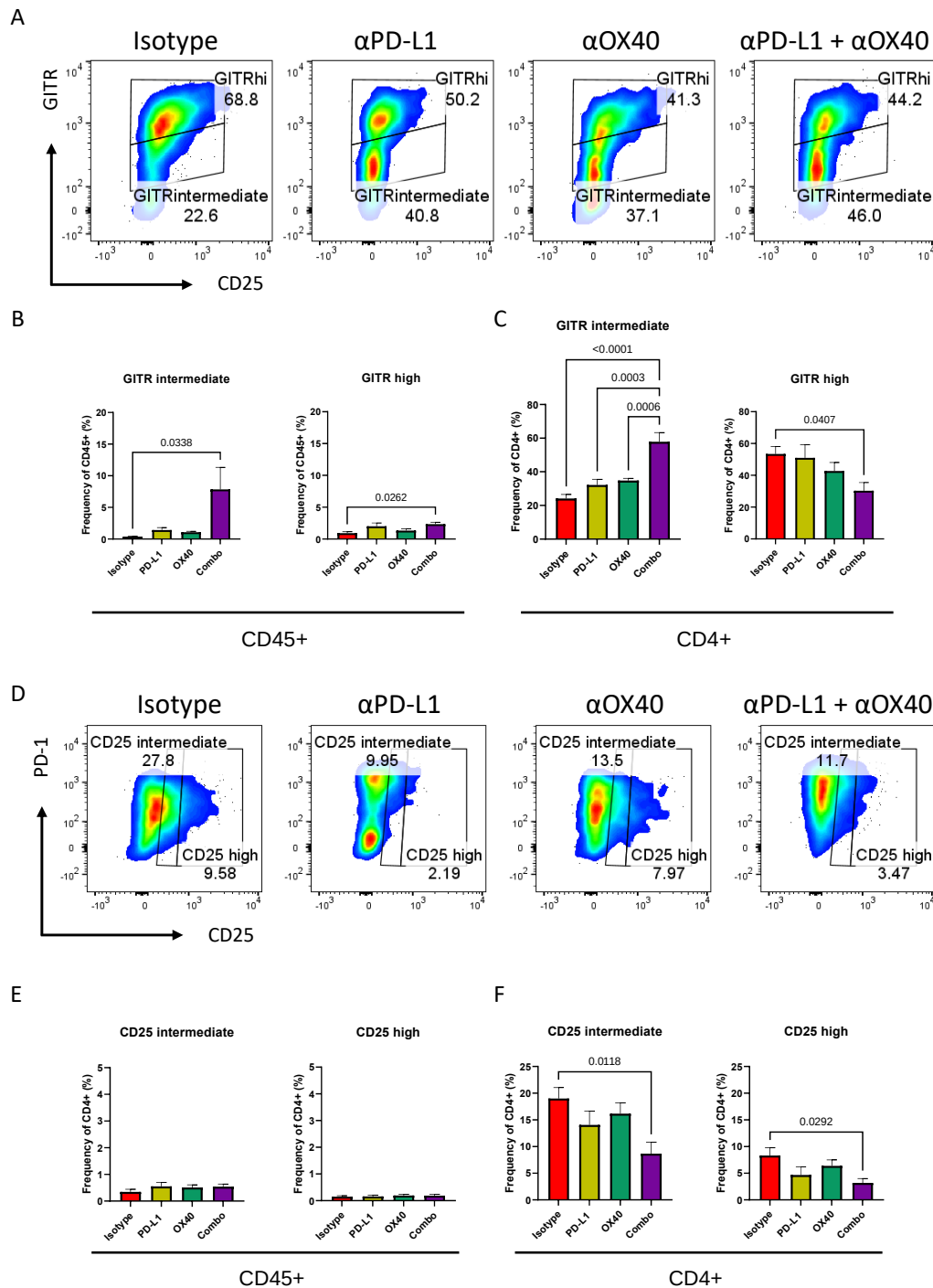


Figure 4.28. *In vivo* αPD-L1 blockade in combination with agonistic OX40 treatment increases GTR+ CD4+ and CD25+ CD4+ T-cells. C57BL/6 mice were implanted s.c. with 250k MC38 tumour cells. Mice were injected i.p. on days 7, 9 and 11 with Isotype controls or ICM treatments as indicated. **A.** Flow plots of GTRhi and GTRintermediate as indicated. **B.** Summary data of population frequencies from A. **C.** Flow plots of CD25hi and CD25intermediate as indicated. **D.** Summary data of population frequencies in C. Data is representative on one experiment. Summary data is mean ± SEM (n=10 per group) showing significant results for an ordinary one-way ANOVA with multiple comparisons of P≤0.05.

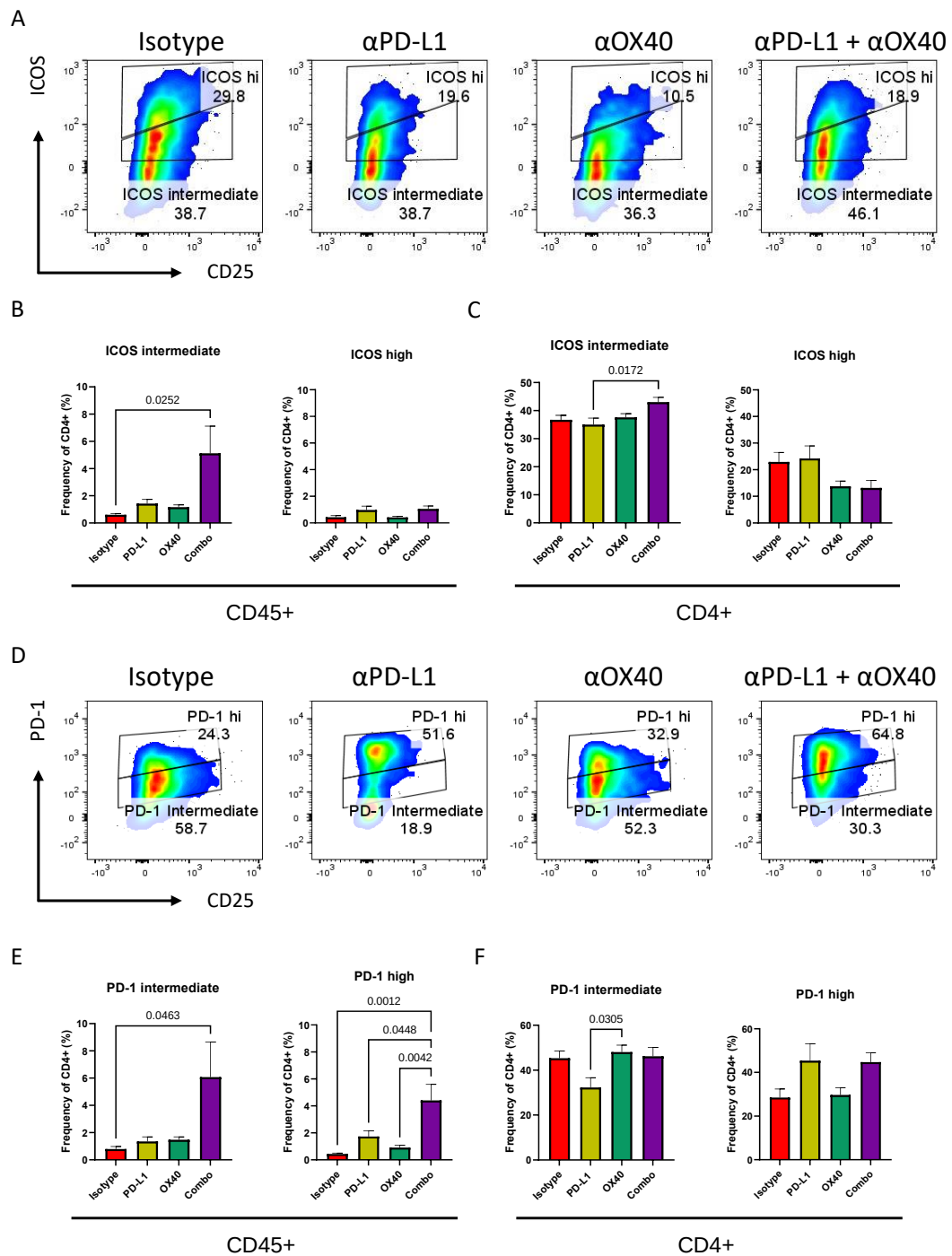


Figure 4.29. *In vivo* αPD-L1 blockade in combination with agonistic OX40 treatment increases PD-1+ CD45+ cells. C57BL/6 mice were implanted s.c. with 250k MC38 tumour cells. Mice were injected i.p. on days 7, 9 and 11 with Isotype controls or ICM treatments as indicated. **A.** Flow plots of ICOS^{hi} and ICOS^{intermediate} as indicated. **B.** Summary data of population frequencies from A. **C.** Flow plots of PD-1^{hi} and PD-1^{intermediate} as indicated. **D.** Summary data of population frequencies in C. Data is representative on one experiment. Summary data is mean ± SEM (n=10 per group) showing significant results for an ordinary one-way ANOVA with multiple comparisons of P≤0.05.

4.3 Discussion

The findings in this chapter demonstrate the use of Tg4 Nr4a3-Tocky and Tg4 Nur77-Tempo models to evaluate the effects of single-agent and combination ICM therapies on peptide-induced TCR-mediated CD4⁺ T-cell activation. Whilst it would have been more appropriately controlled to utilise one reporter model throughout, resource availability of the two strains (Tg4 Nr4a3-Tocky and Tg4 Nur77-Tempo) meant that it was necessary to use the models interchangeably as reporter mice were bred and available. However, all major findings were repeated and confirmed in the Tg4 Nr4a3-Tocky model. To confirm that the models could be used in such a way, both models were evaluated using α CD3-induced activation as a proxy for TCR-induced activation (section 4.2.1), concluding that both models sufficiently report ‘responder’ populations using FT Blue/Red expressions and as such, both models were used throughout.

The Tg4 background of Nr4a3-Tocky and Nur77-Tempo mice provides a unique architecture to study peptide-induced TCR-mediated CD4⁺ T-cell activation using the specific peptide, 4Y-[MBP] (Section 4.2.2). A comparison on moderate (8 μ g) and high (80 μ g) doses of 4Y-[MBP] demonstrated the use of these models to illustrate both quantitative and qualitative effects *in vivo*. The associated changes in expression of co-stimulatory and co-inhibitory receptors in response to 4Y-[MBP]-induced TCR-mediated CD4⁺ T-cell activation is of great importance in this chapter as it is these changes that provide rational for ICM therapy. Stronger primary immunisation of 4Y-[MBP] (80 μ g, later adopting 4 mg/kg to reduce a source of *in vivo* variance) induced the greatest expression of these checkpoint receptors, including ICOS, OX40 and LAG-3 (Figure 4.4). This increased expression of checkpoint receptors associated with

the stronger 4Y-[MBP] dose led to future experiments being conducted with the high 4Y-[MBP] treatment as a primary immunisation.

The use of the adaptive tolerance model (section 4.2.3), developed to explore recalibrating T-cell activation thresholds, shows α PD-L1 blockade as a single-agent therapy is capable of recalibrating T-cell activation thresholds and also induces qualitative effects on the surface of CD4⁺ and responder CD4⁺ T-cells. Findings in section 4.2.3 are consistent with the findings observed by Elliott *et al* (2021), where this adaptive model was utilised to demonstrate that PD-1/PD-L1 pathway blockade via α PD-1 modulation resulted in the enhanced re-emergence of a responder population compared with IgG. Also consistent, this PD-1/PD-L1 pathway modulation was associated with an observed increased expression of OX40. As explained in section 4.2.3, this chapter focuses on antibody-mediated PD-L1 blockade as opposed to PD-1 blockade due to recent observations of unwanted target cell depletion associated with α PD-1 treatment (Polesso *et al.*, 2021).

Though α PD-L1 monotherapy shows promising results *in vivo*, antibody-mediated blockade of PD-L1 alone has not been sufficiently promising in clinical trials. A recent systematic review by Zhao *et al.* (2020) discussed in detail the landscape of clinical trials involving single-agent PD-1/PD-L1 antibody-mediated blockade. Including 160 perspective trials, the overall response rate was 20.21 % and efficacy of single-agent PD-1/PD-L1 pathway blockade varied greatly between cancer types (Zhao *et al.*, 2020). Recently, research has progressed to seek novel combination therapies as opposed to checkpoint monotherapies, with a number of these therapies targeting co-stimulatory receptors using agonists (Beyrend *et al.*, 2019 and van der Sluis *et al.*,

2023). Thus, leading to the rational of α PD-L1 + agonistic therapeutic antibodies targeting OX40 or ICOS. Trialling a range of ICM combinations *in vivo* uses many mice per treatment group, to avoid the unnecessary use of animals, an *in vivo/in vitro* hybrid platform was generated for high throughput screening of a range of ICM combination therapies (see section 4.2.4).

Optimisation of this *in vivo/in vitro* screening platform revealed dose-dependent qualitative changes in FT Blue+ responder CD4+ populations (Figure 4.9). Arguably more relevant to this chapter, dose-dependent quantitative changes in immune checkpoint expression were observed selectively on responder CD4+ T-cells (Figure 4.10). This differential expression of markers observed on responder populations but not on non-responders, highlights the value of utilising FT reporter models like Nr4a3-Tocky and Nur77-Tempo Tg4 mice when studying CD4+ T-cell responses. All markers studied, including ICOS, OX40, PD-1 and GITR, were observed to increase in a dose-dependent manner (Figure 4.11), illustrating that these markers are upregulated in response to TCR-mediated CD4+ T-cell activation.

To explore the effects of checkpoint receptor expression on the surface of CD4+ T-cells in response to ICM treatments, this *in vivo/in vitro* screening platform was used to evaluate α PD-L1 in combination with α OX40 or α ICOS agonistic antibodies (section 4.2.5). Limited treatment effects were observed in frequencies of responders in this system, though dose-dependent effects were still seen (Figures 4.12 and 4.13). More effects were visualised through UMAPs, generated through the expression of all markers and reporters evaluated (Figures 4.14 and 4.15), revealing shared mechanisms of increased activation. One key limitation to this experiment is the

notable use of antibody-mediated OX40 and ICOS agonism, which turn prevents flow cytometric staining of important readouts. It would be interesting to seek different antibody clones that are able to recognise the receptors in the presence of functional antibodies, overcoming this limitation.

As described in section 4.2.5, population One of the UMAPs represent CD4⁺ T-cells that have responded to the primary *in vivo* 4Y-[MBP] immunisation but not the secondary *in vitro* 4Y-[MBP] dose, whilst populations Two and Three both show populations of CD4⁺ T-cells that have responded to the primary and secondary 4Y-[MBP] immunisations. Population Three shows elevated expression of PD-1, CD25, IL-10, CCR6 and GITR, potentially indicating a stronger response to the secondary 4Y-[MBP]. CD25, commonly associated with regulatory and memory T-cell subsets where it is constitutively expressed, is a prominent marker of activation on T-cells, upregulated within 24 hours of stimulation of the TCR/CD3 complex (Reddy *et al.*, 2004). IL-10, an anti-inflammatory cytokine produced by a variety of cell types including regulatory T-cells, has also been observed to be associated with activated T-cells (Trinchieri, 2007). GITR, a costimulatory member of the TNF receptor superfamily, is expressed at low levels on unstimulated T-cells and is upregulated upon activation (Muriglan *et al.*, 2004). Additionally, GITR has recently been observed to be elevated in tandem with Nr4a3-Tocky FT Blue expression at 4 hours with α PD-1 treatment *in vivo* (Elliot *et al.*, 2021). Expression of GITR is also associated with regulatory T-cells, having been linked with their expansion and activation (Ronchetti *et al.*, 2015). This population of CD4⁺ IL-10⁺ T-cells are likely T regulatory type 1 (Tr1) cells (Roncarolo *et al.*, 2018). Peptide-specific upregulation of IL-10 has previously been shown with MBP and it has also been shown to be associated with strong TCR signal strength

(Burkhart *et al.*, 1999 and Elliot *et al.*, 2021). Therefore, the increased expression of CD25, IL-10 and GITR in population Three could be associated either with a stronger response, or with a regulatory T-cell population (Figure 4.14), which warrants further exploration.

When individually quantifying the expression of markers explored on the surface of CD4⁺ T-cells, very limited differences were observed using the *in vivo/in vitro* hybrid system. However, high parameter spectral analysis revealed observed differences in UMAP populations, highlighting the changes observed. The ICM peptide-rechallenge model employed for α PD-L1 blockade in section 4.2.3 was therefore employed to evaluate the α PD-L1 + α OX40 combination (section 4.2.6). Consistent with the UMAPs generated in section 4.2.7, *in vivo* blockade of PD-L1 combined with antibody-mediated agonism of OX40 lowers the activation threshold of CD4⁺ T-cells and increases the proportion of responder CD4⁺ T-cells. This increased responder population is also associated with a significant increase in expression of co-stimulatory and co-inhibitory markers (ICOS, GITR, TIGIT, CD25, PD-1 and CCR6).

Within unpublished data by the Bending lab, CCR6 expression has been observed to be sensitive to α PD-L1 blockade. CCR6⁺ CD8⁺ T-cells have been observed to have characteristics of early effector-memory T-cells (Kondo *et al.*, 2007). Similarly, a study by Kleinewietfeld *et al.* (2005) characterised CCR6 expression on CD25⁺ CD4⁺ T-cells as effector-memory T-cells. In addition, phase I clinical data has revealed an increased proportion of Ki67⁺ CD4⁺ memory T-cells in response to OX40 agonism combined with α PD-L1 (Goldman *et al.*, 2022).

Though expression of PD-1 could suggest a level of exhaustion exhibited, the acute nature of this experiment would be too early for exhaustion, therefore suggesting PD-1 is expressed as an early activation marker. Expression of ICOS, GITR and CCR6 are also likely in favour of T-cell activation. ICOS, which is structurally related to CD28, is a co-stimulatory molecule rapidly induced on the surface of T-cells upon activation (Lohning *et al.*, 2003). *Ex vivo* ICOS⁺ T-cells have been defined as effector T-cells, predominantly secreting IL-10 (Lohnin *et al.*, 2003). GITR, a co-stimulatory molecule also upregulated on the surface of T-cells upon activation, has been observed to induce effector T-cell proliferation (Liao *et al.*, 2010). CCR6⁺ is expressed on CD4⁺ T-cells as antigen-experienced memory cells (Kleinewietfeld *et al.*, 2005), in this case, after exposure to 4Y-[MBP]. Together, the increased expression of these markers in response to α PD-L1 + α OX40 combination therapy suggests that the fraction of responder CD4⁺ T-cells that is observed to increase in response to treatment compared to the isotype, has potential to be an effector T-cell population.

These findings together with recent literature, both pre-clinical and clinical, led to the rationale for trialling α PD-L1 + α OX40 as a potential treatment for cancer (van der Sluis *et al.*, 2023 and Goldman *et al.*, 2022). A recent phase I clinical trial observed an increase of CD8⁺ T-cells within the tumour microenvironment, but intertumoral CD4⁺ T-cells were not evaluated (Goldman *et al.*, 2022). The recent work by van der Sluis *et al* (2023), elegantly explored circulatory CD4⁺ T-cells in response to OX40 agonism and PD-L1 blockade in a murine tumour model, but their work within the tumour microenvironment focuses on CD8⁺ T-cells, though they did note a decrease in regulatory T cells. Despite the recent advances in OX40 agonism combined with PD-1/PD-L1 pathway blockade, the main focus of many of these studies is CD8⁺ T-cells.

Growing evidence highlights the importance of CD4⁺ T-cells in the tumour microenvironment, and the observed effects of α PD-L1 + α OX40 in this chapter suggest that a closer look at intertumoral CD4⁺ T-cells would be of importance.

To explore this therapy in cancer, an MC38-C57BL/6 colorectal tumour model was used, chosen due to its known ICM-sensitive nature. Recent work by van der Sluis *et al.* (2023) shows promising results for PD-L1 + OX40 combination therapy in MC38-tumour bearing WT mice, though their work had a different dosing schedule (days 7, 10 and 13) and included a TLR9 ligand CpG in their OX40 treatment arm which has been shown to augment anti-tumour effects of OX40 agonism. Consistent with the findings presented in this chapter, they observed that α PD-L1 blockade, but not α OX40, delayed tumour growth (van der Sluis *et al.*, 2023). It was observed that PD-L1 + OX40 combination therapy was the most effective treatment, curing most MC38-tumours, consistent with findings presented in section 4.2.7. When exploring the tumour microenvironment in their system, van der Sluis (2023) observed an expansion of functional therapy-responsive CD8⁺ T-cells paired with a decrease in regulatory T-cells. A supplementary finding by van der Sluis (2023) also showed an observed increase in infiltrating CD4⁺ T-cells in the tumour microenvironment, consistent with section 4.2.8. As the research in this chapter focuses primarily on CD4⁺ T-cells, this expansion was further explored.

To do this, the same MC38-tumour model was used as in section 4.2.7, with immunophenotyping taking place at day 14. Consistent with van der Sluis *et al.* (2023), there was a significant expansion of CD4⁺ T-cells in the tumour microenvironment (Figure 4.27B,C). These CD4⁺ T-cells, along with their CD45⁺ gating parents, were

further explored for expression of GITR, CD25, ICOS and PD-1. Interestingly, despite α PD-L1 + α OX40 combination therapy in the adaptive tolerance model being associated with increased ICOS expression (see section 4.2.6), within the tumour microenvironment, CD4⁺ T-cells are observed to decrease expression of ICOS in response to PD-L1 + OX40 combination therapy (Figure 4.29). Furthermore, ICM therapy was observed to decrease the expression of GITR, with a reduction in the GITR^{hi} population and an increase in the GITR intermediate population (Figure 4.28). A decrease in CD25 expression was also observed in response to ICM therapy, a finding consistent with a decrease in regulatory T-cells but further phenotyping would be necessary to define this. CD25, GITR and PD-1 are all markers of activation, but also markers of Tregs. It would therefore be rational to question whether this observed decrease in expression of these markers are associated with Treg depletion, or whether it is an expansion of these CD4⁺ T-cell populations. In order to address this, further analysis would be needed to explore this. Although beyond the scope of this project, an in depth analysis of Tregs in addition to exploring TCR signalling events using Nr4a3-Tocky and Nur77-Tempo FT reporter models would be useful to better understand this observation. The addition of counting beads to obtain cells/mg of tumour would also be advantageous to establish accurate cell counts.

High levels of ICOS in CD4⁺ T-cells has been associated with tumour progression, with low levels of ICOS being associated with tumour control (Meziani *et al.*, 2023). The ICM-induced reduction of ICOS on the surface of CD4⁺ tumour-infiltrating T-cells in section 4.2.8 could therefore aid tumour control and contribute to the mechanism of action for the ICM combination. The finding that agonistic ICOS treatment in

combination with α PD-L1 hinders the effect of α PD-L1 monotherapy would be congruent with this theory (sections 4.2.5 and 4.2.7).

The findings in this chapter suggest that α PD-L1 + α OX40 combination ICM therapy induces anti-tumour effects via modulating expression of co-stimulatory and co-inhibitory surface molecules on the surface of tumour-infiltrating CD4⁺ T-cells. These findings, whilst positive, are restricted to CD4⁺ T-cells in MC38-tumour models, known to be ICM-sensitive. Further tumour models should be explored in their responses to α PD-L1 + α OX40 combination ICM therapy to corroborate the findings here and evaluate the therapy in other tumour models. In addition, further mechanistic studies into the combination therapy would be necessary. It would be important to study the depletion of regulatory T-cells in response to α PD-L1 + α OX40 via FOXP3⁺CD25⁺CD4⁺ expression. It is also important to note that the studies in this chapter are based on immunophenotyping of mixed immune cell populations via flow cytometry, this type of analysis removes the immune cells from the tissue architecture and spatial information is therefore lost. To look at the relationship between the tumour microenvironment and infiltrating immune cells, imaging histology would be necessary. The effectiveness of α PD-L1 + α OX40 treatment in MC38-tumour bearing mice meant that the size of the tumour sample obtained was small, therefore unfortunately histology samples were not taken. A repeat of this experiment with histology as the primary readout would reveal the spatial characteristics of tumour infiltrating lymphocytes, with staining for FOXP3, CD25 and CD4 expression having the ability to demonstrate changes in FOXP3⁺CD4⁺ and FOXP3⁺CD4⁺ regulatory T-cell populations post-ICM treatment.

Though further studies would be necessary to unpin the mechanisms of action, the findings discussed in this chapter suggest that α PD-L1 + α OX40 ICM combination treatment has potential to elicit anti-tumour effects and may work through a CD4-dependent mechanism. It would be appropriate to perform CD4 depletion studies to demonstrate whether CD4 are the key effectors in the MC38 model in response to the combination therapy.

5 FINAL DISCUSSION

Whilst it is important to note that the two research chapters comprised in this thesis are unusually distinct, there is an overall theme of rationally designing a combination strategy for the treatment of cancer. Either by combining oncolytic reovirus with subsequent checkpoint modulation in research chapter 1, or by combining inhibitory α PD-L1 with agonistic α OX40 in research chapter 2.

The human studies in research chapter 1 evaluated NK cells, and these were not explored in the murine *in vivo* models. CD4⁺ and CD8⁺ T-cells were chosen as the focus for *in vivo* studies in research chapter 1 as T-cells are thought to be the key effector cells for checkpoint modulating therapies (Chow *et al.*, 2022). That being said, NK cells have been shown to influence the role of T-cells within anti-tumour immunity by recruiting DCs into solid tumours (Bottcher *et al.*, 2018 and Barry *et al.*, 2018). The findings in research chapter 1 demonstrate that oncolytic reovirus induces the expression of PD-L1 on the surface NK cells. Hsu *et al.* (2018) investigated NK cell activity in response to antibody-mediated PD-1/PD-L1 pathway blockade and observed that treatment not only enhanced NK cell activity but was also crucial for full efficacy of the immunotherapy. It would therefore be of interest to evaluate NK cell activity further.

Research chapter 1 also explores the effects of ICM on CD8⁺ T-cells, whereas research chapter 2 is biased towards CD4⁺ T-cells owing to the nature of the Tg4 TCR transgenic mouse model utilised. This therefore means that CD8⁺ T-cell responses within research chapter 2 are poorly characterised. Whilst this is a limitation, CD8⁺ T-cell responses to immunotherapy have been better characterised to date, whilst CD4⁺

T-cells have gained more traction as important anti-tumour immune effector cells in recent years, in addition to their role in coordinating antigen-specific immune responses (Speiser *et al.*, 2022). CD4⁺ T-cells have been suggested to have a range of functions within the tumour microenvironment, including effector functions (e.g. cytolytic functions) and modulating the tumour microenvironment (e.g. via cytokine secretion). CD4⁺ T-cells have also been demonstrated to be crucial in protective memory, where memory CD8⁺ T-cells mount a defective response in the absence of CD4⁺ T-cells (Sun and Bevan, 2003). In addition, CD4⁺ T-cells have also been shown to aid cancer immunotherapy through a variety of mechanisms, including through helping CD8⁺ T-cells mount effective anti-tumour immune responses (Borst *et al.*, 2018). These findings, in combination with the fact that the presence of CD4⁺ T-cells in tumours is often associated with a favourable response to immunotherapy, suggests that this previously poorly studied cell type warrants further investigation in the setting of cancer (Ling *et al.*, 2016). Therefore, the role of CD4⁺ T-cells in responses to immunotherapy are of particular interest here.

In both research chapters, CD4⁺ T-cells respond to ICM in numerous ways, including their increased activation reported either via CD69 or Nr4a family members, and several co-stimulatory and co-inhibitory markers on their cell surface. One evident limitation to research chapter 1 is the use of CD69 as a marker of activation. Whilst this marker was chosen due to its known characteristic as an early marker of T-cell activation and is routinely used as such, CD69 has been observed in studies to be upregulated in response to a range of stimuli, as opposed to Nr4a family members which are TCR specific (Bremser *et al.*, 2015 and Odagiu *et al.*, 2021). Due to the nature of oncolytic virus treatments selectively infecting tumour cells, their subsequent

lysis would result in several exogenous proteins that would be presented on MHC II by professional APCs to CD4⁺ T-cells or directly via MHC I to CD8⁺ T cells. It would therefore be of interest to utilise the Nr4a3-Tocky or Nur77-Tempo mice models in a reovirus-based model, both *in vitro* and *in vivo*.

In a similar fashion to the transwell co-culture experiments in research chapter 1, murine HCC cell lines (e.g. hep53.4 or 1MEA) could be cultivated in monolayers, with isolated splenocytes from Nur77-Tempo reporter mice co-cultured in the transwells. It would be hypothesised that the treatment of HCC cell lines with oncolytic reovirus would lead to their immunogenic cell death which would be followed by release of DAMPs, PAMPs and exogenous proteins. These proteins could likely pass through the transwell membrane due to typical exogenous proteins having a size of 20 nm (Yoon *et al.*, 2015). The immune cells in this system would be Nur77-Tempo reporter cells, therefore, analysis of CD4⁺ and CD8⁺ T-cells for FT Blue and FT Red expression $\pm$ oncolytic reovirus treatment in HCC cell line co-cultures would allow for analysis of TCR-mediated events between conditions. In addition to *in vitro* models, intravenous delivery of oncolytic reovirus to hep53.4 tumour-bearing Nur77-Tempo reporter mice would allow for analysis of TCR-mediated CD4⁺ and CD8⁺ T-cell activation in the spleen, liver and tumour/TDLNs.

The findings in research chapter 1 strongly support the idea of combining oncolytic reovirus with inhibitory α PD-L1 checkpoint therapy, due to *in vitro* RCM treatment leading to an upregulation of PD-L1 on the surface of both CD4⁺ and CD8⁺ T-cells, in addition to the increased expression of PD-L1 observed on CD8⁺ T-cells in the spleen. Whilst it would be important to repeat the *in vivo* studies, especially to look at PD-L1

expression on CD8+ and CD4+ T-cells in all tissue types, these results suggest that oncolytic reovirus increases PD-L1 expression on the surface of T-cells, a finding consistent with a previous window-of-opportunity study in brain cancer (Samson *et al.*, 2018). These findings suggested that the Hep53.4 *in vivo* tumour model was not sensitive to intravenous reovirus treatment. When repeating these *in vivo* studies, it would be advantageous to explore the effects of reovirus in a model known to be reovirus-sensitive (e.g. the 1MEA-Balb/c model). However, as Tempo/Tocky TCR reporters are on a C57/Bl6 background, other markers would have to be utilised to explore antigen-specific T-cells.

Due to an increased expression of PD-L1 being observed on CD4+ and CD8+ T-cells in response to reovirus in the liver, it would be crucial to explore off target effects before looking at this combination treatment for HCC. The *in vivo* models employed in research chapter 1 for HCC are subcutaneous models. It would be beneficial to explore this treatment in an orthotopic model, particularly one with a background of liver cirrhosis or steatosis (Reiberger *et al.*, 2015 and Kasashima *et al.*, 2020). Utilising a model like this would allow for more accurate evaluation of safety and efficacy of combination therapies given the vast majority of patients with advanced HCC present with a background of liver fibrosis and cirrhosis due to several aetiological factors.

Recently, a PD-L1-expressing orthotopic HCC model was developed to explore immunotherapy treatments (Ou *et al.*, 2019). This model was generated using a BNL-1MEA cell line transfected with PD-L1 plasmids or vector alone. These cells were implanted into the subscapular space of BALB/c mice and growth characteristics of tumours were observed via ultrasound and compared between PD-L1+ and vector. It

was determined that PD-L1 expression did not alter the growth characteristics of liver cancer tumours, however, NanoString analysis confirmed that *ex vivo* PD-L1+ cell-derived tumours had significantly downregulated expression of genes associated with T-cell receptor signalling and activation, highlighting that the presence of PD-L1 in a tumour environment can suppress anti-tumour immunity (Ou *et al.*, 2019). This would suggest that a reovirus-induced increased expression of PD-L1 may also hinder anti-tumour immunity, and subsequent treatment with antibody-mediated PD-L1 checkpoint blockade may augment reovirus monotherapy in a synergistic manner.

Together, the results in research chapter 1 and 2 both suggest that antibody-mediated PD-L1 blockade therapies should be used in combination with other immunotherapies, namely oncolytic reovirus or agonistic α OX40. This finding has implications for future research into combination strategies for cancer. In addition, the findings in research chapter 1 indicate that the timing of subsequent checkpoint blockade could be crucial, e.g. treating with subsequent α PD-L1 blockade once expression is at a high in order to elicit a maximum response.

One crucial aspect to consider when designing combination immunotherapies is treatment-related adverse events. Whilst combination therapies may seem a viable option for enhancing anti-tumour efficacy, off target effects must also be considered. Increased treatment-related adverse events have been demonstrated in the clinical trial for antibody-mediated PD-1 and CTLA-4 combination blockade (Larkin *et al.*, 2015). Although combination treatment demonstrated an increased progression-free survival compared to either monotherapy, grade 3 or higher adverse events were seen in 55 % of patients in the combination group, compared to 16.3 % for PD-1 and 27.3

% in CTLA-4 monotherapies. PD-L1 blockade and OX40 agonism, a treatment combination shown to be promising here, can drive regulatory signatures (e.g. enhanced CD25 and IL-10) and it is likely that increased side effects will be observed.

One consideration here is to develop and screen for biomarkers to predict which patients are likely to respond to ICM, for example, potentially screening for the expression of PD-L1 or OX40 in tumour biopsies (Anagnostou *et al.*, 2022). Presence of these markers could indicate a patient is more likely to respond to ICM, meaning at least that the patients undergoing the treatment will likely have increased benefit outweighing the potential side effects. Another consideration here could be treatment resistance. It has been observed that a number of patients can be initially responsive to ICM (e.g. α PD-L1 blockade) but can develop resistance to the therapy (van Gulijk *et al.*, 2023). In this case, perhaps combination treatment would be of value as a second-line therapy. This is potentially a unique advantage of a combined therapy of anti-PD-L1 with anti-OX40. Given that anti-PD-L1 robustly enhances OX40 levels on CD4⁺ T cells, the agonism of OX40 provides a second boost to responding cells that may aid in reversing treatment resistance.

Lastly, and potentially of most importance, it is crucial to remember that the signalling pathways for the majority of co-inhibitory and co-stimulatory receptors evaluated here are largely poorly understood. It would be critical to explore the diverse network of signalling that unpins how these receptors contribute to T-cell activation and/or inhibition to further develop combination therapies in the future. These pathways may have synergistic, additive or antagonistic effects and only further research into the signalling networks can elucidate this. The screening assay we used (*in vivo*/*in vitro*)

hybrid Tg4 model) could be expanded to incorporate a wide range of potential therapies that can be tested on T-cells undergoing a range of CD4+ T-cell signal strengths. The development of a similar model for CD8+ T-cells would be hugely advantageous as it would permit a high throughput screen for identifying combinations that may boost CD8+ T-cell killing of tumour cells. To this end, the development of *in vitro* assays to generate exhausted CD8+ T-cells may allow a higher throughput determination of the effects of ICM on CD8+ T-cell function (Zhao *et al.*, 2020 and Wu *et al.*, 2023).

The results presented in this thesis are supporting of combining α PD-L1 blockade with other ICM therapies, namely oncolytic reovirus or OX40 agonism. Whilst repeats and further work would be crucial to gain a more mechanistic understanding of these therapies, it is arguable that α PD-L1 has revolutionised the treatment of cancer and rationally combining this therapy with other immune-mediated therapies has potential to sustain and further develop this advancement.

LIST OF ABBREVIATIONS

-	Negative
%	Percentage
(v/v)	Volume per volume
(w/v)	Weight per volume
~	Approximately
+	Positive
=	Equal
>	More than
±	With or without
≤	Less than or equal to
°C	Degrees celcius
µg	Microgram
2D	Two-dimensional
3D	Three-dimensional
4Y-[MBP]	Agonist tyrosine at position 4-myelin basic protein peptide
AF	Autofluoresence
ANOVA	Analysis of variance
AP-1	Activator protein 1
APC	Antigen presenting cell
AWERB	Animal welfare and ethical review body
Bcl2	B-cell lymphoma 2
BCLC	Barcelona-clinic liver cancer
BSF-1	B-cell-stimulating factor 1
BTLA	B and T lymphocyte attenuator
CCL ₄	Tetrachloromethane

CCRn	Chemokine receptor n
CDn	Cluster of differentiation n
CM	Conditioned medium
CO₂	Carbon dioxide
CTLA-4	Cytotoxic T lymphocyte antigen-4
CXCLn	Chemokine ligand n
D	Diversity region
DAG	Diacylglycerol
DAMP	Damage associated molecular pattern
DC	Dendritic cell
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dPBS	Dulbecco's phosphate-buffered saline
e.g.	For example
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EMA	European medicines agency
ER	Endoplasmic reticulum
Erk	Extracellular signal-regulated kinases
FACS	Fluorescence-activated cell sorting
FCS	Foetal calf serum
FDA	Food and drug administration
FGL-1	Fibrinogen-like protein 1
FILMSTAR	Fluorescent intracellular live multiplex signal transduction activity reporter
FM1	Freezing media 1 (see table 2.1)

FM2	Freezing media 2 (see table 2.1)
FMO	Fluorescence minus one
FOXP3	Forkhead transcription factor family
FT	Fluorescent timer
g	Gram
GFP	Green fluorescent protein
GITR	Glucocorticoid-induced TNFR-related protein
GITRL	GITR ligand
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HBC	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
hrs	Hours
HSC	Hepatic stellate cell
HSV	Herpes simplex virus
HVEM	Herpesvirus entry mediator
i.e.	That is
I.P	Intraperitoneal
I.V	Intravenous
ICD	Immunogenic cell death
ICM	Immune checkpoint modulation
ICOS	Inducible T-cell co-stimulator
ICOSL	ICOS ligand
IFN-γ	Interferon-gamma
IgG	Immunoglobulin G
IL-n	Interleukin n
IP₃	Inositol-3-phosphate

ITAM	Immunoreceptor tyrosine-based activation motifs
J	Joining region
K	Lysine
k	Thousand
kg	Killogram
KIR	Killer Ig-like receptors
LAG-3	Lymphocyte-activation gene 3
LCK	Lymphocyte protein tyrosine kinase
LN	Lymph node
LSEC	Liver sinusoidal endothelial cell
mAB	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MBP	Myelin basic protein
MCGF	Mast-cell growth factor
MDSC	Myeloid-derived suppressor cells
MFI	Median fluorescence intensity
mg	Milligram
MHC I	Major histocompatibility complex class I molecule
MHC II	Major histocompatibility complex class II molecule
mins	Minutes
mL	Millilitres
mm	Millimetres
n	To the power n
N/A	Not applicable
NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor kappa B
NK	Natural killer

NKG2D	Natural killer group 2D receptor
Nr4a	Nuclear receptor subfamily 4
OS	Overall survival
OV	Oncolytic virus
OX40	TNF superfamily member 4
OX40L	OX40 ligand
P value	Probability
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PD-1	Programmed cell death 1
PD-L1	PD-L1 ligand
PFA	Paraformaldehyde
pfu	Plaque forming unit
PIP₂	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PLCγ1	Phospholipase C γ 1
PMA	Phorbol myristate acetate
PRR	Pattern recognition receptor
Q1-4	Quadrant 1-4
RAG	Recombinant activating genes
RASGRP1	RAS guanyl nucleotide-releasing protein
RBC	Red blood cell
RCM	Reovirus conditioned medium
RM	Repeated measure
RNA	Ribonucleic acid
RPMI	Roswell park memorial institute

RT	Room temperature
S.C	Subcutaneous
SEM	Standard error of the mean
SHARP	Sorafenib HCC assessment randomised protocol
Shp2	SH2 containing protein tyrosine phosphatase-2
Stage 0	BCLC stage (Very early)
Stage A	BCLC stage (Early)
Stage B	BCLC stage (Intermediate)
Stage C	BCLC stage (Advanced)
Stage D	BCLC stage (End-stage)
STAT1	Signal transducer and activator of transcription 1
T3D	Type 3 dearing
TAA	Tumour associated antigen
TACE	Transarterial chemoembolization
TAP	Transporter associated with antigen processing
TCR	T-cell receptor
TDLN	Tumour draining lymph node
TGF-β	Transforming growth factor beta
Th1	Type 1 helper
Th17	Type 17 helper
Th2	Type 2 helper
TIGIT	T-cell immunoreceptor with Ig and ITIM domains
TIL	Tumour infiltrating lymphocyte
TIM-3	T-cell immunoglobulin and mucin domain-containing protein
TME	Tumour microenvironment
TNF-α	Tumour necrosis factor alpha
Tr1	Type 1 regulatory

Treg	Regulatory T-cell
T-VEC	Talimogene Laherparepvec
UK	United Kingdom
UMAP	Uniform Manifold Approximation and Projection
V	Variable region
VEGF	Vascular endothelial growth factor
WT	Wild type
Y	Tyrosine
Zap-70	Zeta-chain associated protein kinase
α	Alpha
αCD3	Functional antibody-mediated CD3 stimulation
αICOS	Functional antibody-mediated ICOS agonst
αOX40	Functional antibody-mediated OX40 agonist
αPD-L1	Functional antibody-mediated PD-L1 blockade
β	Beta
γ	Gamma
δ	Delta
Δ	Delta
ε	Epsilon
ζ	Zeta
θ	Theta
μg	Microgram
μL	Microlitre
μM	Micromolar
σ	Sigma

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