

ANTIGEN REGULATION OF T CELL RECEPTOR SIGNALLING AND IMMUNE CHECKPOINT EXPRESSION IN HEALTH AND DISEASE

BY

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Abstract

Binding of cognate antigen to the T cell receptor (TCR) results in downstream signalling, which controls T cell activation and effector function. It is still however controversial how antigen dose and affinity, as well as over-expression of immune checkpoints in the tumour microenvironment (TME) affect such processes. The novel Nr4a3-Timer of cell kinetics and activity (Tocky) system was used for the temporal analysis of $CD8^+$ T cell activation through distal TCR signalling analyses. It was first found that expression of two downstream TCR genes respond differently to signalling; Nr4a1 requires shorter, weaker signals than Nr4a3. Using the OTI TCR transgenic system and modified peptides, it was established that TCR affinity affects the antigen dose required to trigger expression of IFNγ and PD-1 as well as affecting maximum response, by altering TCR signalling dynamics. Finally, the *in vivo* effects of the TME on T cell activation, immune checkpoints and IFNγ expression was investigated, resulting in a set of findings that provide an overview of T cell activation and checkpoint expression in response to tumour antigens. This project therefore broadens our understanding of how antigen regulates $CD8^+$ T cell biology and proposes future lines of investigation to understand anti-tumour T cell responses.

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Publications arising from this thesis

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List of abbreviations

Major histocompatibility complex class I / II	MHC I / II
Cluster of differentiation ...	CD..
Antigen presenting cells	APC
T helper cell ($CD4^+$)	T_h
Cytotoxic T cell ($CD8^+$)	T_c
T cell receptor	TCR
Interferon gamma	IFNγ
Double negative cells	DN
Double positive cells	DP
Single positive cells	SP
Immunoreceptor tyrosine activation motif	ITAM
Cytotoxic T-lymphocyte-associated protein 4	CTLA-4
Programmed cell death protein 1	PD-1
Interleukin - …	IL - …
Tumour microenvironment	TME
Nuclear factor of activated T-cells	NFAT
Nuclear receptor 4A	Nr4a
Green fluorescent protein	GFP
Timer of cell kinetics and activity	Tocky
Ovalbumin	ova
Draining lymph node	dLN
Contra-lateral lymph node	cLN

1. Introduction

1.1 T cell role in the immune system

The immune system is an intricate network without which we would not survive. The system is made up of two broad categories; the innate and the adaptive system. The innate system is the body's first line of defence to prevent the spread of pathogens in a non-specific manner. On the other hand, the adaptive system is comprised of specialised cells that kick into action after the innate system in response to a specific pathogen/s. This system can be split into two different categories of lymphocytes that complement each other in the fight against pathogens: B and T cells. This project will be focused on T cells, $CD8^+$ in particular.

B cells are involved in the body's humoral response and have specific membrane bound antibodies (B cell receptors) to recognise pathogenic antigen in its native form. Upon discovering antigen, B cells engulf the pathogen and clonally expand to long lived memory cells and effector plasma cells, which pump out antigen specific antibodies. B cells, dendritic cells and macrophages, also display fragments of pathogenic antigen on their major histocompatibility complex class II (MHC II) and are termed antigen presenting cells (APCs).

By APCs displaying antigen fragments on their MHC II, $CD4^+$ T helper (T_h) cells can encounter the antigen via its T cell receptor (TCR); if it recognises the antigen, the $CD4^+$ T cell becomes activated. Once activated, memory and effector cells, which elicit a multitude of processes by releasing cytokines, are produced. Processes including, but not limited to, activating macrophages and antibody producing B cells, as well as aiding activation of $CD8^+$ cytotoxic T (T_c) cells.

Unlike B cells that are involved with humoral response, $CD8^+$ T cells patrol intracellular immunity. All cells have MHC I on their surface to be able to display endogenous protein to show the cell is functioning normally. However, for example, if a cell becomes cancerous or infected by a virus, peptide fragment of foreign/mutated proteins are displayed on the cell's MHC I which $CD8^+$ T cells can recognise. If the $CD8^+$ T cell TCR recognises antigen on MHC I it becomes activated; producing memory and effector cells. Effector $CD8^+$ T cells kill any cell that is presenting the specific antigen on its MHC by three major mechanisms. The first is the secretion of cytokines TNF-α and type II IFNγ, both of these have pro-inflammatory and anti-tumour effects (Aggarwal *et al.,* 2012; Schroder *et al.,* 2003). The second mechanism is the release of cytotoxins along the immune synapse; perforin, which forms pores in the cell membrane (Kägi *et al.,* 1994) and granzyme, a serine protease that cleaves intracellular proteins (Heusel *et al.,* 1994). The third and most often utilised mechanism is through the binding of Fas ligand (FasL) on activated $CD8^+$ T cells to Fas receptors on the target cell, binding activates the caspase cascade pathway (Juo *et al.,* 1998). All three of these mechanisms cause the target cell to undergo apoptosis and are vital to $CD8^+$ T cells fighting against cancerous and infected cells.

1.2 T cell development

In order for T cells to not become dangerous to our own body and be able to elicit an effective response when faced with pathogen or cancerous cells, the ability to recognise non self-antigen and self-antigen is imperative. This is learnt during T cell development. T cells develop in the thymus gland, which is where they get the name from, and go through a vigorous process so that they can perform their function correctly as either $CD8^+$ or $CD4^+$ T cells. Lymphoid precursor cells leave the bone

marrow and enter the thymus at the corticomedullary boundary as double negative (DN) cells (Lind *et al.,* 2001). The DN cells then go through a four-step process where they become committed to the T cell lineage through interaction with Notch1 (Pui *et al.,* 1999), rearrange their TCR chains, proliferate and become double positive cells (DP) due to cytokine activity (Haks *et al.,* 1999), meaning they express both CD8 and CD4 receptors.

Next in the process is selection, where the DP T cells come into contact with thymic epithelial cells that express high levels of MHCI / II that present intra and extracellular self-peptide to developing cells. Firstly, DP cells undergo positive selection; if they do not recognise self-peptide on MHC, they are signalled to apoptose. However, if the cells recognise self-peptide and MHC, they receive a survival signal and mature from DP to single positive (SP) cells. Depending on which MHC the cell interacts with determines its fate; if TCR binds MHC II this induces expression of ThPOK which reduces Runx3, the protein responsible for driving CD8 expression therefore cells become $CD4^{+}$. On the other hand, if cells do not bind to MHC II strongly, ThPOK will be low and Runx3 will be high, consequently pushing cells into the CD8 lineage (Muroi *et al.,* 2008).

Negative selection (central tolerance) follows next. Thymic epithelial cells express the protein autoimmune regulator (AIRE), which allows the processing and presentation of self-peptide from organs outside of the thymus (Anderson *et al.,* 2005), which is essential as this educates the T cells about self and non-self-peptide from throughout the body. If SP cells have high affinity for self-peptide-MHC they are termed autoreactive and are clonally deleted as these cells are potentially dangerous to self.

A subset of these strongly reactive T cells however can undergo differentiation into Foxp3$^+$ T regulatory cells (Tregs), which are vital for maintenance of tolerance to self-antigens in the periphery. Although it is known that interactions with the TCR are needed for Treg selection, it has also been described that cytokines, in particular IL-2, IL-15 and TGFβ, play a role in the induction and survival of these cells (Perry and Hsieh, 2016). The T cells that make it through all of these stages will then migrate to peripheral lymphoid tissue where they reside until needed by the immune system as specific, specialised naïve CD8$^+$ or CD4$^+$ T cells.

1.3 T cell receptor structure

T cell receptors (TCR) are found on the cell membrane of T cells and are essential for recognising specific antigen displayed on MHC molecules. The TCR is comprised of two chains, either alpha-beta (αβ) or the less common, gamma-delta (γδ) (Kuhns and Badgandi, 2012). Each chain has variable antigen specific regions, constant regions and transmembrane regions. Within the membrane, the TCR associates with CD3 subunits gamma (γ), delta (δ), epsilon (ε) and zeta (ζ), forming the TCR-CD3 complex, where the TCR is situated over the CD3 ectodomains (Birnbaum *et al.,* 2014). Each CD3 subunit has at least one immunoreceptor tyrosine activation motif (ITAM) in the cytoplasmic domain (Figure 1) where various kinases bind and phosphorylate ITAMs to begin receptor signalling, T cell activation and proliferation (Andrés *et al.,* 2018).

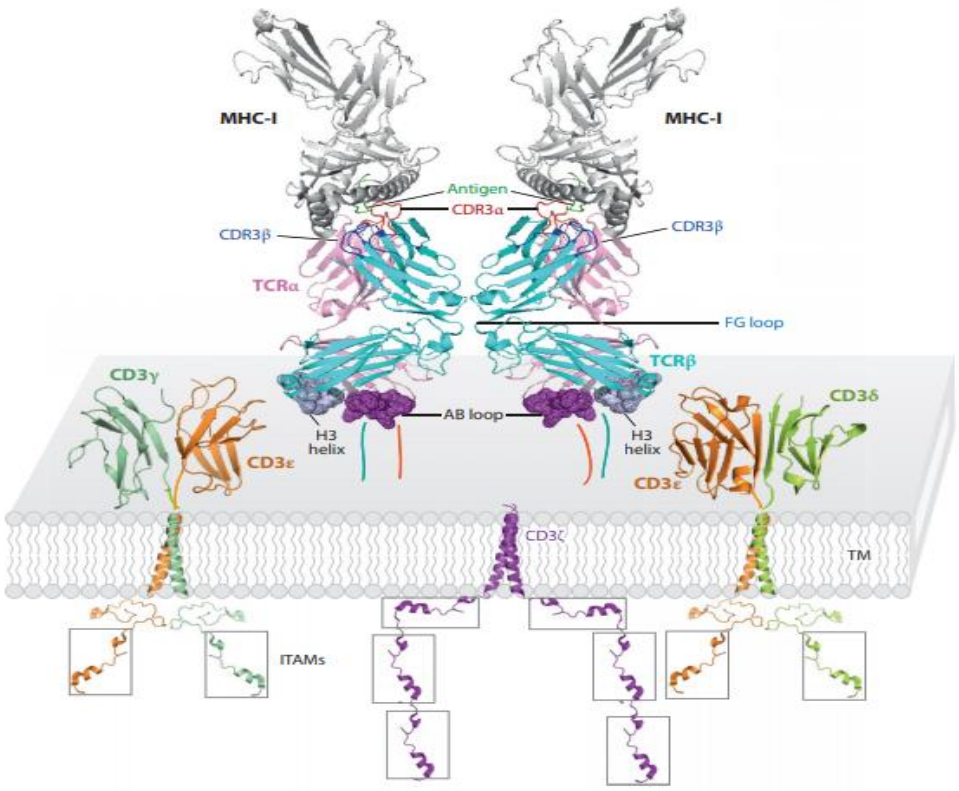


Figure 1. Structural model of TCR-CD3 complex (Andrés *et al.,* 2018)

The ectodomain of TCR αβ chains (*light blue*) can be seen bound to antigen-MHC I (*grey*) and also associated with CD3 subunits in the plasma membrane. The αβ chains each associate with dimers of CD3γε (*left)* or CD3δε (*right)* and CD3ζζ (*middle)*. Each of these subunits have immunoreceptor tyrosine activation motifs which play a vital role in signalling; CD3γε and CD3δε have a single copy however CD3ζζ has three copies (adapted from Andrés *et al.,* 2018).

1.4 T cell receptor binding and the effecting factors

Once the TCR variable regions come across cognate antigen-MHC on APCs and bind, this triggers initial activation. Activation and functional outcome depend on the TCR affinity for antigen-MHC and the dose of antigen, with remarkable sensitivity. T cells are known to use antigen-MHC affinity to determine between self and non-self (Dushek and Merwe, 2014); best demonstrated by the selection process in development. Zehn *et al.,* also describe that when a constant antigen dose is given,

cells with high affinity to antigen-MHC have a greater effector function (including an increase in IFNγ production) and expansion compared to low affinity (Zehn *et al.,* 2009). It is however, unclear if a high dose of antigen from low affinity antigen-MHC can elicit the same response; this is important to understand as antigen abundance can vary depending on the level of immunogenicity the target possesses. Similar to the effect of affinity, dose of antigen plays a role in activation and effector function. Abu-Shah *et al.,* recently describe that although the amount of cytokine detected is linked to a change in dose of antigen, some cytokines exhibit a comparable dose threshold if affinity is kept constant (Abu-Shah *et al.,* 2020). This phenomenon could be seen across a variation of affinities, involving cytokines such as TNF-α, IL-2 and IFNγ, however it is still controversial how dose and also, TCR affinity, effect TCR signalling and cytokine production.

Following initial activation, the T cell co-receptor, either CD8 (T_c) or CD4 (T_h), will also bind to MHC (class I or II, respectively), stabilising the complex and efficiently recruiting Src kinases (Artyomov *et al.,* 2010). In addition to this complex forming, T cells require the action of secondary co-signalling receptors to become fully activated; often termed immune checkpoints. One well described example is an important co-stimulatory signal, CD28. CD28 binds to either CD80 (B7.1) or CD86 (B7.2) on APCs, consequently initiating proliferation and survival of antigen specific T cell (Harding *et al.,* 1992). CTLA-4 also binds to these same receptors with approximately a 20-fold higher affinity than CD28 (Linsley and Ledbetter, 1993) but instead of activating cells, it dampens down the immune response. It does this via many ways, one example being, blocking the formation of Zap-70 containing microclusters in T cells which are essential for transmission of signal from the TCR

(Schneider *et al.,* 2008). There are many other co-signalling receptors that directly affect function and fate of cells by stimulatory or inhibitory action, such as PD-1, Lag-3, TIGIT and OX40, all of which are critical for the regulation of activating T cells and receptor signalling.

1.5 T cell receptor signalling pathways

When TCR binds its cognate antigen-MHC an array of signalling cascades take place intracellularly. An overview of major TCR signalling pathways can be seen in Figure 2 to aid understanding of this section.

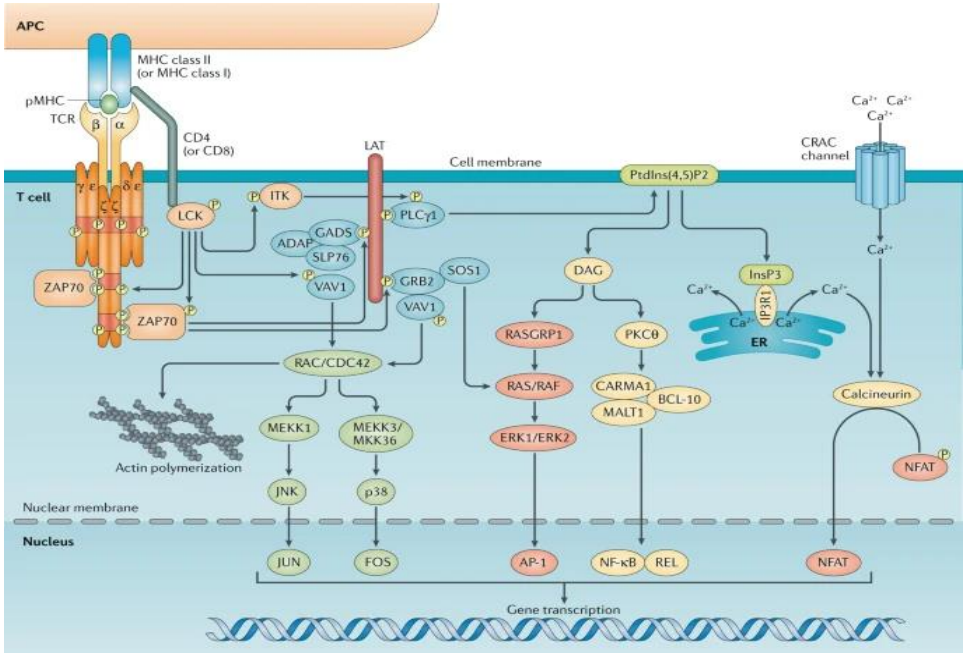


Figure 2. Overview of major T cell signalling pathways (Gaud *et al.,* 2018)

The binding of the TCR to cognate antigen-MHC initiates signalling in T cells. There is a myriad of different signalling pathways however the major ones include; the calcium-calcineurin pathway, the mitogen-activated protein kinase (MAPK) pathway and nuclear factor-κB (NF-κB) pathway. Each play an important role in T cell proliferation, cytokine production, effector function and migration by propagating the translocation of NFAT; REL, NF-κB; FOS, JUN and AP-1, respectively (adapted from Gaud *et al*., 2018). Further details of these pathways can be seen below.

Upon TCR binding, one of the first events to happen is the activation of Src family tyrosine kinases including Lck and Fyn, which consequently phosphorylate ITAMs on the CD3ζζ dimer chains (Furlan *et al.,* 2014). Zap-70 (zeta-chain associated protein kinase) is then recruited to the TCR-CD3 complex where it binds to the ITAMs and gets phosphorylated by Lck (Chan *et al.,* 1992); brought close to the complex by T cell co-receptor (CD4 or CD8) binding MHC (Veillette *et al.,* 1988).

Zap-70 plays a vital role in signalling as it promotes the recruitment and activation of a multitude of downstream adaptors and scaffold proteins. An example is the recruitment and phosphorylation of membrane protein LAT, which associates with GRB2 and SLP-76 to form a large scaffold (Devkota *et al.,* 2015). When Zap-70 phosphorylates SLP-76 this recruits ITK; a tyrosine protein kinase, NCK and GADS; adaptor proteins and Vav; a guanine nucleotide exchange factor. ITK can then go on to phosphorylate phospholipase Cγ1 (PLCγ1), resulting in the production of the secondary messengers, inositol trisphosphate (IP3) and diacylglycerol (DAG), from to the hydrolysis of phosphatidylinositol biphosphate (PIP2) (Koretzky *et al.,* 2006).

DAG is involved in the propagation of the PKCθ pathway; important for several aspects of T cell function. The PKCθ pathway leads to the activation of different transcription factors; AP-1, through the MAPK pathways and NFAT, through promotion of PLCγ1 activity (Hayashi and Altman, 2007). Activation of protein kinase C, by DAG, is also involved in the cascade of phosphorylation events that activate NF-κB (Sun *et al.,* 2000); a major transcription factor in the innate and adaptive system. Additionally, DAG can lead to expression of surface marker CD69 through activation of the Ras-MAPK pathway and is thought to be the cause of its bimodal

expression (Das *et al.,* 2009). IP3 equally has a very important role as it triggers the release of calcium (Ca^{2+}) from the endoplasmic reticulum which then opens Ca^{2+} activated channels. The influx of extracellular Ca^{2+} activates calcineurin, a phosphatase which de-phosphorylates and activates the transcription factor NFAT (Klee *et al.,* 1998); involved in the regulation of a large number of genes including IL-2, FasL and IFNγ by interacting with AP-1 (Macián *et al.,* 2001). These signalling pathways (along with others that are not so well described) are a direct result of both TCR binding and additional surface receptors interactions in order to control T cell function, proliferation and differentiation.

1.6 Studying T cell receptor signalling using the Nr4a receptor family

The Nr4a family of orphan nuclear receptors are widely expressed across a variety of tissues in the body and function mainly by direct stimulation or repression of transcriptional targets to control proliferation, apoptosis and metabolism (Herring *et al.,* 2019). The family consists of three members: Nr4a1 (Nur77), Nr4a2 (Nurr1) and Nr4a3 (Nor1). Both Nr4a1 and Nr4a3 are shown the be upregulated rapidly upon TCR binding cognate antigen-MHC in thymocytes, during development, and peripheral T cells (Cheng *et al.,* 1997). It is well described that all three members of the family are involved in the differentiation of subtypes of T cells, however each also play roles in other pathways.

Nr4a1 is involved in clonal deletion during T cell development; association with regulatory protein Bcl-2 causes apoptosis of self-reactive T cells, a critical stage in negative selection (Thompson and Winoto, 2008). Nr4a1 also plays another role in T cell development; modulating the differentiation of $Foxp3^{+}$ Tregs (Fassett *et al.,*

2012). Nr4a2 is equally described to be able to induce Tregs, as well as being able binding Foxp3 elements to regulate the differentiation of $CD4^+$ T cells into T_h1 cells (Sekiya *et al.,* 2011). Additionally, Nr4a2 has been found to be a proposed regulator in effector function of macrophages; acting as a mediator in chronic inflammation (Ralph *et al.,* 2010). Like Nr4a2, Nr4a3 is described to have a role in the differentiation of monocytes into macrophages or monocyte-derived dendritic cells (MoDCs), the latter of which is required for a proper $CD8^+$ T cell response to bacteria (Boulet *et al.,* 2019). Nr4a3 is further described as being involved in the early programming of $CD8^+$ T cells and memory cells (Odagiu *et al.,* 2020). Similarly, Nr4a1 has been found to control effector function and differentiation of $CD8^+$ T cells with the mechanism of action being linked to repression of transcription factors such as Runx3 and Irf4 (Nowyhed *et al.,* 2015; Nowyhed *et al.,* 2015). These functions are however not fully representative of the Nr4a family. Seo *et al.,* recently found that association of the Nr4a family with transcription factors TOX and TOX2 is critical for T cell exhaustion downstream of NFAT (Seo *et al.,* 2019).

Although the members of this family seem to produce overlapping effects, a new study carried out by Jennings *et al.,* found that Nr4a2 and Nr4a3 are differentially controlled compared to Nr4a1 (Jennings *et al.,* 2020). All three members are described to be Src family kinase dependant, with the MEK/ERK pathway also influencing the family to produce maximum expression. However, it was shown that even though all three members genes bind NFAT1, only Nr4a2 and Nr4a3 are induced by the calcineurin/NFAT pathway, whereas it is redundant for Nr4a1 (Jennings *et al.,* 2020).

Despite this study finding that the Nr4a family members are differentially controlled, data showing they are Src family kinase dependent suggests that induction may be due to Lck, which is directly associated with initial TCR signalling (Jennings *et al.,* 2020). As a result of the family being downstream of the TCR, one member, Nr4a1, is widely used as a reporter in the field, notably as Nr4a1 tagged with green fluorescent protein (GFP) (Moran *et al.,* 2011). This reporter has been used to study a variety of cells; Tregs, iNKT, B cells, haematopoietic stem cells and monocytes, to name a few (Moran *et al.,* 2011; Zikherman *et al.,* 2012; Land *et al.,* 2014; Hanna *et al.,* 2011). Nr4a1-GFP's use in the field is cemented as a marker of antigen specific signalling (Ashouri and Weiss, 2017), however its use for studying temporal dynamics is flawed. GFP has an extended half-life, meaning that once the reporter is first expressed, it can persist from hours to days (Corish and Tyler-Smith, 1999), this consequently does not allow for the analysis of temporal changes that occur after TCR binds cognate antigen. As signalling dynamics play a large role in the control of effector function, differentiation and proliferation of cells a reporter that can track temporal changes is a necessary tool. A new novel reporter that can do this enlists the use of a different family member, Nr4a3.

1.7 Using novel reporter Nr4a3-Tocky to study TCR signalling

A number of pathways are reported to be activated within hours of TCR binding cognate antigen-MHC. In order to study these pathways a novel reporter has been described, named Nr4a3-Tocky (Bending *et al.,* 2018). Like Nr4a1-GFP, this new reporter also consists of a fluorescent protein attached to a member of the Nr4a family, but unlike Nr4a1-GFP, this Timer protein has the ability to shift its emission spectrum from blue to red, consequently allowing for temporal analysis of T cell

activation. Fluorescent Timer proteins have been used in previous different studies including: analysis of protein dynamics and receptor turnover, cell trafficking, neural fibre formation and virus transmission (Khmelinskii *et al.,* 2012; Subach *et al.,* 2009; Verkhusha *et al.,* 2012: Breen *et al.,* 2016).

Through the work of Bending *et al.,* the Nr4a3-Tocky reporter was developed (Bending *et al.,* 2018). The team first confirmed that Nr4a3 is immediately downstream to TCR signalling, with peak expression being within 2 hours of stimulation. They also found that Nr4a3 holds the highest correlation to anti-CD3 mediated activation and *in vivo* signalling in the thymus. After this, a bacterial artificial chromosome (BAC) transgenic reporter mouse was generated, which has the ability to report TCR signalling through the Nr4a3 gene tagged with Timer protein. Cognate antigen binding to the TCR initiates signalling and the transcription of genes. Upon transcription of Nr4a3 and the Timer protein, a short-lived chromophore is formed that emits blue fluorescence (half-life of 4 hours) before forming the mature chromophore which emits red fluorescence. This change in emission is irreversible and can be captured by flow cytometry as a real time tracker of T cell signalling, giving the system its name; Timer of cell kinetics and activity (Tocky). A schematic of how Timer expression can be used to categorise a new, persistent or arrested TCR signal and track temporal changes in T cells can be seen in Figure 3. The Bending team also created a system to study response upon specific antigen recognition by crossing the Nr4a3-Tocky mouse with an OTI mouse ($CD8^+$ T cells express ovalbumin (ova) specific transgenic TCR (Clarke *et al.,* 2000)). This adaptation to the system would be very beneficial for investigating how dose and affinity of antigen effect TCR signalling, an outstanding controversial topic within the field.

During development Bending *et al.,* also describe that this system is stable in different cell subtypes as well as it not being affected by cell division, exogenous IL-2 or co-stimulatory receptor CD28. This reporter system holds great potential for use in the field for investigating the temporal dynamics of T cells and TCR signalling during states of cellular function and dysfunction that can be seen in autoimmune disease, cancer and infections.

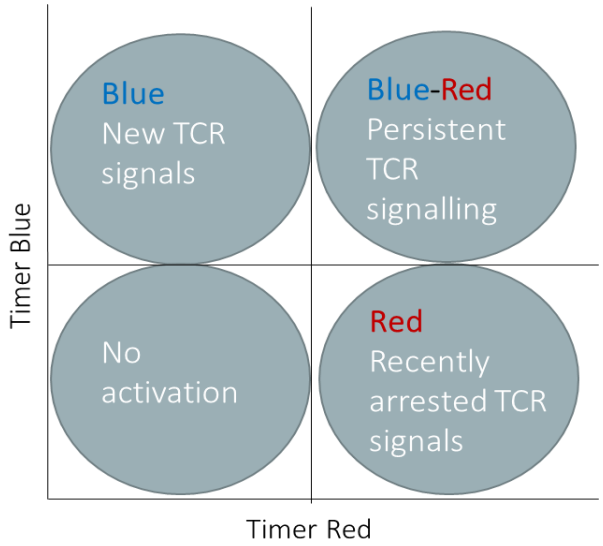


Figure 3. Schematic showing how Timer expression can be used to determine a new, persistent or arrested TCR signal

A schematic of a flow cytometry plot showing Timer red expression versus Timer blue. If there is no TCR signalling, hence no cell activation, no Timer protein is made (bottom left). When new transcription occurs due to TCR signalling, cells acquire blue Timer protein (top left). If signalling persists, blue Timer protein that was initially made matures into red Timer, however new blue Timer is also being made therefore cell acquire a mix of blue-red Timer (top right). When transcription lessens and signalling is arrested no new blue Timer is made, therefore all protein matures to red Timer (bottom right). This is an efficient system to group cells by Timer protein to what type of TCR signal they have received.

1.8 T cell role in disease

As mentioned previously T cells play a critical role in the immune system. If they become infected or fail to respond in their normal fashion there can be devastating effects to the body. Viral infections, such as the human immunodeficiency virus (HIV), is one of the most notorious viruses for affecting T cell function. HIV attacks $CD4^+$ T cells by binding glycoprotein 120 to the CD4 co-receptor, CXCR4 and CCR5 (Madani *et al.,* 1998). This allows the injection of its RNA into the cell and through the use of reverse transcriptase, new pathogenic virus is produced and buds off, consequently killing the cell. As the virus infects more cells there is a steep decline in the amount of $CD4^+$ T cells due to budding and also $CD8^+$ T cell killing, which leads to acquired immune deficiency syndrome (AIDS); a life-threatening illness cause by an immune system that cannot function due to loss of CD4+ T cells.

Unlike viruses that infect and hijack cell mechanisms, autoimmune diseases arise when healthy cells are attacked by the immune system through reasons that are often not known. Some of the most common autoimmune diseases include multiple sclerosis; a neurological condition effecting the spinal cord and brain, rheumatoid arthritis; a chronic inflammatory disorder and type 1 diabetes mellitus (T1DM). T1DM is a chronic disease where insulin producing beta cells in the pancreas are destroyed by immune cells, leading to a dangerous increase in glucose blood levels that can cause a range of serious complications. It is thought that in people with T1DM, allelic variation in HLA DR-DQ (Erlich *et al.,* 2008) and epigenetic regulation (Vafiadis *et al.,* 2001) contribute to suboptimal presentation of pancreatic self-antigen during T cell development, leading to these T cells mistaking pancreatic cells for non-self. It has also been found that some immune checkpoints can be impaired in people with

T1DM, including CTLA-4 and PTPN22 (Pociot *et al.,* 2010); both normally function as inhibitors of TCR signalling.

Immune checkpoints are essential for self-tolerance and regulation in the immune system; they work to either stimulate or inhibit TCR signalling. It has been found that some cancers manipulate the inhibitory immune checkpoints to protect themselves. Cancer arises when mutations in oncogenes and tumour suppressive genes occur, this causes the cell to lose the ability to proliferate and differentiate in a controlled manner. Normally $CD8^+$ T cells would recognise that these cells were not functioning correctly and proceed to kill them, however due to the highly immunosuppressive tumour microenvironment (TME) this does not happen. The TME is comprised not only of immune cells but also fibroblasts, endothelial cells and a robust extracellular matrix (Anderson *et al.,* 2017); all which are in metabolic competition with the demanding tumour cells. These metabolic demands expose T cells to suppressive metabolites whilst also limiting the nutrients available, hence reducing their effector function (Chang and Pearce, 2016). TCR signalling is also highly affected in the TME; chronic antigen stimulation causes T cells to become dysfunctional and 'exhausted'. T cell dysfunction and exhaustion is a state where cells have impaired effector function; they develop epigenetic changes, upregulate inhibitory immune checkpoint receptors and alter the use of key transcription factors (Wherry and Kurachi, 2015). These disruptions to TCR signalling, plus more that are yet to be defined, hinder the ability of T cells to control the proliferation of cancer cells and mount an immune response.

Although cancer remains to be one of the biggest causes of death, there has been some promising advances in therapy in the last few years involving immune checkpoint blockade. As previously mentioned, immune checkpoints are essential for the control of T cell activation and some cancers have found ways of manipulating the inhibitory pathways to stop a T cell response. New drugs named immune checkpoint inhibitors work by blocking these inhibitory pathways being inflicted, consequently allowing $CD8^+$ T cells to be activated and kill cancer cells. There is on-going work to discover the immune checkpoints that seem to be used by different cancers. The CTLA-4 and PD-1 inhibitory pathways have been popular within the field, which has resulted in immune checkpoint blockades being trialled with success for a range of cancers. Ipilimumab is a monoclonal antibody used to target CTLA-4 interaction by binding to the MYPPPY motif (Ramagopal *et al.,* 2017) and has been reported to be effective against a variety of cancers including melanoma, ovarian, prostate and non-small cell lung (Hodi *et al.,* 2010; Hodi *et al.,* 2003; Kwon *et al.,* 2014; Lynch *et al.,* 2012). Similarly, nivolumab is also a monoclonal antibody but is used to target PD-1 and block interaction with its ligands, PD-L1 and PD-L2 (Guo *et al.,* 2017). It has equally been found to be effective against some cancers including melanoma, non-small cell lung, metastatic colorectal cancer and Hodgkin lymphoma (Mahoney *et al.,* 2015; Rizvi *et al.,* 2015; Overman *et al.,* 2017; Ansell, 2017). As both inhibitors were shown to have effect in melanoma patients, trials were conducted, with success, for a combination therapy that is described to be more effective than either agent alone (Larkin *et al.,* 2015). Even though these outcomes look very promising, immune checkpoint blockade therapy still causes many side effects and is reported to have no effect for some cancers. The exact mechanism in which these drugs exert their effect is also still unknown; the improved understanding

of how they affect signalling pathways, dynamics and interactions plus a better understanding of the TME could allow for more optimised treatments.

1.9 Relevance of this project

It is understood that T cells are activated as a result of cognate antigen recognition by the TCR. Consequently, intracellular signalling pathways and transcription factors are activated to control processes such as effector function, differentiation and proliferation**.** Although the effect of antigen dose and antigen-MHC affinity on such processes have been investigated, it is still controversial how these factors affect cell signalling dynamics, gene expression and T cell activation. Zehn *et al*., drew attention to this in their study; it is unclear if a high dose of antigen from low affinity antigen-MHC can elicit the same activation and effector response as high affinity antigen-MHC (Zehn *et al.,* 2009). An additional outstanding topic in the field is how antigen-MHC dose and affinity effects the production of effector molecules, such as IFNγ and PD-1. Understanding these biological effects are vital to broaden the key knowledge of $CD8^+$ T cell function and control, as well as being important for the study of disease. Antigen abundance and affinity for the TCR varies depending on the level of immunogenicity the target holds and ultimately effects the immune response. Knowledge of the downstream effects these factors result in could help in understanding disease and development of treatment. This project aims to address these unknown aspects of T cell biology by investigate how dose, affinity and also time may affect the signalling dynamics leading to gene expression, activation of $CD8^+$ T cells and expression of effector molecules.

Another factor that is known to affect $CD8^+$ T cell response to antigen in disease are the immune checkpoints; some cancers have been described to manipulate these pathways to hide from immune cells. A clinically important immune checkpoint that has become of great interest in the field is PD-1. There is an evolving successful development of using anti-PD-1 as a cancer immunotherapy drug; by blocking PD-1 interacting with its ligands this allows T cells to be activated, hence killing cancer cells (Guo *et al.,* 2017). Although this drug has shown some success in some cancers it is not fully understood how the PD-1 pathway functions to alter signalling, activation and effector function of T cells under normal physiological conditions; this project aims to investigate this. Defining these alterations is important to advance this method of immunotherapy. If signalling dynamics and pathways were defined it may be possible to find any additional pathways that could be targeted using a combination drug so that when the PD-1 pathway is blocked, any recovery or feedback pathways could consequently be blocked.

Along with defining the role of PD-1 to advance the field of cancer immunotherapy, there is also a need to explore the TME effects on T cells function. The way the TME influences T cells to stop an immune response is complex, however this environment is known to be highly immunosuppressive and it has been reported to have the ability to cause T cells dysfunction and exhaustion due to chronic antigen stimulation (Blank *et al.,* 2019). Additionally, it is thought that changes to metabolic demand and epigenetics, as well as the upregulation of immune checkpoints, could be important (Wherry and Kurachi, 2015). It is vital to characterise the effect of this environment on T cells in order to advance the field of immunotherapy; immune checkpoint blockade

may not be as effective if other factors due to the TME overpower its effects. An *in vivo* tumour study will be used in this project in attempt to shed light on this topic.

2. Aims

In order to study antigen regulation of TCR signalling and immune checkpoint expression in health and disease through experiments linked to the above topics, live T cells will be studied through *in vitro* studies and an *in vivo* MC38 tumour model of the microenvironment. The Nr4a1-GFP and Nr4a3-Tocky systems will be used throughout the project; with most experiments enlisting the use of the latter. Use of the novel Nr4a3-Tocky system will allow for temporal analysis of signalling dynamics and $CD8^+$ T cell activation in a range of conditions; the OTI Nr4a3-Tocky system will also give insight when specific antigen is administered. Flow cytometry will be the main method of data collection during the project to analyse the Timer protein and other fluorescent reporters that will be used to fulfil the four main aims that are set out below.

1) Determine how TCR signal strength, duration and affinity for antigen-MHC regulate Nr4a1 and Nr4a3 gene expression in $CD8^+$ T cells.
2) Further investigate how antigen dose, affinity for TCR and time affects the activation dynamics of $CD8^+$ T cells, through TCR signalling analysis, and the expression of IFNγ and PD-1.
3) Examine the *in vitro* functional role of PD-1 in the regulation of cognate $CD8^+$ T cell activation.
4) Explore how T cell activation dynamics (through TCR signalling analysis), immune checkpoints and IFNγ expression are affected by the tumour microenvironment.

3. Materials and methods

3.1 Mice

All animal experiments were performed in accordance with local Animal Welfare and Ethical Review Body at the University of Birmingham (UoB) and under the authority of a Home Office project licence, P18A892E0A. Schedule 1 killing and tissue retrieval was carried out by members of the Bending lab group that hold a PIL licence; Dr David Bending (also carried out *in vivo* mouse work*)* and Thomas Elliot, UoB.

Nr4a3-Tocky mice (Bending *et al.,* 2018), founder line 323 (obtained from Dr. Masahiro Ono, Imperial College London under MTA), were used for the experiments involved with aim 4 of this study. This line was also used to generate F1 OT1 Nr4a3-Tocky Great Smart-17A mice; hereafter known as OTI Nr4a3-Tocky mice. This was done by breeding Nr4a3-Tocky Great-Smart17A (Price *et al.,* 2012) mice to homozygous OTI mice (purchased from Charles River laboratories); mice were used in experiments involved with aims 2, 3 and 4.

Nur77 (Nr4a1)-GFP mice (Moran *et al.,* 2011) were also involved in this project, these mice were bred to Nr4a3-Tocky mice in order to create the Nr4a1-GFP Nr4a3-Tocky line that was used in studies regarding aim 1. Following on from this line, these mice were bred to OTI mice to create the OTI Nr4a1-GFP Nr4a3-Tocky mice, also involved with aim 1.

3.2 Stimulants

In order to stimulate OTI cells, ova peptide was used. OTI T cells have a very high affinity for ova peptide in its native; SII**N**FEKL (Cambridge BioScience), referred to as N4 peptide throughout this project. Two varients that have different amino acids in position 4 are also utilised; SII**Q**FEKL (GL Biochem LTD Shanghai, China); Q4 and SII**V**FEKL (GL Biochem Shanghai, China); V4. Each was chosen to study effect of affinity for TCR; V4 has been shown to be around a 700-fold less potent than N4 with Q4 being in between the two (Zehn *et al.,* 2009). Soluble anti-CD3 (145-2C11, BioLegend) was used for the stimulation of other T cells that don't have ova specific TCR.

3.3 Blockers and inhibitors

Anti-PD1 (IgG2A antibody, clone 29F.1A12, BioLegend) dissolved in 10% RPMI (RPMI1640 + L-glutamine (Gibco) containing 10 % FBS and penicillin/streptomycin (Life Technologies)) was used to block PD-1 interacting with ligands on the T cell surface, consequently allowing for T cell stimulation via TCR signalling. MAC 219 (IgG2A antibody, kind gift from Prof Anne Cooke), also dissolved in 10% RPMI, was used as an isotype to anti-PD1. On the other hand, PP2 (Sigma, 10µM) dissolved in DMSO (Sigma, 0.1%), was used as a Src family kinase inhibitor that results in a diminished TCR signal.

3.4 *In vitro* protocol

In a sterile tissue hood, spleens were forced through and filtered using a 70µm cell strainer (Corning) and 10% RPMI (RPMI1640 + L-glutamine (Gibco) containing 10 % FBS and penicillin/streptomycin (Life Technologies)). The cell suspension was then transferred to a 15ml falcon tube (Corning), centrifuged at 1500 RPM for 5 minutes and the supernatant was removed. A red blood cell (RBC) lysis followed this and was carried out according to manufacturer's instructions (Invitrogen). Cells were then resuspended in 10ml 10% RMPI and a cell count was conducted using a haemocytometer; trypan blue stain was used (Sigma). Cell count was adjusted to 5 million cells/ml.

For all *in vitro* experiments this protocol was followed with adaptations that are highlighted below.

<u>Aim 1 (differential regulation of Nr4a1 and Nr4a3)</u>

For this study either Nur77-GFP Nr4a3-Tocky or OTI Nur77-GFP Nr4a3-Tocky cells were used (see mice section for more details). 100µl of cells were transferred to a 96-well U-bottom plate (Corning) with a range in concentration (that can be seen in figure legends) of stimulant; either 100µl anti-CD3 (145-2C11, BioLegend) or ova peptide, for OTI study; N4 (SIINFEKL, Cambridge BioScience), Q4 (SIIQFEKL, GL Biochem LTD Shanghai, China) and V4 (SIIVFEKL, GL Biochem Shanghai, China). Cells were then incubated at 37°C and 5% CO_2 for 4 hours (anti-CD3) or 5 hours (ova). In order to study the effect of length of signal, PP2 (Sigma, 10µM) inhibitor was added at a final concentration of 10µM with 5µg/ml of anti-CD3. Cells were incubated for a total of 4 hours with PP2 added at the intervals seen in figure legend. Following

incubation, cells were stained using this simple panel for analysis by flow cytometry (BD LSR Fortessa X-20): VD EF780, CD8 BUV395 and CD4 BUV373; further details can be found under flow cytometry analysis. *N.B. mouse line has built in fluorescent markers for Nr4a3-Timer blue, Nr4a3-Timer red and Nr4a1-GFP.*

Aim 2 (effects of peptide dose, affinity and time on $CD8^+$ T cells)

100µl of OTI Nr4a3-Tocky cells were transferred to a 96-well U-bottom plate, along with 50µl of 10% RPMI and 50µl of ova peptide; N4, Q4 or V4. Peptide was added in a range of concentrations for each affinity that can be seen in the figure legends. Cells were additionally plated for an unstimulated sample, unstained sample and a panel of single stains (used in compensation for data analysis); 100µl 10% RPMI was used for these wells. For this study cells were incubated at 37°C and 5% CO_2 for either 4 or 16 hours. After incubation, cells were stained with a cocktail of fluorescent markers and analysis was carried out by flow cytometry. Panel used: VD EF780, CD8 BUV395, Vα2 PerCPCy5.5, CD69 AF700 and PD1 APC; further details can be found under flow cytometry analysis. *N.B. mouse line has built in fluorescent markers for Nr4a3-Timer blue, Nr4a3-Timer red and IFNγ-YFP.*

Aim 3 (functional role of PD-1 in $CD8^+$ T cell activation)

The same protocol was used as aim 2 for this study however, each well contained 100µl of OTI Nr4a3-Tocky cells, 50µl of ova peptide; N4, Q4 or V4 (in varying concentrations) and either 50µl of isotype MAC219 (IgG2A antibody, kind gift from Prof Anne Cooke) or anti-PD1 (IgG2A antibody, clone 29F.1A12, BioLegend) (both used as 20µg/ml). Cells were also only incubated for one time point; 16 hours.

3.5 *In vivo* protocol

In order to study the effect of the TME (aim 4) *in vivo* processes were enlisted. Firstly, the spleen and lymph nodes from OTI Nr4a3-Tocky mice were harvested and a single cell suspension was made. Both tissues were pressed through 70uM cell strainers (Corning), however spleen cells were subject to RBC lysis (as *in vitro* protocol) and then resuspended in 1ml 2% FBS PBS (Sigma), whereas lymph node cells skipped the RBS lysis. All cells were then combined in a 15ml falcon tube and topped up to 10ml for cell count (as described in *in vivo* protocol). The splenocytes were then negatively selected by immunomagnetic separation using the MoJo murine CD8 T cell isolation kit (BioLegend), according to the manufacturer's instructions. 150µl of cells was taken to be analysed by flow cytometry to check the purity of the cell sort. Next these $CD8^+$ T cells were labelled with eFluor (EF)670 proliferation dye (Thermo Fisher); cell density of $1x10^7$ cells/ml stained with 5uM EF670 dye and suspended in sterile PBS (Sigma) ready for transfer to recipient mouse. 2 x 150µl of cells was taken to be analysed by flow cytometry; one to be use as single stain of proliferation dye and one to be stained with full panel as an unstimulated control.

The recipient mice to these cells were Nr4a3-Tocky Great Smart mice, which had previously been injected subcutaneously with MC38-ova tumour cells (colon cancer) that had been allowed to grow for 7 days (Akagi *et al.,* 1997; provided by Prof. David Withers group, UoB; line provided by Dr James Hodge, NIH). The stained OTI Nr4a3-Tocky cells were injected intravenously to these mice by a member of the Withers group.

After 24- and 48-hours tissue was collected; spleen, tumour, draining lymph node and contralateral lymph node. Spleen and lymph nodes were processed as previously described in this protocol. The tumour tissue was first digested using an enzyme mixture of 1.2ml 2% FBS PBS, 12ul of Collagenase D (final concentration 100mg/ml, Sigma) and 12 ul of Dnase (final concentration, 0.1mg/ml Sigma), for each tumour, and incubated in a thermomixer for 20-25 minutes. Digested tumours were then filtered using a cell strainer and 10% RPMI media; resuspended in 200µl of 2% FBS PBS. Cells were then plated (lymph nodes and spleen; 150µl, tumour; 200µl) and 50µl the following staining panel was added for analysis by flow cytometry: VD EF780, CD8 AF700, CD45 BUV395, CD4 BUV737, PD1 PerCP, Lag3 PE-Cy7. *N.B. Nr4a3-Tocky Great Smart (analyse host response) and OTI Nr4a3-Tocky (analyse specific CD8 response to ova-tumour) mouse lines have built in fluorescent markers for Nr4a3-Timer blue, Nr4a3-Timer red and IFNγ-YFP. OTI CD8$^+$ T cells were also stained with EF670 APC proliferation dye before IV injection of cells.*

3.6 Flow cytometry analysis

Following the staining and washing of cells in 96-well U-bottom plates (as describe above), analysis was carried out by flow cytometry using a BD LSR Fortessa X-20 instrument, FACS tubes (BD Biosciences) and 2% FBS PBS as buffer (Sigma). As mentioned previously, the Nr4a3-Tocky and Nr4a1-GFP mouse lines have some built in reporters. Timer blue protein (Tocky) was detected in the blue (450/40 nm) channel; excited off the 405 nm laser. Timer red protein (Tocky) was detected in the mCherry (610/20 nm) channel; excited off the 561 nm laser. IFNγ -YFP and Nr4a1-

GFP were both detected in the FITC/GFP (530/30 nm) channel; excited off the 488 nm laser.

The following directly conjugated fluorescent antibodies were used throughout this project to stain cells and have been stated where relevant in protocols. CD8 BUV395 *(clone 53-6.7, BD Biosciences),* CD8 Alexa Fluor 700 *(clone 53-6.7, BioLegend),* CD4 BUV737 *(clone GK1.5, BD Biosciences),* TCR Vα2 PerCP/Cyanine5.5 *(clone B20.1, BioLegend),* fixable viability dye (VD) eFluor 780 *(Thermo Fisher)*, CD69 Alexa Fluor 700 (*clone H1.2F3, BioLegend)*, CD45 BUV395 (*clone 30-F11, BioLegend)*, PD1 PerCP (*clone 29F.1A12, BioLegend)*, PD1 APC (*clone 29F.1A12, BioLegend)*, Lag3 Pe-Cy7 (*clone C9B7W, BioLegend),* EF670 APC (*proliferation dye, Thermo Fisher).*

3.7 Gating strategy for flow cytometry analysis

Flow cytometry data was collected and subsequently analysed using FlowJo software. Gates were drawn around different populations of cells in order to obtain plots and graphs used within the results section; lymphocytes were first gated, then single cells, live cells (enlisting the use of viability dye) and finally $CD8^+$ T cells; in some experiments these were OTI cells and in others just CD8 cells. The following gating strategies (Figures 4a and 4b) were used to gate cells in order to analyse T cell receptor signalling and cell activation. Setting the axis to CD8 vs various markers (named in flow cytometry analysis section) made it possible to determine how immune checkpoint expression and IFNγ expression were affected under different conditions. Numerical data was pulled out of these plots and manipulated in order to produce graphs.

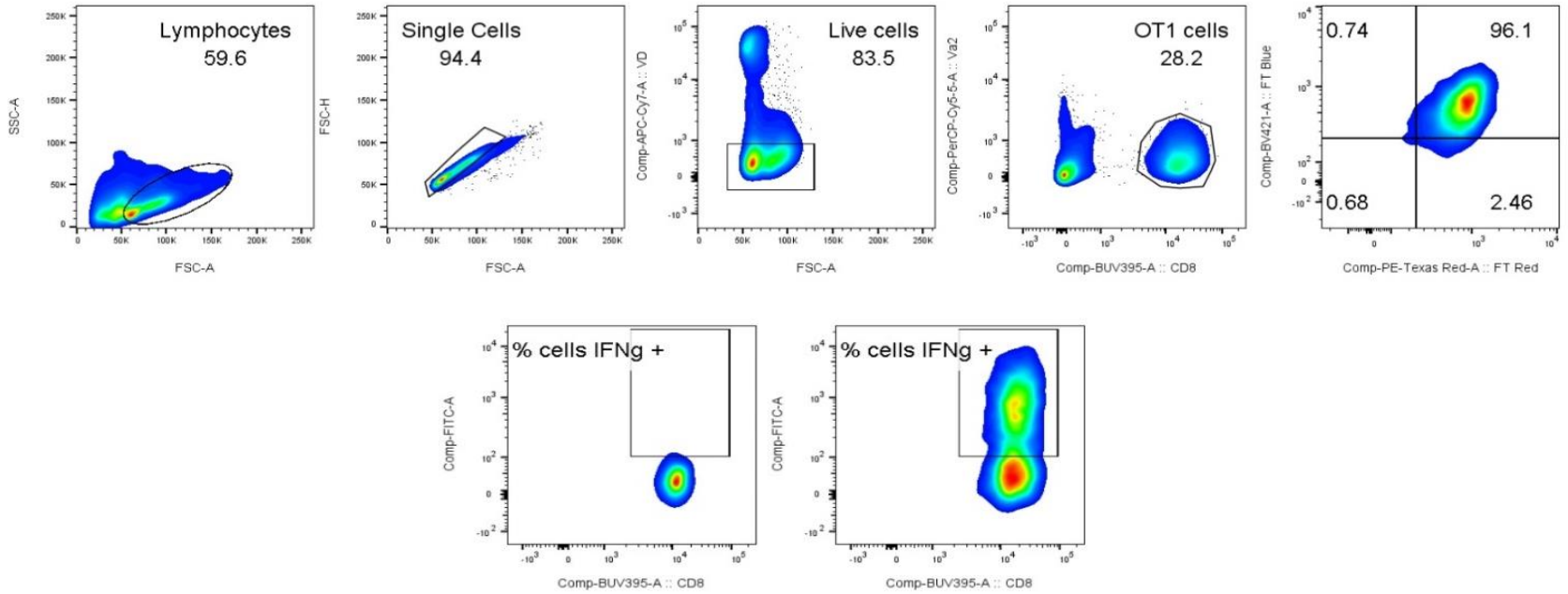


Figure 4a. Gating strategy used for experiments regarding aims 1, 2 and 3

Data collected was entered into FlowJo software and compensated using single stains, unstained and unstimulated samples ran for each experiment. Lymphocytes were first gated using FSC-A vs SSC-A. Then single cells were gated using FCS-A vs FSC-H. Following this, live cells were selected using FSC-A vs EF780 viability dye; if cells die, more dye would enter, therefore cells lower on the plot are live cells compared to higher up. After this, $CD8^+$ T cells were selected; if the experiment was carried out with an OTI mouse line (aims 2 and 3) cells were gated using CD8 BUV395 vs Vα2 PerCPCy5.5 however if it was not axis CD8 BUV395 vs CD4 BUV737 were used. Plots were finally visualised as Timer red vs Timer blue or Timer blue vs Nr4a1-GFP in order to evaluate TCR signalling as well as cell activation. Cross gates were set using an unstimulated sample. The second line of this figure shows how the % of $CD8^+$ T cells that express different markers were gated and analysed by comparison to an unstimulated sample.

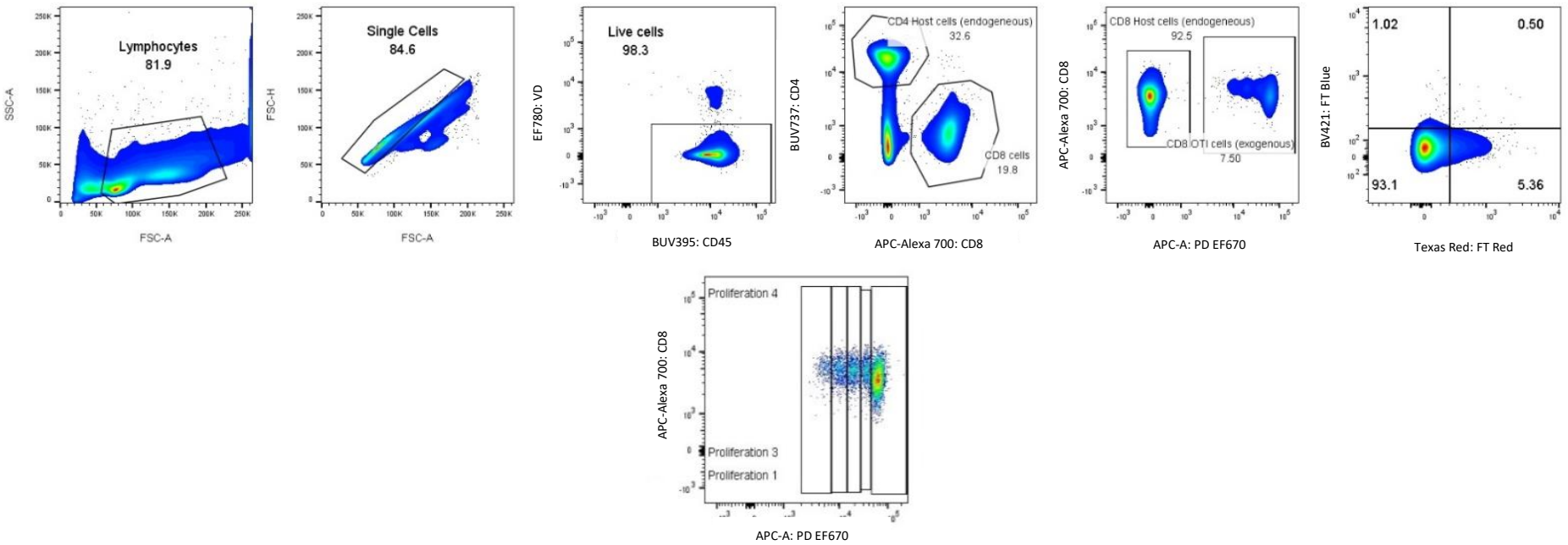


Figure 4b. Gating strategy used for experiment regarding aim 4

Gating is similar to shown in figure 4b however for the selection of live cells, CD45 BUV395 vs EF780 viability dye was used. Also, as this experiment data involved $CD8^+$ OTI cells, $CD8^+$ host T cells and $CD4^+$ host T cells different gates had to be drawn. The different cell populations were drawn out using CD8 AF700 vs CD4 BUV737. $CD4^+$ host T cells were selected straight through this gate to be analysed using Timer red vs Timer blue and other additional markers for expression of molecules. Again, cross gates were set using an unstimulated sample. After the CD8 gate was chosen cells were additionally gated using proliferation dye APC EF670 vs AF700 CD8; OTI cells that were exogenous (IV injected) had proliferation dye, whereas host cells did not. Populations were sorted into host $CD8^+$ T cells and OTI $CD8^+$ T cells. Both populations of $CD8^+$ cells had the timer gates set and molecule expression calculated. Due to the OTI $CD8^+$ cells having a proliferation dye it was also possible to gate each proliferation of cells that was thought to be seen (bottom row of figure).

3.8 Statistical analysis and data visualisation

Statistical analysis and data visualisation were carried out using GraphPad Prism software. To produce dose response curve graphs, data was first normalised using the following equation: ((Response-Background)/(Maximum-Background)) x 100. Then the non-linear regression fit was performed using the [agonist] vs normalised response option. Following this, the EC50 and curve fit statistics were calculated. To calculate fold difference in gene expression the following equation was used: (final value - initial value) / initial value. Two-way ANOVA with Sidak's multiple comparison tests were used for the statistical analysis of all data; comparison of two or more means. Variance is reported as mean + SEM as error bars on all graphs and statistical significance between conditions or tissues is visualised as * p=<0.05, ** p=<0.01, *** p=<0.001, **** p=<0.0001.

4. Results

4.1 Differential expression of Nr4a1 and Nr4a3 is only modestly driven by sensitivity to TCR signal strength.

As mentioned previously, it has been found that Nr4a1 and Nr4a3 are regulated by different TCR signalling pathways; Nr4a3 was shown to be induced by the calcineurin/NFAT pathway whereas Nr4a1 is not (Jennings *et al.,* 2020). This same study also describes the differential expression of Nr4a1 and Nr4a3 in developing thymocytes in the thymus. Jennings *et al.,* described that there was an increase in expression of Nr4a1 during positive selection, notably in the TCRβ^{high} DP fraction, but Nr4a3 was absent. Nr4a3 however was seen to be highly expressed in the SP CD4^{+} CD25^{high} fraction, which is populated by Tregs; a group of cells that are known to receive strong TCR signals (Jennings et al., 2020). The same was found in populations of cells in the periphery; CD8^{+} T cells expressed intermediate levels of Nr4a1 but no Nr4a3, whereas CD4^{+} CD25^{+} Tregs expressed high levels of Nr4a3; previously also reported by Bending *et al.,* (Bending *et al.,* 2018).

These findings collectively suggest that Nr4a1 and Nr4a3 are regulated by different signalling pathways and that the differential expression of the two genes could be due to TCR signal strength. It can be hypothesised that Nr4a1 is the more sensitive of the two genes as it has been shown to be expressed during positive selection, which is associated with low strength TCR signalling events. On the other hand, Nr4a3 has been shown to be expressed in cells that have undergone Treg selection, which is associated with high strength TCR signals and therefore is hypothesised to be the less sensitive of the two.

To investigate the effect of TCR signal strength on gene expression, splenocytes from a dual reporter Nr4a1-GFP Nr4a3-Tocky mouse were used. Cells were stimulated with a dose titration of soluble anti-CD3 for 4 hours, then analysed using flow cytometry to see how gene expression in $CD8^+$ T cells were affected (Figure 5). Figures 5A, B and C show that the proportion of cells that express Nr4a1 GFP increase at a lower dose (0.008µg/ml) than cells expressing Nr4a3 Timer blue (0.04-0.2µg/ml). This indicates that TCR signal strength may have a role in regulating differential gene expression and that Nr4a1 is more sensitive to TCR signals than Nr4a3. It can also be seen that even with the highest dose of anti-CD3 there is a lower proportion of Nr4a3 Timer $blue^+$ cells compared to Nr4a1 GFP^+ cells; this could indicate the effect of signal strength on gene expression however it could also be due to differences in the fluorescent markers. Jennings *et al.,* report a partial quenching of Nr4a3 Tocky in this mouse line, when Nr4a3-Tocky BAC expression is only a single heterozygous block (Jennings *et al.,* 2020 in press).

In order to compare the two systems head to head, I generated dose response curves of Nr4a1 GFP and Nr4a3 Timer blue expression in $CD8^+$ T cells using normalised expression values to equate for differences in the reporter systems (Figure 5D). Figure 5D again shows that there is a big increase in Nr4a1 GFP^+ cells compared to Nr4a3 Timer $blue^+$ cells at a lower dose. Furthermore, there is a high significant difference in the proportions of cells expressing the two reporters between the doses of 0.0016-0.04µg/ml. These findings support the ideas drawn from figures 5A, B and C; Nr4a1 is more sensitive to TCR signal strength than Nr4a3 and TCR signal strength could contribute to differential gene expression. To confirm whether

signal strength could regulate differential gene expression, the EC50 values for each gene expression was calculated; the amount of anti-CD3 needed to achieve 50% of normalised expression. This revealed values that had a 3-fold difference in expression of Nr4a1 and Nr4a3. Although this 3-fold difference and increased sensitivity of Nr4a1 to anti-CD3 suggests that TCR signal strength has an effect on differential gene expression, this modest difference is unlikely to be the sole reason for the different expression pattens of Nr4a1 and Nr4a3 *in vivo*.

Figure 5

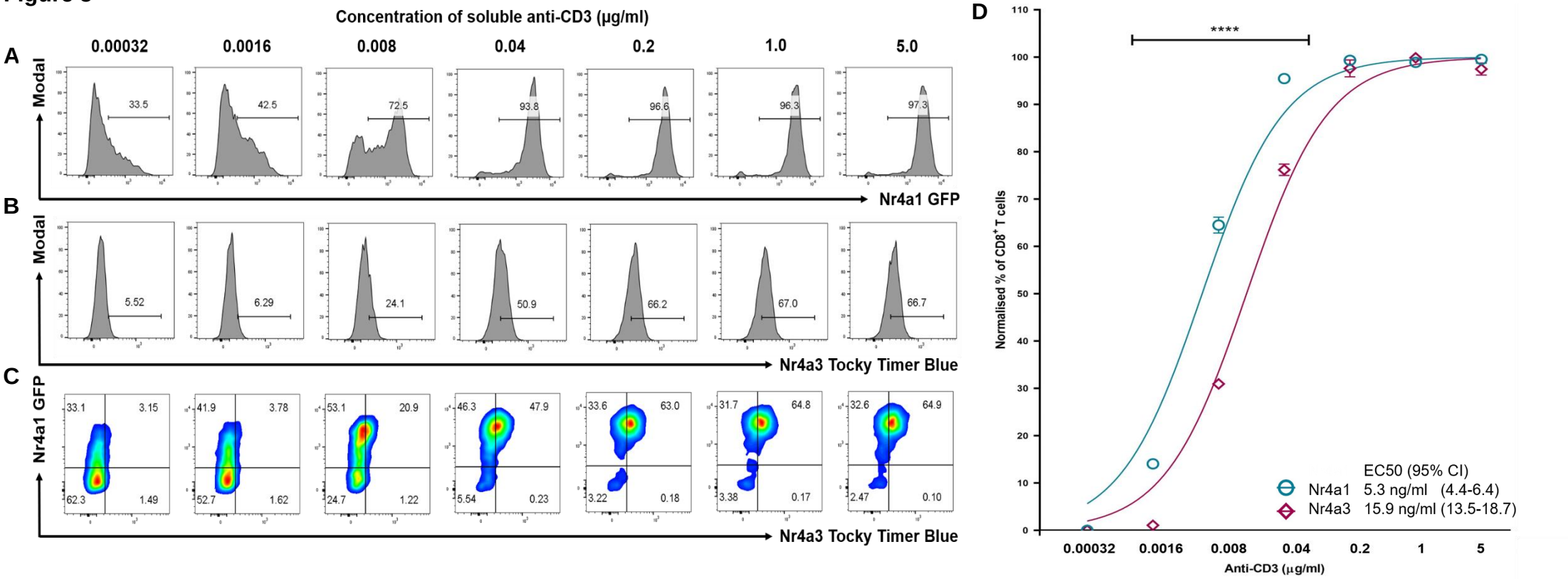


Figure 5. Differential expression of Nr4a1 and Nr4a3 is only modestly driven by sensitivity to TCR signal strength.

Nr4a1-GFP Nr4a3-Tocky (heterozygous mouse line) splenocytes were stimulated with the noted concentrations of soluble anti-CD3 for 4 hours. Live cells were analysed by flow cytometry using the gating shown in figure 4a to investigate $CD8^+$ T cells. Histograms showing the proportion of cells that are either Nr4a1 GFP^+ **(A) or** Nr4a3 Tocky Timer $blue^+$ **(B)** with increasing doses of anti-CD3. **(C)** Plots show expression of Nr4a3 Tocky Timer blue vs Nr4a1 GFP. Cross gates set using lowest dose of stimulant. **(D)** Summary graph showing the normalised % of $CD8^+$ T cells that are either Nr4a1 GFP^+ (*blue line*) or Nr4a3 Timer $blue^+$ (*maroon line*) with increasing doses of anti-CD3. Error bars represent mean+SEM, n=3. Non-linear regression fit, EC50 and 95% confidence intervals (CI) were calculated using Prism. Statistical analysis by two-way ANOVA with Sidak's multiple comparison tests. Significant difference ($p<0.05$) indicated as ****=$p<0.0001$.

4.2 Duration of TCR signal is likely to be responsible for differential expression of Nr4a1 and Nr4a3, with Nr4a1 expression requiring a shorter length of signal.

Duration of TCR signalling has been linked to lineage differentiation during T cell development (Liu and Bosselut, 2004) and is also thought to play a role in the magnitude of primary T cell response (Prlic *et al.,* 2006); processes both Nr4a1 and Nr4a3 are known to be expressed in. Furthermore, Nr4a3 has been shown to be highly and persistently expressed in thymic Tregs (Bending *et al.,* 2018). As these findings link TCR signal duration to differential gene expression, the hypothesis that signal length may regulate differential expression of Nr4a1 and Nr4a3 can be drawn.

To study the effect of TCR duration on gene expression, splenocytes from a dual reporter Nr4a1-GFP Nr4a3-Tocky mouse were again used. Duration of TCR signalling was controlled by the addition of a Src family kinase inhibitor, PP2 (Hanke *et al.,* 1996); both Nr4a1 and Nr4a3 are Src family kinase dependent hence why this inhibitor was chosen. Cells were all stimulated with the same concentration of anti-CD3 (5µg/ml) and PP2 was added at different time intervals to stop TCR signalling; all cells remained in culture for 4 hours to allow time for individual reporter translation and accumulation. Live cells were then analysed using flow cytometry to determine the effect of TCR signal duration on gene expression in $CD8^+$ T cells (Figure 6). Figure 6A, B and C show that with just 2.5-5 minutes of TCR signalling there is an increase in the proportion of Nr4a1 GFP^+ cells, this proportion continues to grow and it can be seen that with 30-60 minutes of TCR stimulation more than 90% of cells express Nr4a1 GFP (Figure 6A). Unlike Nr4a1 GFP^+ cells, the proportion of Nr4a3 Timer $blue^+$ cells only started to increase after 30 minutes of signalling and did not

reach maximum until 120-240 minutes, even then the proportion of cells did not reach the same level as Nr4a1 GFP$^+$ cells. These results highly suggest that TCR signal duration may be responsible for the differential expression of Nr4a1 and Nr4a3. Additionally, it can also be seen that Nr4a1 requires a much shorter signal (2.5 minutes) to be expressed than Nr4a3 (30-60 minutes) (Figures 6A-D).

Figure 6D shows the normalised % CD8$^+$ T cells that express Nr4a1 GFP and Nr4a3 Timer blue. In line with the results shown in figures 6A, B and C, it can again be seen that there is a gradual steep increase in proportion of cells expressing Nr4a1 GFP, whereas proportions of cells expressing Nr4a3 Timer blue stay fairly low with a gradual increase until around the 30-60 minutes mark where the proportion of cells begins to increase. An intermediate significant difference in gene expression can be seen with even 10 minutes of signalling and rises to be a highly significant difference when signal lasts for 15-60 minutes. As this highly significant difference in expression of the two gene in the same time frame that the proportion of Nr4a1 GFP$^+$ cell reach maximum and Nr4a3 Timer blue$^+$ cells only just start to increase (2.5-60 minutes) this strongly supports the idea that TCR signal duration is likely to be the cause of differential gene expression in CD8$^+$ T cells. Along with previous findings of this project it can now be collectively said that expression of Nr4a1 is both more sensitive to TCR signal strength and requires a shorter signal whereas, expression of Nr4a3 is less sensitive to signal strength and needs a much longer signal to be expressed. These new findings support the ideas drawn by Jennings *et al*., that Nr4a1 expression is due to low strength signalling events which are seen during positive selection whereas Nr4a3 expression, seen in Tregs that result from negative selection events, requires a much stronger signal. Building on this premise, it can

now also be suggested that these positive selection events could be as short as 2.5 minutes however events during negative selection that lead to Treg development must remain for 30 minutes or more.

Figure 6

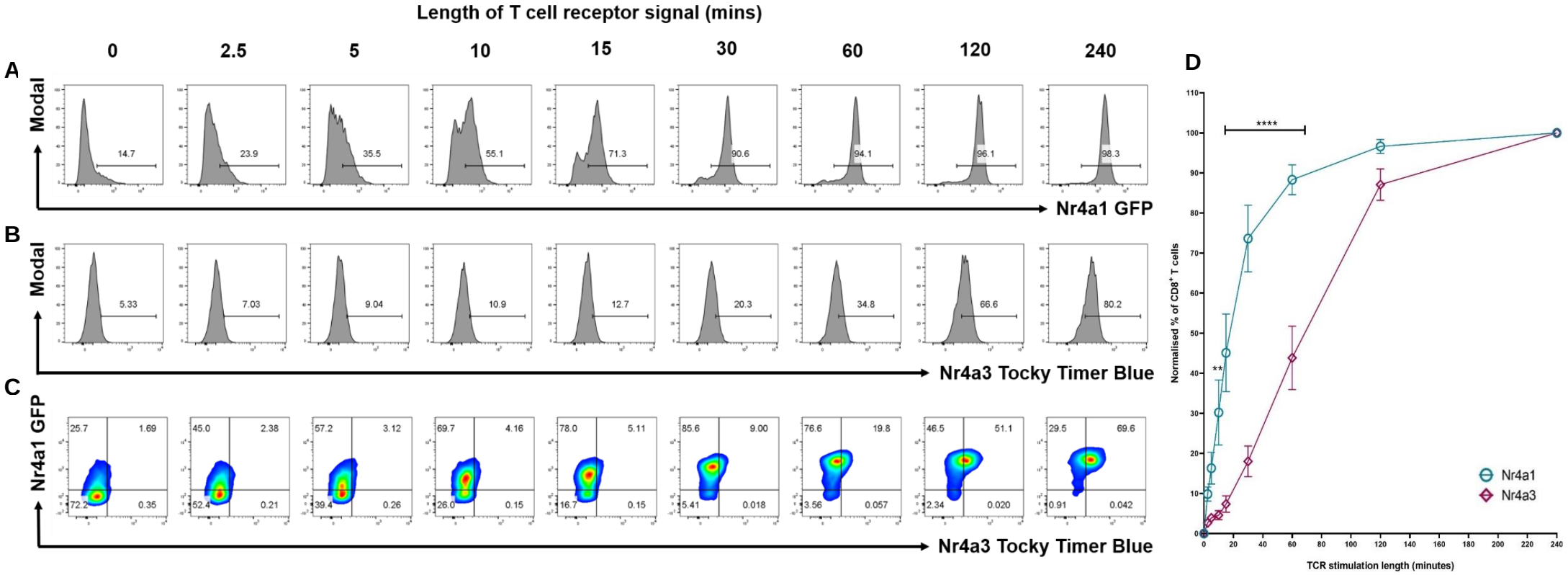


Figure 6. Duration of TCR signal is likely to be responsible for differential expression of Nr4a1 and Nr4a3, with Nr4a1 expression requiring a shorter length of signal.

Nr4a1-GFP Nr4a3-Tocky splenocytes were stimulated with 5µg/ml of soluble anti-CD3. At the indicated time interval, PP2 (Src family kinase inhibitor) was added, at a final concentration of 10µM, to stop TCR signalling. All cells remained in culture for 4 hours to allow fluorescent marker translation and accumulation. Live cells were then analysed using flow cytometry using the gating strategy shown in figure 4a to investigate $CD8^+$ T cells. **(A)** Histograms showing the proportion of cells that are either Nr4a1 GFP^+ **(A) or** Nr4a3 Tocky Timer $blue^+$ **(B)** in response to different lengths of TCR signal. **(C)** Plots show expression of Nr4a3 Timer blue vs Nr4a1 GFP. Cross gates set using 'unstimulated'; PP2 added at 0 minutes. **(D)** Summary graph showing the normalised % of $CD8^+$ T cells that are either Nr4a1 GFP^+ (*blue line*) or Nr4a3 Timer $blue^+$ (*maroon line*) as TCR duration increases. Error bars represent mean+SEM, n=3. Statistical analysis by two-way ANOVA with Sidak's multiple comparison tests. Significant difference ($p<0.05$) indicated as ****=$p<0.0001$, **=p 0.001-0.01.

4.3 TCR affinity for antigen-MHC has a small effect on differential expression of Nr4a1 and Nr4a3, but is linked to an alteration in dose of antigen needed to cause reporter expression.

TCR affinity for antigen-MHC and antigen dose have been shown to be vital for T cell activation and functional outcome. Most notably, affinity for antigen-MHC is relied upon to determine if antigen is self or non-self in the periphery; to control T cell response and in the thymus; to teach developing thymocytes self-tolerance. As affinity plays such a prominent role in the activation of T cells and in thymocyte development, it can be hypothesised that it may also have an effect on the differential expression of downstream TCR signalling genes, such as Nr4a1 and Nr4a3.

In order to study the effect of TCR affinity to antigen-MHC on differential gene expression, a new mouse line was generated. OTI mice express transgenic TCRs that have a high affinity for ovalbumin peptide (ova). OTI mice were crossed with the dual reporter Nr4a1-GFP Nr4a3-Tocky mice to create a model that reports both Nr4a1 GFP and Nr4a3 Tocky in response to ova peptide stimulation. As mentioned, ova peptide has a very high affinity for the TCR epitope (SII**N**FEKL; **N4**), however by mutating the fourth position amino acid, different ova variants can be made with varying levels of affinity: SII**Q**FEKL; **Q4**, intermediate affinity and SII**V**FEKL; **V4**, low affinity. Importantly these do not affect the binding of the peptides to MHC. To investigate how different affinity variants effect gene expression, splenocytes from the dual reporter, ova specific, OTI Nr4a1-GFP Nr4a3-Tocky mice were used. Cells were stimulated with a dose titration of these different affinity ova peptides for 5

hours and subsequently analysed by flow cytometry to see how gene expression in $CD8^+$ T cells was affected (Figure 7).

Figures 7 A-D very clearly shows that affinity of peptide is directly linked to the dose needed to cause gene expression in cells; N4 requires the lowest dose of antigen (10^{-5} µM), Q4 requires a slightly higher dose (10^{-4} µM) and V4 requires a higher dose again (10^{-2} µM). Interestingly it can also be seen in figure 7A that as the proportion of cells that express Nr4a1 GFP increases, so does the proportion of Nr4a3 Timer $blue^+$ cells. This can be seen clearly when cells are stimulated with higher affinity peptides, N4 and Q4, however this simultaneous expression of genes is shown to be slightly different with low affinity V4; the proportion Nr4a1 GFP^+ cells increase before the proportion of Nr4a3 Timer $blue^+$ cells start to rapidly rise. This could indicate that affinity has an effect on differential gene expression however it is more likely that the transient nature of low affinity TCR signalling is responsible (see below).

Figures 7B, C and D support the ideas drawn from figure A. It can be seen that both N4 (Figure 7B) and Q4 (Figure 7C) require a much lower dose of antigen to begin expressing both Nr4a1 GFP and Nr4a3 Timer blue compared to V4 (Figure 7D). This data further shows that stimulation with high doses of N4 (>10^{-3} µM) results in 100% of cells expressing both genes, Q4 (>10^{-2} µM) results in 95% or more and V4 (10^0 µM) results in approximately 90%. Although statistical analysis shows that Nr4a1 and Nr4a3 are differential expressed with high significance at these stimulation doses, dose response curves fitted to the normalised percentage of $CD8^+$ T cells show that cells stimulated with N4, Q4 or V4 have small fold differences in EC50 values (0.32, 0.86 and 2.33, respectively). The slight rise in fold difference and also the significant

expression of Nr4a1 compared to Nr4a3 at 10^{-1}µM of V4 (Figure 7D) is more likely to be due to TCR signal duration rather than effect of affinity, as mentioned previously. As V4 is a low affinity peptide it is thought that signalling is more transient and as previously described in this project, signal duration is a prominent regulator in differential expression of Nr4a1 and Nr4a3, hence it can be concluded that the differential response with V4 is more likely to be caused by duration of TCR signal than affinity. With this reasoning it can be concluded that affinity of the TCR to antigen-MHC seems to have a small effect on Nr4a1 and Nr4a3 differential expression but does however affect the dose of antigen needed to cause gene expression through TCR signalling.

The three studies mentioned in these previous sections have aided in determining how TCR signal strength, duration and affinity for antigen-MHC regulate Nr4a1 and Nr4a3 gene expression in $CD8^+$ T cells (*aim 1)* and the following conclusions can be drawn. The differential expression of Nr4a1 and Nr4a3 is likely due to the duration of the TCR signal rather than signal strength or affinity for antigen-MHC. It however can be suggested that Nr4a1 expression is more sensitive to signal strength and duration than Nr4a3. Additionally, it was also found that affinity for antigen-MHC alters the dose of antigen needed for the expression of both genes.

N.B. The data included in sections 4.1, 4.2 and 4.3 was kindly collected by Dr David Bending and Thomas Elliot of the Bending group (UoB) due to COVID-19 restrictions, however I was involved with planning of experiments and carried out all post-collection data analysis.

Figure 7

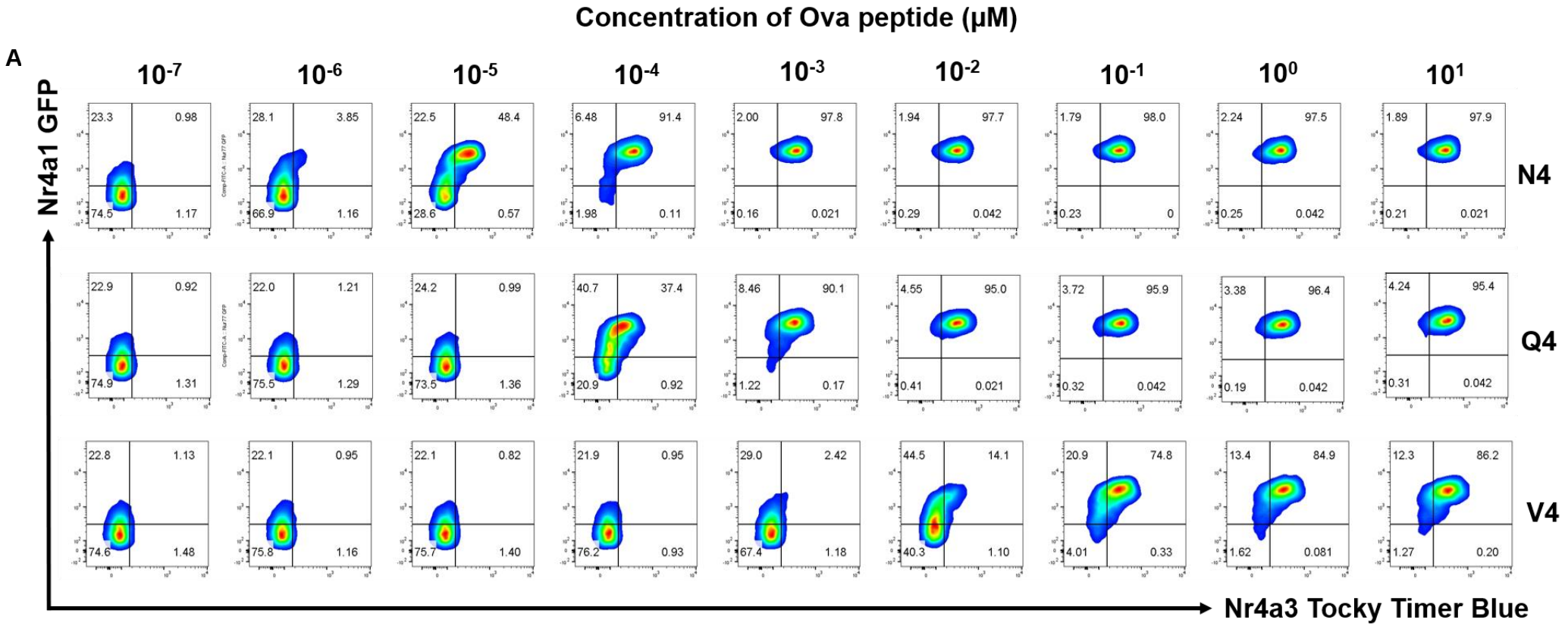

Concentration of Ova peptide (μM)
A
10-7
10-6
10-5
10-4
10-3
10-2
10-1
100
101
Nr4a1 GFP
N4
Q4
V4
Nr4a3 Tocky Timer Blue
23.3 0.98 74.5 1.17
28.1 3.85 66.9 1.16
22.5 48.4 28.6 0.57
6.48 91.4 1.98 0.11
2.00 97.8 0.16 0.021
1.94 97.7 0.29 0.042
1.79 98.0 0.23 0
2.24 97.5 0.25 0.042
1.89 97.9 0.21 0.021
22.9 0.92 74.9 1.31
22.0 1.21 75.5 1.29
24.2 0.99 73.5 1.36
40.7 37.4 20.9 0.92
8.46 90.1 1.22 0.17
4.55 95.0 0.41 0.021
3.72 95.9 0.32 0.042
3.38 96.4 0.19 0.042
4.24 95.4 0.31 0.042
22.8 1.13 74.6 1.48
22.1 0.95 75.8 1.16
22.1 0.82 75.7 1.40
21.9 0.95 76.2 0.93
29.0 2.42 67.4 1.18
44.5 14.1 40.3 1.10
20.9 74.8 4.01 0.33
13.4 84.9 1.62 0.081
12.3 86.2 1.27 0.20

Figure 7 cont.

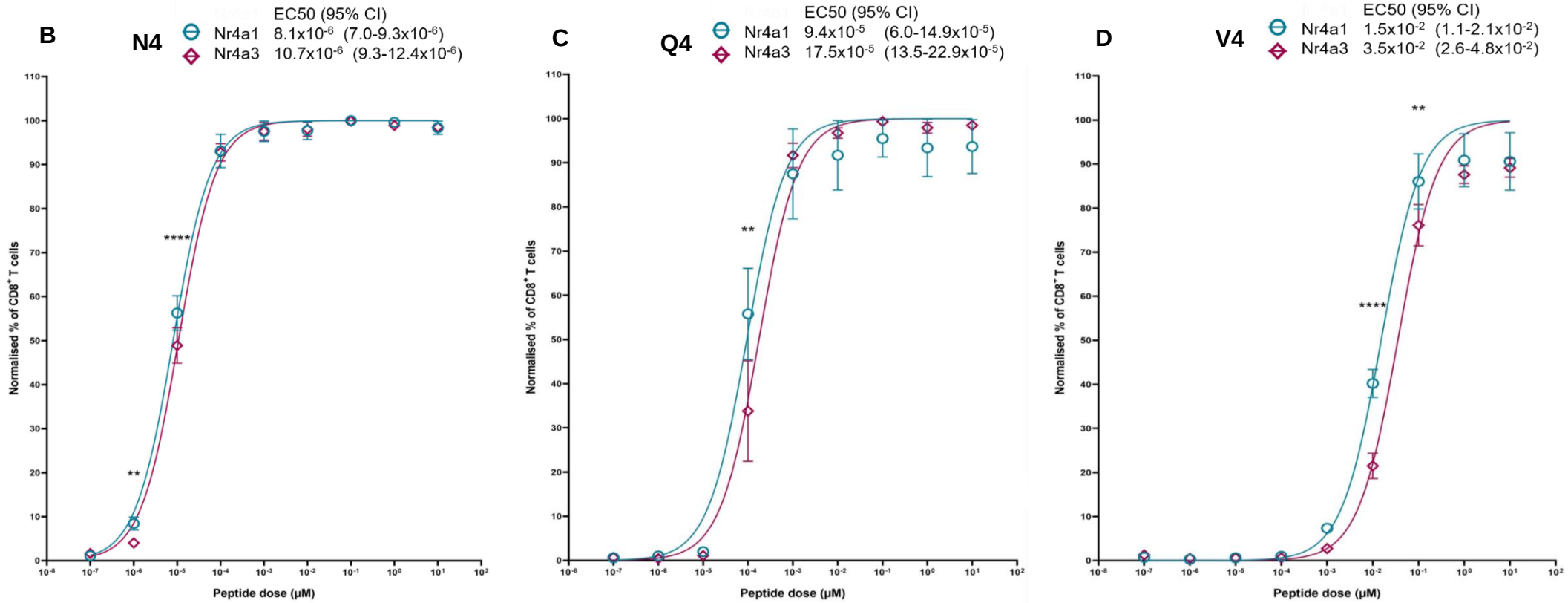


Figure 7. TCR affinity for antigen-MHC has a small effect on differential expression of Nr4a1 and Nr4a3, but is linked to an alteration in dose of antigen needed to cause reporter expression.

OTI Nr4a1-GFP Nr4a3-Tocky splenocytes were stimulated with a dose titration of ova peptide variants N4 (SII**N**FEKL, high affinity for TCR epitope), Q4 (SII**Q**FEKL, intermediate affinity) and V4 (SII**V**FEKL, low affinity) for 5 hours at the concentrations noted. Live cells were then analysed by flow cytometry using the gating shown in figure 4a to investigate OTI $CD8^+$ T cells. **(A)** Plots show expression of Nr4a3 Tocky Timer blue vs Nr4a1 GFP for the range of concentrations tested for each affinity peptide. Cross gates set using lowest dose of stimulant. **(B, C, D)** Summary graphs show the normalised % of $CD8^+$ T cells that are either Nr4a1 GFP^+ (*blue line*) or Nr4a3 Timer $blue^+$ (*maroon line*) with increasing doses of either N4 **(B)**, Q4 **(C)** or V4 **(D)**. Error bars represent mean+SEM, n=2. Non-linear regression fit, EC50 and 95% confidence intervals (CI) were calculated using Prism. Statistical analysis by two-way ANOVA with Sidak's multiple comparison tests. Significant difference ($p<0.05$) indicated as ****=$p<0.0001$, **=p 0.001-0.01.

4.4 Antigen dose, affinity for TCR and time all have an effect on TCR signalling which leads to differential activation of CD8$^+$ T cell and the expression of effector molecules, IFNγ and PD-1.

As concluded from the previous experiments of this project, duration of TCR signal is likely to cause the differential expression of downstream TCR genes Nr4a1 and Nr4a3. It was further found that these genes have different sensitivities to signal strength and duration as well as, affinity for antigen-MHC directly effecting expression depending on dose. It is well established that both dose of antigen and TCR affinity for antigen-MHC are important for activation and effector function of T cells, as mentioned above, however it is still controversial exactly how these factors effect signalling dynamics to result in CD8$^+$ T cell activation and expression of molecules, such as IFNγ and PD-1, to results in effector function.

To investigate this, OTI Nr4a3 Tocky Great Smart mice were used. As this reporter system employs the use of a fluorescent Timer protein this allows for temporal analysis of cell activation following TCR signalling. Expression of the Timer blue protein in cells indicates a new TCR signal however, after 4 hours this blue protein matures into Timer red protein which indicates an arrested signal, a cell expressing both Timer blue and red hence indicates that cells have received a new signal within the last 4 hours (blue) but have also previously received a signal (red); indicates persistent TCR signalling (Figure 3). Additionally, this system has a built-in fluorescent reporter for IFNγ expression (IFNγ-YFP, Price *et al.,* 2012) which allows for the analysis of the cytokine expression following TCR signalling. Furthermore, due to these cells expressing the transgenic OTI TCR it made it possible to study the effects of antigen dose and affinity by using a range in concentrations of different

affinity ova peptides; N4 (high affinity) and Q4 (intermediate) and V4 (low). Cells were stimulated with a dose titration of these different affinity ova peptides for either 4 or 16 hours and then analysed by flow cytometry after being stained with a panel which included fluorescent markers for CD69 (marker of early cell activation) and PD-1 (marker of inhibitory immune checkpoint). Built-in reporters Timer blue and red used to study T cell activation dynamics and IFNγ-YFP used to study production of the pro-inflammatory cytokine. It can be seen when looking at T cell activation dynamics either 4 or 16 hours after stimulation began that there are some similarities due to dose and affinity of antigen, but also some differences.

4.4.1 Activation dynamics of $CD8^+$ T cell after 4 hours.

The 4-hour timepoint analysis in this study was used to observe changes that occur during early T cell signalling and activation. As shown in figures 8A and C there is a very small proportion of cells that are producing Timer red and receiving either a persistent or arrested signal (Figures 8F and G), this however is due to nature of the Timer protein the Tocky system utilises; the blue protein does not begin to mature to red until around 4 hours. On the other hand, figures 8A and B show that a different dose of peptide is required to cause an increase in the proportion of Timer $blue^+$ cells, indicating a new TCR signal (Figure 8E), depending on the affinity of peptide stimulated with. The high affinity cognate peptide, N4 requires just 10^{-5}µM. A small percentages of Timer red^+ cells can be seen at 10^{-6}µM (and also at 10^{-3}µM V4) meaning these cells have arrested signalling (Figure 8G), hence indicating this dose is not enough to cause activation (Figure 8E and F). Intermediate affinity peptide Q4 is shown to require 10^{-4}µM and low affinity peptide V4 requires 10^{-2}µM. These

findings are in line with what was previously seen in study 4.3 and indicate that in order to activate cells through TCR signalling, the affinity of the antigen determines what dose is required. It can also be seen that after the threshold for activation is overcome, a rise in dose of peptide causes a rapid increase the proportion of cells receiving a new TCR signal (Timer blue^{+} cells; Figure 8A and E) and hence activating (Figure 8D). As antigen dose continues to increase, the proportion of Timer blue^{+} cells do too, until a maximum is reached. As seen with activation thresholds, the dose of antigen that is needed for the maximum proportion of Timer blue^{+} cells is also affinity dependant; N4 (10^{-2}µM), Q4 (10^{-1}µM) and V4 (10^{1}µM). Furthermore, figure 8A and B show that affinity also highly significantly effects the proportion of cells that reach maximum Timer blue expression and hence maximum cell activation (Figure 8D) with 4 hours of stimulation, due to new TCR signals (Figure 8E-G). When cells were stimulated with high doses of N4 approximately 90% of cells show to be Timer blue^{+} however with Q4 a maximum of only approximately 75% is reached and even more drastic, with V4 a maximum proportion of only 50% of cells express Timer blue (Figure 8A). It can be seen with N4 and Q4 that these maximal proportion of Timer blue^{+} cells persist and activate for 4 or 3 (respectively) increasing doses of peptide (Figure 8A and D) and it can be thought that V4 would also follow this trend (Figure 8B). These results collectively indicate that although dose of peptide is linked to the proportion of cells that receive a new TCR signal and hence cause CD8^{+} T cell activation, the maximum proportion of cells that can be activated by a new signal depends on affinity of TCR to antigen-MHC.

Figure 8

A

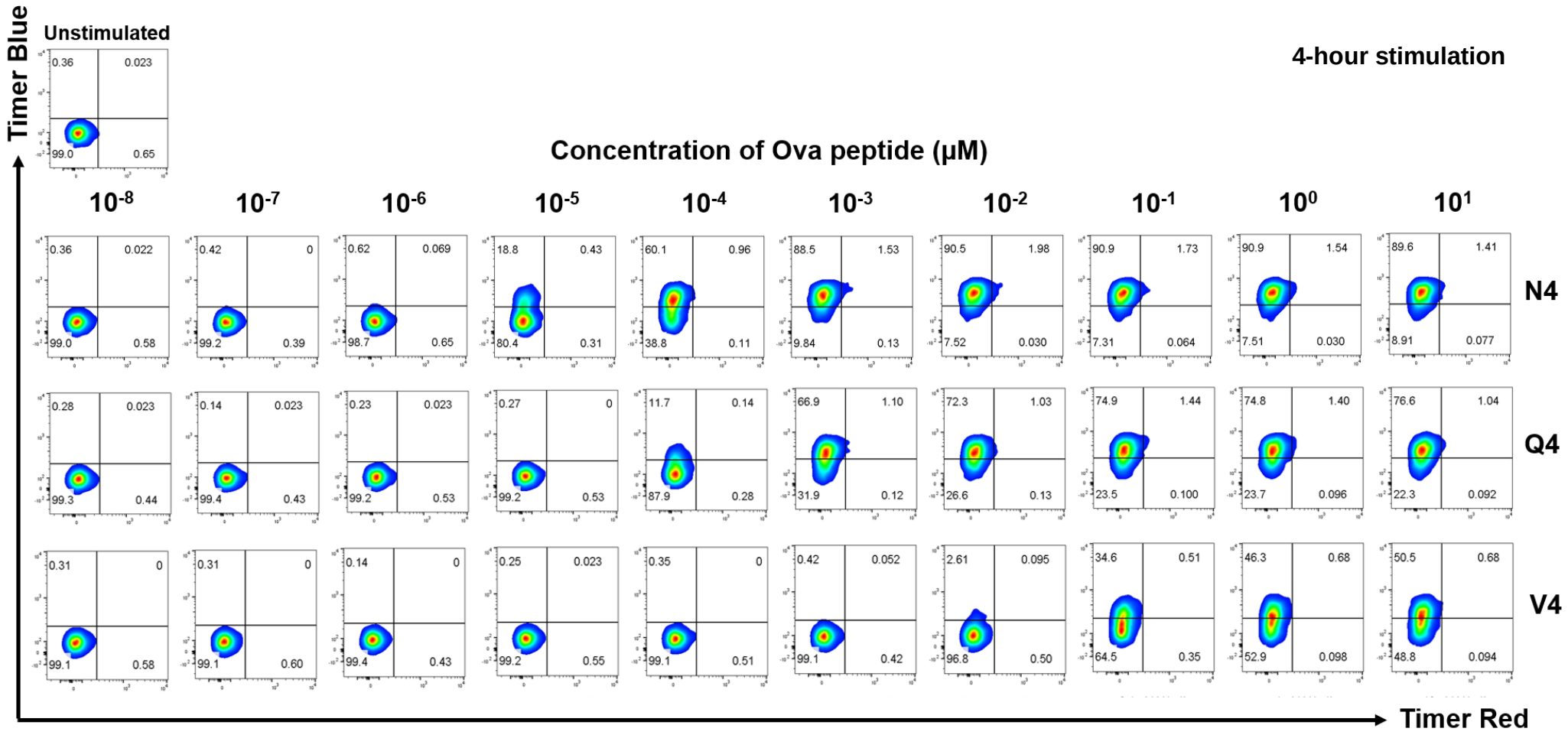

Timer Blue
Unstimulated
0.36
0.023
99.0
0.65
4-hour stimulation
Concentration of Ova peptide (μM)
10-8
10-7
10-6
10-5
10-4
10-3
10-2
10-1
100
101
N4
0.36 0.022 99.0 0.58
0.42 0 99.2 0.39
0.62 0.069 98.7 0.65
18.8 0.43 80.4 0.31
60.1 0.96 38.8 0.11
88.5 1.53 9.84 0.13
90.5 1.98 7.52 0.030
90.9 1.73 7.31 0.064
90.9 1.54 7.51 0.030
89.6 1.41 8.91 0.077
Q4
0.28 0.023 99.3 0.44
0.14 0.023 99.4 0.43
0.23 0.023 99.2 0.53
0.27 0 99.2 0.53
11.7 0.14 87.9 0.28
66.9 1.10 31.9 0.12
72.3 1.03 26.6 0.13
74.9 1.44 23.5 0.100
74.8 1.40 23.7 0.096
76.6 1.04 22.3 0.092
V4
0.31 0 99.1 0.58
0.31 0 99.1 0.60
0.14 0 99.4 0.43
0.25 0.023 99.2 0.55
0.35 0 99.1 0.51
0.42 0.052 99.1 0.42
2.61 0.095 96.8 0.50
34.6 0.51 64.5 0.35
46.3 0.68 52.9 0.098
50.5 0.68 48.8 0.094
Timer Red

Figure 8 cont.

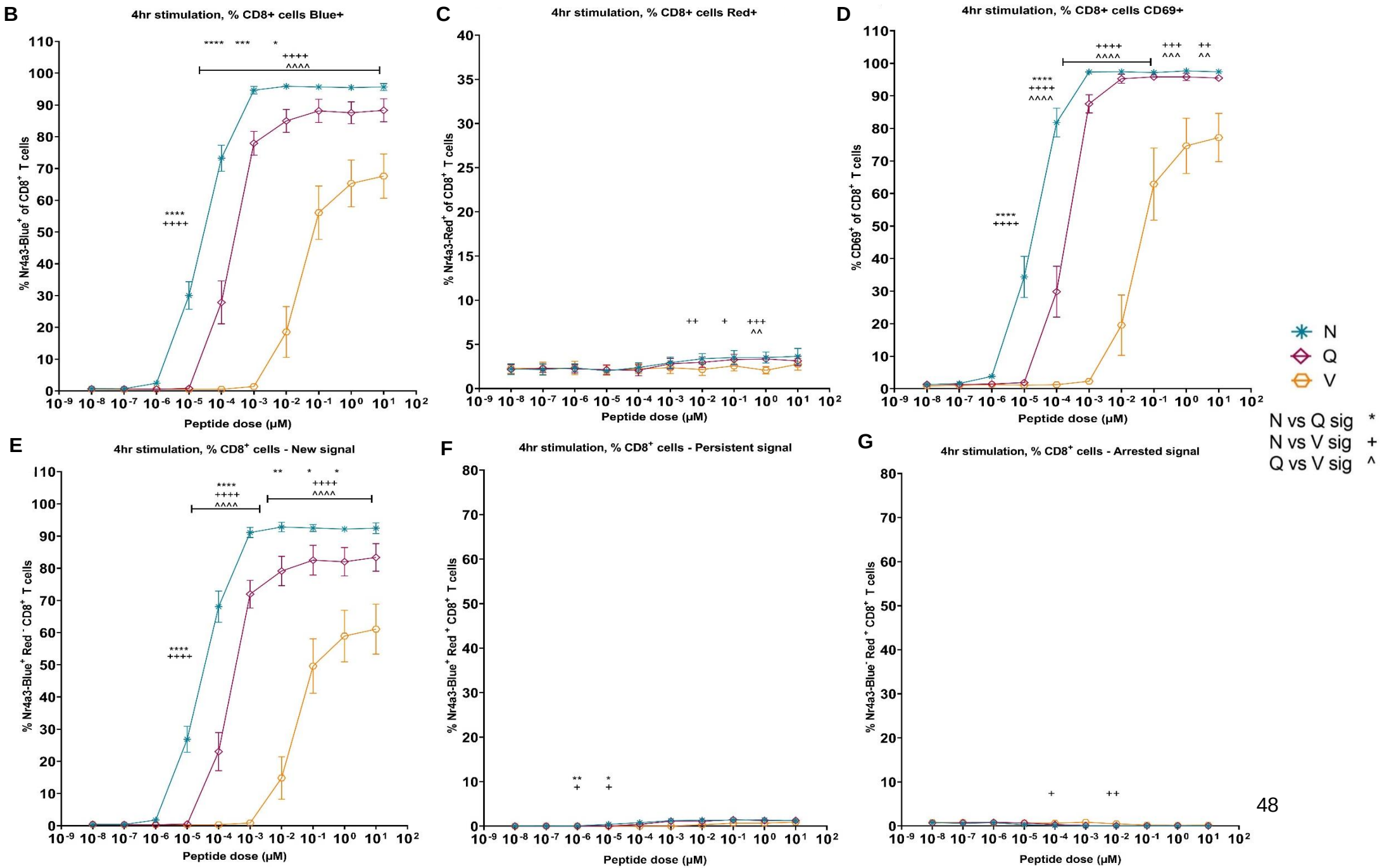


Figure 8. The effect of antigen dose and affinity for TCR on activation dynamics of CD8$^+$ T cell after 4 hours.

OTI Nr4a3 Tocky splenocytes were stimulated with a dose titration of ova peptide variants N4 (SII**N**FEKL, high affinity), Q4 (SII**Q**FEKL, intermediate) and V4 (SII**V**FEKL, low) for 4 hours at the concentrations noted. Live cells were analysed by flow cytometry using gating used in figure 4a to investigate CD8$^+$OTI T cells. **(A)** Plot shows expression of Nr4a3 Timer red vs Timer blue for the range of concentrations tested for each affinity peptide for 4 hours to study activation dynamics following signalling**.** Unstimulated sample was used to set cross gates. **(B-G)** Summary graphs showing the raw % of CD8$^+$ T cells that are positive for Timer protein or CD69 as dose increased of either N4 (*blue)*, Q4 (*maroon)* or V4 (*orange).* **(B)** % Timer blue$^+$ **(C)** % Timer red$^+$ **(D)** % CD69$^+$ **(E)** % Timer blue$^+$ red$^-$, new TCR signal **(F)** % Timer blue$^+$ red$^+$, persistent signal **(G)** % Timer blue$^-$ red$^+$, arrested signal. Error bars represent mean+SEM, n=3. Statistical analysis by two-way ANOVA with Sidak's multiple comparison tests. Significant difference ($p<0.05$) indicated as ****=$p<0.0001$, ***=p 0.0001-0.001, **=p 0.001-0.01, *=p 0.01-0.05, ns = no significance. * N vs Q, + N vs V, ^ Q vs V.

4.4.2 Expression of effector molecules, IFNγ and PD-1 after 4 hours.

It can be further seen that expression of effector molecules is affected by both dose and affinity. Expression of IFNγ (Figure 9A) and PD-1 (Figure 9B) show this same dependency on affinity to determine the threshold of antigen dose needed to cause expression. Additionally, it can also be seen that affinity dictates the maximum proportion of cells expressing each of the effector molecules with high significance; with N4 showing the highest expression, V4 the lowest and Q4 in between. These patterns of effector molecule expression make biological sense as they follow the activation dynamics seen; this could also give reason to the low levels of cells expressing each molecule, in particular the low proportion of cells expressing IFNγ. Contrary to what has been seen previously, although the proportion of PD-1^+ cells increases as the dose of all three affinity peptides also increases (Figure 9B), the proportion of IFNγ^+ cells that increase with doses of high affinity peptide N4 compared to Q4 or V4 shows a big difference. As can be seen in figure 9A, as dose increases with the high affinity N4 peptide, IFNγ^+ cells begin to decrease after they reach the dose that causes maximum proportion of cells to activate (10^{-2}μM), thus producing a bell-shaped curve. This phenomenon has been noted in a study by Lever *et al.,* who found that when $CD8^+$ T cells were stimulated with a 1 million-fold range of different affinity peptides, IFNγ levels in the supernatant (analysed by an ELISA assay after 4 hours of stimulation) also display this same bell-shaped curve for high affinity peptides (Lever *et al.,* 2016). They suggest this is due to a complex model; kinetic proofreading with limited signalling coupled to an incoherent feed-forward loop (KPL-IFF) (see discussion for more details). These results indicate that although increased expression of PD-1 is linked to an increase in antigen dose,

expression of IFNγ does not follow this pattern and shows a decrease in expression when stimulated with high doses of antigen possible due to the complex feedback model, KPL-IFF.

Figure 9

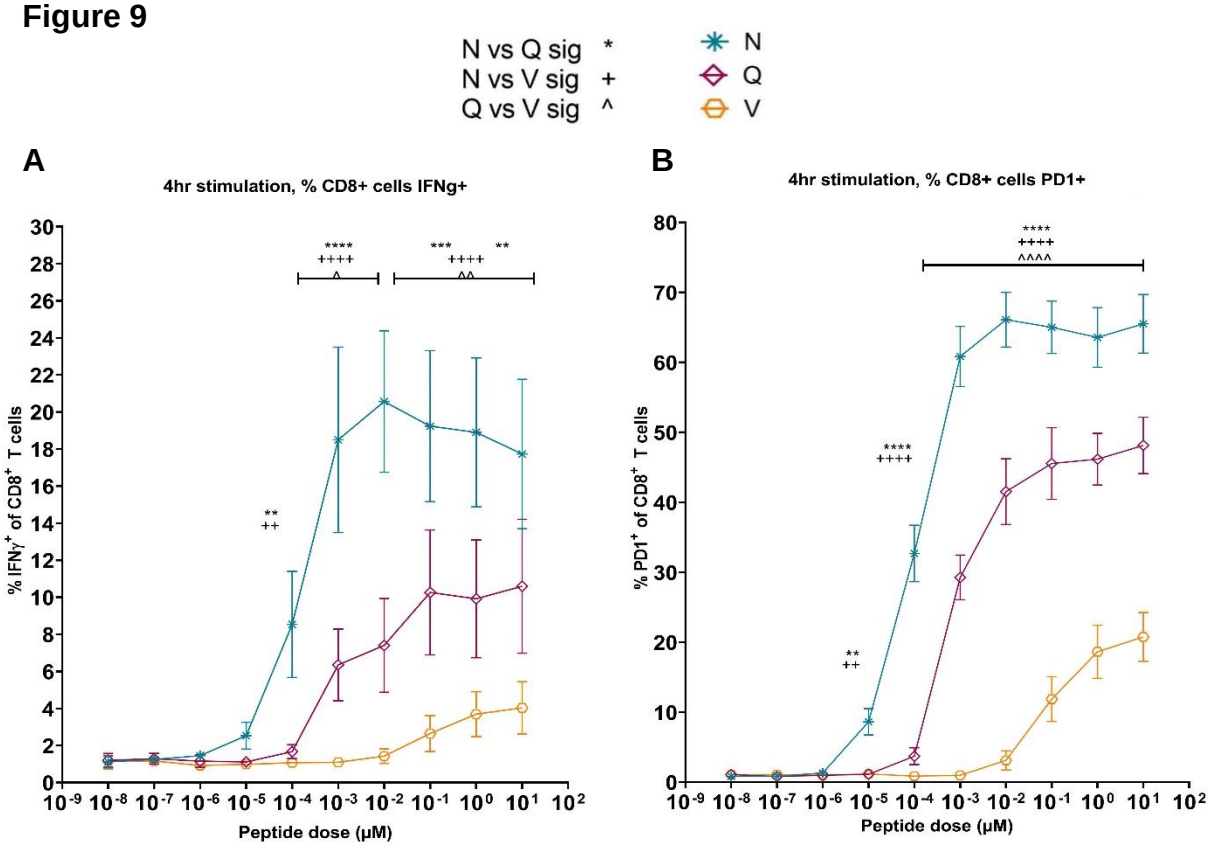


Figure 9. The effect of antigen dose and affinity for TCR on the expression of effector molecules, IFNγ and PD-1, after 4 hours.

OTI Nr4a3 Tocky splenocytes were stimulated with a dose titration of ova peptide variants N4 (high affinity), Q4 (intermediate) and V4 (low) for 4 hours at the concentrations noted. Live cells were analysed by flow cytometry using gating used in figure 4a to investigate OTI $CD8^+$ T cells. **(A-B)** Summary graphs show the raw % of $CD8^+$ T cells that express IFNγ **(A)** and PD-1 **(B)** as dose increased of either N4 (*blue)*, Q4 (*maroon)* or V4 (*orange).* Error bars represent mean+SEM, n=3. Statistical analysis by two-way ANOVA with Sidak's multiple comparison tests. Significant difference ($p<0.05$) indicated as ****=$p<0.0001$, ***=p 0.0001-0.001, **=p 0.001-0.01, *=p 0.01-0.05, ns = no significance. * N vs Q, + N vs V, ^ Q vs V.

4.4.3 Activation dynamics of $CD8^+$ T cell after 16 hours.

The findings described so far are of data that was analysed after 4-hours of stimulation, a 16-hour timepoint was also looked at in order to study slightly longer-term effects of antigen dose and affinity on signalling dynamics, cell activation and effector molecule expression (Figures 10 and 11). As seen with 4-hours of stimulation, it can again be seen that antigen affinity effects the dose needed for cells to activate after receiving a new (Timer $blue^+$) or persistent signal (Timer $blue^+$ red^+) (Figures 10A-E). These doses are also found to be the same as seen previously, hence confirming activation thresholds to be: N4, 10^{-5}µM; Q4, 10^{-4}µM and V4, 10^{-2}µM. Also seen again with this timepoint, is the rapid proportion of cells that activate following a one dose increment after threshold dose. Figure 10A shows that independent of affinity, at these activation threshold doses: a proportion of cells have not received any signal, some have receive a new signal and activated (Timer $blue^+$; Figure 10B, D and E), some have received a persistent signal (Timer $blue^+$ red^+; Figures 10B-D and F) and some have received a signal to activate but have not received further signals to stay activated, hence they have arrested (Timer red^+, Figures 10D-G). This broad response of TCR signalling and hence cell activation is expected to happen at threshold dose and it can be seen that as dose continues to increase past threshold, high proportions of cells become and stay activated, due to persistent TCR signalling (Timer $blue^+$ red^+; Figures 10A-C and F). Consequently, it can be seen that only a small proportion of cells have an arrested signal (Timer red^+; Figure 10A and G) at high doses of peptide. Furthermore, unlike 4-hours of stimulation that captured the early effects, it can be seen that after 16-hours of stimulation, peptide affinity has a much smaller effect on the maximum proportion of

cells activating and receiving a persistent TCR signal (Timer blue^{+} red^{+}). As shown in figure 10A when cells were stimulated with high doses of N4 and Q4 more than 95% of cells are Timer blue^{+} red^{+} indicating that these cells have received persistent TCR signalling to result in CD8^{+} T cell activation (Figure 10F). It can also be seen that stimulation with the highest dose of V4 resulted in more than 90% cells activating. These differences in the maximum proportions of cells displaying persistent signalling and activation could be due to affinity altering signalling dynamics. As seen in response to N4, there is a higher proportion of cells that have had a persistent signal (express Timer blue and red protein) compared to V4, it can be theorised that N4 causes cells to receive new signals quicker (express Timer blue quicker) than what V4 would do, and hence causes the larger increase in proportion of cells that are shown to have had persistent signalling (expressing Timer blue and red) as N4 cells blue protein has had longer to mature to red compared to V4.

Figure 10

A

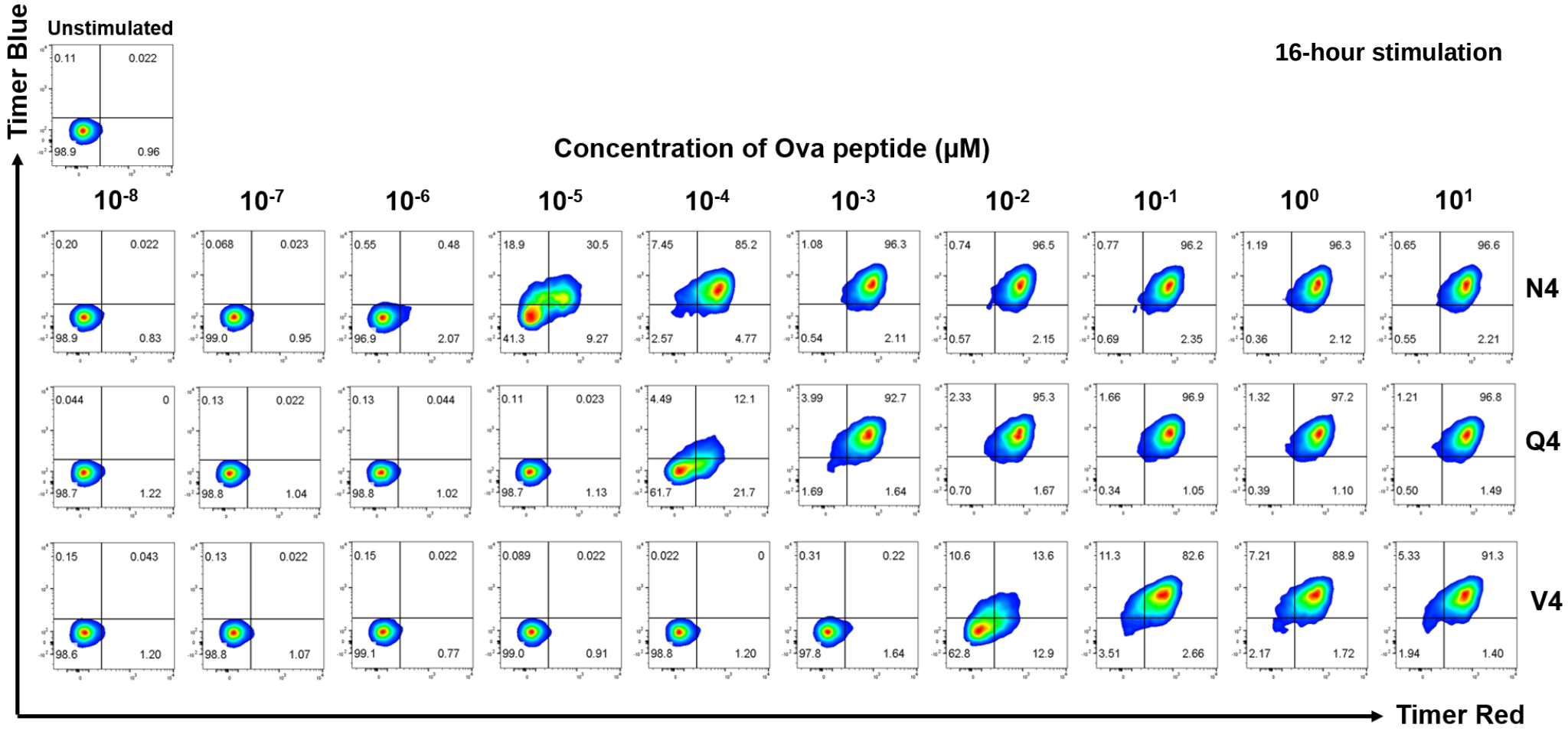

Timer Blue
Unstimulated
0.11
0.022
98.9
0.96
16-hour stimulation
Concentration of Ova peptide (μM)
10-8
10-7
10-6
10-5
10-4
10-3
10-2
10-1
100
101
0.20 0.022 98.9 0.83
0.068 0.023 99.0 0.95
0.55 0.48 96.9 2.07
18.9 30.5 41.3 9.27
7.45 85.2 2.57 4.77
1.08 96.3 0.54 2.11
0.74 96.5 0.57 2.15
0.77 96.2 0.69 2.35
1.19 96.3 0.36 2.12
0.65 96.6 0.55 2.21
N4
0.044 0 98.7 1.22
0.13 0.022 98.8 1.04
0.13 0.044 98.8 1.02
0.11 0.023 98.7 1.13
4.49 12.1 61.7 21.7
3.99 92.7 1.69 1.64
2.33 95.3 0.70 1.67
1.66 96.9 0.34 1.05
1.32 97.2 0.39 1.10
1.21 96.8 0.50 1.49
Q4
0.15 0.043 98.6 1.20
0.13 0.022 98.8 1.07
0.15 0.022 99.1 0.77
0.089 0.022 99.0 0.91
0.022 0 98.8 1.20
0.31 0.22 97.8 1.64
10.6 13.6 62.8 12.9
11.3 82.6 3.51 2.66
7.21 88.9 2.17 1.72
5.33 91.3 1.94 1.40
V4
Timer Red

Figure 10 cont.

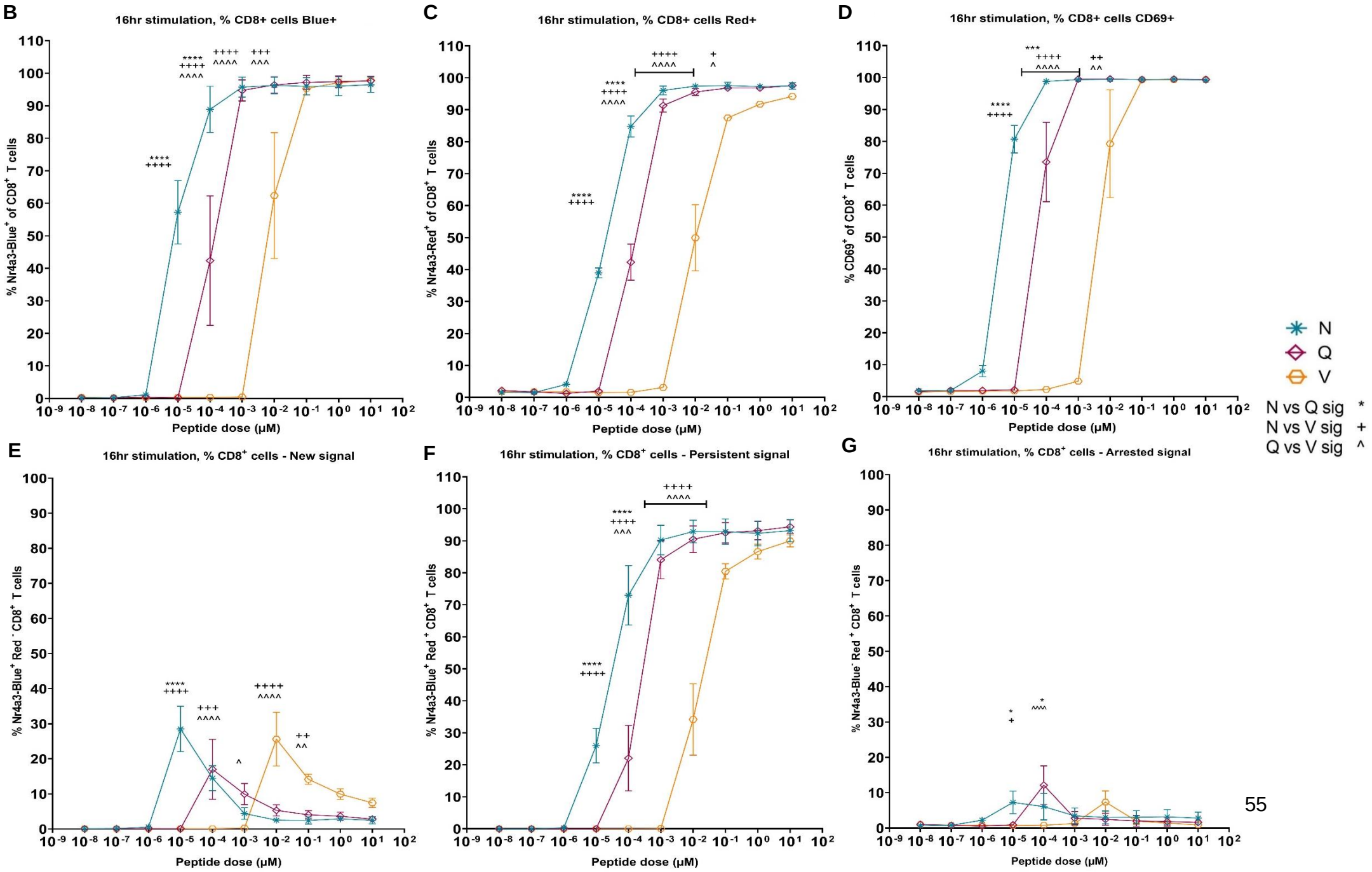


Figure 10. The effect of antigen dose and affinity for TCR on activation dynamics of CD8$^+$ T cell after 16 hours.

OTI Nr4a3 Tocky splenocytes were stimulated with a dose titration of ova peptide variants N4 (high affinity), Q4 (intermediate) and V4 (low) for 16 hours at the concentrations noted. Live cells were analysed by flow cytometry using gating used in figure 4a to investigate CD8$^+$OTI T cells. **(A)** Plot shows expression of Nr4a3 Timer red vs Timer blue for the range of concentrations tested for each affinity peptide for 16 hours to study activation dynamics following signalling**.** Unstimulated sample was used to set cross gates. **(B-G)** Summary graphs showing the raw % of CD8$^+$ T cells that are positive for Timer protein or CD69 as dose increased of either N4 (*blue)*, Q4 (*maroon)* or V4 (*orange).* **(B)** % Timer blue$^+$ **(C)** % Timer red$^+$ **(D)** % CD69$^+$ **(E)** % Timer blue$^+$ red$^-$, new TCR signal **(F)** % Timer blue$^+$ red$^+$, persistent signal **(G)** % Timer blue$^-$ red$^+$, arrested signal. Error bars represent mean+SEM, n=3. Statistical analysis by two-way ANOVA with Sidak's multiple comparison tests. Significant difference ($p<0.05$) indicated as ****=$p<0.0001$, ***=p 0.0001-0.001, **=p 0.001-0.01, *=p 0.01-0.05, ns = no significance. * N vs Q, + N vs V, ^ Q vs V.

4.4.4 Expression of effector molecules, IFNγ and PD-1 after 16 hours.

In line with the findings described that show high proportions of cells activating and receiving persistent TCR signals with high doses of all affinity peptides, an increase in effector molecule expression can also be seen. Figure 11B shows that although dose of antigen needed for cells to begin expressing PD-1 is dependent on affinity after 16-hours of stimulation, it can be seen that as dose of N4 and Q4 increases, the proportion of cells expressing PD-1 reaches a maximum and plateaus, V4 shows this too but slightly less (shows no significant difference). This again indicates that affinity does not have as much effect on effector function of cells receiving persistent signalling compared to new signalling (4-hours). Interestingly, the doses required to reach maximum PD-1 expression are just one increment up from dose threshold, indicating an almost binary expression of PD-1 following activation with higher affinity peptides. Like PD-1 expression, IFNγ expression threshold again seems to depend on affinity of peptide (Figure 11A). It can also be seen in figure 11A that with increasing doses, the proportion of cells expressing IFNγ when stimulated with high affinity N4 starts to forms a bell-shaped curve, which is possibly due to the KPL-IFF model that has been previously mentioned (Lever *et al.,* 2016). It also seems that Q4 could follow this same trend. These collective findings lead one to believe that it is possible for a high dose of low affinity peptide to cause the same response as high affinity peptide; stimulation with low affinity V4 resulted in comparable cell activation and also effector function by expressing PD-1 immune checkpoint and IFNγ.

Figure 11

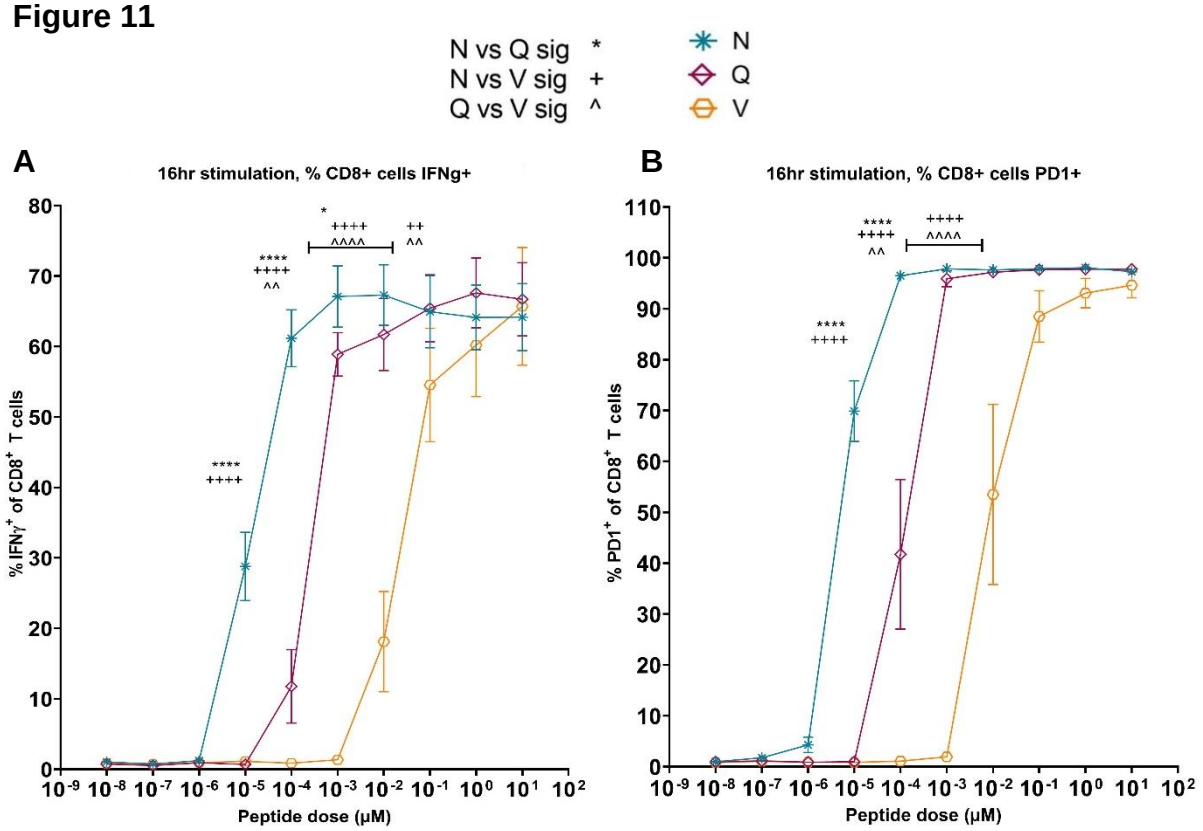


Figure 11. The effect of antigen dose and affinity for TCR on the expression of effector molecules, IFNγ and PD-1, after 16 hours.

OTI Nr4a3 Tocky splenocytes were stimulated with a dose titration of ova peptide variants N4 (high affinity), Q4 (intermediate) and V4 (low) for 16 hours at the concentrations noted. Live cells were analysed by flow cytometry using gating used in figure 4a to investigate OTI $CD8^+$ T cells. **(A-B)** Summary graphs show the raw % of $CD8^+$ T cells that express IFNγ **(A)** and PD-1 **(B)** as dose increased of either N4 (*blue)*, Q4 (*maroon)* or V4 (*orange).* Error bars represent mean+SEM, n=3. Statistical analysis by two-way ANOVA with Sidak's multiple comparison tests. Significant difference ($p<0.05$) indicated as ****=$p<0.0001$, ***=p 0.0001-0.001, **=p 0.001-0.01, *=p 0.01-0.05, ns = no significance. * N vs Q, + N vs V, ^ Q vs V.

4.4.5 Results summary.

The following conclusions can be drawn from this study into how antigen dose, affinity for TCR and time effects the activation dynamics of $CD8^+$ T cells, through TCR signalling analysis, and the expression of IFNγ and PD-1 (*aim 2).* Antigen affinity determines the dose threshold that is required to activate cells and cause expression of both IFNγ and PD-1. Dose of peptide is linked to the proportion of cells that receive a TCR signal and hence cause $CD8^+$ T cell activation, as well as directly effecting expression of PD-1 and IFNγ. Generally, as dose increases so does response however, with high doses of high affinity peptides IFNγ expression decreases, which forms a bell-shaped curve response. Finally, it was also found that affinity seems to affect the maximum proportion of cells that are activated and produce effector molecules with 4-hours of stimulation but not so much after 16-hour stimulation. The differences seen after 16 hours of stimulation in the maximum proportions of cells displaying persistent signalling is likely due to the activation rate for T cells being slower for low affinity peptides.

4.5 In vitro PD1 blockade has minimal effects on the primary activation of $CD8^+$ T cells.

Immune checkpoints are vital in the regulation of $CD8^+$ T cell response to antigen and have been found to be manipulated by some cancers to hide from the immune system. One clinically important checkpoint is PD-1. When PD-1 (expressed on activated T cells) binds to its substrates PD-L1 and -L2 (broad expression on both haematopoietic and non-haematopoietic cells) it inhibits cell activation and function. Therefore, by blocking PD-1 with a monoclonal antibody this allows cells to be activated. Immunotherapy drugs employing this tactic have shown great success in some cancer treatment however also have been seen to cause serious adverse side effects and are reported to not leave lasting effects in some patients. A better understanding of the PD-1 pathway and how it effects $CD8^+$ T cell activation could aid in further developing these drugs.

In order to investigate how PD-1 affects activation and effector function, OTI Nr4a3 Tocky splenocytes were stimulated with a dose titration of the different affinity ova peptides mentioned previously; N4 (high affinity), Q4 (intermediate) and V4 (low). The doses used were chosen from the data shown in 4.4 to select the suspected threshold for activation dose, plus and minus two doses. Cells were incubated with either an isotype (MAC219) or anti-PD1 for 16 hours and then analysed by flow cytometry. Staining panels included: CD69 (marker of early cell activation) and PD-1 (to asses effectiveness of anti-PD1). As well as built-in reporters: Timer blue and red (T cell activation dynamics) and IFNγ-YFP (effector function).

In vitro blockade with anti-PD1 had surprisingly minimal effects on the activation of T cells (Figures 12A and B). It can be seen in the isotype group that there is a high proportion of cells activating and expressing both CD69 (Figures 13-, 14-, 15-D) and IFNγ (Figures 13-, 14-, 15-E), even at doses where PD-1 is active (Figures 13-, 14-, 15-F). This suggests that PD-1 is not causing much of an inhibitory effect on $CD8^+$ T cell activation and effector function *in vitro* during the primary activation of immune cells.

As mentioned previously, affinity of peptide-MHC to TCR effects the dose of antigen needed to activate cells and it seems that although Q4 has shown a constant threshold of 10^{-4}µM (Figures 14A and D), N4 and V4 show some activation but then arrested signalling of cells in between two doses; 10^{-6} - 10^{-5}µM and 10^{-3} - 10^{-2}µM (Figures 13A, C, D and 15A, C, D). It can further be seen that when cells were incubated with anti-PD1, the lower of the dose range shows a subtle broader response of cell activation rather than cells just arresting (Figure 12B). This could suggest that blocking PD-1 lowers the threshold dose of high and low affinity antigen needed to activate cells. However, these changes are not reflected by any significant difference in CD69 expression (Figures 13D and 15D). An important effector function of $CD8^+$ T cells is IFNγ expression. It can be seen that the proportion of cells that are $IFN\gamma^+$ when stimulated with N4 (Figure 13E) and either isotype or anti-PD1 are very comparable; there is a very small but not significant increase at high doses. This can also be seen with Q4 (Figure 14E); at high doses it seems levels are beginning to decrease with anti-PD1 and although impossible to conclude due to dose range, this could be the formation of the bell-shaped curve previously explained in this project. Stimulation with V4 results in a higher proportion of $IFN\gamma^+$ cells with anti-PD1

compared to isotype and even shows some significance at 10^{-1}µM (Figure 15E); indicating that anti-PD1 may affect lower affinity peptides more than higher affinity. However, all the conclusions from this study are to be drawn with caution as levels of PD1's ligands in the culture were not determined.

The following conclusions can be drawn from this study. It was seen that in the isotype group, although PD1 levels were high there was not much inhibition of activation or effector function, possibly due to availability of ligands or the high antigen environment (see discussion for more details). These findings however did suggest anti-PD1 may exert some subtle effects on lower affinity interactions, in particular when assessing T cell effector function as analysed by IFNγ expression.

N.B. Biological repeats of this experiment were kindly carried out by Dr David Bending (UoB) due to COVID-19 restrictions, however I fully planned the experiment and carried out all post-collection data analysis as well as collecting data for the first biological repeat.

Figure 12

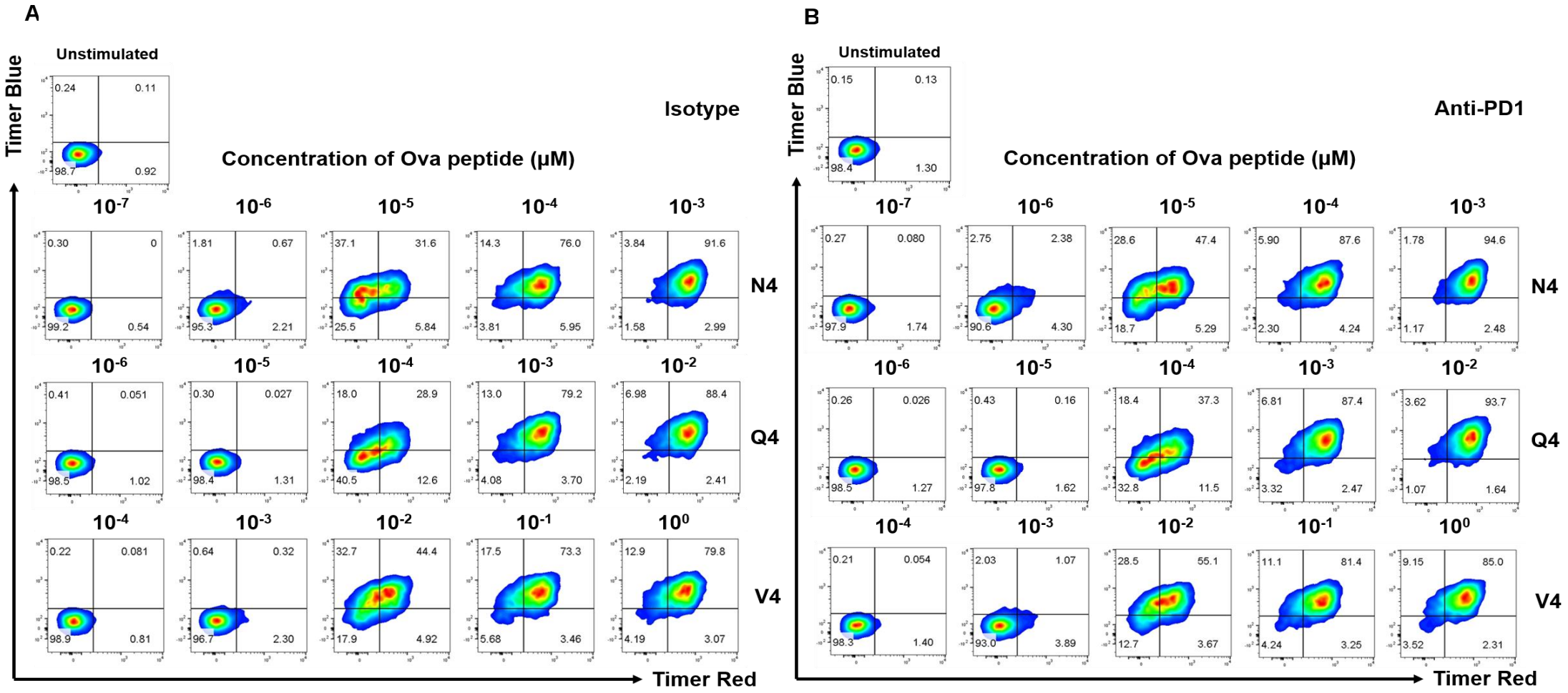


Figure 12. Effect of anti-PD1 on activation of CD8⁺ T cells stimulated with a range of doses of different affinity peptides.

OTI Nr4a3 Tocky splenocytes were stimulated with a dose titration of ova peptide variants N4 (high affinity), Q4 (intermediate) and V4 (low). Doses used were chosen to select the threshold for activation dose, plus and minus two doses. Cells were incubated with either an isotype (MAC219) or anti-PD1 for 16 hours and then analysed by flow cytometry using figure 4a gating strategy to investigate OTI CD8⁺ T cells. **(A, B)** Plots show expression of Nr4a3 Timer red vs Timer blue for the range of concentrations tested for each affinity peptide with either isotype **(A)** or anti-PD1 **(B)** to study activation dynamics following signalling. Unstimulated sample was used to set cross gates.

Figure 13

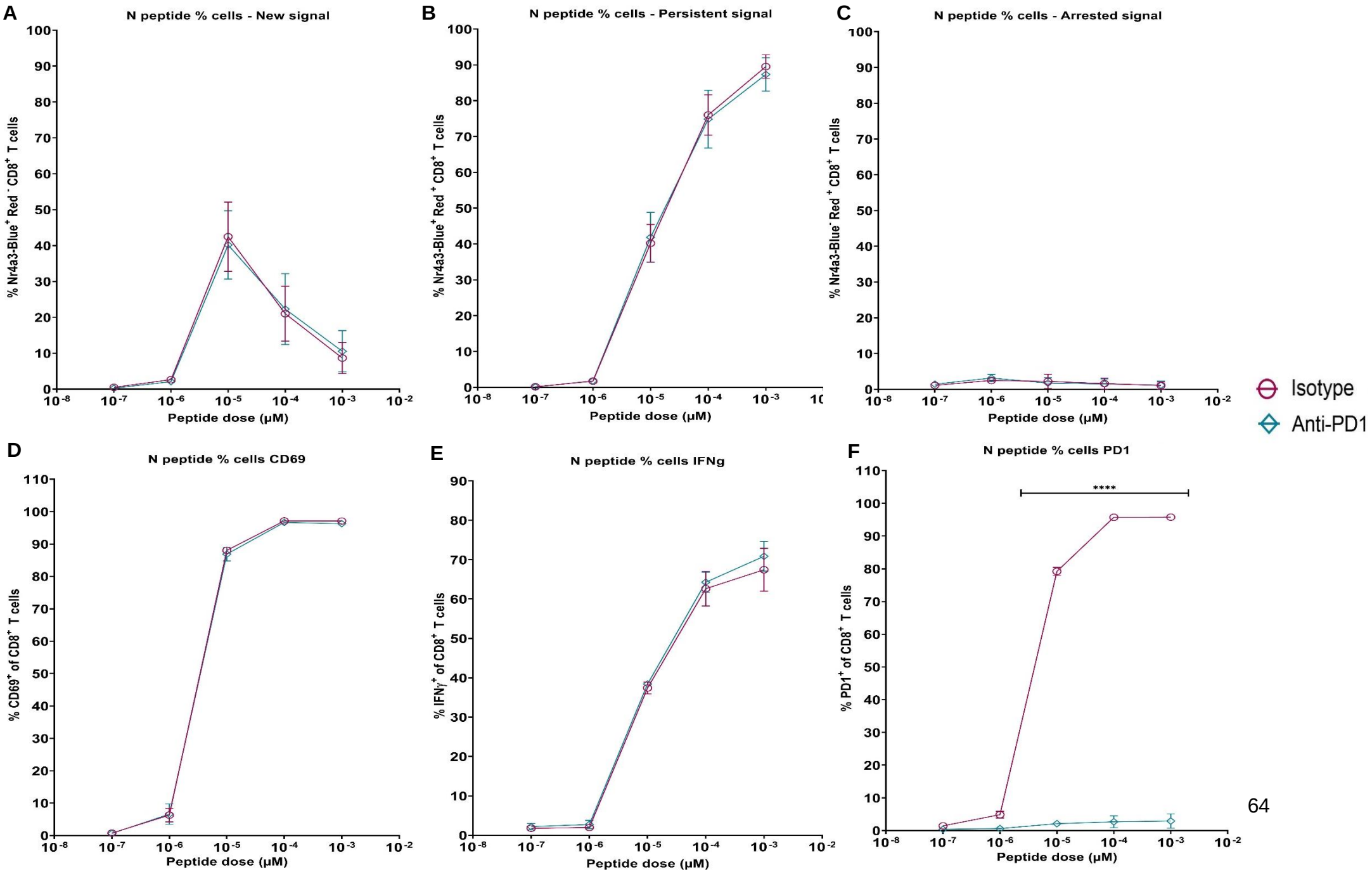

A
N peptide % cells - New signal
% Nr4a3-Blue+ Red- CD8+ T cells
Peptide dose (μM)
B
N peptide % cells - Persistent signal
% Nr4a3-Blue+ Red+ CD8+ T cells
Peptide dose (μM)
C
N peptide % cells - Arrested signal
% Nr4a3-Blue- Red+ CD8+ T cells
Peptide dose (μM)
Isotype
Anti-PD1
D
N peptide % cells CD69
% CD69+ of CD8+ T cells
Peptide dose (μM)
E
N peptide % cells IFNg
% IFNγ+ of CD8+ T cells
Peptide dose (μM)
F
N peptide % cells PD1
****
% PD1+ of CD8+ T cells
Peptide dose (μM)

Figure 13. Effect of anti-PD1 on activation and effector function of $CD8^+$ T cells stimulated with a range of doses of high affinity peptide N4.

OTI Nr4a3 Tocky splenocytes were stimulated with a dose titration of ova peptide variant N4 (high affinity). Doses used were chosen to select the threshold for activation dose, plus and minus two doses. Cells were incubated with either an isotype (MAC219) or anti-PD1 for 16 hours and then analysed by flow cytometry using figure 4a gating strategy to investigate OTI $CD8^+$ T cells. **(A-F)** Summary graphs showing the raw % of $CD8^+$ T cells that are positive for the markers named above each graph, as dose increased, with isotype (*maroon line)* or anti-PD1 (*blue line).* **(A)** % Timer $blue^+$ red^-, new TCR signal **(B)** % Timer $blue^+$ red^+, persistent signal **(C)** % Timer $blue^-$ red^+, arrested signal. **(D)** % $CD69^+$ **(E)** % $IFN\gamma^+$ **(F)** % $PD\text{-}1^+$. Error bars represent mean+SEM, n=3. Statistical analysis by two-way ANOVA with Sidak's multiple comparison tests. Significant difference ($p<0.05$) indicated as ****=$p<0.0001$.

Figure 14

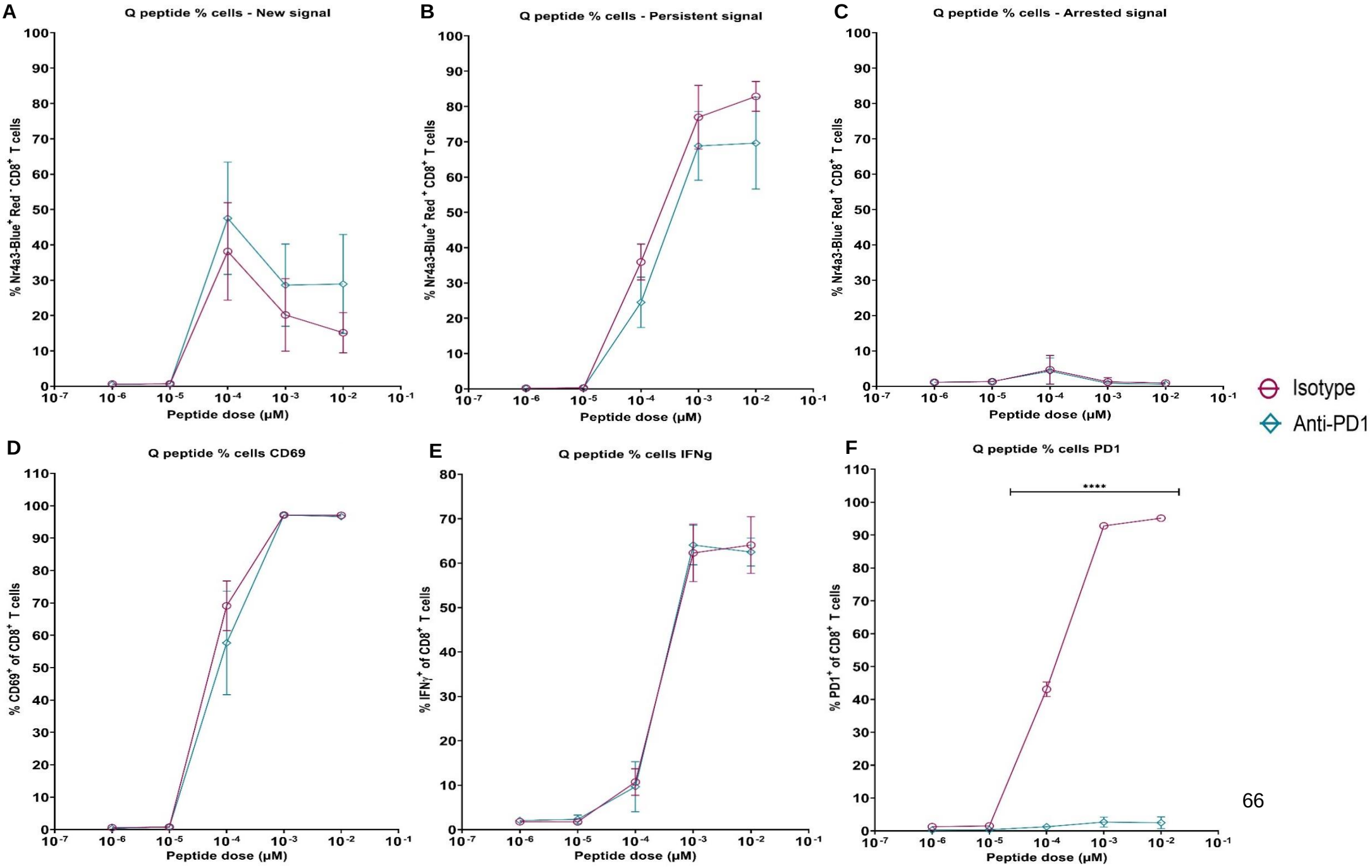

A
Q peptide % cells - New signal
% Nr4a3-Blue+ Red- CD8+ T cells
Peptide dose (μM)
B
Q peptide % cells - Persistent signal
% Nr4a3-Blue+ Red+ CD8+ T cells
Peptide dose (μM)
C
Q peptide % cells - Arrested signal
% Nr4a3-Blue- Red+ CD8+ T cells
Peptide dose (μM)
Isotype
Anti-PD1
D
Q peptide % cells CD69
% CD69+ of CD8+ T cells
Peptide dose (μM)
E
Q peptide % cells IFNg
% IFNγ+ of CD8+ T cells
Peptide dose (μM)
F
Q peptide % cells PD1
****
% PD1+ of CD8+ T cells
Peptide dose (μM)

Figure 14. Effect of anti-PD1 on activation and effector function of $CD8^+$ T cells stimulated with a range of doses of high affinity peptide Q4.

OTI Nr4a3 Tocky splenocytes were stimulated with a dose titration of ova peptide variant Q4 (intermediate affinity). Doses used were chosen to select the threshold for activation dose, plus and minus two doses. Cells were incubated with either an isotype (MAC219) or anti-PD1 for 16 hours and then analysed by flow cytometry using figure 4a gating strategy to investigate OTI $CD8^+$ T cells. **(A-F)** Summary graphs showing the raw % of $CD8^+$ T cells that are positive for the markers named above each graph, as dose increased, with isotype (*maroon line)* or anti-PD1 (*blue line).* **(A)** % Timer $blue^+$ red^-, new TCR signal **(B)** % Timer $blue^+$ red^+, persistent signal **(C)** % Timer $blue^-$ red^+, arrested signal. **(D)** % $CD69^+$ **(E)** % $IFN\gamma^+$ **(F)** % $PD\text{-}1^+$. Error bars represent mean+SEM, n=3. Statistical analysis by two-way ANOVA with Sidak's multiple comparison tests. Significant difference ($p<0.05$) indicated as ****=$p<0.0001$.

Figure 15

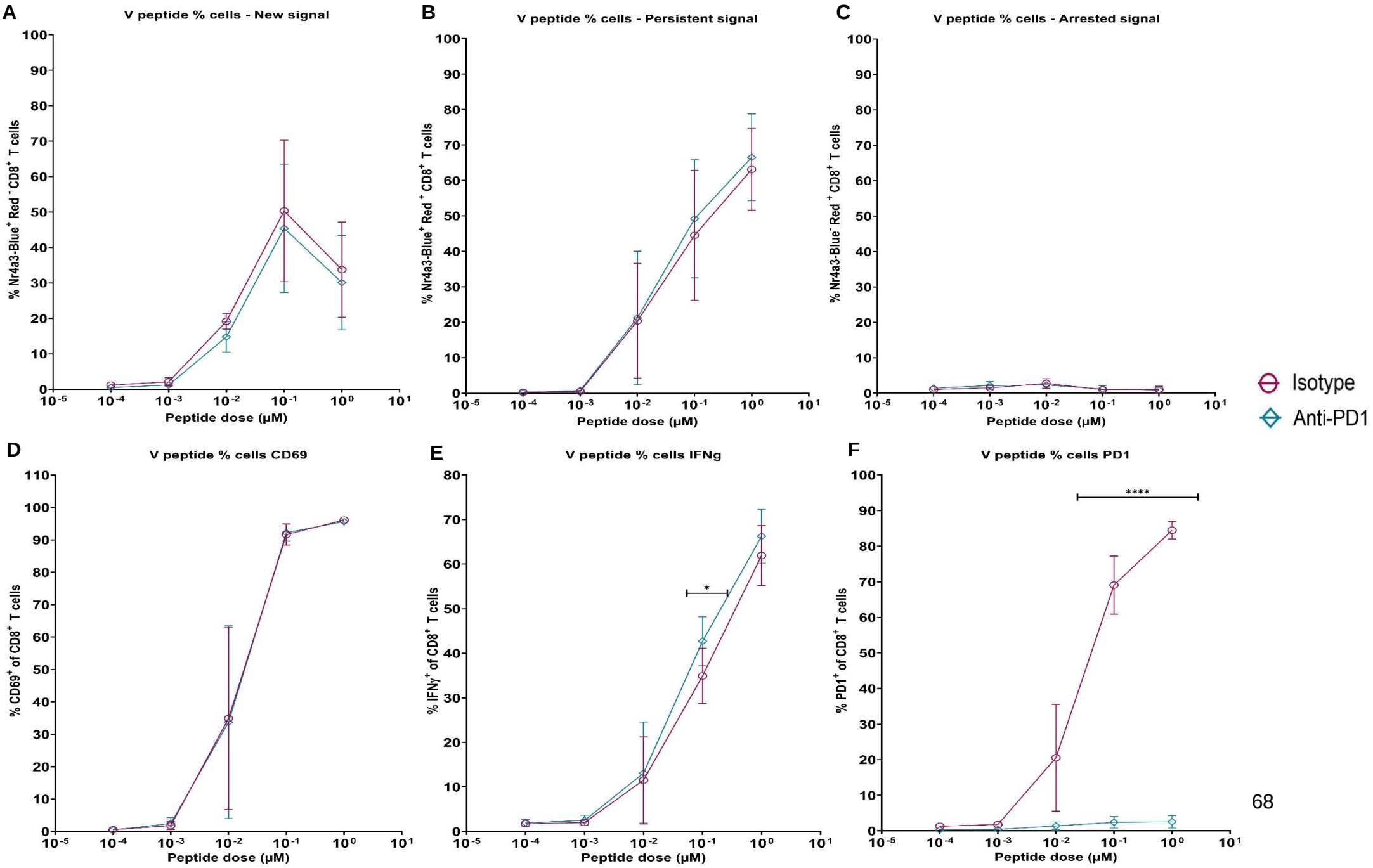

A
V peptide % cells - New signal
% Nr4a3-Blue+ Red- CD8+ T cells
Peptide dose (μM)
B
V peptide % cells - Persistent signal
% Nr4a3-Blue+ Red+ CD8+ T cells
Peptide dose (μM)
C
V peptide % cells - Arrested signal
% Nr4a3-Blue- Red+ CD8+ T cells
Peptide dose (μM)
Isotype
Anti-PD1
D
V peptide % cells CD69
% CD69+ of CD8+ T cells
Peptide dose (μM)
E
V peptide % cells IFNg
% IFNγ+ of CD8+ T cells
*
Peptide dose (μM)
F
V peptide % cells PD1
% PD1+ of CD8+ T cells
****
Peptide dose (μM)

Figure 15. Effect of anti-PD1 on activation and effector function of $CD8^+$ T cells stimulated with a range of doses of high affinity peptide V4.

OTI Nr4a3 Tocky splenocytes were stimulated with a dose titration of ova peptide variant V4 (low affinity). Doses used were chosen to select the threshold for activation dose, plus and minus two doses. Cells were incubated with either an isotype (MAC219) or anti-PD1 for 16 hours and then analysed by flow cytometry using figure 4a gating strategy to investigate OTI $CD8^+$ T cells. **(A-F)** Summary graphs showing the raw % of $CD8^+$ T cells that are positive for the markers named above each graph, as dose increased, with isotype (*maroon line)* or anti-PD1 (*blue line).* **(A)** % Timer $blue^+$ red^-, new TCR signal **(B)** % Timer $blue^+$ red^+, persistent signal **(C)** % Timer $blue^-$ red^+, arrested signal. **(D)** % $CD69^+$ **(E)** % $IFN\gamma^+$ **(F)** % $PD\text{-}1^+$. Error bars represent mean+SEM, n=3. Statistical analysis by two-way ANOVA with Sidak's multiple comparison tests. Significant difference ($p<0.05$) indicated as ****=$p<0.0001$, *=p 0.01-0.05.

4.6 The effects of tumour environment on T cell activation, immune checkpoints and IFNγ expression.

Tumours develop in the body due to the uncontrolled proliferation of cells. These cells have developed mutations in oncogenes and tumour suppressive genes and hence proliferate in an uncontrolled fashion. Under normal conditions the immune system, in particular $CD8^+$ T cells, kill cells that show to have mutations however, cancer cells manipulate T cells in different ways to hide and prevent being killed. The ways in which cancer cells do this is unknown however it is thought that the highly immunosuppressive tumour microenvironment (TME) plays a big role. Within this environment it has been described that T cells become dysfunctional and exhausted; meaning they have impaired activation and effector function due to possible epigenetic changes and upregulation of immune checkpoint receptors. An *in vivo* MC38 tumour model was used in order to investigate how the presence of a tumour effects activation, immune checkpoints and IFNγ expression in different sub groups of T cells in different tissues (including the TME).

Nr4a3-Tocky Great Smart mice were subcutaneous injected with MC38-ova tumour cells (colon cancer) (Akagi *et al.,* 1997), which were allowed to grow for 7 days (tumour was visible at this time). This mouse line enlists the use the Nr4a3-Tocky system and so allows for the analysis of host cell activation dynamics. In order to analyse the initial response of tumour specific T cells, purified $CD8^+$ OTI Nr4a3-Tocky cells stained with a proliferation dye were injected intravenously into the mice displaying tumours. 24- and 48-hours after cells were injected the following tissues were collected: spleen, contralateral lymph node (cLN), tumour draining lymph node

(dLN) and tumour. These tissues were chosen in addition to the tumour as they are heavily involved in the immune response and could be thought to show some differential cell responses that occur due to a tumour being present in the system. Following the processing of each tissue (see 3.5 materials and methods), live cells were staining and analysed by flow cytometry. The mouse line used and additional markers allows for the analysis of the following groups in each tissue at two timepoints: $CD4^+$ T cell host response, $CD8^+$ T cell host response and tumour specific $CD8^+$ OTI T cell host response. Results are divided into theses cell groups for clarity.

4.6.1 $CD4^+$ host T cell response to tumour in the spleen, dLN, cLN and tumour microenvironment.

Although tissue was taken 24- and 48-hours after $CD8^+$ OTI cells were introduced, the host cell response is actually that of a day 8 and 9 timepoint (7 days of tumour growth plus 24 or 48 hours). $CD4^+$ host T cells that have been in the tumour microenvironment are seen to show huge amounts of activation and effector function, which are highly significant differences in to other tissue analysed. It can be seen in figure 16A that cell response in the tumour is far more than any other tissue, with over 60% cells activating at day 8 and over 50% at day 9, which is to be expected. At day 8 it is shown that most cells received either a new or persistent signal (Figures 16D and E) whereas at day 9 there is a shift to cells receiving still a high level of persistent signalling (Figure 16E) but also a significantly higher proportion of cells that have arrested signalling (Figures 16F). This indicates that although cells are being activated and persistently signalled to result in sustained activation in the

tumour, there seems to be a shift in cells receiving a new signal at day 8 to an increase of cells arresting signal at day 9; this could be an effect of the immunosuppressive TME. This effect is reciprocated in expression of IFNγ, PD-1 and Lag-3. Unlike the other tissues investigated, the proportion of cells expressing these molecules is significantly higher. Figures 16G-I show that between days 8 and 9 the proportion of IFNγ^{+} and Lag-3^{+} cells both significantly decrease, however interestingly PD-1^{+} cells significantly increases. This could indicate that the TME is affecting activation of CD4^{+} host T cells through upregulating the immune checkpoint PD-1.

It can further be seen in figure 16A that there is an overall small response of cells in the dLN, cLN and spleen (more than 80% of cells do not activate). Although the response is small, it can be seen at 8 days there are a proportion of cells that are Timer blue^{+} (Figures 16A and B), more so in the dLN and spleen than cLN. This indicates a small proportion of cells have received a new signal and activated in the previous 4 hours (Figure 16D). Additionally, there is also a small proportion of cells that are Timer blue^{+} red^{+}. It can again be seen there is a slightly increased proportion in the dLN and slightly more again in the spleen; indicating this small proportion of cells had a persistent TCR signal and hence stayed activated (Figure 16E). However, the largest proportion of cells are Timer red^{+}, indicating an arrested signal (Figure 16A, C and F). These cells historically had received a TCR signal, but did not receive signals to stay activated. These results collectively indicate that although cells in these tissues may recognise tumour as foreign and some cells are still being activated 8 days after the tumour was introduced, cells either do not stay activated to mount a response or are leaving the lymph node to go to the tumour site. This can

also be seen at day 9 in these tissues; there is a very slight, not significant, decrease in proportion of cells expressing Timer blue; indicating that less cells received a new signal to activate at day 9 (Figure 16D). It can also again be seen that of these cells that did activate, most cells are shown to have arrested signalling (Figure 16A-C and F). Analysis of IFNγ and immune checkpoints, PD-1 and Lag-3, also supports the idea that although cells recognise tumour, they do not mount a sustained response and cells arrest. It can be seen that the proportion of IFNγ$^+$, PD-1$^+$ and Lag-3$^+$ cells are below 5% in the dLN and cLN and PD-1$^+$ and Lag-3$^+$ cells are below 8% in the spleen (Figure 16G-I). Additionally, although it can be seen in figure 16G that the proportion of cells expressing IFNγ$^+$ does significantly increase at day 8 in the spleen compared to dLN and cLN, levels are still very low (approximately 14%) and by day 9 are no longer significant. This increase in IFNγ may be due to the slight increase in Timer blue$^+$ red$^+$ cells at day 8 in the spleen however, as levels of IFNγ decrease (to become not significant) as cell activation is seen to also decrease, the proposed profile that CD4$^+$ cells recognise tumour but do not stay activated to mount a response in these tissues still stands. Additionally, although changes are very subtle, it could be suggested that PD-1 and Lag-3 are involved in causing cells to arrest as the proportion of cells expressing the immune checkpoints at day 8 is slightly (no significance) higher than day 9.

Figure 16

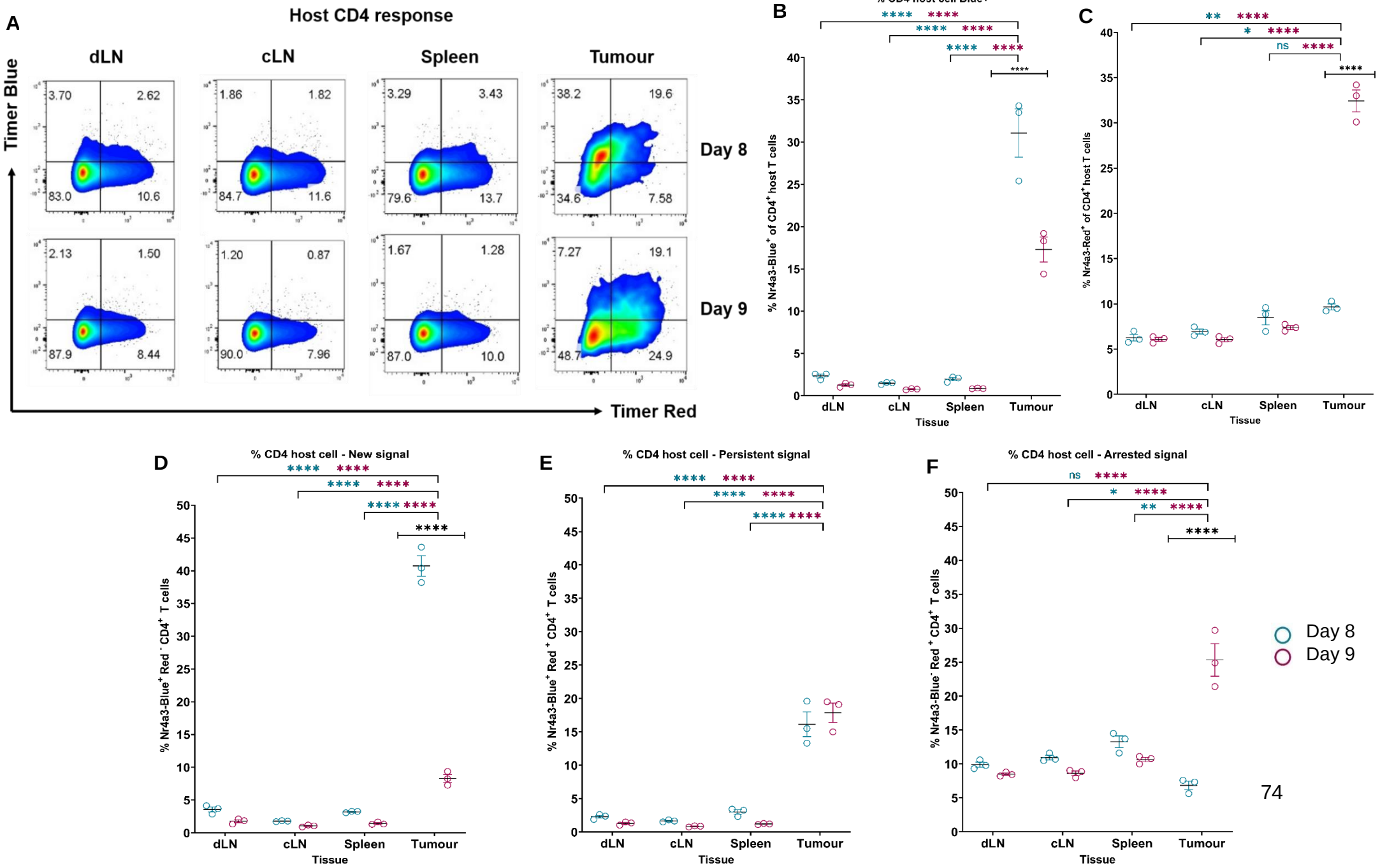

A
Host CD4 response
dLN
cLN
Spleen
Tumour
Timer Blue
Timer Red
Day 8
Day 9
3.70
2.62
83.0
10.6
1.86
1.82
84.7
11.6
3.29
3.43
79.6
13.7
38.2
19.6
34.6
7.58
2.13
1.50
87.9
8.44
1.20
0.87
90.0
7.96
1.67
1.28
87.0
10.0
7.27
19.1
48.7
24.9
B
% CD4 host cell Blue+
% Nr4a3-Blue+ of CD4+ host T cells
Tissue
C
% CD4 host cell Red+
% Nr4a3-Red+ of CD4+ host T cells
Tissue
D
% CD4 host cell - New signal
% Nr4a3-Blue+ Red- CD4+ T cells
Tissue
E
% CD4 host cell - Persistent signal
% Nr4a3-Blue+ Red+ CD4+ T cells
Tissue
F
% CD4 host cell - Arrested signal
% Nr4a3-Blue- Red+ CD4+ T cells
Tissue
Day 8
Day 9

Figure 16 cont.

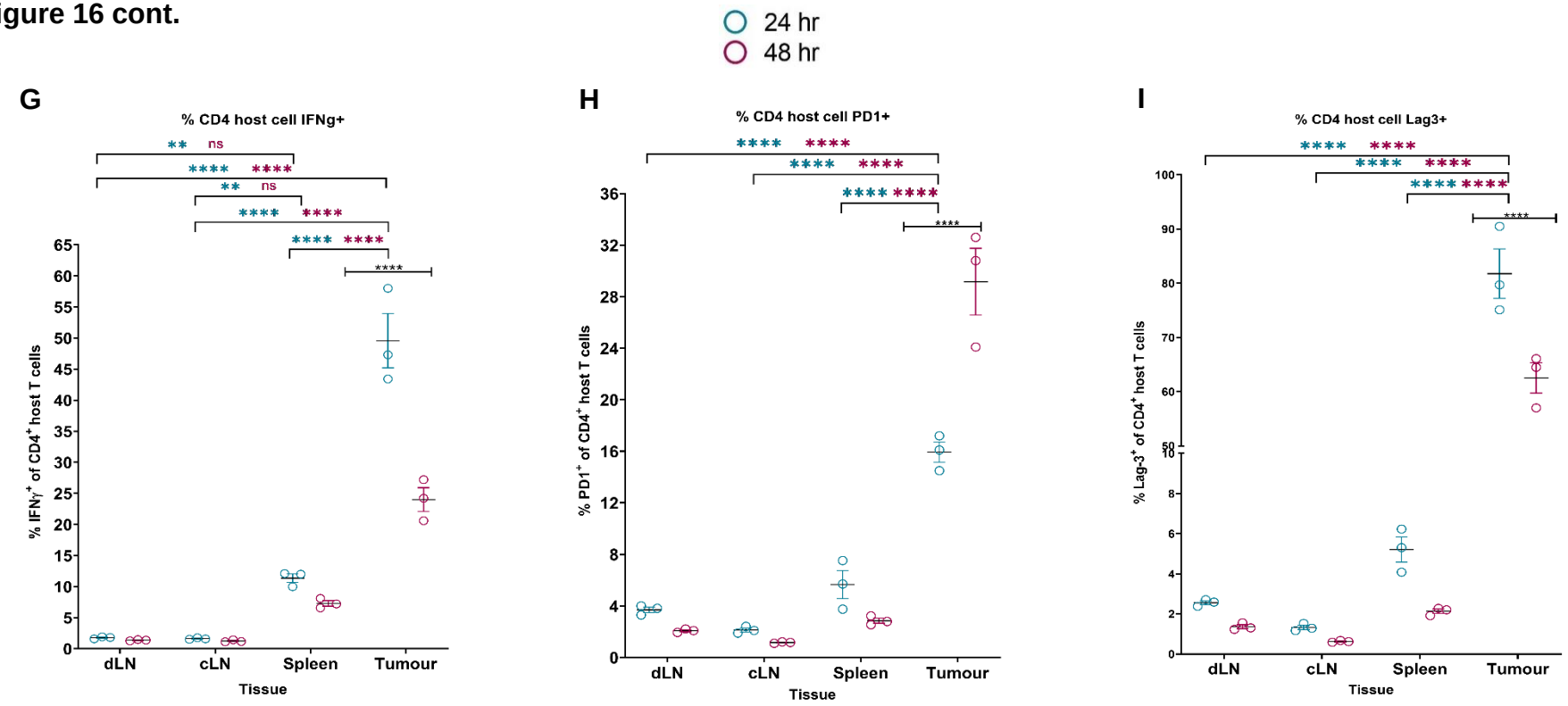


Figure 16. CD4⁺ host T cell response to tumour in the spleen, dLN, cLN and tumour microenvironment.

In vivo study where Nr4a3-Tocky mice were subcutaneously injected with MC38-ova tumour cells. Tumour was able to grow for 7 days and tissues were taken at day 8 and 9. Live cells were analysed by flow cytometry and using gating strategy described in figure 4b CD4⁺ host cells were investigated. **(A)** Plot shows expression of Nr4a3 Timer red vs Timer blue in the range of tissues taken at either day 8 or 9 to study activation dynamics. **(B-I)** Summary graphs showing the raw % of CD4⁺ host T cells that are positive for the markers named above each graph, in each tissue, at either day 8 (*blue circles*) or 9 (*maroon circles*). Each circle represents one mouse; 3 mice used at day 8 and 3 used at day 9. Error bars represent mean+SEM, n=3. Statistical analysis by two-way ANOVA with Sidak's multiple comparison tests. Significant difference ($p<0.05$) indicated as ****=$p<0.0001$, ***=p 0.0001-0.001, **=p 0.001-0.01, *=p 0.01-0.05, ns = no significance. Significance between timepoints shown in black, between tissues is shown in either blue (day 8) or maroon (day 9).

4.6.2 CD8$^+$ host T cell response to tumour in the spleen, dLN, cLN and tumour microenvironment

Like CD4$^+$ host T cells that showed a big difference in activation profile in the tumour, CD8$^+$ host cells also do. It can be seen in figure 17 that a high proportion of cells are Timer$^+$ after 8 and 9 days, significantly more so than response in the other tissues (Figures 17B and C). However, unlike CD4$^+$ host cells that show a significant shift in signalling from new/persistent to persistent/arrested, it can be seen that CD8$^+$ cells received the similar types of signals at days 8 and 9. Most cells had received a new or persistent signal to activate and stay activated, with a slight decrease in new signalling and a slight increase in cells that had arrested signalling at day 9 (Figures 17D-F). This indicates that unlike CD4$^+$ host cells, CD8$^+$ host cells were seen to stay activated in the TME, responding to both new and persistent TCR signals at days 8 and 9. These findings are supported by effector molecule and immune checkpoint expression.

It can be seen that the proportion of IFNγ$^+$, PD-1$^+$ and Lag-3$^+$ cells are significantly higher in the tumour compared to other tissue and it is also seen that all three are expressed at a very high level (Figures 17G-I). This indicates that CD8$^+$ T cell displayed effector function in the tumour whilst activated. It is however also possible these high levels were due to the TME causing increased expression of the immune checkpoints. Furthermore, it was shown that the proportion of cells expressing IFNγ and PD-1 at day 8 and 9 does not change (Figures 17G and H). There was however a significant decrease in the proportion of Lag-3$^+$ cells; however, this decrease still resulted in a high proportion of Lag-3$^+$ cells (Figure 17I). These results therefore

further support the idea that $CD8^+$ host T cell are continuing to stay activated and produce effector function rather than arresting in the TME.

The $CD8^+$ host cell response that can be seen in the dLN, cLN and spleen are, like $CD4^+$ response, very similar. It can be seen in figure 17A that in the dLN and spleen at day 8 and 9, there is a small proportion of cells expressing Timer blue (lowers at day 9), indicative of new TCR signalling (Figure 17B and D). This can also be seen of cells in the cLN but at an even lower level (Figure 17B and D). Although there is this very slight proportion of cells expressing Timer blue, it can be seen than in all three tissues at both day 8 and 9 that most cells that have activated, are expressing Timer red, indicating that cells that had once activated, have arrested TCR signalling (Figures 17A, C and F). This is similar to what $CD4^+$ host T cells showed however, unlike $CD4^+$ cells it can be seen in figure 17A that $CD8^+$ host cells are not expressing Timer $blue^+$ red^+; indicating that they are not receiving any persistent TCR signalling (Figure 17E). Cells in the spleen at day 8 shows some increase in persistent signalling but this is at a very low level. These findings indicate that although some $CD8^+$ host T cells are responding in the dLN, cLN and spleen (small increase Timer $blue^+$ cells) they are not receiving any, or very little, persistent signalling to stay activated and hence have arrested signalling (most cells Timer red^+).

These activation dynamics are again backed up by the overall low expression of IFNγ, PD-1 and Lag-3 (Figures 17G-I). Figure 17G shows the proportion of $IFN\gamma^+$ in the dLN, cLN and spleen are all less than 6%, with no significant change in levels from day 8 to 9. Although very subtle, it can however again be seen the there is a slight increase in $IFN\gamma^+$ cells in the spleen compared to the other two tissues;

possible due to the slight increase in Timer blue^{+} red^{+} cells. No difference can be seen in proportion of PD-1^{+} cells in all three tissues at both timepoints (Figure 17H). However, although levels are low (less than 12%), and differences are not significant, it can be seen in all three tissues that there is a decrease in Lag-3^{+} cells from day 8 to 9 (Figure 17I).

Figure 17

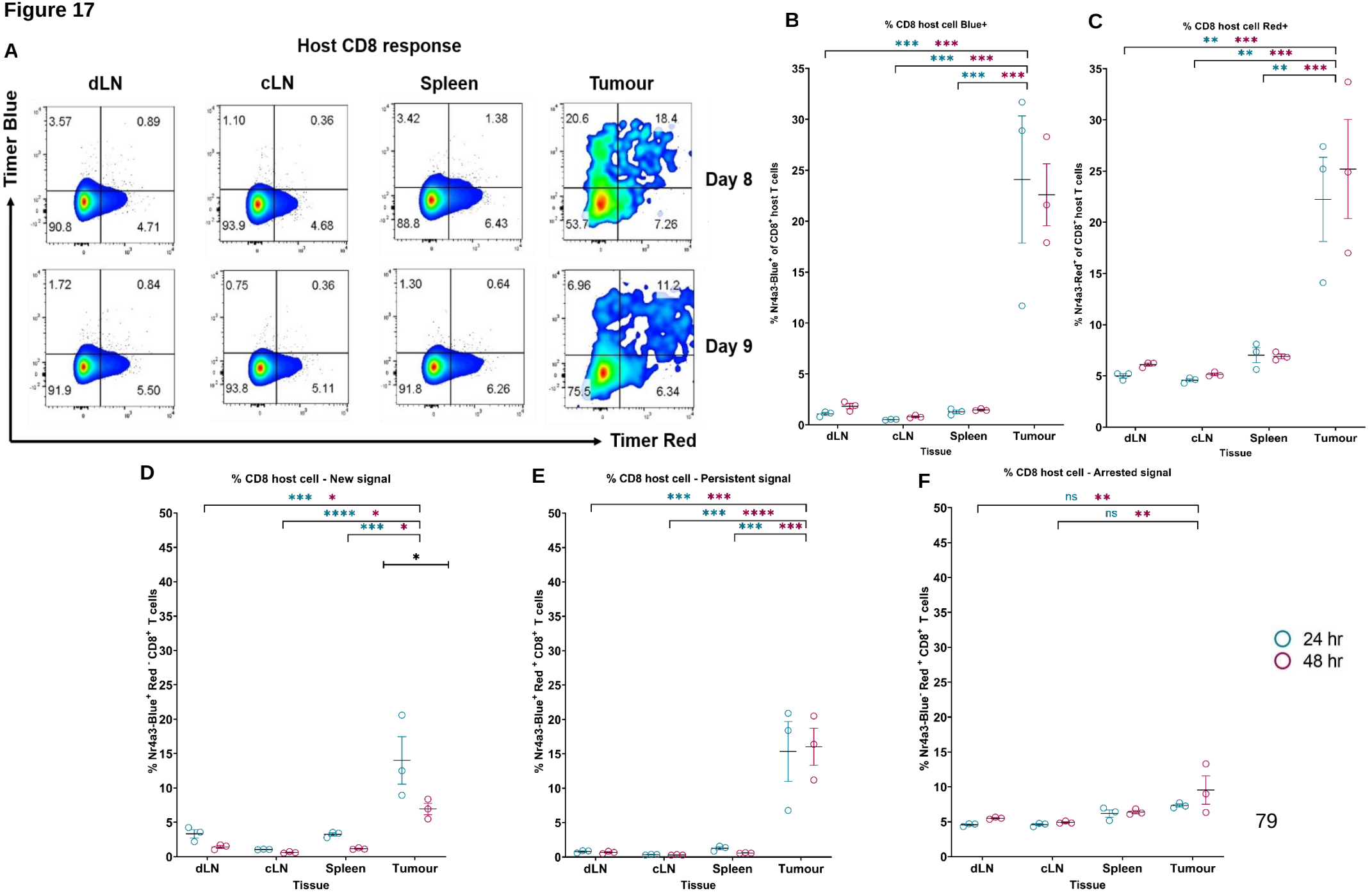

A
Host CD8 response
dLN
cLN
Spleen
Tumour
Timer Blue
Timer Red
Day 8
Day 9
3.57
0.89
90.8
4.71
1.10
0.36
93.9
4.68
3.42
1.38
88.8
6.43
20.6
18.4
53.7
7.26
1.72
0.84
91.9
5.50
0.75
0.36
93.8
5.11
1.30
0.64
91.8
6.26
6.96
11.2
75.5
6.34
B
% CD8 host cell Blue+
% Nr4a3-Blue+ of CD8+ host T cells
C
% CD8 host cell Red+
% Nr4a3-Red+ of CD8+ host T cells
Tissue
D
% CD8 host cell - New signal
% Nr4a3-Blue+ Red- CD8+ T cells
E
% CD8 host cell - Persistent signal
% Nr4a3-Blue+ Red+ CD8+ T cells
F
% CD8 host cell - Arrested signal
% Nr4a3-Blue- Red+ CD8+ T cells
24 hr
48 hr

Figure 17 cont.

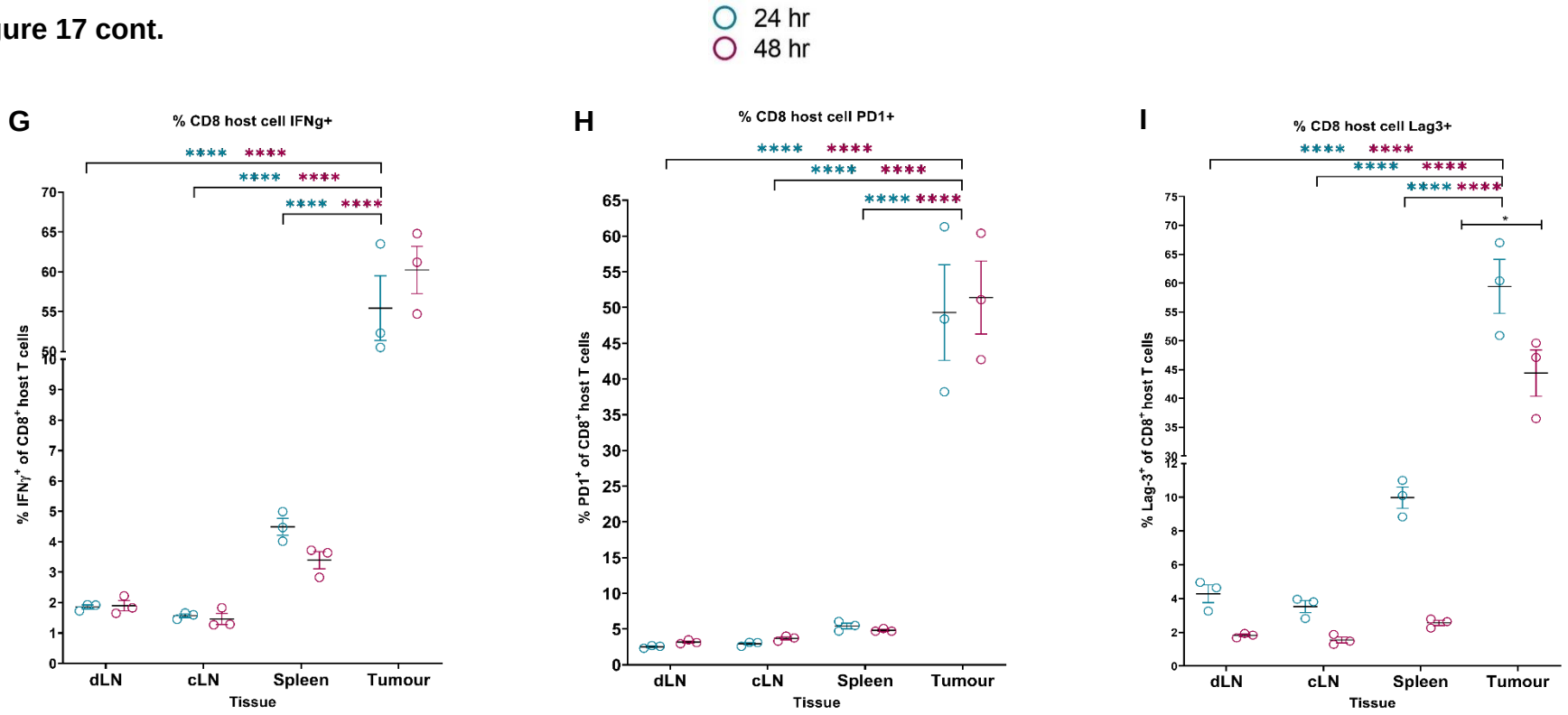


Figure 17. CD8⁺ host T cell response to tumour in the spleen, dLN, cLN and tumour microenvironment.

In vivo study where Nr4a3-Tocky mice were subcutaneously injected with MC38-ova tumour cells. Tumour was able to grow for 7 days and tissues were taken at day 8 and 9. Live cells were analysed by flow cytometry and using gating strategy described in figure 4b CD8⁺ host cells were investigated. **(A)** Plot shows expression of Nr4a3 Timer red vs Timer blue in the range of tissues taken at either day 8 or 9 to study activation dynamics. **(B-I)** Summary graphs showing the raw % of CD8⁺ host T cells that are positive for the markers named above each graph, in each tissue, at either day 8 (*blue circles*) or 9 (*maroon circles*). Each circle represents one mouse; 3 mice used at day 8 and 3 used at day 9. Error bars represent mean+SEM, n=3. Statistical analysis by two-way ANOVA with Sidak's multiple comparison tests. Significant difference ($p<0.05$) indicated as ****=$p<0.0001$, ***=p 0.0001-0.001, **=p 0.001-0.01, *=p 0.01-0.05, ns = no significance. Significance between timepoints shown in black, between tissues is shown in either blue (day 8) or maroon (day 9).

4.6.3 Results summary of host response.

The following conclusions can be drawn from these studies. The response of cells that had been within the TME indicate very different activation dynamics and molecule expression to these other tissues. Both $CD4^+$ and $CD8^+$ cells showed a significantly greater activation in the tumour however each seem to be receiving different types of TCR signals between day 8 and 9. $CD4^+$ host T cells in the tumour were seen to be activated and persistently signalled to result in sustained activation at day 8 however, there seemed to be a shift in signalling at day 9. At day 9, there was both a decrease in new signalling and an increase in persistent signalling and cells arresting; could be an effect of the TME. This is supported by expression levels; it was seen that although expression of all three molecules increased compared to other tissues, $IFN\gamma^+$ and $Lag\text{-}3^+$ cells both significantly decrease from day 8-9, however interestingly $PD\text{-}1^+$ cells significantly increases. This further indicates that the TME may be causing cells to arrest through over expression of the immune checkpoint. Unlike $CD4^+$ cells, $CD8^+$ cells were seen to not arrest in the tumour at these timepoints and are showed to activate and hence respond to the tumour with persistent and new TCR signals at both days 8 and 9. Expression levels of IFNγ and PD-1 did not change between days however there was a slight decrease in Lag-3. Both $CD4^+$ and $CD8^+$ host cells in the dLN, cLN and spleen were shown to have small responses to the tumour. It can be concluded that although cells recognised the tumour as foreign and continued to receive some new TCR signals (Timer $blue^+$) at these timepoints, it was seen that majority of cells had arrested signalling and were not showing any response (Timer red^+). This is supported by the extremely low levels of $IFN\gamma^+$, $PD\text{-}1^+$ and $Lag\text{-}3^+$ cells that can be seen in these tissues.

4.6.4 CD8$^+$ OTI T cell response to tumour in the spleen, dLN, cLN and tumour microenvironment *(ova specific).*

In order to follow the early tumour specific response of T cells, purified CD8$^+$ OTI Nr4a3-Tocky T cells (stained with a proliferation dye) were injected intravenously into mice displaying MC38-ova tumours and tissue was taken 24- or 48-hours after, as mentioned previously. It can be seen that, unlike other tissues, there was not much CD8$^+$ OTI T cell infiltration into the tumour at these timepoints (Figure 18A). It is shown that there is a significant difference between the proportion of Timer blue$^+$ cells in the dLN and cLN to the tumour however, there was no significant difference to be seen between the dLN and tumour for either Timer blue or red (at both timepoints) (Figures 18B and C). This suggests that the activation profiles of cells in both tissues are similar.

As it is known, T cell response to tumours can show great variance due to biological differences and this can be clearly seen to be happening in this investigation looking at the individual biological repeats carried out. This has been taken into consideration when drawing conclusions. It can be seen that at 24-hours, the very few cells detected in the tumour seem to be receiving mainly new TCR signals (Figure 18D), suggesting that cells are beginning to activate in response to tumour antigen. Although figure 18A shows no Timer blue$^+$ red$^+$ or red$^+$ cells, it can be seen in figure 18C that some biological repeats shown an increase in Timer red$^+$ cells. This indicates that CD8$^+$ OTI T cells at 24-hours are also receiving persistent signals while some have arrested (Figure 18E and F). This also gives reason to why no significant difference is seen between tumour and dLN response as they show similar activation

profiles. It can then be seen at 48-hours in figures 18A-C that although the same proportion of cells are Timer blue^{+} (new/persistent), there is a significant increase in Timer red^{+} cells; indicating that by 48-hours cells are beginning to show a broader activation profile where they are being persistently signalled and some have started to arrest (Figure 18E and F). Expression of IFNγ, PD-1 and Lag-3 (Figures 18G-I) support these findings and show that although cell activation profiles of cells in the dLN and tumour are alike, effector function is not (significant difference in all markers between two tissues). Figure 18G-I shows that CD8^{+} OTI T cell activated in the tumour have a significant increase in proportion of cells expressing of IFNγ, PD-1 and Lag-3 compared to other tissues. It can furthermore be seen that although there is no decrease in proportion of IFNγ^{+} and PD-1^{+} cells between timepoints (Figures 18G and H), there is a decrease in Lag-3^{+}; at 48-hours, levels are not significantly different to dLN (Figure 18I). Moreover, even though it is seen that the immune checkpoints are significantly expressed compared to other tissues, levels of PD-1 and Lag-3 are quite low. Hence, it can be suggested that the TME has not yet caused over-expression in this cell subgroup at these timepoints. The sustained levels of IFNγ and the immune checkpoints from 24 to 48 hours (Figure 18G) also indicates that although some CD8^{+} OTI T cells in the tumour are shown to have arrested TCR signalling, there is a high proportion of ova specific cells that are activated and are receiving persistent TCR signals to exert effector function, even though cells are expressing inhibitory immune checkpoints.

It can be seen in figures 18A-F that CD8^{+} OTI cells in both the cLN and spleen do not show any activation; at 24- and 48-hours it is shown that there is very little/no Timer protein being made as well as no IFNγ, PD-1 or Lag-3 expression. Hence indicating

that cells have not received any TCR signals needed to activate (Figures 18D-F) and produce effector function, highlighting that tumour antigens do not spread to non-draining lymphoid tissues. It can however be seen that $CD8^+$ OTI T cells in the dLN show activation and effector function. Figure 18A shows that, at 24-hours there is an increased proportion of cells in the dLN that have receive a new TCR signal (Timer $blue^+$; Figure 18B and D), a small percentage of cells are also shown to have receive persistent signals (Timer $blue^+$ red^+; Figure 18C and E) and it can be seen that almost no cells have also arrested signalling (Figure 18F). Due to the increase in Timer $blue^+$ cells, indicating that majority of activated cells have received a TCR signal 4 hours previous to tissue collection, it can be thought that $CD8^+$ OTI cells had only just recently encountered tumour antigen at 24-hours. This can be further supported by the 48-hour timepoint showing that although there is no significant change in the proportion of cells receiving new or persistent signals (Figure 18B, D and E), there is a significant increase in Timer red^+ cells (Figure 18C), due to an increase in cells arresting signalling (Figure 18F). This further suggests that at 48-hours, cells have begun to respond and recognise tumour antigen as well as also starting to exert effector function; increase in IFNγ^+, PD-1^+ and Lag-3^+ cells (Figures 18G-I). An addition to these findings is the individual cell proliferations of $CD8^+$ OTI T cells (Figure 19). As mentioned before, cells were stained with a proliferation dye before injection and although this was done to be able to gate on the exogenous T cells (Figure 4b), it also makes it possible to look into response after cell proliferation; 48-hours in the dLN is the only time proliferation was able to be picked up. Figure 19A shows that after the first proliferation, were it can be see that there was some increase in Timer $blue^+$ red^+ and Timer red^+ cells, there is a decrease in the proportion of these cells and an increase of Timer $blue^+$ cells with the following

proliferations. This suggests that as cell proliferate, they receive mainly only new TCR signals. When looking at IFNγ, PD-1 and Lag-3 expression (Figures 19B-D), the opposite effect can be seen; as cells proliferation the proportion of cells expressing each marker increases. Therefore, it can be theorised that the decrease in Timer protein is due to a combination of proliferation causing Timer protein to dilute and the cessation of TCR signalling.

Figure 18

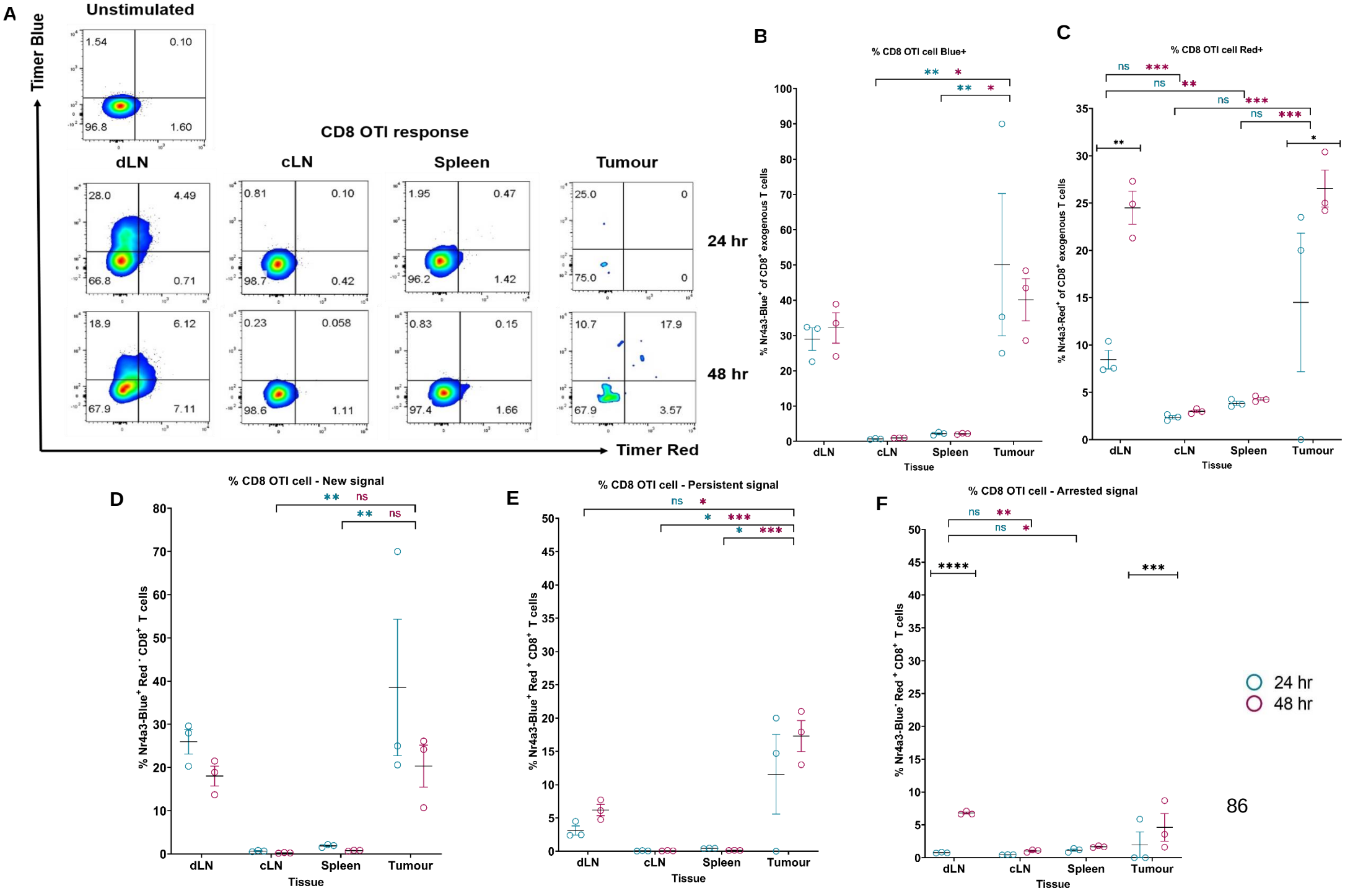

A
Unstimulated
Timer Blue
CD8 OTI response
dLN
cLN
Spleen
Tumour
24 hr
48 hr
Timer Red
1.54
0.10
96.8
1.60
28.0
4.49
66.8
0.71
0.81
0.10
98.7
0.42
1.95
0.47
96.2
1.42
25.0
0
75.0
0
18.9
6.12
67.9
7.11
0.23
0.058
98.6
1.11
0.83
0.15
97.4
1.66
10.7
17.9
67.9
3.57
B
% CD8 OTI cell Blue+
% Nr4a3-Blue+ of CD8+ exogenous T cells
Tissue
C
% CD8 OTI cell Red+
% Nr4a3-Red+ of CD8+ exogenous T cells
Tissue
D
% CD8 OTI cell - New signal
% Nr4a3-Blue+ Red- CD8+ T cells
Tissue
E
% CD8 OTI cell - Persistent signal
% Nr4a3-Blue+ Red+ CD8+ T cells
Tissue
F
% CD8 OTI cell - Arrested signal
% Nr4a3-Blue- Red+ CD8+ T cells
Tissue
24 hr
48 hr
dLN
cLN
Spleen
Tumour

Figure 18 cont.

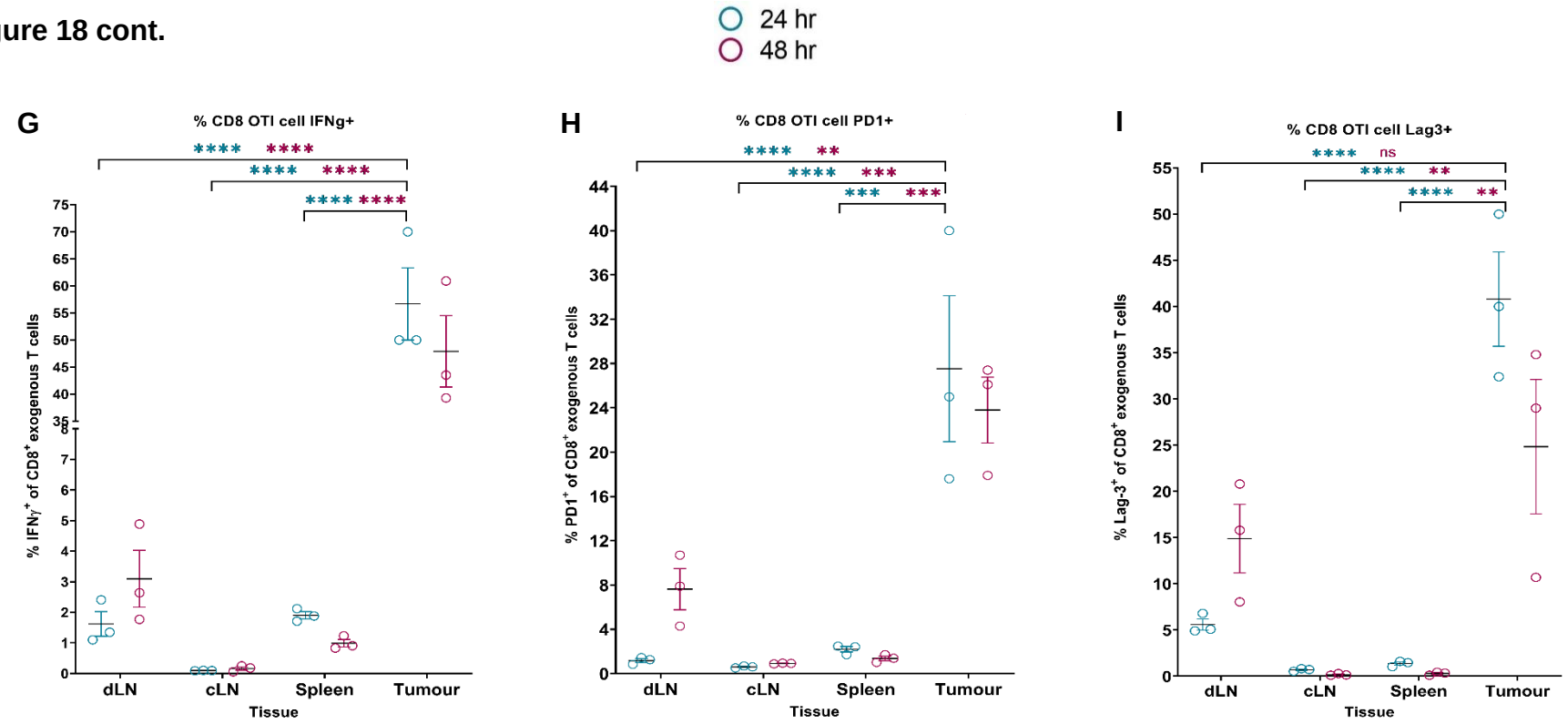


Figure 18. CD8⁺ OTI T cell response to tumour in the spleen, dLN, cLN and tumour microenvironment *(ova specific).*

In vivo study where Nr4a3-Tocky mice were subcutaneously injected with MC38-ova tumour cells. Tumour was able to grow for 7 days before purified CD8⁺ OTI T cells were stained with proliferation dye and intravenously injected. 24- and 48-hours after injection, tissue was taken and live cells were analysed by flow cytometry using the gating described in figure 4b to investigate CD8⁺ OTI T cells. **(A)** Plot shows expression of Nr4a3 Timer red vs Timer blue in the range of tissues taken at either 24- or 48-hours to study early activation dynamics. **(B-I)** Summary graphs showing the raw % of CD8⁺ OTI T cells that are positive for the markers named above each graph, in each tissue, at either day 8 (*blue circles*) or 9 (*maroon circles*). Error bars represent mean+SEM, n=3. Statistical analysis by two-way ANOVA with Sidak's multiple comparison tests. Significant difference ($p<0.05$) indicated as ****=$p<0.0001$, ***=p 0.0001-0.001, **=p 0.001-0.01, *=p 0.01-0.05, ns = no significance. Significance between timepoints shown in black, between tissues is shown in either blue (24 hours) or maroon (48 hours).

Figure 19

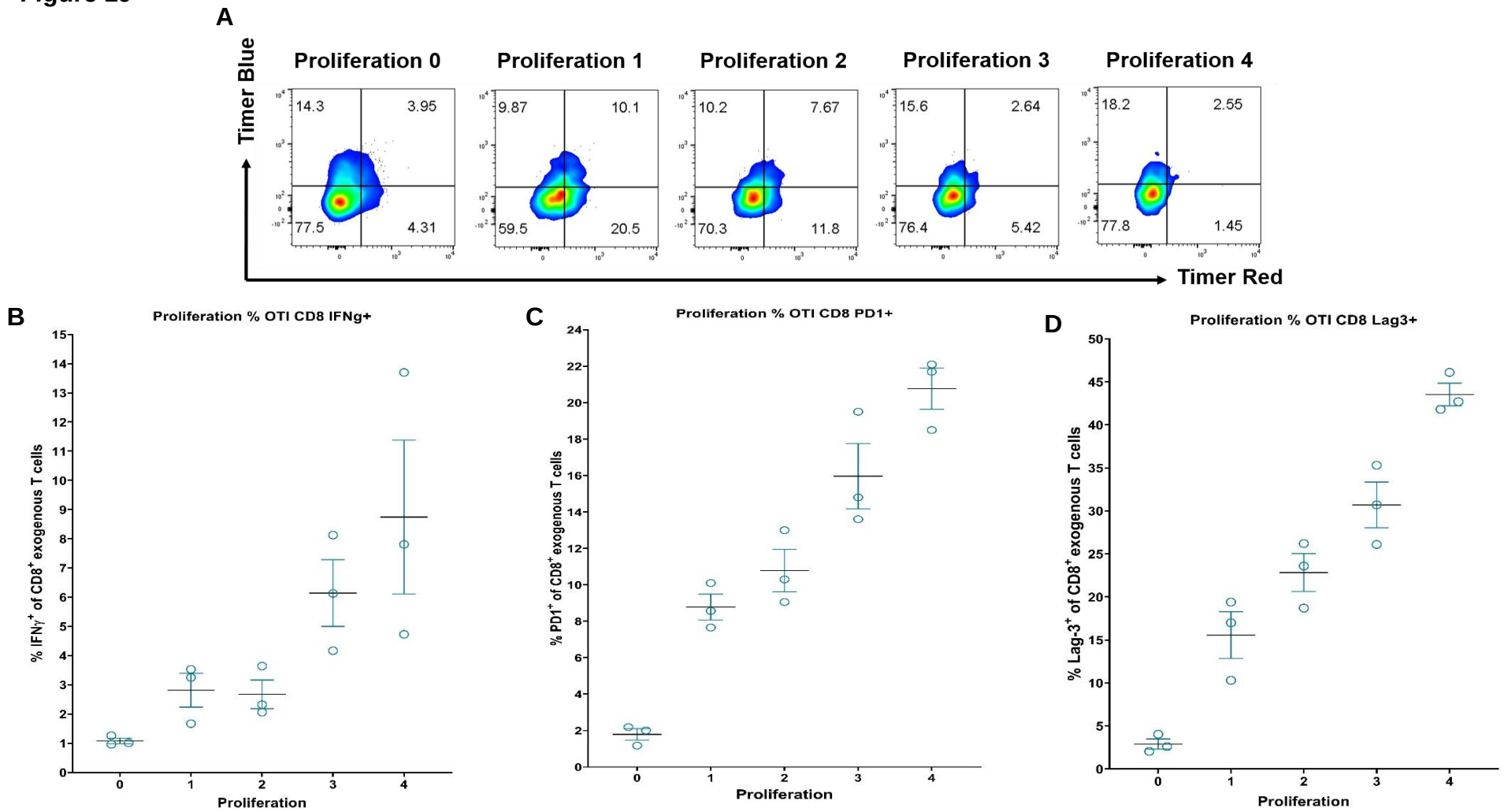


Figure 19. CD8⁺ OTI T cell proliferation in the dLN (48 hours).

In vivo study where Nr4a3-Tocky mice were subcutaneously injected with MC38-ova tumour cells. Tumour was able to grow for 7 days before purified CD8⁺ OTI T cells were stained with proliferation dye and intravenously injected. 24- and 48-hours after injection, tissue was taken and live cells were analysed by flow cytometry using the gating described in figure 4b to investigate CD8⁺ OTI T cells. **(A)** Plot shows expression of Nr4a3 Timer red vs Timer blue as cells proliferated at 48-hours in the dLN. **(B-D)** Summary graphs shows the raw % of CD8+ OTI T cells that are positive for the markers named above each graph as cells in the dLN at 48-hours were shown to proliferate. Error bars represent mean+SEM, n=3.

4.6.5 Results summary of initial ova specific $CD8^+$ T cell response.

The following conclusions can be drawn from this investigation. $CD8^+$ OTI cells in both the cLN and spleen do not show any activation or effector molecule expression 24- or 48-hours after cells were introduced. Cells in the dLN at 24-hours have mainly received new TCR signals and show some persistent signalling but no cells have yet arrested. This indicates that the majority of activated cells have received a TCR signal only 4 hours previous to tissue collection, and so it can be thought that cells had only just recently encountered tumour antigen. By 48-hours more cells express both persistent and arrested signalling indicating cells had begun to respond and recognise tumour in a broader fashion as well as also started to exert effector functions; increase in $IFN\gamma^+$, PD-1^+ and Lag-3^+ cells. It is further seen that at either timepoint there is a decrease in cell infiltration of the tumour however, activation of cells in this tissue compared to cLN and spleen is significant. There is no significant different between tumour and dLN indicating that cells are activated similarly in these tissues. However, it is shown that expression of IFNγ, PD-1 and Lag-3 is significantly different (higher) between tumour and other tissues and hence indicates that although activation profiles seem similar between cells in the dLN and tumour, effector function is not. The increased expression of IFNγ, PD-1 and Lag-3 in the tumour are indicative of T cell activation and suggests that 24- and 48-hours after introduction, although some cells are seen to arrest signalling, high proportions of tumour specific $CD8^+$ OTI T cells are activated, receiving new and persistent signalling resulting in expression of effector molecule such as IFNγ.

5. Discussion and conclusions

This project has been based around the title of 'antigen regulation of T cell receptor signalling and immune checkpoint expression in health and disease'. Four different main aims were investigated, using different stimulations, conditions and mouse models, all of which involved using the novel Nr4a3-Tocky system (Bending *et al.,* 2018). As this reporter system uses Timer protein, this allowed for the real time analysis of the type of signals cells were receiving; whether that was new (Timer blue$^+$, signalled within 4-hours), persistent (Timer blue$^+$ red$^+$, signalled within and previous to 4-hours) or arrested (Timer red$^+$, previously signalled, but not within 4-hours). This consequently also allowed for the temporal analysis of cell activation, as well as IFNγ expression; built-in reporter of Great Smart mice (Price *et al.,* 2012). These readouts that result from using the Nr4a3-Tocky system, alone, as a dual reporter and crossed with OTI cells, suggests that this reporter could be a very useful tool in the future developments to this project and within the field to study phenomenon such as T cell development and self-tolerance as well as advancing cancer immunotherapy.

The first aim of this project looked to determine how TCR signal strength, duration and affinity for antigen-MHC regulate Nr4a1 and Nr4a3 gene expression in CD8$^+$ T cells. Using Nr4a1-GFP Nr4a3-Tocky mice it was found that the proportion of cells that express Nr4a1 GFP increased at a lower dose (0.008µg/ml) than cells expressing Nr4a3 Timer blue (0.04-0.2µg/ml); suggesting that Nr4a1 is more sensitive to TCR signal strength than Nr4a3. Additionally, when EC50 values were calculated there was only a 3-fold difference in expression of the two genes. This collectively suggests that although Nr4a1 shows an increased sensitivity to anti-CD3,

signal strength results in a modest difference in gene expression. As both Nr4a1 and Nr4a3 have been shown to be highly expressed in different subgroups of cells; positive selection during T cell development and Tregs, respectively, it seems unlike this modest effect could control their expression (Jennings et al., 2020; Bending *et al.,* 2018). Following this, the effect of TCR signal duration was investigated. It was found that after 2.5-5 minutes of TCR signalling there was a marked increase in the proportion of Nr4a1 GFP^+ cells, which grew to a maximum, after 30-60 minutes, of 90% reporter positive cells. Unlike this, Nr4a3 Timer $blue^+$ cells only began to increase after 30 minutes of signalling and did not reach the same proportion of reporter positive cells as Nr4a1 GFP even at 12-240 minutes. This suggests that Nr4a1 needs shorter signal to be activated and that duration of TCR signal is likely to be responsible for differential expression.

Finally, the effect of TCR affinity for antigen-MHC was looked into. This study uncovered that like signal strength, affinity has a small effect on differential expression of Nr4a1 GFP and Nr4a3 Tocky; very small fold difference in EC50 values. The increased difference in EC50 with V4 peptide can be thought to be due to the transient nature of low affinity TCR signalling; shorter duration of signal is linked to differential expression, as previously concluded, hence transient signalling is more likely to be the cause rather than affinity. It was however found that affinity is directly linked to the dose needed to cause gene expression in cells. N4 (10^{-5}µM) requires the lowest dose of antigen to show reporter expression, Q4 (10^{-4}µM) requires slightly higher, and V4 (10^{-2}µM) higher again. These findings accumulate into the conclusions that the differential expression of Nr4a1 and Nr4a3 is likely due to the duration of the TCR signal. Affinity for antigen-MHC was also concluded to

alter the dose of antigen needed for the expression of both reporters. It can also further be seen that Nr4a1 expression is more sensitive to both TCR signal strength and duration than Nr4a3. It could be suggested that differential expression is a result of these two genes being downstream to different TCR pathways. Jennings *et al.,* described that although Nr4a3 expression is regulated by the calcineurin/NFAT pathway, Nr4a1 was found not to be (Jennings *et al.,* 2020). Following propagation of this pathway, NFAT has to move into the nucleus to exert effects (Crabtree and Olson, 2002) so, it could be theorised that this is what causes the increased sensitivity of Nr4a1 compared to Nr4a3. This however cannot be concluded as the pathway leading to Nr4a1 expression is not known, therefore it is not possible to comment on possible kinetic differences of downstream signalling. Nr4a1 GFP is a widely relied upon reporter in the field to study TCR signalling in a variety of cells (Moran *et al.,* 2011) however, as it has been described in this project that Nr4a1 GFP can be highly expressed after only a few minutes of TCR signalling it should be used with caution in studies investigating the effect of signal strength and temporal changes. The findings described in this section can be found, in parts, in press, at Cell Reports (Jennings *et al.,* 2020).

The second aim of this project involved further investigating how antigen dose, affinity for TCR and time affects the activation dynamics of $CD8^+$ T cells, through TCR signalling analysis, and the expression of IFNγ and PD-1. Two timepoints were used in this study to look into both the initial (4-hours) and longer (16-hours) effects. It was first concluded that affinity of peptide effects the dose needed to cause activation. This is in line with what I observed in aim 1, and dose thresholds were found to be the same too, at both 4- and 16-hours. At the threshold doses it could be

seen that cells activated due to a broad range of TCR signalling; some cells had not received a signal, some had received a new signal, some received persistent signals and some had arrested signalling (16-hours only, Timer protein not matured at 4-hours). This is what would be expected to be seen at a threshold dose. An interesting conclusion drawn from this study is that affinity affected the maximum proportions of cells that activated (Timer$^+$) both at 4- and 16-hours. At 4-hours it could be seen that with the high affinity N4 peptide, 90% of cells were Timer blue$^+$ however with Q4, 75% were and with V4 only 50% were. This gap closed slightly at 16-hours, with more than 95% Timer blue$^+$ red$^+$ cells, however there was still slightly less again with V4.

A recently study carried out by Conley *et al.,* described that in a similar system testing OTI CD8$^+$ T cells for 24-hours with ova peptide variants N4, T4 and G4, that weak TCR signalling and/or the absence of ITK activation (upregulates IRF4 through the calcineurin/NFAT pathway) resulted in the delayed kinetics of NFAT activation and Irf4 mRNA expression. This hence produced a population of CD8$^+$ T cells that had reduced proliferation capacity in response to infection (Conley *et al.,* 2020). This study supports the theory that the differences in the maximum proportions of cells displaying persistent signalling and activation could be due to affinity altering signalling dynamics. It was shown in response to N4, there was a higher proportion of Timer blue$^+$ red$^+$ cells compared to V4. It can be theorised that if N4 resulted in cells receiving new signals quicker (express Timer blue quicker) than what V4 would do, this would have an effect on the proportion of cells that are shown to have had persistent signalling (expressing Timer blue and red), as blue protein in N4 cells would of had longer to mature to red than what V4 would do, due to slower signalling

dynamics. With the support of Conley *et al.,* findings that weak TCR signalling resulted in the delayed kinetics of NFAT (known to regulate Nr4a3 expression; Conley *et al.,* 2020) it can be concluded that affinity affects signalling dynamics in $CD8^+$ T cells.

The expression of IFNγ and PD-1 were also analysed during this study. Expression showed the same dependency on affinity to determine the threshold of antigen dose needed to cause expression and it was also seen that affinity dictates the maximum proportion of cells expressing each of the effector molecules with high significance; with N4 showing the highest expression, V4 the lowest and Q4 in between. Although these similarities between the two molecules were seen, their expression at high doses couldn't be more different. PD-1 was seen to increase as dose of all three affinity peptides did, at 4-hours. At 16-hours it was seen that expression increased with dose of all three affinity peptides but reached a maximum expression and plateaued, interesting just one dose increment up from threshold (V4 showed this slightly less but still on trend). This could indicate that with higher affinity peptides, PD-1 expression is almost binary. Expression of IFNγ was shown to display a very different pattern of expression. As dose increased with the higher affinity N4 peptide $IFN\gamma^+$ cells begin to decrease after they reach the dose that causes maximum proportion of cells to activate (10^{-2}μM), producing a bell-shaped curve. It also seemed at 16-hours with Q4, expression could be following the same trend. Even though this expression pattern seems very unlikely, the complex feedback model described by Lever *et al.,* KPL-IFF, supports this finding (Lever *et al.,* 2016). They found when $CD8^+$ T cells were stimulated with a 1 million-fold range of different affinity peptides, IFNγ levels also displayed this same bell-shaped curve for high

affinity peptides. Additionally, they also report that affinity also does not affect peak expression levels (Lever *et al.,* 2016), this was found during this project also; maximum proportion of cells expressing IFNγ with high doses show no significant difference and so are comparable, independent of affinity stimulated with. They propose the complex model: kinetic proofreading with limited signalling coupled to an incoherent feed-forward loop (KPL-IFF) (Lever *et al.,* 2016). Where P is linearly proportional to cytokine production and Y is representative of a substrate able to activate P, the incoherent feed-forward loop describes that as well as antigen-MHC-TCR complex directly inhibiting P, P is also inhibited at high concentrations by saturation of Y. Building on this, the kinetic proofreading model describes that when antigen binds, TCR signalling is not immediately triggered so antigen stays bound until it becomes signalling competent; this results in the fact that low affinity peptides induce a lower maximum concentration to become signalling competent. If this concentration is below the level at which Y fully saturates then even high doses of low affinity peptide would not cause inhibition of IFNγ production. Factoring in the limited signalling model to kinetic proofreading (TCR complex only signals for a limited time before turning to a nonsignaling state) further explains that although high affinity peptides remain bound for longer, this does not result in increased IFNγ expression (Lever *et al.,* 2016).

Using findings of this study makes it possible to propose an answer to a big question in the field; can a high dose of low affinity peptide cause the same response as high affinity peptide (Zehn *et al.,* 2009; Conley *et al.,* 2020). The findings of this study can lead one to believe that this is possible; stimulation with low affinity V4 resulted in comparable cell activation and effector function (IFNγ and PD-1 expression) to higher

affinity peptides and any differences could be accounted for due to the effect of affinity of signalling dynamics slowing down activation, rather than controlling it. However, the activation rate is greatly slowed, even when using a high amount of a low affinity peptide, suggesting that they may not achieve the same biological end point. The conclusions drawn from this study are important as they broaden the key knowledge and understanding of CD8+ T cell activation and effector molecule expression as a result of antigen regulation of TCR signalling. They are also important for the study of disease, as it is known that the level of immunogenicity a target has greatly affects the immune response and can also affect the effectiveness of immunotherapy.

The third aim to this project was to examine the *in vitro* functional role of PD-1 in the regulation of cognate $CD8^+$ T cell activation. Blockade of PD-1 interacting with its ligands using anti-PD1 resulted in a surprisingly minimal effect on activation and effector function; high proportions of $CD69^+$ and $IFN\gamma^+$ cells even at doses where PD-1 was active. This suggests that PD-1 did not cause much of an inhibitory effect on cells *in vitro* during the primary activation. It was further seen that the proportion of $IFN\gamma^+$ cells when stimulated with N4 or Q4 and either isotype or anti-PD1 were very comparable. Stimulation with V4 results in a higher proportion of $IFN\gamma^+$ cells with anti-PD1 compared to isotype and even shows some significance at 10^{-1}µM, indicating that anti-PD1 may affect lower affinity peptides more than higher affinity. However, results of this study show overall very minimal response. It can be proposed that the reasons for PD-1 not showing much of an inhibitory effect in vitro stems from experimental design. It is likely that that levels of PD-1 ligands were low in the culture, possibly due to tissue preparation destroying macrophages which are a main

source of binding ligands. Since PD-1 ligands were not present in high numbers, PD-1 could not bind and hence did not exert its inhibitory effect. However, it is possible that there was some ligand available to bind as there was a slight response of low affinity V4 peptide to anti-PD1.

Further plans to advance this study were disrupted by the Covid-19 pandemic. Plan included changes to the protocol that could hopefully solve these problems that arose. As tissues preparation could possibly be the cause to low availability of ligands and hence have caused cells not show much of an inhibitory effect within the isotype group, an alternative method such as digesting tissue rather than forcing through strainers may be advantageous. It was also planned to see how anti-PD1 affected cells on the secondary activation rather than first; by washing away excess peptide and allowing cells to arrest following primary stimulation and then restimulating with a range of doses of different affinity peptides. The effect of anti-PD1 on secondary stimulation would be very interesting, as well as it being clinically important to see the effect of anti-PD1 when encountering different affinity and dose of antigen. If the system was found to work and be representative of anti-PD1 effect, it can be proposed that further studies could be conducted looking into what happens to other immune checkpoints when PD-1 is being blocked. This could give insight to the combinations of immune checkpoints blockades that could be trialled for immunotherapy to optimise therapeutic effects; such as the successful pairing of anti-CTLA4 and anti-PD1 (Larkin *et al.,* 2015) whilst also investigating any unwanted effects; such as seen with anti-OX40 and anti-PD1, sequenced in this order they have therapeutic effect, however in reverse or concurrently, effects are diminished (Messenheimer *et al.,* 2017).

The final aim that was addressed in this project was to explore how T cell activation dynamics (through TCR signalling analysis), immune checkpoints and IFNγ expression are affected by the tumour microenvironment *in vivo*. Additional tissue to the tumours were taken at two different timepoints in order to understand the wider effects being causing on T cells. $CD4^+$ and $CD8^+$ T cell host response as well as exogenous $CD8^+$ OTI (ova specific) cells were studied and showed a variety of activity and effector function in different tissues at different timepoints. It was seen that the $CD4^+$ host response in the tumour was far greater than any other tissue; over 60% cells activated at day 8 and over 50% at day 9. Cells at day 8 were shown to receive mostly new or persistent signalling whereas at day 9, there was a highly significant shift to cells arresting signalling also. This change in signalling indicates that although cells are being activated and persistently signalled to result in sustained activation in the tumour at these timepoints, there seems to be a shift in cells receiving a new signal at day 8 to an increase of cells arresting signal at day 9; this could be an effect of the immunosuppressive TME.

It can be further seen that between day 8 and 9 in the tumour that the proportion of $CD4^+$ host cells that were $IFN\gamma^+$ and Lag-3^+ significantly decreased, whilst PD-1^+ cells significantly increased. This further supports the idea that the change in activation and signalling in the tumour is likely due to the TME causing cell dysfunction and exhaustion, represented by the upregulation of immune checkpoint PD-1. The overall response of $CD4^+$ host cells in other tissues (dLN, cLN and spleen) was seen to be very small, if any response was seen, it was an increase in Timer red$^+$ cells; indicating that although cells had historically received a TCR signal, they had not

received signals to stay activated and hence arrested. IFNγ and immune checkpoints, PD-1 and Lag-3, expression also support this finding; the proportion of cells that were positive for expression were very low, as well as levels showing little to no difference from day 8 to 9.

$CD8^+$ host cell response was also investigated. Like $CD4^+$ host response, it was shown that high proportions of cells were $Timer^+$ (activated) in the tumour after both days 8 and 9. However unlike $CD4^+$ response, $CD8^+$ cells were not shown to shift signalling to mostly arrest signalling at day 9; indicating that this subgroup of cells, at these timepoints, in the tumour, continued to receive both new and persistent signals and hence stayed activated. This can be shown also by the expression of effector molecules. IFNγ, PD-1 and Lag-3 were all shown to be significantly expressed at both timepoints, further indicating that not only are $CD8^+$ host cells still being activated in the tumour, they are also still producing effector function. The high levels of immune checkpoints could indicate the effect of the TME however, these are not taking inhibitory effect yet. The response of $CD8^+$ host cells in other tissues was shown to be the same as $CD4^+$ cell response; very little activity was seen and any activity that was seen was due to arrested signalling. Additionally, there was also very low levels of all three effector molecules, which unlike $CD4^+$ cells did not show a rise in PD-1 day 8 to 9. Collectively these results indicate that although $CD4^+$ and $CD8^+$ host cells in these tissues may recognise tumour as foreign and some cells are still being activated 8-9 days after the tumour was introduced, cells either do not stay activated to mount a response, possibly due to immune checkpoint expression, or are leaving these tissues to go to the tumour site where they show highly activated and functional responses.

Lastly, $CD8^+$ OTI T cell response was investigated. These cells were analysed 24- and 48- hours after being injected into the mouse displaying the tumour, to follow initial response. It could be seen that at these timepoints there was little T cell infiltration into the tumour however, it was seen that the few cells that did enter, after 24-hours were shown to be activated and receiving mainly new Timer $blue^+$ TCR signals, but also some persistent and arrested. This indicates that after 24-hours of $CD8^+$ OTI cells circulating in the system, response in the tumour had just began to activate in the previous 4 hours (approximately 20 hours after injection). By 48-hours, cells are still seen to be responding however there was an increase in Timer red^+ cells, indicating that some cells had received either persistent or arrested signalling by this time; cells display a broader activation profile.

Interestingly it was shown that the activation profile of cells in the dLN and tumour were very similar, showing no significant difference in either Timer blue or red. It was seen that $CD8^+$ OTI cells in the dLN at 24-hours also showed mainly an increase in proportion of cells receiving new signal and also some increase in persistent signalling, however unlike response in the tumour, the proportion of cells that had arresting signalling was lower. This indicates that like cells in the tumour, $CD8^+$ T cells in the dLN also seem to have responded to antigen approximately 20 hours after being introduced however, they are seen to not arresting signalling as much. This difference could possibly be an effect of the TME on cells in the tumour causing them to show increased signal arrest at an earlier timepoint. By 48-hours, cells in the dLN were shown to increase proportion of Timer red^+ cells, like the response of cells in the tumour. Although these findings suggest that activation of cells in the dLN and

tumour are similar, when effector function was investigated, cells display very different expression patterns. In the dLN at 24-hours, the expression of IFNγ, PD-1 and Lag-3 was shown to be very low in cells. Levels started to increase by 48-hours, indicating that as cells showed more activation, they also produced more of an effector function however, this was still at a low level. On the other hand, it can be seen that expression levels in cells from the tumour were significantly higher than that of all other tissues, at both timepoints, and also did not show any decrease in expression as time went on. Levels of PD-1 and Lag-3 were still quite low, suggesting that at 24-48 hours the TME has not yet caused over expression of immune checkpoints in this cell subgroup, like what was seen with $CD4^+$ host T cells in the tumour at day 9. These findings collectively show that although $CD8^+$ OTI T cells in both the dLN and tumour were shown to have some arrested TCR signalling, there was a higher proportion of ova specific cells that were activated and receiving persistent TCR signals in response to tumour antigen. Furthermore, it was also concluded that effector function of cells is altered in the two different tissues; overall, cells in the dLN showed a significantly lower level of IFNγ, PD-1 (at both timepoints) and Lag-3 (at 24-hours) compared to cells in the tumour. It could be thought that this is due to the TME causing cells to over-express immune checkpoint but as levels are still quite low and do not change over time it could be suggested that the environment is not yet causing much effect at this timepoint and levels could be linked to IFNγ role in the PD-1/PD-L1 pathway (Cheng *et al.,* 2007). It was seen that $CD8^+$ OTI T cells in the cLN and spleen show no activation or effector function at either timepoint; indicating that cells in these tissues have not yet encountered tumour antigen (ova-specific) as it does not spread to non-draining lymphoid tissue.

The findings of this study aid in the understanding of how the TME effects $CD4^+$ and $CD8^+$ T cell activation, through TCR signalling, and IFNγ expression of both the polyclonal host and the early ova-specific T cell responses. Analysis of immune checkpoint expression also added to these findings to shed light on how the TME can modulate their expression. Findings relating to other immune related tissues is also very informative as it has been shown in this study that different subgroups of cells, at different timepoints, are capable of recognising and responding to tumour antigen. This study was planned to be expanded on, by investigating the effects of anti-PD1 on cells in the TME however, this got interrupted due to the Covid-19 pandemic. This investigation would be beneficial to carry out as it would allow the analysis of how immune checkpoint blockade effects cell signalling, activation and effector function *in vivo* by comparison to this study of a control group. If this was to take place a longer timepoint would be suggested as well as the ones done in this study, this would allow the analysis of longer-term effect of checkpoint blockade and also possibly be able to give reasoning to why some people do not have long lasting effects of treatment. Another interesting development to this study would be to mutate the ova expressing tumour, so that the tumour antigen displayed had different affinity for TCR. This would hence create a more clinically beneficial model, as the differences in antigen affinity could represent the different levels of immunogenicity tumours can have. Following the successful generation of this model, it would then be very interesting to test combinations of immune checkpoint blockades in different affinity environments to study how cell activation, expression of effector molecules and alternative immune checkpoints are altered. This could be a very useful model in order to investigate the efficacy of immunotherapies and combination therapies while also being able predict possible unwanted secondary effects to blockades.

5.2 Project summary

This project set out to investigate how antigen regulates TCR signalling and immune checkpoint blockade in health and disease. Although the Covid-19 pandemic disrupted some plans, a comprehensive study has taken place to address the aims set out. Firstly, how TCR signal strength, duration and affinity for antigen-MHC regulates the expression of Nr4a1 and Nr4a3 in $CD8^+$ T cells was determined. It was concluded that Nr4a1 requires a shorter, weaker TCR signal than Nr4a3 to be expressed.

This was followed by a further investigation into how antigen dose, affinity for TCR and time effects activation dynamics, through TCR signalling analysis, and the expression of effector molecules, IFNγ and PD-1. The three main conclusions included: 1) Antigen affinity for TCR determined dose threshold to activate cells and cause expression of IFNγ and PD-1. 2) Stimulation with high affinity peptides caused a bell-shaped curve expression of IFNγ. 3) Affinity effected the maximum proportion of cells that activated and produce effector molecules; likely due to changes to TCR signalling dynamics.

Once this had been investigated, the *in vitro* function role of PD-1 was examined, with little success. Finally, an *in vivo* model was used to explore how T cell activation dynamics, immune checkpoints and IFNγ expression were affected by the tumour microenvironment. The response of cells that had been within the TME indicated different activation dynamics and molecule expression to other tissues. Both $CD4^+$ and $CD8^+$ host cells showed a significantly greater activation and effector function in the tumour and were shown to respond to different types of TCR signals. Whereas,

response in the dLN, cLN and spleen were shown to be small and it was concluded that although cells recognised the tumour as foreign, majority of cells had arrested signalling. This was also seen for $CD8^+$ OTI cells in both the cLN and spleen. Activation in dLN and tumour were shown to be similar, but effector function was not. It was further concluded at 24-hours $CD8^+$ OTI cells had only just recently encountered tumour antigen and by 48-hours, cells in the tumour (not dLN) significantly expressed effector function. This study resulted in a broad set of findings that provide an overview of physiological activity in the TME and warrants further exploration.

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