[redacted]

UNIVERSITY OF BIRMINGHAM

UNDERSTANDING THE REGULATORS OF ADAPTIVE TOLERANCE IN CHRONICALLY STIMULATED CD4$^+$ T-CELLS.

BY

REYGN DONE

A thesis submitted to the University of Birmingham for the degree of

MSc BY RESEARCH

Institute of Immunity and Immunotherapy

College of Medical and Health Sciences

The University of Birmingham

September 2024

Abstract

Chronic T-cell receptor (TCR) stimulation is a central driver of $CD4^+$ T-cell hyporesponsiveness. During prolonged stimulation, $CD4^+$ T-cells either enter a state of tolerance, mimicking anergy, or differentiate into type-1 regulatory T-cells (Tr1), a distinct $FoxP3^-$ regulatory T-cell (Treg) subset crucial for maintaining immune tolerance via IL10 production. The specific regulators of adaptive tolerance, and how they differ from $CD8^+$ T-cell exhaustion, remain unclear. Moreover, the signalling dynamics within Tr1-like cells and their environmental modulation are not well understood yet are of significant therapeutic interest. To study chronic-stimulation induced tolerance, Nur77-Tempo mice received escalation dose immunotherapy, allowing us to analyse NFAT-independent TCR signalling. Our study reveals that chronic stimulation-induced tolerance in $CD4^+$ T-cells is associated with increased immune checkpoint (IC) expression and increased Tr1-like selection. These tolerised T-cells display intrinsic signalling defects in multiple T-cell signalling pathways which can be reversed by combined IC blockade (anti-PD-L1 and anti-Lag3) *in-vivo*. Importantly, Tr1-like cells remain responsive to chronic antigen exposure and exhibit heightened sensitivity to IC blockade. While the total and responsive Tr1-like cell populations increase following combination therapy, IL10 expression levels remain stable, indicating that ICs may regulate IL10 production within Tr1-like cells. We conclude that shared biological mechanisms underpin different forms of T-cell dysfunction, including tolerance and exhaustion. ICs are pivotal in enforcing tolerance, yet their function evolves following Tr1 differentiation, supporting the maintenance of a tolerogenic state. These findings suggest a need for further research to understand the impact of IC blockade on Tr1-like cells in various disease contexts, including cancer.

Acknowledgement

Firstly, I sincerely thank my supervisor, Dr David Bending, for his guidance and unwavering support throughout this research journey. His insightful advice and support throughout this project have been instrumental in moulding my work. I am grateful for the opportunity to continue learning and pushing my knowledge boundaries under his mentorship.

I also want to extend a heartfelt thank you to Dr Lozan Sheriff for her invaluable contribution to this project. Her expertise and willingness to provide help when needed and sharing knowledge has been crucial in overcoming challenges faced throughout the project. I have benefited from the extensive discussions we have had, and for this, her support has been indispensable.

I would also like to thank my remaining colleagues in the Bending Lab. This collaborative and supportive environment has inspired and motivated me to complete this project. The collective contributions have also been instrumental in its completion, so for this, I thank everyone.

Contents list

List of Figures and Tables

List of abbreviations

% - Percentage
°C - Degrees Celsius
AhR - Aryl hydrocarbon receptor
AIRE - Autoimmune regulator
ANOVA - Analysis of Variance
APC - Antigen presenting cell
BATF - basic leucine zipper ATF-like transcription factor
Blimp-1 - B lymphocyte-induced maturation protein 1
BMDC - Bone Marrow-derived Dendritic cell
BME - Betamercaptoethanol
Cbl-b - Casitas B-lineage lymphoma proto-oncogene-b
CD - Cluster of Differentiation
CO_2 - Carbon Dioxide
CTLA-4 - Cytotoxic T lymphocyte antigen 4
DC - Dendritic cell
DGKa - Diacylglycerol kinase a
EDI - Escalation Dose Immunotherapy
Egr - Early growth response
FBS - Foetal Bovine Serum
FoxP3 - Forkhead Box P3
FT - Florescent Timer
GITR - glucocorticoid-induced TNFR-related protein
GM-CSF - Granulocyte Monocyte colony stimulating factor
Grail - Gene Related to Anergy In Lymphocytes
HSCT - Haematopoietic Stem Cell Transplant
ICOS - Inducible T-cell Co-stimulator
IFN - Interferon
IL - Interleukin
IRF - Interferon regulatory factor
Lag-3 - Lymphocyte Activation Gene 3
LAT - Linker for Activation of T-cells
LCMV - Lymphocytic choriomeningitis virus
LPS - Lipopolysaccharide
MAPK - Mitogen-activation Protein Kinase
MBP - Myelin Basic Protein
MHC - Major Histocompatibility Complex
mL - Millilitre
mM - millimolar
mTEC - Medullary Thymic Epithelial Cell
NFAT - Nuclear Factor of Activated T-cells
nm - nanometre
Nr4a - Nuclear Orphan receptor 4a
OX40 - Tumour necrosis factor receptor superfamily, member 4
PBS - Phosphate Buffered Saline
PD-1 - Programmed Death receptor 1
PD-L1 - Programmed Death Ligand 1
PKC - Protein Kinase C
PLCy1 - Phospholipase C gamma 1

pMHC - Peptide-loaded Major Histocompatibility complex
RBC - Red Blood Cell
RNA - Ribonucleic Acid
RPM - Rotations Per Minute
RPMI - Roswell Park Memorial Institute
SEM - Standard Error of Mean
SHP - Small Heterodimer Partner
TCR - T-cell receptor
Tempo - Timer rapidly expressed in lymphocytes
Tex - Exhausted T-cell
TGF - Transforming Growth Factor
Th - T-helper
Tigit - T cell immunoreceptor with Ig and ITIM domains
TIM-3 - T-cell immunoglobulin and mucin-domain containing-3
Tocky - Timer of Cell Kinetics and Activty
TolDC - Tolerogenic Dendritic cell
TOX - thymocyte selection-associated high mobility group box
Tpex - Pre-exhausted T-cell
Tr1 - T-regulatory Type-1
TRA - Tissue-restricted antigen
Treg - Regulatory T-cell
µg - Microgram
µL - Microlitre
µm - Micrometre
µM - Micromolar

1. Introduction -

1.1. T-cell activation:

T-cell activation requires two signals; the first is initiated by the ligation of the T-cell receptor (TCR) to peptide-loaded major histocompatibility complexes (pMHC), which is expressed by antigen-presenting cells (APC), such as dendritic cells (DC). The second is mediated by co-stimulatory signals resulting from the ligation of T-cell-derived CD28 to APC-derived CD80/86. After receiving both signals, the T-cell will undergo rapid expansion and differentiation, where it will gain a specific effector function (Fig.1). To fine-tune this process further, T-cells integrate signals mediated by immune checkpoints (IC) and co-stimulatory receptor ligation (1). The dynamic expression of these receptors is exclusive to antigen-experienced T-cells and changes in response to the environment. For example, engagement of novel costimulatory receptors such as inducible T-cell co-stimulator (ICOS) (2,3) and tumour necrosis superfamily member 4 (OX40) (4), ensure optimal co-stimulation upon antigen rechallenge, and are implemented in the generation of immunological memory (5). Conversely, novel ICs, such as programmed death receptor-1 (PD-1) (6), act to provide negative feedback signals to increase the threshold for T-cell activation (7) by targeting TCR (6) and CD28 (7) signalling cascades. Since their discovery, ICs have been therapeutically exploited in clinics, using immunotherapies to ‘release the breaks’ off T-cells, which drive more robust immune responses have revolutionised cancer treatments (8).

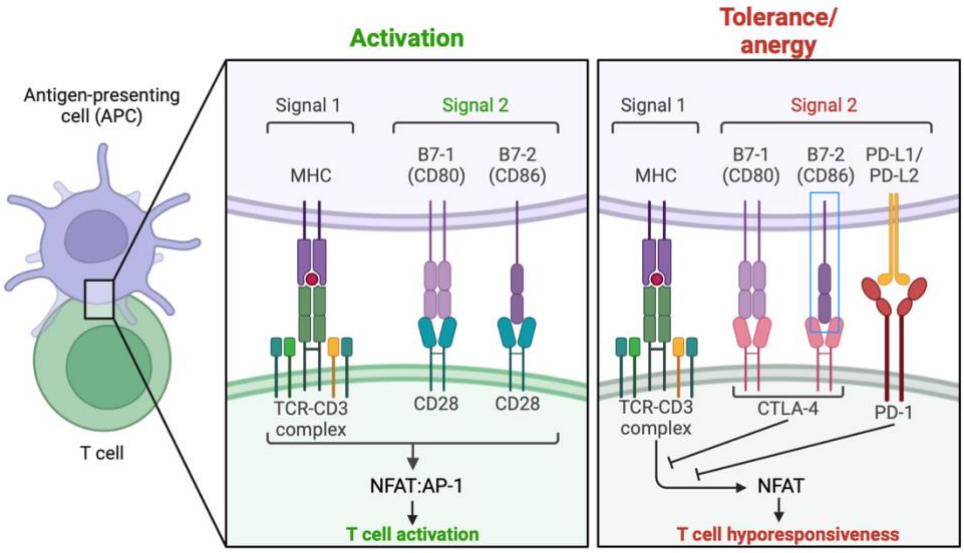

Figure 1 – Signals Required for T-cell Activation vs T-cell Tolerance.

T-cell activation requires 2 signals. Interactions between peptide-loaded MHCII molecules presented by APC's and the TCR initiate signal 1. Interactions between B7-ligands (CD80 and CD86) presented by APC's and the CD28 co-stimulatory receptor initiate signal 2. T-cell anergy/tolerance is induced when signal 1 is provided in the absence of signal 2 or in the presence of excessive co-inhibition (PD-1/PD-L1 interactions). Created using BioRender.com.

1.2. Central tolerance:

The thymus is a primary lymphoid organ (PLO) crucial for developing polyclonal naïve T-cells. Unfortunately, this can be a double-edged sword; in a healthy individual, T-cells can delineate self from non-self and are said to be self-tolerant. In some, self-tolerance is disrupted, forming the basis for autoimmune disorders. To maintain immune homeostasis, T-cells are persistently screened at two sites: 1) the thymus (central tolerance) and 2) in secondary lymphoid organs and the periphery (peripheral tolerance).

T-cell progenitors colonise the thymus following emigration from the bone marrow and interact with a complex, dense network of specialised epithelial cells and DCs (9). Medullary thymic epithelial cells (mTEC) are one population important for this process. mTECs harness the unique ability to ectopically express tissue-restricted

antigens (TRA) within the thymus resulting from the mTEC-specific transcription factor autoimmune regulator (AIRE). Without AIRE (10), mTECs fail to express TRAs and screen for autoreactive thymocytes, permitting the thymic escape of self-reactive T-cells. Fortunately, many other immune subsets exhibit overlapping, non-redundant roles with mTECs, one such population being $CD11c^{+}$DCs (11). Although they lack the intrinsic ability to express TRAs, they co-localise with mTECs, where they obtain TRAs via cooperative antigen transfer (12). Together, medullary APCs test the reactivity of the newly formed TCR to decide the fate of the developing thymocyte based on affinity and avidity. High affinity/avidity recognition of TRAs will drive negative selection, causing developing thymocytes to apoptose. This process, however, is rather complex; described by the affinity model of regulatory T-cell (Treg) selection, the higher the TCR affinity for self-antigen, the higher the likelihood of Treg selection (13–15). However, the point at which Treg selection becomes negative selection remains unclear. Interestingly, this process seems to rely on CD28-mediated co-stimulation (16,17) to increase the efficiency of thymic Treg selection. Conversely, thymocytes interacting with pMHC with a low-moderate affinity can emigrate from the thymus as conventional, naïve $CD4^{+}$ or $CD8^{+}$ T-cells.

1.3. Peripheral tolerance:

Unfortunately, central tolerance is not ‘fool-proof’, deleting only ~60-70% of self-reactive thymocytes (18). Luckily, self-reactive naïve T-cells are again screened within the periphery outside PLOs. This is particularly important as some naïve T-cells exhibit the propensity to recognise innocuous antigens derived from food, gut microbiota, and pregnancy (19,20). If T-cells recognise innocuous antigens, they will be tolerised, the mechanisms of which we discuss more within the following section.

1.3.1 Extrinsic Control of Peripheral Tolerance:

1.3.1.1 Tolerogenic dendritic cells (TolDC):

As previously mentioned, T-cells require simultaneous engagement of their TCR and co-stimulatory receptors to achieve complete activation, mediated by professional APCs. By virtue of constitutive MHCII expression, professional APCs like macrophages, B-cells, and DCs can promote the adaptive arm of the immune response. DCs can act differently depending on their maturation state; for example, immature DCs exhibit an enhanced ability to sample their environment (21) whilst failing to activate T-cells because of reduced co-stimulatory receptor ligand expression (22). Conversely, DCs express CCR7, a chemokine receptor following activation, enabling their migration to the lymph nodes (23). They also lose their ability to sample the environment as efficiently, instead upregulating their expression of MHCII and CD80/CD86, becoming potent T-cell inducers.

Furthermore, some DCs also uphold tolerance, by preventing the activation of potentially self-reactive T-cells. This DC subset – termed tolerogenic DCs (TolDC) – typically exhibits an immature morphology; in fact, TolDCs were initially thought to be DCs that had not matured, although research suggests this is untrue (24). TolDCs can activate to some extent (25), and arise from various stimuli such as IL10 (26,27) and TGFb (28), and have become a promising target for cellular immunotherapy by inducing tolerance in autoimmune settings (29). Like immature DCs, TolDC's lacklustre expression of CD80/86 prevents T-cells' efficient activation and induces tolerance. Beyond CD80/86, TolDCs also express PD-L1 (the ligand for PD-1), which helps induce tolerance (30). Thus, the expression of PD-L1 alone has gained

recognition as a target to promote either T-cell activation (31) or transplant tolerance (32,33). Many studies have also referenced contact-independent mechanisms driven by DC-mediated IL10 expression and secretion (30). Interestingly, a specific subset of IL10-secreting DCs exists – termed DC-10s – and has been implemented to restrict allergic (34) and transplant inflammation (35). Thus, therapeutic targeting of this subset may prove clinically beneficial to restore T-cell responses or promote immune tolerance.

1.3.1.2 FoxP3$^+$ Tregs:

FoxP3$^+$ Tregs can be categorised broadly based on the location of their induction. As previously mentioned, FoxP3$^+$ Tregs can be selected during T-cell development within the thymus and thus are termed thymic Tregs (tTregs). Alternatively, naïve CD4$^+$ T-cells can adaptively differentiate into Tregs by inducing FoxP3 expression to gain suppressive functions – these are termed peripheral Tregs (pTreg). Although their origins differ, the suppressive mechanisms between these populations are conserved and well-defined: 1) Tregs express and secrete immunosuppressive cytokines such as IL10 (36) and TGFβ (37), some of which can be bound to the cell surface (38). Tregs can also express the latent-TGFβ activating integrin αvβ8 (39) and have been implemented to promote the immunoevasion of tumours (40). 2) Tregs constitutively express IC such as CTLA-4 (41,42) to prevent autoimmunity (43). CTLA-4 is a B7-receptor homolog and can bind to APC-derived CD80 and CD86 with a higher affinity than CD28. The ligation of Treg-derived CTLA-4 to APC-derived CD80/CD86 both sequesters and promotes the downregulation of CD80/86 whilst upregulating co-inhibitory receptor ligands, such as PD-L1 (44). Furthermore, the expression of Lag-3, a CD4 homolog, can also limit the maturation of DCs via

MHCII crosslinking (45). Tregs can, however, express many checkpoint receptors and ligands, including PD-1, PD-L1/PD-L2, TIM-3, Tigit and OX40 (46), providing them with excellent immunosuppressive capabilities. 3) Tregs also suppress immune responses via metabolic disruption. Tregs express CD25 in abundance, which acts as a high-affinity IL2 receptor. Like conventional T-cells, Tregs rely on IL-2 signalling for their activation and maintenance (47), but lack the intrinsic ability to express it. Therefore, by virtue of Treg-derived CD25 expression, Tregs can essentially deprive conventional T-cells by sequestering free IL-2 present within the environment (48). Similarly, following activation, Tregs upregulate CD39 and CD73 (49) which work synchronously to convert ATP or ADP into immunosuppressive adenosine. This adenosine can act in an autocrine manner, binding to Treg-derived adenosine receptors and is essential in driving the proliferation and immunosuppressive capabilities of Tregs (50). Alternatively, adenosine acts in a paracrine manner by ligating adenosine receptors on effector T-cells (53), dampening their immune responses.

1.3.1.3 Type 1 regulatory (Tr1) T-cells:

Typically, Tregs are primarily defined by their expression of FoxP3; however, Tregs are much more heterogeneous as FoxP3^{-} Treg populations have been identified in humans (51) and mice (52). This population is termed Type 1 regulatory T (Tr1) cells and are pivotal for upholding immune homeostasis and fostering tolerance across a plethora of chronic inflammatory conditions, including allergy (53,54), allogenic HSCT (55), autoimmunity (56) and even cancer (57). Like conventional FoxP3^{+} Tregs, Tr1s are also antigen-specific (52), and in response to cognate peptide, express and secrete immunosuppressive cytokines such as IL10 and TGFβ – which

is the most prominent non-contact dependent mechanism of Tr1-mediated immunosuppression.

It was proposed that Tr1s emerge from $CD4^+$ T-cells undergoing chronic stimulation, IL10 (52) and TGFβ (58) signalling within a tolerogenic environment. However, recent evidence suggests that IL27 may also act as a principal cytokine driving this process (59,60), as genetic ablation of endogenous *Il10* does not affect Tr1 conversion within the intestine (61), illustrating how other signalling events may converge and compensate for the loss of *Il10.* As previously discussed, DCs can act as a significant source of IL10 and have also been implemented in converting CD4+ T-cells to Tr1s in humans (62,63), with a similar mechanism described in mice (64). Furthermore, like IL10, IL21 is also expressed and secreted by Tr1 cells and is thought to act in an autocrine manner to enhance the stability of the Tr1 phenotype (65).

Unfortunately, the lack of a standardised definition of Tr1 cells creates confusion, and many studies have attempted to define this population phenotypically, with the most promising phenotypic definition to date being the co-expression of CD49b and Lag3 (66,67). However, it is essential to note that the expression of either of these markers is not restricted to the Tr1 lineage. Tr1 cells also co-express many ICs, such as PD-1, CTLA-4, Tigit and TIM-3, with a relatively high magnitude (68–70). However, none of these are Tr1-specific biomarkers; therefore, what remains to be determined is whether the expression of these markers identifies one single Tr1 subset or whether these markers comprise a collection of different subsets bearing similar functional resemblance to ‘actual’ Tr1 cells. Although a direct phenotypic description of Tr1 cells

has not been met, several transcription factors that 'define' Tr1s have been identified. Blimp-1 (71,72), c-Maf (65) and EGR2 (71,73) are all able to transactivate the *Il10* locus and are upregulated in Tr1-like cells. Recently, IRF1 (74), IRF4 (75), BATF (74), Eomes (76) and AhR (72) have all been implemented in Tr1 induction. Still, again, none of these transcription factors are Tr1-specific; instead, they define how the *Il10* gene is transcriptionally regulated in Tr1s rather than defining Tr1 identity.

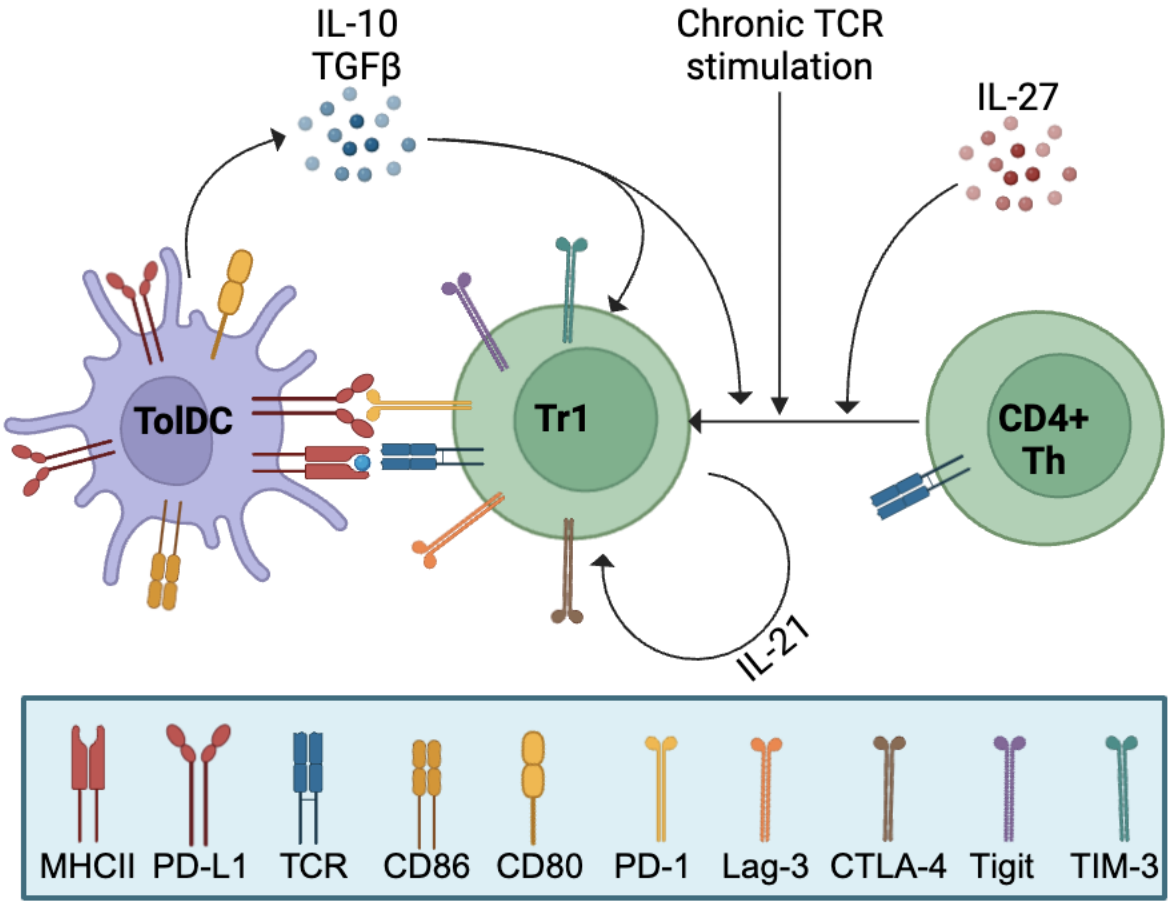

Figure 2 - Induction of Tr1-like CD4+ T-cells.

Tr1-like T-cells differentiate from T helper subsets under a plethora of conditions. Chronic stimulation, IL-27, IL10 and TGFb have all been implemented in their induction. Tolerogenic dendritic cells often provide these signals to Th subsets, and once differentiated, help to maintain the Tr1 phenotype by providing TCR signals under tolerogenic conditions. the Tr1-derived cytokine IL-21 is thought to act in an autocrine manner to reinforce the Tr1 phenotype.

1.3.2 Intrinsic control of peripheral tolerance (Anergy):

T-cell anergy is the most prevalent, non-deletional mechanism of peripheral tolerance, often acting as a crucial checkpoint to avert immunopathology by silencing potentially harmful naïve T-cells. Anergic T-cells are rendered functionally inept via intrinsic inactivation (77) in response to self-antigen. These anergic T-cells seem to exist within the periphery long after they are inactivated, but the reasons for why are unknown. Broadly, anergy can be further separated into clonal anergy (*in-vitro* anergy) and adaptive tolerance (*in-vivo* anergy). The former is thought to arise at the priming stage of naïve T-cells in response to TCR stimulation without co-stimulation. The latter is thought to arise after the priming stage in response to chronic antigenic stimulation. By virtue of this, many experimental systems have been established to investigate the emergence of T-cell anergy, including a-CD3 mediated TCR stimulation in the absence of a-CD28 co-stimulation (78) and repeat administration of immunogenic peptide (79,80). Both emphasise the same findings, including decreased proliferative capacities, effector function and inflammatory cytokine production, with increased anti-inflammatory cytokine and IC expression.

1.3.2.1 Clonal anergy:

Anergy is induced as a direct consequence of unbalanced T-cell stimulation (81) - when signal one is provided in the absence of signal two or the presence of excessive co-inhibition (19,82,83). It is well established that signal transduction mediated via the nuclear factor of activated T-cells (NFAT) and AP-1 transcription factors is crucial for complete T-cell activation. However, the signalling events which bias the induction of anergy are associated with disproportionate calcium signalling, so much so that treatment with ionomycin is sufficient to induce T-cell anergy (84),

and the addition of calcineurin inhibitor - cyclosporin-A is sufficient to prevent anergy (85). Further, analysis of signalling pathways has illustrated that clonally anergic T-cells exhibit defective Ras/MAPK signalling (86), and restoration of this signalling pathway via adenoviral-transduction of constitutively active Ras rescues T-cells from anergy(87).

Given this, it is unsurprising that AP-1 transactivation is also defective in anergic T-cells (88), considering AP-1 resides downstream of Ras. During the induction of anergy, the lack of CD28-mediated co-stimulation diminishes AP-1 transactivation, nuclear translocation (89) and AP-1-mediated chromatin remodelling of effector genes, leaving them hypermethylated, essentially rendering them inaccessible (90). Given that anergic T-cells primarily receive signals via the TCR, NFAT is retained within the nucleus away from AP-1, here it will divert naïve $CD4^+$ T-cell differentiation away from activation and towards inhibition.

This is because the NFAT-biased anergic gene programme upregulates the expression of anergy associated genes such as gene related to anergy in lymphocytes (grail) (91,92) and Egr2 (93,94). Like NFAT, Egr2 and Egr3 can further upregulate the expression of E3 ubiquitin ligases such as Cbl-b (104) and Grail. Interestingly, some research suggests that Cbl-b is also induced in response to CTLA-4 (95) and/or PD-1 ligation (96), highlighting how signals mediated by IC engagement drive anergy. In anergic T-cells, Cbl-b acts as a crucial regulator of T-cell activation via the targeting of TCR and CD28 signal transduction pathways (97), and following the loss of Cbl-b, phenotypically anergic T-cells are rescued (98). Beyond Cbl-b, Egr2 and Egr3 can bind to the transcriptional start site of

diacylglycerol kinase-α (DGKα) (99) and is concurrent with studies illustrating the presence of heightened DGKα in anergic T-cells (84,87,100). Interestingly, forced overexpression of DGKα is sufficient to induce T-cell anergy alone (101), and depletion and/or pharmacological inhibition of DGKα rescues T-cells from anergy (102). This is because DGKα targets diacylglycerol, a vital signalling molecule that activates the Ras/MAPK signalling pathway. By virtue of this, DGKα essentially uncouples TCR signals and AP-1 transcriptional activity via the inhibition of DAG-induced activation of the Ras/MAPK pathway. This data implies that clonally anergic T-cells exhibit their unique transcriptional profile, which negative regulators of T-cell signalling cascades can broadly define and are essential to maintain.

1.3.2.2 Adaptive tolerance:

Adaptive tolerance, or *in-vivo* anergy, refers to a process whereby T-cells undergoing chronic antigenic stimulation adapt by desensitising their responses to antigen. This ultimately results in a functional state mimicking clonally anergic T-cells, although these states remain biochemically distinct. Unlike clonally anergic T-cells, adaptively tolerant T-cells exhibit a primary defect in proximal TCR signalling, specifically at the level of Zap70 (103–105). In response to TCR crosslinking, this crucial protein-tyrosine kinase is responsible for initiating intracellular signalling cascades via hyperphosphorylation of LAT (106). Hyperphosphorylation of LAT allows for a conformational change in its structure, enabling it to act as a docking site linking both proximal and distal signals. To add another layer of complexity, Hundt et al. proposed that following the induction of *in-vivo* anergy, $CD4^+$ T-cells also exhibit defects in LAT (107), which has knock-on effects for other downstream signalling molecules –

specifically PLCγ1 (104). Without PLCγ1 activation, intracellular calcium remains sequestered, and PKCθ remains inactive.

Like clonally anergic T-cells, the expression of Cbl-b also seems to be implemented in maintaining this hyporesponsive state (105). It was initially postulated that Egr-2, a transcription factor downstream of NFAT, is the primary inducer of Cbl-b (93). However, this is unlikely in adaptively tolerised T-cells, considering calcium mobilisation is the primary defect exhibited. Concurrent with these results, adaptively tolerant $CD4^+$ T-cells do not show increased Egr-2 expression (105). Instead, adaptively tolerant T-cells exhibit vast increases in IC expression, and as previously mentioned, engagement of ICs can transactivate Cbl-b. This data demonstrates that although adaptively tolerant and clonally anergic T cells exhibit functional redundancy, both hyporesponsive states differ biochemically. In adaptively tolerant T-cells, the predominant defect is in the calcium/NFAT pathway, whereas clonally anergic T-cells exhibit defects in the Ras/MAPK pathway. It also illustrates how similar immunosuppressive mechanisms may maintain hyporesponsive states, but more research should be conducted on the main mechanisms of immunosuppression within each state. However, it is essential to note that both the CD28 and TCR signalling cascades converge downstream; thus, depending on the experimental mechanism in which anergy is induced, it will indicate experimental specific defects in differential signalling pathways.

1.4 T-cell exhaustion:

T-cell exhaustion is characterised by a stepwise and progressive loss in effector function and proliferative capacity associated with marked upregulation and co-

expression of multiple ICs. Exhaustion was first coined in chronic viral infections (108) and has now been extended to other disease settings such as cancer (109). However, exhaustion is essentially a $CD8^+$ T-cell-centred phenomenon, leaving our current understanding of $CD4^+$ T-cell exhaustive programmes lacklustre. This is surprising given the polyfunctional and plastic nature of $CD4^+$ T-cells, especially in boosting anti-tumour immune responses. Until recently, the predominant function of $CD4^+$ T-cells within the tumour milieu was to provide 'help' to cytotoxic T-lymphocytes, namely by supporting DC antigen cross-presentation (110) and B-cell humoral responses (111) via CD40 ligation.

Similarly, $CD4^+$ T-cells can also act as a reservoir for IL-21 (112) and IL-2 (113), which drives the differentiation and maintenance of cytotoxic anti-tumour $CD8^+$ T-cells, respectively. There is mounting evidence that tumour neoepitopes are restricted to MHCII, which is recognised primarily by $CD4^+$ T-cells in murine models (114) and humans (114,115). Concurrent with these results, studies have identified that tumour-specific $CD4^+$ T-cells may also acquire cytotoxic capabilities upon entry into the tumour (116). Given this information, it is plausible that $CD4^+$ T-cells can be susceptible to exhaustion; however, what CD4+ T-cell exhaustion looks like remains a mystery and, thus, requires relevant investigations on how this impacts health and disease.

The persistent upregulation and co-expression of multiple ICs is one phenotypic hallmark of exhaustion – the primary function of which is to dampen immune responses following T-cell activation. Broadly, these ICs can be categorised into 'classical' (CTLA-4 and PD-1) and 'alternative' (Lag-3, TIGIT and TIM-3) checkpoints,

some of which exhibit overlapping mechanisms to subsequently target the TCR (6) and CD28-co-stimulatory (7) pathways. PD-1 and CTLA-4 can recruit Src homology-2 domain-containing protein tyrosine phosphatase-2 (SHP-2) (117,118) to the TCR to terminate TCR signal transduction to intracellular cascades. Furthermore, CTLA-4 is a CD28 homolog able to sequester CD80/86 (119), albeit with a higher affinity than CD28. Conversely, Lag-3 exhibits vast structural homology with the CD4 co-receptor (120) preventing the primary signal for T-cell activation. Given this, it is unsurprising that the exhaustive state is often maintained via exaggerated and abnormal overactivation of these IC-mediated negative feedback signals. Much like $CD8^+$ T-cells, $CD4^+$ T-cells also express many of these ICs, as depicted in Table 1, in which we can infer that $CD4^+$ T-cells also exhibit traits of phenotypic exhaustion. Further to this point, the more ICs expressed are also associated with later-stage exhaustion, which can indicate a poorer prognosis (121,122). However, it is essential to note that ICs are expressed exclusively by all antigen-experienced T-cells. Therefore, the expression of one IC may be insufficient to both promote and define the exhaustive phenotype (123,124). For example, one study emphasises that the expression of PD-1 in response to chronic Mycobacterium tuberculosis infection (125) represents a population of highly proliferative $CD4^+$ T-cells, which give rise to tuberculosis-specific T-cells. Nevertheless, ICs represent a vital target, especially in the context of cancers. Targeting multiple $CD4^+$ T-cell-derived IC with combination treatment (a-PD-L1 and a-Lag3) can restore the functionality of exhausted $CD4^+$ T-cells (126) by decreasing the TCR signalling threshold (127).

Table 1 - Table showing the expression of immune checkpoints used to define exhausted CD4+ T-cells across different cancers.

Marker	**Primary cancer type**	**Reference**
PD-1	• Subcutaneous lung cancer • Melanoma • Multiple myeloma	(126,128–130)
CTLA-4	• melanoma	(129)
Lag-3	• Melanoma	(126,131)
Tigit	• Melanoma • Head and neck squamous cell carcinoma	(126,132)
TIM-3	• Colorectal cancer • Multiple myeloma • Melanoma • Hepatitis-B associated hepatocellular carcinoma.	(126,130,133,134)

Beyond the expression of ICs, genomic approaches have paved the way for a more comprehensive understanding of T-cell exhaustion and have identified key transcription factors such as Nr4a1 (135,136), NFAT (137), BLIMP-1 and TOX (138,139) as critical drivers implementing the exhaustive programme. As previously mentioned, NFAT activates and is translocated into the nucleus in response to TCR signals, which, alongside AP-1, promote the expression of immune response genes. However, like anergic T-cells, exhausted T-cells exhibit an NFAT transcriptional bias. In the absence of AP-1 transcriptional partners (Fos and Jun), NFAT homodimers increase the expression of ICs (140,141) and other transcription factors implemented in exhaustion, such as Nr4a's, BATF and IRF4 (142).

To add another layer of complexity, many 'exhaustion-related transcription factors' exhibit paradoxical roles largely dependent on T-cells' signalling environment. For example, in one study, the transcriptional circuit formed between NFAT, BATF and IRF4 imposes T-cell exhaustion within chronic contexts (142). In contrast,

overexpression of BATF in CAR T-cells led to functional cooperation with IRF4 and increased resistance to exhaustion (143). Amongst some early immune response genes, Nr4as are expressed in direct proportion to TCR signal strength. Nr4as exhibit many biological functions within T-cells, but within the context of chronic stimulation, the Nr4a family of transcription factors are thought to promote exhaustion directly. This is because Nr4as directly bind to and upregulate immune regulation genes or by sequestering AP-1 proteins and diverting T-cells into an NFAT-dependent exhaustive programme (135). Thus, this highlights the complexity surrounding genomic definitions of exhaustion and how one transcription factor can be implemented in different biological functions, largely dependent on their environment.

Owing to LCMV studies, researchers have realised that the unique lineage fate of T-cell exhaustion exhibits a vast amount of heterogeneity. Because the progressive loss of T-cell function is graded, it has allowed for further characterisation of exhaustive subsets. Those which are terminally exhausted (T_{ex}) and the precursors to these cells (T_{pex}), which are based on the expression of the stem marker TCF1 (encoded by *Tcf7*) (144) and/or CXCR5 (145). T_{pex} cells exhibit the unique ability to self-renew (146), therefore acting as an essential reservoir (147) for tumour-specific T-cells as they traffic between lymphoid tissues and the tumour (147,148). These T_{pex} cells undergo a TCR-dependent transformation into T_{ex} cells (149), which occurs in melanoma-infiltrating $CD4^+$ T-cells upon neoantigen recognition (150). Here, Fu et al. show that melanoma-specific $CD4^+$ T-cells present within lymphoid organs express TFC-1, which is replaced by canonical exhaustive markers PD-1, Lag-3 and TIM-3 following infiltration into the tumour. Like $CD8^+$ T-cells, this process seems to

be augmented by a-PD1/PD-L1 blockade, increasing both the expression of TCF-1 (151) and the proportion of tumour-specific TCF-1^{+}CD4^{+} T-cells within the spleen (150), whilst decreasing the expression of ICs. However, a-PD1/PD-L1 therapy did not seem to augment the proliferative potential of CD4^{+} T_{pex} cells (150) in the same way it augments CD8^{+} T_{pex} cell proliferation (144,152), illustrating a potential discrepancy between T-cell subsets and how they differentially respond to environmental cues. Concurrent with these results, terminally exhausted PD-1^{hi}CD39^{+}CD4^{+} Tumour-infiltrating lymphocytes (TILs) also exhibit an increased expression of TOX (151), which has been identified as an indispensable player driving this exhaustive trajectory (Fig.3). The expression of TOX is associated with more advanced exhaustive states (153), this is because following the onset of terminal exhaustion, Tex have undergone a vast change in their epigenetic landscape (154), and in CD8^{+} T-cells, TOX is identified as the principal transcription factor initiating this change (139). Finally, the transcription factor Blimp-1 (encoded by *Prdm1*) has also been recognised as a critical driver of CD4^{+} T-cell exhaustion (155,156). Conditional deletion of *Prdm1* in CD4^{+} T-cells decreases the expression of ICs (PD-1, Lag-3 and TIM-3) (156) and restores their ability to provide help and prevent exhaustion in CD8+ T-cells in chronic toxoplasmosis (155,156).

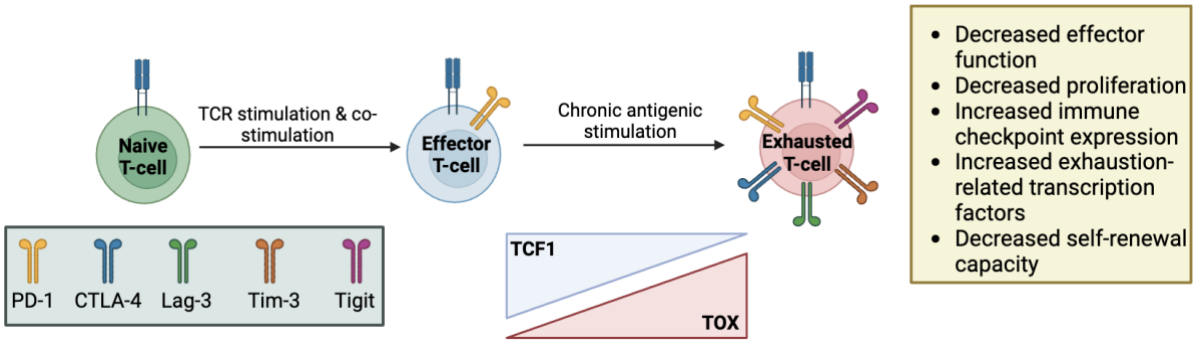

Figure 3 - Induction of Exhaustion in Chronically Stimulated T-cells.

Following acute T-cell activation, T-cells will differentiate into effector subsets. If antigenic challenge isn't removed (cancer or chronic infection), the effector T-cell will continue to be stimulated. Chronic stimulation is one cardinal feature driving T-cell exhaustion and is associated with marked and persistent upregulation of multiple immune checkpoints, increased expression of exhaustion-associated genes and loss of self-renewal capabilities. Following the onset of Terminal exhaustion, T-cells no longer respond to antigen.

Fundamentally, $CD4^+$ and $CD8^+$ T-cells are not equal, often exhibiting non-redundant roles with various functionalities, which is well established. However, extensive research on $CD8^+$ T-cell exhaustion has been instrumental in identifying critical exhaustive mechanisms and patterns, which are somewhat conserved across both subsets. Consequently, leveraging our understanding of $CD8^+$ T-cell exhaustion has allowed for developing therapeutic strategies including, but not limited to, adoptive cell transfer and immune checkpoint blockade (ICB), which are currently being explored as promising avenues for enhancing anti-tumour immune responses.

1.5 Introduction summary:

Many biologically conserved mechanisms utilising immunosuppressive factors are often shared across different cellular states, making it difficult to distinguish between hyporesponsive T-cell states that elicit phenotypic, functional and transcriptional overlap. Chronic antigenic stimulation is one cardinal feature that drives the differentiation of $CD4^+$ T-cells into unique lineage fates such as Tr1-like cells and Tex cells. However, because exhaustion has not yet been defined in $CD4^+$ T-cells, we

often coin hyporesponsive $CD4^+$ T-cell states as simply anergic when this is untrue. Adaptively tolerised, exhausted, and Tr1-like $CD4^+$ T-cells, which functionally resemble anergic T-cells, are not inherently anergic. Therefore, we must understand what cellular and molecular interactions enforce adaptive tolerance and how/if adaptive tolerance and exhaustion are distinct biochemical states.

2. Aims and hypothesis -

Based on existing literature, we hypothesise that ICs are key regulators driving adaptive tolerance in $CD4^+$ T-cells. This hypothesis is supported by the observation that IC expression is consistently associated with different states of T-cell dysfunction. Additionally, we hypothesise that ICs play a crucial role in controlling the function of Tr1-like cells.

We will use the novel Nur77-Tempo reporter mice to test these hypotheses and monitor dynamic changes in TCR signalling. $CD4^+$ T-cells from these mice will be tolerised using the escalation dose immunotherapy protocol established by the Wraith lab (80). Flow cytometry will be employed to assess the expression of specific cell surface markers that we believe are critical in regulating tolerance. We will also use ICB to investigate whether T-cell tolerance can be reversed or restored in this model. Co-culture experiments will be conducted to determine whether tolerance is associated with intrinsic or extrinsic dysfunction. Finally, we will utilise the novel Nr4a3-Tocky line to explore whether NFAT-biased signalling is present in adaptively tolerant T-cells, as reported in the literature for both tolerance and exhaustion. These approaches will allow us to test the following aims:

1) To identify the regulator of adaptive tolerance in $CD4^+$ T-cells undergoing chronic stimulation.
2) To determine the factors controlling Tr1 function when induced in response to chronic versus acute stimulation.
3) To differentiate between adaptive tolerance and exhaustion.

3. Methods -

3.1. Experimental design:

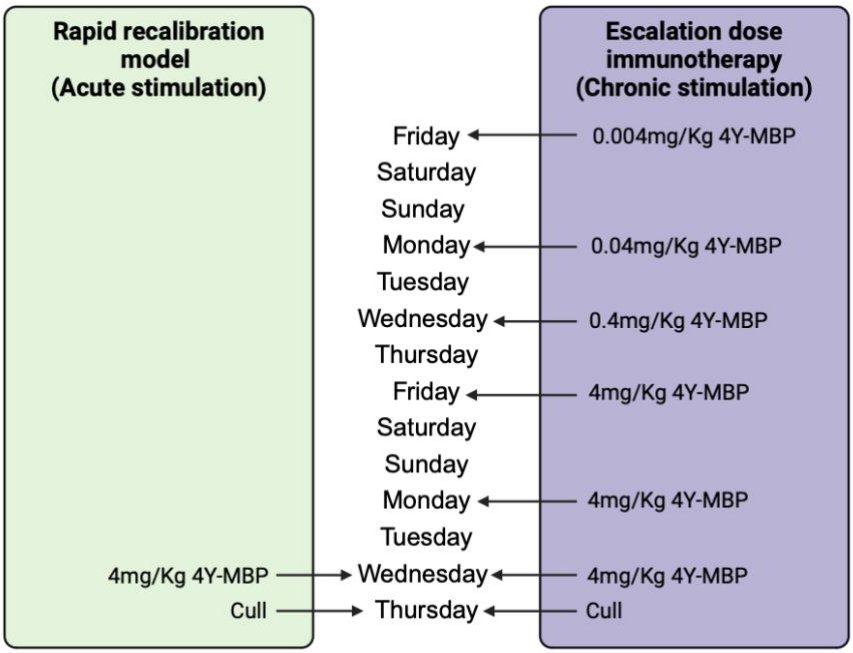

Figure 4 - Protocol for Escalation Dose Immunotherapy and Rapid T-cell Recalibration.

For chronic stimulation and the induction of T-cell tolerance the Escalation dose immunotherapy protocol was followed. Here Nur77-Tempo or Nr4a3-Tocky mice received 6 S.c. immunizations over 2 weeks increasing from 0.004mg/Kg to 4mg/Kg of 4Y-MBP. for acute stimulation, the rapid recalibration protocol was followed here Nur77-Tempo mice received one single high dose (4mg/Kg) dose of 4Y-MBP. This was done on the same day as the final EDI dose. Mice were then culled 24 hours later after the final dose.

3.2. Adaptive tolerance models and immunotherapy:

All animal procedures were carried out at the Biomedical Services Unit at the University of Birmingham and authorised under Dr David Bending's home office Project License PP9984349. All animals were euthanised via schedule one killing – cervical spine dislocation by personal licence holders. For this project, both Tg4 Nur77-Tempo *Il10*-GFP and Tg4 Nr4a3-Tocky *Il10*-GFP mice were utilised. Mice received either one or six immunisations with Myelin Basic Protein (MBP) peptide with an acetylated tyrosine modification at position 4 (4Y-MBP), as outlined in Fig.4. This is because the usual lysine residue at position 4 (4K-MBP) only induces weak

T-cell activation (127). Tg4 Nr4a3-Tocky *Il10*-GFP mice received six doses of 4Y-MBP, the same as chronically stimulated Nur77-Tempo mice (Fig.4).

The Nur77-Tempo model allows us to analyse the dynamic changes in TCR signalling. Nur77 (encoded by Nr4a1) is expressed in response to TCR signals. Here, Nur77 is conjugated to a fluorescent timer protein (157), which, over 4 hours, shifts its emission spectrum from blue to red. This allows us to analyse newly signalling ($Blue^{+}Red^{-}$), persistently signalling ($Blue^{+}Red^{+}$), and arrested ($Blue^{-}Red^{+}$) T-cells, as depicted in Figure 5.

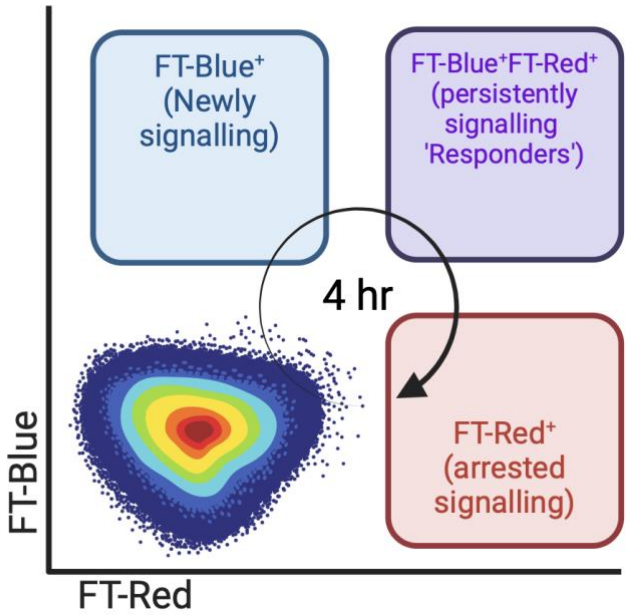

Figure 5 - schematic illustrating how the fluorescent timer works in Nur77-Tempo and Nr4a3-Tocky mice.

Following TCR stimulation, Nur77 is expressed and coupled to the fluorescent timer (FT). initially, the FT is blue and appears in the top left quadrant. Over time, TCR signals are arrested, and the FT protein matures shifting its emission spectrum from blue to red, which will appear in the bottom right quadrant. T-cells which have arrested signals and are restimulated or undergoing persistent signals will appear within the top right quadrant. T-cells which undergo no TCR signals will remain in the bottom left quadrant.

For *in-vivo* blockade experiments, combination antibodies (a-PD-L1 Clone MIH5 and a-Lag-3 Clone C9B7W) or isotype antibodies (Rat IgG2a clone MAC219 and Rat

IgG1 clone MAC221) at concentrations of 0.5mg/Kg were administered via I.p. route 24 hours after the 5th 4Y-MBP immunisation and 24 hours before the 6th immunisation.

3.3. Preparation of tissue:

Whole spleens were harvested from mice and minced before enzymatic digestion in a digest buffer (1mL RPMI + 1mg/mL DNAse I + 0.1mg/mL collagenase-D). 1.2mL of this digest buffer is added to the minced spleen samples in a 1.5mL Eppendorf and spun at 1400 rotations per minute (RPM) for 25 minutes in a thermocytometer at 37°C. The splenocyte suspension is filtered through a 70µm strainer into a sterile 50mL falcon and rinsed with 10% Foetal Bovine Serum (FBS) with 1% Pen-Strep (PS) RPMI to 20mL. The 50mL falcon is centrifuged at 1500 RPM at 4°C for 5 minutes until a pellet has formed. The supernatant is discarded, and the pellet is resuspended in 1mL Red Blood Cell (RBC) lysis buffer (eBioscience) for 2 minutes on ice. Then 10mL 10% FBS 1% PS RMPI is added to the 50mL falcon to stop the reaction before centrifuging again for 5 minutes at 1500 RPM. Once a pellet has formed, the supernatant is discarded and resuspended in 20mL 10% FBS 1% PS RPMI. The number of cells are counted using a 1:1 ratio of Trypan Blue in a haemocytometer. The splenocyte suspension is subsequently made up to a concentration of $1x10^7$ cells/mL; thus, 50µL of the splenocyte suspension will contain ~500,000 cells. This is done within a class II tissue culture hood, following standard aseptic protocols to reduce contamination.

Hind legs were harvested from Tg4 mice, and the muscle was removed from the femur and tibia before washing once in 70% ethanol and twice in PBS. The epiphysis of the femur and tibia were then removed, and the bone marrow was flushed into a

sterile petri dish with a sterile 1mL insulin needle using RPMI. This cell suspension is then aspirated and filtered through a 70um strainer into a sterile 50mL falcon tube. This is then placed into a centrifuge and spun at 1400 RPM for 10 minutes until a pellet has formed. The supernatant is discarded, and the pellet is resuspended in 3mL RBC lysis buffer (eBioscience) for 5 minutes on ice before 10mL RPMI is added to stop the reaction. This suspension is centrifuged for 10 minutes at 1400 RPM until a pellet has formed. The supernatant is then discarded, and the pellet is resuspended in 10mL BMDC media (2mM L-Glutamine, 1% PS, 10% FBS, 10mM HEPES, 50uM BME RPMI), and cells are counted using a 1:1 ratio of trypan blue and a haemocytometer. This bone marrow suspension comprises a final concentration of $1.5x10^6$ cells/mL. To start DC polarization, a 50uM GM-CSF (1:10,000) final concentration is added to the bone marrow suspension. This single-cell suspension is then added to a TC-grade flask, ensuring that the correct size is used for correct CO2 penetration and pH balancing. The flask is added to the incubator for 7-9 days at 37°C. On day 2, an equal amount of BMDC media containing 50uM GM-CSF is added to the flask without disrupting the adherent cells at the base of the flask. At day 4, ~90% of the media is removed, transferred into a sterile 50mL falcon, and centrifuged for 10 minutes at 1400 RPM until a pellet has formed. The supernatant is discarded, and the pellet is resuspended in fresh media with 50uM GM-CSF before being added to the same flask. On day 7, the bone marrow cells should show classic DC morphology under the microscope.

3.4. Cell sorting:

To sort $CD4^+$ T-cells for co-culturing experiments, a single-cell splenocyte suspension is generated following the method outlined above in section 3.3. to

isolate Acute and Chronically stimulated $CD4^+$ T-cells, splenocytes were multiplexed as Acutely stimulated $CD4^+$ T-cells received BUV737-CD4 and chronically stimulated $CD4^+$ T-cells received BUV395-CD4. Both received APC-Cy7 Viability dye before washing with sort buffer and mixing. This allowed for the isolation of live $CD4^+$ T-cells using the FACS ARIA fusion cell sorter (BD Biosciences).

3.5. Preparation of 4Y-MBP peptide:

Aliquots of 4Y-MBP peptide stock at 4mg/mL were used to create titres of the following concentrations: 0.0025μM, 0.01μM and 0.04μM, 0.16μM and 0.64μM. To do this, the 4mM stock solution was diluted using a serial dilution method with 10% FBS 1% PS RPMI. Conditions were initially made at 4X the final concentration to ensure the appropriate final concentration conditions mentioned above are achieved after dilution in culture.

3.6. Preparation of immunotherapy:

Stock solutions of anti-PDL1 (5mg/mL), a-Lag3 (2.49mg/mL), IgG2a (9.15mg/mL), and IgG1 (5mg/mL) were all diluted to a final concentration of 20 ug/mL in 10% FBS 1% PS RPMI. For combination conditions, a-PDL1 and a-Lag3 were mixed, and for isotype conditions, IgG1 and IgG2a were mixed at a 1:1 ratio. Again conditions were initially made at 4X the final concentration to ensure appropriate final concentrations were achieved following dilution in culture.

3.7. Cell culture

In a 96-well U-bottom plate, 500,000 splenocytes were added by transferring 50μL of the splenocyte suspension into each well. Following this, 0.1% β-Mercaptoethanol

(BME) was added to each well by transferring 50µL and was made up by diluting 20µL BME into 5mL 10% FBS 1% PS RPMI. 50µL of the appropriate 4Y-MBP concentration was added to their corresponding cells to rechallenge T-cells. For experiments utilising immunotherapy, 50µL of either isotype or combination treatments were added to their corresponding wells; if the experiment did not require immunotherapy, then 50µL of 10% FBS 1% PS RPMI was added to each well to create a final volume of 200µL. All splenocytes were plated away from the edges to prevent edge effect; any empty wells were then filled with 200µL PBS. Finally, the plate was placed into the incubator for 24 hours at 37°C, 96% humidity and 5% CO_2.

Dendritic cells were generated as discussed in section 2.3 and seeded at 100,000 BMDC per well in a 96 U-bottom well plate. Following this, DCs were activated with 200ng/mL lipopolysaccharide (final concentration) diluted in DC media and pulsed in either the presence or absence of 4Y-MBP (0uM, 0.01uM, 0.04uM, 0.16uM and 0.64uM) for 24 hours. Following the 24-hour incubation period, 100,000 sorted live $CD4^+$ T-cells were co-cultured with dendritic cells, as discussed in section 2.4. If the experiment required immunotherapy, either combination or isotype (as mentioned in section 2.6) was added to the corresponding wells at a final concentration of 20ug/mL. The final volume of each well is 200µL, and empty wells were filled with 200µL PBS. The plate is then added to an incubator for 24 hours at 37°C, 96% humidity and 5% CO2. Again, all procedures were carried out within a class II tissue culture hood, following an aseptic technique to prevent contamination.

3.8. List of Reagents:

Reagent or resource	***Source***
Antibodies	

BUV737-CD4	BD Biosciences
BUV395-TCRvβ8.1.1.2	BD Biosciences
Pecy7-Tigit	BioLegend
PerCp-Lag3	BioLegend
APC-PD1	BioLegend
AF700-ICOS	BioLegend
APC-OX40	BioLegend
PerCP-GITR	BioLegend
PeCy7-CD25	BioLegend
APC-Cy7-Viability Dye	Invitrogen
BUV737-CD86	BioLegend
AF700-MHCII	BioLegend
PeCy7-PD-L1	BioLegend
PerCp-B220	BioLegend
BUV605-F4/80	
BUV395-CD11c	BD Biosciences
APC-CD11b	BioLegend
anti-PD-L1 clone MIH5	
anti-Lag3 clone C9B7W	BioLegend
IgG2a (clone MAC219)	University of Cambridge
IgG1 (clone MAC221)	
Chemicals, peptides, and recombinant proteins	
MBP Ac1-9[4Y] peptide AcASQYRPSQR	GL Biochem Shanghai
Phosphate buffered saline (Ca^{2+} Mg^{2+} free)	ThermoFisher
DNASE I, GRADE II	Roche
Collagenase D	Roche
Foetal Bovine Serum, qualified, heat inactivated, Brazil	ThermoFisher
β-mercaptoethanol	Gibco
Lipopolysaccharide	Invitrogen
GM-CSF	BioLegend
Experimental models	
Mouse: Nr4a3-*Tocky Tg4 Tiger (*Il10-*GFP)*	Jennings et al., 2020
Mouse: Nur77-Tempo Tg4 Tiger (Il10-GFP)	Elliott et al,. 2022

3.9. Flow cytometry:

All samples were run using the BD LSRFortessa X-20, including single stains. The fluorescent timer within the Nur77-Tempo is excited by the 405nm laser to provide blue emission detected within the 450/40nm channel. Following the maturation of the FT protein (4 hours), the FT-protein will shift its emission spectrum to provide red

fluorescence, which is detected in the 610/20nm detection channel. Once all data was collected, samples were compensated for utilising both single stains and unstained samples to correct for both spectral overlapping and leaking into other detection channels. All compensation was conducted after data was collected from the flow cytometer, followed by gating strategies and further analysis using FlowJo 10.10.0. software.

3.9.1. Gating strategies:

Flow cytometry data was collected, as stated in chapter 3.9. For *ex-vivo* analysis of dendritic cells, splenocytes were stained with surface stains, and gates were drawn around populations of interest. First, cells were gated on SSC-A vs FSC-A, then single cells (FSC-H vs FSC-A), live cells (APC-Cy7-V/D vs FSC-A), B-cells ($B220^{+}CD11b^{-}$), and finally dendritic cells ($B220^{-}CD11b^{int}CD11c^{hi}$). The markers MHCII, CD86, and PD-L1 within the DC gate were analysed (Fig.5).

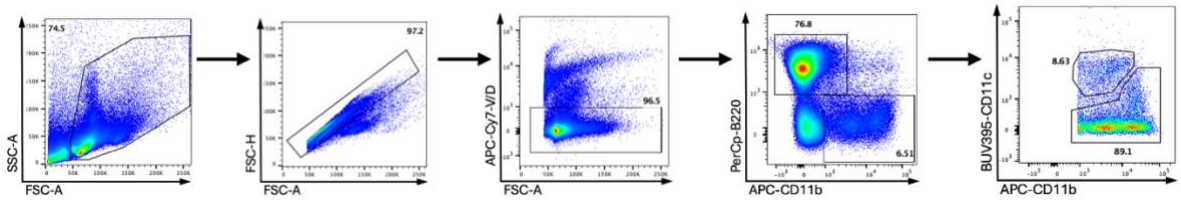

Figure 6 - *Ex-vivo* Gating Strategy of Dendritic Cells.

Data was analysed using FlowJo Software. Compensation was done using single stains and unstained samples. Cells were gated first by comparing SSC-A and FSC-A, then single cells by comparing FSC-H against FSC-A. Viability dye is then used to gate on only live cells. next the B220 and CD11b was used to gate our B-cells. within the CD11b+ gate, DC's were identified by then comparing CD11c and CD11b therefore DC's identified were $B220^{-}CD11b^{int}CD11c^{+}$.

For *ex-vivo* and *in-vitro* analysis of T-cells, splenocytes were stained with surface stains, and gates were drawn around populations of interest. First, lymphocytes were gated (FSC-A vs SSC-A), then single cells (FSC-H vs FSC-A), live cells (APC-Cy7-V/D vs FSC-A), $CD4^{+}$ T-cells ($CD4^{+}TCRv\beta8.1.1.2^{+}$). Respondents were analysed

within this CD4⁺ T-cell gate by comparing the expression of FT-Blue and FT-Red. Furthermore, Tr1-like IL10-expressing populations were analysed by comparing IL10-GFP and CD4. Within the CD4⁺ T-cell gate, the expression of co-inhibitory receptors (PD-1, Lag-3, Tigit) and activation markers (ICOS, OX40, CD25 and GITR) (Fig. 6).

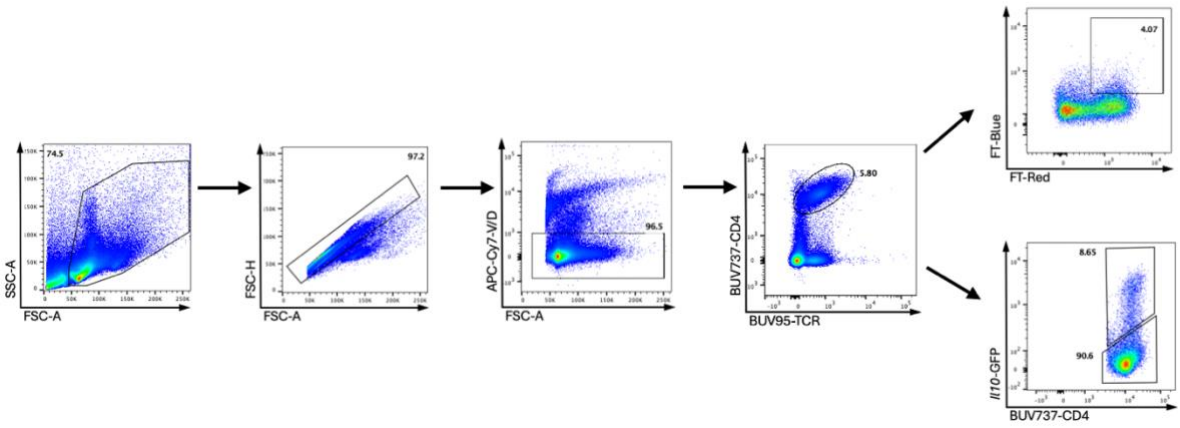

Figure 7 – CD4⁺ T-cell gating strategy.

Data was analysed using FlowJo software. Compensation was done using single stains and unstained samples. Lymphocytes were gates first by comparing SSC-A and FSC-A, then single cells by comparing FSC-H and FSC-A. Viability dye is then used to gate on live cells. Next CD4⁺ T-cells were gated on by comparing CD4 and TCRvb expression. Within this CD4⁺ T-cell gate, the expression of T-cell markers (ICOS, OX40, CD25 and GITR) were analysed and the proportion of responding cells by comparing FT-Blue against FT-Red. For analysis of IL10-expressing Tr1-like CD4⁺ T-cells, IL10^{hi}CD4⁺ populations were gated on within the CD4⁺ T-cell gate.

3.10. Statistical analysis:

All graphs within this project were created using the GraphPad Prism 10 software and utilised for statistical significance. An unpaired t-test was used to compare two means, and Two-way ANOVA was used to compare two independent variables against one another. Any variance is presented as the mean ± SEM, and any p values on the graphs illustrate statistical significance levels *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001.

4. Results -

4.1. CD4$^+$ T-cells are successfully tolerised *in-vivo* following EDI:

Here, mice received either EDI or one 4mg/Kg 4Y-MBP immunisation to chronically or acutely stimulate CD4$^+$ T-cells *in-vivo*, respectively. The spleens were harvested and processed 24 hours after the final immunisation before staining with appropriate antibodies against CD4 and TCRvβ8.1.1.2 to allow for *ex-vivo* analysis of CD4$^+$ T-cells exclusively via flow cytometry. Here, the mean proportion of responders in acute conditions (49.5%) is significantly higher than that of chronic conditions (2.94%) (Fig.8A, 7C). Concomitant with these results, the mean FT-Blue and FT-Red MFI is significantly higher in acutely stimulated CD4$^+$ T-cells (Fig.8B, 8D-E). These results indicate that EDI is sufficient to induce tolerance and renders T-cells unable to respond to peptides *in-vivo*.

To interrogate the phenotype of CD4$^+$ T-cells in each condition *ex-vivo*, splenocytes were also stained with antibodies against ICs: PD-1, Lag3 and Tigit. Interestingly, the expression of all markers was significantly higher in acutely stimulated CD4$^+$ T-cells (Fig.8F-K). these markers are only expressed following T-cell activation and thus indicate that acutely stimulated CD4$^+$ receiving a single, high-dose peptide are more strongly activated than chronically stimulated CD4$^+$ T-cells.

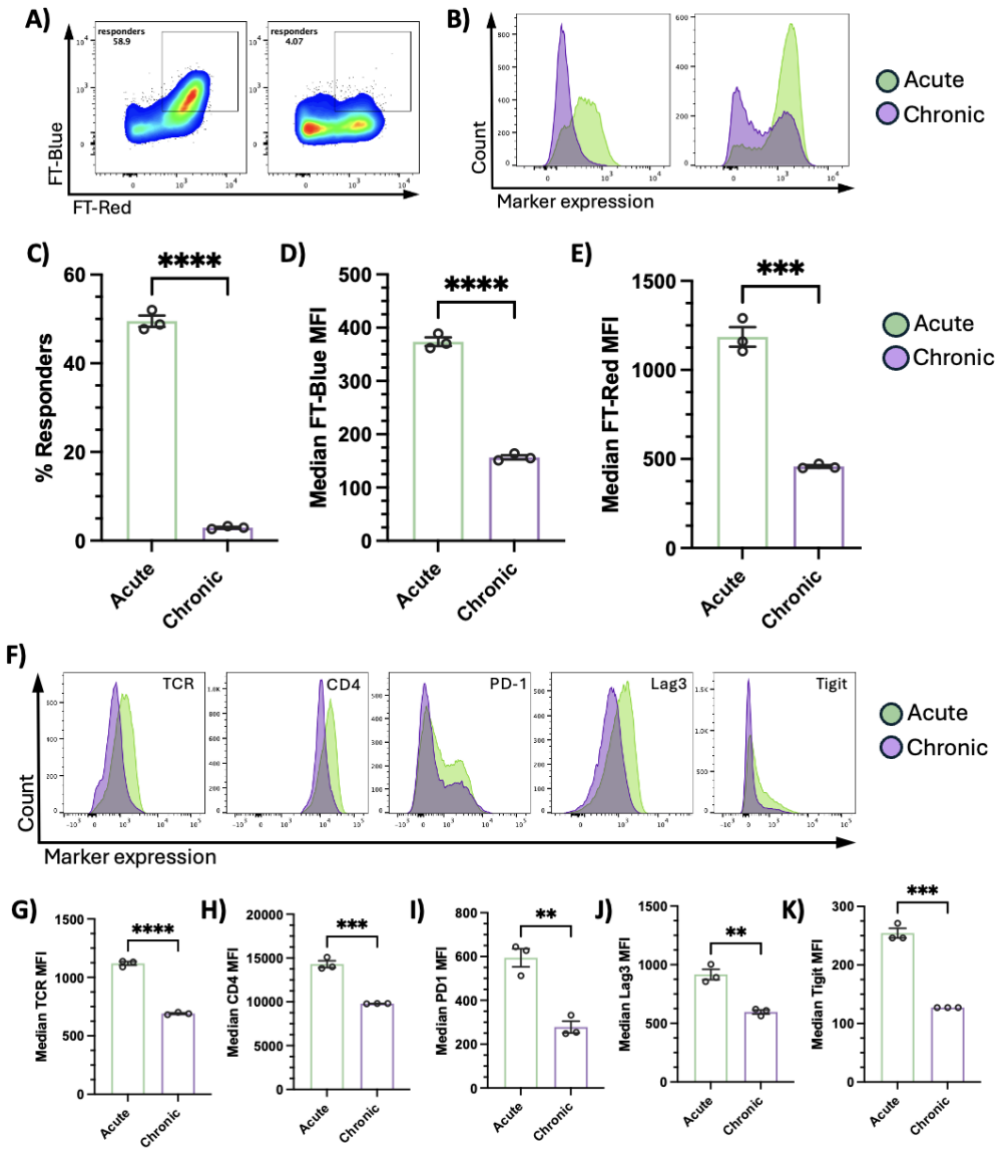

Figure 8 - *Ex-vivo* Analysis of CD4⁺ T-cells from EDI-treated Nur77-Tempo Mice.

A) *Flow cytometry plot of FT-Blue and FT-Red expression.* ***B)*** *Histograms overlaying the expression of FT-Blue and FT-Red between acute and chronically stimulated CD4+ T-cells.* ***C****) Bar chart comparing changes in Blue+Red+ 'Responder' CD4+ populations.* ***D)*** *Bar chart comparing acute and chronically stimulated CD4+ T-cell expression of FT-Blue,* ***E)*** *FT-Red.* ***F)*** *Histograms overlaying the expression of TCR, CD4, PD-1, Lag3 and Tigit between acute and chronically stimulated CD4+ T-cells.* ***G)*** *Bar chart comparing acute and chronically stimulated CD4+ T-cell expression of TCR.* ***H)*** *CD4.* ***I)*** *PD-1.* ***J)*** *Lag3.* ***K)*** *Tigit. Error bars represent mean ± SEM. n=3. Statistical analysis by Unpaired t-test. Significance represented as *p≤0.05, **P≤0.01, ***p≤0.001, ****p≤0.0001.*

To interrogate how the transcriptional profiles of tolerant T-cells differ from those of naïve T-cells, genome-wide RNA-sequencing data, published by Bevington et al. (105), was obtained and re-analysed for markers of interest. Here, adaptively tolerant T-cells at rest exhibited an increase in IC, co-stimulatory receptor, and IL10

expression (Fig.9A-B) compared to naïve T-cells. Together, this implies that at rest, tolerant T-cells maintain a heightened expression of ICs and IL10.

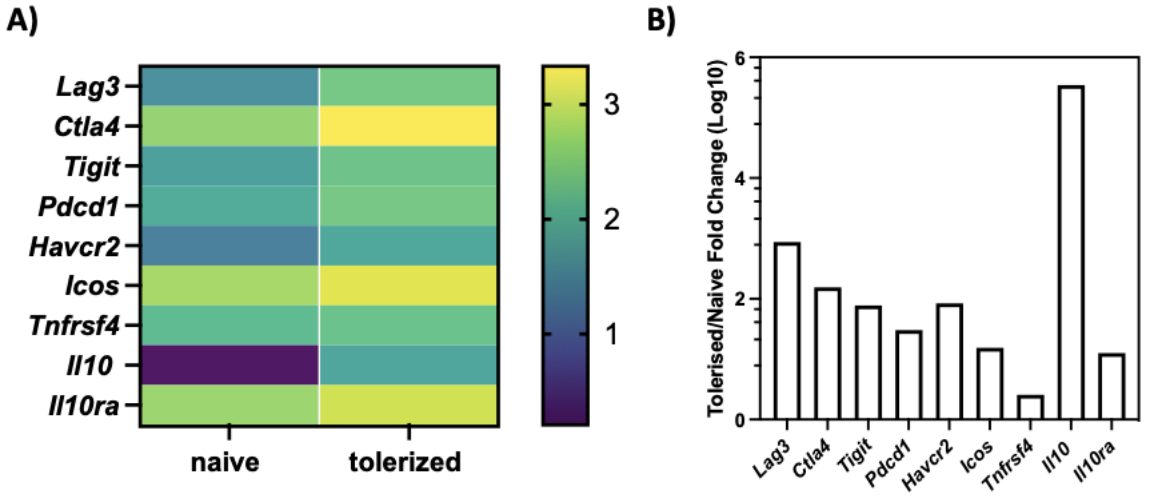

Figure 9 - Changes in Transcriptional Profiles of Naive and Tolerant T-cells.

***A)** Heatmap showing the expression of key markers Lag3, Ctla4, Tigit, Pdcd1 (PD-1), Havcr2 (Tim-3), Icos, Tnfrsf4 (OX40), Il10 and Il10ra. Normalised read counts for genes were Log2 transformed prior to plotting. **B)** Bar chart illustrating the fold change in gene expression between naïve and tolerised T-cells. Data obtained from Bevington et al. (105).*

4.2. Adaptively tolerant CD4⁺ T-cells exhibit increased resistance to peptide re-challenge:

To interrogate the TCR signalling dynamics of CD4⁺ T-cells *in-vitro*, splenocytes from chronically and acutely stimulated mice were cultured with a titration of cognate peptide for 24 hours. Splenocytes were stained with antibodies against CD4, TCRvβ8.1.1.2, ICOS, OX40, CD25 and GITR. Here, CD4⁺ T-cells from both the acutely and chronically stimulated conditions responded to increased peptide concentrations (Fig.10A). It is important to note that chronically stimulated CD4⁺ T-cells consistently exhibited diminished responses to a peptide (Fig.10B) when compared to acutely stimulated CD4⁺ T-cells. Concurrent with these results, the

expression of FT-Blue (Fig.10C, 10E) and FT-Red (Fig.10D, 10F) for each restimulation condition was significantly higher in the acutely stimulated T-cells. Furthermore, the expression of both ICOS (Fig.11A-B) and OX40 (Fig.11A, 11C) form part of the TCR.Strong metric (Fig.11D) was more significant in acutely stimulated CD4⁺ T-cells. Finally, the expression of both CD25 (Fig.11A, 11E) and GITR (Fig.11A, 11F) was also significantly higher in acutely stimulated CD4⁺ T-cells. These data imply that chronically stimulated and thus adaptively tolerised CD4⁺ T-cells exhibit a diminished response and greater resistance to peptide rechallenge *in-vitro*.

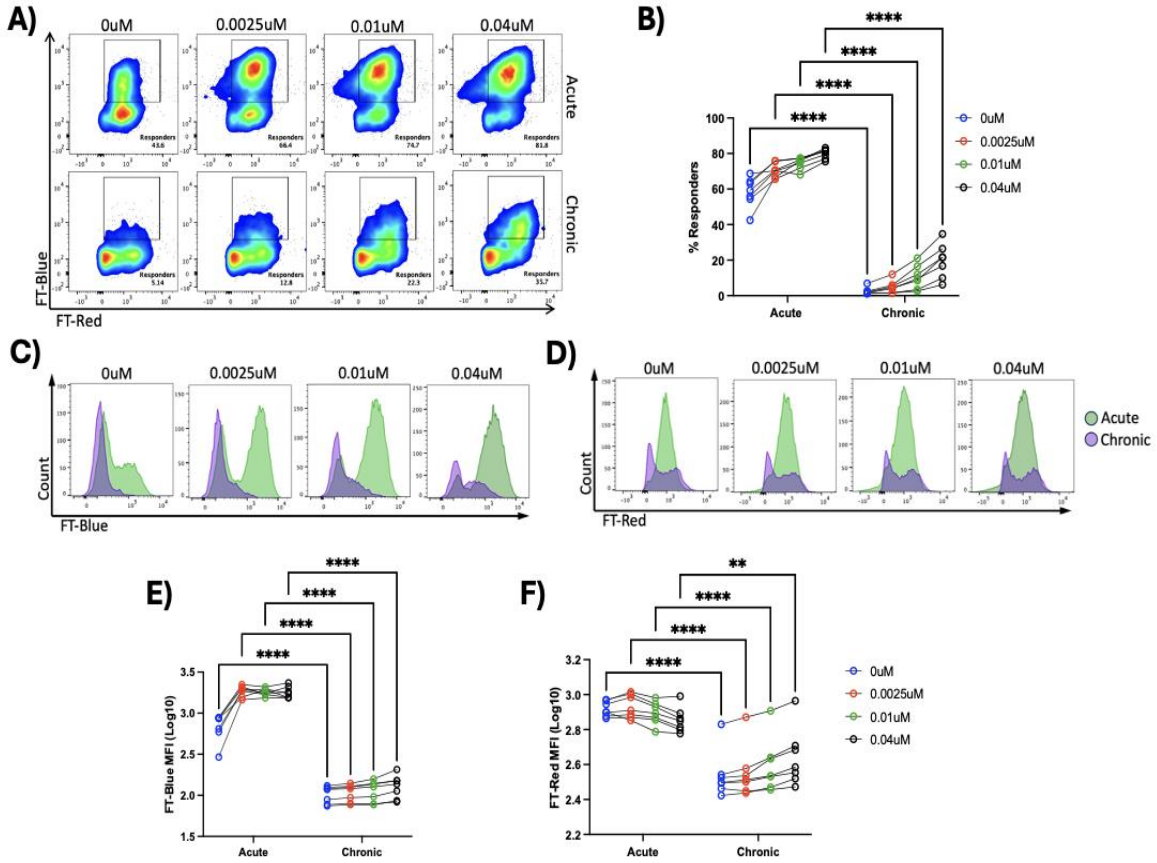

Figure 10 - *In-vitro* quantitative changes in CD4⁺ T-cell responses between acutely and chronically stimulated Nur77-Tempo Mice.

A) *Flow cytometry plot of CD4+ T-cell expression of FT-Blue and FT-Red.* ***B)*** *Scatter graph illustrating changes in Blue+Red+ 'Responder' populations between acute and chronically stimulated CD4+ T-cells.* ***C)*** *Histograms overlaying the expression of FT-Blue and* ***D)*** *FT-Red, between acute and chronically stimulated CD4+ T-cells in response to cognate peptide.* ***E)*** *Scatter graph comparing changes in FT-Blue and* ***F)*** *FT-Red expression between acute and chronically stimulated T-cells in response to cognate peptide. Data representative of 2 independent experiments n=3 and n=4, overall n=7. Statistical analysis by Two-way ANOVA. Significance represented as *p≤0.05, **P≤0.01, ***p≤0.001, ****p≤0.0001.*

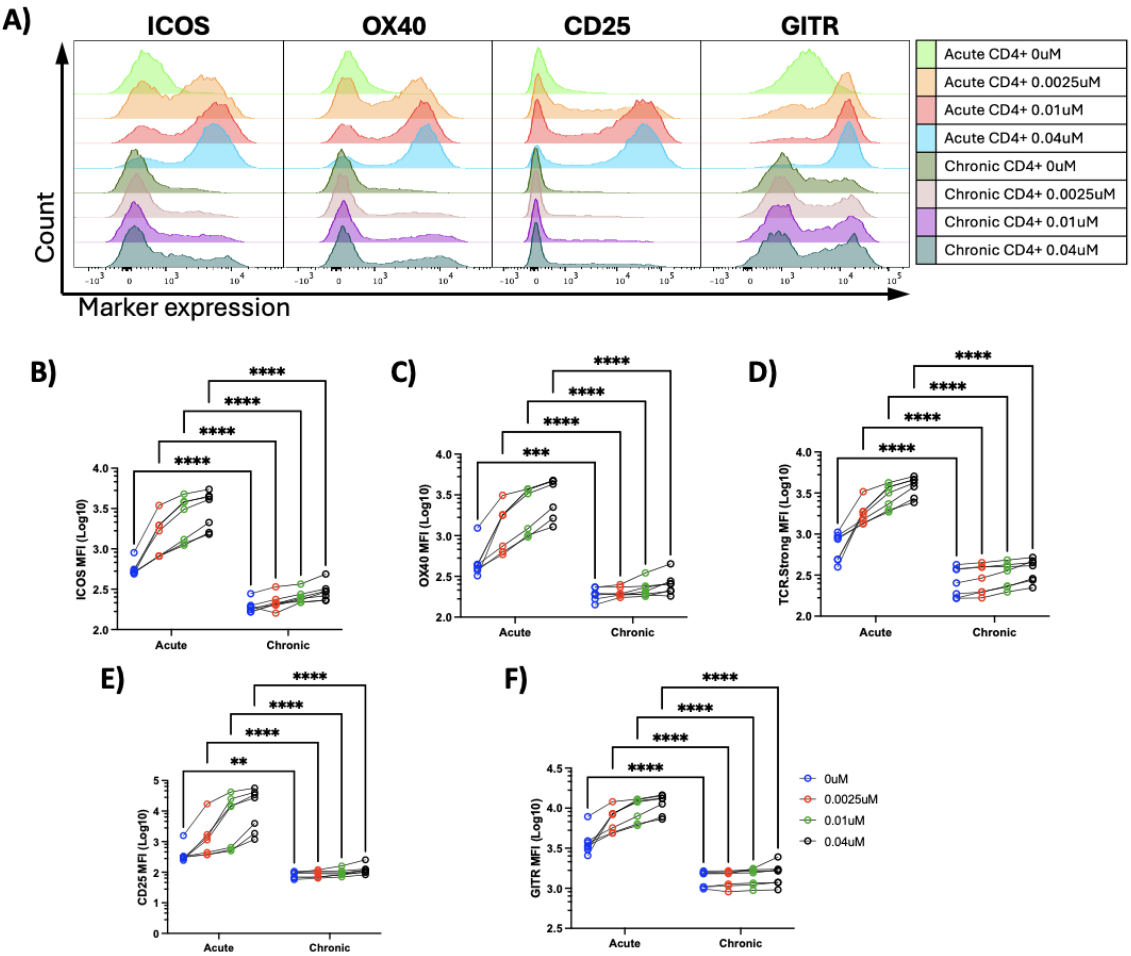

Figure 11 - *In-vitro* Qualitative Changes in CD4⁺ T-cell Phenotype Between Acutely and Chronically Stimulated Nur77-Tempo Mice.

A) *Histograms comparing the expression of ICOS, OX40, CD25 and GITR between acutely and chronically stimulated CD4+ T-cells in response to cognate peptide.* ***B)*** *Scatter graph comparing acute and chronically CD4+ T-cell expression of ICOS.* ***C)*** *OX40.* ***D)*** *TCR.Strong.* ***E)*** *CD25.* ***F)*** *GITR in response to cognate peptide. Data representative of 2 independent experiments n=3 and n=4, overall n=7. Statistical analysis by Two-way ANOVA. Significance represented as *p≤0.05, **P≤0.01, ***p≤0.001, ****p≤0.0001.*

4.3 Phenotypic characterisation of Tolerogenic and Regulatory Immune subsets.

Forming part of the adaptive immune response, CD4⁺ T-cells are primarily reliant on and controlled by signalling inputs from other cells within their microenvironment. Thus, we sought to investigate the phenotype of antigen-presenting cells *ex-vivo*. CD4+ T-cells were chronically or acutely stimulated *in-vivo* to do this, as mentioned in section 3.1. Splenocytes were then prepared and stained with antibodies against

B220, CD11b, CD86, MHCII, and PD-L1. By analysing the B220^{-}CD11c^{int}CD11c^{hi} DC compartment, it is evident that DCs from chronically stimulated mice exhibit a more immature, tolerogenic morphology (Fig. 12A). A trend toward lower MHCII expression reflects this; however, this is statistically insignificant (Fig. 12B), lower CD86 (Fig. 12C) and PD-L1 (Fig. 112D) expression, and heightened IL-10 expression (Fig. 12E). Studies have used the ratio of PD-L1 and CD86 co-expression as a metric to identify largely tolerogenic APC subsets (158,159). DCs from chronically stimulated mice exhibit a heightened PD-L1/CD86 ratio compared to acutely stimulated mice (Fig. 12F). Together, these data imply that EDI-treated mice contain a greater proportion of TolDCs with poor T-cell activation capacity.

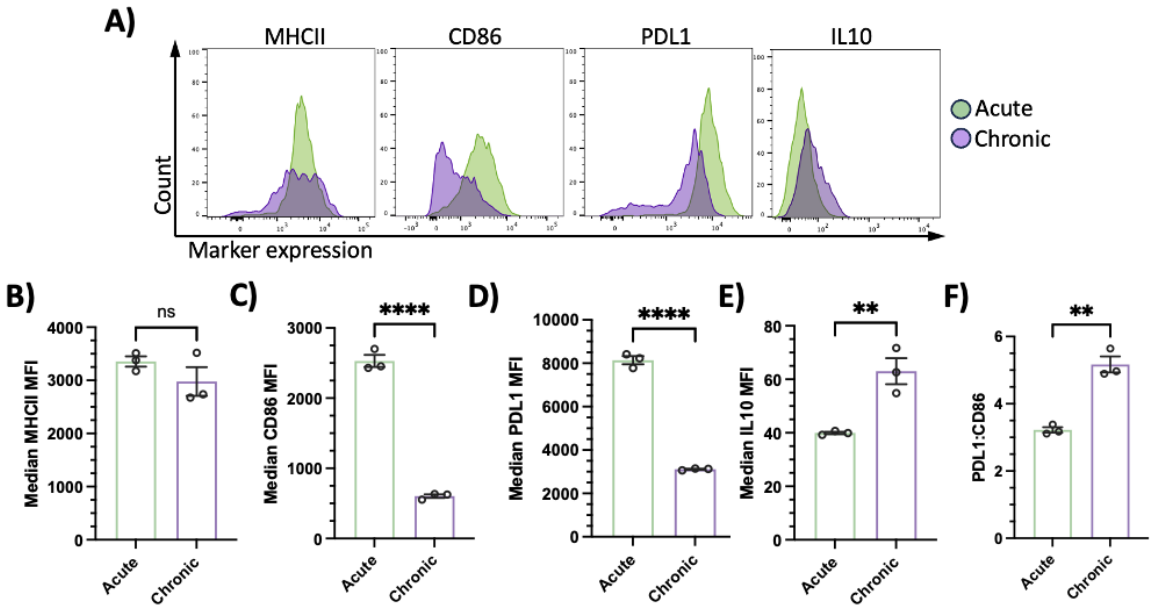

Figure 12 - *Ex-vivo* analysis comparing differences in DC phenotype between acute and chronically stimulated Nur77-Tempo mice.

A) *Histograms comparing the expression of dendritic cell-derived MHCII, CD86, PD-L1 and IL10 between acutely and chronically stimulated Nur77-Tempo mice.* ***B)*** *Bar chart comparing dendritic cell-derived expression of MHCII.* ***C)*** *CD86.* ***D)*** *PD-L1.* ***E)*** *IL10 between acutely and chronically stimulated Nur77-Tempo mice.* ***F)*** *Bar chart comparing the PD-L1/CD86 ratio of dendritic cells from acutely and chronically stimulated Nur77-Tempo mice. Error bars represent mean ± SEM. n=3. Statistical analysis by Unpaired t-test. Significance represented as *p≤0.05, **P≤0.01, ***p≤0.001, ****p≤0.0001.*

To investigate the emergence and phenotype of regulatory $CD4^+$ subsets *ex-vivo*, splenocytes were stained with antibodies against TCRvβ8.1.1.2, CD4, PD-1, Lag3, and Tigit. Flow cytometry analysis revealed a similar Tr1-like regulatory $CD4^+$ T-cell emergence between acutely and chronically stimulated mice (Fig. 13A-B). However, these IL10-expressing T-cells exhibited vastly different phenotypes between the two conditions (Fig. 13C). TCR (Fig. 13D), CD4 (Fig. 13E), and Lag3 (Fig. 13G) are downregulated, whereas PD-1 (Fig. 13F), Tigit (Fig. 13H), and IL10 (Fig. 13I) are upregulated by all IL10-expressing Tr1-like $CD4^+$ T-cells within Acute and Chronic conditions. However, PD-1, IL10 and Lag3 expression is markedly higher in Tr1-like cells within the chronic condition. This data implies that selection into the Tr1-like lineage is associated with a regulatory, tolerising phenotype, which is more pronounced in IL10-expressing Tr1-like cells arising from chronic stimulation.

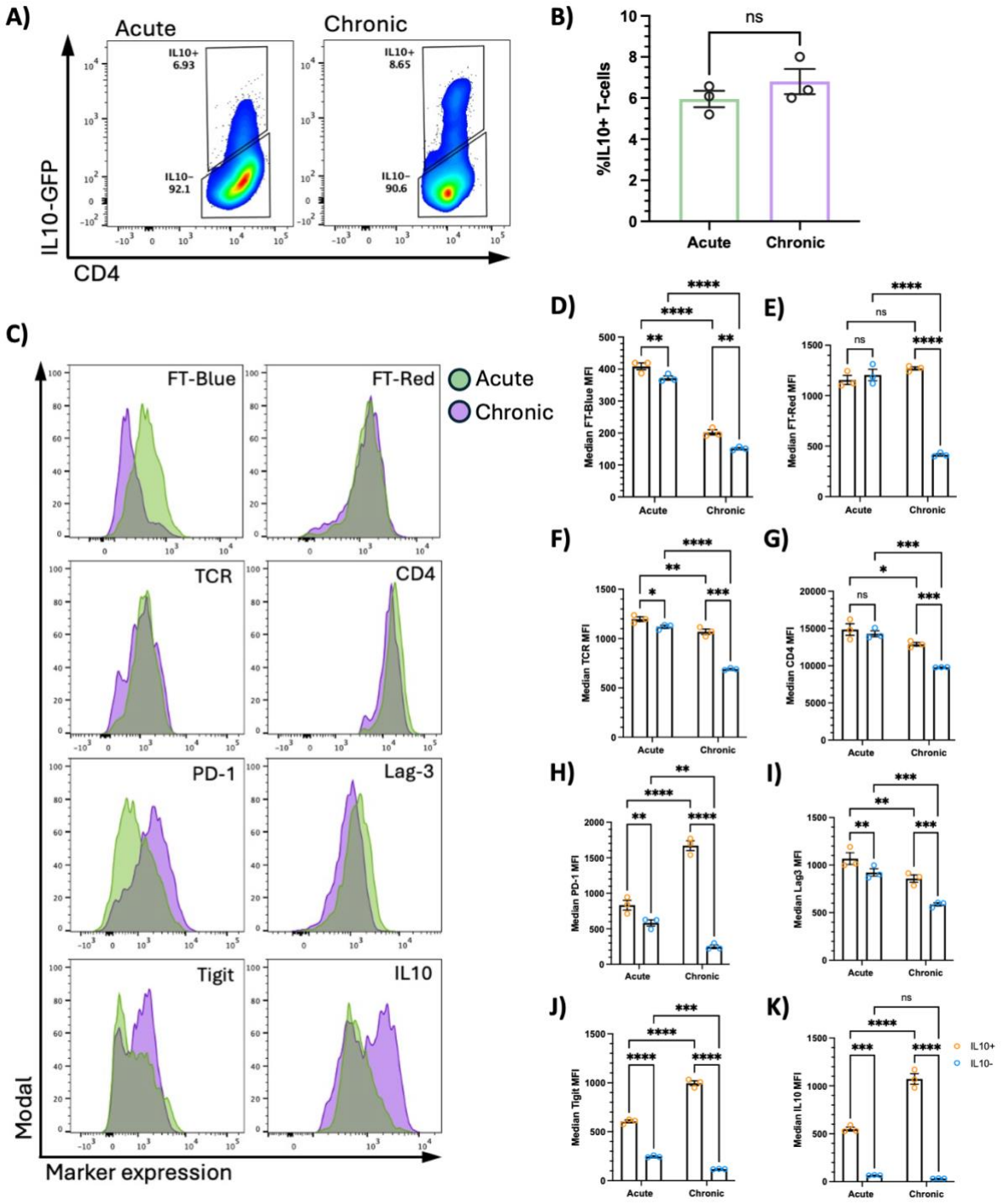

Figure 13 - *Ex-vivo* analysis comparing emergence and phenotype of a regulatory IL10-expressing Tr1-like population between acute and chronically stimulated Nur77-Tempo mice.

A) *Flow cytometry plot comparing IL10- and IL10+ populations between acute and chronically stimulated CD4+ T-cells.* ***B)*** *Bar chart showing the proportion of IL10-expressing Tr1-like cells between acute and chronically stimulated CD4+ T-cells.* ***C)*** *Overlayed histograms comparing the expression of TCR, CD4, PD-1, Lag3, Tigit and IL10 between acute and chronically stimulated CD4+ T-cells.* ***D)*** *Bar charts comparing Acute and chronic IL10+ and IL10- populations expression of TCR.* ***E)*** *CD4.* ***F)*** *PD-1.* ***G)*** *Lag3.* ***H)*** *Tigit.* ***I)*** *IL10. Error bars represent mean ± SEM. n=3. Statistical analysis by Unpaired t-test. Significance represented as *p≤0.05, **P≤0.01, ***p≤0.001, ****p≤0.0001.*

4.4. Regulatory IL10-expressing Tr1-like CD4$^+$ T-cell subsets retain their ability to signal under chronic conditions:

Like conventional T-cells, Tr1-like cells are also antigen-dependent (160), requiring signals via their TCR to activate and thus exert their regulatory functions via IL10 expression and secretion. Given the discrepancy in IL10 expression between IL10-expressing populations in acute and chronic conditions, we wanted to elucidate the TCR signalling dynamics of the IL10-expressing Tr1-like populations present within the culture. To do this, splenocytes were again cultured with a peptide titration as discussed in section 3.7, and 24 hours later, were stained with appropriate antibodies against TCRvβ8.1.1.2, CD4, ICOS, OX40, CD25 and GITR.

Flow cytometric analysis indicates that IL10-expressing populations are increasingly more sensitive to peptide rechallenge (Fig.14A) and express significantly more FT-Blue (Fig.14B) and FT-Red (Fig.14C) than their IL10$^-$ counterparts. Concurrent with these results, the expression of all markers (Fig.14A), such as ICOS (Fig.14D), OX40 (Fig.14E), GITR (Fig.14F), CD25 (Fig.14G), and IL10 (Fig. 14H) are also significantly upregulated. Together, this data implies that following selection into the Tr1-lineage, Tr1-like cells can undergo active TCR signals, which are abolished in the IL10$^-$ populations.

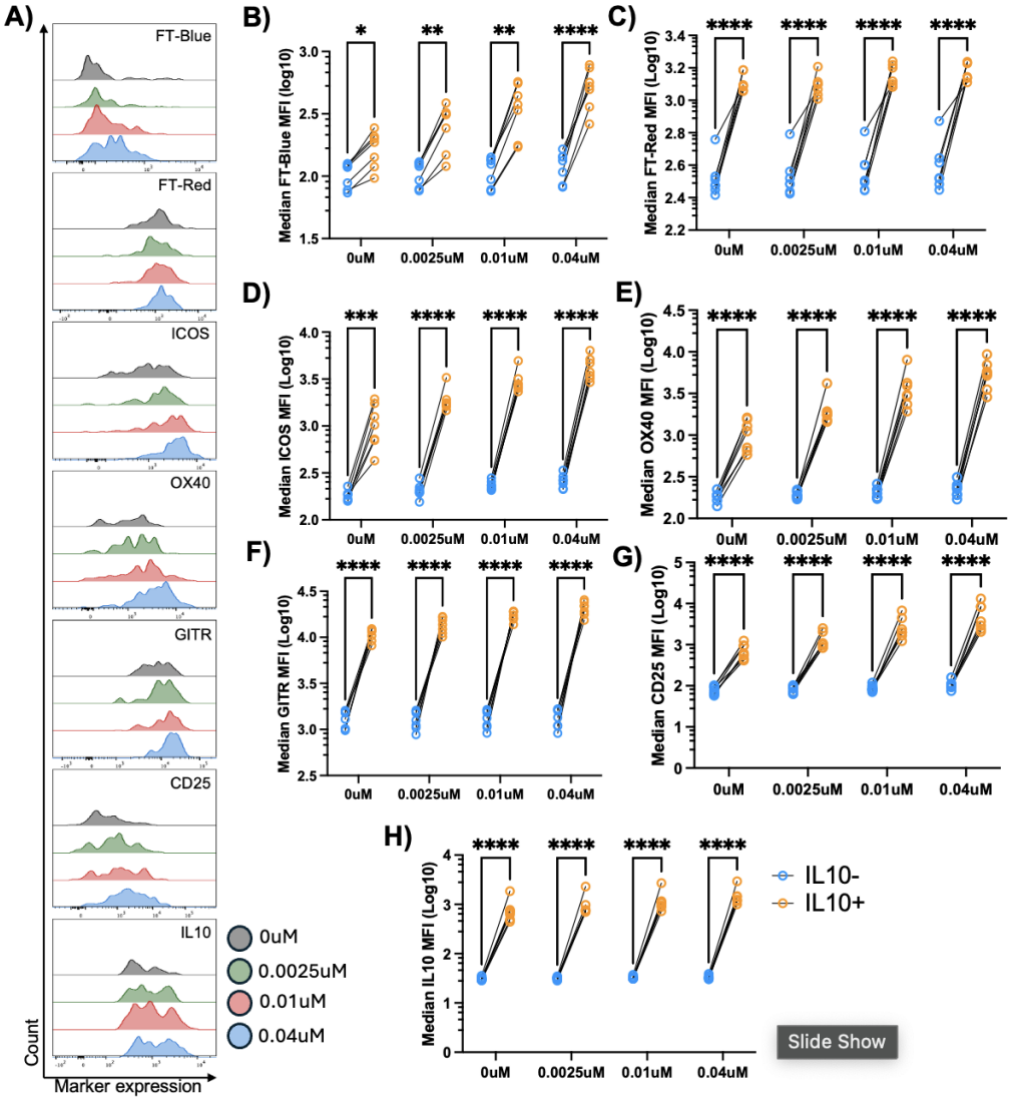

Figure 14 - *In-vitro* analysis comparing the responses of Tr1-like and non-Tr1-like cells from chronically stimulated Nur77-Tempo mice.

A) *Histograms showing the expression of FT-Blue, FT-Red, ICOS, OX40, GITR, CD25 and IL10 from IL10-expressing Tr1-like CD4+ populations.* ***B)*** *scatter graphs comparing changes in FT-Blue,* ***C)*** *FT-Red,* ***D)*** *ICOS,* ***E)*** *OX40,* ***F)*** *GITR,* ***G)*** *CD25 and* ***H)*** *IL10 between chronically stimulated Tr1-like and non-Tr1-like T-cells in response to peptide. Data representative of two independent experiment, n=3 and n=4, Overall n=7. Statistical analysis by Two-way ANOVA. Significance represented as *p≤0.05, **P≤0.01, ***p≤0.001, ****p≤0.0001.*

4.5. Adaptively tolerant CD4$^+$ T-cells are rendered hyporesponsive due to intrinsic defects.

As previously mentioned, DCs can also be potent inducers of T-cell tolerance by virtue of increased co-inhibitory receptors and decreased co-stimulatory receptor engagement. Given the large discrepancy in DC phenotype (Fig.12), we aimed to interrogate how CD4$^+$ T-cell responses may be modified if a stimulatory environment was provided. To do this, stem cells were isolated from the bone marrow of Tg4 mice before culture with GM-CSF to promote DC polarization. BMDCs were cultured in the presence of LPS and titration of 4Y-MBP peptide for 24 hours before co-culture at a ratio of 1:1 with isolated CD4$^+$ T-cells from acute or chronically stimulated mice for another 24 hours. CD4$^+$ T-cells were then stained with the appropriate antibodies against CD4, ICOS, OX40, CD25 and GITR before analysis on the flow cytometer. Both acute and chronically stimulated CD4$^+$ T-cells responded to increased peptide dose (Fig.15A 15D), expressing more FT-Blue (Fig. 15B, 15E) and FT-Red (Fig. 15C, 15F) However, much like the results in section 4.2, chronically stimulated T-cells exhibited diminished responses to rechallenge for the same given peptide dose compared to acutely stimulated T-cells (Fig. 14D).

The expression of ICOS, OX40, CD25 and GITR was analysed to interrogate qualitative changes in the phenotype of these T-cells. Like the results in section 4.2, increased peptide doses promote the expression of all markers (Fig. 16A) in both acute and chronically stimulated T-cells. However, the magnitude of expression for each marker for the same given peptide dose is higher in acutely stimulated T-cells than in chronically stimulated T-cells (Fig. 16B-F). Together, these results indicate

that removing tolerised T-cells from an inhibitory, tolerogenic setting to a stimulatory setting is insufficient for restoring their responses.

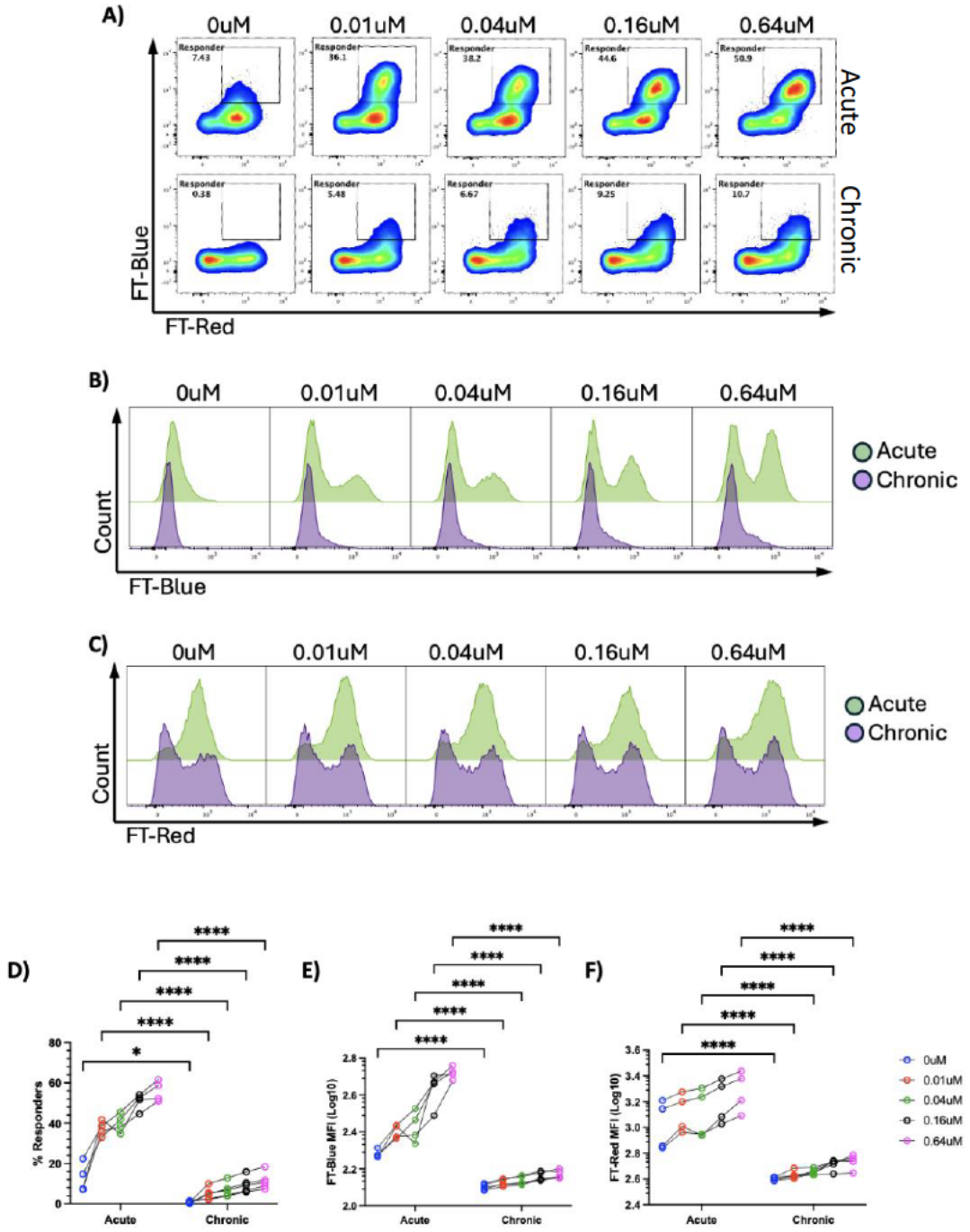

Figure 15 - *In-vitro* quantitative analysis of tolerised CD4⁺ T-cells co-cultured with previously LPS-activated BMDCs.

A) *Flow cytometry plots showing the proportion of Responders (Blue⁺Red⁺) in response to cognate peptide.* ***B)*** *Histogram showing the changes in FT-Blue and* ***C)*** *FT-Red expression between acute and chronically stimulated T-cells at increasing peptide titres.* ***D)*** *Scatter graph illustrating changes in responders,* ***E)*** *FT-blue and* ***F)*** *FT-Red expression. Data representative of 2 independent experiments n=2 and n=3, overall n=5. Statistical analysis by Two-way ANOVA. Significance represented as *p≤0.05, **P≤0.01, ***p≤0.001, ****p≤0.0001.*

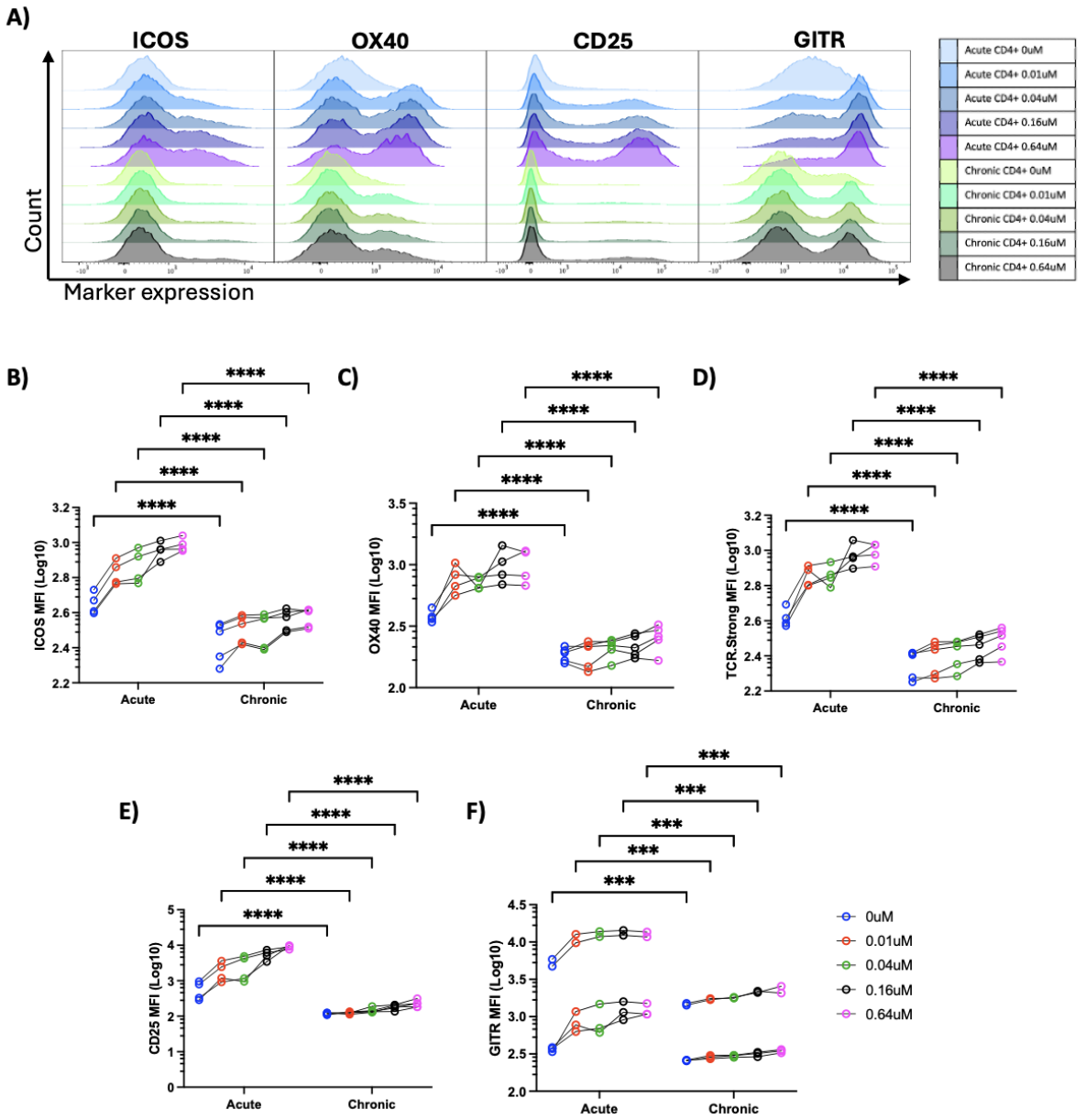

Figure 16 - *In-vitro* qualitative analysis of isolated tolerised CD4^{+} T-cells co-cultured with previously LPS-activated BMDCs.

A) *Histogram overlays showing the change in ICOS, OX40, CD25 and GITR expression between acute and chronically stimulated T-cells in response to increasing peptide titres.* ***B)*** *Scatter graph illustrating changes in ICOS,* ***C)*** *OX40,* ***D)*** *TCR.Strong,* ***E)*** *CD25 and* ***F)*** *GITR expression between acute and chronically stimulated CD4 T-cells. Data representative of 2 independent experiments, n=2 and n=3, overall n=5. Statistical analysis by Two-way ANOVA. Significance represented as *p≤0.05, **P≤0.01, ***p≤0.001, ****p≤0.0001.*

4.6. Adaptively tolerant T-cells do not exhibit NFAT-biased signalling.

Given that adaptively tolerant T-cells exhibit intrinsic defects in intrinsic signalling pathways, we aimed to investigate the affected signalling pathways. To do this we utilised two Nr4a-timer reporters: Nur77-Tempo (157) and the Nr4a3-Tocky (161).

Previous work from our laboratory shows that Nr4a1 (Nur77) expression is NFAT-independent. Conversely, the expression of Nr4a3 is NFAT-dependent (162). Given this discrepancy, we can use the expression of the Nr4a3-Timer as a proxy for NFAT-biased signalling. Thus, Nur77-Tempo and Nr4a3-Tocky mice were EDI-treated to induce CD4$^+$ T-cell tolerance. The mice were culled, and spleens were harvested and processed 24 hours after the final immunisation. For *ex-vivo* analysis, splenocytes were stained immediately with antibodies against TCRvβ8.1.1.2 and CD4. As indicated in Fig. 17A, chronically stimulated CD4$^+$ T-cells from Nr4a3-Tocky mice exhibit responses like those observed in Nur77-Tempo mice. The percentage of CD4$^+$ T-cells responding is increased in CD4$^+$ T-cells from the Nur77-Tempo line (Fig. 17C), but this is the result of the increased brightness of the fluorescent timer protein. Concurrent with this observation, the expression of FT-Blue (Fig.17B, 17D) and FT-Red (Fig. 17B, 17E) is also significantly higher, by virtue of increased brightness.

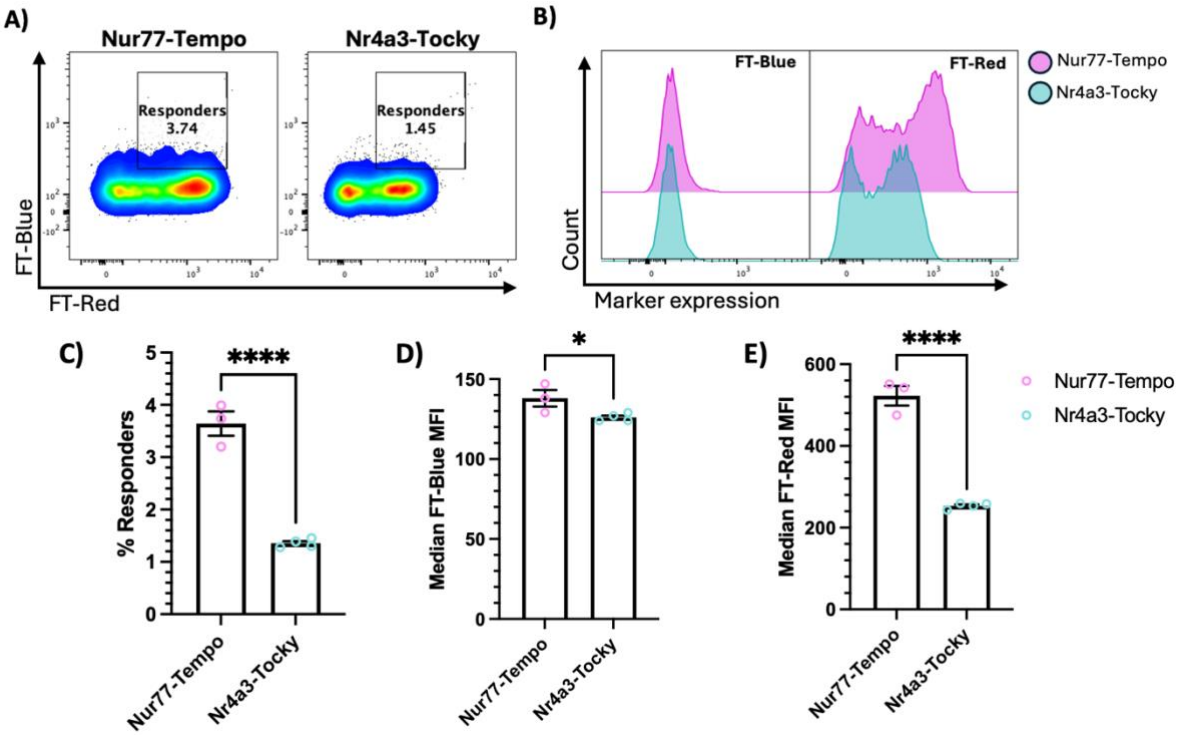

Figure 17 - *Ex-vivo* analysis comparing adaptively tolerant CD4⁺ T-cells from chronically stimulated Nur77-Tempo and Nr4a3-Tocky mice.

A) *Flow cytometry plots showing the proportion of responders (Blue+Red+).* ***B)*** *Histogram overlays comparing the expression of FT-Blue and FT-Red between chronically stimulated CD4+ T-cells from Nur77-Tempo and Nr4a3-Tocky mice.* ***C)*** *Bar chart showing the changes in the proportion of responders,* ***D)*** *FT-Blue and* ***E)*** *FT-Red between chronically stimulated Nur77-Tempo and Nr4a3-Tocky mice. n=7. Statistical analysis by unpaired t-test. Significance represented as *p≤0.05, **P≤0.01, ***p≤0.001, ****p≤0.0001.*

To interrogate if these reporters respond differently to antigenic rechallenge, splenocytes from EDI-treated Tg4 Nur77-Tempo *Il10*-GFP and Tg4 Nr4a3-Tocky *Il10*-GFP mice were cultured in the presence of peptide titrations for 24 hours. Following 24-hour incubation, splenocytes were stained with appropriate antibodies against TCRvβ8.1.1.2 and CD4 for *in-vitro* analysis of CD4⁺ T-cells only. As expected, no significant differences were observed in the proportion of responders between Nur77-Tempo and Nr4a3-Tocky mice (Fig. 18A-B). Concomitant with these results, the expression of FT-Blue also remained similar between these reporters (Fig. 18C-D). Interestingly, FT-Red expression was significantly higher in Nur77-Tempo mice (Fig. 18C, 18E), which results high expression levels of the fluorescent timer protein from the *Nr4a1* allele (157). Together, these results indicate that chronic stimulation in both reporters is associated with greater resistance to peptide rechallenge. Furthermore, given the diminished expression of NFAT-dependent Nr4a3 within the Tg4 Nr4a3-Tocky reporter, following the induction of tolerance, CD4⁺ T-cells don't undergo NFAT-biased signalling in response to chronic antigenic exposure.

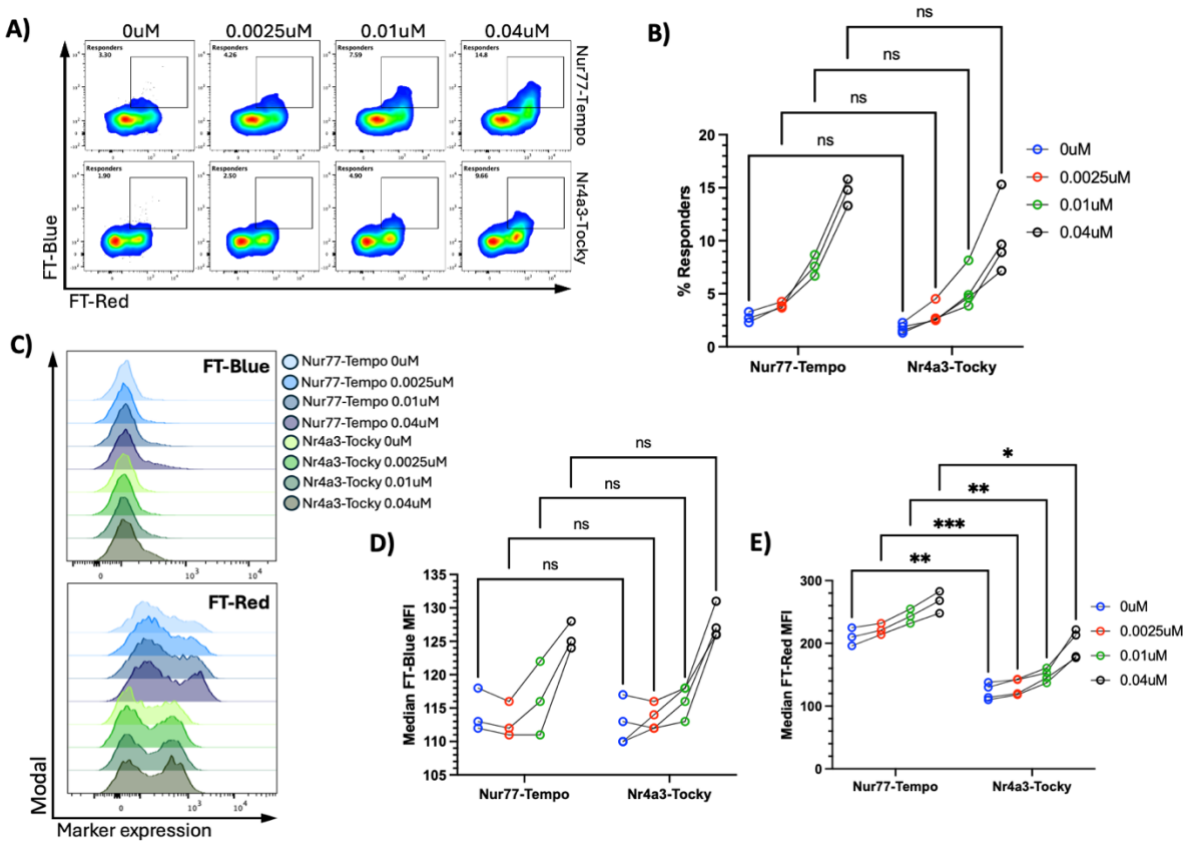

Figure 18 - *In-vitro* analysis comparing quantitative changes in the proportion of responders between chronically stimulated Nur77-Tempo and Nr4a3-Tocky mice.

A) *Flow cytometry plots illustrating the quantitative changes in the proportion of responding (Blue+Red+) CD4+ T-cells in response to peptide.* ***B)*** *scatter graph comparing the changes in the percentage of responders.* ***C)*** *Histogram overlays showing the expression of FT-Blue and FT-Red by chronically stimulated CD4+ T-cells from Nur77-Tempo and Nr4a3-Tocky mice.* ***D)*** *Scatter graph showing changes in FT-Blue and* ***E)*** *FT-Red expression. n=7. Statistical analysis by unpaired t-test. Significance represented as *p≤0.05, **P≤0.01, ***p≤0.001, ****p≤0.0001.*

4.7. Co-blockade of the PD-1 and Lag-3 pathways have little effect on responses of Tolerant T-cells following antigenic rechallenge *in-vitro*:

We had previously shown that antibody blocking of the PD-1 pathway results in greater $CD4^+$ T-cell responses in a model of accelerated adaptive tolerance (127). Building on this, Sheriff et al. show that co-blockade of the PD-1 and Lag3 pathways induce synergistic changes in the proportion of responding T-cells and the phenotype of these T-cells (163). Thus, we decided to interrogate the effect of co-blockade on T-cell activation following the induction of tolerance. To do this, T-cells from Nur77-

Tempo were tolerised following the protocol outlined in section 3.1. The spleens from Nur77-Tempo mice were then harvested, and splenocytes were cultured in the presence of a-PD-L1 and a-Lag3 antibodies, alongside a titration of 4Y-MBP for 24 hours. Splenocytes were stained with the appropriate antibodies against TCRvβ8.1.1.2 and CD4 for exclusive analysis of $CD4^+$ T-cells. As seen previously, tolerant T-cells increased their responses moderately to increased peptide doses (Fig.10A), whilst the addition of combination treatment provided mixed effects on T-cell responses (Fig. 19A-B). Concurrent with these results, the expression of FT-Blue and FT-Red also increased in proportion to increased peptide doses (Fig.19C). It could be argued that there is a moderate trend to increased FT-Blue (Fig. 19D) and FT-Red (Fig. 19E) expression in tolerant T-cells following combination treatment. However, it is essential to note this did not reach statistical significance.

Furthermore, to investigate how the phenotype of tolerant T-cells may have changed following the rechallenge in the presence of combination treatment, antibodies against ICOS, OX40, CD25, and GITR were also utilised. Like the findings above, the expression of these markers, notably ICOS (Fig.19F), was mixed. Again, a slight positive trend for the increased expression of OX40 (Fig.19G), CD25 (Fig.19H) and GITR (Fig.19I) in response to combination treatment could be argued. However, it is essential to note that the expression of markers exhibits variance and a batch effect (Fig. 19I), which did not reach statistical significance. These results imply that co-blockade *in-vitro* does not cause any quantitative or qualitative changes in T-cell responses.

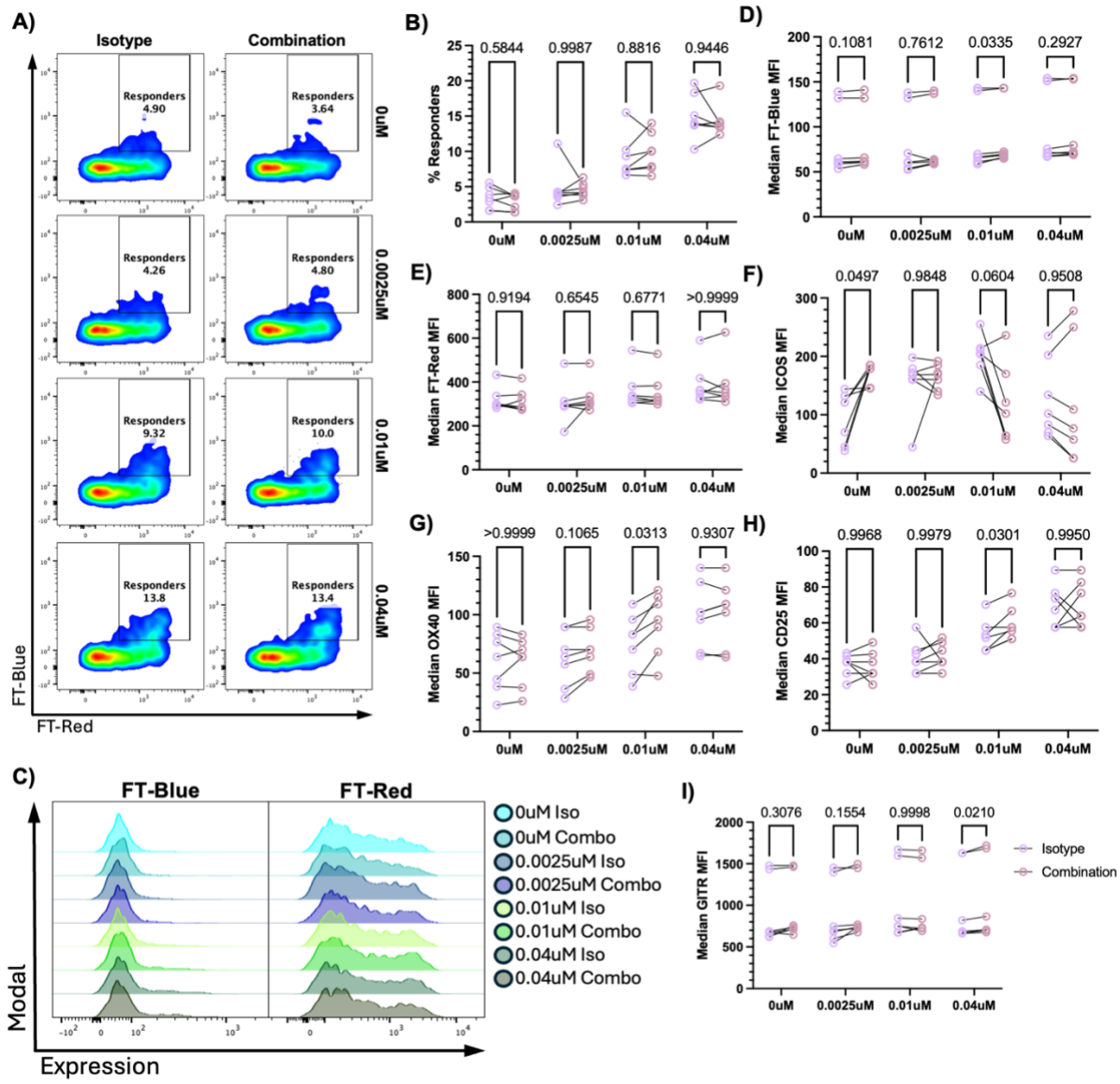

Figure 19 - *In-vitro* analysis comparing changes in tolerant T-cell responses and phenotype following antigenic rechallenge in the presence of combination treatment.

A) *Flow cytometry plots comparing the proportion of Responders following rechallenge in the presence or absence of combination treatment.* ***B)*** *Histogram overlays showing the changes in FT-Blue and FT-Red expression.* ***C)*** *Scatter graphs illustrating the changes in responders,* ***D)*** *FT-Blue,* ***E)*** *FT-Red,* ***F)*** *ICOS,* ***G)*** *OX40,* ***H)*** *CD25 and* ***I)*** *GITR expression following combination treatment. Graphs representative of 2 experiments, n=2 and n=5, overall n=7. Statistical analysis by two-way ANOVA. P values presented on the graphs with significance level represented as P≤0.05.*

4.8. Co-blockade of the PD-1 and Lag-3 pathways restore the responses of tolerant T-cells following antigenic rechallenge *in-vivo*:

Given that the co-blockade of PD-L1 and Lag3 seemed to have little effect *in-vitro*, we wanted to investigate how tolerant T-cells' responses may change in response to

combination treatment during an *in-vivo* environment. To do this, the EDI protocol was followed; however, 24 hours before the final immunisation, either isotype or combination treatment was delivered intraperitoneally to Nur77-Tempo mice. This allowed us to identify combination treatment potency effects following T-cell reactivation *in-vivo*. Tg4 Nur77-Tempo *Il10*-GFP mice were then culled 24 hours following the final immunisation, their spleens were harvested, and splenocytes were stained immediately using appropriate antibodies against TCRvβ8.1.1.2, CD4, ICOS, OX40, CD25 and GITR for *ex-vivo* analysis of $CD4^+$ T-cells exclusively. Contrary to the results from the *in-vitro* assay (section 4.7), combination treatment significantly increased the proportion of responders *in-vivo* (Fig. 20A-B). Concurrent with these results, the expression of all markers increased following rechallenge in the presence of combination treatment *in-vivo* (Fig. 20C). Increased expression of FT-Blue (Fig. 20D) and FT-Red (Fig. 20E) indicates that under normal biological conditions, co-blockade of the PD-1 and Lag-3 pathways restores T-cell signalling in tolerised T-cells. Concurrent with these results, the expression of co-stimulatory markers ICOS (Fig. 20F), OX40 (Fig. 20G) and GITR (Fig.20I) are also significantly increased in response to combination treatment. Finally, the activation marker CD25 is upregulated following combination treatment (Fig. 20G). Together, these results indicate that ICs are essential for enforcing T-cell tolerance *in-vivo* and blocking them restores the functionality of tolerised T-cells.

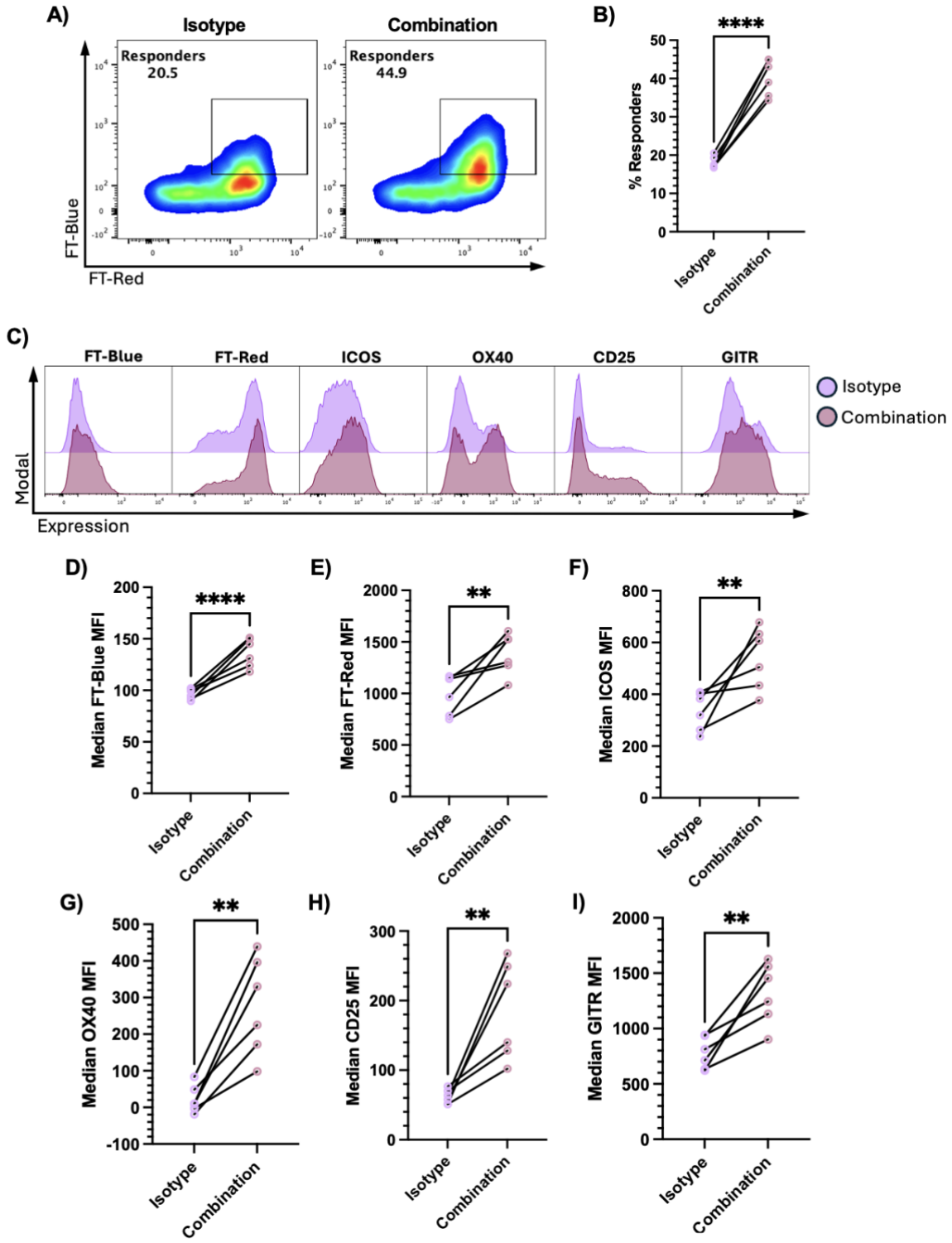

Figure 20 - *Ex-vivo* analysis comparing the effect combination treatment has on restoring functionality in tolerant T-cells.

A) Flow cytometry plots showing the proportion of responding CD4+ T-cells following isotype or combination treatment. **B)** Scatter graph comparing the change in the proportion of CD4+ responders between isotype and combination treated mice. **C)** histogram overlays comparing CD4+ T-cell expression of FT-Blue, FT-Red, ICOS, OX40, CD25 and GITR following combination treatment. **D)** Scatter graph comparing the difference in expression of FT-Blue, **E)** FT-Red, **F)** ICOS, **G)** OX40, **H)** CD25 and **I)** GITR. n=12. Statistical analysis by unpaired t-test. *Significance represented as *p≤0.05, **P≤0.01, ***p≤0.001, ****p≤0.0001.*

4.9. IL10-expressing Tr1-like cells exhibit an increased sensitivity to immune checkpoint blockade.

As previously mentioned, Tr1-like cells exhibit altered signalling dynamics, which enables them to retain their ability to signal under chronic conditions (Fig.14). However, they express heightened amounts of PD-1 (Fig. 13H) and Lag-3 (Fig.13I). To interrogate the effect that combination treatment may have on Tr1-like cell signalling, a combination of a-PD-L1 and a-Lag3 antibodies were i.p. administered to Nur77-Tempo mice 24 hours prior the final peptide immunisation. Interestingly, *ex-vivo* analysis of the IL10-expressing and non-IL10-expressing $CD4^+$ T-cells shows a higher proportion of $CD4^+IL10^+$ populations receiving combination treatment (Fig.21A, 21C). To further interrogate the discrepancy in the amount of Tr1-like $CD4^+$ T-cells responding to antigen the proportion of $Blue^+Red^+$ T-cells within the $CD4^+IL10^+$ gate was analysed (Fig. 21B). Interestingly, a higher proportion of Tr1-like $CD4^+$ T-cells were responding to antigen in the presence of combination treatment (Fig. 21D). Concomitant with these results, the expression of FT-Blue (Fig. 21E-F), FT-Red (Fig. 21E, 21G), OX40 (Fig. 21E, 21I) and CD25 (Fig. 21E, 21J), were significantly upregulated in response to combination treatment. There was also a trend to greater ICOS expression, although this did not reach statistical significance (Fig. 21E, 21H). Interestingly, the expression of both GITR (Fig. 12E, 12K) and IL10 (Fig.12E, 12L) remained the same in response to combination treatment. Together, these results indicate that ICs are essential in controlling Tr1-like cell function and phenotype *in-vivo*, but whether the PD-1 or Lag3 pathway is the most instrumental is to be determined.

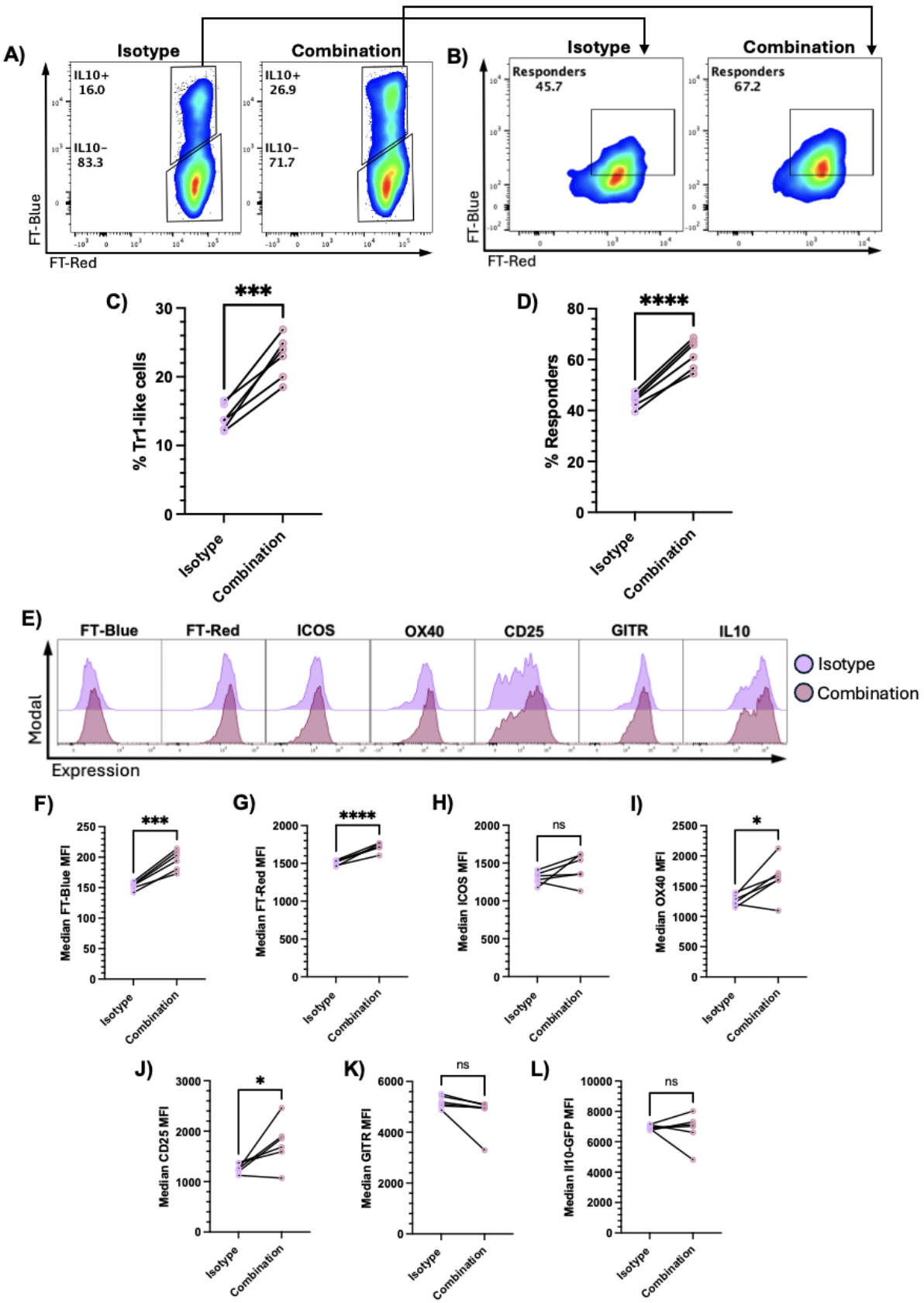
A)
Isotype
Combination
IL10+
16.0
IL10−
83.3
IL10+
26.9
IL10−
71.7
FT-Blue
FT-Red
B)
Isotype
Combination
Responders
45.7
Responders
67.2
FT-Blue
FT-Red
C)
***
% Tr1-like cells
Isotype
Combination
D)
****
% Responders
Isotype
Combination
E)
FT-Blue
FT-Red
ICOS
OX40
CD25
GITR
IL10
Isotype
Combination
Modal
Expression
F)
***
Median FT-Blue MFI
G)
****
Median FT-Red MFI
H)
ns
Median ICOS MFI
I)
*
Median OX40 MFI
J)
*
Median CD25 MFI
K)
ns
Median GITR MFI
L)
ns
Median Il10-GFP MFI

Figure 21 - *Ex-vivo* analysis comparing the sensitivity of IL10-expressing Tr1-like populations to combination treatment.

A) *Flow cytometry plots showing the proportion of IL10-expressing Tr1-like and non-Tr1-like populations following administration of either isotype or combination treatment.* ***B)*** *Flow cytometry plots showing the proportion of Blue+Red+ 'responder' populations within the IL10-expressing Tr1-like populations in response to isotype or combination treatment.* ***C)*** *Scatter graph showing the change in proportion of Tr1-like cells and* ***D)*** *responders following isotype and combination treatment.* ***E)*** *Histogram overlays showing the change in expression of markers following isotype or combination treatment.* ***F)*** *Scatter graph showing change in expression of FT-Blue,* ***G)*** *FT-Red,* ***H)*** *ICOS,* ***I)*** *OX40,* ***J)*** *CD25,* ***K)*** *GITR and* ***L)*** *IL10 expression in response to isotype or combination treatment. n=12. Statistical analysis by unpaired t-test. Significance represented as *p≤0.05, **P≤0.01, ***p≤0.001, ****p≤0.0001.*

5. Discussion -

5.1. Targeting TCR signalling is essential for the rheostat control of T-cell activation in adaptively tolerant T-cells:

The initial findings of this project illustrate that EDI treatment can successfully tolerise $CD4^+$ T-cells, majority of which reside within the timer negative quadrant (Fig.8A), which is indicative of absent immune responses. Furthermore, these chronic-stimulation induced tolerised T-cells fail to express significant amounts of FT-Blue (Fig.8B, 8D) and FT-Red (Fig.8B, 8E), indicating that they are unable to transduce signals from the TCR. These tolerant T-cells do upregulate their expression of key ICs: Pdcd1 (PD-1), Lag3, and Tigit, but to a lesser extent than acutely stimulated $CD4^+$ T-cells (Fig. 8F, 8I-K). Although this is indicative of a tolerised phenotype, acutely stimulated T-cells do not exhibit dysfunction as they can transduce signals from the TCR (Fig. 8C-E, 10C-F) and induce expression of genes associated with an alternative activation signature (TCR.Strong) (Fig. 11B-D). Instead, under acute stimulatory conditions, the expression of ICs is attributed to tight coupling with TCR signal strength and/or duration (127), which explains their dynamic nature. Thus, the expression of ICs within this context may seem redundant; however, it enables T-cells to 'fine tune' their responses to antigen, essentially acting as a switch to avert T-cell hyperactivation. This is supported by one

study whereby $CD4^+$ T-cell activation is associated with rapid upregulation of Pdcd1 (PD-1), which in turn blocked IFNγ production in a negative feedback manner (164). Concurrent with these results, previous work from our laboratory illustrates that $CD4^+$ T-cells, which respond to a primary high-dose immunogenic peptide, remain refractory upon low-dose antigenic rechallenge for at least 24 hours (127). Explaining this discrepancy is the consequent upregulation of ICs, again placing ICs at the epicentre of TCR 'fine-tuning'. Ultimately, within the context of acute T-cell stimulation, the expression of ICs is better suited as a proxy for T-cell activation, which prevents immunopathologies by recalibrating the TCR and 'fine-tuning' T-cell activation thresholds.

By analysing the transcriptional profiles of naïve and adaptively tolerised T-cells, we can delineate the sequential transcriptional modifications that underpin this distinct differentiation state. Notably, the expression of IC genes, including *Pdcd1* (PD-1), *Ctla4*, *Lag3*, and *Tigit*, is markedly elevated in the adaptively tolerised state (Fig. 9A-B). This upregulation occurs independently of robust TCR signal transduction, as evidenced by the reduced expression of FT-Blue (Fig. 8B, 8D) and FT-Red (Fig. 8B, 8E). Explaining this discrepancy is the enhanced accessibility of the *Pdcd1* promoter, facilitated by increased demethylation in peptide-induced tolerant T-cells (165). This epigenetic modification mirrors the mechanisms observed in chronically stimulated $CD8^+$ T-cells during viral persistence (166), where exhaustion is driven by enforced demethylation of the *Pdcd1* locus. Consequently, these ICs become transcriptionally poised, allowing for rapid induction and sustained expression.

Further, by analysing FT-Blue and FT-Red expression, we can infer the signalling history of T-cells. In this context, most adaptively tolerant T cells reside in the timer-negative or arrested quadrant, indicating that these cells have not recently received TCR signals, possibly reflecting defective TCR signal transduction. Studies have shown that ZAP70 activity (104,167) and its localisation to the immune synapse (168) are impaired following tolerance induction. The upregulation of ICs in adaptively tolerised T cells may contribute to this reduction in ZAP70 function. Specifically, ligation of PD-1 (169), CTLA-4 (170), and LAG3 (171) independently disrupt ZAP70 activity.

Furthermore, engagement of these checkpoints can upregulate negative regulators such as Cbl-b (95,96,172), which are found in higher densities at the TCR signalling complex in tolerant T-cells (105). Interestingly, co-blockade of PD-1 and LAG3 in NSCLC-infiltrating CD4+ T cells led to decreased Cbl-b expression. In lung adenocarcinoma models, triple immunotherapy combining PD-1 blockade, LAG3 blockade, and a Cbl-b inhibitor significantly slowed tumour growth and doubled survival (173). Together, this highlights that T-cells exhibit a proximal block in TCR signalling following the induction of tolerance. This is associated with sustained upregulation of ICs, which target the TCR signalosome directly or indirectly via *Cblb* induction.

This increased expression and activity of Cbl-b may also explain the diminished expression of the TCR (Fig. 8G) in tolerised T-cells undergoing chronic stimulation. Following activation, the TCR usually internalises; however, activation cannot explain the discrepancy in TCR expression within this model, as tolerised T-cells remain

refractory *in-vitro* even in culture conditions with activated DCs loaded with excess peptide. It is thought that TCR downregulation is yet another mechanism whereby T-cells 'tune' their response to antigen (174) and has been defined in models of increasing antigenic exposure (175). Interestingly, in this study, concomitant decreases In TCR expression by increased antigenic exposure are also associated with increased expression of Cbl-b. Further, Shamim et al. illustrate that Cbl-b is the most crucial regulator of antigen-induced TCR downregulation, as genetic ablation of *Cblb* significantly reduced TCR downregulation in response to antigenic challenge (176). Thus, mechanistically, the increased exposure to antigen and expression of Cbl-b within tolerised T-cells may account for the decrease in TCRs at the cell surface, keeping tolerant T-cells in a state of hyporesponsiveness. This could be explored in the future through potential Crispr-Cas9 mediated deletion of *Cblb.*

This highlights that many conserved and complex mechanisms are interconnected, initiated by ICs underlying the rheostat control of T-cell activation. Although expressed with a greater magnitude in acutely stimulated T-cells, they remain functionally competent and respond to antigens. Tolerised T-cells express heightened ICs at rest, and their activation further disrupts TCR signal transduction directly via the targeting of TCR signalosome components or indirectly via increasing the expression of Cbl-b. Although the expression of Cbl-b was not investigated within this project, its importance in maintaining $CD4^+$ T-cell tolerance in response to chronic antigenic stimulation is evident. It has been alluded to within studies utilising a similar protocol (105). Whether or not interference of Cbl-b functionality can restore TCR signalling in adaptively tolerised T-cells is yet to be determined. Ultimately, the

intricate balance between checkpoint regulation and TCR tuning underscores immune modulation in various signalling contexts.

5.2. Differential signalling environments impose functional and phenotypic heterogeneity within IL10-expressing Tr1-like populations:

Analysis of Tr1-like subsets reveal significant phenotypic and functional heterogeneity under different signalling conditions. Tr1-like cells from acute stimulation show stronger TCR signals, indicated by increased FT-Blue expression (Fig. 13C-D), consistent with previous findings that strong TCR signals favour the selection of CD4+ T-cells into the Tr1 lineage, alongside upregulated ICs (177). Blocking IC activity under chronic conditions enhances Tr1 selection, increasing IL-10 expressing $CD4^+$ T-cells from 16% to 26.9% (Fig. 21A, 21C). This suggests that chronic stimulation, combined with strong TCR signals, favours Tr1 lineage selection, as IC blockade enhances TCR signalling strength (127). However, more is needed to fully explain the higher IC expression on Tr1-like cells from chronic stimulation (Fig. 13H-J) despite their weaker TCR signals *in-vivo* (Fig. 13D).

Mechanistically, this may be due to increased epigenetic stability of the Tr1 phenotype during chronic stimulation, a phenomenon seen in terminally differentiated subsets exposed to chronic antigenic stimuli (154). Bevington et al. describe how Tr1-like cells undergo epigenomic rewiring upon induction, priming immune regulation genes (*Il10, Pdcd1, Lag3, Tigit)* while silencing immune response genes (*Il2*) (105). This epigenetic modulation is driven by Tr1-like transcription factors IRF4, BATF, and MAF (105) and is associated with heightened levels of ICs: *Pdcd1, Lag3, Tigit*, and *Havcr2* (178), aligning with our results (Fig. 13H-J). This

epigenetic Tr1-like signature appears conserved across different disease settings, such as cancer (179) and chronic infections (180).

This enables Tr1-like cells to establish a regulatory feedback loop, promoting the maintenance of their tolerising phenotype under chronic stimulation. This discrepancy becomes more apparent when comparing $IL10^{+}$ and $IL10^{-}$ populations. Tr1-like cells arising from chronic stimulation retain some responsiveness to antigenic rechallenge compared to their $IL10^{-}$ counterparts (Fig. 14A-C). Although more refractory than Tr1-like cells from acute stimulation (Fig. 22A-B, 22D-E appendix), they maintain heightened suppressive capacities through persistently higher IL10 expression (Fig 22C, 22F appendix). Interestingly, the co-blockade of the PD-L1 and Lag3 pathways enhances Tr1 responsiveness (Fig. 21B, 21D) by boosting TCR signalling strength (Fig. 21F), but this does not increase IL10 expression (Fig. 21L). This suggests that Tr1-derived ICs intrinsically regulate their ability to activate Il10, as blocking these pathways may disrupt this regulatory feedback loop, increasing Tr1-lineage selection but preventing Il10 upregulation.

In this project, blocking antibodies against PD-1 and Lag3 were administered simultaneously, as we had previously shown that their combination synergistically affects T-cell activation and responses (163). However, it remains to be determined which IC is most instrumental in controlling Tr1-mediated Il10 expression and whether ICB in Tr1-like cells induces transcriptional changes in immune response genes known to be sensitive to PD-1 (181).

Furthermore, the timing of Tr1 induction and the duration of exposure of T-cells to Tr1-mediated immunosuppression is known to impair T-cell responses. It could also account for the vast disparity in IL10- and IL10+ responses. Previous studies indicate that Tr1-like cells may mediate immunosuppression via PD-1 and CTLA-4 expression (178), suggesting a conserved mechanism shared with $FoxP3^+$ Tregs, where ICs mediate contact-dependent suppression (182). Moreover, Tr1-derived IL10 is a primary mechanism of Tr1-mediated immunosuppression (160), directly inhibiting $CD4^+$ T-cell function (183) and indirectly targeting APCs. Increased Tr1-derived IL-10 under chronic conditions may lead to an immature/tolerogenic DC phenotype (Fig. 12A-F), preventing effective T-cell activation by inhibiting CD86 upregulation on DCs (184). IL10 has also been implemented in maintaining the suppressive functions of Tr1-like cells as inhibition of IL10ra nullifies their activity *in-vivo* (185). Concomitant with these results, transcriptomic data also illustrates an upregulation of IL10ra in chronic-stimulation-induced Tr1-like cells (Fig. 9A-B). Thus, Tr1-derived IL10 may act like Tr1-derived IL21 (65) in maintaining Tr1 stability. However, whether this acts in an autocrine manner remains to be determined.

In line with IC upregulation on Tr1-like cells (Fig. 13H-J), we also observe significant upregulation of co-stimulatory receptors ICOS (Fig. 14A, 14D), OX40 (Fig. 14A, 14E), and GITR (Fig. 14A, 14F), consistent with findings in DC-10-induced human Tr1 cells (178). While these receptors enhance T-cell activation, their roles in Tr1-like cells appear distinct. The upregulation of OX40 in Tr1-like cells (Fig. 14E) is particularly intriguing, as OX40 ligation is thought to inhibit Tr1 cell induction and function (186) This paradoxical OX40 role may act as a checkpoint, limiting Tr1-mediated suppression when necessary and ensuring controlled immunosuppression.

ICOS and GITR, however, promote Tr1 differentiation and function, with ICOS activation inducing c-Maf (65), IL-21, and IL10 (2,187). GITRL expression on APCs enhances Tr1 lineage selection (188), increasing FoxP3^{-}IL10+ Tr1-like cells in the spleen from 1% to ~7%, with these cells expressing significant IL10 due to GITR ligation (189).

Together, these results show that Tr1-like cells display vast phenotypic and functional heterogeneity, primarily influenced by their signalling environment. Following chronic stimulation, the epigenomic rewiring allows Tr1-like cells to establish a regulatory feedback loop, enabling Tr1-like cells to maintain their suppressive function. Interestingly, ICs play important context-dependent roles in controlling Tr1-phenotype and function. Blocking PD-1 and Lag-3 increases the proportion of IL10-expressing T-cells, concomitant with increased TCR signalling. However, it is not associated with increased suppressive capacity, as seen by no significant change in IL10 expression. Therefore, ICs are likely important in mediating the expression of *Il10.* Still, which ICs are the most instrumental in this process and the mechanism they do this remains to be determined. This begs the question as to whether blockade of the PD-1 and Lag-3 pathways on Tr1-like cells can further augment their functionality. Furthermore, the vast heterogeneity between IL10^{+} and IL10^{-} populations may reflect complex immunosuppressive mechanisms, with both ICs and IL10 playing a central role in Tr1-mediated immunosuppression.

5.3. Adaptively tolerant CD4$^+$ T-cells exhibit global signalling defects in multiple signalling pathways:

As previously highlighted, signals 1 and 2 are crucial to initiating a T lymphocyte-mediated immune response. However, in the absence of signal 2, T-cells enter a state of functional anergy, and is typically induced by TolDCs, which exhibit characteristics closely resembling those of immature DCs. Supporting this, early findings from this project indicate that EDI-treated mice present with DCs displaying immature morphology (Fig. 12A-F) and a tendency to bias towards inhibitory signalling (Fig. 12F). To explore this further, we investigated whether co-culturing chronically stimulated CD4$^+$ T cells with previously LPS-activated DCs could restore their responsiveness. Whilst chronically stimulated CD4$^+$ T cells retained some responsiveness to increased peptide titres, they remained largely refractory compared to acutely stimulated counterparts (Fig. 14A-F). This suggests that an environment rich in co-stimulation is insufficient to reinstate T-cell functionality, indicating that unresponsiveness is intrinsically mediated rather than due to insufficient co-stimulation. The paradoxical roles of NFAT in T-cell activation, tolerance, and exhaustion, may account for this intrinsic control of T-cell activation in this model. The lack of cooperative binding between NFAT and other transcription factor partners, such as AP-1, underscores the vast discrepancy within the functional outcome of NFAT signalling. This prompted us to assess whether chronic stimulation reconfigures TCR signalling towards an NFAT bias. To do this, we utilised two Nr4a reporters, whereby the increased expression of Nr4a3 can be used as a proxy for increased NFAT-biased signalling, given that Nr4a3 is NFAT-dependent (162). Thus,

we hypothesised that the reported NFAT bias in tolerised T-cells would correlate with increased Nr4a3-Timer expression.

Contrary to expectations, adaptively tolerated $CD4^+$ T-cells from Nr4a3-Tocky mice exhibited responses like those in Nur77-Tempo mice, indicating no NFAT signalling bias post-tolerance induction. This is consistent with findings by Chiodetti et al., who reported defects in multiple pathways, including calcium/NFAT, NFκB, and Ras/MAPK (104). However, the most significant impairment was within the calcium/NFAT pathway. Further supporting this, Bevington et al. observed reduced expression and inducibility of both Nr4a1 and Nr4a3 following tolerance induction (105). These results suggest multiple pathways downstream of the TCR and co-stimulatory receptors are compromised following tolerance induction. While tolerance is associated with widespread inhibition across signalling pathways, NFAT remains critical for tolerance induction. However, post-induction, T-cells appear to sustain their hyporesponsive state independently of NFAT signalling, highlighting a distinct difference in the maintenance of dysfunctional states between $CD8^+$ and $CD4^+$ T-cells. Whereas NFAT signalling is essential for both the induction and maintenance of $CD8^+$ T-cell hyporesponsiveness following chronic stimulation (142), its role appears less critical for $CD4^+$ T cells.

In summary, our findings indicate that post-induction of tolerance is associated with intrinsic defects in T-cell signalling, as ensuring an environment rich in co-stimulation and antigen presentation is insufficient at rescuing T-cells from hyporesponsiveness. Interestingly, the maintenance of $CD4^+$ T-cell tolerance occurs independently of NFAT signalling, in contrast to $CD8^+$ T-cells, where NFAT remains crucial. Instead,

the global signalling defects exhibited across multiple signalling pathways indicate that T-cell tolerance is driven by broad signalling impairments rather than one single pathway. For the first time, this provides new insights into the intrinsic regulation of immune responses caused by chronic stimulation.

5.4. Conserved biological mechanisms underpin divergent CD4⁺ T-cell states:

The latter findings from this project suggest the existence of biologically conserved mechanisms across different states of T-cell dysfunction, indicating potential evolutionary conservation of these processes. Prior research from our laboratory demonstrates that combination treatment exerts synergistic effects on CD4⁺ T-cell activation in a model of accelerated adaptive tolerance (163). Specifically, these CD4⁺ T-cells displayed enhanced TCR signalling, leading to increased expression of activation markers ICOS and OX40. This is consistent with the current study, where co-blockade therapy resulted in a higher proportion of CD4⁺ T-cells responding to antigen (Fig. 20A-B), driven by the amplification of TCR signalling (Fig. 20C-E). Furthermore, co-blockade-induced qualitative shifts in T-cell phenotype (Fig. 20C), characterised by the upregulation of ICOS (Fig. 20F), OX40 (Fig. 20G), CD25 (Fig. 20H) and GITR (Fig. 20I). Notably, both ICOS and OX40 have previously been identified as surrogate markers for PD-1 responsiveness (127). These findings were only evident when the assay was conducted *in-vivo*, as combination treatment did not seem to have much effect on both the quantity of responding T-cell (Fig. 19A-B), nor the phenotype of these T-cells (Fig. 19E-I) *in-vitro*. The discrepancy between these findings within the *in-vitro* and *in-vivo* assays highlights how *in-vitro*, the complex microenvironment initiating spatial and mechanical cues present within normal physiological contexts, needs to be improved. This project relies heavily on

these microenvironmental cues, given that the induction of tolerance and exhaustion directly results from the microenvironment. Thus, the *in-vivo* assay is much more representative of normal biological conditions and contexts.

Despite potential differences in the mechanisms underlying tolerance across these models, ICs remain crucial in enforcing tolerance within $CD4^+$ T-cells, particularly under conditions of chronic stimulation during more advanced stages of dysfunction. This suggests parallels exist between exhaustion and tolerance in $CD4^+$ T-cells, where ICs are critical drivers of dysfunction. For example, previous studies have shown that combination therapies in tumour-bearing mice provide enhanced tumour protection and correlate with an upregulation of $CD4^+$ T-cell-derived IFNγ (190). The introduction of IC blockade has revolutionised the therapeutic landscape for solid tumours, with substantial evidence supporting the superiority of combination therapies in eliciting robust anti-tumour immune responses. These enhanced responses are mainly due to the ability of combination therapies to counteract compensatory mechanisms, wherein the upregulation of multiple ICs can compensate for the inhibition of a single checkpoint (191)

Beyond T-cell exhaustion, the tumour microenvironment (TME) also contributed to the induction and maintenance of T-cell tolerance as a mechanism of tumour-immune escape (reviewed in (192)). The TME is a highly immunosuppressive milieu enriched in suppressive moieties and cytokines, which, together with the frequent MHCII restriction of tumour neoantigens (114,115), supports the induction of T-cell tolerance under these conditions. This project provides novel insights suggesting that combination therapies may reverse T-cell tolerance, with possible applications in

tumour immunotherapy. However, this was not directly addressed within this project. Furthermore, in contrast to exhaustion, tolerance is increasingly associated with additional negative regulators such as Cbl-b. Whether IC blockade alone can overcome tumour-induced T-cell tolerance remains to be fully elucidated and warrants further investigation.

5.5. Discussion summary:

This thesis demonstrates that various conserved biological mechanisms are shared across different states of T-cell dysfunction, including exhaustion and tolerance. Importantly, ICs play a crucial role in regulating tolerance, often in collaboration with factors like Cbl-b. ICs likely enforce tolerance by reducing TCR signalling strength in chronically stimulated $CD4^+$ T-cells, though their role shifts in adaptive differentiation into the Tr1 lineage. In this context, ICs maintain the tolerising phenotype and functionality of Tr1-like cells. Despite ICs primarily inhibiting T-cell responses to antigens, adaptive differentiation into the Tr1 lineage creates a unique signalling environment that remains misunderstood. While ICs are involved in T-cell exhaustion, they alone cannot fully define or characterise it, as all antigen-experienced T-cells express them. Therefore, further research is needed to define $CD4^+$ T-cell exhaustion better and determine if adaptive tolerance is a form of $CD4^+$ T-cell exhaustion.

5.6. Future direction:

Future experiments to expand our knowledge of the functional adaptations of $CD4^+$ T-cells will allow us to gain a more comprehensive understanding of the effects of chronic stimulation on $CD4^+$ T-cells. Within the literature, adaptive tolerance and

clonal anergy are distinct biochemical states (104), with adaptive tolerance sharing more features with exhaustion. Given this, it would be interesting to ascertain whether a resting period whereby tolerised T-cells are isolated from splenocytes and placed in culture could reverse their hyporesponsive state. This has been shown to work with exhausted CAR T-cells (193). Further to this point, removing $IL10^{hi}$ Tr1-like populations from the culture before either a resting period or antigenic rechallenge may also restore the ability for $IL10^{-}$ populations to respond.

Further attempts would also consider targeting of multiple T-cell regulators to determine their combined importance in regulating $CD4^{+}$ T-cell hyporesponsiveness under these chronic conditions. For example, Cbl-b has been repeatedly mentioned as an important driver of T-cell tolerance and may work in concert with ICs to maintain hyporesponsiveness. Small molecular inhibitors of Cbl-b have been created (194) and are currently undergoing clinical trials for the treatment of solid tumours (ClinicalTrials.gov ID: NCT05107674). Given that within this thesis we show that ICs are important in mediating tolerance-related hyporesponsiveness, combination of ICB with Cbl-b inhibitors may provide synergistic effects to restore the functionality and signalling of these adaptively tolerised T-cells. Furthermore, subsequent experiments would also consider comparing T-cells under different chronic conditions. For example, in a tumour model, to see whether selection into the Tr1-lineage is as essential, and if so, administering ICB affects these Tr1-like populations the same as in this project. It would also be interesting to conduct transcriptional analysis of these Tr1-like populations following combination therapy, to see if the restored TCR signals induce changes in the phenotype and function of these populations.

6. References -

1. Smith-Garvin JE, Koretzky GA, Jordan MS. T Cell Activation. Annu Rev Immunol. 2009 Apr 1;27(1):591–619.
2. Hutloff A, Dittrich AM, Beier KC, Eljaschewitsch B, Kraft R, Anagnostopoulos I, et al. ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. Nature. 1999 Jan;397(6716):263–6.
3. Beier KC, Hutloff A, Dittrich AM, Heuck C, Rauch A, Büchner K, et al. Induction, binding specificity and function of human ICOS. Eur J Immunol. 2000 Dec;30(12):3707–17.
4. Croft M, So T, Duan W, Soroosh P. The significance of OX40 and OX40L to T-cell biology and immune disease. Immunol Rev. 2009 May 21;229(1):173–91.
5. Gajdasik DW, Gaspal F, Halford EE, Fiancette R, Dutton EE, Willis C, et al. Th1 responses in vivo require cell-specific provision of OX40L dictated by environmental cues. Nat Commun. 2020 Jul 9;11(1):3421.
6. Mizuno R, Sugiura D, Shimizu K, Maruhashi T, Watada M, Okazaki I mi, et al. PD-1 Primarily Targets TCR Signal in the Inhibition of Functional T Cell Activation. Front Immunol. 2019 Mar 29;10.
7. Hui E, Cheung J, Zhu J, Su X, Taylor MJ, Wallweber HA, et al. T cell costimulatory receptor CD28 is a primary target for PD-1–mediated inhibition. Science (1979). 2017 Mar 31;355(6332):1428–33.
8. Brahmer JR, Tykodi SS, Chow LQM, Hwu WJ, Topalian SL, Hwu P, et al. Safety and Activity of Anti–PD-L1 Antibody in Patients with Advanced Cancer. New England Journal of Medicine. 2012 Jun 28;366(26):2455–65.
9. Kurd N, Robey EA. T-cell selection in the thymus: a spatial and temporal perspective. Immunol Rev. 2016 May;271(1):114–26.
10. Anderson MS, Venanzi ES, Klein L, Chen Z, Berzins SP, Turley SJ, et al. Projection of an Immunological Self Shadow Within the Thymus by the Aire Protein. Science (1979). 2002 Nov 15;298(5597):1395–401.
11. Lancaster JN, Thyagarajan HM, Srinivasan J, Li Y, Hu Z, Ehrlich LIR. Live-cell imaging reveals the relative contributions of antigen-presenting cell subsets to thymic central tolerance. Nat Commun. 2019 May 17;10(1):2220.
12. Březina J, Vobořil M, Filipp D. Mechanisms of Direct and Indirect Presentation of Self-Antigens in the Thymus. Front Immunol. 2022 Jun 14;13.
13. Klein L, Robey EA, Hsieh CS. Central CD4+ T cell tolerance: deletion versus regulatory T cell differentiation. Nat Rev Immunol. 2019 Jan 12;19(1):7–18.
14. Kieback E, Hilgenberg E, Stervbo U, Lampropoulou V, Shen P, Bunse M, et al. Thymus-Derived Regulatory T Cells Are Positively Selected on Natural Self-Antigen through Cognate Interactions of High Functional Avidity. Immunity. 2016 May;44(5):1114–26.
15. Lin J, Yang L, Silva HM, Trzeciak A, Choi Y, Schwab SR, et al. Increased generation of Foxp3+ regulatory T cells by manipulating antigen presentation in the thymus. Nat Commun. 2016 Feb 29;7(1):10562.
16. Watanabe M, Lu Y, Breen M, Hodes RJ. B7-CD28 co-stimulation modulates central tolerance via thymic clonal deletion and Treg generation through distinct mechanisms. Nat Commun. 2020 Dec 8;11(1):6264.
17. Hinterberger M, Wirnsberger G, Klein L. B7/CD28 in Central Tolerance: Costimulation Promotes Maturation of Regulatory T Cell Precursors and Prevents Their Clonal Deletion. Front Immunol. 2011;2.

18. ElTanbouly MA, Noelle RJ. Rethinking peripheral T cell tolerance: checkpoints across a T cell's journey. Nat Rev Immunol. 2021 Apr 19;21(4):257–67.
19. Xing Y, Hogquist KA. T-Cell Tolerance: Central and Peripheral. Cold Spring Harb Perspect Biol. 2012 Jun 1;4(6):a006957–a006957.
20. Brown CC, Rudensky AY. Spatiotemporal regulation of peripheral T cell tolerance. Science (1979). 2023 May 5;380(6644):472–8.
21. Leblanc-Hotte A, Audiger C, Chabot-Roy G, Lombard-Vadnais F, Delisle JS, Peter YA, et al. Immature and mature bone marrow-derived dendritic cells exhibit distinct intracellular mechanical properties. Sci Rep. 2023 Feb 3;13(1):1967.
22. STEINMAN RM, HAWIGER D, LIU K, BONIFAZ L, BONNYAY D, MAHNKE K, et al. Dendritic Cell Function *in Vivo* during the Steady State: A Role in Peripheral Tolerance. Ann N Y Acad Sci. 2003 Apr 24;987(1):15–25.
23. Liu J, Zhang X, Cheng Y, Cao X. Dendritic cell migration in inflammation and immunity. Cell Mol Immunol. 2021 Nov 23;18(11):2461–71.
24. Lutz MB, Schuler G. Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity? Trends Immunol. 2002 Sep;23(9):445–9.
25. Lutz MB, Backer RA, Clausen BE. Revisiting Current Concepts on the Tolerogenicity of Steady-State Dendritic Cell Subsets and Their Maturation Stages. The Journal of Immunology. 2021 Apr 15;206(8):1681–9.
26. Steinbrink K, Wölfl M, Jonuleit H, Knop J, Enk AH. Induction of tolerance by IL-10-treated dendritic cells. J Immunol. 1997 Nov 15;159(10):4772–80.
27. Steinbrink K, Graulich E, Kubsch S, Knop J, Enk AH. CD4+ and CD8+ anergic T cells induced by interleukin-10–treated human dendritic cells display antigen-specific suppressor activity. Blood. 2002 Apr 1;99(7):2468–76.
28. Ramalingam R, Larmonier CB, Thurston RD, Midura-Kiela MT, Zheng SG, Ghishan FK, et al. Dendritic Cell-Specific Disruption of TGF-β Receptor II Leads to Altered Regulatory T Cell Phenotype and Spontaneous Multiorgan Autoimmunity. The Journal of Immunology. 2012 Oct 15;189(8):3878–93.
29. Boks MA, Kager-Groenland JR, Haasjes MSP, Zwaginga JJ, van Ham SM, ten Brinke A. IL-10-generated tolerogenic dendritic cells are optimal for functional regulatory T cell induction — A comparative study of human clinical-applicable DC. Clinical Immunology. 2012 Mar;142(3):332–42.
30. Zhuang Q, Cai H, Cao Q, Li Z, Liu S, Ming Y. Tolerogenic Dendritic Cells: The Pearl of Immunotherapy in Organ Transplantation. Front Immunol. 2020 Oct 6;11.
31. Brown JA, Dorfman DM, Ma FR, Sullivan EL, Munoz O, Wood CR, et al. Blockade of Programmed Death-1 Ligands on Dendritic Cells Enhances T Cell Activation and Cytokine Production. The Journal of Immunology. 2003 Feb 1;170(3):1257–66.
32. Ono Y, Perez-Gutierrez A, Nakao T, Dai H, Camirand G, Yoshida O, et al. Graft-infiltrating PD-L1hi cross-dressed dendritic cells regulate antidonor T cell responses in mouse liver transplant tolerance. Hepatology. 2018 Apr 18;67(4):1499–515.
33. Peng W, Ran B, Ma Y, Huang X, Chang Q, Wang X. Dendritic cells transfected with PD-L1 recombinant adenovirus induces T cell suppression and long-term acceptance of allograft transplantation. Cell Immunol. 2011;271(1):73–7.
34. Schülke S. Induction of Interleukin-10 Producing Dendritic Cells As a Tool to Suppress Allergen-Specific T Helper 2 Responses. Front Immunol. 2018 Mar 19;9.
35. Madelon N, Montanari E, Gruaz L, Pimenta J, Muller YD, Bühler LH, et al. Prolongation of rat-to-mouse islets xenograft survival by co-transplantation of autologous IL-10

differentiated murine tolerogenic dendritic cells. Xenotransplantation. 2020 Jul 26;27(4).
36. Rubtsov YP, Rasmussen JP, Chi EY, Fontenot J, Castelli L, Ye X, et al. Regulatory T Cell-Derived Interleukin-10 Limits Inflammation at Environmental Interfaces. Immunity. 2008 Apr;28(4):546–58.
37. Green EA, Gorelik L, McGregor CM, Tran EH, Flavell RA. CD4 + CD25 + T regulatory cells control anti-islet CD8 + T cells through TGF-β–TGF-β receptor interactions in type 1 diabetes. Proceedings of the National Academy of Sciences. 2003 Sep 16;100(19):10878–83.
38. Nakamura K, Kitani A, Strober W. Cell Contact–Dependent Immunosuppression by Cd4+Cd25+Regulatory T Cells Is Mediated by Cell Surface–Bound Transforming Growth Factor β. J Exp Med. 2001 Sep 3;194(5):629–44.
39. Worthington JJ, Kelly A, Smedley C, Bauché D, Campbell S, Marie JC, et al. Integrin αvβ8-Mediated TGF-β Activation by Effector Regulatory T Cells Is Essential for Suppression of T-Cell-Mediated Inflammation. Immunity. 2015 May;42(5):903–15.
40. Lainé A, Labiad O, Hernandez-Vargas H, This S, Sanlaville A, Léon S, et al. Regulatory T cells promote cancer immune-escape through integrin αvβ8-mediated TGF-β activation. Nat Commun. 2021 Oct 28;12(1):6228.
41. Read S, Malmström V, Powrie F. Cytotoxic T Lymphocyte–Associated Antigen 4 Plays an Essential Role in the Function of Cd25+Cd4+ Regulatory Cells That Control Intestinal Inflammation. J Exp Med. 2000 Jul 17;192(2):295–302.
42. Takahashi T, Tagami T, Yamazaki S, Uede T, Shimizu J, Sakaguchi N, et al. Immunologic Self-Tolerance Maintained by Cd25+Cd4+Regulatory T Cells Constitutively Expressing Cytotoxic T Lymphocyte–Associated Antigen 4. J Exp Med. 2000 Jul 17;192(2):303–10.
43. Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, et al. CTLA-4 Control over Foxp3 + Regulatory T Cell Function. Science (1979). 2008 Oct 10;322(5899):271–5.
44. Tekguc M, Wing JB, Osaki M, Long J, Sakaguchi S. Treg-expressed CTLA-4 depletes CD80/CD86 by trogocytosis, releasing free PD-L1 on antigen-presenting cells. Proceedings of the National Academy of Sciences. 2021 Jul 27;118(30).
45. Liang B, Workman C, Lee J, Chew C, Dale BM, Colonna L, et al. Regulatory T Cells Inhibit Dendritic Cells by Lymphocyte Activation Gene-3 Engagement of MHC Class II. The Journal of Immunology. 2008 May 1;180(9):5916–26.
46. De Simone M, Arrigoni A, Rossetti G, Gruarin P, Ranzani V, Politano C, et al. Transcriptional Landscape of Human Tissue Lymphocytes Unveils Uniqueness of Tumor-Infiltrating T Regulatory Cells. Immunity. 2016 Nov;45(5):1135–47.
47. Bachmann MF, Oxenius A. Interleukin 2: from immunostimulation to immunoregulation and back again. EMBO Rep. 2007 Dec;8(12):1142–8.
48. Chinen T, Kannan AK, Levine AG, Fan X, Klein U, Zheng Y, et al. An essential role for the IL-2 receptor in Treg cell function. Nat Immunol. 2016 Nov 5;17(11):1322–33.
49. Antonioli L, Pacher P, Vizi ES, Haskó G. CD39 and CD73 in immunity and inflammation. Trends Mol Med. 2013 Jun;19(6):355–67.
50. Ohta A, Kini R, Ohta A, Subramanian M, Madasu M, Sitkovsky M. The development and immunosuppressive functions of CD4+ CD25+ FoxP3+ regulatory T cells are under influence of the adenosine-A2A adenosine receptor pathway. Front Immunol. 2012;3.

51. Groux H, Bigler M, de Vries JE, Roncarolo MG. Interleukin-10 induces a long-term antigen-specific anergic state in human CD4+ T cells. J Exp Med. 1996 Jul 1;184(1):19–29.
52. Groux H, O'Garra A, Bigler M, Rouleau M, Antonenko S, de Vries JE, et al. A CD4+T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. Nature. 1997 Oct;389(6652):737–42.
53. Głobińska A, Boonpiyathad T, Satitsuksanoa P, Kleuskens M, van de Veen W, Sokolowska M, et al. Mechanisms of allergen-specific immunotherapy. Annals of Allergy, Asthma & Immunology. 2018 Sep;121(3):306–12.
54. Meiler F, Zumkehr J, Klunker S, Rückert B, Akdis CA, Akdis M. In vivo switch to IL-10–secreting T regulatory cells in high dose allergen exposure. J Exp Med. 2008 Nov 24;205(12):2887–98.
55. Bacchetta R, Bigler M, Touraine JL, Parkman R, Tovo PA, Abrams J, et al. High levels of interleukin 10 production in vivo are associated with tolerance in SCID patients transplanted with HLA mismatched hematopoietic stem cells. J Exp Med. 1994 Feb 1;179(2):493–502.
56. Zhou JY, Glendenning LM, Cavanaugh JM, McNeer SK, Goodman WA, Cobb BA. Intestinal Tr1 Cells Confer Protection against Colitis in the Absence of Foxp3+ Regulatory T Cell–Derived IL-10. Immunohorizons. 2023 Jun 1;7(6):456–66.
57. Bonnal RJP, Rossetti G, Lugli E, De Simone M, Gruarin P, Brummelman J, et al. Clonally expanded EOMES+ Tr1-like cells in primary and metastatic tumors are associated with disease progression. Nat Immunol. 2021 Jun 20;22(6):735–45.
58. Chen Z m. IL-10 and TGF- induce alloreactive CD4+CD25- T cells to acquire regulatory cell function. Blood. 2003 Feb 27;101(>12):5076–83.
59. Pot C, Apetoh L, Awasthi A, Kuchroo VK. Induction of regulatory Tr1 cells and inhibition of TH17 cells by IL-27. Semin Immunol. 2011 Dec;23(6):438–45.
60. Vasanthakumar A, Kallies A. IL-27 paves different roads to Tr1. Eur J Immunol. 2013 Apr 16;43(4):882–5.
61. Maynard CL, Harrington LE, Janowski KM, Oliver JR, Zindl CL, Rudensky AY, et al. Regulatory T cells expressing interleukin 10 develop from Foxp3+ and Foxp3− precursor cells in the absence of interleukin 10. Nat Immunol. 2007 Sep 12;8(9):931–41.
62. Gregori S, Tomasoni D, Pacciani V, Scirpoli M, Battaglia M, Magnani CF, et al. Differentiation of type 1 T regulatory cells (Tr1) by tolerogenic DC-10 requires the IL-10–dependent ILT4/HLA-G pathway. Blood. 2010 Aug 12;116(6):935–44.
63. Amodio G, Gregori S. Human tolerogenic DC-10: perspectives for clinical applications. Transplant Res. 2012 Dec 28;1(1):14.
64. Awasthi A, Carrier Y, Peron JPS, Bettelli E, Kamanaka M, Flavell RA, et al. A dominant function for interleukin 27 in generating interleukin 10-producing anti-inflammatory T cells. Nat Immunol. 2007 Dec;8(12):1380–9.
65. Pot C, Jin H, Awasthi A, Liu SM, Lai CY, Madan R, et al. Cutting Edge: IL-27 Induces the Transcription Factor c-Maf, Cytokine IL-21, and the Costimulatory Receptor ICOS that Coordinately Act Together to Promote Differentiation of IL-10-Producing Tr1 Cells. The Journal of Immunology. 2009 Jul 15;183(2):797–801.
66. Gagliani N, Magnani CF, Huber S, Gianolini ME, Pala M, Licona-Limon P, et al. Coexpression of CD49b and LAG-3 identifies human and mouse T regulatory type 1 cells. Nat Med. 2013 Jun 28;19(6):739–46.

67. Chien CH, Yu HC, Chen SY, Chiang BL. Characterization of c-Maf+Foxp3− Regulatory T Cells Induced by Repeated Stimulation of Antigen-Presenting B Cells. Sci Rep. 2017 Apr 12;7(1):46348.
68. Roncarolo MG, Gregori S, Bacchetta R, Battaglia M. Tr1 Cells and the Counter-Regulation of Immunity: Natural Mechanisms and Therapeutic Applications. In 2014. p. 39–68.
69. Anderson AC, Joller N, Kuchroo VK. Lag-3, Tim-3, and TIGIT: Co-inhibitory Receptors with Specialized Functions in Immune Regulation. Immunity. 2016 May;44(5):989–1004.
70. Facciotti F, Gagliani N, Häringer B, Alfen JS, Penatti A, Maglie S, et al. IL-10–producing forkhead box protein 3–negative regulatory T cells inhibit B-cell responses and are involved in systemic lupus erythematosus. Journal of Allergy and Clinical Immunology. 2016 Jan;137(1):318-321.e5.
71. Iwasaki Y, Fujio K, Okamura T, Yanai A, Sumitomo S, Shoda H, et al. Egr-2 transcription factor is required for Blimp-1-mediated IL-10 production in IL-27-stimulated CD4 + T cells. Eur J Immunol. 2013 Apr 26;43(4):1063–73.
72. Neumann C, Heinrich F, Neumann K, Junghans V, Mashreghi MF, Ahlers J, et al. Role of Blimp-1 in programing Th effector cells into IL-10 producers. Journal of Experimental Medicine. 2014 Aug 25;211(9):1807–19.
73. Okamura T, Fujio K, Shibuya M, Sumitomo S, Shoda H, Sakaguchi S, et al. CD4 + CD25 − LAG3 + regulatory T cells controlled by the transcription factor Egr-2. Proceedings of the National Academy of Sciences. 2009 Aug 18;106(33):13974–9.
74. Karwacz K, Miraldi ER, Pokrovskii M, Madi A, Yosef N, Wortman I, et al. Critical role of IRF1 and BATF in forming chromatin landscape during type 1 regulatory cell differentiation. Nat Immunol. 2017 Apr 6;18(4):412–21.
75. Huang W, Solouki S, Koylass N, Zheng SG, August A. ITK signalling via the Ras/IRF4 pathway regulates the development and function of Tr1 cells. Nat Commun. 2017 Jun 21;8(1):15871.
76. Zhang P, Lee JS, Gartlan KH, Schuster IS, Comerford I, Varelias A, et al. Eomesodermin promotes the development of type 1 regulatory T (T R 1) cells. Sci Immunol. 2017 Apr 21;2(10).
77. Schwartz RH. T Cell Anergy. Annu Rev Immunol. 2003 Apr;21(1):305–34.
78. Schwartz RH. A Cell Culture Model for T Lymphocyte Clonal Anergy. Science (1979). 1990 Jun 15;248(4961):1349–56.
79. Burkhart C, Liu GY, Anderton SM, Metzler B, Wraith DC. Peptide-induced T cell regulation of experimental autoimmune encephalomyelitis: a role for IL-10. Int Immunol. 1999 Oct;11(10):1625–34.
80. Burton BR, Britton GJ, Fang H, Verhagen J, Smithers B, Sabatos-Peyton CA, et al. Sequential transcriptional changes dictate safe and effective antigen-specific immunotherapy. Nat Commun. 2014 Sep 3;5(1):4741.
81. Kenison JE, Stevens NA, Quintana FJ. Therapeutic induction of antigen-specific immune tolerance. Nat Rev Immunol. 2024 May 12;24(5):338–57.
82. Fathman CG, Lineberry NB. Molecular mechanisms of CD4+ T-cell anergy. Nat Rev Immunol. 2007 Aug 6;7(8):599–609.
83. Greenwald RJ, Boussiotis VA, Lorsbach RB, Abbas AK, Sharpe AH. CTLA-4 Regulates Induction of Anergy In Vivo. Immunity. 2001 Feb;14(2):145–55.

84. Macián F, García-Cózar F, Im SH, Horton HF, Byrne MC, Rao A. Transcriptional mechanisms underlying lymphocyte tolerance. Cell. 2002 Jun 14;109(6):719–31.
85. Chai J. Immobilized anti-CD3 mAb induces anergy in murine naive and memory CD4+ T cells in vitro. Int Immunol. 1997 Jul 1;9(7):935–44.
86. Li W, Whaley CD, Mondino A, Mueller DL. Blocked Signal Transduction to the ERK and JNK Protein Kinases in Anergic CD4 + T Cells. Science (1979). 1996 Mar;271(5253):1272–6.
87. Zha Y, Marks R, Ho AW, Peterson AC, Janardhan S, Brown I, et al. T cell anergy is reversed by active Ras and is regulated by diacylglycerol kinase-α. Nat Immunol. 2006 Nov 1;7(11):1166–73.
88. Kang SM, Beverly B, Tran AC, Brorson K, Schwartz RH, Lenardot MJ. Transactivation by AP-1 Is a Molecular Target of T Cell Clonal Anergy. Science (1979). 1992 Aug 21;257(5073):1134–8.
89. Yukawa M, Jagannathan S, Vallabh S, Kartashov A V., Chen X, Weirauch MT, et al. AP-1 activity induced by co-stimulation is required for chromatin opening during T cell activation. Journal of Experimental Medicine. 2020 Jan 6;217(1).
90. Thomas RM, Saouaf SJ, Wells AD. Superantigen-induced CD4+ T cell tolerance is associated with DNA methylation and histone hypo-acetylation at cytokine gene loci. Genes Immun. 2007 Oct 2;8(7):613–8.
91. Soto-Nieves N, Puga I, Abe BT, Bandyopadhyay S, Baine I, Rao A, et al. Transcriptional complexes formed by NFAT dimers regulate the induction of T cell tolerance. Journal of Experimental Medicine. 2009 Apr 13;206(4):867–76.
92. Schartner JM, Simonson WT, Wernimont SA, Nettenstrom LM, Huttenlocher A, Seroogy CM. Gene Related to Anergy in Lymphocytes (GRAIL) Expression in CD4+ T Cells Impairs Actin Cytoskeletal Organization during T Cell/Antigen-presenting Cell Interactions. Journal of Biological Chemistry. 2009 Dec;284(50):34674–81.
93. Safford M, Collins S, Lutz MA, Allen A, Huang CT, Kowalski J, et al. Egr-2 and Egr-3 are negative regulators of T cell activation. Nat Immunol. 2005 May 1;6(5):472–80.
94. Harris JE, Bishop KD, Phillips NE, Mordes JP, Greiner DL, Rossini AA, et al. Early Growth Response Gene-2, a Zinc-Finger Transcription Factor, Is Required for Full Induction of Clonal Anergy in CD4+ T Cells. The Journal of Immunology. 2004 Dec 15;173(12):7331–8.
95. Li D, Gál I, Vermes C, Alegre ML, Chong ASF, Chen L, et al. Cutting Edge: Cbl-b: One of the Key Molecules Tuning CD28- and CTLA-4-Mediated T Cell Costimulation. The Journal of Immunology. 2004 Dec 15;173(12):7135–9.
96. Fujiwara M, Anstadt EJ, Clark RB. Cbl-b Deficiency Mediates Resistance to Programed Death-Ligand 1/Programed Death-1 Regulation. Front Immunol. 2017 Jan 26;8.
97. Tang R, Langdon WY, Zhang J. Regulation of immune responses by E3 ubiquitin ligase Cbl-b. Cell Immunol. 2019 Jun;340:103878.
98. Nguyen TTT, Wang ZE, Shen L, Schroeder A, Eckalbar W, Weiss A. Cbl-b deficiency prevents functional but not phenotypic T cell anergy. Journal of Experimental Medicine. 2021 Jul 5;218(7).
99. Zheng Y, Zha Y, Driessens G, Locke F, Gajewski TF. Transcriptional regulator early growth response gene 2 (Egr2) is required for T cell anergy in vitro and in vivo. Journal of Experimental Medicine. 2012 Nov 19;209(12):2157–63.

100. Heissmeyer V, Macián F, Im SH, Varma R, Feske S, Venuprasad K, et al. Calcineurin imposes T cell unresponsiveness through targeted proteolysis of signaling proteins. Nat Immunol. 2004 Mar 15;5(3):255–65.
101. Olenchock BA, Guo R, Carpenter JH, Jordan M, Topham MK, Koretzky GA, et al. Disruption of diacylglycerol metabolism impairs the induction of T cell anergy. Nat Immunol. 2006 Nov 8;7(11):1174–81.
102. Takao S, Akiyama R, Sakane F. Combined inhibition/silencing of diacylglycerol kinase α and ζ simultaneously and synergistically enhances interleukin-2 production in T cells and induces cell death of melanoma cells. J Cell Biochem. 2021 May 5;122(5):494–506.
103. Migita K, Eguchi K, Kawabe Y, Tsukada T, Ichinose Y, Nagataki S, et al. Defective TCR-mediated signaling in anergic T cells. J Immunol. 1995 Dec 1;155(11):5083–7.
104. Chiodetti L, Choi S, Barber DL, Schwartz RH. Adaptive Tolerance and Clonal Anergy Are Distinct Biochemical States. The Journal of Immunology. 2006 Feb 15;176(4):2279–91.
105. Bevington SL, Ng STH, Britton GJ, Keane P, Wraith DC, Cockerill PN. Chromatin Priming Renders T Cell Tolerance-Associated Genes Sensitive to Activation below the Signaling Threshold for Immune Response Genes. Cell Rep. 2020 Jun;31(10):107748.
106. Courtney AH, Lo WL, Weiss A. TCR Signaling: Mechanisms of Initiation and Propagation. Trends Biochem Sci. 2018 Feb;43(2):108–23.
107. Hundt M, Tabata H, Jeon MS, Hayashi K, Tanaka Y, Krishna R, et al. Impaired Activation and Localization of LAT in Anergic T Cells as a Consequence of a Selective Palmitoylation Defect. Immunity. 2006 May;24(5):513–22.
108. Moskophidis D, Lechner F, Pircher H, Zinkernagel RM. Virus persistence in acutely infected immunocompetent mice by exhaustion of antiviral cytotoxic effector T cells. Nature. 1993 Apr 22;362(6422):758–61.
109. Jiang Y, Li Y, Zhu B. T-cell exhaustion in the tumor microenvironment. Cell Death Dis. 2015 Jun 18;6(6):e1792–e1792.
110. Hor JL, Whitney PG, Zaid A, Brooks AG, Heath WR, Mueller SN. Spatiotemporally Distinct Interactions with Dendritic Cell Subsets Facilitates CD4+ and CD8+ T Cell Activation to Localized Viral Infection. Immunity. 2015 Sep;43(3):554–65.
111. Reed CM, Cresce ND, Mauldin IS, Slingluff CL, Olson WC. Vaccination with Melanoma Helper Peptides Induces Antibody Responses Associated with Improved Overall Survival. Clinical Cancer Research. 2015 Sep 1;21(17):3879–87.
112. Zander R, Schauder D, Xin G, Nguyen C, Wu X, Zajac A, et al. CD4+ T Cell Help Is Required for the Formation of a Cytolytic CD8+ T Cell Subset that Protects against Chronic Infection and Cancer. Immunity. 2019 Dec;51(6):1028-1042.e4.
113. Pipkin ME, Sacks JA, Cruz-Guilloty F, Lichtenheld MG, Bevan MJ, Rao A. Interleukin-2 and Inflammation Induce Distinct Transcriptional Programs that Promote the Differentiation of Effector Cytolytic T Cells. Immunity. 2010 Jan;32(1):79–90.
114. Kreiter S, Vormehr M, van de Roemer N, Diken M, Löwer M, Diekmann J, et al. Mutant MHC class II epitopes drive therapeutic immune responses to cancer. Nature. 2015 Apr 30;520(7549):692–6.
115. Linnemann C, van Buuren MM, Bies L, Verdegaal EME, Schotte R, Calis JJA, et al. High-throughput epitope discovery reveals frequent recognition of neo-antigens by CD4+ T cells in human melanoma. Nat Med. 2015 Jan;21(1):81–5.
116. Quezada SA, Simpson TR, Peggs KS, Merghoub T, Vider J, Fan X, et al. Tumor-reactive CD4+ T cells develop cytotoxic activity and eradicate large established melanoma after

transfer into lymphopenic hosts. Journal of Experimental Medicine. 2010 Mar 15;207(3):637–50.
117. Okazaki T, Maeda A, Nishimura H, Kurosaki T, Honjo T. PD-1 immunoreceptor inhibits B cell receptor-mediated signaling by recruiting src homology 2-domain-containing tyrosine phosphatase 2 to phosphotyrosine. Proceedings of the National Academy of Sciences. 2001 Nov 20;98(24):13866–71.
118. Miyatake S, Nakaseko C, Umemori H, Yamamoto T, Saito T. Src Family Tyrosine Kinases Associate with and Phosphorylate CTLA-4 (CD152). Biochem Biophys Res Commun. 1998 Aug;249(2):444–8.
119. Linsley PS, Brady W, Urnes M, Grosmaire LS, Damle NK, Ledbetter JA. CTLA-4 is a second receptor for the B cell activation antigen B7. J Exp Med. 1991 Sep 1;174(3):561–9.
120. Triebel F, Jitsukawa S, Baixeras E, Roman-Roman S, Genevee C, Viegas-Pequignot E, et al. LAG-3, a novel lymphocyte activation gene closely related to CD4. J Exp Med. 1990 May 1;171(5):1393–405.
121. Blaeschke F, Willier S, Stenger D, Lepenies M, Horstmann MA, Escherich G, et al. Leukemia-induced dysfunctional TIM-3+CD4+ bone marrow T cells increase risk of relapse in pediatric B-precursor ALL patients. Leukemia. 2020 Oct 13;34(10):2607–20.
122. Tan J, Yu Z, Huang J, Chen Y, Huang S, Yao D, et al. Increased PD-1+Tim-3+ exhausted T cells in bone marrow may influence the clinical outcome of patients with AML. Biomark Res. 2020 Dec 13;8(1):6.
123. Utzschneider DT, Legat A, Fuertes Marraco SA, Carrié L, Luescher I, Speiser DE, et al. T cells maintain an exhausted phenotype after antigen withdrawal and population reexpansion. Nat Immunol. 2013 Jun 5;14(6):603–10.
124. Odorizzi PM, Pauken KE, Paley MA, Sharpe A, Wherry EJ. Genetic absence of PD-1 promotes accumulation of terminally differentiated exhausted CD8+ T cells. Journal of Experimental Medicine. 2015 Jun 29;212(7):1125–37.
125. Reiley WW, Shafiani S, Wittmer ST, Tucker-Heard G, Moon JJ, Jenkins MK, et al. Distinct functions of antigen-specific CD4 T cells during murine *Mycobacterium tuberculosis* infection. Proceedings of the National Academy of Sciences. 2010 Nov 9;107(45):19408–13.
126. Goding SR, Wilson KA, Xie Y, Harris KM, Baxi A, Akpinarli A, et al. Restoring Immune Function of Tumor-Specific CD4+ T Cells during Recurrence of Melanoma. The Journal of Immunology. 2013 May 1;190(9):4899–909.
127. Elliot TAE, Jennings EK, Lecky DAJ, Thawait N, Flores-Langarica A, Copland A, et al. Antigen and checkpoint receptor engagement recalibrates T cell receptor signal strength. Immunity. 2021 Nov;54(11):2481-2496.e6.
128. Prado-Garcia H, Romero-Garcia S, Puerto-Aquino A, Rumbo-Nava U. The PD-L1/PD-1 pathway promotes dysfunction, but not “exhaustion”, in tumor-responding T cells from pleural effusions in lung cancer patients. Cancer Immunol Immunother. 2017 Jun;66(6):765–76.
129. Rad Pour S, Morikawa H, Kiani NA, Yang M, Azimi A, Shafi G, et al. Exhaustion of CD4+ T-cells mediated by the Kynurenine Pathway in Melanoma. Sci Rep. 2019 Aug 21;9(1):12150.
130. Tan J, Chen S, Huang J, Chen Y, Yang L, Wang C, et al. Increased exhausted CD8+ T cells with programmed death-1, T-cell immunoglobulin and mucin-domain-containing-3

phenotype in patients with multiple myeloma. Asia Pac J Clin Oncol. 2018 Oct;14(5):e266–74.
131. Malandro N, Budhu S, Kuhn NF, Liu C, Murphy JT, Cortez C, et al. Clonal Abundance of Tumor-Specific CD4(+) T Cells Potentiates Efficacy and Alters Susceptibility to Exhaustion. Immunity. 2016 Jan 19;44(1):179–93.
132. Wu L, Mao L, Liu JF, Chen L, Yu GT, Yang LL, et al. Blockade of TIGIT/CD155 Signaling Reverses T-cell Exhaustion and Enhances Antitumor Capability in Head and Neck Squamous Cell Carcinoma. Cancer Immunol Res. 2019 Oct;7(10):1700–13.
133. Sasidharan Nair V, Toor SM, Taha RZ, Ahmed AA, Kurer MA, Murshed K, et al. Transcriptomic Profiling of Tumor-Infiltrating CD4+TIM-3+ T Cells Reveals Their Suppressive, Exhausted, and Metastatic Characteristics in Colorectal Cancer Patients. Vaccines (Basel). 2020 Feb 6;8(1).
134. Li H, Wu K, Tao K, Chen L, Zheng Q, Lu X, et al. Tim-3/galectin-9 signaling pathway mediates T-cell dysfunction and predicts poor prognosis in patients with hepatitis B virus-associated hepatocellular carcinoma. Hepatology. 2012 Oct;56(4):1342–51.
135. Liu X, Wang Y, Lu H, Li J, Yan X, Xiao M, et al. Genome-wide analysis identifies NR4A1 as a key mediator of T cell dysfunction. Nature. 2019 Mar 27;567(7749):525–9.
136. Chen J, López-Moyado IF, Seo H, Lio CWJ, Hempleman LJ, Sekiya T, et al. NR4A transcription factors limit CAR T cell function in solid tumours. Nature. 2019 Mar 27;567(7749):530–4.
137. Wherry EJ, Ha SJ, Kaech SM, Haining WN, Sarkar S, Kalia V, et al. Molecular Signature of CD8+ T Cell Exhaustion during Chronic Viral Infection. Immunity. 2007 Oct;27(4):670–84.
138. Scott AC, Dündar F, Zumbo P, Chandran SS, Klebanoff CA, Shakiba M, et al. TOX is a critical regulator of tumour-specific T cell differentiation. Nature. 2019 Jul 17;571(7764):270–4.
139. Khan O, Giles JR, McDonald S, Manne S, Ngiow SF, Patel KP, et al. TOX transcriptionally and epigenetically programs CD8+ T cell exhaustion. Nature. 2019 Jul 17;571(7764):211–8.
140. Martinez GJ, Pereira RM, Äijö T, Kim EY, Marangoni F, Pipkin ME, et al. The Transcription Factor NFAT Promotes Exhaustion of Activated CD8 + T Cells. Immunity. 2015 Feb;42(2):265–78.
141. Agnellini P, Wolint P, Rehr M, Cahenzli J, Karrer U, Oxenius A. Impaired NFAT nuclear translocation results in split exhaustion of virus-specific CD8 + T cell functions during chronic viral infection. Proceedings of the National Academy of Sciences. 2007 Mar 13;104(11):4565–70.
142. Man K, Gabriel SS, Liao Y, Gloury R, Preston S, Henstridge DC, et al. Transcription Factor IRF4 Promotes CD8+ T Cell Exhaustion and Limits the Development of Memory-like T Cells during Chronic Infection. Immunity. 2017 Dec;47(6):1129-1141.e5.
143. Seo H, González-Avalos E, Zhang W, Ramchandani P, Yang C, Lio CWJ, et al. BATF and IRF4 cooperate to counter exhaustion in tumor-infiltrating CAR T cells. Nat Immunol. 2021 Aug 19;22(8):983–95.
144. Utzschneider DT, Charmoy M, Chennupati V, Pousse L, Ferreira DP, Calderon-Copete S, et al. T Cell Factor 1-Expressing Memory-like CD8+ T Cells Sustain the Immune Response to Chronic Viral Infections. Immunity. 2016 Aug;45(2):415–27.

145. Brummelman J, Mazza EMC, Alvisi G, Colombo FS, Grilli A, Mikulak J, et al. High-dimensional single cell analysis identifies stem-like cytotoxic CD8+ T cells infiltrating human tumors. Journal of Experimental Medicine. 2018 Oct 1;215(10):2520–35.
146. Lee J, Lee K, Bae H, Lee K, Lee S, Ma J, et al. IL-15 promotes self-renewal of progenitor exhausted CD8 T cells during persistent antigenic stimulation. Front Immunol. 2023 Jun 20;14.
147. Connolly KA, Kuchroo M, Venkat A, Khatun A, Wang J, William I, et al. A reservoir of stem-like CD8+ T cells in the tumor-draining lymph node preserves the ongoing antitumor immune response. Sci Immunol. 2021 Oct;6(64):eabg7836.
148. Kennedy BC, Dean I, Withers DR. Migration of stem-like CD8 T cells between tissue microenvironments underpins successful anti-tumour immune responses. Discovery Immunology. 2023 Jan 1;2(1).
149. Miller BC, Sen DR, Al Abosy R, Bi K, Virkud Y V., LaFleur MW, et al. Subsets of exhausted CD8+ T cells differentially mediate tumor control and respond to checkpoint blockade. Nat Immunol. 2019 Mar 18;20(3):326–36.
150. Fu J, Yu A, Xiao X, Tang J, Zu X, Chen W, et al. CD4+ T cell exhaustion leads to adoptive transfer therapy failure which can be prevented by immune checkpoint blockade. Am J Cancer Res. 2020;10(12):4234–50.
151. Balança CC, Salvioni A, Scarlata CM, Michelas M, Martinez-Gomez C, Gomez-Roca C, et al. PD-1 blockade restores helper activity of tumor-infiltrating, exhausted PD-1hiCD39+ CD4 T cells. JCI Insight. 2021 Jan 25;6(2).
152. Im SJ, Hashimoto M, Gerner MY, Lee J, Kissick HT, Burger MC, et al. Defining CD8+ T cells that provide the proliferative burst after PD-1 therapy. Nature. 2016 Sep 15;537(7620):417–21.
153. Alfei F, Kanev K, Hofmann M, Wu M, Ghoneim HE, Roelli P, et al. TOX reinforces the phenotype and longevity of exhausted T cells in chronic viral infection. Nature. 2019 Jul 17;571(7764):265–9.
154. Philip M, Fairchild L, Sun L, Horste EL, Camara S, Shakiba M, et al. Chromatin states define tumour-specific T cell dysfunction and reprogramming. Nature. 2017 May 17;545(7655):452–6.
155. Hwang S, Cobb DA, Bhadra R, Youngblood B, Khan IA. Blimp-1–mediated CD4 T cell exhaustion causes CD8 T cell dysfunction during chronic toxoplasmosis. Journal of Experimental Medicine. 2016 Aug 22;213(9):1799–818.
156. Chihara N, Madi A, Kondo T, Zhang H, Acharya N, Singer M, et al. Induction and transcriptional regulation of the co-inhibitory gene module in T cells. Nature. 2018 Jun 13;558(7710):454–9.
157. Elliot TAE, Jennings EK, Lecky DAJ, Rouvray S, Mackie GM, Scarfe L, et al. Nur77-Tempo mice reveal T cell steady state antigen recognition. Discovery Immunology. 2022 Jan 1;1(1).
158. Zheng J, Liang H, Xu C, Xu Q, Zhang T, Shen T, et al. An unbalanced PD-L1/CD86 ratio in CD14++CD16+ monocytes is correlated with HCV viremia during chronic HCV infection. Cell Mol Immunol. 2014 May 17;11(3):294–304.
159. Shen T, Chen X, Chen Y, Xu Q, Lu F, Liu S. Increased PD-L1 expression and PD-L1/CD86 ratio on dendritic cells were associated with impaired dendritic cells function in HCV infection. J Med Virol. 2010 Jul 25;82(7):1152–9.

160. Roncarolo MG, Gregori S, Bacchetta R, Battaglia M, Gagliani N. The Biology of T Regulatory Type 1 Cells and Their Therapeutic Application in Immune-Mediated Diseases. Immunity. 2018 Dec 18;49(6):1004–19.
161. Bending D, Martín PP, Paduraru A, Ducker C, Marzaganov E, Laviron M, et al. A timer for analyzing temporally dynamic changes in transcription during differentiation in vivo. Journal of Cell Biology. 2018 Aug 6;217(8):2931–50.
162. Jennings E, Elliot TAE, Thawait N, Kanabar S, Yam-Puc JC, Ono M, et al. Nr4a1 and Nr4a3 Reporter Mice Are Differentially Sensitive to T Cell Receptor Signal Strength and Duration. Cell Rep. 2020 Nov;33(5):108328.
163. Lag3 and PD-L1 govern T cell receptor signal duration in adaptively tolerised CD4+ T cells.
164. Honda T, Egen JG, Lämmermann T, Kastenmüller W, Torabi-Parizi P, Germain RN. Tuning of Antigen Sensitivity by T Cell Receptor-Dependent Negative Feedback Controls T Cell Effector Function in Inflamed Tissues. Immunity. 2014 Feb;40(2):235–47.
165. McPherson RC, Konkel JE, Prendergast CT, Thomson JP, Ottaviano R, Leech MD, et al. Epigenetic modification of the PD-1 (Pdcd1) promoter in effector CD4+ T cells tolerized by peptide immunotherapy. Elife. 2014 Dec 29;3.
166. Youngblood B, Oestreich KJ, Ha SJ, Duraiswamy J, Akondy RS, West EE, et al. Chronic Virus Infection Enforces Demethylation of the Locus that Encodes PD-1 in Antigen-Specific CD8+ T Cells. Immunity. 2011 Sep;35(3):400–12.
167. Cornwell WD, Rogers TJ. Uncoupling of T Cell Receptor Zeta Chain Function during the Induction of Anergy by the Superantigen, Staphylococcal Enterotoxin A. Toxins (Basel). 2010 Jun 30;2(7):1704–17.
168. Choi S, Schwartz RH. Impairment of Immunological Synapse Formation in Adaptively Tolerant T Cells. The Journal of Immunology. 2011 Jul 15;187(2):805–16.
169. Sheppard KA, Fitz LJ, Lee JM, Benander C, George JA, Wooters J, et al. PD-1 inhibits T-cell receptor induced phosphorylation of the ZAP70/CD3ζ signalosome and downstream signaling to PKCθ. FEBS Lett. 2004 Sep 10;574(1–3):37–41.
170. Schneider H, Smith X, Liu H, Bismuth G, Rudd CE. CTLA-4 disrupts ZAP70 microcluster formation with reduced T cell/APC dwell times and calcium mobilization. Eur J Immunol. 2008 Jan 21;38(1):40–7.
171. Guy C, Mitrea DM, Chou PC, Temirov J, Vignali KM, Liu X, et al. LAG3 associates with TCR–CD3 complexes and suppresses signaling by driving co-receptor–Lck dissociation. Nat Immunol. 2022 May 18;23(5):757–67.
172. Escors D, Bocanegra A, Chocarro L, Blanco E, Piñeiro-Hermida S, Garnica M, et al. Systemic CD4 Immunity and PD-L1/PD-1 Blockade Immunotherapy. Int J Mol Sci. 2022 Oct 31;23(21):13241.
173. Chocarro L, Blanco E, Fernandez-Rubio L, Garnica M, Zuazo M, Garcia MJ, et al. PD-1/LAG-3 co-signaling profiling uncovers CBL ubiquitin ligases as key immunotherapy targets. EMBO Mol Med. 2024 Jul 19;
174. Gallegos AM, Xiong H, Leiner IM, Sušac B, Glickman MS, Pamer EG, et al. Control of T cell antigen reactivity via programmed TCR downregulation. Nat Immunol. 2016 Apr 22;17(4):379–86.
175. Trefzer A, Kadam P, Wang SH, Pennavaria S, Lober B, Akçabozan B, et al. Dynamic adoption of anergy by antigen-exhausted CD4+ T cells. Cell Rep. 2021 Feb;34(6):108748.

176. Shamim M, Nanjappa SG, Singh A, Plisch EH, LeBlanc SE, Walent J, et al. Cbl-b Regulates Antigen-Induced TCR Down-Regulation and IFN-γ Production by Effector CD8 T Cells without Affecting Functional Avidity. The Journal of Immunology. 2007 Dec 1;179(11):7233–43.
177. 'Lecky DAJ', "Sheriff, LRST", "George, LS", "Drummond, RA", "Wraith, DC", 'Bending, D. Interferon-γ and IL-27 positively regulate type 1 regulatory T-cell development during adaptive tolerance. 2024;
178. Chen PP, Cepika AM, Agarwal-Hashmi R, Saini G, Uyeda MJ, Louis DM, et al. Alloantigen-specific type 1 regulatory T cells suppress through CTLA-4 and PD-1 pathways and persist long-term in patients. Sci Transl Med. 2021 Oct 27;13(617).
179. Cepika AM, Amaya L, Waichler C, Narula M, Mantilla MM, Thomas BC, et al. Epigenetic signature and key transcriptional regulators of human antigen-specific type 1 regulatory T cells. bioRxiv. 2024 Mar 12;
180. Gabryšová L, Alvarez-Martinez M, Luisier R, Cox LS, Sodenkamp J, Hosking C, et al. c-Maf controls immune responses by regulating disease-specific gene networks and repressing IL-2 in CD4+ T cells. Nat Immunol. 2018 May 16;19(5):497–507.
181. Shimizu K, Sugiura D, Okazaki I mi, Maruhashi T, Takegami Y, Cheng C, et al. PD-1 Imposes Qualitative Control of Cellular Transcriptomes in Response to T Cell Activation. Mol Cell. 2020 Mar;77(5):937-950.e6.
182. Kim JH, Kim BS, Lee SK. Regulatory T Cells in Tumor Microenvironment and Approach for Anticancer Immunotherapy. Immune Netw. 2020;20(1).
183. Brooks DG, Walsh KB, Elsaesser H, Oldstone MBA. IL-10 directly suppresses CD4 but not CD8 T cell effector and memory responses following acute viral infection. Proceedings of the National Academy of Sciences. 2010 Feb 16;107(7):3018–23.
184. Bhattacharyya S, Sen P, Wallet M, Long B, Baldwin AS, Tisch R. Immunoregulation of dendritic cells by IL-10 is mediated through suppression of the PI3K/Akt pathway and of IκB kinase activity. Blood. 2004 Aug 15;104(4):1100–9.
185. Brockmann L, Gagliani N, Steglich B, Giannou AD, Kempski J, Pelczar P, et al. IL-10 Receptor Signaling Is Essential for TR1 Cell Function In Vivo. The Journal of Immunology. 2017 Feb 1;198(3):1130–41.
186. Ito T, Wang YH, Duramad O, Hanabuchi S, Perng OA, Gilliet M, et al. OX40 ligand shuts down IL-10-producing regulatory T cells. Proceedings of the National Academy of Sciences. 2006 Aug 29;103(35):13138–43.
187. Bauquet AT, Jin H, Paterson AM, Mitsdoerffer M, Ho IC, Sharpe AH, et al. The costimulatory molecule ICOS regulates the expression of c-Maf and IL-21 in the development of follicular T helper cells and TH-17 cells. Nat Immunol. 2009 Feb 21;10(2):167–75.
188. Carrier Y, Whitters MJ, Miyashiro JS, LaBranche TP, Ramon HE, Benoit SE, et al. Enhanced <scp>GITR</scp> / <scp>GITRL</scp> interactions augment <scp>IL</scp> -27 expression and induce <scp>IL</scp> -10-producing Tr-1 like cells. Eur J Immunol. 2012 Jun 8;42(6):1393–404.
189. Kanamaru F, Youngnak P, Hashiguchi M, Nishioka T, Takahashi T, Sakaguchi S, et al. Costimulation via Glucocorticoid-Induced TNF Receptor in Both Conventional and CD25+ Regulatory CD4+ T Cells. The Journal of Immunology. 2004 Jun 15;172(12):7306–14.

190. Woo SR, Turnis ME, Goldberg M V., Bankoti J, Selby M, Nirschl CJ, et al. Immune Inhibitory Molecules LAG-3 and PD-1 Synergistically Regulate T-cell Function to Promote Tumoral Immune Escape. Cancer Res. 2012 Feb 15;72(4):917–27.
191. Huang RY, Francois A, McGray AR, Miliotto A, Odunsi K. Compensatory upregulation of PD-1, LAG-3, and CTLA-4 limits the efficacy of single-agent checkpoint blockade in metastatic ovarian cancer. Oncoimmunology. 2017 Jan 2;6(1):e1249561.
192. Nurieva R, Wang J, Sahoo A. T-Cell Tolerance in Cancer. Immunotherapy. 2013 May 2;5(5):513–31.
193. Weber EW, Parker KR, Sotillo E, Lynn RC, Anbunathan H, Lattin J, et al. Transient rest restores functionality in exhausted CAR-T cells through epigenetic remodeling. Science (1979). 2021 Apr 2;372(6537).
194. Kimani SW, Perveen S, Szewezyk M, Zeng H, Dong A, Li F, et al. The co-crystal structure of Cbl-b and a small-molecule inhibitor reveals the mechanism of Cbl-b inhibition. Commun Biol. 2023 Dec 16;6(1):1272.

7. Appendices

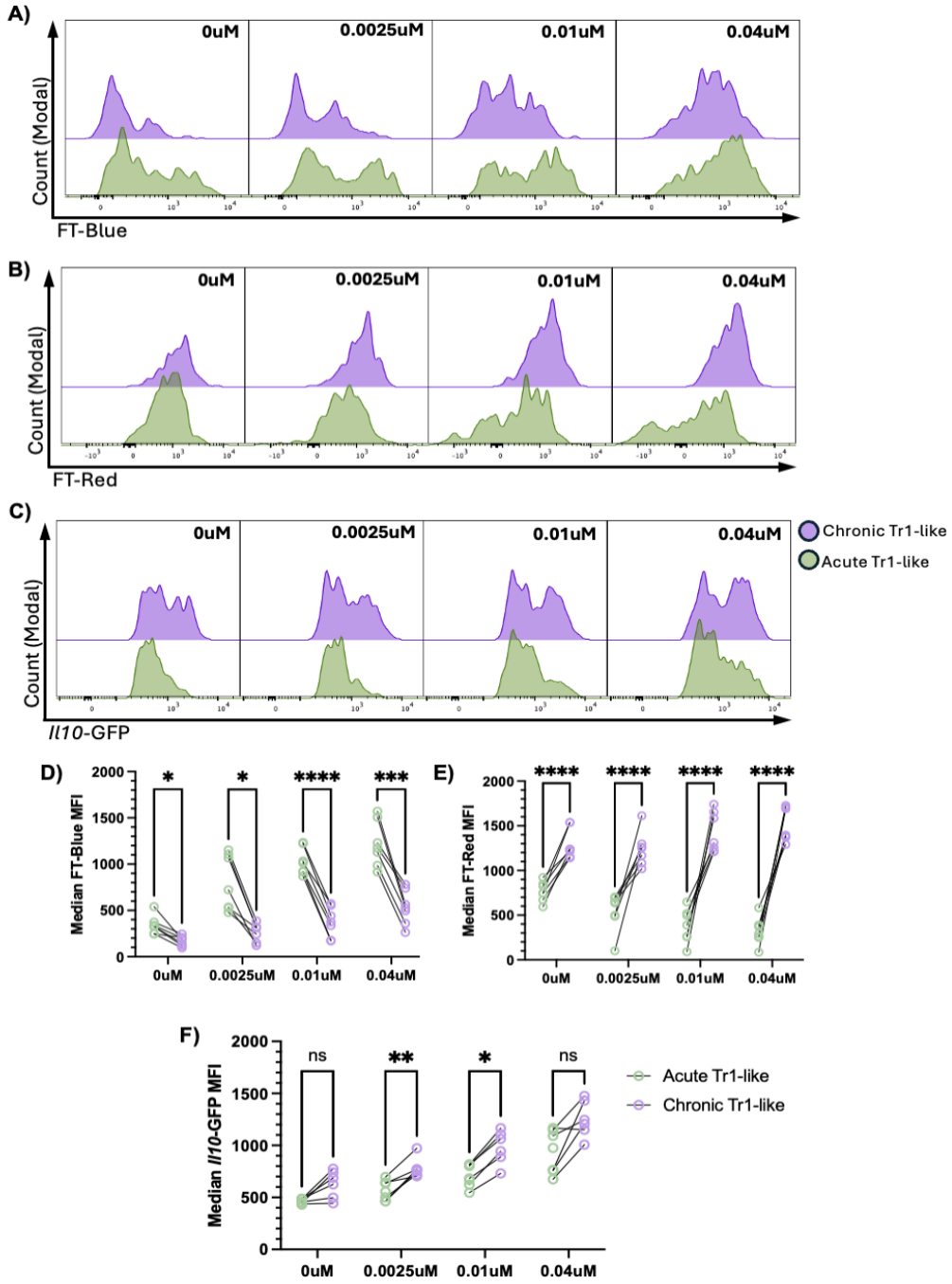

Figure 22 - *In-vitro* analysis comparing Tr1-like population responses within Acute and Chronic conditions.

A) *Histogram overlays comparing the expression of FT-Blue,* ***B)*** *FT-Red and* ***C)*** *Il10-GFP between Tr1-like cells induced in response to acute or chronic stimulation.* ***D)*** *scatter graph showing changes in expression of FT-Blue,* ***E)*** *FT-Red and* ***F)*** *Il10-GFP between Tr1-like populations from Acute and chronic conditions. data representative of two independent experiments, n=3 and n=4, overall n=7. Statistical analysis by Two-way ANOVA. Significance represented as *p≤0.05, **P≤0.01, ***p≤0.001, ****p≤0.0001.*