Clonal selection in CD4 T cells: The role of TCR specificity and avidity

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Declaration

I Julia Eve Merkenschlager, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

The ability to distinguish self from nonself is a fundamental property of the innate and adaptive immune system. For the somatically generated repertoire of T cell and B cell antigen receptors (TCR and BCR, respectively), self–nonself discrimination is primarily achieved through clonal deletion of lymphocytes expressing strongly auto-reactive receptors. Removal of such receptors from the TCR and BCR repertoires causes irreversible self-tolerance. For a T cell, this is a precarious life. During development in the thymus it must adopt a certain degree of self-reactivity in order to pass selection criteria, yet this must fall below a threshold, which would flag it to be negatively selected. This rigorous purging sees only approximately 3-5% of candidate thymocytes making it through the process. Once in the periphery, the quality of TCR signaling continues to be important and is heavily censored by extrinsic factors, which actively shape and condition both the pre-immune and antigen-responding repertoire. Although it is very clear that the signaling capacity of the TCR, whether this be towards self or nonself, is a key determinant in defining the trajectory of a T cell, the degree to which it governs these fate decisions remains ill defined. This thesis examines the relationship that exists between the T cell and its TCR throughout the life of a T cell.

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"All life is an experiment. The more experiments you make the better."
Ralph Waldo Emerson

Table of Contents

Table of Schematics

Table of Figures

List of tables

Abbreviations

Ab	Antibody
AB-IMDM	Air Buffered Iscove's Modified Dulbecco's Media
ACK	Ammonium-chloride-potassium
Aire	Auto-immune regulator
APC	Antigen presenting cells
B cell	B lymphocyte
B6	C57BL/6
BM	Bone marrow
BMDC	Bone marrow derived dendritic cells
bp	Base-pair
BSA	Bovine serum albumin
CD-	Cluster of differentiation
cDNA	Complimentary DNA
CFSE	Carboxyfluorescein succinimidyl ester
CLP	Common lymphoid progenitor
CLR	C-type lectin receptor
cTEC	Cortical thymic epithelial cells
CTL	Cytotoxic T lymphocyte
CTLL	Cytotoxic T lymphocyte lines
D-PBS	Dulbecco's Phosphate Buffered Saline
DAMP	Danger associated molecular pattern
DC	Dendritic cell
DN	Double-negative
DNA	Deoxyribonucleic acid
DP	Double-positive
env	Envelope
ERV	Endogenous retrovirus
F-MLV	Friend murine leukaemia virus
FACS	Fluorescence activated cell sorter
FCS	Foetal calf serum
Foxp3	Forkhead box P3

FV	Friend virus
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony stimulating factor
i.p	Intra-peritoneal
i.v	Intravenously
IFN	Interferon
Ig	Immunoglobulin
IL-	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium
ITAM	Immune-receptor tyrosine-based activation motifs
LCK	lymphocyte protein tyrosine kinase
LDV	Lactate dehydrogenase-elevating virus
LN	Lymph nodes
mAb	Monoclonal antibody
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
mTEC	Medullary thymic epithelial cell
MΦ	Macrophage
NK	Natural killer
NLR	Nod-like receptors
NOD	Non-obese diabetic
PBS	Phosphate-buffered saline
PDBU	Phorbol 12,13-dibutyrate
(**s)pMHCII**	(self)peptide-MHCII complex
PRR	Pathogen recognition receptor
PTA	Peripheral tissue antigen
pTreg	Peripheral Treg
Rag	Recombination activating gene
RLR	RIG-I-like receptors
RNA	Ribonucleic acid
RT	Room temperature
SFFV	Spleen focus-forming virus
SMAC/(p/d/c)	supramolecular activation cluster (peripheral/central/distal)
SP	Single-positive

T cell	T lymphocyte
TCR	T cell receptor
Tfh	T follicular helper
Tg	Transgenic
TGF-β	Transforming growth factor β
Th	T helper type cell
TLR	Toll like receptor
Treg	Regulatory T cell
tTreg	Thymic Treg
WT	Wild-types
YFP	Yellow fluorescence protein
ZAP70	Zeta Chain of T Cell receptor associated protein kinase

Chapter 1. Introduction

1.1 The Immune system

The mammalian immune system has evolved to provide protection against a hugely varied range of pathogens. Here, its architectural framework allows for a sophisticated and multi-layered approach to defence. Although there are many, defence strategies are broadly termed as either being part of the innate or adaptive arms of immunity. These differ both temporally and qualitatively, and whilst each confers its own distinct advantages, efficient protection requires the intimate crosstalk and co-operation between the two.

1.1.1 Innate immunity

Early responses to infection are initiated by components of the innate wing of immunity; these consist of chemical and physical barriers as well as cellular components. Innate immunity plays an important role in providing fast and efficient protection against pathogenic challenges, and in many cases it is sufficient. In other cases, where clearance cannot be achieved alone, innate immune responses act to delay the requirement of the adaptive immune system (Iwasaki and Medzhitov, 2010). Primarily, skin and mucosal surfaces secrete anti-microbial agents and provide a peripheral shield, restricting the free entry of pathogens. However, if these barriers are breached, innate cellular components, such as neutrophils (eosinophils/basophils), natural killer (NK) cells, monocytes/ macrophages, and dendritic cells (DC) are recruited to the compromised sites. The recruitment and activation of innate immune cells is achieved primarily via their expression of pattern recognition receptors (PRR). These receptors either recognise indicators of cellular damage, danger associated patterns (DAMPS), or conserved pathogen associated molecular patterns (PAMPS); which are expressed on the invading pathogen or damaged tissues. PRRs have many functions from opsonisation of invading bodies, to their roles in the initiation and activation of complement pathways, inflammation and cellular death (Vivier and Medzhitov, 2016, Akira et al., 2006, Iwasaki and Medzhitov, 2010). PRRs can be subdivided into four main families; Toll like receptors (TLR), Nucleotide oligomerisation (NLR), C-type Lectin like receptors (CLR), and RIG-1 like receptors (RLR).

Toll receptors play a major role in innate immunity (Janeway and Medzhitov, 2002). Perhaps highlighted by the fact that they are evolutionarily conserved between worm, fly, mouse and human and to this day remain the largest documented family of PRRs (Hoffmann, 2003, Hashimoto et al., 1988, Akira et al., 2006, Belvin and Anderson, 1996, Lemaitre et al., 1997, Irazoqui et al., 2010). So far, 13 different TLRs have been annotated, and collectively they can orchestrate diverse responses towards viruses, bacterial, fungi and parasites (Bordon, 2012, Oldenburg et al., 2012). TLR families are categorised according to their pathogen specificity and their cellular localisation (Akira et al., 2006). Broadly, TLRs 1,2,6 recognise microbial lipids and are positioned at the cell membrane. TLRs 7,8,9 are located intracellulary and predominately recognise nucleic acids, such as bacterial and viral derived genetic material. TLR4 famously recognises bacterial-derived LPS, and forms a heterodimeric complex with MD-2 at the cell membrane (Kim et al., 2007). Briefly CLRs, another family of PRRs, are often endocytic and work in conjunction with TLRs to recognise bacterial and fungal derived PAMPS. Whilst the cytoplasmic PRRs, including NOD and RIG-1 receptors and are mainly responsible for recognising foreign ribonucleic acid (RNA). In addition to their localisation and specificities, PRRs also differ in their function, presumably achieved via their associations with different target genes. Thus downstream signalling of PRRs, allows for the induction of qualitatively and/or quantitatively diverse innate immune responses, according to the flavour of pathogen recognised (Lemaitre et al., 1997).

Upon infection, phagocytes such as the monocytes/macrophages and neutrophils are the first cellular recruits to the scene. These cells utilise their PRRs to sense damage signals that in conjunction with chemokines and chemo-attracts aid in their navigation to the infected tissues. Upon entry, neutrophils come together in tight clusters to isolate the local site and the pathogen, in a behavior which has been described to be reminiscent of the swarming of insects (Lammermann, 2016, Lammermann et al., 2013). In addition to neutrophils, monocytes from the blood are quick to traffic to the infected tissues, where they then differentiate into either inflammatory macrophages or DCs. Once activated, macrophages and neutrophils share common mechanisms of action, from cytokine production to direct pathogen killing either via phagocytosis or via the release of lytic granules or reactive oxygen

species (ROS) (Segal, 2005, Lammermann, 2016). In addition, neutrophils may also selectively release extracellular traps (NETs) in response to large pathogens (Branzk et al., 2014). Activation of DCs at the sites of infection leads a successive wave of cytokine production, which along with those produced by macrophages and neutrophils, act to limit pathogen spread as well as recruit cells of the adaptive immune system (Chiesa et al., 2005, Moretta, 2002). In addition, activation of DCs induces their maturation into highly proficient antigen presenting cells (APCs), capable of priming T lymphocytes (T cells); thus making them critical communicators between the innate and adaptive forms of immunity. However, although cells of the innate immune systems are clearly effective in providing protection against many pathogens, they are limited by the nature of their germline-encoded, and non-clonally distributed receptors. This significantly limits their range of recognition to conserved molecules on pathogens, and caps their capacity to provide protection against a fast evolving pathogen.

1.1.2 Adaptive immunity

Adaptive immunity is characterised by a comparatively broader and more tailored response to infection. Although cells of the adaptive immune system are recruited comparatively later on in responses to infection, forming the basis of immunological memory (a phenomenon first theorized by the ancient Greeks). The cells of the adaptive immune system are the T and B-lymphocytes (T cells/ B cells). Similarly, to innate cells, T and B cells too express PRRs and thus can receive activation signals via these receptors. However, their unique antigen-specificity is derived from their generation and expression of highly diverse and clonally distributed receptors, the T cell receptor and B cell receptor respectively (TCR and BCR). Unlike innate immune cells, these antigen receptors are not fixed by germ-line encoded sequences (Bonilla and Oettgen, 2010); instead they are somatically generated or ‘custom made’ via gene-shuffling events that occur during their development. Although T and B cells are both derived from common hematopoietic stem cells that reside in bone marrow, their later development occurs in separate anatomical locations (Kondo et al., 1997). In this regard B cell development starts and continues in the bone marrow, whilst T cell development requires common lymphoid progenitor (CLP) cells to leave the bone marrow and traffic to the thymus

in order to complete development. For the purpose of this thesis we will focus on T cell development.

1.2 T cell development

1.2.1 Thymocyte development

Self-determination in concert with the generation of diversity, are central principles in immunology. In this regard, the T cell repertoire must be sufficiently varied in order to provide protective coverage to the host against an unpredictable range of antigens, whilst also remaining tolerant to self. This makes lymphocyte development a tricky business, demanding mechanisms capable of manufacturing highly diverse antigen-receptors; coupled with strategies to examine and then remove potentially deleterious receptors. The cross-reactive nature of the TCR toward self-antigens, a property selected for during development but continually necessary during peripheral life, poses an inherent challenge for a T cell; where one must balance the necessity to recognise self with overt self-reactivity. In this regard, and similarly to the Goldilocks principles, a 'model' T cell must be able to recognise self-antigen to just the right amount, neither too much nor too little.

1.2.2 Progenitor to Thymocyte: Initial events

Although the site of T cell development is the thymus, this process requires a constant supply of CLP cells from the bone marrow. Although incompletely understood, the decision of a CLP cell to become a T cell progenitor, versus a B cell progenitor, seems to be associated with Notch derived signalling (Radtke et al., 2013, Germain, 2002, Zuniga-Pflucker, 2004, Pui et al., 1999, Radtke et al., 1999). Here it is thought, that the initiation of Notch signalling in CLP cells, blocks B cell development and favours T cell commitment (Pui et al., 1999, Vicente et al., 2010, Radtke et al., 1999). T cell-committed CLP cells must then leave the bone marrow and navigate the peripheral circulation before seeding the thymus. Still surprisingly little is known about the mechanisms that underpin the successful migration and homing of CLPs to the thymus. Nonetheless, upon their successful arrival to the outer cortical region of the thymus, migrants undergo multiple rounds of cellular divisions driven by interleukin (IL)-7 and Notch signaling which initiates the start of thymic T cell development (Zuniga-Pflucker, 2004, Patra et al., 2013).

Thymic T cell development is a spatially organised process where thymocytes must navigate a full thymic circuit, journeying through the cortex and medullary regions, in order to complete development. During which time, thymocytes must also negotiate strict selection-based obstacles, which are associated with discrete thymic microenvironments, to permit entry in to the next stage of development. This competitive process results in only a lucky few thymocytes that actually succeed to maturity. Fundamental to the process is the step-by-step generation and expression of a functional TCR by a developing thymocyte.

Differentiation can also be visualised by the expression of cell surface markers on developing thymocytes, most notably the expression of cluster of differentiation (CD)-4 and CD8 molecules. The most immature thymocytes, $CD4^{-}CD8^{-}$, are termed double-negative (DN) cells and at this stage express no TCR at all. Instead, these cells harbour non-recombined germline encoded TCR gene segments. At this point, each thymocyte retains the potential to give rise to either the αβ or γδ T cell lineage. DN thymocytes progressively transit through the outer cortex and into the inner cortex, with this migratory pattern accompanied by the oscillating expression of CD44 and CD25. Accordingly, DN cells can be further subcategorised into the following: DN1 ($CD44^{+}CD25^{-}$), DN2 ($CD44^{+}CD25^{+}$), DN3 ($CD44^{-}CD25^{+}$), DN4 ($CD44^{-}CD25^{-}$) (Germain, 2002, Pillai, 2000, Godfrey et al., 1993). At the DN3 stage, thymocytes begin to recombine genetic regions within their TCR β, δ, γ loci. Successful recombination within the TCR δ and TCR γ loci promotes the early assemblage of the γδ TCR complex at the cell membrane. Here the two chains may be tested together. Engagement of a γδ TCR leads to strong expression of extracellular signal-regulated kinase (ERK), early growth response (EGR) and inhibitor of differentiation (ID3) within the cell, which act in concert to drive its commitment towards the γδ T cell lineage (Prinz et al., 2006). It has been noted, that it is this combination of early and strong TCR signalling that is imperative to γδ lineage decisions. Indeed, in certain TCR transgenic mice and as a consequence of the premature expression of TCR transgenes, αβTCRs of sufficient avidity have been shown to instruct for the γδ T cell lineage (Bruno et al.). Conversely, attenuated signaling via a γδ TCR has been described to result in a switch in thymocyte commitment towards the αβ T cell lineage (Haks et al., 2005). These

data highlight the strict temporal and signalling guidelines surrounding γδ T cell development.

Unlike γδ TCR chains, the TCRβ and TCRα chains are expressed successively. This involves the sequential recombination of first the TCRβ chain followed by the TCRα chain and is orchestrated through developmentally associated changes in chromatin conformation and accessibility (Krangel, 2009, Mathieu et al., 2000). Here at the DN3 stage, the expression of a re-arranged TCRβ chain first leads to its pairing with a surrogate invariant alpha chain, to form the pre-TCR complex (van Oers et al., 1995, Born et al., 1985). Successful signalling through the complex, then leads in the allelic exclusion and silencing of the second TCRβ locus; which acts to ensure that each thymocyte expresses only a single recombined TCRβ chain. At this stage thymocytes also undergo several rounds of cellular proliferation fuelled by IL-7 (TCRβ selection) (Robey and Fowlkes, 1994) and also begin to recombine gene segments within their TCRα loci. Next, the productive re-arrangement of the TCRα locus, drives the substitution of the surrogate chain for the newly recombined TCRα chain. This leads to the assemblage of the mature TCR complex, and the expression of CD3, a complex of five polypeptides, CD3 γ,η,δ,ζ,ε (Strominger, 1989). Following its productive surface expression, thymocytes may then proceed to differentiate into, $CD4^+CD8^+$ double positive (DP) cells destined for the αβ lineage. Hence the first fate decision dictated by the TCR is made; to continue as a γδ or αβ T cell.

1.2.3 TCR recombination

The generation of diversity within the TCR repertoire is achieved through several mechanisms. These include somatic recombination, non-templated nucleotide insertions and TCR (α and β) chain coupling; generating combinatorial, junctional and pairing diversity respectively.

The germline TCR locus consists of many back-to-back genetic regions that contain discrete TCR gene families. These are defined as: (C) constant, variable (V) and joining (J) and (in the TCRβ locus) diversity (D) regions. Each family encodes within it many functional genes segments, for example the TCRα locus is

estimated to consist of 70-80 Vα gene segments (Janeway Jr, 2000). However, just one gene segment (from each gene family) is required to make a functional TCR chain. Therefore, site-specific recombination must occur between the multiple V, (D) and J (C) gene segments in order to generate functional VJC_{α} (TCRβα chain) or $VDJC_{\beta}$ (TCRβ chain) TCR transcripts. This 'gene shuffling' process generates combinatorial diversity. The mechanics of this process involves the binding of recombinase proteins (Rag), encoded by the *Rag1* or *Rag2* genes (Mombaerts et al., 1992, Schatz et al., 1989), to recombination signal sequences (RSS) which flank individual gene segments (Dudley et al., 1994). Binding of Rag proteins to these RSS sites, results in double-stranded-breaks within the Deoxyribonucleic acid (DNA) (Oettinger et al., 1990, Sawchuk et al., 1997), which are then resolved by non-homologous end joining (Krangel, 2009). Specific nucleotide sequences, called spacer sequences, are also contained within the RSS. Importantly these differ in length, either consisting of 12 base-pairs (bp) or 23 bp depending on the TCR gene family from which they originate from (van Gent et al., 1996). This discrepancy in spacer sequence length is then utilised during the re-joining process, whereby pairing can only occur between fragments with terminal 12bp spacer sequences and fragments with terminal 23bp spacer sequences (van Gent et al., 1996, Pillai, 2000). This ensures that recombination only occurs between distinct gene segments, which acts to maximise the potential for productive re-arrangements. In addition, during the recombination process the addition of non-template nucleotides to the double-stranded end breaks increases diversity further, this is known as junctional diversification (Janeway Jr, 2000). Lastly, the pairing of different TCRα with different TCRβ chains adds another layer of multeity to the process, and finalizes the TCR signature of an individual T lymphocyte. This quasi-random re-combinatorial process leads to the generation of highly variable complementary determining (CDR) regions within the TCR molecules, which are critical in determining the antigen specificity of each T cell (Davis and Bjorkman, 1988). Specifically, CDR1 and CDR2 regions are thought to be responsible for the binding of the TCRs to exposed areas of the MHC molecule, whereas the CDR3 region binds and determines peptide specificity (Marrack et al., 2008).

1.2.4 Thymocytes to T cell: Selection and Tolerance

The theoretical generation of an almost exhaustive TCR repertoire of up to 10^{15} TCRs is much larger than the diversity observed in the immune system of the host (Davis and Bjorkman, 1988). This discrepancy between the potential TCR diversity and the realised diversity suggests that only a small fraction of TCRs are represented in the periphery at any given time (Attridge and Walker, 2014). Here it is thought that competition, the relatively low success rate of T cell development as well as the physical size of the host organism all contribute in limiting the overall population size (Tough and Sprent, 1994, Smith et al., 2001).

During development, thymocytes are screened against a diverse repertoire of self-peptides and survival is determined by the quality of TCR signalling upheld by a cell (summarised in **Schematic 1**). The ectopic expression of many peripheral tissue antigens (PTA) on thymic APCs is exquisitely controlled by the transcription factor known as autoimmune regulator (Aire), and allows for the high-throughput screening of thymocytes against many self-antigens in the thymus. Here, Aire binds to polymerase complexes, which are associated with a respective genes promoter. Binding then acts to prevent 'RNA polymerase pausing'; normally an inhibitory ('stalling') mechanism that restricts transcription of the downstream gene (Giraud et al., 2012). Once the 'stalled' RNA polymerase is 'unleashed', transcription proceeds, driving the promiscuous expression of many PTA in thymic APCs. Importantly, mutations within the *Aire* gene have been shown to significantly reduce the repertoire of self-antigens presented during thymic selection (Anderson et al., 2002), consequently generating a pathogenic TCR repertoire within such hosts (Venanzi et al., 2008). Thus the recognition of self-antigens forms the basis for the examination and conditioning of the newly generated TCR repertoire in mechanisms known as thymic selection.

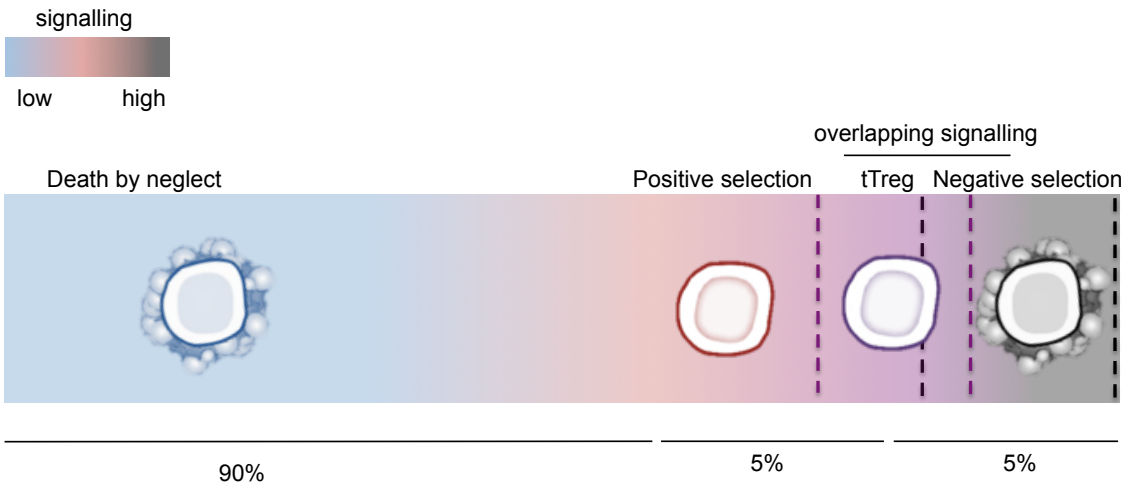
Thymic selection involves two steps, positive and negative selection. This occurs at the DP development stage, and requires each TCR to be tested for its major histocompatibility complex (MHC) restriction and its innate signalling capacity (Surh and Sprent, 1994). Here TCR signalling (or lack of) is used to choreograph three types of developmental outcomes: 1) death by neglect 2) positive selection 3)

negative selection (**Schematic 1**). Positive selection occurs in the cortex, precedes negative selection and is examined by a unique population of thymic APCs called cortical thymic epithelial cells (cTECs). During positive selection, the vast majority of 'auditioning' thymocytes (90%) interact too poorly with cTECs, thus fail to meet TCR signalling requirements and as a result die by neglect (Klein et al., 2014). Thymocytes expressing TCRs whom meet the qualitative requirements, are positively selected for, and transit to the medulla where negative selection events operate. Here thymocytes encounter a distinct and more heterogeneous population of thymic APCs that also present PTAs; these are the medullary thymic epithelial cells (mTEC), thymic dendritic cells (DC) and migratory DCs. The observation that distinct APCs colonise different thymic compartments is suggestive that they each may have specialised roles in thymocyte selection, something discussed in much greater detail within **5.1.3** (Klein et al., 2014, Mandl et al., 2013, Yasutomo et al., 2000). During negative selection, thymocytes with TCRs that recognise self-antigen too strongly are irreversibly removed from the repertoire by clonal deletion, which involves cellular death by apoptosis. In addition to clonal deletion, very strong TCR signaling (overlapping with those normally associated with negative selection) can also lead to the thymic differentiation of regulatory (Treg) cells (Fu et al., 2014). This will be discussed in more depth below.

In addition to the TCR itself, several different molecules may also contribute to the perceived TCR signal strength during thymic selection. In this regard, the expression of such molecules can play an important role in the management of optimal TCR signalling. For example, excessively high signalling can be tuned down, either via the up-regulation of negative regulators of signalling such as CD5 (Mujal and Krummel, 2015, Azzam et al., 1998, Persaud et al., 2014, Mandl et al., 2013, Tarakhovsky et al., 1995) or via the down-modulation of positive regulators of TCR signalling such as CD4/CD8 (Fu et al., 2014). Themis and microRNA 18 are also widely known to negatively and positively regulate TCR signalling (respectively) via the modulation of important cellular phosphatases (Fu et al., 2014). In this way, the fine-tuning of T cell receptor signalling by accessory molecules, can provide a rescue mechanism for otherwise doomed thymocytes. Whether and to what extent the quality of these thymic interactions with self-

antigen may leave a long lasting impression on a T cell, is a question explored in this thesis.

Schematic 1.



An affinity based model for thymocyte selection

This model uses the strength of TCR signalling (colour coded) as a predictor of T cell fate decisions made during thymic selection. To this end, the vast majority of thymocytes receive TCR signalling which is too low to allow for their selection and hence they die as a result of neglect. Conversely, thymocytes receiving the highest TCR signalling are conventionally negatively selected against and die as a result. High levels of TCR signalling, can also (in certain instances) drive clonal deviation, which is the differentiation of thymocytes into thymic regulatory T cells (tTreg). In this model only thymocytes that manage to sustain optimal range of signalling, somewhere positive selection and negative selection are selected to become naïve peripheral T cells.

1.2.5 Lineage fate decisions

Intertwined with TCR signalling during thymic selection is CD4/CD8 lineage commitment. Exactly how DP thymocytes, which express both the above co-receptors, transition into either SP CD4 or SP CD8 T cells has been the subject of much scientific interest and debate. Traditionally, two models the 'instructive' or 'stochastic', have been applied to make sense of how cells may make this decision (Germain, 2002). The instructive model, proposes that distinct signalling pathways are supported by TCRs engaging different classes of MHC molecules, leading to a commitment decision based on the nature of the MHC interacting partner (Boehmer, 1986). In comparison, the 'stochastic' model suggests that lineage commitment is already pre-wired within a thymocyte prior to TCR recombination, with expression of either CD4 or CD8 occurring in a TCR independent fashion. Following which, those cells that chose incorrectly and as a consequence are MHC mismatched are subsequently eliminated (Germain, 2002). An increasing body of evidence now suggests that the decision to become either a CD8 or a CD4 T cell is instructed for by the strength, duration and quality of TCR signalling encountered, collectively defined by the 'strength of signal model'. Here, it is suggested that sustained high levels of TCR signalling are supported by MHCII-mediated interactions, leading to increased intracellular calcium and ERK signaling, in turn instructing for CD4 T cell commitment (Sharp et al., 1997). In contrast low-level signalling for shorter durations of time upon MHCI mediated binding, is suggested to favour CD8 differentiation (Adachi and Iwata, 2002). Irrespective of how these decisions are made, CD4/CD8 commitment must be consummated by the expression of lineage specific (and mutually exclusive) transcription factors. In this regard, ThPOK (He et al., 2005), TOX (Aliahmad and Kaye, 2008, Wilkinson et al., 2002) and GATA3 (Hernandez-Hoyos et al., 2003) are established as key players in authorizing the CD4 T cell lineage; whilst the expression of Runt-related transcription factor 3 (RUNX3) is associated with the CD8 T cell lineage (Taniuchi et al., 2002, Singer et al., 2008). Positively selected mature SP thymocytes then migrate through th perivascular space into the cortico-medullary junction and medulla to eventually become peripheral naive T lymphocytes (Germain, 2002).

1.2.6 T regulatory cell fate

During thymic selection events, a window of 'acceptable' signalling potential, allows for the selection of thymocytes and TCRs of a spectrum of signalling capacities. Thymic Treg cell (tTreg) commitment is thought to be supported by TCR signaling at the higher end of this range (Bains et al., 2013). Although the conditions for tTreg development, remain relatively elusive, it is thought to be a two-step process requiring high avidity TCR mediated interactions to licence Forkhead box P3 (Foxp3) and IL-2 receptor (IL-2R) up-regulation (Burchill et al., 2008, Yoshikura, 1975, Merkenschlager and von Boehmer, 2010). Thus under the right conditions high levels of TCR signalling can result in this CD4 effector differentiation program rather than resulting in clonal deletion. Here it is suggested that IL-7 signalling (Simonetta et al., 2012) and enhanced expression of Bcl-2 (Bouillet et al., 2002) may provide protection to some developing cells from being negatively selected (Tischner et al., 2012, Simonetta et al., 2012). Presumably, this protective blanket allows for continued TCR signalling, driving the expression of the Forkhead box P3 (FoxP3) and IL-2R and successively the acquisition of the Treg cell lineage (Brunkow et al., 2001, Fontenot et al., 2003a, Cabarrocas et al., 2006, Hori et al., 2003).

1.3 Life in the periphery

For the remainder of this thesis we will concentrate on the life of a committed CD4 T cell.

1.3.1 Homeostasis

At the 'steady state' the overall number of peripheral CD4 T cells does not fluctuate greatly. This is perhaps surprising considering the continuous arrival of new T cells from the thymus. However, behind the calm façade the periphery is a highly competitive arena, where the TCR repertoire remains under constant selection pressure. Here, maximal diversity must be maintained but in a limited space. Thus in order to survive, CD4 T cells must actively compete between each other for self-peptide major-histocompatibility-class II (spMHCII) derived and IL-7 mediated

signalling, with the availability of such factors collectively defining the 'niche space' available (Takada and Jameson, 2009, Stockinger et al., 2004). Although responsiveness to self-antigens is largely reduced in the periphery, compared to that observed in the thymus, it continues to play a central role. Here, mature T cells must receive tonic TCR signalling upon recognition of self-antigens, in a process reminiscent of thymic positive selection, in order to stay alive (Takada and Jameson, 2009, Troy and Shen, 2003, Viret et al., 1999). Cells failing to initiate such tonic signalling die or are outcompeted (Martin et al., 2006, Viret et al., 1999, Witherden et al., 2000, Kassiotis et al., 2003). In addition to quantity the quality of these interactions are fundamentally important. In this regard the quality (or avidity) of spMHCII-TCR interactions govern the relative fitness a CD4 T cell, whereby T cells capable of higher avidity interactions with spMHCII will outperform those of lower avidity (Kassiotis et al., 2003).

In addition to TCR derived signalling, IL-7 derived signalling or the expression of IL-7 receptor is also critical for T cell survival in the periphery (Vivien et al., 2001, Kondrack et al., 2003). However, as IL-7 also plays an essential role during early T cell and B cell development, IL-7 or IL7R$^{-/-}$ mice (whom do no generate mature lymphocytes as a consequence of early blocks in lymphocyte development) could not be used to study a role for IL-7 in T cell homeostasis. This had made studying the contribution of IL-7 to the peripheral maintenance of CD4 T cells challenging. This was until experiments, which involved the treatment of adult thymectomized mice with monoclonal anti-IL-7 antibodies, presented the best evidence for a role for IL-7 in this regard. Here, treatment resulted in the rapid decay of polyclonal T cell populations in a strictly anti-IL-7 dosage dependent fashion (Vivien et al., 2001). It is now well recognised that both IL-7 signalling and self-recognition are required for CD4 T cell maintenance homeostatic function. What remains relatively unknown is how downstream MHCII and IL-7 pro survival pathways may integrate and thus contribute to the overall competitive fitness of a CD4 T cell.

1.3.2 Central and Peripheral Tolerance

Although the TCR repertoire is now recognized as being broadly self-reactive, TCR signaling as a consequence of self-recognition must be maintained at sub-threshold levels and be tightly regulated in order to prevent auto-immunity. However, the generation of TCRs is a random process, and thus the products of TCR recombination are unpredictable. Therefore, along with the generation of protective receptors, this process will also generate potentially deleterious and overtly auto-reactive TCRs. In order to limit the potential of such clones, multilayered censorship mechanisms exist known as immune tolerance. These can be subcategorized into either central or peripheral tolerance mechanisms.

Central tolerance involves the irreversible elimination of highly self-reactive T cell clones during development in the thymus (as discussed previously). This involves the negative selection of T cells that elicit TCR signaling above acceptable thresholds. Whilst very efficient, this process is never complete, always allowing for the escape of some auto-reactive T cells. This is impart due to the impossibility of screening every single thymocyte against (and in anticipation of) all the self or food-derived antigens it may encounter. However, this shortcoming may also be strategic: as central tolerance mechanisms, if complete, could prohibitory limit the TCR diversity required for a protective TCR repertoire. Thus, as a compromise, additional tolerance mechanisms (peripheral tolerance) have evolved. These act to continually prune and condition the peripheral TCR repertoire, in attempts to safeguard the host. In addition to clonal deletion, clonal deviation in the thymus (and to some extent in the periphery) is also an effective mode of tolerance. This process removes a population of overtly self-reactive T cells from the conventional T cell (Tcon) repertoire, via their diversion into the Treg lineage. The decision between clonal deletion vs clonal diversion is still not fully understood (Xing and Hogquist, 2012).

Peripheral tolerance exists in many forms, but effectively results in the suppression or inhibition of auto-reactive T cells once in the periphery. Treg T cells are a form of peripheral tolerance, acting as cellular gatekeepers that regulate immune responses elicited by conventional T cells, in response to both self and non-self-

antigens. Ignorance is an additional peripheral tolerance mechanism and involves the incapacity of auto-reactive T cells to respond to self-antigens due to physical barriers that separate TCRs from potential self-ligands *in vivo* (Xing and Hogquist, 2012). In scenarios where T cells are able to recognize a self-antigen to sufficiently high levels, peripheral anergy may act to halt T cell activation. Anergy occurs when TCR signaling is elicited in the absence of appropriate co-stimulatory signals (signals required to license and promote T-cell activation) and results in the T cell becoming functionally inert.

1.4 T cell activation

CD4 T cell activation requires specific cell-to-cell and cognate receptor-ligand interactions. Naïve CD4 T cells continually circulate through the blood to the lymphoid organs and back again; during which time they patrol and sample pMHCII complexes expressed on the APCs that they encounter. In the context of infection, APCs become activated and take up antigen and process it into smaller peptide fragments. These are then loaded onto MHC display platforms and presented to the scanning naïve T cells. CD4 T cell activation requires two signals: 1) TCR recognition of the cognate antigen 2) the simultaneous delivery of co-stimulation. The initial recognition of pMHCII that results in the activation of a naïve T cell is known as T cell priming. The three main professional APCs are DCs, macrophages and B cells, whilst DCs are deemed the main contributors to T cell priming. Importantly different APC types assume different anatomical locations during homeostasis, which may influence their contribution to CD4 T cell responses during early stages of infection. In addition, activation status, co-receptor expression and the level of recruitment of the APC cell types at different points in an infection vary widely, and thus influence an APCs competency accordingly (discussed in more detail in **5.1.3**) (Constant, 1999).

1.4.1 Early events in CD4 T cell activation

T cell activation is initiated by the functional recognition of cognate pMHCII complexes by TCRs. Whilst the intricate signalling framework is still to be fully realised, an early event is this process is the recruitment of kinases to cytoplasmic domains within the TCR complex, immune-receptor tyrosine-based activation

motifs (ITAMs). This initiates the activation of a kinase-signalling cascade involving CD45, lymphocyte protein tyrosine kinase (LCK) and Zeta Chain of T Cell receptor-associated protein kinase (Zap70) and leads to the fluxing of calcium inside the T cell. This in turn regulates the activity of the calcium sensitive phosphatase calcineurin, which mediates the transcription of many genes associated with T cell activation (Fracchia et al., 2013). Activation is also (positively) regulated by the expression of CD28 and (negatively regulated) by cytotoxic T lymphocyte-associated protein (CTLA-4), both expressed at the cell membrane. These co-receptors share common ligands, which are expressed on APCs, CD80 and CD86. Therefore, direct competition for CD80 and CD86 between CD28 and CTLA-4 can regulate to the degree of activation as measured by IL-2 production and clonal expansion (Colombetti et al., 2006).

In addition, TCR triggering leads to the active re-organisation of multiple TCR-pMHC complexes into clusters that form at the T cell-APC interface. These structures eventually form the epicenter of a larger 'bulls-eye' like structure referred to as the supra-molecular activation cluster (SMAC) or the immunological synapse. Around the central clusters of TCR-pMHC (cSMAC), adhesion proteins such as lymphocyte function-associated 1 (LFA1), Talin and intracellular adhesion molecule 1 (ICAM1) are recruited as scaffolding structures that make up the peripheral SMAC (pSMAC). In concert, CD43 and CD45 molecules assemble as a third ring structure surrounding the pSMAC, to make up the distal SMAC (dSMAC) (Dustin, 2009, Grakoui et al., 1999). These synapses are postulated to concentrate and stabiles interactions acting to amplify signaling and regulate T cell activation (Monks et al., 1998).

1.4.2 T cell differentiation

Following early TCR signaling events, a re-landscaping of the transcriptional network allows for the polarization of naïve T cells into diverse T helper (Th) effectors subsets: T helper 1 (Th1), T helper 2 (Th2), T helper 17 (Th17), regulatory T cells (Treg), T follicular helper cells (Tfh) and more recently (Th9) subsets (**Schematic 2**). This requires the coordinated expression of distinct signal

transducers and activators of transcription (STAT) and transcription factors (TFs). Each subset can then be defined by the expression of its lineage specific transcription factor, functionality and by its cytokine-secreting potential. Here it is thought the quality of TCR signaling along with environmental signals such as the cytokine milieu and the competency of the co-operating APC are instructive for different T-helper (Th) lineages.

IL-12 and IFN-γ are essential for the induction of Th1 differentiation, which is marked by the lineage associated transcription factor T-bet. Th1 T cells themselves then go onto make large amounts of IFN-γ, IL-2 as well as varying levels of tumour necrosis factor β (TNF-β). The production of these cytokines, allows these cell to specifically stimulate and activate cellular immunity.

Th2 differentiation requires both IL-4 and IL-2, whilst once activated they produce little to no IL-2 themselves. Instead their hallmark cytokines are IL-4, IL-5, and IL-13. Contrastingly to Th1 cells, Th2 cells mediate humoural immunity, through their close co-operation with B cells, as well as allergic responses. GATA3 is the hallmark transcription factor for the Th2 lineage, whilst STAT5 and STAT6 co-operation is required for the stabilisation of the lineage.

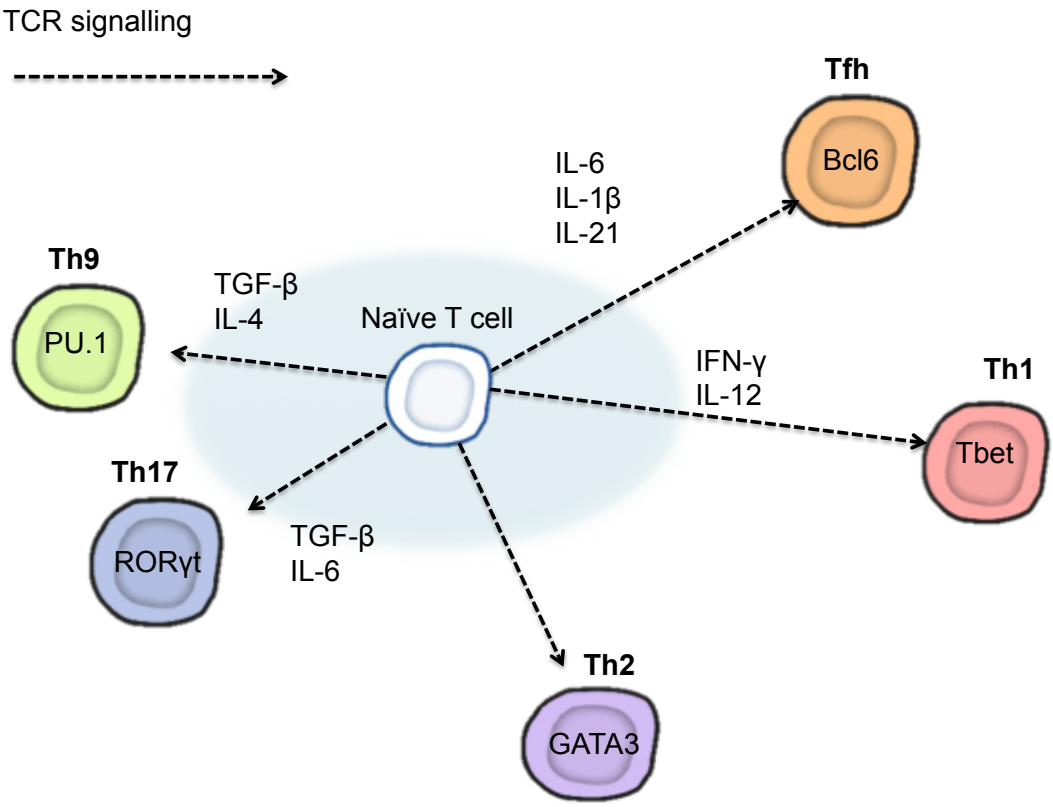
Th17 differentiation can either be promoted through cooperation between TGF-β and IL-6 or TGF-β and IL-21. With differentiated cells then characterised by the hallmark transcription factor RORγt and by their unique expression of IL-17A, IL-17F (Bettelli et al., 2006). Th17 T cells are critical regulators of neutrophils and macrophages and heavily contribute to immune responses against extracellular pathogens.

Tfh differentiation is thought to be promoted by high avidity interactions made between B celrs, and requires IL-6 derived signalling. Lineage commitment is controlled by the transcription factor Bcl-6 which is mutually exclusive to Blimp1, hence Tfh differentiation presents the opposing differentiation program to Th1. Tfh cells are essential in the clonal selection of the B cell response during the germinal centre (GC) reaction and thus play a prominent role in shaping the antibody

repertoire. Central to their role is their production of IL-21 and their expression of CXCR5 (Reinhardt et al., 2009).

Th9 cells are the most recently described CD4 T cell subset, defined by their expression of IL-9 and exhibit pleiotropic functions in immunity and immunopathology (reviewed (Kaplan et al., 2015).

Schematic 2.



CD4 T cell effector subsets
A stylized depiction of CD4 T cell differentiation, which outlines the conventional T cell effector subsets. Here, optimal TCR signal strength is indicated by the length of the dotted line, which are also annotated with the necessary polarizing factors. Lineage defining transcription factors are annotated within the cells types.

1.4.3 Memory cell differentiation

Upon resolution of infection and the clearance of antigen, the vast majority of clonally expanded T cells die via apoptosis. However, some privileged effector cells remain and further differentiate into memory T cells. These cells form the basis of immunological memory, capable of generating fast and efficient responses upon secondary infection. The factors that govern memory selection, allowing for the emergence of a select few from a much larger population, remains somewhat unresolved (discussed in more depth in **1.5.5**.)

1.5 Clonal selection and TCR avidity

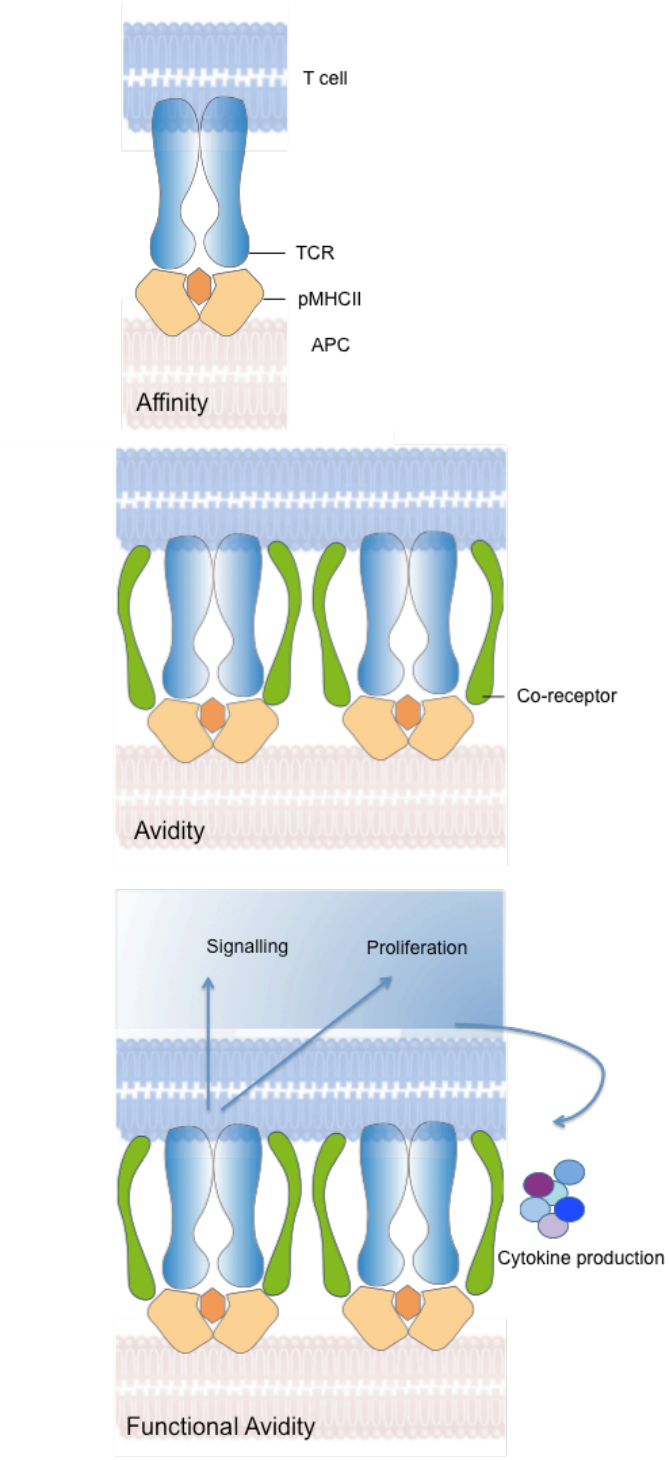
1.5.1 TCR affinity, avidity and functional avidity

TCR affinity, avidity and functional avidity all contribute or refer to aspects of TCR signal strength (**Schematic 3**) (Vigan et al., 2012). Specifically, TCR affinity measures the strength of binding/ interaction made between a single TCR and a pMHCII molecule. Instead, TCR avidity is the sum of multiple interactions between TCRs and pMHCII complexes, but on a single T cell. Next the functional avidity of a T cell clone is a measure of performance or sensitivity towards antigen. In this regard, the functional avidity is inversely proportional to the amount of antigen required to elicit a response. Usually, the amount of peptide antigen that is required to elicit 50% of a T cell clones' maximal response *in vitro* remains the gold standard determinant of functional avidity. Importantly, functional avidity is subject to many cell intrinsic parameters (on top of the TCR itself) that act to additionally tune a T cell's responsiveness. However, the cross talk or relationship between these parameters are often complicated, and how signals are eventually interpreted by a cell remains to be fully understood. For example, two T cell clones may have equivalent overall functional avidities, but very different affinities (clone A: low TCR density but high TCR affinity versus clone B: high TCR density but low TCR affinity) in this way similar TCR strength may be translated into dramatically distinct fates or functional responses. Clearly a better understanding of the factors that contribute to a T cells sensitivity, their mechanisms of action and their relative hierarchy remains important (Martin et al., 2006).

1.5.2 TCR avidity and selection

A set of cells expressing the same TCR (at the protein level), are collectively defined as a clonotype. During homeostasis, but arguably more prominently during antigen-selected responses, those clonotypes most "fit" as opposed to "unfit" are selected for. Fitness is largely dictated by the quality of a T cells TCR. For example, during homeostasis competition between clonotypes for MHC derived signaling acts to weed out the cells least efficient at recognizing spMHC. During infection, competition for growth factors as well as for antigen results in the rapid outgrowth of clonotypes receiving the strongest TCR signals (Tubo et al., 2013a, Thorborn et al., 2014, Tubo and Jenkins, Fasso et al., 2000). Although TCR based selection is well recognized in acute responses, whether it continues to be as important in shaping the composition of protracted responses is not well understood. However, hypothesizing that TCR signaling continues to be the major selecting force throughout the response: would predict the progressive outgrowth of only the highest avidity clonotypes over time. Conceptually, although this would act to focus the TCR response this would also be at expense of immune coverage and could potentially narrow the TCR repertoire to dangerously few clonotypes. Indeed, extensive analysis of evolving TCR repertoires during immune responses has now began to highlight that discrepancies between the clonotypic composition of primary and memory repertoires exists. This would suggest that diverse parameters may exist for effector cell and memory cell recruitment. Therefore, it seems, TCR based clonal selection must be counterbalanced against other mechanisms that are in place to preserve a level of clonotypic diversity in responding repertoires. These will be discussed in more depth in **5.1.2.**

Schematic 3



Affinity, Avidity and Functional Avidity (in brief)
TCR affinity refers to the monomeric interaction between one T cell receptor (TCR) and one peptide loaded major histocompatibility complex molecule (pMHCII). TCR avidity is the sum of multiple interactions between TCRs and pMHCII molecule, on a single T cell. Functional avidity is a measure of the biological responsiveness or sensitivity of a single T cell clone, which is contributed to by both TCR affinity and T cell avidity.

1.5.3 TCR avidity and differentiation

Due to the specialised role of Th subsets in controlling different types of immune challenge, a large interest remains in understanding how commitment to these programs are installed or may be predicted. The TCRs role in this regard can be viewed in two ways, either as a simple on/off switch or as more complex tuning or 'diming' device.

Conventionally, it was thought that effector differentiation was predominately dictated by T cell extrinsic and environmental factors such as the cytokines milieu (Hsieh et al., 1993, Coffman et al., 1991). Thus, the TCR was largely viewed as a simple 'off/on' switch, which simply acted to license activation opposed to having a qualitatively role in determining cell fate decisions. This was largely born out of experiments that were able to polarize polyclonal populations of CD4 T cells into almost every flavour of effector T cell desired, via the addition of exogenous cytokines (Hsieh et al., 1993). Indeed, if the role of the TCR was just to switch the cell into a permissive states to allow for programming by cytokines, you would assume that every TCR should be equally able to produce a given effector subset.

However, more recently the quality of TCR signalling has become recognised as being an important determinant (in addition to the cytokine environment), in tuning the quality of effector T cell responses. Hereof, it has been suggested that high and low avidity interactions may favour Th1 and Th2 cell differentiation respectively (Constant et al., 1995, Rogers et al., 1998). Thus the cell-intrinsic ability of a given TCR to recognize foreign antigen may be used to predict its future effector cell differentiation. This would theoretically result in the segregation of clonotypes into certain subsets according to TCR specificity and TCR avidity. In addition to conventional T cells, it has also been suggested that the strength of TCR signalling, upon recognition of self-antigen rather than foreign-antigen, is a mechanism that also exists in Treg T cells to distribute functional properties between Treg clonotypes (Wyss et al., 2016).

To more accurately determine the role of the TCR in this regard (simple switch vs dimmer switch) the potential of a single T cell clonotype to give rise to distinct

effector T cell populations *in vivo* was accessed. Here, transfer of individual wild-type T cells into allogenic hosts (achieved via limiting dilution adoptive T cell transfer approaches), revealed that although single naïve cells are able to initiate diverse differentiation programs they also have clear preferences towards certain cell fates (Tubo et al., 2013a). This seemed to indicate a potential role for the TCR in effector cell differentiation. In a similar approach but now using TCR transgenic (Tg) CD4 T cells, which have a fixed TCR, the contribution of the TCR in effector cell differentiation was further emphasised. Here, effector populations (derived from single cells) were now less heterogeneous suggesting that intrinsic differences in TCR signalling could predetermine effector T cell fate to some extent (Tubo et al., 2013b). However, although less varied, Tg T cells did also show some variability in their effector cell differentiation, presumably achieved via the presence of other cell intrinsic or cell-extrinsic factors that also contribute to differentiation (Tubo and Jenkins). These data clearly highlight the role for the TCR in this process, whilst also highlighting the importance of other factors in this process also.

1.5.4 TCR avidity towards self

In addition to TCR signalling in response to foreign-antigen, more recent evidence suggests avidity towards self-antigen may be just as important in influencing cell fate decisions. Here, a T cell may be influenced by the quality of TCR signalling received during thymic selection and/or via life-long interactions with spMHCII complexes once in the periphery. Clearly the most pronounced effect of self-antigen avidity is the thymic selection of Treg cells. However, self-recognition may also act to imprint conventional naïve CD4 T cells in a much subtler manner, which will be discussed further in **4.1**.

1.5.5 TCR avidity and memory formation

Upon the clearance of antigen, the fate of the majority of clonally expanded T cells is apoptosis, this is known as clonal contraction. However, some privileged effector cells remain; and may further differentiate into memory T cells. This is known as the linear model of memory differentiation (Ahmed et al., 2009). The factors that govern T cell memory selection and formation remain somewhat of a mystery.

However, early characterization studies of memory T cell populations demonstrated a progressive loss of diversity with memory T cell repertoires enriched for clonotypes with the strongest pMHC tetramer binding (McHeyzer-Williams and Davis, 1995, Savage et al., 1999) or highest functional avidity (Fasso et al., 2000). This would favor an antigen selection model, whereby the strength of TCR signaling continues to be important for the persistence of effector cells and in the selection of memory T cells. Accordingly, T cells capable of the strongest TCR signaling at the peak of infection would be the most likely contributors to the memory pool, as they are also the most efficient clonotypes at responding to antigen when levels are diminishing. Recently studies, which utilized TCR sequencing of the CD4 T cell response to Lymphocytic choriomeningitis virus (LCMV), also demonstrated that high avidity CD4 T cells dominated memory T cell populations (Kim et al., 2013b). However, and unlike in previous studies, these (memory) clonotypes were distinct from those that dominated the acute response. This suggesting that the composition of the primary TCR repertoire cannot be used to predict the clonotypes that will go onto to contribute to T cell memory. To complicate findings further, other studies have shown that the composition of the memory T cell repertoire could not be predicted by TCR avidity to antigen, suggesting that multiple parameters probably exist for memory cell formation (Persaud et al., 2014).

1.6 Friend Virus

1.6.1 The Friend Virus Model

Charlotte Friend first discovered Friend Virus in 1957. Initially, it was described as a disease causing agent that resulted in leukemia in Swiss mice, and was capable of cell-free transmission (Friend, 1957). FV is now known to be complex of two viruses, a replication defective spleen focus forming virus (SFFV) and the replication competent Friend-murine leukaemia virus (F-MLV) (Hasenkrug and Chesebro, 1997). Furthermore, FV has been shown to have different capabilities to induce pathology depending on several host genetic-associated factors. This has allowed FV to be used as a model for a broad range of pathologies, depending on the strain of the murine host it is used in. For example, it provides a model for cancer-related studies when used to infect susceptible hosts whilst it is used as a

model for studying protective immune responses to retroviral infection when studied in resistant murine hosts. Still very little is known about the protective forces driving immunity to retroviral infection in humans, the most obvious example being what mediates defence in long-term survivors of HIV infection. Hence the now well-established model of the FV infection in adult resistant mice, contributes greatly to the understanding of immunity to human retroviruses.

1.6.2 Immunity to Friend Virus

Efficient clearance of FV infection in adult resistant mice, requires three major types of lymphocytes and their associated responses: 1) cytotoxic lymphocyte (CTLs) (Manzke et al., 2013, Chesebro et al., 1990) 2) CD4 Th cells 3) B cells (Dittmer et al., 1999).

1.6.2.1 T cell immunity

The haplotype of the hosts MHCII genes plays an important role in the resistance to FV infection as it controls the quality of the conventional CD4 T cell response. CD4 T cells can recognise two epitope variants derived from the FV envelope glycoprotein 70 (gp70) (Iwashiro et al., 1993). The first is located within the N terminus of the gp70 glycoprotein at amino acids 122 to 141 and is presented in the context of H-2A MHCII alleles. The second epitope, was mapped to the C-terminus of gp70, spanning amino acids 462 to 479 and is associated with presentation by H2-E alleles (Iwashiro et al., 1993). As C57BL/6 (B6/WT) mice have a natural mutation within the H2-E allele, which renders it non-functional, CD4 T cells from B6 mice are limited to recognising a single epitope presented by H-2A alleles ($env_{122\text{-}141}$) (Hasenkrug and Chesebro, 1997). In mice expressing functional forms of both H-2A and H-2E alleles, recovery is better associated with peptide presentation by H-2A. Thus, the expression of the H-2A MHCII allele plays an important role in resistance and recovery of B6 mice to FV infection.

In addition to conventional CD4 T cell responses, more recently it has been suggested that CD4 T cells with cytotoxic potential (CD4 CTL) may also play a role in mediating protection to FV infection in certain settings (Manzke et al., 2013). As

B cells are a major reservoir for FV in persistent infection, the ability for CD4 CTLs to direct killing towards MHCII presenting cells may be critical for limiting viral persistence and spread (Hasenkrug et al., 1998). However, the keys players in cytotoxic responses, in a competent immune system are undoubtedly CD8 T cells. CD8 T cells recognise peptide variants in the context of MHCI alleles and direct killing towards MHCI expressing cells (Hasenkrug and Chesebro, 1997, Chesebro et al., 1990)

1.6.2.2 B cell immunity

B cells elicit their own immune responses to FV infection and heavily contribute to efficient viral clearance, where B cell deficient mice are very poorly protected (Hasenkrug et al., 1995). Their major role in protection is thought to be through their production of virus-specific neutralising antibodies which act to control the level of FV infection and replication (Messer et al., 2004). This was highlighted in studies that showed that treatment of B cell deficient mice with neutralising antibodies compensated for the complete absence of B cell within these animals (Messer et al., 2004). In addition, B cells can also function as APCs capable of influencing T cell responses. Upon activation, antigen specific B cells are able to endocytose antigen 1000 fold more efficiently than non-specific cells by virtue of their specific BCRs (Chen and Jensen, 2008, Schultz et al., 1990). This is achieved through extensive cytoskeleton reorganization that not only allows for increased internalization, but also for the preferential processing and loading of BCR bound antigens onto MHCII molecules. This process acts as an ‘antigen focusing’ mechanism and allows activated B cells to serve as proficient APCs that can contribute to CD4 T cell priming *in vivo*. Therefore during FV infection *in vivo*, B cells may not only contribute to FV control via the production of antibodies but may also contribute to the quality of CD4 T cell immune responses (Schultz et al., 1990).

Chapter 2. Materials & Methods

2.1 Mice

All mouse strains used in this study are listed in Table 1. All animal experiments were approved by the ethical committee of the Francis Crick institute, and conducted according to local guidelines and UK Home Office regulations under the Animals Scientific Procedures Act 1986 (ASPA). Male or female mice were used in separate experiments and were age and gender-matched accordingly. Mice were used at 8–12 weeks of age, with the exception of experiments involving neonatal infections, which were carried out on 1–2 day-old mice.

Table 1 A brief description of the mice utilised in this study

Mouse strains	Descriptions	Reference/source
129S8	An inbred mouse strain which is commonly used in immunological research	Jackson Laboratory (JAX)
3G7A	Mice carry TCRα and TCRβ transgenes derived from a Treg T cell	un-published
3G7A Rag$^{-/-}$	Monoclonal TCRαβ Treg TCR transgenic mouse	un-published
3G7B	Mice carry TCRα and TCRβ transgenes derived from a Treg T cell	un-published
3G7B Rag$^{-/-}$	Monoclonal TCRαβ Treg TCR transgenic mouse.	un-published
A (A/J)	An inbred mouse strain, which is commonly used in immunological research.	Jackson Laboratory (JAX)
B6 CD45.1	Otherwise wild-type mice expressing the CD45.1 allele, which is atypical on the C57BL/6 background, allowing for the marking of transferred cells.	Jackson Laboratory (JAX)
B6 CD45.1/CD45.2	Otherwise wild-type mice that express both a CD45.1 and a CD45.2 allele, and thus can serve as recipients for cell transfer experiments.	Jackson Laboratory (JAX)
B6 EF4.1 Bcl6^{c}	An EF4.1 mouse with addition OX40cre and YFP reporter alleles.	(Merkenschlager et al., 2016)
B6 EF4.1 *Prdm1*^{GFP}	An EF4.1 mouse heterozygote for Prdm1GFP allele.	un-published
B6 EF4.1 TCRβ	TCRβ transgenic mouse strain, with an	(Antunes et al., 2008)

	enriched frequency of env-specific CD4 T cells.	
B6.129H2<dlAB1-Ea>	Mice are homozygous null for MHC class II genes H2-Ab1, H2-Aa, H2-Eb1, H2-Eb2, H2-Ea and thus express no MHCII.	Jackson Laboratory (JAX)
B6.TCRα$^{-/-}$	Mice have a targeted disruption in the TCRα chain gene, and therefore have disrupted T cell development.	Jackson Laboratory (JAX)
C57BL/6 /WT/ B6 CD45.2	Wild-type mice.	Jackson Laboratory (JAX)
C57BL/6 Rag$^{-/-}$	Mice are deficient in recombination activation gene, and are thus are deficient in T and B lymphocytes	Jackson Laboratory (JAX)
CBA	An inbred mouse strain which is commonly used in immunological research	Jackson Laboratory (JAX)
EVa2	Mice carry TCR Vα2 and TCRβ transgenes, derived from a high avidity env-reactive clonotype (red clonotype)	(Merkenschlager et al., 2016)
EVa2 Rag$^{-/-}$	Monoclonal TCRαβ TCR transgenic mouse specific for the env peptide (red clonotype)	
EVa3	Mice carry TCR Vα3 and TCRβ transgenes, derived from a low avidity env-reactive clonotype (blue clonotype)	(Merkenschlager et al., 2016)
EVa3 Rag$^{-/-}$	Monoclonal TCRαβ TCR transgenic mouse specific for the env peptide (blue clonotype)	(Merkenschlager et al., 2016)
L23 Rag$^{-/-}$	Monoclonal TCRαβ TCR transgenic mouse specific for the env peptide (green clonotype)	un-published
MuMT/ Igh6$^{-/-}$	Mice have a targeted disruption in the immunoglobulin heavy chain gene, and therefore have disrupted B cell development	Jackson Laboratory (JAX)
Nur77-GFP	The Cre recombinase/green fluorescent protein fusion protein, driven by the Nr4a1 (Nur77) promoter in these mice	Jackson Laboratory (JAX)

2.1.1 Generation of the TCR transgenic mice

2.1.1.1 EF4.1

The EF4.1 mouse is a TCRβ Tg mouse previously generated in the lab. The fixed TCRβ of the EF4.1 mouse, was originally template from the SB14-31 CD4 T cell clone, which recognising the N-terminal region of the envelope glycoprotein of FV (gp70) (Iwashiro et al., 1993).

2.1.1.2 New TCR transgenic mice

Briefly, cDNAs encoding the TCRα chains of CD4 T cell clones, respectively, were cloned and inserted into the hCD2-VA expression cassette (Antunes et al., 2008). Each of these constructs was then mixed with a construct encoding the TCRβ chain of EF4.1 mice and integrated into the DNA of fertilized B6 oocytes following pronuclear microinjection. Transgenic founders were then identified by flow cytometry and genotyping for the presence of the TCRα and TCRβ transgenes. These new transgenic mice were crossed onto the *Rag1*$^{-/-}$ background, to limit TCR expression to that encoded by the transgenes alone. Following which mice were rendered deficient in Emv2, a single copy endogenous retrovirus, to prevent the spontaneous resurrection or expression of Emv2-derived agents (Young et al., 2012b (Young et al., 2012a)).

2.2 Tissue and cell preparation

2.2.1 Media and culture conditions

Room temperature (RT) cell incubation, antibody staining, cell sorting, and tissue collection was performed with Air-Buffered Iscove's-Modified-Dulbecco's-Medium (AB-IMDM) containing 25mM HEPES buffer and L-glutamine, and supplemented with 0.21% NaCl,60 µg/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). Erythrocyte lysis was performed with Ammonium-Chloride-Potassium (ACK) buffer (0.15 M NH4Cl, 1 mM KHCO3, 0.1 mM EDTA, pH 7.2-7.4). Cells were cultured with complete Iscove's-Modified-Dulbecco's-Medium (IMDM) (Sigma-Aldrich) which was supplemented with 5% heat inactivated foetal calf serum (FCS) (BioSera, Ringmer, UK), 2mM L-glutamine, 100µg/ml penicillin, 100µg/ml streptomycin, and 10-5M

mercaptoethanol. For experiments looking at the role of foetal calf serum (FCS), we used Protein free Hybridoma media (PFHM), or IMDM supplemented with only 2mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10-50M mercaptoethanol (Invitrogen). For experiments requiring the long-term culture of activated primary T cells we used, Roswell Park Memorial Institute medium (RPMI) supplemented with 10% FCS, 2mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10-50M mercaptoethanol and recombinant interleukin 2 (IL-2) (Invitrogen). All flow cytometric straining was performed in fluorescence-activated cell (FACS) sorting buffer (PBS, 2% FCS, 0.1% Azide). Primary cells and cell lines were cultured at 95% humidity, 5% CO_2, and 37°C.

2.2.2 Single cell suspensions

Single cell suspensions were prepared from the spleens, thymi and lymph node of mice via their mechanical disruption through 40um or 70um cell-strainers (Falcon, Becton Dickinson Labware) using a 5mL syringe plug. Spleen suspensions were also treated with ACK for a short time, before being re-suspended in AB-IMDM. Bone marrow cell suspensions were prepared by flushing the bone cavities of the femur and tibiae of donor mice with AB-IMDM. This was followed by treatment with ACK lysis buffer. Live cells were then counted using a Neubauer haemocytometer (Marienfield).

2.2.3 Cell purification and enrichment

Enrichment and isolation of primary cells, was performed using a PE immuno-magnetic selection kit (StemCell EasySep). Single cell suspensions were prepared to a concentration of $1x10^8$ ml in AB-IMDM and then incubated with the required PE- conjugated antibody for 30 minutes in the dark. Cells were then washed once in AB-IMDM (to remove excess antibody) before being re-suspended in the starting volume of AB-IMDM. Following which, the EasyStep Selection cocktail was added (25 µl/ml of cells) and the suspension was incubated at RT for 15-20 mins. EasySep Magnetic Nanoparticle beads were then added (25 µl/ml of cells), and the suspensions was gently re-suspended every 5 minutes to ensure the uniform distribution of the beads. After 20 minutes, the suspension was further diluted X2 in AB-IMDM and transferred into 5ml round bottom polystyrene tubes (StemCell)

before being placed onto the separating magnet (EasySep™ Magnet). The magnet generates a high-gradient magnetic field around the polystyrene tube, allowing for the separation of cells labeled with the EasySep™ Magnetic Particles. For positive selection: After 5 minutes the non-labelled cells were poured off in the supernatant by inverting the magnet and tube. This is repeated 2 more times to increase purity, before the walls of the tube, containing the selected cells, were washed with AB-IMDM. For negative selection of cells: The supernatant was collected with cells captured by the magnet being discarded. Purity can them be ascertained using flow cytometry and was typically over 95%.

2.3 *In vitro*

2.3.1 In vitro T cell activation

Single cell suspensions were prepared from the spleens and lymph nodes of donor mice. Cells were then re-suspended in the required media (as described in **2.2.1**) and plated at a final concentration of $1x10^6$ml in 96-well plates or in Corning T25/T75 cell culture flasks (Sigma Aldrich). Cells were then stimulated with the indicated amount of the index $env_{122-141}$ epitope (DEPLTSLTPRCNTAWNRLKL) or with CD3-CD28 T cell-activating Dyna Beads (Thermo-Fisher).

Alternatively, for the activation of monoclonal populations of CD4 T cells or T cell hybridomas, or to access the capacity of distinct APCs to prime cells, T cells were plated at a 1:1 ratio with pre-prepared APCs of choice (see preparation **2.3.4, 2.3.5**) and subsequently stimulated as descried above.

T cell activation was assessed, 18 hours following stimulation, by flow cytometric detection of early markers of activation; or 3-4 days later by the detection of IL-2 via the CTLL-2 assay (**2.3.2**), cytokine production or cell trace dye dilution by FACS. For long-term culture of T cells following activation, cells were culture in RPMI supplemented with recombinant IL-2 **(2.2.1)**.

2.3.2 IL-2 CTLL-2 assay

Upon activation, CD4 T cells rapidly start to produce IL-2. The production of IL-2, by activated T cell populations, is proportional to the degree of activation they

receive. CTLL-2 cells are mouse-derived cytotoxic T cells, which are dependent on IL-2 for their continued growth and survival and thus their cellular growth can be utilized for an *in vitro* measurement of IL-2 production by recently activated CD4 T cells.

Briefly, CD4 T cell are stimulated overnight with the desired concentration of peptide ($env_{122-141}$) or with CD3-CD28 T cell activating Dyna Beads (Thermo-Fisher). Following which 100ul of supernatant, containing the newly produced IL2, is collected. Supernatants may then be frozen in 100ul aliquots to reduce potential contaminants, or used fresh to re-suspend IL-2 deprived CTLL-2 cells. CTLL-2 cells are plated in 96-well plates at a concentration of $5x10^3$ cells/well in 100µl of the supernatant. Following overnight culture, Alamar blue (Biosoure) is added at 1:10 dilution. Respiration, as a consequence of cellular proliferation, results in a change in the colour on the AlamarBlue reagent, allowing the use of fluorescence intensity collected as a measure of cellular growth and hence the determination of relative concentrations of IL-2. On day 2, fluorescence at 590 nm (excitation at 530 nm) was determined with a Safire2 plate reader (Tecan Group, Switzerland). Results were expressed as fluorescence units, with higher intensity indicating higher CTLL-2 growth and, therefore, higher IL-2 amounts.

2.3.3 CFSE labelling

For carboxyfluorescein succinimidyl ester (CFSE) labelling, cells were washed in Dulbecco's Phosphate Buffered Saline solution (D-PBS) (GIBCO) and re-suspended in CFSE (Molecular Probes). For labeling more than $1x10^6$ cells, CFSE was used at 2.5µM, while for lower cell numbers the concentration was 0.6µM. Cells were incubated for 10 minutes in tissue culture conditions and then washed in culture medium. Dividing cells were identified by CFSE dilution via Fluorescence Activated Cell Sorter (FACS) analysis in the subsequent days.

2.3.4 Preparation of Bone Marrow Derived Dendritic Cells

For the production of bone marrow derived dendritic cells (BMDC), bone cavities of femurs and tibiae from donor mice were flushed with 5ml of complete AB-IMDM media which was supplemented with 10% granulocyte macrophage colony-

stimulating factor (GM-CSF). Suspensions were plated in 10 cm cell culture plates and incubated for 7 days. Dendritic cells were harvested by gently washing the culture plates in AB-IMDM to capture loosely adherent cells.

2.3.5 Preparation of Peritoneal Macrophages

For the preparation of *ex vivo* macrophages, approximately 5ml of AB-IMDM was injected into the peritoneal cavity of euthanized mice. The abdomen of mice was then gently massaged to increase cellular yield, following which the cell-enriched media was then carefully aspirated using a 5ml syringe. The aspirated media was kept on ice before being plated in flat-bottomed tissue culture plates for 1-2 hours at 37°C, thus allowing time for the peritoneal macrophages to adhere to the plastic. Following this time, non-adherent cells were washed away, and the wasted media replenished.

2.3.6 Preparation of Splenic B cells

Enrichment and isolation of primary cells, was performed using an PE-immuno-magnetic selection kit (StemCell EasySep). Single cell suspensions were prepared to a concentration of $1x10^8$ ml in AB-IMDM and then incubated with the B220- PE conjugated antibody for 30 minutes. Cells were then process according to **Cell purification and enrichment methods 2.2.3**

2.3.7 Generation of novel Hybridoma cell lines

The generation of novel T cell hybridoma cell lines involved the fusion of primary T lymphocytes to partner thymoma cells, resulting in the immortalization of the primary T cell. Briefly, activated virus-specific CD4 T cells were sorted *ex vivo* from immunized mice and mixed at a 1:1 ratio with TCRα/β-negative BW5147 thymoma cells (Letourneur and Malissen, 1989). Cells were pelleted and polyethylene glycol (PEG) was added drop-wise to mediate the fusion between partner cells. Fused cells were then re-suspended in Hypoxanthine Aminopterin Thymidine (HAT) selection media at limiting dilutions. Fusion partners were plated on-top of a layer of *ex vivo* peritoneal macrophages (**2.3.5**) which act to phagocytise dead cells and keep the culture conditions optimal. Following 10-14 days, individual hybridoma

colonies could then be harvested and gradually weaned off the HAT selection media and onto complete IMDM. T cell hybridomas were subsequently probed by staining with an anti-Vα2 (clone B20.1) or anti-Vα3.2 (clone RR3-16) monoclonal antibodies, or via the sequencing of expressed *Trav* genes (.

2.4 In vivo

2.4.1 T-cell purification and adoptive transfer

Single-cell suspensions were prepared from the spleens and lymph nodes of donor B6/CD45.1 or B6/CD45.2 mice and CD4 T cells were isolated using CD4-PE immune-magnetic positive selection (**2.2.3**). A total of $1x10^6$ CD4 T cells were intravenously injected (i.v) into B6 CD45.1/CD45.2 recipients. Where indicated, EF4.1 CD4 T cells were instead purified by cell sorting (at > 99% purity), performed on MoFlo cell sorters (Dako-Cytomation, Fort Collins, CO, USA).

2.4.2 Generation of mixed bone-marrow chimeras

Bone marrow cell suspensions were prepared from separate donor mice by flushing the bone cavities of femurs and tibiae in AB-IMDM. Cell suspension were briefly treated in ACK lysis buffer for 2-5 min at room temperature to erupt erythrocyte cells, following which live cells were then counted using a haemocytometer.

For the generation of bone marrow chimeras MHCII-sufficient or -deficient bone marrow cells were injected separately into non-irradiated *Ighm*$^{-/-}$ recipients. In this setting, only the missing lymphocyte population (B cells) was properly reconstituted, creating B-cell-specific loss of MHCII. Each recipient received between $1x10^7$ and $3x10^7$ bone marrow cells. Mice were bled for assessment of reconstitution and were used for infection 8–10 weeks post bone marrow transfer.

For the generation of mixed bone marrow chimeras, B6 CD45.1 and 3G7 *Rag*$^{-/-}$ bone marrow were mixed at the desired ratios and injected together into non-irradiated TCRα$^{-/-}$ recipients. This generating a mixed T cell population derived from both donors in the recipient mice. Each recipient received between $1x10^7$ bone

marrow cells. Mice were bled for assessment of reconstitution and were used for infection 8–10 weeks post bone marrow transfer.

2.5 Retroviral infection and immunization

2.5.1 FV infection

The FV used in this study was a retroviral complex of a replication-competent B-tropic F-MLV and a replication-defective SFFV. Stocks were propagated *in vivo* and prepared from the spleens of infected susceptible mice. A pool of 20 LDV-free BALB/c mice was infected with FV, spleens were isolated 12 days later and homogenized (10% w/v) in PBS. Aliquots of spleen homogenates were frozen and were subsequently used for infection. Mice received an inoculum of ~1,000 spleen focus-forming units of FV by intravenous injection. Stocks of F-MLV-B and F-MLV-N helper viruses were grown in *Mus dunni* fibroblast cells. Mice received an inoculum of ~104 infectious units of F-MLV by intravenous injection. Neonatal F-MLV infection was performed by administering an inoculum of ~4,000 infectious units of F-MLV-B to 1-day-old mice by intraperitoneal injection. All stocks were free of Sendai virus, Murine hepatitis virus, Parvoviruses 1 and 2, Reovirus 3, Theiler's murine encephalomyelitis virus, Murine rotavirus, Ectromelia virus, Murine cytomegalovirus, K virus, Polyomavirus, Hantaan virus, Murine norovirus, Lymphocytic choriomeningitis virus, Murine adenoviruses FL and K87, Mycoplasma sp. and LDV. For co-infection of FV and LDV, a similarly prepared stock of FV additionally containing LDV was also used.

2.5.2 F-MLV copy number analysis

DNA copy numbers of F-MLV were determined by real-time quantitative PCR (qPCR) on DNA samples isolated from the spleen cell suspensions from infected mice, using primers specific to F-MLV env DNA (125 bp product):

forward 5′ -AAGTCTCCCCCCGCCTCTA-3′

Reverse 5′ -AGTGCCTGGTAAGCTCCCTGT-3′

Signals were normalized for the amount of DNA used in the reactions based on amplification of the single-copy Ifnar1 gene (150 bp product) with primers:

Forward 5′ -AAGATGTGCTGTTCCCTTCCTCTGCTCTGA-3′

Reverse 5′ -ATTATTAAAAGAAAAGACGAGGCGAAGTGG-3′

Copy numbers were calculated with a $\Delta\Delta$CT method and are expressed as copies per million cells.

2.5.3 Peptide Immunization

For peptide immunization, WT or *Ighm*$^{-/-}$ recipient mice received an intra-peritoneal (i.p) injection of a total of 12.5 nmol of synthetic env$_{122\text{-}141}$ peptide mixed in Sigma Adjuvant System® (adjuvant was used to provide the necessary stimulus to the immune system to allow CD4 T cell responses).

2.5.4 FBL3 tumour challenge

FBL3 is a FV-induced cell line that expressed the FV CD4 epitipe (gp70) as described (Klarnet et al., 1989). WT or *Ighm*$^{-/-}$ recipient mice were inoculated with 3×10^6 FBL-3 cells via intravenous injected followed by T cell transfer.

2.6 Flow cytometric analyse

2.6.1 Reagents and list of antibodies

Table 2 List of reagents and antibodies

Antibody	Description	Fluorophore	Source	Clone	Dilution
A^b-env$_{125\text{-}135}$ tetramer	Tetramer	APC	Dr Marion pepper	N/A	1 in 25
Alexa Fluor 488 kit	Alexa Fluor 488 amplification kit for fluorescein	Alexa fluro 647	Invitrogen	N/A	1/200
B220	PE-TR Rat Anti-MouseCD45R (B220)	Pe-Texas Red	Invitrogen	RA3-6B2	1/300
B220	Brilliant Violet 605™ anti-mouse/human CD45R/B220 Antibody	BV605	Biolegend	RA3-6B2	1/200
CD19	Anti-Mouse CD19 APC	APC	Life technologies	6D5	1/100
CD2	PE/Cy7 anti-mouse CD2 Antibody	PECY7	Biolegend	RM2-5	1/200
CD25	Anti-Mouse	APC	Ebioscience	PC61.5	1/200

	CD25 APC				
CD38	APC anti-Mouse CD38	APC	Ebioscience	90	1/200
CD38	FITC anti-Mouse CD38	FITC	Ebioscience	90	1/200
CD4	Anti-Mouse CD4 PE-Cyanine7	PE-Texas Red	Ebioscience	GK1.5	1/200
CD4	APC anti-Mouse CD4,	APC	EBioscience	GK1.5	1/200
CD4	PE anti-mouse CD4 (L3T4)	PE	Ebioscience	GK1.5	1/200
CD4	Anti-Mouse CD4 PE-Cyanine7	PE-Cyanine7	Ebioscience	GK1.5	1/200
CD4	FITC anti-Mouse CD4 (L3T4)	FITC	Insight Biotech	RM4-5	1/200
CD43	PE - CD43 Activation-Associated Glycoform	PE	BD	1B11	1/200
CD44	V500 Rat anti-Mouse CD44	V500	BD Biosciences	IM7 (RUO)	1/200
CD44	Pacific Blue® anti-Mouse/Human CD44 (Pgp-1, Ly-24),	Pacific-Blue	Biolegend	IM7	1/200
CD45.1	Anti-Mouse CD45.1 eFluor® 450	Efluoro-450	Ebioscience	A20	1/300
CD45.1	APC/Cy7 anti-mouse CD45.2 Antibody	APCy7	Biolegend	104	1/200
CD45.1	PE anti-Mouse CD45.1	PE	EBioscience	A20	1/200
CD45.2	Anti-Mouse CD25 APC	APC	Ebioscience	PCB1.5	1/200
CD45.2	APC-eFluor® 780 anti-mouse CD45.2	APC-eFluor 780	Ebioscience	104	1/200
CD45.2	APC/Cy7 anti-mouse CD45.2 Antibody	APCy7	Biolegend	104	1/200
CD45.2	APC-eFluor® 780 anti-mouse CD45.2	APC-eFluor® 780	Ebioscience	104	1/200
CD5	Anti-Mouse CD5 PE	PE	Ebioscience	53-7.3	1/500
CD69	FITC anti-Mouse CD69	FITC	EBioscience	H1.2F3	1/200
CD69	Pacific Blue anti-Mouse CD69,	PB	BioLegend (Cambridge Bioscience)	H1.2F3	1/200
CD8α	Anti-Mouse CD8a FITC	FITC	Ebioscience	53-6.7	1/300

Fcγ III	2.4G2 blocking antibody	N/A	NIMR/Francis Crick	N/A	1/200
Foxp3	Anti-Mouse/Rat Foxp3 PE (FJK-16s)	PE	EBiosciences	KIT	1/200
GL7	T- and B-cell activation antigen, FITC-GL7	FITC	BD	GL7	1/200
Glyco-gag	34	FITC	NIMR/Francis Crick	N/A	1/300
IFN-γ	Anti-Mouse IFN gamma	FITC	Biolegend	XMG1.2	1/200
IFN-γ	Anti-Mouse IFN gamma	APC	EBiosciences	XMG1.2	1/200
IgD	PE anti-mouse IgD	PE	Ebioscience	11-26c	1/200
IgD(b)	Brilliant Violet 711™ anti-mouse IgD Antibody	BV711	Biolegend	11-26C.2A	1/200
IgM(b)	FITC anti-mouse IgM Antibody	FITC	Biolegend	RMM-1	1/200
IL-2	Anti-Mouse IL-2 APC	APC	EBiosciences	JES6-5H4	1/200
Ly6c	Biotin Rat Anti-Mouse Ly-6C	Biotin	BD	AL-21	1/200
MHCII	FITC anti-Mouse MHC Class II (I-A/I-E)	Fitc	Ebioscience	M5/114.15.2	1/300
Streptavidin	Streptavidin Pe-Texas Red conjugated	Pe-Texas Red conjugated	Invitrogen	SA1017	1/300
Streptavidin	Streptavidin PE	PE	BD Pharmingen	N/A	1/300
TCR Vα2	Anti-mouse V_2 TCR APC	APC	ebioscience	B20.1	1/200
TCR Vα3.2	FITC Rat ant-Mouse Va 3.2b,c TCR	FITC	BD	RR3-16	1/200
TCR Vα3.2	FITC Rat ant-Mouse Va 3.2	FITC	EBioscience	RR3-16	1/200
TCR Vβ	Vb TCR Screening Panel (kit)	FITC	BD Biosciences	Vb various	1/300
TCRVβ 10b	Vb TCR Screening Panel (kit)	FITC	BD Biosciences	RR3-15	1/200
TCRVβ 11	Vb TCR Screening Panel (kit)	FITC	BD Biosciences	RR3-15	1/200
TCRVβ 12	Vb TCR Screening Panel (kit)	FITC	BD Biosciences	MR11-1	1/200
TCRVβ 13	Vb TCR Screening	FITC	BD Biosciences	MR12-3	1/200

	Panel (kit)				
TCRVβ 14	Vb TCR Screening Panel (kit)	FITC	BD Biosciences	142	1/200
TCRVβ 17a	Vb TCR Screening Panel (kit)	FITC	BD Biosciences	KJ23	1/200
TCRVβ 2	Vb TCR Screening Panel (kit)	FITC	BD Biosciences	B20.6	1/200
TCRVβ 3	Vb TCR Screening Panel (kit)	FITC	BD Biosciences	KJ25	1/200
TCRVβ 4	Vb TCR Screening Panel (kit)	FITC	BD Biosciences	KT4	1/200
TCRVβ 5.1 and 5.2	Vb TCR Screening Panel (kit)	FITC	BD Biosciences	MR9-4	1/200
TCRVβ 6	Vb TCR Screening Panel (kit)	FITC	BD Biosciences	RR4-7	1/200
TCRVβ 7	Vb TCR Screening Panel (kit)	FITC	BD Biosciences	TR310	1/200
TCRVβ 8.1 and 8.2	Vb TCR Screening Panel (kit)	FITC	BD Biosciences	MR5-2	1/200
TCRVβ 8.3	Vb TCR Screening Panel (kit)	FITC	BD Biosciences	MR10-2	1/200
TCRVβ 9	Vb TCR Screening Panel (kit)	FITC	BD Biosciences	B21.5	1/200
TCRβ	Anti-Mouse TCR beta APC-eFluor® 780	Apc-efluro 780	Ebioscience	H57-597	1/200
TCRβ	APC-Cy7 anti-Mouse TCRb	APC-Cy7	Ebioscience	H57-597	1/200
TCRβ	APC anti-Mouse TCRb	APC	Ebioscience	H57-597	1/200
Vα2	FITC anti-Mouse Va2 TCR,	FITC	BD	B20.1	1/200
Vα2	Anti-mouse V_2 TCR APC	APC	Ebioscience	B20.1	1/200

2.6.2 Calculations and absolute numbers

Single-cell suspensions were diluted in AB-IMDM. Live cells were counted using a Neubauer haemocytometer (Marienfield).

2.6.3 Cell surface staining (CSS)

Single-cell suspensions were stained with directly-conjugated antibodies to cell surface markers, obtained from eBiosciences (San Diego, CA, USA), CALTAG/Invitrogen,BD Biosciences (San Jose, CA, USA) or BioLegend (San Diego, CA, USA) and anti-FcR monoclonal antibody (2.4G2) was to block non-specific binding. All staining was performed at 4°C in the dark and were incubated in FACS buffer.

2.6.4 Intracellular cytokine staining (ICS)

For the intracellular detection of cytokines cells were stimulated with phorbol 12,13-dibutyrate (PDBu) and Ionomycin (both at 0.5 µg/ml) at 37°C in AB-IMDM. Here, Ionomycin and PDBu synergise to activate protein kinase C (PKC) and mobilise intracellular calcium, resulting the putative activation of the T cell independently of TCR specificity. After 1 hour, cells were treated with monensin (at 1µg/ml) for a further three hours, to allow for the accumulation of cytokines within the activated T cells. Following this cell were then washed and stained for cell surface antibodies in FACs buffer. Following cell-surface antibody staining cells were then washed and resuspended in fixation buffer (eBioscience) for 20 minutes. Cell were then washed again before being resuspended in permeabilization buffer (eBioscience) supplemented with antibodies for cytokine staining for one hour at RT. After the combined permeabilisation on staining step, cells were washed a further two times before finally being re-suspended in FACS buffer for analysis.

2.6.5 Intra-nuclear Foxp3 staining (INS)

FoxP3 expression was accessed by intranuclear staining using a FoxP3-staining kit (eBiosciences). Following cell-surface antibody staining cells were fixed and permeabilisaton using an anti-mouse/rat FoxP3 staining kit (eBbioscience),

according to manufactures instructions. Cells were washed before being re-suspended in FACS buffer for analysis.

2.6.6 Tetramer staining

Cells were incubated with the conjugated Abenv$_{135\text{-}141}$ tetramers (kindly provided by Dr Marion Pepper) and incubated at 37°C for 3 hours in RPMI. Cells were mixed gently at 30 minute intervals to ensure uniform distribution and staining. Cell surface antibodies were then added and the cells were incubated in the dark at RT for 20 minutes. Cells were washed and re-suspended in FACS buffer before analysis.

2.6.6.1 Acquisition and cell sorting

Data were collected on BD FACSCantoII (BD Biosciences), BD FACSFortessaX20 (BD Biosciences), BD FACSFortessaX30, FACSCalibur (BD Biosciences) and CyAn (Dako, Fort Collins, CO) flow cytometers. Cells were sorted on BD FACSAria II (BD Biosciences), BD Influx (BD Biosciences) or Beckman Coulter MoFlo XDP cell sorters (BD Biosciences) (at purity>98%). Data was processed and analyzed using BD FACSDiva Software, FlowJo v10 (TreeStar) or Summit v4.3 (Dako) analysis software.

2.7 RNA preparation and TCR sequencing

2.7.1 RNA purification and extraction from cells

Total RNA from single cell suspensions was extracted with the RNEasy Mini and Micro Plus kits (Qiagen) according to the manufacturer's instructions or isolated using the QIAcube (QIAGEN, Crawley, UK). Synthesis of cDNA was carried out with the High Capacity Reverse Transcription kit (Applied Biosystems, Carlsbad, US) with an added RNase-inhibitor (Promega Biosciences, Madison, US) according to the manufacturer's instructions. Final RNA concentrations could be quantified using a Nanodrop 1000 (ThermoScientific)

2.7.2 Next-generation sequencing of the TCR repertoire

For TCR sequencing, naive or primed env-specific EF4.1 CD4 T cell subsets were purified by cell sorting (>98%) and RNA was isolated using the QIAcube (QIAGEN, Crawley, UK). Synthesis of cDNA was carried out with the High Capacity Reverse Transcription kit (Applied Biosystems, Carlsbad, CA, USA) with an added RNase-inhibitor (Promega Biosciences, Madison, WI, USA). A final clean-up was performed with the QIAquick PCR purification kit (Qiagen). Purified cDNA was then used as template for the amplification of *Trav14* (encoding Vα2)- or *Trav9* (encoding Vα3)-containing rearrangements, using the following primers:

***Trav14* forward:** **5′-CAAGCTTCAGTCTAGGAGGAATGGAC-3′**

***Trav9* forward:** **5′-CCAAGGCTCAGCCATGCTCCTGGC-3′**

***Trac* common reverse:** **5′-TAACTGGTACACAGCAGGTTCTGGG-3′**

The forward *Trav*-specific primers were located in a promoter region common to all members of the respective *Trav* family and the common reverse was in the common constant region, thus amplifying the entire variable and joining coding region (~500 bp product). Between 4,500 and 43,000 sequences per sample (450 bp median size) were obtained on a GS FLX System by GATC Biotech (Constance, Germany).

2.7.2.1 Analysis

Identification of productive rearrangements, *Trav* and *Traj* gene segment annotation, protein translation and CDR3 segment prediction were performed by the ImMunoGeneTics (IMGT) online tool HighV-QUEST.

2.8 Genomic DNA preparation

2.8.1 Extraction from cells

For the isolation of DNA from cells, cell suspensions were washed in PBS to remove FCS contaminants. Pelleted cells were then re suspended in 500ul of Tail buffer (1% SDS, 0.1M NaCl, 0.1M EDTA, 0.05M Tris,315ml sterile distilled H_2O) and digested with10ul of protease k (20mg/ml) added. This is left at 56°C for 2 hours on a heat block or overnight. To these samples, 0.7 volumes of isopropanol was added and vigorously re-suspended. The material was pelleted, the

supernatant aspirated, and the material re-suspended in 100% ethanol. The material was pelleted, the supernatant aspirated and then the pellet was air dried before being re suspended is distilled H_2O at the desired concentration.

2.8.2 F-MLV copy number analysis

DNA copy numbers of F-MLV were determined by real-time quantitative PCR (qPCR) on DNA samples isolated from the spleen cell suspensions from infected mice, using primers specific to F-MLV env DNA (125 bp product):

forward 5′ -AAGTCTCCCCCCGCCTCTA-3′

reverse 5′ -AGTGCCTGGTAAGCTCCCTGT-3′

Signals were normalized for the amount of DNA used in the reactions based on amplification of the single-copy Ifnar1 gene (150 bp product) with primers:

forward 5′ -AAGATGTGCTGTTCCCTTCCTCTGCTCTGA-3′

reverse 5′ -ATTATTAAAAGAAAAGACGAGGCGAAGTGG-3′

Copy numbers were calculated with a ΔΔCT method and are expressed as copies per million cells.

2.9 Micropipette adhesion frequency assay

Two-dimensional TCR affinities were measured by a micropipette adhesion frequency assay, using the T-cell hybridomas H5, L23 and H18 and Ab-$env_{125–135}$ tetramer. Cells were brought into contact 50 times with the same contact time and area (Ac), and an adhesion frequency (Pa) was calculated. Surface pMHC and TCRβ densities were determined by flow cytometry and BD QuantiBRITE PE Beads for standardization (BD Biosciences). These parameters were then used to calculate two-dimensional affinity using the following equation: Ac × Ka=−ln[1—Pa(∞)] mr−1 ml−1 where mr and ml represent TCR and pMHC surface densities, respectively.

2.10 Statistical analysis

Statistical comparisons were made using SigmaPlot 12.0 (Systat Software, Germany). Parametric comparisons of normally distributed values that satisfied the variance criteria were made by unpaired Student's *t*-tests. Data that did not pass

the variance test were compared with non-parametric two-tailed Mann–Whitney rank sum test. Comparison of Vβ family expression, hierarchical clustering and heat-map production was with Qlucore Omics Explorer (Qlucore, Lund, Sweden).

Chapter 3. TCR specificity and avidity in Treg T cells

3.1 Introduction

Treg T cells play a pivotal role in maintaining tolerance to self and in restraining the pathogenic potential of auto-reactive T cells that exist in the periphery. Although indispensable, these cells also exist as a niche subset, restricted to approximately 10% of the peripheral CD4 T cell population. Clearly too many Tregs, as well as too few, can be detrimental to the host. Understanding how this balance is met and maintained may lie in better defining the parameters surrounding their development and selection. What instructs for the illusive Treg fate remains a very important question.

3.1.1 A brief history

The idea of a T cell population with innate suppressor capacity first came about during the 1960s and 1970s. Here, an initial publication in Science, presented two major findings 1) that neonatal thymectomy in mice lead to their spontaneous development of autoimmunity 2) and that this could be rescued by thymic grafting (Nishizuka and Sakakura, 1969). This work was shortly followed by a study from Gershon and colleagues that demonstrated that some T cells might also suppress antibody mediated responses (Gershon and Kondo, 1970). Two years later, Gershon and Kondo went on to publish their seminal work, the first to propose and define a subset of T cells capable of suppressing antigen-induced responses of other Th subsets (Gershon et al., 1972). These cells were christened the ‘suppressor T cells’, and these findings gave birth to a new field in immunology (Gershon et al., 1972). Following which, interest in the suppressor T cell subset was explosive and they remained intensively studied over the following years. However, the field took a major hit during the 1980s, which saw it virtually collapse in both popularity and integrity. Although this was most likely a result of a combination of failings, it was heavily contributed toward by the absence of lineage specific markers and the lack of the theoretical I-J region, thought to be encoded between the I-A and I-E sub regions of MHCII locus and suggested to be implicit in suppressor function (Kronenberg et al., 1983). However, experiments conducted in

the 1990's, saw the field revised and revitalised, featuring the rebranding of the suppressor T cell as the regulatory T cell. Here, the discovery of markers that could be used to define Treg T cell populations (firstly CD5 and CD45R and then progressively CD25) allowed for their isolation and characterisation (Sakaguchi et al., 2007). Work published by Sakaguchi et al in 1995 then went on to show that antibody-mediated depletion of $CD25^{positive}$ T cells was sufficient to drive spontaneous autoimmunity in previously healthy hosts, pheno-copying the early pathologies observed in thymectomized mice (Sakaguchi et al., 1995). This suggested that although auto-reactive T cells (with the potential to cause immune-pathology) existed in healthy mice, under-normal conditions these could be kept in check by a subset of regulatory $CD25^{positive}$ T cells. In 2001, the disease-associated gene in Scurfy mice, mice that present with fatal lympho-proliferative autoimmune disorder, was identified as being *Foxp3* (Brunkow et al., 2001). By 2003, Foxp3 was shown to be important in the development and function of Treg T cells (Fontenot et al., 2003b, Hori et al., 2003). Moving forward, retroviral transduction of Foxp3 into naïve conventional T cells was shown to be sufficient to illicit the Treg phenotype in those cells, and thus established Foxp3 as the master regulator of the Treg lineage (Hori et al., 2003). The culmination of this work now means that Treg T cells are widely accepted as playing an indispensable role in the maintenance of peripheral tolerance. Whilst many fundamental questions remain unanswered or at least highly debated, popularity in the field is still driven by the prospect that a better understanding of this cell type could have revolutionary consequences on the treatment and management of autoimmune diseases, transplantation and cancer therapeutics.

3.1.2 Origin of T regulatory cells

Thymic generation of Treg T cells seems to require TCR signalling, upon self-recognition, of sufficiently high levels to drive the expression of Foxp3 (as has been discussed previously). In addition, Treg T cells can also be generated via the conversion of conventional T cells once in the periphery, these are known as peripherally converted Treg (pTreg) T cells.

Although the exact events that drive peripheral conversion remain unclear, evidence suggests that the TCR and hence the quality of TCR signaling may be

important (Lathrop et al., 2008, Pacholczyk et al., 2006, Mantel et al., 2006, Long et al., 2011, Sauer et al., 2008).

3.1.3 TCR specificity in T regulatory cells

Still relatively little is known about the TCR requirements of a Treg T cell, whether this be during development, homeostasis or in their peripheral function. However, the TCR repertoire has long been associated with self-reactivity, suggesting that certain traits, associated with select TCRs, may be important for this lineage. In this vein, TCR repertoire analysis, which compared conventional T cell TCR repertoires to Treg TCR repertoires, first highlighted the utilisation of different receptors by the two populations. In addition to being distinct, the Treg TCR repertoire was also reportedly enriched for self-reactive clones (Romagnoli et al., 2002, Hsieh et al., 2004, Hsieh et al., 2006). In line with this, characterisation of Foxp3 deficient mice revealed that the pathogenic T cells in these animals, had overlapping TCR repertoires to Treg T cells found in healthy (Foxp3 sufficient) mice; hence re-installing the self-reactive nature of the Treg TCR repertoire (Hsieh et al., 2006). Treg T cells are also notably absent from conventional double TCRαβTg mice suggesting that specific TCRs are required for Treg T cell development. However, studies have also shown that Treg T cell development can be restored in conventional TCRαβ Tg mice once cognate-antigen is expressed via the introduction of second transgene in the thymus (Jordan et al., 2001). This suggested that it was the self-reactive capacity of a given TCR that allowed it to be instructive for the Treg T cell fate. On the other hand, some studies have also reported substantial overlap between the TCR repertoires of Tcon and Treg T cells (Pacholczyk et al., 2007, Kasow et al., 2004). Again, their self-reactive nature was called into question when it was shown that Treg T cells were also capable of recognising foreign-antigens such as *Mycobacterium Tuberculosis* (Shafiani et al., 2010), *Candida albicans* (Montagnoli et al., 2002) and *Leishmania major* (Belkaid et al., 2002) and *Mycobacterium Tuberculosis* among others (Pacholczyk et al., 2007, Kasow et al., 2004). This has complicated the finer assessment of the TCR requirements necessary for Treg T cell development, maintenance and function.

3.1.4 TCR usage in T regulatory cells

In Tcon cells, TCR signalling is required for both tonic pro-survival signalling and for the initiation of T cell activation in response to infection. However, the role for TCR signalling in Treg T cell homeostasis and peripheral function had remained largely unexplored up until a recent study from Rudensky's lab (Levine et al., 2014). Here, conditional ablation of the TCR on Treg T cells revealed that although the maintenance of the Treg cell–specific gene signature was TCR independent, suppressor function was critically dependent on TCR expression (Levine et al., 2014). However, what remained relatively un-explored was to what degree the quality of the TCR (and its signalling), rather than the presence of any TCR at all, played a role in aspects of Treg T cell fate and function. Whether TCR specificity, avidity and self-reactivity continue to play important roles in maintaining the Treg phenotype once in periphery, remains an interesting question.

3.1.5 Aim

Given the fundamental role of the quality of TCR signalling in many aspects of T cell fate decisions, the instructive capacity of certain TCRs to give rise to Treg T cells was of interest to us. In light of many studies reporting the preferential use of self-reactive TCRs by Treg T cells, we were particularly interested in exploring the role of self-reactivity in their development. To interrogate if Treg T cell lineage commitment was a 'TCR instructive process' and to explore to what degree self-reactivity was implicit in this, we generated Treg TCR TCRαβ transgenic (Tg) mouse strains within the lab.

3.2 Results

3.2.1 Generation of transgenic mouse with a TCR derived from a Treg T cell.

To ask, if the specificity of Treg TCRs, was intrinsically prescriptive for the regulatory program we wanted to study Treg cell development in the Treg derived-TCRαβ transgenic mice. We therefore needed to identify, isolate and subsequently clone TCRs derived from Treg T cells to template for TCRα and TCRβ transgenes.

A major topic in our lab is the study of CD4 T cell immune responses to Friend Virus (FV) infection. In WT mice the precursor frequency of CD4 T cells specific to FV is low, thus limiting the capacity to study CD4 T cell kinetics to FV infection. In order to achieve better resolution of CD4 T cell responses to FV, our studies extensively make use of a TCRβ Tg mouse which was generated within the lab, the EF4.1 mouse (Antunes et al., 2008). The TCRβ transgene expressed by the EF4.1 mouse strain was originally derived from a CD4 T cell clone reactive to the envelope glycoprotein of FV (gp70). Expression of this TCRβ transgene, and its subsequent pairing to endogenous TCRα chains, results in an increased frequency of FV specific CD4 T cells in these mice. Importantly, and in keeping with other observations made in TCRβ Tg mice, the expression of the transgene also allowed for Treg T cell development, although perhaps to a slightly lesser extent than in WT mice (Antunes et al., 2008). In addition, specificity of these Tregs T cells, did not seem to overlap with the pool of FV-reactive T cells (Antunes et al., 2008). This permissiveness for near-normal T cell development in the EF4.1 mice allowed us to use them as a source of Treg T cells.

In order to identify and then isolate Treg T cell clones and their TCRs, the EF4.1 mouse was crossed to a B6.Cg-Foxp3tm1Mal/J ($Foxp3^{GFP)}$ reporter mouse (Wang et al., 2008). This reporter allows for the expression of a green fluorescent protein (GFP) within cells expressing Foxp3. The targeting of the IRES-GFP cassette to exon 11, downstream of the endogenous *Foxp3* 'STOP" codon, allows for the insertion of a GFP reporter without disruption to Foxp3 expression itself. Ultimately this provided a method to allow for the identification of Foxp3 Treg T cells by GFP fluorescence. Using Fluorescence-activated cell sorting, $CD4^{high}$, $CD44^{low}$, $CD25^{high}$, Foxp3-GFP^{high} Treg T cells were sorted from EF4.1 $Foxp3^{GFP}$ reporter mice. These cells were then used to generate Treg T cell hybridomas via their fusion to thymoma partners (BW5147) *in vitro.*

One clone, the 3G7, was selected and used to create the 3G7 A, B, C Treg-TCRαβ transgenic founder strains. These lines were backcrossed onto the C57BL/6 (B6) background for 10 generations, with the exception of the 3G7C strain which did not propagate beyond n=6 generations. Hereon in, we only use the 3G7A and 3G7B founder strains in our analysis. The decision to use two founder strains instead of

just the one, was in foresight of the random integration and hence copy number of TCR transgenes within each founders genome. We reasoned that both the location and the number of successful integrations would influence the levels of transgene/TCR expression. Potentially, this would then allow for the same TCR to have differing signaling potentials between the founder mice as a consequence.

The generation of these transgenic mice, using standard techniques, was all done prior to my arrival in the lab whilst the characterisation of these mice was work conducted by myself.

3.2.2 Reactivity 3G7 T cells with self and foreign antigen.

To study the properties of the 3G7 TCR we utilised the 3G7 hybridoma cell line, propagated from the original fusion event between an *ex vivo* Treg T cell and a thymoma partner. Importantly, although the 3G7 hybridoma retained the TCR specificity of the parental Treg T cell, it did not retain the Treg cell phenotype. This then allowed us to use conventional T cell assays to study T cell reactivity and activation.

Preliminary results showed, the 3G7 hybridoma to spontaneously activate *in vitro* when co-cultured with C57BL/6 splenocytes, as accessed by CD69 up-regulation and later by IL-2 production (Figure 1a-b). To define if this activation was TCR driven, we co-cultured 3G7 hybridoma with MHCII deficient antigen presenting cells (APCs) derived from B6.129H2<dlAB1-Ea> mice. Lack of stimulation in this setting suggested activation to be dependent on TCR-MHCII derived interactions. T cell TCRs can recognise peptide MHCII complexes (pMHCII), however it was possible that the 3G7 TCR could be recognising exposed MHCII backbone residues rather than specific peptides complexed with MHCII. Therefore, we next sought to delineate if cross-reactivity of the TCR to the MHCII backbone, or conventional peptide presentation by MHCII was accounting for 3G7 hybridoma activation. To do so, we co-cultured 3G7 hybridoma with a range of allogenically distinct APCs (Table 3). These experiments showed activation to be restricted to the $H2^b$ haplotype, shared by only 129 and C57BL/6 mice (Figure 1b). This was suggestive that the TCR was not alloreactive, as the 3G7 hybridoma could not tolerate diverse

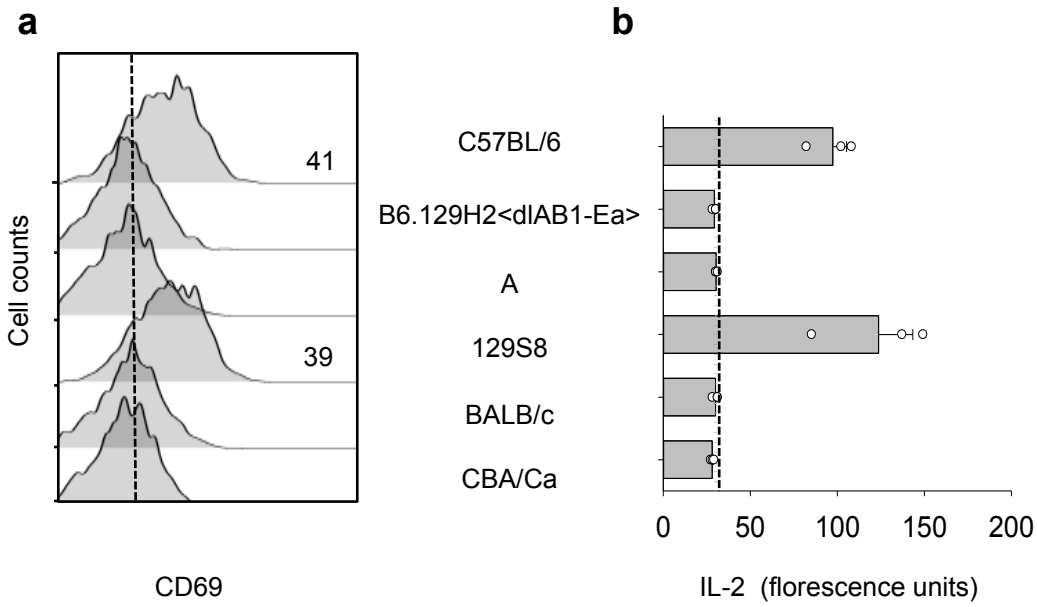
MHCII haplotypes. But without an understanding of whether this restriction was driven by altered peptide presentation between diverse haplotypes, or driven by alterations to the exposed MHCII residues (that the TCR may be recognising) we could not conclusively prove this.

Table 3 MHCII haplotypes

The MHC haplotype in these strains is designated by a small letter.

Mouse strain	MHCII haplotypes
C57BL/6	b
B6.129H2<dlAB1-Ea>	null
A	a
129S8	b
BALB/c	d
CBA/Ca	k

Figure 1 3G7 Hybridoma cells are restricted to MHCII haplotype H2b

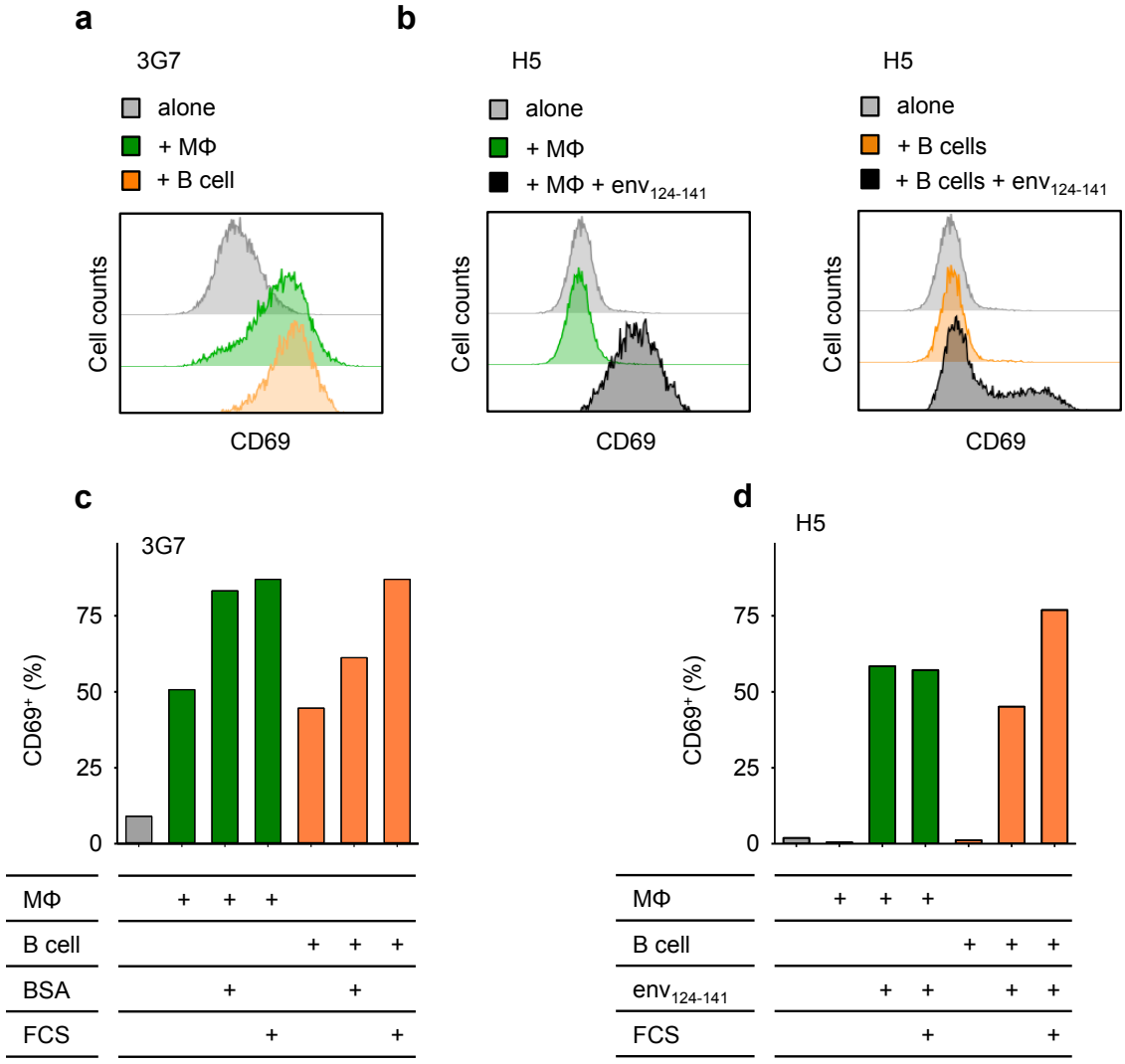


In vitro determination of hybridoma responses upon co-culture with splenocytes of diverse MHCII haplotypes for 18-hours.
(a) Responsiveness of the 3G7 hybridoma cell line, as measured by CD69 induction. Numbering denotes the percentages of cells determined as $CD69^{+}$, where percentage less than 5% are not marked (b) or fluorescence quantitation of AlamarBlue, representing relative concentrations of IL-2 produced by 3G7 hybridoma cells, where the dashed line represents un stimulated control cells. (The statistical significance was compared between non-responding cell and responding cells and was p=0.001 in both instances)

In unimmunized mice the majority of MHCII molecules are filled with endogenous/ self-peptides. It was intuitive to think, especially considering the Treg origin of the receptor, that the TCR was recognising a self-derived peptide presented by the APCs. However, it was also plausible that the TCR could be recognising exogenous peptides derived from the culture conditions, which may also be presented by the APCs. To verify the degree of self-reactivity of the TCR, hybridoma cells were co-cultured with *ex vivo* B cells or peritoneal macrophages in Protein-Free Hybridoma Medium (PFHM). We reasoned that the usage of PFHM, would restrict presentation to endogenously derived peptides only (Figure 2a). For comparison, we used another hybridoma, the H5 (Figure 2b). The H5 TCR was originally templated from a conventional CD4 T cell, and is specific for a F-MLV envelope derived peptide ($env_{122\text{-}141}$). In contrast to the H5, which remained unresponsive to co-culture in the absence of exogenous $env_{122\text{-}141}$ peptide, 3G7 hybridoma activated in the presence of both *ex vivo* B cells and peritoneal macrophages. 3G7 hybridoma activation, in the presence of both cell types, and in the absence of serum components, suggested that the TCR was recognising a self-derived peptide. In addition, levels of CD69 induced in the 3G7 co-cultures were comparable to those exhibited by the H5 hybridoma cells when responding to their cognate peptide, advocating bona fide T cell activation (rather than tonic signalling) in the 3G7 cultures.

Interesting, 3G7 hybridoma cells were even more strongly reactive to the same APCs when cultured in media supplemented with Foetal Calf Serum (FCS) or in the presence of Bovine Serum Albumin supplement (BSA) (Figure 2c). Whilst this could suggest cross-reactivity of the 3G7 TCR to epitopes conserved between BSA and FCS, it was also plausible that these proteins may be indirectly facilitating activation. This was hard to decipher, as although the activated H5 hybridoma showed a preference for FCS containing media (increasing activation by approximately 10%), activation was not affected by the addition of BSA alone (Figure 2d). This potential cross-reactivity of 3G7 TCR to a serum component hindered further analysis of its fine specificity *in vitro*. Nevertheless, the strong reaction of 3G7 hybridoma cells to peritoneal macrophages or B cells in the complete absence of exogenously derived peptides confirmed the self-reactive nature of this TCR.

Figure 2 3G7 and H5 reactivity towards self or foreign-antigens.



(a) Responsiveness of 3G7 T cell hybridoma cells, as measured by CD69 induction, either alone or upon 18-hours of co-culture with C57BL/6 *ex vivo* peritoneal macrophages or splenic B cells. (b) Responsiveness of H5 hybridoma cells, as measured by CD69 induction, either alone or upon 18-hours of co-culture with C57BL/6 peritoneal macrophages or splenic B cells and in the presence of absence of cognate 10^{-5} M $env_{122–141}$ peptide. (c) Responsiveness of 3G7 T cell hybridoma cells, as measured by CD69 induction, either alone or upon 18-hours of co-culture with C57BL/6 peritoneal macrophages or splenic B cells in the presence or absence of the serum components bovine serum albumin (BSA) or foetal calf serum (FCS). (d) Responsiveness of H5 T cell hybridoma cells, as measured by the frequency of cells inducing CD69 expression, either alone or upon 18-hours of co-culture with C57BL/6 peritoneal macrophages or splenic B cells in the presence cognate $env_{122–141}$ peptide and in the presence or absence of serum components.

3.3 Characterisation of the 3G7 TCRαβ Treg transgenic mice.

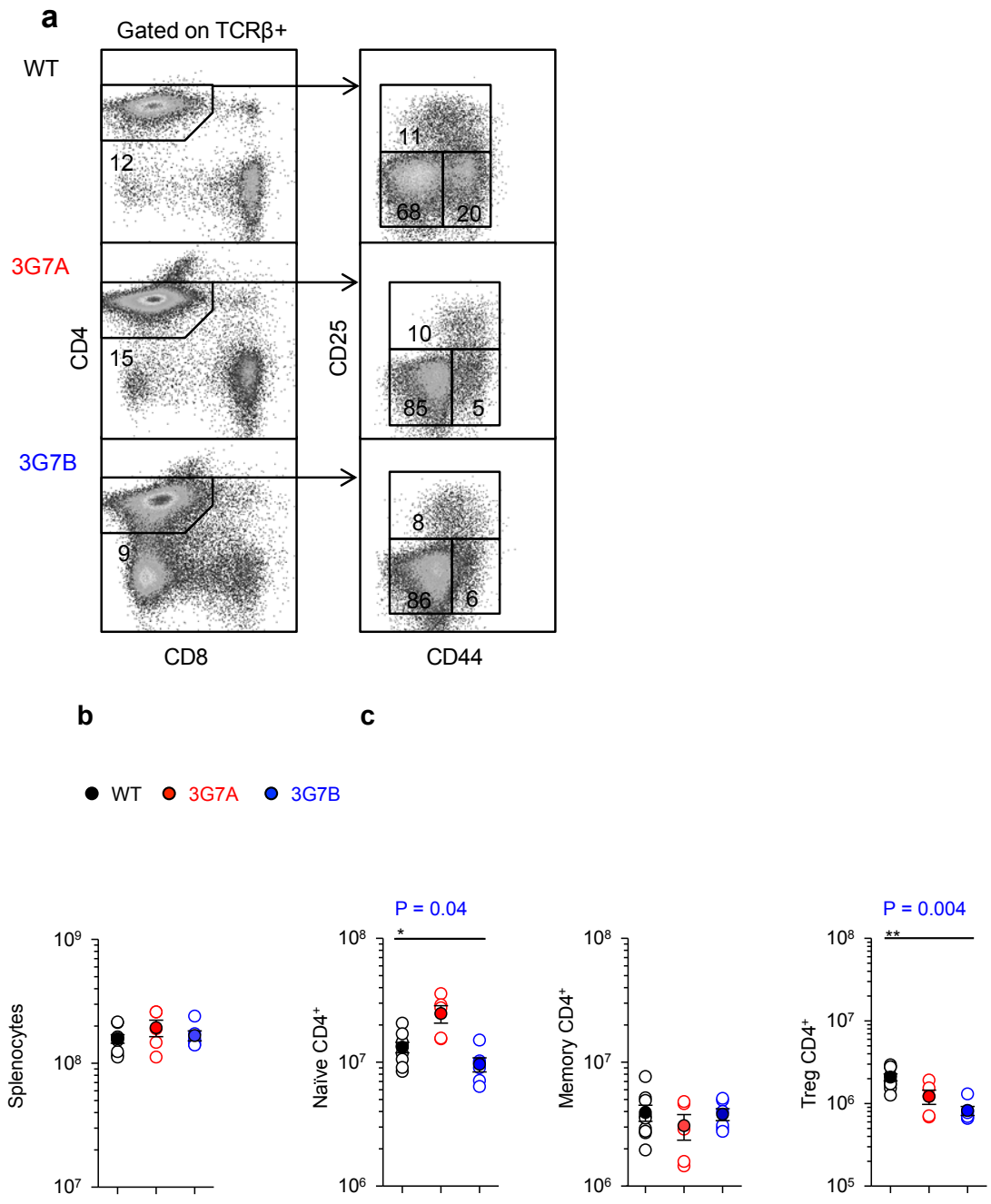
Two Treg TCRαβ transgenic founder lines, expressing the same 3G7 TCR transgenes were generated and backcrossed to the C57BL/6 background. In these mice the presence of functional copies of the recombinase activating genes also allowed for expression of endogenous TCR chains. To limit TCR expression to the transgenes alone, 3G7 mice were subsequently backcrossed onto the *Rag1*$^{-/-}$ background precluding endogenous TCR rearrangements (3G7A/B *Rag1*$^{-/-}$). This allowed us to study the role of expressing a Treg derived TCR in firstly a semi-polyclonal system and secondly in a strictly monoclonal setting.

3.3.1 T cell development in 3G7 TCRαβ transgenic mice.

The 3G7 TCRαβ transgenic strains A and B, were analysed in terms of T cell development. Typical gating for flow cytometric analysis of lymphocytes is shown for WT mice, in direct comparison to Tg mice analysed. Total numbers of each cell type were calculated from their observed frequencies, as ascertained by flow cytometric analysis, multiplied by total cell counts.

Analysis of spleens revealed similar overall cellularity between mice, however closer characterisation of the CD4 compartment revealed that pronounced differences existed between all the three cohorts of mice (Figure 3a-c). In 3G7A mice, as may have been predicted when utilising a TCR originally derived from a CD4 T cell, total CD4 T cell numbers were increased at the expense of CD8 T cells. This increase, was primarily driven by an expanded naïve T cell compartment, as absolute numbers of memory CD4 T cells and Treg T cells were comparable to those observed in WT control mice (Figure 3c). Perhaps, this was an early indication that this TCR, at least expressed in the 3G7A mice, was more conducive with naïve CD4 T cell development rather than memory or Treg T cell development.

Figure 3 Characterisation of lymphocyte development in 3G7 TCRαβ Treg transgenic mice.



(a) Flow cytometric example of the gating applied to define splenic lymphocyte populations from C57BL/6 or 3G7A and 3G7B TCRαβ Treg transgenic mice. Numbering denotes the percentages of cells in each gate (b) Absolute numbers of total splenocytes (b) and naïve ($CD25^{-}CD44^{-}$), memory ($CD25^{-}CD44^{+}$) and Treg ($CD25^{+}CD44^{-}$) CD4 T cell subsets. Closed symbols are the means (±s.e.m.); open symbols are individual mice; numbers within the graph denote the P values accordingly.

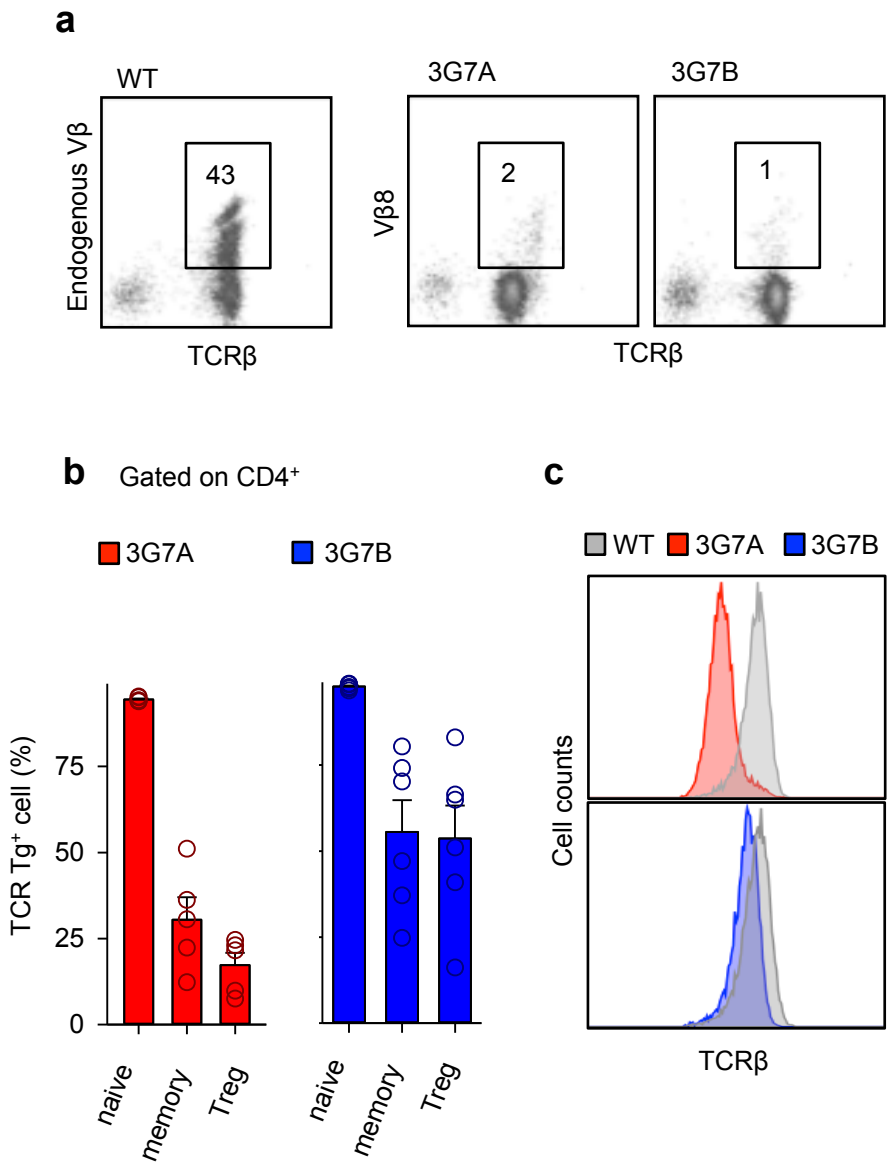
Contrastingly naïve CD4 T cell numbers, in the context of the 3G7B mouse, were significantly reduced as compared to 3G7A comparators and somewhat when compared to WT controls (Figure 3c). Considering the apparent auto-reactive nature of the 3G7 TCR, we wondered if a decreased naïve T cell compartment in 3G7B mice could be explained by an increased capacity of the transgenes to divert cells to other more differentiated lineages. However, this was not evident at least at the population level, as the deficit in naïve CD4 T cells was not recovered by an increase in another CD4 T cell compartment. In fact, it would appear that the expression of the transgenes in this setting was detrimental to all branches of T cell development, although the contribution of endogenous TCR chains must be considered in these experiments.

3.3.2 Expression of the TCRβ transgene in 3G7 TCRαβ CD4 T cells.

Direct determination of the cell fates associated with the TCR transgenes was difficult to access properly thus far, due to the potential contamination of endogenous TCR chains to the repertoire. To more directly look at the compatibility of the transgenes within different T cell compartments we measured the degree of allelic exclusion within CD4 T cells in the Tg mice (Figure 4a-c). In the absence of a specific antibody to stain for the TCRVβ1 transgene, we used a panel of antibodies capable of staining for up to 75% of all possible endogenous TCRVβ families. Considering the efficiency of (TCRβ) allelic exclusion, cells expressing the TCRβ transgene should not express any endogenous TCR chains. Hence the estimated percentage expression of the transgenes could be extrapolated from the percentage expression of endogenous TCRβ chains. In order to do this, we calculated the frequency of CD4 T cells not stained by endogenous TCRβ antibodies in the 3G7A and 3G7B T cells, in comparison with those observed in WT controls, as an estimation of transgene expression. Interestingly, the highest percentage of cells expressing the TCRβ transgene was observed in the naïve compartments of both 3G7 strains, approximately 95-97% of the total (Fig. 4b). In the 3G7A strain, the proportion of CD4 T cells expressing the TCRβ transgene progressively decreased to 31% in the memory compartment and to a further 18% in the Treg T cell compartment. In contrast, within the 3G7B strain, proportions of

cells expressing the TCRβ transgene remained higher at around 55% in both memory and Treg T cell compartments.

Figure 4 Exclusion of endogenous TCRVβ chains in 3G7 TCRαβ Treg transgenic mice.



(a) Expression of endogenous TCRVβ chains in WT, 3G7A and 3G7B TCRαβ Treg transgenic mice. Numbering denotes the percentages of cells expressing endogenous TCRVβ chain as ascertained by flow cytometric analysis. These TCRVβ chains are used by approximately 75% of CD4 T cells in WT mice. (b) The projected percentage expression of the TCR transgenes chains in naïve ($CD25^{-}CD44^{-}$), memory ($CD25^{-}CD44+$) and Treg ($CD25^{+}CD44^{-}$) CD4 T cell subsets in 3G7A and 3G7B TCRαβ Treg transgenic mice. The sum of measured TCR Vβ expression in WT mice was set to 100%, and the expression of endogenous TCRVβ expression in 3G7 TCRαβ Treg transgenic CD4 T cells was then projected accordingly. Bars on the graphs plot the means; open symbols are individual mice (c) Level of TCRβ chain expression in CD4 T cells from 3G7 TCRαβ Treg transgenic mice as compared to WT mice.

3.3.3 Expression of the TCR

Perhaps it was not surprising, considering how the random integration of transgenes can effect TCR expression levels, that such diverse phenotypes existed between the two founder lines. Here, even small differences in the levels of a TCR with intrinsically strong signalling capacity could have large consequences on cellular fate and selection. We therefore wondered, if indeed differing levels of TCR expression between the founder stains could explain the phenotypic differences observed.

TCR expression levels on CD4 T cells was accessed via the intensity of TCRβ chain staining in 3G7A and 3G7B CD4 T cells as compared to physiological levels as set by WT controls (Figure 4c). Importantly, 3G7A T cells expressed only half of WT TCR levels, whilst 3G7B T cells were nearer to 90% of WT TCR levels. Higher levels of TCR expression would clearly increase the signalling potential of the TCR in the setting of the 3G7B mice. Increased TCR signalling capacity may result in an increased propensity for clones to be negatively selected in the thymus, perhaps explaining the capping of naïve T cell numbers observed in the periphery of 3G7B mice (Figure 3b). As well as negative selection, higher levels of TCR signalling during development could also potentially lead to the enrichment of CD4 subsets compatible with higher signalling thresholds. This could perhaps explain the higher usage of the transgenes, within the more differentiated CD4 T cell subsets observed within 3G7B mice compared to 3G7A mice. Of particular interest to us was the increased frequency of Treg T cells expressing the TCRβ transgenes, in 3G7B mice as compared to 3G7A mice (56% and 18% respectively). This was perhaps suggestive that the quality of TCR signal strength was important in the commitment decision towards the Treg T cell lineage.

3.4 Characterisation of 3G7 *Rag1*$^{-/-}$ TCRαβ Treg transgenic mice.

To prevent the confounding effects of the expression of endogenous TCR chains, the 3G7 mice were crossed onto a *Rag1*$^{-/-}$ background (3G7 *Rag1*$^{-/-}$). This prevented TCR recombination within endogenous TCR chains, establishing a monoclonal population of T cells bearing the 3G7 TCR.

3.4.1 T cell development

Using 3G7 monoclonal mice we next examined how the expression of the 3G7 Treg-derived TCR, either expressed at 50% or 90% of WT levels, effected T cell development. The thymi from 3G7A *Rag1*$^{-/-}$, 3G7B *Rag1*$^{-/-}$ and WT mice were harvested and analysed to study T cell development. Flow cytometric gating (as depicted) was used to characterise discrete stages in T cell development (Figure 5a).

Thymic cellularity in both 3G7A *Rag1*$^{-/-}$ and 3G7B *Rag1*$^{-/-}$ was significantly decreased in comparison to WT controls (Figure 5b). However, as CD4/CD8 fate commitment was heavily biased towards the CD4 lineage, with the thymus almost exclusively devoid of single-positive (SP) CD8 thymocytes, overall numbers of SP CD4 thymocytes were comparable to WT controls.

Notably, although CD4 T cell development proceeded normally in 3G7A *Rag1*$^{-/-}$ mice, it was considerably perturbed when the same TCR was expressed at higher levels in the 3G7B *Rag1*$^{-/-}$ mice. Indeed, although total numbers of SP CD4 numbers were normal in the 3G7B *Rag1*$^{-/-}$ mice, the expression pattern of key markers on the developing thymocytes indicated a severe problem with development (as will be discussed).

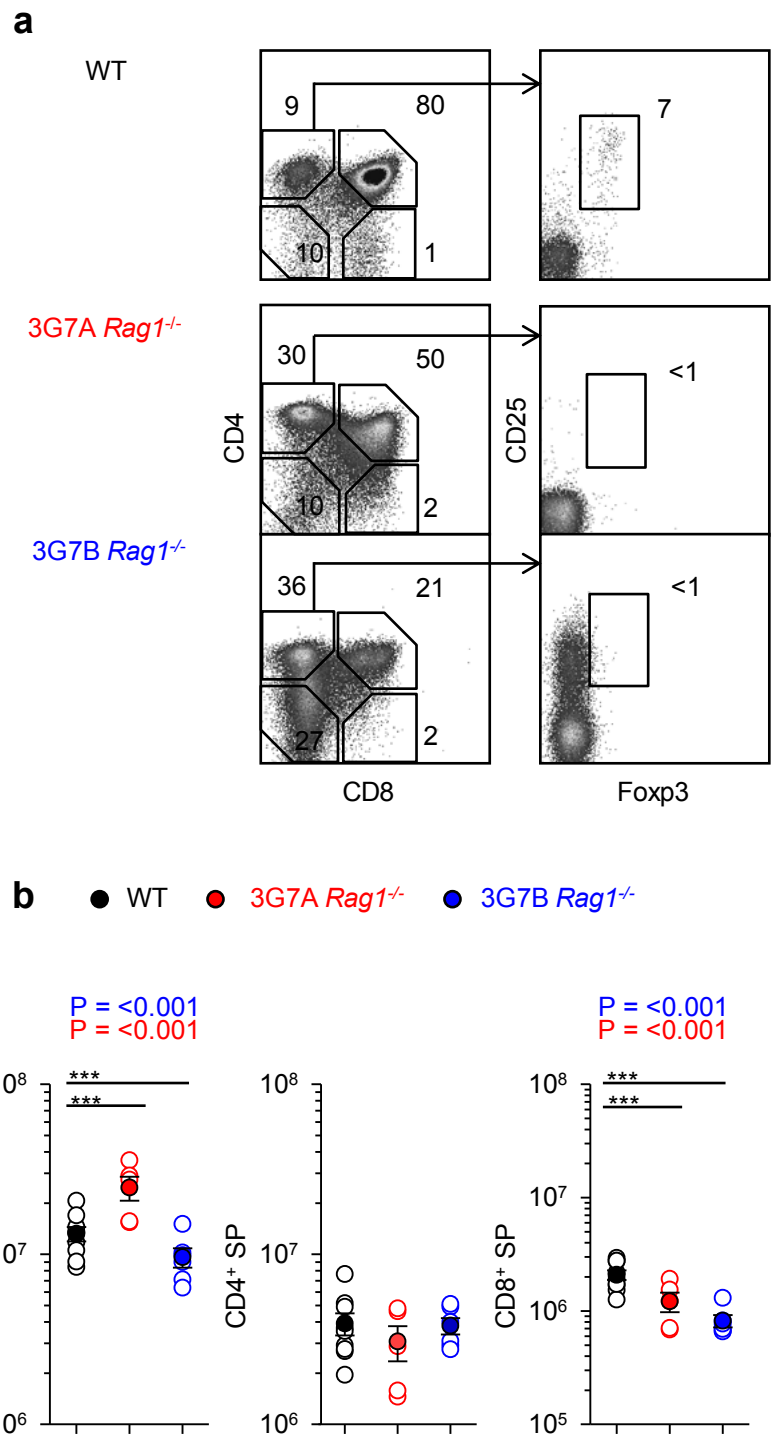
In accordance with T cell development: the proportion of DN ($CD4^{-}CD8^{-}$) thymocytes in 3G7A *Rag1*$^{-/-}$ mice was equivalent to that in the WT, whereas it was largely increased in the 3G7B *Rag1*$^{-/-}$ mice. Subsequently the percentage of double positive DP ($CD4^{+}CD8^{+}$) was largely decreased in 3G7B *Rag1*$^{-/-}$ mice and only

mildly so in the 3G7A *Rag1*$^{-/-}$. This seemed to be symptomatic of a partial block in the transitioning of cells from the double negative DN to DP stage. As this phenotype seemed to titrate with expression levels of the TCR, it was likely a result of heightened and premature signalling by the TCR.

Selection of SP CD4 cells occurred in both the strains, however the higher expression of the TCR in 3G7B *Rag1*$^{-/-}$ mice continued to result in abnormalities. Firstly, expression of CD4 was progressively down modulated during lineage commitment in the B strain (Figure 6a). This seemed to suggest that thymocytes might be actively adjusting levels of co-stimulatory molecules in an attempt to dampen overall TCR signalling. Consistent with this the levels of CD5, a marker of TCR signalling, were heavily distorted in 3G7B *Rag1*$^{-/-}$ thymocytes compared to 3G7A thymocytes (Figure 6c). In addition, CD25 expression was also significantly perturbed again only in the context of the 3G7B *Rag1*$^{-/-}$ mice (Figure 5a). These were likely additional mechanisms employed by developing cells to further curb TCR signalling, presumably in an attempt to evade negative selection.

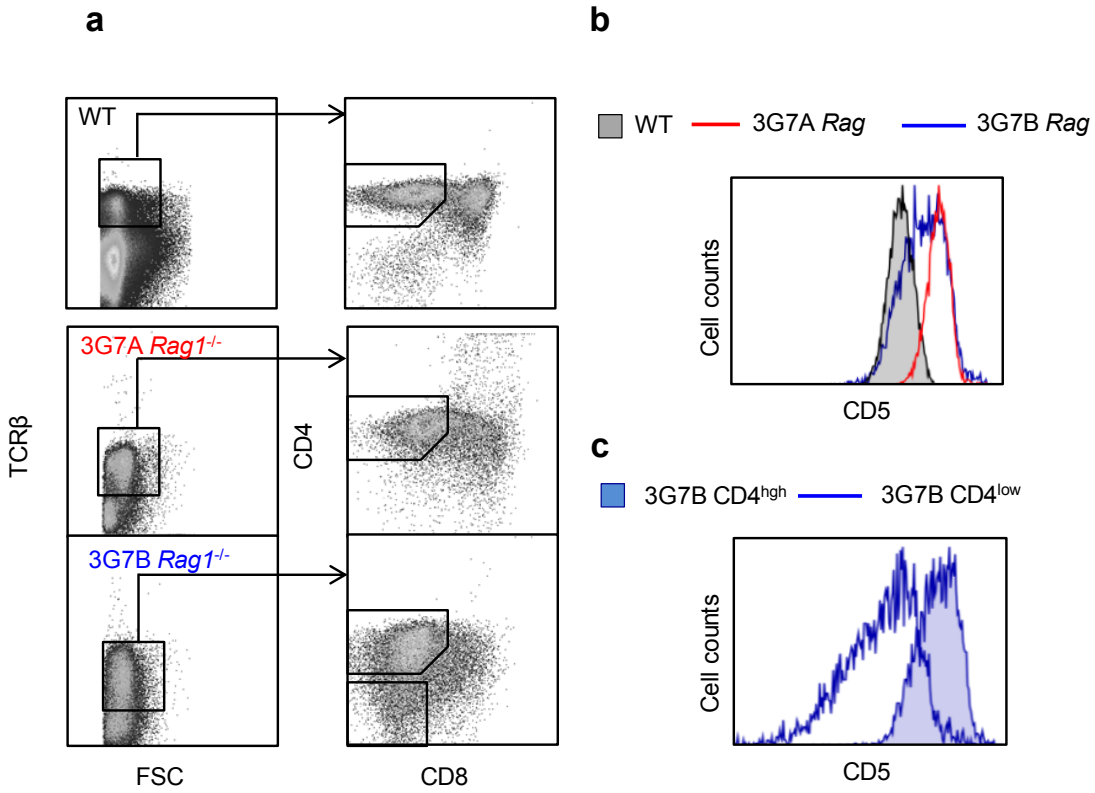
Reassuringly, high levels of CD5 in both 3G7 *Rag1*$^{-/-}$ strains, compared to WT thymocytes seemed to validate the highly self-reactive nature of this TCR (Figure 6b). Despite this, staining for Foxp3 and CD25 revealed the complete absence of tTreg cell development in the thymi of these mice (Figure 5a).

Figure 5 Characterisation of thymocyte development in monoclonal 3G7 TCRαβ Treg transgenic mice



(a) Flow cytometric analysis of thymocyte development in WT, 3G7A *Rag1*$^{-/-}$and 3G7B *Rag1*$^{-/-}$ TCRαβ Treg transgenic mice. Numbering denotes the percentages of cells within each gate. (b) Absolute number of total thymocytes, CD4 single-positive and CD8 single-positive thymocytes in the same mice as in **a.** Closed symbols are the means (±s.e.m.); open symbols are individual mice; numbers within the graph denote the P values accordingly.

Figure 6 Perturbed thymocyte development in monoclonal 3G7 TCRαβ Treg transgenic mice.



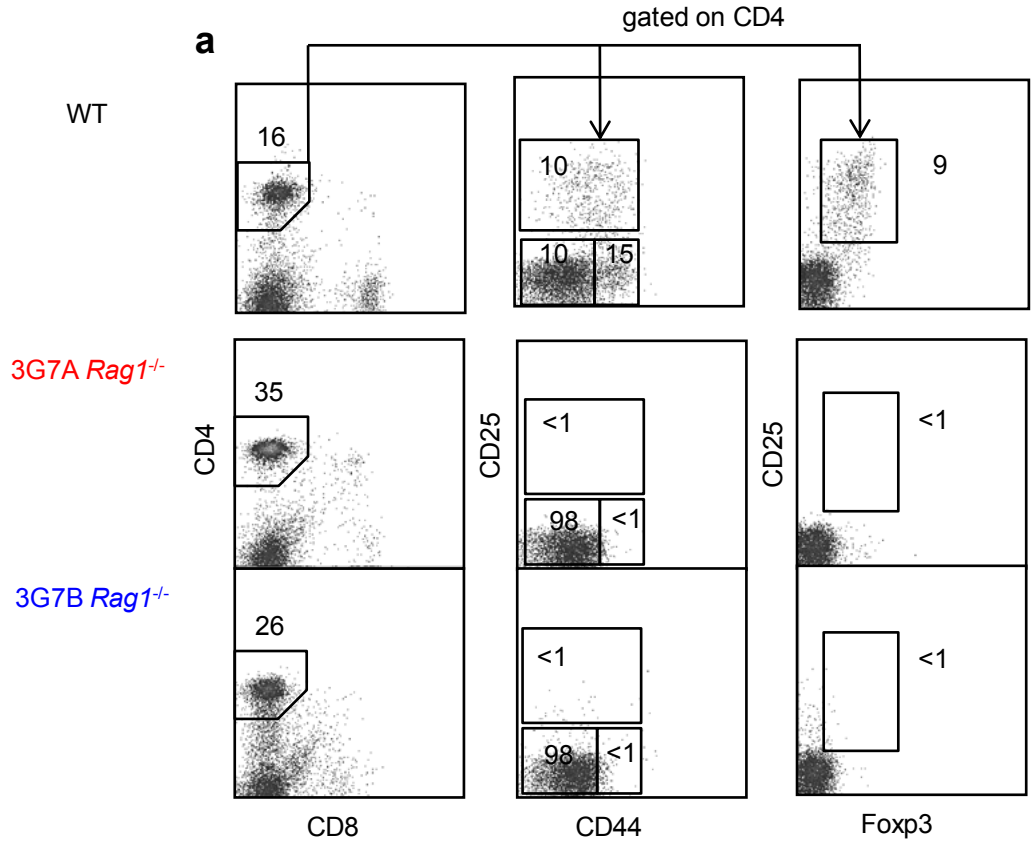
(a) Flow cytometric example of CD4 lineage commitment during thymocyte development in WT, 3G7A *Rag1*$^{-/-}$and 3G7B *Rag1*$^{-/-}$ TCRαβ Treg transgenic mice. (b) CD5 levels in CD4 single-positive thymocytes from the same mice as in **a**. (c) CD5 levels in 3G7B *Rag1*$^{-/-}$ (TCRβ^{high}CD4^{high}) or (TCRβ^{high}CD4^{low}) thymocytes.

3.4.2 Peripheral T cells

We used flow cytometric analysis to look at the peripheral T cell phenotype in the spleens of 3G7A *Rag1*$^{-/-}$ and 3G7B *Rag1*$^{-/-}$ mice (Figure 7). Overall spleen cellularity was much reduced in mice deficient in *Rag1* as is widely reported (Figure 7b). 3G7 *Rag1*$^{-/-}$ mice were devoid of any mature CD8 T cells, as would have been predicted by their absence from the thymus. Profiling of the CD4 T cell populations in these mice showed 3G7 *Rag1*$^{-/-}$ T cells to be mostly naïve in phenotype, and at comparable numbers to WT controls (Figure 7a-c). Analysis of Foxp3 and CD25 expression showed mice to be devoid of Treg T cells in the periphery of these mice, as was the case in the thymus. In addition, and consistent with the thymic phenotype observed in the 3G7B *Rag1*$^{-/-}$ mice, CD4 expression was particularly heterogeneous in these cells compared to its homogenous expression in WT control or 3G7A *Rag1*$^{-/-}$ T cells (Figure 7d).

These results suggested that comparatively higher expression levels of the 3G7 TCR in the B strain led to negative selection of an increased proportion of developing T cells, as well as varying degrees of CD4 down-regulation in the remaining T cells; whereas lower expression of the 3G7 TCR in the A strain had little apparent effect. Neither strain however generated Treg T cells.

Figure 7 Lymphocyte development in monoclonal 3G7 TCRαβ Treg transgenic mice.



(a) Flow cytometric analysis of splenic CD4 subsets in WT, 3G7A *Rag1*$^{-/-}$and 3G7B *Rag1*$^{-/-}$ TCRαβ Treg transgenic mice. Numbering denotes the percentages of cells within each gate (b) Absolute numbers of total splenocytes (c) and naïve (CD25^{-}CD44^{-}), memory (CD25^{-}CD44+) and Treg (CD25^{+}CD44^{-}) CD4 T cell subsets in the same mice as in **a.** (d) Expression levels of CD4 on CD4 T cells from mice as described in **a**. Closed symbols are the means (±s.e.m.); open symbols are individual mice; numbers within the graph denote the P values accordingly. (d) CD4 levels in CD4 splenic T cells in the same mice as in **a.**

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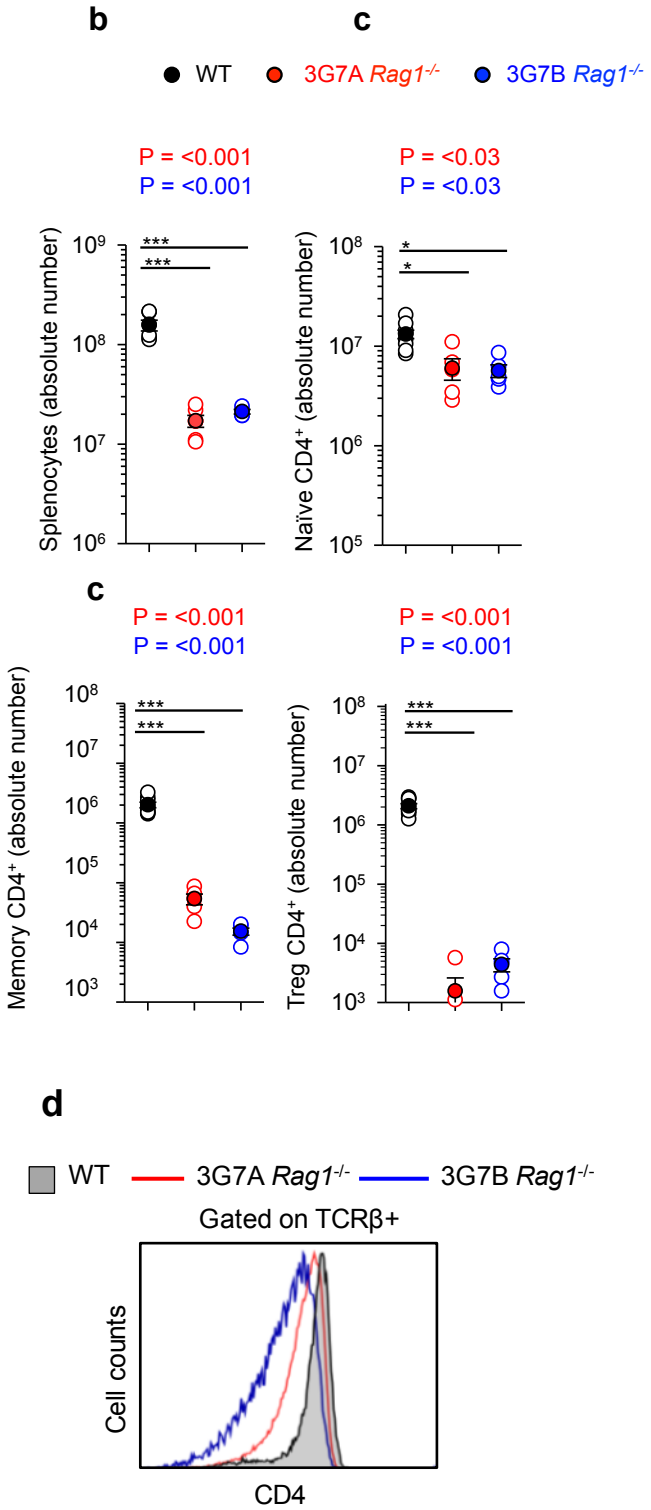

b
c
WT
3G7A Rag1-/-
3G7B Rag1-/-
P = <0.001
P = <0.001
P = <0.03
P = <0.03
Splenocytes (absolute number)
Naïve CD4+ (absolute number)
c
P = <0.001
P = <0.001
P = <0.001
P = <0.001
Memory CD4+ (absolute number)
Treg CD4+ (absolute number)
d
WT
3G7A Rag1-/-
3G7B Rag1-/-
Gated on TCRβ+
Cell counts
CD4

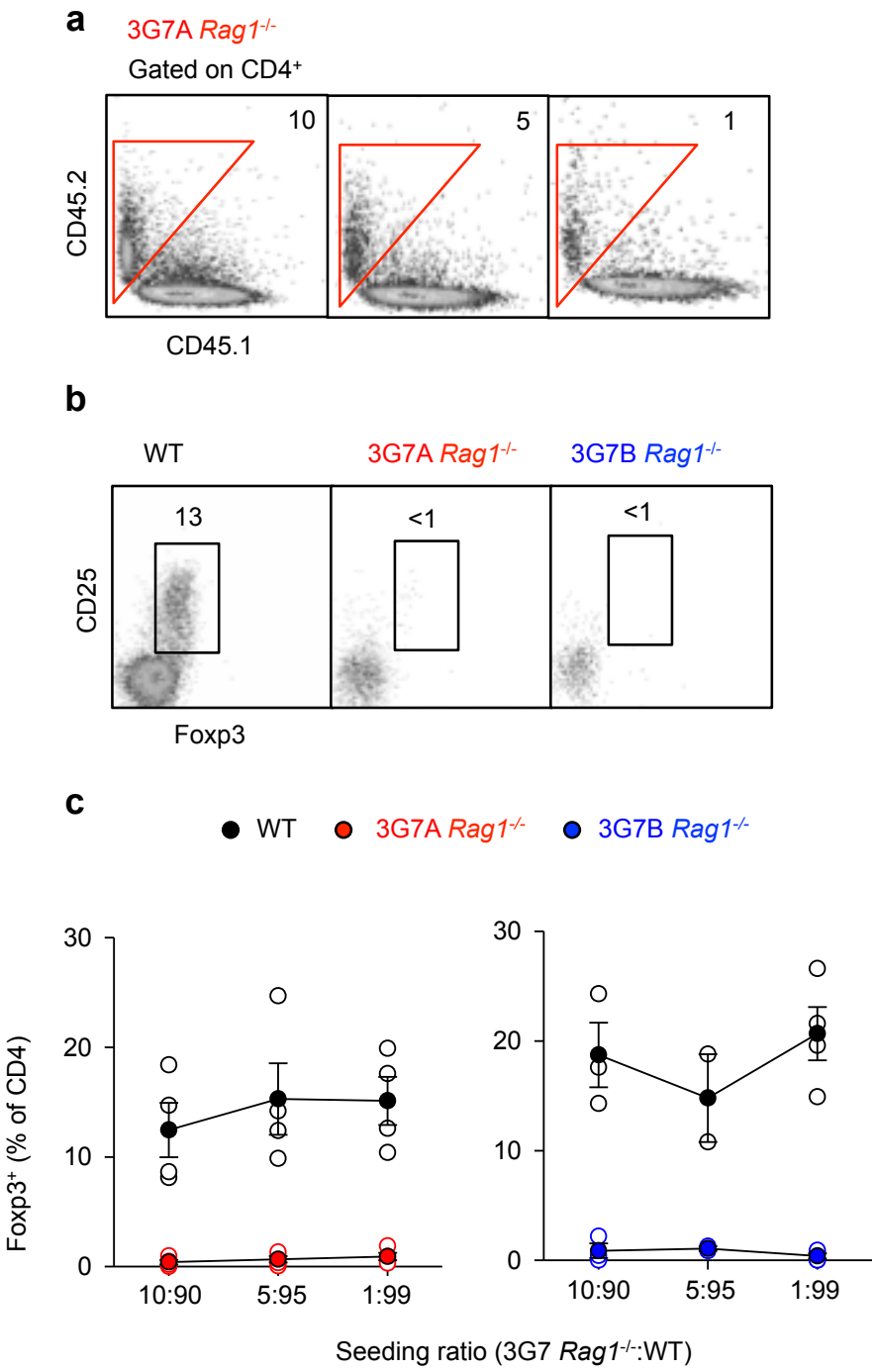
3.5 Effect of precursor frequency of TCRαβ Treg Tg cells on Treg development.

Aware that competition among a clonal repertoire may limit the ability of any receptor to give rise to Treg T cells, we next adjusted the precursor frequency of 3G7 progenitor cells to more-physiological levels; in an attempt to reduce intraclonal competition (Bautista et al., 2009, Leung et al., 2009).

3.5.1 Treg potential was evident in polyclonal control T cells but not from 3G7, even at adjusted precursor frequencies.

Mixed bone marrow chimeras were generated whereby 3G7 *Rag1*$^{-/-}$ bone marrow was diluted by allotypically-marked WT bone marrow. Mixed bone marrow suspensions were then injected into T cell deficient (TCRα$^{-/-}$) hosts. Upon identification of CD4 T cells in the peripheral blood of recipient mice, mice were culled and flow cytometry was used to look for Treg development from within the different donor populations (Fig. 8a-c). In both sets of data for 3G7A *Rag1*$^{-/-}$ or 3G7B *Rag1*$^{-/-}$ strains, we saw that reconstitution was successful with a titration of the 3G7 progeny according to the input ratio (10%, 5% or 1%) (Figure 8a). In addition, we observed that 3G7A *Rag*$^{-/-}$ reconstitution was more complete than in 3G7B *Rag1*$^{-/-}$, consistent with the propensity for negative selection in the latter. Although Treg T cells were present in all recipients, they were exclusively of WT origin (Figure 8b-c). Thus, even when environmental and competitive settings were adjusted to allow for Treg T cell development, the 3G7 TCR was still not instructive for Treg T cell selection.

Figure 8 Treg-TCR potential is not restored upon adjusted precursor frequencies



3G7 *Rag1*$^{-/-}$ TCRαβ Treg transgenic bone marrow was mixed with congenically marked WT bone marrow at various ratios (10%, 5%,1%) and injected into T cell deficient hosts (TCRα$^{-/-}$). Recipient mixed were analyzed 8 weeks following bone marrow transfer (a) Flow cytometric analysis showing the relative proportions of 3G7A *Rag1*$^{-/-}$ CD4 T cells from mixed bone marrow chimeras when seeded at various input ratios (10%, 5%,1%). Numbers denote the starting input percentages of 3G7A *Rag1*$^{-/-}$ bone marrow (b) Flow cytometric analysis of Treg T cell development from WT, 3G7A *Rag1*$^{-/-}$ or 3G7B *Rag1*$^{-/-}$ donor derived bone marrow within individual recipients. (c) Graphs depict the ratio of 3G7 *Rag1*$^{-/-}$: WT bone marrow versus the frequency of Foxp3 Treg cells generations. Closed symbols are the means (±s.e.m.); open symbols are individual mice. (P=>0.001 between WT and 3G7 *Rag1*$^{-/-}$ conditions

3.6 Exploring Treg potential

For completeness we wanted to study if 3G7 primary T cells responded atypically upon *in vitro* activation. It remained a possibility that the Treg derived TCR, although not instructive for thymic Treg T cell development, may still be instructive for the acquisition of this cell fate following T cell activation. To study the magnitude of the response we quantified CD69 induction and CFSE dilution at various time points following 3G7 T cell activation. To profile the quality of effector cell differentiation within the same cells, we also measured cytokine production and Foxp3 expression 3-4 days following T cell activation. For comparison we used WT primary T cells activated with CD3/CD28 *in vitro*.

3.6.1 Spontaneous activation of 3G7 T cells in vitro

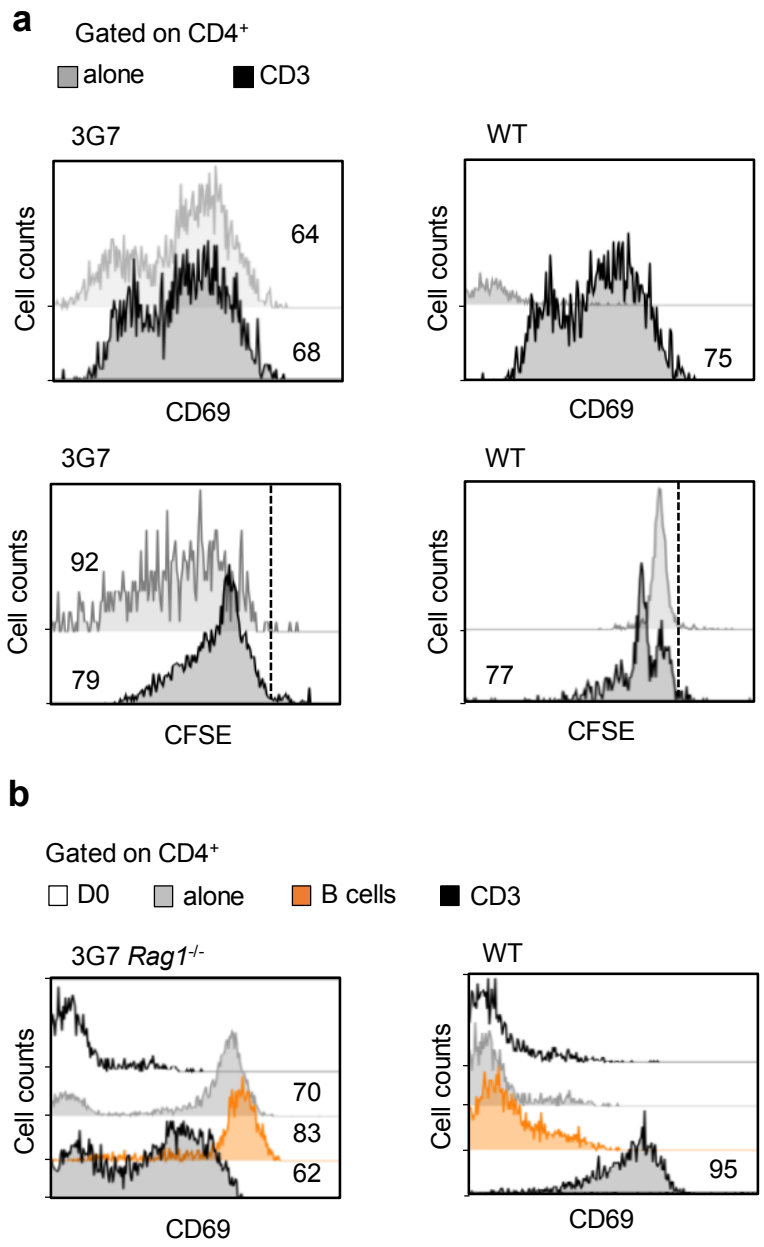
In contrary to the evidence for the auto-reactive nature of the 3G7 TCR, peripheral T cells in 3G7 mice remained wholly naïve in phenotype. However, we observed that upon culturing single cell suspensions from the spleens of 3G7 mice *in vitro*, T cells would now spontaneously activate (Figure 9a). The levels of CD69 on 3G7 CD4 T cells from these splenic cultures mirrored those levels expressed by 3G7 CD4 T cells activated in the presence of CD3/CD28 beads, and thus was in accordance with authentic activation. In addition, CFSE dilution peaks showed 3G7 T cells went on to actively proliferate. Expectedly, this spontaneous activation was not supported in single cell suspensions from WT mice, suggesting this was a property unique to the 3G7 TCR.

In order to limit responses observed to those mediated by the 3G7 TCR/T cells alone we next plated single-cell-spleen suspensions from 3G7 *Rag1*$^{-/-}$ splenocytes. Although notably devoid of any B cells these cultures were also able to support spontaneous activation, however activation was further increased when cultures were supplemented with WT B cells (Figure 9b). This transition from the naïve state seen *in vivo* to full activation *in vitro* seemed to result from the mechanical disruption of the splenic architecture that would normally segregate T cells from APCs during homeostasis. Presumably, forcing interactions between these cell types, through the disruption of their spatially distinct niches, lead to T cell

activation *in vitro*. Although, here the role of exogenous ligands introduced as a consequence of culture conditions cannot be entirely eliminated.

Irrespective of the ligand, the functional consequence of 3G7 activation on effector differentiation could be accessed via cytokine production and Foxp3 expression on day 4 following T cell activation (Figure 9c-d). However, percentages of cells producing IL-2 and IFN-gamma were not statistically different between activated WT or 3G7 *Rag1*$^{-/-}$ T cell cultures, whilst the percentage of Foxp3, CD25^{high} cells was actually statistically lower in 3G7 *Rag1*$^{-/-}$ cultures. Thus activated 3G7 T cells, either via self-recognition or via CD3 stimulation, did not seem to behave atypically and displayed no bias in differentiation towards the Treg lineage.

Figure 9 Spontaneous activation of 3G7 TCRαβ Treg transgenic primary T cells *in vitro*



Flow cytometric analysis of (a) Responsiveness of 3G7 or WT spleen suspensions, following 1 or 4 days in culture either alone or in response to CD3-CD28 stimulation (as measured by CD69 induction or CFSE dilution respectively) (b) Responsiveness of spleen suspension from 3G7 monoclonal mice or WT mice, as measured by the frequency of cells inducing CD69 expression after 18 hours, when cultured alone or with purified WT splenic B cells (B220+) or following CD3-CD28 stimulation. Numbers denotes the percentage of cells which are deemed $CD69^{positive}$ or $CFSE^{-}$ (with percentages less than 5% not being annotated) (c) Flow cytometric detection of intranuclear or intracellular staining of Foxp3, IFN-gamma or IL-2 in cells following 3 days of activation as described in **b.** (d) Graphs depict the percentage of CD4 T cells expressing Foxp3, IFN-gamma and IL2 from 3G7 monoclonal or WT spleen suspension following 3 days of activation *in vitro*. Closed symbols are the means (±s.e.m.)

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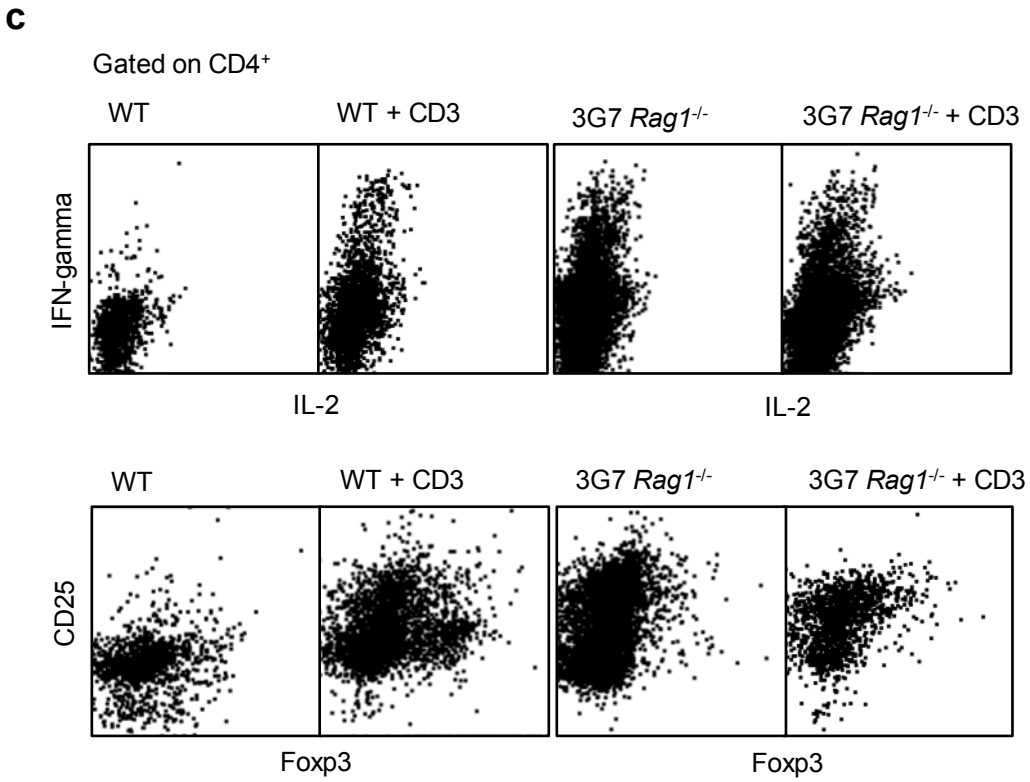
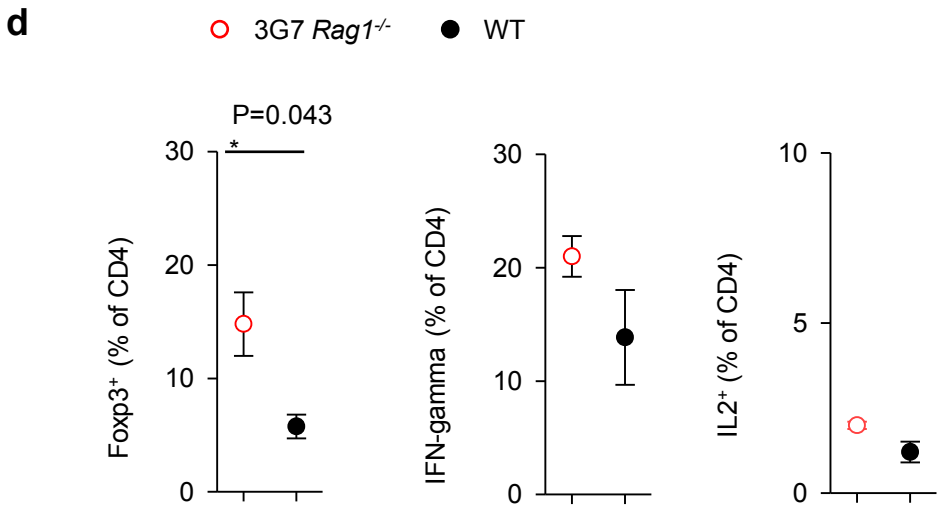

c
Gated on CD4+
WT
WT + CD3
3G7 Rag1-/-
3G7 Rag1-/- + CD3
IFN-gamma
IL-2
IL-2
WT
WT + CD3
3G7 Rag1-/-
3G7 Rag1-/- + CD3
CD25
Foxp3
Foxp3


d
3G7 Rag1-/-
WT
P=0.043
*
Foxp3+ (% of CD4)
IFN-gamma (% of CD4)
IL2+ (% of CD4)
30
20
10
0
30
20
10
0
10
5
0

Discussion

Treg T cell development had not been observed in conventional TCR transgenic mice. This was an early indication that TCR specificity maybe crucial for thymic Treg T cell development. Critically, Treg T cell development could be restored in conventional TCR Tg mice when cognate antigen was expressed via a second transgene which emulated the expression of self-peptides. This suggested that increased specificity to self-derived-antigen was a crucial determinant for Treg T cell selection. Considering this, we decided to explore the effect of expressing a Treg TCR on Treg T cell development in two TCRαβ Tg mice. Although the TCR sequences were identical between the two strains, TCR avidities were different (high or low) as a consequence of differing TCR expression levels. Using 3G7 hybridoma cells we were primarily able to confirm the auto-reactive nature of this Treg derived receptor. Thus, without the need to decipher the 3G7 TCR specificity or having to introduce antigen via a second transgene, we used the differing levels of TCR expression levels exhibited between the transgenic strains, to manipulate the quality of TCR signalling received. This gave us a model to look at the instructive capacity of both TCR specificity and TCR avidity in Treg T cell commitment.

However, despite the Treg origin of 3G7 TCR, neither of the TCRαβ Tg strains generated Treg T cells. As stated, differing levels of surface TCR between the strains allowed us to look at a spectrum of signalling potential from the 3G7 TCR. These results suggested that the higher levels of TCR signalling in 3G7B mice led to increased negative selection and advanced CD4 down regulation rather than being instructive towards the Treg T cell lineage. Noticeably, even when TCR signalling was intrinsically tuned down in 3G7B thymocytes, and thus the quality of the signalling broadened, this did not allow for a window of signalling opportunity which was instructive for Treg cell development. Lower expression of the 3G7 TCR in the A strain carried with it minimal deleterious consequence in terms of CD4 development however nor did it lead to Treg T cell development. Previously, groups had attempted to produce monoclonal Treg TCR transgenic mice, with only the minority of receptors giving rise to Treg T cells. Further investigation associated the lack of Treg T cell development to the artificiality of using a monoclonal system

rather than a failing associated with the encoded TCRs, whereby the observed low frequency of Foxp3 cells in these settings was suggested to be a result of clonal competition. In these studies, the instructive capacity of a given Treg TCR could be visualised once the precursor frequency of these receptors was adjusted to more physiological levels. However, adjusting the clonal frequency of transgenic T cells in our system did not restore the instructive capacity of the 3G7 TCR for the Treg T cell lineage. Therefore, our data would suggest that not all Treg TCR Tg models can be manipulated to recapitulate tTreg development. We can conclude that although Treg T cell development may indeed include a ‘TCR instructive’ step, the specificity of the TCR alone is not enough to prescribe for the Treg lineage.

Chapter 4. A role for self-reactivity in CD4 T cell responses to foreign-antigens

4.1 Introduction

It is now understood that the TCR repertoire is broadly self-reactive. In the periphery, naïve T cell survival requires IL-7 signaling and spMHCII recognition (as discussed previously). However, interactions made between different TCRs and spMHCII molecules in the periphery are not equal, with some clonotypes seeing self-antigens better than others. This has lead to an increasing interest in determining if sub-threshold TCR signaling events play a role beyond just homeostatic maintainence and survival.

4.1.1 Clonotypic selection and Avidity

As a result of antigenic challenge, T cells undergo activation initiated by specific interactions made between their TCRs and pMHCII molecules on APCs. As described earlier, these interactions drive the clonal expansion of specific clonotypes that are able to recognise the given pathogen. During the acute phase of an immune response, the strength of TCR signalling seems to be the major determinant in clonal selection and features the outgrowth of the highest avidity clones. This bias allows for avidity maturation, at least at the population level, despite the fact that individual TCRs are fixed (Busch and Pamer, 1999). Following the peak of infection, a large proportion of the responding population die by apoptosis leaving behind a much smaller pool of T cells. What controls the clonotypic composition of this remaining population remains somewhat of an unknown and although there is evidence for the continued selective expansion of the highest avidity clones there is also evidence for the extensive reshaping of the TCR repertoire. Understanding which clonotypes persist and why remains a complex but fundamental question in T cell immunology. Here we will consider a role for self-reactivity in the clonotypic selection of CD4 T cell immune responses.

4.1.2 A role for self-reactivity in dictating reactivity to cognate antigen

The specificity and sensitivity of a T cells TCR is vital for its role in immune responses. Although the generated of the TCR repertoire is based around the recognition of self-antigens, the main purpose of the TCR repertoire is to recognise foreign-antigens. At first this strategy of selection appeared to be highly counter-intuitive, however recently we have started to understand the relevance of self-antigens in dictating T cell responses to foreign-antigens.

It is firstly important to recognise the highly cross-reactive nature of the TCR; where one TCR can recognise many different antigens, sometimes even simultaneously. In fact, studies have been able to visualise the recruitment and accumulation of self-antigen at the immunological synapse, during responses to foreign antigen (Wulfing et al., 2002, Irvine et al., 2002). In such scenarios, recognition of self-antigens in concert with the recognition of foreign-antigens has been suggested to provide a unique mechanism to increase the intrinsic sensitivity of a T cell and the likelihood of subsequent activation. This could theoretically occur in multiple ways: Firstly, the binding of TCRs to spMHCII (in conjunction to pMHCII) may act to structurally stabilise the partnership between a T cell and an APC, leading to a better quality interaction. In addition, spMHCII interactions may help to increase the recruitment of TCR signalling molecules to the synapse, something that may be particularly beneficial when foreign antigen is limiting. Thus the physical accumulation of self-antigen to the SMAC presents at least one mechanism of how self-peptide may contribute to T cell activation especially in scenarios where foreign antigen may be sparce (Krogsgaard et al., 2007).

In other cases, recognition of self (prior to antigen encounter) may act to increase TCR sensitivity to future antigens. Here high avidity interactions with self-antigen during thymic selection or in the periphery, may maintain the TCR signalling components in a state of partial phosphorylation (Stefanova et al., 2002, Persaud et al., 2014). In this regard, low level basal signalling achieved through self-recognition may act to polarize the downstream components of the TCR signalling pathways toward a state of responsiveness which would allow for increased TCR sensitivity upon TCR triggering (Mandl et al., 2013). Recently, evidence for such a

role for spMHCII mediated interactions has been confirmed both *in vitro* and *in vivo* and by multiple laboratories. Briefly, experiments that cultured naïve T cells for set periods of time *in vitro,* and in the absence of MHCII contacts, demonstrated the rapid loss of pre-existing partial TCR ζ-chain phosphorylation or Erk phosphorylation, associated with an overall decrease in sensitivity (Stefanova et al., 2002, Persaud et al., 2014). A role for spMHCII in the modification of the TCR signaling components was also confirmed *in vivo* through the administration of a monoclonal antibodies blocking MHCII (Stefanova et al., 2002) or via the transfer of CD4 T cells into MHCII deficient host (Smith et al., 2001, Kassiotis et al., 2003); both of which resulted in a decrease in T cell sensitivity at the population level. Thus T cells with enhanced capacity to recognise self, may be poised to respond with greater sensitivity upon antigen recognition (Persaud et al., 2014). In this way positive selection can be viewed as a mechanism that selects for T cells that will continue to see 'self' in a biologically meaningful and advantageous way once in the periphery (Vrisekoop et al., 2014). Sufficient levels of signalling as a consequence of self-antigen recognition may also partially influence the differentiation state of a CD4 T cell, leading to the acquisition of a more differentiated phenotype. The most obvious example of this is the Treg T cell, which assumes an 'antigen experienced' state in the absence of exposure to foreign antigen but instead as a consequence self-antigen-recognition.

Another hypothesis is that T cells capable of recognising self to a sufficient degree may recognise foreign antigen similarly well (Mandl et al., 2013). In this way positive selection may provide a mechanism to select for TCRs with intrinsic capacity to recognise antigen broadly, and thus self-reactivity may correlate with antigen-reactivity (Stefanova et al., 2002). Although structural data (which could be used to conclusively confirm such a hypothesis) does not yet exist, functional studies have highlighted a direct relationship between the cells of the highest avidity in terms of selection in the thymus, and those cells that are the highest avidity clones during acute responses to foreign-antigen (Mandl et al., 2013). Here CD4 T cell clones, that exhibited TCR signalling at the upper end of the spectrum during selection, contributed most greatly during responses to infection.

4.1.2.1 A role for self-reactivity in memory formation

One model for memory formation, suggests that TCR clonotypes are selected to form memory based on TCR avidity for foreign antigen (Mandl and Germain, 2014). However more recently another model for memory formation has been proposed, suggesting that the capacity to become a memory T cell is determined prior to the onset of the response. Here naïve CD4 T cells were sorted according to their levels of self-reactivity and then high versus low (self-reactive) cells were competed against each other in response to infection (Mandl et al., 2013). This study highlighted clonotypes that were most self-reactive contributed to a greater extent, than clonotypes comparatively lower in self-reactivity to the memory T cell pool.

4.1.3 Measuring self-reactivity

A single TCR has the potential to recognise many different, un-characterised self-peptides. In addition, these interactions are typically of low avidity as otherwise they would fuel autoimmunity. This means that we can rarely directly measure the quality (or self-reactivity) of TCR-spMHCII interactions. Instead surrogate markers of self-reactivity have conventionally been employed to provide a read-out for the inferred self-reactive nature of a T cell clone. Perhaps the best described of these markers is CD5. A more comprehensive overview of such markers and their uses is described in **4.3**.

4.2 Aims

The TCR is critical in determining the future of a T cell, but how and to what extent is still not fully understood. Although TCR specificity to self-antigen is well studied during thymic development, conventionally CD4 T cell differentiation has become more directly associated with exogenous antigen recognition. In this regard, TCR specificity, the strength and duration of this signaling, as well as the cytokine environment have been shown to collaborate in determining different CD4 T cell fates. However, such interactions between a TCR and its foreign cognate ligand are extremely rare. Instead the vast majority of TCR engagements are with self-peptides. Whilst most work has centered on establishing the fundamental role of self-recognition during T cell development, much less has focused on a role for

self-reactivity in the periphery. Given the fundamental role of the strength of TCR signaling to foreign peptide in determining cell fate decisions, we aimed to elucidate how recognition of self-peptides may influence future CD4 T cell responses.

4.3 Results

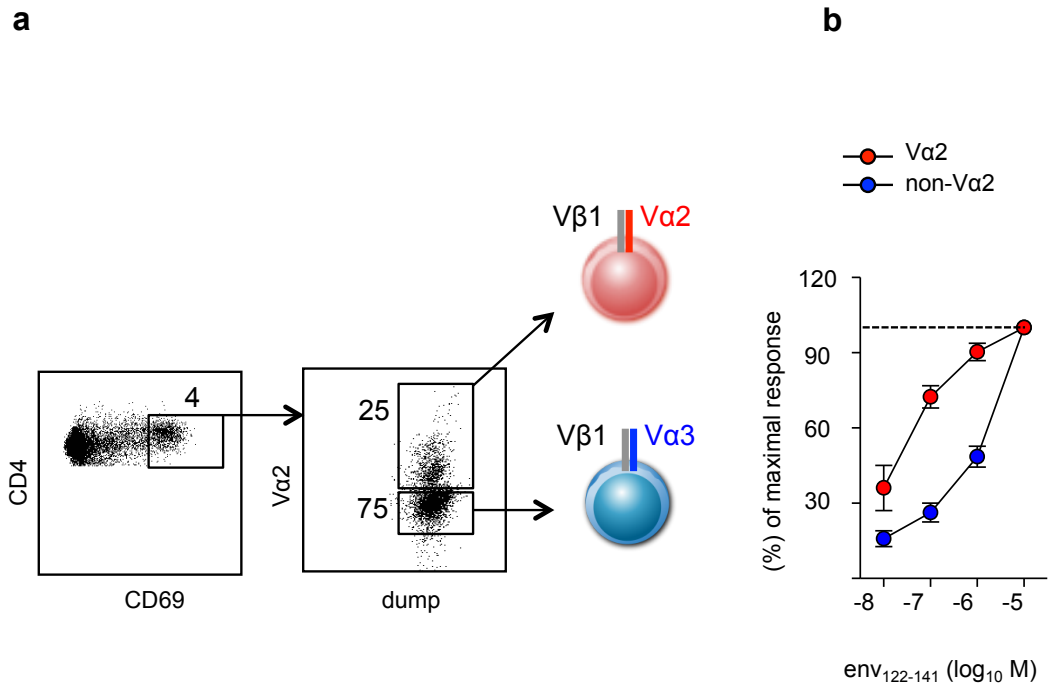
4.3.1 EF4.1 FV infection model

Here, we used a well-characterized model to study the factors that are involved in the evolution of the CD4 T cell response to retroviral infection. We used infection of C57BL/6 (B6) mice with Friend virus (FV), a retroviral complex of Friend murine leukemia virus (F-MLV) and spleen focus-forming virus (SFFV), to achieve a protracted infection that is eventually cleared. Due to the limiting precursor frequencies of FV reactive T cells that exist within a polyclonal repertoire, an in-house TCRβ-transgenic strain (EF4.1) was used as a source of virus-specific CD4 T cells. Adoptive transfer of purified EF4.1 CD4 T cells, into host mice at the time of infection allowed us to track and study CD4 T cell responses to FV infection.

4.3.2 Functional avidity of Vα2 and Vα3 *in vitro*.

The association of the Vβ1 Tg TCRβ chain with endogenous TCRα chains in EF4.1 T cells generates a semi-polyclonal repertoire, enriched for TCR clonotypes reactive with the F-MLV $env_{122\text{-}141}$ epitope (Antunes et al., 2008). *In vitro* peptide stimulation of EF4.1 T cells with $env_{122\text{-}141}$, revealed a multiclonal virus-specific population. Collectively, the responding population made up ~4% of total EF4.1 CD4 T cells (Figure 10a). Responding T cell clones could be sub-categorized into two families, according to their usage of TCRα chains; with T cells either expressing TCR Vα2 chains (encoded by *Trav14* gene segments) or Vα3 chains (encoded by *Trav9* gene segments). The composition of virus specific T cells within the pre-immune repertoire expressing either a Vα2 or Vα3 chain, was 25% or 75% respectively (Figure 10a) (Antunes et al., 2008). *In vitro* activation of EF4.1 T cells also revealed that TCRVα usage could segregate T cells in respect to their functional avidity. Importantly, the pairing of the Vβ1 transgene with either Vα2 or Vα3 chains created clonotypes with higher or lower functional avidity, respectively (Figure 10b) (Antunes et al., 2008, Young et al., 2012b).

Figure 10 TCR Vα usage in env$_{122-141}$–specific EF4.1 TCRβ-transgenic CD4 T cells



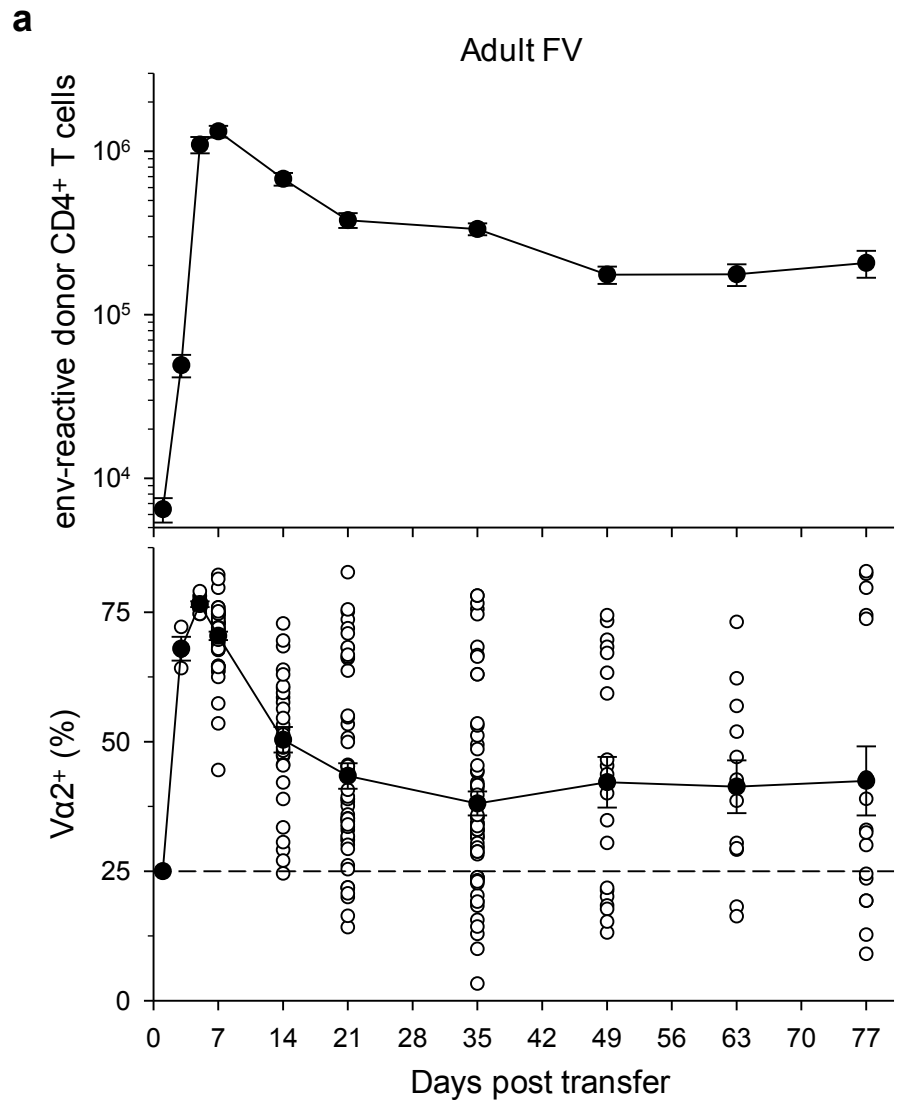
(a) Flow cytometric gating showing TCR Vα usage in activated (CD69^{high}) EF4.1 TCRβ transgenic CD4 T cells responding following 18 hours stimulation with the 10^{-5} M env$_{122-141}$ peptide. (b) Characterisation of the functional responsiveness of Vα2 or non Vα2 (Vα3) env-reactive donor T cells following 18 hours of env$_{122–141}$ peptide stimulation (at varying doses). Responses were measured by CD69 induction and are plotted as percentage of maximal response. Dashed line indicates 100% of maximal response. Closed symbols are the means (±s.e.m.) and are representative of more than 5 independent experiments.

4.3.3 Switch between Vα2 and Vα3 families

As mentioned, in order to unequivocally identify virus-specific CD4 T cells over the course of infection, we used an adoptive transfer system where WT mice received allotypically marked EF4.1 TCRβ-transgenic CD4 T cells at the time of FV infection (Antunes et al., 2008). This system not only allowed us to track the expansion of total virus-specific EF4.1 CD4 T cells but also allowed us to assess the relative contribution of Vα2 (high avidity cells) or Vα3 (low avidity cells) at different points in the response. Here, we used the measured functional avidities as a read-out for a T cells avidity.

Following transfer, EF4.1 T cells exhibited typical kinetics, expanding over 200-fold during the first 7 days (the peak of infection). Expectedly, this expansion was followed by the sharp contraction of the response over the following weeks as virus was cleared (Figure 11). As has been previously published, although initially outnumbered in the pre-immune repertoire, virus-specific Vα2 T cells homogenously dominated during the primary response (Thorborn et al., 2014). This saw Vα2 clonotypes climb from ~25% to ~75% in all mice by day 7. This would suggest that avidity was a key determinant in shaping the clonotypic hierarchy within the primary response, but what happens past the peak was not known. Here, we followed the ensuing response and found, that on average the frequency of Vα2 clonotypes progressively declined, although this was wildly variable between individual hosts (Fig. 11). Nevertheless, the clear evolution of the CD4 T cell repertoire suggested that distinct forces existed for the recruitment of clonotypes into primary or memory responses.

Figure 11 Evolution of the Vα virus-specific CD4 T cell response over the course of FV infection



(a) Absolute numbers and (b) Vα composition of env-reactive donor EF4.1 CD4 T cells in the spleens of recipient mice after adoptive T cell adoptive transfer and FV infection Closed symbols are the means (±s.e.m.); open symbols are individual mice; n=9–61 mice per time point; P<0.001 between Vα2 frequency on day 7 and any later time point, Mann–Whitney rank sum test.

Dominance of a high avidity Vα2 clones at the peak of infection seemed to agree with the clonal selection theory, whereby the T cells receiving the highest TCR signalling would proliferate the most, outcompeting the rest. However, the late dominance of lower avidity Vα3 clones, in most of the mice, was harder to rationalise. Although our data, measuring the functional responsiveness of EF4.1 T cells, had suggested that Vα3 F-MLV reactive clones were of lower avidity than Vα2 clones, this was limited to behavior observed in bulk Vα2 and Vα3 populations *in vitro*. It was important to consider that within these families there existed the potentially for much diversity. It was also possible that clonotypes activated under *in vitro* conditions may be distinct from those that are recruited *in vivo*. We therefore wanted to look at the behaviour of individual clonotypes that were responsible for driving the responses observed *in vivo*.

4.3.4 Diversity of virus-specific T cell clonotypes increases over time.

To examine whether the observed changes in clonotypic composition were indeed caused by differences in TCR reactivity between responding CD4 T cells, we first identified the frequency of unique TCR clonotypes. This was achieved via the deep sequencing of the endogenous TCRα chains in virus-specific EF4.1 CD4 T cells, followed by bioinformatics analysis. Exclusive usage of the Vβ1 transgene, by all virus-specific T cells allowed for TCRα sequencing to be sufficient to determine the identity of the TCR clones. In addition, to validate that the observed changes were not the result of the expression of a second TCR by responding clones we used EF4.1 *TCRα*$^{-/-}$ mice as donors for T cell transfer and sequencing. Other members of the lab jointly collaborated to do this work.

For determination of the pre-immune repertoires, a cohort of naïve CD4 T cells from the respective EF4.1 donor mice used were purified and processed. Following which, the remaining EF4.1 CD4 T cells were transferred into mice on the same day as being infected with FV. Then at different time points following FV infection, activated donor EF4.1 CD4 T cells ($CD4^+CD45.2^+CD44^+$) were sorted from individual hosts. Total RNA was then isolated from these populations, before being reverse transcribed into cDNA. Purified cDNA was then used as template for the

amplification of *Trav14* (encoding Vα2)- or *Trav9* (encoding Vα3)-containing rearrangements. Due to the diversity exhibited between TCR sequences, primers had to be designed toward conserved sequences between the *Trav* families. To this end, forward primers were targeted to *Trav* promoter regions and reverse primers to common *Trac* constant regions. This resulted in a PCR product of approximately 500bp in length, following which these amplicons could then be sequenced using 454 sequencing.

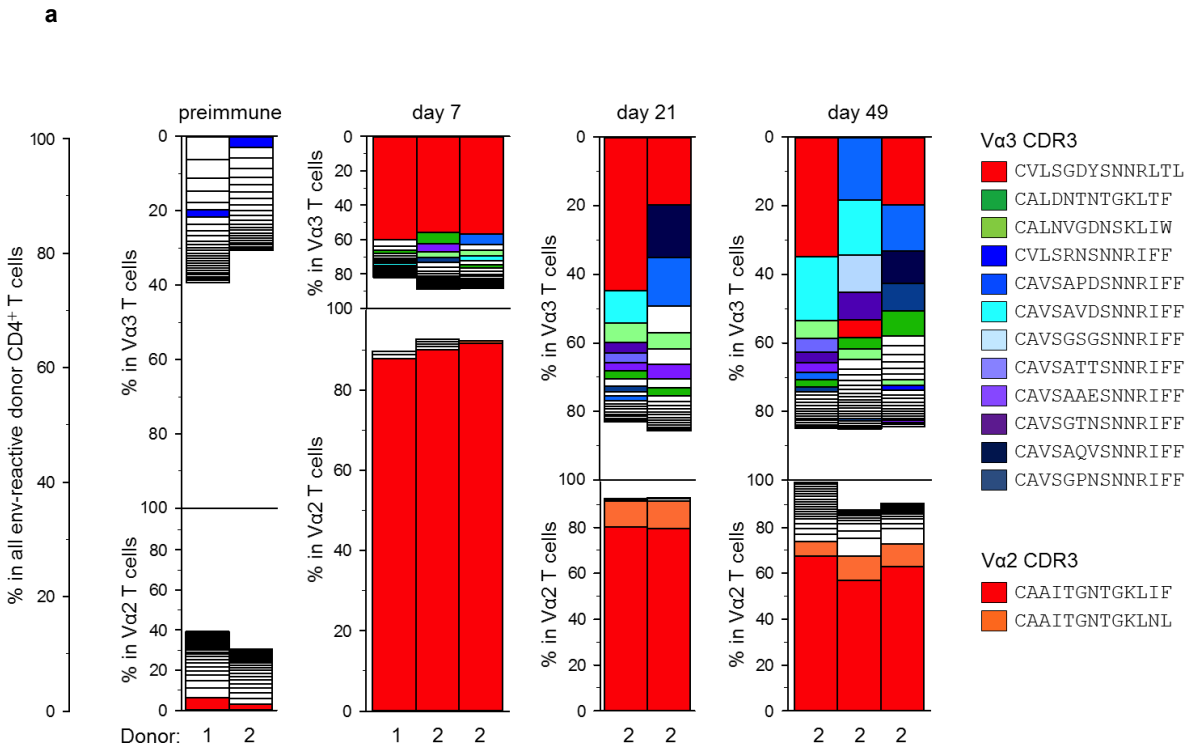
454 sequencing can process comparatively longer reads than Illumina sequencing. This allowed for the sequencing of the full PCR product (representative of a single TCR), without the need for its fragmentation. Firstly, adaptor sequences were added to each end of the PCR product. Addition of such adaptors targeted the PCR product to associate with 'capture' beads, themselves coated with complementary adaptor sequences. 'Bead-capture' conditions were such to limit the attachment of just one DNA strand per bead. Bead-associated DNA strands could then be amplified via emulsion PCR guided by primers specific for the protruding adaptor sequences. Following this, beads coupled with the amplified product were sorted into wells, thus in this way each well would contain information derived from a single TCR. TCR sequences could then be determined via pyro sequencing. Between 4,500 and 43,000 nucleotide sequences per sample were obtained. Nucleotide sequences were then filtered for productive rearrangements. The protein sequence was predicted and annotated by the ImMunoGeneTics (IMGT) online tool HighV-QUEST. Nucleotide sequences giving rise to identical CDR3s at the protein level were classified as clonotypes. Grouping clones into clonotypes of conserved amino acid sequences allowed us to trace (identified) clonotypes throughout the infection. Considering that TCR repertoires between mice are never identical, due of the random nature of TCR recombination, we used two EF4.1 donors to ask if clonotypes (and their behavior) were conserved (public) or exclusive (private) between mice.

Subsequent sequencing of the pre-immune repertoires of donor EF4.1 CD4 T cells revealed initial diversity of Vα2 and Vα3 compartments to be comparable (Figure 12). Analysis of the TCR repertoires at the peak of infection (day 7) revealed that the virus-specific Vα2 response was almost exclusively (~90%) dominated by a

single clonotype (CDR3 sequence CAAITGNTGKLIF), indicated in red. Notably, this Vα2 'red' clonotype was also the single largest clone within the Vα2 pre-immune repertoire, and was conserved between mice; perhaps indicating an increased propensity for this clonotype to be selected and maintained during T cell development. Interestingly, the peak of the virus-specific Vα3 response was also dominated by a single clonotype, similarly annotated in red due to its corresponding behavior (CDR3 sequence CVLSGDYSNNRLTL) (Fig). However, this clonotype was less prolific, only comprising ~55% of total Vα3 cells on day 7. This allowed for a greater level of diversity within the responding Vα3 repertoire. In addition, and in contrast to its Vα2 counterpart, this particular red clonotype was very rare (<0.5%) in the pre-immune repertoire.

Past the peak of infection, the substitution of the Vα2 family for the Vα3 family, was again evident at the population level. Despite this, the red Vα2 clonotype remained high (>60%) within virus-specific Vα2 cells, although this was slightly reduced in comparison with its peak due to the emergence of another clonotype (indicated in orange). In contrast, the newly dominant Vα3 family was considerably more polyclonal, containing distinct sub families of clonotypes. Notably, the virus-specific Vα3 response was characterized by the emergence of two new families of distinctive behaviors. The first group was comprised of two 'public' clonotypes (indicated by green colours; CDR3 sequences CALDNTNTGKLTF and CALNVGDNSKLIW), which together made up ~10% of all virus-specific Vα3 cells. Notably, their frequency remained remarkably consistent throughout the course of infection. The second group comprised of multiple clonotypes (indicated by blue-purple colours), all sharing the same SNNRIFF motif in their CDR3 sequences, created by the use of the *Traj31* segment. This group contained clonotypes that were not always conserved between mice, but collectively increased in frequency to 30–50% of the virus-specific Vα3 cells by day 49 of infection.

Figure 12 Clonotypic diversity of virus-specific CD4 T cells increases over the course of FV infection.



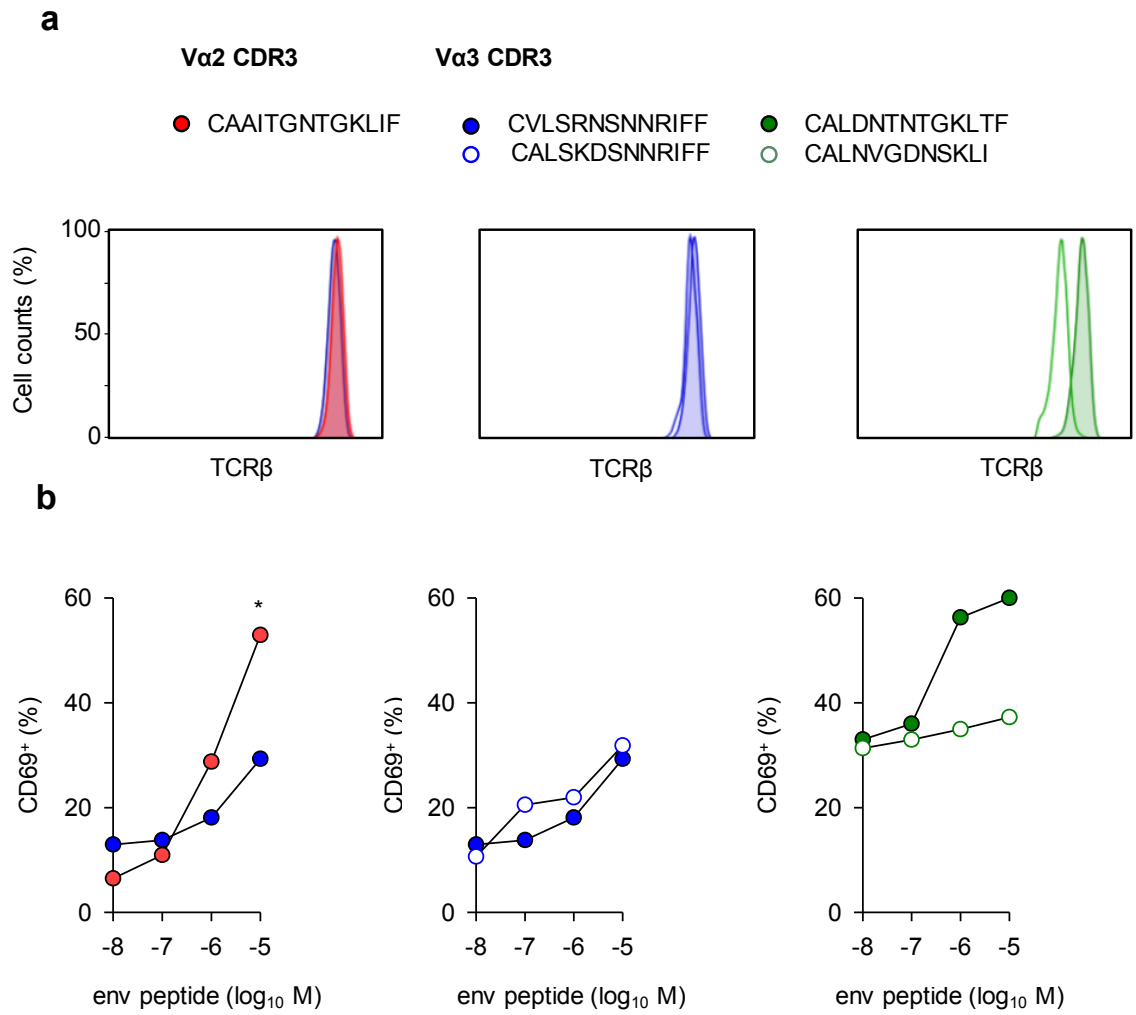
(a) Frequency of various Vα2 and Vα3 clonotypes in total EF4.1 CD4 T cells from two uninfected donor mice (pre-immune; donors 1 and 2) or in env-reactive EF4.1 CD4 T cells from the spleens of recipient mice after adoptive T
cell transfer from the indicated donor and FV infection. Each bar graph represents an individual mouse. Clonotypes with frequencies <0.5% are not plotted for simplicity. Identical clonotypes within Vα2 and Vα3 subsets are marked with the same color, with red color indicating the dominant Vα2 and Vα3 clonotypes. The scale of the Vα2 and Vα3 plots are adjusted to the relative ratio of the two subsets at a given time point.

These findings revealed three distinct types behaviour, which correlated with different CDR3 TCR motifs. The first was exemplified by the 'red' clonotypes that universally dominated the primary response. Importantly clonotypes exhibiting this behaviour existed within both the Vα2 and Vα3 populations. The second, exemplified by green clones, was unique due to the proportional stability of the clones throughout the response. The third was exemplified by the more diverse blue clonotypes, which collectively dominated at later time points driving the clonotypic switch from the Vα2 family to Vα3 family. Thus, the clonotypic diversity of virus-specific EF4.1 CD4 T cell population increased over time owing to the decrease in the proportion of initially dominant red clonotypes in conjunction with the proportional increase in blue clonotypes from within the Vα3 population. Although common motifs within CDR3 regions could be used to discriminate and group clonotypes, they could not predict the respective avidities of distinct clonotypes.

4.3.5 Generation of hybridoma cell lines.

To interrogate differences between clonotypes we measured their functional avidities alongside accurate measurements of TCR affinity. To this end, we generated multiple hybridoma cell lines representing the three families of clonotypes, 'red' Vα2, 'blue' Vα3 and 'green' Vα3 (Figure13). Most of the hybridoma clones expressed comparable levels of TCR apart from one of the green clonotypes (CDR3 CALDNTNTGKLTF) (Figure 13a). Levels of activation achieved following *in vitro* peptide stimulation at a range of peptide doses, was then used to calculate functional avidities and hence infer the relative avidities of the different hybridomas cell lines. Reassuringly, different hybridomas from the same family behaved almost identically to each other in functional assays, suggesting that they were representative of their larger clonotypic families. Furthermore, *in vitro* stimulation of 'red' and 'blue' hybridomas reproduced the differences in functional avidity observed between the bulk EF4.1 env-specific Vα2 and Vα3 populations (Figure 13b). Notably the green clonotypes exhibited higher functional avidities than other Vα3 clonotypes.

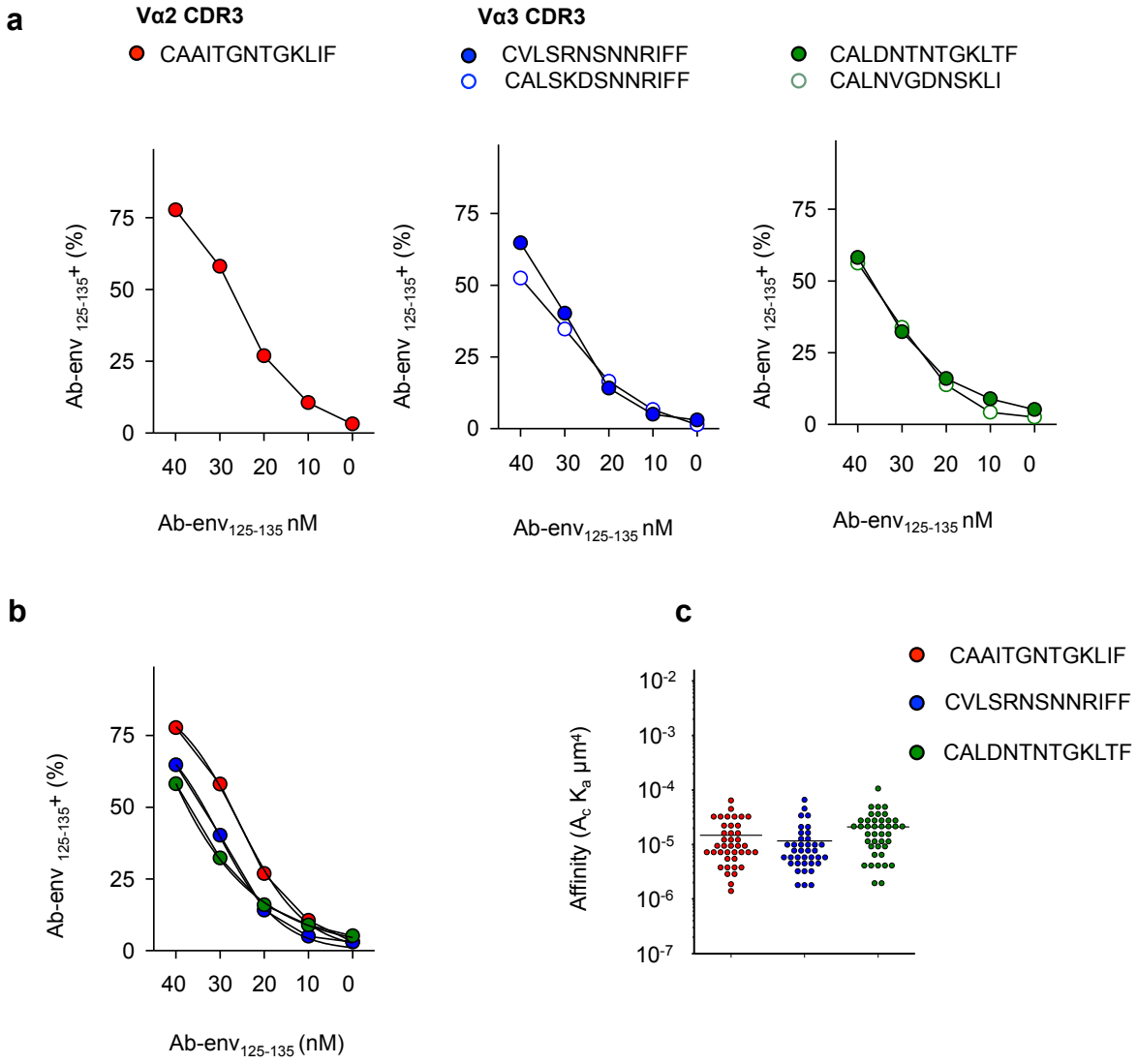
Figure 13 Generation and characterisation Vα2 'red', Vα3 'blue' and Vα3 'green' T cell hybridoma cell lines.



(a) CDR3 sequences and TCRβ expression levels in newly generated hybridoma cells lines representing the indicated Vα2 'red', Vα3 'blue' and Vα3 'green' clonotypes (b) Responsiveness of the same hybridoma cells lines as in **a** to overnight stimulation with varying amounts of $env_{122–141}$ peptide, as measured by CD69 induction. Closed symbols are the means (±s.e.m.) and are representative of more than 3 independent experiments.

We reasoned that differences in the functional responsiveness of clonotypes, could arise from underlying differences in TCR affinity for pMHCII. We decided to explore this via the use of MHCII tetramers. MHC tetramer technology was first introduced in 1996 as a way to identify antigen-specific T lymphocytes (Altman et al., 1996). Tetramers are multimeric structures consisting of fluorescently tagged pMHCII molecules. Binding of cognate TCRs to tetramer complexes, allows for the detection of specific clones via the identification of fluorescence carried by the tetramer complex. In addition to detecting antigen-specific cells, tetramers have also been used as a way to measure the strength of TCR-pMHCII interactions, where it is assumed that TCRs of high affinity will be more efficient at binding tetramer than comparatively lower affinity TCRs. Therefore, we decided to employ the use of env-pMHC tetramers, generated by our collaborators, to interrogate the affinities of the different hybridoma cell lines, Surprisingly however, the measured functional avidity of the clones correlated weakly with binding affinity towards cognate antigen, as measured in this way. Indeed, when stained with titrated amounts of an A^b-$env_{125-135}$ tetramer, assuming higher concentrations of tetramers are required to measure lower affinity interactions, the apparent affinity of Vα3 ‘blue’ clones and Va3 ‘green’ clones was only marginally lower than that of the Vα2 ‘red’ clones (Figure 14a-b).

Figure 14 Affinity of Vα2 'red', Vα3 'blue' and Vα3 'green' T cell hybridoma cell lines.



(a) CDR3 sequences representing the indicated Vα2 'red', Vα3 'blue' and Vα3 'green' clonotypes and the frequency of Ab-env$_{125–135}$ tetramer-positive cells, at different tetramer concentrations, (b) The frequency of Ab-env$_{125–135}$ tetramer-positive cells, at different tetramer concentration, following staining of the three selected hybridoma cells lines (lines or best fit are plotted) Closed symbols are the means (±s.e.m.) and are representative of more than 3 independent experiments. (c) Effective two-dimensional TCR affinities for Ab-env$_{125–135}$ of T-cell hybridomas as in **b** measured by the micropipette adhesion frequency assay and normalized by TCR surface density. Each individual data point represents the affinity of a single T cell. Numbers in the plot represent the effective affinity geometric mean of the population. (n.s)

However, although tetramers have been widely used as a correlate of TCR affinity, some studies have suggested this correlation to be tenuous or limited. More recently, micropipette adhesion assays have been applied to specifically study TCR-ligand interactions, and has allowed for more accurate measurements of affinity. Specifically, this technique allows for the assessment of 2-dimensional (2D) interactions, rather than three-dimensional (3D), between receptors and ligands, both anchored to apposing surfaces (Chesla et al., 1998). Additionally, and unlike multimeric tetramer approaches, this assay interrogates a single receptor ligand bond (Chesla et al., 1998). In this assay, red blood cells (RBC) are immobilized on a pressurized micropipette, having been loaded with pMHCII. Each RBC thus acts as a surrogate APC in this system. Next an immobilized T cell, on a second pipette, is forced into contact with the RBC, enabling TCR:pMHC interactions between the two cells. The T cell is then pulled away from the contact and the force required to break this interaction is measured. This is repeated many times, allowing for a measurement of the frequency of productive bonds, in addition to the mechanical force needed to break them (Chesla et al., 1998). Thus to validate our findings from tetramer based approaches, we sent our hybridoma clones to collaborators who performed this assay. However, measurements of 2-dimensional TCR affinity for A^b-$env_{125-135}$ using this micropipette adhesion frequency assay, again revealed only minor differences in effective TCR affinities between the clonotypes (Figure 14c) (Huang et al., 2010) This data suggested that functional avidity did not correlate well with TCR affinity towards antigen.

4.3.6 Clonotypic evolution of virus-specific CD4 T cells depends on pre- and post-infection selection events

Precise measurement of TCR affinity towards antigen had only revealed minor differences between clonotypes. In addition to TCR affinity for antigen, recent studies have also indicated a role for self-reactivity in determining CD4 T cell responses toward cognate antigen (Mandl et al., 2013, Malherbe et al., 2008, Tubo et al., 2013a, Persaud et al., 2014). We therefore wanted to investigate if clonotypic behaviour correlated better with the levels of self-reactivity than with foreign antigen. It was possible that increased self-reactive potential could set intrinsic sensitivity towards antigen, thus dictating the different responsiveness of the

clonotypes. It was also possible, that an enhanced ability to recognise spMHC, once antigen availability had become limiting, could drive the preferential maintenance of the most self-reactive clones.

4.3.7 Ly6c expression distinguishes Vα2 and Vα3 at the population level.

Due to the unknown nature of the self-peptides recognised by each clonotype, we could not use the hybridoma cell lines to measure respective TCR affinities towards self-antigens. In order to compare self-reactivity, and without the knowledge of the fine self-specificity of each TCR, we used surrogate markers of self-reactivity. Initially we looked for evidence for differences in self-reactivity between the bulk en-reactive Vα2 and Vα3 families from within EF4.1 mice. Here, the self-reactive capacity of a T cell population was accessed according to the expression of Ly6c, CD44 and CD5.

Perhaps the best-described marker for self-reactivity is the expression of CD5 on naïve CD4 T cells. CD5 itself, is a negative regulator of TCR signalling, and is first expressed during T cell development in the thymus. Here, its expression is thought to be proportional to the degree of TCR signalling received (Azzam et al., 1998, Tarakhovsky et al., 1995, Mandl et al., 2013). With higher levels of CD5 expression on thymocytes, indicative of higher levels of TCR signalling encountered by thymocytes during positive selection. CD5 expression is not set indefinitely in the thymus and requires continued maintenance in the periphery, achieved via MHCII derived signalling. In this regard, the expression of CD5 on peripheral CD4 T cells is used to report the intensity of recent self-pMHC engagements (Azzam et al., 1998, Smith et al., 2001, Mandl et al., 2013). Although currently the best-characterised and most sensitive marker for self-reactivity, distribution of CD5 is relatively narrow when accessed by antibody staining. Therefore, perceivably small differences in the expression between cells may equate to relatively large differences in their potential self-reactivity. Beyond CD5, work has shown that Ly6c expression on naïve CD4 T cells can also serve as a marker for self-reactivity. In fact, Ly6c expression can split naïve CD4 T cells into two distinct $Ly6c^{high}$ or $Ly6c^{low}$ fractions. Here, naïve CD4 T cells present a comparatively greater range in distribution of Ly6c expression, than that observed using CD5. Additionally, and

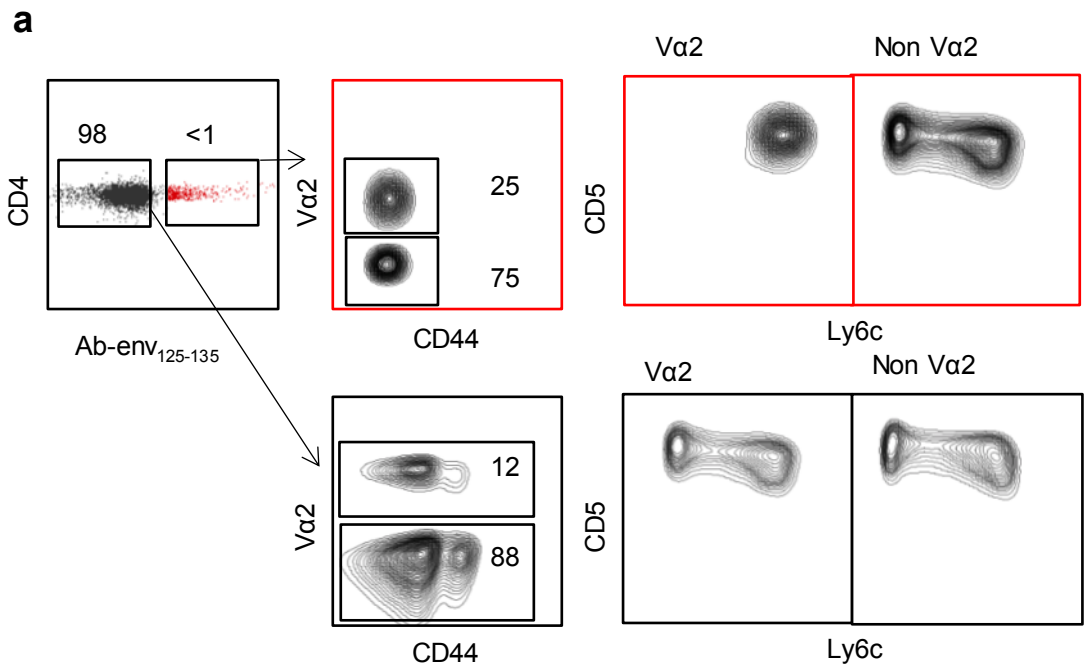
unlike with CD5, Ly6c expression is inversely proportional to a clones self-reactivity (Martin et al., 2013). Again unlike CD5, CD4 T cells also only acquire Ly6c expression as they leave the thymus (McHeyzer-Williams and McHeyzer-Williams, 2004). After which, loss of Ly6c expression is proportional to the ability of a clone to recognise self-antigens in the periphery (McHeyzer-Williams and McHeyzer-Williams, 2004). Thus, it is established that $Ly6c^{low}$ naïve T cells are comparatively more self-reactive than $Ly6c^{high}$ naïve T cells. Heightened self-reactivity may also be reflected in the expression of CD44, on CD4 T cells. CD44 is conventionally used as a marker of T cell activation as it is recruited to the immunological synapse during T cell activation. The heterogeneity of CD44 expression within the peripheral CD4 T cell pool, suggests that sufficiently strong TCR signalling to self-antigen can drive CD44 expression in the absence of foreign-antigen-activation. However, its need for strong TCR signalling perhaps restricts its ability to report for only the most self-reactive clones.

4.3.7.1 Heterogeneity of the naïve pool: Ly6c expression

Staining with labelled cognate pMHCII tetramer allowed us to detect naïve F-MLV env-specific CD4 T cells from within the naïve CD4 repertoire of EF4.1 mice. Additional staining for TCR Vα2 chains, allowed us to distinguish high and low avidity clones accordingly (Figure 15). TCR Vα2 antibody staining was used, as Vα3.2 antibodies did not capture all the Vα3 family members. Although this approach didn't allow for resolution of the individual ‘red’, ‘blue’ and ‘green’ clonotypes, it did reveal differences in Ly6c expression between high and low avidity families; the Vα2 and non-Vα2 tetramer positive CD4 populations respectively. Tetramer bound non-Vα2 cells were heterogeneous in Ly6c expression, and comparable to the bulk (non-specific) CD4 T cell population. In contrast, Vα2 tetramer positive cells were all high for Ly6c expression. This property seemed unique to Vα2 F-MLV env-specific cells as tetramer negative Vα2 cells were again comparable to the bulk CD4 T cell population. As the degree of Ly6c expression is thought to be inversely proportion to self-reactivity, this result predicted the Vα3 population to contain more self-reactive cells than the Vα2 population. However, this particular tetramer seemed to bind some $env_{122\text{-}141}$-

specific T cell clones but not others as it only allowed for the detection of ~5% of all the env-specific cells available. Since this tetramer-based strategy was severely limited to the visualisation of only a minority of FV-specific clones, we wanted to confirm this result using additional approaches.

Figure 15 Heterogonous expression of Ly6c on Vα2 or non-Vα2 virus-specific naive EF4.1 CD4 T cells

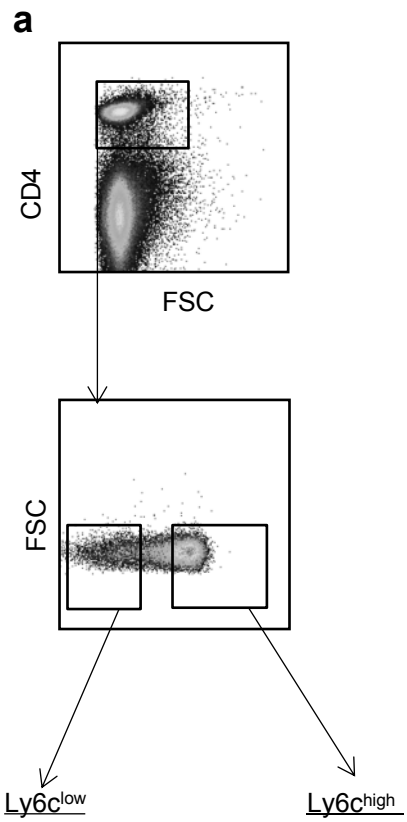


(a) Flow cytometric example of Ly6c expression on Vα2 or non-Vα2 (Vα3) virus-specific (Ab-env$_{125-135}^{+}$) on non-specific (Ab-env$_{125-135}^{-}$) on EF4.1 CD4 T cells that were not stimulated. Numbering denotes the percentages of cells in each gate.

To identify all env-reactive T cells, we sorted naïve ($CD44^{low/int}$ $CD25^{-}$) CD4 T cells from EF4.1 mice into $Ly6c^{low}$ or $Ly6c^{high}$ fractions and subsequently stimulated them *in vitro* (Figure 16). Gating on activated cells, prior to the first cell division, we next accessed differences in the Vα composition between these fractions. Interestingly the activated $Ly6c^{low}$ fractions consisted of mainly Vα3 cells, with minimal representation from the Vα2 population. In contrast the activated $Ly6c^{high}$ fraction was heavily enriched for Vα2 cells, comprising ~40% of the response, compared to the 25% observed in unsorted cells. Next by calculating the relative distribution of Vα2 or Vα3 T cell clones, between the $Ly6c^{high}$ and $Ly6c^{low}$ cells we estimated that 6% of Vα2 cells compared to 23% of Vα3 cells lay within the $Ly6c^{low}$ fraction. This was in agreement with the tetramer data, suggesting that the Vα3 population contained more self-reactive clones. Next we aimed to resolve the self-reactivity of the individual clonotypes from within Vα3 population using TCR based sequencing techniques.

EF4.1 CD4 T cells were sorted into three fractions, $CD44^{low}Ly6c^{high}$ (most naïve), $CD44^{low}Ly6c^{low}$ (intermediate) and $CD44^{high}$ (memory-phenotype), spanning the spectrum from the most naive to most self-reactive T cells (Figure. 17a). We then sequenced these fractions and asked how the Vα3 ‘red’, ‘blue’ and ‘green’ clonotypes where distributed between them. TCR sequencing revealed that the majority of cells from the ‘red’ or ‘blue’ Vα3 sub-families were $CD44^{low}Ly6c^{high}$, the most naïve in phenotype. In sharp contrast, the vast majority of ‘green’ clonotypes were in the intermediate $CD44^{low}Ly6c^{low}$ fraction, with the remaining split between naïve and memory-phenotypes (Figure 17b). These findings suggested that perhaps the remarkable proportional stability of ‘green’ Vα3 clonotypes was due to their pre-differentiated phenotype (Tubo et al., 2013a, Persaud et al., 2014). However, differences in self-reactivity, as reflected by CD44 and Ly6c levels, could not account for the disparate behaviour exhibited by the ‘red’ and ‘blue’ using Vα2 or Vα3 clonotypes.

Figure 16 Distribution of Vα2 or non-Vα2 virus-specific EF4.1 CD4 T cells into Ly6^{high} or Ly6c^{low} fractions.



(a) Flow cytometric example of the sort strategy used to isolate naïve EF4.1 CD4 T cells into Ly6c^{high} (least self-reactive) or Ly6c^{low} (more self-reactive) fractions (purity >98%). Sorted fractions where then activated with 10^{-5}M of $env_{122–141}$ peptide *in vitro* (b) Flow cytometric gating to show the frequency of Vα2 env-reactive cells (CD69^{+}) in Ly6c^{high} or Ly6c^{low} (as in **a**) fraction following 18 hours of $env_{121-142}$ peptide stimulation. Number denote the frequency of Vα2 cells in each gate (c) Graphs show the percentage or distribution of env-reactive Vα2 or Va3 CD4 EF4.1 T cell into activated Ly6c^{high} or Ly6c^{low} fractions. Bars on the graphs plot the means; open symbols are individual experiments.

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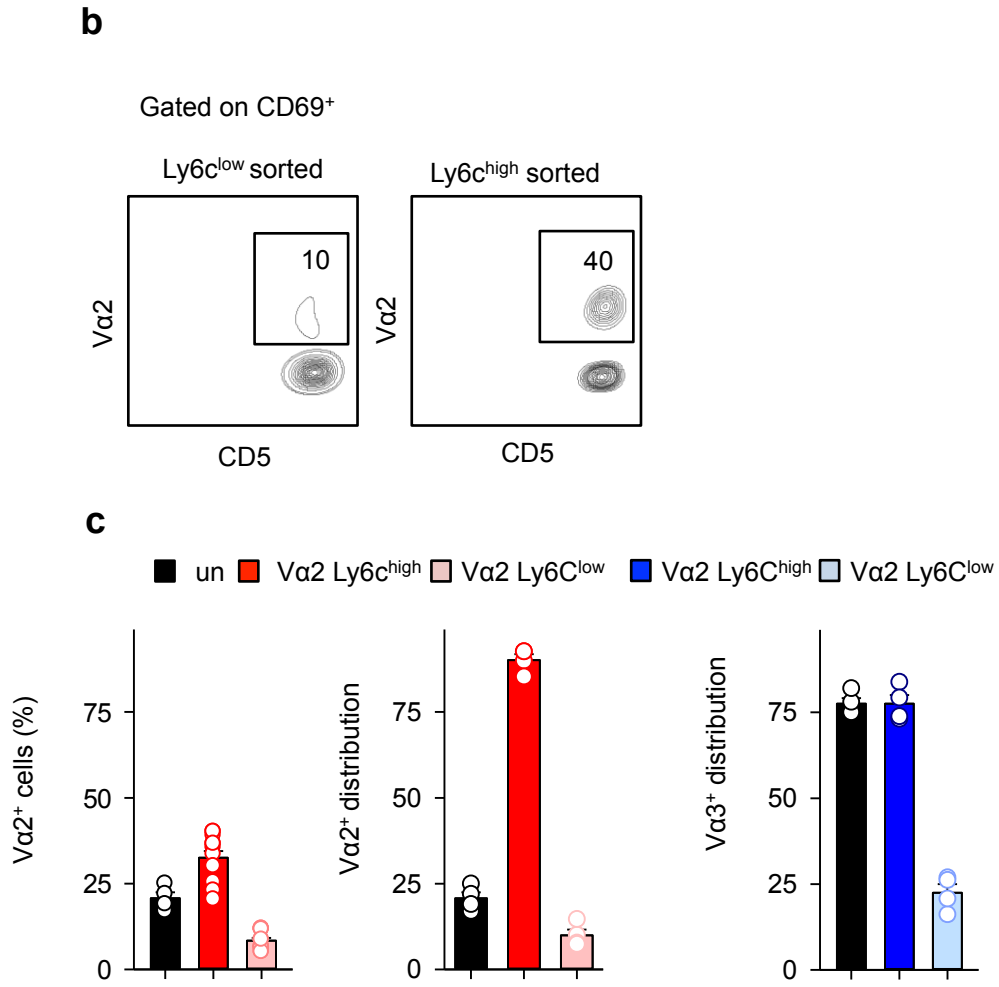
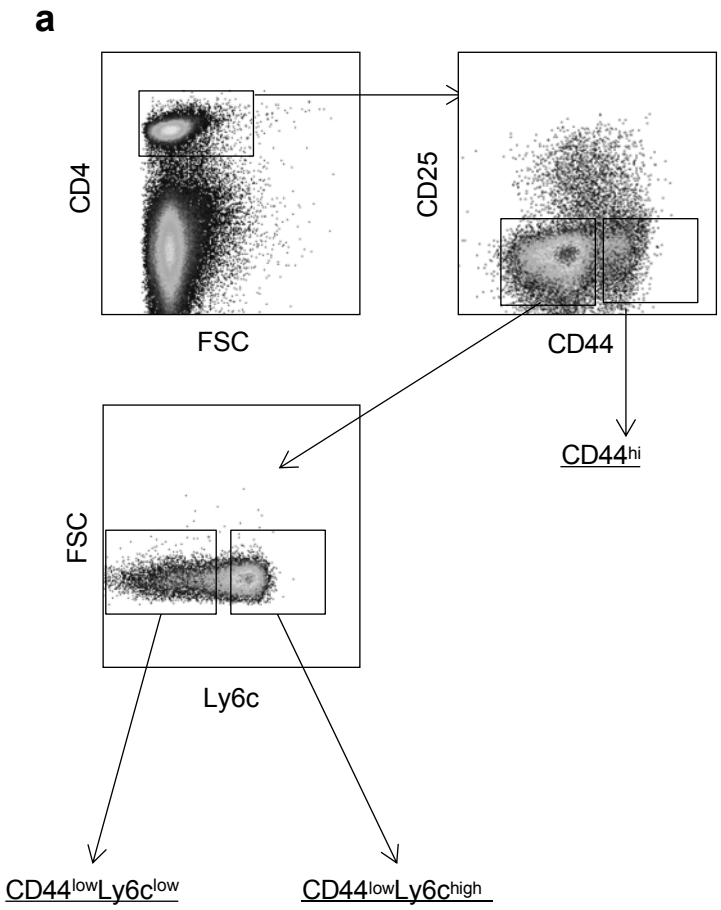

b
Gated on CD69+
Ly6clow sorted
Ly6chigh sorted
10
40
Vα2
Vα2
CD5
CD5
c
un
Vα2 Ly6chigh
Vα2 Ly6Clow
Vα2 Ly6Chigh
Vα2 Ly6Clow
Vα2+ cells (%)
Vα2+ distribution
Vα3+ distribution
75
50
25
0

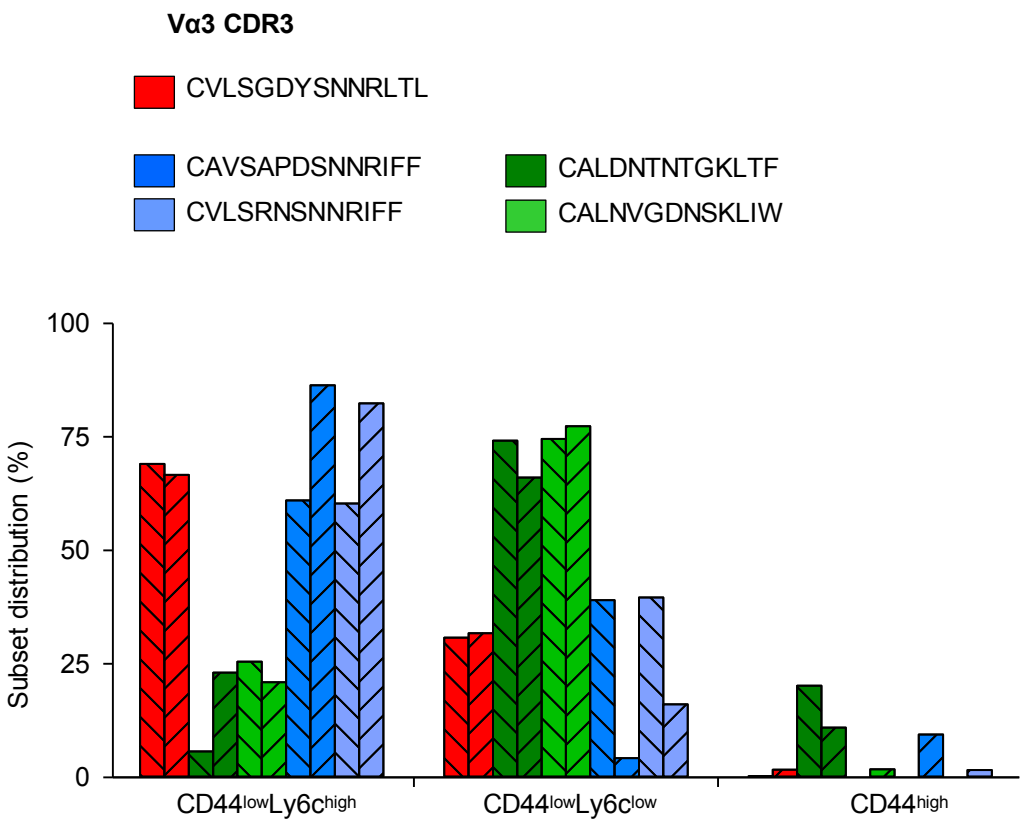
Figure 17 Vα3 'green' clonotypes are overtly self-reactive in the pre-immune repertoire



EF4.1 CD4 T cell were sorted into three fractions, $CD44^{low}Ly6c^{high}$ (most naïve), $CD44^{low}Ly6c^{low}$ (intermediate) and $CD44^{high}$ (memory-phenotype). These populations then were sequenced to analyses the how Vα3 red, blue and green clonotypes where distributed between them. (a) Flow cytometric example of Ly6c and CD44 expression in total EF4.1 CD4 T cells. Cells were sorted as $CD44^{low}Ly6c^{high}$, $CD44^{low}Ly6c^{high}$ or $CD44^{high}$. (b) Frequency of the indicated Vα3 clonotypes was determined by next generation TCR sequencing within the fraction. The fractions were purified from two separate EF4.1 donor mice and processed separately. Bars on the graphs represent individual mice.

Continued on the next page

b


Vα3 CDR3
CVLSGDYSNNRLTL
CAVSAPDSNNRIFF
CVLSRNSNNRIFF
CALDNTNTGKLTF
CALNVGDNSKLIW
Subset distribution (%)
100
75
50
25
0
CD44lowLy6chigh
CD44lowLy6clow
CD44high

4.3.8 Generation and characterisation of monoclonal mice and hybridoma cell lines.

Analysis of the pre-immune differentiation states did not reveal any major discrepancies between 'red' and 'blue' clonotypes. Due to the weak nature of TCR-spMHC interactions, the finer dexterity of self-recognition by a clone might not be fully reflected by these markers. Developing thymocytes have been reported to display hyper-sensitivity to self-ligands, in part due to their ectopic expression of a specific voltage gated sodium channel in thymocytes (Hogquist and Jameson, 2014). This allows for what are considered as 'weak' self-mediated interactions in mature T cells to induce strong selection signals in thymocytes (Lo et al., 2012). Perhaps then, finer differences in self-reactivity could be better accessed during development where sensitivity to self is heightened. We therefore reasoned that assessments of self-reactivity between clonotypes may be better studied through the generation of TCRαβ Tg mice which would allow us to study thymocyte development. Furthermore, since TCR signaling can differ between hybridoma cell lines and primary T cells, we reasoned affinity measurements for antigen, previously performed on hybridomas cells, would also be better accessed on primary T cells generated in these mice.

4.3.9 Clonotypic behaviour correlates better with self-reactivity than affinity

To this end, one 'red' and one 'blue' hybridoma cell line was used to template for TCRs to generate two TCRαβ doubly transgenic mouse strains EVα2 (Vα2-red clone) and EVα3 (Vα3-blue clone) respectively. To limit TCR expression to that of the transgenic TCR chains alone, mice were crossed onto a *Rag1*$^{-/-}$ background, preventing the expression of endogenous TCR chains. In addition, mice were then rendered free from the presence of the endogenous retrovirus *Emv2*.

Emv2, a relic of previous retroviral infection, is a replication-defective, single-copy endogenous retrovirus (ERV) present in B6 mice (King et al., 1988). *Emv2* shares 80% homology with F-MLV, and importantly expresses an envelope-derived epitope almost identical to the F-MLV derived epitope. Previous work in the lab showed sequence identity between the two epitopes to be corresponding, with the exception of a single amino acid substitution, a Y instead of an L at position 128

(Young et al., 2012b). Although *emv2* is normally replicative defective, due to mutations, its stable integration allows for *emv2*-derived products to be expressed to the immune system in a similar manner to self-derived proteins. In addition, in immunodeficent *Rag1*$^{-/-}$ mice, it has been reported that *emv2* can recombine with other dormant endogenous viruses (Young et al., 2012a). This can result in replication competent virus, with the potential to affect MLV-specific T-cell development and subsequent response. Thus to prevent the potential of *emv2* to serve as a self-ligand or to contribute to spontaneous resurrection of endogenous viruses, it was crossed out of the B6 germline.

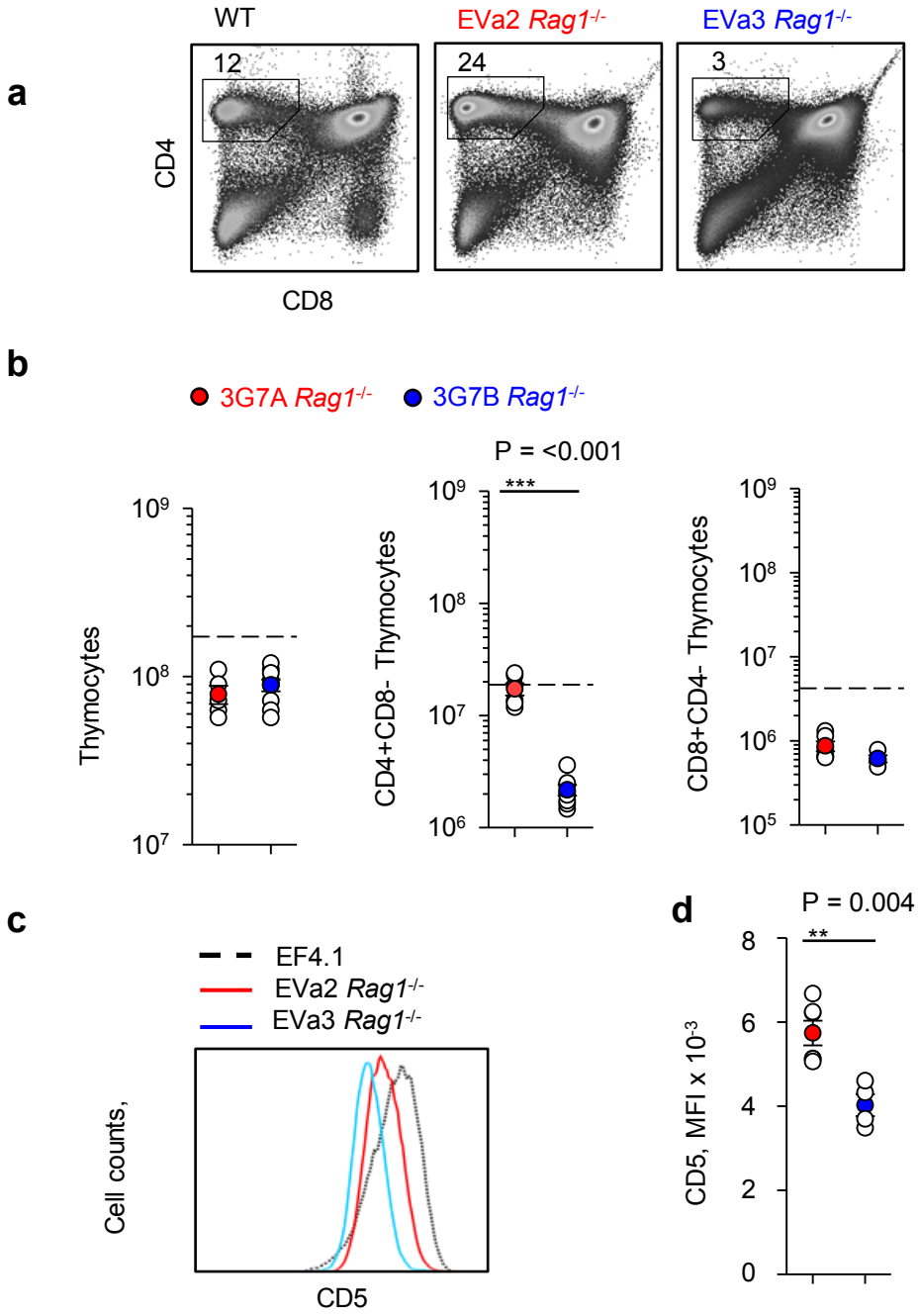
4.3.9.1 Characterisation of the EVα2 and EVα3 mice.

T cell development proceeded normally in both the EVα2 and EVα3 mice. Although in both instances T cell development was considerably skewed towards the CD4 T cell lineage, this was considerably more pronounced in the former (Figure 18a). These differences were also reflected in a statistically significant increase in both the frequency and in the absolute numbers of SP CD4 T cells in EVα2 mice as compared to EVα3 mice, indicative of more efficient positive selection as a result of stronger TCR signaling (Figure 18b). Furthermore, levels of CD5, which correlates with self-reactivity, were significantly higher in EVα2 than in EVα3 SP CD4 T cells. Collectively suggesting EVα2 T cells were able to recognize self-ligands to a greater extent (Figure 18b-c).

Peripheral CD4 T cell numbers, which also reflect the degree of self-reactivity (Kassiotis et al., 2003), were comparable to WT levels in EVα2 mice; whilst being significantly reduced in EVα3 mice (Figure 19). Although mature CD4 T cells in both monoclonal strains were all naïve in phenotype ($CD44^{low}CD25^{low}$), a greater proportion of cells were $Ly6c^{low}$ in EVα2 mice, signifying a higher degree of self-reactivity (Figure 20a). Although CD5 expression was comparatively lower in peripheral T cells compared to that in thymocytes, mature EVα2 T cells still maintained significantly higher levels than mature EVα3 T cells, confirming continued heightened self-reactivity in the periphery. These pronounced phenotypic differences in self-reactivity between the monoclonal EVα2 and EVα3 T cells

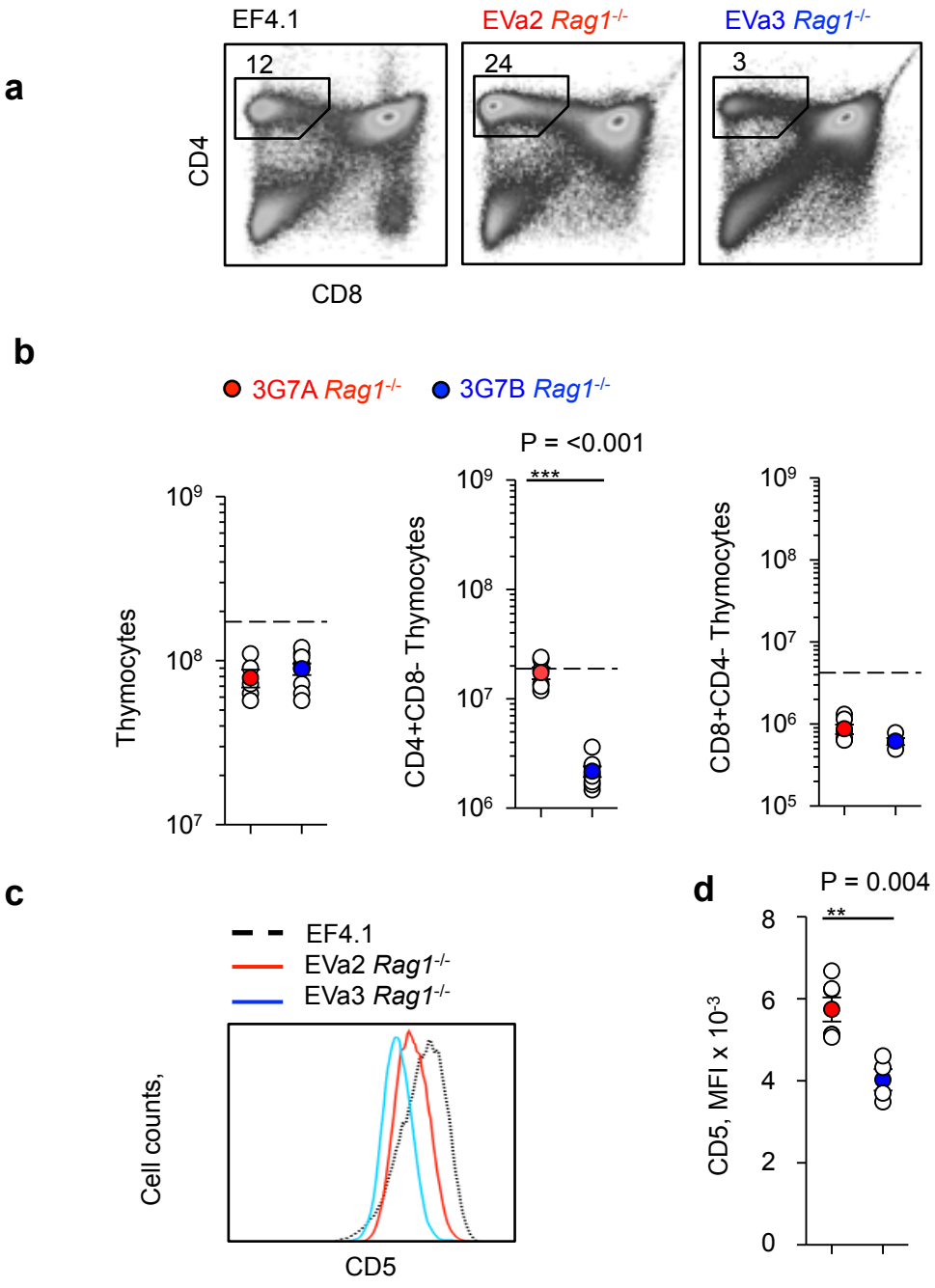
correlated remarkably well with their inferred avidities toward env-peptide stimulation *in vitro*. And although two-dimensional TCR affinity measurements for cognate peptide did reveal differences in the recognition of cognate peptide between the clones, this sensitivity only differed by a factor of two, arguably too small to account for the large disparity in their functional avidities (Figure 20e). Thus we concluded that although small differences in antigen affinity did exist, the functional avidity of ‘red’ EVα2 and ‘blue; EVα3 T cells, better correlated with self-reactivity.

Figure 18 Characterisation of thymocyte development in monoclonal EVα2 and EVα3 TCR transgenic mice



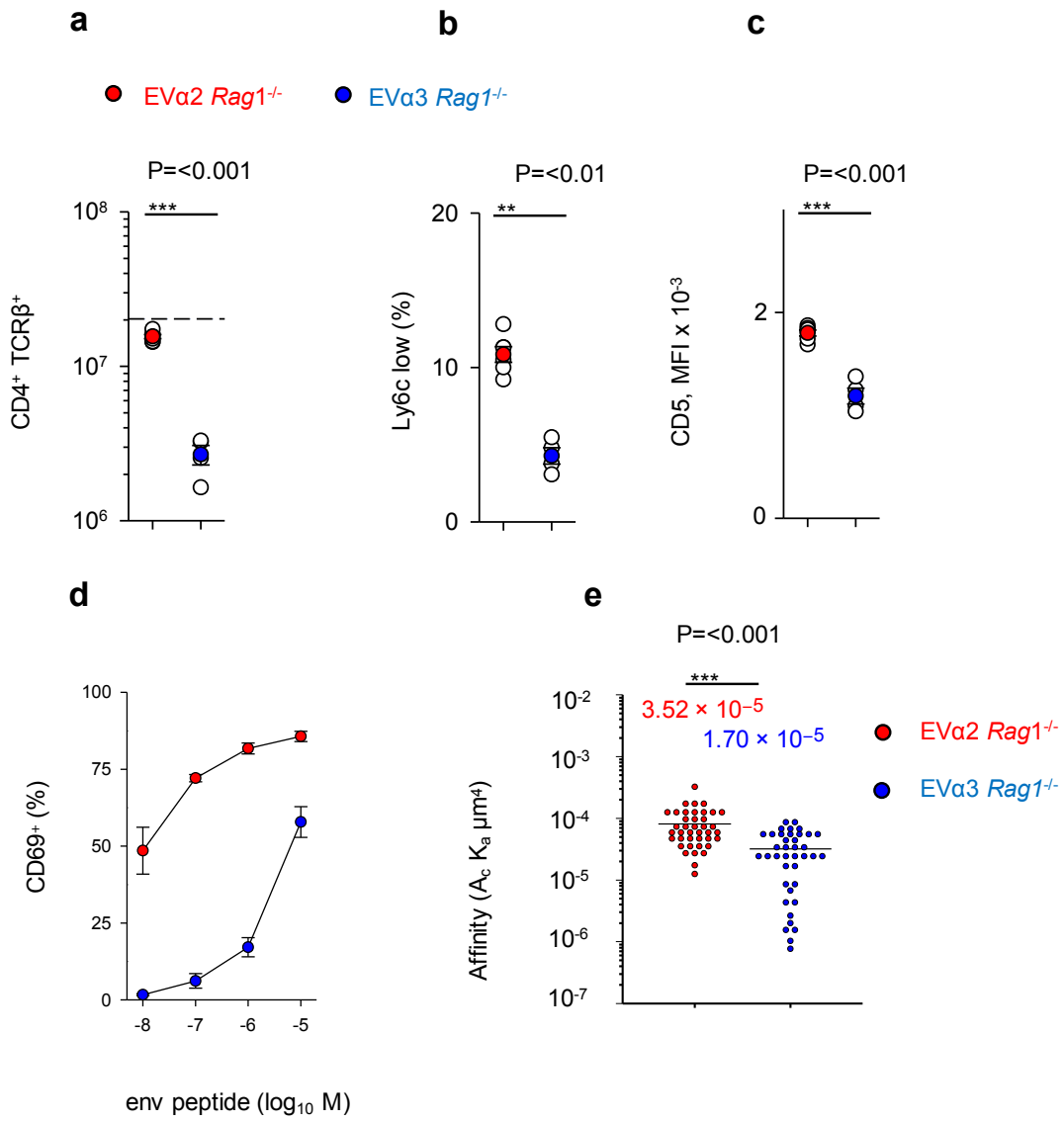
(a) Flow cytometric analysis of thymocyte development in EF4.1, EVα2 and EVα3 TCR transgenic mice. Number denote the percentage of cells CD4-single positive thymocytes (b) Absolute number of thymocytes, CD4 single-positive and CD8 single-positive thymocytes in the same mice as in **a** (c) CD5 levels (d) and MFI in post-selection CD4 single-positivee thymocytes from the same mice as in **a** Closed symbols are the means (±s.e.m.); open symbols are individual mice; numbers within the graph denote the P values accordingly; horizontal dashed lines represent the mean values for control EF4.1 mice.

Figure 19 Characterisation of lymphocyte development in monoclonal EVα2 and EVα3 TCR transgenic mice



(a) Flow cytometric analysis of splenic CD4 subsets in EF4.1, EVα2 and EVα3 transgenic mice. Numbers denote the percentage of cells within each gate (b) Flow cytometric example of Ly6c distribution in naive splenic CD4 T cells from EF4.1, EVα2 and EVα3 mice. Horizontal dashed lines represent the mean values for control EF4.1 mice. (c) and of CD5 expression levels in naive splenic CD4 T cells from the same mice.

Figure 20 Characterisation of reactivity to self and foreign-antigen of monoclonal EVα2 and EVα3 TCR transgenic mice



(a) Absolute number of naïve splenic CD4 T cells in EVα2 and EVα3 mice. (b) Ly6c (c) and CD5 mean fluorescence intensities (MFI) to infer levels of self-reactivity in CD4 T cells from within the same mice as in **a.** Closed symbols are the means (±s.e.m.); open symbols are individual mice; numbers within the graph denote the P values accordingly; horizontal dashed lines represent the mean values for control EF4.1 mice. (d) Responsiveness of EVα2 and EVα3 T cells to overnight $env_{122–141}$ peptide stimulation. Responses were measured by CD69 induction and are plotted as means (±s.e.m., n=3–4). (e) Effective two-dimensional TCR affinities for Ab-$env_{125–135}$ of primary T cells measured by the micropipette adhesion frequency assay and normalized by TCR surface density. Each individual data point represents the affinity of a single T cell. Numbers in the plot represent the effective affinity geometric mean of the population.

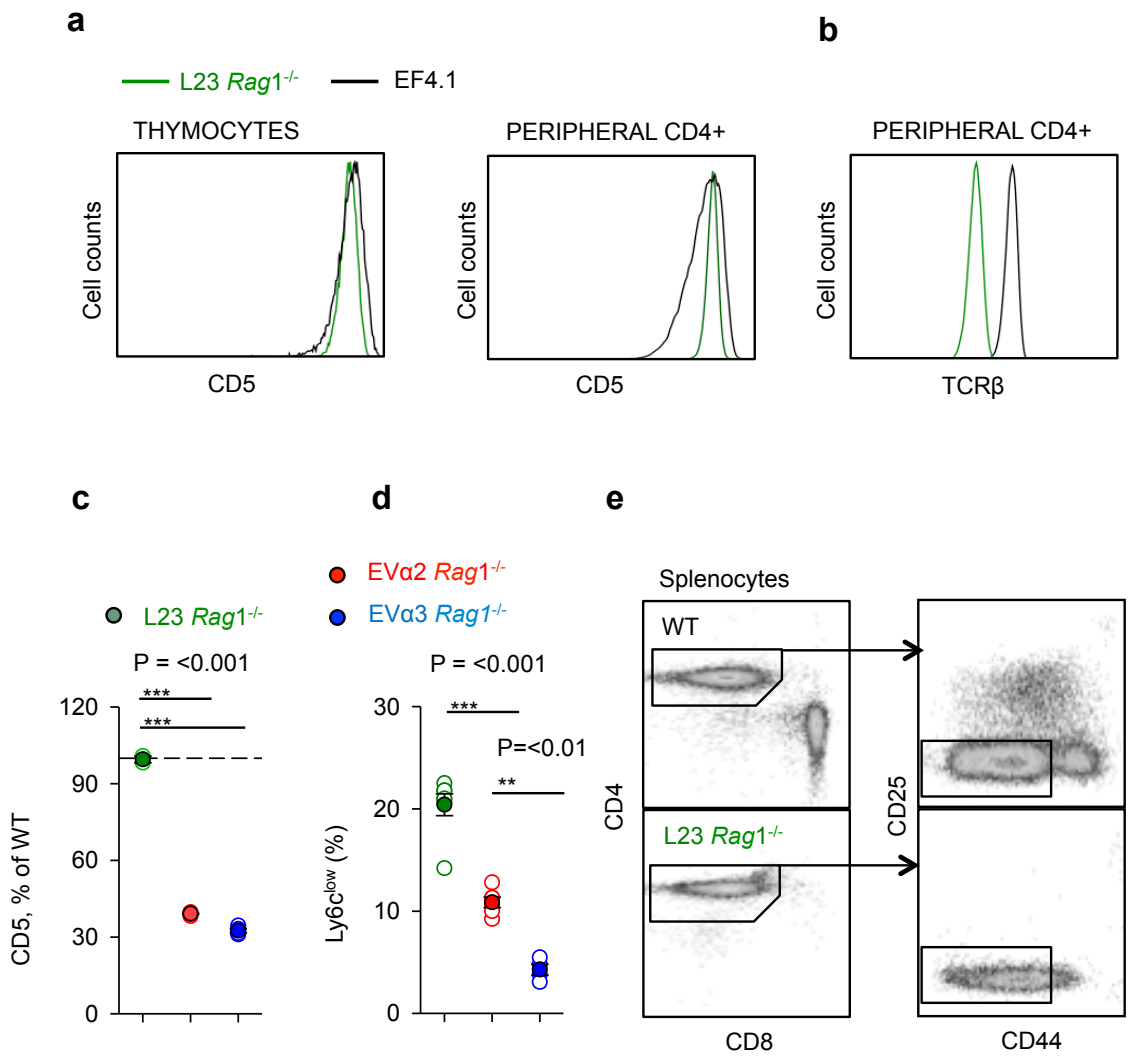
4.3.10 Generation of a 'green' TCR monoclonal mouse

Thus the behaviour of all three clonotypes correlated better with sensitivity to self than toward foreign antigen. The most overtly self-reactive TCRs seemed to belong to the 'green' family of clonotypes. Therefore, for completeness we also generated a TCR L23 monoclonal mouse templated from a 'green' clonotypes. Subsequent characterisation of the L23 mice, as described below, showed strong evidence for the self-reactive nature of this clonotype family.

4.3.10.1 Characterisation of the L23 'green' clonotypes mice

Characterization of the L23 monoclonal mice, revealed that L23 T cells expressed CD5 at significantly higher levels than those observed in EVα2 and EVα3 T cells. In addition, loss of Ly6c marked a much larger fraction of peripheral T cells in the L23 mice as compared to EVα2 and EVα3 T cells. Further still although the majority of peripheral L23 T cells were naïve ($CD44^{low}CD25^{-}$) in phenotype, there also existed an intermediate ($CD44^{int}CD25^{-}$) population suggesting the acquisition of a slightly more differentiated state. This was despite the extremely low expression levels of TCR in L23 T cells. Perhaps, itself hampered in expression, as a consequence of its overt self-reactivity. However, such low levels of TCR precluded direct comparisons between L23, EVα2, EV3 and EF4.1 T cells in any *in vitro* or functional assays. Collectively these data confirmed our previous findings attributing the stability of the 'green' Vα3 clonotypes to their pre-immune differentiation state, established by their overtly self-reactive nature.

Figure 21 Characterisation of the monoclonal L23 TCR transgenic mice re-affirms the overtly self-reactive nature of the green clonotypes.



(a) Flow cytometric analysis of CD5 levels in thymocytes and peripheral naïve splenic CD4 T cells in EF4.1 and L23 monoclonal mice (b) Levels of TCRβ on peripheral naïve splenic CD4 T cells from the same mice as in **a.** (c) Expression levels of CD5 on peripheral naïve CD4 T cells from EVα2, EVα3 and L23 monoclonal mice, expressed as a percentage of those observed in control EF4.1 CD4 T cells. Horizontal dashed lines represent the mean values for control EF4.1 mice. (d) Expression of Ly6c on peripheral naïve CD4 T cells from EVα2, EVα3 and L23 monoclonal mice. (e) Flow cytometric analysis of splenic CD4 subsets in WT and L23 monoclonal transgenic mice. Closed symbols are the means (±s.e.m.); open symbols are individual mice.

4.4 Discussion

Our findings highlight the prominent role for the TCR in dictating the clonotypic-composition of CD4 T cell responses. Most studies attribute the ability of a T cell clonotype to perform to the affinity of its given TCR towards cognate peptide. However, our study lends support towards to a growing field of evidence, supporting the importance of self-reactivity is setting sensitivity towards antigen, over just affinity for antigen alone. Further still, although it has been well established that clonal selection and dominance are key features of primary T cell responses, it remains unknown as to whether these remain key features in memory repertoire selection. Our analysis of the evolution of the CD4 TCR repertoire in responses to retroviral infection clearly highlighted differences between the primary and memory TCR repertoires. Using deep sequencing of the TCRα chains of TCRβ Tg EF4.1 T cells, we were able to show the progressive diversification of the T cell response over time, rather than repertoire narrowing. TCR repertoire evolution was driven by the proportional loss of high avidity early responders, allowing for the emergence of the lower avidity clonotypes later in the response. Our results support a prominent role for TCR self-reactivity in determining responsiveness to antigen however the molecular basis behind this remains to be defined. Furthermore, the TCR extrinsic factors driving the diversification of the clonotypic response remained to be resolved. We next wanted to understand the selective forces which were driving the proportional rise in cells of lower avidities at later time points in FV infection, and to establish why this differed so wildly between different mice.

Chapter 5. The role of APCs in the diversification of the CD4 T cell response.

5.1 Introduction

5.1.1 Aim

It is now established that clonal selection and clonal dominance are key features of primary T cell responses. However, extensive analysis of TCR repertoires throughout immune responses has highlighted differences between the primary and memory CD4 T cell repertoires; suggestive that distinct criteria may exist for each. In this study, we have explored this question by using TCR clonotypic analysis at different time-points during chronic retroviral infection. Previously, we were able to show that discrete families of clonotypes dominated at different phases in the response, and that distinct clonotypic behaviours segregated with their functional avidity. In this regard, clonotypes of the highest functional avidity (red clones) responded rapidly and dominated the early response, whereas the comparatively lower avidity clonotypes (blue clones) were favored at later time points. This ultimately led to the diversification, rather than the progressive restriction, of the responding CD4 T cell repertoire. In addition, data showed that clonotypic sensitivity and functional responsiveness to F-MLV better correlated with self-reactivity than with affinity towards foreign-antigen. However, the mechanisms or forces behind the clonotypic evolution remained un-resolved. Moreover, why in some mice the clonotypic switch was observed but in others not, remained unclear. We therefore wanted to explore T cell extrinsic factors, that may differ between individual mice, and that may be influencing the clonotypic evolution of the evolving CD4 T cell response.

5.1.2 Clonal Selection and Diversity

Although TCR avidity is critical in establishing the clonotypic hierarchy in many CD4 T cell responses, other parameters must exist in order to balance clonal selection with diversity. These parameters presumably have the potential to narrow the gap between higher and lower avidity clonotypes by overhauling TCR avidity hierarchies.

5.1.2.1 Interclonal competition

Competition for limited resources allows for the evolution of a given population, this is equally true for CD4 T cell populations (Raff, 1992). The ability of a T cell to compete will not only be affected by its own ability to recognise antigen but also by their competitors ability (McHeyzer-Williams and Davis, 1995). Here clonotypes of the same specificity but differing avidities or functional avidities may not only compete for access to antigen but also for growth factors and the physical space available. Therefore, the faster a clone is able to respond the less prohibited its expansion will be due to the relative availability of such factors. Progressively as the available space is filled, expansion ceases, limiting the recruitment of clones that saw antigen less well or later. Therefore, the faster the response the more the initial advantage of high avidity clonotypes is retained (Thorborn et al., 2014). Conversely, slower responses, and hence slower completion, may permit clonotypes of a broader range of avidities to be recruited. Thus the clonotypic composition of the CD4 T cell repertoire may be largely dictated by the speed of the response (Thorborn et al., 2014).

Competition can also be linked to the precursor frequency of a particular clonotype. In order to become activated a T cell must first find an APC presenting its cognate peptide (Vrisekoop et al., 2014). These events can be rare, especially when considering the vastness of the periphery in respect to a single T cell. Therefore. increasing the number of T cells utilising a given TCR (precursor frequency) will act to significantly increase the likelihood and decrease the time taken for a clonotype to make contact with its cognate-peptide (Vrisekoop et al., 2014). In this way, clonotypes with higher precursor frequencies presumable already have a head start in the race. Although the parameters that dictate the size of clonal families is not established, presumable clonotypes with heightened self-reactivity may be better represented in the peripheral pool due to their ability to ascertain pro-survival signalling.

5.1.2.2 Antigen availability

Antigen availability will alter the degree of clonal competition between clonotypes. For example, increasing amounts of antigenic pMHCII complexes will prime an

increasing number of clonotypes. This will presumably lesson competition and allow for a greater range and diversity of clonotypes to be recruited. Conversely limiting availability of antigen will enforce fierce interclonal competition, selecting for the cells most efficient at recognising antigen, presumably the cells of highest avidities. In addition, the abundance or density of pMHCII complexes expressed at the level of a single APC may too be important; where high densities of pMHCII will trigger a more intense TCR signal allowing for the recruitment of lower avidity clonotypes in conjunction with higher avidity clonotypes. Conversely, too much antigen may also limit the success of certain clonotypes potentially rendering the cells anergic or leading to their elimination from the response via activation induced cell death (AICD). Presumably, this would most impact those clonotypes with highest sensitivity toward antigen. In these ways the levels and availability of antigen can influence the ensuing CD4 T cell response.

5.1.2.3 APC availability

As CD4 T cells cannot recognise antigen in its native form, the availability of antigen will be intrinsically limited by the availability of APCs at any given time. In this way the number of APCs presenting antigen can influence the clonal composition. Presumably the greater the availability of APCs and pMHCII, the more clonotypes recruited and the greater the diversity of the responding pool. Furthermore, different types of APCs have been associated with instructing for divergent CD4 Th cell subsets. As different Th subsets, specifically germinal centre associated Tfh, have been associated with differences in long-term longevity, APCs may also effect long-term representation of certain clonotypes.

5.1.3 Antigen presentation: Quality, Quantity and Location.

As discussed any variability in antigen load or availability of antigen presenting cells could impact the clonotypic composition of a given CD4 T cell response. Thus the potential for different APCs as critical regulators of T cell fate should be considered more generally.

A role for different APCs in dictating T cell fate is first evident when studying T cell development in the thymus. Very early experiments, using reconstitution of

irradiated hosts with donor bone marrow, showed that subsequent T cells that emerged from the thymus (now seeded with donor APCs) were newly restricted by the MHCII haplotype of the donor cells. This not only highlighted the role of APCs in mediating the quality of the selected T-cell repertoires but also suggested that distinct APCs had the capacity to contribute to this process: in this scenario donor bone-marrow derived cells in addition to host thymic epithelial cells (TECS) (Longo and Schwartz, 1980). It is now well established that different types of antigen presenting cell colonise distinct parts of the thymus. In the thymic cortex, cTECs are the main APC type. Here they mediate positive selection events. Studies have suggested that these cells may exhibit special proteolytic antigen processing pathways (Hogquist and Jameson, 2014), that allows them to generate and present unique peptide repertoires suggested to be essential for efficient positive selection (Klein et al., 2014, Marrack and Kappler, 1987). In addition, cTECs promote clusters of 'nurse cells' to envelope developing thymocytes, generating a special micro-environment thought to optimize T-cell selection and promote proliferation (Nakagawa et al., 2012, Shortman et al., 1986), although the necessity of these architectures remains to be fully understood (Pezzano et al., 2001). Negative selection occurs in the medulla, and involves distinct thymic APCs, these being mainly mTECs and thymic DCs whose main role is in mediating negative selection. In addition, various thymic APC subsets have been associated with different capacities in their ability to promote tTreg cell differentiation (Lee et al., 2011).

The organisation of different APC types into discrete anatomical niches has become a subject of much interest and debate (Matzinger and Guerder, 1989). When trying to understand why this patterning exists *in vivo*, much research has suggested that thymic APCs differ in their ability to perform certain tasks necessary for TCR repertoire selection, which in turn determines their contribution and attendance at positive or negative selection events during T cell development (Wilkinson et al., 1995, Klein et al., 2009). On the other hand, research has also contested this theory, concluding that diverse thymic APC types are equally efficient at mediating positive and negative selection as well as in the selection of tTreg T cells (Yasutomo et al., 2000, Martinic et al., 2003, Proietto et al., 2008). These studies proposed that the spatial organization of APCs (*in vivo*) dictated the

capacity of a certain APCs to perform and not the other way round. Clearly, when some of these anatomical or numerical barriers were abolished, for example in *in vitro* aggregate cultures, where cells are randomly positioned, diverse APCs could fulfill roles not conventionally associated with them *in vivo* (Yasutomo et al., 2000) However, whilst different APCs may appear to support efficient thymocyte selection *in vitro* (as accessed by the numerical output of SP thymocytes), the quality of the selected repertoires are most likely drastically impacted. This could have large implications of the protective capacities of these selected repertoires which is something better accessed *in vivo* rather than *in vitro*. Nonetheless, experiments comparing the efficiency of non-thymic APCs versus thymic APCs at mediating negative selection, conclusively showed the former to be far less efficient in this regard (Wilkinson et al., 1995). As availability of MHC derived signaling was not limited in these experiments, this suggested that this difference was mediated by a qualitative rather than a quantitative difference conclusively highlighting the potential for diverse APCs in influencing CD4 T cell fate.

Different APCs have also been suggested to assume different roles in the clonal selection of CD4 T cells in the periphery. Exogenous antigen, is mainly internalized by three types of professional APCs in the periphery 1) DCs 2) B cells and 3) Macrophages. Antigen is then processed by the APCs and subsequently displayed to CD4 T cells in the form of pMHCII complexes. Diverse APCs have been shown to differ in the way they capture and process exogenous antigen and in their expression of co-stimulatory molecules, as well as in factors that promote the cellular adherence between T cells and APCs (Hamilos, 1989). All such factors co-operate to install qualitatively distinct signals in the partnered CD4 T cells, potentially instructing for diverse cells fates accordingly. In this regard, differences in the type of APC a T cell receives its signal from, may go some way in explaining the observations from several studies which showed relatively surprising plasticity of a single T cell clone, although other factors must be considered here too (Tubo et al., 2013a, Stemberger et al., 2007, Gerlach et al., 2010). In addition to the qualitative differences that may exist between professional APCs, they also vary wildly in their absolute numbers and anatomical locations. These spatial and numerical considerations likely influence the capacity of certain cell types or subsets to perform as proficient APCs. Classically, DCs are thought to be the main

antigen presenting cells, responsible for priming T cells during an immune challenge. However, in some regards B cells have perhaps been unfairly dismissed. Studies have shown that activated B cells (Malynn et al., 1985), but not naive B cells, were able to prime and expand T cell populations (Lassila et al., 1988). This seemed to 'put to bed' the idea that B cells might significantly contribute to early T cell responses, as although they vastly dominant over DCs in terms of absolute numbers, low precursor frequencies of antigen-specific B cells and a dependency on T cell help to expand them, would presumably hinder them in this capacity. However, B cells can also become activated independently of the BCR, which could perhaps unleash them from the numerical, anatomical and qualitative limitations associated with naïve B cells; as clearly *in vitro* B cell can perform as proficient APC when given the opportunity.

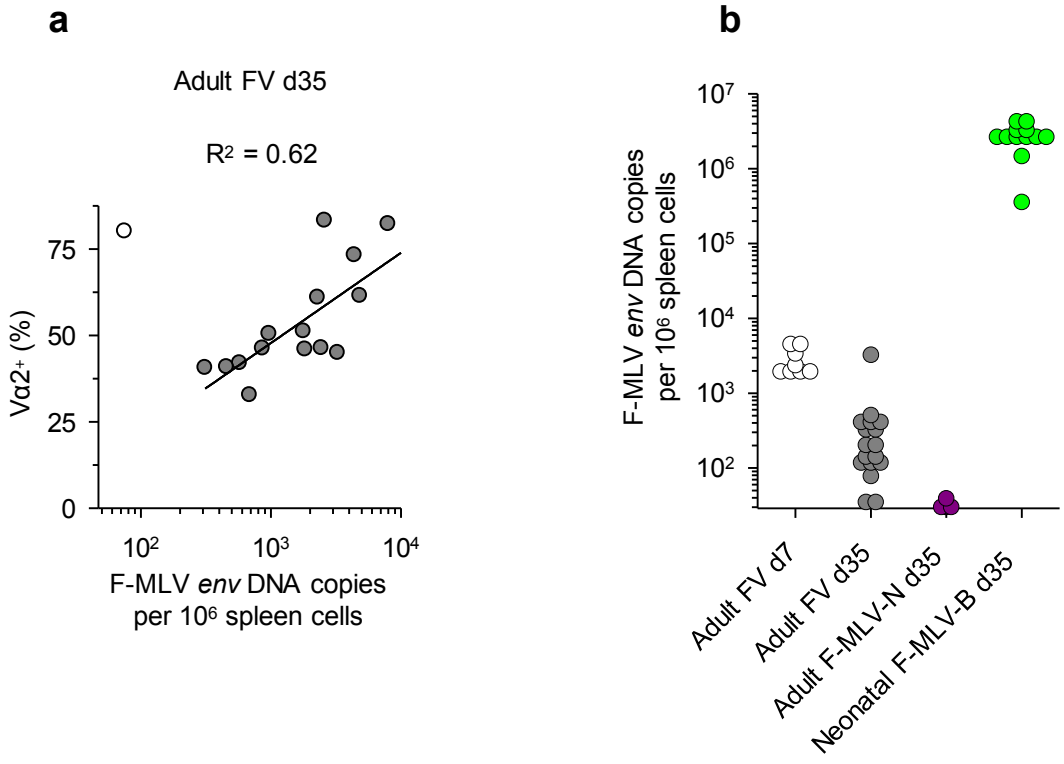
Another obvious example for the role for different APCs in impacting CD4 T cell responses was revealed by studies that investigated the conditions that permitted Treg T cell proliferation (Walker, 2004). Treg T cells are often termed as occupying an anergic phenotype *in vitro,* i.*e.* they remain unresponsive when activated via their TCR. However, experiments that interrogated various sources of APCs, APC types and maturation states, revealed that in fact Treg T cell cells could proliferate extensively when stimulated in the context of the right APC. In these experiments the direct expansion of functional Treg T cells could be only be achieved in the presence of antigen loaded mature $CD86^{high}$ DCs, by not isolated splenic B cells or $CD86^{low}$ DCs (Yamazaki et al., 2003). Thus highlighting the potential for diverse APCs in shaping the clonotypic composition of many types of CD4 T cell responses.

5.2 Results

5.2.1 Effect of infection kinetics on the composition of virus specific T cells.

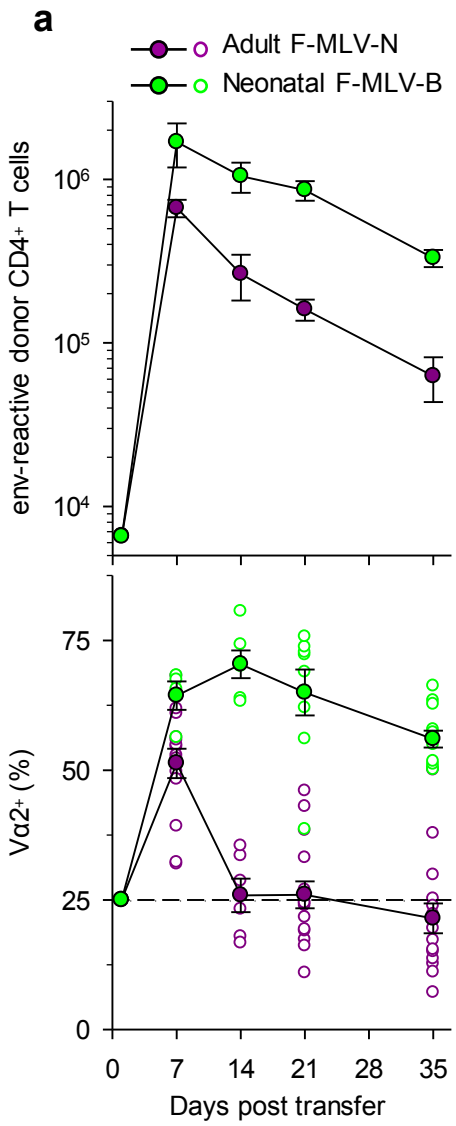
Although B6 mice are resistant to FV, with all mice eventually controlling the virus, individuals vary in their efficiency to do so. Viral loads were measurement by the copy number of F-MLV per $1x10^6$ spleen cells from FV infected mice. Viral copy number was determined by real-time qPCR, using primers that specifically amplified the envelope of F-MLV. Measuring F-MLV copy number revealed that although mice had near identical viral titres at day 7, by d35 viral loads where highly variable (Figure 22b). Interestingly, this was reflected in a corresponding divergence in the quality of the CD4 T cell repertoires between mice. Thus, we considered that viral load might be directly influencing the clonotypic composition of the responding cells. Since strong signalling can lead to cell death or anergy it was possible that antigen, if persistently high, could eventually become detrimental; presumably with greatest negative impact on the most sensitive clonotypes (Persaud et al., 2014). Such a relationship was suggested by a strong correlation between F-MLV copy numbers, from the spleens of infected mice and the corresponding frequency of virus-specific Vα2 clones within those mice. However, a positive correlation between the two argued against antigen compromising the clones of highest avidity (Vα2) (Figure 22a). Rather, this suggested higher viral loads favoured the maintenance of the Vα2 clones. To better understand if CD4 clonotypic responses were associated with the abundance of antigen, we decided to manipulate viral kinetics to study how this subsequently influenced the responding CD4 T cell repertoire. To this end, we either infected adult B6 mice with N-tropic F-MLV (F-MLV-N), or neonatal B6 mice with B-tropic F-MLV (F-MLV-B).

Figure 22 Viral load correlates with the persistence of Vα2 virus-specific CD4 T cells.



(a) Frequency of Vα2 env-reactive donor EF4. 1 CD4 T cells plotted against copies of F-MLV env DNA in the spleens of recipient mice 35 days after adoptive T cell transfer and FV infection. Each symbol is representative of an individual mouse; the open symbol, represents a single mouse which was excluded from the regression analysis. (b) Copies of F-MLV env DNA in the spleens of recipient mice that were infected as adults with FV or F-MLV-N or as neonates with F-MLV-B. In all case, EF4.1 CD4 T cells were transferred into adult recipients and then analysed in the days following T cell transfer. Each symbol is an individual mouse. The dashed line denotes the detection limit.

Figure 23 Effect of infection kinetics on the clonal composition of virus-specific CD4 T cells.



(a) Absolute numbers (top) and Vα composition (bottom) of env-reactive donor EF4.1 CD4 T cells in the spleens of recipient mice after T cell adoptive transfer into adult mice either infected with F-MLV-N at the time of T-cell transfer or with F-MLV-B as neonates (n=4–15 mice per time point; $P<0.001$ between the two types of host on days 14–35, Mann–Whitney rank sum test). Closed symbols are the means (±s.e.m.); open symbols are individual mice; the dashed line represents the frequency of Vα2 cells in pre-immune env-reactive EF4.1 CD4 T cells

Murine leukaemia viruses can be divided into distinct groups according to their tropism: B tropic, N tropic, and NB tropic (Yoshikura, 1975). Viral tropism relates to the specificity of the virus towards a given host or a host tissue. Thus N and B tropic viruses preferentially grow in N or B mouse types, whereas NB-tropic viruses can propagate in both (Yoshikura, 1975). B6 are a B type mouse strain and thus are particularly non permissive to F-MLV-N, specifically due to its restriction by the host *Fv1*^{b} allele. Therefore, F-MLV-N is quickly eliminated in adult B6 mice (Figure 22b). This leads to lower levels of virus, as accessed by qPCR, and for shorter durations of time. Importantly, F-MLV-N viral tropism is determined by a pair of adjacent amino acids within the viral capsid, and therefore does not alter the quality of the T cell envelope epitope recognised (Stevens et al., 2004). Contrastingly, prolonged infection with inflated viral loads can be achieved through the infection of neonatal B6 mice with WT F-MLV-B (Figure 22b). Although adult B6 mice are resistant to F-MLV-B, its delivery into young mice, results in immunological tolerance and thus persistent infection (Erlebacher, 2013). In order to study CD4 T cell responses in this setting, EF4.1 T cells were subsequently transferred into neonatal-infected hosts when they reached adulthood. These distinct infection regimès allowed us to study the effect of low-level infection (F-MLV-N) or non-resolving (neonatal F-MLV-B infection) infection on the clonotypic composition.

Initial expansion of total EF4.1 T cells was proportional to the degree of viral replication exhibited between the two infections (Figure 23 top panel). At day 7, Vα2 clones dominated the response in all the neonatal F-MLV-B infected hosts. Whilst on average this was also true for F-MLV-N infected hosts, there was a much greater degree of heterogeneity between mice already by day 7 (Figure 23 bottom panel). In fact, past day 7, the frequency of Vα2 clones rapidly declined homogenously in the non-persistent infection. This was in sharp contrast to the exclusive maintenance of Vα2 clones in the non-resolving infection. This data advocated that the clonotypic composition was directly influenced by antigen, whereby continually high levels maintained clones of the highest avidity, thus preventing the clonotypic switch towards clones of lower avidity. This was perhaps surprising considering that higher avidities and affinities are normally favored at low levels of antigen, as the more sensitive the TCR presumably also the more efficient

they are at recognizing antigen at low densities (Walker et al., 2010). In contrast, low affinity and avidity T cells have been shown to be better established in the context of higher levels of antigen (Leggatt, 2014). We therefore reasoned a distinct parameter must be responsible for selecting for lower avidity clones besides antigen.

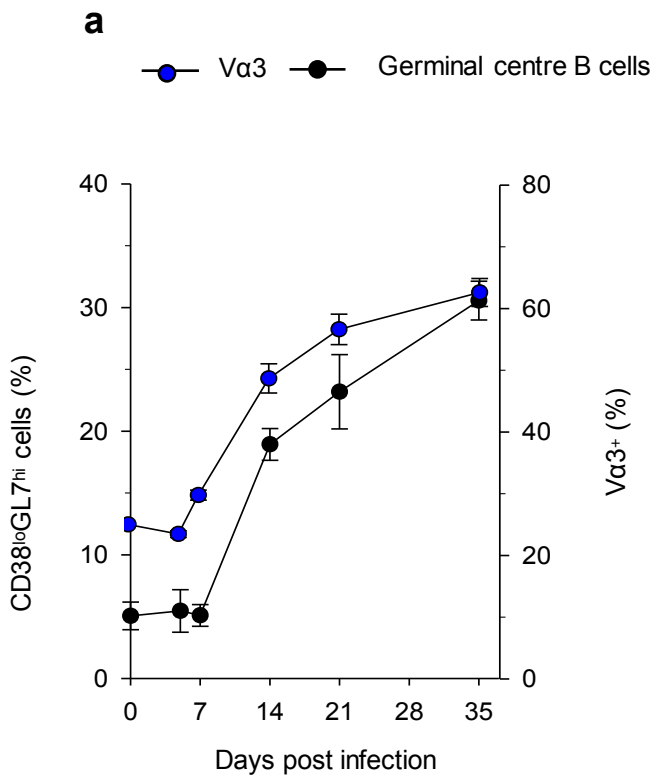
5.2.2 Clonal replacement requires cognate B cell interactions

Induction of CD4 T cell responses require interactions with APCs, as T cells can only recognise antigen in the context of MHC. Thus we reasoned, the effect of higher viral loads on CD4 T cell responses must be mediated through APCs. It was therefore important to consider the impact of viral load on the quality and quantity of both antigen and the APCs themselves. Non-specific and specific activation of B cells is not only critical for the initiation of B cell responses to infection, but also for their ability to perform as proficient APCs. Conventionally, antigen presentation to CD4 T cells by B cells is considered a heavily one-sided interaction, allowing B cells to obtain T cell help. However, we considered that these T-B cell collaborations may be equally important for the CD4 T cell response. Thus we decided to study the B cell response to FV infection.

To this end, host mice were infected with FV, following which the frequency of $CD38^{low}GL7^{hi}$ cells within $IGD^{low}B220^{high}$ B cells (germinal centre phenotype), were measured at the indicated time-points during infection (Figure 24a). We observed that the B cell response to FV, compared to the bulk CD4 T cell response, was temporally distinct in regards to its activation kinetics. This saw, the proportion of germinal centre cells ($CD38^{low}GL7^{hi}$ cells within $IGD^{low}B220^{+}$ B cells) remaining unchanged at day 7, the peak of the CD4 response. In fact, the frequency of activated B cells only started to increase following day 10 post infection, following which it then continued to steadily increase throughout the course of infection. Although B cell activation was delayed when compared to the bulk CD4 T cell response, it in-fact temporally matched the late proportional rise of low avidity Vα3 clonotypes (Figure 24a). To better understand the relationship between these two responses, we decided to manipulate the kinetics of B cell activation to study how this might impact the clonotypic replacement of Vα2 for Vα3 clonotypes. To this

end, we transferred EF4.1 T cells into mice co-infection with F-MLV and Lactate Dehydrogenase Virus (LDV).

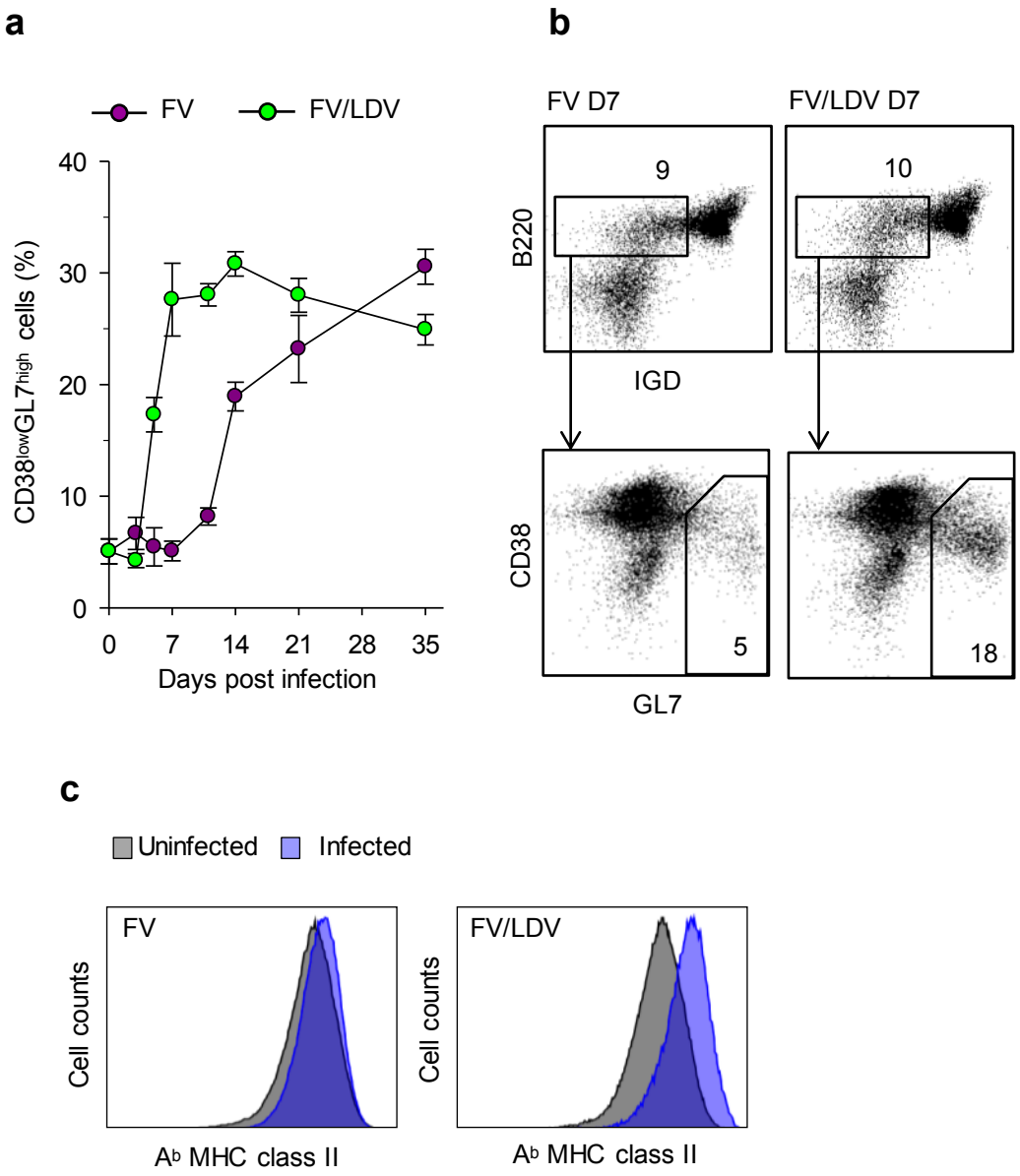
Figure 24 B cell responses in FV infected hosts, temporally match the proportional rise Vα3 virus-specific clonotypes



(a) Frequency of $CD38^{low}GL7^{high}$ of $B220^{high}IgD^{low}$ splenic B cells in recipient mice over the course of FV infection and following T cell adoptive transfer (b) and (left) the Vα3 composition of env-reactive donor EF4.1 CD4 T cells in the spleens of recipient mice after T cell adoptive transfer. Closed symbols are the means (±s.e.m.).

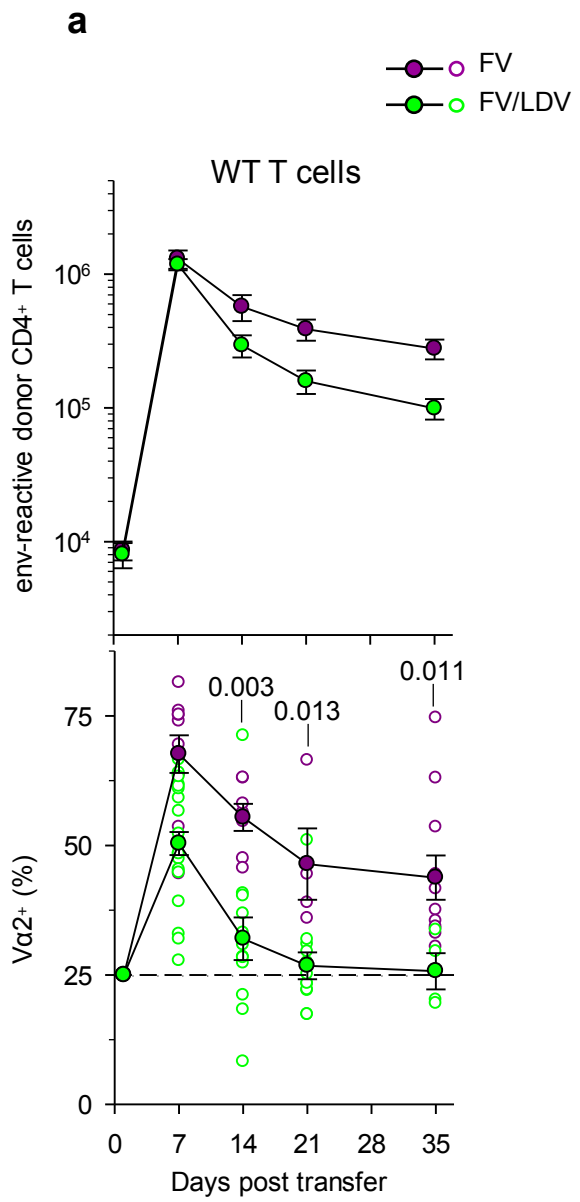
LDV infection is known to result in the polyclonal activation of B cells. As this activation is T cell independent, it reportedly allows for rapid and mass proliferation of cells irrespective of their BCR specificity (Montes et al., 2007). Indeed, we observed that in comparison to infection with FV alone, B cell responses to co-infection were greatly accelerated, rapidly expanding from day 3 through until day 7 and reaching their peak at d10 (Figure 25a-b). Co-infection also induced higher levels of MHCII expression on B cells by day 7 as compared to FV infection alone (Figure 25c). Critically, this accelerated B cell response was mirrored by a faster decline in Vα2 clonotypes within responding EF4.1 T cells (Figure 26). An increase in their numerical response, alongside their heightened levels of MHCII would presumably enhance the capacity of B cells to contribute to antigen presentation in co-infected mice. It was therefore tempting to suggest that the progressive contribution of B cells acting as APCs, may be influencing the clonotypic evolution of the CD4 T cell repertoire. However, as infection of LDV has dramatic effects beyond just the polyclonal activation of B cells, we could not exclude a role for other APCs in this system (Robertson et al., 2008). Therefore, we next examined the specific requirement for T-B cell interactions in mediating the clonotypic switch.

Figure 25 Accelerated and polyclonal B cell activation is achieved through FV/LDV co-infection.



(a) Frequency of $CD38^{low}GL7^{high}$ of $B220^{high}IGD^{low}$ in splenic B cells over the course of FV infection or FV/LDV co-infection. Closed symbols are the means (±s.e.m.)(b) Flow cytometric analysis of the frequency of $CD38^{low}GL7^{high}$ from $B220^{high}IgD^{low}$ populations in splenic B cells on day 7 post FV infection or FV/LDV co-infection. Numbers denote the percentage of cells in the gates(c) MHCII expression on total B cells ($B220^{+}$) from the same mice at 7 days post infection, compared with uninfected control mice.

Figure 26 Accelerated clonal replacement of virus-specific Vα2 CD4 T cells is achieved in FV-LDV co-infected mice



(a) Absolute numbers (top) and Vα composition (bottom) of WT env-reactive donor EF4.1 CD4 T cells in the spleens of recipient mice after adoptive T-cell transfer and FV infection (n=3–8 mice per time point) or FV/LDV co-infection (n=3–9 mice per time point). Closed symbols are the means (±s.e.m.); open symbols are individual mice

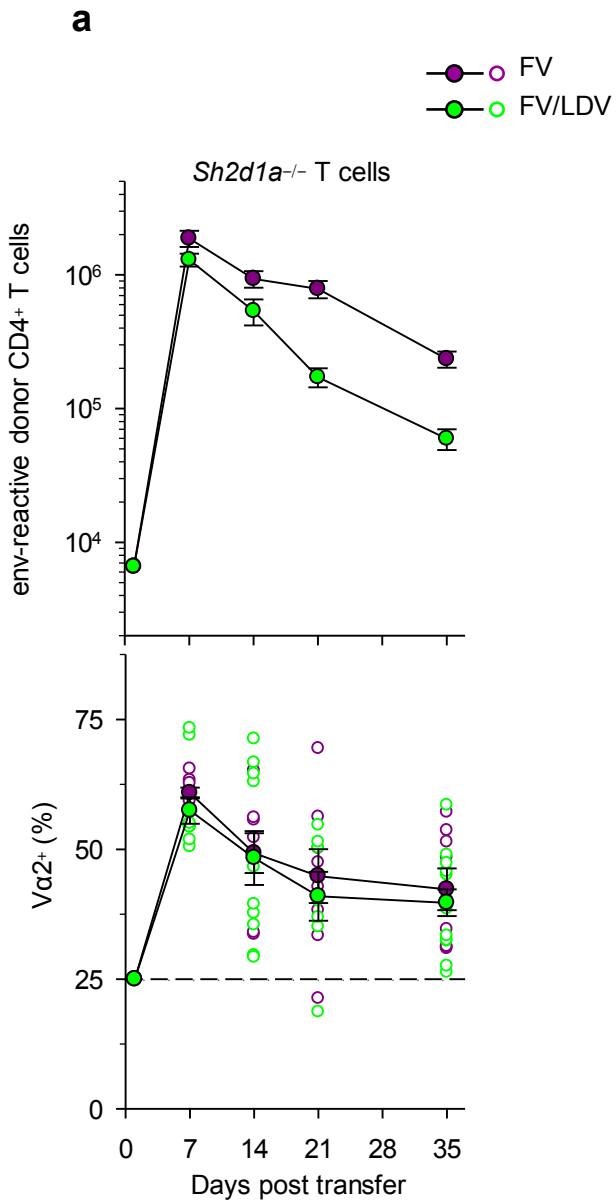
Signaling lymphocytic activation molecule (SLAM)-associated protein (SAP), encoded by the *Sh2d1a* gene, is required for the formation of prolonged T–B cell conjugates. Thus, the lab generated EF4.1 T cells deficient in this molecule, in order to delineate a requirement for interactions mediated by SAP. *Sh2d1a* deficient or WT EF4.1 T cells were transferred in WT hosts, which had either been infected with FV or co-infected with FV and LDV (Figure 27a). The clonotypic compositions of the respective CD4 T cell populations were then compared over the course of the infections.

The magnitude and activation kinetics of *Sh2d1a* deficient EF4.1 T cells, in response to both infections, were comparable to those observed in WT EF4.1 T cells. However, the use of *Sh2d1a* deficient EF4.1 T cells resulted in the exclusive maintenance of Vα2 clonotypes throughout the course of infection. This was in sharp contrast to the clonal replacement of Vα2 clonotypes observed when WT EF4.1 T cells were transferred into co-infected mice (Figure 27a). Thus, via the attenuation of T-B cell conjugates, as a result of *Sh2d1a* deficiency, we negated the accelerated decline in Vα2 cells previously observed in co-infected hosts (Figure 26). However, as *Sh2d1a* deficiency only reduces the efficiency of T-B interactions, it did not exclude the possibility of these conjugates forming. Therefore, to deny these interactions absolutely, lab members repeated these experiments using transfer of WT EF4.1 T cells into B cell deficient hosts (*Ighm*$^{-/-}$). It was observed that T cell transfer, into infected *Ighm*$^{-/-}$ but not into WT hosts, resulted in the stable maintenance of virus-specific Vα2 clonotypes, irrespective of the infection setting (Figure 27b).

Collectively this data suggested, that T-B cell interactions were somehow mediating the clonotypic switch. However, it was hard to delineate the exact role played by B cells in this process due to their multifaceted roles during infection. Although cognate interactions between B cells and T cells are known to be responsible for the clonal selection of B cells, via the provision of T cell help, a role for B cells in reciprocally influencing the clonal selection of the CD4 T cell response had not been reported. To try and dissociate a role for B cells as APCs, from other B cell dependent processes, the lab next reconstituted *Ighm*$^{-/-}$ hosts with MHCII sufficient or deficient bone marrow (Figure 27c). Reconstitution of *Ighm*$^{-/-}$ hosts by the

respective donor bone marrow, either generated B cells sufficient or deficient in MHCII and therefore capable or incapable of B cell mediated antigen presentation. These bone marrow chimeric mice were then used as hosts for infection and EF4.1 T cell transfer. On day 14 post infection, the clonotypic composition of responding CD4 EF4.1 T cells were analysed. It was observed that following FV-LDV co-infection; Vα2 high avidity clonotypes were now only maintained in hosts where B cells lacked expression of MHCII (Figure 27c). Contrastingly, hosts with MHCII sufficient B cells, capable of antigen presentation, significantly reduced the frequency of Vα2 clones, in a dose-dependent manner (Figure 27c). Therefore, we concluded that the clonotypic replacement of CD4 T cell clonotypes was driven by antigen presentation by B cells.

Figure 27 Clonal replacement of virus-specific Vα2 CD4 T cells requires cognate B-cell interaction



(a) Absolute numbers (top) and Vα composition (bottom) of Sh2d1a$^{-/-}$ (right) env-reactive donor EF4.1 CD4 T cells in the spleens of recipient mice after adoptive T cell transfer and FV infection (n=3–8 mice per time point) or FV/LDV co-infection (n=3–9 mice per time point). (b) Absolute numbers (top) and Vα composition (bottom) of env-reactive donor EF4.1 CD4 T cells in the spleens of WT or *Ighm*$^{-/-}$ recipient mice after adoptive T-cell transfer and FV infection (*n*=4–11 mice per time point) or FV/LDV co-infection (*n*=4–23 mice per time point). (**c**) Absolute numbers (top) and Vα composition (bottom) of env-reactive donor EF4.1 CD4+ T cells in the spleens of *Ighm*$^{-/-}$ mice previously reconstituted with WT (*n*=18) or *H2-Ab1*−/− B cells (*n*=14) 14 days after adoptive T-cell transfer FV/LDV co-infection, plotted against the level of B-cell reconstitution. Closed symbols are the means (±s.e.m.); open symbols are individual mice

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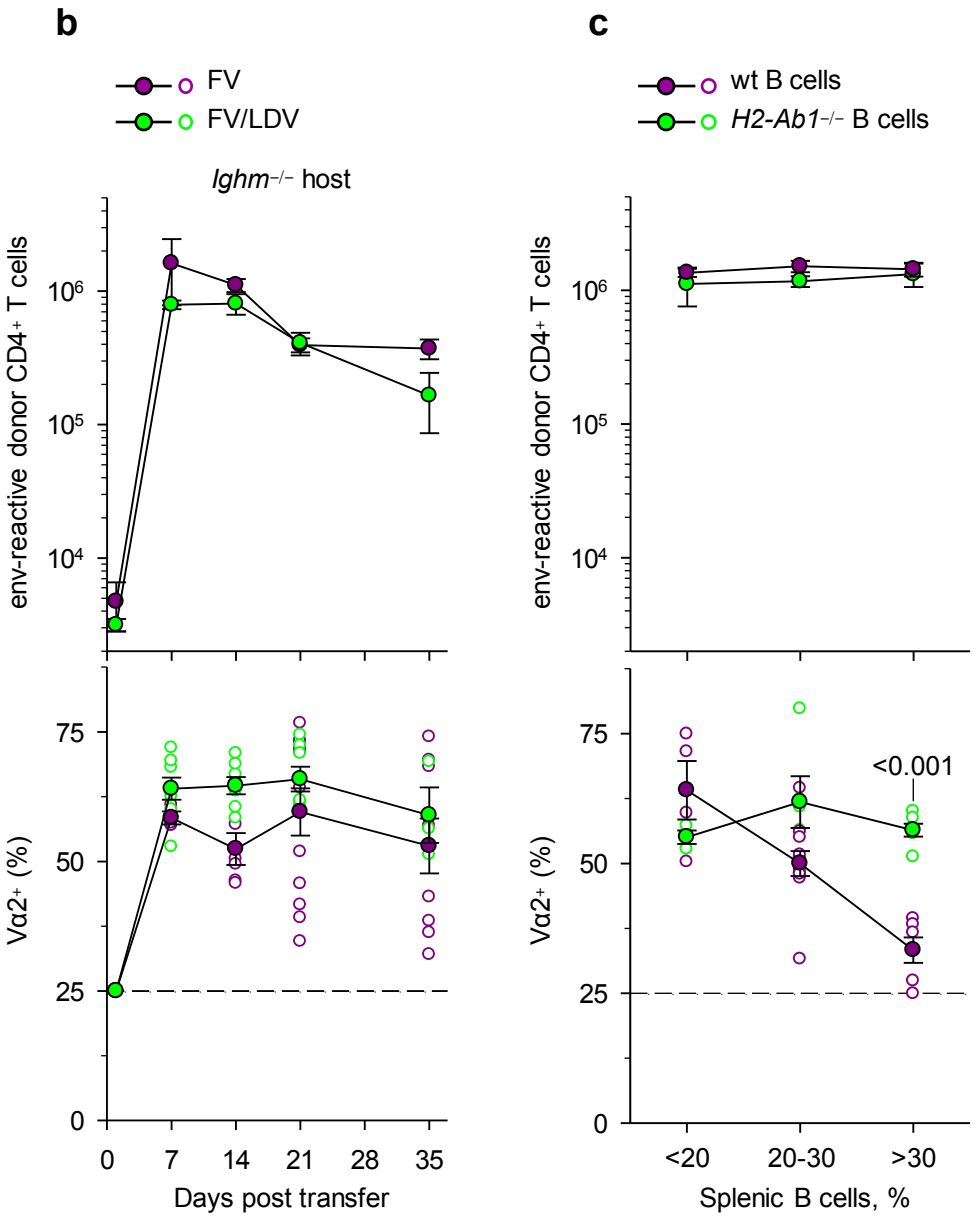

b
c
FV
FV/LDV
wt B cells
H2-Ab1-/- B cells
Ighm-/- host
env-reactive donor CD4+ T cells
Vα2+ (%)
Days post transfer
Splenic B cells, %
<0.001
<20
20-30
>30

5.2.3 Clonal replacement is independent of differing T effector differentiation potential

We next wanted to examine exactly how antigen presentation by B cells could be mediating this. We considered that T-B cell interactions might be qualitatively different from interactions made between T cells and other APC types. Clearly, the overall quality of the TCR signal induced in individual naïve clonotypes is dependent on both the TCR and the nature of the partner APC. We next considered how these factors could influence the evolution of the virus-specific CD4 T cell response.

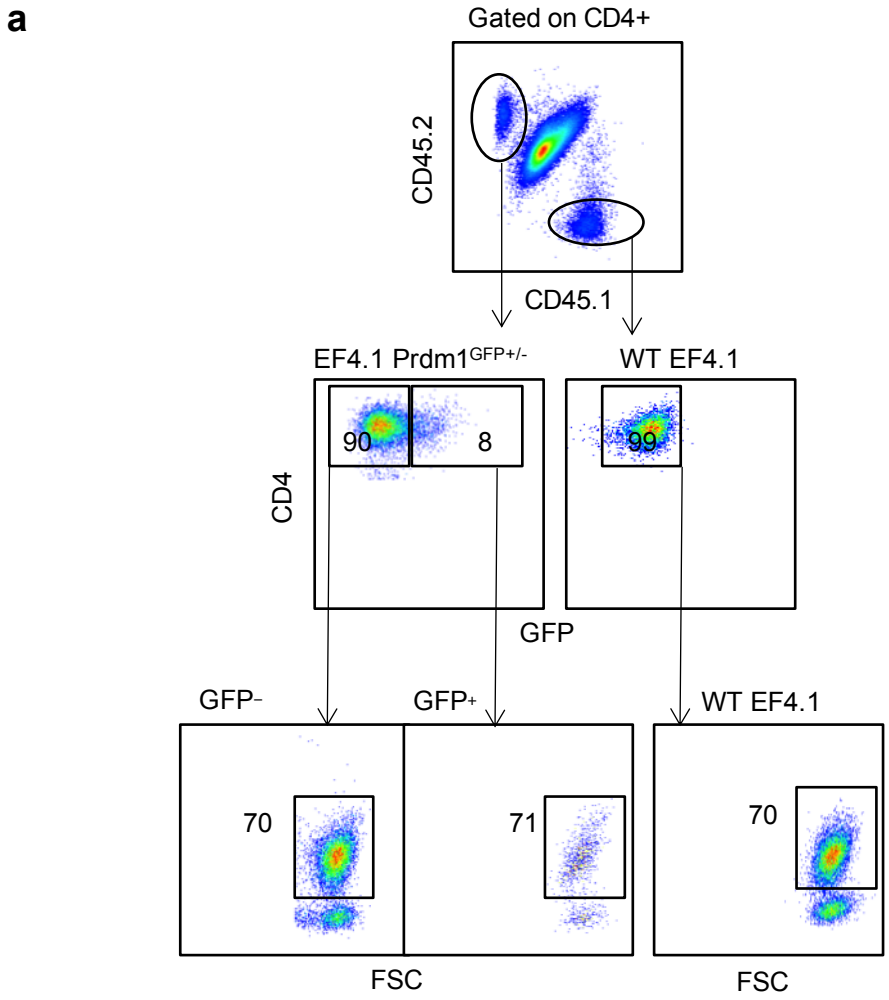
Many studies have reported a role for the strength of TCR signaling in determining the quality of effector cell differentiation, with high and low avidity interactions favoring Th1 and Th2 cellular differentiation respectively. In addition, strong TCR signaling in the context of T-B cellular interactions has been shown to elicit Tfh cell differentiation (King et al., 2008, Fazilleau et al., 2009). Thus, we considered that each different clonotype might have a tendency to produce certain types of effector cells (Tubo et al., 2013a). In this way, the long-term representation of a clonotype may be intrinsically linked to the stability of its acquired Th lineage; with some effector populations potentially being more stable than others. For example, Tfh cells are thought to be dependent on interactions made with germinal center B cells, thus their maintenance is presumably associated with the continuation of the germinal centre reaction. It was therefore possible that B cells promoted the differentiation of particular clonotypes into different effector populations, affecting both their functionality and longevity. We therefore hypothesised that high avidity interactions between Vα2 clonotypes and B cells could promote preferential differentiation into (potentially short lived) Tfh cells. In contrast, lower avidity interactions exhibited by Vα3 clonotypes may instead favour their differentiation into potentially longer-lived Th1 cells. To confirm if distinguishing patterns of differentiation could be responsible for the persistence of a given clonotype we accessed the representation of the different clonotypes within different effector cell populations during FV infection.

5.2.3.1 Clonotypic replacement affects the Th1 subset.

To first examine the distribution of virus-specific Vα2 or Vα3 clonotypes within Th1 effector cell populations we used *Prdm1*^{GFP} reporter EF4.1 T cells. Here, EF4.1 TCRβ-transgenic CD4 T cells also carried a GFP reporter in the *Prdm1* locus. As the introduction of the GFP cassette into the locus disrupts normal gene function, cells homozygous for the *Prdm1*^{GFP} reporter allele are deficient in B lymphocyte–induced maturation protein-1 (Blimp1), encoded for by *Prdm1*. Blimp1 is thought to directly repress Bcl6 and is therefore thought to be crucial for Th1 differentiation. Therefore, EF4.1 T cells where kept heterozygous for the allele, allowing for both GFP and *Prdm1* expression (from distinct alleles), and thus concomitantly preserving and marking Th1 differentiation.

Congenically marked EF4.1 *Prdm*1$^{GFP+/-}$ T cells and WT EF4.1 T cells (serving as internal controls) were transferred in B6 recipients at the time of infection. The clonotypic compositions of these responding populations were then accessed throughout the course of infection. Following FV challenge, only a minority of donor env-specific EF4.1 *Prdm*1$^{GFP+/-}$ cells expressed GFP, perhaps as a consequence of the heterozygosity of the reporter allele (Figure 28a). Nonetheless, the composition of Vα2 cells remained consistent between GFP^{+} and GFP^{-} *Prdm*1$^{GFP+/-}$ T cells as well as consistent to those observed in activated WT EF4.1 cells from within the same mouse (Figure 28b). This seemed to argue against preferential differentiation of certain clonotypes into the Th1 lineage. However, the visualisation of just a minority of total Th1 cells, by GFP expression severely limited this approach. Thus, in order to further examine evidence for clonotypic segregation between Th lineages, we targeted our approach to Tfh differentiation.

Figure 28 Clonal replacement in virus-specific CD4 T cells occurs independently of Blimp-1 dependent Th1 differentiation.



(a) Flow cytometric analysis and detection of virus-specific WT or Prdm1(GFP$^+$) or (GFP-) EF4.1 CD4 T cell donor populations from the same recipient. Numbers denote the percentage of cells contained in each gate. (b) Vα2 frequency in GFP$^+$ and GFP$^-$ Prdm1 or WT EF4.1 CD4 T cell donor populations over the course of FV infection. Closed symbols are the means (±s.e.m.)

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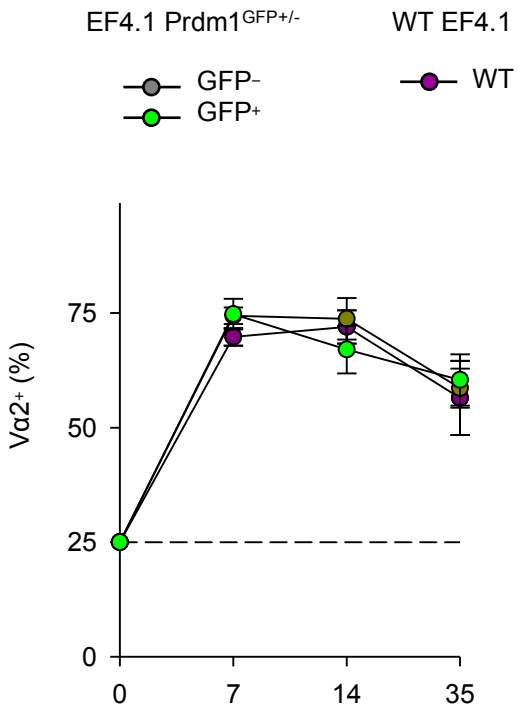
b



5.2.3.2 Clonotypic replacement affects the Tfh subset

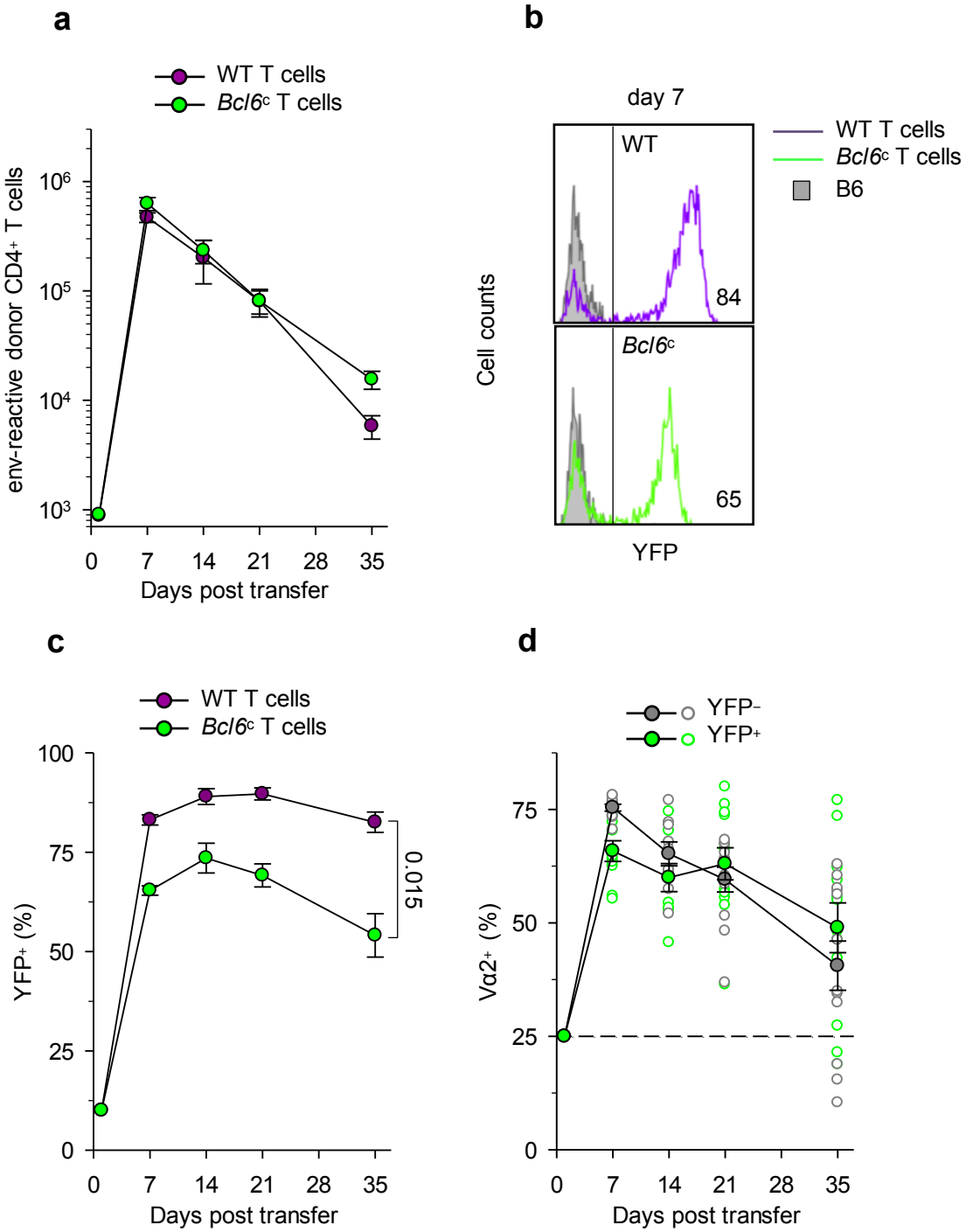
To examine if clonotypic replacement of Vα2 clones was due to their preferential differentiation into Tfh cells, we targeted deletion of *Bcl6* to EF4.1 T cells.

Bcl6 is thought to be the master transcription factor for Tfh cells; therefore, *Bcl6* deficient T cells cannot become Tfh cells. We reasoned that is Tfh cell differentiation was indeed critical for mediating the clonotypic evolution of the CD4 T cell response then its abrogation would prevent the switch. To this end, EF4.1 T cells were crossed to carry an OX40-Cre allele, in conjunction with Cre conditional *Bcl6* (*Bcl6*^{c}) and YFP (Gt(ROSA)26SorYFP) reporter alleles. As OX40 is up regulated upon strong TCR engagement, Cre expression and activity could be targeted to activated T cells. This approach would, theoretically, result in Cre mediated excision of the *Bcl6*^{c} genes (guided by the recognition of loxP sites) in all activated *Bcl6*^{c} EF4.1 T cells. However, Cre conditional systems are rarely 100% efficient, either due to the ectopic expression of Cre or its incomplete deletion of target genes. In an attempt to monitor the efficiency of Cre expression, we also used T cells carrying Gt(ROSA)26SorYFP reporter alleles. This reporter works through the incorporation of YFP into the constitutively expressed Rosa26 locus, which is preceded by a loxP flanked 'STOP' cassette. In the absence of Cre, the 'STOP' cassette prevents the transcription of YFP. Conversely, expression of Cre, allows for the excision of the stop cassette, and hence YFP expression. This acts to permanently mark cells, and their descendants, whom have expressed Cre. In this regard we used the YFP reporter to not only track cells that have expressed Cre, but also to mark activated *Bcl6*^{c} EF4.1 T cells that had presumably also deleted *Bcl6*. As 'WT' controls, EF4.1 CD4 T cells carrying just the OX40cre and YFP reporter alleles (Gt(ROSA)26SorYFP) but not the *Bcl6*^{c} allele were used. In these cells T cell activation lead to Cre mediated YFP expression, whilst the Bcl6 allele remained undisturbed and functional.

Numerically, responses elicited by WT or *Bcl6*^{c} EF4.1 T cells were equivalent (Figure 29 top). However, the proportion of YFP^{-} cells within the activated *Bcl6*^{c} EF4.1 T cell population, statistically increased over time. The proportional increase of this YFP^{-} population contrasted to its relative stability in WT EF4.1 T cells,

suggesting Tfh cell differentiation to be important to at least some of the responding clones. However, whilst the overall *Bcl6*^{c} YFP^{-} fraction increased in representation, the composition of Vα2 cells remained consistent between YFP^{+} and YFP^{-} fractions within the same host. Equal distribution of clonotypes between these fractions, suggested Vα2 clones were no more likely to promote Tfh cell differentiation than Vα3 clones. Moreover, as clonotypic replacement was evident in both the YFP^{+} and YFP^{-} fractions we concluded that disallowing Tfh differentiation did not prevent the clonotypic switch between Vα2 and Vα3 cells. Collectively this data argued that clonal replacement was not a consequence of the selective differentiation of clonotypes into divergent Th lineages.

Figure 29 Clonal replacement in virus-specific CD4 T cells occurs independently of Bcl6-dependent Tfh differentiation

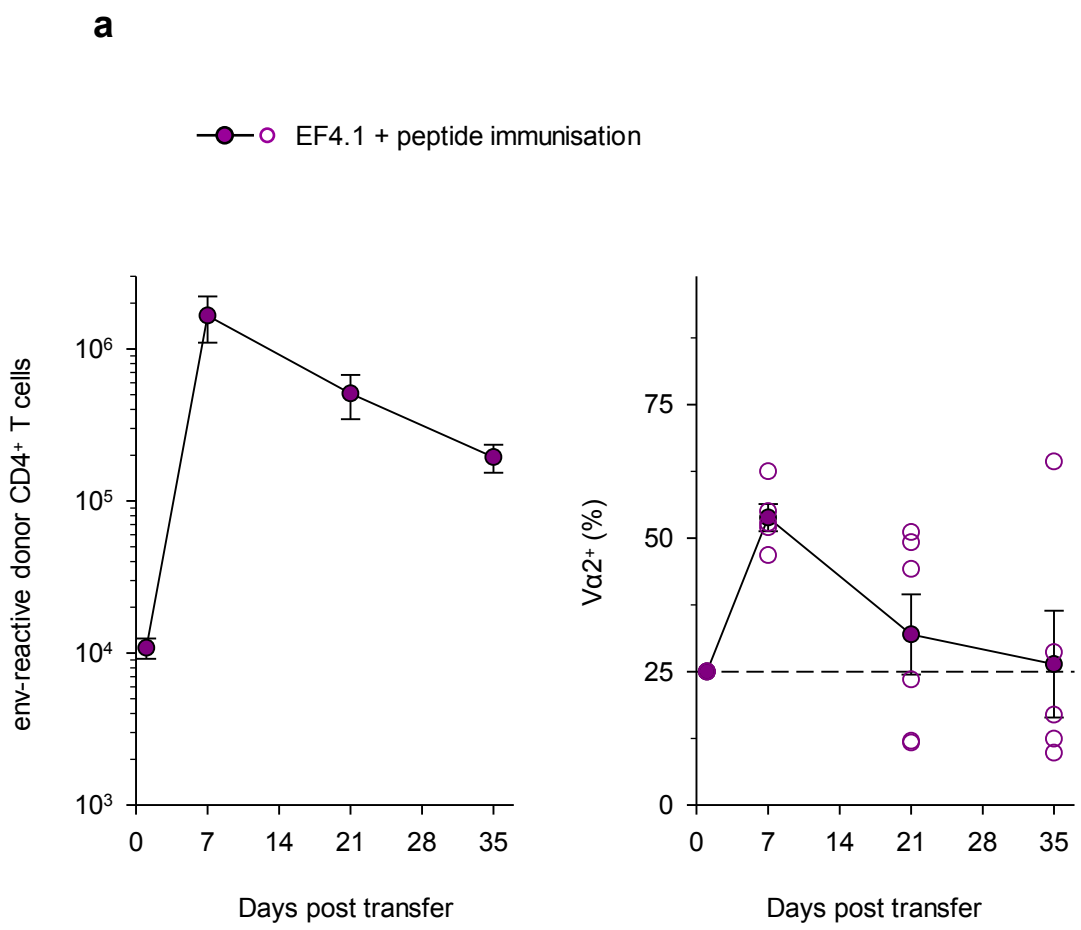


(a) Absolute numbers of env-reactive donor WT or Bcl6^c EF4.1 CD4 T cells in the spleens of recipient mice after adoptive T cell transfer and FV infection (*n*=3–9 mice per time point). (b) YFP expression on day 7 in the same env-reactive donor EF4.1 CD4+ T cells as in **a** (c) Frequency of YFP$^+$ cells overtime, within WT (see text) or Bcl6^c EF4.1 CD4 T cells in the spleens of recipient mice. (d) Vα2 frequency in YFP$^+$ and YFP$^-$ Bcl6^c env-reactive donor EF4.1 CD4 T cells over the course of FV infection. Closed symbols are the means (±s.e.m.); open symbols are individual mice.

5.2.4 Clonal evolution is not driven by alternative forms of the epitopes.

Variability in the nature of the proteases, cathepsins and differences in the cellular processing machinery between different APCs can result in distinct epitope determinants being presented by different cell types. It was therefore possible that differences in processing of antigen by B cells could lead to a change in the quality of peptides presented. Such a shift in the quality of peptides presented could theoretically reverse the functional avidities of the responding clonotypes, potentially favoring different clones accordingly. To investigate such a role, we restricted the CD4 T cell response to one pre-processed epitope alone, by transferring EF4.1 T cells into peptide immunized WT mice. However, the clonotypic replacement of Vα2 virus-specific CD4 T cells was still observed within these mice; suggesting that clonal replacement of Vα2 clones was not mediated by the presentation of alternative forms of the env epitope later in the response (Figure 30).

Figure 30 Clonal replacement in virus-specific CD4 T cells is not driven by epitope variation.



(a) Absolute numbers (left) and Vα composition (right) of env-reactive donor EF4.1 CD4 T cells in the spleens of $env1_{22-141}$ peptide immunised mice after adoptive T-cell transfer (n=6 mice per time point) Closed symbols are the means (±s.e.m.); open symbols are individual mice.

5.2.5 Clonal replacement is driven by asynchronous expansion of high and low avidity clonotypes.

We next considered an alternative hypothesis, to explain the clonotypic replacement of high avidity clones for low avidity clones (Figure 31). We reasoned, that instead of all virus-specific clones responding synchronously, but with distinct abilities to persist, clonotypes could respond with identical kinetics but instead may be asynchronously recruited. In this model, 'red' high avidity clones would expand early during infection followed by the delayed expansion of 'blue' low avidity clonotypes. Considering their already established role, we suspected that antigen presentation by B cells could be mediating a second wave of recruitment. However, the heterogeneous nature of the EF4.1 Vα3 population hindered our ability to visualise evidence for the temporal recruitment of blue clonotypes. Therefore, to test this we used EVα3 TCR Tg mice, where the TCR transgene was originally templated from a public blue clonotype, as a source of monoclonal blue T cell clonotypes.

Figure 31 Synchronous versus Asynchronous expansion models.

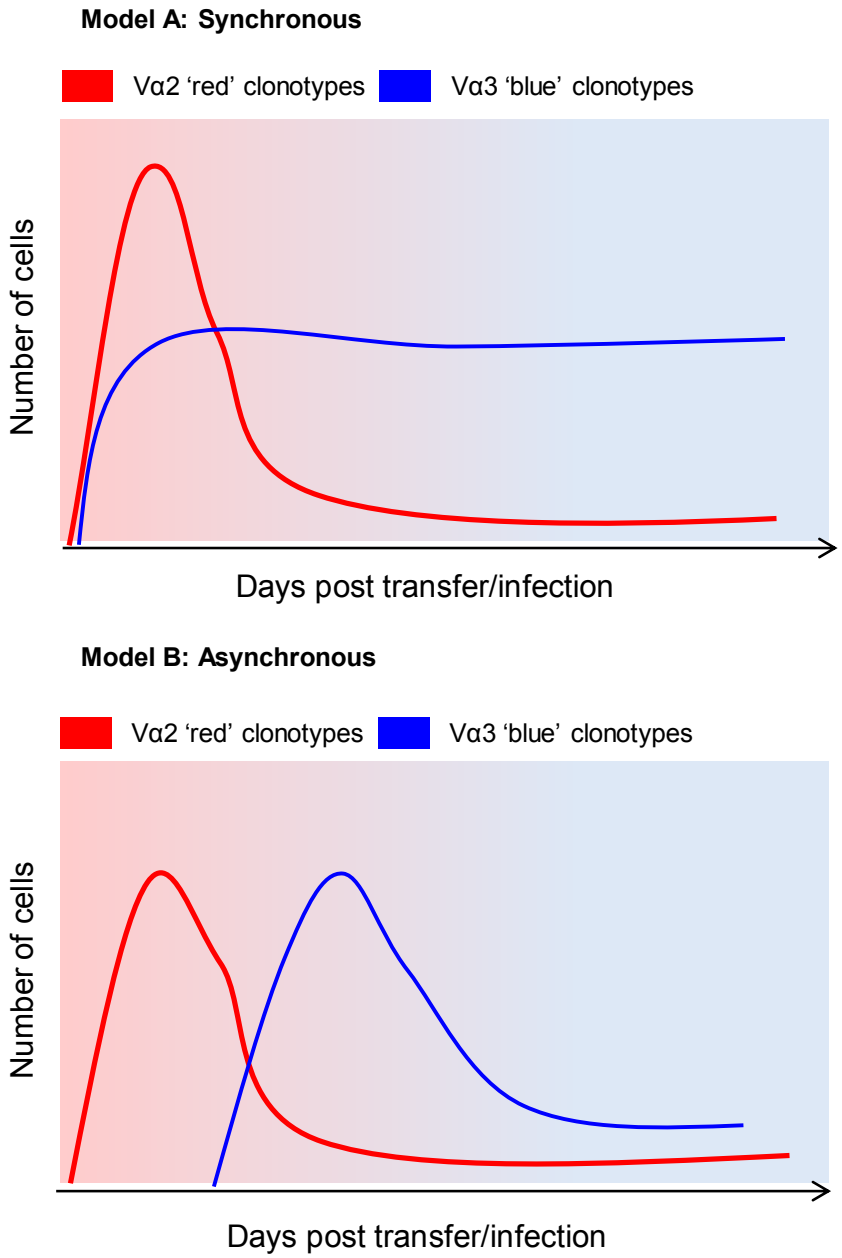


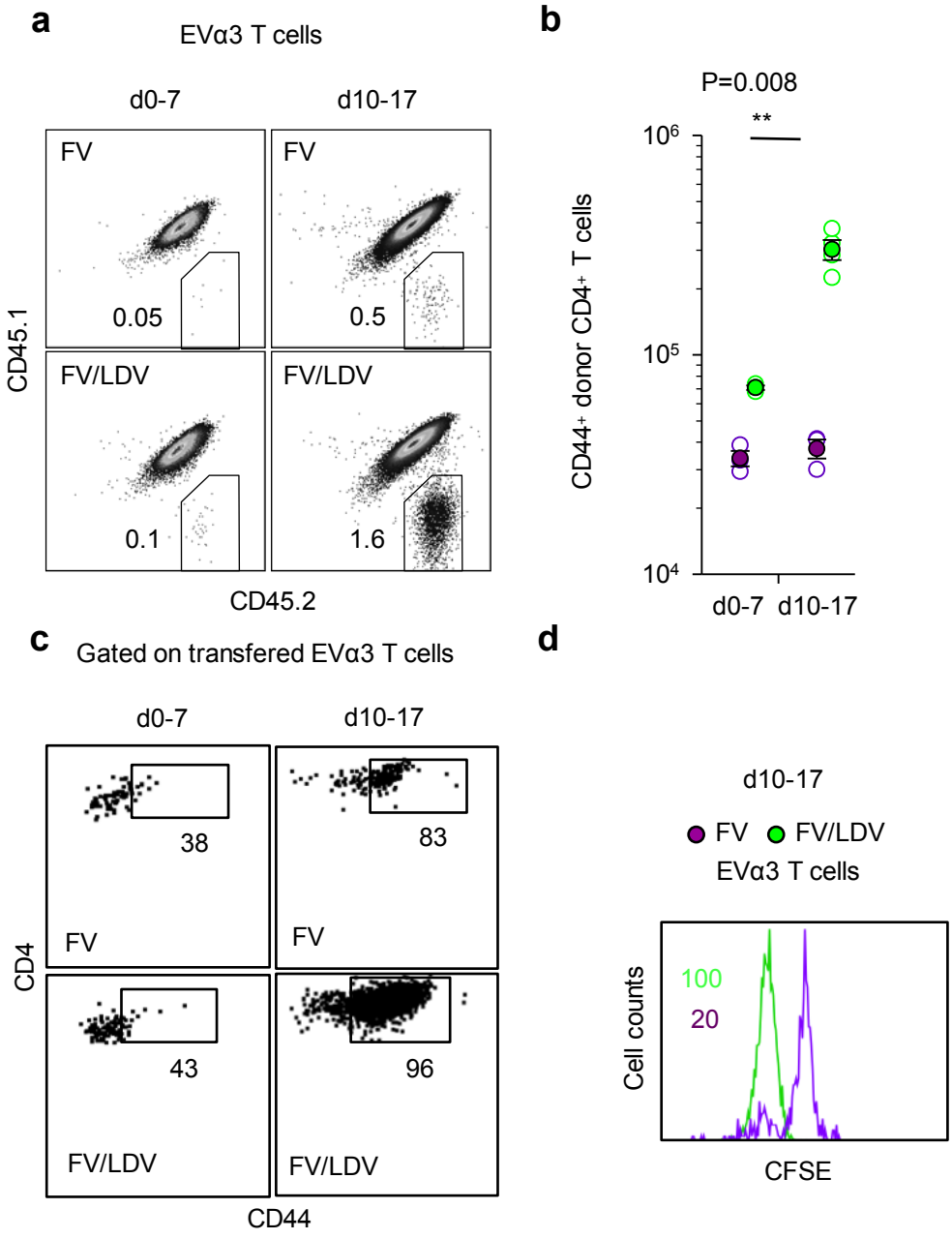
Figure 31 Synchronous and Asynchronous expansion models.
Both models could be applied to explain the clonal replacement of the Vα2 'red' clonotypes for the Vα3 'blue' clonotypes over the course of FV infection.
Model A Synchronous: In this model, we assume that both Vα2 and Vα3 clonotypes expand at the same time. To explain the clonotypic switch we assume Vα2 clonotypes would expand significantly more, than the Vα3 initially. However, at later time points clonal contraction would also be more pronounced in the Vα2 population, leaving the Vα3 to increase in their proportional representation as a consequence.
Model B Asynchronous: In this model, we suggest that Vα2 and Vα3 clonotypes are temporally recruited in accordance with their functional avidities. Here, it is not until the Vα2 (high functional avidity) red clonotypes have expanded and began to contract until the (lower functional avidity) Vα3 blue clonotypes are recruited. This would see two waves of clonal recruitment and would explain why Vα3 populations monopolize the CD4 T cell responses at later time points in FV infection.

To study the efficiency of recruitment at distinct time points, marked EVα3 T cells were either transferred into WT hosts at the time of infection or into hosts which had been infected 10 days prior (Figure 32). The efficiency of recruitment of EVα3 T cells was then measured 7 days later. In addition, and to access the role for B cell mediated recruitment, we also used hosts co-infected with FV and LDV. Again, we either delivered EVα3 T cells on the day of infection or 10 days post infection, when the B cell response was at its peak. When T cells were transferred on the same day that mice had received FV challenge, we observed only a minimal expansion of EVα3 T cells (Figure 32a-b). Here, the absolute numbers of activated EVα3 T cells recovered were used to measure the level of recruitment. Total numbers were calculated from the observed frequencies of activated EVα3 T cells ($CD45.2^+$,$V\alpha3^+$,$CD44^+$), as ascertained by flow cytometric analysis, multiplied by total cell counts (Figure 32b). Similarly, low numbers of expanded EVα3 T cells were also observed in mice which had been co-infected with FV and LDV on the same day as T cell transfer. Lack of expansion seemed to be due to inefficient activation of EVα3 T cells, with the majority of recovered cells not up-regulating the activation marker CD44 (Figure 32c). Furthermore, EVα3 T cells transferred on day 10 of FV infection, only expanded marginally better than in the previous conditions. However, the majority of EVα3 T cells were now $CD44^{high}$ in phenotype, indicating improved levels of activation (Figure 32c). Strikingly, expansion of EVα3 T cells was largely improved when T cells were delivered on day 10 of co-infection with FV and LDV (Figure 32a-b). This was also reflected in homogenously high CD44 expression (Figure 32c). Notably, these mice had received EVα3 T cells at the peak of their B cell response. This suggested that the degree of recruitment was dependent on the availability of activated B cells capable of presenting antigen. To highlight this, we labelled EVα3 T cells with CFSE before their transfer into hosts pre-infected with FV or FV and LDV (Figure 32d). CFSE is a cell permeable fluorescent dye that allows for flow cytometric assessment of cellular proliferation due to the progressive halving of CFSE fluorescence within daughter cells following each cell division (Lyons and Parish, 1994). The complete loss of CSFE dye, owing to levels of proliferation that exceeded the number of cell divisions CFSE can

visualise was only achieved when EVα3 T cells were transferred into mice at the peak of the B cell response.

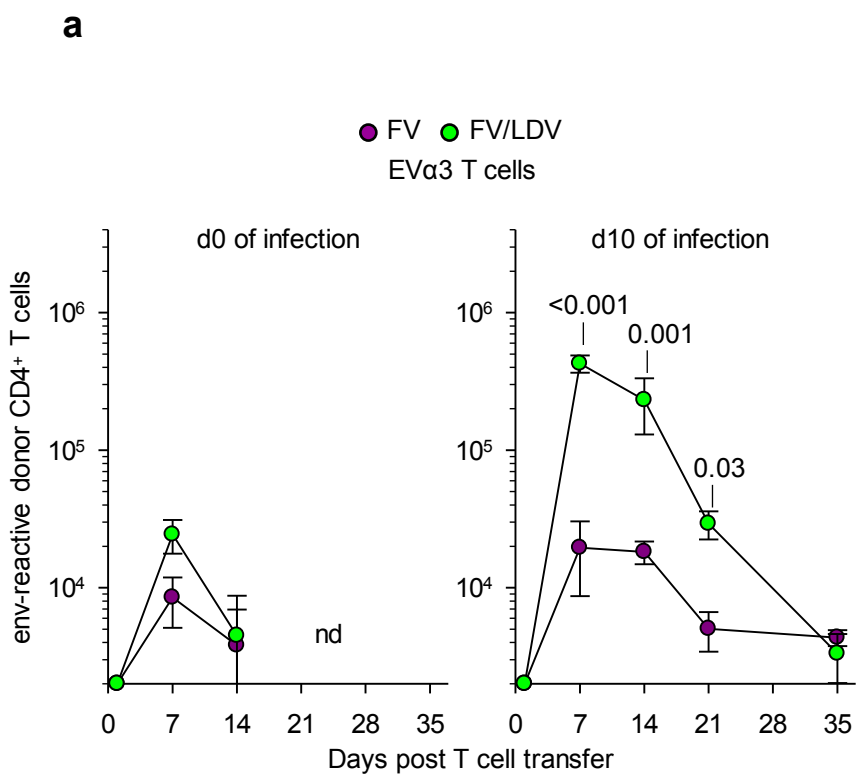
Importantly, this pattern of recruitment contrasted with the strong expansion of Vα2 clonotypes within the first 7 days of infection, proving families of clonotypes were temporally recruited. We continued to monitor the response of transferred EVα3 T cells, following their initial expansion, to study their ability to persist. However, similarly to the Vα2 response, expansion was followed by the sharp contraction in the absolute number of responding EVα3 T cells (Figure 32a). This suggesting that once activated, EVα3T cells did not behave atypically. These results demonstrated that clones of lower avidity/'blue' clonotypes were recruited later than clones of higher avidities/'red' clonotypes, in a B cell dependent manner.

Figure 32 Asynchronous expansions of higher- and lower-avidity clonotypes.



(a) Flow cytometric detection of CD45.2 donor EVα3 *Rag1*$^{-/-}$ *Emv2*$^{-/-}$ CD4 T cells and CD45.1/CD45.2 host CD4 T cells following transfer either on the day of infection (d0 of infection, d0–7) or 10 days after infection (d10 of infection, d10–17) with FV or FV/LDV. In both setups, T-cell expansion was analysed 7 days after transfer (*n*=5–9). (b) Absolute number of activated (CD44^{high}) EVα3 CD4 T cells recovered from the spleens of FV or FV/LDV co-infected recipients 7 days after transfer either on the day of infection (d0–7) or 10 days after infection (d10–17). Closed symbols are the means (±s.e.m.); open symbols are individual mice (c) Flow cytometric detection of CD44 levels on CD4 T cell population gated as in **a**. (d) Responsiveness, as measured by CFSE dilution, of EVα3 *Rag1*$^{-/-}$ *Emv2*$^{-/-}$ CD4 T cells following transfer into recipients 10 days after infection (d10 of infection, d10–17) with FV or FV/LDV. Numbers denote the percentage of cells in each gate or deemed CFSE^{-}

Figure 33 Following their late recruitment, lower-avidity clonotypes then display identical kinetics to higher-avidity clonotypes.

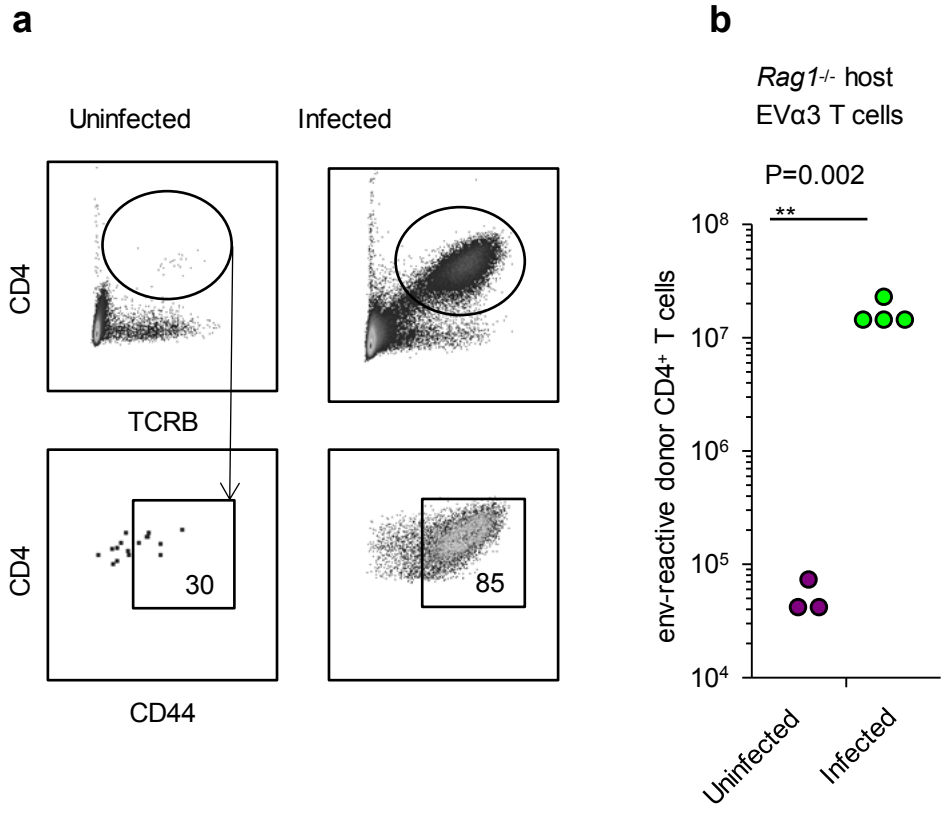


(a) Absolute number of env-reactive donor EVα3 CD4 T cells recovered from the spleens of FV or FV/LDV co-infected recipients 7 days after transfer either on the day of infection (left) or 10 days after infection (d10–17) and then over the course of FV infection or FV/LDV co-infection (nd, not detected). Closed symbols are the means (±s.e.m.).

5.2.5.1 Diversification of the immune response is not driven by a unique mode of antigen presentation by B cells

Recruitment and expansion of clonotypes of lower avidities seemed to be driven by B cell mediated antigen presentation. To understand if qualitative differences in antigen presentation between B cells and other APCs were responsible for this, we tested if DCs could also recruit EVα3 T cells. To this end, we transferred marked cohorts of EVα3 T cells into F-MLV infected B cell deficient *Rag1*$^{-/-}$ hosts (Figure 34). Here, and in contrast to the minimal expansion of EVα3 T cells in WT F-MLV infected hosts, EVα3 T cells expanded considerably in infected *Rag1*$^{-/-}$ hosts. To control for expansion that would be driven by homeostatic-proliferation we also transferred EVα3 T cells into un-infected *Rag1*$^{-/-}$ hosts, but this resulted in comparatively little expansion. Thus, by alleviating interclonal competition we also alleviated the dependency of EVα3 T cells on priming by B cells. Henceforth, we propose a quantitative rather than qualitative role for B cells in our system, in which increased numbers of activated B cells, participating in antigen presentation, acts to relax interclonal competition. This effectively allows lower avidity cells a window of opportunity to expand, resulting in second wave of recruitment which subsequently drives the evolution and diversification of the CD4 T cell response.

Figure 34 Low-avidity clonotypes can be primed by diverse APCs in the absence of interclonal competition.



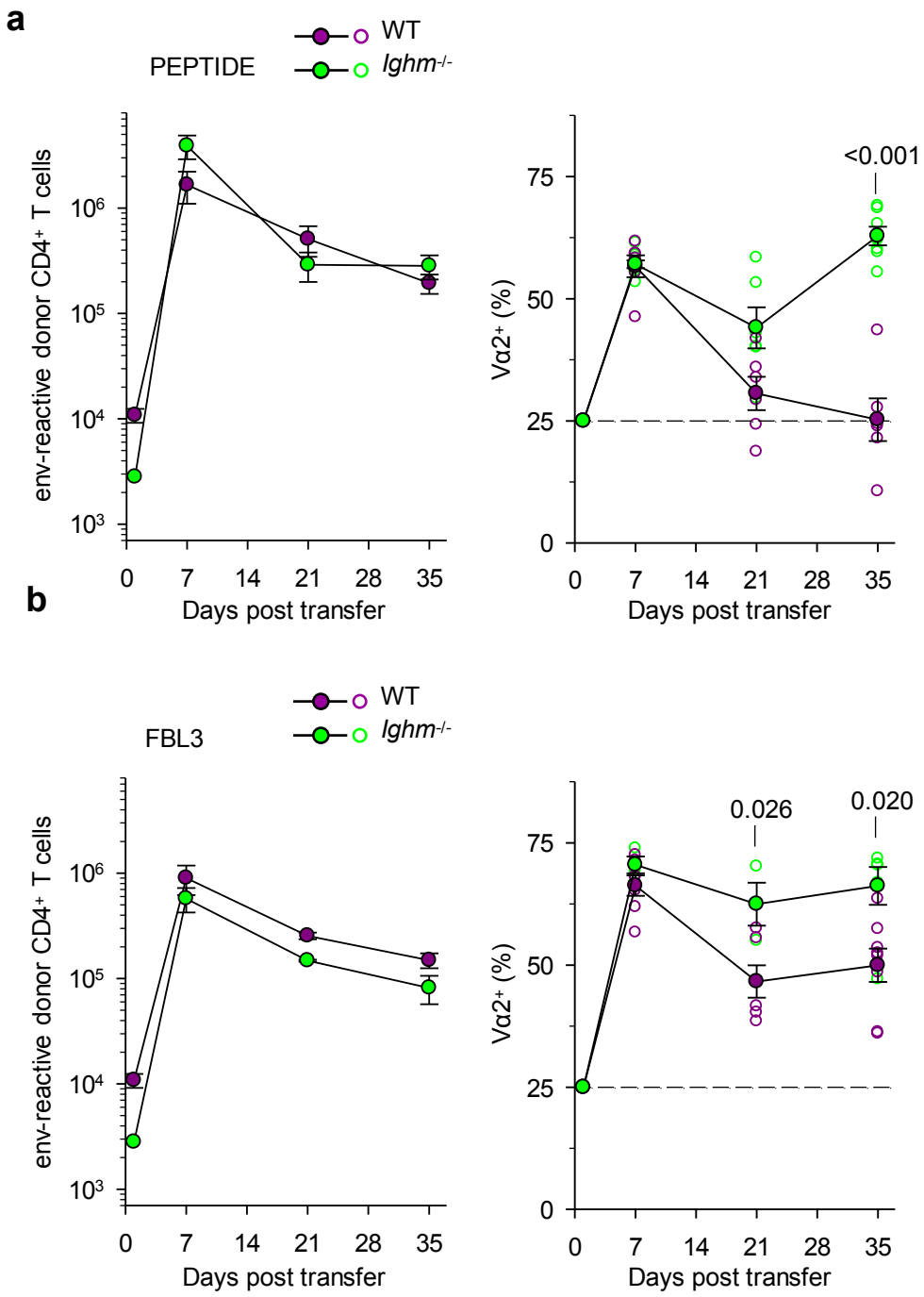
(a) Flow cytometric detection of EVα3 CD4 T cells recovered 7 days after transfer into lymphocyte-deficient Rag1$^{-/-}$ Emv2$^{-/-}$ recipients that were either left uninfected or were F-MLV-B-infected. Each symbol represents an individual mouse (b) Absolute numbers of EVα3 CD4 T cells recovered from the spleens of recipient mice describe in **a** Each symbol is an individual mouse.

5.2.6 B cells promote TCR diversity in various CD4 T cell responses.

Our model would predict that settings that feature polyclonal activation of B cells or B cells as dominant APCs, would also feature the induction of lower-avidity clonotypes in addition to higher-avidity clonotypes. Vaccine strategies often involve the co-administration of adjuvants, which are essential for engaging the adaptive immune response towards vaccine-associated antigens. Different adjuvants will elicit different responses/effects, some of which involve the polyclonal activation of B cells and the up-regulation of MHCII. Thus, we wanted to examine if B cells played a similarly important role in conditioning the composition of CD4 T cell responses in a vaccine regimen.

To test this prediction, we compared the EF4.1 CD4 T cell response in WT or in *Ighm*$^{-/-}$ mice immunized with $env_{121-142}$ peptide. Interestingly the clonotypic replacement of Vα2 T cells was only observed in B cell sufficient hosts, but not in B cell deficient *Ighm*$^{-/-}$ hosts (Figure 35a); highlighting the requirement for B cells in the process. We further tested this model in another immunization setting where mice were challenged with the tumor cell line FBL-3 cells. Critically, this FV induced tumor cell line expresses the F-MLV envelope epitope but does not produce infectious viral particles (Klarnet et al., 1989). EF4.1 CD4 T cells were either transferred into tumor challenged WT or *Ighm*$^{-/-}$ mice. Again the replacement of Vα2 virus-specific CD4 T cells was only observed in B cell sufficient WT, but not in *Ighm*$^{-/-}$ hosts (Figure 35b). Thus, following peptide vaccine immunization and tumor challenge, B cells were required for the realization of the full clonotypic diversity of the CD4 T cell response.

Figure 35 B-cell-dependent expansion of lower avidity CD4 T-cell clonotypes occurs in diverse infection or immunization settings



(a) Absolute numbers (left) and Vα composition (right) of env-reactive donor EF4.1 CD4 T cells in the spleens of WT or *Ighm*$^{-/-}$ (B cell deficient) recipient mice after adoptive T cell transfer and env$_{122–141}$ peptide immunization (*n*=6–9 mice per time point). (b) Absolute numbers (left) and Vα composition (right) of env-reactive donor EF4.1 CD4 T cells in the spleens of WT or *Ighm*$^{-/-}$ (B cell deficient) recipient mice after adoptive T-cell transfer and FBL-3 tumor challenge (n=3–8 mice per time point) Closed symbols are the means (±s.e.m.); open symbols are individual mice.

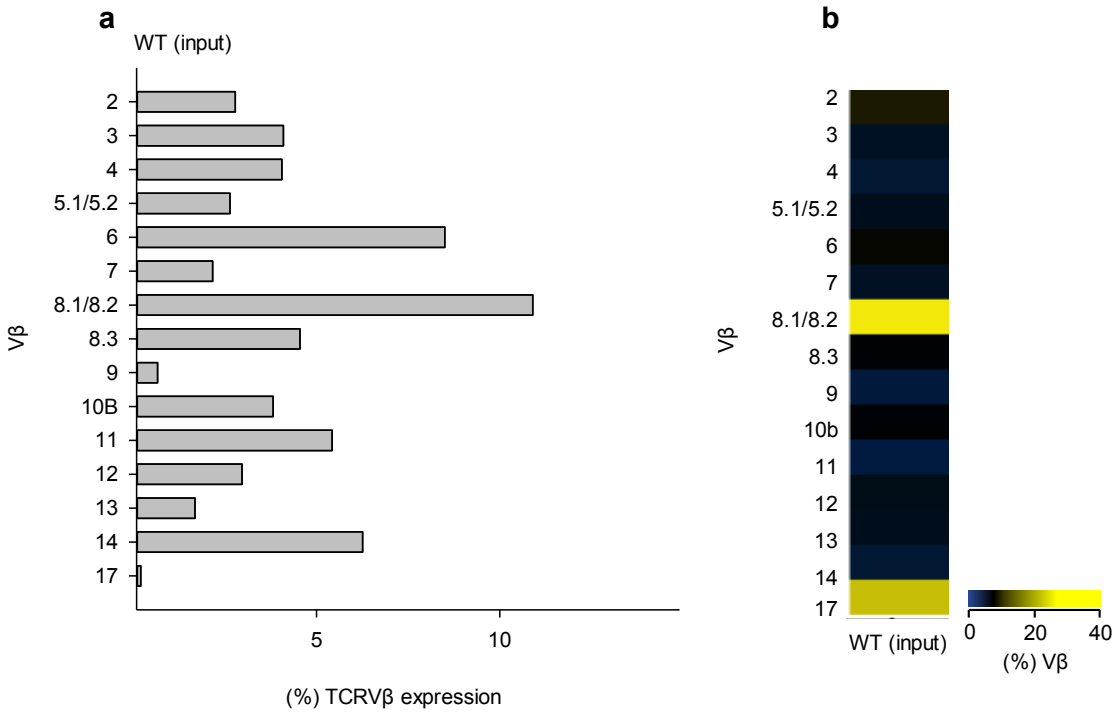
5.2.7 B cells balance TCR diversity

The naïve T cell repertoire is under constant selective pressure, whereby T cells must use their TCRs to host interactions with spMHC in order to be maintained. Cells neglecting to do so die or are outcompeted by clones whom presumably have a higher avidity for spMHC. This competition among CD4 T cells is essential for the tuning, maintenance and self-limitation of the naïve T cell repertoire. We therefore considered whether B cells might play a similar role in maintaining the diversity of the TCR repertoire during homeostasis

Clearly, antigen presentation by B cells played an important role in relaxing clonal competition during infection, subsequently allowing for the diversification of the responding CD4 T cell repertoire. We therefore predicted that B cells would play a similarly important role in supporting a diverse TCR repertoire during homeostasis, maintaining lower avidity clones alongside higher avidity clones. To test this, we asked if the diversity of a polyclonal TCR repertoire was preserved in the absence of B cells. Although TCR repertoires cannot be predicted, due to the random nature of TCR recombination, B6 mice have a preferred usage of TCRVβ gene families (Figure 36). We could therefore use this highly reproducible TCRVβ fingerprint to measure TCR repertoire maintenance upon transfer of polyclonal WT CD4 T cells into hosts either lacking just T cells (*TCRα*$^{-/-}$) or both T and B cells (*Rag1*$^{-/-}$*).* Representation of TCRVβ members was ascertained using a panel of fluorescently labelled antibodies that stain up to 75% of all possible endogenous TCRVβ. Therefore, the percentage expression and relative representation of certain TCRVβ families (TCRVβ signature) could be measured. TCRVβ signatures were compared between the input and expanded populations in the respective hosts, following 21 days of reconstitution. Relative distribution/representation of the TCRVβ families within individual mice was displayed as heat maps, with similarities between TCRVβ signatures accessed by unsupervised hierarchal clustering (Figure 36b/37). Strikingly, the TCRVβ usage between the input (WT mice) and the reconstituted *TCRα*$^{-/-}$ host mice were indistinguishable by clustering analysis, suggesting that TCRVβ diversity was perfectly preserved during reconstitution in the presence of B cells (Figure 37). In sharp contrast, the majority of mice reconstituted in the absence of B cells (*Rag1*$^{-/-}$ hosts*)*, exhibited considerable skewing away from the

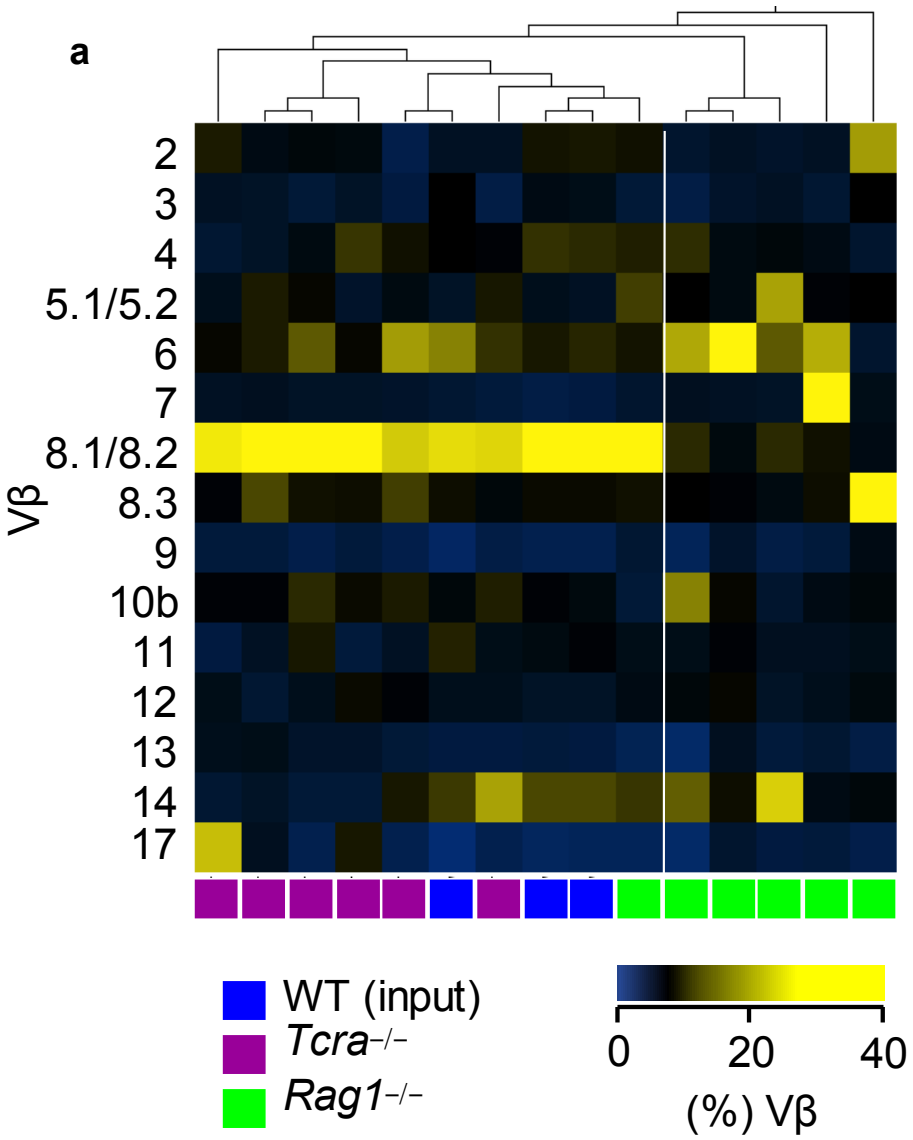
input TCRVβ signature. This resulted in these *Rag1*$^{-/-}$ hosts clustering away from all other hosts (Figure 37). Furthermore, these TCRVβ usage profiles were unique to each *Rag1*$^{-/-}$ host, presumably driven by the private expansion of individual clones in the individual mice, indicative of a TCR driven process.

Figure 36 Expression of endogenous TCRVβ chains in WT polyclonal CD4 T cells.



(a)The percentage of T cells expressing TCR Vβ2, Vβ3, Vβ4, Vβ5.1/5.2, Vβ6, Vβ7,Vβ8.1/8.2, Vβ8.3, Vβ9, Vβ10b, Vβ11, Vβ12, Vβ13, Vβ14 or Vβ17a in WT CD4 T cells as determined by flow cytometric analysis.(b) Heat-map depicting the same data as in **a**.

Figure 37 TCR repertoire skewing, during CD4 T cell reconstitution, in the absence of B cells



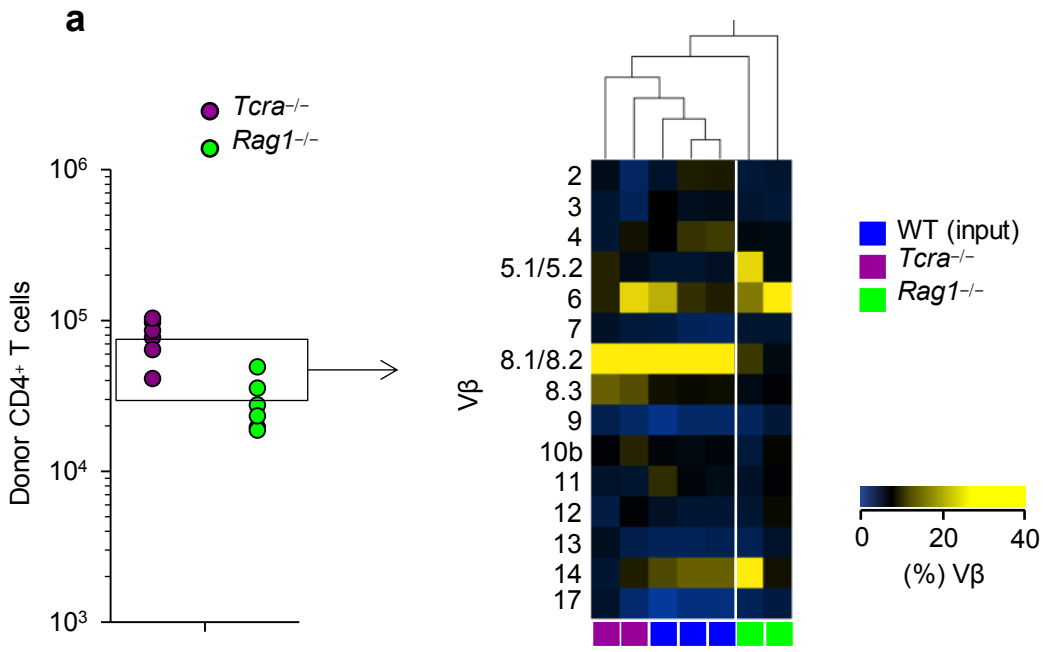
(a) Heat-map of hierarchically-clustered WT donors and *Tcrα*$^{-/-}$ (T cell deficient) or *Rag1*$^{-/-}$ (B cell and T cell deficient) recipients of purified WT CD4 T cells according to the frequency of the indicated Vβ family before (WT (input)) or 21 days after T-cell reconstitution (*Tcrα*$^{-/-}$ or *Rag1*$^{-/-}$) Each column is an individual mouse.

A polyclonal repertoire will contain a range of clones, some engaging spMHC better than others. Our model would predict that reconstitution of *TCRα*$^{-/-}$ hosts would result in the retention of a greater range of TCRs avidities. Conversely it would predict, that reconstitution of *Rag1*$^{-/-}$ hosts would lead to a reduction in TCR repertoire diversity driven by the out competition of lower avidity clones by higher avidity clones in conditions where MHCII derived signaling was limiting. A direct comparison of the avidity of T cells selected in the presence or absence of B cells was difficult to access; however, we observed that CD5 levels were significantly reduced in the most expanded TCRVβ families of *Rag1*$^{-/-}$ hosts (Figure 39). Maintenance of CD5 expression, by naive CD4 T cells, is known to be regulated by MHCII contacts made in the periphery (Smith et al., 2001). Studies which have experimentally deprived CD4 T cells of MHCII contact, showed T cells to sensory adapt CD5 to permit for increased TCR sensitivity (Smith et al., 2001). As the reconstitution of lymphopenic hosts, is promoted by MHCII recognition, we reasoned that reduced CD5 expression was reflective of insufficient MHCII availability in the absence of B cells. A reduction in the overall numbers of reconstituted T cells between hosts was also indicative of this. However importantly, TCRVβ usage skewing was not a direct result of the reconstitution efficiency as it also characterized hosts with comparable numbers of expanded CD4 T cells (Figure 38).

Lower expression levels of CD5 within *Rag1*$^{-/-}$ reconstituted CD4 T cells, could also be driven via the selective outgrowth/advantage of individual clones that were originally CD5^{low} in phenotype. In the absence of TCR repertoire sequencing of the expanded clonotypes in these hosts, we were unable to unanimously discredit this. However, CD5 levels were equally down regulated in the most negatively skewed TCRVβ families within *Rag1*$^{-/-}$ host mice, arguing against this. In addition, selective outgrowth of previously CD5^{low} cells would also presumably be driven by their heightened capacity to signal. However usage of the Nur77^{GFP} reporter for TCR signaling (Moran et al., 2011) revealed that *Rag1*$^{-/-}$ reconstituted CD4 T cells received significantly lower overall TCR signals (Figure 38). Here T cells express GFP, due to its integration into the immediate early gene *Nr4a1* (Nur77) locus, following antigen receptor stimulation (Moran et al., 2011). Furthermore, expression of GFP directly correlates with the strength of TCR signaling. Therefore, the

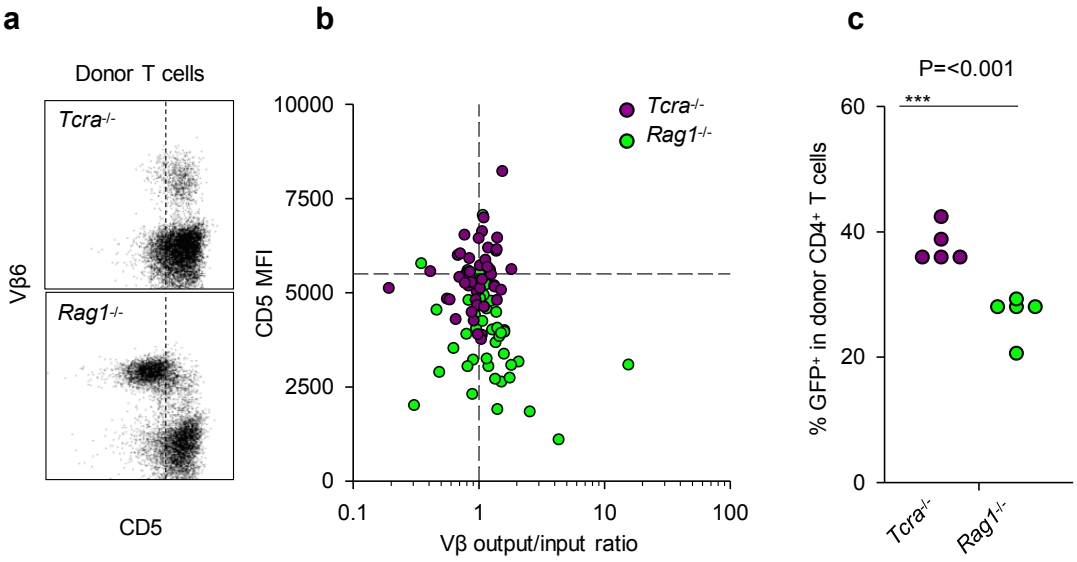
comparatively higher signaling capacity of reconstituted TCRα$^{-/-}$ T cells, suggested that B cells were necessary to provide optimal signaling. Optimal signaling would therefore be most important for the expansion of the lowest avidity clonotypes. We therefore concluded that B cells are also important for the maintenance of TCRVβ repertoire diversity during reconstitution of lymphopenic host.

Figure 38 TCR repertoire skewing in T cell-reconstituted *Rag1*$^{-/-}$ hosts independently of the extent of reconstitution.



(a) Numbers of donor CD+ T cells 21 days after reconstitution of *Tcrα*$^{-/-}$ or *Rag1*$^{-/-}$ Emv2$^{-/-}$ recipients with purified WT CD4 T cells. Each symbol is an individual mouse. Mice selected for comparable T cell reconstitution (rectangle) were then analyzed for Vβ usage. (b) Heat-map of hierarchically-clustered WT donors and *Tcrα*$^{-/-}$ or *Rag1*$^{-/-}$ Emv2$^{-/-}$ recipients from (a), according to the frequency of the indicated Vβ family before (WT (input)) or 21 days after T cell reconstitution (*Tcrα*$^{-/-}$ or *Rag1*$^{-/-}$) Each column is an individual mouse.

Figure 39 Balanced TCR diversity during CD4 T-cell reconstitution requires B cells.



(a) Flow cytometric detection of CD5 and Vβ6 expression in donor CD4 T cells from *Tcra*$^{-/-}$ (T cell deficient) or *Rag1*$^{-/-}$ (B cell and T cell deficient) recipients of purified WT CD4 T cells. (b) CD5 median fluorescent intensity (MFI) plotted against the divergence from the input frequency of each detected Vβ family in donor CD4+ T cells from the same recipients as in a. Only three recipients are plotted for clarity. The horizontal dashed line represents the CD5 MFI in control cells (P<0.001 between CD5 levels in T cells from the two types of host, Mann–Whitney rank sum test). (c) Frequency of GFP^{+} cells in donor CD4 T cells 21 days after T-cell reconstitution of *Tcra−/−* or *Rag1*$^{-/-}$ recipients with purified CD4 T cells from Nur77-GFP transgenic donors. Each symbol represents an individual mouse.

5.3 Discussion

Here we show that the clonal replacement of higher avidity clonotypes for lower avidity clonotypes was dependent on antigen presentation by B cells. Our results support a model whereby the degree of B cell activation and hence capacity to act as proficient APCs, determines the recruitment of lower avidity T cell clonotypes later in the response. Thus, this leads to the progressive diversification of the antigen-selected TCR repertoire. Further still, we report that B cell-mediated clonotypic selection of CD4 T cells is also important in shaping the TCR repertoire during T cell reconstitution of lymphopenic recipients. Thus B cell mediated antigen presentation plays fundamental roles TCR repertoire evolution during viral infection, and preservation during T cell homeostasis.

Chapter 6. Discussion

6.1 Brief summary of finding

This study was aimed at analysing the role of TCR specificity and avidity in mediating the selection of CD4 T cell repertoires, both during development and in antigen-selected responses.

6.2 Role of TCR specificity and avidity in Treg cell commitment

We initiated this study by first investigating a role for TCR specificity and avidity during T cell development. We reasoned that one of the most pronounced effects of TCR signalling (and hence specificity and avidity) during development was the thymic generation of Treg T cell populations. Thus we began this work by investigating the prescriptive capacity of a Treg derived TCR, expressed in various settings, at mediating thymic Treg T cell differentiation. To gauge the importance of TCR specificity in this regard, we used a self-reactive Treg derived TCR to template for a Treg TCRαβ Tg mouse. To explore a role for TCR avidity, we propagated two Treg TCRαβ founder strains, 3G7A and 3G7B. Here, although TCR specificities were identical between the strains, TCR expression differed by a factor of two and thus allowed for different intrinsic signalling capacities between the mice. This gave us a model to look at the instructive capacity of both TCR specificity and avidity in Treg T cell commitment.

6.2.1 In the thymus

Although in the thymus, TCR-specificity to self-antigen appears to be a primary determinant for tTreg cell commitment we found that TCR-specificity and self-reactivity alone were not sufficient to instruct for this lineage. Indeed, analysis of the 3G7 Treg TCR Tg mice showed the complete absence of thymic Foxp3 Treg T cells. Instead T cell development was increasing distorted in an avidity dependent fashion. This was most pronounced in the 3G7B *Rag*$^{-/-}$ mice, when the TCR was expressed at higher levels and was clonally distributed, leading to

increased negative selection and a decreased thymic output in conjunction with abnormal expression of many developmental markers. These findings highlighted some of the diverse conservation mechanisms that have evolved to limit and manage overtly self-reactive thymocytes during development. In the 3G7A *Rag*$^{-/-}$, where TCR levels were comparatively lower, signalling was now at optimal levels for CD4 lineage commitment, however the mice remained completely devoid of tTreg T cells.

6.2.2 In the periphery

Despite the Treg origin of the 3G7 TCR receptor, but consistent with the thymic phenotype, the periphery of these mice was also devoid of Treg T cells. An interesting observation was that despite the clear auto-reactive nature of the 3G7 TCR, peripheral T cells in 3G7 mice remained wholly naïve in phenotype. In the 3G7 *Rag* proficient mice, it was plausible that immune-tolerance was maintained despite this, by the presence of a Treg T cell population (which presumably utilised endogenously derived TCRs) in these mice. However, when 3G7 mice were backcrossed onto the *Rag1*$^{-/-}$ background, hence excluding the possible contribution of other TCRs and abrogating Treg T cell development, we did not observe spontaneous auto-immunity. In addition, 3G7 T cells were not anergic, as spontaneous activation could be observed when culturing single-cell spleen suspensions *in vitro* overnight in PFHM. We suggested that the transition from the naïve state (as observed *in vivo)* to full activation (as observed *in vitro)* resulted from the mechanical disruption of the splenic architecture that would normally segregate T cells from certain antigens or APCs. Thus we reasoned that 3G7 T cells remained in a state of ignorance *in vivo*, which clearly when disrupted could lead to T cell activation *in vitro*. However, we did not conclusively test this hypothesis, via disrupting the splenic architecture or antigenic niches *in vivo*.

6.2.3 In vitro

There is evidence that Tcon cells can convert to the Treg lineage later in life. Although the role for TCR signalling in this process has been explored, whereby

TCR signalling is thought to open the Foxp3 promoter region, a role for TCR specificity is less well understood. TCR repertoire studies, which have compared the TCR repertoires of tTreg cells and total systemic Treg T cells have shown them to be largely overlapping. The conclusion from these studies was that the major contributor to the mature Treg pool where tTreg T cells, suggesting that the contribution of peripherally converted Tregs to be was minimal (Pacholczyk et al., 2007). A second hypothesis to explain this observation is that certain TCRs may be instructive toward both tTreg and pTreg T cell development. Thus some TCRs may poise T cells to become Treg cells, if not in the thymus than once in the periphery. Whilst TCR repertoire analysis comparing peripherally converted pTreg T cells and thymically generated tTreg T cell populations would be required to most appropriately explore such a relationship we decided to explore the potential for this in our system. Specifically, we asked is Tcon cells expressing the 3G7 Treg derived TCR would be prone to differentiate into Treg cells upon activation. We observed that despite activation induced by self-antigen or by CD3/CD28 mediated activation and at titrating amounts, 3G7 T cells were not prone to differentiate into regulatory T cells.

6.2.4 Lessons from the Treg TCR Transgenics.

Two studies, which interrogated the TCR ‘instructive’ hypothesis for Treg T cell commitment, through the generation of Treg TCR Tg mice, observed very few to no Treg T cells in these mice (Leung et al., 2009, Bautista et al., 2009). Whilst these observations could have led to the dismissal of the TCR-instructive model, instead it was proposed that stunted Treg T cell potential was probably a result of the prohibitively high precursor frequencies in monoclonal TCR Tg animals, rather than a failing of the TCR itself. It was therefore reasoned, that the intrinsic capacity of given Treg TCRs to encode the Treg cell fate could then be restored through adjusting the precursor frequencies in monoclonal mice to physiological levels. Indeed, in many, but notably not in all, seeding Treg TCR Tg progenitor cells into polyclonal T cell environments restored the Treg T cell potential of these TCRs. This suggested that there is a thymic 'niche' size which acts to limit Treg cell development *in* vivo; perhaps itself an important mechanism to regulate the contribution of Treg T cells to the polyclonal repertoire (Hogquist and Moran, 2009).

However, adjusting the clonal frequency of transgenic T cells in our system did not restore the instructive capacity of the 3G7 TCR for the Treg lineage. Thus our data suggest that not all Treg TCR Tg models can be manipulated to recapitulate tTreg T cell development. Overall these results suggested that although Treg T cell development may be a TCR-instructive process, this can be overridden by a variety of factors including precursor frequency and niche availability, suggesting TCR specificity alone is simply not enough.

6.3 Role of TCR specificity and avidity during antigen selected responses

Next we aimed to understand the role for TCR signalling in the clonal selection of CD4 T cells during retroviral infection. Furthermore, we aimed to decipher the factors that contributed to TCR sensitivity and the overall functional responsiveness of a T cell. To this end, we used a TCRβ transgenic mouse strain, with an increased frequency of virus-specific CD4 T cells, to specifically track the CD4 responses during FV infection. In addition, as the usage of different Vα chains by virus-specific CD4 T cells, marked cells according to their functional avidity this allowed us to study a role for the quality of TCR signaling in clonal selection. Specifically, this model allowed us to assess the relative contribution of Vα2 (high avidity cells) or Vα3 (low avidity cells) at different points in the response.

In brief, our findings supported a critical role for TCR signalling in determining clonal selection during CD4 T cell responses, as has been reported previously (Hogquist and Jameson, 2014, Tubo and Jenkins, Cohen et al., 2002, Moran et al., 2011, Busch and Pamer, 1999, Kim et al., 2013a). However, it also highlighted some un-anticipated results, revealing a hierarchy of factors that contribute to setting the intrinsic sensitivity of a T cell when responding to foreign-antigen. The critical findings can be summarised into three main points and will be discussed accordingly:

- We showed evidence for the clonal evolution, and the diversification of CD4 T cell responses over the course of retroviral infection.

- We showed evidence that T cell sensitivity to antigen better correlated with self-reactivity than TCR affinity toward foreign-antigen.

- We highlighted an essential role for B cells, as APCs capable of mediating the clonal selection of CD4 T cell repertoires.

6.3.1 Clonal evolution: Three types of clonotypic behavior

A characteristic feature of the B cell immune response is affinity maturation. Here, B cells progressively enhance the affinity of their immunoglobulin receptors during the course of infection. This involves multiple rounds of somatic recombination events, which act to diversify their antigen receptors, followed by affinity discrimination that positively selects the most successful recombinants. Unlike B cells, a T cells antigen receptor is fixed and hence cannot undergo further modifications at the sequence level. Instead, it has been suggested that TCR repertoires may undergo affinity maturation at the population level, via the selective expansion of the highest affinity clones. This has been suggest to act to focus the TCR repertoire (Busch and Pamer, 1999). However, it must be considered that this 'repertoire focusing' would be at the expensive of repertoire richness and diversity, which could be potentially catastrophic when the antigen receptor is fixed (ie there is no going back). Thus we wanted to understand if the same, or if diverse TCR clonotypes contributed to the acute or memory phases during retroviral infection.

As has been previously published, EF4.1 CD4 T cell responses to FV infection were characterised by the dominance of clones of highest avidity at the peak of infection (Antunes et al., 2008, Thorborn et al., 2014). However, tracking of the protracted response revealed (quite unexpectedly), the clonal replacement of previously dominant high avidity cells for clonotypes of comparatively lower avidities. We therefore aimed to understand, the driving forces behind this.

TCR repertoire analysis revealed that indeed the clonotypic switch observed at the population level was driven by diverse families of clonotypes coming from within

the larger Vα2 and Vα3 populations. As diverse behaviours correlated with distinctive CDR3 regions, as accessed at the protein level, this suggesting this was also a TCR driven process. We then went on to categorise the three types of clonotypic behaviours accordingly:

The first was exemplified by clonotypes that universally dominated the primary response, annotated as 'red' clonotypes. Indeed, the 'red' clonotypes showed the lowest diversity both within the Vα2 and Vα3 subsets. At least within the Vα2 population, they remained the dominant population (>60%) at all times, although their relative contribution to the overall response progressively decreased over the course of the infection. Presumably these clonotypes would be responsible for progressive enrichment of high avidity CD4 T cell and concomitant loss of diversity in conditions where engagement of additional clonotypes is prevented.

The second type of clonotypic behaviour (green clones) was unique due to the relative stability of these clonotypes throughout the response. Thus a green clonotypes' representation within the total responding population was minimally affected by the behaviour of other competing clonotypes. Closer characterisation of the 'green family' members revealed their remarkable stability correlated with their established differentiation state, with evidence for an acquired maturation phenotype prior to the infection. This was initially reflected in the loss of Ly6c and with the acquisition of CD44 expression on green clonotypes derived from a naïve semi-polyclonal population and was re-affirmed via the characterisation of monoclonal TCR Tg mice bearing a representative TCR. It is currently unclear if this apparent pre-activation state of the 'green' stems from higher than average self-reactivity or from cross-reactivity with environmental antigens. Nevertheless, the results support the notion that the fate of at least some clonotypes following infection is predetermined by earlier TCR encounters.

The third type of clonotypic behaviour, and potentially the most pronounced was that exhibited by a diverse family of the *Traj31*-using 'blue' clonotypes. Although, no one family member dominated the response, collectively they monopolised the CD4 T cell response at later time points and thus were responsible for the clonotypic switch from the Vα2 family to Vα3 family.

Clearly different clonotypes has distinct temporal behaviours associated with their TCRs and presumably with the quality of TCR signalling they supported.

6.4 Avidity, Affinity and Self-reactivity

As all the clonotypes shared the same antigen reactivity, recognising the gp-70 envelope glycoprotein of FV, we next sought to understand what parameters associated with their TCRs were driving differences in their functional avidities (as observed *in vitro*) and their responsiveness as observed (*in vivo).*

6.4.1 Limitations in the field

Defining the binding properties associated with a TCR, and understanding how they impact the overall sensitivity of a T cell, should provide a better understand of how many T cell fate decisions are encoded by TCR signalling. Indeed, many studies have tried to understand the structural, biochemical and biophysical framework that underpins these interactions (Stone et al., 2009). However, this have been very challenging, predominately as the nature of these interactions fall at the lower detection limits of current biophysical techniques (Cole et al., 2007, Cole et al., 2014). Another important and confounding factor, is the incorrect usage and definition of these properties within the literature. In this regard, TCR affinity and avidity are often interchangeably used and incorrectly described. This coupled with limitations in the tools available to measure them, makes accessing their real contributions to cellular fate hard to decipher. A clear example of this is the widespread use of TCR tetramers to define properties of the TCR.

The use of MHCII tetramers has undoubtedly revolutionised CD4 T cell biology, allowing for the visualisation of antigen-specific CD4 T cells from heterogeneous repertoires, as well as being used as a proxy for TCR affinity measurements (Stone et al., 2009). However, recent work has suggested their use in the characterisation of finer properties of the TCR as well as their breadth of recognition (the number of specificities that can be assessed simultaneously by a single tetramer) may both be severely limited (Sabatino et al., 2011, Newell et al., 2009). In regard to their usage in affinity measurements: affinity is defined as the strength of a 1:1 interaction/monomeric interaction. However, due to the relatively weak nature of a

TCR-pMHCII interactions, tetramers must be multimeric complexes in order to permit the visualisation of T cells bearing cognate TCRs (Matsui et al., 1991). Thus tetramers are neither a true measurement of affinity nor of avidity, as although they potentially measure multiple TCR interactions rather than a simple one, it remains impossible to determine exactly how many TCRs they truly engage. In addition, the weak nature of TCR-pMHCII interactions, may also limit the capacity of tetramers to visualize low affinity or avidity TCRs and thus tetramers may only allow for the visualization of the highest affinity or avidity TCRs. This means that the contribution of less 'fit' CD4 T cells to antigen selected responses may be vastly underestimated as a consequence (Sabatino et al., 2011). Further still, the responsiveness of a given T cell can rely on more than just the affinity and avidity of TCR-pMHCII interactions, and can be strongly influenced by the organization of components of the TCR signaling cascade (Viola and Lanzavecchia, 1996). These additional factors complicate clean measurements of individual parameters further (Huang et al., 2010). However, more sophisticated techniques are evermore becoming available, which should act to enrich the immunologists' toolbox. These in combination with many of the traditional approaches should aid a better understanding of the parameters that shape TCR signaling quality and downstream CD4 T cell fate decisions. In this study, we used a combination of new and old approaches in order to first accurately capture the entire responding CD4 T cell repertoire and then to compare affinity, functional avidity and self-reactivity at the level of individual clonotypes.

6.4.2 Appreciating a role for self-reactivity

Although there is general consensus for the role of TCR signal strength in dictating responses to antigen, the underlying factors responsible for functional differences are still a matter of debate. Traditionally, TCR affinity towards foreign-antigens have been considered as the dominant factor in the clonal selection of the CD4 T cell response, where it was assumed clonotypes of higher affinities performed better. Thus one possible explanation for the differences between the functional responses observed between Vα2 and Vα3 clonotypes was differing TCR affinities to foreign antigen. We imagined, that in the case for 'red' clonotypes, higher TCR

affinity for antigen may increase their functional responsiveness and thus the likelihood of red clonotypes to expand upon exposure to antigen. However, precise measurements of TCR affinity towards cognate antigen revealed little difference between the diverse clonotypes. Instead our findings supported an important role for self-reactivity in setting the clonotypic hierarchy during the CD4 T cell response (McHeyzer-Williams and Davis, 1995; Savage et al., 1999; Weber et al., 2012; Corse et al., 2011).

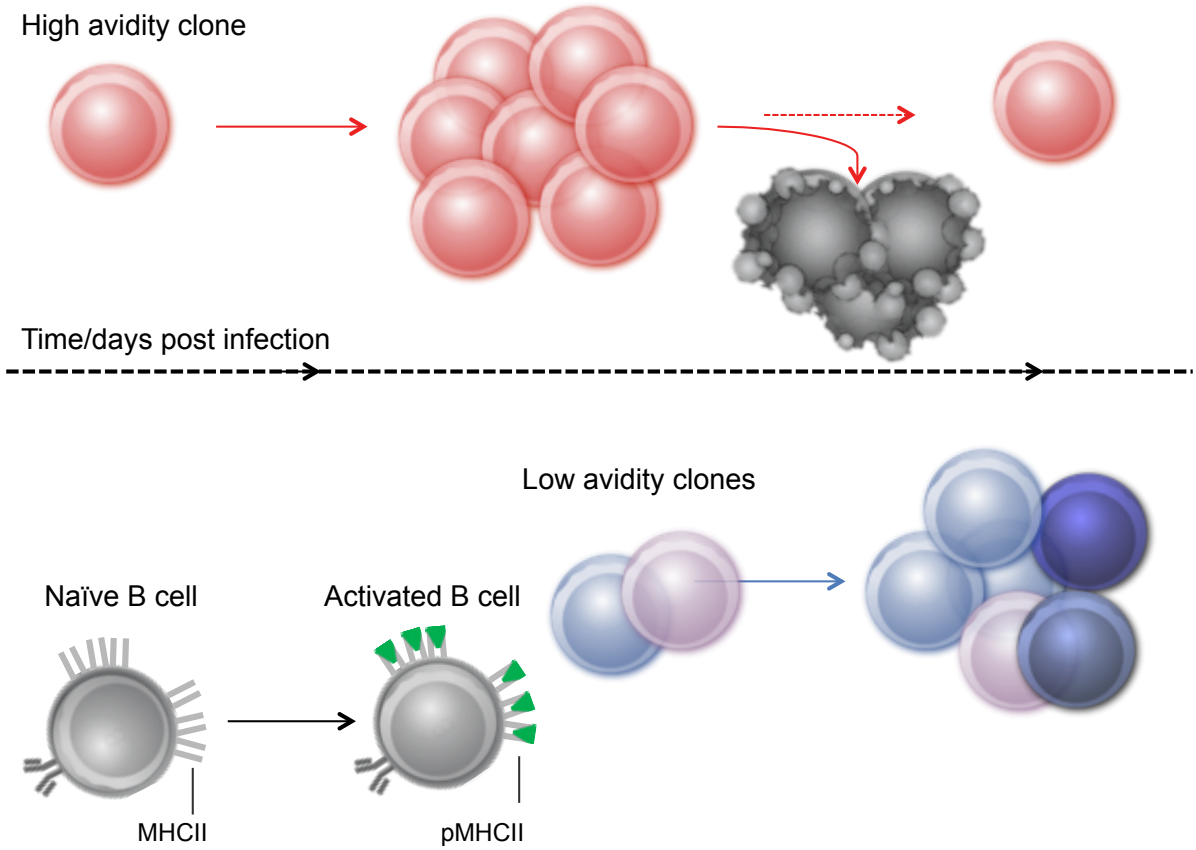
Studies of functional responses of CD4 T cells upon immune challenge have largely dismissed a role for self-recognition. Although these interactions are comparatively weaker, the vast majority of interactions a TCR will make is with self-antigen rather than with foreign-antigen. As with foreign-antigen, different TCRs recognise self-antigen with varying affinity and avidities. Here, even small differences in signalling encode vastly different cell fates; clearly exemplified in positive and negative selection events during thymocyte development. However, only recently have these interactions been suggested to contribute in setting the intrinsic sensitivity of a T cell to foreign-antigens (Mandl et al., 2013, Vrisekoop et al., 2014, Wulfing et al., 2002). Indeed, our study lends support to these recent findings, highlighting the importance of self-reactivity, rather than affinity for antigen, in determining a CD4 T cells responsiveness during infection. To date the most compelling models for how TCR self-reactivity may influence responses to foreign-antigen are as follow: One model which suggests that strong self-reactivity during positive selection acts to select and predict TCRs that also have similarly heightened binding kinetics to antigenic pMHC (Mandl et al., 2013). A second, which suggests that self-recognition prior to antigen encounter poises a T cell in a sensitized state allowing more efficient responses upon TCR triggering (Persaud et al., 2014). Our results are more consistent with the latter notion as responding clonotypes were similar in their affinities toward F-MLV. However, as a direct mechanistic link between self-reactivity and antigen sensitivity was not examined in this current study, we are unable to validate this assumption. Thus the question still remains: how does TCR recognition of self-ligands instruct for such diverse cellular fates?

6.5 B cell as critical regulators of CD4 T cell responses

We also report that the progressive diversification of the CD4 T cell response to FV infection was driven by the asynchronous expansion of virus-specific clonotypes, in a B cell dependent fashion (**Schematic 4**). This highlighted two major findings, first that clonotypes were recruited in successive waves according to their avidity; and second that the late recruitment of clonotypes was depended the availability of antigen-presenting B cells. Thus our findings uncover an additional dimension to the antigen-presenting contribution of B cells, in the clonotypic selection of CD4 T cells.

Studies have long reported a role for the quality of different APC types, in influencing CD4 T cell fate decisions. However, these have mainly centered on qualitative differences between APCs. For example, variations in TCR signalling imparted by the nature of the APC or its expression of co-receptors and co-stimulatory molecules, have been described to influence the functionality or longevity of an activated CD4 T cell. Instead, our results favor a more simplistic role for B cells acting as APCs. Rather than exhibiting a unique trait, we suggest that B cells may influence the clonal selection of CD4 T cell responses via their numerical contribution to CD4 T cell priming. In this study we observed that the degree of B cells that contributed to antigen presentation, rather than the role of a specific subset, directly correlated with the recruitment of lower avidity ‘blue’ clonotypes. In addition, and in the absence of high avidity clonotypes, diverse subsets of APCs were sufficient in priming blue clonotypes of lower avidity. This clearly argued against a unique type of antigen presentation exhibited only by B cells. Thus we concluded that asynchronous expansion of lower avidity clonotypes, which was dependent on the timing of B cell engagement in the response, coupled with waning response of higher avidity clonotypes were the major forces underlying TCR repertoire evolution during FV infection. In addition, we suggest that rather than providing qualitatively distinct signals, antigen presentation by B cells simply acts to increase the availability of pMHCII complexes and provide the necessary abundance of antigenic stimulation to allow priming and expansion of all clonotypes irrespective of TCR avidity. In this way antigen presentation by B cells acts to diversify the responding CD4 T cell repertoire

Schematic 4



B cells diversify the antigen-selected CD4 T cell repertoire

High avidity ('red') clonotypes are recruited early in the response to infection. Lower avidity ('blue') clonotypes are recruited progressively later in the response and in a B cell dependent manner. Here, antigen presentation by B cells serves to narrow the (avidity) gap between the 'red' and 'blue' clonotypes by reducing clonal competition. This then allows for the progressive diversification of the antigen-selected CD4 T cell response to Friend Virus infection.

6.5.1 B cell regulate the quality of TCR repertoires during homeostasis

Our data also novelly show that B cell-mediated clonotypic selection of CD4 T cells shaped the TCR repertoire during T cell reconstitution of lymphopenic recipients. This suggested that B cells might not only be an important determinant in TCR repertoire evolution during viral infection, but also during T cell homeostasis. Our prediction would be that B cells also heavily contribute to the availably of MHCII derived signalling at the 'steady-state', known to be critical for T cell homeostasis. Thus they act to relax interclonal competition, resulting in the maintenance of clones that recognise self-antigen to lesser extents alongside clones that recognise self-antigen increasing well. Although the overall avidity of clonotypes supported in B cell proficient versus deficient hosts could not be directly measured due to the unknown of the selecting self-antigens, differential expression of markers of self-reactivity were in alignment with this prediction.

Although not explored in this study, the maintenance of a more diverse range of TCRs may not always be advantageous. Although diversity has the obvious advantage of achieving a higher probability of clones that may be able to recognise any given pathogen, it also increases the potential for auto-reactive T cells in the repertoire. This coupled with the consideration that interactions between TCRs and auto-antigen are typically described to be within the lower range of avidities, suggests B cells may be key facilitators in the onset of T cell mediated autoimmunity. Indeed, there are many studies that may support a role for B cells in selecting for a pathogenic TCR repertoire (Serreze et al., 1996). For example, in non-obese diabetic (NOD) mice a broad repertoire of auto-reactive T cells contribute to the development of diabetes and autoimmunity in these mice. Interesting NOD MuMT mice, NOD mice deficient in B cells, are disease free. However upon B cell reconstitution of NOD muMT mice, spectra-typing of the TCR repertoire revealed the progressive re-instalment of the diabetogenic TCR repertoire, and the subsequent re-establishment of autoimmunity in these mice (Vong et al., 2011). Although in this context deleterious, this study further supports a role for B cells in the diversification of the T cell repertoire during homeostasis.

Furthermore, we were able to confirm a role for B cells in promoting TCR diversity in various T cell immune responses. Our data would suggest that in infections or immunizations where B cells are dominant APCs, T cells of lower avidity would be recruited alongside T cells of higher avidity, making it an important consideration in vaccine design. Perhaps in support of this, a study which examined the evolution of polyclonal CD4 T cell repertoires in response to combined TLR agonists, peptide or protein vaccination were able to show the nature of the vaccination regime was able to predictably shape TCR repertoires (Baumgartner et al., 2012). Whereby peptide vaccines preserved high avidity clones whilst protein vaccines skewed repertoires towards low avidity clones. The mechanism for the selective preservation of low avidity cells over high avidity cells was not elucidated, although a key role for CD27 derived signalling was suggested as necessary. Interesting CD27 is a T-B cell co-stimulatory molecule, with CD27-CD70 interactions shown to be critical in order to regulate B-cell activation by T cells (Kobata et al., 1995). Considering the role B cells play in our system, perhaps the difference in repertoires can be explained by the degree of involvement of B cells acting as APCs between the vaccine regimes. At least in our study we were able to show that the availability of B cells acting as APCs in vaccination setting, directly influenced the diversity and range of avidities recruited. Clearly deciphering some of these underlying principles involved could revolutionise vaccine development and drug design.

6.6 Implications for this work

Diverse, specific T cell repertoires are required for sufficient protection against future infections or for controlling escape mutants in chronic infections. Understanding how to elicit stable and diverse repertoires would be important in targeted vaccine design. DCs have conventionally been associated with being the major contributors to T cell priming and as a result have promoted many DC based vaccination strategies. An important finding of our study, is the relationship between the degree of antigen presentation by B cells and the associated diversification of the CD4 T cell response. B cells appear to be largely dependent on their activation state in order to perform as professional APCs. Limiting precursor frequencies of antigen specific B cells at the start of an infection, and

hence the assumed time it would take to expand these initially few to sufficient numbers that would allow them to significantly contribute to CD4 T cell priming, has largely lead to their dismissal as major contributors to this process. However, B cells can capture, process and present antigen for which they are not specific for and can be activated in multiple ways besides directly through the BCR (such as via TLRs). This would override the numerical limitations associated with the low precursor frequencies of antigen-specific B cells. In fact, B cells have been calculated to outnumber DCs at the steady-state by two orders of magnitude (Breton et al., 2015), hence their recruitment as APCs through BCR independent mechanisms of activation, could make them tour de force and provide a mechanism which would allow for the predictable diversification of CD4 T cell repertoires. Thus targeted manipulation of B cell activation or antigen presentation to CD4 T cells, might provide an amenable way of controlling the quality of the antigen-specific TCR repertoire during infection, vaccination or autoimmunity.

In addition, it is also important to consider that outside of the laboratory, natural host immune systems are never entirely naïve. In fact, the mammalian immune system is always fluctuating in its degree of activation even at an apparent ‘steady state’. Indeed, in non-sterile environments, even asymptomatic hosts, carry multiple viruses and bacteria as part of their microbiome, all of which may be recognised by the immune system (Virgin). The maintenance of this equilibrium between host and microbiome as well as the continual and often simultaneous challenge of the host by infectious agents has been shown to significantly alter future responses to pathogens. Hence symbiotes as well as past and present pathogens are able to physiologically imprint a host, and thus partially dictate disease severity and the immune control of future pathogens, this is known as the genotype/phenotype relationship (Virgin). Thus considering our findings, a host in which B cells are pre-activated may result in the selection of qualitatively distinct TCR responses accordingly. This may go some way to explain why disease progression can be so radically different in otherwise very similar hosts. Again, this further highlights the need for our better understanding of T cell extrinsic factors capable of influencing the quality of CD4 T cell responses in a given host at a given time.

Finally, the observation that self-reactivity correlated better with a CD4 T cells functional responsiveness than with TCR affinity toward antigen, should have major implications for future adoptive T cell based therapeutics. Conventionally, TCR gene transfers have been used to generate engineered T cells of given specificities and avidities towards foreign antigen, with the aim that their delivery may enrich existing TCR repertoires and aid functional responses to pathogenic challenge. However, in light of a role for self-reactivity, rather than cellular transfer approaches, conditioning of the existing repertoire via self-like proteins could be used to optimize the functional avidity of the endogenous repertoire. Indeed previous work from our lab has already demonstrated that self-like proteins, derived from endogenous retroviruses rather than exogenously introduced, can heavily influence TCR repertoire formation and optimize responses to retroviral infection (Young et al., 2012b). Taking inspiration from the endogenous retroviruses in this instance, it would be possible to introduce synthetic antigens, whose expression would mimic that of self-derived proteins, at various stages or places during T cell development as a method to tailor CD4 T cell repertoires. Thus therapeutic conditioning of the T cell repertoire via the introduction of self-like antigens may provide a tractable way to select for and to sensitise endogenous clonotypes in the pre-immune repertoire of individuals, with the aim that they then go on to perform better upon infection.

6.7 Concluding Remarks

Perhaps the popular discussion of nature vs nurture can be recycled to look at the relationship that exists between the T cell and its TCR. This analogy can be used to describe how cell fate is dependent on not only the intrinsic properties of the cell, the genetically encoded TCR ("nature"), but also by extrinsic events such as the APC is meets (“nurture”). This body of work suggest a prominent role for the TCR but also uncovers the powerful influence of additional T cell-intrinsic and -extrinsic factors that can reverse TCR-based hierarchies. These include both the maturation state of a CD4 T cell in the pre-immune repertoire and, importantly, differential recruitment or expansion of CD4 T cell clonotypes by diverse APC types.

6.8 Future work

Although we were able to recognise a role for self-reactivity in influencing CD4 T cell responses to cognate antigen, a question which has not yet been addressed by this study and remains largely un-answered in the field is exactly howTCR recognition of self-ligands can sensitises and set T cell responses to foreign-antigen. A model whereby the self-ligand repertoire of a given clonotype in addition to its antigen specificity were known would provide an elegant system whereby you could selectively withdraw the availability of self-ligand at different stages in T cell development, without influencing the entire TCR population. This would aid our knowledge of how, when and where self-recognition matters in terms of the conditioning of the CD4 T cell response.

In addition, although we were able to understand that the diversification of the CD4 T cell response to FV infection was achieved via the late recruitment of low avidity cells, beyond this point we did not characterise the capacity of each different clonotype family to give rise to long-term memory. Therefore in the future, we would like to follow the long-term memory repertoire to understand which clonotype persist in the host and why. As well as this, we aim to further characterise the different clonotypes potentials to contribute to different memory subsets. Considering the fact that functional responsiveness of a clonotype correlated better with the self-reactive potential of each naïve T cell clone, than its affinity toward cognate-antigen, we are also interested in understanding if these original differences are preserved in antigen experienced cells and during recall responses. In this regard we would like to test which clonotypes (red, blue or green) are recruited upon secondary exposure to antigen. Such a study would allow us to track which clonotypes respond upon antigen re-encounter and allow us to ask if antigen-experienced clonotypes retain behaviours from their past, or if following antigen encounter their sensitivity (originally set by self) is newly adjusted, now making them more or less equivalent.

Another outstanding question is how multiple cell fate decisions are encoded by TCR signalling. Where TCR signalling is equal, but affinities are not, an interesting question is how a T cell then interprets this. For example, do T cells count the

number of triggered TCRs, like a signaling ‘Morse code’, or does the sum signal elicited by all the triggered TCRs matter more? Furthermore, the question of how TCR signaling results in genetic and epigenetic re-programming and mapping of T cell fate remains largely un-resolved in the field. Indeed, this signalling network remains somewhat of the missing link in the biology of T cell development. Understanding these intricacies should fuel our basic understanding of the T cell and unlock rational development of vaccination and immunotherapeutic approaches improving our weaponry against infectious disease, cancer and autoimmunity.

Appendix

PUBLICATIONS

Merkenschlager,J., Ploquin, M., Kassiotis, G. (2016) Stepwise B-cell-dependent expansion of T helper clonotypes diversifies the T-cell response. Nature communications (doi:10.1038/ncomms10281)

Merkenschlager,J., Kassiotis, G. Unpublished. A cell-intrinsic role for Major Histocompatibility Class II molecules (MHCII) in restraining proliferation.

Merkenschlager,J., Kassiotis, G. (2015) Narrowing the Gap: Preserving Repertoire Diversity Despite Clonal Selection during the CD4 T Cell Response. Frontiers Immunology (doi.10.3389/fimmu.2015.00413)

Mavrommatis, B., Baudino,L., Levy,P., **Merkenschlager,J**.,Eksmond,U., Donnarumna,T., Young,G., Stoye,J.,Kassiotis,G., (2016) Dichotomy between T cell and B cell tolerance to neonatal retroviral infection permits T cell therapy. Journal of Immunology (In press)

Donnarumma,T., Young, G. ,**Merkenschlager,J**.,Eksmond,U., Nutt,S.,Ditmer,U., Bayer,W.,Kassiotis,G. (2016) Opposing development of cytotoxic and follicular helper CD4 T cells controlled by the TCF-1–Bcl6 nexus. Cell Reports (Under peer review)

Eksmond,U., Jenkins,B., **Merkenschlager,J**., Mothes,W., Stoye, J., Kassioitis,G. (2016) Mutation of the Putative Immunosuppressive Domain of the Retroviral Envelope Glycoprotein Reveal a Critical Function in Virus Exit (Under peer review)

CONFERENCE ATTENDANCE

April 2016 Cold Spring Harbour: Gene Expression and Signaling in the Immune System.
Abstract selection for a Poster presentation

March 2016 CSC workshop: Experimental and Computational biology.

January 2015 Keystone Symposia: Viral Immunity.
Abstract selected and a poster presentation

July 2015: UCL: Annual Infections Symposium.
Abstract selected for a poster presentation.

Summer 2014: UCL: Student symposium.
Abstract selected for a talk.

Summer 2013: ENII EFIS/EJI: Immunlogy summer school

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