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Tissue-specific butyrophilin-like proteins are $\gamma\delta$ TCR selecting ligands distinct from antigens

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Tissue-specific butyrophilin-like proteins
are $\gamma\delta$ TCR selecting ligands distinct from
antigens

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A thesis submitted for the degree of
Doctor of Philosophy
King's College London
January 2020

Declaration

I Iva Zlatareva 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.

The majority of the data presented in chapters III, IV and V were published in Melandri, et al., 2018 and Willcox et al., 2019.

Abstract

Tissue-resident $\gamma\delta$ T cells are a population of unconventional lymphocytes able to mount rapid immune responses following tissue damage. It has long been debated whether selecting elements for $\gamma\delta$ T cells exist, akin to MHC for $\alpha\beta$ T cells. In mice, $\gamma\delta$ T lymphocytes show tissue-restricted T cell receptor (TCR) repertoires, which are dependent, at least in the epidermis and gut, on the tissue-specific expression of butyrophilin-like proteins (Btl). Hence, we hypothesised that the Btl family of proteins are physiological ligands modulating $\gamma\delta$ T cell selection and function. We have previously established *in vitro* that BTNL3 and BTNL8, specifically expressed in the human intestinal epithelium, induce selective TCR-dependent responses in a subset of colonic $\gamma\delta$ T cells. The aim of this project was to investigate the mechanism of interaction between BTNL3 plus BTNL8 and the TCR of responding cells.

To this end, responding, human colonic intraepithelial lymphocytes (IELs) co-cultured with BTNL3 plus BTNL8-expressing cells were single cell-sorted and sequenced. Majority of responding cells were clonally diverse $V\gamma 4^+$ lymphocytes and several paired $\gamma\delta$ TCR sequences were expressed in TCR-deficient Jurkat cells. In co-culture assays, $V\gamma 4$ -expressing Jurkats downregulated their TCR and upregulated CD69 when exposed to BTNL3 plus BTNL8. Site-directed mutagenesis showed unexpectedly, that the hypervariable loop 4 (HV4) of the $V\gamma 4$ chain was critical for BTNL3-specific recognition. Intriguingly, CD1c-phosphatidylcholine-reactive $V\gamma 4V\delta 1$ and endothelial protein C receptor (EPCR)-reactive $V\gamma 4V\delta 5$ cells were capable of mounting a response against BTNL3 plus BTNL8 deeming them dually reactive. Importantly, CD1c and EPCR recognitions were CDR3-mediated.

Thus, human $V\gamma 4^+$ TCRs appear to have two spatially distinct regions involved in antigen recognition. The adaptive, non-germline encoded $\gamma\delta$ CDR3 loops are required for binding to clonally-restricted ligands whereas the innate, germline-encoded HV4 γ loop is required for interaction with the tissue-specific complex BTNL3/8. The same phenomenon is true for murine intestinal $V\gamma 7^+$ cells which require Btl1/4/6 for their development and tissue retention. Although these molecules are not direct human orthologues, an evolutionarily conserved mechanism emerges. BTNL3/8 are perhaps selecting elements of intestinal $V\gamma 4^+$ IELs and future studies should investigate the biological significance of these interactions in humans. Of note, early studies presented in this thesis suggest that BTNL3 and BTNL8 heteromeric complex is partially depleting the $V\gamma 4$ TCR from the lymphocyte surface, suggesting an immunoregulatory role for this interaction.

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Finally, I am grateful to all the brave patients who gifted pieces of their gut in the name of science, despite undergoing an incredibly uncomfortable procedure!

Dedication

This thesis is dedicated to the strongest woman that I know - my mother, Kristina Zlatareva.
Thank you for all your love and support in the pursuit of my dreams.

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Abbreviations

-/-	Knock-out
α GalCer	α -galactosylceramide
ADCC	Antibody-dependent cell-mediated cytotoxicity
Akt	Protein kinase B
aly/aly	Alymphoplasia
APC	Antigen presenting cell
APRIL	A proliferation-inducing ligand
BAFF	B cell-activating factor
BCR	B cell receptor
Blk	B lymphocyte kinase
BTN	Butyrophilin
BTNL	Butyrophilin-like
C	Constant
CCL	Chemokine C-C motif ligand
CCR	C-C chemokine receptor type
CD	Cluster of differentiation
cDC	Conventional dendritic cell
CDR	Complementarity determining region
CMV	Cytomegalovirus
CNV	Copy number variation
CRISPR	Clustered regularly interspaced short palindromic repeats
cTEC	Cortical thymic epithelial cell
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
CX3CR	CX3C chemokine receptor
D	Diversity
DAG	Diacylglycerol
DETC	Dendritic epidermal T cell
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific ICAM-3 grabbing non-integrin
DMEM	Dulbecco's Modified Eagle's Medium
DN	Double-negative
DNA	Deoxyribonucleic acid
DOXP	1-Deoxy-d-xylulose 5-phosphate

DP	Double-positive
DSS	Dextran sodium sulfate
E	Embryonic day
EclF1	<i>E. coli</i> translation initiation factor 1
Eomes	Eomesodermin homolog
EPCR	Endothelial protein C receptor
ER	Endoplasmic reticulum
Erk	Extracellular signal-regulated kinase
ERMAP	Erythroblast membrane associated protein
ESCRT	Endosomal sorting complexes required for transport
EV	Empty vector
FAE	Follicle-associated epithelium
FoxP3	Forkhead box P3
FPP	Farnesyl diphosphate
FR3	Framework region 3
GALT	Gut-associated lymphoid tissue
GFD	Gluten-free diet
GFP	Green fluorescent protein
GI	Gastrointestinal
HBSS	Hank's balanced salt solution
HIV-1	Human immunodeficiency virus 1
HLA	Human leukocyte antigen
HMBPP	(E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate
HSCT	Hematopoietic stem cell transplant
HSPC	Hematopoietic stem and precursor cell
HV4	Hypervariable loop 4
IBD	Inflammatory bowel disease
IEL	Intraepithelial lymphocyte
IFN γ	Interferon γ
Ig	Immunoglobulin
IgH	Immunoglobulin heavy chain
IgL	Immunoglobulin light chain
IgSF	Immunoglobulin super-family
IL	Interleukin

ILC	Innate lymphoid cell
ILF	Isolated lymphoid follicle
iNKT	Invariant natural killer T
IP ₃	Inositol-1,4,5-triphosphate
IPP	Isopentenyl pyrophosphate
IRES	Internal ribosome entry site
ISP	Immature single positive
ITAM	immunoreceptor tyrosine-based activation motif
J	Joining
KGF	Keratinocyte growth factor
I1	Btnl1
L3	BTNL3
I6	Btnl6
L8	BTNL8
LAT	Linker for activation of T cells
LB	Lysogeny broth
Lck	Lymphocyte-specific protein tyrosine kinase
LP	Lamina propria
LPS	Lipopolysaccharide
LSSR	Lymphoid stress surveillance response
M cell	Microfold cell
MAIT	Mucosal-associated invariant T
MART1	Melanoma antigen recognized by T cells 1
MHC	Major histocompatibility class
MHCp	Major histocompatibility class-peptide loaded
MICA	MHC Class I Polypeptide-Related Sequence A
MICB	MHC Class I Polypeptide-Related Sequence B
MLN	Mesenteric lymph node
moDC	Monocyte-derived dendritic cell
MOG	Myelin oligodendrocyte protein
MR1	Major histocompatibility complex class I-related gene protein
mRNA	Messenger ribonucleic acid
Myd88	Myeloid differentiation primary response 88
mTORC	Mammalian target of rapamycin complex

NCBI	National Centre for Biotechnology Information
NFAT	Nuclear factor of activated T-cells
NF κ B	Nuclear factor kappa B
NK	Natural killer
NKG2A	Natural killer group 2A
NKG2D	Natural killer group 2D
NKR	Natural killer receptor
NKT	Natural killer T
NOD	Non-obese diabetic
<i>nu/nu</i>	Nude athymic
ORAI1	Calcium release-activated calcium channel protein 1
OVA	Ovalbumin
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
PDB	Protein data bank
PDK	Phosphoinositide-dependent protein kinase
pDC	Plasmacytoid dendritic cell
PE	Phycoerythrin
pIgR	Polymeric immunoglobulin receptor
PI3K	Phosphoinositide 3-kinase
PIP ₂	Phosphatidylinositol-4,5-bisphosphate
PKC	Protein kinase C
PLC γ	Phospholipase C γ
PMA	Phorbol myristate acetate
PP	Peyer's patch
Rae-1	Retinoic acid early inducible 1
RAG	Recombination-activating gene
RhoB	Ras homolog gene family, member B
RNA	Ribonucleic acid
SCID	Severe combined immunodeficiency
SE	Staphylococcal enterotoxin

SEA	Staphylococcal enterotoxin A
SEB	Staphylococcal enterotoxin B
SED	Subepithelial dome
Skint	Selection and upkeep of intraepithelial T cells
SKINTL	Selection and upkeep of intraepithelial T cells-like
SP	Single-positive
SPR	Surface plasmon resonance
sTCR	soluble T cell receptors
STIM	Stromal interaction molecule
Syk	Spleen tyrosine kinase
TCR	T cell receptor
TGF β	Transforming growth factor β
Th	T helper
TLR	Toll like receptor
TNF α	Tumour necrosis factor α
Treg	T regulatory cell
TRIM	tripartite motif
tRNA	Transfer ribonucleic acid
TSG101	Tumour susceptibility gene 101
TSST-1	Toxic shock syndrome toxin 1
UC	Ulcerative colitis
ULBP	UL16 binding protein
V	Variable
VPS4	Vacuolar protein sorting-associated protein 4
Zap70	Zeta-chain-associated protein kinase 70

Chapter I. Introduction

1.The mucosal immune system in the gut

The intestine's primary functions are digestion, nutrient and water absorption, and waste elimination. It is continually exposed to a high load of antigens coming from the diet, commensal microbiota, and pathogenic microbes and toxins. Potentially reflecting this, the intestinal tract contains the body's largest immune compartment which simultaneously protects from pathogenic insults, limits inflammatory responses to commensal bacteria, and provides tolerance to food antigens (Mowat and Agace, 2014).

There are three distinct mucosal immune microenvironments of the intestine: the gut-associated lymphoid tissue (GALT), the loosely-packed connective tissue termed "lamina propria" (LP) and the overlying epithelium (Mowat and Agace, 2014; Gibbons and Spencer, 2011).

1.1. GALT – the induction site of mucosal antigen-specific responses

The GALT is an organised lymphoid tissue located along the gastrointestinal (GI) tract. In humans, it consists of Peyer's patches (PPs) and isolated lymphoid follicles (ILFs) which serve as induction sites for antigen-specific immune responses (Mowat and Agace, 2014).

PPs are the best characterised GALT structures found in the small intestine. Their numbers increase from the duodenum to the ileum and they are important inductive sites for intestinal immunoglobulin A (IgA) production. Reflective of this, they are rich in B, T and dendritic cells (DCs). They are highly organised and comprise numerous B cell follicles associated with adjacent T cell zones (Fig.1.1). Specialised follicle-associated epithelium (FAE) overlays PPs (Neutra et al., 2001; Reboldi and Cyster, 2016). The FAE has several distinct features: low amounts of mucus and digestive enzymes production compared to other epithelial cells; a distinct glycosylation pattern; no expression of the polymeric Ig receptor which is required for the transport of protective IgA from the interstitium into the gut lumen; and a porous basal membrane. Collectively, these characteristics promote the local contact of microbes with the FAE and facilitate antigen transportation to the underlying immune cells (Neutra et al., 2001).

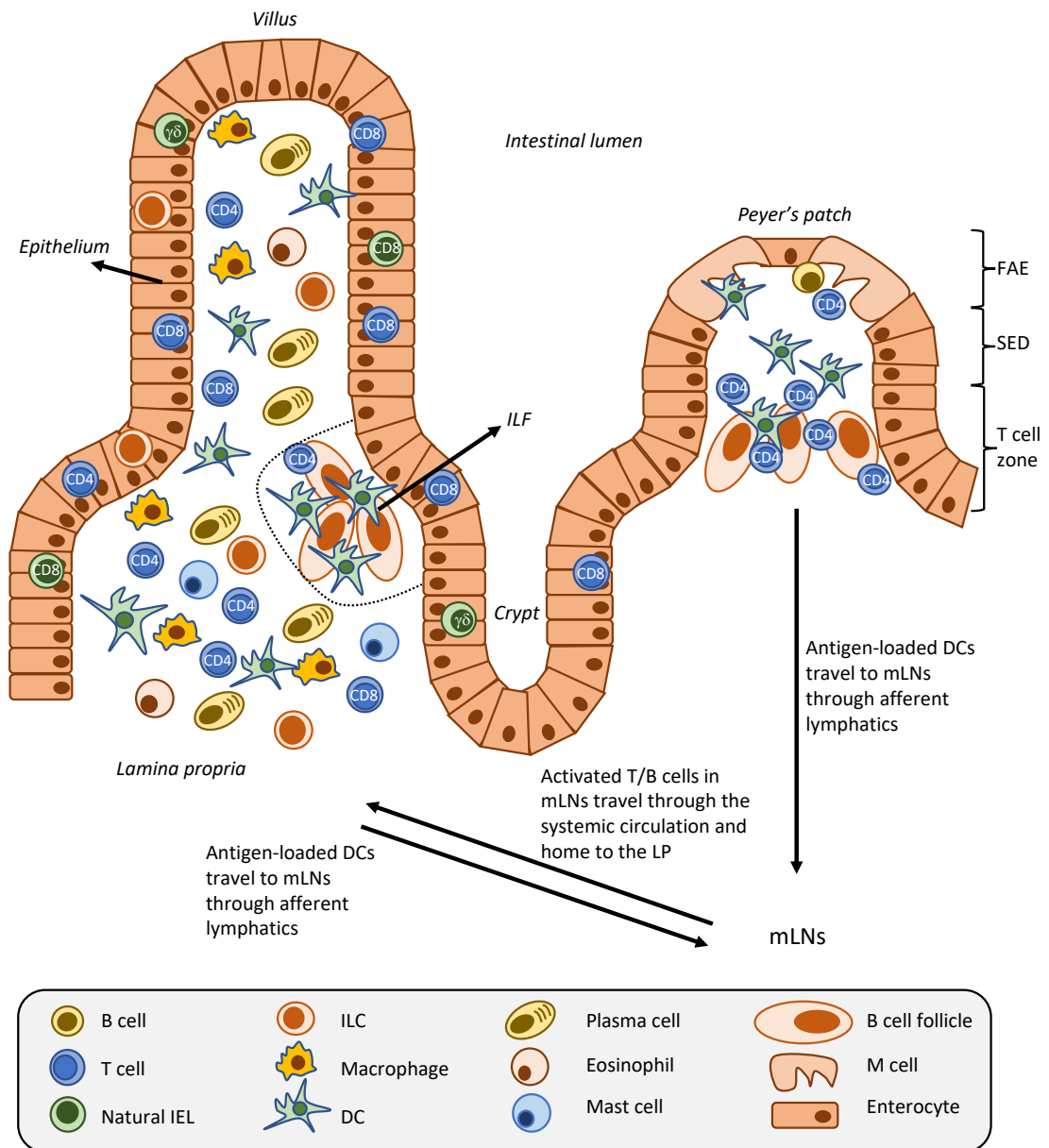


Figure 1.1. Simplified diagram of the intestinal immune system. Antigen which has gained access through the Peyer's patches is processed by the underlying antigen-presenting cells and presented to CD4⁺ T lymphocytes found adjacently or in the T cell zone where B cell follicles are subsequently generated. Alternatively, antigen-loaded DCs can travel through the afferent lymphatics and enter the mLN where consequent T cell recognition occurs. Activated B cells and T cells exit the mLN and Peyer's patches and enter the systemic circulation from where they preferentially home to the intestinal LP. In the LP, B cells differentiate into IgA-producing plasma cells. In contrast, ILFs are the site of T cell-independent IgA class-switching. LP DCs can also capture antigen, which has gained access directly through the villus intestinal epithelial barrier, and prime T cells locally or migrate to the mLN to induce antigen-specific responses. In addition, the LP is rich in macrophages, (legend continues on the next page)

(Figure 1.1. legend continued)

ILCs, eosinophils and mast cells. IELs such as natural $CD8\alpha\alpha^+ \alpha\beta$ T cells and $\gamma\delta$ T cells as well as conventional $CD8\alpha\beta^+ \alpha\beta$ T cells and ILCs populate the epithelial barrier where they can provide immediate tissue surveillance. For further details see text. DC – dendritic cell; ILC – innate lymphoid cell; FAE – follicle-associated epithelium; SED – subepithelial dome; IEL – intraepithelial lymphocyte; mLN – mesenteric lymph node; LP – lamina propria; ILF – isolated lymphoid follicle. *Adapted from Mowat, 2003.*

The FAE is particularly enriched for specialized microfold cells (M cells) which sample the gut lumen and transcytose intact antigens which are presented to the closely associated DCs and B cells (Jung et al., 2010). In turn, B cells can process and present the acquired antigens to adjacent T cells while DCs migrate away from the subepithelial dome region and into the T cells zones where they carry out their function as antigen presenting cells (APCs) (Neutra et al., 2001). Together, DCs and primed T cells can then activate naive B cells in the underlying follicles and proliferation, immunoglobulin IgM-to-IgA class switch recombination and affinity maturation are triggered in the germinal centres. In 1971, seminal experiments undertaken in rabbits demonstrated PPs to be important for the generation of IgA-producing cells (Craig and Cebra, 1971). This is largely a T cell-dependent process since $TCR\beta/\delta$ deficient mice exhibit approximately 75% reduction in intestinal IgA (Macpherson et al., 2000; Macpherson et al., 2004).

In contrast to PPs, ILFs are considered to be the site of T-cell independent IgA class-switching based on mouse models (Tsuji et al., 2008). Consistent with this, ILFs contain DCs and B cells organised in germinal centres with no clear T cell zone. ILFs numbers increase from the jejunum towards the ileum and from the ascending towards the rectosigmoid colon (Mowat and Agace, 2014; Junker et al., 2009). *In vitro* data suggests that the process of T-cell independent class-switching can be promoted by transforming growth factor β (TGF β), B cell-activating factor (BAFF) and a proliferation-inducing ligand (APRIL) secreted by $CD11b^+CD11c^+$ macrophages/DCs and stromal cells (Tsuji et al., 2008).

In sum, GALT is a major site for the induction of intestinal B cell. This ultimately leads to the production of IgA which plays a crucial role in the host's tolerance to the intestinal microbiota (Fagarasan et al., 2002).

1.2. LP – the effector site of mucosal antigen-specific responses

The LP is the effector site of the intestinal immune system. Accordingly, it harbours a myriad of immune cell types of both myeloid and lymphoid origin (Fig.1.1) which are described below.

1.2.1. LP myeloid cells

Major myeloid populations resident in the LP include macrophages, DCs, eosinophils and mast cells which employ multiple strategies to maintain tissue homeostasis.

1.2.1.1. Macrophages

The GI tract is the largest reservoir of macrophages in the body (Smythies et al., 2005). Interestingly, these cells are profoundly anergic to inflammatory signals in the healthy human intestinal mucosa but retain their function as professional phagocytes specialized in the clearance of invading microbes and dying cells (Smythies et al., 2005; Zhou et al., 2016). Unlike in other tissues, a large proportion of LP intestinal macrophages are continuously replenished from blood monocytes. Once migrated, monocytes undergo several stages of differentiation towards an anti-inflammatory phenotype characterised by a decrease in TNF α production and an increase in IL-10 secretion (Bain et al., 2013). They become progressively more phagocytic despite acquiring Toll-like receptor (TLR) hypo-responsiveness (Smythies et al., 2005; Bain et al., 2013; Bujko et al., 2018). Additionally, *in vitro* phagocytosis assays with these cells have demonstrated a lack of specific pro-inflammatory IL-1, IL-6, TNF α and IL-8 cytokine release which are normally produced following phagocytosis by blood-derived monocytes (Smythies et al., 2005). Finally, LP macrophages promote tissue integrity through the secretion of tissue-remodelling metalloproteinases and factors such as prostaglandin E2 and Wnt ligands which support the proliferation and survival of intestinal crypt stem cells (Bain and Schridde, 2018).

1.2.1.2. Dendritic cells

DCs in the LP are thought to be central in determining whether intestinal T cell responses will be inflammatory or tolerogenic. They can be subdivided into plasmacytoid (pDCs), monocyte-derived (moDCs), and conventional (cDCs). In mice, cDCs are the most efficient in promoting naive T cell activation and can be further subdivided into CD8 α ⁺CD103⁺ cDC1, specialized in antigen cross-presentation to CD8⁺ T lymphocytes, and CD11b⁺ cDC2, which promote CD4 T helper differentiation (Granot et al., 2017). In humans, cDC2 are the dominant population in the intestinal LP, followed by cDC1 and pDC (Granot et al., 2017). At steady state in the human intestine, DCs express low levels of TLR2 and TLR4 required for pathogen recognition, and low levels of CD40, CD80 and CD86 required for T cell priming and activation (Bell et al., 2001; Hart et al., 2005). Furthermore, colonic DCs were shown to produce primarily the regulatory cytokine IL-10 (Hart et al., 2005). Accordingly, intestinal DCs were demonstrated to have reduced ability to function as APCs when compared to DCs from skin or blood (Mann et al., 2012). In the absence of inflammation and epithelial barrier disruption, mucus-secreting goblet cells found within the

epithelial barrier can deliver luminal antigen to the underlying LP CD103⁺ DCs (McDole et al., 2012). Subsequently, antigen-loaded CD103⁺ DCs migrate to the mesenteric lymph nodes (MLNs) where they predominantly induce the differentiation of IL-10-producing regulatory CD4⁺ T cells (Tregs) which contribute to the maintenance of an anti-inflammatory environment (Sun et al., 2007; Jaensson et al., 2008). In contrast, during tissue stress, this immune tolerance is lost as DCs upregulate co-stimulatory molecules and promote pro-inflammatory T cell development.

1.2.1.3. Eosinophils and mast cells

Eosinophils and mast cells are also common in the healthy intestinal mucosa (Bischoff et al., 1996; Mowat and Agace, 2014). Mast cells produce mediators such as histamine and leukotrienes which regulate mucus production, peristalsis and vascular tone, and interact with the enteric nervous system (Mowat and Agace, 2014). Eosinophil accumulation is a common feature of numerous GI disorders including food allergy, inflammatory bowel disease (IBD) and eosinophilic gastroenteritis (Rothenberg et al., 2001). However, very little is known about the biological and pathological significance of eosinophils in humans. A recent study has shown that eosinophil deficient mice have reduced levels of serum and intestinal IgA and impaired generation and maturation of IgA-producing plasma cells (Chu et al., 2014). *In vitro* experiments revealed these cells to be a major source of TGFβ in the LP. Therefore, the development of CD103⁺ DCs and CD103⁺ CD4⁺ T cells, including forkhead box P3⁺ (Foxp3) Treg cells, was also affected in eosinophil deficient mice (Chu et al., 2014). The authors of the study reported an altered intestinal microbiota, with outgrowth of Gram-negative bacteria, but no other intestinal barrier perturbations at steady state. Considering the tolerogenic role of CD103⁺ DCs and Tregs, it could be hypothesised that there will be exacerbated inflammatory immune response following tissue stress in eosinophil-deficient mice.

1.2.2. Lamina propria lymphoid cells

Lymphoid populations found in the LP include B cells, T cells and innate lymphoid cells (ILCs).

1.2.2.1. B cells and plasma cells

Once B cells are activated in the GALT, they migrate to the MLNs, circulate through the body and home to the intestinal LP where they become antibody-secreting plasma cells or memory B cells. This process is dependent on CCL25 and CCL28 expression by the epithelium which directs immune cell expressing CCR9 and CCR10 towards the small intestinal or colonic LP, respectively (Mowat and Agace, 2014). More than 80% of plasma cells in the LP secrete polymeric IgA which binds to the polymeric Ig receptor (pIgR) to be transported across the intestinal epithelium and

into the gut lumen where it regulates bacterial populations (Spencer and Sollid, 2016). The rest of the plasma cells in the human gut secrete IgM and were recently shown to arise from a population of LP IgM⁺ memory B cells. This was in stark contrast to the murine intestinal tract where IgM-producing cells are found in negligible numbers (Magri et al., 2017).

1.2.2.2. T cells

Once naive T cells have been primed by DCs in the GALT or the MLNs, they acquire gut-tropic receptors CCR9/CCR10 and $\alpha_4\beta_7$, circulate through the body and home to the intestinal LP where they become CD69⁺ CD45RO⁺ effector-memory cells (Jaensson et al., 2008; Schieferdecker et al., 1992; De Maria et al., 1993). LP CD4 helper and CD8 cytotoxic T cells are found at approximately 2:1 ratio (Schieferdecker et al., 1992). While 20-60% of LP T cells are naive in paediatric donors, these numbers decline to 5-10% in young adults (Thome et al., 2016), reflecting the high-level of antigen exposure driving T cell differentiation, activation and memory formation in the gut.

Classically, CD4⁺ lymphocytes have the capacity to differentiate into T helper 1 (Th1), Th2 or Th17 effector cells depending on the cytokine milieu and the presented antigen (Fig.1.2). Th1 cells develop in response to activated DCs which produce IL-12 and present antigens derived from intracellular pathogens. In turn, the primed Th1 cells secrete IFN γ , TNF α and IL-12 which amplify the immune response, stimulate macrophages and promote IgG_{2a} class switching in B cells (Weaver et al., 2007). DCs presenting antigens derived from parasitic helminths drive the differentiation of Th2 lymphocytes which produce IL-4, IL-5 and IL-13. IL-4 itself is required for Th2 polarization but the exact source during infection is unclear and various innate immune subsets such as eosinophils, mast cells and NKT cell are thought to be involved (Weaver et al., 2007). Th2 responses promote IgG₁ and IgE class switching as well as eosinophil recruitment and mucus hypersecretion (Weaver et al., 2007). Finally, DCs presenting antigens produced by extracellular bacteria and fungi induce Th17 responses. Accordingly, IL-6 and IL-23 produced by the activated DCs and TGF β derived from the tissue environment collectively polarize CD4 lymphocytes towards a Th17 phenotype characterized by the production of IL-17, IL-21 and IL-22 (Weaver et al., 2007).

The healthy human intestinal mucosa is dominated primarily by Th1 and Th17 responses (Maynard and Weaver, 2009; Hovhannisyan et al., 2011; personal observations). At homeostasis, these are counteracted by the large number of LP-resident anti-inflammatory Foxp3⁺ and IL-10-producing CD4 Treg cells which function to dampen the immune responses (Maynard and Weaver, 2009; Thome et al., 2016).

A small number of unconventional lymphocytes can also be found in the human intestinal LP such as natural killer T (NKT), $\gamma\delta$ and mucosal-associated invariant T (MAIT) cells (O’Keeffe et al., 2004, Dusseaux et al., 2011). Of these, most common are the $CD161^{high}CD4^{+}CD8^{-}$ MAIT cells which account for $\sim 10\%$ of $CD3^{+}$ lymphocytes, express a semi-invariant $V\alpha 7.2-J\alpha 33$ T cell receptor (TCR) and recognise vitamin B metabolites presented by the MHC class I-like protein MR1 (Dusseaux et al., 2011). The functions of these unconventional lymphocytes are currently not fully understood.

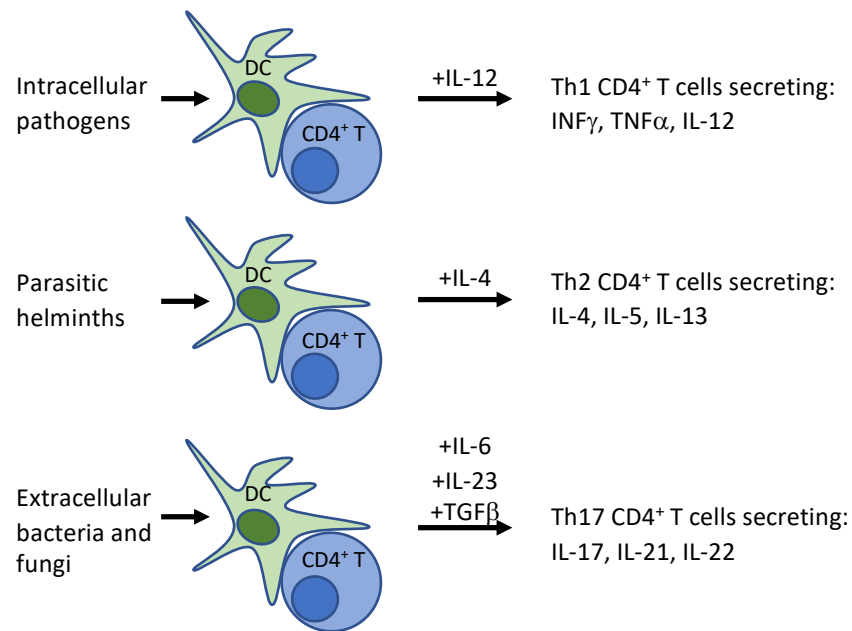


Figure 1.2. Classical T helper responses induced by pathogens. Antigen-loaded DC activate naive $CD4^{+}$ T cells. Depending on the nature of the pathogen and the cytokine milieu, lymphocytes differentiate into Th1, Th2 or Th17 effector cell. Each T effector subset can then produce cytokines specific for the type of inflammatory response.

1.2.2.3. ILCs

In recent years, newly-discovered ILCs have become a focus of intense research. Natural killer (NK), ILC1, ILC2 and ILC3 develop from a common lymphoid progenitor and are considered the innate counterparts of $CD8$ cytotoxic lymphocytes, Th1, Th2 and Th17 $CD4$ helper T cells, respectively (Eberl et al., 2015). They do not express somatically recombined antigen receptors and thus do not undergo clonal expansion. Instead, they respond to cytokine signals produced by myeloid and non-hematopoietic cells in tissues by producing effector cytokines that activate the local innate and adaptive immune system (Eberl et al., 2015).

Studies in mice show these populations are important for the maintenance of tissues homeostasis and their dysregulation can lead to aggravated inflammatory responses (reviewed in Zeng et al., 2019). In the human intestinal tract, ~1% of CD45⁺ lymphocytes are a mix of ILC1/2/3 and ~1% are NK cells. CD127⁺ILC1 account for more than half of the ILC population in the duodenum, followed by ILC3, with negligible proportions of ILC2, while in the ileum and colon ILC3 become the dominant population (Krämer et al., 2017). The exact function of these cells in the human intestine is not well defined. Interestingly, a recent study investigating ILC populations in human infant gut reported NK cells as a major ILC population in early life (Sagebiel et al., 2019). They accounted for approximately 15% of LP CD45⁺ cells in the first three months of life when the adaptive immune system is still immature and rapidly declined within a year to 5%. This period is characterised by a large exposure to microbial and dietary antigens and Eomes⁺ cytotoxic T cells were shown to gradually increase in the intestinal tissue (Sagebiel et al., 2019). The authors suggest that ILCs may provide important effector functions in early life when the immune system is still developing.

In sum, the LP harbours a large number of effector lymphocytes generated in response to the intestinal microflora and food antigens. Their function, however, is kept in check by the maintenance of an anti-inflammatory environment generated by: factors secreted by myeloid cells such as IL-10 and TGFβ; the preferential generation of Treg cells in the absence of active inflammation; and by restricting the sensitivity of the innate immune compartment to microbial products. In addition, the LP contains a dominant population of IgA-producing plasma cells. The generation and transportation of IgA across the epithelial barrier further promotes intestinal homeostasis as IgA sequesters bacterial invasion into the tissue.

1.3. Intraepithelial lymphocytes (IELs)

The intestinal epithelium consists of a single layer of columnar epithelial cells which are involved in nutrient uptake and provide a barrier against harmful pathogens. TCR⁺ and TCR⁻ lymphocytes can be found within the epithelium (Olivares-Villagomez and Van Kaer, 2018). Recently, NK cells were shown to account for 20-30% of the intraepithelial CD45⁺ cells in the first year of life, and in adults, they decrease to ~5% (Sagebiel et al., 2019). By contrast, the dominant population in the epithelium comprises TCR⁺ lymphocytes which can be subdivided into two types that have been described by various terms, including “type A” and “type B” (Hayday et al., 2001) or

“induced” and “natural” T cells (Olivares-Villagomez and Van Kaer, 2018). Induced T cells are derived from conventional T lymphocytes that home to the intestinal epithelium upon antigen recognition in the systemic circulation and have an effector-memory phenotype (Masopust et al., 2001; Mayassi and Jabri, 2018). Approximately 70% of human IELs are $\alpha\beta\text{TCR}^+\text{CD8}\alpha\beta^+$ and 10% are $\alpha\beta\text{TCR}^+\text{CD4}^+$ (Jarry et al., 1990).

In contrast, natural IELs are $\alpha\beta\text{TCR}^+\text{CD8}\alpha\alpha^+$ or $\gamma\delta\text{TCR}^+\text{CD8}\alpha\alpha^{+/+}$ and are believed to home to the epithelium during or immediately after their development (Olivares-Villagomez and Van Kaer, 2018). At steady-state, natural IELs express the activation and tissue-resident marker CD69, high levels of NK receptors, and cytotoxic molecules such as granzyme B. However, the cells express little perforin, exhibit very low basal cytokine production and little proliferation at steady state (Shires et al., 2001; Lutter et al., 2018; Mayassi and Jabri, 2018; our unpublished observations). Thus, natural IELs have been described as having an “activated yet resting” phenotype: positioned, primed and equipped to provide rapid immune responses to local tissue dysregulation.

In both mice and humans, adaptively induced IEL TCR repertoires are oligoclonal in nature (Helgeland et al., 2004; Balk et al., 1991; Van Kerckhove et al., 1992). In germ-free mice, there is a significant reduction in $\alpha\beta\text{TCR}^+\text{CD8}\alpha\beta^+$ and $\alpha\beta\text{TCR}^+\text{CD4}^+$ IELs accompanied by an increase in TCR clonality (Kawaguchi et al., 1993) which further supports the notion of antigen-driven selection and development of these cells. In contrast, $\gamma\delta\text{IEL}$ numbers are essentially preserved in germ-free animals (Bandeira et al., 1990; Di Marco Barros et al., 2016) and are the dominant natural IELs found in mice and humans (Di Marco Barros et al., 2016; Mayassi and Jabri, 2018). Despite the evident importance in immune-protection and immuno-regulation of $\gamma\delta\text{IELs}$ (discussed below), their antigen recognition and development are very poorly understood and are the topic of this study. In mice, these cells have conspicuously limited repertoires and preferentially express the TCR $V\gamma 7$ chain (Itohara et al., 1990). Similar phenomenon is observed in other tissues such as the skin epidermis where essentially a monoclonal population of $V\gamma 5V\delta 1$ dendritic epidermal T cells (DETCs) resides (Itohara et al., 1990). Humans lack DETCs and the extent to which there is preferential expression of $\gamma\delta\text{TCR}$ chain-pairs at mucosal sites has long been debated, although there is some evidence for the difference in repertoires between intestinal tissue and blood (discussed below).

2. $\gamma\delta$ T cells

$\gamma\delta$ T cells were first identified about 35 years ago (Hayday et al., 1985; Brenner et al., 1986). They are unconventional, non-MHC restricted T lymphocytes, which have been described as a link between the innate and adaptive immune responses. Similar to $\alpha\beta$ T cells, $\gamma\delta$ lymphocytes undergo V-(D)-J gene segment rearrangement to generate diverse sets of T cell receptors (TCRs). Indeed, together with $\alpha\beta$ T cell and B cells, they make up the three evolutionary conserved lineages that deploy somatic gene rearrangement for diversification (Hirano et al., 2013; Vantourout and Hayday, 2013). While they represent 5-10% of adult human peripheral blood T cells (Esin et al., 1996), they localise in higher proportions (20-30%) in non-lymphoid tissues such as skin, uterus and intestine (Bos et al., 1990; Mincheva-Nilsson et al., 1992; Di Marco Barros et al., 2016). Although $\gamma\delta$ cells can play major protective roles in various viral, bacterial and parasitic infections (Carding et al., 1993; Ferrick et al., 1995; Hamada et al., 2008; Lee and Kasper, 2004; Nishimura et al., 2004; Seixas and Langhorne, 1999; Takano et al., 1998; Usami et al., 1995; Wang et al., 2003) and contribute to anti-tumour immunity (Choudhary et al., 1995; Couzi et al., 2010; D'Asaro et al., 2010; Fisher et al., 2014; Gao et al., 2003; Girardi et al., 2001; Viey et al., 2005; Gentles et al., 2015; Wu et al., 2019), the mechanisms by which they do this, and the contribution of the $\gamma\delta$ T cell receptor ($\gamma\delta$ TCR) to these outcomes are largely uncharacterised.

2.1. The $\gamma\delta$ T cell receptor

The $\gamma\delta$ TCR is a membrane-bound heterodimeric protein consisting of a γ and a δ chain which, similarly to the $\alpha\beta$ TCR, is expressed in an octameric complex with the CD3 molecules.

2.1.1. The $\gamma\delta$ TCR genes

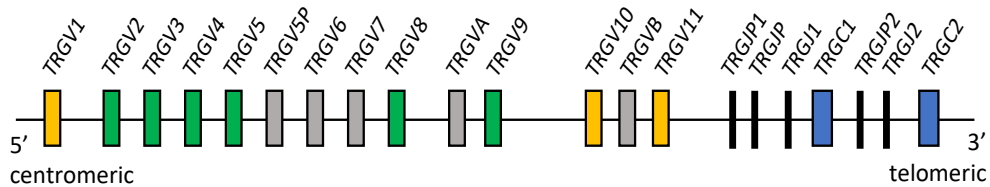
2.1.1.1. The $\gamma\delta$ locus organisation in humans

The γ chain gene locus, located on chromosome 7, consists of V [variable], J [joining] and C [constant] gene segments (Fig.1.3A). There are six functional human *TRGV* genes (*TRGV2-5*, 8 and 9), five *TRGJ* genes (*TRGJP1*, *TRGJP*, *TRGJ1*, *TRGJP2*, *TRGJ2*) and two *TRGC* genes (*TRGC1-2*). *TRGC1* has three exons. *TRGC2* is almost identical to *TRGC1* except for a duplicated exon 2 or, in certain populations, triplication of the same exon (Lefranc et al., 1986; Buresi et al., 1989).

The δ chain locus, situated within the *TCR α* chain locus on chromosome 14, is composed of V, D [diversity], J and C gene segments (Fig.1.3B). There are eight *TRDV* genes (*TRDV1-8*), three *TRDD* genes (*TRDD1-3*), four *TRDJ* genes (*TRDJ1-4*) and one *TRDC* gene. There are only three “true”

TRDV genes, *TRDV1-3*. The remaining genes encode for TCR α chain variable regions but are frequently found in δ chain rearrangements. Thus, *TRAV14*, *TRAV29*, *TRAV23*, *TRAV36*, and *TRAV38* are also known as *TRDV4*, *TRDV5*, *TRDV6*, *TRDV7* and *TRDV8*, respectively (Kazen and Adams, 2011).

A TRG locus on chromosome 7



B TRA-TRD locus on chromosome 14

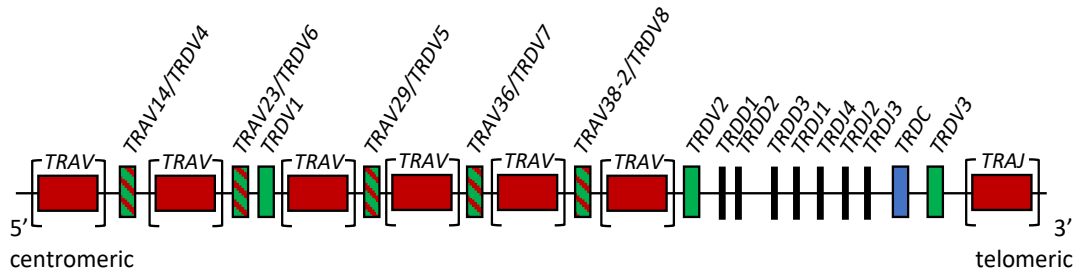


Figure 1.3. Simplified diagram of human TCR γ and TCR δ chain loci. **A.** TCR γ chain genes are situated on chromosome 7. **B.** TCR δ chain genes are found within the TCR α gene locus on chromosome 14. Green boxes – functional V genes; blue boxes – functional C genes; yellow boxes – open reading frames; grey boxes – pseudogenes; black boxes – J and D gene segments; red boxes with brackets – collection of a varying number of *TRAV* or *TRAJ* genes; red/green striped boxes – TCR variable gene segments used by both TCR α and TCR δ chains.

$\gamma\delta$ TCRs are generated *via* recombination-activating gene (RAG)-mediated V(D)J recombination (Schatz and Swanson, 2011). The nested organisation of the δ locus within the α locus prevents the simultaneous rearrangement of both chains on the same chromosome. Thus, the δ gene segments are irreversibly deleted from the chromosome following $V\alpha$ - $J\alpha$ gene recombination events (Krangel et al., 1998).

There are four key features which contribute to the generation of antigen receptor diversity during the assembly of the TCR hetero-dimeric complex. The first contributor to diversity is a quasi-random chain pairing – each TCR γ chain can be paired with any of the TCR δ chains. Next, any V gene can be recombined to any J segment, for TCR γ , or any D and J segments, for TCR δ ,

via RAG-mediated somatic gene rearrangement. The quasi-random fusion of single V(D)J segments creates the complementarity determining region 3 (CDR3) in which diversity is further increased by the deletion and incorporation of non-germline nucleotides. The final contributor to antigen receptor diversity is unique to the $\gamma\delta$ TCR. The small number of *TRGV* and *TRDV* genes suggests there is lower $\gamma\delta$ TCR diversity compared to $\alpha\beta$ T cells. Nonetheless, the TCR δ chain is able to fuse multiple D-gene segments that can encode protein in all three reading frames. This confers higher potential for CDR3 diversity on TCR δ than exists for either of the other antigen receptor chains that include D segments: TCR β or immunoglobulin heavy chain (IgH) (Elliott et al., 1988).

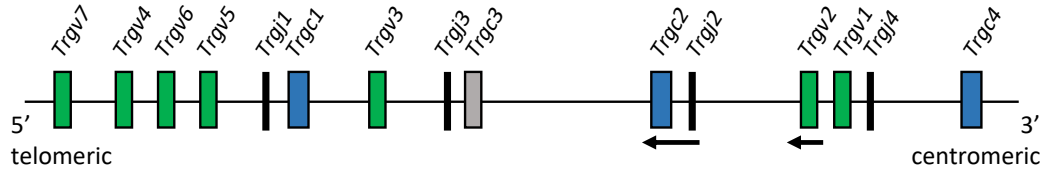
CDR1, CDR2 and CDR3 were first described as regions of high variability within IgH and IgL chains which were hypothesised to contribute to antigen binding (Wu and Kabat, 1970). These form distinct loops on the binding surface of the antigen receptor and can be identified in antibodies, $\alpha\beta$ TCR and $\gamma\delta$ TCR. CDR1 and CDR2 are germline-encoded in the V regions of the TCR. The CDR3 regions are considered the principal antigen recognition sites in the context of $\alpha\beta$ TCRs, while CDR1 and CDR2 predominantly interact with the MHC molecule although this is not a strict rule and contributions can vary between the various TCR-pMHC systems (Cole et al., 2014; Burrows et al., 2010; Manning et al., 1998; Borg et al., 2005).

2.1.1.2. The $\gamma\delta$ locus organisation in mice

The murine γ chain gene locus is located on chromosome 13 and consists of seven V genes (*Trgv1-7*), all of which are functional, four J segments (*Trgj1-4*) and four C segments (*Trgc1-4*), of which three are functional (Fig.1.4A).

The murine δ chain gene locus is located on chromosome 14 and is situated within the TCR α chain locus. It consists of 16 *Trdv* genes (*Trdv1*, *Trdv2-1*, *Trdv2-2*, *Trdv3*, *Trdv4*, *Trdv5*, *Trdv6-1*, *Trdv6-2*, *Trdv6D-1*, *Trdv6D-2*, *Trdv7*, *Trdv8*, *Trdv9*, *Trdv10*, *Trdv11*, *Trdv12*), two *Trdd* genes (*Trdd1,2*), two *Trdj* (*Trdj1,2*) and one *Trdc* gene segments (Fig.1.4B). There are only five “true” *Trdv* genes – *Trdv1*, *Trdv2-1*, *Trdv2-2*, *Trdv3*, *Trdv4*, *Trdv5*, of which *Trdv3* is a pseudogene. The remaining genes encode for TCR α chain variable regions but are frequently found in δ chain rearrangements. Thus, *Trav15D-1*, *Trav15D-2*, *Trav16D*, *Trav14D-3*, *Trav15-1*, *Trav15-2*, *Trav4-4*, *Trav6-7*, *Trav13-4*, and *Trav21* are also known as *Trdv6D-1*, *Trdv6D-2*, *Trdv11*, *Trdv8*, *Trdv6-1*, *Trdv6-2*, *Trdv10*, *Trdv9*, *Trdv7* and *Trdv12*, respectively.

A *Trg* locus on chromosome 13



B *Tra-Trd* locus on chromosome 14

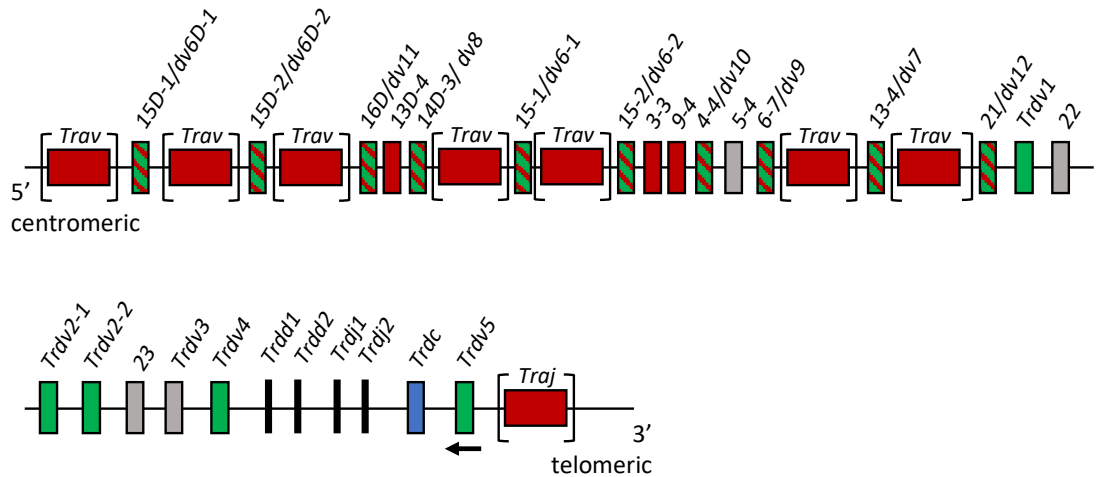


Figure 1.4. Simplified diagram of mouse TCR γ and TRC δ chain loci. **A.** TCR γ chain genes are situated on chromosome 13. **B.** TCR δ chain genes are found within the TCR α gene locus on chromosome 14. Green boxes – functional V genes; blue boxes – functional C genes; grey boxes – pseudogenes; black boxes – J and D gene segments; red boxes with brackets – collection of a varying number of *Trav* or *Traj* genes; red/green striped boxes – TCR variable gene segments used by both TCR α and TCR δ chains; arrows under boxes – indicate genes which are transcribed in the opposite direction to the entire locus.

2.1.1.3. Anatomical distribution of specific $\gamma\delta$ chain pairings

Despite their potential for high diversity, the repertoires of $\gamma\delta$ T cells are skewed by preferential $V\gamma/V\delta$ pairings, which appear to be in part tissue-specific. This is especially true in mice where monoclonal $V\gamma 5V\delta 1$ and $V\gamma 6V\delta 1$ TCRs are found in the epidermis and uterus, respectively; and oligoclonal $V\gamma 7^+$ TCR chains are dominant in the murine intestinal epithelium (Itoharu et al., 1990; Allison and Havran, 1991; Goodman and Lefrancois, 1989; Takagaki et al., 1989; Kyes et al., 1989;). In humans, the dominant blood $\gamma\delta$ T cells are $V\gamma 9V\delta 2^+$ while the majority of tissue-resident $\gamma\delta$ lymphocytes are $V\delta 1^+$ (Landau et al., 1995). The observed tissue-specificity of skewed

TCRs might reflect the different nature of pathogens encountered at different sites or the recognition of tissue-specific antigens.

2.1.2. Structure of the $\gamma\delta$ TCR

The $\gamma\delta$ TCR is a member of the immunoglobulin super-family (IgSF). Structurally, it is similar to $\alpha\beta$ TCRs and antibody Fab domains (Allison et al., 2001). A major difference exists within the C domain where the inter-chain disulphide bond between the TCR γ and TCR δ chains is positioned differently in $\gamma\delta$ TCRs compared to $\alpha\beta$ TCRs. The distance between the last ordered C α and C β residues and the interchain disulphide bond is 7 and 2 amino acids, respectively (Allison et al., 2001). In the $\gamma\delta$ TCR, this distance is increased to 12 and 23 amino acids in C γ and C δ , respectively. In the case of *TRGC2* gene usage, the inter-chain disulphide bond is completely absent (Allison et al., 2001). This feature of the $\gamma\delta$ TCR has been proposed to lead to altered association with the CD3 complex and the assembly of a unique signalling machinery (Allison et al., 2001). Indeed, the use of *TRGC2* would lead to an extension of the TCR γ chain by 16 amino acids and in the case of the allele with exon triplication – by 32 amino acids, which could affect pairing with the CD3 chains. To date, no published studies have investigated the functional consequences of different C γ usage.

The CD3 complexes, which the $\alpha\beta$ and $\gamma\delta$ TCRs associate with, consist of four chains – CD3 γ , CD3 δ , CD3 ϵ and TCR ζ (CD247). Studies in knockout mice for each of these chains has revealed CD3 ϵ , CD3 γ and TCR ζ to be essential for $\gamma\delta$ TCR assembly as few or no $\gamma\delta$ thymocytes or peripheral T cells were detected in these animals (Malissen et al., 1995; Haks et al., 1998; Liu et al., 1993; Hayes et al., 2003). In contrast, mice deficient in CD3 δ have arrested development of $\alpha\beta$ T cells but normal $\gamma\delta$ T cells (Dave et al., 1997). *Ex vivo* analysis of murine $\gamma\delta$ T cells has shown that CD3 δ is actively excluded from the TCR complex (Hayes and Love, 2002; Hayes et al., 2003). Consequently, the stoichiometry of two CD3 $\gamma\epsilon$ chain pairs associated with two CD247 chains and the $\gamma\delta$ TCR was proposed (Fig.1.5A).

Case studies of patients with severe combined immunodeficiency (SCID) confirm that CD3 ϵ and TCR ζ chains are essential for the $\gamma\delta$ TCR assembly (de Saint Basile et al., 2004; Fischer et al., 2005; Roberts et al., 2007). However, patients with CD3 δ deficiency display complete lack of $\gamma\delta$ T cells (Dadi et al., 2003; de Saint Basile et al., 2004) in contrast to CD3 γ deficient people who exhibited only mild immunodeficiency and minor reduction in $\gamma\delta$ TCR⁺ blood lymphocytes (Fischer et al., 2005; Siegers et al., 2007). Interestingly, CD3 γ haploinsufficient individuals also display reduced surface $\gamma\delta$ TCR expression (Munoz-Ruiz et al., 2013). Therefore, the preferred organisation in

humans is $CD3\epsilon\gamma + \gamma\delta TCR + CD3\epsilon\delta + TCR\zeta\zeta$ (Fig.1.5B). $CD3\epsilon\delta$ could partially compensate when $CD3\gamma$ availability is limited, although this would lead to less stable TCR complexes (Siegers et al., 2007; Munoz-Ruiz et al., 2013).

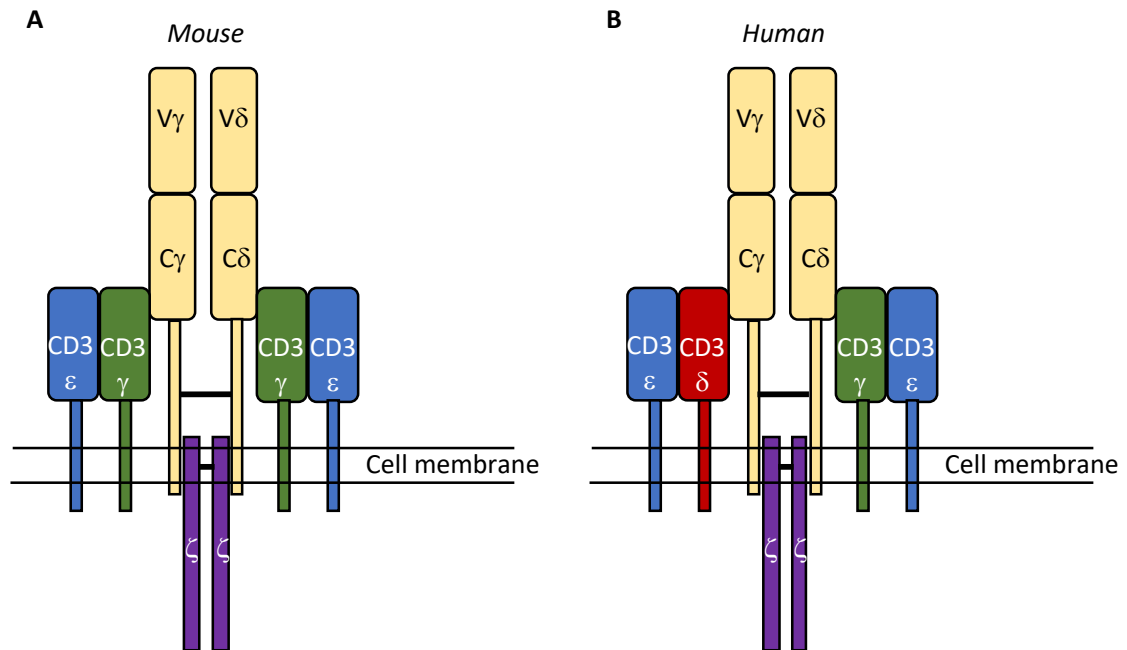


Figure 1.5. Structure of the $\gamma\delta$ TCR-CD3 octameric complex. **A.** The mouse $\gamma\delta$ TCR associates with two $CD3\epsilon\gamma$ dimers and two TCR ζ chains. **B.** The human $\gamma\delta$ TCR associates with one $CD3\epsilon\gamma$ dimer, one $CD3\epsilon\delta$ dimer and two TCR ζ chains.

2.1.3. Proximal $\gamma\delta$ TCR signalling

In conventional $\alpha\beta$ T cells, engagement of the TCR and either CD4 or CD8 results in the initiation of signalling (Fig.1.6) (Cantrell, 2015). The intracellular domain of the co-receptors recruits the Src family kinase Lck which phosphorylates the immunoreceptor tyrosine-based activation motifs (ITAMs) of the CD3 subunits. This leads to the recruitment and activation of Zap70. In turn, Zap70 phosphorylates the transmembrane adaptor protein LAT. Phosphorylated LAT is then able to recruit numerous proteins, forming a multimolecular signalosome. Among these is $PLC\gamma$ which generates inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) from the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2). IP_3 triggers the release of calcium ions from the endoplasmic reticulum (ER). Increased intracellular calcium levels stimulate the calcineurin-NFAT pathway. On the other hand, DAG is able to activate the Ras-Erk and PKC-NF κ B pathways. The phosphorylation of PIP_2 by PI3K and the generation of PIP_3 is another outcome of TCR triggering, although the exact mechanisms or PI3K recruitment to the cell membrane are unclear (Cantrell, 2015). PIP_3 promotes the recruitment of PDK and Akt to the plasma membrane

where PDK phosphorylates Akt and activates the Akt-mTORC1 signalling pathway. The outcome of these signalling events, together with the input from other co-stimulatory or co-inhibitory molecules and cytokine signals, governs conventional T cell responses such as proliferation, migration, exhaustion, and effector functions (Cantrell, 2015).

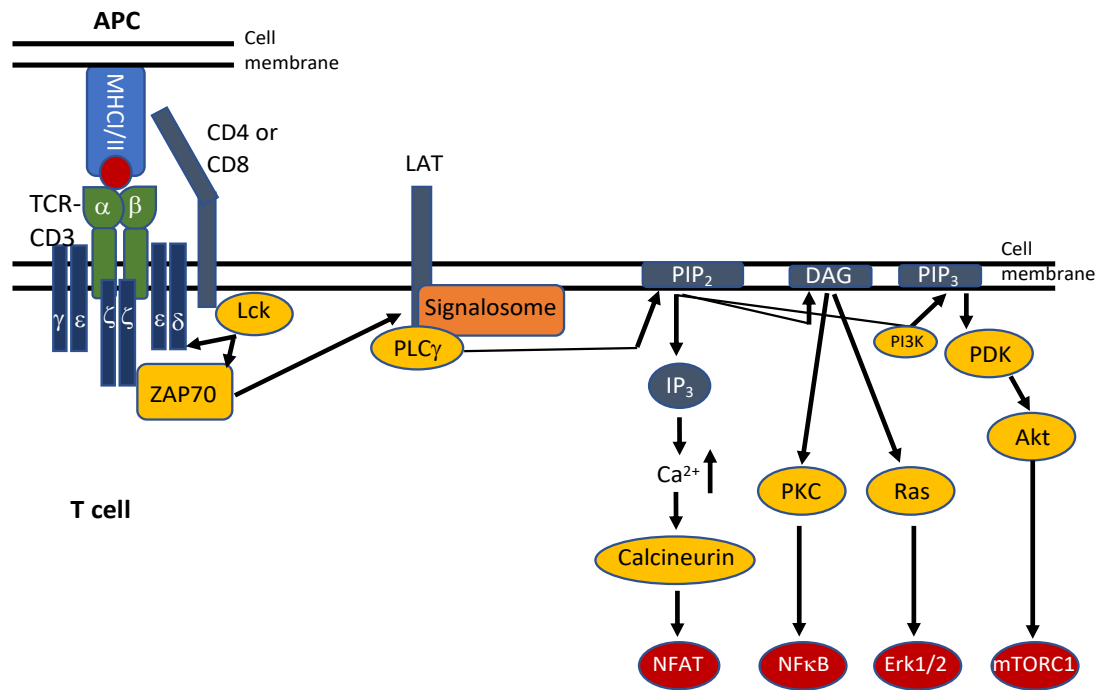


Figure 1.6. Simplified diagram of proximal $\alpha\beta$ TCR receptor signalling. For detailed explanation, see text. The different outcomes of TCR signalling depend on the regulation by feedback mechanisms (not depicted) at several points within these pathways. The incorporation of signals from other receptors further regulates cellular responses and may trigger additional pathways not shown here. *Adapted from Muro et al., 2019.*

Murine studies of $\gamma\delta$ lymphocyte development suggest that key differences exist between proximal signalling employed by $\alpha\beta$ TCRs and $\gamma\delta$ TCRs. For example, the knockout of *Lck* results in the almost complete block of $\alpha\beta$ T cell development while $\gamma\delta$ T cells can still be detected at near-normal levels in the periphery of mice. The double knockout of *Lck* and *Fyn* (another Src family kinase capable of partially compensating for *Lck* deficiency) is required for complete loss of both populations (Groves et al., 1996). However, DETCs are severely affected by the loss of *Lck* alone (Kawai et al., 1995). In contrast, intestinal $\gamma\delta$ IELs were still present in *Lck*^{-/-}*Fyn*^{-/-} albeit with reduced frequencies and unaffected in *Lck*^{-/-} animals (Page et al., 1997). Zap70 deficiency results in severely reduced numbers of gut $\gamma\delta$ IEL and somewhat reduced DETC with abnormal morphology (Kadlecek et al., 1998). Importantly, no such effects were reported in lymph node,

spleen and thymic $\gamma\delta$ cells (Kadlecek et al., 1998). The development of $\alpha\beta$ lymphocytes in *Zap70*^{-/-} animals is blocked at the CD4⁻CD8⁻ double-negative stage. LAT deficiency completely prevents the development of $\alpha\beta$ TCR⁺ cells and impairs that of $\gamma\delta$ T cells which exhibit an immature Th2 skewed phenotype and accumulate in the secondary lymphoid organs (Nunez-Cruz et al., 2003). B cell receptor (BCR) signalling molecules Blk and Syk have also been implicated in $\gamma\delta$ TCR signalling (Laird et al., 2010; Muro et al., 2018; Mallick-Wood et al., 1996). Blk deficiency was shown to compromise the functional development of IL-17-producing $\gamma\delta$ T cells in the thymus and lymph nodes (Laird et al., 2010) while early studies in chimeric mice with *Syk*^{-/-} lymphocytes demonstrated an impaired development of DETC and $\gamma\delta$ IELs (Mallick-Wood et al., 1996).

Murine $\gamma\delta$ T cells develop with pre-programmed effector functions and cells utilising different TCR γ chains exhibit preferential cytokine productions (discussed below). For example, DETC and intestinal V γ 7⁺ IELs are IFN γ producers while V γ 6⁺ and some V γ 4⁺ cells secrete IL-17 (Prinz et al., 2013). Furthermore, the TCR signalling strength has been proposed as the driving force of IFN γ vs IL-17 cell-fate decision (Jensen et al., 2008; Turchinovich and Hayday, 2011; Sumaria et al., 2017; Zarin et al., 2015). Thus, the complex phenotypes emerging from knockout studies may reflect different signalling pathways utilized by the two types of $\gamma\delta$ lymphocytes. Indeed, recently it was proposed that Syk-PI3K-Akt signalling pathway was dominant in $\gamma\delta$ cells adopting an IL-17 fate while Zap70/Syk-LAT-Erk signalling was favoured by IFN γ -producing cells (Muro et al., 2018).

A patient with a structurally destabilizing mutation in the kinase domain of Lck was reported to have dramatically expanded absolute numbers of blood $\gamma\delta$ lymphocytes (Hauck et al., 2012). The blood $\gamma\delta$ compartment of this person consisted of approximately 50% V δ 1⁺ and 50% V δ 1⁻V δ 2⁻ cells rather than a prevailing V δ 2⁺ lymphocyte population characteristic of healthy individuals. Immunoscope analysis demonstrated the presence of a dominant V γ 5V δ 1 clone (Hauck et al., 2012). A similar expansion of $\gamma\delta$ lymphocytes was observed in two patients with a loss-of-function mutation in LAT, which resulted in the truncation of the protein and the loss of all the phosphorylation sites associate with signalling (Keller et al., 2016). The phenotyping of one of those patients revealed that 25% of the $\gamma\delta$ TCR⁺ cells were also V δ 1⁺ and 74% were V δ 1⁻V δ 2⁻. Finally, a Zap70-deficient patient was reported to have normal percentage blood $\gamma\delta$ T cell of total CD3⁺ lymphocytes but with a reduction in *TRGV9* and *TRGV2* usage as determined by immunoscope analysis (Hauck et al., 2015). One may hypothesise that the preferential expansion of V δ 2⁻ cells may reflect differential signalling requirements of these cells and ultimately different biology of V γ 9V δ 2⁺ versus V γ 9V δ 2⁻ cells.

2.1.4. Ligand recognition of the $\gamma\delta$ TCR

Antigen presentation to $\gamma\delta$ TCRs is not well understood. The few ligands that have been identified are primarily recognised by small subpopulations of $\gamma\delta$ T cells or individual clones, and are commonly considered to be recognised directly rather than being presented (Hayday and Vantourout 2013; Luoma et al., 2014; Willcox et al., 2012; Marlin et al., 2017). In this regard, antigen recognition by the $\gamma\delta$ TCR may resemble more that of an Ig rather than that of an $\alpha\beta$ TCR. This is further supported by the analysis of the $\gamma\delta$ CDR3 loops (Rock et al., 1994).

CDR3 δ is on average the longest receptor loop among TCRs and antibodies, with a mean length of 18 amino acids. It is closely followed by immunoglobulin heavy chain (IgH) with mean CDR3 length of 16 amino acids. The CDR3 loops of $\alpha\beta$ TCRs appear to be very similar – on average 13 amino acids. Finally, CDR3 γ and immunoglobulin light chain (IgL) have a mean of 11 and 10 amino acids, respectively. Furthermore, the IgH and TCR δ chains were found to have the most variable CDR3 lengths, whereas their respective paired chains were more restricted (Rock et al., 1994). The contrast between $\alpha\beta$ TCR and antibody CDR3 loops could be explained by the nature of their antigen recognition. The long and diverse distribution of CDR3 length in IgH was suggested to be the result of direct, unprocessed antigen recognition. On the other hand, $\alpha\beta$ TCR loops were constrained by the requirement for MHC binding (Rock et al., 1994). Thus, the principal antigen binding site in $\gamma\delta$ TCRs is reminiscent of that in antibodies. It may suggest that the $\gamma\delta$ TCR recognises whole proteins rather than antigens on a presenting molecule.

2.1.4.1. TCR specificities of non-V γ 9V δ 2 cells

CD1 molecules have been reported by several groups to engage V δ 1⁺ $\gamma\delta$ TCRs and crystallography data has provided insight into the binding mechanism (Luoma et al., 2014; Hayday and Vantourout, 2013). They are MHC-I-related proteins which present lipids on the cell surface and are required for the development of CD1-restricted $\alpha\beta$ T cells such as type I invariant NKT cells (iNKT) and type II diverse NKT cells. There are five CD1 genes in humans – *CD1A*, *CD1B*, *CD1C*, *CD1D* and *CD1E*, of which only *Cd1d* is conserved in mice. CD1a-c are predominantly expressed on antigen-presenting cells, whereas CD1d has a more general expression. CD1e is required intracellularly for the processing and editing of lipids (Van Kaer et al., 2016).

There are reports for human $\gamma\delta$ clones binding to all four surface CD1 molecules (summarised in Hayday and Vantourout, 2013). Of note, Russano et al. (2007) demonstrated that CD1 reactivity was far more common in intestinal $\gamma\delta$ (8 out of 12) than among $\alpha\beta$ (3 out of 76) T cell clones. Bai et al. (2012) stained the peripheral blood of healthy donors with CD1d tetramers loaded with

sulfatide, a self-lipid abundant in the brain. Less than one per cent of circulating V δ 1⁺ cells were positive and they represented clonal populations. Two clones were investigated further – a V γ 4V δ 1 clone, DP10.7; and a V γ 5V δ 1 clone, AB18.1. Interestingly, clone AB18.1 comparably bound unloaded CD1d and CD1d loaded with the microbial glycolipid α -galactosylceramide (α GalCer). In contrast, DP10.7 cells were stained exclusively by CD1d-sulfatide tetramers. Binding of the TCRs of these clones was further confirmed by surface plasmon resonance (SPR) and a single-chain version of the DP10.7 clone TCR was co-crystallised with a CD1d-sulfatide molecule (Luoma et al., 2013). The DP10.7 TCR – CD1d-sulfatide interaction was exclusively δ chain mediated resulting in a tilted docking mode (Fig.1.7). CDR1 δ , CDR2 δ and CDR3 δ all contributed to CD1d binding whereas CDR3 δ also contacted the cargo. This docking mode was highly reminiscent of an earlier report of a murine $\gamma\delta$ TCR clone G8 bound to its MHCIb ligand, T22 (Fig.1.7) (Adams et al., 2005). T22 does not have a human homologue and ~0.09-0.6% of murine $\gamma\delta$ IEL and spleen lymphocytes are T22 reactive and share a CDR3 δ sequence motif which is required for antigen binding (Adams et al., 2005; Shin et al., 2005). Nevertheless, the two crystal structures suggested of a conserved mechanism of antigen recognition by the $\gamma\delta$ TCR.

In another study, a proportion of blood-derived V δ 1⁺ CD1d- α GalCer-reactive lymphocytes were also found to recognise unloaded CD1d as well as CD1d loaded with various other lipids (Uldrich et al., 2013). A V γ 5V δ 1 TCR was cloned (9C2) and co-crystalized with CD1d- α GalCer. CD1d contact was dominated by CDR1 δ and to some degree by CDR3 δ , while CDR3 γ was binding the lipid (Fig.1.7).

The crystal structures of the DP10.7 TCR and the 9C2 TCR in complex with CD1d reveal very different modes of interaction. In contrast to the iNKT TCR which was shown to adopt a more parallel conformation during CD1d- α GalCer recognition, the two $\gamma\delta$ TCRs were positioned orthogonally relative to the CD1d lipid pocket (Fig.1.7) (Luoma et al., 2014). Moreover, the two $\gamma\delta$ TCR structures contained non-overlapping contact residues. Only three germline residues of the V γ 5 chain were involved in CD1 binding by 9C2. Two of them are conserved in V γ 4. Therefore, it is likely that the difference in $\gamma\delta$ CDR3 lengths was a major contributing factor to the observed discrepancies. Nevertheless, both studies demonstrated that $\gamma\delta$ T cells are seemingly autoreactive against CD1d and the nature of the cargo only modified the binding affinity. Interestingly, such CD1-centric mechanism of binding has also been described for NKT TCRs recognising CD1a and CD1c (de Jong et al., 2014; Birkinshaw et al., 2015; Wun et al., 2018; Cotton et al., 2018) suggesting it may be a common phenomenon across autoreactive, unconventional T cells.

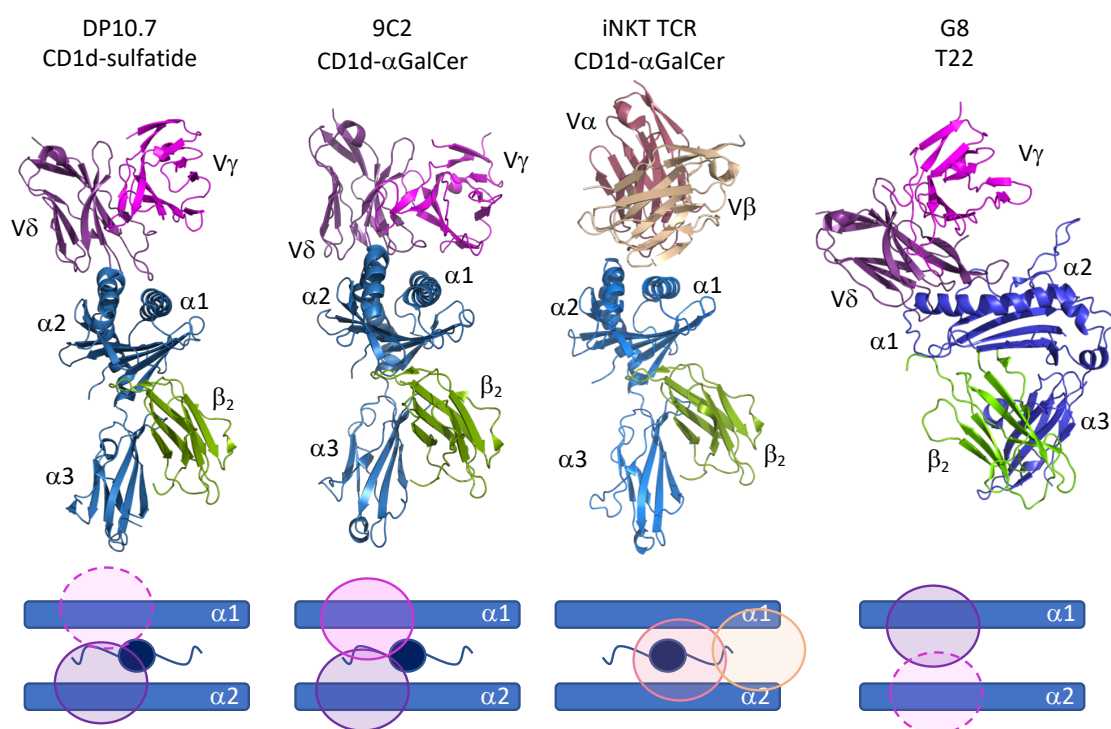


Figure 1.7. Comparisons of $\gamma\delta$ and invariant NKT (iNKT) TCR docking modes. The crystal structures of the respective TCRs bound to their ligand were downloaded from the PDB database and displayed in PyMOL. From left to right: side view of the DP10.7 clone bound to CD1d-sulphatide (PDB code: 4MNG); side view of the 9C2 clone bound to CD1d- α GalCer (PDB code: 4LHU); side view of an iNKT TCR bound to CD1d- α GalCer (PDB code: 2PO6); frontal view of the murine clone G8 bound to T22 (PDB code: 1YPZ). Only the variable domains of the TCRs are shown. Bottom schematics represent the respective TCRs docking onto the CD1d/T22 surface. View is looking down upon the CD1d/T22 surface. Each TCR chain is depicted as a circle. Dotted line indicates TCR chain does not contact CD1d-lipid/T22. TCR chain colours are the same as in the crystal structures. CD1d lipid ligands are also depicted in the schematics. *Adapted from Luoma et al., 2014.*

Recently, another MHC class I-like molecule, MR1, was also shown to be recognised by less than 1% of human peripheral $\gamma\delta$ lymphocytes (Le Nours et al., 2019). MR1 is known to present vitamin B metabolites to MAIT cells. However, Le Nours et al. (2019) isolated multiple $\gamma\delta$ clones, carrying diverse $V\gamma$ and $V\delta$ chains, which stained with MR1 tetramers loaded with 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU). A $V\gamma 9V\delta 1$ TCR (clone G7) was co-crystallized in complex with MR1. Surprisingly, the binding mode revealed a very unusual docking orientation. While the MAIT TCR normally docks above the MR1 antigen binding cleft, the $\gamma\delta$ TCR recognised the $\alpha 3$ domain and thus bound the underside of the protein. Moreover, the

interaction was mediated primarily by the V δ 1 chain, thus evoking parallels with DP10.7 TCR – CD1d-sulphatide and G8 – T22 binding mechanisms (Le Nours et al., 2019).

Another crystal structure was resolved of a human V γ 8V δ 1 TCR (clone 5F3) bound to HLA-A2 presenting the melanoma-associated antigen MART-1 (Benveniste et al., 2018). In this instance, the TCR docked in an orientation similar to that of classical $\alpha\beta$ TCR binding to MHC-peptide (MHCp). The CDR1 $\gamma\delta$ and CDR2 $\gamma\delta$ loops were positioned over the two chains of the MHC molecule while CDR3 $\gamma\delta$ were centrally positioned over the peptide. Only CDR3 δ and CDR1 γ made contacts with the peptide. Overall, the $\gamma\delta$ TCR was less peptide-centric than control HLA-A2/MART1-specific $\alpha\beta$ TCRs. Importantly, alanine scanning of the HLA-A1 molecule and TCR footprinting analysis suggested that another V γ 8V δ 1 TCR (clone 3C2) used a different docking strategy to recognise the same MHCp complex (Benveniste et al., 2018). It is unclear what is the physiological relevance of this ligand recognition as the $\gamma\delta$ clones were generated *in vitro* after a long culture period and continual MART1 antigen stimulation.

MICA (MHC Class I Polypeptide-Related Sequence A), EPCR (Endothelial protein C receptor) and Annexin A2 are stress and infection-regulated antigens recognised by $\gamma\delta$ T cell clones (Xu et al., 2011; Willcox et al., 2012; Marlin et al., 2017).

MICA is a highly polymorphic, stress-induced ligand for the NKG2D receptor expressed on NK, $\gamma\delta$ T and CD8 $\alpha\beta$ T cells. The interaction triggers cytolytic granule release which leads to targeted cell killing of transformed or infected cells (Frazao et al., 2019). Early studies used soluble MICA tetramers to stain NKG2D⁺ $\gamma\delta$ TCR-transduced cell lines to demonstrate direct interaction (Wu et al., 2002). Subsequently, SPR data determined the binding affinity of MICA for a V γ 4V δ 1 TCR to be weaker (110-900 μ M) than that for the NKG2D receptor (0.3-21 μ M) (Xu et al., 2011). Moreover, the same MICA-reactive $\gamma\delta$ clone was subsequently demonstrated to bind CD1d-sulfatide with much higher affinity (33.9 μ M) in biolayer interferometry experiments (Luoma et al., 2013). Therefore, the interaction of MICA with the $\gamma\delta$ TCR may not be the main route of recognising dysregulated cells (Xu et al., 2011).

EPCR, structurally similar to CD1 molecules, is involved in the coagulation pathway, although more recently it was found to be overexpressed in epithelial cancers (Lal et al., 2017). A single V γ 4V δ 5 (LES) clone represented 25% of all T cells in the blood of a CMV-infected transplant recipient (Lafarge et al., 2005). It was shown to recognise certain tumour cells and CMV-infected endothelial cells. The LES TCR was found to directly recognise a conformational antigenic epitope on EPCR in a CDR3 γ -dependent, lipid- and glycosylation-independent manner (Willcox

et al., 2012). The affinity of the interaction was determined to be $\sim 90 \mu\text{M}$ by SPR, a value which fits in the low-affinity end of the range of K_d values reported for $\alpha\beta\text{TCR-pMHC}$ binding ($\sim 1\text{--}100 \mu\text{M}$). However, not every EPCR-expressing cell line was able to activate JRT3 cells transduced with the LES TCR, indicating that additional factors are required (Willcox et al., 2012). CMV infection of some of these cells induced the expression of co-stimulatory molecules such as CD54 and CD58 which aided in the recognition by the LES clone (Willcox et al., 2012).

Intracellular annexin A2 has pleiotropic roles including a role in exocytosis, endocytosis and membrane trafficking. Under stress conditions, it can be translocated to the cell membrane and is commonly overexpressed in many cancer cell lines (Wang and Lin, 2014). A $V\gamma 8V\delta 3$ clone (73R9) was recently validated via SPR to bind annexin A2 with a calculated K_d value of $3 \mu\text{M}$ (Marlin et al., 2017). A small proportion of polyclonal blood $V\delta 2^+ \gamma\delta$ T cells were also shown to proliferate in response to this protein and sequencing revealed diverse $\gamma\delta$ chain usage. Of these, a $V\gamma 4/8/9V\delta 3^+$ clone 24.2 specifically reacted to soluble annexin A2. In contrast, a $V\gamma 4V\delta 1^+$ clone 33.2 reacted to both soluble annexin A2 and A6, suggesting it recognised a conserved sequence between the two proteins (Marlin et al., 2017). The low frequency of annexin A2-reactive $\gamma\delta$ T cells implies an adaptive-like mechanism of recognition, requiring clonal expansion in specific situations (Marlin et al., 2017).

Other reports of $\gamma\delta\text{TCR}$ ligands include the human histidyl-tRNA synthetase recognised by a $V\gamma 3V\delta 2$ M88 clone isolated from polymyositis lesion, although direct biochemical evidence is lacking (Wiendl et al., 2002; Dornmair et al., 2004; Bruder et al., 2012). The TCR was cloned and expressed in a mouse T cell $\alpha\beta\text{TCR}^-$ hybridoma BW58 (Wiendl et al., 2002). The extracts from lysed but not intact myoblasts and the human rhabdomyosarcoma cell line TE671 were able to induce IL-2 production by the M88 transfectants and not by $V\alpha 22V\beta 9$ transfectants (Wiendl et al., 2002). Therefore, the recognised antigen was not cell-surface expressed. Moreover, mutagenesis of the $\gamma\delta\text{CDR3}$ loops indicated these regions were required for a response in the stimulation assay (Wiendl et al., 2002; Bruder et al., 2012). Subsequent studies discovered that cytosolic extracts from *Escherichia coli* also stimulated the $V\gamma 3V\delta 2^+$ hybridoma and identified several potential binding partners such as lysyl- and asparagine-tRNA synthetase, and the *E. coli* translation initiation factor 1 (EclF1) (Bruder et al., 2012). Human recombinant histidyl-tRNA synthetase was shown to stimulate IL-2 production by the M88 transfected cell line and the effects were completely blocked by a polyclonal anti-histidyl-tRNA synthetase antibody (Bruder et al., 2012). Thus, the M88 TCR was able to recognise multiple functionally and structurally unrelated proteins from evolutionary diverse species.

A small proportion of human, murine and bovine $\gamma\delta$ T cells recognise phycoerythrin (PE), a fluorescent protein from cyanobacteria and red algae (Zeng et al., 2012). TCR-deficient Jurkat cell lines were transduced with PE-reactive human $\gamma\delta$ TCRs and stained with PE to confirm binding (Zeng et al., 2012). In addition, SPR confirmed direct recognition by murine soluble PE-specific MA2 TCR clone and a K_d affinity constant of $\sim 2.7 \mu\text{M}$ was calculated (Zeng et al., 2012).

2.1.4.2. TCR specificities of V γ 9V δ 2 cells

Response to low molecular mass phosphoantigens by human V γ 9V δ 2 T cells has been widely established (Constant et al., 1994; Morita et al., 1995; Morita et al., 2007). Phosphoantigens are naturally occurring low-molecular-mass alkyl phosphates. The most potent is hydroxymethyl-but-2-enylpyrophosphate (HMBPP), an intermediate of the non-mevalonate DOXP pathway found in most bacteria and some protozoa (Fig.1.8) (Morita et al., 2007). Infection with such microbes is associated with expansion and activation of V γ 9V δ 2 peripheral T cells (Abo et al., 1990; Kabelitz et al., 1991; Behr and Dubois, 1992; Roussilhon et al., 1994; Guo et al., 1995). V γ 9V δ 2 lymphocytes are also activated by the self-phosphoantigen isopentenyl pyrophosphate (IPP) which is an intermediate of the mevalonate pathway conserved in prokaryotes and eukaryotes (Fig.1.8). Aminobisphosphonate drugs are commonly used in osteoporosis and metastatic bone disease. They inhibit farnesyl diphosphate (FPP) of the mevalonate pathway which results in the accumulation of intracellular IPP. In cancer settings, overproduction of IPP can sensitize tumour cells to V γ 9V δ 2 cytotoxicity (Morita et al., 2007).

A direct interaction between phosphoantigens and the TCR has not been proven, although TCR transfer and mutagenesis studies have established the requirement for the V γ 9V δ 2 TCR (Bukowski et al., 1995; Wang et al., 2010). Alanine scanning has determined that recognition is critically dependent on a V γ 9-J γ P chain with limited CDR3 γ sequence diversity and length (Wang et al., 2010). Mutations of a lysine residue at position 108 in the J γ P region and several mutations in CDR1 γ , CDR2 γ and CDR2 δ were shown to be detrimental to phosphoantigen reactivity. In contrast, the CDR3 δ region of reactive clones preferentially had a hydrophobic residue at position 97 but exhibited variable amino acid sequences and length distributions (Wang et al., 2010). Cell-to-cell contact is also necessary (Morita et al., 1995). The wide area of germline-encoded elements of the V γ 9V δ 2 TCR involved in phosphoantigen reactivity suggested that an antigen-presenting protein was involved (Wang et al., 2010). Initial studies suggested the ectopic expression of mitochondrial F1-ATPase could act as a presenting molecule (Scotet et al., 2005; Mookerjee-Basu et al., 2010). Subsequently, the activation of V γ 9V δ 2 T cells by phosphoantigens was shown to be critically dependent on butyrophilin 3A1 (BTN3A1) (Harly et

al., 2012) which was proposed to be the phosphoantigen-presenting element (Vavassori et al., 2013).

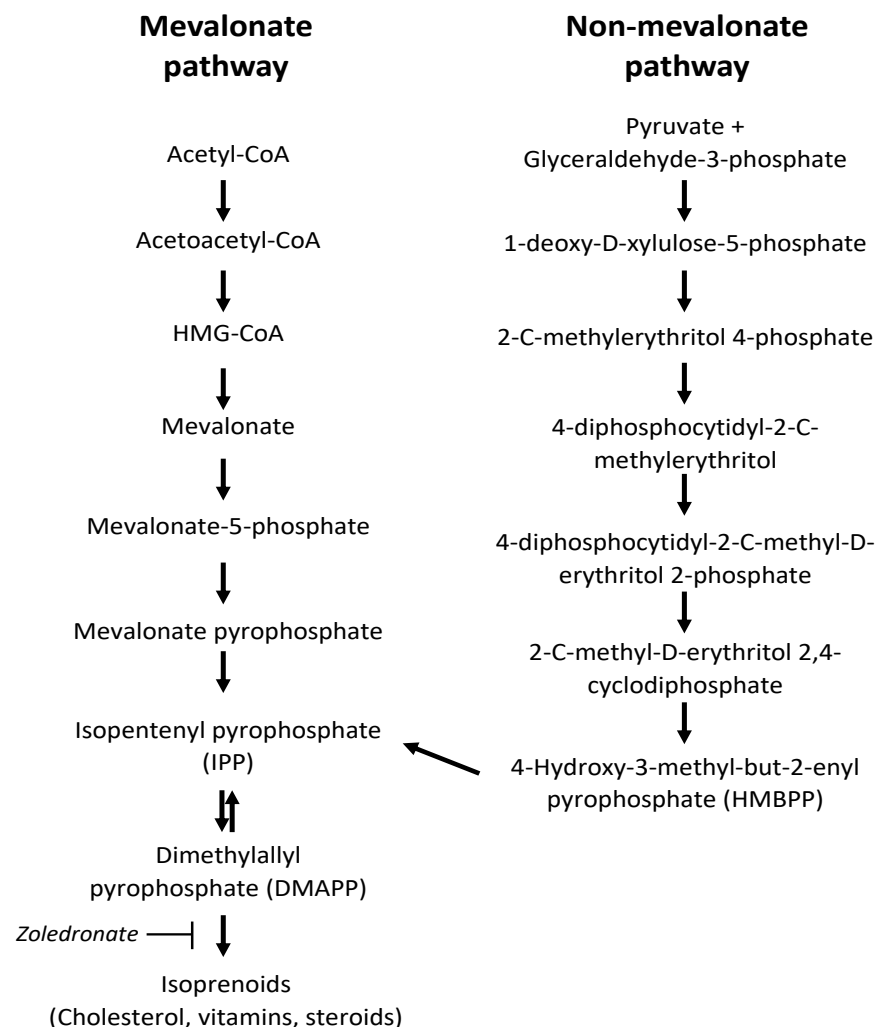


Figure 1.8. Simplified schematic of the mevalonate and non-mevalonate pathways. The mevalonate metabolic pathway is found in eukaryotes and some bacteria and leads to the synthesis of isoprenoids such as cholesterol, vitamin K and steroid hormones. IPP and its isomer DMAPP are used as the building blocks of isoprenoids. The bisphosphonate drug zoledronate, which is frequently used to activate V γ 9V δ 2 cells in cell culture assays, blocks this conversion. The non-mevalonate pathway is an alternative metabolic pathway for the generation of IPP and DMAPP found in most bacteria and protozoa. *Adapted from Meraviglia et al., 2008.*

In summary, V γ 9V δ 2 lymphocytes respond to the presence of low-molecular-mass alkyl phosphates likely presented by a protein. In contrast, the nature of the described non-V γ 9V δ 2 ligands strongly supports the notion that these $\gamma\delta$ TCRs recognise conformational epitopes, rather than linear peptides – an antigen binding mechanism similar to that of antibodies

2.2. $\gamma\delta$ T cell function

TCR $\gamma\delta$ knockout mice display increased tumour load in inducible epithelial cancer models (Girardi et al., 2001; Matsuda et al., 2001), impaired wound healing (Jameson et al., 2002) and enhanced dissemination of enteric bacteria (Dalton et al., 2006; Ismail et al., 2011) demonstrating a role for $\gamma\delta$ T cells in both tissue integrity as well as host protection from invading pathogens.

2.2.1. *Innate and adaptive $\gamma\delta$ T cells*

The textbook model of an inflammatory response during microbial infections comprises of rapid myeloid cell activation (innate immunity) and delayed mobilization of antigen-specific lymphocytes (adaptive immunity) (Fig.1.9A). Conserved pathogen-associated molecular patterns (PAMPs) bind TLRs expressed on myeloid populations. This triggers their effector responses and their subsequent migration to local lymphoid tissues where they present antigens to cognate T lymphocytes (Medzhitov and Janeway, 1998). Consequently, activated lymphocytes clonally expand, differentiate and migrate to affected tissues where they elicit pathogen-specific responses (Medzhitov and Janeway, 1998). In addition, some lymphocytes commit to a memory fate and respond more readily to secondary challenges.

$\gamma\delta$ T lymphocytes possess several characteristics of innate immune cells. Thus, they have been considered key players in the innate lymphoid stress surveillance response (LSSR) which is orchestrated by tissue-resident lymphocytes such as NK, CD8⁺ memory T cells and unconventional lymphocytes, of which $\gamma\delta$ T cells are the prototype (Fig.1.9B) (Hayday, 2009). They are not delayed by clonal expansion and *de novo* differentiation. Instead, they can sense tissue dysregulation directly, via TLRs (Martin et al., 2009), inflammatory cytokine receptors (Sutton et al., 2009) and natural killer receptors (NKR) such as NKG2D (Shafi et al., 2011; Strid et al., 2008).

The NKG2D axis is a model for the LSSR. Molecules such as MICA, MICB, ULBP1-6 in humans, and Rae-1, Mult-1, H60 in mice, become upregulated during tissue stress facilitating their recognition by the NKG2D activating receptors expressed on NK, $\gamma\delta$ T and some CD8 $\alpha\beta$ T cells (Gleimer and Perham, 2003). The recognition induces in the lymphocytes a cytolytic effector mechanism directed against transformed or infected cells (Hayday, 2009). However, cytotoxicity is not the only effector outcome. Illustrating this in the context of the LSSR mode, epidermis-specific upregulation of Rae-1 in mice was sufficient to initiate a rapid, NKG2D-dependent DETC response which promptly induced the activation of local NKG2D⁺ Langerhans cells, and the

recruitment of $\alpha\beta$ T lymphocytes into the epidermis (Strid et al., 2008). This mechanism could be particularly important in the context of tumorigenesis. Indeed, mice selectively deficient for the DETC population were more susceptible to tumour development (Strid et al., 2008). In humans, NKG2D ligation could act in a co-stimulatory manner, by enhancing TCR-mediated activation (Das et al., 2001), as well as to directly activate blood-derived V γ 9V δ 2 cells (Rincon-Orozco et al., 2005; Lanca et al., 2010; Shafi et al., 2011). The significance of this axis is further implied by the many routes of immune-evasion employed by tumours and viruses to suppress NKG2D-mediated lymphocyte activation (Fernandez-Messina et al., 2010; Waldhauer et al., 2008; Wilkinson et al., 2008).

Other functions such as antigen presentation on MHCII and cross-presentation on MHCI have also been described for activated human V γ 9V δ 2 T cells *in vitro* (Brandes et al., 2005; Brandes et al., 2009; Meuter et al., 2010). In addition, Wu et al. (2009) have reported that $\gamma\delta$ lymphocytes are capable of professional phagocytosis.

Collectively, these results highlight that $\gamma\delta$ T cells possess hallmark features of the innate myeloid lineage such as recruitment of immune cells to the site of dysregulation, direct sensing and removal of foreign substances, and antigen presentation.

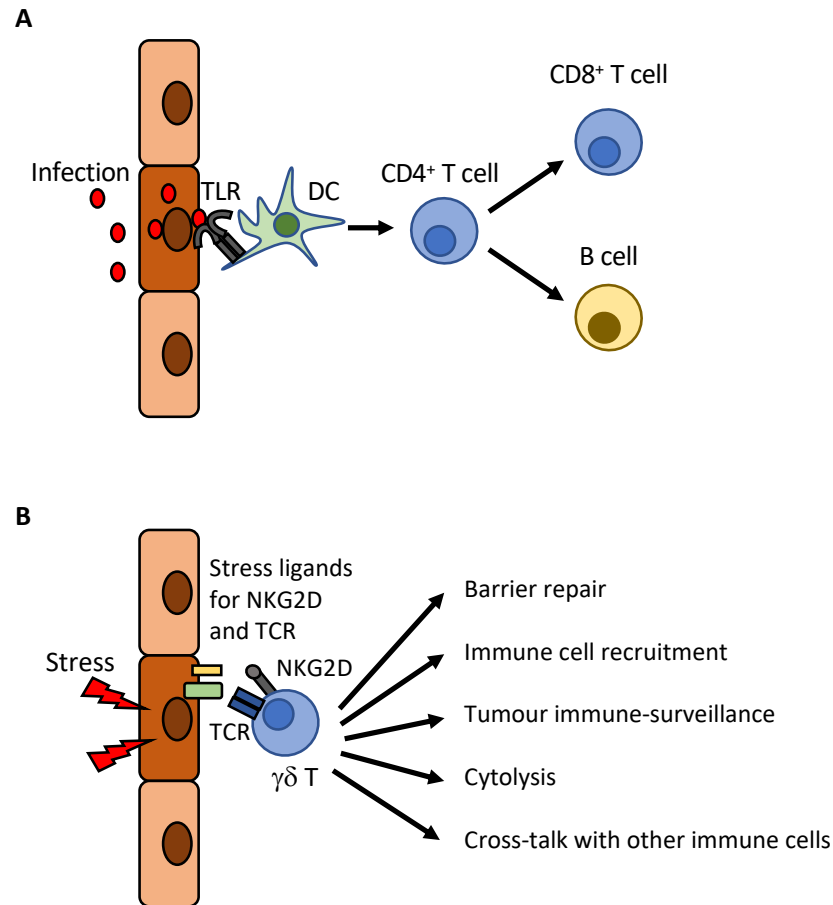


Figure 1.9. Tissue stress surveillance. **A.** Conventional immune responses to infection initiated by myeloid cells. Pathogen-derived molecular patterns bind Toll-like receptors (TLRs) on the surface of myeloid cells. Activated myeloid cells produce effector cytokines and chemokines required for recruitment and activation of additional leukocytes, and migrate to the peripheral tissues where they present antigens to cognate T lymphocytes. Activated T lymphocytes undergo clonal expansion and differentiation into T helper or cytotoxic T lymphocytes. T helper cells can subsequently aid in the activation of antigen-specific B lymphocytes. **B.** The $\gamma\delta$ T cells lymphoid stress surveillance response (LSSR) to tissue dysregulation. Stress ligands for the NKG2D receptor, other natural killer receptors and the TCR become upregulated following non-microbial as well as microbial tissue stress. They engage their cognate receptors on the surface of the lymphocyte. Activation of the LSSR response has been implicated in direct cytolysis, maintenance of barrier integrity, tumour surveillance, leukocyte recruitment and cross-talk with downstream effector immune cells. *Adapted from Hayday, 2009.*

On the other hand, $\gamma\delta$ clonal expansion, the hallmark of an adaptive response, has been documented in humans upon infection (Ravens et al., 2017). Human cytomegalovirus (CMV) infection is generally asymptomatic but can cause major complications in immunocompromised individuals. Thus, it was first observed in kidney transplant patients that CMV contagion induces the proliferation of $V\delta 2^-$ cells (Déchanet et al., 1999). Recently, a longitudinal study of the $\gamma\delta$ TCR repertoire in hematopoietic stem cell transplant (HSCT) patients provided compelling evidence for the clonotypic expansion of $V\delta 2^-$ cells associated with CMV re-activation (Ravens et al., 2017). In healthy individuals, the repertoire was stable over a period of 90 days suggesting that a strong immunological event is required to alter its composition. In HSCT patients, it took approximately 30-60 days for the $\gamma\delta$ compartment to be established, after which it remained stable in the course of 180 days. However, in a subset of patients where re-activation of CMV was triggered, there was a substantial clonal proliferation of a small number of $V\gamma 9^-$ and $V\delta 2^-$ T cells consistent with an adaptive response to the viral infection (Ravens et al., 2017). In addition, Vermijlen et al. (2010) showed that while the $\gamma\delta$ TCR repertoire is generally unfocused and polyclonal at time of delivery, it becomes highly restricted upon congenital CMV infection *in utero*, with a specific expansion of a public $V\gamma 8V\delta 1$ clone. In another study, the blood $V\delta 1$ repertoire in healthy individuals varied in terms of clonality, irrespective of the CMV status (Davey et al., 2017). Interestingly, the unfocused adult $V\delta 1$ compartment was characterised by a $CD27^{hi}CCR7^+CD28^+IL-7R\alpha^+CD62L^+$ naive phenotype, similar to cord blood $V\delta 1^+$ cell. Conversely, individuals with more clonally expanded populations displayed $CD27^{low/-}CCR7^-CD62L^{low}CD28^-IL-7R\alpha^-$ $V\delta 1$ phenotype analogous to clonally restricted CD8 $\alpha\beta$ T effectors (Davey et al., 2017). Thus, the expansion of such effector cells may be suggestive of immunological memory in the $\gamma\delta$ T cell compartment. Such evidence was recently described for murine $\gamma\delta$ T cells as well, which mount an adaptive-like response to infection with CMV (Khairallah et al., 2015; Sell et al., 2015) and malaria (Mamedov et al., 2018).

2.2.2. $\gamma\delta$ IEL and intestinal homeostasis

Based on their preferential location at the barrier between the external environment and the body, it can be hypothesised that $\gamma\delta$ IEL will play key roles in intestinal homeostasis and protection, possibly through both innate and adaptive mechanisms.

Intravital imaging in mice showed $\gamma\delta$ IEL to be highly migratory, rapidly moving in the space between the epithelial layer and the basement membrane *via* occludin and IL-15-dependent

mechanisms (Edelblum et al., 2012; Hoytema van Konijnenburg et al., 2017; Hu et al., 2018). Following infection with *T. gondii* or *S. typhimurium*, $\gamma\delta$ IEL slowed down their migratory speed and prolonged their localization to epithelial cells in close proximity to bacteria (Edelblum et al., 2015). Further studies revealed that a $\gamma\delta$ IEL surveillance-like migratory phenotype was controlled by the commensal microbiota (Hoytema van Konijnenburg et al., 2017). Germ-free and antibiotic-treated animals displayed altered $\gamma\delta$ IEL migratory behaviour characterised by reduced unique basolateral epithelial surface area covered by individual lymphocytes per hour. The migratory pattern of $\gamma\delta$ IELs was found to be dependent on epithelial cells sensing pathogens and the microbiota via Myd88 signalling (Hoytema van Konijnenburg et al., 2017), an adaptor protein used by Toll-like receptors (TLRs) and a key mediator of innate immunity signal transduction (Deguine and Barton, 2014).

Other functions of $\gamma\delta$ IEL demonstrated in mice models include keratinocyte growth factor (KGF) secretion following activation which promotes epithelial cells proliferation and tissue healing (Boismenu and Havran, 1994; Chen et al., 2002), secretion of antimicrobial RegIII γ and lysozyme following mucosal injury by dextran sodium sulfate (DSS) (Ismail et al., 2009), and promotion of oral tolerance (Fujihashi et al., 1999).

Despite the numerous studies in mice, relatively little is known about $\gamma\delta$ IEL function in humans, in part due to limited tissue access. Nevertheless, analysis of samples from patients with pathological conditions such as IBD and coeliac disease provide insight into the biology of human $\gamma\delta$ T cells. Of note, a regulatory function has been described for $\gamma\delta$ IELs in patients with coeliac disease (Bhagat et al., 2008). Coeliac disease is a gastrointestinal inflammatory disorder, triggered by the exposure to gluten. It is characterised by polyclonal expansion of IEL populations, of which $\gamma\delta$ IEL frequencies remain high even after resolution of symptoms and the retraction of pro-inflammatory $\alpha\beta$ T cells following a gluten-free diet (GFD) (Jabri and Sollid, 2009). Bhagat et al. (2007) demonstrated through a series of *in vitro* experiments that CD8⁺NKG2A⁺ $\gamma\delta$ IEL were capable of inhibiting the cytotoxic potential of $\alpha\beta$ T cells in a TGF β -dependent mechanism. However, NKG2A expression appears to be lost in active disease and NKG2A⁻ $\gamma\delta$ IEL were less able to regulate the immune response by $\alpha\beta$ lymphocytes. In a more recent study, $\gamma\delta$ IEL from active disease were shown to acquire expression of IFN γ , consistent with a Th1 phenotype (Mayassi et al., 2019). Furthermore, the same study characterised V δ 1⁺ cells in the duodenum of control patients to be innate-like and potentially cytotoxic. They expressed the NK receptors NKp44 and NKp46, and no TNF α or IFN γ at steady state, but readily produced granzyme B. Transcriptional analysis of these cells showed upregulated expression of

genes involved in tissue repair in steady-state *versus* active disease. Thus, similar to their murine counterparts, human $\gamma\delta$ IEL appear to be ideally positioned to actively contribute to tissue homeostasis.

2.3. $\gamma\delta$ T cell ontogeny

T cells develop largely in the thymus from a common hematopoietic progenitor for both $\alpha\beta$ and $\gamma\delta$ lymphocytes (Fig.1.10) (Petrie et al., 1992; Dudley et al., 1995; Rothenberg et al., 2008; Joachims et al., 2006;). T cell progenitors infiltrate the thymus at the cortico-medullary junction as TCR⁻, CD4⁻CD8⁻ double-negative (DN) cells and undergo several stages of differentiation (DN1-4) according to their surface expression of CD25 and CD44 in mice, and CD34, CD38 and CD1a in humans (DN1: CD25⁻CD44⁺/ CD34⁺CD38⁻CD1a⁻ → DN2: CD25⁺CD44⁺/ CD34⁺CD38⁺CD1a⁻ → DN3: CD25⁺CD44⁻/ CD34⁺CD38⁺CD1a⁺ → DN4: CD25⁻CD44⁻/ CD34⁻CD38⁻CD1a⁻) (Rothenberg et al., 2008; Joachims et al., 2006). Thymocytes subsequently enter the immature single positive (ISP) CD4⁺ stage, the double-positive (DP) CD4⁺CD8⁺ stage and, finally, reach the mature single-positive (SP) stage expressing an $\alpha\beta$ TCR and either CD4 or CD8 co-receptors (Rothenberg et al., 2008). Lineage commitment to $\alpha\beta$ or $\gamma\delta$ cell fate begins in DN3 thymocytes but the exact mechanisms underlying it are not understood. The process of β selection, whereby the thymocytes commit to an $\alpha\beta$ cell fate, occurs after a productive TCR β has been rearranged and paired with pre-T α to form the pre-TCR. Pre-TCR signalling promotes proliferation, survival and maturation from DN4 towards DP cells (Gascoigne et al., 2016). At the DP stage, TCR α rearrangement is completed and with that the full commitment to an $\alpha\beta$ cell fate as the TCR δ genes are irreversibly deleted from the chromosome. The newly formed TCRs are tested for their ability to recognise self-MHC on cortical thymic epithelial cells (cTEC). After TCRs have been positively selected to recognise self-MHCI or self-MHCII, they become SP CD8 or CD4 T cells, respectively. Negative selection occurs in the thymic medulla where SP cells that strongly recognise self-antigens presented by medullary DCs are deleted (Gascoigne et al., 2016).

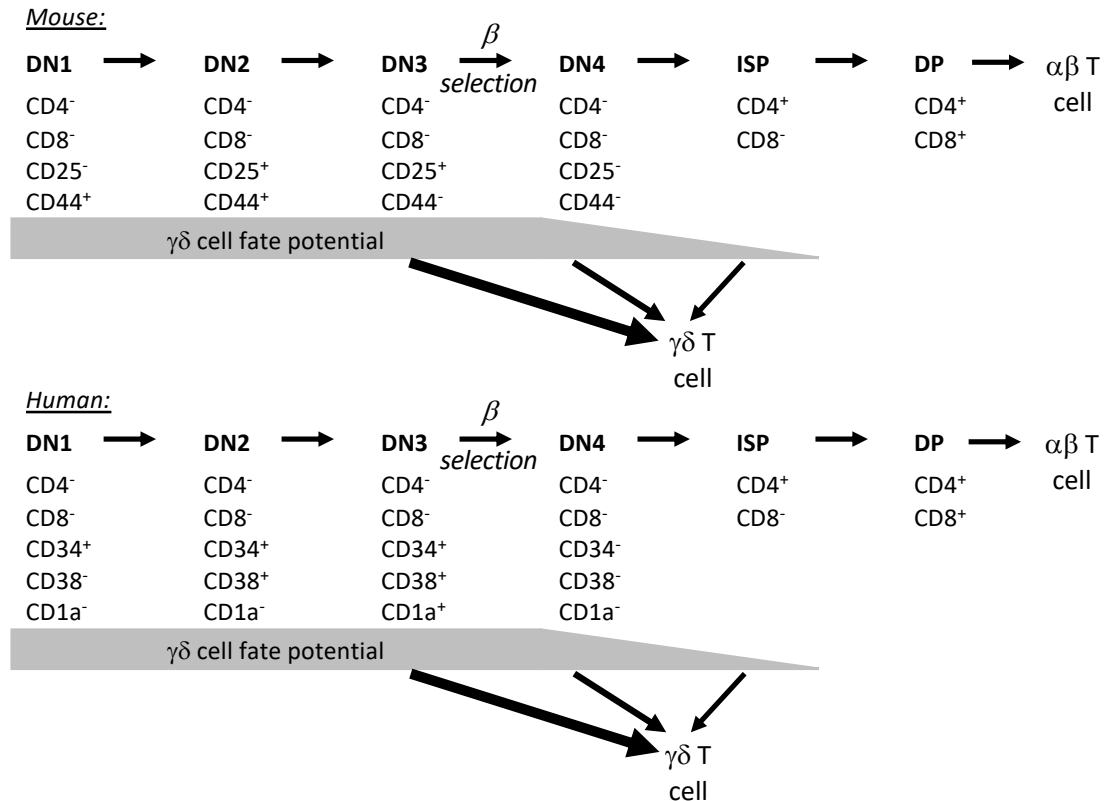


Figure 1.10. Developmental stages of T cell progenitors. $\alpha\beta$ and $\gamma\delta$ T cells develop from a common lymphoid progenitor. They undergo several stages of differentiation as CD4⁻CD8⁻ double-negative (DN) cells expressing different combination of the indicated surface markers (CD25 and CD44 in mice; CD34, CD38 and CD1a in humans). Following β selection and the full commitment to an $\alpha\beta$ T cell, lymphocytes become immature single-positive (ISP) CD4⁺ cells and subsequently become double-positive (DP). Around the time of the DN3-DN4 stages, if a successful $\gamma\delta$ TCR has been expressed, the cells can commit to a $\gamma\delta$ cell fate. Adapted from Joachims et al., 2006.

It is not clear at what point of this developmental process does $\gamma\delta$ lineage commitment occurs. As a result, three models have been developed based on mouse studies. The pre-commitment model suggests that lineage fate is determined prior to TCR expression, and TCR signalling only reinforces the previously established fate decision. Evidence for this comes from transgenic mice for TCR β , TCR γ and TCR δ (Gerber et al., 2004). The thymocytes of triple-transgenic mice generated a $\gamma\delta$ compartment comparable to that of double-transgenic for TCR γ and TCR δ whereas the $\alpha\beta$ compartment was comparable to that of TCR β transgenic mice (Gerber et al., 2004). The instructional model proposes that lineage decision is determined by the successful rearrangement of an $\alpha\beta$ or $\gamma\delta$ TCR. Support for this hypothesis comes from the study of TCR γ and TCR δ rearrangements in the $\alpha\beta$ lymphocytes and their progenitors of wild-type and TCR δ

knockout mice (Dudley et al., 1995). These cells were shown to be depleted of productively rearranged TCR γ and TCR δ chains in normal mice suggesting that the successful rearrangement of an $\alpha\beta$ TCR drove their development (Dudley et al., 1995). In TCR $\delta^{-/-}$ mice, the δ chain locus can still be productively rearranged but does not lead to the translation of a functional protein due to a deletion within the δ constant region. In these animals, $\alpha\beta$ lymphocytes contained productive rearrangements for the TCR δ chain, consistent with the hypothesis that the failed generation of a $\gamma\delta$ TCR would lead to an $\alpha\beta$ fate decision (Dudley et al., 1995). Finally, the signal strength model suggests that strong TCR signal in DN cells drives $\gamma\delta$ lineage commitment and weak signalling by the pre-TCR drives cells towards an $\alpha\beta$ fate. Evidence for this comes from transgenic $\gamma\delta$ TCR thymocytes where ligand availability or downstream signalling capacity were manipulated (Hayes et al., 2005; Haks et al., 2005; Lee et al., 2014).

The TCR chain loci in human thymocytes were found to rearrange in an established order – TCR δ recombines first, followed by TCR γ and TCR β , with TCR α rearranging last (Dik et al., 2005). TCR sequencing of human thymocytes showed that TCR γ chain was rearranged on both alleles in nearly all cells, irrespective of lineage choice, whereas TCR β remained largely in germline configuration in $\gamma\delta$ cells and the few TCR β rearrangements were primarily out-of-frame (Joachims et al., 2006; Sherwood et al., 2011). Approximately 30% of the rearranged TCR γ chain sequences were productive in $\alpha\beta$ lymphocytes but TCR δ chain was predominantly out-of-frame. Thus, the $\alpha\beta$ subset rarely harboured both γ and δ productively rearranged sequences (Joachims et al., 2006). These results suggest the majority of $\alpha\beta$ lymphocytes developed from progenitors which failed to produce a functional $\gamma\delta$ TCR. Furthermore, they indicate that cell fate is largely determined prior to TCR β rearrangement but after TCR γ rearrangement which argues against both the signal strength and the pre-commitment models in human $\gamma\delta$ T cell development. Instead, this data is consistent with the instructive model where the rearrangement of a functional $\gamma\delta$ TCR drives fate decision.

2.3.1 Developmental waves in $\gamma\delta$ T cell ontogeny

The murine foetal thymic development of $\gamma\delta$ T cells is organised in waves of progenitors defined by their V γ usage and their residence in a particular anatomical site (Fig.1.11A) (Carding and Egan, 2002). The first wave of progenitors consists of V γ 5V δ 1 cells which develop between embryonic day 13 (E13) until approximately E17 and home to the epidermis (Prinz et al., 2013). The second wave of V γ 6V δ 1 lymphocytes develops from E14 until birth and populate the uterus, lung and tongue. Finally, V γ 1⁺ and V γ 4⁺ cells, mainly found in secondary lymphoid organs and blood, begin to develop around E16 and continue to be produced through adulthood (Prinz et

al., 2013). $V\gamma 7^+$ cells are thought to develop extrathymically around the time of birth (see below). Some murine $\gamma\delta$ lymphocytes acquire their effector functions during thymic development and commit to an IFN γ - or IL-17-producing cell fate (Prinz et al., 2013). These develop prenatally and express (semi)invariant TCRs. Thus, the first developmental wave is associated with the generation of IFN γ -producing monoclonal $V\gamma 5V\delta 1$ DETCs. The second wave leads to the generation of IL-17-secreting monoclonal $V\gamma 6V\delta 1$ lymphocytes. Oligoclonal $V\gamma 1^+$ and $V\gamma 4^+$ cells generated prenatally were also demonstrated to acquire IL-17 effector function (Haas et al., 2012) and were termed “natural” IL-17-producing $\gamma\delta$ lymphocytes (Chien et al., 2013). Cells produced postnatally are functionally naive, with diverse CDR3 repertoires and thus more adaptive-like (Chien et al., 2013; Vermijlen and Prinz, 2014). Early studies suggested that antigen experienced $\gamma\delta$ thymocytes become IFN γ producers and antigen inexperienced cells become IL-17 producers (Jensen et al., 2008; Turchinovich and Hayday, 2011). More recently, it was demonstrated that TCR signal strength determines effector fate decision – strong TCR signalling inhibits the development of IL-17-secreting $\gamma\delta$ thymocytes and promotes their progression along the IFN γ pathway (Munoz-Ruiz et al., 2017; Sumaria et al., 2017).

$\gamma\delta$ TCR gene rearrangements can first be detected in human foetal liver at week 6 of gestation, and by week 8 in human foetal thymus, liver and gut (McVay et al., 1998), and protein expression has been reported by week 9.5 in the foetal thymus (Haynes et al., 1988). At the beginning of the second trimester approximately 75% of the developing peripheral blood $\gamma\delta$ lymphocytes express the canonical blood $V\gamma 9V\delta 2$ TCR pairing (Dimova et al., 2015). Thus $V\gamma 9V\delta 2$ -expressing cells seemingly represent the first wave of $\gamma\delta$ T lymphocytes in the developing human (Fig.1.11B). Indeed, in a recent study $CD34^+$ hematopoietic stem and precursor cells (HSPCs) isolated from human foetal liver predominantly generated $V\delta 2^+$ cells in OPD-L1 *in vitro* cultures and were very inefficient at differentiating into $V\delta 1^+$ lymphocytes. This was in stark contrast to $CD34^+$ HSPCs derived from cord and adult blood which were most efficient at producing $V\delta 1^+$ cells in the same experimental conditions (Tieppo et al., 2020). This is highly reminiscent of the murine system where DETC can only be established in a foetal thymic organ culture system from HSPCs of foetal but not adult origin (Ikuta et al., 1990; Havran and Alisson, 1990).

Interestingly, at birth, the $V\delta 1^+$ lymphocytes, more commonly found in mucosal sites, predominate the blood $\gamma\delta$ compartment (Morita et al., 1994; Dimova et al., 2015) and within the first year of life their absolute counts rapidly decline (De Rosa et al., 2004). While the percentage of thymic $V\delta 1^+$ and $V\delta 2^+$ cell remains stable with time, the proportion of $V\delta 2^+$ $\gamma\delta$ lymphocytes in the blood increases with age (Parker et al., 1990), most likely due to the

combination of expansion driven by antigen encounter and the sequestration of V δ 1⁺ cell in tissues. Recent sequencing analysis of foetal and postnatal V γ 9V δ 2 thymocytes and peripheral blood lymphocytes indicated that adult blood V γ 9V δ 2 were most likely generated from the postnatal thymus rather than representing an expanded and long-lived population of prenatal thymocytes (Papadopoulou et al., 2019). Thus, the adult V γ 9V δ 2 thymocytes and peripheral blood lymphocyte repertoires were fundamentally different from their prenatal counterparts and contained on average more non-germline-encoded additional nucleotides and longer CDR3 γ and CDR3 δ sequences (Papadopoulou et al., 2019).

Unlike CD8 $\alpha\beta$ and V δ 1⁺ lymphocytes, circulating V γ 9V δ 2 T cells become rapidly activated within the first year of life, acquiring CD45RO expression, and readily produce IFN γ and perforin upon *in vitro* stimulation with phorbol myristate acetate (PMA) and ionomycin (De Rosa et al., 2004). The functional competence of these neonatal $\gamma\delta$ T cells is acquired *in utero* unlike that for $\alpha\beta$ T cells which produce very little IFN γ at birth (Gibbons et al., 2009). However, $\gamma\delta$ thymocytes from paediatric donors were found to be immature, produced negligible amounts of IFN γ and required IL-2 or IL-15 to complete their functional differentiation (Ribot et al., 2014). Collectively, these findings suggest that similarly to $\alpha\beta$ T cell, human postnatal $\gamma\delta$ lymphocytes complete their functional maturation in the periphery. On the other hand, recent transcriptional analysis of foetal $\gamma\delta$ thymocytes demonstrated these cells to have programmed IFN γ effector functions which were not present in foetal $\alpha\beta$ and postnatal $\gamma\delta$ and $\alpha\beta$ thymocytes (Tieppo et al., 2019). Thus, parallels may exist with murine system where IFN γ - and IL-17-producing effector functions are acquired in the thymus before birth (Jensen et al., 2008; Turchinovich and Hayday, 2011; Sumaria et al., 2017) in contrast to the subsequent generation of functionally immature $\gamma\delta$ lymphocytes (Chien et al, 2013; Vermijlen and Prinz, 2014).

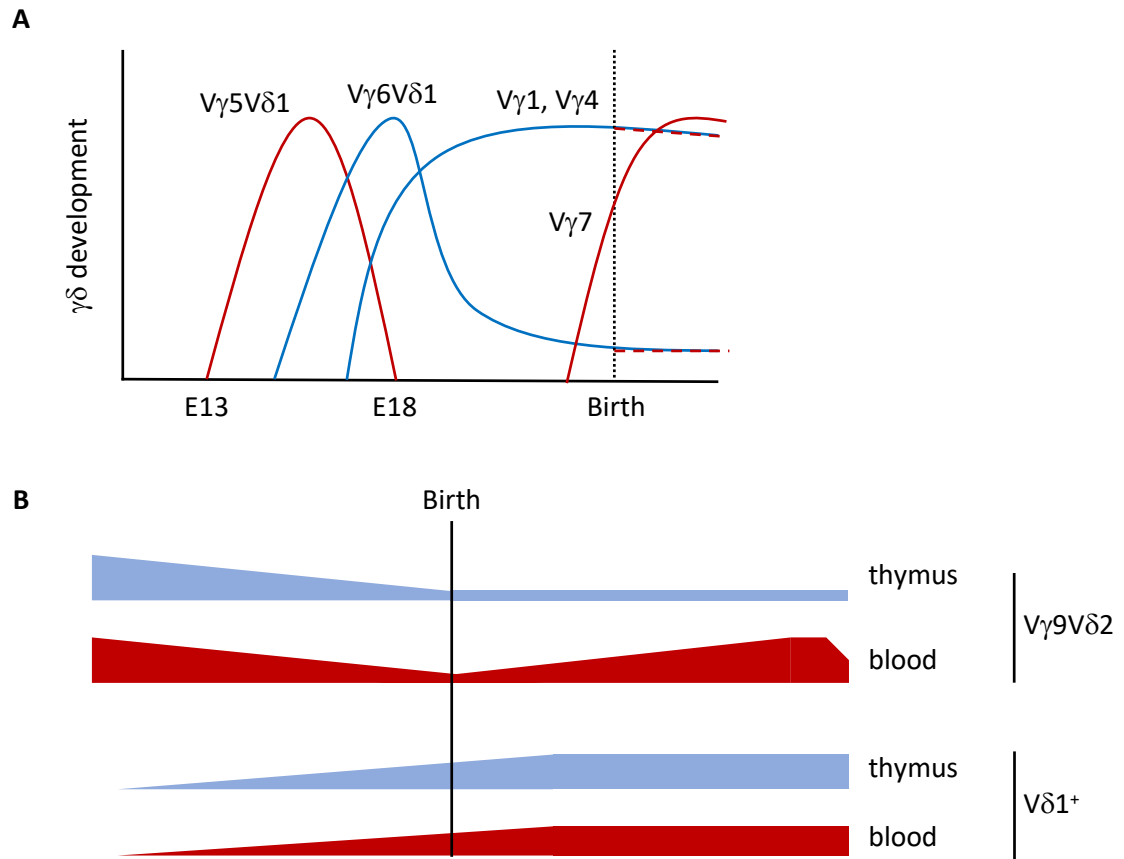


Figure 1.11. Developmental waves of $\gamma\delta$ lymphocytes in mice and humans. **A.** Murine $\gamma\delta$ lymphocytes development. Red lines indicated IFN γ effector function. Blue lines indicate IL-17 effector function. Dashed red and blue lines indicate the development of functionally naive populations expressing the indicated V γ chains. (Adapted from Carding and Egan, 2002). **B.** Human $\gamma\delta$ lymphocytes development. Illustration of thymic production, persistence and export into blood of the indicated subsets. *Adapted from Vermijlen and Prinz, 2014.*

2.3.2. Intestinal $\gamma\delta$ IEL development

The intestinal $\gamma\delta$ IEL compartment in mice develops perinatally and seemingly does not require a thymus. In athymic (*nu/nu*) mice, $\gamma\delta$ T cells develop in nearly normal numbers in contrast to $\alpha\beta$ IEL which are almost completely absent (Bandeira et al., 1991; Di Marco Barros et al., 2016). Furthermore, *nu/nu aly/aly* double-mutant mice, which lack a thymus, all lymph nodes, PPs and ILFs, also successfully develop normal gut $\gamma\delta$ IEL pool suggesting these sites are dispensable for extrathymic development (Nonaka et al., 2005). Instead, intestinal cryptopatches, GALT structures present only in mice and which develop normally in *nu/nu aly/aly* mice, were proposed to be the source of $\gamma\delta$ T cells (Saito et al., 1998).

Nevertheless, the origins of gut IELs remain controversial. GFP expression was not detected in the gut of mice expressing GFP under the control of the *Rag2* promoter. Instead, GFP⁺ cells were only observed outside the thymus after irradiation-induced lymphocyte depletion (Guy-Grand et al., 2003) demonstrating extrathymic development may only occur when thymic output is compromised.

In humans, there are no cryptopatches in the gastrointestinal tract and thus, no parallels can be drawn with the mouse model. However, *RAG1* and *RAG2*, normally expressed during lymphocyte development, have been detected in the gut epithelium (Lynch et al., 1995; Bas et al., 2003).

IELs were first detected in the foetal human intestine at week 11 of gestation. Of these, a negligible proportion were $\gamma\delta$ T cells until week 18 when they underwent dramatic expansion, coming to represent approximately 20% of the IELs (Spencer et al., 1989). The TCR δ repertoire was polyclonal during mid-gestation, increasing in diversity until birth. This diversity was lost in adolescence indicating that there was marked selection in the first years of life (Holtmeier et al., 1997).

2.3.3. Butyrophilins as selecting antigens in $\gamma\delta$ T cell development

It has long been established that $\gamma\delta$ lymphocytes develop normally in MHCII and β_2 -microglobulin-deficient mice (Bigby et al., 1993; Correa et al., 1992). Instead, BTN and butyrophilin-like (BTNL) molecules (discussed in more details below) have recently emerged as regulators of $\gamma\delta$ T cell development.

2.3.3.1. The *Skint* genes and skin $\gamma\delta$ T cells

The dominant population of immune cells in the murine epidermis consists of an essentially monoclonal V γ 5V δ 1 population with dendritic morphology, also known as DETCs (Asarnow et al., 1988). The limited receptor diversity suggested that the TCR was positively selected on a self-agonist and although this was highly disputed (Asarnow et al., 1993), the hypothesis was proven when *Skint1* was identified by our laboratory as a selecting component in the development of DETC (Lewis et al., 2006; Boyden et al., 2008; Barbee et al., 2011; Turchinovich and Hayday 2011). Although Skint-1 does not have a functional human homolog, it belongs to the extended B7 family of co-stimulatory receptors together with the BTN(L) proteins (Boyden et al., 2008).

Skint1 is specifically expressed by medullary thymic epithelium and keratinocytes. Mice harbouring a natural mutation resulting in a premature stop codon in this gene are severely

depleted of V γ 5V δ 1 T cells in the epidermis while other T lymphocyte compartments are unaffected (Lewis et al., 2006; Boyden et al., 2008; Barbee et al., 2011). The transcriptome profiling of embryonic E15 and E16 thymi isolated from wild type and *Skint1*-mutated (FVB.Tac) mice showed that *Skint1* promoted the functional differentiation of embryonic V γ 5V δ 1 T cells away from an IL-17-producing phenotype and towards an IFN γ -producing phenotype. Thus, at E15 V γ 5V δ 1 thymocytes of *Skint1*-mutant FVB.Tac mice display enhanced production of IL-17A and migrate to the uterus and peritoneum where the dominant V γ 6V δ 1 population has an IL-17 phenotype (Turchinovich and Hayday, 2011). Interestingly, the ectopic expression of this gene does not lead to accumulation of DETCs in other tissue compartments (Barbee et al., 2011). Mice have 11 *Skint* genes (Boyden et al., 2008) and the full locus knockout phenotype recapitulates that of *Skint1*^{-/-} mice (Narita et al., 2018). Therefore, it is conceivable that *Skint1* partners with other *Skint* molecules, perhaps in heteromeric interactions, to regulate the skin epidermal $\gamma\delta$ T cell compartment. Indeed, *Skint2*^{-/-} mice phenocopy FVB.Tac mice in that they do not develop the canonical V γ 5V δ 1 DETC population (Anett Jandke, unpublished data). Thus, *Skint1* and *Skint2* do not compensate for each other and may form heteromers in order to regulate the development of epidermal $\gamma\delta$ lymphocytes. Furthermore, *Skint3* and *Skint9* expression levels were upregulated following tissue damage. They were reportedly required for the prompt re-epithelialization as well as appropriate DETC behaviour and recruitment at the edge of the wound (Keyes et al., 2016). Therefore, different combinations of *Skint* proteins may be required to orchestrate the behaviour of DETC in different circumstances.

Direct interaction between *Skint1* and the DETC TCR has not been formally proven. However, Chodaczek et al. (2012) reported that the murine V γ 5V δ 1 TCR engages a self-antigen expressed by keratinocytes at steady state. Their study demonstrated that the majority of DETC dendrites pointing at the apical epidermis contained phosphorylated TCR clusters, the earliest event following TCR engagement. The data argue strongly for the interaction of the $\gamma\delta$ TCR with a physiological ligand present on keratinocytes and the authors suggested *Skint1* as a likely candidate.

Phylogenetic analysis showed that *SKINTL* in hominoids, such as human, chimpanzee, gorilla and orangutan, has multiple inactivating mutations consistent with pseudogenization (Mohamed et al., 2015). In contrast, other old-world monkeys, such as olive baboons, cynomolgus macaques and rhesus macaques, appear to have a functional copy of this gene. Interestingly, *SKINTL* in cynomolgus macaques is specifically expressed in the skin and thymus, and 40% of the epidermal

T cells consist of a semi-invariant V γ 10V δ 1 population which is highly dendritic (Mohamed et al., 2015).

In summary, *Skint* genes encode skin-specific proteins which are required for the regulation of skin-specific $\gamma\delta$ T cell populations.

2.3.3.2. Murine gut V γ 7⁺ IELs and *Btnl1*, *Btnl4* and *Btnl6*

Another evidence for the regulation of tissue-specific $\gamma\delta$ subsets by tissue-specific Btl molecules comes from the development of the murine intestinal $\gamma\delta$ IEL compartment.

The essentially restricted expression of *Btnl1*, *Btnl4* and *Btnl6* in the small and large intestine (Bas et al., 2011) and the establishment of the Btl-related *Skint1* as a DETC selecting element (Lewis et al., 2006; Boyden et al., 2008; Barbee et al., 2011; Turchinovich and Hayday 2011) prompted our laboratory to investigate the role of Btl1/4/6 in regulating intestinal $\gamma\delta$ T cells (Bas et al., 2011; Di Marco Barros et al., 2016). Btl1 was initially shown to inhibit immune cell activation (Yamazaki et al., 2010;). Recently, we demonstrated that it is required for the development and phenotypic maturation of the gut V γ 7⁺ IEL compartment (Di Marco Barros, 2016). Mice lacking *Btnl1* were deficient in V γ 7⁺ IELs but no other T cell types. Furthermore, Btl1 acted extra-thymically as athymic *nu/nu* mice had a relatively normal V γ 7⁺ IEL pool whereas *Btnl1*^{-/-} *nu/nu* double-transgenics were severely depleted in these cells. Bone marrow derived from both wild type and *Btnl1*^{-/-} mice efficiently reconstituted the $\gamma\delta$ IEL compartment of irradiated *TCR δ* ^{-/-} animals suggesting that Btl1 selected in trans. Importantly, irradiated *Btnl1*^{-/-} hosts, in contrast to wild type mice, failed to support the efficient reconstitution and maturation of V γ 7 cells. Induced global *Btnl1* expression did not drive ectopic V γ 7 development in other tissues, akin to the *Skint1* experiments (Di Marco Barros, 2016; Barbee et al., 2011), suggesting that heteromeric Btl complexes may regulate the V γ 7 IEL compartment.

Btnl4^{-/-} mice had no evident perturbations of the IEL compartment, despite *Btnl4* being expressed in the gut from embryonic day E15 when *Btnl1* and *Btnl6* were not detected yet (Di Marco Barros, 2016). Nevertheless, Btl4 and Btl6 enhanced Btl1 surface expression in co-transfection experiments of murine enteric cell line MODE-K (Di Marco Barros, 2016). Btl6 had a poor surface expression on its own and required a heteromeric interaction with Btl1 to reach the cell surface (Lebrero-Fernandez et al., 2016a; Di Marco Barros, 2016). Thus, Btl1 and Btl6 jointly induced TCR-specific responses in intestinal V γ 7⁺ cells, as judged by the down-regulation of the TCR and CD122, and the up-regulation of CD25 in co-cultures with MODE-K cells expressing Btl1+6 (Di Marco Barros, 2016). Furthermore, those experiments were undertaken

with IELs isolated from Nur77.gfp mice in which GFP expression is upregulated by nuclear factor of activated T cells (NFAT) activation downstream of TCR signalling: all IELs, which upregulated CD25 in response to Btnl1+6 became GFP⁺. Notably, no other $\gamma\delta$ T cells or $\alpha\beta$ T cells responded to Btnl1+6, consistent with this biology being highly selective for V γ 7⁺ IELs (Di Marco Barros, 2016).

3. Butyrophilin (BTN) and Butyrophilin-like (BTNL) proteins

BTN(L) molecules are conserved between mouse and human. They are part of the immunoglobulin superfamily and members of the extended B7 family of co-stimulatory molecules (Linsley et al., 1994; Compton et al., 2004). Some of the best characterised B7 proteins include CD80 (B7-1), CD86(B7-2), PD-L1(B7-H1) and PD-L2 (B7-DC). CD80/CD86 provide co-stimulatory signals through CD28 ligation during antigen recognition by T cells and inhibitory signals following T cell activation via ligation to CTLA-4 (Schildberg et al., 2016). PD-L1/2 ligation to PD-1 expressed on T cells inhibits TCR signalling, and is key in regulating T cell activation threshold, T cell tolerance, resolution of inflammation and T cell exhaustion (Schildberg et al., 2016).

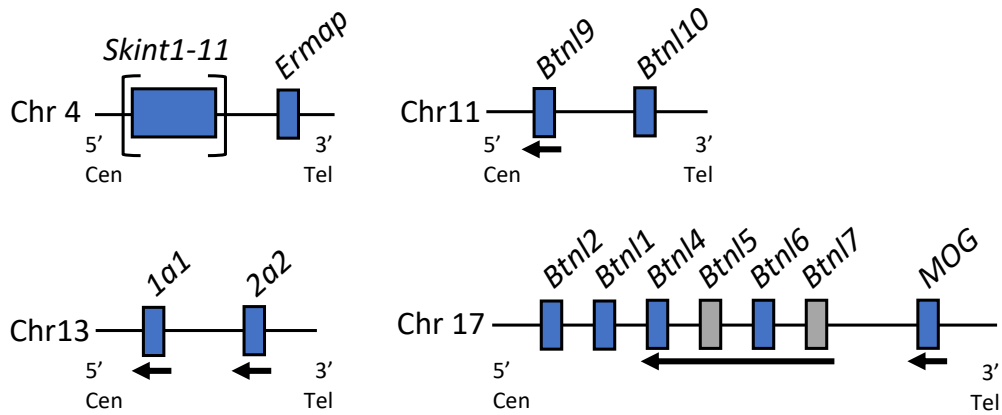
3.1. Structure and gene loci organisation of BTN and BTNL proteins

A comprehensive phylogenetic analysis illustrated that the *BTN(L)* genes evolved by gene duplication, deletion, diversification and pseudogene formation. Some genes, such as *Btn3* in rodents, were lost in certain lineages, while others underwent extensive gene duplication, for instance, the *Skint* genes in mice and *BTN2* and *BTN3* in humans (Afrache et al., 2012). Thus, the BTN(L) family is rather divergent across the mammalian species (Fig.1.12).

Humans have 7 known *BTN* genes – *BTN1A1*, *BTN2A1-3*, *BTN3A1-3*, and 6 known *BTNL* genes - *BTNL2*, *BTNL3*, *BTNL8*, *BTNL9*, *BTNL10* and *SKINTL*. Of these, *BTN2A3* and *SKINTL* are pseudogenes. *Erythroid membrane-associated protein (ERMAP)* and *myelin oligodendrocyte glycoprotein (MOG)* are conserved between mouse and human and belong to the butyrophilin family as well (Arnett and Viney, 2014).

ERMAP, *SKINTL* and *BTNL10* are located on chromosome 1. *BTNL3*, *BTNL8* and *BTNL9* are found in a cluster on chromosome 5. *BTNL2* is situated within the MHCII locus on chromosome 6. *MOG* and all the *BTN* genes are in close proximity to the MHCI locus on chromosome 6 (Fig.1.12) (Afrache et al., 2012).

Mouse:



Human:

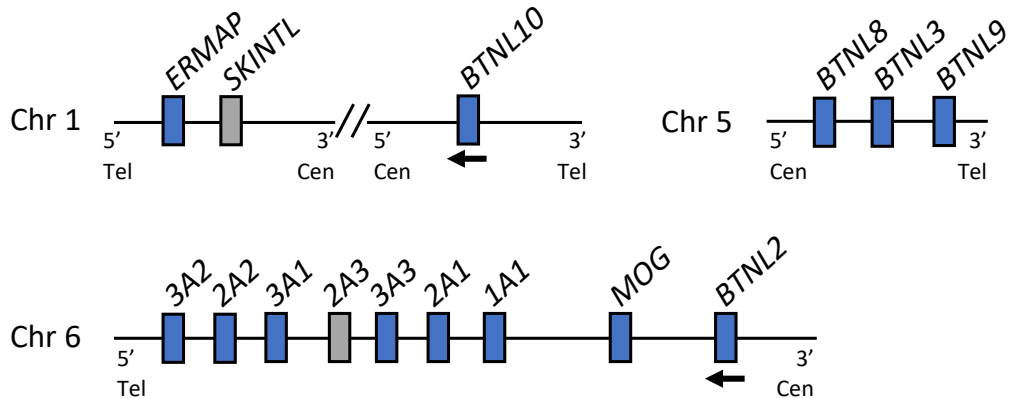


Figure 1.12. Organisation of the Btn(l)/BTNL(l) loci in mouse and human. Blue boxes – functional genes; grey boxes – pseudogenes; blue box with brackets – collection of eleven murine Skint genes; arrows under boxes – indicate genes which are transcribed in the opposite direction to the locus; Tel – telomeric; Cen – centromeric. Distances between genes are not to scale. Human ERMAP and SKINTL are found on chromosome 1p. Human BTNL10 is situated on chromosome 1q.

A copy number variation (CNV) occurs in the human *BTNL3/8/9* locus, which is overrepresented in European and American populations and underrepresented in Oceanic, central Asian and Sub-Saharan African populations (Aigner et al., 2013). The allele frequency in Europeans is 34% while in Asians, Africans and Oceanic populations it is 29%, 9% and 3%, respectively. The CNV removes ~56 kilobase pairs of genomic sequence between intron four of *BTNL8* and intron four of *BTNL3*, while maintaining a functional open reading frame. Thus, the new *BTNL8*3* fusion gene consists of exons one to four of *BTNL8* and exons five to eight of *BTNL3*, and it is successfully transcribed and translated (Fig.1.13) (Aigner et al., 2013; our unpublished data).

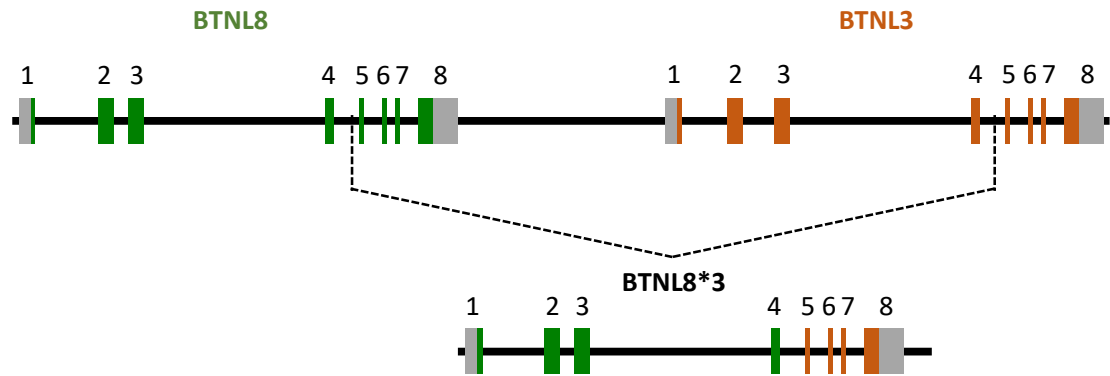


Figure 1.13. Exon organisation of BTNL8*3 fusion gene. The genomic region between exon 5 of *BTNL8* and exon 4 of *BTNL3* are deleted in certain individuals which results in a new fusion gene *BTNL8*3* bearing exons 1-4 from *BTNL8* and exons 5-6 of *BTNL3*. Green boxes – exons of *BTNL8*; orange boxes – exons of *BTNL3*; grey parts of boxes – untranslated regions of the exon.

Classically, BTN(L)s consist of extracellular immunoglobulin variable (IgV) and constant (IgC) domains, a transmembrane domain and a cytoplasmic B30.2 domain (Fig.1.14) (Afrache et al., 2012). However, variations exist. BTNL2 has a duplication of the IgV and the IgC domains and no B30.2 domain. ERMAP and MOG have no exons coding for an IgC domain. MOG has an extra transmembrane domain and no B30.2 domain. BTNL9 has lost the conserved cysteine residues required for the stabilization of an Ig-like folding and thus, has been proposed to lack an IgC domain (Afrache et al., 2012). BTNL3A2 has no B30.2 domain (Afrache et al., 2012). Finally, the reported BTNL8*3 protein would have the IgV, IgC and transmembrane domain sequences of BTNL8 and the intracellular B30.2 domain of BTNL3, thus retaining the classical structure.

In the human genome, the B30.2 domain is characteristic of the BTN(L) and tripartite motif (TRIM) family of proteins. It represents a fusion of SPRY and PRY domains (Rhodes et al., 2005) and has classically been considered to mediate protein-protein interactions (D’Cruz et al., 2013).

While *SKINTL* has several premature stop codons (Boyden et al., 2008), murine Skint locus has undergone extensive gene expansion event. Skint molecules have one or two Ig domains, three to five transmembrane regions and no B30.2 domain (Afrache et al., 2012).

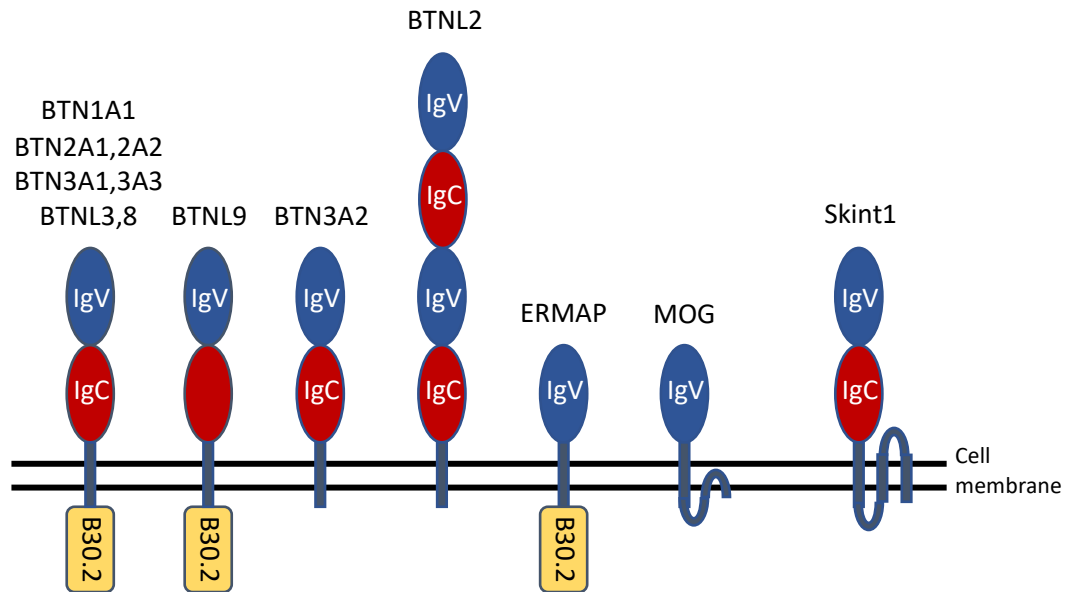


Figure 1.14. Overview of the domain organisation of different BTN and BTNL proteins. Majority of the family members consist of one IgV and one IgC domain, a transmembrane domain and an intracellular B30.2 domain. Note that for simplicity BTNL9 was included in the list of proteins with the classical structure. However, the cysteine residues required for a disulphide bridge and an IgC fold have been mutated in this protein. Skint1 belongs to the murine Btn(I) family. The only *SKINTL* gene in humans is a pseudogene. *Adapted from Rhodes et al., 2016.*

3.2. Function of human BTN and BTNL proteins

Btn(I)/BTN(L) proteins appear to be profound regulators of the development, tissue retention and/or effector functions of specific $\gamma\delta$ subsets in mice (Section 2.3.3.). The mechanisms by which this is achieved are not understood. One hypothesis, which will be investigated in this thesis, is that they directly interact with the $\gamma\delta$ TCR. Alternatively, they may act as co-stimulatory or co-inhibitory receptors akin to other B7 members. In this regard, a spectrum of data which relates to the immunoregulatory potential of several Btn(I)s/BTN(L)s shall be reviewed next. Much of our current understanding of the Btn(I)/BTN(L) family comes from studies using homomeric Fc-fusion proteins. Therefore, the results need to be considered cautiously due to the evidence that these molecules require the formation of heteromeric complexes for optimal function (Di Marco Barros et al., 2016; Vantourout et al., 2018; Rigau et al., 2020). Additionally, such constructs lack the transmembrane and intracellular domains which may be functionally important. Finally, this section will end with review of the literature providing evidence for the regulation of human $\gamma\delta$ T cell by the BTN(L) proteins.

3.2.1. Immunoregulatory potential of BTN(L) molecules

3.2.1.1 BTN1A1

BTN1A1 is the founding member of the butyrophilin family, first identified on the apical surface of milk-secreting cells in bovine mammary tissue and as a major component of the membrane of milk fat globules (Franke et al., 1981). It is conserved across species and preferentially expressed in the mammary tissue (Afrache et al., 2012). *Btn1a1* knockout mice exhibited abnormal lipid secretion in milk during lactation which led to reduced weight and survival of pups fed by *Btn1a1*^{-/-} mice (Ogg et al., 2004). Lipid accumulated in the mammary epithelium and large lipid droplets with fragmented outer membrane were secreted. This regulatory function was proposed to be mediated via the binding of the B30.2 domain to xanthine oxyreductase which led to the stabilization of the milk fat globule (Jeong et al., 2009).

Furthermore, *Btn1a1* was shown to ameliorate MOG-induced experimental autoimmune encephalomyelitis via molecular mimicry. It was proposed that the consumption of milk, of which *Btn1a1*/BTN1A1 is a major component, may modulate pathogenic autoimmune responses to MOG (Steffner et al., 2000). Furthermore, recombinant murine IgV of *Btn1a1* inhibited antigen-induced lymphocyte proliferation *in vitro*. Splenocytes from animals pre-treated with the protein exhibited reduced Th1 responses and increased IL-10 production in response to MOG (Mana et al., 2004). Collectively, these findings also suggest a direct immunosuppressive function of *Btn1a1*. The significance of this in humans is uncertain due to unclear role of anti-MOG antibodies in human pathology (Reindl and Waters, 2018) and the rapid degradation of human BTN1A1 at gastric pH<4 (Peterson et al., 1998).

Nevertheless, subsequent murine studies have confirmed an immunosuppressive potential for *Btn1a1*. *Btn1a1*-Fc fusion protein bound to activated but not resting T cells and was able to suppress CD3-induced CD4 and CD8 T cells proliferation (Smith et al., 2010). Moreover, the same authors demonstrated *Btn1a1* mRNA expression in an array of murine tissues, although there was a clear 10-fold enrichment in the mammary glands. Low levels of *BTN1A1* were also detected in the healthy human gut and the expression was significantly upregulated in whole tissue biopsies from patients with ulcerative colitis (UC) (Lebrero-Fernandez et al., 2016b). It is unclear whether this was the result of changes in the epithelium or due to immune infiltration but the results suggest an immune association for this gene in humans as well.

3.2.1.2 BTN2

BTN2A1, *BTN2A2* and *BTN2A3* are paralogous genes, generated through duplication events of an ancestral gene after the branching of primate and rodent lineages (Afrache et al., 2012). Mice possess a single orthologous *Btn2a2* gene. Due to the appearance of multiple stop codons, *BTN2A3* has become a pseudogene. *BTN2A1* and *BTN2A2* are seemingly expressed ubiquitously (Malcherek et al., 2007; Smith et al., 2010).

A *BTN2A1*-Fc fusion protein was shown to specifically stain human monocyte-derived DCs. The putative counterreceptor was downregulated following DC activation with LPS or TNF α and was identified as DC-SIGN (dendritic cell-specific ICAM-3 grabbing non-integrin). Importantly, the binding to *BTN2A1* was dependent on appropriate carbohydrate modifications on this protein and was suggested to be important in tumour immune surveillance (Malcherek et al., 2007). This hypothesis has not been investigated further.

BTN2A1 single nucleotide polymorphisms have also been associated with predisposition to hypertension, myocardial infarction and dyslipidaemia, although a mechanism for this has not been proposed (Murakata et al., 2014; Horibe et al., 2014).

Like *Btn1a1*, murine *Btn2a2*-Fc fusion protein also bound to activated but not resting T cells and suppressed CD3-induced CD4 and CD8 T cells proliferation (Smith et al., 2010). This was mediated through inhibition of CD3 ϵ , Zap70, PI3K, Akt and Erk1/2 phosphorylation (Ammann et al., 2013). Furthermore, its protein expression was upregulated in antigen-presenting cell - B cells, peritoneal macrophages and bone marrow-derived DCs, after activation with LPS (Ammann et al., 2013). Engagement of *Btn2a2* in conjunction with anti-CD3 and anti-CD28 treatment of naive T cells induced Foxp3 expression and differentiation towards a T regulatory phenotype *in vitro* (Ammann et al., 2013). This was further investigated *in vivo* in *Btn2a2* knockout mice (Sarter et al., 2016). Knockout animals were developmentally normal, with no immune perturbations in any immune compartment at steady state. However, there were enhanced T cell responses upon challenge with OVA antigen. Irradiated wild type mice, receiving *Btn2a2*^{-/-} bone marrow, developed more IFN γ ⁺ T cells and had reduced frequencies of Foxp3 T regulatory cells following OVA immunization (Sarter et al., 2016).

The function of human *BTN2A2* has been poorly studied. Its expression was detected in healthy human colon, it was moderately upregulated in UC (Lebrero-Fernandez et al., 2016b) and significantly upregulated in several cancers (Oncomine database).

3.2.1.3. BTNL2

This gene is conserved and located in the MHC regions in several species (Afrache et al., 2012). It has wide tissue expression pattern in humans, including high expression in the small intestine and B cells but is almost undetectable in the human colon (Valentonyte et al., 2005; Arnett et al., 2009; Lebrero-Fernandez et al., 2016b). Its expression is induced on activated T cells (Subramaniam et al., 2015). Many genome-wide association studies have implicated BTNL2 in the control of several immune disorders such as inflammatory bowel disease (Silverberg et al., 2009; Pathan et al., 2009; Prescott et al., 2015), sarcoidosis (Valentonyte et al., 2005; Rybicki et al., 2005; Spagnolo et al., 2007; Milman et al., 2011) and rheumatoid arthritis (Mitsunaga et al., 2013). Nonetheless, due to the location of the *BTNL2* gene within the MHCII locus and its strong linkage disequilibrium with HLA-DR, few studies had the power to show an association independent of the HLA genes (Valentonyte et al., 2005; Pathan et al., 2009; Mitsunaga et al., 2013; Prescott et al., 2015).

Murine Btl2-Fc was shown to specifically stain resting B cells. The expression of the putative Btl2 receptor was further upregulated on activated B cell and began to be expressed on activated CD4 and CD8 T cells (Nguyen et al., 2006; Smith et al., 2010). Plate bound Btl2-Fc efficiently inhibited anti-CD3 induced T cell proliferation and moderately affected IL-2 production (Nguyen et al., 2006; Smith et al., 2010). However, when used in soluble form, the immunosuppressive effects were lost, suggesting a requirement for crosslinking in order to overcome TCR stimulation. Subsequent studies showed that murine Btl2-Fc was capable of inhibiting human T cell proliferation indicating the BTNL2/Btl2 receptor is conserved across species (Arnett et al., 2007). Furthermore, Btl2-Fc, in combination with recombinant B7-2 and anti-CD3, inhibited Akt signalling and cytokine production, without an effect on proliferation. T cells efficiently differentiated towards induced Tregs when cultured in the presence of IL-2, TGF β , and the recombinant proteins (Swanson et al., 2013).

Therapeutic potential for Btl2-Fc has recently been demonstrated in non-obese diabetic (NOD) mice. Treatment with the recombinant protein delayed the onset and reduced the incidence of type one diabetes in NOD mice. This was associated with increased generation of Foxp3⁺ Tregs and inhibition of T cell activation in pancreas islet infiltrates and spleen (Tian et al., 2019). These results agree with earlier *in vitro* data and indicate Btl2 has an immunosuppressive role. Whether this holds true in humans, remains to be investigated.

3.2.1.4. BTNL8

In vitro experiments with human BTNL8-Fc have suggested that this protein has co-stimulatory function following suboptimal TCR stimulation (Chapoval et al., 2013). Questionable studies in mice have also reported that animals immunised with BTNL8-Fc and ovalbumin had boosted IgG₁ production during the primary immune response compared to those injected with ovalbumin alone, suggesting BTNL8 enhanced the immune response *in vivo* as well (Chapoval et al., 2013). These observations are highly disputable considering the fact that there is no murine *BTNL8* orthologue, and that BTNL8 and BTNL3 function as heteromers (Di Marco Barros et al., 2016; Vantourout et al., 2018).

3.2.1.5. BTNL9

This gene is conserved across several species, including mice, and shares a common ancestral gene with *BTNL3* and *BTNL8*.

Murine Btl9-Fc stained several immune cell types including macrophages, bone marrow-derived DCs, B cells and T cells, and staining was increased following activation of these cells (Yamazaki et al., 2010). However, the fusion protein failed to induce any functional modulation in the immune populations.

Based on the NCBI Gene database and our unpublished observations, human *BTNL9* expression is enriched in adipocytes and stromal cells. Its expression has also been demonstrated specifically in B cells (Arnettai et al, 2009). In lymphoblastoid cell lines derived from human subjects, BTNL9 protein expression was nearly absent in lines homozygous for the *BTNL8*3* fusion suggesting that the deleted genomic region contains regulatory sequences of *BTNL9* gene expression (Aigner et al., 2013). BTNL9 downregulation was also reported in several cancers including uveal melanoma and breast cancer (Jiang and Liu, 2019, Bao et al., 2019; Oncomine database). The significance of these findings is unclear as the function of BTNL9 has not been elucidated.

3.2.2. Evidence for $\gamma\delta$ regulation by *BTN(L)* molecules in humans

3.2.2.1. BTN3A1, BTN3A2 and BTN3A3 as key regulators of human blood V γ 9V δ 2

The molecular mechanisms of blood V γ 9V δ 2 activation by phosphoantigens have been unclear for many years. The first crystal structure of this TCR proposed that a small molecule binding pocket involving CDR3 γ and CDR2 δ was present on the protein (Allison et al., 2001). However, there is no evidence for direct interaction between the two. Subsequent mutagenesis analysis

revealed that the area of the V γ 9V δ 2 TCR required for phosphoantigen reactivity was significantly larger than the proposed binding site (Wang et al., 2010). This strongly indicated the engagement of a larger epitope, possibly from a presenting molecule. A major breakthrough in the field was the discovery by Harly et al. (2012) that V γ 9V δ 2 phosphoantigen reactivity was critically dependent on BTN3A1 expression.

The BTN3 gene cluster arose *via* duplication of an ancestral gene which was lost in rodents but conserved in primates (Afrache et al., 2012). BTN3 transcripts are present in lymphoid tissues and all immune cells (Compte et al., 2004). Protein expression has been detected in all immune cell types as well as on breast, ovary, pancreatic and endothelial cell lines (Compte et al., Yamashiro et al., 2010).

Early *in vitro* experiments using pan-BTN3 antibody (clone 232-5) demonstrated BTN3 molecules to be inhibitors of cellular immune responses. Anti-CD3+rIL-2 induced T cell proliferation and IFN γ production were downmodulated by the addition of clone 232-5 (Yamashiro et al., 2010). In contrast, the use of another anti-BTN3 clone (20.1), in combination with anti-CD3, induced enhanced T cell proliferation, IFN γ production, and TCR signalling (Messal et al., 2011).

An observation that soluble 20.1 antibody massively expanded *ex vivo* $\gamma\delta$ T cells from peripheral blood mononuclear cells (PBMCs), led to the discovery of a role for BTN3A1 in V γ 9V δ 2 activation (Harly et al., 2012). Anti-BTN3 induced *ex vivo* cytotoxic degranulation, as judged by increased CD107a surface expression, production of IFN γ and TNF α , and upregulation of CD69 by blood $\gamma\delta$ T cells without the requirement for anti-CD3 stimulation. Moreover, the effects were shown to be specific to V γ 9V δ 2 and not to other $\gamma\delta$ lymphocytes. The 20.1 clone sensitized target cells to V γ 9V δ 2 killing independently of antibody-dependent cell-mediated cytotoxicity (ADCC) through Fc receptors. Knockdown studies showed that phosphoantigen reactivity of V γ 9V δ 2 was dependent on BTN3 expression by target cells. It was therefore proposed that 20.1 induces a conformational change in the BTN3 molecules, similar to the one presumably induced by phosphoantigens. Although re-introduction of each BTN3 in the knockdown cell lines recovered 20.1-mediated degranulation, only BTN3A1 was capable of restoring soluble phosphoantigen reactivity.

Exactly how BTN3A1 regulates V γ 9V δ 2 phosphoantigen reactivity remains uncertain and two models have been proposed (Fig.1.15A, B).

The simplest one suggests that phosphoantigens are presented via the extracellular domain of BTN3A1 (Fig.1.15A). Evidence in support of this came from Vavassori et al. (2013) who showed

that recombinant BTN3A1-IgV stimulated V γ 9V δ 2 cells in the presence of IPP and HMBPP. They demonstrated using mass spectrometry that the small molecules bound BTN3A1-IgV (Vavassori et al., 2013). BTN3A1 and BTN3A2 have identical IgV domains. Therefore, the authors proposed that the B30.2 domain of BTN3A1 may be required for the physiological activation of V γ 9V δ 2 cells. The domain, which is absent in BTN3A2, may be required for trafficking the presenting molecule to the appropriate intracellular compartments where it is loaded with IPP. Alternatively, the B30.2 domain may be required for compartmentalization of BTN3A1 at the plasma membrane which would facilitate efficient activation of the $\gamma\delta$ lymphocytes (Vavassori et al., 2013).

The second model proposes that phosphoantigens bind intracellularly to the B30.2 domain of BTN3A1 and induce a conformational change in an inside-out signalling mode (Fig.1.15B) (Gu et al., 2018). Harly et al. (2012) first alluded to the importance of the intracellular domain. The first crystal structure of BTN3A1 B30.2 domain revealed a negative pocket which could accommodate the small molecules, but no binding was detected (Wang et al., 2013). The authors speculated that additional proteins may be required. Subsequently, a crystal structure of a phosphoantigen-bound B30.2 domain was solved but SPR failed to detect V γ 9V δ 2-BTN3A1-IgVlgC interaction (Sandstrom et al., 2014). Crystallographic studies of full length extracellular BTN3A1 demonstrated the protein existed as homodimers in solution, most likely in a V-shaped conformation (Palakodeti et al., 2012). Accumulation of structural and biochemical studies of the extracellular and intracellular portions of the BTN3A1 strongly support the view of a conformational change in the ectodomains induced upon intracellular phosphoantigen binding (Palakodeti et al., 2012; Sandstrom et al., 2014; Gu et al., 2017; Salim et al., 2017; Nguyen et al., 2017; Yang et al., 2019).

Nevertheless, early on Riaño et al. (2014) showed that BTN3A1 was required but not sufficient for V γ 9V δ 2 phosphoantigen activation. Zoledronate treated CHO cells transfected with BTN3A1 alone failed to activate $\gamma\delta$ lymphocytes and required the presence of human chromosome 6 in these cells. Thus, a human-specific protein is required. RhoB and periplakin have been reported to mediate phosphoantigen recognition by V γ 9V δ 2 cells via intracellular associations with BTN3A1 (Sebestyen et al., 2016; Rhodes et al., 2015). However, they are conserved across species and absent from human chromosome 6. On the other hand, the other two genes from the *BTN3* locus, *BTN3A2* and *BTN3A3*, are found in humans but not rodents (Karunakaran et al., 2014).

Data from our lab has recently demonstrated the importance of these proteins (Vantourout et al., 2018). Earlier studies used small hairpin RNAs to knock down each BTN molecule to elucidate their roles (Harly et al., 2012). This approach does not ensure the complete removal of the genes. Therefore, a residual expression of BTN3A2 and BTN3A3 may have rescued the phenotype of knockdown cells. In contrast, Vantourout et al. (2018) used CRISPR-Cas9-mediated deletion of each gene and the complete locus in HEK293T cells to investigate the role of each protein. BTN3A2 deletion significantly impaired the response of polyclonal V γ 9V δ 2 lines to zoledronate pre-treated cells implicating it in modulating BTN3A1 activity. The joint deletion of BTN3A2 and BNT3A3 completely abolished activation and the reconstitution with BTN3A1 alone did not restore normal phosphoantigen reactivity. While BTN3A2 was required for optimal functional response, BTN3A3 could partially compensate but was not itself necessary in the presence of BTN3A2 (Vantourout et al., 2018). BTN3A1 was found to form heteromeric interactions with both BTN3A2 and BTN3A3 mediated via the IgC domain. BTN3A2 regulated BTN3A1 ER dwelling time which appears to be an important quality-control step perhaps required for interaction with a cargo (Vantourout et al., 2018).

Most recently, the BTN2 member BTN2A1 was shown to co-localise with BTN3A1 and appeared to be necessary for V γ 9V δ 2 phosphoantigen reactivity (Rigau et al., 2020). The data demonstrated that the expression of BTN2A1 on its own or BTN3A1 plus BTN3A2 in murine or hamster cell lines did not support the activation of *in vitro* expanded $\gamma\delta$ T cells. In contrast, the co-expression of BTN3A1 plus BTN2A1 was necessary and the presence of BTN3A2 further enhanced $\gamma\delta$ lymphocyte activation in the assay (Rigau et al., 2020). SPR experiments detected binding of the BTN2A1 ectodomain to a V γ 9V δ 2 TCR (K_d ~40 μ M) as well as to a V γ 9V δ 1 TCR (K_d ~50 μ M). However, HMBPP and IPP did not interact with the B30.2 domain of BTN2A1 (Rigau et al., 2020). Thus, a third model of phosphoantigen recognition may be proposed, whereby BTN3A1 participates in hetero-dimeric or -trimeric complexes with BTN2A1 and BTN3A1 (Fig. 1.15C).

Finally, phylogenetic studies suggest a co-evolution of the *BTN3* genes, *TRGV9* and *TRDV2* in placental mammals, further supporting a functional relationship between BTN3 proteins and V γ 9V δ 2 cells (Karunakaran et al., 2014).

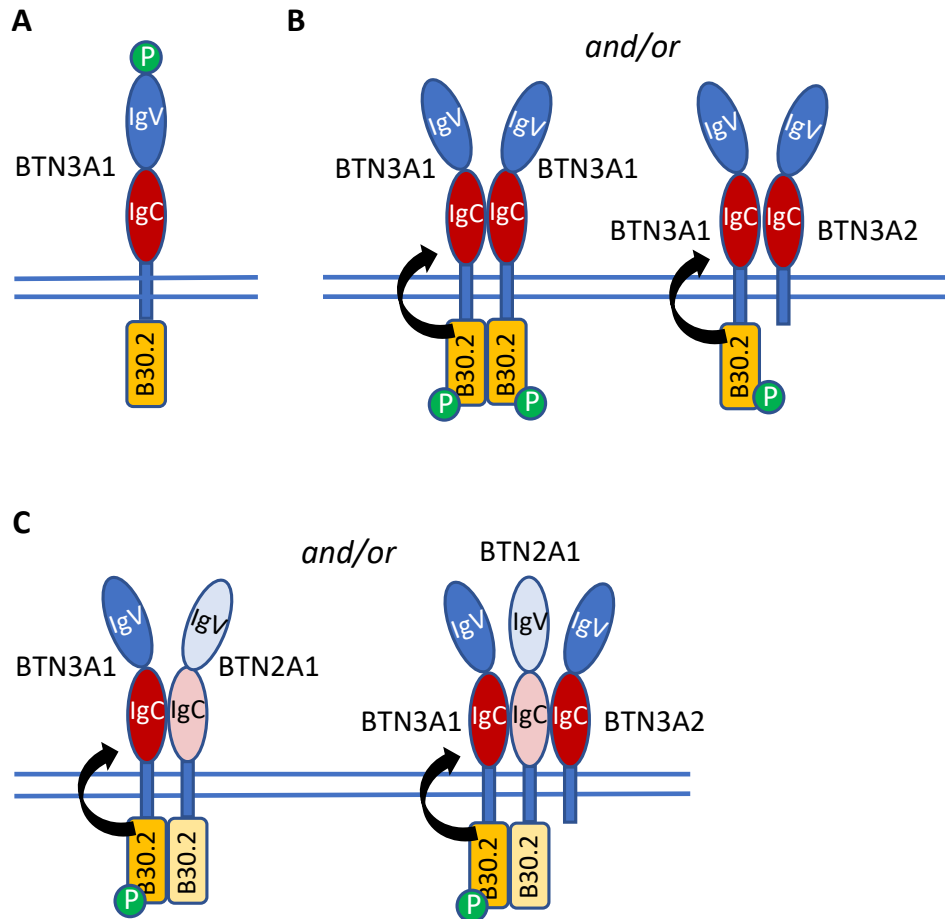


Figure 1.15. Models of phosphoantigen binding to BTN3A1. **A.** The antigen presenting model. BTN3A1 displays phosphoantigens (green circle) extracellularly *via* its IgV domain to the V γ 9V δ 2 TCR. **B.** Inside-out signalling model. BTN3A1 forms homodimers and/or heterodimers with BTN3A2. Phosphoantigens bind intracellularly to the B30.2 domain of BTN3A1 and induce a conformational change (black arrow) which is propagated to the extracellular portions of the protein and allows it to interact with the V γ 9V δ 2 TCR. **C.** The data generated by Rigau et al., (2020) suggests BTN3A1 may also exist in heterodimers with BTN2A1 or heterotrimers with BTN3A2 and BTN2A1.

3.2.2.2. Human gut V γ 4⁺ IEL and BTNL3 and BTNL8

BTNL3 and *BTNL8* have been lost in the rodent lineage during evolution (Afrache et al., 2012). Both genes are highly enriched in the human intestinal epithelium (Di Marco Barros et al., 2016; Lebrero-Fernandez et al., 2016b), although their expression has also been detected in neutrophils (Arnett et al., 2009).

BTNL3 and *BTNL8* are proposed to be the functional equivalents of murine *Btl1* and *Btl6* (Di Marco Barros et al., 2016). *BTNL3* requires heteromerization with *BTNL8* for efficient cell surface

expression (Di Marco Barros et al., 2016; Vantourout et al., 2018). Together, they elicit TCR-dependent responses in a subset of human colonic $\gamma\delta$ T cells, namely TCR downregulation and CD25 upregulation (Di Marco Barros et al., 2016). Thus, $V\delta 2^-$, but neither $V\delta 2^+$ nor $\alpha\beta$ IELs, phenocopied murine $V\gamma 7^+$ lymphocytes when co-cultured with HEK293T cells transduced with BTNL3+8 (Di Marco Barros et al., 2016). Moreover, the response was specific for gut IELs stained with a $V\gamma 2/3/4^-$ specific antibody. $\gamma\delta$ T cells from skin and peripheral blood had few $V\gamma 2/3/4^+$ cells and did not respond in the culture conditions (Di Marco Barros et al., 2016). Early reports suggested that $V\gamma 4$ is the dominant chain used by human gut $\gamma\delta$ IEL (Landau et al., 1995). This, together with the deep sequencing analysis of one donor and the relatively low frequency of $V\gamma 5/3^+$ IELs across many patients, led us to hypothesise that $V\gamma 4^+$ cells were the population specifically responding to BTNL3+8. More recently, *TRGV4* was shown to be the dominant transcript in duodenal biopsies from control patients in two studies of coeliac disease (Mayassi et al., 2019; Eggesbo et al., 2019). In contrast, loss of *BTNL8* epithelial expression was associated with a loss of *TRGV4* transcripts in people with active disease (Mayassi et al., 2019). Collectively, these and our observations imply that BTNL3 and BTNL8 proteins may be required for the shaping of the intestinal $\gamma\delta$ IEL compartment in humans.

4. Summary and aim of the study

In conclusion, $\gamma\delta$ T cells are evolutionarily conserved, unconventional T lymphocytes which preferentially localise to epithelial barriers where they contribute to both innate and adaptive immune responses. They exhibit limited receptor diversity and preferential V γ /V δ chain pairings depending on the tissue. Murine studies suggest that tissue-resident $\gamma\delta$ T cells are regulated by organ-specific BTNLs. In humans, the functional capacity of blood-specific V γ 9V δ 2 cells is regulated by ubiquitously expressed BTN3 proteins and gut-specific BTNL3 and BTNL8 elicit TCR-dependent responses in a subset of $\gamma\delta$ IELs.

This thesis seeks to bring clarity in the $\gamma\delta$ field by asking a simple fundamental question:

Is the impact of BTNL3 and BTNL8 on human colonic $\gamma\delta$ lymphocytes mediated primarily by the TCR, and if so, what are the requirements for this?

Addressing this question can provides us key mechanistic insight into how human intestine-resident $\gamma\delta$ T cell subsets are regulated by BTNL proteins in the healthy mucosa and how these lymphocytes might differentiate physiological from stress-induced antigens.

Chapter II. Materials and Methods

1. Cell lines

Human cell lines HEK293T, HeLa and HT29, murine MODE-K and 3T3, and hamster CHO cells were from ATCC and were maintained in DMEM (10% FBS and 100U/mL Penicillin-Streptomycin). Human TCR β ⁻ reporter cell line JRT3 NFAT-*G/luc* was a gift from Dr Salah Mansour, University of Southampton. The cells were transduced with a vector expressing the Gaussia luciferase under the control of the NFAT promoter and maintained in RPMI 1640 (10% FBS and 100U/mL Penicillin-Streptomycin) supplemented with 50ng/ml G418. Human TCR β ⁻ JRT3-T3.5 (ATCC) and TCR $\alpha\beta$ ⁻ J76 (gift from Dr Patricia Barral, King's College London) cell lines were cultured in RPMI 1640 (10% FBS and 100U/mL Penicillin-Streptomycin).

Cells transduced with any combination of EV, BTNL3, BTNL8 and EPCR were cultured in DMEM (10% FBS, 100U/mL Penicillin-Streptomycin) supplemented with 1 μ g/mL puromycin.

2. Human samples

Up to 12 endoscopy biopsies were obtained from macroscopically healthy mucosa from the ascending colon of adult patients undergoing diagnostic colonoscopy after informed consent and in compliance with local ethical approval (Study number: 16/LO/0642) from the NHS Health Research Authority (London – Fulham Research Ethics Committee).

At the time of collection, samples were placed in Hanks balanced salt solution (HBSS) (ThermoFisher) supplemented with 10mM HEPES and processed within 4h as described below. One biopsy was placed in RNeasy lysis buffer (Qiagen) for RNA/DNA extraction.

3. Isolation of primary cells

Primary gut lymphocytes were obtained following a previously described protocol (Di Marco Barros et al, 2016) adapted from (Clark et al., 2006). In brief, autoclaved Cellfoam matrices (Cytomatrix PTY Ltd) were incubated in 100mg/mL rat tail collagen I (Corning) in PBS for 30 min at room temperature and washed twice in PBS. Biopsies were washed for 30 min in 5mL wash medium (RPMI 1640 10% FBS, β -mercaptoethanol, penicillin 500U/mL, streptomycin 500 μ g/mL,

metronidazole 5µg/mL (Pharmacy Department, Guy's Hospital), gentamicin 100µg/mL (Sigma-Aldrich), and amphotericin 12.5µg/mL (ThermoFisher)). One biopsy was placed on top of each matrix and into a 24-well plate (1 per well), covered with 2mL "gut medium" (RPMI 1640 supplemented with 10% FCS, β-mercaptoethanol, penicillin 100U/mL, streptomycin 100µg/mL, metronidazole 1µg/mL, gentamicin 20µg/mL, amphotericin 2.5µg/mL), IL-2 100U/mL (Pharmacy Department, Guy's Hospital) and IL-15 10ng/mL (Biolegend). 1mL of the medium was aspirated every second day and replaced with complete medium containing 2x concentrated cytokines.

4. Co-culture assays

4.1. Co-culture assay with primary lymphocytes

Primary lymphocytes were harvested after 5-7 days of culture on the grids. The cell suspension was passed through a 70µm nylon cell strainer, centrifuged at 400g for 5 min and resuspended in gut medium without additional cytokine and used for the experiments immediately. Lymphocytes were co-cultured in 2:5 ratio with HEK293T cells stably expressing either empty vector or BTNL3 and BTNL8. Typically, 2.5×10^5 HEK293T cells were plated in U-bottom 96-well plates and centrifuged at 400g for 1 min. The supernatant was removed and 1×10^5 IELs were added in 100µL of gut medium without cytokines. Cells were mixed, centrifuged at 400g for 1 min and incubated at 37°C, 5% CO₂ overnight. Experiments were acquired on BD LSRFortessa.

4.2. Co-culture assay with Jurkat cell lines

Cell lines were co-cultured in 1:2 ratio of Jurkat TCR-expressing cell line to EV/BTNL/EPCR-expressing cell line, in U-bottom 96-well plates and at 37°C, 5% CO₂. For each experiment, unless otherwise stated in the figure legend, transduced cell lines were used.

4.2.1. Assessing BTNL3, BTNL8, Btl1, Btl6 and EPCR reactivity

J76 or JRT3 NFAT-*Gluc* cells were mixed with EV/BTNL/Btl/EPCR-expressing cell lines and co-cultured for 5h. As a control, Jurkat cell lines were stimulated with 10 µg/ml of soluble anti-CD3ε (OKT3; BioLegend) or 'pan' antibody to γδTCR (B1; BioLegend), and IgG isotype-matched control antibody (MOPC-21; BioLegend). Experiments were acquired on BD FACS Canto II.

4.2.2. Using antibodies to block *BTNL3* plus *BTNL8* or *EPCR* responses

293T cells were pre-incubated for 45 min with 10 µg/ml antibody to EPCR (AF2245; R&D Systems) or goat IgG (AB-108-C; R&D Systems) at 4°C, subsequently mixed with Jurkat cell lines and co-cultured for 3h at 37°C, followed immediately by staining and acquisition on BD FACS Canto II.

Cell lines were pre-incubated for 30 min with 10µg/ml anti-FLAG (L5, Biolegend), anti-HA (16B12, Biolegend) or IgG control antibody (MOPC-21, Biolegend) at 4°C, co-cultured for 3h at 37°C, followed immediately by staining and acquisition on BD FACS Canto II.

4.2.3. TCR trans-endocytosis experiments

Indicated cell lines were co-cultured for 1h. Samples were subsequently split for surface and intracellular staining or underwent the latter alone. Cells were stained with Zombie NIR or Aqua Viability kit (Biolegend), fixed, permeabilized and stained. The experiment was acquired on BD FACS Canto II or BD LSRFortessa same day or the following day.

Where fixed cell lines were used in the co-culture experiment, 293T.L3L8, 293T.EV and J76-hu17 were fixed for 15 min with BD CellFIX (BD Biosciences) and washed three times with RPMI. Subsequently, the live cell line was added in the well.

4.2.4. ImageStream co-culture assay

Cell lines were pre-incubated with 10nM Bafilomycin A for 30 min at 37°C and co-cultured for 1h, followed by incubation with Zombie NIR viability dye for 15 min at room temperature, fixation, permeabilization and staining. The experiment was acquired same day or the following day on Amnis ImageStream MARK II. Data was analysed using the IDEAS software.

5. Sequencing

5.1. Single-cell TCR sequencing

5.1.1 Single-cell TCR sequencing of *BTNL3/8*-responding $\gamma\delta$ IELs

Human primary IEL were single-cell sorted using BD FACS Aria II. Cells were sorted based on $\gamma\delta$ TCR downregulation ($V\gamma 2/3/4^{\text{low/-}}$ -TCR $\gamma\delta^{\text{low/-}}$ or $V\gamma 2/3/4^{\text{low/-}}$ -CD25 $^{\text{high}}$) in 5µl of PBS in 96-well PCR plates and immediately frozen at -80°C. Subsequently, a plate was thawed on ice, incubated at 65°C for 6 min and placed back on ice. The reverse transcription and first round of PCR were performed using the qScript XLT One-Step RT-PCR Kit (Quanta Biosciences) following the

manufacturer's recommendations with slight modifications. In brief, five different primers (V γ 2/4 F, V δ 1 F, V δ 3 F, C γ 1/2 R and C δ R) were used in a 20 μ L reaction with 167nM of each forward primer and 250nM of each reverse primer. PCR conditions were as follows: 20 min at 50 °C, directly followed by 3 min at 94 °C; 15s at 94 °C, 30s at 68 °C and 1 min 30s at 72 °C, for 35 cycles; and 2 min at 72 °C. The PCR product of this step was diluted 1 in 5 and used in a second round of nested PCR with an internal set of primers (hV γ 2-8int F, hV δ 1int F, hV δ 3int F, hC γ 1/2int R, hC δ int R). The second 20 μ L PCR reaction included Phusion HF Buffer (5x), 0.4 μ L DMSO, 500nM MgCl₂, 200nM final concentration of dNTP solution mix (NEB), 167nM of each forward internal primer, 250nM of each reverse internal primer and 0.2 μ L of Phusion® High-Fidelity DNA Polymerase (NEB). PCR conditions were as follows: 3 min at 98 °C; 20s at 98 °C, 15s at 64 °C and 15s at 72 °C, for 35 cycles; 2 min at 72 °C. The second-round PCR product was run on 2% agarose gel and bands at 500-750bp were excised and purified using the QIAquick Gel Extraction Kit following manufacturer's instructions. The purified bands were sent for Sanger sequencing to Eurofins Genomics (Germany) using custom hDCseqR and hGCseqR primers. Primer sequences are listed in Chapter VIII Appendix.

5.1.2. Single-cell TCR sequencing of skin $\gamma\delta$ T cells

Live/CD3⁺V δ 2⁻/ $\gamma\delta$ TCR⁺V γ 2/3/4⁺ cells from a human skin-derived polyclonal $\gamma\delta$ T cell line were single-cell sorted in 5 μ L of PBS in 96-well PCR plates and immediately frozen at -80°C. Single-cell PCR was done as above.

5.1.3. Single-cell TCR sequencing of CD1c-PC-reactive $\gamma\delta$ IELs

Human primary IEL were single cell-sorted using BD FACS Aria II. Cells were sorted based on CD1c-phosphatidylcholine (PC) dextramer positive staining (CD1c-PC⁺V δ 1⁺) in 5 μ L of PBS in 96-well PCR plates and immediately frozen at -80°C. Plates were processed as above with several alterations. The first round of PCR included the following primers: hV γ 2-8ext F, V δ 1 F, C γ 1/2 R and C δ R. In the second round, the nested PCRs for gamma and delta chains were split into two separate reactions with hV γ 2-8int F and hC γ 1/2int R primers, or hV δ 1int F and hC δ int R primers, respectively. Primer sequences are listed in Chapter VIII Appendix. CD1c-PC was generated and provided by Salah Mansour laboratory, University of Southampton.

5.2. TCR deep sequencing

AllPrep DNA/RNA Mini kit (Qiagen) was used to extract mRNA and genomic DNA from whole donor biopsies following manufacturer's instructions. Qiagen TissueLyser II was used to

homogenise tissue samples. Messenger RNA was sent for $\gamma\delta$ TCR-chain deep sequencing using the IlluminaMiSeq platform with short-read 100/150 PER primers (iRepertoire).

6. Cloning

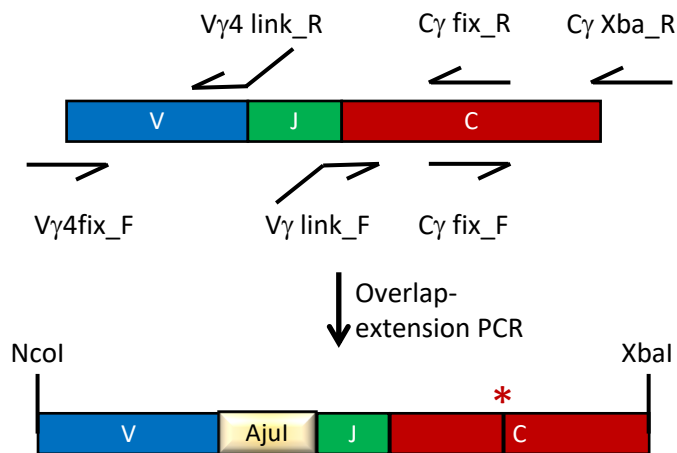
All plasmids in this study were transformed into chemically competent bacteria following 45s heat shock at 42°C and plated on ampicillin (100µg/ml) LB agar plates at 30°C. Colonies were picked and grown overnight at 30°C in 6mL of LB supplemented with 100µg/ml ampicillin. Plasmid DNA was extracted using the QIAprep Spin Miniprep kit and sent for sequencing with Eurofins Genomics (Germany) or Genewiz (UK).

6.1. TCR cloning

6.1.1. Backbone generation

Full-length gamma and delta chains were modified and cloned into self-inactivating lentiviral vector pCSIGPW (SFFV promoter – Multiple Cloning Site [MCS] – IRES-GFP – CMV promoter – PuromycinR-WPRE) (described in Di Marco Barros et al., 2016) or pCSIGW, lacking the puromycin resistance gene. PCR primers were used to introduce several modifications into the TCR chains (Fig.2.1) that would allow for the subsequent cloning of CDR3 $\gamma\delta$ sequences. Primers are listed in Chapter VIII Appendix. An overlap-extension PCR was undertaken to join the fragments from each PCR modification reaction. Final TCR γ and TCR δ chain products were double-digested with NcoI/XbaI and XhoI/NotI, respectively. pCSIGPW/pCSIGW was digested with XhoI and XbaI to allow for the cloning of the TCR δ chain downstream of the SFFV promoter and the TCR γ chain upstream of the puromycin resistance gene (in pCSIGPW) or the WPRE sequence (in pCSIGW). In a second reaction, pCSIGPW/pCSIGW was digested with NotI and NcoI and the internal ribosome entry site (IRES) was gel-purified and cloned between the gamma and delta chain sequences. The final map of the expression vector is shown in Fig.2.2. There are two gene segments for C γ . C γ_1 has three exons and C γ_2 has four, and an allele of C γ_2 carries five exons (Buresi et al., 1989). Thus, constructed backbones carried a combination of V γ X-J $_{1/2}$ -C γ_1 , V γ X-J $_{1/2}$ -C $\gamma_{2.1}$ or V γ X-J $_{1/2}$ -C $\gamma_{2.2}$, where V γ X can be V $\gamma_2/3/4/5/8$, and V δ_1 -J $_1$ -C δ or V δ_3 -J $_1$ -C δ TCR chains. An additional backbone was constructed carrying the V γ_9 and V δ_2 chains with no AjuI/BaeI restriction sites modifications.

Gamma chain



Delta chain

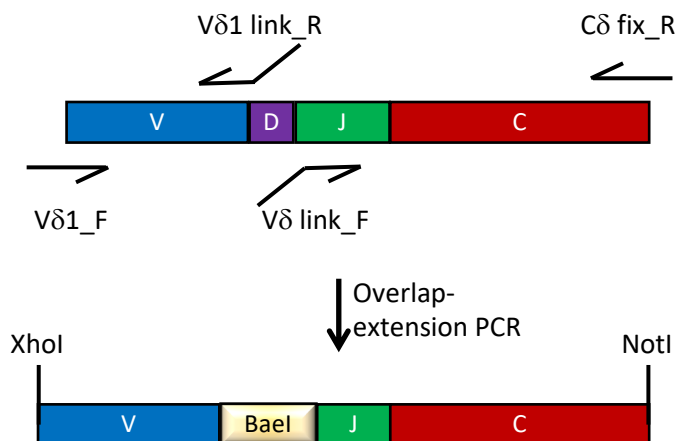


Figure 2.1. PCR site-directed mutagenesis of the TCR γ and TCR δ chains. The indicated primers were used to introduce NcoI and XbaI restriction sites in TCR γ chain and XhoI and NotI in TCR δ chain. Primers binding to the CDR3 region of each chain were used to introduce Ajul and Bael restriction sites before the J segment. Internal primers in C γ were used to introduce a silent point mutation (indicated by a vertical black line) to abolish a naturally occurring Bael site and allow for a double-digest of the expression vector in subsequent experiments.

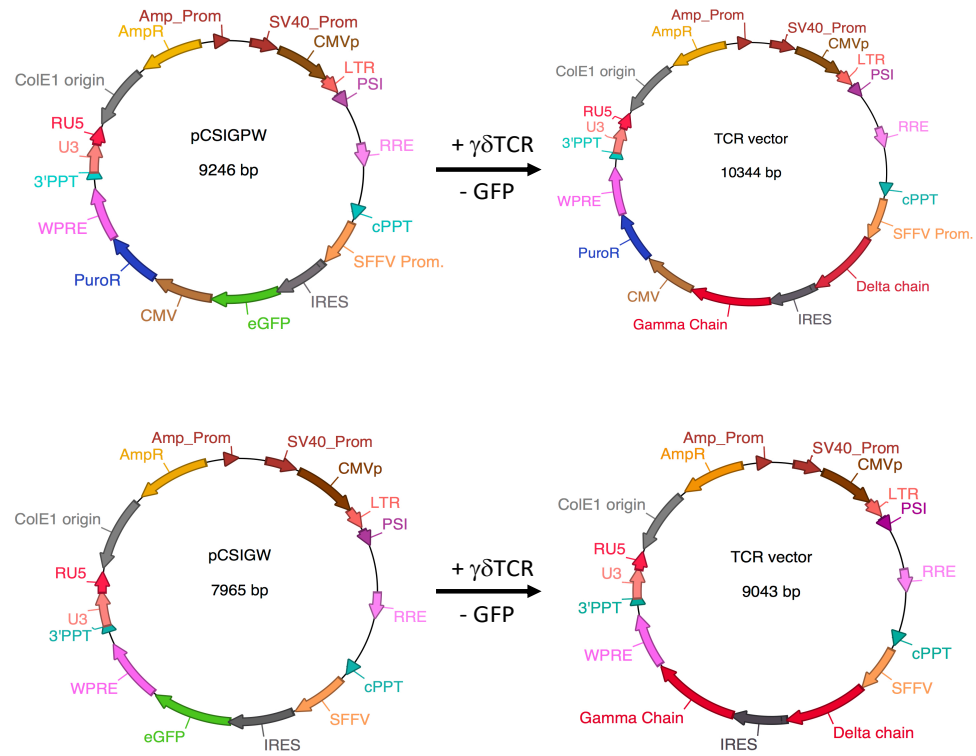


Figure 2.2. Map of the lentiviral expression vectors used in the study. Top row – pCSIGPW before and after cloning of the modified $\gamma\delta$ TCR chains. Bottom row – pCSIGW before and after cloning of the modified $\gamma\delta$ TCR chains. In both cases, the GFP cassette was removed. The final size of the vectors is shown in the middle of each map. The maps were visualised using the ApE sequence viewer (version 2). SV40_Prom - simian vacuolating virus 40 promoter sequence; CMVp – cytomegalovirus promoter; LTR - long terminal repeat; PSI - Psi packaging element; RRE - Rev response element; cPPT - central polypurine tract; SFFV Prom - spleen focus-forming virus promoter sequence; IRES - internal ribosome entry site; eGFP - enhanced green fluorescence protein gene; PuroR – puromycin resistance gene; WPRE - Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element; 3'PPT – 3' polypurine tract; ColE1 origin - colE1 origin of replication sequence; AmpR – ampicillin resistance gene; Amp_Prom – ampicillin resistance promoter sequence.

6.1.2 Single-cell TCR cloning

Identified CDR3 γ and CDR3 δ sequences from the single-cell sequencing were synthesized using Eurofins Genomics Custom DNA Oligos services. 1 μ L from each forward and reverse oligos (100 μ M stock) were dimerized and phosphorylated in a 10 μ L reaction using T4 Polynucleotide Kinase (NEB) under the following conditions: 30 min at 37°C, 5 min at 95°C finally ramping down to 25°C at 0.1°C/s. The reaction product was diluted 1 in 200 and 1 μ L was used in a four-way ligation reaction using T4 ligase (NEB) with ~9kb and ~1.5kb products of the Bael/Ajul double-digested pCSIPW/pCSIW backbone vector carrying the relevant V γ and V δ chains.

6.1.3 TCR chimaeras

Overlap-extension PCR was used to generate TCR chimaeras and swap relevant amino acids in the variable region of the gamma chain. Primers are listed in Chapter VIII Appendix.

6.2 BTNL3 and BTNL8 cloning

Plasmids encoding BTNL protein tagged at the amino terminus were described (Di Marco Barros et al., 2016). BTNL3 and BTNL8 were cloned from Caco-2. FLAG and HA tags were added downstream of the putative leader sequences of BTNL3 and BTNL8 respectively, by overlap PCR.

6.3 EPCR cloning

Messenger RNA was extracted from HT29 cells using the RNeasy Mini kit (Qiagen) following the manufacturer's instructions and converted into cDNA using SuperScript II Reverse transcriptase (ThermoFisher). Full-length EPCR was amplified by PCR. The reaction was run on 2% agarose gel, ~1kb bands were cut and purified using the QIAquick Gel Extraction Kit following manufacturer's instructions. The PCR product was digested with XhoI/NotI and ligated into pCSIGPW vector upstream of the IRES-GFP site. Primers are listed in Chapter VIII Appendix.

7. Transfections

HEK293T cells were plated at 1×10^6 per well in 6-well plates and allowed to adhere overnight. 12 μ g PEI (1mg/ml) (Polysciences) was mixed with 4 μ g of total plasmid to be transfected in 200 μ l of Opti-MEM (ThermoFisher) and incubated for 40 min at room temperature. The transfection mixture was then added dropwise on top of the HEK293T cells. Media was replaced after 24h. Cells were used for experiments 48h post-transfection.

8. Lentiviral transduction

8.1 Protocol I

1×10^6 HEK293T cells were plated in 6-well plates and transfected with 600ng of pHIT/G (MLV env) (Fouchier et al., 1997), 1600ng of expression vector and 1800ng Gag-Pol pCMV Δ R8.91 (Zufferey et al., 1997), as described in section 7. After 48h, the medium was collected, centrifuged at 800g for 5 min and supernatant used to transduce $1-2.5 \times 10^5$ J76 or JRT3-T3.5 cells. Cells were mixed with supernatants, incubated for 1h at 37°C, centrifuged for 1h at 800g

and plated in 12-well plates. Cells were assessed for TCR expression after 2-7 days. Plasmids used in this protocol were purified with QIAGEN Plasmid Mini or Midi Kit.

8.2 Protocol II

Alternatively, HEK293T cells were transfected with 600ng of pHIT/G (MLV env), 1600ng of TCR expression vector pCSIW and 1800ng Gag-Pol pCR/V1 (Zennou et al., 2004) using the transfection protocol described in section 7. After 48h, viral supernatant was collected and filtered through 0.45µm nylon mesh. 1×10^5 Jurkat cell lines were resuspended in 500-1000µl of transduction medium and centrifuged at 1200g for 30 min, plated in 48-well plates overnight, and subsequently transferred to 12-well plates. Cells were assessed for TCR expression after 2-7 days. Plasmids used in this protocol were purified with NucleoBond Xtra Midi EF kit (Macherey-Nagel).

BTNL3/8 and EPCR transgenic cell lines were established following this protocol. 24h post-transfection, target cell lines were plated in 12-well plates. At 48h, the viral supernatant was collected, filtered and added to the 12-well plates. Puromycin selection started after another 48h. Where a combination of plasmids was transduced, viral supernatants were mixed in a 1:1 ratio. Cells were bulk-sorted 1 week post transduction.

9. Western blot

Cell lines were kept overnight in 'starving medium' (RPMI and 0.5% FBS), then harvested, washed with 'starving medium' and allowed to 'rest' in suspension for 1 h at 37°C and 5% CO₂. 2.5×10^5 J76-hu17 or JRT3-LES cells were mixed with 6.25×10^5 stimulatory cells. Cells were spun down at 600g for 1 min and incubated at 37°C for 5 min, 10 min and 20 min. 1 ml ice-cold PBS was then added and samples were spun down at 600g for 2 min at 4°C. Pellets were re-suspended in 100 µl ice-cold lysis buffer (50 mM Tris, pH 8, 150 mM NaCl, 0.5% NP-40, and 1x Protease and Phosphatase Inhibitor Cocktail (ThermoFisher)). Lysates were incubated for 20 min on ice and were spun at 20,000 g for 20 min at 4°C. Supernatants were subsequently mixed with NuPAGE LDS Sample Buffer supplemented with β₂-mercaptoethanol (2.5% final concentration), then separated by electrophoresis through NuPAGE 4–12% Bis-Tris protein gels (ThermoFisher) and were transferred onto nitrocellulose membranes. Membranes were incubated in blocking solution (PBS 0.1% Tween20 and 3% BSA) for 90 min at room temperature and were subsequently cut in several strips to allow for the separate blotting of multiple proteins from the same membrane. Thus, membranes were cut along the 100kDa, 50kDa and 25kDa ladder

markers and each strip was incubated with a primary antibody (1:1000 dilution in blocking solution) overnight at 4 °C. Membranes were then washed 3x15 min in PBS 0.1% Tween20, incubated for 1 h at room temperature with secondary antibodies (1:5000 dilution in blocking solution), washed again 3x15 min and developed with Clarity Max Western ECL Blotting Substrate (BioRad). Anti-CD3 ϵ (D7A6E), anti-LAT phosphorylated at Tyr191 (3584), anti-PLC γ_1 phosphorylated at Tyr783 (14008/2821), HRP-linked antibody to rat IgG (7077) and HRP-linked antibody to rabbit IgG (7074) were all from Cell Signalling Technologies.

10. Generation of EPCR knockout cell lines

HEK293T EPCR knockout cell lines were generated by transient transfection of the CRISPR/Cas9 lentiviral vector pLG2C (described in Vantourout et al., 2018) with custom made guide oligonucleotides targeting the EPCR gene. 24h post-transfection, cells were single-cell sorted in flat-bottom 96-well plates based on GFP expression. Clones that grew up were screened for EPCR expression by flow cytometry and validate by reverse transcriptase (RT)-PCR. Oligonucleotides were synthesised using Eurofins Genomics Custom DNA Oligos services. Sequences are listed in Chapter VIII Appendix.

11. Luciferase assay

The JRT3 NFAT-*Gluc* reporter cell line was transduced with $\gamma\delta$ TCRs, and 2×10^5 cells were co-cultured at 37°C, 5% CO₂ with 5×10^5 HEK293T cells expressing EV or L3L8. Alternatively, JRT3 lines were stimulated with 10 ng/ml PMA (phorbol 12-myristate 13-acetate) and 1 μ g/ml ionomycin. After 24 h, supernatants were collected, and luciferase activity was measured using the BioLux Gaussia Luciferase Assay Kit (NEB) following the manufacturer's instructions. Luminescence was acquired on an EnVision plate reader (PerkinElmer). Background levels were measured from untransduced reporter cell lines.

12. Flow cytometry

Flow cytometry was performed using the following antibodies, coupled to the indicated fluorochromes. These antibodies were purchased from Biolegend unless otherwise stated; viability dye Aqua was from Invitrogen. Viability dye Zombie NIR was from Biolegend. The

following antibodies against human molecules were used: CD69-AF647 (FN50), CD3-BV421 (OKT3), CD3-BV786 (OKT3), CD3-AF647 (OKT3), $\gamma\delta$ TCR-PeCy7 (IMMU510; Beckman Coulter), V δ 1-FITC (TS8.2, ThermoFisher), V δ 1-APC (REA173; Miltenyi), V δ 2-PerCpCy5.5 (B6), CD25-BV421 (BC96), CD45RA-PE (HI100) CD45-PacBlue (HI30). The biotin-conjugated antibody against V γ 2/3/4 (23D12) (Kabelitz et al., 1994) was detected by conjugation to PE-streptavidin. Other antibodies were as follows: DYKDDDDK-PE (L5), DYKDDDDK-APC (L5), HA-DyLight 650 (2-2.2.14, Invitrogen), HA-BV421 (16B12), HA-AF647 (16B12), HIS-APC (J095G46), EPCR-PE (RCR-16), EPCR-APC (RCR-16), $\gamma\delta$ TCR-PeCy7 (GL3).

For intracellular staining, cells were incubated with viability dye for 20 min, washed with FACS buffer (PBS, 2% FBS, 2mM EDTA), fixed for 10 min in 1x BD CellFIX and permeabilized for 10 min with 1x Intracellular Staining Permeabilization Wash Buffer (Biolegend). These steps were done at room temperature. Cells were then incubated with antibodies diluted in the Permeabilization Wash Buffer for 35 min at 4°C, washed two times with Permeabilization Wash Buffer, once with FACS buffer and acquired.

Flow cytometry data analysis was performed on FlowJo (Version 10).

13. Soluble recombinant TCRs

13.1. Staining

Soluble $\gamma\delta$ TCRs were described (Melandri et al., 2018). 1×10^5 HEK293T cells transduced with EV or BTNL3 variants plus BTNL8 were incubated with soluble TCRs for 45 min at 4°C, followed by washing and incubation with anti-HIS antibody (J095G46, Biolegend) for 45 min at 4°C. Cells were washed twice and acquired on BD FACS Canto II.

13.2. Soluble TCR blocking assay

1×10^5 BTNL3 plus BTNL8 or EV transduced HEK293T cell lines were pre-incubated with various concentrations of anti-FALG (L5), anti-HA or IgG control antibody (MOPC-21) for 45 min at 4°C. In parallel, 10 μ g/ml of soluble V γ 4V δ 1 TCR and 1:200 dilution of anti-HIS-APC (J095G46) antibody were incubated in FACS buffer. Subsequently, cells were washed twice, 40 μ l of APC-labelled soluble TCR were added to each well and incubated for 45 min at 4°C. Cells were washed twice and acquired on BD FACS Canto II.

13.3. Detection of BTNL3/8 downregulation

1 x 10⁵ BTNL3 plus BTN8 or EV transduced HEK293T cell lines were pre-incubated at 37°C or 4°C for 10 min. Various concentrations of soluble TCRs were then added to the medium and incubation continued for another hour at the respective temperatures. Detection with secondary antibody anti-HIS (1:200) was carried out at 4°C for 35 min. In some experiments, cell lines were incubated with 10µg/ml anti-BTNL3 (ARP46769_P050, Aviva Systems) or anti-FLAG (L5) in parallel. Detection with secondary antibodies anti-mouse-APC (1:1000) and anti-rabbit-APC (1:500) was carried out at 4°C for 35 min.

14. Organoids

14.1. Establishing of organoid cultures

8 biopsies were minced with a sharp sterile blade and collected in 10ml of digestions medium (DMEM:F12 supplemented with 10µg/ml hyaluronidase IV-S from bovine testes (SigmaAldrich), 1mg/ml collagenase II (SigmaAldrich) and 10µM Y-27632 (SigmaAldrich)) and incubated shaking horizontally for 1 hour at 37°C. Subsequently, cells were passed through 70µm nylon mesh and any remaining pieces were pushed through with several washes with DMEM:F12+++ (15mM HEPES, 1x GlutaMAX (ThermoFisher) and 1x penicillin/streptomycin (ThermoFisher)). 5% FBS was added to the filtrate and cells were spun down for 10 min with the break on 3 at 350g. Cell pellets were washed three times with DMEM:F12++. Finally, pellets were resuspended in 150µl of Cultrex RGF Basement Membrane Extract (BME), type 2 (R&D Systems) on ice and 40µl or suspension were placed in the centre of a well in a 24-well plate. Plates were placed in an incubator for 15 min and subsequently, 500µL of IntestiCult™ human organoid growth medium (STEMCELL) was added supplemented with 10µM Y-27632 (Sigma-Aldrich), 3µM CHIR99021 (SigmaAldrich) and 100µg/mL primocin (InvivoGen). Media was changed every 2-3 days. Organoids were split once a week.

14.2. Splitting organoids

BME domes were broken by pipetting up and down and everything was collected in a 15ml falcon tube on ice. Wells were washed with DMEM:F12+++ to collect any residual cells. Cells were centrifuged at 4°C for 5 min at 850rpm, the supernatant was removed, pellets were resuspended in 1ml of TrypLE-Express and incubated at 37°C water bath for 3 min. Subsequently, falcons were placed immediately on ice, 4ml of cold DMEM:F12+++ 5% FBS was added on top and cells were centrifuged at 4°C for 5 min at 850rpm. The medium was aspirated off, pellets were resuspended in fresh BME on ice and plated as above.

14.3. Organoid differentiation

Human organoids were differentiated according to STEMCELL IntestiCult™ Organoid Growth Medium (OGM) protocol. In brief, following a passage, cultures were seeded as described above and maintained in standard medium for 4 days. Medium change was performed on day 2 and day 4 after seeding. On day 5, standard OGM medium was replaced with differentiation medium (OGM component A and DMEM:F12 15mM HEPES at 1:1 ratio and supplemented with 10μM Y-27632 (SigmaAldrich), 3μM CHIR99021 (SigmaAldrich) and 100μg/ml primocin (InvivoGen)). Media change was performed on days 7 and 9. Organoids were collected for qPCR analysis on day 10.

15. qPCR

RNA was extracted using the RNeasy mini kit (Qiagen) following manufacturer instructions and 1μg of RNA was converted into cDNA using SuperScript II Reverse transcriptase (ThermoFisher). 4μl of cDNA (1:50 diluted) were mixed with 5μl of PowerUP SYBR Green Master Mix (ThermoFisher) and 1μl of primer mix containing forward and reverse primers at 400nM concentration each. The reaction was run on a QuantStudio™ 5 qPCR System. The PCR cycles were as follows: 2 min at 50°C, 2 min at 95°C, then 40 cycles of 15 sec at 95°C, 15 sec at 60°C, 1 min at 72°C, followed by a melting curve stage of 15 sec at 95°C, 1 min at 60°C and 15 sec at 95°C. Primers are listed in Chapter VIII Appendix.

16. Statistical analysis and data representation

GraphPad Prism (version 7) was used to plot data and perform statistical analysis. *P* values were determined by the paired two-tailed Student's *t*-test or a one-way ANOVA with Tukey's test for correction for multiple comparisons. The *n* values and error bars are defined in each figure legend.

BTNL3 and BTNL8 heteromeric complex model was displayed in PyMOL (version 2).

Chapter III. Identification and cloning of BTNL3/8-reactive IELs

Early studies (Landau et al., 1995) and sequencing data (Di Marco Barros et al., 2016; Mayassi et al., 2019; Eggesbo et al., 2019) suggested *TRGV4* may be a dominant chain expressed in the human intestinal epithelium. Furthermore, our initial findings demonstrated that intestinal IELs detected by an antibody specific for the $V\gamma 2/3/4$ chains (from here termed $V\gamma 2/3/4^+$ cells) responded to HEK293T expressing BTNL3 and BTNL8 (293T.L3L8) in the absence of exogenous cytokines. The response consisted of strong TCR downregulation and variable levels of CD25 upregulation (Di Marco Barros et al., 2016) – two events frequently associated with direct TCR engagement and T cell activation (San Jose et al., 2000; Reddy et al., 2004). Therefore, we sought to determine whether the TCR was necessary for a response to BTNL3 and BTNL8.

To this end, we aimed to determine whether the BTNL3 plus BTNL8 reactivity was polyclonal or monoclonal. A polyclonal response was most probable, given that large numbers of $V\gamma 2/3/4^+$ cells responded to 293T.L3L8 (Di Marco Barros et al., 2016). This would suggest that the clonotypic TCR CDR3 regions were perhaps not directly involved. However, polyclonal responsiveness might not preclude direct interactions with the TCR.

In addition, responding TCRs were isolated and cloned into TCR-deficient cell line J76 to determine if BTNL3 and BTNL8 reactivity could be conferred. J76 is a Jurkat E6.1 T cell subline which has both TCR α and TCR β chains mutated and is thus devoid of surface TCR-CD3 complex expression (Heemskerk et al., 2003). These cells have been widely used to evaluate TCR antigen recognition without the problem of mispairing with endogenous TCR chains (Kjer-Nielsen et al., 2012; Uldrich et al., 2013; Birkinshaw et al., 2015; Nakatsugawa et al., 2016; Roskopf et al., 2018)

1. Single-cell sorting and sequencing of responding TCRs

Intestinal immune cells were isolated according to a previously established protocol (Di Marco Barros et al., 2016). Several colonic biopsies from three apparently healthy donors (GN08, GN09 and GN17) were placed on grids and cultured for up to one week in media supplemented with IL-2 and IL-15 before single-cell sorting.

1.1. Donor phenotypes

On day two of cell culture, some of the biopsies were harvested and the $\gamma\delta$ lymphocyte compartment composition assessed by flow cytometry (Fig.3.1A). Approximately 80% of all live cells were $CD3^+\gamma\delta TCR^- \alpha\beta$ T cells (range 71.7-90%) and 6% were $\gamma\delta TCR^+$ (range 3.6-10%). As expected, $V\delta 1^+$ cells were the predominant among $\gamma\delta$ T cells (average of 56%). Nearly a quarter of the $\gamma\delta$ lymphocytes were negative for the $V\delta 1$ and $V\delta 2$ antibodies, most likely expressing $V\delta 3$ or $V\delta 5$. Within the γ chain repertoire, 78% (range 68-92%) of the $V\delta 2^-$ compartment stained for the $V\gamma 2/3/4$ antibody, thus establishing $V\gamma 2/3/4$ cells as the dominant $\gamma\delta$ populations in the gut. Most of these (72%) were also $V\delta 1^+$ (Fig.3.1A).

On day five of cell culture, additional biopsies were harvested. Isolated lymphocytes were co-cultured overnight with HEK293T cells expressing empty vector (293T.EV) or BTNL3 plus BTNL8 (293T.L3L8) in cytokine-free media, to evaluate the capacity of donor lymphocytes to respond to 293T.L3L8 prior to single-cell sorting. $V\gamma 2/3/4^+V\delta 2^-$ cells downregulated their TCR by approximately 50% in response to 293T.L3L8 relative to 293T.EV while $V\gamma 2/3/4^+V\delta 2^+$ cells (expressing $V\gamma 5,8$ or 9), $V\delta 2^+$ cells (most likely paired with $V\gamma 9$), and $\alpha\beta$ T cells ($CD3^+\gamma\delta TCR^-$) did not (Fig.3.1B). In addition, two donors (GN09 and GN017) also upregulated CD25 expression (Fig.3.1B). While the response was a property of $V\gamma 2/3/4$ cells, the δ chain seemed less relevant since $V\gamma 2/3/4^+V\delta 1^-$ and $V\gamma 2/3/4^+V\delta 1^+$ responded (Fig.3.1C).

In sum, each donor harboured a dominant $V\gamma 2/3/4^+V\delta 1^+$ compartment which was reactive to BTNL3 plus BTNL8, and that was therefore suitable for further experiments.

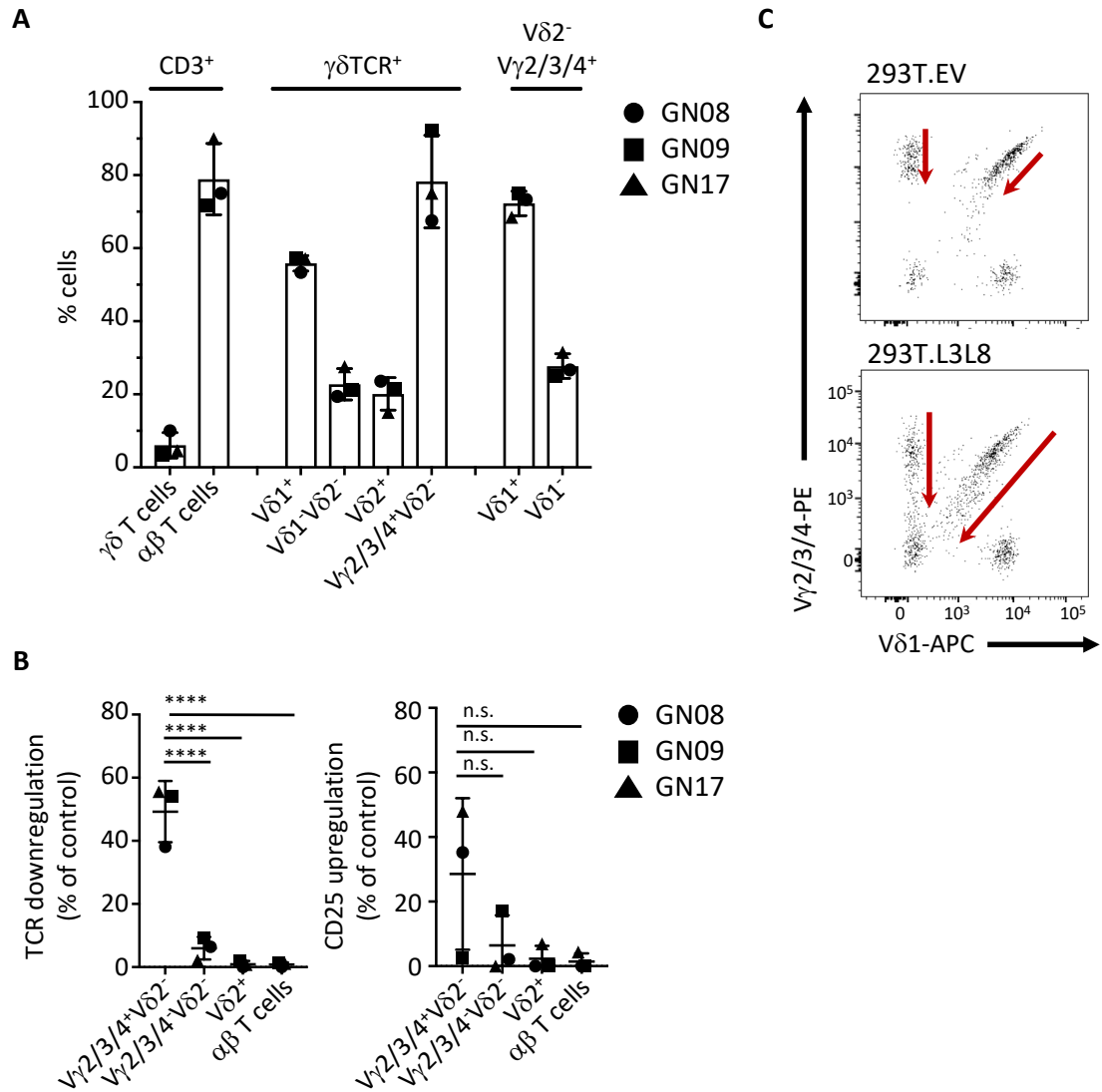


Figure 3.1. Characterisation of the $\gamma\delta$ compartment in three donors used for single-cell sorting. **A.** Flow-cytometric analysis of TCR $\gamma\delta$ chain usage across three donors – GN08, GN09 and GN17 (key). Lines above bars indicate the parental population. Data expressed as mean $\pm$ s.d. **B.** Flow-cytometric analysis of TCR downregulation (left) and CD25 upregulation (right) in different donor immune subsets (x-axis) co-cultured overnight with 293T.L3L8 or 293T.EV. Geometric mean of the fluorescence intensity (gMFI) of CD3 or CD25 on the indicated lymphocyte subsets co-cultured with 293T.L3L8 were normalized to their counterpart cells co-cultured with 293T.EV. Key of donors as in **A**. Data expressed as mean $\pm$ s.d (n = 3), ****p<0.0001. Analysed by one-way ANOVA. **C.** Representative flow cytometry plots for GN17 from the experiment in **B**. The surface expression of V γ 2/3/4 and V δ 1 on live V δ 2⁻CD3⁺ $\gamma\delta$ TCR⁺ singlets after overnight co-culture in the specified conditions is shown. Red arrows indicated the shift in TCR staining.

1.2. Single-cell sorting and sequencing of responding cells

On day seven of cell culture, the remaining biopsies were harvested and live, CD3⁺γδTCR⁺Vδ2⁻ cells were single-cell sorted based on Vγ2/3/4 downregulation (donor GN08) or Vγ2/3/4 downregulation and CD25 upregulation (donors GN09, GN17) (Fig.3.2A).

Single-cell nested PCR was performed on sorted cells. The PCR primers were designed to amplify the CDR3 region of TCR Vγ2, Vγ3 and Vγ4, and TCR Vδ1 and Vδ3 chains in a single-tube reaction. The choice of primers was based on the observation that only Vγ2/3/4⁺ cells respond when exposed to BTNL3 plus BTNL8 as well as the fact that Vδ1 and Vδ3 are the most common tissue delta chains (see below).

Generally, δ chain amplification was visualized following gel electrophoresis for more than 75% of the samples. However, detection of γ products proved more variable, with an average 14.6% recovery efficiency (Fig.3.2B). Therefore, only lanes which appeared to have a band corresponding to amplified CDR3γ were subsequently processed.

19 unique pairs of γδTCR chains were sequenced across the three donors (Table 3.1). Additionally, 15, 6 and 13 unpaired unique sequences were obtained from GN08, GN09 and GN17, respectively. (Table 3.2). Sequences varied in length and composition (Table 3.1 and 3.2). 10 out of 46 unique TCRδ chains used a *TRDV3* gene segment while the remaining used *TRDV1*. Vγ2 accounted for only four out of the 26 unique TCRγ sequences recovered, while the remaining 22 utilized the *TRGV4* gene segment (Table 3.1 and 3.2). Several of the γδCDR3 regions appeared more than once within the same plate. However, none of the recovered CDR3 sequences was shared across donors. Thus, responding TCRs were polyclonal and predominantly expressed a Vγ4 chain paired to either Vδ1 or Vδ3.

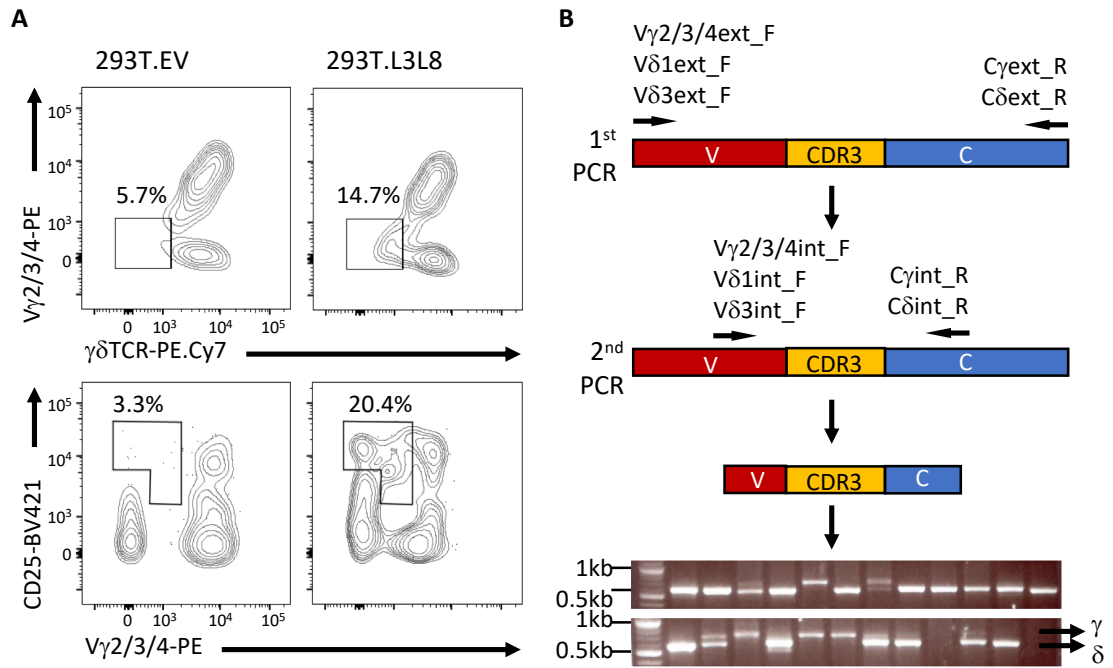


Figure 3.2. Single-cells sorting and PCR of responding $\gamma\delta$ T cells. **A.** Flow cytometry analysis of the expression of $V\gamma 2/3/4$ and $\gamma\delta$ TCR (GN08) or $V\gamma 2/3/4$ and CD25 (GN09 and GN17) by human colonic lymphocytes after co-culture overnight with 293T.EV or 293T.L3L8 cells. Lymphocytes were pre-gated on live $CD3^+V\delta 2^- \gamma\delta$ TCR $^+$ singlets. The indicated gates were used for single-cell sorting. **B.** Outline of the single-cell PCR process. In the first PCR step, external primers were used to amplify the full-length $\gamma\delta$ TCR chains. In the second PCR step, internal primer sets were used. These were designed to bind to part of the $V\gamma/\delta$ and $C\gamma/\delta$ sequences most proximal to the CDR3 region. Final PCR product was run on an agarose gel. A representative image is shown from donor GN17. Expected bands: ~700bp for TCR γ and ~600bp for TCR δ .

Donor	Clone	V usage	CDR3 γ			CDR3 δ		
			AA sequence	Freq.	Rank	AA sequence	Freq.	Rank
GN08	hu1	V γ 4V δ 1	ATWD P GWFKI	n.f	n.f	ALGE I GYWGI H RVNKL	n.f	n.f
	hu2	V γ 4V δ 3	ATWD W GYYKKL	n.f	n.f	A SGD T TDKLI	n.f	n.f
	hu3	V γ 4V δ 3	ATW A GYYKKL	n.f	n.f	A AMGV P FL E GD T GP K LI	n.f	n.f
	hu4	V γ 2V δ 1	ATW K SSDWIKT	n.f	n.f	ALGE L GYPDKLI	0.02%	1118
GN09	hu5	V γ 4V δ 1	ATWDG A CTTGWFKI	n.f	n.f	ALGE K MGP N KLI	n.f	n.f
	hu6	V γ 4V δ 1	ATWDG A CTTGWFKI	n.f	n.f	ALG P YRVRLIDKLI	n.f	n.f
	hu7	Vγ4Vδ1	ATWDGPWNYYKKL	0.13%	88	ALGERGYWGLGDKLI	n.f	n.f
	hu8	V γ 4V δ 3	ATW A PYYKKL	0.21%	49	A FCSVYWGI C TDKLI	3.30%	6
	hu9	V γ 4V δ 3	ATWDG P NYKKL	11%	2	A FFFGWG I RFYTDKLI	42.70%	1
GN17	hu10	V γ 4V δ 3	ATW E TYYKKL	3.40%	4	A FMFP P VG L LI	36.60%	1
	hu11	V γ 4V δ 1	A I A NYKKL	0.04%	216	ALGE L LYVGG I DKLI	n.f	n.f
	hu12	Vγ4Vδ1	ATWVMAHYKKL	3.60%	3	ALGERESLYKLI	7.50%	2
	hu13	V γ 4V δ 1	ATWDG P VL	0.80%	17	ALGE S TGPYWGI R GYTDKLI	2.00%	13
	hu14	V γ 4V δ 1	ATW V PGWFKI	0.44%	36	ALGE L REWGT G VYTDKLI	1.20%	18
	hu15	V γ 4V δ 1	ATWDG R GATGWFKI	0.63%	28	ALG C QYWGI Q ADKLI	2.20%	12
	hu16	V γ 2V δ 3	ATWDG P HYKKL	10.40%	2	A FMFP P VG L LI	36.60%	1
	hu17	Vγ4Vδ1	ATWDGSKKL	0.20%	72	ALGESSLGYWGLADKLI	n.f	n.f
	hu18	V γ 4V δ 1	ATWD A FGWFKI	n.f	n.f	ALGE L ELRLK P TDKLI	3.90%	6
	hu19	V γ 4V δ 3	ATWD C RYKKL	n.f	n.f	A FL P YWGI R KGS D TLTDKLI	n.f	n.f

Table 3.1. $\gamma\delta$ TCR chain pairs recovered from the single-cell PCR analysis. Amino acid sequences and chain usage of CDR3 γ and CDR3 δ sequenced from colonic lymphocytes responding to overnight stimulation with 293T.L3L8 are shown. Bold rows (hu7, hu12 and hu17) indicated $\gamma\delta$ TCR chain pairs successfully transduced into J76 cells in subsequent experiments. Non-germline amino acids are highlighted in red. Rank and frequency of each CDR3 in the deep sequencing dataset are also indicated (n.f: not found).

Donor	V usage	AA sequence
GN08	Vδ1	ALGELGRRRLDLGDTWSTDKLI
	Vδ1	ALGEWARYWGRRTDKLI
	Vδ1	ALGELKSYWFWGILGFDKLI
	Vδ3	CALSVWYWGIRVPTYTDKLI
	Vδ1	ALGENPKRWARILEPLI
	Vδ1	ALGSLPHGAYWGRYTDKLI
	Vδ1	ALGPNQGSPSYVTRADKLI
	Vδ1	ALGETRVYPWGINTDKLI
	Vδ3	ALWWGIRPGLTTYTDKLI
	Vδ1	ALGEPTFLRRFSDKLI
	Vδ1	ALGNRHLLPLYWGFISRADKLI
	Vδ1	ALGELFIDHPLHYWGIFNTDKLI
	Vδ1	ALGETRPSSCRTDKLI
	Vδ1	ALGEPFSLKLI
	Vδ1	ALGELVPFFDWGILPDKLI
GN09	Vγ2	ATWDGHEKL
	Vγ4	ATWDGLYKKL
	Vδ3	AFLHPTAGGSFYDKLI
	Vδ1	ALGELGGGYWGERMDKLI
	Vδ1	ALGEPLSPSYVWAGGYMYTDKLI
	Vδ1	ALGELTFFVLGTTSKLI
GN17	Vγ4	ATWDGLGGGWFKI
	Vγ2	ATWDGPHYKKL
	Vγ4	ATWDRATGWFKI
	Vγ4	ATWDGLGGGWFKI
	Vγ4	ATWDASGWFKI
	Vδ1	ALGESSFSMPYINWGIRDKLI
	Vδ1	GAPGEPLPPDKLI
	Vδ1	ALGELVLLFTGGYRGRLI
	Vδ1	WGTSGGPLHPDKLI
	Vδ1	ALGESFFVSGIPPYTDKLI
	Vδ1	ALGERFELVLGGPIRVTDKLI
	Vδ1	ALGVPLFTDKLI
	Vδ1	ALGERYDGGWPDKLI

Table 3.2. Individual TCR chains recovered from the single-cell PCR analysis. Amino acid sequences of CDR3γ and CDR3δ sequenced from colonic lymphocytes responding to overnight stimulation with 293T.L3L8. Non-germline amino acids are highlighted in red.

2. Deep sequencing of the $\gamma\delta$ TCR repertoire in responding donors

One freshly-excised biopsy from each of the donors used for single-cell sorting was placed in RNAlater. Total RNA was subsequently extracted and sent for deep sequencing of the $\gamma\delta$ TCR repertoire.

2.1 TCR gene usage across donors

From the deep sequencing study, the three most common TCR δ chains found in the intestinal biopsies were V δ 1, V δ 2 and V δ 3 of which *TRDV1* sequences accounted for nearly 70% of the raw productive reads (Fig.3.3). Furthermore, nearly all TCR δ chains utilized the *TRDJ1* gene segment (Fig.3.3).

TRGV4 accounted for approximately 20-30% of all reads, with *TRGV2*, *TRGV4* and *TRGV5* reads almost equally represented, while *TRGV8* was the least frequent read (Fig.3.3). Nearly 80% of all reads contained *TRGJ2* gene segment (Fig.3.3). The fact that *TRGV2*, *TRGV4* and *TRGV5* were comparably represented emphasises that there is some diversity in the γ chain usage of human gut $\gamma\delta$ cells. From this compartment, lymphocytes expressing V γ 4 are enriched in 293T.L3L8-responsive cells, as judged by the single-cell analysis shown above.

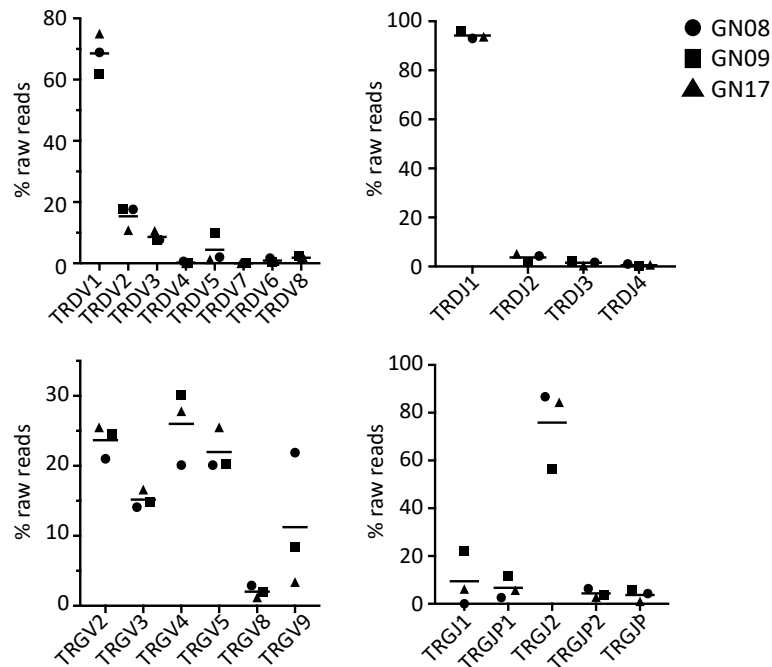


Figure 3.3. Deep sequencing analysis of TCR gene segments. Gene usage was calculated as a per cent of all raw productive reads. Top left – V δ genes. Top right – J δ genes. Bottom left – V γ genes. Bottom right – J γ genes. Lines represents average across three donors – GN08, GN09 and GN17 (key).

2.2. Analysis of the CDR3 sequences

The CDR3 repertoires of donors GN09 and GN17 were dominated by a high number of expanded clones, while that of GN08 was more diverse, with few clonal expansions, albeit that one γ rearrangement was strongly represented (Fig.3.4A). Owing to the preferential expression of *TRGV9* and *TRDV2* in blood $\gamma\delta$ lymphocytes, and the low representation of *TRGV8* and *TRDV4-8*, those sequences were not subjected to further CDR3 analysis.

CDR3 γ length of each analysed γ chain was focused, on average 11 amino acids long after correction for clonal expansion. In contrast, *TRDV1* and *TRDV3* CDR3 δ regions were longer and lacked focusing. There were clear oligoclonal expansions demonstrated by several peaks at various CDR3 δ lengths in the raw copy number analysis which were absent in the number of unique CDR3 sequences analysis (Fig.3.4B).

Since the preferred CDR3 γ length was 11 amino acids, relative diversity analysis was conducted on these sequences. Diversity was similar across different TCR γ chains and donors (Fig.3.4C). The direct fusion of *TRGV* to *TRGJ* would result in a CDR3 region of 11 amino acids. Thus, it was not unexpected for the first four and last four amino acids to be highly conserved across TCR γ chains and donors (Fig.3.4C). In contrast, the middle of the region would be more diverse due to imprecision in the joining of the two genes segments – excision and replacement of germline nucleotides with non-germline nucleotides at the end of the V and the beginning of the J. Of note, approximately half of the CDR3 sequences from the 19 TCR pairs recovered by single-cell PCR could also be identified at variable frequencies in the deep sequencing datasets (Table 3.1).

In sum, V δ 1 was the dominant intestinal TCR δ chain, but there was a comparable representation of several *TRGV* genes, each of which displayed similar relative diversity and focused CDR3 lengths. The representation in the deep sequencing data of the TCRs isolated from the single responding $\gamma\delta$ cells showed that those cells were derived from both common and rare clones. Thus, the capacity to respond to 293T.L3L8 did not correlate with clonal dominance in the whole population, consistent with it being a polyclonal response.

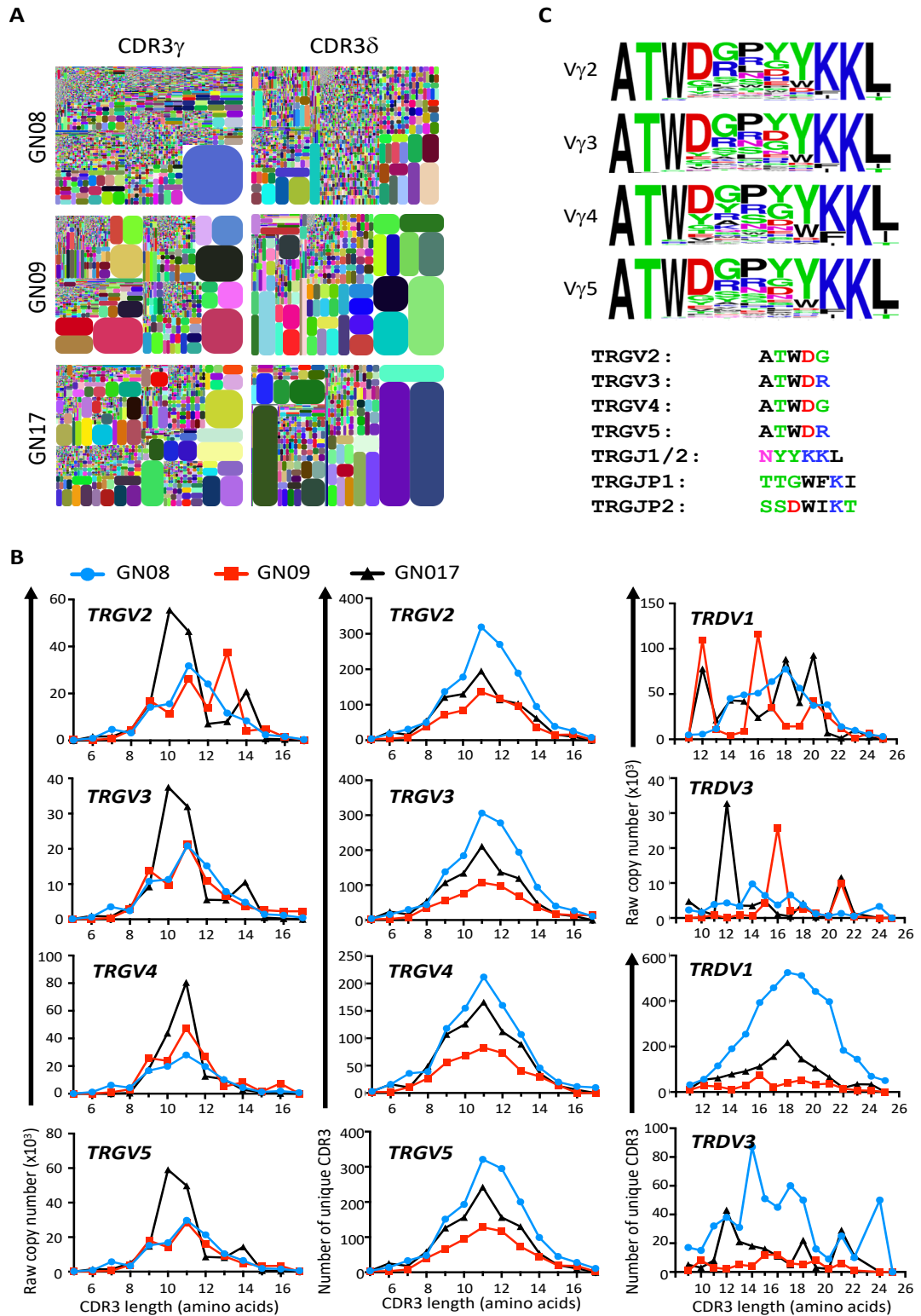


Figure 3.4. Deep sequencing of donor $\gamma\delta$ TCR repertoires. **A.** Tree maps representing the degree of polyclonality of intestinal $\gamma\delta$ lymphocytes across three donors. Each spot on the plot represents a unique CDR3 entry and its size denotes the relative frequency in the dataset. Plots on the left represent the clonality of CDR3 γ sequences and those on the right (legend continues on the next page)

(Figure 3.4 legend continued)

– of CDR3 δ sequences. **B.** CDR3 amino acid length distribution for the indicated *TRGV* or *TRDV* genes represented as raw reads or corrected for clonal expansion as indicated on the y axis. **C.** Relative amino acid composition for the most common CDR3 length (11) across the indicated TCR γ chains in donor GN09. Representative of all donors. Graphs were generated *via* the WebLogo application (black, hydrophobic; green, polar; red, acidic; blue, basic). The germline V γ and J γ amino acids, which form part of the CDR3, are shown for reference below.

3. Cloning of responding TCRs

3.1. TCR cloning strategy

TCRs hu2, hu3, hu7, hu8, hu9, hu12, hu17 and hu19 were chosen for subsequent cloning experiments owing largely to the nature of the expression vectors, described in Materials and Methods. More specifically, J γ 1/2 was the J γ segment used in all plasmids, and hence the chosen TCRs were limited to those utilising J γ 1/2. Furthermore, *AjuI* and *BaeI* were used to clone CDR3 γ and CDR3 δ oligos into the expression vectors. These restriction enzymes are double-cutters and produce sticky ends which impose a limit to the type of CDR3 sequences that can be cloned. Thus, CDR3 γ sequences which can be ligated in the vector must start with the amino acids ATW (alanine, threonine, tryptophan) and end with L (leucine). V δ 1 CDR3 must begin with an A (alanine) and end on KLI (lysine, leucine, isoleucine) and V δ 3 CDR3 must end on KLI.

3.2 Optimization of TCR transductions

The transduction efficiency of TCR $\alpha/\beta^{-/-}$ J76 cells with control pCSIGPW plasmid (empty vector, EV) was poor, and it was even lower for V γ 9V δ 2-pCSIPW encoding a peripheral blood-derived V γ 9V δ 2 TCR (huPB) (Fig.3.5A,B). Therefore, a series of troubleshooting and optimisation steps were carried out.

A JRT3-T3.5 cell line, which is deficient for the TCR β chain, was used as an alternative host. However, this did not improve transduction efficiency which was essentially zero for huPB and 4.7% for EV (Fig.3.5C). Puromycin selection for 7 days failed to enrich for TCR-expressing cells to satisfactory levels (Fig.3.5D). Next, electroporation was used as a means of delivering the target genes but this also did not lead to improved expression (Fig.3.5E). Changing the SFFV promoter for that of CMV yielded similarly disappointing results (Fig.3.5F). Sequencing of the V γ 9V δ 2 chains and IRES did not reveal any mutations (data not shown). Furthermore, transient

transfection of HEK293T cells with all the human CD3 chains and the huPB-pCSIPW vector resulted in the successful expression of a TCR complex on the cell surface (Fig.3.5G). These data suggested that the issue lay in the transduction process and not in the sequences *per se*.

The V γ 9V δ 2-pCSIPW vector size is 10.3kb of which 6.7kb are packaged into the viral particles and incorporated into the host cell line genome. When the sequence to be packaged and incorporated is larger than 6kb, lentiviral particle production and transfection efficiency may dramatically decrease (Cante-Barrett et al., 2016). Therefore, the puromycin resistance gene was removed. This resulted in a 9kb huPB-pCSIW vector of which 5.4kb are packaged and incorporated into the host cell line genome. In initial experiments, 5.6% of the J76 cells transduced with huPB-pCSIW were $\gamma\delta$ TCR⁺ compared to 0% of the cells transduced with huPB-pCSIPW (Fig.3.5H). Based on this encouraging development, several other additional modifications were introduced. *Gag-Pol* encoding vector pCR/V1 was used instead of pCMV Δ R8.91 and the plasmid purification kit was changed from Qiagen Miniprep Kit to Macherey-Nagel NucleoBond Xtra Midi EF kit. The new kit yielded higher purity plasmids which significantly improved transduction efficiency, resulting in >95% TCR⁺ and GFP⁺ Jurkats 72h post-transduction (Fig.3.5I).

Subsequently, V γ 4V δ 1 TCRs hu7, hu12 and hu17 cloned into pCSIW were successfully transduced into J76 cells (Fig.3.6A). However, V γ 4V δ 3 TCRs hu2, hu3, hu8, hu9 and hu19 failed to express (data not shown). Resequencing of the chains and IRES did not reveal any mutations. Transient transfection of HEK293T cells with human CD3 chains and several of the V γ 4V δ 3 vectors did not result in surface expression of a TCR complex. In contrast, TCRs hu7, hu17 and huPB were again successfully expressed (Fig.3.6B). Occasionally, during plasmid preparation, bacteria may recombine regions of the vector resulting in mutations. Therefore, hu2 was digested with XbaI/XhoI and V δ 3-IRES-V γ 4 were transferred into the backbone of hu17. The new plasmid was tested via transduction and transfection. There was still no expression detected on J76 or HEK293T cells (data not shown). Therefore, subsequent experiments were carried out with the V γ 4V δ 1 TCRs: hu7, hu12 and hu17.

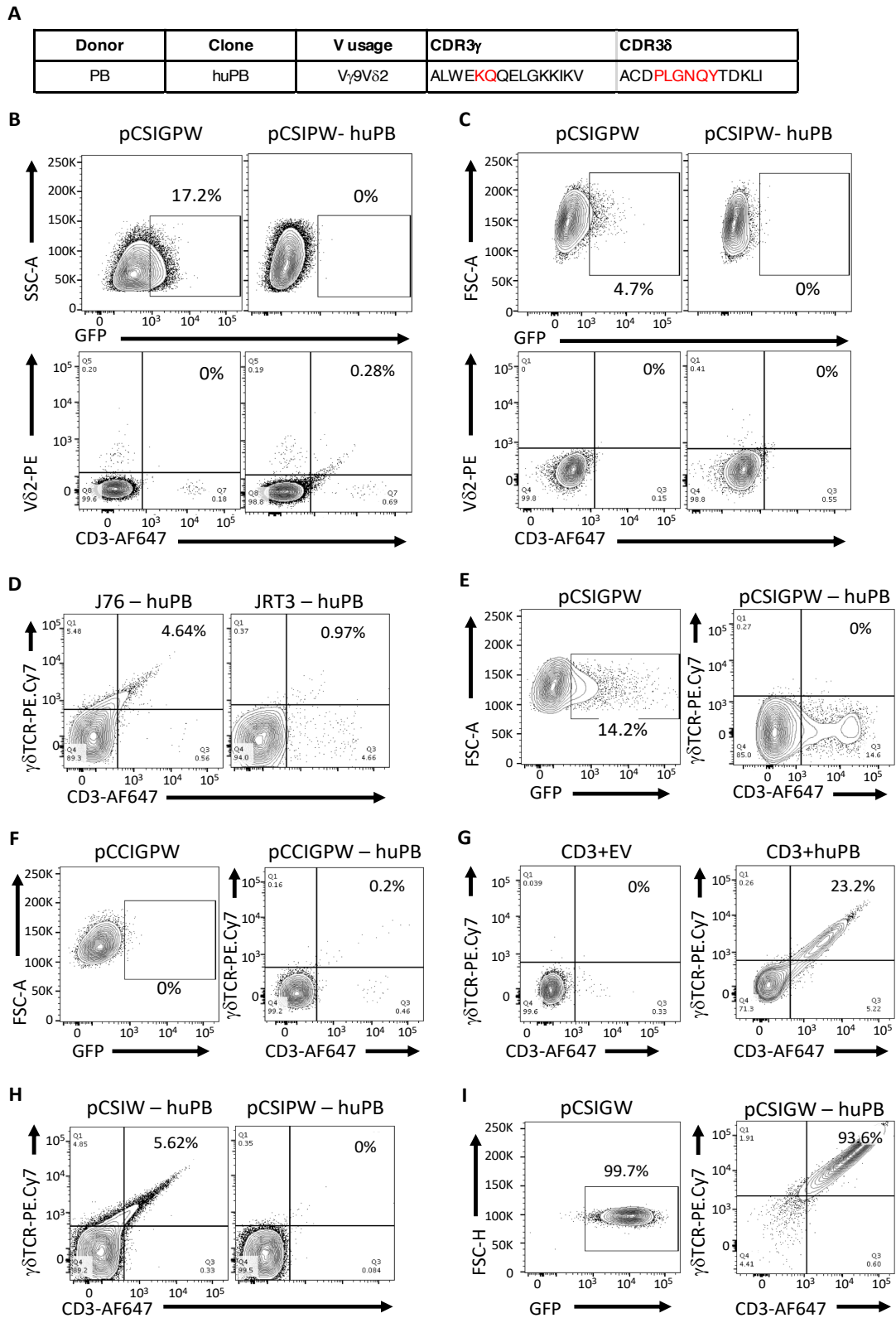


Figure 3.5. TCR transduction troubleshooting and optimisation.

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Figure 3.5. TCR transduction troubleshooting and optimisation. **A.** CDR3 $\gamma\delta$ amino acid sequence of control TCR V γ 9V δ 2 clone huPB. Red – non-germline encoded amino acids. **B.** J76 cells were transduced with pCSIGPW or huPB-pCSIPW and expression was checked 72h later. Top row – GFP expression. Bottom row – TCR expression. **C.** JRT3-T3.5 cells were transduced with pCSIGPW or huPB-pCSIPW and expression was checked 72h later. Top row – GFP expression. Bottom row – TCR expression. **D.** J76 (left) and JRT3-T3.5 (right) were selected with puromycin for 7 days before TCR expression was checked again. Puromycin was added 72h post-transduction. **E.** J76 cells were electroporated with pCSIGPW or huPB-pCSIPW and expression was checked 48h later. Left – GFP expression. Right – TCR expression. **F.** J76 cells were transduced with pCCIGPW or huPB-pCCIPW and expression was checked 72h later. Left – GFP expression. Right – TCR expression. **G.** HEK293T cells were transfected with pCSIGHW vector encoding all human CD3 chains and either pCSIGPW (EV) or huPB-pCSIPW. TCR expression was checked 48h post-transfection. **H.** J76 cells were transduced with huPB-pCSIW (left) or huPB-pCSIPW (right) and expression was checked 72h later. **I.** J76 cells were transduced with pCSIGW (left) or huPB-pCSIW (right) following protocol II from Materials and Methods. Expression was checked 72h post-transduction. All plots are representative of at least two independent transduction or transfection experiments.

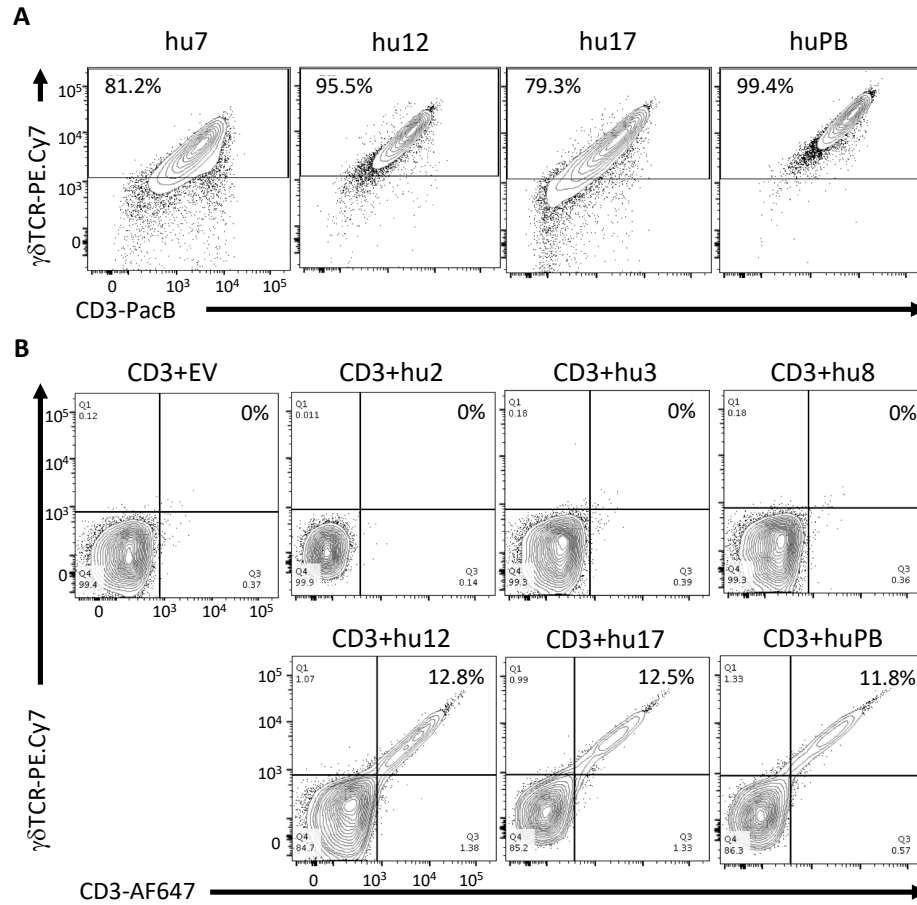


Figure 3.6. Expression of $V\gamma 4^+$ TCRs. **A.** $V\gamma 4V\delta 1$ (hu7, hu12 and hu17) and $V\gamma 9V\delta 2$ (huPB) TCR surface expression in J76 cells 72h post-transduction using protocol II from Materials and Methods. **B.** HEK293T cells were transfected with pCSIHW vector encoding all human CD3 chains and pCSIGW (EV) or the indicated TCR vectors. TCR expression was checked 48h post-transfection. All plots are representative of at least two independent transduction or transfection experiments.

4. Co-culture of TCR-transduced Jurkats with cells expressing BTNL3 and BTNL8

Hu7, hu12 and hu17 displayed diverse CDR3 γ and CDR3 δ sequences with different representation in the deep-sequencing dataset (Table 3.1). J76 transduced with each of these displayed strong TCR downregulation in response to either anti-CD3 stimulation or co-culture with 293T.L3L8 cells. CD69 was upregulated following the same stimuli, although the magnitude was more variable (Fig.3.7A). In stark contrast, J76 expressing huPB (J76-huPB), responded to anti-CD3 stimulation but did not downregulate their TCR or upregulate CD69 when exposed to 293T.L3L8 (Fig.3.7A).

In addition, a JRT3-NFAT reporter cell line was transduced with hu12 or huPB. These cells express the Gaussia luciferase under the control of NFAT which can be activated downstream of TCR signalling. Thus, NFAT activity was measured in the resultant transductants after overnight stimulation with 293T.EV, 293T.L3L8 or PMA (phorbol-12-myristate-13-acetate) and ionomycin. NFAT activity was specifically detected when 293T.L3L8 cells were co-cultured with JRT3-hu12 and not JRT3-huPB (Fig.3.7B). In addition, PLC γ ₁ and LAT phosphorylation were upregulated in J76-hu17 cells co-cultured with 293T.L3L8 (Fig.3.7C).

Likewise, J76-hu12 and J76-hu17 were co-cultured with cell lines of various species and tissue origin transduced with human BTNL3 and BTNL8. Failure to respond to any of the tested cell lines would suggest that additional, unidentified proteins may be required for interaction. This was likely not the case, as J76-hu12 and J76-hu17 responded to BTNL3 plus BTNL8 expressed by murine intestinal MODE-K and fibroblast-derived 3T3 cells, Chinese hamster ovary (CHO) cells and human T lymphocyte-derived J76 and cervical epithelium-derived HeLa cells (Fig.3.8A, B). However, the magnitude of the response appeared to depend on the BTNL expression levels which varied across the cell lines (Fig.3.8B, C). For example, J76 and 3T3 had lower BTNL expression efficiency and thus a weaker response was seen in these co-cultures (Fig.3.A-C).

Finally, two V γ 4V δ 1 TCR clones, sk1 and sk2, were isolated from skin-derived single-cell sorted $\gamma\delta$ T cells (Fig.3.9A). Both J76-sk1 and J76-sk2 transductants showed TCR downregulation and CD69 upregulation when co-cultured with 293T.L3L8 (Fig.3.9B).

In sum, all the data obtained supported the conclusion that the transfer of a V γ 4⁺ TCR to TCR-deficient Jurkat cell line was sufficient to conferred reactivity to BTNL3 plus BTNL8 expressed on cells from different species and tissue origins.

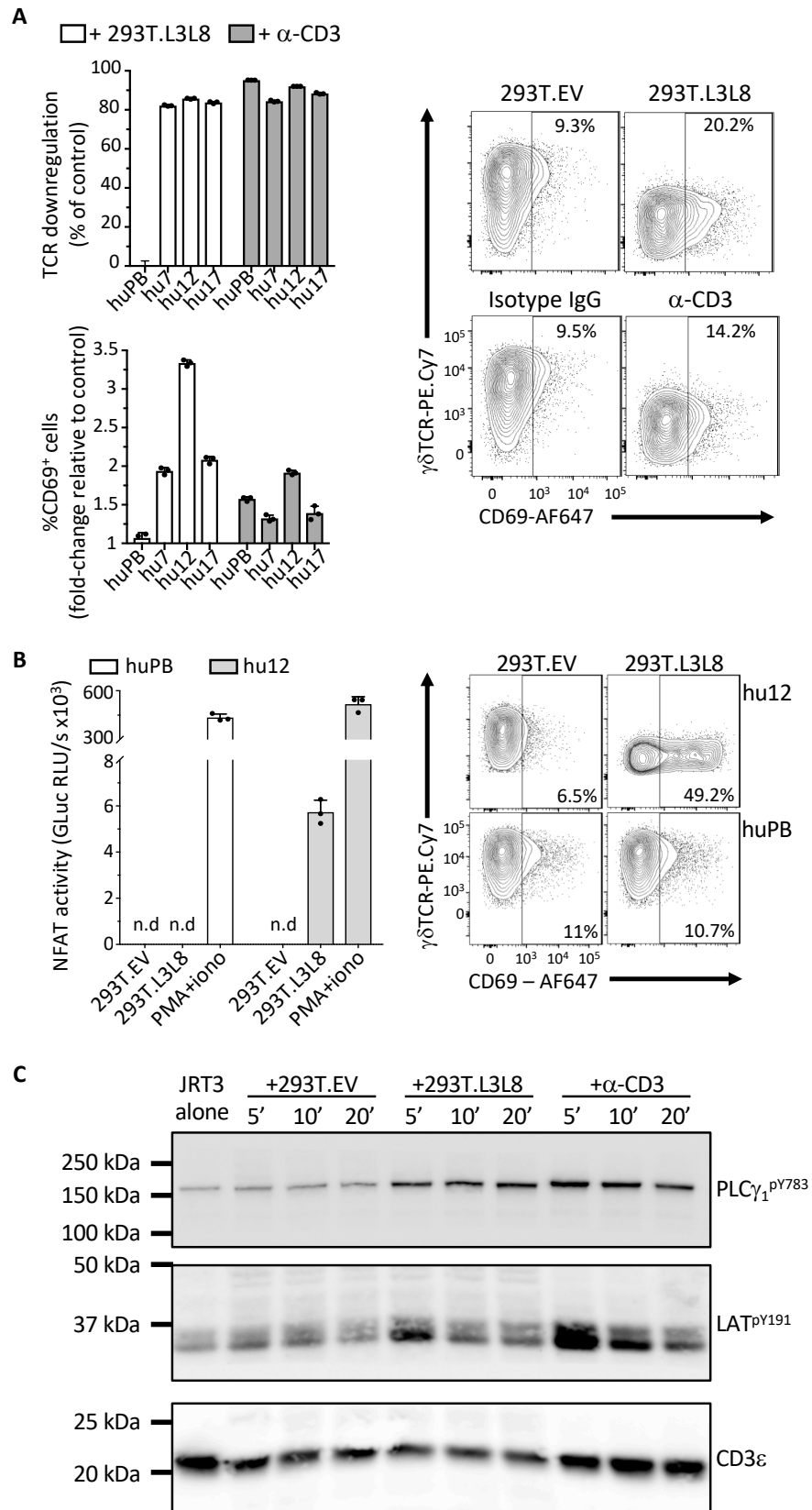


Figure 3.7. The transfer of responding TCRs to J76 cells confers BTNL3 plus BTNL8 reactivity.

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Figure 3.7. The transfer of responding TCRs to J76 cells confers BTNL3 plus BTNL8 reactivity. **A.** Flow-cytometry analysis of $\gamma\delta$ TCR downregulation (top bar chart) and CD69 upregulation (bottom bar chart) in J76 cells transduced with various TCRs (x-axis, details in Table 3) and co-cultured for 5 h with anti-CD3, or 293T.L3L8 cells; results were normalized to those obtained for control co-cultures with IgG or 293T.EV, respectively. Data expressed as mean $\pm$ s.d (n = 3). Each symbol represents an individual co-culture done on the same day. Data are representative of more than three independent experiments. Representative flow plots from J76-hu17 co-cultures in the indicated conditions are shown on the right. **B.** Measurement of NFAT promoter activity (left) in JRT3 reporter cell lines transduced with the indicated TCRs and co-cultured with 293T.EV, 293T.L3L8 or stimulated with PMA and ionomycin for 24h. Gaussia luciferase (GLuc) was measured in supernatants (RLU/s, relative light units per second). Data expressed as mean $\pm$ s.d of three independent co-cultures done on the same day; n.d, non-detectable above background. Cells were analysed in parallel by flow cytometry for $\gamma\delta$ TCR and CD69 expression (right) as a positive control of the response to 293T.L3L8 cells. Representative of two independent experiments. **C.** Western blot analysis of PLC γ_1 and LAT phosphorylation in V γ 4V δ 5⁺ JRT3-LES cells (discussed in detail in Chapter V) unstimulated, co-cultured with the indicated cell lines or stimulated with 10 μ g/ml crosslinked anti-CD3 (UCHT1) for 5min, 10min and 20min at 37°C. CD3 ϵ , loading control.

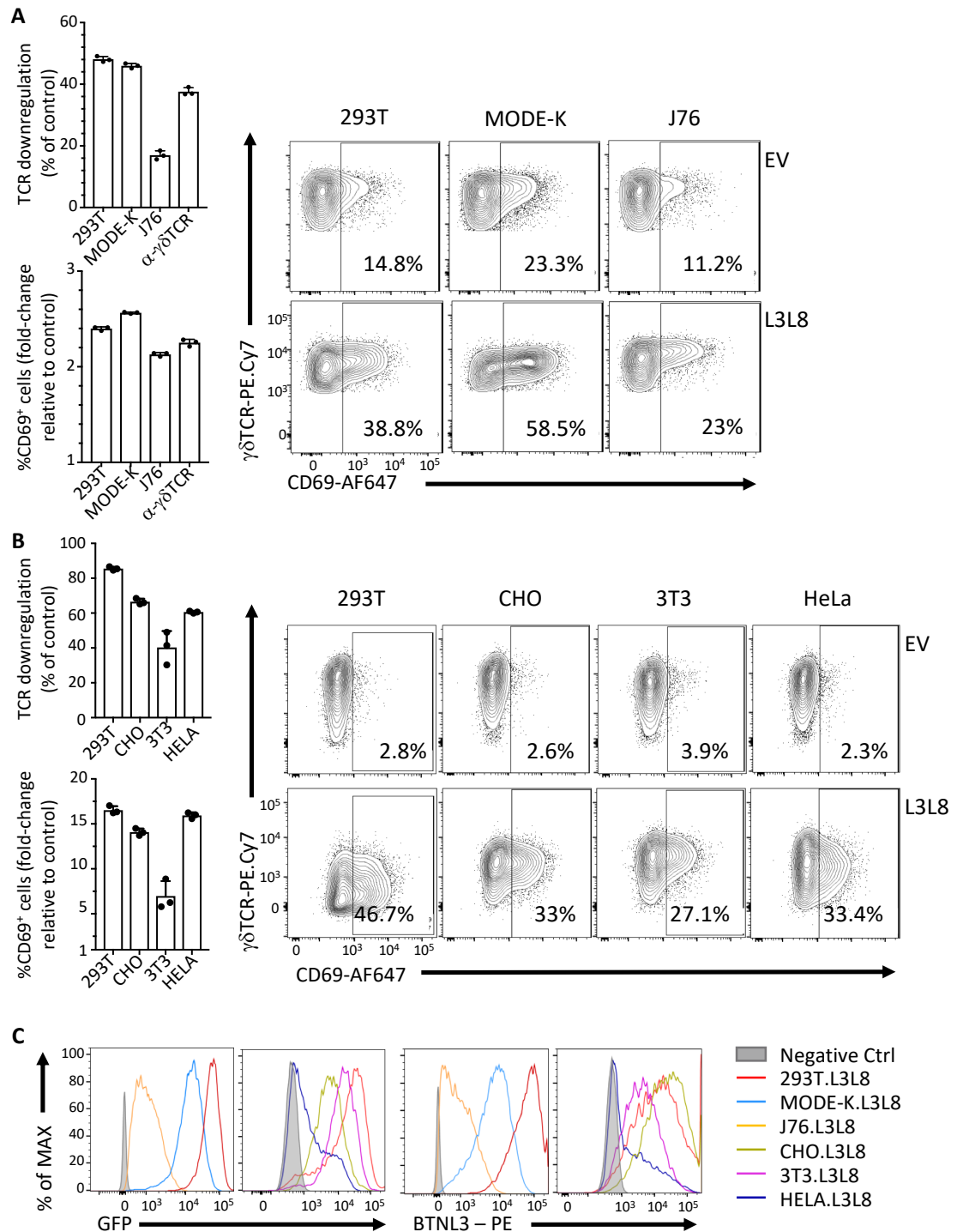


Figure 3.8. $V\gamma 4^+$ J76 cells respond to BTNL3 plus BTNL8-expressing cell lines of various origins. **A.** Flow-cytometry analysis of $\gamma\delta$ TCR downregulation (top bar chart) and CD69 upregulation (bottom bar chart) in J76-hu12 co-cultured for 5 h with anti- $\gamma\delta$ TCR, or the indicated cell lines expressing BNTL3 plus BTNL8; results were normalized to those obtained for control co-cultures with IgG or the respective cell lines expressing EV. Data expressed as *(legend continues on the next page)*

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mean $\pm$ s.d of three independent co-cultures done on the same day. Data are representative of two (J76.L3L8) or three (MODE-K.L3L8, 293T.L3L8) independent experiments. Representative flow plots are shown on the right. **B.** Flow-cytometry analysis of $\gamma\delta$ TCR downregulation (top bar chart) and CD69 upregulation (bottom bar chart) in J76-hu17 co-cultured for 20 h with anti- $\gamma\delta$ TCR, or the indicated cell lines expressing BNTL3 plus BTNL8; results were normalized to those obtained for control co-cultures with IgG or the respective cell lines expressing EV. Data expressed as mean $\pm$ s.d of three independent co-cultures done on the same day. Data are representative of two independent experiments. Representative flow plots are shown on the right. **C.** Flow cytometry analysis of GFP and BTNL3 expression levels in cell lines expressing BNTL3 plus BTNL8 used in **A.** and **B.**

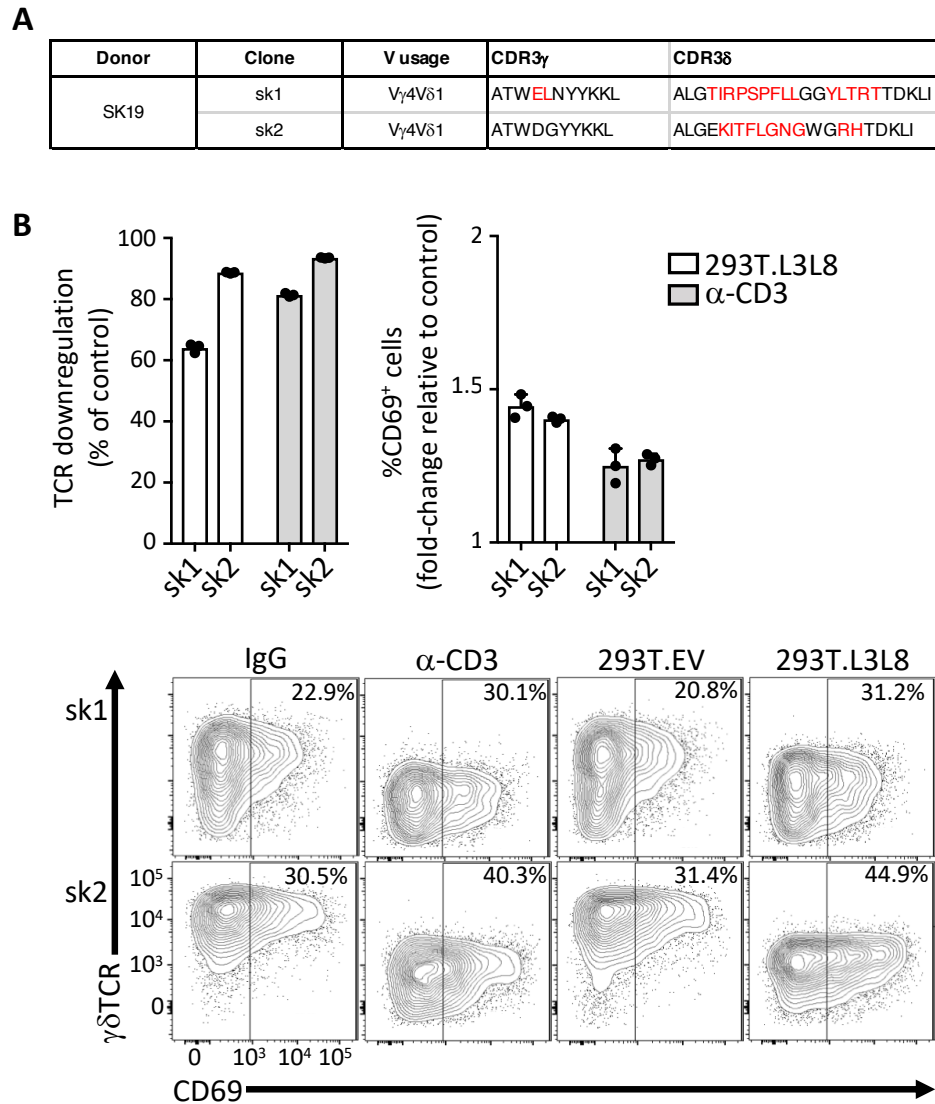


Figure 3.9. Expression of skin-derived V γ 4V δ 1 TCRs confers BTNL3 plus BTNL8 reactivity. A. Amino acid sequences and chain usage of CDR3 γ and CDR3 δ sequenced from single-cell sorted skin $\gamma\delta$ lymphocytes. **B.** Flow-cytometry analysis of $\gamma\delta$ TCR downregulation (left bar chart) and CD69 upregulation (right bar chart) in J76 transduced with the indicated TCRs (x-axis) and co-cultured with and co-cultured for 5 h with anti-CD3, or 293T.L3L8 cells; results were normalized to those obtained for control co-cultures with IgG or 293T.EV, respectively. Data expressed as mean $\pm$ s.d of three independent co-cultures done on the same day. Data are representative of three independent experiments. Representative flow plots are shown below the bar charts.

5. Conclusions

This study demonstrates that the $\gamma\delta$ T cells responding to BTN13 plus BTN18 primarily express the V γ 4 chain paired to V δ 1 or V δ 3. This provides formal confirmation of a prediction made by Di Marco Barros et al. (2016) that was based largely on the exclusion of several other $\gamma\delta$ T cell types. Additionally, the current study presents data that the response is polyclonal, with responsive V γ 4⁺ cells displaying diverse CDR3 regions and variable TCR δ usage.

5.1. Conclusions from population analyses

In mice, the skin is populated by monoclonal V γ 5V δ 1 population and the intestinal epithelium – by oligoclonal V γ 7⁺ IELs. (Asarnow et al., 1988, Kyes et al., 1989). It was shown that these compartments are selected on Btl-family molecules (Boyden et al., 2008, Di Marco Barros et al., 2016). Furthermore, the *in vitro* parallels seen in the Btl/BTNL responses of mouse V γ 7⁺ and human V γ 4⁺ IELs, respectively, suggested that human BTN13 and BTN18 might shape a dominant $\gamma\delta$ compartment similar to that in mice. However, deep sequencing analysis of human intestinal $\gamma\delta$ TCRs in this study revealed a diverse repertoire with almost equal representation of *TRGV2*, *TRGV4* and *TRGV5*, a situation that seems different to the dominance of the mouse IEL compartment by V γ 7⁺ cells.

Certain limitations in the nature of the experiments should be considered. First, the deep sequencing was applied to total mRNA which was extracted from whole tissue in order to gain insight into the status of the cells *in vivo*. This precluded the normalisation of the samples to cell numbers. Hence, the read numbers may reflect either the frequency of the cell expressing the relevant gene or the level of expression of that gene in the cell. Thus, it is possible that *TRGV2*, *TRGV3* and *TRGV5* reads were overrepresented because of this. To circumvent this, genomic DNA might be used as a starting material, but this was not possible for V γ chain estimation since many $\alpha\beta$ T cells carry productively recombined TCR γ chains (Sherwood et al., 2011). Additionally, isotypic exclusion is not efficient at the TCR γ locus, and many $\gamma\delta$ cells harbour productively rearranged mRNAs for more than one V γ chain (Heilig and Tonegawa, 1987).

In this context, it is useful to revisit the data that we (Di Marco Barros et al., 2016) and others (Landau et al., 1995; Mayassi et al., 2019; Eggesbo et al., 2019) obtained using mRNA from sorted intestinal $\gamma\delta$ IELs to characterise their TCR γ usage. While Mayassi et al. (2019) found that *TRGV4* was highly enriched in healthy individuals, 2/8 control patients within their cohort exhibited diverse TCR γ usage with no enrichment for *TRGV4*. Thus, interindividual diversity in the V γ 4 chain usage apparently exists.

Additional differences need to be considered when comparing the data of this study with others in which V γ 4 appeared more dominant. For example, Mayassi et al. (2019) used duodenal biopsies while the experiments here focused on colonic tissue. Of note, studies of the murine gut $\gamma\delta$ compartment is normally focus on the small intestine. Furthermore, they used *ex vivo* isolated sorted V δ 1⁺ and V δ 1⁻ IELs. The deep sequencing data obtained here was from whole tissue biopsy which includes the epithelium, the LP, and other organised lymphoid structures, that together with T cell populations, may vary along the GI tract length (Mowat and Agace, 2014; Thome et al., 2016). Nonetheless, $\gamma\delta$ TCR⁺ lymphocytes are found in relatively low numbers in the LP (Ullrich et al., 1990) and other lymphoid structures. Therefore, these factors are not expected to greatly complicate the data. Finally, the next-generation deep sequencing analysis detects a larger scope of unique CDR3 sequences. In contrast, the cloning and sequencing approach used by others (Mayassi et al., 2019) would only reliably detect common dominant clones. Overall, it appears appropriate to conclude that there is a bona fide V γ chain diversity in the human colonic $\gamma\delta$ compartment, but that in most individuals V γ 4 chain usage is common and is experimentally enriched for when cells are selected based on BTNL3 plus BTNL8 responsiveness. More definite resolution of this issue should in the future be provided by the development and application of a V γ 4-specific monoclonal antibody.

The CDR3 diversity of productively rearranged V γ 4 sequences was not unique, with no evidence for specific amino acids enrichments, compared to other TCR γ chains. Both common (present in the deep sequencing analysis) and rare (absent from the deep sequencing analysis) TCR chains were recovered from the single-cell PCR of 293T.L3L8-responsive cells. Moreover, skin-derived V γ 4V δ 1 TCRs were also BTNL-responsive. Taken together, these data suggest that the CDR3 loops are not critically involved in BNTL3 plus BTNL8 reactivity and that every V γ 4⁺ cell, irrespective of the delta chain or of its anatomical origin, may have the capacity to respond. Moreover, the fact that primary V δ 1⁻V δ 2⁻ V γ 2/3/4⁺ lymphocytes were capable of responding to the BTNL molecules clearly demonstrates no obligate requirement for V δ 1 usage.

5.2. Conclusions from single TCR analyses

The transfer of BTNL3 plus BTNL8-responding TCRs to J76 cells formally demonstrated that a V γ 4⁺ TCR could confer reactivity. TCR downregulation was the clearest readout while CD69 upregulation was always present but more variable across experiments, both in response to the BTNL proteins and in response to anti-CD3. This could be the result of TCR downregulation acting as a negative feedback mechanism, dampening downstream TCR signalling responses to varying degrees. CD69 baseline levels of cultured J76 also affected the outcome of the co-culture assay,

stronger upregulation generally occurring for transductants with lower background levels. Of note, basal CD69 expression tended to be greater when J76 transductants were maintained at higher confluency. This might be the result of increased stress levels due to reduced nutrients in the medium. Alternatively, the transfer of a $\gamma\delta$ TCR may also confer some level of auto-reactivity, not present in the untransduced Jurkats. Consequently, higher confluency would lead to a higher chance of cell-to-cell contact and interaction with yet unidentified antigen.

The presence of a response to BTNL-expressing cell lines from different species and tissue origin strongly supported the notion that the TCR may not only be necessary but also be sufficient for BTNL3 plus BTNL8 reactivity. While there was some variability in the level of response, this could be attributed to the variable efficiencies with which the BTNL molecules were expressed on the cell surface. An equal expression could not be achieved even after cell sorting as some cell lines either showed a natural tendency to silence BTNL3 and BTNL8 (J76 cells) or failed to efficiently express the proteins on the cell surface (3T3 cells). The capacity of different cell lines from different species to support BTNL3 plus BTNL8 reactivity compares to the BTNL3A1-dependent phosphoantigen response of peripheral blood V γ 9V δ 2 cells. In that setting, *BTNL3A1*-transduced mouse cells could not provoke a phosphoantigen response unless they were co-transduced with *BTNL2A1* (Rigau et al., 2020). In both cases a combination of BTNL/BTNL genes drives T cell responses defined by the use of a specific V γ chain. However, whereas phosphoantigen response is also strongly influenced by CDR3 γ and the use of V δ 2, our results argue that V γ 4 may be the primary and/or sole determinant of BTNL3 plus BTNL8 reactivity. In this regard, the occurrence of V γ 2 sequences in the single-cell PCR analysis will be considered as part of the next chapter.

Chapter IV. Dissecting the requirements for TCR-BTNL interaction

Having established the capacity of $V\gamma 4^+$ TCRs to confer BTN3 plus BTN8 reactivity on Jurkat cells, the underlying mechanism was investigated next. The antigen specificity of immunoglobulins and TCRs lies primarily in the CDR3 regions. However, the data presented in the preceding chapter showed that reactivity was a property of cells with diverse CDR3 lengths and sequence composition. This is somewhat in contrast to murine $\gamma\delta$ TCRs regulated by Btnls as they display an essentially monoclonal repertoire in the skin (Asarnow et al., 1988; Lewis et al., 2006; Boyden et al., 2008; Barbee et al., 2011; Turchinovich and Hayday 2011) and restricted diversity in the gut (Kyes et al., 1989; Di Marco Barros et al., 2016). Likewise, we have shown (Chapter III) that the response to BTN3 plus BTN8 did not require a specific $V\delta$ chain. Collectively, these results argue that the determining feature of BTN3 plus BNL8 reactivity may lie in the germline encoded sequence of the $V\gamma 4$ chain which distinguish it from other *TRGV* genes. This is investigated in this chapter, primarily by the use of TCR mutagenesis.

1. BNL-responsiveness is dependent on sequences within the CDR2 and HV4 loops of the $V\gamma 4^+$ chain

Two of the 19 unique TCR chain pairs recovered from the single-cell PCR expressed $V\gamma 2$ which differs by only nine amino acids from $V\gamma 4$ (Fig.4.1A). Hence, it was important to determine whether $V\gamma 2$ could also confer reactivity to BTN3 plus BTN8. However, the $V\gamma 2$ TCR sequences were not suitable for cloning into our expression system, for practical reasons described in the previous chapter. Therefore, to test whether $V\gamma 2^+$ TCRs were also able to confer-BTN3 plus BTN8 reactivity, a hu17 chimeric receptor was generated in which a $V\gamma 2$ chain replaced $V\gamma 4$ (hu17. $V\gamma 2$) (Fig.4.1B). The full-length TCR δ chain, CDR3 γ and C γ were identical across hu17 and hu17. $V\gamma 2$ (Fig.4.1B). The resultant J76-hu17. $V\gamma 2$ transductants showed stable expression of the introduced TCR but that was not downregulated in response to co-culture with 293T.L3L8 cells, nor was CD69 upregulated (Fig.4.1C). In contrast, hu17 TCR expressing J76 cells predictably downregulated their TCR and upregulated CD69 (Fig.4.1C). Such results are from this point expressed by plotting TCR downregulation *versus* CD69 upregulation (Fig.4.1C-F). Thus, $V\gamma 2^+$ TCRs recovered from the single-cell sort most likely derived from cells that at the time of the sorting were expressing low TCR levels for reasons other than BTN3 plus BTN8-dependent

downregulation. It may be concluded that reactivity is specific for V γ 4⁺ cells. Moreover, the very few sequence differences between V γ 4 and V γ 2 offered an opportunity to establish the determinants of V γ 4 reactivity.

Three of the amino acid differences between V γ 2 and V γ 4 are found within CDR1 and CDR2. In $\alpha\beta$ TCRs, these loops are known to contact MHC proteins (Cole et al., 2014). Therefore, the involvement of CDR1 and CDR2 in BTNL-responsiveness was investigated. A single amino acid change was introduced in CDR1 of hu17 to generate a chimeric V γ 4 receptor with a V γ 2 CDR1 (hu17.V γ 2^{CDR1}). Similarly, two amino acid changes were introduced in CDR2 of hu17 to generate a V γ 4 TCR with a V γ 2 CDR2 (hu17.V γ 2^{CDR2}) (Fig.4.1B). Additionally, both regions were mutated to generate hu17.V γ 2^{CDR1+2} (Fig.4.1B). The resultant J76 transductants successfully downregulated TCR expression and upregulated CD69 when co-cultured with 293.L3L8 cells (Fig.4.1C). Thus, the failure of V γ 2 to confer BTNL3 plus BTNL8 responsiveness is not due to its CDR1 and/or CDR2 sequences.

Four of the nine amino acid differences between V γ 2 and V γ 4 are found in a solvent-exposed region between CDR2 and CDR3 that is often called hypervariable loop 4 (HV4). HV4 is highly divergent across the TCR γ chains (Fig.4.1A). Therefore, HV4 chimeric hu17 TCRs were generated, in which all four amino acids were swapped for their V γ 2 chain counterparts (hu17^{DGKM>YANL}) or in which mutations were introduced in pairs (hu17^{DG>YA} and hu17^{KM>NL}) (Fig.4.1B). J76-hu17^{DGKM>YANL} completely failed to respond to co-culture with 293T.L3L8 cells, phenocopying J76-hu17.V γ 2 transductants (Fig.4.1D). In fact, exchanging just the first two amino acids (DG>YA) in the sequence was sufficient to abolish the response, while a change in the second pair (KM>NL) had a minor effect on TCR downregulation (Fig.4.1D). Importantly, the exchange of YA for DG in the V γ 2 chain (hu17.V γ 2^{YA>DG}) was sufficient to confer BTNL3 plus BTNL8 reactivity (Fig.4.1B, E).

The V γ 3 chain is even more divergent from V γ 4 than V γ 2 is (Fig.4.1A). Thus, hu17 TCR chimaeras encoding a V γ 3 chain (hu17.V γ 3) predictably failed to respond in co-cultures with 293T.L3L8 (Fig.4.1B, E). The replacement of the V γ 3 HV4 with that of the V γ 4 HV4 (hu17.V γ 3-V γ 4^{HV4}) partially restored reactivity (Fig.4.1E). When considering what might be required for full wild-type response, we reasoned that the high sequence similarity between V γ 2 CDR2 and V γ 4 CDR2 (Fig.4.1A) might have masked the contribution of this loop to BTNL responsiveness when V γ 2-V γ 4 chimaeras were assessed. Indeed, because V γ 3 CDR2 is more divergent, a hu17 TCR was generated, expressing a V γ 3 chain in which the entire region including CDR2 to HV4 sequences was replaced with that of a V γ 4 chain (hu17.V γ 3-V γ 4^{CDR2+HV4}) (Fig.4.1B). J76-hu17.V γ 3-V γ 4^{CDR2+HV4}

transductants completely phenocopied wild type J76-hu17 cell when co-cultured with 293T.L3L8 cells (Fig.4.1F).

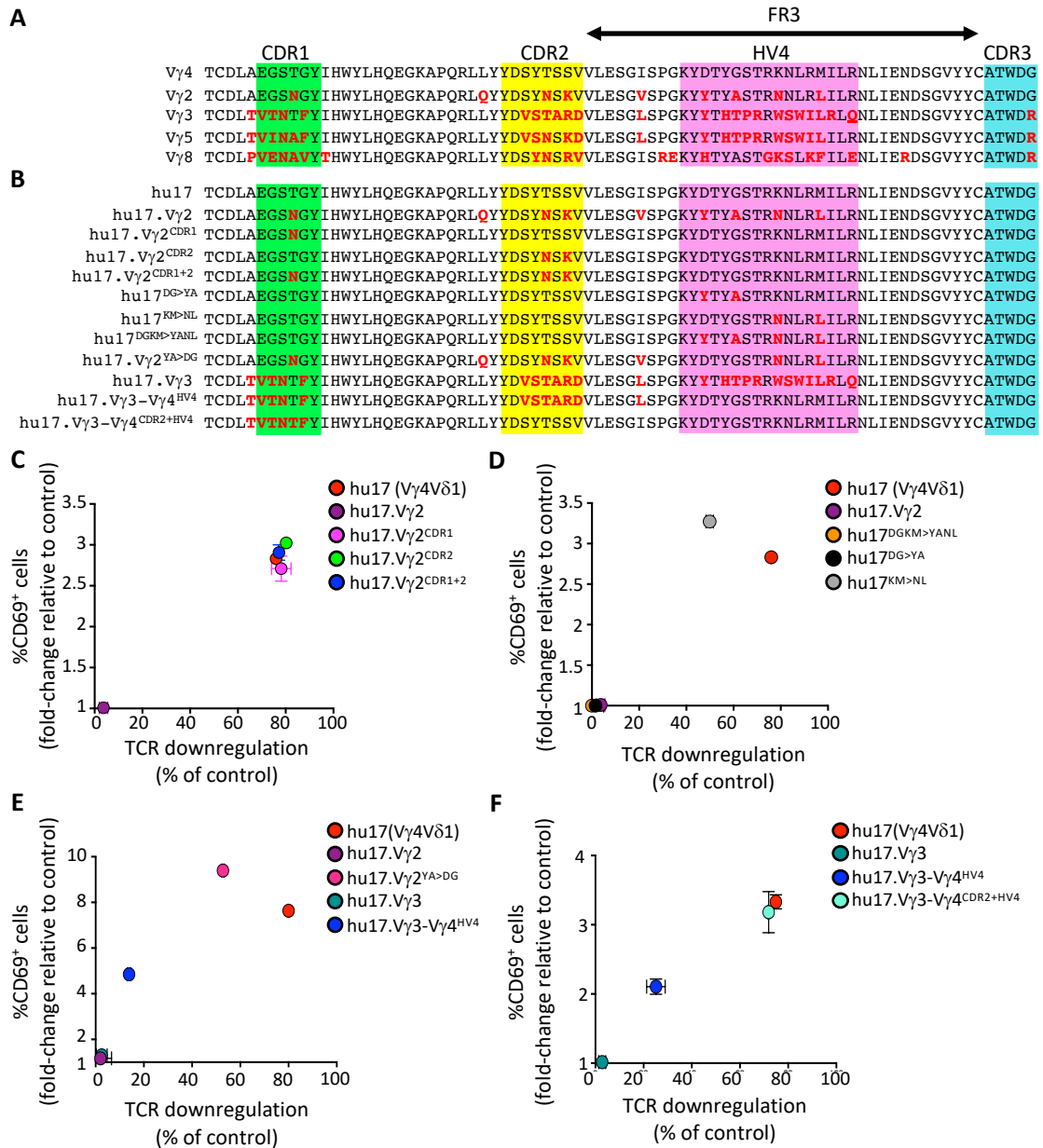


Figure 4.1. BTNL responsiveness is mediated by the TCR V γ 4 CDR2-HV4 loops. **A.** Alignment of the amino acid sequences of human TCR γ chains: red font indicates divergence from V γ 4; gaps, asterisks (*), periods (.) and colons (:) follow the standard ClustalW designations for amino acid comparisons; shading indicates variable regions (green, CDR1; yellow, CDR2; pink, HV4; blue, CDR3); horizontal arrow (above) delineates FR3. **B.** Alignment of the h17 TCR chimaeras tested in **C-F**. All TCRs had identical CDR3 γ , C γ and full-length TCR δ chain. Colour coding as in **A**. **C-F.** Flow-cytometry analysis of TCR downregulation (horizontal axis) plotted against that of CD69 upregulation (vertical axis) in J76 cells transduced with wild- (legend continued on the next page)

(Figure 4.1. legend continued)

type hu17 or its variants (key) and co-cultured for 5 h with 293T.L3L8 cells; results were normalized to those obtained by co-culture with 293T.EV cells. Data are representative of two (C), three (D and F) or four (E) independent experiments (mean $\pm$ s.d. of three independent co-cultures done on the same day).

Several of the hu17 constructs were also introduced into the JRT3-NFAT reporter cell line and NFAT activity was measured. The results were largely consistent with those obtained from J76 transductants, although the response of hu17.V γ 3 was almost completely rescued by V γ 4 HV4 (hu17.V γ 3-V γ 4^{HV4}), emphasising the value of examining constructs in different experimental settings (Fig.4.2). Finally, a chimeric receptor was constructed, in which the human V γ 4 HV4 sequence was used to replace the counterpart HV4 sequence in a V γ 7⁺ TCRs (mo5) derived from murine IEL (mo5.huV γ 4^{HV4}). The data showed that the human HV4 sequence was sufficient to abolish the reactivity of J76 transductants toward mouse Btln1 plus Btln6, but to confer some level of responsiveness (particularly CD69 upregulation) to human BTNL3 plus BNTL8 (Fig.4.3A, B). Taken together, these data establish the amino-terminal portion of V γ 4 HV4 as the most critical element mediating BTNL3 plus BTNL8 reactivity of V γ 4⁺ cells. HV4 was sufficient to confer BTNL3 plus BTNL8 responsiveness to the other TCRs, although CDR2 also made some contribution. Importantly, HV4 and CDR2 are both germline-encoded sequences present in all V γ 4 chains, consistent with BTNL3 plus BTNL8 eliciting a polyclonal response from V γ 4⁺ lymphocytes. Moreover, molecular modelling based on several TCR structures places HV4 spatially adjacent to CDR2, offering a potential interaction face for binding to BTNL3 plus BTNL8 (Melandri et al., 2018).

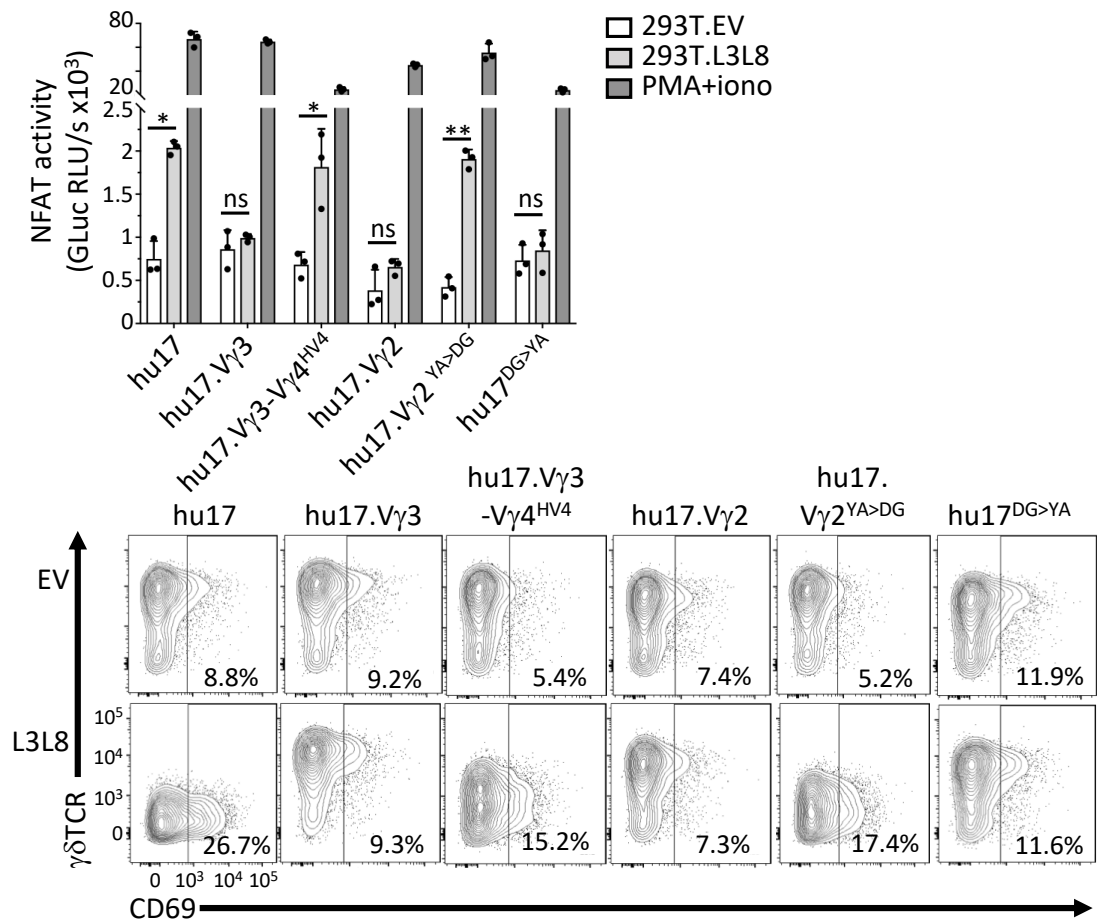


Figure 4.2. NFAT activity in JRT3 cells transduced with the hu17 TCR and its variants. Measurement of NFAT promoter activity (top) in JRT3 reporter cell lines transduced with the indicated TCRs and co-cultured with 293T.EV, 293T.L3L8 or stimulated with PMA and ionomycin for 24h. Gaussia luciferase (GLuc) was measured in supernatants (RLU/s, relative light units per second). Data expressed as mean±s.d of three independent co-cultures. Cells were analysed in parallel by flow cytometry for γδTCR and CD69 expression (bottom) as a positive control of the response to 293T.L3L8 cells. *P < 0.05; **P < 0.01; ns, not significant. Analysed by paired two-tailed Student's t-test.

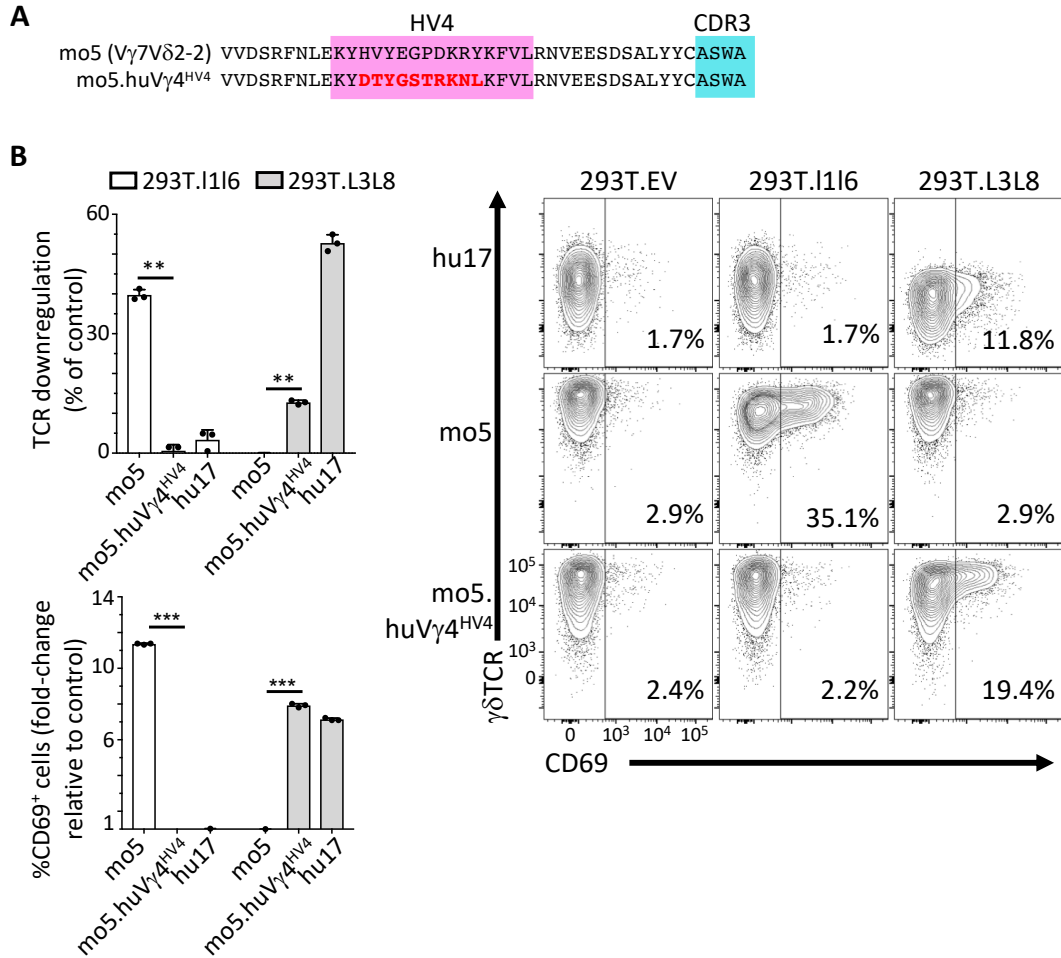


Figure 4.3. BTNL3/8 and Btln1/6 reactivity of human-murine TCR chimaeras. **A.** Alignment of mouse V γ 7V δ 2-2 clone mo5 and its human chimeric TCR sequence. Both TCRs had identical CDR3 γ , C γ and full-length TCR δ chain. Pink, HV4; blue, CDR3. Red bolded font indicates divergence from V γ 7. **B.** Flow-cytometry analysis of $\gamma\delta$ TCR downregulation (top bar chart) and CD69 upregulation (bottom bar chart) in J76 cells transduced with various TCRs (x-axis) and co-cultured for 5 h with 293T.L3L8 cells or 293T.L1I6 cells (key); results were normalized to those obtained for control co-cultures with 293T.EV. Data expressed as mean $\pm$ s.d of three independent co-cultures. Each symbol represents an individual co-culture done on the same day. Data are representative of more than three independent experiments. Representative flow plots are shown on the right. **P < 0.01 and ***P < 0.001. Analysed by paired two-tailed Student's t-test. (*Generation of 293T.L1I6, cloning and expression of mo5 and mo5.huV γ 4^{HV4} were done by Daisy Melandri, King's College London*)

2. V γ 4⁺ cell responses are dependent on sequences in the CFG face of BTN3L

2.1. V γ 4V δ 1⁺ J76 co-cultures with wild-type and BTN3L CFG-mutant 293T.L3L8

Normally, BTN3L and BTN8 require each other in order to be efficiently expressed on the cell surface (Di Marco Barros et al., 2016; Vantourout et al., 2018). Nevertheless, under strong transduction or transfection efficiencies, it is possible to force the expression of BTN3L and BTN8 individually on the cell surface of HEK293T cells (293T.L3 and 293T.L8). J76-hu17 transductants co-cultured with 293T.L3 showed partial TCR downregulation together with CD69 upregulation while co-cultures with 293T.L8 did not result in a response (Fig.4.4A). This suggested that BTN3L might have a more direct impact on the TCR than BTN8, consistent with a previously proposed model where one BTN(L) is “functional” and one is “accessory” (Vantourout et al., 2018). Based on this consideration, extracellular regions of BTN3L versus BTN8 were investigated for their potential to mediate responses.

In work undertaken by Pierre Vantourout and our collaborators, Raphael Chaleil and Paul Bates, a model for BTN3L-BTN8 heterodimer was derived from the crystal structure of a BTN3A1 homodimer (PDB accession code 4F80) using the homology program 3D-JIGSAW (Bates et al., 2001). Candidate solvent-exposed motifs which differed in the IgV domains of BTN3L versus those of BTN8 were identified (Fig.4.4B, C). The IgV domain is composed of several β -strands (A, B, C, C', C'', D, E, F, G) (Fig.4.4B, C) distributed between two β -sheets with topology and connectivity which forms a β -barrel. In this context, NQFHA from the C strand, EDWESK from the C'' strand, WF from the F strand and DEEAT from the G strand from BTN3L were replaced by their BTN8 counterparts GQFSS, KDQPFM, RI and YQKAI, respectively. Each of these chimaeras was co-expressed on the cell surface with wild-type BTN8 to similar levels (Melandri et al., 2018) and the resultant cell lines were co-cultured with J76-hu17. Only the 293T.L3^{KDQPFM}L8 cell line provoked a wild-type response in J76-hu17 cells, whereas co-cultures with the other BTN3L variants completely abolished the response (Fig.4.4D). Therefore, amino acids in the CFG face of the BTN3L IgV domain mediate V γ 4 reactivity toward BTN3L plus BTN8. Of note, this part of the immunoglobulin fold is a well-established facilitator of protein-protein interactions (Holness and Simmons, 1994).

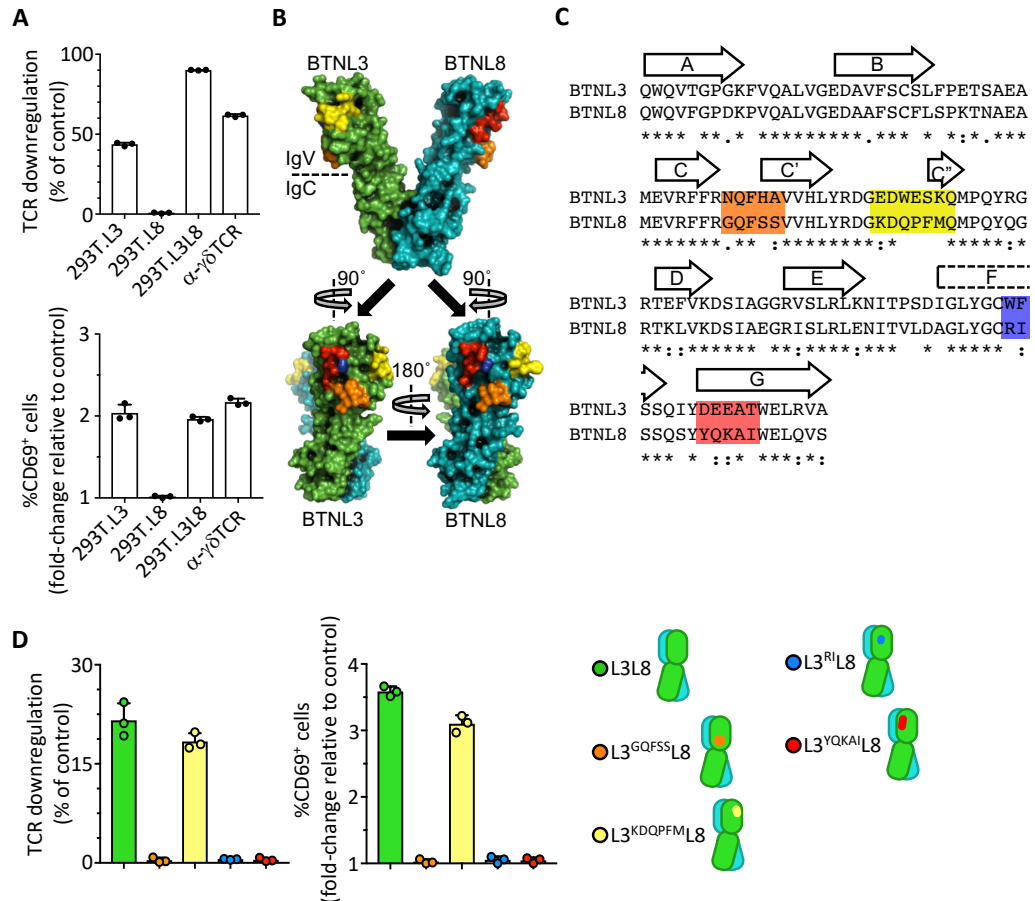


Figure 4.4. BTNL3 CF face mediates responses in $V\gamma 4^+$ Jurkats. **A.** Flow-cytometry analysis of TCR downregulation (top) and CD69 upregulation (bottom) in J76-hu17 cells co-cultured with the indicated cell lines or and anti- $\gamma\delta$ TCR antibody (x-axis) for 5h. Results were normalized to those obtained for control co-cultures with 293T.EV or isotype control. Data expressed as mean $\pm$ s.d of three independent co-cultures done on the same day. Representative of three independent experiments. **B.** Heterodimeric model of BTNL3 (green)–BTNL8 (teal), derived from a BTN3A1 homodimer (PDB accession code 4F80) using the 3D-JIGSAW online homology modelling tool; orange, yellow, blue and red indicate candidate motifs described in **C.** (*The model was generated by Dr Raphaël Chaleil, The Francis Crick Institute, see Melandri et al., 2018*) **C.** Alignment of BTNL3 and BTNL8 IgV domains. Canonical immunoglobulin fold β -strands are indicated (A, B, C, C', C'', D, E, F). Orange (NQFHA-GQFSS), yellow (EDWESKQ-KDQPFMQ), blue (WF-RI) and red (DEEAT-YQKAI) highlight solvent-exposed regions which are divergent across the two proteins. **D.** Flow-cytometry analysis of TCR downregulation (left) and CD69 upregulation (right) in J76-hu17 cells co-cultured for 5h with HEK293T transfected with wild-type BTNL8 together with wild-type BTNL3 or the indicated mutants (key). Results were normalized to those obtained for control co-cultures with 293T.EV. Data expressed as mean $\pm$ s.d of three independent co-cultures done on the same day. Representative of three independent experiments. (*Experiment was performed by Dr Pierre Vantourout, King's College London*)

2.2. Soluble TCR staining of wild-type and BTNL3 CFG-mutant 293T.L3L8

The above experiments allowed for the identification of putative interacting regions of V γ 4 and BTNL3, but they do not provide direct evidence for the two proteins' binding. To address this by a different method, we employed soluble, recombinant monomeric V γ 4V δ 1, V γ 4V δ 2, V γ 2V δ 1 and V γ 8V δ 1 TCRs. Strikingly, the former two soluble TCRs (sTCRs) stained 293T.L3L8 cells in a dose-dependent manner, whereas the latter two did not (Fig.4.5A, B). Furthermore, the staining was abolished when HEK293T cells expressed chimeric BTNL3^{GQFSS}, BTNL3^{RI} and BTNL3^{YQKAI} (Fig.4.5C) which was consistent with the functional data obtained for these constructs (Fig.4.4D). Of note, TCR staining was decreased but not absent on BTNL3^{KDQPFM} expressing cells (Fig.4.5C) consistent with them retaining the capacity to elicit responses in J76-hu17 cells (Fig.4.4D).

In sum, these findings establish a strong correlation between the capacity of V γ 4⁺ cells to respond to BNTL3 plus BTNL8 and the capacity of V γ 4⁺ TCRs to physically engage BTNL3-expressing target cells. This correlation could best be explained by direct binding of V γ 4 by BTNL3 *via* sequences on the C, F and G strands of BTNL3. Although the sequence EDWESK from the C'' strand was not so strongly implicated, it appeared to influence the interaction of BTNL3 and V γ 4. Taking this further, an analysis of the BTNL3-BTNL8 heterodimer model revealed that the N-termini of BTNL3 and of BTNL8 are in close proximity to their respective CFG faces (Fig.4.6A). As BTNL3 and BTNL8 expressed in HEK293T cells were FLAG- and HA-tagged, respectively, at the N-terminus, anti-FLAG and anti-HA antibodies were tested for their ability to interfere with BTNL-V γ 4 interactions. Anti-FLAG efficiently blocked soluble V γ 4V δ 1 binding to 293T.L3L8 cells in a dose-dependent manner, while neither anti-HA nor isotype control had a similar effect (Fig.4.6B). Likewise, anti-FLAG, but not anti-HA or isotype control, was able to inhibit J76-hu17 TCR downregulation in response to co-culture with HEK293T transiently expressing BNTL3 plus BTNL8 (Fig.4.6C). There was also a limited effect on CD69 upregulation, probably reflecting the fact that the concentrations of anti-FLAG antibody used in these experiments did not completely abolish TCR downregulation, thereby permitting downstream signalling and CD69 upregulation (Fig.4.6C). Nevertheless, the differential impacts of anti-FLAG and anti-HA are completely consistent with a model in which an N-terminus proximal region of BTNL3 mediates interactions preferentially with the HV4-CDR2 region of V γ 4.

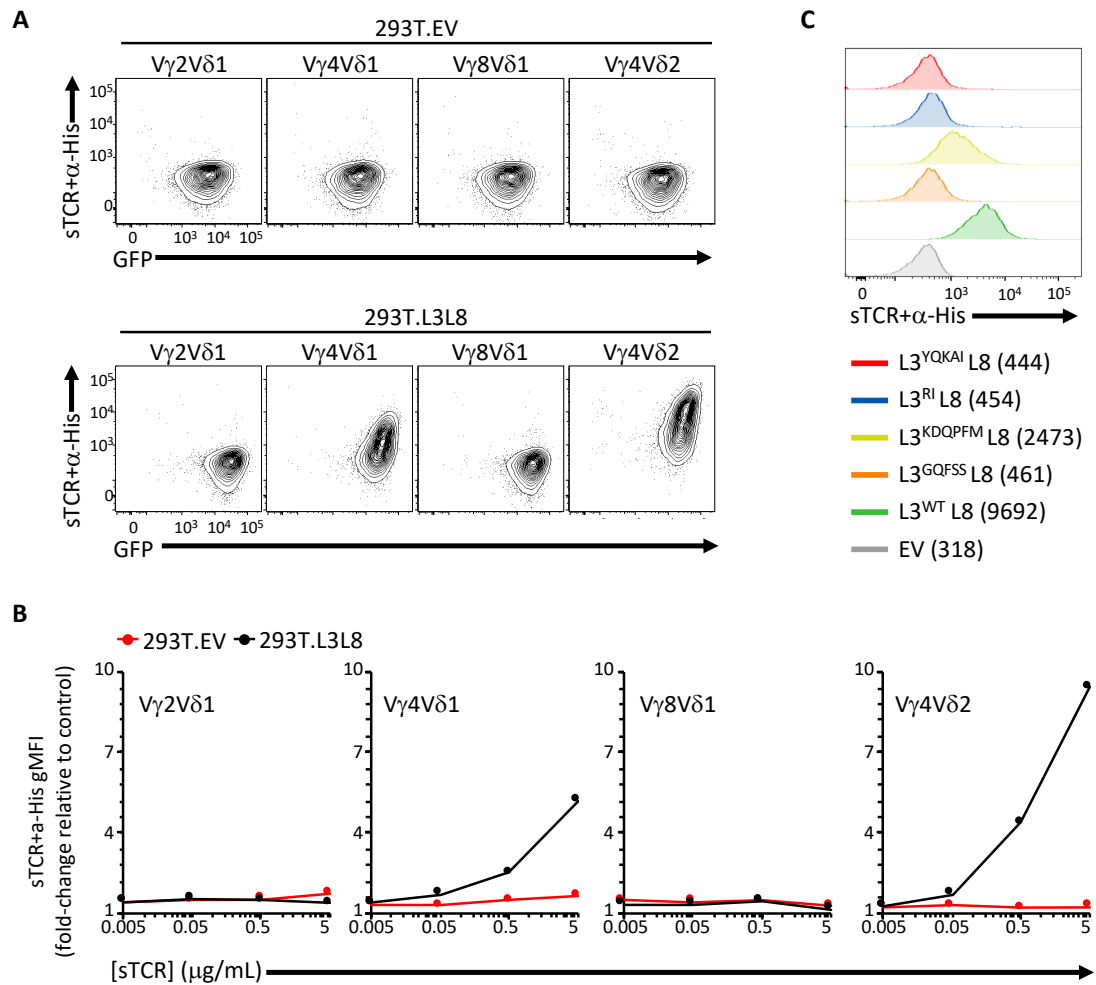


Figure 4.5. Soluble V γ 4⁺ TCRs bind BTN1L3. **A.** Soluble TCRs (indicated above plots) were pre-incubated with anti-His tag antibody and used to stain 293T.EV (top) or 293T.L3L8 (bottom) cells at 4°C for 1h. Flow cytometry plots are shown. Data are representative of three independent experiments (*Experiments were performed by Dr Pierre Vantourout, King's College London*) **B.** Flow-cytometry analysis of 293T.EV or 293T.L3L8 cells (key) stained by various concentrations (x-axis) of soluble TCRs (top left in the plot; pre-incubated as in **A**); results are presented as gMFI of staining with the soluble TCR plus antibody to histidine, normalized to staining with the secondary antibody to histidine alone. Data are presented as mean $\pm$ s.d. of three individual stainings. (*Experiments were performed by Dr Pierre Vantourout, King's College London*). **C.** Flow-cytometry analysis of HEK293T cells transduced with empty vector (EV), or different BTN1L3 constructs and wild-type BTN1L8 (key below plot) and stained with 10 μ g/ml of soluble V γ 4V δ 2. Numbers in brackets represent the gMFI for sTCR+anti-His-tag staining.

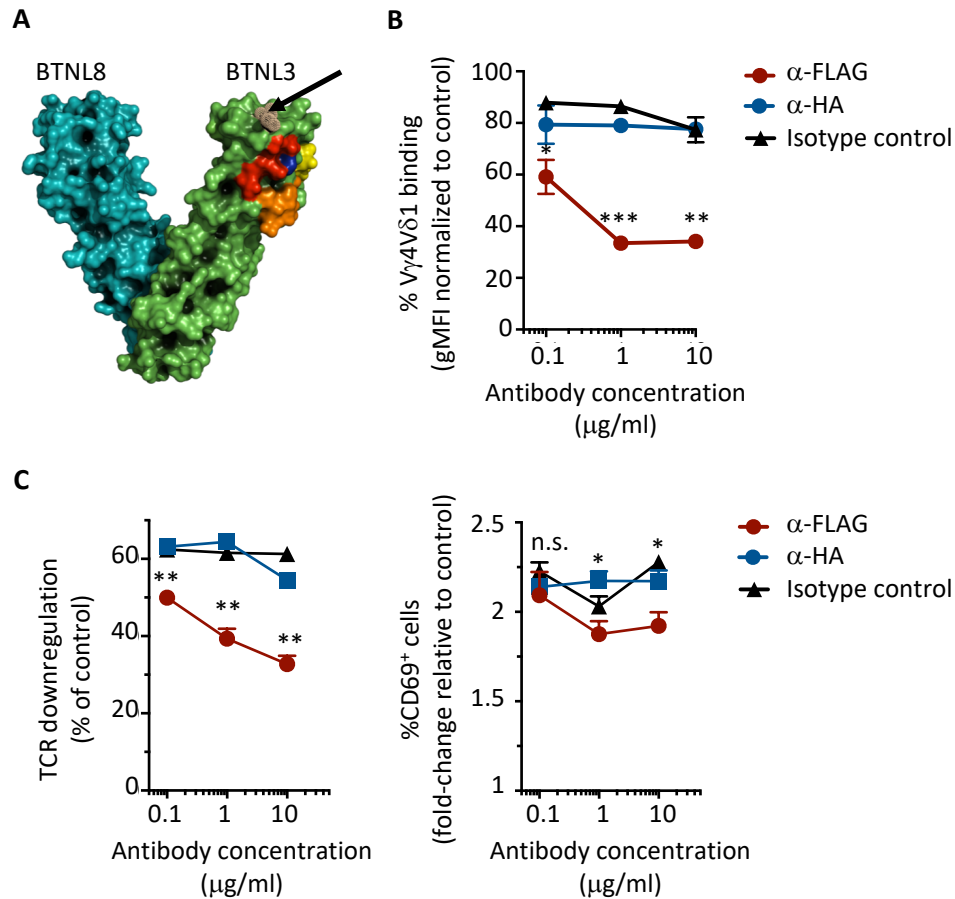


Figure 4.6. The use of anti-FLAG and anti-HA as blocking reagents. A. Heterodimeric model of BTNL3 (green)–BTNL8 (teal), derived from a BTN3A1 homodimer (PDB accession code 4F80) using the 3D-JIGSAW online homology modelling tool; orange, yellow, blue and red indicate candidate motifs described in Fig.4.4. Arrow indicates the amino terminus of BTNL3 where the protein is FLAG-tagged. **B.** Flow cytometry analysis of 293T.L3L8 cells pre-incubated with various concentrations (x-axis) of the indicated antibodies (key) and stained with 10 μ g/ml of soluble V γ 4V δ 1 pre-incubated with anti-His. V γ 4V δ 1+anti-His gMFI was normalized to that of 293T.L3L8 not pre-incubated with any antibodies. Data expressed as mean $\pm$ s.d of three independent stainings done on the same day. Representative of three independent experiments. *P=0.0218, **P=0.0032, ***P=0.0004. Analysed by paired two-tailed Student's t-test. **C.** Flow cytometry analysis of TCR downregulation (left) and CD69 upregulation (right) in J76-hu17 cells co-cultured for 3h with 293T.L3L8 pre-incubated with various concentrations (x-axis) of the indicated antibodies (key). Results were normalized to those obtained for control co-cultures with 293T.EV. Data expressed as mean $\pm$ s.d of three independent co-cultures done on the same day. Representative of three independent experiments. **P=0.0013 (10 μ g/ml), **P=0.0045 (1 μ g/ml), **P=0.0012 (0.1 μ g/ml), *P=0.0219 (10 μ g/ml), *P=0.0345 (1 μ g/ml), n.s. – not significant. Analysed by paired two-tailed Student's t-test where anti-FLAG was compared to isotype control conditions.

3. Conclusions

Collectively, these experiments provide evidence consistent with the hypothesis that the V γ 4 chain of the $\gamma\delta$ TCR directly interacts with the CFG face of BTNL3, thereby initiating a response in V γ 4⁺ cells. In particular, there was a striking correlation between constructs that mediated biological responsiveness (e.g. TCR downregulation) and those that mediated direct binding of V γ 4⁺ sTCRs to BTNL3 plus BTNL8-expressing cells.

The interaction was shown to be critically dependent on amino acids in the amino-terminal of the HV4 loop but was also modulated by sequences in the CDR2 loop that is spatially adjacent. This mode of interaction is in stark contrast to that of conventional $\alpha\beta$ TCRs binding to pMHC, in which CDR1 $\alpha\beta$ and CDR2 $\alpha\beta$ predominantly contact the MHC while CDR3 $\alpha\beta$ interact with the presented peptide (Cole et al., 2014).

Nevertheless, precedents exist describing the TCR HV4 loop as a binding site. Thus, the binding of microbial endogenous “superantigens” to $\alpha\beta$ TCRs commonly relies in part on the HV4 region of TCR α or TCR β (Saline et al., 2010; Rödström et al., 2014; Rödström et al., 2015). Consistent with this, superantigens elicit polyclonal responses from $\alpha\beta$ T cells, akin to those induced in $\gamma\delta$ T cells by BTNL3 plus BTNL8.

In some settings, HV4 engagement is integrated with the conventional antigen binding site in which the CDR3 loop is prominent. Thus, HV4 on the TCR α chain of rat and mouse invariant NKT cells has been implicated in their binding to CD1d- α GalCer (Paletta et al., 2015). Recently, the crystal structure of an IgE Fab region was shown to simultaneously utilise conventional CDR1-3 binding and superantigen-like binding modes to interact with a grass pollen-derived antigen. The superantigen-like binding was mediated by framework region 3 (FR3) in which the HV4 loop is found (Mitropoulou et al., 2018). While these datasets emphasise that HV4 might contribute to multiple types of antigen binding, they are distinct from BTNL3 plus BTNL8 interactions since those are seemingly independent of both CDR3 and the TCR δ chain. Indeed, V γ 8V δ 1, V γ 2V δ 1 and V γ 4V δ 1 sTCRs carried an identical V δ 1 chain, but only V γ 4V δ 1 sTCRs stained 293T.L3L8 cells. Moreover, the ability of both V γ 4V δ 1 and V γ 4V δ 2 to bind to 293T.L3L8 cells further supports the notion that the TCR γ chain alone is necessary and sufficient for mediating the responses to BTNL3 plus BTNL8.

Finally, the data demonstrates that BTNL3 is the functional partner, mediating the interaction with TCR V γ 4⁺ cells. BTNL8 may play an important chaperone-type role in the heteromeric complex. Studies from our laboratory have shown that BTNL3 and BTNL8 contain ER association

motifs and that the co-expression enhances ER exit and cell surface expression (Vantourout et al., 2018). Thus, the function of BTNL8 may primarily be to facilitate efficient surface expression of BTNL3. Conversely, it remains possible that BTNL8 interacts *via* another sub-region of the TCR complex, but that this interaction is not critical to the functional outcomes measured in this study.

Chapter V. Dual reactivity of the V γ 4⁺ TCR

The preceding chapters have described an interaction specifically mediated by the V γ 4 chain of human intestinal $\gamma\delta$ TCRs and the BTNL3 component of BTNL3 plus BTNL8 complexes. The interaction is likely to be of considerable physiological importance, given that analogous interactions between the murine V γ 7 chain and the Btl6 component of Btl1 plus Btl6 complexes are necessary for the establishment of the wild-type gut $\gamma\delta$ IEL compartment (Di Marco Barros et al., 2016; Melandri et al., 2018). Once the respective intestinal $\gamma\delta$ populations are established in humans and mice, they may presumably make clonotypic responses to antigens mediated by the conventional recognition site that integrates CDR3 $\gamma\delta$ with CDR1 $\gamma\delta$ and CDR2 $\gamma\delta$. In this Chapter, the potential for BTNL3 plus BTNL8-responsive TCRs to engage clonotypic antigens is investigated.

Antigen recognition by $\gamma\delta$ TCRs is not well understood, particularly for those $\gamma\delta$ subsets that are not phosphoantigen-reactive. Of the few ligands described, most are unprocessed self-antigens such as CD1, EPCR, MICA, annexin A2 and histidyl-tRNA synthetase (reviewed by Vantourout and Hayday, 2013). CD1 proteins are the most frequently reported ligands of $\gamma\delta$ TCRs, with reports of CD1a, CD1b, CD1c and CD1d-reactive clones (reviewed by Hayday and Vantourout, 2013). CD1 molecules naturally present endogenous and exogenous lipids to unconventional $\alpha\beta$ NKT lymphocytes. Human type I invariant NKT cells express V α 24-J α 18 paired to a limited repertoire of V β chains and recognise CD1d- α GalCer. Type II NKT cells have diverse TCR repertoires and do not bind CD1d- α GalCer (Salio et al., 2014). Among the few examples of CD1-reactive $\gamma\delta$ T cells, the docking footprints of V δ 1⁺ TCRs bound to CD1d- α GalCer (clone 9C2) or to CD1d-sulfatide (clone DP10.7) are highly similar to that of a type II NKT TCR bound to CD1d-sulfatide (Adams et al., 2015). Of note, DP10.7 is a V γ 4V δ 1 TCR, suggesting that it might have a dual reactivity to CD1d-sulfatide and BTNL3 plus BTNL8.

EPCR is a CD1-related protein involved in the coagulation pathway. Analogous to CD1, EPCR has a lipid-binding groove, occupied by phosphatidylcholine (PC) or phosphatidylethanolamine (Oganessian et al., 2002). The V γ 4V δ 5 TCR of a T cell clone LES, found to be massively expanded in a transplant patient, was demonstrated to recognise a conformational antigenic epitope on EPCR independently of glycosylation and distal to the lipid-binding site (Willcox et al., 2012). Again, the possibility of dual reactivity (EPCR and BTNL3 plus BTNL8) existed. This was investigated here.

1. The EPCR-reactive clone LES responds to BTNL3 plus BNTL8

JRT3-LES and JRT3-hu12 were co-cultured with HEK293T cells transduced with EPCR (293T.EPCR), BTNL3 plus BTNL8 (293T.L3L8) or EPCR plus BTNL3 plus BTNL8 (293T.EPCR+L3L8). Both Jurkat cell lines responded with strong TCR downregulation and CD69 upregulation when exposed to 293T.L3L8 or 293T.EPCR+L3L8 (Fig.5.1A). On the other hand, 293T.EPCR elicited weak TCR downregulation and strong CD69 upregulation only in JRT3-LES, but not in JRT3-hu12 cells. Similar results were obtained when the JRT3 TCR transductants were co-cultured with the cell line HT29, which was originally used to identify EPCR as the specificity of the LES clone (Willcox et al., 2012) (Fig.5.1A). Thus, EPCR responses were specific to the LES TCR and co-expression with BTNL3 plus BTNL8 did not appear to either diminish BTNL-reactivity or to have an additive effect. Furthermore, anti-EPCR antibody blocked EPCR-mediated responses elicited by co-culture with transiently transfected HEK293T cell expressing EPCR or EPCR plus BTNL3 and BTNL8, but not BTNL3 plus BTNL8 alone (Fig.5.1B, C).

HEK293T were described initially as an EPCR-expressing non-stimulating cell line in the context of the LES TCR (Willcox et al., 2012). Nevertheless, EPCR was deleted from the genome of HEK293T cells to rule out its contribution to the BTNL-responsiveness of JRT3-LES cells (Fig.5.1D). Expanded single-cell EPCR wild-type and knockout clones were transduced with EPCR or BTNL3 plus BTNL8 and co-cultured with JRT3-LES or JRT3-hu17 cells (Fig.5.1E). The deletion of EPCR did not affect BTNL reactivity of either JRT3-LES or JRT3-hu17 cells (Fig.5.1E). Thus, JRT3-LES displayed both clonotypic and non-clonal TCR reactivities towards EPCR and the BTNL molecules, respectively. Importantly, the reactivity to EPCR was previously mapped to the conventional antigen binding site of the LES TCR with major contributions by CDR3 γ and CDR3 δ (Willcox et al., 2012; Willcox et al., 2019).

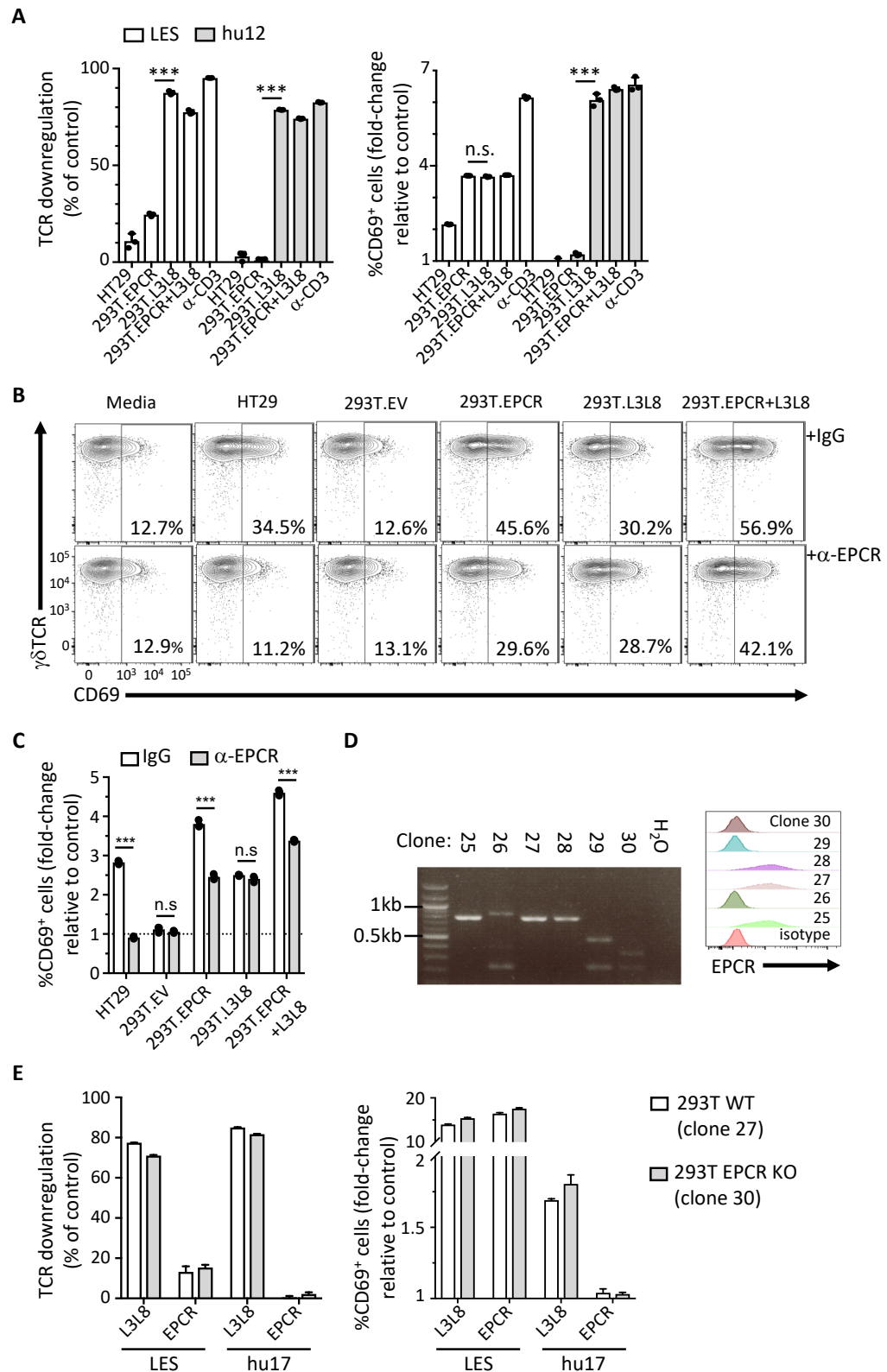


Figure 5.1. Dual reactivity of the EPCR-reactive clone LES. **A.** Flow cytometry analysis of TCR downregulation (left) and CD69 upregulation (right) of JRT3-LES and JRT3-hu12 co-cultured for 5h with the indicated cell lines or anti-CD3 antibody (x-axis); results were normalized to (legend continues on the next page)

(Figure 5.1 legend continued)

those obtained by co-culture with 293T.EV cells, isotype control antibody or unstimulated JRT3-LES. Data are expressed as mean $\pm$ s.d. of three independent co-cultures done on the same day. Plots are representative of two independent experiments. ***P<0.001, n.s. – not significant. Analysed by paired two-tailed Student's t-test. **B.** Flow cytometry analysis of TCR and CD69 expression on JRT3-LES co-cultured for 3h with the indicated cell lines (above plots) in the presence of control IgG or anti-EPCR. **C.** Summary graph for CD69 upregulation from the experiment in **B.** Data is expressed as mean $\pm$ s.d. of three independent co-cultures done on the same day. Plots are representative of two independent experiments. ***P<0.001, n.s. – not significant. Analysed by paired two-tailed Student's t-test. **D.** PCR (left) and flow cytometry (right) analysis of EPCR knockout HEK293T single-cell clones. Expected band size of wild-type EPCR, 750bp. **E.** Flow cytometry analysis of TCR downregulation (left) and CD69 upregulation (right) of JRT3-LES and JRT3-hu17 (lines below plots) co-cultured for 5h with the indicated cell lines (key) expressing BTNL3 plus BTNL8 or EPCR (x-axis); results were normalized to those obtained by co-culture with 293T.EV cells. Data are expressed as mean $\pm$ s.d. of three independent co-cultures done on the same day. Plots are representative of three independent experiments.

2. CD1c-reactive clone hu20 responds to BTNL3 plus BTNL8

Next, we sought to investigate whether there are BTNL3 plus BTNL8-responsive $\gamma\delta$ cells that also recognised CD1 molecules. To this end, we collaborated with Dr Salah Mansour (University of Southampton) who provided CD1c tetramers loaded with phosphatidylcholine (CD1c-PC) which were used to stain primary colonic $\gamma\delta$ lymphocytes from a healthy donor.

0.115% of live CD3⁺ $\gamma\delta$ TCR⁺V δ 2⁻ cells were CD1c-PC-reactive (Fig.5.2A). These were single-cell sorted and subjected to single-cell PCR using a protocol which was modified in several ways that permitted nearly 100% recovery of the TCR γ and TCR δ chains (Fig.5.2A). First, a new pan-TCR γ chain forward primer was used which was able to amplify V γ 2,3,4,5 and 8. Second, V δ 3 primers were not included in these PCRs because only V δ 1⁺ cells were single-cell sorted. Lastly, the second PCR step was split into two reactions, amplifying TCR γ and TCR δ separately. These steps ensured minimal interference that may have occurred between the primer sets which allowed for the more efficient amplification of each chain.

24 band pairs were sent for sequencing from the single-cell PCR. Of these, 21 sequences were V γ 5V δ 1⁺, two were V γ 8V δ 1⁺ and one was a V γ 4V δ 1⁺ TCR (clone hu20) (Fig.5.2B). To determine whether a CD1c-reactive V γ 4⁺ clone would also respond to BTNL3 plus BTNL8, J76 cells were

transduced with the hu20 TCR. The CD1c-PC specificity was validated using tetramer staining (Fig.5.2C). J76-hu20 cells, expressing the CD1c-PC-reactive V γ 4V δ 1 TCR, stained specifically with CD1c-PC tetramers whereas control J76-hu17 cells did not (Fig.5.2C). However, both transductants responded in co-culture with 293T.L3L8 cells (Fig.5.2D). Thus, J76-hu20 displayed both clonotypic and non-clonal TCR reactivities towards CD1c-PC and BTNL3 plus BTNL8, respectively.

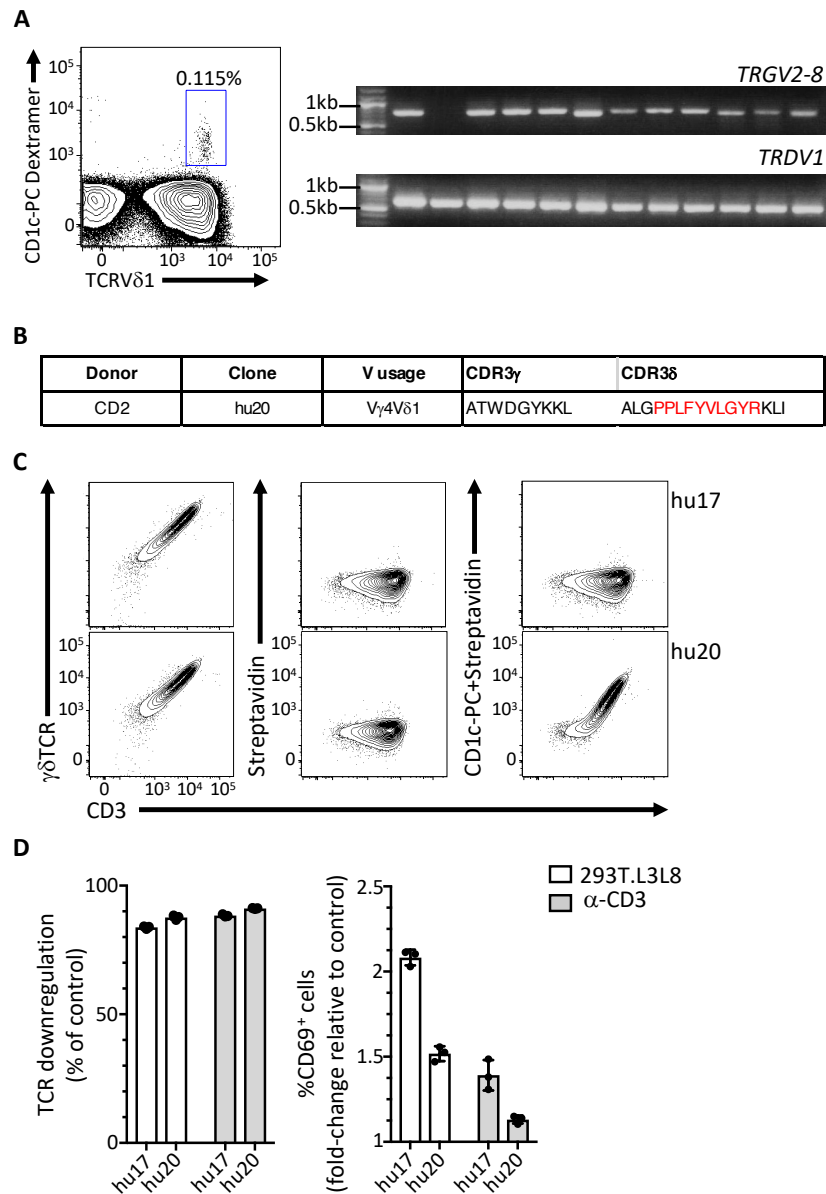


Figure 5.2. Dual reactivity of the CD1c-reactive clone hu20. **A.** Flow cytometry analysis (left) of CD1c-phosphatidylcholine (PC)-reactive V δ 1⁺ human colonic lymphocytes (donor CD2). Lymphocytes were pre-gated on live CD3⁺V δ 2⁺γ δ TCR⁺ singlets. The indicated gate was used for single-cell sorting. Representative image of the single-cell PCR is shown on the right. Top, (legend continues on the next page)

(Figure 5.2 legend continued)

gamma chain PCR; bottom, delta chain PCR. Expected bands: ~700bp for TCR γ and ~600bp for TCR δ . **B.** Clone hu20 amino acid composition of CDR3 γ and CDR3 δ sequenced from single-cell sorted CD1c-PC reactive colonic $\gamma\delta$ lymphocytes. **C.** Flow cytometry analysis of TCR surface expression (left), streptavidin alone stainings (middle) and CD1c-PC+streptavidin tetramer stainings (right) of J76-hu17 and J76-hu20 transductants. Representative of three independent stainings. (*Experiment was performed by Dr Pierre Vantourout, King's College London; recombinant CD1c-PC was generated by Dr Andrew Chancellor, University of Southampton*). **D.** Flow cytometry analysis of TCR downregulation (left) and CD69 upregulation (right) of J76-hu17 and J76-hu20 (x-axis) co-cultured for 5h with 293T.L3L8 or anti-CD3 antibody (key); results were normalized to those obtained by co-culture with 293T.EV cells or isotype control antibody. Data are expressed as mean $\pm$ s.d. of three independent co-cultures done on the same day. Plots are representative of three independent experiments.

3. Conclusions

The results of this Chapter demonstrate that V γ 4⁺ cells can have dual reactivity. While in Chapter IV BTNL-responsiveness was shown to be mediated *via* germline CDR2 γ and HV4 γ sequences, EPCR recognition was demonstrated to depend on CDR3 γ and CDR3 δ (Willcox et al., 2012, Willcox et al., 2019). Likewise, Dr Pierre Vantourout from our laboratory has shown that the substitution of either CDR3 γ or CDR3 δ of the hu20 TCR for that of the hu12 TCR was sufficient to abolish CD1c-PC tetramer staining, thus implicating those regions in CD1c-PC binding (unpublished data). Collectively, the two examples suggest that V γ 4⁺ cells use distinct regions of the $\gamma\delta$ TCR in order to recognise clonally restricted antigens *versus* the BTNL3 plus BTNL8 complex. Whether these regions can be simultaneously and independently bound by the two types of ligands is unclear.

CD1 proteins have emerged as major clonal determinants of $\gamma\delta$ T cells (Hayday and Vantourout, 2013; Luoma et al., 2013) and the work presented here adds another example to this concept. Two crystal structures have shown that this antigen recognition is dependent on the $\gamma\delta$ CDR3 loops, although the mechanisms of interaction were not identical (Uldrich et al., 2013; Luoma et al., 2013). One of these structures was a V γ 4V δ 1 TCR bound to CD1d-sulfatide which showed a binding mode largely dependent on CDR1-3 of the V δ 1 chain (Luoma et al., 2013). This structure was used in docking experiments with the predicted structure of the BTNL3-BTNL8 heteromer (Chapter IV, Fig.4.4B). The data suggested that the TCR might be able to

simultaneously engage BTNL and CD1 proteins (Melandri et al., 2018) which opened the possibility that other or all clonally restricted $V\gamma 4^+$ TCRs would have similar potential. However, results from experiments involving EPCR- $V\gamma 4$ TCR interaction argue against the latter being customary.

The simultaneous overexpression of EPCR and BNTL3 plus BNTL8 did not have additive or cancelling effect in terms of the responses of JRT3-LES and JRT3-hu12 cells. Notably, EPCR exposure alone induced significantly less TCR downregulation compared to BNTL3 plus BNTL8 in JRT3-LES cells while 293T.EPCR+L3L8 responses were similar to those induced by 293T.L3L8. Others have previously shown that the level of TCR downregulation positively correlates with the affinity for the stimulating antigen. (Cai et al., 1997; Gallegos et al., 2016). Therefore, the $V\gamma 4^+$ TCR likely has a higher affinity for the BTNL molecules than for the clonally restricted antigen. Indeed, we recently established in SPR experiments that the LES TCR directly interacts with BNTL3 IgV ($K_d \sim 21 \mu\text{M}$) and not BNTL8 IgV (Willcox et al., 2019). This binding was of higher affinity compared to the previously established EPCR affinity constant of $\sim 90 \mu\text{M}$ (Willcox et al., 2012). In a competition assay, the addition of increasing concentrations of BNTL3 IgV, and not BNTL8 IgV, successfully inhibited EPCR binding to immobilised LES TCRs in concentration-dependent manner (Willcox et al., 2019). Together, these results strongly support the hypothesis that the two antigens are not simultaneously engaged, at least in the context of the EPCR-reactive clone LES.

Nonetheless, a partial block of response was seen in 293T.EPCR+L3L8 co-cultured with JRT3-LES cells in the presence of an anti-EPCR antibody. In those experiments HEK293T were transiently transfected with the three vectors which would result in a heterogeneous mixture of BNTL3 plus BNTL8 and/or EPCR-expressing cells. Therefore, the partial attenuation of 293T.EPCR+L3L8-elicited CD69 upregulation with an anti-EPCR antibody may be attributed to responses blocked specifically in a number of JRT3-LES cell which have encountered predominantly EPCR and not the BTNL complex on HEK293T cells.

Finally, the $V\gamma 4V\delta 5$ LES data provides another example of a $V\gamma 4^+V\delta 1^-$ TCR which is able to respond to BNTL3 plus BNTL8, further supporting the notion that reactivity is largely independent of the TCR δ chain. Thus, the single-cell PCR analysis (Chapter III), the soluble TCR staining (Chapter IV), and the results here demonstrated that $V\gamma 4^+$ TCRs paired to $V\delta 1$, $V\delta 2$, $V\delta 3$ or $V\delta 5$ can recognise the BNTL3 plus BNTL8 complex. Moreover, the LES TCR was isolated from a peripheral-blood $\gamma\delta$ clone, suggesting again that reactivity is largely independent of the tissue origin.

Chapter VI. Some consequences of BTNL-TCR interactions

The B7 family of molecules are involved in both stimulatory and inhibitory immune processes. CD80 and CD86, the prototypic members of this family, can be recognised by CD28 or CTLA-4. Interaction with the former leads to co-stimulation and T cell activation while binding to the latter results in inhibition of the immune responses (Schildberg et al., 2016). CD28 is constitutively expressed on the surface of resting T cells. In contrast, CTLA-4 expression is induced following activation and is constitutively expressed by Tregs (Walker and Sansom, 2011). The mode of action of CTLA-4 has been a matter of debate (reviewed by Walker and Sansom, 2011). However, a particularly interesting mechanism has been described whereby CTLA-4 binding leads to the physical removal of its ligands from APCs and their transfer into the CTLA-4-expressing cell (Qureshi et al., 2011; Hou et al., 2015). This process of trans-endocytosis was specific to the CTLA-4 protein, as its expression was sufficient to confer this function to several cell types (Qureshi et al., 2011).

In fact, protein transfer across the immunological synapse is not uncommon and has been referred to by other names such as absorption, internalization and trogocytosis (reviewed by Davis, 2007). Of note, the transfer of target cell membrane onto V γ 9V δ 2 cells has been demonstrated before. This process was dependent on TCR signalling since it was blocked by the Src family inhibitor PP2 (Espinosa et al., 2002). Therefore, experiments were undertaken which sought to determine if similar mechanisms might operate in co-cultures of V γ 4⁺ JRT3 with 293T.L3L8 cells. In addition, organoid cultures from human colonic epithelium were set up, in which findings from the co-culture assays might be further validated and in which BTNL- $\gamma\delta$ TCR cross-talk could in the future be investigated at more physiological levels.

1. Trans-endocytosis of the $\gamma\delta$ TCR

To investigate whether BTNL binding might provoke the uptake of the V γ 4⁺ TCR by metabolically active cells, we were able to make use of a set of recombinant, His-tagged soluble TCRs (sTCRs), a unique reagent provided by collaborators at Gamma Delta Therapeutics. Thus, 293T.L3L8 cells were incubated with soluble monomeric V γ 4V δ 2 TCRs at 37°C or at 4°C to suppress metabolic activity. Subsequently, a secondary stain with an anti-His antibody was performed. If the sTCRs were internalized, then the signal from the secondary staining would decrease. Indeed, although

the sTCRs clearly bound to the 293T.L3L8 cells, there was a strong loss of V γ 4V δ 2 staining at 37°C but not at 4°C across a range of sTCR concentrations (Fig.6.1A). To confirm this was not due to reagent degradation at higher temperatures, sTCRs were pre-incubated with wild-type HEK293T cells at 37°C for one hour and supernatants were subsequently used to stain 293T.L3L8 cells at 4°C. This resulted in only a minor decrease in sTCR gMFI, compared to >50% loss of staining in cultures at 37°C (Fig.6.1B). By contrast to the binding of the sTCRs to BTNL3 plus BTNL8, incubation of 293T.L3L8 cells at 37°C with anti-BTNL3 and anti-FLAG antibodies, both of which bound to BTNL3, resulted in only 2% and 14% decreases in gMFI, respectively, relative to the 4°C control staining (Fig.6.1C). Collectively, these data suggest that a V γ 4⁺ TCR binding likely induces BTNL downregulation from the cell surfaces.

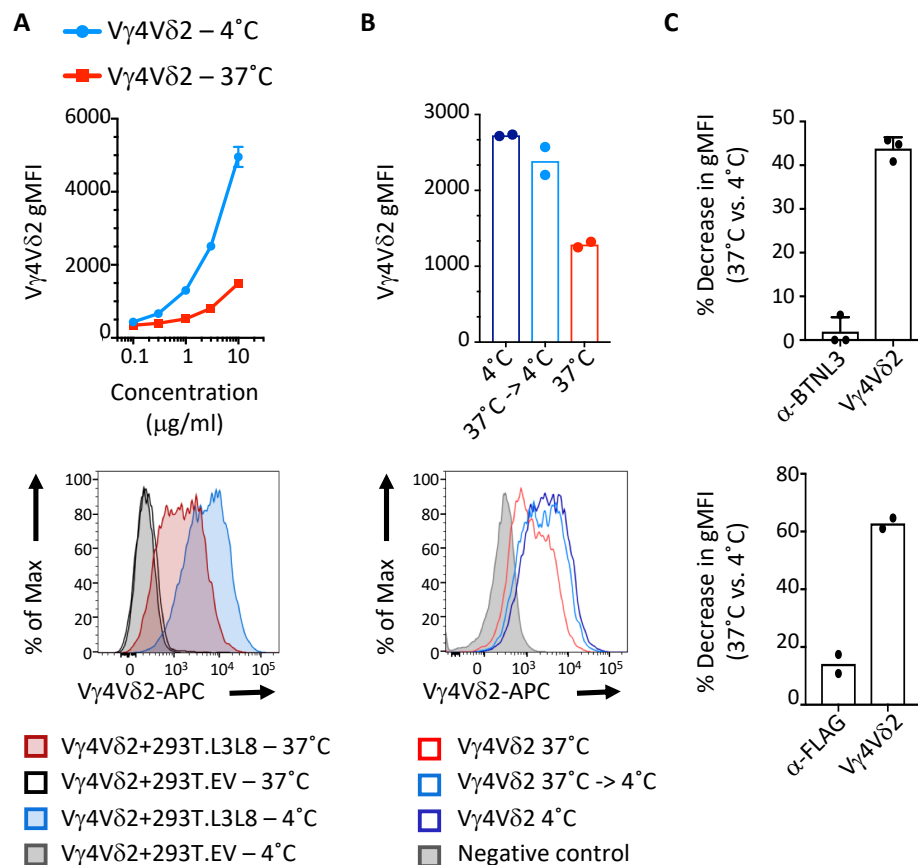


Figure 6.1. BTNL downregulation induced by soluble V γ 4⁺ TCRs. **A.** Summary plot (top) of flow cytometry analysis of 293T.L3L8 cells incubated for 1 hour with various concentrations of soluble monomeric V γ 4V δ 2 TCRs (x-axis) at 4°C or 37°C (key) and subsequently stained with secondary anti-His tag antibody against the soluble TCRs. Data expressed as mean $\pm$ s.d. of three independent stainings. Example histograms for the 10mg/ml data points are shown below the graph. **B.** Summary plot (top) of flow cytometry analysis of 293T.L3L8 cells incubated for 1h with 10mg/ml soluble V γ 4V δ 2 at 37°C or 4°C (x-axis) and detected as in **A.** (legend continues on the next page)

(Figure 6.1 legend continued)

37°C->4°C denotes samples where the staining reagent was pre-incubated at 37°C for 1 hour and subsequently used at 4°C. Example histograms are shown below. Representative of two independent stainings. **C.** Summary graphs of anti-BTNL3 and V γ 4V δ 2 (top) or anti-FLAG and V γ 4V δ 2 (bottom) stainings of 293T.L3L8 cells at 37°C, detected as in **A**. Results are normalised to those obtained from control conditions at 4°C. Data are expressed as mean $\pm$ s.d. of three (anti-BTNL3) independent stainings or as the mean of two independent stainings (anti-FLAG).

We next focussed on the response to TCR engagement of the BTLN3 and BTLN8 molecules. Co-culture with JRT3-hu17 cells resulted in strong BTLN3 and BTLN8 downregulation in HEK293T cells transfected with these proteins (Fig.6.2A). By contrast, JRT3 cells expressing the hu17.V γ 3 chimeric TCR did not induce such a response (Fig.6.2A). Considering the reports of CTLA-4-CD80/CD86 trans-endocytosis, the possibility of similar mechanisms operating during BTLN-TCR interaction were investigated. Strikingly, CD3 expression was reproducibly detected on the surface of 293T.L3L8 cells by flow cytometry following co-culture with JRT3-hu17 but not JRT3-hu17.V γ 2 cells (Fig.6.2B), arguing for its transfer from the T cell surface. Furthermore, CD3 appeared to be endocytosed by HEK293T cells as there was consistently more CD3⁺ HEK293T cells detected following intracellular staining (Fig.6.2B, C). Indeed, 40-50% of the BTLN3 plus BTLN8-expressing cells became CD3⁺ after co-culture with JRT3-hu17 but not JRT3-hu17.V γ 2 cells (Fig.6.2B, C). In striking contrast, there was little BTLN3 transfer to JRT3 cells, as judged by anti-FLAG antibody staining (Fig.6.2C).

The CD3 transfer could also be observed in CHO.L3L8 and HELA.L3L8 cells co-cultured with JRT3-hu17 cells (Fig.6.2D), albeit to a lower degree, perhaps reflecting the tendency for weaker transfection efficiencies and lower BTLN expression (Fig.3.8C). Importantly, this process seemed to be CD3-specific as CD45 trans-endocytosis was not observed in these experiments (Fig.6.2D; top panels). Interestingly, when JRT3-hu17 transductants were co-cultured with fixed 293T.L3L8 cells, the TCR downregulation on the JRT3 cells was 35% weaker (Fig.6.2E), and there was no acquisition of CD3 staining by the HEK293T cells (Fig.6.2F). These data suggest that TCR downregulation, a primary measure of V γ 4 TCR engagement by BTLN3 plus BTLN8, may reflect a combination of specific TCR signalling in the JRT3 cell line and TCR trans-endocytosis by BTLN-expressing cells.

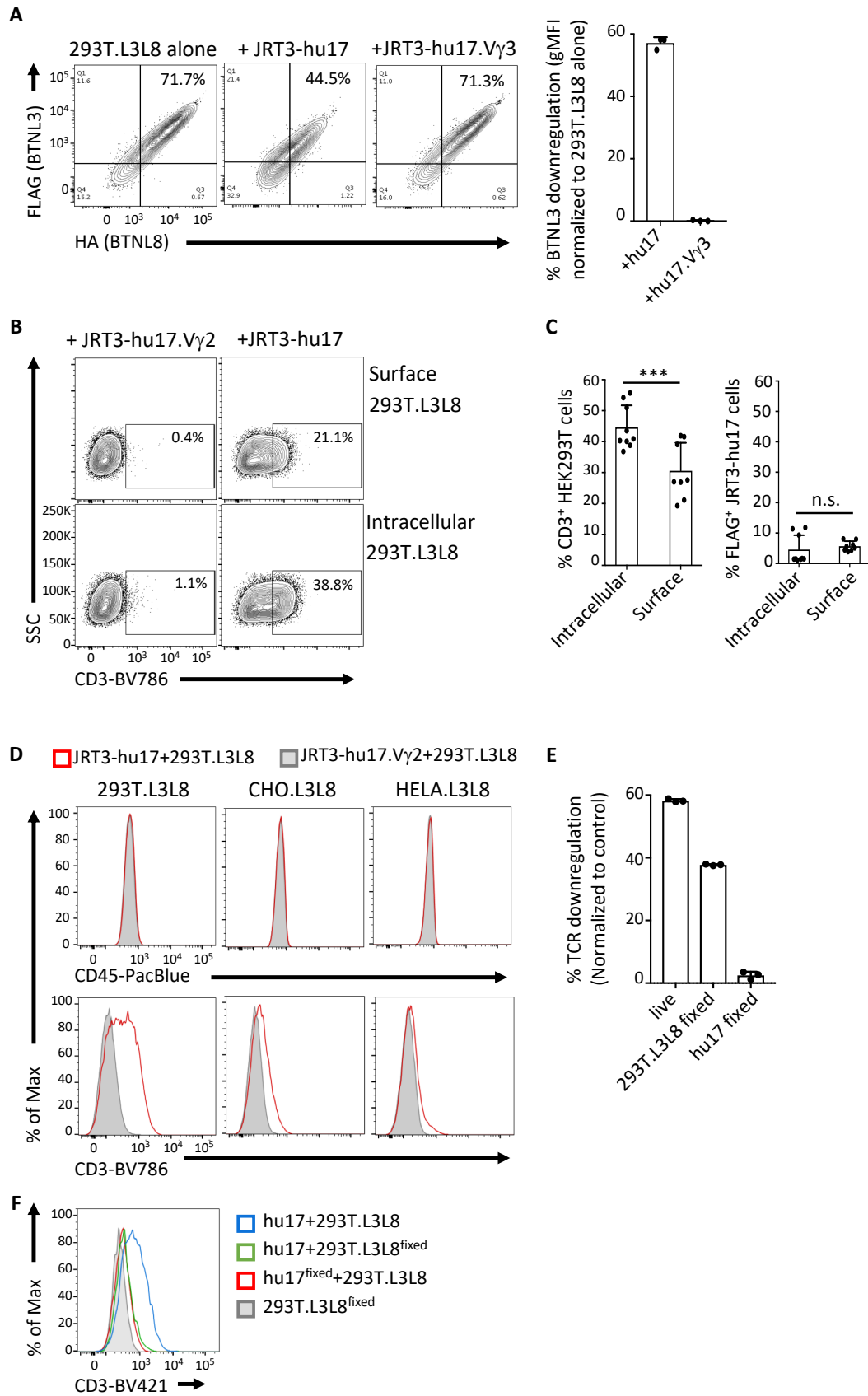


Figure 6.2. Flow cytometry analysis of $\gamma\delta$ TCR trans-endocytosis in the co-culture assay.
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Figure 6.2. Flow cytometry analysis of $\gamma\delta$ TCR trans-endocytosis in the co-culture assay. **A.** Flow cytometry analysis of BTNL3 and BTNL8 expression on transfected HEK293T cells after 1 hour of co-culture with JRT3-hu17 or JRT3-hu17.V γ 3 or without other cells. Representative flow cytometry plots are shown on the right. Summary graph of BTNL3 downregulation is shown on the left. Results for anti-FLAG gMFI were normalized to those obtained for 293T.L3L8 cultured alone. Data expressed as mean $\pm$ s.d. of three independent co-cultures done on the same day. Representative of three independent experiments. **B.** Representative flow cytometry plots of surface (top) and intracellular (bottom) anti-CD3 staining of L3L8 transfected HEK293T after 1 hour of co-culture with JRT3-hu17 or JRT3-hu17.V γ 2 (above plots). **C.** Surface and intracellular flow cytometry analysis (x-axis) of CD3⁺ HEK293T (left) or FLAG⁺ JRT3-hu17 cells (right). Data expressed as mean $\pm$ s.d. (n=8). Representative of three independent experiments. ***P<0.001; n.s. – not significant. Analysed by paired two-tailed Student's t-test. **D.** Intracellular flow cytometry analysis of CD45 and CD3 staining on HEK293T, CHO and HELA cells transduced with BTNL3 plus BTNL8 (above plots) and co-cultured with JRT3-hu17 or JRT3-hu17.V γ 2 (key) for 1 hour. Representative of three independent experiments. **E.** Flow cytometry analysis of TCR downregulation in JRT3-hu17 cells co-cultured with 293T.L3L8 for 5 hours. Both cell lines were live in the co-culture, or fixed 293T.L3L8 were co-cultured with live JRT3, or live 293T.L3L8 were co-cultured with fixed JRT3 cells (x-axis). Data expressed as mean $\pm$ s.d. of three independent co-cultures. **F.** Representative histograms of intracellular 293T.L3L8 anti-CD3 staining from the experiment in **E**.

Image stream analysis showed punctate CD3 staining in HEK293T cells transiently transfected with BTNL3 plus BTNL8 and co-cultured with JRT3-hu17 (Fig.6.3A). The specificity of this was implied by the fact that staining was limited to GFP⁺ HEK293T cells by comparison to GFP⁻ HEK293T cells that were present in the same cultures but which were evidently not transfected. Furthermore, CD3 staining partially overlapped with the signal for CD107a, a marker for late endosomes/lysosomes. Consistent with the flow cytometry data, no CD3 staining was seen in co-cultures with JRT3-hu17.V γ 2 cells (Fig.6.3A). To further demonstrate that the $\gamma\delta$ TCR complex becomes endocytosed, 293T.L3L8 cells were first incubated with pre-labelled soluble V γ 4V δ 2 TCRs at 4°C or 37°C, and subsequently treated with trypsin or left in plain medium (Fig.6.3B). Image stream analysis revealed that following trypsin treatment of cells incubated at 4°C, soluble TCR detection was completely lost (Fig.6.3B). By contrast, trypsin treatment of 293T.L3L8 cells stained at 37°C resulted in only partial loss of the V γ 4V δ 2 TCR staining, suggesting that the remaining signal originated from endocytosed molecules which were protected from extracellular enzymatic degradation (Fig.6.3B).

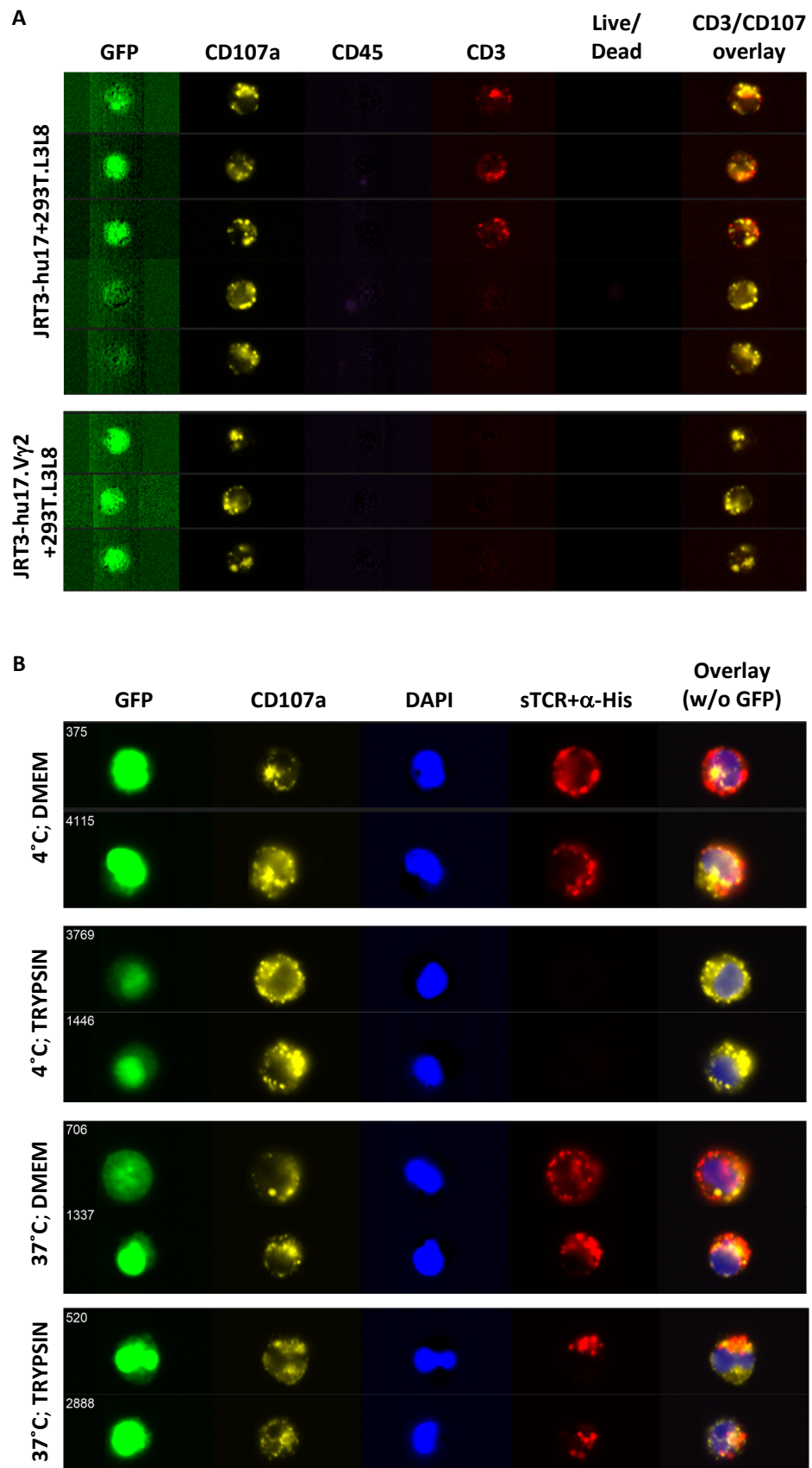


Figure 6.3. Image stream analysis of $\gamma\delta$ TCR trans-endocytosis in the co-culture assay.
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Figure 6.3. Image stream analysis of $\gamma\delta$ TCR trans-endocytosis in the co-culture assay. A. Image stream analysis of 293T transfected with BTNL3 plus BTNL8 and co-cultured for 1 hour with JRT3-hu17 (top panel) or JRT3-hu17.V γ 2 (bottom panel). Cells were fixed and permeabilised before staining for CD107a, CD45 and CD3. Live, CD45⁺ events are shown. Representative of one experiment. **B.** Image stream analysis of 293T.L3L8 stained with soluble V γ 4V δ 2 for 1 hour at 4°C and subsequently not treated (top panel) or treated (second panel) with trypsin; or at 37°C and subsequently not treated (third panel) or treated (bottom panel) with trypsin. Soluble V γ 4V δ 2 was pre-incubated with anti-His antibody before staining 293T.L3L8. Cells were fixed and permeabilised before staining for CD107a and DAPI. Representative of two independent experiments (*Experiments were performed by Dr Pierre Vantourout, King's College London*).

2. Establishing organoids as a model to study the $\gamma\delta$ -BTNL axis in a more physiological context

The data presented in this thesis was generated in systems where the $\gamma\delta$ TCRs, BTNL3 and BTNL8 were overexpressed in cell lines. In order to start investigating the consequences of their interaction, a more physiological system is required. The recent development of organoid cultures may offer a unique opportunity to do so, and have several advantages over conventional gut-derived cell lines. Organoids are 3D epithelial *in vitro*-cultured structures. They can be derived from the stem cell niche of the intestinal epithelium from healthy donor biopsies which permits the cells to self-renew for extended periods (Sato et al., 2011). In addition, such cultures are genetically stable and can be manipulated using all standard laboratory techniques such as transfection, transduction and CRISPR-Cas9 gene-modification (Schutgens and Clevers, 2019). Finally, their differentiation can be induced following the retraction of several stem-cell promoting factors (Sato et al., 2011), offering the prospect of culturing various cell types which form the intestinal barrier – goblet cells, Paneth cells, enteroendocrine cells and epithelial cells. Thus, we wanted to investigate whether BTNL3 and BTNL8 are expressed in differentiated organoid cultures. If so, this would make the system a suitable model for future studies such as, for example, a more physiological co-culture set-up and investigating the regulation of BTNL expression by stress.

To this end, organoid cultures were established from macroscopically normal human colonic epithelium from individuals with no known history of cancer. After 5 days of culture in differentiation medium, organoids strongly downregulated the stem-cell marker *LGR5* (Fig.6.4A)

and upregulated the transcription factor *CDX2*, a master regulator of gut differentiation (Fig.6.4B). Furthermore, *HNF4A*, a transcription factor which regulates *BTNL3* and *BTNL8* expression (Dr Pierre Vantourout, unpublished data), was also upregulated, along with *BTNL3* and *BTNL8* (Fig.6.4C-E). Importantly, the expression levels were comparable to those seen in whole gut biopsies from the corresponding donors (Fig.6.4A-E). Thus, the data suggests that organoid cultures can be successfully differentiated and may represent a suitable system in which human BTNL expression, downstream signalling and interaction with $\gamma\delta$ lymphocytes are investigated in the future.

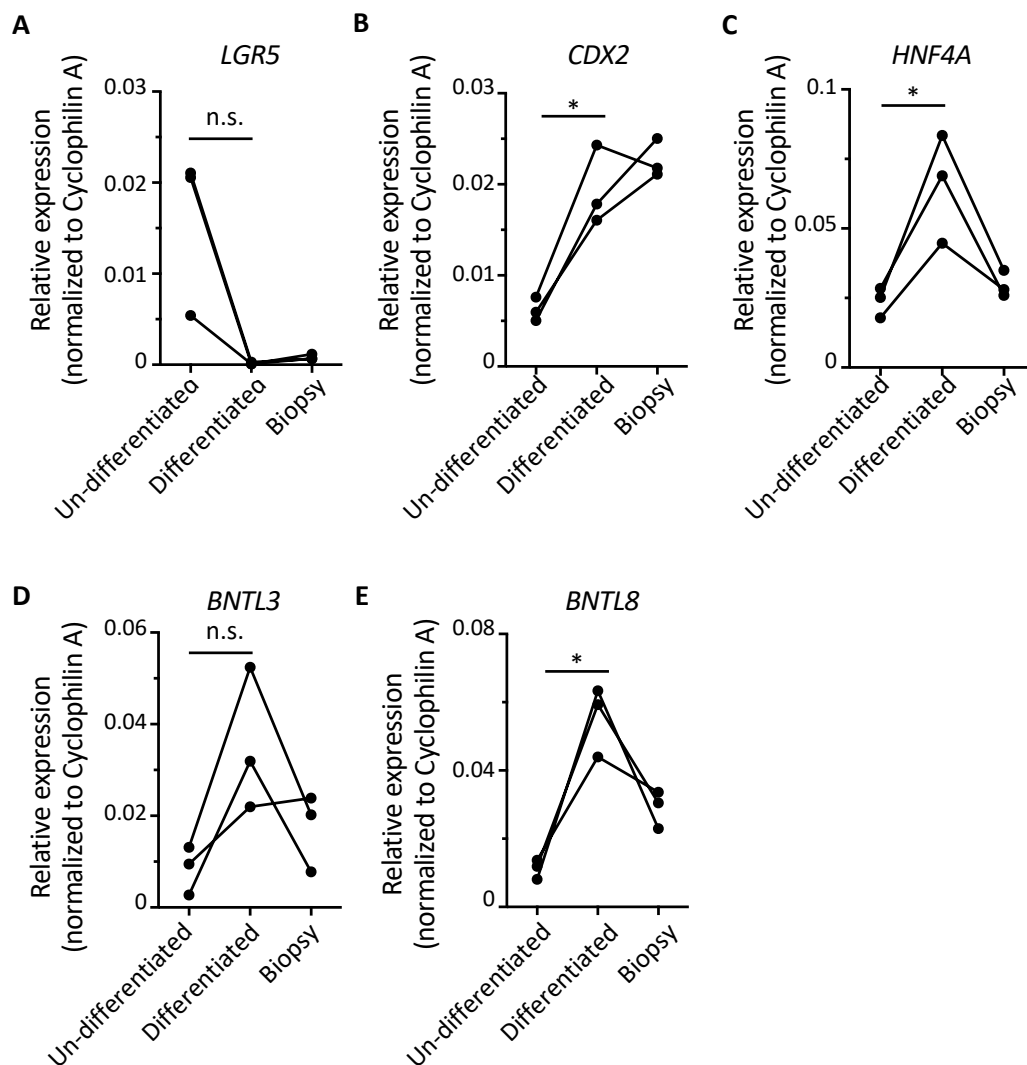


Figure 6.4. Differentiation of human colonic organoid cultures. qPCR analysis of *LGR5* (A), *CDX2* (B), *HNF4A* (C), *BTNL3* (D) and *BTNL8* (E) expression in un-differentiated versus differentiated human colonic organoid cultures or whole tissue biopsies (x-axis). Data from three independent donors are shown. *P<0.05; n.s. – not significant. Analysed by paired two-tailed Student's t-test.

3. Conclusions

Collectively, the results of this Chapter demonstrate that upon binding, the $V\gamma 4^+$ TCR becomes internalized in BTNL3 plus BTNL8 expressing cells. The $\gamma\delta$ TCR complex was clearly endocytosed as judged by image stream analysis of 293T.L3L8 cells incubated with soluble $V\gamma 4V\delta 2$ since following enzymatic treatment the sTCR staining could still be observed in HEK293T cells. Importantly, the signal was punctate which suggested that the sTCRs were sequestered in an endosomal compartment, likely as a result of specific endocytosis. Interestingly, this process operates in the opposite direction compared to the trans-endocytosis of CD80/CD86 by CTLA-4-expressing lymphocytes. Thus, rather than the B7 family members (i.e. BTNL3 and BTNL8) being transferred into the T lymphocyte, the $\gamma\delta$ TCR was actively transported into the epithelial cell line. It is unclear how the process of trans-endocytosis occurs and what its function is, but roles in both suppression and augmentation of immune responses have been proposed (Davis, 2007; Ahmed and Xiang, 2011). How this may be significant in the context of the cross-talk between $\gamma\delta$ IELs and the epithelium is discussed in the next Chapter.

Finally, the organoid *in vitro* system represents potentially an appropriate one to investigate at more physiological levels the regulation of BTNL3/BTNL8 expression, and the consequences of $\gamma\delta$ TCR-BTNL interaction within the epithelial cells. However, protein expression should also be confirmed, albeit there are currently no suitable, commercially available monoclonal BTNL3- and BTNL8-specific antibodies and the anti-BTNL3 antibody used in these studies failed to stain organoid-derived epithelial cells by flow cytometry. Nonetheless, differentiated organoid cultures merit further investigation and use in the future. For example, future experiments may aim to validate soluble $\gamma\delta$ TCR internalisation by the organoid-derived epithelium and should also aim to establish organoid-lymphocyte co-cultures in order to examine the communication between $\gamma\delta$ T cells and the epithelium by looking at, for example, the responses of primary $V\gamma 4$ cells or looking at BTNL downstream signalling events.

Chapter VII. Discussion

The primary goal of this study was to determine whether the $\gamma\delta$ TCR mediates the profound impact of BTN3 and BTN8 proteins on human $\gamma\delta$ IELs. Single-cell PCR of reactive cells and deep sequencing analysis of the intestinal $\gamma\delta$ TCR repertoire collectively revealed that the cells responding to BTN3 and BTN8 comprise a polyclonal population of $V\gamma 4^+$ lymphocytes, and this conclusion was validated by the cloning of TCRs from single responding cells. Through extensive mutagenesis experiments, the $V\gamma 4$ HV4 loop was identified as critical for mediating the biological effects of BTN3 plus BTN8, although some contributions were made by the adjoining CDR2. For the butyrophilins, BTN3 emerged as a key mediator of interactions with critical contributions made by the BTN3 CFG face. By contrast, the BTN8 protein may fulfil a chaperone-like role, regulating the appropriate intracellular trafficking of the BTN3 plus BTN8 complex (Vantourout et al., 2018). Analogous results relating to Btl1 plus Btl6-mediated regulation of $V\gamma 7^+$ IEL (Di Marco Barros et al., 2016; Melandri et al., 2018) strongly argue that the results described in this thesis reflect an evolutionary conserved biology.

Although this thesis stopped short of direct biochemical studies between purified proteins, the fact that soluble TCRs (sTCRs) specifically stained cells expressing BTN3 plus BTN8 strongly supports the prospect that the interaction of $V\gamma 4$ and BTN3 is direct. This has since been demonstrated by SPR and isothermal calorimetry experiments conducted in collaboration with Willcox and colleagues at the University of Birmingham (Willcox et al., 2019).

Intriguingly, the use of sTCRs revealed a surprising downstream consequence of interactions with the BTNL molecules. Thus, in the co-culture assay and upon TCR binding, the BTNL molecules capture some of the TCR and trans-endocytose it into the epithelial cell line. It was striking that trans-endocytosis occurred to a much lesser degree for antibodies specific to BTNL proteins. Finally, the results of this thesis also showed that a $V\gamma 4^+$ chain has the capacity to interact with clonotypic antigens, such as EPCR and CD1c, using spatially distinct regions from those used to mediate responses to BTN3 plus BTN8. Collectively, these data have important implications for the development of tissue-resident $\gamma\delta$ T cells, for the general mechanisms of $\gamma\delta$ TCR ligand recognition, for the regulation of $\gamma\delta$ T cells at steady-state, and for their potential to transition into responding to tissue stress.

1. BTNL3-BTNL8 heteromer binding is not clonally restricted

1.1. The $\gamma\delta$ TCR repertoire of colonic $\gamma\delta$ lymphocytes

A major goal of the deep sequencing analysis (Chapter III, Fig.3.3-4, Table 3.1) was to determine if single-cell sequenced TCRs from responding $\gamma\delta$ lymphocytes represented clonally expanded populations. Additionally, the data provided a more general overview of the $\gamma\delta$ TCR repertoire in the human colon. To our knowledge, there are no studies which have used next-generation deep-sequencing to evaluate human $\gamma\delta$ TCR diversity at this anatomical site.

CDR3 $\gamma\delta$ length distribution (Chapter III, Fig.3.4B) clearly matched earlier findings in other tissues (Rock et al., 1994; Davey et al., 2017), with CDR3 γ being markedly shorter and less diverse compared to CDR3 δ which makes the principle antigen recognition site of the $\gamma\delta$ TCR more similar to that of antibodies than of $\alpha\beta$ TCRs. The overall clonality of the $\gamma\delta$ TCR repertoire was variable, as has been reported for human peripheral blood (Davey et al., 2017). Thus, there were persons with highly oligoclonal intestinal compartments (GN09 and GN17) and those with more polyclonal repertoires (GN08) (Chapter III, Fig.3.4A). In peripheral blood, an unfocused repertoire has been associated with a naive $\gamma\delta$ T cell pool, while a focused one has been shown to be more reflective of antigen encounter, memory formation and an adaptive T cell biology (Davey et al., 2017). Likewise, Holtmeier et al. (1997) observed a progressive decline in the human intestinal $\gamma\delta$ repertoire diversity from birth to adolescence, suggesting strong, site-specific selective signals, that might include dietary and microbial ligands and/or endogenous stress antigens. Although this study did not attempt to identify correlations between markers of antigen experience and clonality in the human colonic $\gamma\delta$ compartment, our data confirm that mature repertoires are highly donor specific, strongly suggesting that they are not simply the product of non-clonal BTNL-driven responses.

1.2. Every V γ 4⁺ cell will likely have the capacity to respond to BNTL3 plus BTNL8

Approximately half of the paired $\gamma\delta$ TCR sequences were found in the deep sequencing dataset with variable frequencies (Chapter III, Table 3.1). The rest of the sequences were not found, seemingly reflecting rare clones. Owing to the nature of the starting material, sequencing depth could not be accurately predicted. Nevertheless, these results argue that every V γ 4⁺ lymphocyte, be it abundant or rare, has the capacity to respond to BTNL3 plus BTNL8, a conclusion supported

by the lack of a clear CDR3 motif associated with the *TRGV4* reads (Chapter III, Fig.3.4C), and by the fact that skin-derived $V\gamma 4V\delta 1$ TCRs also responded to BTNL3 plus BTNL8 (Chapter III, Fig.3.9).

Nonetheless, a recent study challenged this conclusion (Mayassi et al., 2019). TCR sequencing analysis of healthy donors and patients with coeliac disease showed enrichment of CDR3 γ reads with a histidine-J γ 1 (H-J γ 1) signature in active disease compared to data from controls and people in remission after a gluten-free diet. From a coeliac patient, a $V\gamma 4V\delta 1$ H-J γ 1 TCR was cloned and expressed but it failed to upregulate Nur77 and downregulate its TCR in co-culture assays with HEK293T cells expressing BTNL3 plus BTNL8. However, the cumulative data from three experiments clearly demonstrated a reduction in CD3 MFI compared to co-cultures with untransduced or BTNL8-transduced HEK293T cells. The authors acknowledged data from this thesis which has also been published (Melandri et al., 2018). Since TCR hu12 also carries the H-J γ 1 motif but it responds to BTNL3 plus BTNL8, they reasoned that the exceptionally long CDR3 δ of their clone may be contributing to impaired responsiveness. While it is possible that CDR3 δ is involved in modulating the affinity of the interaction and that a long loop might have caused steric hindrance, this does not seem to be the case for skin-derived TCRs, sk1 and sk2, which have CDR3 δ lengths of 24 and 21 amino acids, respectively (Chapter III, Fig.3.9A). Furthermore, other sequences derived from the single-cell sorting of responding cells also displayed long CDR3 δ loops, for example, hu3, hu7, hu13, hu17 and hu19 (Chapter III, Table 3.1). Possibly, the exact CDR3 δ amino acid sequence was impacting upon BTNL3 plus BTNL8-responsiveness, but the authors did not provide this information in their manuscript. Nevertheless, it seems very probable that the assay employed by Mayassi et al. (2019) was of insufficient sensitivity to detect the capacity of all $V\gamma 4^+$ TCRs to respond, as we recently considered (Willcox et al., 2019).

In sum, the data of this study establish that the $V\gamma 4$ chain is the primary, non-clonal determinant of BTNL3 plus BTNL8 responsiveness. Moreover, contemporaneous studies of mouse $V\gamma 7^+$ IEL responses to Btl1 plus Btl6 strongly suggest that this biology is highly conserved (Melandri et al., 2018; Willcox et al., 2019).

2. The $V\gamma 4^+$ chain binds BTNL3 in a superantigen-like manner

Superantigens are microbial products of viral or bacterial origin, which elicit strong, non-clonal immune activation in a large proportion of $\alpha\beta$ T cells depending on which $V\beta$ and/or $V\alpha$ they express (Marrack and Kappler, 1990). Staphylococcal enterotoxins (SE) are among the most extensively studied superantigens produced by *Staphylococcus aureus* (Stiles and Krakauer,

2005). This pathogenic bacterium can colonize the skin or mucosal surfaces and produces various virulent factors that include SEs among which there is toxic shock syndrome toxin 1 (TSST-1). As the name suggests, TSST and other SEs can promote exacerbated inflammatory immune responses, although these are commonly followed by induction of anergy or deletion of T cell subsets expressing specific V β or V α genes (Stiles and Krakauer, 2005; Saline et al., 2010). The mechanisms by which superantigens achieve this involves simultaneous binding directly to MHCII molecules on APCs and directly to the TCR on T cells (Fields et al., 1996; Li et al., 1998; Sundberg et al., 2002; Saline et al., 2010; Rödström et al., 2014). For several SEs, the CDR2 and HV4 loops of the TCR β chain are critical interaction sites (Fields et al., 1996; Li et al., 1998; Sundberg et al., 2002; Rödström et al., 2014), with variable contributions depending on the nature of the superantigen (Kreiß et al., 2004). More recently, superantigens have also been reported to enhance B7-1/2 and CD28 synapse formation, possibly by inducing allosteric changes and increasing the affinity of these molecules for each other (Levy et al., 2016; Popugailo et al., 2019).

Thus, the CDR2-HV4 region of TCR β has a clear biological significance. Considering the close evolutionary relationship between TCR β and TCR γ (Rast et al., 1997), the involvement of V γ 4 CDR2-HV4 loops in mediating BTNL3 responsiveness (Chapter IV, Fig.4.1-3) seems to present a striking parallel to superantigen-mediated T cell regulation.

Interestingly, superantigen binding has also been reported in the context of V γ 9V δ 2 T cell (Loh et al., 1994; Morita et al., 2001). SEA, which was initially characterised as binding to V β 5.2, was also found to bind the V γ 9 chain independent of CDR3 $\gamma\delta$ (Loh et al., 1994; Morita et al., 2001). Importantly, SEA binding to V γ 9 and V β 5.2 was mediated through different portions of the toxin – the amino-terminus and the carboxyl-terminus, respectively (Morita et al., 2001). In both cases the presence of MHCII-expressing APCs was required for SEA reactivity. However, this does not imply that the cells had cognate reactivity to MHCII and it is important to note that activated $\gamma\delta$ T cells express MHCII (Brandes et al., 2005). Other SEs such as SEB, SEC3, SED and SEE did not stimulate $\gamma\delta$ T cells consistent with specific superantigens having preferential TCR chain targets (Morita et al., 2001). Hence, a single molecule can use two distinct molecular mechanisms to non-clonally target V γ 9⁺ and V β 5.2⁺ cells. While this phenomenon is rare among known superantigens, it directly extends the superantigen concept to V γ -specific regulation of $\gamma\delta$ cells. It is appropriate to consider several key features which distinguish superantigens from clonally-restricted $\alpha\beta$ T cell antigens:

- (1) generally, they can directly bind the TCR in a spatially-distinct region outside the pMHC-binding groove;
- (2) they exert biological effects as intact proteins without any obvious requirement for processing;
- (3) their effects are largely not restricted to specific MHC alleles;
- (4) they are dependent on specific TCR V regions ($V\beta$, $V\alpha$, $V\gamma$);
- (5) they can promote augmented immune responses followed by anergy.

One can consider that BTNL3 plus BTNL8 engagement by the $V\gamma 4^+$ TCR shares at least the first four of these characteristics. It may likewise be true that the fifth property is shared, based on studies in mice where *Btnl1* transgene was used to rescue $V\gamma 7^+$ IEL development in *Btnl1*^{-/-} animals (Di Marco Barros et al., 2016). *Btnl1* re-expression initially drove potent IEL proliferation, thereby filling the compartment, and afterwards the cells existed in an “activated yet resting” state (Di Marco Barros et al., 2016; Shires et al., 2001), that is possibly a form of reversible anergy. This may be maintained by the sustained expression of *Btnl1* and *Btnl6* by enterocytes in the steady state (Di Marco Barros et al., 2016).

SEB challenge in $CD4^+$ naive T cells induced anergy characterised by loss of IL-2 production and no proliferation, while there was no effect seen on primed $CD4^+$ Th cells (Lussow and MacDonald, 1994). Subsequent studies showed that SEA-treated $CD4^+$ T cells lose the ability to produce IL-2, IL-4 and IFN γ upon subsequent antigenic challenge, which was partially mediated by increased production of IL-10 and TGF β as well as impaired Erk signalling (Miller et al., 1999). In addition, SEB-treated $CD4^+$ memory T cells exhibited reduced PLC γ_1 , LAT and Zap70 phosphorylation relative to naïve $CD4^+$ lymphocytes (Watson and Lee, 2006). Lck phosphorylation in memory cell was rapidly induced and extinguished within two minutes, which led to inefficient recruitment of Zap70 to the TCR (Watson and Lee, 2006). Intriguingly, another study showed that Lck was dispensable for superantigen-induced signalling which could alternatively utilize PLC β -mediated pathways (Bueno et al., 2006). Clearly, these results collectively offer a framework for investigating potential parallels between BTNL-induced and superantigen-induced signalling, that may elucidate the molecular and biological consequences of $\gamma\delta$ -BTNL cross-talk.

Of note, the changes in phosphorylation and activation of signalling molecules which lead to defective signal transduction in T cells during adaptive tolerance and superantigen encounter

are more similar compared to those that develop in TCR signalling during clonal anergy (Choi and Schwartz, 2007). Data generated by Dr Robin Dart in our laboratory suggest that BTNL3 and BTNL8 engagement does not lead to an obvious effector activation and cytokine production in primary $\gamma\delta$ T cells. Therefore, superantigen-like signalling in BTNL-stimulated $V\gamma 4^+$ lymphocytes would possibly limit these potentially self-reactive T cells by inducing a state of tolerance which is essential in a heavily antigenic environment such as the gastrointestinal tract. In this vein, it is important to consider that all characterised superantigens are encoded by bacteria, viruses, or endogenous proviruses, whereas BTNL3 and BTNL8 are encoded by *bona fide* human genes. This raises an intriguing possibility that there are as yet unelucidated human genes that interact with $V\beta$ or $V\alpha$ regions in order to select and/or regulate $\alpha\beta$ T cells within tissues, and that superantigen binding reflects microbial exploitation of that biology.

3. Dual reactivity of the $V\gamma 4^+$ TCR

3.1. BTNL3 and BTNL8 are perhaps selecting agonists for intestinal $V\gamma 4^+$ lymphocytes

Murine gut $V\gamma 7^+$ IEL development is critically dependent on *Btnl1* expression (Di Marco Barros et al., 2016). A mature $\gamma\delta$ IEL compartment is established within 3-4 weeks post-birth. This coincides with the upregulation of *Btnl1* and *Btnl6* in the murine intestinal epithelium, phenotypic changes in the $\gamma\delta$ lymphocytes, and a proliferative burst around week 2-3 after which $V\gamma 7^+$ IELs are essentially quiescent. *Btnl1* knockout mice exhibit severely reduced $V\gamma 7^+$ IEL numbers and any residual cells do not exhibit phenotypic maturation (Di Marco Barros et al., 2016). Thus, *Btnl1* is a selecting element required for the establishment of a dominant $V\gamma 7^+$ IEL compartment. Furthermore, parallel studies in mice also demonstrated the $V\gamma 7$ HV4 loop to be involved in an interaction primarily mediated by *Btnl6* which is normally expressed together with *Btnl1* (Melandri et al., 2018; Di Marco Barros et al., 2016). Given this, it is reasonable to hypothesise that BTNL3 plus BTNL8 heteromers are perhaps selecting agonists for human intestinal $V\gamma 4^+$ IELs. Conventional $\alpha\beta$ T cells undergo positive and negative selection based on TCR specificity which leads to an MHC-restricted nonself-reactive repertoire. However, self-reactive T cells such as Tregs, NKT cells and intestinal IELs are believed to undergo agonist selection due to their antigen-experienced phenotype (Stritesky et al., 2012).

3.2. BTNL3 and BTNL8 interaction may communicate a status of “normality” to V γ 4⁺ lymphocytes

Although they are actually resting, murine intestinal $\gamma\delta$ IELs display some noticeable properties of prior activation. Thus, they have high levels of granzyme and chemokine transcripts (Shires et al., 2001), high basal intracellular Ca²⁺ levels (Malinarich et al., 2010), high basal Erk1/2 activity which is independent of microbial colonization (Sydora et al., 1993) and are hypo-responsive to *in vitro* TCR-stimulation (Mosley et al., 1991; Sydora et al., 1993; Malinarich et al., 2010; Di Marco Barros et al., 2016). Of note, injection of anti- $\gamma\delta$ TCR antibody was able to diminish basal intracellular Ca²⁺ flux in intestinal $\gamma\delta$ IELs which indicated that the cells may be constantly activated through their TCR at steady state (Malinarich et al., 2010).

Interestingly, calcium signalling was shown to be particularly important in the development of other self-reactive agonist-selected T cells (Oh-Hora et al., 2013). Following TCR triggering, the endoplasmic reticulum (ER) stores provide a rapid flux of intracellular Ca²⁺. Their depletion leads to the activation of ORAI1 channels by ER-localised STIM1 and STIM2 which induces store-operated Ca²⁺ entry and the activation of the Ca²⁺-calmodulin-NFAT signalling pathway (reviewed in Trebak and Kinet, 2019). *Stim1*^{-/-}*Stim2*^{-/-} mice have almost no Tregs, iNKT cells and TCR $\alpha\beta$ ⁺ CD8 $\alpha\alpha$ ⁺ IELs due to impaired proliferation and functional maturation. In contrast, there was only a moderate effect on the negative selection of conventional T lymphocytes (Oh-Hora et al., 2013). The knockout of *Stim1* alone was sufficient to hamper iNKT development and similar observations were made in a patient with STIM1 deficiency (Oh-Hora et al., 2013; Fuchs et al., 2012). The consequences of *Stim1* and *Stim2* knockout on $\gamma\delta$ T cells have not been reported but it can be speculated that their development will be affected as well, a hypothesis that should be tested in the future.

Murine dendritic epidermal $\gamma\delta$ T cells (DETCs) have also been described to have atypical Ca²⁺ flux and reduced TCR responsiveness (Wencker et al., 2014). Normal DETC development is dependent on the Btl-related protein Skint1, expressed by medullary thymic epithelial cells (mTEC) (Boyden et al., 2008). This process was found to be TCR-dependent as the majority of DETC progenitors from *Nur77*.GFP mice were GFP⁺ whereas this was not seen in mice bred on a *Skint1* hypomorphic background known as FVB.Tac (Wencker et al., 2014). At E15 DETC progenitors displayed normal Ca²⁺ flux and TCR responsiveness which was rapidly lost by E16 in wild-type but not FVB.Tac mice (Wencker et al., 2014). The authors of the study demonstrated that $\gamma\delta$ TCR signalling was initiated in *Skint1*-dependent manner and coincided with the commencement of hypo-responsiveness which was sustained throughout life. However, DETCs

are not irreversibly anergic as they rapidly respond to tissue dysregulation (Strid et al., 2008; Strid et al., 2011). Moreover, their TCRs are constantly clustered in phosphotyrosine-rich aggregates located on their dendrites and are activated *in vivo* as judged by constitutive CD247 (CD3 ζ) and Zap70 phosphorylation (Chodaczek et al., 2012).

In the murine intestinal epithelium, $\gamma\delta$ IELs also appear to be hypo-responsive to *in vitro* stimulation with anti-CD3. Interestingly, the V γ 7⁺ IELs from *Btnl1*^{-/-} mice displayed improved anti-CD3 responses compared to V γ 7⁺ cells isolated from wild-type mice (Di Marco Barros et al., 2016). Furthermore, the V γ 7⁻ lymphocytes from wild-type animals, which have not been selected on Btnl1, also appeared to be more responsive to anti-CD3 stimulation compared to the Btnl1-selected V γ 7⁺ cells (Di Marco Barros et al., 2016).

Taken together, the data support a hypothesis whereby the basis for $\gamma\delta$ T cell-mediated tissue immune surveillance is the constitutive, TCR-mediated interactions with Btnl/BTNL molecules expressed by the epithelium. Thus, BTNL3 and BTNL8 interaction may promote agonist selection and then sustain the IELs in an appropriate state, communicating to them a state of tissue normality.

In support of this hypothesis, BTNL3 and BTNL8 are constitutively expressed by differentiated intestinal epithelium (Mayassi et al., 2019; Dr Robin Dart, unpublished data). They become downregulated following tissue stress in conditions such as UC (Lebrero-Fernandez et al., 2016b), active coeliac disease (Mayassi et al., 2019) and cancer (Lebrero-Fernandez et al., 2016b; Oncomine database). BTNL3 has further been reported to become epigenetically silenced via CpG methylation in the intestinal mucosa of treatment-naïve UC patients (Taman et al., 2018). On the other hand, certain CD1 molecules and EPCR become upregulated following infection and in tumour settings, respectively. For example, CD1a, CD1b and CD1c were upregulated on monocytes following *in vitro* infection with *Mycobacterium tuberculosis* (Roura-Mir et al., 2005); CD1c expression was upregulated following *Staphylococcus aureus* Cowan I bacteria treatment or BCR cross-linking of tonsil-isolated B cells (Allan et al., 2011); and the treatment of human monocytes with lipids derived from *Borrelia burgdorferi*, the causative agent of Lyme disease, resulted in upregulated CD1a, CD1b and CD1c expression (Yakimchuk et al., 2011). Finally, EPCR overexpression has been reported in colorectal cancer (Lal et al., 2017). Collectively, these examples show that the two types of TCR-dependent regulators of V γ 4⁺ lymphocytes have inversely correlated expression patterns: non-clonal (BTNL) regulators reflect normality while clonotypic regulators reflect dysregulations (Chapter V, Fig.5.1-2). In this light, the

interindividual variation in IEL clonality considered above may reflect variable episodes of gut epithelial dysregulation due to microbial infections.

3.3. A model for antigen recognition by intestinal $V\gamma 4^+$ lymphocytes

Considering the antibody-like mechanisms of antigen-binding by $\gamma\delta$ T cells and the physiological expression of BTNL3 and BTNL8, we propose a “molecular switch” model to explain the nature of $V\gamma 4$ -BTNL3 interactions (Fig.7.1). Thus, BTNL3 plus BTNL8 heteromeric complex engages the $V\gamma 4^+$ TCR in a healthy tissue, induces a semi-anergic state and communicates a signal of “normality” to the lymphocytes. In contrast, during tissue dysregulation, BTNL3 and BTNL8 become downregulated and the $V\gamma 4^+$ TCR is free to recognise stress-induced clonally-restricted self-antigens, to expand locally, and to exert effector functions such as cytokine secretion and cytotoxicity.

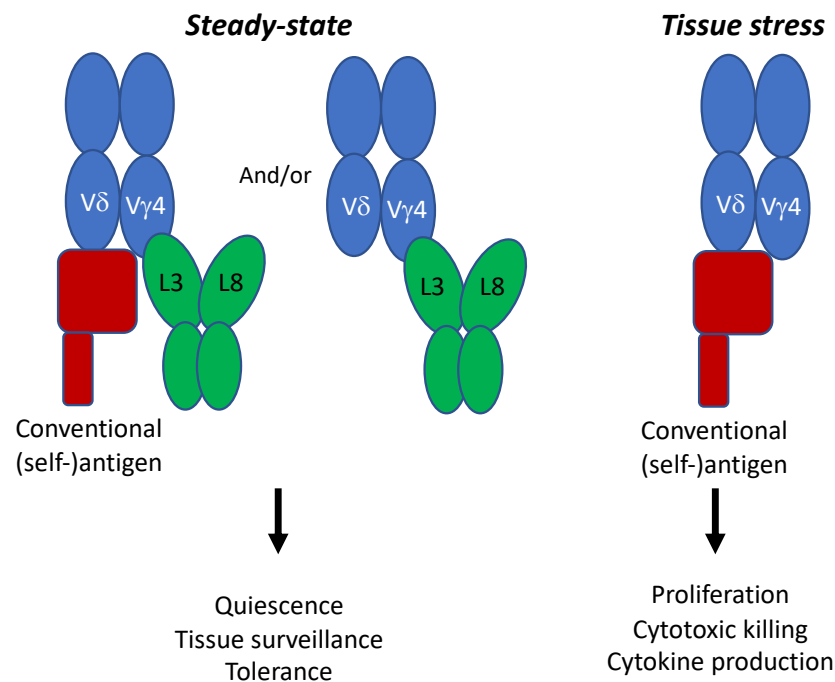


Figure 7.1. A proposed model of BTNL3 plus BTNL8 interaction with the $\gamma\delta$ TCR. At steady-state, the $V\gamma 4^+$ TCR is constantly engaging the BTNL3 and BTNL8 heteromeric complex which induces a $\gamma\delta$ IEL functional program associated with tissue surveillance, homeostasis and overall quiescence. The TCR may also simultaneously contact a clonally-restricted antigen which would not be able to override the BTNL-induced signals. When the epithelium becomes stressed, BTNL3 and BTNL8 are downregulated and the TCR is free to sample its environment for stress-induced ligands of endogenous and/or exogenous origin. In turn, this would induce activation, proliferation and cytotoxic activity of the $V\gamma 4^+$ lymphocyte.

In silico analysis of V γ 4V δ 1-CD1d-BTNL3-BTNL8 binding suggests that the $\gamma\delta$ TCR might simultaneously engage both antigens, were they both to be expressed simultaneously (Melandri et al., 2018). Considering the level of TCR downregulation elicited by the two types of ligands, the interaction with a BTLN3 plus BTLN8 complex may be stronger and of higher affinity for the TCR in the case of direct TCR binding, as we recently showed (Willcox et al., 2019; Cai et al., 1997; Gallegos et al., 2016). Therefore, BTLN3 plus BTLN8-induced signalling outcomes might dominate, irrespective of whether both antigens are bound to the receptor as it is suggested by Chapter V, Fig.5.1A. Conversely, extensive mutagenesis analysis revealed a discrete patch of the β -sheet face on the side of the EPCR molecule to be crucial for recognition by the LES clone (Willcox et al., 2012). This orientation might not permit simultaneous ligation of the TCR to the BTLN heteromeric complex and EPCR. Similarly, a V γ 9V δ 1 TCR was shown to bind on the underside of MR1, with the V δ chain contacting the α 3 domain of MR1 and the V γ chain pointing towards the cell membrane (Le Nours et al., 2019). Therefore, it is possible that the $\gamma\delta$ TCR engages one ligand at a time. In fact, we recently confirmed in SPR experiments that the BTLN3 IgV domain outcompetes EPCR for binding to the LES TCR (Willcox et al., 2019).

4. What is the functional significance of a $\gamma\delta$ TCR trans-endocytosis?

Surface protein transfer between immune and target cells appears to be a widespread phenomenon, sometimes termed “trogocytosis” (reviewed by Davis, 2007). Its biological significance is not clear yet but it occurs rapidly after synapse formation, and seems to play a role in regulating immunological responses. There are several examples. Thus, T cells were shown to donate several surface proteins to B cells via exosome secretion. The ability of B cells to promote proliferation in co-culture assays with responder T cells was impaired by pre-treatment of the B lymphocytes with exosomes secreted by anergic T cells, although the exact nature of the proteins which were transferred and conferred these effects was not elucidated (Nolte-’t Hoen et al., 2004). In addition, the transfer NKG2D from NK cells to MICB-expressing target cells was shown to correlate with subsequently reduced cytotoxicity potential of NK cells (Roda-Navarro et al., 2006). More recently, a proposed mechanism of action of CTLA-4-mediated T cell inhibition has gained substantial interest related to the development of cancer immunotherapies targeting this receptor. Thus, it was demonstrated that CTLA-4 binds CD80/CD86 which it depletes from the surface of APCs through trans-endocytosis (Qureshi et al., 2011). Hence, the APC can no longer drive CD80/CD86-CD28-mediated T cell co-stimulation. The authors distinguish this process from “trogocytosis” due to the observations that

CD80/CD86 become internalized in the recipient cell rather than being merely transferred onto the cell surface. Collectively, these examples suggest that diverse mechanisms exist whereby the exchange of effector molecules can regulate immune responsiveness.

Experiments in this and other studies have primarily used TCR downregulation as a metric of specific engagement with BTN(L) and BTN(L)-related molecules. However, using fixed 293T.L3L8 cells, it seems that approximately one third of the observed TCR downregulation is mediated *via* TCR transfer into 293T cells (Chapter VI, Fig.6.2E-F). TCR downregulation represents a negative feedback signal which constrains T cell effector function (Gallegos et al., 2016). Therefore, the process of trans-endocytosis observed in the studies here (Chapter VI, Fig.6.1-3) could represent an additional immunosuppressive mechanism through which epithelial cells limit antigen recognition by the $V\gamma 4^+$ TCR via its depletion from the lymphocyte cell surface.

Importantly, the transfer of molecules was specific to the TCR complex and both the TCR and the CD3 could be trans-endocytosed (Chapter VI, Fig.6.3). In contrast, the same was not seen for CD45 which is part of the immunological synapse but is actively excluded from the TCR microclusters (Varma et al., 2006). Thus, the observed effects were not simply due to non-specific membrane exchange at the site of interaction.

The majority of reports in the literature investigated the transfer of molecules towards the T cell and although some of the experiments show that TCR signalling is required, the transfer of the TCR itself was not usually involved (Davis, 2007). Nevertheless, a precedent exists for $\alpha\beta$ TCR transfer to B cells (Choudhuri et al., 2014). In these experiments, small vesicles enriched with TCRs were secreted primarily at the site of the immunological synapse but could also be found distally. Importantly, they were captured by the B cells and were able to induce antigen-dependent BCR signalling suggesting an immunostimulatory function (Choudhuri et al., 2014).

5. Future directions

There are now several examples $\gamma\delta$ T cells regulation by Btn(I)s/BTN(L)s: murine DETC and $V\gamma 7^+$ IELs, and human $V\gamma 9V\delta 2$ and $V\gamma 4^+$ lymphocytes. However, the field has failed to resolve whether these are true ligands. The work here, in particular the specificity of sTCR binding to 293T.L3L8 cells (Chapter IV, Fig.4.5) and the fact that $V\gamma 4^+$ TCR transfer was necessary and sufficient to confer BTN(L) plus BTN(L) responsiveness in J76/JRT3 cells (Chapter III), is completely consistent with this hypothesis. However, it has now been formally established by us and our collaborators that the $V\gamma 4^+$ TCR directly binds BTN(L) of the BTN(L) plus BTN(L) heteromeric complex (Willcox

et al., 2019). Thus, future studies should aim to elucidate the biological consequences of this ligand-receptor interaction.

5.1. What is the nature of the BTNL-induced TCR signalling?

It was technically challenging to investigate TCR signalling in our co-culture system. Detection of phospho-PLC γ_1 , phospho-LAT, and NFAT-activity (Chapter III, Fig.3.7B, C) strongly implicated a direct TCR ligation. Nevertheless, those changes do not necessarily reflect the true signalling events found in primary lymphocytes and compensatory mechanisms may occur in the $\alpha\beta$ lymphocyte-derived Jurkat cell line. As discussed in the introduction, the $\alpha\beta$ TCR and the $\gamma\delta$ TCR utilize similar but not identical signalling pathways and heterogeneity exists within the $\gamma\delta$ T cell sub-populations. Moreover, murine $\gamma\delta$ IELs express high levels of the signalling molecules *Lyn* and *Lat2*, and low levels of *Lat* in contrast to gut-derived and splenic conventional CD8 $\alpha\beta^+$ $\alpha\beta$ T cells (Denning et al, 2007). While *Lat* is known to be involved in TCR signalling, the related *Lat2* protein is expressed in B cells, mast cells, NK cells and monocytes but not resting $\alpha\beta$ T cells (Iwaki, Jensen and Gilfillan, 2008). Likewise, *Lyn* is known to regulate the BCR signalling cascade (Seda and Mraz, 2014). In addition, according to the ImmGen database *Plcg2*, normally involved in BCR but not TCR signalling, is highly expressed in V γ 7 $^+$ IELs and not in splenic $\gamma\delta$ or other $\alpha\beta$ subsets. Collectively, these examples demonstrate that key molecules of the BCR transduction pathway are also implicated in murine intestinal $\gamma\delta$ TCR signalling. Thus, future experiments should aim to investigate whether human intestinal V γ 4 cells utilize a conventional, antigen-dependent Lck-Zap70-LAT signalling pathway in response to BTNL3 plus BTNL8 or whether they employ alternative signalling molecules. The prospect of the latter is strongly suggested by the parallels with superantigens and the evidence from mouse studies considered above.

A major practical issue currently hindering further studies of signalling in human samples is the availability of material. As our current source is intestinal biopsies, the number of cells that are extracted from a tissue digest are not sufficient for *ex vivo* studies. Analysis of *in vitro* IL-2 and IL-15-expanded cells would be confounded by the fact that these cytokines promote activation and cytotoxicity of $\gamma\delta$ T cells (Correia et al., 2011; Van Acker et al., 2016). One approach to simplify this situation would be to utilise soluble recombinant BTNL3 plus BTNL8 complexes, if they can be shown to mimic co-culture with BTNL3 plus BTNL8 expressing cells. Conversely, since BTNL3 plus BTNL8 interaction site of human V γ 4 is the major sequence discrimination of V γ 4 and V γ 2 (which does not respond to BTNL3 plus BTNL8) the possibility exists that signalling might be mimicked by a V γ 4-specific monoclonal antibody that we have just obtained in collaborative studies with Gamma Delta Therapeutics.

5.2. What is the role of the B30.2 domain?

The B30.2 domain has emerged relatively recently in evolution and is found only in vertebrates with an adaptive immune system (Rhodes et al., 2005). It is an extremely common fold, shared with other protein families such as the TRIM genes. It has no known enzymatic function but it is believed to be involved in protein-protein interactions (Perfetto et al., 2013). A proportion of the B30.2-containing TRIM proteins also possess an E3 ubiquitin ligase activity which suggests that this domain may be required for binding to specific substrates (Ozato et al., 2008). Several of the TRIM proteins have also been implicated in antiviral immunity and innate immune responses (D'Cruz et al., 2013). For example, the B30.2 domain of rhesus monkey TRIM5 α mediates anti-retroviral effects by binding to the capsid of human immunodeficiency virus-1 (HIV-1) and targeting it for proteasomal degradation in Old World monkey cells (Stremlau et al., 2006). Thus, the biological function of the BTN(L) proteins might also be regulated by their intracellular portion via interaction with endogenous or exogenous factors.

In line with this view, the B30.2 fold of BTN1A1 was shown to bind the xanthine oxidoreductase which in turn stabilized the formation of milk fat globules (Jeong et al., 2009). Furthermore, the most extensively studied B30.2 domain in the BTN(L) family is that of BTN3A1. Initial depletion, domain swapping and mutation experiments have implicated it in V γ 9V δ 2 phosphoantigen reactivity (Harly et al., 2012; Sandstrom et al., 2014). The B30.2 domains of BTN3A1 and BTN3A3 display 87% sequence identity. However, BTN3A3 alone poorly supports phosphoantigen-mediated activation of V γ 9V δ 2 cells, only in the presence of very high doses of zoledronate (Vantourout et al., 2018). The swap of BTN3A3 B30.2 domain for that of BTN3A1 was sufficient to confer full function, while its deletion from BTN3A1 abolished V γ 9V δ 2 activation (Harly et al., 2012). Originally, BTN3A2 was considered a decoy receptor due to its lack of a B30.2 sequence. However, our laboratory recently showed that BTN3A2 is essential for optimal BTN3A1 function and that it mediates heteromeric interactions with BTN3A1 *via* its IgC domain (Vantourout et al., 2018). The transfer of BTN3A1 B30.2 domain to BTN3A2 was not sufficient to confer function and the juxtamembrane portion was required as well to recapitulate BTN3A1 expression pattern and function (Vantourout et al., 2018).

Thus, the extracellular domains of BTN3A1 and BTN3A2 are seemingly interchangeable, with BTN3A1 function depending upon its intracellular determinants that include the B30.2 domain. It was proposed that phosphoantigens bind the B30.2 domain and induce a conformational change in an inside-out signalling model (Harly et al., 2015). The most recent crystal structure data supports this by demonstrating that HMBPP binds in a highly basic pocket found on the

B30.2 fold which led to a conformational change propagated to the juxtamembrane region (Yang et al., 2019). In addition, the C terminus of BTN3A2 was shown to contain ER retention motifs which were required for the efficient heteromeric expression with BTN3A1, and function. Similar sequences were also described for BTNL3 and BTNL8 (Vantourout et al., 2018). Thus, the intracellular part of the BTN(L) molecules has clear biological significance. Future studies should further investigate how the different intracellular sequences of BTNL3 and BTNL8 regulate their expression and contribute to their cross-talk with $V\gamma 4^+$ cells. Would the deletion of either or both B30.2 domains in the BTNL3 plus BTNL8 complex disrupt $\gamma\delta$ TCR binding or trans-endocytosis? Do any intracellular proteins associate with the heteromers? Are there phosphorylation-mediated downstream signalling events?

The development of specific BTNL3 and BTNL8 antibodies has been hindered by their high overall sequence similarity. Nevertheless, reagents are being developed and currently there are two commercially available antibodies targeting the N-terminal regions of BTNL3 and BTNL8, respectively. The BTNL3 antibody was validated in our laboratory. However, it appears to be of low affinity and does not stain primary epithelial cells (data not shown). On the other hand, anti-BTNL8 was shown to stain duodenal tissue sections (Mayassi et al., 2019). For example, such reagents may be used in co-immunoprecipitation experiments using primary gut epithelial cell in order to determine whether any intracellular proteins interact with the B30.2 domain.

5.3. Further investigation of the $\gamma\delta$ TCR trans-endocytosis pathway

BTNL3 plus BTNL8-mediated $V\gamma 4^+$ TCR trans-endocytosis was an unexpected and intriguing observation. Future studies may compare the fate of the sTCR LES engaging 293T.L3L8 vs 293T.EPCR vs 293T.L3L8+EPCR cell lines, so as to determine whether the phenomenon is a specific result of BTNL3 plus BTN8 but not EPCR ligation, and whether the presence of the latter has any effect on BTNL-mediated endocytosis. Antibodies to BTNL3 plus BTNL8 were not efficiently trans-endocytosed in these experiments. However, it would be interesting to see whether an anti-BTNL3 antibody can be obtained which closely mimics the binding mode of the $V\gamma 4$ TCR chain, and to determine whether this reagent would be internalised. Likewise, the potential role of the B30.2 domain in the process of trans-endocytosis should also be examined.

Importantly, the prospect for $V\gamma 4^+$ TCR trans-endocytosis to have an immunoregulatory function, requires its further validation in more physiological systems, such as organoids.

5.4. Towards the use of more physiological systems

While cell culture experiments facilitate high-throughput biochemical, cell and molecular biological experiments to be conducted at scale, there is the persistent concern that cell lines fail to recapitulate physiological processes occurring *in vivo*. In this regard, Caco2 and T84 human colonic cell lines have historically been used to study intestinal epithelial cell layer functions because at confluence they can spontaneously differentiate *in vitro* towards mature enterocytes with an apical brush border and tight junctions (Bolte et al., 1997; Sambuy et al., 2005; Hurley et al., 2016; Devriese et al., 2017). However, their differentiation is time-consuming and they have high genetic imbalance due to polyploidy reflective of their tumour origin. In preliminary experiments, Caco2 and T84 failed to upregulate *BTNL3* and *BTNL8* to levels observed in whole biopsy mRNA, despite successfully upregulating *CDX2*, *HNF4A* and downregulating the stem cell marker *LGR5* (data not shown).

As an alternative, the rapidly evolving field of human organoids has permitted differentiated cultures to be established from tissues such as intestine, lung, brain and kidney (Sato et al., 2011; Camp et al., 2015; Combes et al., 2019). Human intestinal organoids can be differentiated within five days and contain all epithelial cell types found within the native tissue (Sato et al., 2011). Furthermore, transcriptomic analysis of colonic, duodenal and ileal organoids indicates they largely maintain tissue-specific gene expression patterns (Kozuka et al., 2017). In our hands, as human colonic organoids differentiated, *BTNL3* and *BTNL8* mRNA expression reached levels similar to the tissue of origin (Chapter VI, Fig. 6.4). Ideally, upregulation of *BTNL3* and/or *BTNL8* protein should also be demonstrated. Nevertheless, the system may represent an excellent model to study several aspects of *BTNL*- $\gamma\delta$ IEL biology. Thus, organoid cultures can be used to study the regulation of *BTNL3* and *BTNL8* expression during the differentiation process of enterocytes and colonocytes, or during tissue stress. Furthermore, $\gamma\delta$ TCR trans-endocytosis might be further validated in this system, first with the use of the sTCRs and perhaps at a later stage by introduction of $V\gamma 4^+$ TCR transductants or of primary $V\gamma 4^+$ lymphocyte. In addition, organoids can be used to investigate the B30.2 domain signalling, and transcriptional changes in the epithelium and the $\gamma\delta$ lymphocytes upon *BTNL3*- $V\gamma 4$ interaction in co-culture experiments.

Of note, murine intestinal organoids were reportedly co-cultured with $\alpha\beta$ and $\gamma\delta$ IELs in the organoid-supporting matrix Matrigel, facilitating the study of IEL motility within the epithelium (Nozaki et al., 2016). Human monocyte-derived DCs were shown to spontaneously migrate towards gastric organoids through the Matrigel and this interaction was enhanced by *Helicobacter pylori* infection (Sebrell et al., 2019). The co-culture of epithelial tumour organoids with peripheral lymphocytes was used to generate tumour-reactive T cells in a Matrigel-free

assay (Dijkstra et al., 2018). Human primary macrophages have also been used in a co-culture model with an enteric monolayer derived from small intestinal organoids (Noel et al., 2017). Finally, epithelial cell monolayer growth conditions have been developed for the differentiation of organoid-derived cells (Kozuka et al., 2017). While no co-culture was attempted in the experiments described here, our current protocols may further be optimised for such a purpose.

5.5. Are V γ 4⁺ IELs special?

An important question which remains to be answered is whether V γ 4⁺ TCRs are unique among the gut TCR repertoire in interacting with BTN13 plus BTN18. One can envision several scenarios. First, selected V γ 4⁺ T cells may be unique, expressing distinct gene expression patterns that reflect their distinct physiological roles. Alternatively, other $\gamma\delta$ IELs may have similar functional potential, reflecting their selection by elements that are as of yet unelucidated. To gain insight into this, RNAseq experiments will be undertaken, exploiting the recent development of V γ 4-specific antibodies by Gamma Delta Therapeutics, which we have now fully validated. IEL sub-populations isolated according to their reactivities to combinations of antibodies, including anti-V γ 4, anti-V γ 2/3/4, anti-V γ 9 and anti- $\gamma\delta$ TCR will be compared at the transcriptome level by RNAseq.

Likewise, further experiments should use the V γ 4-specific antibody to determine the frequency and phenotype of V γ 4⁺ lymphocytes within the intestinal epithelium in contrast to other tissues such as skin, lung, adipose tissue, blood and thymus. An enrichment in the intestinal tissue would strongly support the notion that these cells are being selected and preferentially retained at this anatomical site akin to the Btln1 plus Btln6-dependent enrichment of murine V γ 7⁺ cells within the IEL population. Additionally, the comparison of foetal, infant/adolescent and adult samples could provide insight into the ontogeny of the development and phenotypic maturation of the $\gamma\delta$ IEL compartment throughout the human lifetime.

Finally, the contribution of dysregulated V γ 4⁺ lymphocytes to inflammatory bowel conditions should also be investigated. To this end, we are currently recruiting patients with UC and Crohn's disease so as to examine the frequencies and phenotypes of V γ 4⁺ IELs in paired inflamed and non-inflamed tissue samples. Mayassi et al. (2019) demonstrated a persistent loss of V γ 4 cells in coeliac disease and changes in the gene expression pattern of V δ 1⁺ cells even in patients with

remission. Hence, the analysis of IBD can provide comparisons with healthy controls and with those affected with a different type of intestinal allergic inflammation. It is tempting to hypothesise that any setting in which the dominant V γ 4 IEL compartment is diminished or lost will have pathological consequences as the capacity to maintain and/or restore local epithelial homeostasis is lost.

6. Conclusion

In sum, the present study demonstrates that the regulation of human colonic IEL by BTNL3 plus BTNL8 is mediated essentially exclusively via the V γ 4 chain of the TCR, a highly represented TCR V region in the human gut. Our identification of key regions on the V γ 4 and BTNL3 required for the interaction, coupled with direct binding of soluble V γ 4⁺ TCRs to cells specifically expressing BTNL3 plus BTNL8 has strongly suggested that BTNL3 and V γ 4 interact directly, a hypothesis now validated by SPR and isothermal calorimetry experiments to which we contributed (Willcox et al., 2019).

However, unlike the conventional engagement of clonally restricted antigens, the binding to BTNL3 plus BTNL8 is *via* a germline-encoded region present on all V γ 4 chains. This is reminiscent of TCR V β and TCR V α engagement by superantigens, albeit that no endogenous non-viral gene has been identified as a V β or V α regulator akin to the BTNL/Btnl proteins.

As well as acting as IEL repertoire selecting agonists, the constitutive expression of BTNL3 and BTNL8 at steady state might provide essential signals indicating “normality” to V γ 4⁺ cells, thereby constraining their potential for activation. In contrast, the proteins’ downregulation during tissue stress may facilitate IEL responses to stress-associated antigens, leading to functional effector activation. $\gamma\delta$ IELs are ideally positioned to contribute to immune-protection and immune-regulation in a heavily antigenic environment such as the intestine and their stringently regulated cross-talk with the epithelium would be critical for this. While many questions remain to be answered, this study has provided clear evidence that the epithelium is a profound regulator of local $\gamma\delta$ T cell biology *via* direct communication mediated by BTNL molecules.

Chapter VIII. Appendix

1. Culture media and buffers

Phosphate Buffered Saline (PBS), pH 7.4 – Thermo Fisher, 10010023

Hanks' Balanced Salt Solution (HBSS) with 10mM HEPES – STEMCELL, 37150

Gut wash medium

500mL	RPMI 1640 – Thermo Fisher, 52400025
10%	Foetal Bovine Serum (FBS) – Thermo Fisher, 16000044
3.5µL	β-mercaptoethanol – Thermo Fisher, 21985023
500U/mL	Penicillin
500µg/mL	Streptomycin
5µg/mL	Metronidazole
100µg/mL	Gentamicin
12.5µg/mL	Amphotericin B

Gut medium

500mL	RPMI 1640 – Thermo Fisher, 52400025
10%	FBS
3.5µL	β-mercaptoethanol
100U/mL	Penicillin
100µg/mL	Streptomycin
1µg/mL	Metronidazole
20µg/mL	Gentamicin
2.5µg/mL	Amphotericin B
100U/mL	IL-2
10ng/mL	IL-15

Adherent cell lines culture medium

500ml	DMEM – Thermo Fisher, 41966029
10%	FBS
100U/mL	Penicillin
100µg/mL	Streptomycin

Jurkats culture medium

500mL	RPMI 1640 – Thermo Fisher, 52400025
10%	FBS
100U/mL	Penicillin
100µg/mL	Streptomycin

Organoid culture medium*

50mL	IntestiCult OGM Human Component A – STEMCELL, 06011
50mL	IntestiCult OGM Human Component B – STEMCELL, 06012
10µM	Y-27632
3µM	CHIR99021
100µg/mL	Primocin

*antibiotics and chemicals were added fresh to reconstituted IntestiCult OGM before each medium change

Organoid digestion medium

500mL	DMEM/F12 with 15mM HEPES – Thermo Fisher, 11330057
10µg/ml	Hyaluronidase IV-S from bovine testes
1mg/ml	Collagenase II
10µM	Y-27632

Organoid differentiation medium*

5mL	IntestiCult OGM Human Component A – STEMCELL, 06011
5mL	DMEM/F12 with 15mM HEPES – Thermo Fisher, 11330057
10µM	Y-27632
3µM	CHIR99021
100µg/mL	Primocin

*antibiotics and chemicals were added fresh to reconstituted IntestiCult OGM before each medium change

DMEM/F12+++

500mL	DMEM/F12 with 15mM HEPES – Thermo Fisher, 11330057
5mL	GlutaMAX – Thermo Fisher, 35050061
100U/mL	Penicillin
100µg/mL	Streptomycin

FACS buffer

500mL	PBS
2%	FBS
2mM	EDTA

Western blot lysis buffer

50mM	Tris, pH 8
150mM	NaCl
0.5%	NP-40
1x	Protease and Phosphatase inhibitor cocktail

Western blot running buffer

100mL	NuPAGE MES SDS Running Buffer – Thermo Fisher, NP0002
1900mL	Deionized water

Western blot transfer buffer

100mL	NuPAG Transfer Buffer – Thermo Fisher, NP0006
10%	Methanol
1700mL	Deionized water

Western blot blocking buffer

1L	PBS
0.1%	Tween20 – Thermo Fisher, P9416
3%	Bovine serum albumin – Thermo Fisher, A9647

Western blot wash buffer

1L	PBS
0.1%	Tween20 – Thermo Fisher, P9416

2. List of reagents

Antibiotics	Supplier	Catalogue number
Pnicillin/Streptomycin	Thermo Fisher	10378016
Metronidazole	Pharmacy Department, Guy's Hospital	N/A
Gentamicin	Sigma-Aldrich	G1397
Amphotericin B	Thermo Fisher	15290018
Puromycin Dihydrochloride	Thermo Fisher	A1113803
G418	Thermo Fisher	11811023
Primocin	InvivoGen	ant-pm-1
Cytokines	Supplier	Catalogue number
IL-2	Pharmacy Department, Guy's Hospital	N/A
IL-15	Biolegend	570304
Chemicals	Supplier	Catalogue number
Y-27632	Sigma-Aldrich	688001
CHIR99021	Sigma-Aldrich	SML1046
Bafilomycin A	Sigma-Aldrich	B1793
Polyethylenimine (PEI)	Polysciences	23966-1
Enzymes	Supplier	Catalogue number
XbaI	New England Biolabs	R0145L
XhoI	New England Biolabs	R0146L
NcoI	New England Biolabs	R0193L
NotI	New England Biolabs	R0189L
AjuI	Thermo Fisher	ER1951
BaeI	New England Biolabs	R0613L
Collagenase II	Sigma-Aldrich	C6885
Hyaluronidase IV-S from bovine testes	Sigma-Aldrich	H3884
T4 Polynucleotide Kinase	New England Biolabs	M0201L
T4 DNA Ligase	New England Biolabs	M0202L

Kits	Supplier	Catalogue number
BioLux Gaussia Luciferase Assay Kit	New England Biolabs	E3300
RNAeasy mini kit	Qiagen	74106
QIAquick PCR Purification kit	Qiagen	28106
QIAquick Gel Extraction kit	Qiagen	28706
AllPrep DNA/RNA Mini kit	Qiagen	80204
QIAprep Spin Miniprep kit	Qiagen	27106
QIAGEN Plasmid Midi kit	Qiagen	12145
NucleoBond Xtra Midi EF kit	Macherey-Nagel	740420.5
PowerUP SYBR Green Master Mix	Thermo Fisher	A25778
SuperScript II Reverse transcriptase kit	Thermo Fisher	18064014
qScript XLT One-Step RT-PCR kit	Quanta Biosciences	95143-200
Phusion High-Fidelity PCR kit	New England Biolabs	E0553L
Clarity Max Western ECL Blotting Substrate	BioRad	1705060
Other	Supplier	Catalogue number
RNAlater Stabilization Solution	Thermo Fisher	AM7020
NuPAGE 4–12% Bis-Tris protein gels	Thermo Fisher	
BD CellFIX	BD Biosciences	340181
Zombie NIR Fixable Viability Kit	Biolegend	423105
LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit	Thermo Fisher	L34965
Intracellular Staining	Biolegend	421002
Permeabilization Wash Buffer		
dNTPs	New England Biolabs	N0447S
MAX efficiency Stbl2 competent cells	Thermo Fisher	10268019
Opti-MEM Reduced Serum Medium	Thermo Fisher	31985070
Rat Tail Collagen I	Corning	354236
Cultrex RGF Basement Membrane Extract	R&D	3433-005-01
Protease and Phosphatase inhibitor cocktail	Thermo Fisher	78444
Precision Plus Protein Kaleidoscope Prestained Protein Standards	BioRad	1610375

Antibody	Clone	Company	Catalogue Number
<u>Stimulation and blocking</u>			
EPCR	AF2245	R&D Systems	AF2245
DYKDDDDK	L5	Biolegend	637301
HA	16B12	Biolegend	901514
BTNL3	ARP46769_P050	AvivaSystemBiology	ARP46769_P050
Mouse IgG1	MOPC-21	Biolegend	400166
Mouse IgG2a	MOPC-173	Biolegend	400224
Rabbit isotype	Poly29108	Biolegend	910801
Goat isotype	AB-108-C	R&D Systems	AB-108-C
$\gamma\delta$ TCR	B1	Biolegend	331204
CD3	OKT3	Biolegend	317315
<u>Western blot</u>			
anti-LAT	N/A	CST	3584T
anti-PLCg	D6M9S	CST	14008S
anti-CD3e	CD3-12	CST	4443T
HPR-anti-rat	N/A	CST	7077S
HPR-anti-rabbit	N/A	CST	7074
<u>Flow cytometry</u>			
CD69-AF647	FN50	Biolegend	310918
CD3-BV421	OKT3	Biolegend	317344
CD3-BV786	OKT3	Biolegend	317330
CD3-AF647	OKT3	Biolegend	317312
$\gamma\delta$ TCR-PeCy7	IMMU510	Beckman Coulter	B10247
V δ 1-FITC	TS8.2	Thermo Fisher	TCR2730
V δ 1-APC	REA173	Miltenyi	130-100-519
V δ 2-PerCpCy5.5	B6	Biolegend	331424
CD25-BV421	BC96	Biolegend	302630
CD45RA-PE	HI100	Biolegend	304108
CD45-PacBlue	HI30	Biolegend	304029
V γ 2/3/4	23D12	Kabelitz et al., 1994	N/A
EPCR-PE	RCR-16	Biolegend	351904
EPCR-APC	RCR-16	Biolegend	351906
$\gamma\delta$ TCR-PeCy7	GL3	Biolegend	118124
DYKDDDDK-PE	L5	Biolegend	637310
DYKDDDDK-APC	L5	Biolegend	637308

HA-DyLight 650	2-2.2.14	Thermo Fisher	26183-D650
HA-BV421	16B12	Biolegend	682405
HA-AF647	16B12	Biolegend	682404
HIS-APC	J095G46	Biolegend	362605
anti-mouse-APC	N/A	Thermo Fisher	A-21235
anti-rabbit-APC	N/A	Thermo Fisher	A-31573
streptavidin-PE	B243518	Biolegend	405204

3. Oligos and primers

Single-cell PCR external primers

hV γ 2-8ext_F	TGCCAGTCAGAAATCTTCCAAC
V γ 2/4_F	ATATCTCGAGGCCGCCACCATGCAGTGGGCCCTAGCG
V δ 1_F	ATATCTCGAGGCCGCCACCATGCTGTTCTCCAGCCTGCTG
V δ 3_F	ATATCTCGAGGCCGCCACCATGATTCTTACTGTGGGCTTTAGCTTTTTG
C γ 1/2_R	ATATGCGGCCGCTTATGATTCTCTCCATTGCAGCAG
C δ _R	ATATGCGGCCGCTTACAAGAAAAATAACTTGGCAGTCAAGAG

Single-cell PCR internal primers

hV γ 2-8int_F	ATATCCTGCAGGCACTGGTACCTACACCAGGAGG
hV δ 1int_F	ATATGGCGCGCCGGTACAAGCAACTTCCCAGCAAAG
hV δ 3int_F	ATATGGCGCGCCACCGGATAAGGCAAGATTATTC
hC γ 1/2int_R	ATATGGCGCGCCGGAGGAGGTACATGTAATATGCAGAG
hC δ int_R	ATATGCGGCCGCGGCAGCTCTTTGAAGGTTGC

Backbone generation primers

V γ 4fix_F	ATATCCATGGCGTGGGCCCTAGCGGTG
V γ 4 link_R	CTTATACCAACACTGACTTCCCCATCCCAGGTGGCAC
V γ link_F	TGGGGAAGTCAGTGTGGTATAAGAACTCTTTGGCAGTGGAAAC
C γ fix_R	GTTTTTATTATTCTCATGTCTGATATACATCTGTCTTTT
C γ fix_F	CAAAGAAGACAGATGTATATCAGACATGAGAATAATAAAAAC
C γ Xba_R	ATATTCTAGATTATGATTTTTCTCCATTGCAGCAG
hV δ 3link_R	GATGAGTTTATCGGTGGTACAGTCGTAAAGGCACAGTAGTAAGTGGCACT
V δ 1 link_R	GATGAGTTTATCGGTGGTACAGTCGTTTCCCCAAGAGCACAAAAGTACTT

Vδ link_F	ACGACTGTACCACCGATAAACTCATCTTTGGAAAAGG
Cδ fix_R	ATATGCGGCCGCTTACAAAAAATAACTTG
Vγ9Fix_F	ATATACCATGGTGTCACTGCTCCACACATC
Vδ2_F	ATATCTCGAGGCCGCCACCATGCAGAGGATCTCCTCCCTCAT

Modifying Vγ4 into Vγ2

Vγ4fix_F	<i>See above</i>
Vγ4CDR3hu17_R	GCCAAAGAGTTTCTTACTGCCGTCCCAGGTGGC
Vγ4CDR3hu17_F	CGGCAGTAAGAACTCTTTGGC
Cγ Xba_R	<i>See above</i>

Modifying Vγ4 into V 3

Vγ4fix_F	<i>See above</i>
Vγ4CDR3hu17_R	GCCAAAGAGTTTCTTACTGGGGTCCCAGGTGGCACAGTAATAG
Vγ4CDR3hu17_F	<i>See above</i>
Cγ Xba_R	<i>See above</i>

Modifying Vγ4 CDR1/2 into Vγ2

CDR1_F	CTGAAGGAAGTAACGGCTACATCC
CDR1_R	GGATGTAGCCGTTACTTCCTTCAG
CDR2_F	GACTCCTACAACCTCCAAGGTTGTG
CDR2_R	CACAACCTTGGAGTTGTAGGAGTC

Modifying Vγ4-HV4 into Vγ2

YANL_F	GAAGTATTATACTTACGCAAGCACAAGGAACAACTTGAGATTGATACT
YANL_R	AGTATCAATCTCAAGTTGTTCTTGCTTGCGTAAGTATAATACTTC
YA_F	GGAAGTATTATACTTACGCAAGCACAAGG
YA_R	CCTTGTGCTTGCGTAAGTATAATACTTCC
NL_F	GCACAAGGAACAACTTGAGATTGATAC
NL_R	GTATCAATCTCAAGTTGTTCTTGTC

Gain of function mutants

Vg2 YA>DG_F	GAAGTATGATACTTACGGAAGCACAAG
Vg2 YA>DG_R	CTTGTGCTTCCGTAAGTATCATACTTC

Vg3>Vg4 HV4_F

GAAGCACAAGGAAGAACTTGAGAATGATACTGCGAAATCTTATTGAAAATGATTC

Vg3>Vg4 HV4_R CTCAAGTTCTTCCTTGTGCTTCCATAAGTATCATACTTCTGGACTGAGTC**Vg3>Vg4 CDR2-HV4_F** CAGCGTCTTCTGTACTATGACTCCTACACCTCCAGCG**Vg3>Vg4 CDR2-HV4_R** GTCATAGTACAGAAGACGCTG***Other cloning primers***

EPCR_XhoI_F ATATCTCGAGGGAGCCTCAACTTCAGGATG**EPCR_NotI_R** ATATGCGGCCGCTTAACATCGCCGTCCACC***qPCR primers***

Cyclophilin A_F ATGCTGGACCCAACACAAAT**Cyclophilin A_R** TCTTTCACCTTGCCAAACACC**HNF4a_F** CCAAGTACATCCCAGCTTTCTG**HNF4a_R** GAGCAGCACGTCCTTGAAC**CDX2_F** CTGGAGCTGGAGAAGGAGTTT**CDX2_R** CTTTGCTCTGCGGTTCTGAA**LRG5_F** CATCTCTTCCTCAAACCGTCTGCAATC**LRG5_R** AAGACGACAGGAGGTTGGACGATAG**MS4A12_F** CTGGTGTCACAGGGTTGCTTTC**MS4A12_R** GTGATTCTGCTGCTGGTGGATATGTGC**BTNL3_F** GGTTCAAGTCCCAGATTACGATGAGG**BTNL3_R** AGGTGAGGGCTGGAAAAACGTC**BTNL8_F** GGCTCAGTTCCTCTCATTTCCATCAC**BTNL8_R** TGGTAGCCAGGTGCCACGATAT***EPCR CRISPR oligos***

EPCRint1_F1 ACAGCATCTGTGTCTGCAGCGTTTT**EPCRint1_R1** GCTGCAGACACAGATGCTGTGCGGTG**EPCRint1_F2** GATCGCCCATAAAGTTCTATTCGGTG**EPCRint1_R2** GATCGCCCATAAAGTTCTATTCGGTG**EPCRint3_F1** AGCAACAAGAGGCCACAGCGTTTT**EPCRint3_R1** GCTGTGGGCCTCTTGTGCTCGGTG**EPCRint3_F2** CCAAAGCGTTTCTGCCCCAGGTTTT**EPCRint3_R2** CTGGGGCAGAAACGCTTTGGCGGTG

Single-cell TCR oligos

hu2 gamma_F	GGACTGGGGGTATTATAAGAAACT
hu2 gamma_R	CTTATAATACCCCCAGTCCCAGGT
hu2 delta_F	CTGTGCCTCGGGGGATACACCGATAAACTC
hu2 delta_R	TATCGGTGGTATCCCCGAGGCACAGTAGTA
hu3 gamma_F	GGCAGGTTATTATAAGAAACT
hu3 gamma_R	CTTATAATAACCTGCCCAGGT
hu3 delta_F	CTGTGCGGCTATGGGGGTTCCGTTTTTAGAGGGGGATACAGGTCCAAAACCTC
hu3 delta_R	TTGGACCTGTATCCCCCTCTAAAAACGGAACCCCCATAGCCGCACAGTAGTA
hu7 gamma_F	GGATGGGCCGTGGAATTATTATAAGAAACT
hu7 gamma_R	CTTATAATAATTCCACGGCCCATCCCAGGT
hu7 delta_F	TCTTGGGGAGCGTGGGTACTGGGGGATTTGGGCGATAAACTC
hu7 delta_R	TATCGCCCAAAATCCCCCAGTACCCACGCTCCCCAAGAGCACA
hu8 gamma_F	GGCTCCCTATTATAAGAAACT
hu8 gamma_R	CTTATAATAGGGAGCCCAGGT
hu8 delta_F	CTGTGCCTTTTGTCTCCGTCTACTGGGGGATTTGCACCGATAAACTC
hu8 delta_R	TATCGGTGCAAATCCCCCAGTAGACGGAGCAAAAGGCACAGTAGTA
hu9 gamma_F	GGATGGGCCGAATTATAAGAAACT
hu9 gamma_R	CTTATAATTGGGCCCATCCCAGGT
hu9 delta_F	CTGTGCCTTTTTTTTCGGATGGGGGATACGCTTTTACACCGATAAACTC
hu9 delta_R	TATCGGTGTAAAAGCGTATCCCCCATCCGAAAAAAAAAGGCACAGTAGTA
hu12 gamma_F	GGTAATGGCCCATTTATAAGAAACT
hu12 gamma_R	CTTATAATGGGCCATTACCCAGGT
hu12 delta_F	TCTTGGGGAAAGGGAGTCTCTTTATAAACTC
hu12 delta_R	TATAAAGAGACTCCCTTTCCCCAAGAGCACA
hu17 gamma_F	GGATGGGAGTAAGAAACT
hu17 gamma_R	CTTACTCCCATCCCAGGT
hu17 delta_F	TCTTGGGGAATCCTCACTCGGGTACTGGGGGATACTTGCCGATAAACTC
hu17 delta_R	TATCGGCAAGTATCCCCCAGTACCCGAGTGAGGATTCCCCAAGAGCACA
hu19 gamma_F	GGATTGCCGGTATAAGAAACT
hu19 gamma_R	CTTATACCGCAATCCCAGGT
hu19 delta_F	CTGTGCCTTCCTCCCCTACTGGGGGATACGAAAGGGTCCGACACCTTAACCGATAAACTC
hu19 delta_R	TATCGGTAAAGGTGTCGGAACCTTTTCGTATCCCCCAGTAGGGGAGGAAGGCACAGTAGTA

hu20 gamma_F	GGATGGATATAAGAAACT
hu20 gamma_R	CTTATATCCATCCCAGGT
hu20 delta_F	TCTTGGGCCCCCCTATTCTACGTACTGGGCTATCGAAAACCTC
hu20 delta_R	TTCGATAGCCCAGTACGTAGAAATAGGGGGGGCCCAAGAGCACA
sk1 gamma_F	GGAACCTAATTATTATAAGAAACT
sk1 gamma_R	CTTATAATAATTAAGTTCCCAGGT
sk1 delta_F	TCTTGGGACGATCCGGCCTTCCCCTTTTTTACTGGGGGGCTACCTAACCCGTACCACCGATAAACTC
sk1 delta_R	TATCGGTGGTACGGGTTAGGTAGCCCCCAGTAAAAAAGGGGAAGGCCGGATCGTCCCAAGAGCACA
sk2 gamma_F	GGATGGATATTATAAGAAACT
sk2 gamma_R	CTTATAATATCCATCCCAGGT
sk2 delta_F	TCTTGGGGAAAAAATAACCTTCCTAGGAAATGGCTGGGGGAGACACACCGATAAACTC
sk2 delta_R	TATCGGTGTGTCTCCCCCAGCCATTCCTAGGAAGGTTATTTTTTCCCAAGAGCACA

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