



**1) INVESTIGATION OF THE CLATHRIN-DEPENDENT
INTERNALISATION OF CYTOTOXIC T LYMPHOCYTE
ANTIGEN-4**

**2) ANALYSIS OF THE ANTIGEN-SPECIFIC CYTOKINE
RESPONSES OF CD4⁺ T-LYMPHOCYTES**

This thesis is submitted by Marie Ann Voice to The University of
Birmingham for the degree of:
MRes Biomedical Research

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INVESTIGATION OF THE CLATHRIN-DEPENDENT INTERNALISATION OF CYTOTOXIC T LYMPHOCYTE ANTIGEN-4

By Marie Ann Voice
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Supervisor: Dr David Samson

Abstract

Cytotoxic T Lymphocyte Antigen-4 (CTLA-4) is a key inhibitory regulator of T-cell function. Its expression is predominantly intracellular however, many theories surrounding its inhibitory action do not account for its endocytic behaviour. Recent reports have suggested a process of trans-endocytosis through which CTLA-4 internalises the ligand (CD80/CD86) from antigen presenting cells and prevents ligand binding to its stimulatory homologue CD28. The endocytic pathway of CTLA-4 is currently ill-defined and has a central role in this novel theory. Previous evidence suggests CTLA-4 is internalised via clathrin-dependent endocytosis (CDE). However observations in a transfected CHO cell model have suggested internalisation independent of this pathway. In this study CTLA-4 showed the same endocytic recycling behaviour when transfected into a HeLa cell line. Immunostaining showed a strong degree of co-localisation between internalising CTLA-4 and the transferrin receptor (which is classically associated with CDE). Co-localisation with Adaptor-Protein 1 suggested exocytosis from the trans-Golgi network to the plasma membrane was also clathrin-dependent, however, no association was observed with clathrin directly. Little CTLA-4 co-localisation to a lysosomal marker suggests that only a small proportion of the cytosolic receptor is targeted for degradation. CDE was inhibited by transfection of shRNA for Adaptor Protein 2 (AP-2). In CTLA-4⁺ CHO cells knock down of AP-2 resulted in decreased intracellular CTLA-4 but this was not statistically significant. This data supports but does not confirm that CTLA-4 can internalise independent of CDE.

1.0 Introduction

1.1 Functional characteristics of Cytotoxic T-Lymphocyte Antigen-4 (CTLA-4)

Cytotoxic T-Lymphocyte Antigen-4 (CTLA-4) is an inhibitory receptor expressed in both activated T-cells [1, 2] and T-regulatory cells (T_{reg}) [3, 4]. T-cells are activated by T-cell receptor ligation to antigenic peptide bound to Major Histocompatibility Complex (MHC) which is expressed by antigen presenting cells (APC). Additional co-stimulation by associated receptors such as CD28 (which binds CD80/CD86 on APC) is also required to potentiate a full response [5]. Conversely, inhibitory receptors prevent excessive or self-reactivity and are required to maintain immune homeostasis (reviewed in [6, 7]). CTLA-4 is the structural homologue of CD28 and paradoxically shares the same ligands, yet has an antagonistic effect upon binding [8]. Its critical role is exemplified in CTLA-4^{-/-} deficient mice which develop fatal neonatal lymphoproliferative disease characterised by chronic lymphocytic infiltration into multiple organ systems [9, 10]. In addition, genetic polymorphisms in the CTLA-4 gene are associated with autoimmune diseases such as diabetes mellitus [11, 12] and rheumatoid arthritis [13] [14], thus demonstrating its role in maintaining self-tolerance and promoting clonal anergy [15].

The mechanisms through which CTLA-4 elicits its inhibitory functions are yet to be elucidated and many theories as to its mechanism of action are proposed (some notable concepts are listed in Table 1.1). However, many of these do not consider the unusual endocytic behaviour of the receptor or account for its existence. In contrast to CD28 which is expressed readily at the plasma membrane the surface expression of CTLA-4 is limited and the receptor is predominantly located within endosomal compartments [16]. This contradicts

the long-standing theory that CTLA-4 and CD28 simply compete for their common ligands and other such theories that assume significant receptor surface expression.

Cell intrinsic (intracellular signalling)	Cell extrinsic
Inhibition of IL-2 expression via reduced NF-AT activity (reduced proliferation) [17]	Direct competition with CD28 for their common ligands [18]
Reduction in inflammatory transcription factor activity (NF-κB) [17]	Induction of IDO by APC (reduced T-cell proliferation through tryptophan metabolite depletion) [19]
T _{reg} modulation: enhanced expression of the regulatory transcription factor FOXP3 and the anti-proliferative factor TGF-β [20, 21]	inhibition of immune synapse formation through disruption of lipid raft [22, 23]
	Increased threshold for TCR activation [24]

Table 1.1 Reported evidence for the inhibitory mechanism of CTLA-4 function. APC; Antigen Presenting Cell, CD28; Cluster of Differentiation 28, FOXP3; Forkhead box P3, IDO; Indoleamine 2,3-Dioxygenase, IL-2; Interleukin 2, NF-AT; Nuclear Factor of Activated T-cells, NF-κB; Nuclear Factor-κB, TCR; T-cell receptor, TGF-β; Transforming growth factor β. (Table adapted from [25]).

With regards to cell-intrinsic mechanisms, despite numerous reports there is no definitive association of CTLA-4 with inhibitory signalling pathways. This is consistent with the structure of the receptor, which has a short cytoplasmic domain with no immunoreceptor tyrosine-based activation motif (ITAM) [26]; classically associated with immune receptor signalling pathways. Given that 1) small changes in CTLA-4 surface expression have profound effects on T-cell activity [25], 2) Increases in surface expression are shown to be independent of CTLA-4 mRNA transcription [27], 3) There is differential surface expression of CTLA-4 in different T-cell subsets [28], it is highly likely that CTLA-4's endocytic behaviour has a central role in its functionality. Consistent with this a novel mechanism of action for CTLA-4 is reported accounting for such recycling characteristics. CTLA-4 was shown to 'rip' CD80/CD86 from neighbouring cells and internalise it; thus depleting the ligand and making it unavailable for association with CD28 [29]. In light of this finding it is

important to further investigate the receptors internalisation and patterns of endosomal trafficking.

1.2 Internalisation of surface CTLA-4 is attributed to the clathrin-dependent endocytic pathway.

Numerous publications suggest that CTLA-4 surface expression is restricted through rapid clathrin-mediated endocytosis (CDE) [30-32]; the most classically well-defined endocytic pathway. Clathrin is a structural triskelion protein involved in the coating and rounding of invaginating endosomes at the plasma membrane and other organelles [33]. The basic internalisation process mediated by clathrin is shown in Figure 1.1 and requires recruitment by adaptor protein-2 complex (AP-2) and action of accessory proteins [34].

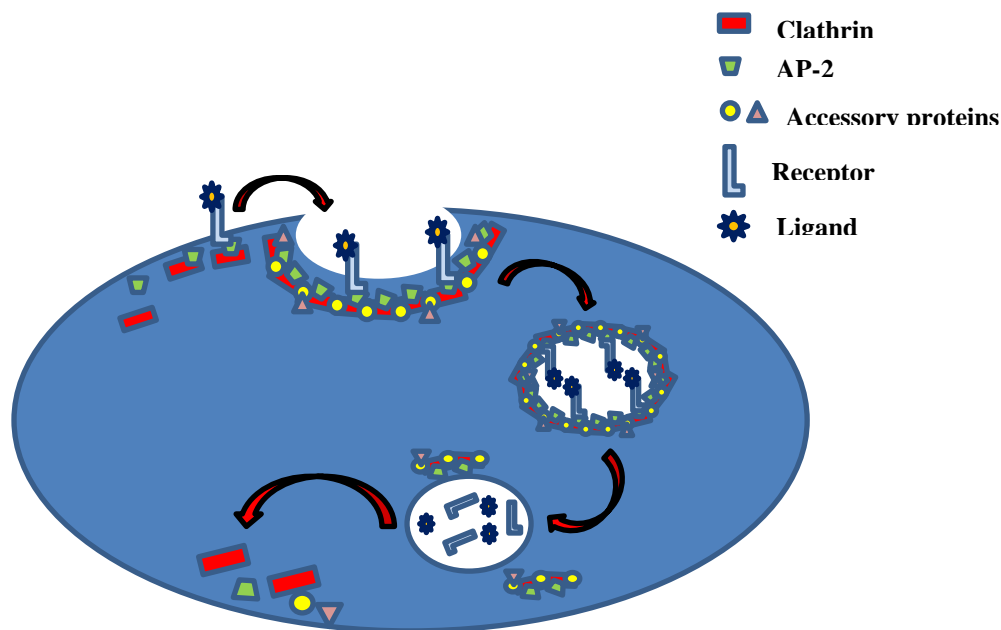


Figure 1.1 Schematic representation of clathrin-mediated endosome formation. Clathrin is recruited to receptor microdomains by Adapter Protein-2. This binds a YVKM amino acid motif in the cytoplasmic domain of the receptor; thus linking clathrin to receptor (and bound ligand). Clathrin forms a structural coat which stabilises the membrane invagination. Accessory proteins such as epsins and endophilin facilitate bending and curvature of the membrane to form a clathrin-coated pit. Accessory proteins such as dynamin and amphiphysins are required for membrane cleavage and the pit is pinched off to form a coated vesicle. Upon internalisation the early endosome is uncoated and the components recycled back to the plasma membrane.

AP-2 recruitment is the first stage of vesicle formation forming the link between transmembrane receptors and clathrin. The cytoplasmic tail of CTLA-4 contains a variant of a conserved tyrosine-based amino acid motif which binds the μ -subunit of the AP-2 (Tyrosine-Valine-Lysine-Methionine) [27, 31, 35]. It is previously observed that this interaction facilitates CTLA-4 internalisation, whereas alternative amino acids around this motif in the cytoplasmic tail of CD-28 are restrictive [36]. Additionally TCR activation is shown to upregulate phosphorylation of Tyr²⁰¹ via src kinases which prevents binding of AP-2 and permits binding of phosphatidylinositol-3-kinase and SHP-2 kinases (kinases associated with signal transduction). Potentially this promotes both the surface retention and activity of CTLA-4, which suggests through AP-2 CDE is involved in the balance of surface activity versus internalisation [35, 37] [38-40].

Chimeric protein studies have showed increased internalisation of CD28 when engineered to express the cytoplasmic domain of CTLA-4 and increased surface retention of a CTLA-4 construct expressing the cytoplasmic domain of CD28 [16]. However intracellular CTLA-4 is still observed when mutated at Tyr²⁰¹ which suggests it can be internalised in an alternative fashion independent of clathrin [41, 42]. In support of this evidence has shown 1) CTLA-4 may internalise through a non-clathrin-coated mechanism involving the reorganisation of F-actin microfilaments. 2) Mutant CTLA-4 expressing the transmembrane and cytoplasmic domain of CD28 can still trans-endocytose ligand, suggesting only the extra-cellular domain is necessary for its function (both unpublished data). This is consistent with studies in CTLA-4^{-/-} mice where re-introduction of CTLA-4 lacking the cytoplasmic domain promotes the restoration of a wild type phenotype [43].

As this body of evidence suggests CDE is not necessary for CTLA-4 function the significance of the CDE route requires clarification and alternative or redundant endocytic pathways must be characterised.

1.3 Overview of CDE in intracellular trafficking and regulation of CTLA-4 expression

Once internalised the fate of CTLA-4 and the proportions of receptor targeted for different cellular compartments are not completely defined (potential trafficking pathways are shown schematically in Figure 1.2).

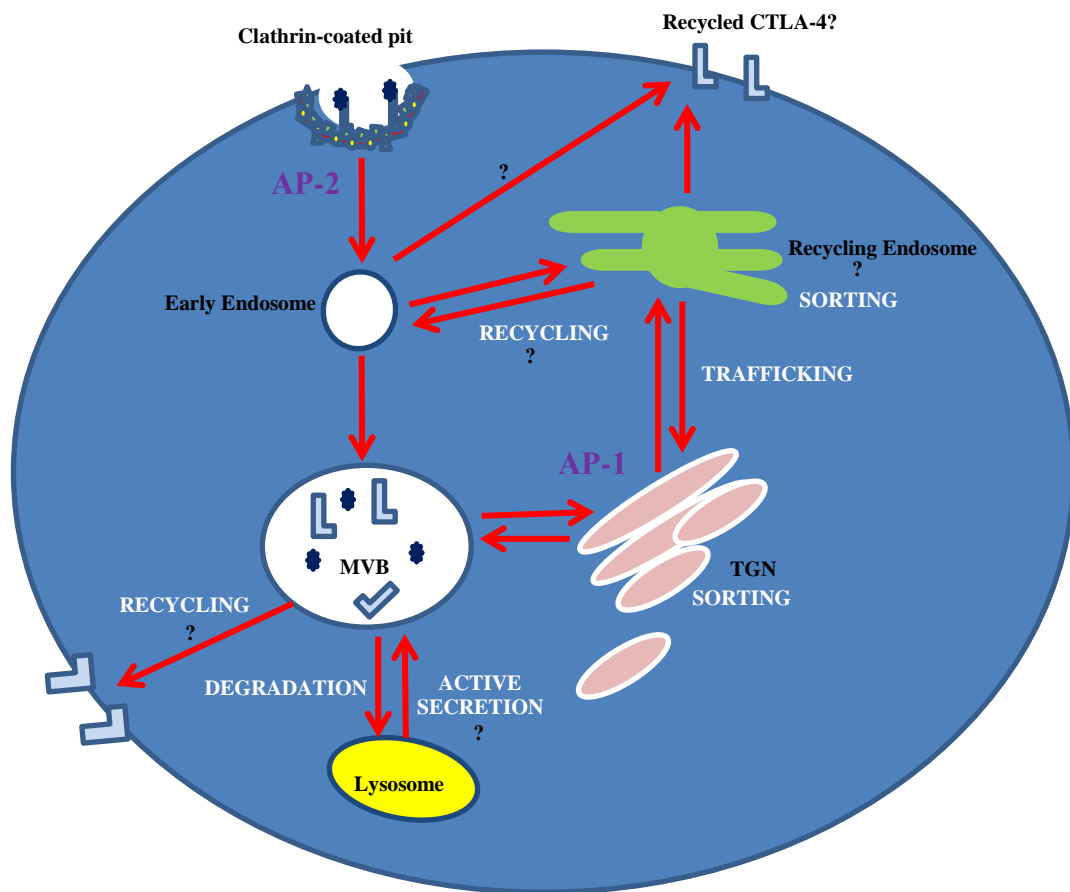


Figure 1.2 Potential endocytic trafficking pathways of CTLA-4. Once internalisation clathrin-coated vesicles uncoat and fuse to form early endosomes. From here CTLA-4 may be recycled back to the surface directly or via sorting endosomes. Alternatively early endosomes may mature to form Multi-Vesicular Bodies (MVB). From here CTLA-4 may be recycled via the *trans*-Golgi network (TGN) where protein sorting occurs. Clathrin-coated vesicle formation at the surface requires AP-2 whereas transport from the TGN requires AP-1 (reviewed in [34]). The TGN may regulate overall receptor expression through trafficking to lysosomes via MVB [44] . Within lysosomes CTLA-4 may be degraded or recycled through active secretion (Image adapted from [45]).

CDE has been most extensively defined by studies of the transferrin receptor (CD71); an archetypal CDE recycling receptor involved in the cellular uptake of Fe^{2+} and Fe^{3+} -bound transferrin (reviewed in [45]). When considering classical CDE in context of CTLA-4 trans-endocytosis the documented similarities and differences between CD71 behaviour must be considered. For CD71 iron molecules (cargo) dissociate in the acidic environment of the early endosome. Following geometry-based sorting processes (through an increased stoichiometric surface:volume ratio of narrowing microtubules in the recycling endosomes) over 99% of CD71 is recycled back to the cell surface [46]. Similar to CD71 it is plausible to consider that CTLA-4 separates from cargo (trans-endocytosed ligand) and is recycled as this is energetically favourable. However in contrast its ligand would be targeted for lysosomal degradation whereas $\text{Fe}^{2+/3+}$ would traffic to where required. Supporting this reported work using the lysosomal inhibitor bafilomycin A results in increased internalised CD80/CD86 but no concurrent increase in CTLA-4 [29]. This suggests the ligand is targeted for lysosomal degradation whereas the receptor is diverted along the pathway. However, this is not consistent with other studies that have identified both the presence of CTLA-4 and its active secretion from the lysosomal compartment [41]. Another observation which suggests the trafficking of CTLA-4 is more complex than CD71 is that the receptor is shown to accumulate in the perinuclear area close to the microtubule organising centres and be resistant to degradation [47, 48]. It also accumulates in late endosomes (multi-vesicular bodies), and as a specific signals (for example ubiquitination [49, 50]) are required for sorting into these mature compartments it may suggest CTLA-4 trafficking is subject to additional regulatory steps not associated with classical CDE.

CDE is associated with both endocytic and exocytic events and although largely beyond the scope of this investigation it is important to note this exocytic pathway in context of the regulation of CTLA-4 surface expression. AP-1 binds the same cytoplasmic motif as AP-2 but

is associated with the trans-Golgi network; where newly synthesised proteins are sorted into different cellular compartments [51]. From here the receptor may be trafficked to the cell surface via recycling endosomes or be targeted to lysosomes via MVB depending signals as yet to be elucidated.

1.4 Experimental aims

Previously the transfection of CTLA-4 into a Chinese Hamster Ovary (CHO) cell line has been used to characterise the endocytic behaviour of CTLA-4 *in vitro*. In light of the implications of trans-endocytosis on CTLA-4 functionality, it is important to more precisely investigate its endosomal behaviour and challenge whether pathways are specifically clathrin-dependent. Association of CTLA-4 to CDE will be analysed through observing co-localisation of the receptor with known CDE-associated cellular markers. This will require the progression of the CHO cell model into a human cell line as such markers are not available for the hamster species. Transfection into a human HeLa cell line will also be a step progression towards a more physiological system. It will then also be examined whether CTLA-4 can internalise via pathways independent of clathrin. shRNA (small hairpin RNA) is a useful technique for silencing genes of specific of interest and this will be used to specifically knock down AP-2, thus preventing the formation of clathrin-coated pits and blocking CDE.

2.0 Materials and Methods

2.1.1 Culture media, solutions and reagents

Mammalian Cell Culture

Complete DMEM	Dulbecco's Modified Eagle Medium (DMEM) [Invitrogen, UK], 10% Fetal Bovine Serum (FBS) [BioSera, Sussex, UK], 1% Penicillin and 1% Streptomycin [both Invitrogen]
G418	Gentamycin 50µg/ml [Invitrogen]
PBS	Phosphate Buffered Saline (8g/l NaCl, 0.26/l KCl, 1.15g/l Na_2HPO_4 , 0.2g/ml KH_2PO_4); 1 PBS tablet added per 100ml distilled H_2O [Oxoid, Basinstoke, UK]
Trypsin	Trypsin 0.05% EDTA [Invitrogen]

Bacterial Cell Culture

Circle Grow	0.04 w/v in dH_2O [MP biosciences, Cambridge, UK]
Select Agar	0.015 w/v in circle grow [Sigma, UK]
SOC Medium	Super Optimal Broth, [New England Biolabs, Herts, UK]
Chloramphenicol	30µg/ml supplemented in agar plates

Additional Reagents

Lipofectamine	Lipofectamine 2000 transfection system [Invitrogen]
Mounting Medium	Vectashield mounting medium with DAPI [Vector Laboratories, Peterborough, UK]
PFA	3% PFA in PBS [Sigma, UK]
Saponin	0.1% in PBS [Acros Organics]
Plasmid purification kit	QIAprep spin miniprep kit [QIAGEN, Sussex, UK]
Electroporation Solution	AMAXA nucleofector kit T [Lonza International]
Cholera Toxin	Red Fluorescent Protein labelled Cholera Toxin (RFP)

2.1.2 Fluorescence Staining Reagents

	Target	Antibody	Species	Dilution	Company
Primary	CTLA-4	ticilimumab	Human	1/10000	Pfizer
		C19	Goat	1/250	Santa Cruz
		11G1	Mouse	1/100	-
	Clathrin	ab6316-200	Goat	1/500	Abcam
Secondary	γ -adaptin	ab21980	Mouse	1/250	Abcam
	α -human IgG	Alexa Fluor-488	Mouse	1/500	Invitrogen
	α -mouse IgG	Alexa Fluor-488	Donkey	1/500	Invitrogen
	α -goat IgG	Alexa Fluor-647	Donkey	1/500	Invitrogen
Direct Conjugates	CTLA-4	CTLA-4 PE	Mouse	1/500	BD Biosciences
		CTLA-4 APC	Mouse	1/500	BD Biosciences
	Transferrin Receptor	Transferrin-647	Human	1/100	Invitrogen
	Lysosome	LAMP1-Cy5	Rabbit α -human	1/250	Abcam

Table 2.1. Antibodies and fluorescently-labelled conjugates used for immunostaining in confocal and flow cytometry experiments.

2.2 Cell Culture and transfection of CTLA-4 into a HeLa cell line

HeLa cells were cultured in complete DMEM medium at 37°C, 5% CO₂. Upon reaching 90-95% confluency, cells were washed with PBS, split using 0.05% trypsin EDTA and replenishing the DMEM culture medium. A pre-established Chinese Hamster Ovary (CHO) cell line expressing human CTLA-4 in a pCDNA3.1 expression vector was cultured in the same manner. To induce CTLA-4 expression in HeLa cells this construct was transfected using Lipofectamine 2000 Transfection Reagent [Invitrogen] according to the manufactures guidelines.

To identify the optimum ratio of DNA to Lipofectamine reagent an enhanced green fluorescent protein (pmaxGFP) control plasmid construct [AMAXA] was transiently

transfected into HeLa cells cultured on glass coverslips in a 24-well plate. 1-3 μ g DNA and 1-3 μ l Lipofectamine were transfected and cells cultured for up to 48h. DNA and Lipofectamine were diluted in 50 μ l of opti-MEM (Invitrogen) and incubated for 5min. These were then combined for 20min, 100 μ l added per 0.5x10⁶ cells and incubated at 37°C 5% CO₂ for 4h. The solution of DNA/Lipofectamine complexes was then aspirated and replaced with 500 μ l of DMEM medium.

To establish a stable cell line the transfection was performed in 25cm² flasks and the reagents scaled up accordingly. Transfected cultures were then treated with 500 μ g/ml G418 in DMEM culture medium for 7 days. Positive clones were also selected using a BD FACSAria cell sorter. Flow cytometry was performed pre- and post-cell sort to determine CTLA-4 expression levels.

2.3.1 CTLA-4 internalisation assay

CTLA-4⁺ HeLa or CHO cell cultures were trypsinized and resuspended at ~ 1x10⁶ cells/ml. 500 μ l of cell suspension was then cultured on poly-L-lysine coated coverslips for 16h. Cells were washed with PBS and incubated with a primary α -CTLA-4 antibody for 30min. Surface staining was performed at 4°C, whereas the recycling pool was stained at 37°C. Cells were then washed with PBS and fixed for 15min in 3% PFA. Cells were washed with PBS and permeabilised with 0.1% saponin. An appropriate secondary fluorescently-conjugated antibody diluted in 0.1% saponin was used to stain the pool of internalised receptor (Table 1). Cells were then washed with PBS and the coverslips allowed to air dry. Coverslips were mounted on glass slides with mounting medium containing the nuclear stain DAPI and sealed with varnish. An alternative version of this procedure was performed in some HeLa cell experiments, where surface and intracellular CTLA-4 was stained simultaneously. Here cells were labelled with primary α -CTLA-4 antibody for 30min 37°C. They were then washed three

times in ice cold DMEM and surface receptor stained with Alexa Fluor-488 conjugated antibody. Cells were then fixed and permeabilised as described above and CTLA-4 internalised from the cell surface stained with an Alexa Fluor-555 secondary conjugate.

Recycling CTLA-4⁺ HeLa cells was also co-stained with the transferrin receptor (CD71) at 37°C to identify commonality between their internalisation pathways. CD71 internalisation was tracked using fluorescently tagged transferrin which is internalised bound to the receptor upon ligand binding. For this experiment cells were serum starved for 15min prior to staining to prevent competitive binding with the transferrin within FCS.

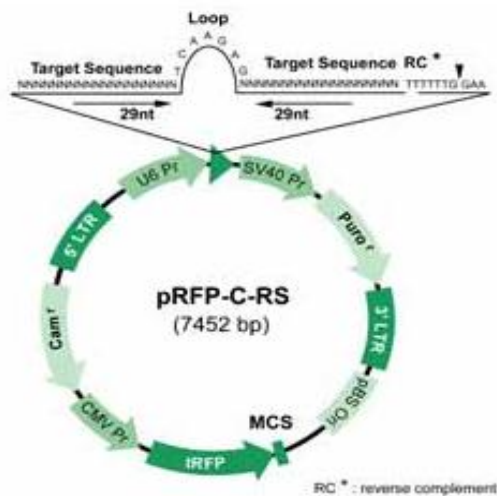
2.3.2 Co-localisation of CTLA-4 with endosomal markers

To co-stain for CTLA-4 with endosomal markers CTLA-4⁺ HeLa cells were fixed and permeabilised prior staining to allow access of primary antibodies to the different intracellular compartments. Both a cells surface and intracellular CTLA-4 expression was therefore identified in these experiments. Cells were stained for clathrin to identify direct CTLA-4 co-association with clathrin-mediated pathways. They were also stained for the γ -adaptin subunit of AP-1 to identify clathrin-mediated transport from the TGN to the plasma membrane. Cy-5 conjugated Lysosomal-Associated Protein-1 (LAMP-1) was used as a marker for lysosomes in conjunction with both clathrin and γ -adaptin.

2.4.1 Cloning of shRNA plasmid constructs

Plasmid constructs encoding shRNA for the μ -subunit of AP-2 (AP2M1 gene) and two control constructs were obtained from Origene (Fig 4). 2 μ l of reconstituted DNA was added to 80 μ l of chemically competent DH5- α e.coli on ice for 30min. Bacteria were then heat shocked at 42°C for 45sec and transferred back to ice immediately. 250 μ l of SOC medium was added and cultures transferred to a rotary shaker for 1h.

A)



B)

Construct	shRNA sequence	Abbreviation
TR30014	None	C1
TR30015	Scrambled	C2
FI1358997	TTCCACCAGTGTGTGCGACTCAGCAAGTT	97
FI1358998	AGCAAGTTTGACTCTGAACGCAGCATCAG	98
FI1358999	TGGTGATGAAGAGCTACCTGAGTGGCATG	99
FI1359000	GATGTGCTGGAGAGTGTGAACCTGCTCAT	90

Fig 2.1 HuSH shRNA construct against AP2M1 in a pRFP-C-RS vector (OriGene). A) shRNA was ready-cloned into the vector to be expressed via a U6 promotor. Vector contained chloramphenicol and puromycin resistance genes for selection of transfected clones. Red fluorescence protein (RFP) was expressed under a CMV promotor and used to visually identify transfected cells. B) The shRNA insert sequence for two controls and four AP2M1 shRNA constructs.

Bacteria were then grown overnight on 30µg/ml chloramphenicol agar plates at 37°C, 5% CO₂. Plasmid DNA was purified using a QIAprep spin miniprep kit according to the manufacturers' specification [QIAGEN] and stored at -20°C. The plasmid DNA concentration was quantified by a nanodrop spectrophotometer [Thermo Scientific, Wilmington, USA].

2.4.2 Transient transfection of shRNA into CTLA-4⁺ CHO and CTLA-4⁺ HeLa cell lines.

Cells were trypsinized and resuspended at ~ 1x10⁶ cells/ml. CTLA-4⁺ CHO cells were transfected with 5µg of each shRNA plasmid using an Amaxa cell line nucleofector kit T. An Amaxa nucleofector electroporation device set to program U-023 was used for high efficacy transfection of the CHO cell line. To identify the most efficiently transfected construct cells were initially cultured overnight in 6-well plates, trypsinized, and flow cytometry performed to quantify expression. For imaging experiments 50µl of electroporated cells were added to coverslips in a 24-well plate and made up to 500µl with complete DMEM. Cells were incubated for 24h at 37°C, 5% CO₂ prior to staining.

CTLA-4⁺ HeLa were transiently transfected on coverslips according to the transfection optimisation protocol described in section 2.2. Concentrations of DNA and lipofectamine were chosen consistent with the result of the assay optimisation.

2.5 Flow cytometry

All fluorescently stained samples were stored in the dark at -2 °C prior to flow cytometry experiments, which were performed using either a FACSCalibur [Becton Dickinson] or a Dacko cyAn_{ADP} device and data collected by Cell Quest Pro v5.2.1 and Summit v4.3 software respectively. Data was analysed using FlowJo v8.5.3 or Summit v4.3.

2.6 Confocal microscopy

Imaging was performed using either a Zeiss LSM 510 or a Zeiss LSM 780 inverted laser confocal microscope under the supervision of Dr Omar Qureshi. All images were collected under a X100 oil immersion objective lens. ImageJ [Wayne Rasband, NIH] software was used to process and analyse collected images. ImageJ was also used to calculate the mean fluorescence intensity of staining for different fluorochromes. This was an automated calculation following the selection of a desired area of image.

3.0 Results

3.1 Characterisation of the endocytic nature of CTLA-4 in a transfected CHO cell line.

We firstly aimed to demonstrate the endocytic recycling of Cytotoxic T-Lymphocyte Antigen-4 (CTLA-4) using an established CTLA-4 transfected Chinese Hamster Ovary (CHO) cell line. Staining for CTLA-4 at 4°C could identify protein solely expressed at the plasma membrane as cells are metabolically inactive at this temperature and endocytic pathways are halted. At a physiological temperature (37°C) the cells cellular transport is permitted, thus the recycling pool of CTLA-4 can be demonstrated. Flow cytometry data showed that at 4°C surface stained CTLA-4 expression was ten-fold lower than at 37°C. This suggests that CTLA-4 is expressed at the plasma membrane and that endocytic pool of CTLA-4 is directed to the cell surface where it is able to bind fluorescently-conjugated antibody (Fig 3.1A). The staining intensity at 37°C was very similar to that when the total CTLA-4 was stained indicating the majority of CTLA-4 within a cell is recycling. Additionally the fluorescence intensity of labelled CTLA-4 increases in a near linear fashion over time. This further suggests a continual endocytic turn-over of receptor trafficked to the plasma membrane, fluorescently labelled and then internalised (Fig 4B). To support this theory confocal microscopy was performed to visualise the cellular localisation of the recycling pool of CTLA-4. When CHO cells were stained with anti-CTLA-4 for at 37°C images showed the receptor was predominantly located in discrete intracellular vesicles dispersed throughout the cytosol (Fig 3.2). There was little staining of the receptor at the cell surface indicating the majority is expressed within endosomes at physiological temperature.

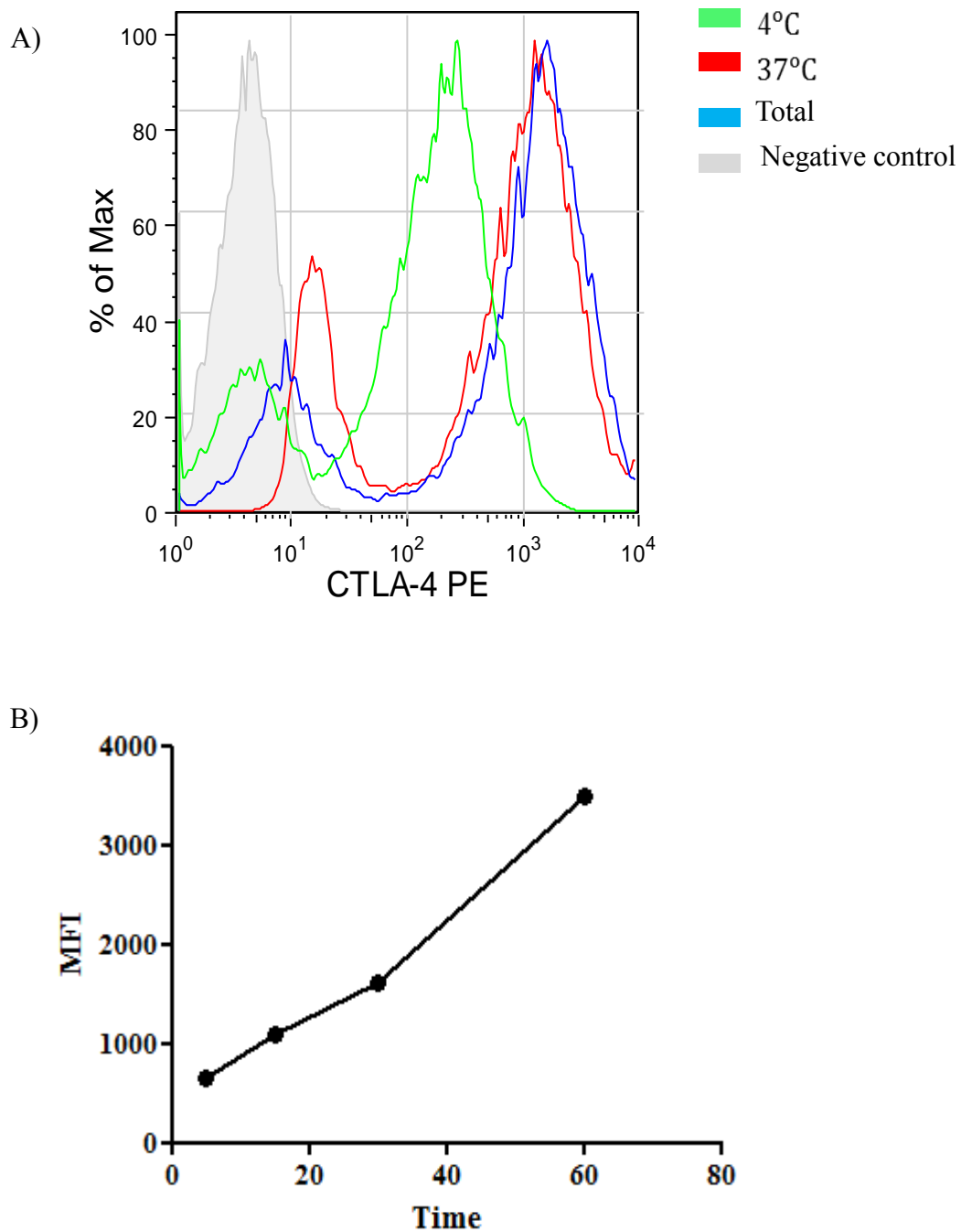


Figure 3.1 Characterization of the surface and intracellular expression of CTLA-4 in CHO cells by flow cytometry. A) CHO cells transfected with wild type CTLA-4 were incubated with anti-CTLA-4 antibody for 30min at 4°C (surface pool) and 37°C (recycling pool). The total pool was also stained by fixing and permeablising the cells prior to staining. B) Cells were incubated with anti-CTLA-4 antibody over a 1 hour time course at 37 °C. Mean fluorescence intensity (MFI) of CTLA-4 positive cells increases with time. mouse IgG2ak isotype was used as a negative control. n=1 for both experiments.

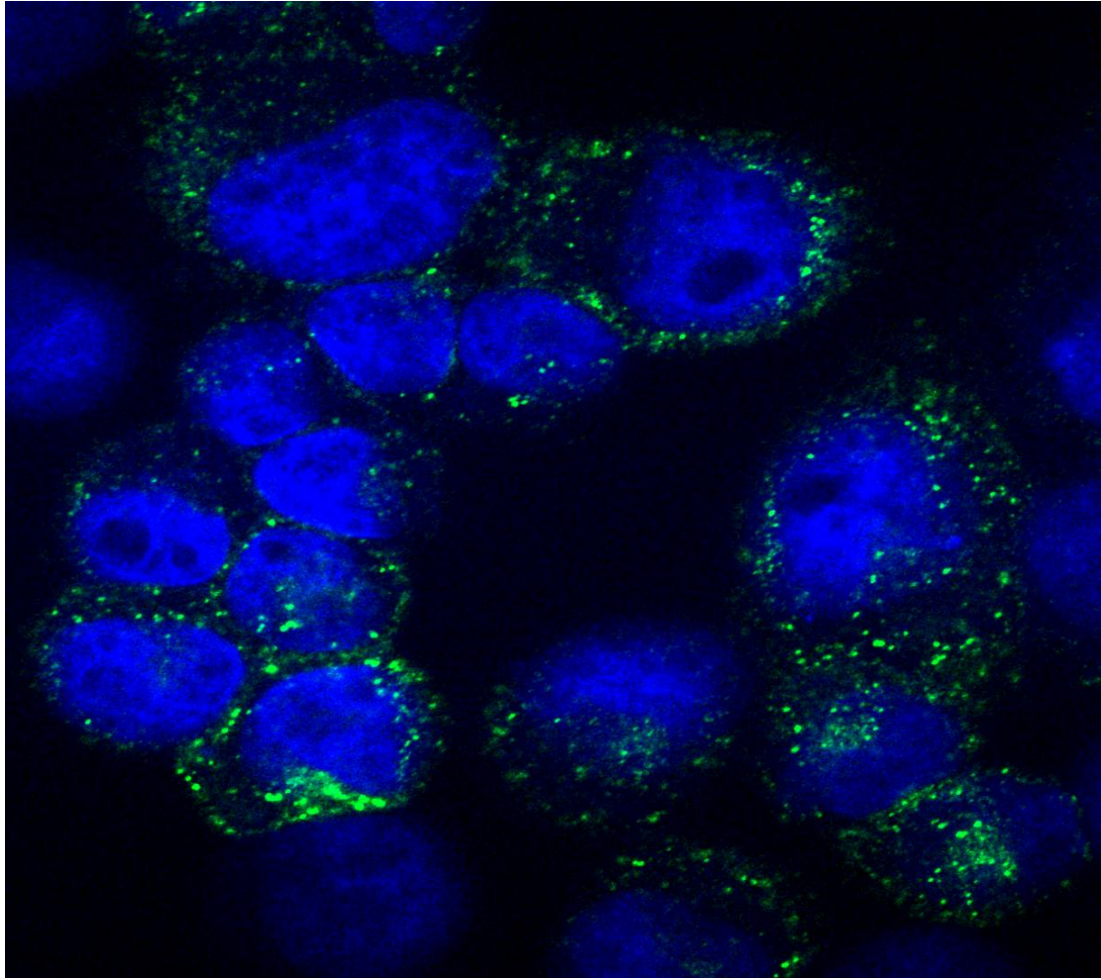


Figure 3.2 The cellular localisation of CTLA-4 in CHO cells. CHO cells expressing CTLA-4 were stained with anti-CTLA-4 antibody at 37°C. Cells were then fixed, permeabilised and stained with a FITC-conjugated secondary antibody (green). Mounting medium containing DAPI was used to stain cell nuclei (blue). Image shows the recycling pool of CTLA-4 in intracellular vesicular endosomes. Image representative of n=2 independent experiments.

3.2 Transfection of CTLA-4 into HeLa cells

The association of CTLA-4 with clathrin-mediated endocytosis (CDE) could be identified using fluorescently-labelled antibodies to various endosomal markers of the pathway and analysing co-localisation. As these antibodies were not applicable for a CHO cell species the model was transferred to a human HeLa cell line. To optimise transfection using Lipofectamine reagent a CTLA-4-GFP construct was used to allow easy visualisation of successful transfectants via confocal microscopy. Varying the ratio of Lipofectamine to DNA indicated that the optimal ratio was 1:1 as the highest proportion of CTLA-4 positive cells with the brightest GFP signal was seen at this ratio (Fig 3.3). A greater proportion of Lipofectamine caused a greater degree of membrane disruption and blebbing (DNA:LF 1:2), along with increased cell death. A higher ratio of DNA did not improve transfection and efficacy was poor (DNA:LF 2:1). Transfections were also carried out over either one or two days, with a two day transfection assay giving optimal results (data not shown). However no numerical data for this evidence was produced and findings were based on microscope visualisation at the time of investigation.

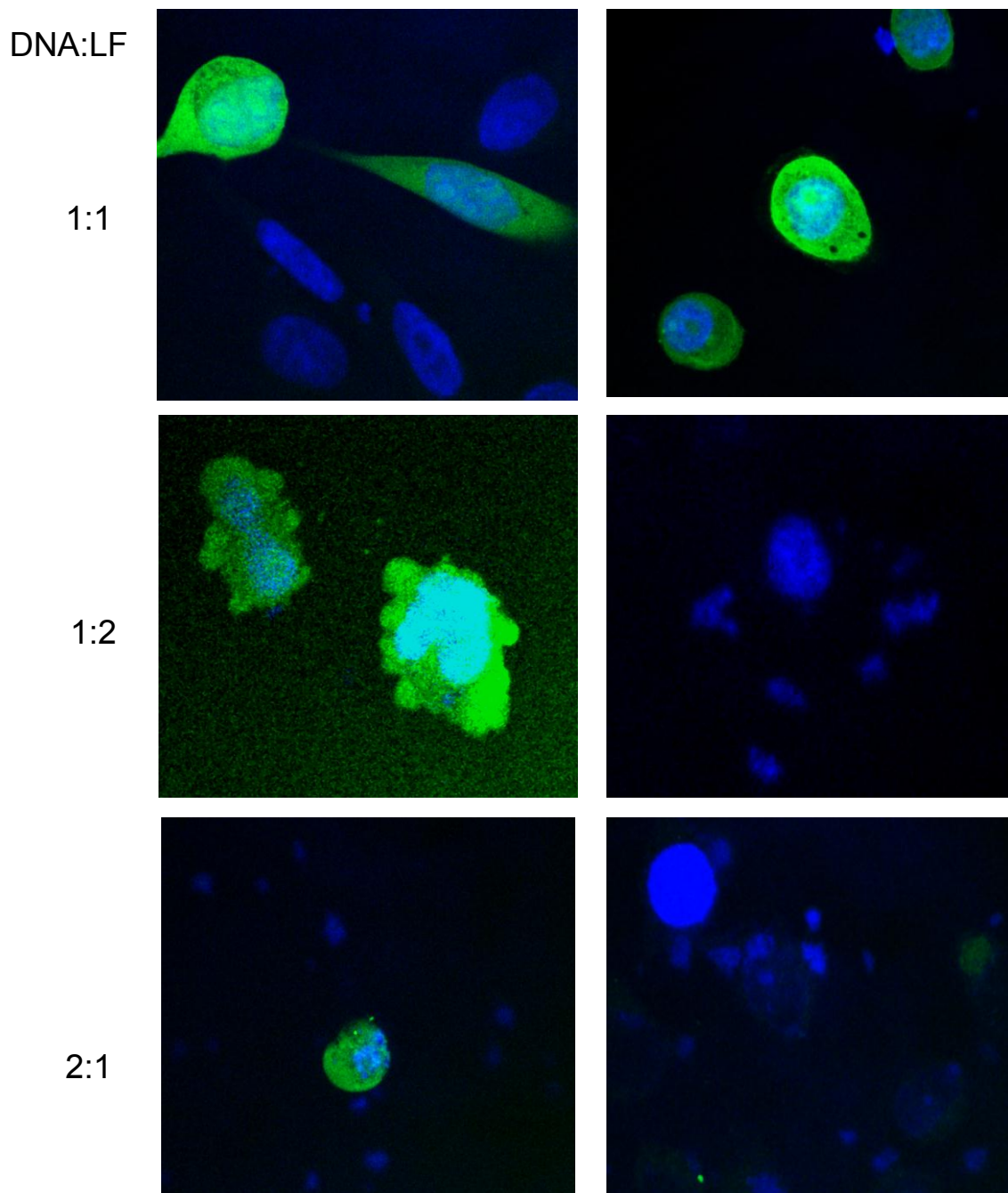


Figure 3.3 Confocal microscopy images representing the optimization of CTLA-4 transfection into HeLa. Expression of CTLA-4 GFP by HeLa cells was induced by transfection of a plasmid construct via the lipofectamine 2000 (LF) transfection system (Invitrogen). Optimisation of the protocol was achieved by varying the DNA/lipofectamine ratio. Two representative images for each ratio is shown. Mounting medium containing DAPI (blue) was used to stain cell nuclei.

Flow cytometry was performed on the newly transfected cell line to confirm and characterise CTLA-4 expression (Fig 3.4B). Staining for the surface and recycling pool of CTLA-4 revealed a trend consistent with that seen in CHO cells (Fig 3.1), with the majority of CTLA-4 within a cell recycling and labelled at the cell surface at the physiological temperature. There was also a ten-fold difference seen between this pool and the surface pool at 4°C. However, despite successful transfection, CTLA-4 expression was low with the majority of cells CTLA-4^(low). The cell line also showed a high degree of cell death. Therefore the cells were sorted for CTLA-4^(hi) expression, and flow cytometry confirmed >95% of cells now expressed high levels of the receptor (Fig 3.4A).

In order to more accurately quantify the amount of surface versus intracellular expression of CTLA-4 the two pools were co-stained simultaneously using secondary antibodies conjugated to two different fluorochromes (Fig 3.5). This technique could later be used to quantify the knock down effect on clathrin-mediated endocytosis. Confocal images showed CTLA-4 expression in HeLa cells had a similar distribution to the established CHO cell line, with a higher fluorescence intensity of the intracellular portion. The mean fluorescence intensity (MFI) showed the surface to intracellular ratio of CTLA-4 was approximately 1:3 in both HeLa and CHO cells, which added further evidence that CTLA-4 is predominantly intracellular in both cell lines.

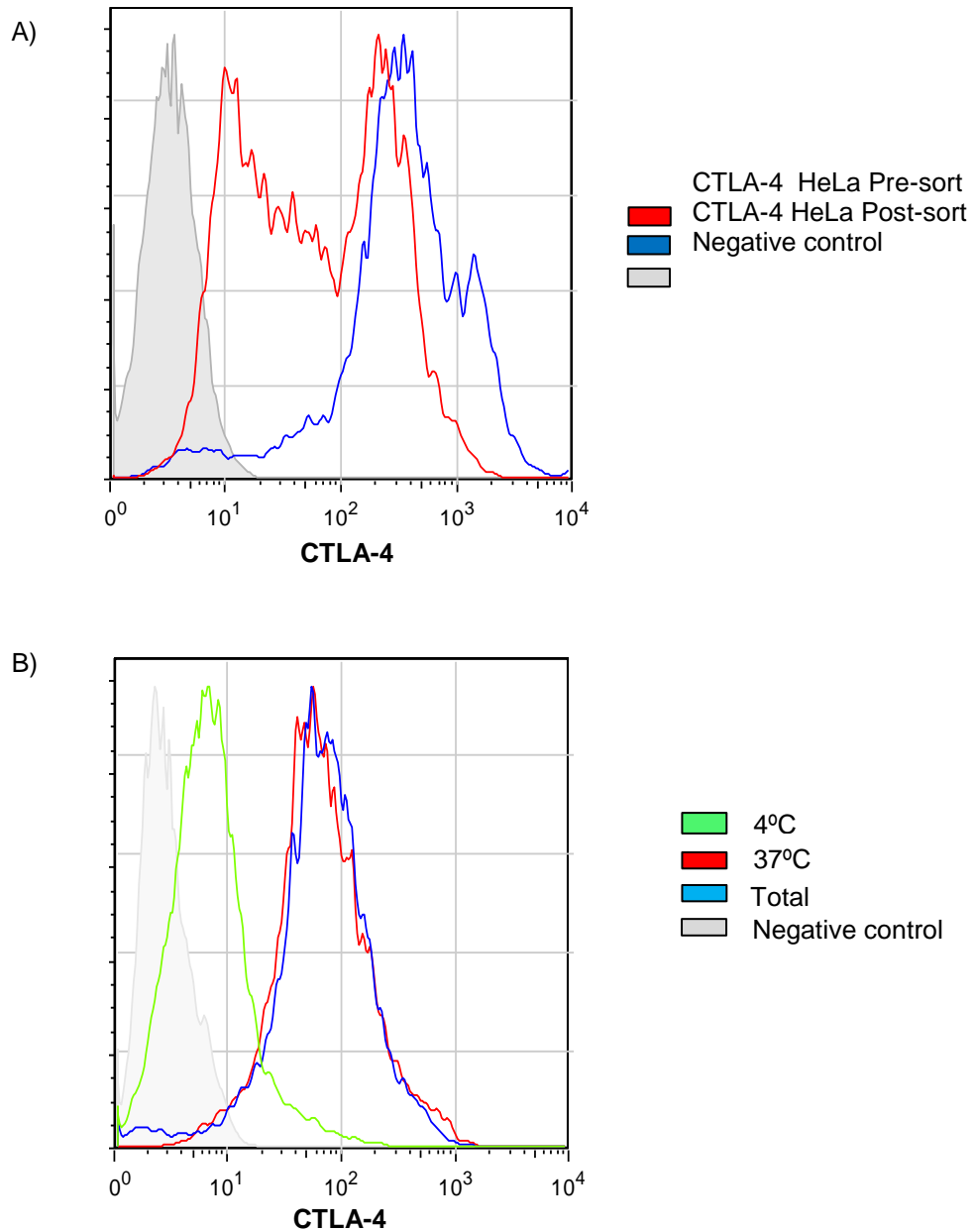


Figure 3.4 Characterisation of CTLA-4 expression in transfected HeLa cells by flow cytometry. A) Transfected HeLa were sorted by FACS for CTLA-4^(hi). B) HeLa cells transfected with wild type CTLA-4 were incubated with anti-CTLA-4 antibody for 30min at 4°C (surface pool) and 37°C (recycling pool). The total pool was also stained by fixing and permeablising the cells prior to staining.

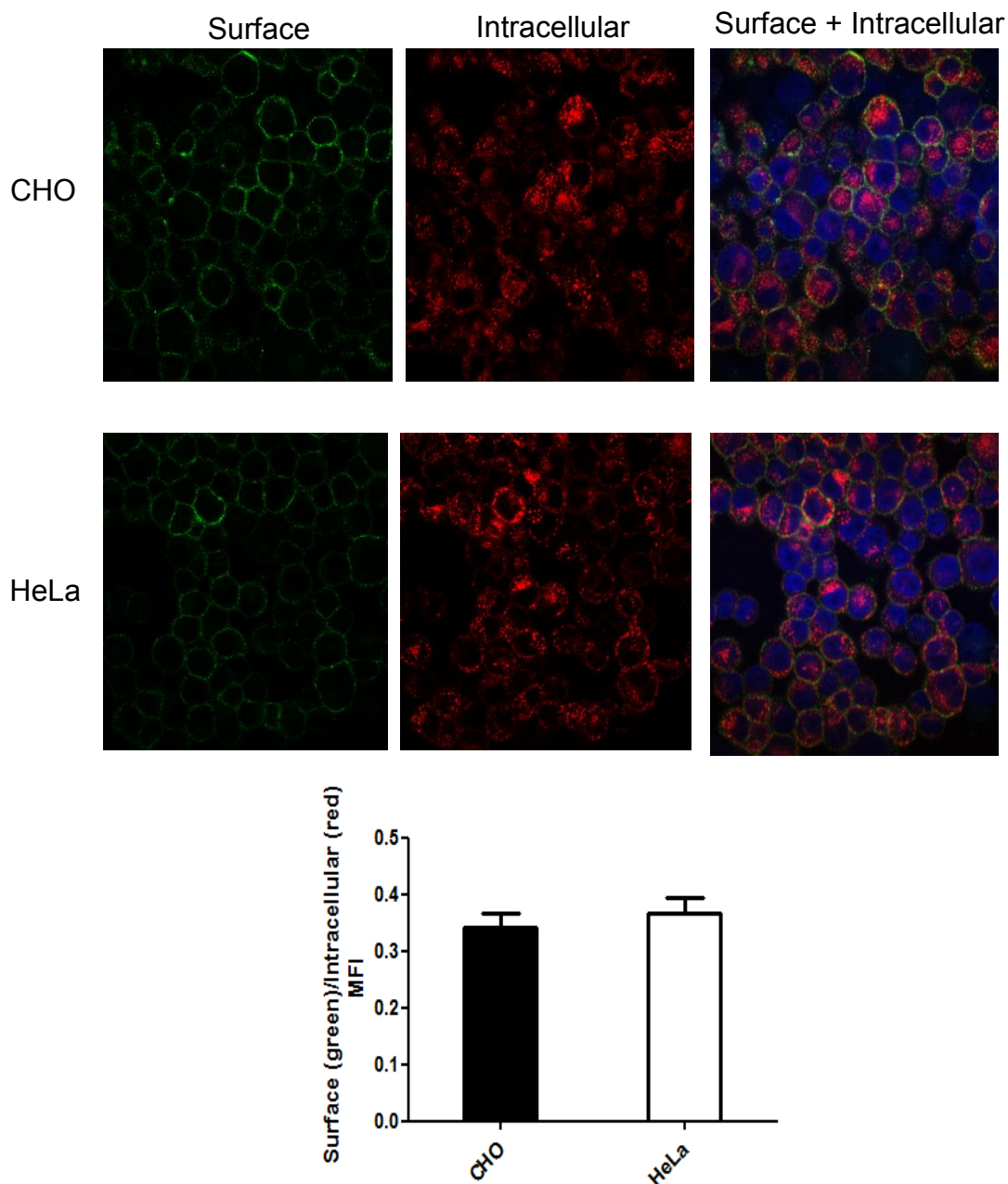


Figure 3.5 Double staining of surface and intracellular CTLA-4 in CHO and HeLa cells. Cells were labeled with anti-human CTLA-4 primary antibody at 37°C. Surface CTLA-4 was stained with Alexa Fluor 488-conjugated secondary (green). Cells were then fixed, permeabilised and intracellular CTLA-4 stained with Alexa Fluor 647-conjugated antibody (red). Mounting medium containing DAPI was used to stain cell nuclei (blue). Graph shows the surface/intracellular ratio of CTLA-4 using the mean fluorescence intensity of individual cells. The MFI was found to be 0.34 ± 0.025 and 0.366 ± 0.288 for HeLa and CHO cells respectively ($n=20$).

3.3 Identification of the endocytic pathway of internalised CTLA-4 in HeLa cells.

To investigate the fate of endocytosed CTLA-4 HeLa cells were surface stained with anti-CTLA-4 antibody at 5min, 30min & 60min and the location of labeled receptor viewed at three time points (Fig 3.6). At 5min the intensity of CTLA-4 staining was greatest at the surface in some cells. However endocytic vesicles containing CTLA-4 could be observed at this time point indicating rapid and constitutive internalization of the receptor (Fig 3.6A). At 30min vesicles containing CTLA-4 could be seen dispersed throughout the cytoplasm with less visible staining at the cell surface (Fig 3.6). At 60min there was an increased intensity of CTLA-4 staining around the perinuclear area, with an accumulation within centrally localized vesicles (Fig 3.6C).

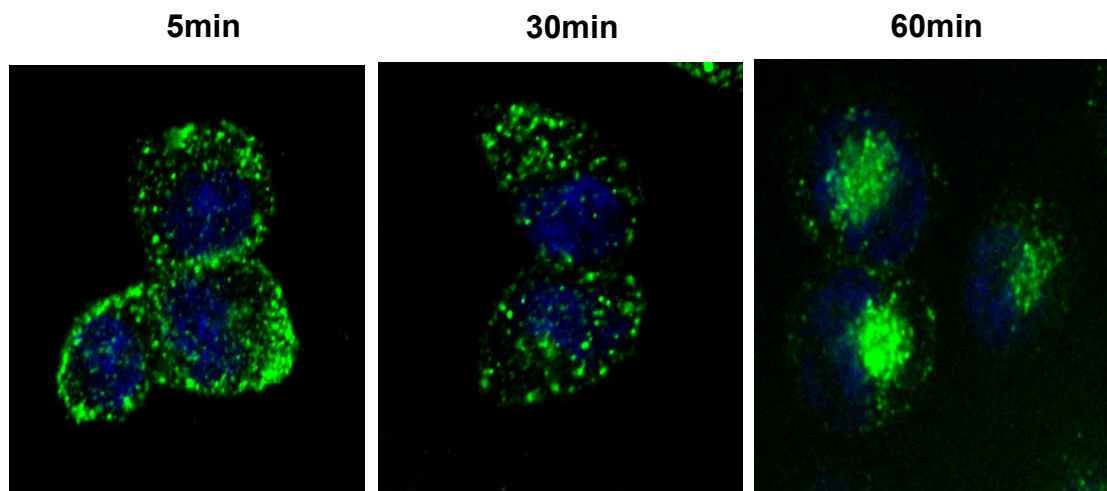


Figure 3.6. Cellular localisation of internalised CTLA-4 in HeLa cells. HeLa cells transfected with CTLA-4 were stained with α -CTLA-4 antibody for a 5-60min duration. Following fixation and permeabilisation cells were stained with a secondary 488-conjugated antibody (green). Images representative of n=1 experiment.

To identify any geometry based association between CTLA-4 and clathrin-mediated endocytosis (CDE) transfected CTLA-4 was firstly co-stained with clathrin itself. A lysosomal marker LAMP-1 (lysosome-associated membrane protein-1) was also added to identify CTLA-4 and clathrin within this subcellular compartment (Fig 3.7).

Co-staining identified little co-localisation when CTLA-4 with clathrin (Fig 3.7A). Clathrin staining was restricted to the plasma membrane (blue) whilst consistent with Fig 3.5 little CTLA-4 was stained at the cell surface and a strong signal shown within vesicular bodies (green). There appeared some degree of convergence between the lysosomal marker and CTLA-4 (as shown by yellow vesicles in Fig 3.7B, Fig 3.7C). Areas of co-localisation were identified towards the centre of the cell but more predominantly at the periphery. This suggests a small but notable proportion of intracellular CTLA-4 is targeted for lysosomal degradation, however, no clathrin was associated with this endosomal compartment.

Cells were also co-stained for the transferrin receptor (CD71): an archetypal endocytic receptor known to internalise specifically in a clathrin-dependent manner (Fig 3.8). Surface staining of both receptors at physiological temperature would identify any commonality or convergence of their internalisation pathways, thus linking CTLA-4 to CDE. Confocal microscopy images identified a strong degree of co-localisation between CTLA-4 (green) and the CD71 (red) in some cells (yellow vesicles Fig 3.8). These were particularly prominent around the perinuclear area where the staining intensity was greatest, and support a link between CTLA-4 and CDE. However, singularly stained vesicles indicated there remains some disparity in the endosomal pathways of each receptor. It was noted that some cells appeared to express high levels of CTLA-4 did not stain well for CD71 and cells expressing little CTLA-4 seemed to have more internalised CD71 in some instances. This indicated a

preferential internalisation of either one of the receptors, and suggested their uptake may have influences on each other.

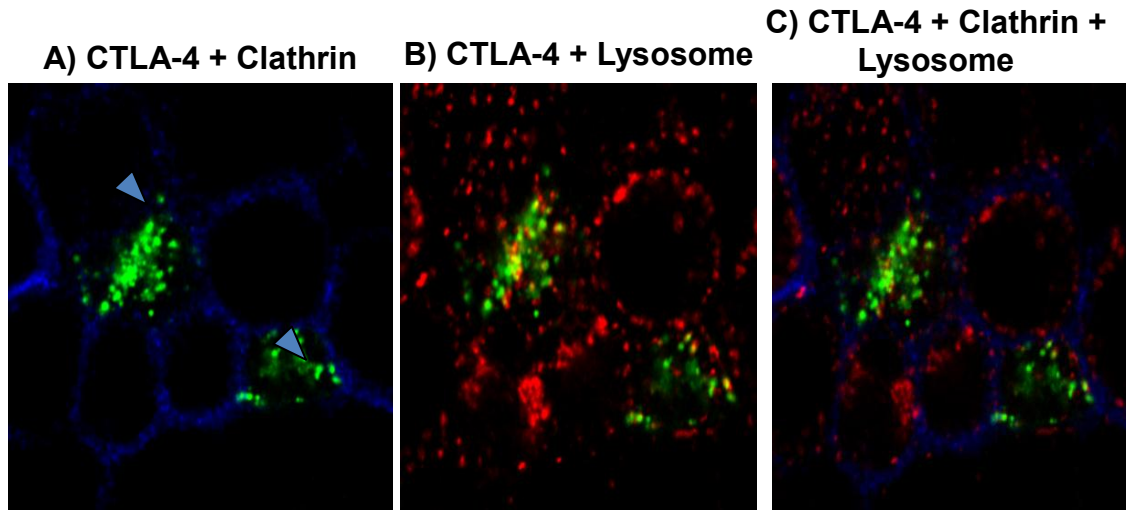


Fig 3.7 Co-staining of CTLA-4 and endocytic markers in CTLA-4 transfected HeLa cells. CTLA-4 positive HeLa cells were fixed, permeabilised and stained with anti-CTLA-4 and anti-clathrin antibodies for 30min. A) CTLA-4 was labelled with a Alexa-Fluor-488 secondary antibody (green). Arrows show transfected cells. Clathrin was labelled with a Alexa Fluor-647 (blue). B) Cells were also co-stained for the lysosomal marker LAMP-1 with Cy5-conjugated anti-LAMP-1 antibody (Red). C) Staining for CTLA-4 and both endosomal markers. Images representative of n=1 experiment.

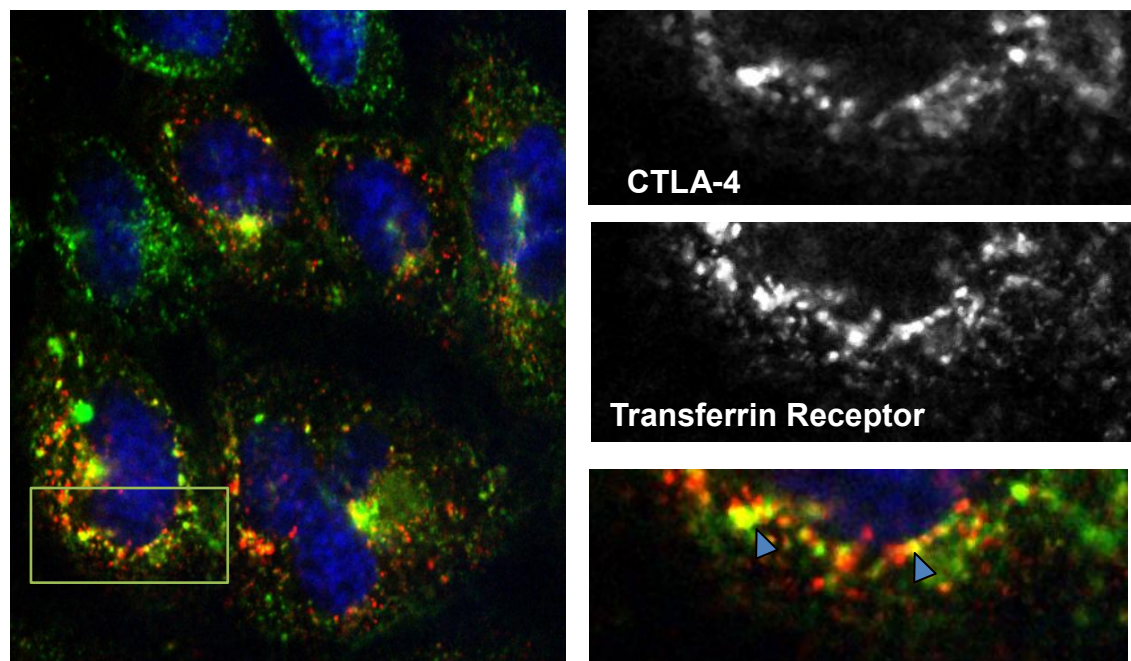


Figure 3.8 Co-Localisation of CTLA-4 and the transferrin receptor in HeLa cells. HeLa cells expressing CTLA-4 were surface labelled with anti-CTLA-4 antibody and fluorescently labelled transferrin. Cells were fixed, permeabilised and stained for internalised receptor. Mounting medium was used to stain cell nuclei (blue). Confocal microscopy image (left) shows vesicles containing internalised CTLA-4 (green) and transferrin (red). Right panel highlights a degree of co-localisation between CTLA-4 and transferrin within intracellular vesicles (yellow) in the enlarged area indicated left. Images representative of one independent experiment.

To identify association of CTLA-4 with a clathrin-mediated intracellular sorting pathway it was also co-stained with γ -adaptin. This subunit of the Adaptor Protein-1 (AP-1) complex facilitates the formation of clathrin-coated vesicles budding from the *trans*-Golgi network (TGN) (Fig 3.9A). LAMP-1 was also stained to show whether CDE is involved in transporting CTLA-4 from the TGN to lysosomes directly (Fig. 3.9B, 3.9C). There was a small degree of co-localisation of CTLA-4 and γ -adaptin, particularly in areas of intense CTLA-4 staining towards the centre of the cell (sky blue vesicles Fig 3.9A), This may represent the newly synthesised pool of CTLA-4 or receptor transported to the TGN from different endosomal compartments for sorting. This is evidence that the transport of the

receptor from the TGN involves CDE. As demonstrated in Fig 3.7B, in this separate investigation a small proportion of CTLA-4 was identified as lysosomal by association with the lysosomal marker LAMP-1 (magenta vesicles Fig 3.9B). γ -adaptin was also shown to associate with lysosomes (orange vesicles Fig 3.9C) which suggested its involvement in receptor transport from the TGN to lysosomes.

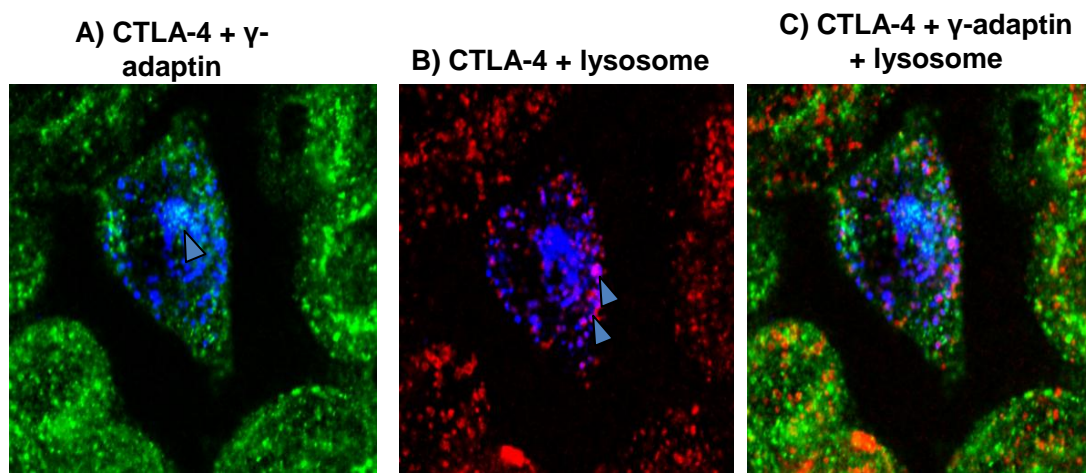


Fig 3.9 Co-staining of CTLA-4 and endocytic markers in CTLA-4 transfected HeLa cells. CTLA-4 positive HeLa cells were fixed, permeabilised and stained with anti-CTLA-4 and anti- γ -adaptin antibodies. Arrows highlight areas of co-localisation A) CTLA-4 was labelled with a Alexa-Fluor-350 secondary antibody (blue). γ -adaptin was labelled with Alexa Fluor-488 (green) B) CTLA-4 was also co-stained for the lysosomal marker LAMP-1 with Cy5-conjugated anti-LAMP-1 antibody (Red).. C) Staining for CTLA-4 and both endosomal markers. Images representative of n=1 experiment.

3.4 Pharmacological blockade of clathrin-dependent endocytic pathways

Examining whether CDE is essential for CTLA-4 internalisation required a CDE-specific blockade of this route without disruption of other modes of endocytosis. We therefore tested whether hypertonic blockade of endocytosis by sucrose solution was sufficient to achieve this specific CDE inhibition. CD71 internalisation is clathrin-dependent whereas cholera toxin hijacks multiple endocytic routes [52, 53], and both were seen within intracellular vesicles at 30min without sucrose (Fig 3.10). Consistent with current literature, cholera toxin accumulated in the centre of the cell around the location of the Golgi apparatus [53]. Cells adopted a very elliptical morphology with a smooth membrane in the presence of sucrose and both internalisation of CD71 and cholera toxin was abrogated. Therefore sucrose disrupted both clathrin-dependent and independent pathways and was insufficient for the purposes of this investigation.

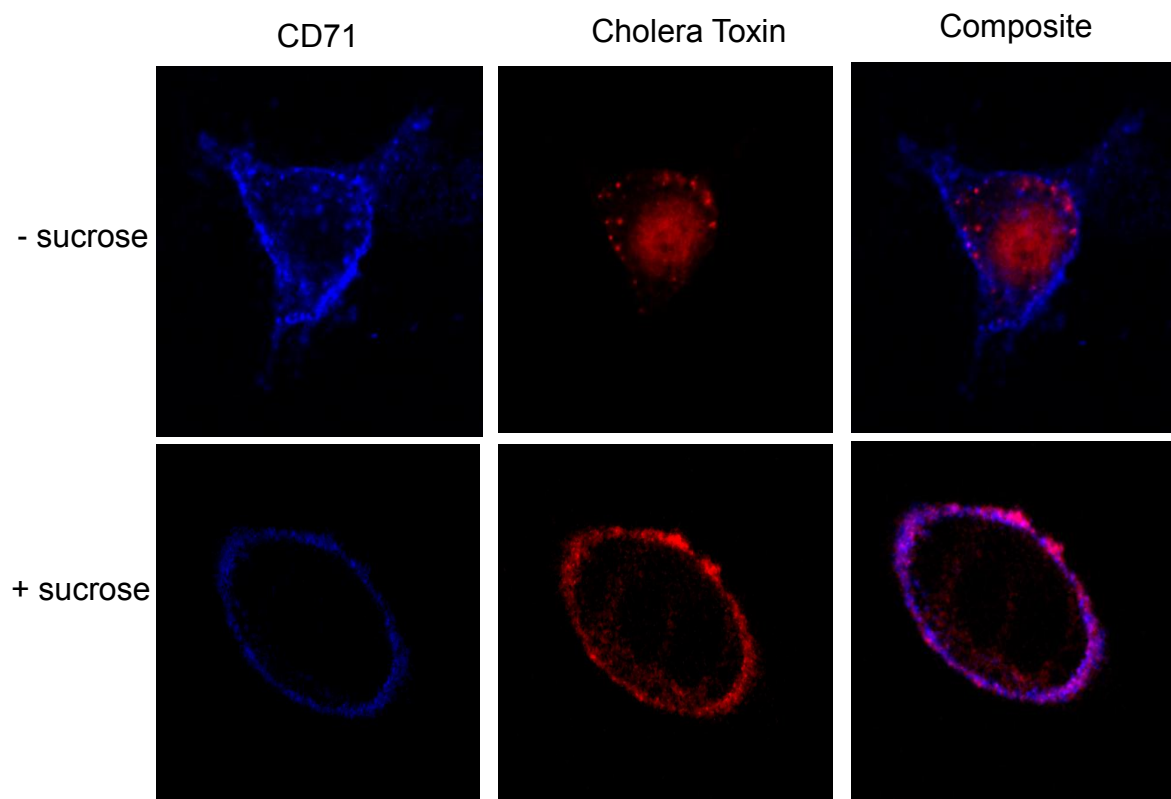


Figure 3.10 Block of transferrin receptor and cholera toxin internalisation by sucrose. HeLa cells were incubated with Alexa Fluor-647 conjugated α -CD71 antibody (blue) and RFP-conjugated cholera toxin (red) +/- the presence of 0.4M sucrose at 37°C. Presence of sucrose abrogated the internalisation of both CD71 and cholera toxin. Image is representative of n=2 independent experiments.

3.5 shRNA knock down of the AP-2 μ -subunit in CTLA-4 CHO and HeLa cell lines.

To produce more specific CDE inhibition expression of an essential component of clathrin-coated pit formation was abrogated. This was achieved through transient transfection of an AP2 μ 2-subunit shRNA plasmid construct with a red fluorescent protein (RFP) reporter. Firstly the procedure was optimised in CHO cells expressing CTLA-4. Four constructs (abbreviated to 97, 98, 99 and 90) against different segments of μ 2 mRNA were available and flow cytometry was performed to see which construct was most highly expressed upon transfection (Fig 3.11). Transfection of all four constructs revealed RFP⁺ cells relative to untransfected, which confirmed successful shRNA transfection

A control construct with scrambled shRNA insert was the most efficiently transfected (Fig 3.11B), however of the AP-2 constructs the efficiency of 98 was the greatest, with both the highest percentage of CTLA-4⁺ shRNA⁺ cells (46.5%) and the highest RFP mean fluorescence intensity (Fig 3.11A and 3.11B). This construct was then taken forward for further knock down experiments. Interestingly the CTLA-4 MFI for shRNA positive cells also increased following transfection with all constructs.

Following the confirmation of successful shRNA transfection the effect of AP-2 knock down on CTLA-4 recycling was investigated in CHO and HeLa cells. CHO cells were transiently transfected with shRNA and receptor internalising from the cell surface labelled at 37°C as previously described. Cells stained red (RFP⁺) could be identified as cells successfully transfected with shRNA (Fig 3.12A). In some CHO cells there appeared a reduced number vesicles containing CTLA-4 (green) in AP-2 knock down cells when compared to untransfected cells, and what receptor was labelled appeared to be restricted to the surface.

However, this was not true of all shRNA⁺ cells. The mean fluorescence intensity of CTLA-4 staining in these cells was calculated and found to be lower than that of both the positive

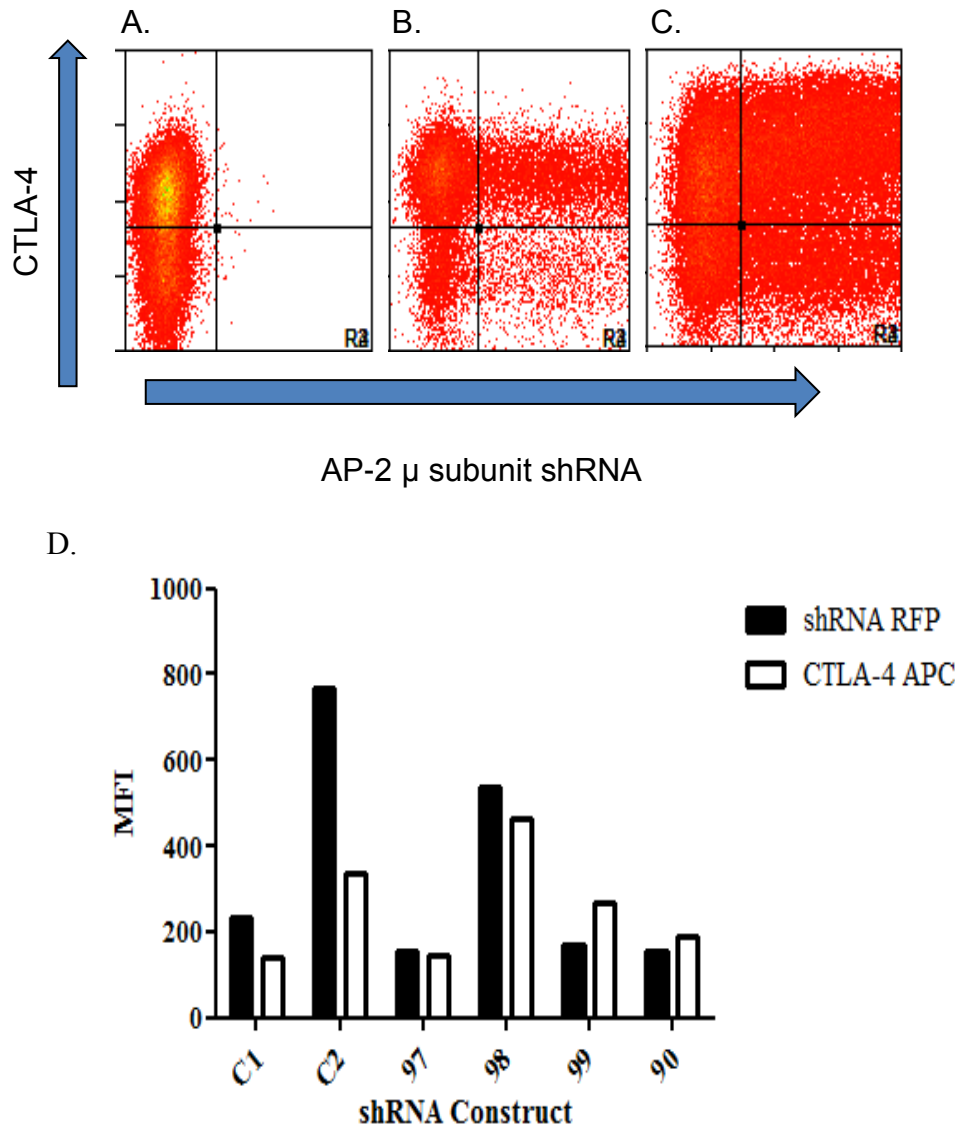
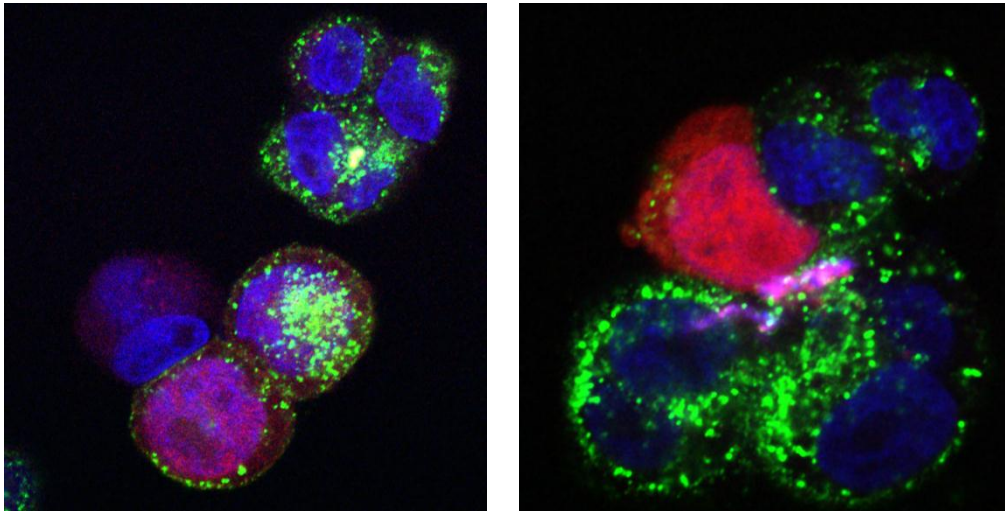


Figure 3.11. Transfection of shRNA constructs into CHO cells. Four RFP positive plasmid constructs containing shRNA inserts for the μ -subunit of AP-2 were transiently transfected into CTLA-4 positive CHO cells and incubated overnight. Constructs lacking the shRNA insert those containing scrambled shRNA were used as controls (C1 and C2 respectively). Following cell staining with anti-CTLA-4 antibody flow cytometry was performed to identify the most efficiently expressed shRNA construct. A) CHO cells singularly transfected with CTLA-4. B) Positive control (C1) shows a population of CTLA-4 positive RFP positive cells which confirms successful transient transfection. C) 98 was the most efficiently transfected construct, with the highest percentage of RFP⁺ cells. D) Shows the mean fluorescence intensity (MFI) of CTLA-4 positive shRNA positive cells for each transfected construct (n=1).

controls and the untransfected cells (Fig 3.12B). This suggested the blockade of clathrin-mediated endocytosis was inhibiting the CTLA-4 internalisation, however no statistical inferences could be drawn from this data due to lack of experimental replicates.

HeLa cells were then transfected with shRNA and co-stained for both surface and intracellular CTLA-4 for more accurate quantification as previously described (Fig 3.5). Cells successfully transfected with shRNA via the previously optimised lipofectamine protocol were identified (red). However unlike in CHO cells the transfection did not visibly seem to decrease the proportion of intracellular CTLA-4 when compared to untransfected cells or control constructs (blue). In contrast an increased staining intensity was observed (blue panel Fig 3.13). There was an observable increase in the intensity of CTLA-4 staining at the cell surface (green panel). However the RFP-signal from shRNA transfected cells was so intense it caused considerable cross-over into the Alexa fluor-488 emission spectrum causing a false positive CTLA-4 signal (Fig 3.13C). It was therefore impossible to accurately calculate the MFI of CTLA-4 and no quantitative data for the surface or intracellular CTLA-4 expression.

A)



B)

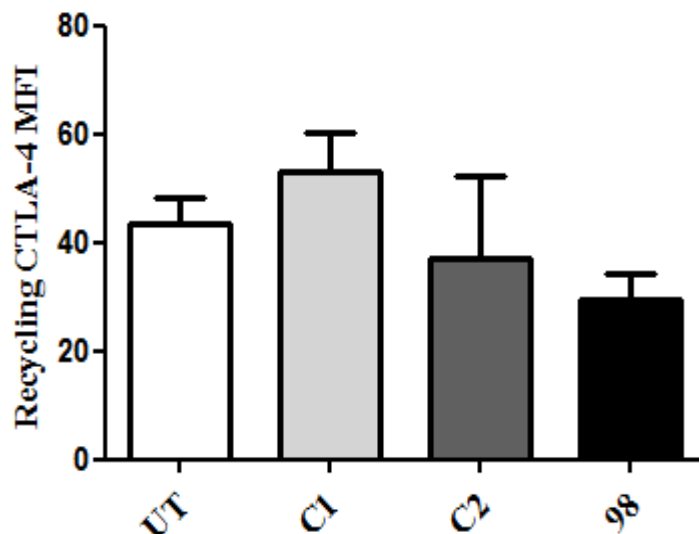


Figure 3.12 The cellular localisation of CTLA-4 in transfected CHO cells following shRNA knock down of the μ subunit of adapter protein 2 (AP-2). CTLA-4 positive CHO cells were transiently transfected with an shRNA-RFP construct to disrupt clathrin-mediated endocytosis. Cells were labelled with human CTLA-4 primary antibody at 37°C. Fixed and permeabilised cells were stained with Alexa fluor 488-conjugated anti-human to show internalised CTLA-4. Mounting medium containing DAPI was used to stain cell nuclei (blue). A) Two confocal microscopy images showing CTLA-4 localisation (green) in cells transfected with AP-2 shRNA (magenta) and those shRNA negative (blue). B) The mean fluorescence intensity (MFI) of CTLA-4 in shRNA negative cells (UT), those expressing two positive control constructs (C1 and C2) and shRNA positive cells (98). Data representative of 5-11 cells in n=1 experiment.

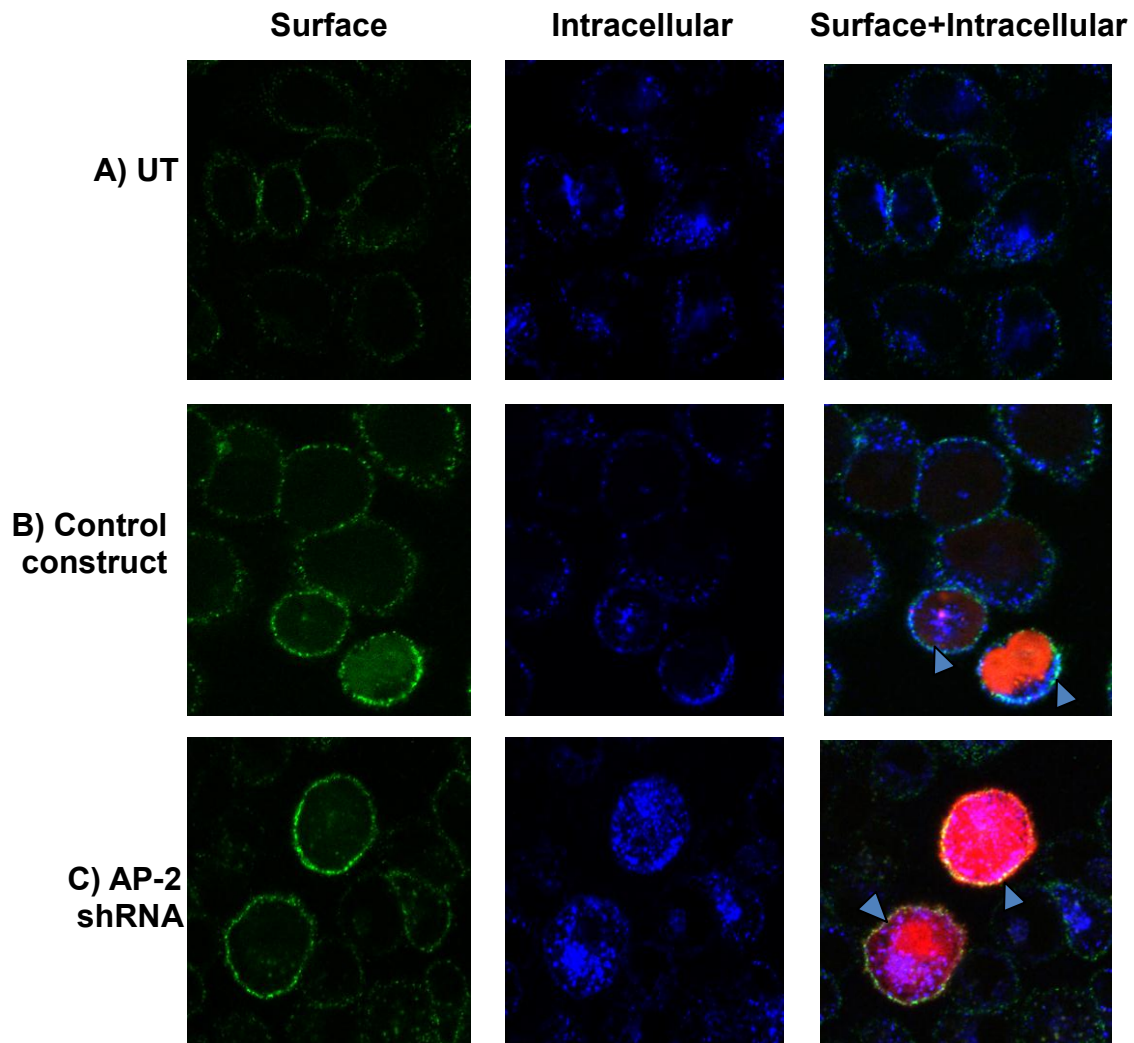


Figure 3.13 Surface and intracellular expression of CTLA-4 in transfected HeLa cells following shRNA knock down of the μ subunit of adapter protein 2 (AP-2). HeLa cells expressing CTLA-4 were transiently transfected with an shRNA-RFP construct to disrupt clathrin-mediated endocytosis. Cells were incubated with anti-CLTA-4 antibody at 37°C for 30min. Surface CTLA-4 was stained with Alexa Fluor 488-conjugated secondary antibody at 4 °C for 30min (green). Cells were fixed, permeabilised and intracellular CTLA-4 stained with Alexa Fluor 647-conjugated antibody at 37 °c (blue). A) Confocal microscopy images showing CTLA-4 localisation in CTLA-4 positive AP-2 shRNA negative HeLa. B) CTLA-4 localisation of cells transfected with an shRNA-RFP control construct (arrow heads). C) CTLA-4 localisation in cells transfected with AP-2 shRNA-RFP construct (arrow heads). Images representative of n=1 experiment. UT: Untransfected

4.0 Discussion

Given recent observations suggesting the endosomal behaviour of Cytotoxic-T-Lymphocyte Antigen-4 (CTLA-4) has a fundamental role in its mechanism action, the objective of this investigation was to examine its endocytic behaviour. Determining whether or not CTLA-4 internalisation was clathrin-dependent (CDE) was central to the study, due to conflicting reports as to its endosomal pathway (reviewed in [25]).

Initially, previous observations of the receptors endocytic behaviour in a transfected CHO cell line were successfully replicated. Flow cytometry showed the surface expression (at 4°C) of the receptor was limited but when staining at physiological temperature cytoplasmic CTLA-4 was trafficked to the plasma membrane where it bound conjugated antibody. The fluorescence intensity increased over time and the fact there is finite space at the cell surface leads to the conclusion labelled receptor is internalised and represented a recycling pool of receptor (Fig 3.1). This was confirmed by confocal microscopy images which showed intracellular vesicles containing CTLA-4 labelled at the cell surface (Fig 3.2). Notable in flow cytometry experiments was when the total expressed receptor was stained it was very similar to the recycling pool. This suggests that the majority of CTLA-4 is recycling, however the surface staining was performed live and total stains performed fixed. Therefore it is possible that the chemical stress of the fixing and permeabilisation process negatively affected the staining procedure and leads to an under-representation of receptor.

To associate the trafficking pathway of CTLA-4 with CDE this model was transferred into a human (HeLa) cell line, so endosomal markers of the CDE pathway could be obtained. HeLa cells were transfected using lipofectamine reagent. Optimisation showed that the optimum ratio of DNA to lipofectamine was 1:1 (1.5µg:1.5µl) cultured for 2 days leading to a transient process. However, further refinement and more quantitative analysis would have been beneficial as the cationic reagent was still considerably cytotoxic causing morphology

changes and considerable cell death in culture. This was demonstrated when it was used at higher concentrations in the optimisation process (Fig 3.3). Once transfected the CTLA-4 HeLa cells were initially unstable and the expression of the receptor was low. However, once sorted for CTLA-4(hi) a stable line with a high percentage expression was established (Fig 3.4A). CTLA-4 expression in HeLa showed very similar recycling patterns as seen in CHO cells, confirming consistency between the two models. This consistency was demonstrated by the similar CTLA-4 staining intensities of the surface and recycling pool of CTLA-4 by flow cytometry (Fig 3.4B) and confocal microscopy images where the ratio of surface to intracellular CTLA-4 expression was comparable (Fig 3.5).

Staining for the recycling pool over 60min in HeLa was analysed by confocal microscopy and it may have been favourable to support this with flow cytometry data as performed in CHO cells. However, images showed an accumulation of surface stained receptor in the perinuclear area at the hour time point (Fig 3.6) which is consistent with published reports that the receptor accumulates here around the microtubule organising centre and is resistant to degradation [41, 47, 54] . What was not possible to determine from this experiment is whether or not internalised CTLA-4 recycles back to the cell surface directly and in what proportion. This could be achieved by surface staining with anti-CTLA-4 fluorescently-conjugated antibody and surface staining again with an anti-antibody with a different fluorophore at set time points. By comparing the mean fluorescence intensities of the labelled and double-labelled CTLA-4 the proportion of receptor that had recycled at least once could be established.

In contrast to CHO cells transfection of CTLA-4 into HeLa allowed for its internalisation to be compared with transferrin receptor (CD71): classically associated with the clathrin-dependent endocytic route. The fact there was a strong degree of co-localisation between

CTLA-4 and CD71 in some intracellular vesicles is evidence of a commonality between their endocytic pathways (Fig 3.8). This is suggestive that the endocytic pathway of CTLA-4 is clathrin-mediated. However, the fact there was also significant expression of each receptor in separate intracellular compartments may suggest their pathways merge or converge at different points. It may be informative to repeat the experiment on live cells and record the location of each receptor over a given time. What was also noted is that some cells which strongly expressed internalised CTLA-4 did not appear to show much internalised transferrin and visa versa (Fig 3.8). This could possibly suggest that the artificially high expression of CTLA-4 in HeLa results in competition between the receptor and CD71 for clathrin, membrane and associated machinery at the cell surface. This competition could further suggest CTLA-4 and CD71 share a common CDE internalisation pathway. Despite this the observation was not supported by a decrease in CD71 uptake in CTLA-4 HeLa compared to untransfected HeLa in flow cytometry experiments. However, a decrease in CD71 uptake was observed in experiments comparing a CTLA-4⁺ Jurkat T cell line and untransfected Jurkat cells.

Despite the evidence supporting a common pathway between the two receptors it must be considered that CD71 internalisation requires direct ligand binding, whereas in contrast CTLA-4 is constitutively recycles and transiently upregulates in response to T-cell receptor (TCR) ligation in activated T-cell [55, 56]. More appropriate analogies between CTLA-4 and the glucose uptake transporter (GLUT4) receptor have been drawn [57]. GLUT4 is actively mobilised and recycles to the membrane in response to insulin binding to the insulin receptor [58], in the way CTLA-4 mobilises through TCR activity. In addition to CD71 it may therefore be interesting to compare CTLA-4 to receptors whose response is indirectly dependent on the activity of another plasma membrane receptor.

When CTLA-4 was co-stained with clathrin to directly identify an association with CDE no apparent co-localisation was observed (Fig 3.7). This was surprising given published evidence of a positive association with clathrin coated-pit forming machinery [26, 30-32] and this data showing co-localisation of the receptor with CD71. It was also surprising that clathrin staining was restricted to the plasma membrane despite cell permeabilisation given its involvement in vesicular budding from the Golgi apparatus. Further investigation is required before the cause of this pattern of expression and lack of geographical connection can be ascertained.

AP-1 is the adapter protein which associates CTLA-4 to clathrin and is essential for the clathrin-dependent endosomal transport originating from the trans-Golgi network (TGN) [34]. When vesicles containing CTLA-4 pinch off from the TGN it has been shown they can be either exported to the cell surface or traffic to mature endosomal compartments. In this way CDE may have an important role in regulation of surface and overall expression. To link CTLA-4 to CDE in transport from the TGN pathway the γ -subunit of AP-1 was fluorescently labelled in transfected HeLa (3.9). A notable degree of co-localisation was found towards the centre of cells. This is consistent with expected subcellular location of the Golgi apparatus i.e. adjacent to the nucleus, and supports previous work showing CLTA-4 association with AP-1 [27, 59]. To confirm this it would be beneficial to additionally label a marker for the exocytic pathway from the TGN (for example the TGN marker syntaxin) to further confirm CTLA-4 associated with AP-1.

The quantity or origin of CTLA-4 targeted to lysosomes is not completely defined. In light of recent reports from the lab which have suggested CTLA-4 rips its ligand from antigen presenting cells (APC) through transendocytosis, it is important to further investigate whether internalised receptor bound ligand is targeted to lysosomes for degradation. In this study some CTLA-4 was found geographically co-localised to lysosomes (as shown by yellow vesicles in

Fig 3.7 and magenta vesicles in Fig 3.9). As CTLA-4 was stained intracellularly and not at the surface the source of lysosomal receptor was not established and given that lysosomes are the primary site of degradation, some lysosomal receptor may be as expected. However, the receptors co-localisation with lysosomes does not necessarily correlate to its breakdown. For example published data has suggested CTLA-4 can by-pass degradation through active secretion from lysosomes [27]. It would therefore be informative to visualise whether CTLA-4 stained at the cell surface and trafficked to lysosomes could recycle to other endosomal compartments or back to the plasma membrane.

Once an association between CTLA-4 and CDE had been established through endosomal markers the aim was to attenuate the CDE pathway and examine whether this abolished CTLA-4 internalisation. In this way an alternative internalisation independent of clathrin could be identified. Historically hypertonic sucrose solution has been used to block of this pathway (reviewed by Andrei I. 2008). Studies suggest this is through disruption of clathrin assemblage is shown by a reduced number of clathrin coated pits [60]. In line with this theory sucrose did abrogate the internalisation of CD71 in HeLa cells suggesting clathrin-dependent machinery was inhibited (Fig 3.10). However changes in cell morphology suggested other effects consistent with osmotic changes and inhibition of any membrane invagination. Cholera toxin binds to GM1 gangliosides which are lipid receptors associated with lipid rafts [52]. These lipid rafts are then internalised via clathrin-coated pits, caveolae (non-coated) or clathrin/caveolin-independent mechanisms. As cholera toxin internalisation was also inhibited by sucrose and in keeping with previous findings it is suggested hypertonic blockade inhibits all major endocytic pathways (Fig 3.10). It was therefore insufficient for our purpose of targeting clathrin-dependent routes specifically. Intriguingly a proposed mechanism of action

of CTLA-4 is through disruption of lipid raft formation through association with GM1 and it is not implausible to also consider its role in endocytic regulation [61].

shRNA is a useful tool for blocking specific elements of a pathway of interest, and this was used to block the formation of clathrin-coated pits through knock down of the AP-2 complex. In CTLA4⁺ CHO cells transfection of shRNA resulted in a decrease in intracellular CTLA-4 when compared to controls, indicating that internalisation of surface receptor was repressed (Fig 3.12). This finding would suggest that the receptors major endocytic route is clathrin-mediated. However there was no statistical difference in CTLA-4 mean fluorescence intensity given the number of cells analysed and further refinement of the transfection procedure may yield a higher number of shRNA⁺ cells for analysis. To draw any inferences from this data it would be necessary confirm complete knock down of AP-2 by staining for CD-71 to ensure the clathrin pathway was fully inhibited. It can be noted that in a previous study assessing the completeness of AP-2 knock down by shRNA in HeLa cells the complex was still identified in clathrin-coated pits after shRNA transient transfection [62, 63]. In this study despite incomplete knock down internalisation of CD-71 was significantly reduced, but highlights the limitations of using interference RNA technologies.

In HeLa cells analysis of shRNA⁺ cells was hindered by the intensity of the RFP signal associated with transfected cells (Fig 3.13C). It is probable that the increased intensities of CTLA-4 staining observed in this experiment was a result of cross-emission of the different fluorophores, therefore no affirmative conclusion could be drawn from this data. This phenomenon could be improved by 1) transfecting less shRNA 2) selecting an alternative shRNA construct that expressed less readily. 3) electronic compensation by staining with each fluorochrome individually to correct for spectral bleed-through.

In both shRNA knock down experiments considerable morphological changes were noted in transfected cells (Fig 3.12A). Cells appeared clustered together and were often multinucleated. As clathrin-mediated vesicular transport is shown to be important in the late phases of mitosis [63] it was considered that AP-2 knock down caused a significant disruption to cytokinesis. However changes were also observed in cells transfected with control constructs. Therefore it is more likely these changes were a negative consequence of the transfection procedure, again emphasising the importance of further optimisation in this system.

In summary the endocytic behaviour of CTLA-4 was successfully compared to intracellular markers in a HeLa cell model and further repetition is required to draw more affirmative inferences on the acquired observations. Inhibition of clathrin-dependent endocytosis by shRNA knock down showed its potential for investigating the internalisation of CTLA-4, but at this stage could not support previous observations in our lab that the receptor can internalise via alternative endocytic pathways.



ANALYSIS OF THE ANTIGEN-SPECIFIC CYTOKINE RESPONSES OF CD4⁺ T-LYMPHOCYTES

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requirements for the award of the Mres Biomedical
Research

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Abstract

The phenotype of a T-lymphocyte can be defined by its expression of secretory cytokines. However, characterising the different T-cell lineages is challenging given the numerous subsets and significant overlap in cytokine profiles. Additionally the stability of a T-cell once fully differentiated is currently under review, with identifiable functional plasticity between different subsets. I investigated whether the recall cytokine response of CD4⁺ T-cells is influenced by the nature of the antigenic stimuli presented. PBMC were stimulated *ex vivo* with bacterial (SEB, PPD, Tetanus Toxoid), viral (EBV) and fungal antigens (yeast). IFN γ , TNF α , IL-17, IL-21 and IL-10 were stained intracellularly and expression analysed by flow cytometry. Results identified stimulus specific IFN γ , TNF α and IL-17 responses in the CD4⁺ CD45RO⁺ memory population for all antigens tested; however no detectable IL-21 and IL-10 secretion was established. There was variability in the ratio of IL-17⁺ : IFN γ ⁺ expression between different antigens indicating a potential Th₁₇ or Th₁ bias respectively. The ratio of TNF α ⁺:IFN γ ⁺ expressing cells was also variable, highlighting the potential significance of a TNF α ⁺IFN γ ⁻ CD4⁺ population. Low number of responsive cells prevented comprehensive analysis of double positive cytokine secreting cells. However the proportion of IFN γ ⁺ cells also TNF α ⁺ following PPD stimulation was notably but not significantly higher than the positive control (SEB), whereas the IL-17⁺IFN γ ⁺ proportions were similar. This study suggests but does not confirm that different antigenic stimuli cause an enrichment of differential T-cell subsets. Such findings are highly applicable to multiple disciplines involving cell-mediated immunity.

1.0 Introduction

1.1 T-cell response to the local cytokine milieu drives differentiation and defines subset populations.

T-lymphocytes have a central role in cell-mediated immunity and memory responses to previously encountered pathogens. Through selection processes in the thymus they are broadly divided into CD4 or CD8 subsets, dependent on their MHC-class restriction. Whereas CD8⁺ cells predominantly have a cytotoxic function and are involved in the elimination of virally infected or malignant cells, CD4⁺ are termed 'T helper cells' (Th), and co-ordinated the type and severity of a recall response to a particular pathogen (reviewed in [64]). In order to acquire effector function naïve CD4⁺ cells both proliferate and differentiate following stimulation of the TCR. This is initiated by cognate interaction between the TCR and specific antigenic peptide bound to major histocompatibility complex (MHC) (expressed by antigen presenting cells) in conjunction with various co-stimulatory signals [65]. Cytokines are small immunomodulatory signalling molecules involved in cell-cell communication and directing a cells response to a particular environment. The recognition of these molecules by cytokine receptors expressed by T-cells initiates an intracellular signalling cascade that modulates the expression of particular target genes. This is achieved through changes in transcription factor activity, thus a cells cytokine profile is also often co-associated with specific transcriptional regulators [66].

Upon stimulation naïve T-cells secrete interleukin (IL)-2, which through a positive feedback loop induces the proliferation and the expansion of a cell responsive to a specific antigen. The expanded CD4⁺ population then differentiates into subpopulations dependent on the cytokines secreted by APC and other cells resident in the environment. The cytokine milieu itself is dependent on the nature of the pathogen through its interaction with pattern

recognition receptors, for example Toll-Like Receptors (TLR) [67]. In this way the CD4+ lineage is polarised towards the subset most effectively capable of dealing with the particular pathogenic agent. Historically there were two characterised subsets of CD4+ cells [68, 69]; Th₁ are induced in response to IL-12 & IFN γ and Th₂ which predominantly differentiate in response to IL-4 and IL-2 (reviewed in [70]). IL-12 produces a Th₁ phenotype through activity of the transcription factors STAT4 (signal transducer and activator of transcription-4) and IFN γ potentiates its own expression through Tbet induction by STAT1 [71]. In contrast IL-4 signalling is via STAT6 [72] and GATA3 [73] (trans-acting T-cell specific transcription factor-3) activity. Th₁ cells are predominantly characterised by their secretion of interferon- γ (IFN γ) and a notable secretion of tumour necrosis factor- α (TNF α). Their function is associated with the eradication of intracellular pathogens such as viruses [74] and *Mycobacterium* species [75, 76]. Amongst many properties IFN γ is a potent inhibitor of viral replication and increases macrophage and NK cell activity whereas TNF α is an important systemic pro-inflammatory mediator. Th₂ are defined by their expression of IL-4, IL-5 and IL-13, and promote a more humoral response through stimulation of B-cell activation, proliferation and antibody class switching [64] .

In this Th₁/Th₂ model the polarisation towards a particular phenotype is potentiated through cytokines of one branch inhibiting the actions of another, for example, IL-4 induced GATA3 expression transiently inhibits IFN γ production; thus blocking Th₁ differentiation [77] . However, significant imbalance has been shown to be potentially pathogenic, with a Th₂ strong bias associated with increased susceptibility to both infection with intracellular pathogens and inflammatory allergic conditions. The latter is partially through Th₂ mediated increase in antibody class switching to IgE and eosinophil activity [78]. In children with allergic asthma the exposure to Th₁ polarising antigens such as bacterial endotoxins has been shown to be protective, which demonstrates the importance of exposure to environmental

antigens in immune regulation. Although an attractive model in reality this Th₁/Th₂ theory is now known to be over-simplistic given the identification of multiple other subsets of Th cell, whose phenotype are also characterised by distinctive cytokine and transcription factor profiles. Examples of these include T regulatory cells (T_{reg}) defined by expression of the transcription factor FOXP3 and CD25^(hi)CTLA-4^(hi) surface markers (reviewed in [6]), and the recently identified pro-inflammatory Th₁₇ subset [79, 80].

Th₁₇ differentiate through a pathway independent of Th₁ and Th₂, with IL-6 [81] and TGFβ shown to be key determinants of the lineage [80, 82]. IL-23 is also associated, and as it is also secreted by Th₁₇ may be additionally involved in maintaining the stability of the phenotype. This phenotype is predominantly characterised by the expression of the IL-17, IL-21 and IL-23 through activation of the transcription factor RORC (RORγt in mice) [83]. Secretion of IL-17 (predominantly IL17A and IL-17F) induces the secretion of other pro-inflammatory mediators and chemotactic agents which target numerous cell types. Th₁₇ populations have shown to be responsive to extracellular pathogens and are shown to have particular importance in maintaining mucosal immunity [84, 85]. This subset are also notably responsive to fungal antigens, with clonal expansions of the populations been shown upon stimulation with *Candida albicans* [86] and other strains of yeast [87]. In a model of oropharyngeal candidiasis mice deficient in Th₁₇ but not Th₁ were shown to have an increased pathological susceptibility to opportunistic candida infection, thus demonstrating the subsets protective mucosal function [88, 89]. However, Th₁₇ are also associated with inflammatory pathologies at these sites, and the neutrophil infiltration, mucosal inflammation and chronic airway remodelling observed in asthmatic patients has been partially attributed to Th₁₇ activity [90, 91]. Additionally several autoimmune and inflammatory disorders were previously characterised through a dominant Th₁ response, as blocking IL-12 ameliorates disease. However, IL-12 shares the p40 subunit with IL-23 and it was later found blocking IL-23 and

not IL-12 was required to abrogate disease in experimental models, thus linking Th₁₇ to autoimmunity [92]. Knock out studies in mice have reported increased self-reactive Th₁₇ activity in models of arthritis [93], inflammatory bowel diseases and experimental autoimmune encephalomyelitis [92]. Additionally in humans and increased peripheral blood Th₁₇ frequency has been associated with both rheumatoid arthritis [94] and Chron's disease [95].

1.2 Functional plasticity of CD4⁺ T-cell lineages

Precisely identifying a cell phenotype is increasingly complex given the plethora of known cytokines and considerable overlap between the more recently identified subsets. For example both Th₁ and Th₂ have been shown to secrete the anti-inflammatory cytokine IL-10 in addition to T_{reg}. In addition to this historically it was thought that differentiation of the CD4⁺ lineage was permanent, and there was no re-differentiation or switching of phenotypes between each subset. However the existence of subsets expressing an atypical cytokine profile of a combination of each subtype is increasingly being reported [80] (as shown in Figure 1.1). A population of cells expressing both IL-17 and IFN γ have recently been identified, and these cells express both Tbet and RORC transcription factors in mouse models. The question then arises, are these cells Th₁ cells that have switched on IL-17 expression, or Th₁₇ cells which have switched on IFN γ expression? In light of current understanding it appears these cells are of the Th₁₇ lineage and also secrete IFN γ , and it has been shown that this IFN γ production by Th₁₇ cells is associated with various pathologies, such as the inflammatory bowel disorder in Chron's disease. The stability of each lineage has therefore come under particular scrutiny. It is yet to be demonstrated whether Th₁ cells can be converted into Th₂ or Th₁₇ cells *ex vivo*, but evidence suggests that both Th₂ or Th₁₇ can be converted towards a Th₁ phenotype under the IFN γ –rich polarising conditions [96]. In addition a population of IL-4/IL-17 dual secreting

cells have also been shown to be enriched in the lungs of chronic asthmatics, suggesting a role for Th₂/Th₁₇ hybrids in the inflammatory disorder [96]. Due to this apparent plasticity between T-cell lineages it is therefore extremely important to identify the initiator of such atypical gene expression, as the transcriptional regulators of such processes are yet to be elucidated. As a response to a specific pathogen can be polarised towards a specific T-cell lineage, this study aims to investigate whether recall responses to different pathogens can identify expansions of these novel subsets in an antigen-specific manner; thus demonstrating a pathogen-associated polarisation towards this differential gene expression. Therefore the frequency of these atypical dual cytokine secreting cells responsive to various bacterial, fungal and viral antigens will be investigated. Such research can potentially be applied to reverse phenotypes associated with disease, or drive differentiation of therapeutic benefit. The latter is analogous to processes within vaccine design, where agents such as novel adjuvants are being investigated to polarise a response to give the most effective long-lasting immunity to a particular pathogen. The significance of IL-17 responses in vaccine induced immunity have been investigated in animal models (reviewed in [97]). Adjuvants which provoke strong Th₁₇ based immunity (such as cholera toxin) have been administered in conjunction with established vaccines to enhance the activity of this lineage to improve vaccine performance.

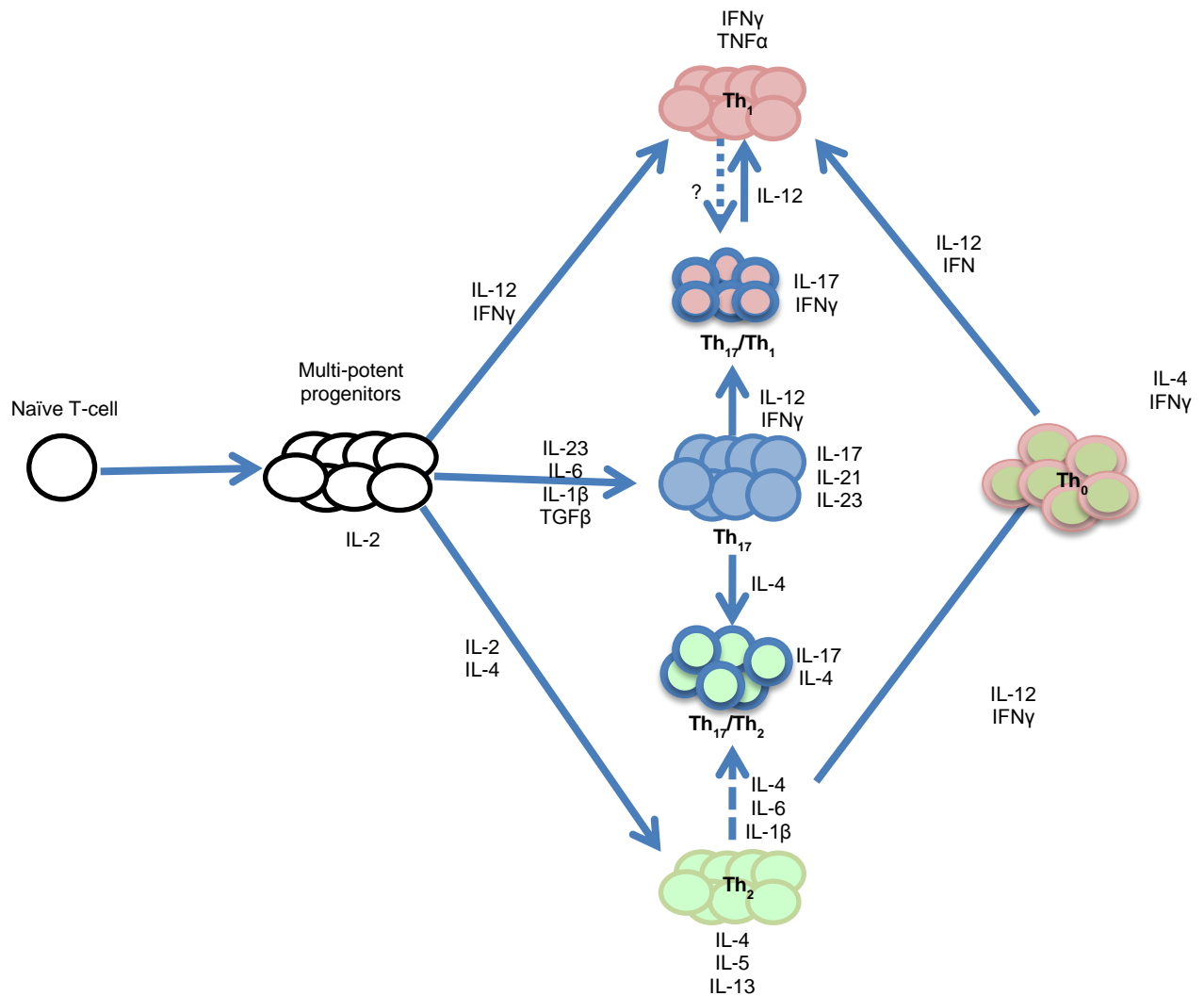


Figure 1.1 Differentiation and plasticity of CD4⁺ Th₁, Th₂ and Th₁₇ cell lineages. Naïve T-cells proliferate to a pool of multi-potent progenitors through IL-2 expression. The cytokine milieu polarises differentiation towards a Th₁, Th₂ or Th₁₇ phenotype; each defined by their own specific cytokine profile. Th₁₇ can induce expression of Th₁ and Th₂ cytokines to form dual secreting populations expressing both classically-associated transcription factors. Th₁₇ have been shown to revert to a fully Th₁ phenotype secreting IFN γ alone. Some evidence suggests Th₂ cells revert to a Th₁ phenotype with persistent IFN γ stimulation (image adapted from [98]).

1.3 Phenotypic analysis of infrequent cell populations

As during a typical immune response only 0.001-0.0001% of T-cells are responsive to a specific antigen, the measuring of such small cell populations is challenging. Given that Th₁₇ cells represent such a small percentage of the T-cell population (and dual-secreting cells even less) this poses even further difficulties. ELISpot (enzyme-linked immunosorbent spot [99, 100]) is a variation of the ELISA technique commonly used to characterize cells by the nature of their cytokine secretion (Figure 1.2). ELISpot is extremely sensitive, and can measure cytokine secretion at the level of individual cells. Cytokines secreted by cells stimulated *ex vivo* are captured extracellularly by specific antibodies coated to a plate. Binding of a second cytokine-specific reporter antibody (for example attached to a fluorochrome) produces a series of spots upon the plate that is proportional to the level of cytokine secretion of each responsive cell in the vicinity. However, the precise identification of the particular cells producing cytokine is limited due to the restrictions on multi-parameter staining for associated phenotypic markers. Additionally the number of cytokines that can be measured for particular cell is limited, making accurate phenotyping increasingly problematic. Therefore, staining for cytokine intracellularly (by blocking their secretion) and performing analysis by flow cytometry is an attractive alternative for phenotyping antigen-specific responses of T-cells [101]. Using this technique multiple parameters can be measured simultaneously, so a fuller cytokine profile of individual cells can be established. In addition, cells can be accurately phenotyped by staining for identifiable cell surface markers. In this investigation flow cytometry will be used to measure the antigen-specific cytokine responses of CD4⁺ T-cells in peripheral blood. This will be used to identify and characterize any antigen-induced expansion of cell populations expressing an atypical cytokine profile, for example cells secreting both IL-17 and IFN γ .

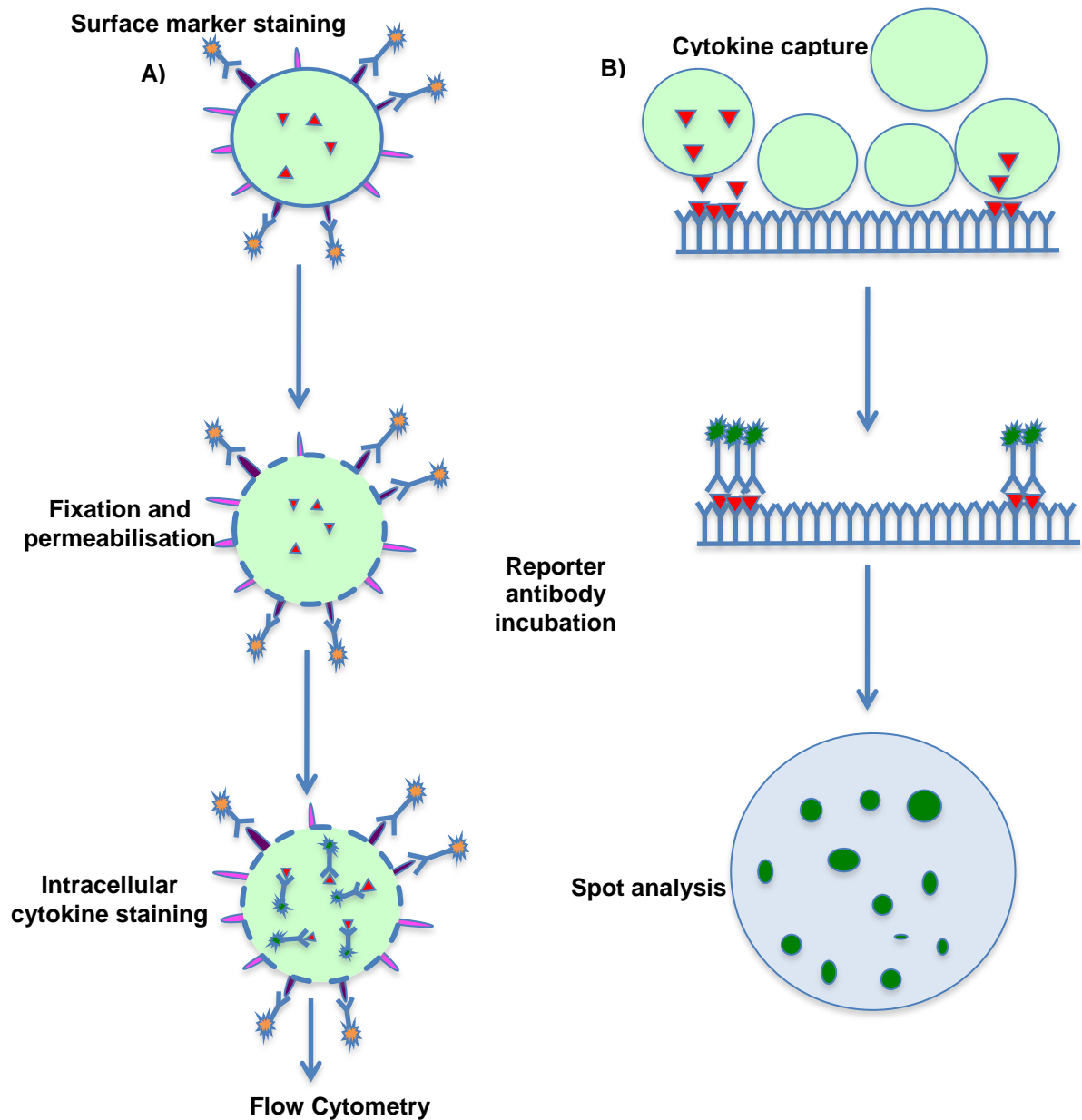


Fig 1.2 Cytokine detection by flow cytometry and ELISpot. A) Intracellular cytokine staining. Cells are stimulated and treated with a reagent to block exocytosis and trap expressed cytokines within the cell. They are then phenotyped by staining for associated surface markers. Fixing and permeabilisation allows antibody access to intracellular cytokines. These are then stained with using different fluorophores and analysed by flow cytometry. B) Extracellular cytokine staining. Cytokine-specific antibody is coated on a polyvinylidene fluoride plate and non-specific binding blocked with serum. Cells and stimulant are incubated on the plate and cytokine secreted is captured by antibody in the immediate vicinity. Following washing the plate is incubated with a second antigen specific antibody conjugated to a reporter (fluorophore or biotin-streptavidin system). Plates are analysed with each spot representing cytokine secreted from an individual cell.

1.4 Aims

Given the recently recognised plasticity between T-cell lineages [98] [102], it is important to further understanding of what environmental triggers influence each subsets gene expression. Additionally cells expressing an atypical cytokine profile are increasingly associated with autoimmune pathologies; thus demonstrating a functional significance of this expanded population in disease development. Such atypical cells are also candidate cellular biomarkers for particular diseases. For example, cells expressing both IFN γ and TNF α are shown to be elevated in active *Mycobacterium tuberculosis* infection compared to inactive disease, and can be used to distinguish between particular disease states [103]. This study aims to identify populations of CD4⁺ cells both IFN γ ⁺TNF α ⁺, and IFN γ ⁺IL-17⁺, and investigate whether they are expanded in recall response to various antigenic agents. The antigen-specific polarisation of a lineage towards such a dual-secreting phenotype will be demonstrated using flow cytometry, so multiple-phenotypic markers can be used to accurately define each cell line. As antigen-specific responses can polarise the lineage of a responsive cell on initial encounter, it may be anticipated that the phenotypes of cells responsive to a particular antigen may vary dependent upon the nature of the re-stimulation.

2.0 Materials and Methods

2.1.1 Culture medium

RPMI medium: RPMI (Roswell Park Memorial Institute) 1640 [Sigma-Aldrich Irvine, UK] supplemented with 1% GPS (2mM l-glutamine, 100U/ml penicillin, 100ug/ml streptomycin) [HyClone, Northumberland, UK] and 1% HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) [Sigma-Aldrich]

RPMI 10% HIFCS: RPMI 1640 medium supplemented with 10% Heat Inactivated Fetal Calf Serum (HIFCS) [Biosera, Ringmer, UK]

RPMI 5% HS: RPMI 1640 medium supplemented with 5% Heat Inactivated Human Serum (HIHS) [HS; HD Supplies, Aylesbury, UK]

2.1.2 Antigens

Abbreviation	Antigen
PMA/Ionomycin	Phorbol 12-myristate 13-acetate (PMA) Ionomycin (IONO) [both Sigma-Aldrich]
SEB	Staphylococcus Enterotoxin B [Sigma-Aldrich Irvine, UK]
PPD	Purified Protein Derivative: Heat inactivated supernatant from cultures of virulent <i>Mycobacterium tuberculosis</i> [Statens Serum Institut, Copenhagen, Denmark]
TetTox	Toxoid from <i>Clostridium tetani</i> [EMD Chemicals Group, Darmstadt, Germany].
EBV	Epstein Barr Virus: Antigenic peptides and lysates (kindly supplied by Prof Alan Rickinson group, Birmingham, UK).
YEAST	Heat-inactivated cells from overnight culture of <i>Cryptococcus neoformans</i> (capsular and Acapsular), and <i>Cryptococcus gattii</i> (kindly supplied by Dr Robin May group, Birmingham, UK).

Table 2.1 Antigens used to stimulate isolated peripheral blood mononuclear cells

Surface Markers	Company	Catalogue No.	Dilution
CD4 PE-Cy7	BD Biosciences	557852	1/20
CD8 V500	BD Biosciences	560774	1/20
CD45RO PETR	Beckman-Coulter	IM2712U	1/20
Cytokine			
IFN γ eFluor450	eBioscience	48-7319-42	1/80
TNF α PerCP-Cy5.5	eBioscience	42-7349-42	1/40
IL-17A FITC	eBioscience	53-7179-42	1/20
IL-21 PE	eBioscience	12-7029-82	1/40
IL-10 alexa fluor 647	eBioscience	51-7108-73	1/20

Table 2.2 Antibodies used to characterise cells by flow cytometry. PE (Phycoerythrin), Cyanine 7 (Cy7), Phycoerythrin Texas Red (PETR), Peridinin-chlorophyll protein (PerCP), Cyanine 5.5 (Cy5.5), Fluorescein isothiocyanate (FITC), Allophycocyanin (APC). All antibodies were raised in mouse the alexa fluor 647 conjugate which was raised in rat.

2.1.3 Additional Reagents

Brefeldin A [Sigma,UK]

Heparin Sodium [Leo Laboratories LTD, Bucks, UK]

Ficoll-Paque Plus: [GE Healthcare Bioscience, Amersham, UK]

PBS: Phosphate Buffered Saline (8g/l NaCl, 0.26/l KCl, 1.15g/l Na_2HPO_4 , 0.2g/ml KH_2PO_4); 1 PBS tablet added per 100ml distilled H_2O [Oxoid, Basinstoke,UK]

PBS 2%BSA: PBS, 2% Bovine Serum Albumin (BSA) [Sigma-Aldrich]

Cell Permeabilization Kit: [Caltag-Medsystems Limited, Bucks, UK]

Compensation beads: Anti-mouse and Anti-rat IgK/Negative control (FBS) compensation
Particle set [BD Biosciences]

2.2 Isolation of Peripheral Blood Mononuclear Cells (PBMC)

Blood was extracted from the median cubital vein of healthy donors into a syringe. This was transferred into a 50ml falcon tube containing heparin sodium at 5IU/ml blood. Blood was diluted at a 1:1 ratio with RPMI and carefully layered over 7ml of Ficoll-Paque Plus into a 25ml universal tube. Tubes were centrifuged at 400g with brake 0 for 30min. The individual layer was then extracted and transferred to a tube containing either RPMI 10% HIFCS or RPMI 5% HHS. Cells were washed twice at 300g before being counted using a haemocytometer. After a final wash cells were resuspended at 1×10^6 cells per 50 μ l of supplemented medium.

2.3 Antigenic Stimulation of Isolated PBMC

2.3.1 Stimulation with PMA/Ionomycin

1×10^6 PBMC were added to wells of a 96-well plate in 50 μ l of RPMI 10% HIFCS. Unstimulated wells were used as a negative control and made up to 200 μ l with medium. For stimulated wells PMA and Ionomycin were added both at the concentration of 750ng/ml and the wells made up to 200 μ l. 2 μ g/ml of Brefeldin A was added to both stimulated and unstimulated wells to prevent cytokine release. Cells were incubated at 37°C and 5% CO₂ for three hours.

2.3.2 Stimulation with T cell receptor specific antigens.

PBMC required a more prolonged incubation for stimulation by antigens requiring T cell receptor engagement. Therefore 1×10^6 PBMC were resuspended in 50 μ l of RPMI 5% HHS prevent any reactivity xenoantigens in HIFCS. Unstimulated wells were made up to 200 μ l with RPMI 5% HHS. The superantigen SEB was added to wells at 1 μ g/ml. The bacterial antigens PPD and TetTox were titrated between 0.01-50 μ l and 0.1-10 μ g/ml respectively. For viral stimulation a mixture of nine known EBV antigenic peptides was used at 20 μ g/ml. In addition a lysate of whole virus isolated and enriched from EBV infected cells was used at 10 μ g/ml [Virusys Corporation, US]. Yeast antigen was prepared by washing the cell cultures twice in PBS and counting using a haemocytometer. Between 1×10^3 - 1×10^7 cells were then added to the PBMC. A yeast lysate was also prepared by repeatedly freezing and thawing the cells every 30min between -80 $^{\circ}$ C and 37 $^{\circ}$ C. The lysate of 1×10^6 cells was then added per well. All stimulated wells were made up to 200 μ l with RPMI 5% HHS and incubated for 16 hours at 37 $^{\circ}$ C and 5% CO₂.

2.4 Staining of cell surface markers and intracellular cytokines

Cells were centrifuged in the 96-well plate for at 4 $^{\circ}$ C for 4min at 300g. The supernatant was flicked away and the cells were resuspended in 50 μ l of surface antibody in PBS 2%BSA. The plate left on ice in the dark for 15min. The cells were washed by addition of 150 μ l of PBS 2%BSA and the centrifugation and supernatant removal repeated. Cells were then fixed for 15min in the dark at room temperature by adding 50 μ l of Reagent A from the Catlag cell permeabilisation kit. The wash step was repeated and cytokine antibodies added in 50 μ l of permeabilisation Reagent B. The plate was incubated at room temperature in the dark for 15min. After a final washing the cells were resuspended in 100 μ l of PBS 2%BSA and stored

at -2°C in preparation for flow cytometry. Prior to analysis cells were resuspended in a total of 300µl.

2.5 Flow cytometry and statistical analysis

Cells were analysed for their surface marker and cytokine profiles on a Dako-Cyan flow cytometer using Summit v4.3 software. Compensation beads stained with each conjugated antibody individually were used to correct for spectral overlap. Positive cytokine signal was based above a threshold set using unstimulated cells. Due to the low cell numbers the absolute cell counts were taken and expressed as a percentage to two decimal places to improve accuracy. Statistical analysis was performed using GraphPad Prism 5. A non-parametric Wilcoxon matched pairs signed rank test was used to analyse differences in cytokine expression between the memory (CD45RO⁺) and naive (CD45RO⁻) lymphocyte populations. Friedman analysis of variance was used to compare the number of IL-17⁺ to IFNγ⁺ and the number of TNFα⁺ to IFNγ⁺ secreting cells for each antigenic stimulation.

3.0 Results

3.1 Optimisation of stimulation assay for detection of antigen specific responses

It was important to optimise the stimulation assay to maximise detection of the very small T cell populations responsive to a specific antigen. It was also essential to minimise the background or non-specific signals to discern true cytokine expression. Following PBMC isolation and analysis by flow cytometry the lymphocyte population was gated on to reduce contamination by monocytes and other cytokine producing cells (Fig 3.1A). The pulse width signal produced as cells pass through the flow cytometer was used to exclude cells stuck together as the cytokine profiles of these doublets are unrepresentative of individual cells. CD4 surface receptor was used to identify T helper subsets and CD45RO was used as a marker for memory antigen experienced cells (Fig 3.1B and Fig 3.1C). Any positive cytokine signal produced by naïve CD45RO⁻ lymphocytes was used as a measure of non-antigen specific background stimulation; this population was assumed to be quiescent and negative for the expression of IFN γ , IL-17, TNF α , IL-21 and IL-10.

Initially 1×10^6 PBMC were stimulated per condition, but this number proved insufficient to achieving responses distinguishable from background for such small T-cell populations. We therefore investigated the effect of increasing the number of cells stimulated per well of a 24-well microtiter plate and analysed the IFN γ secreting population (Fig 3.2). Incubation with the superantigen SEB induced a population of IFN γ secreting CD4⁺CD45RO⁺ cells significantly more numerous than seen for unstimulated PBMC (Fig 3.2B-D). Increasing the number of stimulated PBMC did not significantly affect the percentage of IFN γ CD45RO⁺ cells, but did reduce the signal in the naïve populations (Fig 3.2E). The signal to background ratio was therefore more favourable at higher cell numbers and the protocol was adapted to allow for

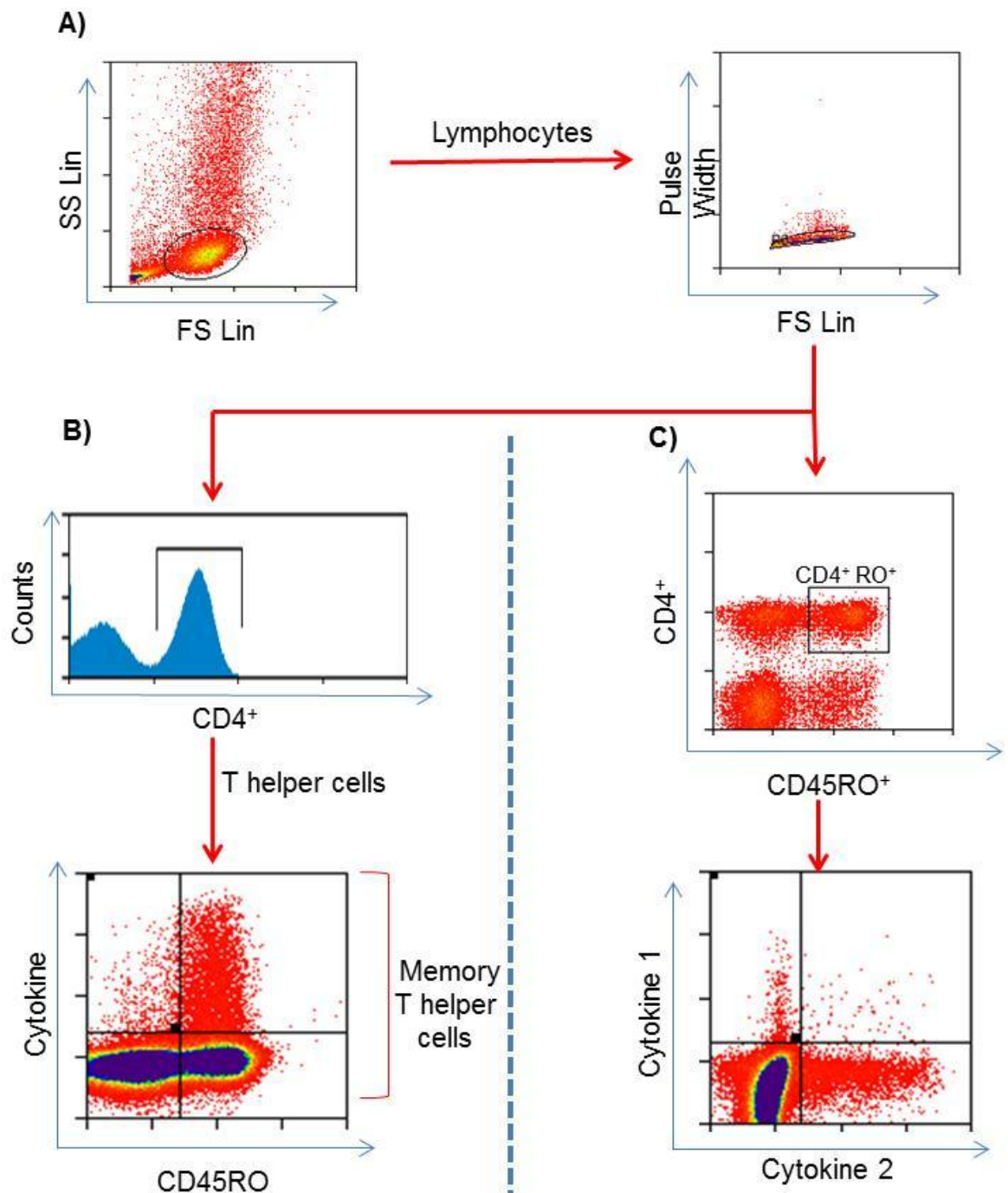


Figure 3.1 Gating strategy for cytokine profiling of peripheral blood mononuclear cells (PBMC) by flow cytometry A) Gating on the lymphocyte population of isolated PBMC using their forward and side scatter profile. Pulse width was used as a doublet discriminator to exclude cells stuck together. B) Gating strategy to investigate cytokine secretion by the memory T-helper cell population in response to specific antigen. CD4 surface receptor was used as a T-helper cell marker and CD45RO was used to show a memory phenotype. Cytokine producing memory cells appear in the top right quadrant. C) Gating strategy to investigate dual cytokine secretion. The memory T-helper cell population was gated on and two cytokines plotted against each other to identify cells secreting both (top right quadrant).

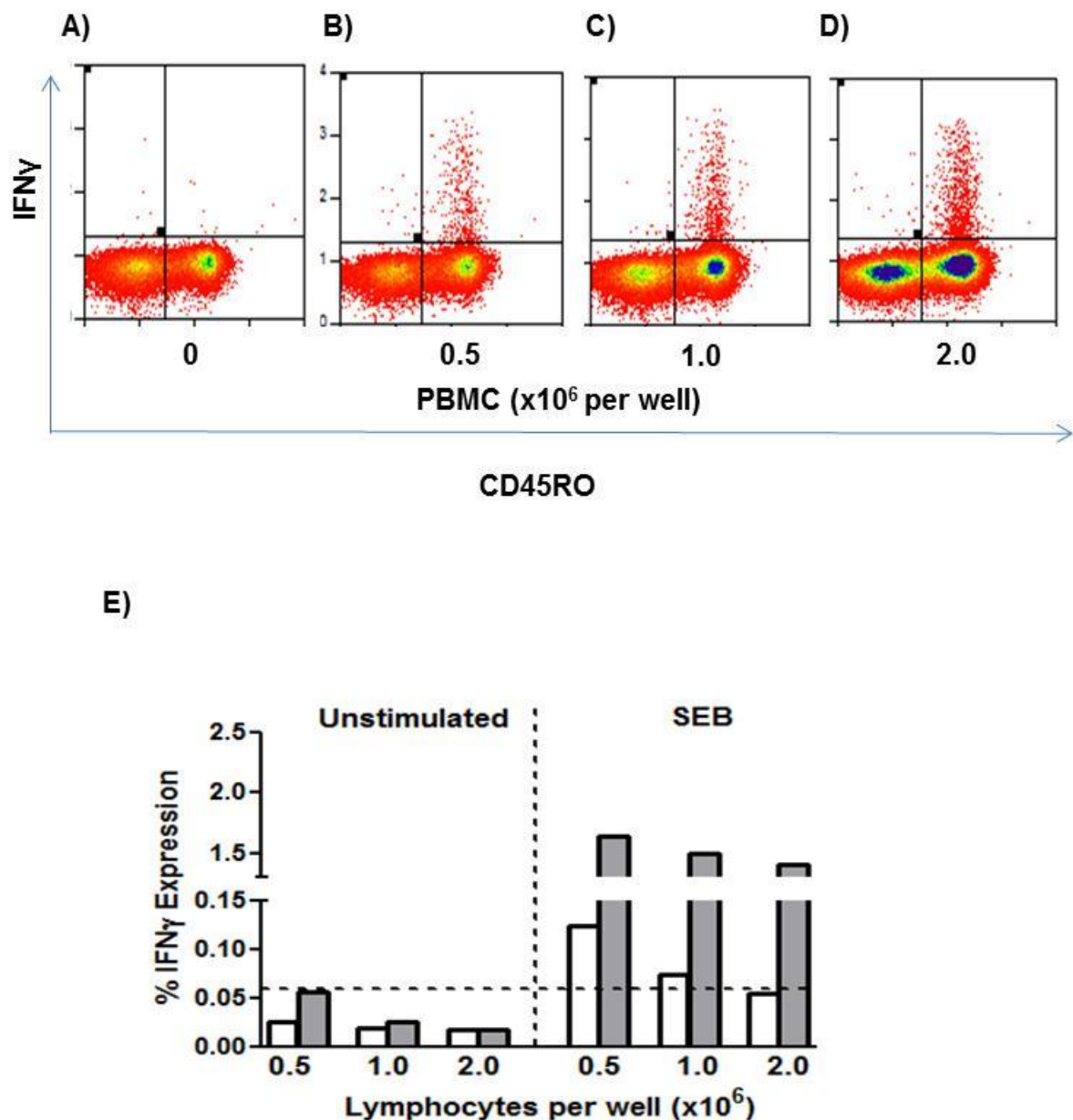


Figure 3.2. Optimisation of stimulation assay through increased cell numbers. Increasing numbers of PBMC were stimulated with Staphylococcus Enterotoxin B (SEB) for 16h. The percentage of IFN γ producing CD4⁺ CD45RO⁺ T-helper cells was used as a measure of stimulation. Percentage of IFN γ producing naïve (CD45RO⁻) CD4⁺ cells was used to measure background and non-antigen specific stimulation. A) IFN γ production by 1 $\times 10^6$ unstimulated lymphocytes. B-D) IFN γ production by 0.5-2 $\times 10^6$ lymphocytes in response to SEB stimulation. E) Percentage of IFN γ producing naïve and memory lymphocytes following stimulation with SEB. Data is representative of n=1 experiment.

the stimulation of more cells. Multiple wells were also stimulated and pooled prior to flow cytometry to further increase cell numbers.

It was necessary to titrate the two chosen bacterial antigens (PPD and tetanus toxoid) to identify the optimal concentration to detect antigen specific responses. The pro-inflammatory cytokines IFN γ and IL-17 were used as a measure of successful stimulation. Tenfold dilutions of PPD induced IFN γ and IL-17 expression undistinguishable from background below the concentration of 1 μ g/ml (Fig 3.3D and E). However there was a dose dependent increase in cytokine production by the CD45RO⁺ population with greatest percentage expressing both cytokines at 10 μ g/ml (Fig 3.3C-E) which was the concentration used in subsequent experiments. Stimulation at 50 μ g/ml abrogated cytokine expression although it was not determined whether this was artifactual as the experiment was not repeated.

The number of IFN γ and IL-17 responsive cells following tetanus toxoid stimulation was much less than seen with PPD stimulation. However ten-fold dilutions showed the optimal concentration to be 10 μ g/ml for IFN γ secretion with little influence in IL-17 response (Fig 3.4). This concentration was considered adequate and no concentrations greater than this were tested due to the availability of the toxoid.

3.2 Cytokine profiling of CD4⁺ T-helper lymphocytes following ex vivo stimulation

A panel of monoclonal antibodies fluorescently labelled to different fluorochromes was then used to label IL-21, IL-10 and TNF α in addition to IFN γ and IL-17. This would identify variation in cells individual cytokine profile when stimulated with various antigens. When stimulated with the SEB the memory CD4⁺ cells produced significantly more of all five cytokines than the naïve CD4⁺ cells (Fig 3.5B and Fig 3.6A). This confirmed the limited ability of non-antigen experienced cells to secrete cytokine. As expected given the polyclonal nature of SEB activation the percentage of cytokine producing cells was significantly greater

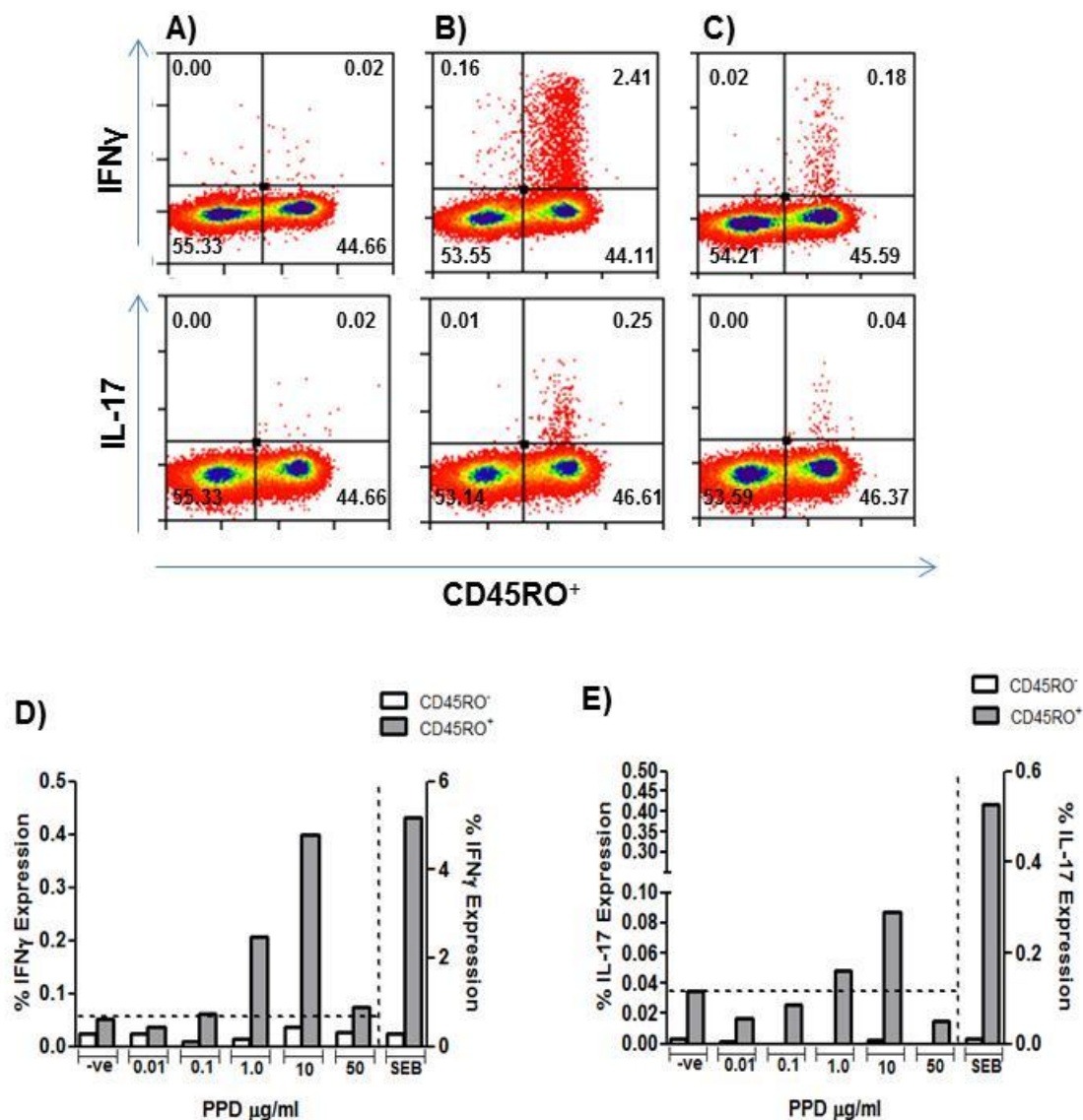


Figure 3.3 Dose dependent cytokine response of CD4 $^{+}$ T helper lymphocytes upon ex vivo stimulation with Purified Protein Derivative (PPD). Isolated PBMC were stimulated with increasing concentrations of PPD for 16h and analysed for IFN γ and IL-17 secretion by flow cytometry. Lymphocytes are gated on the CD4 $^{+}$ T-helper cell population and a memory phenotype (CD45RO $^{+}$) plotted against cytokine expression A) Unstimulated lymphocytes were analysed as a negative control to quantify background IFN γ and IL-17. B) Staphylococcus Enterotoxin B. C) Antigen specific response to 10 μ g/ml PPD showing a population of IFN γ and IL-17 producing memory T-helper cells (top right quadrants). D) Titration of PPD antigen using the percentage of IFN γ producing memory (CD45RO $^{+}$) lymphocytes as a measure of stimulation. Percentage of IFN γ producing naïve (CD45RO $^{-}$) CD4 $^{+}$ cells were used to measure background and non-antigen specific stimulation. E) The percentage of IL-17 secreting cells with increased dose of PPD antigen. Horizontal lines represent the level of background signal. Data is representative of n=1 experiment.

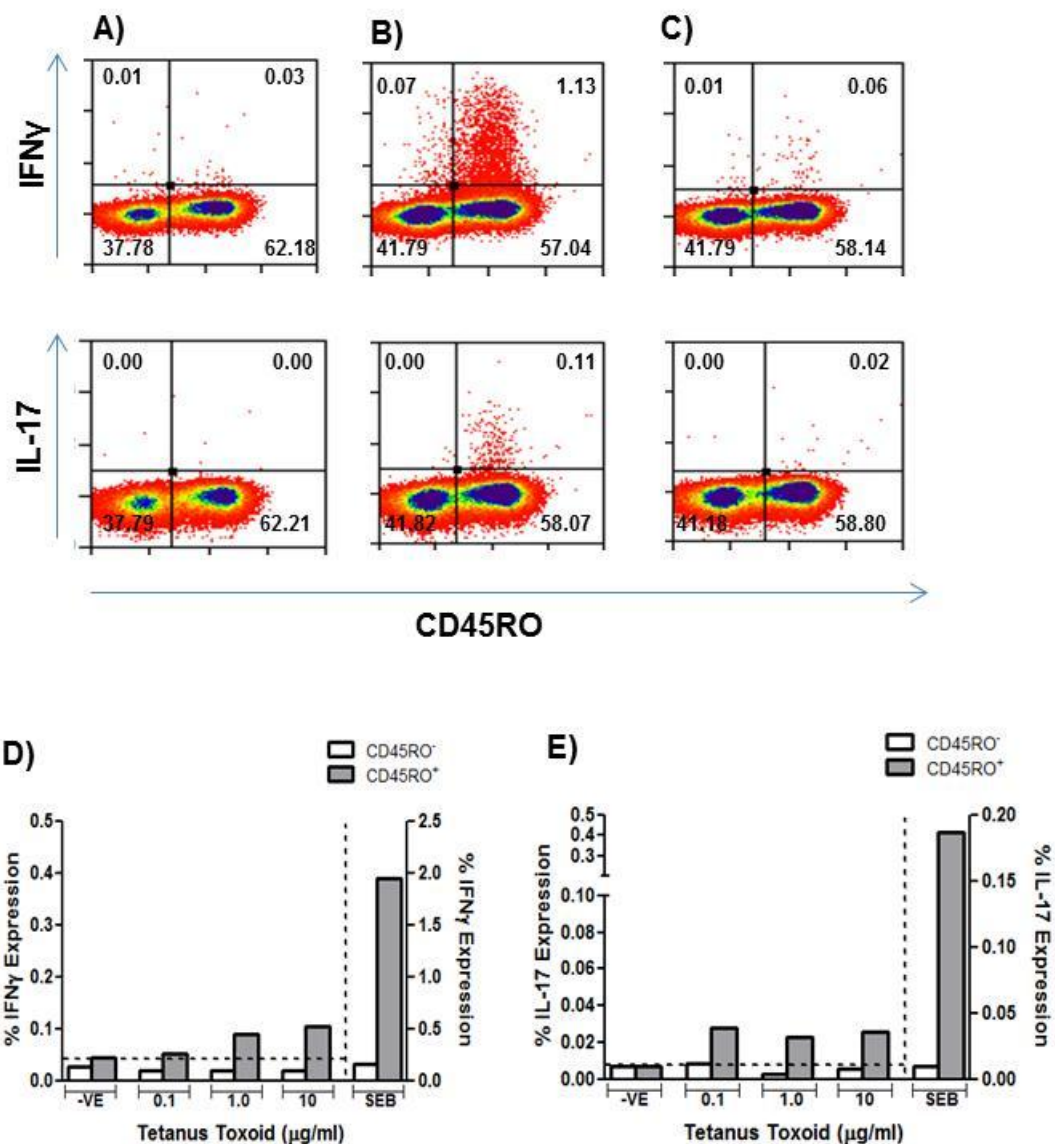


Figure 3.4 Dose dependent cytokine response of CD4⁺ T helper lymphocytes upon ex vivo stimulation with tetanus toxoid. Isolated PBMC were stimulated with increasing concentrations of tetanus toxoid for 16h and analysed for IFN γ and IL-17 secretion by flow cytometry. Lymphocytes are gated on the CD4⁺ T-helper cell population and memory phenotype (CD45RO⁺) plotted against cytokine expression A) Unstimulated lymphocytes were analysed as a negative control to quantify background IFN γ and IL-17. B) Staphylococcus Enterotoxin B was used as a positive control to confirm successful stimulation. C) Antigen specific response to 10 μ g/ml of tetanus toxoid showing a small population of IFN γ and IL-17 producing memory T-helper cells (top right quadrants). D) Titration of tetanus toxoid antigen using the percentage of IFN γ producing memory (CD45RO⁺) lymphocytes as a measure of stimulation. Percentage of IFN γ producing naïve (CD45RO⁻) CD4⁺ cells were used to measure background and non-antigen specific stimulation. E) The percentage of IL-17 secreting cells with increased dose of tetanus toxoid. Horizontal lines represent the level of background signal. Data is representative of n=1 experiment.

than the other antigens tested and this was particularly notable for IFN γ expression. This was then used as a positive control in further experiments and compared with the antigen specific stimulations. Antigen induced IFN γ , IL-17 and TNF α responses significantly different from naïve CD4 $^{+}$ lymphocytes were detected in memory CD4 $^{+}$ CD45RO $^{+}$ populations following stimulation with PPD, tetanus toxoid and EBV lysates (Fig 3.6 B,C,D). Of these antigens tetanus toxoid stimulated the least proportion of cells to secrete all three cytokines, whereas PPD induced cytokine secretion from the greatest proportion of memory cells (Fig 3.5C, 3.5E, 3.6B, 3.6D). PPD and EBV lysate caused a similar very small percentage of memory cells to secrete IL-17 but PPD provoked a greater production of TNF α producers than EBV stimulation (Fig 3.5C-D,3.6B-C). Yeast lysate stimulation caused the largest increase in IFN γ expression (Fig 3.5F,3.6E), however this was in both naïve and memory s and there was repeatedly a smearing of low level IFN γ signal on the borderline of the negative vs positive gate (Fig 3.5F). However the IL-17 and TNF α response for memory cells was significantly different from naïve following yeast lysate stimulation.

Excluding SEB there was no discernible difference in IL-21 and IL-10 secretion between naïve and memory CD4 $^{+}$ cells for all four antigens tested. For both cytokines the percentage of positive cells was extremely low in both populations.

3.3 Polarisation of CD4 $^{+}$ CD45RO $^{+}$ cytokine responses by different antigenic stimuli

It was then addressed whether different antigens polarised memory T-helpers cells to produce a different profile of cytokines upon stimulation. This was achieved by dividing the number of cells which expressed IL-17 or TNF α and dividing it by the number expressing IFN γ to give a cytokine¹:cytokine² ratio. For all antigens IFN γ was the cytokine produced by the largest number of cells. Compared to SEB it was found cells stimulated with PPD, EBV, Tetanus toxoid and yeast had a higher IL-17 : IFN γ ratio, with tetanus toxoid inducing a

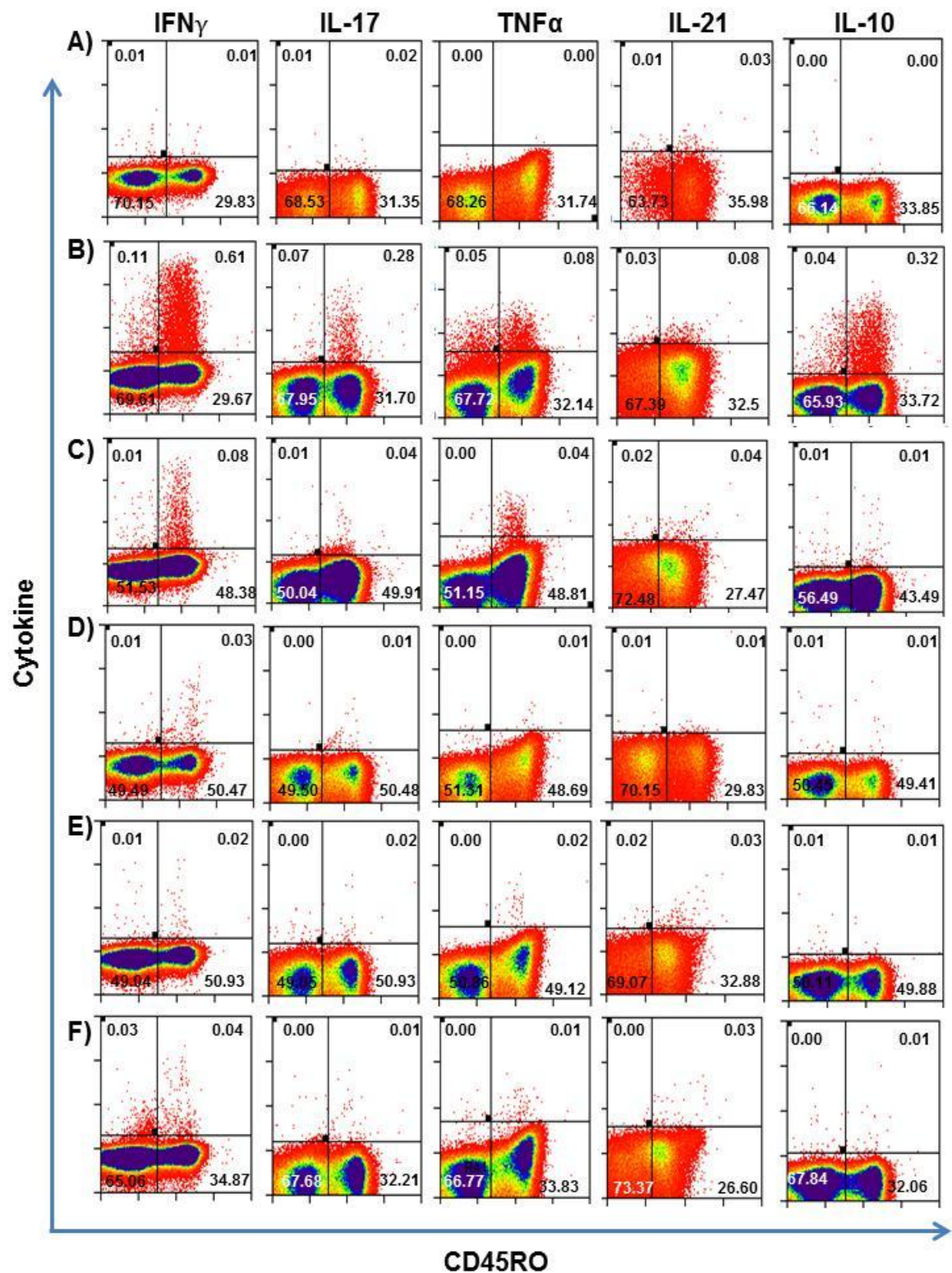


Figure 3.5 Antigen specific cytokine responses of CD4⁺ T helper lymphocytes following ex vivo stimulation. Isolated PBMC were stimulated with five antigens (B-F) and profiled for the expression of five cytokines by flow cytometry (left to right). Numbers represent the percentage of lymphocytes per quadrant. A) Unstimulated lymphocytes B) Staphylococcus Enterotoxin B C) Protein Derived Derivative (PPD) D) Epstein-Barr Virus E) Tetanus toxoid F) Yeast lysates. Images are representative of $0.5\text{--}0.75 \times 10^6$ CD4⁺ lymphocytes in n=7 independent experiments

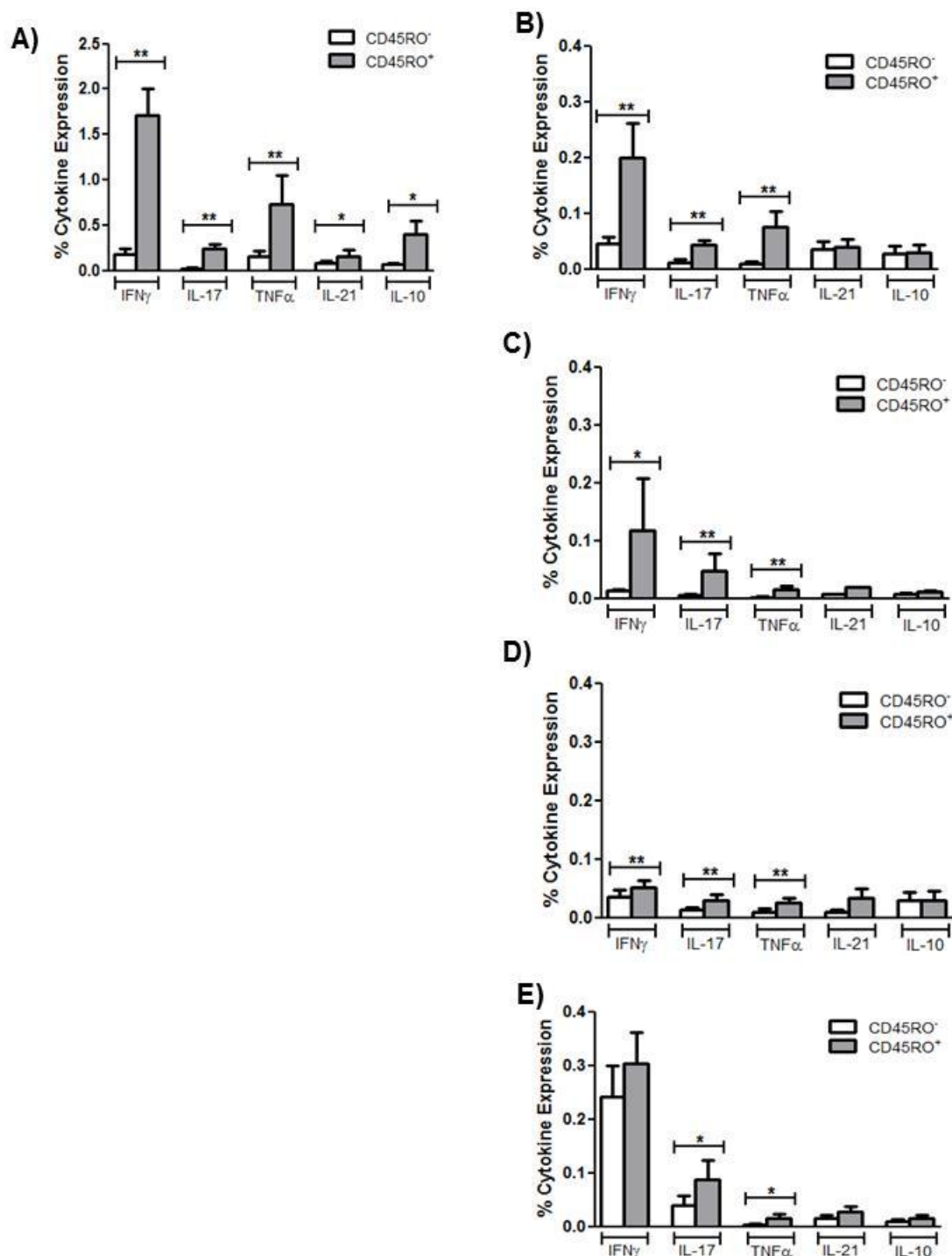


Figure 3.6 Memory specific CD4⁺ cytokine responses vary between different antigenic stimulations. Isolated PBMC were stimulated with five antigens (A-E) and profiled for the expression of five cytokines by flow cytometry. Data shows the percentage of memory (CD45RO⁺) and naïve (CD45RO⁻) cells expressing each cytokine A) Staphylococcus Enterotoxin B (SEB) B) Protein Derived Derivative (PPD) C) Epstein-Barr Virus (EBV) lysate D) Tetanus toxoid E) Yeast lysates. Wilcoxon matched pairs sign test showed *p<0.05 **p<0.01 indicating significant difference between naïve and memory populations. Data is representative of n=3-7 independent experiments.

statistically greater proportion of IL-17 expressing cells than SEB (Fig 3.7A, 3.7C). The low ratio seen with SEB indicates the response is dominated by IFN γ expression. There was also notable but not statistical differences in the IL-17 : IFN γ ratio between the different antigens. This may suggest polarisation toward Th₁₇ driven IL-17 secretion and away from Th1 driven IFN γ secretion in recall responses dependent on the nature of the original stimuli (Fig 3.7A, 3.7C).

There was also a difference between antigens in the proportion of TNF α secreting cells compared to IFN γ secretors. Tetanus toxoid and PPD stimulation produced the highest proportion of TNF α positive cell and the ratio of TNF α : IFN γ expressing cells was ~1:2 and ~1:2.5 for these antigens respectively. There was a statistical difference in ratio between these antigens and stimulation with yeast (characterised predominantly by IFN γ expression) which was the only significant difference between antigens. However EBV also did not seem to drive TNF α secretion from cells as much as PPD and tetanus toxoid and IFN γ producers were proportionally greater (Fig 3.7D). The EBV response was therefore characterised by an IL-17 bias and limited TNF α production, whereas tetanus toxin and PPD appeared to have a more mixed repertoire of cytokine production less polarised towards IFN γ production.

3.4 Dual cytokine secretion by stimulated CD4⁺CD45RO⁺ lymphocytes

The proportion of stimulated IFN γ ⁺ cells which co-expressed IL-17 or TNF α was calculated to identify differences in dual secretion under different stimulation conditions. As the population of double positive cells was so small SEB and PPD were the only antigens that simulated adequate cell numbers to analyse. The number of dual secretors was divided by the total number of cells expressing IFN γ . Under SEB stimulation ~10% of IFN γ expressing cells also expressed TNF α whereas ~30% secreted both cytokines when stimulated with PPD (Fig 3.7E), however this finding was not statistically significant at $p < 0.05$. The number of IFN γ ⁺

cells co-expressing IL-17 was comparable for SEB and PPD stimulations and represent a very small proportion of the IFN γ ⁺ (~3%). This very small population was the most difficult to discern from background staining.

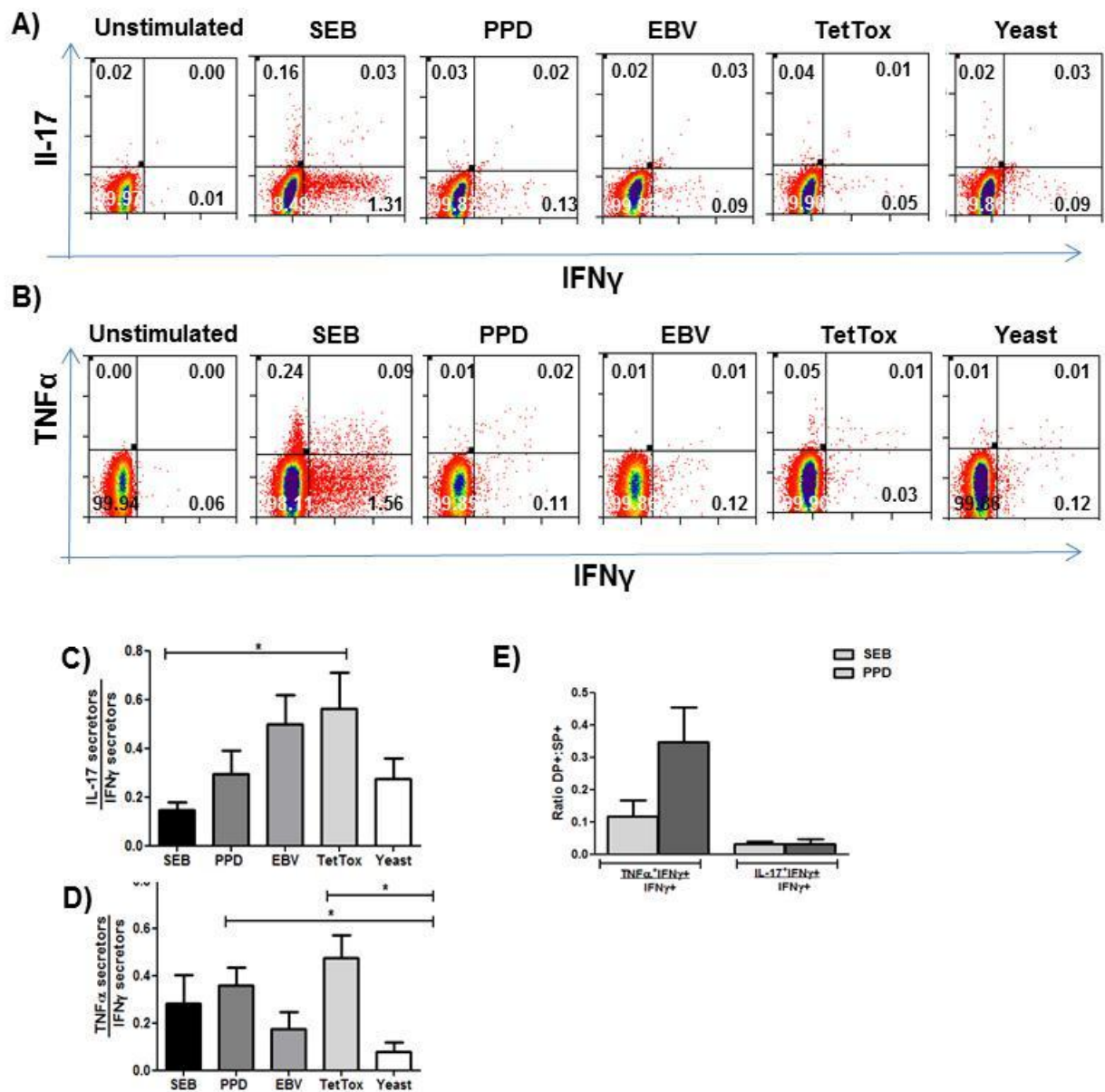


Figure 3.7 CD4⁺ CD45RO⁺ T helper cell cytokine profiles in response to specific antigenic stimulation. Isolated PBMC were stimulated with five antigens and the memory lymphocyte population analysed for the expression of IFN γ , IL-17 and TNF α by flow cytometry. A) Shows populations of IFN γ ⁺ and IL-17⁺ single cytokine secreting lymphocytes and a population of IFN γ ⁺ IL-17⁺ double secretors (top right quadrant) for each antigenic stimulation. B) Shows populations of IFN γ ⁺ and TNF α ⁺ single cytokine secreting lymphocytes and a population of IFN γ ⁺ TNF α ⁺ double secretors (top right quadrant). C) The ratio of IL-17 producing cells to cells producing IFN γ for each stimulation. D) The ratio of TNF α ⁺ cells to those producing IFN γ for each stimulation. *p<0.05 indicating significant difference between SEB and TetTox. E) The proportion of IFN γ producing cells which also co-express IL-17 or TNF α expressed as a ratio of double positive to single positive cells. SEB (Staphylococcus Enterotoxin B), PPD (Protein Derived Derivative), EBV (Epstein-Barr Virus lysate), TetTox (tetanus toxoid), Yeast (mixed yeast lysates). Data is representative of n=3-7 independent experiments. * P<0.05 where indicated.

4.0 Discussion

ELISpot is a sensitive method for analysing cytokine secretion at the level of individual cells and is commonly used to measure antigen-specific recall responses due to the minimal number of responsive lymphocytes. Therefore for such purposes it is reported that ELISpot techniques are favourable over flow cytometry analysis for low level detection [104]. However the latter is advantageous as each individual cytokine secreting cell can be precisely identified. Additionally more comprehensive cytokine profiling is permitted as multiple parameters per cell can be measured simultaneously, thus such work is represented in current published literature [105]. By optimising a flow cytometry protocol it was possible to measure differential expression of three out of five tested cytokines in response to ex vivo re-stimulation with four antigens. However the study has also highlighted the difficulties of measuring antigen specific responses, and suggested the method may not be appropriate for all antigenic stimuli. On evaluating findings the biological variability influencing T-cell responses was potentially a limitation given the number of experimental repeats, with the following being the most notable: 1) genetic immune variability 2) vaccination history 3) age 4) previous infection (symptomatic or asymptomatic) 5) geographical exposure to environmental antigens. For example following stimulation with PPD 0.55% of one individuals CD4⁺ cells showed an antigen specific IFN γ response, whereas only 0.09% responded in another participant. This demonstrates significant biological variability given the small cell numbers measured.

The CD4⁺ and CD8⁺ T lymphocyte populations were identified and analysis was focused on the CD4⁺ T helper subset, as these are central in orchestrating the nature of memory responses on subsequent antigenic challenges. A Th₁ phenotype was characterised by expression of the classically associated cytokine IFN γ and a Th₁₇ phenotype was characterised by IL-17

expression. The expression of transcription factors associated to a lineage in conjunction with cytokine profile can also add evidence to a lymphocytes true subset. In preliminary flow experiments it was found that nearly all $\text{IFN}\gamma^+$ cells co-expressed the archetypal Th_1 transcription factor Tbet, IL-5^+ cells co-expressed the classic Th_2 transcription factor GATA3 (<97% when compared to matched isotype controls) and IL-17^+ cells co-expressed the Th_{17} associated transcription factor RORC. Despite this such categorisation is not definitive and is generalised given the complex overlap in cytokine profiles with other T-cell lineages, for example the multiple T regulatory subsets. However in addition to $\text{IFN}\gamma$ and IL-17 the expression of proinflammatory cytokines $\text{TNF}\alpha$, IL-21 and anti-inflammatory IL-10 was also measured for a more complete perspective on CD4^+ responses.

The superantigen SEB was used as a positive control for successful stimulation and induced a significantly greater population of memory CD4^+ cells to secrete all five cytokines when compared to naive. Naïve T-cell secrete IL-2 and not inflammatory cytokines on TCR peptide-HLA ligation as the necessary genes are not expressed without prior antigenic exposure. SEB cross links the $\text{V}\beta$ chain of the T cell receptor to HLA molecules on antigen presenting cells resulting in polyclonal activation of both CD4^+ and CD8^+ T-cell [106]. This explains why the number of responsive T-cell is significantly greater than stimulation with more specific antigenic agents. However not all T-cell are SEB responsive as the antigen only binds specific variants of the $\text{V}\beta$ chain with $\text{V}\beta 8$ being the most notable [107]. An individual's T-cell response to SEB will therefore vary depending on their specific $\text{v}\beta$ repertoire. In keeping with the literature it was found SEB induced the most CD4^+ T-cell to produce $\text{IFN}\gamma$ in preference to other cytokines, suggesting a dominant Th_1 response. There was also significant amounts of $\text{TNF}\alpha$ production which the systemic inflammation caused by the enterotoxin is physiologically partially attributed to [108]. SEB also stimulated a population of memory CD4 cells to produce IL-17 suggesting the antigen activates the small

population of Th₁₇ memory cells. The ratio of IL-17⁺: IFN γ ⁺ was low as expected given the Th₁₇ frequency (under the assumption that the frequency of SEB binding V β chains is equal within both Th₁ and Th₁₇ subsets).

IL-21 secretion has recently been associated with Th₁₇ differentiation and effector function [109-111] . However SEB stimulation was the only circumstance in which detectable differences between naïve and memory populations for IL-21 could be distinguished (no correlation between IL-21 and IL-17 secretion was noted). With specific antigen stimulation this may have been because A) Given the minimal cell numbers and only <0.05% of memory CD4⁺ responding to IL-21 secretion this could not be distinguished from background. B) The quality of staining with the IL-21 antibody was not sufficient to distinguish between truly positive cells and non-specific binding. This scenario was also true for analysis of IL-10 secretion, and both cytokines were therefore excluded from further analysis of the four antigens. However the positive control did produce an IL-10 response which may be attributed the intensive stimulation by the superantigen as IL-10 is shown to be upregulated in response to excessive or chronic stimulation [112].

The CD4⁺ cytokine responses to the two bacterial stimuli tested were different in both degree and nature. PPD and tetanus toxoid were chosen to increase the probability of prior exposure given the routine childhood vaccination program in the UK. The fact some individuals in the cohort may not have been exposed to vaccine (or environment antigen in the case of PPD) has to be considered as no vaccination history was taken, which may make the average results under-representative.

PPD induced more cells to secrete IFN γ and TNF α than tetanus toxin, but the difference between IL-17 secretion was not statistically significant. The reason re-challenge with PPD may produce a stronger response than tetanus toxoid is immunisation against mycobacterium

tuberculosis is a live attenuated vaccine which evidence shows provides long lasting immunity. This may be through increased initial expansion of $CD4^+$ populations or prolonged antigenic stimulation by persistence of the attenuated strain [113]. Toxoid vaccinations do not provide lasting immunity and require ten yearly boosters during adulthood. There is evidence adult immunity to tetanus declines with age due to lack of uptake of necessary booster vaccines [114], and this may contribute to the average toxoid response being marginal even at the optimal concentration tested. Despite this there was a small notable difference in the ratio of $IL17^+ : IFN\gamma^+$ producing cells between the two antigens. The ratio on tetanus toxoid stimulation was elevated, perhaps suggesting either there is a greater clonal expansion of Th_{17} on vaccination, or those Th_{17} cells persist longer than the Th_1 population. $TNF\alpha^+ : IFN\gamma^+$ ratios were also varied yet it is difficult to speculate on both the phenotype of this population of cells which secretes $TNF\alpha$ without $IFN\gamma^+$ and the significance of the differential ratios given the limited cytokines analysed. However the significance of this $TNF\alpha^+ IFN\gamma^-$ population has recently been highlighted in context of *M. Tuberculosis* infection. A. Harari *et al* a significantly elevated population of $TNF\alpha^+ IFN\gamma^-$ cells in active compared to latent *M. Tuberculosis* infection. The sensitivity of this distinction was substantial enough that the $TNF\alpha^+ IFN\gamma^-$ population is candidate cellular biomarker for active tuberculosis.

To test polarisation of cytokine profiles in response to fungal antigens we used two strains of *Cryptococcus* found in soil (the opportunistic *Cryptococcus Neoformans* and *Cryptococcus Gatii* which infects immunocompetent hosts). It is reported cryptococcal strains are found world-wide [115], however significant exposure of the cohort could not be presumed given current epidemiological evidence. It is also reported the major epitope is a glucuronoxylomannan [116], a component of the polysaccharide capsule surrounding the organism. However conflicting reports suggest major epitopes are in fact hidden beneath the

capsule masking them from immune surveillance and making the coat a virulence factor in itself [117, 118]. Yet despite using an acapsular strain of the species stimulation with whole organisms proved ineffective in a preliminary experiment, so a mixed lysate was created to increase antigen availability. Lysate stimulation produced considerable low level IFN γ signal in both the naïve and memory CD4⁺ pools. This may be non-specific stimulation by the lysate or non-specific binding of the staining antibodies to components of the yeast cells in the prep. The latter is less likely given the same background was not seen with the IL-17 or TNF α antibodies and a significant number of memory T-cell were stimulated when compared to naïve. It is therefore possible there is some specific IFN γ signal masked by the considerable background. Given this IFN γ signal unreliability it is not possible to draw any inferences from the ratios with other cytokines as the value would be a considerable under representation. However it can be noted that the IL-17 signal was strong when compared to other antigens and may support evidence in the literature that Th₁₇ cells have a key role in mucosal immunity against fungal infections . It is also demonstrated that IL-17 and the associated IL-23 have a role in preventing opportunistic infections. In mice challenged with *Cryptococcus Neoformans* deficiency in both Th₁₇ and Th₁ is significantly more lethal than Th₁ deficiency alone. In further support impaired Th₁₇ derived immunity has been associated with pathogenic candidiasis through infection with *Candida Albicans*, another fungal opportunistic infection.

EBV is largely endemic with <80% of individuals latently infected. Much work has focused around the CD8⁺ cytotoxic response of the virus but little is known about the CD4⁺ responses, particularly in latency. Initially we used a combination of nine EBV-derived peptides for a specific HLA type and stimulated PBMC from a HLA matched donor. However this achieved little response and had the added constraint of HLA haplotyping every participant in the cohort. A whole EBV lysate was then tested and a significant number of responsive EBV specific memory CD4⁺ cells were identified, with ~0.1% responding with IFN γ expression.

This may be expected given the potent antiviral properties of the molecule and its importance in control of intracellular pathogens. This is in keeping with evidence that CD4⁺ response to Epstein-Barr Nuclear Antigen 1 (the major latency antigen) is strongly polarised towards a Th₁ phenotype. However there are conflicting reports all dependent on the nature of stimulatory peptides examined. In the case of Herpes Simplex Virus; another virus with a latent and lytic cycle, IFN γ secretion around infected neuronal ganglion is essential for maintaining the virus in its latent phase and preventing reactivation. This is not restricted to CD8⁺ cytotoxic cells, and CD4⁺ cells have been shown to have a particularly important role in the latency phase when the density of viral antigens is less dense.

I identified very few TNF α ⁺ CD4⁺ memory cells despite an apparent EBV specific IFN γ response (particularly compared to PPD simulation). It may be speculated the cytokine profile of EBV CD4⁺ T-cell is deliberately skewed away from TNF α expression to prevent pathological damage given the persistence of the virus. IL-17 was also produced by a small but notable number of cells, which supports the suggestion IL-17 has a role in maintaining persistent viruses in the latency phase.

In this study it is important to note that changes in cytokine profiling cannot be attributed to differential TCR signalling as both antigen presenting cells and T-cell have an array of other pathogen pattern recognition receptors, for example Toll-Like Receptors. These substantially contribute to lineage polarisation of naïve T-cell and potentially influence memory T-cell phenotype upon re-stimulation. The contribution of the TCR could be equated by functionally blocking HLA molecules on APC to prevent peptide presentation on stimulation. The cytokine contribution from the non-TCR specific portion could then be subtracted. This would be particularly useful when using lysates and vaccine preparations which may contain contaminants or adjuvants. Alternatively tetrameric MHC-peptide complexes could be used

for stimulation purposes but this requires HLA haplotyping and sequence information of stimulatory peptide fragments.

The contribution antigenic milieu receptor combination is important when considering when considering the plasticity of particular lineage and what drives a subset to express an atypical cytokine. SEB and PPD were the only antigens stimulating adequate cell numbers to analyse the co-secretion of TNF α or IL-17 with IFN γ . Only around 10% of IFN γ producing cells also produced TNF α following SEB secretion compared to 30% with PPD. This possibly represents a population of Th₁ co-secretors and supports the association of TNF α with a Th₁ phenotype for the purpose of activating macrophages and other inflammatory cells. However it can be as easily speculated these are an unidentified subset of TNF α secretors that have switch on IFN γ expression. Evidence gathered supported the existence of an IL-17/IFN γ co-secreting population and their numeracy suggested this combination of cytokines is extremely rare. However not enough antigens were tested to speculate whether different antigenic stimuli influenced either population of co-secretors examined.

In summary this study has demonstrated the feasibility that different antigenic stimuli can cause differential cytokine profiles in CD4⁺ cells. Given these changes can be detected in healthy individuals without any clonal expansion associated with *in vivo* re-challenge or reactivation this is useful for comparison of non-pathological versus pathological presentation. For example, in the case of EBV infection it is possible to compare the T-cell cytokine profiles of seropositive individuals who don't present with infectious mononucleosis to those who develop the disease . Additionally longitudinal of IM patients during primary infection, subsequent symptomatic presentation and when the EBV virus is in a non-reactive state could identify any changes in CD4 T-cell phenotype. This is important given the historical black and white Th1 Vs. Th2 classification is no longer considered applicable given

the discover of numerous sublineages and overlap in cytokine profile. The approach is also applicable for demonstrating the plasticity and stability of a specific sublineage which itself adds an additional layer of complexity phenotypic analysis. Such approaches are also applicable to vaccine design where it is desirable to polarise a T-cell response to provide the most effective immunity against a pathogen of interest.

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