Subset analysis of the EBV-specific CD4+ T cell response in primary and persistent infection

and

Characterisation of CD8+ T cell responses against CMV derived antigens protected in wild type strains of the virus

By Louise Hosie

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University of Birmingham

UNIVERSITY OF BIRMINGHAM

Institute of Cancer Research UK

School of Cancer Sciences

University of Birmingham

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Louise Hosie

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UNIVERSITY OF BIRMINGHAM

Institute of Cancer Research UK

School of Cancer Sciences

University of Birmingham

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I hereby declare that the work contained in this thesis is of my own, unless directly stated, under the direct supervision of Dr Heather Long and Dr Graham Taylor.

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List of Abbreviations

Ag –Antigen

BL – Burkitts Lymphoma

E – Early Protein

EBNA – Epstein-Barr Virus Nuclear Antigen

EBV – Epstein-Barr Virus

HLA – Human leukocyte antigen

IE – Immediate Early protein

IFN – Interferon

IL –Interleukin

IM – Infectious Mononucleosis

LCL – Lymphoblastoid Cell Line

LMP – Latent Membrane Protein

MHC – Major Histocompatibility Complex

mTF – Master Transcription Factors

PBMCs – Peripheral Blood Mononuclear Cells

PTLD – Post transplant lympoproliferative disease

PFA – Paraformaldehyde

TAP – Transporter Associated Protein

TCR – T Cell Receptor

Th – T Helper

Tfh – T Follicular Helper

ThCTL – T Helper Cytotoxic

Abstract

CD4+ T cell responses are critical components of the immune response to viral infections, yet unlike their CD8+ counterparts, CD4+ responses to many viral agents remain uncharacterised. The CD4+ T cell subsets involved in controlling primary and persistent EBV infection (causative agent of IM) have not been extensively studied. This investigation attempted the first systematic analysis of the CD4+ T cell response to epitopes from across the EBV genome in a subset-specific manner. It combined MHC II tetramer technology (nine epitopes) with intracellular staining for transcription factors of the Th1/Th2/Treg subsets to analyse 8 healthy and 2 IM donors. Although the analysis of Th1/Th2 subsets requires further optimisation, MHC II tetramer staining and intracellular staining were successfully combined for FOXP3. Evidence is presented for the presence of EBV-specific Treg responses, within healthy persistent carriers but the absence of such responses in acute primary IM patients. The results implicate that a lack of Tregs, in combination with other genetic factors, may play a role in IM development. Preliminary Th1 responses and negative Th2 responses in healthy and IM patients were observed. Further understanding the subsets involved in controlling EBV, will help focus therapeutic applications towards EBV-associated malignancies at specific subsets.

CHAPTER 1 - INTRODUCTION

1.1 – Epstein-Barr Virus and lifecycle

Epstein-Barr Virus (EBV) is a widespread γ-herpesvirus with a double stranded (ds) DNA genome that infects >90% of the world's adult population (Reviewed in[1]). EBV is typically acquired in childhood via saliva where it is associated with an asymptomatic infection in the vast majority of cases.

Oral transmission (via saliva) of EBV into a naive host results in the initiation of a lytic infection within the epithelial cells of the oropharynx characterised by high rates of viral shedding, which is slowly brought under control by the mounted immune response. Parallel to this lytic infection, the virus infects circulating mucosal B cells and initiates its latent growth-transforming programme (Figure 1). EBV expresses a set of latent proteins that then drive the proliferation of infected B cells and their differentiation into a pool of memory cells. This initial proliferation results in up to as many as 1-10% of the infected host's total B cell count being EBV infected within the blood - which decreases quickly as the infection is limited by the T cell response. Within healthy long term carriers, the number of EBV positive B cells within the blood are rare at around 1-50 per 10^6 B cells[2].

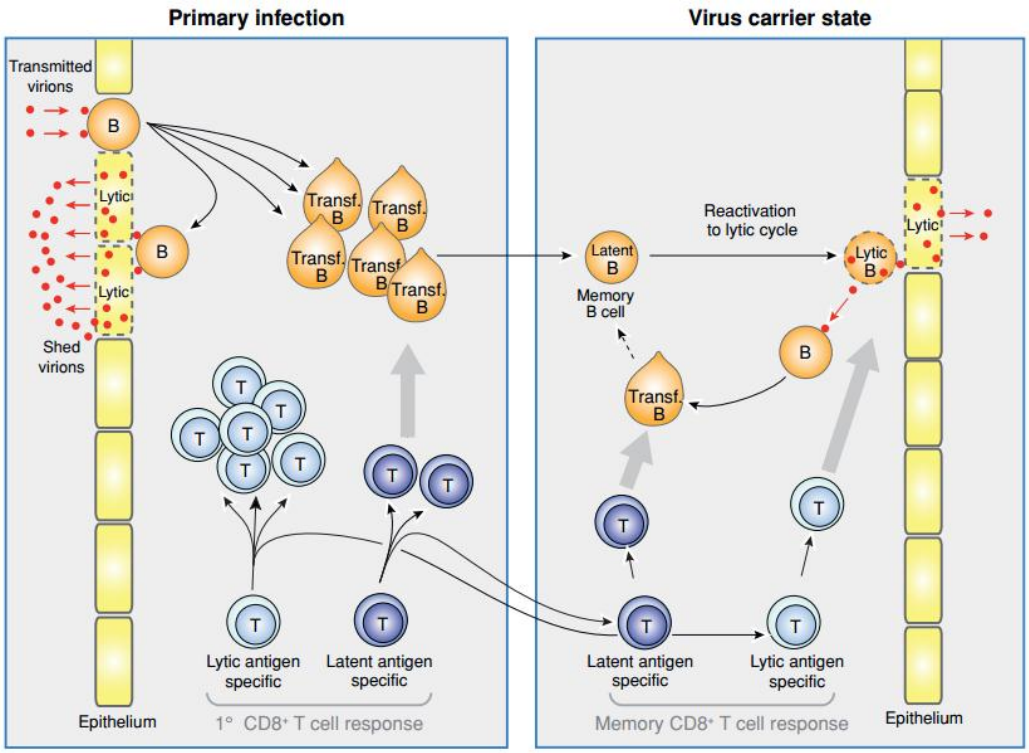


Figure 1 - A schematic representation of the primary replication of EBV, long term carriage and reactivation of EBV. EBV replicates within the nasopharynx inducing a large CD8 T cell response parallel to infecting local B mucosal cells to establish latency. Latent B cells may enter the lytic phase and shed virions into the saliva[3].

EBV undergoes two different forms of infection: lytic and latent. During lytic infection the full cohort of ~60 genes become expressed progressively during the infection separated into: immediate early (IE), early (E), and late (L) proteins according to the time of their expression. This cohort allows the virus to fully replicate and produce new progeny. The first viral gene product to be expressed is the viral transactivator BZLF1 which controls the expression of the viral genome. Functions of example of proteins used during the current project are shown in Table 1.

Parallel to this lytic infection, the virus expresses a separate smaller cohort of 9 genes that are involved in establishing the latent infection within B cells. All of the latent and lytic proteins expressed by EBV are all immunogenic to the host which results in a robust immune response that eventually brings the infection under control forming the EBV-specific memory

T cell response of the host for life. The loss of this immune control, specifically the adaptive T cell response results in clinical disease by EBV.

Once a pool of latent EBV infected B cells is formed the virus switches off of all gene expression to avoid detection by EBV specific T cells and to maintain an infected B cell pool that can spontaneously revert to the lytic cycle during the hosts lifetime. Throughout the lifetime of the carrier, these latently infected B cells can recirculate back to the nasopharynx where they re-establish the lytic cycle of infection by spontaneous reactivation. New progeny in the saliva can then be transmitted to a naive host (Figure 1). These subsequent reactivations are controlled by the memory T cell response.

As well as its acute capabilities, EBV is the causative agent of the B cell malignancies - Hodgkin's lymphoma and Burkitt's lymphoma - as a result of its B cell growth transforming capabilities[2]. Within these different B cell malignancies, EBV infected B cells exhibit a different latency programme (described in Table 2) distinguished by the expression of a limited and differing panel of the 9 latency genes.

Protein	Infection Stage	Function
BZLF1	Lytic IE	Viral Transactivator
BMRF1	" E"	DNA polymerase activity [4]
BARF1	" E"	Suspected oncogene activity [5]
BFRF1	" E"	Recruits nuclear envelope components at the nuclear membrane for nuclear release [6]
EBNA1	Latent	Binds to the viral origin of replication on the viral genome episome to ensure the propagation of EBV DNA to daughter cells during host cell division
EBNA2	'' ''	Transactivator that is the first viral protein expressed during latency and essential for cellular transformation

Table 1 – Examples and Functions of EBV lytic and latent proteins[2; 7; 8]. These proteins were selected as examples due to the MHC class II tetramers investigated within this project presenting an epitope from these proteins (Table 3).

Type of Infection	Growth Programme	Genes Expressed	Associated Malignancy
Latent	Latency 0	No significant gene expression	-
Latent	Latency I/Latency Programme	EBNA 1	Burkitts lymphoma
Latent	Latency II/Default Programme	LMP1/2 EBNA 1	Hodgkin's lymphoma and Nasopharyngeal carcinoma
Latent	Latency III/Growth Programme	LMP1/2 EBNA1/2/3A/3B/3C	PTLD

Table 2 - Growth programmes of EBV and their gene expression combinations[9]

1.2 - Infectious mononucleosis and EBV association with malignancies

In the Western world, as a result of improved sanitation, primary infection can be delayed to adolescence in up to 25% of patients where a self-limiting acute disease known as 'Infectious Mononucleosis' (IM) presents. Symptoms of IM are generally mild including fever, malaise, fatigue, sore throat, lymphadenopathy and splenomegaly that all typically resolve after 1 month. IM provides us with a rare opportunity to follow a genetically stable viral agent from primary infection to the establishment of life long latency. This has provided us invaluable information on the development of virus specific T cell responses, the kinetics of these responses and how these responses change over the course of primary to persistent infection. The CD8+/CD4+ T cell responses to EBV have been well documented to alter in their magnitude, breadth and immunodominance in IM compared to chronic infection[10]. Within IM, it is known that a large expansion of the CD8+, and to a lesser extent the CD4+ T cells occurs[11]. This expansion is thought to be responsible for the clinical symptoms.

1.3 - CD8+ T cell response in IM and Healthy Donors

To date, the most interest has been directed towards the EBV-specific CD8+ T cell response primarily due to the large expansions of these cells during IM. In 1996, the development of MHC class I tetramers where fluorescently labelled quadramers of HLA class I molecules present a peptide from a known EBV epitope, have allowed the extensive and direct visual analysis of EBV-specific CD8+ T cells from IM patients to epitopes derived from lytic and latent antigens, at the single cell level[12]. There is a marked preference for particular antigens of the lytic and latent viral protein classes and the particular immunodominance of CD8+ T cells towards epitopes derived from the EBNA3 protein family and the BZLF1 and BRLF1 IE proteins is observed. With regards to latent antigens, the CD8+ T cell response appears to be broadly directed across all nine latent proteins. The responses to lytic antigens compared

to latent are generally much larger. Studies have revealed that the EBV-specific CD8+ T cell responses in IM may be oligoclonal for a single epitope of the viral genome and that responses for an individual lytic epitope can represent up to 40% of the total T cell response during IM. The immunodominance hierarchy is a result of the immunoevasive proteins that impair the MHC class I (MHC I) antigen presenting pathway (Table 3) and therefore only the antigens expressed before the immunevasion proteins interact with MHC I are recognised well[13; 14; 15 16].

1.4 - CD4 T cell response

CD4+ T cells are recognised for their functions in helping the immune system in response to viral infections [Reviewed in[17; 18]]. They promote class switching and affinity maturation of antibodies; induce the phagocytic ability of antigen presenting cells (APCs) and increase CD8+ T cell cytoxicity. Enhancing the CD8+ T cell cytotoxic effect by the CD4+ T cells involves the supply of co-stimulatory molecules, production of pro inflammatory cytokines/chemokines and direct cytotoxic effector functions upon target virus infected cells themselves. In order to perform these 'helper roles' activated CD4+ T cells undergo a process of differentiation into distinct effector subsets[18; 19] (Figure 2). Each subset expresses a characteristic cytokine and master regulator transcription factor (mTF) although it is now clear that there is plasticity between the subsets. The two best studied subsets are the T helper T cells (Th) 1 and 2. Th1 cells are defined by IFN-γ and TNF-α production and expression of the mTF TBet. Their main effector function is promoting a proinflammatory response by activating macrophages to attract homing lymphocytes. Responses to viral antigens are predominantly of the Th1 response with classically high levels of IFN-ɣ production. Th2 cells express Interleukin (IL)-4, mTF GATA3 and are associated with allergic and responses to extracellular pathogens. T Regulatory cells (Tregs) are important in the suppression of over

robust immune responses (both CD4 and CD8) and express the mTF FOXP3. However, FOXP3 is not solely expressed in Tregs but as yet is considered the most characteristic marker[20]. As a result of their suppressive functions, some viral pathogens are thought to promote the differentiation of CD4+ T cells into Tregs in order to hinder the antiviral response that may lead to its elimination from the host [21]. Two types of Tregs have been identified: The first known as the 'natural' Tregs produced within the thymus and the second known as the 'adaptive' Tregs which are induced to express the characteristic markers CD25 and FOXP3 once activated[20]. Th17 cells, characteristically producing IL-17 and the RORγ mTF have been implicated in the immunopathology associated with viral infections. These T cells are normally generated in the presence of IL-6 and TGF-β. IL-23 is associated with the development of the pathogenic effects of these cells[22]. Follicular helper cells (Tfh) are thought to reside in niches within the germinal centres providing help for B cell class switching. However it is as yet unclear whether these are a true distinct subset or rather a state that is imposed upon the other subsets. Tfh express IL-21 and Bcl6. Finally it has now been demonstrated in several contexts that CD4+ T cells can acquire a cytotoxic phenotype. Such cytotoxic CD4+ T cells (ThCTL) are reported to be Th1 like producing IFN-γ and exert their apoptotic effects on their targets via the release of perforin and granzyme[17]. In the context of CD4+ T cells, the presence of IFN-γ during activation favours the development of Th1 cells over Th2. The CD4+ T cell subsets are very fluid and as a result may express more than one master regulator and thus express different cytokine combinations. It is therefore important to fully characterise the EBV-specific CD4+ T cell response for comparison to that of characterised and successful viral vaccine responses. This may facilitate the development of an EBV-vaccine that induces a broad and polyfunctional CD4+ /CD8+ responses.

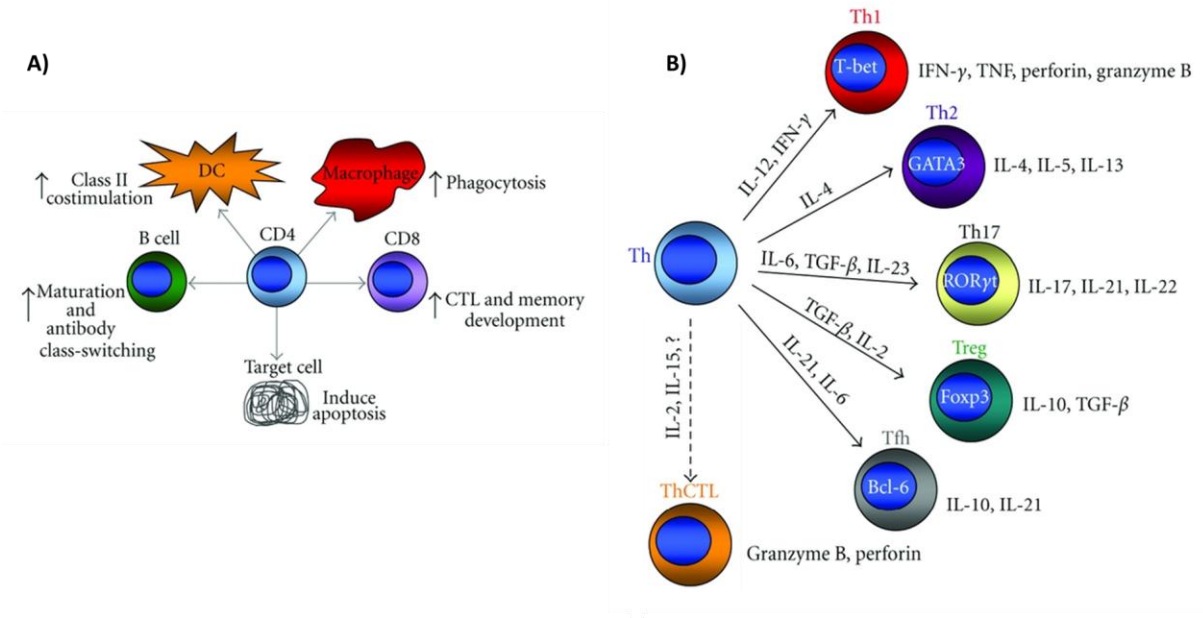


Figure 2 - The Roles and subsets of CD4 T cells. A) The helper functions of the CD4 T cells. CD4 T cells promote antiviral immunity via many direct and indirect interactions with immune cells. B) The differentiated effector subsets of activated CD4 T cells. Subsets are influenced by the cytokine milieu in combination with the mater regulator[17].

1.5 - CD4+ T cell response to EBV in primary and persistent infection

To date, the dissection of the CD4+ T cell response to EBV during primary infection has been hindered as a consequence of lack of knowledge of individual epitopes and their restricting HLA alleles and no available reagents to detect the low frequencies of circling EBV-specific CD4+ T cells. As a result, much less is known about the CD4+ EBV T cell response regarding its epitope specificity, magnitude and kinetics as compared to the CD8+ T cell response. Furthermore, the CD4+ T cell response is slightly more complicated due to the presence of the several distinct effector subsets. Interest in the CD4+ T cell response to EBV is now increasing following observations that many EBV-specific CD4+ T cells are able to directly recognise EBV-infected cells [24] and the EBV associated lymphomas are MHC class II (MHC II) positive. Within a phase II clinical trial, 33 Post Transplant Lymphoproliferative Disease (PTLD) patients were treated with a cytotoxic T cell therapy, more positive responses were observed when the T cell transplants contained higher >5% CD4+ T cells[25].

Current analyses of the EBV-specific CD4+ T cell responses have, until recently, been carried out using the use of *ex vivo* functional assays that have mostly monitored the production of IFN-γ. This cytokine is characteristic of the Th1 response restricting the analysis of the CD4+ T cell response to any given EBV antigen to Th1 cells. However, where studied, functional assays have indicated that CD4+ T cell responses towards the latency proteins are of the cytotoxic Th1 subset[26]. These functional assays coupled with epitope mapping studies (using peptides of 20mers from a range of lytic and latent antigens) have allowed for the isolation of EBV specific CD4+ T cell clones and the subsequent characterization of their restricting HLA alleles[27]. Thus, recent knowledge of epitopes, TCR specificity, restricting MHC (Table 3) and advances in technology have opened the way for the generation of MHC II:EBV peptide tetramers. MHC II tetramers display epitopes derived from EBV proteins within their peptide binding grooves, allowing characterisation of epitope-specific T cells by flow cytometry on a single cell level and removing the reliance on functional assays [Reviewed in[28; 29; 30; 31; 32]].

The uses of MHC II tetramers and IFN-γ readout assays have provided us with the following important findings. It has been documented that within IM patients, 2.7% of the circulating CD4+ T cells are specific for EBV and directed towards both the lytic and latent classes of EBV Ags[33]. Therefore in the context of IM, the EBV-specific CD4+ T cell responses are now known to also expand during IM, albeit at a 10 fold lower magnitude compared to the CD8+ T cell response with any given antigen[11]. CD4+ T cell responses to lytic antigens do not show marked immunodominance towards particular lytic antigens expressed in a particular phase, but rather, a broad response against the range of lytic antigens[34]. In contrast to the CD8+ response, CD4+ responses against the latent antigens (EBNA3C and EBNA1) are in higher frequency than those against the lytic. The responses to lytic Ags are detectable within the

peripheral blood of IM patients and rapidly decline during disease convalescence, whereas the CD8+ responses to latent Ags are rarely detected at primary infection and increase over the subsequent weeks. With surprising efficiency, CD4+ T cell clones generated against EBV epitopes have been shown to be directly cytotoxic against LCLs presenting the correct epitope[34; 35; 36]. EBV-specific CD4+ T cells have been demonstrated to recognise virus-infected cells such as Burkitt lymphoma cell lines suggesting a potential for these CD4+ T cell clones in the treatment of MHC II positive EBV-associated malignancies[37].

Phase of Lifecycle	**Protein**	**Region**	**Epitope**	**MHC restricting allele**
Lytic	BZLF1	61-75	LTA	DR52b
	BMRF1	136-150	VKL	DR17
	BARF1	185-199	SRD	DR7
	BFRF1	125-139	MLG	DR7
Latent	EBNA1	474-493	SNP	DR51
	EBNA1	509-528	VYG	DR7
	EBNA2	276-295	PRS	DR7
	EBNA2	276-295	PRS	DR52b
	EBNA2	301-320	PAQ	DR17

Table 3 - CD4 epitopes of EBV lytic and latent antigens. Epitopes are denoted by three letters indicating the first three amino acids of their primary sequence[11].

1. 6 - Aims

We now have the tools that allow us to analyse the EBV-specific CD4+ T cell response at a single cell level. Through using MHC II tetramers, we already have an understanding of the frequencies and phenotypic characteristics of EBV-specific CD4+ T cells present during primary and persistent infection. In this study it was aimed to further characterise these cells by investigating the CD4+ T cell subsets present. Understanding the CD4+ T cell subsets involved in the control of IM and healthy persistence may help to facilitate the design of immunotherapies directed toward EBV proliferative diseases.

The aims of this investigation were as follows:

1. To optimise intracellular staining protocols for antibodies against the master transcription factors of the major CD4+ T cell subsets: Tbet (Th1), GATA3 (Th2) and FOXP3 (Treg)

2. To analyse the percentage of CD4+ T cells expressing Tbet, GATA3 and FOXP3- within the total CD4+ T cell pool and EBV MHC II tetramer-positive populations in healthy donors

3. To conduct preliminary studies on IM donors to analyse the percentage of CD4+ T cells expressing Tbet, GATA3 and FOXP3 within total CD4+ T cells and EBV MHC II tetramer-positive populations

CHAPTER 2 – MATERIALS AND METHODS

Transcription Factor Antibodies	**Host**	**Clone**	**Company**	**Volume/test**
Alexa Fluor 488 Mouse anti-human FOXP3	Mouse	259D/C7	BD Biosciences	20 µl
Alexa Fluor 488 Mouse anti GATA3	Mouse	L50-823	BD Biosciences	20 µl
Alexa Fluor 488 Mouse IgG k Isotype Control	Mouse	P3.6.2.8.1	BD Biosciences	20 µl
Anti-Mouse/Human TBet PE	Mouse	4B10	eBiosciences	2.5 µl
Anti-Mouse/Human TBet PerCP-Cy5.5	Mouse	4B10	eBiosciences	1.25 µl

Table 4 **- Antibodies used for the staining of intracellular proteins**

Antigen	**Fluorchrome**	**Company**	**Volume/test**
CD45RO	Pacific Blue	Biolegend	2 µl
CD4	PE	BD	2 µl
CD3	APC	Invitrogen	0.5 µl
CD4	FITC	BD Pharmingen	2 µl
CD4	ECD	Beckman Coulter	1 µl
CD4	PerCp-Cy5.5	eBiosciences	2 µl

Table 5 **-Concentrations of the fluorochromes used for surface staining and or compensation of the multi-colour flow cytometry**

Reagent	**Company**
FCS	Gibco
1 X PBS	Dulba
4% Paraformaldehyde in PBS	Sigma
RPMI 1640	Gibco
Saponin	Sigma (4% stock in PBS was made in house)
Saponin 0.5% FACS buffer	In House
MACS Buffer (1X PBS, 0.5% BSA, 2mM EDTA)	In House
FACS buffer	In House

Human Sera (HuS)	TCA
Goats Sera (Heat Inactivated Normal Goat Serum)	Gibco
Lymphoprep	PAA
PBS-NGS (1%)-NGS (1%)	In House
Kit	**Company**
FOXP3 fix/perm staining kit	eBiosciences
Intracellular Fixative (IC)	eBiosciences

Table 6 – Reagents used throughout the investigation

2.1 - Preparation of PBMC's from healthy donors

Heparinised (1:100) blood was collected from EBV-sero positive donors, diluted 1:1 with fresh RPMI and layered onto 15 ml of Lymphoprep in a 50ml falcon tube. The tubes were centrifuged at 2000 rpm for 30 minutes, at 22^{o}C with the brakes off. The lymphocytes were carefully aspirated off the interface between the lymphoprep and plasma/RPMI. 'Peripheral Blood Mononuclear Cells' (PBMCs) were washed twice in 30ml RPMI and centrifuged at 2000 rpm for 10 minutes at 22^{o}C to remove the ficoll and the PBMCs were counted on a haemocytometer. PBMCs were then centrifuged at 1600 rpm for 7 minutes and the pellet resuspended in 10% DMSO freezing medium. 1ml resuspended PBMCs were aliquoted to a cryovial, left at -80^{o}C overnight before transfer to liquid nitrogen for long term storage.

2.2 - MHC Class II Tetramer Staining

Tetramer staining of PBMCs was done prior to the surface and intracellular staining for transcription factors. PBMCs were thawed from liquid nitrogen in 1ml of RPMI (10% FCS and P/S) and centrifuged at 2000 rpm for 5 minutes at 22^{o}C. The PBMCs were then counted on a haemocytometer and distributed to FACS tubes. For tetramer staining, a minimum of 1 x 10^6 cells per sample were required (1.5 x 10^6 cells per sample were used to compensate for cell loss during the subsequent washes). Cells were resuspended in 10ml RPMI (8% FCS), counted and CD8 depleted using CD8 DynaBeads for 30 minutes on a rotating shaker in a cold room. For IM patient samples, 3 times the amount of CD8 depletion beads were used, compared to the healthy donor PBMCs, to accommodate for the large CD8 expansion. PBMCs were re-counted and 1.5 x 10^6 CD8 depleted cells transferred to the FACS tubes and 0.5 x 10^6 for control tubes. Cells were washed in 500µl of human serum before adding 1µl of the relevant tetramer, for the peptide under investigation, to each tube and incubating for 2 hours at 37^{o}C.

Following tetramer staining, the cells were viable and then surface stained (vital dye binds only dead cells allowing their elimination on the LSR II). Vital dye (diluted 1:100) was added at 1.5µl to the relevant tubes (excluding unstained control tubes) and incubated for 20 minutes at room temperature in the dark. Cells were washed once in PBS, surface antibodies (specific for CD3 and CD4 table 5) were added and incubated for 30 minutes on ice to prevent antibody internalisation. Excess antibodies were removed by one wash in PBS. Cells were fixed and permeabilised according to one of the protocols in 2.3, 2.4 and 2.5.

2.3 - Paraformaldehyde (PFA) method for intracellular staining

Following surface staining, cells were fixed for 20 minutes in 2% PFA (1:1 dilution of 4% PFA stock to PBS) at room temperature. After fixation, the cells were washed once in PBS/2%BSA and then once in 500µl saponin buffer (0.5% final concentration from a 4% stock diluted in PBS). The cells were incubated within the residual saponin buffer for 20 minutes at room temperature before intracellular antibodies were added and the cells further incubated for 1 hour in the dark at room temperature. Antibody concentrations used were 0.5µg for the TBet PE, 0.25µg for the TBet PerCP-Cy5.5, and 20µl for both the FOXP3 and GATA3 antibodies (Table 4). Finally, the cells were washed twice in PBS/2% BSA, resuspended in 300µl and kept on ice/4^{o}C in the dark until analysis on the LSR II.

2.4 – EBiosciences FOXP3 staining kit

Following surface staining, the cells were resuspended in 1ml fix/perm buffer (stock 1 in 4 with diluent) and left on ice for 1 hour in the dark. Cells were washed twice in 1ml of perm buffer (at a 1 in 10 dilution with H_20) and centrifuged at 1600 rpm for 4 minutes. All subsequent wash centrifugations were done at 1600 rpm for 4 minutes. To block any unspecific binding sites, the cells were incubated for 15 minutes at room temperature with 100µl of perm buffer with mouse serum (diluted 1 in 10 with the perm buffer). After incubation, intracellular antibodies were added at the concentrations given in 2.3 and incubated for 1 hour at room temperature in the dark. Following incubation, the cells were washed twice in 1ml perm buffer, resuspended in 300µl PBS and kept on ice/4^{o}C in the dark until analysis on the LSR II.

2.5 - EBiosciences intracellular fixative kit for intracellular staining

Following surface staining, 100µl of eBiosciences intracellular fixative (IC) was added to the cells, vortexed well and incubated for 1 hour on ice in the dark (and vortexed after 30 minutes of the incubation period). After incubation, 100µl of 0.2% Triton-X 100 was added to the tubes (final concentration 0.1%) and incubated for 30 minutes on ice in the dark. Cells were subsequently washed once in 2ml PBS-NGS-NHS (materials), resuspended in 300µl PBS and kept on ice/4^{o}C in the dark until analysis on the LSR II.

2.6 - Flow Cytometry

The LSR II FACs machine (BD biosciences) was used for analysing multi-colour flow cytometry experiments. Compensation beads stained with antibodies (Table 4) were used to compensate the different colours involved in each experiment. One drop of each of negative and positive beads were added to each compensation tube and stained for 30 minutes on ice. Data was analysed via FlowJO/DIVA software.

CHAPTER 3 - RESULTS

3.1 – Optimisation of the intracellular staining protocol

The first step in this investigation was to determine the optimum concentration of each antibody that enabled visualisation of cells expressing the relevant transcription factor in the absence of non-specific staining. Therefore titrations of the three antibodies were carried out by flow cytometry.

The gating strategy used for analysing CD4+ T cells from PBMCs is shown in Figure 3. PBMCs were stained with vital dye and then surface antibodies for the CD3+/CD4+ molecules. Firstly, lymphocytes were gated upon on the plot showing side scatter against forward scatter of the total CD8-depleted PBMC population (Figure 3A), and live cells gated upon within the lymphocyte population (Figure 3B) and finally the CD3+CD4+ T cells gated upon from the lymphocyte population (Figure 3C). All subsequent analyses in this study were gated in this manner, and further analysed the cells falling within the CD3+CD4+ gate.

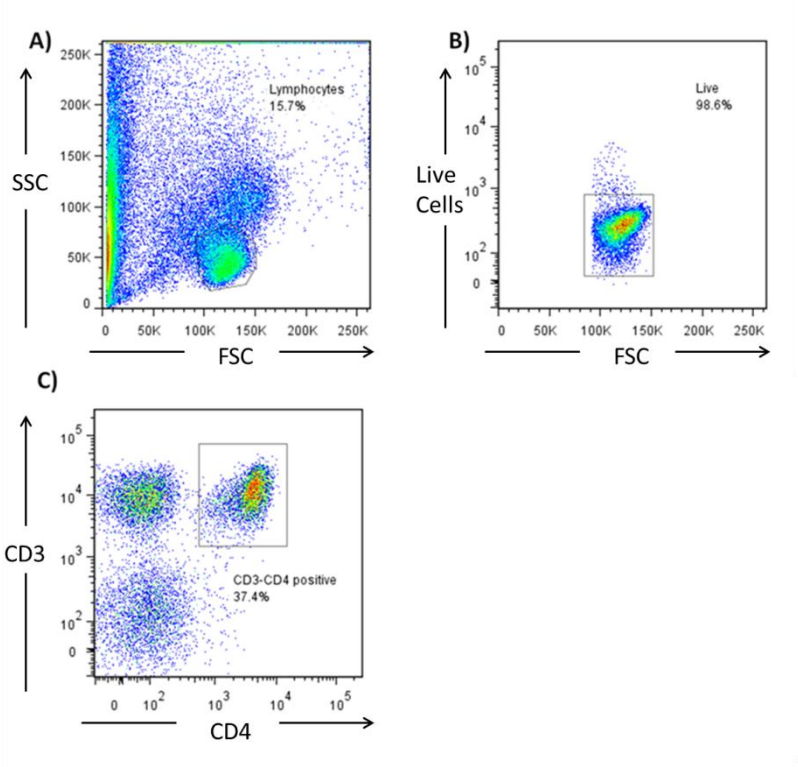


Figure 3 - Gating strategy for analysis of CD4 positive T cells from PBMCs of healthy donors. A) The gated region represents the lymphocyte population. B) The gated region represents live, viable cells that had not bound to the vital dye. The viability dye irreversibly labels dead cells and fluoresces within the pacific blue region. C) The gated region represents CD3+CD4 T cells that were contained within the live cell gate shown in B.

For the intracellular staining of transcription factors and titrations of the transcription factor-specific antibodies, three individual methods/kits for cell fixation and permeabilisation were compared to determine the optimal method for use with each antibody. The three methods will be referred to as the 'PFA method' involving in house preparation of all the reagents as described in the materials and methods, the 'eBiosciences method' which utilised a commercialised fixing reagent purchased from eBiosciences and the 'FOXP3 staining kit method' which involved fixing/permabilising reagents provided in a commercially available kit (see methods).

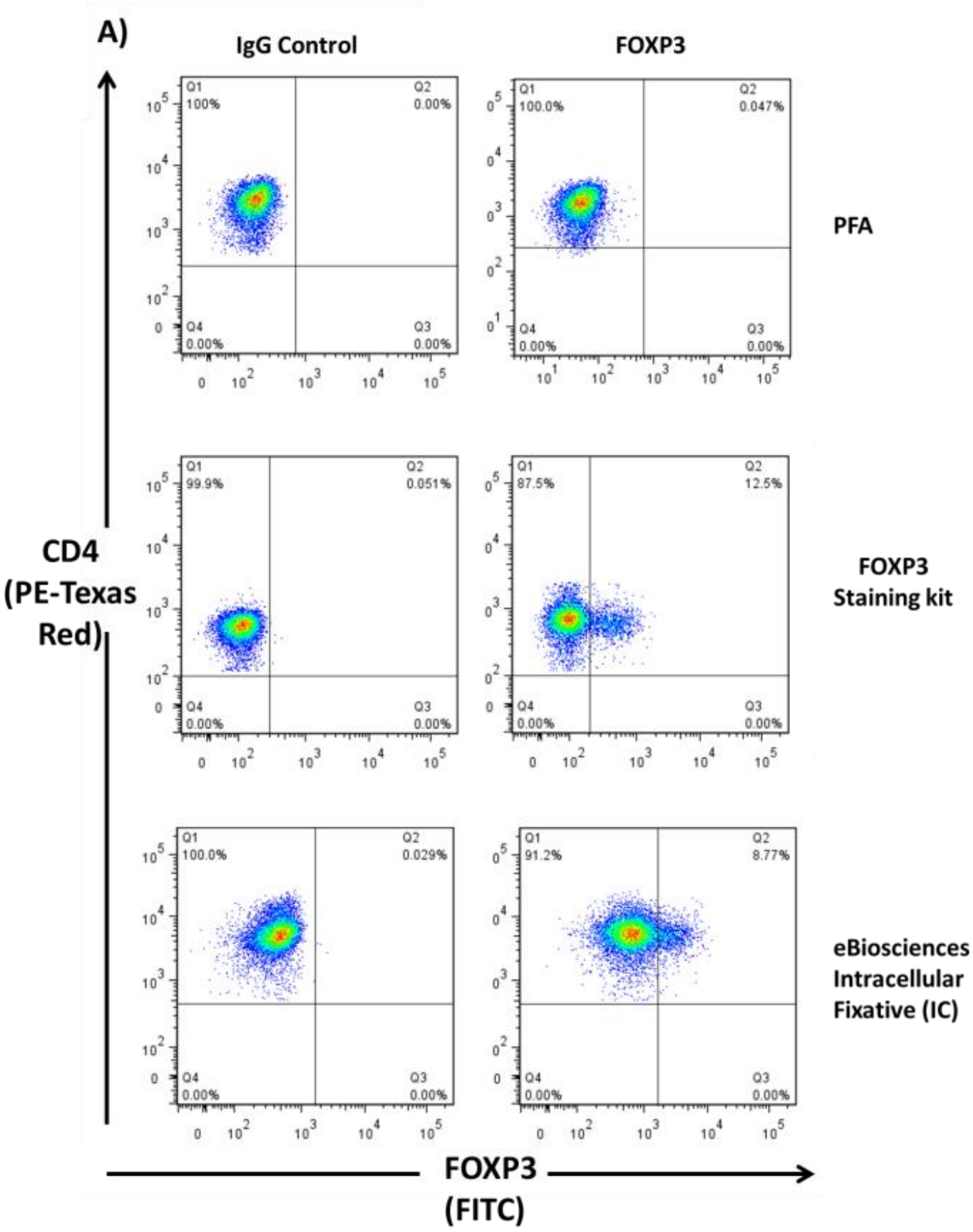
The results for the FOXP3 antibody used in combination with the three methods are shown in Figure 4A. In the top panel, very little staining was observed within the CD4+ T cells when fixed/permeabilised using the PFA method. However when the cells were fixed and permeabilised using the FOXP3 staining kit method, a discrete population of

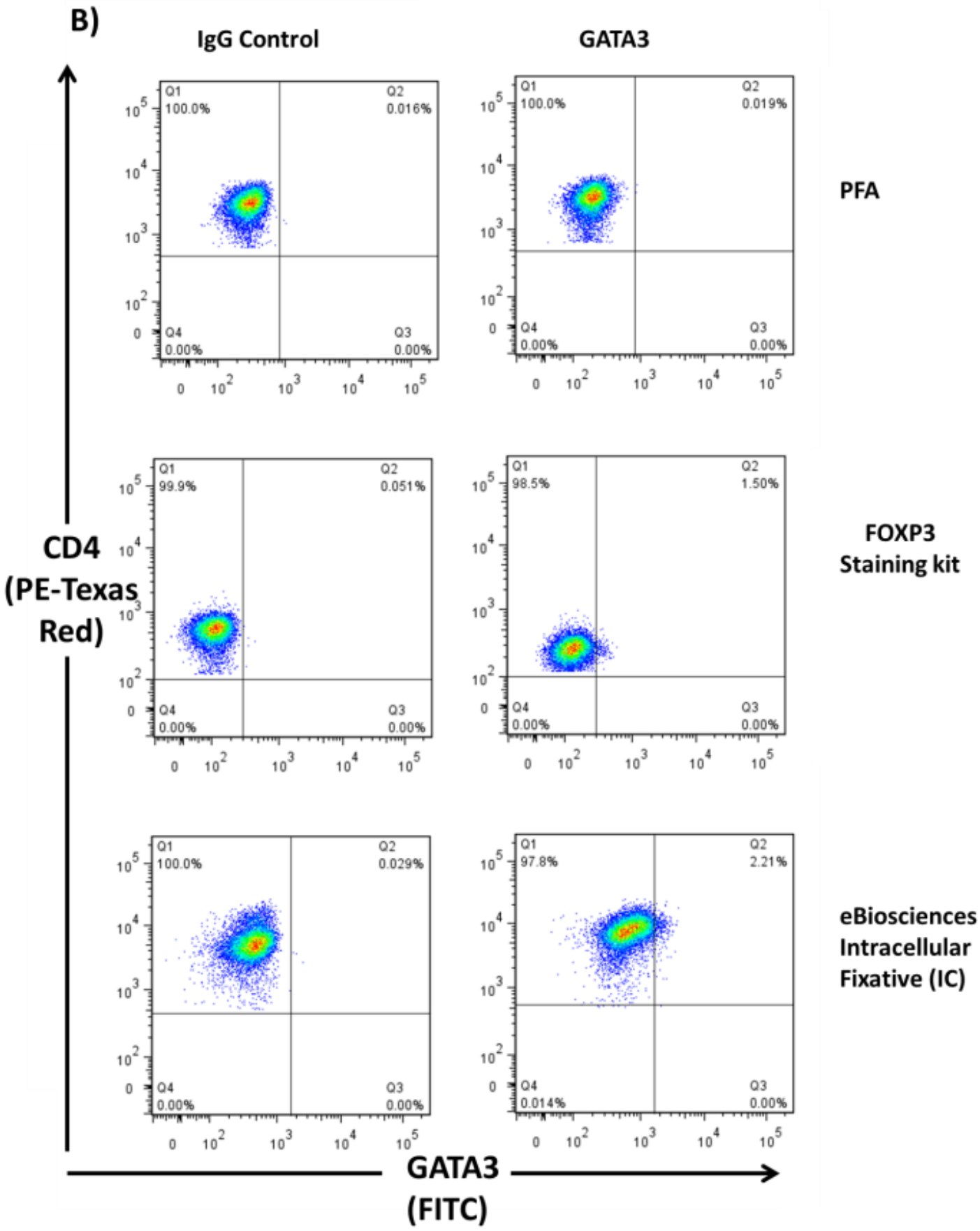
12.5% of total CD4+ T cells were identifiable as expressing FOXP3 (middle panel), which is in line with previous frequencies of FOXP3 positive cells in the blood[38]. Although FOXP3 expression was detected in 8.77% of CD4+ T cells using the eBioscience method (bottom panel), the positive population was not as discrete as that seen using the FOXP3 staining kit, and an overall increase in the MFI of the total CD4+ population indicated significant non-specific binding.

Similar to the FOXP3 results, the GATA3 antibody showed very little staining with the PFA method (top panel Figure 4B). However, the most discrete population expressing GATA3 was observed with the eBiosciences method at 2.21% of the total CD4+ T cell population (bottom panel) and a small less defined positive population with the FOXP3 staining kit at 1.5% (middle panel).

The TBet antibody (Figure 4C) presented with a very small TBet expressing population of the total CD4+ T cell population at 0.564% (middle panel) or negative staining (bottom panel) with the FOXP3 staining kit and eBiosciences methods respectively. Positive TBet staining was observed with the PFA method (top panel) with 8.17% of the total CD4+ T cell population detected as expressing TBet. However, even after this optimisation, staining was weak and was unlikely to reflect the true percentage of TBet positive populations which was reported to be 20% of total CD4+ T cells[39]. The TBet antibody is available on different fluorochromes and was tested conjugated to PE - which is reported to be a brighter fluorochrome (BD Biosciences Fluorochrome laser reference). The results yielded a larger more defined TBet expressing CD4+ T cell population (data not shown) however, as the MHC II tetramers are on PE, these two antibodies could not be used in

combination. Therefore, the combination of the TBet antibody conjugated to PerCP-Cy5.5 and the PFA protocol was optimal within this investigation.




B)
IgG Control
GATA3
PFA
FOXP3 Staining kit
eBiosciences Intracellular Fixative (IC)
CD4 (PE-Texas Red)
GATA3 (FITC)
Q1 100.0%
Q2 0.016%
Q4 0.00%
Q3 0.00%
Q1 100.0%
Q2 0.019%
Q4 0.00%
Q3 0.00%
Q1 99.9%
Q2 0.051%
Q4 0.00%
Q3 0.00%
Q1 98.5%
Q2 1.50%
Q4 0.00%
Q3 0.00%
Q1 100.0%
Q2 0.029%
Q4 0.00%
Q3 0.00%
Q1 97.8%
Q2 2.21%
Q4 0.014%
Q3 0.00%

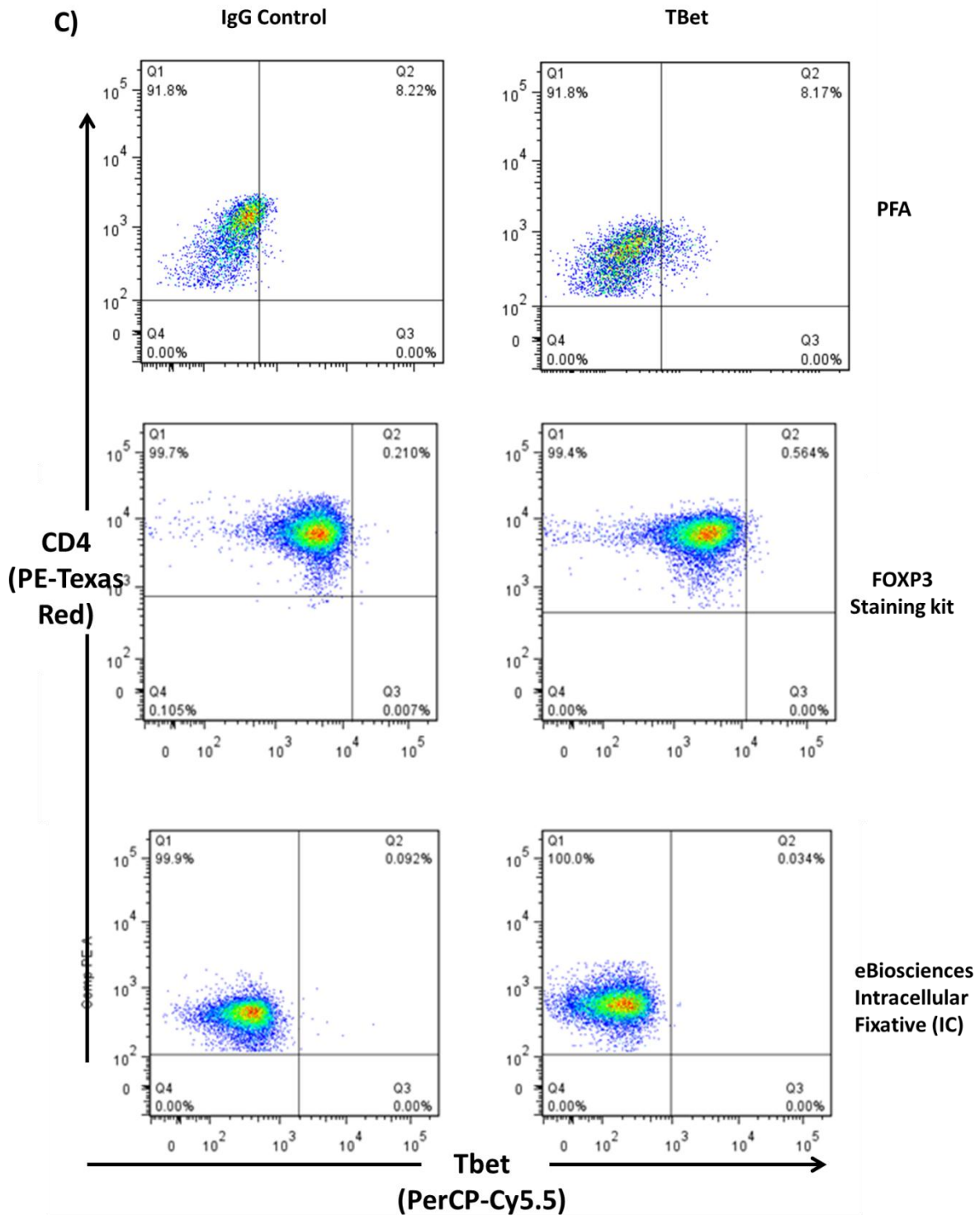


***Figure 4 - Optimisation of the FOXP3, GATA3 and TBet intracellular cytokine antibodies using the three intracellular staining methods* for the optimisation of the intracellular staining protocols.** A) Dot plots of the FOXP3 antibody using all three intracellular methods. FOXP3 positive CD4 cells are within Q2. The IgG control and FOXP3 antibodies were used at 20µl. The CD4+FOXP3+ cells were gated upon as described in Figure 3) Dot plots of the GATA3 antibody. The IgG and GATA3 antibodies were used at 20µl. The GATA3 antibody is also conjugated to FITC. C) Dot plots of the TBet antibody. The IgG and TBet antibodies were used at 1.25µl. The TBet antibody is conjugated to PerCP-Cy5.5. Note that all dot plots are from a minimum of 30 000 events. The CD4+ T cells were gated upon as described in Figure 3 and displayed is CD4+ T cells vs. transcription factor. The left hand panels show the IgG isotype controls and the right hand panels the transcription factor.

After selecting the optimal protocol for the three transcription factor-specific antibodies, each was then titrated with their respective method. The FOXP3 titration data is shown as an example where the volumes used per test ranged from 2.5-40µl (Figure 5). The optimal volume of each antibody was selected based on the largest positive population and no unspecific binding (i.e. the entire population shifted to the right). As shown for FOXP3 there were increasing positive populations: 1.76%, 3.24%, 5.68%, 36% and 77.9% for the volumes 2.5-40µl respectively (Figure 5B). However, the volume of 40µl produced a non-specific binding shift (increase in MFI) of the entire CD4+ T cell population. Therefore a volume of 20µl was selected for the FOXP3 antibody as it provided the largest and most discrete positive population with no unspecific shifting.

Subsequently, the GATA3 and TBet antibodies were titrated in the same way, however using the following volumes (2.5-40µl for GATA3 and 0.5-2.5µl for TBet). With the previous criteria, the optimum volumes selected were as follows: 1.25µl for TBet and 20µl for GATA3.

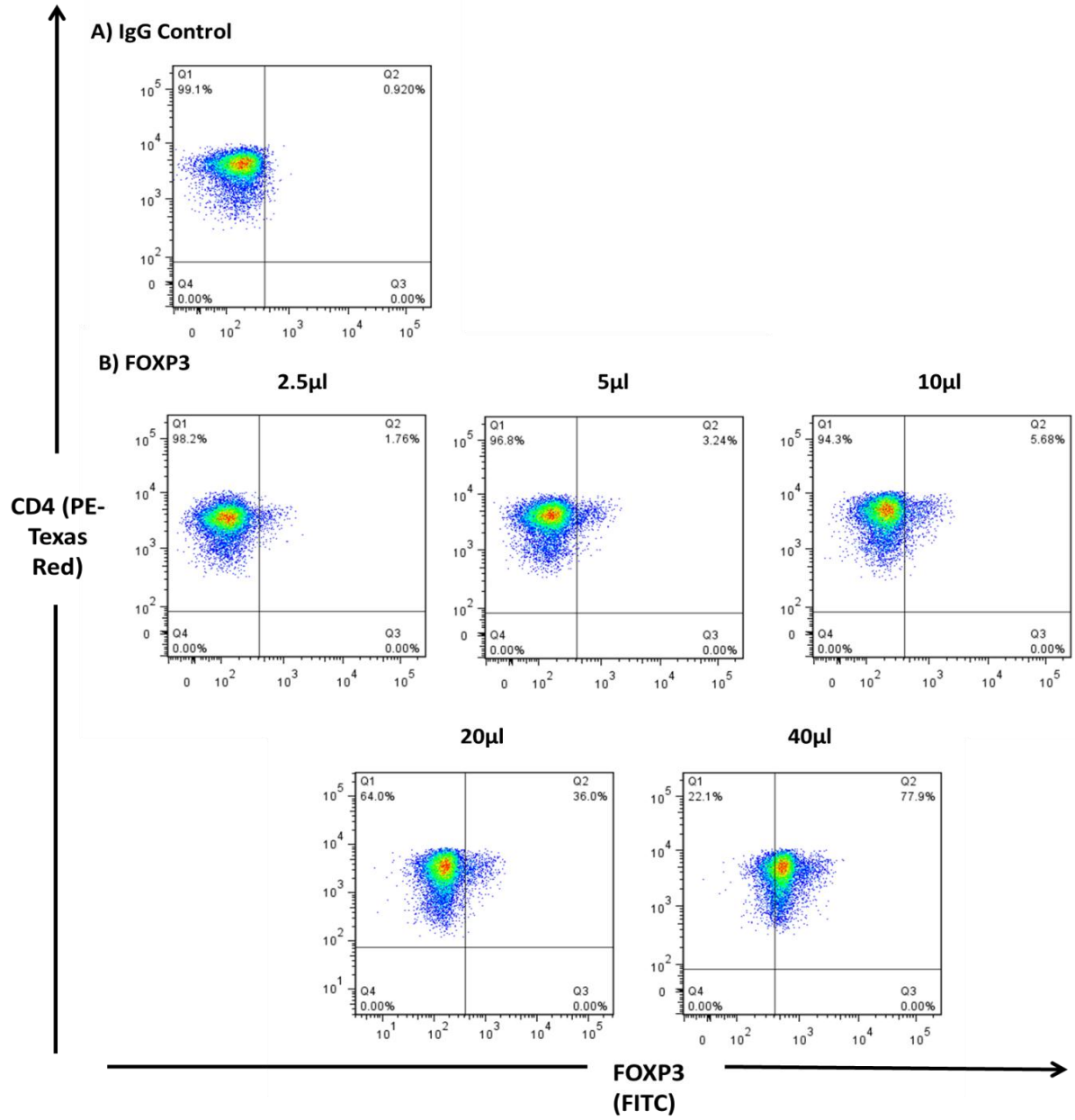


Figure 5 - The optimal titration of the FOXP3 intracellular antibody using the eBiosciences FOXP3 staining kit. Panel A represents the IgG control used at 20µl, the same as the optimal amount for the FOXP3 antibody. Panel B represents the titrations series of the FOXP3 antibody at 2.5, 5, 10, 20 and 40µl. The CD4+ cells were gated as described in Figure 3. The FOXP3 positive cells are represented in the Q2 quadrant and were gated upon the CD4+ cells using FITC as described in Figure 4.

To summarise, the optimal fixation and permeabilisation methods selected for use with the TBet, FOXP3 and GATA3 antibodies were the PFA, FOXP3 staining kit from eBiosciences and the intracellular fixative (IC) from eBiosciences respectively. Although, further optimisation of the TBet staining is required, due to time constraints this was not possible. The analysis of the subsets of EBV epitope-specific CD4+ T cells present within CD4+ T cell populations in healthy donor peripheral blood was then carried out.

3.2 – The percentage of FOXP3, TBet and GATA3 expression amongst EBV epitope-specific CD4+ T cells in healthy carriers

After optimising the intracellular staining protocol for the transcription factors of the three major CD4+ T cell subsets (Th1, Th2 and Tregs) the optimised protocols were used to analyse the presence of these subsets within EBV epitope-specific populations in the peripheral blood of healthy carriers.

PBMC's were isolated from eight seropositive healthy donors and stained with relevant MHC II tetramers (Table 3), as described in the methods, prior to intracellular staining for transcription factor expression utilising the relevant protocols as selected in 3.1.

The EBV MHC II tetramer-specific CD4+ T cells within the CD3+CD4+ T cells (gated as in Figure 3) were analysed as shown for two example donors in Figure 6, using MHC II tetramers specific for each donor's individual HLA type. The CD4+/tetramer+ cells were discriminated via PE-texas red (CD4) vs. PE (MHC II tetramer). Donor 1 was MHC DR52b and DR7 positive and as shown in the left panel no positive staining was observed in the absence of tetramer. The percentage of the total CD4+tetramer+ T cell population observed was larger with the LTA lytic tetramer (0.041%) compared to the latent PRS and VYG tetramers (0.014% and 0.012% respectively). Donor 2 was also DR52B and DR7 positive and the results were similar with a negative result in the absence of tetramer and the latent LTA tetramer+ percentage (0.022%) being larger than the latent PRS tetramers (0.168% and 0.023% respectively).

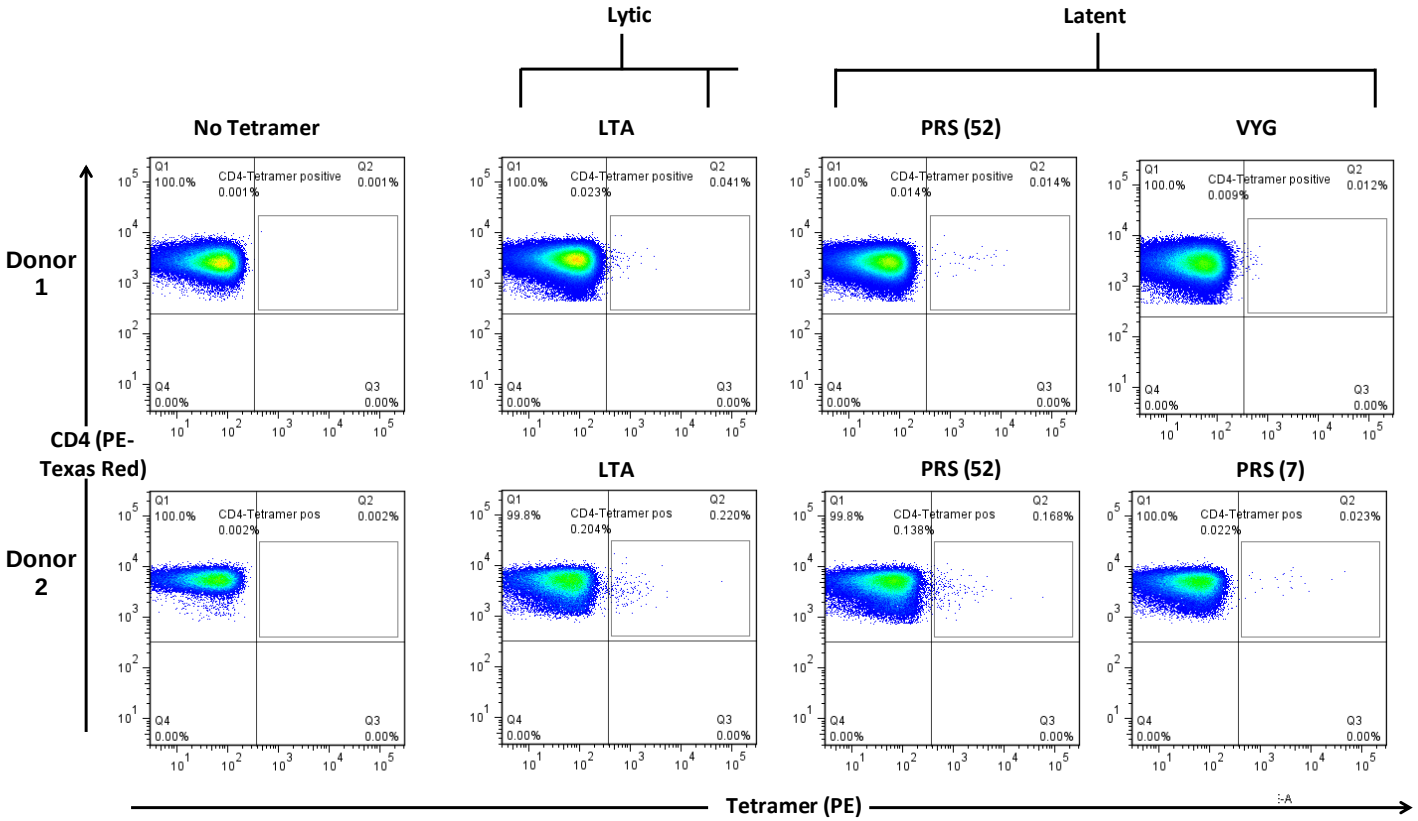


Figure 6 - The gating strategy for the determination of the % of tetramer positive CD4 T cells from the total population of CD4 cells. All plots are gated on CD3+CD4+ as described in Figure 3. CD4+/Tetramer+ cells (Q2) were gated upon the total CD4 population by PE-texas Red (staining CD4) and PE (staining the tetramers). The far left panel represents the 'no tetramer' control for each separate donor. Each subsequent panel represents staining with the indicated tetramer. The top panel and bottom panels are from two separate donors and two separate experiments. The gating for the CD4 cells was as described in Figure 3. LTA, PRS, SNP and VYG epitopes are as described in Table 3. Note that the PRS (7) and PRS (52) epitopes are from the same EBV antigen but are restricted through HLA-DR7 and HLA-DR52b alleles respectively. Each dot plot represents a minimum of 1, 000, 000 events for the top panel donor and 600 000 events for the bottom panel donor.

The tetramer+ cells falling within the gates shown on Figure 6 were then analysed for expression of TBet, GATA3 and FOXP3 antibodies by intracellular staining. The results from two donors are shown in Results Figure 7 and 8 showing examples of the staining obtained with the antibodies characterising Th1/Th2 and Tregs, within both the total CD4+ T cell pool and within MHC II tetramer-staining cells. The results shown for donor 1 (Figure 7) are from CD4+ T cells stained with the MHC tetramer presenting the PRS epitope from the EBNA2 protein (DR7 restricted) or for donor 2 with the PAQ tetramer also from the EBNA2 protein (DR17 restricted) (Figure 8).

The staining for donor 1 indicated that the % of TBet+ CD4+ T cells was the largest positive population at 16.1% of the total CD4+tetramer+ T cell population (Figure 7 bottom panel). This was followed by 8.65% for the FOXP3 expressing CD4+tetramer+ T cells (top panel) and a negative GATA3 staining (middle panel). The staining for donor 2 opposed the FOXP3 results for donor 1 by presenting with a negative FOXP3 response (Figure 8). However, the staining also indicated TBet expressing CD4+tetramer+ T cells (8.1%) and a negative GATA3 staining.

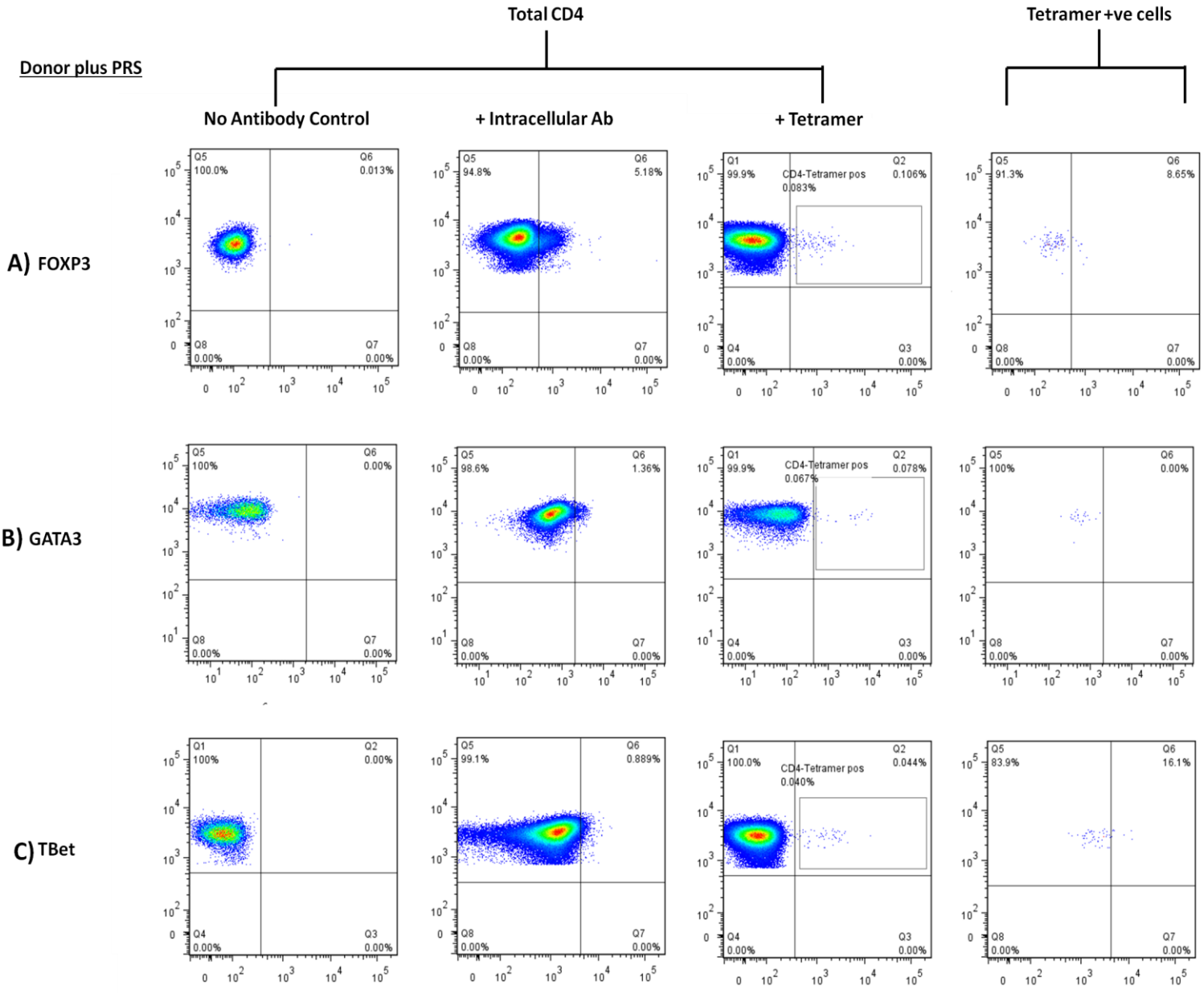


Figure 7 - % of tetramer positive cells within a healthy donor. The far left column represents the no TF antibody and no tetramer control. The second column represents the TF antibody+ and tetramer negative control. The third column represents the tetramer+ and TF antibody negative samples. The final column represents the tetramer+/ TF antibody+ cells. The gate representing the tetramer+ and TF+ population was set as described in Figure 6. The PRS epitope are as described in Table 3. The top panel represents the FOXP3 responses, the middle panel the GATA3 responses and the bottom panel the TBet responses. All dot plots are from one individual experiment and one donor.

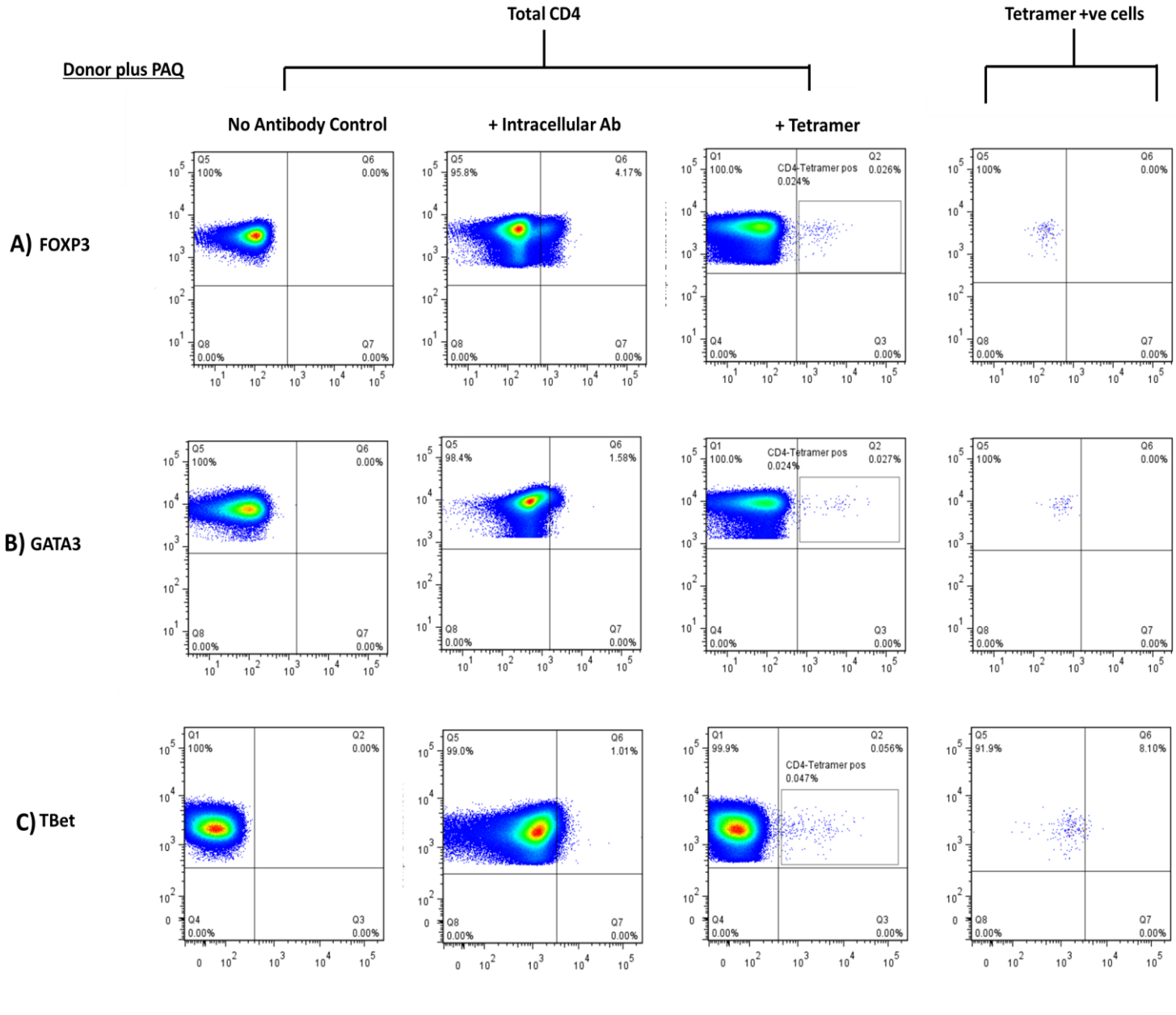


Figure 8 - % of tetramer positive cells within a second healthy donor. Results in this figure were analysed as for Figure 5 but with a separate donor and the PAQ epitope as described in Table 3.

Overall, the results did indicate the presence of virus specific TBet expressing CD4+ T cells. However, the overall results obtained for GATA3 and TBet expression in the total CD4+ T cell populations of the 8 healthy donors (Appendix) were not as high as was expected based upon previous literature and the expected number of cells of those subsets[31; 37]. It has been reported that 20% of the total CD4+ T cell response within healthy adults (aged 25-50) are Th1 cells[39]. Thus the weak staining obtained with the TBet and GATA3 antibodies was likely underestimating the % of Th1 and Th2 cells present in the MHC II tetramer positive cells, from this point on, the donors were then screened for EBV-specific CD4+tetramer+ T cells expressing FOXP3 only. The summary of the FOXP3 staining detected in the total and MHC II tetramer+ CD4+ T cells from eight healthy donors is provided in Figure 9A/B.

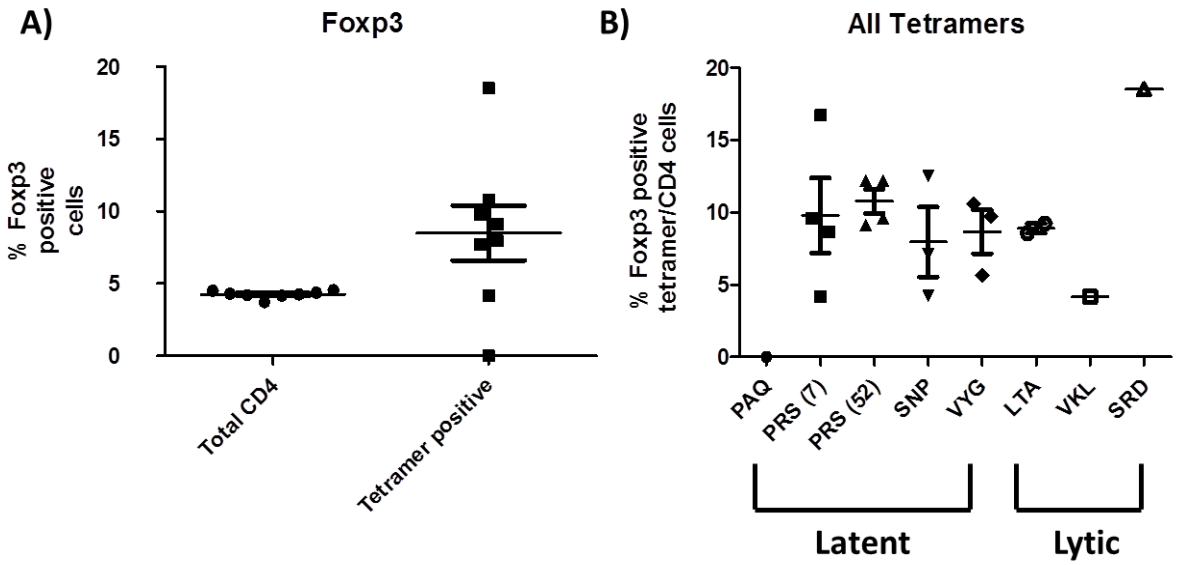


Figure 9 - Column graphs of the % of tetramer positive cells and transcription factor positive cells within all healthy donors. A) The Total CD4 population gate represents the total % of cells expressing FOXP3 as described in Figure 4. The tetramer positive cells represent the % of TF+ CD4+ T cells as described in Figure 6. The % of EBV-specific FOXP3+ CD4+ T cells for each epitope rested in this investigation.

Tregs have been described as comprising 2-5% of the total CD4+ T cells within healthy donor PBMCs. The results in this investigation demonstrated that the percentage of FOXP3 expressing cells within the total CD4+ T cell population of healthy donors falls within a remarkably small range (2-5.18%) which is in line with the published data (Figure 9A). The data summarised in Figure 9A, indicate that there are in fact EBV-specific CD4+ T cells expressing FOXP3, to the epitopes tested in this investigation from both the lytic and latent phases. The percentages of FOXP3 expressing CD4+tetramer+ T cell populations are spread with one high value (18.5%) and one negative response. The EBV-specific FOXP3 expressing CD4+ T cells did not appear to be focused towards either the latent or lytic antigens (Figure 9B) with the mean percentages being rather similar (excluding epitopes tested once). Furthermore there does not appear to be one epitope in particular that elicits a large EBV-specific FOXP3 expressing CD4+ T cell population. There was one high % (18.5%) of EBV-specific CD4+ T cells expressing FOXP3 within the SRD positive population but this epitope was tested once only.

3.3 – The percentage of Th1, Th2 and Tregs responding to EBV epitopes in IM patients

After testing a panel of healthy donors for EBV-specific FOXP3+ CD4+ T cells to epitopes described in table 3, preliminarily experiments were conducted to investigate the presence of FOXP3+ expressing EBV-specific CD4+ T cells during primary virus infection using previously collected peripheral blood samples from two IM patients (Figure 10 and 11).

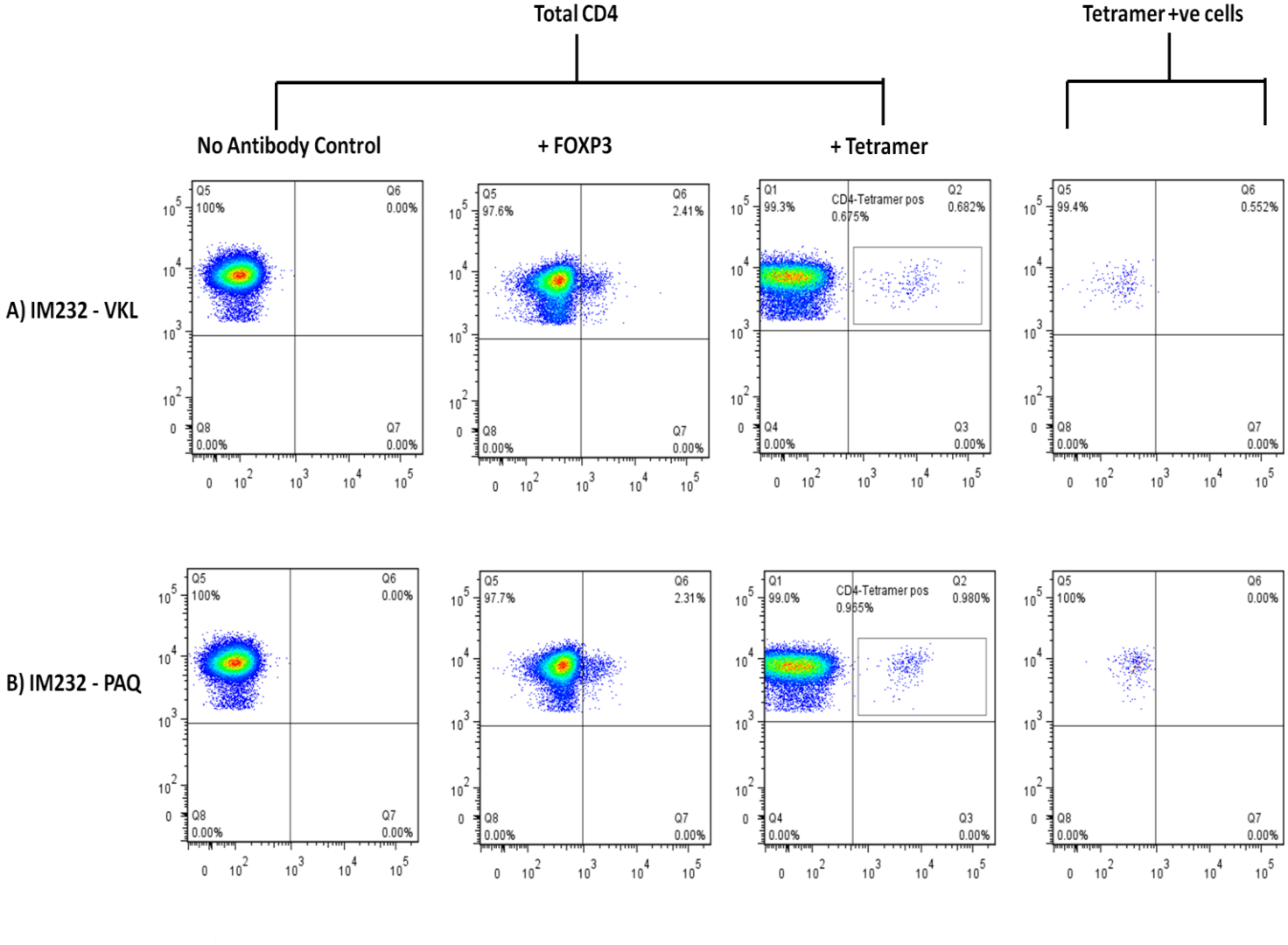


Figure 10 - Preliminary intracellular staining data for IM patient 232. Panel A shows the flow cytometry dot plots for the lytic VKL tetramer positive CD4 cells. Panel B shows the flow cytometry dot plots for the latent PAQ tetramer positive CD4 cells. The transcription factor positive cells were gated on as described in Figure 4. The tetramer positive cells were gated on as described in Figure 6. The tetramer and transcription factor positive cells were gated upon as described in Figure 7. The population in the Tetramer positive cell panel represents the population gated in the Total CD4 + Tetramer panel. Note that this figure represents data from one IM donor and one experiment.

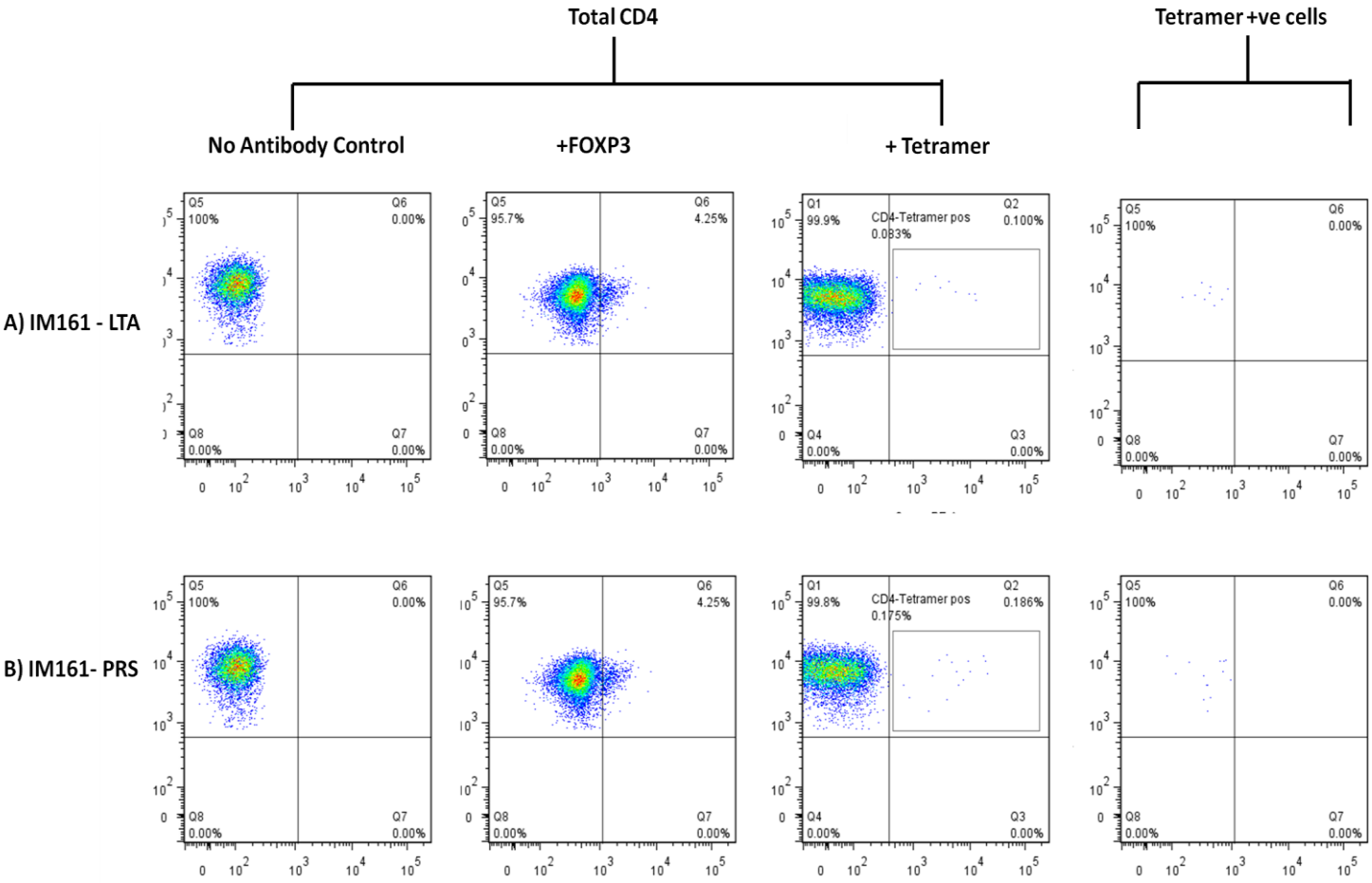


Figure 11 - Preliminary intracellular staining data for IM patient 161. Panel A shows the flow cytometry dot plots for the lytic LTA tetramer positive CD4 cells. Panel B shows the flow cytometry dot plots for the latent PRS tetramer positive CD4 cells. The transcription factor positive cells were gated on as described in Figure 4. The tetramer positive cells were gated on as described in Figure 6. The tetramer and transcription factor positive cells were gated upon as described in Figure 7. The population in the Tetramer positive cell panel represents the population gated in the Total CD4 + Tetramer panel. Note that this figure represents data from one IM donor and one experiment.

Although the data is preliminary, it is clear that in the two IM donors analysed in Figure 10 and 11 whilst FOXP3+ expressing cells could be found within the total CD4+ T cell population (second column Figure 10 and 11), all the epitope-specific CD4+ T cells from both donors were FOXP3 negative (fourth column Figure 10 and 11). This contrasts with the

healthy donor data where 7 out of 8 donors appeared to have a range of EBV-specific CD4+ T cells expressing FOXP3 present within the tetramer positive populations of the epitopes tested (Figure 7, 8 and 9). The total FOXP3 expressing CD4+ T cells appear to be diminished within IM patient 232 (Figure 10) as compared to the two healthy donors (Figure 7 and 8). The investigation into more IM patients will provide a clearer picture whether a reduction of EBV-specific FOXP3 positive cells is a phenomenon of IM, or a feature of the two patients analysed in the present project.

CHAPTER 4 - DISCUSSION

The CD8+ T cell response to EBV has been well-documented over several decades[3]. The EBV-specific CD4+ T cell response has been far less well characterised at the single cell level as a result of the lack of tools for the analysis of virus-specific CD4+ T cells. Recent advances in knowledge of proteins and epitopes targeted by the CD4+ T cell immune system have facilitated the development of MHC II tetramers[29]. These have for the first time, enabled the visualisation of changes, within healthy donors, of the EBV-specific CD4+ T cell response from primary infection to long term carriage[11]. CD4+ T cells are clearly of importance in controlling virus infection since patients with EBV-induced PTLD who receive therapeutic EBV-specific T cells have been shown to achieve greater clinical success if those preparations contains >5% CD4+ T cells[25]. Further understanding the characteristics of CD4+ T cells that are important for the control of EBV in healthy individuals may define what is important for including within therapeutic preparations to enhance T cell-based treatments for EBV malignancies. Complicating the matter, the CD4+ immune response comprises multiple subsets with separate functions and cytokine production (Figure 2)[19]. The results from this investigation may represent a model platform for increasing what is known about the CD4+ T cell response to viruses other than EBV.

4.1 – Optimisation of the ICS for nuclear transcription factors

In order to optimise staining, three cell fixing/permeabilising protocols for each antibody - 'PFA', 'FOXP3 staining kit' and 'eBiosciences' were investigated for the intracellular staining of three transcription factor-specific antibodies TBet GATA3 and FOXP3. Surprisingly, optimal intracellular staining was detected with separate protocols – TBet with the PFA, GATA3 with the eBiosciences and FOXP3 with the FOXP3 staining kit. However,

throughout the investigation the TBet staining was very weak (8.1% Figure 4C) based on results obtained previously in the literature, in which ~20% of the total CD4+ T cell pool are reported to express TBet[39]. GATA3 has been demonstrated within our lab to be expressed in low % of the total CD4+ T cell pool (unpublished data); however the staining obtained in this investigation (2.31% Figure 4B) was lower still. Due to time restrictions it was not possible to further optimise ICS for TBet and GATA3. However, the optimisation of FOXP3 staining was of greater success. The % of FOXP3 expressing CD4+ T cells obtained was in the range of 5-12% which is in line with previous literature of 5-10%[38]. The difference in staining obtained was unexpected given that all three are specific for nuclear transcription factors. This may be a result of the differing affinities of the antibodies.

4.2 - MHC II tetramer staining within healthy donors

Having optimised intracellular staining for transcription factors within total CD4+ T cells, these protocols were combined with surface staining using MHC II tetramers to analyse the CD4+ T cell subsets present within EBV-specific populations.

The frequencies of tetramer positive CD4+ T cells detected within the blood of healthy donors in this investigation specific for epitopes PRS (0.106%, 0.076% and 0.044% (Figure 7) and PAQ (0.026%, 0.027% and 0.056% Figure 8) were within the range as previously published for the same epitopes[11], demonstrating successful combination of the MHC class II staining with the transcription factor staining.

Similar to the optimisation experiments, the range of frequencies of the total CD4+ T cell population expressing TBet was 0.56 to 7.26% (Appendix Table 2 and 3). Unexpectedly, the percentage of TBet expressing CD4+tetramer+ cells detected was higher, and comparable to the 20% previously published[39]. For example 24.1% for PRS-specific cells (restricted through

DR52b), 23.5% for LTA and 16.1% and 14.7% for PRS (restricted through DR7) (Table A1 and A2). However these percentages may be inaccurate due to the low numbers of tetramer-positive cells involved in the analysis and low intensity of the TBet staining. For the GATA3 staining, the % of the total CD4+ T cell population expressing this TF ranged from 0.8 to 2.21% (Table A3 and A4) and the CD4+tetramer+ cells from 1.67 to 11.1% (Table A1 and A2) again similar to the frequencies seen in the optimisation experiments.

From previous literature it was expected to find that the majority of responses within healthy donors would be of the Th1 response[30; 31]. Bickham et al. detected IFN-γ producing CD4+ T cell responses to the EBNA1 protein in 18 /19 healthy donors. Leen et al. identified Th1 responses to the EBNA1, EBNA3C, LMP1 and LMP2 proteins with decreasing frequencies. Given the limitations of the TBet and GATA3, and the discrepancies seen with the expected frequency of TBet positive CD4+ T cells detected, the remainder of the analyses concentrated on determining the presence of FOXP3 expressing CD4+tetramer+ T cells only.

No systematic study of the presence of epitope specific FOXP3 expressing cells whether in healthy or IM donors, has yet been carried out. FOXP3 is a forked helix transcription factor that is very closely associated with the development of Treg CD4+ T cells[20]. This subset of CD4+ T cells has previously been shown, within an experimental setting, to control the proliferation of viral specific CD4+/CD8+ T cells generated from HIV, HCV and HBV infections[40; 41; 42]. This establishes them as key components of the development and maintenance of viral chronic infections. The manipulation of Tregs may represent a potential mechanism of controlling viral infections.

Overall in this study, in 8 donors, a mean frequency of 9.11% FOXP3-expressing cells within MHC II tetramer+ cells was detected. The range had a much larger spread than that of the

total CD4+ T cell population expressing FOXP3 (Figure 9A) from 4.17% to 16.7% and was in line with published results[43]. The total CD4+ T cell population remained~5% expressing FOXP3. This could be a result of different characteristics of T cells specific for the different epitopes or intrinsic properties of the proteins the epitopes are derived from, but is more likely due to the fact it is hard to define the true % of the FOXP3 expressing CD4+ tetramer+ T cells as this is limited by the lower number of tetramer positive cells within the total CD4+ T cell population. Despite this limitation, it is clear that FOXP3-expressing EBV epitope-specific CD4+ T cells are present in the blood of healthy EBV carriers.

A limited number of previous studies have attempted to analyse the presence of Tregs in the blood of healthy donors. A study by Voo et al, identified IL-10 production by CD4+ T cell lines specific for an epitope within the EBNA 1 protein[44]. These lines were also able to suppress CD4+/CD8+ T cells, indicating the possibility that some EBV epitopes could induce a Treg response[44]. Previous studies to detect the presence of FOXP3+ Treg cells against the LMP1 protein have also been carried out within the persistent infection setting[45]. The study utilised the PRG epitope from the LMP1 protein to stimulate PBMCs from seropositive patients. They found high production of IL-10 by CD4+ T cells specific for the PRG epitope within seropositive patients able to suppress IFN-γ production.

4.3 – MHC II tetramer staining within IM patients

The final part of this study involved preliminary analysis of the presence of FOXP3-expressing EBV-specific CD4+ T cells in the blood of two IM patients. The % of tetramer positive CD4+ T cells detected within both IM donors in this investigation (Figure 9) for epitopes VKL (0.682%) and PAQ (0.98%) in IM232 and for epitopes LTA (0.1%) and PRS (0.186%) in IM161 were in a similar range to previously published frequencies for these

epitopes in primary infection[11]. The study by Long et al. also observed higher staining with the latent than lytic epitopes and the % of CD4+ T cells that stained with the relevant tetramer. Interestingly, within both IM donors, a lack of EBV-specific CD4+ T cells expressing FOXP3 was observed within the tetramer positive population (Figure 10 and 11).

A further intriguing finding within this investigation was a decrease in the percentage of the total CD4+ T cell population that expressed FOXP3 within IM donor 232 compared to the healthy donors (Figure 7 and 8). If EBV-specific CD4+ T cells found in IM donors truly do not contain any FOXP3 positive cells, then it could be speculated that the sum of the expanded CD4+ T cell responses seen during IM may be large enough to alter the overall percentage of FOXP3-expressing CD4+ T cells within the peripheral blood. This could account for the smaller frequency of FOXP3-positive cells detected within the total CD4+ population of IM232 rather than an actual deduction in the Treg population.

It must be stressed that these results are based on the analysis of two EBV epitope-specific populations in two donors, and further analyses are required to confirm this finding. However, the results are backed up by two previous studies[46; 47]. Whilst neither study was able to analyse Tregs at an epitope level as in the present study, they both demonstrated that Tregs were decreased within the blood of IM patients as compared to non IM patients. Furthermore, the same frequencies were reflected in the tonsillar distribution of these cells demonstrating the decrease in peripheral Tregs could therefore not be accounted for by an accumulation within the tonsils (the site of primary EBV infection). The results of this project are supported by the second study by[47] where the authors describe a large Th1 response during the acute phase of disease with recovery being associated with the development of regulatory responses in four IM patients.

The results presented here have expanded upon previous studies by carrying out a systematic analysis of CD4+ T cells specific for peptides from a range of lytic and latent EBV proteins within 8 healthy and 2 IM patients using MHC II technology. The data indicate that EBV-specific FOXP3+ Treg cells may be present within healthy donors and absent in IM patients where T cell control of the virus is principally lost. This finding suggests a possible role for Tregs, within the context of primary EBV infection, in controlling the proliferation of EBV specific CD4+/ CD8+ T cells. Tregs have been identified as important in the suppression of over robust immune responses from both CD4+/CD8+ T cells, it may be logical to speculate that the lack of Tregs during IM might contribute to significant expansions of both these T cell subsets seen.

This investigation may provide us with a model hypothesis for the development of IM and indicate why some patients of similar ages develop IM and not others. It is possible that Tregs become activated early during acute primary infection and block the proliferative expansion of CD8+/CD4+ T cells that are characteristically seen in IM. The failure of this protective Treg response may play a part in combination with other genetic factors known to be linked with symptomatic infection such as NK cells and HLA type[48; 49] to the progression to IM.

To gain further insight into the EBV-specific CD4+ T cell subsets, cytokine staining should be combined with ICS for the nuclear transcription factors. CD4+ T cell subsets are particularly fluid and pleotropic and as a consequence some cells express more than one master regulator and thus express multiple combinations of cytokines. This combination could definitively aid the definition of the subsets present.

4.4 - Future Research

One of the objectives of this project was to understand the CD4+ T cell subsets involved in the control of EBV in healthy donors in order to facilitate the design of future immunotherapies for patients with EBV-associated disease. Although effective, around 50% of the EBV-associated malignancy patients treated with chemotherapy develop serious side effects, tumour relapse or secondary tumours [reviewed in[50]]. Each year, ~200 000 cases worldwide of EBV positive malignancies are diagnosed (Table 6). The reduction in cases arising from EBV associated malignancies via the use of an EBV prophylactive or therapeutic vaccine would undoubtedly improve public health and economic infrastructure.

Malignancy:	Cell of Origin	% Associated with EBV	Cases associated with EBV:
Burkitt Lymphoma: Developed Undeveloped	B cell	Undeveloped: AIDS 30-40% African 90% Developed: Sporadic 10-170%	6700: 100 6600
Gastric Carcinoma	Epithelial cell	10%	84 050
Hodgkin Lymphoma	B cell	40-80%	28 600
Nasopharyngeal Lymphoma	Squamous epithelial cell	90%	78 100
Total:			**196, 450**

Table 7 – The annual estimated new cases of EBV associated malignancies worldwide[50]

Clarification of the role of CD4+ T cell subsets in the control of EBV by identifying those peptides which stimulate Th1, Th2 or Treg responses during IM could facilitate opportunities for the manipulation of early EBV infection. Similarly, further understanding the characteristics of the CD4+ T cells present in healthy donors might help to direct future therapies to target epitopes with the greatest potential for controlling EBV patients with EBV-associated disease

4.4 – Final conclusion

To conclude the clarification of the range of the CD4+ T cell subsets responding to EBV during primary vs. persistent infection will provide opportunities to develop new therapies directed towards EBV associated malignancies. The success of therapy directed towards EBV associated malignancies may be enhanced by the manipulation of the immune response towards the ratios of T cell subsets seen in healthy carriers.

CHAPTER 5 - APPENDIX

Donor;	% Tetramer/TF positive CD4 cells														
	PAQ			PRS 7			PRS 52			SNP			VYG		
Latent	Tbet	Foxp3	Gata3	TBet	Foxp3	Gata3	Tbet	Foxp3	Gata3	TBet	Foxp3	Gata3	TBet	Foxp3	Gata3
1	-	-	-	-	9.6	-	-	-	-	-	-	-	-	10.6	-
2	-	-	-	-	-	-	24.1	12.2	0	18.7	7.14	0	-	-	-
3	-	-	-	-	-	-	5.74	9.11	7.29	17.5	12.5	1.75	-	-	-
4	-	-	-	-	-	-	-	-	-	2.91	4.23	1.26	-	-	-
5	8.1	0	0	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	0	16.7	5.26	4.94	12.21	3.41	-	-	-	0	9.71	1.67
7	-	-	-	16.1	8.65	0	-	-	-	-	-	-	0	5.67	11.1
8	-	-	-	14.7	4.17	6.67	2.08	9.59	6.42	-	-	-	-	-	-
Mean %:	**8.1**	**0**	**0**	**10.27**	**9.78**	**3.9767**	**9.215**	**10.778**	**5.7067**	**13.04**	**7.9567**	**1.0033**	**0**	**7.69**	**6.385**

Table A1 – Raw values of the % of CD4 T cells that are tetramer positive and transcription factor positive responding to the latent epitopes

Donor;	% CD4 tetramer/TF positive CD4 cells								
	LTA			VKL			SRD		
Lytic	TBet	Foxp3	Gata3	TBet	Foxp3	Gata3	TBet	Foxp3	Gata3
1	-	-	-	-	-	-	-	-	-
2	23.5	8.55	1.72	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-
5	-	-	-	10.5	4.17	1.89	-	-	-
6	3.03	9.22	3.57	-	-	-	-	-	-
7	-	-	-	-	-	-	0	18.5	6.67
8	6.67	9.59	5.8	-	-	-	-	-	-
Mean %:	**11.07**	**9.12**	**3.6967**	**10.5**	**4.17**	**1.89**	**0**	**18.5**	**6.67**

Table A2 – Raw values of the % of CD4 T cells that are both tetramer and transcription factor positive responding to the lytic epitopes

Donor;	% CD4/TF+ Cells (Total CD4)														
	PAQ			PRS 7			PRS 52			SNP			VYG		
Latent	TBet	Foxp3	Gata3	TBet	Foxp3	Gata3	Tbet	Foxp3	Gata3	TBet	Foxp3	Gata3	TBet	Foxp3	Gata3
1	-	-	-	-	2.03	-	-	-	-	-	-	-	-	2	-
2	-	-	-	-	-	-	0.76	4.85	1.2	0.573	3.95	1.28	-	-	-
3	-	-	-	-	-	-	0.517	4.54	1.24	0.935	4.24	1.24	-	-	-
4	-	-	-	-	-	-	-	-	-	7.26	3	2.21	-	-	-
5	1.01	4.17	1.58	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	2.33	4.1	1.42	4.6	5.02	1.42	-	-	-	2.85	4.78	1.46
7	-	-	-	0.889	5.18	1.36	-	-	-	-	-	-	0.562	4.31	0.797
8	-	-	-	2.18	3.85	0.869	1.76	3.64	0.694	-	-	-	-	-	-
Mean %:	**1.01**	**4.17**	**1.58**	**1.79967**	**4.3767**	**1.2163**	**1.909**	**4.5125**	**1.1385**	**2.9227**	**3.73**	**1.5767**	**1.706**	**4.545**	**1.1285**

Table A3 – Raw values of the % of CD4 T cells expressing the relevant transcription factor

Donor;	% CD4/TF+ Cells (Total CD4)								
	LTA			VKL			SRD		
Lytic	TBet	Foxp3	Gata3	TBet	Foxp3	Gata3	TBet	Foxp3	Gata3
1	-	-	-	-	-	-	-	-	-
2	0.696	4.63	1.06	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-
5	-	-	-	1.16	4.26	1.29	-	-	-
6	2.51	4.46	1.72	-	-	-	-	-	-
7	-	-	-	-	-	-	1.07	4.31	1.02
8	1.45	3.54	1.02	-	-	-	-	-	-
Mean %:	**1.552**	**4.21**	**1.2667**	**1.16**	**4.26**	**1.29**	**1.07**	**4.31**	**1.02**

Table A4 – Raw values of the % of CD4 T cells expressing the relevant transcription factor

CHAPTER 6 - BIBLIOGRAPHY

[1] RICKINSON, A. B.; E., K. Epstein-Barr virus. In: KNIPE, D. M.;HOWLEY, P. M., *et al* (Ed.). ***Field's Virology*** 5th Edition. Philadelphia: Lippincott, Williams & Wilkins, 2007.

[2] KUPPERS, R. B Cells Under Influence: Transformation of B Cells By Epstein-Barr Virus. ***Nature Reviews Immunology*** v. 3, p. 801-811, 2003.

[3] HISLOP, D. A. et al. Cellular Responses to Viral Infection in Humans: Lessons from Epstein-Barr Virus. ***Annual Reviews of Immunology,*** v. 25, p. 587-617, 2007.

[4] NEUHIERL, B.; DELECLUSE, H.-J. The Epstein-Barr Virus BMRF1 Gene Is Essential for Lytic Virus Replication. ***The Journal of Virology*** v. 80, p. 5078-5081, 2006.

[5] FIORINI, S.; OOKA, T. Secretion of Epstein-Barr Virus-encoded BARF1 oncoprotein from latently infected B cells. ***Virology Journal*** v. 5, p. 1-4, 2008.

[6] LEE, C.-P. et al. The ESCRT Machinery Is Recruited by the Viral BFRF1 Protein to the Nucleus-Associated Membrane for the Maturation of Epstein-Barr Virus. ***The Public Library of Science Pathogens,*** v. 8, p. 1-18, 2012.

[7] KRAUER, K. G. et al. The EBNA-3 gene family proteins disrupt the G2/M checkpoint. ***Oncogene,*** v. 23, p. 1342-1353, 2004.

[8] LONG, H. M.; TAYLOR, G. S.; RICKINSON, A. B. Immune defence against EBV and EBV-associated disease. ***Current Opinion in Immunology*** v. 23, p. 258-264, 2011.

[9] CALLAN, M. F. The immune response to Epstein-Barr virus. ***Microbes and Infection*** v. 10, p. 937-945, 2004.

[10] AMYES, E. et al. Characterization of the CD4+ T cell response to Epstein-Barr virus during primary and persistent infection. ***Journal of Experimental Medicine*** v. 198, p. 903-911, 2003.

[11] LONG, H. M. et al. MHC II tetramers visualize human CD4+ T cell responses to Epstein-Barr virus infection and demonstrate atypical kinetics of the nuclear antigen EBNA1 response. ***The Journal of Experimental Medicine*** v. 210, p. 933-950, 2013.

[12] ALTMAN, J. D. et al. Phenotypic Analysis of Antigen-Specific T Lymphocytes. ***Science,*** v. 274, p. 94-96, 1996.

[13] CROFT, P. et al. Stage-Specific Inhibition of MHC Class I Presentation by the Epstein-Barr Virus BNLF2a Protein during Virus Lytic Cycle. ***Public Library of Science*** v. 5, p. 1-13, 2009.

[14] ROWE, M.; ZUO, J. Immune responses to Epstein-Barr virus: molecular interactions in the virus evasion of CD8+ T cell immunity. ***Microbes and Infection*** v. 12, p. 173-181, 2010.

[15] ZUO, J. et al. The Epstein-Barr virus-encoded BILF1 protein modulates immune recognition of endogenously processed antigen by targeting major histocompatibility complex class I molecules trafficking on both the exocytic and enocytic pathways. ***The Journal of Virology*** v. 85, p. 1604-1614, 2011.

[16] PUDNEY, V. A. et al. CD8+ immunodominance among Epstein-Barr virus lytic cycle antigens directly reflects the efficiency of antigen presentation in lytically infected cells. ***The Journal of Experimental Medicine,*** v. 201, p. 349-360, 2005.

[17] MARSHALL, N. B.; SWAIN, L. S. Cytotoxic CD4 T Cells in Antiviral Immunity. ***Journal of Biomedicine and Biotechnology,*** v. 2011, p. 1-8, 2011.

[18] SWAIN, S. S.; MCKINSTRY, K. K.; STRUTT, T. M. Expanding roles for CD4+ T cells in immunity to viruses. ***Nature Reviews in Immunology,*** v. 12, p. 136-148, 2012.

[19] NAKAYAMADA, S. et al. Helper T cell diversity and plasticity. ***Current Opinion in Immunology*** v. 24, n. 297-302, 2012.

[20] O'GARRA, A.; VIEIRA, P. Regulatory T cells and mechanisms of immune system control. ***Nature Medicine,*** v. 10, n. 801-805, 2004.

[21] ROBERTSON, S. J.; HASENKRUG, K. J. The role of virus-induced regulatory T cells in immunopathology. ***Springer Seminars in Immunopathology*** p. 51-62, 2006.

[22] MCGEACHY, M. J. et al. Interleukin 23 receptor is essential for terminal differentiation of effector T helper type 17 cells in vivo. ***Nature Immunology*** v. 10, p. 314-324, 2009.

[23] MAKEDONAS, G.; BETTS, M. R. Polyfunctional analysis of human t cell responses: importance in vaccine immunogenicity and natural infection. ***Springer Seminars in Immunology*** v. 28, p. 209-219, 2006.

[24] KHANNA, R. et al. Class I processing-defective Burkitt's lymphoma cells are recognized efficiently by $CD4^+$ EBV-specific CTLs. ***The Journal of Immunology*** v. 158, p. 3619-3625, 1997.

[25] HAQUE, T. et al. Allogenic cytotoxic T-cell therapy for EBV-positive posttransplantation lymphoproliferative disease: results of a phase 2 multicenter clinical trial. ***Blood*** v. 110, p. 1123-1131, 2007.

[26] MORALES, O. et al. EBV Latency II-derived Peptides Induce A Specific CD4+ Cytotoxic T-cell Activity and Not A CD4+ Regulatory T-cell Response. ***The Journal of Immunotherapy*** v. 35, p. 254-266, 2012.

[27] LONG, H. M. et al. CD4+ T-Cell Responses to Epstein-Barr Virus (EBV) Latent-Cycle Antigens and the Recognition of EBV-Transformed Lymphoblastoid Cell Lines. ***Journal of Virology*** v. 79, p. 4896-4907, 2005.

[28] GUILLAUME, P.; DOJCINOVIC, D.; LUESCHER, I. F. Soluble MHC-peptide complexes: tools for the monitoring of T Cell responses in clinical trials and basic research. ***Cancer Immunology,*** v. 9, p. 7-12, 2009.

[29] NEPOM, G. T. MHC Class II Tetramers. ***The Journal of Immunology,*** v. 188, p. 2477-2482, 2012.

[30] LEEN, A. et al. Differential Immunogenicity of Eptsein-Barr Virus Latent-Cycle Proteins for Human CD4+ T-Helper 1 Responses. ***Journal of Virology*** v. 75, p. 8649-8659, 2001.

[31] BICKHAM, K. et al. EBNA1-specific CD4+ T cells in healthy carriers of Epstein-Barr virus are primarily Th1 in function. ***The Journal of Clinical Investigation*** v. 107, p. 121-130, 2001.

[32] ATTARBASCHI, T. et al. T cell cytokine profile during primary Epstein-Barr virus infection (infectious mononucleosis). ***European Cytokine Network,*** v. 14, p. 34-39, 2003.

[33] PRECOPIO, M. L. et al. Differential Kinetics and Specificity of EBV-Specific CD4+ and CD8+ T cells during Primary Infection. ***The Journal of Immunology*** v. 170, p. 2590-2598, 2003.

[34] LONG, M. H. et al. Cytotoxic CD4+ T cell Responses to EBV Contrast with CD8 Responses in Breadth of Lytic Cycle Antigen Choice and in Lytic Cycle Recognition. ***The Journal of Immunology*** v. 187, p. 92-101, 2011.

[35] WILSON, A. D. et al. Virus-specific cytotoxic T cell responses are associated with immunity of the cottontop tamarin to Epstein-Barr virus (EBV). ***Clinical and Experimental Immunology,*** v. 103, p. 199-205, 1996.

[36] MISKO, I. S. et al. Composite response of naive T cells to stimulation with the autologous lymphoblastoid cell line is mediated by CD4 cytotoxic T cell clones and includes an Epstein-Barr virus-specific component. ***Cellular Immunology*** v. 132, p. 295-307, 1991.

[37] PALUDAN, C. et al. Epstein-Barr Nuclear Antigen 1-Specific CD4 + Th1 Cells Kill Burkitt's Lymphoma Cells. ***The Journal of Immunology*** v. 169, p. 1593-1603, 2002.

[38] SAKAGUCHI, S. et al. Immunogenic self-tolerance mantained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. ***Journal of Immunology*** v. 155, p. 1151-1164, 1995.

[39] DURAMAD, P. et al. Flow Cytometric Detection of Intracellular Th1/Th2 Cytokines Using Whole Blood: Validation of Immunologic Biomarker for Use in Epidemiologic Studies. **Cancer Epidemiology, Biomarkers & Prevention,** v. 13, p. 1452-1458, 2004.

[40] KINTER, A. L. et al. CD25(+)CD4(+) regulatory T cells from the peripheral blood of asymptomatic individuals regulate CD4(+) and CD8(+) HIV-specific T cell immune responses in vitro and are associated with favourable clinical markers of disease status. ***Journal of Experimental Medicine*** v. 200, p. 331-343, 2004.

[41] RUSHBROOK, S. M. et al. Regulatory T cells suppress in vitro proliferation of virus-specific CD8+ T cells during persistent hepatitis C virus infection. ***Journal of Virology*** v. 79, p. 7852-7859, 2005.

[42] XU, D. et al. Circulating and liver resident CD4+CD25+ regulatory T cells actively influence the antiviral immune response and disease progression in patients with hepatitis B. ***Journal of Immunology*** v. 177, p. 739-747, 2006.

[43] SAKAGUCHI, S. et al. Foxp3 CD251 CD4 natural regulatory T cells in dominant self tolerance and autoimmune disease. ***Journal of allergy and Clinical Immunology*** v. 212, p. 8-27, 2006.

[44] VOO, K. S. et al. Functional characterisation of EBV-encoded nuclear antigen 1-specific CD4 helper and regulatory T cells elicited by in vitro peptide stimulation. ***Cancer Research,*** v. 65, p. 1577-1586, 2005.

[45] MARSHALL, N. A.; VICKERS, M. A.; BARKER, R. N. Regulatory T cells secreting IL-10 dominate the immune response to EBV latent membrane protein 1. ***Journal of Immunology*** v. 170, p. 6183-6189, 2003.

[46] WINGATE, P. J. et al. Regulatory T Cell Activity in Primary and Persistent Epstein-Barr Virus Infection. ***Journal of General Virology,*** v. 81, p. 870-877, 2009.

[47] MARSHALL, N. A. et al. CD4(+) T-cell responses to Epstein-Barr virus (EBV) latent membrane protein 1 in infectious mononucleosis and EBV associated non-Hodgkin lymphoma: Th1 in active disease but Tr1 in remission. ***British Journal of Heamatology*** v. 139, p. 81-89, 2007.

[48] MCAULAY K. A. et al. HLA class I polymorphisms are associated with development of infectious mononucleosis upon primary EBV infection. ***Journal of Clinical Investiagtion*** v. 117, p. 3042-3048, 2007.

[49] WILLIAMS, H. et al. The immune response to primary EBV infection: a role for natural killer cells. **British Journal of Haematology,** v. 129, p. 266-274, 2005.

[50] COHEN, J. I. et al. Epstein-Barr Virus: An Important Vaccine Target for Cancer Prevention. ***Science Translational Medicine,*** v. 3, p. 1-3, 2011.

Characterisation of CD8+ T cell responses against CMV derived antigens protected in wild type strain infection

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Louise Hosie

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UNIVERSITY OF BIRMINGHAM

Institute of Cancer Research UK

School of Cancer Sciences

University of Birmingham

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I hereby declare that the work contained in this thesis is of my own, unless directly stated, under the direct supervision of Dr Annette Pachnio and Prof. Paul Moss

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List of Abbreviations

AIDS – Acquired Immunodeficiency Syndrome

CMV – Cytomegalovirus

DC –Dendritic cell

DMSO – Dimethyl sulfoxide

E – Early Protein

ER – Endoplasmic reticulum

ELISA – Enzyme linked immunosorbent assay

ELISPOT – Enzyme linked immunospot assay

ICS – Intracellular staining

IE – Immediate Early protein

IFN – Interferon

IL – Interleukin

IRP – Immune risk phenotype

HIV – Human immunodeficiency virus

HLA – Human leukocyte antigen

MHC – Major Histocompatibility Complex

NK – Natural killer

ORF – Open reading frame

PBMCs – Peripheral Blood Mononuclear Cells

PFA – Paraformaldehyde

SEB – Staphylococcus enterotoxin B

TCR – T Cell Receptor

TNF – Tumour necrosis factor

WT – Wild type

Abstract

In CMV-seropositive elderly donors virus-specific CD8+ T cells against the pp65 and IE-1 proteins can occupy up to 40% of the total CD8+ T cell pool. CMV encodes for several immunomodulatory proteins that interfere with the MHC class I antigen presenting pathway. Studies using an immunevasion-deleted virus strain (RV798) revealed a large proportion of CMV-specific T cell responses are evaded by cells infected with WT virus, but are primed *in vivo*. This study undertook the first functional and phenotypical characterisation of CD8+ T cells in young and elderly donors responding to CMV-derived epitopes identified using the deletion mutant. These CD8+ T cells seem to accumulate within the elderly reaching up to 32%, against a HLA-C restricted epitope, of the total CD8+ T cell pool producing IFN-γ, TNF-α and little IL-2. The CD8+ T cells were CD45RA+/CD57+/CD28-/CCR7- and therefore of the EMRA compartment. *In vitro* they recognised their antigen in the context of RV798 infection, but not wild type (WT) infection. In summary the CD8+ T cells specific for the 'protected' antigens studied here display very similar characteristics compared to previously studied CMV-specific CD8+ T cells. What is currently unclear is the role *in vivo* they play in controlling the virus.

CHAPTER 1 - INTRODUCTION

1.1 – Cytomegalovirus

Cytomegalovirus (CMV) is a member of the β-herpesviruses, a sub family of the *Herpesviridae* (Reviewed[1; 2]). It is a large double stranded DNA virus whose genome is enclosed by a capsid surrounded by a tegument layer and enveloped by a bilipid membrane containing glycoprotein complexes. The hCMV genome is ~230 Kb of DNA in size encoding for >160 proteins. Around 60% of the adult population are infected with this virus (up to 90% depending upon geographic location and socioeconomic status) with primary infection usually being asymptomatic. CMV is able to infect a wide range of host cells such as: endothelial cells, epithelial cells, fibroblasts and dendritic cells[3]. Persistence is established in CD34+ hematopoietic progenitor cells [4] and maintained in the myeloid lineage. Reactivation of the virus is very closely linked with the differentiation of dendritic cells (DCs) and their activation status[5]. Transmission into naïve hosts occurs via bodily fluids such as saliva, urine and also via breastfeeding or intrauterine. In immunocompetent individuals this is kept under control by the immune system. However, this virus causes serious and even fatal disease and organ damage within the immunocompromised host (such as AIDS or transplant patients and during congenital infection) as a result of uncontrolled viral replication. The use of the murine model and studies within humans have identified T cell immunity, in particular CD8+ T cells, as the critical component in controlling CMV infection, limiting virus spread and reducing clinical manifestations[1]. Even though infection is generally asymptomatic in immune competent individuals, hCMV is now increasingly being accepted as a major contributor to the development of senescence of the immune

system[6], poor responses to viral vaccines such the influenza vaccine [7] and the development of cardiovascular diseases[8].

1.2 – Immunity to CMV

Both the innate and adaptive arms of the host immune system play a recognised role in controlling CMV dissemination and replication. These include type I interferons produced by dendritic cells and macrophages which leads to the activation of Natural killer (NK) cells[9], production of neutralising antibodies[10] and both CD4+/CD8+ T cells [11]. The T cell mediated immune response to hCMV seems to play a major role in the control of the virus as their loss due to immune suppression frequently leads to viral reactivation[12].

All of the hCMV encoded proteins are potential targets for the CMV-specific T cell response. The most extensive study of the CMV-specific T cell response to date was done by Sylwester et al, which involved the analysis of antigen-specific T cell activation using 213 ORFs encoded by the hCMV genome. This for the first time provided a global picture of the virus-specific T cell responses and an idea of the extent to which it occupies the overall peripheral T cell pool[13]. Of these 213 ORFs screened, a total of 151 ORFs induced T cell responses by either CD4+ or CD8+ T cells or both as measured by IFN-γ production. The average total CD8+ T cell responses to CMV was 7.6%, with each donor recognising an average of 21 ORFs.

Despite the large number of potential antigenic targets for the CD8+ response, most studies to date have focused on two immunodominant proteins namely IE-1, as it is one of the first proteins expressed in the virus life cycle, and pp65, the major tegument protein which comprises a large part of the virion. There have been recent development of efficient and more sensitive methods, such as MHC class I (MHC I) tetramer technology, the enumeration of *ex vivo* cytokine responses post-stimulation with overlapping peptides and 'Enzyme-linked immunospot assays' (ELISPOT). These have allowed the direct detection and a more detailed study of antigen-specific CD8+ T cells. Thus, knowledge regarding the breadth of the hCMV-specific CD8+ T cell response has increased considerably.

It is now known that hCMV infection occupies a large percentage of the peripheral T cell pool[14; 15; 16] and that this expansion accumulates substantially with age[17]. The study by Vescovini et al. demonstrated that the expansion of CMV-specific Th1 like CD8+ T cells is further amplified within the extremely elderly (90-100 years). Within healthy middle aged patients CMV infection can occupy up to 40% of the total T cell pool compared to 2-4% within younger patients. This accumulation termed 'memory inflation'[15] has been most extensively demonstrated within murine models of CMV infection[2]. Utilising this model T cells specific for certain epitopes have been demonstrated to increase with time and stabilise at high frequencies – known as 'inflationary epitopes' (e.g. M38 and IE3 proteins). Other epitopes have been shown to elicit small responses that remain so over time – known as 'stable epitopes' (e.g. M45 and M57 proteins)[16]. It is currently not very clear whether the same distinction can be made in humans between stable and inflationary epitopes.

The responses to CMV epitopes have been shown within both mice and humans to have a characteristic phenotype which is marked by the expression of CD45RA and CD57 and the loss of CD28 and CCR7 expression. It is difficult to analyse the primary response to CMV as the time point of natural infection is hard to detect due to the asymptomatic nature of primary infection. A study by Van de Berg et al. utilised CMV negative renal transplant recipients to monitor their first encounter with the virus and phenotypically characterise the responding T cells overtime[8]. A change in surface phenotype from $CD45RA^{-}CD27^{+}CCR7^{-}$ (central memory) to the loss of CD28/CD27 and the regaining of the naïve isoform CD45A (revertant memory) was observed.

As CMV-specific CD8+ T cells lack the expression of the co-stimulatory molecules CD27 and CD28 they produce reduced levels of IL-2 and have previously been thought to be dysfunctional[18]. Khan et al. showed that 90% of CMV-specific CD8+ T cells within the elderly did not express CD69, CD38 or HLA-DR which are characteristic of activated T cells but were perforin positive and therefore cytotoxic[19]. As these CMV-specific T cells still express significant amounts of effector molecules, others have hypothesised that these cells represent 'late stage effector cell' rather than being dysfunctional. Khan et al. also demonstrated that within elderly patients, there was a marked 32% increase in oligoclonality of the CMV-specific expansions[19]. The oligoclonality has been proposed to be the result of repeated antigen exposure throughout the patient's life time. Further supporting the functionality of these expansions, CD8+ T cells expressing IFN-γ and TNF-α were largely increased within responses to the CMV

protein pp65[20]. It is now generally accepted that these inflated CMV-specific CD8+ T cell responses are extensively differentiated but retain their functional capabilities[8; 16; 19; 20; 21].

Memory inflation has been hypothesised to restrict the naive CD8+ pools and consequently impair the inability of individuals to mount a sufficient primary adaptive response to new antigens. CMV-seropositivity has been correlated with inefficient responses to the influenza vaccine[7]. Furthermore, the accumulation of $CD57^{+}/CD28^{-}$ CD8+ T cells has been associated as one of the major indicators of immune senescence within the elderly and this in combination with CMV-seropositivity has been identified as an 'Immune Risk Phenotype' (IRP) that has been linked to premature death with patients >80 years of age. It has been further hypothesised that the large number of cytotoxic CD8+ T cells within seropositive individuals can enhance the severity of vascular diseases for example atherosclerosis (discussed in[8]).

1.3 CMV mediated immune evasion

CMV is not cleared from the host but persists for life. Therefore a fine balance between the virus and host has evolved over millions of years. Despite being recognised by the immune system, CMV has developed multiple mechanisms to evade both innate and adaptive recognition[22].

One of the main strategies of CMV to evade innate immunity is to avoid activation of NK cells. As numerous mechanisms work to down-regulate MHC I and II molecules from the surface this would lead to NK cell activation based on the missing-self theory. Ways the virus employs to circumvent this include expression of MHC I homologues like UL18[23] on the surface of infected cells or binding of ligands of NKG2D – an activating NK cell receptor – by UL16 to retain them intracellularly[24]. Additionally, hCMV encodes an IL-10 homologue that has been reported to lead to a long term decrease in the pools of NK cells allowing the virus to establish its persistent infection[25].

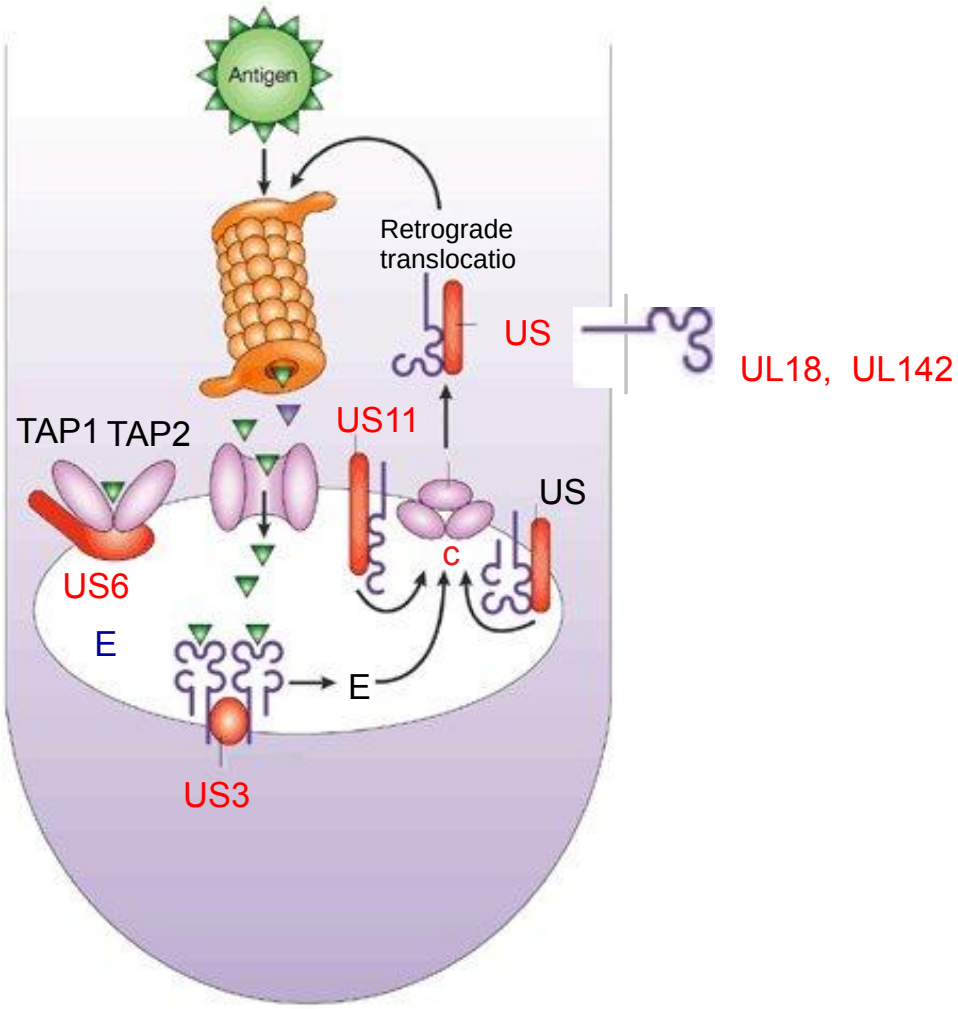


Figure 1 – Immunevasion mechanisms of the US2-US11 genes encoded by hCMV. Adapted from[1]. The US genes are indicated by red rods or in the case of USE a red circle. MHC I heavy chains are indicated in dark purple. TAP1and TAP2 gene products are indicated in light purple.

However, best understood to date is CMV immunevasion of CD8+ T cell recognition of infected cells. Cells infected with hCMV have down regulated levels of 'Major Histocompatibility Class I' (MHC I) as a consequence of the US2-11 genes encoding for immunosubversive proteins. The US2-US11 gene products act in concert to down-regulate MHC I from the cell surface and to prevent the generation of CMV peptide loaded complexes (Figure 1). The overall effect is to limit presentation of viral antigens to the CD8+ T cells (individual functions summarised in Table 1).

Antigen	Function
US2	Relocation of MHC I heavy chains from the ER for proteasomal degradation
US3	Retention of MHC I heavy chains within the ER
US6	Prevents the translocation of peptides from the cytosol to the ER via TAP
US11	Relocation of MHC I heavy chains from the ER for proteasomal degradation

Table 1 – MHC Class I subverting immunoevasins encoded by hCMV[22]

A CMV-strain deleted for US2-US11termed 'RV798'[26] was used by Paul Moss' and Stanley Riddell' labs to study T cell responses against virus infected cells. This was compared to T cell responses induced by fibroblasts infected with WT strains[27; 28]. Results showed that the responses after stimulation with the RV798 strain were up to 10-fold and 40-fold (Khan and Manley labs respectively) increased compared to those with the WT.

Stan Riddell and colleagues hypothesised that the presence of the US genes 'protected' certain CMV antigens from presentation to the CD8+ T cells during natural infection. They then employed the RV798 strain to further analyse CMV-specific CD8+ T cell responses to proteins from across the hCMV genome[28]. Using this CMV strain, Riddell et al. identified new peptide-epitopes derived from a number of CMV-encoded proteins, confirming that responses are directed against a broad range of antigens expressed in the early and late phases of the virus life cycle (see Table 3 Materials and Methods). Their results demonstrate that the down-regulation of MHC I affects the recognition of CMV-epitopes by CD8+ T cells within infected cells.

1.4 Aims

Based on the study using the CMV-strain RV798 which is deleted of the immune evasion proteins, Riddell at al. identified a number of peptide epitopes which are 'protected' from T cell recognition in WT infected cells. Currently very little is known about the characteristics of these T cell responses in carriers of CMV. Therefore, the aims of the study were as follows:

1. To determine whether CD8+ T cells specific for these peptides can be detected in healthy individuals

2. To study whether the magnitude of these responses differs between young and older individuals i.e. accumulate with age

3. To characterise peptide specific CD8+ T cells with regard to functionality (cytokine production) and phenotype

4. To determine whether T cells specific for these peptides are able to recognise processed antigen

5. To phenotypically characterise these CMV-specific CD8+ T cells to assert whether they have the same characteristic phenotype as previously described for CD8+ expansions to other epitopes

6. To address whether these newly identified epitopes are recognised by cognate CD8+ T cell clones during WT CMV infection of fibroblasts vs. infection with the RV798 mutant virus.

Cryopreserved PBMCs from young and elderly seropositive donors were stimulated with the relevant epitopes and the CD8+ T cell responses measured by the detection of intracellular IFN-γ, TNF-α and IL-2 production.

CHAPTER 2 – MATERIALS AND METHODS

Reagent	**Company**
1X Phosphate Buffered Saline (PBS)	Dulba
4% Para formaldehyde in PBS	Sigma
RPMI 1640	Gibco
Penicillin/Streptamycin	Gibco
Saponin 4% stock in PBS	Sigma
MACS Buffer (1X PBS, 0.5% BSA, 2mM EDTA)	In House
Fetal Calf Serum (FCS)	PAA
Human Serum (HuS)	TCS
Lymphocyte Separation Media	PAA
MLA144 cells	Rabin et al 1981
Media	**Components**
Culture media	RPMI 1640 10% FCS 1% P/S
T cell media	RPMI 1640 30% MLA 10% FCS 1% HuS 1% P/S 50U/ml IL-2
Cloning media	RPMI 1640 10% FCS 1% HuS 1% P/S
ELISA reagents	**Company**
Anti IFN-γ antibody	Thermo Scientific
Biotinylated anti IFN-γ antibody	Thermo Scientific
ExtraAvidin Peroxidase	Sigma
TMB	Rockland
Blocking Buffer – 1 x PBS, 0.05% TWEEN, 1% BSA	In House
Wash Buffer – 1 x PBS, 0.05% TWEEN	In House

Antigen	**Fluorchrome**	**Company**	**Volume/test**	**Clone**
CD3	APC	Invitrogen	2 µl	S4.1
CD8	AmCyan	BD Biosciences	1 µl	SK1
CD4	PE	BD Pharmingen	2 µl	RPA-T4
CD57	PE	Biolegend	3 µl	HCD57
CD3	Pacific Blue	eBioscence	0.5 µl	OKT3
CCR7	FITC	R & D	10 µl	150503
CD27	FITC	BD Pharmingen	2 µl	M-T271
CD4	Pe Cy7	eBioscience	1 µl	RPA-T4
CD8	PerCp-Cy5.5	eBioscience	2 µl	RPA-T8
CD28	PerCP-Cy5.5	BD Biosciences	2 µl	L293
IFN-γ	FITC	Biolegend	2 µl	4S.B3
TNF-α	Pe Cy7	eBioscience	1 µl	MAb11
IL-2	PE	Biolegend	10 µl	MQ1-17H12
Viability Dye	**Fluorchrome**	**Company**	**Volume/test**	
LIVE/DEAD Fixable Dead Cell Stain	Violet fluorescent reactive dye	Invitrogen	1µl of 1:100 dilution	

Table 2 – Regents used throughout the investigation

CMV Antigen	location	function	CTL Epitope	HLA Restricting Allele	Kinetics of Expression
$UL16_{162-170}$	glycoprotein	NK cell evasion	YPRPPGSGL	B0702	E
$UL17_{12-21}$	unknown		RPRHCRLEML	B0702	E
$UL52_{349-358}$	matrix or tegument	unknown	SPSRDRFVQL	B0702	L
$UL69_{569-578}$	tegument	cell-cycle arrest (G1 – S) transport of unspliced RNA from nucleus	RTDPATLTAY	A0101	E/L
$UL105_{715-723}$	?	proposed helicase activity, DNA replication	YADPFFLKY	A0101	E
$US23_{65-73}$	matrix or tegument	unknown	IPHNWFLQV	B5101	E
$UL122_{466-475}$	IE2	replication, inhibition of apoptosis	NEGVKAAWSL	B4403	IE
$UL56_{503-511}$	matrix or tegument	involved in DNA encapsidation	DARSRIHNV	B5101	E
$UL36_{51-60}$	(matrix or) tegument	inhibitor of caspase activity (apoptosis)	RSALGPFVGK	A1101	E
$UL36_{7-21}$			TLMAYGCIAIR AGDF	B5101	E
$UL36_{78-88}$			HPFGFVEGPGF	B3501	E
$UL23_{34-42}$	tegument	unknown	WPKDRCLVI	B5101	?
$UL23_{162-171}$			RATYRGRLMV	Cw1502	?
$UL28_{327-335}$	unknown	unknown, encodes spliced mRNA?	FRCPRRFCF	Cw0702	?
$UL33_{120-128}$		putative chemokine receptor (G-coupled receptor) --> signalling	SYRSTYMIL	Cw0702	E
$UL24_{120-134}$	tegument		YLCCQTRLAFV GRFV	Cw1601	E/L
$UL84_{3-11}$		suggested role in DNA-synthesis	RVDPNLRNR	A0301	E

Table 3 – HLA restricted CMV epitopes (Stan Riddell – unpublished data).

2.1 - Preparation of PBMC's from healthy donor blood

15 young donors aged 27 to 59 years and 16 elderly donors from the '1000 elders cohort' aged 68 to 86 years were analysed (Appendix Table 7 and 8). Written consent was taken.

'Peripheral blood mononuclear cells' (PBMCs) were isolated from whole blood by density gradient centrifugation. Seropositive heparinised blood was diluted 1:1 with RPMI and layered onto 15ml of Lymphoprep. This was centrifuged at 1800 rpm for 30 minutes at 22^{o}C with the brakes off. Lymphocytes were very carefully aspirated off the interface between the lymphoprep and plasma/RPMI and washed with 30ml of fresh RPMI to remove ficoll (spun at 2000 rpm for 10 minutes and 22^{o}C). Cells were washed again in RPMI and resuspended in culture medium (materials) for counting on a haemocytometer. The pellet was resuspended in freezing medium containing 10% FCS and 1 x 10^6 cells transferred to a cryovial. Cryovials were kept at -80 overnight before transfer into liquid nitrogen for long term storage.

2. 2 - Peptide stimulation of PBMCs

To assess T cell responses, peptide stimulations were set up. For this, PBMCs were thawed in culture medium, centrifuged at 1600 rpm for 5 minutes and resuspended within 1-2ml for counting. 1 x 10^6 cells per stimulation were used in a total volume of 500µl culture medium. Relevant peptide was added to the tubes at a final concentration of 1µg/ml. 10µl of 'Staphylococcus enterotoxin B' (SEB) served as a positive control (final concentration 0.2µg/ml) and one tube was left unstimulated

(negative control). Brefeldin A (BfA; 10µg/ml final concentration) was added to block cytokine secretion. The tubes were incubated at 37^{o}C for 6 hours.

2.3 Intracellular cytokine staining

Following stimulation, PBMCs were stained to detect the production of cytokines by activated T cells. PBMCs were washed once in PBS and centrifuged at 2000rpm at 10^{o}C for 5 minutes (all washes were done in 4ml and centrifuged as just described). Viability dye (materials) was added and tubes left to incubate for 15 minutes room temperature in the dark before one wash in PBS and one wash in MACS buffer. Antibodies staining surface molecules were added and tubes incubated for 15 minutes at 4^{o}C in the dark after which they were washed in MACS buffer. To fix the cells, 4% PFA was added, vortexed well and incubated for 15 minutes at room temperature in the dark. The PFA was washed off once with MACS buffer followed by permebilisation with with 0.5% saponin. After 5 minutes, intracellular antibodies were added and left to incubate for 30 minutes at room temperature in the dark. Cells were washed with MACS buffer and left at 4^{o}C until analysis on the LSR. Data analysis was performed using BD FACS Diva analysis software.

2.4 CD8 T cell cloning

CD4 depleted PBMC were stimulated with the relevant peptide (final concentration 1µg/ml) for 3 hours to induce activation and IFN-γ production of specific T cells. These were then enriched using an IFN-γ- secretion assay cell enrichment and detection kit (Miltenyi Biotec) before limiting dilution cloning.

2.4.1 IFN-γ capture assay

After the 3 hour stimulation, cells were washed twice in culture medium. The supernatant was removed and the pellet resupsended in 120µl of cold medium and 30µl of IFN-γ catch reagent added. These were incubated on ice for 5 minutes before addition of 10ml warm cloning medium and further 45minutes incubation at 37^{o}C while rotating.

Cells were then washed with 15ml cold MACS buffer and centrifuged at 2000 rpm for 5 minutes at 4^{o}C. The pellet was resuspended in 120µl MACS buffer. 30µl of anti IFN-γ catch reagent conjugated to PE added and left to incubate at 4^{o}C for 10 minutes in the dark.

Following this, cells were washed as before and resuspended in 120µl MACS buffer and 30µl of anti PE microbeads added and left to incubate for 20 minutes at 4^{o}C in the dark.

Unbound microbeads were washed off and labelled cells resuspended in 500µl of MACS buffer. IFN-γ producing cells were then enriched using a MACS-separator according to manufacturer's instructions. Cells of the positive fraction were collected and counted before use in limited dilution cloning.

2.4.2 Limiting Dilution Cloning

PBMCs isolated from Buffy preparations were PHA treated (final concentration 10µg/ml) for 1 hour before irradiation with 4000 rads – these served as a feeder layer

in the cloning procedure. Autologous LCLs were peptide pulsed (with the corresponding peptide) for 1 hour at 37^{o}C before also being irradiated with 4000 rads to prevent proliferation – these served as antigen-presenting cells.

Per 10 plates, 100ml of cloning mixture (materials) were prepared consisting of:

- 100 x 10^6 Buffy cells
- 10 x 10^6 LCLs

100µl/well were plated out.

For the limiting dilution cloning of cells retrieved in the positive fraction (in section 2.4.1), 10 plates (96-well round bottom) with 0.3 cells/well and 10 plates with 3 cells/well were seeded for each specificity.

The limiting dilution cultures of LCLs and T cells were then 'fed' on day 2 with 100µl/well media (60% MLA, 10% FCS, 1% HuS, 1% P/S and 100U/ml IL-2) and left at 37^{o}C for 2 weeks.

2.5 – Assessment of peptide specificity following limiting dilution cloning

2 weeks after cloning ,wells were identified that showed cell expansion and tested for peptide specificity. For this, 50µl was taken from the selected wells and co cultured with peptide-pulsed or DMSO-pulsed autologous LCL at 37^{o}C overnight in duplicate.

ELISA plates were coated over night with an anti IFN-γ antibody. The following day, plates were blocked for 1 hour with blocking buffer (materials) at room temperature then washed three times in wash buffer (materials). 50µl supernatant from the overnight co-cultures was added to the relevant wells and left to incubate for 2 hours at room temperature. The IFN-γ standard was also added to the wells at this point. A

second biotinylated antibody that binds IFN-γ was then added to the wells at 3.75µl per plate and left to incubate for 2 hours at room temperature. A 1:2 dilution series of IFN-γ starting at 2000pg/ml served as a standard curve. Following six washes in wash buffer a biotinylated anti IFN-γ antibody was added and left to incubate for 1 hour at room temperature. After this, plates were washed six times in wash buffer and 50µl of peroxidase-conjugated ExtraAvidin (1:1000 in wash buffer) per well for 30 minutes at room temperature. This was washed off in wash buffer eight times; TMB substrate solution was added and allowed to develop for up to 30 minutes before stopping the colour development by the addition of IM hydrochloric acid. Absorbance was measured at 450nm.

Specific clones were transferred to and propagated on 24 well plates for further use and cryopreservation. The clones were maintained in 2ml T cell media (materials) with the addition of 1 x 10^6 PHA treated, irradiated Buffy cells and 1 x 10^5 peptide pulsed, irradiated LCLs.

2.6 – Fibroblast Recognition Assay

1.5 x 10^4 fibroblasts expressing HLA-A1 per well of a 96 well plate were seeded and left to adhere overnight. Fibroblasts were then infected with either the AD169 or RV798 strains at an M.O.I. of 0.5 or 0.05 for 48 hours. After 48 hours, RTD and YAD-specific CD8+ T cells were added at 10000 cells per well and left overnight. T cell recognition was assessed by IFN-γ detection in an ELISA of the supernatant of the co-cultures.

CHAPTER 3 - RESULTS

3.1 – Titrations of the intracellular cytokine antibodies

The first aim of the study was to identify CD8+ T cells which respond to the investigated epitopes by cytokine production. These were detected by flow cytometry using intracellular cytokine staining (ICS). For two of the three cytokine-specific antibodies, anti-IFN-γ and anti-TNF-α it was necessary to determine the appropriate concentration to stain with. For this purpose PBMCs were stimulated with SEB, stained with CD8-PerCP-Cy5.5 and decreasing concentrations of the cytokine-specific antibodies ranging from 5µl (manufacturer's recommendation) to 0.5µl. Results of the antibody titrations are depicted in Figure 2.

For the analysis, single cells were gated on followed by selection of the lymphocytes (Figure 2A). The lymphocyte gate was then further analysed for the expression of CD8 and production of cytokine (Figure 2B). To calculate the response size of CD8+ T cells, the percentage of cytokine producing CD8+ T cells within the total CD8+ T cell population was calculated. These are displayed as the percentage of CD8+ T cells in Figure 2B and C.

The criteria for selection of the appropriate antibody concentration was the detection of a distinct positive population which was on the scale of detection. For the anti-IFNγ antibody this was achieved with the use of 2µl per test, for anti-TNFα, the chosen volume of antibody per test was 1µl (Figure 2B and C). These were used in all subsequent experiments.

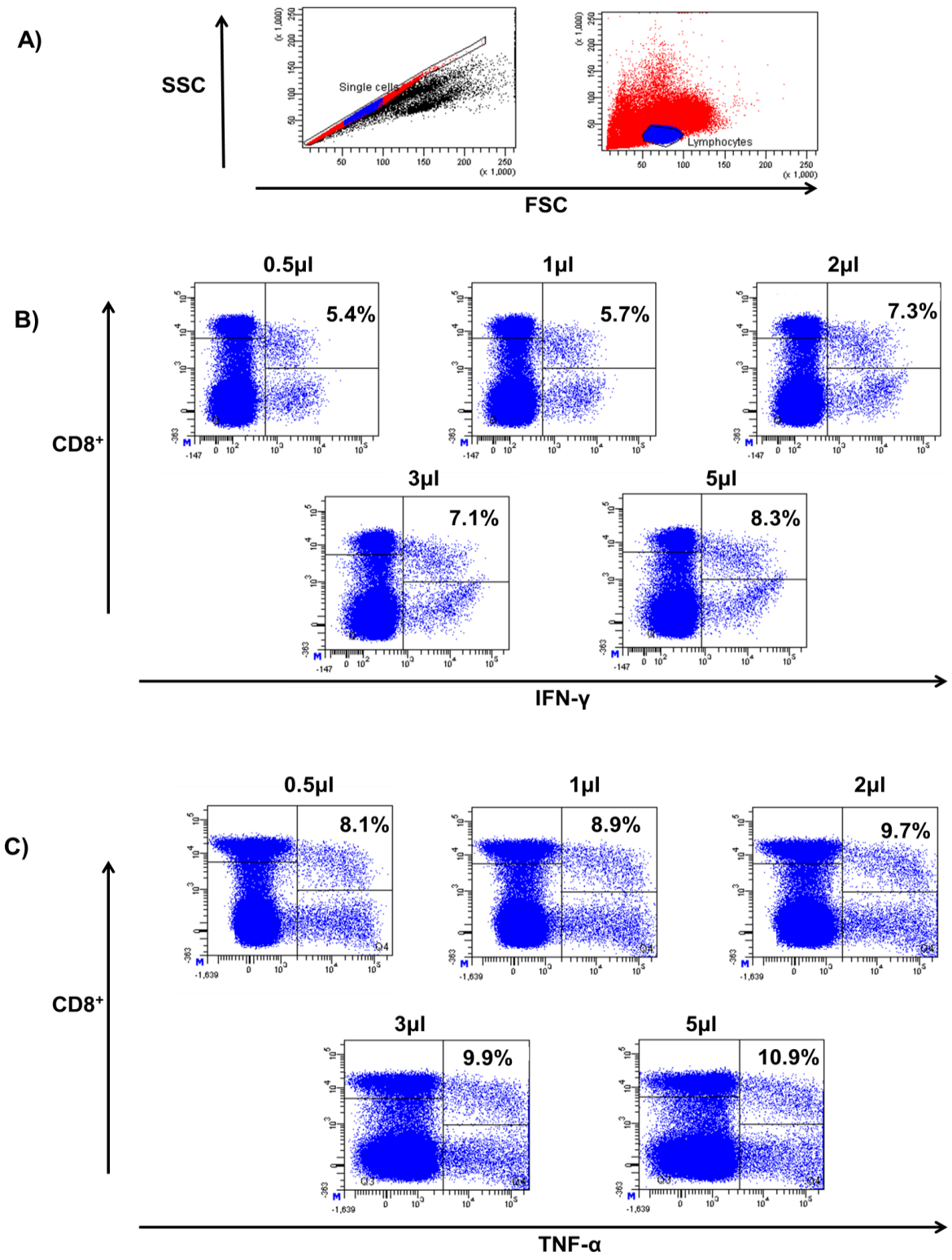


Figure 2 – Titration of cytokine-specific antibodies. A) Gating Strategy. Initially single cells were gated upon. Of these, the lymphocyte population was gated using FSC vs. SSC. Analysed then was the cytokine production (B) IFN-γ or (C) TNF-α of CD8+ T cells with increasing amounts the antibody to be tested Indicated are the percentage of the total CD8+ T cells producing cytokine in response to stimulation with SEB.

3.2 – Detection of CD8+ T cells specific for CMV-derived epitopes in young healthy donors

The peptide epitopes studied in this project have recently been identified using a CMV-strain termed RV798 which is deleted for the immunevasion molecules. To date, T cell responses to these epitopes have not been characterised in detail. In the first instance, 15 young CMV seropositive donors (aged from to 27-59) were chosen to analyse CD8+ T cell responses to these CMV-derived antigens. PBMCs were stimulated with peptide epitopes according to HLA-restriction matched with the donor's HLA-type. Details for the donors can be found in the appendix - Table A1. Figure 3A demonstrates the gating strategy used throughout the study for the analysis of ICS. Single, viable, CD3+ lymphocytes were identified and then analysed for the frequency of cytokine producing CD8+ T cells within this population. In all subsequent ICS, the CD8+ T cells were gated for in this manner. Example dot plots and the percentage of CMV-specific CD8+ T cells responding to a selection of the tested epitopes are shown in Figure 3B.

Examples of four epitope-specific CD8+ T cell responses in young donors are shown in Figure 3B. The largest CD8+ T cell responses seen are against the HLA-Cw16 restricted FRC (2.09% IFN-γ and 2.25% TNF-α) and the HLA-B44 restricted NEG (2.31% IFN-γ and 3.98% TNF-α) epitopes. The RTD (0.03% IFN-γ and 0.04% TNF-α) and YAD (0.16% IFN-γ and 0.27% TNF-α) epitopes (both HLA-A1 restricted) elicited the smallest CD8+ T cell responses. These results demonstrate the potential of these peptides to induce CD8+ T cell responses. Overall the antigen-specific T cells mainly produce IFN-γ and TNF-α and little IL-2 as has been previously described for CMV-specific CD8+ T cells.

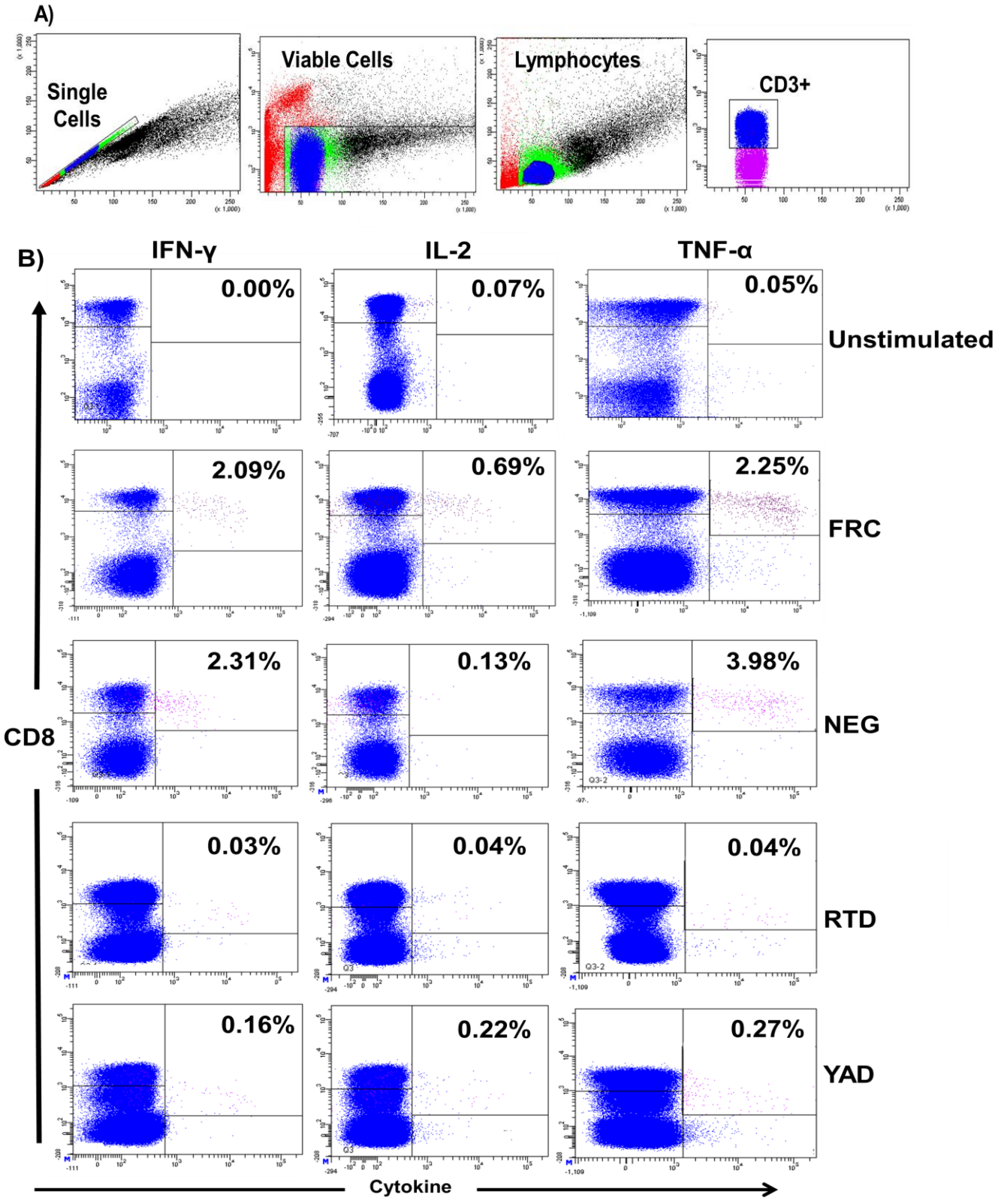


Figure 3 – Cytokine production of CD8+ T cells in response to CMV-epitopes in young donors. A) Gating strategy. Single viable lymphocytes were gated on. CD3+ cells were identified and for these CD8+ T cells against cytokine production were plotted (B). The percentage of the total CD8+ T cell population producing IFN-γ, IL-2 and TNF-α are indicated. In all subsequent ICS, CD8+ T cells were gated for in this manner. B) Examples of T cell responses of varying magnitude to selected peptide-epitopes studied. The top row shows unstimulated cells served as a control. Each row represents staining from an individual donor.

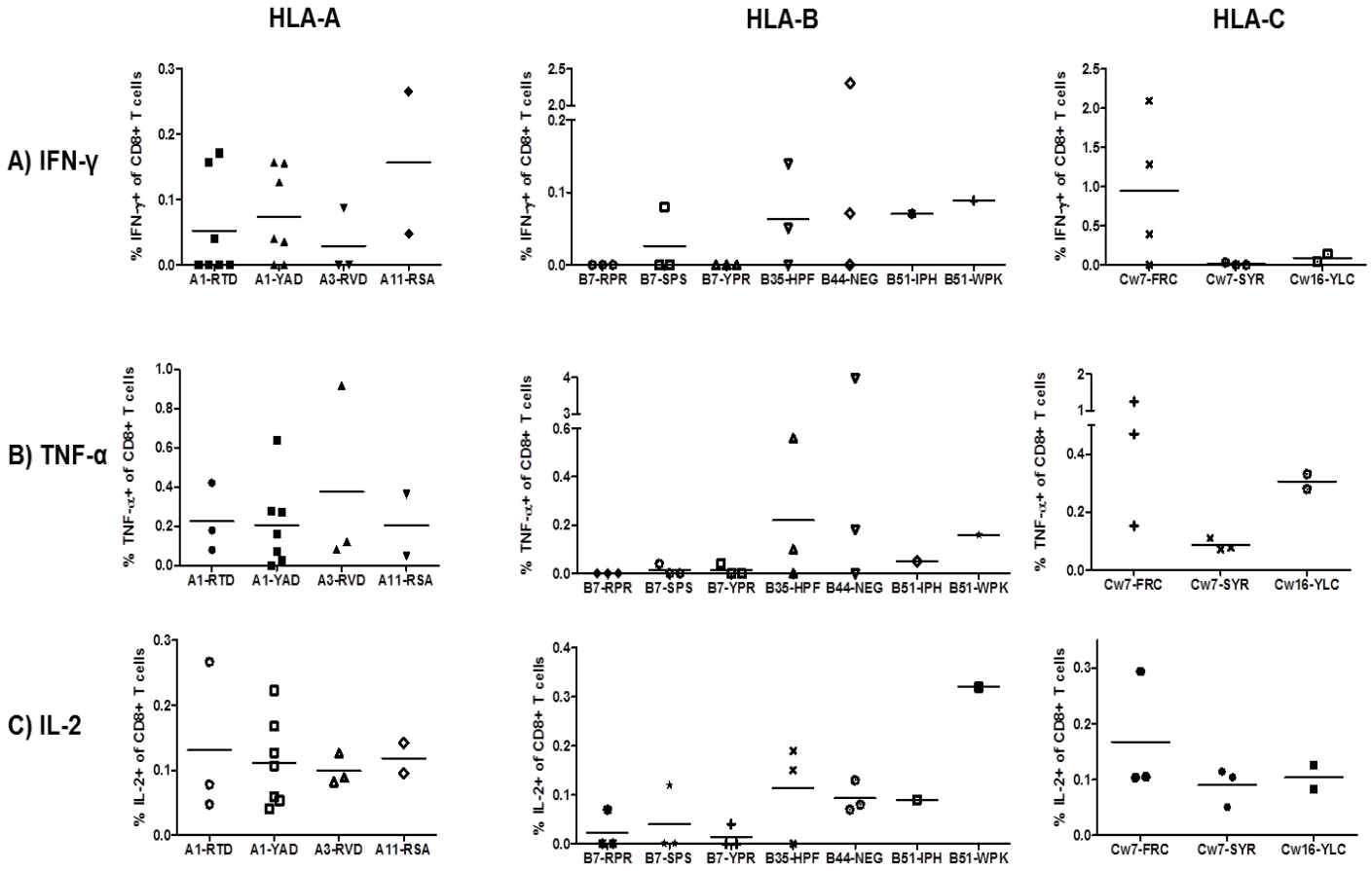


Figure 4 – Summary of screening results for all young donors tested A) IFN-γ production. B) IL-2 production. C) TNF-α production. The left hand column represents T cell responses to HLA-A restricted epitopes, the middle column HLA-B restricted epitopes and the right hand column HLA-C restricted epitopes. The means are indicated by horizontal bars. Note the different scales between cytokines and HLA types. Each symbol indicates the response from an individual donor.

Figure 4 summarises the screening results from all 15 young donors. CD8+ T cell responses were detected against all 14 of the epitopes tested however with varying magnitudes. The size of the CD8+ T cell responses ranged from 0.02 to 2.25%. A general trend was observed where the CD8+ T cell responses to the HLA-C restricted epitopes are larger than both the HLA-A and HLA-B. The HLA-Cw7-restricted FRC epitope exhibited a larger TNF-α response than the other two cytokines and elicited

the largest overall cytokine responses**.** The HLA-B responses (in particular the HLA-B7 restricted RPR epitope) were almost all negative, except for the NEG epitope who elicited comparable IFN-γ and TNF-α production (2.31% and 3.98% respectively) to the FRC epitope and the HLA-B51 restricted WPK epitope (tested once). The TNF-α responses ranged from 0.02% to 2.25%, the IFN-γ from 0.02-2.09% and the IL-2 production was very small across all HLA-A, HLA-B and HLA-C restricted epitopes tested, ranging from 0.04-0.69%.

3.3 – Detection of CD8+ T cell specific for CMV-derived epitopes in elderly donors

Previous studies have shown increasing magnitudes of CMV-specific T cell responses in elderly donors. However, these were mainly against pp65 or IE-1 derived epitopes. Next it was therefore investigated whether a similar increase could be observed for these newly identified peptide-epitopes. PBMCs from 15 elderly donors (aged 68– 86 years) were screened in the same fashion as the young donors.

Example dot plots of four epitope-specific T cell responses in elderly donors are shown in Figure 5, demonstrating that a range of response magnitudes against these CMV epitopes can be detected. In one donor the CD8+ T cell response to FRC takes up 32% and 31% of the CD8+ T cell pool producing IFN-γ and TNF-α respectively, whereas the response to the RTD epitope is much smaller with only about 0.15% and 0.17% of cells producing IFN-γ and TNF-α respectively (Figure 5). As seen with the young donors, the T cells are largely producing IFN-γ and TNF-α in response to antigen stimulation with very little or no IL-2 production.

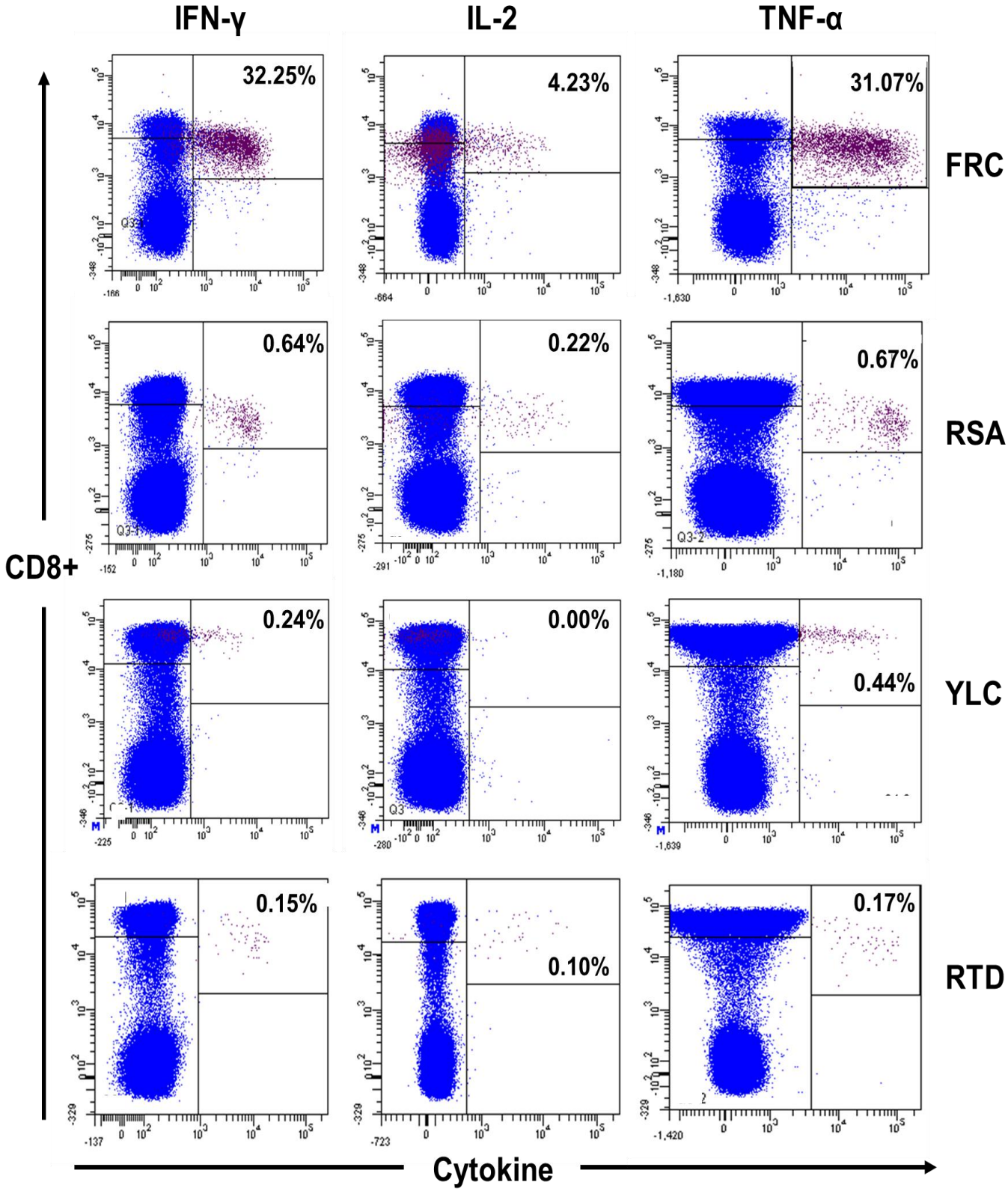


Figure 5 – Frequency of CD8+ T cells responding to CMV epitopes in elderly donors. Shown are example dot plots of four selected epitopes. PBMCs were stimulated with peptide and stained to identify IFN-γ, IL-2 and TNF-α producing CD8+ T cells. Each row represents a CD8+ T cell response detected in an individual donor.

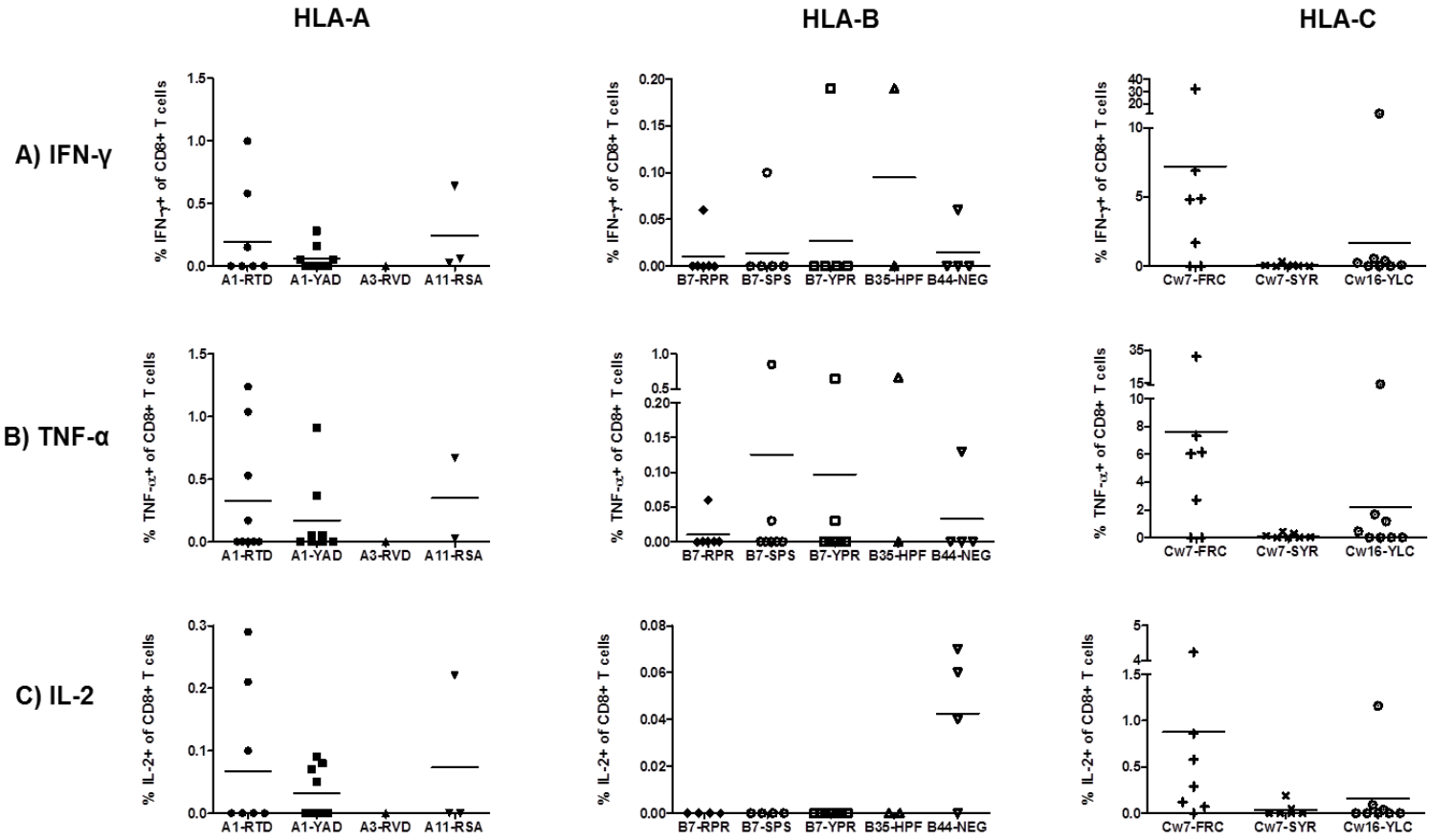


Figure 6 - % CD8+ T cells producing cytokines after stimulation with 14 CMV peptide epitopes within 15 elderly donors A) IFN-γ production. B) IL-2 production. C) TNF-α production. This is figure is as described for the summary of young responses in Figure 3.

CD8+ T cell responses against all 12 epitopes tested were detected in the elderly donors varying in magnitude (Figure 6). Similar to what was observed within the young donors (Figure 4), responses within the elderly to the HLA-B restricted epitopes elicited no IL-2 (except the NEG epitope at 0.04-0.07%) production and very little IFN-γ. However, the HLA-B7 SPS, YPR and HLA-B35 HPF epitopes elicited good TNF-α production in one donor tested with those epitopes (0.85%, 0.65% and 0.66% respectively). Also similar to observed with the young donors the responses to the HLA-A epitopes were a good size ranging from 0.05 to 1.24%. The FRC epitope, as in the young donors, elicited the strongest response for all three cytokines at 32.25%, 31.07% and 4.23% for IFN-γ, TNF-α and IL-2 production respectively.

3.4 – Comparison of detected CD8+ T cell responses between young and elderly donors

Although only a small number of donors were screened for T cell responses against these new epitopes, frequencies of TNF-α, IFN-γ and IL-2 producing CD8+ T cells were compared between young and elderly donors.

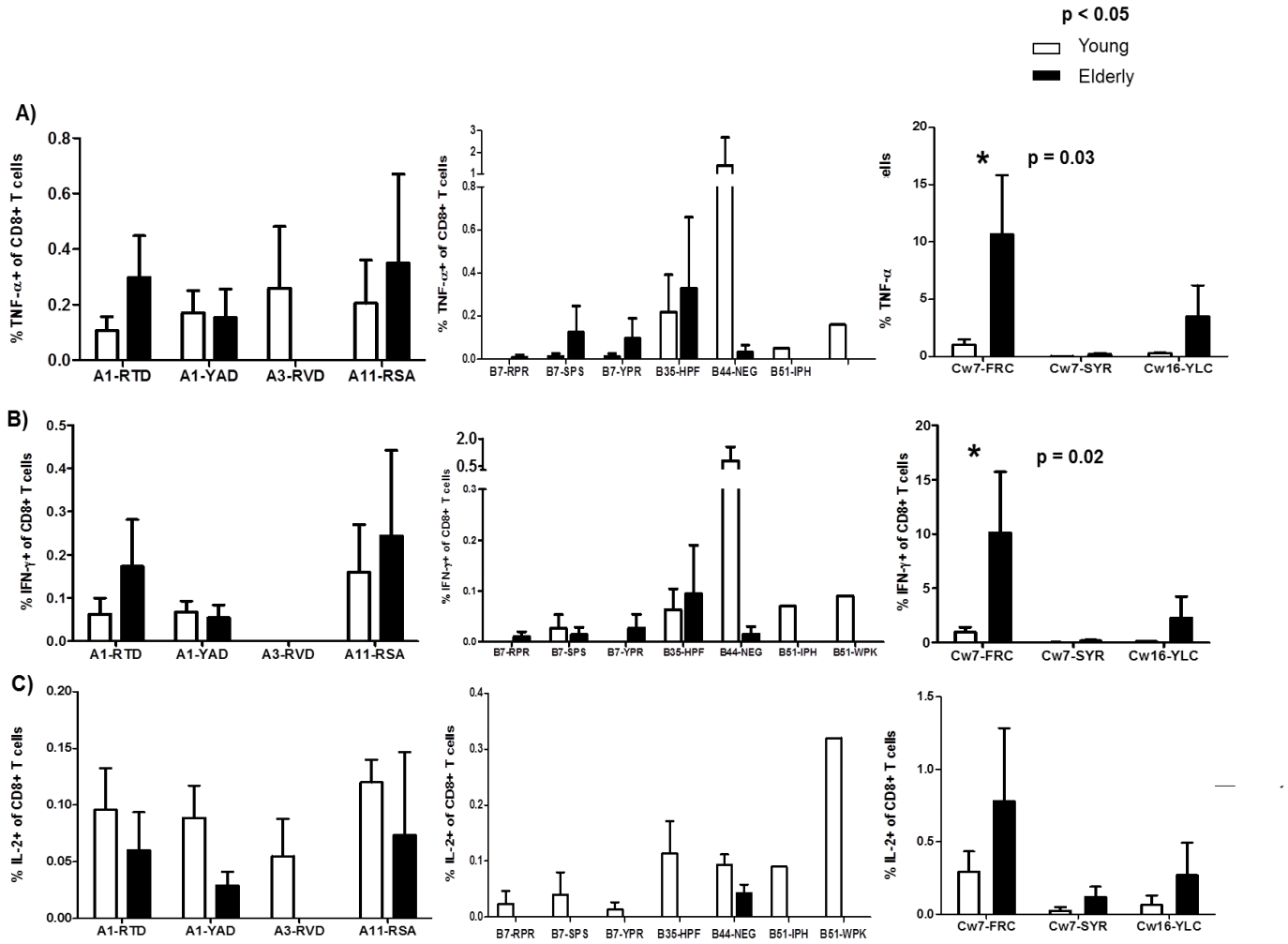


Figure 7 - Comparison of the CMV-specific CD8+ T cell responses between young and elderly donors A) TNF-α+ CMV-specific CD8+ T cell response to the epitopes listed. B) IFN-γ+ CMV-specific CD8+ T cell responses to the epitopes listed. C) IL-2+ CD8+ T cell responses to the epitopes listed. Represented are mean response sizes with SEM. Responses significantly different between the young and elderly donors are indicated by an asterisk and have a p value < 0.05. Note the difference in scale between the IFN-γ, TNF-α and IL-2 graphs.

The comparison of the average response to each epitope tested in the young vs. elderly is shown in Figure 7. The overall results indicate that within the elderly donors, there is a trend towards higher frequencies of cells producing IFN-γ and TNF-α for the majority of epitopes. However, for IL-2 responses the reverse seems to be true with the exception of the HLA-C restricted epitopes. The IL-2 responses within the elderly are still very small as within the young and do not represent a significant % of the total CD8+ T cell pool.

The HLA-C restricted epitopes induced some very large CD8+ T cell responses in both young and elderly donors in particular the HLA-Cw0702 restricted epitope FRC. It elicits a significantly larger IFN-γ and TNF-α response in the elderly at a ~9 fold increase (Figure 7A and B right hand column).

The YLC epitope also elicits CMV-specific CD8+ T cell responses larger than the responses seen within the young donors (2-4 fold) but to a lesser extent (Figure 3B and 5). The RTD response appeared to have a larger magnitude (0.28 vs. 1% IFN-γ and 0.42% vs. 1.24% TNF-γ within the young and elderly respectively) and a more defined positive population (Figure 3B and Figure 5).

The raw data can be found within the appendix Tables A3-A8

3.5 – Phenotypic analysis of the CMV-specific CD8 T cells towards the newly identified antigens

The majority of CMV-specific T cell responses analysed to date display a very characteristic phenotype marked by the loss of CCR7 and co-stimulatory molecules like CD28 and gain of CD57 expression. In addition they re-express CD45RA even though they are antigen experienced.

To assess the phenotype of CD8+ T cells specific for these newly identified epitopes, intracellular staining for TNF-α production (to identify the CMV-specific CD8+ T cells) was combined with staining for phenotypic surface markers. The co-expression of CCR7 and CD45RA was used initially to distinguish cells of distinct memory cell compartments (Figure 8A and B).

In general, T cells are separated into four subsets as follows: naïve (CD45RA+, CCR7+), central memory (CD45RA-, CCR7+), effector memory (CD45RA-, CCR7-) and effector memory RA revertant (EMRA) cells (CD45RA+CD27-) (See schematic diagram in Figure 8A).

Here four different epitopes were chosen and analysed in two young and one elderly donor. In addition to CCR7 and CD45RA, PBMCs were surface stained for CD57, and CD28 to further discriminate their differentiation status (Figure 8C).

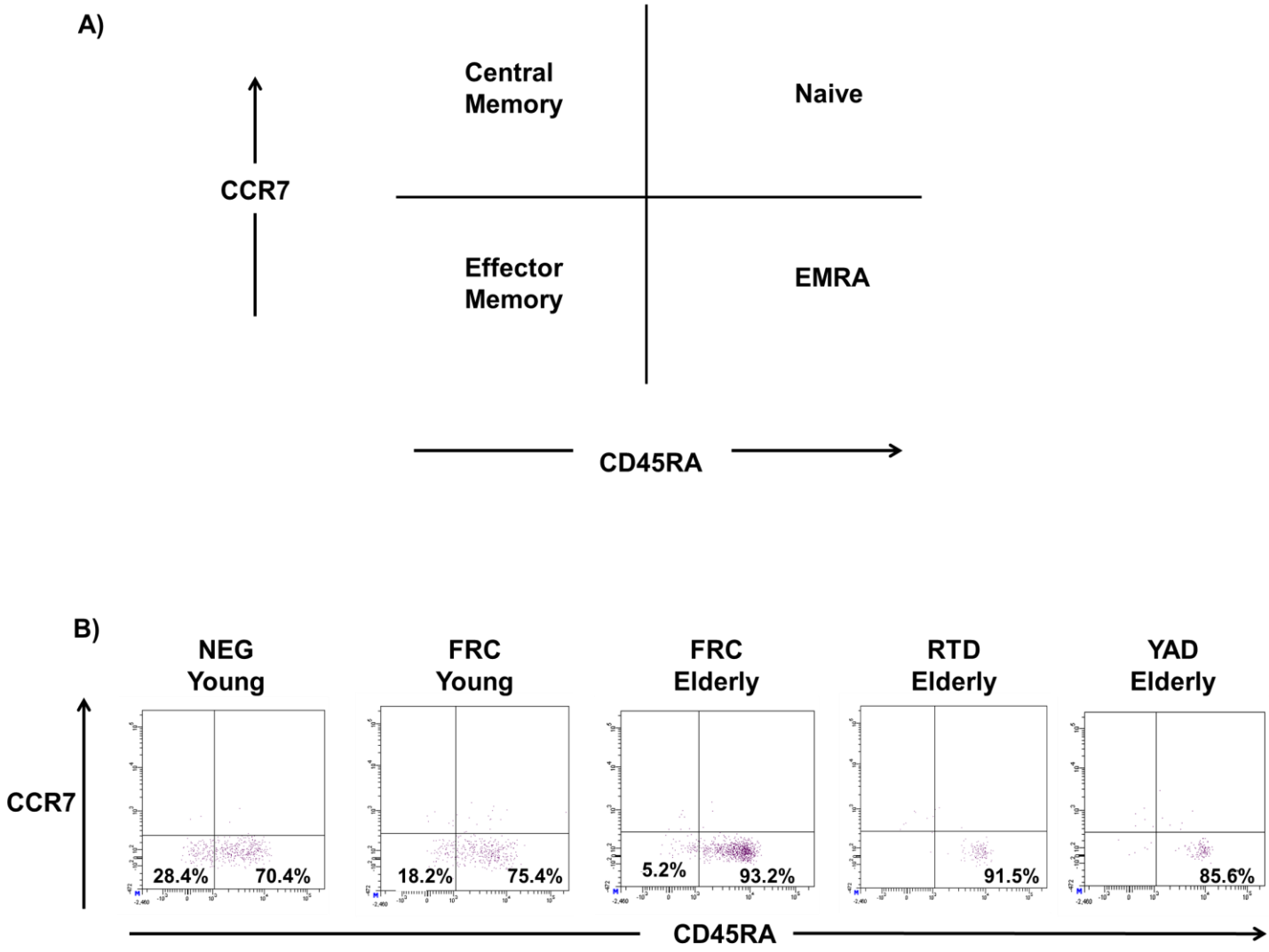


Figure 8 – Identification of T cell memory subsets: A) Schematic diagram showing the co-expression of CCR7 and CD45RA used to identify T cell memory subsets. B) FACS plots showing expression of CCR7 and CD45RA on epitope-specific T cells. PBMCs were stimulated with the peptide indicated, followed by ICS. CD8+ T cells producing TNF-α in response are gated on and displayed here.

As shown in Figure 8B, for all epitopes tested the vast majority of the virus-specific CD8+ T cells displayed an effector memory phenotype, in particular that of EMRA cells (on average 83% of responding CD8+ T cells). The two responses detected in young donors contained a slightly lower proportion of EMRA cells, with 70.4% and 75.4% respectively. This is line with previous findings for pp65 and IE-1 derived epitopes.

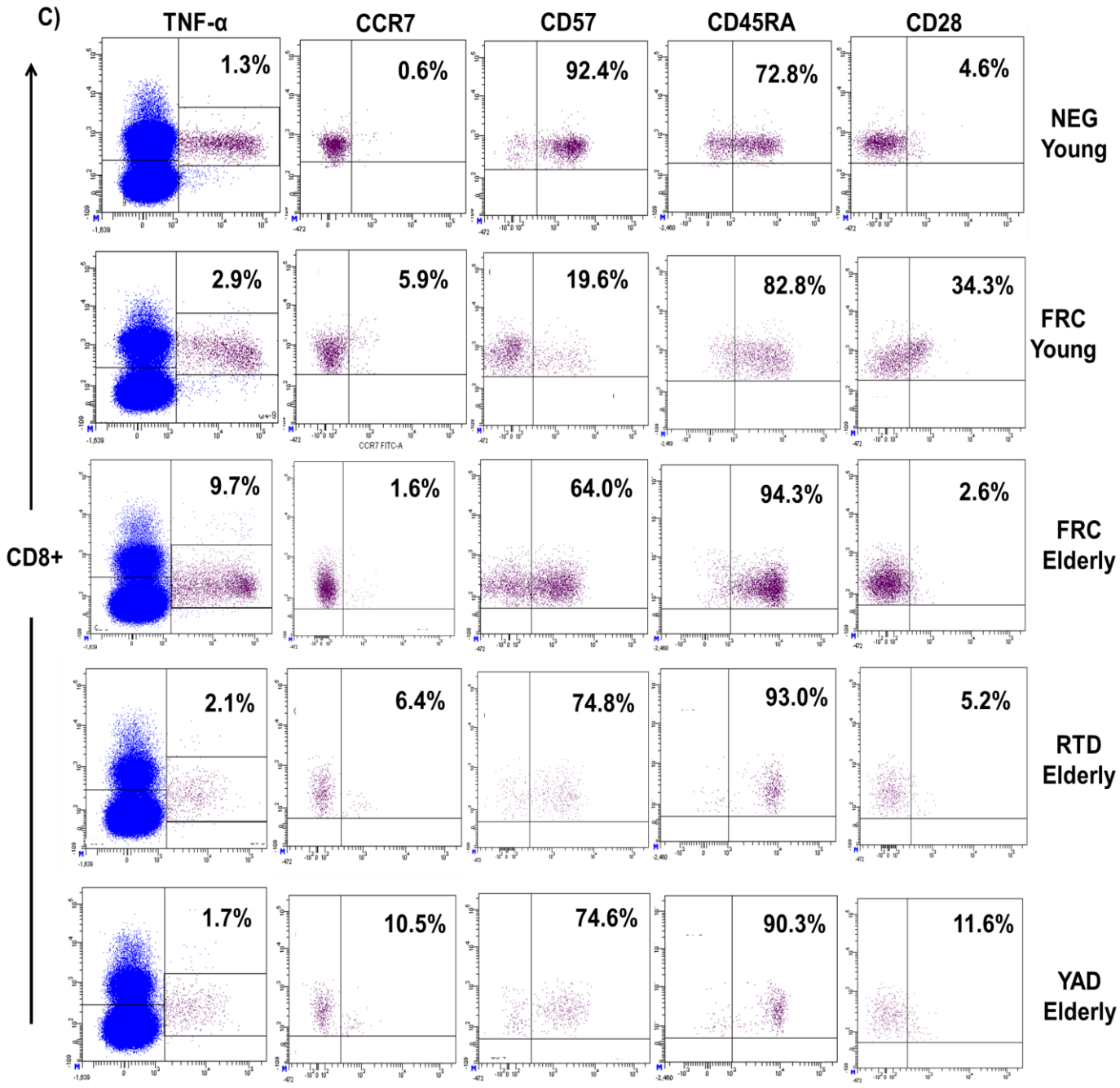


Figure 9 - Phenotype of the CD8+ T cells responding to the new CMV epitopes. Example FACS plots demonstrating the surface levels of CCR7, CD57, CD45RA and CD28 on antigen-specific T cells. CMV-specific CD8+ T cells are discriminated by TNF-α production. (left hand column). The subsequent columns show the percentage of CMV-specific CD8+ T cells expressing the particular surface molecule. Each row represents data for one specific T cell epitope as indicated.

In addition to CCR7 and CD45RA, expression of CCR7 and CD28 was analysed on TNF-α producing CD8+ T cells. Results are presented in Figure 9. The CMV-specific CD8+ T cells from both the two young and single elderly donor largely lacked expression of CD28 and had gained expression of CD57. An exception is the FRC CD8+ T cell response within one younger donor. Here a larger proportion of responding cells still had CD28 on their surface (34%) and the frequency of CD57 expressing cells was very low with 19.6%. This was in contrast to CD8+ T cells responding to the same epitope within the elderly donor (2.6% and 64.0% respectively).

3.6– Cloning of CD8+ T cells specific for CMV-epitopes

In order to analyse whether 'protected epitopes' are recognised within the context of virus-infection, two of the detected epitope-specific CD8+ T cell responses were cloned. This was done utilising an IFN-γ capture cytokine assay followed by limiting dilution cloning (see materials and methods). By IFN-γ ELISA peptide-specific clones were detected following overnight co-culture of T cells with autologous peptide-pulsed or DMSO-pulsed LCL. DMSO serves as a solvent for the peptides and therefore as a control. Figure 10 shows an example result for one such screening experiment.

For donor YD4, twenty clones specific for the RTD epitope were identified (clones c10, c14, c15, c20, c23, c37, c39, c40, c45, c47, c48, c51, c58, c63, c66, c67, c68, c73, c74, c75, c79, c81, c86, c90, c103, c104, c117 and c118) and for donor YD5, four specific clones for the YAD epitope were identified (clones c2, c13, c23 and c32). RTD specific clones c63, c90 and c104 as well as YAD specific clones c23 and c32 were grown up in culture and used for further experiments.

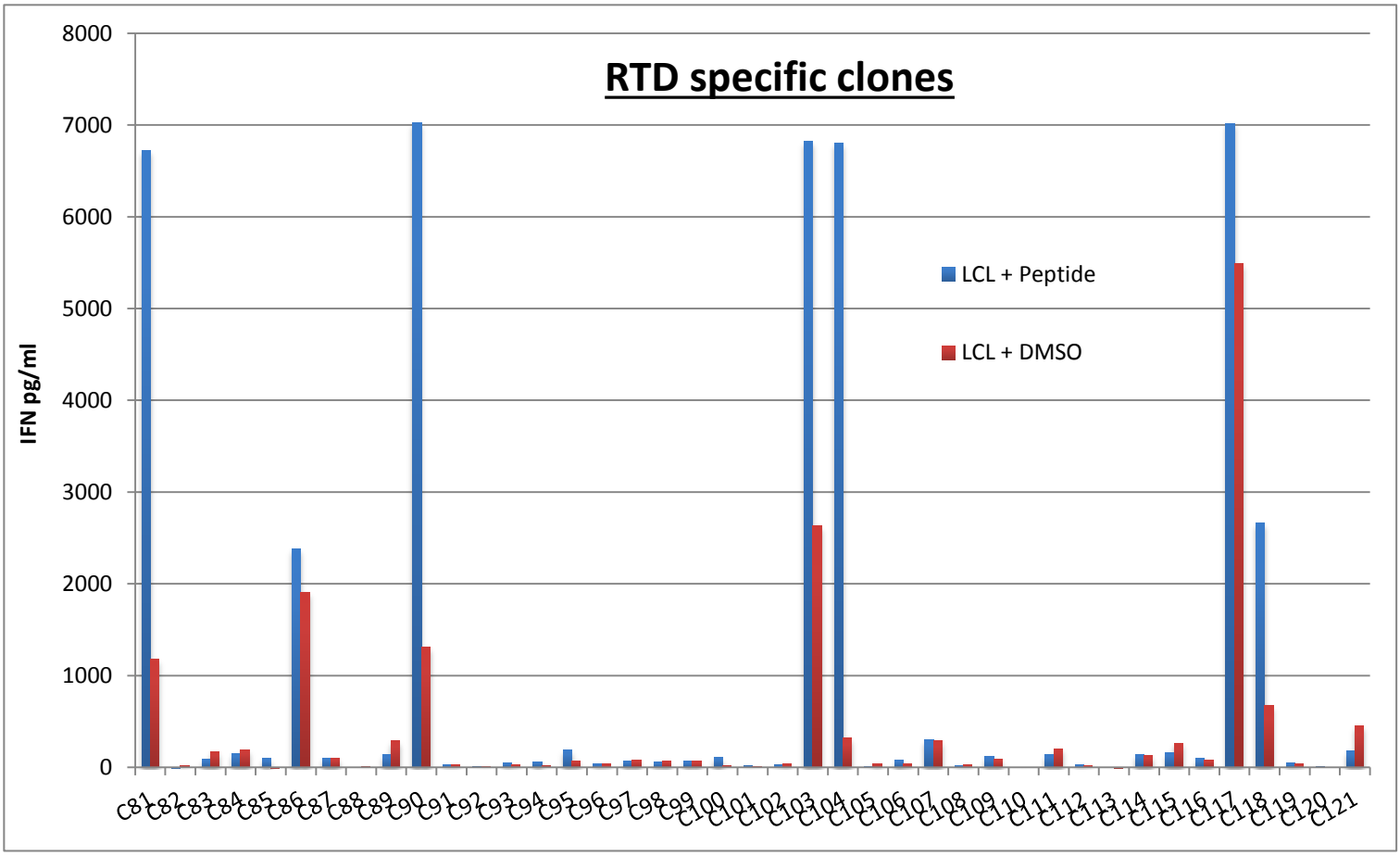
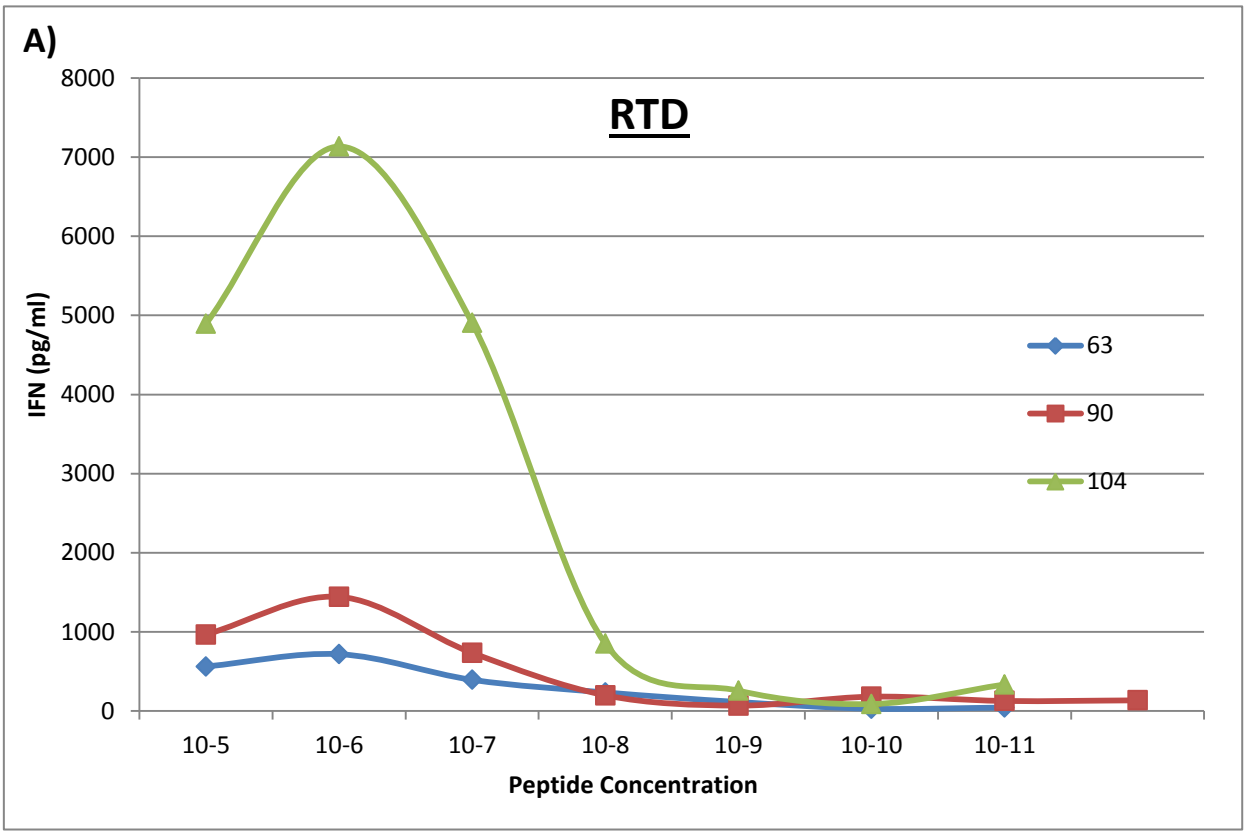


Figure 10 – Representative results of an IFN-γ ELISA to identify peptide-specific T cell clones. IFN-γ was measured in the supernatant of co-cultures of potential T cell clones with peptide-pulsed or DMSO-pulsed autologous LCLs.

3.7 – Determination of peptide avidity of CD8+ T cell clones

To determine the avidity of the CD8+ T cell clones, peptide titrations were performed. For this, autologous LCLs were pulsed with a dilution series of the appropriate peptide, co-cultured with the T cell clones and IFN-γ detected within the supernatant by ELISA.

As shown in Figure 11, all clones tested had a very high avidity for their respective peptides. Peptide dilutions as low as 10^{-8} were able to induce a cytokine response by the clones tested. Findings were very similar for all clones, even though the magnitude of the response differed considerably between them. This was comparable to previous findings in our lab. This was comparable to previous findings for CMV-specific CD8+ T cell responses.



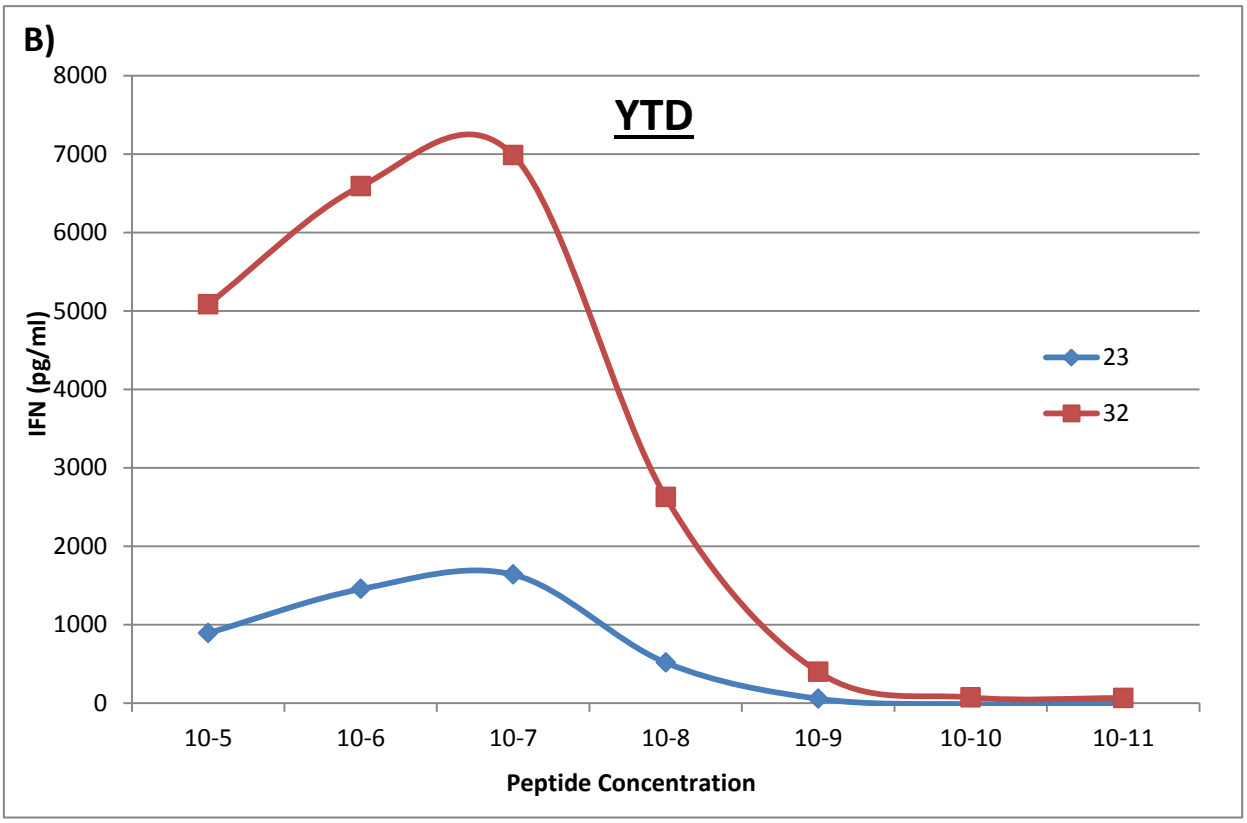


Figure 11 - Measurement of peptide avidity of RTD and YAD-specific CD8+ T cell clones. A) Peptide avidity of clones c63, c90 and c104 specific for the RTD epitope. B) Peptide avidity of clones c23 and c32 specific for the YAD epitope was determined. The peptide concentration is given along the X-axis. The amount of IFN-γ produced is given along the Y-axis in pg/ml.

3.8 – CD8+ T cell recognition of CMV-infected fibroblasts

In the final experiment of this study, it was analysed whether the epitope-specific CD8+ T cells were able to recognise naturally processed and presented antigen. It was also investigated whether these two epitopes are presented by cells infected with WT-CMV strain (AD169) in comparison to the deletion mutant RV798.

Fibroblasts were infected for 48hours with either AD169 or RV798 virus strains at an M.O.I. of 0.5 and 0.05. This time was chosen as it allows the US gene products to exert their MHC I down regulatory effects. After 48 hours, CD8+ T cells specific for the relevant epitope were added overnight and IFN-γ production measured in an ELISA (Figure 12).

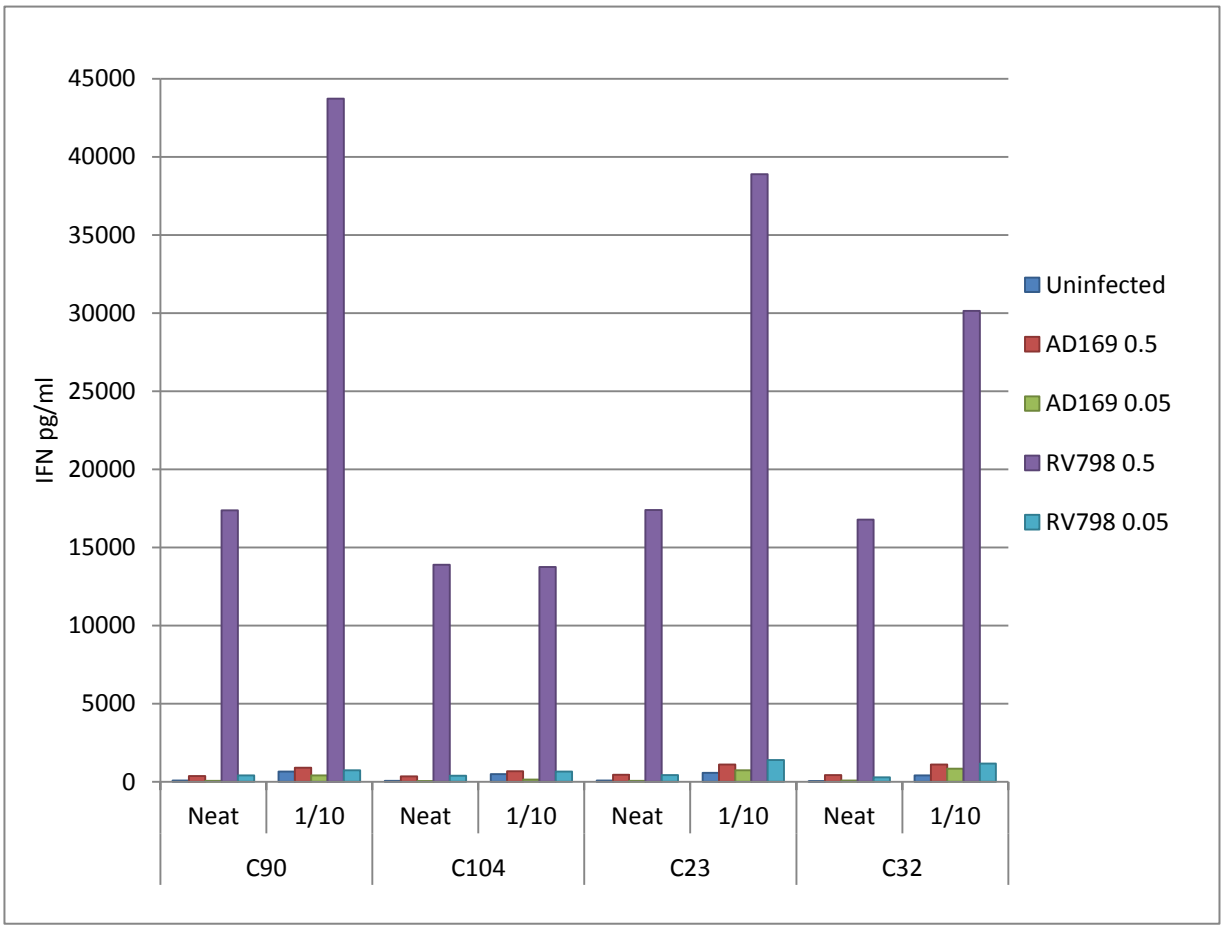


Figure 12 – T cell recognition of virus-infected fibroblasts. Clones c90, c104 (RTD-specific) and c23 and c32 (YAD-specific) were stimulated with uninfected fibroblasts, AD169 at M.O.I. 0.5, AD1659 at M.O.I. 0.05, RV798 at M.O.I 0.5 and RV798 at M.O.I. 0.05. IFN-γ in the supernatant was detected by ELISA. Supernatants were used neat or 1/10 diluted to ensure accurate measurements of the cytokine. The amount of IFN-γ produced is given along the Y-axis in pg/ml.

For both specificities the results indicate that the epitopes are only sufficiently presented by fibroblasts during infection with deletion virus RV798 (Figure 12). No CD8+ T cell recognition was observed when the cells were infected with the WT strain AD169 which expressed the immune evasion proteins. Recognition of RV798 infection was only seen with an M.O.I. of 0.5.

CHAPTER 4 - DISCUSSION

CMV-driven proliferation and accumulation of CD8+ T cells within the elderly (termed memory inflation) can take up substantial fractions of the host total CD8+ T cell pool. This has been hypothesised to impact upon the health of elderly donors. CMV encodes for several immumomodulatory genes that protect CMV-derived antigens from CD8+ T cell recognition. However CMV-specific responses to such antigens can be detected within the peripheral blood of young and elderly individuals when PBMCs are co-cultured *in vitro* with fibroblasts infected with a CMV-strain deleted for immunoevasins. Further understanding the characteristics, functionally and phenotypically of the CD8+ T cell responses to these 'protected' CMV-antigens and how they change over time may allow us to address whether these cells behave differently *in vivo* in comparison to 'non-protected' responses and also indicate, whether in humans, stable or inflationary epitopes can be discriminated as they have been within the murine model. The results from this investigation support the previous findings of memory inflation of CMV-specific CD8+ T cells, the same characteristic phenotype and identified epitopes that may dominate the immune response as seen for some pp65 and IE-1-derived epitopes.

4.1 – Detection and magnitude of CD8+ T cell responses in young vs. elderly donors

In an attempt to characterise the CMV-specific CD8+ T cell response to newly identified CMV-derived peptide-epitopes (Stan Riddell, unpublished), PBMCs of 15 young and 15 elderly individuals were screened. Peptide stimulation was combined with intracellular cytokine staining for IFN-γ, IL-2 and TNF-α production to identify activated T cells. Immune responses were detected to all epitopes tested within this study (Table 3) derived from the UL16, UL17, UL23, UL24, UL28, UL33, UL36,

UL52, UL56,UL84, UL105, UL122 proteins encoded by the CMV genome. The 15 young donors responded to all 14 epitopes tested and the elderly to all 12 however at varying magnitudes (raw frequencies of cytokine responses and the mean values are provided in the appendix). This investigation presents evidence for CD8+ T cell responses specific for antigens derived from across the CMV genome. Furthermore, it supports the memory inflation hypothesis of CMV-driven accumulation of virus-specific CD8+ T cells.

The largest T cell responses detected within this study were against the epitope FRC (4.23 to 32% of CD8+ T cells) from elderly donors (Figure 5 and 6). Even within the young, the responses to this epitope were also the largest (0.69 to 2.09% of CD8+ T cells). The FRC epitope is HLA-Cw0702 restricted and derived from the UL28 protein whose function is currently unknown. It is therefore difficult to extrapolate why this protein was so immunogenic within these donors.

Large responses were also detected against another HLA-C restricted epitope, YLC, derived from the UL24 protein which forms part of the tegument (Table 1) and as seen for the FRC epitope, seem to accumulate with age (Figure 7). Cytokine response frequencies within the young donors ranged from 0.04-0.15% (IFN-γ), 0.13% (IL-2) and 0.28-0.33% (TNF-α). In the elderly these responses ranged from 0.09-12.14% (IFN-γ), 0.04-1.16% (IL-2) and 0.44-14.22% (TNF-α). The HLA-A1 restricted epitope RTD presented with an age-related accumulation with an average IFN-γ and TNF-α response within the young and elderly – 0.06%/0.11% and 0.17%/0.30% respectively. The IL-2

production remained similar (0.08 and 0.06% respectively). The RTD epitope is derived UL69 protein forming part of the tegument. The responses to YLC and RTD epitopes could be generated when the virus enters the body, before the US2-11 proteins prevent their presentation to the CD8+ T cell response. The primed CD8+ T cells may then be driven to proliferate when the virus reactivates during the host's lifetime.

The CD8+ T cell responses detected against the HLA-B restricted epitopes were rare within the donors here investigated and it is therefore difficult to draw any conclusions from the data obtained. For example, the WPK and IPH epitopes were tested once within young donors but could not be tested within the elderly as none were HLA-B51 positive. The HLA-B restricted epitopes (in particular HLA-B7) in the majority induced very little or no cytokine producing CD8+ T cell responses in both age groups analysed here (Figure 4, 6 and 7) with very minimal IFN-γ (0.05-0.14% young and 0.06-0.19% elderly), IL-2 (0.04-0.19% young and 0.04-0.07% elderly) and TNF-α (0.04-0.19% young and 0.04-0.56% elderly). This could be as a result of their negative CD28 surface expression (Figure 8C) which will be further discussed in 4.2. It is surprising that the immune responses to the YPR and SPS epitopes were low as they are derived from a glycoprotein and the tegument respectively (Table 1) and should theoretically elicit larger response's than was observed as these antigens are brought in as part of the virus particle. However, these antigens may be presence in a small abundance of the virion and therefore not be particularly immunogenic. These low CD8+ T cell responses may also be down to the way these antigens are processed for antigen-presentation *in vivo*. Studies by Wolkers et al. demonstrate clear differences in the priming efficiency depending upon the epitopes position within the protein[29].

Most virus-specific CD8+ T cells responses to date are restricted through the HLA-A and HLA-B molecules. These alleles are known for their presentation of viral peptides to the CD8+ T cell population to elicit a CTL response[30]. The HLA-C alleles are also able to present peptide[31], however, HLA-C alleles have been attributed a lesser role in the generation of virus-specific CTL responses due to their much lower surface expression level (~10% of HLA-A/B)[32] and in addition they are less stable. It is now generally accepted that their main role is as an inhibitory ligand for killer-cell immunoglobulin-like (KIRs) present on NK cells[33]. This poses a question to why the CMV-specific CD8+ T cell responses to the HLA-C restricted epitopes in this study, in particular the FRC epitope, are so large.

The down-regulation of HLA-A and HLA-B but not HLA-C has been described for the HIV virus by the virus-encoded protein nef[34]. This is in an attempt to evade CTL recognition but still remain resistant to NK cell recognition. It is plausible that CMV also may down-regulate HLA-C alleles to a lesser extent from the surface of infected cells to achieve the same evasion. Studies indicate that this indeed may be the case[35]. This may contribute to the particular immunodominance of the HLA-C restricted CD8+ T cells responses seen in previous literature and within this investigation. It may also provide a mechanism explaining the smaller CMV-specific CTL responses towards the HLA- and HLA-B restricted epitopes as these HLA genes have reduced surface expression on CMV-infected cells caused by the US gene products.

Within the elderly, a general trend towards increased CMV-specific CD8+ T cell responses was seen (Figure 4 and 7) which provides early evidence for age-dependent accumulation of CD8+ T cell responses to these newly characterised epitopes. This correlates with the previous published data on CMV memory inflation[2; 14; 17; 19]. This might be slightly surprising as one could think these epitopes would be presented at lower levels *in vivo* by WT-infected cells and therefore represent 'stable' epitopes.

It would be extremely interesting to develop HLA-C tetramers containing CMV-derived epitopes, especially the FRC epitope. This would allow studying them directly *ex vivo* and further characterising these T cell responses.

4.2 – Phenotype of CD8+ T cells to the newly characterised epitopes

The surface phenotype of the epitope-specific CD8+ T cells analysed within the scope of this study correlates with the phenotype that has previously been published for CMV-specific CD8+ T cell responses (Figure 8C)[2; 14; 15; 16]. The results did suggest that the CD8+ T cell responses to the FRC epitope acquire CD57 over time which fits in with the previous data (19.6% and 64.0% from a young and a separate elderly donor respectively, Figure 8C). However, this was investigated in only one donor. Furthermore, using CCR7 vs. CD45RA to distinguish memory compartments, the majority of virus-specific CD8+ T cells in young and elderly donors display an EMRA phenotype (Figure 8A/B). Studies by Carrasco et al. have shown that antigen-experienced T cells can revert to the CD45RA isoform, normally associated with naive T cells, when they have not encountered antigen for an extended period of time[36]. This might be an explanation for the re-expression of CD45RA on CMV-specific CD8+ T cell expansions. The fact that

these CMV-specific CD8+ T cells lack CD28 expression could provide an explanation for little IL-2 production seen in response to all the epitopes tested (Figure 8C). The loss of CD28 on murine CD8+ T cells has previously been correlated with a reduction in IL-2 production showing that co-stimulation is essential for induction of this cytokine[37]. There appeared to be no major difference in the surface phenotype of the CD8+ T cells responding to the four epitopes tested, between the young and elderly donors. The next step in the investigation would first of all be to extend the phenotype study to more young and elderly donors to allow more definite comparisons to be made. The second would be to perform cytotoxicity assays to further assess the functionality of these CMV-specific CD8+ T cells.

4.3– Recognition of processed and presented CMV peptide epitopes during WT infection

The results from the fibroblast recognition assay show that *in vitro* the WT virus is masking the T cells from recognising HLA-A1 restricted epitopes RTD and YAD. However, as this investigation has demonstrated CMV-specific CD8+ T cell responses against these two epitopes can be found *in vivo*, which opens the question to how these CD8+ T cell responses are being generated. The first explanation could be that these CD8+ T cell responses recognise these epitopes before the US gene products impair MHC I presentation. To investigate this possibility fibroblast recognition assays should be repeated with shorter infection times. This will shed light on whether the CD8+ T cell clones can recognise epitopes during WT infection before the MHC I evasion gene products have exerted their effects.

A second explanation could be cross-priming of the CD8+ T cells by DCs. Exogenous antigens can be taken up by professional APCs, enter the MHC I pathway and be presented to CD8+ cells[38]. This has been hypothesised to contribute to the CMV-specific CTL priming *in vitro.* CTL responses to pp65 have been observed in the context of non-permissive DCs that have ingested CMV-infected fibroblasts[39]. *In vivo* studies with MCMV by Busche et al. confirm that cross-presentation plays an important role in the priming of virus-specific CD8+ T cell responses[40].

4.4 - Future research and final Conclusion

Altogether, the evidence indicates that CMV infection drives the adaptive immune response towards oligoclonality responsible in part for immune senescence and is, to date, the largest documented response against a viral agent. Understanding the magnitude and kinetics of both the CD4+ and CD8+ responses within young vs. elderly donors will help to identify CMV-derived epitopes that elicit stable vs. inflationary CD8+ T cell responses as has been characterised within the murine model. To date it still remains to be investigated whether the accumulation of CMV-specific CD8+ T cells occurs with other antigens from the CMV genome and this investigation made a first attempt. Preliminary experiments within this study indicate that the CD8+ T cell responses to the newly identified CMV peptide epitopes display the same characteristic phenotype being $CCR7^-/CD28^-/CD45RA^+/CD57^+$ and responses to some epitopes accumulate with age. However, it is not known which role they play *in vivo* in controlling the virus. It might therefore be interesting to study T cell responses to these 'protected' epitopes in an immune suppressed setting.

CHAPTER 5 - APPENDIX

		HLA Type		
Donor	**Age**	**HLA-A**	**HLA-B**	**HLA-C**
YD1	**48**	A2 **A11**	B50 **B35**	Cw0401, Cw0602
YD2	**48**	**A1** A29	**B44** B57	Cw0602, **Cw1601**
YD3	**59**	**A1**	**B8**	**Cw7**
YD4	**27**	**A1** A32	**B8** B14	Cw701, Cw0802
YD5	**53**	**A1 A3**	**B8** B18	Cw0501, **CW0701**
YD6	**49**	A29, A30	**B7** B45	Cw06, **Cw07**
YD7	**30**	**A3** A24	B58 **B35**	Cw0302 Cw 0401
YD8	**52**	**A1** A2	**B8** B27	Cw0101, Cw0701
YD9	**47**	A2 **A3**	B15 B60	Cw9, Cw3
YD10	**49**	**A1** A2	B12(**44**) B17	Cw0401
YD11	**45**	**A1 A11**	**B51** B37	Cw0401, Cw0602
YD12	**44**	A2 A24	B27 **B35**	Cw0202, Cw04
YD13	**52**	**A3** A29	**B7**	**Cw0702**
YD14	**29**	**A3** A31	**B7 B44**	**Cw0702 Cw1601**
YD15	**30**	**A1** A2	**B8** B15	Cw0701 Cw0704
	Average 44			

Table A1 – HLA types and age of the 15 young donors

		HLA Type		
Donor	**Age**	**HLA-A**	**HLA-B**	**HLA-C**
OD33	**68**	A1	B8 B57	Cw6
OD04	**78**	**A1** A2	B8 **B44**	Cw0701 Cw1601
ODO7	**86**	**A1 A11**	**B7** B8	Cw0701 Cw0702
OD01	**80**	**A3** A29	B8 **B44**	Cw7 Cw1601
OD08	**83**	**A1** A2	B8	-
OD12	**86**	A24 A68	**B7 B44**	Cw0702 Cw1601
0D21	**77**	**A1** A2	B38 B60	-
OD26 (CMV neg)	**-**	**-**	-	-
OD28	**81**	**A1** A2	B8 B62	Cw0701
OD10	**not known**	A11/CW		-
OD11	**79**	**A1** A2	**B7, B35**	Cw0702
OD27	**81**	A2 **A11**	**B7 B44**	Cw0702
OD14	**80**	A2 A33	B39 B65	-
OD25	**79**	**A1 A11**	B8 **B35**	Cw0701
OD24	**74**	**A2**	**B7**	-
OD30	**71**	A2 **A11**	**B7** B27	Cw0702
	Average 74			

Table A2 – HLA types and age of the 16 elderly donors

IFN

Donor:	A1-RTD	A1-YAD	A3-RVD	A11-RSA	B7-RPR	B7-SPS	B7-YPR	B35-HPF	B44-NEG	B51-IPH	B51-WPK	Cw7-FRC	Cw7-SYR	Cw16-YLC
YD1	-	-	-	0.05	-	-	-	0.05	-	-	-	-	-	-
YD2	0.00	0.04	-	-	-	-	-	-	2.31	-	-	-	-	0.04
YD3	0.28	0.05	-	-	-	-	-	-	-	-	-	-	-	-
YD4	0.03	0.00	-	-	-	-	-	-	-	-	-	-	-	-
YD5	0.00	0.16	0.00	-	-	-	-	-	-	-	-	0.00	0.00	-
YD6	-	-	-	-	0.00	0.08	0.00	-	-	-	-	1.28	0.04	-
YD7	-	-	0.00	-	-	-	-	0.14	-	-	-	-	-	-
YD8	0.00	0.16	-	-	-	-	-	-	-	-	-	-	-	-
YD9	-	-	0.00	-	-	-	-	-	-	-	-	-	-	-
YD10	0.00	0.00	-	-	-	-	-	-	0.07	-	-	-	-	-
YD11	0.17	0.13	-	0.27	-	-	-	-	-	0.07	0.09	-	-	-
YD12	-	-	-	-	-	-	-	0.00		-	-	-	-	-
YD13	-	-	0.00	-	0.00	0.00	-	-	-	-	-	2.09	0.00	-
YD14	-	-	-	-	0.00	0.00	0.00	-	0.00	-	-	0.39	0.00	0.14
YD15	0.02	0.00	-	-	-	-	-	-	-	-	-	-	-	-
Average	**0.06**	**0.07**	**0.00**	**0.16**	**0.00**	**0.00**	**0.00**	**0.06**	**0.79**	**0.07**	**0.09**	**0.94**	**0.01**	**0.09**

Table A3 - Frequencies of IFN-γ producing CD8+ T cell responses in young healthy donors

IL-2

Donor:	A1-RTD	A1-YAD	A3-RVD	A11-RSA	B7-RPR	B7-SPS	B7-YPR	B35-HPF	B44-NEG	B51-IPH	B51-WPK	Cw7-FRC	Cw7-SYR	Cw16-YLC
YD1	-	-	-	0.10	-	-	-	0.15	-	-	-	-	-	-
YD2	0.04	0.04	-	-	-	-	-	-	0.13	-	-	-	-	0.13
YD3	0.00	0.05	-	-	-	-	-	-	-	-	-	-	-	-
YD4	0.04	0.00	-	-	-	-	-	-	-	-	-	-	-	-
YD5	0.19	0.22	0.13	-	-	-	-	-	-	-	-	0.10	0.00	-
YD6	-	-	-	-	0.07	0.12	0.04	-	-	-	-	0.29	0.10	-
YD7	-	-	0.09	-	-	-	-	0.19	-	-	-	-	-	-
YD8	0.00	0.17	-	-	-	-	-	-	-	-	-	-	-	-
YD9	-	-	0.00	-	-	-	-	-	-	-	-	-	-	-
YD10	0.00	0.00	-	-	-	-	-	-	0.07	-	-	-	-	-
YD11	0.27	0.13	-	0.14	-	-	-	-	-	0.09	0.32	-	-	-
YD12	-	-	-	-	-	-	-	0.00	-	-	-	-	-	-
YD13	-	-	0.00	-	0.00	0.00	0.00	-	-	-	-	0.69	0.00	-
YD14	-	-	-	-	0.00	0.00	0.00	-	0.08	-	-	0.10	0.00	0.00
YD15	0.07	0.10	-	-	-	-	-	-	-	-	-	-	-	-
Average	**0.08**	**0.09**	**0.05**	**0.07**	**0.02**	**0.04**	**0.01**	**0.10**	**0.14**	**0.09**	**0.32**	**0.30**	**0.03**	**0.06**

Table A4 – Frequencies of IL-2 producing CD8+ T cell responses in young healthy donors.

TNF

Donors	A1-RTD	A1-YAD	A3-RVD	A11-RSA	B7-RPR	B7-SPS	B7-YPR	B35-HPF	B44-NEG	B51-IPH	B51-WPK	Cw7-FRC	Cw7-SYR	Cw16-YLC
YD1	-	-	-	0.05	-	-	-	0.10	-	-	-	-	-	-
YD2	0.16	0.28	-	-	-	-	-	-	3.98	-	-	-	-	0.33
YD3	0.05	0.00	-	-	-	-	-	-	-	-	-	-	-	-
YD4	0.04	0.00	-	-	-	-	-	-	-	-	-	-	-	-
YD5	0.16	0.27	0.12	-	-	-	-	-	-	-	-	0.15	0.00	-
YD6	-	-	-	-	0.00	0.04	0.04	-	-	-	-	1.25	0.07	-
YD7	-	-	0.92	-	-	-	-	0.56	-	-	-	-	-	-
YD8	0.00	0.64	-	-	-	-	-	-	-	-	-	-	-	-
YD9	-	-	0.00	-	-	-	-	-	-	-	-	-	-	-
YD10	0.00	0.00	-	-	-	-	-	-	0.18	-	-	-	-	-
YD11	0.42	0.16	-	0.36	-	-	-	-	-	0.05	0.16	-	-	-
YD12	-	-	-	-	-	-	-	0.00	-	-	-	-	-	-
YD13	-	-	0.00	-	0.00	0.00	0.00	-	-	-	-	2.25	0.00	-
YD14	-	-	-	-	0.00	0.00	0.00	-	0.00	-	-	0.47	0.00	0.28
YD15	0.02	0.02	-	-	-	-	-	-	-	-	-	-	-	-
Average	**0.11**	**0.17**	**0.26**	**0.18**	**0.00**	**0.01**	**0.01**	**0.28**	**2.08**	**0.05**	**0.16**	**1.03**	**0.02**	**0.31**

Table A5 – Frequencies of TNF-α producing CD8+ T cell responses in young healthy donors

IFN

Donor	A1-RTD	A1-YAD	A3-RVD	A11-RSA	B7-RPR	B7-SPS	B7-YPR	B35-HPF	B44-NEG	Cw7-FRC	Cw7-SYR	Cw16-YLC
OD33	1.00	0.16	-	-	-	-	-	-	-	-	-	-
ODO4	0.00	0.00	-	-	-	-	-	-	0.00	-	-	0.00
OD07	0.00	0.00	-	-	0.00	0.00	0.00	-	-	32.25	0.00	12.19
OD01	-	-	0.00	-	-	-	-	-	0.00	0.00	0.05	0.09
OD08	0.58	0.00	-	-	0.00	0.00	0.00	-	-	0.00	0.34	0.00
OD12	-	-	-	-	0.00	0.00	0.00	-	0.06	1.68	0.00	0.00
OD21	0.00	0.00	-	-	-	-	-	-	-	-	-	-
OD26 (CMV neg)	0.00	0.00	-	-	-	-	-	-	-	-	-	-
OD28	0.15	0.05	-	-	-	-	-	-	-	-	-	-
OD10	0.00	0.00	-	-	-	-	-	-	-	-	-	-
OD11	0.00	0.28	-	-	-	0.10	0.19	0.19	-	4.88	0.08	0.40
OD27	-	-	-	0.06	0.06	0.00	0.00	-	0.00	6.91	0.00	0.24
OD14	-	-	-	-	-	-	-	-	-	-	-	-
OD25	0.00	0.05	-	0.03	-	-	-	0.00	-	-	-	-
OD24	-	-	-	-	0.00	0.00	0.00	-	-	-	-	-
OD30	-	-	-	0.64	0.00	0.00	0.00	-	-	4.81	0.00	0.56
Average	**0.17**	**0.05**	**0.00**	**0.24**	**0.01**	**0.01**	**0.03**	**0.10**	**0.01**	**3.61**	**0.03**	**0.96**

Table A6 - Frequencies of IFN-γ producing CD8+ T cell responses in elderly donors.

IL-2

Donor:	A1-RTD	A1-YAD	A3-RVD	A11-RSA	B7-RPR	B7-SPS	B7-YPR	B35-HPF	B44-NEG	Cw7-FRC	Cw7-SYR	Cw16-YLC
OD33	0.29	0.09	-	-	-	-	-	-	-	-	-	-
ODO4	0.00	0.07	-	-	-	-	-	-	0.00	-	-	0.00
OD07	0.00	0.00	-	-	0.00	0.00	0.00	-	-	4.23	0.00	1.16
OD01	-	-	0.00	-	-	-	-	-	0.04	0.07	0.05	0.04
OD08	0.21	0.00	-	-	0.00	0.00	0.00	-	-	0.00	0.19	0.00
OD12	-	-	-	-	0.00	0.00	0.00	-	0.06	0.12	0.00	0.00
OD21	0.00	0.00	-	-	-	-	-	-	-	-	-	-
OD26 (CMV neg)	0.00	0.00	-	-	-	-	-	-	-	-	-	-
OD28	0.10	0.05	-	-	-	-	-	-	-	-	-	-
OD10	0.00	0.00	-	-	-	-	-	-	-	-	-	-
OD11	0.00	0.00	-	-	-	-	0.00	0.00	-	0.29	0.00	0.00
OD27	-	-	-	0.00	0.00	0.00	0.00	-	0.07	0.86	0.00	0.00
OD14	-	-	-	-	-	-	-	-	-	-	-	-
OD25	0.00	0.08	-	0.00	-	-	-	0.00	-	-	-	-
OD24	-	-	-	-	0.00	0.00	0.00	-	-	-	-	-
OD30	-	-	-	0.22	0.00	0.00	0.00	-	-	0.58	0.00	0.09
Average	**0.06**	**0.03**	**0.00**	**0.07**	**0.00**	**0.00**	**0.00**	**0.00**	**0.04**	**0.44**	**0.02**	**0.09**

Table A7 - Raw % frequencies of IL-2 producing CD8+ T cell responses in elderly donors.

TNF

Donor:	A1-RTD	A1-YAD	A3-RVD	A11-RSA	B7-RPR	B7-SPS	B7-YPR	B35-HPF	B44-NEG	Cw7-FRC	Cw7-SYR	Cw16-YLC
OD33	1.24	0.37	-	-	-	-	-	-	-	-	-	-
ODO4	0.00	0.00	-	-	-	-	-	-	0.00	-	-	0.00
OD07	0.00	0.00	-	-	0.00	0.00	0.00	-	-	31.07	0.00	14.22
OD01	-	-	0.00	-	-	-	-	-	0.00	0.00	0.12	0.00
OD08	0.53	-	-	-	0.00	0.00	0.00	-	-	0.00	0.29	0.00
OD12	-	-	-	-	0.00	0.00	0.00	-	0.13	2.71	0.00	0.00
OD21	0.00	0.00	-	-	-	-	-	-	-	-	-	-
OD26 (CMV neg)	0.00	0.00	-	-	-	-	-	-	-	-	-	-
OD28	0.17	0.05	-	-	-	-	-	-	-	-	-	-
OD10	0.00	0.00	-	-	-	-	-	-	-	-	-	-
OD11	1.04	0.91	-	-	-	0.85	0.65	0.66	-	6.03	0.41	1.16
OD27	-	-	-	0.03	0.06	0.03	0.03	-	0.00	7.34	0.03	0.44
OD14	-	-	-	-	-	-	-	-	-	-	-	-
OD25	0.00	0.05	-	-	-	-	-	0.00	-	-	-	-
OD24	-	-	-	-	0.00	0.00	0.00	-	-	-	-	-
OD30	-	-	-	0.67	0.00	0.00	0.00	-	-	6.15	0.00	1.67
Average	**0.30**	**0.14**	**0.00**	**0.35**	**0.01**	**0.13**	**0.10**	**0.33**	**0.03**	**3.81**	**0.06**	**1.25**

Table A8 - Frequencies of TNF-α producing CD8+ T cell responses in elderly donors

Chapter 6 – Bibliography

[1] REDDEHASE, M. J. Antigens and Immunoevasins: Opponents in Cytomegalovirus Immune Surviellance. ***Nature Reviews in Immunology*** v. 2, p. 831-844, 2002.

[2] SNYDER, C. M. Buffered memory: a hypothesis for the maintenance of functional, virus specific CD8+ T cells during cytomegalovirus infection. ***Immunology Research*** v. 51, p. 195-204, 2011.

[3] SINZGER, C.; DIGEL, M.; JAHN, G. Cytomegalovirus cell tropism. ***Current Topics in Microbiology and Immunology*** v. 325, p. 63-83, 2008.

[4] SINDRE, H. et al. Human cytomegalovirus suppression of and latency in early hematopietic progenitor cells. ***Blood,*** v. 88, p. 4526-4533, 1996.

[5] SINCLAIR, J. Human cytomegalovirus: Latency and reactivation in the myeloid lineage. ***Journal of Clinical Virology,*** v. 41, p. 180-185, 2008.

[6] WIKBY, A. et al. An immune risk phenotype, cognitive impairment, and survival in very late life: impact of allostatic load in Swedish octogenarian and nonagenarian humans. ***Journal of Gerontology Series A: Biological Sciences and Medical Sciences,*** v. 60, p. 556-565, 2005.

[7] TRZONKOWSKI, P. et al. Association between cytomegalovirus infection, enhanced proinflammatory response and low level of anti-hemagglutinins during the anti-influenza vaccination - an impact of immunosenescence. ***Vaccine*** v. 21, p. 3826-3836, 2003.

[8] VAN DE BERG, P. J. E. J. et al. A fingerprint left by cytomegalovirus infection in the human T cell compartment. ***Journal of Clinical Virology*** v. 41, p. 213-217, 2008.

[9] BIRON, C. A. et al. Natural killer cells in antiviral defense: function and regulation by innate cytokines. ***Annual Reviews in Immunology*** v. 17, p. 189-220, 1999.

[10] GERNA, G. et al. Human cytomegalovirus serum neutralizing antibodies block virus infection of endothelial/epithelial cells, but not fibroblasts, early during primary infection. ***Journal of General Virology*** v. 89, p. 853-865, 2008.

[11] ROSA, C. L.; DIAMOND, D. J. The immune response to CMV. ***Future Virology*** v. 7, p. 279-293, 2012.

[12] CHAKRABARTI, S. et al. High incidence of cytomegalovirus infection after nonmyeloablative stem cell transplantation: potential role of Campath-1H in delaying immune reconstitution. ***Blood*** v. 99, p. 4357-4363, 2002.

[13] SYLWESTER, A. W. et al. Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory compartments of exposed subjects. ***The Journal of Experimental Medicine,*** v. 202, p. 673-685, 2005.

[14] MOSS, P.; KHAN, N. CD8+ T-Cell Immunity to Cytomegalovirus. ***Human Immunology,*** v. 65, p. 456-464, 2004.

[15] WALLER, E. C. P. et al. Dynamics of T cell memory in human cytomegalovirus infection. ***Medical Microbiology and Immunology*** v. 197, p. 83-96, 2008.

[16] SNYDER, C. et al. Memory Inflation during Chronic Viral Infection Is Maintained by Continuous Production of Short-Lived, Functional T cells. ***Immunity*** v. 29, p. 650-659, 2008.

[17] VESCOVINI, R. et al. Massive load of Functional Effector CD4+ and CD8+ T Cells against Cytomegalovirus in Very Old Subjects. ***The Journal of Immunology*** v. 179, p. 4283-4291, 2007.

[18] OUYANG, Q. et al. Dysfunctional CMV-specific CD8(+) T cells accumulate in the elderly. ***Experimental Gerontology*** v. 39, p. 607-613, 2004.

[19] KHAN, N. et al. Cytomegalovirus Seropositivity Drives the CD8 T Cell Repertoire Toward Greater Clonality in Healthy Elderly Individuals. ***The Journal of Immunology*** v. 169, p. 1984-1992, 2002.

[20] LACHMANN, R. et al. Polyfunctional T cells Accumulate in Large Human Cytomegalovirus-Specific T Cell Responses. ***Journal of Virology,*** v. 86, p. 1001-1009, 2012.

[21] GILLESPIE, G. M. A. et al. Functional Heterogeneity and High Frequencies of Cytomegalovirus-Specific CD8+ T Lymphocytes in Healthy Seropositive Donors. ***Journal of Virology*** v. 74, p. 8140-8150, 2000.

[22] JACKSON, S. E.; MASON, G. M.; WILLS, M. R. Human cytomegalovirus immunity and immune evasion. ***Virus Research*** v. 157, p. 151-160, 2011.

[23] BECK, S.; BARRELL, B. G. Human cytomegalovirus encodes a glycoprotein homologous to MHC-class I antigens. ***Nature*** v. 331, p. 269-272, 1988.

[24] EAGLE, R. A. et al. ULBP6/RAET1L is an additional human NKG2D ligand. ***European Journal of Immunology*** v. 39, p. 3207-3216, 2008.

[25] CHANG, W. L. W.; BARRY, P. A. Attenuation of innate immunity by cytomegalovirus IL-10 establishes a long term deficit of adaptive antiviral immunity. ***Proceedings of the National Academy of Sciences USA*** v. 107, p. 22647-22652, 2010.

[26] JONES, T. R.; SUN, L. Human Cytomegalovirus US2 Destablizes Major Histocompatibility Complex Class I Heavy Chains. ***Journal of Virology*** v. 71, p. 2979-2979, 1997.

[27] KHAN, N. et al. Identification of Cytomegalovirus-Specific Cytotoxic T Lymphocytes In Vitro Is Greatly Enhanced by the Use of Recombinant Virus Lacking the US2-US11 Region of Modified Vaccinia Virus Ankara Expressing Individual Viral Genes. ***Journal of Virology*** v. 79, p. 2869-2879, 2005.

[28] MANLEY, T. J. et al. Immune evasion proteins of human cytomegalovirus do not prevent a diverse CD8+ cytotoxic T-cell response in natural infection. ***Blood,*** v. 104, p. 1075-1082, 2004.

[29] WOLKERS, M. C. et al. Antigen bias in T cell cross-priming. ***Science*** v. 304, p. 1314-1317, 2004.

[30] RAMMENSEE, H. G.; FALK, K.; ROTZSCHKE, O. Peptides Naturally Presented by MHC Class l Molecules. ***Annual Review of Immunology*** v. 11, p. 213-244, 1993.

[31] FALK, K. et al. Allele-specific peptide ligand motifs of HLA-C molecules. **Proceedings of the National Academy of Science USA,** v. 90, p. 12005-12009, 1993.

[32] SNARY, D. et al. Molecular structure of human histocompatibility antigens: the HLA-C series. ***European Journal of Immunology*** v. 7, p. 580-585, 1977.

[33] COLONA, M. et al. HLA-C is the inhibitory ligand that determines dominant resistance to lysis by NK1- and NK2-specific natural killer cells. ***Proceedings of the National Academy of Science USA*** v. 90, p. 12000-12004, 1993.

[34] COHEN, G. B. et al. The selective downregulation of class l major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells ***Immunity*** v. 10, p. 661-671, 1999.

[35] SCHUST, D. J. et al. Trophoblast class l major histocompatibility complex (MHC) products are resistant to rapid degradation imposed by the human cytomegalovirus (HCMV) gene products US2 and US11. ***Journal of Experimental Medicine*** v. 188, p. 497-503, 1998.

[36] CARRASCO, J. et al. CD45RA on human CD8 Tcells is sensitive to the time elapsed since the last antigenic stimulation. ***Blood*** v. 108, p. 2897-2905, 2006.

[37] SHAHINIAN, A. et al. Differential T Cell Costimulatory Requirements in CD28-Deficient Mice. ***Science*** v. 161, p. 609-612, 1993.

[38] KURTS, C.; ROBINSON, B. W. S.; KNOLLE, P. A. Cross-priming in health and disease. ***Nature Reviews in Immunology*** v. 10, p. 403-414, 2010.

[39] ARRODE, G. et al. Cross-Presentation of Human Cytomegalovirus pp65 (UL83) to CD8+ T Cells Is Regulated by Virus-Induced, Soluble-Mediator-Dependent Maturation of Dendritic Cells. ***Journal of Virology*** v. 76, p. 142-150, 2002.

[40] BUSCHE, A. et al. Priming of CD8+ T cells against cytomegalovirus-encoded antigens is dominated by cross-presentation. ***Journal of Immunology*** v. 190, p. 2767-2777, 2013.