

Functional Competence of CD8+ T cell Responses specific to Human Cytomegalovirus in Common Variable Immunodeficiency

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By

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Declaration of authorship

I declare that the work in the thesis was carried out in accordance with the regulations of the University College London. The work is original, except where indicated by special references in the text, and no part of the thesis has been submitted for any other academic award.

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Abstract

This PhD investigated the role of human cytomegalovirus (HCMV) in inflammatory disease associated with common variable immunodeficiency (CVID) by examining the functional competence of HCMV specific CD8⁺ T cell responses. The project was based on the hypothesis that HCMV is a major factor driving the expansion of CD8⁺ T cells that contribute to the inflammatory pathology.

HCMV specific CD8⁺ T cell frequencies were significantly elevated in inflammatory patients compared to non-inflammatory patients or healthy subjects. The frequency of EBV (GLC) epitope specific CD8⁺ T cells did not differ between patient groups. HCMV CD8⁺ T cells from inflammatory patients displayed a distinct cytokine expression profile with the majority of cells producing IFN- γ only or IFN- γ and TNF- α in response to antigen stimulation. These cells did not show evidence of exhaustion, with low PD-1 expression; rather, they showed high functionality, high TCR avidity and high proliferative potential. CD8⁺ T cells from inflammatory patients but not non-inflammatory patients or healthy donors expressed high levels of Ki-67 and proliferated in response to antigen stimulation *in vitro* without co-stimulation. Further phenotypic analysis revealed striking correlations between the frequencies of HCMV specific CD8⁺ T cells expressing PD-1 or granzyme B and the overall frequency of CD8⁺ CD27⁻ CD28⁻ T cells. Consistent with their hypothesized role in the inflammatory disease, the CD8⁺ T cells from inflammatory patients expressed reduced levels of the anti-inflammatory marker CD73. Further support for the involvement of HCMV in driving the inflammatory pathology came from collaborative work in which viral antigen was detected at the sites of inflammation.

The results support the hypothesis that HCMV and HCMV-specific T cells are key factors in CVID associated inflammation. They explain previously reported T cell ‘abnormalities’ seen in CVID and provide an evidence base for clinical trials of anti-TNF therapy and/or antiviral therapy in these patients.

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I owe a great deal of loving thanks to my mother and father for all their unlimited support, love throughout my life, and providing me with perhaps the greatest gift of all: an education! Therefore, I would like to take this opportunity to dedicate this PhD thesis to them, my sisters and my brothers for great support, encouragement, and love I have received from them.

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Glossary

Adenosine Triphosphate	ADP
Antibody-dependent cell-mediated cytotoxicity	ADCC
Allophycocyanin	APC
Antigen	Ag
Antigen Determining Region	AD
Antigen Presenting Cells	APCs
Bone Marrow Transplant	BMT
Bovine serum albumin	BSA
Burkitt's lymphoma	BL
Carboxyfluorescein succinimidyl ester	CFSE
Cluster of Differentiation	CD
Common Variable Immunodeficiency	CVID
Complement control protein	CCP
Cytotoxic T lymphocyte	CTL
Delayed early	DE
Dendritic Cells	DC
Deoxyribonucleic Acid	DNA
Endoplasmic Reticulum	ER
Epidermal growth factor receptor	EGFR
Epstein-Barr virus	EBV
Epstein-Barr nuclear antigen	EBNA
Fas ligand	FasL
Fluorescein isothiocyanate	FITC
Fluorescence Activated Cell Sorter	FACS
Foetal calf serum	FCS
Ganciclovir	GCV
Glycoprotein B	gB

Guanosine monophosphate	GMP
Guanosine Triphosphate	GTP
Heparin Sulphate Proteoglycan	HSPG
Hodgkin lymphoma	HL
Human Cytomegalovirus	HCMV
Human Herpes Virus 4	HHV4
Human Herpes Virus 5	HHV5
Human Herpes Virus 6	HHV6
Human Immunodeficiency Virus	HIV
Human Leukocyte Antigen	HLA
Inducible costimulator	ICOS
International committee on Taxonomy of viruses	ICTV
Immediate Early	IE
Immune risk phenotype	IRP
Immunoglobulin	Ig
Immunoreceptor tyrosine-based activation motif	ITAM
Immunoreceptor tyrosine-based inhibitory motif	ITIM
Infectious mononucleosis	IM
Inflammatory bowel disease	IBD
Interferon gamma	IFN- γ
Interleukin	IL
Intravenous	IV
Inverted Repeats	IR
Killer inhibitor receptors	KIR
Kilo Dalton	KDa
Latent membrane protein	LMP
Lymphoblastoid cell line	LCL
Major Capsid Protein	MCP
Minor Capsid Protein	mCP

Major Histocompatibility Complex	MHC
Major Immediate Early	MIE
Major Immediate Early Promoter	MIEP
Matrix metalloproteinase	MMP
Milliliter	ml
Monocyte derived macrophage	MDM
Murine Cytomegalovirus	MCMV
Nasopharyngeal carcinoma	NC
Natural Killer (cell)	NK
Nuclear factor kappa B	NFKB
Open Reading Frame	ORF
Origin of lytic	OriLyt
Pathogen-associated molecular pattern	PAMP
Pattern recognition receptor	PRR
Peptide loading complex	PLC
Peptide nucleic acid	PNA
Peridinin chlorophyll protein	PerCP
Peripheral Blood Mononuclear Cell	PBMC
Post-transplant lymphoproliferative disease	PTLD
Precursor assembly protein protease	pAP
Phosphate Buffered Saline	PBS
Phycoerythrin	PE
Protein Kinase C	PKC
Small Capsid Protein	SCP
T-cell Receptor	TCR
Tissue inhibitor of metalloproteinase	TIMP
TNF-related apoptosis inducing ligand	TRAIL
Toll-like receptor	TLR
Transporter associated with antigen processing	TAP

Trypan blue	TB
Tumour necrosis factor alpha	TNF- α
UL 16-binding protein	ULBP
Unique Long Region	ULR
Unique Short Region	USR

Chapter 1

1 Introduction

1.1 History of cytomegalovirus

It was in 1881 that Ribbert first noted large “protozoan-like” cells in the section of the kidney and the parotid glands of children. He published his observation in 1904 although he was not able to interpret them until he noticed the report by Jesionek and Kiolemenoglou, who described similar cells in the lungs, kidneys and liver of an 8 month luetic, old name for syphilis, fetus. The eccentrically placed nuclei of these large cells contained a “central nuclear body” surrounded by two well defined zones, an inner dark and an outer clear halo. Additional instances were reported in 1910 and again in 1914 by Smith and Weidman, but they concluded that they were dealing with protozoa until 1921 when Goodpasture and Talbot reported the similar structures in the lungs, liver, and kidneys of a 2 months’ old infant and they concluded that they were dealing with a new kind of abnormal cytomorphosis to which they gave the name of *cytomegalia*. These findings appear to be the first description of cytomegalic cells containing intranuclear inclusions (Farber and Wolbach, 1932). In 1925, Von Glahn and Pappenheimer noted that Lipschuetz had also discovered these intranuclear inclusion containing cells four year earlier from a man infected by herpes zoster. Therefore, like Goodpasture and Talbot, they doubted that these cells were related to protozoa and they believed that these unusual cells might be related to a group of related viruses, now known as the *Herpesviridae* (Ho, 2008). The story of detecting the large cells with similar structures (containing typical intranuclear inclusions) from variety of organs carried out until the name of “generalized cytomegalic inclusion disease (CID)” had been suggested by Wyatt et al, although the virological etiology of CID was not yet known (Wyatt and Saxton, 1950). It was 1953 when in a case of CID, Minder first saw

by electron microscopy the particles of 199nm size, suggestive of a virus, in the pancreatic cells containing intranuclear inclusions (Minder, 1953).

However, discovery of human embryonic cell cultures provided a breakthrough for the field of virology and many virologists who were eager to apply these techniques. Tomas Weller, who was a pediatrician and parasitologist, applied the newly discovered cell culture techniques to culture the protozoa *Toxoplasma* and to clinical cases of CID. This work together with two other independent studies headed by Smith and Rowe finally led to the identification of a virus that later proposed to name as cytomegalovirus by Weller (Craig et al., 1957;Weller, 1970).

Human cytomegalovirus (HCMV) or human herpes virus 5 (HHV5) is the prototype member of the *Betaherpesvirinae* subfamily of the *Herpesviridae* family. The recent availability of extensive nucleotide sequence data has led to the establishment of a new order, *Herpesvirals*, by the Herpesvirus Study Group of the International Committee on Taxonomy of Viruses (ICTV) (Pellett and Roizman, 2007).

In general, species-specificity is one of the main characteristics of the *Betaherpesvirinae* and therefore each species has their own uniquely adapted CMV. The fact that human cytomegalovirus does not infect animals is one of the limitation of studying CMV. Although beyond the scope of the present study, there are several animal models (Figure 1-1) for studying various aspects of CMV in addition to *in vitro* models such as AD169 (a commonly used laboratory strain of human CMV which is derived from the clinical strains as a result of *in vitro* passage) (Wynn and Khanna, 2006).

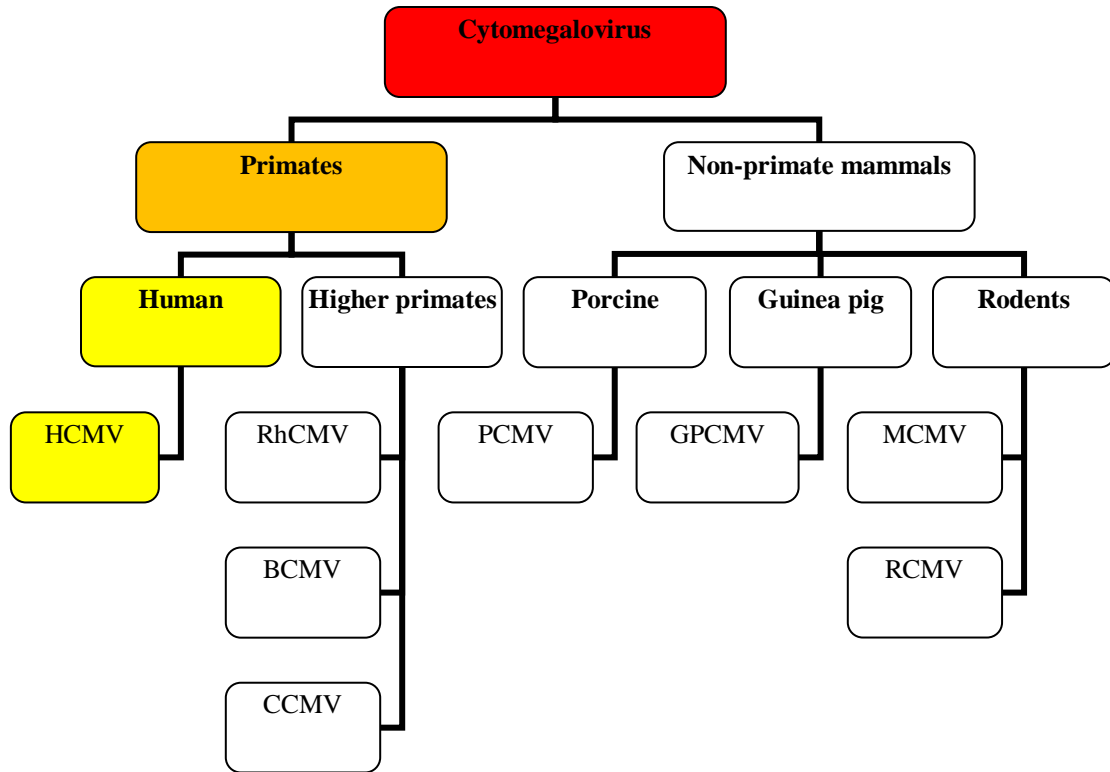


Figure 1-1: Schematic representation of animal models used for studies of cytomegalovirus.

The focus of this study is HCMV as shown in coloured boxes. The figure is modified from(Wynn and Khanna, 2006). *Abbreviations: RhCMV, rhesus CMV; BCMV, baboon CMV; CCMV, chimpanzee CMV; PCMV, porcine CMV; GPCMV, guinea pig CMV; MCMV, murine CMV, RCMV, rat CMV.*

1.2 Epidemiology of Human Cytomegalovirus

Naturally, transmission of HCMV infection occurs both vertically and horizontally through direct contact with infectious body fluids including blood, saliva, breast milk, and cervical secretions, indicating the intimate contact requirement for its spread. Contact with children and sexual activity are two of the most common types of horizontal modes of transmission (Pereira et al., 1990; Sohn and Oh, 1991; Coonrod et al., 1998).

Sero-epidemiological studies carried out in different populations show that human cytomegalovirus is ubiquitous among the population with seroprevalance levels ranging from 60-100% based on numerous factors including living circumstances, socio-economic status, and hygiene. Many of HCMV infections are acquired during early childhood and the overall prevalence of HCMV infection generally increases with age. However, the influence of seasonal variation on the prevalence of HCMV infection has not been proven (Gratacap, 1998; Mocarski et al., 2007).

1.3 Replication cycle of HCMV

1.3.1 Structure and genome organization

Similar to other members of *Herpesviridae*, HCMV morphologically consists of a virion structure at 200 to 300nm diameter containing a double-stranded linear DNA core which is enclosed in an icosahedral capsid at 100-110nm size. The capsid is composed of 162 capsomers which is embedded in a tegument containing a number of virus-encoded proteins which are mostly phosphorylated. The tegument is surrounded by a host cell

derived lipid bilayer containing more than 20 virus encoded surface glycoproteins which are incorporated during the maturation process (Figure 1-2) (Emery and Griffiths, 1990; Mocarski et al., 2007; Crough and Khanna, 2009).

The terminally redundant genome is composed of two covalently linked segments so-called long (L) and short (S), each consisting a unique long (UL) and unique short (US) domains, which are flanked on one end by terminal repeated sequences (TRL and TRS) and on the other end by internal repeats (IRL and IRS) (Figure 1-2) (Davison et al., 2003; Dolan et al., 2004).

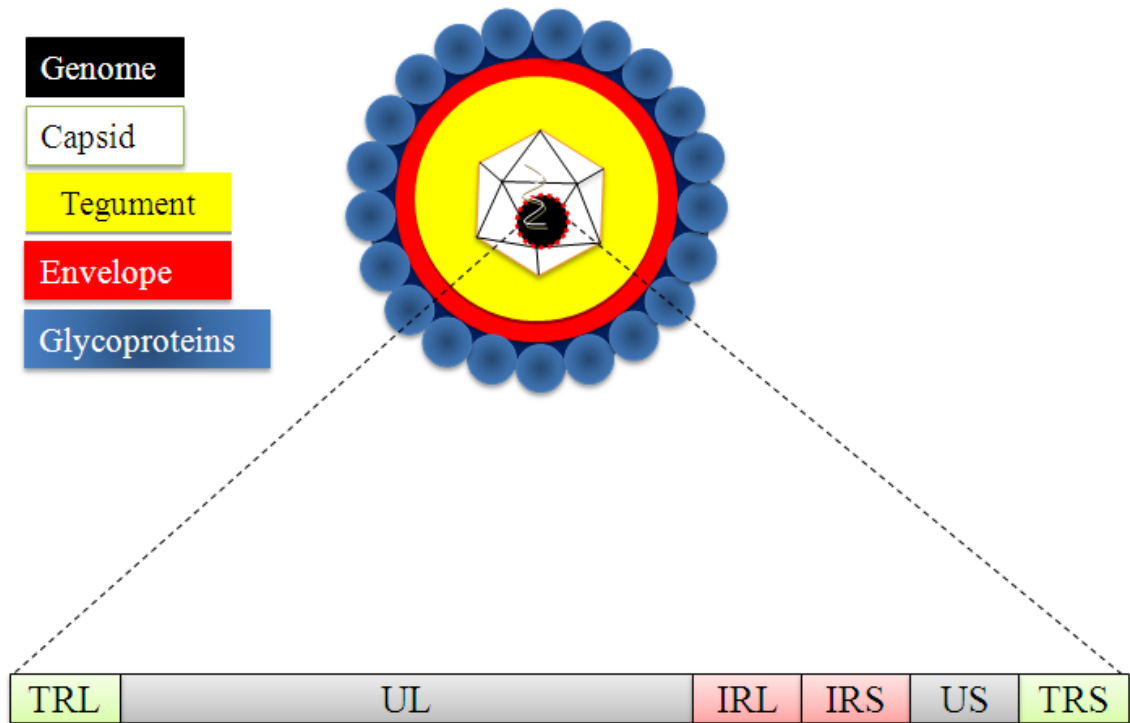


Figure 1-2: Schematic representation of the structural features as well as genome organizations of HCMV (not to scale).

The lettering within the individual segments of the genome indicates the following features: terminal repeat long (TRL); unique long (UL); internal repeat long (IRL); internal repeat short (IRS); unique short (US); and terminal repeat short (TRS).

The life cycle of HCMV initiates with a series of distinct steps including attachment, cell penetration, gene transcription and DNA replication which is followed by virion assembly and egress (Compton, 2004; Mocarski et al., 2007).

1.3.2 Attachment and Entry

To initiate infection, the virus must attach to the cell surface. The cell entry of HCMV can be triggered in at least two ways, resulting in different pathways of entry. Fusion of virus envelope with the plasma membrane is considered to be the main pathway which is dependent on multiple receptor-ligand interaction on the cell surface (Compton and Fiere, 2006). The second pathway involves endocytosis of the enveloped capsid with the membrane of the endocytic vesicle (Ryckman et al., 2006) (Figure 1-3). Many different cell surface molecules can serve as receptors for the virus entry. Initial binding is believed to be mediated through engagement of glycoprotein B (gB) which binds to cell surface heparan sulphate proteoglycan (HSPG) moieties, a relatively conserved feature of herpesvirus entry pathways (Mocarski et al., 2007). Glycoprotein B, encoded by the UL55 gene, is the major structural protein of the viral envelope which is present on the surface of infected cells, involving in all stages of virus entry (Emery and Shannon-Lowe, 2007). Interaction of gB with heparan sulphate is believed to initiate a signaling cascade that involves other cellular receptors and viral entry mediators (Boyle and Compton, 1998) such as heterodimeric complexes of glycoproteins H, L, M, and N (gM: gN, gH: gL). The role of gB and gH/gL as the main HCMV entry molecules has been supported by most studies (Compton et al., 1993; Compton and Fiere, 2006; Mocarski et al., 2007). It has recently been shown that HCMV infects cells through interacting with epidermal growth factor receptor (EGFR), indicating additional cell receptors for virus.

In response to human cytomegalovirus, EGFR is phosphorylated, inducing signaling events through activating the phosphatidylinositol 3-kinase (PI-3 kinase), Akt (serine/threonine protein kinase), and releasing intracellular calcium stores (Wang et al., 2003).

Following attachment, virus penetration occurs at the cell surface via direct fusion such as in fibroblasts or via an endocytic route in endothelial cells. Based on cell type, it seems the functional requirement involved in attachment and penetration vary, suggesting the various minor envelope glycoproteins of HCMV may play a part in entry into different cell types. Once the virus nucleocapsid containing the viral genome and tegument proteins is deposited into the cytoplasm, the capsid is transported along cytoplasmic microtubules and then translocated to the nucleus where viral DNA is entered through nuclear pores (Mocarski et al., 2007).

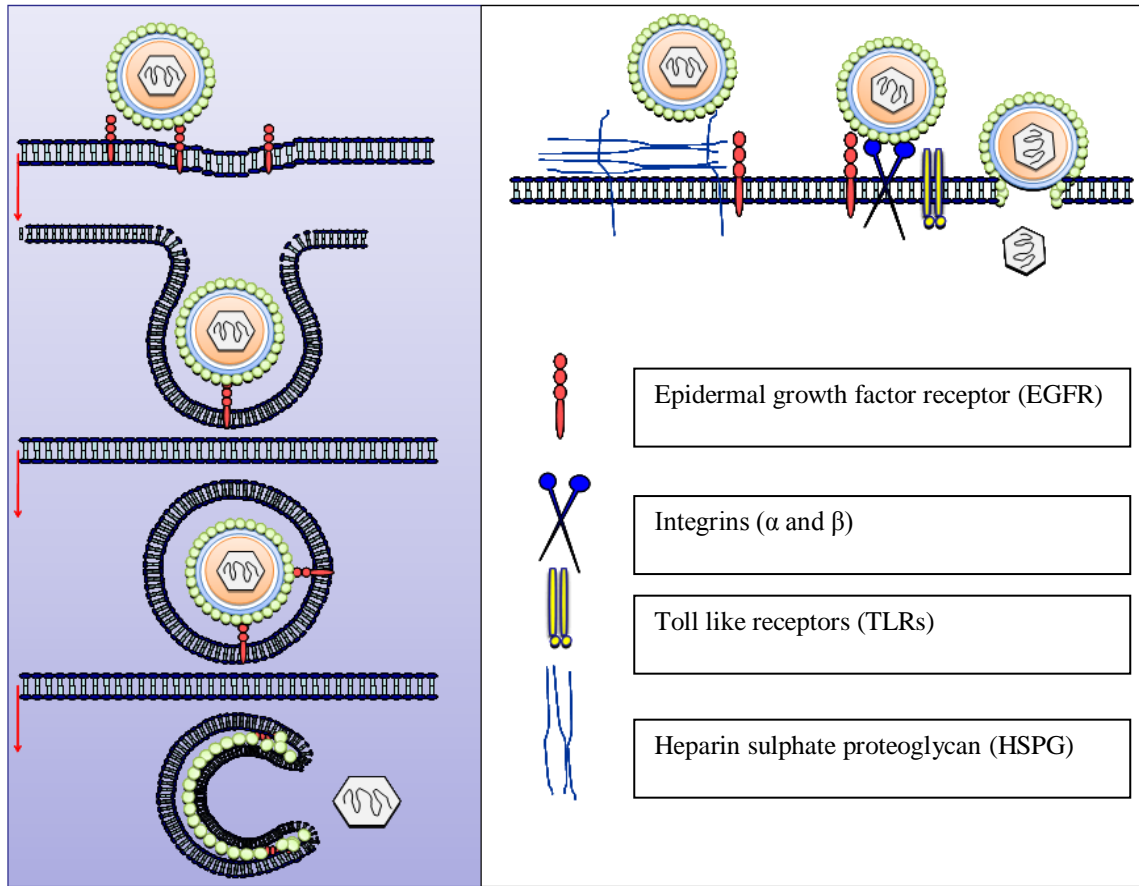


Figure 1-3: Schematic representation of working models of human cytomegalovirus entry into the cells.

Depending on cell type, receptor-dependent endocytosis (shaded box) or receptor-dependent fusion are main entry pathways. This figure is modified from (Compton, 2004;Milne et al., 2005)

1.3.3 Viral gene expression and DNA replication

Following the deposit of the HCMV nucleocapsid into the cytoplasm, it translocates to the nucleus where viral DNA is released. It appears that cytoplasmic microtubules facilitate the translocation of viral genome to the nucleus (Mocarski et al., 2007). Expression of IE genes starts immediately following the entry of viral genome to the nucleus. HCMV gene expression like other members of the *Herpesviridae* is temporally regulated, with three classes of gene products namely immediate-early (IE) (α), delayed-early (DE) (β) and late (L) (γ) (Figure 1-4) (Emery and Shannon-Lowe, 2007). Initial transcription occurs in the absence of protein synthesis and is not affected by the expression of other viral genes while the expression of DE genes requires the expression of functional products of IE genes. Following the interaction of the viral envelope with the cell membrane and entering the viral genome to the nucleus, IE gene expression occurs by regulating the activity of HCMV major IE promoter (MIEP). Although the regulation of IE gene expression is highly complex, many gene products including IE1 and IE2, UL36 and UL37, and TRS1/IRS1 have been shown to be important for the regulation and the activities of MIEP. These genes give rise to multiple transcripts and gene products. IE1 (UL123/IE72) and IE2 (UL122/IE86) are two major immediate early transactivators which play a central role in the activation of DE and L gene expression. In addition, ppUL123 and ppUL122 are shown to be essential transactivators for DNA replication (Mocarski et al., 2007). UL36 and UL37 are two other gene products that influence gene expression during replication. It has also been shown that these two genes have anti-apoptotic functions (Mocarski et al., 2007). In addition to being a transactivator, the gene products of TRS1 and IRS1 are both shown to counteract with

the host cell antiviral response pathways by blocking IFN-inducible protein kinase R (PKR) (Child et al., 2004; Mocarski et al., 2007).

The DE step of HCMV replication initiates following the expression of major immediate early (MIE) regulatory proteins when a large proportion of the viral genome becomes transcriptionally active. This stage starts by 6 hours post infection (hpi), lasting around 18 to 24 hpi when synthesis of viral DNA starts (Yu et al., 2003). Several genes such as UL112/UL113 and UL54 are involved in regulation of the DE stage which encodes viral transcripts or gene products required for DNA replication or stages of capsid maturation. The expression of the UL54 gene encoding DNA polymerase catalytic subunit has been shown to start early but reaches much higher levels at late (L) times of infection. Despite their importance, little information is available regarding late gene expression in HCMV infected cells (Mocarski et al., 2007).

Although HCMV replication is a slow event *in vitro* and takes 48 to 72 hours before the release of progeny virions, in the human host active replication of HCMV is highly dynamic (with doubling times around 1 day) although the rate of replication depends on several factors such as target cell availability and the functional capacity of the specific immune system (Emery et al., 1999). The HCMV genome contains one origin of lytic (*oriLyt*) and DNA synthesis of virus depends on transcriptional activation of *oriLyt* which is a structurally complex region. The core region of *oriLyt* includes repeat elements and transcription factor binding sites (Prichard et al., 1998). Viral DNA synthesis have been shown to be dependent on a set of HCMV proteins, including UL54-UL44 complex with polymerase activity (UL54: the DNA polymerase catalytic subunit; UL44: the polymerase accessory protein), single stranded (ss) DNA binding

protein (UL57), and the heterotrimeric complex of helicase-primase (HP) which consist of HP1 (UL105), HP2 (UL70), and UL102-encoded HP3 (McMahon and Anders, 2002). However, HCMV DNA replication occurs on the circularized genome and create a concatameric DNA structure that needs to be cleaved into unit genome lengths prior to encapsidation (Mocarski et al., 2007).

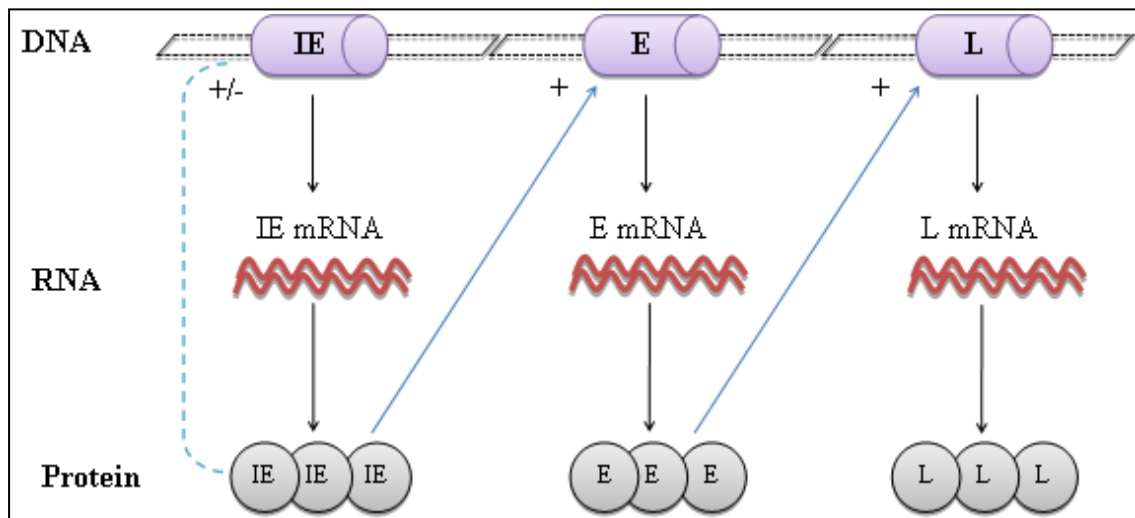


Figure 1-4: Schematic representation of virus structure and temporal hierarchy of gene expression by HCMV.

IE, E, and L indicate immediate early, early, and late, respectively. The IE genes are expressed within 2-4 hours of infection followed by the expression of early genes which starts within 8-24 hours post infection before initiation of late gene expression. The image is provided and modified with permission from Professor Vincent Emery.

1.3.4 Viral Capsid Assembly and Virion Egress

Capsid maturation, DNA encapsidation, and release from the cells are crucial functions which are carried out by late and many delayed-early genes. Following accumulation of L proteins, viral DNA is encapsidated in the nucleus and matures by moving to the cytoplasm (Penfold and Mocarski, 1997). Studies from most herpesviruses suggest the involvement of a complex two-stage process (envelopment/ de-envelopment/ re-envelopment) in the budding processes of newly formed virions. This process occurs in different subcellular compartments and finally leads to virion release by exocytosis at the plasma membrane. The first step (or envelopment process) starts at the inner nuclear membrane and it has been shown that two HCMV-encoded proteins of UL50 and UL53 genes are crucial (Mettenleiter, 2004). This process delivers viral particles to the pre-nuclear space between the inner and outer nuclear membranes where it is likely to be followed by a de-envelopment event. A secondary process (or final envelopment) occurs in cytoplasm in close proximity to the Golgi apparatus. Whereas some virion tegument proteins are associated with the nucleocapsid during the first envelopment process, it appears that the bulk of these proteins found in mature virions are added to the nucleocapsid during final or secondary envelopment as it travels through the cytoplasm (Mocarski et al., 2007). The endpoint of the final envelopment process is an enveloped virion within a secretory vesicle which is then transported to the plasma membrane where it fuses, resulting in the release of mature virions from the infected cells (Mettenleiter, 2004).

1.4 Role of different cell types in replication of HCMV

HCMV has strict host specificity and is limited to humans; however, within the human host the virus can target an exceptionally broad range of cell types including endothelial cells, connective tissue cells, mucosal epithelial cells and smooth muscle cells, macrophages and dendritic cells. In immunocompromised hosts, different cell types from various organs including liver, gastrointestinal tract, lung, kidneys, salivary glands, retina and brain have been shown to be sites of infection with HCMV, with the endothelial and epithelial cells being the primary target for virus infection (Sinzger et al., 2008; Revello and Gerna, 2010).

In cell culture, primary cultures of human foreskin or embryonic lung fibroblasts (HELFL) are the most commonly used cell type for propagation of HCMV which yield relatively high titer stocks (Revello and Gerna, 2010). However, having a finite life span and not being predominantly infected *in vivo* are two main disadvantages of primary human fibroblasts that limits the study of HCMV replication (Bresnahan et al., 2000; Emery, 2001). Several other cell types including vascular smooth muscle cells (Tumilowicz et al., 1985), placental trophoblast cells (Halwachs-Baumann et al., 1998), retinal pigment epithelial cells (Tugizov et al., 1996), hepatocytes (Sinzger et al., 1999), macrophages and monocytes (Ibanez et al., 1991), astrocytoma cell line (Kari et al., 1992), a teratocarcinoma cell line (LaFemina and Hayward, 1986), and endothelial cells have been shown to support the replication of the virus *in vitro*. However, none of these cell lines produce high-titer stocks or plaques when infected with HCMV (Bresnahan et al., 2000). The entry of HCMV into certain cell types such as epithelial and endothelial cells have been shown to be influenced to large extent by the unique long b' (ULb')

region of the HCMV genome, encoding particularly UL131A, UL130 or UL128 genes which form a complex with gH/gL (Hahn et al., 2004; Wang and Shenk, 2005; Revello and Gerna, 2010).

Since the immediate-early phase of viral gene expression is dependent on transcription factors in the host cell, it was thought that cell type-specific differences in IE transcription of HCMV might be the reason behind differences in permissiveness to infection in most cultured cell types, even those that are not permissive for virus infection but high levels of transcription by the major IE promoter have been observed (Fritschy et al., 1996). The ability of HCMV to persist in the face of strong T and B cell immunity is likely related to its properties such as latency, cell association of virions and a large number of virus encoded gene products that allow the virus to escape the host immune system through different mechanisms (see later) (Reeves and Sinclair, 2008; Sinclair, 2010). Persistent virus replication usually occurs in epithelial cells of salivary glands as well as kidneys which shed sporadically in body fluids throughout life. Reactivation of virus occurs when an endogenous virus reactivates in a seropositive individual (Emery et al., 2000; Rubin, 2007)..

1.4.1 Latency and virus reactivation

HCMV like other members of herpes family establishes lifelong latency within the host and as a result, the ability to reactivate from latency is a common feature of this virus. Viral latency can be defined as the ability of the viral genome to maintain itself in the absence of production of infectious virions. Under certain conditions, however, the viral genome is able to reactivate (Sinclair, 2008). Genomic viral DNA has been detected in

various cell types including monocytes and macrophages (Taylor-Wiedeman et al., 1991; Soderberg et al., 1993), endothelial cells (Sinzger et al., 1995), lymphocytes (Schrier et al., 1985), and CD34+ bone marrow cells (Mendelson et al., 1996). Although the exact site of virus latency has not yet been determined, cells of myeloid lineage appear to be the main site of latency (Soderberg-Naucler et al., 1997b; Sinclair, 2008). Active viral replication appears to be directly related to the state of differentiation of the permissiveness of cells such as myeloid cells. However, HCMV can enter the undifferentiated cells but these cells are not permissive for viral replication (Soderberg-Naucler et al., 1997b; Sissons et al., 2002).

Regarding the establishment of virus latency, at least three possible pathways have been proposed. The first possibility is the entrance of virus (following attachment) directly into a latent state without expressing any de novo viral genes. A second option is that following entry the virus initiates productive infection which is interrupted prematurely which may finally lead to latency. The third possibility is the expression of a subset of viral genes unrelated with productive infection but appear to be necessary for the successful establishment of latency (Kondo et al., 1994; Goodrum et al., 2002; Reeves et al., 2005a; Cheung et al., 2006; Crough and Khanna, 2009).

It is becoming increasingly clear that the repression of the major immediate early promoter by cellular transcription factors as well as post-transcriptional of histone modifications is the key event in latency (Sinclair, 2010; Reeves, 2010). Therefore, desilencing of the MIE promoter or enhancer is shown to be crucial for triggering the reactivation of virus from latency (Reeves et al., 2005b; Zydek et al., 2010). In addition, it has been suggested that cytokines particularly TNF- α can play a role in reactivation of

HCMV infection (Docke et al., 1994;Fietze et al., 1994). In this context, it has been shown that more than 25 gene products of HCMV play some part in modulation of both innate and adaptive immune responses either directly or via a proinflammatory process based on cytokines such as TNF- α which binds to TNF- α receptor on latently infected cells, stimulating the latently infected cells to differentiate and support virus reactivation probably via initiating a signaling process which involves activation of NF- κ B (Pleskoff, 2005;Mocarski et al., 2007;Rubin, 2007). Reactivation of HCMV has also been shown in response to inflammation or even stress (Prosch et al., 2000;Crough and Khanna, 2009), but the precise mechanisms involved are not fully elucidated. It seems that stress catocholamines, epinephrine and norepinephrine are, at least in part, responsible for HCMV reactivation via inducing cyclic AMP-dependent signaling pathways and finally stimulating the reactivation process (Reinke, 1999).

1.5 Pathogenicity and Clinical Manifestation

Human cytomegalovirus disease can follow primary infection or reactivation. In immunocompetent individuals, primary (and recurrent) infection with HCMV is usually asymptomatic, indicating a healthy immune system has the ability to control virus. Although rare, severe infection with HCMV even in the immunocompetent host has been reported (Eddleston et al., 1997). Additionally, congenital CMV disease, which is an important medical and public health problem, occurs in the normal host (Mocarski et al., 2007). Clinical symptoms of newborns with congenital HCMV disease are included petechiae, microcephaly, purpura, hepatospelenomegaly, prematurity, and jaundice.

However, when the host immune system is unable to function properly due to either infection such as HIV, immaturity as seen in neonates, or through iatrogenic intervention following organ transplantation, HCMV becomes potentially pathogenic. In immunocompromised individuals particularly in transplant patients HCMV infection can arise from reactivation virus, reinfection, or primary infection (Emery, 2001). Viral replication and viral load have been shown to be important factors in pathogenesis in neonates, AIDS patients, and transplant populations. It has also been demonstrated that there is a direct correlation between peak viral loads and CMV disease (Cope et al., 1997; Hassan-Walker et al., 1999; Emery et al., 2000; Regoes et al., 2006). Accordingly, high levels of virus replication in the T cell immunocompromised patients such as HIV-infected individuals has been observed to be related with a variety of end-organ diseases caused by HCMV (Regoes et al., 2006).

In the immunocompromised host, HCMV infection can cause a range of clinical manifestations (such as gastrointestinal, retinitis, and pneumonitis) which can vary between immunocompromised patient groups (Table 1-1). Pneumonitis, for example, is the most common clinical manifestation of HCMV in bone marrow recipients, whereas hepatitis and retinitis are the most frequent clinical features in liver transplant recipients and patients with AIDS, respectively (Emery, 2001).

Clinical Manifestations	Immunocompromised host		
	SOT	HST	AIDS
Fever	*	*	*
Hepatitis	**	*	
Gastrointestinal	*	*	*
Retinitis	*	*	**
Pneumonitis	*	**	
Encephalopathy			*
Polyradiculopathy			*
Myelosuppression		**	
Immunosuppression	*		
Rejection	*		
Addisonian		*	

Table 1-1: Clinical manifestation of cytomegalovirus in immunocompromised host.

The table is reproduced from (Emery, 2001). Abbreviations: *SOT*, solid organ transplantation; *HST*, haematopoietic stem cell transplantation; *AIDS*, Acquired immune deficiency syndrome.

1.6 Immune responses to HCMV

Our knowledge of the exact mechanisms behind the viral latency is not complete; however, there is no doubt that immune responses are crucial in keeping HCMV replication, and as a consequence life threatening disease, in check. Although beyond the focus of this study, there are several animal models available (described in section 1.1) to investigate different functional aspects of immune response against cytomegalovirus (Wynn and Khanna, 2006). For example, MCMV is the most commonly used animal model to address many fundamental aspects of immunology such as the cellular (Sierro et al., 2005) and humoral immune responses to CMV (Wilson et al., 2008). However, for several reasons defining the crucial features of protective responses to relevant viruses in human can not be directly applied by the findings from the mouse model systems. Because deliberate infection of humans is impossible and symptoms of primary infection may not be recognized or only develop weeks following infection, it is difficult to identify the exact time point of infection in humans which is contrary to what can be achieved in mouse models. Additionally, humans encounter a variety of pathogens throughout their life some of which (such as herpesviruses) can reach a latent stage in the host; as a consequence, different viral-specific T cells can present and be active simultaneously whereas in mouse models it is usual to study singular pathogens. Another issue that needs to be considered in this context is the fact that, compared to the human immune system providing lifelong protection, many studies performed in mice usually last for few weeks. Furthermore, in immunocompetent mice the immune system has the ability to clear the majority of pathogens whereas even in immunocompetent

individuals, many of the persistent viruses are not eliminated and have to be continuously kept in check by the immune system (van Leeuwen et al., 2006).

It appears that innate immunity, such as NK cells, and adaptive immune responses including CD8, and CD4 T cells, all play important roles in containing HCMV (Khan, 2007). In a study carried out by Emery et al, preexisting HCMV immunity has been shown to reduce both the replication rate and the peak of virus load during HCMV active infection (Emery et al., 2002). According to many studies protective immunity is T cell mediated, although in most settings especially in vertical transmission, antibody responses play an important role (Plotkin et al., 1989;Gonczol et al., 1989;Mocarski et al., 2007).

1.6.1 Innate immunity

The innate immune system is essential for the initial detection of invading viruses and subsequent activation of adaptive immunity. Recruiting immune cells such as NK cells and phagocytes to sites of infection, recognition of foreign pathogens by several innate immune receptors expressed either at the host cell surface or within cells, activation of the complement cascade as well as the adaptive immune system through antigen presentation are major functions of innate immune system.

Natural Killer Cells: natural killer cells (NK) are an integral part of innate immunity that serve as a first line of defense to prevent viral invasion (Redpath et al., 2001). NK cells are widespread throughout lymphoid and non-lymphoid tissues and represent a minor fraction of total lymphocytes (from 2% to 18% in human peripheral blood). Through various activatory (ITAMs: immunoreceptor tyrosine-based activation motifs)

and inhibitory (ITIMs: immunoreceptor tyrosine-based inhibition motifs) cell surface receptors, NK cells recognize a vast array of molecules (Vivier et al., 2008). These cells mediate their killing activity via different mechanisms (Lee et al., 2007), including release of cytokines (Orange et al., 1995), utilizing cytolytic granules such as perforin (Loh et al., 2005), and induction of cell death through TNF-related apoptosis inducing ligand (TRAIL) (Mirandola et al., 2004). In addition, NK cells are known to express several cell surface activating complexes that are formed by noncovalent association between distinct transmembrane ligand-binding and signaling adaptor polypeptides such as Fc γ receptor. These receptors enable NK cells to recognize the antibody-coated target cells and thereby facilitate antibody-dependent cell cytotoxicity (ADCC) and cytokine production (Vivier et al., 2004).

The role of NK cells in the clearance of experimental MCMV infection has been demonstrated as the adoptive transfer of NK cells can provide protection against MCMV (Bukowski et al., 1985; Polić et al., 1998b; Crough and Khanna, 2009). Little is known about the role of these cells in the immune defense of humans against HCMV. Data supporting a key role for these cells in HCMV infection came from the increasing numbers of viral gene products blocking NK cell-mediated recognition (see section 1.7). In addition, the MHC class I down regulation which the virus uses to escape T cell response likely increases the susceptibility of infected-cells to lysis by NK cells (Falk et al., 2002; Mocarski, 2004). An increase of NK cell activity has also been shown during both primary and recurrent HCMV infection of renal transplant patients (Venema et al., 1994).

Pattern recognition receptors: Another innate immune component which has recently received extensive attention is Toll-like receptors (TLRs) and their critical role in host defense against infection. It was originally thought that innate immunity recognize antigens nonspecifically, however, the discovery of TLRs in the mid-1990s showed that pathogen recognition by the innate immune system instead occurs in a specific manner. This specific recognition is receptor mediated and relies on germ line-encoded pattern-recognition receptors (PRRs) that have evolved to detect components of foreign pathogens referred to as pathogen-associated molecular patterns (PAMPs). Based on the recognition patterns there are several members of the TLR family (10 and 12 functional TLRs in human and mice, respectively). Toll-like receptors are type I transmembrane proteins with multiple leucine-rich ectodomains that mediate the recognition of PAMPs and are related to IL-1 receptors based on the similarity in the cytoplasmic portions. It appears that TLRs are essential for protective immunity against infection. However, inappropriate TLR responses can contribute to systemic autoimmune diseases as well as inflammation (Kawai and Akira, 2010).

The role of TLRs in sensing innate responses to viral pathogens including HCMV is becoming increasingly clear. Following stimulation, TLRs activate signal transduction pathways which can induce the secretion of a broad range of inflammatory cytokines that are important for the activation of adaptive immune response (Compton et al., 2003;Boehme and Compton, 2004). It has been documented that HCMV can trigger inflammatory cytokine production through the interaction of certain virus envelope glycoproteins (such as gB) and TLR2 (Compton et al., 2003;Boehme et al., 2006;Juckem et al., 2008). In this regard, the HCMV envelope glycoproteins B and H

have been shown to activate TLR2 and both gB and gH physically associate with TLR2 as shown via co-immunoprecipitation experiments. In response to HCMV, it appears that TLR2 exerts its functional activity together with TLR1 as a heteromeric complex. In support of this, it has been shown that gB and gH also co-precipitate with TLR1 (Boehme et al., 2006).

Complement system: The complement-mediated immune system is another facet of the first-line immunological defense which includes inflammatory proteins, proteolytic enzymes, cell surface receptors and proteins, causing apoptosis via osmotic lysis after insertion into biological membranes such as virus-infected cell surfaces (Hengel et al., 1998). HCMV is able to escape this system probably by producing some gene products which express a surface Fc-receptor that is distinct from endogenously produced Fc-receptors. Using mutant analysis, it has been identified that HCMV encodes two distinct Fc γ receptor (Fc γ R) homologs, including vFc γ R gp68 (encoded by UL119-UL118) and vFc γ R gp34 encoded by TRL11/IRL11 (Atalay et al., 2002).

Fc receptors (FcRs) constitute a family of cell-surface receptors expressed on most cells of the immune system and play a key role in host defenses against pathogens via linking humoral and cellular immune responses. Multiple components of the immune system can interact with the Fc domain of immunoglobulins via distinct binding sites such as complement (Ravetch and Bolland, 2001).

Moreover, by inducing FC receptors it has been shown that HCMV infection can upregulate the cell surface expression of CD46 and CD55, two complement control proteins (CCPs), in some cell types (Spiller et al., 1996). The increased CD55 expression enhances the capacity of infected cells to regulate C3 deposition and thus protects

infected cells from complement-mediated lysis. Furthermore, the incorporation of CD55, CD59 and CD46 into virus particles may also be a potential tool for HCMV to escape from host defenses probably via downregulation of HLA molecules at the infected cell surface (Spear et al., 1995; Spiller et al., 1997).

1.6.2 Acquired Immunity: Humoral Responses

Although the focus of the response to HCMV is typically cell mediated, clinical and experimental evidence indicate that humoral immune responses to HCMV may play a role in defence against infection (Weber et al., 1993). Initial infection with HCMV induces a primary immune response and following establishment of long-term immunity, this serves to control the replication of virus following reactivation. Although impairment of cellular immunity can enhance the severity of HCMV infection, a synergistic role for the humoral response in keeping HCMV loads below critical thresholds cannot be discounted. Indeed, it has been shown in clinical studies that the humoral response has a beneficial role in preventing the blood borne spread of the virus (Schoppel et al., 1998). This is also supported by evidence that the probability of transmission of viral infection from mother to fetus significantly increased if the antibody response to HCMV is of low avidity and of poor neutralizing activity (Fowler et al., 1992; Schleiss et al., 2004). Recent studies have shown that treating pregnant women with HCMV-specific hyperimmune globulin not only increased virus-specific IgG concentrations and avidity but also considerably lowered the risk of congenital HCMV infection and disease (Nigro et al., 2005). In addition, recently published results of a phase 2, placebo-control and randomized trial have showed that HCMV

glycoprotein B vaccine has the potential to reduce the incidence of maternal and congenital infection (Pass et al., 2009).

Studies in guinea pig models have previously demonstrated the efficacy of gB, either purified from CMV virions (Harrison et al., 1995) or produced as a recombinant protein (Schleiss et al., 2003), as an effective vaccine (Schleiss et al., 2007). Moreover, passive immunization with hyperimmune anti-gB to pregnant guinea pigs has also been shown to be protective against congenital infection (Chatterjee et al., 2001).

HCMV is a structurally complex virus encoding around 168 unique proteins, a large number of which can generate antibody responses. Although, *in vivo*, the tegument and capsid proteins are inaccessible to antiviral antibodies because they are enclosed within the virion envelope, interestingly, protein components of the tegument and capsid elicit the most intense and durable antibody responses, serving often as the basis for diagnostic serological assays (Britt, 2007). Figure 1-5 illustrate the virion proteins and glycoproteins that have been experimentally defined. Neutralizing antibody responses have been detected against many viral surface glycoproteins such as gB (UL55), gM (UL100), gN (UL73), and gH (UL75) (Mocarski et al., 2007; Britt, 2007). Glycoproteins B and H (gB and gH) are two main glycoproteins in the virion envelope that have significant immunogenicity in humans and to be targeted by antiviral antibodies (Rasmussen et al., 1991). In addition to being involved in cell attachment and penetration, gB is the major target for neutralizing antibodies to HCMV (Britt et al., 1990; Marshall et al., 1992). Glycoprotein H is another target which induces potent virus-neutralizing antibodies as well as being involved in the fusion of the viral envelope with the host cell membrane (Rasmussen et al., 1991).

In HCMV, gM forms a heterodimeric complex through disulfide bonds with gN. The gM/gN complex is the most abundant glycoprotein components of the HCMV virion envelope that can elicit a virus-neutralizing antibody response in HCMV infected individuals. Antibodies derived from convalescent human serum have been shown to react with the gM/gN complex and neutralize virus infectivity (Shimamura et al., 2006;Britt, 2007).

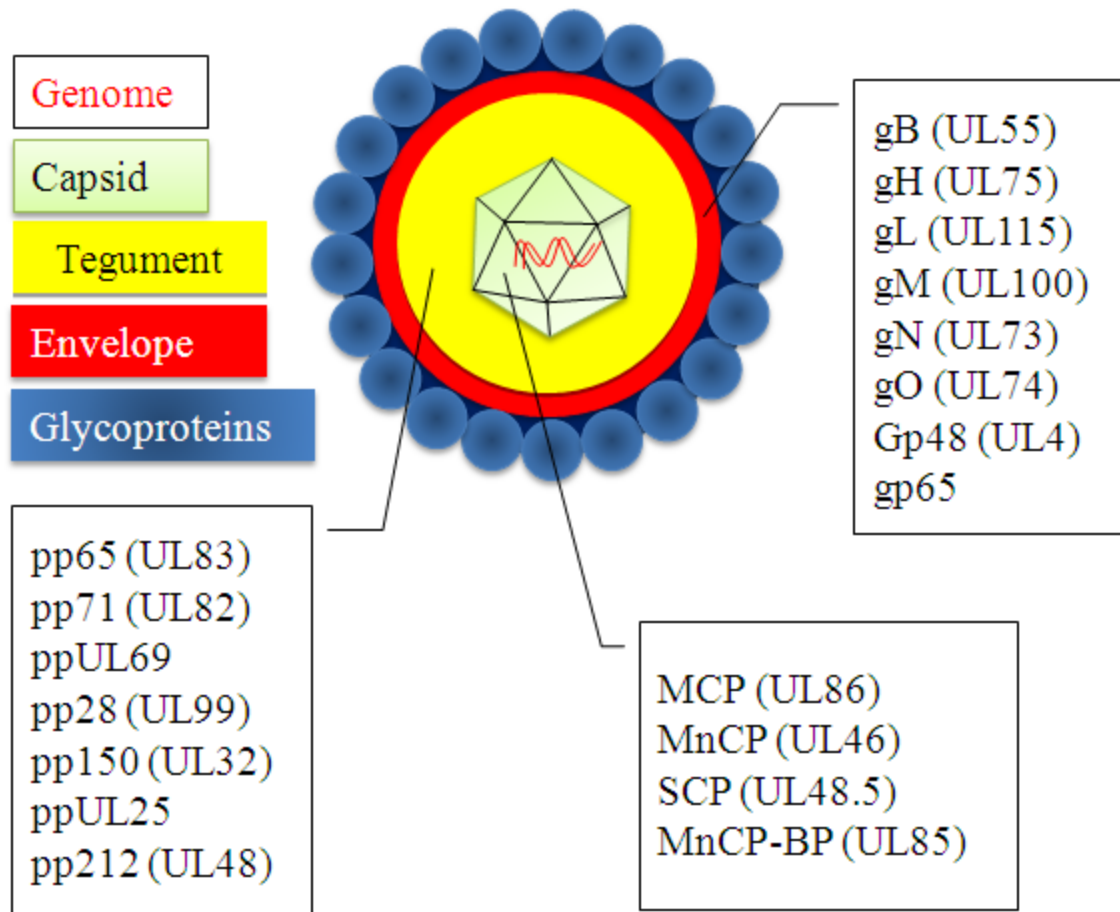


Figure 1-5: Schematic representation of protein components of the HCMV virion.

The most immunogenic envelope proteins (gB and gH) are located in envelope as coloured in red. This figure reproduced from (Emery and Shannon-Lowe, 2007). *Abbreviations:* gB, glycoprotein B; pp65, phosphoprotein 65; MCP, major capsid protein; MnCP, minor capsid protein; SCP, small capsid protein.

1.6.3 Acquired Immunity: Cellular Responses

Antigen-specific T cells are essential components of the immune response against a large number of pathogens. It has also been shown that T cell responses have an important role in limiting viral replication as well as controlling virus-associated diseases (Pantaleo and Harari, 2006).

1.6.3.1 CD4+ T cell response

There is increasing data which emphasizes an important role for CD4+ T cell responses in the control of infection (Khan, 2007) and these cells are likely to be important in aiding control of HCMV (Nebbia et al., 2008). CD4+ T cell activation is dependent on antigen presentation by MHC-II molecules on antigen presenting cells. Unlike CD8+ T cells which recognise endogenous antigens (presented directly from cytosol), antigen recognition by CD4+ T cells usually occurs when antigen has been taken up by an antigen-presenting cell (Landsverk et al., 2009). Therefore, the cytotoxic potential of the CD4+ T cell response for viral infections could be limited as it might be impossible for virally infected cells to present antigens through MHC class II molecules (Crompton et al., 2008). However, recent evidence has indicated that HCMV glycoprotein B (gB) can be presented directly from infected cells without the need for cross-presentation (Hegde et al., 2005). Cross-presentation, which was first described by Bevan four decades ago, is an important factor for the development of CD8+ T cell responses specific to viruses that are not able to directly infect antigen-presenting cells (APCs). It appears that dendritic cells (DCs) are the main potent APCs for cross-presentation (Ackerman and Cresswell, 2004; Singh and Cresswell, 2010).

Evidence supporting the importance of CD4⁺ T cells in controlling of HCMV infection is obtained from studies of transplant patients undergoing a primary infection. In patients undergoing bone marrow transplantation, although CD8⁺ T cell infusions are able to protect patients from HCMV disease, the maintenance of effective CD8⁺ T cell response was shown to be dependent on the presence of CD4⁺ T cells specific to HCMV (Walter et al., 1995; Einsele et al., 2002; Waller et al., 2008). In addition, an increased incidence of recurrent MCMV infection has been reported following selective depletion of CD4⁺ T cells in mice infected with MCMV (Poliç et al., 1998a).

The effector mechanisms of CD4⁺ T cells include cytokine secretion, facilitating the differentiation and proliferation of antigen (Ag)-specific CD8⁺ T cells, activation of dendritic cells by ligation of CD40, and providing signals for virus-specific B cells. Accumulating evidence proposes that CD4⁺ T cells can directly act as effectors against virus-infected cells or help virus-specific CD8⁺ T cells to expand and become cytotoxic (Gamadia et al., 2004; van Leeuwen et al., 2004; Hegde et al., 2005; Wills and Sissons, 2007). Moreover, it has also reported that patients who have higher numbers of IFN- γ producing CD4⁺ T cells specific for HCMV clear the virus faster and exhibit fewer clinical symptoms (Sester et al., 2001; Gamadia et al., 2003).

The analysis of HCMV specific CD4⁺ T cell responses is not as intensive as for CD8⁺ T cells because of, in part, difficulties in producing MHC class II tetramers, however, CD4⁺ T cells often respond to the same HCMV ORFs as CD8⁺ T cells (Waller et al., 2008). Similar to the CD8⁺ T cell response, a high frequency of HCMV specific CD4⁺ T cells (an average of 10% and in some donors even 40%) has been reported in healthy seropositive individuals (Sester et al., 2002; Sylwester et al., 2005).

1.6.3.2 Gamma delta \square T cells

The $\gamma\delta$ T lymphocytes comprises a small fraction (0.5-6%) of peripheral blood; however, it represents a more substantial fraction of lymphoid cells in areas of the body exposed to the external milieu, such as the intestinal mucosa (Dechanet et al., 1999b). An important role of the $\gamma\delta$ T cells in host immunity to viral infections including HSV-1 and MCMV has been examined using mouse models (Sciammas et al., 1997). Additionally, a protective role of circulating $\gamma\delta$ T cells has been suggested in renal transplant patients as marked expansion of these cells was shown to be co-incident with active HCMV infection (Dechanet et al., 1999a).

1.6.3.3 CD8+ T cell response

The importance of CD8+ T cell responses in the elimination of pathogens and tumors is well known. Several studies support the importance of CD8+ T cell in control of HCMV infection (Gandhi and Khanna, 2004). This notion initially came from the observation that HCMV disease is a typical problem in patients with normal levels of antibody but who have impaired T cell immunity such as solid organ transplant recipients, stem cell transplant recipients and since 1983 patients with HIV infection (Mocarski et al., 2007). Accordingly, it has been shown that adoptive transfer of virus-specific CD8+ T cells into allogeneic bone marrow transplant recipients is effective in controlling HCMV replication (Riddell et al., 1992; Walter et al., 1995).

Virus-specific CD8+ T cells recognize and respond to the short viral-encoded peptides (with 8-10 amino acid residues in length) presented by MHC class I molecules on either infected cells or antigen presenting cells (Daniels et al., 2001; Haring et al., 2006) as

shown in Figure 1-6. Endogenously synthesized proteins are cleaved in the proteasome and transported into the lumen of the endoplasmic reticulum (ER) via heterodimeric transporter associated with antigen processing (TAP) proteins which is composed of TAP1 and TAP2 subunits. The newly formed MHC class I molecules are transported to the lumen of the ER where they associate with calnexin (a chaperon protein). Once the β_2 -microglobulin associates with the MHC-I, calnexin is displaced by a set of different chaperone proteins including calreticulin (a lectin-like chaperone that binds to the heavy chain of the MHC class I), Erp57, and tapasin to form the peptide loading complex (Purcell and Elliott, 2008). Erp57 is a thiol oxidoreductase that associates with calreticulin noncovalently and is disulfide-linked to tapasin, which is necessary for optimization of the MHC class I peptide cargo (Williams et al., 2002; Radcliffe et al., 2002). The peptide/MHC-I complex is finally transported via the Golgi apparatus to the surface of infected cells where they can be targeted by the T cell receptor (TCR) of CD8+ T cells (Purcell and Elliott, 2008).

In addition to the interaction between the TCR and the peptide-MHC complex, subsequent co-stimulatory signals provided by molecules such as CD28 are required for optimal activation of naïve T cells. It has been shown that the engagement of CD28 as a co-stimulatory molecule with its ligand B7, which is present on the surface of antigen presenting cells, provides an important co-stimulatory signal for optimal T cell activation (Shahinian et al., 1993).

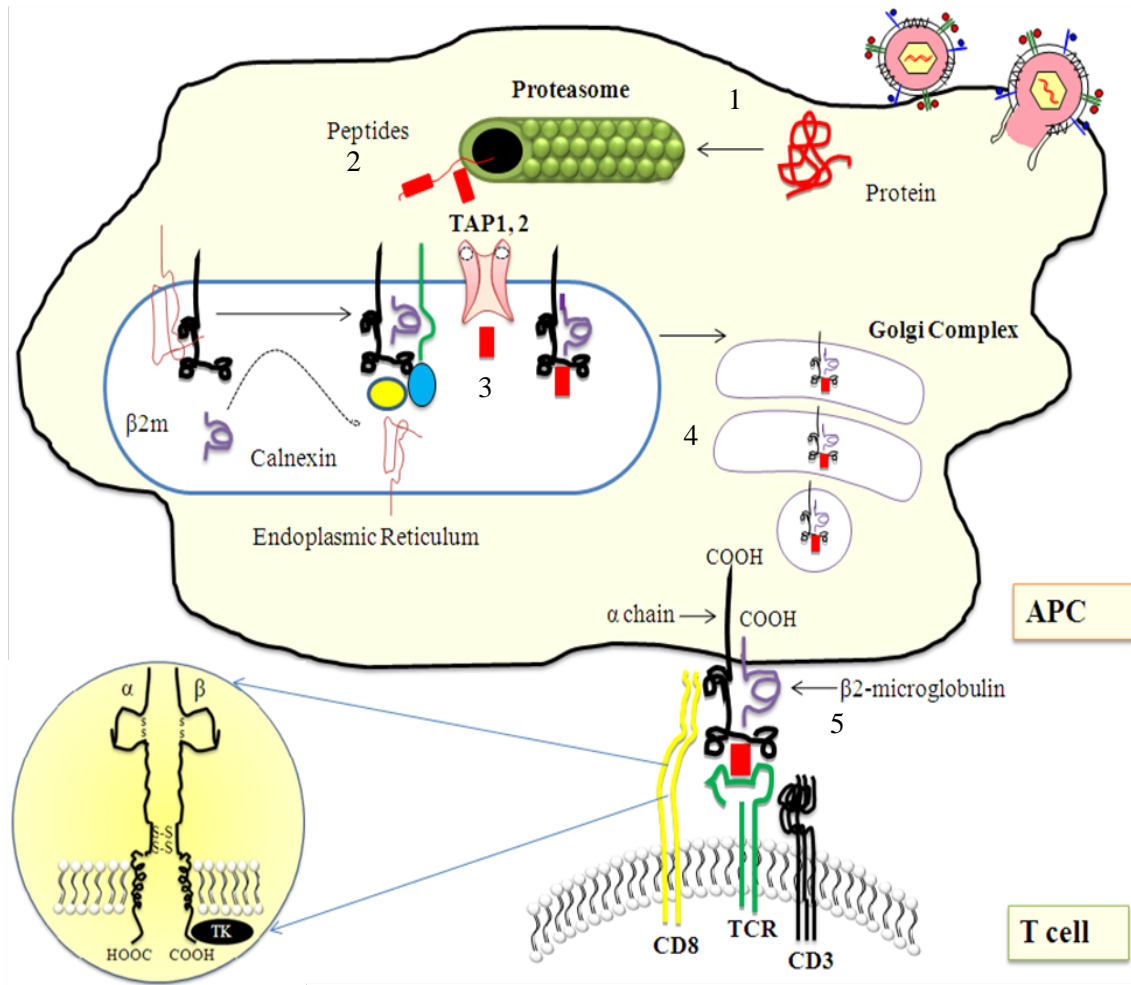
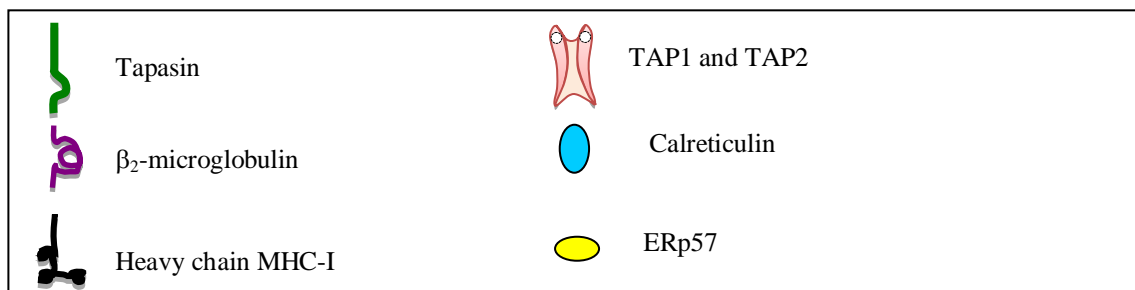


Figure 1-6: Schematic representation of MHC-I antigen presentation to CD8+ T cells.

Degradation of proteins by proteasome results in generation of eight to ten amino acid peptides that (1) translocate via TAP to the endoplasmic reticulum (2). Nascent MHC-I molecules form the peptide loading complex in association with tapasin, calreticulin, and ERp57 to facilitate the peptide loading into the peptide binding groove (3). Peptide-MHCI complex then transports via Golgi apparatus to the cell membrane (4) where interacts with TCR (5). Zoomed circle shows the structure of CD8 involving in MHC-TCR interaction. *Abbreviations: TCR, T cell receptor; β 2m, β 2-microglobulin; TAP, transported associated with antigen processing, APC, antigen presenting cell; TK, tyrosine kinase; MHC-I, major histocompatibility complex class I.*



In response to viral antigens, naive CD8⁺ T cells become activated and virus specific T cells undergo an extensive clonal expansion, differentiating into a heterogeneous effector CD8⁺ T cell pool, acquiring effector functions and the capability of tissue-trafficking which together with many other mediators can control viral infection. After antigen clearance or neutralization of the pathogen, the expanded T cells specific to antigen contracts substantially and fewer than 10% of the remaining antigen stimulated T cells differentiate into memory T cells conferring enhanced protection following reinfection (Klenerman and Hill, 2005; Harty and Badovinac, 2008; Cui and Kaech, 2010).

Recognition of specific epitopes on the surface of infected cells allows the activated CD8⁺ T cells to lyse them via different mechanisms such as exocytosis of granules containing granzymes and perforin. Granzyme B is a 32 KDa serine protease which has been shown to induce rapid cell death of a virus infected target cell through the cleavage of downstream substrates as well as the activating cleavage of caspases (Poe et al., 1991; Sutton et al., 1997; Bird et al., 1998; Waugh et al., 2000). Following recognition and binding to virus infected or other target cells, cytotoxic lymphocytes (or NK cells) release perforin (a Ca²⁺-dependent pore-forming protein which multimerizes in the plasma membrane of the target cells) and granzyme B which can provide protease access to the cytosol of target cells (Kagi et al., 1994; Liu et al., 1995; Boivin et al., 2009). Upon target cell recognition and release into the cytoplasm, granzyme B can target substrates in the cytosol as well as the nucleus, inducing cell death (Boivin et al., 2009).

Engagement of death receptors is another complementary mechanism by which cytotoxic T cells induce apoptosis in infected cells. The engagement of cell receptors

occurs via a group of cell surface molecules of the tumor necrosis factor (TNF) receptor superfamily such as Fas (CD95) which bind to the relevant ligand (FasL) expressed on the cell surface of activated CTLs predominantly CD8⁺ T cells (Lowin et al., 1994).

However, it is becoming clear that cytotoxic CD8⁺ T cells can control viral infections through noncytolytic mechanism by secretion of antiviral cytokines such as IFN- γ and TNF- α , thereby limiting the extent of the infection (Guidotti and Chisari, 2001). IFN- γ and TNF- α are the main cytokines produced by differentiated CD8⁺ effector T cells and have a critical role in CD8⁺ T cell mediated immunity and control of viral infection by inducing intracellular pathways that activate an anti-viral state or apoptosis (Kaech et al., 2002b; Glimcher et al., 2004). In addition, these cytokines, which have often been considered as a signature of a proinflammatory environment, play an important role in cellular proliferation and differentiation, inflammation, and cytotoxic activity (MacEwan, 2002; Kelchtermans et al., 2008).

1.6.3.3.1 CD8⁺ T cell response to HCMV

The proportion of CD8⁺ T cell responses against HCMV antigens can be quite large particularly in elderly individuals. Unlike the response to many viruses such as influenza virus and varicella-zoster virus, which reduces with progressing age (Asanuma et al., 2000; Deng et al., 2004), there is an accumulation of HCMV-specific CD8⁺ T cells with age, eventually representing up to 40% of the CD8⁺ T cell pool (Khan, 2004; Crough and Khanna, 2009). This rise in virus-specific CTLs, so called ‘memory inflation’, has also been described in the CD8⁺ T-cell immune response to MCMV (Karrer et al., 2003). During persistent infection, memory CD8⁺ T cell responses specific to HCMV

have been shown to be highly dynamic in terms of both function and absolute number (Crough et al., 2007).

Recent data using peptides (of 15 amino acids in length) which represent the entire HCMV proteome reveal that many proteins are targeted by the host CD8⁺ or CD4⁺ T cell responses. In the study carried out by Sylwester et al, the majority (70.9%) of HCMV proteins (151 out of 213 HCMV ORFs) were shown to elicit CD8⁺ and CD4⁺ T cell response in at least one donor (as illustrated in Figure 1-7) and UL48, UL83 (pp65), and UL123 (IE-1) were three ORFs that recognized by more than half of the cohort (Sylwester et al., 2005).

Regardless of targeting many HCMV proteins by CD8⁺ T cell responses; however, pp65 and IE-1 are two HCMV proteins that have been extensively studied in terms of the immunology of CD8 T cell response (Jackson et al., 2010). It has been well documented by several studies that the pp65 phosphoprotein is the dominant target antigen of cytotoxic T lymphocytes as well as being a target of both the humoral and cellular immune system (Grefte et al., 1992; Laughlin-Taylor et al., 1994; Beninga et al., 1995; Wills et al., 1996). The UL83-encoded pp65 phosphoprotein is the major constituent of HCMV particles (Kalejta, 2008) and is dispensable for growth in cell culture (Schmolke et al., 1995). In addition, it has been shown that pp65 is able to mediate the phosphorylation of viral IE proteins, thereby preventing their proteasomal processing and subsequent presentation by MHC class I molecules (Gilbert et al., 1996).

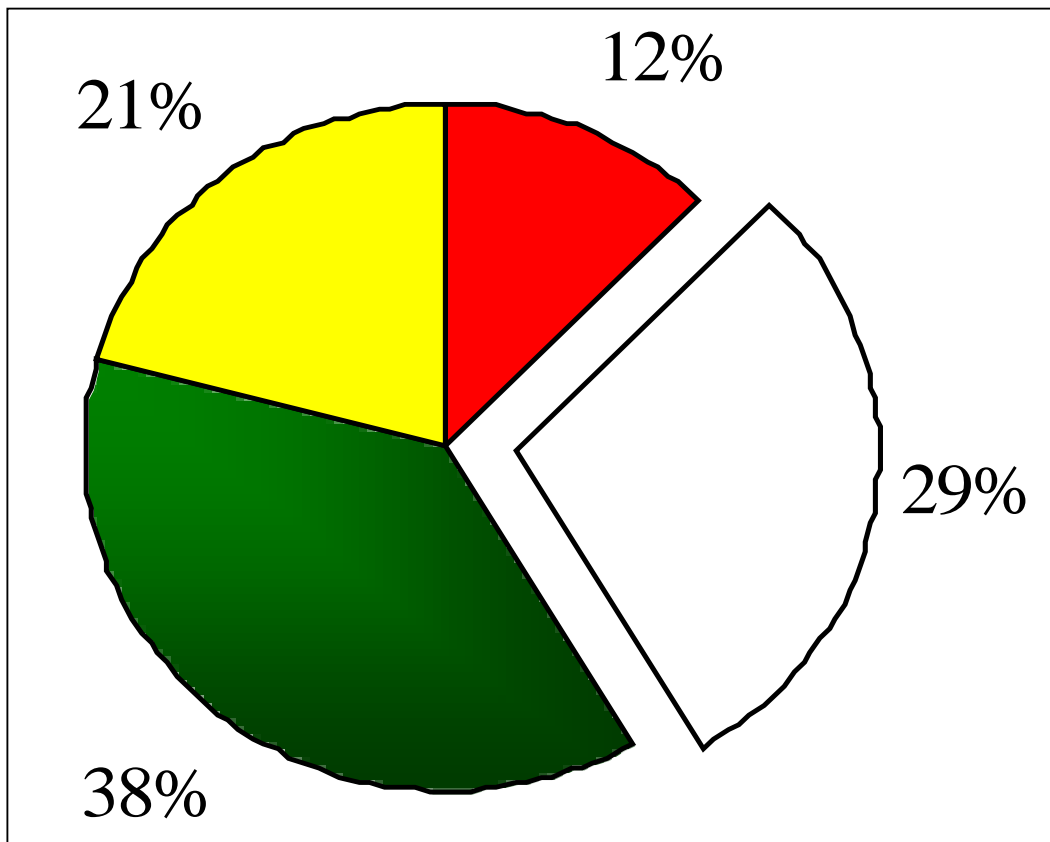
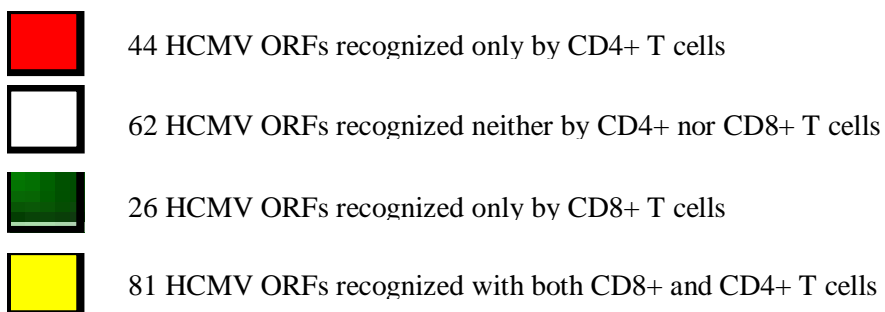


Figure 1-7: Identification of CD8+ and CD4+ T cell responses specific to HCMV ORFs in HCMV-seropositive adults.

The image is modified from (Sylwester et al., 2005).



The expansion of CD8⁺ T cells specific to HCMV is usually oligoclonal although in some instances can be monoclonal (Price et al., 2005; Day et al., 2007; Wynn et al., 2008). Moreover, it has been shown that the immune response to a given HCMV peptide is characteristically highly focused as up to 23% of oligoclonal expansions of CD8⁺ memory cells in elderly individuals are shown to be directed against a single HCMV epitope (Khan et al., 2002; Waller et al., 2008). The clonal repertoire of HCMV-specific CD8⁺ T cell memory response is known to be distinct in latent HCMV infection from that observed during acute infection (Karrer et al., 2003). Although primary HCMV infection generates a diverse virus-specific TCR repertoire which comprises clones with a range of affinities, analysis of the clonal composition of the memory CD8⁺ T cells repertoire specific to pp65 epitopes shows a high degree of clonal focusing on a few or even a single TCR (Day et al., 2007; Jackson et al., 2010).

Furthermore, data from longitudinal-ageing studies indicate the association of HCMV seropositivity with a cluster of immune parameters (a so-called “immune risk phenotype”) such as an inverted CD4⁺/CD8⁺ T cell ratio due to increased levels of CD8⁺ T cells and the presence of CD8⁺ T cell clonal expansions. In very elderly individuals over 80 years of age, immune risk phenotype (IRP) is considered as predictive of increased mortality (Olsson et al., 2000; Khan et al., 2002; Akbar and Fletcher, 2005; Crough and Khanna, 2009).

1.6.3.3.2 Dynamics of HCMV specific CD8⁺ T cells

Transition from naive to effector and effector to memory CD8⁺ T cell populations are associated with distinct changes in gene expression which results in the expression of signature genes central to CD8⁺ effector T cell function, including genes that encode

cytokines and genes associated with cytolysis (Kaech et al., 2002a). Human memory T cells were originally assigned into two broad classes named effector memory T cells (T_{EM}) and central memory T cells (T_{CM}) based on the differential expression of the lymph-node homing receptors CC-chemokines receptor 7 (CCR7) and CD62 ligand (CD62L), immediate effector capability, and ability to proliferate after subsequent encounter with antigen (Sallusto et al., 1999). Human T_{CM} cells are CD45RO⁺ memory cells that express CCR7 and CD62L and proliferate strongly in response to antigen. Human T_{EM} cells are memory cells that lack CCR7 and CD62L, and enriched in peripheral tissues such as lung, liver, and gut. T_{EM} cells have immediate effector functions but proliferate less strongly in response to antigen (Wherry et al., 2003b; Sallusto et al., 2004).

The leukocyte common antigen, CD45, is a transmembrane tyrosine phosphatase that regulates signaling through the T-cell receptor (TCR)-CD3 complex (Thomas, 1989; Trowbridge, I, 1994; van Lier et al., 2003). It is expressed as distinct isoforms with different molecular weights as a result of differential splicing of three exons (A, B and C) (Streuli et al., 1987). High molecular weight isoform CD45 which contains exons A, B and C (or A and B) are referred to as CD45RA, whereas the low molecular weight form, CD45RO, lacks any of the variable exons (Wills and Sissons, 2007).

It has previously been suggested that naïve CD8⁺ T cells express CD45RA (the long isoform of CD45) and the expression of CD45RO (the short isoform of CD45) marks memory and effector T cells. However, it has been shown that following resolution of HCMV viremia, a subset of these effector memory CD8⁺ T cells specific to HCMV re-express CD45RA being therefore known as “revertant” memory cells (T effector

memory CD45RA cells, T_{EMRA}). Most of these cells (T_{EMRA}) lack the chemokine receptor CCR7 and do not express the costimulatory receptors CD27 and CD28 (Wills and Sissons, 2007; Waller et al., 2008; Cantisan et al., 2010). Although antigen specific T cells display some degree of heterogeneity; they exhibit unique profiles based on their viral specificity. For example, it has been shown that HIV- specific T cells preferentially exhibit an so called intermediate phenotype (CCR7-CD27+CD28-CD45RA-), whereas HCMV and EBV are shown to be frequently CCR7-CD27-CD28-CD45RA+ and CCR7-CD27+CD28+CD45RA-, respectively (Appay et al., 2002). The presence of HCMV specific T cells with a CCR7-CD27-CD28-CD45RA+ phenotype has been shown to be directly correlated with HCMV infection but not with previous exposure to other viruses such as EBV or VZV (Kuijpers et al., 2003; Waller et al., 2008). CD45RA expression on HCMV-specific effector memory CD8+ T cells was also associated with the kinetics of HCMV replication after transplantation (Cantisan et al., 2010).

It is well known that the frequency of memory CD8+ T cells specific to HCMV is very large and in elderly HCMV seropositive individuals this frequency is often striking. These cells do not express CD27 or CD28 however many of them express CD57 (Waller et al., 2008). Moreover, it has been previously shown that there is an association between the presence of CCR7-CD27-CD28-CD57+T cells and HCMV infection (Gillespie et al., 2000; van Lier et al., 2003; Brenchley et al., 2003). CD57+ cells, which have also been called leu-7, are principally defined by reactivity to the antibody HNK-1 (Abo and Balch, 1981c). The name HNK-1 is originally derived from the ability of the antibody to recognize human NK cells. HNK-1's epitope, CD57 is a carbohydrate epitope that is observed in many glycoproteins and glycolipids in various tissues

(Uusitalo and Kivela, 2001). It is also found on NK cells and CD8⁺ T lymphocytes (Abo and Balch, 1981b). While the exact function of CD57 on immune cells has not yet been elucidated, experiments have begun to produce some ideas about the function of these cells. Lymphocytes that express CD57 are clonally expanded (Wood et al., 2009) and large oligoclonal expansions have been described in the CD8⁺CD57⁺ population (Morley et al., 1995). In healthy individuals, 5-20% of total CD8⁺ lymphocytes express CD57 (Abo and Balch, 1981a; Morley et al., 1995; Wood et al., 2009). Although the exact mechanism or the nature of the stimulus behind this expansion is not yet clear, some viral or tumor antigens may play an important role as it is well documented that expansion of CD8⁺ T cells can occur in response to chronic viral infections (Wood et al., 2009). To support this, expansions of the CD8⁺CD57⁺ cell subset has been shown to be associated with both infections with HCMV (Wang et al., 1993; Rossi et al., 2007) and HIV (Wood et al., 2005).

Unlike acute viral infections where the combination of antigen-independent responses and generation of memory CD8⁺ T cell responses confer long-term protective immunity and control replication, virus specific CD8⁺ T cells during many persistent virus infections often fail to differentiate into fully functional memory CD8⁺ T cells. Substantial alterations in the phenotype and functionality of memory CD8⁺ T cells are often observed following persistent infections compared with acute infection (Klenerman and Hill, 2005; Harty and Badovinac, 2008). These phenotypic and functional changes may result in the exhaustion of their effector functions or even the deletion of CD8⁺ T cells of certain specificities (Wherry and Ahmed, 2004). The exhaustion of CD8⁺ T cells during persisting infection, which is likely to have an

important impact on viral control, was first described by Barber et al in persistently infected mice with LCMV. Using LCMV specific T cells from both acute and chronic LCMV infection models, they performed a genome-wide gene profiling and showed a significant upregulation of PD-1 in a subset of exhausted cells. Additionally *in vivo* blockade of PD-1 and PD-L1 interactions was shown to enhance the clearance of infection and improve the functionality of the memory CD8⁺ T cells (Barber et al., 2006). The finding that exhausted T cells exhibit high expression of the programmed cell death 1 (PD-1) has been a centre of several studies analyzing the expression of PD-1 on CD8⁺ T cells specific to HIV (Trautmann et al., 2006;Day et al., 2006;Petrovas et al., 2006) and HCV (Urbani et al., 2006;Penna et al., 2007). These studies have demonstrated that the expression of PD-1 is higher on CD8⁺ T cells specific to HIV and HCV. However, looking at T cells specific to, for example, nonpersisting viruses such as vaccinia and influenza has showed that this phenomenon is not uniform as low PD-1 expression was also found on these virus-specific T cells (Urbani et al., 2006;Day et al., 2006;Sharpe et al., 2007).

The immunoreceptor PD1, also called CD279, is a negative regulator of T cell activation and is expressed by many cells including T cells, B cells, NK cells, and myeloid derived cells (Greenwald et al., 2005;Sharpe et al., 2007). The PD-1 receptor, which was originally isolated as a transcript up-regulated in apoptotic cells, is a 288 amino acid cell surface monomer. Although this receptor shares structural properties with the members of CD28 family, it is located on a different region of the same chromosome (2q37). Two ligands with different expression patterns have been described for PD-1, PD-L1 and PD-L2. PD-L1 (also known B7-H1 and CD274) is broadly distributed on various cell types

such as B cells, DCs, macrophages, mesenchymal stem cells as well as a wide variety of nonhematopoietic cell types. PD-L2 (also known B7-DC and CD273), by contrast, is limited to professional antigen presenting cells including macrophages, DCs, and cultured bone marrow-derived mast cells (Augello et al., 2005; Okazaki and Honjo, 2006; Keir et al., 2007; Sharpe et al., 2007).

1.7 Manipulation of immune system by HCMV

Because of selective pressures imposed by the host immune response, viruses have developed numerous strategies in order to subvert and also down regulate the host immune surveillance and defense by both the innate and adaptive facets of the immune system, thereby allowing the virus to survive (Galluzzi et al., 2010).

As described previously, T cells play a key role in eliminating virus infected cells by recognition of viral antigens in the context of MHC class I. In the endoplasmic reticulum (ER), MHC class I molecules as well as viral peptides undergo several maturation stages in order to be presented on the cell surface. Particular specialized proteins including calreticulin, tapasin, ERp57, and the transporter associated with antigen processing (TAP) play a critical role in this scenario (Hansen and Bouvier, 2009). Therefore, it is not surprising that viruses target key stages of the antigen presentation pathways by MHC class I. The immunomodulatory roles of gene products encoded by HCMV have been largely characterized and because of the multitude of immune-modulatory strategies, HCMV is considered as a prototype for viral immune evasion with the capability of modulating both the innate and adaptive immune response. Figure 1-8

illustrates the HCMV-encoded immune evasion genes as well as targeting of the MHC-I pathway by particular gene products of HCMV (gpUS2, gpUS3, gpUS6, and gpUS11).

It is well documented that numerous proteins of HCMV are involved in manipulating of immune system; however, US2, US3, US6, and US11 are the four most extensively studied proteins that target the key stages of the MHC class I antigen presentation pathways (Gewurz et al., 2001; Mocarski, Jr., 2002; Hansen and Bouvier, 2009; Mueller, 2010).

US3 is a type I transmembrane glycoprotein that is preferentially expressed during the immediate early phase of virus infection. By directly binding to tapasin, it is able to block the expression of MHC class I presenting HCMV peptides on cell surface via inhibiting the optimization of the peptide cargo and thereby preventing the translocation of MHC-I from the endoplasmic reticulum to the Golgi apparatus (Lee et al., 2000; Park et al., 2004). In the peptide loaded complex (PLC), tapasin stabilizes the groove of MHC class I against irreversible denaturation and plays a crucial role for the optimal expression of MHC class I molecules on cell surface (Ortmann et al., 1997; Chen and Bouvier, 2007).

US6 (21-KDa) is another type I transmembrane glycoprotein expressed during both early and late phases of HCMV infection. US6 can interact directly with the ER lumen side of TAP (Ahn et al., 1997; Lehner et al., 1997). By stimulation of ATP hydrolysis, it has been suggested that TAP provides required energy for peptide translocation (Hansen and Bouvier, 2009). By inhibiting ATP binding and thereby depriving the energy source from TAP, US6 inhibits the peptide translocation into ER (Ahn et al., 1997; Kyritsis et al., 2001).

US2 and US11 are two small gene products of HCMV that have been shown to play a role in the destruction of MHC class I heavy chains (Jones et al., 1995). The human cytomegalovirus proteins US11 and US2 are both ER-resident membrane glycoproteins, and induce the dislocation of MHC class I heavy chains from the ER into the cytosol for degradation by ubiquitin-dependent proteasome. It seems that ubiquitin plays an important role in this case (Wiertz et al., 1996; Story et al., 1999; Rehm et al., 2002). Using trophoblast cells, it has also been shown that these proteins can mediate degradation of Human histocompatibility leukocyte antigens (HLA)-A and B locus products but not HLA-C and G (Schust et al., 1998). Moreover, it was shown that gpUS2 and gpUS11 downregulate the MHC class I presentation of HCMV infected fibroblast cells in an antigen dependent manner (Besold et al., 2009).

In addition to MHC class I down regulation, it has also been demonstrated that HCMV can down regulate the expression of MHC-II. In this context, inhibition of the MHC class II transactivator (CIITA) early in infection and cytokine-mediated mechanisms probably through viral IL-10 are two proposed strategies by which virus can down regulate MHC-II expression (Silacci et al., 1994; Zhou and Glimcher, 1995; Mocarski, Jr., 2002).

Considering the important role of NK cells during innate phase of the immune response against HCMV, it is not surprising that the virus has evolved strategies to modulate NK cell activities (Lodoen and Lanier, 2005). It has been shown that HCMV encodes at least six gene products including UL18, UL16, UL40, UL83, UL141, UL142, and a micro RNA that are capable of interfering with NK cell function using different mechanisms (Wilkinson et al., 2008; Loewendorf and Benedict, 2010).

UL18 is one of genes encoded by HCMV that is involved in NK cell immune evasion. Similar to classical host MHC class I molecules gpUL18 can express as trimeric complex associated with β 2-microglobulin and peptide (Browne et al., 1990). It has been shown that gpUL18 binds specifically to the NK cell inhibitory receptor, leukocyte immunoglobulin-like receptor 1 (LIR1/ILT2), expressed predominantly on monocytes, B cells, and NK cells. Binding affinity of LIR1 for gpUL18 was shown to be much higher than (>1000 fold) for endogenous MHC class I (Chapman et al., 1999;Cosman et al., 1999).

UL16 glycoprotein is another virus gene product that has a potent NK cell immune evasion function by impeding the expression of NKG2D ligands. NKG2D is an activating receptor that is expressed on almost all NK cells and CD8+ T cells (Wilkinson et al., 2008). In addition to MHC-I chain related A and B proteins (MICA and MICB), human NK cells express at least six other ligands from another family of UL16-binding proteins (ULBP1-6) which recognize NKG2D receptor. The UL16 protein of HCMV has been shown to bind MICB, ULBP1, 2, and 6 (Cosman et al., 2001;Kubin et al., 2001;Welte et al., 2003).

UL40, an early HCMV protein, induces protection against NK cell activation via upregulating the cell surface expression of HLA-E independent of TAP as constructed adenovirus encoding US6, an efficient inhibitor of the TAP, was shown to downregulate the surface expression of MHC-I however it did not affect the capacity of gpUL40 to upregulate the surface expression of HLA-E (Tomasec et al., 2000). HLA-E that is a non-classical MHC class I molecule that is recognized by the NK cell inhibitory receptor complex CD94/NKG2A (Braud and McMichael, 1999;Wilkinson et al., 2008).

UL83 is another HCMV gene that encodes an abundant tegument protein (pp65). Regardless of it being major target for T cell responses it has also been shown that pp65 can bind directly to the NK cell activating receptor NKp30 (Arnon et al., 2005).

UL141 is a highly conserved gene between HCMV isolates and it can be readily detected from 24 h post-infection and it accumulates into the late phase of the virus replicative cycle. UL141 has the ability to evade the NK cell lysis by sequestering CD155 in the endoplasmic reticulum. CD155 is a cell surface ligand interacting with activating receptors of the NK cells, CD226 and CD96. Multiple cellular functions have been attributed to CD155 including transendothelial migration, adhesion, and endocytosis (Tomasec et al., 2005).

UL142 is an MHC-I like molecule that has been shown to inhibit the NK cell lysis of virus infected cells (Wills et al., 2005) via interfering with cell surface expression of ULBP3, a ligand for the NKG2D receptor (Bennett et al., 2010).

There is also accumulating evidence that HCMV encodes additional membrane proteins that interact with host proteins such as chemokine receptors (Streblow et al., 1999), a viral TNF receptor (UL144, is a type I transmembrane glycoprotein that is expressed early after infection of fibroblasts) (Benedict et al., 1999; Rahman and McFadden, 2006), a viral CXC-1 inducing the chemotaxis of human peripheral blood neutrophils (UL146) (Penfold et al., 1999), and UL111A encoded interleukin 10 (IL10) homologue that likely reduces cell-surface expression of MHC class I and II proteins (Kotenko et al., 2000).

The role of small, non-coding microRNAs (miRNAs) in both gene silencing and stress responses has been recently recognized (Leung and Sharp, 2007). In addition to proteins it has been shown that HCMV can express an array of mirRNAs (Grey et al., 2005; Dunn

et al., 2005). Mir-UL112 is one of the microRNAs encoded by HCMV that has been shown to specifically suppress cell surface expression of MICB, therefore reducing the sensitivity of NK cell recognition (Stern-Ginossar et al., 2007; Wilkinson et al., 2008).

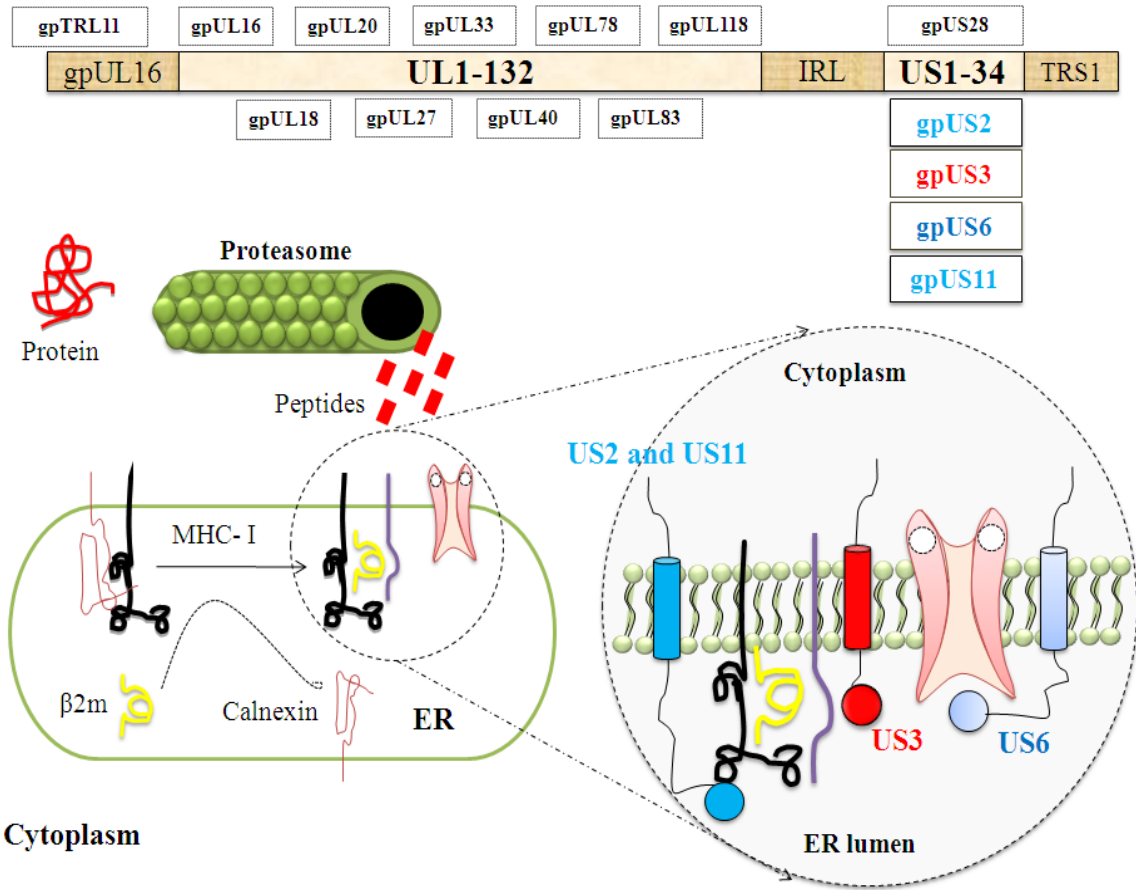
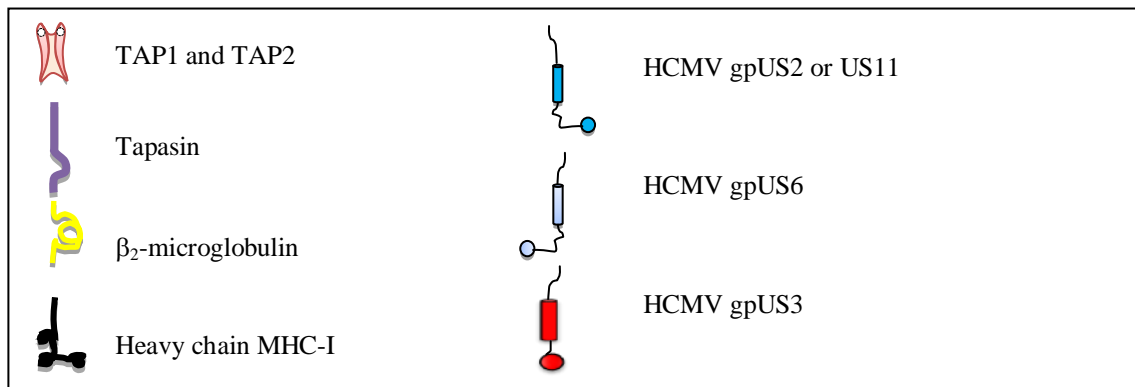


Figure 1-8: Schematic representation of HCMV-encoded immune evasion genes targeting MHC-I pathway with HCMV proteins (gpUS2, gpUS3, gpUS6, and gpUS11).

Cleaved endogenously derived cytosolic proteins via proteasome translocate into the ER through TAP. HCMV gene products US2 and US11 redirect nascent MHC-I molecules to the cytosol where it is degraded. By binding to tapasin, US3 retain MHC-I complexes in the ER, and US6 blocks TAP via directly interacting with the ER lumen side of TAP. *Abbreviations: ER, endoplasmic cytoplasm; TAP, the transporter of antigen processing; IRL, internal repeat long; TRS, terminal repeat short; UL, unique long; US, unique short; gp, glycoprotein.*



The focus of this thesis has been on human cytomegalovirus, however, Epstein-Bar virus (EBV) was used as control in my studies. EBV is another member of herpes family that is characterized by latency and reactivation. The biology of virus is described in next section.

1.8 Epstein-Barr virus

EBV is a member of herpes virus family which was the first in the family to be completely sequenced. Like other herpesviruses, EBV has a linear double-stranded 184kb DNA enclosed in a nucleocapsid, an outer envelope, and a tegument between envelope and nucleocapsid. The genome encodes around 85 genes (Baer et al., 1984; Kieff and Rickinson, 2007).

Primary infections occur mostly during the first years of life and are usually subclinical (Amon and Farrell, 2005). However, in some individuals (particularly in adolescence) acute primary infection can manifest as a self-limiting lymphoproliferative disease known as infectious mononucleosis (IM) (Kutok and Wang, 2006).

Several malignancies including endemic Burkitt lymphoma (BL), nasopharyngeal carcinoma (NC), post-transplant lymphoproliferative disease (PTLD) and subsets of Hodgkin lymphoma (HL) have been shown to be associated with persistent EBV infection. EBV-related lymphoproliferative disorders have been shown to arise frequently in the setting of iatrogenic suppression in organ transplantation or in AIDS (Kutok and Wang, 2006). Additionally, EBV infection in the immunocompromised host may result in uncontrolled virus-driven activation and proliferation of B cells with the development of lymphomatous diseases ranging from polyclonal B cell lymphoproliferation to monoclonal B cell lymphomas. The tumors are an increasing problem, affecting both the transplant population and those with HIV infection (Rowe and Zuo, 2010). EBV is also implicated in T-cell lymphoma, adult T-cell leukemia,

Natural Killer cell leukemia, and various other lymphoid and epithelial malignancies (Kutok and Wang, 2006).

1.8.1 EBV infection and persistence

EBV preferentially infects B lymphocytes (Kuppers, 2003). However, in some circumstances virus can also infects other cell types including T cells and thymocytes (Watry et al., 1991;Kelleher et al., 1996;Dreyfus et al., 1996), epithelial cells(Shannon-Lowe et al., 2009), NK cells (Kanegane et al., 1996), and monocytes (Revoltella et al., 1989;Savard et al., 2000).

B cell infection is preferentially mediated through the binding of the EBV glycoprotein gp350/220 to the C3d complement receptor CD21 (Fingerth et al., 1984) as virus entry can be blocked using purified CD21 (Nemerow et al., 1990). However, successful immortalization of cells with recombinant virus lacking gp 350/220 indicates other alternative viral entry pathways (Janz et al., 2000). It appears that a complex of three additional envelope glycoproteins including gH, gL and gp42 is necessary for virus entry into B cells whereas entry of EBV into epithelial cells is shown to be mediated by the gH/gL complex but not gp42. It has been shown that gp42 interacts with HLA class II, which functions as a co-receptor. B cells express high levels of HLA class II whereas epithelial cells do not express significant levels of HLA class II (Wang et al., 1998;Borza and Hutt-Fletcher, 2002).

EBV is potent in transforming infected B cells *in vitro* (resulting in B-lymphoblastoid cell lines (B-LCLs)) and autonomously proliferating lymphoblasts *in vivo*. This ability has been shown to be associated with a set of latent genes encoding six nuclear antigens,

EBNAs 1, 2, 3A, 3B, 3C, LP, and three latent membrane proteins, LMPs 1, LMP2A, and LMP2B (Hislop et al., 2007b; Rowe et al., 2009). Therefore, keeping the virus under control relies on efficient immune control *in vivo*.

Based on the location and differentiation state of the infected cells *in vivo*, EBV establishes a complex set of latency programs as well as lytic infection (Kutok and Wang, 2006). Table 1-2 shows the patterns of latent infection gene expression in EBV infected cells. Latency type 0 is associated with no detectable latent gene expression whereas in latency type I, gene expression is restricted to only Epstein-Barr virus nuclear antigen 1 (EBNA1) expression and is seen in Burkitt lymphoma (Niller et al., 2008; Halder et al., 2009). EBNA1 is a DNA binding protein that mediates genome maintenance (Lee et al., 1999) and plays a central role in latency by binding to sequences in viral origin of replication termed *OriP*, therefore promoting initiation of episomal DNA replication. Moreover, EBNA1 has been shown to play an important role in plasmid segregation of dividing cells through its ability to bind both the viral genome and metaphase host chromosomes (Yates et al., 1985; Sears et al., 2004; Speck and Ganem, 2010).

Latency type II is another viral gene expression pattern that is frequently detected in both Hodgkin's lymphoma and nasopharyngeal carcinomas. Type II latency is associated with the expression of EBNA1, latent membrane protein 1 (LMP-1), LMP-2A, and LMP-2B (Niller et al., 2008; Halder et al., 2009). LMP-1 is a member of TNF superfamily that is capable of activating NF- κ B to promote cell survival (Mosialos et al., 1995).

Latency type III is mostly seen in immunocompromised individuals developing lymphoproliferative diseases and lymphoblastoid cell lines transformed by EBV. It is characterized by the expression of EBV-encoded nuclear antigens (EBNA1, 2, 3A, 3B, 3C), latent membrane proteins (LMP-1 and LMP-2), and Epstein-Barr virus nuclear antigen leader protein (EBNA-LP) (Niller et al., 2008; Halder et al., 2009). EBNA-LP has been shown to play a key role in upregulating B cell gene expression (Mannick et al., 1991).

Although the viral genes expressed during latency are not clustered in a single region of the genome, the promoters and cis-elements that control these genes are shown to be clustered in a relatively small region spanning the fused terminal repeats (TRs) of the viral genome (Speck and Ganem, 2010).

Latency program	Disease association	Genes expressed
Latency 0	-	Un detectable genes
Latency I	BL	EBNA1
Latency II	HL, NC	EBNA1, LMP1, LMP2A, LMP2B
Latency III	IM, PTLD	EBNA1, EBNA2, EBNA3 (A, B, and C), EBNA-LP, LMP1, LMP2A

Table 1-2: Patterns of latency and genes expressed during EBV infection.

Abbreviations: EBNA, Epstein-Barr virus nuclear antigen; LMP, Latent membrane protein; NC, Nasopharyngeal carcinomas; BL, Burkitt's lymphoma; HL, Hodgkin lymphoma; IM, infectious mononucleosis; PTLD, Post-transplantation lymphoproliferative disorders.

1.8.2 The immune response to EBV

Like other members of herpes family, EBV establishes a life-long infection and more than 90% adult human population worldwide are infected with the virus (Rowe and Zuo, 2010). Both cellular and humoral immune systems are important to keep the infection under control (Thorley-Lawson and Gross, 2004). Most of the information available on the primary antibody response to EBV is provided through patients with infectious mononucleosis which is an acute but self-limiting illness observed in approximately 25% of adolescent seroconversions. The early phase of acute IM is associated with generally increased levels of total serum IgM, IgG, and IgA in addition to antibody responses specific to EBV (Crawford et al., 2006; Kieff and Rickinson, 2007). The role of NK cells (as a main component of innate immune responses) in the case of EBV is controversial. It has been shown that the inhibition of EBV-induced transformation of resting B cells can occur *in vitro* when NK cells added within few days of infection, however the clinical evidence suggest a less important role for these cells as in recipient of T-depleted stem cell transplantation, the most commonly seen symptom is EBV-driven lymphoproliferative disease (LPD) (Hislop et al., 2007b).

Studies following individual IM patients showed large numbers of CD8⁺ T cells during acute infection probably through either a polyclonal response to a virus encoded or virus induced superantigen or a nonspecific bystander phenomenon (Sutkowski, 2001; McNally and Welsh, 2002; Hislop et al., 2007b). It has also been shown that these CD8⁺ T cells from acute IM individuals are functional in terms of cytokine expression as well as cytotoxic activity (Steven et al., 1997; Catalina et al., 2001; Catalina et al., 2002). In healthy individuals, a significant proportion of the circulating CD8⁺ T cell

pool is devoted to control virus infection. In the case of EBV lytic and latent epitopes, it has been shown that individual epitope-specific populations usually account for 0.2%-2% and 0.05%-1% of CD8⁺ T cells, respectively (Benninger-Doring et al., 1999;Saulquin et al., 2000;Bihl, 2006;Hislop et al., 2007b). Although the proportion of EBV-specific CD8⁺ T cells appears to be stable over time (Crough et al., 2005), there is evidence indicating the presence of an age-related inflation over a longer time frame. This inflation of EBV-specific CD8⁺ T cells occurs in elderly people infected with EBV alone and can reach levels of ~14% of total CD8⁺ T cells (Ouyang et al., 2003;Hislop et al., 2007b). However, in older people caring both EBV and HCMV it appears there is no inflation of CD8⁺ T cells specific to EBV unlike massive expansion of HCMV specific CD8⁺ T cells. It seems that HCMV may overtake EBV in terms of CD8⁺ T cell inflation although it is not fully clear how this phenomenon occurs (Khan, 2004).

During acute IM, the majority of EBV specific CD8⁺ T cells express the activation markers HLA-DR and CD38 and the memory marker CD45RO. The expression of CD28 and CD57 on CD8⁺ T cells specific to EBV varies depending on the donor and ranges from 9% to 86% for CD28 and 2% to 37% for CD57 (Appay et al., 2002;Radziewicz et al., 2007). However, in the latency or memory stage, EBV-specific memory T cells have been found to have an early phenotype (CCR7-CD27⁺CD28⁺CD45RA⁻) (van Lier et al., 2003).

Until recently, EBV was not considered as a major virus in manipulating the immune system in contrast to the multiple immune evasion strategies described for HCMV. However, many EBV genes have been recently characterized that manipulate the immune response (Hansen and Bouvier, 2009). EBV nuclear antigen 1(EBNA1) is the

best known example that can specifically interfere with the proteasomal processing which plays a central role in generation of peptides for antigen presentation pathways through MHC class I (Levitskaya et al., 1995; Rensing et al., 2008). In addition, BARF1, BZLF2, BILF1, and BNLF2a are at least four other genes of EBV that are known to have immune-modulatory functions (Rowe and Zuo, 2010). By encoding a secreted protein with the capability of binding to colony stimulating growth factor-1, BARF1 has been shown to inhibit macrophage activation (Strockbine et al., 1998).

BZLF2 encodes gp42 which mediates immune evasion through MHC class II molecules. Mechanistically, it has been shown that gp42 associates with MHC-II molecules in the endoplasmic reticulum and accompanies the MHC-II complexes to the cell surface, blocking the proper interaction with T cell receptors of CD4+ T cells (Rensing et al., 2003; Rensing et al., 2005).

BILF1 is a seven transmembrane segment G-protein receptor (GPCR) that shares structural and functional characteristics with chemokine receptors. Although the precise mechanisms have yet to be defined, BILF1 targets and downregulates MHC class I expression (Beisser et al., 2005; Rensing et al., 2008). BNLF2a is one of the EBV-encoded lytic phase proteins that contributes to the MHC class I downregulation via inhibiting TAP-mediated peptide transport, resulting in a diminished peptide supply into the ER lumen. BNLF2a is a small membrane-associated protein that interferes with both ATP and peptide binding to TAP (Hislop et al., 2007a; Horst et al., 2009).

1.9 Common Variable Immunodeficiency

Common variable immunodeficiency (CVID), first described by Janeway in 1953 (Janeway et al., 1953), is a heterogeneous disease characterized by defective antibody production, resulting in recurrent bacterial infections of the respiratory and gastrointestinal tract (Park et al., 2008;Chapel and Cunningham-Rundles, 2009). After selective IgA deficiency, CVID is the second most frequent primary immunodeficiency disease and, worldwide, has an incidence between one in 50000 and one in 200000 (Bayry et al., 2005;Lopes-da-Silva and Rizzo, 2008;Park et al., 2008).

The number of circulating B lymphocytes can be reduced or normal; however , these cells fail to terminally differentiate into plasma cells secreting antibody (Cunningham-Rundles and Ponda, 2005). It has also been documented that 50-75% of CVID patients have low numbers of class-switched memory B cells (CD27+IgM-IgD-) (Tangye et al., 1998;Piqueras et al., 2003;Alachkar et al., 2006;Park et al., 2008). Phenotypic heterogeneity of CVID disease makes the proper classification difficult. In the last few years three different classifications including Freiburg (Warnatz et al., 2002), Paris (Piqueras et al., 2003), and EUROclass (Wehr et al., 2008) have been suggested for CVID patients based on the assessment of Ig synthesis in vitro and immunophenotyping of B-cell subsets.

CVID has a broad and heterogeneous phenotype including sinopulmonary and gastrointestinal complications, inflammatory lesions, autoimmune disease, and malignancies, mainly non-Hodgkin's lymphoma (Park et al., 2008;Chapel and Cunningham-Rundles, 2009). Although monogenic defects including mutations in

inducible T cell co-stimulator (ICOS) (Grimbacher et al., 2003;Salzer et al., 2004), TACI (transmembrane activator and calcium-modulating cyclophilin ligand interactor) (Castigli et al., 2005;Salzer et al., 2005;Pan-Hammarstrom et al., 2007), CD19 (van Zelm et al., 2006), and BAFFR (B-cell activating factor of the TNF family receptor) have been described in 5-10% of CVID patients, the vast majority of these patients have no identifiable genetic defect.

While CVID is categorized as a B-lymphocyte-related disease, reports have described alterations in both the phenotype and function of T cells (Webster, 2001) such as the impaired function of dendritic cells, Th1 skewing and decreased production of IL-2 (Bayry et al., 2004b;Isgro et al., 2005a). In addition, the reduced expression of surface molecules (for example: CD40L, L-selectin and attractin) (Farrington et al., 1994), defective Vav expression, and impaired F-actin organization (Paccani et al., 2005) have also been described. Beside the reduction in the absolute numbers of T cells as well as inversion of the CD4:CD8 ratio due to reduced CD4⁺ T cell numbers (Isgro et al., 2005b), in some studies disturbed function of monocytes or monocyte-derived dendritic cells have also been reported in a subset of CVID patients (Cambronero et al., 2000;Bayry et al., 2004a;Cunningham-Rundles and Ponda, 2005).

CVID patients are known to be susceptible to recurrent infections such as multisystem inflammatory granuloma although the exact mechanisms are unknown (Chapel and Cunningham-Rundles, 2009). Although CVID patients have not been historically considered at risk of serious viral infections, patients with diminished specific antibody production and concurrent disturbances in T lymphocytes, infection with particular viruses have been reported such as entroviral infection (Halliday et al., 2003), HCV

(Yap et al., 1994), HSV-1 (Dray et al., 2006), HHV-8 (Wheat et al., 2005), EBV (Le et al., 2003), and HCMV (Tahan et al., 2000; Witte et al., 2000; Stack et al., 2004). The role of persistent viral infections in CVID patients is still not clear. However using CD4 responses to HCMV lysate as a marker of HCMV infection, 60% of CVID patients have been found to be HCMV-infected (Raeiszadeh et al., 2006). Little is known about T cell-mediated immunity to viral antigens in these patients and understanding the viral and host immuno-regulatory mechanisms that are involved in the development of disease is critical for developing new and improved treatments.

A significant proportion of CVID patients suffer from inflammatory lesions within a variety of organs. The role of HCMV infection in inflammatory diseases and long term complications such as atherosclerosis after solid organ transplantation is supported by data from epidemiological, interventional and mechanistic studies (Soderberg-Naucler, 2006). Table 1-3 shows the most common clinical manifestation of inflammatory disease in CVID patients with inflammation.

Clinical Manifestations	Organs Involved
Granulomatous disease	Lungs, liver, spleen, skin, and gastrointestinal tract
Pulmonary complications	Lung and airways
Gastrointestinal disease	Gut and intestine
Infections	Lung, liver, intestine, sinuses, middle ear, and spleen
Autoimmune diseases	Red blood cells and platelets
Neoplasias	Breast, prostate, ovary, skin, and colon

Table 1-3: Clinical phenotypes and organs involved in CVID patients.

1.10 HCMV and Inflammation

Human cytomegalovirus infection has, historically, been an important clinical problem after organ transplantation, leading to a broad range of direct and indirect clinical symptoms such as hepatitis, pneumonitis, enteritis, acute and chronic organ rejection. Morbidity and mortality attributed to HCMV has been reduced significantly in recent years due to the development of prophylactic and pre-emptive therapeutic strategies. High level viral replication is a dominant and well documented risk factor in HCMV disease (Emery, 2001). In addition, studies have shown a direct association between the functional impairment of CD8+ T cells specific to HCMV in interferon gamma production and increased virus replication and disease (Crough et al., 2007; Mattes et al., 2008). Regardless of the direct immunosuppressive effects of HCMV in transplant

patients for instance, increasing *in vitro* evidence is accumulating that suggests HCMV infection has many other effects on the host's immune response (Soderberg-Naucler, 2006;Freeman, Jr., 2009). There is substantial anecdotal evidence suggesting the role of infectious agents such as HCMV as potential contributors in the pathogenesis of vascular disease (Freeman, Jr., 2009). For example, the role of HCMV in atherogenesis through specific effects on matrix metalloproteinase 9 (MMP-9) activities has been suggested. To support this, it was found that MMP-9 can prevent the development of atherosclerotic plaques in mice (Straat et al., 2009). Moreover, the potential role of HCMV and p53 interaction in coronary restenosis has been examined (Speir et al., 1995). More recently, HCMV has been found in atherosclerotic plaques of patients with peripheral vascular disease and it appears that endothelial cells infected with virus play a central role in this scenario (Bentz and Yurochko, 2008).

Active HCMV replication has been frequently found in the site of chronic inflammation in rheumatoid arthritis and inflammatory bowel disease (IBD), in the vascular lesions of coronary artery transplant vasculopathy, systemic lupus erythematosus (SLE), several autoimmune diseases such as Sjögren's Syndrome and Hashimoto's Thyroiditis (Soderberg-Naucler, 2008). HCMV has also been implicated in contributing to the net state of immunosuppression seen in cancer patients (Harkins et al., 2002;Miller, 2009;Michaelis et al., 2009). HCMV-induced proinflammatory state could play the formation or progression of these diseases.

In the light of these studies linking HCMV with inflammatory conditions the potential role of HCMV infection and the CD8⁺ T cell responses in CVID patients with inflammatory disease provided the major focus for my PhD studies.

1.11 Project Aims

The aim of this PhD project was to investigate several aspects of functional competence of CD8⁺ T cell responses induced against HCMV compared to EBV in CVID patients (with and without inflammation) and to compare them with healthy HCMV and EBV seropositive individuals. The whole project was based on the hypothesis that HCMV may be a major factor driving CD8⁺ T cell expansion and inducing an aberrant HCMV specific CD8⁺ T cell response in CVID patients with inflammation.

Determining the sero-status against previously encountered viral pathogens in these patients is not possible because they do not produce immunoglobulins. Previous work in the laboratory used analysis of T cell response to determine the exposure of CVID patients to HCMV and EBV and this project builds upon these data to provide a comprehensive phenotype of these cells and to investigate their association with the pathology observed in CVID patients.

The aims of the thesis were as follows:

1. To assess the total CD8⁺ T cell response against epitopes specific to HCMV and EBV using virus-specific peptide stimulation in inflammatory CVID patients compared to both non-inflammatory patients as well as healthy age-matched controls.
2. To investigate the polyfunctional signature of CD8⁺ T cells specific to both HCMV and EBV in CVID patients with or without concurrent inflammatory disease as well as healthy controls to probe in more detail whether HCMV-

specific CD8+ T cells in CVID patients with inflammation are characterized by unique features.

3. To evaluate the phenotypic analysis of HCMV and EBV specific-CD8+ T cells and pentamer stained T cells in CVID patients and healthy individuals with regard to phenotypic maturation, cytotoxicity, and cell exhaustion.
4. To assess functional aspects of CD8+ T cells, with respect to proliferative capacity following single virus-specific peptide stimulation.
5. To assess the avidity of CD8 binding and anti-inflammatory potential of CD8+ T cells in CVID patients (with and without inflammation).

Chapter 2

2 Materials and Methods

2.1 Study population

A cohort of thirty two CVID patients who had an HLA type for which a ppUL83 class I HLA pentamer was available, and 14 HLA- and age matched healthy controls were recruited to the study. Because HLA-A2 is relatively common in the UK population and class I HLA pentamers for HCMV and EBV was available the study focused on this group of patients. CVID was defined according to the International Union of Immunological Societies (IUIS) criteria (Chapel et al., 2003;Chapel and Cunningham-Rundles, 2009). There was no significant differences in either age or gender between patients (40-69 years (median: 55years); 13 female and 12 male) and the healthy group (34-60 years (median: 49 years); 8 female and 6 male). Seven of the CVID patients were excluded because of either being HCMV negative (as judged by CD4+ T cell responses against HCMV lysates or not having an HLA type for which an HLA multimer was available. The CVID group (n = 25) were all HCMV positive using previous criteria (Raeiszadeh et al., 2006). Prior exposure to HCMV was determined based upon CD4+ T cell responses to a cytomegalovirus cell infected lysate (Autogen Bioclear, UK). For EBV, prior exposure was determined using pentamer staining as described in section 2.4.

All HCMV positive CVID patients were further sub-divided by the treating clinicians into patients with inflammatory disease (n = 9) and those with no inflammation (n =16). Inclusion in the inflammatory disease group was based on fulfillment of one or more of the following criteria: i) hepatitis without evidence of hepatitis B or hepatitis C virus infection; (ii) splenomegaly; (iii) clinical evidence and a biopsy indicative of chronic

enteropathy involving small and/or large bowel not explained by infection or gluten sensitivity; (iv) interstitial lung disease not explained by bacterial infection. All CVID patients regularly received standard immunoglobulin therapy.

The study was approved by the local research ethics committee of the Royal Free Hospital.

2.2 Cell culture

2.2.1 Isolation of PBMC from peripheral blood

Fresh whole blood (20-25mL) was collected from CVID patients and healthy volunteers using the monovette system, containing heparin. The blood was then carefully overlaid onto Ficoll-Paque (GE Healthcare, UK) at a 1:1 volume ratio in 50 ml sterile Falcon universal tubes (Fisher, UK). Peripheral blood mononuclear cells (PBMCs) were separated by centrifugation (778g, 22 min at room temperature) with slow acceleration and without braking. Following centrifugation, the interphase containing mononuclear cells, created between the plasma and the lymphocyte separation medium, was carefully removed and placed into a fresh 25ml universal tube and the volume made up to 25ml with unsupplemented RPMI-1640 (Gibco, UK) and centrifuged at 600g (1800 rpm) for 10 minutes (with fast acceleration and brake) to remove any residual Ficoll. After centrifugation, the supernatant was discarded and the resultant PBMC pellet was resuspended in an additional 10ml of RPMI-1640 and centrifuged extra time at 500g (1500rpm) for 5 min. Following the final wash, the PBMC pellet was resuspended in 5ml RPMI-1640 supplemented with 2-mM glutamine and 10% fetal calf serum (FCS).

Viable cells were stained with trypan-blue (Sigma) and counted using a haemocytometer and either used immediately or stored in liquid nitrogen.

2.2.2 Freezing/thawing PBMC

Isolated PBMCs were centrifuged at 500g for 5 minutes and the pellets were resuspended at concentration of 3×10^6 to 5×10^6 per ml in pre-cooled 90% FCS and 10% dimethyl sulphoxide (DMSO) (Sigma, UK). Resuspended cells were then transferred in 1ml aliquots into labeled 1.5ml cryovials (Starlab, UK) and placed in an isopropanol freezing container (previously equilibrated to 4°C) prior to transfer into liquid nitrogen storage for long term storage. For thawing PBMC, frozen cells were taken from liquid nitrogen storage and immediately placed the cryovials in a water bath (37°C). When the cells had almost completely thawed they were then transferred to a 15ml Falcon tube and resuspended gently in 9ml RPMI-1640 containing 10% FCS. Cells were then centrifuged at 500g for 5 minutes to remove residual DMSO. Following additional washing, cells were assessed for both number and viability using the Trypan Blue (TB) (Sigma, UK) exclusion method (to stain dead cells) and counted via a haemocytometer.

2.3 Intracellular cytokine staining

Following PBMCs separation, cells were resuspended in RPMI-1640 supplemented with 10% FCS at a density of 1×10^6 cells/0.5ml in sterile capped 12x75mm polypropylene tubes (Marathon Lab Sup, UK). Cells were stimulated with $5\mu\text{g/ml}$ of viral peptides (shown in table 2-1) or left unstimulated as a negative control in the presence of $5\mu\text{g/ml}$ co-stimulatory monoclonal antibody, anti-CD28 (Becton Dickinson, UK). Tubes containing cells were vortexed gently to mix and then incubated for two hours in a

humidified incubator at 37⁰C with 5% CO₂. Following 2 hours stimulation, suspensions were mixed by adding 0.5ml supplemented RPMI-1640 (total volume 1ml per tube) containing freshly thawed Brefeldin A (Sigma St Louis, MO, US) at a concentration of 10µg/ml and incubated for an extra 14 hours at 37⁰C 5% CO₂.

Viral peptides	Virus	Proteins	Stock Preparation
NLVPMVATV	HCMV	Derived from pp65 (UL83) protein	1mg lyophilized peptide dissolved in 50µl DMSO + 950µl sterile dH ₂ O, and frozen in aliquots at -20 ⁰ C.
GLCTLVAML	EBV	Derived from lytic protein of BMLF1	
Pp65 pool peptide	HCMV	Tegument protein (UL83)	Lyophilized powder dissolved in 50µl of DMSO + 200µl sterile dH ₂ O and stock frozen in aliquots at -20 ⁰ C.
IE-1	HCMV	Non-structural protein	

Table 2-1: Viral peptides used for intracellular cytokine stimulation assay.

All purchased antibodies and antigens used in this study are listed with catalogue number and shown in appendix 1. Matched peptides including NLV and GLC peptides were purchased from Proimmune and pp65/IE-1 pool peptides were purchased from JPT technologies PepMix.

Following 16 h stimulation, tubes were vortexed to mix, centrifuged at 500g (1500rpm) for 5 minutes and the supernatant discarded. The resuspended cells in residual media were subjected to surface staining by adding 10µg/ml anti-CD8 (or anti-CD4) conjugated with Peridinin-chlorophyll proteins (PerCp cy5.5) to each tube, and incubated for 15 minutes at room temperature (RT) followed by washing in 2ml phosphate buffered saline (PBS) containing 0.1% sodium azide (NaN₃) and centrifugation at 500g for 5 minutes. The cells were then fixed/permeabilised for 15

minutes at RT by adding 100µl Caltag medium A (Invitrogen) whilst vortexing. For intracellular staining, cells were washed again in 2ml PBS containing 0.1% NaN₃, permeabilised with 100ml Caltag medium B (Invitrogen) while vortexing and stained with fluorochrome antibodies against IFN- γ (10µg/ml), TNF- α (10µg/ml), and IL-2 (2µg/ml) conjugated with Allophycocyanin (APC), Phycoerythrin (PE), and Fluorescein (FITC) (BD Biosciences, UK), respectively. Stained cells were then incubated in the dark at RT for 15 minutes followed by a final wash as described above. Following the final wash, cells were resuspended in 100µl of 4% paraformaldehyde in PBS (Sigma, UK) to fix and either directly acquired using Fluorescence Activated Cell Sorter (FACS) Calibur (BD Biosciences, UK) or stored at 4⁰C in the dark until acquisition. 50,000 CD8 cells were acquired using Cell Quest software and four-colour FACSCalibur. All further data analysis was done using the FlowJo software (Tree Star, Inc, US). Background subtraction was used in all cases. For this purpose, number of cytokine producing cells measured in unstimulated PBMC was subtracted from the cells producing the same cytokines following peptide stimulation to yield the final frequencies. Figure 2-1 illustrates an example of representative FACS density plots of cytokine producing CD4⁺ T cells in response to CMV lysate stimulation and background level of unstimulated cells.

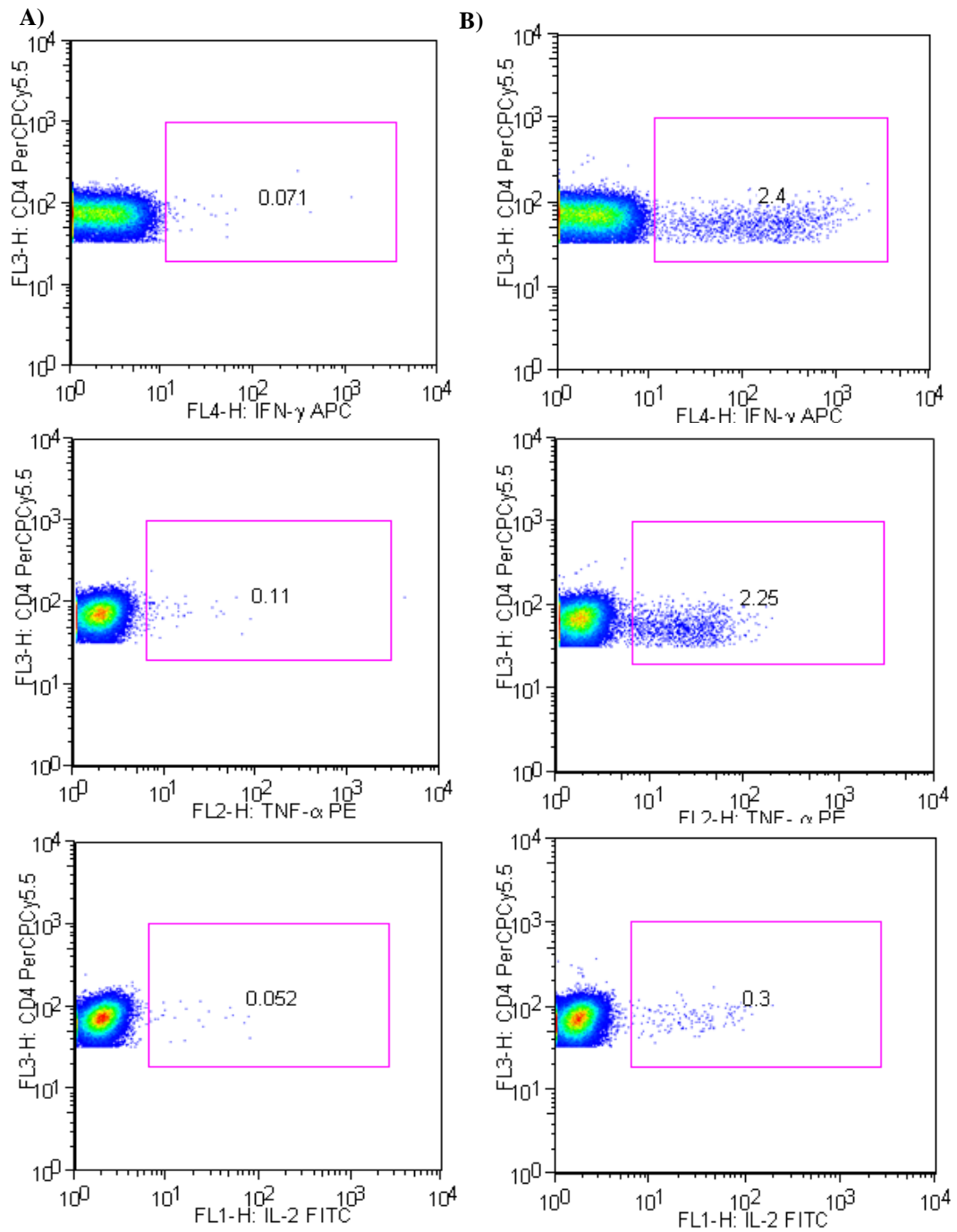


Figure 2-1: Representative FACS plots of cytokine expressing CD4⁺ T cells in response to no stimulation as background staining (A) or CMV lysate (B).

For polyfunctional analysis, cytokine co-expression subsets were further determined using the Boolean gating function in the FlowJo software. Figure 2-2 illustrates a schematic of the polyfunctional analysis and combination analysis of the seven potential subsets. For this purpose as shown in Figure 2-2, lymphocytes (A) were gated based on forward scattering (FSC) and side scattering (SSC) and CD8⁺ T cells (B) were then analyzed for individual cytokine production including IFN- γ (C), TNF- α (D), and IL-2 (E). Polyfunctional analysis for cytokine co-expression profiles were determined using the Boolean gating function available in the FlowJo software to measure the frequencies of different expression profiles corresponding to triple producers (F), double producers (G, H, and I), and single producers (J, K, and L).

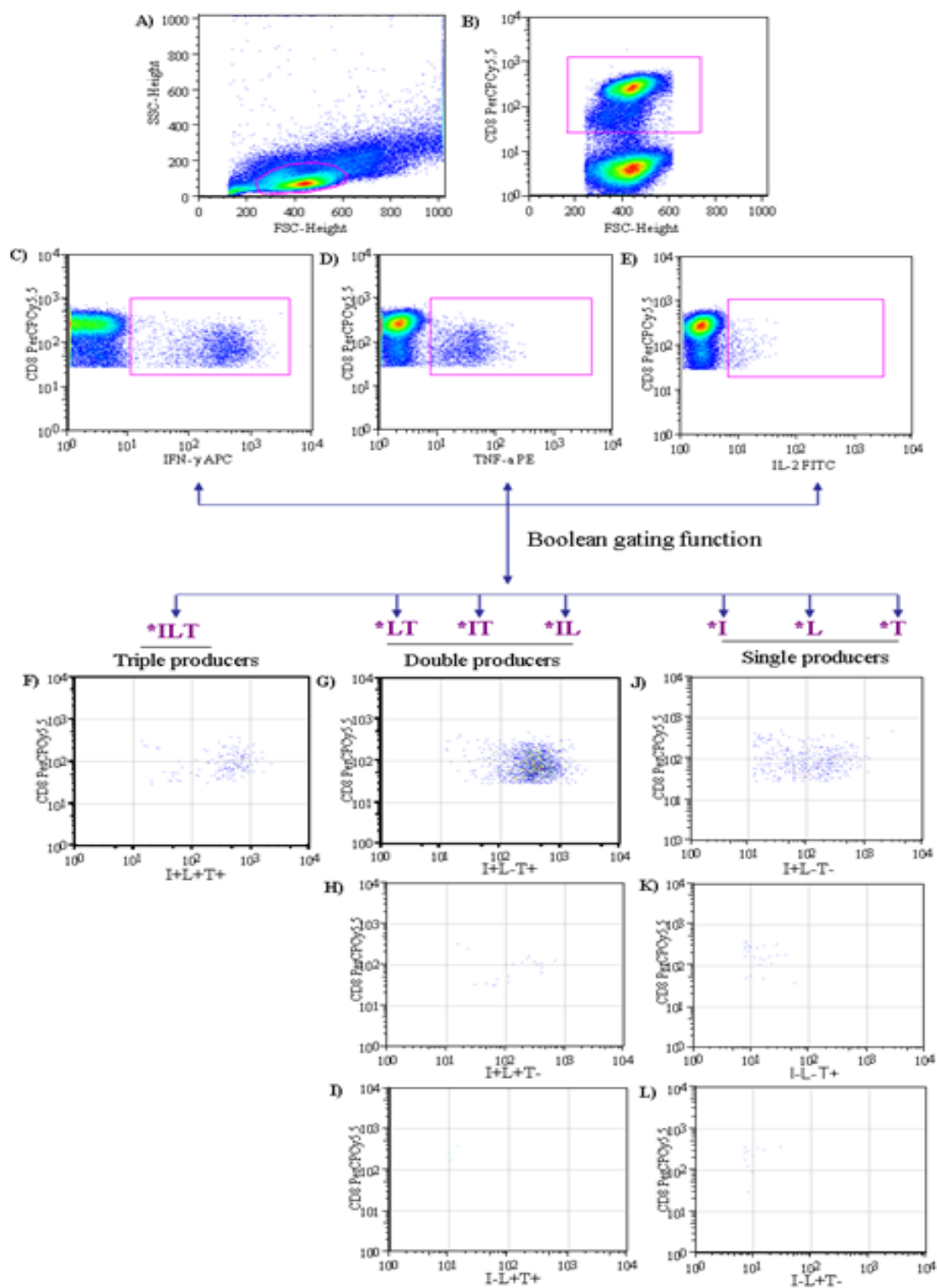


Figure 2-2: Schematic representation for the analysis of cytokine co-expression profiles of CD8⁺ T cells in response to HCMV peptide stimulation.

*ILT: IFN- γ , IL-2, and TNF- α ; *LT: IL-2 and TNF- α ; *IT: IFN- γ and TNF- α ; *IL: IFN- γ and IL-2; *I: IFN- α ; *L: IL-2; *T: TNF- α .

2.4 *Ex vivo* staining

Both fresh and frozen PBMCs were counted and placed in FACS tubes (12×75mm polypropylene tubes) at a concentration of 1×10^6 cells/0.5ml (supplemented RPMI-1640 in 10% FCS) and centrifuged at 500g for 5 minutes. Following centrifugation, the supernatant was discarded and cells resuspended in residual fluid and then stained for 15 minutes at RT (protected from light) with PE conjugated pentamers (A*0201: NLVPMVATV derived from pp65 (ppUL83) of HCMV and for EBV A*0201: GLCTLVAML derived from lytic protein of BMLF1) (Proimmune, UK). After incubation, cells were washed for 5 minutes at 500g with 2ml PBS containing 0.1% NaN₃. Following discarding the supernatant, cell pellets were resuspended and stained for 15 minutes in RT with 10µg/ml of conjugated surface markers including, CD8 PerCP Cy5.5, CD57FITC, CD45RO FITC, CD45RA APC, PD-1 FITC, IgG1k isotype control FITC (all from BD Biosciences, UK), and CD73 APC (eBiosciences, UK). Following 15 minutes incubation, cells were washed (all washes consisted of adding 2ml PBS/0.1% NaN₃ and centrifuging at 500g for 5 minutes) and then resuspended in 100µl of 4% paraformaldehyde in PBS and stored at 4⁰C until acquisition. Surface staining was carried out at room temperature for 15 minutes. The cells were washed with PBS/ 0.1% azide as above, and. For some markers such as granzyme B (Invitrogen, UK) and Ki-67 (BD Biosciences, UK), after surface staining with pentamer and CD8, cells were fixed by adding 100µl Caltag medium A fixative reagent and further incubated for 15 minutes at room temperature. Cells were washed again, permeabilised using 100µl Caltag medium B and co-incubated for 15 minutes with 5µg/ml and 10µg/ml of granzyme B-APC or Ki67-FITC, respectively. Cells were washed a final time and resuspended in

100µl of 4% paraformaldehyde and were stored at 4⁰C until FACS acquisition. 50,000 CD8+ T cells were acquired on a FACS Calibur.

2.5 Carboxyfluorescein diacetate succinimidyl ester (CFSE) labelling assay

CFSE is a fluorescent dye with the ability of permanently labeling the cells, providing a valuable tool for monitoring the cell proliferation. CFSE powder (Molecular Probes, UK) was dissolved in DMSO to a final concentration of 5mM and stored in suitably sized aliquots (6µl) at -20°C. CFSE labeling was performed according to a standard protocol (Quah et al., 2007). For this purpose, freshly isolated PBMCs were re-suspended in PBS containing 5% FCS at a concentration of $5-10 \times 10^6$ cells/ml, and CFSE labeled by adding 2.5µM of CFSE into cell suspension for 5 minutes in the dark at room temperature. Cells were then washed three times with 5% FCS in PBS and subsequently re-suspended in RPMI 1640 supplemented with 10% FCS. CFSE-labeled cells were then cultured in 96-well plates at a concentration of $5-10 \times 10^5$ cells/200 µl per well for 5 days in 37⁰C 5% CO₂. In each set of experiments, CFSE-treated cells were stimulated with 10 µg/ml of HCMV pp65-derived peptide (A2: NLVPMVATV), EBV BMLF1-derived peptide (A2: GLCTLVAML), or Phytohemagglutinin (PHA) (Sigma, UK) at a final concentration of 10 µg/ml. As a negative control, labeled cells were left unstimulated. On day 5, cells were harvested, centrifuged and surface stained with 5µg/ml of PE conjugated pentamers as described in section 2.4. Following 15 minutes incubation at room temperature and washing with PBS/0.1% sodium azide, cells were subsequently stained for the surface marker CD8 using anti-CD8 PerCP Cy5.5. Finally cells were washed and fixed by suspending in 100µl of 4% paraformaldehyde in PBS.

2.6 Supernatant transfer assay

Freshly isolated PBMCs from inflammatory CVID patients were CFSE labeled, as mentioned in section 2.5, with 2.5 μ M of CFSE. 5×10^5 CFSE labeled cells in 200 μ l RPMI-1640 medium supplemented with 10% FCS were added per well of a 96 well plate following stimulation with a final concentration of 10 μ g/ml HCMV pp65-derived peptide (A2: NLVPMVATV), EBV BMLF1-derived peptide (A2: GLCTLVAML), or no peptide stimulation as a negative control for 5 days. In addition, a HCMV pp65 peptide pool covering the whole ORF (JPT Peptide Technologies GmbH, Germany) was included in this setting to check that any response to HCMV was specific. On day two, cell cultures were collected and centrifuged for 5 minutes at 500g. Supernatants were then collected in pre-labeled tubes and filtered using 0.2 μ m filters.

For supernatant transfer experiments, PBMCs were taken from both healthy donors and non-inflammatory patients and the same procedure was followed; cells were CFSE labeled and treated with the same panel of antigens. Stimulated cells at a concentration of 5×10^5 labeled cells/100 μ l were cultured in 96 well plates in the absence and presence of 100 μ l supernatants collected (and filtered using 0.2 μ m filter) from the inflammatory CVID samples. Plates were incubated at 37⁰C 5% CO₂ for 5 days before surface staining with PE-labeled pentamers and anti-CD8-PerCP Cy5.5. Figure 2-3 shows a schematic of the supernatant transfer assay.

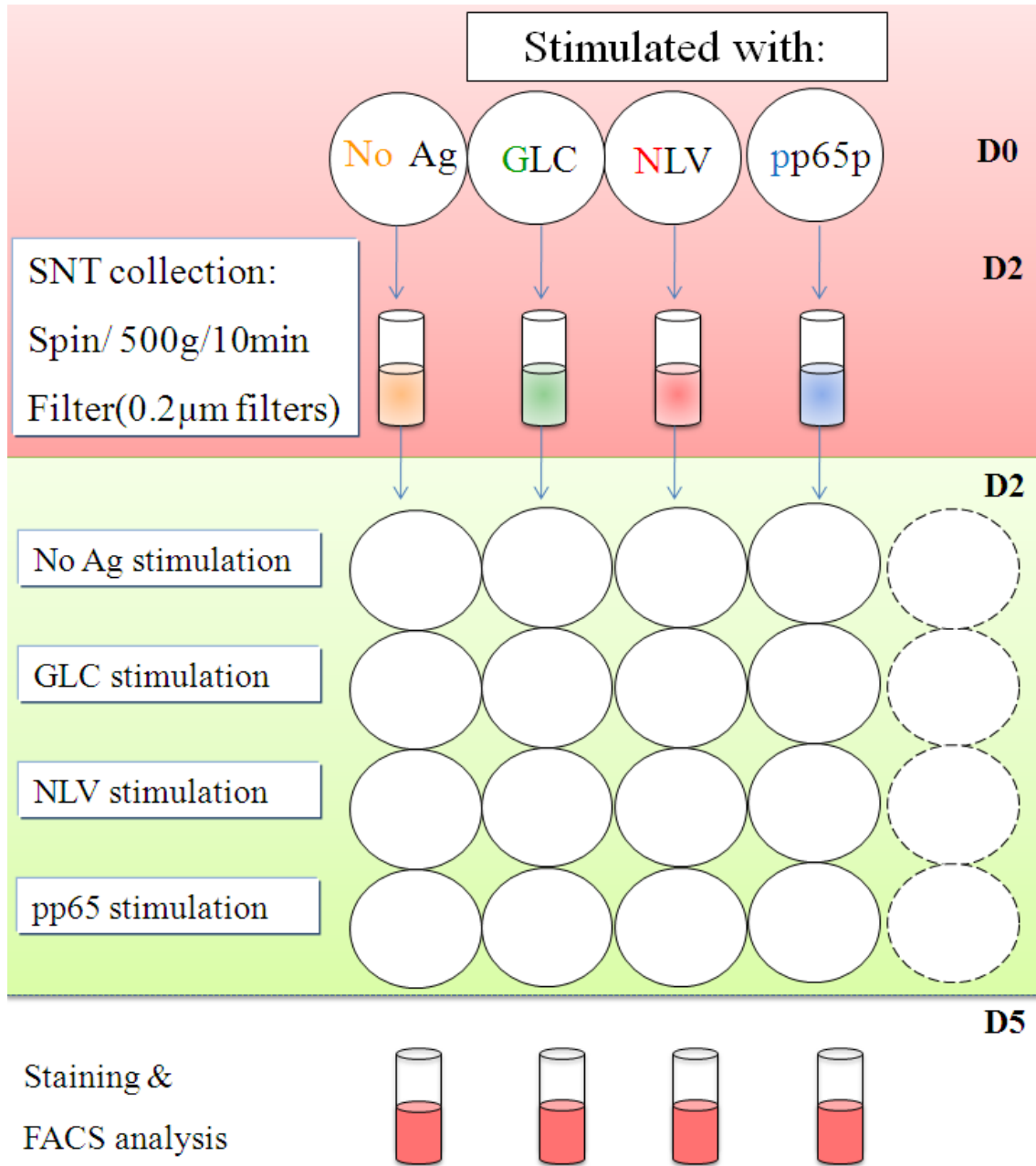


Figure 2-3: Schematic representation of supernatant transfer assay.

PBMCs taken from inflammatory CVID patients (light red box) were CFSE labeled and stimulated with (GLC, NLV, pp65 pool) and without antigens. On day 2 (D2), supernatants were collected from cell cultures, centrifuged, filtered, and transferred into sterile tubes (shown as different colors) for supernatant transfer assay (SNT). Before SNT, PBMCs were taken from healthy or non-inflammatory CVID patients (light green) and cells were CFSE labeled and stimulated as above. Cell cultures were then incubated for 5 days in the absence (dashed circles) and presence of supernatants. After 5 days, cell cultures were collected, washed, and stained for FACS analysis.

2.7 Cytokine blocking assay

Freshly isolated PBMCs from COVID patients with inflammation were CFSE labeled as described in the previous section. CFSE labeled cells were stimulated with 10 μ g/ml of GLC, NLV, or pp65 peptide pool peptides or left unstimulated as a control. Cells were then seeded in a 96 well plate at a concentration of 5×10^5 cells in 200 μ l medium (RPMI-1640) supplemented with 10% FCS. Different concentrations of anti-human TNF- α (0-0.2 μ g/ml) and anti-IFN- γ (0-0.26 μ g/ml) purified polyclonal antibodies (Peprotech, UK) were used for cytokine inhibition to determine their effect on proliferation. Prior to transfer into the cell cultures, polyclonal antibodies were reconstituted in sterile water to a concentration 0.1-1.0mg/ml. On day five, cell cultures were collected, washed and surface stained at room temperature with PE-conjugated pentamers and CD8 PerCP Cy5.5 as described in sections 2.4 and 2.5.

2.8 Production and use of differential avidity class I HLA tetramers

Monomers of CMV pp65 NLV epitope specific tetramers with differential avidity were synthesized in E-coli, purified and refolded in the presence of β_2m by the Klenerman laboratory at Oxford. For tetramerisation, monomers (25 μ g) were diluted to 0.5 μ g/ μ l in PBS and kept on ice or at 4 $^{\circ}$ C. Using a 1:20 molar ratio of streptavidin: monomer, PE-conjugated extravidin at a final concentration of 25 μ g/ml (Sigma) was added 10 times to the vial containing monomer with 15 minute intervals between additions. The CD8 null tetramer has reduced avidity for peptide bearing targets because of a mutated CD8 binding domain (Wooldridge et al., 2007) as shown in Figure 2-4. Once prepared,

tetramers were stored in the dark at 4⁰C for a maximum of one month. For staining, 1 × 10⁶ cells were stained by adding 4μg of PE-labeled tetramers (null and normal) for 20 min at 4⁰C. Cells were washed in PBS and then surface stained with 10μg/ml of anti-CD8 PerCP Cy5.5 for further 20 minutes at 4⁰C. Finally cells were washed in PBS, fixed with 100ml of 4% paraformaldehyde in PBS and acquired using FACSCalibur as described in section 2.4.

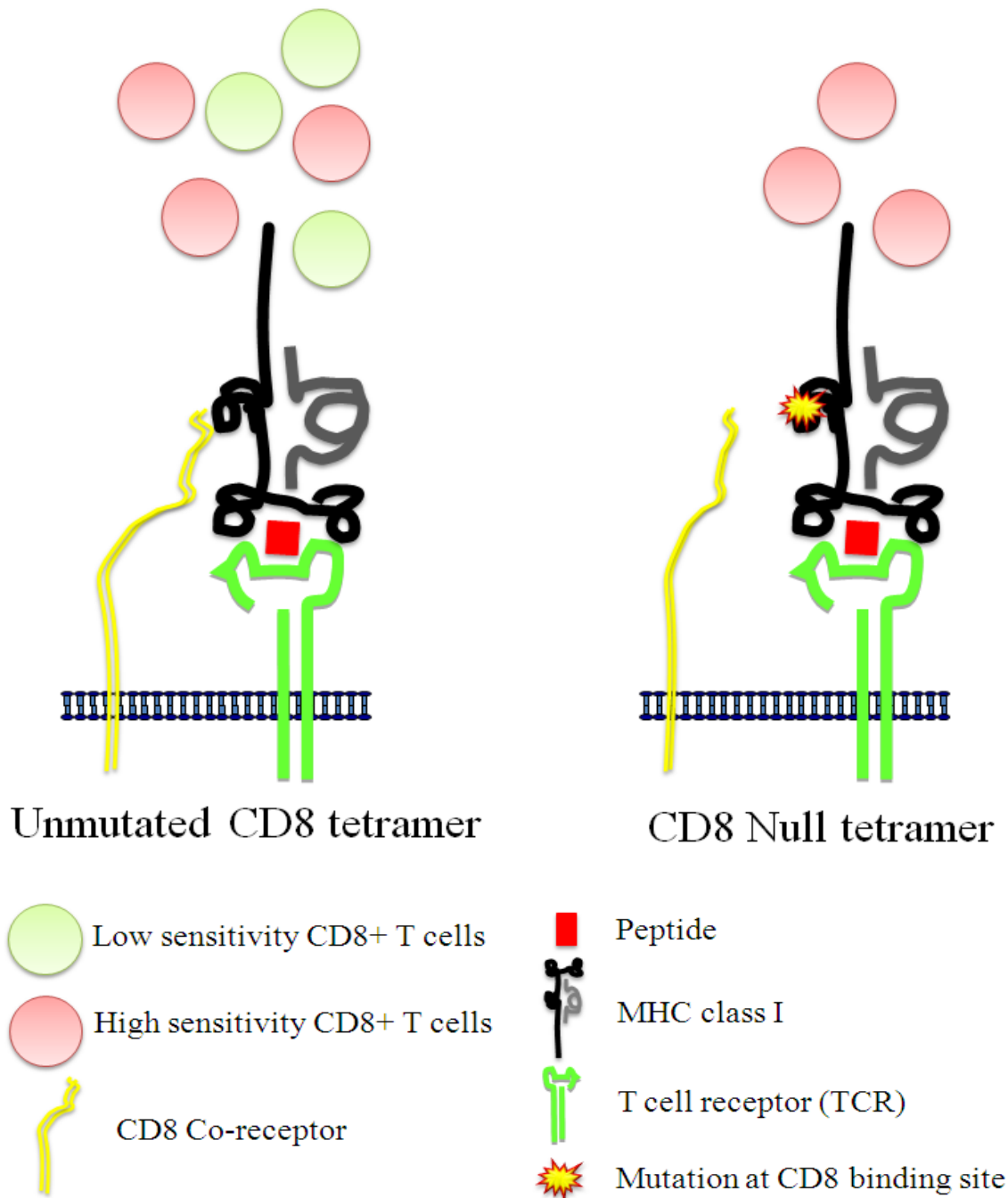


Figure 2-4: Schematic representation of HLA class I tetramers with differential avidity

For simplicity tetramers is drawn as monomers. Unmutated CD8 tetramer does allow CD8 co-receptor ligation and thereby both high (light red balls) and low (light green balls) sensitivity cells CD8+ T cells can interact with pMHC-TCR complex. Because of a mutation in CD8 binding site, null tetramers only detect high avidity CD8+ T cells.

2.9 Telomere length measurement

Telomere length of PBMCs in the cohort of CVID patients with and without inflammation was measured using flow-fluorescence in situ hybridization (FISH) method (Plunkett et al., 2001; van de Berg et al., 2010b). Frozen PBMCs were thawed in RPMI-1640 supplemented with 10% FCS (as described in section 2.2.2) and washed in phosphate-buffered PBSA (PBS plus bovine serum albumin, BSA, 0.5%) (Lonza, UK). Cells were then surface stained with 15 μ l of CD8-biotin (BD Biosciences) for 15 min at RT followed by washing with PBSA for 5 minutes. Surface staining was carried out by adding 0.2 μ g (per 1 \times 10⁶ cells) streptavidin-Cy3 (Cedarlane Laboratories, Burlington, Canada) for 15 min at RT before washing cells in PBS (not PBSA) for 5 min at 688g (1800rpm). Following cell surface staining, cells were fixed using 2mM BS3 solution (BS3, Bis [sulfosuccinimidyl] suberate, is a water-soluble, non-cleavable and membrane impermeable crosslinker that crosslinks cell-surface proteins prior to cell lysis and immunoprecipitation. As it is unstable, 2mM BS3 solution was prepared immediately before use by adding 200 μ l of PBS into one BS3 'no-weigh' powder aliquot from 4⁰C). Cell pellets were resuspended in 100 μ l of PBS and 100 μ l BS3 solution then incubated for 30 minutes at 4⁰C. The fixation process was followed by quenching the BS3 by adding 1ml of 50mM Tris-HCL pH 7.2 in PBS and incubating for 20 min at RT in the dark before washing with PBS. For hybridisation, while vortexing, 1ml of hybridisation buffer was added to the cell pellet, centrifuged at 778 \times g for 7 min and cells were resuspended in 300 μ l hybridisation solution (refer to the tables in this section). After washing in hybridisation buffer, cells were subjected to telomere-Cy5 (3 μ l human telomere PNA (peptide nucleic acid) probe + 97 μ l hybridisation solution per tube) and

were heated for 10 min at 82⁰C in a water bath, rapidly cooled on ice, and hybridized for 1 h at room temperature in the dark. Samples were washed twice with 1ml of post-hybridisation solution (refer to the tables 2.1 and 2.2) for 7 min at 778×g. Following the final wash in PBSA (5 minutes at 478×g) cells were analyzed immediately by flow cytometry. Two cryopreserved PBMC samples with known telomere fluorescence were used as standards to ensure consistency of the results. Results were obtained as median fluorescence intensity values. The following tables indicate the reagents used and solutions for the telomere length studies.

Hybridisation Buffer:

Formamide	7ml	10.5ml	14ml
Tris. HCL	0.2ml of 1M stock	0.3ml of 1M stock	0.4ml of 1M stock
BSA	2.5ml of 4% stock	3.75ml of 4% stock	5ml of 4% stock
NaCl	0.3ml of 5M stock	0.45ml of 5M stock	0.6ml of 5M stock
Total Volume	10ml	15ml	20ml

Table 2-2: Hybridisation buffer ingredients.

4% BSA:

2g BSA dissolved in 50ml sterile distilled water (SDW) (store at 4⁰C)

5M NaCl:

29.22g in 100ml SDW (steriled by autoclaving)

50mM Tris in PBS:

5ml 1M Tris + 10 ml 10x PBS + 85ml SDW

Post – Hybridisation Buffer:

Formamide	7ml	14ml	28ml
Tris. HCL	0.1ml of 1M stock	0.2ml of 1M stock	0.4ml of 1M stock
BSA	0.25ml of 4% stock	0.5ml of 4% stock	1ml of 4% stock
Tween20	0.1ml of 10% stock	0.2ml of 10% stock	0.4ml of 10% stock
NaCl	0.3ml of 5M stock	0.6ml of 5M stock	1.2ml of 5M stock
H₂O	2.25ml	4.5ml	9ml
Total Volume	10ml	20ml	40ml

Table 2-3: Post-Hybridisation buffer ingredients.**10% Tween20:**

1ml Tween20 in 10ml SDW (stored at 4⁰C)

2mM BS3 solution:

2mM BS3 solution was prepared immediately before by reconstituting BS3 powder in 200µl of PBS and transferring to a tube containing 1550µl of PBS (1750µl total volume). This gave enough 2mM solution for 17 samples. BS3 was purchased from Pierce (Thermo Fisher Scientific Inc, US).

2.10 Statistical analysis

GraphPad Prism was used for plotting graphs and for statistical analysis, Student's t test, 2 way ANOVA, and paired t-test were used as appropriate. P-values ≤ 0.05 were regarded as significant.

Chapter 3

3 CD8+ T cell responses to HCMV and EBV in CVID patients

3.1 Introduction

T cell responses and antibodies comprise a key part in the natural history of the host response to self-antigens and various pathogens such as viruses. As a result, both T cells and antibodies could potentially be useful as markers of disease for clinical as well as diagnostic purposes. Most importantly, of course, they play an essential role in protection against a variety of infections. The role of CD8+ T cells, as the second wave of the cellular immune counterattack, has been extensively studied in mouse model systems (Pantaleo and Harari, 2006). Although the direct role of CD8+ T cells in human antiviral immunity is difficult to establish, adoptive transfer of virus-specific CD8+ T cells has been shown to be effective against HCMV, EBV, and HIV-1 (Walter et al., 1995; Brodie et al., 1999; Khanna et al., 1999; Yewdell and Hill, 2002).

Viral antigens are initially presented to CD8+ T cells as peptide-MHC Class I complexes on the surface of infected cells, particularly by professional antigen presenting cells (pAPC) (Tewalt et al., 2009). CD8+ T cells exert their principal antiviral effects via the localized secretion of lytic molecules in close vicinity to the virus-infected APCs. The killing process is mediated by releasing perforin and granzymes. In addition, nearly all CD8+ T cells secrete IFN- γ and TNF- α , which induce a potent antiviral state in cells (Yewdell and Hill, 2002).

The focus of this chapter is on the CD8+ T cell responses to HCMV and EBV in a population of CVID patients. Defective antibody production and therefore failure to control microbial infections are the main characteristic features of these patients (Park et al., 2008). The inability to produce immunoglobulin against invading pathogens

provides an important clinical model for the study of various aspects of the adaptive immune response. This study aimed to focus on T cells because of their projected role in the control of established chronic viral infections (Ahmed and Gray, 1996; Klenerman and Hill, 2005). Because CVID patients are unable to mount a humoral immune response, determining whether they have been previously exposed to virus has been problematic. The recent development of standardized T cell stimulation assays has allowed consistent and reproducible measurement and characterization of antigen-specific memory T cells in human whole blood, indicative of previous infection (Breinig et al., 2006; Pantaleo and Harari, 2006).

Data available from studies of HCMV and tuberculosis have demonstrated the importance of CD8+ and CD4+ T cell responses in the control of these infections and verified that quantitative measures of T-cell function (for instance IFN- γ - secretion) are clinically applicable and can be an important tool in monitoring of disease activity (Fishman and Rubin, 1998; Lalvani et al., 2001; Bunde et al., 2005; Lazarevic et al., 2005; Pantaleo and Harari, 2006). Much of this work has been made possible by the advancement of virus specific tetramer and peptide technology.

Fluorescence labeled, peptide-MHC multimers (which is usually known in the field of T cell immunology as MHC ‘tetramers’ or ‘pentamers’) was first described by Altman et al in 1996. Unlike functional assays involving *ex vivo* re-stimulation, MHC tetramers allow direct and specific staining of the T cell without further *in vitro* manipulation. They also allow for simultaneous characterization of multiple functions through co-staining for various cell surface markers, enabling a broad assessment of the magnitude,

phenotype and therefore functional capacity of T-cell effector functions (Perfetto et al., 2004; Leisner et al., 2008).

Taken together, the fact that CVID patients have dysfunctional humoral immunity and the growing body of evidence supporting the development of T cell phenotyping as a marker of disease, characterizing T cells in these patients may provide new insights into disease progression and the role of viral infections. This could ultimately lead to improved monitoring and patient management (Pantaleo and Harari, 2006).

Until fairly recently it was not clear how common HCMV infection was in CVID patients. However, using HCMV lysate and measuring CD4+ T cell response in CVID patients the incidence of HCMV among these population was previously shown to be around 60%. Additionally, no significant difference was found when CD4+ T cell response against HCMV was compared between CVID patients and healthy controls (Raeiszadeh et al., 2006).

The work discussed in this chapter, aimed to investigate the role of CD8+ T cell responses to HCMV and EBV in CVID patients, with the focus on a subset of patients who experienced inflammatory diseases compared to non-inflammatory patients. The first aim of this chapter was to determine whether these patients mount CD8+ T cell responses to HCMV and EBV. If they do, is there any difference between inflammatory CVID patients and those without inflammation. The second aim was to check whether any elevation in CD8+ T cell responses was a generalized phenomenon to the virus.

Although a variety of different cytokines can be used to measure the CD8+ T cell immune response to viral peptide stimulations, three well studied cytokines; IFN- γ , TNF- α , and IL-2, were chosen because measures of T cell function cannot be based the

quantification of an individual cytokine. In terms of assessing effector functions, the role of IFN- γ and TNF- α in the clearance of various microbial infections and the induction of cellular antiviral proteins has been well documented (Bogdan et al., 1990; Liew et al., 1990). However, to define a relatively good antigen-elicited response against specific infections that require T cells for protection, IL-2 was included in the cytokine panel. IL-2 has little direct effector function, but it is important in promoting the expansion of T cells to amplify effector T cell responses (Seder et al., 2008).

3.2 Comparison and analysis of antigen-specific pentamer populations in HCMV/EBV infected CVID patients and healthy controls

Ex vivo staining using pentamers specific for HCMV and EBV (A2: NLVPMVATV, A2: GLCTLVAML, respectively) was used to determine the frequency of HCMV and EBV specific CD8+ T cells in CVID and healthy individuals.

HCMV ppUL83 NLV specific CD8+ T cell frequencies were significantly increased in CVID patients compared to healthy age matched controls (Mean \pm SD; CVID: 2.6% \pm 0.49%, n = 25; Healthy: 0.6% \pm 0.2%, n = 14; P= 0.005). No such difference was observed in the frequency of EBV BMLF1 GLC-specific CD8+ T cells between the two groups (CVID: mean frequency 0.18% \pm 0.04%, n = 25; Healthy: mean frequency 0.32% \pm 0.19%, n = 14; P> 0.05) (Figure 3-1).

Within the CVID patient group, the frequency of HCMV-specific CD8+ T cells was significantly higher than the frequency of EBV-specific cells with an average difference of 14.4-fold (HCMV: mean frequency 2.6% \pm 0.49%; EBV: mean frequency 0.18% \pm 0.04%; P< 0.0001). This was much greater than the difference observed between the responses to these epitopes in healthy individuals (2.1-fold) (HCMV: mean frequency 0.66% \pm 0.2%; EBV: mean frequency 0.32% \pm 0.19%; P> 0.05) (Figure 3-1).

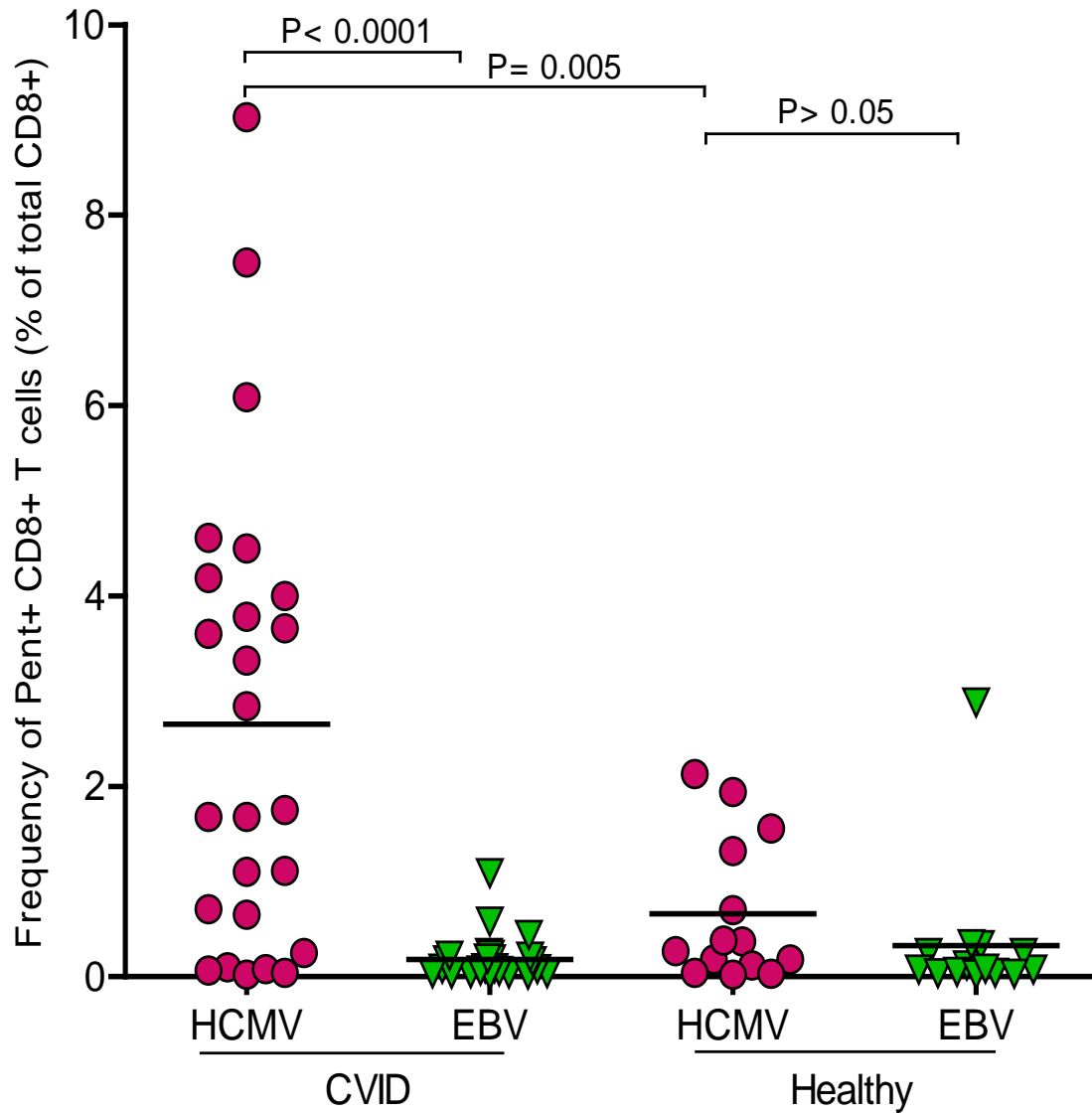


Figure 3-1: Mean percentage frequency of antigen-specific CD8+ T cells.

HCMV-specific CD8+ T cell population was significantly higher in CVID patients compare to healthy controls. Bars indicated mean values. Each point represents a single subject.

3.3 Cytokine production of HCMV-specific CD8+ T cells in CVID patients and healthy controls

The magnitude of the CD8+ T cell response is a basic characteristic used to describe T cell responses. However, measuring a single parameter for the magnitude of the response, for example IFN- γ production, does not reflect the full functional potential of T cells. Determining which functions to measure is important and can broaden our understanding of T cell function (Seder et al., 2008).

To gain a preliminary insight into the functional capacity of CD8+ T cells, the production of IFN- γ , TNF- α , and IL-2 was measured. To determine the frequency of cytokine producing CD8+ T cells, PBMCs isolated from the CVID patients and healthy controls were stimulated with the A2/NLV peptide (for HCMV). The proportion of cytokine producing CD8+ T cells were enumerated by intracellular cytokine staining and FACS analysis and background levels of cytokine production measured in unstimulated PBMC which were then subtracted from the stimulated cells to yield the final frequencies. Figure 3-2 shows an example of representative FCAS plots of CD8+ T cells producing cytokines (IFN- γ , TNF- α , and IL-2). CD8+ T cells from CVID patients were characterized by a significant increase in the frequency of cells releasing IFN- γ (Mean \pm SD; CVID: mean frequency 1.6% \pm 0.29%, n = 25; healthy: mean frequency 0.49% \pm 0.14%, n = 14; P = 0.0006) and TNF- α (CVID: mean frequency 0.86% \pm 0.13%, n = 25; healthy: mean frequency 0.26% \pm 0.07%, n = 14; P = 0.002) as compared to healthy controls (Figure 3-3) However, IL-2 production by CD8+ T cells showed no significant difference between the two groups (CVID: mean frequency 0.24% \pm 0.05%, n = 25; healthy: mean frequency 0.11% \pm 0.03%, n = 14; P > 0.05) (Figure 3-3).

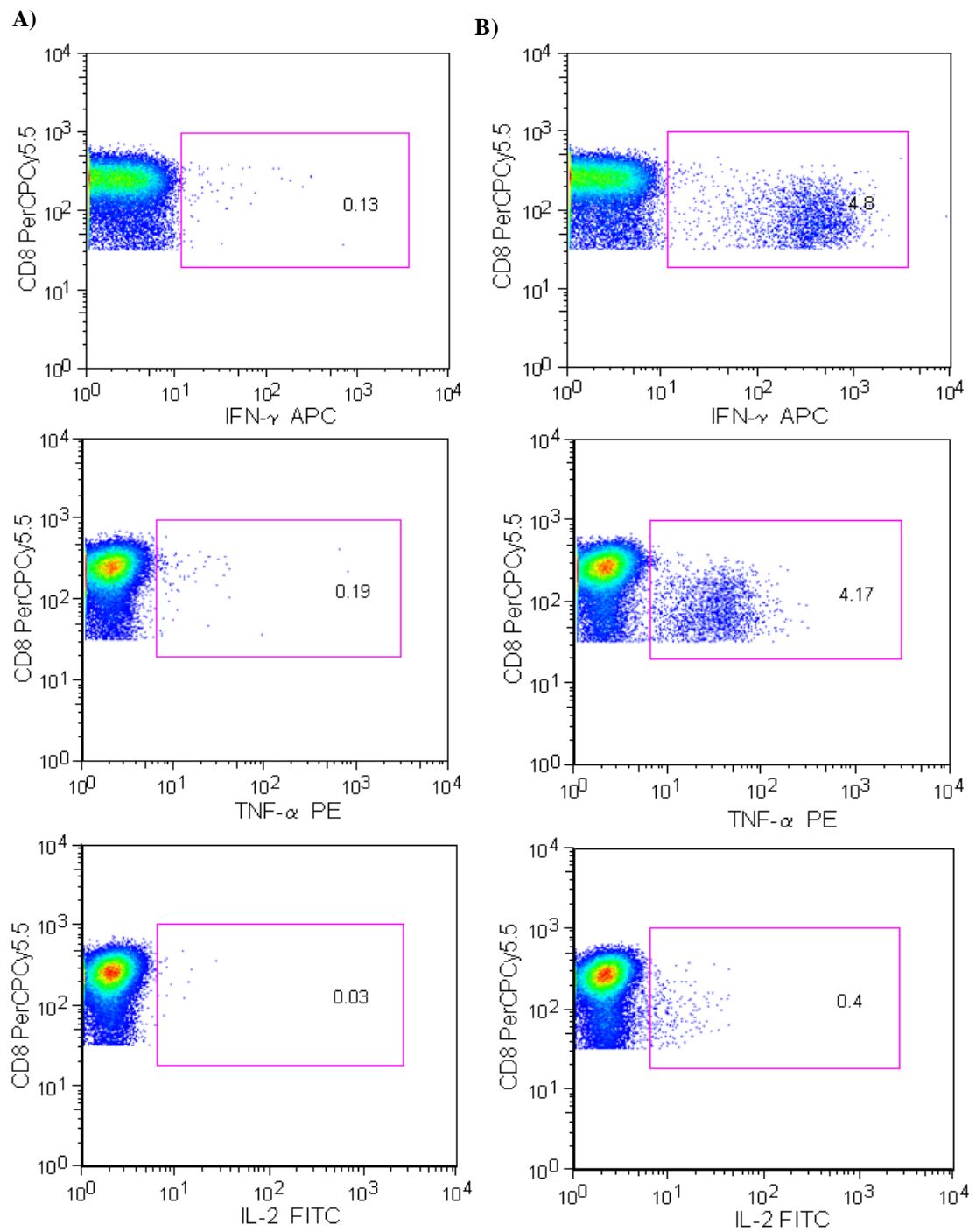


Figure 3-2: Representative FACS density plots of cytokines producing CD8+ T cells.

IFN- γ , TNF- α and IL-2 productions after overnight incubation are shown for unstimulated cells (A) and after Ag stimulation (B).

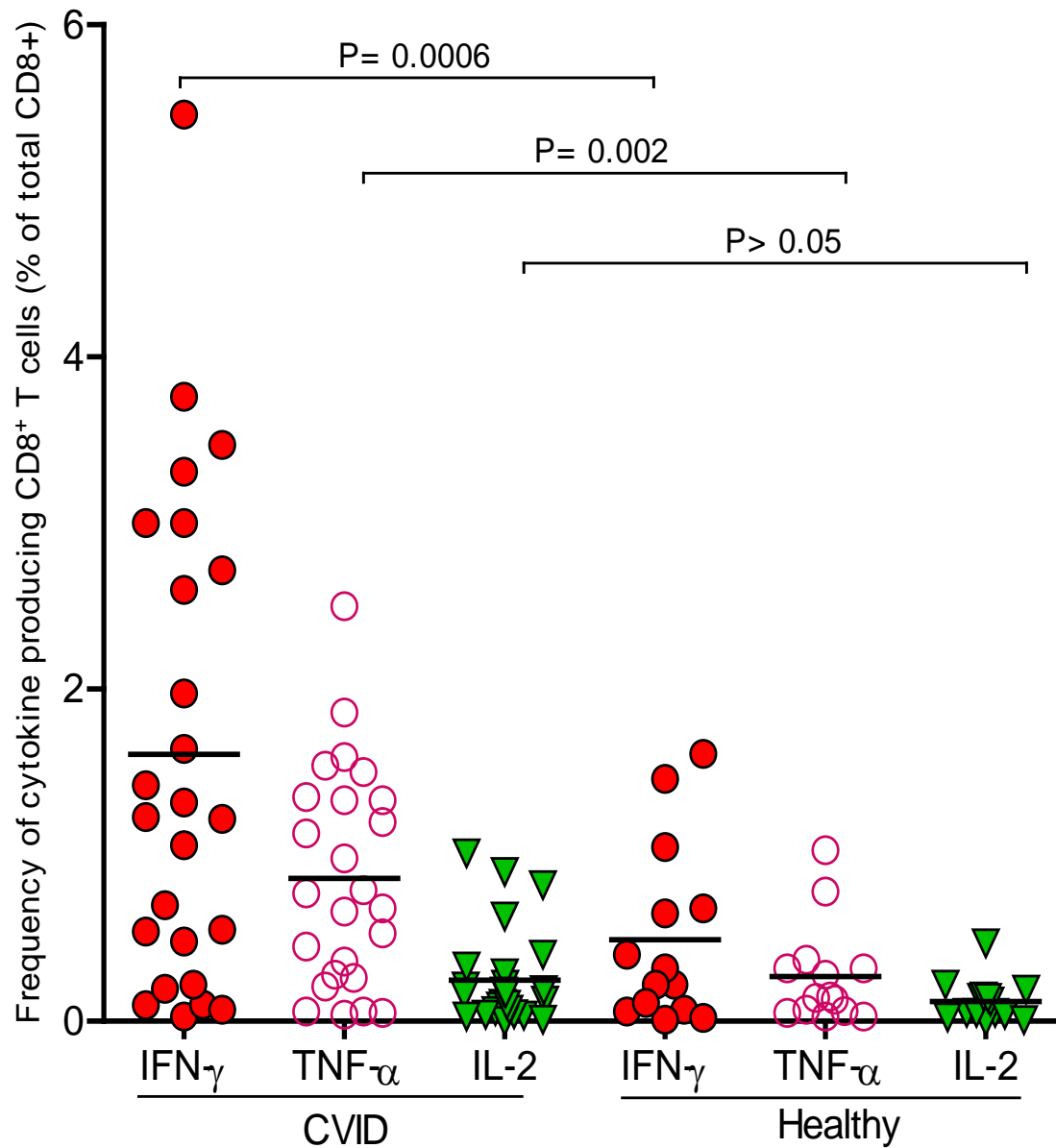


Figure 3-3: Cytokine production of CD8+ T cells in response to HCMV (NLV) peptide.

In CVID patients, CD8+ T cells produced significantly higher IFN- γ and TNF- α , but not IL-2, compared to healthy controls. Bars indicated mean values. Each point represents a single subject.

3.4 Cytokine production of EBV-specific CD8+ T cells in CVID patients and healthy controls

To determine whether the data obtained with HCMV CD8+ T cells was unique or reflected a more general phenomenon, the response to another herpesvirus, EBV, was investigated as an internal control. Although CVID manifests as an antibody deficiency, CD20+ EBV-associated lymphoma has been described in the context of CVID (Le et al., 2003) and patients still have B cells although they are compromised in antibody production, so validating the use of this virus as a control.

Intracellular cytokine staining to enumerate the frequency of CD8+ T cells producing IFN- γ , TNF- α , and IL-2 after stimulation with the EBV GLC peptide, showed a marked contrast to the results seen with HCMV NLV peptide stimulation. For EBV, no significant differences were observed between the frequencies of CD8+ T cells producing any of the three cytokines in CVID patients and healthy individuals (Mean \pm SD; CVID; IFN- γ : 0.31% \pm 0.08%; TNF- α : 0.29% \pm 0.08%; IL-2: 0.18% \pm 0.05%) (Healthy; IFN- γ : 0.44% \pm 0.17%; TNF- α : 0.41% \pm 0.1%; IL-2: 0.23% \pm 0.06%) ($P > 0.05$) (Figure 3-4). In CVID patients, there was significant differences in the percentage of cells producing IFN- γ and TNF- α on stimulation with HCMV peptide compared to EBV peptide stimulation (IFN- γ : mean frequency 1.6% \pm 0.29% vs. 0.31% \pm 0.08%; $P < 0.0001$) (TNF- α : 0.86% \pm 0.13% vs. 0.29% \pm 0.08%; $P = 0.0007$). However, no such difference was found in the frequency of IL-2 production in these patients (HCMV: 0.24% \pm 0.05%; EBV: 0.18% \pm 0.05%; $P > 0.05$) (Figure 3-5).

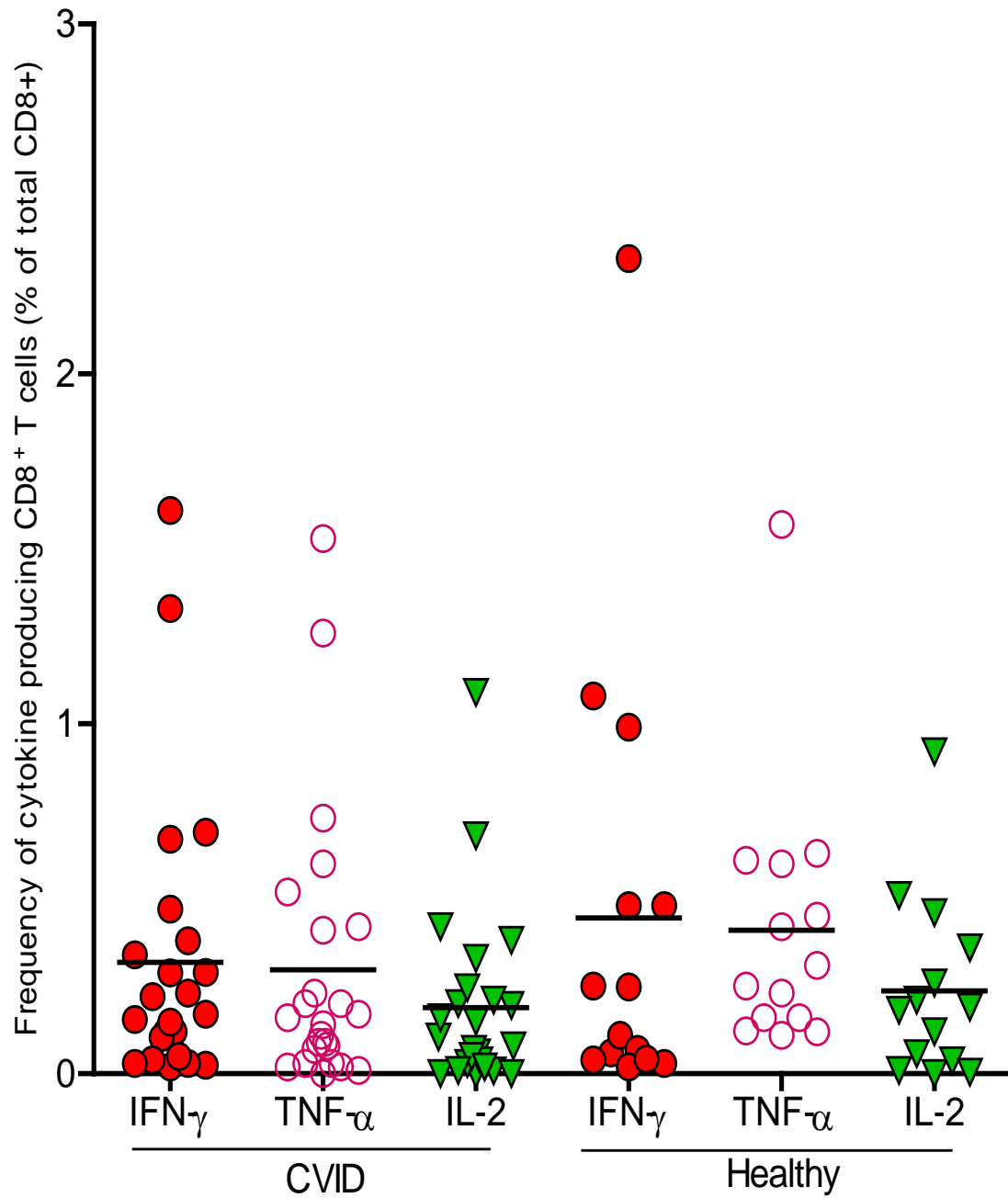


Figure 3-4: Cytokine production of CD8+ T cells in response to EBV (GLC) peptide.

Statistically there was no significant difference between CVID patients and healthy controls. Bars indicated mean values. Each point represents a single subject.

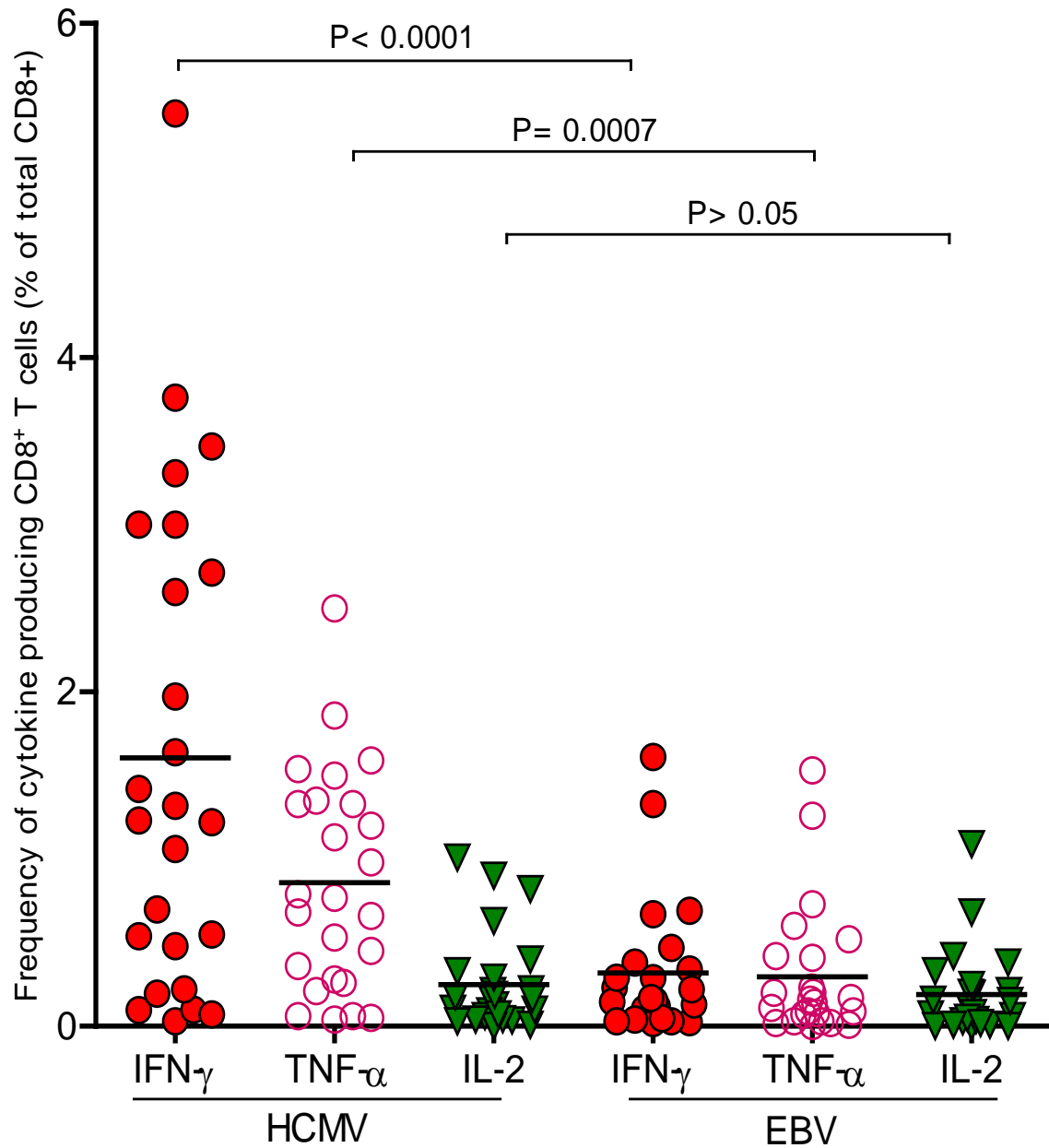


Figure 3-5: Cytokine production of CD8+ T cells of CVID patients in response to HCMV and EBV viral peptides.

CD8+ T cells produced significantly higher IFN-γ and TNF-α in response to HCMV pp65 derived epitope (NLV) compared to corresponding cells specific to EBV (GLC). Bars indicated mean values. Each point represents a single subject.

3.5 Differences in HCMV CD8+ T cell responses in CVID patients with and without inflammation

As a subset of the CVID patients was suffering from inflammatory lesions in different organs, they were subdivided into two groups: patients with (n = 9) and without (n = 16) inflammatory disease. Inclusion in the inflammatory disease group was determined by the treating clinicians, based on fulfillment of one or more of the following criteria: (i) hepatitis without evidence of hepatitis B or hepatitis C virus infection; (ii) splenomegaly; (iii) clinical evidence and a biopsy indicative of chronic enteropathy involving small and/or large bowel not explained by infection or gluten sensitivity; (iv) interstitial lung disease not explained by bacterial infection; (v) granulomatous disease.

The data described in 3.2-3.4 were then analyzed in the context of inflammatory diseases. CVID patients with inflammatory disease had a significantly higher frequency of HCMV NLV-specific CD8+ T cells compared to CVID patients without inflammation and healthy controls (Mean \pm SD; inflammatory: 4.81% \pm 0.77%, n = 9; non-inflammatory: 1.44% \pm 0.39%, n = 16; P= 0.0002) (4.81% \pm 0.77% inflammatory vs. 0.66% \pm 0.2% healthy, n = 14; P<0.0001) (Figure 3-6; panel A). Furthermore, comparison of the frequency of HCMV (NLV)-specific CD8+ T cells between the non-inflammatory CVID patients and healthy individuals showed no significant difference (non-inflammatory patients: 1.44% \pm 0.39%, n = 16; Healthy: 0.66% \pm 0.2%, n = 14; P> 0.05) (Figure 3-6; Panel A).

As shown in figure 3-6 panel B, the frequencies of EBV GLC-specific CD8+ T cells were similar in all groups (inflammatory: 0.2% \pm 0.07%; non-inflammatory: 0.17% \pm 0.06%; healthy: 0.32% \pm 0.1; P> 0.05).

Based on the striking difference observed in the frequency of HCMV NLV-specific CD8⁺ T cells in CVID patients with inflammatory diseases, the inflammatory disease profile was then superimposed on the cytokine expression data in 3.3. After NLV peptide stimulation, CVID patients with inflammatory disease had significantly higher frequencies of IFN- γ and TNF- α producing CD8⁺ T cells than the non-inflammatory group and healthy controls (IFN- γ : 3.04% \pm 0.4% inflammatory vs. 0.79% \pm 0.2% non-inflammatory and 0.49% \pm 0.1% healthy; $P < 0.0001$) (TNF- α : 1.46% \pm 0.17% inflammatory vs. 0.52% \pm 0.11% non-inflammatory and 0.26% \pm 0.07% healthy; $P < 0.0001$) (Figures 3-7 and 3-8; panel A). Frequencies of IFN- γ and TNF- α producing CD8⁺ T cells in the CVID patients without inflammatory disease were similar to those observed in healthy controls (IFN- γ : 0.79% \pm 0.2% non-inflammatory vs. 0.49% \pm 0.14% healthy; TNF- α : 0.52% \pm 0.11% non-inflammatory vs. 0.26% \pm 0.07% healthy; $P > 0.05$) (Figures 3-7A and 3-8A).

IL-2 producing CD8⁺ T cells showed no significant difference between the two groups of CVID patients ($P > 0.05$). Similarly, the difference between non-inflammatory patients and healthy controls was not statistically significant ($P > 0.05$). However, the difference in IL-2 production was significantly elevated in inflammatory patients compared to healthy controls ($P = 0.03$) (Figure 3-9A).

Figures 3-7B, 3-8B, and 3-9B illustrate the frequencies of cytokine-producing CD8⁺ T cells after EBV peptide stimulation in both CVID patients (with and without inflammation) and healthy controls. No significant differences were observed in the frequencies of IFN- γ (inflammatory: 0.37% \pm 0.15%; non-inflammatory: 0.29% \pm 0.09%; healthy: 0.44% \pm 0.17%) or TNF- α (inflammatory: 0.32% \pm 0.14%; non-

inflammatory: $0.28\% \pm 0.09\%$; healthy: $0.41\% \pm 0.1\%$) producing CD8+ T cells between any of the patient groups ($P > 0.05$) (Figures 3-7B and 3-8B, respectively) and frequencies of cells producing these cytokines were similar to healthy controls. IL-2 production also showed no significant difference between the two subgroups of CVID patients or the healthy control group ($P > 0.05$) (Figure 3-9B).

An obvious concern was that this elevated CD8+ T cell response observed in CVID patients with inflammation is specific to HCMV NLV peptide rather than a generalized phenomenon of CD8+ T cell response to HCMV. To address this, PBMCs from 6 inflammatory CVID and 8 non-inflammatory CVID patients were stimulated with a pool of peptides encompassing the entire pp65 ORF. Consistent with NLV peptide specific CD8+ T cell response, the frequency of both IFN- γ and TNF- α production, but not IL-2, was significantly elevated in inflammatory CVID patients compared to non-inflammatory patients in response to pp65 peptide pool stimulation (IFN- γ : $1.9\% \pm 0.3\%$ inflammatory vs. $0.7\% \pm 0.1\%$ non-inflammatory, $P = 0.005$; TNF- α : $1.34\% \pm 0.3\%$ inflammatory vs. $0.6\% \pm 0.1\%$ non-inflammatory, $P = 0.05$) (Figure 3-10), that indicates this enhanced response was not a feature of NLV peptide stimulation. It was still possible that this result was restricted to responses to pp65 peptide stimulation because NLV is part of pp65 pool peptide.

However, stimulation with another peptide pool encompassing the IE-1 ORF, (a non-structural protein of HCMV), still showed the same pattern indicating the generalized elevated CD8+ T cell response against HCMV in CVID patients with inflammation (IFN- γ : $2.2\% \pm 0.3\%$ inflammatory vs. $0.7\% \pm 0.2\%$ non-inflammatory, $P = 0.004$; TNF- α : $1.5\% \pm 0.4\%$ inflammatory vs. $0.6\% \pm 0.2\%$ non-inflammatory, $P = 0.05$) (Figure 3-

11). Consistent with previous data, the frequency of IL-2 production, no significant difference was found between two subsets of patients as shown in Figure 3-12.

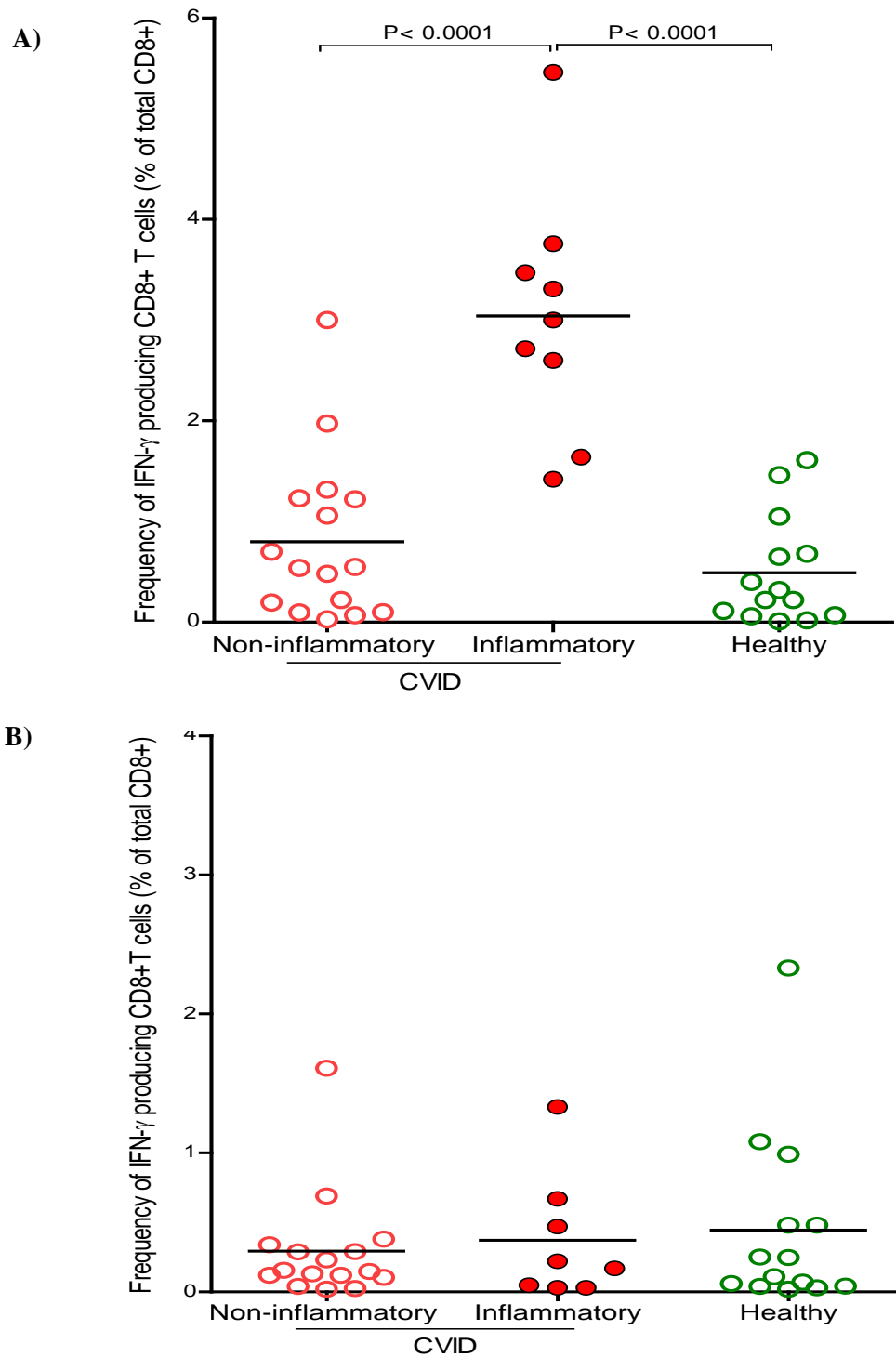


Figure 3-7: Frequency of IFN- γ producing CD8+ T cells in response to HCMV and EBV specific peptides.

In response to NLV peptide, inflammatory CVID patients produced significantly higher IFN- γ compared to non-inflammatory patients as well as healthy controls (A). No difference was found between groups in response to EBV (B). Bars indicated mean values. Each point represents a single subject.

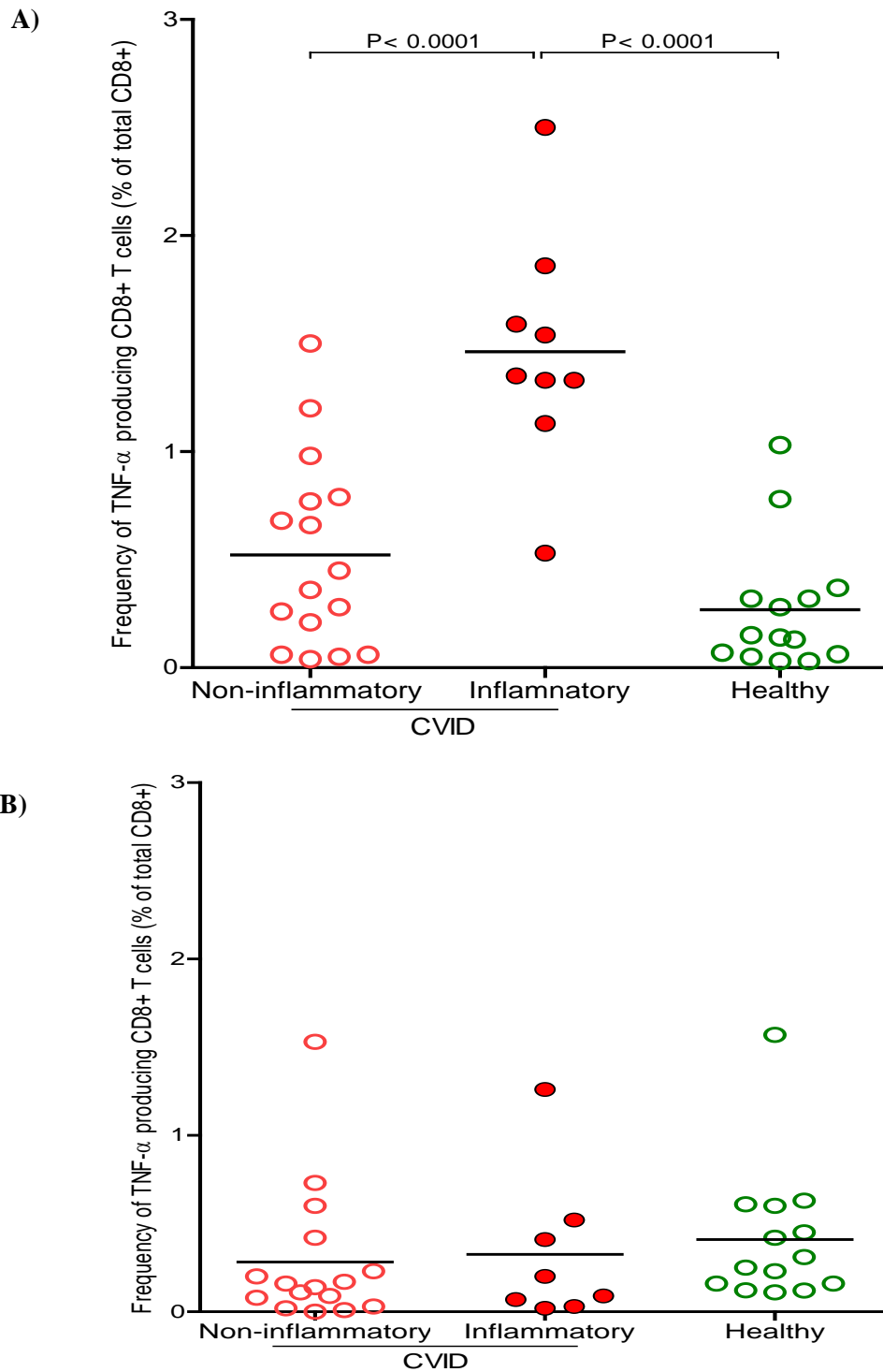


Figure 3-8: Frequency of TNF- α producing CD8+ T cells in response to HCMV (NLV) and EBV (GLC) peptides.

In response to NLV peptide, inflammatory CVID patients produced significantly higher TNF- α compared to non-inflammatory patients as well as healthy controls (A). No difference was found between groups in response to EBV (B). Bars indicated mean values. Each point represents a single subject.

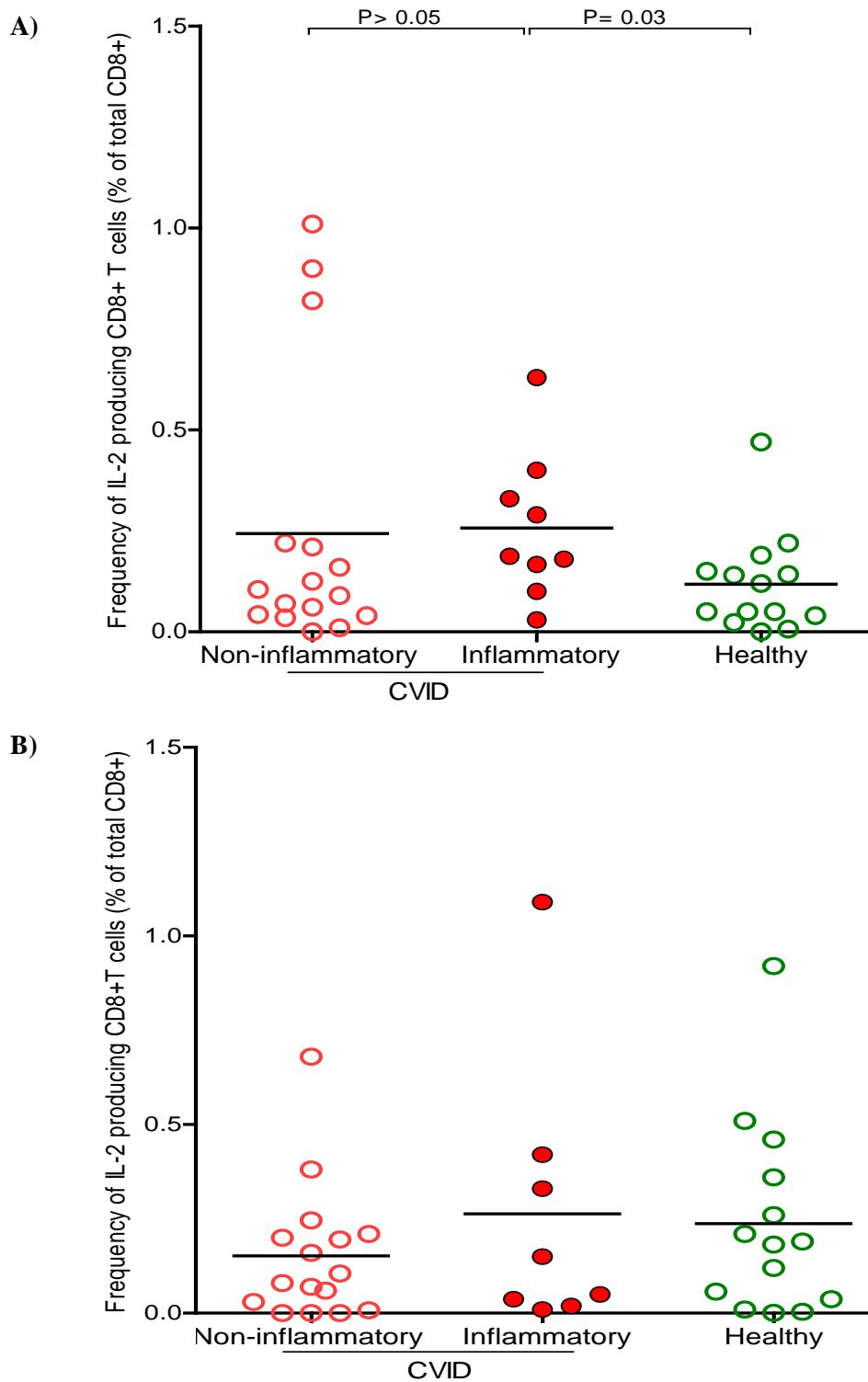


Figure 3-9: Frequency of IL-2 producing CD8+ T cells in response to HCMV (NLV) and EBV (GLC) peptides.

No significant difference was found between patient groups as well as healthy controls in response to (A) NLV or (B) GLC peptides. Bars indicated mean values. Each point represents a single subject.

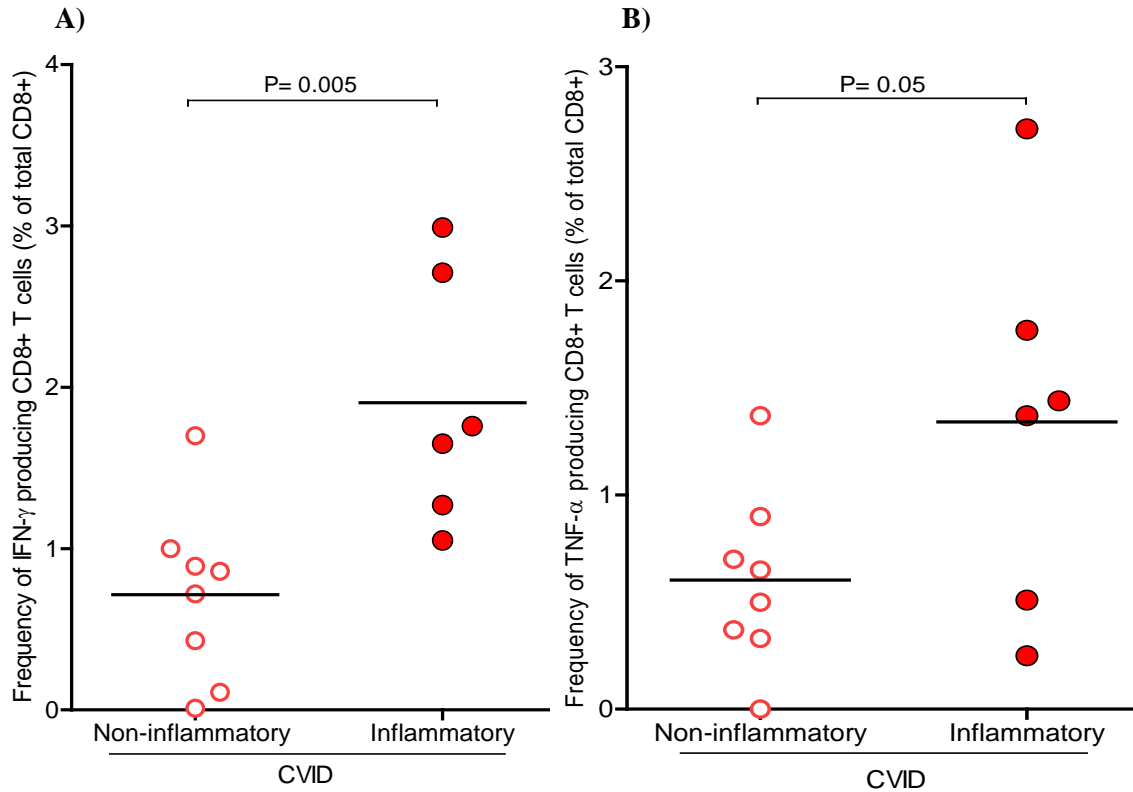


Figure 3-10: Frequency of cytokine-producing CD8+ T cells in response to pp65 pool peptide of HCMV.

Frequency of (A) IFN- γ and (B) TNF- α producing CD8+ T cells in patients with and without inflammation. CVID patients with inflammation produced significantly higher IFN- γ and TNF- α compared to non-inflammatory patients. Bars indicated mean values. Each point represents a single subject.

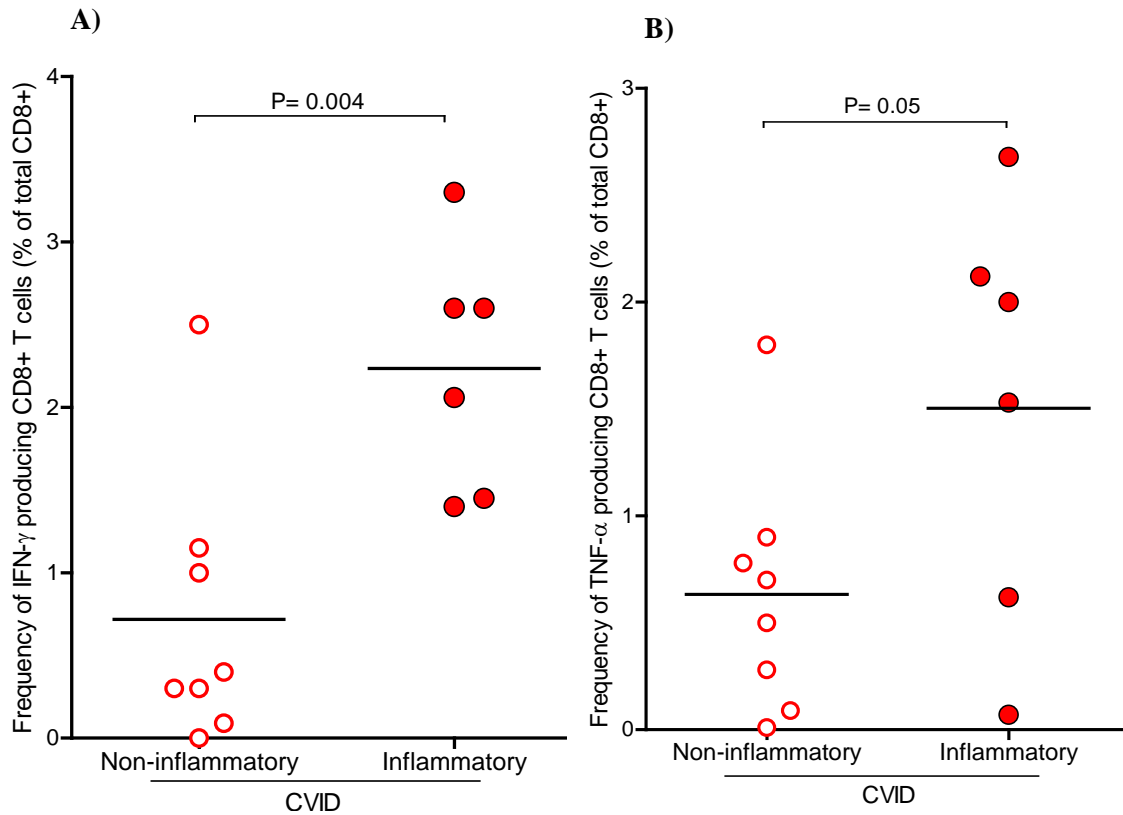


Figure 3-11: Frequency of cytokine-producing CD8+ T cells in response to IE-1 pool peptide of HCMV.

Frequency of (A) IFN- γ and (B) TNF- α producing CD8+ T cells in patients with and without inflammation. CVID patients with inflammation produced significantly higher IFN- γ and TNF- α compared to non-inflammatory patients. Bars indicated mean values. Each point represents a single subject.

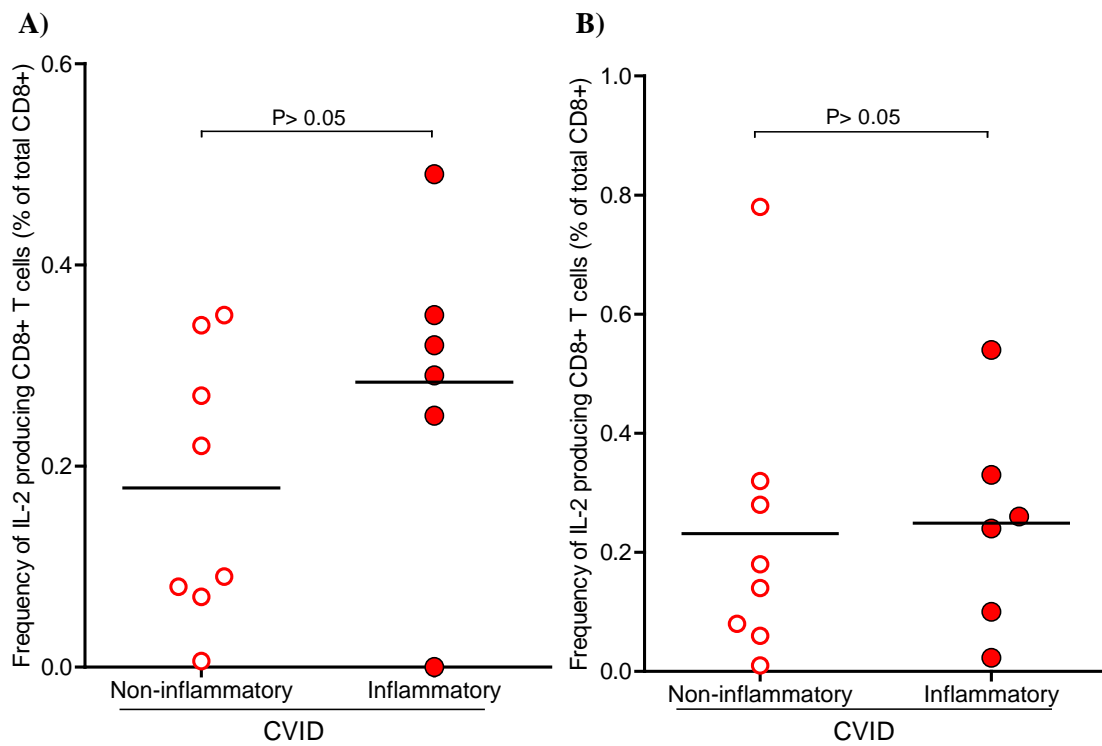


Figure 3-12: Frequency of IL-2 producing CD8+ T cells in response to pp65 and IE-1 pool peptides of HCMV.

No significant difference was found between CVID patients with and without inflammation in the frequency of IL-2 producing CD8+ T cells in response to (A) pp65 and (B) IE-1 pool peptides. Bars indicated mean values. Each point represents a single subject.

3.6 Discussion

In this chapter, a distinct functional immune signature of HCMV NLV-specific CD8+ T cells was found in the context of CVID patients with inflammation. HCMV-specific CD8+ T cells were highly elevated in CVID patients compared to healthy controls. However, when CVID patients were sub-divided according to the presence of inflammatory diseases, the elevated HCMV-specific CD8+ T cells predominantly segregated with the patients with inflammatory disease whereas the frequency of these cells in non-inflammatory CVID patients was comparable to healthy controls. This elevation was specific to HCMV because EBV GLC-specific CD8+ T cell frequencies were similar in these two groups of patients and healthy controls.

HCMV-specific CD8+ T cells were found to be functionally competent as measured by the pentamer reagent and by secretion of three cytokines after peptide stimulation. Interestingly, CD8+ T cells of inflammatory CVID patients were produced significantly higher pro-inflammatory cytokines (IFN- γ and TNF- α), but not IL-2, in response to HCMV peptide stimulation. This elevation was not seen in either non-inflammatory CVID patients or healthy controls. Similarly, no differences were observed in the cytokine expression profiles for EBV-specific CD8+ T cells after peptide stimulation between CVID patients with and without inflammatory disease or when compared to healthy controls.

HCMV immune responses in CVID patients are aberrant compared to normal individuals, but this is not universal and may implicate HCMV as an important pathogenic factor. However, these data contrast with what has been shown in renal

transplant recipients where risk of high level HCMV replication and disease after transplantation is associated with a HCMV pentamer+ CD8+ T cell population with a reduced ability to produce IFN- γ after peptide stimulation (Crough et al., 2007; Mattes et al., 2008).

In elderly persons, a high type 1 cytokine production such as IFN- γ coupled with low levels of type 2 cytokine production such as IL-2 can change the cytokine microenvironment in lymphatic tissues and trigger ubiquitous inflammatory processes (Almanzar et al., 2005). Moreover, it appears pro-inflammatory cytokines play an important role in facilitating the functional pathology and disease course of some age-related disorders such as atherosclerosis (Almanzar et al., 2005). Therefore, it is tempting to speculate that in a subset of CVID patients destined to suffer inflammatory disease the unknown underlying genetic lesions result in an aberrant HCMV-specific T cell phenotype that is much more pro-inflammatory than those normally produced leading to either the formation of the inflammation or exacerbation of an existing inflammatory condition. Consistent with this observation, recent data suggests a role for pro-inflammatory cytokines including IFN- γ and TNF- α in the pathogenesis of inflammatory bowel diseases as well as autoimmune arthritis (Neurath and Finotto, 2006a; Kim and Moudgil, 2008a). Additionally, treatment of CVID patients with the anti-TNF antibody Infliximab has been shown to reduce the inflammatory symptoms (Chua et al., 2007b).

Taken together, the work in this chapter suggest that the pronounced response to HCMV could be a homeostatic T cell expansion that attempts to compensate for the failure or lack of endogenous specific antibodies in these patients. Data from mouse models have

shown that in the absence of the T cell subsets (either CD4+ or CD8+), cell loss can be compensated by the remaining cellular subset (Freitas and Rocha, 2000). Indeed, mice which lack CD4 T cells, and therefore have a marked decrease in helper cell activity for antibody response, have been shown to develop normal functional CD8+ T cells with cytotoxic activity against viruses (Rahemtulla et al., 1991).

Chapter 4

4 Polyfunctional analysis of antigen-specific CD8+ T cell responses in patients with CVID

4.1 Introduction

Antiviral CD8+ T cell responses play a critical role in the control of many viral infections. Production of cytokines such as IFN- γ , TNF- α , and IL-2 are important markers of anti-viral T cell function. The magnitude of a T-cell response is generally represented as the expression of a specific effector function, such as cytokine production or the frequency of antigen-specific T cells. Nevertheless, the magnitude of a T-cell response as expressed by a single parameter such as IFN- γ production probably underestimates the full functional potential of the response. In this regard, multiple functions can be expressed in complex combinations that collectively defined as the quality of the T-cell response. This can be expressed on an individual T cell basis or for a population of antigen-specific T cells (Perfetto et al., 2004;Seder et al., 2008).

There are several lines of evidence indicating that multi-cytokine producer T cells are important in control of viral infections (Bogdan et al., 1990;Lichterfeld et al., 2004;Nebbia et al., 2008). For example, Betts et al have shown a strong association between the maintenance of polyfunctional HIV-specific CD8+ T cells and virus control (Betts et al., 2006), indicating that the quality of antigen-specific CD8+ T cells is a clinical correlate of protection from disease progression.

This chapter aimed to evaluate the cytokine co-expression profiles of HCMV and EBV-specific CD8+ T cells from CVID patients (with and without inflammation) and healthy controls for the expression of the three cytokines (IFN- γ , TNF- α , and IL-2) to better understand the functional quality of antigen-specific CD8+ T cells in different settings particularly in the context of inflammatory disease. For these purpose, lymphocytes were

gated based on forward scattering (FSC) and side scattering (SSC), and CD8+ T cells were gated and analyzed for expression of IFN- γ , TNF- α , and IL-2 cytokines. Cytokine co-expression profiles were then determined using the Boolean gating function of Flow Jo software to quantify the magnitude of expression profiles corresponding to the seven different subsets consisting of triple producers (TP), double producers (DP), and single producers (SP) based on their expression of IFN- γ , TNF- α , and IL-2 (see Chapter 2, section 2.3).

Boolean gating is a flow cytometric data analysis technique and based on this technique cells are divided into all possible combinations of the functions. These functions are measured via the Boolean operations 'and' and 'not' on analysis gates which is applied to those measurements (Seder et al., 2008).

After subtracting the background values (from unstimulated cells), the proportion of each of the seven subsets was expressed as a percentage of total cytokine-positive cells. These included: single producer state (only producing one cytokine such as IFN- γ or IL-2), double producer state (expression of two cytokines), and triple producer state which measures expression of three cytokine including IFN- γ , IL-2, and TNF- α leading seven functional states or subsets.

Additionally, in order to get a better reflection of the total functional response for a population of cytokine producing cells, all single cytokine expression profiles were also analyzed using integrated median fluorescent intensities (iMFI), a metric term incorporating both the magnitude and quality of a response that can be derived by multiplying the frequency by the MFI (Darrah et al., 2007). Moreover, by assessing all combination of three cytokines at the single cell level it should be possible to define the

quality of T cell cytokine response. The relative amount of an individual cytokine produced per cell can be measured for each functional population using the MFI which is related to the quantitative expression of each functional parameter on a per cell basis (Precopio et al., 2007).

4.2 Frequencies of cytokine positive cells specific for HCMV and EBV in CVID patients

The pie charts in Figure 4-1 show that the proportions of CD8+ T cells producing triple, double, or single cytokines were different for CVID patients compared to healthy individuals. In the healthy group, 65% of CD8+ T cells specific for HCMV produced more than one cytokine. Of the total response, 2% consisted of triple producers, 63% of double producers, and 35% of single producers. In this healthy group, a similar cytokine expression pattern was found for EBV-specific CD8+ T cells (3% TP, 56% DP, and 41% SP).

However, the patterns of cytokine expression and the relative proportion of Ag-specific CD8+ T cells positive for the three cytokines were different between the inflammatory (n = 9) and non-inflammatory CVID patients (n = 16) and with the healthy group (n = 14). The proportion of HCMV specific CD8+ T cells producing three cytokines in CVID patients were in the same range (2% for inflammatory and 4% for non-inflammatory) of healthy controls (2%). The proportion of triple cytokine producers in response to EBV peptide stimulation was found to be slightly higher in CVID patients with (5%) and without inflammation (9%) compared to healthy controls (3%) although these differences did not reach statistical significance ($P > 0.05$). In CVID patients the proportion of CD8+ T cells producing three cytokines in response to EBV was slightly higher compared to those detected after HCMV peptide stimulation, but again this did not reach statistical significance ($P > 0.05$).

Of the total CD8+ T cell response specific to HCMV, the proportion of double cytokine producers decreased in CVID patients with and without inflammation compared to

healthy group (DP: 63% healthy vs. 48% non-inflammatory vs. 34% inflammatory). Interestingly, when the proportion of single cytokine producers in response to HCMV was compared between different clinical settings, the CD8+ T cells producing only one cytokine constituted a large proportion of the specific response in inflammatory CVID patients (64%) compared to non-inflammatory CVID patients (48%) and the healthy group (35%).

In the context of EBV responses, although the proportion of single cytokine producers was higher in CVID patients with (55%) and without inflammation (63%) compared to the healthy group (41%), the pattern of this individual cytokines was different in CVID patients when compared in response to HCMV.

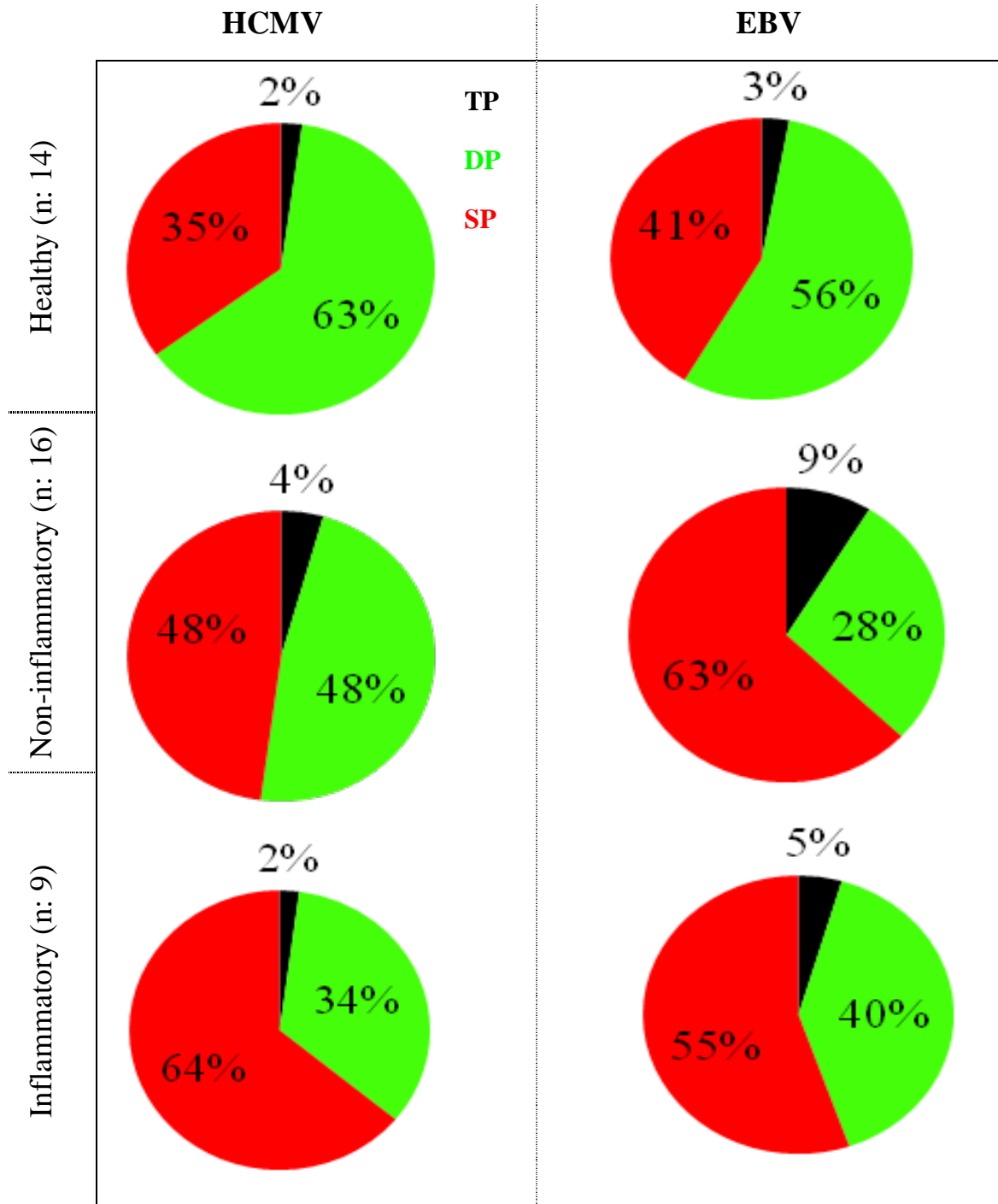


Figure 4-1: The quality of CD8+ T cell response specific to HCMV and EBV.

Pie charts represent the fraction of total responses in each group (Healthy, non-inflammatory, and inflammatory CVID patients). The responses comprise of cells expressing all three cytokines (IFN- γ , TNF- α , and IL-2), any two cytokines, or any one cytokine. **TP:** Triple cytokine producer (shown as black); **DP:** Double cytokine producer (shown as green); **SP:** Single cytokine producer (shown as red).

4.3 Cytokine co-expression pattern of CD8+ T cell responses specific to HCMV and EBV

The co-expression of IFN- γ , TNF- α , and IL-2 production by HCMV and EBV specific CD8+ T cells was then investigated to produce a polyfunctional CD8+ T cell signature in these patients.

In CVID patients with inflammatory disease, the CD8+ T cell responses to HCMV (NLV) peptide stimulation was dominated by IFN- γ producing cells, either as single producers (I) or as IFN- γ /TNF- α double producers (IT) as shown in Figure 4-2. These two subsets comprise 79.8 percent of the total population of HCMV-specific CD8+ T cells. In contrast, the frequency distribution in the non-inflammatory CVID patients was essentially the same as that seen after HCMV stimulation in healthy controls.

In healthy individuals, no significant differences were seen in the distribution of the seven subsets after HCMV stimulation compared to EBV stimulation (Figure 4-3). In contrast to the HCMV data, the response to the EBV peptide stimulation also showed no significant differences between the two CVID patient groups (inflammatory vs. non-inflammatory) (Figure 4-4). However, when compared to healthy individuals, both CVID patient groups showed significantly lower frequencies of IFN- γ single producers after EBV peptide stimulation. All other subsets were similar following stimulation with the EBV peptide.

For a more refined assessment of the differences in cytokine expression an integrated median fluorescent intensity (iMFI) analysis was performed which incorporates the frequency and MFI providing an estimate of the total amount of cytokine in the system.

Figures 4-5 and 4-6 show a worked example of iMFI calculation. Only single cytokine producing CD8+ T cell subsets were included for the purpose of iMFI analysis (Figure 4-7). The predominance of single-producing IFN- γ HCMV NLV specific CD8+ T cells in CVID patients with inflammatory disease was reflected in the iMFI analysis, emphasizing the prominence of IFN- γ in this response (Figure 4-7; panel A). Interestingly, in the case of EBV the iMFI profile of IFN- γ producing CD8+ T cells was much lower than the same responding cells in both non-inflammatory CVID patients and healthy controls (Figures 4-7; panel B).

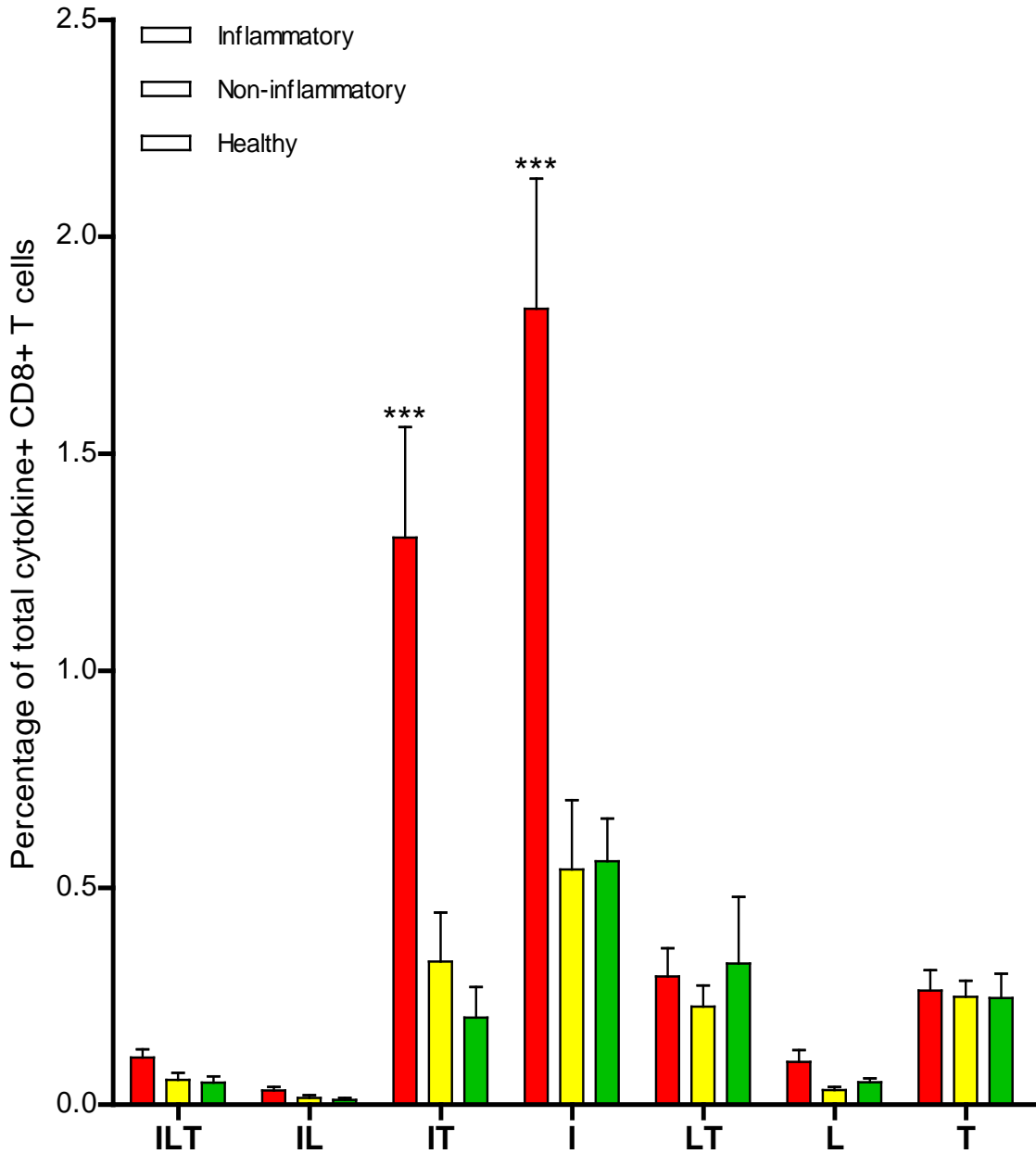


Figure 4-2: Cytokine co-expression profile of HCMV (NLV) specific CD8+ T cells.

The quality of response and cytokine subsets (as a percentage of total cytokine positive CD8+ T cells) in inflammatory CVID patients (red; n = 9) compared to non-inflammatory patients (yellow; n = 16) and healthy controls (green; n = 14). In response to HCMV (GLC) peptide stimulation, CD8+ T cells of inflammatory CVID patients produced significantly higher IFN- γ (as a single producers or co-producer with TNF- α) compared to non-inflammatory CVID patients and healthy controls. Data were analyzed using 2way ANOVA. Asterisks (***) indicate $P < 0.0001$. **ILT**: IFN- γ , TNF- α , and IL-2; **IL**: IFN- γ and IL-2; **IT**: IFN- γ and TNF- α ; **I**: IFN- γ ; **LT**: IL-2 and TNF- α ; **L**: IL-2; **T**: TNF- α .

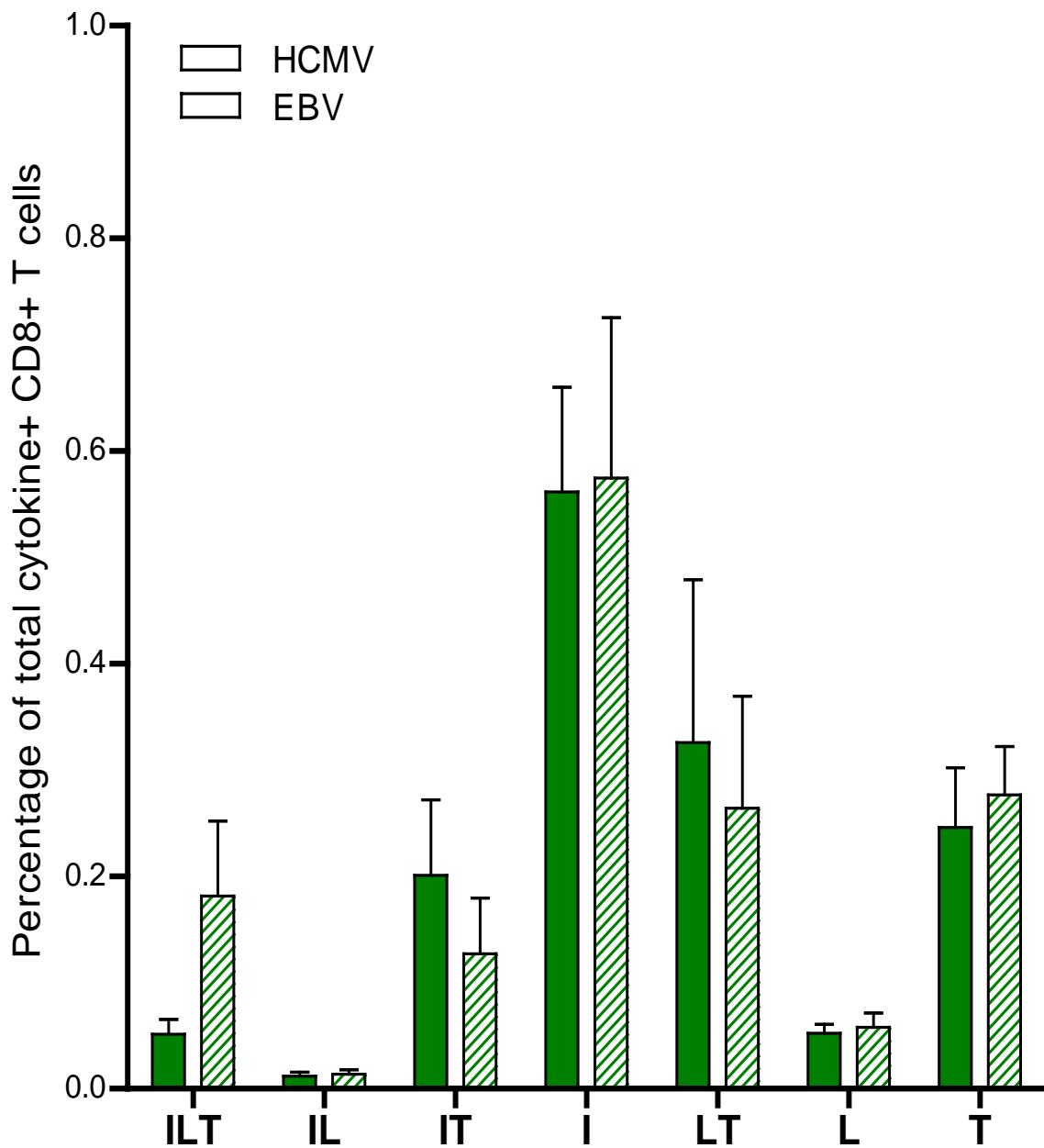


Figure 4-3: Cytokine co-expression profile of antigen-specific CD8+ T cells in healthy controls (n = 14).

The quality of response (as a percentage of total cytokine positive CD8+ T cells) and cytokine subsets of CD8+ T cells in response to HCMV (NLV) and EBV (GLC) peptide stimulation. Data were analyzed using 2way ANOVA. **ILT:** IFN- γ , TNF- α , and IL-2; **IL:** IFN- γ and IL-2; **IT:** IFN- γ and TNF- α ; **I:** IFN- γ ; **LT:** IL-2 and TNF- α ; **L:** IL-2; **T:** TNF- α .

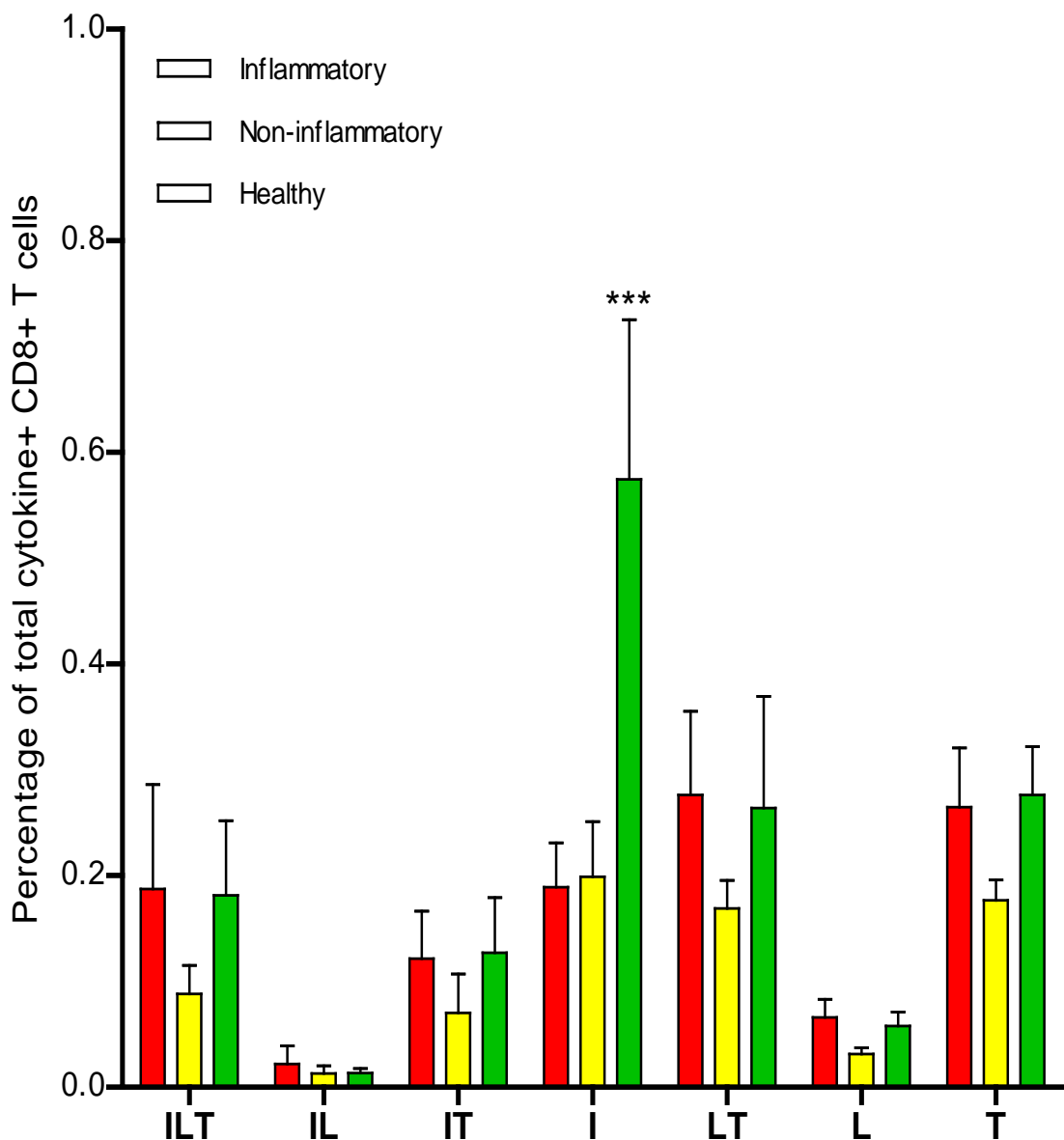


Figure 4-4: Cytokine co-expression profile of EBV-specific CD8+ T cells.

The quality of response (as a percentage of total cytokine positive CD8+ T cells) and cytokine subsets of CD8+ T cells in inflammatory CVID patients (red; n = 9) compared to non-inflammatory patients (yellow; n = 16) and healthy controls (green; n = 14). In response to EBV (GLC) peptide stimulation, CD8+ T cells of healthy controls produced significantly higher IFN- γ (as a single producers or I subset) compared to CVID patients (with and without inflammation). Data were analyzed using 2way ANOVA. Asterisks (***) indicate $P < 0.0001$. **ILT:** IFN- γ , TNF- α , and IL-2; **IL:** IFN- γ and IL-2; **IT:** IFN- γ and TNF- α ; **I:** IFN- γ ; **LT:** IL-2 and TNF- α ; **L:** IL-2; **T:** TNF- α .

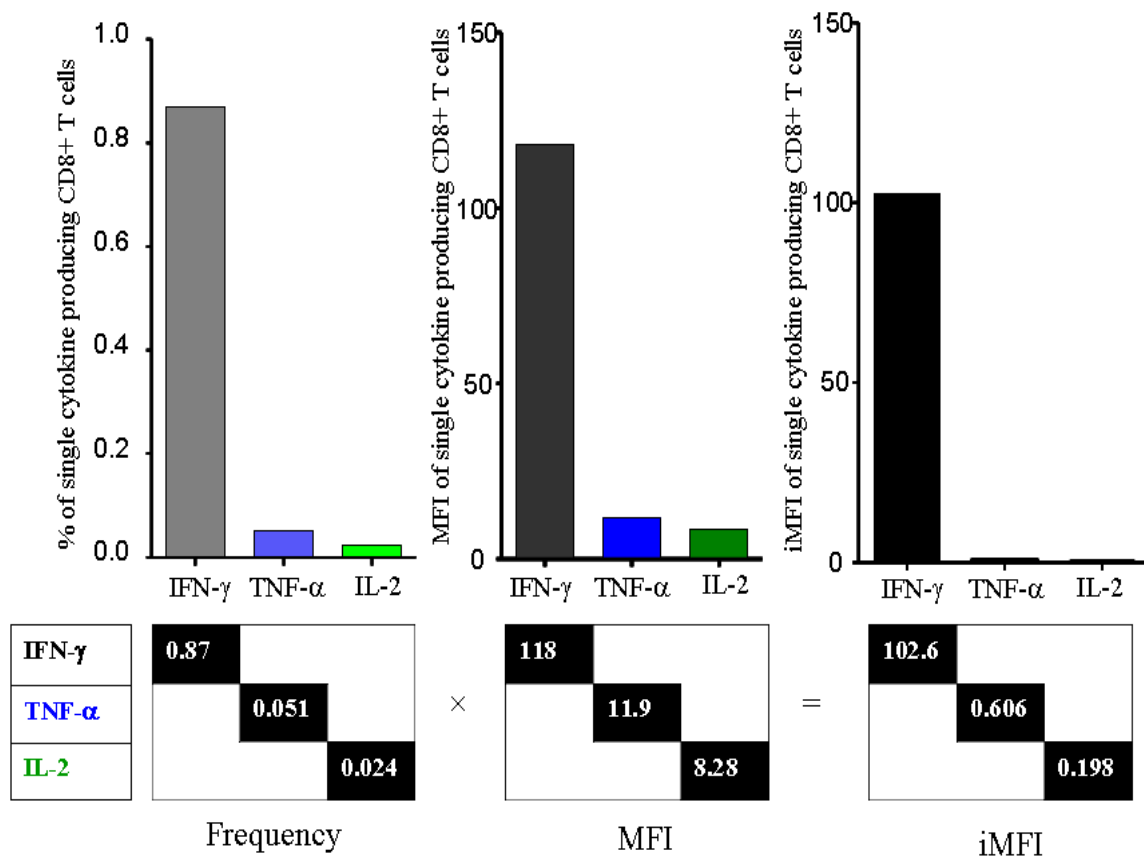


Figure 4-5: A worked example of integrated median florescent intensities (iMFI), which is the product of the frequency of a subset and its MFI.

Bar charts are showing the total frequency (left panel), MFI (middle panel) and iMFI (right panel) of single cytokine producing CD8+ T cells, respectively. iMFI values were calculated by multiplying the frequency by MFI as shown in tables.

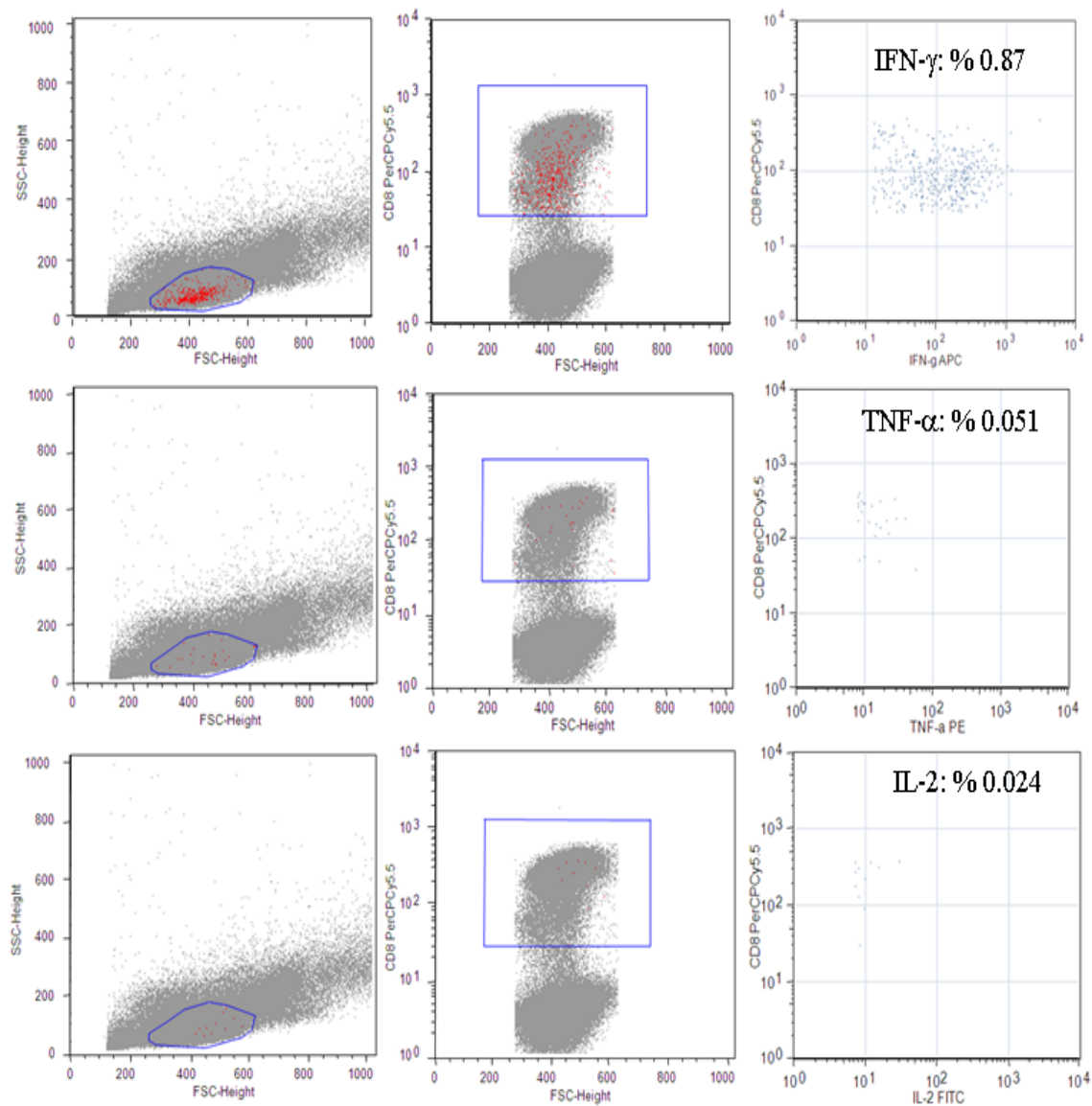


Figure 4-6: Representative FACS plots showing an example of the frequency of single cytokine producing CD8+ T cell subsets in response to HCMV (NLV) peptide stimulation.

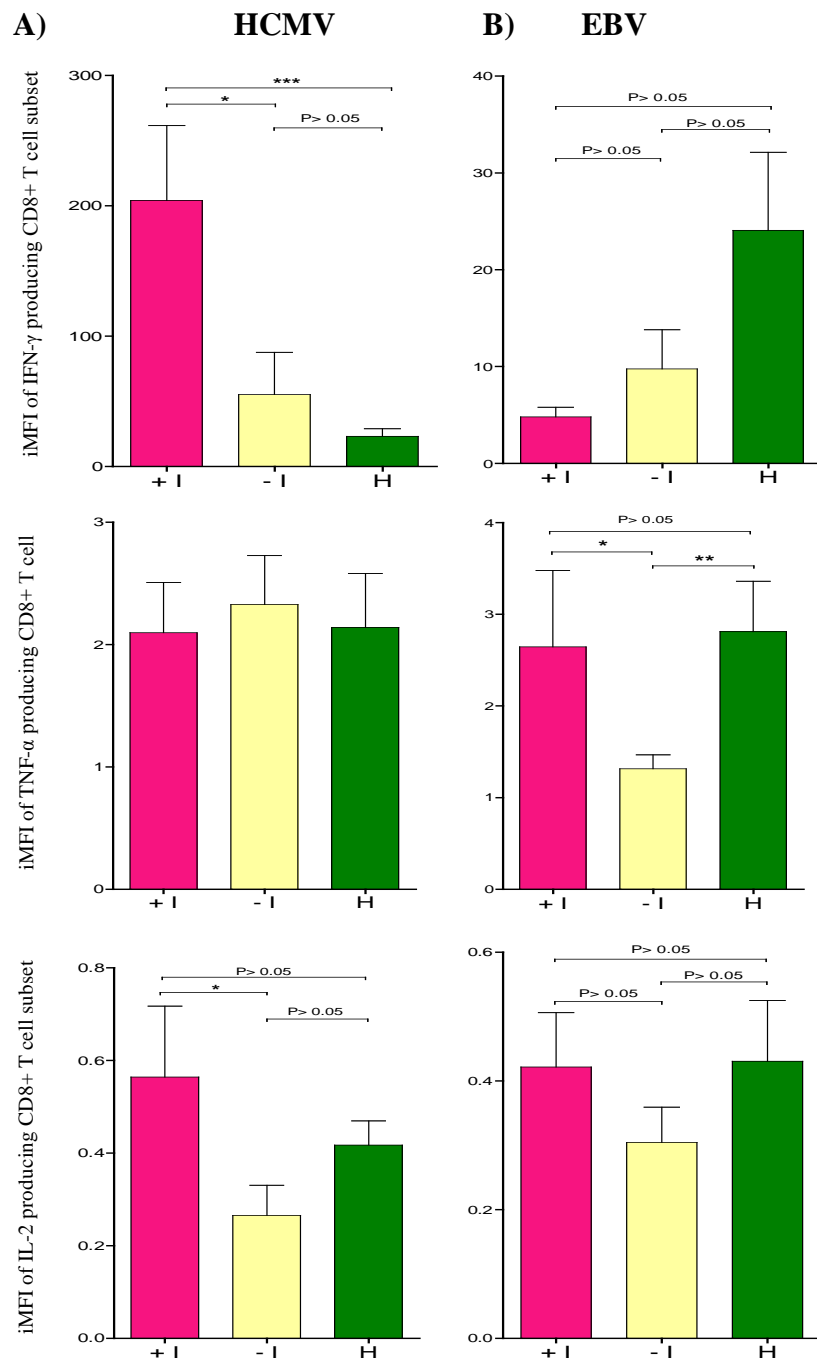


Figure 4-7: integrated median florescent intensities (iMFI) of single cytokine expression profile of HCMV and EBV-specific CD8+ T cells (A and B panels, respectively).

The quality of response and single cytokine subsets of CD8+ T cells in CVID patients with inflammation (+I; red bars; n = 9) compared to non-inflammatory patients (-I; yellow bars; n = 16) and healthy controls (H; green bars; n = 14). In response to HCMV, CD8+ T cells of inflammatory patients produced significantly higher IFN- γ (as a single producer) compared to non-inflammatory or healthy controls. Data were analyzed using unpaired t test. Asterisks (***) and (*) indicate $P < 0.0001$ and $P < 0.05$, respectively.

4.4 Level of cytokine production per cell in different CD8+ T cell subsets

The seven functional subsets of CD8+ T cells from CVID patients (with and without inflammation) and healthy controls were analyzed to investigate whether the amount of cytokine expressed per cell differed after HCMV or EBV stimulation. This was determined based on the median fluorescence intensity for each cytokine per subset.

In most cases, the triple producers expressed higher amounts of cytokine per cell than the corresponding double and single producers after stimulation with HCMV ($P < 0.05$). Although no significant differences were seen between double and single producers in any of the groups (inflammatory, non-inflammatory or healthy), there was a trend toward higher MFIs amongst the double producers (Figure 4-8).

In contrast to HCMV peptide stimulation, the MFI of IFN- γ production by triple cytokine producers of EBV-specific CD8+ T cells found to be much lower in both inflammatory CVID patients and healthy individuals compared to non-inflammatory CVID patients. In addition, these triple cytokine producers also had a lower MFI for IFN- γ production compared to the MFI of the double producers. With this exception, MFI patterns after EBV stimulation are similar to those observed after HCMV stimulation (Figure 4-9).

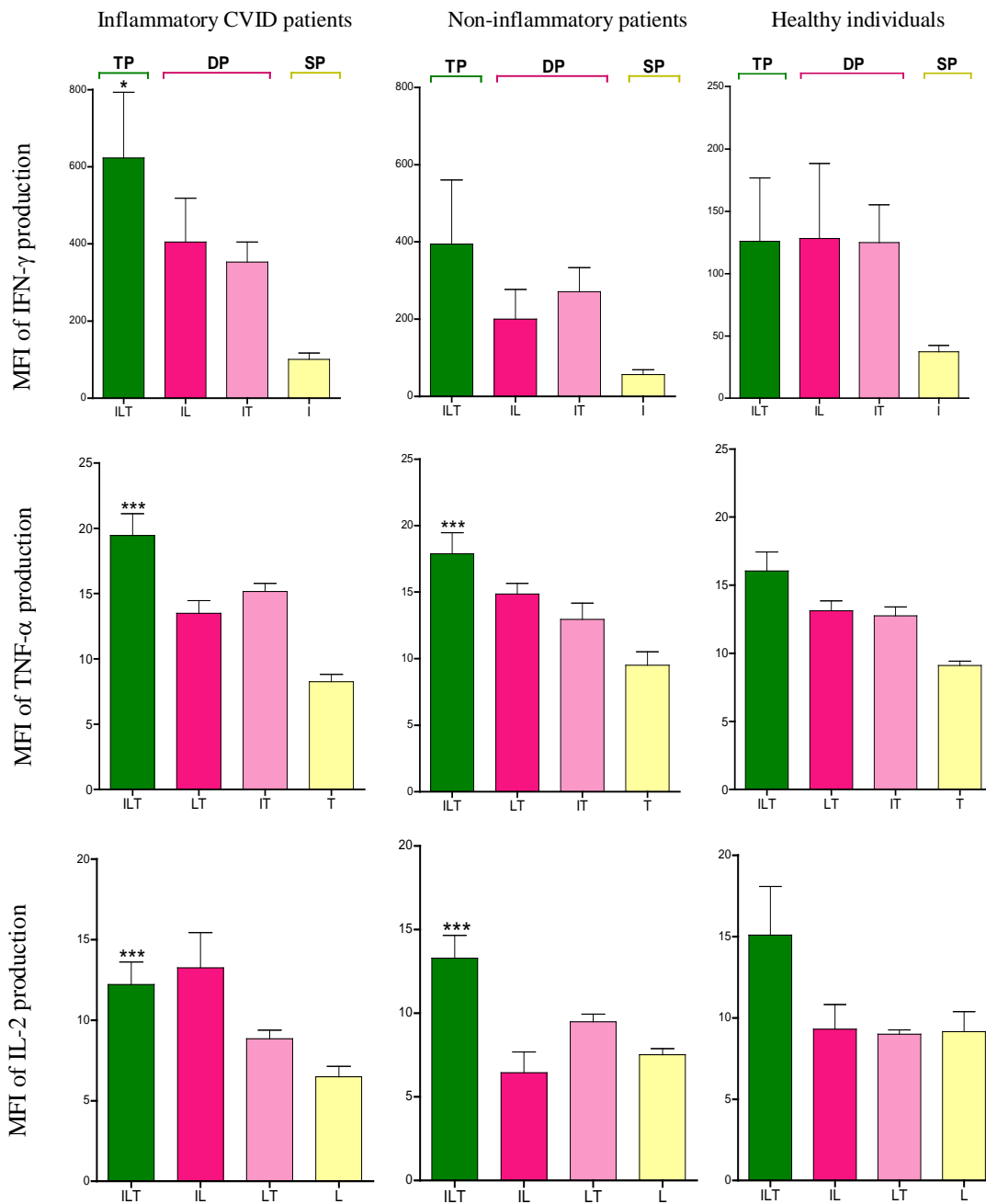


Figure 4-8: Median fluorescent Intensities (MFI) of HCMV (NLV)-specific CD8+ T cells.

MFI of HCMV-specific CD8+ T cell triple producers (TP), double producers (DP), and single producers (SP) in inflammatory CVID patients (n = 9), non-inflammatory patients (n = 16) and healthy controls (n = 14). A paired t test was used for comparing cytokine MFI of triple producers and single producers. Asterisks (***) and (*) represents $P < 0.0001$ and $P < 0.05$, respectively. **ILT**: IFN- γ , TNF- α , and IL-2; **IL**: IFN- γ and IL-2; **IT**: IFN- γ and TNF- α ; **I**: IFN- γ ; **LT**: IL-2 and TNF- α ; **L**: IL-2; **T**: TNF- α .

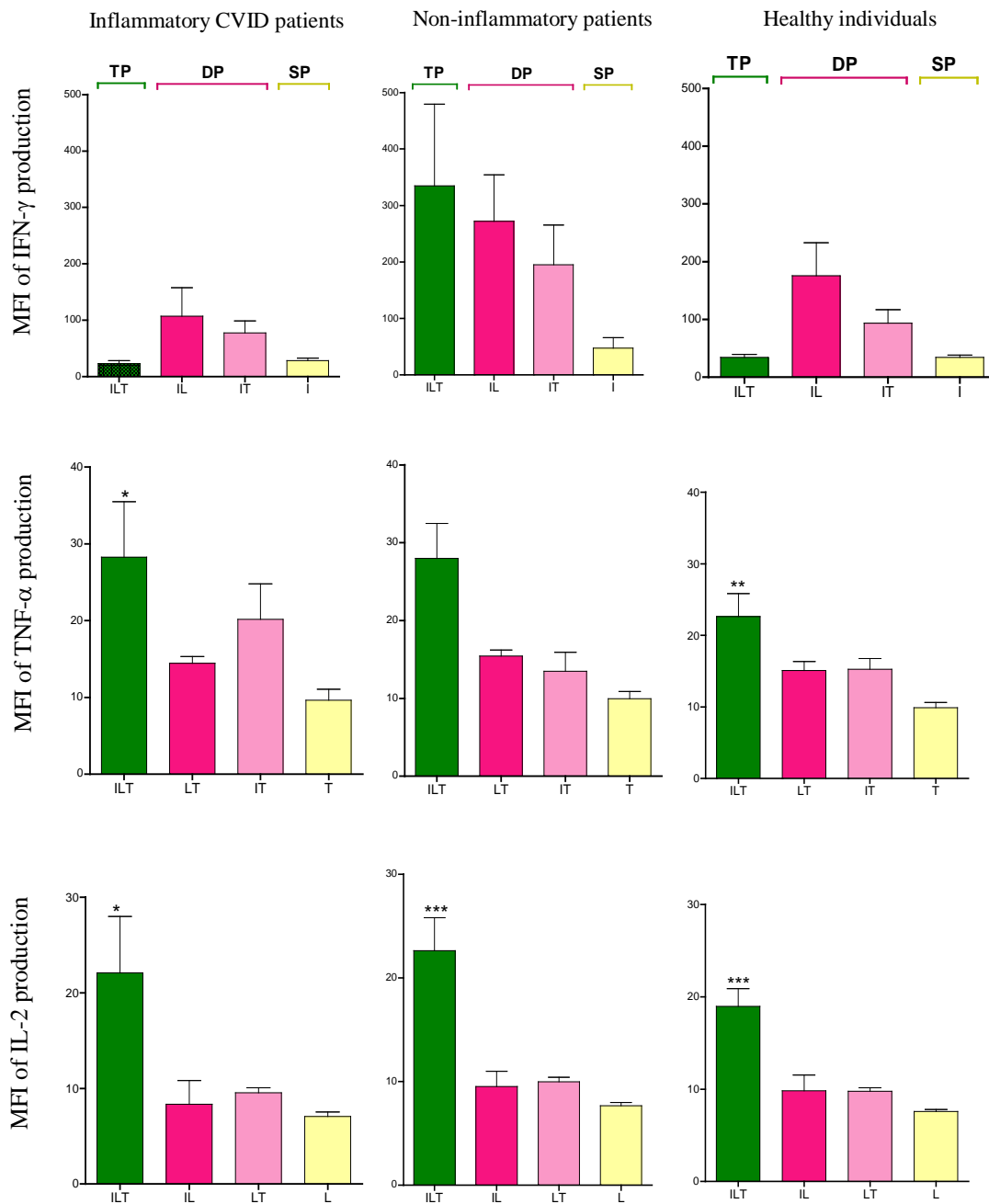


Figure 4-9: Median fluorescent Intensities of EBV (GLC)-specific CD8+ T cells.

MFI of EBV-specific CD8+ T cell triple producers (TP), double producers (DP), and single producers (SP) in inflammatory CVID patients (n = 9), non-inflammatory patients (n = 16) and healthy controls (n = 14). A paired t test was used for comparing cytokine MFI of triple producers and single producers. Asterisks (***), (**), and (*) represents P < 0.0001, P < 0.001, and P < 0.05, respectively. **ILT**: IFN-γ, TNF-α, and IL-2; **IL**: IFN-γ and IL-2; **IT**: IFN-γ and TNF-α; **I**: IFN-γ; **LT**: IL-2 and TNF-α; **L**: IL-2; **T**: TNF-α.

4.5 Discussion

In Chapter 3, the frequencies of antiviral CD8+ T cells were evaluated by measuring IFN- γ , TNF- α , and IL-2 separately. Following stimulation with HCMV antigens (but not EBV) a significant elevation of proinflammatory cytokines (IFN- γ and TNF- α) was found only in CVID patients with inflammation compared to non-inflammatory patients and healthy individuals. However, as both the magnitude and the quality of CD8+ T cell are crucial for the control of viral infection, this chapter further evaluated the quality of CD8+ T cells specific to HCMV and EBV in CVID patients. For this purpose, the cytokine co-expression profiles of antigen-specific CD8+ T cells as well as the magnitude of cytokine production per cell were assessed using polyfunctional cytokine analysis in CVID patients (with and without inflammation) compared to healthy individuals. This was the first study in which the simultaneous analysis of three cytokines at the single-cell level for CD8+ T cells specific to HCMV and EBV in CVID patients has been performed in the context of inflammatory disease.

Analysis of data from this chapter showed cytokine co-expression profiles are not only diverse in response to different antigens but they are also different in the various settings such as inflammatory disease. Interestingly, a distinct functional signature of virus specific CD8+ T cells producing single, double or triple cytokines was found in CVID patients with inflammation compared to both non-inflammatory and healthy subjects. These differences were also observed when the cytokine co-expression profiles were analyzed for HCMV-specific CD8+ T cells compared to the same responding cells specific to EBV.

However, in healthy individuals and non-inflammatory CVID patients, cytokine co-expression profiles induced by HCMV-specific CD8+ T cells overlapped with EBV-induced cytokine co-expression profiles. In contrast, the particular cytokine profiles observed in response to HCMV peptide stimulation were found to be distinctively skewed in inflammatory CVID patients. HCMV-specific CD8+ T cell responses in these patients consisted predominantly of IFN- γ producing cells (single producers, 'I' subset) and IFN- γ - and TNF- α -coproducing cells ('IT' subset) (Figure 4-2). This profile was different from responding CD8+ T cells responding to EBV where polyfunctional analysis showed no differences between the two CVID patient groups. Importantly, when iMFI analysis was applied to all single cytokine expression profiling of Ag-specific CD8+ T cells in both CVID patients (with and without inflammation) and healthy controls, the results of iMFI confirmed the patterns observed following data analysis measuring the magnitude by frequency.

Although the majority of cytokine-positive CD8+ T cells in CVID patients were single and double cytokine producers, triple-cytokine producers (IFN- γ , TNF- α , and IL-2) expressed more cytokine per cell than single producers of the same cytokine. It appears that the quality of T cells specific for persistent viral infections to be different from non-persistent viral infections. To support this, although analyzing antigen-specific CD4+ T cells, it has been shown that higher proportion of the total cytokine response to vaccinia and flu viruses (representing non-persistent viruses) consisted of triple cytokine producers (IFN- γ , TNF- α , and IL-2) compared to the same responding cells to HCMV (Kannanganat et al., 2007).

It is not yet clear what contributes to these differences and also why HCMV-specific CD8+ T cell responses in inflammatory CVID patients are skewed predominantly toward single IFN- γ producing cells and IFN- γ - and TNF- α -coproducing cells. It is tempting to speculate that antigen exposure and the distinct interaction of this virus in response to the adaptive or innate immune system may play an important role in this scenario. It has been shown that antigen exposure can influence the cytotoxic-granule profiles as well as cytotoxic activity of CD8+ T cells (Harari et al., 2009). Moreover, a rapid elimination of intracellular parasites (such as *Lieshmania major*) was shown to be mediated by TNF- α in the presence of IFN- γ which was highly significant compared to IFN- γ alone, indicating the crucial role of IFN- γ in TNF- α -mediated killing of *Lieshmania major* parasites (Bogdan et al., 1990). In addition, in response to HIV-1 peptide stimulation a strong cytotoxic activity has been found by HIV-1 specific CD8+ T cells producing both IFN- γ and TNF- α (Lichterfeld et al., 2004).

Collectively, and consistent with the results in Chapter 3, data from this Chapter suggest a distinctive functional signature of HCMV-specific CD8+ T cells in inflammatory CVID patients. Although T cell responses to viral antigens or immunizations are broad and complex, the results presented in this chapter indicate that a single response by looking at individual cytokines is probably inadequate to fully characterize the CD8+ T cell immune responses; Therefore, optimal measurements of functional competence of immune responses to pathogens require the coordinated measurement of more multiple cytokines (De Rosa et al., 2004).

Chapter 5

5 Phenotypic characteristics of antigen-specific CD8+ T cells in CVID patients

5.1 Introduction

Results from Chapters 3 and 4 showed a clear functional immune signature of HCMV-specific CD8+ T cells only in CVID patients with inflammatory disease but not in non-inflammatory patients or healthy controls. The aim of the work described in this chapter was to perform a phenotypic analysis of HCMV and EBV-specific CD8+ T cells and pentamer stained T cells in CVID patients (with and without inflammation) and healthy individuals with regard to markers of maturation, cytotoxicity, and T cell exhaustion.

Monitoring fluctuations in T cell responses over time together with quantitative measurement of phenotypic markers (serving as surrogate markers of virus replication) can produce a reliable method to measure aspects of the clinical course of virus-associated diseases (Pantaleo and Harari, 2006). Over the last two decades, a large number of phenotypic markers have been described to both define different populations of antigen-specific CD8+ T cells and identify functionally different stages of T cell differentiation (Pantaleo and Harari, 2006).

In this chapter a panel of different cell surface markers (as explained later) were used to assess the phenotype of CD8+ T cell responses in CVID patients and healthy controls using *ex vivo* staining as described in Chapter 2 (section 2.4). The panel of cell surface markers used included CD57, granzyme B, PD-1, CD45RO, CD45RA, CD27, and CD28.

1) CD57

As previously mentioned in chapter 1 (section 1.63.3.2), CD57 can be expressed mainly by T cells and CD16+ NK cells. In healthy individuals 5-20% of CD8+ T cells express

CD57 (Morley et al., 1995). Although CD57 has been extensively studied in different contexts, its exact function on an immune cell is unknown (Wood et al., 2009). Initially, an increase in CD8+ T cells expressing CD57 was described in elderly individuals (Ligthart et al., 1986;McNerlan et al., 1998). Although expansion is seen with aging, the stimulus behind the expansion is unclear. It is possible that viral or tumor antigens are responsible for this expansion. It is well acknowledged that chronic viral infections and persistent antigen stimulation can lead to expansion of CD8+ lymphocytes. Both, HCMV and human immunodeficiency virus (HIV) infections have been shown to be associated with expansions of the CD8+CD57+ lymphocyte subset (Wang et al., 1993;Rossi et al., 2007;Wood et al., 2009).

However, clonal expansion of CD57 expressing lymphocytes can also occur in some rheumatologic or autoimmune conditions such as rheumatoid arthritis (Wang et al., 1993). Moreover, clonal expansion of CD8+CD57+ lymphocytes has been reported in HCMV positive transplant recipients such as renal transplant patients, cardiac transplant patients, and bone marrow transplant recipients (BMT) (Sabnani et al., 2006;Wood et al., 2009).

2) Granzyme B

Granzyme B is a 32 kDa serine protease, which has been shown to induce rapid cell death of a virus infected target cell through the cleavage of downstream substrates as well as activating cleavage of caspases (Poe et al., 1991;Sutton et al., 1997;Bird et al., 1998;Waugh et al., 2000). Following recognition and binding to virus infected or other target cells, cytotoxic lymphocytes (or NK cells) release perforin, a Ca²⁺-dependent pore-forming protein which multimerizes in the plasma membrane of the target cells,

forming a pore allowing granzyme B access to the cytosol of target cells (Kagi et al., 1994; Liu et al., 1995; Boivin et al., 2009). Upon target cell recognition and releasing into the cytoplasm, granzyme B can target substrates such as autoantigens in the cytosol or in the nucleus, inducing apoptosis via several pathways (Boivin et al., 2009).

The importance of granzyme B in infection, chronic diseases, and cancer has recently emerged with the discovery of new immune and non-immune cell sources of the protease. During chronic inflammation, the levels of granzyme B have been found to be elevated due to infiltrating activated cytotoxic immune cells such as lymphocytes to damaged and inflamed areas. An elevation of extracellular concentrations of granzyme B has also been shown in bodily fluids (such as synovial fluid and serum) of various diseases including Rheumatoid arthritis (Trapani and Sutton, 2003; Goldbach-Mansky et al., 2005; Chowdhury and Lieberman, 2008; Boivin et al., 2009; Darrah and Rosen, 2010).

3) PD-1

The immunoreceptor, programmed death (PD)-1 (also called CD279) is a negative regulator of T cell activation. PD-1 can be expressed by many cells including T cells, B cells, NK cells, and myeloid derived cells. The PD-1 receptor, which was originally isolated as a transcript up-regulated in apoptotic cells (Ishida et al., 1992), is a 288 amino acid cell surface monomer. Although it shares structural properties with the members of CD28 family, the PD-1 gene is located on a different region of the same chromosome (2q37). Two ligands with different expression patterns have been described for PD-1, PD-L1 and PD-L2. PD-L1 (also known B7-H1 and CD274) is broadly distributed on various cell types such as B cells, DCs, macrophages, mesenchymal stem cells as well as a wide variety of nonhematopoietic cell types. PD-L2 (also known B7-

DC and CD273), by contrast, is limited to professional antigen presenting cells including macrophages, DCs, and cultured bone marrow-derived mast cells (Augello et al., 2005; Okazaki and Honjo, 2006; Keir et al., 2007; Sharpe et al., 2007).

In general, resting T cells from healthy individuals express barely detectable levels of PD-1. However, TCR engagement on these cells induces PD-1 expression via transducing an inhibitory signal through recruiting Src homology phosphatase (SHP)-1 and SHP-2, blocking PI3K activation, and interfering with glucose uptake (Riley and June, 2007; Sharpe et al., 2007). In mouse models, it has been shown that the interaction of PD-1 and its ligands contributes directly to T cell dysfunction and lack of viral control in established chronic infection. For example, during the first week of infection with lymphocytic choriomeningitis virus (LCMV), the level of PD-1 expression on virus-specific CD8+ T cells increases, consistent with the upregulation of PD-1 on activated T cells. In the case of clearance of infection, functional memory T cells are generated following the rapid reduction of PD-1 expression. In contrast, PD-1 expression in infection with the LCMV clone 13 strain (that causes chronic infection) remains high and T cell function decreases (Barber et al., 2006; Sharpe et al., 2007). Applying these observations to humans, PD-1 expression has been shown to be elevated on HIV-specific T cells (Trautmann et al., 2006), T cells specific to hepatitis B virus (HBV) (Boettler et al., 2006) and hepatitis C virus (HCV) (Urbani et al., 2006). Indeed, in the case of viremic HIV patients, not only was PD-1 expression found to be higher on CD8+ T cells specific to HIV, but also its expression on T cells correlated with viral load. It seems that high PD-1 expression is not a general phenomenon as T cells specific for

non-persisting viruses such as influenza or vaccinia express very low levels of PD-1 (Sharpe et al., 2007).

However, it is possible that similar regulatory mechanisms may also apply for other persistent viral infections in humans (Klenerman and Hill, 2005; Sester et al., 2008). Investigating the functional exhaustion of CD4+ T cells specific to HCMV, Sester et al found a significantly higher proportion of PD-1 expression in HCMV-specific CD4+ T cells when they compared viremic renal transplant recipients with nonviremic transplant patients, dialysis patients or controls (Sester et al., 2008). Additionally, significantly higher PD-1 expression has been found on both total and HCMV specific CD8+ T cells in transplant recipients who developed HCMV disease compared to the HCMV positive healthy controls (La Rosa et al., 2008).

4) CD45RO, CD45RA, CD27, and CD28

To investigate the differentiation state of antigen-specific CD8+ T cells in CVID patients with and without inflammation, PBMCs were surface stained for the expression of CD45RO, CD45RA, CD27, and CD28. As previously described in Chapter 1 (section 1.6.3.3.2), CD45RA, CD45RO, CCR7, CD27, and CD28 are the most-commonly used cell surface markers (among various cell surface markers) for defining the memory stage of antigen-specific T cells as differential expression of these molecules allows the distinction between different subsets of antigen-experienced T cells (Appay et al., 2008).

Although antigen specific T cells display some degree of heterogeneity; they exhibit distinct profiles based on their viral specificity. For example, during latent infection with HCV, HIV, HCMV, or EBV cells are predominantly CCR7+CD27+CD28+, CCR7-

CD27+CD28-, CCR7-CD27-CD28-, or CCR7-CD27+CD28+, respectively (Appay et al., 2002). Nevertheless, it is important to note that many of these observations do not hold in a setting of T cell activation or inflammation. Many T cell attributes can change rapidly upon activation, such that activated T cells will behave differently from their resting state. In the circulating pool, for instance, activated CD8+ T cells can be found during the acute response to HCMV, EBV, and HIV with a relatively early memory phenotype (i.e., CCR7-CD28+CD27+) that still display high cytolytic potential (Gamadia et al., 2003; Appay et al., 2008; Waller et al., 2008). It has been shown that persistent infections often cause substantial alterations in the phenotype and functionality of memory CD8+ T cells (Wherry et al., 2003a; Klenerman and Hill, 2005; Harty and Badovinac, 2008).

As explained above, the importance of these functional markers is well documented either in the context of antigen or disease. However, there is limited data available regarding these functional markers in the context of CVID patients and antigen-specific CD8+ T cells. Although a plethora of T cell abnormalities, including increased expression of CD57, CD45RO, HLA-DR and perforin or reduced expression of CD45RA, have been previously reported in CVID patients (Wright et al., 1990; Baumert et al., 1992; Piqueras et al., 2003; Raeiszadeh et al., 2006; Viallard et al., 2006), none of these studies looked at these T cell abnormalities in the context of inflammatory disease experienced by a subset of CVID patients.

To get a more complete picture of the phenotypic characteristic of antigen-specific CD8+ T cells of CVID patients with and without inflammation this chapter finally aimed to analyze data obtained from functional markers such as PD-1 in the context of the total

cell population phenotyping analysis which was performed by staff in the diagnostic clinical laboratory. Samples from CVID patients were routinely subjected to extensive immunological phenotyping (markers listed in table 5-1) as part of standard clinical management.

T cell phenotyping panel (Frequency)	B cell phenotyping panel (Frequency)	Immunodeficiency panel (Count & Frequency)
CD3+	CD19+ B cells	CD3 count & %
CD3+ CD4+	CD27- IgD+ (naïve)	CD4 count & %
CD3+ CD8+	CD27+ IgD+ (IgM memory)	CD8 count & %
CD4+ CD45RO+	CD27+ IgD- (Sw. memory)	CD19 count & %
CD4+ CD45RO+ CXCR5+	CD21- CD38-	CD16+ CD56 count & %
CD8+ CD27- CD28-	Transitional B cells	CD4/CD8 ratio
CD8+ CD27+ CD28-	Plasmablasts	
CD4- CD8-		
CD4+ CD45RA+		
CD4+ CDRA+ CD31+		
CD127 ^{low} CD25+		

Table 5-1: The panel of markers used for regular immunophenotyping of CVID patients.

5.2 Frequency of antigen-specific CD8+ T cells expressing CD57 in CVID patients

Total CD8+ T cell populations as well as antigen-specific CD8+ T cells expressing CD57 were surface stained with pentamers restricted to HCMV and EBV as previously described in Chapter 2 (section 2-4). Figure 5-1 illustrates an example of representative FACS plots showing the CD57 expression on both total CD8+ T cells and Ag-specific CD8+ T cells.

As shown in Figure 5-2, a significant increase was found in the frequency of CD8+ T cells expressing surface marker CD57 in inflammatory CVID patients compared to non-inflammatory CVID patients and healthy controls (Mean \pm SD; 65.08% \pm 3.7% inflammatory vs. 40.03% \pm 6.01% non-inflammatory; P= 0.007) (65.08% \pm 3.7% inflammatory vs. 37.37% \pm 6.2% healthy; P= 0.003).

Consistent with CD8+ T cell responses specific to EBV (described in Chapters 3 and 4), analyzing the frequency of EBV-specific CD8+ T cells expressing CD57 showed no significant difference between inflammatory CVID patients compared to non-inflammatory patients and healthy controls (28.25% \pm 8.1% inflammatory vs. 21.14% \pm 4.7% non-inflammatory; P> 0.05) (28.25% \pm 8.1% inflammatory vs. 23.07% \pm 5.1% healthy; P> 0.05) (Figure 5-3).

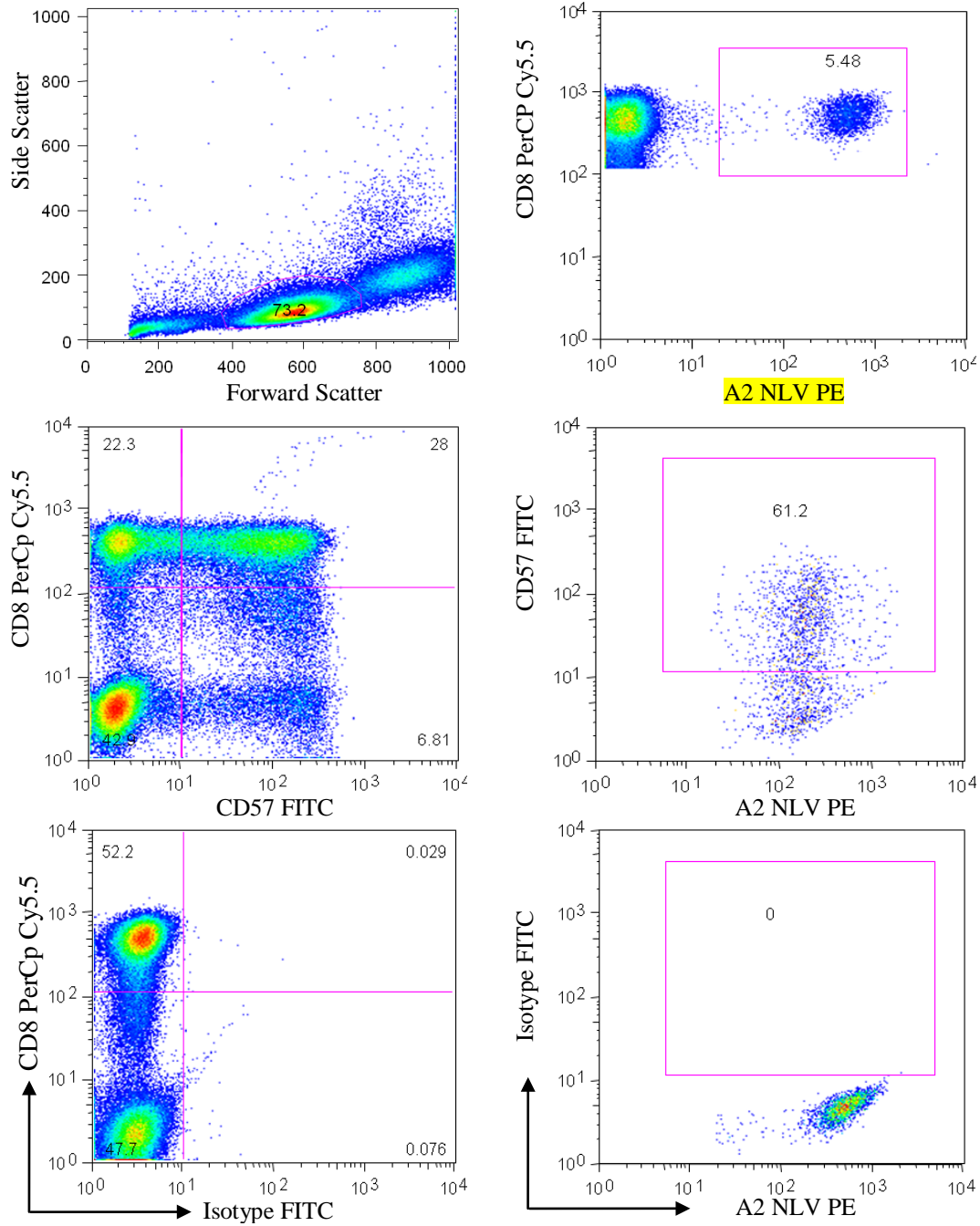


Figure 5-1: A representative FACS plots of CD57 expressing CD8+ T cells from an inflammatory CVID patient.

CD57 expression was gated on both total CD8+ T cells (gated as quadrant) and NLV-specific CD8+ T cells (gated as box). Gates were adjusted according to the appropriate isotype controls.

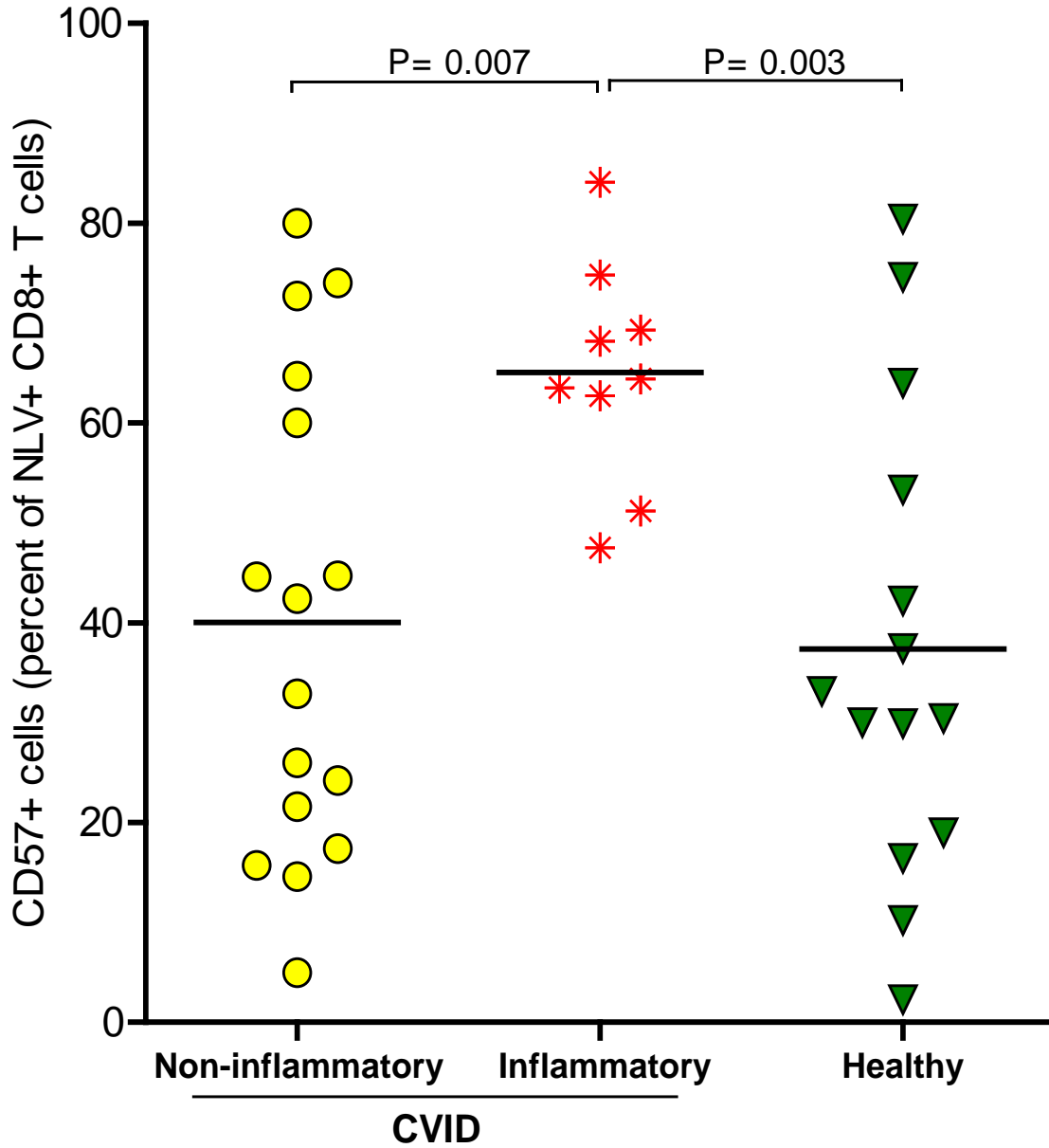


Figure 5-2: Frequency of CD57 expressing HCMV (NLV)-specific CD8+ T cells.

HCMV-specific CD8+ T cells expressed significantly higher CD57 in inflammatory CVID patients compared to non-inflammatory patients as well as healthy individuals. Bars indicated mean values. Each point represents a single subject.

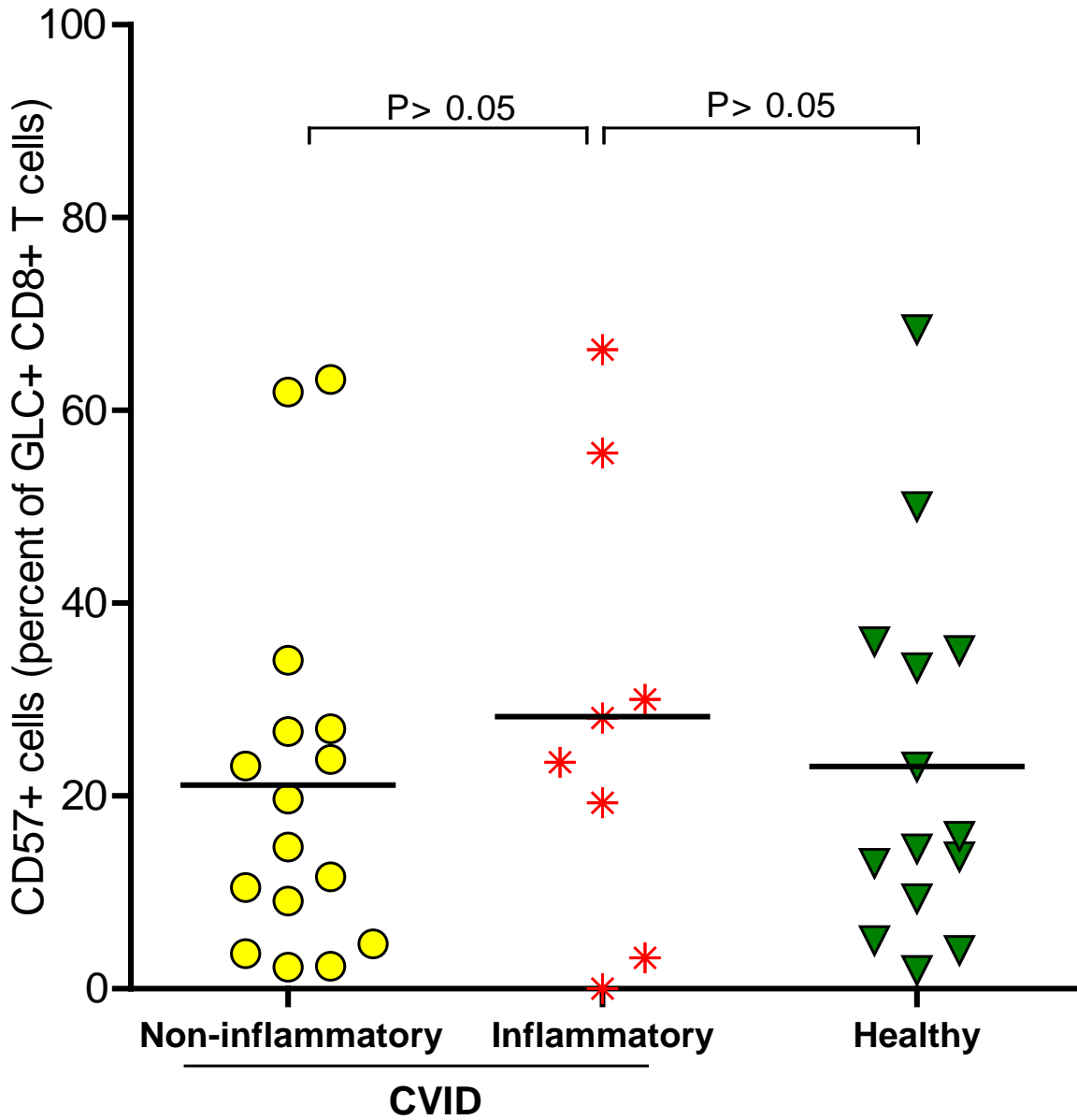


Figure 5-3: Frequency of CD57 expressing EBV (GLC)-specific CD8+ T cells.

No significant difference was found in the frequency of CD57 expression between CVID patients (with and without inflammation) and healthy control group. Bars indicated mean values. Each point represents a single subject.

5.3 Frequency of antigen-specific CD8+ T cells expressing granzyme B in CVID patients

The expression of Granzyme B was examined as a marker of cytotoxicity. Figure 5-4 shows an example of representative FACS plots of the expression of granzyme B on both total- and Ag-specific CD8+ T cells in CVID patients (with and without inflammation) and healthy individuals.

Although there was no significant difference between inflammatory CVID patients and non-inflammatory ones, a statistically significant difference was found in the frequency of granzyme B expressing CD8+ T cells specific to HCMV in CVID patients (with and without inflammation) compared to healthy controls (Mean \pm SD: 31.9% \pm 4.6% inflammatory vs. 13.13% \pm 3.1% healthy; $P= 0.002$) (33.06% \pm 4.7% non-inflammatory vs. 13.13% \pm 3.1% healthy; $P= 0.002$) (Figure 5-5).

EBV-specific CD8+ T cells in CVID patients with and without inflammation expressed nearly same levels of granzyme B (18.2% \pm 4.6% inflammatory vs. 15.7% \pm 3.7% non-inflammatory; $P> 0.05$). Although the frequency of granzyme B expression by EBV-specific CD8+ T cells were slightly higher in CVID patients compared to healthy controls, the difference was not statistically significant (18.2% \pm 4.6% inflammatory vs. 8.4% \pm 2.4% healthy; $P= 0.05$) (15.7% \pm 3.7% non-inflammatory vs. 8.4% \pm 2.4% inflammatory; $P> 0.05$) (Figure 5-6).

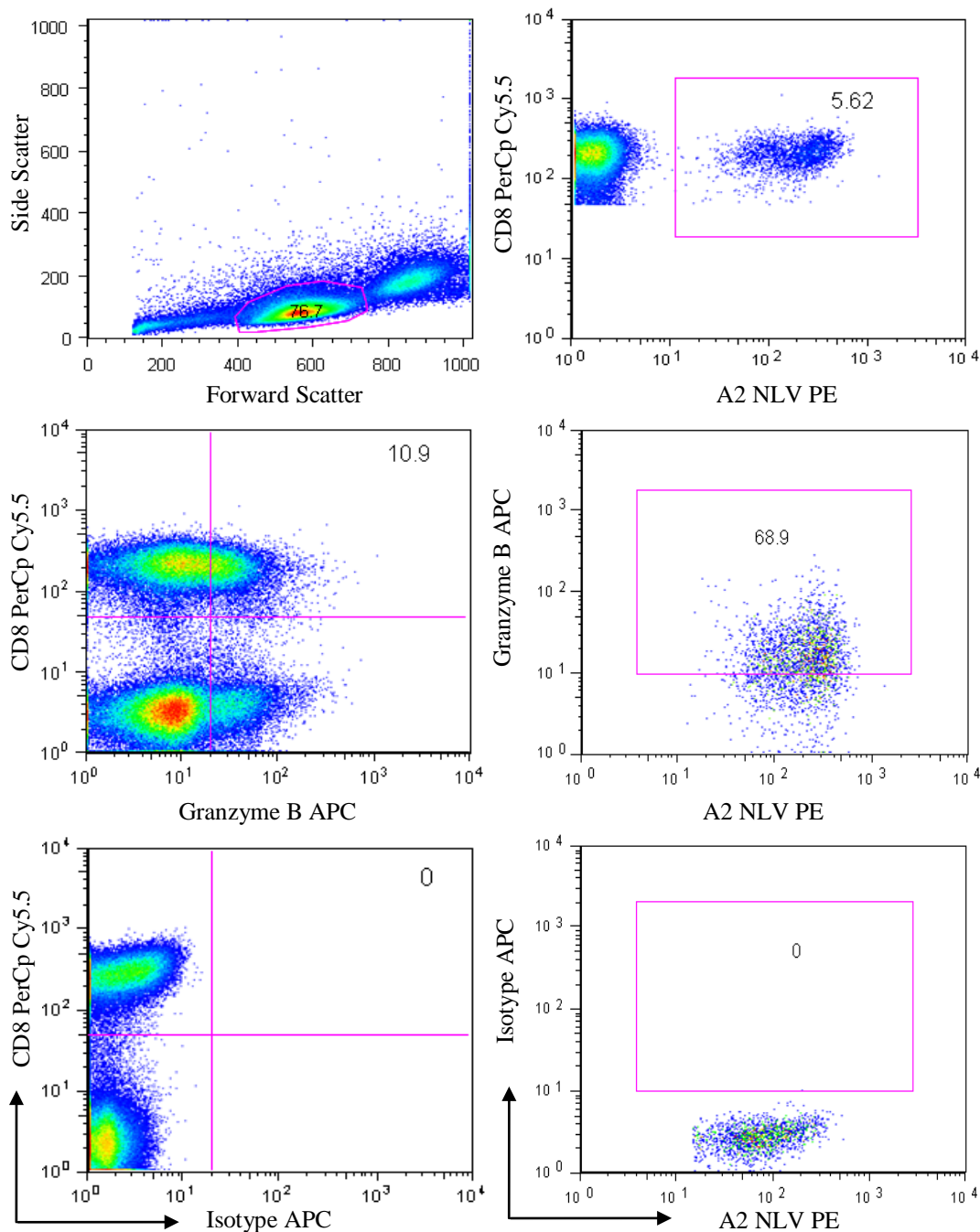


Figure 5-4: Representative FACS plots of granzyme B expressing CD8+ T cells from an inflammatory COVID patient.

CD57 expression was gated on both total CD8+ T cells (gated as quadrant) and HCMV (NLV)-specific CD8+ T cells (gated as box). Gates were adjusted according to appropriate isotype controls.

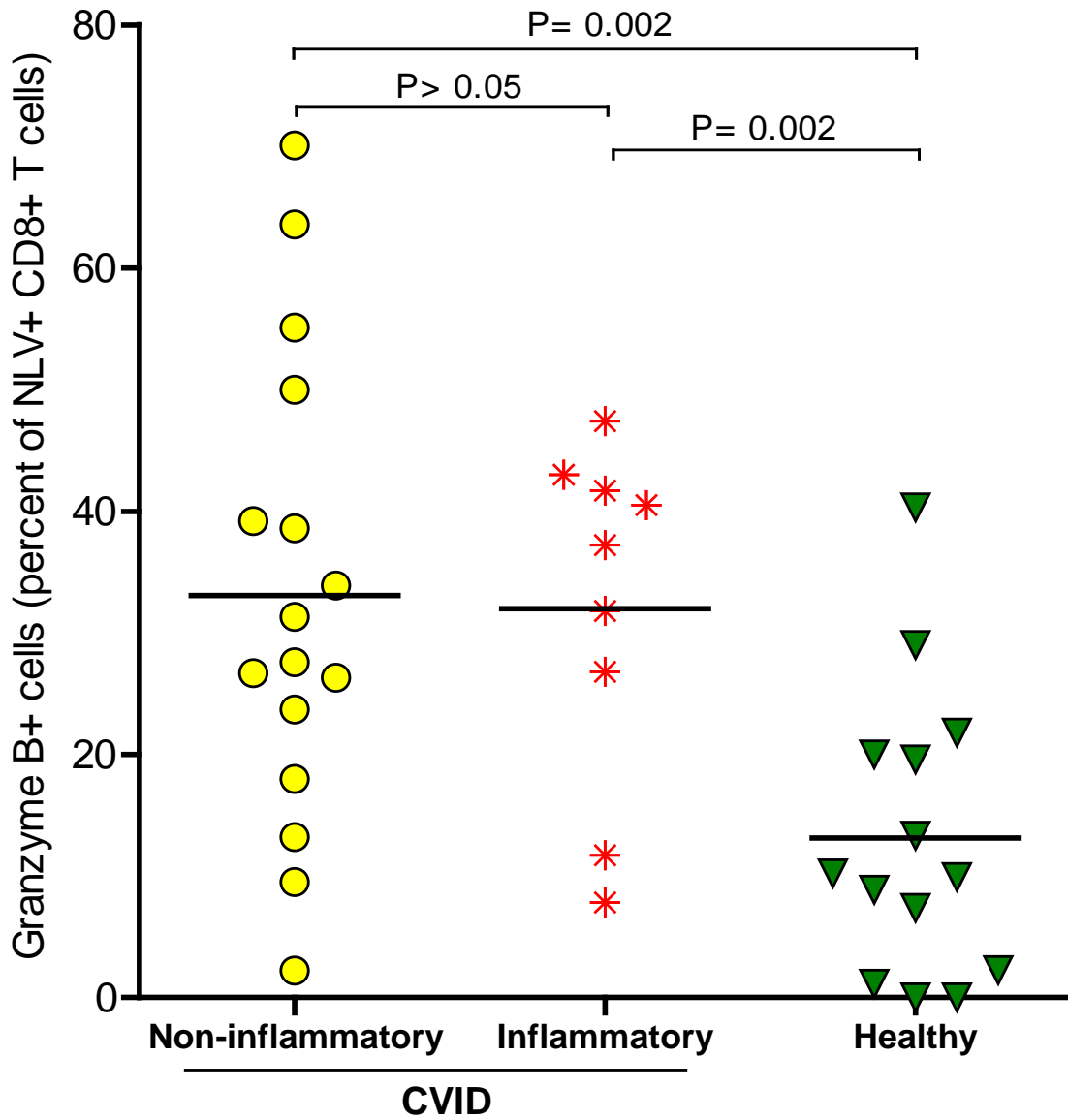


Figure 5-5: Frequency of granzyme B expressing HCMV (NLV)-specific CD8+ T cells.

HCMV-specific CD8+ T cells in CVID patients (with and without inflammation) expressed significantly higher granzyme B compared to healthy controls. Bars indicated mean values. Each point represents a single subject.

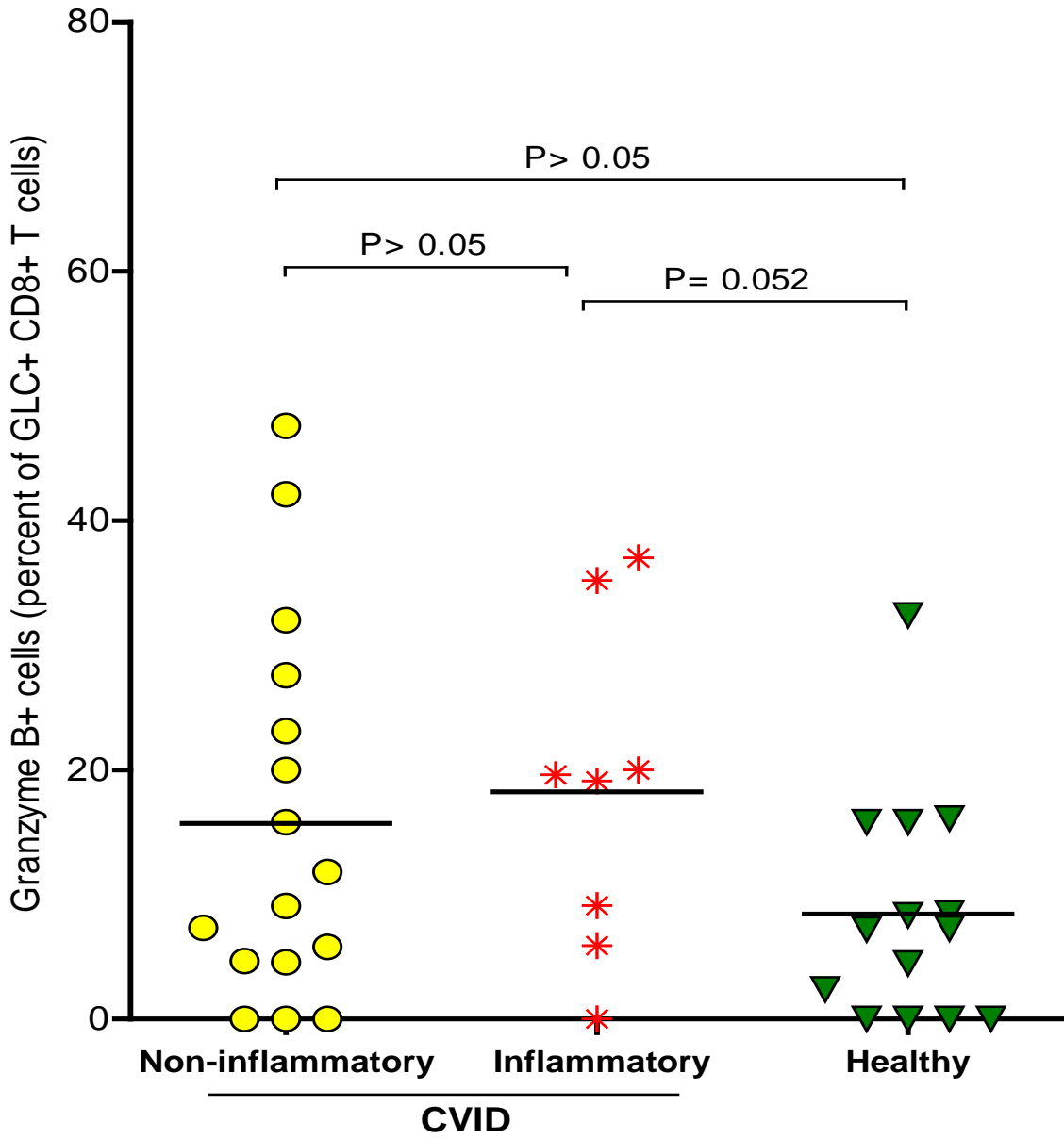


Figure 5-6: Frequency of granzyme B expressing EBV (GLC)-specific CD8+ T cells.

EBV-specific CD8+ T cells in CVID patients (with and without inflammation) expressed significantly higher granzyme B compared to healthy controls. Bars indicated mean values. Each point represents a single subject.

5.4 Frequency of PD-1 expression by antigen-specific CD8+ T cells in CVID patients

The analysis of PD-1 in the context of Ag-specific CD8+ T cells has not been carried out in CVID patients. Therefore, PD-1 expression was analyzed, on both total CD8+ T cells and Ag-specific T cells from CVID patients with and without inflammation and compared to healthy HCMV-seropositive individuals. Figure 5-7 shows an example of FACS plot analysis of a CVID patient and the gating strategy which was applied to all data sets.

Unlike CD57 and Granzyme B in which expression in inflammatory CVID patients was significantly higher than healthy controls, the levels of PD-1 expression in these patients, although marginally higher than healthy controls, was generally very low. As shown in Figure 5-8, the percentage of PD-1 expressing HCMV-specific CD8+ T cells was higher in non-inflammatory patients compared to both inflammatory patients and healthy controls. This difference was statistically significant for the comparison with healthy controls (Mean \pm SD; 34.1% \pm 7.4% non-inflammatory vs. 11.1% \pm 3.7% healthy; $P= 0.01$) but not for inflammatory patients (34.1% \pm 7.4% non-inflammatory vs. 19.6% \pm 9.5% inflammatory; $P> 0.05$). Similarly, the MFI of PD-1 expression on HCMV-specific CD8+ T cells was higher in non-inflammatory CVID patients; however, there was no significant difference between different groups (60.5 units \pm 19.6 non-inflammatory vs. 32.4 units \pm 10.03 inflammatory and 32.8 units \pm 5.6 healthy) ($P> 0.05$) (Figure 5-9). No difference was seen when comparing PD-1 expression on total CD8+ T cells.

Additionally, the same set of PD-1 analyses was performed on EBV-specific CD8+ T cells in the CVID patients as well as healthy individuals (Figures 5-10 and 5-11). No significant difference was found in the levels of PD-1 expression on CD8+ T cells specific to EBV between non-inflammatory and inflammatory CVID patients. The mean percentage of PD-1 expression levels on EBV-specific CD8+ T cells was slightly higher in non-inflammatory patients compared to inflammatory patients but not significantly so ($32.6\% \pm 5.1\%$ non-inflammatory vs. $22.08\% \pm 7.2\%$ inflammatory; $P > 0.05$). Nevertheless, these levels were significantly higher compared to healthy subjects ($32.6\% \pm 5.1\%$ non-inflammatory vs. $14.6\% \pm 5.2\%$ healthy; $P = 0.03$) (Figure 5-10). In this regard, analysis of PD-1 expression based on MFI revealed no significant difference between different groups (70.4 units ± 20.7 non-inflammatory vs. 49.7 units ± 24.3 inflammatory and 49.8 units ± 11.5 healthy) ($P > 0.05$) (Figure 5-11).

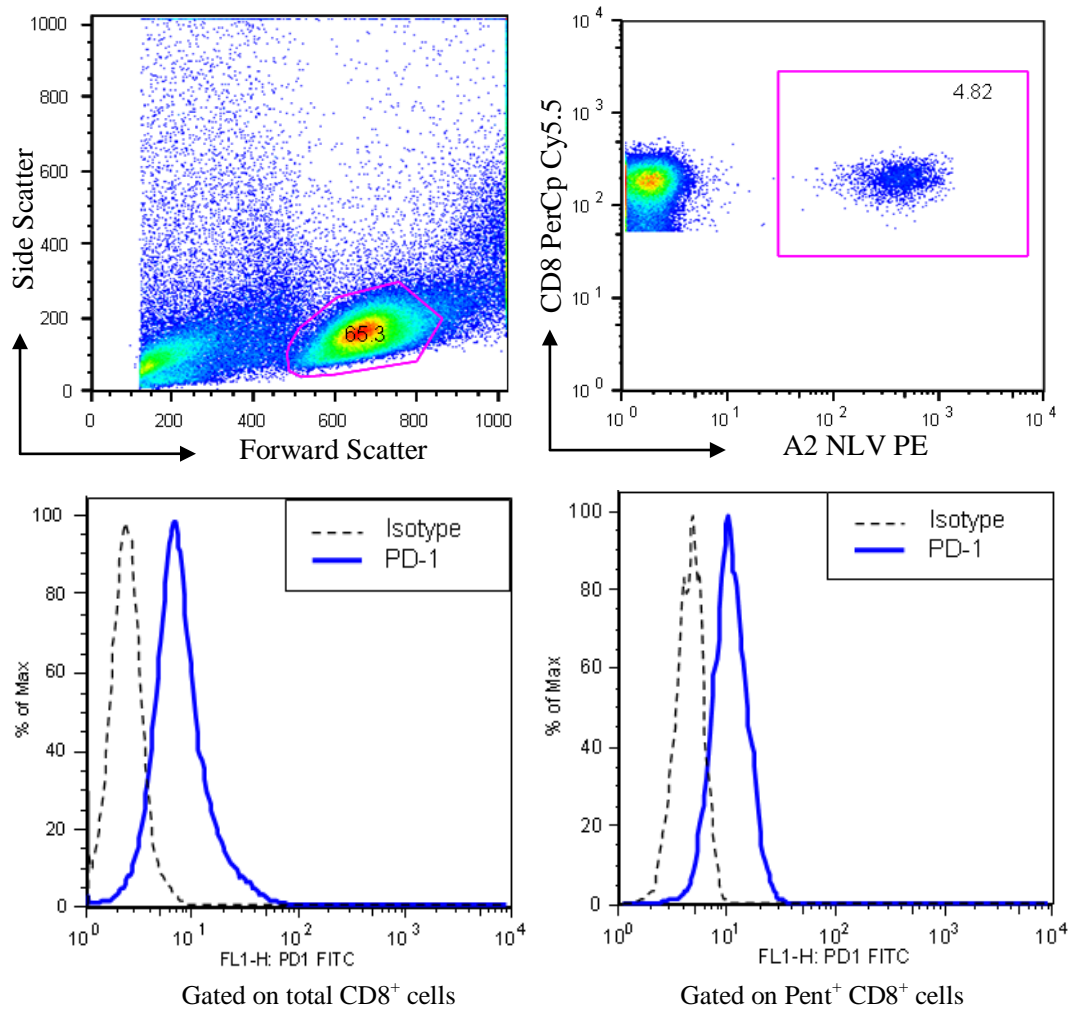


Figure 5-7: Representative FACS plots of PD-1 expression on total CD8+ T cells and HCMV-specific CD8+ T cells.

The lower panels (Histogram) show the isotype control (IgG1 κ ; dash line) and anti-PD1 FITC antibody (blue line). For PD-1 analysis, positive populations were gated on total CD8+ T cells and HCMV-specific CD8+ T cells.

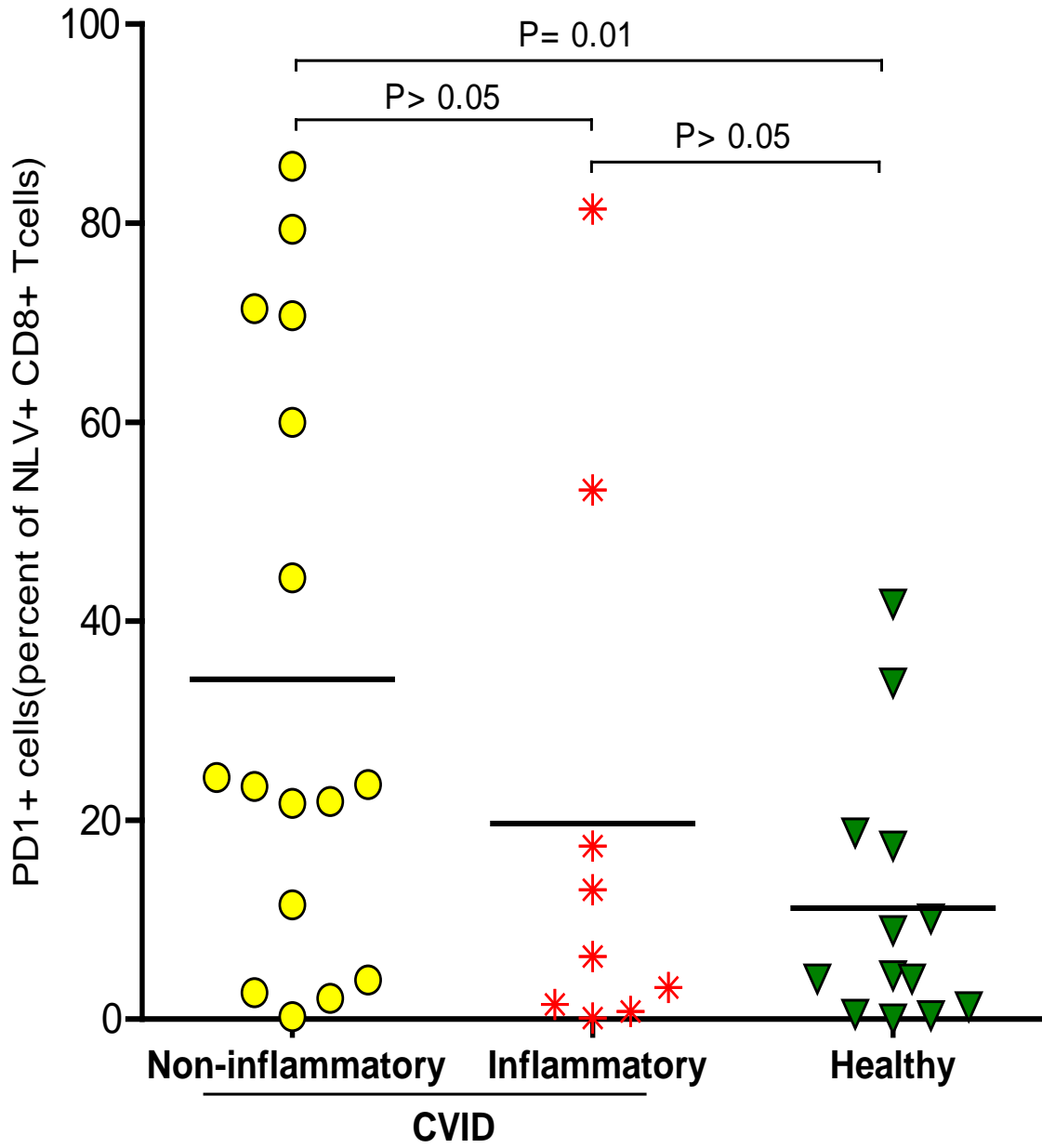


Figure 5-8: Frequency of PD-1 expressing HCMV (NLV)-specific CD8+ T cells.

HCMV-specific CD8+ T cells of non-inflammatory CVID patients expressed higher PD1 compared to inflammatory patients and healthy controls although this difference was significant for healthy group. Bars indicated mean values. Each point represents a single subject.

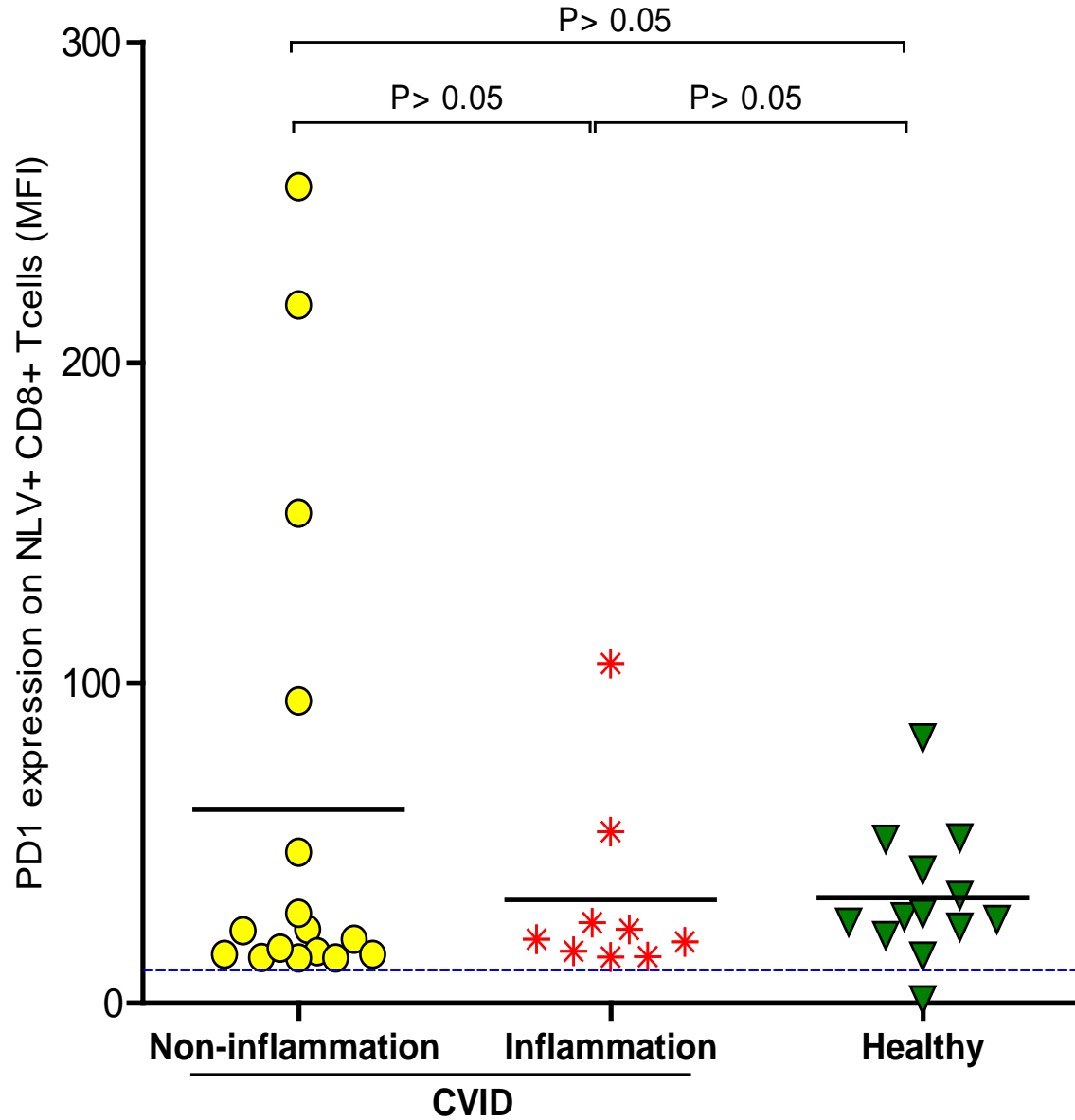


Figure 5-9: Median fluorescent intensity (MFI) of PD-1 expression on HCMV (NLV)-specific CD8+ T cells.

Comparing the MFI of PD1 expression on CD8+ T cells specific to HCMV showed no significant difference between any of three groups (non-inflammatory CVID patients, inflammatory patients, and healthy controls). The blue dashed line shows the MFI of isotype controls. Each point represents a single subject.

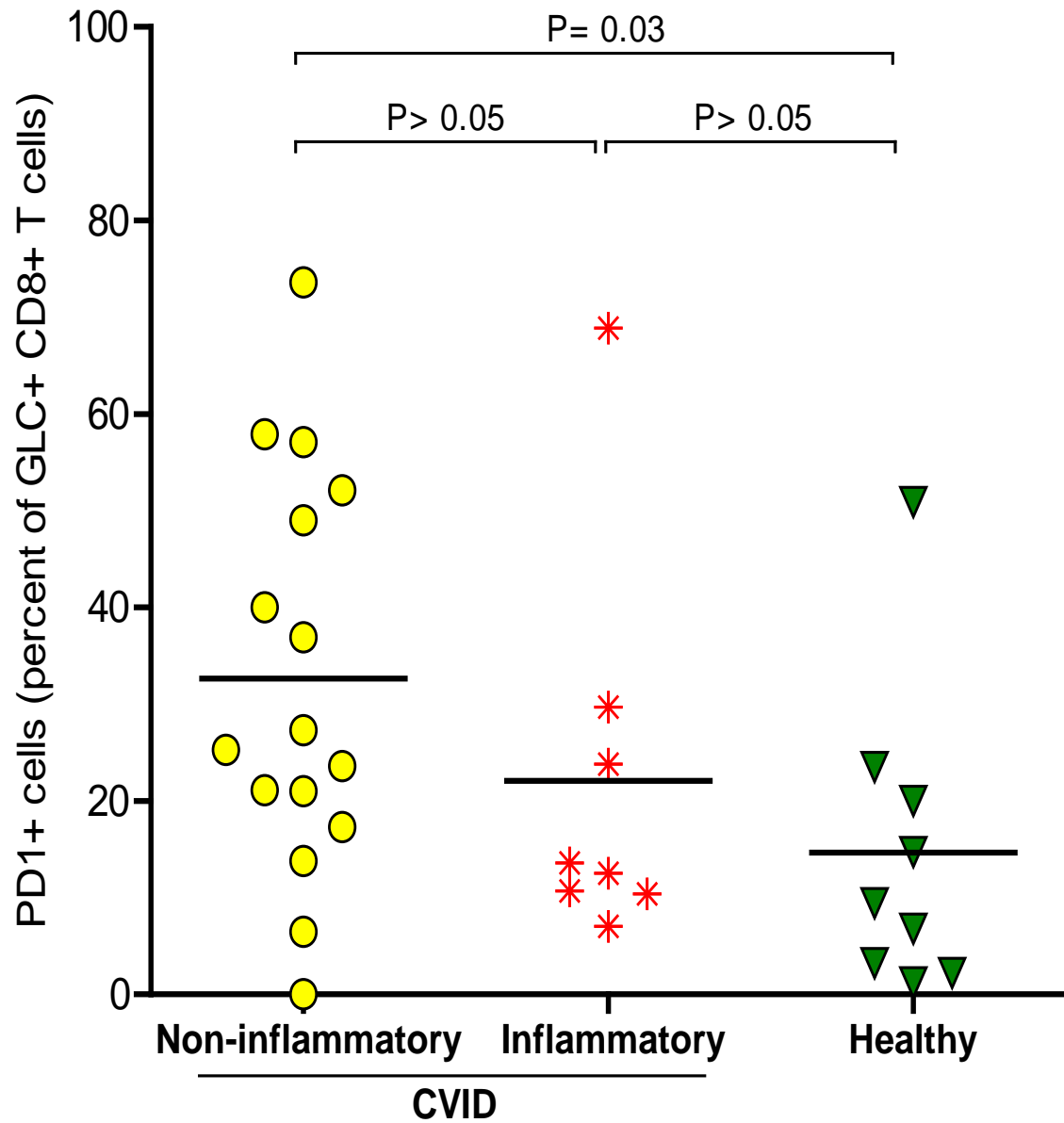


Figure 5-10: Frequency of PD-1 expressing EBV (GLC)-specific CD8+ T cells.

EBV-specific CD8+ T cells of non-inflammatory CVID patients expressed higher PD1 compared to inflammatory patients and healthy controls although this difference was significant for healthy group. Bars indicated mean values. Each point represents a single subject.

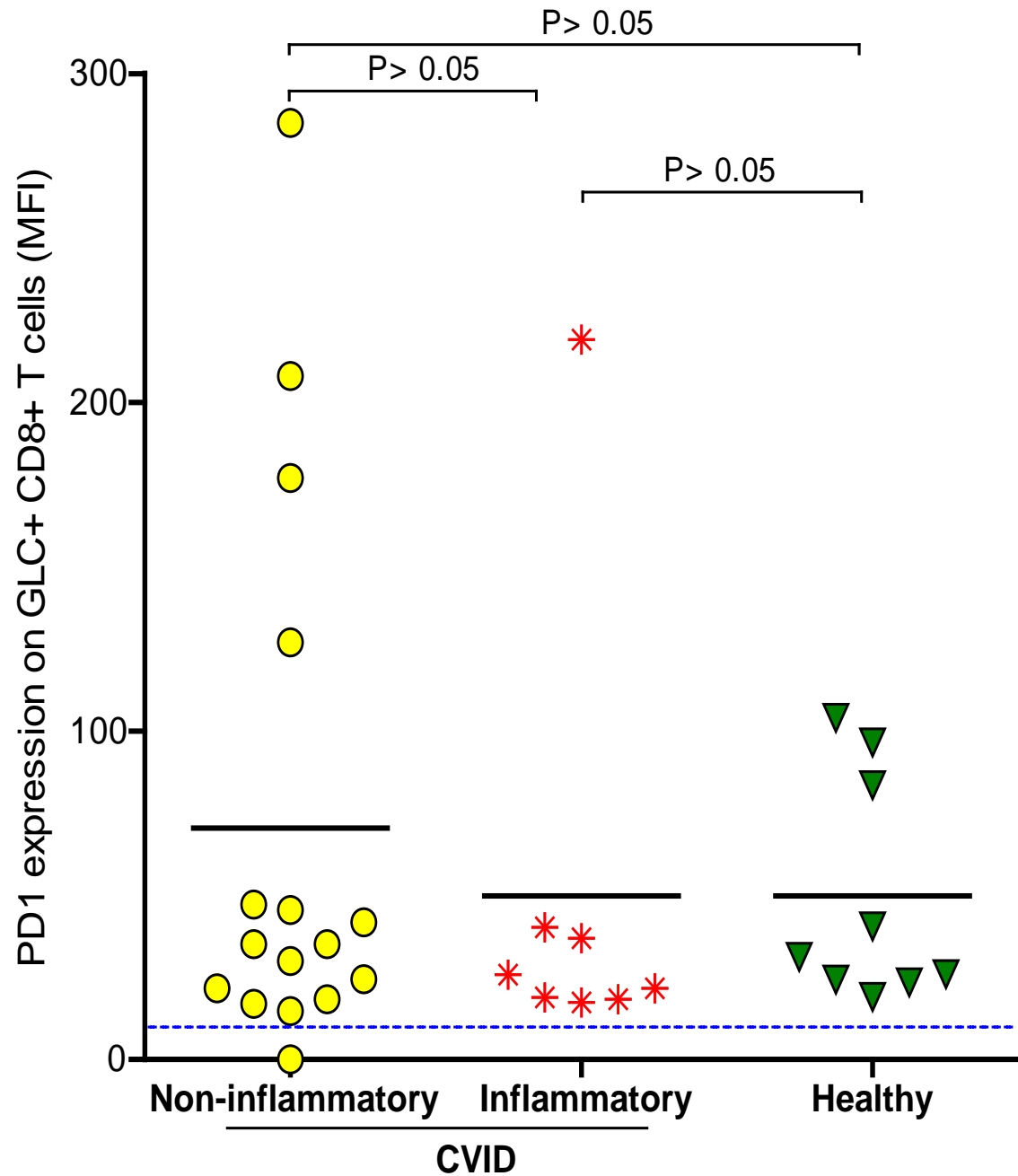


Figure 5-11: Median fluorescent intensity (MFI) of PD-1 expression on EBV (GLC)-specific CD8+ T cells.

No significant difference was found in the comparisons made for the MFI of PD1 expression on CD8+ T cells specific to EBV between any of three groups (non-inflammatory CVID patients, inflammatory patients, and healthy controls). The blue dashed line shows the MFI of isotype controls. Each point represents a single subject.

5.5 Phenotypic characteristics of CD8+ T cells in CVID patients

CD45RO, CD45RA, CD27, and CD28 can be used to distinguish naïve cells from Ag-experienced CD8+ T cells. Expression of these markers was analyzed on both total CD8+ T cells and Ag-(HCMV and EBV) specific CD8+ T cells of inflammatory CVID patients compared to non-inflammatory patients using *ex vivo* staining. Only samples CVID patients with and without inflammation were analysed for the purpose of this part of chapter since the phenotypic characterization of HCMV and EBV specific CD8+ T cells has already been described for healthy controls in a same range of age (Colonna-Romano et al., 2007).

Figure 5-12 illustrates an example of representative FACS plots of CD45RO and CD45RA expression on both total CD8 and NLV+ CD8+ T cells from an inflammatory CVID patient. As shown in Figure 5-13, the levels of CD45 (RO and RA) expression were heterogeneous and no significant difference was found comparing the mean percentage frequency of CD45RO expressing total CD8+ cells or NLV-specific CD8+ T cells in inflammatory CVID patients compared to non-inflammatory patients (Total CD8: 17.2% ± 3.8% inflammatory vs. 7.8% ± 3.1% non-inflammatory; NLV+ CD8+ T cells: 12.5% ± 7.7% inflammatory vs. 26.8% ± 5.5% non-inflammatory; $P > 0.05$). CD8+ T cells of inflammatory patients possessed significantly higher frequency of CD45RA cells compared to non-inflammatory patients (15.8% ± 4.7% inflammatory vs. 4.3% ± 2.4% non-inflammatory; $P = 0.03$) although this elevation was not statistically significant when the CD45RA expressing CD8+ NLV+ T cells were analyzed (32.5% ± 10.2% inflammatory vs. 11.7% ± 5.05% non-inflammatory; $P > 0.05$).

Additionally, in order to appropriately define the memory state of Ag-specific CD8+ T cells in CVID patients with and without inflammation, samples were also analyzed for the surface expression of CD27 and CD28. As an example, a representative FACS plot for CD27 and CD28 expression on both total CD8+ cells and NLV+ CD8+ T cells is shown in Figure 5-14. With the exception of one individual, CD8+ T cells of CVID patients (with and without inflammation) expressed low or negligible levels of CD28 (Mean \pm SD; 5.7% \pm 1.8% inflammatory vs. 5.2% \pm 2.5% non-inflammatory; $P > 0.05$). However, CD8+ T cells, although heterogeneous, expressed high levels of CD27 and this elevation was statistically significant in CVID patients with inflammation compared to non-inflammatory patients (23.4% \pm 2.3% inflammatory vs. 15.3% \pm 1.8% non-inflammatory; $P = 0.01$) (Figure 5-15; Panel A). However, this difference was not found when CD8+ T cells specific to HCMV (NLV+) were analyzed for CD27 expression (43.4% \pm 8.3% inflammatory vs. 47.2% \pm 6.5% non-inflammatory; $P > 0.05$). In the same cell population, although not significant, CD28 expression was higher in non-inflammatory patients than other patients with inflammation. Indeed, in latter group, NLV+CD8+ T cells were mostly negative for CD28 expression (14.2% \pm 5.5% vs. 3.4% \pm 1.7%; $P > 0.05$) (Figure 5-15; Panel B).

In order to examine whether the phenotypic patterns of Ag-specific CD8+ T cells in CVID patients are influenced by viral infection, HCMV and EBV specific CD8+ T cells were further analyzed for the expression of CD45RO, CD45RA, CD27, and CD28. Table 5-2 shows the results of this analysis on CD8+ T cells specific to both HCMV and EBV. There were no statistically significant differences between HCMV and EBV-specific CD8+ T cells with regard to any of the measured markers. However, EBV-

specific CD8+ T cells showed a trend towards higher levels of expressing for both CD27 and CD28 compared cells specific to HCMV (CD27: 60.05% \pm 4.3% EBV vs. 46.4% \pm 5.2% HCMV; $P > 0.05$) (CD28: 20.01% \pm 6.09% EBV vs. 10.4% \pm 3.8% HCMV; $P > 0.05$). Additionally, HCMV and EBV specific CD8+ T cells were analyzed in the context of patients with inflammation and without inflammation and in this regard, none of the comparisons between inflammatory and non-inflammatory patients reached statistical significance.

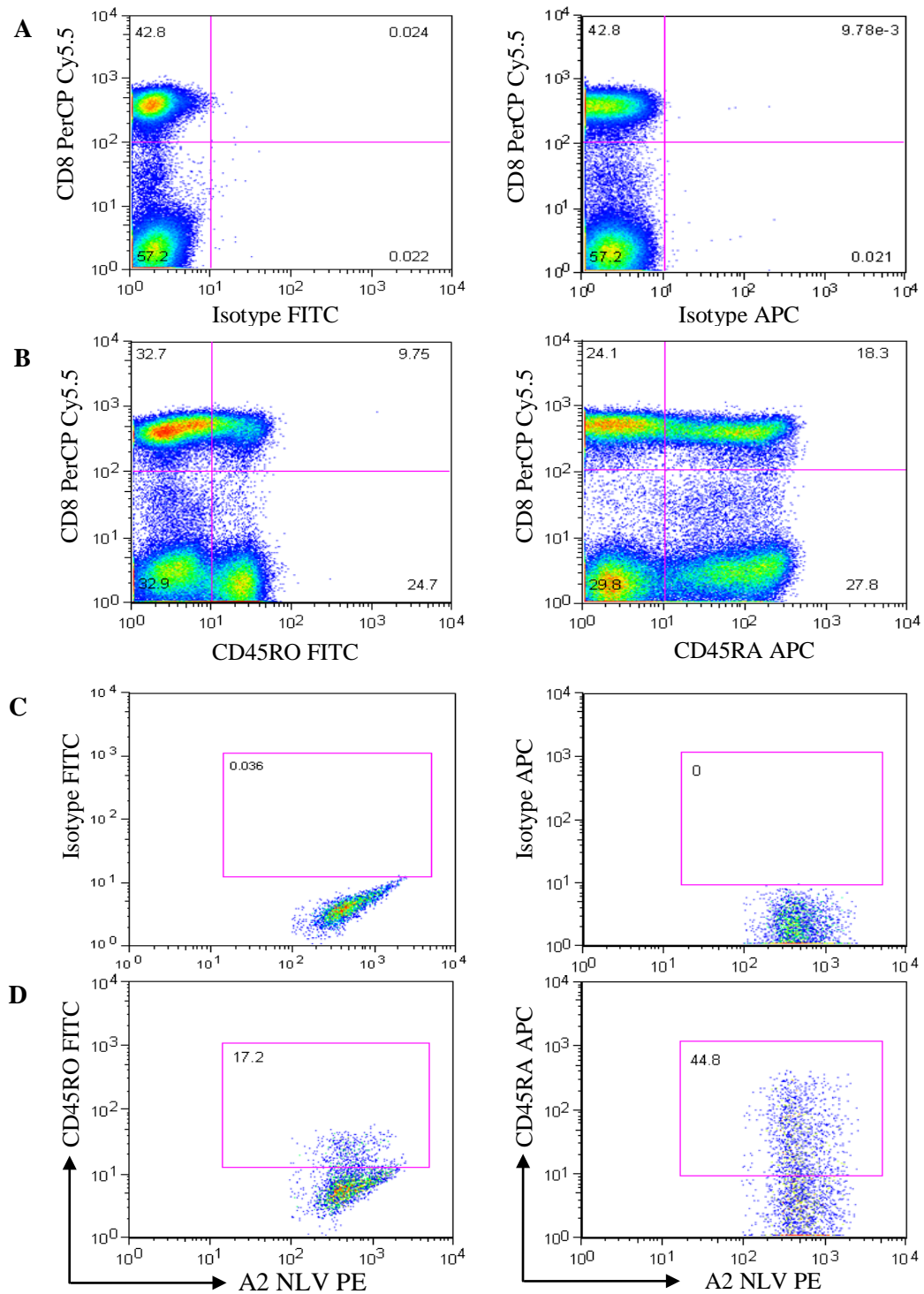


Figure 5-12: Representative FACS plots of CD45 (RO and RA) expressing CD8+ (NLV+) T cells from an inflammatory CVID patient.

For the expression of CD45 RA and RO, cells were gated on both total CD8+ T cells (Panels A and B) and NLV-specific CD8+ T cells (Panels C and D). Gates were adjusted according to the appropriate isotype controls.

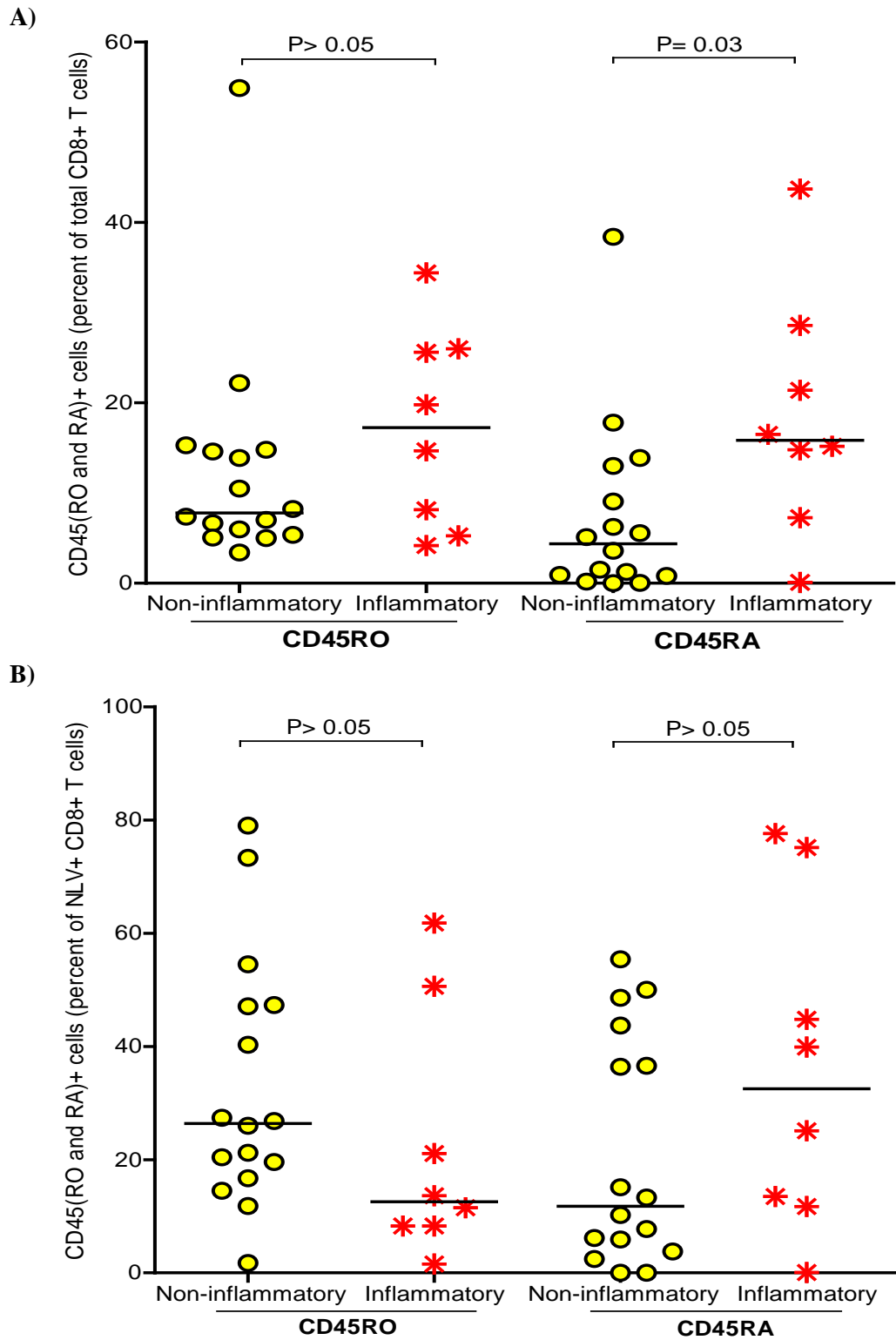


Figure 5-13: Mean percentage of CD8 (A) and NLV- specific CD8+ T cells (B) expressing CD45RO and CD45RA in CVID patients with (stars) and without inflammation (open circles).

Horizontal bars represent the median values. Each point represents a single subject.

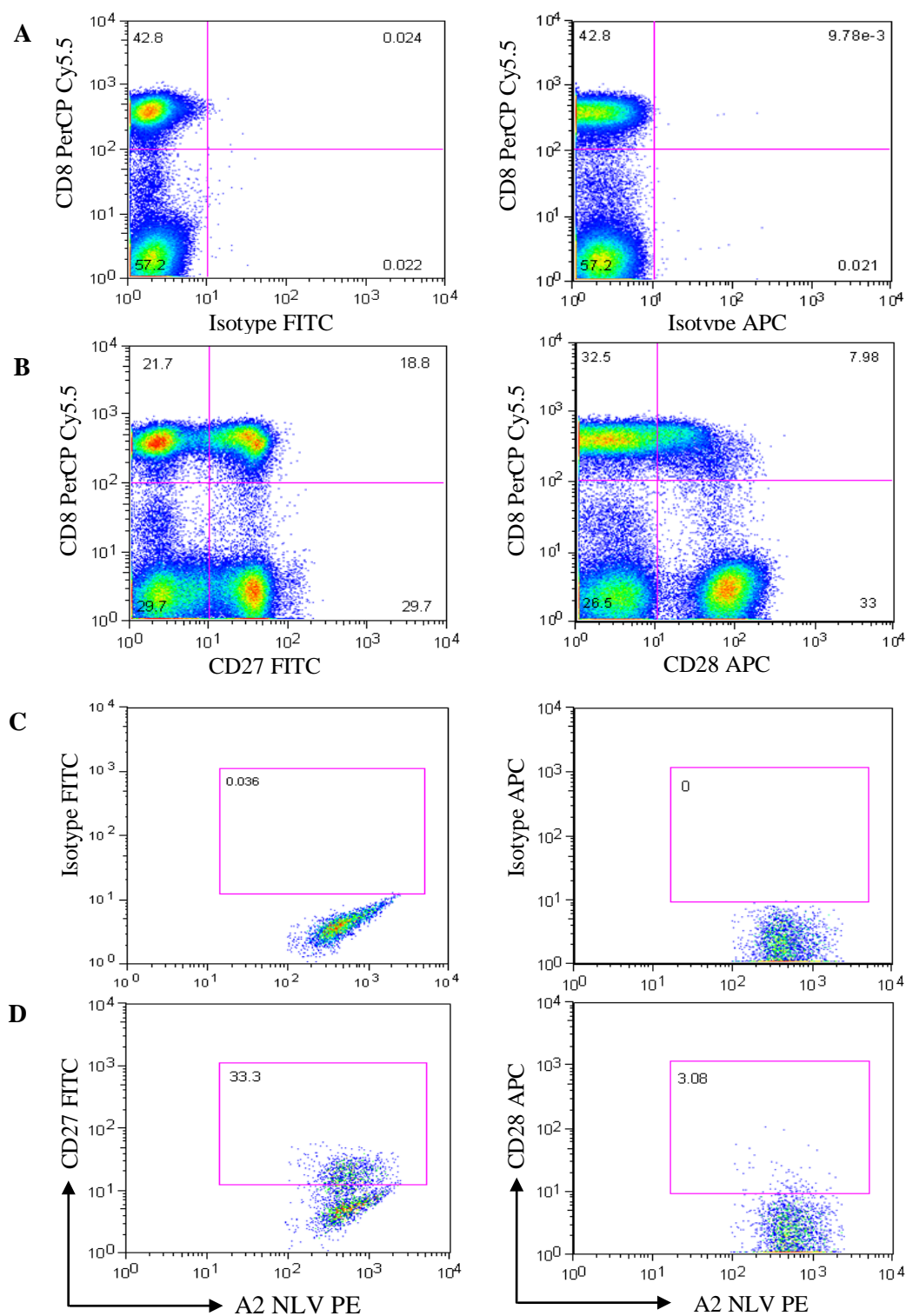


Figure 5-14: Representative FACS plots of CD27 and CD28 expressing CD8+ (NLV+) T cells from an inflammatory CVID patient.

For the expression of CD27 and CD28, cells were gated on both total CD8+ T cells (Panels A and B) and NLV-specific CD8+ T cells (Panels C and D). Gates were adjusted according to the appropriate isotype controls.

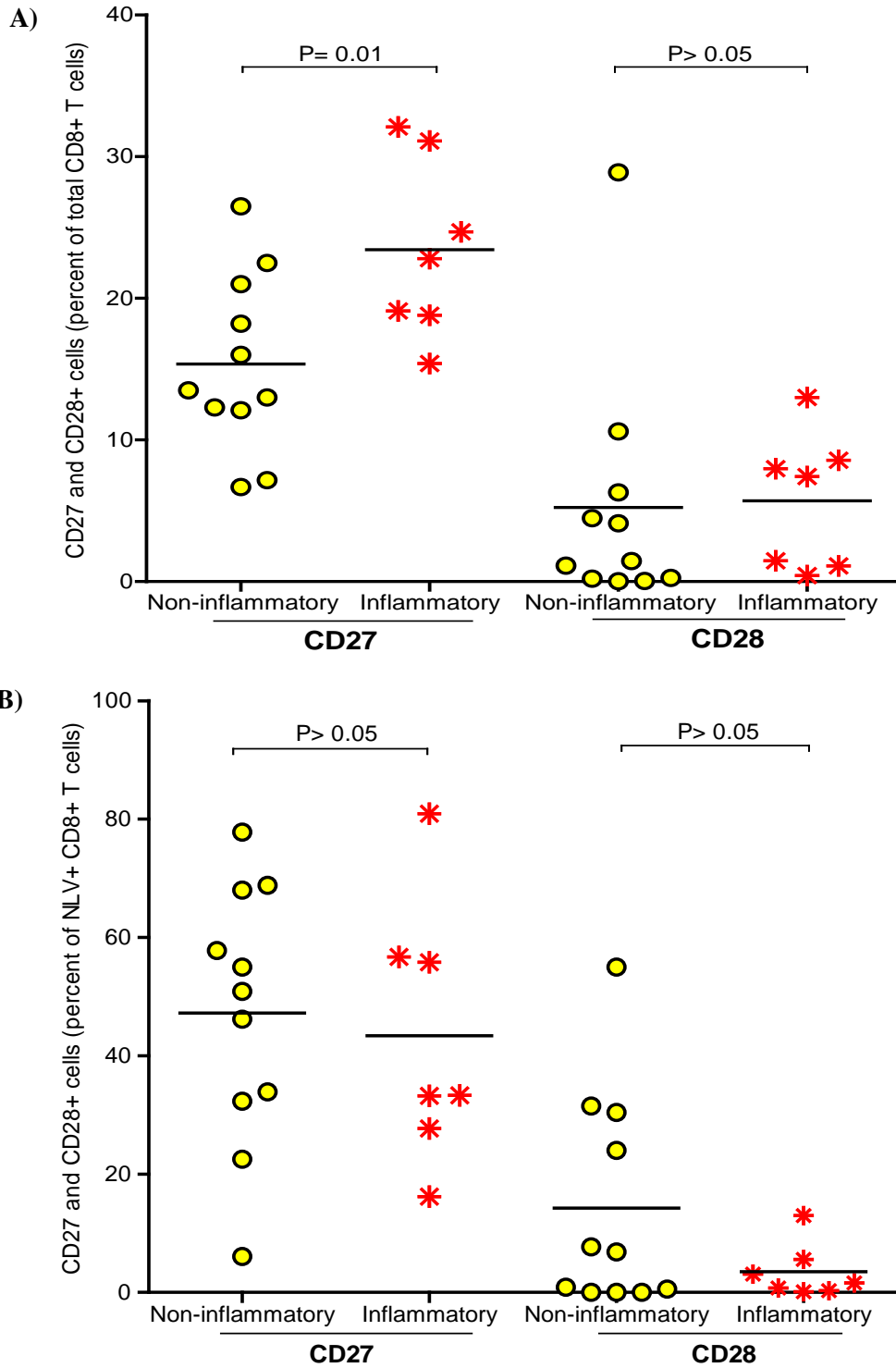


Figure 5-15: Mean percentage of total CD8 and NLV-specific CD8+ T cells expressing CD27 and CD28.

Mean percentage of total CD8 (A) and NLV specific CD8+ T cells (B) expressing CD27 and 28 in CVID patients with (stars) and without inflammation (open circles). Horizontal bars represent the mean values. Each point represents a single subject.

Marker	Pentamer	% Frequency (Mean \pm SD)	P value
CD45RO	HCMV	29.35 \pm 4.52	P > 0.05
	EBV	26.62 \pm 3.79	
CD45RA	HCMV	25.96 \pm 4.89	P > 0.05
	EBV	34.14 \pm 8.01	
CD27	HCMV	46.46 \pm 5.26	P > 0.05
	EBV	60.05 \pm 4.30	
CD28	HCMV	10.48 \pm 3.81	P > 0.05
	EBV	20.01 \pm 6.09	

Table 5-2: Evaluation of functional phenotypic markers on antigen-specific CD8+ T cells in CVID patients.

The table below shows the mean percentage frequency of each marker (CD45RO, CD45RA, CD27, and CD28) on HCMV and EBV-specific CD8+ T cells from CVID patients (with and without inflammation).

5.6 T cell phenotyping of CVID patients with and without inflammation

Samples from all patients were routinely subjected to extensive immunological phenotyping by staff in the diagnostic immunology laboratory as part of standard clinical management. As the data were available, I included them in my analysis.

For most of the parameters measured, there was no significant difference between the inflammatory and non-inflammatory groups; however, as shown in Figure 5-16 the overall percentage of CD8+ CD27- CD28- cells was significantly higher in patients with inflammatory diseases compared to those without inflammation ($P < 0.01$).

Analysis of these total cell population phenotyping data in the context of my own functional observations on HCMV-specific responses revealed a statistically significant inverse correlation between the proportion of NLV+ CD8+ T cells expressing PD-1 or granzyme B and the percentage of CD8+ CD27- CD28- cells (PD-1: $r^2 = 0.83$, $P < 0.0001$; Granzyme B: $r^2 = 0.36$, $P = 0.009$) (Figure 5-17). No correlation was seen with either the EBV-specific cells or between the percentages of CD8+ CD27- CD28- cells and the proportion of total CD8+ cells expressing PD-1 or granzyme B.

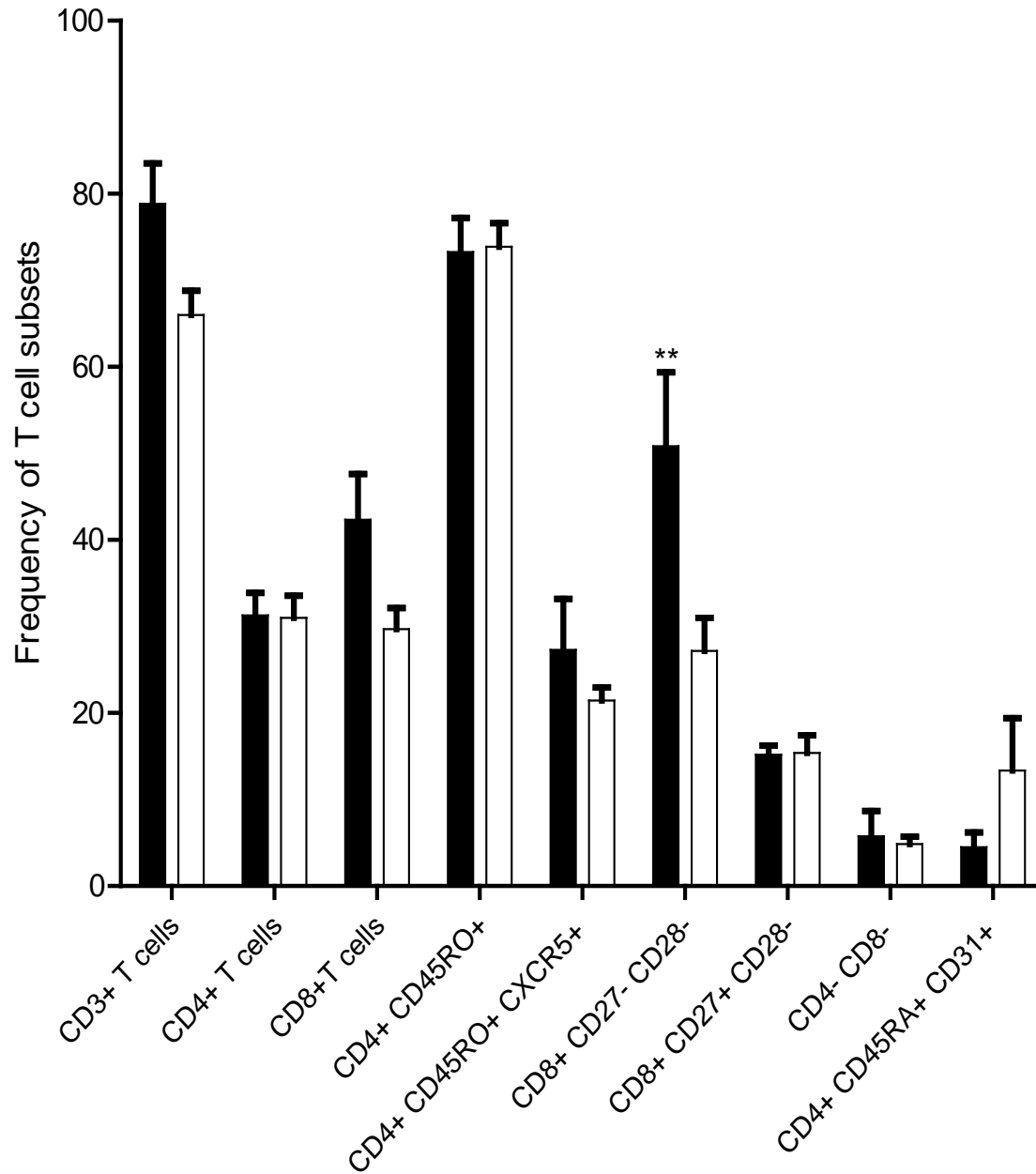


Figure 5-16: Phenotypic analysis of T cell markers in CVID patients with (black bars) and without inflammation (white bars).

Data were analyzed using 2-way ANOVA and significant values are shown with asterisks (** indicates $P < 0.01$).

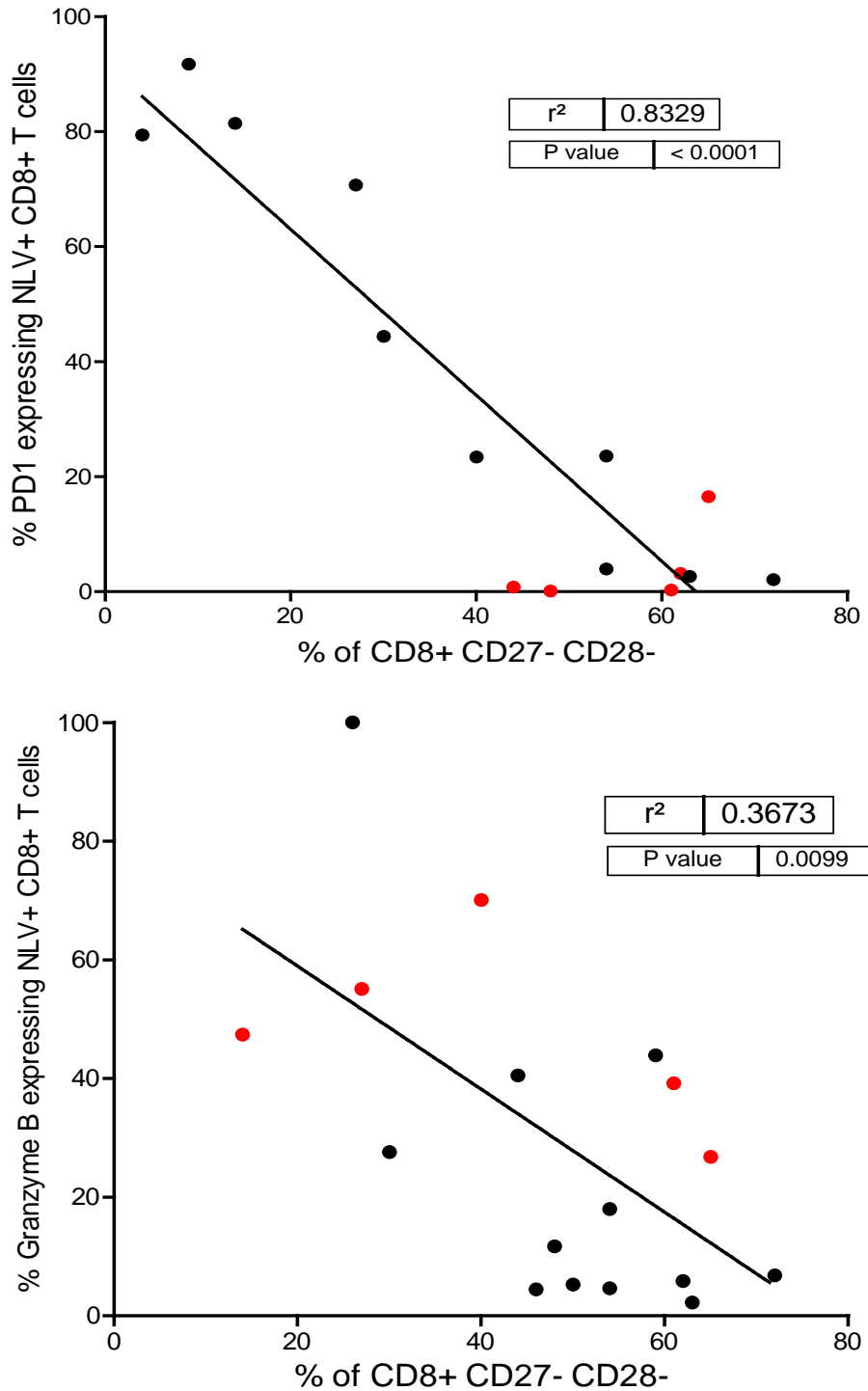


Figure 5-17: Reverse correlation between the expression of functional markers (PD-1 and granzyme B) on HCMV-specific CD8+ T cells and the proportion of CD8+CD27-CD28- cells of CVID patients with (red dots) and without inflammation (black dots).

Linear regression, r^2 , and P values were determined in Graphpad Prism.

5.7 Discussion

The aim of this chapter was to further investigate the phenotypic characteristics of HCMV and EBV-specific CD8+ T cells in CVID patients with and without inflammation. On this basis, CD8 cells were described using a broad panel of surface (CD57, PD1, CD45RO, CD45RA, CD27, and CD28) and intracellular (granzyme B) markers. This is the first example of the phenotypic signature of virus-specific T cells being investigated in inflammatory CVID patients compared to non-inflammatory CVID patients.

CVID patients with inflammatory disease, but not those without inflammatory disease, had significantly elevated frequencies of CD57+ HCMV-specific CD8+ T cells ($P=0.007$). In the non-inflammatory group there was a wide range of CD57+ frequencies. In contrast to CD57 expression on HCMV-specific CD8+ T cells, the frequency of CD57 expression on EBV-specific CD8+ T cells in CVID patients (with and without inflammation) were comparable with healthy subjects ($P>0.05$).

Although, the exact role of CD57+ cells is not fully understood; it has been shown that there is a correlation between HCMV seropositivity and the number of CD8+CD57+ cells in transplant patients and healthy elderly subjects, suggesting the involvement of persistent viral antigen stimulation behind the expansion of CD8+ CD57+ T cells. For example, a dose dependent rise in the number of CD57+ cells has been observed following the addition of both IL-2 and HCMV supernatant to allogenic fibroblasts. Interestingly, after day 13 this increase in the frequency of CD57+ CD8+ T cells has been found only in the presence of HCMV (Rowbottom et al., 2000).

Although the molecular mechanisms and signaling pathways of CD57 are not yet fully understood, follow up studies will determine whether a high frequency of CD57+ CD8+ T cells in patients without inflammatory disease is predictive of developing inflammatory disease. Moreover, higher frequencies of CD8+ T cells expressing CD57 in these patients may have occurred as a direct consequence of repeated episodes of past virus replication, resulting perhaps from the lack of specific antibodies. Although asymptomatic, this may have led to an expansion of mature antigen-specific populations which may be necessary for control of the infection. It may also be possible that in this impaired immune setting, the inflammation as a direct or indirect consequence of repeated antigen exposure leads to a marked elevation of CD8+ T cells expressing CD57 in CVID patients with inflammation.

Analysis of intracellular levels of granzyme B which is an indicator of cytolytic capacity of T cells also yielded similar results. A significant elevation was observed in the levels of granzyme B on both total CD8+ T cells and HCMV-specific CD8+ T cells of CVID patients compared to healthy individuals ($P= 0.002$). No such difference was found with CD8+ T cells specific to EBV ($P> 0.05$). High level expression of granzyme B is indicative of the cytotoxic capacity of CD8+ T cells specific to HCMV in these patients. It is yet not clear why HCMV and EBV-specific CD8+ T cell populations followed different patterns of CD57 and granzyme B expression. It may reflect the differing nature of latency associated with these pathogens or reflect persistent presence of HCMV antigen (this will be discussed in Chapter 6).

Unlike CD57 and Granzyme B in which expression in inflammatory CVID patients was significantly higher than non-inflammatory patients as well as healthy controls, the

levels of PD-1 expression (on both total CD8+ T cells and Ag-specific CD8+ T cells) in these patients although marginally higher than healthy controls, was generally very low.

This is the first study looking at the PD-1 expression of CD8+ T cells in CVID patients. Together, these results indicate that compared to non-inflammatory patients, CD8+ T cells in CVID patients with inflammation not only display a distinctive phenotype that is characterized by a high frequency of CD57 expression and low levels of PD-1 expression and this phenotype also was specific to HCMV. It appears that HCMV-specific CD8+ T cells seen in this particular group of patients are functionally competent as they are characterized by high expression of CD57 and low PD-1 expression. In addition, as described in Chapter 3, HCMV stimulated CD8+ T cells of this group of CVID patients produce high levels of IFN- γ and TNF- α after overnight peptide stimulation.

Both groups of CVID patients had high frequencies of granzyme B-expressing HCMV-specific CD8+ T cells but expressed very low levels of PD-1. High levels of PD-1 expression have been associated with poor proliferative capacity of T cells in a number of chronic viral infections (Trautmann et al., 2006; Golden-Mason et al., 2007) and in the case of HCMV, high PD-1 expression on CD8+ T cells has been observed in liver transplant patients during periods of HCMV replication and disease and also in renal transplant patients experiencing high level HCMV replication (Sester et al., 2008; La Rosa et al., 2008). So this implies that HCMV CD8+ T cell in inflammatory patients are destined to have high proliferative potential.

The association found between the proportion of NLV+ CD8+ T cells expressing PD-1 (or granzyme B) and the overall percentage of CD8+ CD27- CD28- cells was

interesting. PD-1 is considered as a negative regulator of T cell function, yet, by all measures, the HCMV specific response in both subsets of CVID patients is functional. The association of this marker with lack of CD27 and CD28 expression is perhaps more consistent with direct role in the control of T cell differentiation.

The statistically significant elevation in the frequency of CD8+CD27-CD28- cells in the inflammatory CVID patients was of interest in the context of a possible role for HCMV in the causation or potentiation of the inflammatory disease. This late-differentiated effector cell type is typically enriched in HCMV+ individuals (Waller et al., 2008) and is a key contributor to the functional deficit associated with immunosenescence (Akbar and Vukmanovic-Stejic, 2007), thought to be a consequence of multiple re-exposures to antigen via repeated episodes of virus reactivation (Cantisan et al., 2009).

HCMV infection has been known to increase the magnitude of circulating CD27-CD28- T cells and it has been suggested that these cells may play a role in the immunosuppression associated with HCMV infection (Tovar-Salazar et al., 2010) or might be a reflection of continuous stimulation of HCMV-specific CD8+ T cells over long period of times (Sacre et al., 2005). Regarding CVID, it is not yet clear whether this HCMV associated phenomenon has any role in attenuating immune response against other pathogens such as EBV.

Various other cell surface markers including CD45RA (and CD45RO), CD27, and CD28 were also used to further characterize the heterogeneity and functional signature of HCMV and EBV-specific CD8+ T cells in the CVID patients with and without inflammation. Phenotypic analysis of T cells in CVID samples showed a marked inter-individual variability of effector/memory cell population that expressed varying levels of

CD45RO, CD45 RA, and CD27. This was seen with both HCMV and EBV specific cells in inflammatory as well as non-inflammatory patients. CD28 expression which was low (or negative) on both total and Ag-specific CD8+ T cells was the only exception. Although based on the combined expression of various effector (or memory) markers expressed by CD8+ T cells, anatomical origins, and the state of infection (acute or latent), different differentiation models for memory CD8+ T cells have been proposed (Appay et al., 2002;van Lier et al., 2003;van Leeuwen et al., 2006), it seems that there is considerable inter-individual variability indicating the complexity of direct extrapolation of memory state of Ag-specific immune responses. However, low PD-1 expression combined with the CD27+CD28- phenotype suggests that HCMV-specific CD8+ T cells in CVID patients are not late memory cells and are not in a stunned or memory inflated state, rather, they resemble intermediate memory T cells. However, it is worth noting that further analysis will be needed to give us a better understanding of the role of these specific T cell subsets in these patients.

Taken together, the data presented in this chapter showed that in CVID patients with inflammatory disease CD8+ T cells specific to HCMV (but not EBV) exhibit a distinct phenotypic functional signature as determined by high expression of CD57 and granzyme B, but low PD-1 expression with no sign of exhaustion. These cells tend to consist of an effector-memory phenotype. In line with the distinct cytokine profile of HCMV - specific CD8+ T cell responses seen in inflammatory CVID patients (Chapters 3 and 4) these data support the hypothesis that persistent activation of CD8+ T cells caused by reactivation of virus may act as a trigger of the inflammatory process in this particular subset of patients.

Chapter 6

6 Proliferative capacity of CD8+ T cells in CVID patients

6.1 Introduction

In the presence of growth factors, T lymphocytes naturally proliferate upon being exposed to mitogen or antigens. What is not yet clear is the nature of the factors controlling T cell proliferation and whether these can be regulated differentially in T cell subsets (Migliaccio et al., 2006). The relative composition and size of the memory CD8+ T cell population has been shown to be influenced by various factors including the interaction of T cell receptor (TCR) with peptide–MHC complexes (signal 1), co-stimulatory molecules (signal 2), and inflammatory cytokines (signal 3) (Mescher et al., 2006a; Wiesel et al., 2009) as shown in Figure 6-1.

The level of antigen available *in vivo* seems to be an important element in governing the extent of T cell proliferation. For example, by infecting mice with recombinant vaccinia strains producing either high or low quantities of an ovalbumin (OVA) epitope, it has been shown that the size of the responding CTL population was proportional to epitope abundance (Wherry et al., 1999; Kaech et al., 2002c). Moreover, it has been suggested that the inflammatory environment associated with infection can contribute to high levels of proliferation of Ag-specific CD8+ T cells. In addition to the crucial role of proinflammatory cytokines in stimulating antimicrobial defenses, the importance of proinflammatory cytokines such as IFN- γ for optimal CD8+ T cell proliferation during virus infection has also been studied (Masopust et al., 2004; Whitmire et al., 2005; Haring et al., 2006; Jameson and Masopust, 2009). Extensive CD8+ T cell proliferation has been observed during viral and intracellular bacterial infections although this response is quite heterogeneous depending on different factors such as type of antigen, cytokines and co-

stimulator molecules (Callan, 1996;Wills et al., 1996;Kaech and Ahmed, 2001;Migliaccio et al., 2006). Understanding the mechanisms behind this expansion as well as its regulation could provide important therapeutic opportunities to enhance or abrogate immune responses.

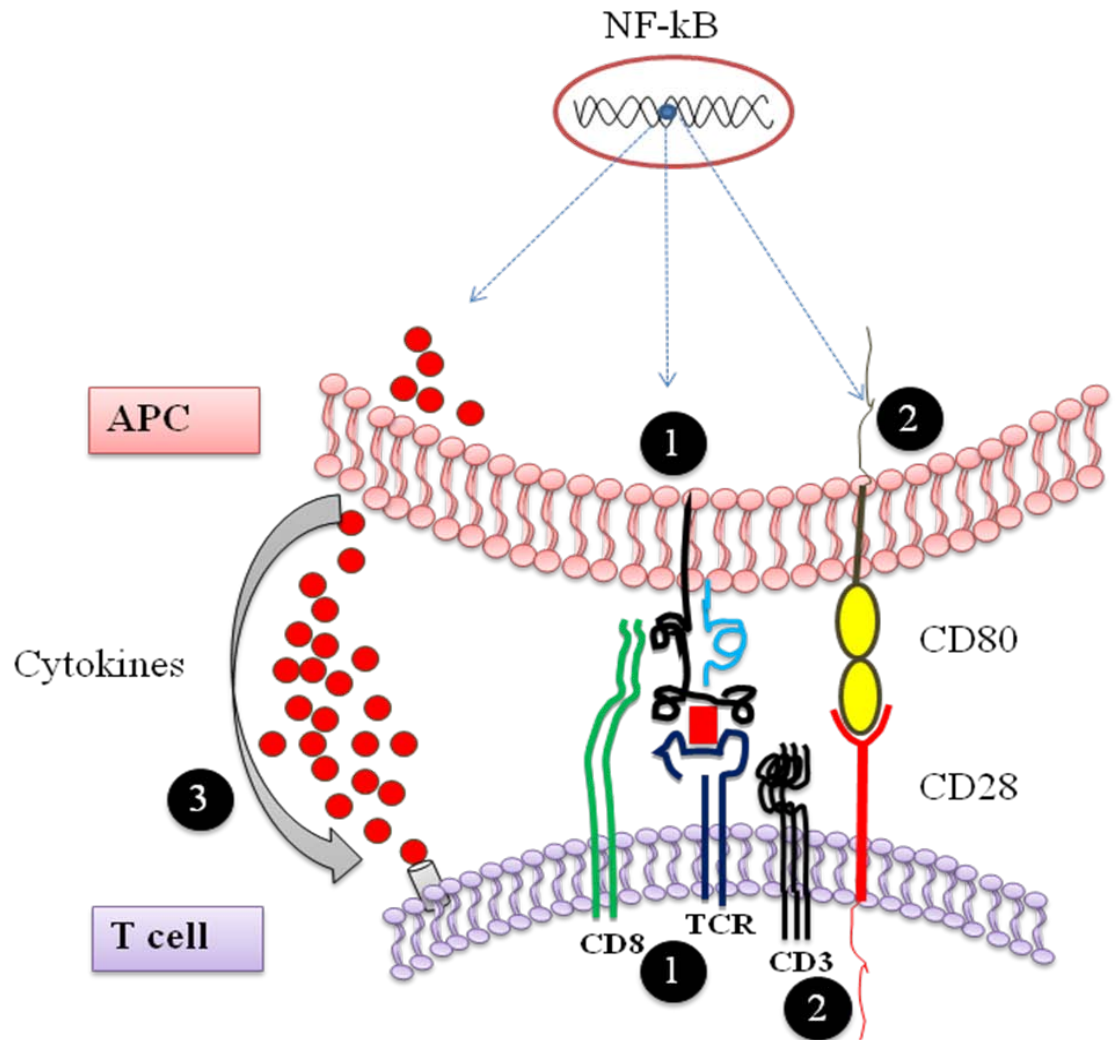


Figure 6-1: Schematic representation of signals (black circles) involved in T cell activation.

Interaction of T-cell receptor with antigen presented in the context of MHC class I is the key stage (also termed signal 1) in T cell activation. Co-stimulatory molecules and proinflammatory cytokines form signals 2 and 3, respectively.

The initial aim of this chapter was to investigate the proliferative capacity of Ag-specific CD8+ T cells to ask whether these cells in CVID patients with and without inflammation could proliferate following specific peptide stimulation in the absence of cytokines and co-stimulators. In a conventional assay using 5 days stimulation of CFSE labeled PBMCs with the pp65 peptide pool in the presence of exogenous IL-15, the expansion of HCMV specific CD8+ T cells from CVID patients has been demonstrated (Raeiszadeh et al, personal communication). In the case of proliferation, the chapter further aimed to study expression levels of Ki-67, as a marker of *in vivo* cycling, and activation marker HLA-DR to assess the specificity of proliferation phenomenon.

Ki-67, a nuclear protein which is widely accepted as an intracellular marker associated with cellular proliferation, was first described by Gerdes et al (1991). The name of Ki-67 is derived from both Kiel (the city of origin) and the number of the original clone in the 96-well plate (Gerdes et al., 1991;Shedlock et al., 2010). Using immunohistochemistry or flow cytometry staining techniques, Ki-67 has been frequently measured as a marker of the proliferative capacity and for determining the growth fraction of a given cell population (Brown and Gatter, 2002;Shedlock et al., 2010).

A number of factors such as the level of T cell response, the number of targeted epitopes, functionality of the responses, and the longevity of the response have been suggested to be important in determining the outcome of disease in viral infections. In both murine models and human infections, CD8+ T cells capable of recognizing very low amounts of antigen have been shown to be more efficient in keeping viruses in check (Alexander-Miller et al., 1996;Almeida et al., 2007;Walker et al., 2010). Recent developments in tetramer technology have made it possible to assess the avidity of T cell

receptors (TCRs) on a T cell via modifications in the binding interaction of the peptide-MHC: CD8. This approach, which allow an immediate *ex vivo* analysis of CD8 binding, utilizes T cell binding tetramers where CD8 interaction is abrogated (termed CD8-null tetramers) to identifying CD8+ T cells of high avidity (Walker et al., 2010).

There is no data available regarding functional (HCMV specific-) CD8+ T cell avidity in CVID patients. Therefore, this chapter aimed to study the binding avidity of CD8+ T cells in a subset of CVID patients with and without inflammation. Additionally, this chapter further aimed to investigate the association between activated cycling HCMV-specific CD8+ T cells and inflammatory CVID by measuring the expression of CD73 (an ecto-5-nucleotidase which is known to have anti-inflammatory function) on both total CD8+ and HCMV-specific CD8+ T cells. CD73 is abundantly expressed on endothelial cells and leukocytes. As a component of an ectoenzyme cascade on lymphocytes, CD73 converts extracellular nucleoside-5'-monophosphates (AMP and IMP) into nucleosides such as adenosine and inosine. By acting on G protein-coupled receptors, adenosine can induce anti-inflammatory cellular responses (Zimmermann, 1992;NiemeLa et al., 2004;Grunewald and Ridley, 2010). It has been suggested that adenosine not only has anti-inflammatory properties in many physiological and pathological events (Cronstein, 1994;Ohta and Sitkovsky, 2001;McPherson et al., 2001;Grunewald and Ridley, 2010) but also acts, through adenosine receptors on T cells, to stimulate the production of T-regulatory cells and mediate immune suppression (Kobie et al., 2006;Deaglio et al., 2007). An ischemic representation of CD73 expression and its anti-inflammatory activities is illustrated in Figure 6-2.

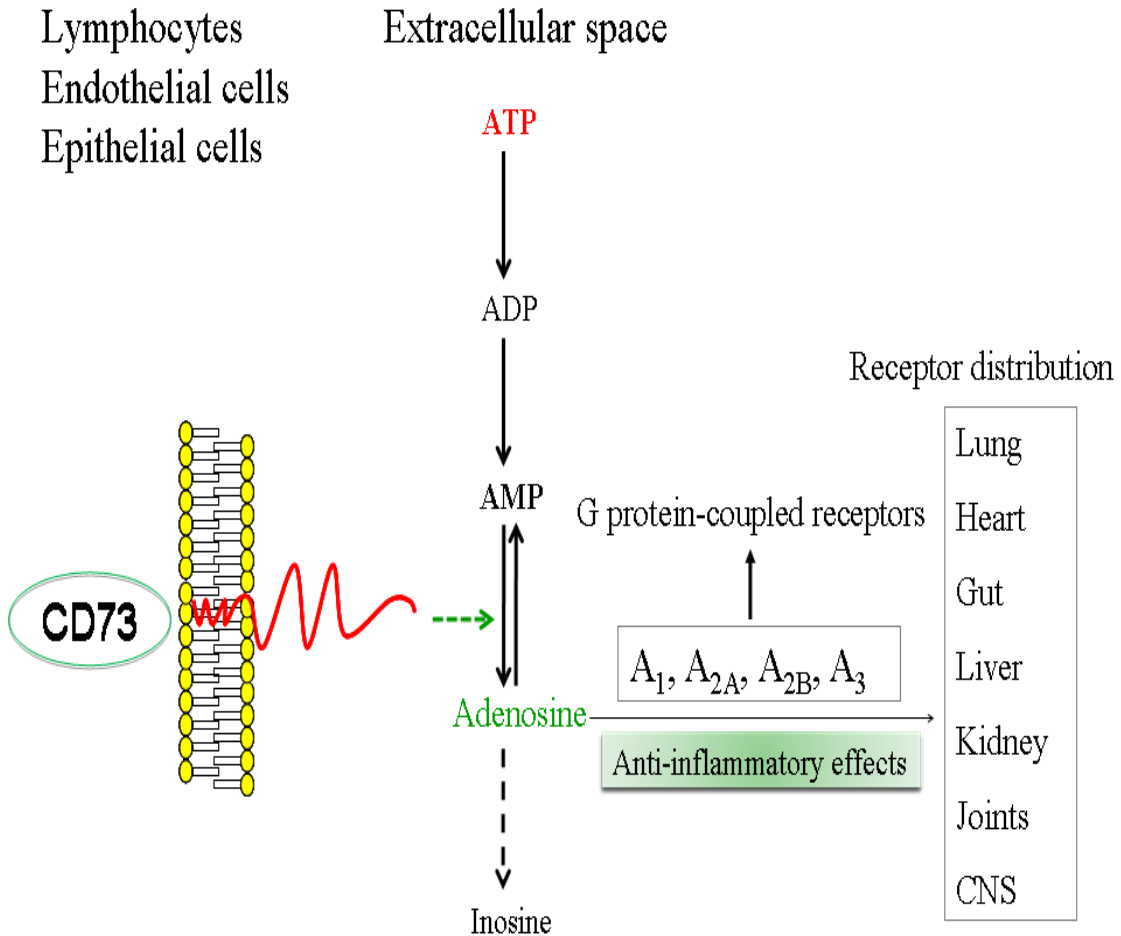


Figure 6-2: Schematic representation of CD73 expression and its anti-inflammatory potential.

CD73 is a 70-kDa cell surface enzyme expressed on many cell types including subsets of lymphocytes, endothelial cells and epithelial cells. During inflammation and the initiation of primary immune responses, ATP is released from damaged target cells into the extracellular environment and is converted to AMP by CD39. This AMP is converted to extracellular adenosine by the catalytic action of CD73. Why adenosine is important? Extracellular adenosine has potent immunosuppressive and anti-inflammatory effects, mediated through its four G protein-coupled receptors: A₁, A_{2A}, A_{2B}, and A₃. The image is redrawn from *Nat Rev Immunol*; 2005 (5): 760 – 71.

The final aim of this chapter was to investigate the telomere length of CD8+ T cells in a FACS based Flow Fish as described in Chapter 2 (section 2.9). Telomeres are gene-poor regions that are located at the end of chromosomes and contain long repeats of TTAGGG sequences protecting chromosomes from inappropriate DNA damage and recombination. Telomeres shorten after each cell division because DNA polymerase cannot duplicate the 3' end of linear DNA. After a critical length is reached, telomeres cannot function properly, and cells will either become senescent or apoptotic (Verdun and Karlseder, 2007;van de Berg et al., 2010b). Although large inter-individual variations are seen among people of the same age it has been shown that telomere length steadily decreases in somatic cells with aging. The exact origins of this variation have not been elucidated, but the role of genetic factors, oxidative stress, and chronic inflammatory challenges have been implicated (Epel et al., 2004;van de Berg et al., 2010b). In this context, it has also been shown that short telomere length in leukocytes can be a risk factor for age-related diseases such as cardiovascular disease and Alzheimer's disease (Samani et al., 2001;Brouillette et al., 2003;Cawthon et al., 2003). Spontaneous hyperactivity of the immune system such as autoantibody production, inflammation, and oxidative stress are a characteristic of many autoimmune and inflammatory diseases. Although the mechanisms behind these processes are not yet fully understood, interestingly, inflammation as well as increased leukocyte renewal have been associated with telomere shortening suggesting a link between the telomere (or telomerase) system and these diseases (Georgin-Lavialle et al., 2010). Telomere length erosion of the circulating T cell pool has also been found to be exacerbated following cytomegalovirus infection suggesting that immune response to HCMV can be

a major cause in variation of telomere length in healthy subjects (van de Berg et al., 2010b).

Overall, this chapter aimed to assess the functional aspects of CD8+ T cells, with respect to proliferative capacity following single virus-specific peptide stimulation, CD8 binding avidity and anti-inflammatory potential of CD8+ T cells in COVID patients (with and without inflammation).

6.2 *Ex vivo* Proliferative potential of CD8⁺ T cells in CVID patients

PBMCs from a subset of samples from CVID patients (with and without inflammation; n = 6 and n = 8, respectively), and healthy individuals (n = 4) were CFSE labeled before being stimulated with PHA (positive control), HCMV A*0201 restricted NLV peptide, a HCMV pp65 peptide pool, and EBV A*0201 restricted GLC peptide in the absence of any exogenous cytokines or co-stimulators. To determine background levels of proliferation, cells were CFSE stained and incubated without stimulation. After 5 days, cells were examined by FACS for a decrease in CFSE fluorescence indicating proliferation.

No expansion was observed in PBMC from non-inflammatory patients or healthy controls (Figure 6-3A). Interestingly, CD8⁺ T cell proliferation was found in all six PBMCs from inflammatory CVID patients following HCMV peptide stimulation but not following stimulation with the EBV GLC peptide (Figure 6-3B). Figure 6-4 illustrates an example of representative FACS plots showing CFSE-labeled cells from a representative non-inflammatory CVID patient (A) and an inflammatory CVID patient (B) after 5 days stimulation with and without antigens.

Two inflammatory and two non-inflammatory patients had subsequent samples (2-3 samples per patient) and these were re-tested over several months to determine whether these observations were consistent over time. The proliferation seen not only was consistent between inflammatory CVID patients but it also was consistent over time (Figure 6-5A). In addition, the frequency of HCMV-specific CD8⁺ T cells from same inflammatory patient was monitored over a long period of time to test whether this

heterogeneity seen in proliferation is associated with the levels of CD8+ T cells specific to HCMV. As shown in Figure 6-5B (bar chart), the frequency of HCMV-specific CD8+ T cells remained relatively stable and consistent over long period of time.

Moreover, using ModFit software the extent of CD8+ T cell proliferation was equivalent to 5 divisions consistent with a doubling time of approximately 24 hours (Figure 6-6).

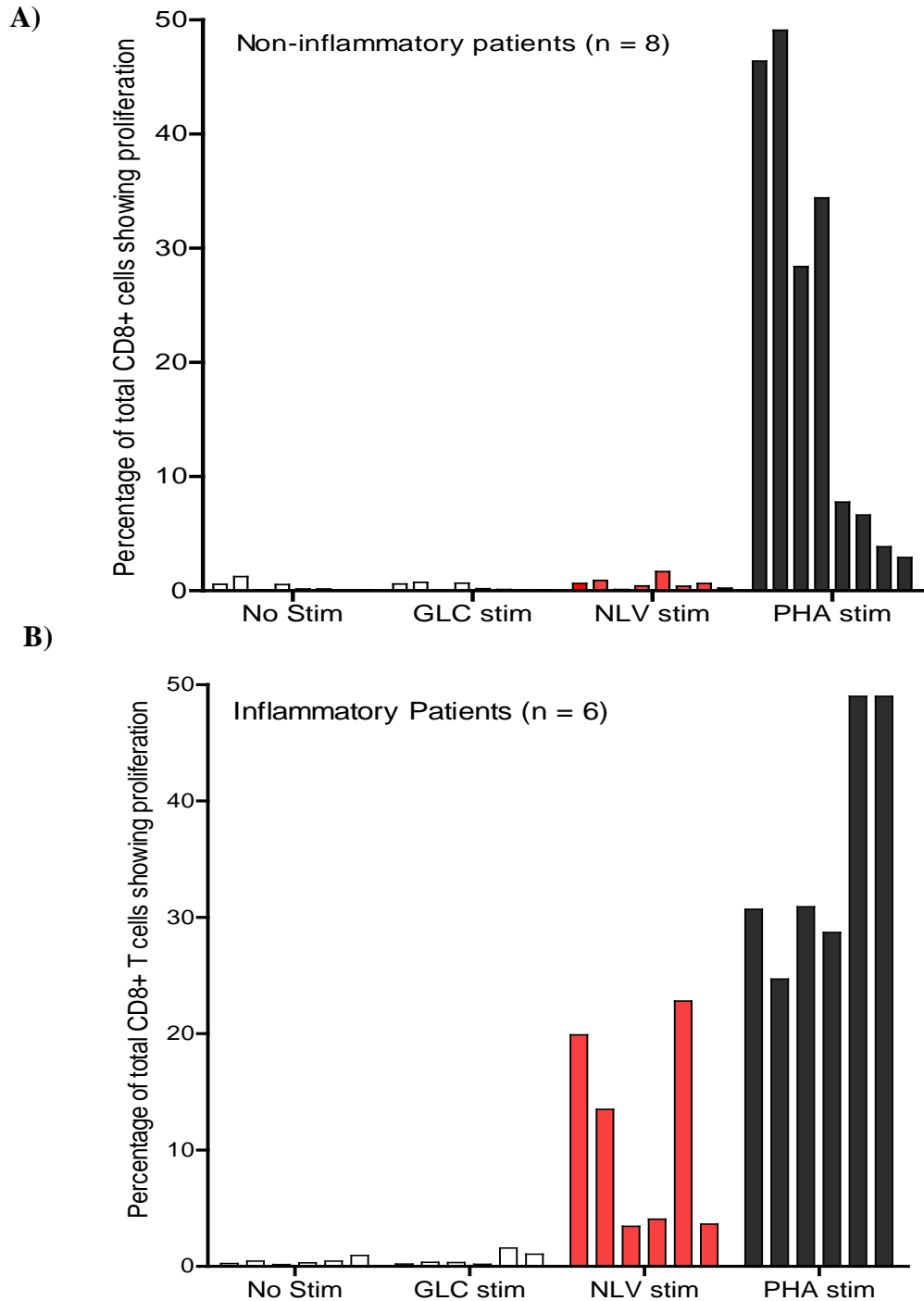


Figure 6-3: The proliferation response of CD8+ T cells to 5 days stimulation (shown as stim) with and without specific antigens.

Bars indicate the proportion of CD8+ T cells that had expanded (judged by low CFSE staining) 5 days after stimulation. Each bar on the graphs shows the results from a single patient. Except for PHA stimulation, in non-inflammatory CVID patients (n = 8) there was no proliferation of CD8+ T cells in response to both HCMV (NLV) and EBV (GLC) peptide stimulation (A). In contrast, all CFSE-labeled CD8+ T cells from inflammatory patients (n = 6) proliferated in response to NLV peptide stimulation (B).

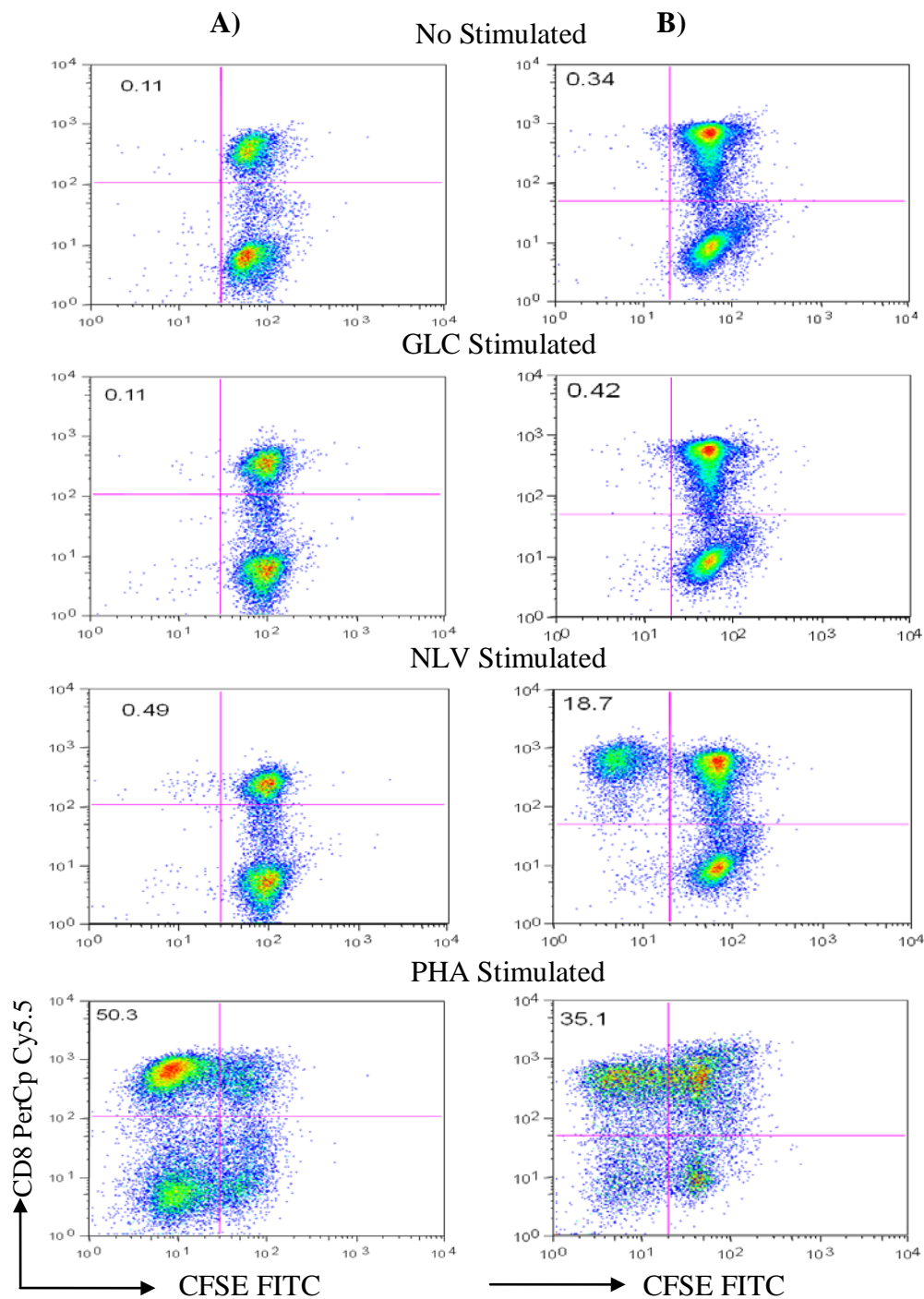


Figure 6-4: Representative FACS scatter plots demonstrating the response of CFSE labeled CD8+ T cells to 5 days stimulation with and without specific antigens.

Gates were set on the live T lymphocyte population and CD8+ T cells. Numbers in the top left quadrant indicate the proportion of proliferating cells expressed as the percentage of total CD8+ T cells. Panel A illustrates the CFSE labeled T cells of a representative non-inflammatory CVID patient compared to an inflammatory CVID patient shown in panel B.

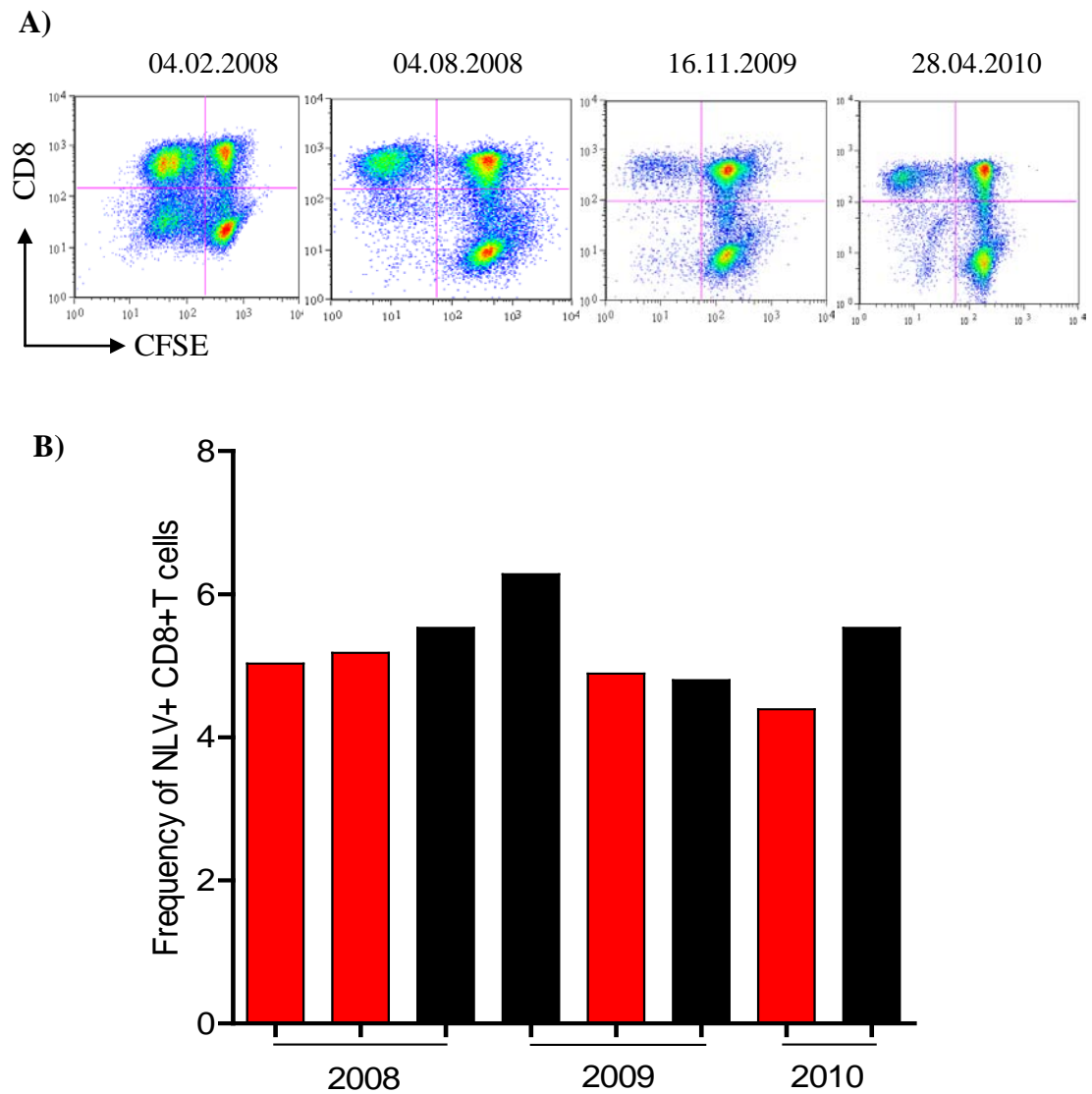


Figure 6-5: Proliferation of CD8+ T cells at different time points from an inflammatory CVID patient.

Proliferation of CFSE labeled CD8+ T cells of inflammatory CVID patient in four different time points (A). Gates were set on the live T lymphocyte population and CD8+ T cells. The proliferation was consistent although the extent of proliferation was heterogeneous. The frequency of HCMV (NLV)-specific CD8+ T cells from same inflammatory CVID patient is shown over different time points (B). Red bars indicate the frequency of NLV-specific CD8+ T cells related to the indicated FACS plots.

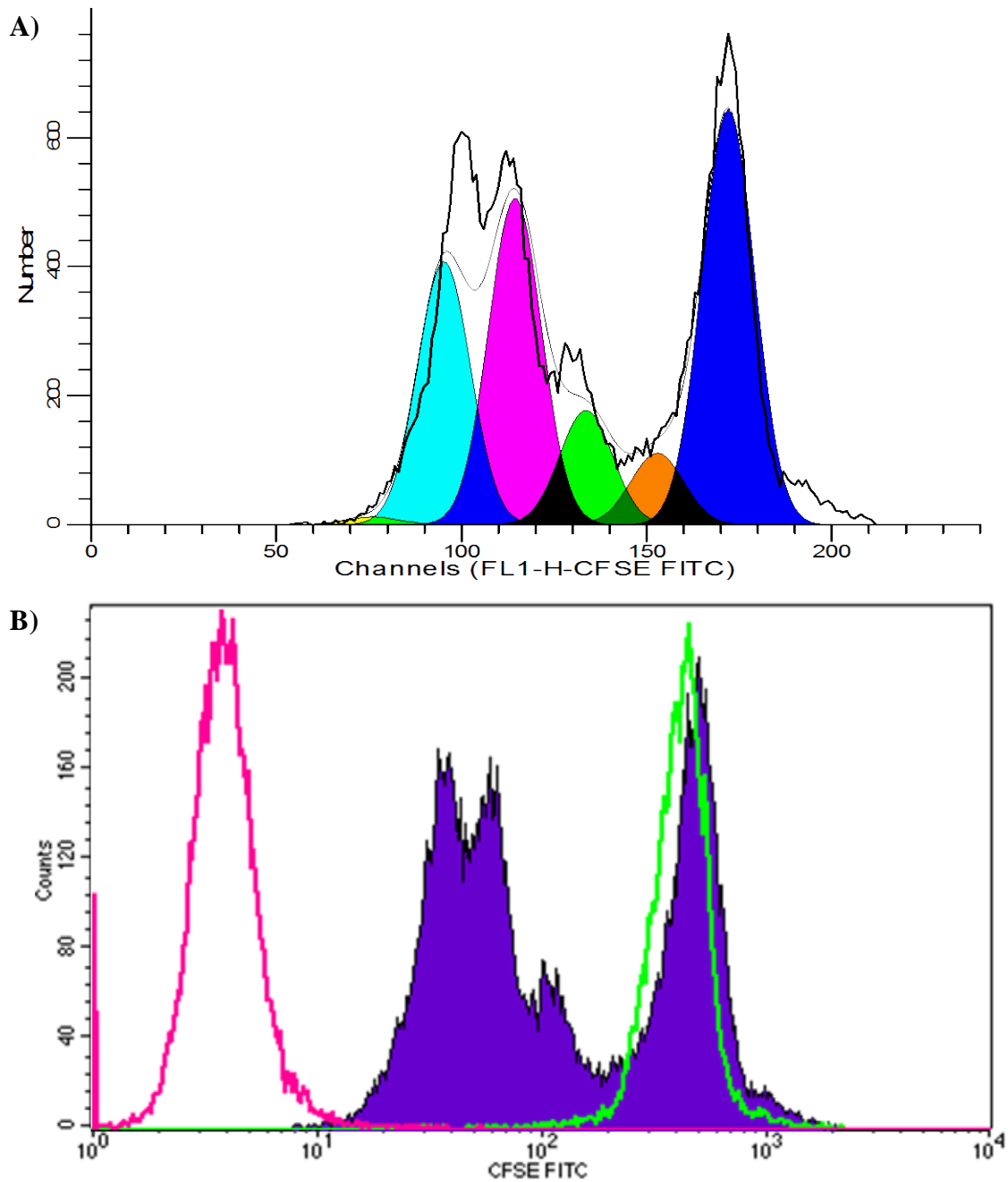


Figure 6-6: Generational analysis of proliferation of a representative NLV peptide stimulated inflammatory sample.

A) Using ModFit software the expanded cells were analyzed to assess how many generation they went through. B) The same expanded CD8+ T cell population is plotted (as histogram) against control samples: unstimulated cells and unlabeled cells which are shown as green and red lines, respectively.

6.3 Investigation of soluble factors mediating proliferation of antigen-specific CD8+ T cells in CVID patients with inflammation

The proliferation described above was only seen in HCMV-specific CD8+ T cells of inflammatory CVID patients. This phenomenon presented a potential model system for investigating the control of T cell proliferation in these patients. As a first step to assess whether soluble factors were involved in the proliferation process two approaches were adopted. In the first, PBMCs from inflammatory patients were stimulated with a mixture, containing different ratios, of the HCMV and EBV peptides to determine whether HCMV proliferating cells produced a soluble factor capable of rendering the proliferation of EBV-specific CD8+ T cells.

The results shown in Figure 6-7A show that when cells were stimulated with mixtures of NLV and GLC peptides, the majority of the proliferated cells were NLV specific. Thus, GLC specific cells did not proliferate after cognate peptide stimulation even in an environment favouring proliferation of NLV specific cells. To address whether these expansions were antigen specific in the mixed population stimulated cells, the expanded cells were surface stained with class I HLA pentamers for the HCMV and EBV A*0201 restricted epitopes. The majority (>90%) of the proliferated cells were HCMV specific in all cases indicating that the EBV-specific CD8+ T cells did not proliferate even in an environment favouring proliferation (Figures 6-7B). Figure 6-8 illustrates representative FACS plots demonstrating the response of CFSE labelled CD8+ T cells from inflammatory CVID patient in response to mixed peptide stimulation (NLV+GLC).

The second approach involved the transfer of supernatants from the proliferating cells of inflammatory CVID cultures (n = 4) at 2 days post stimulation into cell cultures taken from non inflammatory (n = 1) as well as healthy controls (n = 3). The results indicated that supernatant transfer from NLV stimulated PBMCs from inflammatory CVID patients enhanced NLV-peptide driven proliferation of HCMV-specific CD8+ T cells of both healthy and non-inflammatory patients in a dose dependent fashion (Figure 6-9). Although the expansion was not as substantial as that observed in the T cell cultures derived from the CVID inflammatory disease patients, it was clearly HCMV specific as no expansion was found in EBV (GLC) stimulated CD8+ T cells following supernatant transfer (Figure 6-10A). To determine whether these expanded CD8+ T cells were HCMV specific, labeled cells were surface stained with NLV class I HLA pentamers and anti-CD8 antibodies. More than 80% of expanded CD8 cells were found to be HCMV (NLV) specific. Stimulation with a pp65 peptide pool produced similar results to those observed with NLV peptide stimulation (Figure 6-10B).

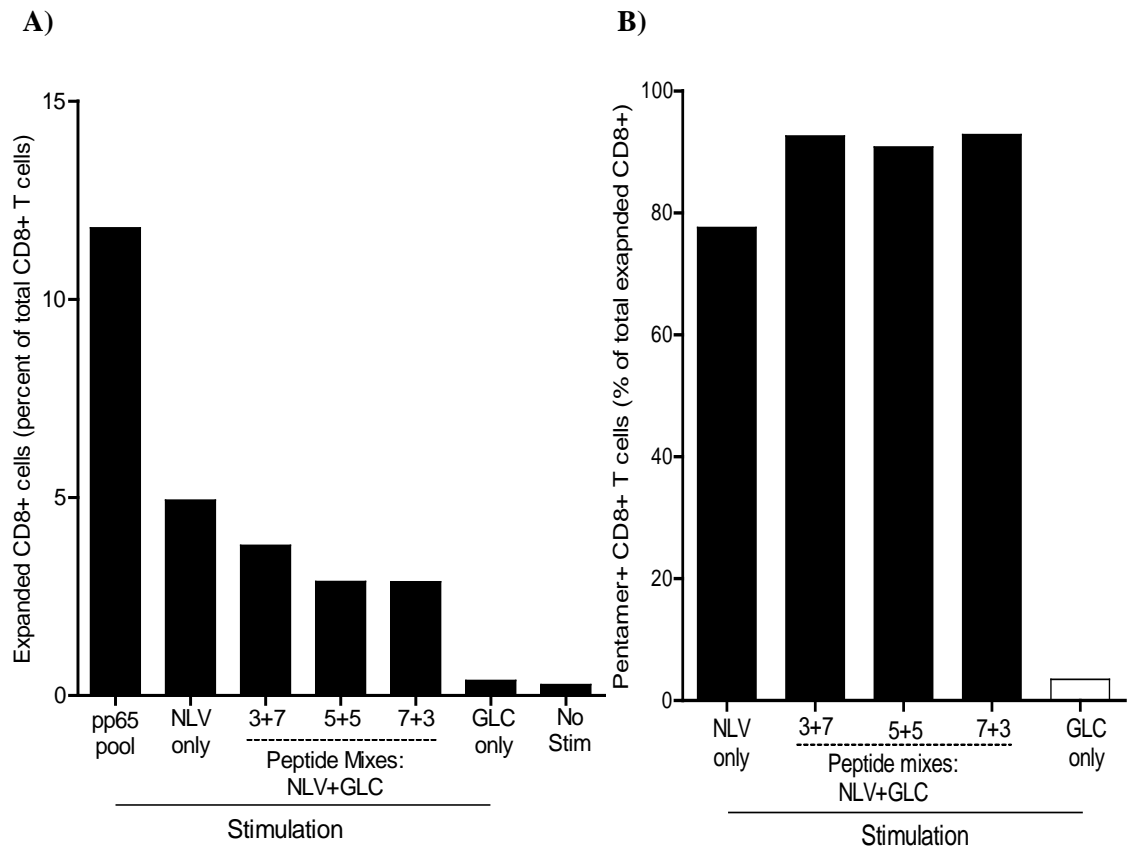


Figure 6-7: CD8+ T cell proliferation measured 5 days post-stimulation with HCMV (NLV) and EBV (GLC) peptides mixed in the indicated ratios ($\mu\text{g/ml}$).

The percentage of total CD8+ T cells assayed 5 days after stimulation that had proliferated is given in each case (A). Panel B shows the antigen specificity of expanded CD8+ T cells from the experiment shown in panel A. The proportion of expanded CD8+ T cells that stained with the HCMV NLV (black bars) or EBV GLC (white bar) pentamers is indicated (B).

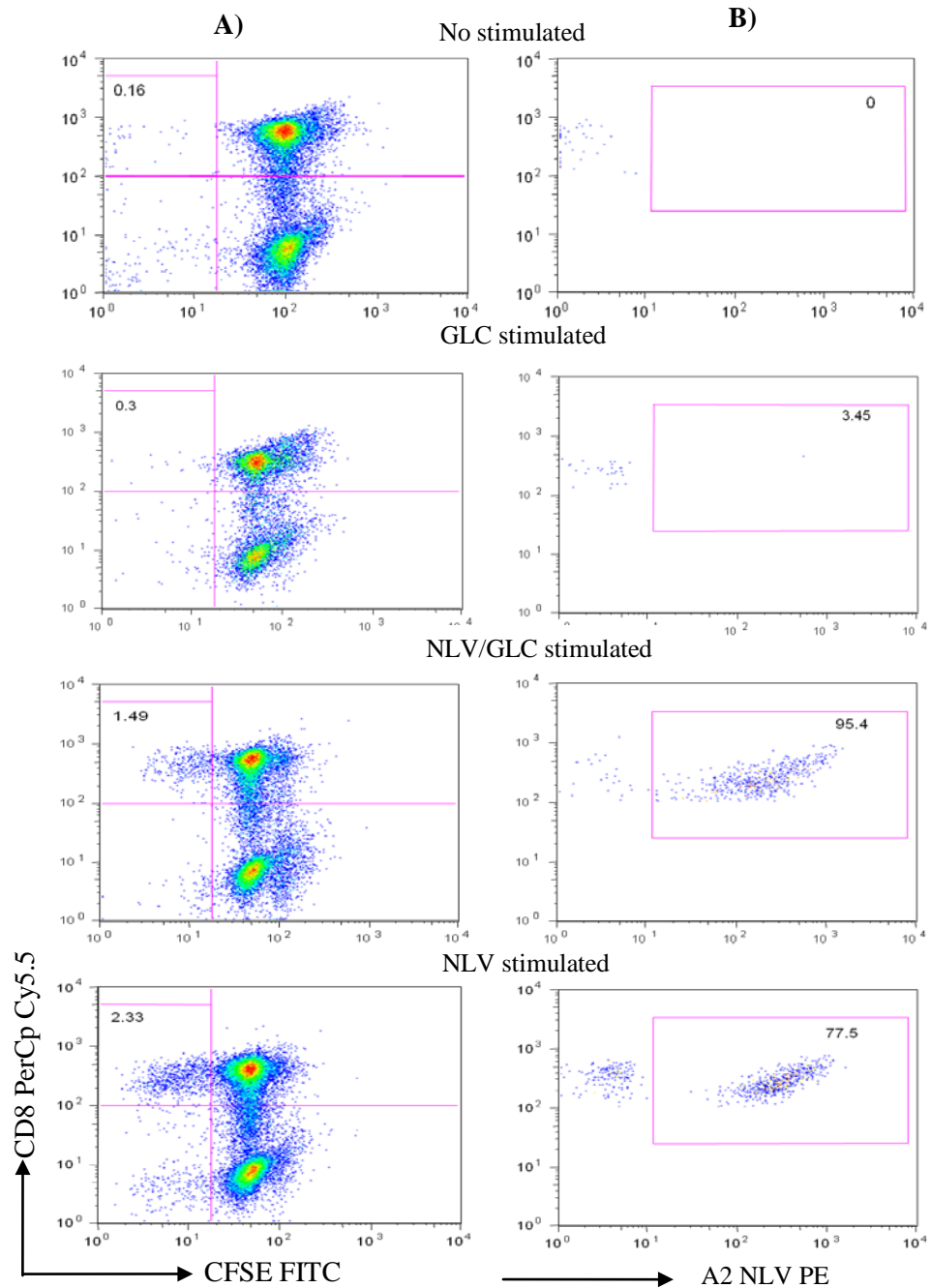


Figure 6-8: Representative FACS scatter plots of CFSE labeled CD8+ T cells in response to mixed peptide stimulation.

The response of CFSE labelled CD8+ T cells from an inflammatory CVID patient to 5 days post-stimulation with (GLC, NLV/GLC, and NLV) and without antigen (A). Gates were set on the live T lymphocyte population and CD8+ T cells. (B) Antigen specificity of expanded CD8+ T cells from the experiment shown in panel A.

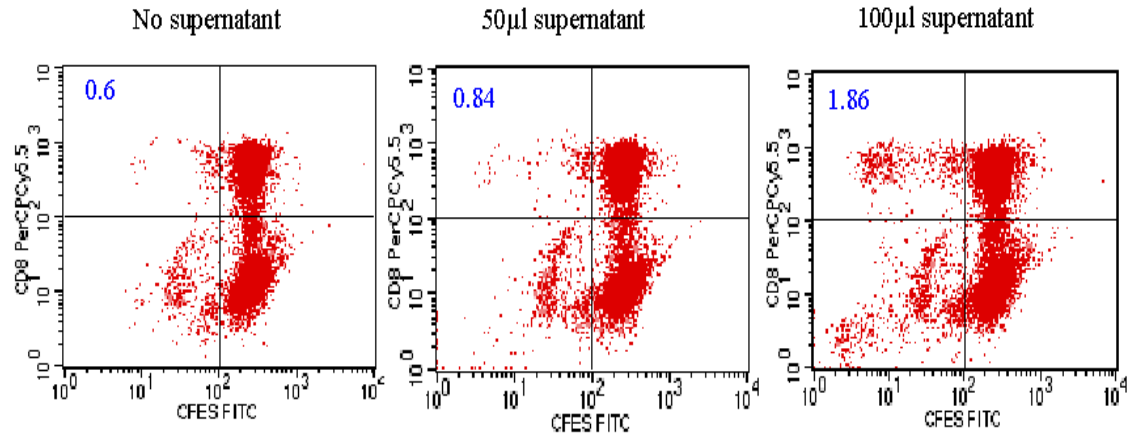


Figure 6-9: Dose-dependent proliferation of NLV-stimulated cells.

To determine the optimal volume of cell cultured supernatant for supernatant transfer assay, PBMCs from an inflammatory CVID patient were labeled with CFSE and cultured in the absence and presence of NLV peptide stimulation. On day 2, supernatants were collected, centrifuged, and filtered as explained in chapter 2. Two different volumes (50µl and 100µl) of supernatant were transferred to the pre-labeled NLV-stimulated PBMCs of a non-inflammatory CVID patient and incubated for 5 days. A dose-dependent enhancement was found in the proliferation of NLV-stimulated CD8+ T cells from non-inflammatory patient following transfer of increased volume of supernatant.

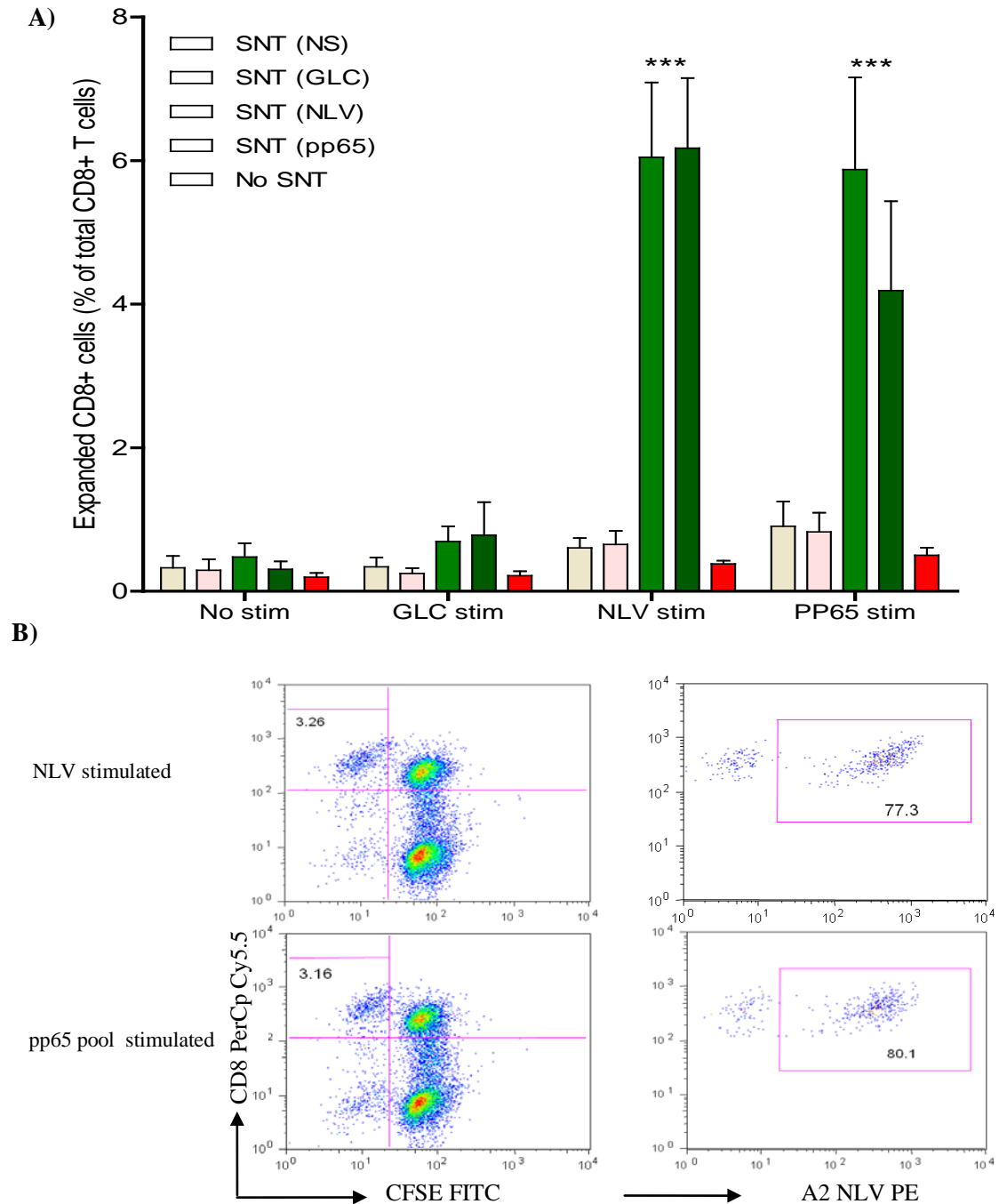


Figure 6-10: Supernatant transfer to illustrate the role of soluble factors in proliferation.

A) Proliferation of antigen stimulated (GLC, NLV or pp65) CD8+ T cells from non-inflammatory and healthy subjects in the presence or absence of supernatant (SNT) from HCMV stimulated inflammatory CVID patient cells. B) Representative FACS plots showing specificity of expanded CD8+ T cells. HCMV NLV peptide or pp65 peptide pool stimulated PBMCs (from a healthy control subject) were analyzed 5 days after supernatant transfer. CD8+ T cells were stained with HCMV NLV pentamer. The gated values show: proliferated CD8+ T cells (as a % of total CD8+) and % of the proliferated CD8+ T cell population defined in that was HCMV NLV pentamer positive.

6.4 Use of anti-cytokine antibodies to Block proliferation

Since the cytokine profile of the stimulated NLV-specific CD8+ T-cells in CVID patients with inflammation showed a high frequency of TNF- α and IFN- γ double producing cells (see Chapter 3) I reasoned that these cytokines may be important as drivers of expansion in the proliferation assays described in previous sections. Therefore, the proliferation assay was repeated in the presence of increasing concentrations of antibodies against TNF- α (0-0.02 μ g/ml) and IFN- γ (0-0.26 μ g/ml) either alone or in combination. Anti-IFN- γ (Figure 6-11) and anti-TNF- α (Figure 6-13) inhibited proliferation by 50% and 80% respectively and a combination of the two antibodies inhibited proliferation by 85% (Figure 6-15). As shown in Figure 6-12, anti-IFN- γ antibody reduced both the proportion of expanded NLV specific-CD8+ T cells (91.7% to 50.6%) and total expanded cells (3.55% to 0.33%). Similarly, following addition of anti-TNF- α antibody the proportion of total expanded cells reduced from 4.93% to 0.72% with a commensurate reduction of NLV specific-CD8+ T cells from 77.6% to 22.3% (Figure 6-14).

Although these results indicated that IFN- γ and TNF- α were both major contributing cytokines to the proliferation and it remains possible that other cytokines are involved either at the initial stages of proliferation or during the ongoing proliferation stages.

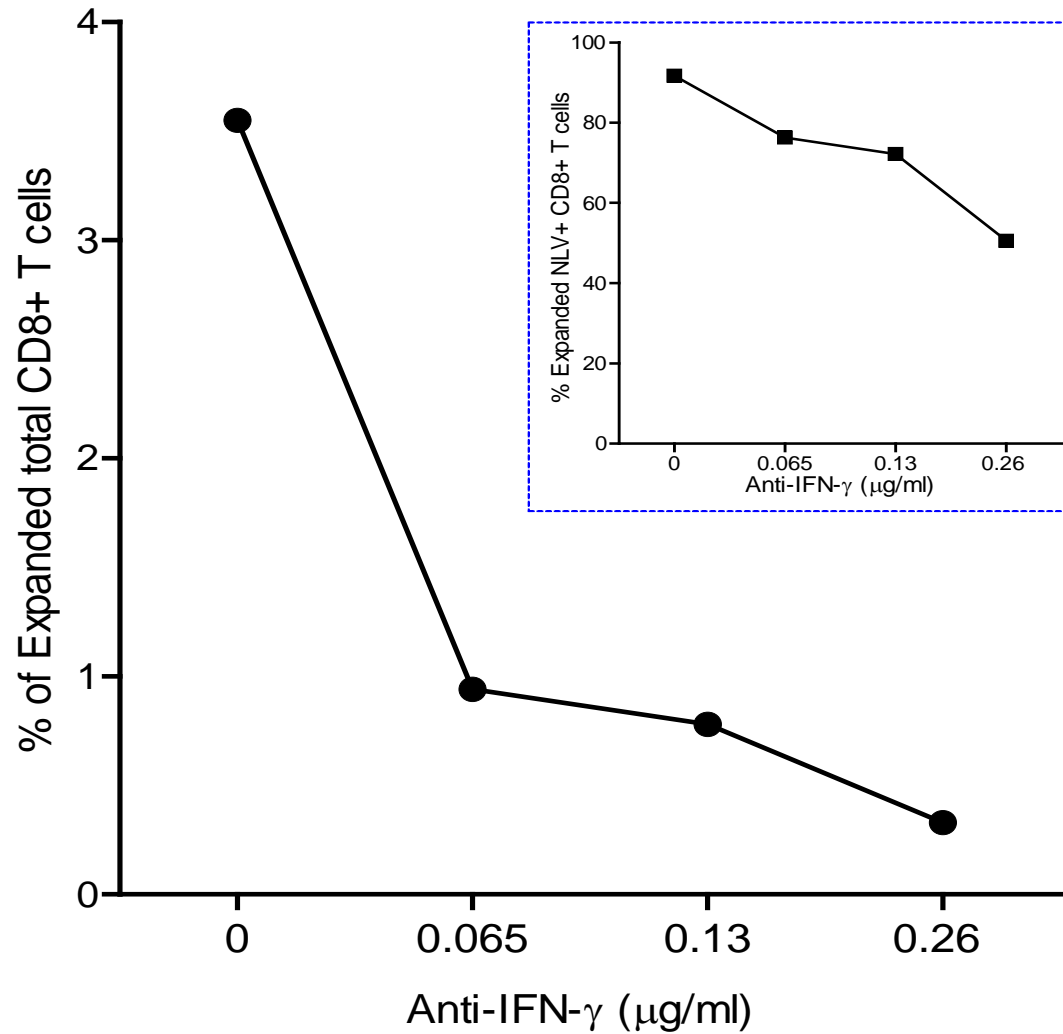


Figure 6-11: Dose-dependent reduction of CD8+ T cell proliferation following addition of soluble anti-IFN- γ .

The dose-dependent reduction of total expanded CD8+ T cells as well as expanded NLV-specific CD8+ T cells (inset) in the absence or presence of different concentrations of anti-IFN- γ (0- 0.26 $\mu\text{g/ml}$).

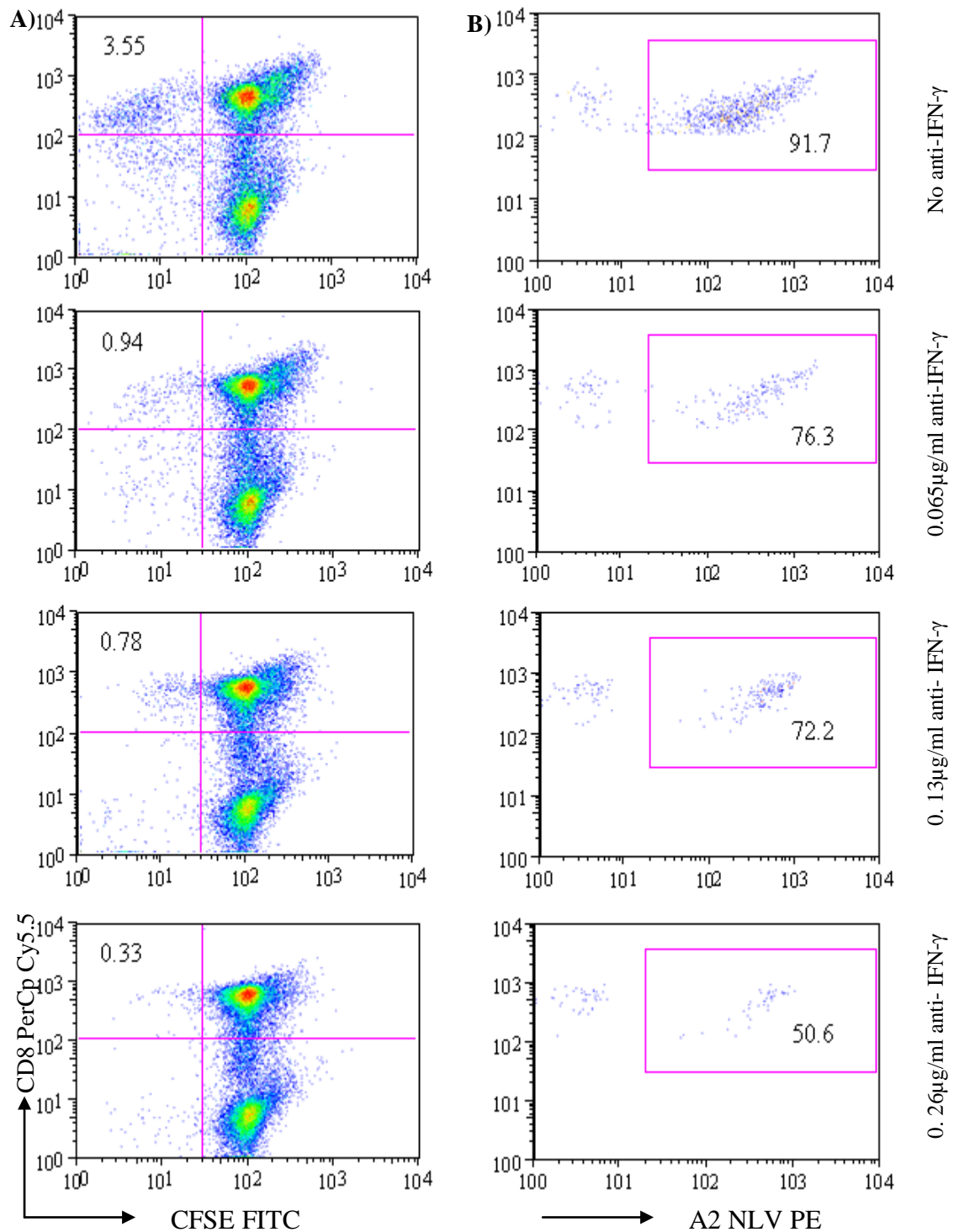


Figure 6-12: Representative FACS plots of dose-dependent reduction of CD8+ T cell proliferation following addition of soluble anti-IFN- γ antibody.

Dose-dependent reduction of A): expanding CD8+ T cells and B): expanded NLV-specific CD8+ T cells in the absence and presence of different concentration of anti-IFN- γ antibody (0 - 0.26 μ g/ml).

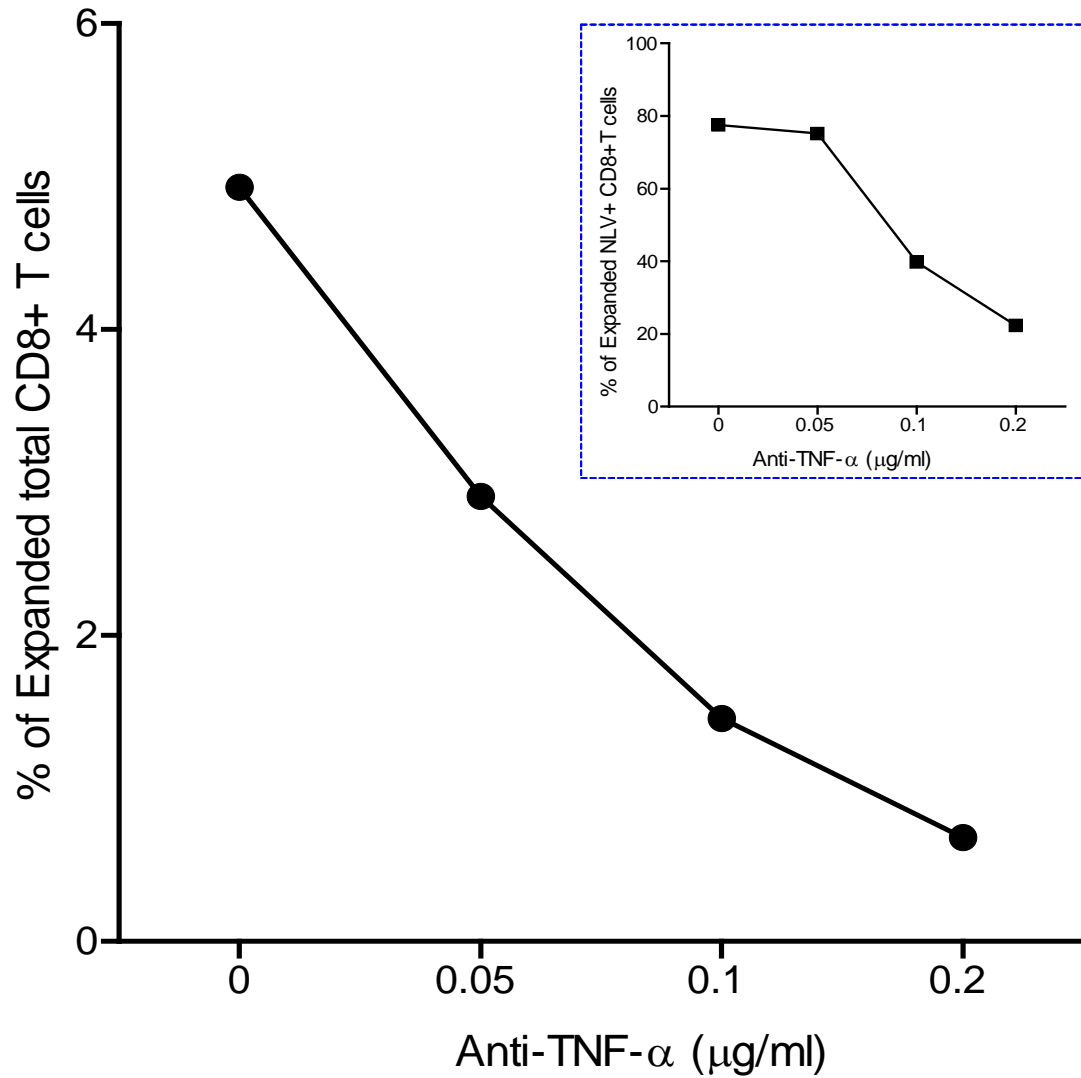


Figure 6-13: Dose-dependent reduction of CD8+ T cell proliferation following addition of soluble anti-TNF- α antibody.

The dose-dependent reduction of total expanded CD8+ T cells as well as expanded NLV-specific CD8+ T cells (inset) in the absence or presence of different concentrations of anti-TNF- α antibody (0 - 0.2 μ g/ml).

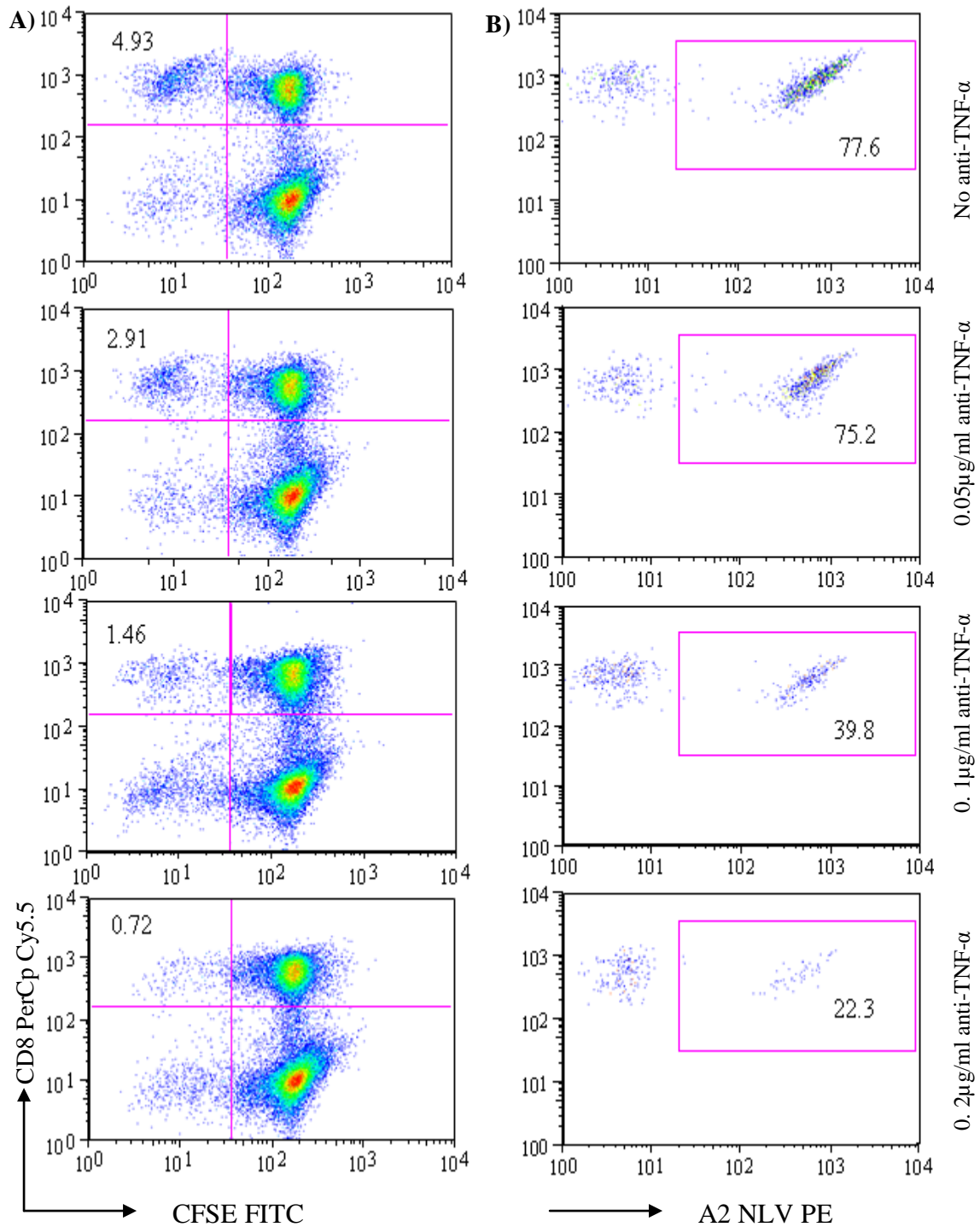


Figure 6-14: Representative FACS plots of dose-dependent reduction of CD8+ T cell proliferation following addition of soluble anti-TNF- α antibody.

Dose-dependent reduction of A): expanding CD8+ T cells and B): expanded NLV-specific CD8+ T cells in the absence and presence of different concentration of anti-TNF- α antibody (0 - 0.2 μ g/ml).

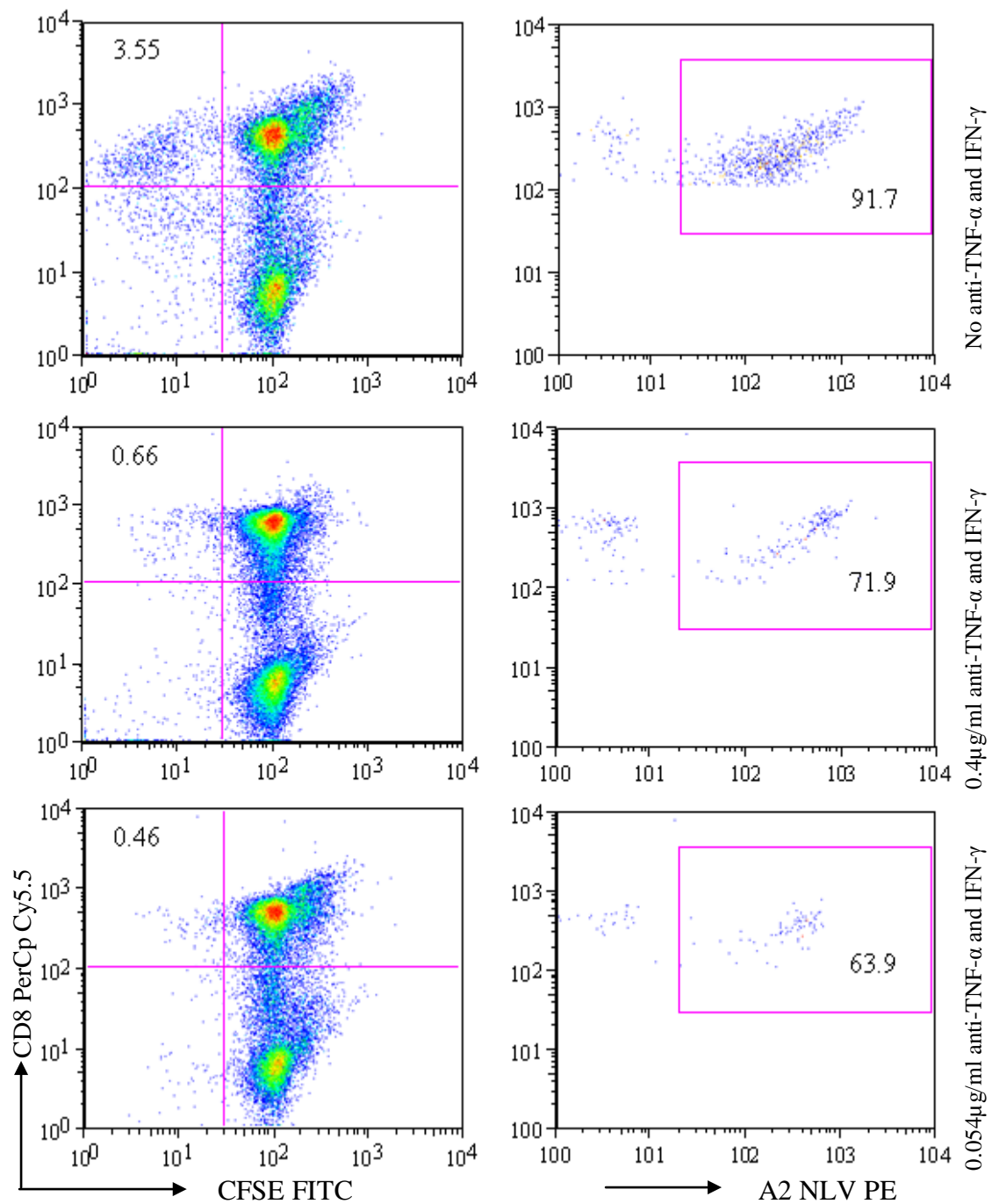


Figure 6-15: Representative FACS plots of dose-dependent reduction of CD8+ T cell proliferation following addition of soluble anti-TNF- α and anti-IFN- γ antibodies (0.05 μ g/ml and 0.4 μ g/ml).

6.5 Activation profile of HCMV specific CD8+ T cells in CVID patients

Since CD8+ T cells of inflammatory CVID patients were found to proliferate following HCMV NLV peptide stimulation but not with EBV stimulation and had a low levels of PD-1, the activation status of HCMV specific CD8+ T cells further assessed in CVID patients with and without inflammation using Ki-67 and HLA-DR expression.

As mentioned in previous sections, high proliferative capacity was observed on HCMV-specific CD8+ T cells of CVID patients with inflammation compared to non-inflammatory patients (Figures 6-3 and 6-4). Interestingly, consistent with the proliferation results, CD8+ T cells of CVID patients with inflammation expressed significantly higher levels of Ki-67, when compared to non-inflammatory CVID patients and healthy controls (Mean \pm SD; 2.4% \pm 0.37% inflammatory vs. 0.75% \pm 0.15% non-inflammatory; P= 0.0002) (2.4% \pm 0.37% inflammatory vs. 0.32% \pm 0.03% healthy; P= 0.002) (Figure 6-16). This distinction was greatly enhanced when Ki-67 expression in HCMV (NLV)-specific CD8+ T cells was analyzed, with cells from inflammatory CVID patients expressing significantly higher levels of ki-67 compared to non-inflammatory patients and healthy controls (36.8% \pm 7.4% inflammatory vs. 1.86% \pm 0.7% non-inflammatory; P< 0.0001) (36.8% \pm 7.4% inflammatory vs. 0.68% \pm 0.4% healthy; P= 0.003) (Figures 6-17 and 6-18).

A similar result was found when the frequency of HLA-DR expression by CD8+ T cells was investigated (Mean \pm SD; 34.5% \pm 8.03% inflammatory vs. 11.3% \pm 3.05% non-inflammatory; P= 0.005; 34.5% \pm 8.03% inflammatory vs. 3.8% \pm 2.2% healthy; P= 0.01). Consistent with total CD8+ T cells, HCMV-specific CD8+ T cells from

inflammatory CVID patients expressed significantly higher frequencies of HLA-DR compared with non-inflammatory patients and healthy controls (61.1% \pm 7.03% inflammatory vs. 36.4% \pm 6.3% non-inflammatory: P= 0.03; 61.1% \pm 7.03% inflammatory vs. 21.4% \pm 3.2% healthy: P= 0.002) (Figures 6-19 and 6-20).

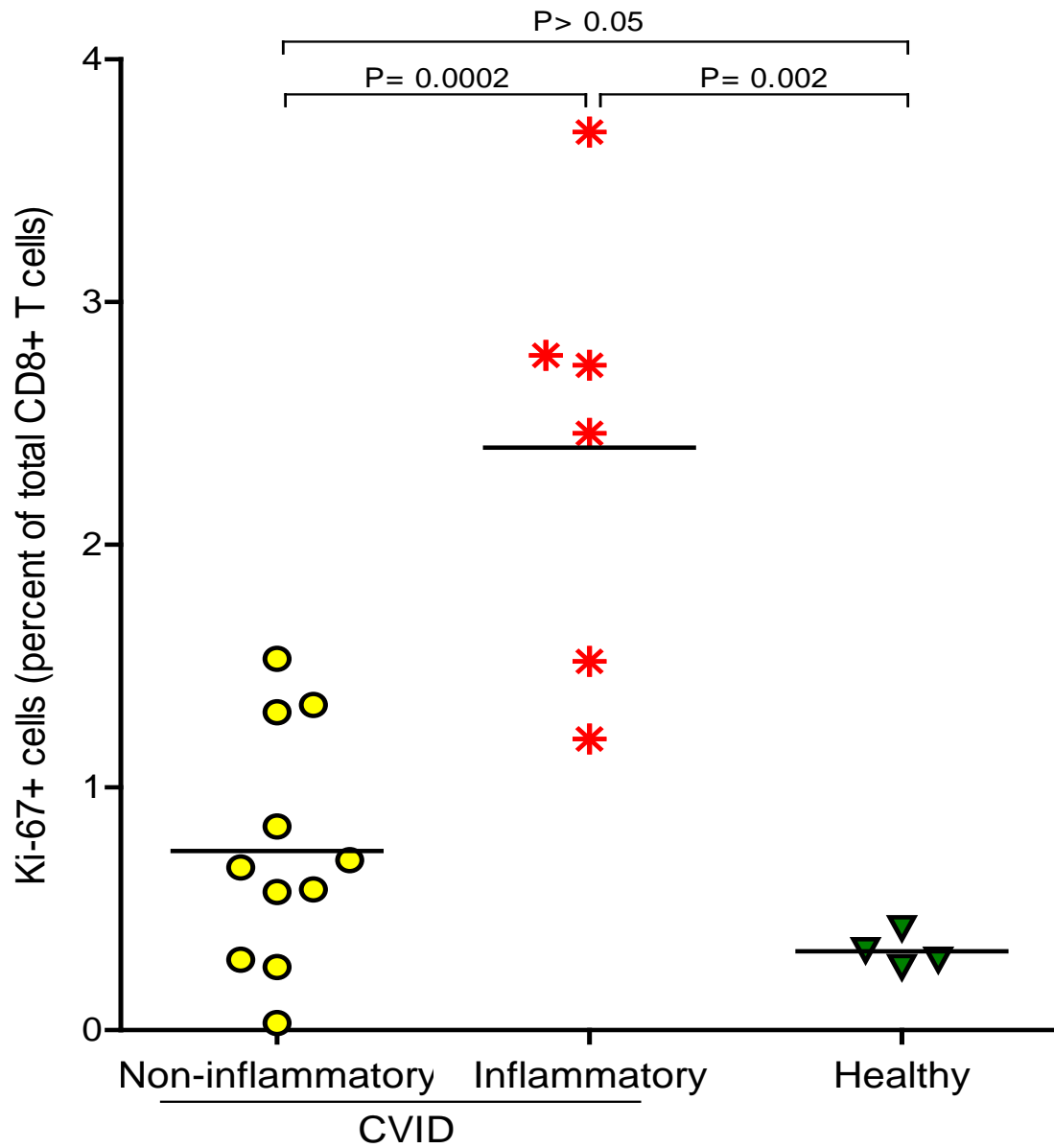


Figure 6-16: Frequency of total CD8+ T cells expressing Ki-67.

CD8+ T cells from inflammatory CVID patients (n = 6) expressed significantly higher levels of Ki-67 compared to non-inflammatory patients (n = 11) as well as healthy controls (n = 4). Bars indicated mean values. Each point represents a single subject.

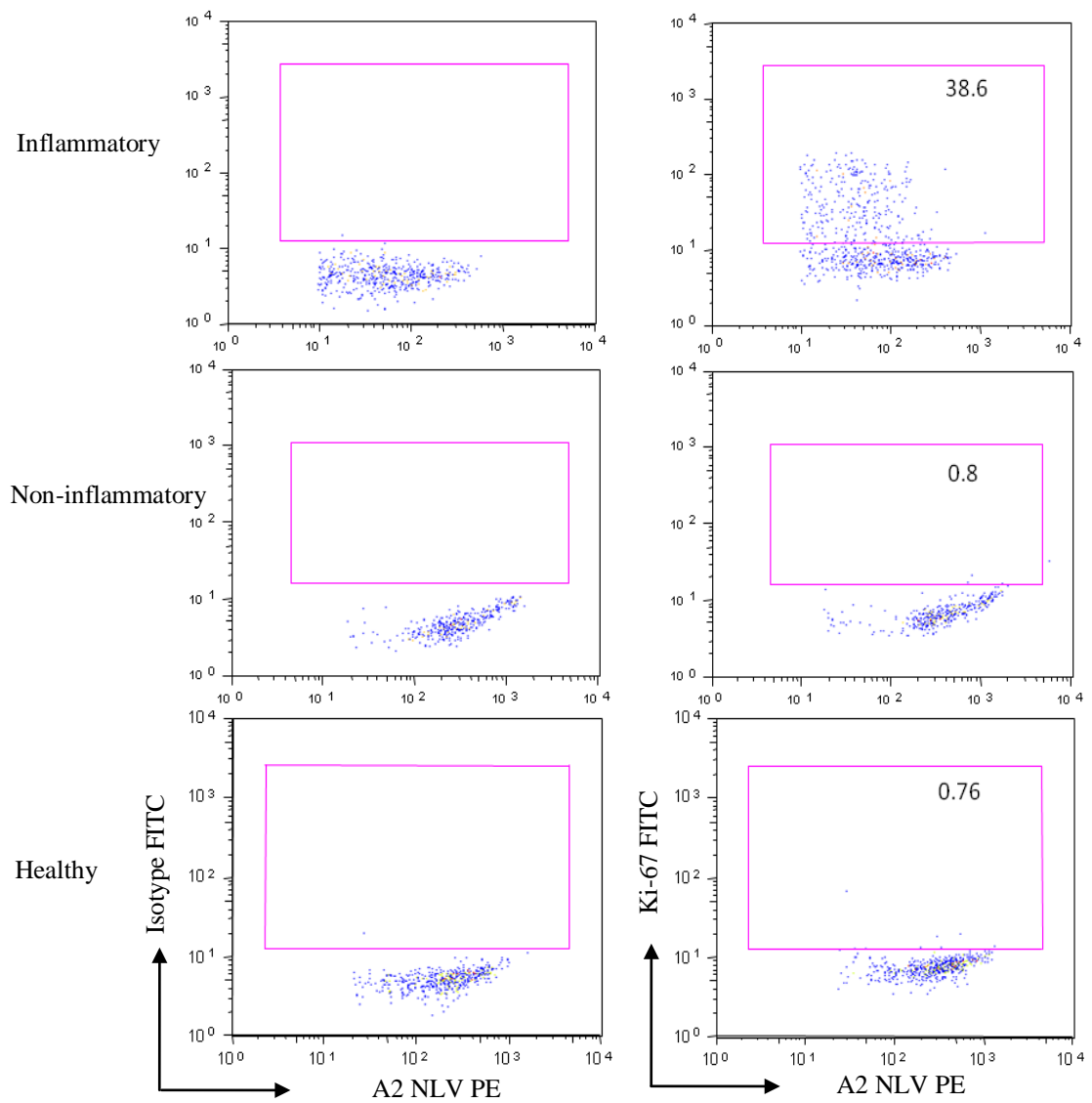


Figure 6-17: Representative FACS plots showing Ki-67 expression on HCMV (NLV)-specific CD8+ T cells.

HCMV-specific CD8+ T cells expressing Ki-67 is shown for an inflammatory CVID patient (upper panels), a non-inflammatory patient (middle panels), and a healthy subject (lower panels). Gates were adjusted based on appropriate isotype control.

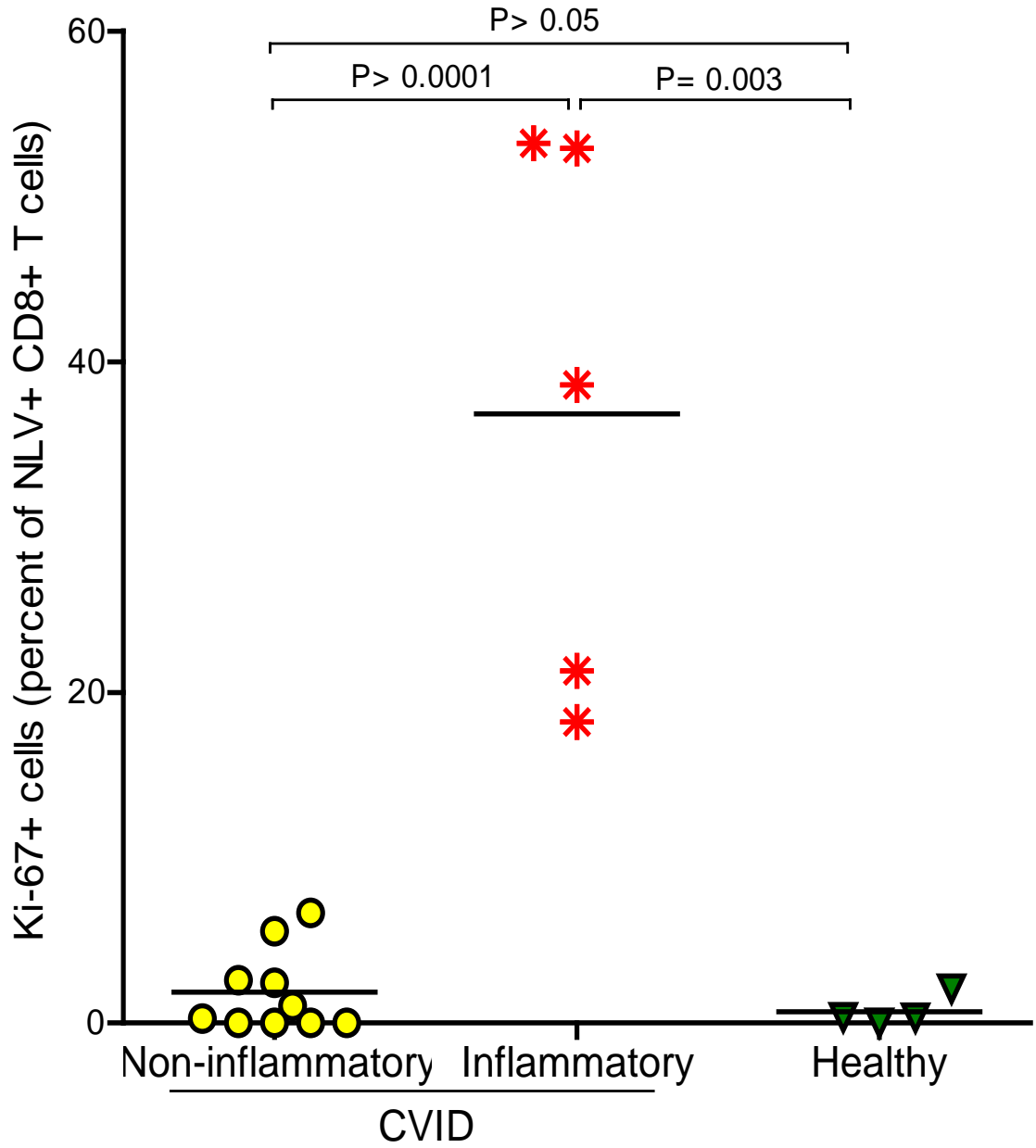


Figure 6-18: Frequency of HCMV (NLV)-specific CD8+ T cells expressing Ki-67.

HCMV-specific CD8+ T cells from inflammatory CVID patients (n = 5) expressed significantly higher levels of ki-67 compared to non-inflammatory patients (n = 10) as well as healthy controls (n = 4). Bars indicated mean values. Each point represents a single subject.

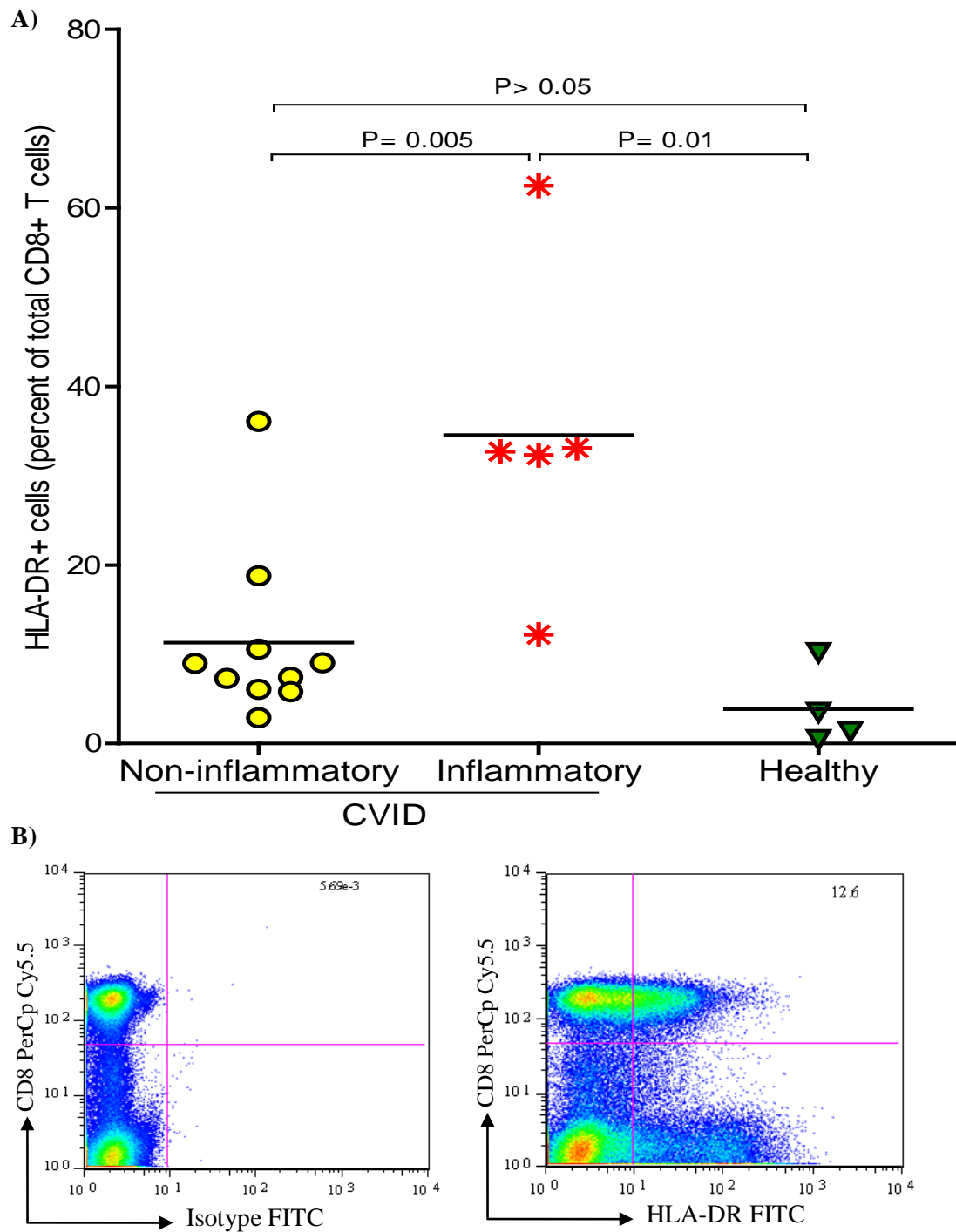


Figure 6-19: Frequency of total CD8+ T cells expressing HLA-DR.

A) CD8+ T cells from inflammatory CVID patients ($n = 5$) expressed significantly higher levels of HLA-DR compared to both non-inflammatory patients ($n = 10$) and healthy controls ($n = 4$). Bars indicated mean values. Each point represents a single subject. Representative FACS plots of HLA-DR expression on total CD8+ T cells from CVID patient with inflammation (B).

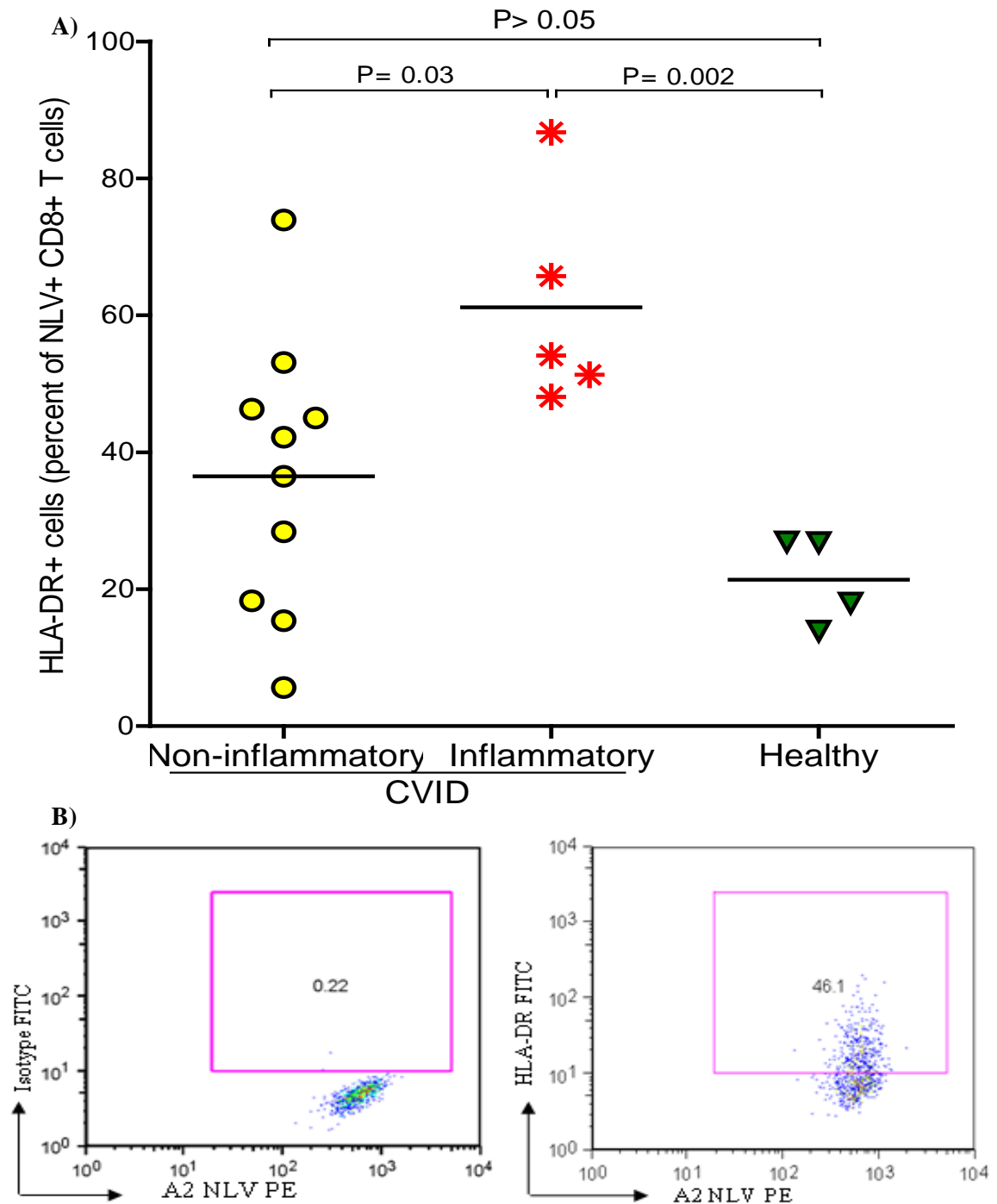


Figure 6-20: Frequency of HCMV (NLV) - specific CD8+ T cells expressing HLA-DR.

A) HCMV-specific CD8+ T cells from inflammatory CVID patients (n = 10) expressed significantly higher levels of HLA-DR compared to non-inflammatory patients (n = 5) as well as healthy controls (n = 4). Bars indicated mean values. Each point represents a single subject. Representative FACS plots of HLA-DR expression on NLV specific CD8+ T cells from a CVID patient with inflammation (B).

6.6 Avidity of HCMV specific CD8+ T cells in CVID patients

The capacity of HCMV (NLV) - specific CD8+ T cells from inflammatory patients to undergo autonomous proliferation in response to peptide stimulation may indicate an enhanced functional sensitivity (Walker et al., 2010). Therefore, using point-mutated soluble tetrameric class I HLA complexes refolded with the pp65 A*0201 epitope, the avidity of HCMV-specific CD8+ T cells in a subset of CVID patients with (n = 5) and without inflammation (n = 5) was assessed.

As shown in Figure 6-21, in 4 of 5 inflammatory CVID patients studied, the normal and CD8-null HCMV tetramers identified similar numbers of cognate CD8+ T cells, indicating that in these patients, CD8+ T cell responses specific to HCMV exhibit a high degree of avidity for this peptide antigen. In contrast, in all five noninflammatory CVID patients tested there was a dominant population of low avidity CD8+ T cells. The frequency of HCMV-specific CD8+ T cells identified by CD8 null tetramers was remarkably low when compared with normal tetramers (3.05% vs. 0.8%; 0.58% vs. 0.3%; 4.82% vs. 0.14%; 1.25% vs. 0.28%; 0.37% vs. 0.081%), indicating the low avidity of HCMV-specific CD8 cells in this group of patients (Figure 6-22).

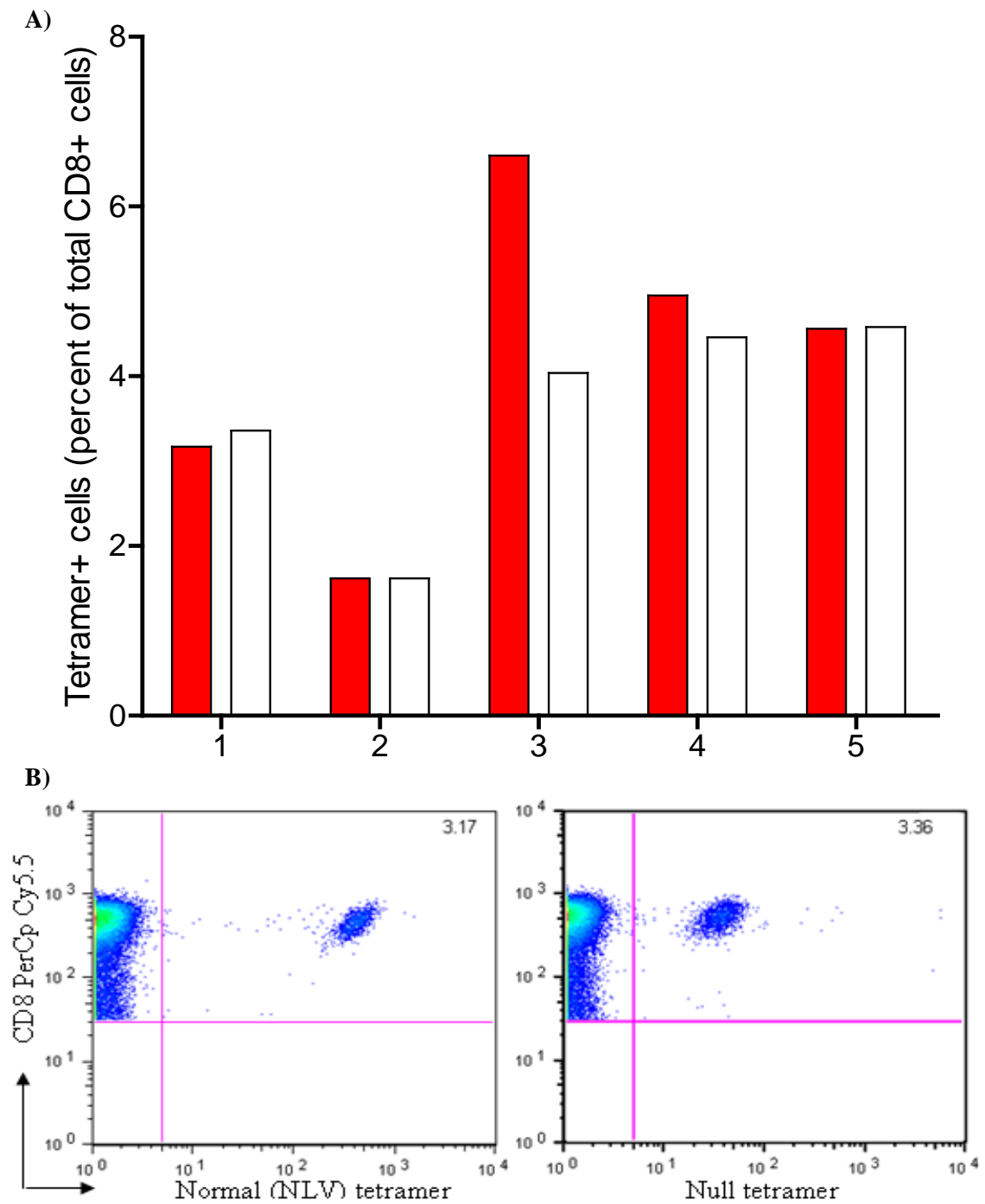


Figure 6-21: The avidity binding of HCMV (NLV)-specific CD8+ T cells from inflammatory CVID patients (n = 5).

A) Bar graphs depicts the frequency of HCMV-specific CD8+ T cells stained with the normal (red bars) and null (white bars) class I HLA tetramers. B) A representative FACS plot of tetramer (normal and null) staining patterns for an inflammatory CVID patient.

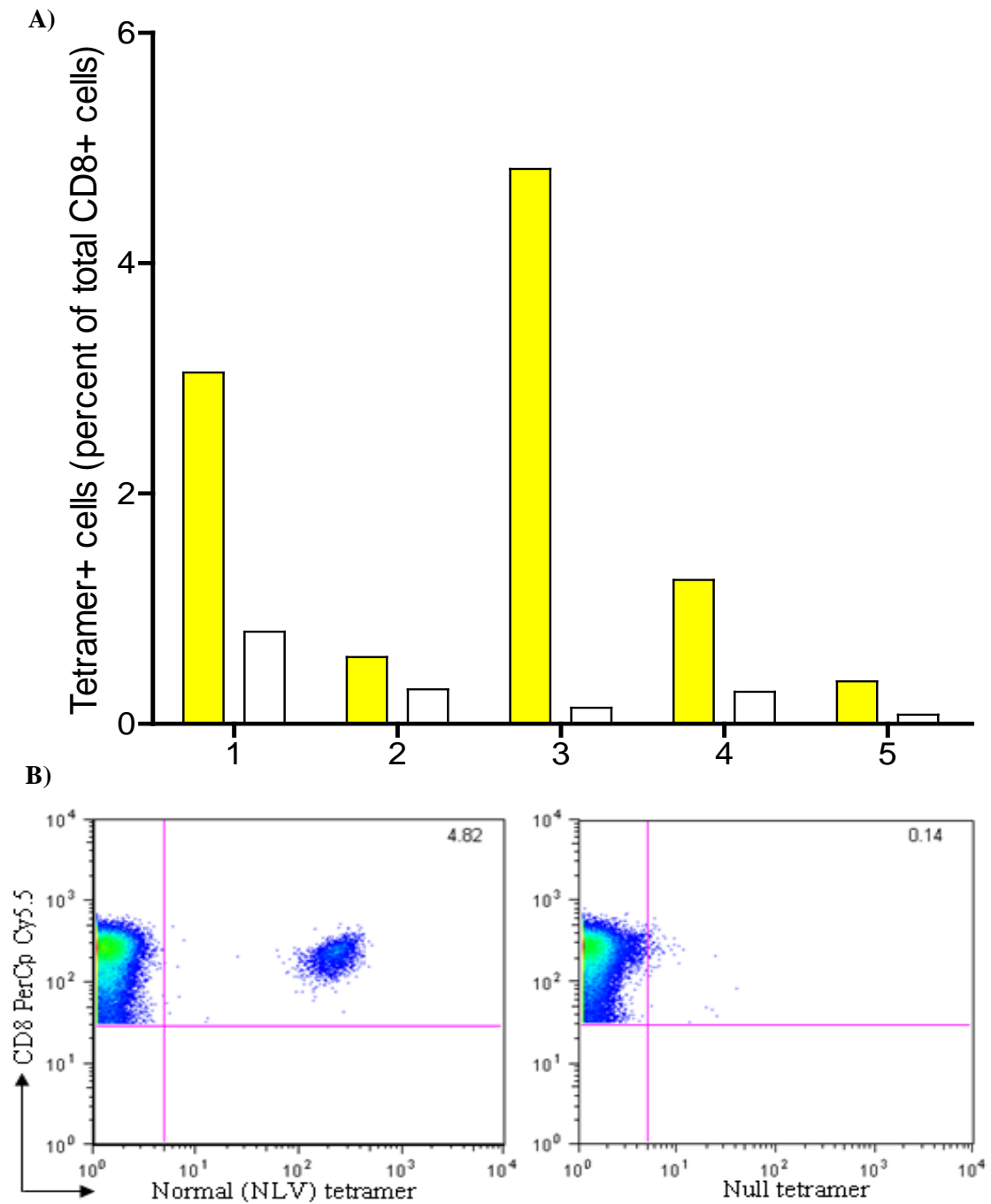


Figure 6-22: The avidity binding of HCMV (NLV)-specific CD8+ T cells from non-inflammatory CVID patients (n = 5).

A) Bar graphs depicts the frequency of HCMV-specific CD8+ T cells stained with the normal (red bars) and null (white bars) class I HLA tetramers. B) A representative FACS plot of tetramer (normal and null) staining patterns for a non-inflammatory CVID patient.

6.7 Frequency of CD73 expressing CD8+ T cells in CVID patients

The role of CD73 as a potent anti-inflammatory molecule has been described in many studies as mentioned in section 6.1. The *ex vivo* expression of CD73 on both total- and HCMV-specific CD8+ T cells in inflammatory (n = 6) and non-inflammatory CVID patients (n = 12) was investigated. There was no significant difference in the frequency of CD73 expression on total CD8+ T cell population between the two groups of CVID patients and healthy controls although the expression level in the inflammatory patients was slightly higher (Mean \pm SD: 3.8% \pm 1.6% non-inflammatory vs. 5.07% \pm 1.06% inflammatory vs. 6.7% \pm 1.18% healthy; $P > 0.05$) (Figure 6-23). However, when CD73 expression on CD8+ T cells specific to HCMV was analyzed, a significant difference was found between inflammatory CVID patients and non-inflammatory patients. Indeed, the frequency of CD73 expressing HCMV-specific CD8+ T cells from inflammatory CVID patients was significantly lower compared to non-inflammatory patients and healthy controls (1.67% \pm 0.8% inflammatory vs. 42.01% \pm 8.05% non-inflammatory: $P = 0.004$) (1.67% \pm 0.8% inflammatory vs. 19.8% \pm 7.9% healthy: $P = 0.03$) (Figure 6-24).

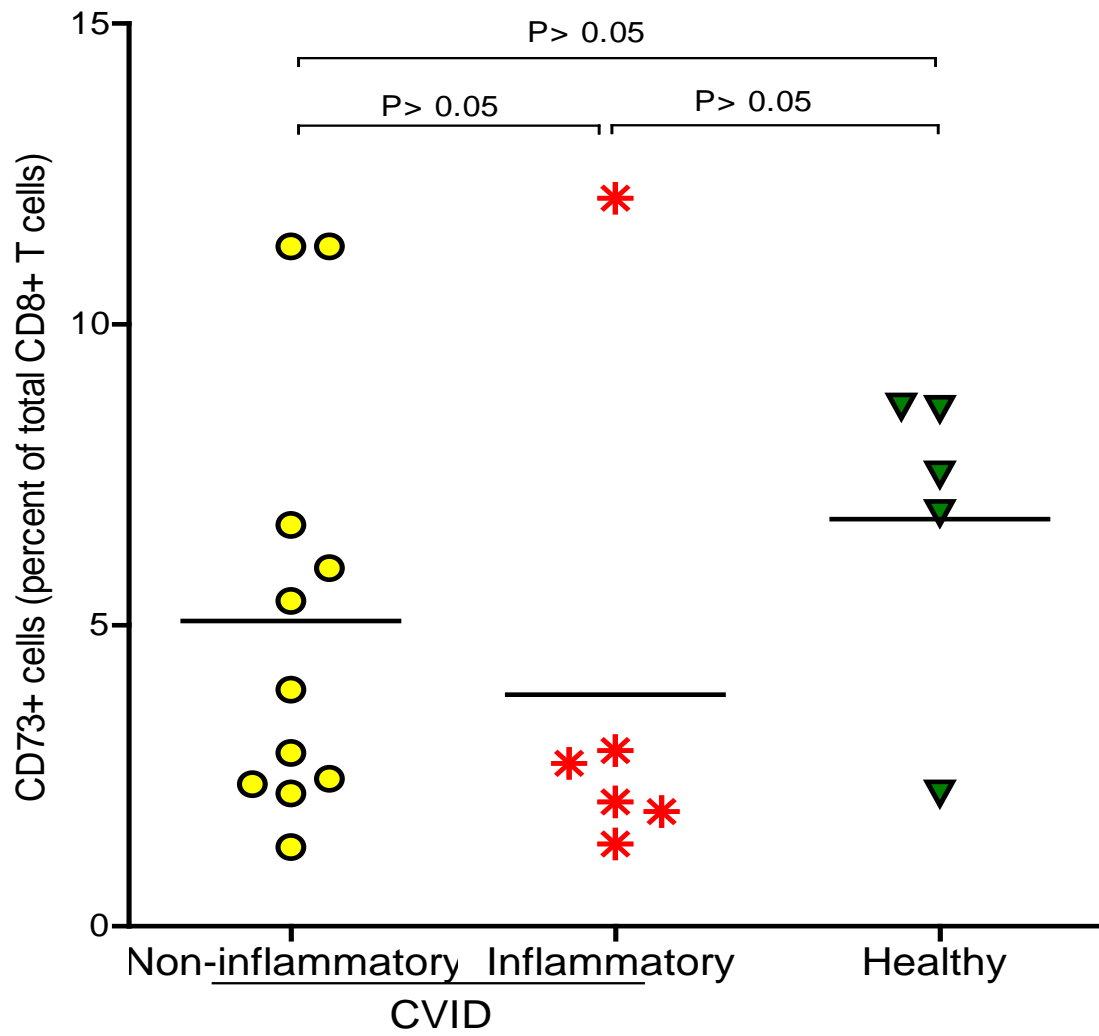


Figure 6-23: The frequency of CD73 expression on total CD8+ T cells.

CD73 expressing CD8+ T cells from inflammatory CVID patients (n = 6) compared to non-inflammatory patients (n = 11) as well as healthy controls (n = 5). Bars indicated mean values. Each point represents a single subject.

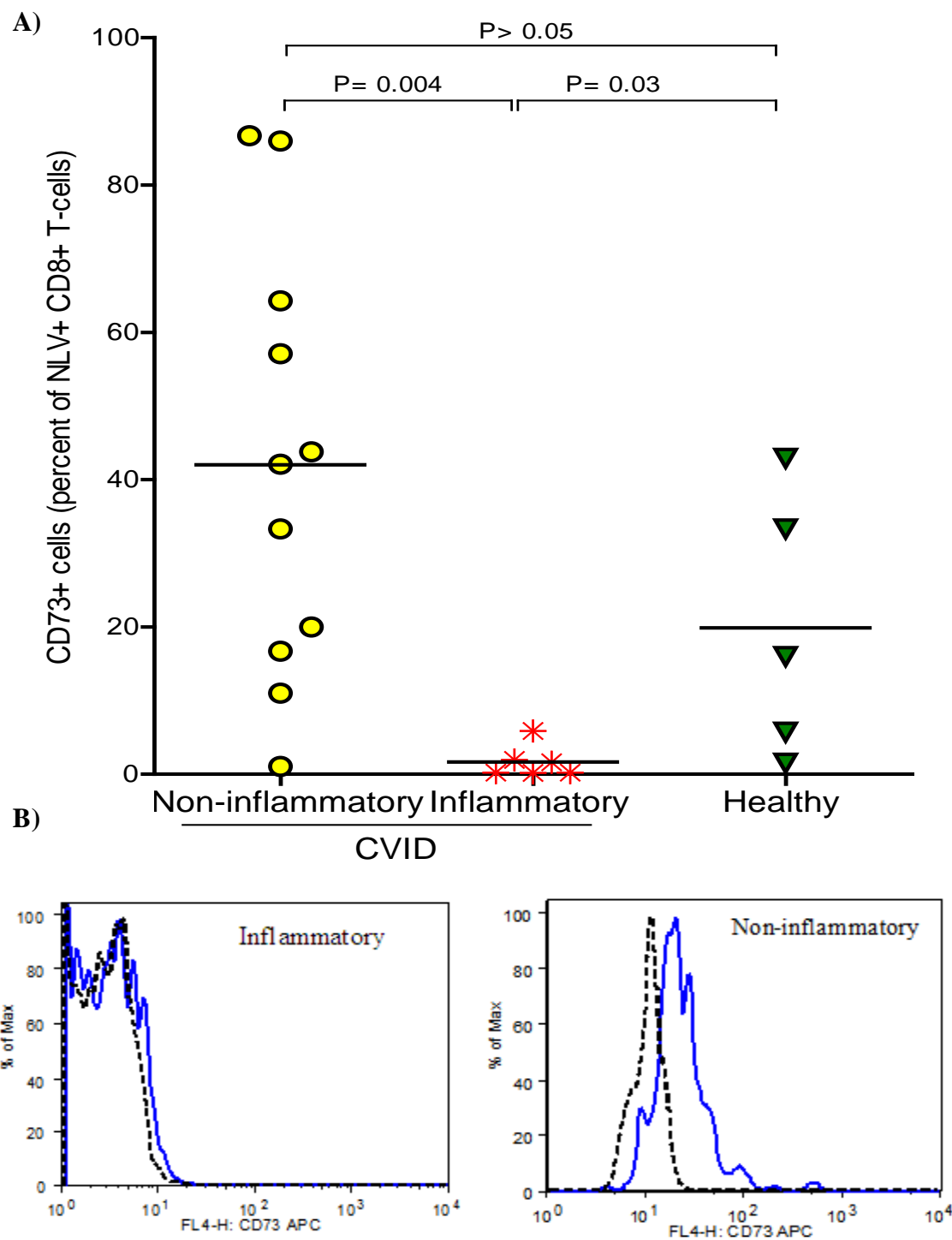


Figure 6-24: The frequency of CD73 expression on HCMV (NLV)-specific CD8+ T cells.

A) CD73 expression on CD8+ NLV+ T cells in inflammatory CVID patients (n = 6) compared to non-inflammatory patients (n = 11) and healthy controls (n = 5). Bars indicated mean values. Each point represents a single subject. Representative histograms of isotype control antibody (dash line) and anti-CD73 antibody (bold blue line) binding to NLV+ CD8+ T cells in an inflammatory and a non-inflammatory CVID patient (B).

6.8 Measurement of telomere length of CD8+ T cells in CVID patients

It has been shown that the immune response to HCMV can be a major cause in variation of telomere length of T cells in healthy subjects (van de Berg et al., 2010b). Because the CVID patients with inflammation showed elevated levels of HCMV-specific CD8+ T cells producing proinflammatory cytokines, the telomere lengths of CD8+ T cells in this group of patients was investigated.

CD8+ T cells from ten CVID patients with (n = 5) and without inflammation (n = 5) were subjected to a FACS based FlowFish assay as described in Chapter 2 (section 2.9). Table 6-1 shows telomere length values for CVID patients with and without inflammation determined using FlowFish. The table compares these experimental values with predicted telomere length values based on previously published values for healthy individuals of the same age. Consistent with previous studies (van de Berg et al., 2010b), there was an inverse correlation between increase of age and telomere length in the CVID patients ($r^2 = 0.85$; $P = 0.0001$) (Figure 6-25). Shorter telomere length values were found in CVID patients (with and without inflammation) when compared to the predicted telomere length values of healthy subjects at the same age (Table 6-1).

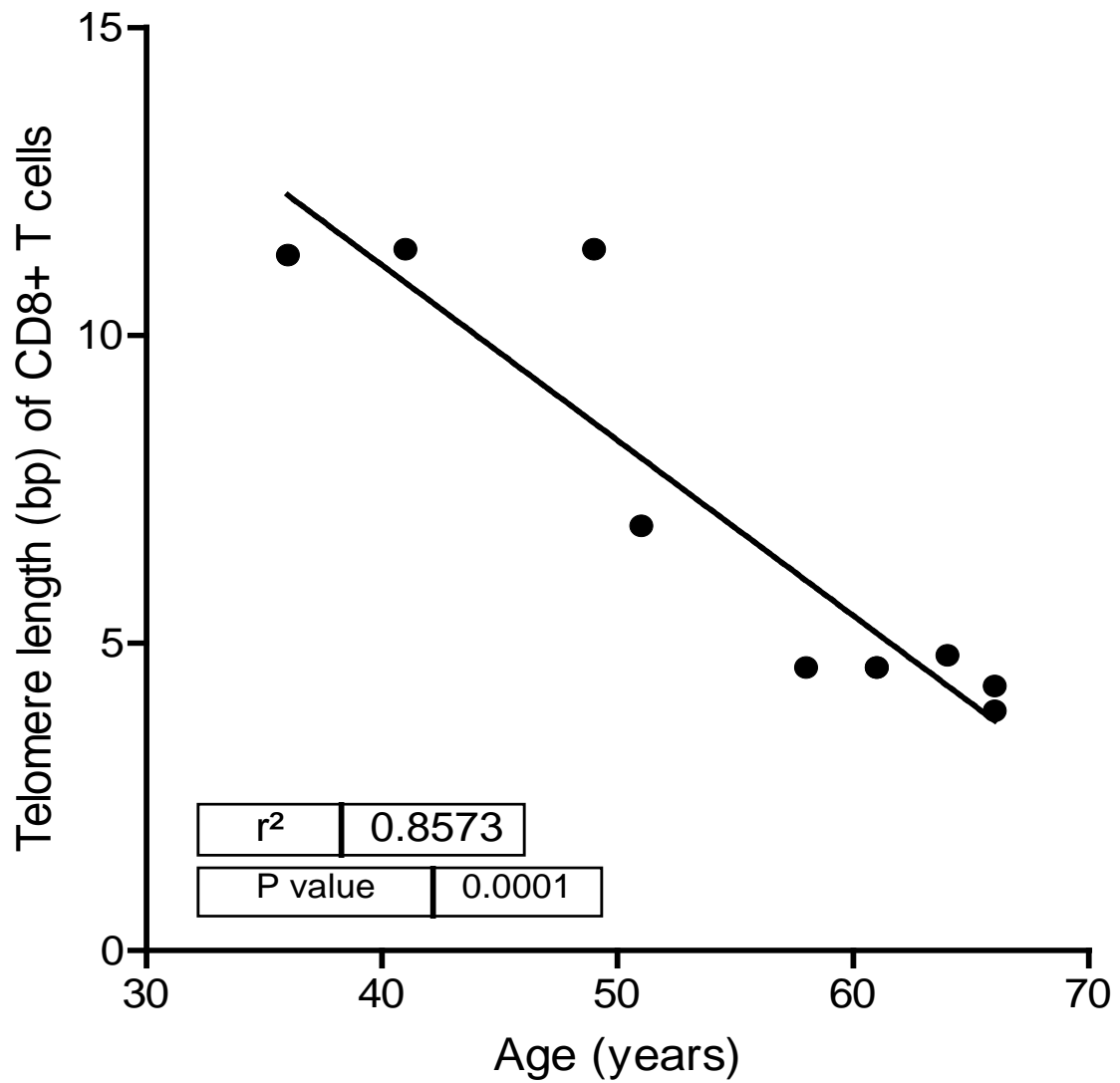


Figure 6-25: Correlation of telomere length reduction with age.

A strong inverse correlation was found between telomere length on CD8+ T cells of ten CVID patients (with and without inflammation) and age. Linear regression, r^2 , and P values were determined in Graphpad Prism.

Patients	No	Age (years)	Actual values - CVID (bp)	Predicted values - Healthy (bp)*	Summary
Inflammatory	1	58	4.6	6.5	Shorter
	2	64	4.8	5.9	Shorter
	3	61	4.6	6.2	Shorter
	4	36	11.3	8.5	Longer
	5	61	4.6	6.2	Shorter
Non-inflammatory	6	66	4.3	5.7	Shorter
	7	41	11.4	8.1	Longer
	8	51	6.9	7.1	Shorter
	9	66	3.9	5.7	Shorter
	10	49	11.4	7.3	Longer

Table 6-1: Summary of actual and predicted telomere length (TRL) on CD8+ T cells.

Actual values of TRL were determined in 5 inflammatory CVID patients (1-5) and 5 noninflammatory ones (6-10). Predicted TRL values (column labeled with asterisk*) for healthy individuals of the same age were extracted based on previously published data (van de Berg et al., 2010b). The summary column indicates the telomere length of CVID patients compared to Healthy subjects.

6.9 Discussion

The work presented in this chapter demonstrated for the first time that HCMV-specific CD8+ T cells from inflammatory CVID patients show evidence of proliferation *in vivo* and respond rapidly to antigen *in vitro*. In CVID patients with inflammatory disease, HCMV-specific CD8+ T cells proliferative *in vitro* following stimulation with HCMV antigens alone whereas this phenomenon was not observed in non-inflammatory patients or in healthy individuals. HCMV-specific CD8+ T cells in inflammatory patients showed no evidence of exhaustion, with low levels of PD-1 (as presented in Chapter 5) and high TCR avidity. Rather, they showed features consistent with high *in vivo* functionality and proliferative activity.

T cell exhaustion, especially of high avidity cell populations, has been described in HIV, HCV and LCMV infections (Alexander-Miller et al., 1996; Derby et al., 2001; Anderton, 2001). However, in inflammatory CVID patients the majority of HCMV-specific CD8+ T cells were found to be high avidity (Figure 6-21) for cognate antigen as assessed using the modified tetramers (Walker et al., 2010) whereas this property was not observed for non-inflammatory patients (Figure 6-22) where a substantial proportions of cells were CD8 dependent, indicating lower avidity.

Interestingly, in inflammatory CVID patients, the majority of HCMV-specific CD8+ T cells expressed HLA-DR and were able to proliferate and maintain full functionality. To investigate this further, Ki-67 expression which is indicative of *in vivo* proliferation was analyzed and its expression was much more frequent in CD8+ T cells from inflammatory patients. These suggest that 20-30% of the circulating CD8+ T cells were

in cycle, indicating a very high degree of turnover in these particular patients, compared to non-inflammatory patients or healthy controls (1-2%). The increased peripheral proliferation of T cells may also be indicative of compensatory adaptation for the ineffective thymic output in adults. Although I did not evaluate the thymic output in these patients, others have shown reduced expression of CD31, a marker of thymic output, in a large proportion of CVID patients (Giovannetti et al., 2007). The levels of proliferation of EBV (GLC)-specific CD8+ T cells were also low in the CVID patients.

Consistent with the Ki-67 expression pattern observed *in vivo*, T cells from inflammatory patients showed substantial proliferative capacity *in vitro* after NLV peptide stimulation. Unlike healthy individuals where minimal cell proliferation is seen in the absence of exogenous cytokines or co-stimulation, in inflammatory CVID patients, a remarkable expansion of CD8+ T cells was found in response to HCMV peptide stimulation alone. In this setting, it appears that both the inflammation milieu and particular antigen stimulation are important because the proliferation of CD8+ T cells only occurred in patients suffering from inflammation. Under these assay conditions, addition of exogenous peptide did not lead to the proliferation of HCMV specific CD8+ T cells in either healthy donors or CVID patients without inflammatory disease.

Additionally, CD8+ T cells only proliferated in response to HCMV (NLV/pp65) peptide stimulation but not with EBV (GLC) peptide stimulation in parallel experiments, indicating the antigen specificity of proliferated CD8+ T cells observed in inflammatory CVID patients with the majority of (~90-95%) of T cells being HCMV specific using HCMV-specific pentamers. These data confirm the substantial proliferative capacity of

the CD8+ T cells in CVID patients and underline the fact that the cellular response is not exhausted. Although the mechanism behind the enhanced proliferative capacity in these experiments is not clear, it could include differences in avidity, cytokine production (which could have an autocrine effect *in vitro*) and response to endogenous co-stimulation.

Since proliferation of HCMV specific CD8+ T cells, but not EBV specific cells, was only observed in CVID patients with inflammation, the possibility that soluble factors produced either by specific CD8+ T cell subsets or bystander cells such as CD4+ T cells and B cells was investigated using two approaches. In the first, combined stimulation using different ratios of the HCMV and EBV peptides showed that the majority (> 90%) of the proliferating cells were HCMV specific i.e. the EBV-specific CD8+ T cells did not proliferate even in an environment of HCMV proliferating cells. Secondly, when supernatants from the proliferating cells of inflammatory CVID cultures were transferred to cultures of cells from non-inflammatory patients as well as healthy controls, HCMV specific cells were driven to proliferate although it was not as substantial as that observed in the T cell cultures derived from the CVID inflammatory disease patients. This suggests that soluble factors are mediating the proliferation enhancement of HCMV-specific CD8+ T cells of both healthy and non-inflammatory patients. Interestingly, transfer of supernatants from the proliferating cultures was only able to enhance expansion of HCMV-specific CD8+ T cells in non-inflammatory patients and healthy controls but had no effect on EBV-specific CD8+ T cells indicating that the cytokine receptors available on HCMV-specific CD8+ T cells may be particularly geared towards a specific cytokine profile.

Since the cytokine profile of the HCMV-specific CD8+ T cells in CVID patients with inflammation showed a high frequency of TNF- α and IFN- γ producing cells it seemed reasonable to suggest that these cytokines rather than IL-2 may be the primary driver of expansion. This hypothesis was tested using anti-TNF- α and IFN- γ antibodies, alone or in combination. Adding anti-TNF- α and anti-IFN- γ inhibited proliferation by 80% and 50% respectively and a combination of the two antibodies inhibited proliferation by 85% showing that the major cytokines mediating this expansion were IFN- γ and in particular TNF- α . Proinflammatory cytokines have been shown to play an important role not only in the control of viral infections (by influencing the antigen level) but also contribute in CD8+ T cells priming, resulting in survival, modulation of proliferation, and effector function acquisition (Steinman and Hemmi, 2006; Mescher et al., 2006b; Wiesel et al., 2009).

As mentioned in section 6-1, in an optimal CD8 stimulation three signals initiate a complex course of proliferation; however, it appears in CVID patients with inflammation the quality of both the original signal provided by HCMV antigen exposure and inflammatory cytokines may have a critical impact on subsequent CD8+ T cell proliferation. It is not yet clear whether sequential stimulation or a relatively short encounter with virus antigens could launch the CD8+ T cells into successive rounds of proliferation. Perhaps the most striking distinctions between the two CVID groups was seen when the frequencies of CD8+ T cells expressing the potent anti-inflammatory molecule CD73 was measured. Interestingly, when looking at CD73 expressing HCMV-specific CD8+ T cells, a significant increase was found in the levels of CD73 expression in non-inflammatory CVID patients compared to inflammatory patients (Figure 6-24).

Since this difference was not observed when comparing total CD8+ T cells expressing CD73, it suggests a connection between HCMV infection or HCMV antigen stimulation and CD73 expression. Beside anti-inflammatory properties (Ohta and Sitkovsky, 2001) and mediating immune suppression (Deaglio et al., 2007), adenosines generated by CD73 suppress T cell proliferation (Erdmann et al., 2005). A recent study documented joint swelling after *Borrelia garinii* infection of CD73^{-/-} mice and suggested that low levels of adenosine may favour local inflammatory responses and persistent infection (Yegutkin et al., 2010). There is very little data on CD73 expression on antigen-specific CD8+ T cells in other infections, but intuitively it seems likely that the enzyme is downregulated during antigen driven proliferation to allow adequate T cell expansion for control of the infection.

Since telomeric erosion of PBMC has been already shown in several diseases such as systemic lupus erythematosus, rheumatoid arthritis, sarcoidosis, and type I diabetes (Schonland et al., 2003; Guan et al., 2007; Fyhrquist et al., 2010; Georjin-Lavialle et al., 2010), in this chapter, telomere length values were also measured on CD8+ T cells taken from a subset of CVID patients with and without inflammation. Telomere lengths were shown to be shorter in CVID patients compared to age matched controls. Whether this telomeric shortening is connected to CVID disease is not clear. It has been suggested that accumulative oxidative damage during aging or repeated activation of specific T cells could be potentially underlie telomere attrition (von Zglinicki, 2002; Akbar and Vukmanovic-Stejić, 2007). However, in addition to aging several other factors including inflammation, oxidative stress, and increased leukocyte renewal have been suggested to

be main environmental factors associated with accelerated telomere shortening (von Zglinicki, 2002;Georgin-Lavialle et al., 2010).

Taken together, data from this chapter showed: firstly, HCMV (NLV) specific CD8+ T cells from inflammatory CVID patients showed evidence of substantial proliferation *in vivo* and respond rapidly to antigen *in vitro*. Second, in this subset of patients HCMV specific CD8+ T cells were characterized by high levels of avidity for cognate antigens whereas the equivalent cells from non-inflammatory patients included substantial proportions where binding was CD8 dependent, indicating lower avidity. Third, while there was no significant difference in CD73 expressing CD8+ T cells in both groups of patients, HCMV-specific CD8+ T cells of inflammatory CVID patients expressed significantly lower levels of CD73 compared to non-inflammatory patients. Fourth, compared to telomere length values available for age matched healthy controls, CVID patients generally had a reduced telomere length. Additionally a strong correlation was found between telomere length reduction and age.

Chapter 7

7 General Discussion

This study, as a PhD project, aimed to investigate various aspects of the functional capacity of CD8+ T cells against human cytomegalovirus infection in CVID patients with a focus on the subset of patients who experienced inflammatory diseases within a variety of organs. The study comprised four experimental chapters based on the hypothesis that HCMV is a major factor driving CD8+ T cell expansion and is associated with an aberrant HCMV specific CD8+ T cell response in CVID patients with inflammation.

CVID is the most common primary antibody immunodeficiency and is characterized by defective antibody production and consequent increased susceptibility to some infections (Park et al., 2008). Although monogenic defects have been described in a minority of CVID cases (Yong et al., 2008), for the remaining population the etiology is unknown. Importantly, a significant proportion of these patients suffer from inflammatory complications that are expressed either as organ specific involvement that may lead to fibrosis and granuloma formation or, less frequently, as multisystem granulomatous disease. The organs most commonly affected are the lung, liver, spleen, skin, and gastrointestinal tract. Persistent as yet undefined viral infection(s) has been suggested as a potential cause of granulomatous disease in CVID (Chapel and Cunningham-Rundles, 2009). Historically, CVID patients have not been considered at risk of serious viral infections. However, a small number of patients develop persistent entroviral infection of the central nervous system with significant morbidity and mortality (Halliday et al., 2003). In addition, some CVID patients infected with HCV by contaminated immunoglobulin batches have an aggressive clinical course of their hepatitis (Yap et al., 1994).

The present PhD project investigated the functional capacity of HCMV-specific CD8+ T cells in CVID patients in more detail. There are several important lines of evidence supporting the rationale for investigating the role of this virus in these patients:

1- Although HCMV rarely causes disease in immunocompetent individuals, it exerts its full pathogenic potential when the immune system is not functioning properly. The two most common settings are in HIV infected individuals and transplant recipients where there is a pathogenic and therapeutic immunosuppression, respectively (Emery, 2001). By triggering chronic inflammation even subclinical active HCMV infection may have critical pathophysiological consequences as shown for chronic allograft injury (Reinke et al., 1994).

2- The role of HCMV infection in inflammatory diseases and some of the long term complications of solid organ transplantation such as atherosclerosis is supported by data from epidemiological, interventional and mechanistic studies (Soderberg-Naucler, 2006). For example, HCMV infection has been shown to differentially modulate the levels of matrix metalloproteinases and leukotrienes (Bolovan-Fritts et al., 2007; Bolovan-Fritts and Spector, 2008). Additionally, HCMV is detected frequently in the gut of patients with inflammatory bowel disease and systemic lupus erythematosus (Soderberg-Naucler, 2006) as well as in tumors of the colon (Harkins et al., 2002) and the virus has been implicated in contributing to the net state of immunosuppression seen in cancer patients (Miller, 2009).

3- The ability to elicit a remarkable antiviral immune response (Sylwester et al., 2005) in addition to the great capability of this virus in modulating the immune system particularly in immunocompromised settings.

Until fairly recently it was not recognised how common HCMV was in CVID patients. However, since antibody measurement is uninformative due to underlying B cell deficiencies as previously reported, prior exposure to HCMV and EBV in the CVID patients can be documented by using CD4+ T cell assays (Raeiszadeh et al., 2006). Previous work leading up to this work showed a highly significant association between HCMV exposure and inflammatory complications. Thirty one of 43 (72%) HCMV exposed CVID patients had one or more features of chronic inflammation as compared to 8 of 31 (26%) HCMV naïve patients (Raeiszadeh M, personal communication).

Although CVID manifests as an antibody deficiency, CD20+ EBV-associated lymphoma has been described in the context of CVID (Le et al., 2003) validating the use of this virus as a control for my studies of HCMV. Until the work in this thesis, HCMV-specific CD8+ T cell responses in the context of inflammatory CVID disease had not been investigated. Cytotoxic CD8+ T cells are a fundamental component of the immune response to viral infections and play an important role in immunosurveillance. Therefore, this study first aimed to investigate the functional capacity of CD8+ T cell responses specific to HCMV and EBV in CVID patients (with and without inflammatory disease) and healthy controls. This functional assessment of antigen-specific T cells was mainly performed on the basis of cytokine production (IFN- γ , TNF- α , and IL-2) (Chapter 3) and the pattern of cytokines secreted as described in Chapter 4.

HCMV-specific CD8+ T cell frequencies were significantly elevated in CVID patients compared to healthy controls whereas EBV-specific CD8+ T cell frequencies were similar in these two groups. However, when the CVID patients were sub-divided according to the presence of inflammatory diseases, the elevated HCMV responses

predominantly segregated with the patients with inflammatory diseases. These elevations were observed for both HCMV pentamer+ CD8+ T cells and for HCMV-specific CD8+ T cells producing IFN- γ and TNF- α after NLV peptide stimulation but were not seen in the subset of CD8+ T cells producing IL-2. Thus, in CVID patients with inflammatory disease the IFN- γ and TNF- α producing HCMV-specific CD8+ T cell populations are expanded without a commensurate increase in IL-2 producing cells. In contrast, no difference was observed in the cytokine expression profiles for EBV-specific CD8+ T cells after peptide stimulation between CVID patients with or without inflammatory disease or when compared to healthy controls. In these analyses, the frequencies of HCMV NLV-specific CD8+ T cells measured by the pentamer reagent and by intracellular cytokine staining after peptide stimulation (as described in Chapter 2) were comparable indicating that these T cells are functionally competent at least in cytokine production. Although these data describe the response to a single epitope in HCMV ppUL83, the same elevation was observed in IFN- γ production from CD8+ T cells in CVID patients with inflammatory diseases after stimulation with peptide pools for ppUL83 and also for the HCMV major immediate-early antigen suggesting that CD8+ T cells targeted against multiple HCMV epitopes have a similar phenotype.

Recently it has been shown that latent HCMV infection in healthy individuals can induce a sustained systemic inflammatory response with a type 1 cytokine signature (van de Berg et al., 2010a) which is in agreement of the data presented here where a significantly elevated inflammatory response was found in CVID patients with inflammation. However, these data contrast with what have been shown in renal transplant recipients where risk of high level HCMV replication and disease after

transplantation is associated with a HCMV-specific CD8⁺ T cell population with a reduced ability to produce IFN- γ after peptide stimulation (Crough et al., 2007; Mattes et al., 2008).

In addition to analysing typical effector functions such as single cytokine production, results can be integrated in polyfunctional analysis and the term "polyfunctional" has been used to define T cell immune responses (Pantaleo and Harari, 2006; Seder et al., 2008; Cellerai et al., 2010). It has been shown in some cases, at least, that cells producing two or three cytokines are functionally superior to single cytokine producers (Kannanganat et al., 2007). In Chapter 4, the co-expression of IFN- γ , TNF- α and IL-2 by HCMV- and EBV-specific CD8⁺ T cells was investigated in the target populations to produce a polyfunctional CD8⁺ T cell signature associated with these infections in the different clinical settings. Within the subset of CVID patients with inflammatory disease, the CD8⁺ T cell response to the HCMV (NLV) peptide showed a remarkable focus on IFN- γ producing cells, either as single producers or as IFN- γ / TNF- α double producers (notably both subsets also failed to produce significant levels of IL-2), these two subsets comprising an average 79.8 percent of the total population of HCMV NLV-specific CD8⁺ T cells. This distribution was reflected in the iMFI analysis, which combines frequency and MFI providing an estimate of the total amount of cytokine in the system (Darrah et al., 2007), emphasizing the prominence of IFN- γ in this response. In contrast, the frequency distribution in the non-inflammatory patients was essentially the same as that seen in healthy controls. Analysis of the response to the EBV peptide showed no differences between the two CVID patient groups emphasizing that the focus seen in the HCMV-specific response in the CVID patients with inflammatory

disease does not reflect a general elevation in IFN γ producing CD8 $^+$ T cell populations in these patients. In fact, the EBV specific responses in both CVID patient groups mirrored those of the healthy controls. Importantly, the polyfunctional signatures obtained after HCMV and EBV peptide stimulation in the healthy donors were essentially the same.

HCMV-specific CD8 $^+$ T cells in CVID patients with inflammatory disease displayed a distinctive intracellular cytokine expression profile (TNF- α high, IFN- γ high, IL-2 low), with the majority of cells producing IFN- γ only (47%) or IFN- γ and TNF- α (33%), a feature indicating good functional capacity (Casazza et al., 2006; Kannanganat et al., 2007). These results might indicate active antigen stimulation in CVID patients with inflammation. It has already been shown that T cell responses can be influenced substantially by the antigen load (Pantaleo and Harari, 2006). Additionally, higher IFN- γ production was shown to be directed by recent and stronger *in vivo* T cell activation (Schlingmann et al., 2009). It has been also suggested that IFN- γ and TNF- α play an important role in HCMV reactivation (Soderberg-Naucler et al., 1997a). For example, it has been shown that TNF- α can stimulate the immediate early enhancer/ promoter of HCMV in the transfected granulocyte/monocyte progenitor-like cell line HL-60 (Prosch et al., 1995) and immature monocytic cells (Stein et al., 1993). Thus, the T cell response to HCMV in inflammatory CVID patients may paradoxically enhance HCMV replication. Such antigen-induced immune activation by triggering proinflammatory cytokines as found in inflammatory CVID patients may be of crucial pathophysiological importance in these particular patients by contributing to the immunopathogenesis of inflammatory injuries.

Since the HCMV CD8⁺ T cells in the CVID patients with inflammatory disease expressed pro-inflammatory cytokines after stimulation, this thesis then aimed to further define the phenotype of these CD8⁺ T cells *ex vivo* (as presented in Chapter 5) and identify functional profiles that may correlate with protective CD8⁺ T cell responses as has already been characterized in other settings (Cellerai et al., 2010).

It has been already shown that during HCMV latency, a pool of highly differentiated HCMV-specific CD4⁺ and CD8⁺ T cells is maintained after resolution of the primary infection and this late-differentiated effector cell type is typically enriched in elderly HCMV seropositive individuals and is a key contributor to the functional deficit associated with immunosenescence, thought to be a consequence of multiple re-exposures to antigen via repeated virus reactivations (Appay et al., 2002; Waller et al., 2008). However, CVID patients with inflammatory disease, but not those without inflammatory disease, had significantly elevated frequencies of CD57⁺ HCMV-specific CD8⁺ T cells. The range of CD57⁺ frequencies in the latter group encompasses almost all the frequency values seen in the CVID with inflammation group. Moreover, CVID patients had high frequencies of granzyme B-expressing HCMV-specific CD8⁺ T cells. Follow up studies will be needed to determine whether a high frequency of CD57⁺ cells in patients without inflammatory disease is predictive of development of inflammatory disease.

Studies in healthy HCMV-seropositive individuals have shown that HCMV-specific CD8⁺ T cells express high levels of CD57 (Chong et al., 2008) that are oligoclonally derived (Wang et al., 1995) and as effector memory T cells capable of immediate functional activity with limited proliferative capacity and shortened telomere length

(Appay et al., 2002). However, recently it has been shown in healthy donors that CD8⁺CD57⁺ T cells are capable of rapid expansion (Chong et al., 2008) and induction of IFN- γ and TNF- α synthesis in response to HCMV peptides (Kern et al., 1999), thereby challenging the use of CD57 molecule as a marker of terminal differentiation or replicative senescence (Petrovas et al., 2009). Studies looking at the differential association of CD57 and PD-1, a molecule found in association with “exhausted” populations in LCMV and HIV infection (Barber et al., 2006;Day et al., 2006), have shown that the majority of CD8⁺ T cells specific to HIV exhibit a characteristic phenotype of PD-1^{hi}CD57^{hi} (Petrovas et al., 2009). High level PD-1 expression has been associated with poor proliferative capacity of T-cells in a number of chronic viral infections (Trautmann et al., 2006;Golden-Mason et al., 2007) and in the case of HCMV, high PD-1 expression on CD8⁺ T cells has been observed in liver transplant patients during periods of HCMV replication and disease and also in renal transplant patients experiencing high level HCMV replication (Sester et al., 2008;La Rosa et al., 2008).

In CVID patients with inflammatory disease, HCMV-specific CD8⁺ T cells expressed low levels of PD-1 and, indeed, the expression levels or expression frequencies of PD-1 observed on HCMV or EBV- specific CD8⁺ T cells were not significantly different between any of the groups, with, in general, all CD8⁺ T cells expressing low levels of PD-1. The striking correlation observed between the proportion of NLV pentamer⁺ cells expressing granzyme B, or PD-1, and the overall percentage of CD8⁺, CD27⁻, and CD28⁻ cells was surprising (refer to the Figure 5-17). PD-1 is considered as a negative regulator of T cell function, yet, by all measures, the HCMV specific response in both

groups of CVID patients is functional. The inverse association of this marker with lack of CD27 and CD28 expression is perhaps more consistent with a direct role in the control of T cell differentiation. The statistically significant elevation in frequency of CD8⁺ CD27⁻, CD28⁻ cells in the inflammatory group was of interest in the context of a possible role for HCMV in causation or potentiating of the inflammatory disease. Studies have shown that following resolution of HCMV viremia, a certain percentage of effector memory CD8⁺ T cells re-express CD45RA (Waller et al., 2008; Cantisan et al., 2010), and are therefore named EMRA (Romero et al., 2007). Additionally, it has been found that the majority of EMRA CD8⁺ T cells hardly express CD27 and CD28, and are specific to HCMV (Appay et al., 2002; van Lier et al., 2003; Waller et al., 2008). Until recently, these cells were considered by some groups as fully differentiated with defect in proliferation (Rufer et al., 2003; Cantisan et al., 2010). However, this notion has been challenged by other studies where these revertant memory CD8⁺ T cells were shown to proliferate perfectly well in response to HCMV peptide (Wills et al., 2002; van Leeuwen et al., 2002; Carrasco et al., 2006; Waller et al., 2008).

The data presented in Chapters 3-5 defined a distinct polyfunctional phenotype of HCMV specific CD8⁺ T cells that accumulate in CVID with inflammatory disease that is characterized by the CD57^{hi}, PD-1^{lo}, granzyme B^{hi} phenotype able to produce both high levels of IFN- γ and IFN- γ +TNF- α after peptide stimulation. Since persistent HCMV replication in inflamed tissues could lead to an environment in which HCMV specific CD8⁺ T cells are driven to immune exhaustion, in Chapter 6, the thesis then sought to investigate the proliferative capacity of the CD8⁺ T cells from CVID patients with inflammation compared to non-inflammatory patients. T cell exhaustion, especially

of high avidity cell populations, has been described in HIV, HCV and LCMV and in other settings (Alexander-Miller et al., 1996; Derby et al., 2001; Anderton, 2001). In spite of significantly elevated frequency of HCMV pp65-NLV specific CD8⁺ T cells in inflammatory patients, these cells showed no evidence of exhaustion, with low levels of PD-1 and high levels of TCR avidity, indicating that these cells are functionally competent in this subset of patients. These data are consistent with the data obtained from healthy individuals although it has been noted that CD8⁺ T cells with high avidity are more prone to cell death in an environment of excess antigen load (Alexander-Miller et al., 1996; Price et al., 2005). Although avidity in healthy individuals may be variable (Price et al., 2005) these may suggest that chronic HCMV replication in the CVID patients with inflammatory disease is not associated with loss of high avidity populations.

Not only did HCMV-specific CD8⁺ T cells in inflammatory patients not show any evidence of an exhaustion phenotype, rather, they showed features consistent with high *in vivo* functionality and proliferative activity. Indeed, the majority of HCMV specific CD8⁺ T cells also expressed HLA-DR, considered as an activation marker. In addition, Ki-67 expression on CD8⁺ T cells was very high in inflammatory patients compared to both non-inflammatory patients and healthy controls. Interestingly, this distinction was greatly enhanced when Ki-67 expression in HCMV-specific CD8⁺ T cells was analyzed, with healthy controls and non-inflammatory patients showing less than 2% and inflammatory patients around 40% Ki-67 positive implying a high proportion of the HCMV T cells were actively cycling. To support this, when PBMC were cultured with HCMV peptides in the absence of exogenous cytokines such as IL-15, HCMV-specific

CD8⁺ T cells from CVID patients with inflammation proliferated with a doubling time of approximately 1 day whereas cells from the other groups showed no proliferation under these conditions. Interestingly, there was no proliferation in response to EBV peptides in parallel experiments indicating that this was a HCMV specific effect. Additionally, transfer of supernatants from the proliferating cultures enhanced proliferation of CD8⁺ T cells specific to HCMV from non-inflammatory and healthy controls but had no effect on EBV specific- CD8⁺ T cells. This may indicate that the cytokine receptors available on HCMV specific CD8⁺ T cells are particularly geared towards a specific cytokine profile or that these cells are intrinsically better able to respond to the relevant cytokines such as IFN- γ and TNF- α . The *ex vivo* proliferation required both IFN- γ and TNF- α since adding anti-TNF- α and anti-IFN- γ inhibited proliferation to 80% and 50%, respectively. Although these results indicated that IFN- γ and TNF- α were both major contributing cytokines to the proliferation it was still possible that other cytokines such as IL-15 and IL-2 could be involved. The role of IL-15 as a growth factor for T cell proliferation has been documented by both *in vitro* and *in vivo* studies (Li et al., 2001; Judge et al., 2002; Schluns and Lefrancois, 2003).

Whether IFN- γ and TNF- α induce T cell proliferation in these inflammatory patients by causing the release of other cytokines, such as IL-15, that then act directly on T cells is not clear. However, it is tempting to speculate that these proinflammatory cytokines, at least in part, may stimulate other cell types, either directly or indirectly, to produce secondary cytokines that then act directly on T cells. To support this, studies have shown that like IFN type I, IFN γ and IL-12 can induce IL-15 production by antigen-presenting

cells and that IL-15 causes selective stimulation of purified CD44^{hi} CD8⁺ T cells *in vitro* (Zhang et al., 1998; Judge et al., 2002).

Consistent with this observation, recent data suggests a role for proinflammatory cytokines including IFN- γ and TNF- α in the pathogenesis of inflammatory bowel diseases as well as autoimmune arthritis (Neurath and Finotto, 2006b; Kim and Moudgil, 2008b). Additionally, treatment of CVID patients with the anti-TNF antibody Infliximab has been shown to reduce the inflammatory symptoms (Chua et al., 2007a). Interestingly, a similar effect has been found with the antiviral drug ganciclovir that inhibits HCMV replication (Raeiszadeh et al., 2006). Indeed, although preliminary, a marked reduction in HCMV (NLV) pentamer⁺ CD8⁺ T cell frequencies (4% of CD8⁺ T cells pre-therapy to 0.3% post-therapy) was observed that parallels clinical improvement in an inflammatory CVID patient undergoing antiviral therapy (Figure 7-1).

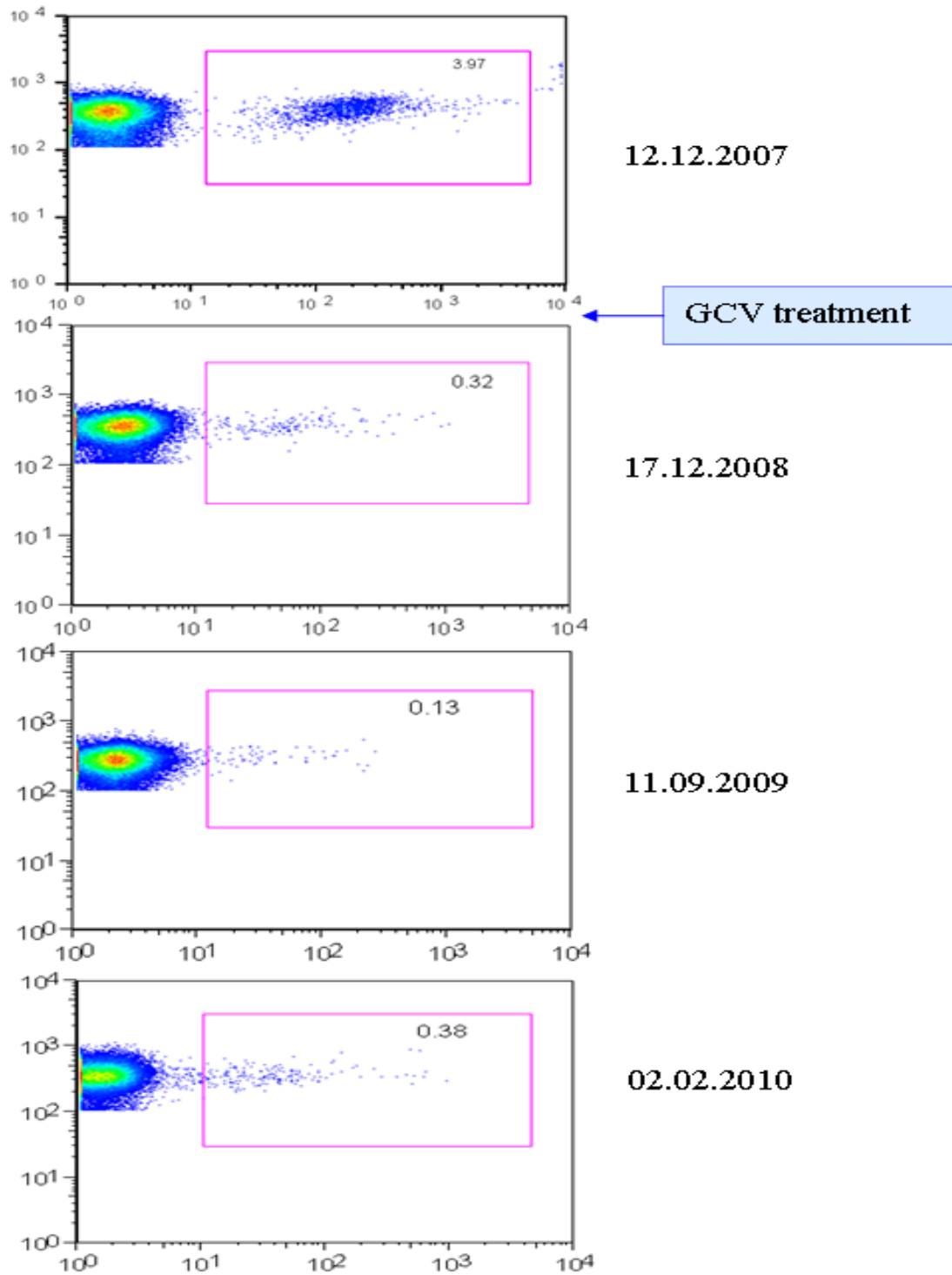


Figure 7-1: Frequency of HCMV (NLV)-specific CD8+ T cells in one inflammatory CVID patient before and after gancyclovier (GCV) treatment.

These data confirm the substantial proliferative capacity of the CD8⁺ T cells in CVID patients and underline the fact that the CD8⁺ T cell response is not exhausted. Although the mechanism behind the enhanced proliferative capacity in these experiments is unknown, it could include differences in avidity, cytokine production (which could have an autocrine effect in vitro), cytokine responsiveness and response to endogenous co-stimulation or protection against cell death.

Further supporting a role of HCMV-specific CD8⁺ T cells in the inflammatory disease seen in CVID patients was the observation that the frequency of CD73 expression on HCMV-specific CD8⁺ T cells was significantly reduced in inflammatory patients. CD73 is an ecto-5-nucleotidase which acts in the purine salvage pathway to generate nucleosides such as adenosine, which has anti-inflammatory properties (Colgan et al., 2006; Grunewald and Ridley, 2010). Indeed, in a recent study, *Borrelia garinii* infection of CD73^{-/-} mice was shown to be associated with joint swelling and the authors suggested that low levels of adenosine may favour local inflammatory responses and persistent infection (Yegutkin et al., 2010). Interestingly, infection of endothelial cells with CMV has been shown to increase the expression and activity of CD39 (ecto-ATP) and CD73 (Kas-Deelen et al., 2001).

Data presented in Chapter 6 indicated: first, HCMV (NLV) specific CD8⁺ T cells in inflammatory CVID patients were characterized by high levels of avidity for cognate antigens whereas the equivalent cells from non-inflammatory patients included substantial proportions where binding was CD8 dependent, indicating lower avidity. Second, while there was no significant difference in CD73 expressing CD8⁺ T cells in both groups of patients, HCMV-specific CD8⁺ T cells of inflammatory CVID patients

expressed significantly lower CD73 compared to non-inflammatory patients. Third, compared to telomere length values available for age matched healthy controls, CVID patients tested were shown to have reduced telomere values. Additionally a strong correlation was found between telomere length reduction and age.

It appears that HCMV can induce a chronic proinflammatory state not only in immunosuppressed patients but also in healthy individuals as a correlation was found between serum C-reactive protein (CRP) and IFN- γ levels during latent HCMV infection in healthy individuals, suggesting the involvement of both acute phase response proteins and type 1 cytokines in generating a proinflammatory state following HCMV infection (van de Berg et al., 2010a). There is increasing evidence indicating the potential role of HCMV in inflammation either directly or indirectly (Soderberg-Naucler, 2008).

None of the CVID patients in this study had HCMV DNAemia in whole blood implying that T-cell responses were sufficient to control replication even in the absence of antibody production and ruling out any assessment of the relationship of persistent antigen expression to PD-1 expression. Although more data are needed to investigate the presence of HCMV in the sites of inflammatory disease, it has been previously shown by our group that in a CVID patient with chronic gut inflammation HCMV DNA was present in lesions at moderate levels (~10,000 genomes/150,000 cell equivalents) in association with a high frequency of HCMV epitope-specific CD8⁺ T cells (~5%) among cells eluted from the biopsy (Raeiszadeh et al., 2006). The presence of viral DNA may indicate persistent low level replication that would be consistent with the elevated HCMV-specific responses observed in these patients.

Until now there has been no rational explanation for the frequent occurrence of many of the inflammatory complications associated with CVID (Fasano et al., 1996; Webster ADB, 1999; Malamut et al., 2008), or the persistent lymphocyte activation or oligoclonal expansions of activated CD8⁺ T cells described in a subset of CVID patients (Wright et al., 1990; Jaffe et al., 1993; Serrano et al., 2000; Carbone et al., 2006). This has been compounded by the inability to provide evidence of pathogens in inflamed organs using conventional histology/immunohistochemistry despite gene array analysis of PBMCs suggesting that the T-cells in these CVID patients are responding to a persistent stimulus (Holm et al., 2004). However, by utilising an enhanced immunohistochemistry approach (Samanta et al., 2003; Soderberg-Naucler, 2008), further support for the involvement of HCMV in driving the inflammatory pathology came from the collaborative work in which HCMV antigens were detected at the sites of inflammation (Figure 7-2), providing the first direct evidence of actively replicating virus in the inflamed tissues and supporting the conclusion that CMV infection is a major factor accounting for the aforementioned observations. Further investigations are needed to reveal the extent of HCMV infection and HCMV specific CD8⁺ T cell infiltration at sites of inflammation and whether this HCMV CD8⁺ T cell phenotypic signature is the same as that found in peripheral T cell population.

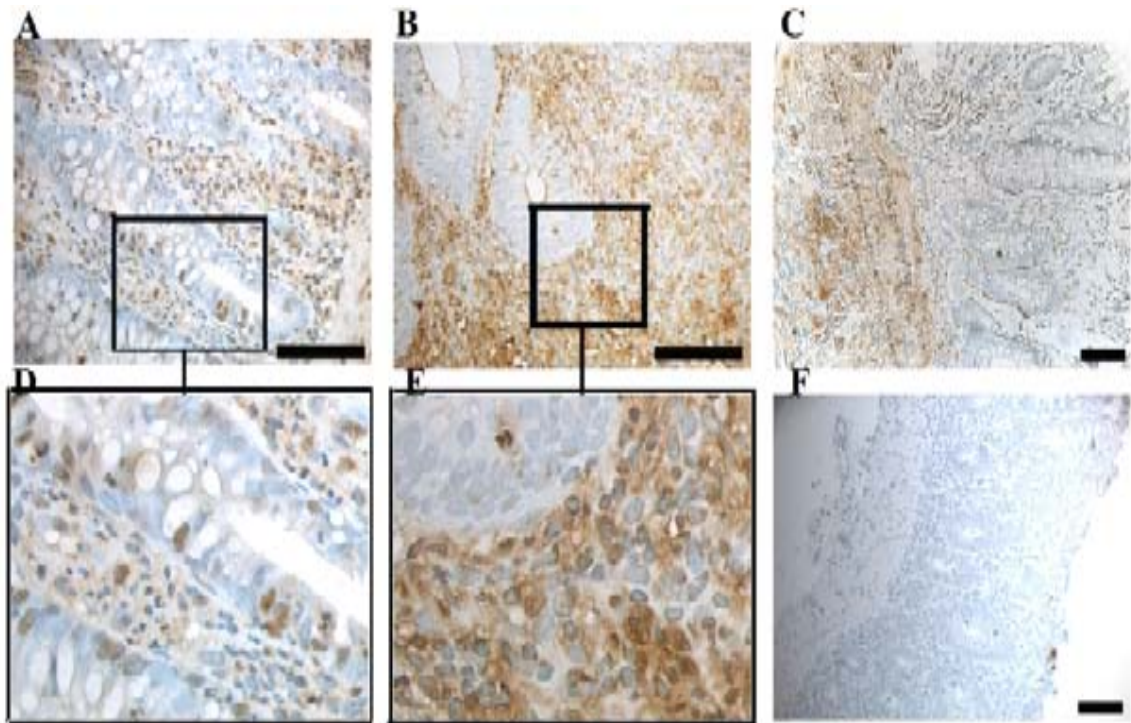


Figure 7-2: Immunohistochemical detection of CMV antigens in colon biopsy from inflammatory CVID patient.

In collaborative work with Karolinska institute, sections were stained for CMV IE-1 (Panels A and D) or L (Panels B and E) antigen; α -actin (Panel C); or as processed without a primary antibody (Panel F). Panels D and E are enlargements of the sections of the sections highlighted in panels A and B. Bars are 50 μ m. The image is provided with the permission of Professor Vincent Emery.

Taken together, the data presented above show that the HCMV-specific CD8⁺ T cells that accumulate in CVID with inflammatory disease display a distinctive polyfunctional phenotype that is characterized by a high frequency of CD57 expression and low level PD-1 expression. The cells are granzyme B⁺ and produce high levels of IFN- γ or of IFN- γ and TNF- α after peptide stimulation. Further investigations are needed to reveal the extent of HCMV infection and HCMV-specific CD8⁺ T cell infiltration at sites of inflammation and whether this HCMV-specific CD8⁺ T cell signature is associated with increased proliferative capacity and is an early marker of patients destined to develop inflammatory disease.

In conclusion, this PhD thesis provide compelling evidence base that HCMV infection is the stimulus for persistent T cell activation in many CVID patients and a specific HCMV CD8⁺ T cell phenotype is likely to be directly, or indirectly responsible for most of the chronic inflammatory complications in CVID patients suffering from inflammation. As the marked increase of proinflammatory cytokines was only found in these subset of patients, it is more likely that these cytokines are involved in changing the cytokine microenvironment to trigger ubiquitous inflammatory processes of local tissues in this group of patients, as there are some recently published data emphasizing the role of proinflammatory cytokines including IFN- γ and TNF- α in the pathogenesis of inflammatory bowel diseases as well as autoimmune arthritis (Neurath and Finotto, 2006b; Kim and Moudgil, 2008b). Thus, removal of the antigen or suppression of the inflammatory T-cell response can be effective in controlling inflammatory disease in this setting. The on-going longitudinal follow-up of CVID patients without inflammatory disease but with the characteristic CD8⁺ T cell signature of patients with inflammatory

disease will determine the temporal relationship between expression of immune markers and the onset of symptoms. In addition, the data presented in this PhD thesis suggest that the CD8⁺ T cell response to HCMV in CVID, and in particular the differences observed between the two CVID patient groups in the response to HCMV, but not EBV, provides a powerful and tractable model of immune regulation.

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8 References

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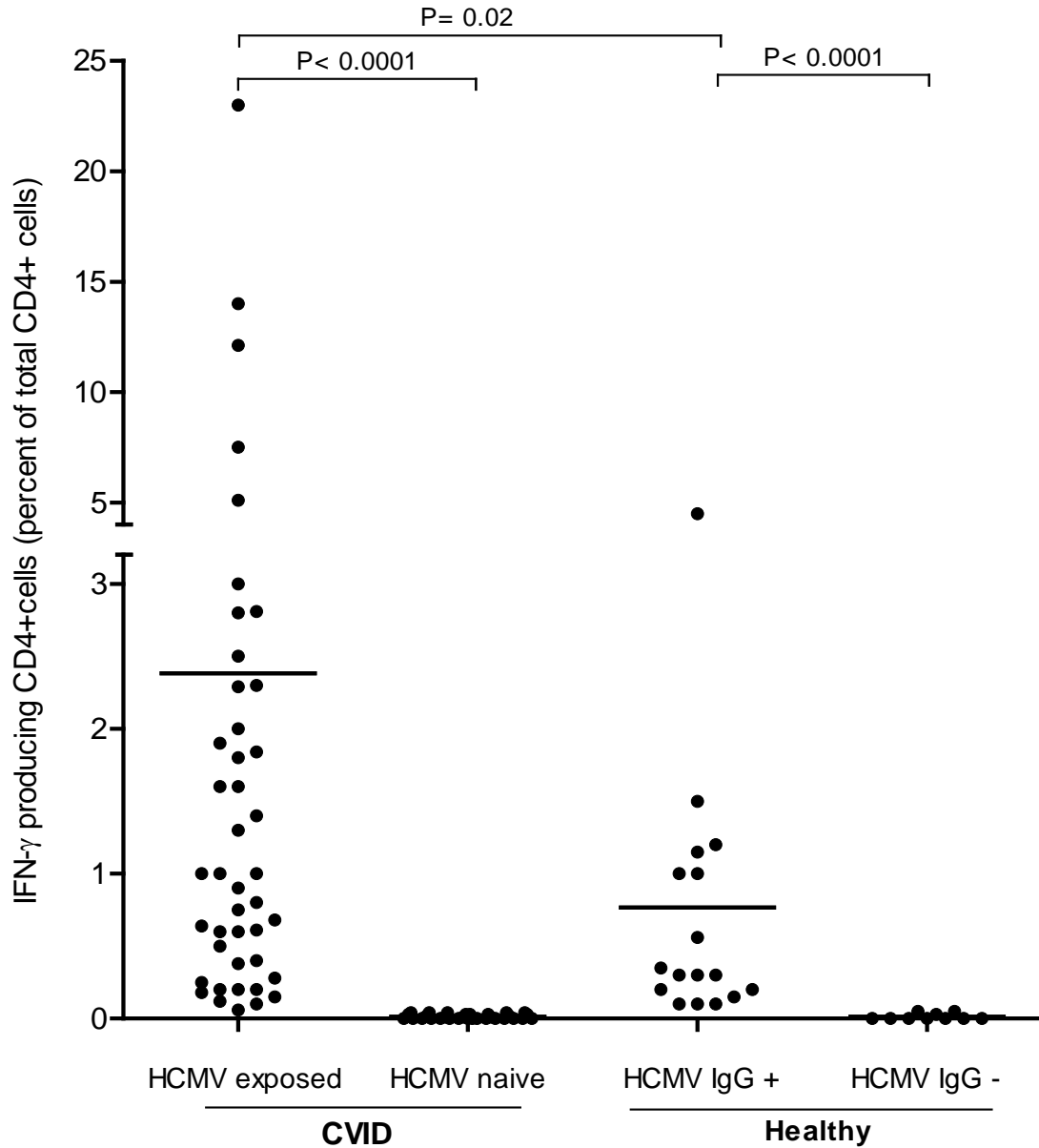
Appendices

Antibody	Conjugate	Cat. No	Company
CD8	PerCP Cy5.5	341050	BD Biosciences
CD4	PerCP Cy5.5	332772	BD Biosciences
IFN- γ	APC	554702	BD Biosciences
TNF- α	PE	559321	BD Biosciences
IL-2	FITC	340448	BD Biosciences
CD57	FITC	333169	BD Biosciences
Geranzyme B	APC	GRB05	Invitrogen
CD45RO	FITC	555492	BD Biosciences
CD45RA	APC	550855	BD Biosciences
CD27	FITC	555440	BD Biosciences
CD28	APC	559770	BD Biosciences
PD-1	FITC	557860	BD Biosciences
Ki-67	FITC	556026	BD Biosciences
HLA-DR	APC	FAB4869A	R&D
CD73	APC	17-0739-42	e-Biosciences
Isotype IgG1k	FITC	555909	BD Biosciences
Isotype IgG1k	APC	IC002A	R&D
Isotype IgG2ak	FITC	IC003F	R&D
CD28	Pure	348040	BD Biosciences
Caltag medium A	Fixation	GA50015-100	Invitrogen
Caltag medium B/ Perm.	Permeabilisation	GA50025-100	Invitrogen

Appendix 1: The list of antibodies and reagents used in this thesis for *ex vivo* or intracellular staining.

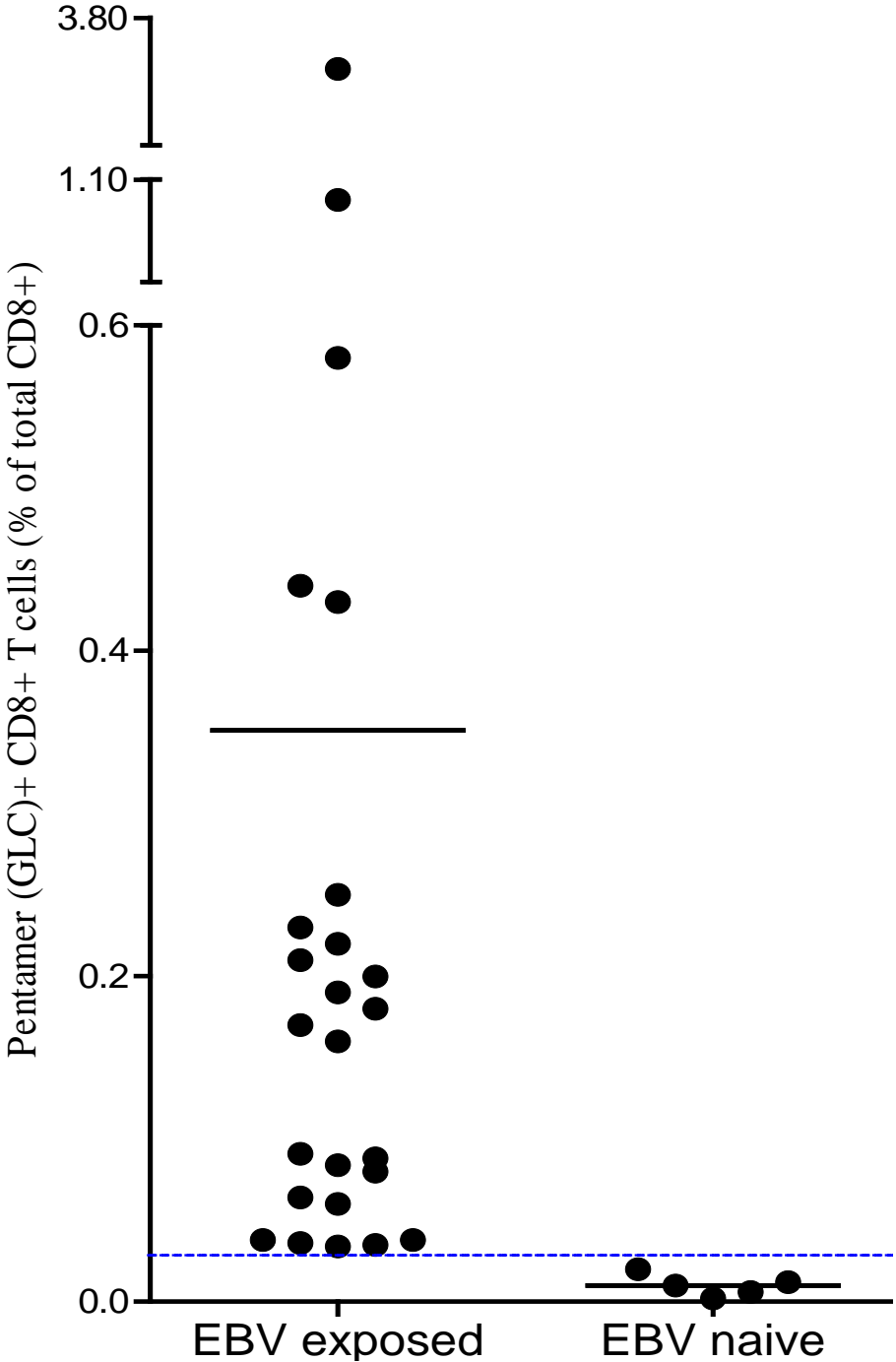
Item and sequences	Cat. No	Company
NLV pentamer/ A0201/ NLVPMVATV/ R-PE	F2B-008	Proimmune
GLC pentamer/ A0201/ GLCTLVAML/ R-PE	F2A00	Proimmune
NLV peptide/ A0201/ NLVPMVATV	P008-0B-G	Proimmune
GLC peptide/ A0201/ GLCTLVAML	POA001	Proimmune
PepMix HCMVA (pp65)/ UL83/ 561 aa MESRGRRCP E.....CIAS TP KKHR G	P06725	JPT
PepMix HCMVA (IE-1)/ UL123/ 491aa MESSAKRKMD.....HPMVTRSKAD Q	P13202	JPT
HCMV Lysate	10-144-000	Autogen bioclear

Appendix 2: The list of pentamers and peptides used in this thesis.



Appendix 3: Setting up the cut-off for T cell response positivity and prior exposure to HCMV (this graph originally was made by Mohammad Raeiszadeh).

Serological confirmation of HCMV infection in CVID patients is not practical. Therefore, the presence of CD4+ T cells expressing IFN- γ was measured following stimulation with HCMV infected cell lysates as a marker of prior exposure to HCMV infection. To set the appropriate cut-off for this analysis, 27 healthy donors (17 HCMV IgG positive, 10 HCMV IgG negative) were tested and found that in the HCMV IgG negative individuals, the mean frequency of CD4+ T-cells responding to HCMV lysate was 0.013% (SD = 0.021%). CD4+ T cell frequencies greater than 2 standard deviations above this value was used to distinguish HCMV exposed and HCMV naïve CVID patients. In this thesis only HLA-A2 subjects (25 patients and 14 healthy controls) were included.



Appendix 4: Setting up the cut-off for prior exposure to EBV in CVID patients with and without inflammation.

CD8+ T cells were stained *ex vivo* with EBV (GLC) pentamer and CD8+ T cell frequencies greater than 2 standard deviations above the mean frequency of EBV-specific CD8+ T cells in EBV naïve patients were considered as a cutoff (blue dashed line) to distinguish EBV exposed and EBV naïve CVID patients.

No	CVID patients	HLA types	
		HLA-A	HLA-B
1	√	0201/01L/07/09/11/15N – 0101/04N	0801/02/08N
2	√	0201/01L/02/07/09/14/15N – 0205/06	5101/03/09
3	√	0201/01L/04/07/15N/18/20 – 0301/39	1501/01N
4	√	0201/04/07/09/12/43N – 2601/02/08	1501/12/14
5	√	0201/07/09/18/20 – 3201/05	1501/12/14
6	√	0201/04/07/09/20 – 0202/04/15	1501/12/14/51
7	√	0201/07/09/20/24 – 1101/02/03	2703/05/09/17
8	√	0201/01L/07/09/15N – 0101/04N/06	0801/04/08N
9	√	0201/07/09/25 – 0301/03N/04	0702/10
10	√	0201/02/04/07/32N	4402/05/14/17
11	√	0201/01L/04/07/09 – 2402/02L/03	0801/04/08N/81
12	√	0201/04/12/13/31 – 2402/11N/13	-
13	√	0201/07/09/21 – 0101/04N/02/06	4001/14/22N
14	√	0201/01L/04/07/09/20/24/42	4501
15	√	0201/04/13/27 – 0301/02/03N/04	0702/10
16	√	0201/1L/07/15N/49 – 0101/04N	4402/23/33
17	√	0201/01L/04/07 – 2402/02L/03/08	1501/01N
18	√	0201/01L/07/09/20/24	5101/03/09
19	√	0201/04/07/09/12/16 – 0301/02/03N	1501/12/14/19
20	√	0201/01L/07/09/15N – 0101/04N/06	0702/04/05
21	√	0201/04/07/09/17/18/20/24	4402/03/07
22	√	0201/02/07 – 0205/06	-
23	√	0201/07/09/18/20/22	1501/12/14/19
24	√	0201/04/12/13 – 2402/03/06	2703/05/09
25	√	0201/01L/07/09/15N	5801/04

Appendix 5: HLA-phenotyping of CVID patients with and without inflammatory patients.

No	Healthy controls	HLA types	
		HLA-A	HLA-B
1	√	0201	0801
2	√	0201 - 0101	1501
3	√	0201 - 2301	4901
4	√	0201/02/04/07 – 2301/03	0702
5	√	0201/07/09 – 1101/02/03	3501/07
6	√	0201 - 2402	0801
7	√	0201/04/07/09 – 2601/02	1501/12
8	√	0201	-
9	√	0201/01L/07 – 0301/01N	4402
10	√	0201 - 0101	0702
11	√	0201/02/04 – 3101/05	1501
12	√	0201 - 2402	2703
13	√	0201	-
14	√	0201	-

Appendix 6: HLA-pehontyping of healthy controls.