Humoral responses to Cytomegalovirus glycoprotein B vaccine with MF59 adjuvant

A thesis submitted to the University College London for the degree of Ph.D.
by

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"The only true wisdom is in knowing you know nothing."

— Socrates

Declaration

I, Ilona Anna Baraniak, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Solid organ transplant (SOT) patients are at risk of end-organ diseases such as pneumonitis, hepatitis or enteritis caused by HCMV. HCMV infection can occur via primary infection of a seronegative recipient or upon reinfection or reactivation in a seropositive transplant recipient. Seronegative recipients have the greatest risk of viraemia and disease, showing that pre-existing natural immunity provides substantial protection. This, in turn, underpins vaccination as a viable strategy to control HCMV in the transplant setting. To test this, a clinical trial with a vaccine based on HCMV glycoprotein B (gB) antigen plus MF59 adjuvant was performed in SOT awaiting transplantation. The study showed that antibody titres against the gB antigen were significantly increased in both seropositive and seronegative recipients of the vaccine in comparison to the patients who received placebo, and importantly, higher titres correlated directly with reduced viraemia post-transplant. The aim of my thesis was to identify the component of the specific humoral response responsible for this effect. In comparison with placebo recipients, I could find no evidence for the protection being due to induction of antibodies that mediate neutralisation, antibody dependent cellular cytotoxicity, or prevent cell to cell spread of virus in culture. In contrast, analysis of antigenic domains of gB bound by the antibodies revealed that vaccination of seropositive individuals enhanced antibody responses against antigenic domain 2 and that these correlated with reduced viraemia post-transplant. Antibodies against three other antigenic domains were induced by the vaccine, but did not correlate with protection. These results suggest that antigenic domain 2 should be an important component of future HCMV vaccines to boost pre-existing immune responses that protect from HCMV infection. The protection afforded to seronegatives remains unidentified, but could be explained if another antigenic domain on gB remains to be discovered.

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Abbreviations

aa- amino acid

Ab- antibody

AD- antigenic domain

AIDS- Acquired Immune Deficiency Syndrome

ADCC- Antibody Dependent Cellular Cytotoxicity

ADCP- Antibody Dependent Cellular Phagocytosis

APC- Antigen presenting cell

bnAb- broadly neutralising antibody

bp- base pairs

BSA- Bovine serum albumin

CD4- Cluster of Differentiation 4

CD8- Cluster of Differentiation 8

CHO- Chinese hamster ovary

CI- Confidence Interval

CMV- Cytomegalovirus

CNS- Central Nervous System

CPE- Cytopathic effect

CTL- Cytotoxic T-Lymphocyte

D- Donor

DAG- Diaglycerol

DB- Dense Bodies

DC- Dendritic cells

DMEM- Dulbecco/Vogt modified Eagle's minimal essential medium

DMSO- Dimethyl Sulfoxide

Dpi- Days post infection

DNA- Deoxyribose nucleic Acid

dsDNA- double stranded DNA

E- Early

EBV- Epstein Barr Virus

E. coli- Escherichia coli

EDTA- Ethylenediaminetetraacetic acid

EFF- Ets-2 repressor

EFG- Epidermal Growth Factor

EFGR- Epidermal Growth Factor Receptor

ELISA- enzyme-linked immunosorbent assay

Env- Envelope

ER- Endoplasmic reticulum

FACS- Fluorescence-activated cell sorting

FBS- Fetal bovine serum

FCS- Fetal calf serum

FITC- fluorescein isothiocyanate

FMO- Fluorescence minus one

gB- glycoprotein-B

GCV- Ganciclovir

Gfi-1- Growth factor independent-1

GFP- Green Fluorescence Protein

gH- glycoprotein-H

gL- glycoprotein-L

gM- glycoprotein-M

gN- glycoprotein-N

GST- Glutathione S-transferase

GVHD- graft versus host disease

HAART- Highly active antiretroviral therapy

HCMV- Human cytomegalovirus

HCV- Hepatitis C virus

HD- High Dose

HFF- Human Foreskin Fibroblasts

HHV- Human herpesvirus

HIG-Hyperimmune globulin

HIV- Human Immunodeficiency Virus

HLA- Human leukocyte antigens

Hpi- Hours post infection

HSC- Hematopoietic stem cell

HSCT- Hematopoietic stem cell transplant

HSPG- heparan sulphate proteoglycan

HSV- Herpes simplex virus

IE- Immediate Early

IE1- Immediate Early protein 1

IFγ- Interferon gamma

Ig- Immunoglobulin

IL- Interleukin

IPV- Inactivated Polio Vaccine

IRL- Internal Repeat Long

IRS- Internal Repeat Short

IU- International Unit

IVIG- Intravenous immunoglobulin

kbp- kilo base pairs

KIR- Killer-cell immunoglobulin-like receptors

L- Late

LAMP- Lysosome associated membrane protein

LD- Low Dose

Log- logarithm

MCP- Major Capsid Protein

MCMV- murine cytomegalovirus

MHC- Major Histocompatibility Complex

MEM- Minimal Essential Medium

miRNA- micro RNA

mM- Millimolar

µM- Micromolar

MMR- Measles, Mumps, Rubella

MnCP- Minor Capsid Protein

MOI- Multiplicity of Infection

M_r- the relative formula mass

MUSC- The Medical University of South Carolina

MWW- Mann-Whitney-WIlcoxon

MVA- Modified Vaccinia Ankara

MYA- Million Years Ago

n- Number

Nab- Neutralizing antibody

NF-κB- Nuclear Factor kappa-light-chain-enhancer of activated B cells

NGS- Next Generation Sequencing

NHS- National Health Service

NK- Natural Killer cells

nNab- non-neutralizing antibody

ns- Not significant

OD- optical density

OPV- Oral Polio vaccine

ORF- open reading frame

OriLyt- Origin of replication

PBMC- peripheral blood mononuclear cell

PBS- Phosphate-buffered saline

PCR- Polymerase Chain Reaction

PCR2- Polycomb repressor complex 2

PDGFR- Platelet-derived growth factor receptors

pDNA- plasmid DNA

PE- Phycoeryerthyrin

PEG- Polyethylene glycol

PerCP- Perydinin chlorophyll protein

PFA- Paraformaldehyde

plac- placebo

PKC- Protein kinase C

PMA- phorbol 12-myristate 13-acetate

Pol- Polymerase

Pp65- phosphoprotein 65

Pp28- phosphoprotein 28

R- Recipient

Rb- Retinoblastoma

RFH- Royal Free Hospital

RhCMV- Rhesus cytomegalovirus

RNA- Ribonucleic Acid

RPE- Retinal Pigment Epithelium

RT- Room Temperature

rtPCR- reverse transcriptase Polymerase Chain Reaction

U- Unique

S- Short

SCP- Small Capsid Protein

SD- Standard deviation

SEM- Standard error of mean

SIV- Simian immunodeficiency viruses

SNHL- Sensorineural hearing loss

SOT- Solid Organ Transplant

TCID50- Tissue Culture Infective Dose

TLR- Toll-like receptor

TMB- Tetramethylbenzidine

TNF-α- Tumor necrosis factor alpha

TNFRI- TNF-α receptor 1

T. pallidum- Treponema pallidum

TRL- Terminal Repeat Long

TRS- Terminal Repeat Short

TRIM28- Tripartite motif-containing 28

Tx- Transplant

UK- United Kingdom

UL- Unique Long

µl- Microlitre

US- Unique short

USA- United States of America

WHO- World Health Organisation

V- Variable

v- Visit

vacc- vaccinated

Vir- viraemia

VPR- alphavirus like particles

vs- Versus

VZV- Varicella Zoster Virus

YY-1- Ying-Yang-1

1. Introduction.

1.1. The history of human cytomegalovirus discovery.

Early observations on the histopathological alterations caused by HCMV were made in 1881 by a German clinician, Prof Ribbert. The examination of a stillborn suspected to be infected with *T. pallidum* (syphilis) puzzled the clinician, as he could not find an explanation for the unusual looking, enlarged cells in the kidney. A few years later (1904), similar observations were made following histopathological examination of organ biopsies from babies born premature; including salivary glands of these children. The researchers reported finding the enlarged 20-30μm in diameter altered “owl’s-eye” cells with intranuclear inclusions. Nevertheless, these cellular pathologies were thought to be associated with bacterial or protozoal infection [1, 2]. In 1921 two researchers Ernest Goodpasture and Fritz Talbert suggested for the first time, that the cellular alterations that they called: ‘Cytomegalia infantum’ may be a result of a viral infection [3]. This hypothesis was based on the observation that the ‘cytomegalia’ that they noticed displayed similarities to the pathologies in skin lesions caused by varicella [4]. The development of new histopathological methods; such as, *e.g.:* exfoliative cytology in the 1950’s facilitated progress in the field of cellular pathology. Then, for the first time, murine CMV was isolated and propagated in murine fibroblasts (by Margaret Smith in 1954) [5]. Shortly after, three groups of researchers independently reported isolation of the human virus: Smith in 1956 [6] Rowe and co-workers in 1956 [7], and Weller and co-workers in 1957 [8]. The term “cytomegalovirus" was coined in 1960 by Weller and co-workers to describe the cytopathic effect of human CMV (HCMV).

1.2. Classification of human cytomegalovirus.

HCMV belongs to the *Herpesviridae* family, *Betaherpesvirinae* subfamily, genus Cytomegalovirus and species Human herpesvirus 5 (HHV-5). The *Herpesviridae* family members have characteristics distinct from other viruses; herpesviruses possess a linear, dsDNA genome packaged in an icosahedral capsid. Their capsid is surrounded by tegument (a protein matrix); and the outer membrane consists of the lipid bilayer with membrane-associated proteins (glycoproteins). Historically, the herpesviruses were assigned into one of the three categories: α, β, γ based on their biological properties such as growth kinetics or host and tissue tropism.

β herpesviruses have the largest genome amongst the *Herpesviridae* family, ranging from 145kbp for HHV-7 to approximately 241kbp for chimpanzee cytomegalovirus. The viruses that belong to this subfamily are generally cell-associated and do not cross host-species barriers. Such a high species-specificity indicates a long period of co- evolution with the host. Interestingly, the base of the phylogenetic tree of the *Herpesviridae* family has been estimated at 400 million years ago (based on detailed tree-building models); (Figure 1.1; [9]). Moreover, due to the fact that the herpesviruses co-evolved with their mammalian hosts; they have been used as surrogates to track mammalian – including human – evolution and migration [10].

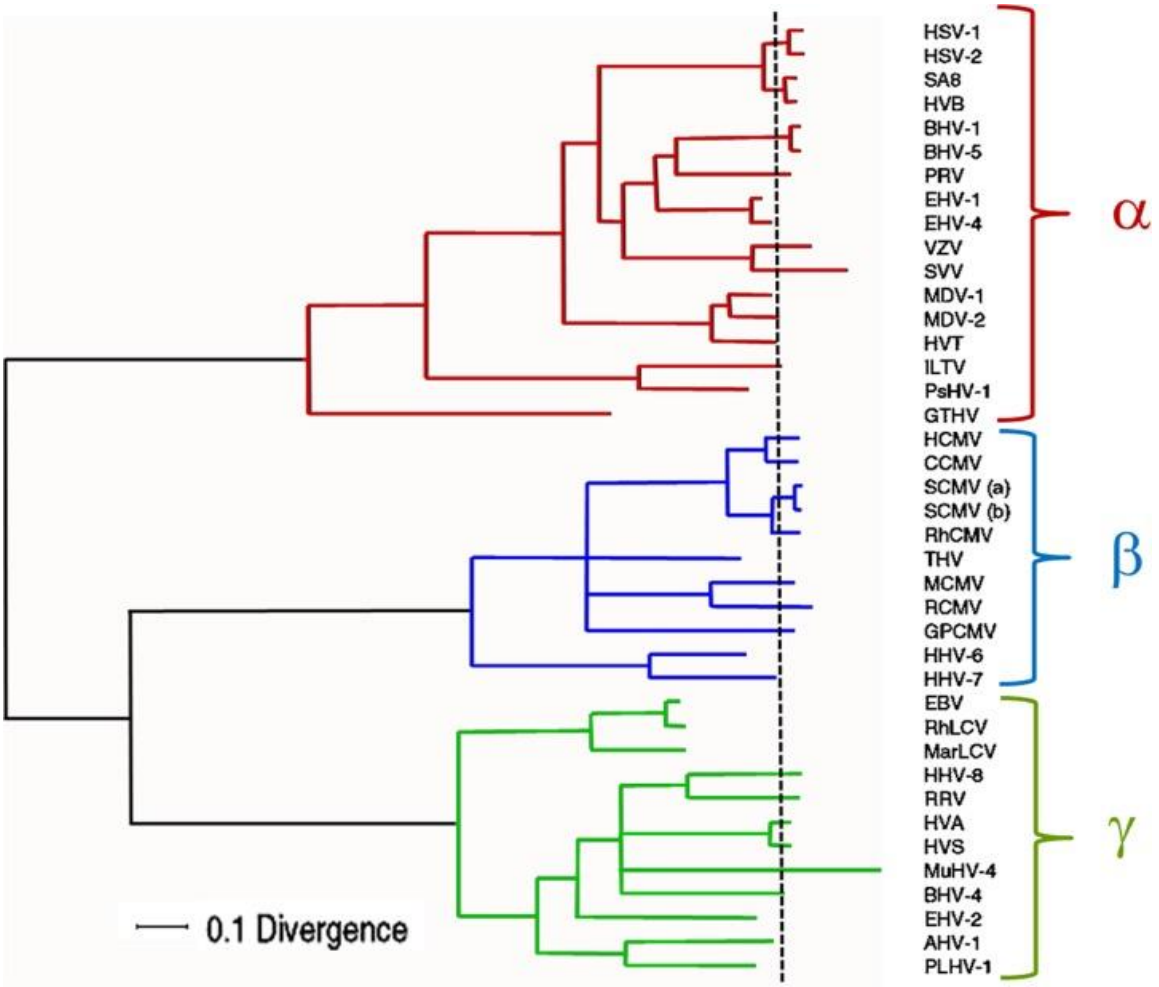


Figure 1.1. Phylogenetic tree for the family Herpesviridae.

A phylogenetic tree was constructed based on an alignment of amino acid sequences for six shared genes from 40 different herpesviral species within all three subfamilies. The genes included the orthologs of VZV ORF28, ORF29, ORF30, ORF31, ORF40, and ORF42. Initial tree evaluation utilized a Bayesian Monte Carlo Markov chain process. The root of the tree was estimated as the midpoint between the mean tip positions of terminal branches in the alphaherpesviruses (α) and those in the betaherpesviruses (β) plus gammaherpesviruses (γ). The mean tip position is marked with a vertical dashed line. A divergence scale is shown at the bottom of the figure. Abbreviations for the 40 viruses are as follows: HSV-1, herpes simplex virus 1; HSV-2, herpes simplex virus 2; SA-8, simian agent 8; HVB, herpesvirus B; BHV-1, bovine herpesvirus 1; BHV-5, bovine herpesvirus 5; PRV, pseudorabies virus; EHV-1, equid herpesvirus 1; EHV-4, equid herpesvirus 4; VZV, varicella-zoster virus; SVV, simian varicella virus; MDV-1, Marek's disease virus type 1; MDV-2, Marek's disease virus type 2; HVT, herpesvirus of turkeys; ILTV, infectious laryngotracheitis virus; PsHV-1, psittacid herpesvirus 1; GTHV, green turtle herpesvirus; HCMV, human cytomegalovirus; CCMV, chimpanzee cytomegalovirus; SCMV (a) and SCMV (b), simian cytomegalovirus; RhCMV, rhesus cytomegalovirus; THV, tupaiid herpesvirus; MCMV, murine cytomegalovirus; RCMV, rat cytomegalovirus; HHV-6, human herpesvirus 6; HHV-7, human herpesvirus 7; EBV, Epstein-Barr virus; RLV, rhesus lymphocryptovirus; MarLCV, marmoset herpesvirus; HHV-8, human herpesvirus 8; RRV, rhesus rhadinovirus; HVA, herpesvirus ateles; HVS, herpesvirus saimiri; MuHV-4, murid herpesvirus 4; BHV-4, bovine herpesvirus 4; EHV-2, equid herpesvirus 2; AHV-1, alcelaphine herpesvirus 1; PLHV-1, porcine herpesvirus 1. Reprinted from *Virus Research* [9].

1.3. Structure of HCMV.

The human cytomegalovirus virion is very large in comparison to other viruses measuring approximately 300nm in diameter. The outer membrane-lipid bilayer contains glycoprotein complexes that play important roles in viral entry. Enclosed within this lipid bilayer is an icosahedral capsid consisting of 162 capsomers that encapsidate the genetic material. Electron cryomicroscopy at 18Å resolution coupled with computer reconstruction demonstrated that the icosahedral capsid consists of 12 pentons, 150 hexons and 320 triplexes arranged on a T=16 icosahedral lattice which resembles the organisation of other, distant herpesviruses such as HSV (an alpha herpesvirus) [11].
The tegument is located between the icosahedral capsid and the envelope. Electron cryomicroscopy analyses revealed that this structure consists of 960 copies of filamentous densities that form a thin, net-like shell, enclosing the icosahedral nucleocapsid and interacting with the capsid proteins. The structure of the tegument is also ordered icosahedrally [11].

Another virus-like particle was detected during *in-vitro* cell culture. These so-called dense bodies (DB) are spherical particles surrounded by a membrane similar to the viral envelope. Their most important feature is lack of nucleocapsids, therefore containing little or no DNA. However, DB had most, but not all of the structural polypeptides of HCMV (Figure 1.2 D) [12, 13].

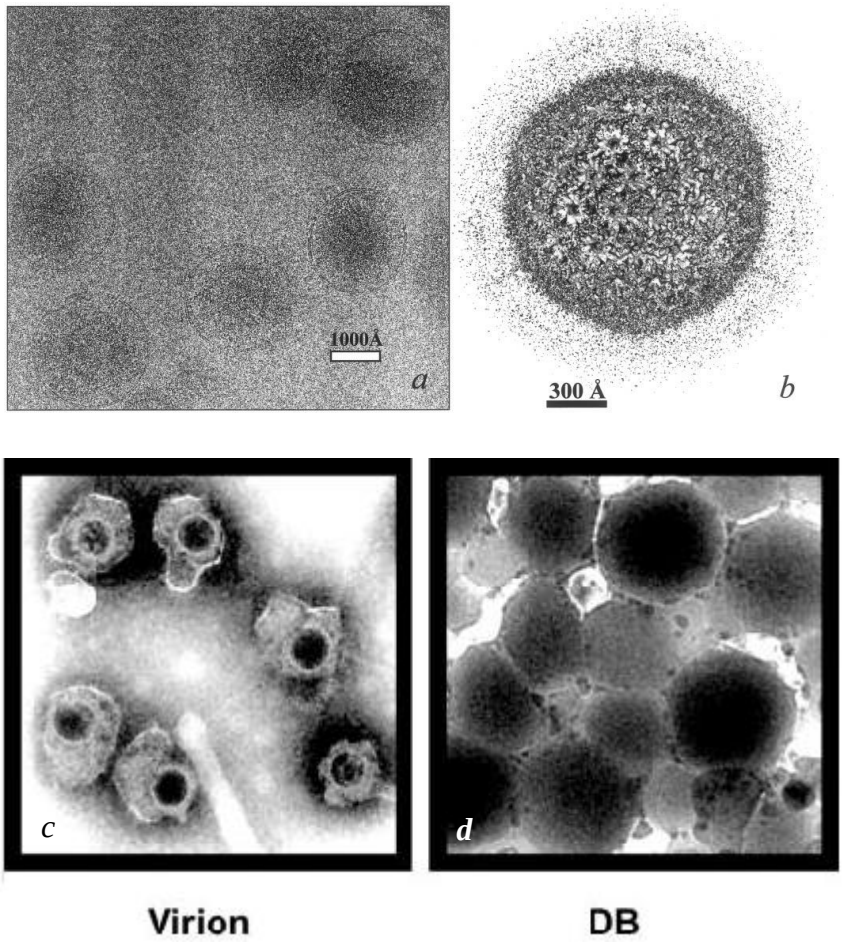


Figure 1.2. Characterisation of HCMV particles and dense bodies (DB).

(a) Electron cryomicrograph of intact HCMV particles embedded in vitreous ice; the electron micrographs of ice-embedded HCMV particles were recorded at 400 kV in a JEOL 4000 electron cryomicroscope at 30,000 magnification using a dosage of; 6 electrons/Å^2. The underfocus value of this image was determined to be 1.8mm. (b) Shaded surface representation of the 18-Å resolution 3D reconstruction of the icosahedrally ordered portion of the intact HCMV particles as viewed along a three-fold symmetry axis. The map is contoured at 0.8 s (standard deviation). Reprinted from [11]. Electron microscopy of negatively stained HCMV virion (c) and dense body (d) preparations. Magnification, ×8,400 (c,d). Reprinted from [13].

1.4. The genetic sequence of the HCMV genome.

The first annotated sequence of an HCMV strain was reported for the laboratory-adapted strain AD169 [14]. The genome of this strain contained 208 non-overlapping open reading frames (ORFs) A subsequent comparison with the Toledo genome revealed that the AD169 strain was missing around 30kb of genome [15]– now referred to as ULb'. The loss of this region is likely due to the extensive passage of AD169 in culture. Furthermore, the depletion of these genes contributed to its attenuation, which in turn allowed its development as a vaccine strain [16, 17].

However, the coding potential of HCMV is constantly revisited using newer sequencing approaches as they become available. These modern techniques have resulted in the deletion of 37 previously annotated ORFs from the genome map with at least nine previously unrecognized ORFs being added into the map of the AD169 genome. In total, the presence of 192 ORFs in this clinical strain was reported by E. Murphy and his co-workers in follow up study [18].

Also, viral genomes of four clinical isolates (Toledo, FIX, PH, and TR) and two laboratory strains (AD169 and Towne) were cloned as infectious bacterial artificial chromosomes (BAC) and subsequently sequenced [18]. DNA of the 5^{th} clinical strain Merlin was sequenced uncloned [19]. This extensive and comparative sequencing analysis showed that the clinical strains might contain 252 ORFs (maximum predicted number) that potentially encode functional proteins and are conserved amongst these isolates [20], although more recent prediction suggests that only approximately 150 ORFs are likely to encode proteins [21]. Moreover, the alignment of the sequences between laboratory and clinical strains revealed that these clinical strains possess additional sequence spanning the region UL133-UL155 and an additional IR_L sequence in comparison to the laboratory adapted strains [20].

As stated above, the difference in the number of ORFs between laboratory strains and clinical isolates is consistent with the fact that these laboratory adapted strains of the virus have undergone considerable genome re-arrangements. Most of the missing genes encode immune-evasive proteins and factors that determine cellular tropism, both of which are unnecessary for growth *in vitro* in fibroblasts. Thus, selective pressure results in the mutation and elimination of these genes.

Clinical isolates passaged minimally in fibroblasts		**Laboratory strains** highly passaged and adapted to grow in cell-culture	
Toledo	Isolated from urine of a congenitally infected child [22].	**AD169**	Attenuated due to extensive passaging *in vitro;* first HCMV strain to be sequenced [16].
FIX	(derivate of VR1814)-isolated from a pregnant woman with primary HCMV infection [23].	**Towne**	Recovered from the urine of a congenitally infected new born, was attenuated through 125 passages in human embryonic lung fibroblast [24].
PH	Isolated from a bone marrow transplant patient [25].		
TR	Ganciclovir and cidofovir-resistant ocular isolate from an AIDS patient with retinitis [26].		
Merlin	Isolated from the urine of a congenitally infected child [19].		

Table 1.1. List of HCMV strains.

Both clinical and laboratory adapted strains of the virus have been identified and isolated.

1.5. Genome organization.

The genome size of HCMV is approximately 235kbp. The genome organisation is characterized by the presence of long (L) and short (S) genome segments that can be inverted relative to each other. Each of these genome segments contains a unique region (U_L and U_S) that is flanked by repeated segments internally (IR_L and IR_S) or at the ends (TR_L and TR_S). These regions contain: TR_L: multiple repeats of a (a_n) and b, TR_S multiple repeats of a (a_n) and c; and IR_L-IR_S –b'a'$_n$ c'
Therefore the whole genome is organized as:

a_nb-U_L- b'a'$_n$ c'-U_S-a_nc

The general organisation of the genome is thus: TR_L-U_L-IR_L-IR_S-U_S-TR_S [27]; (Figure 1.3). The a_n sequences can recombine with the internal a' sequences therefore this complex organisation (E genome) enables four different isomers of the genome to form with equal frequency. However, the significance of this genome organization is not fully understood yet [28-31]. The terminal regions "a" contain repetitive 'head to tail' motifs [32] which include the cis-acting sequences *pac-1* and *pac-2* [31].These short sequences contain an AT rich core flanked by GC rich motifs that are recognized by DNA cleavage and encapsidation machinery [31]. The cleavage of the sequences adjacent to *pac-1* and *pac-2* leaves a single *3'* base overhang; which in turn facilitates subsequent circularization of the linear dsDNA upon the entry.

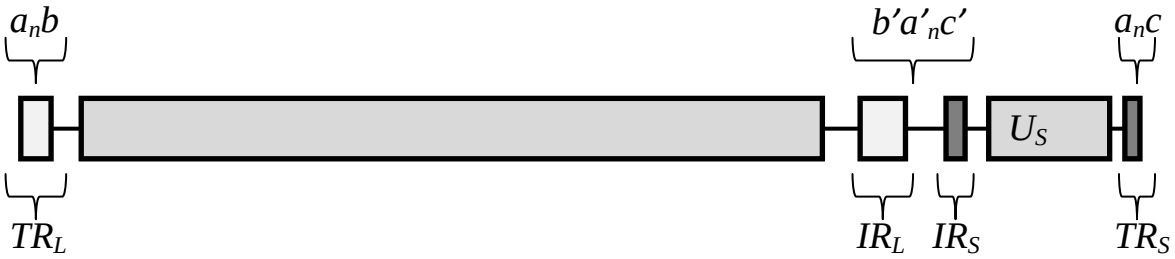


Figure 1.3. Schematic representation of the HCMV genome organization.

1.6. Genetic diversity and antigenic polymorphism in HCMV.

Infection and reinfection with multiple cytomegalovirus (CMV) strains is shown to occur frequently in immunocompromised individuals, sexually transmitted disease clinic attendees, and children attending day care centres [33-36]. Therefore there have been an increasing number of studies that have attempted to address whether strain variation and re-infection is a major route of morbidity in previously infected individuals. Despite this research emphasis there is still only limited evidence on such putative correlations.

Sijmons *et al*., conducted high-resolution analysis of inter-host genome diversity, providing an unbiased insight into cytomegalovirus variability and evolution. This study concluded that "cytomegalovirus is significantly more divergent than all other human herpesviruses. Importantly, "75% of strains are not genetically intact but contain disruptive mutations in a diverse set of 26 genes, including the immunomodulatory genes UL40 and UL111A" [37]. The implication of divergence has been challenged by other studies. For example, Lassalle *et al.* suggested that diversity was restricted to specific loci – with the RL13 locus being particularly heterogeneous between strains [38]. Similarly, other large-scale analysis of HCMV sequences sampled from human hosts revealed the existence of hot and cold spots of genetic variability [39]. The estimates of HCMV genome-wide mutation rates reported in this study was approximately an order of magnitude higher than those reported of a closely related herpesvirus, HSV-1 [39]. However, the high level of mutation for HCMV reported in this work remains highly controversial. Indeed, it has been speculated that such analyses could be confounded by events such as superinfection with different strains of HCMV that frequently happen in seropositive individuals. Whilst the major claims of this manuscript still remain to be corroborated; this report did demonstrate that viral genetic diversity is "unevenly distributed across three host compartments and show that HCMV populations of vascular compartments are genetically constrained while enriched for polymorphisms of glycoproteins and regulatory proteins" [39].

Other studies also confirm high HCMV diversity in healthy seropositive individuals; with one report showing that 93.7% of the seropositive individuals in their cohort contained multiple gN and/or gB genomic variants [40]. Reasons for such high rates of genetic polymorphism of glycoproteins of HCMV remain unknown. It is tempting to speculate that genetic variation in genes encoding glycoproteins may play a role in modifying HCMV tropism *in vivo* and/or could be driven by adaptive responses of the host and subsequent attempts to evade them [39, 41].

The genotypes of gB are classified into four different categories, based on sequence variation observed in the UL55 gene (that encodes gB). Differentiation into four distinct gB genotypes was based on the nucleotide sequence of variable region of gB that is known to encompass the protease cleavage site [42-44]. One study reported the distribution of HCMV gB in a cohort of immunocompromised patients, including both transplant recipients and non-recipients to be as follows: gB1, 28.9% of patients; gB2, 19.6%; gB3, 23.7%; gB4, 2.0%; and mixed infection, 25.8%. In contrast to patients infected with a single gB genotype, patients infected with multiple gB genotypes developed progression to HCMV disease, had an increased rate of graft rejection, had higher HCMV loads, and were statistically more likely to be infected with other herpesviruses [45, 46]. Similarly, a vast number of studies with seropositive pregnant women and HIV patients co-infected with HCMV looking at the relationships between gB serotypes and the development of HCMV syndrome were conducted. However, while some reports show correlation between particular gB serotypes and worse outcome, others indicate no association [47-54]. Due to these conflicting results it is impossible to draw a firm conclusion on whether the genotype of gB is correlated with the clinical outcome of the patients and this subject should be further investigated.

1.6.1. Potential impact of genetic diversity and antigenic polymorphism in HCMV on vaccine development.

An increasing body of evidence indicates that it is very important to consider that these high rates of polymorphism and mutation in glycoproteins could impact on the development of successful antivirals and vaccine strategies based on the targeting of these viral components. In the context of this increasing evidence it has to be considered that a vaccine strategy based on a single strain of HCMV or subunit genotype may not be completely effective. For example, the humoral response elicited to vaccine antigens might not be sufficient to cover the broad range of highly polymorphic gB and genetically variable strains of the virus, as even the point mutations may completely alter presentation of the protective epitopes. Consequently, it will be important to determine whether immune responses against conserved domains of viral glycoproteins provide better protection from HCMV infection.

1.7. The virus entry process.

The entry process is usually divided into two phases: tethering and post attachment/fusion events [55].

The initial entry step is tethering of the virions to heparan sulfate proteoglycans (HSPG). This helps stabilize the virion on the cellular surface and is mediated by electrostatic forces (under physiological conditions the ester and amide sulphate groups of HSPG are deprotonated and attract positively charged amino acid motifs on the glycoproteins (gB, gM/gN). Following this first phase, virions are promptly re-located to the heparin-resistant binding site [56], where the entry of the pathogen occurs. The experimental data suggest that gB mediates fusion of plasma membranes (pH independent) in fibroblasts, however the exact mechanism is not fully understood yet. Recently, a set of analyses with recombinant virus in which the UL55 gene was replaced with galK (pAD/CreΔUL55) demonstrated that the null gB mutants (ΔUL55) were unable to enter the cells, however this was rescued by the addition of membrane fusion agent- polyethylene glycol (PEG). This clearly indicates that HCMV glycoprotein B

is essential for entry of the virus into cells and dissemination of the virus from cell-to-cell. However, it is not essential for virion attachment, assembly and egress [57].

So far, several different binding interactions were proposed in this second post–attachment step, with most evidence suggesting interactions between gB-β1 integrin (α2β1, α6β1, αVβ3) [58, 59], gB-EGFR [60]- still more data needed to confirm this finding (the latest reports suggest that EFGR is not the crucial entry receptor for HCMV- [61-63]) and most recently proposed- platelet-derived growth factor-alpha receptor PDGFR-alpha [64]. Other receptors have also been identified (such as Annexin II) and it is proposed that they may play an auxiliary role in the entry of the virus [65-67].

Generally, such a wide cellular tropism of HCMV is consistent with the virus either using multiple/and or variable receptors and/or entry targets that are ubiquitously distributed cell-surface molecules. It seems likely that the entry mechanism of this pathogen might vary depending on the cell-type and more data are needed to fully understand this process. However, gB is unquestionably one of the key proteins that mediate viral entry and contributes to the pathogenicity of this virus.

1.8. HCMV lytic life cycle.

As with all the herpesviruses, HCMV has two distinct life- cycles: lytic and latent. Lytic infection is associated with the release of progeny virus from the infected cell and it is accompanied by cell death. Herpesviruses also establish latent infection within specific tissues, which are characteristic for each virus reflecting the unique tissue tropism of each member of this family [68].

1.8.1. Organization of HCMV-expressed genes.

The gene expression of HCMV occurs in a distinct temporal manner [69]. The genes are grouped according to the time of their transcription [70]: Immediate-early genes- (IE), Early genes- (E) and Late genes- (L). The expression of the IE group is initiated by tegument proteins pp71 (ppUL82) and ppUL69 that act as trans- activators of viral gene expression [71-75]. The pp71 protein is known to enhance the transcription of the major immediate early (MIE) gene region via degradation or/and relocalization of transcriptional repressors such as: Daxx and ATRX [76]. The ppUL69 protein shuffles between cytoplasm and nucleus and its function is to enhance expression of MIE region [77]. Also, the pp65 protein (ppUL82) enhances activation of the MIE promoter via its interaction with the IFI16 protein [78]. These events take place prior to *de novo* viral protein synthesis, as shown in experiments with cyclohexamide (used to block *de novo* viral protein synthesis) or in the presence of phosphonoformate or ganciclovir (GCV) that are used to block HCMV DNA synthesis [79].

These genes encode multiple proteins that are crucial for:

1) Viral DNA synthesis and regulation of replication [80, 81]:

- UL122-123 (IE1/IE2) gene region- different splicing of this region produces trans-activator proteins:

  -The IE72 (IE1) - protein is crucial for efficient replication of viral DNA *in vivo*; has anti- apoptotic and regulatory effects; facilitates immune- evasion and promotes cell cycle progression to facilitate viral expression [82-87];

  -The IE86 (IE2) - is an essential, multifunctional viral protein that auto-regulates the MIE enhancer-containing promoter. The role of this protein is absolutely crucial as it trans- activates the expression of the early genes, binding to- and activating the UL54 promoter that controls expression of the viral polymerase, inactivates the Rb repressor, thereby promoting transition of the cell cycle from G_0/G_1 to G_2/M and interfering with the activation of innate antiviral mechanisms; reviewed in [88];
- TRS1/IRS1- take a role in viral replication [89, 90].

2) Immune-evasion:

- US3- non-regulatory protein that binds and sequesters major histocompatibility complex class I (MHC I) heavy chains in the endoplasmic reticulum leading to inhibition of antigen presentation [79, 80].

3) Inhibition of apoptosis:

- UL36- 38: trans- activator proteins, the UL36 and UL38 gene products have a crucial role in the inhibition of apoptosis [79, 91].

Next, the early- phase genes (E genes) are expressed at approximately 4– 48hpi. These mainly encode proteins that are involved in DNA replication and further transcriptional regulation [70].

Subsequently, late genes are expressed. This occurs strictly after the onset of replication (shown in the experiments with GCV and phosphonoformate- that block DNA replication) up to viral egress (usually detectable at approximately 72-96hpi). These genes mainly encode structural components of the virions [70, 92].

1.8.2. Replication of the viral genome.

The HCMV lytic replication is an extremely complex and well- regulated process that is not fully understood yet. It is observed that this pathogen replicates dynamically in the human host with a doubling time of approximately 24h [93].

The genome of HCMV contains cis-acting elements that direct DNA replication; packaging and transcription. The HCMV replication origin- ori*Lyt* is mapped to an approximately 1500bp domain that is located in the middle of the U_L region (between ORFs 57 and 69). The core of this replication origin consists of multiple various repeated elements and sites that 1) respond to UL84 and IE2; 2) enable RNA/DNA contact [94, 95].

Although the exact mechanism of the initiation of replication is unknown, it is well established that the protein encoded by UL84 plays a key role in this event. This is a unique (no known homolog), multifunctional protein that governs regulation of the transcriptional activation mediated by IE2 by associating with it and supressing its transcriptional activation function. The binding of UL84-IE2 complex to the promoter (found in the essential region I within ori*Lyt)* initiates the replication process. However, at least six additional core proteins are involved in this process as well:
-UL44- DNA processivity factor (dsDNA binding protein pUL44);
-UL54- DNA polymerase;
-UL57- a single stranded DNA binding protein;
-a helicase-primase complex encoded by the genes UL70 (primase), UL102 (primase-associated factor) and UL105 (helicase).

The interaction of the pUL84 protein and IE2 is believed to influence activity of both proteins. As mentioned above the pUL84/IE2 complex binding to the ori*Lyt* is the first step of the lytic replication process; this is followed by subsequent binding of the helicase-primase complex and auxiliary protein pUL57 (unwinding the helical DNA at the replication fork, priming for synthesis of the lagging strand and

preventing the re- anneling of the unwound single stranded DNA sequences). This creates a so-called “bubble” at which the polymerase itself (product of UL54 gene expression) attaches and proceeds to unwind the double-stranded DNA and continues replicating single stranded DNA [96]. Interestingly, current therapies for HCMV infection are mostly based on antivirals that target this polymerase and therefore prevent replication of this virus. Interestingly, although the virus encodes its own DNA polymerase (UL54), it uses the RNA polymerase II (Pol II) of the host [97]. DNA replication of herpesviruses is thought to proceed as a biphasic process involving theta followed by rolling circle mechanisms [98, 99]. Origin-specific initiation on a circularized input genome leads to an early, theta mechanism that later undergoes a switch to a rolling-circle type of replication [98, 100-104]. It is proposed that viral DNA production during lytic infection is achieved mostly though the latter mechanism- the rolling-circle replication. Moreover, the switch between these two modes of replication may be a key step that governs this process, however this is not fully elucidated yet [98].

1.8.3. DNA packaging and virion egress.

The assembly of betaherpesviruses, specifically cytomegaloviruses, is extremely complex and poorly understood. The elaboration of this process was even compared to “that of some cellular organelles” [105]. Much of the information on HCMV virion maturation was originally based on the studies of the assembly of alphaherpesviruses (especially HSV) [105, 106]. Later studies indicated that although most of the structural proteins of CMVs and other betaherpesviruses share extensive structural and functional homology with the HSV capsid proteins, there are also significant differences between these subfamilies. The cryoelectron microscopic data indicated that although HCMV capsid structure is similar, it is not identical to HSV (or other herpesviruses) [105, 107-109]. More recent analyses showed that tegument is the most variable compartment amongst herpesviruses and it contains many betaherpesvirus- and HCMV-unique proteins [105, 110]. One of the most significant functional differences between these subfamilies of herpesviruses is that betaherpesviruses do not abrogate host cell protein synthesis even at late phases of replication, which is in contrast to

alphaherpesviruses. It is predicted that the assembly of betaherpesviruses may either compete with host cell protein synthesis and/or target and modulate host cellular pathways to optimize viral protein synthesis and transport [105].

The process of viral egress can be divided into phases:

- Assembly of the capsid

The initial steps of capsid assembly involve formation of a scaffold for the generation of the capsid subunit and the pre-capsid structures. The major components of the capsid are: major capsid protein- MCP (UL86) and small capsid protein- SCP (UL48A) and the scaffolding proteins. The scaffolding proteins of HCMV have been identified as gene products of UL80a and UL80.5 ORFs and contain MCP-binding domain. Interactions between the MCP and UL80a and UL80.5, and possible interactions between MCP and SCP were reported (Table 1.2). These interactions take place in the cytoplasm, followed by translocation of these complexes (MCP-SCP and SCP/MCP/UL80a/80.5) to the nucleus.

Once the complexes are translocated from the cytoplasm to the nucleus, the interaction between the domains of the products of the UL80a (assemblin precursor) or the UL80.5 (assembly protein) leads to formation of pentons and hexons and, as a consequence, generation of the capsid scaffold [105, 111-113].

Protein	HCMV
Major capsid protein	**MCP (UL86)∗**
Small capsid protein	**SCP (UL48A)∗**
Minor capsid protein	**MnCP (UL85)∗**
Minor capsid protein	**MnCP-bp (UL46)∗**
Assembly protein	**Assembly protein (UL80.5)**
Assembly protein precursor	**Assemblin precursor$_{COOH}$ (UL80a)**
Assembly protein precursor	**Assemblin (UL80a)∗**
Portal protein	**UL104**

Table 1.2. Proteins of the capsid of β-herpesviruses (HCMV).

The ∗ indicates capsid proteins that have been demonstrated in infectious virions. Pre-capsids are thought to contain products of both the UL80a and UL80.5 ORFs, mature virions contain products of the UL80a ORF, but not UL80.5. Modified and reprinted from [105].

- Capsid maturation and DNA packaging

In this next step the immature form of the capsid undergoes various modifications. For HSV, capsid maturation takes place in the absence of viral DNA, and it is predicted that the same holds true for betaherpesviruses. The final stages of capsid maturation involve proteolytic cleavage of the C-terminal MCP-binding domain of UL80.5 and UL80a. Next, the pentons and hexons interact with the triplex formed between MnCP/MnCP-bp and create the pre-capsid.

Moreover, the depletion of the UL80.5 encoded scaffolding structures appears to be coupled to viral DNA packaging [105, 113, 114]. Once the pre-capsid is formed, the unit length of viral DNA is packed into this structure. This process is facilitated mainly by virus-encoded proteins (products of UL56 and UL89 ORFs; these proteins govern packaging and cleavage of viral DNA) and the crucial point is the recognition of two conserved motifs, the *pac-1* and *pac-2* sequences (located in the terminal repeat "*a"* sequences at each end of the viral genome) [110]. These proteins interact together with the UL104 portal protein and as a consequence, viral DNA is inserted into the capsid [105, 115-117]. However, this process is not fully understood yet and it is very likely that other viral and cellular proteins may also play an important role.

- Nuclear tegumentation and nuclear egress

The nuclear tegumentation of HCMV is a poorly understood process. As mentioned before tegument is the least conserved compartment among herpesviruses, that contains many betaherpesvirus- and HCMV-unique proteins [105, 110]. Therefore, it seems very likely that the nuclear egress of this pathogen may vary from the mechanisms that were described for alphaherpesviruses (these involve three phases: nuclear tegumentation, de-tegumentation, and a final tegumentation) [105, 113, 118].

Two models of nuclear egress of the HCMV capsid have been proposed:

1) The first model predicts that the accumulation of the intra-nuclear capsids surrounded by domains containing viral proteins encoded by UL50 and UL53 occurs in proximity to the inner nuclear membrane. Concomitantly, the cellular protein kinase C is recruited for phosphorylation and dissolution of the nuclear lamina. These events evoke the transient disruption of nuclear membrane that is followed by nuclear cytoplasmic mixing and ultimately, viral capsid release into the cytoplasm. However, it is predicted that the nuclear envelope rapidly reseals, resulting in only transient nuclear/cytoplasmic mixing [119].

2) The second model predicts that the newly-assembled and aggregated capsids interact with yet unidentified cellular- or virus-encoded proteins. Such an interaction would potentially cause a fusion with the inner nuclear envelope. This is followed by a secondary event- budding of the capsids into the cytoplasm. This mechanism is strongly supported by the reports describing the assembly of alphaherpesviruses [58, 66, 71] [120].

- Cytoplasmic tegumentation and envelopment

It is well established that the final stages of tegumentation, envelope glycoprotein trafficking and envelopment of the virons take place exclusively in the cytoplasm of the infected cell [105]. This is confirmed by several studies that demonstrated the presence of both tegument and envelope proteins- including processed glycoprotein-B (gB, UL55) in this cellular compartment [121-123]. However, the exact mechanisms that regulate tegumentation and envelopment of the infectious betaherpesvirus particles are not fully understood yet [105].Unfortunately, there are no existing models of HCMV tegument assembly.

However, several studies described some features of intracellular trafficking of the structural tegument protein pp28 (UL99). In virus infected cells, pp28 is transported to the assembly compartment, where it localizes with envelope glycoproteins and with other tegument proteins including pp150 [105, 123, 124]. It seems that the correct trafficking and incorporation of this tegument protein into viral particles is crucial, as the deletion of UL99 gene produced exclusively non-infectious particles [124]. It remains to be established what are the actual roles of this and the remaining tegument proteins in the assembly of the HCMV particle.

1.9. Latency and reactivation.

One of the most important characteristics of herpesviruses is their ability to establish both lytic infection in majority of cell-types and life-long latency in specific host cells (reviewed in [125]).

Primary infection (productive- lytic cycle) of a healthy immune-competent individual is usually asymptomatic due to the quick and robust immunological responses of the host and is followed by the establishment of life-long latency. These periods of latency are broken by periodic reactivation events, which enable the spread of the virus within the population. This strategy aids the evolutionary success of these viruses, as it allows the life-long carriage of this pathogen within the host, despite the constant challenge with multiple immune control measures for productively infected cells.

As well as with primary infection (especially in congenital HCMV, described in 1.15.2.1), a profound burden of HCMV disease is also associated with reactivation of infectious virus within latently infected individuals. Despite the fact that the latent virus does not cause the HCMV-associated disease itself, due to the high prevalence of the virus within the populations (up to 100% in developing countries) it is re-activation events that mainly cause the increase in the rates of HCMV-related mortality and morbidity; particularly in allograft bone marrow transplant patients [126] and also in congenital HCMV. Although it is estimated that pre-existing immunity substantially lowers the risk of congenital infection (by 69% [127]), the recent meta-analysis of congenital CMV infection rates in developing

countries identified that the “CMV birth rates prevalence ranged from 0.6% to 6.1%, which is higher than the range of 0.2–2.0% (average of 0.65%) most often reported for developed countries” [33].

This finding is extremely important because the world's largest populations and highest birth rates are reported in developing countries. Although the exact numbers are unknown, it is very likely that “the aggregate number of children born with congenital HCMV infection in these regions is likely to be enormous” [33]. Due to the high seroprevalence in these underprivileged regions it is predicted that practically all congenital HCMV infection cases are caused by non-primary maternal infection [33, 125].

Progress in the development of highly sensitive and specific molecular techniques enabled researchers to adequately address this phenomenon of latency/reactivation [128]. Examples include nested-PCR; establishment of an *in vitro* model of latency (isolation and propagation the progenitor cells of the myeloid lineage such as CD34+ cells and their CD14+ derivatives primary cells in cell culture) and reactivation (*ex vivo* differentiation of latently infected primary myeloid cells to dendritic cells and macrophages). Due to the fact that a large number of studies on this topic were conducted in the past decade, the mechanisms that govern the fate of the viral infection and progression towards either lytic or latent infection are now becoming clearer [129].

1.9.1. Establishment of latency.

The initial steps in this process involve repression of viral lytic gene expression. This is achieved by repression of MIEP and blockage of expression of early genes that are crucial for viral replication. It is now well established that regulation of the MIEP during latency is a complex process that “involves multi- faceted integration of viral and cellular functions that act concomitantly to generate a phenotype that promotes latent infection” [125]. One of the crucial steps to repress this promoter is sequestration of pp71 (potent viral trans activator) in the cytoplasm by yet unknown cellular factor(s), as well as the epigenetic repression of chromatin in the MIEP region. The heterochromatization is believed to be mediated by cellular transcription repression factors such as: YY1 (ying-yang-1) [130], EFF (ets-2 repressor) [131] and Gfi-1 (growth factor independent-1) [132]; as well as heterochromatic modifications of the histones (binding of HP1 protein) [133], enzymes that mediate these modifications, and some adaptor proteins that may recruit co-repressor complexes [129, 134, 135].

1.9.2. Maintenance of latency.

Once latency is established, certain strategies are employed by the virus to maintain this “status quo” and effectively hide from immune-surveillance of the host. Latent infection is associated with a restricted viral transcription programme with expression of only a small number of viral genes in comparison to the lytic cycle [136-138]. However; many viral transcripts, long non-coding RNAs, were detected recently during latent infection, such as:

-beta 2.7- predominant transcript in naturally latent CD14+ cells; its function is to modulate the metabolic viability of the infected host and it is also hypothesized to be important for the protection of neuronal progenitor cells from cell stress [139, 140];

-Inc.4.9- is hypothesized to interact with members of polycomb repressor complex 2 (PRC2) that is involved in histone binding and histone methylation, which in consequence would enhance the epigenetic silencing of the MIEP promoter [139, 141, 142].

Many virally encoded micro RNAs (miRNAs) were also identified in latently infected cells, however the exact role of these latent transcripts is still unclear (reviewed in [125] and [143]). It is hypothesized that these miRNA have mostly regulatory functions, as they have both viral and cellular targets [101]. Perhaps the most studied latently expressed miRNA is: miR-UL112.1- that inhibits the expression of IE72 expression- one of the major activators of MIEP by targeting the UL123 mRNA [125, 144].

Additionally, few virally encoded proteins are expressed during latency [136-138]; these are mainly immune-modulators (Table 1.3).

Interestingly, a product derived from the MIE region was also detected in latently infected cells- the IE1ex4 (exon 4 of the MIE). Most importantly, the expression of its transcript is under the control of a cryptic promoter (distinct from the MIEP). It is hypothesized that the IE1ex4 gene product has an important tethering function (similarly to IE72 in lytic infection) [125, 145].

1.9.3. Reactivation.

One of the key characteristics of the herpesvirus family is that the latent viral genome can be reactivated and re-enter a replicative cycle if the right conditions are met [146]. A key event for reactivation is the induction of lytic gene expression from previously silenced promoters. Differential regulation of IE gene expression is considered central to latency and reactivation and, consequently, many studies have focused on the mechanisms that regulate these promoters. In the case of HCMV, it has been demonstrated that MIEP activity is regulated by higher order chromatin structure whereby on reactivation there is marked increase in histone acetylation at the MIEP - a correlate of transcriptional activity. This model is true of all herpes viruses and thus the key question was what drives the post-translational modification of histones required to allow viral gene expression [133, 141, 146].

With HCMV it appears two events are critical: the activation of cell signalling pathways in response to cytokines and cellular differentiation of the myeloid progenitor to a mature DC or macrophage. Specifically, it has been demonstrated that IL-6 activation of ERK-MAPK signalling in DCs is central to this process. Importantly, it was demonstrated Il-6/ERK activity triggered the activation of a specific cellular kinase MSK family [147] which, in turn, was responsible for the phosphorylation of two proteins; CREB and histone H3. The phosphorylation of histones was demonstrated to be crucial for reactivation as it acted as a key intermediate in the transition from a methylated promoter to an acetylated promoter [148]. It has subsequently been hypothesised that the reactivation of HSV and KSHV is dependent on the same mechanisms although HSV utilises stress activated JNK, and not MSK, to mediate histone phosphorylation [149].

It remains to be determined why the activation of Il-6/ERK does not trigger HCMV reactivation in myeloid progenitors since this pathway is ubiquitous in all cell types. Clearly, myeloid cell differentiation induces changes in the cell proteome which dictates a response that is pro-reactivation. One possibility is the concomitant activation of multiple pathways. Indeed, recent work from the Goodrum laboratory suggests that two viral gene products work antagonistically to regulate EGFR signalling which may be crucial for controlling HCMV reactivation [150]. It is not inconceivable that the IL-6 and EGFR pathways are acting in concert in DCs to promote a cellular milieu supportive of viral reactivation.

Latently expressed protein:	Function:
US28	enables modification of the chemokine environment of the cell by sequestration of CC chemokines [151];
ORF94	involved in downregulation of cellular innate responses by inhibition of 2',5'-oligoadenylate synthetase expression and function [152];
UL144	Activator of NFκB-induced expression of the chemokine CCL22. Upregulation of CCL22 attracts Th2 and regulatory T cells, which are believed to lower the host Th1 immune responses [153];
UL138	upregulates TNFR1 surface expression to sensitize latently infected cells to TNF-α-mediated reactivation of HCMV [154].

Table 1.3. List of proteins expressed during latency.

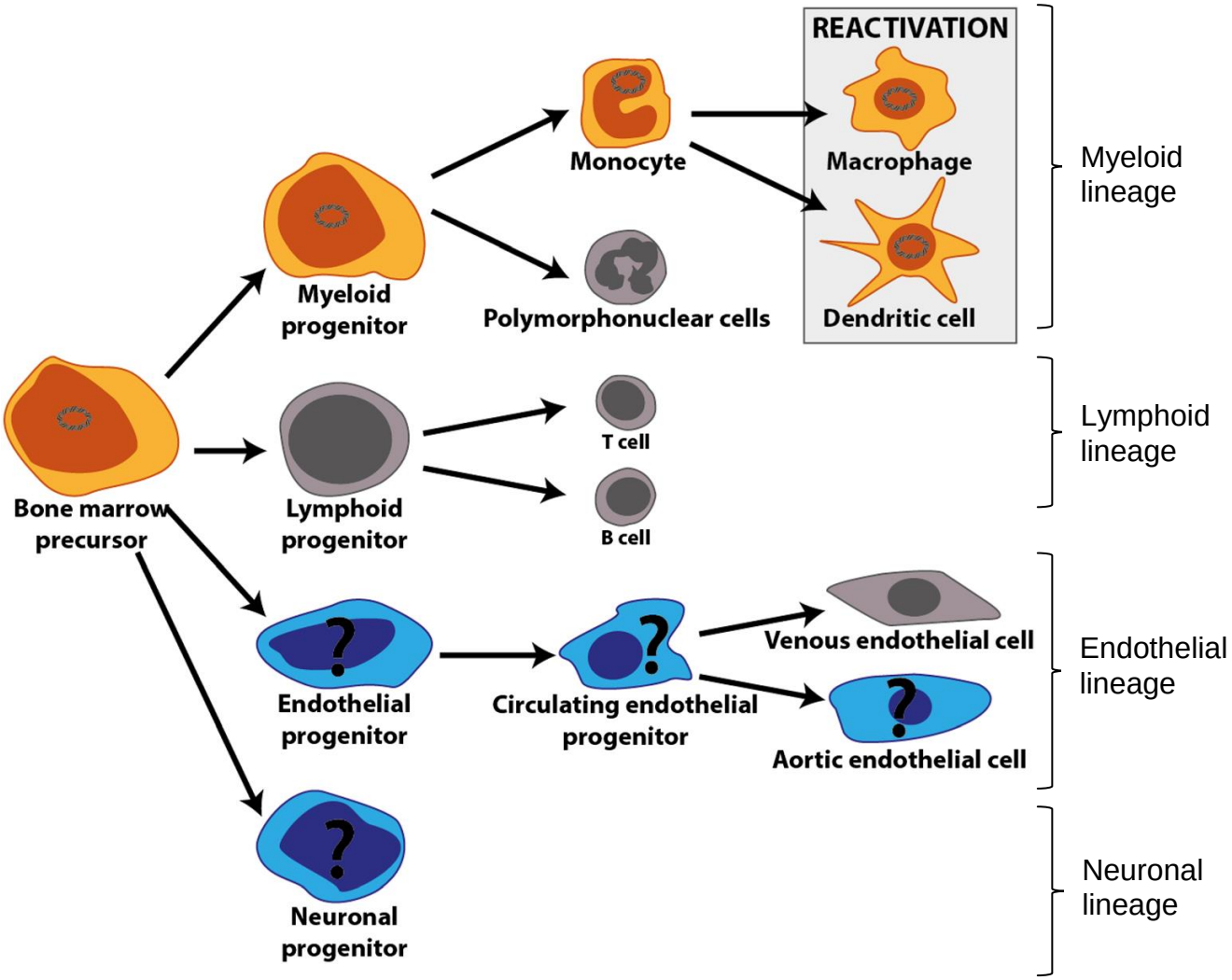


Figure 1.4. Human cytomegalovirus natural latency in cell lineages.

Viral latency is established in the haematopoietic progenitor's resident in the bone marrow, and the carriage of viral genomes has been defined in the monocyte/myeloid lineage with reactivation occurring in the terminally differentiated myeloid macrophages and DCs (Orange cells). In contrast, the viral genome is not carried in the lymphocyte population nor is there any evidence for viral latency in venous endothelial cells (grey cells). Experimental infection data suggest that endothelial and neuronal progenitor cells may also be sites of latency, although no data from natural latency currently exists (blue cells). Reprinted from [125].

1.10. Glycoprotein B.

One of the major components on the envelope of HCMV is glycoprotein B (gB). This protein is a major target of humoral and cell-mediated immune responses. gB is a disulphide linked glycoprotein complex consisting of a component of M_r 116,000- and a component of M_r 55,000 termed gp116 and gp55 respectively (with the latter sometimes called gp58). The protein has high homology with gB in HSV and EBV and thus is a conserved gene throughout the *Herpesviridae.* The sequence of the gB gene was published in 1986 [155], soon after it was proposed that the viral glycoproteins might serve as targets of protective responses due to their high immunogenicity. Consistent with this, immunological responses evoked by this protein were reported [155, 156]. All these data indicated that it is necessary to investigate the actual role of this protein in virus infectivity as well as host derived protective immunity.

1.10.1. The immunogenicity of glycoprotein B.

In order to reveal the immunological characteristics of this envelope protein and its role in virus infectivity, two approaches were employed; expression of this protein in prokaryotic (*E.coli*) and in mammalian cells infected with a recombinant vaccinia virus. These studies demonstrated that the function of gB is strongly influenced by the presence of carbohydrate modifications (pattern of glycosylation). The analysis of the gB expressed in *E. coli* indicated that this protein is present in its non-glycosylated form, however the epitopes recognized by the anti-gB monoclonal antibodies were conserved in the prokaryote-derived protein [157]. Production of this protein in both prokaryotic and eukaryotic systems allowed preliminary characterization of the relative immunogenicity of both preparations in animal models. Both prokaryotic and eukaryotic derived recombinant gB induced virus-binding antibodies in animals. However, quantitative (higher titre of virus binding antibodies by 3-4 fold) and qualitative (significantly higher levels of complement dependent neutralizing antibodies) differences were detected in the animals that were vaccinated with the protein expressed in the eukaryotic cells. The results

indicated that, in the absence of post-translational modifications such as glycosylation, only complement-independent neutralizing antibodies were induced, likely due to the lack of proper conformation. The expression of this major envelope protein in both systems allowed researchers to begin the characterization of its antigenic properties and its role in the pathogenesis of HCMV infections. Nevertheless, at that stage it was still impossible to directly determine the influence of immunization of mice with gB of HCMV on resistance to disease. Further studies extended the immune response to HCMV gB at the clonal level from the immunized mouse system to the naturally infected human host [157-161].

The aim of one of these pioneering studies on humoral responses to gB was to investigate the antibody profiles against gB in healthy seropositive individuals. These analyses demonstrated that there are no qualitative differences in the anti-gB antibody repertoire and the responses were stable over time. However, quantitative alterations were noticed in some of the seropositive donors. Moreover the investigation of humoral responses following primary infection with the virus revealed that there is a delay of 50-100 days in the synthesis of antibodies specific for this glycoprotein, in contrast to the antibodies that were elicited towards other HCMV-specific antigens that appeared shortly after infection. Additionally, both reactivation and reinfection resulted in the concomitant synthesis of the antibodies [162]. Importantly, some of the later studies revealed that the majority of the serum-neutralizing activity against HCMV was directed towards gB: 40-70% [161]. It was hypothesized that this biased response towards gB might be a result of the abundance of this protein within the envelope in comparison to other glycoproteins, its expression on the surface of the infected cells and its immunogenicity, which might be connected with the extensive glycosylation as it was shown that the gB expressed in prokaryotic cells was less immunogenic [161] [157]. Nevertheless, this finding indicated that gB may be the major target of humoral responses elicited towards HCMV following infection. Therefore many extensive analyses of this protein have been conducted in order to fully understand the functions of this protein and ultimately utilize the immunogenic properties of gB in the development and production of vaccines and therapeutic monoclonal antibodies.

1.10.2. The synthesis of glycoprotein B.

The mounting evidence about the importance of this glycoprotein complex in the structure of the envelope, its abundance and its immunogenicity *in vitro* and *in vivo,* indicated that a complete understanding of its structure, including its intracellular transport, is essential to define the native forms of this protein complex as well as to identify novel targets of future vaccines. In addition, such studies were fundamental to understand the morphogenesis of the complex envelope structure of HCMV.

Human immune responses to HCMV have been characterized by use of virus-infected cells expressing gB protein on their surface and the gp55, gp116 and gp170 components were reported to be present on the surface of the infected cell. Most importantly, gp170 represented a minor component within preparations of extracellular virion proteins. Therefore its relative abundance on the cell surface indicated that its transport to the surface was more efficient than its incorporation into virions. Moreover, the presence of uncleaved protein indicated that proteolytic cleavage was unnecessary for cell surface transport [163].

Additionally, studies on the kinetics of the protein post-translational modifications were conducted. It has been estimated that the first, rapid step of processing this protein is the glycosylation of the approximately M_r 105,000 polypeptide (non-glycosylated form) to M_r 150,000 precursor protein (gp150) containing only simple, high-mannose sugars. This precursor form is then transported from the endoplasmic reticulum (ER) into the Golgi apparatus where terminal sugar modifications are made, and finally this gp150 is processed to M_r 165,000-170,000 species (gp165-170) [163, 164]. The fully glycosylated form, gp165-170 possesses simple and complex carbohydrates, which indicate that transport through the Golgi complex is essential for complete processing of this protein. Moreover, this process was also described as ‘relatively slow’; this step is followed by the endoproteolytic cleavage into the gp116 and gp55 components (Fig. 1.5) [161, 163, 165].

Previous studies of other herpesviruses, *e.g*. HSV, described the oligomerization of the gB envelope glycoprotein as a mechanism of delayed transport of this molecule [166]. The analysis of gB from HCMV revealed that this protein undergoes similar processing- The half maximal time of the gp150 formation was only slightly faster than the estimated half-maximal time of oligomerization (20 vs 25min), suggesting that the oligomerization occurred nearly coincidentally with synthesis of gB.

The experiments with conformation-specific monoclonal antibody demonstrated that the folding of this protein takes place shortly after oligomerization. This post-oligomerization folding appeared to be necessary for transport to the cellular membranes of the virally infected cells and the half maximal-time of formation of this form was estimated to be up to 8 times longer than that of oligomerization. Moreover, oligomerization and folding were not absolutely dependent on glycosylation of the precursor polypeptide, suggesting that all the necessary information for the oligomerization and folding of HCMV gB was represented within the primary sequence.

The precursor was processed into the mature products (between 80 and 160 min). This event was indicated by the initial appearance of the cleavage products gp55-116. This process of folding was relatively slow and was consistent with the previously reported prolonged processing time required for transport, terminal glycosylation and cleavage of the precursor gp150 to the final components of the gp55-116 complex (Fig. 1.5) [167].

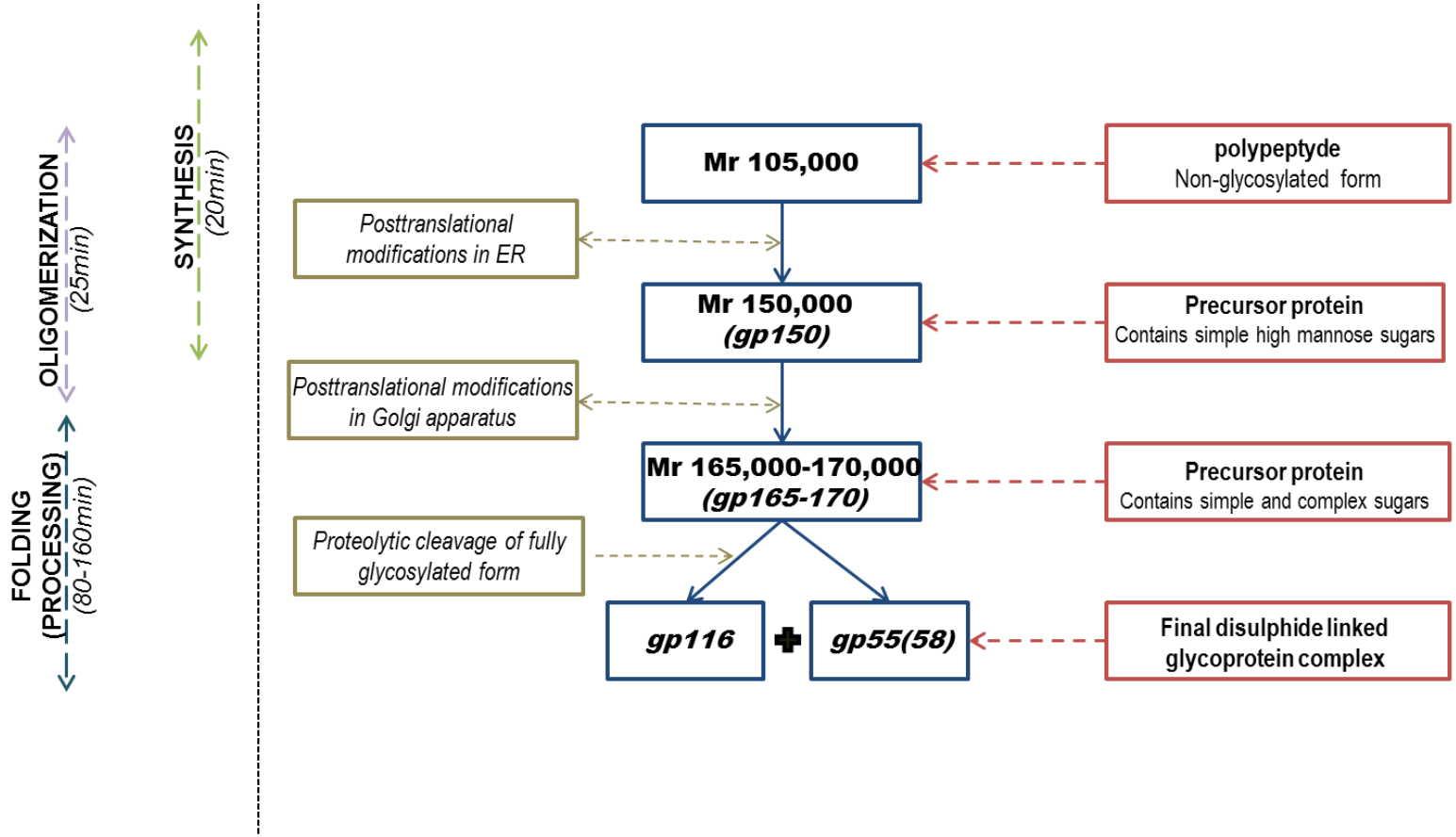


Figure 1.5. Schematic representation of synthesis and processing of glycoprotein B.

1.10.3. The role of glycoprotein-B in virus entry, broad tissue tropism and pathogenesis.

Membrane bound glycoproteins are important mediators of entry of the virus into cells. So far around 19 different glycoproteins have been identified on the viral envelope [13]. It is proposed that of those, only five of them are necessary for viral entry: gM/gN (UL100/UL73); gB (UL55); gH/gL (UL75/UL115).

The gM protein was found to be the most abundant, accounting for ten percent of the total virion mass [13], and, in complex with gN, acts as an attachment receptor [168-170]. Glycoprotein H is a fusion receptor, while glycoprotein L acts as a chaperone and most likely facilitates localization of receptors on the cellular membranes [171-173]. gH/gL acts together with gO (entry to fibroblasts) or UL128-UL131A (together called the ‘five member-complex”; entry to endothelial and epithelial cells) [174]. Glycoprotein B -the second most abundant glycoprotein [13] plays crucial role at the entry events as it mediates the fusion [56, 168, 175].

The virus can only replicate in living cells of the host; therefore it is the range of permissive cell and tissue types that is crucial for efficient spread of the virus within the host and within population. Importantly, one of the most important characteristics of HCMV is its exceptionally broad cellular and tissue tropism [176, 177]. It has been shown that HCMV can enter nearly every single cell-type of the host such as:

-fibroblasts (standard cell culture system for propagation of HCMV replication); epithelial, endothelial and smooth muscle cells are the predominant sites of viral replication [178-180];

-epithelial cells support inter-host transmission of the pathogen [177, 181];

-endothelial cells and hematopoietic cells facilitate systemic spread of the virus within the host [177, 182].

The only cell types that are known to block HCMV replication are lymphocytes and polymorphonuclear leukocytes [183]. Therefore, as a consequence of such a wide spectrum of susceptible cells we can observe an array of clinical manifestations of this viral infection. Due to the strictly host-specific nature this pathogen, it is only possible to examine the pathological changes of the tissue and organs of the infected individuals from diagnostic samples of the patients or autopsies (post-mortem analyses). The only easily accessible site is the blood compartment, thus, majority of the assessment of viral kinetics were done with blood samples collected from patients [184]. Thus, broad cellular tropism of the virus is inevitably linked to its evolutionary success, as it allowed the virus to be easily transmitted and therefore widely spread within populations. On the other hand; and most importantly from the medical point of view- the cellular tropism of the virus determined its pathogenicity.

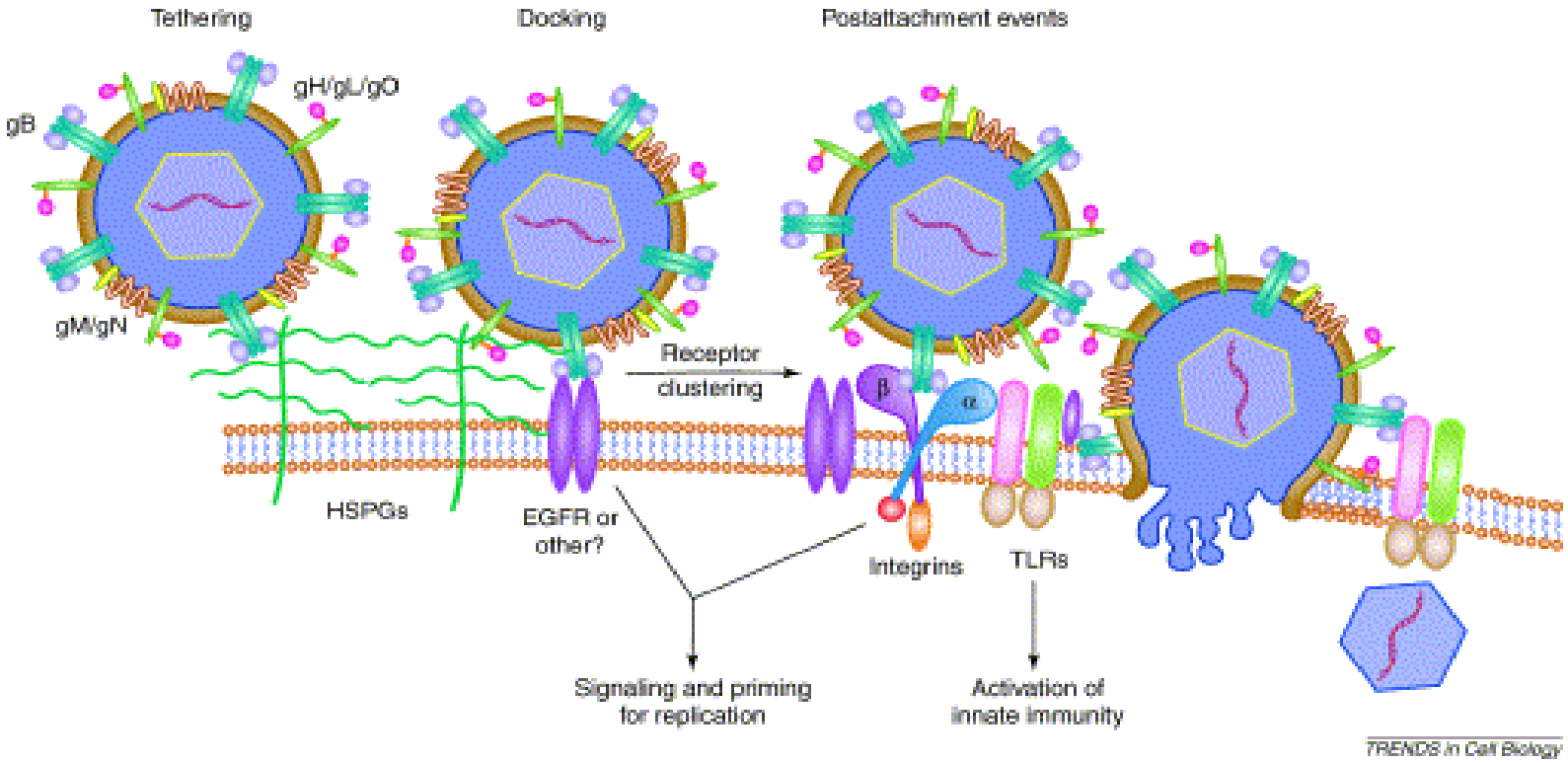


Figure 1.6. Working model for human cytomegalovirus (HCMV) entry into cells.

HCMV initially attaches in a tethering step to heparan sulfate proteoglycans (HSPGs) through gM/gN and/or gB glycoproteins. In a stable docking step, gB interacts with the epidermal growth factor receptor (EGFR) on many HCMV-permissive cell types or with as yet unidentified receptors in hematopoietic cells. Other interactions between the glycoproteins of the HCMV envelope and cellular integrins promote receptor clustering. At least one of these interactions triggers fusion that leads to the internalization of virion components. Signal transduction events are initiated through EGFRs and/or integrins and these events are hypothesized to prime and facilitate downstream steps in the virus lifecycle such as nuclear translocation of the capsid and efficient viral gene expression. Toll-like receptors (TLRs) detect an HCMV-displayed pathogen-associated molecular pattern during the entry of the virus leading to distinct signalling events and the activation of innate immunity.
Reprinted from: T. Compton; http://dx.doi.org/10.1016/j.tcb.2003.10.009 [185].

1.11. Targets of humoral responses to HCMV.

Although gB represents an immuno-dominant target of the humoral response it is quite clear that during primary infection with HCMV a broad spectrum of antibodies specific for multiple viral antigens are elicited in the host. The role of antibodies in conferring protection against and control of this common pathogen has long been debated. However, a mounting body of evidence suggests that humoral immunity plays a crucial role in restricting viral dissemination and most likely contribute to minimizing the clinical manifestations of the disease [127, 186-188].

The spectrum of humoral responses against HCMV includes:

- structural tegument proteins (*e.g*., pp65 and pp150),
- envelope glycoproteins (predominantly gB and gH and gH/gL, pentameric complex),
- non-structural proteins (such as the IE1 protein).

Interestingly, one of the pioneering studies on the humoral response to HCMV has shown a delay of 50-100 days for the appearance of glycoprotein-specific antibodies during primary HCMV infection. Conversely antibodies specific to antigens other than glycoproteins were synthesized quickly following the infection. In contrast, a synchronized production of antibodies was reported during reactivation or reinfection of this pathogen [162].

Historically, gB has been a major focus of studies of the humoral immune response as antibodies specific to this antigen could be easily detected in nearly all naturally infected individuals [162, 189]. An early study on neutralization demonstrated that approximately 40 to 70% of the total serum virus-neutralizing activity of a group of seropositive individuals was directed against this single envelope glycoprotein [161]. This was demonstrated by using HeLa cells infected with the gp55-116 recombinant vaccinia virus as a specific immunosorbent that allowed the depletion of gB antigen specific antibodies from seropositive sera [161]. The antibody response to gB is

polyclonal and thus further analyses sought to investigate whether there were immune- dominant epitopes that predominated the anti-gB humoral response. The AD1 domain was recognized as a major target of humoral responses towards gB since nearly 100% of sera from HCMV healthy seropositive donors had antibodies directed towards this region [162, 189, 190]. Subsequently, a wide range of neutralizing capacity amongst the antibody responses against AD-1 that developed after natural infection with HCMV was reported [158, 191]. Potential competition between non-neutralizing and neutralizing antibodies was also noticed in the early 1990s [158, 161, 192] (see chapter 6.1). Indeed, the recognition of gB as a dominant target of humoral immune responses [161] led it to be considered the candidate most suitable for a vaccine antigen and the work on subunit vaccine using gB as a single antigen was initiated in the beginning of the 90' [165].

However, more recently, subsequent work does not support the immune- dominance of gB reported by previous studies. One Korean study reported in 2000 that only "19-50% of the total virus-neutralizing activity of sera with past HCMV infections was derived from anti-gB antibody" [193]. It remains to be further investigated whether the substantial differences between this study and the previous ones could be due to differences in methodology or perhaps are reflecting distinct populations. The results showing much lower level of neutralization by antibodies specific to gB are more in line though with latter analysis of the B-cell specific repertoire to this viral antigen. Furthermore, Potzch *et al.* revealed that the vast majority (>90%) of gB-specific antibodies secreted from B-cell clones do not have virus neutralizing activity [160].

Although the reasons for these discrepancies remain largely unknown it is a consideration that historically, the determination of the neutralizing activity of human sera from HCMV-infected individuals has been carried out in *in vitro* assay systems using exclusively human fibroblasts and laboratory adapted strains of this virus. More recently, this conventional experimental methodology has been challenged. Many groups have demonstrated that the entry pathway in fibroblasts differs from that in endothelial, epithelial and myeloid cells [194]. In particular, the pentameric glycoprotein complex (pentamer) formed between gH/gL and the UL128, UL130, and UL131A proteins complex has been shown to be required for infection of both

endothelial and epithelial cells [174, 195], while the glycoprotein complex gH/gL/gO is required for infection of human fibroblasts (described in detail in chapter 4.1) [169, 196-198]. Given that the majority of studies of humoral responses in the past were solely focused on potent anti-gB antibodies using laboratory adapted strains of the virus (that do not possess functional pentameric complex) and fibroblasts coupled with the discovery of the role of pentameric complex, it is now clear that such an *in vitro* setting could not adequately reflect much more complex situation *in vivo*. Therefore, the true extent of neutralization by HCMV-neutralizing antibodies must be carefully revaluated as the full impact of neutralizing antibodies against HCMV remains relatively poorly characterized and requires further investigation [199].

HCMV is a highly complex virus harbouring more than 20 different glycoproteins in its envelope [13]. Although a large percentage of the antibodies elicited by natural infection is directed towards gB, other antigens such as: the gM/gN complex, the gH/gL complex and the pentameric complex have also been identified as highly immunogenic.

Britt *et al.* reported in 2000 that gM-gN complex is highly immunogenic. This study revealed that while most sera failed to react with either gM or gN when expressed alone, 62% of sera from HCMV seropositive individuals were positive for the gM-gN complex [170]. Also, studies with a murine monoclonal antibody against gN in the gM-gN complex showed that this antibody could effectively neutralize infectious virus. Therefore, based on these results, it seems likely that the gM-gN complex may also represent a major antigenic target of antiviral antibody response [170]. Other researchers focused on immunogenicity of gH/gL complex as it is well established that those glycoproteins are essential for the entry of the virus into all cell-types and are also abundant on cellular membranes of the cells infected with this pathogen. Consistent with this, antibodies against gH have been reported in 96% of sera from HCMV seropositive individuals. Moreover, the decrease of total virus neutralizing activity between 0% and 58% was reported in the sera depleted of the antibodies against this glycoprotein. Such a result strongly argues that gH must be one of the major antigens for the induction of neutralizing antibodies during natural infection [200].

However, the discovery of the crucial role of the pentameric complex in the entry of the virus to the endothelial, epithelial and myeloid cells as well as the role in the cell-associated spread of the virus (described in detail in chapter 4.1); suggests that this pentameric complex might be also an important target of functional and neutralizing antibodies.

Indeed, studies in past decade revealed that sera from infected individuals possess very potent antibodies against this complex that were capable of neutralizing HCMV infection of epithelial and endothelial cells [201]. Highly potent HCMV-neutralizing monoclonal antibodies that were isolated from the HCMV specific memory B-cell repertoire were reported to be specific for this pentamer complex. Also it was shown that this complex is the main target of the neutralizing humoral response to HCMV infection in epithelial/endothelial cells [202, 203]. Lanzavecchia *et al.,* reported that those "unusually potent neutralizing antibodies might be used for passive immunotherapy and identifies, through the use of such antibodies, novel antigenic targets in HCMV for the design of immunogens capable of eliciting previously unknown neutralizing antibody responses" [201]. The preliminary studies with animal models confirmed these *in vitro* studies, as the immunization with the pentameric complex elicited a strong neutralizing antibody response in mouse, rabbit, and rhesus macaque models [204-206]. Several studies with soluble pentamers are currently ongoing to investigate whether the vaccine based on this complex would be capable in conferring protection against this pathogen [207].

1.12. Mechanisms of acquired immunity to HCMV and mechanisms that the virus evolved divert these immune responses.

HCMV is sometimes considered the master of immune evasion. A substantial part of its genome is dedicated to the expression of proteins that subvert the immunological responses of the host. Thanks to this elaborate mechanism of immune-evasion this pathogen managed to achieve extraordinary evolutionary success, as it is able to persist indefinitely within the host and has been reported to infect almost 100% of the population in developing countries. Although the host is eliciting very potent

immunological responses, both humoral and cellular, the virus is clearly able to evade them sufficiently to persist.

It is likely that the enormous success of this pathogen is a result of a long co-evolution with its host. As the new mechanisms of immunological responses were evolving within host, the virus was co-evolving to produce new or refined mechanisms of evasion/ countermeasure of those host responses or, alternatively, by hijacking those new immunological mechanisms for its own benefit. Due to the nature of evolution itself- selection of better adjusted and well adapted progenies, the virus has upper hand over its human host, as doubling time of the virus is approximately 24h (once it is replicating) [184] in comparison to decades between new generations in human population.

1.12.1. Humoral responses of the host.

The 235kb HCMV genome encodes approximately 200 open reading frames [208]. Therefore, unsurprisingly, the spectrum of antibodies specific for multiple viral antigens is very broad (as described in greater detail in section: 1.11). The majority of antibody responses are directed against viral glycoproteins. Although historically the glycoprotein B (gB) has been implicated as principal target of virus neutralizing antibody [161, 192], more recent data points to the humoral response against the pentameric complex being important, as it elicits very potent neutralizing antibodies (described in 4.1 and 1.11) [199, 201-203]. Also antibodies against gM/gN complex [170] and phosphoproteins *e.g.*: phosphoprotein 65 (pp65) are abundant in the sera of seropositive individuals [209]. Proteins recognized in healthy individuals, furthermore, include the tegument proteins, such as *e.g.*: pp28 (UL99) [210] and pp150 [211, 212].

Although it is clear that humoral immunity plays a crucial role in restricting viral dissemination and alleviates severity of the disease [127, 186-188] the actual extent of the role of antibodies in conferring protection is still contentious. HCMV, unlike other congenitally transmitted viruses (*e.g.*: Rubella virus) may be transmitted to the fetus of already seropositive mothers. Therefore, it is clear that naturally occurring

antibodies do not completely interrupt transmission of the virus [36]. This study, although controversial at that time implied that the natural immunity may not be sufficient to completely prevent the infection. However, the subsequent data form that laboratory has shown substantial reduction in the rate of transmission of the virus to the fetus (67%) in seropositive mothers [127] arguing thathumoral immunity could play an important role. Similarly, in studies of transplant patientsit has been demonstrated that there is a clearbenefit when pooled Ig from HCMV seropositive donors is administered [213]. Furthermore, the newest report from a clinical trial testing infusion of monoclonal antibodies against gH plus UL131 in SOT showed significant protection in a treated cohort in comparison to placebo patients [186]. All these data clearly suggest that the antibody responses against HCMV do have important roles albeit it is unclear why the relatively abundant antibody response that develops following the infection with HCMV is not sufficient to prevent re-infection or re-activation.

1.12.2. Evasion of humoral responses.

HCMV has evolved many ways to avoid host humoral responses.. One of the most important mechanisms is the route of transmission and systemic spread of the virus. During the course of my own studies Murrell *et al.*, [194] shows that genetically wild type virus spreads mainly in a cell associated form in vitro. They propose that, if this was recapitulated *in vivo*, this way of transmission allows the virus to effectively avoid contact with neutralizing antibodies present in the sera of seropositive individuals, as the viral particle will not leave the cell to the extracellular compartment. Thus preventing exposure to the antibody response provides a mechanism of evasion.

Another way of evading humoral immunity includes the expression of Fc-like decoy receptors on the surface of infected cells. This allows binding and subsequent internalization of antibodies which, as a consequence, incapacitates these host humoral responses. This clever way of evading IgG responses of the host is common for both alpha and beta herpesviruses and was discovered in early 70’s [214-217]. Recognition of IgGs by surface receptors (FcγRs) for the Fc domain of

IgG (Fcγ) can trigger both humoral and cellular immune responses. Two human cytomegalovirus (HCMV) - encoded type I transmembrane receptors with Fcγ-binding properties (vFcγRs): gp34 and gp68, have been identified on the surface of HCMV-infected cells [218, 219]. gp34 and gp68 were recently demonstrated to be encoded by independent genes, *TRL11/IRL11* and *UL119-UL118* respectively. Both vFcγRs, gp34 and gp68, were shown to be cell surface proteins that bind to Fcγ [219, 220]. gp34 and gp68 share binding properties with gE-gI, the HSV-1 vFcγR, in that each is specific for human IgG but not human IgA or IgM. The HCMV vFcγRs, however, bind all four human IgG subclasses (IgG1, IgG2, IgG3, and IgG4) [219, 221]; whereas gE-gI does not bind IgG3 [222].

Although these virally encoded Fc receptors were extensively studied in the past, their exact role still remains debated. It seems that apart from evading potent humoral responses, such as *e.g.:* neutralization, they may also enable the infected cells to avoid or modulate antibody effector functions, such as, for example, Antibody Dependent Cellular Cytotoxicity (ADCC) - which is discussed in more detail in chapter 5.

Lastly, it remains a consideration that the virus might also influence the fine specificity of the antibodies elicited shortly after the infection so that they will become less effective in combating the infection. HCMV may achieve that though multiple mechanisms, such as altering the exposition of protective epitopes in the way that they are less available for recognition by host immunity. Whether this could be achieved via heavy glycosylation or partial masking of those regions by highly immunogenic but not protective antigenic domains, is still unclear. This subject was also studied in this thesis, and the detailed description is in chapter 6.

1.12.3. Cellular responses of the host.

Cellular immunity to HCMV is a topic of extensive research for more than three decades now. The early studies on this subject were almost solely focused on the "classic" cytotoxic alpha/beta (α/β) $CD8^+$ T cells recognizing lytic-phase HCMV antigens. Therefore, for a long time the prevalent opinion was that those classical T cell responses are the most important effector responses against HCMV [223, 224]. However this view is constantly being challenged with numerous reports focusing on other cells, most importantly $CD4^+$ T cells, but also gamma/delta (γ/δ) T cells and natural killer cells. It is clear now that a vast spectrum of different subsets of T cells is critically involved in the cellular immune response to HCMV [225].

A pan-genomic evaluation of HCMV immunogenicity determined the overall T-cell responses to HCMV. This cytokine flow cytometry based analyses utilized 13,687 overlapping 15mer peptides comprising 213 predicted HCMV open reading frames (ORFs) - the entire genome of this pathogen. The results showed that "151 HCMV ORFs were immunogenic for $CD4^+$ and/or $CD8^+$ T cells, more specifically: $CD4^+$ T cells recognized 125 proteins, $CD8^+$ T cells recognized 107 proteins, and 81 proteins were recognized by both and that ORF immunogenicity was influenced only modestly by ORF expression kinetics and function". Interestingly, the HCMV- specific T cell responses were mainly targeting regions that are highly conserved between different HCMV strains [226]. This robust analysis revealed that total HCMV- specific T cell responses in seropositive subjects "were enormous, comprising on average ~10% of both the $CD4^+$ and $CD8^+$ memory compartments in blood" [226]. This landmark report demonstrated the extreme complexity and breadth of the T cell responses that are elicited by the human host in response to natural infection [226]. This discovery was in contrast to precedent literature, as it was previously thought that two T cell responses VLE (IE72) and NLV (pp65) were immuno- dominant. However, this report [226] and the follow up studies demonstrated the diversity of T cell responses is variable for different people and actually changes all the time in percentage of T cells directed against different epitopes [227].

Thus, it seems very likely that such an enormous cellular response elicited by the host could have some clinical implications. Indeed, much of a current focus on cellular immunity is directed towards understanding the impact of a long-life infection with HCMV on the profile of the immune system. It is clear that the long-term control of HCMV requires considerable effort from the host immune system [228, 229]. One hallmark of HCMV infection is the maintenance of large populations of CMV- specific memory $CD8^+$ T cells — a phenomenon termed memory inflation [230]. Recent data suggest that HCMV infection is associated with impaired immunity in the elderly, mainly due to large, biased HCMV- specific T cell responses that are neither required nor beneficial but actually cause significant damage [230-234]. Despite having clear correlation between memory inflation and worse clinical outcomes in elderly, it is still unclear to what extent this is related to HCMV-specific immunity [230].

Although huge efforts from the scientific community have revealed many characteristics of these cellular responses to HCMV, far less is still understood about the nature and threshold level of HCMV-specific T cell responses required for long-term HCMV control [225]. However, it is clear that cellular immunity is crucial for containment of HCMV, as it is well documented that the adoptive T cell transfer therapy is successful in preventing acute HCMV-related complications after bone marrow transplantation [235-237]. Therefore full understanding of viral control by cellular immunity would be extremely beneficial as it would allowed identifying susceptible individuals and provide a specific target for immunotherapeutic approaches.

1.12.4. Evasion of cellular responses.

Host cellular responses to HCMV are countered by many virally encoded proteins. The role of those viral immuno-evasive mechanisms, although still not fully understood, is to effectively disrupt the recognition of this pathogen by $CD8^+$ and $CD4^+$ T lymphocytes and consequently, allows the pathogen to “hide” from these robust cellular host responses [238].

One of the most important features of cellular immunity is priming of $CD8^+$ and $CD4^+$ T cells for future recognition of endogenous and exogenous antigens. Those antigens, such as virally encoded proteins, are presented by MHC I and MHC II molecules at the cell surface of either infected cells or antigen-presenting cells. The presentation of those viral antigens via MHC I and MHC II molecules promotes either direct killing the infected cells or initiates other specific immune responses. Therefore, processing of viral antigens via MHC I and MHC II pathway and recognition of MHC proteins by $CD4^+$ and $CD8^+$ lymphocytes became the most important targets for HCMV to evade effective clearance from the host [238, 239].

Viral immunoevasins exploit diverse cellular processes to interfere with host antiviral functions (discussed in detail in: [240]). One of the strategies employed by HCMV to achieve this goal is targeting the central components of the antigen presentation machinery, especially at the earliest stages of assembly and intracellular trafficking in the biosynthetic pathway. It is well documented that every cellular protein that resides in the membranous system of the cell has specific features that determine its intracellular localization. Therefore the disruption of these elaborate cellular trafficking mechanisms, specifically perturbation of the endosomal route of molecules that participate in immune recognition, may effectively inhibit antigen presentation to T cells (reviewed in [47-50]). Some of the immune-evasive proteins that act at multiple stages of this process are encoded within the US2-US11 region [241]. These proteins disrupt various aspects of the major histocompatibility complex (MHC) class I and class II antigen presentation

pathways, functioning in cytoplasmic membranes to cause retention, degradation, or mislocalization of MHC proteins (discussed in [242]).

- Inhibition of the MHC class I pathway:

An effective mechanism for detection of putative pathogens by the immune system is crucial as it facilitates control of the infection.. A key event in the adaptive immune response is the detection and presentation of virus-derived peptides at the cell surface by MHC I molecules and subsequent elimination of those virally infected cells by cytotoxic T lymphocytes (CTLs). Therefore, unsurprisingly, MHC I antigen presentation pathway is a prime target for viral immune evasion, as it is an efficient way of displaying foreign and potentially dangerous antigens to the immune system of the host.

In this pathway, the viral antigens loaded onto surface of MHC I molecules are products of the proteasomal degradation of viral proteins that were present in the cytosol. Subsequently, those viral peptides are translocated by the Transporter associated with Antigen Processing (TAP) into the endoplasmic reticulum (ER), where they are loaded onto newly synthesized MHC I molecules. Peptide-loaded MHC I proteins are displayed at the cell surface and monitored by TCR/CD3 complexes on $CD8^+$ T lymphocytes. Alternatively, specialized antigen presenting cells, such as dendritic cells can also uptake the viral peptides from their environment upon specific recognition and then present those antigens to CTLs (reviewed in [242, 243]). However, HCMV evolved in a way to tackle this mechanism of adaptive response of the host. It is well established now that the product of the HCMV US6 gene attacks the TAP complex and blocks peptide loading onto MHC I molecules in the ER [244], while products of the US3 and US11 genes dislocate newly synthesized MHC I heavy chains from the ER into the cytosol for degradation [245, 246]. Additionally, the remaining MHC I proteins that managed to escape the recognition by US3 and US11 and then destruction are captured by the US3 gene product and retained in the ER [247, 248]. The cooperative activity of HCMV immune evasive molecules would result in a gradual loss of MHC I from the cell surface [239] (Fig. 1.7).

Thus, viral products inhibit MHC class I antigen processing and presentation via three major pathways [243]:

-inhibition of major histocompatibility complex (MHC) class I expression on cells,

-blockade of peptide trafficking and loading on MHC class I molecules,

-inhibition of peptide generation in host cells.

- Inhibition of the MHC class II pathway:

In addition, HCMV has also evolved mechanisms that disrupt presentation of antigens via MHC II pathway. This immune evasion strategy is based on remodelling of the endosomal route of MHC II molecules that sample exogenously derived peptide antigens. Peptide-loaded MHC II proteins are exposed at the cell surface and constitutively cycle in the endosomal system (Reviewed in [239]). Lee *et al*., demonstrated that infection with HCMV does not alter the early stages of endosomal transport (including recycling), entry of the MHC II into a peptide-loading compartment and assembly and peptide loading of MHC II. However, the blockage in the transport of those MHC II molecules was seen at the latest stages of their transport. Peptide-loaded MHC II is retained in the perinuclear area and sequestered in an enlarged peptide-loading compartment with defective trafficking of MHC II-loaded vesicles toward the periphery [249, 250]. Indeed, the downregulation of the expression of MHC II molecules was then subsequently reported for many different HCMV infected cell types: in dendritic cells [251]; monocytes/macrophages [252, 253] and professional antigen presenting cells (mature Langerhans cells) [250].

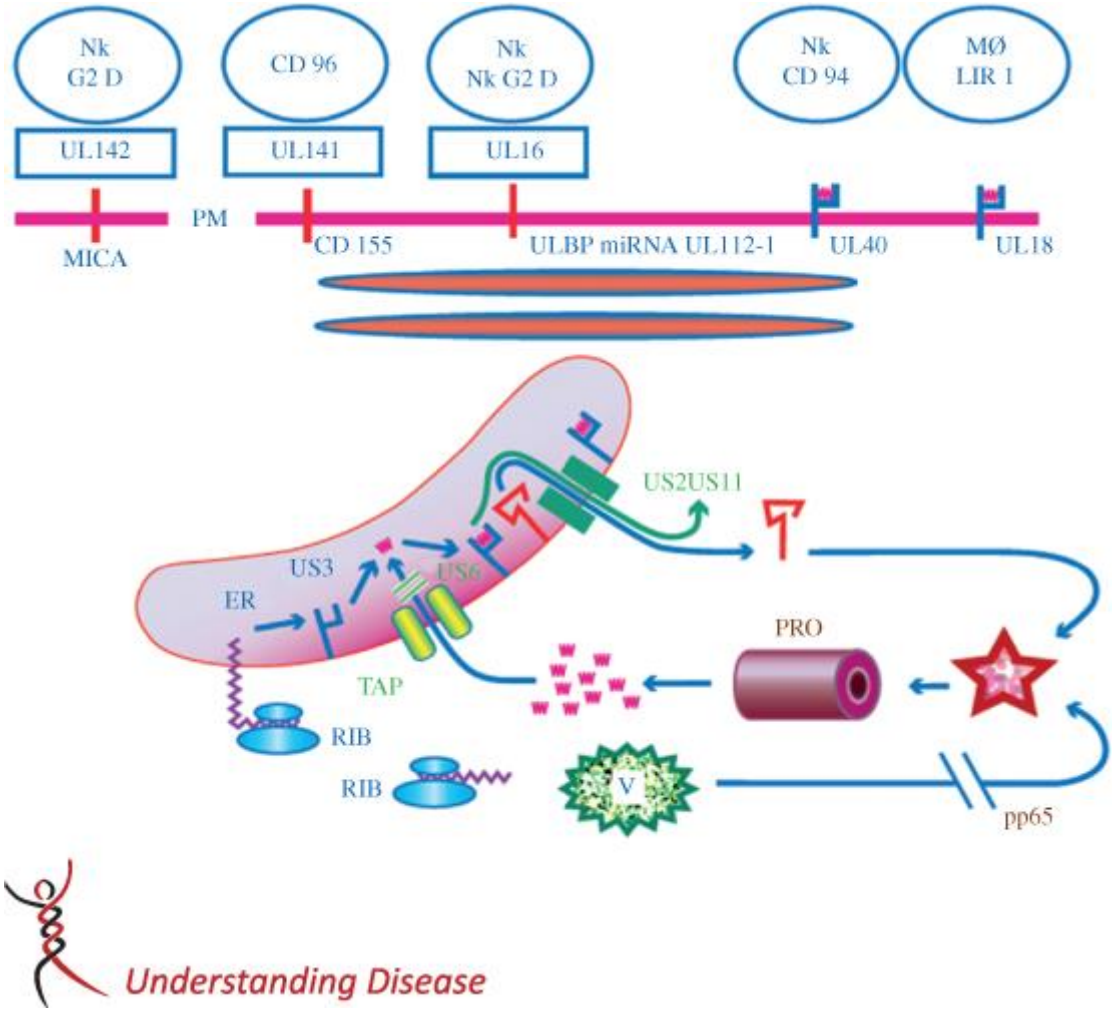


Figure 1.7. Mechanisms used by HCMV to interfere with cell-mediated immunity.

Synthesis of a protein on a ribosome (RIB) is shown, followed by digestion in the proteasome (PRO), transport of peptides into the endoplasmic reticulum (ER) by the transporter associated with antigenic peptides (TAP) and display, together with class I HLA molecules, at the plasma membrane. HCMV proteins pp65, UL97, US3, US6, US2 and US11 reduce display of mature class I complexes. HCMV proteins UL18 and UL40 present decoy signals to prevent immune attack. HCMV proteins UL16, UL141, UL142 plus micro-RNA UL112-1 block the display of stress ligands, which would otherwise precipitate immune attack. Reprinted from [254].

1.13. Passive immunity.

Passive immunity refers to the process of providing IgG antibodies to protect against infection. It gives immediate, but short-lived protection—several weeks to 3 or 4 months at most. Passive immunity is usually classified as natural or acquired [255].

Nnatural passive immunity occurs, for example,when maternal antibodies (mainly IgG) cross the placenta and provides some level of protection for the fetus and then new born baby for several weeks/months until such antibody is degraded and lost. In contrast, “acquired passive immunity refers to the process of obtaining serum from immune individuals, pooling this, concentrating the immunoglobulin fraction and then injecting it to protect a susceptible person” [255].

HCMV can be transmitted from mother to fetus even when the mother is known to have been infected prior conception, as well as when primary infection occurs during pregnancy [127]. However, in congenital HCMV infection there is plenty of evidence that maternal immunity offers some level of protection to the fetus. Fowler *at al*. has shown 67% reduction of fetal transmission in seropositive mothers’ cohort in comparison to seronegative mothers. Additionally, although the pre-existing antibody responses do not completely prevent the transmission of HCMV, their presence lessens the severity of congenital disease once the infection happens [127]. These observations alone demonstrate that there is a clear benefit of passive immunity in this cohort of the patients. Because of this, many studies addressed this clinical problem. Interestingly, in 2005, the first study (non-randomized) suggested that the administration of HCMV-specific hyper immune globulin to pregnant women with primary infection resulted in a significant decrease in the rate of mother-to-fetus transmission from 40% to 16% ($P=0.04$), and the risk of congenital disease also decreased significantly, from 50% to 3% ($P<0.001$) [256]. However, despite these positive results, subsequent randomized studies have not confirmed these findings - no significant reduction in the rate of transmission of HCMV infection among women receiving hyperimmune globulin as compared with women receiving placebo has been reported since [257]. Currently

there are two randomized, phase 3 studies of the prevention of congenital infection ongoing.

The possibility that preformed antibodies present at the time of transplant could reduce transmission of HCMV from the donor organ has also been evaluated in multiple randomised controlled trials comparing placebo with the infusion of anti-HCMV monoclonal antibodies (mAbs) or pooled IgGs preparations. Available data suggests that CMVIG, when associated with antiviral therapy, may provide an additional benefit in preventing HCMV disease and manifestations of chronic rejection in transplant recipients [258]. One meta-analysis of several clinical trials testing the efficacy of immunoglobulins demonstrated a beneficial effect following the prophylactic use of CMVIG on total survival [RR (95% confidence interval; CI): 0.67 (0.47-0.95)] and prevention of HCMV-associated death [RR (95% CI): 0.45 (0.24-0.84)] in solid organ transplant recipients except kidney transplant recipients [RR (95% CI): 0.35 (0.12-1.04)]. HCMV disease was significantly reduced in all recipients receiving prophylactic CMVIG [RR (95% CI): 0.697 (0.57-0.85)]. However, CMVIG had no impact on HCMV-infections [213, 259]. The benefit of the prophylactic use of CMVIG appears particularly apparent in the highest-risk group D+/R− SOT transplant patients [258-263].
Importantly, such results again clearly demonstrate that humoral immunity can reduce the severity of the disease caused by this virus.

1.14. Cytomegalovirus vaccine vectors.

Viral vectors have been developed and intensely studied as potential tools to deliver vaccines as they present many advantages over traditional vaccines in a way that they stimulate a broad range of immune responses including cell mediated immunity [264]. Novel HCMV vectors have “emerged during the past several years as promising vectors for HIV-1 and other pathogens, owing to their immunogenicity and protective efficacy in stringent non-human primate challenge studies” [265].

Despite the extensive number of immune-evasive mechanisms that are encoded by HCMV which enable the virus to persist throughout the lifetime of its host (as described in section 1.12); it is very interesting to note that HCMV based vaccines themselves are highly immunogenic. This clearly suggests a model where HCMV infection triggers potent humoral and cellular responses of the host and thus, in turn, requires has to encode a repertoire of countermeasures to evade them However, utilizing the natural immunogenicity of this virus as a HCMV based viral vector approach has been hypothesized to be a clever way to stimulate the immune system of vaccine recipients.

One of the most important characteristic of this HCMV vaccine vector approach is:

- high immunogenicity;
- can maintain systemic high-frequency, circulating and tissue-resident effector memory T cell (T_{EM} cell) responses that have immediate antiviral effector activity and thereby clear and control viral challenges in 50% of vaccines early after exposure [265];
- can be repeatedly used in individuals with pre-existing immunity to HCMV;
- can be programmed to elicit unusually broad $CD8^+$ T cell responses that recognize conventional and/or unconventional epitopes (including major histocompatibility complex class II (MHC II)-restricted $CD8^+$ T cells);
- can be modified to express multiple vaccine inserts, of 6kbp (or more) of exogenous sequence, using endogenous promoters to control insert expression;
- can be greatly attenuated without loss of immunogenicity or efficacy.

This is generally in contrast to conventional T cell-targeted vaccines as they have only limited ability to manifest effective antiviral effector T cell activity immediately after the onset of viral infection; before systemic spread of the virus. Thus, HCMV vectors have clear advantages over the conventional approaches as they are able to maintain systemic high-frequency of T_{EM} cells [266].

Some researchers believe that this original HCMV based approach may greatly facilitate the development of a successful HIV/AIDS vaccine. This HCMV-based HIV/AIDS vaccine is currently being investigated and the preliminary results are very promising [266-268].

1.15. Vaccines against HCMV.

The discovery of highly immunogenic regions and the major targets of humoral responses in the late 1980's [157, 161] provided a rationale and facilitated the development of the first vaccines against this common human pathogen. Such vaccines might be administered to high risk groups to lessen the severity of HCMV disease, as well as to the general population to interrupt transmission of the virus within the population that might ultimately lead to the elimination of this virus.

Over the last four decades many efforts have been made to produce such vaccines, some of them were evaluated in pre-clinical studies and then in clinical trials. First attempts were made with live attenuated vaccines (based on laboratory passaged HCMV strains: Towne and AD169 [16, 24, 269-272]. Apart from the unsatisfactory immunogenicity levels reached by these vaccines, one of the major concerns over the use of attenuated-whole-virus vaccines is the possibility of establishment of latency post-vaccination in vaccine recipients. Therefore, to overcome this obstacle, new vaccination strategies, including subunit and vectored vaccines, were developed as reviewed elsewhere [273]. Despite many attempts, there is no CMV vaccine available yet for clinical use. Several vaccine formulations are currently under phase-2 clinical development, including cytomegalovirus (CMV)-modified vaccinia Ankara (MVA) triplex vaccine (NCT02506933) and CMVpp65-A*0201 peptide vaccine (NCT02396134);

(described in this section). To date, one vaccine candidate has reached phase-3 clinical trials- the ASP0113 vaccine (NCT01877655).

1.15.1. The rationale for vaccine development.

Infection with HCMV is very common. It has been estimated that approximately 60% of the population in developed countries and up to 100% of the population in developing countries is infected with this virus [274]. Although the infection with this pathogen is usually asymptomatic and the immune system in healthy individuals is able to control the virus; it has been estimated that life-expectancy for the seropositive population is shorter than for seronegative individuals [275]. In some settings though, the virus might impose a high risk for HCMV disease development. The consequence of the infection with this virus may be very severe, even life threatening in several subgroups of a population: immunocompromised individuals such as SOT and haematopoietic stem cell transplants (HSC), infants infected in utero and late stage HIV patients (AIDS sufferers). The socioeconomic burden associated with HCMV disease is enormous, so a vaccine is predicted to be cost-effective and even cost-saving [276]. Therefore the importance of vaccine development is widely recognised and it has been called ‘a top priority’ and a very important health problem by the World Health Organisation (WHO) [277].

1.15.2. The target populations.

1.15.2.1. Women of childbearing age.

It is well known that the burden associated with this congenital infection is extremely high: infection during pregnancy may damage the central nervous system (CNS) of the fetus and cause life-long neurodevelopmental sequelae, such as sensorineural hearing loss (SNHL), mental retardation, cerebral palsy and many other pathologies [278]. It is well established that the presence of maternal anti-HCMV immunity partially alleviates the severity of disease, as it reduces transmission of the virus from mother to the fetus. Interestingly, one study reported that naturally acquired immunity results in a 69% reduction of the risk of congenital

HCMV infection in future pregnancies [277]. Although the protective effect of pre-existing immunity is widely recognized, many of the congenitally infected babies are born to seropositive mothers due to their high abundance in the population [187, 279]. However primary infection in pregnant mothers is associated with more severe sequelae of congenital HCMV infection [127].

Although some of these infants are treated with antiviral drugs such as valganciclovir to reduce progressive deterioration of SNHL, such therapy is associated with adverse effects and, in general, damage done by the virus prior to antiviral drug administration is irreversible [280, 281]. Thus, this should not be seen as a cure for congenital HCMV disease. Furthermore, there is no available medical intervention that could decrease transmission of the virus from the infected mother to the infant [282]. Therefore development of a vaccine that could be administered to adolescent girls and women of childbearing age remains an urgent need. To complement this, a vaccine is needed for toddlers to reduce exposure of their mothers to HCMV [283].

1.15.2.2. Solid Organ Transplant and Haematopoietic Stem Cell Transplant patients.

Historically, primary infection, re-infection and re-activation of HCMV were a major cause of mortality in transplant patients; however, the introduction of anti-viral drugs resulted in a significant decrease of the risk associated with HCMV disease, such as death, rejection of allograft, viremia, pneumonitis, retinitis [284-290]. Although nowadays the mortality rate in transplant patients is much lower than in the past, in the HSC setting we can still observe a higher number of deaths in HCMV seropositive transplant recipients. Similarly, in SOT patients we can also observe an increase in the number of patients who develop viraemia and HCMV disease when seronegative transplant recipients receive an organ from a seropositive donor (primary infection). The same is true when seropositive patients receive an organ from a seropositive donor (re-infection) or from a seronegative donor (re-activation) as compared with seronegative patients who received grafts from seronegative individuals [188, 291].

Moreover, the infection of transplant patients with HCMV increases the risk of opportunistic fungal and bacterial infections as well [289, 292]. Therefore an HCMV vaccine that could be administered to these patients is very important.

*1.15.2.3. Patients co-infected with HIV***.**

Reactivation of HCMV in HIV carriers is a serious medical problem, as the prevalence of HCMV in the population is very high– up to 100% in developing countries. In addition, patients may be re-infected. Many studies suggested that the vast majority of HIV patients are co-infected with HCMV [293, 294]. The most common disease is retinitis, affecting up to 85% of these patients [295] [296], encephalitis [297], pneumonitis [298, 299] and many other complications. Fortunately, the introduction of highly active antiretroviral therapy (HAART) decreased the mortality rate and the disease disadvantage amongst AIDS sufferers in such way that their reconstituted immune system prevents HCMV disease. Despite such a promising outcome of HAART, the problem has not yet been solved, as many of the HIV patients have no- or restricted access to this antiviral therapy. Thus, undoubtedly, a vaccine that could be administrated to the general population to prevent HCMV exposure of this high risk group would be enormously beneficial [293].

1.15.3. The vaccine candidates.

The vaccine candidates that were already evaluated and are currently being investigated in clinical trials can be generally divided into two categories:
-live, attenuated vaccines (whole attenuated viruses),
-subunit vaccines (targeting only one particular protein, or protein complex of interest)
However, recently the repertoire of the vaccines against this pathogen that are under development became even more diverse. The lack of a licensed vaccine fuelled the development of new technologies and new vaccine administration strategies such as:

- dense body vaccines

- peptide based vaccines
- DNA vaccines expressing gB and pp65
- alphavirus replicon particles (VRPs) expressing gB; pp65 and IE-1.
- Whole virus, non-replicating vaccine
- self-replicating RNA
- soluble pentamer

-novel administration strategies, such as:

- prime-boost regimen

1.15.3.1. Live attenuated HCMV vaccines.

Live attenuated strains of HCMV were utilized in the production of the pioneering vaccine candidates against HCMV. The first vaccine against this pathogen that was used in studies with humans was based on the laboratory adapted strain AD169 [16]. Although this vaccine elicited some humoral responses in seronegative recipients, adverse effects were reported as well, such as significant injection-site and systemic reactogenicity. Thus, the development of this vaccine was abandoned.

Next, vaccines against HCMV were constructed with the attenuated Towne strains [24]. The Towne vaccine was given to kidney transplant patients and seronegative women that were at higher risk of acquiring primary infection (in comparison to the general healthy population). The studies on these vaccines revealed that both humoral and cellular immunity were elicited against this pathogen and moreover, the safety profile of this vaccine was very good. Although a correlation between immune responses and decreased risk of severe HCMV disease development in SOT patients was found (in fact a reduction of ~85% in severe disease was achieved, similar to the protection provided by prior natural infection) [300], the vaccine failed to protect renal transplant recipients from infection itself- vaccinees acquired HCMV infection at the same rate as did placebo control subjects [301]. Similarly, vaccination did not protect women from primary infection with the virus [270]. Due to this rather disappointing result with the live attenuated vaccine in

women of childbearing age this vaccine was no longer the major focus of the HCMV vaccinology field. Subsequent studies with this vaccine aimed to reveal the cause of this low immunogenicity. Latter phase I studies with healthy volunteers suggested that the relatively low immunogenicity of this vaccine was caused by the impaired responses of CD4+ and CD8+ T-cells in antigen-specific IFN gamma production [271] [269, 300, 301].

Several different approaches have been subsequently proposed to increase the immunogenic properties of this vaccine; one of them was co-administration of the Towne vaccine with recombinant IL-12. In order to address this hypothesis a phase-1 clinical study was conducted and it has shown better immunogenicity profiles in vaccines who also received IL-12 [272]. Another solution that was proposed was to replace some of the regions of the genome of the attenuated Towne strain with the corresponding regions of the genome from the less attenuated Toledo strain. To date, four independent vaccines with such chimeric structures have been manufactured and tested in clinical trials. This approach demonstrated an increase in immunogenicity in comparison to the vaccine constructed on the backbone of the attenuated Towne strain only. Moreover these vaccines proved to be relatively safe and are currently being further analysed [302, 303].

ALVAC vaccine:

The phase-1 study of the canarypox-HCMV pp65 recombinant vaccine aimed to investigate whether this vaccine elicits potent cellular (HCMV pp65-specific CTL, helper T lymphocytes) and humoral responses. The vaccine was given at three different doses to healthy seronegative adults.

The analyses of immunogenicity revealed that pp65 specific cellular responses were present following administration of the third dose of the vaccine. This study demonstrated for the first time that the recombinant vaccine elicited cellular immune responses towards HCMV. This vaccine was not further developed due to the low immunogenicity [304].

1.15.3.2. Subunit vaccines.

The vast majority of the efforts to develop a subunit vaccine are focused on the dominant humoral target on the envelope gB and the molecules IE1 and pp65 that induce T-cell responses. Recombinant subunit vaccines based on these proteins are currently being tested in clinical trials.

1.15.3.2.1. pp65 subunit vaccines.

The recovery from HCMV disease is correlated with the development of HCMV-specific cytotoxic T lymphocytes (CTL). Therefore the major viral target antigens that induce cellular immunity were evaluated as attractive candidates for vaccine development. The majority of the efforts to induce cellular immunity though vaccination has been so far focused on pp65 tegument protein. pp65 is known to be a significant target antigen for CD8+ class I major histocompatibility complex (MHC)-restricted HCMV-specific CTL. One study revealed that between 70% and 90% of all CTL recognizing HCMV-infected cells were pp65 specific [305, 306]. Moreover, the studies conducted with bone marrow transplants revealed that passive transfer of T-cell clones specific for pp65 is correlated with better prognosis in this group of patients. This therapy was pronounced to be a ‘safe and effective way to reconstitute cellular immunity against HCMV’ following transplantation [307].

Pp65 peptide vaccine:

A recent, small study with healthy adult volunteers evaluated two candidate HCMV peptide vaccines composed of the HLA A*0201 pp65(495-503) cytotoxic CD8(+) T-cell epitope fused to two different universal T-helper epitopes (either the synthetic pan DR epitope [PADRE] or a natural tetanus sequence) for safety and ability to elicit pp65 T cells in HLA A*0201 healthy volunteers. This vaccine proved to be safe and evoked the expansion of pp65 (495-503) specific T cells in 30% of healthy volunteers when administered with the adjuvant. Most importantly, the safety profiles were generally good, although the addition of PF03512676 (1mg) adjuvant substantially raised reactogenicity [304]. This vaccine is currently being evaluated in phase 2 clinical trial.

1.15.3.2.2. IE subunit vaccines*.*

IE1 gene product has been recognized as a very important target of CD8+ responses against HCMV. The analysis of T-cell responses in seropositive healthy individuals revealed that approximately 40% of them have CD8+ T cells specific to IE1, but no CD4+ -IE1 specific responses [308]. The vaccine with IE1 only as a target has never been investigated- this antigen has been used in combination with other potent antigens: gB and pp65 proteins as a trivalent vaccine formulation.

1.15.3.2.2. Combined vaccines.

DNA vaccines:

-Bivalent DNA vaccine:

The effects of the bivalent DNA vaccines that are encoding gB and pp65 of HCMV were tested in animal models. Both DNA constructs proved to be highly immunogenic when delivered separately. However, when these constructs were administrated together (in PBS), the immune responses elicited by this vaccine formulation were less prominent. The outcome was improved when this bivalent vaccine was formulated with the poloxoamer adjuvant (VF-P1205-02A) [309]. Based on encouraging data from pre-clinical studies and good safety profiles, this bivalent DNA vaccine entered human clinical trials. The phase -1 clinical trial aimed to investigate safety and immunogenicity of this vaccine formulation. In total 44 healthy seropositive and seronegative individuals were enrolled in the study and different dosages and immunisation schedules were applied. In general the vaccine proved to be safe; only mild and moderate adverse effects were seen, all of which were short-term [310]. The immunotherapeutic effect of this vaccine was studied in a randomised, double-blind, placebo-controlled phase-2 clinical trial with haematopoietic stem-cell transplant (HSCT) patients, as they are at very high risk of HCMV reactivation due to their compromised T-cell responses. Although the percentage of patients who required antiviral therapy following transplantation was similar between placebo and vaccine group of patients, follow-up studies revealed that the occurrence and recurrence of cytomegalovirus viraemia as well as the time-to-event for viraemia episodes improved compared with placebo [311]. This vaccine was generally well tolerated and is now undergoing a phase-3 study.

-Trivalent DNA vaccine:

This vaccine formulation resembles the bivalent DNA vaccine except that this vaccine contains an additional plasmid that encodes IE1. Moreover, PBS was used instead of poloxomer. This formulation was tested in a prime-boost regime

together with the live attenuated Towne strain vaccine in seronegative healthy volunteers that were divided into three groups. Different immunization schedules (with or without boost with Towne strain) and different dosages of the vaccine were administered to patients in each of these groups. Regardless of the approach applied, the vaccine did not elicit potent immune responses. It has been reported that not more than 20% of vaccine recipients in each group responded to the vaccine. Interestingly though, differences in the time of immune response developments were noticed. The time to mount a positive anti-HCMV gB IgG antibody response and a positive ELISPOT response to HCMV pp65 and/or gB antigen stimulation was much shorter amongst the patients who received a boost with the live-attenuated Towne strain approximately a year after administration of the DNA priming [312].

-Trivalent Alphavirus replicon particles expressing gB and pp65-IE1:

The vaccine based on an alphavirus replicon particle with gB or a pp65/IE1 fusion protein was evaluated in a randomized, double-blind phase-1 clinical trial with healthy seronegative volunteers. The vaccine was delivered at two different doses. The analyses of the clinical material from the individuals involved in this study revealed that this vaccine induces both humoral and cellular immunity. Neutralizing antibody responses were found in all recipients of the high dose and 93% of recipients of the low dose of the vaccine. Moreover, the peak titres of these neutralizing antibodies in the patients who received high dose of the vaccine were on average 2-fold lower than the average titre of neutralizing antibodies in seropositive sera from patients following natural infection with this pathogen. The neutralizing antibody responses decreased over time and this effect was more profound in the recipients of the low dose. Peak cellular responses were found after administration of the second dose of the vaccine and it has been estimated that approximately 90% of vaccine recipients elicited T-cell specific responses to all three antigens. Detailed analysis of cellular responses revealed that the majority of the $CD8^+$ T cells were specific for pp65 following vaccination. Conversely, $CD4^+$ T cells targeted all three antigens with similar frequencies. This vaccine was assessed to be relatively safe and may be further developed [313].

1.15.3.2.3. gB-subunit vaccines.

This protein has been recognized as a major target of humoral responses-nearly 100% of individuals infected with the virus produced antibody against this protein and 40-70% of these antibodies had neutralizing properties [161]; moreover this protein is very abundant on the surface of infected cells and viral particles. The prototype vaccines with this glycoprotein were studied in animal models; administration of the recombinant gB-vaccine decreased the rate of virus transmission in pregnant guinea pigs and, as a result, a decline in mortality amongst the new-borns was seen. This study indicated for the first time that vaccination with this recombinant protein could be effective in preventing congenital HCMV (reviewed in [314]). Such results provided a rationale to further develop this vaccine and test its efficacy in clinical trials.

Over the past two decades one of the gB vaccine formulations has been studied extensively in several clinical trials with different target populations.

The protein used in the production of this vaccine was expressed in the eukaryotic- Chinese Hamster Ovary (CHO) cell line and the gB gene that was utilized in the production of this protein was derived from Towne strain of HCMV (Chiron) [315, 316]. Some alterations were introduced to this gB in comparison to the native protein in order to ease the expression and purification gB protein *in vitro* (Figure 1.8); [317]:
-the cleavage site was mutated in order to prevent the cleavage of this protein;
-a stop codon was introduced prior to the hydrophobic region of this protein in the transmembrane domain.

Such changes produced a truncated protein that, although closely resembling the native protein, could potentially induce a different repertoire of humoral responses (Figure 1.8). There are no available data on those potential differences and the extent of them between the immunogenicity of the native gB and the recombinant protein that was used as a vaccine antigen. It is very difficult to predict to what level the modification introduced to the recombinant gB could alter epitope presentation and glycosylation patterns. Although it is highly speculative; it seems

important to consider that those differences in immunogenicity may at least partly influence the antibody profiles of seropositive and seronegative vaccine recipients.

Safety profiles, immunogenicity and the immunisation schedule of this vaccine were evaluated in several studies that involved the participation of women of childbearing age in the late 90's. The optimal antigen dose and immunization regimens of this recombinant subunit were investigated in phase I study with healthy individuals [318]. Another phase I study, a randomized, double-blind, placebo-controlled trial, was designed to study the effect of the adjuvants alum and MF59; the results indicated that the vaccine formulation with MF59 was more immunogenic than that with alum [319]. Most importantly though, a randomized, double-blind, placebo-controlled phase 1 trial in seronegative toddlers determined the reactogenicity and immunogenicity of this vaccine. The researchers demonstrated that immunization with this vaccine formulation (subunit, recombinant gB with MF59) was generally safe as no serious side effects were seen following the administration of the vaccine. It was speculated that vaccination with this formulation could possibly result in the decline of child-to-child and child-to-adult transmission of the virus [320].

These findings prompted the initiation of Phase II randomized, placebo-controlled studies to test the efficacy of this vaccine that were performed with young mothers, solid organ transplant patients (chapter 1.16) and adolescents. The study with young women was initiated in 1999 and approximately 400 participants were involved in the studies. After a minimum of 1 year of follow-up, there were 49 confirmed infections, 18 in the vaccine group and 31 in the placebo group. Kaplan-Meier analysis showed that the vaccine group was more likely to remain uninfected during a 42-month period than the placebo group (P=0.02). Vaccine efficacy was 50% (95% confidence interval, 7 to 73) on the basis of infection rates per 100 person-years. One congenital infection among infants of the subjects occurred in the vaccine group, and three congenital infections occurred in the placebo group. Although more side-effects were reported in the vaccinated group in comparison to the placebo, generally these symptoms were mild and had short duration [321]. These studies show the potential of this vaccine to decrease transmission of the virus from the infected mother to the fetus; however the

efficacy was estimated to be only 50%. Nevertheless, although the effect observed in this study is only modest, such an outcome may still be appealing, especially for prevention of HCMV infection in future mothers at high risk of HCMV infection, as no other alternative therapies are accessible at the moment [322]. Moreover, the researchers speculated that this result could be biased by the choice of this particular population that was characterised to have an intense exposure to HCMV and a high rate of congenital HCMV infection.

More recently the same vaccine formulation was administered to adolescent girls in a phase 2 clinical trial (197 vaccine and 207 placebo recipients). Although the vaccine was proven to be safe, immunogenic and generally well-tolerated, the efficacy was lower than expected as the protection did not reach conventional level of significance [323]. Phase 3 clinical trials with congenital infection as the primary endpoint might be conducted in the future [324]. The major goal of such study would be to address whether the efficacy of this vaccine could be higher in a population that has less exposure to the virus [321, 322].

Another phase-2 study of this vaccine enrolled 150 seropositive women who were at increased risk of reactivation of the virus or reinfection with a different strain. The aim was to investigate whether this recombinant gB vaccine with MF59 adjuvant is able to boost both cellular and humoral immune responses. The analyses revealed that the vaccine is capable of stimulating both arms of the immune system; induction of CD4+ T-cell responses and boosting of gB-specific antibodies, were reported. However, these responses were transient. It seems plausible that the CD4+ T-cells that were generated in response to the vaccination facilitated the increase in the level of gB-specific antibody [325].

1.16. The soluble recombinant subunit glycoprotein B (gB) with MF59 adjuvant Vaccine Study in SOT.

Prior to my studies a phase-2 clinical trial with the soluble recombinant subunit glycoprotein B (gB) with MF59 adjuvant was conducted at the Royal Free Hospital in London, UK [188] which I describe in this section.

SOT patients are at risk of end-organ diseases such as pneumonitis, hepatitis, enteritis or retinitis caused by HCMV. The patients can be assigned into four different categories based on their donor- and recipient serostatus for HCMV: D+R-; D+R+; D-R+; D-R-. In SOT the highest incidence of HCMV viraemia and disease occurs in the D+R- followed by the D+R+ patients. In the D+R- subset, seronegative recipients lacking pre-existing immunity towards the virus acquire HCMV from positive donors causing primary infection in these individuals. Seropositive recipients in the D+R+ category are also at risk of developing HCMV infection due to reinfection from a different strain of the virus acquired from the donor or reactivation of latent virus; however, the incidence of the disease is lower in these groups. Seropositive recipients in the D-R+ category are only at risk of developing HCMV infection due to reactivation of latent virus and have the lowest incidence of disease. Thus, in the context of SOT, pre-existing natural immunity provides substantial protection from HCMV viraemia and disease, supporting vaccination as a viable strategy to control HCMV in the transplant setting [35].

In order to provide the dynamic and quantitative measurements of viremia (number of genome copies per mL) and other virological parameters, such as peak HCMV load in donor/recipient groups, HCMV replication kinetics and antiviral therapy, a natural history study was performed prior to vaccine clinical trial. The consecutive blood samples that were collected from 689 solid organ transplant patients (liver and renal) were analysed and the levels of HCMV DNA were measured by quantitative PCR. All the patients that were enrolled into this analysis were managed by pre-emptive antiviral therapy and no patient received antiviral prophylaxis. All the transplants were performed and/or followed up at the Royal Free Hospital, London, between July 2002 and the end of January 2010 [35].

This study was crucial to establish the necessary virological parameters that could enable the interpretation of the results from the subsequent vaccine clinical trials. These natural history analysis provided very useful data on the indicators of risk of HCMV primary infection (D+R-); reactivation (D+R+; D-R+) and reinfection (D+R+); and associated kinetics of replication (Figure 1.9; Table 1.4).

Most importantly, these quantifications were used as a reference point for the phase-2 vaccine trial with subunit, recombinant gB vaccine [2]. Once the virological parameters were established, it was possible to investigate whether the vaccination could block transmission of the virus from the donor and confer protection in this group of patients.

To test this, a clinical trial with a vaccine based on HCMV glycoprotein B (gB) antigen plus MF59 adjuvant was performed in patients awaiting kidney or liver transplantation. 67 patients received the vaccine and 73 received placebo, all of whom were evaluable for the analysis (Figure 1.10). Following administration of the vaccine or placebo, gB antibody titres were measured by enzyme immunoassay.

In order to perform the pharmacodynamic assessment of the clinical samples obtained from the patients who participated in this clinical trial, the glycoprotein B antibody titres were measured and correlated with the virological parameters that were established in the natural history studies described above. Antibody titres against the gB protein were significantly increased in both seropositive and seronegative recipients of the vaccine in comparison to the patients who received placebo (Figure 1.11). The viral load was evaluated by PCR in those who proceeded to transplant. The duration of viraemia and number of days of ganciclovir treatment were inversely correlated with gB antibody titres in the group of vaccinated patients who developed viraemia suggesting that vaccine was having a protective effect (Figure 1.12); [188]. Although not all of the differences observed between vaccinated and placebo patients reached statistical significance, due the fact that numbers of the patients in each groups were very small (Figure 1.13; Table 1.5), the glycoprotein-B antibody titres correlated inversely with duration of viraemia in both seropositive and seronegative patients (Figure 1.12). Such a result strongly suggests that the vaccination had a protective effect as all the values of virological parameters were reduced in vaccine recipients [188].

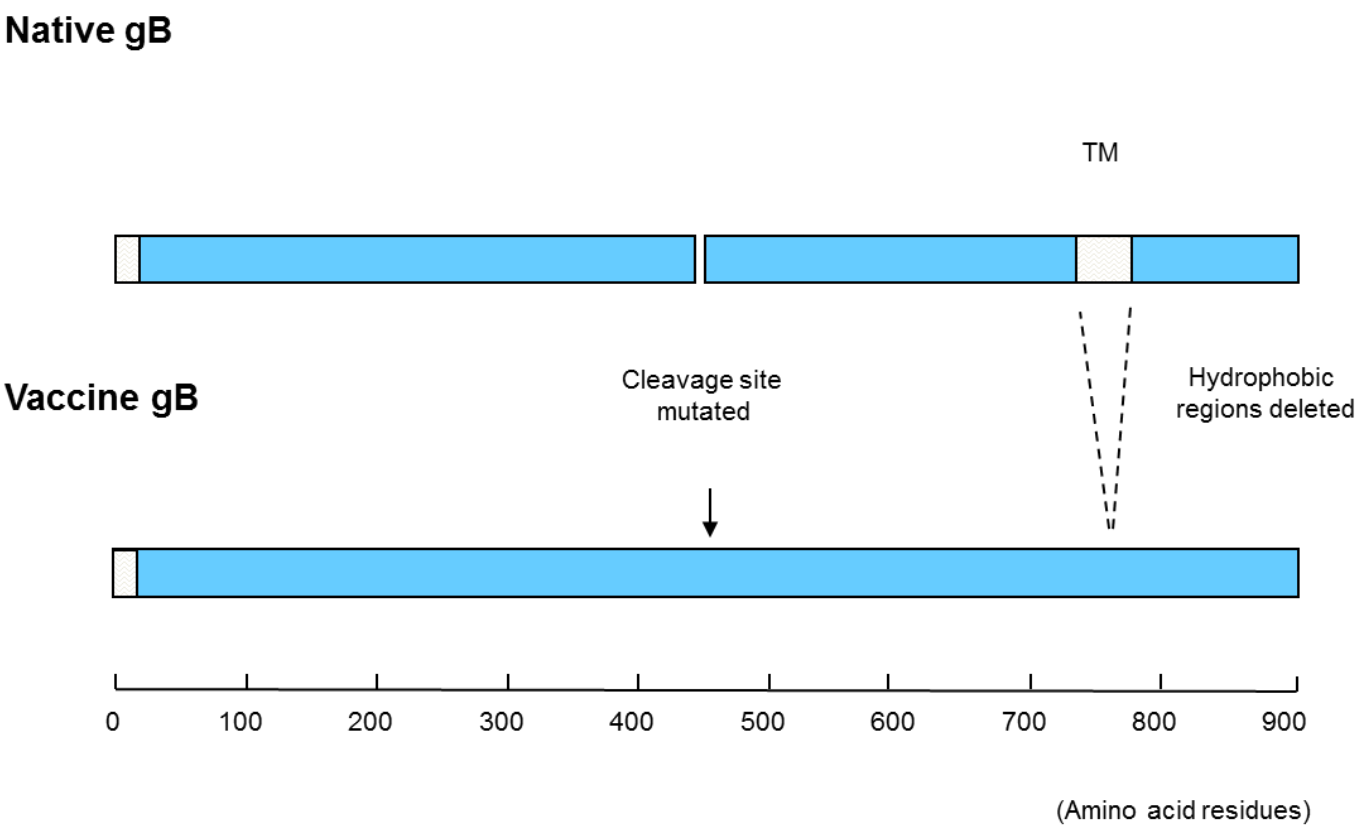


Figure 1.8. Schematic representation of the differences between the native gB and the recombinant gB.

The recombinant protein was used in the production of the subunit vaccine with MF59 adjuvant used in the phase-2 clinical trial (NCT00299260) [293].
* TM-transmembrane domain.

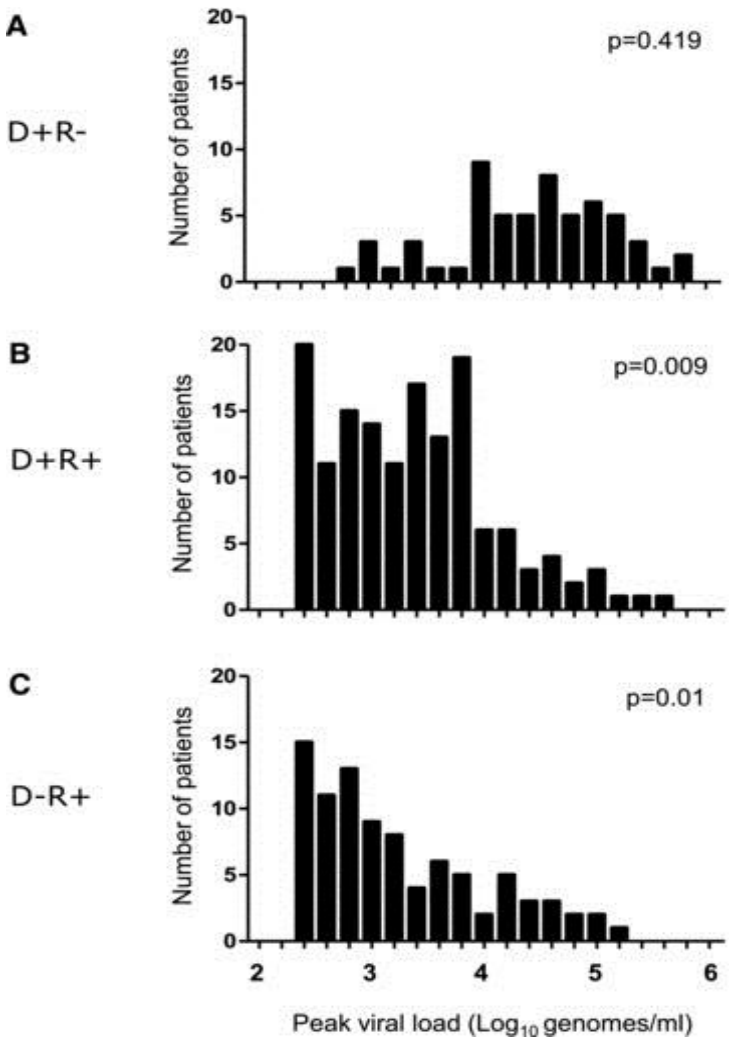


Figure 1.9. Frequency distribution plots of the values of peak viral load among the three DR groups of patients at risk of HCMV infection.

Bin size is 0.2 Log 10 genomes. P-Values indicate difference from Gaussian distribution and were calculated with the D'Agostino & Pearson omnibus K2 normality test. Reprinted from [35].

HCMV donor/recipient status	% of patients who developed viremia	% of patients who required treatment
D+R-	78% (58/74)	69% (51/74)
D+R+	54% (147/270)	23% (62/270)
D-R+	40% (89/222)	13% (29/222)
D-R-	0% (0/123)	0% (0/123)

Table 1.4. HCMV viraemia and treatment in subgroups of transplant patients with defined donor and recipient serostatus [35].

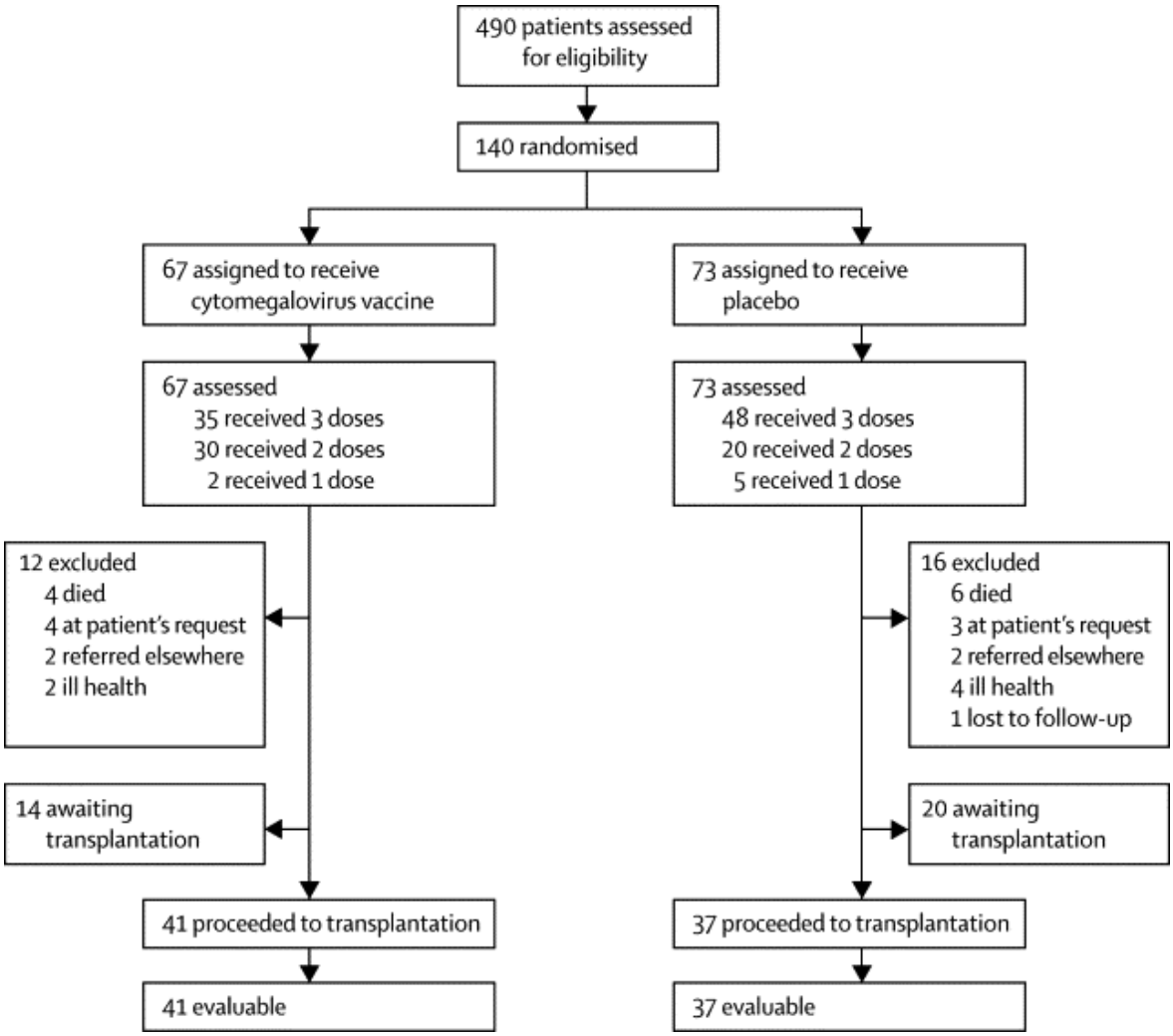


Figure 1.10. Trial profile at the time of analysis.

Disposition of the solid organ transplant patients who participated in the phase-2 clinical trial (NCT00299260) with subunit glycoprotein-B vaccine with MF-59 adjuvant [188].

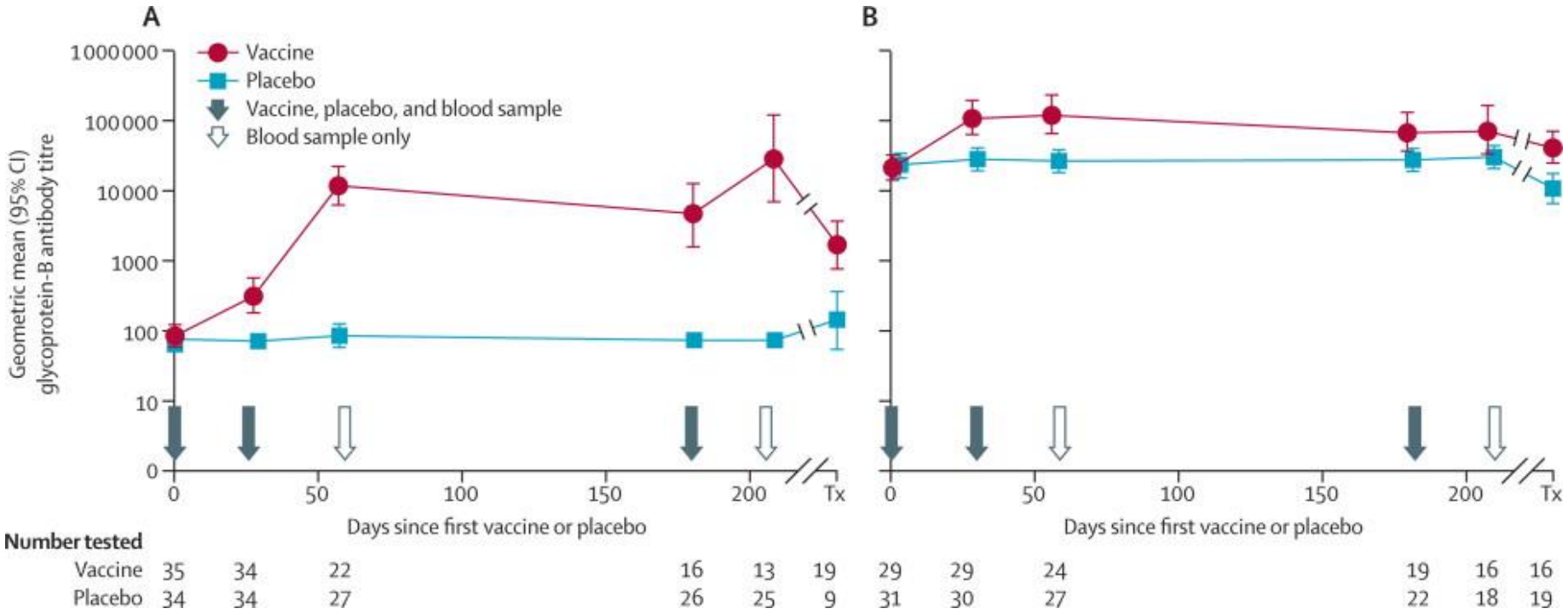


Figure 1.11. Geometric mean (95% CI) antibody titres measured by glycoprotein-B enzyme-linked immunoassay.

(A) Seronegative recipients. (B) Seropositive recipients. Tx indicates the geometric mean titres found at the time of transplantation. Reprinted from [188].

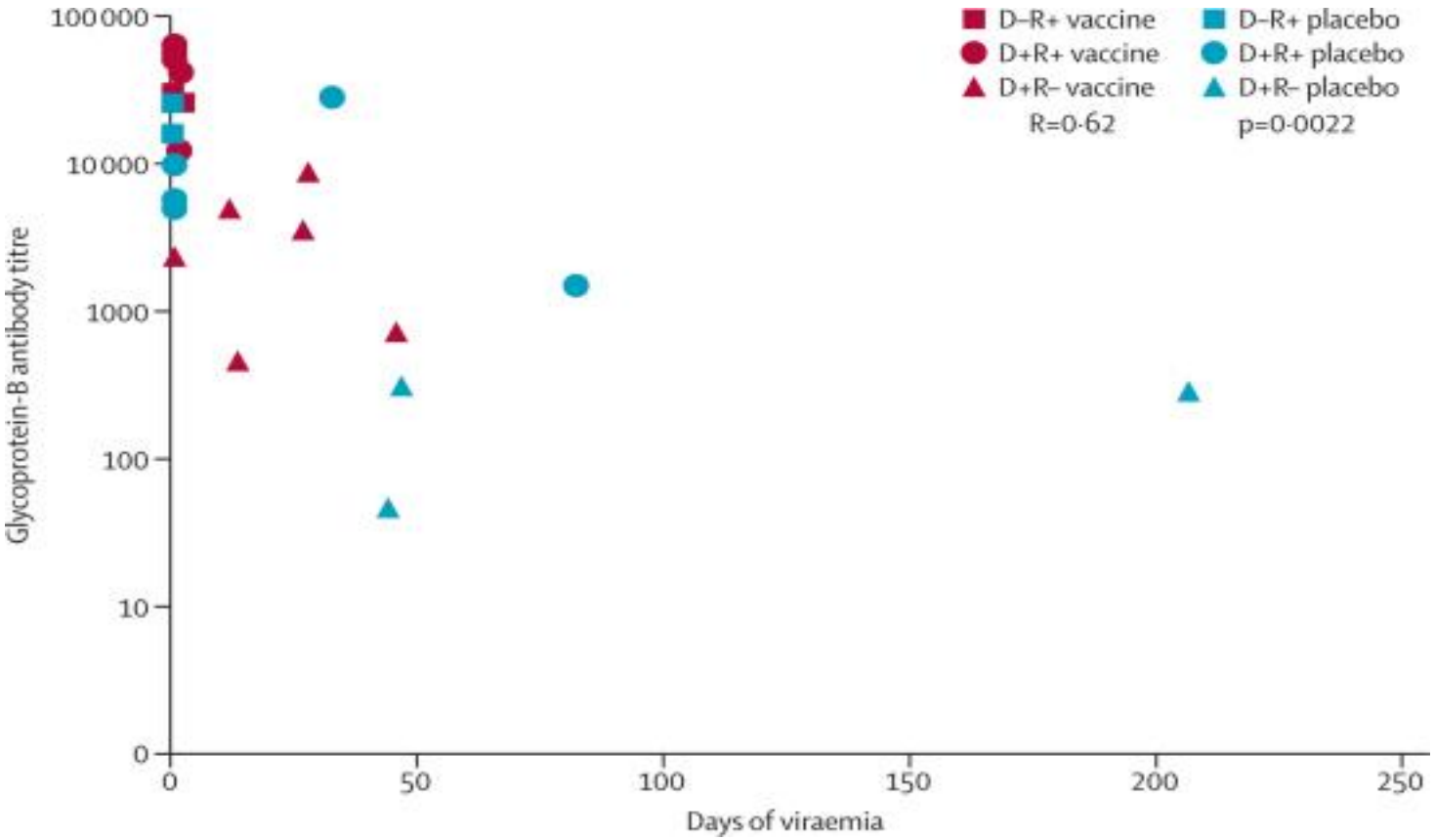


Figure 1.12. Inverse correlation of titre of antibodies against glycoprotein B present at the time of transplantation with duration of viraemia after transplantation.

The titre of anti-gB antibodies were measured by ELISA. Only patients with viraemia at any time were selected for this analysis. D−=cytomegalovirus seronegative donor. R−=cytomegalovirus seronegative recipient. D+=cytomegalovirus seropositive donor. R+=cytomegalovirus seropositive recipient. Reprinted from [188].

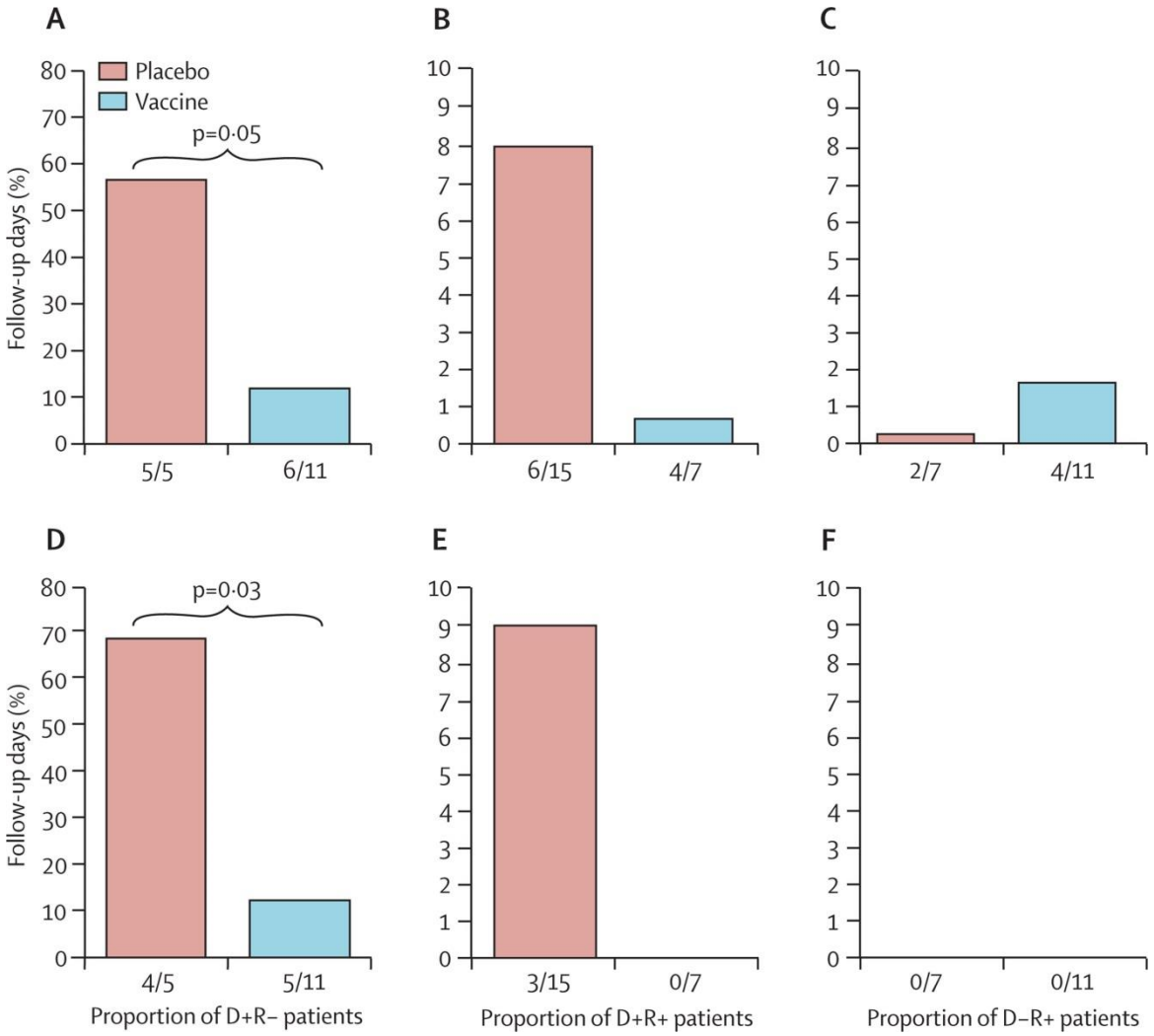


Figure 1.13. Proportion of days that patients in the three subgroups at risk of CMV infection spent with viraemia or received antiviral treatment.

A,B, and C show the duration of viraemia. D,E, and F show the duration of antiretroviral therapy. D+R−, D+R+, and D−R+ are three groups at risk of primary infection, reinfection, and reactivation, respectively. The numbers below each column indicate the number of patients with viraemia (or treatment) divided by the number in the subgroup. Note the different values on the Y-axes of panels A and D compared with panels B, C, E, and F. D−=cytomegalovirus seronegative donor. R−=cytomegalovirus seronegative recipient. D+=cytomegalovirus seropositive donor. R+=cytomegalovirus seropositive recipient. Reprinted from [188].

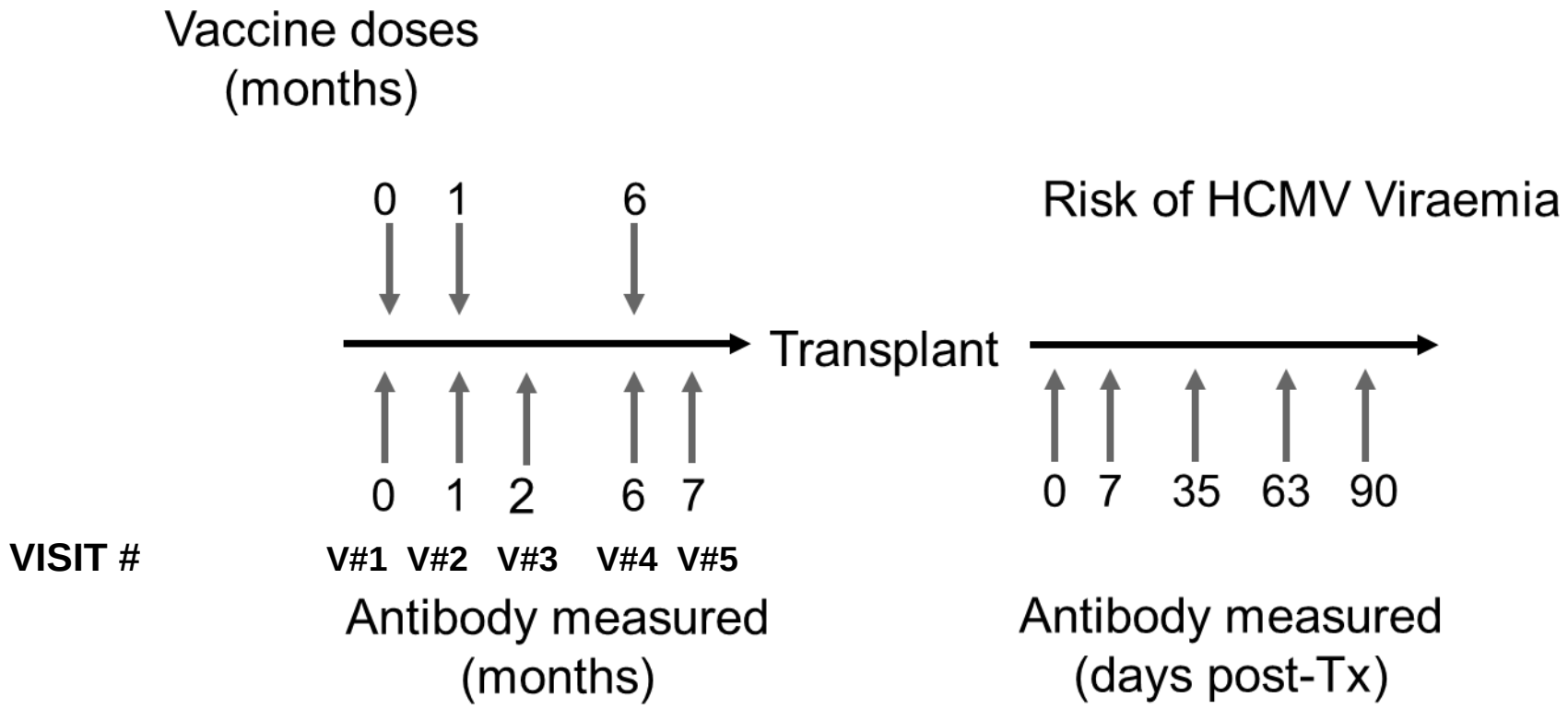


Figure 1.14. Vaccine administration schedule.

Upper panel: vaccination and placebo administration schedule (months). Lower panel sample collection time points months (prior to transplantation; days (following the transplantation).

	Viraemia >200 genomes per mL	Number treated	Median days of follow-up (range)	Median number of samples (range)	Days PCR positive/ total person-days of follow-up (%)*	Days treated/total person-days of follow-up (%)*
Seronegative donor, seronegative recipient (n=22)						
Placebo (n=10)	0	0	96 (51–115)	13 (6–25)	0/915 (0%)	0/915 (0%)
Vaccine (n=12)	0	0	105 (15–138)	15 (3–26)	0/1204 (0%)	0/1204 (0%)
p value	..	..	..	..	1·00	1·00
Seronegative donor, seropositive recipient (n=18)						
Placebo (n=7)	2	0	95 (65–157)	20 (8–25)	2/696 (<1%)	0/696 (0%)
Vaccine (n=11)	4	0	93 (89–176)	20 (4–26)	21/1209 (2%)	0/1209 (0%)
p value	..	..	..	..	1·00	0·59
Seropositive donor, seropositive recipient (n=22)						
Placebo (n=15)	6	3	95 (45–173)	17 (8–26)	119/1489 (8%)	135/1489 (9%)
Vaccine (n=7)	4	0	93 (91–228)	14 (7–21)	6/803 (<1%)	0/803 (0%)
p value	..	..	..	..	0·51	0·22
Seropositive donor, seronegative recipient (n=16)						
Placebo (n=5)	5	4	91 (20–278)	19 (5–62)	339/599 (57%)	415/599 (70%)
Vaccine (n=11)	6	5	94 (90–122)	19 (10–24)	128/1069 (12%)	142/1069 (13%)
p value	..	..	..	..	0·0480	0·0287
N=78	27	12	..	..	..	..

*Total number of person-days during which any participant had viraemia higher than 200 genomes per mL or received treatment divided by total number of days of follow-up for all participants who underwent a transplantation. The proportion of days of post-transplantation follow-up spent with viraemia (or receiving treatment) was calculated for each individual. These values were then compared between vaccine and placebo with a Mann-Whitney *U* test. Comparison of proportion of days of viraemia in (all) vaccine versus (all) placebo p=0·99.

Table 1.5. Cytomegalovirus viraemia and treatment in subgroups of transplant patients with defined donor and recipient serostatus. Reprinted from [188].

Aims of the thesis:

The pharmacodynamic approach described above allowed evaluation of the efficacy of the gB/MF59 vaccine despite the lack of information on the correlate of protection.

Therefore, the remit of my studies was to identify the component of the specific humoral response that appears responsible for improving HCMV outcomes in SOT recipients.

2. General Materials and Methods.

2.1. Patient population.

The population from whom samples have been evaluated and described in this work are the cohort of solid organ transplant patients who were enrolled in the phase-2 randomised and double-blinded placebo controlled cytomegalovirus glycoprotein-B vaccine with MF59 adjuvant trial [188]. In total 140 adult renal and liver transplant patients participated in the study; 73 of them received vaccine and 67 received placebo for HCMV (Figure 1.10). The vaccine or placebo was given in three doses: at day 0 (baseline), 1 month and 6 months later (Fig.1. 14). Following vaccination, the blood samples from patients were obtained consecutively. The first five blood samples were collected in order to measure the antibodies (qualitatively and quantitatively) at baseline, and after 1, 2, 6 and 7 months. The patients who subsequently underwent transplantation were followed up for 90 days during which serial blood samples were obtained around days 0, 7, 35, 63, 90 (Figure 1.14). The level of viral DNA was also tested by measuring HCMV DNA by real-time quantitative PCR (RT-qPCR) [188]. Exclusion criteria included: pregnancy (a negative pregnancy test was required before each vaccine dose); receipt of blood products (except albumin) in the previous 3 months, and simultaneous multi-organ transplantation [188].

All the patients that were enrolled into this analysis were managed by pre-emptive antiviral therapy and no patient received antiviral prophylaxis. For the strategy of pre-emptive therapy, no patient receives prophylaxis and drug is only administered to those where surveillance samples detect viraemia above a threshold value defined by real time polymerase chain reaction (PCR) [326]. Pre-emptive therapy is typically stopped when a patient has two consecutive blood samples where HCMV DNA is undetectable [326]. Surveillance for infection continues and some patients develop a second episode of viraemia which is again treated until viraemia becomes undetectable.

The study was approved by the Research Ethics Committee and all patients gave written informed consent [188] (Appendix).

	Vaccine group		Placebo group	
	Cytomegalovirus positive	Cytomegalovirus negative	Cytomegalovirus positive	Cytomegalovirus negative
Total number of patients	32	35	38	35
Organ awaiting transplantation				
Liver	10 (31%)	15 (43%)	13 (34%)	16 (46%)
Kidney	22 (69%)	20 (57%)	25 (66%)	19 (54%)
Sex				
Male	16 (50%)	22 (63%)	17 (45%)	27 (77%)
Female	16 (50%)	13 (37%)	21 (55%)	8 (23%)
Age (years)	55 (12)	49 (12)	52 (12)	48 (13)
Race				
Caucasian	24 (75%)	32 (91%)	22 (58%)	33 (94%)
Black	1 (3%)	0 (0%)	7 (18%)	1 (3%)
Asian	5 (16%)	3 (9%)	5 (13%)	0 (0%)
Other	2 (6%)	0 (0%)	4 (11%)	1 (3%)
Number of vaccinations received				
1	1 (3%)	1 (3%)	4 (11%)	1 (3%)
2	12 (38%)	18 (51%)	12 (32%)	8 (23%)
3	19 (59%)	16 (46%)	22 (58%)	26 (74%)
Days from vaccine 1 to vaccine 2 (median, range)	32 (21–118) n=31	35 (22–274) n=34	30 (21–119) n=34	31 (23–241) n=34
Days from vaccine 1 to vaccine 3 (median, range)	186 (154–416) n=19	188 (147–224) n=16	188 (167–298) n=22	188 (151–375) n=26
Total number of patients who proceeded to transplantation during study period	18	23	22	15
Organ transplanted				
Liver	8 (44%)	11 (48%)	10 (46%)	10 (67%)
Kidney	10 (56%)	12 (52%)	12 (55%)	5 (33%)
Sex				
Male	7 (39%)	15 (65%)	7 (32%)	13 (87%)
Female	11 (61%)	8 (35%)	15 (68%)	2 (13%)
Age at transplantation (years)	53 (12)	50 (13)	50 (12)	49 (12)
Race				
Caucasian	12 (67%)	22 (96%)	14 (64%)	15 (100%)
Black	1 (6%)	0 (0%)	2 (9%)	0 (0%)
Asian	4 (22%)	1 (4%)	3 (14%)	0 (0%)
Other	1 (6%)	0 (0%)	3 (14%)	0 (0%)
Number of doses of vaccine or placebo received before transplantation				
1	0 (0%)	1 (4%)	1 (5%)	0 (0%)
2	9 (50%)	16 (70%)	8 (36%)	6 (40%)
3	9 (50%)	6 (26%)	13 (59%)	9 (60%)
Days from vaccine 1 to transplantation (median, range)	216 (40–636)	123 (22–604)	199 (8–1134)	262 (36–1231)
Immunosuppressive drugs administered*				
Basiliximab	5 (28%)	9 (39%)	7 (32%)	3 (20%)
Tacrolimus	14 (78%)	23 (100%)	22 (100%)	13 (87%)
Azathioprine	5 (28%)	6 (26%)	7 (32%)	4 (27%)
Mycophenolate mofetil	10 (56%)	17 (74%)	16 (73%)	10 (67%)
Prednisolone	13 (72%)	21 (91%)	19 (86%)	11 (73%)
Methylprednisolone	10 (56%)	16 (70%)	18 (82%)	9 (60%)
Available follow-up by PCR since transplantation (days)				
Median (range)	93 (89-228)	97 (15–138)	95 (45–173)	95 (20–278)
Cytomegalovirus status of donor				
Positive	7 (39%)	11 (48%)	15 (68%)	5 (33%)
Negative	11 (61%)	12 (52%)	7 (32%)	10 (67%)
Transplantation type				
Cadaver	13 (72%)	17 (74%)	16 (73%)	14 (93%)
Live person	5 (28%)	6 (26%)	6 (27%)	1 (7%)

Table 2.1. Baseline characteristics according to patients' cytomegalovirus status and randomisation group. Reprinted from [188].

2.2. Processing of the blood samples from the clinical trial with cytomegalovirus glycoprotein-B vaccine with MF59 adjuvant.

Blood samples (5ml) were collected in sterile tubes (without anticoagulant) and then left in a standing position for approximately half an hour in order to allow enough time for blood to clot, then the samples were centrifuged at RT at 1500g for 15min. Following centrifugation, the serum fraction was separated from the clot. Serum samples were stored at -78°C prior to analysis.

2.3. Isolation of Peripheral Blood Mononuclear Cells (PBMCs).

PBMCs were retrieved from venous blood samples by centrifugation (20mins 850g, brake off in 20°C) on Histopaque-1077 (Sigma Aldrich). After centrifugation, the top blood plasma layer was removed and discharged. The interface created between the plasma layer and the lymphocyte separation medium was carefully removed to avoid contamination with adjacent layers and placed into fresh tubes. The volume was made up to 30ml by addition of RPMI 1640 and the cell suspension was centrifuged at 550g for 10min. The supernatant was discarded and the pellet was re-suspended in RPMI 1640. Cells were washed twice by centrifugation at 450g for 5mins.
Cellular pellet was finally re-suspended in 20ml of PBS and cells were counted using haemocytometer.

2.4. Detection of viraemia.

Blood samples after SOT were tested by real-time quantitative PCR (RT-qPCR) for cytomegalovirus DNA. HCMV PCR was done on a routine basis with an in-house TaqMan (ABI)–based method as described in detail in [327] and [328]. HCMV viraemia was defined as one or more positive HCMV PCR results (cut-off, 200 genomes/mL of whole blood, equivalent to 168 IU/mL). If viraemia higher than 3000 genomes per mL was detected (equivalent to 2520 IU/mL), the patient was treated with antiviral drugs as described in [188].

2.5. Cell culture.

Human Foreskin Fibroblast cell line.

Human foreskin fibroblasts (HFF) were maintained in DMEM media (Dulbecco) supplemented with 10% fetal bovine serum (FBS) (Gibco by Life Technologies) 2mM L-Glutamine, 50UI/ml penicillin (Sigma-Aldrich) and 50UI/ml streptomycin solution (Sigma-Aldrich). Cells were passaged as follows: the cell monolayer was washed with 10ml of PBS pH 7.7 (IX) [-] $CaCl_2$[-]$MgCl_2$ (Gibco by Life Technologies) and incubated with 5mL of trypsin (0.25% Trypsin-EDTA (IX) Gibco by Life Technologies) to detach the monolayer and then either used for experiments or split, typically, into 3x T175 flasks. The cells were incubated at 37°, 5%CO_2 with 100% humidity.

2.6. Virus.

The low passage strain of the virus Merlin was utilized in the experimental part of this thesis and is the clinical reference strain for the sequence of HCMV [329].
Low passage HCMV strain Merlin (a gift of Dr Richard Stanton, University of Cardiff) was cultured in HFF cells. The cells were grown to 80% confluency in 30ml of DMEM media in T-175 flasks. The medium was removed and the monolayer of cells was washed with PBS. Cells were inoculated with Merlin (0.05 MOI) and virus allowed to adhere to the cells for 1h at 37°C. The viral inoculum was removed, and the cell monolayer was washed and replaced with fresh DMEM media. The cells were incubated at 37°C until the formation of cytopathic effect was observed (approximately 12 days). The supernatant containing the virus was then removed and centrifuged at 1000g for 5 minutes in order to remove remaining cellular debris. The supernatant was stored in 1mL aliquots at -78°C.

2.7. Staining of the HFF cells for immediate early proteins.

To stain for IE protein expression, 100µl of ice-cold 100% ethanol (Sigma-Aldrich) was added to each well and plates were left in -20°C for 30 min. Following the incubation, ethanol was discarded, plates were washed 3x with PBS (200µl of

PBS to each well) and incubated with PBS at RT for 5min. 100µl of primary antibody: (mouse anti-CMV IE antigen monoclonal antibody MAB8131, Millipore, 1:1000 dilution in PBS) was added to each well and incubated for 1h at RT. Following the incubation, plates were washed twice with PBS. Then 100µl of secondary antibody- goat anti-mouse Alexa Fluor-568 (Life Technologies; 1:1000 dilution in PBS) plus DAPI (Life Technologies, 10ng/ml) was added to each well and plates were left in the dark for 1h at RT. After the incubation, the dilution of secondary antibody and DAPI was discarded, plates were washed 3x with PBS (200µl of PBS to each well) and incubated with PBS at RT for 5min. 100µl of fresh PBS was added to each well. Plates were covered with plastic lids and stored at 4°C prior to analysis.

2.8. Cell imaging.

WiScan®- plates were analysed by the Hermes WiScan® 3.4 cell-imaging system developed by IDEA Bio-Medical Ltd. and The Medical University of South Carolina (MUSC) This high throughput screening system consists of WiScan (data generator) and WiSoft (analysis software) and enables cell imaging under physiological conditions. Multiparametric analysis of multicolour data was performed using this method.

-IE positivity and nuclear staining (chapter 3 and 4, 6 and 7);
-GFP positivity and nuclear staining (chapter 4),

In these experiments the focus speed was slow and the lens magnification was 10x and 20x.

Leica DMI4000B- Microscopic analysis was performed by Automated Inverted Microscope Leica DMI4000B with motorized excitation manager and FIM (Fluorescence Intensity Manager) and Leica DFC365 FX Fluorescence Camera. The system was particularly designed to work efficiently even with weakly fluorescing or rapidly fading specimens. The lens magnification used in these experiments was 10x and 20x.

2.9. Analysis of the results.

The analysis of the results in chapters 3 and 4 was performed by MetaMorph® Microscopy Automation & Image Analysis Software v7.8. This software provided a tool to:

- count the number of IE72/86 positive cells and total amount of cells (nuclear staining) in chapter 3;
- count the number of IE86-GFP positive cells and total amount of cells (nuclear staining) in chapter 4.

The raw data were analysed then by Graph Pad Prism®-software. This programme provided the statistical analysis and graphs for all chapters.

2.10. Statistical analysis of the results.

The analysis of the results from the cohort of patients who participated in a phase-2 clinical trial (NCT00299260) of subunit glycoprotein-B vaccine with MF-59 adjuvant was performed by Graph Pad Prism®-software. An overview of the statistical tests used and why they were chosen is given below.

Wilcoxon signed ranks test- this is a non-parametric test used when data are not normally distributed. The comparison is made between matched observations on a single sample. Secondly, each pair is chosen randomly and independently. The data are measured at least on an ordinal scale (cannot be nominal). This test was used to calculate the statistical differences between the mean value of the percentage of infection between the samples obtained from the patients at the day of vaccine/placebo administration and their corresponding samples collected at the day of transplantation in the neutralization assays (chapter 3) and in the viral spread assay (chapter 4). This test takes into account the magnitude of the differences within the analysed specimen instead of just assessing whether some value is less or greater than the median value (in comparison to the Sign test) therefore it has been chosen for this analyses.

Mann-Whitney U test also called the Mann–Whitney–Wilcoxon (MWW):

Wilcoxon rank-sum test (WRS), or Wilcoxon–Mann–Whitney test- this test measures the differences between groups of observations that are not related to each other. The Mann-Whitney test was developed to evaluate data that are measured on a continuous scale, but do not follow a Gaussian distribution. The Mann-Whitney calculations convert the values to ranks and then the sum of ranks (U) is computed by Graph Pad Prism® software. The smallest value in these populations is assigned the number “1” and the highest value in these two compared groups receives the rank “n” (*n* is the total number of values in the two groups). The software calculates then the mean of the ranks in each group, and p value reflects the difference between the means in these populations. Statistical differences were obtained from the Mann Whitney test between the mean value of the percentage of infection between the samples obtained from the vaccinated and placebo patients at the time of transplantation in the neutralization assays (chapter 3) and in the viral spread assay (chapter 4). This test was also used to calculate the statistical differences in the level of CD107a expression between groups of patients and healthy donors and to calculate the statistical differences between the percentage of infection between cells infected with the virus and the cells infected with the virus and incubated with either monoclonal antibodies or sera (in chapter 5); and in chapter 6 to calculate the statistical differences between the mean value of optical densities (ODs) for particular antigenic domains (ADs) between the populations of patients: vaccinated vs placebo and viraemia vs no viraemia.

3. Neutralizing antibody responses.

3.1. Introduction.

Neutralization can be defined as a biological phenomenon whereby antibodies can inhibit the infectivity of a pathogen or toxicity of a toxin molecule. Neutralizing antibodies (Nab) usually work by blocking site(s) on bacteria or viruses that are used during the entry process into the target cell [330, 331]. In addition, neutralization can occur following internalisation of virion- antibody complexes. For example, the recently discovered cytosolic IgG receptor- tripartite motif-containing 21 (TRIM21), was shown to have higher affinity for the Fc portion of antibodies than any other IgG receptor in the human body. Importantly, this receptor binds to antibody-virus complexes in the cytoplasm and delivers them to the proteasome via its E3 ubiquitin ligase activity where it is degraded. This recently discovered mechanism of adaptive immunity provides protection against pathogens by blocking the transcription of virally encoded genes and extends our understanding of the role of neutralizing antibodies in conferring protection not only to the extracellular but also to the cytoplasmic compartment [332].

It has long been considered that the neutralization of pathogens by antibodies is the major mechanism of protection following vaccination [333, 334]. Indeed, for many licensed vaccines the correlate of protection (if known) is a robust neutralizing antibody response. For example, the first vaccine that was developed (against smallpox) conferred protection though neutralizing antibody responses with the data from animal and human studies indicating that the protective titre of neutralizing antibody was approximately 1/20 [333, 335, 336]. Similarly, neutralizing antibodies provided the best correlate of immunity after administration of other successful vaccines such as: inactivated (IPV) and oral (OPV) polio vaccine [337] and MMR (measles [338, 339], mumps, and rubella) vaccine [333, 334, 337, 339-341]. Therefore, inducing a neutralizing antibody response that prevents initiation of infection is highly desirable and it is one of the most important goals of vaccination [342]. It has even been said that ‘A vaccine that generates

broadly neutralizing antibodies (bnAbs) has been the 'holy grail' of HIV vaccine research' [343].

Several vaccine candidates against HCMV have been evaluated in clinical trials in the past three decades, including live attenuated (based on laboratory-adapted AD169 strain and laboratory adapted-clinical strain Towne), subunit vaccines (gB) and plasmid vaccines (pp65, gB). Although live attenuated vaccines may elicit neutralizing antibody responses, protection against infection with the virus was not as potent as that following natural infection [344]. However, in the phase-1 trials of subunit gB with MF59 adjuvant it was observed that neutralizing antibody titres, measured by standard plaque-reduction assay, were significantly higher in the sera following vaccination in comparison to the titres of neutralizing antibodies in the serum samples obtained prior to vaccination [318, 319].

The aim of the current study was to investigate the correlate of protection associated with the gB MF59 vaccine delivered to solid organ transplant candidates in a phase II trial. Preliminary studies of the neutralizing antibody responses in the sera from the solid organ transplant patients who took part in this randomised study have been already investigated and the results published (Table 3.1); [188]. In those experiments sera from the patients were heat inactivated and serial dilutions of the specimen were prepared prior to analysis. The HCMV strain Towne RC256, which expresses β galacatosidase was used and the target cells were human fetal foreskin fibroblasts. Guinea pig complement (5% volume/volume) was included in the protocol and incubated with heat-inactivated and diluted sera. The final read-out in these assays was infectivity, determined colourimetrically with X-gal as a substrate for β galacatosidase. Final results were shown as a geometric mean of the neutralising antibody titre for each group of patients (patients were grouped based on the categories: day of sample collection; vaccine/placebo status; HCMV serostatus); (Table 3.1); [188].

Using this approach, limited evidence of a functional Nab response was detected in vaccinated seronegative recipients. The Nab response in seropositive recipients was boosted significantly but the response was only transient, because the antibody titres declined quickly with time.

Objectives:

The aim of the current study was to extend these observations and investigate in more detail, utilising the wild type Merlin strain, whether Nabs were a critical component of the protective immune response elicited by gB vaccination in this trial.

	Vaccine				Placebo			
	Cytomegalovirus positive		Cytomegalovirus negative		Cytomegalovirus positive		Cytomegalovirus negative	
	n	GMT (95% CI)	n	GMT (95% CI)	n	GMT (95% CI)	n	GMT (95% CI)
	32		35		37		35	
Day 0	29	67 (40–111)	35	4 (4–4)	31	65 (40–105)	34	4 (4–4)
Day 28	29	272 (150–494)	34	4 (–)*	30	82 (54–125)	34	4 (4–4)
Day 56	24	261 (129–526)	22	5 (4–6)	27	78 (50–122)	27	4 (–)*
Day 180	19	120 (59–244)	16	4 (4–5)	22	78 (45–137)	26	4 (–)*
Day 208	16	137 (58–326)	13	17 (6–45)	18	80 (43–149)	25	4 (–)*

Table 3.1. Titre of neutralising antibody according to time from first dose of vaccine.

*Insufficient variation to compute a 95% CI. 50% neutralising titres were calculated by graph pad prism. A dilution of 1 in 8 was the lowest used: for this analysis, negative sera were assigned a titre of 4 (Griffiths et al., 2011[188]).

3.2. Materials and Methods.

3.2.1. Patient population.

The neutralizing antibody responses were measured in solid organ transplant patients who participated in a phase-2 clinical trial with gB/MF59 (described in chapter 1.16). Samples evaluated in these assays were obtained from the patients at the day of administration of the first dose of the vaccine (seronegative patients: n=26; seropositive patients n=18) or placebo (seronegative patients: n=16; seropositive patients n=24); and at the day of transplantation (challenge with the virus). Some of these patients developed HCMV viraemia post-transplant.

3.2.2. Neutralisation Assay.

Confluent HFF cells were detached from the surface of culture flasks by addition of trypsin (0.25% Trypsin-EDTA (IX) Gibco by Life Technologies). The cell suspension was centrifuged at 400g for 5 min, trypsin and media were discharged and the cellular pellet was re-suspended in fresh DMEM (with 10%FBS, 5%pen/strep) media. Cells were plated at the density of 10^4 HFFs/well (96-well plate format). All serum samples were heat inactivated (1h at 56°C) and diluted in the ratio 1:10 in DMEM media (with 10%FCS, 5%pen/strep). 30µl of each heat inactivated serum sample was added to 270µl of media and aliquoted into 100µl. All samples were stored at -78°C prior to analysis.

To study the impact of sera on HCMV infection, the Merlin strain of HCMV was incubated with either the aliquoted serum samples (100µl) or characterised antibodies that recognise the gB protein: ITC88 (Ohlin) [345]; 2F12 (Abcam), HCMV37 (Abcam), or isotype matched controls: IgG1 (Abcam) and IgG2a (Abcam);or with DMEM medium only (negative control), for 1h at 37°C and then used to inoculate HFFs at the MOIs ranging from 0.5-1 as indicated in figure. Cells were then incubated with the mix of antibodies (or serum) and virus for 3h at 37°C; 5% CO_2. Following incubation, the DMEM media with serum and low passage HCMV strain (Merlin) was removed and replaced with fresh DMEM media (100µl per well/96-well plate). Plates were incubated for 24h in 37°C (for IE staining) or

96h in 37°C (for pp28 staining). Following the incubation, media was discarded and plates were washed twice with PBS, then the cells were fixed with 70% ethanol (Sigma-Aldrich) for 1 hour at -20°C and subsequently washed twice with PBS. Cells were stained either for IE72/86 expression (Mouse Anti-CMV IE Antigen Monoclonal antibody, clone 6F8.2 (1:1000; Millipore)) or pp28 expression (Mouse Anti-CMV pp28 Antigen Monoclonal antibody 1:500, Santa Cruz Biotech) for 1 hour, washed in PBS and then incubated with secondary antibody (goat anti-mouse alexa fluor-568, LifeTechnologies1:1000 dilution). Nuclei were counterstained using DAPI- 1:1000 (Sigma-Aldrich) in the secondary antibody incubation. Wells were washed with PBS and plates were stored at 4°C prior to analyses.

All the error bars shown on the graphs in this chapter representing patients indicate standard deviation (SD) and the error bars shown on the summary graphs (representing the groups of patients indicate standard error of mean (SEM)).

3.2.3. Cell imaging and analysis of the results.

Cells were visualised by WiScan® 3.4 cell-imaging system (described in chapter 2.8). 20 images were captured per well. Each experimental condition was repeated three times, and the mean value was calculated from these images by Metamorph software. These mean values are represented on the figures: 3.4-3.12. The summary graphs show the mean of the values representing each patient in the given population (Fig. 3.13).

The pp28- positive cells were counted manually by randomly selecting fields of view, twenty for each well and counting 100 cells and taking an average (Figures: 3.9- 11).

3.3. Results.

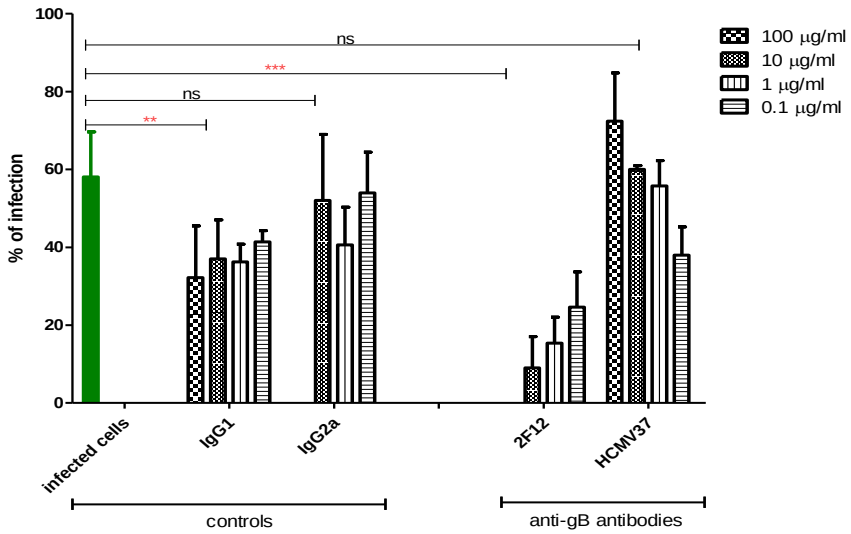
3.3.1. Establishing experimental conditions to detect neutralising antibody activity.

In order to test the sera for neutralising antibodies, I first established an assay that would allow me to measure neutralizing activity. To do this, a panel of commercially available antibodies, 2F12 and HCMV37 which recognise gB of HCMV, were tested at different concentrations for their ability to neutralise infection (100µg/ml- 0.1µg/ml), when compared with their respective isotype controls (IgG1 and IgG2a) at corresponding dilutions. Analysis of infection by IE staining showed that the antibody 2F12 proved to be a very potent neutralizing antibody- completely blocking the infection at 100µg/ml concentration and was still highly effective at 10µg/ml. In contrast, the HCMV37 antibody failed to neutralize HCMV infection (Figures 3.1 A and 3.2).

Next, I tested antibody ITC88– a known neutralising antibody- in my assay alongside 2F12 antibody. Again the antibodies were tested at different concentrations: 10 µg/ml; 1 µg/ml; 0.1µg/ml; together with isotype control- IgG1 at corresponding dilutions.
Both antibodies were confirmed to effectively block infection in a dose dependent manner; the highest neutralization was observed with the highest concentrations of antibodies (Figures 3.1.B. and 3.3).

These data show that neutralization of HCMV infection can be effectively measured using this assay and that for future assays the anti-gB antibody ITC88 at the concentration 10µg/ml was used as a positive control.

A)



B)

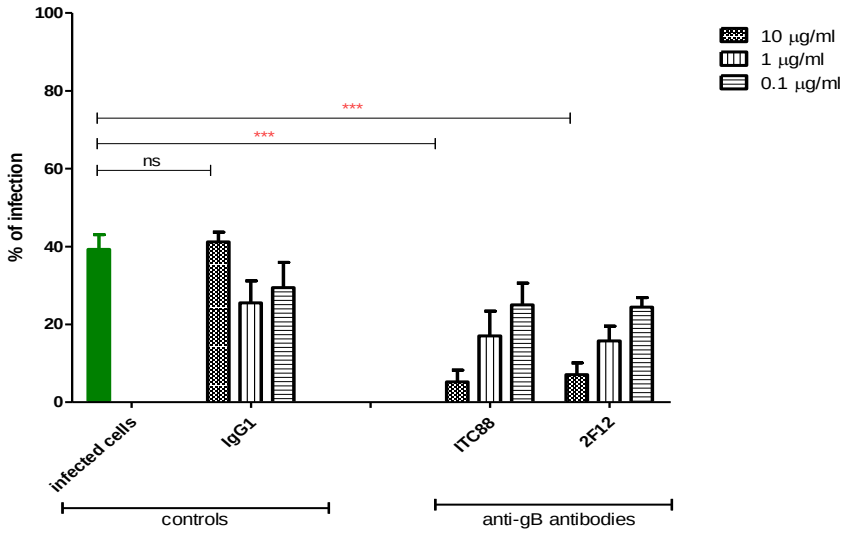


Figure 3.1. Commercially available antibodies display different abilities to block HCMV infection.

A) Merlin (MOI=1) was incubated with 2F12, HCMV37 or the isotype controls IgG1 and IgG2a, respectively; at the concentrations: 100µg/ml; 10µg/ml; 1µg/ml; 0.1µg/ml. After 24 hours cells were stained for IE to quantify infection. B) Merlin (MOI=1) was incubated with 2F12, ITC88 or isotype control IgG1 at the concentrations: 10µg/ml; 1µg/ml; 0.1µg/ml. After 24 hours cells were stained for IE to quantify infection. The differences in the decrease of % of infection between groups were assessed by Mann-Whitney U test. ns (not significant):p>0.05; **:p<0.01; ***p<0.001.

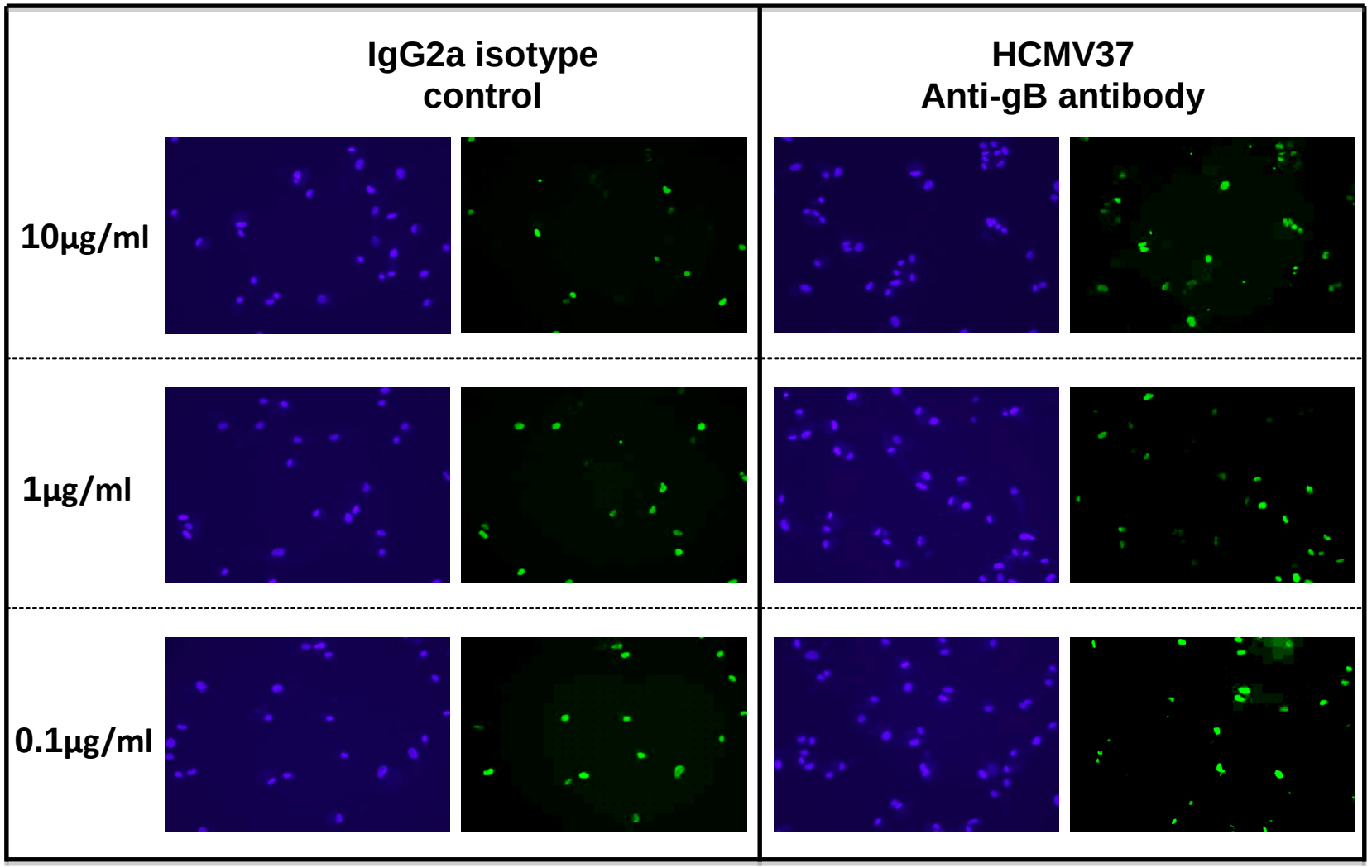


Figure 3.2. The HCMV37 antibody does not block HCMV lytic infection.

Merlin was incubated with either HCMV37 or IgG2a isoptye control at different concentrations (10- 0.1ug/ml) for 1 hour and then used to infect HFFs (MOI=1). Cells were then fixed and immuno-stained for IE gene expression 24hpi (green). Nuclei were counterstained with DAPI (blue).

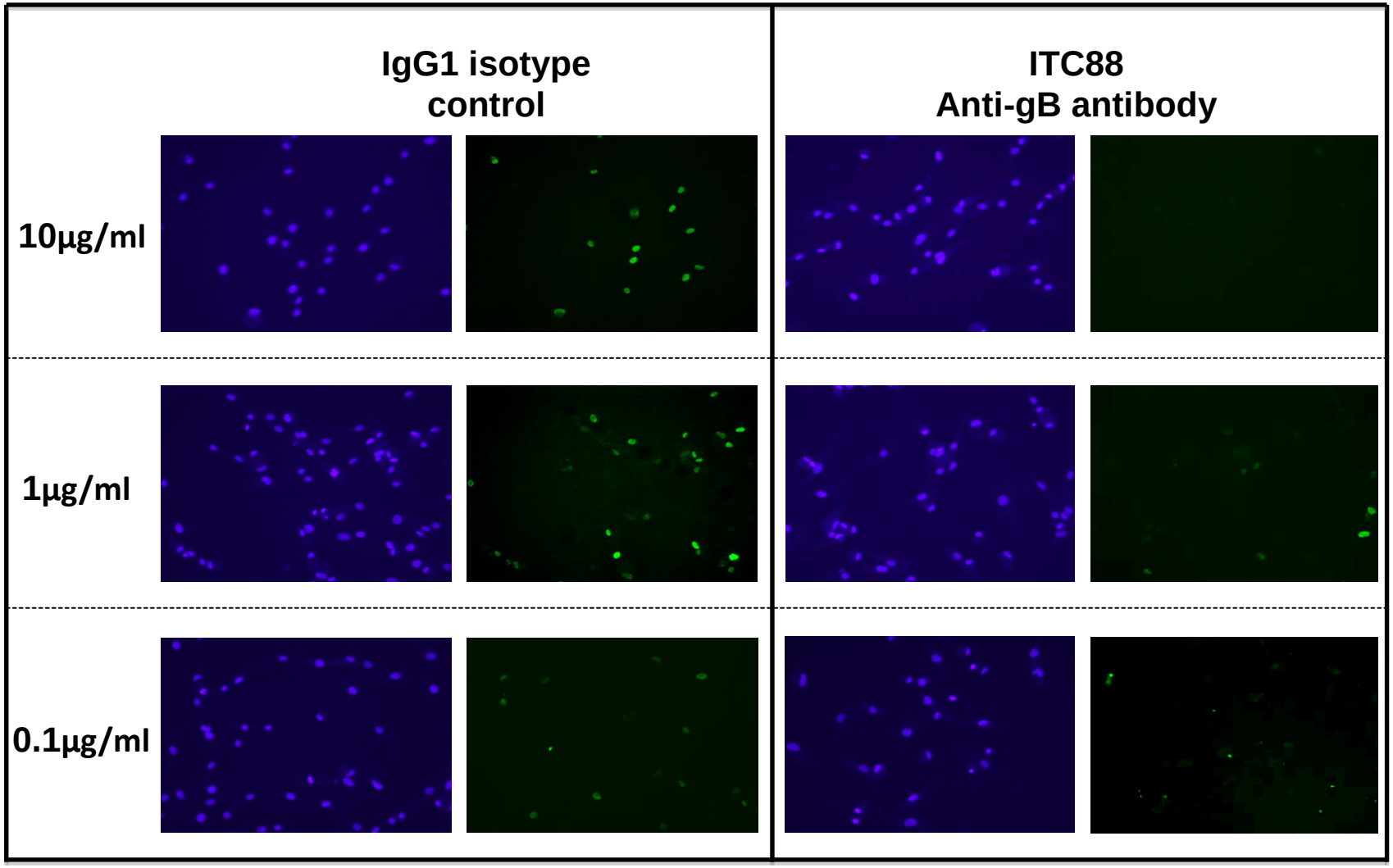


Figure 3.3. ITC88 antibody blocks HCMV lytic infection.

Merlin was incubated with either ITC88 or IgG1 isoptye control at different concentrations (10- 0.1ug/ml) for 1 hour and then used to infect HFFs (MOI=1). Cells were then fixed and immuno-stained for IE gene expression 24hpi (green). Nuclei were counterstained with DAPI (blue).

3.3.2. Testing neutralizing antibody responses in sera from the patients enrolled in the phase-2 clinical trial with recombinant subunit glycoprotein-B vaccine with MF59 adjuvant.

Having established a protocol and controls for studying the ability of antibodies to block HCMV infection as a surrogate measure of neutralisation, I next tested whether similar activity could be measured in serum samples from liver and renal transplant recipients who participated in the phase-2 clinical trial.

3.3.2.1. Sera from seropositive patients display variable levels of neutralizing activity but this is independent of vaccination status.

My first experiments analyzed the sera from the seropositive patients. Merlin virus was pre-incubated with sera from seropositive patients pre vaccination (baseline) and at post vaccination (day of transplant) and then used to infect HFFs. The data show that pre-incubation with sera from seropositive individuals reduced the level of infection (Figures 3.4 and 3.5) which, in many instances, was comparable with the ITC88 control. Specifically, sera from the majority of the patients were able to neutralize virus as effectively as the positive control- ITC88 at the concentration 10µg/ml; although I did observe that sera from some seropositive patients were less effective (e.g. 003-0008; 003-0003, 003-00015, 001-00046, 001-00051); (Figures 3.4, and 3.5). However, the data also show that no differences were observed between paired sera pre and post vaccination (Figure 3.13). There were also no statistical differences in the mean value of percentage of infected cells between placebo and vaccinated patients who did and did not develop viraemia after transplantation (Figure 3.12).

Moreover, there were no differences between the mean value of the percentage of infection between the serum samples obtained from the patients at the day of vaccine administration (pre-vaccination) and at the day of transplantation in the seropositive group of solid organ transplant patients (Figure 3.12). Thus, although HCMV seropositive individuals display a neutralizing antibody response against HCMV, vaccination with gB conferred no increased protection versus those administered the placebo control. Furthermore, no correlation between neutralizing activity and protection from viraemia was observed. Both groups (viraemia vs non-viraemia) responded similarly in this assay which suggests that the neutralizing antibody responses that were evoked mainly by natural infection and possibly boosted with the vaccine (Table 3.1; [188]) may not confer protection against the onset of viraemia.

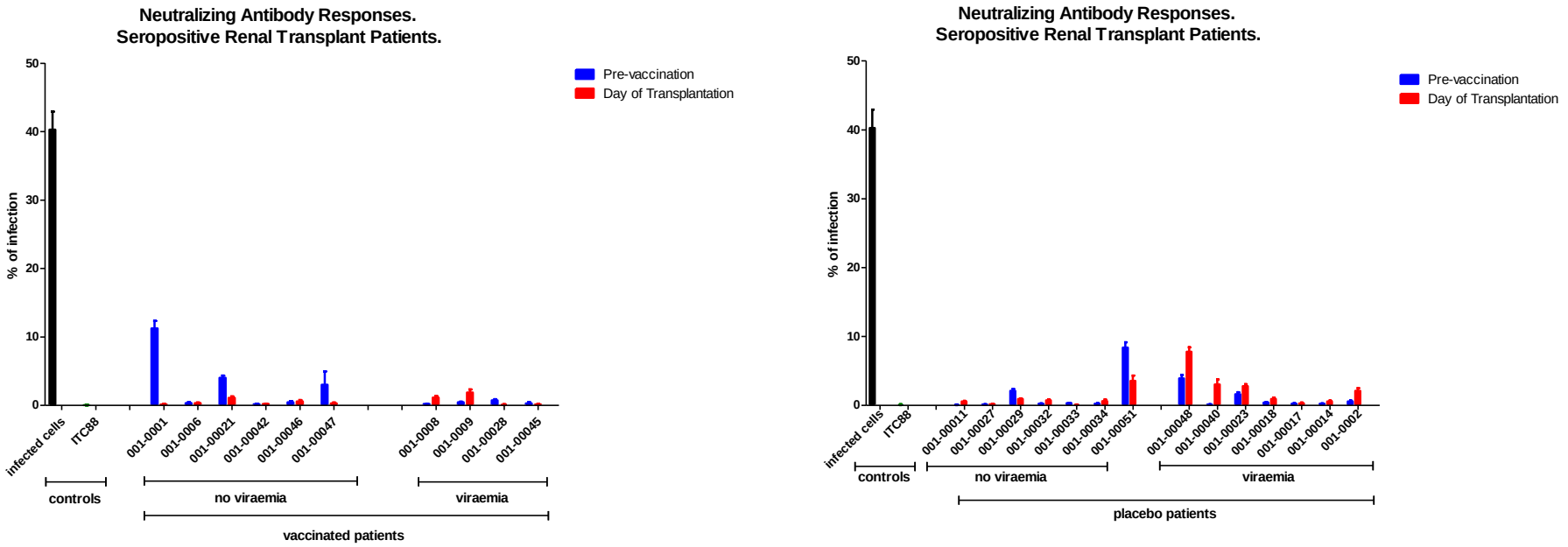
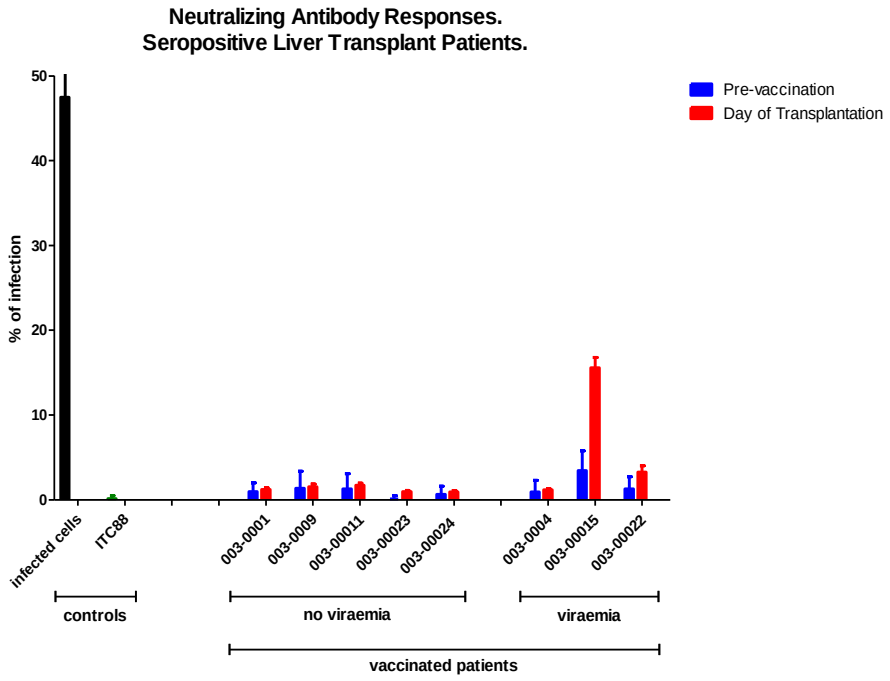


Figure 3.4. Sera from seropositive renal transplant patients reduces HCMV infection in vitro but is not enhanced by vaccination.

Merlin was incubated with sera from seropositive renal patients, or an ITC88 positive control, and used to inoculate HFFs *in vitro* (MOI=1). Infection was assayed by IE immunostaining 24hpi and the proportion of infected cells calculated by counterstaining nuclei with DAPI. Sera isolated pre-vaccination (baseline – blue bars) or post vaccination (day of transplant – red bars) was tested in triplicate. Sera from patients vaccinated with gB (A) or placebo (B) are shown and are subdivided into patients who went onto display evidence of viraemia post-transplant.

A)



B)

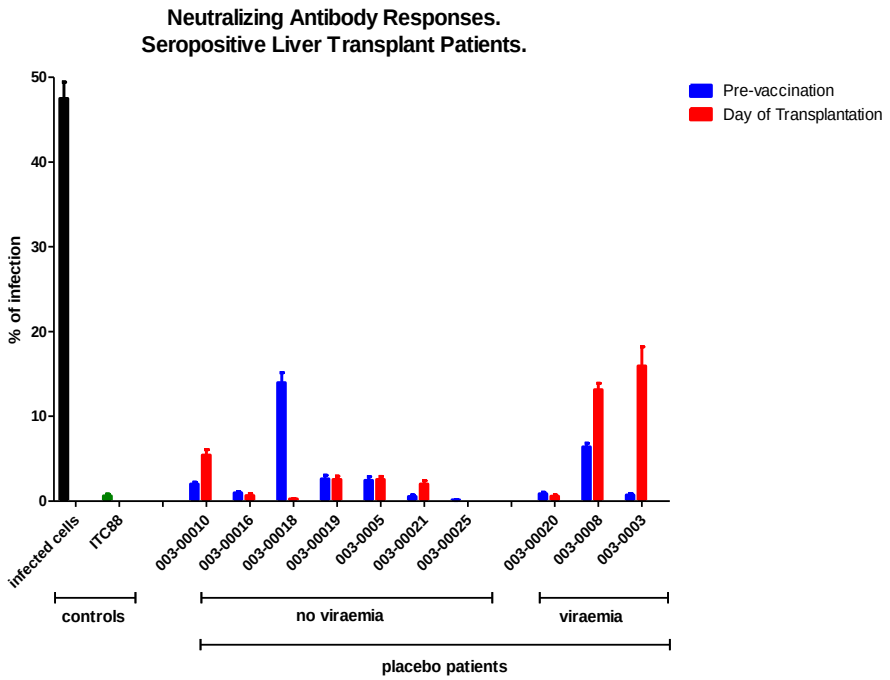


Figure 3.5. Sera from seropositive liver transplant patients reduces HCMV infection in vitro but is not enhanced by vaccination.

Merlin was incubated with sera from seropositive liver patients, or an ITC88 positive control, and used to inoculate HFFs *in vitro* (MOI=1). Infection was assayed by IE immunostaining 24hpi and the proportion of infected cells calculated by counterstaining nuclei with DAPI. Sera isolated pre-vaccination (baseline – blue bars) or post vaccination (day of transplant – red bars) was tested in triplicate. Sera from patients vaccinated with gB (A) or placebo (B) are shown and are subdivided into patients who went onto display evidence of viraemia post-transplant.

3.3.2.2. Sera from the seronegative patient cohort display minimal evidence of neutralizing antibody activity after vaccination.

A. Neutralization measured as decrease in the percentage of IE positive cells.

Next, I performed analysis of the seronegative cohort sera samples. As before, neutralization assays measured the decrease in the proportion of infected cells (by IE gene expression). Here I could find only minimal evidence to support the hypothesis that a neutralizing antibody response directed towards gB in sera from seronegative patients could be mediating the protective effect seen following vaccination. Specifically, the percentage of HCMV infected cells incubated with sera from seronegative patients was comparable with the percentage of HCMV infected cells in negative controls (Figures 3.6 and 3.7). The data show no differences in the percentage of infection between vaccinated and placebo patients. Moreover, there is no distinction between the levels of neutralizing antibodies in patients who developed viraemia and those who did not. Finally, there are no observable changes between the percentage of infected cells using virus that had been pre-incubated with sera harvested pre- placebo/vaccine administration and with sera harvested at the time of challenge with the virus (transplantation); (Figures 3.6, 3.7 and 3.12).

Importantly, repetition of the study showed that the observations were consistent. A combined analysis of patient sera based on vaccination status and transplant outcome of either renal or liver transplant patients showed no differences in the level of neutralization between pre-vaccination (depicted in blue) and the day of transplantation (depicted in red) samples in these vaccinated seronegative patients (Figures 3.8A and 3.8C). The level of neutralization in vaccinated individuals was similar to placebo group in both renal (Figures 3.8A and 3.8B) and liver (Figures 3.8C and 3.8D) patients. Additionally, no differences in the level of neutralization were found between patients who experienced viraemia and those who did not following challenge with the virus at time of transplantation.

B. Neutralization measured as decrease in the percentage of pp28 expressing cells.

Previously, phase-1 studies with this vaccine formulation clearly indicated that neutralising antibodies were generated based on plaque assays that were used in those analyses. To address this discrepancy, I investigated whether my readout of IE gene expression could be an explanation. I investigated the pp28 positivity of cells infected with the virus because pp28 is a late protein and therefore a marker of the ability of the virus to complete the viral lifecycle. Any observed difference in the level of expression of the early and late genes would suggest that the neutralization may be occurring post-infection. In these sets of experiments sera from seronegative renal transplant patients (vaccinated n=12, placebo: n=8) were incubated with virus (Merlin) and subsequently added to confluent fibroblasts. Cells were stained 96h post infection and the proportion of pp28 positive cells was counted manually. The vaccinated cohort was compared to the corresponding placebo group and respective controls (Figures 3.9 and 3.11).

This analysis showed that the number of pp28 positive cells was generally comparable to the number of IE positive cells in corresponding samples from this seronegative renal transplant group (Figure 3.10). Importantly, the level of pp28 did not change following the administration of the vaccine (Figures: 3.9-3.11) supporting my previous finding with IE that vaccination did not elicit neutralizing antibody responses in seronegative vaccine recipients.

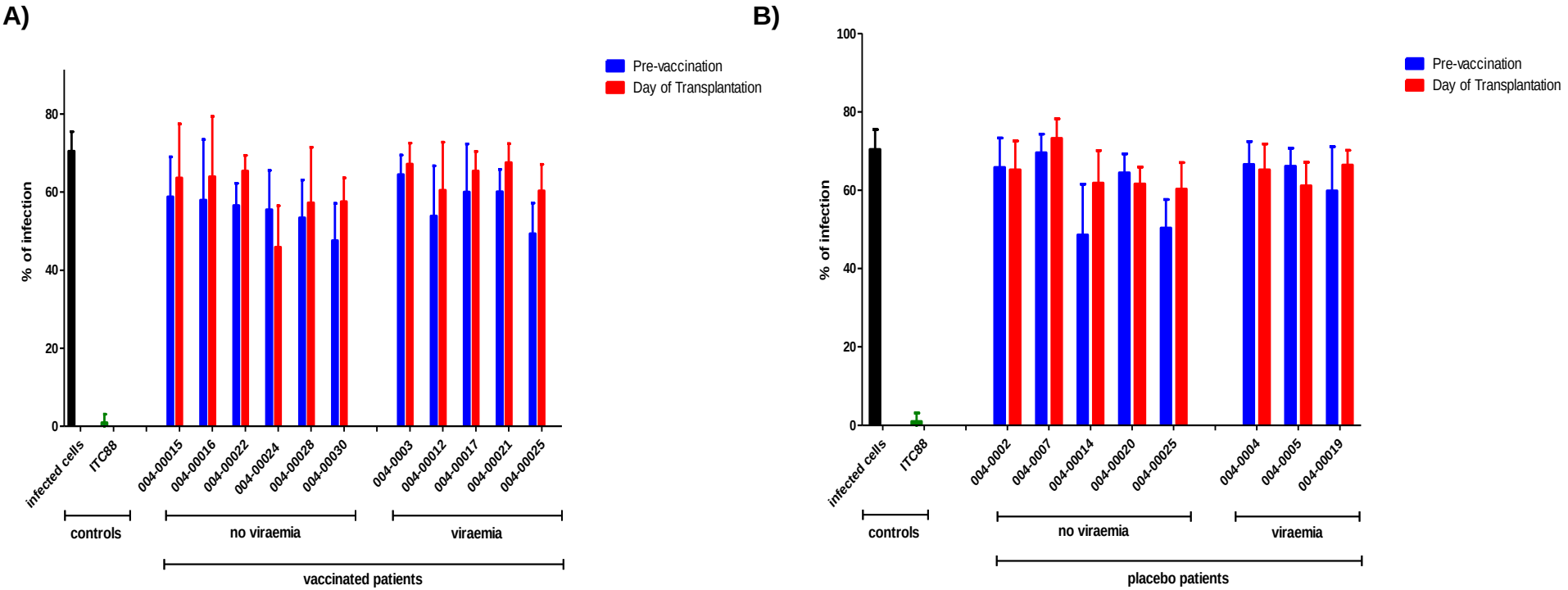


Figure 3.6. Sera isolated from seronegative liver transplant recipients do not reduce HCMV infection *in vitro*.

Merlin was incubated with sera from seronegative patients, or an ITC88 positive control, and used to inoculate HFFs *in vitro* (MOI=1). Infection was assayed by IE immunostaining 24hpi and the proportion of infected cells calculated by counterstaining nuclei with DAPI. Sera isolated pre-vaccination (baseline – blue bars) or post vaccination (day of transplant – red bars) was tested in triplicate. Sera from patients vaccinated with gB (A) or placebo (B) are shown and are subdivided into patients who went onto display evidence of viraemia post-transplant.

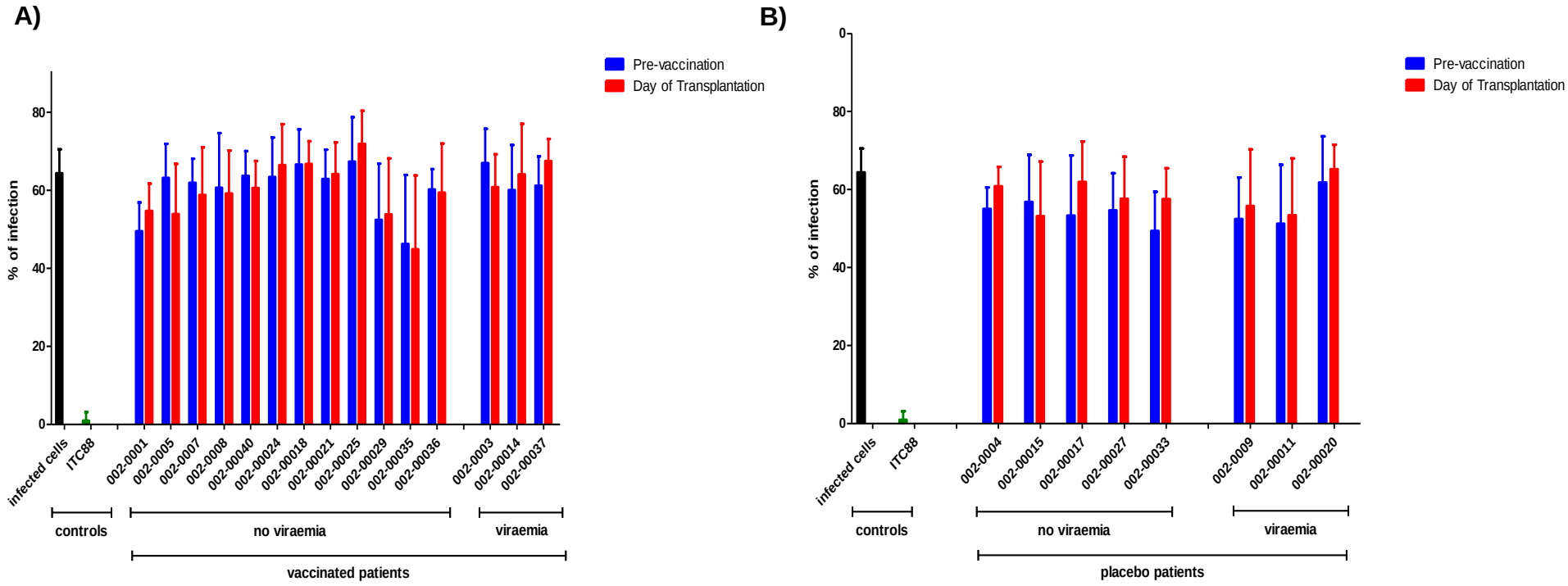


Figure 3.7. Sera isolated from seronegative renal transplant recipients do not reduce HCMV infection *in vitro*.

Merlin was incubated with sera from seronegative patients, or an ITC88 positive control, and used to inoculate HFFs *in vitro* (MOI=1). Infection was assayed by IE immunostaining 24hpi and the proportion of infected cells calculated by counterstaining nuclei with DAPI. Sera isolated pre-vaccination (baseline – blue bars) or post vaccination (day of transplant – red bars) was tested in triplicate. Sera from patients vaccinated with gB (A) or placebo (B) are shown and are subdivided into patients who went onto display evidence of viraemia post-transplant. .

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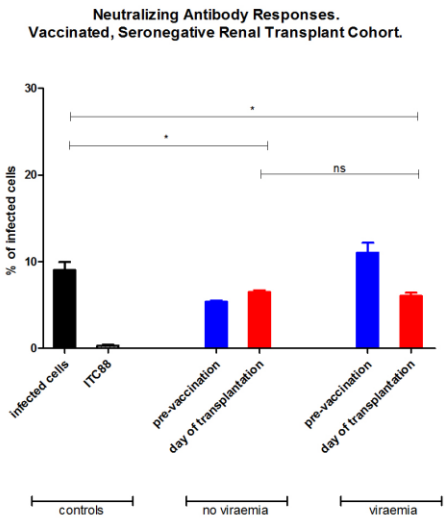

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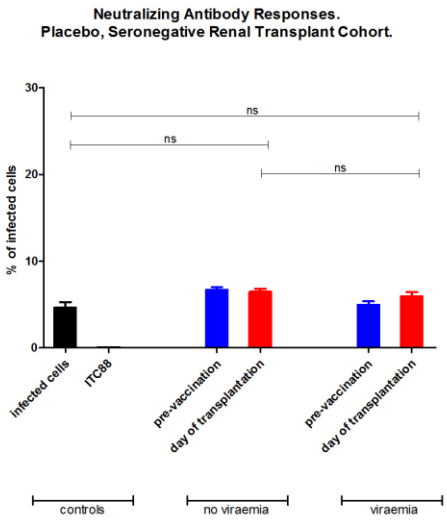

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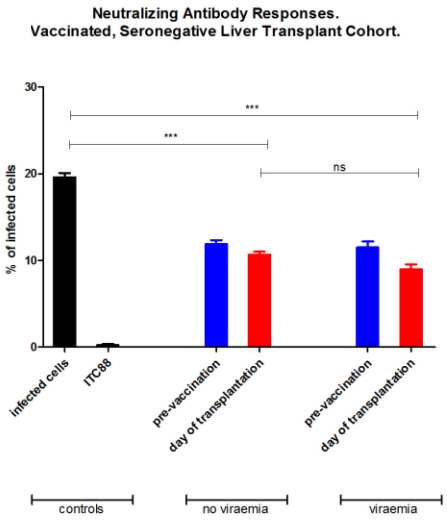

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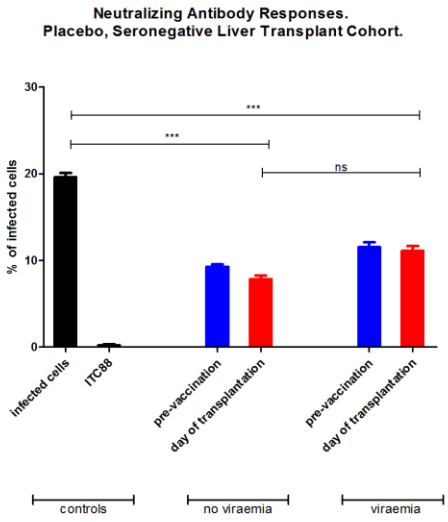
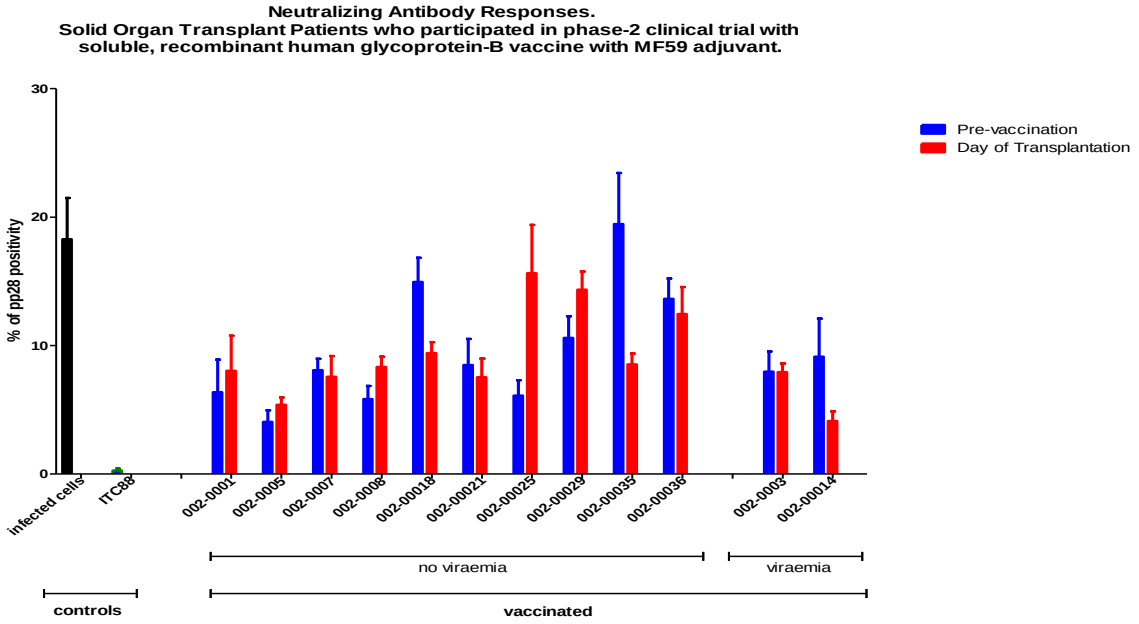


Figure 3.8. No difference in the level of neutralization (measured as a decrease in % of infectivity) between the sera isolated from seronegative transplant recipients who did and did not experience viraemia post-transplant.

Merlin was incubated with sera from seronegative renal transplant patients (vaccinated=12; placebo: n=7), sera from seronegative liver transplant patients (vaccinated=10; placebo: n=9) or an ITC88 positive control, and used to inoculate HFFs *in vitro* (MOI=1). Infection was assayed by IE immunostaining 24hpi and the proportion of infected cells calculated by counterstaining nuclei with DAPI. Sera isolated pre-vaccination (baseline – blue bars) or post vaccination (day of transplant – red bars) was tested in triplicate. Sera from patients vaccinated with gB (A,C) or placebo (B,D) are shown and are subdivided into patients who went onto display evidence of viraemia post-transplant. The differences in the level of IE positivity between patients who developed viraemia and those who were protected post-transplant were not statistically significant as p-value for differences in the level of infection at the day of transplantation were P>0.05; *: p<0.05; ***: p<0.001. Results were obtained from the Mann Whitney test.

A)



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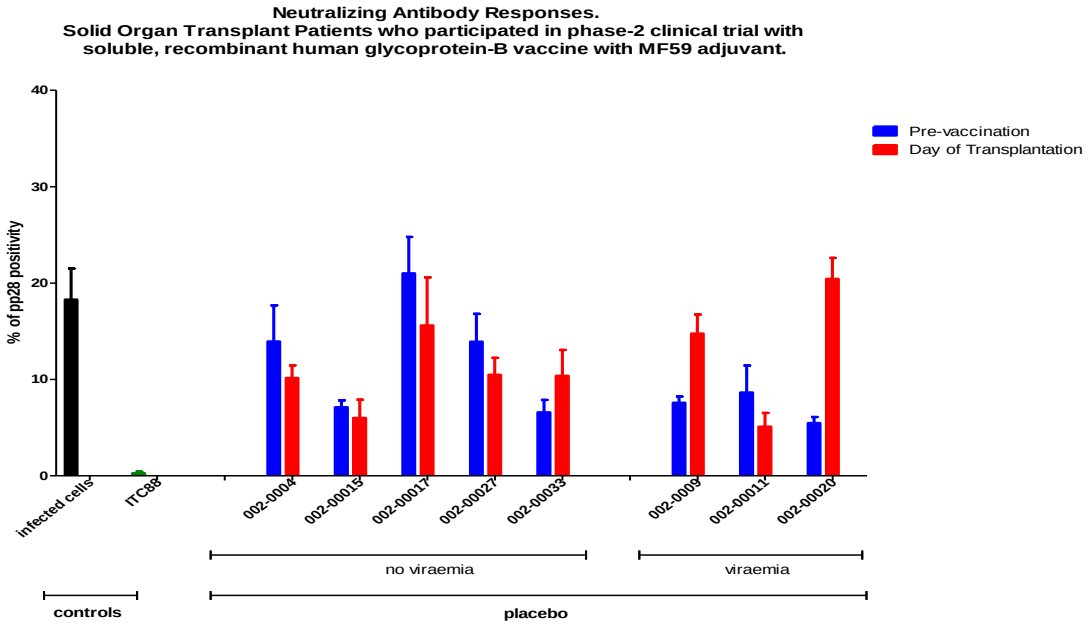
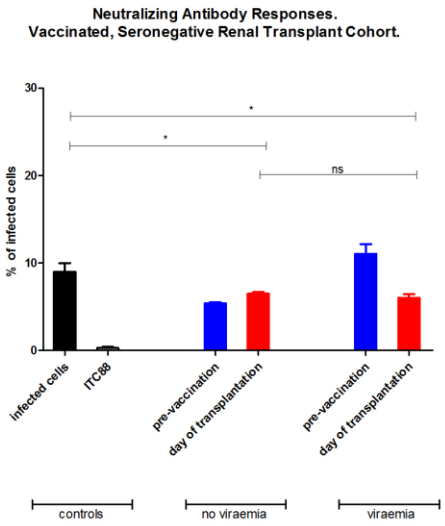


Figure 3.9. Pre-incubation of HCMV with seronegative sera generally does not reduce the detection of pp28 positive cells.

Merlin was incubated with sera from seropositive renal patients, or an ITC88 positive control, and used to inoculate HFFs *in vitro* (MOI=1). Infection was assayed by pp28 immunostaining 96hpi and the proportion of infected cells calculated by counterstaining nuclei with DAPI. Sera isolated pre-vaccination (baseline – blue bars) or post vaccination (day of transplant – red bars) was tested in triplicate. Sera from patients vaccinated with gB (A) or placebo (B) are shown and are subdivided into patients who went onto display evidence of viraemia post-transplant.

A)



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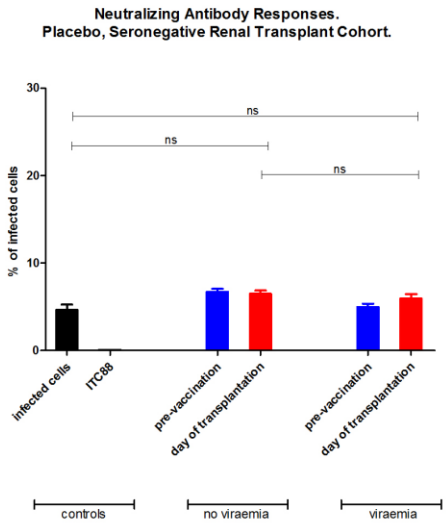

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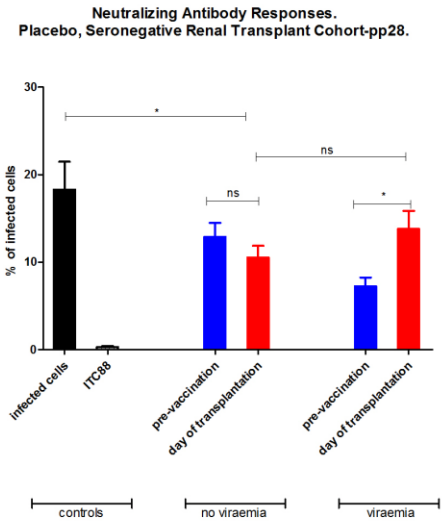

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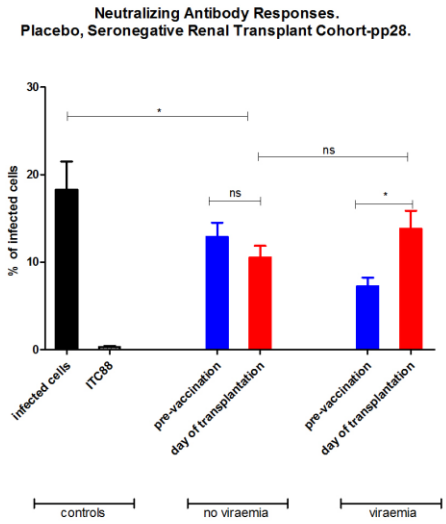


Figure 3.10. Sera isolated from seronegative renal transplant patients do not display differential activity against HCMV when IE positivity is measured or minimal differences when pp28 positivity is measured.

A) and B) represent IE staining; and C) and D) represent pp28 staining in corresponding groups of seronegative renal transplant patients. The differences in the level of IE and pp28 (vaccinated=12; placebo: n=7) positivity between patients who developed viraemia and those who were protected post-transplant were not statistically significant (at the day of transplant) as p-value for differences in the level of infection at the day of transplantation were ns: P>0.05; *. Results were obtained from the Mann Whitney test.

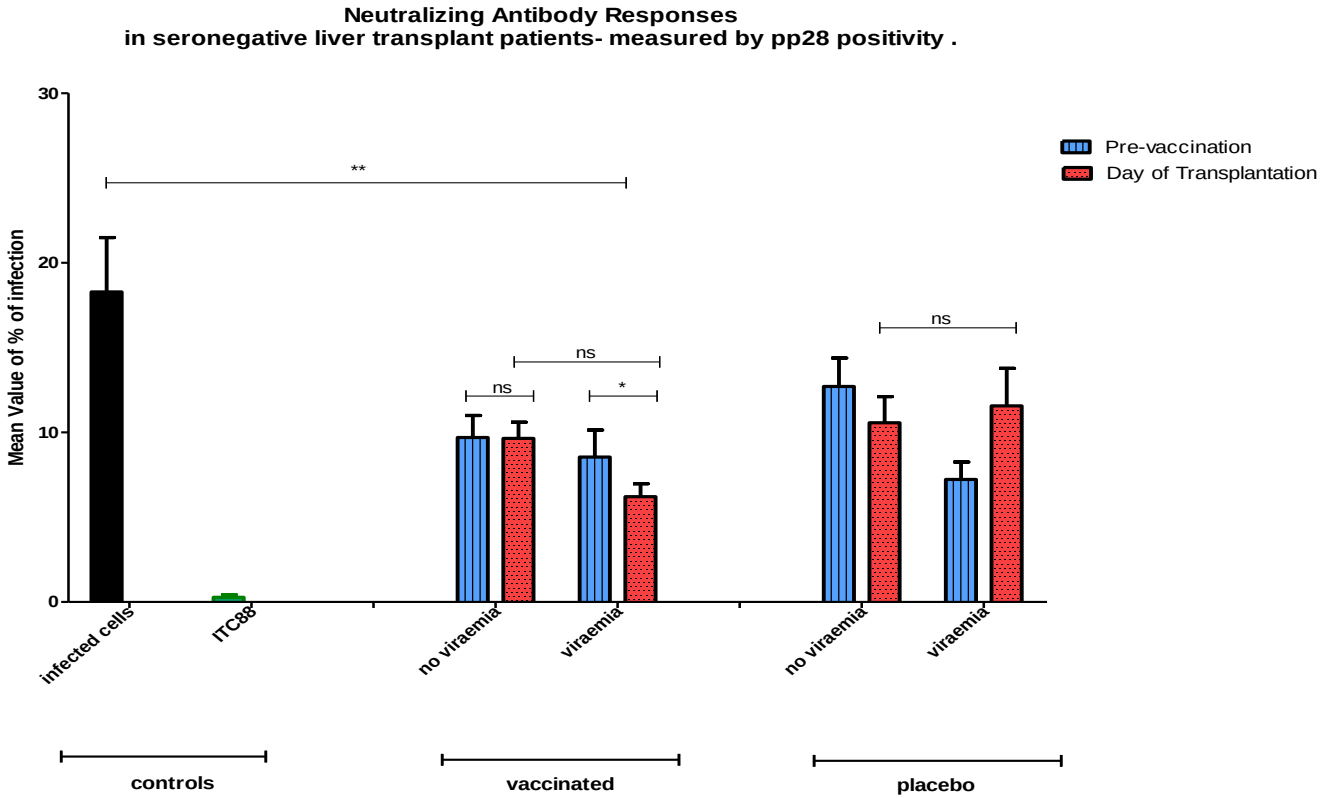


Figure 3.11. Vaccination has no significant impact on the number of pp28-positive cells post infection.

Merlin was incubated with sera from seropositive renal patients (vaccinated=12; placebo: n=7), or an ITC88 positive control, and used to inoculate HFFs *in vitro* (MOI=1). Infection was assayed by pp28 immunostaining 96hpi and the proportion of infected cells calculated by counterstaining nuclei with DAPI. Sera isolated pre-vaccination (baseline – blue bars) or post vaccination (day of transplant – red bars) was tested in triplicate. Sera from patients vaccinated with gB or placebo are subdivided into patients who went onto display evidence of viraemia post-transplant. Positivity between patients who developed viraemia and those who were protected post-transplant were not statistically significant (at the day of transplant) as p-value for differences in the level of infection at the day of transplantation were ns: P>0.05; *: p<0.05; **: p<0.01. Results were obtained from the Mann Whitney test.

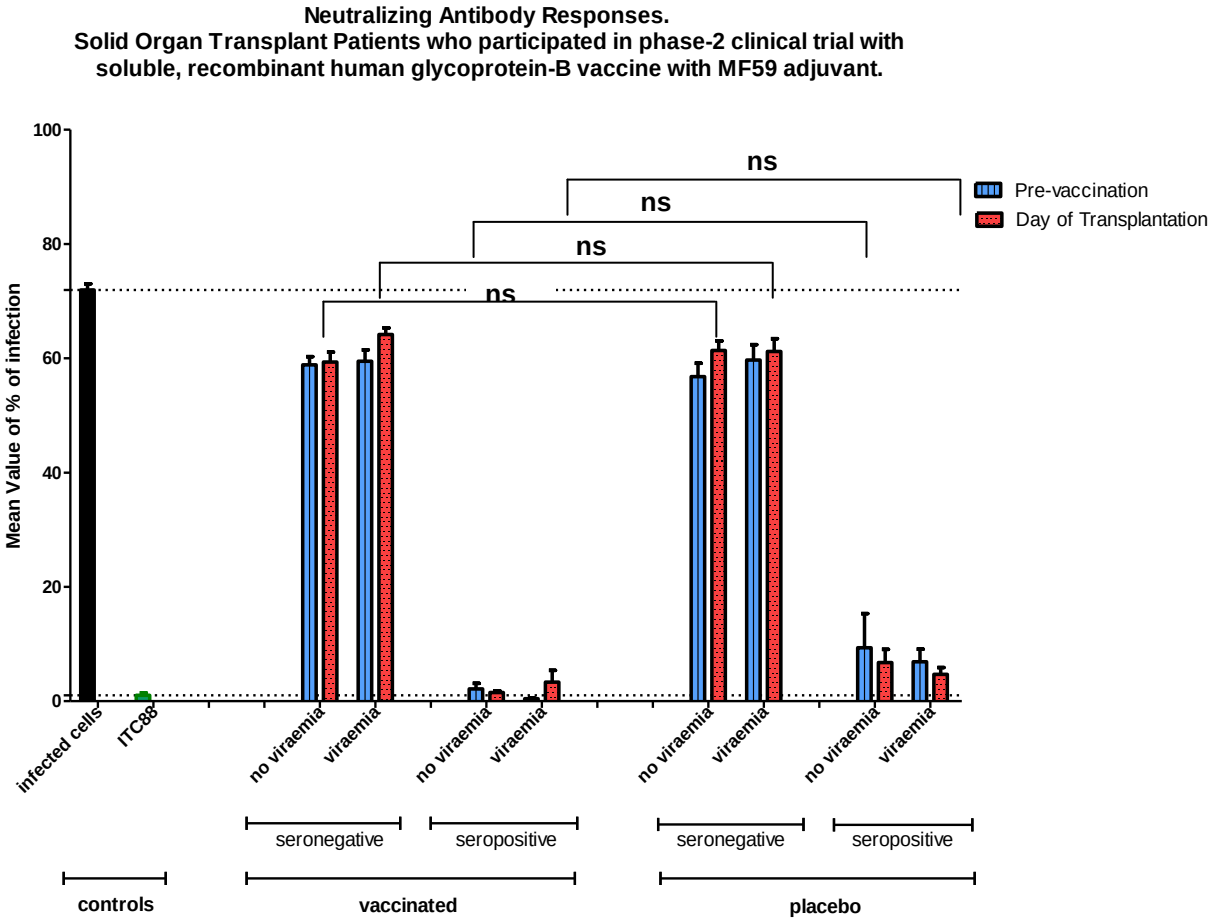


Figure 3.12. Summary of neutralizing antibody responses in Solid Organ Transplant Patients.

Merlin was incubated with sera from seropositive renal patients, or an ITC88 positive control, and used to inoculate HFFs *in vitro* (MOI=1). Infection was assayed by IE immunostaining 24hpi and the proportion of infected cells calculated by counterstaining nuclei with DAPI. Sera isolated pre-vaccination (baseline – blue bars) or post vaccination (day of transplant – red bars) was tested in triplicate. Sera from patients vaccinated with gB or placebo are subdivided into patients who went onto display evidence of viraemia post-transplant; vaccine: (seronegative patients: n=26; seropositive patients n=18); placebo (seronegative patients: n=16; seropositive patients n=24). The differences in the level of IE positivity between vaccinated and corresponding placebo groups were not statistically significant as p-value for differences in the level of infection (Vaccinated vs. Placebo) at the day of transplantation were P>0.05. seronegative, no viaremia (vaccinated vs placebo) p=0.6513; seronegative, viaremia (vaccinated vs placebo) p= 0.345; seropositive, no viraemia (vaccinated vs placebo) p=0.76; seropositive, viraemia (vaccinated vs placebo) p=0.53. Results were obtained from the Mann Whitney test.

3.4. Discussion.

Neutralization is commonly defined as the ability of antibodies to prevent infection. The mechanism can involve neutralizing antibodies binding to the pathogen and promoting the recruitment of complement to lyse the virus. Alternatively, and importantly for the assays I performed here in the absence of complement, they can also function to block the attachment of the pathogen and subsequent entry into susceptible cells. Therefore, evoking neutralizing antibodies through vaccination is highly desirable. It has been demonstrated that in the case of many licensed vaccines neutralization is the major immunological mechanism that confers protection [334, 342]. Thus I asked whether this gB/MF59 vaccine elicited neutralizing antibodies and whether the protection seen in vaccinated patients correlated with the high titres of these Nabs.

These studies support the findings published before [188]. Although the methodology (immunostaining) and materials (strain of the virus) used in these experiments were different, I now demonstrate in a separate assay that the glycoprotein-B vaccine did not evoke protective neutralizing antibody responses in seronegative vaccine recipients. Importantly, my assay utilized the wild type strain of HCMV, Merlin, which represents the HCMV strain used *in vitro* which most likely represents the virus circulating *in vivo* [329]. The proportion of infected cells following incubation of the inoculum virus with sera from vaccinated seronegative patients was very high, comparable with the negative control, cells infected with virus alone. Such a result suggests that the vaccination did not elicit potent neutralizing antibody responses that could inhibit the entry of the HCMV into the fibroblasts in these seronegative vaccine recipients.

These observations are consistent with those previously published by our group, where neutralization in sera from the same cohort of vaccinated/placebo seronegative transplant patients [188] showed only minimal level of Nabs in seronegative patients (Figure 3.1, Table 3.1). However, this is in contrast to a previous phase-1 study with seronegative children vaccinated with this soluble recombinant gB with MF59 vaccine by Mitchell *et al.* [320]. According to that report the pediatric group mounted much higher levels of neutralizing antibodies in

comparison to the level of these antibodies in naturally infected individuals and adult populations who participated in other phase-1 trials with this vaccine formulation [318, 319].

It is worth noting that these vaccinated populations are very diverse which could potentially explain some of the differences in the level of neutralization- it is well known that the immune system of young children is more efficient and able to produce much higher levels of neutralizing antibodies than the immune system of adults, and this ability maybe further decreased in generally unwell transplant populations who are aged around 50 years on average. There is a precedent, in that patients receiving renal dialysis (and so may be candidates for renal transplantation) mount poor responses to hepatitis B vaccine [346, 347].

These apparent discrepancies between previous studies and my own led me to investigate further. My original assay measured whether IE gene expression (which occurs within 3hpi) was detectable as a means to assess lytic infection. Thus we could not exclude possible effects on post-IE gene expression. Therefore, I performed additional analysis for pp28 positivity in cells infected with HCMV (Merlin) that was pre-incubated with seronegative renal transplant patients' sera obtained at time of vaccine/placebo administration and time of transplantation. This protein (pp28) is known to be expressed in late stages of viral life cycle and is indicative of successful viral replication. The aim was to formally rule out the possibility that HCMV antibodies could promote abortive viral infections which would leave cells as IE positive but unable to produce infectious virus (as measured by a plaque assay).

The results showed no difference in the expression of pp28 between vaccinated and placebo cohort and also no differences in the level of expression of pp28 between pre-vaccination and day of transplantation in vaccinated patients. These results correspond well to the levels of IE positivity which suggests that the viral particles that are not neutralized are able to undergo all phases of lytic cycle gene expression and thus likely produce new progeny which subsequently infect other cells.

In contrast to the seronegatives, the read-outs in vaccinated seropositive solid organ recipients are far more complex to analyze due to the pre-existing neutralizing antibody responses. It is interesting to note that the level of neutralization is variable amongst the seropositive patients. Although serum from the majority of patients was very effective in neutralizing the virus and preventing infection, sera from others were less efficient. However, when I analyzed the combined data from all seropositive patients only small differences were seen between placebo and vaccinated patients. Most importantly, I found little evidence to suggest that the vaccine increased the pre-existing neutralizing immune responses in seropositive patients that would be still present at the day of transplantation (when analyzed at the population level- no differences in the proportion of infected cells were observed between the day of vaccination and the time of challenge with the virus -transplantation). Anti-gB antibodies with neutralizing capacity are presumably present in most of these individuals following natural infection with the virus; however their efficiency in neutralizing the virus may differ significantly.

Furthermore, although the vaccine was based on gB, seropositive individuals may have pre-existing Nabs directed against the pentameric complex of HCMV. This complex consisting of gH/gL and UL128/130/131A is a major target of the neutralizing antibody responses due to the potency of antibodies against these antigens, shown in both human and animal studies [203, 348-350]. The pentameric complex is necessary for viral entry into endothelial, epithelial cells, dendritic cells and presumably some other cell types as well. However, the infection of fibroblasts requires only the presence of the heterotrimer gH/gL/gO [197, 351, 352]. One study demonstrated that antibodies that target the UL128/130/131A failed to block infection of fibroblasts but have exceptionally high potency in neutralizing HCMV infection of endothelial, epithelial cells and myeloid cells [201]. Nevertheless, many recent studies have demonstrated that antibodies against gH/gL are very effective in neutralizing the virus and preventing infection of fibroblasts. It has been hypothesized that gH might be ‘a universal’ antigen if anti-gH antibodies could effectively interfere with binding to the conformational epitopes on the pentameric complex and inhibit the infection of epithelial and endothelial cells as well as inhibit the infection of fibroblasts [201]. In my assays,

where I have not used complement, it is likely that in the seropositives, the presence of antibodies against gB and the gH/gL/gO trimer are likely mediating the effects we are observing given the importance of these glycoproteins for HCMV entry into fibroblasts [203, 350, 353, 354].

It is likely that effective neutralization seen in some seropositive individuals could be due to antibodies targeted against antigens other than gB (*e.g.:* gH). To test this hypothesis I would incubate the sera with the gB vaccine protein prior to the neutralization assay in order to deplete the anti-gB antibodies from seropositive sera: if under these conditions no impact on the ability of sera to neutralize HCMV infection was observed it would provide further supporting evidence that the vaccine induced gB responses are not important for the neutralization phenotype we observe *in vitro.*

Our analyses of seropositive sera illustrate that it is possible to boost neutralizing antibodies directed against HCMV using our assay. Thus it remains difficult to explain why the vaccination failed to induce potent neutralizing antibody responses in seronegative individuals. One well-known mechanism that is utilized by many viruses is the glycosylation of the highly protective antigenic domains and epitopes and shielding them from our immune system (preventing antibody binding due to steric hindrance). This common strategy of immune-evasion was noticed and well described in other viruses, such as: influenza [355] and HIV [356, 357]. The recently published crystal structure of HCMV gB gives us a first insight on how the antigenic domains might be presented on this protein [358]. Based on this crystal structure it became clear that the antibody domains: AD4, AD5 and site 1 of AD2 that are believed to elicit potent neutralizing responses are heavily glycosylated, which is in contrast to the ADs that do not elicit potent neutralizing antibody responses- especially AD1 [158, 161, 191] (described in chapter 6). As mentioned before, this recombinant vaccine protein was expressed in mammalian cell line (CHO). Therefore the patterns of glycosylation on this recombinant gB should remain similar to those observed with the native protein. Consequently, future experiments should investigate whether the protective antigenic domains that are present on this recombinant vaccine protein are also heavily glycosylated, in a similar manner to those described on the crystalized gB. Such a strategy

would impede the immune surveillance of the host and could, at least partly, explain poor neutralizing antibody responses in these seronegative vaccine recipients.

Other documented ways to evade the immune system of the host include:
-selective exposure of domains to prevent recognition of protective epitopes and subsequent binding of potent neutralizing antibodies during entry (strategy used by HIV, reviewed elsewhere [359]);
-using sequential multiple receptors that shorten the exposure of protective epitopes (*e.g.*: HCV);
-virus-specific neutralizing antibody- driven sequence evolution of glycoproteins (observed during acute HCV infection *in vivo* [360]).

Alternatively, antibodies that are able to effectively neutralize virus might be targeting only the pre-fusion form of this protein and be partly/completely inefficient in neutralizing the post-fusion form. Indeed, it is possible that the vaccine antigen is presented as the post-fusion form of this protein as the modifications that were introduced to this vaccine antigen in order to increase stability and solubility, as well as facilitate expression in CHO cells, were very similar to the modifications reported on the crystallized gB protein structure which represents the post-fusion state [358]. Therefore, the antibody responses that were elicited or boosted by vaccination may have decreased efficacy in comparison to those that were elicited following natural infection as only the post-fusion form of the protein would be exposed to the immune system of seronegative, vaccinated patients. If this holds true, then this could potentially explain poor responses of the seronegative patients towards these antigenic domains although the overall antibody response to gB does not appear to be impaired [188]. It remains to be further investigated to what extent the virus presents the pre-fusion form of this antigen to the immune system and whether the exposure of antigenic domains from the post- fusion state might be an immune-evasive strategy used by the virus to effectively avoid protective immunological responses.

To conclude: in the case of this vaccine antigen (recombinant gB protein) it is difficult to predict the actual conformation of this protein, how known antigenic domains are presented on this recombinant gB and the potential differences from the native protein. Without having a resolved crystal structure of this recombinant protein it is impossible to predict the accuracy of the presentation of the antigenic domains. However, potential alterations of the structure and antigen presentation of this recombinant protein (in comparison to the native one) could, at least partly, explain why the vaccine induced only minimal neutralizing antibody responses in these seronegative patients.

One of the biggest limitations of this study is the small number of analyzed samples which has an impact on the power of statistical analyses. Although there were some statistically significant differences detected in the assay between the day of vaccination and the day of transplantation in the groups of placebo seronegative and placebo seropositive patients (figure 3.12) the confirmation of biological relevance would require the study of further samples.

Nevertheless, although classically neutralisation has been thought to be the major mechanism that protects vaccinated individuals, recent data published by many groups suggest that non-neutralizing antibodies (nNab) that do not possess neutralizing activity *in vitro* may be crucial in conferring protection against pathogens *in vivo* [342]. The dogma that the induction of a potent neutralizing antibody response is the pre-requisite of protective immunity following vaccination has been challenged by data published recently on HIV-1. These results suggest that other functional, but non-neutralizing, antibodies may also play an important role and contribute to the protection afforded by vaccines against the pathogen [342, 343]. A pharmacodynamic assessment of sera from the patients who participated in the RV144 HIV vaccine trial demonstrated the presence of antibodies that bind to the HIV envelope protein whereas, in contrast, the cytotoxic T cell responses evoked in the vaccinated patients were weak. Although this was consistent with the humoral response being important for protection, in-depth analysis of the clinical material revealed that the antibodies that were elicited by the vaccine failed to neutralize varied strains of this virus- and thus did not possess broadly neutralizing activity [342, 361] leading to the hypothesis that the

modest efficacy seen in this clinical trial was afforded by other immune responses, either alone or in combination with neutralizing antibodies [362].

In order to investigate this concept of non-neutralising antibodies further, detailed analysis of the robust antibody response generated by this HIV vaccine was performed. These data revealed subtle differences in the immunoglobulin subclasses amongst the patients. Specifically, there was an increase in the level of IgG3 in vaccine recipients and an inverse correlation between the IgG3-mediated non-neutralising antibodies (nNab) against the first and the second variable domain (V1 and V2) of the surface glycoprotein envelope (Env) and the risk for HIV acquisition. However this was a short-term effect and the level of IgG3 decreased rapidly after administration of the vaccine [363-365]. The IgG3 antibody subclass is well known to have a high affinity for the Fcγ receptor and IgG3 can fix complement. It has already been shown that IgG3 had a role in mediating protection against some pathogens [366] [367].Thus, it was postulated that these binding, but non-neutralizing, antibodies were capable of inhibiting the transmission of the virus and decreasing infectivity via alternative mechanisms, e.g. using effector cells to kill infected cells such as Antibody Dependent Cellular Cytotoxicity (ADCC), Antibody Dependent Cellular Phagocytosis (ADCP) or opsonisation [368, 369]. Moreover, a direct correlation between IgA-mediated antibodies and increased risk for infection with the virus was reported. Follow-up studies of humoral responses showed that ADCC was inversely correlated with risk when the plasma IgA Env-specific antibodies were at low level [370] [371, 372].Thus, these studies of immune correlates argue that functional antibodies that do not possess neutralizing abilities in vitro might still contribute to protection following administration of the vaccine [342, 343].

To conclude, my data, supported by findings published before [188], provides no strong evidence that a potent neutralising antibody response is responsible for mediating the protection afforded by this vaccine. Consequently, this suggests that additional humoral effector functions are important.

4. Antibody mediated inhibition of viral spread.

4.1. Introduction.

Human cytomegalovirus infection produces cell-free and cell associated virus. It can be hypothesised that viral spread in cultures could be inhibited by humoral responses through three distinct mechanisms [373]:

-by direct neutralization of cell-free virus,
-by interruption of cell-to-cell transmission of the virus,
-by the induction of antibody effector functions that eliminate virally infected cells.

It is hypothesised that a large proportion of HCMV *in vivo* is disseminated by the transmission of cell associated virus (Figure 4.1) [195]. Arguably, virus that transmits from cell to cell could be effectively hidden from neutralising antibody responses providing further support for a role for alternative mechanisms of the humoral response being important for controlling HCMV *in vivo*. In 1992 Navarro β galacatosidase reported for the first time that monoclonal anti-gB antibodies that were able to neutralize cell-free herpes simplex virus in a complement independent manner were also capable of blocking the cell-to-cell spread of infection in HSV [374]. Parallel studies on humoral responses against HCMV gB found that a vast majority of antibodies that exerted very potent neutralizing activities in *in vitro* assays, some of them were also able to block dissemination of the virus from cell-to-cell [175]. Follow up studies by another group showed that the ability of antibodies to block the spread of HCMV was not correlated with their neutralizing capacities [375]. In contrast to these observations, a further study suggested that sera from seropositive HSC transplant patients failed to block spread of the virus [376] suggesting that *in vivo* observations may not reflect data obtained *in vitro*. Moreover, more recent detailed analysis of a panel of monoclonal anti-gB antibodies and CMV-Ig demonstrated that they failed to control the spread of the virus in epithelial and fibroblast models *in vitro* [373, 377]. Although the reasons for these discrepancies are unknown, it is clear that the

understanding of viral cell-to-cell spread and the immune responses of infected individuals that target this route of viral transmission are very important.

In order to model the impact of the sera on the long term culture of HCMV *in vitro* a viral spread assay was developed. In principle, HFFs are infected at a low MOI (0.05) and then the growth of the virus is monitored over time. As the virus replicates it will infect an increasing number of cells with each replication cycle I can assess the level of infection by monitoring plaque formation or use IE immunofluorescence to quantify more precisely the number of infected cells. Furthermore, I can manipulate the growth kinetics of the HCMV strains via genetic modification. Recent studies have shown that genetically wild type HCMV that encodes a functional pentameric complex (the pentameric complex comprising the products of 5 genes: gH/gL, UL128-131A that are required for entry into non-fibroblast cells [174]) exhibits a greater percentage of cell associated virus [194]. Under laboratory conditions, growth in HFFs selects for mutations in this region that favours production of cell free virus. To control for this, I used genetically modified Merlin-IE2 GFP (4.4.2) that grows mostly as cell associated virus unless the virus is grown in specialised HFFs expressing the Tet repressor protein.

Objectives:

The objectives for this chapter were to investigate whether sera from the patients who participated in this clinical trial (described in chapter 1.16) possess antibodies that can effectively inhibit the spread of the virus from cell-to-cell.

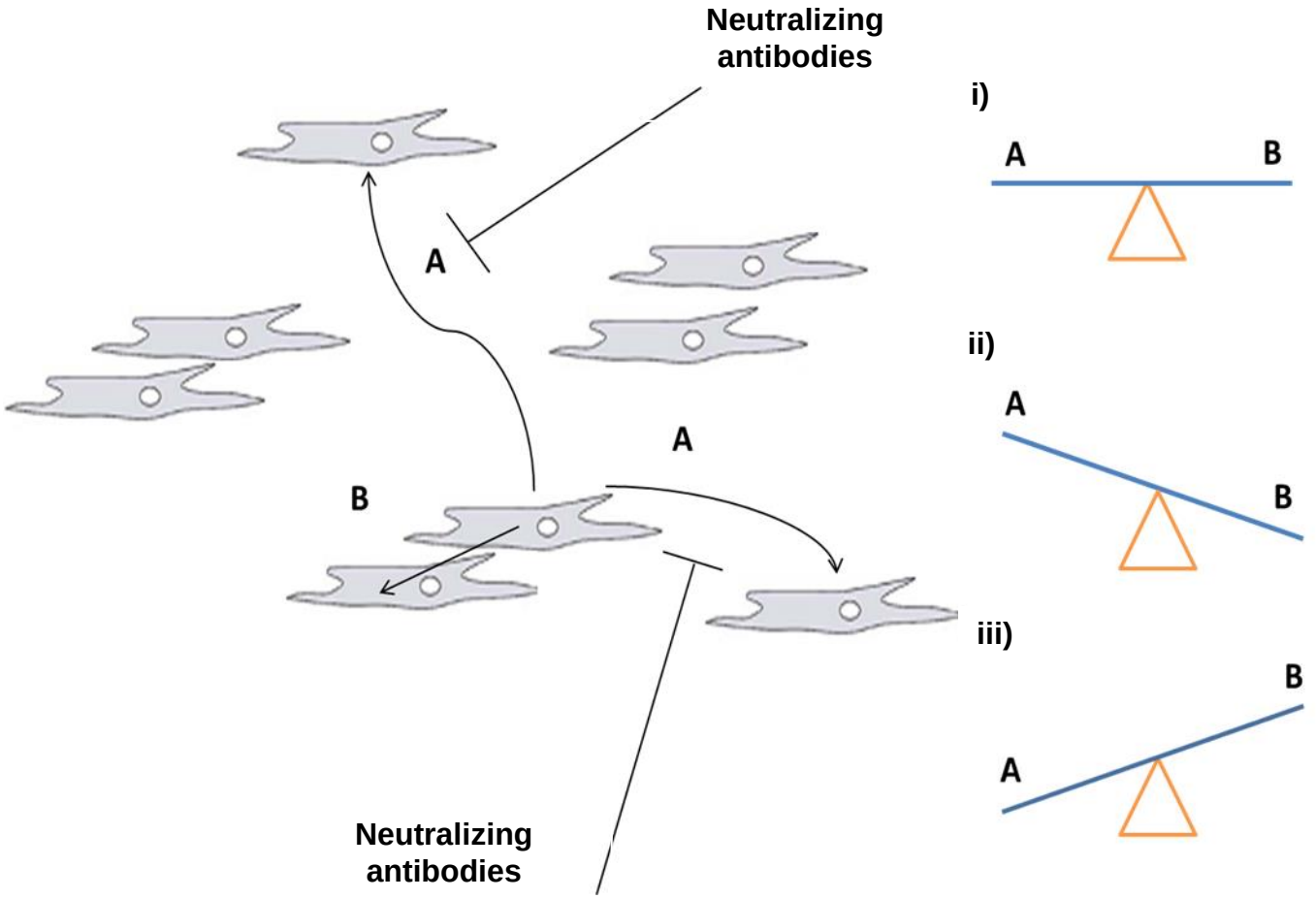


Figure 4.1. Schematic representation of possible routes of HCMV infection.

A) via the production of extracellular virus which can then infect new cells, B) direct spread of the virus from cell to cell. The clinical isolates use both routes of infection and this is represented by: i) and iii) scenario; laboratory adapted strains are predominantly the cell-free, represented by: ii) scenario.

4.2. Materials and Methods.

4.2.1. Patient population.

The serum samples from SOT patients (chapter 1.16) were examined in these novel viral spread assays. Samples that were tested in this experiment were obtained from the patients at the day of first vaccine (seronegative patients: n=25; seropositive patients n=17) or placebo (seronegative patients: n=14; seropositive patients n=23) administration and the corresponding samples collected on the day of transplantation (equivalent to challenge with the virus).

4.2.2. Viruses.

Two viruses were used for these analyses. Firstly, the Merlin clinical isolate was used (a gift of R. Stanton, University of Cardiff) – this virus is considered the reference strain in HCMV [329]; (described in chapter 2.6). The Merlin strain, when extensively passaged through fibroblasts, will grow as a cell free virus due to an accumulation of mutations in the UL128-131 locus of the virus. Therefore, a second strain of Merlin was used. This virus was also Merlin but contained two key features that allowed me to use and analyse it as a cell associated virus: (an IE2-GFP virus) which was less accessible to neutralizing antibody mediated responses (Figure 4.1) [174].

To allow analyses of cell associated virus in real time we used an IE2-GFP virus that was constructed in the Merlin backbone (a gift of Dr R. Stanton, University of Cardiff). Importantly, this virus contains a Tet repressor sequence upstream of the UL128 promoter. The UL128 gene along with gH/gL/UL130 and UL131A forms the pentameric complex that is required for the infection of epithelial and endothelial cell types [174]. However, the expression of the pentameric complex in HFFs is also considered to block the release of cell-free virus. Thus, infection of HFFs with a virus expressing wild type pentamer will result in a predominantly cell associated infection. Importantly, the inclusion of the Tet repressor allows the growth of the virus as a cell free virus through propagation of the virus in HFF-Tet expressing cells and prevents the selective pressure that forces the mutation of this region

when grown *in vitro.* This generates a virus that is phenotypically a lab strain (due to a lack of pentamer) but retains the genetic sequence of wild type Merlin in the UL128-131A locus. However, infection of normal HFFs will allow the expression of the UL128-131A locus which (due to no pressure to mutate in culture) is essentially wild type in sequence at this locus. This results in a highly cell-associated viral infection. Additionally, the presence of the GFP-tag allows tracking of infection in real time (Figure 4.2). Thus we now have a system to assess the impact of serum on cell free and cell associated viral spread in my assays.

To propagate cell free stocks of IE2-GFP virus it was cultured in HFF-tet cells for 12-14 days. Briefly, the HFF-tet cells were infected with Merlin IE2-GFP at low MOI (0.25) and incubated at 37°C for 24h in order to establish an infection. Following this incubation period the media was discarded and replenished with fresh media. Approximately 5 days post infection, the supernatant containing cell-free virus was aspirated and replenished with fresh media. Once cytopathic effect (CPE) was observed (using confocal microscopy), usually 10-12 days post infection, the supernatant containing cell-free virus was collected, stored in aliquots and fresh media added to the culture. This step was repeated four times (every second day following day 10 post infection). All the viral stocks were stored in -78°C. In order to assess the concentration of the virus the $TCID_{50}$ value (the tissue culture infectious dose which will infect 50% of the cell monolayers challenged with the defined inoculum) was determined.

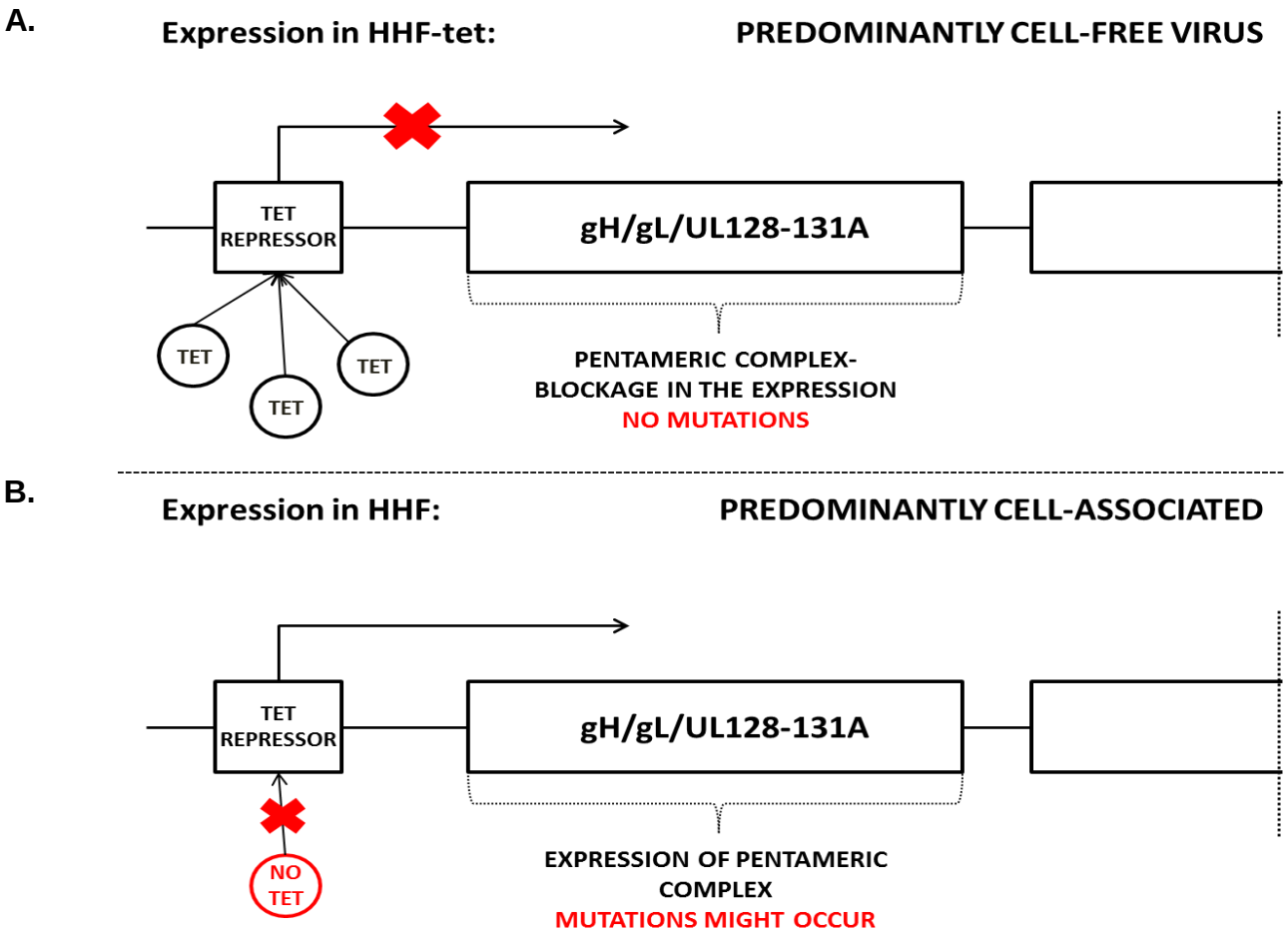


Figure 4.2. Schematic representation of the genome expression of IE2-GFP tagged virus.

A) Expression of viral genome of predominantly cell-free virus in HFF that constitutively express tet protein, the pentameric complex is not expressed, the virus remains genetically wild-type. B) Expression of viral genome of predominantly cell-associated virus in HFF (lack of tet expression), the pentameric complex is expressed, and due to the selective pressure the mutations in this region might occur.

4.2.3. Viral spread Assay.

Confluent HFF cells were detached from the surface of culture flasks by addition of trypsin (0.25% Trypsin-EDTA (IX) Gibco by Life Technologies). The cell suspension was centrifuged at 700g for 5 min, trypsin and medium were aspirated and the cellular pellet was re-suspended in fresh DMEM (supplemented with 10% FBS, 5% pen/strep) media. Cells were plated at the density of 10^4 HFFs/well (96-well plate format).

All patient serum samples were heat inactivated (1h at 56°C) prior to use and diluted at 1:10 in DMEM media (with 10% FCS, 5% pen/strep). 30µl of each heat inactivated serum sample was added to 270µl of media and stored in 100µl aliquots at -78°C prior to analysis.

The HFF cells were then infected with Merlin IE2-GFP at low MOI (0.05) or low passage Merlin strain at low MOI (0.05) and incubated at 37°C for 24h in order to establish an infection. Following this incubation period the cells were incubated in fresh DMEM and supplemented as described in results with serum or antibodies. Routinely, serum samples and antibodies were added 24hpi and replenished 7days post infection. Cells were fixed with 2% PFA (paraformaldehyde, Sigma-Aldrich) solution in PBS prior to automated numeration of infected cells.

4.2.4. Cell imaging and analysis of the results.

Cells were visualised and counted by WiScan® 3.4 cell-imaging system described in 2.8. 20 images were captured per well and the average proportion of infection was calculated. Each experimental condition was repeated three times, and the mean value was calculated from the percentage of infected cells. These mean values are represented on the figures: 4.4- 4.12.

4.3. Results.

4.3.1. Establishment of the viral spread assay.

To investigate the bioactivity of the sera against HCMV undergoing multiple cycles of replication and infection I established a viral spread assay. The data in Figure 4.3 show a typical assay with Merlin HCMV set up in multiple wells to allow IE staining at different time-points of culture. An analysis at 24hpi reveals very few cells are IE positive consistent with a low multiplicity of infection. However, as the infection proceeds we see an increase in IE positive cells in the cultures over time. By 14dpi I observed a substantial increase in the number of infected cells which reflects multiple rounds of viral replication and infection.

4.3.2. Experimental validation of the assay with low passage Merlin strain.

I next asked whether a neutralizing antibody could block the spread of low passage Merlin HCMV in this assay. We tested the anti-gB antibody ITC88 at a concentration where we observed significant neutralization previously (100µg/ml). As shown before, fibroblasts infected with HCMV showed high levels of IE positivity at 14 dpi consistent with the spread of virus in the culture. In contrast, addition of the ITC88 antibody reduced the mean value of the percentage of infection in the cells incubated with the antibody in comparison to the infection control (Figure 4.4). Next we tested whether sera could also block viral spread in this assay. To do this, serum from a healthy HCMV seropositive donor was diluted at 1:10, 1:100 and 1:1000. This study showed that serum from a healthy seropositive could significantly reduce the number of infected cells when used at a 1:10 dilution – an effect that could be diluted so that no effect was seen with the 1:1000 dilution. Importantly, this effect was specific to seropositive serum as parallel analysis of serum from a seronegative healthy donor revealed that no control on viral spread was exerted (Figure 4.5).

4.3.3. Experimental validation of the assay with the genetically engineered GFP tagged Merlin strain.

The low passage Merlin virus that was used in these initial analyses is able to infect the cells via at least two different mechanisms (described in chapter 4.1), (Figures 4.1 and 4.2). Therefore we next investigated the effect of the sera and antibodies on the growth of Merlin strain (Merlin IE2-GFP) engineered to grow predominantly via cell to cell spread. Firstly, two neutralising antibodies (described in chapter 3): 2F12 and ITC88 were tested at different concentrations: 100μg/ml; 10μg/ml, 1 μg/ml. These antibodies are known to have a very good neutralizing activity against gB in *in vitro* assays including my own (Figure 3.1B); (described in chapter 3). Additionally, antibody ITC88 proved to be highly effective in inhibition of the low passage Merlin strain at high concentration in my viral spread assay (described in section 4.3.1.).

I first asked whether neutralising antibodies ITC88 and 2F12 could block the spread of cell-associated GFP-tagged Merlin. The data show that the percentage of infection (as measured by IE2-GFP positivity) was not significantly affected by the antibodies (Figure 4.6). Specifically, the antibody 2F12 exhibited no effect on the level of infection at the lowest dose -1 μg/ml a result comparable with the negative control-cells infected with the virus only. Although at higher doses (100μg/ml 10 μg/ml) some block to spread was observed. Similarly, ITC88 also failed to inhibit spread of the virus at the lower doses, but caused some decline in the percentage of infected cells at the highest dose: 100μg/ml. Although both antibodies appeared to be inefficient in blocking the spread of the cell associated GFP tagged virus, some degree of protection was observed and this effect was dose dependent (Figure 4.6) but, importantly, far less effective than observed against Merlin (Figure 4.4).

Next, the sera from healthy donors were tested in this viral spread assay with the GFP tagged Merlin strain with serum from a seronegative donor used as a negative control for the assay. As observed with the monoclonal antibodies, a decline in the percentage of infected cells following incubation with sera from

healthy individual was observed although not as high as in the assay with low passage Merlin (Figure 4.7). Although the sera diluted at 1/5 inhibited the spread of the virus quite efficiently, the dilution of 1/100 did not have any impact on the inhibition of the GFP-tagged Merlin. Sera from a seropositive donor diluted at 1/10 had a moderate effect on the inhibition of the spread of the virus (Figure 4.7), but again the decrease in the percentage of infected cells was less prominent than observed with the low passage Merlin strain (Figure 4.5).

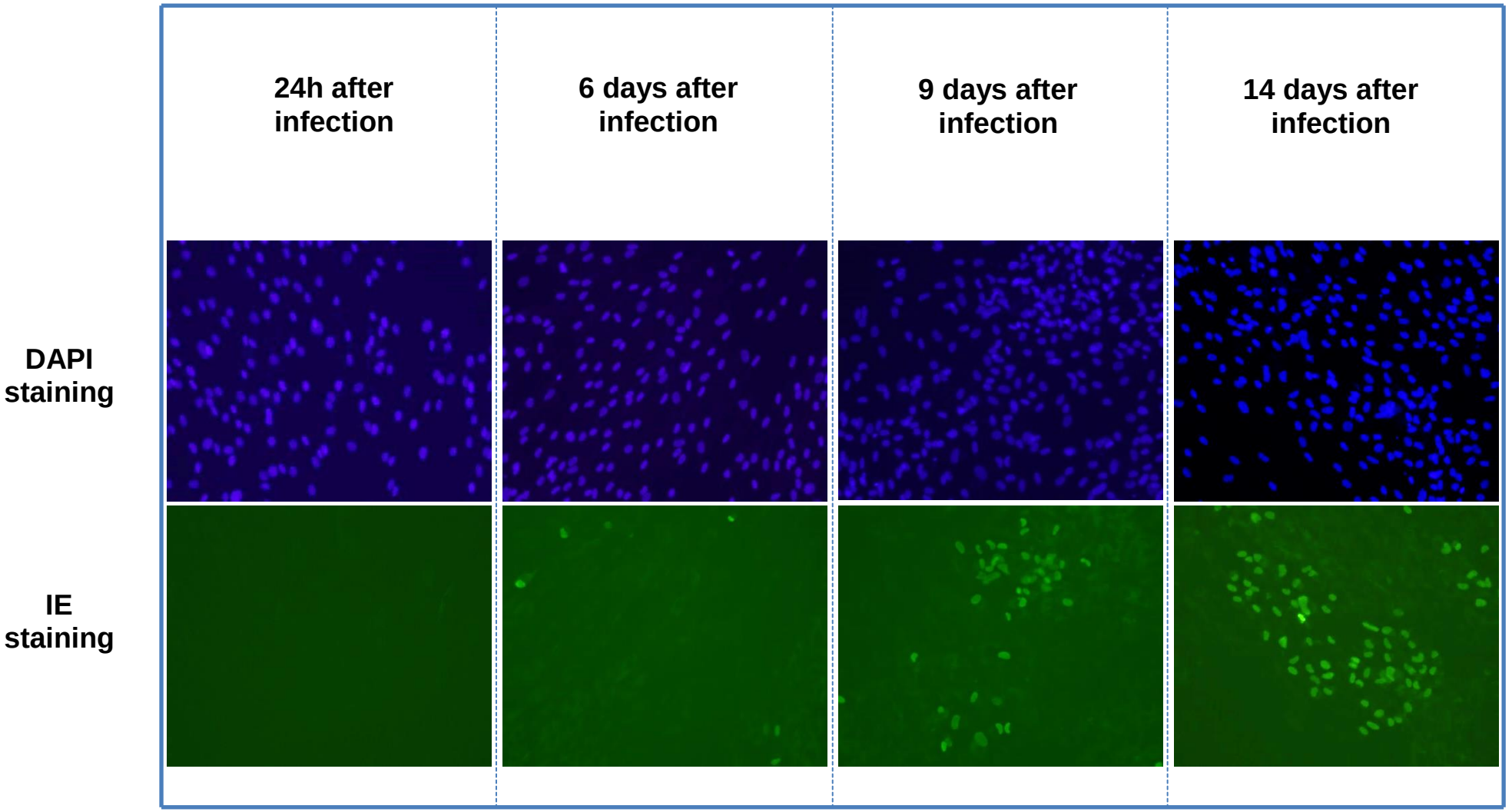


Figure 4.3. Example of the spread of the low passage strain of HCMV virus-Merlin thought the cell culture (HFFs).
Lower panel: cells were stained for IE positivity 24h, 6, 9, and 14 days after the infection. Upper panel: nuclear staining-DAPI.

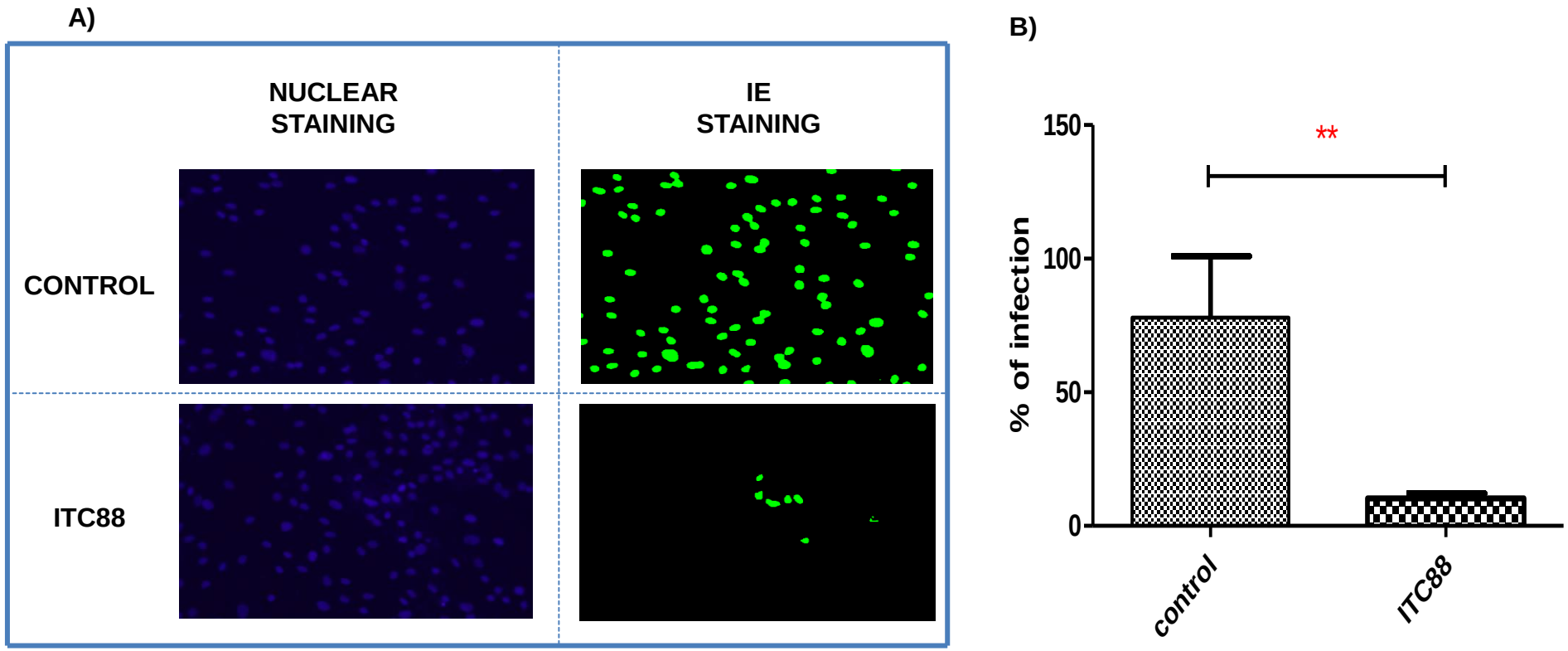


Figure 4.4. The neutralizing antibody ITC88 blocked spread of the virus in in vitro assays.

A) Immunostaining of the cells infected with the virus (Merlin) and incubated with the commercially available anti-gB ITC88 at the concentration: 100 µg/ml, 14 days post infection. The left panel represents DAPI (nuclear) staining and the right panel represents IE staining B) Testing the commercially available anti-gB ITC88 at the concentration 100µg/ml. The control is fibroblasts infected with the virus (Merlin). The mean values of the percentage of infection are presented with the error bars indicating the standard deviation. Statistical significance obtained from the Mann Whitney test, p value=0.008.

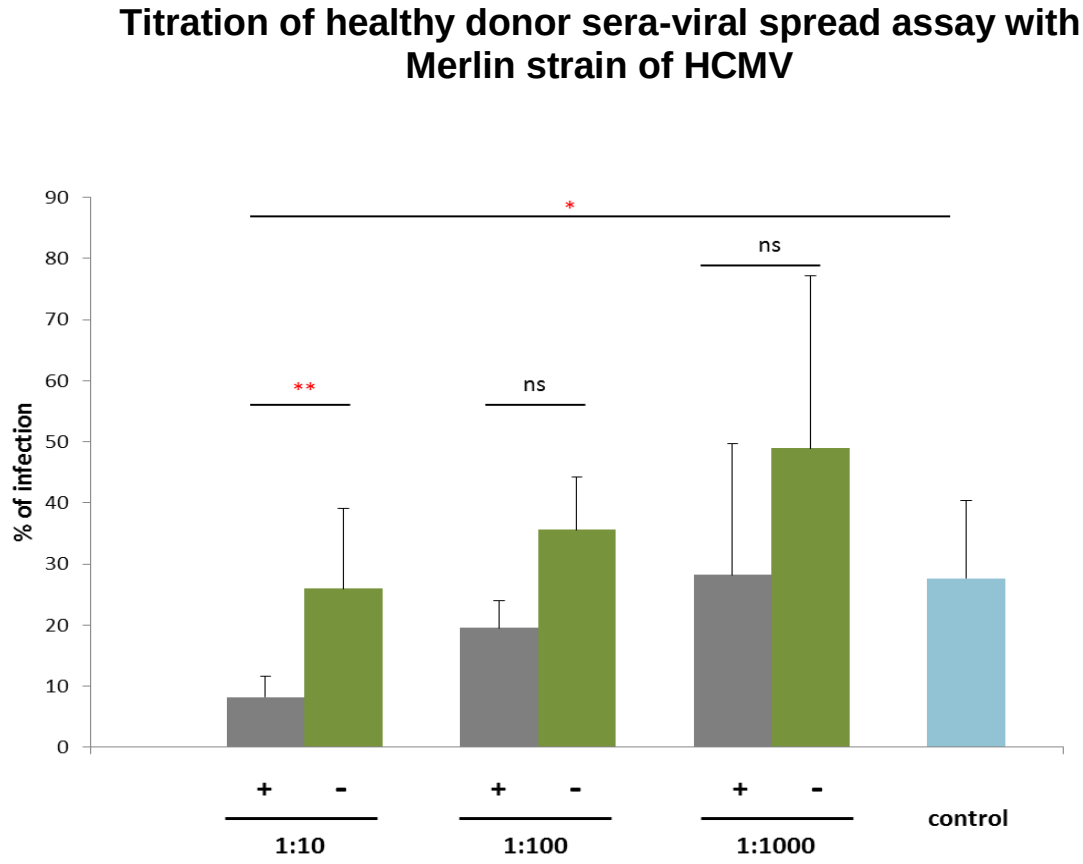


Figure 4.5. Serum from a healthy HCMV seropositive donor blocks spread of the Merlin strain of the virus.

HFF cells were infected with low passage HCMV strain-Merlin and the proportion of infection was calculated based on IE positivity 14 days post infection. Serum was diluted: 1/10, 1/100 and 1/1000. Control was infected cells only. The mean values of the percentage of infection are presented with error bars indicating the standard deviation. Statistical significance was obtained from the Mann Whitney test, ns: p value>0.05; **: p=0.004; *: p=0.018.

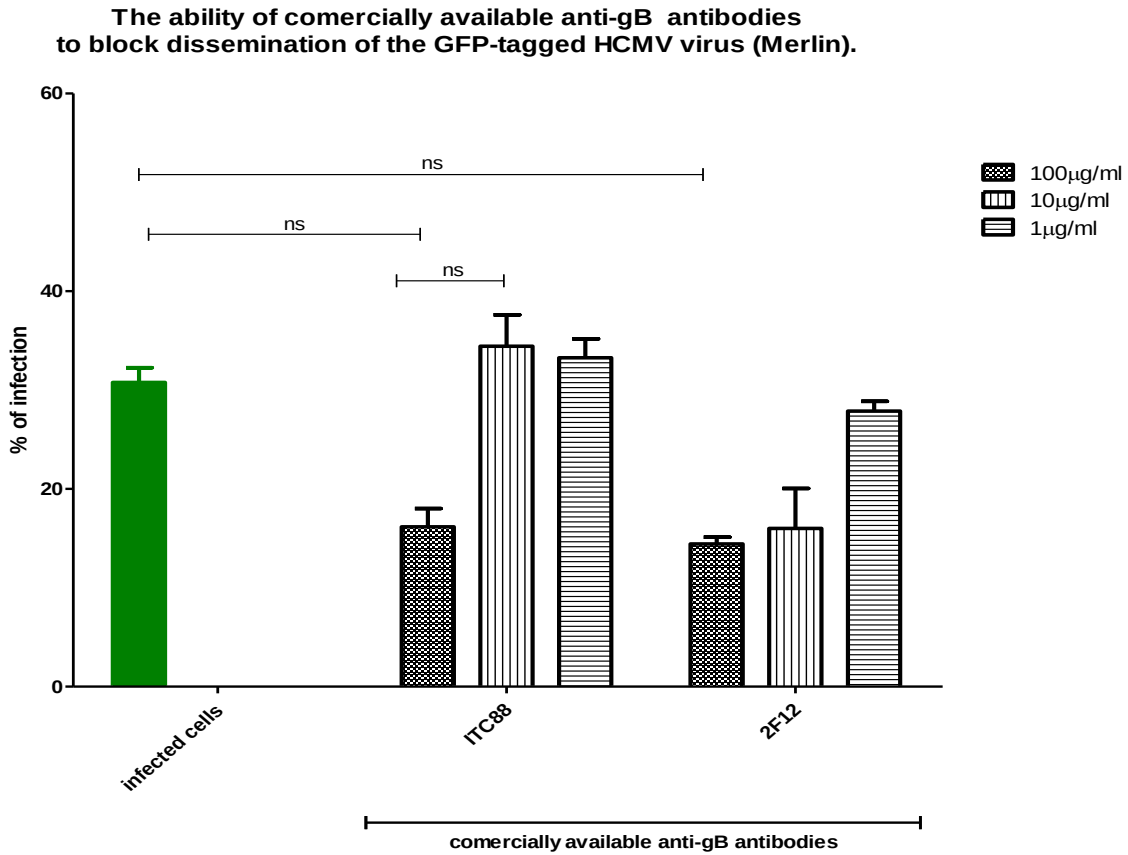


Figure 4.6. Neutralising anti-gB antibodies 2F12 and ITC88 have only limited ability to block the dissemination of the GFP-tagged HCMV virus (Merlin).

The antibodies were used at the concentrations: 100 μg/ml 10 μg/ml, 1 μg/ml. The negative control is fibroblasts infected with the GFP-tagged HCMV virus. The mean values of the percentage of infection are presented with the error bars indicating the standard deviation. Statistical differences –two tailed p-values were obtained from the Mann-Whitney U test; ns: $p>0.05$.

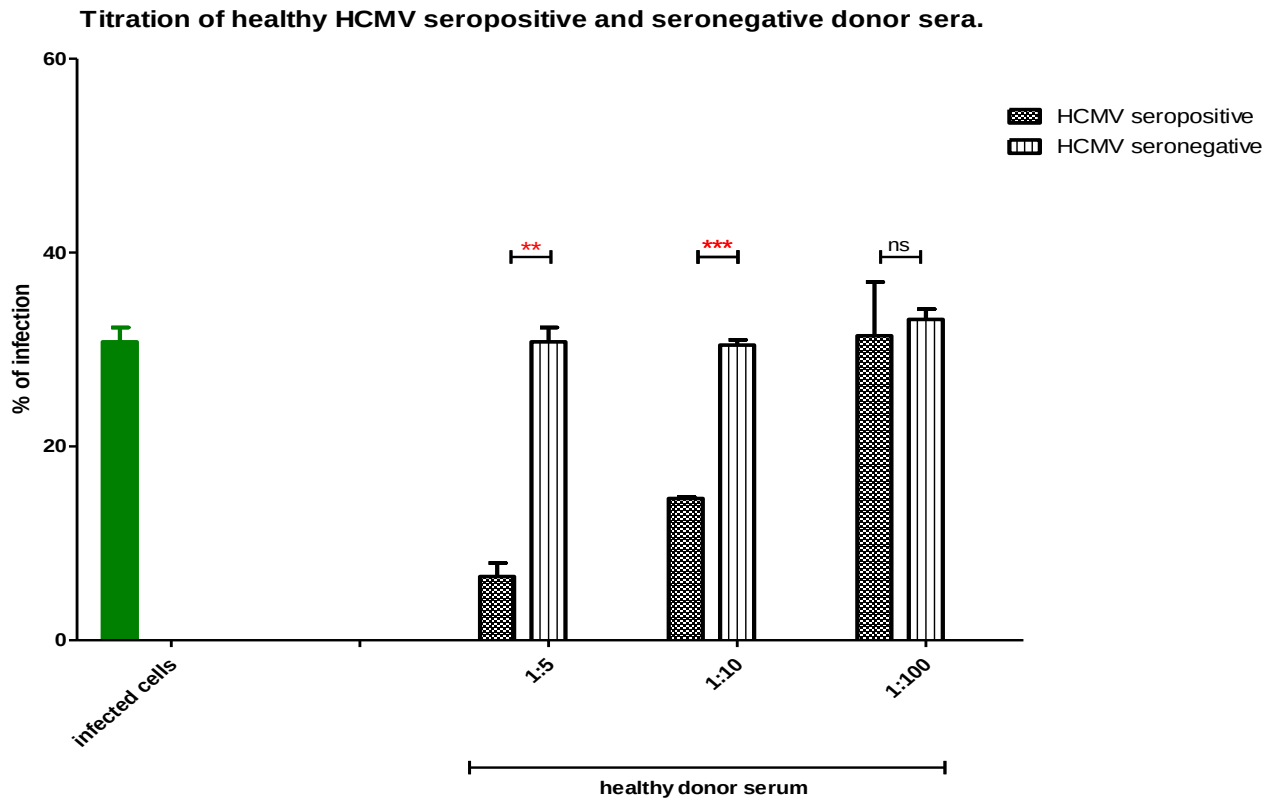


Figure 4.7. Serum from a healthy HCMV seroposive donor can block spread of the virus at low dilutions.

The serum samples from HCMV seropositive and seronegative individuals were used at the dilutions: 1/5; 1/10 and 1/100 to block dissemination of the GFP-tagged Merlin. The negative control is fibroblasts infected with the virus- GFP-tagged Merlin. The mean values of the percentage of infection are presented with the error bars indicating the standard deviation. Statistical differences –two tailed p-values were obtained from the Mann-Whitney U test; ns: p=0.83; **: p=0.007; ***: p=0.0004.

4.3.4. Testing capability of sera from the patients in the gB/MF59 clinical trial to inhibit spread of the virus though cell culture.

Having established an assay I next tested the sera from patients who participated in the gB/MF59 trial (described in section: 1.11). The samples that were analyzed in this assay were collected on the day of first vaccine (seronegative patients: n=25; seropositive patients n=17) or placebo (seronegative patients: n=14; seropositive patients n=23) administration and their corresponding samples obtained at the day of the transplantation- time of the challenge with the virus. Controls for this assay were cells infected with the GFP-tagged HCMV virus (Merlin) to allow us to establish a baseline for the spread of HCMV. To control for non-specific serum effects, cells infected with the GFP-tagged HCMV virus (Merlin) were incubated with serum from a HCMV seronegative healthy individual diluted 1/10. As a positive control for these assays, cells infected with the GFP-tagged HCMV virus (Merlin) and incubated with sera from HCMV seropositive healthy individual at the dilution of 1/10 were used since I have assessed their impact on viral spread previously (Figure 4.7).

My first analysis was of the sera from seronegative SOT patients. Generally, I did not detect any consistent block to viral spread with any of the donors' sera. The analysis of patients as vaccinated versus placebo revealed no differences in the ability of sera to block viral spread. Finally, the analyses of individual donor serum pairs pre- and post- vaccination again revealed no evidence to suggest that vaccination improved the control of viral spread by these donor sera (Figure 4.8. and Figure 4.9).

The joint analysis of these data was performed which confirmed that indeed, there were no differences in the percentage of the infected cells between vaccinated and placebo patients and that the level of the infection in these groups was similar to those observed in the negative controls. Moreover, there was no distinction between the viral spread observed when the cultures were incubated with sera from patients who developed viraemia and those who did not. Lastly, no

differences in the percentage of infected cells were seen in assays which compared sera collected pre- placebo/vaccine administration and the sera harvested at the day of transplantation (Figure 4.12).

Next I analyzed the more complicated samples from the seropositive recipients. In this cohort I observed that the incubation of sera from seropositive renal and liver patients with the cells that were infected with this cell-associated virus decreased the percentage of infected cells in comparison to the negative controls. Again, the responses measured in this group of patients differed significantly between individuals whereby the incubation of serum samples from some of those individuals had a greater impact on viral spread than others (Figure 4.10 and Figure 4.11). However, a joint analysis of this set of data showed that no difference in the activity of the sera against HCMV viral spread was observed when comparing sera from vaccinated and placebo patients. Similarly, no differences in the mean value of the percentage of infection were found between the sera obtained from the patients at the day of vaccine/placebo administration and at the time of the challenge with the virus. Moreover, no differences were found between the groups of patients who developed viraemia versus those who did not. Interestingly, the mean value of the percentage of infection of the cells that were incubated with the sera from these seropositive patients is very similar to the mean value of the percentage of infection seen in the positive control (Figure 4.12).

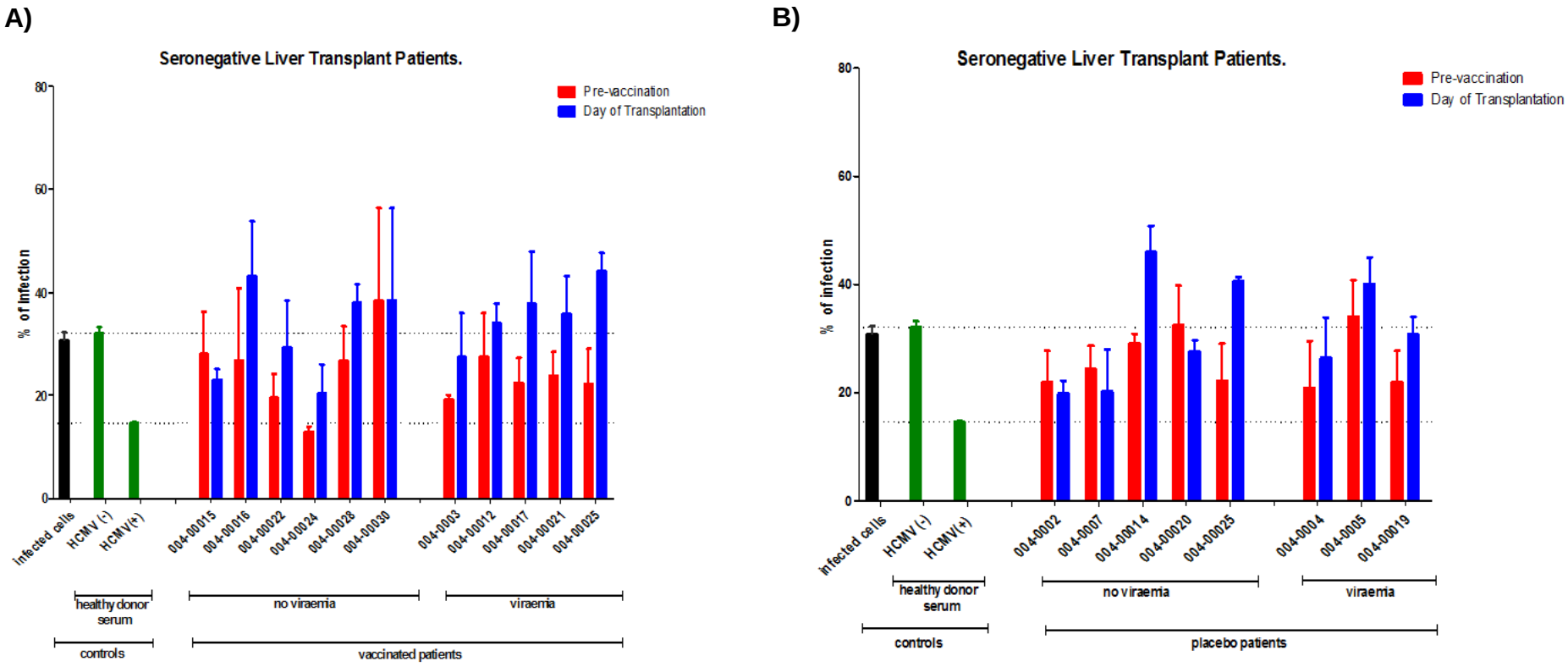


Figure 4.8. Sera from seronegative liver transplant recipients had minimal impact on the inhibition of viral spread.

GFP-tagged HCMV virus (Merlin) was incubated with sera from seronegative liver transplant patients, healthy donor sera (seronegative and seropositive individual) or an ITC88 positive control, and used to inoculate HFFs in vitro (MOI=0.25). Infection was assayed by GFP positivity 14dpi and the proportion of infected cells calculated by counterstaining nuclei with DAPI. Sera isolated pre-vaccination (baseline – red bars) or post vaccination (day of transplant – blue bars) was tested in triplicate. Sera from patients vaccinated with gB (A) or placebo (B) are shown and are subdivided into patients who went onto display evidence of viraemia post-transplant.

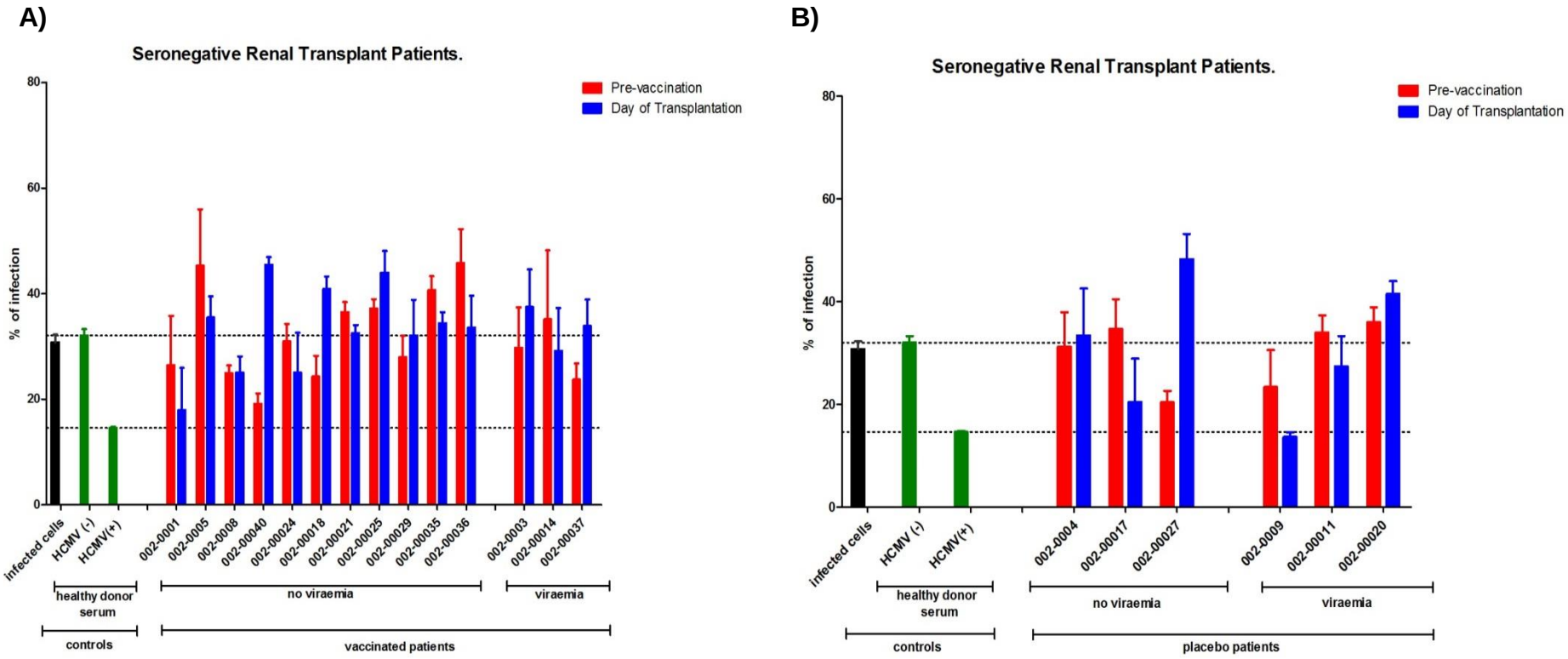


Figure 4.9. Sera from seronegative renal transplant recipients had minimal impact on the inhibition of viral spread.

GFP-tagged HCMV virus (Merlin) was incubated with sera from seronegative renal transplant patients, healthy donor sera (seronegative and seropositive individual) or an ITC88 positive control, and used to inoculate HFFs in vitro (MOI=0.25). Infection was assayed by GFP positivity 14dpi and the proportion of infected cells calculated by counterstaining nuclei with DAPI. Sera isolated pre-vaccination (baseline – red bars) or post vaccination (day of transplant – blue bars) was tested in triplicate. Sera from patients vaccinated with gB (A) or placebo (B) are shown and are subdivided into patients who went onto display evidence of viraemia post-transplant. .

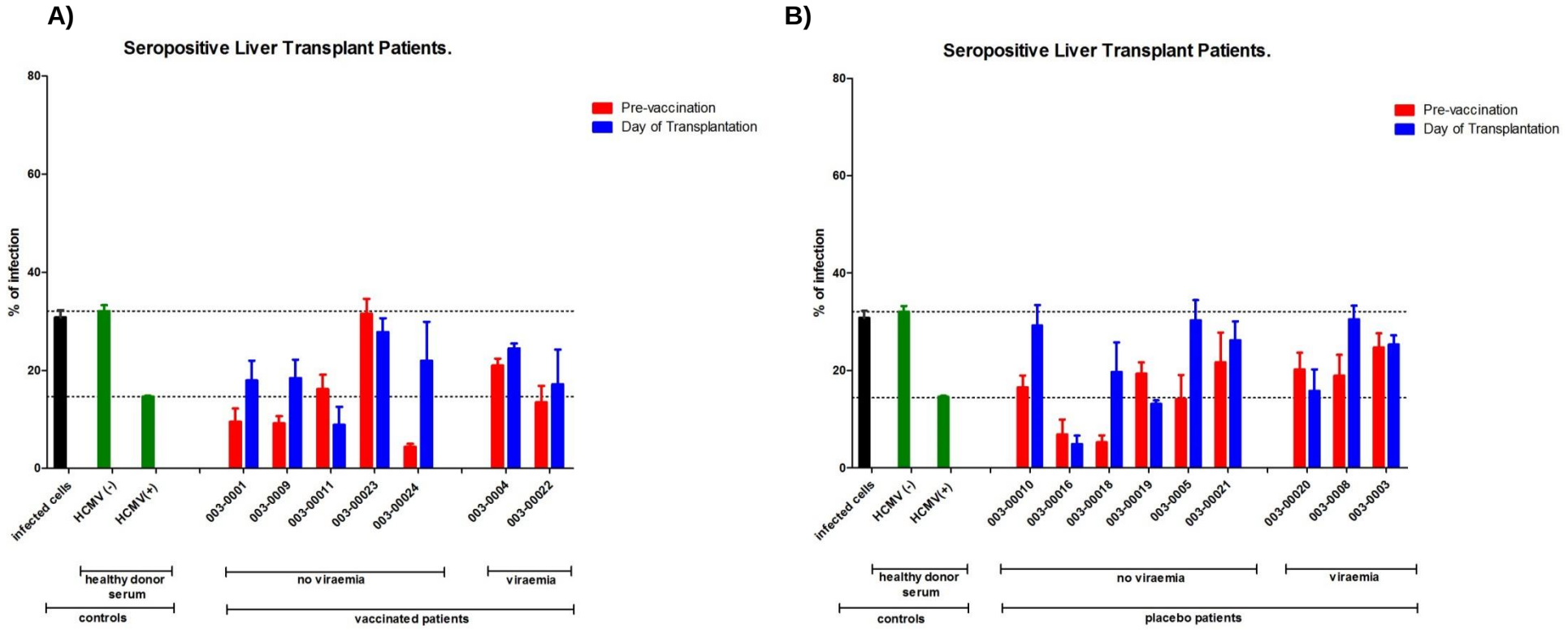


Figure 4.10. Sera from seropositive liver transplant recipients inhibit spread of the virus.

GFP-tagged HCMV virus (Merlin) was incubated with sera from seropositive liver transplant patients, healthy donor sera (seronegative and seropositive individual) or an ITC88 positive control, and used to inoculate HFFs in vitro (MOI=0.25). Infection was assayed by GFP positivity 14dpi and the proportion of infected cells calculated by counterstaining nuclei with DAPI. Sera isolated pre-vaccination (baseline – red bars) or post vaccination (day of transplant – blue bars) was tested in triplicate. Sera from patients vaccinated with gB (A) or placebo (B) are shown and are subdivided into patients who went onto display evidence of viraemia post-transplant. .

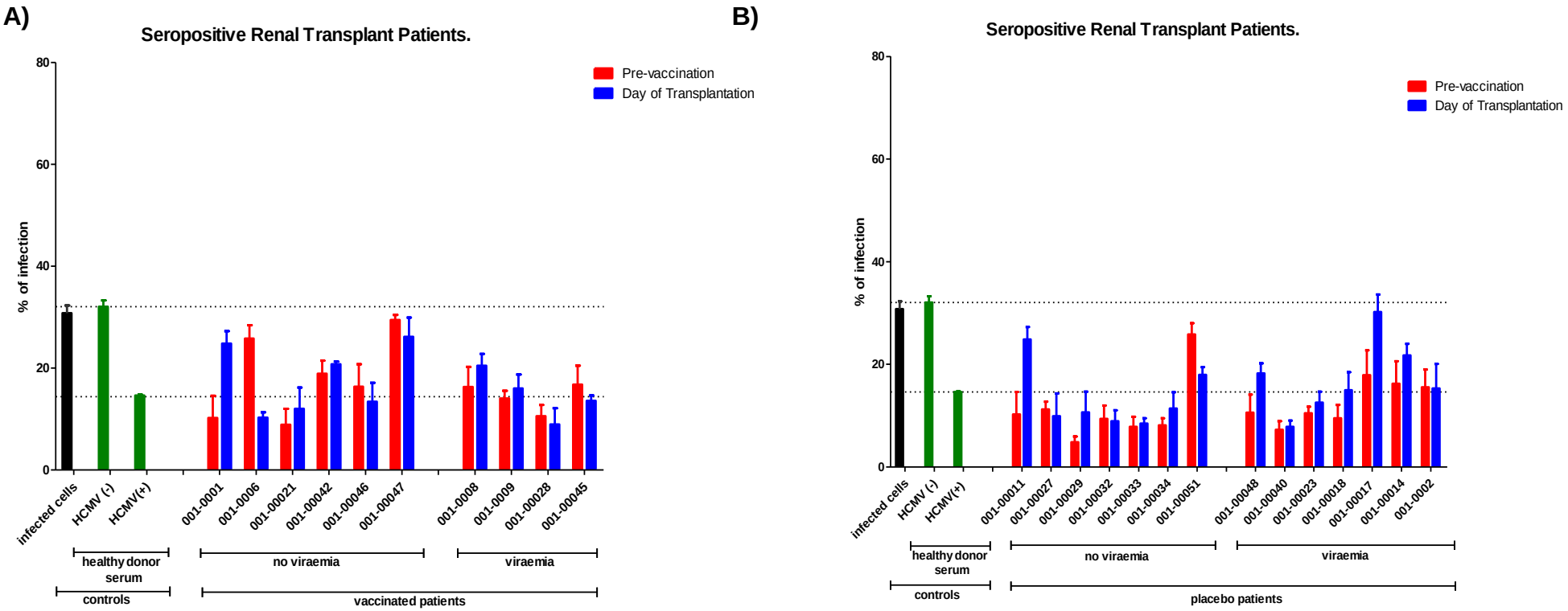


Figure 4.11. Sera from seropositive renal transplant recipients inhibit spread of the virus.

GFP-tagged HCMV virus (Merlin) was incubated with sera from seropositive liver transplant patients, healthy donor sera (seronegative and seropositive individual) or an ITC88 positive control, and used to inoculate HFFs in vitro (MOI=0.25). Infection was assayed by GFP positivity 14dpi and the proportion of infected cells calculated by counterstaining nuclei with DAPI. Sera isolated pre-vaccination (baseline – red bars) or post vaccination (day of transplant – blue bars) was tested in triplicate. Sera from patients vaccinated with gB (A) or placebo (B) are shown and are subdivided into patients who went onto display evidence of viraemia post-transplant.

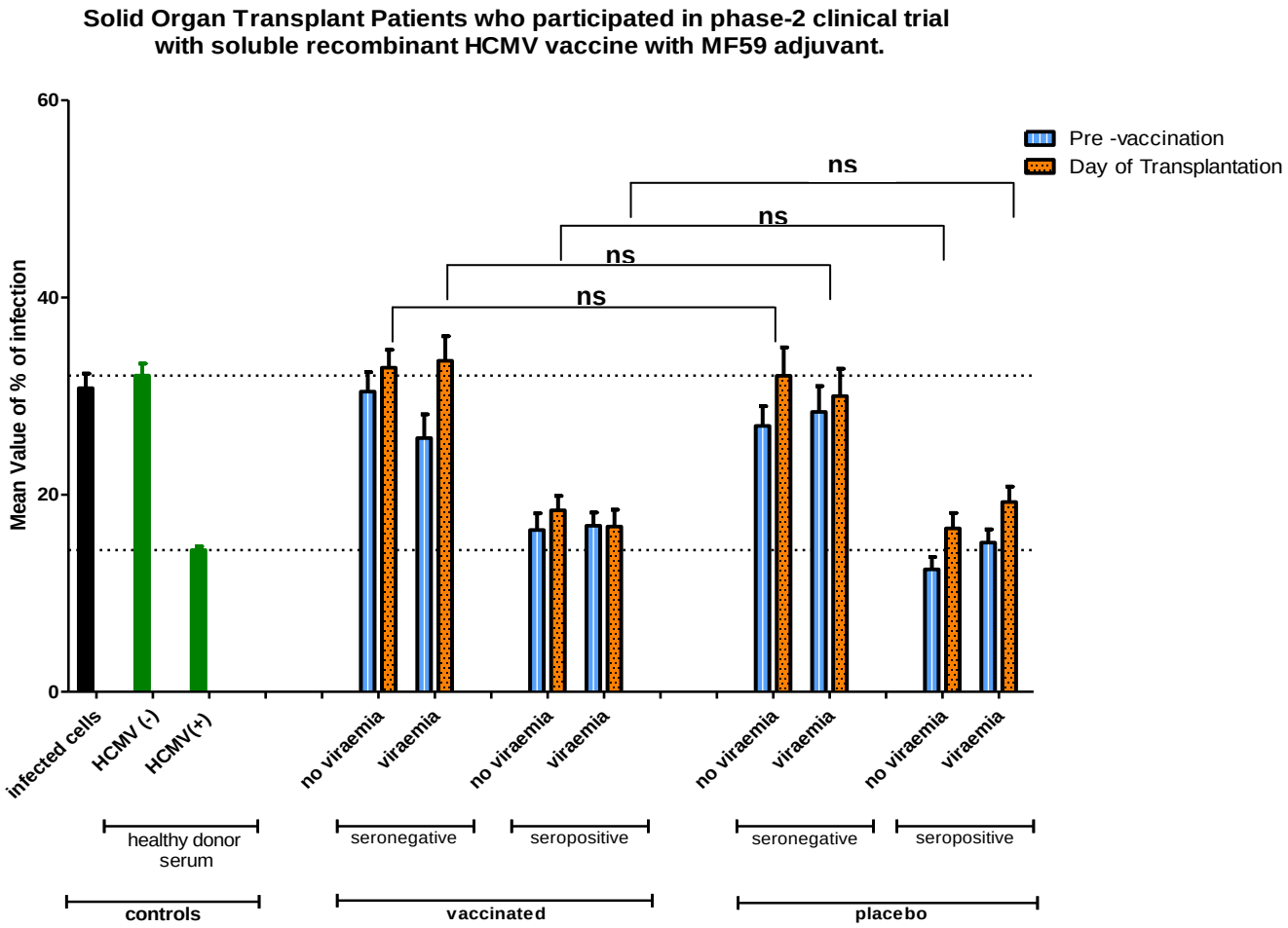


Figure 4.12. Vaccination failed to induce humoral responses that would inhibit spread of the cell-associated virus in the in-vitro assay in seronegative Solid Organ Transplant Patients (de novo) and did not boost these responses in seropositive vaccine recipients.

Seronegative patients: n=25; seropositive patients n=17) or placebo (seronegative patients: n=14; seropositive patients n=23) administration (Pre-vaccination, depicted in blue) and at the time of the challenge with the virus (day of the transplantation, depicted in orange). All patients' serum samples were diluted 1/10. The inhibition of the spread of the virus as the decrease in the % of the cells infected with the virus. The error bars indicate the standard error of mean. Negative control are cells infected with the GFP-tagged HCMV virus (Merlin) and the cells infected with the GFP-tagged HCMV virus (Merlin) and incubated with sera from HCMV seronegative healthy individual (dilution 1/10) the and positive control are the cells infected with the GFP-tagged HCMV virus (Merlin) and incubated with sera from HCMV seropositive healthy individual at the dilution of 1/10. No statistically significant differences were observed between the mean value of the percentage of infection between the samples obtained from the vaccinated and placebo patients at the time of transplantation. Statistical differences were obtained from the Mann Whitney test; seronegative, no viraemia (vacciated, n=13 vs placebo, n=8) p=0.9549; seronegative, viraemia (vacciated, n=7 vs placebo, n=6) p=0.3420; seropositive, no viraemia (vaccinated, n=11 vs placebo, n=13) p=0.2941; seropositive, viraemia (vacciated, n=6 vs placebo, n=10).

4.4. Discussion.

The understanding of viral cell-to-cell spreading and the immune responses of the infected individuals that target this route of viral transmission is important, however many aspects of viral dissemination remain still unclear. The detailed knowledge about this phenomenon could be used to develop new therapeutic strategies that could block spread of the virus *in vivo.* The results presented in the literature are inconsistent: some reports showed reduction of the viral spread in culture by seropositive sera while others showed no impact of the antibodies on cell-to-cell spread. The reasons for these discrepancies are unclear but one possible explanation is the identity of the virus isolate used – many studies have utilised laboratory strains which grow more efficiently as cell free viruses. Potentially, this cell-free growth phase renders the virus more sensitive to neutralization and other effector responses that require direct binding to the virus particle. Therefore, for the purpose of this research I have used more clinically relevant Merlin strain (Chapters: 2.6; 4.2.2) and, furthermore, utilised a genetically modified version of Merlin that enhances the growth of the virus as a cell associated virus.

The sera from the patients participating in the gB/MF59 trial were assayed for their ability to impact on viral spread in my in vitro assay. Similarly to the neutralization assays, the majority of the sera samples from seronegative patients had minimal impact on HCMV growth in this assay. Furthermore, no protective effect could be observed using the sera of patients post vaccination. Thus no evidence was found that this vaccine elicited gB antibody responses in seronegative patients that could interfere and block the spread of the cell associated, genetically engineered GFP tagged Merlin strain because the mean value of the percentage of HCMV infected cells incubated with sera from seronegative patients was comparable with percentage of HCMV infected cells in control wells.

In contrast, the percentage of infected cells (which is a measure of viral spread in my assay) is highly variable amongst the different seropositive patients' serum samples tested; the majority of sera displayed anti-HCMV activity in the assay

whereby spread of HCMV appeared to be impaired when compared to the negative control sera. However, when the data from all seropositive patients were analyzed and the mean value for the percentage of infection of the cells was calculated for the placebo and vaccinated group, no differences were observed. Additionally, no differences in the rate of spread of the virus were observed when sera collected from the patients prior to vaccination were compared with the corresponding patients' samples obtained at the time of challenge with the pathogen (day of transplantation). Finally, there was no correlation between the decrease in the percentage of infected cells and viraemia development. In summary, this analysis provided no evidence that the vaccine induces antibody responses in seropositive patients that would be capable of blocking dissemination of the virus over and above those that already exist. Although I could observe that the HCMV seropositive patients possess antibodies in their sera that interfere with the spread of the virus to some extent, the controls show that these antibodies were developed as a response to the natural infection and that vaccination with gB/MF59 failed to boost these pre-existing antibody responses.

Taken together, these data show that vaccination of individuals with the gB/MF59 vaccine did not elicit humoral immune responses sufficient to effectively control the spread of HCMV alone in this assay. Although it is clear that prior natural infection with HCMV (*e.g.:* the seropositive recipients) does elicit an immune response that is partially effective in this assay, the decrease in the infectivity of the virus I observe in these individuals is independent of any vaccine induced responses but instead, is due to endogenous responses present in seropositive individuals' serum.

Importantly, I observed that the anti-viral effect seen was more effective against the Merlin rather than the GFP-Merlin virus. A plausible explanation for this difference is that the endogenous antibody response neutralizes the cell-free component of HCMV in the viral spread assay which would be consistent with the neutralization data.

The question remains whether there are components of the humoral immune response that are elevated in vaccinated individuals that make them better equipped to control viral dissemination of cell-associated virus. Glycoprotein B is expressed on the surface of infected cells and therefore is a target for anti-gB antibody binding. Whether antibodies can bind to gB on the plasma membrane and direct immune responses against infected cells remains to be determined. For example, rather than through a direct impact on virions it is possible that binding of plasma membrane gB by antibodies can recruit further immune functions to these cells i.e. NK cells and complement fixation. Alternatively, there is some debate over the role glycoproteins B, H and L play in the fusion of cells in culture – with a potential role in the transmission of cell associated virus between cells. Thus if antibody binding to gB on the cell surface could impede this fusion process it could also impact on the spread of a cell associated virus under these culture conditions. Additionally, it would also be interesting to investigate whether the sera from vaccinees could block the spread of the virus in other cell lines; especially epithelial and endothelial cells. The viral entry into these cells requires the presence of pentameric complex and as stated before it has been shown that clinical strains that do express functional pentameric complexes are predominantly cell-associated.

In summary, I have established a mechanism to analyze the viral spread of HCMV in culture and to define whether the spread is via cell associated or cell-free virus. Thus, these data now position me to interrogate other aspects of the humoral immune response that may be important for controlling HCMV *in vivo*. Clearly, the serum from vaccinated patients does not control viral spread alone suggesting that it, if it is the source of protection in the vaccinated patients, might need to act in concert with other facets of the immune response.

5. Antibody Dependent Cell Mediated Cytotoxicity.

5.1. Introduction.

Natural killer (NK) cells are crucial components of the innate immune system that comprise approximately 15% of all circulating lymphocytes. A significant feature of NK cells is their ability to lyse target cells without prior sensitization (natural cytotoxicity) [378].

Antibody-dependent cell-mediated cytotoxicity (ADCC) is a distinct immunological mechanism that relies on both NK cell-mediated immune defence as well as humoral immunity. Inhibition of infection, decrease in disease incidence and severity are conferred via ADCC through antibodies (humoral immune response) that bind to and activate the effector cells (NK cell-mediated immunity). Moreover, this type of response depends on prior existence of antibodies, thereby linking the innate and adaptive immune responses.

In this immunological mechanism, antibodies bind to foreign antigens expressed on the surface of a target cell. Subsequently, the Fc receptor of the effector cell of the immune system binds to the Fc portion of antibody-coated target cells. This event induces the degranulation of the effector cell, release of cytolytic granules such as perforins and granzymes and inflammatory mediators such as cytokines and chemokines (Figure 5.1); [379, 380]. This immunological mechanism is predominantly mediated by NK cells; however it has also been reported that neutrophils, macrophages and eosinophils can act via ADCC as well [381-384].

Human NK cells are a subpopulation of lymphocytes characterized by the expression of the cell adhesion marker CD56 and lack of the T-cell receptor CD3 ($CD56^{+}CD3^{-}$) [385]. Based on their phenotypic properties, human NK cells are divided into two subsets as follows [378]:
-$CD56^{dim}$ $CD16^{bright}$ (>95% of $CD56^{dim}$NK cells are $CD16^{bright}$)
-$CD56^{bright}$ $CD16^{low}$ (approximately ≈50–70%of $CD56^{bright}$ do not express CD16 and the remaining cells exhibit a low-density expression of this receptor).

Along with this phenotypic characterisation, functional studies show that the $CD56^{dim}$NK-cell subset is highly cytotoxic. In contrast, the $CD56^{bright}$ subset has the capacity to produce abundant cytokines following activation by monocytes, but has low natural cytotoxicity [378].

Three different Fcγ receptor classes have been discovered in humans: the high affinity FcγRI (CD64), medium-low affinity FcγRII (CD32), and FcγRIII (CD16).
NK cells express exclusively FcγRIII and none of the other activating FcγRs; FcγRIII (CD16) receptor class is believed to be the most important mediator of ADCC [380]. Thus, the level of expression of CD16 is strongly correlated with the level of ADCC. Consistent with this, the subset of $CD56^{dim}$NK cells characterized by high-level of CD16 expression, exhibit much higher level of ADCC than the $CD56^{bright}$ subset [378].

The subset of $CD56^{dim}$NK cells (cytotoxic NK cells) contain high numbers of secretory granules in their cytoplasm which have the characteristics of lysosomes and are sometimes referred to as “secretory lysosomes”. One of their functions is to store and release pro-apoptotic proteins, amongst which perforin and granzyme are the most abundant. Indeed, human NK cells are known to express perforin and granzymes A and B constitutively [386]. These lytic proteins are released into the immune synapse in an exocytic way in a process known as degranulation.

The major role of the membrane-disruptive protein perforin is to form a pore either on the endosome or plasma membrane of the target cell. Such an event promotes the delivery of serine proteases known as granzymes into the target cell [387,

388]. Eleven granzymes have been reported so far, all of them are lymphocyte granule serine proteases belonging to the trypsin family. The function of this protein family is to initiate apoptosis of the target cell [389-392].

In order to elicit cytotoxic functions, NK cells must be activated from their resting state. The integration of multiple signals from both activating and inhibitory receptors on the surface of NK cells can promote degranulation of the secretory granules [378, 393]. The granule core is surrounded by a lipid bilayer containing lysosomal-associated membrane glycoproteins (LAMPs) including CD107a (LAMP-1), CD107b (LAMP-2), and CD63 (LAMP-3)- ubiquitous, highly glycosylated, integral membrane proteins of largely unknown function [394]. These proteins account for about 50% of the total protein content in the lysosomal membrane [394] and are transiently expressed on the NK cell surface following degranulation [395]. Recent studies reported that LAMP-1(CD107a) is significantly upregulated on the surface of stimulated NK cells. Moreover, a correlation between CD107a expression and NK cell-mediated lysis of target cells was found [395]. Other studies revealed that the expression of CD107a on the cell surface was strongly upregulated following stimulation in concordance with a loss of perforin. Despite a clear understanding of the phenotypic changes associated with CD107a localisation, the actual role of this membrane protein in NK-cell biology remains largely unknown. Inhibition of expression of this protein blocks NK-cell cytotoxicity, due to the failure in granzyme B delivery to target cells. Moreover, decreased expression of CD107a impaired movement the lytic granules and reduced levels of perforin inside these cellular structures [396]. Despite the limited understanding of the functions of this protein, transient CD107a expression at the membrane surface was concluded to be a good indicator of ADCC activity [395].

Given the wealth of evidence, CD107a (LAMP1) is widely used as an indirect marker for NK-cell degranulation [395, 397-399] and a positive correlation between CD107a expression and NK cell cytotoxic activity has been recently described in many reports [400-403].

The functional impact of NK cytotoxic activity has been extensively studied in the past decade. An increasing body of evidence indicates that ADCC contributes to the control of HIV and SIV infection and inversely correlates with progression of the disease [404-409]. Studies on seasonal influenza trivalent inactivated vaccine revealed that infection with a seasonal influenza virus elicits ADCC-mediating antibodies that can target conserved regions of influenza virus proteins and recognize a broad spectrum of influenza strains. The authors concluded that cross-reactive ADCC may provide a contributory mechanism for reducing the severity of divergent influenza virus infection [397-399, 402].

As well as being important for controlling viral infections, ADCC might be an important mechanism of action of therapeutic monoclonal antibodies against malignantly transformed cells. The activation of apoptosis and effector-cell-mediated cytotoxicity were studied in an experimental mouse model with the use of therapeutic agents: trastuzumab (Herceptin®) and rituximab (Rituxan®). The anti-tumor activities of such therapeutic antibodies are related to the engagement of Fcγ receptors on effector cells and this mechanism is believed to be the dominant component of the *in vivo* activity of antibodies against tumours [410].

In light of the evidence that ADCC plays a role in the clearance of viral infections and confers protection after vaccination, I investigated whether any ADCC humoral immune responses were generated in patients who participated in the HCMV gB/MF59 trial [188]. In order to do this, CD107a activation marker on NK cells in the presence of anti-HCMV antibodies was measured as an indirect assessment of the ADCC response. In preliminary experiments, the level of CD107a expression on activated NK cells in whole PBMC and isolated NK cell samples was measured and no significant differences between samples were found [401, 402]. Therefore in these experiments total PBMCs from healthy donors were utilized in the majority of the assays.

Objectives:

The objectives of this chapter were to: assess NK cell effector function in seropositive and seronegative SOT patients who received placebo (natural history data); study whether vaccination elicited ADCC responses in seronegative patients and boosted pre-existing ADCC responses in seropositive patients; correlate ADCC antibody levels with decrease in the incidence of viraemia; investigate potential differences in the level of ADCC responses between total PBMCs and purified NK cells.

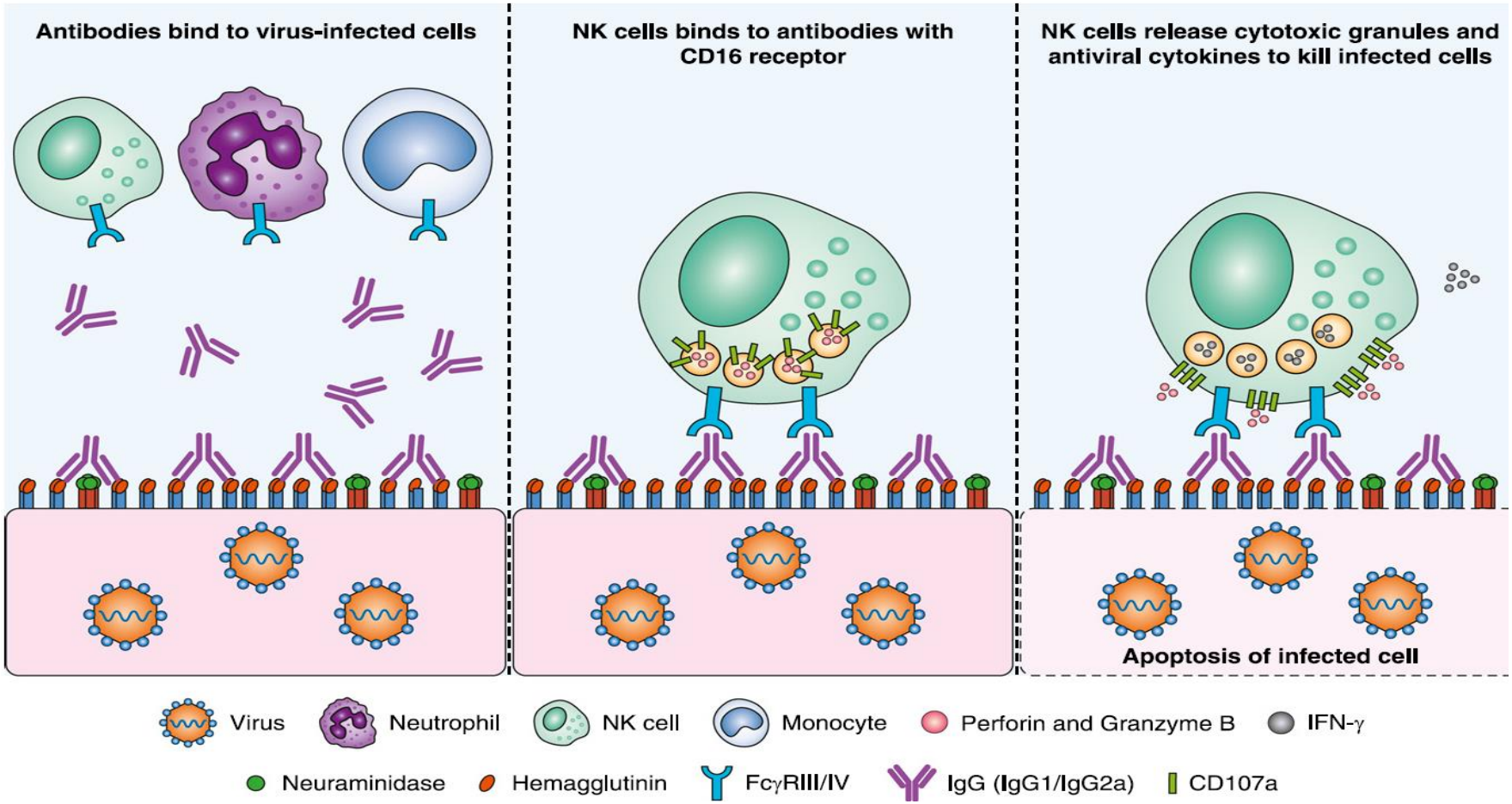


Figure 5.1. Schematic diagram of ADCC.

IgG Abs (IgG1/IgG3 in humans or IgG2a/IgG2b in mice) bind to viral Ags expressed on the surface of virus-infected cells (*left panel*). Effector cells, such as NK cells (also neutrophils and monocytes), bind to the Ab Fc-region using their FcγRIII receptor (in mice FcγRIV*, middle panel*. Upon Ab ligation, cytotoxic granules are released and antiviral cytokines are expressed. This results in apoptosis of the infected cells and a reduction of viral replication. Reproduced from reference: Jegaskanda, S., P.C. Reading, and S.J. Kent, *Influenza-specific antibody-dependent cellular cytotoxicity: toward a universal influenza vaccine.* J Immunol, 2014. 193(2): p. 469-75.

5.2. Materials and Methods.

5.2.1. Patient population.

Control sera were obtained from healthy seropositive and seronegative HCMV donors, whose HCMV serostatus had been confirmed by standard NHS diagnostic serology procedures (Chapter 2.1. and 2.2.). All samples were anonymous.

In addition, samples were used from the cohort of HCMV- seropositive and seronegative renal and liver transplant patients enrolled in the gB/MF59 trial (described in 1.16), who had undergone transplantation. These patients had given written consent to use their serum samples for research purposes.
These blood samples were obtained from the patients prior to transplantation (five samples) and the blood sample collected at the day of transplantation or 7 days after transplantation (Figure 1.14).

Details of the sample processing were described in chapter 2.2. In principle, all samples were heat-inactivated (56°C for 1h) prior to analysis to destroy complement. Depending on the assay, serum samples were either used neat or diluted in PBS-pH 7.7 [-]$CaCl_2$ [-]$MgCl_2$ (Gibco by Life Technologies) at the ratio 1/5, 1/10, 1/20.

5.2.2. Isolation of Peripheral Blood Mononuclear Cells (PBMCs) and NK cells.

Donor PBMCs were retrieved from venous blood samples by centrifugation (20mins 850xg, brake off at 20°C) on Histopaque-1077 (Sigma Aldrich). After centrifugation, the top blood plasma layer was removed and discarded. The interface created between the plasma layer and the lymphocyte separation medium was carefully removed to avoid contamination with adjacent layers and placed into fresh tubes. The volume was made up to 30ml by addition of RPMI 1640 and the cell suspension was centrifuged at 550g for 10min. The supernatant was discarded and the pellet was re-suspended in 20ml of RPMI 1640. Cells were washed twice by centrifugation at 450g for 5mins. After the last wash, cells were re-suspended in 10ml of RPMI medium (Dulbecco) supplemented with 10% fetal calf serum (FCS). Viable cells were stained with 4% trypan-blue and counted using a haemocytometer. Cells were stored at -78°C. NK cells were isolated from PBMC fraction by use of the NK Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's instructions. The principle of this isolation kit is to isolate untouched NK cells by depletion of non-target cells that are indirectly magnetically labelled with a cocktail of biotin-conjugated antibodies against lineage specific antigens and a cocktail of MicroBeads prior to a separation step. Isolation of the NK cell fraction occurred in the magnetic field of the separator. Labelled cells were retained on the magnetic column and the flow-through contained the unlabelled NK cells. Cells were stored at -78°C.

5.2.3. Thawing of frozen PBMCs and NK cells.

Vials of frozen PBMCs and purified NK cells were thawed in a 37°C water bath for approximately 30secs (until they began to thaw but there was still ice visible). The cells were transferred to 40mL pre-warmed RPMI medium (Dulbecco) supplemented with 20% FCS and thawed quickly. The cell suspension was centrifuged at 450g for 5mins (brake off) at 20°C and the supernatant discarded. The cellular pellet was re-suspended in 10mL of RPMI medium (Dulbecco) supplemented with 10% FCS.

5.2.4. Cell culture.

The highly undifferentiated K562 human erythroleukemic cell line (Sigma-Aldrich) was used in some of the assays (5.3.1.2). The major feature of this cell line is the lack of expression of MHC class I molecules. It is well established that NK cells mediate non-major histocompatibility complex (MHC)-restricted cytotoxicity and antibody-dependent cytotoxicity [411-413]. The loss or down-regulation of self-MHC class I molecules triggers the activation of NK cells. This phenomenon was described in the 1980's and termed the '*missing-self hypothesis*'[414, 415]. However, it has recently been discovered that there are also other MHC class I molecule independent mechanisms that activate the cytotoxic functions of NK cells [416]. NK cells are able to lyse K562 tumour cells due to the lack of expression of major MHC class I molecules by this cell line. CD107a marker is significantly upregulated on the surface of NK cells following stimulation with K562 cells [395] and the optimal effector to target ratios for detecting CD107a expression range from 1:1 and 10:1 [395, 400, 401, 403]. It has also been reported that simultaneous incubation of PBMCs with both phorbol 12-myristate 13-acetate/ionomycin (PMA/I) and K562 enhance the release of cytotoxic molecules and expression of CD107a marker on activated NK cells [400].

Prior to thawing, 10ml of media warmed to 37°C was added to a 15ml falcon and 5mls of warm media to two T25 flasks. Then flasks were placed in 37°C 5%CO_2 incubator. Once the vial was removed from liquid nitrogen, it was thawed immediately in 37°C water bath; then the vial content was transferred to the 15ml falcon with warm media. Thawed cells were centrifuged at 300g for 5mins at RT. Supernatant was discarded and pellet resuspended in 10ml of fresh warm media. Following this, 5ml of suspension was transferred to each of the two T25 flasks. Following thawing these cells reach log phase in 5 to 7 days. Once the cell density reached 0.8x10^6%cells/ml the culture was split to approximately 0.4x10^6%cells/ml with fresh, warm media. From this point, the cells double on average every 24 hours. 2. The cells were splitted when the density reached approx. 0.75%million/ml. The cell culture was maintained in RPMI 1640 (Dulbecco) supplemented with 10% FBS, 2mM L-glutamine and 50UI/ml penicillin; at 37°, 5%CO_2 with 100% humidity.

5.2.5. Antibody Dependent Cell Mediated Cytotoxicity (ADCC) Assay.

In order to investigate the ADCC responses in patients who participated in the gB/MF59 study, an indirect in vitro assay was developed:

1. **gB vaccine protein coating onto 96 well plates.**

The gB vaccine protein (Sanofi Pasteur) was diluted in 1x ELISA coating buffer (Biolegend) at the ratio: 1:1299 (0.77μL of the protein in 1mL of 1x ELISA coating buffer) in order to achieve a final concentration of 0.75μg/mL. 100μL of the solution was added per well to the 96-well ELISA plates (Microtest 96 well clear ELISA plate, BD). Plates were covered using a plate sealer (Seal Plate- plate sealer films, Elkay) and incubated overnight at 4°C in 5% CO_2, then subsequently washed 1x with 200ul and 4x with 100ul of PBS-pH 7.7 [-]$CaCl_2$ [-]$MgCl_2$ (Gibco by Life Technologies). Washes were followed by addition of 100μL ELISA Blocking Solution-5% BSA (Sigma-Aldrich) in sterile PBS (Gibco by Life Technologies). Plates were covered by using a plate sealer and incubated for 2 hrs at room temperature, then subsequently washed 1x with 200μl and 4x with 100μl of PBS-pH 7.7 [-]$CaCl_2$ [-]$MgCl_2$ (Gibco by Life Technologies).

2. **Incubation with serum.**

Glycoprotein-B immobilised on the solid surface was incubated with serum as follows: 50μL of respective heat-inactivated, autologous, healthy donor HCMV seropositive or seronegative serum or serum from SOT patients enrolled in the vaccine study (neat or diluted 1:5, 1:10, 1:20) were added to the corresponding wells. Plates were covered using a plate sealer and incubated overnight at 4°C.

3. Incubation with PBMCs or purified NK cells.

Following the incubation with serum, plates were washed 1x with 200µl and 4x with 100µl of PBS-pH 7.7 [-]$CaCl_2$ [-]$MgCl_2$ (Gibco by Life Technologies) in order to remove non-specific antibodies. The anti-gB antibodies bound to gB on the solid surface were then incubated with PBMCs or purified NK cells. PBMCs and purified NK cells were isolated from healthy HCMV positive and negative blood donors; (method is described in the section: 5.2.2 and 5.2.3). Cells were counted and re-suspended at $10x10^6$/mL in RPMI medium (Dulbecco) supplemented with 10% FCS $0.5x10^6$ of the PBMC's (50µL of $10x10^6$/mL suspension) or 10^4 of the purified NK cells (50µL of $2x10^5$/mL suspension) and 50µL of RPMI medium (Dulbecco) supplemented with 10% FCS added to each well. In order to stimulate the PBMC cells (positive control): PMA (phorbol 12-myristate 13-acetate; 50ng/mL) and ionomycin 500ng/mL were added to respective wells (5.3.1.1).

4. Stimulation of the PBMCs and cell-surface staining.

CD107a FITC (BD) used at the concentration 5µl/mL and final dilution of 1/5 and RPMI medium supplemented with 10% FCS were added to each well and incubated for 4.5h at 37°C. Plates were covered with a cell culture 96-well plate lid and incubated at 37°C. After 1h of incubation, 50µL per well of the mix of: monensin (BD GolgiStop; used at the concentration 6µg/mL and final dilution 1/333); Brefeldin A (BD; used at the concentration 5µl/mL and final dilution of 1/200); were added to each well and plates were placed back in incubator for remaining 3.5h. Monensin and brefeldin A are protein transport inhibitors that block the intracellular protein transport processes. This results in the accumulation of cytokines and/or proteins in the Golgi complex. The increased accumulation of cytokines in the cell enhances the detectability of cytokine-producing cells in the assay.

Following the incubation, cells were transferred to 96 well V-bottom polystyrene plates. 100µL of PBS with 2% heat inactivated-FCS was added to each well, and then plates were centrifuged at 800g for 5 min, 4°C. 100µL of PBS with 10% heat inactivated-FCS was added to each well and incubated for 15 minutes on ice in order to block non-specific binding sides. After the blocking step 100µL of PBS with 2% heat inactivated-FCS was added to each well and plates were centrifuged at 800g for 5 min, 4°C. 100µL of the Cell Surface Antibody mix was added to all wells: CD3 PerCP (BD) used at the concentration 12.5µl/mL and final dilution of 1/5 CD56 AF647 (BD) used at the concentration 50µl/mL and final dilution of 1/50 or CD56 APC used at the concentration 12µl/mL and PBS with 2% heat inactivated-FBS. Cells were incubated for 20 minutes on ice in the dark.

Following cell surface staining, 100µL of PBS with 2% heat inactivated-FCS was added to each well, and then plates were centrifuged at 800gfor 5 min, 4°C. Cellular pellet was re-suspended in 200µL/well of 100µL of PBS with 2% heat inactivated-FCS and transferred to mini tubes for data acquisition. Stained cells were stored at 4°C in the dark prior to FACS acquisition.

5. Intracellular staining.

To assess intracellular staining, following the cell surface staining, 200µL of Cytofix/Cytoperm (BD) was added to each well and incubated for 20minutes on ice; plates were centrifuged at 800g for 5 min, 4°C and then incubated with Perm/Wash for 20min on ice in the dark.

IFNγ AF700 (BD) antibody was used at the concentration 50µl/mL and diluted in 1xPerm/Wash (BD). Plates were incubated for 20min on ice in the dark and then centrifuged at 2000rpm for 5 min, 4°C. Cellular pellet was re-suspended in 200µL/well of 100µL of PBS with 2% heat inactivated-FCS and transferred to mini tubes for FACS acquisition.

Solutions used in the assays:

-5x Wash Buffer- pH 6.6-6.8 (for 1L): 101.0g NaCl (Sigma Aldrich); 1.0g KH_2PO_4 (Potassium Phosphate, monobasic, Sigma Aldrich); 4.585g Na_2HPO_4 (Sodium Posphate, dibasic, Sigma Aldrich); 2.5mL Tween20 (Sigma Aldrich) and deionized water. All reagents were dissolved on a magnetic stirrer, in approximately 800mLs of deionized water. Volume was adjusted to 1L with deionized water; pH was verified by pH indicator strips (Sigma-Aldrich). Solution was stored at 2-10°C up to 2 months.

-Recombinant HCMV gB protein (Sanofi Pasteur): Stored at -70°C in 250uL aliquots at 0.975mg/mL(=975 ug/mL). Used at a final concentration of 0.75ug/ml: 0.77uL in 1mL of 1x ELISA coating buffer. The maximum number of freeze/thaw cycles per aliquot was 3.

-ELISA coating buffer (Biolegend) 5x concentrate, diluted 1:5 in deionised water. Stored undiluted at 4°C.

-ELISA blocking buffer: 1% BSA (Sigma Aldrich) in sterile PBS, filtered and stored at 4°C.

-PMA (Sigma-Aldrich) Lab stock 100µg/mL in ethanol. Used at 50ng/mL final concentration: stock was diluted 1/10 then uses at 1/200. Stored at -20°C.

-Ionomycin (Ionomycin) Lab stock 100µg/mL in ethanol. Used at 500ng/mL: stock was diluted at 1/10 then used at 1/20. Stored at -20°C.

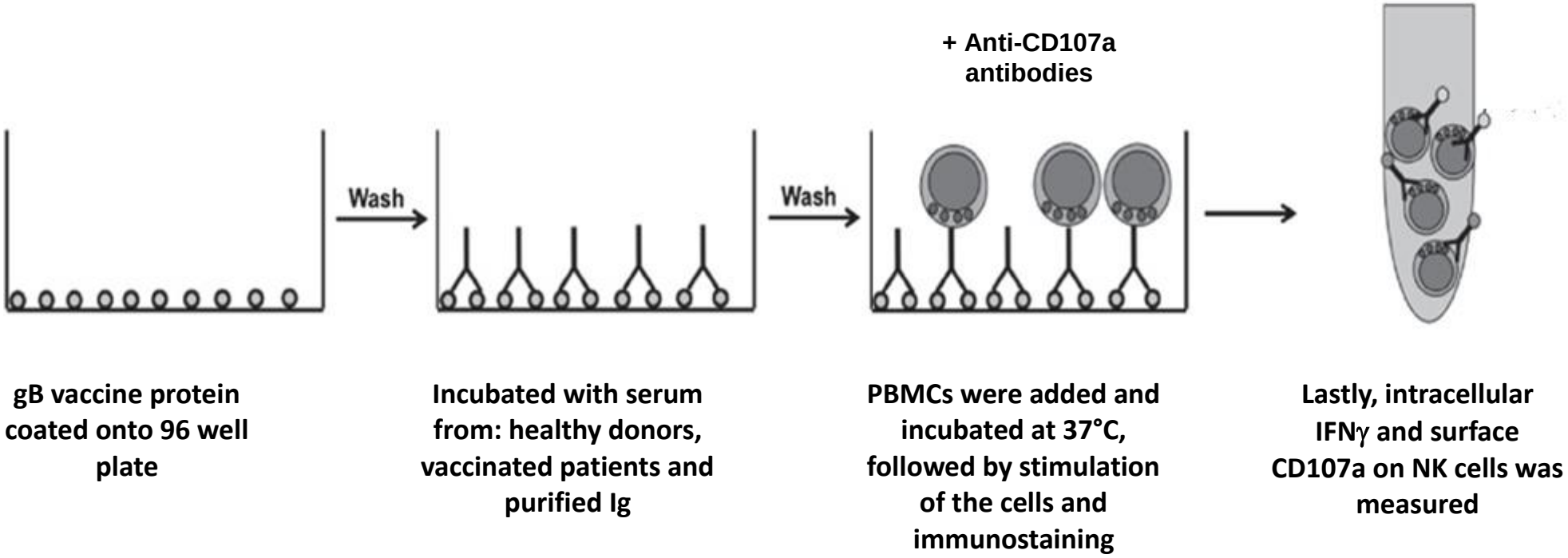


Figure 5.2. Schematic representation of the indirect ADCC assay.

Adapted from: Jegaskanda S. et al., *J Immunol*. 2013; 190: 1837-1848 [402].

5.2.6. FACS Acquisition.

Following stimulation and staining of the cells, the cell suspensions were acquired using a four laser LSR Fortessa flow cytometer (BD Bioscience, Oxford, UK). FACS analyses were performed using FlowJo Data Analysis software, version 10 (FlowJo LCC, OR, USA).

5.2.6.1. Gating strategy.

Initial gating to identify the lymphocyte population was performed using forward and side scatter. A secondary gate was placed around the CD3-CD56+ population to identify NK cells. The threshold fluorescence intensity for CD107a (and interferon gamma in some initial assays) positive cells was set using unstimulated control cells (Figure 5.3).

Compensation values (the simultaneous solution of the equations for the contributions of the spectral overlaps of each of the colours into every detector) are used by the flow cytometer to subtract out the contributions of non-primary colours overlapping into a given detector. In order to determine the spectral overlap the CompBead presenting single-stained controls (BD) were used.

The CompBeads were stained as follows: one drop each of positive and negative CompBeads was added to a FACS tube and centrifuged for 3mins at 1000g. The CompBeads were stained as per Table 1, and then incubated for 10min in the dark at room temperature. 2ml of PBS with 2% heat inactivated-FCS was added to samples and centrifuged at 1000g for 3 min. The supernatant was discarded and 200μL of PBS with 2% heat inactivated-FCS was added and mixed well. Stained CompBeads were stored at 4°C in the dark until acquired by FACS.

Antibody	Company	Concentration	Work concentration	µL/ test
CompBeads	BD	N/A	1 drop	Neg + Pos
mIgG1 PerCP	Invitrogen	100µg/mL	1/100	1
CD56 APC	BD	12µg/mL	1/50	2
mIgG1 FITC	BD	50µg/mL	1/50	2

Table 5.1. Antibodies for compensation beads (BD CompBeads).

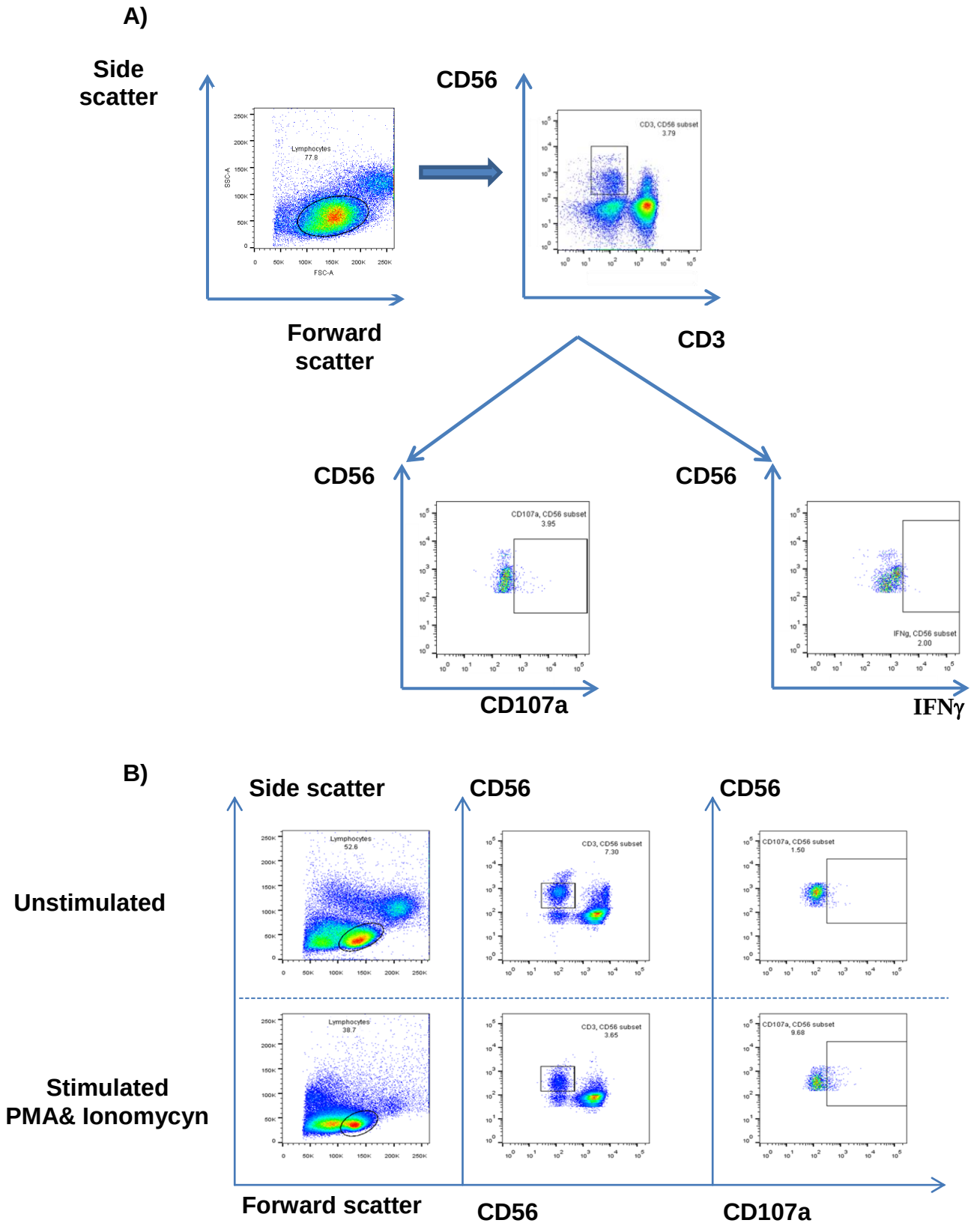


Figure 5.3. Gating strategy for the assay.

A) Representative staining for all markers used in this chapter B) Representative staining for positive and negative control for the assay (positive control: PBMCs stimulated with PMA/I and negative control: unstimulated PBMCs).

5.2.7. Optimization of the assay.

5.2.7.1. Fluorescence minus one (FMO).

In order to correctly identify and gate cells in the context of data spread due to the multiple fluorochromes in a given panel, FMO control experiment was conducted.
In this FMO control, one PBMC sample was stained with all the flurochromes (FITC, PerCP, AF647 and AF7004 colour staining).
In this panel four fluorochromes were used, therefore there were four separate FMO controls-PBMC cells stained with all the fluorochromes minus one fluorochrome that was being tested.
This allowed any spread of fluorochrome(s) into the unlabelled channel to be taken into account and allowed the gate to be set in the correct place (Figure 5.4).

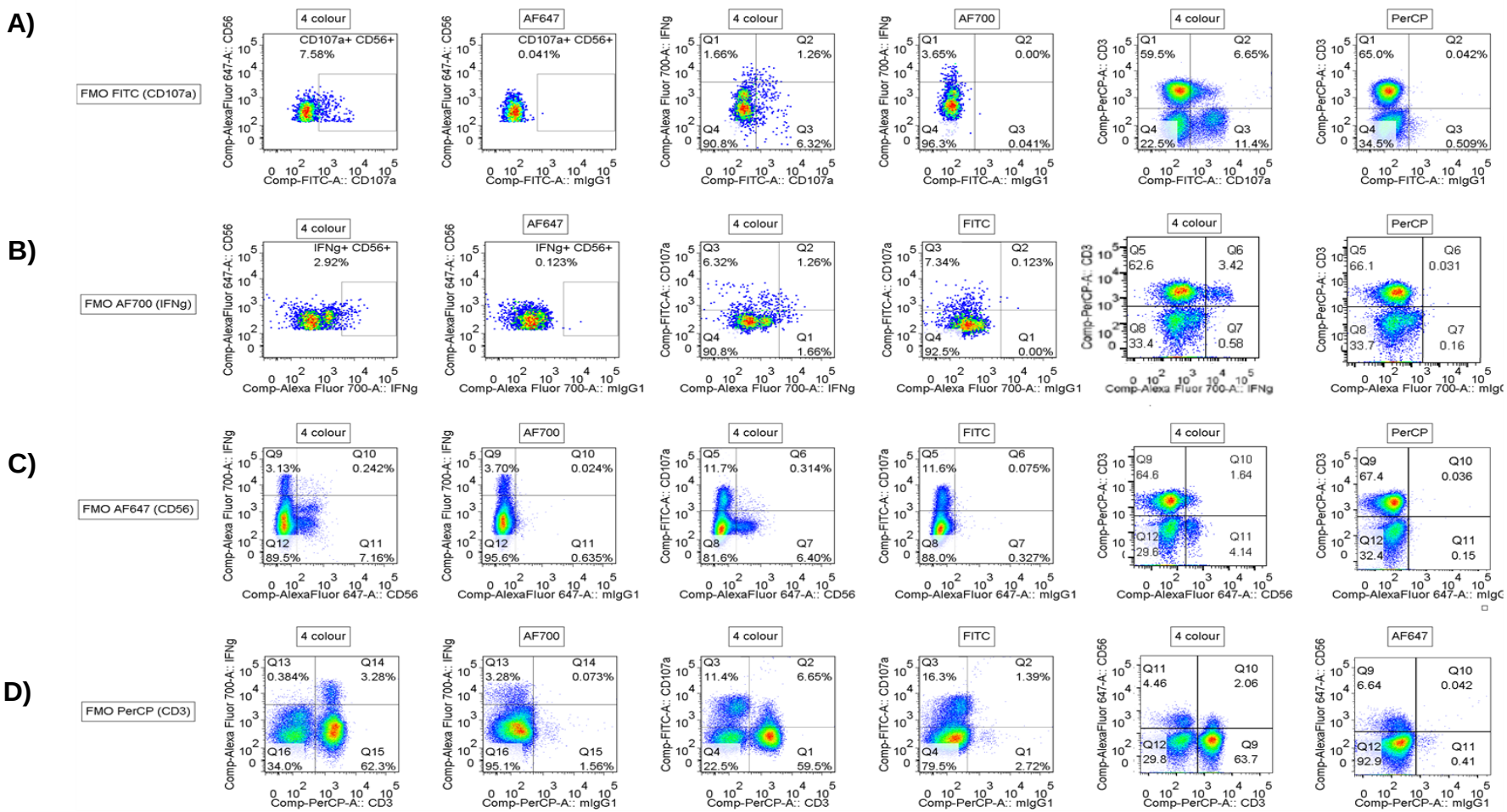


Figure 5.4. Fluorescence minus one.

Four separate FMO controls-PBMC cells stained with all but one fluorochrome that was being tested: A) FITC (for CD107a staining) B) AF700 (for IFNγ staining), C) AF647 (for CD56 staining) and D) PerCP (for CD3 staining). Positive control were PBMCs stained with four colours (depicted on the graph as “four colours”. Control sample is followed by test sample in order to compare the proportion of positive cells in both control and test samples in the same settings.

5.3. Results.

5.3.1. Validation of ADCC system.

In order to ensure that my assay was working effectively I first established the conditions for a robust positive control. Thus the first series of experiments was conducted with different concentrations of PMA/Ionomycin (a potent activator of NK cells) and a K562 target cell line at different effector to target ratios.

5.3.1.1. Stimulation of PBMC with PMA/I.

Firstly, it was tested whether stimulation of PBMC with PMA/I upregulated the expression of ADCC marker (CD107a) on activated NK cells. PMA (Phorbol-Myristate-Acetate) is a substitute for DAG (diacylglycerol), one of the adaptor molecules that induce the signal transduction enzyme protein kinase C (PKC) [417, 418]. Ionomycin is a selective calcium ionophore whose major role is to raise the intracellular level of calcium (Ca^{2+}), [419]. The combination of these molecules (PMA/I) aids the activation of PKC and facilitates an influx of intracellular calcium; both events are required to induce signalling pathways for degranulation [401, 417];

PBMCs were stimulated with PMA/I at the following concentrations [395, 400, 401];
-PMA 50ng/mL and ionomycin 100ng/mL;
-PMA 50ng/mL and ionomycin 500ng/mL;
-PMA 2500ng/mL and ionomycin 500ng/mL**.**

PBMC stimulation with PMA/ionomycin resulted in a significant increase in the level of expression of CD107a and IFNγ on activated NK cells at the concentrations of 50ng/mL PMA + 100ng/mL ionomycin and 50ng/mL PMA + 500ng/mL ionomycin. At the highest concentration: 2500ng/mL PMA + 500ng/mL ionomycin an upregulation of the expression of CD107a was observed, but interestingly the IFNγ production was lost. The highest level of the expression of both markers was observed with the cells stimulated with a concentration of: PMA 50ng/mL and ionomycin 500ng/mL (Figure 5.5).

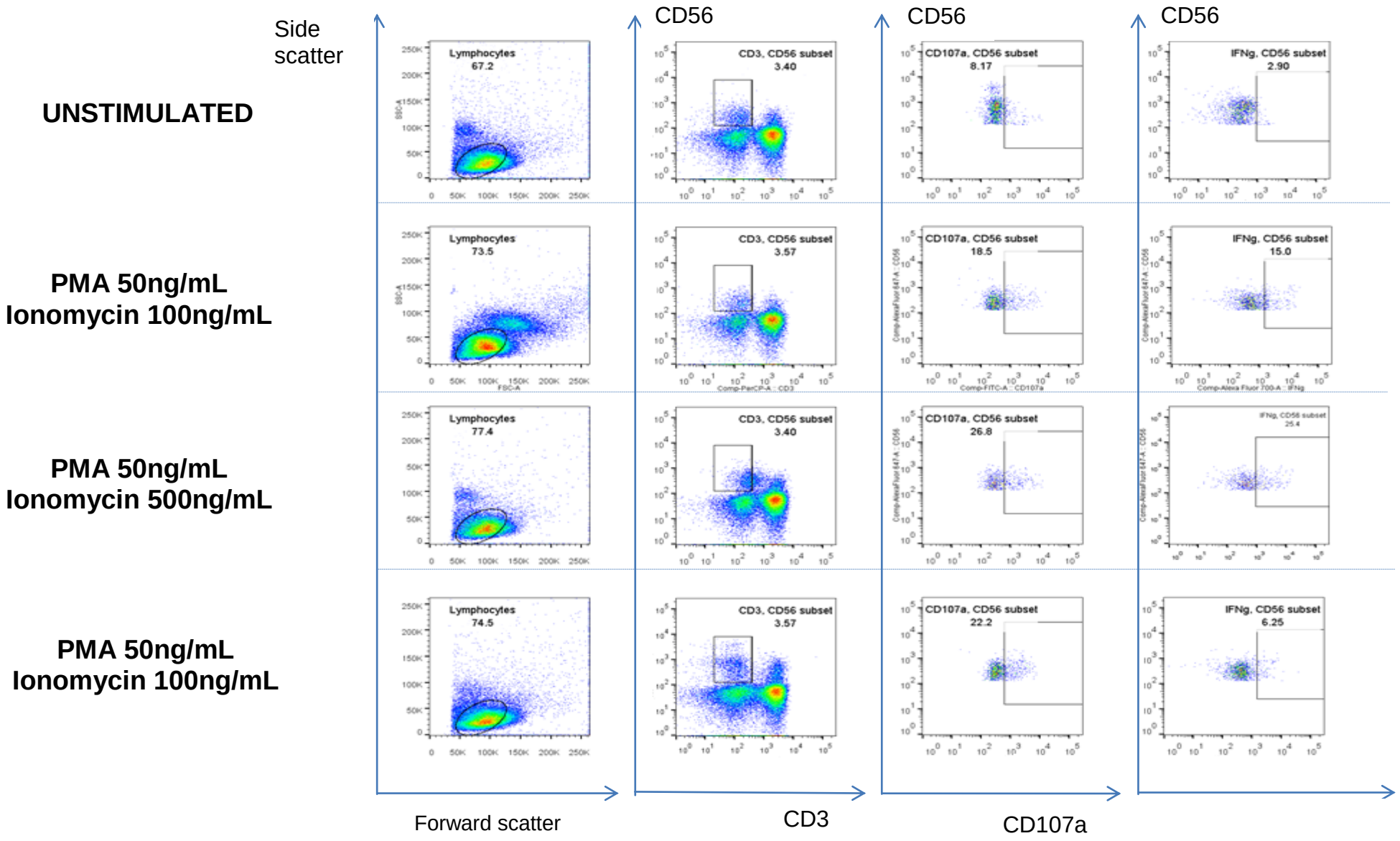


Figure 5.5. Level of expression of CD107a and IFNγ on CD3-CD56+ cells following stimulation of PBMCs with PMA/I. PMA/I was used at three different concentrations: i) PMA: 50ng/mL, ionomycin: 100ng/mL; ii) PMA: 50ng/mL, ionomycin: 500ng/mL; iii) PMA: 2500ng/mL, ionomycin: 500ng/mL. The unstimulated cells are negative control for this assay .

5.3.1.2. Stimulation of PBMC with K562 cell line.

In order to investigate whether incubation of NK cells with K562 would result in higher level of CD107a expression in comparison to the stimulation with PMA/I several assays were performed. In this set of experiments cells were co-incubated at effector to target ratio (NK: K562): 1:1 or 5:1 with or without concomitant stimulation with PMA/I. Negative controls were unstimulated PBMC, and unstimulated K562 cells (Figure 5.6).

As expected, activation of NK cells was observed following the stimulation with K562. The increase in the level of CD107a expression was seen at both E:T ratios where 9.2% and 8.2% of CD3-CD56+ cells were expressing CD107a (5:1; 1:1 respectively). However, only 5.26% of CD3-CD56+ cells were expressing CD107a in the negative control (unstimulated PBMCs).
The concomitant stimulation of PBMCs with K562 and PMA/I also activated NK cells. I observed an increase in the number of cells expressing CD107a compared to unstimulated PBMCs. Specifically 10.2% and12.9% of NK cells were expressing CD107a (5:1; 1:1, neg. control, respectively).

In summary, stimulation of PBMCs with PMA/I only, at the concentration of PMA 50ng/mL and ionomycin 500ng/mL appeared to have the most profound effect on NK cell activation (16.2% of CD3-CD56+ cells were expressing CD107a); (Figure 5.6). Consequently, it has been confirmed that PBMC stimulation with PMA/I only was sufficient to induce high levels of CD107a degranulation. Based on these experiments, this dosage was chosen as a positive control for the future degranulation assays.

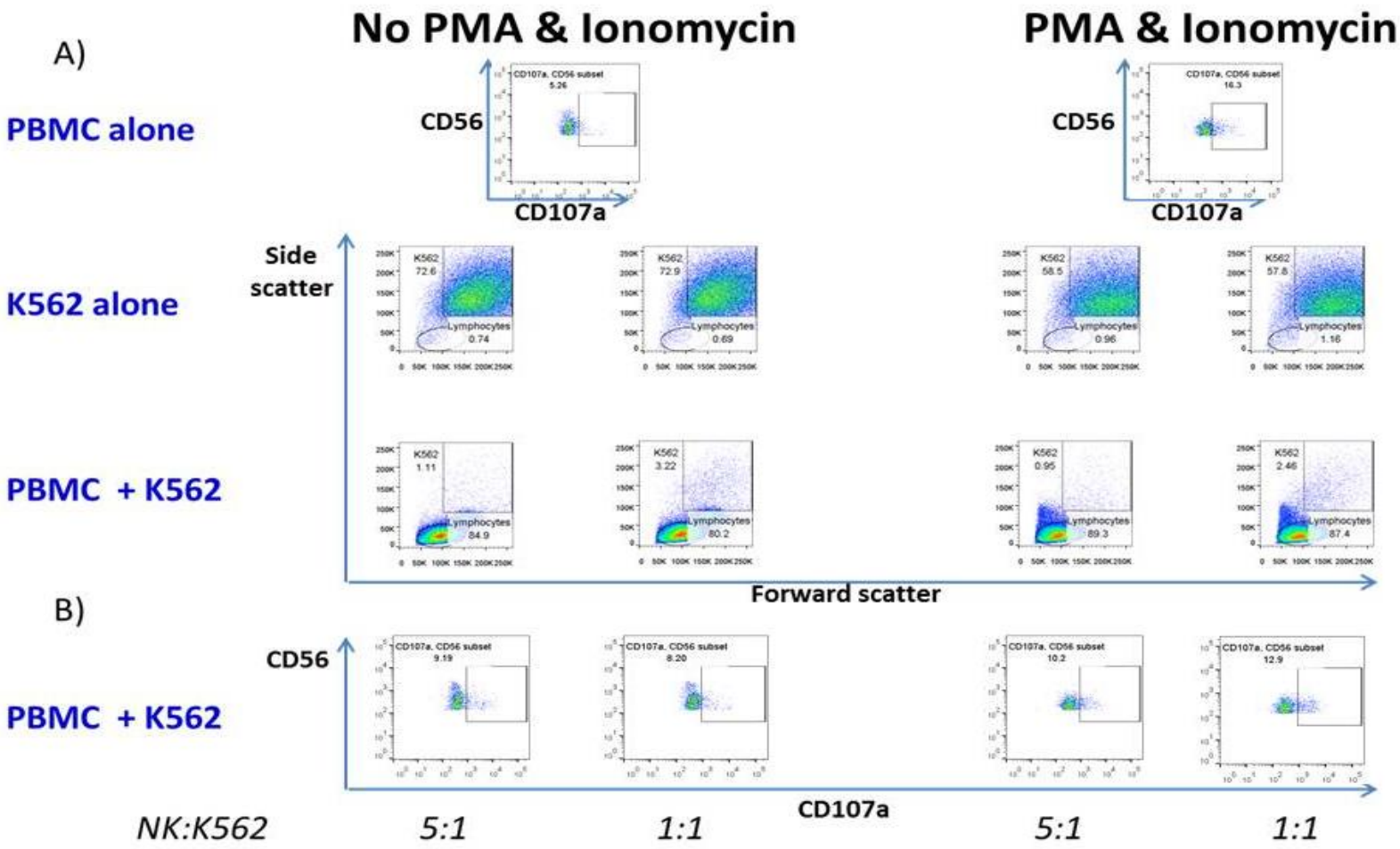


Figure 5.6. The level of CD107a marker expression following PBMC stimulation with K562 human erythroleukemic cell line.

A) without concomitant PMA/I stimulation and B) with concomitant PMA/I stimulation; at different effector to target ratios: 5:1 and 1:1.

5.3.2. The effect of heat inactivation of serum samples from healthy donor HCMV seropositive and HCMV seronegative patients on the level of CD107a expression by NK cells in total PBMCs.

Heat inactivation of serum is required to eliminate the influence of various immune factors, particularly serum complement, which may have an impact on the outcome of the experiments. In order to examine whether there is any evidence of beneficial effect of serum heat- inactivation in the assay; ten neat HCMV seropositive and ten HCMV seronegative sera from healthy donor patients were tested with and without prior heat-inactivation. The expression of CD107a marker on activated NK cells ($CD3^{-}CD56^{+}$) was measured.

The data from this indirect assay show that there is a statistically significant difference in the expression of the CD107a protein on activated NK cells in whole PBMC between seropositive and seronegative HCMV healthy serum donors irrespective of whether the sera were heat-inactivated or not. However, the difference in the expression of CD107a was higher and more statistically significant between seropositive and seronegative serum donors when the samples were heat– inactivated prior to analysis (Figure 5.7).

In summary, these data show that heat inactivation of the serum samples enhances the difference in the level of the CD107a de-granulation between HCMV seropositive and seronegative patients. Thus, for all future experiments measuring ADCC only heat inactivated serum samples were used in order to preserve the integrity of these immunoassays and eliminate confounding effects of complement in the patient serum.

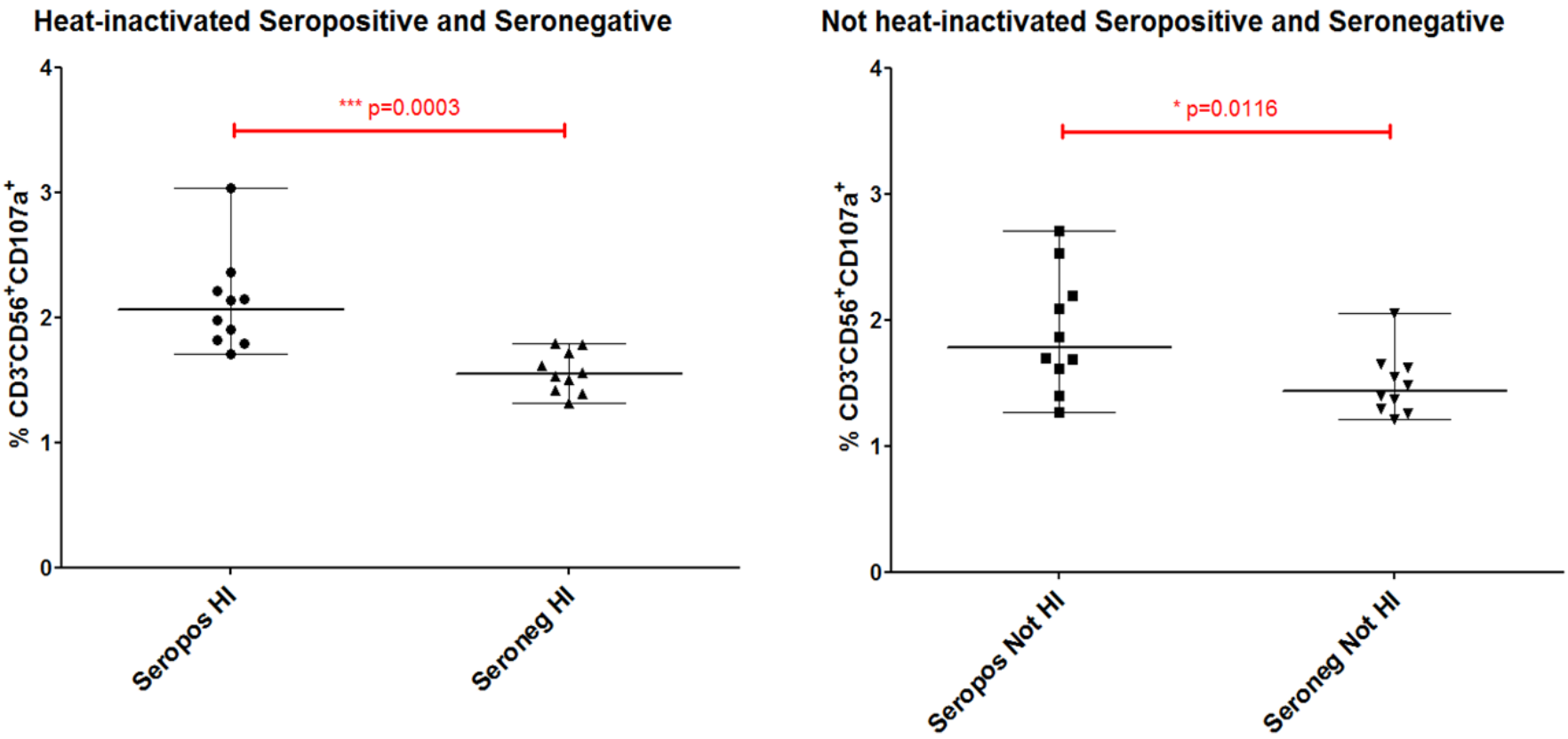


Figure 5.7. The effect of heat inactivation of serum on the level of CD107a expression by CD3-CD56+ (NK) cells.

Sera were obtained from healthy HCMV seropositive (n=10) and seronegative (n=10) donors. The differences between groups were assessed by Mann Whitney U test and significant differences are depicted in red. The horizontal lines indicate the median value and the range.

5.3.3. The effect of serum dilution on the level of CD107a expression by NK cells in total PBMCs and the establishment of the optimal serum dilution.

In order to maximise the use of the precious biological material from the gB/MF59 clinical trial (described in chapters: 1.11. and 2.1.) the effect of serum dilution on the expression of CD107a was studied to determine the highest dilution of serum that can be used without losing bioactivity in the assays.

To address this, neat and diluted (1/5, 1/10, 1/20) heat-inactivated sera from healthy HCMV seropositive (=10) and seronegative (n=10) donors were tested in the ADCC assay. As expected, no expression of CD107a protein on NK cells analysed from a HCMV seronegative donor was detected when the PBMC were incubated with seronegative sera (Figures 5.8C and 5.9C) confirming that the assay is not detecting non-specific reactions. However, when the NK cells from HCMV seronegative donor were incubated with seropositive donors' sera cell surface expression of CD107a was observed. Interestingly, there was a substantial variability in the level of CD107a expression in response to the different donors' serum. Although incubation with sera from some donors failed to induce higher level of CD107a expression, addition of sera from the others resulted in high level of NK cell degranulation (Figures 5.8A and 5.9A).

Interestingly, when the same HCMV seronegative serum samples were incubated with PBMC from a HCMV seropositive donor, expression of the CD107a protein on NK cells was observed (Figures 5.8D and 5.9D). Moreover, the incubation of serum from HCMV seropositive donors with NK cells from HCMV seropositive donor resulted in similar level of CD107a expression as with seronegative sera (Figures 5.8B and 5.9B).

In summary, there were no statistically significant differences in the expression of CD107a by activated NK cells between the dilutions of serum in both HCMV seronegative and seropositive groups of patients when incubated with PBMCs from healthy HCMV seronegative (Figures: 5.8A, C and Figures: 5.9A, C) and healthy HCMV seropositive donors of serum (Figures: 5.8B, D and Figures: 5.9B, D). However, a trend was observed amongst the seropositive patients whose sera were incubated with NK cells from HCMV seronegative donor- the number of patients displayed a higher level of CD107a expression when incubated with diluted sera (1/5 and 1/10). Therefore, it was decided that all the patients' serum samples should be diluted 1/5 prior to analysis.

5.3.4. The effect of PBMC donor serostatus on the expression of CD107a by NK cells.

The differential effects in response to serum observed with PBMCs isolated from seropositive and seronegative donors (described in section 5.3.3) suggested that serostatus of the PBMC donor might influence the responsiveness of NK cells and therefore impact on the fidelity of the ADCC assay I intended to use in my assays of the vaccinees' sera.

Thus I wished to investigate the effect of PBMC donor HCMV serostatus on the expression of CD107a by NK cells in more detail. PBMCs from heathy HCMV seropositive- and seronegative donors were incubated with serum taken from both seropositive (n=10) and seronegative (n=10) donors. Additionally, the HCMV seropositive and seronegative PBMCs were tested against their autologous serum.

The first analyses concerned the incubation of seropositive donor PBMC with serum from both seropositive and seronegative individuals. The data show that no differences in CD107a expression were observed under all experimental conditions (neat and diluted sera). Thus irrespective of HCMV serostatus, seropositive PBMCs incubated with serum displayed evidence of elevated CD107 expression (Figure 5.10). Interestingly, mean values of CD107a expression of

seronegative donor samples at all different dilutions were higher than the expression of CD107a in negative control (unstimulated seropositive PBMCs); (Figure 5.8 A, C). In contrast, when PBMCs from a HCMV seronegative healthy donor were incubated with sera, the differences in the expression of CD107a following incubation with seropositive and seronegative donors' serum were statistically significant under all conditions (neat-seropositive vs neat-seronegative serum: p=0.0003; 1/5 diluted seropositive vs 1/5 diluted seronegative serum: p=0.0018; 1/10 diluted seropositive vs 1/10 diluted seronegative serum: p=0.0031; 1/20 diluted seropositive vs 1/20 diluted seronegative serum: p=0.0018); (Figure 5.11). The data show that evidence of ADCC activation (e.g. CD107 expression) was only observed when the PBMC were incubated with seropositive donor sera.

In order to investigate this, additional studies of the seropositive and seronegative purified NK cells and total PBMCs were performed. The results of these serological analyses (of sera from both healthy HCMV seropositive and seronegative donors) revealed no major differences in the level of CD107a expression measured between NK cells from seropositive and seronegative donors (Figure 5.12). I could observe that the expression of CD107a on seropositive NK cells (Figure 5.12B) resembles the expression patterns on seronegative NK cells (Figure 5.12C). Moreover, I could observe similar results with total PBMCs from seropositive donor (Figure 5.12A and D). In both scenarios (purified NK cells and NK cells in total PBMCs) I could see clear differences in the level of CD107a expression between seropositive and seronegative sera (Figure 5.12).

These results did not confirm previous findings therefore a third analysis was performed (Figure 5.13). Again, I could observe clear differences in the level of CD107a expression between seropositive and seronegative sera; with the level of CD107a expression on the latter ones being similar to negative controls in both purified NK (Figure 5.13C and D) cells and total PBMCs (Figure 5.13A and B) from seropositive and seronegative donors.

The reasons for this discrepancy were unclear. However, in order to proceed with the analyses of the sera from the clinical trial I decided to use the NK cells in total PBMCs from seronegative donor only (excluding PBMC from seropositive donor) to avoid any potential confounding factors (see 5.3.7).

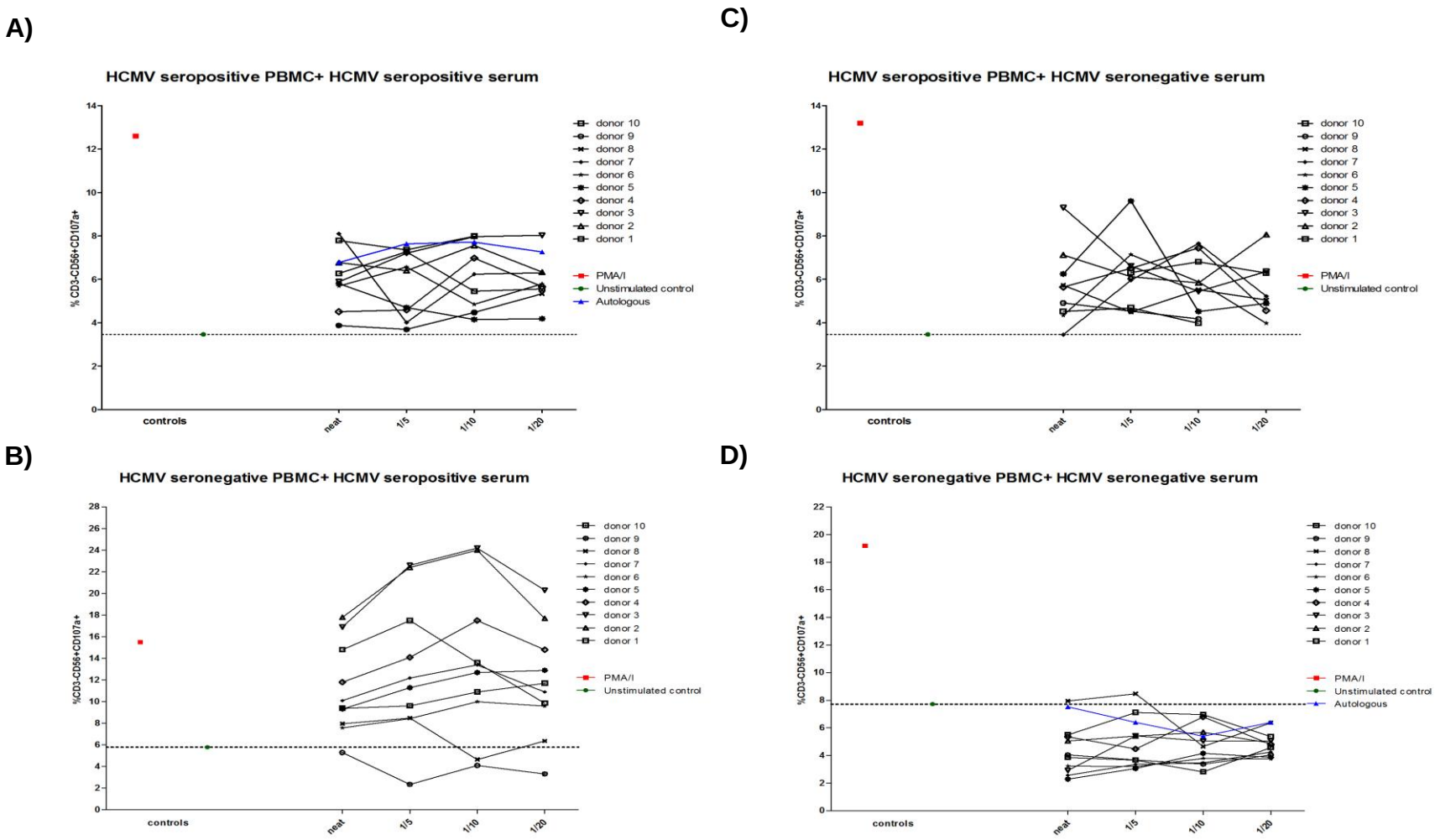


Figure 5.8. Level of CD107a expression on NK cells in whole PBMCs incubated with different dilutions of sera.

A) PBMCs from HCMV one seronegative healthy donor incubated with HCMV seropositive (n=10) and C) seronegative (n=10) neat and diluted (1/5; 1/10; 1/20) sera from healthy donors; B) PBMCs from one HCMV seropositive healthy donor incubated with HCMV seropositive (n=10) and D) seronegative (n=10) neat and diluted (1/5; 1/10; 1/20) sera from healthy donors.

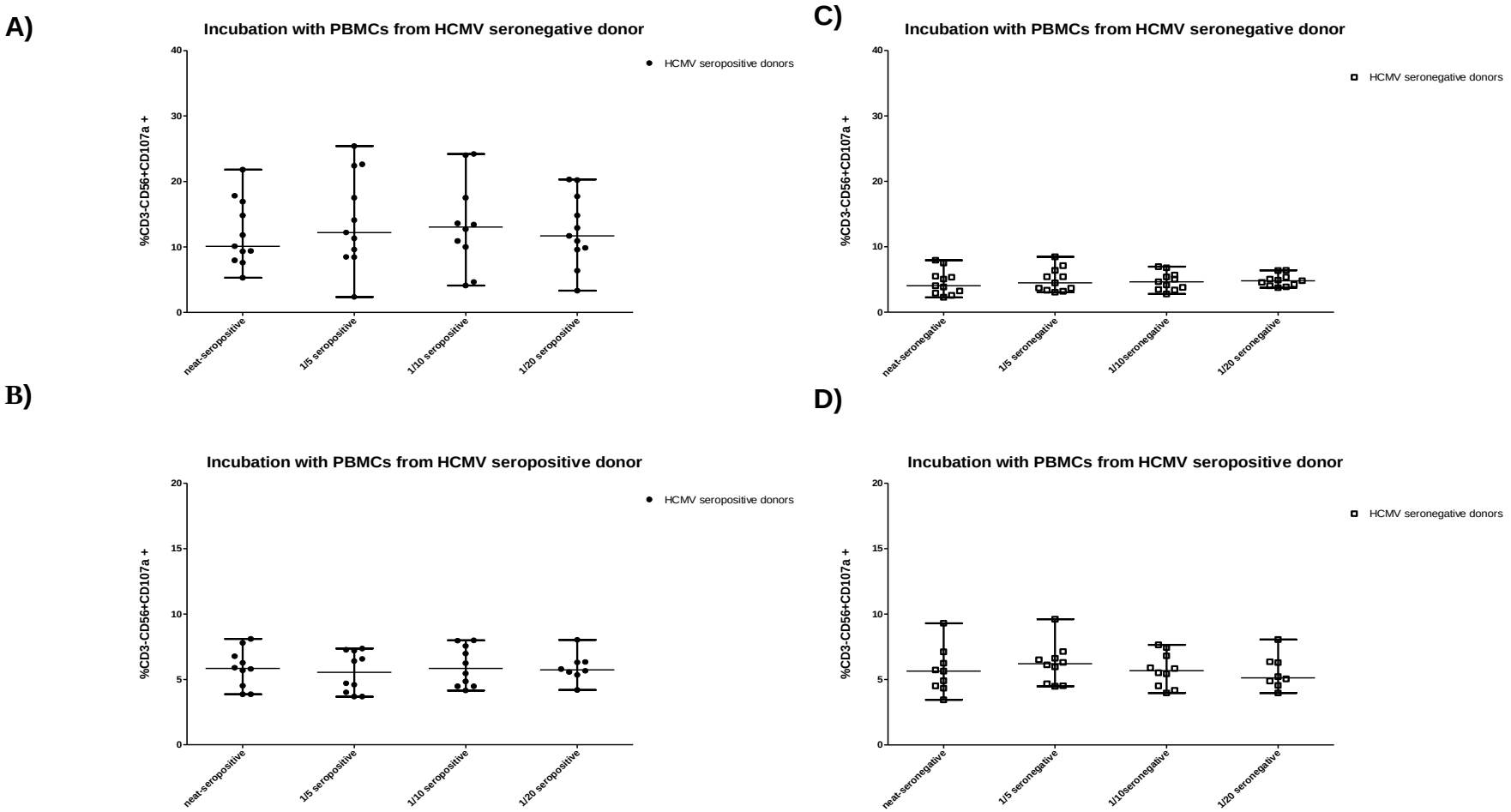


Figure 5.9. Minimal effect of serum dilution on the level of CD107a expression.

A). Incubation of PBMCs from HCMV seronegative donor with sera from HCMV seropositive (n=10) healthy donors C). Incubation of PBMCs from HCMV seronegative donor with sera from HCMV seronegative (n=10) healthy donors. B) Incubation of PBMCs from HCMV seropositive donor with sera from HCMV seropositive (n=10) healthy donors D) Incubation of PBMCs from HCMV seropositive donor with sera from HCMV seronegative (n=10) healthy donors. The horizontal lines indicate the median value and the range. All differences in the expression of CD107a between compared groups were not statistically significant (ns; p>0.05). P values were calculated by Mann Whitney U test.

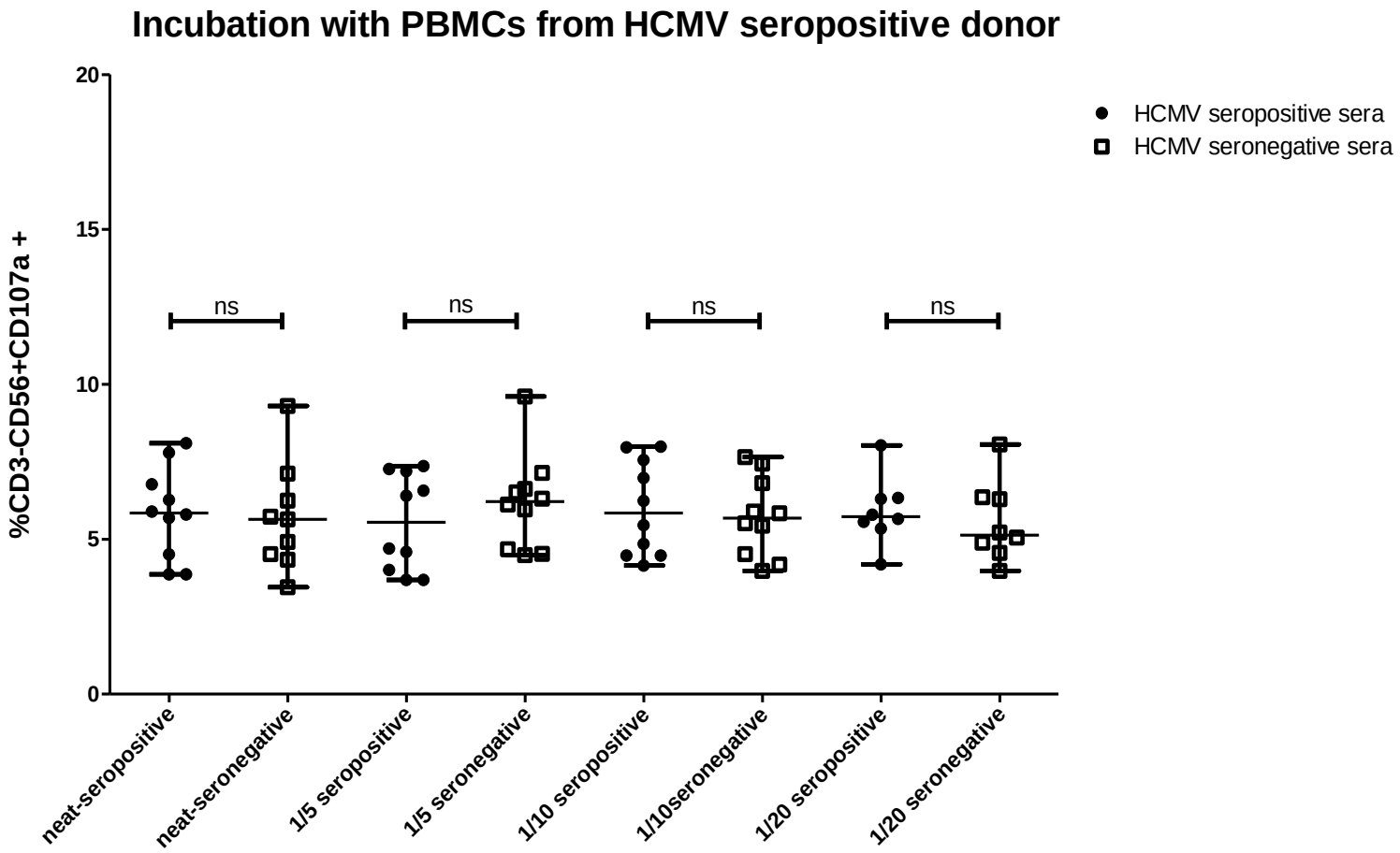


Figure 5.10. Minimal differences in the level of CD107a expression on PBMCs from one HCMV seropositive healthy donor when incubated with HCMV seropositive and seronegative sera.

The seropositive (n=10) and seronegative sera (n=10) were obtained from healthy donors. The horizontal lines indicate the median value and the range. All differences in the expression of CD107a between compared groups were not statistically significant (ns; p>0.05). P values were calculated by Mann Whitney U test.

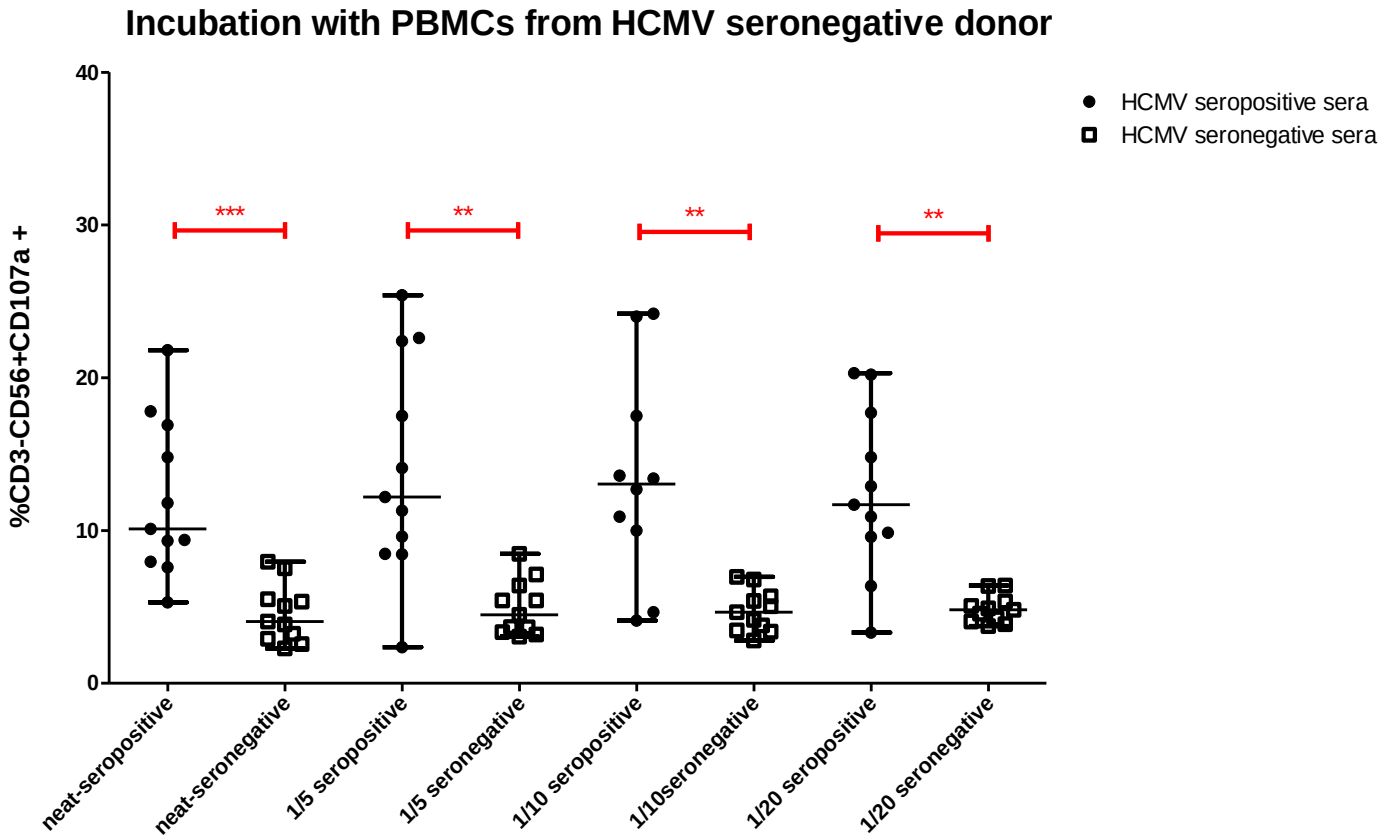


Figure 5.11. Significant differences in the level of CD107a expression on PBMCs from one HCMV seronegative healthy donor when incubated with HCMV seropositive and seronegative sera.

The seropositive (n=10) and seronegative sera (n=10) were obtained from healthy donors. The horizontal lines indicate the median value and the range. The differences between groups were assessed Mann Whitney U test and P values were depicted in red. All the differences in the expression of CD107a between compared groups were statistically significant (neat-seropositive vs neat-seronegative serum: p=0.0003; 1/5 diluted seropositive vs 1/5 diluted seronegative serum: p=0.0018; 1/10 diluted seropositive vs 1/10 diluted seronegative serum: p=0.0031; 1/20 diluted seropositive vs 1/20 diluted seronegative serum: p=0.0018).

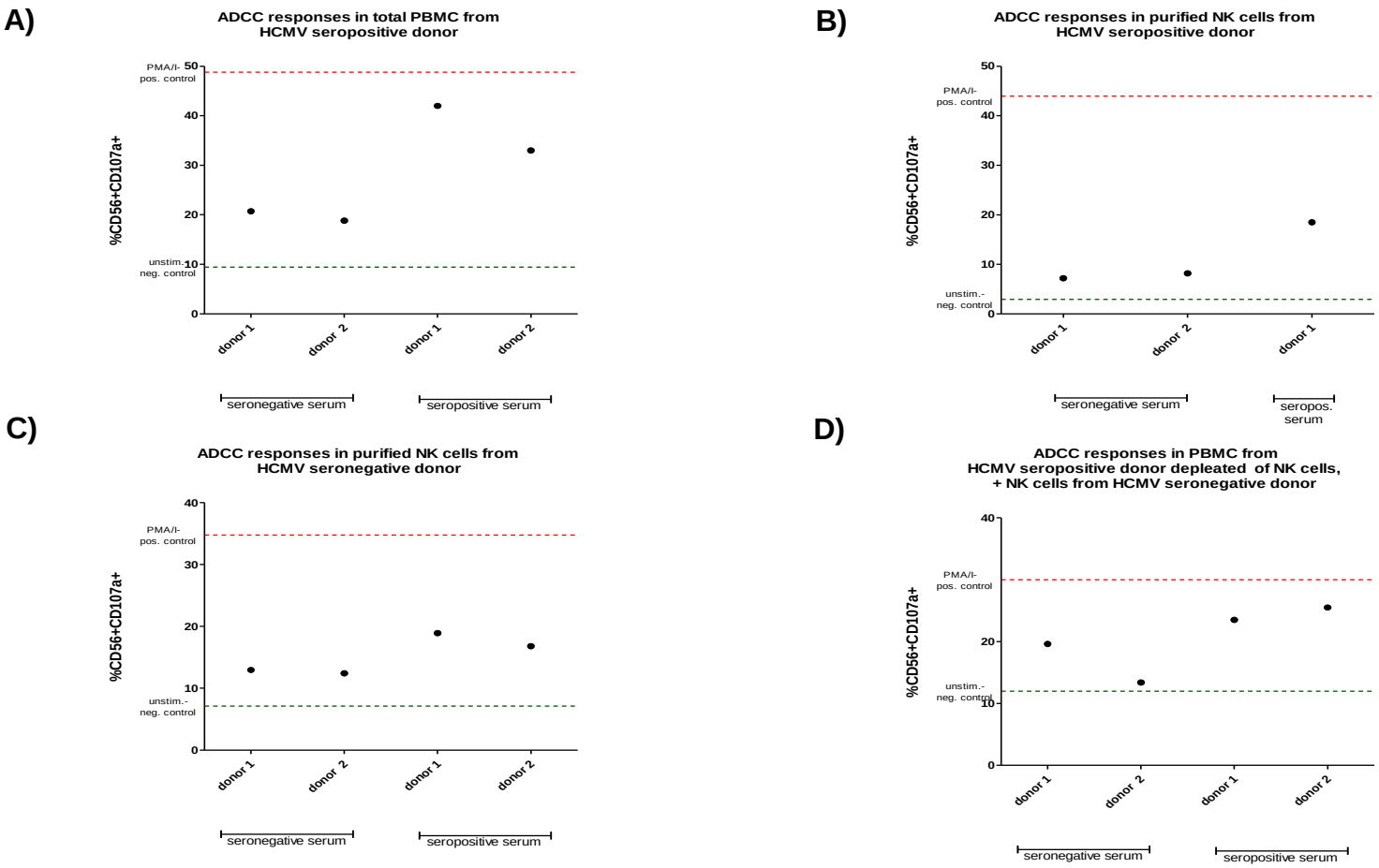


Figure 5.12. Similar pattern of ADCC responses between NK cells in total PBMCs from seropositive donor and purified NK cells from seropositive and seronegative donors.

Level of CD107a expression on purified NK from healthy HCMV seropositive donor (B); healthy seronegative donor (C) and on NK cells in total PBMCs isolated from healthy HCMV seropositive individual (A) and in total PBMCs isolated from healthy HCMV seropositive individual depleted from NK and incubated with NK cells isolated from healthy HCMV seronegative individual (D); when incubated with sera from seropositive and seronegative healthy donors.

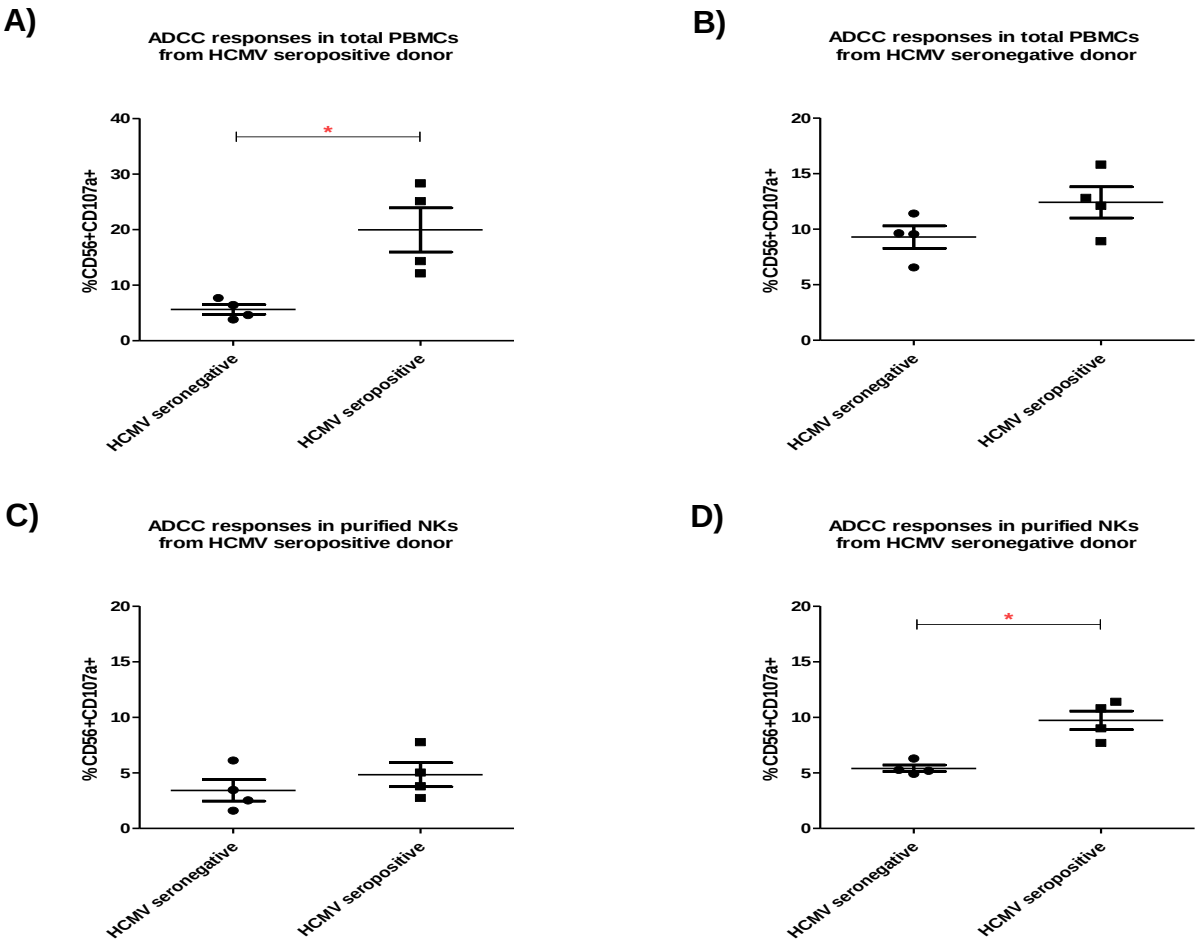


Figure 5.13. Similar pattern of ADCC responses between NK cells in total PBMCs and purified NK cells.

The ADCC responses were measured by the CD107a expression on: NK cells in total PBMCs isolated from healthy HCMV seropositive individual (A) NK cells in total PBMCs isolated from healthy HCMV seronegative individual (B); purified NK from healthy HCMV seropositive donor (C); purified NK from healthy HCMV seronegative donor (D); when incubated with sera from seropositive and seronegative healthy donors.

5.3.5. Investigating the influence of PBMCs versus purified NK cells and their donor serostatus on the level of CD107a expression.

In order to test whether the addition of purified NK cells instead of total PBMCs would influence the level of ADCC activity measured by this indirect assay, serum samples from renal transplant patients who participated in the gB/MF59 trial were analysed with purified NK cells from a HCMV seronegative healthy donor. The level of CD107a expression was used as a marker of ADCC activity and measured as described in 5.2.5. Purified NK cells obtained from a HCMV seronegative healthy donor were incubated with sera from HCMV seropositive (n=8) and seronegative (n=8) renal transplant patients. As in previous analyses, NK cells stimulated with PMA/I served as a positive control for the assay. NK cells were also tested against their autologous sera which, alongside unstimulated NK cells, served as a negative control for the assays (Figure 5.14 A, B; Figure 5.15 A, B).

The analyses of CD107a expression on purified NK cells from healthy seronegative donor incubated with seronegative sera showed similar results to those obtained in the previous assays with total PBMCs from seronegative donor. Besides one outlier, the level of CD107a responses in the remaining read-outs was below- or comparable to the level of this marker expression in negative controls (Figure 5.14 A, B). Lack of expression of CD107a in this assay is consistent with the previous finding- the lack of ADCC stimulating antibody responses in seronegative patients (Figure 5.8 D). Interestingly, when I performed similar analysis with the sera from seropositive renal transplant patients I could observe a range of ADCC responses (Figure 5.15 A, B). The level of CD107a expression greatly varies between the patients, as it was observed in the previous experiments with total PBMCs (Figure 5.8B) and generally the level of CD107a responses in the sera from these vaccinees corresponds well with the outcomes from the concomitant analyses of these patients with total PBMCs Therefore, due to economical and practical reasons I decided to test sera from the clinical trial with the NK cells in total PBMCs.

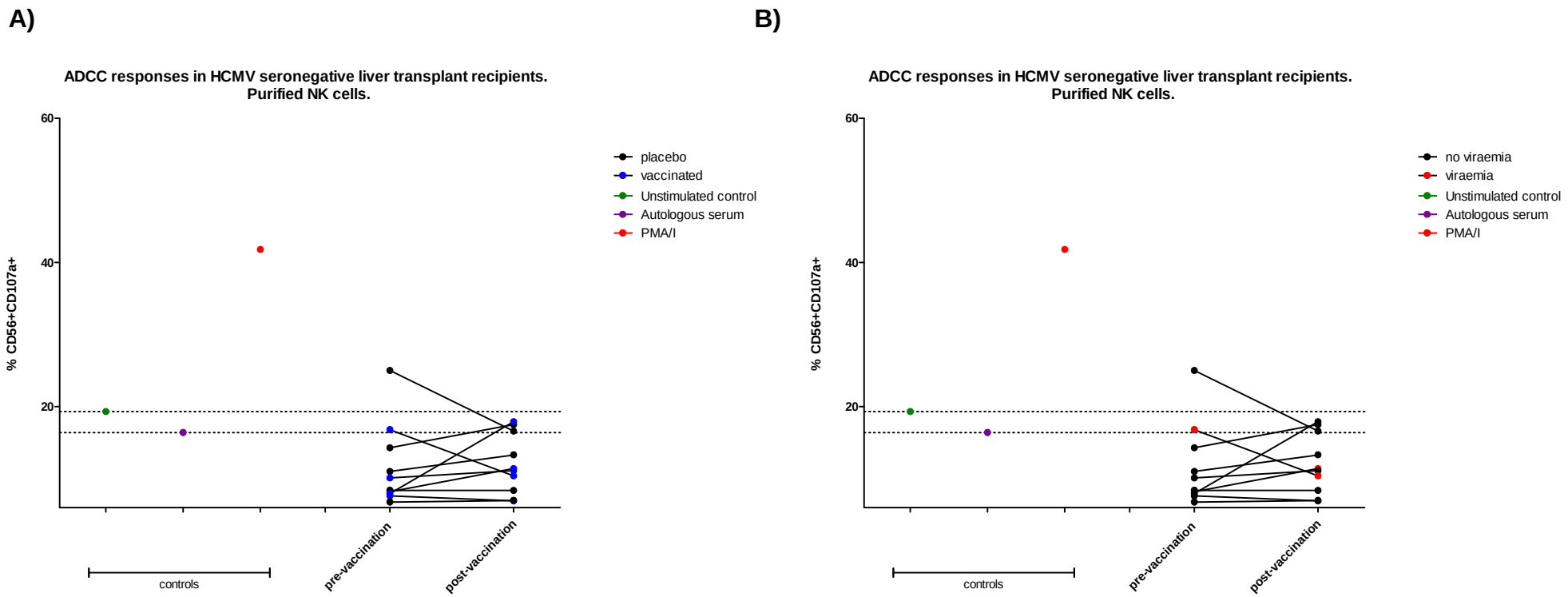


Figure 5.14. No evidence for vaccine-induced ADCC responses in seronegative liver transplant patients.

The level of the CD107a expression on activated, purified NK cells was a marker of ADCC activity. Sera used in this assay were collected from seronegative renal transplant patients who participated in phase-2 clinical trial (NCT00299260) subunit glycoprotein-B vaccine with MF-59 adjuvant at the day of vaccine or placebo administration (pre-vaccination) and at the day of transplantation (post-vaccination). A. Patients who received vaccination are depicted in blue (n=4); patients who received placebo are depicted in black (n=4). B. Patients who developed viraemia are depicted in red (n=2) and patients without viraemia are depicted in black (n=6).

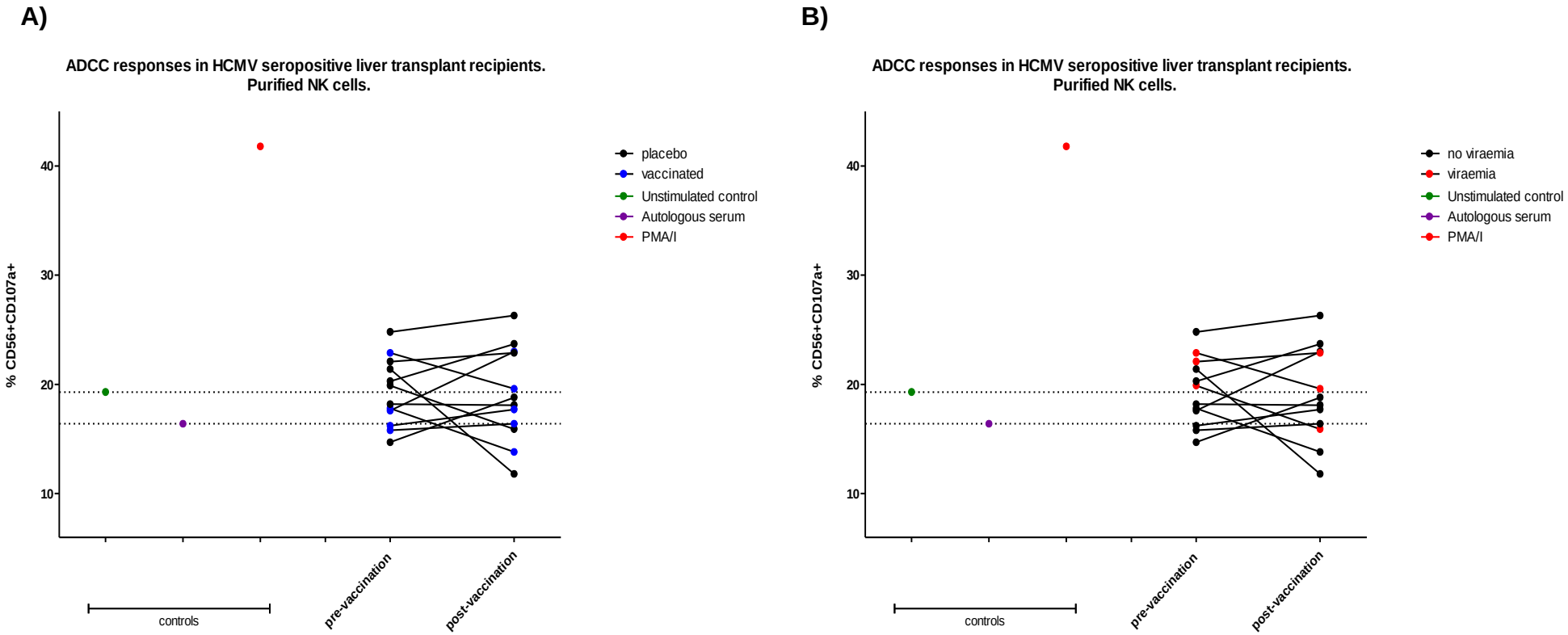


Figure 5.15. High variability of the ADCC responses in seronegative liver transplant patients.

The level of the CD107a expression on activated NK (purified) cells was a marker of ADCC activity. Sera used in this assay was collected from seropositive liver transplant patients who participated in phase-2 clinical trial (NCT00299260) subunit glycoprotein-B vaccine with MF-59 adjuvant at the day of vaccine or placebo administration (pre-vaccination) and at the day of transplantation (post-vaccination). A. Patients who received vaccination are depicted in blue (n=4); patients who received placebo are depicted in black (n=4). B. Patients who developed viraemia are depicted in red (n=3) and patients without viraemia are depicted in black (n=5).

5.3.6. Testing serum from the patients enrolled in the gB/MF59 clinical trial.

Having established conditions where the ADCC response to vaccine gB could be measured indirectly through CD107a cell surface expression, I was confident that the analysis of the serum samples collected from the transplant recipients was feasible.

Sera from patients were tested in this indirect ADCC assay and the level of the CD107a expression by activated NK cells was measured according to the procedure described in 5.2.5. Serum samples were processed and stored according to the NHS diagnostic serology procedure prior to analysis (described in section 2.2). Throughout my analyses I confirmed that CD107a expression could be induced on the NK cells using PMA/I as stimulation as a positive control.
Additionally, the PBMCs were also tested against their autologous serum which showed, as expected, that the level of CD107a on PBMCs stimulated with autologous sera (which is seronegative) was very low-similar to the level of expression of CD107a on unstimulated PBMCs (Figures:5.26- 5.29). Therefore, measuring the level of the CD107a expression by activated NK cells against autologous sera provided an additional negative control for the experiments.

I first analysed the sera from seropositive transplant recipients. These data showed that the incubation of PBMCs with gB and sera from seropositive renal and liver transplant patients resulted in higher levels of CD107a expression on the NK cell population in comparison to the level of CD107a expression on unstimulated NK cells (negative control); (Figures: 5.16; 5.20-5.22). Generally, a wide range of ADCC responses was observed prior and post vaccination. However, an analysis of the samples post vaccination provided no evidence that supported the hypothesis that the vaccine boosted pre-existing ADCC responses. No differences in the level of CD107a expression were observed between samples collected at different time points post vaccination- (v#1: v#5 and D0/D7); (Figures: 5.16; 5.20-5.22).

A comparison of the difference in expression of CD107a on NK cells in total PBMCs stimulated with sera from vaccinated and placebo groups of seropositive liver transplant patients at the time of transplantation also revealed no statistically significant differences (Figure 5.18). In fact, in the seropositive renal vaccine recipient samples when the sera from those patients was incubated with NK cells lower levels of CD107a expression were observed in comparison to the sera from placebo recipients (Figure 5.24). Furthermore, although evidence of ADCC promoting antibodies was observed in the seropositive patients there was no correlation between the level of CD107a expression (i.e. level of ADCC promoting antibodies) and patient outcome -no statistically significant differences in the expression of the CD107a on NK cells in total PBMCs incubated with sera from patients who developed viraemia vs patients without viraemia was observed in both liver and renal transplant patients (Figures 5.18 and 5.24). Generally, no differences were found between groups of seropositive patients when two variables were analysed in this seropositive cohort of patients (placebo/vaccine status and viraemia/no viraemia incidence).

I next analysed the serum samples from seronegative liver and renal transplant recipients who received vaccine or placebo in my ADCC assay. Interestingly, throughout these analyses I could detect no evidence of CD107a activation on NK cells in response to seronegative serum irrespective of vaccination or not. The level of CD107a expression in all the read-outs was comparable with the level of CD107a expression in negative control-unstimulated PBMCs (Figures: 5.26-5.29).

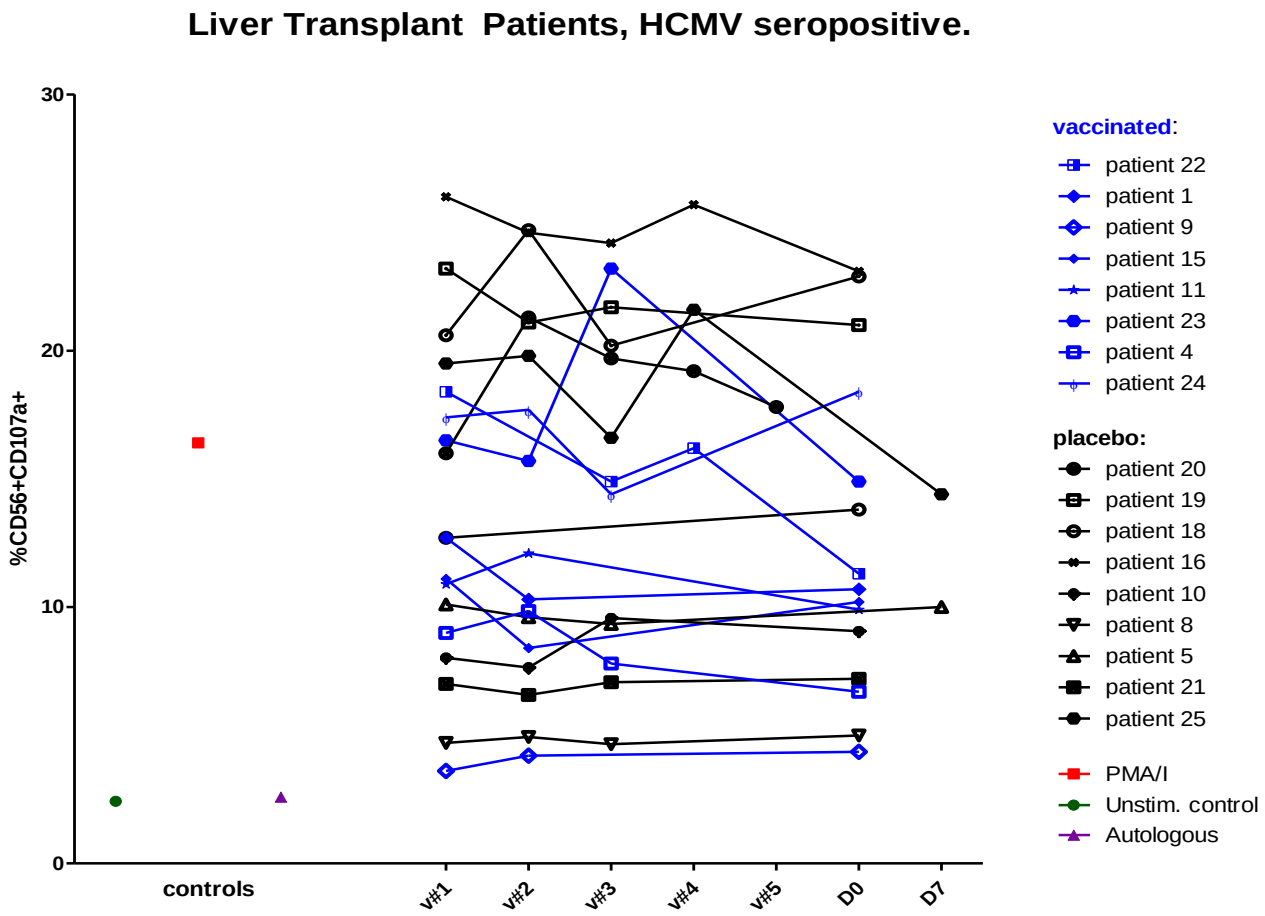


Figure 5.16. High variability in the level of the CD107a expression on activated CD3-CD56+ cells in seropositive liver transplant patients.

Sera were obtained from individuals who participated in the trial with gB/MF59 vaccine at the day of vaccine or placebo administration (v#1), 1week following the vaccination: 1month (v#2), 2 months(v#3), 6months (v#4), 7months (v#5) and at the day of transplantation (D0) or following the transplantation (D7). Vaccinated patients (n=8) are depicted in dark blue and placebo patients (n=9) are depicted in black

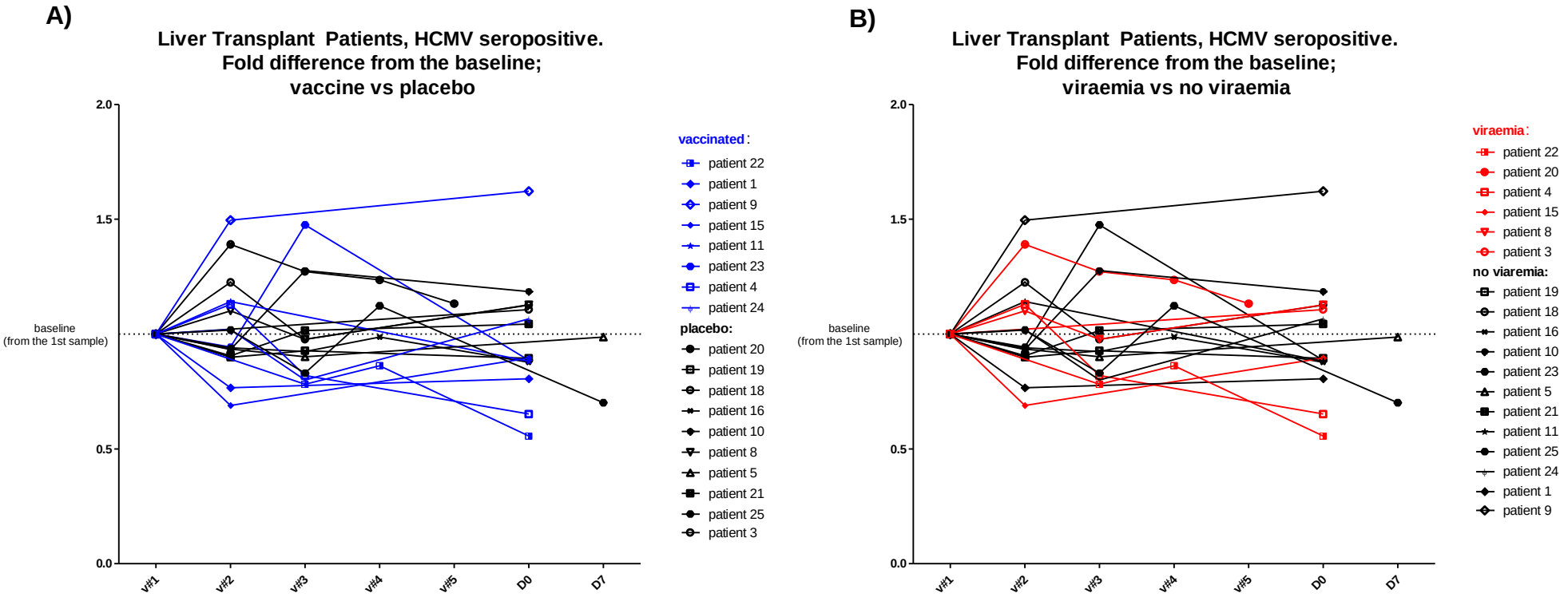


Figure 5.17. Fold difference from the baseline (v#1) in seropositive liver transplant patients shows no segregation in the level of CD107a expression on activated NK cells between groups of patients.

Sera from individuals (n=17) who participated in the trial with gB/MF59 vaccine were collected at the day of vaccine or placebo administration (v#1), 1week following the vaccination: 1month (v#2), 2 months(v#3), 6months (v#4), 7months (v#5) and at the day of transplantation (D0) or following the transplantation (D7). A) Vaccinated patients (n=8) are depicted in dark blue and placebo patients (n=9) are depicted in black. B) Patients who had viraemia (n=6) are depicted in red and patients with no viraemia (n=12) are depicted in black.

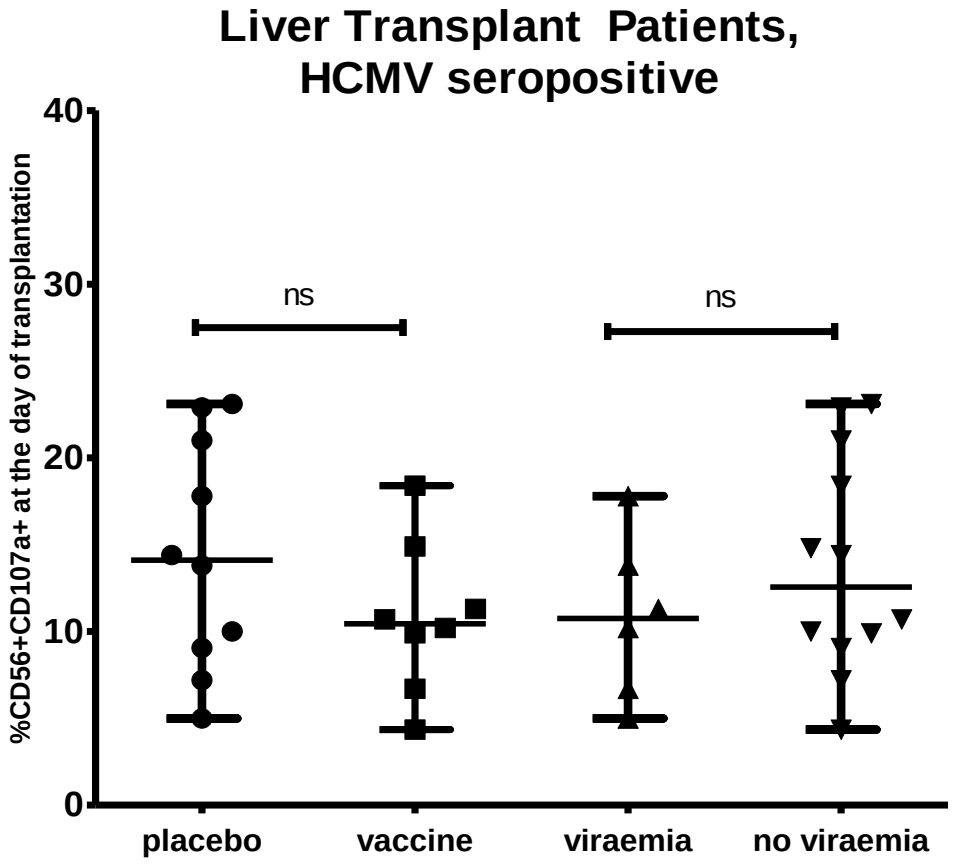


Figure 5.18. Comparable levels of CD107a expression on activated CD3-CD56+ (NK) between different groups of seropositive liver transplant patients.

Sera were obtained from individuals (n=17) who participated in gB/MF59 trial at the day of transplantation (D0) or 1 week following the transplantation (D7). The differences between groups were assessed by Mann-Whitney U test. The horizontal lines indicate the median value and the range. All the differences in the expression of CD107a between compared groups were not statistically significant (placebo vs vaccine: p=0.36; viraemia vs no viraemia p=0.426).

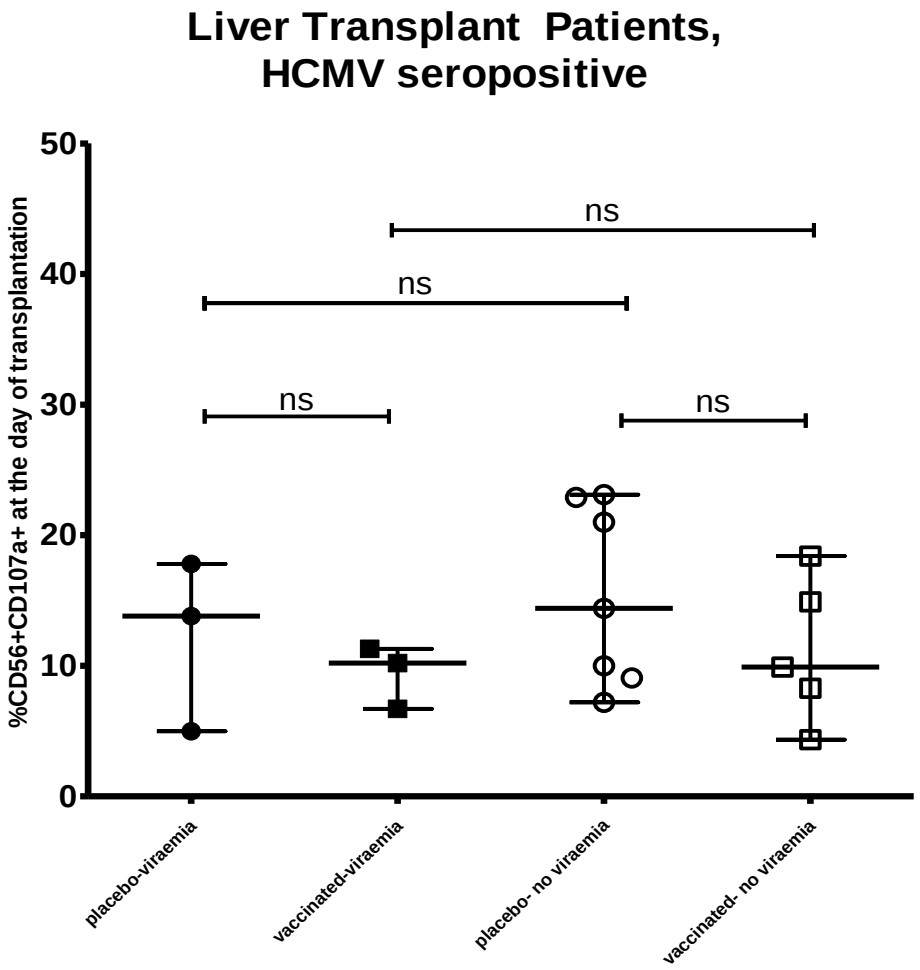


Figure 5.19. Minimal differences in the levels of the CD107a expression on activated CD3-CD56+ (NK) cells between different groups of seropositive liver transplant patients.

Sera from seropositive individuals (n=17) who participated in gB/MF59 trial at the day of transplantation (D0) or 1 week following the transplantation (D7). The horizontal lines indicate the median value and the range. All the differences in the expression of CD107a between compared groups were not statistically significant $p>0.05$. The differences between groups were assessed by Mann-Whitney U test.

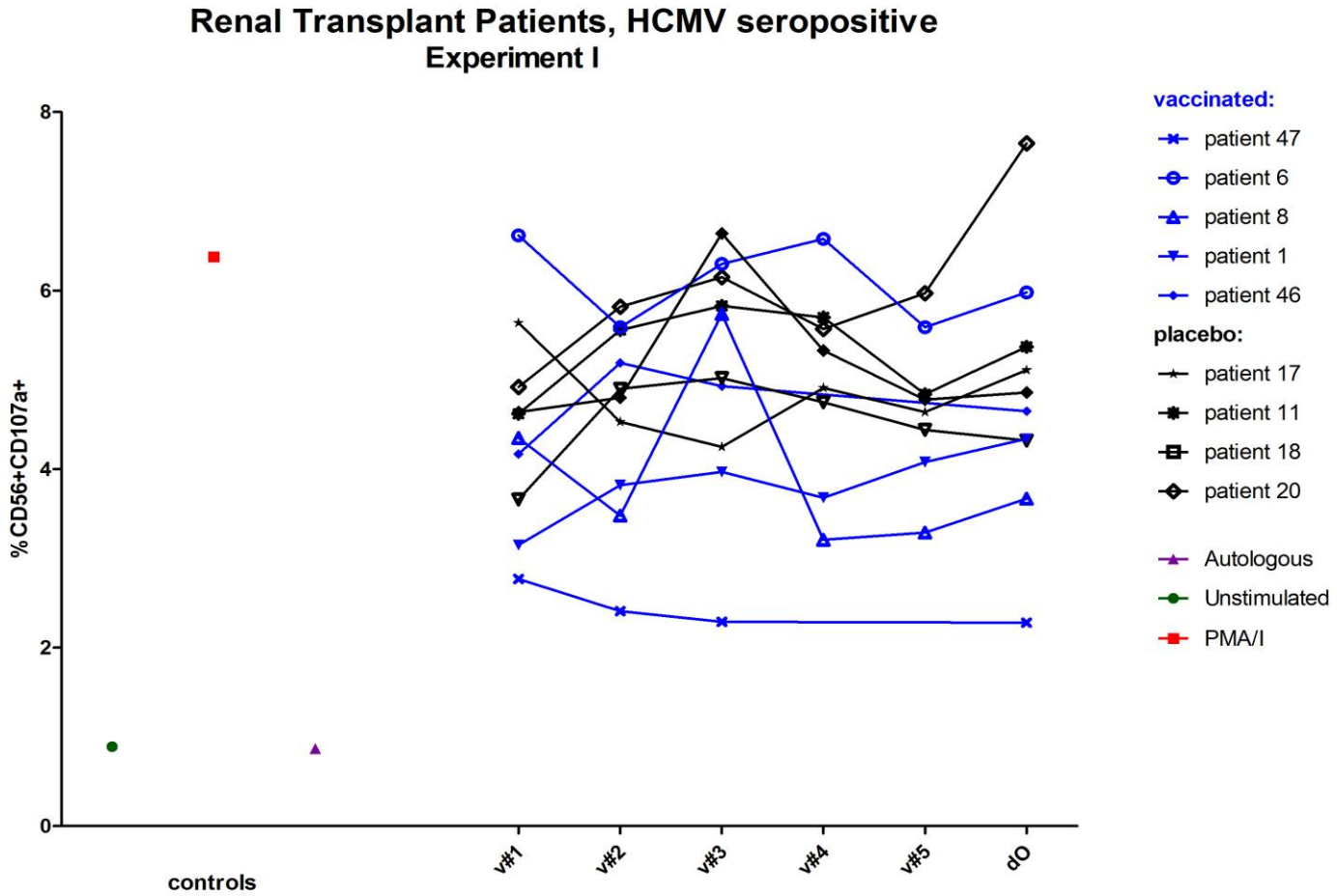


Figure 5.20. High variability in the level of the CD107a expression on activated CD3-CD56+ cells in seropositive renal transplant patients-experiment I.

Sera were obtained from individuals who participated in the trial with gB/MF59 vaccine at the day of vaccine or placebo administration (v#1), 1week following the vaccination: 1month (v#2), 2 months(v#3), 6months (v#4), 7months (v#5) and at the day of transplantation (D0) or following the transplantation (D7). Vaccinated patients (n=5) are depicted in dark blue and placebo patients (n=4) are depicted in black.

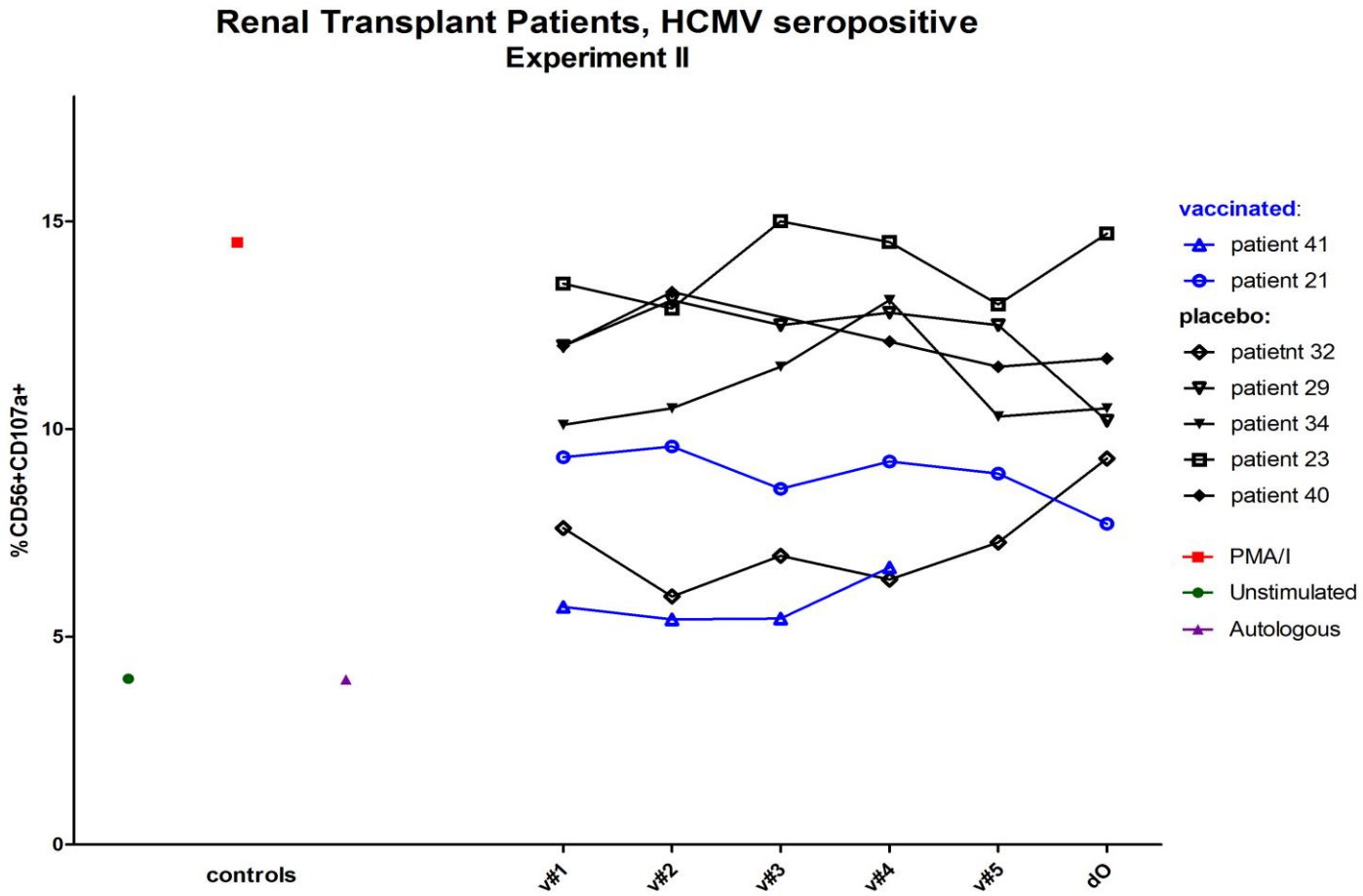


Figure 5.21. High variability in the level of the CD107a expression on activated CD3-CD56+ cells in seropositive renal transplant patients-experiment II.

Sera were obtained from individuals who participated in phase-2 clinical trial with gB/MF59 vaccine at the day of vaccine or placebo administration (v#1), 1week following the vaccination: 1month (v#2), 2 months(v#3), 6months (v#4), 7months (v#5) and at the day of transplantation (D0) or following the transplantation (D7). Vaccinated patients (n=2) are depicted in dark blue and placebo patients (n=5) are depicted in black.

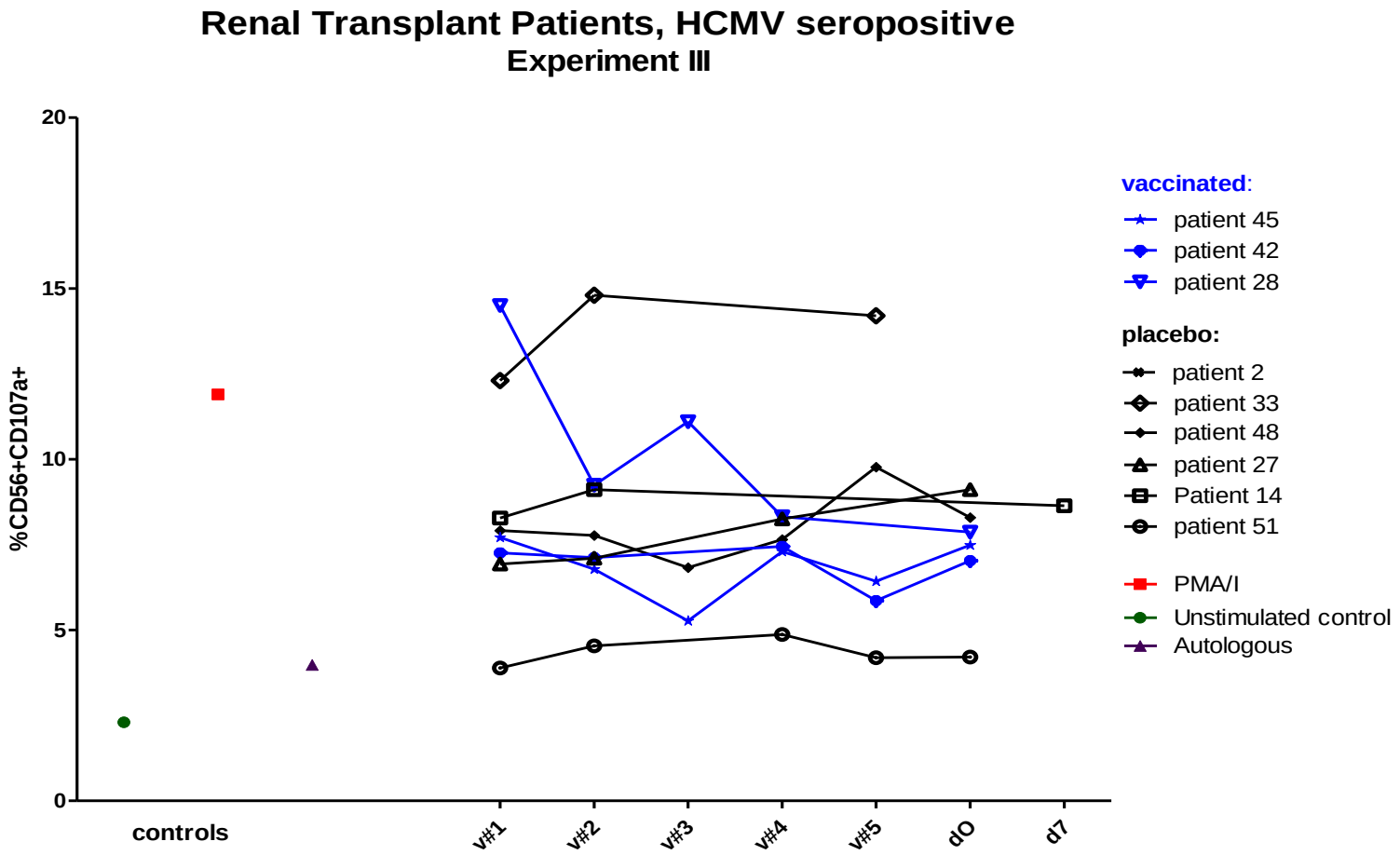
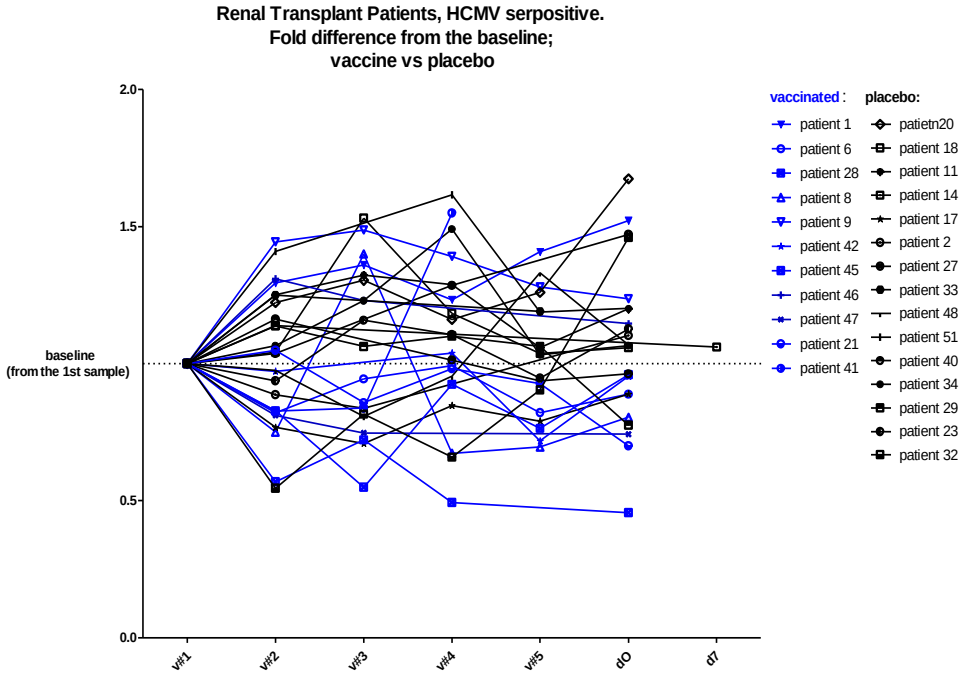


Figure 5.22. High variability in the level of the CD107a expression on activated CD3-CD56+ cells in seropositive renal transplant patients- experiment III.

Sera were obtained from individuals who participated in phase-2 clinical trial with gB/MF59 vaccine at the day of vaccine or placebo administration (v#1), 1week following the vaccination: 1month (v#2), 2 months(v#3), 6months (v#4), 7months (v#5) and at the day of transplantation (D0) or following the transplantation (D7). Vaccinated patients (n=3) are depicted in dark blue and placebo patients (n=6) are depicted in black.

A)



B)

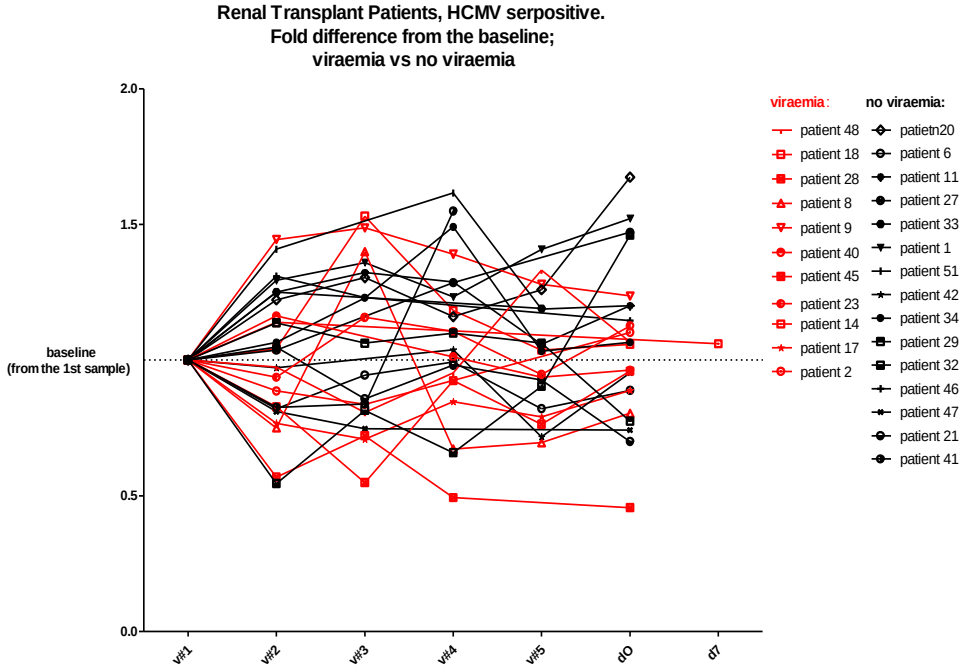


Figure 5.23. Fold difference from the baseline (v#1) in seropositive renal transplant patients shows no segregation in the level of CD107a expression on activated NK cells between groups of patients.

Sera from individuals (n=26) who participated in phase-2 clinical trial with gB/MF59 vaccine at the day of vaccine or placebo administration (v#1), 1week following the vaccination: 1month (v#2), 2 months(v#3), 6months (v#4), 7months (v#5) and at the day of transplantation (D0) or following the transplantation (D7). A) Vaccinated patients (n=11) are depicted in dark blue and placebo patients (n=15) are depicted in black. B) Patients who had viraemia (n=11) are depicted in red and patients with no viraemia (n=15) are depicted in black.

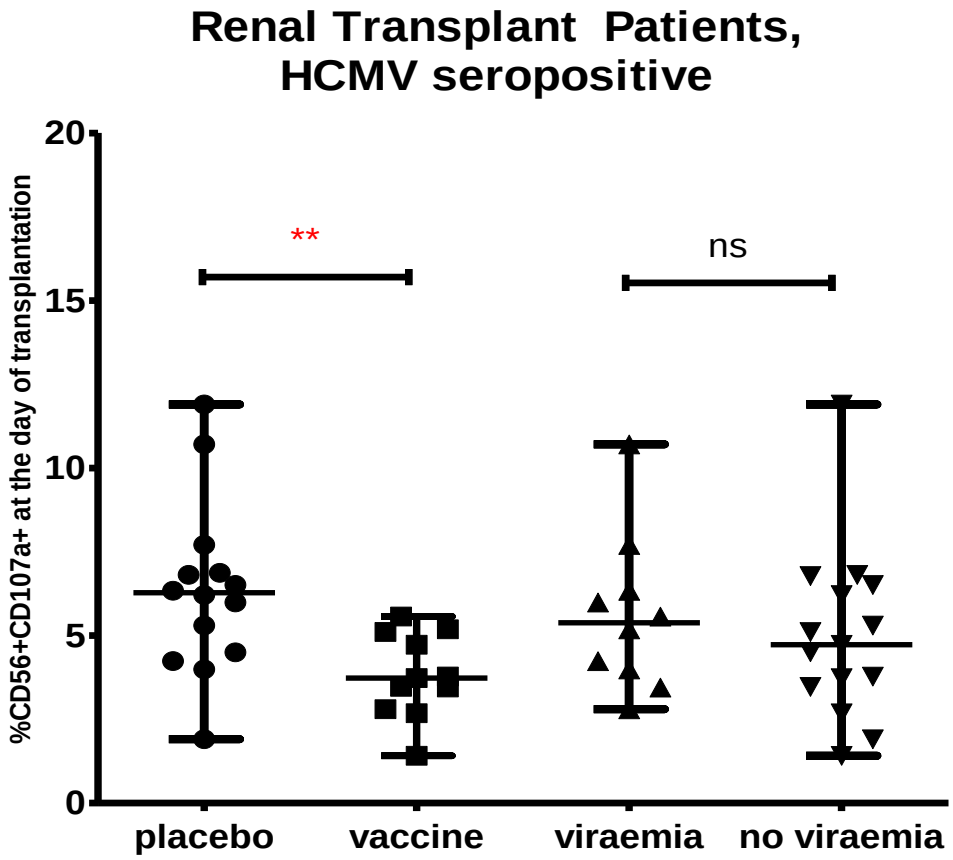


Figure 5.24. No evidence that the vaccine enhanced pre-existing ADCC responses; lack of correlation between the level of the CD107a expression and onset of viraemia.

Sera were obtained from seropositive individuals (n=26) who participated in phase-2 clinical trial with gB/MF59at the day of transplantation (D0) or 1 week following the transplantation (D7). The differences between groups were assessed by Mann-Whitney U test. The horizontal lines indicate the median value and the range. The differences in the expression of CD107a between compared groups were: placebo vs vaccine: p=0.0034; viraemia vs no viraemia p=0.56.

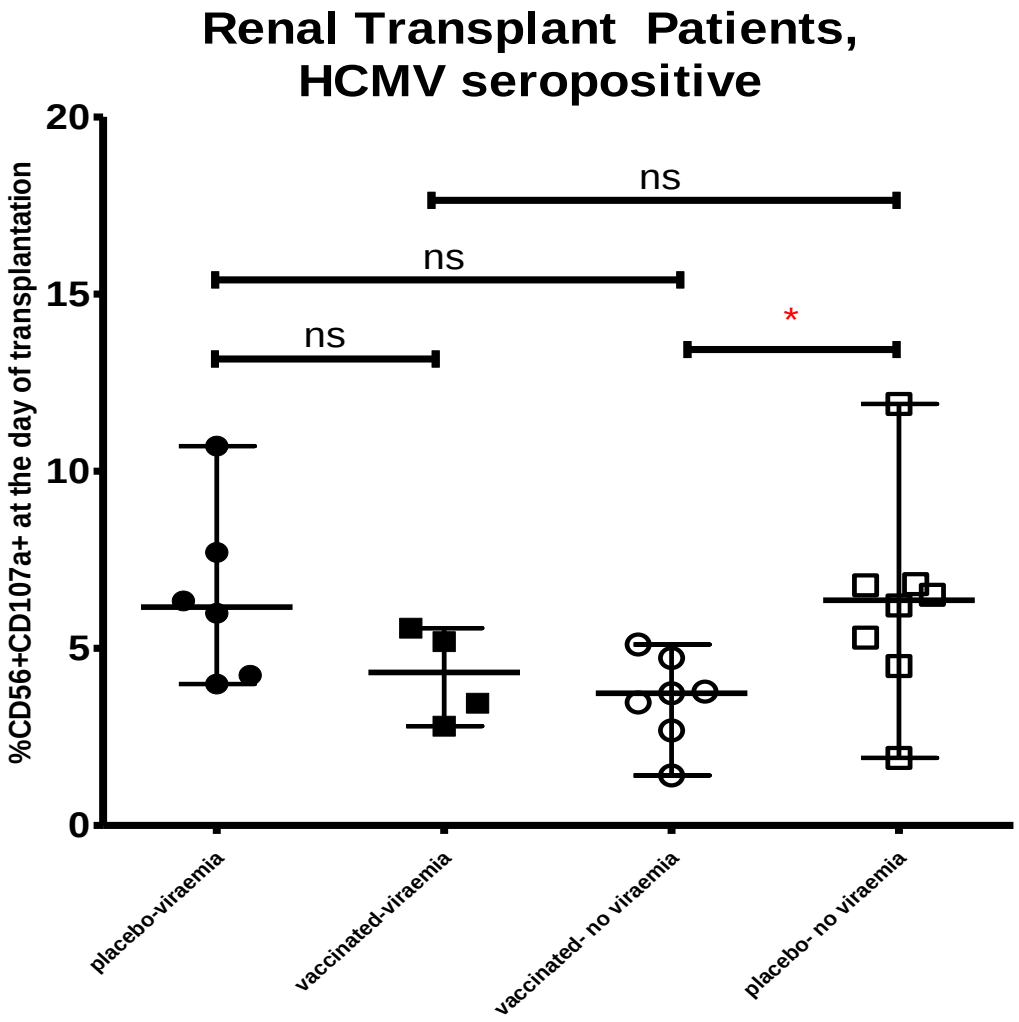


Figure 5.25. Similar levels of the CD107a expression on activated CD3-CD56+ (NK) cells between different groups of seropositive renal transplant patients.

Sera from seropositive individuals (n=26) who participated in gB/MF59 trial at the day of transplantation (D0) or 1 week following the transplantation (D7). The horizontal lines indicate the median value and the range. The differences between groups were assessed by Mann-Whitney U test. The differences in the expression of CD107a between compared groups were: placebo-viraemia (n=6) vs placebo-no viraemia (n=8) p=0.949; vaccinated-viraemia (n=4) vs vaccinated- no viraemia (n=7) p=0.52; placebo-viraemia (n=6) vs vaccinated viraemia (n=4) p=0.114; vaccinated- no viraemia (n=7) vs placebo- no viraemia (n=8) p=0.02.

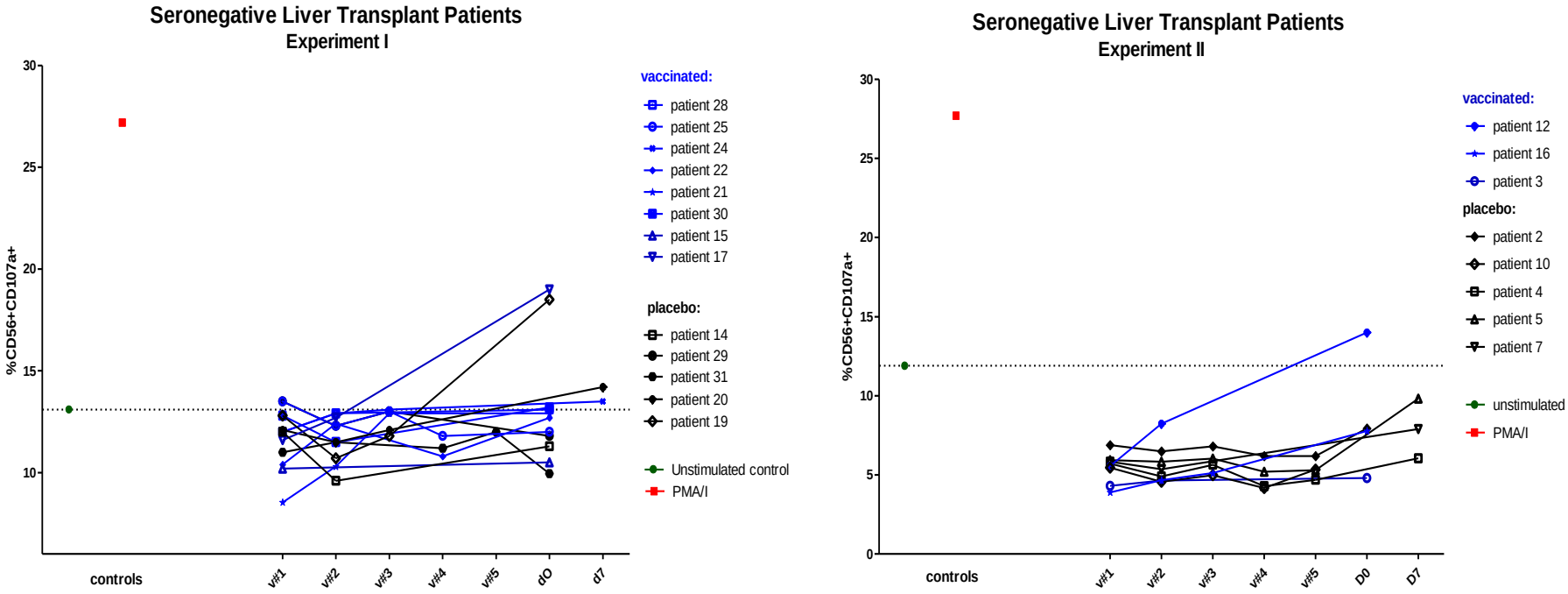


Figure 5.26. No evidence of vaccine induced ADCC responses in seronegative liver transplant patients. Level of the ADCC responses was measured by surrogate marker CD107a expression on activated CD3-CD56+ cells.

Sera tested in this assay were obtained from seropositive liver transplant patients who participated in gB/MF59 trial at the day of vaccine or placebo administration (v#1), 1week following the vaccination: 1month (v#2), 2 months(v#3), 6months (v#4), 7months (v#5) and at the day of transplantation (D0) or following the transplantation (D7). Vaccinated patients (n=11) are depicted in dark blue and placebo patients (n=10) are depicted in black.

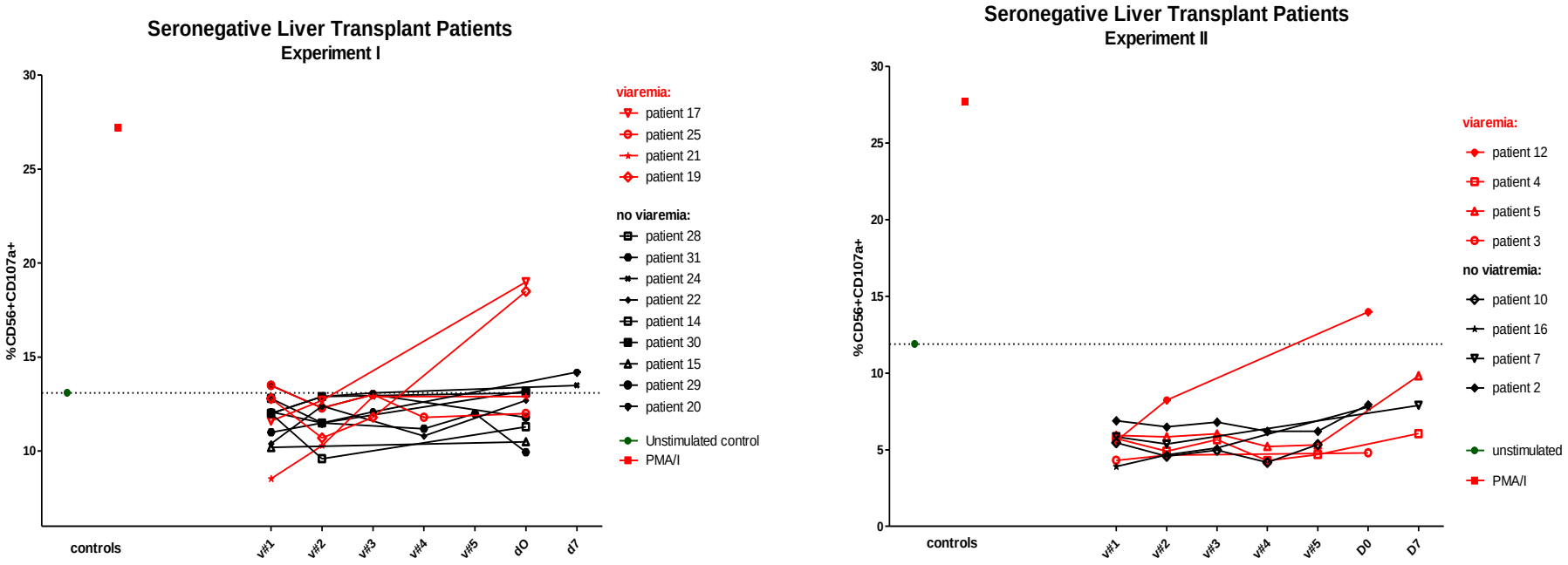


Figure 5.27. No evidence of ADCC responses preventing onset of viraemia following vaccination in seronegative liver transplant cohort.

Level of the CD107a expression on activated CD3-CD56+ cells in seropositive liver transplant patients who participated in gB/MF59 trial at the day of vaccine or placebo administration (v#1), 1week following the vaccination: 1month (v#2), 2 months(v#3), 6months (v#4), 7months (v#5) and at the day of transplantation (D0) or following the transplantation (D7). Patients who developed viraemia are depicted in red (n=8) and patients without viraemia are depicted in black (n=13).

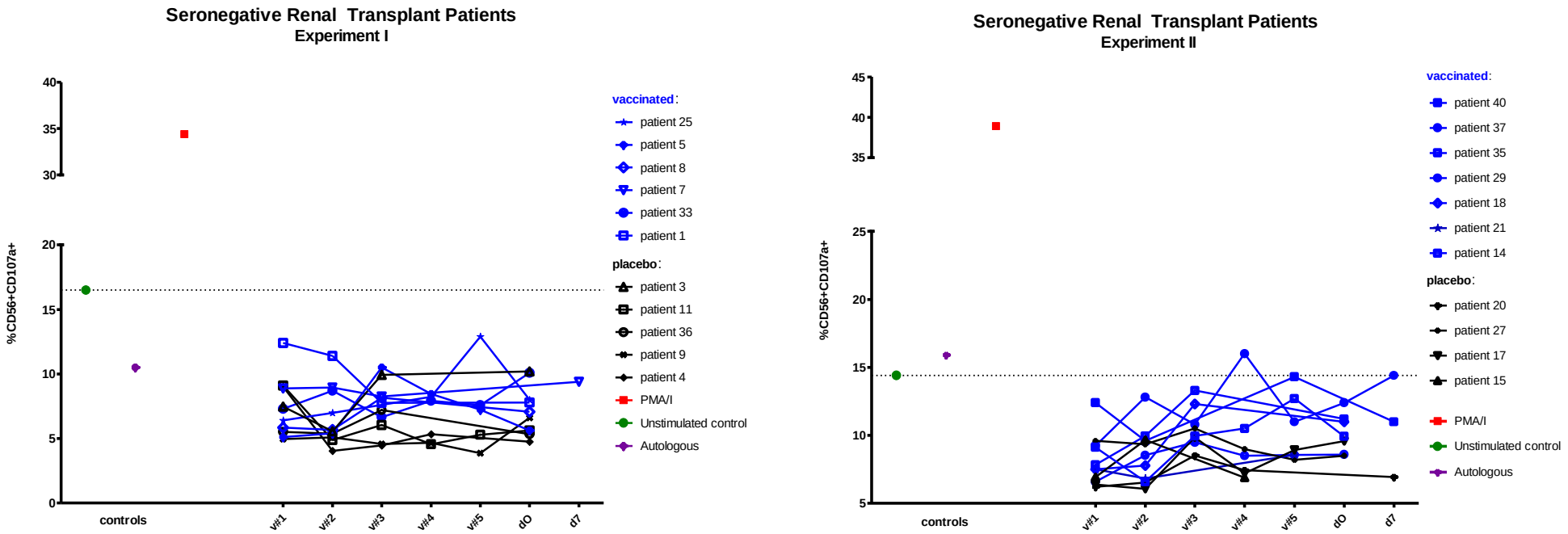


Figure 5.28. No evidence of vaccine induced ADCC responses in seronegative renal transplant patients. Level of the ADCC responses was measured by surrogate marker CD107a expression on activated CD3-CD56+ cells.

Sera tested in this assay were obtained from seropositive renal transplant patients who participated in gB/MF59 trial at the day of vaccine or placebo administration (v#1), 1week following the vaccination: 1month (v#2), 2 months(v#3), 6months (v#4), 7months (v#5) and at the day of transplantation (D0) or following the transplantation (D7). Vaccinated patients (n=13) are depicted in dark blue and placebo patients (n=9) are depicted in black.

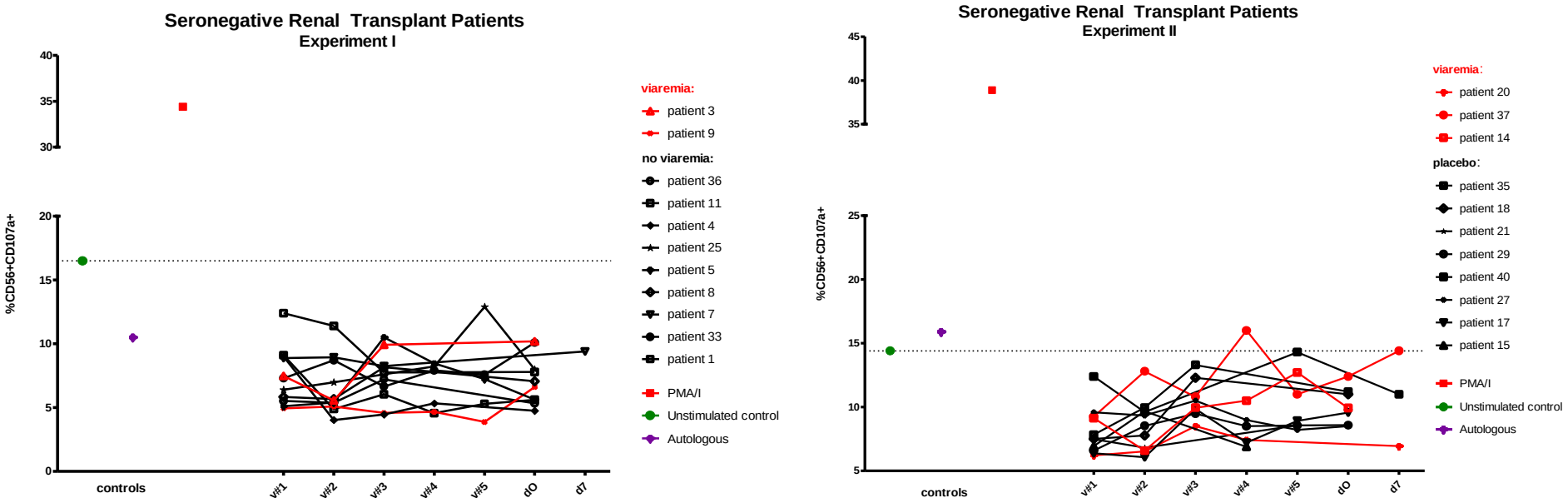


Figure 5.29. No evidence of ADCC responses preventing onset of viraemia following vaccination in seronegative renal transplant cohort.

Level of the CD107a expression on activated CD3-CD56+ cells in seropositive renal transplant patients who participated in gB/MF59 trial at the day of vaccine or placebo administration (v#1), 1week following the vaccination: 1month (v#2), 2 months(v#3), 6months (v#4), 7months (v#5) and at the day of transplantation (D0) or following the transplantation (D7). Patients who developed viraemia are depicted in red (n=5) and patients without viraemia are depicted in black (n=17).

5.4. Discussion.

The results from previous sections provide no evidence for vaccination inducing protection via intrinsic ability of the sera only (chapter 3-neutralization and chapter 4-inhibition of viral spread from cell to cell). Therefore, next I wished to investigate the importance of ADCC responses in the patients who participated in the phase-2 clinical trial with the soluble recombinant subunit gB vaccine with the MF-59 adjuvant (NCT00299260). In order to perform this kind of analysis an assay for measuring ADCC using CD107a was established and optimised. Using this assay the correlation between the levels of CD107a expression with the incidence of viraemia was sought.

In one of the preliminary, experiments I investigated whether the serostatus of the PBMCs donor can influence the level of ADCC (when incubated with both, seropositive and seronegative sera). The initial analysis showed significant differences in the level of expression of ADCC marker between the same serum specimens when incubated with PBMCs from seropositive and seronegative individuals. This finding suggested that serostatus of the PBMC donor might influence the responsiveness of NK cells. In order to investigate this in more detail, I decided to perform additional sets of analyses in which I tested the seropositive and seronegative donor sera against seropositive and seronegative purified NK cells and total PBMCs.

I hypothesized that two potential outcomes were possible:

1) If purified NK cells respond in the same way as was reported for total PBMC – that is similar level of CD107a expression on NK cells irrespective of donor serostatus- then that suggests that NK cells from a seropositive individual exhibit different biological activities. Although, classically, NK cells were considered to be a part of the innate immune system, some recent publications demonstrate that NK cells undergo preferential clonal expansion following HCMV infection [420]. Several groups have recently reported that NK cells from HCMV infected individuals possess different subsets of memory-like NK cells. It has been reported

that clonal-like expansion of NK cells in response to HCMV infection causes stable imprints in the human KIR repertoire which is skewed and a bias for self-specific inhibitory KIRs. [421]. Others revealed that HCMV is capable of shaping the NK cell receptor repertoire inducing the expansion of an NK cell subset expressing the activating NKG2C receptor [422]. Moreover, it has been reported that the memory-like NK cell repertoire is modulated and maintained by a mechanism that relies on both epigenetic modification of gene expression and antibody-dependent expansion [421, 423, 424]. Thus it seems plausible that the development of NK cells and the distribution of NK cell receptors can be deeply influenced by HCMV infection. The presence of so called “memory-like” NK cells in the infected individuals argues that immunological responsiveness of these cells could be different to the NK cells from uninfected subjects.

2) Alternatively, if the pattern of CD107a expression on isolated NK cells reflects that observed with PBMCs from a seronegative individual (i.e. only evidence of ADCC with seropositive donors’ serum) this would indicate that other immunological mechanisms not intrinsic to the NK cells were engaged. In these experiments total PBMC, that contains cells such as cytotoxic T lymphocytes (CTL), antigen presenting cells (APC), monocytes, were used. Additionally an antigen (gB) was present in the system. Thus, it seems possible that the responses of the NK cells from the seropositive donor could have been driven by some cytokine mediated effects underpinned by antigen presenting events occurring in the experimental set up.

In order to investigate this, additional studies of the seropositive and seronegative purified NK cells and total PBMCs were performed. The results of these serological analyses did not confirm the previous findings and I could not see the difference in the level of CD107a expression between the total PBMCs and purified NK cells from seropositive and seronegative donor when incubated with seropositive and seronegative sera. However, I order to avoid any potential immunological confounders I decided to utilize only PBMCs from seronegative donors.

Interestingly, I observed that the incubation of seronegative PBMCs with recombinant gB protein and sera from healthy seropositive individuals resulted in higher level of CD107a expression on NK cells (in total PBMCs) when compared with unstimulated controls - indicative of the presence of ADCC inducing antibodies in the serum of seropositive individuals. I also noted that the level of ADCC appears to be highly variable amongst seropositive patients, indicating a natural diversity in the ADCC responsiveness within the population which further reflects the heterogeneity of the antibody response against gB and. Nevertheless, this finding strongly supports the presence of ADCC inducing antibodies against gB in the sera of healthy seropositive individuals that has been induced by natural infection. However, despite the presence of ADCC promoting antibodies in seropositives these results provide evidence that the protective effect elicited by the gB subunit vaccine against HCMV is not dependent on the induction of ADCC stimulating antibody responses. Although I could see wide range of ADCC responses among seropositive patients, regardless of their vaccine or placebo status, the level of ADCC responses was not correlated with the viraemia incidence among the patients who received the vaccine and proceeded to transplantation. The data presented here clearly showed that the administration of the vaccine also did not increase any pre-existing responses in this cohort. Such result does not support the hypothesis that the ADCC responses might have a protective role in vaccinated transplant patients. Moreover a comparison of vaccine versus placebo in liver transplant group showed that the level of CD107a was generally lower in the vaccinated patients. Nevertheless, the statistical power of this study is limited by the small number of samples; therefore this correlation might not be biologically relevant. The results in seronegative patients clearly show that the vaccination failed to induce ADCC stimulating responses. Besides a few outliers, the level of CD107a expression in the read-outs are below or comparable to the level of the expression of CD107a protein in negative controls-unstimulated NK cells in total PBMCs. Such result again argues against the protective humoral responses generated by the vaccine being through the production of ADCC inducing antibodies against gB.

Importantly, however, I assessed ADCC using PBMC isolated from healthy seronegative donor and these analyses provided the majority of the experimental analyses. Thus I also investigated whether there is a difference in the responsiveness of NK cells depending whether they are used in the assays as total PBMCs or as purified cells. In order to investigate the potential confounding effects in total PBMCs I analysed the ADCC responses incubating sera from healthy donors and vaccine cohort. Generally, this analysis allowed the level of ADCC responses to be compared between the assays with purified NK cells and total PBMCs. As I expected, the pattern of ADCC responses was similar in both types of assays and there were no major differences in the expression of CD107a when I used purified NK cells instead of total PBMCs suggesting that the data I have accrued is representative of the general consensus that ADCC against gB has little or no impact on conferring protection. Such results justify testing sera from this vaccine cohort using total PBMCs instead of purified NK cells as it is a more practical and economical approach.

Taken together, the current data suggest that another mechanism of protection must be elicited by the vaccine. Using this approach it was demonstrated that the vaccine alone did not generate ADCC inducing responses in seronegative patients nor did the vaccine boost the pre-existing ADCC responses in seropositive patients. However, it has to be considered that the assay is an indirect measure of ADCC. Thus, although the approach is a well-established measure of ADCC activity, it does not preclude NK cells as being important cells required for the control of HCMV infection.

6. Epitope specific humoral responses to recombinant CMV gB vaccine.

6.1. Introduction.

Once gB was recognized as a dominant target of humoral immune responses [161] many extensive examinations were conducted to establish whether or not this protein is capable of eliciting potent neutralizing antibodies that would exert a protective effect. It is hypothesised that gB plays important roles in the entry of different herpes viruses as the proteins are highly conserved across the family. These functional constraints placed on gB may render it less able to undergo substantial mutation to avoid deleterious immune responses.

Initially, researchers mapped epitopes via expression in prokaryotes. This approach has limitations but some observations were made [161]. The first highly conserved linear neutralizing epitope was identified on gB by use of murine monoclonal antibodies [192]. The newly identified region (608- 625aa) was a component of AD1-dominant antigenetic determinant (AD1: 560-640 aa). A detailed analysis of human convalescent sera and monoclonal antibodies (human and mouse) discovered that AD-1 comprises approximately 80aa between positions 560 and 640 of gB (gp58) based on the sequence of the AD169 strain [425]. AD1 was recognized as a major target of humoral responses towards gB since nearly 100% of sera from HCMV healthy seropositive donors had antibodies recognising this antigenic domain [162, 189, 190] Interestingly, the antibody responses against AD-1 that developed after natural infection with HCMV had a wide range of neutralizing capacity [158, 191]. Potential competition between non-neutralizing and neutralizing antibodies against this antigenic domain was reported for the first time in the 1990s [158, 161, 192]. Subsequently, it was proposed that the competitive binding of these antibodies could be a manifestation of an immune-evasive mechanism that the virus utilizes to avoid neutralization of cell free virus, since the neutralizing activity of the polyclonal human antibodies against the AD-1 that were obtained from healthy seropositive donors did not exceed 50% in *in vitro* studies. This finding also showed that natural infection with

the virus induces both neutralizing and competing non-neutralizing antibodies that are specific to this immune-dominant antigenic domain [191]

The studies of sequence homology between the Towne and AD169 strains of HCMV led to the discovery of AD2 (which is located in gp116 between 50-77aa). Although the gB homology between these laboratory strains is very high (-95%), the distribution of the differences appeared to be not random across the whole molecule-interestingly; it was revealed that the level of homology in the N-terminal part of the molecule was only approximately 55%. Thus both monoclonal antibodies and sera isolated from healthy seropositive individuals were incubated with this amino-terminal portion of gB that was expressed in prokaryotic systems and synthetic peptides. The examination of the binding capacities of the antibodies to both strains indicated that there are two binding sites:

-site I- located between 68-77aa and conserved amongst the strains. The antibodies that bound to this site exhibited neutralizing capacities. It has been estimated that approximately 50% of infected individuals have antibodies against this site I of AD2 [162, 426, 427].
-site II- located between 50-54aa, not conserved amongst the strains. The antibodies that bound to this site were incapable of neutralizing the virus [426].

Another linear epitope, AD-3, was discovered when the C-terminal part of gB was mapped using synthetic peptides. The incubation of sera from HCMV seropositive healthy individuals with gp58 revealed that most of these donors possessed antibodies that bound to the sequence localized in the intraluminal part of this molecule (between 798 to 805aa). Such localization however suggests that the antibodies that are directed towards this epitope may not have the capacity to neutralize cell-free virus *in vivo* as this region may not be exposed to the immune system of the host [160, 428].

The relatively large size of the gB protein suggested that there might be other, additional binding sites for antibodies that are elicited following infection with the virus. In order to complete the epitope map of this glycoprotein, analysis of humoral responses with the use of gB-specific memory B cells isolated from healthy HCMV-seropositive individuals and clonally expanded was performed. Experiments in which antibodies secreted from these B-cells were examined demonstrated that the majority of antibodies that were elicited against HCMV following natural infection (90%) did not possess the capacity to neutralize the virus in *in vitro* assays. Most importantly, two previously unknown antibody domains that bound the majority of the antibodies released by these clonal B cells were discovered. Moreover, many of the antibodies against these domains did prove to have very potent neutralizing activities:

-domain I (AD5)- located between 133–343 aa- approximately 50% of seropositive individuals developed antibodies that bound to this antigenic site [160]; although more recent data suggest that the incidence of AD5 responses in seropositives is higher (personal communication with Prof Michael Mach).
-domain II (AD4) - a discontinuous domain that recognizes a structural motif defined by regions 121–132 aa and 344–438aa. Approximately 90% of seropositive individuals elicit antibody responses against this epitope [160].

Although a number of AD have been identified that give rise to strong humoral responses *in vivo* it is not entirely clear which domains are important targets for the control of HCMV infection. AD1 is recognized as a major target of humoral responses towards gB since nearly 100% of sera from HCMV healthy seropositive donors have antibodies that bind to this antigenic domain [162, 189]. However, it was shown before that this domain induces a mixture of neutralizing and non-neutralizing specificities. It is suggested that antibodies to AD2 may confer better protection against HCMV infection because a strong neutralising antibody response is directed against this epitope. However, given that only 50% of people have AD2 responses and that neutralising antibody responses against AD4 and AD5 are detectable this hypothesis needs further consideration [429].

Analysis of the antibody profiles towards each antigenic domain can potentially reveal that the protection against the pathogen is correlated with specific antibody responses and moreover, such analysis could provide further clues to the protective nature of the vaccine response since these epitopes have been linked with different aspects of immune control of HCMV infection. Additionally, such experiments will allow the antibody responses elicited by the vaccine to be compared with those following natural infection. The changes that were introduced to facilitate expression and production of this recombinant vaccine protein could potentially alter antigen presentation and influence the fine specificity of these humoral responses that may be important for the protection of vaccinated patients.

In the phase-2 study in SOT patients the duration of viraemia was inversely correlated with the anti-gB antibody titre measured by ELISA which suggests that humoral responses may be protective [188]. Therefore, in this set of analyses, the binding activity of these sera to 4 (of 5) key antigenic domains was assessed. The AD1, AD2, and AD5 epitopes that have been identified so far on gB were tested by ELISA.

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Objectives:

The aim of the present study was to provide detailed analysis of the antibody repertoire following natural infection and vaccination with gB/MF59 and to determine whether any correlations between AD specific antibody responses and protection from viraemia following the transplantation could be identified as a potential basis for the protective effect observed with gB/MF59 vaccination.

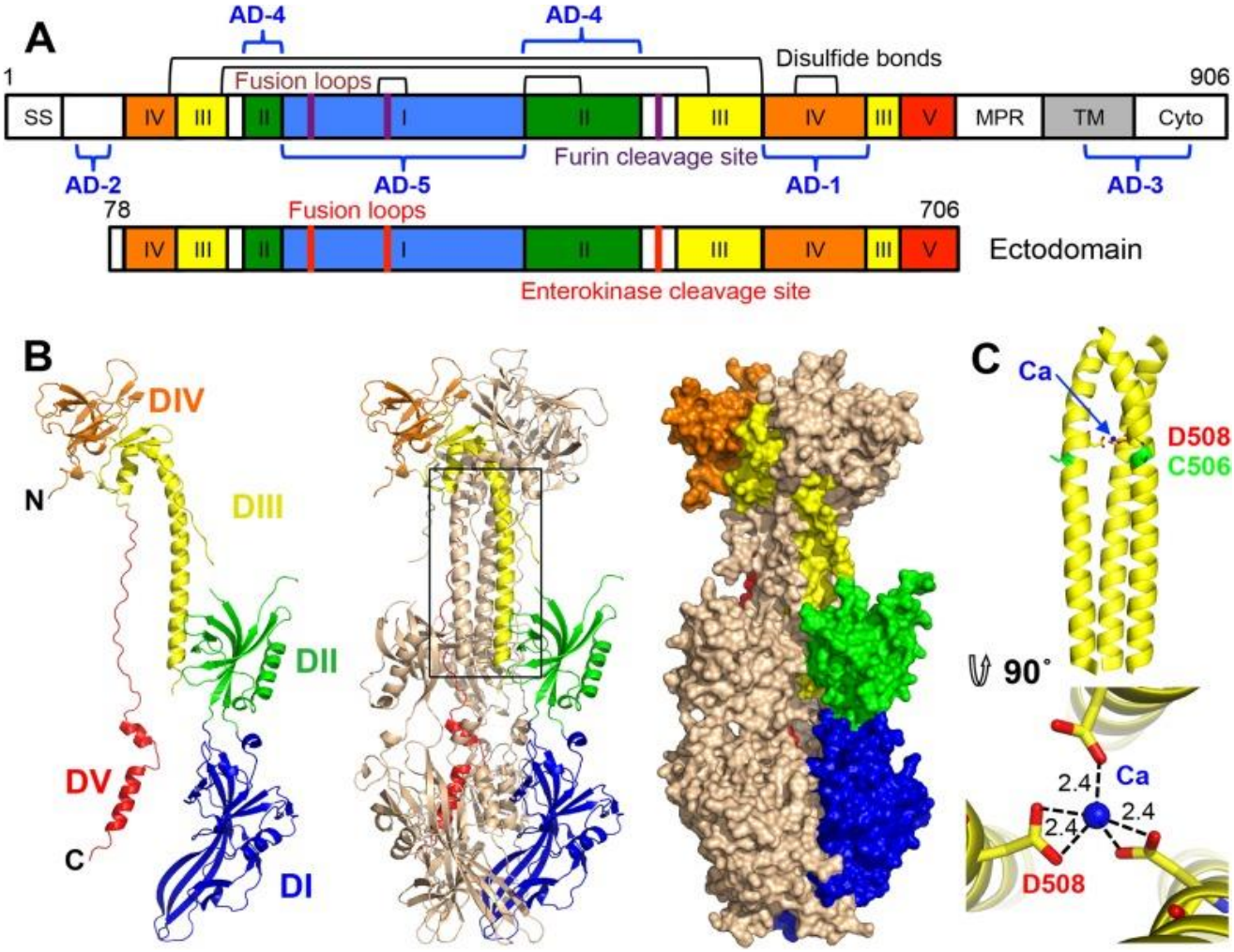

Figure 6.1. Schematic representation of gB from HCMV- ectodomain structure.

ADx are representing the locations of the antigenic domains domains mapped onto the gB. (A) Schematic representation of the full-length HCMV gB (top) and the crystallized construct, gB78-706-7M-E (bottom). Disulfide bonds are represented as black brackets, antigenic domains (AD-1-5) are indicated in blue brackets, and mutations are shown using red bars. Structural domains, are colored as follows: domain I = blue, II = green, III = yellow, IV = orange, V = red, as in [430, 431]. SS = signal sequence, MPR = membrane proximal region, TM = transmembrane domain, and Cyto = cytoplasmic domain. Numbers denote construct boundaries. (B) The crystal structure of the HCMV gB ectodomain is shown as a protomer and a trimer in cartoon representation as well as a trimer in surface representation. Chain B is colored by domain as in (A). (C) Side and top down view of the coiled coil in DIII with a coordinated calcium ion (CA) (blue sphere). Side chains of D508 (yellow) with carboxyl oxygens (red) and C506 (green) are also shown. Dashed lines indicate distances between the carboxyl oxygens in D508 and the calcium ion. All structure figures were made in Pymol (http://www.pymol.org). Reprinted from Burke H.G. et al., 2015; doi: 10.1371/journal.ppat.1005227 [358].

6.2. Materials and methods.

6.2.1. Antigens.

For this component of the work, I collaborated with Professor Michael Mach in Erlangen who has prepared and evaluated the complex antigens required. The assays were performed by his staff in Germany and I analysed all the results.

The following gB-specific antigens, derived from HCMV strain AD169, were used: AD-1, containing aa 484-650, AD-2 containing aa 68-80 (Cambridge research biochemicals), AD-4 containing a fused polypeptide of aa 121-132 and 344-438 and AD-5 containing aa133 to 343. The ELISA tests for AD-1 and AD-2 have been described in detail by Schoppel et al. [162]. For determination of AD-4-specific antibodies a purified GST-AD-4 fusion protein was used as antigen as described by Spindler et al [432]. AD-5-specific antibodies were determined in a capture ELISA using a mammalian cell derived AD-5 polypeptide containing a HA-epitope tag at the amino terminus of the protein as described elsewhere [433]. To capture the antigen, an anti-HA monoclonal antibody (clone HA-7, Sigma-Aldrich) was diluted to 1 µg/ml in 0.05 M sodium carbonate buffer; pH 9.6, and 50 µl/well was used to coat polystyrene 96-well plates (NuncImmuno™) overnight at 4°C.

6.6.2. Enzyme-linked immunosorbent assay (ELISA) tests.

All of the following reactions were performed at 37°C. Reaction wells were rinsed with PBS supplemented with 0.1%Tween then the reaction wells were blocked with PBS containing 2% fetal calf serum for 1 h, washed three times with PBS plus 0.1% Tween 20 and incubated with comparable amounts of AD-5 wt or mutant proteins for 2 h. The plate was washed three times with PBS containing 0.1% Tween 20 and human serum was added at a dilution of 1:100 for 1 h. Dilution of all sera was done in PBS with 2% FCS. Unbound antibody was removed by washing three times and peroxidase-conjugated secondary antibody (goat-anti-human IgG, Dianova) was added for 1 h. After three washing steps with 100 µl of tetramethylbenzidine peroxidase substrate was added for 3.5 min, diluted 1:1 in

peroxidase substrate solution B (KPL, USA). The reaction was stopped by adding 100 µl of 1 M phosphoric acid. The optical density at 450 nm (OD450) was determined using an Emax microplate reader (Eurofins MWG Operon, Germany).

6.2.3. Statistical analyses.

The analysis of the results was performed by Graph Pad Prism®-software. Statistical differences between the mean value of the OD of the samples obtained at the same time points between populations of patients: vaccinated vs placebo and viraemia vs no viraemia were obtained from Mann Whitney Test (ns: not significant; *: $P < 0.05$; **: $P < 0.005$; ***: $P < 0.005$). Geometric mean values (±95%CI) were represented by horizontal lines.

6.3. Results.

6.3.1. Antibody responses towards AD1.

The AD1 specific antibody responses were measured by ELISA at five different time points: day of vaccine/placebo administration (month 0); day of administration of the second (month 1) and third dose (month 6), and 3 and 7 months post vaccination. As expected, nearly all HCMV seropositive individuals possessed detectable antibody responses against this antigenic domain (Figure 6.2 A, B). Furthermore, vaccination of HCMV seropositive SOT candidate patients with gB/MF59 increased pre-existing antibody responses against AD1 (Figure 6.2 A and C). However, although a boost of these antibody responses was observed there was no evidence that there was reduced occurrence of viraemia among the patients who had high level of these antibody responses and underwent transplantation (Figure 6.2.D).

The same analyses of sera from the seronegative cohort revealed that vaccination elicited antibody responses only in a minority of patients (Figure 6.3). Furthermore, it was observed that seronegative patients who developed good responses against AD1 were equally likely to develop viraemia following transplantation than those that did not have AD1 responses.

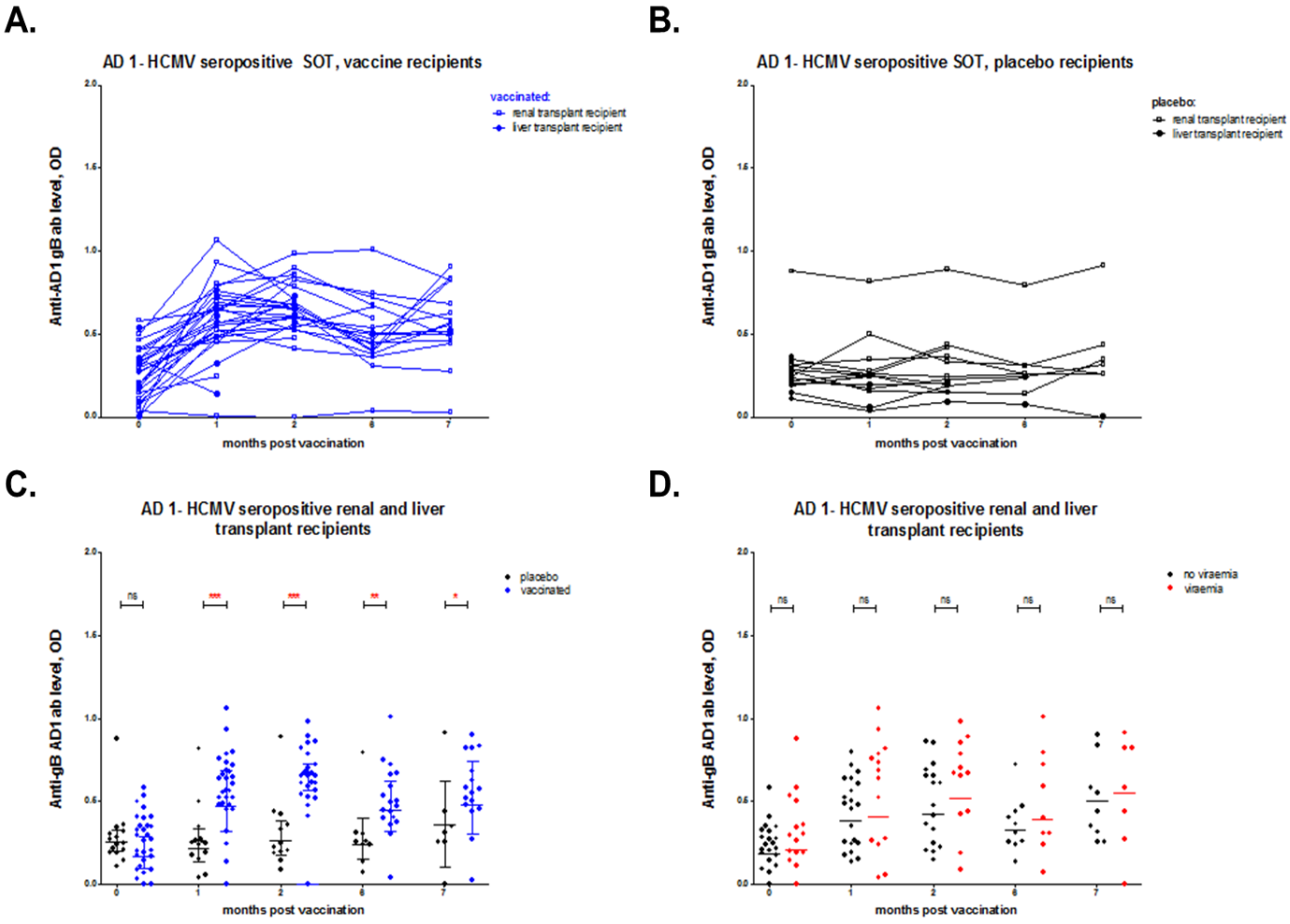

Figure 6.2. Vaccination boosted AD1 antibody levels in HCMV seropositive patients but higher AD1 levels did not correlate with protection from viraemia following transplantation.

AD1 responses are represented as OD values at different time-points: day of first vaccine/placebo administration (month 0); day of administration of the second (month 1) and third dose (month 6), and 2 and 7 months post vaccination. (A) AD1 responses in HCMV seropositive vaccine recipients represented as OD values (B) AD1 responses in HCMV seropositive placebo recipients represented as OD values (C) Comparison between antibody levels against AD1 in the sera from vaccinated and placebo patients. Horizontal lines represent geometric mean values (±95%CI). (D) Comparison of antibody levels against AD1 between patients who developed viraemia versus patients who did not develop viraemia following transplantation. Horizontal lines represent geometric mean values. Statistical differences between the mean value of ODs between the populations of patients: vaccinated vs placebo and viraemia vs no viraemia were obtained from Mann Whitney Test (ns: not significant; *: $P < 0.05$; **: $P < 0.005$; ***: $P < 0.005$).

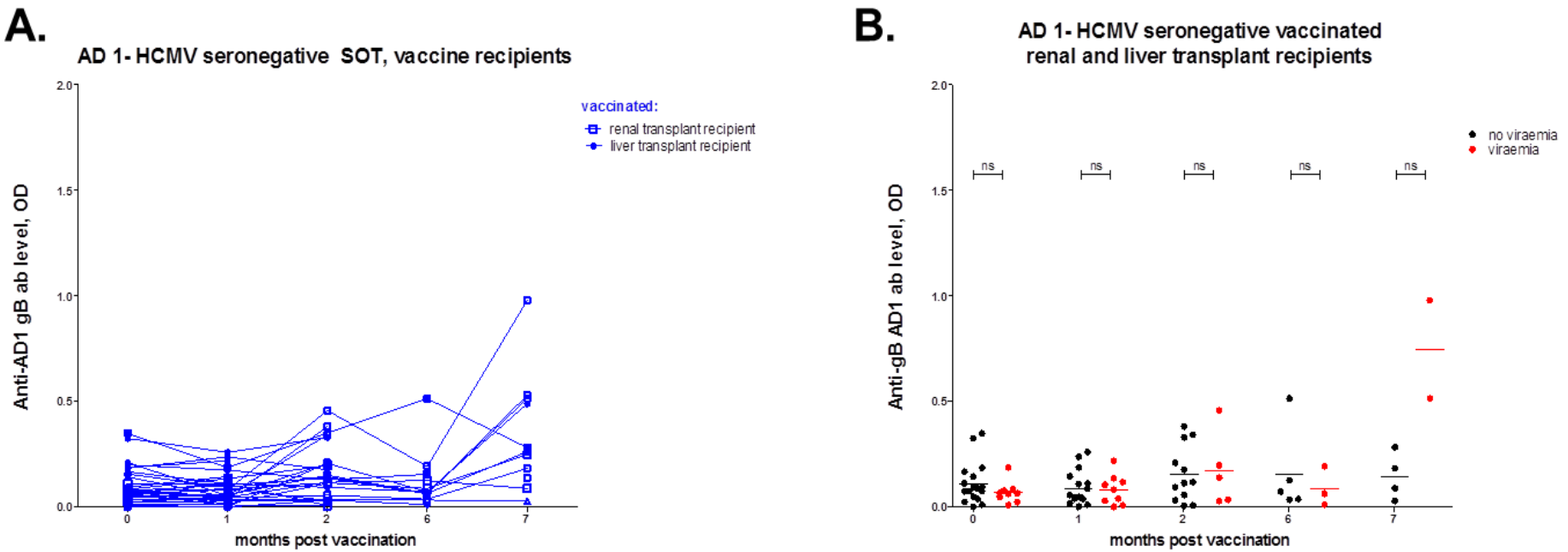

Figure 6.3. Vaccination of HCMV seronegative patients induced variable responses against AD1.

AD1 responses are represented as OD values at different time-points: day of first vaccine/placebo administration (month 0); day of administration of the second (month 1) and third dose (month 6), and 2 and 7 months post vaccination. (A) AD1 responses in HCMV seropositive vaccine recipients represented as OD values (B) Comparison of antibody levels against AD1 between patients who developed viraemia versus patients who did not develop viraemia following transplantation. Horizontal lines represent geometric mean values. Statistical differences between the mean value of ODs between the populations of patients (vaccinated vs placebo were obtained from Mann Whitney Test; ns: not significant).

6.3.2. Antibody responses towards AD2.

I next performed the same analyses on AD2 responses. Consistent with previous studies [160, 189] approximately 50% of seropositive patients had detectable levels of anti-AD2 antibodies prior to vaccination (Figure 6.4 A and B). Administration of the first dose of vaccine boosted pre-existing antibody responses against AD2 in HCMV seropositive SOT patients (Figure 6.4 A and C, Figure 6.5). However, there was no evidence that the vaccine induced de novo anti-AD2 responses in seropositive individuals who did not possess anti-AD2 responses at baseline (Figure 6.4 A). Interestingly, in patients with an AD2 response there was a direct correlation between higher levels of AD2 antibodies and reduced viraemia post-transplant (Figure 6.4.D).

In seronegative patients there was no evidence that the vaccine induced de novo anti-AD2 responses (Figure 6.6).

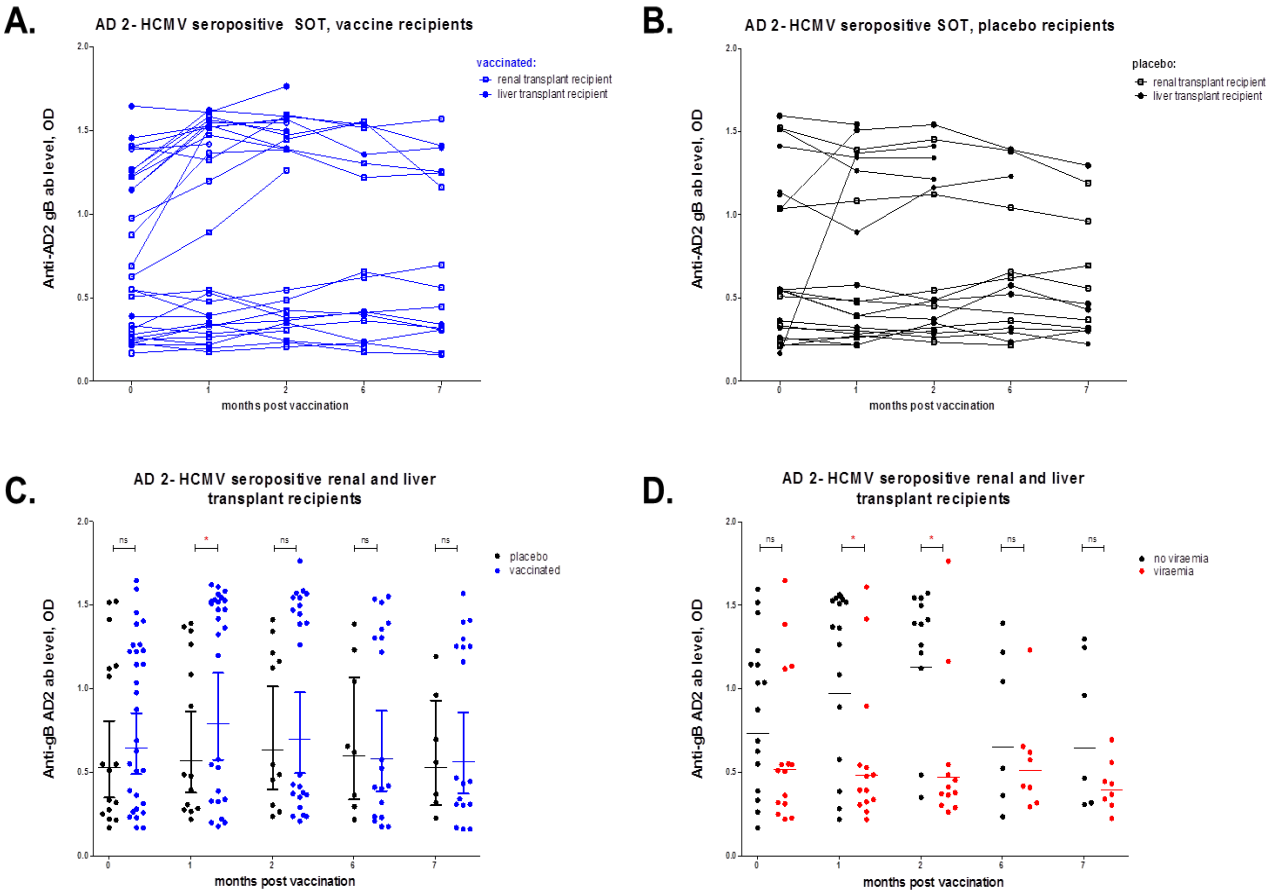

Figure 6.4. Vaccination boosted pre-existing antibody responses against AD2 in HCMV seropositive SOT patients that correlated with protection.

AD2 responses are represented as OD values at different time-points: day of first vaccine/placebo administration (month 0); day of administration of the second (month 1) and third dose (month 6), and 2 and 7 months post vaccination. (A) AD2 responses in HCMV seropositive vaccine recipients represented as OD values (B) AD2 responses in HCMV seropositive placebo recipients represented as OD values (C) Comparison between antibody levels against AD2 in the sera from vaccinated and placebo patients. Horizontal lines represent geometric mean values (±95%CI). (D) Comparison of antibody levels against AD2 between patients who developed viraemia versus patients who did not develop viraemia following transplantation. Horizontal lines represent geometric mean values. Statistical differences between the mean value of ODs between the populations of patients: vaccinated vs placebo and viraemia vs no viraemia were obtained from Mann Whitney Test (ns: not significant).

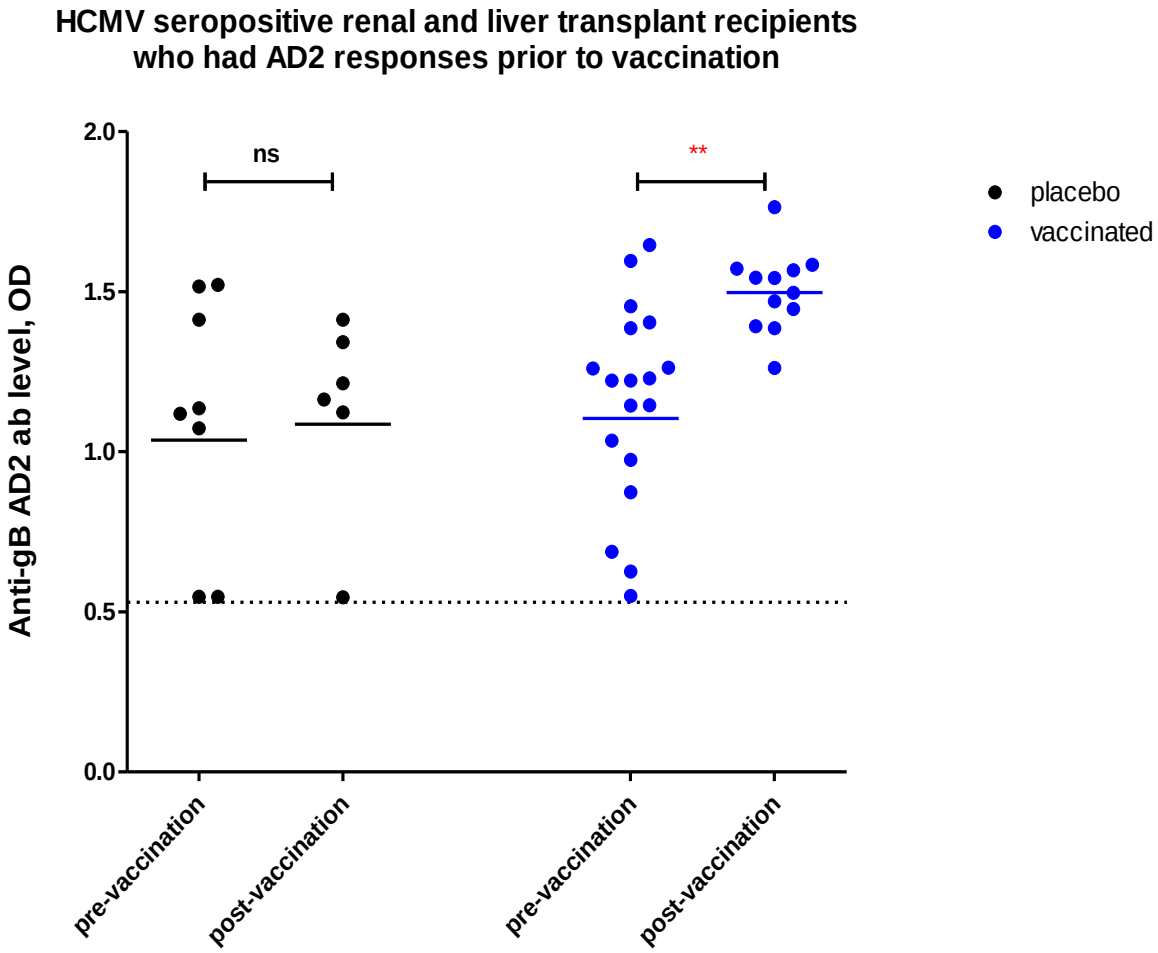

Figure 6.5. Vaccination with the subunit glycoprotein-B vaccine with MF-59 adjuvant boosted pre-existing antibody responses against AD2 in HCMV seropositive patients.

AD2 responses are represented as OD values at day of first vaccine/placebo administration (pre-vaccination) and 2 months following the vaccination (post-vaccination). The dotted line represents a cut-off value (the highest OD value in seronegative group at the time of vaccine administration). Horizontal lines represent geometric mean values. Statistical differences between the mean value of ODs between the populations of patients: vaccinated vs placebo were obtained from Mann Whitney Test (ns: not significant; **: $P < 0.005$).

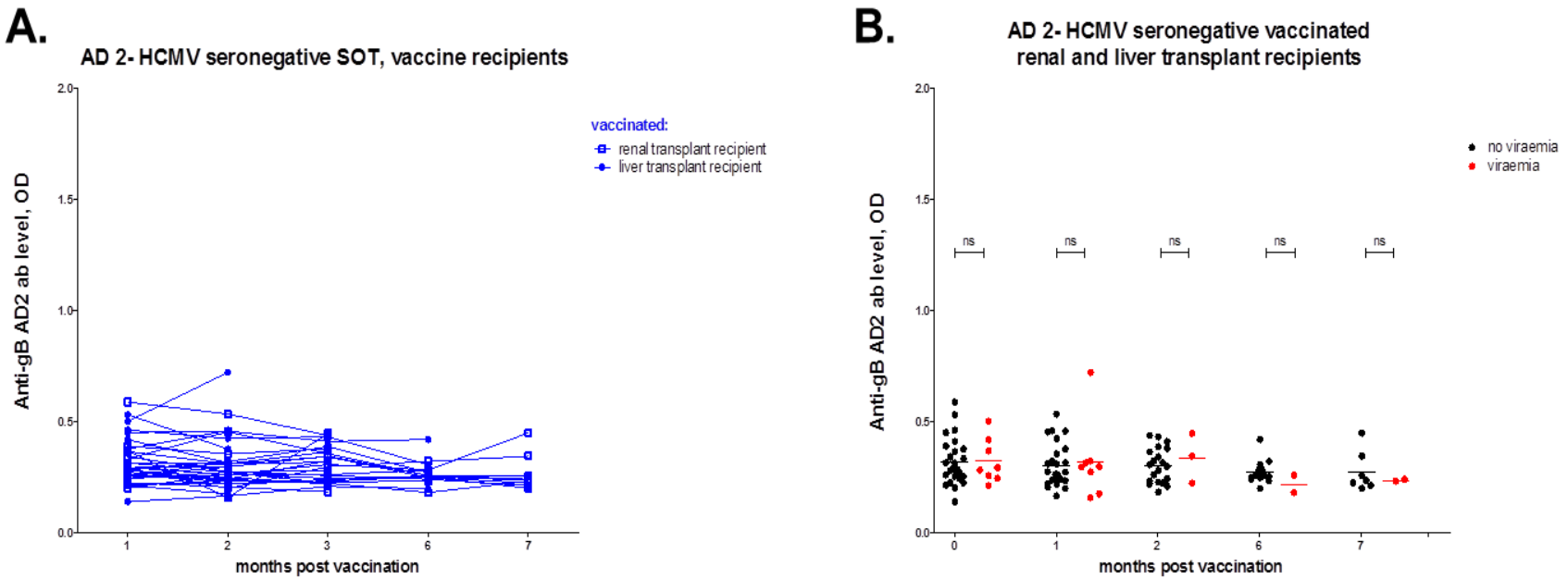

Figure 6.6. Vaccination of HCMV seronegative patients did not elicit detectable antibody responses against AD2.

AD2 responses are represented as OD values at different time-points: day of first vaccine/placebo administration (month 0); day of administration of the second (month 1) and third dose (month 6), and 2 and 7 months post vaccination. (A) AD2 responses in HCMV seronegative vaccine recipients represented as OD values (B) Comparison of antibody levels against AD2 between patients who developed viraemia versus patients who did not develop viraemia following transplantation. Horizontal lines represent geometric mean values. Statistical differences between the mean value of ODs between the populations of patients (vaccinated vs placebo were obtained from Mann Whitney Test; ns: not significant.

6.3.3. Antibody responses towards AD4.

Next I studied antibody responses against AD4. Most seropositive patients had detectable antibody levels against AD4 prior to first vaccination (Figure 6.7 A and B). Furthermore, vaccination of HCMV seropositive patients awaiting SOT significantly boosted the level of antibody against AD4 in HCMV seropositive SOT patients who had high anti-AD4 antibody levels at baseline (Figure 6.7 A and C). Of the few seropositive patients with low level AD4 antibody responses an increase in anti-AD4 antibody level post vaccination was seen in some individuals but not all (Figure 6.7 A). Furthermore, an analysis of AD4 levels and outcome revealed that there was a trend for patients who had higher levels of AD4 specific antibody responses to be less likely to develop viraemia (Figure 6.7 D) although this did not reach statistical significance.

In contrast to my observations with seropositive patients, the immunization of seronegative patients failed to induce any detectable antibody responses towards AD4 (Tables 6.1 and 6.2).

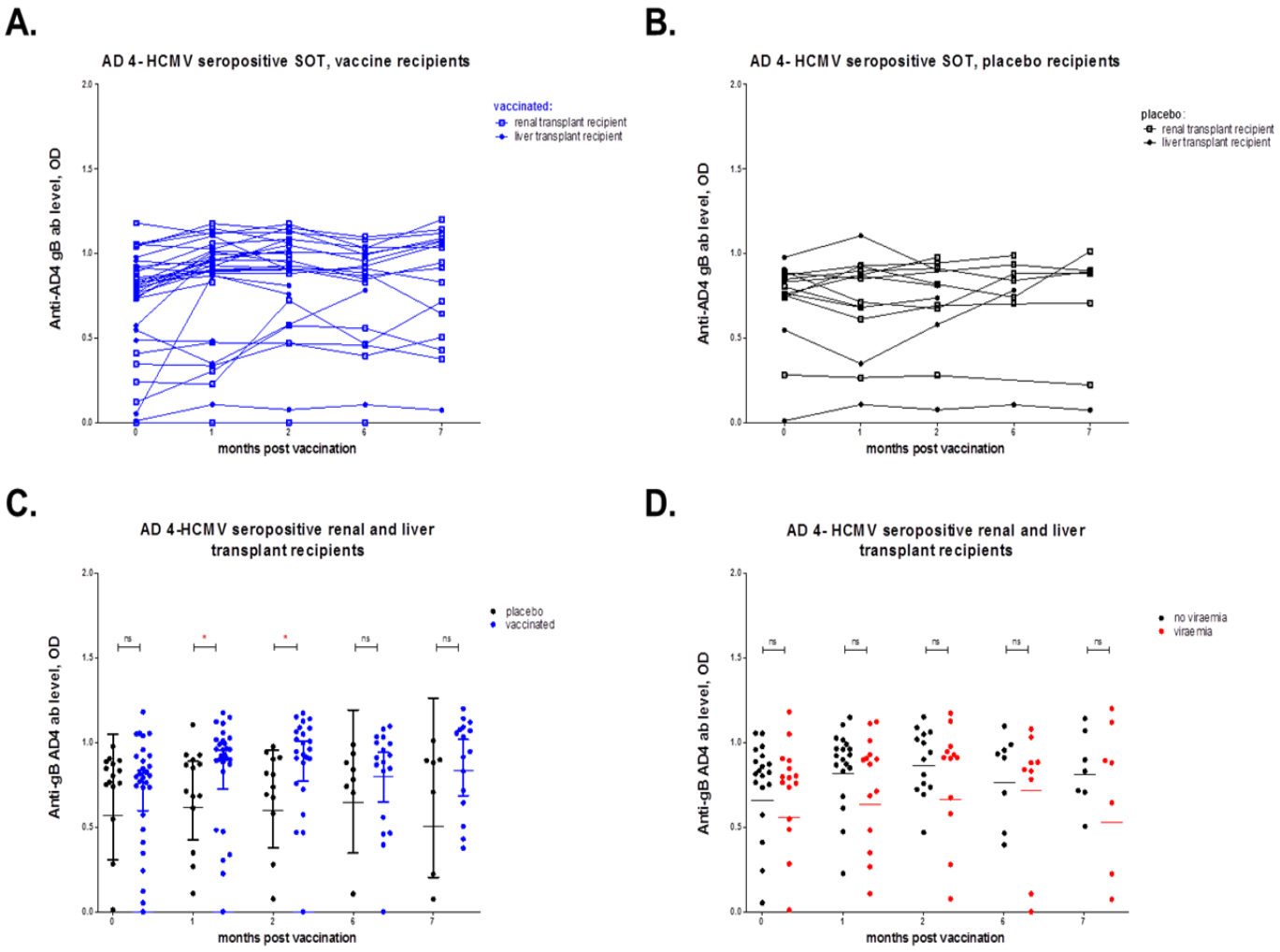

Figure 6.7. Vaccination of HCMV seropositive patients increased antibody levels against AD4.

AD4 responses are represented as OD values at different time-points: day of first vaccine/placebo administration (month 0); day of administration of the second (month 1) and third dose (month 6), and 2 and 7 months post vaccination. (A) AD4 responses in HCMV seropositive vaccine recipients represented as OD values (B) AD4 responses in HCMV seropositive placebo recipients represented as OD values (C) Comparison between antibody levels against AD4 in the sera from vaccinated and placebo patients. Horizontal lines represent geometric mean values (±95%CI). (D) Comparison of antibody levels against AD4 between patients who developed viraemia versus patients who did not develop viraemia following transplantation. Horizontal lines represent geometric mean values. Statistical differences between the mean value of ODs between the populations of patients: vaccinated vs placebo and viraemia vs no viraemia were obtained from Mann Whitney Test (ns: not significant; *: $P < 0.05$).

6.3.4. Antibody responses towards AD5.

The final analysis was of antibody responses to AD5. Sera from nearly all seropositive patients contained antibodies against AD5 (Figure 6.8 A and B) and vaccination increased pre-existing antibody levels against AD5 in all patients (Figure 6.8 A and C). Despite this boost, these AD5 specific responses did not correlate with protection from viraemia following transplantation (Figure 6.8 D).

In seronegatives, post-administration of the second dose of the vaccine, detectable antibody responses against AD5 in the majority of the patients were observed. However, similar to my observations in the seropositive group, these responses did not correlate with protection from viraemia (Figure 6.9).

6.3.5. Correlation between AD1 and AD2 responses.

Lastly I attempted to establish whether there is a correlation between AD1 and AD2 responses, as it was suggested previously in the literature. There were three possible relationships between the AD1 and AD2 OD levels in vaccinated seropositive SOT recipients: I) competition; II) additive effect; III) no interaction (Figure 6.10). Analysis of the observed antibody responses revealed that there was no correlation between the AD2 and AD1 OD levels in seropositive SOT recipients- post vaccination.

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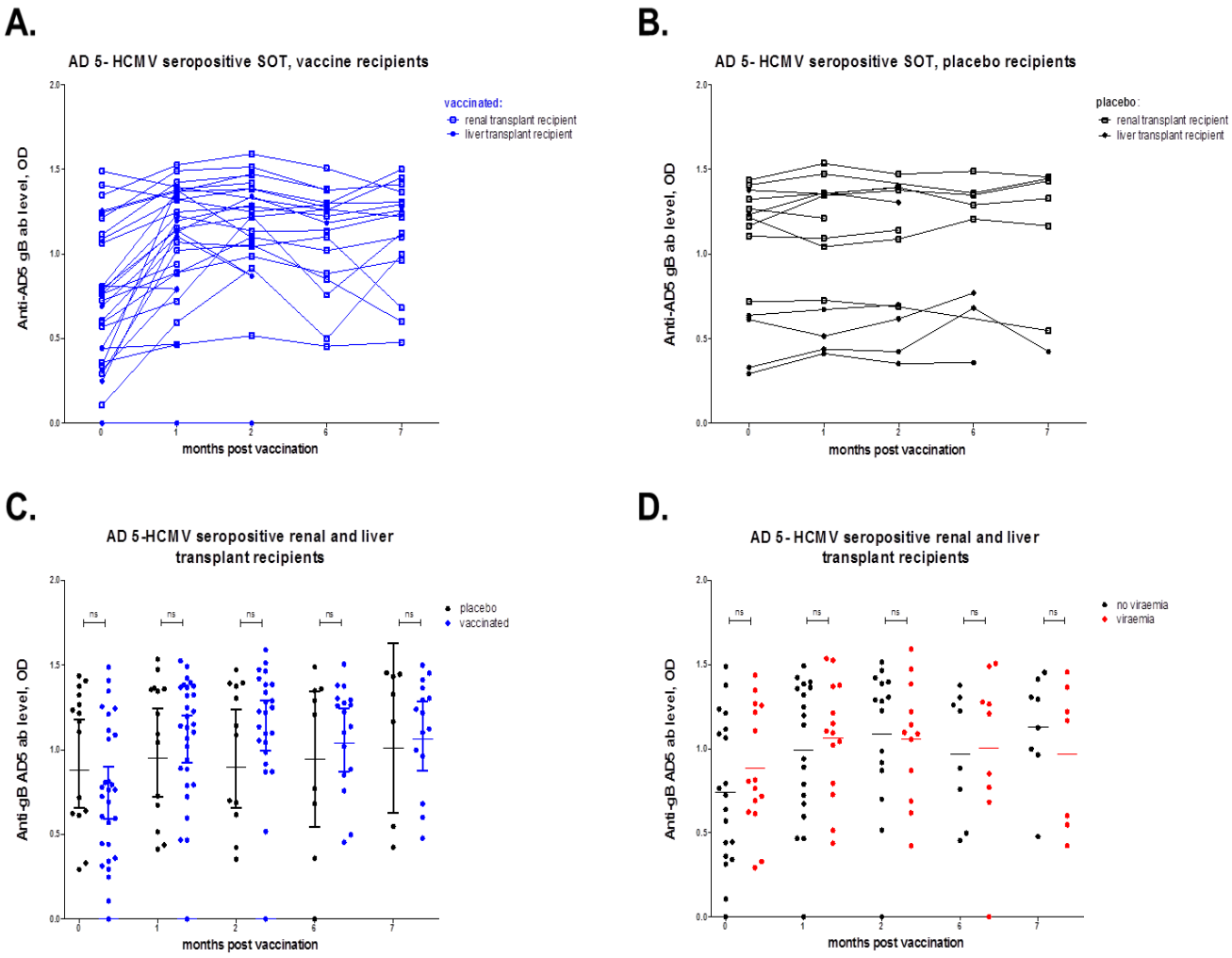


Figure 6.8. Vaccination of HCMV seropositive patients boosted antibody responses against AD5 but did not correlate with protection from viraemia following transplantation.

AD5 responses are represented as OD values at different time-points: day of first vaccine/placebo administration (month 0); day of administration of the second (month 1) and third dose (month 6), and 2 and 7 months post vaccination. (A) AD5 responses in HCMV seropositive vaccine recipients represented as OD values (B) AD5 responses in HCMV seropositive placebo recipients represented as OD values (C) Comparison between antibody levels against AD5 in the sera from vaccinated and placebo patients. Horizontal lines represent geometric mean values (±95%CI). (D) Comparison of antibody levels against AD5 between patients who develop viraemia versus patients who did not develop viraemia following transplantation. Horizontal lines represent geometric mean values. Statistical differences between the mean value of ODs between the populations of patients: vaccinated vs placebo and viraemia vs no viraemia were obtained from Mann Whitney Test (ns: not significant).

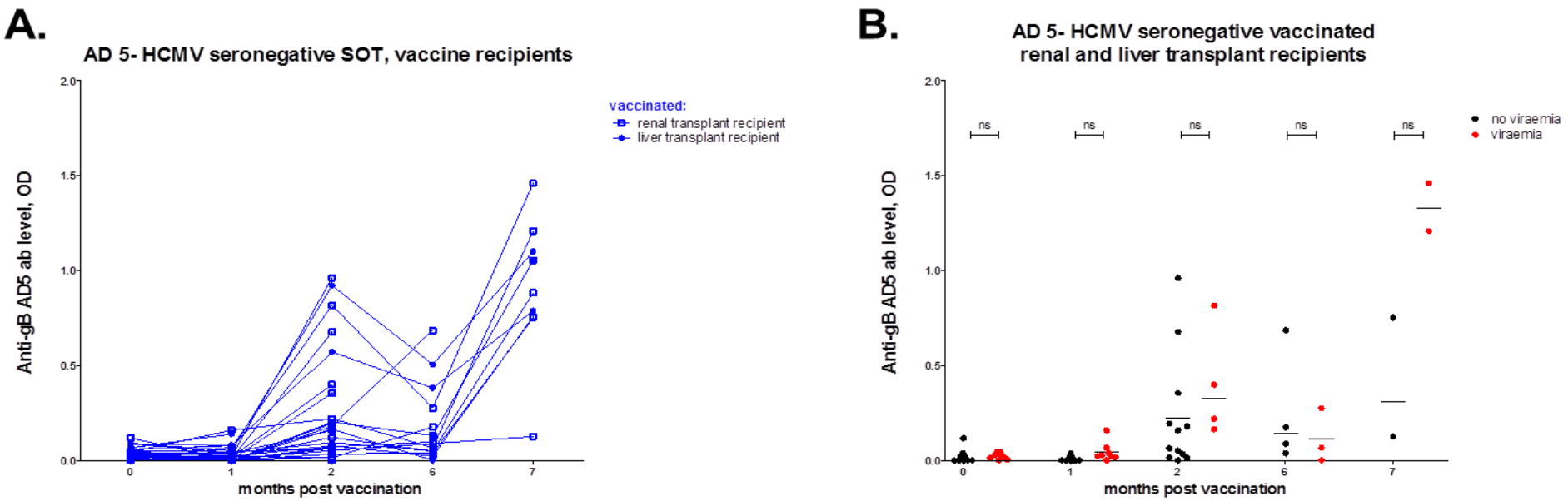


Figure 6.9. Vaccination of HCMV seronegative patients promoted antibody responses against AD5 but did not correlate with protection from viraemia following transplantation.

AD5 responses are represented as OD values at different time-points: day of first vaccine/placebo administration (month 0); day of administration of the second (month 1) and third dose (month 6), and 2 and 7 months post vaccination. (A) AD5 responses in HCMV seronegative vaccine recipients represented as OD values (B) Comparison of antibody levels against AD5 between patients who developed viraemia versus patients who did not develop viraemia following transplantation. Horizontal lines represent geometric mean values. Statistical differences between the mean value of ODs between the populations of patients (vaccinated vs placebo were obtained from Mann Whitney Test; ns: not significant).

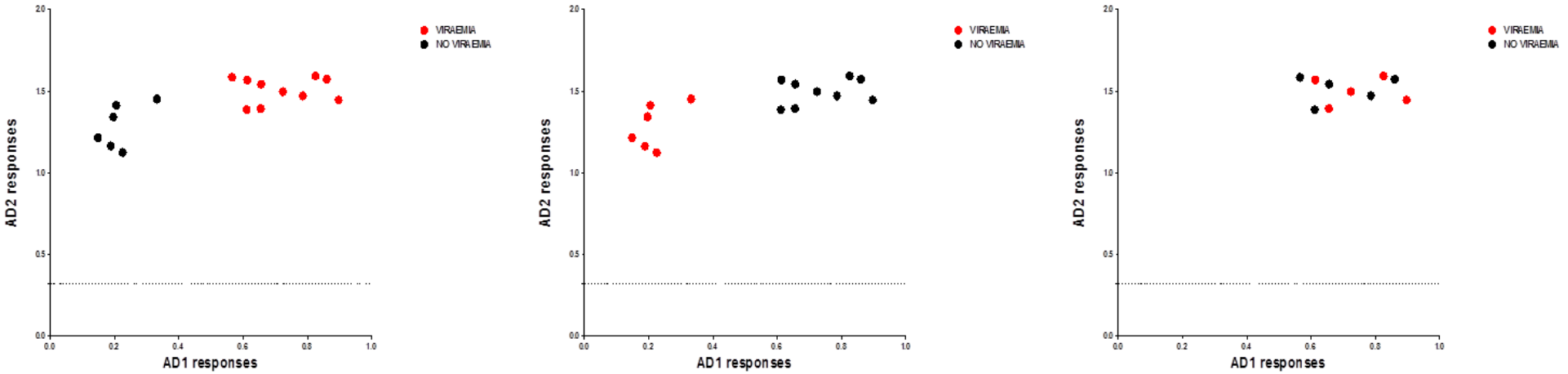


Figure 6.10. Hypothetical models of possible relationships between the AD1 and AD2 OD levels in vaccinated seropositive SOT recipients.

I) competition; II) additive effect; III) no interaction.

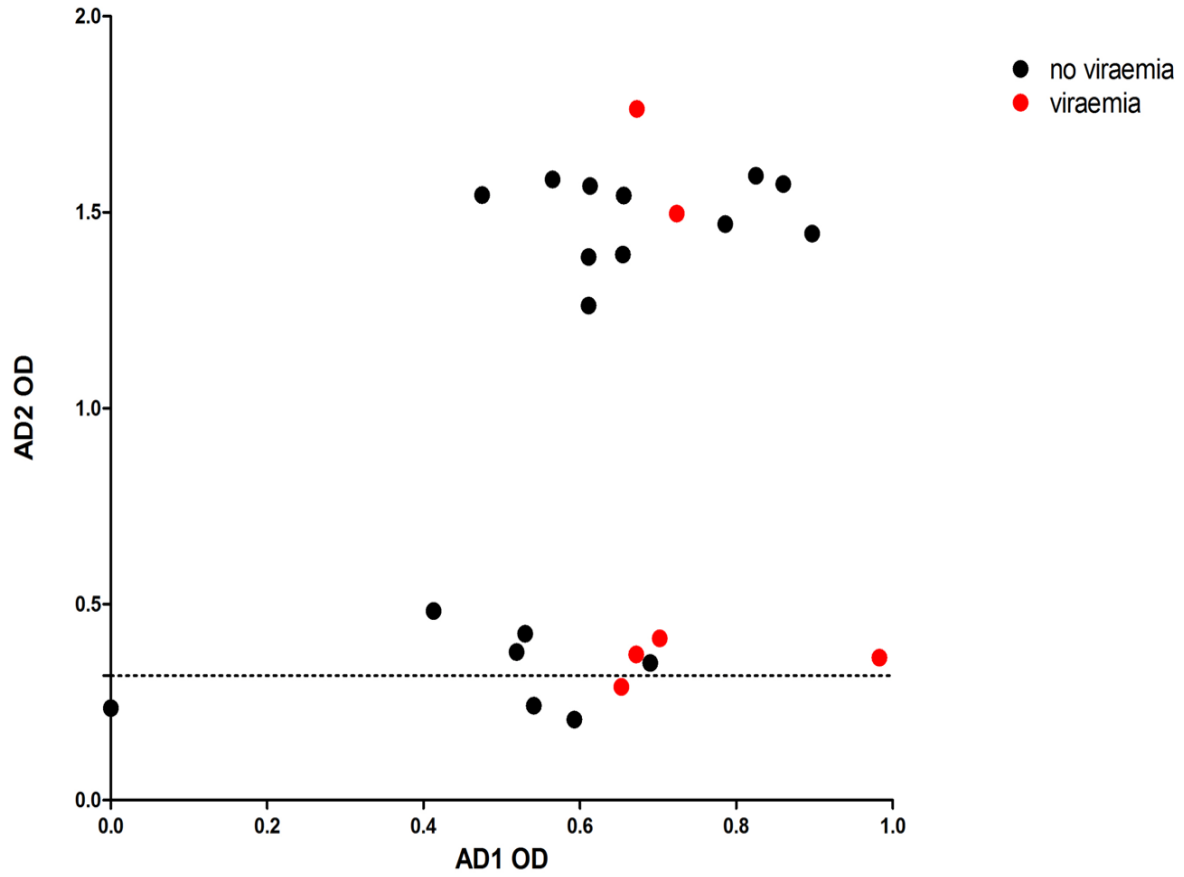


Figure 6.11. No correlation between the AD2 and AD1 OD levels in seropositive SOT recipients- post vaccination.

Antigenic domain	HCMV seropositive vaccine recipients		HCMV seronegative vaccine recipients
	Induction of antibody responses de novo	Boost of pre-existing responses	Induction of antibody responses
AD1	**Yes (Figure 6.2)**	**Yes (Figure 6.2)**	**Some (Figure 6.3)**
AD2	**No (Figure 6.5; Figure 6.5)**	**Yes (Figure 6.5; Figure 6.5)**	**No (Figure 6.6)**
AD4	**No (Figure 6.7)**	**Yes (Figure 6.7)**	**No (data not shown)**
AD5	**Yes (Figure 6.8)**	**Yes (Figure 6.8)**	**Yes (Figure 6.9)**

Table 6.1. Summary of antibody responses in sera from patients vaccinated with the subunit glycoprotein-B vaccine with MF-59 adjuvant against four key antigenic domains mapped onto gB.

Antigenic domain	HCMV seropositive vaccine recipients			HCMV seronegative vaccine recipients	
	% positivity prior to vaccination	% positivity following vaccination	protection from viraemia	% positivity following vaccination	protection from viraemia
AD1	**86.4% (38/44)**	**93.8% (15/16)**	**No**	**36% (4/11)**	**No**
AD2	**50% (23/46)**	**50% (9/18)**	**Yes**	**0%**	**No**
AD4	**98% (43/44)**	**93.8% (15/16)**	**Trend**	**0%**	**No**
AD5	**97.7 (43/44)**	**95.8 (23/24)**	**No**	**62.5% (10/16)**	**No**

Table 6.2. Summary of serologic results of sera from patients vaccinated with the subunit glycoprotein-B vaccine with MF-59 adjuvant against four key antigenic domains mapped onto gB and correlated with protection from viraemia following transplant.

6.4. Discussion.

The initial remit of this study was to identify whether any specific antibody responses against four known epitopes of gB correlated with protection against viraemia. To be classified as a correlate of protection in seronegative transplant recipients, any immunological responses would need to be induced by the vaccine and to correlate with protection against viraemia. In the seropositive donors the analysis was more complex: firstly, I could address whether any pre-existing immune responses to gB correlated with protection; secondly, whether vaccine administration enhanced pre-existing immune responses; thirdly, whether the vaccine elicited new responses which again correlated with protection. In this study I sought to find such a correlate that could explain the observed partial protection from viraemia seen following the administration of this vaccine formulation [188].

This analysis illustrates the complexity of studying immune responses to HCMV. For example, HCMV establishes latency from which it periodically reactivates which could alter the pattern of immunological responses seen at any time of analysis irrespective of any external vaccine administration. To control for this, I examined not only vaccinated patients but also recipients of placebo at the same time points. This allowed me to follow natural changes in the composition of the humoral immune response in seropositive transplant recipients. Here I aimed to provide more insight into the protective nature and fine specificity of the humoral responses against gB evoked either by vaccination with gB/MF59 or natural infection.

A major observation in this study was boosting of pre-existing AD-2 responses by gB/MF59. I demonstrated that the antibody responses towards this antigenic domain correlated with a level of protection in our patient cohort. The immunogenicity of AD-2 was variable, which is consistent with the published literature and extends the observation in natural infection to a human challenge model. Previous studies showed that only approximately 50% of infected individuals developed antibodies against the site I of AD-2 following natural

infection [162, 189, 426, 427] and the data I presented here are consistent with these observations.

This study also shows that the administration of the first dose of the subunit glycoprotein-B vaccine with MF-59 adjuvant boosted pre-existing antibody responses against AD2 in HCMV seropositive SOT patients (Figures 6.4 A and 6.5). These elevated antibody responses correlated with a lower number of patients who developed viraemia following transplant consistent with the hypothesis that antibodies against AD2 are protective (Figure 6.4 D). However, I noted that the vaccine failed to induce de novo anti-AD2 responses in both seronegative patients (Figure 6.6) and in seropositive individuals who did not possess anti-AD2 responses prior to vaccination (Figure 6.4 A and C). Taken together these data suggest that whilst pre-existing AD2 responses can be enhanced, the vaccine does not induce novel AD2 responses. Thus although evidence for a protective role for AD2 responses in a subset of patients could be postulated it clearly cannot explain the protection seen in patients who did not show evidence of an AD2 response.

Structural and immunochemical analyses suggest that the anti-AD-2 specific immunological responses may be created though a cascade of rare and very specific immunoglobulin gene re-arrangement events [429, 434]. Therefore, it is likely that the variable response towards this epitope following both natural infection and vaccination with gB/MF59 and Towne based vaccines is a consequence of the low probability of developing antibodies that require recombination of two well-conserved human germline V elements (IGHV3-30 and IGKV3-11), and IGHJ4 and the possibility of antigen competition through the easier production of AD-1 antibodies [435]. Antibodies against AD-2 are also characterized by specific substitutions at certain positions that seem to be crucial for high affinity binding to this epitope [159, 429, 436, 437]. Although only a proportion of infected individuals develop these rare AD-2 antibodies, they seem to play an important role in neutralizing the virus and controlling infection [427, 437-439]. Thus an immunogen that can enhance or generate de novo responses against AD-2 is a good candidate for a new HCMV vaccine. Importantly, my data suggest that, whilst pre-existing AD-2 responses established at the time of primary infection or in response to the reactivation of the virus from latency can be

enhanced, the gB/MF59 vaccine does not induce AD-2 responses in those lacking them at baseline. Interestingly, I observed an increase in the level of AD-2 responses in one recipient of placebo and suggest that this might be a response to reactivation of latent virus or even a re-infection event in this patient prior to transplant. In summary, although evidence of a protective role for AD-2 responses was seen in a subset of patients and boosting of these responses with a vaccine in seropositive patients correlated with protection, the failure to induce de novo AD-2 responses in both seropositive and seronegative patients suggests a target that newer vaccines should aim for.

In contrast to the variability in individuals displaying AD-2 responses, the vast majority of HCMV infected individuals develop antibodies against AD-4 following natural infection. This discontinuous domain is highly immunogenic, since approximately 90% of seropositive individuals possessed humoral responses against this structure in a previous report [160]. Here I confirm that indeed nearly all seropositive patients possess antibodies against AD-4. These AD-4 specific antibodies have a high neutralizing capacity at the post-adsorption step which suggests that evoking these immunological responses by vaccination may help control the virus [160].

Furthermore, the vaccination of HCMV seropositive patients awaiting solid organ transplant boosted significantly the level of antibody against AD4 in HCMV seropositive SOT patients who had high anti-AD4 antibody levels prior to vaccination (Figure 6.7 A and C). Of the few seropositive patients with low level AD4 antibody responses I noted an increased anti-AD4 antibody level post vaccination in some individuals but not all (Figure 6.7 A). However, I also observed small increases in these AD4 specific responses in some placebo recipients, too, which may be indicative of natural fluctuation in the AD4 responses in healthy seropositives possibly due to re-infection or reactivation of latent infection occurring in these individuals during the period of analysis (Figure 6.7 B). Although the results did not reach statistical significance, there was a clear trend that the patients who had higher level of AD4 specific antibody responses were less likely to develop viraemia (Figure 6.7 D). This trend indicates that the antibody responses against AD4 may play an important role in protection.

In contrast to my observations with seropositive patients, the immunization of seronegative patients failed to induce any antibody responses towards AD4 (Table 6.1 and 6.2).

Here it is interesting to note potential analogies with other herpesviruses. Antibodies that bind to the AD-4 corresponding sequence on HSV-gB inhibit the interaction of gB with gH/gL complex with a downstream effect on viral fusion [440]. Although the actual role of this antigenic domain in HSV entry into cells remains unclear, a similar effect on HCMV could have marked impact on the ability to infect cells. Although the specific activity of the antibody response against AD-4 on infection remains unclear I cannot exclude from the small number of serum samples available to me that the antibody responses against AD-4 may also provide a certain level of protection. Importantly, similar to AD-2 responses, vaccination boosted only the pre-existing responses towards this potentially protective epitope. It remains to be established why vaccination could not elicit de novo AD-2 and AD-4 responses in seropositives not possessing these responses prior to vaccination or induce these responses in seronegative individuals. One possibility is that the structural form of the gB delivered in the vaccine occludes the production of good antibody responses against these potentially protective epitopes. One future approach to address this may utilise fragments of gB for vaccination to determine whether AD-2 or AD-4 responses can be induced de novo when presented alone or prior to vaccination with full gB.

In contrast to my observations with AD-2 and AD-4 directed antibody responses, serological analysis of this vaccine cohort revealed that the antibody responses towards two further domains, AD-1 and AD-5, did not correlate with protection. It is well documented that the humoral responses against the immune- dominant region AD-1 that develop after natural infection with HCMV have a wide range of neutralizing capacity [158, 191]. However, competition between non-neutralizing and neutralizing antibodies against AD-1 was reported soon after the discovery of this antigenic region [158, 161, 192]. I could find no supporting evidence that high levels of AD-1 antibodies increased the chance of viraemia but the lack of a protective effect could argue for its removal from a future vaccine. Thus, if we consider the hypothesis that the virus is selected to benefit from immune

responses directed against AD-1 because of impaired generation of robust responses against key neutralizing epitopes, and then elimination of AD-1 from gB may lead to better protective antibody responses. What was not clear from my initial study was whether AD-1 binding antibodies have a negative impact through competition with other neutralizing antibodies. It seemed possible that the antibodies against AD-1 could potentially block the AD-2 epitope due to conformational masking as both of these antigenic domains are in close proximity on gB [358]. However, attempts to engineer gB without AD-1 proved to be difficult as AD-1 is necessary for oligomerization of gB and lack of this domain significantly compromised the structure of this protein [441]. In order to establish whether there is a competition between AD1 and AD2 antigenic domains and their binding capacities due to steric proximity I performed additional set of analyses in which I have shown no interactions between these antibody responses (Figure 6.11).

Based on the date presented here, it can be hypothesised that future vaccines could be developed by using immunogens encoding AD-2 and AD-4 domains only. The most straightforward way to do this would be with peptides. However, a pre-clinical animal study on peptide-based vaccine specific to the HCMV gB AD-2 region showed that such vaccine formulation elicits only poor neutralizing antibody responses [442]. Such an outcome emphasises that there are many challenges in developing potent immunogenic vaccines against single antigenic domains presented on a large parent molecule.

I also analysed humoral responses against the recently described AD-5. Importantly, the antibodies that target this domain neutralize the virus in *in vitro* studies [160] [433] which suggests that these responses may play an important role in protection. The first report on AD-5 immunogenicity revealed that approximately 50% of seropositive individuals developed antibodies that bound to this antigenic site [160]. However, using second generation antigens and tests, seropositivity rates in healthy HCMV infected individuals were found to be in the range of 90% (A. Wiegers, M. Mach, personal communication). Consistent with this, we showed that sera from nearly all seropositive patients contain antibodies against AD5 (Figure 6.8 A and B) which is in line with the more recent estimates. Interestingly, similarly to AD1 responses, analyses showed no correlation between

increased level of these AD5 specific responses and reduction in viraemia. Whether HCMV exploits AD1 and AD5 domains to evade the immune system by masking the epitopes that may be protective, or by directing the attention of the immune system towards poorly functional domains, remains to be further investigated.

The previous report from my group revealed that the vaccine elicited high titres of anti-gB antibodies in seronegative patients [188]. However, the immunogenicity of the vaccine against these four well-defined antigenic domains used in this study is surprisingly low amongst the vaccinated seronegative cohort. I could observe only modest responses in seronegative patients against AD1 and AD5 that did not correlate with protection. It is worth noticing that the AD1 and AD4 antigenic domains were produced in bacterial expression systems which could potentially alter glycosylation patterns and at least partly explain poor recognition of the antibodies towards these epitopes. However, the antibody responses in seropositive individuals prior to vaccination are well recognised by these antigenic domains. The reasons why this vaccine boosted pre-existing antibody responses in seropositive patients but failed to induce these responses de novo in both seronegative and seropositive patients, remains unclear. Clearly, a substantial response against gB is made, [188] suggesting the possibility that some other, currently unknown epitopes on gB are presented effectively in the vaccine.

7. Longitudinal analyses of humoral antibody responses elicited by vaccination.

7.1. Pharmacodynamic assessment of the post-transplant sera.

Pharmacodynamics is the study of the effect of a particular drug or chemical compound exhibited on a living organism. This approach focuses on the duration and magnitude of response observed in relation to the concentration of the drug at an active site in the organism. In contrast, pharmacokinetic approaches investigate how the drug or chemical compound is absorbed and distributed ("biotranslocation"), what chemical alterations a drug may undergo in the body, ("biotransformation") and ultimately- how the drug is stored within the living organism and eliminated from it. Put succinctly, they can be described as: "pharmacodynamics is study of what a drug does to the body, whereas pharmacokinetics is the study of what the body does to a drug".
Consequently, pharmacokinetics requires a specific knowledge about the mechanism of action of the drug. Therefore it is not possible to use this approach for medical conditions where the protective component has not been identified. On the other hand, the pharmacodynamic approach offers a potential unbiased, open-ended way to a discovery as long as a robust biomarker is available as the read out of successful control of a disease process.

These principles can be applied to HCMV. It is known that the immune system controls replication of this virus, but the precise components of the immune system that provide control have not been identified. In patients whose immune system is compromised, HCMV replicates to high levels and can be detected in the blood. Thus, serial quantitative measures of viraemia (described in chapter 1.16; Table 1.4, Fig. 1.9) can be interpreted as a natural biomarker of HCMV activity. This quantification was used as a reference point for the phase-2 vaccine trial with subunit, recombinant gB vaccine [2] (described in chapter 1.16). Most importantly; this systematic analysis could then be applied to immunocompromised patients randomised to receive an experimental vaccine or a matching placebo. If the vaccine reduces the viraemia biomarker, then correlations can be sought with a variety of potential immune effectors.

In order to perform the pharmacodynamic assessment of the clinical samples obtained from the patients who participated in the clinical trial, the glycoprotein-B antibody titres were measured and correlated with the virological parameters that were established in the natural history studies described previously. This approach allowed evaluation of the efficacy of the vaccine despite the lack of prior information on the correlate of protection (see chapter 1.16, [188]).

7.2. Interruption of viral transmission.

A major characteristic of herpesviruses like HCMV is their ability to undergo both lytic and latent types of infection [34, 275]. Typically, after primary infection and initial immune response, HCMV establishes life- long latency that may be followed by periods of reactivation of lytic infection [443]. Although HCMV is controlled through the life-time by the immune system of healthy individuals, there might be severe outcomes of reactivation from the latent stage in immunocompromised individuals such as HIV patients, [293-296, 298, 299] individuals that require immunosuppressive therapy, SOT patients, hematopoietic stem cell transplant recipients [188, 284-291]; and possibly even the elderly population whose immune system is less efficient due to immune senescence [444]. The reactivation of latent virus in these vulnerable groups is associated with increased mortality and morbidity rates [284, 286]. The maintenance of latency requires constant evasion of the robust immune-surveillance of the host. In order to survive in the hostile environment, HCMV evolved to become a master of immune-evasion, encoding many proteins that target and downregulate the effectors of the host's immunological system [445-447]. Such interference is associated with an immunosuppressive effect, thus it is very likely that it makes the host more susceptible to other infections [448]. Additionally, an increasing body of evidence indicates that the replication of HCMV is also associated with inflammation and several autoimmune-diseases. However, some of those links between inflammation, auto-immunity and the role of HCMV infection are controversial and not fully understood yet [448-450].

Interestingly, over the last few years a number of reports showed that HCMV plays a more active role during latency than was previously thought as many virally encoded genes are now known to be expressed during this dormant stage. However, the role of these latently expressed proteins is not fully elucidated yet. Indisputably, having both latent and lytic types of infection the strategy common to all herpes viruses, made them very successful in persistence and transmission within the human population and it is important to investigate whether vaccination is able to interrupt transmission of virus from the organ donor during SOT.

Therefore, as a last step of this follow up analysis, I investigated whether this vaccine could block transmission of the virus from HCMV infected donor to seronegative graft recipient. It was well established in our previous studies that patients in this group (D+R-) are at the highest risk of onset of viraemia and HCMV disease [35, 188]. Although the number of patients in the highest risk D+R- group was small, I was interested whether humoral immunity induced by vaccination in these patients had an impact on reducing the transmission rate of HCMV from donor to recipient and how these humoral responses conferred protection in some of these vaccinated patients.

Objectives:

The analyses thus far have focused on characterising the humoral immune response up to the point of transplantation in an attempt to identify the protective element. In this section my approach is to analyse the sera post-transplant to determine whether there is evidence of differential responses in individuals who have received the vaccine versus placebo. Finally, I will investigate, as a proof of concept, whether I can establish an assay to measure whether the vaccine completely blocks transmission as well as reducing measures of viraemia.

7.3. Materials and methods.

7.3.1. Clinical trial: conduct and patients.

Randomisation, masking, vaccination schedule and conduct of this phase-2 placebo-controlled trial clinical trial were described in detail elsewhere (chapter 2.1) [188]. Briefly, the vaccine or placebo was given in three doses: at day 0 (baseline), 1 month and 6 months later. Blood samples were collected on each of these 3 times as well as 1 month after dose 2 (Month 2) and one month after the third dose (Month 7) [188]. The patients who subsequently underwent transplantation were followed up for 90 days during which serial blood samples were obtained at days: 0, 7, 35, 63 and 90 (Figure 1.14). Some patients who completed this study gave informed consent to obtain their blood samples at the times of their follow-up visits (approximately 2 years post vaccination, end of the study-July, 2016). The population from whom samples have been evaluated and described in this work are HCMV seronegative solid organ transplant patients who participated in this follow-up study.

7.3.2. Detection of Latency.

PCR was performed using 2x PCR Master Mix (ThermoFisher). Forward and reverse primers (Invitrogen) were present at 2ng/µl in each reaction. The nested UL138 PCR used 30 cycles for the first reaction, then 15 for the nested PCR (Table 7.1. A).

A nested PCR that amplified the UL138 region was conducted according to standard procedures as follows: 25µl of PCR MasterMix (Promega): 1µl (1:10 diluted in sterile water) of forward primer for UL138 (Integrated DNA Technologies); 1µl (1:10 diluted in sterile water) of reverse primer for UL138 (Integrated DNA Technologies) made up to a final volume of 45µl with sterile, nuclease-free water (Promega). Then, 5µL of extracted DNA from clinical samples were added and all clinical samples were analysed in duplicate. The extremely low proportion of genome positive cells in natural latency (1 per 10,000 cells [128]) often requires the use of nested PCR approach to detect it. Therefore a nested PCR was performed on 5µL of the first round PCR product, 25µl of PCR

MasterMix (Promega),1µl of forward primer for nested UL138 (Thermofisher); 1µl of reverse primer for nested UL138 (Thermofisher) and made up to a final volume of 45µl with sterile, nuclease-free water (Promega). As a control the DNA samples were amplified with primers against the house- keeping gene 18S (1:10 diluted in sterile water) (Thermofisher), conditions as indicated above. PCR cycling conditions were: 10min at 95°C; and 30 cycles of 40s at 95°C; 40s at 55°C and 90s at 72°C and 10min at 72°C (for both standard and nested PCR).

A)

	Stage	Temperature	Duration
1 x	Initial Denature	95^{o}C	60 seconds
30 x	Denature	95^{o}C	40 seconds
	Annealing	55^{o}C	40 seconds
	Extension	72^{o}C	90 seconds
1 x	Final Extension	72^{o}C	600 seconds

B)

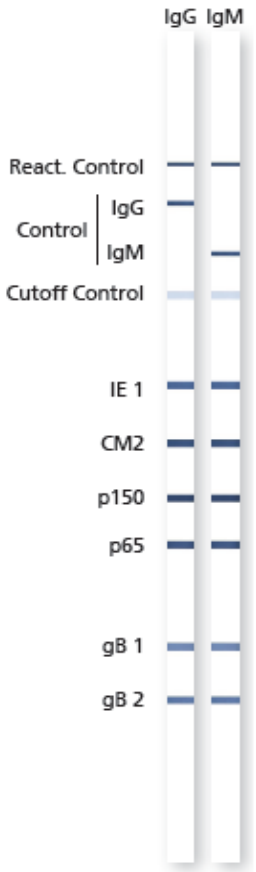
Product	Forward	Reverse
UL138	TGCGCATGTTTCTGAGCTAC	ACGGGTTTCAACAGATCGAC
UL138 Nest	GAGCTGTACGGGGAGTACGA	AGCTGCACTGGGAAGACACT
18S	GTAACCCGTTGAACCCCA	CCATCCAATCGGTAGTAGCG

Table 7.1. PCR specification. A) PCR workflow; B) Primer sequences.

7.3.3. Serological analysis.

Serological analyses were performed using commercially available line immunoassay kit recomLine CMV IgG and recomLine CMV IgM (Mikrogen Diagnostik) according to manufacturer's instruction. This stripe test contains recombinant antigens to detect IgG and IgM antibodies directed against HCMV from human sera. The antigens included in the test were IE1 (IE1 protein); CM2 (p52 protein, UL44, UL57); p150 (pp150 protein, UL32); p65 (pp65 protein, UL83); gB1 (gB protein, UL55) and gB2 (gB protein, UL55). Statistical analysis was not performed here due to the small number of samples.

A)



B)

Antigen	Reading frame / protein	Natural function	Size [kDa]
IE 1	UL123 / IE 1/1	Non structure protein, „immediate-early"-protein	53
CM2	UL44, UL57 / p52 (DBP)	Non structure protein	45
p150	UL32 / pp150	Tegument protein	50
p65	UL 83 / pp65	Tegument protein	31
gB 1	UL 55 / gB	Membrane glycoprotein gB	25
gB 2	UL 55 / gB	Membrane glycoprotein gB	18

Figure 7.1. Commercially available line immunoassay kit recomLine CMV IgG and recomLine CMV IgM (Mikrogen Diagnostik).

A) Schematic representation of the stripe test that contains recombinant antigens to detect IgG and IgM antibodies directed against human cytomegalovirus from human sera and B) table containing the list of gB antigens that are incorporated into this test and their short characterization.

7.4. Results.

7.4.1. Pharmacodynamic assessment of the post-transplant sera from the D+R- group.

In order to investigate the magnitude of humoral responses post-transplantation, total level of antibodies against gB were measured by enzyme immunoassay and the viral load was evaluated by PCR (Table 7.2). Unfortunately, the number of patients in this D+R- who proceeded to transplant was very limited. We had only four placebo and eleven vaccine recipients. Interestingly; all four placebo patients (100%) experienced viraemia in comparison to six out of eleven vaccinated patents (55%); (Table 7.2). Moreover, three placebo patients had antibody levels below the baseline (negative cut-off) throughout the period of analysis and only one placebo patient developed quickly a high level of anti-gB antibodies. However, this did not protect this individual from the onset of viraemia. In general, the vaccinated patients (when compared to placebo): had higher levels of anti-gB antibodies, and required less time to produce these high titres of antibodies (Table 7.2).

I also analysed virological parameters other than viraemia occurrence such as total viraemia duration, occurrence and duration of subsequent viraemia episodes, peak viral load and duration of antiviral therapy. The detailed analysis of the duration of viraemia in both vaccine and placebo recipients revealed interesting differences between these two groups of patients. A decrease in the total duration of viraemia in vaccinated group was observed (Figure 7.2.A). Due to the small number of samples it is difficult to assess whether there are any differences in the duration of the first episode of viraemia in those patients who developed viraemia. (Figure 7.2.B). However, when I looked at the subsequent episodes of viraemia, it became clear that vaccine prevented its occurrence. None of the vaccinated patients experienced second episodes of viraemia (0/11) in comparison to three out of four patients (placebo recipients) who had more than one episode of viraemia (Figure 7.2.C). Moreover, I could see an inverse relationship between the baseline antibody titre and viraemia occurrence (Figure 7.3.A) and duration (Figure 7.3.B, [188]) in seronegative patients who received organs from

seropositive donors. Interestingly though, it seems that vaccination did not have impact on the other virological parameter: peak viral load, which is defined as a maximum number of viral copies per ml of blood that was detected during the follow-up period in each patient (Figure 7.4).

Overall, despite sample number limitations, these results suggest that vaccine recipients were immunologically primed to combat and clear viral infection more effectively in comparison to the placebo group.

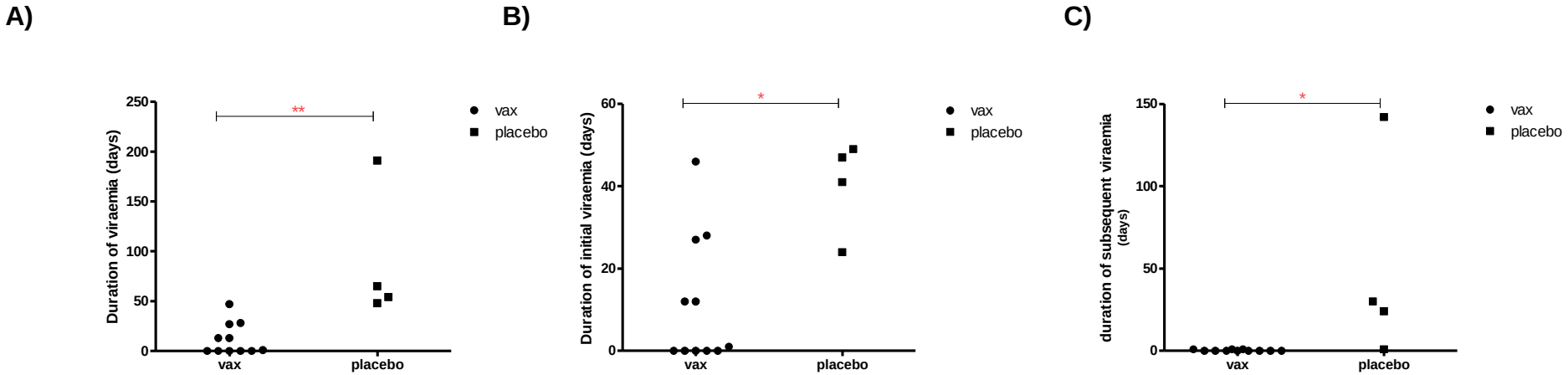


Figure 7.2. Duration of viraemia post- transplantation in seronegative patients who received organs from seropositive donors is shorter in vaccinated group.

A) Total duration of viraemia in vaccine vs placebo groups of patients. B) Duration of first episode of viraemia in vaccine vs placebo groups of patients. C) Duration of subsequent episodes of viraemia in vaccine vs placebo group of patients. Statistical differences were obtained from Mann Whitney Test; *: $p<0.05$; **: $p<0.01$).

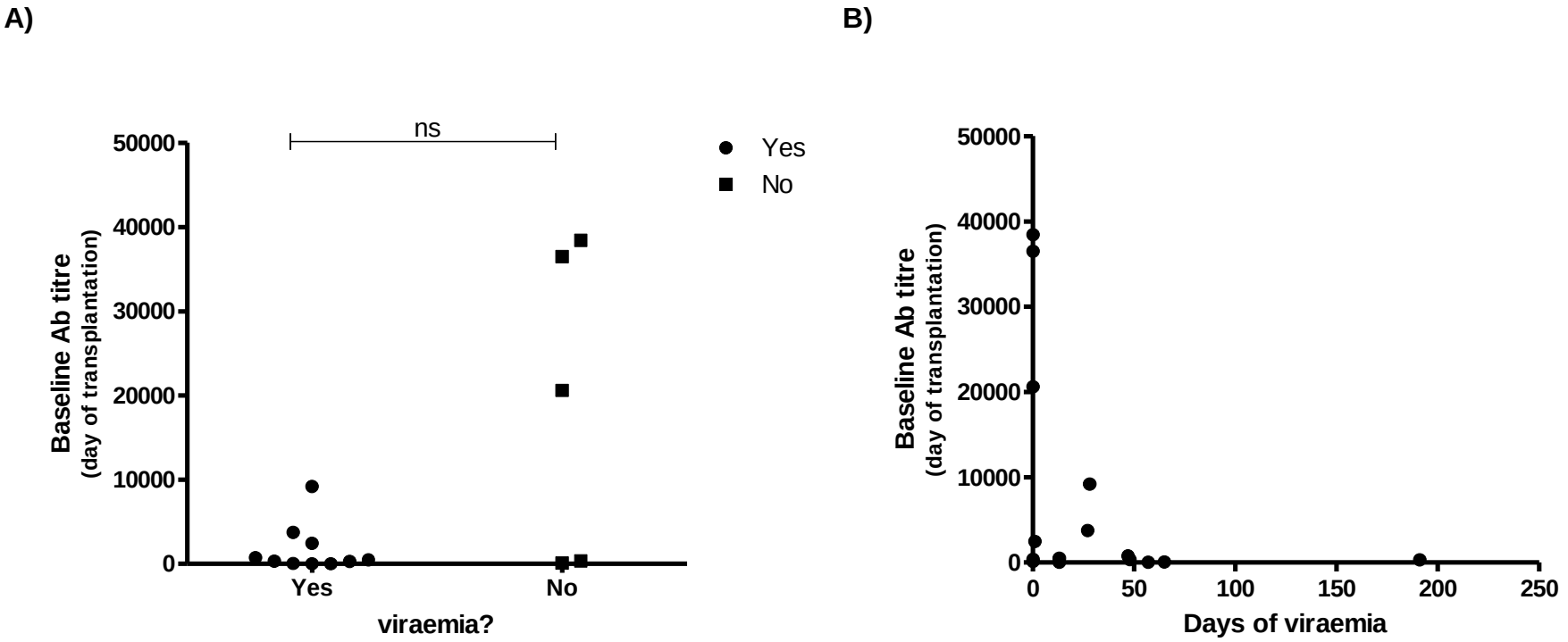


Figure 7.3. Inverse relationship between the baseline antibody titre at the day of transplantation and viraemia occurrence and duration in seronegative patients who received organs from seropositive donors (D+R-).

A) Inverse correlation between the baseline antibody concentration and viraemia occurrence B) Inverse correlation between the baseline antibody concentration and duration of viraemia. Statistical differences were obtained from Mann Whitney Test; ns: p>0.05).

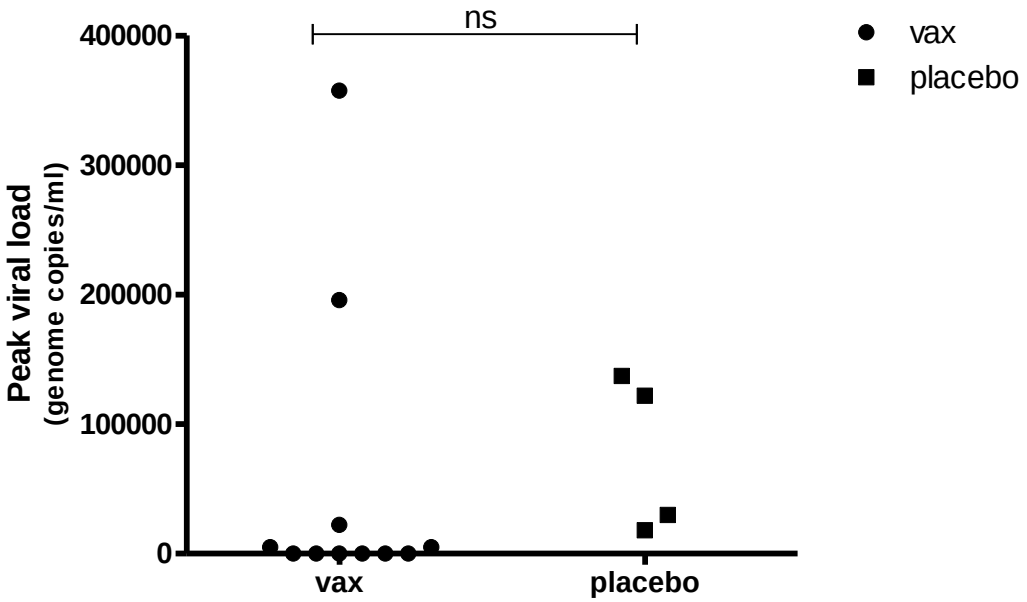


Figure 7.4. Peak viral load in vaccinated and placebo seronegative patients who received organs from seropositive donors.

Peak viral load is the highest value of genome copies per ml of blood that was detected during the surveillance time (up till 90 days post vaccination). Statistical differences were obtained from Mann Whitney Test; ns: p>0.05).

No	vaccine/ placebo	donor status	viraemia	days viraemia	gB last sample before tx	gB D0	gB D7	gB D35	gB D63	gB D90
002-009	placebo	POS	yes	191d	395	305	233↓	149	184	160
002-020	placebo	POS	yes	54d	<50		<50	<50↓	257	3140
004-004	placebo	POS	yes	65d	<50		106305↓	71785	114106	229984
004-019	placebo	POS	yes	48d	<50	323	209↓	1324	9131	5400
002-003	vax	POS	yes	47d	737	764	472	277↓	42038	
002-008	vax	POS	no		1243	355	151	318	283	281
002-018	vax	POS	no		37858	38426	17108	16815	10840	5177
002-035	vax	POS	no		10425	36487	15869	14798	11879	9261
002-040	vax	POS	no		41612	20590	21590	21725	14464	12355
004-003	vax	POS	yes	1d	<50	2458	1593↓	1234	2287	1898
004-012	vax	POS	yes	27d	1107	3737	6844↓	8330	37606	42677
004-015	vax	POS	no		137	103	186	243	663	340
004-017	vax	POS	yes	13d	<50	474	1393↓	1048	13019	77544
004-021	vax	POS	yes	13d	<50		↓	660262	1732390	284393
004-025	vax	POS	yes	28d	49961	9170	7833↓	15024		

Table 7.2. The database of the D+R- cohort.

The table contains both: virological parameters (viraemia occurrence and duration) and the serological data – total level of the anti-gB antibodies measured by ELISA in the samples collected at different time-points prior to and post transplantation. Red arrows indicate the onset of viraemia.

7.4.2. Serology.

7.4.2.1. Analyses of the antibody repertoire following vaccination.

The suggestion of prompt anti-gB responses in the R- population led me to investigate whether a humoral response was raised against other HCMV antigens post- transplant. I performed longitudinal analyses of serological responses (IgG Table 7.3 and IgM Table 7.4) towards key HCMV-antigens IE1 (IE1 protein); CM2 (p52 protein, UL44, UL57); p150 (pp150 protein, UL32); p65 (pp65 protein, UL83); gB1 (gB protein, UL55) and gB2 (gB protein, UL55) in follow-up serum samples from seronegative HCMV gB-MF59 vaccine recipients collected approximately two years following transplantation. Patients from the highest risk group D+R- (n=7) are highlighted in yellow; patients from non-risk group D-R- (n=3; served as control group in these analyses) are highlighted in green. Interestingly, this analysis revealed that all patients from D+R- group had good IgG antibody responses towards HCMV. All patients had antibody responses towards the vaccine antigen- gB- as expected, but also there was clear evidence of antibody responses towards other HCMV antigens. For example, pp150 responses were detected in all subjects. All 7 D+R- patients had viraemia post-transplant and no follow up samples were available from D+R- patients without viraemia. In contrast, in the control group (D-R-) no antibody responses were detected against HCMV proteins.

As well as analysis of IgG I also investigated the IgM antibody repertoire. In contrast to the IgG response only two out of seven D+R- and none of control D-R- patients had detectable levels of IgM.

patient no	IE1	CM2	p150	pp65	gB1	gB2
002-00010	yes	yes	yes	no	yes	yes
004-00004	no	no	yes	no	yes	yes
004-00012	yes	yes	yes	yes	yes	no
004-00017	yes	yes	yes	yes	yes	yes
004-00019	yes	yes	yes	yes	yes	yes
004-00021	no	no	yes	no	yes	yes
002-00014	no	no	yes	no	yes	no
002-00027	no	no	no	no	no	no
002-00028	no	no	no	no	no	no
004-00020	no	no	no	no	no	no

Table 7.3. Analyses of IgG responses towards key HCMV-antigens in the sera from gB-MF59 seronegative vaccine recipients that were collected approximately 2 years following transplantation.

Patients from the highest risk group D+R- (n=7) are highlighted in yellow, patients from non-risk group D-R- (n=3) highlighted in green served as control group in these analyses.

patient no	IE1	CM2	p150	pp65	gB1	gB2
002-00010	yes	yes	yes	no	no	no
004-00004	no	no	no	no	no	no
004-00012	no	no	no	no	no	no
004-00017	no	no	no	no	no	no
004-00019	no	no	no	no	no	no
004-00021	no	no	no	no	no	no
002-00014	no	no	yes	no	no	no
002-00027	no	no	no	no	no	no
002-00028	no	no	no	no	no	no
004-00020	no	no	no	no	no	no

Table 7.4. Analyses of IgM responses towards key HCMV-antigens in the sera from gB-MF59 seronegative vaccine recipients that were collected approximately 2 years following transplantation.

Patients from the highest risk group D+R- (n=7) are highlighted in yellow, patients from non-risk group D-R- (n=3) highlighted in green served as control group in these analyses.

7.4.2.2. The impact of the duration from the time of receiving the last vaccination dose to the time of challenge with the virus (transplantation) and the number of vaccine doses received on development of viraemia.

The next parameter I wished to investigate was time. Specifically, I was interested to determine whether the duration from the time of receiving the last vaccination dose to the time of challenge with the virus had any impact on the virological parameters- *e.g*. probability of viraemia occurrence; longer duration of viraemia or anti- viral treatment. Secondly, I was interested whether the number of vaccine doses administered to the patients is inversely correlated with the duration of viraemia.

The results are shown in the Table 7.5. Due to the small number of patients in this high risk group (D+R-) it is difficult to assess whether the duration from the time of receiving the last vaccination dose to the time of challenge with the virus was correlated with the values of these virological parameters. Similarly, it is difficult to conclude whether the number of vaccine doses received impacted the outcome, as the majority of the patients in this group received only two doses of the vaccine prior to transplantation.

No	*vaccine/ placebo*	*donor status*	*viraemia*	*days viraemia*	*No. of doses*	*Period between vaccination and transplantation*
002-009	placebo	POS	yes	191d	3	3 months
002-020	placebo	POS	yes	54d	3	3 months
004-004	placebo	POS	yes	65d	3	3 years, 7 months 2 weeks
004-019	placebo	POS	yes	48d	2	2 weeks
002-003	vax	POS	yes	47d	2	2 weeks
002-008	vax	POS	no		2	4 months 2 weeks
002-018	vax	POS	no		*2*	*2 weeks*
002-035	vax	POS	no		2	1 month
002-040	vax	POS	no		3	3 months 3 weeks
004-003	vax	POS	yes	1d	2	1 month
004-012	vax	POS	yes	27d	2	2 weeks
004-015	vax	POS	no		1	1 month
004-017	vax	POS	yes	13d	2	2 weeks
004-021	vax	POS	yes	13d	2	1 month 3 weeks
004-025	vax	POS	yes	28d	3	3 weeks

Table 7.5. Database of the D+R- cohort.

The table contains virological parameters (viraemia occurrence and duration); the total number of vaccine/placebo doses received and the duration between vaccination and transplantation**.**

7.3.3. Detection of latent infection.

Finally, I wished to investigate whether I could detect latent viral genomes in the monocytes of seronegative patients in post-transplant follow up samples. To do this, DNA was harvested from 10^5 CD14+ monocytes which had been isolated from peripheral blood from previously R- transplant recipients. DNA was then amplified using a nested PCR against viral DNA. A PCR analysis of three placebo recipients (D+R-); two vaccine recipients (D+R-) and a D-R+ positive control was performed. Interestingly, viral DNA could be amplified from all samples (except water control) including one D+R- sample that did not exhibit any evidence of viraemia post-transplant. Although this signal was less (lane 5) the PCR amplification the cellular DNA gene 18S suggested that this was likely due to lower levels of input DNA in the PCR.

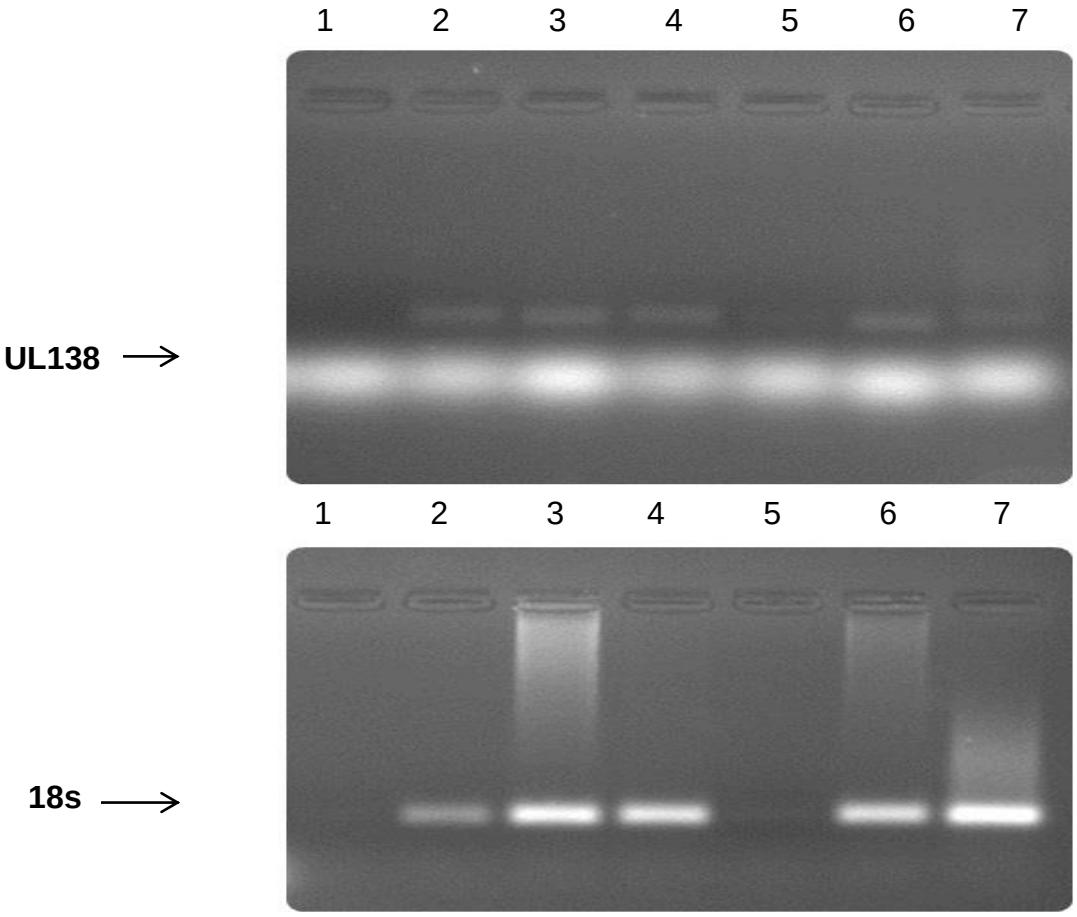


Figure 7.5. Detection of HCMV UL138 gene as a marker of latent infection.

Lane 1) water (as a negative control), 2) D+R-; placebo recipient (002-00010); 3) D+R-; vaccine recipient (002-00014); 4) D+R-; placebo recipient (004-00004); 5) D+R-; vaccine recipient (004-00015); 6)D+R-; placebo recipient (004-00019); 7) D-R+ vaccine recipient (001-00053)- as a positive control. Upper panel indicates UL138 (pointed by arrow) and lower indicates control- 18s (pointed by arrow).

patient	vaccine/ placebo	donor/ recipient status	Viraemia (>200 genome copies/mL blood)	PCR positive-latency
002-00010	plac	D+R-	yes	yes
002-00014	vacc	D+R-	yes	yes
004-0004	plac	D+R-	yes	yes
004-00015	vacc	D+R-	no	yes
004-00019	plac	D+R-	yes	yes
001-00053	*vacc*	*D-/R+*	*no*	*yes*

Table 7.6. Detection of HCMV UL138 sequences as a marker of latent infection.

7.4. Discussion.

A long duration of antibody responses elicited by vaccination could be important for seronegative transplant candidates who are waiting to be challenged with the virus transmitted from a seropositive donor. These recipients do not possess any pre-existing immune responses to the virus other than those made in response to vaccination and would elicit impaired antibody responses towards the HCMV from the positive organ due to the immunosuppressive drug regimen. Therefore, this serological analysis illustrates the advantages of conducting longer-term follow up studies to correctly assess the humoral responses evoked by vaccination and their full potential to protect from this pathogen.

In this analysis I aimed to investigate the humoral responses in follow up samples (post-transplant) of this highest risk group D+R-. Firstly, I looked at the duration of these antibody responses and analysed sera of D+R-patients that were collected approximately 2 years after transplantation. I could detect good and broad antibody responses in all of the D+R- individuals. Unfortunately, a very limited number of samples were available and it was not possible to differentiate the quality of responses between placebo/ vaccine group and most importantly- between patients who experienced viraemia and those who did not. All the patients whose sera were analysed here had viraemia post-transplantation and, therefore, it is not surprising that all these patients had broad antibody responses which must have developed following exposure to other viral antigens. However, it would be very interesting to see whether the sera from patients who did not develop viraemia also contained these anti-CMV antibodies and that analysis should be built into future studies of CMV vaccines. The detection of antibody responses against multiple antigens other than gB (vaccine antigen) would suggest that, although there was transmission of the virus from the donor, the infection was controlled by the immune responses of the host. Alternatively, if further analyses of the non-viraemic D+R- patient cohort revealed that IgGs were only raised against gB it would argue that vaccination was successfully interrupting HCMV at the point of transmission.

Of course, a similarly plausible explanation of only antibodies against gB being present in these non-viraemic D+R- patients could be a lack of virus exposure following transplant (natural history data: Table 1.4: not all but 78% of D+R- experienced viraemia).

The pharmacodynamic analysis of the post-transplant sera revealed some differences between placebos and vaccinated D+R- patients. Although I cannot conclude whether there is a difference in the duration of the initial period of viraemia and other virological parameters such as peak viral load (Figure 7.1. A, B and Figure 7.3) between these groups of patients due to the small sample size, it is clear that vaccinated patients did not experience subsequent episodes of viraemia (Figure 7.1.C). This, together with generally much higher titres of anti-gB antibodies in these vaccinated individuals suggests that the vaccination might prime the immune system of vaccinees prior to challenge with the virus following transplant. It seems possible that, although I could not detect specific antibody responses towards particular antigenic domains in these seronegative patients (see chapter 6) prior to transplantation, patients could possess memory B-cells specific for these antigens. However, due to the lack of exposure to the pathogen, the level of the antibodies could remain low or below the level of detection. However, upon exposure to the virus (i.e. at the time of transplantation) the immune-system could react quicker and produce the protective antibodies more efficiently.

In any vaccine studies it is important to identify whether the vaccine provides sterilising immunity or just protects from viraemia. This becomes particularly important with herpes viruses since they have the capacity to establish lifelong latent infections that likely contribute to transmission in the population and can result in pathogenesis in immune-compromised patients upon reactivation. Thus I reasoned that it would be interesting to ask whether vaccination of R- individuals receiving an organ from a seropositive donor prevented the establishment of latent infection as this would be good evidence that the vaccine had prevented transmission of the virus.

Unfortunately, although we had originally 16 patients in D+R- group (11 vaccine and 5 placebo recipients) who completed the phase-2 study and consented to participate in follow-up study, we received in total only 5 follow up samples from D+R- patients (2 vaccine recipients, one of whom experienced viraemia post transplantation). Interestingly, all of these patients had detectable levels of HCMV DNA present in their monocyte fraction (Figure 7.5 and Table 7.6). The detection of HCMV DNA sequences in the monocytes of the D+R- transplant patients who experienced viraemia is perhaps not surprising. Unfortunately, we obtained only one sample of the most informative case-scenario: vaccinated patient from high-risk D+R-group who did not experience viraemia after viral challenge, which if were proven to be genome-negative could potentially suggest interruption of viral transmission by vaccination. The detection of DNA in the monocytes isolated from that patient would be consistent with latency in that individual. Thus, although no viraemia was observed in this patient there is direct evidence that they have become DNA- positive suggesting the virus was transmitted, albeit at a low level. However this observation remains anecdotal and could reflect the acquisition of HCMV post-transplant. Thus it would be of interest in future studies to carry out a more systematic analysis of non-viraemic D+R- patients post-transplant to ask whether they had established latent infections.

Of course, it also remains possible that the antibodies detected in samples of the transplant patients collected approximately 2-years post transplantation could be elicited by infection with other strain of HCMV (than the donor strain) or even multiple strains of the virus (superinfection), that were acquired in a period between transplantation and follow up studies. Therefore, I cannot exclude the possibility that the humoral responses in those long-term follow up samples were made only in response to the vaccine antigen and possibly- virus from seropositive organ. In order to have more insight into this, I performed the analyses of IgM response profiles in this follow up study. Of course, the presence of IgM antibodies would indicate that the infection was acquired recently. I detected that 2 out of 7 D+R- patients had IgM responses to at least one viral antigen in this follow-up analysis (Table 1.4). This suggests that those patients experienced either re-infection with different strain or re-activation of their latent HCMV in the past few months. This method, although offers some insight into the nature of immune

responses of those patients, it has also certain limitations; *e.g.*: it is impossible to determine whether vaccine successfully prevented subsequent infections with different strains. To ultimately determine whether the antibody repertoire in those follow up samples was elicited by the viral strain acquired during transplantation or by subsequent re-infections with different HCMV strains during the follow up period; it would be necessary to genotype viral strains of the donors prior to transplantation and viral strains of the patients. Such approach would offer a possibility to compare the sequences of the donor strains and the strains of the patients in the follow-up period. If they match, that would clearly indicate that although vaccine did not prevent transmission at the time of transplantation, it conferred some protection against re-infections with different strains of this pathogen.

Despite the severe limitations of sample size that have limited many of the follow up analyses presented here, what they do show is that as a proof of concept we can interrogate sera and cell samples post-transplant with standard laboratory techniques. As such there would be great power in ensuring that in future studies part of the focus was to include a more comprehensive recruitment and analysis post transplantation.

8. Final discussion.

The results in this thesis illustrate the complexity of trying to discover vaccines able to control HCMV infection. Despite the fact that this virus was isolated for the first time over 60 years ago [5-8], there are still no vaccines available to control this important pathogen [344]. Although several vaccine candidates for HCMV have been trialled to date, none of them is approaching licensure [273].

First attempts were made with live attenuated vaccines (based on laboratory passaged CMV strains: Towne and AD169) [16, 24, 269-272]. Apart from the unsatisfactory immunogenicity levels induced by these vaccines, one of the major concerns over the use of live whole-virus vaccines is the possibility of establishment of latency post-vaccination. Therefore, to overcome this obstacle, new vaccination strategies including subunit and vectored vaccines were developed (reviewed elsewhere [273]). Several vaccine formulations are currently under phase-2 clinical development, including modified vaccinia Ankara (MVA) CMV triplex vaccine (NCT02506933) and CMVpp65-A*0201 peptide vaccine (NCT02396134). To date, only one vaccine candidate has reached phase-3 clinical trials- the DNA plasmid ASP0113 vaccine (NCT01877655). There are several reasons why the development of vaccines against HCMV has proved to be difficult. One of the most important features of HCMV is the natural history that is much more complex than viruses that have previously been controlled by immunisation, for example rubella [36, 127]. Due to the similarities and the analogy in the mode of infection, transmission of the virus from newly infected pregnant women to the fetus (especially in the first trimester of pregnancy) - many of the initial conclusions about HCMV were extrapolated from studies on rubella. The general dogma was that HCMV could be controlled by preventing primary infection [451]. However, in the case of infection with HCMV, the situation proved to be much more complex. For example, in addition to causing primary infection, HCMV can reactivate from latency and individuals can be re-infected with new strains [35, 125, 126, 188]. The first observation suggesting that some of the congenitally infected babies were born to seropositive mothers and that the burden of the disease is associated with the quantity of the virus (viral load) came

from natural history studies of new-borns conducted in 1970s in Alabama, USA [36]. This finding was initially controversial and hard to accept by many researchers in these very early days of HCMV research. It seemed odd to many, that maternal immunity is not always able to prevent the virus from damaging the fetus [127], as it was in case of infection with rubella virus. The mounting evidence ultimately showed that pre-existing immunity substantially lowers the risk of congenital infection (by 69% [127]). However, due to the high prevalence of this virus, up to 100% in developing countries- many infections occur due to reactivation or reinfection of the mother [33]. This complex natural history of HCMV makes development of a vaccine more challenging, as it needs to protect not only from primary infection but also reactivation of latent virus and reinfection with a different strain. Interestingly, all of the extensive number of proteins encoded by HCMV is recognised by the immune system [452] but, despite this, reactivation and reinfection still occur, which suggests that mimicking natural immunity through vaccination may not be sufficient to prevent transmission of this virus and ultimately, control disease.

Furthermore, although virus neutralisation is conventionally regarded as a correlate of immune protection mediated by vaccination [333, 334], this again seems to be much more complex in case of the HCMV infection [301]. The reactivation and reinfection with HCMV occur in individuals who already have neutralising antibodies, which shows that the presence of neutralizing antibodies does not always confer protection. This very complex natural history may be the result of HCMV encoding a large number of immune evasion genes which target cell-mediated and humoral immunity [453-455]. Alternatively, it may be explained by the transmission of virus in a cell associated form, which would not normally be expected to be susceptible to classical virus neutralising antibodies. Furthermore, the type of inoculum of virus transmitted from person to person may be different in distinct patient groups such as transplant patients or women of childbearing age [274]. Also, the absence of relevant animal models that could imitate the human immune responses following infection with HCMV is another practical hurdle in vaccine development [344].

Despite all these uncertainties, the results described in this thesis show the advantages of applying pharmacodynamics to discover vaccines with activity against HCMV infection in transplant patients. This approach uses biomarkers of viral load that were validated for each patient group in a previous natural history study [35]. My results are based upon a detailed analysis of one phase-2 randomised controlled trial in candidates awaiting transplantation of a kidney or liver [35]. Strength of this approach is that the date of challenge with virus that is transferred between humans is known, because it is the day of transplant. A weakness of this approach is that the strain of HCMV present in the donor organ cannot be controlled, nor can the effective inoculum transmitted at the time of transplant. Furthermore, the number of patients studied in a proof of concept study is necessarily small, so limiting the definitive conclusions that can be reached. Nevertheless, detailed examination of the samples available to me offers some conclusions with the potential of helping design further studies to address some novel hypotheses.

I sought to identify correlates of humoral immune protection in these patients. To address the precise nature of the protective humoral response a number of assays were performed. Firstly, I focused on the intrinsic abilities of the sera from vaccinated patients to decrease infectivity of cell free virus and to block the spread of the virus from cell to cell. These assays were developed to investigate whether vaccine administration had an impact on cell-free virus neutralization and inhibition of viral dissemination. Although, classically, many vaccine responses have relied on the induction of neutralising antibody responses, my results provide no evidence for vaccination inducing an effective neutralising antibody response against HCMV in seronegative vaccine recipients. Moreover, relatively good neutralizing responses in seropositive vaccine recipients were not correlated with decreased incidence of viraemia.

It should be pointed out that gB is not the only target of neutralizing antibody responses, as there is a much broader spectrum of antigens: *e.g.:* pentameric complex and different glycoproteins that are present on viral envelope. This would be consistent with the literature that suggests gH/gL and the pentameric complex being the targets of potent neutralizing antibodies. These antigens are not presented to the immune system of seronegative vaccine recipients; therefore could not be developed following the vaccination.

Next, I sought to explain why there were such good antibody responses in seropositive vaccine recipients in the *in vitro* assays but this was not correlated with protection *in vivo*. The recently published data in the literature suggest that *in vitro* the virus is predominantly spreading from cell to cell [195]. In this case, these neutralizing antibody responses would have decreased efficacy (as they would target only cell- free virus). As I could observe in further analysis, sera from the seropositive vaccine recipients were indeed far less effective in the inhibition of cell-associated than the inhibition of the cell-free virus. This finding fits with the above hypothesis that *in vitro* the virus is predominantly cell-associated.

However, in the *in vitro* assay that was optimized to detect neutralizing antibodies I could see good responses in seropositive vaccine recipients. Although the role of these neutralizing antibody responses cannot be underestimated as, in principle, potent neutralizing antibodies could be very effective in lowering or even completely disrupting transmission of secretions containing cell-free virus. However, the results described in this thesis suggest that eliciting high titres of neutralizing antibody responses may not be fully sufficient to achieve a satisfactory level of protection. This suggests that a successful vaccine may induce potent neutralizing antibodies to protect against the cell-free mode of viral transmission, but those neutralizing antibody responses might not be sufficient to provide sterilizing protection *in vivo* because of cell-to-cell transmission of HCMV. This is a very important finding, as many researchers are still relying on induction of high titres of neutralizing antibody responses by vaccination as a guide to successful vaccination.

Although I could observe some level of the protection in the sera from seropositive vaccine recipients, this was not correlated with the vaccine/placebo status. Moreover, sera from seronegative vaccine recipients had no impact on viral spread. Taken together, the results described in my thesis strongly indicate that serum alone is unable to control the virus and other mechanisms must be evoked by vaccination than those mediated by intrinsic ability of the sera.

Therefore the next step was to investigate the indirect mechanisms of humoral responses- such as induction of antibody effector functions that induce apoptosis of infected cells. An increasing body of evidence supports ADCC as a mechanism for conferring protection after vaccination in the cases of HIV Thai trial and natural viral infections: *e.g.* Influenza) [397-399, 404-406, 409]. Interestingly, I found no evidence that the protective effect elicited by the CMV gB/MF59 vaccine involves the induction of ADCC stimulating antibody responses in either seronegative or seropositive vaccine recipients. The vaccine did not induce ADCC responses in seronegative vaccine recipients and did not boost the level of already existing ADCC-mediating antibody responses in seropositives. The relatively high level of ADCC response that was present at the time of vaccine administration (following natural infection) in seropositive individuals was not correlated with improved outcome, which suggests that ADCC may not be the predominant mechanism of protection following natural infection with HCMV.

Additionally, I performed an epitope mapping analysis of the sera from SOT patients who participated in the gB/MF59 clinical trial. The binding activity of the sera to 4 (of 5) epitopes that have been identified so far on the gB molecule was tested by ELISA. The analysis of the antibody profiles towards each antigenic domain could potentially reveal that the protection against the pathogen is correlated with the specific antibody responses towards one of these antigenic domains and could provide further clues to the protective nature of the vaccine response since these epitopes have been linked with different aspects of immune control of HCMV infection. Moreover, this analysis allowed antibody responses elicited by the vaccine to be compared with those following natural infection.

Based on all of my results, described in detail in the results section of this thesis, I can propose a series of conclusions that can be formally tested in the future.

First, I propose that at least some of the protection seen in seropositive individuals is due to them having antibodies against AD2 epitope. This immediately draws attention to our lack of understanding of this component of the natural history of HCMV infection; namely, why do only 50% of people with natural immunity against HCMV have antibodies that recognise this epitope [162, 189, 190, 426]? Studies of the immunoglobulin gene rearrangements show that these antibodies are not found in the germline DNA, but have to go through a cascade of rare and very specific immunoglobulin gene re- arrangement events [429, 434]. The fact that only a proportion of infected individuals develop these rare AD-2 antibodies following natural infection could be a consequence of the low probability of developing antibodies that require such specific recombination events [435]. Moreover, these rare immunoglobulins are also characterized by specific substitutions at certain positions that seem to be detrimental to achieve high affinity binding to this epitope, which is an additional factor that contributes to their limited availability [159, 429, 436, 437]. Importantly, my data suggest also that, whilst pre-existing AD-2 responses established at the time of primary infection or in response to the reactivation of the virus from latency can be enhanced, the gB/MF59 vaccine does not induce AD-2 responses in those lacking them at baseline. Interestingly, I observed an increase in the level of AD-2 responses in one recipient of placebo (Figure 6.4.B) and suggest that this might be a response to reactivation of latent virus or even a re-infection event in this patient prior to transplant. It is tempting to speculate that these responses develop with time as individuals reactivate HCMV from latency and present antigen repeatedly.

Although publications in the literature have suggested the desirability of vaccines that induce AD2 antibodies [429], I believe my thesis contains the first results to substantiate these suggestions, because I present strong evidence that these antibodies correlate with protection against viraemia. Thus, the results presented in this thesis suggest that an immunogen that can enhance or generate *de novo* responses against AD2 is a good candidate for a new HCMV vaccine.

These results can be presented as objectives for the future: we need new vaccines that can induce AD2 antibodies in both seronegative and seropositive individuals who currently lack these antibodies. Based on my results, the necessary studies can now be performed in phase-1 immunogenicity studies, which are much quicker and cheaper to perform than phase-2 proof of concept studies. Now that the gB crystal structure is available [358], appropriate immunogens can be easily designed. However, reaching satisfactory immunogenicity levels using short peptide vaccines may not be a simple task. A recently published study on animal models (mice and rabbit) with peptide-conjugate vaccines targeting AD2 and its flanking regions showed that although vaccines could produce strong binding titers of antibodies specific to AD2, they failed to induce significant levels of neutralization [456]. This pioneering work suggests that using simple peptides as an antigens may not be sufficient for this purpose.

On the other hand, it is very important to bear in mind that immunogenicity is not, on its own, sufficient to prioritise an immunogen for vaccine development; responses against AD1 were common, but were not associated with protection against viraemia. Furthermore, having an immunodominant AD1 epitope might facilitate immune evasion by HCMV if the gB molecule also contained an epitope (AD2) that could induce protective immunity. The literature contains suggestions that antibodies against AD1 may interfere with the putative protective antibodies against AD2, due to its spatial location on this gB molecule, but my thesis provides no evidence for this. This observation is very important, because it may be necessary to retain substantial portions of the gB molecule in order to have an immunogen that induces protective antibodies against AD2.

In contrast to the results in HCMV seropositive cohort, my analyses did not identify a correlate of protection for HCMV seronegative vaccine recipients. On the contrary, in my study I could see only very limited humoral responses in those vaccinated seronegative transplant patients. It is a surprising result, considering that the previously reported levels of anti-gB antibodies in those seronegative vaccine recipients (measured by ELISA assays with the immobilized vaccine antigen- recombinant gB on the solid surface) were relatively high, approaching the level of anti-gB antibodies seen in seropositive cohort (Fig. 1.11) [188]. Despite these previous, encouraging results showing high level of anti-gB antibodies elicited by vaccine in seronegative recipients; I could not find strong evidence that these antibodies were functional, as I observed only minimal effects of these anti-gB antibodies in my assays. The reasons for this discrepancy are unclear. However; there are several plausible explanations to this paradoxical observation:

i) One possible explanation is that the antibodies elicited by the recombinant gB (vaccine antigen) may have either decreased, or even completely lost affinity to the native gB. This would be possible in case the recombinant gB used in vaccine contains epitopes that are not presented during natural infection or that the epitopes were presented differently in the context of vaccine and native antigen. This is definitely a possibility, because there were several changes introduced into this recombinant gB in order to increase its stability and solubility [165, 316]. Nevertheless, it is impossible to speculate to what extent such changes could potentially change the antigen/epitope presentation to the immune system of vaccine recipients. Although the crystal structure of this molecule was published recently [358], presumably it represents only the post-fusion form of this protein. Of course, during natural infection the two forms of gB are present (pre- and post- fusion), therefore, the humoral responses are elicited against both forms following the natural infection. The crystal structure of the vaccine antigen is not available so the direct comparison between both specimens is not possible. However, such a putative differences in the antigen presentation could at least partly explain the discrepancy between my- and the previously

published work [188]. Interestingly though, I can clearly see that the vaccine is boosting immune responses of seropositive patients. Thus, it is clear that at least to some extent the vaccine antigen is recognized by the memory B-cells that evolved in response to previously acquired natural infection. Also, the exposure to vaccine antigens could have been insufficient in those seronegative individuals. This would result in a very low probability of developing those very specific rearrangements required to produce rare, protective antibodies in naïve individuals. On the other hand such antigen presentation could still potentially suffice to boost pre-existing immunity in seropositives. This possibility, although speculative, further illustrates the potential advantage of using pharmacodynamics rather than trying to mimic natural immunity when designing vaccines.

ii) Secondly, the influence of potential antigenic polymorphism between the gB used in some of the assays (with Merlin strain) and the vaccine gB (that was derived from Towne strain) could also play a role. The increasing body of data demonstrates that the glycoproteins of HCMV have very high rates of genetic polymorphism; with one report showing that 93.7% of the seropositive individuals in their study group had multiple gB genomic variants [40]. Although the reasons for such high rate of polymorphism in glycoproteins are unknown, it is hypothesised that this could be a potential way of evading immune responses of the host [39, 41]. Of course, such a high rate of antigenic polymorphism between strains could affect the recognition of epitopes through some conformational changes (being a result of the mutations or mismatches between the sequences of the strains) or decreased affinity of the antibodies against different strains of the virus. However, again this seems less likely, as I have used not only gB derived from Merlin (in neutralization and cell-cell spread assays), but also vaccine recombinant gB (ADCC assays). In both cases I see very limited responses in the sera form seronegative patients. Therefore, it seems likely that although antigenic polymorphism between stains and potential

mismatch between the vaccine derived gB and the gB that I have used in some of the assays, could play role in decreasing the affinity of antibodies, it is unlikely that it played a crucial role in the recognition, as can I see good antibody responses in seropositive patients.

iii) Thirdly, it is possible that the gB vaccine primed helper T-cell and memory B-cell responses to have a more rapid response when they encountered HCMV in the donor organ. I could see that although patients in both groups: placebo and vaccine recipients experienced viraemia (Fig. 7.2.A, B); the vaccinees did not develop any subsequent episodes (Fig.7.2.C). This result clearly suggests that these patients were able to mount substantial immunity against the virus much quicker than corresponding placebo group. When I looked at the overall humoral responses towards vaccine antigen in those seronegative transplant patients, I could see that generally the level of anti-gB antibodies raised to much higher levels after the exposure to the virus (at the time of transplant) in vaccine recipients; and this possibly explains why this group of patients was more efficient at preventing subsequent episodes of viraemia (Table 7.2). While the number of patients available precludes definitive conclusions, this possibility should be examined when further phase-2 vaccine studies are performed. Although my results provided some evidence that vaccine recipients were immunologically primed to respond quicker to and more effectively in comparison to the placebo group, it would be important to investigate in more detail what immunological mechanism was responsible for this. One way of investigating this would be to collect samples from the patients not only post-vaccination but also following challenge with the virus (day of transplant). If there are much more rapid responses and higher titres of protective antibody responses in those post-transplant samples, in comparison to responses detected in pre-transplant samples- that would support vaccine priming of the immune system of seronegatives. Of course, future studies should not only focus entirely on those humoral responses but also investigate possible synergistic effect of T-cell helper responses primed by the vaccine.

From my results I can also propose some criteria that could be applied in future proof of concept studies. I argue that the small-sized study described in my thesis highlights the importance of longitudinal studies of immunological responses evoked by vaccination and that coupling it with molecular analysis of latency could prove informative in the future when considering markers that reflect a block to transmission of HCMV. The investigation of the putative changes in rate of viral transmission following vaccination is indisputably highly relevant. This seems to be particularly important for this patient group as it would decrease potential risk not only for primary infection but also reactivation and reinfection; all of which occur more frequently in transplant recipients than in the healthy population and are associated with increased morbidity. It will be important to differentiate between a vaccine that can completely prevent transmission of HCMV from donor to recipient and a vaccine that just reduces the effective inoculum. Both may be clinically useful, but the former has the advantage that the individual does not have latent HCMV that can reactivate at a later date. Although the number of samples available to me was very small, the results (Figure 7.5) show that detecting transcripts from latent genes offers a potential approach to this problem. Of course, acquisition of the virus at a later date post-transplant could not be ruled out in this type of analysis- (the positivity for latent HCMV genome) but if the rate of acquisition was higher than the natural rate of infection in the population (about 1% per annum)- that would provide supporting evidence for transmission at the time of transplant. Consideration of this point is important when powering future studies that seek to address transmission as such analyses would require much larger cohorts of patients in order to reach statistical significance.

To support future studies attempting to interrupt transmission, genotyping of the viral strains from the donor could be performed prior to transplantation and then compared with the genotype of HCMV strain from the respective recipients in the follow-up samples. If the strains match, that would provide strong evidence for the transmission of virus from the donor organ. In addition, antibody responses against proteins not incorporated in the vaccine could be used to identify individuals who have become re-infected with wild-type HCMV despite having received a vaccine. This may have little practical significance for the transplant patient population (except perhaps for those who need a second transplant), but

would be very important where a vaccine is intended to give long term protection spanning the childbearing years.

I also suggest that any future study should incorporate analysis of memory B-cell repertoire by using single cell sequencing with optimized next generation sequencing (NGS) technologies. This approach can provide a high resolution of cellular differences (prior to and following vaccination) and enable analysis of vaccine-induced antibody responses (paired heavy and light chain sequencing). This could be particularly important for development of diagnostic and therapeutic antibodies as well as new generation of vaccines.

Also, I did consider the possibility that the titre of antibody made against gB may be a correlate of immunity, but not a mechanistic correlate of immunity [457]. For example, the titre of antibody might be an indirect marker of good CD4 T-cell response to a vaccine with T-helper activity explaining the high titres of antibody produced. Recent results from a randomised controlled trial proposed by my department are therefore of interest [186]. Seronegative patients destined to receive a kidney from seropositive donors were randomised to receive infusions of monoclonal antibodies specific for HCMV or a matching placebo. The results showed significantly less CMV viraemia in the recipients of monoclonal antibodies. This study proves that humoral immunity can reduce transmission of virus from donor to recipient. The study was performed with monoclonal antibodies reactive against glycoprotein H and UL130 in combination rather than gB, because clinical grade gB monoclonals were not available [186]. This suggests two possibilities; even better results might be obtained if gB monoclonals were added to the existing combination and monoclonal antibodies reactive with any CMV protein found on the surface of infected cells [458] could be investigated. These suggestions could be tested directly.

Despite all the complexities of the natural history of HCMV, its immune evasion genes and the detailed results presented in this thesis when trying to identify correlates of protective immunity, it is salutary to note that all the manufacturers

attempting to produce vaccines against HCMV are still using induction of neutralising antibodies as a marker of success in phase-1 studies. My results suggest that a more sophisticated, formal analysis of correlates of immunity should be incorporated into all future phase-2 randomised controlled trials.

9. References.

1. Jesionek A., K.B., *Uber einen befund von protozoenartigen gebilden in den organen eines heriditarluetischen fotus. .* Munch. Med. Wochenschr. , 1904(51): p. 1905–1907.
2. D., R., *Uber protozoenartige zellen in der niere eines syphilitischen neugoborenen und in der parotis von kindern. .* Zentralbl. Allg. Pathol. , 1904. 15: p. 945–948.
3. Goodpasture E. W., T.F.B., *Concerning the nature of "proteozoan-like" cells in certain lesions of infancy.* Am. J. Dis. Child., 1921. 21: p. 415–421.
4. E.E., T., *Varicella-zoster.* Philipp. J.Sci. , 1906. 1: p. 349-353.
5. M.G., S., *Propagation of salivary gland virus of the mouse in the tissue culture .* Proc.Soc. Exp. Biol. Med 1954. 92: p. 424-430.
6. Smith, M.G., *Propagation in tissue cultures of a cytopathogenic virus from human salivary gland virus (SGV) disease.* Proc Soc Exp Biol Med, 1956. 92(2): p. 424-30.
7. Rowe, W.P., et al., *Cytopathogenic agent resembling human salivary gland virus recovered from tissue cultures of human adenoids.* Proc Soc Exp Biol Med, 1956. 92(2): p. 418-24.
8. Craig, J.M., et al., *Isolation of intranuclear inclusion producing agents from infants with illnesses resembling cytomegalic inclusion disease.* Proc Soc Exp Biol Med, 1957. 94(1): p. 4-12.
9. McGeoch, D.J., F.J. Rixon, and A.J. Davison, *Topics in herpesvirus genomics and evolution.* Virus Res, 2006. 117(1): p. 90-104.
10. Kolb, A.W., C. Ané, and C.R. Brandt, *Using HSV-1 Genome Phylogenetics to Track Past Human Migrations.* PLoS ONE, 2013. 8(10): p. e76267.
11. Chen, D.H., et al., *Three-dimensional visualization of tegument/capsid interactions in the intact human cytomegalovirus.* Virology, 1999. 260(1): p. 10-6.
12. Sarov, I. and I. Abady, *The morphogenesis of human cytomegalovirus.* Virology, 1975. 66(2): p. 464-473.
13. Varnum, S.M., et al., *Identification of proteins in human cytomegalovirus (HCMV) particles: the HCMV proteome.* J Virol, 2004. 78(20): p. 10960-6.

14. Chee, M.S., et al., *Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169.* Curr Top Microbiol Immunol, 1990. 154: p. 125-69.
15. Cha, T.A., et al., *Human cytomegalovirus clinical isolates carry at least 19 genes not found in laboratory strains.* J Virol, 1996. 70(1): p. 78-83.
16. Elek, S.D. and H. Stern, *Development of a vaccine against mental retardation caused by cytomegalovirus infection in utero.* Lancet, 1974. 1(7845): p. 1-5.
17. Prichard, M.N., et al., *A review of genetic differences between limited and extensively passaged human cytomegalovirus strains.* Rev Med Virol, 2001. 11(3): p. 191-200.
18. Murphy, E., et al., *Reevaluation of human cytomegalovirus coding potential.* Proc Natl Acad Sci U S A, 2003. 100(23): p. 13585-90.
19. Dolan, A., et al., *Genetic content of wild-type human cytomegalovirus.* J Gen Virol, 2004. 85(Pt 5): p. 1301-12.
20. Murphy, E., et al., *Coding potential of laboratory and clinical strains of human cytomegalovirus.* Proc Natl Acad Sci U S A, 2003. 100(25): p. 14976-81.
21. Yu, D., M.C. Silva, and T. Shenk, *Functional map of human cytomegalovirus AD169 defined by global mutational analysis.* Proc Natl Acad Sci U S A, 2003. 100(21): p. 12396-401.
22. Quinnan, G.V., Jr., et al., *Comparative virulence and immunogenicity of the Towne strain and a nonattenuated strain of cytomegalovirus.* Ann Intern Med, 1984. 101(4): p. 478-83.
23. Hahn, G., et al., *The human cytomegalovirus ribonucleotide reductase homolog UL45 is dispensable for growth in endothelial cells, as determined by a BAC-cloned clinical isolate of human cytomegalovirus with preserved wild-type characteristics.* J Virol, 2002. 76(18): p. 9551-5.
24. Plotkin, S.A., et al., *Candidate cytomegalovirus strain for human vaccination.* Infect Immun, 1975. 12(3): p. 521-7.
25. Rice, G.P., R.D. Schrier, and M.B. Oldstone, *Cytomegalovirus infects human lymphocytes and monocytes: virus expression is restricted to immediate-early gene products.* Proc Natl Acad Sci U S A, 1984. 81(19): p. 6134-8.

26. Smith, I.L., et al., *Clinical failure of CMV retinitis with intravitreal cidofovir is associated with antiviral resistance.* Arch Ophthalmol, 1998. 116(2): p. 178-85.
27. Davison, A.J., et al., *The human cytomegalovirus genome revisited: comparison with the chimpanzee cytomegalovirus genome.* J Gen Virol, 2003. 84(Pt 1): p. 17-28.
28. Kilpatrick, B.A. and E.S. Huang, *Human cytomegalovirus genome: partial denaturation map and organization of genome sequences.* J Virol, 1977. 24(1): p. 261-76.
29. Kilpatrick, B.A. and E.S. Huang, *Structural organization of human cytomegalovirus DNA.* IARC Sci Publ, 1978(24 Pt 1): p. 105-12.
30. Wilkie, N.M. and R. Cortini, *Sequence arrangement in herpes simplex virus type 1 DNA: identification of terminal fragments in restriction endonuclease digests and evidence for inversions in redundant and unique sequences.* J Virol, 1976. 20(1): p. 211-21.
31. Kemble, G.W. and E.S. Mocarski, *A host cell protein binds to a highly conserved sequence element (pac-2) within the cytomegalovirus a sequence.* J Virol, 1989. 63(11): p. 4715-28.
32. Tamashiro, J.C. and D.H. Spector, *Terminal structure and heterogeneity in human cytomegalovirus strain AD169.* J Virol, 1986. 59(3): p. 591-604.
33. Lanzieri, T.M., et al., *Systematic review of the birth prevalence of congenital cytomegalovirus infection in developing countries.* Int J Infect Dis, 2014. 22: p. 44-8.
34. Staras, S.A., et al., *Seroprevalence of cytomegalovirus infection in the United States, 1988-1994.* Clin Infect Dis, 2006. 43(9): p. 1143-51.
35. Atabani, S.F., et al., *Cytomegalovirus replication kinetics in solid organ transplant recipients managed by preemptive therapy.* Am J Transplant, 2012. 12(9): p. 2457-64.
36. Stagno, S., et al., *Congenital Cytomegalovirus Infection.* New England Journal of Medicine, 1977. 296(22): p. 1254-1258.
37. Sijmons, S., et al., *High-throughput analysis of human cytomegalovirus genome diversity highlights the widespread occurrence of gene-disrupting mutations and pervasive recombination.* J Virol, 2015.

38. Lassalle, F., et al., *Islands of linkage in an ocean of pervasive recombination reveals two-speed evolution of human cytomegalovirus genomes.* Virus Evolution, 2016. 2(1): p. vew017-vew017.
39. Renzette, N., et al., *Limits and patterns of cytomegalovirus genomic diversity in humans.* Proceedings of the National Academy of Sciences, 2015. 112(30): p. E4120-E4128.
40. Novak, Z., et al., *Cytomegalovirus strain diversity in seropositive women.* J Clin Microbiol, 2008. 46(3): p. 882-6.
41. Zhou, M., et al., *Comparative analysis of gO isoforms reveals that strains of human cytomegalovirus differ in the ratio of gH/gL/gO and gH/gL/UL128-131 in the virion envelope.* J Virol, 2013. 87(17): p. 9680-90.
42. Pignatelli, S., et al., *Genetic polymorphisms among human cytomegalovirus (HCMV) wild-type strains.* Rev Med Virol, 2004. 14(6): p. 383-410.
43. Chou, S.W. and K.M. Dennison, *Analysis of interstrain variation in cytomegalovirus glycoprotein B sequences encoding neutralization-related epitopes.* J Infect Dis, 1991. 163(6): p. 1229-34.
44. Chou, S., *Comparative analysis of sequence variation in gp116 and gp55 components of glycoprotein B of human cytomegalovirus.* Virology, 1992. 188(1): p. 388-90.
45. Coaquette, A., et al., *Mixed cytomegalovirus glycoprotein B genotypes in immunocompromised patients.* Clin Infect Dis, 2004. 39(2): p. 155-61.
46. Manuel, O., et al., *Impact of genetic polymorphisms in cytomegalovirus glycoprotein B on outcomes in solid-organ transplant recipients with cytomegalovirus disease.* Clin Infect Dis, 2009. 49(8): p. 1160-6.
47. Rycel, M., et al., *Mixed infections with distinct cytomegalovirus glycoprotein B genotypes in Polish pregnant women, fetuses, and newborns.* Eur J Clin Microbiol Infect Dis, 2015. 34(3): p. 585-91.
48. Steininger, C., et al., *Cytomegalovirus genotypes present in cerebrospinal fluid of HIV-infected patients.* Aids, 2005. 19(3): p. 273-8.
49. Barbi, M., et al., *CMV gB genotypes and outcome of vertical transmission: study on dried blood spots of congenitally infected babies.* J Clin Virol, 2001. 21(1): p. 75-9.
50. Bale, J.F., Jr., et al., *Intrauterine cytomegalovirus infection and glycoprotein B genotypes.* J Infect Dis, 2000. 182(3): p. 933-6.

51. Mewara, A., et al., *Cytomegalovirus glycoprotein B gene polymorphism and its association with clinical presentations in infants.* Southeast Asian J Trop Med Public Health, 2009. 40(4): p. 759-64.
52. Picone, O., et al., *Cytomegalovirus (CMV) glycoprotein B genotype and CMV DNA load in the amniotic fluid of infected fetuses.* Prenat Diagn, 2004. 24(12): p. 1001-6.
53. Roubalova, K., et al., *[Genotyping of viral glycoprotein B (gB) in hematopoietic stem cell transplant recipients with active cytomegalovirus infection: analysis of the impact of gB genotypes on the patients' outcome].* Epidemiol Mikrobiol Imunol, 2010. 59(2): p. 92-9.
54. Zawilinska, B., et al., *[UL55 genotype diversity of cytomegalovirus strains isolated from newborns and infants hospitalized in southern Poland].* Przegl Epidemiol, 2011. 65(3): p. 409-13.
55. Isaacson, M.K.L.K.J. and T. Compton, *Virus Entry and Innate Immune Activation*, in *Human Cytomegalovirus*, T.E.S.M.F. Stinski, Editor. 2008, Springer. p. 85-100.
56. Compton, T., D.M. Nowlin, and N.R. Cooper, *Initiation of human cytomegalovirus infection requires initial interaction with cell surface heparan sulfate.* Virology, 1993. 193(2): p. 834-41.
57. Isaacson, M.K. and T. Compton, *Human Cytomegalovirus Glycoprotein B Is Required for Virus Entry and Cell-to-Cell Spread but Not for Virion Attachment, Assembly, or Egress.* Journal of Virology, 2009. 83(8): p. 3891-3903.
58. Feire, A.L., H. Koss, and T. Compton, *Cellular integrins function as entry receptors for human cytomegalovirus via a highly conserved disintegrin-like domain.* Proc Natl Acad Sci U S A, 2004. 101(43): p. 15470-5.
59. Wang, X., et al., *Integrin alphavbeta3 is a coreceptor for human cytomegalovirus.* Nat Med, 2005. 11(5): p. 515-21.
60. Wang, X., et al., *Epidermal growth factor receptor is a cellular receptor for human cytomegalovirus.* Nature, 2003. 424(6947): p. 456-61.
61. Isaacson, M.K., A.L. Feire, and T. Compton, *Epidermal growth factor receptor is not required for human cytomegalovirus entry or signaling.* J Virol, 2007. 81(12): p. 6241-7.

62. Fairley, J.A., et al., *Human cytomegalovirus infection inhibits epidermal growth factor (EGF) signalling by targeting EGF receptors.* J Gen Virol, 2002. 83(Pt 11): p. 2803-10.
63. Cobbs, C.S., et al., *Human cytomegalovirus induces cellular tyrosine kinase signaling and promotes glioma cell invasiveness.* J Neurooncol, 2007. 85(3): p. 271-80.
64. Soroceanu, L., A. Akhavan, and C.S. Cobbs, *Platelet-derived growth factor-alpha receptor activation is required for human cytomegalovirus infection.* Nature, 2008. 455(7211): p. 391-5.
65. Pietropaolo, R.L. and T. Compton, *Direct interaction between human cytomegalovirus glycoprotein B and cellular annexin II.* J Virol, 1997. 71(12): p. 9803-7.
66. Raynor, C.M., et al., *Annexin II enhances cytomegalovirus binding and fusion to phospholipid membranes.* Biochemistry, 1999. 38(16): p. 5089-95.
67. Pietropaolo, R. and T. Compton, *Interference with annexin II has no effect on entry of human cytomegalovirus into fibroblast cells.* J Gen Virol, 1999. 80 (Pt 7): p. 1807-16.
68. RJ., W., *Herpesviruses.* 4th edition ed. Medical Microbiology, ed. Baron S. 1996, Galveston (TX): University of Texas Medical Branch at Galveston;.
69. Roizman, B., et al., *Herpesviridae. Definition, provisional nomenclature, and taxonomy. The Herpesvirus Study Group, the International Committee on Taxonomy of Viruses.* Intervirology, 1981. 16(4): p. 201-17.
70. Wathen, M.W. and M.F. Stinski, *Temporal Patterns of Human Cytomegalovirus Transcription: Mapping the Viral RNAs Synthesized at Immediate Early, Early, and Late Times After Infection.* Journal of Virology, 1982. 41(2): p. 462-477.
71. Winkler, M. and T. Stamminger, *A specific subform of the human cytomegalovirus transactivator protein pUL69 is contained within the tegument of virus particles.* J Virol, 1996. 70(12): p. 8984-7.
72. Winkler, M., S.A. Rice, and T. Stamminger, *UL69 of human cytomegalovirus, an open reading frame with homology to ICP27 of herpes simplex virus, encodes a transactivator of gene expression.* J Virol, 1994. 68(6): p. 3943-54.

73. Bechtel, J.T. and T. Shenk, *Human cytomegalovirus UL47 tegument protein functions after entry and before immediate-early gene expression.* J Virol, 2002. 76(3): p. 1043-50.
74. Schierling, K., et al., *Human Cytomegalovirus Tegument Proteins ppUL82 (pp71) and ppUL35 Interact and Cooperatively Activate the Major Immediate-Early Enhancer.* Journal of Virology, 2004. 78(17): p. 9512-9523.
75. Tomtishen Iii, J.P., *Human cytomegalovirus tegument proteins (pp65, pp71, pp150, pp28).* Virology Journal, 2012. 9: p. 22-22.
76. Penkert, R.R. and R.F. Kalejta, *Tale of a tegument transactivator: the past, present and future of human CMV pp71.* Future Virol, 2012. 7(9): p. 855-869.
77. Kalejta, R.F., *Tegument proteins of human cytomegalovirus.* Microbiol Mol Biol Rev, 2008. 72(2): p. 249-65, table of contents.
78. Cristea, I.M., et al., *Human cytomegalovirus pUL83 stimulates activity of the viral immediate-early promoter through its interaction with the cellular IFI16 protein.* J Virol, 2010. 84(15): p. 7803-14.
79. Tenney, D.J. and A.M. Colberg-Poley, *Human cytomegalovirus UL36-38 and US3 immediate-early genes: temporally regulated expression of nuclear, cytoplasmic, and polysome-associated transcripts during infection.* J Virol, 1991. 65(12): p. 6724-34.
80. Colberg-Poley, A.M., *Functional roles of immediate early proteins encoded by the human cytomegalovirus UL36-38, UL115-119, TRS1/IRS1 and US3 loci.* Intervirology, 1996. 39(5-6): p. 350-60.
81. Isomura, H. and M.F. Stinski, *The Human Cytomegalovirus Major Immediate-Early Enhancer Determines the Efficiency of Immediate-Early Gene Transcription and Viral Replication in Permissive Cells at Low Multiplicity of Infection.* Journal of Virology, 2003. 77(6): p. 3602-3614.
82. Greaves, R.F. and E.S. Mocarski, *Defective growth correlates with reduced accumulation of a viral DNA replication protein after low-multiplicity infection by a human cytomegalovirus ie1 mutant.* J Virol, 1998. 72(1): p. 366-79.
83. Jault, F.M., et al., *Cytomegalovirus infection induces high levels of cyclins, phosphorylated Rb, and p53, leading to cell cycle arrest.* J Virol, 1995. 69(11): p. 6697-704.

84. Taylor, R.T. and W.A. Bresnahan, *Human cytomegalovirus IE86 attenuates virus- and tumor necrosis factor alpha-induced NFkappaB-dependent gene expression.* J Virol, 2006. 80(21): p. 10763-71.
85. Taylor, R.T. and W.A. Bresnahan, *Human cytomegalovirus immediate-early 2 protein IE86 blocks virus-induced chemokine expression.* J Virol, 2006. 80(2): p. 920-8.
86. Lang, D., et al., *Functional interaction between the human cytomegalovirus 86-kilodalton IE2 protein and the cellular transcription factor CREB.* J Virol, 1995. 69(10): p. 6030-7.
87. Mocarski, E.S., et al., *A deletion mutant in the human cytomegalovirus gene encoding IE1(491aa) is replication defective due to a failure in autoregulation.* Proc Natl Acad Sci U S A, 1996. 93(21): p. 11321-6.
88. M.F. Stinski, D.T.P., *Functional Roles of the Human Cytomegalovirus Essential IE86 Protein*. Current topics in Microbiology and Immunology ed. T.E.S. M.F.Stinski. 2008: Springer
89. Braggin, J.E., S.J. Child, and A.P. Geballe, *Essential role of protein kinase R antagonism by TRS1 in human cytomegalovirus replication.* Virology, 2016. 489: p. 75-85.
90. Marshall, E.E., et al., *Essential role for either TRS1 or IRS1 in human cytomegalovirus replication.* J Virol, 2009. 83(9): p. 4112-20.
91. McCormick, A.L., et al., *Differential function and expression of the viral inhibitor of caspase 8-induced apoptosis (vICA) and the viral mitochondria-localized inhibitor of apoptosis (vMIA) cell death suppressors conserved in primate and rodent cytomegaloviruses.* Virology, 2003. 316(2): p. 221-33.
92. Chevillotte, M., et al., *Fluorescence-Based Assay for Phenotypic Characterization of Human Cytomegalovirus Polymerase Mutations Regarding Drug Susceptibility and Viral Replicative Fitness.* Antimicrobial Agents and Chemotherapy, 2009. 53(9): p. 3752-3761.
93. Emery, V.C., *Viral dynamics during active cytomegalovirus infection and pathology.* Intervirology, 1999. 42(5-6): p. 405-11.
94. Anders, D.G., et al., *Boundaries and structure of human cytomegalovirus oriLyt, a complex origin for lytic-phase DNA replication.* J Virol, 1992. 66(6): p. 3373-84.

95. Borst, E.M. and M. Messerle, *Analysis of human cytomegalovirus oriLyt sequence requirements in the context of the viral genome.* J Virol, 2005. 79(6): p. 3615-26.
96. Pari, G.S., *Nuts and Bolts of Human Cytomegalovirus Lytic DNA Replication*, in *Human Cytomegalovirus*, T.E.S.a.M.F. Stinski, Editor. 2008.
97. Snaar, S.P., M. Vincent, and R.W. Dirks, *RNA polymerase II localizes at sites of human cytomegalovirus immediate-early RNA synthesis and processing.* J Histochem Cytochem, 1999. 47(2): p. 245-54.
98. Courcelle, C.T., et al., *Requirement for uracil-DNA glycosylase during the transition to late-phase cytomegalovirus DNA replication.* J Virol, 2001. 75(16): p. 7592-601.
99. Lehman, I.R. and P.E. Boehmer, *Replication of herpes simplex virus DNA.* J Biol Chem, 1999. 274(40): p. 28059-62.
100. Ben-Porat, T. and S.A. Tokazewski, *Replication of herpesvirus DNA II. Sedimentation characteristics of newly synthesized DNA.* Virology, 1977. 79(2): p. 292-301.
101. Igarashi, K., et al., *Construction and properties of a recombinant herpes simplex virus 1 lacking both S-component origins of DNA synthesis.* J Virol, 1993. 67(4): p. 2123-32.
102. Jacob, R.J., L.S. Morse, and B. Roizman, *Anatomy of herpes simplex virus DNA. XII. Accumulation of head-to-tail concatemers in nuclei of infected cells and their role in the generation of the four isomeric arrangements of viral DNA.* J Virol, 1979. 29(2): p. 448-57.
103. St Jeor, S.C. and R. Hutt, *Cell DNA replication as a function in the synthesis of human cytomegalovirus.* J Gen Virol, 1977. 37(1): p. 65-73.
104. Stinski, M.F., *Sequence of protein synthesis in cells infected by human cytomegalovirus: early and late virus-induced polypeptides.* J Virol, 1978. 26(3): p. 686-701.
105. Britt, W., *Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis.*, ed. C.-F.G. Arvin A, Mocarski E, et al. 2007, Cambridge: Cambridge University Press.
106. Newcomb, W.W., et al., *Assembly of the herpes simplex virus procapsid from purified components and identification of small complexes containing the major capsid and scaffolding proteins.* J Virol, 1999. 73(5): p. 4239-50.

107. Zhou, Z.H., et al., *Visualization of tegument-capsid interactions and DNA in intact herpes simplex virus type 1 virions.* J Virol, 1999. 73(4): p. 3210-8.
108. Zhou, Z.H., et al., *Seeing the herpesvirus capsid at 8.5 A.* Science, 2000. 288(5467): p. 877-80.
109. Butcher, S.J., et al., *Structure of the human cytomegalovirus B capsid by electron cryomicroscopy and image reconstruction.* J Struct Biol, 1998. 124(1): p. 70-6.
110. Mocarski E. S., C., *Cytomegaloviruses and their replication.* 4th ed. In Fields Virology, ed. W.a. Wilkins;. Vol. 2. 2001, Philadelphia: Lippincott. 2629–2673.
111. Welch, A.R., L.M. McNally, and W. Gibson, *Cytomegalovirus assembly protein nested gene family: four 3'-coterminal transcripts encode four in-frame, overlapping proteins.* J Virol, 1991. 65(8): p. 4091-100.
112. Welch, A.R., et al., *A herpesvirus maturational proteinase, assemblin: identification of its gene, putative active site domain, and cleavage site.* Proc Natl Acad Sci U S A, 1991. 88(23): p. 10792-6.
113. Gibson, W., *Structure and assembly of the virion.* Intervirology, 1996. 39(5-6): p. 389-400.
114. Lee, J.Y., A. Irmiere, and W. Gibson, *Primate cytomegalovirus assembly: evidence that DNA packaging occurs subsequent to B capsid assembly.* Virology, 1988. 167(1): p. 87-96.
115. Bogner, E., K. Radsak, and M.F. Stinski, *The gene product of human cytomegalovirus open reading frame UL56 binds the pac motif and has specific nuclease activity.* J Virol, 1998. 72(3): p. 2259-64.
116. Scholz, B., et al., *Identification of the ATP-binding site in the terminase subunit pUL56 of human cytomegalovirus.* Nucleic Acids Res, 2003. 31(5): p. 1426-33.
117. Komazin, G., L.B. Townsend, and J.C. Drach, *Role of a mutation in human cytomegalovirus gene UL104 in resistance to benzimidazole ribonucleosides.* J Virol, 2004. 78(2): p. 710-5.
118. Mettenleiter, T.C., *Herpesvirus assembly and egress.* J Virol, 2002. 76(4): p. 1537-47.
119. Muranyi, W., et al., *Cytomegalovirus recruitment of cellular kinases to dissolve the nuclear lamina.* Science, 2002. 297(5582): p. 854-7.

120. Roffman, E., et al., *Putative site for the acquisition of human herpesvirus 6 virion tegument.* Journal of Virology, 1990. 64(12): p. 6308-6313.
121. Sanchez, V., et al., *Accumulation of virion tegument and envelope proteins in a stable cytoplasmic compartment during human cytomegalovirus replication: characterization of a potential site of virus assembly.* J Virol, 2000. 74(2): p. 975-86.
122. Dal Monte, P., et al., *Analysis of intracellular and intraviral localization of the human cytomegalovirus UL53 protein.* J Gen Virol, 2002. 83(Pt 5): p. 1005-12.
123. Sanchez, V., E. Sztul, and W.J. Britt, *Human cytomegalovirus pp28 (UL99) localizes to a cytoplasmic compartment which overlaps the endoplasmic reticulum-golgi-intermediate compartment.* J Virol, 2000. 74(8): p. 3842-51.
124. Silva, M.C., et al., *Human cytomegalovirus UL99-encoded pp28 is required for the cytoplasmic envelopment of tegument-associated capsids.* J Virol, 2003. 77(19): p. 10594-605.
125. Dupont, L. and M.B. Reeves, *Cytomegalovirus latency and reactivation: recent insights into an age old problem.* Rev Med Virol, 2016. 26(2): p. 75-89.
126. Boeckh, M. and W.G. Nichols, *The impact of cytomegalovirus serostatus of donor and recipient before hematopoietic stem cell transplantation in the era of antiviral prophylaxis and preemptive therapy.* Blood, 2004. 103(6): p. 2003-8.
127. Fowler, K.B., et al., *The outcome of congenital cytomegalovirus infection in relation to maternal antibody status.* N Engl J Med, 1992. 326(10): p. 663-7.
128. Slobedman, B. and E.S. Mocarski, *Quantitative analysis of latent human cytomegalovirus.* J Virol, 1999. 73(6): p. 4806-12.
129. Poole, E. and J. Sinclair, *Sleepless latency of human cytomegalovirus.* Med Microbiol Immunol, 2015. 204(3): p. 421-9.
130. Liu, B. and M.F. Stinski, *Human cytomegalovirus contains a tegument protein that enhances transcription from promoters with upstream ATF and AP-1 cis-acting elements.* J Virol, 1992. 66(7): p. 4434-44.
131. Wright, E., et al., *Ets-2 repressor factor recruits histone deacetylase to silence human cytomegalovirus immediate-early gene expression in non-permissive cells.* J Gen Virol, 2005. 86(Pt 3): p. 535-44.

132. Zweidler-Mckay, P.A., et al., *Gfi-1 encodes a nuclear zinc finger protein that binds DNA and functions as a transcriptional repressor.* Mol Cell Biol, 1996. 16(8): p. 4024-34.

133. Reeves, M.B., et al., *An in vitro model for the regulation of human cytomegalovirus latency and reactivation in dendritic cells by chromatin remodelling.* J Gen Virol, 2005. 86(Pt 11): p. 2949-54.

134. Reeves M, S.J., *Aspects of human cytomegalovirus latency and reactivation*. Current Topics in Microbiology and Immunology, ed. T.S.M. Stinski. 2008.

135. Liu, X.F., et al., *Epigenetic control of cytomegalovirus latency and reactivation.* Viruses, 2013. 5(5): p. 1325-45.

136. Jenkins, C., A. Abendroth, and B. Slobedman, *A novel viral transcript with homology to human interleukin-10 is expressed during latent human cytomegalovirus infection.* J Virol, 2004. 78(3): p. 1440-7.

137. Reeves, M.B. and J.H. Sinclair, *Analysis of latent viral gene expression in natural and experimental latency models of human cytomegalovirus and its correlation with histone modifications at a latent promoter.* J Gen Virol, 2010. 91(Pt 3): p. 599-604.

138. Bego, M., et al., *Characterization of an antisense transcript spanning the UL81-82 locus of human cytomegalovirus.* J Virol, 2005. 79(17): p. 11022-34.

139. Rossetto, C.C., M. Tarrant-Elorza, and G.S. Pari, *Cis and trans acting factors involved in human cytomegalovirus experimental and natural latent infection of CD14 (+) monocytes and CD34 (+) cells.* PLoS Pathog, 2013. 9(5): p. e1003366.

140. Reeves, M.B., et al., *Complex I binding by a virally encoded RNA regulates mitochondria-induced cell death.* Science, 2007. 316(5829): p. 1345-8.

141. Reeves, M.B., et al., *Latency, chromatin remodeling, and reactivation of human cytomegalovirus in the dendritic cells of healthy carriers.* Proc Natl Acad Sci U S A, 2005. 102(11): p. 4140-5.

142. Saffert, R.T., R.R. Penkert, and R.F. Kalejta, *Cellular and viral control over the initial events of human cytomegalovirus experimental latency in CD34+ cells.* J Virol, 2010. 84(11): p. 5594-604.

143. Grey, F., *Role of microRNAs in herpesvirus latency and persistence.* J Gen Virol, 2015. 96(Pt 4): p. 739-51.
144. Grey, F., et al., *A human cytomegalovirus-encoded microRNA regulates expression of multiple viral genes involved in replication.* PLoS Pathog, 2007. 3(11): p. e163.
145. Tarrant-Elorza, M., C.C. Rossetto, and G.S. Pari, *Maintenance and replication of the human cytomegalovirus genome during latency.* Cell Host Microbe, 2014. 16(1): p. 43-54.
146. Knipe, D.M., et al., *Snapshots: Chromatin Control of Viral Infection.* Virology, 2013. 435(1): p. 141-156.
147. Reeves, M.B. and T. Compton, *Inhibition of inflammatory interleukin-6 activity via extracellular signal-regulated kinase-mitogen-activated protein kinase signaling antagonizes human cytomegalovirus reactivation from dendritic cells.* J Virol, 2011. 85(23): p. 12750-8.
148. Kew, V.G., et al., *Mitogen and stress activated kinases act co-operatively with CREB during the induction of human cytomegalovirus immediate-early gene expression from latency.* PLoS Pathog, 2014. 10(6): p. e1004195.
149. Cliffe, Anna R., et al., *Neuronal Stress Pathway Mediating a Histone Methyl/Phospho Switch Is Required for Herpes Simplex Virus Reactivation.* Cell Host & Microbe. 18(6): p. 649-658.
150. Buehler, J., et al., *Opposing Regulation of the EGF Receptor: A Molecular Switch Controlling Cytomegalovirus Latency and Replication.* PLoS Pathog, 2016. 12(5): p. e1005655.
151. Bodaghi, B., et al., *Chemokine Sequestration by Viral Chemoreceptors as a Novel Viral Escape Strategy: Withdrawal of Chemokines from the Environment of Cytomegalovirus-infected Cells.* The Journal of Experimental Medicine, 1998. 188(5): p. 855-866.
152. Tan, J.C., et al., *Inhibition of 2',5'-oligoadenylate synthetase expression and function by the human cytomegalovirus ORF94 gene product.* J Virol, 2011. 85(11): p. 5696-700.
153. Poole, E., et al., *The UL144 gene product of human cytomegalovirus activates NFkappaB via a TRAF6-dependent mechanism.* Embo j, 2006. 25(18): p. 4390-9.

154. Montag, C., et al., *The latency-associated UL138 gene product of human cytomegalovirus sensitizes cells to tumor necrosis factor alpha (TNF-alpha) signaling by upregulating TNF-alpha receptor 1 cell surface expression.* J Virol, 2011. 85(21): p. 11409-21.
155. Cranage, M.P., et al., *Identification of the human cytomegalovirus glycoprotein B gene and induction of neutralizing antibodies via its expression in recombinant vaccinia virus.* Embo j, 1986. 5(11): p. 3057-63.
156. Pellett, P.E., et al., *Epstein-Barr virus genome may encode a protein showing significant amino acid and predicted secondary structure homology with glycoprotein B of herpes simplex virus 1.* Journal of Virology, 1985. 56(3): p. 807-813.
157. Britt, W.J., L. Vugler, and E.B. Stephens, *Induction of complement-dependent and -independent neutralizing antibodies by recombinant-derived human cytomegalovirus gp55-116 (gB).* J Virol, 1988. 62(9): p. 3309-18.
158. Ohlin, M., et al., *Fine specificity of the human immune response to the major neutralization epitopes expressed on cytomegalovirus gp58/116 (gB), as determined with human monoclonal antibodies.* J Virol, 1993. 67(2): p. 703-10.
159. Axelsson, F., et al., *Humoral immunity targeting site I of antigenic domain 2 of glycoprotein B upon immunization with different cytomegalovirus candidate vaccines.* Vaccine, 2007. 26(1): p. 41-6.
160. Pötzsch, S., et al., *B Cell Repertoire Analysis Identifies New Antigenic Domains on Glycoprotein B of Human Cytomegalovirus which Are Target of Neutralizing Antibodies.* PLoS Pathogens, 2011. 7(8): p. e1002172.
161. Britt, W.J., et al., *Cell surface expression of human cytomegalovirus (HCMV) gp55-116 (gB): use of HCMV-recombinant vaccinia virus-infected cells in analysis of the human neutralizing antibody response.* J Virol, 1990. 64(3): p. 1079-85.
162. Schoppel, K., et al., *The humoral immune response against human cytomegalovirus is characterized by a delayed synthesis of glycoprotein-specific antibodies.* J Infect Dis, 1997. 175(3): p. 533-44.

163. Britt, W.J. and L.G. Vugler, *Processing of the gp55-116 envelope glycoprotein complex (gB) of human cytomegalovirus.* J Virol, 1989. 63(1): p. 403-10.
164. Gretch, D.R., R.C. Gehrz, and M.F. Stinski, *Characterization of a human cytomegalovirus glycoprotein complex (gcI).* J Gen Virol, 1988. 69 (Pt 6): p. 1205-15.
165. Spaete, R.R., et al., *Sequence requirements for proteolytic processing of glycoprotein B of human cytomegalovirus strain Towne.* J Virol, 1990. 64(6): p. 2922-31.
166. Manservigi, R., et al., *Protection from herpes simplex virus type 1 lethal and latent infections by secreted recombinant glycoprotein B constitutively expressed in human cells with a BK virus episomal vector.* J Virol, 1990. 64(1): p. 431-6.
167. Britt, W.J. and L.G. Vugler, *Oligomerization of the human cytomegalovirus major envelope glycoprotein complex gB (gp55-116).* J Virol, 1992. 66(11): p. 6747-54.
168. Kari, B. and R. Gehrz, *A human cytomegalovirus glycoprotein complex designated gC-II is a major heparin-binding component of the envelope.* J Virol, 1992. 66(3): p. 1761-4.
169. Compton, T., R.R. Nepomuceno, and D.M. Nowlin, *Human cytomegalovirus penetrates host cells by pH-independent fusion at the cell surface.* Virology, 1992. 191(1): p. 387-95.
170. Mach, M., et al., *Complex formation by human cytomegalovirus glycoproteins M (gpUL100) and N (gpUL73).* J Virol, 2000. 74(24): p. 11881-92.
171. Keay, S. and B. Baldwin, *Anti-idiotype antibodies that mimic gp86 of human cytomegalovirus inhibit viral fusion but not attachment.* J Virol, 1991. 65(9): p. 5124-8.
172. Kinzler, E.R. and T. Compton, *Characterization of Human Cytomegalovirus Glycoprotein-Induced Cell-Cell Fusion.* Journal of Virology, 2005. 79(12): p. 7827-7837.
173. Huber, M.T. and T. Compton, *Characterization of a novel third member of the human cytomegalovirus glycoprotein H-glycoprotein L complex.* J Virol, 1997. 71(7): p. 5391-8.

174. Wang, D. and T. Shenk, *Human cytomegalovirus UL131 open reading frame is required for epithelial cell tropism.* J Virol, 2005. 79(16): p. 10330-8.
175. Navarro, D., et al., *Glycoprotein B of human cytomegalovirus promotes virion penetration into cells, transmission of infection from cell to cell, and fusion of infected cells.* Virology, 1993. 197(1): p. 143-58.
176. C.A. Alford, W.J.B. and R.W. B. Roizman, C. Lopez (Eds.), Human Herpesviruses, , *Cytomegalovirus*, in *Raven Press* (1993),. p. pp. 227-255.
177. C. SIzinger, M.D., G. Jahn eds: T.E. Shenk, M. F. Stinski, *Cytomegalovirus Cell Tropism*. Current Topics in Microbiology and Immunology. 2008: Springer-Verlag. 63-84.
178. Sinzger, C., et al., *Fibroblasts, epithelial cells, endothelial cells and smooth muscle cells are major targets of human cytomegalovirus infection in lung and gastrointestinal tissues.* J Gen Virol, 1995. 76 (Pt 4): p. 741-50.
179. Tumilowicz, J.J., et al., *Replication of cytomegalovirus in human arterial smooth muscle cells.* J Virol, 1985. 56(3): p. 839-45.
180. Roberts, W.H., et al., *Cytomegalovirus infection of gastrointestinal endothelium demonstrated by simultaneous nucleic acid hybridization and immunohistochemistry.* Arch Pathol Lab Med, 1989. 113(5): p. 461-4.
181. Hamprecht, K., et al., *Epidemiology of transmission of cytomegalovirus from mother to preterm infant by breastfeeding.* Lancet, 2001. 357(9255): p. 513-8.
182. Kahl, M., et al., *Efficient lytic infection of human arterial endothelial cells by human cytomegalovirus strains.* J Virol, 2000. 74(16): p. 7628-35.
183. Sinzger, C., et al., *Tissue macrophages are infected by human cytomegalovirus in vivo.* J Infect Dis, 1996. 173(1): p. 240-5.
184. Emery, V.C., et al., *The dynamics of human cytomegalovirus replication in vivo.* J Exp Med, 1999. 190(2): p. 177-82.
185. Compton, T., *Receptors and immune sensors: the complex entry path of human cytomegalovirus.* Trends in Cell Biology, 2004. 14(1): p. 5-8.
186. Ishida, J.H., et al., *Phase 2 Randomized, Double-Blind, Placebo-Controlled Trial of RG7667, a Combination Monoclonal Antibody, for Prevention of Cytomegalovirus Infection in High-Risk Kidney Transplant Recipients.* Antimicrob Agents Chemother, 2017. 61(2).

187. Boppana, S.B., et al., *Intrauterine transmission of cytomegalovirus to infants of women with preconceptional immunity.* N Engl J Med, 2001. 344(18): p. 1366-71.
188. Griffiths, P.D., et al., *Cytomegalovirus glycoprotein-B vaccine with MF59 adjuvant in transplant recipients: a phase 2 randomised placebo-controlled trial.* Lancet, 2011. 377(9773): p. 1256-63.
189. Kropff, B., M.P. Landini, and M. Mach, *An ELISA using recombinant proteins for the detection of neutralizing antibodies against human cytomegalovirus.* J Med Virol, 1993. 39(3): p. 187-95.
190. Schoppel, K., et al., *Antibodies specific for the antigenic domain 1 of glycoprotein B (gpUL55) of human cytomegalovirus bind to different substructures.* Virology, 1996. 216(1): p. 133-45.
191. Speckner, A., et al., *Antigenic domain 1 of human cytomegalovirus glycoprotein B induces a multitude of different antibodies which, when combined, results in incomplete virus neutralization.* J Gen Virol, 1999. 80 (Pt 8): p. 2183-91.
192. Utz, U., et al., *Identification of a neutralizing epitope on glycoprotein gp58 of human cytomegalovirus.* J Virol, 1989. 63(5): p. 1995-2001.
193. Park, J.W., et al., *Little role of anti-gB antibodies in neutralizing activity of patient's sera with human cytomegalovirus (HCMV) infection.* J Korean Med Sci, 2000. 15(2): p. 133-8.
194. Murrell, I., et al., *The pentameric complex drives immunologically covert cell-cell transmission of wild-type human cytomegalovirus.* Proc Natl Acad Sci U S A, 2017. 114(23): p. 6104-6109.
195. Wilkinson, G.W., et al., *Human cytomegalovirus: taking the strain.* Med Microbiol Immunol, 2015. 204(3): p. 273-84.
196. Wu, Y., et al., *Human cytomegalovirus glycoprotein complex gH/gL/gO uses PDGFR-α as a key for entry.* PLoS Pathogens, 2017. 13(4): p. e1006281.
197. Wille, P.T., et al., *A human cytomegalovirus gO-null mutant fails to incorporate gH/gL into the virion envelope and is unable to enter fibroblasts and epithelial and endothelial cells.* J Virol, 2010. 84(5): p. 2585-96.
198. Zhou, M., J.M. Lanchy, and B.J. Ryckman, *Human Cytomegalovirus gH/gL/gO Promotes the Fusion Step of Entry into All Cell Types, whereas*

gH/gL/UL128-131 Broadens Virus Tropism through a Distinct Mechanism. J Virol, 2015. 89(17): p. 8999-9009.

199. La Rosa, C. and D.J. Diamond, *The immune response to human CMV.* Future virology, 2012. 7(3): p. 279-293.
200. Urban, M., et al., *Glycoprotein H of human cytomegalovirus is a major antigen for the neutralizing humoral immune response.* J Gen Virol, 1996. 77 (Pt 7): p. 1537-47.
201. Macagno, A., et al., *Isolation of human monoclonal antibodies that potently neutralize human cytomegalovirus infection by targeting different epitopes on the gH/gL/UL128-131A complex.* J Virol, 2010. 84(2): p. 1005-13.
202. Freed, D.C., et al., *Pentameric complex of viral glycoprotein H is the primary target for potent neutralization by a human cytomegalovirus vaccine.* Proceedings of the National Academy of Sciences, 2013. 110(51): p. E4997-E5005.
203. Fouts, A.E., et al., *Antibodies against the gH/gL/UL128/UL130/UL131 complex comprise the majority of the anti-cytomegalovirus (anti-CMV) neutralizing antibody response in CMV hyperimmune globulin.* J Virol, 2012. 86(13): p. 7444-7.
204. Wussow, F., et al., *A vaccine based on the rhesus cytomegalovirus UL128 complex induces broadly neutralizing antibodies in rhesus macaques.* J Virol, 2013. 87(3): p. 1322-32.
205. Wen, Y., et al., *Human cytomegalovirus gH/gL/UL128/UL130/UL131A complex elicits potently neutralizing antibodies in mice.* Vaccine, 2014. 32(30): p. 3796-804.
206. Fu, T.M., et al., *Restoration of viral epithelial tropism improves immunogenicity in rabbits and rhesus macaques for a whole virion vaccine of human cytomegalovirus.* Vaccine, 2012. 30(52): p. 7469-74.
207. Ciferri, C., et al., *Structural and biochemical studies of HCMV gH/gL/gO and Pentamer reveal mutually exclusive cell entry complexes.* Proceedings of the National Academy of Sciences, 2015. 112(6): p. 1767-1772.
208. Greijer, A.E., et al., *Molecular fine-specificity analysis of antibody responses to human cytomegalovirus and design of novel synthetic-peptide-based serodiagnostic assays.* J Clin Microbiol, 1999. 37(1): p. 179-88.

209. Ohlin, M., et al., *Human antibody reactivity against the lower matrix protein (pp65) produced by cytomegalovirus.* Clin Diagn Lab Immunol, 1995. 2(3): p. 325-9.
210. Meyer, H., et al., *Identification and procaryotic expression of the gene coding for the highly immunogenic 28-kilodalton structural phosphoprotein (pp28) of human cytomegalovirus.* J Virol, 1988. 62(7): p. 2243-50.
211. Landini, M.P., et al., *Human cytomegalovirus structural proteins: immune reaction against pp150 synthetic peptides.* J Clin Microbiol, 1991. 29(9): p. 1868-72.
212. Plachter, B., et al., *Detection of cytomegalovirus antibodies by an enzyme-linked immunosorbent assay using recombinant polypeptides of the large phosphorylated tegument protein pp150.* J Clin Microbiol, 1992. 30(1): p. 201-6.
213. Bonaros, N., et al., *CMV-hyperimmune globulin for preventing cytomegalovirus infection and disease in solid organ transplant recipients: a meta-analysis.* Clin Transplant, 2008. 22(1): p. 89-97.
214. Frey, J. and B. Einsfelder, *Induction of surface IgG receptors in cytomegalovirus-infected human fibroblasts.* Eur J Biochem, 1984. 138(1): p. 213-6.
215. Furukawa, T., et al., *Demonstration of immunoglobulin G receptors induced by human cytomegalovirus.* J Clin Microbiol, 1975. 2(4): p. 332-6.
216. Keller, R., et al., *An IgG-Fc receptor induced in cytomegalovirus-infected human fibroblasts.* J Immunol, 1976. 116(3): p. 772-7.
217. Rahman, A.A., et al., *Appearance of IgG (Fc) receptor(s) on cultured human fibroblasts infected with human cytomegalovirus.* J Immunol, 1976. 117(1): p. 253-8.
218. Sprague, E.R., et al., *The Human Cytomegalovirus Fc Receptor gp68 Binds the Fc C(H)2-C(H)3 Interface of Immunoglobulin G.* Journal of Virology, 2008. 82(7): p. 3490-3499.
219. Atalay, R., et al., *Identification and expression of human cytomegalovirus transcription units coding for two distinct Fcgamma receptor homologs.* J Virol, 2002. 76(17): p. 8596-608.

220. Lilley, B.N., H.L. Ploegh, and R.S. Tirabassi, *Human cytomegalovirus open reading frame TRL11/IRL11 encodes an immunoglobulin G Fc-binding protein.* J Virol, 2001. 75(22): p. 11218-21.
221. Antonsson, A. and P.J. Johansson, *Binding of human and animal immunoglobulins to the IgG Fc receptor induced by human cytomegalovirus.* J Gen Virol, 2001. 82(Pt 5): p. 1137-45.
222. Wiger, D. and T.E. Michaelsen, *Binding site and subclass specificity of the herpes simplex virus type 1-induced Fc receptor.* Immunology, 1985. 54(3): p. 565-72.
223. Quinnan, G.V., Jr., et al., *HLA-restricted cytotoxic T lymphocytes are an early immune response and important defense mechanism in cytomegalovirus infections.* Rev Infect Dis, 1984. 6(2): p. 156-63.
224. Quinnan, G.V., Jr., et al., *Cytotoxic t cells in cytomegalovirus infection: HLA-restricted T-lymphocyte and non-T-lymphocyte cytotoxic responses correlate with recovery from cytomegalovirus infection in bone-marrow-transplant recipients.* N Engl J Med, 1982. 307(1): p. 7-13.
225. Terrazzini, N. and F. Kern, *Cell-mediated immunity to human CMV infection: a brief overview.* F1000Prime Reports, 2014. 6: p. 28.
226. Sylwester, A.W., et al., *Broadly targeted human cytomegalovirus-specific CD4^{+} and CD8^{+} T cells dominate the memory compartments of exposed subjects.* The Journal of Experimental Medicine, 2005. 202(5): p. 673.
227. Gillespie, G.M., et al., *Functional heterogeneity and high frequencies of cytomegalovirus-specific CD8(+) T lymphocytes in healthy seropositive donors.* J Virol, 2000. 74(17): p. 8140-50.
228. Steininger, C., *Clinical relevance of cytomegalovirus infection in patients with disorders of the immune system.* Clin Microbiol Infect, 2007. 13(10): p. 953-63.
229. Griffiths, P.D., *CMV as a cofactor enhancing progression of AIDS.* J Clin Virol, 2006. 35(4): p. 489-92.
230. Klenerman, P. and A. Oxenius, *T cell responses to cytomegalovirus.* Nat Rev Immunol, 2016. 16(6): p. 367-377.

231. Pawelec, G., et al., *Immunosenescence and Cytomegalovirus: where do we stand after a decade?* Immun Ageing, 2010. 7: p. 13.
232. Derhovanessian, E., et al., *Hallmark features of immunosenescence are absent in familial longevity.* J Immunol, 2010. 185(8): p. 4618-24.
233. Eriksson, P., et al., *Expansions of CD4+CD28- and CD8+CD28- T cells in granulomatosis with polyangiitis and microscopic polyangiitis are associated with cytomegalovirus infection but not with disease activity.* J Rheumatol, 2012. 39(9): p. 1840-3.
234. Morgan, M.D., et al., *CD4+CD28- T cell expansion in granulomatosis with polyangiitis (Wegener's) is driven by latent cytomegalovirus infection and is associated with an increased risk of infection and mortality.* Arthritis Rheum, 2011. 63(7): p. 2127-37.
235. Cobbold, M., et al., *Adoptive transfer of cytomegalovirus-specific CTL to stem cell transplant patients after selection by HLA-peptide tetramers.* J Exp Med, 2005. 202(3): p. 379-86.
236. Riddell, S.R. and P.D. Greenberg, *T cell therapy of human CMV and EBV infection in immunocompromised hosts.* Rev Med Virol, 1997. 7(3): p. 181-192.
237. Riddell, S.R., P. Reusser, and P.D. Greenberg, *Cytotoxic T cells specific for cytomegalovirus: a potential therapy for immunocompromised patients.* Rev Infect Dis, 1991. 13 Suppl 11: p. S966-73.
238. Horst, D., et al., *Viral evasion of T cell immunity: ancient mechanisms offering new applications.* Current Opinion in Immunology, 2011. 23(1): p. 96-103.
239. Lucin, P., et al., *Cytomegalovirus immune evasion by perturbation of endosomal trafficking.* Cell Mol Immunol, 2015. 12(2): p. 154-169.
240. Lilley, B.N. and H.L. Ploegh, *Viral modulation of antigen presentation: manipulation of cellular targets in the ER and beyond.* Immunol Rev, 2005. 207: p. 126-44.
241. Huber, M.T., et al., *Human Cytomegalovirus US7, US8, US9, and US10 Are Cytoplasmic Glycoproteins, Not Found at Cell Surfaces, and US9 Does Not Mediate Cell-to-Cell Spread.* Journal of Virology, 2002. 76(11): p. 5748-5758.

242. Tortorella, D., et al., *Viral subversion of the immune system.* Annu Rev Immunol, 2000. 18: p. 861-926.
243. Hewitt, E.W., *The MHC class I antigen presentation pathway: strategies for viral immune evasion.* Immunology, 2003. 110(2): p. 163-9.
244. Hengel, H., et al., *A viral ER-resident glycoprotein inactivates the MHC-encoded peptide transporter.* Immunity, 1997. 6(5): p. 623-32.
245. Wiertz, E.J., et al., *The human cytomegalovirus US11 gene product dislocates MHC class I heavy chains from the endoplasmic reticulum to the cytosol.* Cell, 1996. 84(5): p. 769-79.
246. Wiertz, E.J., et al., *Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction.* Nature, 1996. 384(6608): p. 432-8.
247. Ahn, K., et al., *Human cytomegalovirus inhibits antigen presentation by a sequential multistep process.* Proc Natl Acad Sci U S A, 1996. 93(20): p. 10990-5.
248. Jones, T.R., et al., *Human cytomegalovirus US3 impairs transport and maturation of major histocompatibility complex class I heavy chains.* Proc Natl Acad Sci U S A, 1996. 93(21): p. 11327-33.
249. Cebulla, C.M., et al., *Human cytomegalovirus disrupts constitutive MHC class II expression.* J Immunol, 2002. 169(1): p. 167-76.
250. Lee, A.W., et al., *Human cytomegalovirus alters localization of MHC class II and dendrite morphology in mature Langerhans cells.* J Immunol, 2006. 177(6): p. 3960-71.
251. Raftery, M.J., et al., *Targeting the function of mature dendritic cells by human cytomegalovirus: a multilayered viral defense strategy.* Immunity, 2001. 15(6): p. 997-1009.
252. Odeberg, J. and C. Soderberg-Naucler, *Reduced expression of HLA class II molecules and Iinterleukin-10- and transforming growth factor beta1-independent suppression of T-cell proliferation in human cytomegalovirus-infected macrophage cultures.* J Virol, 2001. 75(11): p. 5174-81.
253. Spencer, J.V., et al., *Potent immunosuppressive activities of cytomegalovirus-encoded interleukin-10.* J Virol, 2002. 76(3): p. 1285-92.
254. Griffiths, P., I. Baraniak, and M. Reeves, *The pathogenesis of human cytomegalovirus.* J Pathol, 2015. 235(2): p. 288-97.

255. Baxter, D., *Active and passive immunity, vaccine types, excipients and licensing.* Occupational Medicine, 2007. 57(8): p. 552-556.
256. Nigro, G., et al., *Passive immunization during pregnancy for congenital cytomegalovirus infection.* N Engl J Med, 2005. 353(13): p. 1350-62.
257. Revello, M.G., et al., *A Randomized Trial of Hyperimmune Globulin to Prevent Congenital Cytomegalovirus.* New England Journal of Medicine, 2014. 370(14): p. 1316-1326.
258. Rea, F., et al., *Cytomegalovirus Hyper Immunoglobulin for CMV Prophylaxis in Thoracic Transplantation.* Transplantation, 2016. 100(Suppl 3): p. S19-S26.
259. Snydman, D.R., et al., *Cytomegalovirus prevention and long-term recipient and graft survival in pediatric heart transplant recipients.* Transplantation, 2010. 90(12): p. 1432-8.
260. Snydman, D.R., *Cytomegalovirus immunoglobulins in the prevention and treatment of cytomegalovirus disease.* Rev Infect Dis, 1990. 12 Suppl 7: p. S839-48.
261. Snydman, D.R., et al., *Cytomegalovirus immune globulin prophylaxis in liver transplantation. A randomized, double-blind, placebo-controlled trial.* Ann Intern Med, 1993. 119(10): p. 984-91.
262. Fisher, R.A., et al., *The association between cytomegalovirus immune globulin and long-term recipient and graft survival following liver transplantation.* Transpl Infect Dis, 2012. 14(2): p. 121-31.
263. Valantine, H.A., et al., *Impact of cytomegalovirus hyperimmune globulin on outcome after cardiothoracic transplantation: a comparative study of combined prophylaxis with CMV hyperimmune globulin plus ganciclovir versus ganciclovir alone.* Transplantation, 2001. 72(10): p. 1647-52.
264. HQ, W., *WHO informal consultation on characterization and quality aspect of vaccines based on live viral vectors*. 2003, WHO: Geneva.
265. Barouch, D.H. and L.J. Picker, *Novel vaccine vectors for HIV-1.* Nat Rev Micro, 2014. 12(11): p. 765-771.
266. Hansen, S.G., et al., *Cytomegalovirus Vectors Violate CD8⁺ T Cell Epitope Recognition Paradigms.* Science, 2013. 340(6135).

267. Hansen, S.G., et al., *Immune clearance of highly pathogenic SIV infection.* Nature, 2013. 502(7469): p. 100-104.
268. Hansen, S.G., et al., *Profound early control of highly pathogenic SIV by an effector memory T-cell vaccine.* Nature, 2011. 473(7348): p. 523-7.
269. Plotkin, S.A., et al., *Multicenter trial of Towne strain attenuated virus vaccine in seronegative renal transplant recipients.* Transplantation, 1994. 58(11): p. 1176-8.
270. Adler, S.P., et al., *Immunity induced by primary human cytomegalovirus infection protects against secondary infection among women of childbearing age.* J Infect Dis, 1995. 171(1): p. 26-32.
271. Jacobson, M.A., et al., *Antigen-specific T cell responses induced by Towne cytomegalovirus (CMV) vaccine in CMV-seronegative vaccine recipients.* J Clin Virol, 2006. 35(3): p. 332-7.
272. Jacobson, M.A., et al., *Safety and immunogenicity of Towne cytomegalovirus vaccine with or without adjuvant recombinant interleukin-12.* Vaccine, 2006. 24(25): p. 5311-9.
273. Lilja, A.E. and P.W. Mason, *The next generation recombinant human cytomegalovirus vaccine candidates—Beyond gB.* Vaccine, 2012. 30(49): p. 6980-6990.
274. Cannon, M.J., D.S. Schmid, and T.B. Hyde, *Review of cytomegalovirus seroprevalence and demographic characteristics associated with infection.* Rev Med Virol, 2010. 20(4): p. 202-13.
275. Simanek, A.M., et al., *Seropositivity to cytomegalovirus, inflammation, all-cause and cardiovascular disease-related mortality in the United States.* PLoS One, 2011. 6(2): p. e16103.
276. Institute of Medicine Committee to Study Priorities for Vaccine, D., *The National Academies Collection: Reports funded by National Institutes of Health*, in *Vaccines for the 21st Century: A Tool for Decisionmaking*, K.R. Stratton, J.S. Durch, and R.S. Lawrence, Editors. 2000, National Academies Press (US)

277. Arvin, A.M., et al., *Vaccine development to prevent cytomegalovirus disease: report from the National Vaccine Advisory Committee.* Clin Infect Dis, 2004. 39(2): p. 233-9.
278. Cheeran, M.C.J., J.R. Lokensgard, and M.R. Schleiss, *Neuropathogenesis of Congenital Cytomegalovirus Infection: Disease Mechanisms and Prospects for Intervention.* Clinical Microbiology Reviews, 2009. 22(1): p. 99-126.
279. Townsend, C.L., et al., *Long-term outcomes of congenital cytomegalovirus infection in Sweden and the United Kingdom.* Clin Infect Dis, 2013. 56(9): p. 1232-9.
280. Kimberlin, D.W., et al., *Effect of ganciclovir therapy on hearing in symptomatic congenital cytomegalovirus disease involving the central nervous system: a randomized, controlled trial.* J Pediatr, 2003. 143(1): p. 16-25.
281. Kimberlin, D.W., et al., *Valganciclovir for symptomatic congenital cytomegalovirus disease.* N Engl J Med, 2015. 372(10): p. 933-43.
282. *Hyperimmune Globulin to Prevent Congenital CMV Infection.* New England Journal of Medicine, 2014. 370(26): p. 2543-2545.
283. Griffiths, P.D., *Burden of disease associated with human cytomegalovirus and prospects for elimination by universal immunisation.* The Lancet Infectious Diseases. 12(10): p. 790-798.
284. Erard, V., et al., *Reduced Mortality of Cytomegalovirus Pneumonia After Hematopoietic Cell Transplantation Due to Antiviral Therapy and Changes in Transplantation Practices.* Clin Infect Dis, 2015. 61(1): p. 31-9.
285. Boeckh, M., et al., *Valganciclovir for the prevention of complications of late cytomegalovirus infection after allogeneic hematopoietic cell transplantation: a randomized trial.* Ann Intern Med, 2015. 162(1): p. 1-10.
286. Viot, B., et al., *Two-year post-transplantation cytomegalovirus DNAemia in asymptomatic kidney transplant recipients: incidence, risk factors, and outcome.* Transpl Infect Dis, 2015.
287. Matsumoto, A., et al., *Development of CMV retinitis in an antigenemia-negative infant after cord blood transplantation.* Rinsho Ketsueki, 2015. 56(5): p. 506-10.

288. Liu, J., et al., *Patients with refractory CMV infection following allo-HSCT are at high risk for CMV disease and non-relapse mortality.* Clin Microbiol Infect, 2015.
289. Yalci, A., et al., *Evaluation of Infectious Complications in the First Year After Kidney Transplantation.* Transplant Proc, 2015. 47(5): p. 1429-1432.
290. Cohen, L., et al., *Risk factors and prognostic scale for cytomegalovirus (CMV) infection in CMV-seropositive patients after allogeneic hematopoietic cell transplantation.* Transpl Infect Dis, 2015.
291. Sagedal, S., A. Hartmann, and H. Rollag, *The impact of early cytomegalovirus infection and disease in renal transplant recipients.* Clinical Microbiology and Infection, 2005. 11(7): p. 518-530.
292. Ariza-Heredia, E.J., L. Nesher, and R.F. Chemaly, *Cytomegalovirus diseases after hematopoietic stem cell transplantation: A mini-review.* Cancer Letters, 2014. 342(1): p. 1-8.
293. Griffiths, P., *Cytomegalovirus infection of the central nervous system.* Herpes, 2004. 11 Suppl 2: p. 95a-104a.
294. Mujtaba, S., S. Varma, and S. Sehgal, *Cytomegalovirus co-infection in patients with HIV/AIDS in north India.* Indian J Med Res, 2003. 117: p. 99-103.
295. Kestelyn, P.G. and E.T. Cunningham, Jr., *HIV/AIDS and blindness.* Bull World Health Organ, 2001. 79(3): p. 208-13.
296. Biswas, J., et al., *Ocular lesions associated with HIV infection in India: a series of 100 consecutive patients evaluated at a referral center.* Am J Ophthalmol, 2000. 129(1): p. 9-15.
297. Bilgrami, M. and P. O'keefe, *Chapter 90 - Neurologic diseases in HIV-infected patients*, in *Handbook of Clinical Neurology*, B. José and M.F. José, Editors. 2014, Elsevier. p. 1321-1344.
298. Hsiao, N.Y., et al., *Cytomegalovirus viraemia in HIV exposed and infected infants: prevalence and clinical utility for diagnosing CMV pneumonia.* J Clin Virol, 2013. 58(1): p. 74-8.
299. Polaczek, M.M., et al., *[Pneumocystis pneumonia in HIV-infected patients with cytomegalovirus co-infection. Two case reports and a literature review].* Pneumonol Alergol Pol, 2014. 82(5): p. 458-66.

300. Plotkin, S.A., et al., *Towne-vaccine-induced prevention of cytomegalovirus disease after renal transplants.* Lancet, 1984. 1(8376): p. 528-30.
301. Plotkin, S.A., *Vaccination against cytomegalovirus, the changeling demon.* Pediatr Infect Dis J, 1999. 18(4): p. 313-25; quiz 326.
302. Heineman, T.C., et al., *A phase 1 study of 4 live, recombinant human cytomegalovirus Towne/Toledo chimeric vaccines.* J Infect Dis, 2006. 193(10): p. 1350-60.
303. Adler, S.P., et al., *A Phase 1 Study of 4 Live, Recombinant Human Cytomegalovirus Towne/Toledo Chimera Vaccines in Cytomegalovirus-Seronegative Men.* J Infect Dis, 2016. 214(9): p. 1341-1348.
304. La Rosa, C., et al., *Clinical evaluation of safety and immunogenicity of PADRE-cytomegalovirus (CMV) and tetanus-CMV fusion peptide vaccines with or without PF03512676 adjuvant.* J Infect Dis, 2012. 205(8): p. 1294-304.
305. Wills, M.R., et al., *The human cytotoxic T-lymphocyte (CTL) response to cytomegalovirus is dominated by structural protein pp65: frequency, specificity, and T-cell receptor usage of pp65-specific CTL.* J Virol, 1996. 70(11): p. 7569-79.
306. McLaughlin-Taylor, E., et al., *Identification of the major late human cytomegalovirus matrix protein pp65 as a target antigen for CD8+ virus-specific cytotoxic T lymphocytes.* J Med Virol, 1994. 43(1): p. 103-10.
307. Walter, E.A., et al., *Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor.* N Engl J Med, 1995. 333(16): p. 1038-44.
308. Slezak, S.L., et al., *CMV pp65 and IE-1 T cell epitopes recognized by healthy subjects.* J Transl Med, 2007. 5: p. 17.
309. Selinsky, C., et al., *A DNA-based vaccine for the prevention of human cytomegalovirus-associated diseases.* Hum Vaccin, 2005. 1(1): p. 16-23.
310. Wloch, M.K., et al., *Safety and immunogenicity of a bivalent cytomegalovirus DNA vaccine in healthy adult subjects.* J Infect Dis, 2008. 197(12): p. 1634-42.
311. Kharfan-Dabaja, M.A., et al., *A novel therapeutic cytomegalovirus DNA vaccine in allogeneic haemopoietic stem-cell transplantation: a randomised,*

double-blind, placebo-controlled, phase 2 trial. Lancet Infect Dis, 2012. 12(4): p. 290-9.

312. Jacobson, M.A., et al., *A CMV DNA vaccine primes for memory immune responses to live-attenuated CMV (Towne strain).* Vaccine, 2009. 27(10): p. 1540-8.
313. Bernstein, D.I., et al., *Randomized, double-blind, Phase 1 trial of an alphavirus replicon vaccine for cytomegalovirus in CMV seronegative adult volunteers.* Vaccine, 2009. 28(2): p. 484-93.
314. Schleiss, M.R., et al., *Protection against congenital cytomegalovirus infection and disease in guinea pigs, conferred by a purified recombinant glycoprotein B vaccine.* J Infect Dis, 2004. 189(8): p. 1374-81.
315. Spaete, R.R., *A recombinant subunit vaccine approach to HCMV vaccine development.* Transplant Proc, 1991. 23(3 Suppl 3): p. 90-6.
316. Spaete, R.R., et al., *Human cytomegalovirus strain Towne glycoprotein B is processed by proteolytic cleavage.* Virology, 1988. 167(1): p. 207-25.
317. Rasmussen, L., et al., *Antibody response to human cytomegalovirus glycoproteins gB and gH after natural infection in humans.* J Infect Dis, 1991. 164(5): p. 835-42.
318. Frey, S.E., et al., *Effects of antigen dose and immunization regimens on antibody responses to a cytomegalovirus glycoprotein B subunit vaccine.* J Infect Dis, 1999. 180(5): p. 1700-3.
319. Pass, R.F., et al., *A subunit cytomegalovirus vaccine based on recombinant envelope glycoprotein B and a new adjuvant.* J Infect Dis, 1999. 180(4): p. 970-5.
320. Mitchell, D.K., et al., *Immunogenicity of a recombinant human cytomegalovirus gB vaccine in seronegative toddlers.* Pediatr Infect Dis J, 2002. 21(2): p. 133-8.
321. Pass, R.F., et al., *Vaccine prevention of maternal cytomegalovirus infection.* N Engl J Med, 2009. 360(12): p. 1191-9.
322. Pass, R.F., *Development and Evidence for Efficacy of CMV Glycoprotein B Vaccine with MF59 Adjuvant.* Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology, 2009. 46(Suppl 4): p. S73-S76.

323. Bernstein, D.I., et al., *Safety and efficacy of a cytomegalovirus glycoprotein B (gB) vaccine in adolescent girls: A randomized clinical trial.* Vaccine, 2016. 34(3): p. 313-9.
324. Krause, P.R., et al., *Priorities for CMV vaccine development.* Vaccine, 2013. 32(1): p. 4-10.
325. Sabbaj, S., et al., *Glycoprotein B vaccine is capable of boosting both antibody and CD4 T-cell responses to cytomegalovirus in chronically infected women.* J Infect Dis, 2011. 203(11): p. 1534-41.
326. Atabani, S.F., et al., *Cytomegalovirus replication kinetics in solid organ transplant recipients managed by preemptive therapy.* Am.J.Transplant., 2012. 12(9): p. 2457-2464.
327. Mattes, F.M., et al., *Kinetics of Cytomegalovirus Load Decrease in Solid-Organ Transplant Recipients after Preemptive Therapy with Valganciclovir.* Journal of Infectious Diseases, 2005. 191(1): p. 89-92.
328. Fox, J.C., et al., *Longitudinal analysis of cytomegalovirus load in renal transplant recipients using a quantitative polymerase chain reaction: correlation with disease.* J Gen Virol, 1995. 76 (Pt 2): p. 309-19.
329. Stanton, R.J., et al., *Reconstruction of the complete human cytomegalovirus genome in a BAC reveals RL13 to be a potent inhibitor of replication.* J Clin Invest, 2010. 120(9): p. 3191-208.
330. Janeway CA Jr, T.P., Walport M, et al. New York: Garland Science; Glossary. , *Immunobiology: The Immune System in Health and Disease. .* 2001. 5th edition.: p. Glossary. Available from: http://www.ncbi.nlm.nih.gov/books/NBK10759/.
331. Biology-Online., *Neutralizing antibody.* 2008.
332. Mallery, D.L., et al., *Antibodies mediate intracellular immunity through tripartite motif-containing 21 (TRIM21).* Proc Natl Acad Sci U S A, 2010. 107(46): p. 19985-90.
333. Plotkin, S.A., *Correlates of protection induced by vaccination.* Clin Vaccine Immunol, 2010. 17(7): p. 1055-65.
334. Plotkin, S.A., *Complex correlates of protection after vaccination.* Clin Infect Dis, 2013. 56(10): p. 1458-65.

335. Mack, T.M., J. Noble, Jr., and D.B. Thomas, *A prospective study of serum antibody and protection against smallpox.* Am J Trop Med Hyg, 1972. 21(2): p. 214-8.
336. Sarkar, J.K., A.C. Mitra, and M.K. Mukherjee, *The minimum protective level of antibodies in smallpox.* Bull World Health Organ, 1975. 52(3): p. 307-11.
337. du Châtelet, I.P., et al., *Serological response and poliovirus excretion following different combined oral and inactivated poliovirus vaccines immunization schedules.* Vaccine, 2003. 21(15): p. 1710-1718.
338. Chen, R.T., et al., *Measles antibody: reevaluation of protective titers.* J Infect Dis, 1990. 162(5): p. 1036-42.
339. Polack, F.P., et al., *Successful DNA immunization against measles: neutralizing antibody against either the hemagglutinin or fusion glycoprotein protects rhesus macaques without evidence of atypical measles.* Nat Med, 2000. 6(7): p. 776-81.
340. Puligedda, R.D., et al., *Human monoclonal antibodies that neutralize vaccine and wild-type poliovirus strains.* Antiviral Research, 2014. 108(0): p. 36-43.
341. Weibel, R.E., et al., *Long-term follow-up for immunity after monovalent or combined live measles, mumps, and rubella virus vaccines.* Pediatrics, 1975. 56(3): p. 380-7.
342. Excler, J.L., et al., *Nonneutralizing functional antibodies: a new "old" paradigm for HIV vaccines.* Clin Vaccine Immunol, 2014. 21(8): p. 1023-36.
343. Hope, T.J., *Moving ahead an HIV vaccine: To neutralize or not, a key HIV vaccine question.* Nat Med, 2011. 17(10): p. 1195-1197.
344. Schleiss, M.R. and T.C. Heineman, *Progress toward an elusive goal: current status of cytomegalovirus vaccines.* Expert Rev Vaccines, 2005. 4(3): p. 381-406.
345. Reeves, M.B., A. Breidenstein, and T. Compton, *Human cytomegalovirus activation of ERK and myeloid cell leukemia-1 protein correlates with survival of latently infected cells.* Proc Natl Acad Sci U S A, 2012. 109(2): p. 588-93.
346. Schillie, S.F., P.R. Spradling, and T.V. Murphy, *Immune Response of Hepatitis B Vaccine Among Persons With Diabetes: A systematic review of the literature.* Diabetes Care, 2012. 35(12): p. 2690-2697.

347. Tong, N.K., et al., *Immunogenicity and safety of an adjuvanted hepatitis B vaccine in pre-hemodialysis and hemodialysis patients.* Kidney Int, 2005. 68(5): p. 2298-303.
348. Fouts, A.E., et al., *Mechanism for neutralizing activity by the anti-CMV gH/gL monoclonal antibody MSL-109.* Proc Natl Acad Sci U S A, 2014. 111(22): p. 8209-14.
349. Wang, H., et al., *Complete protection of mice against lethal murine cytomegalovirus challenge by immunization with DNA vaccines encoding envelope glycoprotein complex III antigens gH, gL and gO.* PLoS One, 2015. 10(3): p. e0119964.
350. Auerbach, M.R., et al., *A neutralizing anti-gH/gL monoclonal antibody is protective in the guinea pig model of congenital CMV infection.* PLoS Pathog, 2014. 10(4): p. e1004060.
351. Vanarsdall, A.L., et al., *Human Cytomegalovirus gH/gL Forms a Stable Complex with the Fusion Protein gB in Virions.* PLoS Pathog, 2016. 12(4): p. e1005564.
352. Vanarsdall, A.L., M.C. Chase, and D.C. Johnson, *Human cytomegalovirus glycoprotein gO complexes with gH/gL, promoting interference with viral entry into human fibroblasts but not entry into epithelial cells.* J Virol, 2011. 85(22): p. 11638-45.
353. Ciferri, C., et al., *Antigenic Characterization of the HCMV gH/gL/gO and Pentamer Cell Entry Complexes Reveals Binding Sites for Potently Neutralizing Human Antibodies.* PLoS Pathog, 2015. 11(10): p. e1005230.
354. Gerna, G., et al., *Monoclonal Antibodies to Different Components of the Human Cytomegalovirus (HCMV) Pentamer gH/gL/pUL128L and Trimer gH/gL/gO as well as Antibodies Elicited during Primary HCMV Infection Prevent Epithelial Cell Syncytium Formation.* J Virol, 2016. 90(14): p. 6216-23.
355. Eggink, D., P.H. Goff, and P. Palese, *Guiding the Immune Response against Influenza Virus Hemagglutinin toward the Conserved Stalk Domain by Hyperglycosylation of the Globular Head Domain.* Journal of Virology, 2014. 88(1): p. 699-704.
356. Mascola, J.R. and D.C. Montefiori, *HIV-1: nature's master of disguise.* Nat Med, 2003. 9(4): p. 393-394.

357. Wei, X., et al., *Antibody neutralization and escape by HIV-1.* Nature, 2003. 422(6929): p. 307-312.
358. Burke, H.G. and E.E. Heldwein, *Crystal Structure of the Human Cytomegalovirus Glycoprotein B.* PLoS Pathog, 2015. 11(10): p. e1005227.
359. Wilen, C.B., J.C. Tilton, and R.W. Doms, *HIV: Cell Binding and Entry.* Cold Spring Harbor Perspectives in Medicine, 2012. 2(8): p. a006866.
360. Dowd, K.A., et al., *Selection Pressure from Neutralizing Antibodies Drives Sequence Evolution during Acute Infection with Hepatitis C Virus.* Gastroenterology, 2009. 136(7): p. 2377-2386.
361. Kim, J.H., J.L. Excler, and N.L. Michael, *Lessons from the RV144 Thai phase III HIV-1 vaccine trial and the search for correlates of protection.* Annu Rev Med, 2015. 66: p. 423-37.
362. Montefiori, D.C., et al., *Magnitude and breadth of the neutralizing antibody response in the RV144 and Vax003 HIV-1 vaccine efficacy trials.* J Infect Dis, 2012. 206(3): p. 431-41.
363. Haynes, B.F., et al., *Immune-correlates analysis of an HIV-1 vaccine efficacy trial.* N Engl J Med, 2012. 366(14): p. 1275-86.
364. Rolland, M., et al., *Increased HIV-1 vaccine efficacy against viruses with genetic signatures in Env V2.* Nature, 2012. 490(7420): p. 417-420.
365. Yates, N.L., et al., *Vaccine-Induced Env V1-V2 IgG3 Correlates with Lower HIV-1 Infection Risk and Declines Soon After Vaccination.* Science Translational Medicine, 2014. 6(228): p. 228ra39-228ra39.
366. Roussilhon, C., et al., *Long-Term Clinical Protection from Falciparum Malaria Is Strongly Associated with IgG3 Antibodies to Merozoite Surface Protein 3.* PLoS Med, 2007. 4(11): p. e320.
367. Kam, Y.W., et al., *Early appearance of neutralizing immunoglobulin G3 antibodies is associated with chikungunya virus clearance and long-term clinical protection.* J Infect Dis, 2012. 205(7): p. 1147-54.
368. Bonsignori, M., et al., *Antibody-dependent cellular cytotoxicity-mediating antibodies from an HIV-1 vaccine efficacy trial target multiple epitopes and preferentially use the VH1 gene family.* J Virol, 2012. 86(21): p. 11521-32.
369. Pollara, J., et al., *High-throughput quantitative analysis of HIV-1 and SIV-specific ADCC-mediating antibody responses.* Cytometry A, 2011. 79(8): p. 603-12.

370. Zolla-Pazner, S., et al., *Analysis of V2 Antibody Responses Induced in Vaccinees in the ALVAC/AIDSVAX HIV-1 Vaccine Efficacy Trial.* PLoS ONE, 2013. 8(1): p. e53629.
371. Haynes, B.F., et al., *Immune-Correlates Analysis of an HIV-1 Vaccine Efficacy Trial.* New England Journal of Medicine, 2012. 366(14): p. 1275-1286.
372. Tomaras, G.D., et al., *Vaccine-induced plasma IgA specific for the C1 region of the HIV-1 envelope blocks binding and effector function of IgG.* Proceedings of the National Academy of Sciences, 2013. 110(22): p. 9019-9024.
373. Jacob, C.L., et al., *Neutralizing antibodies are unable to inhibit direct viral cell-to-cell spread of human cytomegalovirus.* Virology, 2013. 444(1-2): p. 140-7.
374. Navarro, D., P. Paz, and L. Pereira, *Domains of herpes simplex virus I glycoprotein B that function in virus penetration, cell-to-cell spread, and cell fusion.* Virology, 1992. 186(1): p. 99-112.
375. Frenzel, K., et al., *Antiviral function and efficacy of polyvalent immunoglobulin products against CMV isolates in different human cell lines.* Medical Microbiology and Immunology, 2012. 201(3): p. 277-286.
376. Sinzger, C., et al., *Effect of serum and CTL on focal growth of human cytomegalovirus.* J Clin Virol, 2007. 38(2): p. 112-9.
377. Schröer, J. and T. Shenk, *Inhibition of cyclooxygenase activity blocks cell-to-cell spread of human cytomegalovirus.* Proceedings of the National Academy of Sciences, 2008. 105(49): p. 19468-19473.
378. Cooper, M.A., T.A. Fehniger, and M.A. Caligiuri, *The biology of human natural killer-cell subsets.* Trends in Immunology, 2001. 22(11): p. 633-640.
379. Caligiuri, M.A., *Human natural killer cells.* Blood, 2008. 112(3): p. 461-9.
380. Nimmerjahn, F. and J.V. Ravetch, *Fcgamma receptors as regulators of immune responses.* Nat Rev Immunol, 2008. 8(1): p. 34-47.
381. F Dallegri, F.P., G Holm, G Gahrton, and C Sacchetti, *Neutrophil-mediated antibody-dependent cellular cytotoxicity against erythrocytes. Mechanisms of target cell destruction.* Clinical and Experiamental Immunology, 1983. 52((3)): p. 613–619.

382. Clark, A.J., et al., *Calicum Microdomains form within Neutrophils at the Neutrophil-Tumor Cell Synapse: Role in Antibody-Dependent Target Cell Apoptosis.* Cancer Immunology, Immunotherapy, 2010. 59(1): p. 149-159.
383. Joshi, T., et al., *The PtdIns 3-Kinase/Akt Pathway Regulates Macrophage-Mediated ADCC against B Cell Lymphoma.* PLoS ONE, 2009. 4(1): p. e4208.
384. Hallam, C., et al., *Rat eosinophil-mediated antibody-dependent cellular cytotoxicity: investigations of the mechanisms of target cell lysis and inhibition by glucocorticoids.* Clinical and Experimental Immunology, 1982. 48(3): p. 641-648.
385. Romee, R., et al., *NK cell CD16 surface expression and function is regulated by a disintegrin and metalloprotease-17 (ADAM17).* Blood, 2013. 121(18): p. 3599-608.
386. Fehniger, T.A., et al., *Acquisition of murine NK cell cytotoxicity requires the translation of a pre-existing pool of granzyme B and perforin mRNAs.* Immunity, 2007. 26(6): p. 798-811.
387. Podack, E.R., J.D. Young, and Z.A. Cohn, *Isolation and biochemical and functional characterization of perforin 1 from cytolytic T-cell granules.* Proceedings of the National Academy of Sciences, 1985. 82(24): p. 8629-8633.
388. Tschopp, J. and M. Nabholz, *Perforin-mediated target cell lysis by cytolytic T lymphocytes.* Annu Rev Immunol, 1990. 8: p. 279-302.
389. Cullen, S.P. and S.J. Martin, *Mechanisms of granule-dependent killing.* Cell Death Differ, 2008. 15(2): p. 251-62.
390. Grossman, W.J., et al., *The orphan granzymes of humans and mice.* Current Opinion in Immunology, 2003. 15(5): p. 544-552.
391. Russell, J.H. and T.J. Ley, *Lymphocyte-mediated cytotoxicity.* Annu Rev Immunol, 2002. 20: p. 323-70.
392. Kam, C.M., D. Hudig, and J.C. Powers, *Granzymes (lymphocyte serine proteases): characterization with natural and synthetic substrates and inhibitors.* Biochim Biophys Acta, 2000. 1477(1-2): p. 307-23.
393. Cerwenka, A. and L.L. Lanier, *Ligands for natural killer cell receptors: Redundancy or specificity.* Immunological Reviews, 2001. 181: p. 158-169.

394. Winchester, B.G., *Lysosomal membrane proteins.* European Journal of Paediatric Neurology, 2001. 5, Supplement 1(0): p. 11-19.
395. Alter, G., J.M. Malenfant, and M. Altfeld, *CD107a as a functional marker for the identification of natural killer cell activity.* Journal of Immunological Methods, 2004. 294(1–2): p. 15-22.
396. Krzewski, K., et al., *LAMP1/CD107a is required for efficient perforin delivery to lytic granules and NK-cell cytotoxicity.* Blood, 2013. 121(23): p. 4672-4683.
397. Jegaskanda, S., et al., *Age-associated cross-reactive antibody-dependent cellular cytotoxicity toward 2009 pandemic influenza A virus subtype H1N1.* J Infect Dis, 2013. 208(7): p. 1051-61.
398. Jegaskanda, S., et al., *Antibody-Dependent Cellular Cytotoxicity Is Associated with Control of Pandemic H1N1 Influenza Virus Infection of Macaques.* Journal of Virology, 2013. 87(10): p. 5512-5522.
399. Jegaskanda, S., P.C. Reading, and S.J. Kent, *Influenza-specific antibody-dependent cellular cytotoxicity: toward a universal influenza vaccine.* J Immunol, 2014. 193(2): p. 469-75.
400. Aktas, E., et al., *Relationship between CD107a expression and cytotoxic activity.* Cell Immunol, 2009. 254(2): p. 149-54.
401. Teilah, H.K., *Natural Killer Cell Cytotoxic Activity: Measurement of the Apoptotic Inducing Mechanisms.* Clinical and Experimental Medical Sciences, 2013. 1(8): p. 373-386.
402. Jegaskanda, S., et al., *Cross-reactive influenza-specific antibody-dependent cellular cytotoxicity antibodies in the absence of neutralizing antibodies.* J Immunol, 2013. 190(4): p. 1837-48.
403. Dons'koi, B.V., V.P. Chernyshov, and D.V. Osypchuk, *Measurement of NK activity in whole blood by the CD69 up-regulation after co-incubation with K562, comparison with NK cytotoxicity assays and CD107a degranulation assay.* J Immunol Methods, 2011. 372(1-2): p. 187-95.
404. Ahmad, A., et al., *Evidence for a defect of antibody-dependent cellular cytotoxic (ADCC) effector function and anti-HIV gp120/41-specific ADCC-mediating antibody titres in HIV-infected individuals.* J Acquir Immune Defic Syndr, 1994. 7(5): p. 428-37.

405. Forthal, D.N., et al., *Antibody-dependent cellular cytotoxicity independently predicts survival in severely immunocompromised human immunodeficiency virus-infected patients.* J Infect Dis, 1999. 180(4): p. 1338-41.
406. Forthal, D.N., G. Landucci, and B. Keenan, *Relationship between antibody-dependent cellular cytotoxicity, plasma HIV type 1 RNA, and CD4+ lymphocyte count.* AIDS Res Hum Retroviruses, 2001. 17(6): p. 553-61.
407. He, X., et al., *Compromised NK Cell-Mediated Antibody-Dependent Cellular Cytotoxicity in Chronic SIV/SHIV Infection.* PLoS ONE, 2013. 8(2): p. e56309.
408. Jia, M., et al., *Impaired natural killer cell-induced antibody-dependent cell-mediated cytotoxicity is associated with human immunodeficiency virus-1 disease progression.* Clinical and Experimental Immunology, 2013. 171(1): p. 107-116.
409. Wren, L.H., et al., *Specific antibody-dependent cellular cytotoxicity responses associated with slow progression of HIV infection.* Immunology, 2013. 138(2): p. 116-123.
410. Clynes, R.A., et al., *Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets.* Nat Med, 2000. 6(4): p. 443-6.
411. Di Santo, J.P., *Natural killer cell developmental pathways: a question of balance.* Annu Rev Immunol, 2006. 24: p. 257-86.
412. Kiessling, R., et al., *"Natural" killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell.* Eur J Immunol, 1975. 5(2): p. 117-21.
413. Kiessling, R., E. Klein, and H. Wigzell, *"Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype.* Eur J Immunol, 1975. 5(2): p. 112-7.
414. Karre, K., et al., *Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy.* Nature, 1986. 319(6055): p. 675-8.
415. Kim, S., et al., *Licensing of natural killer cells by host major histocompatibility complex class I molecules.* Nature, 2005. 436(7051): p. 709-13.

416. Kumar, V. and M.E. McNerney, *A new self: MHC-class-I-independent natural-killer-cell self-tolerance.* Nat Rev Immunol, 2005. 5(5): p. 363-74.
417. Anel, A., G.V. Richieri, and A.M. Kleinfeld, *A tyrosine phosphorylation requirement for cytotoxic T lymphocyte degranulation.* J Biol Chem, 1994. 269(13): p. 9506-13.
418. Castagna, M., et al., *Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters.* J Biol Chem, 1982. 257(13): p. 7847-51.
419. Liu, W.S. and C.A. Heckman, *The sevenfold way of PKC regulation.* Cell Signal, 1998. 10(8): p. 529-42.
420. Goldberger, T. and O. Mandelboim, *The use of microRNA by human viruses: lessons from NK cells and HCMV infection.* Semin Immunopathol, 2014. 36(6): p. 659-74.
421. Beziat, V., et al., *NK cell responses to cytomegalovirus infection lead to stable imprints in the human KIR repertoire and involve activating KIRs.* Blood, 2013. 121(14): p. 2678-88.
422. Della Chiesa, M., et al., *Impact of HCMV Infection on NK Cell Development and Function after HSCT.* Front Immunol, 2013. 4: p. 458.
423. Lee, J., et al., *Epigenetic modification and antibody-dependent expansion of memory-like NK cells in human cytomegalovirus-infected individuals.* Immunity, 2015. 42(3): p. 431-42.
424. Schlums, H., et al., *Cytomegalovirus infection drives adaptive epigenetic diversification of NK cells with altered signaling and effector function.* Immunity, 2015. 42(3): p. 443-56.
425. Wagner, B., et al., *A continuous sequence of more than 70 amino acids is essential for antibody binding to the dominant antigenic site of glycoprotein gp58 of human cytomegalovirus.* J Virol, 1992. 66(9): p. 5290-7.
426. Meyer, H., et al., *Glycoprotein gp116 of human cytomegalovirus contains epitopes for strain-common and strain-specific antibodies.* J Gen Virol, 1992. 73 (Pt 9): p. 2375-83.
427. Meyer, H., Y. Masuho, and M. Mach, *The gp116 of the gp58/116 complex of human cytomegalovirus represents the amino-terminal part of the precursor molecule and contains a neutralizing epitope.* J Gen Virol, 1990. 71 (Pt 10): p. 2443-50.

428. Silvestri, M., et al., *Characterization of a major antigenic region on gp55 of human cytomegalovirus.* J Gen Virol, 1991. 72 (Pt 12): p. 3017-23.
429. McLean, G.R., et al., *Recognition of human cytomegalovirus by human primary immunoglobulins identifies an innate foundation to an adaptive immune response.* J Immunol, 2005. 174(8): p. 4768-78.
430. Backovic, M., R. Longnecker, and T.S. Jardetzky, *Structure of a trimeric variant of the Epstein-Barr virus glycoprotein B.* Proc Natl Acad Sci U S A, 2009. 106(8): p. 2880-5.
431. Roche, S., et al., *Crystal structure of the low-pH form of the vesicular stomatitis virus glycoprotein G.* Science, 2006. 313(5784): p. 187-91.
432. Spindler, N., et al., *Characterization of a discontinuous neutralizing epitope on glycoprotein B of human cytomegalovirus.* J Virol, 2013. 87(16): p. 8927-39.
433. Wiegers, A.K., et al., *Identification of a neutralizing epitope within antigenic domain 5 of glycoprotein B of human cytomegalovirus.* J Virol, 2015. 89(1): p. 361-72.
434. Thomson, C.A., et al., *Germline V-genes sculpt the binding site of a family of antibodies neutralizing human cytomegalovirus.* Embo j, 2008. 27(19): p. 2592-602.
435. Schrader, J.W. and G.R. McLean, *Location, location, timing: analysis of cytomegalovirus epitopes for neutralizing antibodies.* Immunol Lett, 2007. 112(1): p. 58-60.
436. Ohlin, M., *A new look at a poorly immunogenic neutralization epitope on cytomegalovirus glycoprotein B. Is there cause for antigen redesign?* Mol Immunol, 2014. 60(2): p. 95-102.
437. Lantto, J., J.M. Fletcher, and M. Ohlin, *Binding characteristics determine the neutralizing potential of antibody fragments specific for antigenic domain 2 on glycoprotein B of human cytomegalovirus.* Virology, 2003. 305(1): p. 201-9.
438. Ishibashi, K., et al., *Lack of antibodies against the antigen domain 2 epitope of cytomegalovirus (CMV) glycoprotein B is associated with CMV disease after renal transplantation in recipients having the same glycoprotein H serotypes as their donors.* Transpl Infect Dis, 2011. 13(3): p. 318-23.

439. Ohizumi, Y., et al., *Neutralizing mechanisms of two human monoclonal antibodies against human cytomegalovirus glycoprotein 130/55.* J Gen Virol, 1992. 73 (Pt 10): p. 2705-7.
440. Atanasiu, D., et al., *Bimolecular complementation defines functional regions of Herpes simplex virus gB that are involved with gH/gL as a necessary step leading to cell fusion.* J Virol, 2010. 84(8): p. 3825-34.
441. Britt, W.J., et al., *Antigenic Domain 1 Is Required for Oligomerization of Human Cytomegalovirus Glycoprotein B.* Journal of Virology, 2005. 79(7): p. 4066-4079.
442. Finnefrock, A.C., et al., *Preclinical Evaluations of Peptide-Conjugate Vaccines Targeting the Antigenic Domain-2 of Glycoprotein B of Human Cytomegalovirus.* Hum Vaccin Immunother, 2016: p. 0.
443. Taylor-Wiedeman, J., et al., *Monocytes are a major site of persistence of human cytomegalovirus in peripheral blood mononuclear cells.* J Gen Virol, 1991. 72 (Pt 9): p. 2059-64.
444. Pawelec, G., et al., *Cytomegalovirus and human immunosenescence.* Rev Med Virol, 2009. 19(1): p. 47-56.
445. Gardner, T.J. and D. Tortorella, *Virion Glycoprotein-Mediated Immune Evasion by Human Cytomegalovirus: a Sticky Virus Makes a Slick Getaway.* Microbiol Mol Biol Rev, 2016. 80(3): p. 663-77.
446. Brinkmann, M.M., et al., *Cytomegalovirus immune evasion of myeloid lineage cells.* Med Microbiol Immunol, 2015. 204(3): p. 367-82.
447. Noriega, V., et al., *Diverse immune evasion strategies by human cytomegalovirus.* Immunologic Research, 2012. 54(1): p. 140-151.
448. Freeman, R.B., Jr., *The 'indirect' effects of cytomegalovirus infection.* Am J Transplant, 2009. 9(11): p. 2453-8.
449. Nieto, F.J., *Viruses and atherosclerosis: A critical review of the epidemiologic evidence.* American Heart Journal, 1999. 138(5, Supplement): p. S453-S460.
450. Dolcino, M., et al., *Infections and autoimmunity: role of human cytomegalovirus in autoimmune endothelial cell damage.* Lupus, 2015. 24(4-5): p. 419-32.
451. Stern, H. and S.M. Tucker, *Prospective study of cytomegalovirus infection in pregnancy.* Br Med J, 1973. 2(5861): p. 268-70.

452. Sylwester, A.W., et al., *Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory compartments of exposed subjects.* J Exp Med, 2005. 202(5): p. 673-85.
453. Jackson, S.E., G.M. Mason, and M.R. Wills, *Human cytomegalovirus immunity and immune evasion.* Virus Res, 2011. 157(2): p. 151-60.
454. Powers, C., et al., *Cytomegalovirus immune evasion.* Curr Top Microbiol Immunol, 2008. 325: p. 333-59.
455. Noriega, V., et al., *Diverse immune evasion strategies by human cytomegalovirus.* Immunol Res, 2012. 54(1-3): p. 140-51.
456. Finnefrock, A.C., et al., *Preclinical evaluations of peptide-conjugate vaccines targeting the antigenic domain-2 of glycoprotein B of human cytomegalovirus.* Hum Vaccin Immunother, 2016. 12(8): p. 2106-2112.
457. Plotkin, S.A. and P.B. Gilbert, *Nomenclature for immune correlates of protection after vaccination.* Clin Infect Dis, 2012. 54(11): p. 1615-7.
458. Weekes, Michael P., et al., *Quantitative Temporal Viromics: An Approach to Investigate Host-Pathogen Interaction.* Cell, 2014. 157(6): p. 1460-1472.

Appendix.

Royal Free Hampstead NHS
NHS Trust

Royal Free Local Research Ethics Commitee
Chief Executives office
Pond Street
London
Hampstead
London
NW3 2QG

04-107
Original LREC approval
(1st approval)

28 February 2002

Prof Paul D Griffiths
Department of Virology
The Royal Free & University Medical School
Pond Street
Hampstead
London
NW3 2QG

Dear Prof Griffiths

Evaluating a CMV Vaccine for Transplant Patients

Ethics Reference: 5476 (Please quote on ALL correspondence)

I refer to your recent application to the Ethics Committee regarding the above project and am pleased to inform you that the project was approved at the committee meeting on 27th February 2002 .

This approval is for one year from the date of this letter. We also require to be notified of the completion of the project and to be sent a copy of any subsequent publication. Extension of this period will be dependent on the submission of a brief synopsis of the progress of the project together with an estimation of the time required for its ultimate completion.

In addition we require that:

(a) You inform the committee immediately of any information received by yourself or of any information of which you become aware which would cast doubt upon, or alter, any information contained in the original application, or any amended later application, submitted to the committee which would raise questions about the safety and/or continued contact of the research. This would include the reporting of all "adverse events" of which you become aware. These "adverse events" should also be reported to the person who provided independent review of the original application.

(b) All those involved in the study appreciate the importance of maintaining confidentiality and that they comply with the Data Protection Act 1984.

(c) All proposed amendments to the protocol, that have a bearing on the treatment or investigation of patients or volunteers, are submitted to the committee for approval.

(d) The conduct of the study complies with good clinical research practice as outlined in the ICH GCP guidelines.

(e) A copy of the patient consent form and information sheet be lodged in the clinical notes.

Please note that ethical committee approval does not mean that the study may commence. The study

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may only commence following approval by the Trust through the office of the Director of Research & Development (please contact Zoe Spyvee on extn. 8304).

Yours sincerely [redacted]

[redacted]

Dr. Michael Pegg
Chairman
Royal Free Local Research Ethics Committee

Documents received:

Application form received	Yes
Consent form	Yes
Patient information sheet	Yes

NRES Committee London - Hampstead
Skipton House
Ground Floor, NRES/HRA
80 London Road
London
SE1 6LH

Telephone: 020 797 22580

17 June 2013

Prof Paul Griffiths
Professor of Virology
Royal Free Hospital Medical School
Dept Virology
Rowland Hill Street
NW3 2PF

Dear Prof Griffiths

Study title:	**Long term antibody response to CMV gB vaccine in patients requiring liver or renal transplant. A Phase II open, single-site study, in participants who received CMV gB vaccine or placebo in previous trial (CTA ref no 20363/0238/001-0010; REC ref no 5476; UCL sponsor no 05/009).**
REC reference:	**13/LO/0335**
Protocol number:	**12/0161**
EudraCT number:	**2012-002767-95**
IRAS project ID:	**112951**

Thank you for your letter of 07 May 2013, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

We plan to publish your research summary wording for the above study on the NRES website, together with your contact details, unless you expressly withhold permission to do so. Publication will be no earlier than three months from the date of this favourable opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to withhold permission to publish, please contact the Co-ordinator Hayley Fraser NRESCommittee.London-Hampstead@nhs.net

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation [as revised], subject to the conditions specified below.

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites listed in the application, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Non-NHS sites

The Committee has not yet been notified of the outcome of any site-specific assessment (SSA) for the non-NHS research site(s) taking part in this study. The favourable opinion does not therefore apply to any non-NHS site at present. We will write to you again as soon as one Research Ethics Committee has notified the outcome of a SSA. In the meantime no study procedures should be initiated at non-NHS sites.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

Clinical trial authorisation must be obtained from the Medicines and Healthcare products Regulatory Agency (MHRA).

The sponsor is asked to provide the Committee with a copy of the notice from the MHRA, either confirming clinical trial authorisation or giving grounds for non-acceptance, as soon as this is available.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Covering Letter	from Emily Rothwell	18 February 2013
Evidence of insurance or indemnity	Arthur J Gallagher International	30 July 2012
GP/Consultant Information Sheets	1.0	13 April 2012
Investigator CV	Prof Paul Griffiths	14 February 2013
Investigator's Brochure	14.0	14 November 2012
Letter from Sponsor	Gemma Jones-CT operations manager	15 February 2013

Letter from Statistician	Dr Colette Smith - UCL	12 February 2013
Letter of invitation to participant	1.0	14 February 2013
Other: Letter from David Wilson confirming UCL insurance		26 June 2012
Other: Copy of IRAS- NHS REC Application Form	Print Version	14 February 2013
Other: CMV gB vaccine long term antibody response Consent Form	1.0	20 July 2012
Participant Information Sheet	1.0	02 July 2012
Participant Information Sheet: Parts 1 and 2	V1.1, track changed	07 May 2013
Participant Information Sheet: Parts 1 and 2	1.1, clean copy	07 May 2013
Protocol	1.0	13 November 2012
REC application		08 March 2013
Response to Request for Further Information	Letter from Emily Rothwell	07 May 2013

Statement of compliance

This Committee is recognised by the United Kingdom Ethics Committee Authority under the Medicines for Human Use (Clinical Trials) Regulations 2004, and is authorised to carry out the ethical review of clinical trials of investigational medicinal products.

The Committee is fully compliant with the Regulations as they relate to ethics committees and the conditions and principles of good clinical practice.

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

13/LO/0335	Please quote this number on all correspondence

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

With the Committee's best wishes for the success of this project.

Yours sincerely

[redacted]

Miss Stephanie Ellis
Chair

Email: NRESCommittee.London-Hampstead@nhs.net

Enclosures: "After ethical review – guidance for researchers" *[SL-AR1]*

Copy to: *Adedayo Akinyemi, University College London*
Muhammad Rahman, R&D