Characterisation of immune responses to varicella vaccination in relation to clinical outcome

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DECLARATION:

I declare that the work presented in this thesis is my own.

Signed:

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ABSTRACT:

This thesis examines both humoral and cellular adaptive immune responses to varicella vaccination (up to 18 months post immunisation), in an ethnically diverse population of healthcare workers. Using two parameters of humoral immunity at six weeks post first vaccination; (avidity readings ≥60%, and a TRFIA reading ≥400mIU/mL) a cut-off of 130mIU/mL was defined for a more sensitive in house immuno assay (TRFIA), in this vaccinated adult population. Using these cut-offs, three patterns of antibody responses were identified; primary responders who seroconverted following vaccination, secondary responders who had pre-existing immunity and subjects who responded poorly to vaccination. Demographic and immunological characteristics of each subset were examined. An association between black ethnicity and lower antibody titre to vaccination in primary responders was identified, whilst Caucasians were more likely to have a history and pre-existing immunity, in keeping with the epidemiology of chickenpox in temperate climates. The follow-up study revealed that affinity maturation to VZV can take longer than 18 months in response to vaccination. At follow-up, 25% of subjects recruited at this time point were seronegative by TRFIA. Seroconversion after two doses of vaccine and a TRFIA titre of <500mIU/ml after two doses were significantly associated with waning antibody titre over time. Positive IFN-γ ELISPOT responses at 18 months did not necessarily correspond with TRFIA seropositive status.

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ABBREVIATIONS:

aa	Amino acid
A	Adenine
ACIP	Advisory committee on immunization practices
AEC	3-amino-9-ethyl-carbazole
Af	African
AID	Activation-induced deaminase
APC	Antigen presenting cell
APC	Allophycocyanin (fluorochrome)
ADCC	Antibody dependent cellular cytotoxicity
AP-1	Activator protein 1
BHV-1	Bovine herpes virus-1
BLT	Bart's and the London NHS Trust
BSA	Bovine serum albumin
BSA	Bystander activation
bp	Base pair
C	Cytosine
C	Constant region
Cauc	Cucasian
Cbn	Carribbean
CCR	Chemokine Receptor
CD	Cluster of differentiation
CD11a/CD18	See LFA-1
CD28	Expressed on T cells, costimulatory molecule, binds to B7.1 and B7.2
CD80	B7.1, a costimulatory molecule, binds to CD28 and CTLA-4
CD83	Involved in antigen presentation
CD86	B7.2, a co-stimulatory molecule, binds to CD28 and CTLA-4
CD95	See Fas
CD152	See CTLA-4
CDR	Complementarity-determining regions
CHO	Chinese hamster ovary
CIITA	MHC-II transactivator
CLA	Cutaneous leukocyte antigen
CMI	Cell-mediated immunity

CMV	Cytomegalovirus (human)
CPE	Cytopathic effect
CTL	Cytotoxic T lymphocyte
CTL	Clinical Transplant Laboratory, Bart's and the London
CTLA-4	Cytotoxic T-lymphocyte antigen 4 (CD152)
CXCL13	Chemokine (C-X-C motif) ligand 13
CXCR5	(C-X-C motif) receptor 5 also known as (Burkitt lymphoma receptor 1; BLR1)
D	Diversity gene segment
dATP	Deoxyadenine triphosphate
DC	Dendritic cell
dCTP	Deoxycytosine triphosphate
DELFIA	Dissociation-enhanced lanthanide fluorescent immunoassay
dGTP	Deoxyguanine triphosphate
DMF	N,N-Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DRG	Dorsal root ganglia
DTT	Dithiothreitol
dTTP	Deoxythymidine triphosphate
E (genes)	Early (genes)
EBV	Epstein-Barr virus
ECM	Extra cellular matrix
EDTA	Ethylenediaminetetraacetic Acid
EIA	Enzyme Immunoassay
eIF-2α	Eukaryotic initiation factor 2
EHV	Equine herpes virus
ELCHA	East London and the city health authority
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunosorbent spot assay
EM	Electron microscopy
ER	Endoplasmic reticulum
ERK	Extracellular Signal-Regulated Kinase
EU/mL	European units/mL
F	Forward (primer)

FAb	Antigen binding fragment of antibody
Fas	(CD95) Apoptosis inducing receptor; interacts with Fas ligand on activated T cells.
FAMA	Fluorescent antibody membrane antigen
FACS	Fluorescent activated cell sorter
FBS	Foetal bovine serum
Fc	Constant fragment of antibody
FcR	Fc receptors (FcαR binds IgA; FcγR bind IgG and so)
FDC	Follicular dendritic cell
FITC	Fluorescein isothiocyanate (fluorochrome)
G	Guanine
g	Gravitational force
gB	Glycoprotein B
gC	Glycoprotein C
GC	Germinal centre
gD	Glycoprotein D
gE	Glycoprotein E
gH	Glycoprotein H
gI	Glycoprotein I
gK	Glycoprotein K
gL	Glycoprotein L
gM	Glycoprotein M
gN	Glycoprotein N
GMT	Geometric mean antibody titre
GPEF	Guinea pig embryo lung fibroblast
GpELISA	Glycoprotein-based enzyme-linked Immunosorbent assay
GSK	Galaxosmithkline
GST	Glutathione-S-transferase
h	hour
H	Heavy chain (antibody)
HBV	Hepatitis B virus
HBV	Herpes B virus
HCMV	Human cytomegalovirus
HCV	Hepatitis C virus
HCW	Health care worker
HELF	Human embryo lung fibroblast
HFL	Human Foetal lung fibroblasts

HHV-1	Human herpes virus 1 (HSV-1)
HHV-2	Human herpes virus 2 (HSV-2)
HHV-3	Human herpes virus 3 (VZV)
HHV-4	Human herpes virus 4 (EBV)
HHV-5	Human herpes virus 5 (HCMV)
HHV-6	Human herpes virus 6
HHV-7	Human herpes virus 7
HHV-8	Human herpes virus 8 (KSHV)
HI	Heat Inactivated
HiB	*Haemaophilus Influenzae* B
HIV	Human immunodeficiency virus
HFDL	Human fibroblast diploid lung (cells)
HLA	Human leukocyte antigen
HNR	Vaccine humoral non-responder
HPV	Human papiloma virus
HRP	Horseradish peroxidase
HSV	Herpes simplex virus
HSPG	Heparan sulphate proteoglycan
HVS	Herpesvirus Saimiri
HZ	Herpes zoster
IE (genes)	Immediate early (genes)
ICAM-1	Intercellular adhesion molecule-1 (CD54)
ICFC	Intracellular cytokine flow cytometry
ICMS	Institute of Cell and Molecular Science
ICOS	Inducible costimulator
ICP	Infected cell protein (HSV protein nomenclature)
IDE	Insulin-degrading enzyme
IFN	Interferon
Ig	Immunoglobulin
IκB	Inhibitor of NFκB
IL	Interleukin
Ind (Ind Sub)	Indian subcontinent
IQR	Interquartile range
IRF-1	Interferon regulatory factor -1
IRL	Internal repeat long
IRS	Inverted repeat short
IU	International units

J	Joining segment
JAK	Janus kinases
JNK/SAPK	Jun NH_2-terminal kinase/stress-activated protein kinase
Kb	Kilo bases
KCl	Potassium chloride
KDa	Kilo Dalton
KOAc	Potassium acetate
KSHV	Kaposi sarcoma herpes virus
L (genes)	Late (genes)
L	Light chain (antibody)
LA	Latex agglutination
LAT	Latency associate transcript
LFA	Leukocyte function associated antigen
LPA	Lymphocyte proliferation assay
M	Molar
mAb	Monoclonal antibody
Man 6-P	Mannose-6-phosphate
MAPK	Mitogen-activated protein kinase
MCMV	Murine cytomegalovirus
MDV	Marek's Disease Virus
MEM	Minimum Essential (Eagles) Medium
MeWo	Melanoma cell line
MFI	Medain/mean fluorescence intensity
$MgCL_2$	Magnesium chloride
$Mg(OAc)_2$	Magnesium acetate
MHC	Major histocompatability complex
MIP-3β	Macrophage inflammatory protein-3β
mIU/mL	Mili international unit/mililitre
mL	Mililitre
mM	Millimolar
MMR	Measles, Mumps, Rubella (vaccine)
MPR	Mannose 6-phosphate receptor
MRC_5	Human lung fibroblast cell line
mRNA	Messenger ribonucleic acid
NA	Not applicable
NHS	National Health Service
NFκB	Nuclear Factor κB

ng	Nanogram
NI	Negative-immune (Negative baseline TRFIA, secondary responder)
NK	Natural killer cell
NLR	Negative (TRFIA baseline) Low Responder
NSC1	Negative (TRFIA baseline) Seroconverted after one dose of vaccine
NSC2	Negative (TRFIA baseline) Seroconverted after two doses of vaccine
nm	Nanometer
OBP	Origin of replication binding protein
Oct-1	Octamer-binding protein 1
OD	Optical density
ORF	Open reading frame
Ori_s	Origin of replication (short)
p38/MAPK	p38 mitogen-activated protein kinase
PACS-1	Phosphofurin acidic cluster sorting protein 1
P'Blue	Pacific Blue (fluorochrome)
PBMC	Peripheral blood mononuclear cell
PBG	Peptide binding groove
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCT	Primary care trust
PE	Phycoerythrin (fluorochrome)
PerCP	Peridinin-chlorophyll-protein (fluorochrome)
pfu	Plaque forming unit
PHA	Phytohaemoglutinin (T cell mitogen)
Phil	Filipino
PHLS	Public health laboratory service
PHN	Postherpetic neuralgia
pi	Post infection
PI	Positive (TRFIA baseline) Immune (secondary responder)
PKR	Double-stranded RNA-activated protein kinase
PLR	Positive (TRFIA baseline) Low Responder
PM	Plasma membrane
PMSF	Phenylmethanesulphonylfluoride
POka	Parental Oka strain of VZV

PRV	Pseudorabies virus
R	Reverse (primer)
R	Retained (TRFIA ≥130mIU/mL at 12 weeks and 18 months)
RAG	Recombination activating
RAI	Relative avidity index
RANTES	Regulated on activation normal T cell expressed and secreted (chemokine)
RCF	Responder cell frequency
RER	Rough endoplasmic reticulum
RNA	Ribonucleic acid
ROC	Receiver (or relative) operating characteristic
ROVE	Response to Oka vaccine evaluation (study)
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RT	Room Temperature
R1-5	Repeat region 1-5
SAPE	Streptavidin (SA)/phycoerythrin (PE)
SC	Seroconverted
SCID-Hu	Severe combined immunodeficient mouse with human xenograft
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SER	Smooth endoplasmic reticulum
SFUs	Spot forming units
SHM	Somatic hypermutation
SIEVE	Society of independent European vaccination experts
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
Sp1	Specificity factor 1
STAT	Signal transducers and activators of transcription
SVV	Simian varicella virus
T	Thymine
Tc	Cytotoxic T cell
T_{CM}	Central memory T cell
TCR	T Cell Receptor
TGN	Trans-Golgi network

TEM	Transmission electron microscopy
T_{EM}	Effector memory T cell
T_{FH}	$CD4^+$ T follicular helper cell
Th1	$CD4^+$ T helper 1 cell
Th2	$CD4^+$ T helper 2 cell
TK	Thymidine kinase
TLR	Toll like receptor
TNFα	Tumour Necrosis Factor α
T_{reg}	T regulatory cell
TRFIA	Time resolved fluorescence immunoassay
TRL	Terminal repeat long
TRS	Terminal repeat short
U	Uracil
U_L	Unique long (also used for HSV protein nomenclature)
U_S	Unique short
USA	United States of America
USF	Upstream stimulatory factor
UV	Ultraviolet
V	Variable region
V1	Visit 1 of the ROVE study (baseline)
V2	Visit 2 of the ROVE study (six weeks post first dose of vaccine)
V3	Visit 3 of the ROVE study (12 weeks post 1st dose, six weeks post second dose of vaccine)
V4	Visit 4 of the ROVE study (approximately 18 month follow up, post first dose)
VCAM	Vascular cell adhesion molecule
Visit 1	See V1
Visit 2	See V2
Visit 3	See V3
Visit 4	See V4
VLP	Virus like particle
VOka	Oka vaccine virus
VP	Virion Protein (HSV protein nomenclature)
VRE	Vaccine related event
VTM	Viral transport medium
VZIG	Varicella zoster immune globulin

VZV	Varicella zoster virus
WFS	Withdrew from study
WT	Wild type
7AAD	7-aminoactinomycin D (fluorochrome)

CHAPTER 1: INTRODUCTION

1.1 Varicella Zoster Virus in Context:

Varicella zoster virus (VZV) is a highly contagious ubiquitous human pathogen that causes varicella (chickenpox) upon primary infection. In temperate climates approximately 90% of the population are seropositive by 10-14 years of age (Vyse *et al.,* 2004; Waclawski *et al.,* 2002; Vandersmissen, *et al.,* 2000; Nardone, 2003). The virus establishes life long latency in dorsal root ganglia and in around 25% of infected individuals will later reactivate along the ganglia to cause herpes zoster (shingles). About 15% of all zoster patients experience postherpetic neuralgia (PHN); defined as the dermatomal pain associated with shingles persisting beyond one month after resolution of the rash.

1.2 Classification and Virus Structure:

Varicella zoster virus (VZV), also known as human herpesvirus 3 (HHV3), is one of eight members of the herpesviridae family known to infect humans. The family is classified into three subfamilies *alphaherpesviriae*, *betaherpesviriae* and *gammaherpesviriae* (figure 1.1 and table 1.1). Members of the *alphaherpesviriae* subfamily infect birds, reptiles, amphibians, molluscs and mammals, whereas *betaherpesvirinae* and *gammaherpesviriae* are members are only known to infect mammalian hosts.

Classification of the *Herpesviridae* family was originally based on the biological characteristics of members of the same subfamilies; in particular their sites of latency. Alphaherpesviruses are characterized by establishment of latency in host ganglia; betaherpesviruses in host haematopoietic cells and gammaherpesviruses in host lymphocytes, (Winkler *et al.,* 2000; Baxi *et al.,* 1995; Borchers *et al.,* 1999; Welch *et al.,* 1992; Kennedy *et al.,* 2004; Romero *et al.,* 2003; Croen *et al.,* 1988; Huff and Barry, 2003; Hahn *et al.,* 1998; Koffron *et al.,* 1998; Kondo *et al.,* 2002; Kempf *et al.,* 1998; MacSween *et al.,* 2003; Tibbetts *et al.,* 2003 and

Schulz, 2000; Cohen *et al.,* 2007b). Gene content and sequence similarities are now also used to define classification (Roizman, 1992).

VZV is a member of the alphaherpesvirus subfamily, which is further characterized by a relatively short reproductive cycle, rapid cell-to-cell spread and cytolysis (Mori and Nishiyama, 2005; Arvin, 2001c; Cohen *et al.,* 2007b). Based on phylogenetic analysis of conserved herpesvirus genes the mammalian alphaherpesvirus subfamily is delineated as α1 and α2 lineages, corresponding to the genera simplexvirus and varicellovirus, respectively (McGeoch, 1989; McGeoch *et al.,* 1995; McGeoch *et al.,* 2000), (see table 1.1).

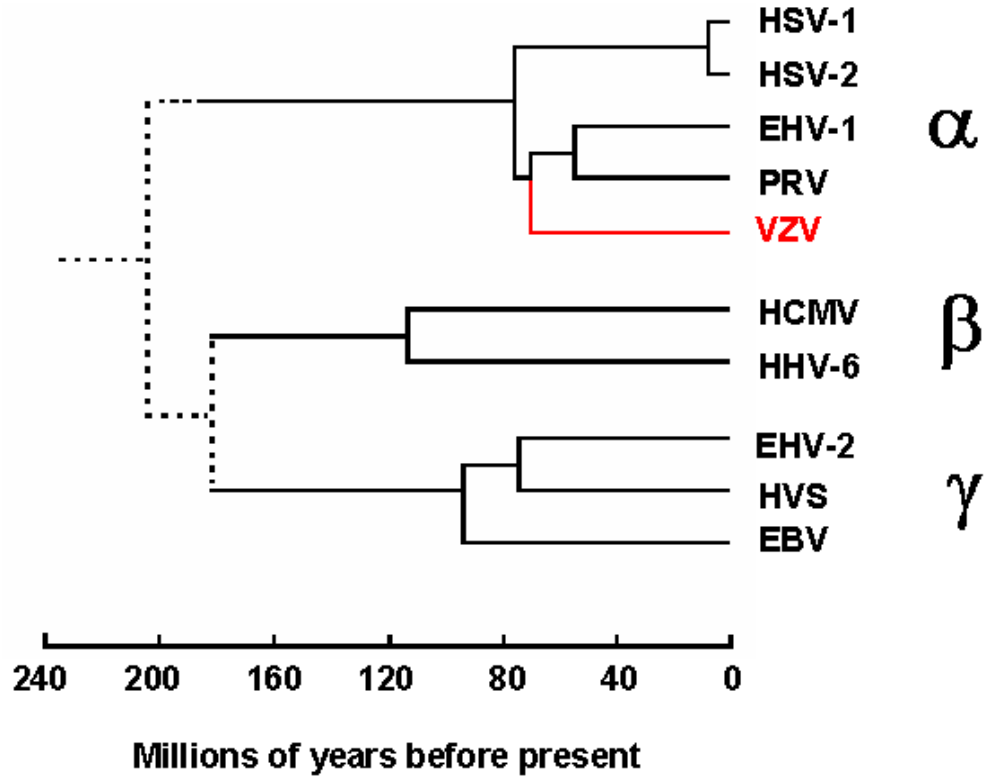


Figure 1.1: Phylogenetic Tree for Selected Mammalian Herpesviruses.
This was deduced by McGeoch *et al.,* (1995) from the combined amino acid sequences encoded by several well conserved genes. A proposed timescale is shown inferred from host palaeontology. The oldest part of the tree is shown as a broken line, indicating lower confidence. Key: HSV-1, herpes simplex virus 1; HSV-2, herpes simplex virus 2; HCMV, human cytomegalovirus; HHV-6, human herpes virus 6; HVS, herpesvirus saimiri; EBV, Epstein-Barr virus (Adapted from Davison, 2000; with permission).

Sub Family	Genus and Lineage	Species	Other Names
Alphaherpesviriae	Simplexvirus (α1 lineage)	Human Herpesvirus 1 (HHV1)	Herpes Simplex Virus 1 (HSV-1)
		Human Herpesvirus 2 (HHV2)	Herpes Simplex Virus 2 (HSV-2)
		Bovine Herpesvirus 2 (BHV2)	Bovine Mamillitis Virus, Allerton Virus, Pseudolumpy Skin Disease Virus
		Herpes B Virus (HBV)[a]	Cercopithecine Herpesvirus 1 Herpes Simiae virus 3
	Varicellovirus (α2 lineage)	Human Herpesvirus 3 (HHV3)	Varicella-Zoster Virus (VZV)
		Bovine Herpesvirus 1 (BHV1)	Infectious Bovine Rhinotracheitis Virus
		Equid Herpesvirus 1 (EHV1)	Equine Abortion Herpesvirus
		Equid Herpesvirus 4 (EHV4)	Equine Rhinopneumonitis Virus
		Simian Varicella Virus (SVV)	
		Pseudorabies Virus (PRV)	Suid Herpes Virus 1 Aujeszky's Disease Virus
Betaherpesviriae	Cytomegalovirus (CMV)	Human Herpesvirus 5 (HHV5)	Human Cytomegalovirus (HCMV)
	Muromegalovirus	Mouse Cytomegalovirus 1 (MCMV1)	Murid Herpesvirus
	Roseolovirus	Human Herpesvirus 6A (HHV6A)[b]	
		Human Herpesvirus 6B (HHV6B)[b]	
		Human Herpesvirus 7 (HHV7)	
Gammaherpesviriae	Lymphocryptovirus	Human Herpesvirus 4 (HHV4)	Epstein-Barr Virus (EBV)
	Radinovirus	Human Herpesvirus 8 (HHV8)	Kaposi's Sarcoma-Associated Herpesvirus

Table 1.1: Classification of the Eight Human Herpes Virus, Along with Some of the Most Common Mamalian Herpesviruses. *NB*: [a]HBV which is endemic in macaque monkeys is the only one of 35 identified non-human primate herpes viruses that is highly pathogenic for humans (Whitley and Hillard, 2007). [b]Although not formally recognized as such, the HHV-6 variants (HHV-6A and HHV-6B) satisfy the requirements for recognition as separate herpes viruses (Dominguez *et al.,* 1999). (Adapted from Quinlivan and Breuer, 2006; with permission).

Varicella-zoster virus is the only member of the varicellovirus genus that infects humans (Davison, 2000). Other members of this genus include bovine herpesvirus 1 (BHV1), equid herpesvirus 1 (EHV1), equid herpesvirus 4 (EHV4), simian varicella virus (SVV) and pseudorabies virus (PRV). The simplexvirus genus consists of human herpesvirus 1 and 2 (HHV1, HHV2) (otherwise known as herpes simplex virus 1 and 2 (HSV-1, HSV-2)), bovine herpesvirus 2 (BHV2) and herpes B virus (HBV), (table 1.1).

1.2.1 Structure and Morphology of the Varicella Zoster Virus:

The VZ virus is approximately 175nm in diameter (Nagler and Rake, 1948; Rake *et al.,* 1948). VZV, like all herpesviruses, consists of four major elements: The core, nucleocapsid, tegument and envelope (see figure 1.2). The core contains one copy of the linear double stranded DNA genome (approximately 125kb) (Davidson and Scott, 1986), which is incorporated into each virion. This has been described as a loose fibrillar cage of strands surrounding a dense cylindrical core of DNA fibers (Puvion-Dutilleul *et al.,* 1987). The nucleocapsid is an assemblage of 162 capsomeres with a 5:3:2 axial symmetry in which pentameric proteins form the vertices of an 80-120nm icosahedron and hexameric elements comprise its facets (Almeida *et al.,* 1962). The tegument is an amorphous protein material that bridges the nucleocapsid and the envelope. The tegument also includes enzymes controlling virus replication and immediate early proteins, (IE proteins) encoded by open reading frame (ORF) ORF4, ORF62 and ORF63 (Kinchington *et al.,* 1992) as well as numerous other proteins (see table 1.2). The nucleocapsid assembles within patches of host cellular membranes, that have been modified to display virally encoded glycoproteins (in order of abundance: gE, gB, gH, gI, gC, and gL) to form its envelope (Asano *et al.,* 1980a). VZV encodes nine membrane glycoproteins, which in addition to those listed above include gK, gM and gN, (Davison and Scott 1986, Davison *et al.,* 1986; Grose, 1990; Gomi *et al.,* 2002; Storlie *et al.,* 2008; Yamagishi *et al.,* 2008). These three glycoproteins have not been fully characterised, but recently gM has been shown to be expressed in the viral envelope

(Yamagishi *et al.,* 2008), whilst gK is postulated to be incorporated into the virion envelope, although this is yet to be experimentally determined (Hall *et al.,* 2007).

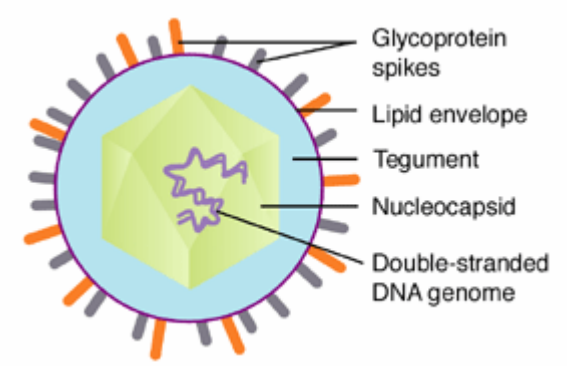


Figure 1.2: Structure of the Varicella Zoster Virus Particle.
Note: glycoproteins are of various lengths. (Quinlivan and Breuer, 2005; reprinted with permission).

1.3 Genomic Structure and Organisation:

The complete genome of the Dumas strain of VZV, which was isolated in the Netherlands, was determined in 1986 by Davison and Scott. VZV has the smallest genome of all the human herpesviruses; the virus is comprised of 72 ORFs, of which at least 69 are unique. These 72 ORFs actually encode only 71 genes as the transcripts of ORFs 42 and 45 are spliced and thus translate into a single protein product (Davison and Scott, 1986). The genome is a linear double stranded DNA molecule, 124,884bp in length.

The genome consists of a unique long region (U_L) of 104,836bp which is flanked by the inverted repeat regions: The terminal repeat long (TRL) and internal repeat long (IRL) both of which are 88.5bp in length; and a unique short region (U_S) of 5,232bp. This in turn is flanked by the internal repeat regions terminal repeat short (TRS) and the inverted repeat short (IRS), both of which are 7,319.5bp in length, (see figure 1.3). The inverted repeat structure of the VZV genome has been verified using electron microscopy of VZV DNA molecules (Ecker and Hyman, 1982; Gilden *et al.,* 1982b; Straus *et al.,* 1982). The viral genome is usually present as a linear molecule within the virion, but there are four isotypes; of which two

predominate (Kinchington *et al.,* 1985; Straus *et al.,* 1981; Davison 1984) (see figure 1.4).

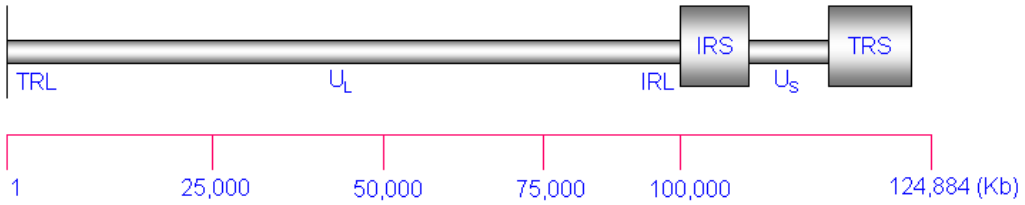


Figure 1.3: A Schematic of the VZV Genome. Key: TRL-Terminal repeat long, U_L-unique long, IRL-Internal repeat long, IRS-inverted repeat short, U_S-unique short region, TRS-terminal repeat short. (Adapted from Cohen *et al.,* 2007, with permission).

The genome contains five regions with repeat elements R1-R5 (Davison and Scott, 1986; Hondo and Yogo, 1988); see figure 1.5. R1-R4 are guanine-cytosine (G-C) rich and R1, 2 and 3 regions are located in U_L segments of genomes. The R1 region is located within ORF11 and consists of four separate repeated elements (Kinoshita *et al.,* 1988). The R2 region, present within the glycoprotein C gene consists of 42bp repeats (which differ among different strains of VZV) (Kinchington *et al.,* 1986). The R3 region is the largest repeat region, about 1,000bp in length and encoded by ORF22 (Davison, 1984). The R4 region is located in both IRS and TRS regions between ORFs 62 and 63 and ORFs 70 and 71 and consists of multiple 27bp elements (Casey *et al.,* 1985). Finally the R5 region is located between ORFs 60 and 61 and consists of 88 and 24bp repeats and is adenine-thymine (A-T) rich (Hondo and Yogo, 1988). The Dumas strain of VZV has only one copy of the R5 repeats whereas other strains of VZV have multiple R5 repeats. Indeed the R1-5 repeats differ in length and have been used to distinguish different strains of VZV by restriction endonuclease analysis (Hayakawa *et al.,* 1986; Hondo and Yogo, 1988; Straus *et al.,* 1983). The variability in the number of repeats implies that the different strains of VZV have differing overall lengths of their genome.

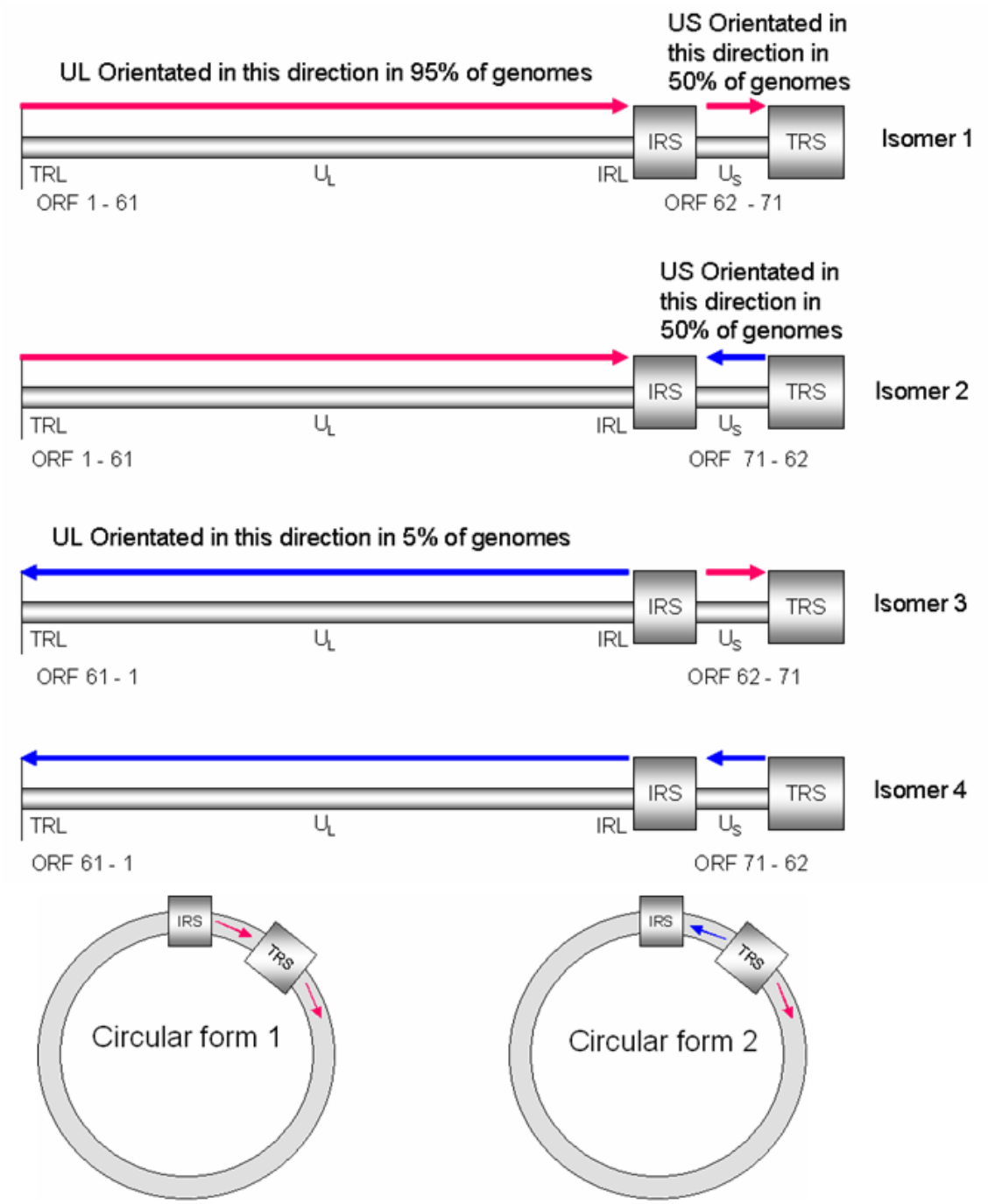


Figure 1.4: VZV Isomers. Two linear isomeric forms predominate; circularized forms are rare. The direction of the U_S and U_L sequences differs between isotypes. Each end of the linear genome has a single unpaired complementary nucleotide (C-G), which may base pair to form circular isoforms.

As mentioned previously, the genome contains at least 69 unique genes, three of which are present in two copies in the IRS and TRS regions (see figure 1.5). Eleven of the VZV genes are thought to have overlapping reading frames (Davison and Scott, 1986).

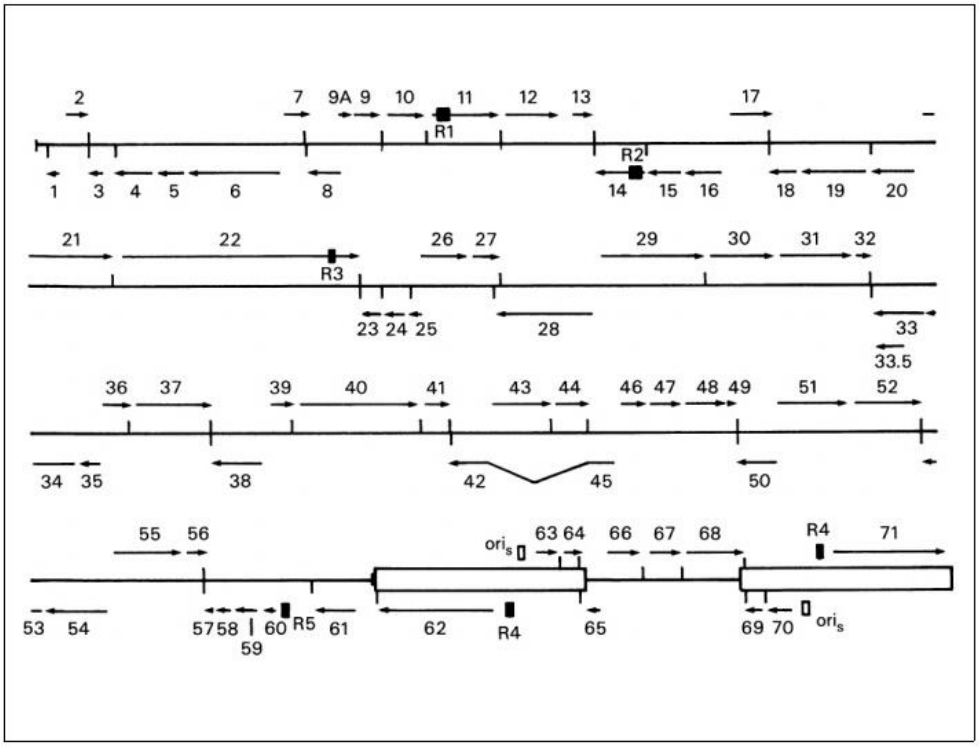


Figure 1.5: VZV Open Reading Frames (ORFs). ORF transcripts are numbered and shown as arrows. ORFs 69 to 71 are duplications of ORFs 62 to 64. Repeats (R1-R5) are indicated by filled rectangles. The origins of DNA replication (ori$_s$ are shown.) (Reprinted from Cohen *et al.,* 2007b, with permission).

A large portion of the VZV genome is colinear with HSV-1 genome (see table 1.2). Only five VZV genes in the U_L regions do not have HSV-1 homologues: ORFs 1, 2, 13, 32 and 57. Four ORFs (1, 2, 32 and 57) have homologues in equine herpesvirus 1 (Telford *et al.,* 1992) whilst 29 VZV genes have EBV homologues (Baer *et al.,* 1984; McGeoch and Davison, 1986; Davison and Taylor, 1987). These conserved genes include transcriptional, regulatory, viral enzyme and glycoprotein genes (Davison and Taylor, 1987). The gene which is encoded by ORF13 is the viral enzyme thymidylate synthase, which is not present in any other alphaherpesvirus (Honess *et al.,* 1986, Richter *et al.,* 1988).

Gene	Function	HSV-1 homologue
1	Membrane protein	No homologue
3	Unknown function	UL55
4	Transcriptional activator, multifunctional regulatory protein ♦ ♦ ♦	UL54
5	Glycoprotein K (gK) ♦	UL53
6	DNA replication; DNA helicase/primase component	UL52
7	Myrisolated protein ♦	UL51
8	Nucleotide metabolism; Deoxyuridine triphosphatase (dUTPase)	UL50
9	Complexes with gE, Interacts with IE62 ♦	UL49
9A	Stearoylated membrane protein (syncytia formation) (gN)	UL49A
10	Transcriptional activator ♦	UL48
11	Unknown ♦	No homologue
12	Unknown ♦	UL46
13	Thymidylate synthetase	No homologue
14	Glycoprotein C (gC) ♦	UL44
15	Integral membrane protein	UL43
16	DNA replication; Associated with DNA polymerase	UL42
17	Host shut-off	UL41
18	Ribonucleotide reductase small subunit	UL40
19	Nucleotide metabolism; Ribonucleotide reductase large subunit	UL39
20	Minor capsid binding protein	UL38
21	Associated with developing nuclearcapsid ♦ ♦	UL37
22	Large tegument protein ♦	UL36
23	Small capsid binding protein, Hexon tips	UL35
24	Role in nuclear egress	UL34
25	DNA packaging protein (putative)	UL33
26	Capsid protein; DNA packaging protein (putative)	UL32
27	Nuclear maturation, capsid nuclear egress	UL31
28	DNA replication; DNA polymerase	UL30
29	DNA replication; Single stranded DNA binding protein ♦	UL29
30	Virion maturation; DNA cleveage; terminase subunit homologue	UL28
31	Glycoprotein B (gB) ♦	UL27
32	Capsid scaffold	No homologue
33	Capsid scaffold protease	UL26
33.5	Scaffold/Assembly protein	UL26.5
34	Capsid associated tegument protein; DNA packaging protein (putative)	UL25

Table 1.2: Genes of VZV to Which a Function has Been Assigned, (continued; 1 of 2)

Gene	Function	HSV-1 homologue
35	Nuclear protein, role in cell-to-cell fusion	UL24
36	Pyrimidine deoxyribonucleotide kinase	UL23
37	Glycoprotein H (gH)♦	UL22
38	Unknown♦	UL21
39	Virion egress, Co-localizes with gK, suggested role in envelopment	UL20
40	Major nucleocapsid antigen♦	UL19
41	Minor capsid protein	UL18
42	DNA packaging protein; terminase subunit homologue	UL15
43	DNA packaging protein♦	UL17
44	Unknown♦	UL16
45	Terminase subunit homologue	
46	Myristoylated protein ♦	UL14
47	Protein kinase ♦	UL13
48	Virion maturation; Deoxyribonuclease	UL12
49	Myristoylated protein ♦	UL11
50	Glycoprotein M (gM)	UL 10
51	Origin of replication (ori) binding protein	UL9
52	DNA replication; Component of DNA helicase/primase	UL8
53	Tegument protein ♦	UL7
54	Minor capsid protein; DNA packaging protein (putative)	UL6
55	DNA replication; Component of DNA helicase/primase	UL5
56	Unknown function	UL4
57	Virion egress♦	No homologue
58	Unknown	UL3
59	Nucleotide metabolism; Uracil-DNA glycosylase	UL2
60	Glycoprotein L (gL)♦	UL1
61	Transcriptional activator/repressor♦	ICPO
62, 71	Transcriptional activator ♦♦♦	ICP4
63, 70	Transcriptional activator ♦♦♦	US1
64	Unknown♦	US10
65	Membrane protein, axonal transport;	US9
66	Protein kinase ♦	US3
67	Glycoprotein I (gI)♦	US7
68	Glycoprotein E (gE)♦	US8

Table 1.2: Genes of VZV to Which a Function has Been Assigned. (2 of 2)

Key: immediate early protein; early protein; late protein, class unknown, (virion-associated protein ♦), (tegument protein; ♦), (transcribed during latency♦), (McDonald and Breuer, 2008; adapted with permission).

1.4 Functions and Properties of Key VZV Proteins:

This section summarizes what is known about the function of some of the VZV proteins which are known to be of biological importance. For some VZV proteins, functional studies have been carried out, whilst for others, functions have been extrapolated from what is known of their HSV homologues. Table 1.3 summarises some of the main characteristics of these proteins. For some proteins, little else is known besides these main characteristics and thus these proteins are not discussed further in the text. Whether VZV proteins are essential for infection and replication (either *in vitro* or in the SCID-Hu mouse model), or the establishment of latency (in the cotton rat model) is discussed in section 1.6.1.2 and section 1.6.2.1.

1.4.1 Glycoproteins and Structural Proteins:

VZV glycoproteins, like their HSV-1 homologues, are involved in virion attachment, entry, envelopment, cell-to-cell spread, and egress (Cohen *et al.,* 2007b), discussed in section 1.5. Glycoprotein B, the second most abundant glycoprotein, is important for entry into the cell (Keller *et al.,* 1986) and binds cell surface heparan sulphate (Jacquet *et al.,* 1998). This glycoprotein, (along with gC, gE and gH); is a type-1 membrane protein which contains a signal peptide. This particular protein is a heterodimer, linked by disulphide bonds and has been shown to have great fusogenic properties in the presence of gE (Maresova *et al*., 2001). The DNA sequence for gB encodes for a polypeptide of 868aa residues, comprising as mentioned above; an 8aa signal peptide, the main body of the protein, and a hydrophobic anchor region (aa 699-743), followed by a positively charged C-terminal domain (Keller *et al.,* 1986). Two tyrosine based motifs within the cytoplasmic domain of gB are required for endocytosis into the Golgi network (Heineman and Hall, 2001; Heineman *et al.,* 2000), a process during which gB associates with clathrin, (Pasieka *et al*., 2003a), discussed in more detail in section 1.5.1. It is the 36aa at the carboxy-terminus of gB that are required for normal trafficking of this glycoprotein to the golgi and for normal viral egress (Heineman and Hall, 2002).

Gene Classification IE, E, L	ORF	Name	Size (kDa)	HSV-1 Homologue	Number of aa	Transcribed During Latency	Location within virus	Additional References
IE	4	IE4	51	ICP27	452	Yes	Tegument	Cohen *et al.,* 2005b; Sadzot-Delvaux and Rentier, 2001
	61	IE61	62-65	ICP0	467		Not tegument	Stevenson *et al.,* 1992
	62/71	IE62	175	ICP4	1,310	Yes	Tegument	Cohrs and Gilden, 2006
	63/70	IE63	45	US1.5 is expressed colinearly with ICP22 (US1)	278	Yes	Tegument	Kennedy *et al.,* 2000, Lungu *et al.,* 1998, Mahalingam *et al.,* 1996
E	29	DNA binding protein	130	ICP8 (UL29)	1,204	Yes		Cohen *et al.,* 2007a
	47	Serine/threonine protein kinase	54	UL13	510		Tegument	Kinchington *et al.,* 1992
	66	Serine/threonine protein kinase	48	US3	393	Yes	Tegument	

Table 1.3: Summary of Properties of Some Key VZV Proteins. (1 of 2)

Key: NR; non-reducing conditions, R; reducing conditions, IE; immediate early, E; Early, L; Late, NCP; Nuclear capsid protein, abbreviations in parentheses are the former names of glycoproteins. Referenced from Davison and Scott, 1986.

Gene Classification IE, E, L	ORF	Name	Size (kDa)	HSV-1 Homologue	Number of aa	Transcribed During Latency	Location within virus	Additional References
L	5	gK	40	UL53	340		Postulated as envelope	Govero *et al.,* 2007; Mo *et al.,* 1999
	10		50	VP16 (UL48)	410		Tegument	Moriuchi *et al.,* 1993b
	14	gC (gpV)	80-170 (Dependent on strain)	UL44	560		Envelope	Kinchington *et al.,* 1990; Kinchington *et al.,* 1986; Ling *et al.,* 1991
	31	gB (gpII)	20-140 (NR) 60-70 (R)	UL27	868		Envelope	Keller *et al.,* 1986
	37	gH (gpIII)	118	UL22	841		Envelope	Keller *et al.,* 1987
	40	NCP	155	UL19	1,396		Capsid	
	50	gM	20	UL10	435		Envelope	Yamagishi *et al.,* 2008
	60	gL (gpVI)	20	UL1	159		Envelope	
	67	gI (gpIV)	62	US7	354		Envelope	
	68	gE (gpI)	98	US8	623		Envelope	

('Properties of Some Key VZV Proteins') continued 2 of 2

The size of glycoprotein C ranges between VZV strains from 80–170kDa, which as mentioned above, is due to the different number of R2 repeat units located in the coding sequence of this protein. The amount of mRNA transcribed, along with the level of protein expressed within infected cells also varies depending on the strain of VZV (Kinchington *et al.,* 1990; Kinchington *et al.,* 1986; Ling *et al.,* 1991).

The most abundantly produced glycoprotein in VZV infected cells is gE (Montalvo *et al.,* 1985). Expression of this glycoprotein in epithelial cells induces formation of tight junctions whilst enhancing establishment of trans-epithelial resistance, which results in increased cell-to-cell contact (Mo *et al.,* 2002). This glycoprotein has been shown to bind to the Fc fragment of IgG (Edson *et al.,* 1985a; Litwin *et al.,* 1992; Litwin *et al.,* 1990; Yao *et al.,* 1993b) and is known to form a complex with gI by a non-covalent link (Vafai *et al.,* 1989; Yao *et al.,* 1993a). Glycoprotein I is required for normal maturation of gE and for efficient distribution of gE to the cell surface. A study by Kimura (*et al.,* 1997) demonstrated that the entire gE:gI complex could be immunoprecipitated using monoclonal antibodies to either gE or gI and this complex requires the amino terminus of gI for its interaction (Kimura *et al.,* 1997); along with residues 168-208 in the cysteine-rich region of the gE ectodomain (Berarducci *et al.,* 2009). The unique N-terminal region of gE is required for viral replication, cell-to-cell spread and secondary envelopment (Berarducci *et al.,* 2006). This region also binds to the VZV receptor insulin-degrading enzyme (IDE) (Li *et al.,* 2007). The C-terminal is essential for TGN and plasma membrane trafficking (Moffat *et al.,* 2004). Recently gE has been shown to form a complex with ORF9 protein *in vitro* (Che *et al.,* 2008).

Glycoprotein H is the third most abundant VZV glycoprotein and unlike other VZV glycoproteins, is secreted into the medium of cells in culture (Shiraki and Takahashi, 1982). This glycoprotein associates with gL to form the gH:gL complex (Forghani *et al.,* 1994), which allows processing in the Golgi network and subsequent transport to the cell surface (Duus *et al.,* 1995). VZV gH is a fusogen that is important for cell-to-cell spread of

the virus (Rodriquez *et al.,* 1993); gE enhances fusion mediated by gH (Maresova *et al.,* 2001), and these glycoproteins can also form a complex in virus infected cells (Maresova *et al.,* 2005; Pasieka *et al.,* 2004). VZV glycoprotein H has two hydrophobic domains, one at the N-terminus and the other at the C-terminus (Keller *et al.,* 1987). Like gB, gH endocytosis is clathrin dependent (Pasieka *et al*., 2003a).

Glycoprotein K is conserved among alphaherpesviruses but the highly hydrophobic nature of the protein has made it difficult to study and thus it has not been fully characterized. This glycoprotein is a multiply inserted type-3 membrane protein which consists of four transmembrane domains, containing a signal peptide (Govero *et al.,* 2007). It is found in enveloped virions and is evenly distributed in the cytoplasm in infected cells (Mo *et al.,* 1999). During infection, the ORF39 protein and gK tightly co-localize with VZV envelope glycoproteins B, E and H (Govero *et al.,* 2007). The half-life of gK is considerably shorter than that of other VZV glycoproteins including gB, gE and gH (Hall *et al.,* 2007).

Knowledge of glycoprotein M is limited, but recent studies using gM deletion mutants in MRC_5 cells demonstrated that this protein was expressed on the viral envelope, and functions in virus cell-to-cell spread (Yamagishi *et al.,* 2008).

The VZV ORF10 protein is a structural component of the viral tegument (Moriuchi *et al.,* 1993b). Following transfection into cells it is expressed diffusely in the cytoplasm, but is localized to the TGN with either co-transfection of gI or after viral infection (Wang *et al.,* 2001). VZV ORF10 transactivates the IE VZV ORF62 gene promoters in transient expression assays but is unable to transactivate the VZV IE ORF4 and ORF61 promoters (Che *et al.,* 2007).

1.4.2 Proteins Involved in Replication:

The ORF4 protein is a transcriptional activator that influences several promoters from each of the three classes of genes (Defechereux *et al.,* 1993). The ORF4 protein transactivates expression of its own promoter as well as VZV IE promoters ORF61, ORF62, early promoters ORF29, ORF36 and late promoter ORF67. ORF4 does not transactivate expression of IE ORF63 or early ORF28 promoters, (Defechereux *et al.,* 1996; Defechereux *et al.,* 1997). The ORF4 protein works cooperatively with ORF62 to function at both transcriptional and post-transcriptional levels (Schoonbroodt *et al.,* 1996). Furthermore, in transient expression assays ORF4 synergizes with ORF62 to transactivate expression of IE promoters (ORF4, ORF61), early promoters (ORF36) and late promoters (ORF14, ORF67 and ORF68) (Moriuchi *et al.,* 1994a). The ORF4 protein has recently been shown to be essential *in vitro* (Zhang *et al.,* 2007) and have an important role in the establishment of latency (Cohen *et al.,* 2005b; Sadzot-Delvaux and Rentier, 2001).

The ORF29 protein promoter binds to the gI promoter *in vitro* (Boucaud *et al.,* 1998). The ORF29 protein is secreted from VZV infected cells *in vitro*, where it can be endocytosed by neurons (Annunziato *et al.,* 2000). Absence or over-expression of the ORF29 protein impairs late gene expression and reduces latency in a rodent model (Cohen *et al.,* 2007a). BAG3, Hsp70/Hsc70 and Hsp90 (cytosolic molecular chaperones which prevent protein misfolding and aggregation) co-localize with the ORF29 protein in nuclear transcription/replication factories during lytic replication of VZV and this interaction is crucial for viral replication (Kyratsous and Silverstein 2007). Localization of the ORF29 protein to the nucleus or cytoplasm correlates with whether VZV infection is lytic or latent respectively and the ubiquitination and degradation of this protein implicates the proteasome as one of the determinants of this protein's localization (Stallings *et al.,* 2006). Proteins expressed during latency are discussed in more detail in section 1.6.2.1.

The protein product of ORF47 is a serine/threonine protein kinase and tegument component. The ORF47 protein autophosphorylates and phosphorylates the major IE transactivator proteins IE62 (Kinchington *et al.,* 1992; Ng *et al.,* 1994) and IE63 (Kenyon *et al.,* 2001), gE (ORF68) (Kenyon *et al.,* 2002, Montalvo and Grose, 1986b) and ORF32 (Reddy *et al.,* 1998a). Glycoprotein E requires ORF47 phosphorylation to mediate cell fusion and TGN trafficking for virion assembly (Kenyon *et al.,* 2002). Recently, ORF47 kinase was shown to be important in the activation of the Akt signaling cascade (Rahaus *et al.,* 2007); which has an important role in the regulation of cell survival.

The ORF61 protein optimizes the availability of cellular factors involved in gene expression and can either enhance or repress activation of other VZV genes by IE4 and IE62, but is dependent on the cell lines and transfection conditions used (Moruchi *et al.,* 1993a; Nagpal and Ostrove, 1991; Perera *et al.,* 1992b). This protein has been shown to contain a RING finger domain which is required for its transregulatory functions (Moriuchi *et al.,* 1994b). Phosphorylation occurs in the N- and, to a lesser extent, C-terminal portions of this protein. The C-terminal region directs transport of the protein to the nucleus, whereas the N-terminal region, which contains a potential zinc-binding domain, is responsible for a punctate distribution (Stevenson *et al.,* 1992). This protein has recently been shown to facilitate the nuclear import of IE63 within guinea pig enteric neurons (Walters *et al.,* 2008). The VZV ORF61 protein has been suggested to play an important role in the fine-regulation and activation of the MAPK pathways and their downstream targets (Rahaus *et al.,* 2005).

IE62 is also known to transactivate VZV immediate early (ORF4, ORF61), early (ORF36) and late (ORF67, 68) gene promoters in transient expression assays (Inchauspé and Ostrove, 1989; Perera *et al.,* 1992a; Perera *et al.,* 1992b). Co-expression of the IE62 tegument protein with the ORF66 protein results in cytoplasmic accumulation of IE62 (Kinchington *et al.,* 2000) and is required for localization within virions in cultured cells, (Kinchington *et al.,* 2001) but not VZV-infected primary T cells (Schaap-

Nutt *et al.,* 2006). IE62 is known to activate its own promoter in lymphocytes and neural cells but represses its own promoter in simian cells (Disney and Everett, 1990; Felser *et al.,* 1988; Perera *et al.,* 1992a). This protein has also been shown to be transcribed during latency (Cohrs and Gilden, 2006). This protein is a substrate for CDK1/cyclin B1 and recent data indicate that binding to IE62 leads to the incorporation of cellular kinase into the tegument (Leisenfelder *et al.,* 2008). IE62 also binds to ORF9 at IE62 residues 1-43 (Cilloniz *et al.,* 2007) and to the proteins encoded by ORF47 (Besser *et al.,* 2003) and ORF4 (Spengler *et al.,* 2000).

IE63 binds to, and co-localizes with, IE62 in infected cells (Lynch *et al.,* 2002). The IE62 binding site that lies within IE63 has been mapped to IE63 amino acids 55 to 67, with R59/L60 being critical residues (Baiker *et al.,* 2004b). In IE63 mutant viruses, ORF47 kinase protein and glycoprotein E (gE) synthesis were reduced, indicating that IE63 contributed to optimal expression of early and late gene products (Baiker *et al.,* 2004b). IE63 is important in the establishment of latency (see section 1.6.2.1).

The ORF66 product is a serine/threonine protein kinase that phosphorylates nuclear egress lamina proteins and mediates phosphorylation of cellular and viral proteins, including IE62 (Eisfeld *et al.,* 2006). This leads to the cytoplasmic accumulation of IE62 late in infection (Cohrs *et al.,* 2003b). ORF66 affects several host cell signalling pathways, which have been shown to play an important role in viral immune evasion, (Abendroth *et al.,* 2001a; Eisfeld *et al,* 2007; Schapp *et al.,* 2005; Rahaus *et al.,* 2007; Schaap-Nutt *et al.,* 2006; Schaap *et al.,* 2005) which is discussed in section 1.7.

1.5 VZV Infection at a Cellular Level:

Knowledge of VZV replication is somewhat limited, but extrapolation from what is known for other human herpes viruses, along with mammalian alphaherpesviruses, has helped with our understanding of this process. In order to replicate the virus must first attach to the host cell surface to facilitate entry. The viral genome must then be released from the capsid and translocate into the nucleus, where replication (aided by the host's own cellular machinery) commences. Newly synthesised DNA is then packaged within newly synthesised nucleocapsids and exported from the nucleus. The encapsidated DNA must then acquire tegument proteins and an envelope before the resulting progeny virus exits the host cell. Kinetic studies of VZV-infected cells which were used to inoculate uninfected cells, demonstrated that viral proteins were expressed as early as 1h post infection (Reichelt *et al.,* 2009) and progeny virus spreads to neighbouring cells as early as 8h post infection (Asano and Takahashi, 1980).

1.5.1 Cell Attachment and Entry:

Like other herpesviruses, VZV particles are presumed to enter cells by fusion of the virion envelope with the plasma membrane or by endocytosis, (Cohen *et al.,* 2007b). As with all of the human herpes viruses, VZV glycoproteins are presumed to be involved in virion attachment and entry, (as well as envelopment, cell-to-cell spread, and egress), (Roizman and Knipe 2001; Heldwein and Krummenacher, 2008). VZV attaches to the host cell surface via an interaction between gB and heparan sulphate constituents on the host cell surface proteoglycans (heparan sulphate proteoglycan (HSPG)), (Zhu *et al.,* 1995; Jaquet, 1998), see figure 1.6A. Glycoproteins H and I which are embedded in the outer envelope of the virus also appear to be important in the interaction of VZV with HSPG (Cohen and Nguyen, 1997; Rodriguez *et al.,* 1993). VZV entry is likely to be a two-stage process in which initial binding to HSPG stabilizes VZV on the plasmalemma of target cells, enabling subsequent viral entry.

It has been proposed that following attachment, mannose 6-phosphate (Man 6-P) residues in N-linked complex oligosaccharide chains (Gabel *et al.,* 1989) (present on at least four of the viral glycoproteins ectodomains: gB, gE, gH and gI) interact with Man 6-P receptors on the host cell surface and initiate viral entry (Wang *et al.,* 2000; Gershon *et al.,* 1994b), see figure 1.6B. Chen (*et al.,* 2004) demonstrated that the cation-independent mannose 6-phosphate receptor (MPR^{ci}) is required for infection of cell-free VZV, but not cell-associated VZV. MPR^{ci} is a type-I transmembrane protein with multiple functions, one of which is the delivery of proteins from the trans-Golgi network (TGN) and extracellular space to late endosomes. Endosomes are thought to play a role in several stages of viral replication and are discussed in the text where appropriate.

It was suggested that gE may also be involved in viral entry and cell-to-cell spread because gE deletion viruses are not stable (Mo *et al.,* 2002) and gE antibodies neutralize virus *in vitro* (Lowry *et al.,* 1992). Li (*et al.,* 2006) found that IDE is a cellular receptor for both cell-free and cell-associated VZV. Although IDE is located in the cytoplasm of cells, it is (along with MPR^{ci}), also expressed on the cell surface and within endosomes, see figure 1.6B and C. The region of VZV gE required to bind IDE has recently been defined as residues 24-71 in the extracellular domain, located immediately after the predicted signal peptide, (Li *et al.,* 2007). Chimeric HSV-2/VZV gE proteins used in the study also demonstrated that the secondary structure of gE is critical for its interaction with IDE. As IDE is a soluble protein it would appear to require a membrane-anchoring adaptor molecule (or co-receptor); but such a molecule has yet to be identified. It has been suggested that MPR^{ci} and IDE may interact to facilitate virus infection, and that there are also likely to be unidentified receptors involved in the process of viral entry, as blocking IDE results in only a 25-75% reduction of VZV infection and cell-to-cell spread (Li *et al.,* 2006).

Recent work by Hambleton (*et al.,* 2007) has highlighted the importance of membrane cholesterol in the establishment of VZV infection *in vitro*. Cholesterol is a major constituent of lipid rafts and entry of both EBV and HSV have previously been shown to be cholesterol dependent, (Bender *et al.,* 2003; Katzman and Longnecker, 2003). Based on analogy to HSV entry, it is possible that lipid rafts are also involved in VZV entry through the participation of gB in the fusion of the viral envelope with cellular membranes. This glycoprotein has been shown to play a role in VZV-induced membrane fusion, (Maresova *et al.,* 2001) and is one of the most conserved amongst the human herpesviruses. In the case of HSV, a physical association between gB and host cell lipid rafts has been demonstrated and it has been suggested that cholesterol plays a role in that fusion process (Bender *et al.,* 2003).

The work of Hambleton and colleagues also suggested that clathrin-mediated endocytosis is a major route of viral entry into several human cell types *in vitro*. Although MPR^{ci} trafficking is generally clathrin mediated, and depletion of cholesterol has been demonstrated to affect the distribution of MPR^{ci} (Miwako *et al.,* 2001), the internalization of the MPR^{ci} from the cell surface does not require cholesterol (Hambleton *et al.,* 2007). The researchers proposed that after binding to MPR^{ci}, VZV undergoes receptor-mediated endocytosis and is subsequently delivered to an intracellular compartment (see figure 1.6B and C). Here, fusion is triggered by unknown factors, possibly co-receptors, such as IDE. Recently it has shown that VZV entry into Chinese hamster ovary (CHO) cells is dependent on an acidic compartment (Finnen *et al.,* 2006) and thus it has been proposed that there is a possibility that the same endosomal conditions that have been shown to inactivate outgoing virions, could be responsible for triggering the fusion between incoming viruses and the endosomal membrane.

Once the virus has entered the cell, to spread within this new environment, it is believed that VZV utilizes proteins within the host cells cytoskeleton (Cilloniz *et al.,* 2007; Che *et al.,* 2008), (this process is also believed to be used during cell-to-cell spread; see section 1.5.5). The cytoskeleton is composed of three types of protein structures, known as microfilaments, microtubules and the intermediate fibre system, (Henderson and Weber, 1981). Microfilaments mainly consist of the globular protein actin, which once assembled into fibres are important for the maintenance of cell morphology. Microtubules, which are comprised of α- and β-tubulin subunits, perform general functions within cells including organelle movement and cargo transport (Nogales, 2000). During the VZV replication cycle *in vitro*, tubulin and actin networks undergo significant changes including fibre elongation (Khun *et al.,* 2005). It was demonstrated that when these networks were destroyed intentionally, viral replication was diminished, which suggested that these systems are vital for efficient infection and viral replication (Khun *et al.,* 2005). Recently it was demonstrated that the ORF9 protein binds to β-tubulin, suggesting that this protein may attach to microtubules and play a role in the intracellular transport of non-enveloped virions (Cilloniz *et al.,* 2007). Many herpes viruses including HSV and CMV are also known to use the host cells microtubular network during replication (Sodeik *et al.,* 1997; Dohner *et al.,* 2004).

The contents of the viral particle are released into the host cell cytoplasm via fusion of the viral and endosomal envelopes. Tegument proteins and the nucleocapsid migrate to and enter the host cell nucleus (see figure 1.6D and E). Controversy surrounds the mechanisms that are utilized by herpesviruses to translocate DNA into the nucleus (Cohen *et al.,* 2007b), and the mechanisms for VZV are not known. However, with HSV-1 it has been demonstrated that capsids are transported from the site of de-envelopment to the nuclear pore along the microtubular network (Kristensson *et al.,* 1986; Sodeik *et al.,* 1997; Topp *et al.,* 1994). The capsid then binds to the nuclear pore and releases viral DNA in the

presence of an energy source (Ojala *et al.,* 2000). Simultaneously tegument proteins are released into the cytosol, of which VP1-2 (UL36) (the VZV homologue ORF22) migrates with the capsid to the nuclear pore, whilst VP16 (UL48) (the VZV homologue ORF10) travels to the nucleus independently (Batterson and Roizman, 1983). Upon entry of the viral genome into the nucleus, DNA replication commences.

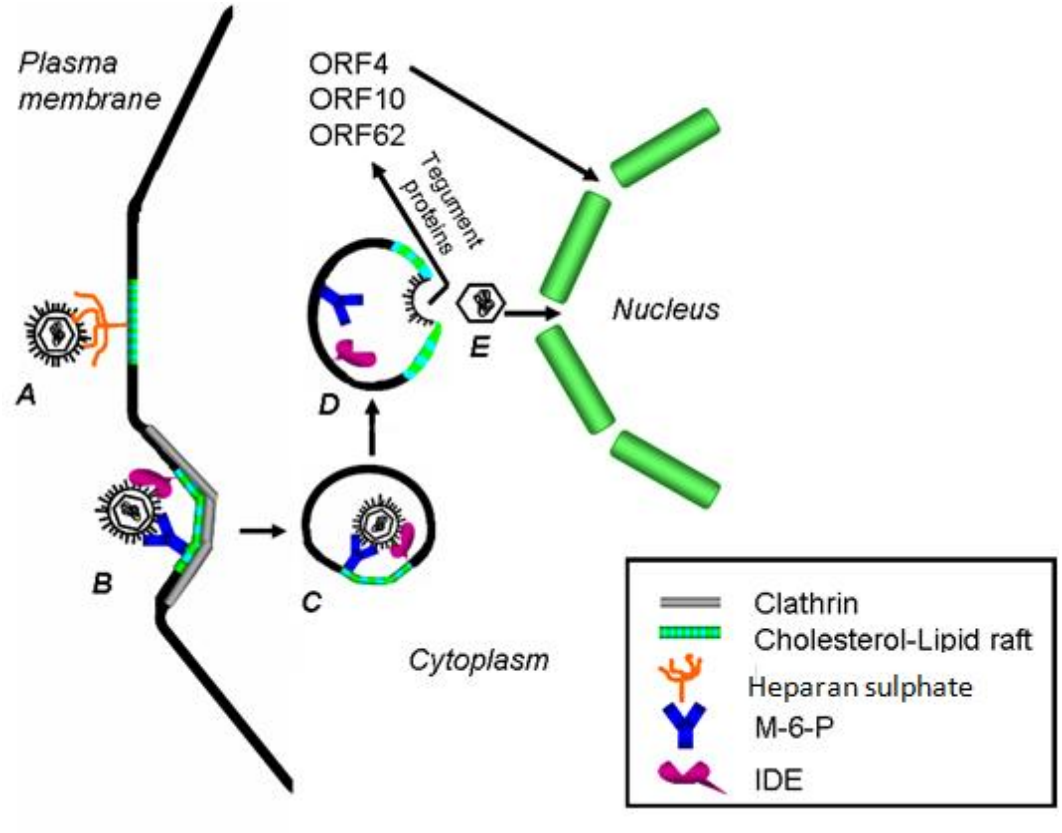


Figure 1.6: Viral Attachment and Cell Entry. VZV enters the cells by attaching to HSPG (A) and binding to receptors ($M6PR^{ci}$ via gB, IDE via gE) on the plasma membrane, and undergoes clathrin dependent endocytosis (B). The virus is engulfed in an endosome (C), in which the virus and the endosome undergo membrane fusion (D), which releases tegument proteins and the nucleocapsid. *NB:* nuclear entry via fusion with the nuclear membrane is extrapolated from the HSV model (E). Not to scale; adapted from Hambleton *et al.,* 2007 and Cohen *et al.,* 2007 with permission.

1.5.2 Initial Events in Viral Replication:

Transactivation can be defined as stimulation of transcription, by transcription factors binding to DNA and activating adjacent proteins. Five viral proteins are capable of transactivating (and/or transrepressing) specific VZV gene promoters. These proteins are encoded by genes of the immediate early (IE) class that lie within ORF4, ORF62, ORF63, and ORFs 10 and 61, (Baudoux *et al.,* 2000). Apart from the IE61, all of these proteins are structural components of the virion tegument (Kinchington *et al.,* 1992; Kinchington *et al.,* 1995), see figure 1.7A. The IE62 protein is the major viral transactivator and is known to induce expression of all classes of VZV genes (Disney *et al.,* 1990; Inchauspe *et al.,* 1989; Perera *et al.,* 1992b). In addition to the viral transactivators, several cellular transcription factors have been shown to play an important role in viral gene transactivation, including specificity factor 1 (Sp1), upstream stimulatory factor (USF), activator protein 1 (AP-1), octamer-binding protein 1 (Oct-1), and TATA element (Jones *et al.,* 2005; Meier and Straus, 1995; Moriuchi *et al.,* 1995b; Rahaus and Wolff, 1999; Ruyechan *et al.,* 2003). USF is particularly important for VZV replication, due to the direct physical interaction that occurs between this cellular transcription factor and IE62 (Meier and Straus, 1995; Rahaus *et al.,* 2003; Yang *et al.,* 2004). Recently it was demonstrated that the activation domain of USF1 is sufficient for synergistic activation with IE62 (Yang *et al.,* 2006). In conjunction with the IE62 protein, USF transactivates the promoters of IE4 and ORF10 in reporter plasmid assays (Michael *et al,* 1998). Approximately 25% of the putative regulatory elements that control expression of the 71 VZV ORFs contain USF consensus binding sites, indicating that USF is likely to play a key role in VZV replication (Ruyechan *et al.,* 2003).

1.5.2.1 Viral DNA Replication:

The machinery that enters the nucleus and replicates viral DNA is composed of early proteins; including the DNA helicase/primase (encoded by ORF6, 52 and 55); the large subunit of DNA polymerase encoded by ORF28; (the small subunit is thought to be encoded by ORF16) and the

single stranded DNA binding protein (encoded by ORF29), (Zweerink *et al.,* 1981); see table 1.4 and figure 1.7C. By analogy with HSV; VZV ORF6, 52 and 55 are predicted to form a heterotrimeric complex exhibiting both helicase and primase activity. The model of VZV DNA replication is based on elements of a general mechanism proposed for HSV DNA replication and on VZV specific data. Recently it has been demonstrated *in vitro* that formation of DNA replication compartments begins 4-6h post infection (Reichelt *et al.,* 2009). Unwinding of the DNA origin is thought to occur via the ORF29 encoded single-stranded DNA binding protein (Kinchington *et al.,* 1988), see figure 1.7B. Replication is then initiated at one of two origin of replication (short) (Ori_S) loci by binding of the ORF51 encoded origin of replication binding protein (OBP) (Stow *et al.,* 1990; Chen and Olivo, 1994) and possibly cellular factor(s). The remainder of the replication proteins are then recruited to the origin; see figure 1.7C. Five additional VZV DNA binding proteins have been identified ranging from 21-175kDa (Roberts *et al.,* 1985). Like HSV-1, the VZV genome has single nucleotide extensions (Mocarski and Roizman 1982; Davison, 1984). These extensions are presumed to mediate circularisation of the genomes via direct ligation of the termini (Davison, 1984), after their entry into the host cell nucleus, (see figure 1.7C). Viral protein synthesis (discussed in section 1.5.3), is initiated by tegument proteins binding to the circularised viral DNA, see figure 1.7D. By analogy with HSV; a nick introduced into the replicating DNA shifts replication into a rolling circle mechanism, (Ruyechan and Hay, 2000). This form of replication generates head-to-tail concatamers which are cleaved by specific nucleases to generate linear DNA for packaging (see figure 1.7E). Based on sequence comparison to HSV, VZV encodes homologues for each of the seven essential genes whose protein products are responsible for the cleavage and packaging of viral DNA into capsids. The putative DNA encapsidation genes are encoded by VZV ORFs 25, 26, 30, 34, 43, 45/42 and 54 (Sheaffer *et al.,* 2001). Capsid formation is discussed in section 1.5.4.1 and figure 1.7F.

VZV Gene	Function	HSV Homologue
ORF6	Primase	UL52
ORF16	Polymerase processivity Factor (small subunit)	UL42
ORF28	Polymerase catalytic (large) subunit	UL30
ORF29	Single-stranded DNA Binding	UL29
ORF51	Origin Binding Protein	UL9
ORF52	Helicase/Primase accessory	UL8
ORF55	Helicase	UL5

Table 1.4: VZV Proteins Involved in DNA Replication. The functions of the proteins encoded by VZV ORFs 6, 16, 52 and 55 are based on the properties of their HSV homologues.

1.5.3 Protein Synthesis:

As mentioned above, viral protein synthesis is initiated by tegument proteins that bind to the circularised VZV DNA. By analogy with cells infected with HSV and other herpesviruses, VZV protein expression is believed to be a tightly regulated cascade of events that occurs in three stages which are classified based on the time course of their expression after virus entry (Cohen *et al.,* 2007b). Each stage is dependent on the host cell ribonucleic acid (RNA) polymerase and may also be regulated by the RNA cleavage activity of VZV ORF17 (Sato *et al.,* 2002a). The first genes transcribed are the immediate early genes. Products of these genes include transcriptional activator and transcription repressor proteins. These proteins are expressed in the host cell cytoplasm but are translocated back into the nucleus to down regulate further immediate early gene transcription and to initiate synthesis of the early proteins. Early proteins enter the nucleus to facilitate viral DNA replication (see figure 1.7C). The third class of VZV proteins to be expressed are the late proteins. These encode the structural proteins that act as the building blocks for the assembly of new virions and include the viral glycoproteins (Davison and Scott, 1986), see figure 1.7.

As achievable titres of cell-free VZV are too low to permit synchronous infections of cultured cells, kinetic studies which have been conducted for other herpesviruses are lacking. However, a recent study was conducted that examined the kinetics of VZV protein expression *in vitro* using different fluorescent cell dyes to label VZV-infected inoculum cells and

uninfected cells (Reichelt *et al.,* 2009). Newly infected human fibroblast cells were evaluated by confocal immunofluorescence and electron microscopy at specific time points, at the single-cell level. This study revealed that IE62 and IE61 appeared within 1h post infection whilst late proteins such as the ORF23 capsid protein were detectable at 9h. Surprisingly, although gE is classified as a late protein, accumulation in the Golgi compartment was seen as early 4h post infection, whilst substantial gE expression on plasma membranes was only evident at 9h.

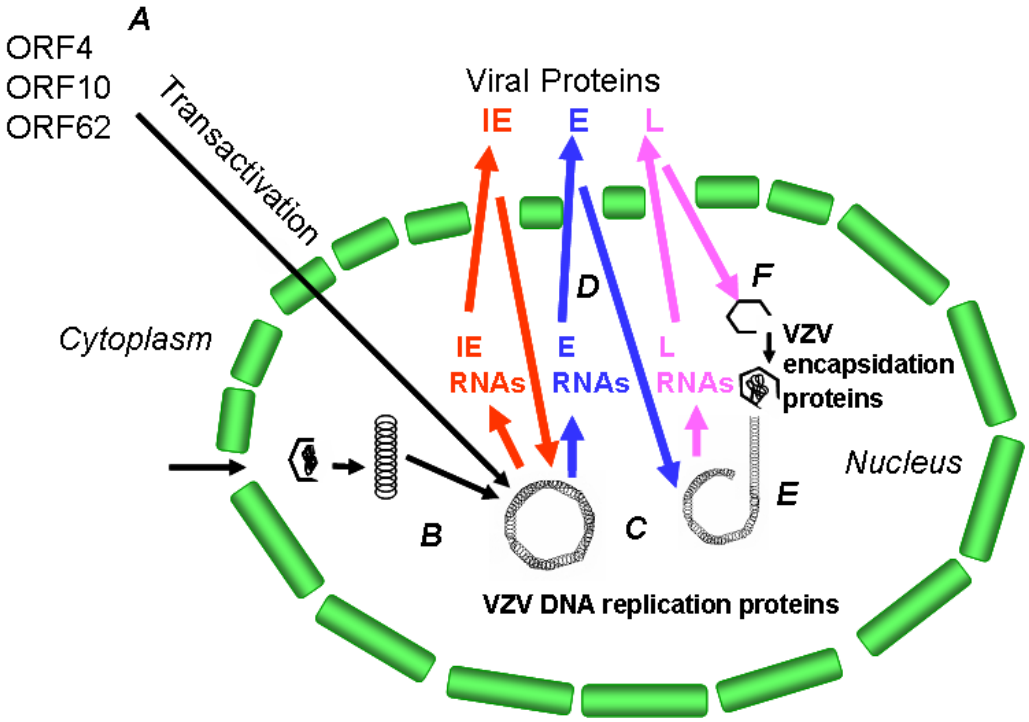


Figure 1.7: An Overview of Events in the Nucleus; Viral DNA Replication, Protein Synthesis and DNA Packaging. (A): Transactivation by both viral and cellular transcription factors occurs; (B): Unwinding of DNA is facilitated by ORF29 protein, binding of ORF51 initiates DNA replication and recruitment of other DNA replication proteins encoded by ORFs 6, 16, 28, 52 and 55; (C): Rolling circle replication ensues; (D): Messenger RNAs are transcribed, transported to the cytoplasm and translated. The encoded proteins are then transported back to the nucleus; (E): Head-to-tail concatamers are cleaved by specific nucleases to generate linear DNA for packaging. The putative DNA encapsidation proteins are encoded by VZV ORFs 25, 26, 30, 34, 43, 45/42 and 54; (F): Nucleocapsids are assembled in the nucleus. Key: IE; immediate early, E; early, L; late, RNA; ribonucleic acid. Not to scale, adapted from Cohen *et al.,* 2007, with permission.

1.5.4 Virion Assembly:

1.5.4.1 The Nucleocapsid:

The nucleocapsids of VZV (Gershon *et al.,* 1994; Harson and Grose, 1995); and indeed HSV (Nii *et al.,* 1968), and PRV (Whealy, 1991), are assembled in the nuclei of infected cells. Recent kinetic experiments *in vitro* have shown that these structures are present within the nucleus at 9h post infection (Reichelt *et al.,* 2009). Capsid assembly is a complex process involving interactions between multiple proteins in both the cytoplasm and in the nucleus. Like other alphaherpesviruses, the formation of VZV capsids is predicted to require the products of VZV ORFs 20, 23, 33, 33.5, 40, and 41 (Gibson and Roizman, 1972; Kut and Rasschaert, 2004). Direct information about VZV capsid proteins is limited, but assuming conservation of function with HSV (see table 1.5 below), the outer capsid shell comprises of ORF20, ORF23, ORF40 and ORF41.

VZV Capsid Protein	HSV Capsid Protein Homologue	Function and Property of HSV Homologue
ORF20	VP19C (U_L38)	Forms a triplex complex with VP23; part of the outer capsid; interacts and stabalizes adjacent capsomers
ORF23	VP26 (U_L35)	Smallest capsid protein (SCP), covers the top of hexons of the outer surface of the capsid shell; homologue of UL48/49 in HCMV
ORF33	VP24/VP21	Minor scaffolding proteins
ORF33.5	Pre-VP22a	Scaffolding protein, protease which self cleaves
ORF40	VP5 (U_L19)	Major HSV-1 capsid protein;
ORF41	VP23 (U_L18)	Forms all capsomers; and thus the outer capsid; see VP19C

Table 1.5: VZV Capsid Proteins Closely Related to HSV Capsid Proteins. (Additional HCMV reference form Lai and Britt, 2003).

Like HSV-1, (Rixon *et al.,* 1996), not all of the VZV capsid proteins have a nuclear localization capacity. This is restricted to the ORF23 and ORF33.5 protein products and has been mapped to residues 229-234 in the C-terminus of the ORF23 protein product (Chaudhuri *et al.,* 2008). The ORF40 protein is predominantly translocated to the nucleus by the ORF23 protein, and to a lesser extent by the ORF33.5 protein *in vitro*. However as ORF23 has been shown to be essential for replication *in vivo*, ORF33.5 cannot compensate for the loss of ORF23 *in vivo* (Chaudhuri *et al.,* 2008). It is also noteworthy that although ORF23 is not essential for replication in melanoma cells *in vitro*, growth kinetics and capsid formation are severely disrupted in the absence of this protein; however, this was not the case in a fibroblast cell line. Using recombinant capsid protein GFP fusion constructs the intracellular trafficking of the individual proteins revealed that neither ORF23 nor ORF33.5 could facilitate the transport of ORF20 or ORF41 from the cytoplasm to the nucleus. Chaudhuri and colleagues have proposed a model based on analogy with HSV capsid assembly (see figure 1.8). In HSV-1, the ORF20 and ORF41 homologues are known to form a trimeric complex, which is transported into the nucleus upon binding to VP5; the major capsid protein and ORF40 homologue. Thus, this may also be the case for VZV. A similar trimeric complex may form in the cytoplasm, composed of ORF20 and ORF41, bound to ORF40. This larger complex may then translocate into the nucleus to allow for the final assembly of capsomeres through ORF40 binding to either ORF23 or ORF33.5.

Encapsidated viral DNA then exits the nucleus, and in doing so, acquires a primary envelope as it passes through the inner nuclear membrane. This enveloped structure then enters the perinuclear cisterna, which is continuous with the RER, (this mechanism is accepted for all of the alphaherpesviruses, (Mettenleiter, 2002)). In order to achieve intimate contact with the inner nuclear membrane, the nuclear lamina has to be softened and at least partially dissolved; in HSV-1, PRV, EBV and MCMV, this requires two virally encoded proteins, which are both structurally and functionally conserved in *Herpesviridae*, (Reynolds *et al.,* 2004; Fuchs *et*

al., 2002; Gonnella *et al.,* 2005; Lotzerich *et al.,* 2006). The VZV homologues of these two proteins (pUL31 and pUL34) are ORFs 27 and 24 respectively. Homologues of pUL34 are predicted type-II transmembrane proteins that complex with pUL31. Complex formation is important for correct positioning at the nuclear membrane, which is a prerequisite for primary envelopment. In the absence of either protein, nuclear egress has been demonstrated to be drastically impaired. For the alphaherpesviruses examined to date (HSV-1 and BHV-1) nucleocapsids subsequently gain access to the cytoplasm by fusion of their primary envelope with the outer nuclear membrane, however the exact mechanisms for this are still unclear (Leuzinger *et al.,* 2005).

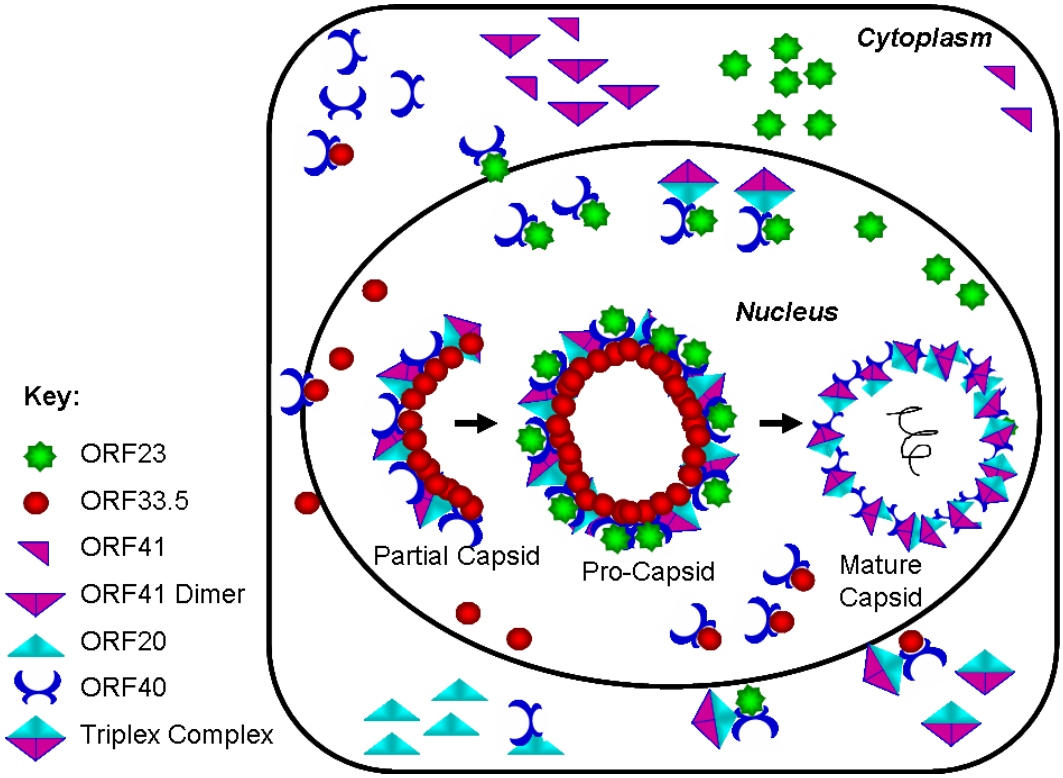


Figure 1.8: A Proposed Model of VZV Capsid Assembly. (Based on analogy of HSV capsid assembly); not to scale; adapted from Chaudhuri *et al.,* 2008, with permission.

Following encapsidation and subsequent egress from the nucleus, acquisition of both the tegument and a glycoprotein studded envelope in the cytoplasm must occur. Two models which incorporate these concepts have been proposed (Gershon *et al.,* 1994; Harson and Grose 1995) and will be discussed in detail in section 1.5.4.4.

1.5.4.2 Acquisition of Tegument Proteins:

Knowledge of the events that occur during VZV tegumentation is limited, but in the case of HSV-1, tegumentation (and secondary envelopment) are driven by a highly ordered network of protein–protein interactions in the cytoplasm (Vittone *et al.,* 2005), and the proteins involved are conserved in the *Herpesviridae* family. Final tegumentation of HSV-1 can initiate at two distinct sites; the capsid and the future envelope, which then combine to produce a mature virion. The capsid-proximal tegument contains the conserved pUL36 and pUL37 (homologous to VZV ORFs 22 and 21), which form a physical complex, pUS3 (VZV ORF66), and presumably, also contains the conserved pUL25 (VZV ORF34). This structure is either reassembled immediately after nuclear egress, or remains associated with the capsid during this process (Wolfstein *et al.,* 2006; Luxton *et al.,* 2005; Zhou *et al.,* 1999).

In the case of VZV, the mechanism by which the IE62 protein is incorporated into the viral tegument is largely unknown. However, the viral kinases present in the viral tegument (encoded by VZV ORF47 and ORF66) have been shown to be capable of phosphorylating IE62. ORF47 kinase function is known to be important for normal VZV virion assembly (Besser *et al.,* 2004), is critical for replication in human immature Dendritic cells (DCs), (Hu and Cohen, 2005) and has been shown to form a stable complex with IE62 (Besser *et al.,* 2003; Kenyon *et al.,* 2003). Phosphorylation of IE62 by ORF66 kinase is required for the redistribution and exclusion of IE62 from the nucleus to the cytoplasm. Recently it was suggested that the ORF9 protein may also play a role in viral tegument formation via the recruitment of IE62 and possibly ORF47 kinase to complexes within the cytoplasm (Cilloniz *et al.,* 2007; Che *et al.,* 2008).

ORF9, which is essential for replication *in vitro* (Che *et al.,* 2008), interacts with IE62 via the central portion of the ORF9 protein (aa 117 to 186) and the N-terminal half of the IE62 acidic activation domain (Cilloniz *et al.,* 2007). In the cytoplasm, IE62 co-localizes with the ORF9 protein in filamentous structures leading to the incorporation of IE62 into the viral particle (Kinchington *et al.,* 2000; Cilloniz *et al.,* 2007).

The process of VZV tegumentation is not addressed in the Harson and Grose model, but according to the Gershon model, nucleocapsids lose their nuclear derived envelopes when they fuse with the RER, releasing naked nucelocapsids into the cytoplasm (figure 1.9A); which is consistent with what is seen with HSV. Subsequently, naked nucelocapsids are enveloped in the TGN where they acquire both tegument proteins and a mature envelope (see section 1.5.4.4 for more details). The Gershon model was based upon transmission electron microscopy (TEM) studies and recent TEM experiments in HELF cells (Reichelt *et al.,* 2009) add support to the model of tegumentation (and envelopment) of viral nucleocapsids by the c-shaped cisternae of the TGN.

1.5.4.3 Glycoprotein Synthesis and Trafficking;

Both models agree that the VZV glycoproteins are synthesized and processed in the rough endoplasmic reticulum (RER) and are transported to the Golgi apparatus independently of the newly assembled nucleocapsids, (Gershon *et al.,* 1994; Harson and Grose, 1995). Once transcribed, glycoprotein mRNA is translocated out of the nucleus and into the cytoplasm where it is translated by polyribosomes associated with the RER. The glycoprotein polypeptides then cross the RER membrane and enter the RER lumen. As the peptides migrate through the RER, they become glycosylated and acquire Man 6-P (Gabel *et al.,* 1989), (see figure 1.9A). Recent experiments by Storlie (*et al.,* 2006) have shown that gE, gI, gH, and gB are synthesized within 4-6h postinfection in cultured cells, and travel via the TGN to the outer cell membrane.

For their transport to the TGN, some glycoproteins use TGN-specific aa targeting patches in their cytoplasmic tails. For gE, gB and gI, these sequences have been identified as AYRV and a second acidic aa rich sequence, the putative signal patch, (Zhu *et al.,* 1996), YSRV and YMTL (Heineman *et al.,* 2000), and TIREE (Wang *et al.,* 1998) respectively. The acidic cluster of gE interacts with the connector protein, phosphofurin acidic cluster sorting protein 1 (PACS-1), and directs VZV gE from endosomes to the TGN, (Wang *et al.,* 1998 and Wang *et al.,* 2001). VZV gE is phosphorylated on its cytoplasmic tail (Grose *et al.,* 1989, Montalvo and Grose 1986b, Olson *et al.,* 1997) and differential phosphorylation of the gE acidic cluster targets endocytosed gE to either the TGN for viral assembly or to the cell surface for cell-to-cell spread (Kenyon *et al.,* 2002). Glycoprotein E is an essential protein *in vitro* (Mo *et al.,* 2002) and both the YAGL motif and the first 16 residues of the unique N-terminal region of this protein have been shown to be essential for VZV replication *in vitro* (Moffat *et al.,* 2004; Berarducci *et al.,* 2006). However, the gE-AYRV mutant which interferes with TGN targeting was found to be associated with substantial attenuation in skin (Moffat *et al.,* 2004).

Because those VZV glycoproteins that lack targeting sequences (gC, gH and gL) concentrate in the TGN of infected cells, it has been proposed that gE and gI, serve as navigators, forming complexes that direct the signal-deficient glycoproteins to the TGN (Gershon and Gershon 1999; Alconda *et al.,* 1998; Duus and Grose, 1996). These glycoproteins also rely on these mechanisms for their transport to the infected cell membrane and/or retrieval from this site back to the TGN for their incorporation into new virions (Heineman and Hall 2002; Alconda *et al.,* 1998; Gershon and Gershon, 1999; Duus and Grose, 1996; Duus *et al.,* 1995; Kenyon *et al.,* 2002; Pasieka *et al.,* 2003). Duus and Grose (1996) have reported that gE can interact with gH, and Pasieka (*et al.,* 2004) reported on a gE:gH complex shuttling gH to the TGN after internalisation of the complex through the trafficking motif in the long gE tail. Preliminary observations have suggested that gI can also redirect the gH:gL complex to the TGN (Wang *et al.,* 2000).

VZV gE is transported to the cell plasma membrane (PM) from where it is selectively retrieved by endocytosis, and returned to the TGN, before being recycled back to the PM (Olson and Grose 1997; Olson and Grose 1998; Wang *et al.,* 2000). As a recombinant VZV genome lacking only endocytosis-competent gE cannot replicate, this supports the conclusion that endocytosis is an essential glycoprotein trafficking mechanism (Maresova *et al.,* 2005, Moffat *et al.,* 2004). Glycoprotein K is also transported to the PM of infected cells where it is efficiently endocytosed (Hall *et al.,* 2007), however, the half-life of gK is considerably shorter than that of other VZV glycoproteins including gB, gE and gH (Hall *et al.,* 2007). Glycoprotein I is also transported to the PM, but in some cells, some gI is retained in the TGN (Wang *et al.,* 1998). Glycoprotein I can, but does not always form a complex with gE. Complex formation, which is presumed to occur in the RER, (Gershon and Gershon, 1999) requires the gI N-terminus (Kimura *et al.,* 1997) whilst the gI binding domain on gE was recently mapped to aas 163-208 (Li *et al.,* 2007). Complex formation also enhances the exit of gI from the RER and the targeting of gI to the TGN (Gershon and Gershon, 1999; Wang *et al.,* 1998; Olson and Grose, 1998). In the absence of gI, VZV-infected cells show an unusual punctuate distribution of gE on plasma membranes, reduced syncytium formation and diminished synthesis of the mature form of gE (Mallory *et al.,* 1997).

1.5.4.4 Envelopment and Virion Morphogenesis:

The hypothesis of VZV envelopment in the TGN is similar to that originally proposed for PRV and later extended to HSV and other herpes viruses (Whealy, 1991; Card *et al.,* 1993; Mettenleiter, 2006). According to the Gershon model (1994b), the sacs of the TGN flatten and curve to acquire the shape of the letter ‘c’ which envelops a VZV nucleocapsid (figure 1.9B). Viral glycoproteins are segregated to the inner face of these cisternae, while MPRs and other cellular proteins are restricted to the opposing outer face of the same c-shaped cisternae (Gershon *et al.,* 1994), (figure 1.9B). The cytoplasmic tails of all three major VZV glycoproteins, gE, gH, and gB, harbour functional tyrosine-based endocytosis motifs that mediate internalization (Maresova *et al.,* 2005).

Figure 1.9: Proposed Assembly and Envelopment of VZV.

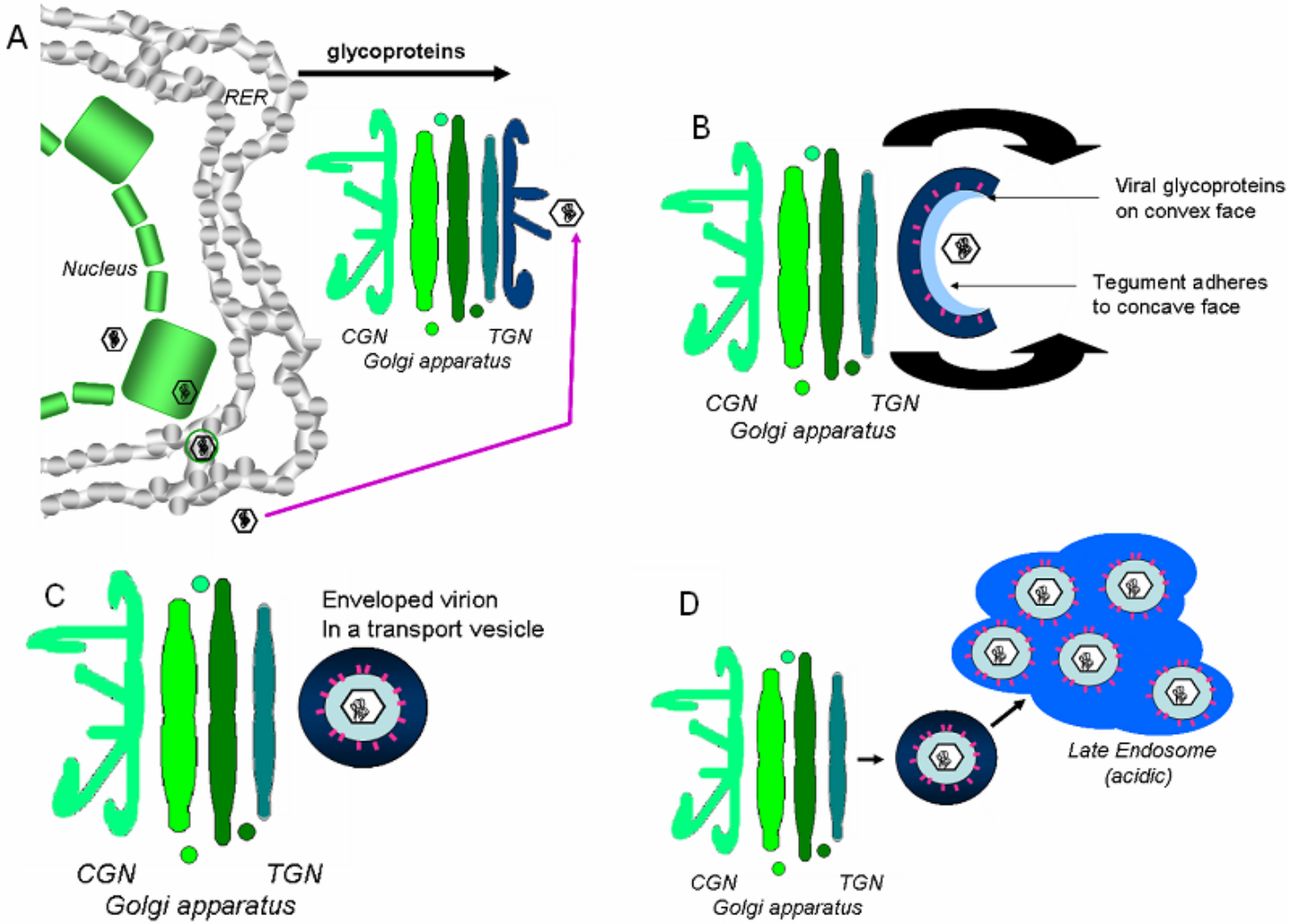
(A) Nucleocapsids lose their nuclear derived envelopes when they fuse with the RER, releasing naked nucleocapsids into the cytoplasm. Glycoproteins are synthesized on the RER and delivered to the TGN independently of nucleocapsids and tegument.

(B) The TGN forms C-shaped cisternae, each of which envelops a VZV nucleocapsid (for ease of diagrammatic representation, only one C-shaped cisterna is shown). Viral glycoproteins are segregated to the inner (concave) face of these cisternae, while MPRs and other cellular proteins are restricted to the outer (convex) face of the same C-shaped cisterna.

(C) The encroaching arms of the TGN fuse with the creation of a double membrane bound structure. The inner (concave) face becomes the glycoprotein-containing-VZV-envelope, while the outer (convex) face functions as a transport vesicle.

(D) In most cell types the transport vesicle facilitates MPR-dependent delivery of VZV to late endosomes.

Key: CGN: Cis-Golgi network; TGN: trans-Golgi newtork; PM; Plasma membrane; RER; rough endoplasmic reticulum, MPR; Mannose-6- phosphate receptor. Not to scale; adapted from Hambleton *et al.,* 2004, with permission.


A
glycoproteins
RER
Nucleus
CGN
TGN
Golgi apparatus
B
Viral glycoproteins
on convex face
Tegument adheres
to concave face
CGN
TGN
Golgi apparatus
C
Enveloped virion
In a transport vesicle
CGN
TGN
Golgi apparatus
D
Late Endosome
(acidic)
CGN
TGN
Golgi apparatus

This segregation of viral glycoproteins and MPRs may serve to organise viral assembly and egress and, possibly, to permit cellular MPRs to interact with glycoproteins of the VZV envelope (Gershon *et al.,* 1994b, Zhu *et al.,* 1995).

The encroaching arms of the TGN fuse, creating a double membrane bound structure. The inner membrane becomes the glycoprotein-containing-VZV-envelope, while the outer membrane functions as a transport vesicle (see figure 1.9C). Accumulation of MPRs on the outer membrane of the wrapping cisternae is thought to route viral particles from the cell secretory pathway to endosomes (Dahms *et al.,* 1989; Westlund *et al.,* 1991; Gershon *et al.,* 1994b), (figure 1.9D). TGN-derived vesicles containing MPRs are preferentially sorted to late endosomes (Dahms *et al.,* 1989; Griffiths *et al.,* 1988) and VZV-containing vacuoles have been found to contain a lysosomal acid phosphatase enzyme (Gershon *et al.,* 1973). The de-envelopment and subsequent re-envelopment of VZV has been proposed to be a mechanism which enables tegument proteins that lack signal sequences and thus are synthesised in the cytosol, to be incorporated into mature viral particles.

The model proposed by Harson and Grose (1995), (see figure 1.10), was formulated from experiments in melanoma cells (MeWo) and lung diploid cells (MRC_5) and suggests that nucleocapsids acquire and then retain their envelopes as they bud through the inner nuclear membrane, (figure 1.10A). Although these authors acknowledged the presence of naked nucleocapsids in the cytoplasm, they stated this to be a rare event. From here they travel through the perinuclear space into a SER vacuole which pinches off and enters the cytoplasm, (figure 1.10B). The virion envelopes are glycosylated when the SER vacuole fuses with Golgi-derived vesicles, laden with glycoproteins, (figure 1.10C and 1.10D). This model also suggests that late in infection, some particles re-enter the nucleus. Whilst residing in the perinuclear space, some particles appear to travel back through the inner nuclear membrane, and thus also acquire their envelope

from the inner membrane, (Figure 1.10F); accounting for the phenomenon that enveloped capsids are seen within the nucleus.

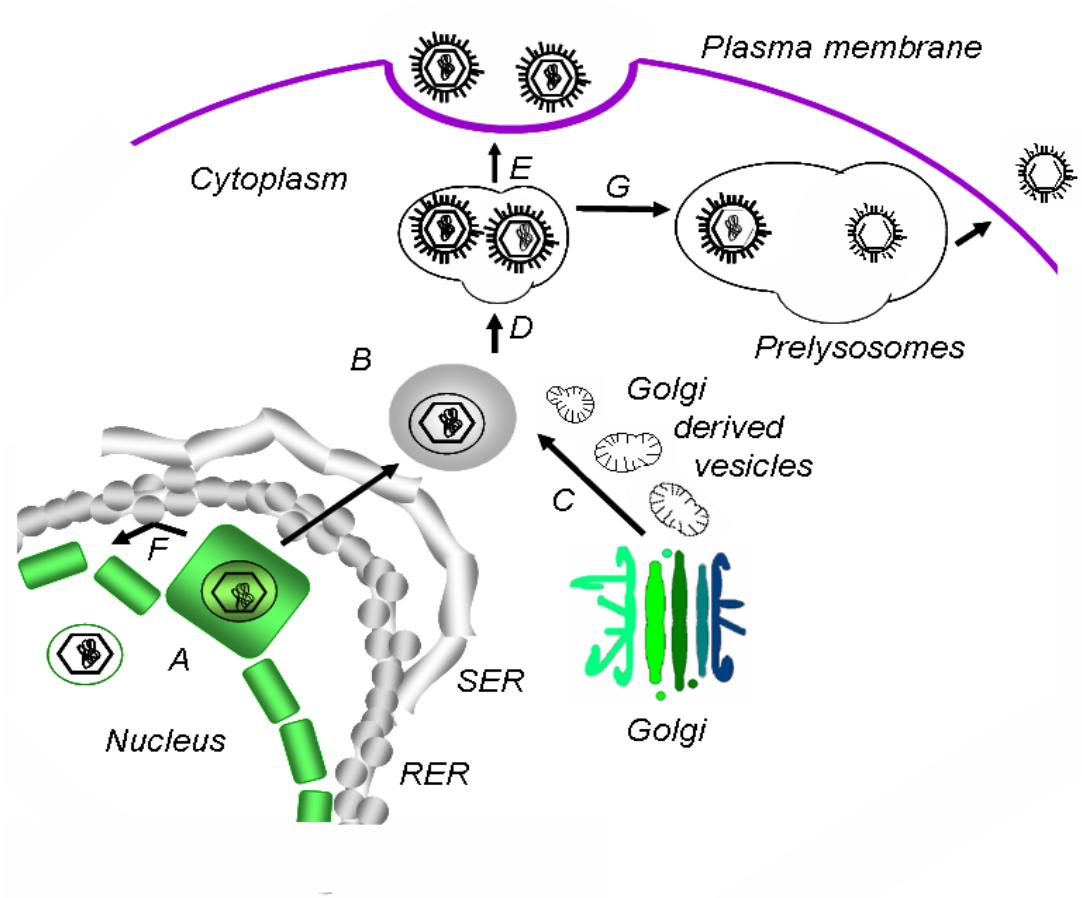


Figure 1.10: The Harson and Grose Model of Envelopment and Egress of VZV Particles. (A) Nucleocapsids in the nucleoplasm bud through the inner nuclear membrane and in doing so, acquire an envelope and travel through the perinuclear space into the Smooth Endoplasmic Reticulum (SER) (B) The SER vacuole pinches off and enters the cytoplasm. (C) Golgi derived vesicles containing VZV glycoproteins fuse with the SER vacuole. (D) The vesicles fuse to form a large vacuole containing complete viral particles. (E) Some vacuoles fuse with the Plasma membrane and enveloped viruses emerge on the surface of the infected cell. (G) Some vacuoles fuse with prelysosomes, leading to partial degradation of viral particles. (F) Late in infection, some particles re-enter the nucleus. Key: RER; Rough endoplasmic reticulum, SER; smooth endoplasmic reticulum. Not to scale; adapted from Harson and Grose, 1995 and Cohen *et al.,* 2007b with permission.

It is important to note that the main discrepancy between these two models (ie: whether there is a de-envelopment and subsequent re-envelopment of VZV) may be explained by the difference of virion morphogenesis seen in different cell types *in vitro*. It has thus been suggested that VZV envelopment is not a uniform process in all susceptible cell types. The Harson and Grose model is based on the hypothesis that the melanocyte is a preferred cell type for replication as the virus usurps the cell machinery used for melanin production.

1.5.5 Egress and Cell-to-Cell Spread:

According to both models, most particles are either transported to the cell surface for release (figure 1.10E), or to prelysosomes (figure 1.10G), within which the particles are partially degraded before release. This leads to an abundance of defective progeny VZV being released. Recently it has been demonstrated that aberrant 'light' particles that lack a core capsid, but contain equivalent gB, gE and gI content to infectious progeny, comprise the majority (75 to 85%) of all egressed particles from melanoma cells *in vitro* (Carpenter *et al.,* 2008) and in comparison to infectious virus particles are produced at a ratio of approximately 40,000:1 (Carpenter *et al.,* 2009). This implies a dissociation between events in the nucleus related to capsid formation and the subsequent envelopment processes in the cytoplasm of VZV infected cells, (see figure 1.11 for a proposed model of light particle assembly and release). Although controversy surrounds both the site of envelopment and the pathway leading to the release of the VZV particle from the cell, unenveloped nucleocapsids in the cytosol of infected cells are a feature of infection with any alphaherpesvirus (Mettenleiter, 2002). With both models, although virion degradation takes place, pathways that avoid prelysosomes must exist in humans for efficient spread of infection.

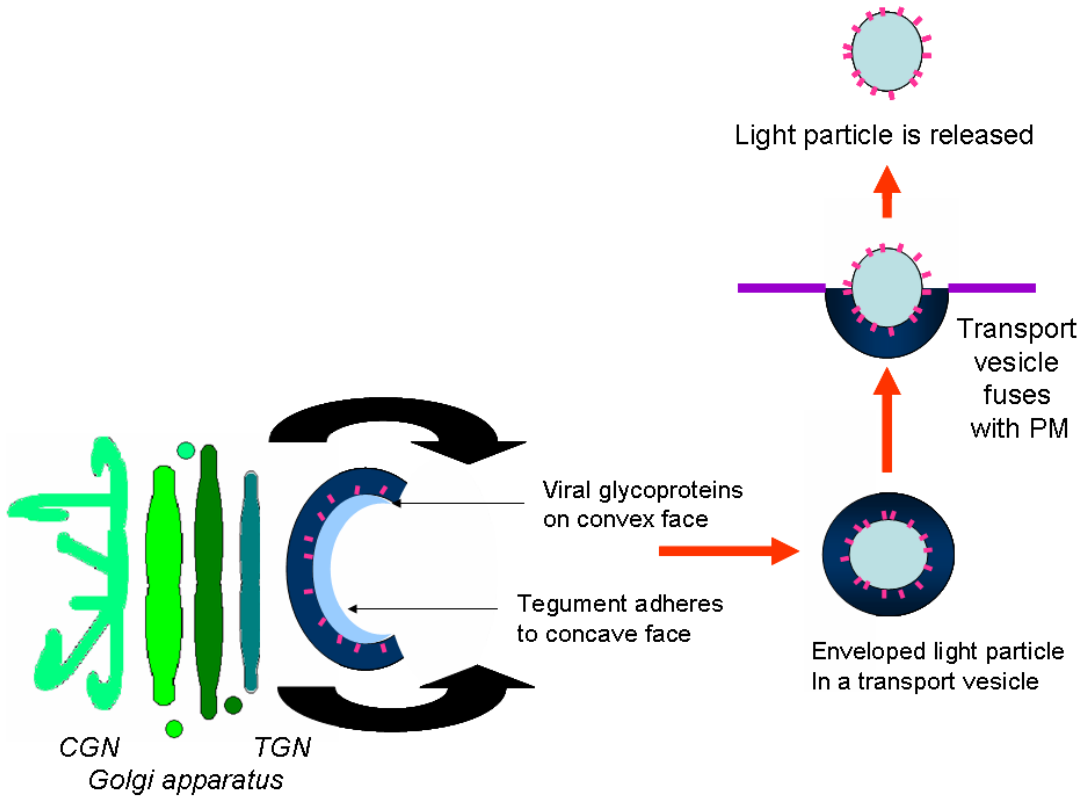


Figure 1.11: Proposed Model for the Formation and Release of 'Light' Particles During Viral Replication. This is based on the HSV model and recent work by Carpenter *et al.,* 2008. Key: CGN: Cis-Golgi network, TGN; trans-Golgi newtork; PM; Plasma membrane. Not to scale; adapted from Mettenleiter *et al.,* 2006, with permission.

Fusion between the TGN-derived transport vesicle membrane and the plasma membrane releases mature virions from the cell surface (Johnson and Huber, 2002). No unique combination of membrane proteins has been identified as a minimum fusion unit. Instead, several VZV proteins, individually and in various combinations, have been observed to induce cellular fusion. Cell-to-cell fusion has been suggested as the prominent mechanism by which VZV spreads to new host cells (Cole and Grose, 2003), but although syncytia formation is a hallmark of VZV, recent experiments in HELF cells demonstrated that initial infection of neighbouring cells does not require cell-to-cell fusion (Reichelt *et al.,* 2009). However, cell-to-cell fusion (which was first seen *in vitro* at 9h post infection), is likely to substantially amplify VZV replication. Membrane fusion is mediated by gH and gL or gB and gE (Cole and Grose, 2003;

Rodriguez *et al.,* 1993; Duus and Grose, 1996). While expression of gH or gB alone induce a modest amount of fusion, expression of gE alone is not sufficient for fusion unless it is co-expressed with gB (Maresova *et al.,* 2001). Cell fusion has been shown to be regulated by VZV gH endocytosis (Pasieka *et al.,* 2004) and VZV gK (based on analogy to its HSV-1 homologue) is believed to participate in membrane fusion (and cytoplasmic virion morphogenesis), (Maresova *et al.,* 2005; Hall *et al.,* 2007). As mentioned earlier, virus spreads to neighbouring cells as early as 8-12h post infection (Asano and Takahashi, 1980; Reichelt *et al.,* 2009).

It is also believed that VZV utilize proteins within the host cells cytoskeleton in order to facilitate its cell-to-cell spread (Mo *et al.,* 2000; Cilloniz *et al.,* 2007; Che *et al.,* 2008). Expression of VZV gE has been shown to induce changes in the organization of microfilaments in polarized epithelial cells and it has been hypothesized that it is the rearrangement of the actin filaments that facilitates the cell-to-cell spread of VZV (Mo *et al.,* 2000). Co-immunoprecipitation experiments have recently demonstrated that ORF9 binds to gE, (Che *et al.,* 2008) as well as to β-tubulin (Colloniz *et al.,* 2007).

Experiments carried out in MeWos demonstrated that VZV emerges from an infected cell surface in a distinctive pattern described as viral highways (Harson and Grose, 1995; Padilla *et al.,* 2003). These structures consist of thousands of particles arranged in linear pathways across the syncytial surface. This egress pattern has not been seen with the five other human herpesviruses investigated to date or with PRV (Padilla *et al.,* 2003).

1.6 The Natural History of VZV Infection:

VZV is highly contagious and causes annual epidemics among susceptible individuals during late winter and spring in temperate climates (Cohen *et al.,* 2007b). Unique amongst the human herpes viruses, VZV is thought to be spread by inhalation of aerosolized virus particles (Brunell, 1989; Tsolia *et al.,* 1990; Sawyer *et al.,* 1994; Arvin *et al.,* 1996). The virus is transmitted in the human population, (its only natural reservoir), either by direct contact with infectious virus in varicella or zoster skin lesions, or by respiratory secretions (Arvin *et al.,* 1996; Cohen *et al.,* 2007b).

Primary VZV infection is associated with viraemia and a diffuse cutaneous rash recognized as varicella, (chickenpox). VZV establishes latency in sensory ganglia and during periods of impaired cellular immunity, reactivation from latency allows the virus to migrate down the nerves to the skin (Esiri and Tomlinson, 1972) and cause herpes zoster (shingles).

1.6.1 Pathogenesis of Varicella:

The restricted infectivity of VZV for non-human species has been a major obstacle in the study of VZV pathogenicity and disease progression. Originally theories of VZV pathogenesis was based upon similarities to the mousepox model (Grose, 1981b). According to this model (see figure 1.12), VZV is presumed to infect mononuclear cells in regional lymph nodes, causing a primary viraemia that carries the virus to reticuloendothelial organs, such as the liver, for a phase of viral amplification, which is followed by a secondary viraemia in the late incubation period that results in VZV transport to the skin.

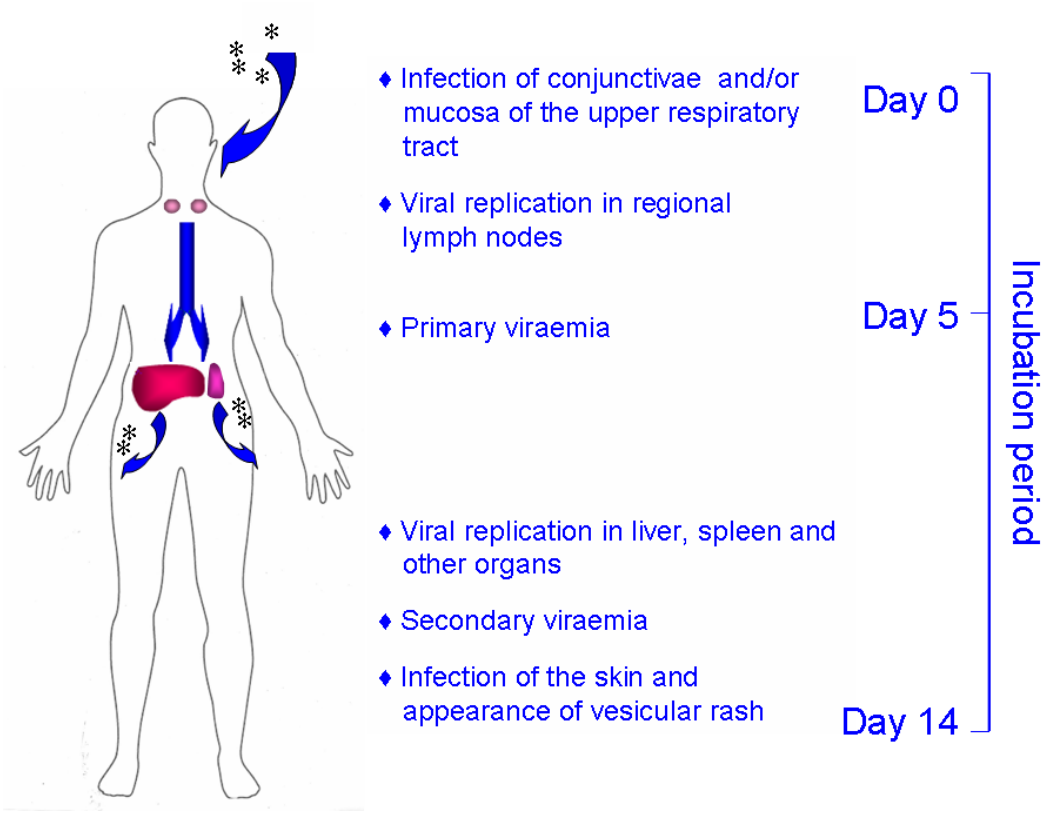


Figure 1.12: Pathogenesis of Varicella; Based on the Mouse Pox Model. According to this model, the incubation period includes two episodes of viraemia: Following virus replication at a local focus, primary viraemia seeds internal organs. A more prolonged secondary viraemia leads to widespread cutaneous infection and characteristic exanthema, which occurs when high titre virus is released from internal sites of multiplication. Not to scale. (Adapted from Grose 1981b, with permission).

Clinical observations of varicella have indicated that VZV is transported from respiratory sites of inoculation (Sawyer *et al.,* 1994) to the skin via a cell associated viraemia (Ozaki *et al.,* 1984; Asano *et al.,* 1990; Ozaki *et al.,* 1994). As with the mousepox model, these observations have also suggested that this viraemia was biphasic, with an asymptomatic phase preceding the onset of the skin rash. Viraemia leads to the dissemination of the virus to the lungs, spleen, liver and other organs, and can cause life-threatening varicella in immunocompromised children, (Asano *et al.,* 1993; LaRussa *et al.,* 2000). Using culture techniques, viraemia has been detected in patients between five days prior and one day post, rash development in several studies (Asano *et al.,* 1985a; Asano *et al.,* 1985b; Ozaki *et al.,* 1984). Detection immediately prior to disease onset was suggested to correspond to secondary viraemia. In the same studies,

viraemia disappeared two days after disease onset and was undetectable when specific immunity to VZV developed (Asano *et al.,* 1985a; Ozaki *et al.,* 1986). However, using PCR amplification viral DNA was detected for longer periods (Ozaki *et al.,* 1994). Although virus could not be isolated from the blood early in the incubation period using culture techniques, viral DNA was detected at the same stage by PCR amplification and was assumed to represent primary viraemia, (Ozaki *et al.,* 1994).

The relatedness of VZV and simian varicella virus (SVV) and the similarities in the clinical symptoms and pathogenesis of human and simian varicella, make SVV infection of non-human primates an excellent animal model to investigate VZV pathogenesis (and latency), (Gray, 2004). SVV is a neurotropic alphaherpesvirus that causes a natural, varicella-like disease in several monkey species including African Green (Gray, 2003; Mahalingam *et al.,* 1998b; Kennedy *et al.,* 2004), St. Kitts vervet (Gray *et al* 1998; Ou *et al.,* 2007) and Cynomolgus monkeys (Mahalingam *et al.,* 2007) as well as Rhesus macaques (Kolappaswamy *et al.,* 2007). The genomes of SVV and VZV are similar in size and structure, co-linear with respect to gene organization and share 70-75% DNA homology. Experimental infection of African Green monkeys is followed by a 7-10 day incubation period during which a viraemia disseminates the virus throughout the host (Gray, 2003). Clinical disease is characterized by fever and a vesicular skin rash and is highly contagious. Pneumonia and hepatitis may occur during more severe infections. Examination of acutely infected tissues reveals histopathology including necrosis and haemorrhage in the skin, lung, liver, and spleen. Host immune responses are induced which resolve the acute infection within 14-21 days (Mahalingam *et al.,* 2007). However, research with this model has only been carried out in a limited number of laboratories, due to both the lack of availability of appropriate facilities and the economic burden associated with working with such a model.

In addition to simian models, several small laboratory animals (rats, rabbits and guinea pigs) have been used as models of VZV pathogenesis; (Myers *et al.,* 1980; Matsunaga *et al.,* 1982). However, animal-to-animal spread, viraemia, and exanthem have only been demonstrated in guinea pigs, (Myers and Connelly, 1992). The combined use of guinea pigs and guinea pig adapted viral strains is a good model of VZV pathogenesis as (providing the guinea pigs body temperature (39.3^{o}C) is kept below the viral replication shut-off temperature (39^{o}C)), replicating viruses can reach the skin and cause lesions (Myers and Connelly, 1992; Yamanishi *et al.,* 1980). Various guinea pig strains including Weanling, Hartley, strain-2 and hairless have proved useful (Yamanishi *et al.,* 1980; Lowry *et al.,* 1993; Sabella *et al.,* 1993) and viraemia is detectable during primary infection in about half of animals used regardless of the strain of guinea pig. Experiments in Weanling guinea pigs demonstrated that animals inoculated intranasally or subcutaneously with VZV shed virus from the nasopharynx (Yamanishi *et al.,* 1980).

However, the development of the SCID-Hu mouse model for VZV (Moffat *et al.,* 1995) greatly enhanced knowledge of VZV pathogenicity, and has led to a new model being proposed (see figure 1.13). Due to their immunodeficient state, SCID-Hu mice cannot reject human tissue xenografts and also lack the capacity to develop an adaptive immune response against the VZV infection within the engrafted tissues. This has made it possible to evaluate VZV interactions in human target cells located within an intact tissue microenvironment. Recently the use of this model was combined with the emerging technique of live whole-animal imaging (Zhang *et al.,* 2007; Oliver *et al.,* 2008). This technique allows for the measurement of proteins expressed *in vivo* using luminescence and will undoubtedly lead to a greater understanding of VZV pathogenesis in the future.

However thus far, the use of the VZV SCID-Hu model has demonstrated that lesions in skin xenografts involve the epidermis and dermis; typical of the patterns observed in biopsies of varicella and zoster lesions. In addition, both human $CD8^+$ and $CD4^+$ T cells in human fetal thymus/liver xenografts support VZV replication (Moffat *et al.,* 1995) as well as epithelial cells in human skin xenografts. By analogy to the mousepox model (Grose, 1981b) it is believed following inhalation by the host, the virus infects the mucous membranes in the respiratory tract. Respiratory epithelial cells cover tonsil surfaces and penetrate into tonsillar tissues, forming 'crypts'. These crypts are adjacent to foci of mononuclear cells which contain activated memory T cells. Ku (*et al.,* 2002) found that tonsil T cells in the SCID-Hu mouse model are highly susceptible to VZV infection. Furthermore, memory $CD4^+$ T cells that express skin homing markers, cutaneous leucocyte antigen (CLA) and chemokine receptor 4 (CCR4), are most likely to be infected. As well as tonsils, other lymphoid tissues of the upper respiratory tract, such as the adenoids, contain this subset of T cells, which may become infected when VZV infects respiratory mucosa (Ku *et al.,* 2004).

Memory T cells that express these receptors carry out immune surveillance by trafficking through the skin, thus the increased susceptibility of this subset of memory T cells to VZV infection may further enhance their role in VZV pathogenesis (Ku *et al.,* 2004). According to the model in figure 1.13 tonsillar T cells infected with VZV enter the circulation and transport the virus to the skin shortly thereafter; it is the viral spread to the skin that causes the lesions that constitute the characteristic rash of varicella (Arvin *et al.,* 1996). When SCID-Hu mice were inoculated via the tail vein with VZV-infected tonsil T cells, it took only 24 hours for the human T cells to appear in the skin xenografts, with an early distribution around the hair follicles (Ku *et al.,* 2004).

As a clinical correlate, VZV dissemination to skin may be modulated in young children, who have few memory T cells, whereas adults with varicella who have <75% memory $CD4^+$ T cells usually develop many cutaneous lesions (Arvin, 2001c). This is also seen with vaccine-related rashes (discussed in section 1.8); in most children, cases consist of fewer than 10 lesions, but in adults this can be up to 50 (Weibel *et al.,* 1984; Arbeter *et al.,* 1986).

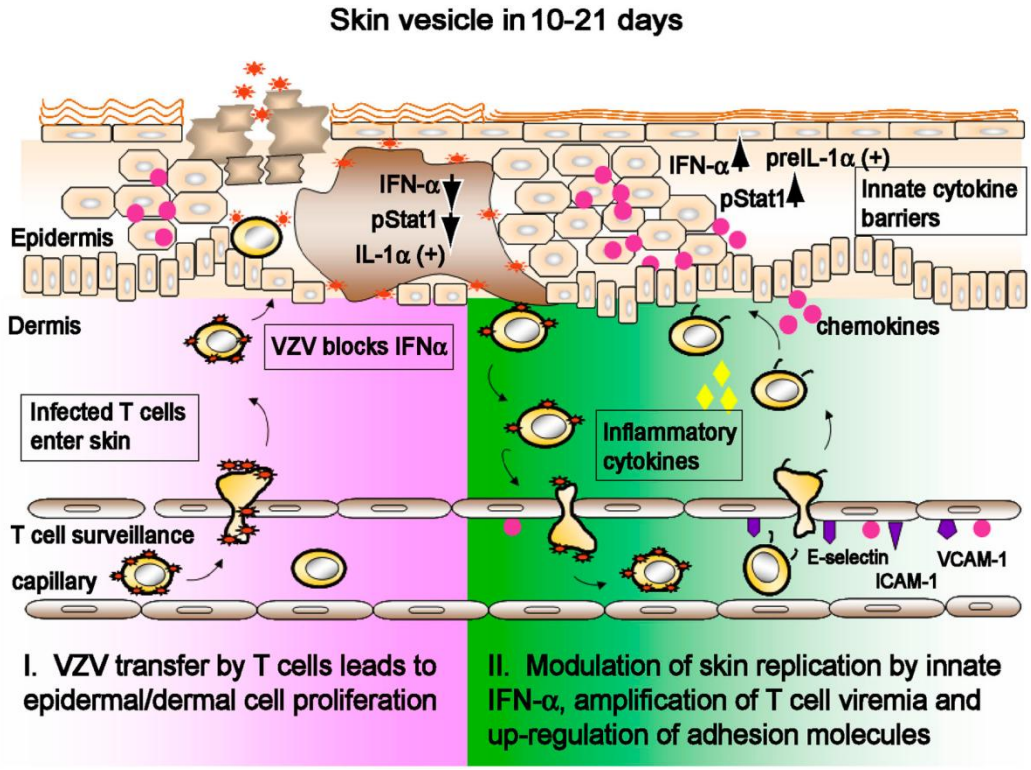


Figure 1.13. A Model of the Pathogenesis of Primary VZV Infection. T cells within the local lymphoid tissue of the respiratory tract may become infected by transfer of VZV from its initial site of inoculation in respiratory epithelial cells. T cells may then traffic the virus to the skin immediately and release infectious VZV. The remainder of the 10–21 day incubation period appears to be the interval required for VZV to overcome the innate IFN-α response in enough epidermal cells to create the typical vesicular lesions containing cell-free virus at the skin surface. The signaling of enhanced IFN-α production in adjacent skin cells may prevent a rapid, uncontrolled cell-to-cell spread of VZV. Secondary "crops" of varicella lesions may result when T cells traffic through early stage cutaneous lesions become infected and produce a secondary viraemia. Intact host immune responses appear to be required to trigger up-regulation of adhesion molecules, facilitating the clearance of VZV by adaptive immunity. Not to scale. (Ku *et al.,* 2004, reprinted with permission)

Recent data from Sri Lankan adults hospitalized with varicella demonstrated that the clinical disease severity score increased significantly with advancing age and that there was a positive correlation between age and peripheral blood viral load during disease. Patients with >500 skin lesions had significantly higher viral loads than patients with fewer lesions, and thus the number of skin lesions appears to be a good clinical indicator of viral loads in peripheral blood (Malavige *et al*., 2008b). The number of lesions has also been demonstrated to correlate with the maximum body temperature and duration of fever (Asano *et al.,* 1990).

In healthy individuals, the estimated proporrtion of PBMCs infected varies depending on the experimental technique but ranges from 0.01% to 0.001% (Koropchak *et al.,* 1989; Koropchak *et al.,* 1991; Mainka *et al.,* 1998). In guinea pig models the frequency of VZV-infected PBMCs is estimated to be at least 1 in 200,000, which is comparable to that observed in human infection (Lowry *et al.,* 1993). Data from *in vitro* studies has shown that VZV also infects B cells, monocytes, (Koenig and Wolff, 2003) macrophages, (Arbeit *et al.,* 1982), hepatocytes (Shiraki *et al.,* 2003) as well as mature (Morrow *et al.,* 2003) and immature (Abendroth *et al.,* 2001b) dendritic cells (DCs). These observations have led to the suggestion that it may be the ability of the virus to infect immature DCs of the respiratory mucosa, which subsequently transport virus to the T cell-rich draining lymph nodes, which results in T-lymphocyte infection and subsequent dissemination of virus to other sites (Abendroth *et al.,* 2001b), see figure 1.14.

1.6.1.1 Rash Formation and the Host Immune Response:

The characteristic chickenpox rash develops after an incubation period of 10 to 21 days (Arvin, 2001c; Cohen *et al.,* 2007b). The remainder of this incubation period could be the interval required for VZV to overcome the innate IFN-α response in enough epidermal cells to create the typical vesicular lesions at the skin surface (Ku *et al.,* 2004). (See section 1.7.6.1 for more details on the role of IFN-α in VZV infection). Signalling of

enhanced IFN-α production in adjacent skin cells prevents a rapid, uncontrolled cell-to-cell spread of VZV. Additional "crops" of varicella lesions may result when T cells traffic through early-stage cutaneous lesions, become infected, and produce a secondary viraemia. This process continues until host immune responses trigger the up-regulation of adhesion molecules (such as ICAM-1, VCAM-1 and E-selectin) and mediate the clearance of the virus by VZV-specific antiviral T cells.

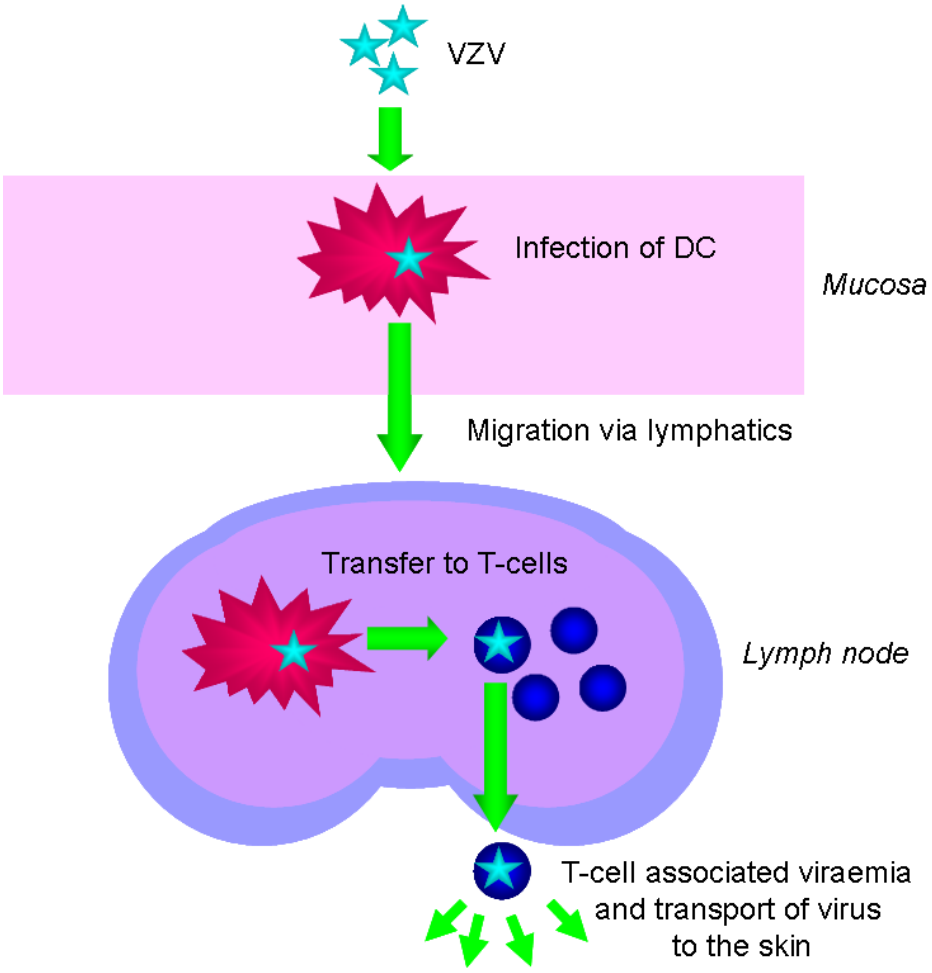


Figure 1.14: A Model of VZV Dissemination Following Primary Inoculation. The virus enters the host at respiratory mucosa and subsequently infects dendritic cells which are then triggered to migrate to the regional lymph nodes. It is here that the infected DCs interact with T cells, resulting in virus transmission and productive infection of these T cells. Following T cell infection the virus continues to replicate and is disseminated to other sites of the body, infecting cutaneous epithelial cells with the formation of the characteristic vesicular rash of varicella. Not to scale. (Bosnjak *et al.,* 2005; adapted with permission)

Data on the incidence and severity of vaccine-related rashes in immunocompromised compared to immunocompetent children also lends support to the role of the host immune system in controlling rash formation. The frequency of vaccine-related-rashes in healthy children is about five percent, but most consist of fewer than 10 lesions (Weibel *et al.,* 1984). However up to 40-50% of leukaemic children receiving chemotherapy develop a vaccine associated rash, (usually maculopapular and vesicular) with around 80 lesions, although there have been cases of up to 1,000 lesions (Arvin and Gershon 1996; Gershon and Steinberg 1989; Gershon *et al.,* 1992; LaRussa *et al.,* 1996). In addition to this, severe, disseminated life-threatening infections have occurred when the varicella vaccine has been inadvertently administered to immunocompromised children (Kramer *et al.*, 2001; Sharrar *et al.,* 2000; Ghaffar *et al.,* 2000; Levy *et al.,* 2003).

1.6.1.2 The Role of Individual VZV Proteins in Varicella Pathogenesis:

Table 1.6 summarizes what is known to date about the requirement of different VZV proteins for replication either *in vitro* or in skin and T cells in the SCID-Hu mouse model (*in vivo*). Of the VZV proteins investigated so far in the SCID-Hu mouse model, products of ORF23, IE62, gE and gI are essential for skin replication, and without these proteins virus cannot be recovered, whilst ORF47, IE62, IE63, gE and gI are essential for replication in T cells, whilst numerous proteins are important 'virulence factors' for growth and replication (see table 1.6 for references). If VZV pathogenesis depends on the assembly and release of virions from T cells then the infected cells must survive long enough to transport VZV from respiratory sites of inoculation to the skin. ORF66 protein kinase appears to have a unique role in viral replication in T cells. This protein supports VZV T cell tropism by contributing to immune evasion and enhancing survival of infected T cells (Schaap *et al.,* 2005). Preventing ORF66 protein kinase expression impairs the growth of virus in SCID-Hu T cell xenografts, and increases the susceptibility of infected T cells to apoptosis (Schaap *et al.,* 2005). For more details on the role of ORF66 in immune evasion see section 1.7.3.

Findings by Besser (*et al.,* 2004) using ORF47 deletion mutant viruses suggest a differential requirement for cell fusion and virion formation in the pathogenesis of VZV infection in skin and T cells. Evaluation of two recombinants of the Oka strain of VZV showed that ORF47 kinase function was necessary for optimal viral replication in human skin xenografts in SCID mice, but not T cells. Although virion assembly was reduced and no virion transport to cell surfaces was observed, epidermal cell fusion persisted, and VZV polykaryocytes were generated by both mutants in skin.

Normal VZV virion assembly requires ORF47 kinase function. Thus, cell fusion was induced by ORF47 mutants in skin, and cell-to-cell spread occurred even though virion formation was deficient. VZV-infected T cells did not undergo cell fusion, and impaired virion assembly by ORF47 mutants was associated with a complete elimination of T cell infectivity. However, ORF35 has also been demonstrated to be required for efficient infection of both skin and T cell xenographs, but is dispensable for viral replication (Ito *et al.,* 2005).

ORF63 is expressed in neurons during both productive and latent infection. Most mutations of ORF63 made with the VZV cosmid system prove lethal for infectivity. The few IE63 changes that are tolerated result in VZV mutants with an impaired capacity to replicate *in vitro*. However, the IE63 mutants are attenuated in skin but not T cells in the SCID-Hu mouse model indicating that the contribution of the IE63 tegument/regulatory protein to VZV pathogenesis depends upon the differentiated human cell type which is targeted for infection within the intact tissue microenvironment (Baiker *et al.,* 2004b). A recent study by Hood (*et al.,* 2006) showed that ORF63 can suppress apoptosis of neurons. This may play a significant role in viral pathogenesis by promoting neuron survival during primary and reactivated infections.

ORF	Function	Replication in T cells		Replication in Skin		Virus Transfer from T cells to Skin	Establishment of Latency	References
		In vitro	*In vivo*	*In vitro*	*In vivo*			
0	Putative membrane Protein	Not Investigated	Not Investigated	Virulence factor[2]	Virulence factor[2]	Not Investigated	Not Investigated	Zhang *et al.,* 2007
1	Membrane protein	No[1]	Not Investigated	No[1/2]	No[2]	No[1]	No	Sato *et al.,* 2003b; Soong *et al*., 2000; Cohen and Seidel, 1995; Zhang *et al*., 2007
2	Unknown	Not Investigated	Not Investigated	No[2]	No[2]	Not Investigated	No	Sato *et al.,* 2002b; Zhang *et al.,* 2007
3	Unknown	Not Investigated	Not Investigated	No[2]	No[2]	Not Investigated	Not Investigated	Zhang *et al.,* 2007
4	Transcriptional regulator	Not Investigated	Not Investigated	Yes[2]	Not Investigated	Not Investigated	No (Impaired)*	Cohen *et al.,* 2005b; Sato *et al*., 2003d; Zhang *et al*., 2007
5	Glycoprotein K	Not Investigated	Not Investigated	Yes[2]	Not Investigated	Not Investigated	Not Investigated	Mo *et al.,* 1999; Cohen and Seidel, 1994b
9	Virion	Yes	Not Investigated	Yes	Not Investigated	Not Investigated	Not Investigated	Tischer *et al*., 2007; Che *et al.,* 2008
10	Transcriptional regulator	No[a]	No[1/2]	No[1/a]	Virulence factor[a]	Not Investigated	No	Che *et al.,* 2006; Sato *et al*., 2003b; Cohen and Seidel, 1994a

Table 1.6 'Essentiality of VZV ORFs for Replication in Skin and T Cells' Continued (1of 5)

ORF	Function	Replication in T cells		Replication in Skin		Virus Transfer from T cells to Skin	Establishment of Latency	References
		In vitro	*In vivo*	*In vitro*	*In vivo*			
11	Unknown	No[a]	Not Investigated	No[a]	Virulence factor[a]	Not Investigated	Not Investigated	Che *et al.,* 2008
12	Unknown	No[a]	Not Investigated	No[a]	(No)[a]	Not Investigated	Not Investigated	Che *et al.,* 2008
13	Thymidylate synthetase	No[1]	Not Investigated	No[1]	Not Investigated	No[1]	No	Sato *et al.,* 2003b; Soong *et al*., 2000; Cohen and Seidel, 1993
14	Glycoprotein C	Not Investigated	Not Investigated	No[1/2]	Virulence factor [2]	Not Investigated	No	Moffat *et al.,* 1998a; Grinfield *et al*., 2004b
19	Ribonucleoltide reductase	Not Investigated	Not Investigated	Virulence factor[2]	Not Investigated	Not Investigated	Not Investigated	Heineman and Cohen, 1994
21	Unknown	Not Investigated	Not Investigated	Not Investigated	Not Investigated	Not Investigated	No	Xia *et al.,* 2003
23	Capsid protein	Not Investigated	Not Investigated	No (Capsid assembly disrupted)	Yes	Not Investigated	No	Chaudhuri *et al.,* 2008
29	SS-DNA binding protein	Not Investigated	Not Investigated	Yes[2]	Not Investigated	Not Investigated	No (Impaired)♦	Cohen *et al.,* 2007a

Table 1.6 'Essentiality of VZV ORFs for Replication in Skin and T Cells' Continued (2of 5)

ORF	Function	Replication in T cells		Replication in Skin		Virus Transfer from T cells to Skin	Establishment of Latency	References
		In vitro	*In vivo*	*In vitro*	*In vivo*			
32	Unknown	No[1]	Not Investigated	No	Not Investigated	No[2]	No	Sato *et al.,* 2003b; Soong *et al*., 2000; Ito *et al*., 2005; Reddy *et al*., 1998
35	Unknown	No[2]	Not Investigated	Yes[2]	No[2]	Not Investigated	Not Investigated	Ito *et al.,* 2005
47[£]	Serine-threonine kinase	Virulence factor	Yes[b/1]	No[1]	Virulence factor [1/4]	Virulence factor	No	Sato *et al.,* 2003b; Soong *et al*., 2000; Moffat *et al*., 1998b; Heinman and Cohen, 1995; Reddy *et al*., 1998a; Besser *et al.,* 2003; Besser *et al*., 2004
50	gM	Not Investigated	Not Investigated	Virulence factor; MRC_5	Not Investigated	Not Investigated	Not Investigated	Yamagishi *et al.,* 2008
57	Unknown	No[1]	Not Investigated	No[2]	Not Investigated	No[2]	No	Soong *et al.,* 2000;Cox *et al*., 1998; Sato *et al*., 2003b

Table 1.6 ‘Essentiality of VZV ORFs for Replication in Skin and T Cells’ Continued (3 of 5)

ORF	Function	Replication in T cells		Replication in Skin		Virus Transfer from T cells to Skin	Establishment of Latency	References
		In vitro	*In vivo*	*In vitro*	*In vivo*			
58	Unknown	Not Investigated	Not Investigated	No[2] (MRC_5)	Not Investigated	Not Investigated	Not Investigated	Yoshii *et al.,* 2008
61	Transcriptional regulator	Not Investigated	Not Investigated	Virulence factor	Not Investigated	Not Investigated	No	Cohen and Nguyen, 1998; Sato *et al.,* 2003c
62/71[c]	Transcriptional regulator	Not Investigated	Yes[2]	Yes[2]	Yes[2/♠]	Not Investigated	Not Investigated	Sato *et al.,* 2003a;
63	Transcriptional regulator	No[2]	No[7]	Yes[7/2]	Virulence factor[7]	Not Investigated	Yes	Cohen *et al.,* 2005a; Baiker *et al*., 2004b;
63/70	Transcriptional regulator	Not Investigated	Yes[2]	Yes[2]	Not Investigated	Not Investigated	Yes[§]	Cohen *et al.,* 2004; Baiker *et al*., 2004b; Sommer *et al*., 2001
64	Unknown	No	Not Investigated	Not Investigated	Not Investigated	Not Investigated	Not Investigated	Sommer *et al.,* 2001
64/69[d]	Unknown	No	Not Investigated	Not Investigated	Not Investigated	Not Investigated	Not Investigated	Sommer *et al.,* 2001
65	Unknown	Not Investigated	No[2]	No[2]	No[2]	Not Investigated	Not Investigated	Cohen *et al.,* 2001; Niizuma *et al*., 2003

Table 1.6 'Essentiality of VZV ORFs for Replication in Skin and T Cells' Continued (4 of 5)

[a]Deletion mutant virus lacking either; ORF 10, 11, 12 or (10/11); (11/12); or (10/11/12) were dispensible for replication in skin *in vitro*; ORFs 11, 12 or (10/11/12) were dispensable T cell tropism *in vitro*, ORF 11, (10/11) and (11/12) were virulence factors in skin *in vivo*, ORF12 alone was dispensable for replication in skin *in vivo*, but (10/11/12) was essential for skin infection *in vivo* (Che *et al.,* 2008).

[b]Also important for replication in immature DCs;

[c]At least one copy of diploid gene required;

[d]Neither copy of diploid gene required;

*Cotton rats inoculated with VZV lacking ORF4 showed reduced frequency of latency compared to animals infected with the parental or ORF4-rescued virus. Ganglia were infected at the same rate as WT, but lack the ability to persist in ganglia, (Cohen *et al.,* 2005b).

[£]ORF47 kinase activity which is essential for infection and replication in human skin *in vivo*, is due to the formation of complexes between the ORF47 and IE62 proteins (Besser *et al.,* 2003).

♠Viruses with both genes deleted, but with rescued vOka62 or pOka62 were not infectious *in vivo* or replicated to a very limited extent in only one implant of all of the skin xenografts that were inoculated with these viruses (Sato *et al.,* 2003a).

[ξ]19 of 22 changes in the coding sequence were lethal for VZV replication *in vitro*

♣ORF63 is not required for VZV to enter ganglia but is critical for establishment of latency.

♦Cotton rats infected with the ORF29 deletion mutant, or a virus over-expressing ORF29 had markedly reduced frequency of latent infection in dorsal root ganglia compared to those infected with parental virus, indicating that regulation of ORF29 at appropriate levels is critical for VZV latency in the rodent model (Cohen *et al.,* 2007a).

ORF	Function	Replication in T cells		Replication in Skin		Virus Transfer from T cells to Skin	Establishment of Latency	References
		In vitro	*In vivo*	*In vitro*	*In vivo*			
66	Serine-threonine kinase	Virulence factor	Virulence factor[1]	No[1/2]	Virulence factor[1]	Virulence factor[1]	No	Soong *et al.,* 2000; Sato *et al.,* 2003b; Schaap *et al.,* 2005; Schaap-Nutt *et al.,* 2006; Moffat *et al.,* 1998b; Heinman *et al.,* 1996
67	Glycoprotein I	No[3]	Yes[2]	No[2/8]	Yes[2/3]	Not Investigated	No	Grinfield *et al.,* 2004b; Cohen *et al.,* 1997; Mo *et al.,* 2002; Moffat *et al.,* 2002; Ito *et al.,* 2003; Mallory *et al.,* 1997;
68	Glycoprotein E	Not Investigated	Yes[2/5]	Yes[6]	Yes[6]	Not Investigated	Not Investigated	Mo *et al.,* 2002; Mallory *et al.,* 1997; Berarducci *et al*., 2006; Moffat *et al.,* 2004a

Table 1.6 Essentiality of VZV Open Reading Frames for Replication in Skin and T Cells *In Vitro and In Vivo* (SCID-Hu Mouse Model). (5 of 5)

Key: **Yes:** gene is essential (no virus is recovered if this gene is deleted, or stop codon inserted); **No;** gene is not essential (very little effect, if any, on viral growth and replication); **Virulence factor**: Infection/replication/growth is possible without this gene, but is severely reduced/impaired; [1]Stop codon inserted into gene; [2]Gene deleted; [3]Mutation in promoter site that binds cellular transcription factors; [4]Point mutation in conserved kinase motif; [5]Point mutation in endocytotic signal motif/deletion of C-terminal domain; [6]Mutation in gE trans-Golgi network targeting motif/gE phosphorylation motif; [7]Mutation in IE63 single putative phosphorylation sites or deletion of IE63 nuclear localisation signal. [8] VZV gI is not essential for viral replication *in vitro*, but impaired cell-to-cell spread and altered posttranslational modification and trafficking of gE were shown in cells infected with VZV gI deletion mutant.

The ORF10 protein is required for efficient viral replication, virion assembly, and cell-to-cell spread in human skin in the SCID-Hu mouse model, but is not required for replication in T cells in this model, (Che *et al.,* 2006). The cellular transcription factor USF is required as a co-factor with IE62 to produce the ORF10 protein in infected cells *in vitro* and is a direct determinant of VZV skin virulence in the SCID-Hu mouse model (Che *et al.,* 2007). *In vitro*, ORFs 10, 11 and 12 are dispensable for replication in melanoma and T cells and appear to have no role in nucleocapsid formation and virion assembly. Deletion of all three ORFs simultaneously has no effect on growth kinetics or infectivity *in vitro*. However, in the SCID-Hu mouse model ORF12 is also dispensable, whereas ORF11 is an important virulence factor for skin. When both ORF10 and ORF11 were deleted along with ORF12, this resulted in a complete loss of infectivity. This indicates that compensating mechanisms, which permit some replication when ORF10 and ORF11 are deleted, do not overcome the incremental effect of deleting ORF12 from the VZV genome. However ORF9 is essential for replication *in vitro*. It has been suggested that this maybe because ORF9 has critical functions at different steps of VZV virion assembly; through its interactions with gE as well as IE62, (Che *et al.,* 2008).

1.6.2 Pathogenesis of Latency and Reactivation:

VZV establishes latency in the primary afferent neurons of dorsal root and cranial nerve ganglia, (Hope-Simpson, 1965) and in neurons of the enteric nervous system (Gershon *et al.,* 2008). The distribution of zoster lesions typically involves trigeminal and thoracic dermatomes which are in the anatomical regions where the majority of varicella lesions appear (on the face and trunk), (Ragozzino *et al*, 1982). This clinical observation indicates that during varicella, the virus spreads directly by retrograde axonal transport from infected epidermal and dermal tissues to the dorsal root ganglia (Straus and Oxman, 1999). In addition, studies of vaccine-associated zoster have demonstrated that reactivation commonly occurs at or near the vaccine injection site, suggesting that latency was established in sensory ganglia serving that particular dermatome (Hardy *et*

al., 1991; Lawrence *et al.,* 1988). However in cases of fatal varicella, analysis of ganglia recovered at autopsy, suggest that the virus may reach the dorsal root ganglia via systemic viraemia (Cheatham *et al.,* 1956). In such cases, satellite cells positive for virus have been found surrounding neurons which are virus negative (Croen *et al.,* 1988). This suggests that had the virus travelled through the sensory neuron and spread through the ganglia to the neighbouring satellite cells, both cell populations would have been virus positive.

However, recent data from a model of lytic infection, latency and reactivation in enteric neurons from adult guinea pigs and foetal mice, suggested the former hypothesis of retrograde spread from the epidermis to be correct, (Gershon *et al.,* 2008). In this newly developed model, latency was established when neurons were infected by cell-free virus in the absence of fibroblasts or other cells. Whilst in contrast, lytic infection occurred either when fibroblasts were present, or when the enteric neurons were infected by cell-associated VZV. Based on these observations, and previous studies (Chen *et al.,* 2004), the authors suggested that as abundant cell-free virus is only produced in the epidermis, and the sensory nerves which serve this site are present as naked nerve fibres; in VZV-infected regions of skin, nerve fibres would be infected by the cell-free virus, (Gershon *et al.,* 2008).

As mentioned earlier, the onset of zoster is caused by reactivation of latent VZV from the dorsal root ganglia, where the virus multiplies and spreads within the ganglion. This causes neuronal necrosis and intense inflammation, often resulting in severe neuralgia. The virus then spreads antidromically down the sensory nerve, causing intense neuritis. The virus is subsequently released from sensory nerve endings in the skin where it spreads to produce the characteristic cluster of zoster vesicles. Typically, this results in a unilateral vesicular rash within a single cutaneous dermatome. The rash is often accompanied by debilitating acute pain that can last for several weeks.

Latency (and in some cases reactivation) have been studied in various models, including SVV in the simian model. SVV establishes life-long latent infection, as indicated by detection of viral DNA within neural ganglia. Subsequently, SVV may reactivate to cause secondary disease and spread the virus to susceptible monkeys (Mahalingam *et al.,* 2007). Studies of guinea pig enteric neurons (Chen *et al.,* 2003; Stallings *et al.,* 2006; Walters, 2008) have also been carried out and have demonstrated that VZV gene sequences are detectable in ganglia tissue for at least 80 days after infection (Lowry *et al.,* 1993). More recently human ganglionic explants in SCID-Hu mice have been used (Zerboni *et al.,* 2005b; Zerboni *et al.,* 2007) but a vast quantity of knowledge has been gained from earlier studies in the cotton rat model.

In the cotton rat model, latency is characterized by a long-lasting presence of the viral genome and selected viral gene transcription in the dorsal root ganglia (Sadzot-Delvaux *et al.,* 1990). Many similarities with latency in humans have been found, including the persistence of the virus in the ganglia for at least two years (Grinfeld *et al.,* 2004a), the largely neuronal localization of the virus, and the expression of a similar subset of genes in both systems during ganglionic latency (Kennedy *et al.,* 2000; Kennedy *et al.,* 2001; Sadzot-Delvaux *et al.,* 1995). However, it is important to note that there are many differences. For example, more satellite cells are found to be positive in infected ganglia in the rat model than in human latency, (Kennedy *et al.,* 2001) as well as in axons (Debrus *et al.,* 1995), something not observed in human ganglia. Unlike in humans, the late VZV gene encoded by ORF40 has been shown to be expressed in the rat model (Kennedy *et al.,* 2001). VZV reactivation has not been obtained in rats *in vivo* which is an important criterion of viral latency, but can be induced *ex vivo* after repeated stresses (Sadzot-Delvaux *et al.,* 1995). Despite the shortcomings of this model it has been widely used to evaluate the contribution of virus genes in the establishment of putative ganglionic latency (Grinfeld *et al.,* 2004b, Cohen *et al.,* 2005a, Cohen *et al.,* 2005b; Grinfeld *et al.,* 2007a; Annunziato *et al.,* 2001; Sato *et al.* 2003c and 2003d; Hoover *et al.,* 2006; Cohen *et al.,* 2007).

As mentioned above, the SCID-Hu mouse model has also been used to study latency; when human dorsal root ganglia (DRG) were successfully transplanted, clinical isolates of VZV persisted in the ganglia without any requirement for VZV-specific adaptive immunity (Zerboni *et al.,* 2005b). This was associated with continued transcription of the ORF63 regulatory gene. The live attenuated varicella vaccine virus exhibited the same pattern of short-term replication, persistence of viral DNA and prominent ORF63 transcription, comparable to clinical isolates. VZV-infected T cells transferred virus from the circulation into DRG, suggesting that VZV lymphotropism facilitates its neurotropism.

Recently a more detailed study of the cellular tropisms and mechanisms of neuropathogenesis during acute infection of human DRG xenografts has been carried out in this model using multiscale correlative immunofluorescence-electron microscopy (Reichelt *et al.,* 2008). The observations from this study suggest a model in which the first event in the acute infection of sensory ganglia, caused by VZV reactivation from latency is viral replication in a single neuron. This leads to polykaryon formation with adjacent satellite cells and the subsequent release of virions which infect nearby satellite cells which surround other neuronal cell bodies. This process leads to further polykaryon formation and it is polykaryon formation between these secondarily infected satellite cells and their neuronal cell bodies which enhances access of virions to neuronal axons for transport to skin sites of replication. This model predicts that most neurons become infected by virions that are released into the DRG by productively infected satellite cells, not by the simultaneous reactivation of latent VZV genomes harboured by multiple neurons within the affected ganglion.

Another model to aid in our understanding of latency is infection in human intact explant DRG which has recently been developed (Gowrishankar *et al.,* 2007). This model enables VZV infection of ganglionic cells to be studied in the context of intact DRG, but results in cell free VZV being

released from infected DRG, rather than remaining highly cell associated (Gowrishankar *et al.,* 2007).

1.6.2.1 The Role of Individual VZV Proteins in the Pathogenesis of Latency:

Various studies designed to determine the extent of VZV transcription in latently infected human ganglia have yielded different results. *In situ* hybridization using gene-specific oligonucleotide probes detected transcripts from VZV genes 4, 18, 21, 29, 40, 62, and 63 but not genes 28 or 61 (Kennedy *et al.,* 1999; Kennedy *et al.,* 2000). However, using sequence analysis from RNA extracted from latently infected human ganglia, Cohrs and colleagues detected transcripts mapping to only five VZV genes: 21, 29, 62, 63, and 66 but not to VZV genes 4, 10, 40, 51, or 61 (Cohrs *et al.,* 1996; Cohrs and Gilden, 2003). Proteins encoded by ORFs 4, 21, 29, 62 and 63 have been detected in human ganglia in a separate report (Lungu *et al.,* 1998). In studies to date only the ORF21 protein has not been shown to induce an immune response; see section 3.1.3 (chapter 3) and 4.1.4 (chapter 4) for more details. Prevalence and abundance of multiple VZV gene transcripts in human ganglia has also produced conflicting results (Cohrs *et al.,* 2000, Cohrs and Gilden, 2007). Looking at ganglia samples from the same individuals, the 2000 study found VZV gene 63 transcripts were detected in either or both ganglia from all of 11 subjects (100%), VZV gene 29 transcripts were detected in 2 of 11 (18%), and VZV gene 21 transcripts were detected in 4 of 11 (36%) subjects. Compared to VZV gene 21 transcripts, VZV gene 63 and 29 transcripts were 22 and 26 fold more abundant. In the more recent study, analysis of 28 trigeminal ganglia (from 14 humans) revealed that the most prevalent transcript found was VZV gene 63 (78%), followed by gene 66 (43%), gene 62 (36%), and gene 29 (21%), (Cohrs and Gilden, 2007). Interestingly, despite the use of PCR primers and probes identical to those used in a previous study, VZV gene 21 transcripts were not detected in any of the 28 ganglia in this study. Both studies confirmed that VZV gene 63 is the most prevalent and abundant transcript, and revealed that VZV gene 29 transcripts are detected more often than VZV gene 21 transcripts.

This suggests that gene 63 may be more important for the maintenance of virus latency than the less abundantly transcribed and randomly detected VZV genes 21, 29, 62, and 66.

Thus far only ORFs 4 and 63 have been shown to be essential to the establishment of latency (Cohen *et al.,* 2005b; Cohen *et al.,* 2004). This was demonstrated via use of the cotton rat model infected with mutant viruses, with either the ORF 4 gene or >90% of both copies of the ORF63 genes respectively deleted. ORFs 2, 14, 21, 47, 61, 66 and 67 have been shown to be dispensable for the establishment of latency: (Xia *et al.,* 2003; Sato *et al.,* 2003c; Sato *et al.,* 2002b; Grinfeld *et al.,* 2004b; Sato *et al.,* 2003d), whilst ORF29 has recently been shown to be dispensable for the initial infection of DRG, but important in the establishment of latency in the cotton rat model (Cohen *et al.,* 2007). Some establishment of latency still occurs with ORF29 deleted VZV mutants, but this is markedly reduced compared to that seen in the parental virus. The proteins transcribed during latency are localised in the cytoplasm of infected neurons of dorsal root ganglia (Lungu *et al.,* 1998). This sharply contrasts with the nuclear localisation of these VZV proteins during reactivation, (Lungu *et al.,* 1998). ORF21 is the only transcript which has not been shown to invoke an immune response.

Some of these VZV proteins may restrict regulatory proteins from entering the nucleus, thereby maintaining a latent state in the presence of the essential immediate-early protein product of ORF62, an efficient transactivator of VZV gene transcription. The protein kinase encoded by ORF66 (66-pk), phosphorylates the protein product of ORF62, leading to its cytoplasmic accumulation (Cohrs *et al.,* 2003b). Prevention of IE62 import to the nucleus by VZV 66-pk phosphorylation is one possible mechanism by which VZV latency is maintained (Cohrs *et al.,* 2003b). Another mechanism is the ability of ORF63 to downregulate ORF62 transcription, which may play an important role in virus replication and latency (Hoover *et al.,* 2006). ORF61 is also thought to play an important role in viral reactivation. In the rodent enteric neuron model, when latency

was established, reactivation was accomplished by expression of ORF61p (or its HSV homologue ICP0), (Gershon *et al.,* 2008).

Recent experiments in the SCID-Hu mouse model demonstrated that DRG xenografts with a VZV mutant that lacked gI resulted in highly restricted VZV replication, demonstrating the effect of attenuated cell-to-cell spread in this model (Zerboni *et al.,* 2007). The authors suggested that altered intracellular trafficking of gE (when gI is lacking), is likely to be the mechanism for impaired VZV infection of DRG, as gE–gI complexes have been shown to potentiate polykaryon formation (Maresova *et al.,* 2001). Infection with this particular mutant also caused a prolonged infectious process during which VZV DNA synthesis, transcription and translation of both IE and late genes, and production of infectious virions continued for at least 70 days. This is in contrast to DRG infected with intact VZV, where there was an early transition to persistence (Zerboni *et al.,* 2007).

1.7 Host Immune Responses to VZV, and Immune Modulation and Evasion by the Virus:

Adaptive cell mediated immunity and antibody responses to VZV are discussed at length in chapters 3 and 4. Here, innate immune responses and subsequent modulation of the innate (and the adaptive) immune response are discussed. Innate immune responses are non-specific and lack the ability to improve with each successive exposure. The cellular response is produced primarily by leucocytes such as natural killer (NK) cells, macrophages and neutrophils. The humoral arm of the innate immune system is composed mainly of complement and innate cytokines (Murphy *et al.,* 2008). The early host responses to viruses in particular, involve NK and cytokines such as type-I interferons. They function to restrict virus replication and spread (Arvin, 1996a). However, the innate immune system is unable to prevent progressive disease. The adaptive immune response is subsequently activated with the clonal expansion of VZV-specific T and B cells that interfere with viral replication and spread of the virus, (see chapters 3 and 4).

1.7.1 NK Cell Mediated Immunity and VZV Induced Immune Modulation:

NK cells are a population of innate lymphocytes, which are also referred to as large granular lymphocytes (LGLs) and express neither B nor T cell receptors. These cells have the capacity to recognise the surface changes that occur on virally infected cells which have down regulated their MHC. These cells are able to kill target cells coated with IgG antibodies via their IgG Fc receptors (FcγRIII: CD16) a process known as antibody dependent cellular cytotoxicity (ADCC) which is discussed in more detail in chapter 3, section 3.1.1.5.

NK cells have been shown to lyse VZV-infected fibroblasts *in vitro* and these cytotoxic effects are enhanced by incubating NK cells with IL-2 (Bowden *et al.,* 1985; Ihara *et al.,* 1984; Hayward *et al.,* 1986a). In one report, IFN-α (secreted by HLA-DR$^+$ cells) was found to contribute to NK cell-mediated lysis of VZV infected cells (Oh, *et al.,* 1987). Granulysin is an antiviral protein produced by NK cells (and CTLs) and has been shown to have potent activity against VZV-infected cells (Hata *et al.,* 2001). Granulysin acts to overcome VZV-induced inhibition of apoptosis in infected cells, which can be expected to reduce viral load during acute infection (Hata *et al.,* 2001).

Severe herpes virus infections, including varicella, have been reported in cases of NK deficiency (Biron *et al.,* 1989). Indeed, recurrent acute varicella has proved fatal in the case of a child who had an NK cell deficiency (Etzioni *et al.,* 2005) and in a separate study of five cases of life threatening varicella; reported in children with no history of recurrent infection or immunodeficiency, it was found that NK cells (and CD8$^+$ T cells) were absent from their circulation during active infection (Vossen *et al.,* 2005a). However, in the four patients who recovered both subsets of cells reappeared in the circulation, suggesting that VZV has the unique capability to sequester NK (and CD8$^+$ T cells) from the circulating lymphocyte pool.

1.7.2 Immune Modulation of Dendritic Cells:

The impact of VZV infection on Dendritic cell (DC) maturation and function is emerging as another immune evasion tactic adopted by VZV. In brief, DCs are professional antigen presenting cells that play an important role in the induction of adaptive immune responses to protein antigens. Immature DCs can be found in the epithelia of skin and respiratory sites and express low levels of surface molecules such as MHC and co-stimulatory molecules which are needed to efficiently activate T cells. The main function of immature DCs is to capture antigen and translocate to the nearest draining lymph node. During migration to the lymph node, the immature DCs mature to become extremely efficient at antigen presentation. In their mature state, DCs are capable of stimulating naïve T cells (Murphy *et al.,* 2008). Huch and colleagues (Huch *et al.,* 2006) reported that VZV infection of immature DCs prevented DC maturation *in vitro*. Even LPS induced maturation was strongly inhibited by VZV infection, as demonstrated by a failure to upregulate the cell surface expression of MHC-I, CD86, CD80 and CD83 (CD86; also known as B7.2, and CD80; also known as B7.1, are both essential co-stimulatory molecules which bind to CD28 (or CTLA-4), whilst CD83 is involved in antigen presentation). Induction of cytokines IL-12, IL-10 and TNFα was inhibited compared to mock infected counterparts, and VZV infection also resulted in a significant decrease in DC migration in response to chemokines such as macrophage inflammatory protein-3β (MIP-3β). In addition the authors found that CCR7 expression was absent on the majority of VZV infected DCs, thus they would be unable to migrate to the nearest draining lymph node. VZV infected mature DCs have also been shown to down regulate co-stimulation molecules CD80, CD83 and CD86 (Morrow *et al.,* 2003). More importantly, VZV infection of this cell type significantly reduced the ability of the infected DCs to stimulate the proliferation of allogeneic T cells (Morrow *et al.,* 2003).

1.7.3 Modulation of Apoptosis:

Apoptosis is a process of cell death characterised by nuclear condensation and fragmentation and plasma membrane blebbing which leads to phagocytosis of the cell without inducing an inflammatory response (Murphy *et al.,* 2008). VZV has been shown to elicit anti-apoptosis within various infected cells *in vitro*. Hu and Cohen (2005) found that VZV-infected DCs have reduced cell surface expression of Fas (CD95); an apoptosis inducing receptor, which would thus reduce the likelihood of apoptosis of the cell. Recently, several viral proteins have been shown to play a role in anti-apoptosis *in vitro*. VZV infection of T cells is associated with modulation of apoptosis via the ORF66 protein (Schaap *et al.,* 2005). Preventing the protein's expression increased the susceptibility of infected T cells to apoptosis (and reduced the capacity of the virus to interfere with induction of the interferon pathway by exposure to IFN-γ). ORF66 protein appears to have a unique role that supports VZV T cell tropism by contributing to immune evasion and enhancing survival of infected T cells (Schaap *et al.,* 2005). A later study in human neuronal cells demonstrated that mutant viruses lacking one copy of the diploid gene encoded by ORF63, induced apoptosis, whilst cells infected with parental virus did not undergo apoptosis. The authors suggested that IE63 may play a significant role in viral pathogenesis by promoting neuron survival during primary and reactivated infections (Hood *et al.,* 2006).

It has also been shown that VZV is able to suppress apoptosis induced by the ERK signalling pathway. Pro-apoptotic functions of a key protein in the ERK pathway (Bad) are suppressed by its phosphorylation, and a 10 fold increase of phosphorylation was seen in VZV infected melanoma cells (Rahaus *et al.,* 2006). The role of VZV in signalling pathways is discussed in section 1.7.6-1.7.7.

1.7.4 Immune Modulation of Adhesion Molecules:

Some of the major adhesion molecules expressed on T cells are members of the integrin family. The major functions of T cell integrins are to mediate adhesion to professional APCs, endothelial cells and extra cellular matrix (ECM) proteins. There are two subfamilies of the integrin family; β1 and β2. The β2 family consists solely of LFA-1 (Leukocyte function associated antigen-1, or CD11a/CD18), which is expressed on 90% of mature T cells, B cells, granulocytes and monocytes. A ligand of LFA-1 is Intercellular adhesion molecule-1 (ICAM-1; CD54). ICAM-1 is a membrane glycoprotein that is expressed on a variety of cells including B- and T cells, fibroblasts, keratinocytes and endothelial cells. Surface expression of ICAM-1 has been shown to be down regulated in VZV-infected keratinocytes, despite the expression of pro-inflammatory cytokines such as IFN-γ and TNFα within cutaneous lesions (Nikkels *et al.,* 2004). This may hinder interactions of infected cells with LFA-1-bearing T cells. *In vitro* VZV infection is able to inhibit the expression of ICAM-1 in response to TNFα in infected melanoma cells and fibroblasts and this has been shown to be caused by VZV interfering with the NFκB pathway (El-Mjiyad *et al.,* 2007). In addition, treatment of VZV-infected keratinocytes with IFN-γ *in vitro* has demonstrated that ICAM-1 is upregulated to a much lesser extent than in uninfected keratinocytes (Black *et al.,* 2009).

1.7.5 VZV Modulation of the Major Histocompatibility Complexes (MHC-I and MHC-II):

Interference in the expression of MHC-I and MHC-II proteins (required for $CD4^+$ and $CD8^+$ T cell recognition; see chapter 4, section 4.1.1) has been postulated to be a mechanism by which VZV delays the clearance of virus infected cells (Cohen, 1998; Abendroth *et al.,* 2000; Abendroth *et al.,* 2001a). MHC-I is present on nearly all nucleated cells of the body, (some nucleated cells located in immune privilege sites lack MHC expression). Experiments in the SCID-Hu mouse have demonstrated that MHC-I expression is down regulated on VZV infected T cells in this model (Abendroth *et al.,* 2001a). Down regulation of MHC-I has also been seen

in vitro on mature DCs (Morrow *et al.,* 2003), as well as human melanoma fibroblasts (Eisfeld *et al.,* 2007). Recent experiments in a human derived keratinocyte cell line infected with both parental and vaccine strains of VZV, demonstrated that upregulation of MHC-I following IFN-γ treatment was severely impaired, compared to uninfected controls (Black *et al.,* 2009) and infected keratinocytes were unable to stimulate antigen specific $CD8^+$ T cell responses.

Cohen (1998) demonstrated that there was a reduction in class-I heavy chains on the surface of infected cells and suggested this was due to a defect in posttranslational processing. Pulse chase experiments and immunoprecipitation revealed that VZV down regulates MHC-I by impairing its transport from the Golgi to the plasma membranes of infected cells (Abendroth *et al.,* 2001a). Transient expression of the ORF66 protein kinase has been associated with this decrease in cell surface expression (Abendroth *et al.,* 2001a) and more recent experiments *in vitro* in human melanoma fibroblast and T cells, have illustrated that ORF66 kinase activity actually delays MHC-I transport through the *cis/*medial-Golgi complex (Eisfeld *et al.,* 2007).

In addition, VZV has ORF66-independent mechanisms that contribute to reduced MHC-I surface expression that are yet to be identified (Eisfeld *et al.,* 2007). A panel of plasmids expressing VZV ORFs 0, 1, 2, 7,9, 11, 17, 21, 23, 32, 40, 44, 46, 58, 64, and 65 revealed no ability for any one protein to downregulate MHC-I, in fibroblasts *in vitro*. Further investigations into VZV ORF9a protein (putative gN); the orthologue of the BHV-1 UL49.5 (a transporter associated with antigen processing inhibition) revealed no effects on MHC-I expression in a T cell line. As gN and gM complex in PRV the authors investigated the combined effect on VZV ORFs 9a:50 (gM) expression, but again found no effect on MHC-I expression (Eisfeld *et al.,* 2007).

MHC-II expression is predominantly restricted to professional antigen presenting cells (APCs) such as B cells, DCs and monocytes/ macrophages. IFN-γ causes the upregulation of MHC-II on APCs and can actually induce the expression of MHC-II on non-professional APCs, such as fibroblasts (Collins *et al.,* 1984). During primary infection, NK cells produce IFN-γ, and as the VZV-specific T cell response develops, $CD4^+$ T cells that produce IFN-γ are induced (Abendroth *et al.,* 2001). Thus inhibiting IFN-γ induced MHC-II upregulation would allow a window in which VZV could replicate in skin. Abendroth and colleagues (2000) demonstrated that fibroblasts infected with VZV and treated with IFN-γ expressed less that half the amount of MHC-II than non-infected fibroblasts, and similar results have recently been demonstrated in a human keratinocyte cell line, (Black *et al.,* 2009). In addition, when skin biopsies of varicella lesions were analyzed by *in situ* hybridization, MHC class-II transcripts were detected in areas around lesions but not in cells that were infected with VZV. A later study by Nikkels (*et al.,* 2004) in herpes zoster biopsies again showed that MHC-II transcripts were found in keratinocytes around vesicles, but not in VZV-infected cells. In addition, VZV-infected keratinocytes treated with IFN-γ have an impaired ability to stimulate antigen specific $CD4^+$ T cells *in vitro* compared with infected or uninfected keratinocytes that had not been treated with IFN-γ and this has been shown to be due to inhibition of the IFN-γ signalling pathway (Black *et al.,* 2009).

The engagement of a cytokine (such as IFN-γ) to its appropriate receptor (such as the IFN-γ receptor) results in the activation of intracellular signalling pathways. This leads to either the induction or inhibition of transcription of cytokine regulated genes, (such as MHC-II). In brief, cytokines initiate intracellular signalling through the ligand induced aggregation of their receptor components. The common outcome of receptor aggregation is the activation of kinases associated with the cytoplasmic domains of these receptors, followed by phosphorylation of the cellular substrates. Some of the ultimate cellular responses to a

cytokine include cell growth, differentiation and proliferation, apoptosis and up or down regulation of surface molecules. The unique function a cytokine has on a particular cell type is related to the signalling pathway which it activates and the interplay between these pathways (Murphy *et al.,* 2008).

A signalling pathway frequently used by IFN-γ (and type-I Interferon) receptors involves enzymes called Janus kinases (JAKs) and transcription factors called signal transducers and activators of transcription (STATs). Thus the pathway is called the JAK/STAT pathway. There are four members of the JAK family; JAK-1, JAK-2, JAK-3 and Tyk2 and seven STATs (1-5, -6a, and -6b). Receptor-associated inactive JAK enzymes, which are loosely attached to the cytoplasmic domains of the receptors, become activated via transphosphorylation. This leads to the phosphorylation of tyrosine residues in the cytoplasmic portions of the clustered receptors. STAT proteins bind to the receptors via phosphotyrosine residues and are subsequently phosphorylated by the receptor-associated JAK kinases. This results in the bound STAT proteins dissociating from the receptor, allowing the STAT dimer to migrate to the nucleus and activate gene transcription via NFκB (Murphy *et al.,* 2008).

Analysis of regulatory events in the IFN-γ signalling pathway demonstrated that VZV infection inhibited transcription of both interferon regulatory factor -1 (IRF-1) and the MHC-II transactivator (CIITA), thus enabling the virus to inhibit IFN-γ induction of cell surface MHC-II expression (see figure 1.15). STAT-1α is known to induce the expression of CIITA and IRF-1 by IFN-γ treatment (Meraz *et al.,* 1996, Piskurich *et al.,* 1998). Abendroth and colleagues (2000) found that VZV infection inhibited the expression of both STAT-1α and JAK2 proteins but had little effect on JAK1. HCMV and MCMV also inhibit MHC-II expression at the level of transcription. In the case of HCMV, the virus inhibits MHC-II expression (in human fibroblasts *in vitro*) by blocking JAK/STAT signal transduction through a specific decrease in JAK-1 expression (Miller *et al.,* 1998). In contrast, MCMV

inhibits IFN-γ stimulated MHC-II expression (in murine macrophages *in vitro*) by a mechanism that does not involve JAK/STAT signal transduction (Heise *et al.,* 1998). The fact that these three herpesviruses all employ mechanisms to reduce MHC-II antigen presentation is indicative that they have been exposed to a similar immunological selection pressure.

1.7.6 Cytokines Induced by VZV Infection, and the Immune Modulation of Cytokine Pathways by VZV:

Cytokines are small soluble proteins (approximately 8-80kDa) that usually act in an autocrine or paracrine, (rather than endocrine), manner to alter the behaviour or properties of cells. These proteins are part of an extracellular signalling network that controls immune responses. Cytokines produced during an innate immune response include TNFα, IL-1, IL-6, IL-12, IFN-α, IFN-β, IFN-γ and various chemotactic cytokines (chemokines) such as IL-8. Interleukins have a variety of functions, mainly directing other cells to divide and differentiate. Interferons are particularly important in limiting the spread of viral infections; type-I interferons (IFN-α and IFN-β) are produced by virally infected cells early in infection and induce a state of antiviral resistance in uninfected cells. Type-II Interferon (IFN-γ) is produced by NK cells (and activated T cells).

VZV has been shown to modulate host immune responses by modifying several signalling pathways such as the Jun NH_2-terminal kinase/stress-activated protein kinase (JNK/SAPK) pathway and the p38 mitogen-activated protein kinase (p38/MAPK) pathway, (Desloges *et al.,* 2008a).

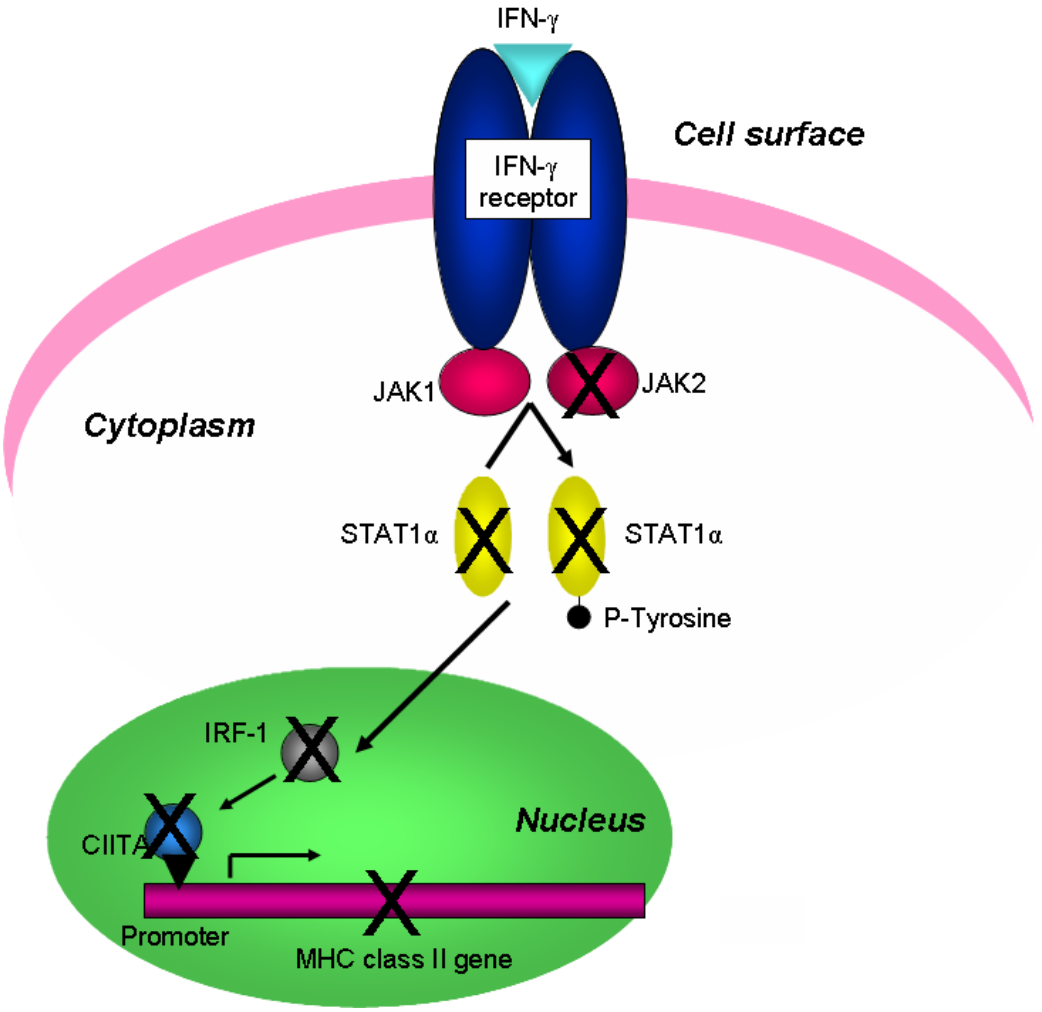


Figure 1.15: Schematic of the Sites of VZV-Mediated Disruption of IFN-γ Induced MHC-II Expression. The proteins which are affected in VZV infected cells are crossed out. Key: CIITA - MHC-II transactivator, IFN – interferon, IRF-1 - interferon regulatory factor -1, JAK - Janus kinases, Stat - signal transducers and activators of transcription. Not to scale. (Adapted from Abendroth and Arvin 2001b, with permission).

A range of cytokines are produced in response to VZV infection, and have either been measured from patients *ex vivo*, *in vitro*, or in skin xenografts on the SCID-Hu mouse model. For many of these cytokines, VZV-induced modulation of the signalling pathways which lead to their transcription, have been investigated.

PBMCs obtained from patients during the acute phase of varicella were shown to produce significantly higher amounts of IFN-γ, TNFα and IL-12 compared to healthy individuals (Torigo *et al.,* 2000). Levels of IL-6 and IFN-γ have been shown to be slightly raised in the serum of zoster patients at the time of acute disease compared with a group of normal healthy subjects (Zak-Prelich *et al.,* 2003). Human monocytes, macrophages and keratinocytes have been shown to produce IL-6 (Wang *et al.,* 2005, Nikkels *et al.,* 2004). In VZV-infected monocytes, the production of this cytokine has been shown to be via Toll Like Receptor 2 (TLR-2) dependent activation of NFκB (Wang *et al.,* 2005). IL-6, IL-1 and TNFα are cytokines which are crucial to the induction of acute phase proteins and the acute phase response, a key part of an inflammatory response. IL-1 and TNFα are pyrogens which play an important role in the induction of adhesion molecule expression on endothelial cells and the stimulation of chemokine production (Murphy *et al.,* 2008).

Interleukin-8 (IL-8) is a pro-inflammatory cytokine and a potent neutrophil attractant. VZV infection has been shown to induce the secretion of IL-8 in melanoma cells, fibroblasts, a T cell line (Desloges *et al.,* 2008b) and human monocytes (Wang *et al.,* 2005) *in vitro*. Inhibition of VZV DNA replication has no effect on IL-8 release *in vitro*, suggesting that virus entry, release of tegument proteins and/or expression of immediate early or early genes are sufficient for the induction of IL-8 secretion (Desloges *et al.,* 2008b). The presence of IL-1β or TNFα caused a significant induction of IL-8 secretion in infected melanoma and fibroblast cells, but had no effect on infected T cell line. Treating cells prior to infection with inhibitors of NFκB, JNK/SAPK or p38/MAPK, revealed that IL-8 expression was not dependent on NFκB activation in VZV-infected melanoma and T cells. However, the JNK/SAPK pathway is required for secretion of IL-8 in VZV infected melanoma cells, whilst both the JNK/SAPK and p38/MAPK pathways are necessary for IL-8 secretion in a VZV-infected T cell line (Desloges *et al.,* 2008b).

VZV infection is also associated with the induction of IL-1α production in epidermal cells in SCID-Hu mice (Ku *et al.,* 2004). In this model, it was demonstrated that Interleukin-1α was expressed constitutively in the cytoplasm of epidermal cells in uninfected skin. In VZV infected skin xenografts IL-1α was translocated to the nuclei of infected cells but remained in the cytoplasm of adjacent, uninfected cells (Ku *et al.,* 2004).

1.7.6.1 Type I (IFN-γ) and Type II (IFN-α and IFN-β) Interferons:

IFN-α has been detected in the serum of healthy children with varicella and administration of exogenous IFN-α to immunocompromised children with varicella has been shown to reduce disease severity (Arvin *et al.,* 1982). Resolution of herpes zoster rash is accompanied by local production of IFN-α detected in vesicle fluid from cutaneous lesions (Stevens *et al.,* 1975). Binding of IFN-α to its receptors induces phosphorylation of STAT-1 by JAK kinases (Darnell *et al.,* 1994). Unless STAT-1 is phosphoylated, it does not translocate to the nucleus and IFN-α production is blocked. In VZV infected skin xenografts in the SCID-Hu mouse model, phosphorylated STAT-1 was localised to nuclei in neighbouring uninfected epidermal cells, but it was not detected in cells expressing VZV proteins. STAT-1 was not phosphorylated and remained cytoplasmic, as did IFN-α, in uninfected skin xenografts. VZV replication therefore appears to result in expression of a gene product(s) that interferes with STAT-1 activation and thereby inhibits antiviral IFN-α production in foci of infected skin cells *in vivo* (Ku *et al.,* 2004). Although this study found that IFN-α was down regulated in VZV-infected cells it was prominently expressed in neighbouring uninfected epidermal cells (Ku *et al.,* 2004). Experiments with neutralizing antibodies against the IFN-α/β receptor confirmed the role of type-I interferon in immune modulation (Ku *et al.,* 2004).

IFN-α confers cellular resistance against virus infection through at least two cellular proteins, double-stranded RNA-activated protein kinase (PKR) and 2'-5'oligoadenylate synthetase. VZV has been shown to counteract the IFN type-1 mediated host defence effectively by circumventing the induction of PKR- and RNase L dependent antiviral systems (Desloges *et al.,* 2005a, Desloges *et al.,* 2005b). A recent study by Ambagala and Cohen (2007) indicates that VZV ORF63 interferes with the PKR pathway. The authors found that ORF63 deletion mutant viruses (in which over 90% of both copies of ORF63 were deleted) are hypersensitive to human IFN-α compared to parental virus. Activated PKR phosphorylates the α-subunit of eukaryotic initiation factor 2 (eIF-2α), thereby preventing initiation of translation. Cells infected with the VZV ORF63 deletion mutant had increased levels of phosphorylated eIF-2α (eIF-2α-P) compared to those infected with parental virus, suggesting that expression of the ORF63 protein is necessary to overcome virus-induced phosphorylation of eIF-2α. In addition, transient expression of IE63 diminished basal levels of eIF-2α-P.

In an *in vitro* study of lymphocytes the induction of IFN-γ production by VZV was found to be associated with IFN-α production and phosphorylation of both STAT-1 and STAT-4 (Yu *et al.,* 2005). IFN-γ can restrict VZV replication by its direct antiviral effects, and can be detected in the serum of healthy subjects with acute varicella (Arvin *et al.,* 1986b; Wallace *et al.,* 1994). IFN-γ and IFN-β inhibit the replication of VZV *in vitro* (Desloges *et al.,* 2005a). IFN-β was found to be more effective than IFN-γ, but the level of inhibition of VZV replication achieved by the combination of both IFNs was more than additive; thus these two cytokines acted synergistically (Desloges *et al.,* 2005a). In the presence of IFN-β but not IFN-γ, expression of the IFN-induced, double-stranded RNA-activated protein kinase PKR and its phosphorylation were increased, explaining in part the inhibition of virus replication by IFNs (Desloges *et al.,* 2005a).

1.7.7 Modulation of the NFκB Transactivation Pathway:

NFκB possesses the ability to promote the expression of numerous proteins involved in innate and adaptive immunity and thus regulates the immune response following various stimuli, including infections. NFκB activation during early stages of viral infection, leads to the expression of immune response genes such as those for proinflammatory cytokines (IFN-β, TNFα, IL-6, and IL-8), chemokines (RANTES), and adhesion molecules (ICAM-1 and VCAM-1). NFκB also strongly induces MHC-I and CD80/86 expression on antigen-presenting cells, thus increasing T cell activation. Several viruses, including HSV and CMV, have also developed strategies to interfere with NFκB activation (Amici *et al.,* 2006; Montag *et al.,* 2006).

Members of the nuclear factor kappa B (NFκB) family of proteins which includes NFκB1 (p105 processed to p50), NFκB2 (p100 processed to p52), Rel A (p65), Rel B, and c-rel are key cellular transcription factors. The most abundant form of NFκB is a heterodimer composed of p50 and p65/RelA. In resting cells, NFκB proteins are inactive, as the nuclear localization signals of p50 and p65 are bound to an inhibitor of NFκB (IκB) known as IκBα and are sequestered in the cytoplasm (Richmond, 2002). The family of IκBs also includes IκBα, -β, -ε, p100, and p105. Following signalling, the IκB proteins dissociate from the NFκB complex as they are phosphorylated by the IκB kinases (IKKs), ubiquitinated, and degraded by the proteasome, thus allowing the activated transcription factor (NFκB) to translocate to the nucleus where it can bind the unique DNA sequence in the promoter region of cytokine responsive genes (Karin and Ben-Neriah, 2000).

DNA microarrays demonstrated that many NFκB responsive genes are down regulated in VZV-infected fibroblasts (Jones *et al.,* 2003; Jones, 2005). VZV has been shown to inhibit the NFκB pathway by sequestering p50 and p65 in the cytoplasm of the infected cells after a transient nuclear

translocation *in vitro* (Jones *et al.,* 2006). It was also demonstrated that VZV has the ability to inhibit the degradation of IκBα after exogenous stimulation of the NFκB pathway by TNFα signalling. In human xenografts on SCID mice, NFκB was retained in the cytoplasm of VZV-infected epidermal cells (Jones *et al.,* 2006). VZV inhibits innate responses mediated through the NFκB pathway *in vitro* and *in vivo,* whereas these cellular defences are upregulated in adjacent uninfected skin (Jones *et al.,* 2003; Jones *et al.,* 2006a).

As mentioned previously, VZV induced modulation of ICAM-1 expression is now known to be due to the disruption of the NFκB signalling pathway (El Mjiyad *et al.,* 2007). In melanoma and fibroblasts *in vitro* VZV is able to inhibit NFκB nuclear translocation by destabilizing the NFκB p65-p50 heterodimers which are crucial for the TNFα induction of the *icam-1* gene. The NFκB subunits p65, p52 and c-Rel are still induced upon VZV infection, but the subunit p50 does not translocate in response to TNFα. Nuclear p65 is no longer associated with p50 and is unable to bind the proximal NFκB site of the *icam-1* promoter. VZV also induces the nuclear accumulation of the NFκB inhibitor p100 (El Mjiyad *et al.,* 2007).

1.8 Prevention and Treatment:

1.8.1 Antiviral Therapy:

There are several drugs which are currently licensed for the treatment of VZV disease, including aciclovir (and its prodrug valaciclovir) penciclovir (and its prodrug famciclovir), ganciclovir, cidofovir, brivudin and foscarnet (Simpson and Lyseng-Williamson, 2006; Boeckh, 2006; Whitley and Gnann, 1999). Both valaciclovir and famciclovir are converted within the body into their active forms aciclovir and penciclovir respectively. Aciclovir is administered either by the oral route or intravenously, depending on the severity of the illness. Zoster can also be treated successfully with the oral medications famciclovir and valaciclovir. There is a high correlation between early treatment (within 24h of onset of rash) and a successful outcome (Balfour *et al.,* 1983). Antiviral therapy for varicella does not

prevent latent VZV infection. These drugs have different modes of action, but all act by inhibiting VZV DNA replication (Whitley and Gnann, 1999).

1.8.2 Varicella Vaccination:

1.8.2.1 Development of the Varicella Oka Vaccine:

The live attenuated varicella vaccine was developed by Takahashi and co-workers in the early 1970s (Takahashi *et al.,* 1974) and is the only licenced vaccine available against a herpes virus. Similar live attenuated vaccines have been widely used and proven extremely effective for immunization against a variety of viruses including polio (Sabin, 1957; Sabin and Boulger, 1973), measles (Katz *et al.,* 1960), mumps (Hilleman *et al.,* 1968) and rubella (Plotkin *et al.,* 1968; Plotkin, 1972).

The original parental wild type strain of VZV was isolated in Japan in 1971 from the vesicle fluid of a three year old boy, of the surname Oka who had typical chickenpox but was otherwise healthy (Takahashi *et al.,* 1974). Attenuation of the virus was achieved at the Biken Institute in Osaka, Japan using the classical method of propagation at low temperature *in vitro*, in cells from a species different from that of the natural host.

In addition to the Biken vaccine, Merck and GSK have since produced their own forms of the Oka VZV vaccine. Both are derived from the Oka strain developed by Takahashi and co-workers. Figure 1.16 summarises the differences in propagation between the three brands of vaccine.

1.8.2.2 Comparison of POka and VOka Viruses:

The vaccine strain (vOka) is not clonal, but instead contains a mixture of substrains that differ from one another genetically (Gomi *et al.,* 2002; Quinlivan *et al.,* 2004; Quinlivan *et al.,* 2005). Comparison between the parental strain (Biken pOka) from which all the mixture of vaccine strains derive, has been carried out (Gomi *et al.,* 2000; Gomi *et al.,* 2001; Argaw *et al.,* 2000) and revealed that the Oka vaccine virus differs from the parent virus at 46 loci (Gomi *et al.,* 2002); only a 0.034% sequence difference. The majority of these differences occur in the form of single

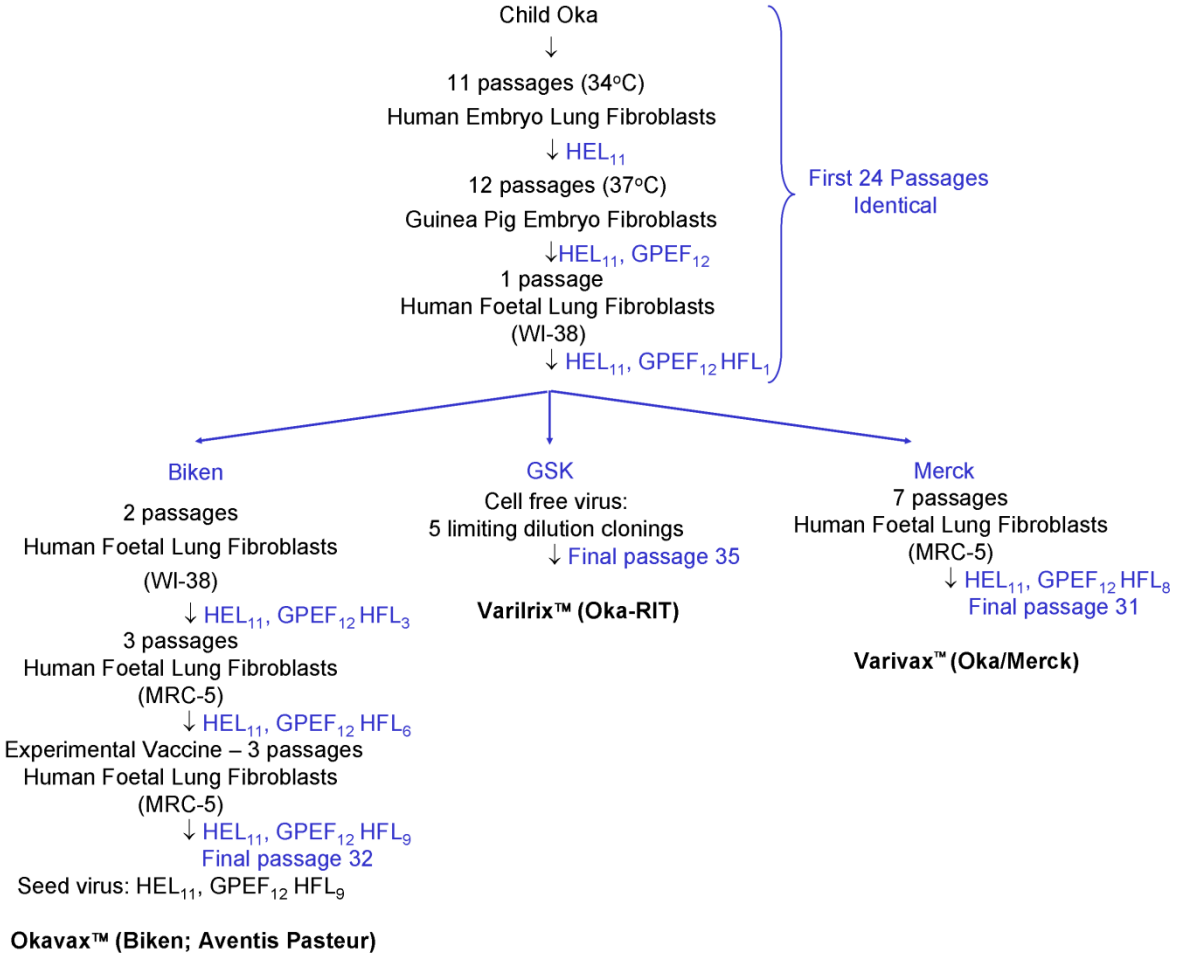


Figure 1.16: Attenuation of P-Oka to V-Oka and the Differences in Attenuation Between the Biken, GSK and Merck Vaccines. Attenuation of the virus was achieved using the classical method of propagation at low temperature *in vitro*, in cells from a species different from that of the natural host. Key: HEL-human embryo lung fibroblasts, GPEF-guinea pig embryo fibroblast, HFL-human Foetal lung fibroblasts.

nucleotide polymorphisms (SNPs), but also include nucleotide additions and deletions in variable repeat regions of the genome (Gomi *et al.,* 2002). There are only 42 base substitutions, resulting in 20 amino acid (aa) conversions and length differences in tandem repeat regions (R1, R3, and R4) and in an origin of DNA replication. Amino acid substitutions exist in ORFs 6, 9A, 10, 21, 31, 39, 50, 52, 55, 59, 62, and 64. Direct sequencing of the vaccine genome revealed that at the majority of the 46 loci, both a novel vaccine nucleotide and the parental wild-type virus nucleotide were present, (Gomi *et al.,* 2002).

1.8.2.3 Vaccine Virus Attention:

Although both vOka and pOka have been shown to replicate well *in vitro*, the SCID-Hu mouse model has facilitated studies of the attenuation of vOka *in vivo* (Moffatt *et al.,* 1998a; Moffatt *et al.,* 1998b). VOka and pOka have been shown not to differ in their infectivity for human T cells in thymus/liver xenografts *in vivo* (Moffatt *et al.,* 1998b) and recently it has been demonstrated in human keratinocytes *in vitro* that pOka and vOka have the same capacity to inhibit the IFN-γ signaling pathway (Black *et al.,* 2009). However in skin xenografts, vOka was shown to have a reduced capacity to replicate in differentiated human epidermal cells, with lower yields of infectious virus, decreased viral protein synthesis and slower progression of cutaneous lesions compared to the parental strain (and a low passage clinical isolate) which were fully virulent, (Moffatt *et al.,* 1998a; Moffatt *et al.,* 1998b).

Further investigations of the genetic basis of vOka attenuation have been carried out using cosmids to generate chimeric pOka and vOka recombinant viruses, which have been evaluated in SCID-Hu skin xenografts (Zerboni *et al.,* 2005a). Plaque morphologies and the growth kinetics of pOka, vOka and six chimeric pOka/vOka viruses, did not differ *in vitro*, but the pOka/vOka chimeric viruses showed variable virulence in skin xenografts. Within vaccine strains there are 15 base substitutions, (leading to eight aa differences), located in gene 62/71, but these

experiments indicated that ORF62 vaccine changes were not sufficient to diminish vOka virulence in skin. In addition, the presence of pOka ORFs 30–55 in chimeric viruses was sufficient to maintain wild-type infectivity in skin, (Zerboni *et al.,* 2005a).

1.8.2.4. Uses of the Varicella Vaccine:

The Oka Biken vaccine was approved for administration to both high risk and healthy children in Japan in 1986, and is also licensed in Korea (Asano *et al.,* 1994; Asano, 1996) and Taiwan (Tseng *et al.,* 2003) for widespread administration to susceptible children.

VARIVAX™ (Merck & Co., Inc, USA) is currently marketed worldwide in 46 countries, with around 55 million doses distributed to date, (Galea *et al.,* 2008). This brand of vaccine was licensed in the US in 1995 for vaccination of healthy children, healthy adults (Wise *et al.*, 2000; Sharrar *et al.,* 2000), children with leukaemia (LaRussa *et al.,* 1996), and in some HIV positive children (Advisory Committee on Immunization Practices, 1999). Since 1996, universal vaccination of all children aged 12-18 months has been recommended in the US (National Advisory Committee on Immunization, 1999). To further reduce varicella disease burden, a routine two-dose varicella vaccination recommendation has been approved by the Centers for Disease Control and Prevention (CDC) Advisory Committee on Immunization Practices (ACIP) since 2006; with the first dose administered to children aged 12–15 months and a second dose at 4–6 years of age (Marin *et al.,* 2007).

Universal vaccination against varicella in the UK is not practised. However, there is targeted vaccination of groups at risk of severe varicella (Department of Health, 2003). In 2003 the VARILRIX™ and VARIVAX™ vaccines were licensed for use in the UK in health care workers (Nardone, 2003). Licensure and vaccination strategies in Europe are varied (reviewed in Sadzot-Delvaux *et al.,* 2008), however, the Society of Independent European Vaccination Experts (SIEVE) recommends that the immunization of susceptible adolescents needs to be urgently

implemented throughout Europe, in addition to the current recommendations targeting high-risk patients, their close contacts with a negative history of varicella and seronegative health-care workers. A universal policy, optimally incorporating a two-dose schedule, will be needed to finally reduce the burden of disease of varicella from a societal point of view. The SIEVE recommends the implementation of such a policy as soon as financially and practically possible, (reviewed in Sengupta *et al.,* 2007).

1.8.2.5 Vaccine Efficacy:

Before vaccine licensure, only one double-blind, placebo-controlled efficacy study of the Oka/Merck varicella vaccine was conducted, (Weibel *et al.,* 1984). Almost 1,000 healthy children in the United States were immunized with one dose (>17,000pfus) and followed up for several years. After nine months of follow-up, the vaccine was found to be 100% effective, with only children vaccinated with the placebo developing varicella. The children who were given the placebo were then vaccinated, and after two years, efficacy was 98% overall. At seven years, 95% had remained free from disease. Since the varicella vaccine (which contains a minimum of 1,350pfu/dose) was licensed in the United States, several effectiveness studies have been carried out (reviewed in Seward *et al.,* 2008). Data collected from the 19 studies found that overall, one dose of varicella vaccine was 84.5% effective (range, 44%–100%) in preventing varicella disease and 100% effective in preventing severe disease.

1.8.2.6 The Aim of Universal Vaccination and Achievements Ten Years Post Introduction:

In the long term, it is hoped that with universal vaccination against VZV in childhood, varicella and subsequently zoster will be prevented. Before implementation of the varicella vaccination program in the States, VZV infection resulted in an average of 11,000–13,500 hospitalizations and 100–150 deaths each year (Galil *et al.,* 2002a; Ratner, 2002; Davis *et al.,* 2004; Meyer *et al.,* 2000). Since 1996, the annual incidence of varicella in

USA has fallen by over 70% in areas of high coverage and associated hospital admission rates have also dramatically decreased (Seward *et al.,* 2002; Zhou *et al.,* 2005; Davis *et al.,* 2004). In Germany use of an age-structured analytical model has estimated that a routine varicella vaccination programme targeting healthy children could prevent 82.7% of varicella cases and over 4,700 major complications per year in Germany provided the coverage level was 85%. Under these conditions, the elimination of varicella is predicted to be achievable within 18 years (Wutzler *et al*., 2002).

Data accumulated 10 years after the varicella vaccine was introduced in the States suggests its use so far has been highly effective (Hambleton and Gershon, 2005a; Hambleton and Gershon, 2005b; Seward *et al.,* 2008). A number of recent studies relate increasing vaccine coverage in the US to significant reductions in varicella-related consultations, hospitalizations and deaths (Davis *et al.,* 2004; Patel *et al.,* 2004; Reynolds *et al.,* 2008), in addition to various indicators of reduced varicella incidence and VZV transmission, (Hambleton and Gershon, 2005a). The safety record of the vaccine also remains after its administration to millions of individuals (Wise *et al.,* 2000, Sharrar *et al.,* 2000; Galea *et al.,* 2008; Chaves *et al.,* 2008).

1.8.2.7 The Effect of Varicella Vaccination on Rates of Zoster:

The effects of varicella vaccination on zoster are not yet known and this represents one of the major areas of uncertainty in relation to its widespread use (Wagenpfeil *et al.,* 2004). Vaccine strain virus can certainly establish latent infection and can also reactivate to cause zoster (LaRussa *et al.,* 2000; Sharrar *et al.,* 2000), but at a rate that appears significantly lower than wild type virus (at least in the immunocompromised), (Hardy *et al.,* 1991). Alongside this potential protective effect in vaccinees however, must be considered the possibility that overall zoster incidence may increase in highly vaccinated populations because of the loss of boosting exposures to varicella (Gershon, 1996, Thomas *et al.,* 2002, Brisson *et al.,* 2002). This has been demonstrated in

a Spanish study (Pérez-Farinós *et al.,* 2007), where the vaccine was first introduced in 1997 for use in high risk patients, and recommended for vaccination of children of 12 months of age in 1999. Data from this sentinel study, conducted in Madrid between 1997-2004, indicated that there was a sustained and significant rise in annual incidence of zoster during this time period, particularly among the oldest age groups (Pérez-Farinós *et al.,* 2007).

However, within the ten year time frame since the vaccine has been licensed in the USA, the incidence of zoster has remained stable whilst the incidence of varicella has decreased (Jumaan *et al.,* 2005). These findings revealed that the vaccination-associated decrease in varicella disease has not resulted in an increase in the incidence of zoster thus far (Jumaan *et al.,* 2005), but these early findings will have to be confirmed as the incidence of varicella disease continues to decrease. However, in a recent long-term follow-up study on healthy young adults who had been vaccinated 10-26 years previously, the incidence of herpes zoster was similar to that of the US population in the pre-vaccine era (Hambleton *et al.,* 2008).

1.8.3 Development of the Zoster Vaccine:

Following on from the success of the varicella vaccination, a vaccine aimed at boosting pre-existing immunity to prevent herpes zoster was developed. The zoster vaccine (ZOSTAVAX™; Oka/Merck) was licensed in the United States in May 2006, and was recommended for use in adults aged ≥60 years by ACIP in October 2006. It has been estimated that in the United States, the vaccine has the potential to prevent 300,000 outpatient visits, 375,000 medication prescriptions, 9,700 emergency department visits, and 10,000 hospitalizations per million vaccine recipients (Pellissier *et al.,* 2007).

In a randomized, double-blind, placebo-controlled trial of almost 40,000 adults, 60 years of age or older, the use of the zoster vaccine reduced the incidence of postherpetic neuralgia (PHN) by 66.5 percent ($p<0.001$), and

the incidence of herpes zoster by 51.3 percent ($p<0.001$) (Oxman *et al.,* 2005). The minimum potency (19,400 pfus/dose) administered was 14 times greater than that of the minimum potency of VARIVAX™ (minimum of 1,350pfu). Preliminary studies indicated that potencies of this magnitude are required to elicit a significant increase in the cell mediated immunity to VZV among older adults (Oxman *et al.,* 2005). There are no data to suggest that the vaccines aimed at varicella would be efficacious in protecting older adults from zoster or PHN.

CHAPTER 2: MATERIALS AND METHODS

2.1 List of Buffers and Reagents:

Complete Growth Medium	Culture medium, 10% human serum or FBS; 1% penicillin/streptomycin, 1% glutamine
ELISPOT:	
Blocking solution	Complete cell culture medium
Coating buffer	1× PBS
Dilution buffer	1× PBS, 10% FBS
Substrate solution	BD™ AEC Substrate Reagent Set (AEC (3-amino-9-ethyl-carbazole) stock solution: 100mg AEC in 10mL DMF (N,N-Dimethylformamide).
Wash buffer I	1× PBS, 0.05% Tween-20
Wash buffer II	1× PBS
Freezing media	90% FBS, 10% DiMethyl SulfOxide (DMSO)
Maintenance media	Culture medium, 2% FBS, 1% Penicillin-Streptomycin–L-glutamine
PBS	137mM NaCl, 10mM Na_2HPO_4, 3mM KCL, 2mM, K_2HPO_4, pH 7.4
VTM	Modified Hank's balanced salt solution, pH 7.3 (+/- 0.2) 20mM HEPES, 2% BSA, Amphotericin B (4mg/mL) and vancomycin (100mg/mL)
Western blot	
Blocking buffer	5% w/v dried skimmed milk powder, 0.05% v/v Tween 20 in PBS
Wash buffer	0.05% Tween 20 in PBS

2.2 Patient Recruitment and Sample Collection:

2.2.1 ROVE (Response to Oka Vaccine Evaluation) Study:

The following research study is based on the introduction of the Oka vaccination programme at Bart's and the London (BLT) NHS Trust with the cooperation of the Trust Occupational Health and BLT Virology departments.

In June 2005 the occupational database (dating back to March 2000) consisted of 1,308 employment screening blood results that were serologically negative or equivocal to VZV by standard serology testing (Diamedix, USA,). 260 staff were invited to take part in our study: Response to Oka Vaccine Evaluation (ROVE). Ethical permission was granted by the East London and the City Health Authority Local Research Ethics Committee 3 (05\Q0605\1). A total of 110 BLT staff members were recruited for the ROVE study (with thanks to Mrs Fiona Scott). The study protocol can be found in appendix 7.1.

Healthcare workers (HCW) who were eligible for varicella vaccination according to the Department of Health guidelines (Department of Health, 2003) were asked to attend the ROVE clinic three times. This included HCWs who had a negative history for varicella and were negative by serological testing and those who had recently migrated from the tropics with a positive history, but were negative by serological testing. On the first visit, the volunteers answered a short questionnaire on demographics and history of varicella (see appendix 7.2). Exclusion criteria included immunocomprised, e.g: by steroid treatment, and (where applicable) pregnancy and/or breastfeeding. Baseline samples were taken. These samples consisted of 3mL of EDTA blood (7.5% 0.072mL), 5mL of clotted blood, and 18mL of heparinised blood (sodium heparin 170IU/10mL tube). After samples were collected, the HCW was administered subcutaneously either the GlaxoSmithKline Oka-RIT (VARILRIX^{TM}) vaccine or the Oka/Merck Aventis Pasteur (VARIVAX^{TM}) vaccine (see table 2.1 and appendix 7.3). Each 0.5mL dose of VARIVAX$^{®}$ contained a minimum of 1,350 plaque forming units (pfus) of live attenuated varicella virus, and the following inactive ingredients: Sucrose, hydrolysed gelatine, urea, sodium chloride, monosodium-L-glutamate, anhydrous disodium phosphate, potassium dihydrogen phosphate and potassium chloride. (Aventis Pasteur MSD Limited, 2003). Each 0.5mL dose of VARILRIX^{TM} contained 1,995 ($10^{3.3}$) pfus of live attenuated varicella virus, and the following

inactive ingredients: Amino acids, human albumin, lactose, neomycin sulphate sorbitol and mannitol (GlaxoSmithKline, 2006).

At the second visit (approximately six weeks after the first visit/vaccination) HCWs answered a questionnaire relating to whether any symptoms were associated with the previous vaccination, and whether they had come into contact with either chickenpox or shingles, (see appendix 7.2). The same set of blood samples were collected and the second dose of vaccine (from the same manufacturer as the corresponding first dose) was administered subcutaneously (see table 2.1 and appendix 7.3). On the third visit, (approximately 12 weeks after the first vaccination, and six weeks after the second vaccination) the HCW again answered a questionnaire on symptoms and contacts and the full repertoire of samples was collected. No vaccine was administered at the third visit. Subsequently, 75 study participants were recruited for a 1-2 year follow up visit, where again, the full set of samples were collected, and a questionnaire was answered by the HCW on contacts with chickenpox and shingles since their last visit.

Appendix table 7.4 documents the weeks elapsed between visits for each HCW, and thus those individuals who participated in a fourth visit.

Visit Number	Timescale	Aim	Vaccination Strategy	Samples
Visit 1 (V1)	Baseline	Examine pre-existing immunity	First dose of vaccine administered	Blood samples collected
Visit 2 (V2)	Six weeks post first dose	Examine immune response to first dose	Second dose of vaccine administered	Blood samples collected
Visit 3 (V3)	12 weeks post first dose, Six weeks post second dose	Examine immune response to second dose	No vaccine administered	Blood samples collected
Visit 4 (V4)	12-18 months	Examine waning immunity and boosting	No vaccine administered	Blood samples collected

Table 2.1: Summary of ROVE Visits Timings, Vaccine Schedule and Sample Collection.

2.2.2 Sample Collection in the Case of ROVE Rash Development, Post Vaccination:

If at any point during the study a HCW developed symptoms which they thought could be attributed to a varicella rash (one or more cutaneous lesions) or suspected a vaccine related event (VRE) (see appendix 7.2) the ROVE research nurse was contacted directly by the HCW. If lesions were present the research nurse would carry out a visual examination and vesicle fluid from all lesions would be collected using swabs. Swabs were then stored in viral transport media (VTM) (see list of buffers and reagents) and DNA was extracted within 2h. A full set of blood samples were collected, and processed within 2h.

2.3 Sample Processing and Storage:

2.3.1 ROVE Blood Samples:

All ROVE blood samples were transported back to the laboratory at room temperature (RT) and processed within 2h of being taken.

2.3.2 Whole Blood:

From the 5mL EDTA tube, 200μL of blood was removed for DNA extraction (see section 2.3.6).

2.3.3 Plasma Isolation:

The remaining contents of the EDTA tube were spun at 1,800rpm (500g) in a Jouan CR 412 centrifuge at RT for 5min. The resulting plasma layer was then aspirated within a class 2 hood using a sterile Pasteur pipette into 2 aliquots. The remaining pellet was discarded according to the correct safety procedures.

2.3.4 Serum Isolation:

The 5mL clotted blood tube was spun along side the EDTA tube at 1,800rpm (500g) in a Jouan CR 412 centrifuge at RT for 5min. The resulting serum layer was then aspirated (as described for the plasma sample), into 3 aliquots. The remaining pellet was discarded according to correct safety procedures.

2.3.5 Peripheral Blood Mononuclear Cells (PBMC) Separation:

The heparinized blood was processed aseptically in a class 2 Trimat hood. 18mL of heparinized blood was added to equal volumes of phosphate buffered saline (PBS) (Sigma, UK) in a sterile 50mL Falcon™ tube. The sample was slowly layered onto 15mL of ficoll (Amersham Pharmacia Biotech AB, Sweden). This was centrifuged immediately (25min, 1,800rpm (500g), RT, Jouan CR 412). Three quarters of the upper serum layer was discarded, and the PBMCs layer was carefully aspirated with a sterile Pasteur pipette. 40mL of PBS was added to the PBMCs, mixed and centrifuged (12min, 1,500rpm, (350g), RT). The PBS was discarded, and the remaining cell pellet gently resuspended in 10mL of PBS and centrifuged (5min, 1,500rpm (350g), RT). This step was repeated and the final cells pellet was then frozen (see section 2.3.7).

2.3.6 DNA Extraction:

DNA was extracted from a 200μL aliquot of whole blood (see section 2.3.2) using a QIAamp® DNA Mini Kit (Qiagen LTD., UK). For each sample, 20μL of Protease K, the 200μL of sample and 200μL of Buffer AL were sequentially placed into a 1.5mL microcentrifuge tube. Tubes were pulse-vortexed (Vortex 2 Genie, Scientific industries) for 15 seconds and incubated at 56^{o}C for 10min in a heating block (Stuart Scientific Test Tube Heater Sht 10). All tubes were then briefly centrifuged (Heraeus Biofuge Pico, Thermo Fisher Scientific, UK) to remove drops from the inside of the lids, after which, 200μL of molecular biology grade ethanol (100%) (Sigma, UK) was added. Tubes were again pulse vortexed for 15 seconds and briefly centrifuged to remove drops from the inside of the lids. The contents of the tube was transferred to a QIAamp spin column which were then centrifuged (8,000rpm; 5,000g), 1min. The spin column was transferred to a fresh collection tube and the collection tube containing the AL filtrate was discarded according to correct safety procedures. 500μL of buffer AW1 was added to the spin column, which was centrifuged (8,000rpm (5,000g), 1 min). The spin column was transferred to a fresh collection tube and the collection tube containing the AW1 filtrate was

discarded according to correct safety procedures. 500μL of buffer AW2 was added to the column, which was centrifuged (14,000rpm (15,000g), 3min), placed into a new collection tube and re-centrifuged (14,000rpm (15,000g), 1min) to remove any residual buffer AW2. The column was then placed into the pre-labelled 1.5mL microcentrifuge tube into which the final DNA sample was to be collected. 200μL of buffer AE was added to the spin column and incubated at RT for 5min. The column was then centrifuged (8,000rpm (5,000g), 1min) to elute the DNA, which was stored as a single aliquot as mentioned above. The spin column was the disposed of according to correct safety procedures. When more than one sample was processed at a time, a negative control was employed by replacing sample material with 200μL of molecular biology grade water (Sigma).

2.3.7 Cell Freezing:

Cell pellets were resuspended in 500μL of ice cold freezing media and transferred to a 1.8mL cryovial (Nunc). The vial was then immediately transferred to an isopropanol bath (Mr Frosty Nalgene^{TM} Cryo 1^{o}C freezing container) and frozen at -70^{o}C. After 24h the cells were placed into liquid nitrogen storage.

2.3.8 Thawing Cell Aliquots

Cells were thawed rapidly in a water bath at 37°C. The cell suspension was transferred into a 50mL Falcon tube and washed twice (cells were pelleted at 1,600rpm (400g), (Jouan CR 412 centrifuge) for 10min at RT) with 50mLs of complete medium. The cells were then resuspended in complete medium to the desired concentration.

2.3.9 Viable Cell Counts:

To determine cell viability, cell counts were carried out by diluting samples 1/10 in cell culture media or PBS, and subsequently further diluting in trypan blue (Sigma) 1/2. Cells were mounted on either a haemocytometer, or for ELISPOT and tetramer assays, were counted using disposable

'Fastread' counting slides (Immune Systems Limited, UK). Cells were enumerated by examination on a light microscope and use of a cell counter.

2.3.10 Sample Storage:

All processed samples from the ROVE study were labelled with patient number, visit number, date of visit and a description of the sample (e.g. serum, plasma, DNA or PBMCs). DNA was stored in 1.5mL eppendorfs at -20^{o}C, PBMCs in sterile 1.8mL NUNC cryovials in liquid nitrogen and all other blood samples in sterile 1.8mL NUNC cryovials at -70^{o}C.

2.4 Serology Based Assays:

2.4.1 ELISA Based Assays:

2.4.1.1 VZV IgG TRFIA.

Details of the VZV IgG TRFIA assay have been published (Maple *et al.,* 2006). In brief, this assay used VZV enzyme-linked immunosorbent assay grade antigen (The Binding Site, Birmingham, United Kingdom) which was a sucrose density gradient centrifugation-purified extract of human embryo lung (HEL) cultured VZV strain Ellen. The coating concentration of antigen was that which gave a europium count of 400,000 to 600,000 with British standard VZV antibody (NIBSC, South Mimms, United Kingdom) at a concentration of 50mIU/mL. DELFIA (Dissociation-Enhanced Lanthanide Fluorescent Immunoassay) microtiter plates (Perkin Elmer, Cambridge, United Kingdom) were coated with antigen at concentrations of 1.0 to 2.0μg/mL (depending on batch) prepared in 0.05M carbonate/bicarbonate buffer, pH 9.6. The plates were stored overnight at 4°C and washed four times with DELFIA wash buffer (Perkin Elmer, United Kingdom) using a DELFIA plate washer (Perkin Elmer, United Kingdom). Sera for testing were diluted 1 in 50 in DELFIA assay buffer (Perkin Elmer, United Kingdom), and 100μL was loaded into appropriate wells. A standard curve was run on each plate, prepared from British standard VZV antibody diluted in DELFIA assay buffer at concentrations ranging from 50mIU/mL to 0.39mIU/mL. The plates were sealed and incubated in a humid chamber

for 2h at 37°C and then washed four times, as before. Europium labeled anti-human IgG conjugate (Perkin Elmer, United Kingdom) diluted 1 in 500 in DELFIA assay buffer was added at 100μl per well using a multichannel pipette. The plates were then incubated for 1h at 37°C and washed four times, as before, and 150μL DELFIA enhancement solution (Perkin Elmer, United Kingdom) was added to all wells. Following 15min of rotating incubation at room temperature, in the dark, the plates were read using a DELFIA 1234 reader (Perkin Elmer, United Kingdom), and data were analyzed using Multicalc software, version 2000 (Wallac Oy, Finland). Interpolated antibody concentrations were expressed in mIU/mL. With thanks to Dr. Chris Maple, HPA, London, for assistance.

2.4.1.2 VZV IgG Avidity (EUROIMMUN) Assay:

VZV IgG avidity assays were undertaken using the EUROIMMUN (Lubeck, Germany) VZV IgG avidity microplate kit assay according to the manufacturer's instructions. In brief, patient samples were diluted 1:101 in sample buffer and 100μL was loaded, in duplicate, into VZV antigen coated wells of a 96 well microtitre plate. Control samples of high and low avidity human anti-VZV IgG were included with the kit and run on each plate. After incubation for 30min, (RT), the wells were manually washed once, with 450μL working strength wash buffer. 200μL of urea solution was added to one set of sample wells and PBS to the others and the plate left at RT for 10min. The plate was then washed three times and horseradish peroxidise-labelled rabbit anti-human IgG added and the plate incubated at RT for 30min. Following x3 washes, 100μL of chromogen/substrate (tetramethylbenzidine/ hydrogen peroxide) solution was added and the plate incubated for 15min at RT in the dark. The reaction was stopped by the addition of stop solution (0.5M sulphuric acid) to each well and after gentle shaking the plate read at 450nm (reference 620nm) using a Multiskan Ascent™ microplate photometer (Thermo Fisher Scientific, USA). The VZV IgG relative avidity index (RAI) of samples was calculated by dividing the extinction of the sample with urea treatment by the extinction of the sample without urea treatment and

expressed as a percentage. According to the manufacturer RAI <40% indicates low avidity antibodies, RAI 40% - 60% values are equivocal and RAI >60% represents high avidity antibodies. Samples with VZV specific IgG levels below OD: <0.150 after incubation with urea treatment were excluded from analyses.

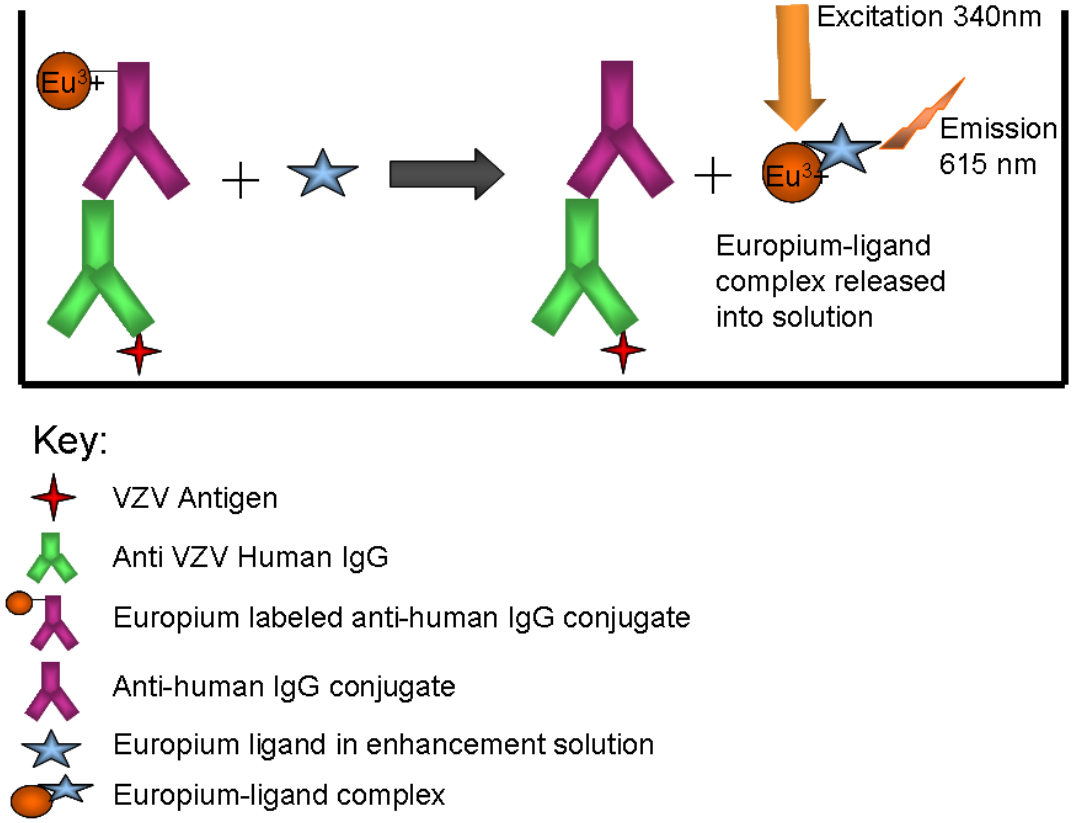


Figure 2.1 Schematic of TRFIA Reaction. In the final stages of the assay an enhancement solution is added which contains a Europium ligand. This causes the Europium bound to IgG to dissociate. Subsequent excitement by UV light causes the Europium-ligand complexes to fluorescence. The half life of this fluorescence is of the order of several hundredths of a micro-second compared to the background fluorescence which is of the order of nanoseconds. Once background fluorescence has decayed, a time resolved fluorimeter measures the fluorescence of the Europium-ligand complex, enabling highly sensitive detection.

2.4.2 SDS-PAGE and Western Blotting:

NuPage® LDS 4x Sample Buffer, containing 5% (v/v) β-Mercaptoethanol (Sigma, UK) was added to the lysates at a ratio of 1:3 and heated at 70°C for 10min. Uninfected vero cell lysate and VZV-infected vero cell lysate (both Advanced Biotechnologies Inc., USA), were used at concentration of

7μg/well. 1μl/well of MagicMark XP western standard (Invitrogen, UK), diluted 1/10 in LDS buffer and 5μl/well of Precision plus protein™ standard (Bio-Rad) were used as markers in all gels, to define protein band size. Samples and markers were loaded onto a NuPage® 10% Bis-Tris SDS gel (Invitrogen, UK) and run in a X-Cell Sure Lock™ Novex Mini Cell (Invitrogen) at 200 volts (constant); (300mAmps), for 50 min in NuPage® MOPS SDS running buffer, which had 0.25% v/v NuPage® Antioxidant (Invitrogen) added just prior to electrophoresis. After separation on the gel, proteins were transferred to Immobilon-P™ PVDF Transfer membrane 0.45μm (Millipore) using an X-Cell Sure Lock™ Novex Mini Cell transfer device (Invitrogen). Transfer was performed in NuPage® transfer buffer (Invitrogen) and a current of 30 volts (constant), (300mAmps) applied for 1h. Following transfer the membrane was washed in blocking buffer (PBS; (Gibco) containing 0.05% v/v Tween 20 and 5% w/v dried skimmed milk powder) for 1h at RT or overnight at 4^{o}C, under constant agitation. The membranes were then incubated with either a commercial mouse monoclonal antibody mixture, containing antibodies specific for seven VZV epitopes (Acris Antibodies, Germany), (2mg/mL; see individual results for dilution), see chapter 3, section 3.3.5 for protein sizes), or ROVE sera diluted in 5% blocking buffer; (see individual results for dilutions), for 1h at RT, under constant agitation. Membranes were then then washed 3 times for 15min in wash buffer (PBS containing 0.05% v/v Tween 20). After washing, the appropriate secondary antibody diluted in 5% blocking buffer; (see individual results for dilutions), was applied and incubated with the membrane for 1h at RT. The membrane was then washed three times for 15min in wash buffer and drip-dried. The membrane was then incubated for between 1-5min at RT in ECL advance^{TM} (Amersham GE Healthcare UK). The membrane was again drip-dried and covered with saran wrap and placed in a Kodak cassette prior to exposure to Hyperfilm^{TM} (Amersham GE Healthcare UK).

2.4.2.1 Coomassie Staining:

Following SDS-PAGE, NuPage® 10% Bis-Tris SDS gels (Invitrogen, UK) were stained in 40mLs/gel of EZBlue Brilliant blue gel saining reagent (Sigma, UK), overnight at RT under constant agitation. The following day gels were washed x3 in distilled water for up to 1h. Images were then visualised on a G:Box™ gel documentation machine (Synegene, UK), using GeneSnap acquisition software (Synegene, UK).

2.4.2.2 Bradford Assay:

Assays were carried out following the manufactures instructions using 'Quick Start Bradford Dye Reagent' (Bio-Rad, USA). BSA (Sigma, UK) was used as a standard and (two fold serial dilutions in PBS), starting at concentration of 4mg/mL. Standards and 'test proteins' were assayed in triplicate. Assays were read on a Versamax™ tunable microplate reader (Molecular Devices, USA), and standard curves were plotted using Excel, (Microsoft 2003, Reading, UK).

2.4.3 Fluorescent Antibody to Membrane Antigen (FAMA) Technique:

The FAMA assays were performed in the laboratory of Professor Anne Gershon according to the method described (Williams *et al.,* 1974). Incubations were carried out for 30min at RT in a humidifying chamber. 48h post-VZV infection, (90% CPE) 25μL of unfixed live human embryonic lung cells (at a concentration of approximately $1x10^5$ cells/mL) were incubated as specified above with 25μL of ROVE sera in 96-well u-bottomed polyvinylchloride plates (Dynex Technologies, USA). Samples were diluted in Minimal essential (Earle's) medium, (Invitrogen) and ranged from 1:2 to 1:128 for each sample. Positive and negative controls consisted of sera from a seropositive lab donor and PBS respectively. Following x3 washes in PBS, 25μL of Fluorescein-conjugated goat anti–human IgG (Kirkegaard & Perry Laboratories, USA), diluted 1:10 was added and incubated as specified above. Cells were then washed, mounted on slides, and examined by fluorescence microscopy (Lietz Ortholux) in a blinded fashion. The end-point titre was the highest dilution

that yielded a positive response. There were no positive cells when serum from a susceptible person was tested. Fluorescence at a 1:2 dilution of test serum and higher was considered positive. With thanks to Sharon Steinberg, Columbia University, USA.

2.5 Cell Based Assays:

2.5.1 IFN-γ ELISPOT:

2.5.1.1 Ex vivo Overnight Stimulation on ROVE HCW PBMCs Using Whole VZV Lysate as Antigen:

Human IFN-γ ELISPOT plates were purchased from BD (UK) and the assay was carried out according to manufacturer's instructions. Briefly, coating (capture) antibody was diluted (according to the certificate of analysis) in coating buffer, and 100μL of diluted antibody solution was added to each well of an ELISPOT plate, which was stored at 4ºC overnight. The coating antibody was discarded and wells were washed with 200μL/well blocking solution. This was discarded and a further 200μL/well blocking solution was added and incubated for 2h at RT.

The blocking solution was discarded and 100μL/well of either media alone, uninfected vero cell lysate (20μg/mL) (Advanced Biotechnologies Inc., USA); (negative controls), PHA (5μg/mL) (Sigma, UK) (positive control) or VZV lysate (20μg/mL) (Advanced Biotechnologies Inc., USA) was added to triplicate wells/study sample. PBMCs were resuspended as detailed in section 2.3.8, diluted to ($2x10^6$/mL) and stored on ice for a maximum of 30min prior to 100μL being added to each well ($2x10^5$/well), to each assay condition, in triplicate. Cells were stored on ice to help minimize cell aggregation. The plate was incubated overnight (16h) at 37ºC, in a 5% CO_2, humidified incubator. When more than one plate was assayed, plates were placed individually on incubator shelves, and not stacked, to allow even distribution of heat. Cell culture stages of the ELISPOT were carried out under aseptic conditions in a class-II Trimat hood.

Following overnight incubation, the cell suspension was then aspirated, and wells were washed twice with distilled water. The wells were allowed to soak for 5min at each wash step. This was to lyse and remove any remaining cells. The wells were then further washed 3x with 200μL/well wash buffer I. Detection antibody was then diluted in dilution buffer according to the certificate of analysis and added at 100μL per well. The plate was incubated for 2h at RT. Detection antibody solution was discarded and wells were washed x3 with 200μL/well wash buffer I. Wells were allowed to soak for 2min at each wash step. Enzyme Conjugate (Streptavidin-HRP) was then diluted in dilution buffer according to the certificate of analysis. 100μL/well diluted enzyme reagent was added and incubated for 1h at RT. The enzyme conjugate solution was discarded and wells were washed x4 with 200μL/well wash buffer I and allowed to soak for 2min at each wash step. Wells were then washed twice with 200μL/well wash buffer II. 100μL of Final Substrate Solution was added to each well.

Spot development was monitored for 1h, and substrate reaction was stopped by washing wells with distilled water. The plate was air-dried at RT overnight until completely dry. Plates were stored in a sealed plastic bag in the dark, until analysis was perforrmed using an automated ELISPOT plate reader (Biosystem 5000, BioSys, Germany). The number of spot forming units/10^6 PMBCs was calculated by subtracting the total number of spots seen in all negative control wells (if any), from the total number of spots in the VZV-stimulated well. The mean of the triplicate wells was calculated and presented as the number of SFU/10^6PBMCs. Study participants were classified as having a positive VZV-specific IFN-γ response if the number of SFUs in the VZV lysate stimulated wells was greater than the (mean+3SD) of all the negative control wells combined.

2.5.1.2 Assessing PBMC Aggregation at Various Incubation Temperatures for ELISPOT Optimisation:

PBMCs from a healthy donor which had been stored in liquid nitrogen for the same period of time as many of the earlier ROVE study time points

were thawed rapidly and counted using a disposable counting slide. The cells were resuspended in 6mL RPMI and mixed thoroughly by pipetting up and down five times. The homogeneous cell suspension was split into three equal volumes (comparable to the volumes of which cells would be resuspended for use during ELISPOT assays) and incubated at either at 4^oC (on ice), RT and 37^oC (5% CO_2), for 1h. Cells were then recounted.

2.5.2 DRB1*1501 Specific Peptide Stimulation of PBMCs; T Cell Lines:
The following assay was carried out under aseptic conditions in a class-II Trimat hood. PBMCs were thawed as described previously (section 2.3.8) and resuspended cells were seeded in a 24 well plate (Nunc, UK) at $4x10^6$/well in 2mL complete RPMI 1640 medium. Peptides were added at a final concentration of 4μg/mL. The plate was then incubated at 37^oC in a humid incubator, 5% CO_2. The PBMCs were fed on day 3 and day 7 by carefully removing 1mL of media from each well and replacing with 1mL of complete RPMI containing 200IU/mL IL-2 (Sigma, UK). Plates were examined under the microscope daily and flow cytometry analysis was carried out on day 10.

2.5.3 Flow Cytometry Analysis:
*2.5.3.1 Flow Cytometry Analysis of DRB1*1501 Tetramer and Surface Staining on ROVE PBMCs:*
DRB1*1501$^+$ ROVE PBMCs were resuscitated as specified in section 2.3.8 and the cell pellet was resuspended in 1mL of RPMI and split between FACs tubes (100μL for compensation tubes, 300μL for tetramer staining). Cells were repelleted by centrifuging at 1,500rpm (350g) for 5min at RT. The supernatant was discarded and in the dark, 2μL of either gE (TSPLLRYAAWTGGLA) or IE63 (QRAIERYAGAETAEY) tetramer (Beckman Coulter, iTAg™ MHC Class II Human SAPE DRB1501/PE) was added to each sample and incubated for 1h at 37^oC (5% CO_2) in the dark. Each sample was then washed with 1mL of PBS (Gibco, UK) and centrifuged at 1,500rpm (350g) for 5min at RT. Surface staining was then carried out by adding the quantity of fluorochrome conjugated antibody

specified in table 2.2 and incubating for 20min at RT in the dark. Each sample was then washed and resuspended in 300μL of 0.5% PFA (Sigma, UK). Samples were stored at 4^{o}C overnight in the dark.

Samples were analysed the following day on a Cyan ADP (Dako) and were first gated according to size and granularity (forward and side scatter) to select lymphocytes. This was then gated further on live T lymphocytes, (viaprobe/7AAD, CD14 and CD19 antibody negative staining). This population was then plotted by CD4 and tetramer positivity. Unstained cells and fluorochrome compensation tubes were used for each run. Data was analysed using Flow Jo (version 8.1.1 Ashland, Oregon, USA).

Specificity	Amount	Flourochrome conjugate	Manufacturer
7 AAD	2μL	PerCP	eBioscience
CCR4	2μL	PE-Cy 7	BD Pharmigen
CCR6	2μL	FITC	R and D
CCR7	2μL	PE-Cy7	BD
CD14	2μL	PerCP	BD Biosciences
CD19	2μL	PerCP	BD Biosciences
CD27	2μL	FITC	BD Pharmigen
CD28	2μL	APC	BD Biosciences
CD3	2μL	FITC	BD Pharmigen
CD3	2μL	PerCP	BD Biosciences
CD3	2μL	APC	BD Pharmigen
CD4	1μL	P'Blue	Biolegend
CD4	2μL	PerCP	BD
CD4	2μL	PE	BD
CD45R0	2μL	APC	BD
CD62L	2μL	APC	BD
CLA	2μL	FITC	BD
Viaprobe	5μL	PerCP	BD Biosciences

Table 2.2: Antibodies Used for Surface Staining of PBMCs Prior to Flow Cytometry Analysis. Key: 7AAD: 7-amino-actinomycin D (viability stain); CCR; CC Chemokine receptor, CD; cluster of differentiation, CLA; Cutaneous lymphocyte associated, antigen (skin homing marker), viaprobe; 7-amino-actinomycin D (viability stain), PerCP; Peridinin chlorophyll protein; PE: Phycoerythrin; Cy; Cyanin, FITC; Fluorescein isothiocyanate, APC; Allophycocyanin, P'Blue; Pacific blue. NB: commonly expressed PBMC markers (CD3 or CD4) were used for compensation staining.

2.6 PCR Based Assays:

2.6.1 PCR on Vaccine Associated Rash DNA:

A VZV-vaccine specific PCR was carried using the VZV reference laboratory methodology, (with thanks to Mr Gavin Wall), on DNA extracted from the vesicles taken from ROVE 1052, three days after the onset of rash.

2.7 Statistical Analysis:

2.7.1 Mixture Modelling:

Following $\log_{10}$ transformation, mixture modelling was performed in Excel (Microsoft 2003, Reading, UK)) using the solver function to fit two normal distributions to the data by maximum likelihood. A proportion (p) were assumed to be part of low or negative distribution with mean (m1) and standard deviation (SD1) and a proportion (1-p) to be part of a positive distribution with mean (m2) and standard deviation (SD2). Using the fitted distributions, the sensitivity and specificity of different cut-offs could be estimated based on the proportions of the two distributions, above (or below) the cut-off.

After allocating samples as negative or positive based on the post vaccination avidity the mean and SD ($\log_{10}$ scale) was calculated and the sensitivity and specificity of different cut-offs calculated using the observed proportions that were assigned as negative and positive that were above and below the cut-off.

2.7.2 ROC Analysis:

ROC analysis was performed using Minitab Inc. (2006, Pennsylvania, USA). The 'gold standard' was taken as avidity six weeks (visit 2), with ≤40% being negative and ≥60% being positive. Samples in between these cut-offs (n=3); and individuals with missing values for either six week avidity or baseline TRFIA were removed from analysis (n=9); thus ROC analysis was carried out using 98 samples. The diagnostic test to be considered was $\log_{10}$ TRFIA values at baseline, with the relevant cut-off (< cut-off being negative, ≥ cut-off being positive). Cut-offs were applied in

$\log_{10}$ 0.02 increments from a value of 1.18 up to 4.8, covering the entire range of the data.

2.7.3 Calculating the Accuracy of Serological Assays:

Serological assay parameters were calculated using the following formulas:

		Reference Assay	
		Positive	Negative
'Test' Assay	Positive	TP	FP
	Negative	FN	TN

Key: Positive assay reading: TP = true positive FP = false positive
Negative assay reading: FN = false negative TN = true negative

- Sensitivity = TP/(TP+FN)
- Specificity = TN/(TN+FP)
- Positive predictive value = TP/(TP+FP)
- Negative predictive value = TN/(TN+FN)

2.7.4 All Other Statistical Analysis:

All other analysis was carried out in SPSS (version 15, Chicago; Illinois, USA). All p-values were generated by independent 2 tailed t-tests unless otherwise stated. Standard deviations were calculated in Excel, (Microsoft XP, Reading, UK).

Fisher's exact test was carried out at the following website: http://www.langsrud.com/fisher.htm

Chi squared tests were carried out at the following website: http://www.quantpsy.org (Preacher, 2001).

CHAPTER 3: HUMORAL IMMUNE RESPONSES TO VACCINATION

Chapter Summary:

In this study I have examined the antibody responses of a cohort of healthcare workers (HCWs) immunised with the Oka vaccine. The relationship between post immunisation antibody titres and avidity was used to evaluate how accurately a new in-house enzyme immunoassay was able to discriminate those who were truly non-immune at enrolment (true negatives). Further characterisation of the nature of the humoral immune response was used to identify groups of subjects, including those who may be at risk of waning immunity. The role of ethnicity in the magnitude of humoral immune responses to natural infection was also investigated.

3.1 INTRODUCTION:

3.1.1 Antibody Structure and Function:

Unlike the innate response, the adaptive immune response is highly specific and has the ability to improve with each successive exposure. Adaptive immune responses are mediated by T and B cells, the latter of which produce antibodies (Murphy *et al.,* 2008). Antibodies are B cell receptors secreted by terminally differentiated B cells known as plasma cells.

3.1.1.1 Antibody Structure and Primary Diversification of Antibody Repertoire:

Antibodies are Y-shaped molecules consisting of three equally sized portions which are bound by a flexible tether (see figure 3.1). Antibodies are constructed from paired heavy (50kDa) and light (25kDa) polypeptide chains, bound by non-covalent and disulphide bonds. The two arms of the Y (F'ab fragment) contain the variable (V) region of the antibody, which varies between different antibody molecules. The antigen binding sites are found at the tips of the Y and are identical on each arm. The antigen specificity of the antigen binding site is determined by complementarity-determining regions (CDRs), three of which are found in each of the heavy

and light chains of the variable region. It is the combination of the CDRs from both regions that contribute to the antigen binding site and thus determine the antigen specificity. The antigen binds via interactions with amino acids within the CDRs. The stem of the Y (Fc fragment) is composed solely of the constant (C) region which is considerably less variable and interacts with effector cells.

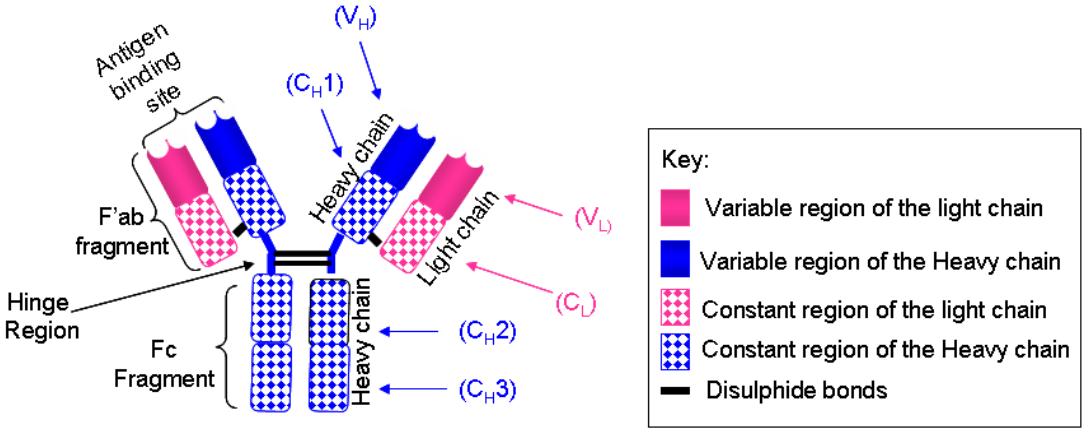


Figure 3.1: Structure of Immunoglobulin. Molecules are composed of Heavy and Light chains, made up of 4 and 2 domains respectively (V_H, C_H1-C_H4 and V_L, C_L), which are bound by a hinge region. Each chain contains variable and constant domains and the variable domains of both the heavy and light chain contain the antigen binding site.

The variation in the antigenic binding sites of each antibody cannot be encoded for in full in the genome, as this would require more genes than there are in the entire genome. Instead the variable region is coded for in gene segments, which are assembled in the developing lymphocyte by somatic DNA recombination to form a complete V sequence. This process is called gene rearrangement. For the light chain the V domain is encoded by two DNA segments; a variable (V) gene segment and a joining (J) gene segment. The joining of the V and J segment creates an exon which encodes the whole of the light chain V region. The heavy chain V domain is encoded by three gene segments. In addition to a V and J segment, an additional diversity (D) gene segment is encoded, which lies between the other two segments. The recombination activating genes (RAG) *RAG-1* and *RAG-2* which encode the recombinase proteins RAG-1 and RAG-2, are essential for catalyzing immunoglobulin gene rearrangement. There are actually numerous copies of all gene segments within the germline

DNA. Random selection of just one gene segment (of each type) accounts for the vast diversity of V domains found in immunoglobulins.

3.1.1.2 Secondary Diversification of the Antibody Repertoire:

When the B cell receptor of a naïve B cell encounters the appropriate antigen, this results in the upregulation of specific chemokine receptors, causing the B cell to migrate to the T-cell-rich area (known as the T-cell zone) of lymphoid tissues. In the T cell zone, B cells become fully activated as a result of their interaction with $CD4^+$ T helper cells and antigen presenting cells.

The secondary phase of antibody diversification occurs in activated B cells and includes antibody class (isotype) switching and affinity maturation via somatic hypermutation (SHM); (discussed in more detail in section 3.1.1.2.1). These processes take place in highly specialized and dynamic microenvironments known as germinal centres (GC). During T-cell-dependent antibody responses to exogenous antigen, GCs are formed by proliferating B cells in the follicles of peripheral lymphoid tissues (such as the tonsils, the spleen, lymph nodes and Peyer's patches), (see figure 3.2 for a schematic of a GC in the context of a lymph node). The formation of GCs and the processes which occur inside of them are yet to be fully elucidated (Elgueta *et al.,* 2009). However it is know that the formation of these structures requires the interaction of co-stimulatory B cell surface receptors with ligands expressed by T cells; specifically CD40, (expressed by all B cells), and its ligand CD154 expressed by helper T cells. CD40 signalling of B cells promotes GC formation; survival of GC-B cells; secondary diversification of the antibody repertoire and the formation of long-lived plasma cells and memory B cells (Elgueta *et al.,* 2009).

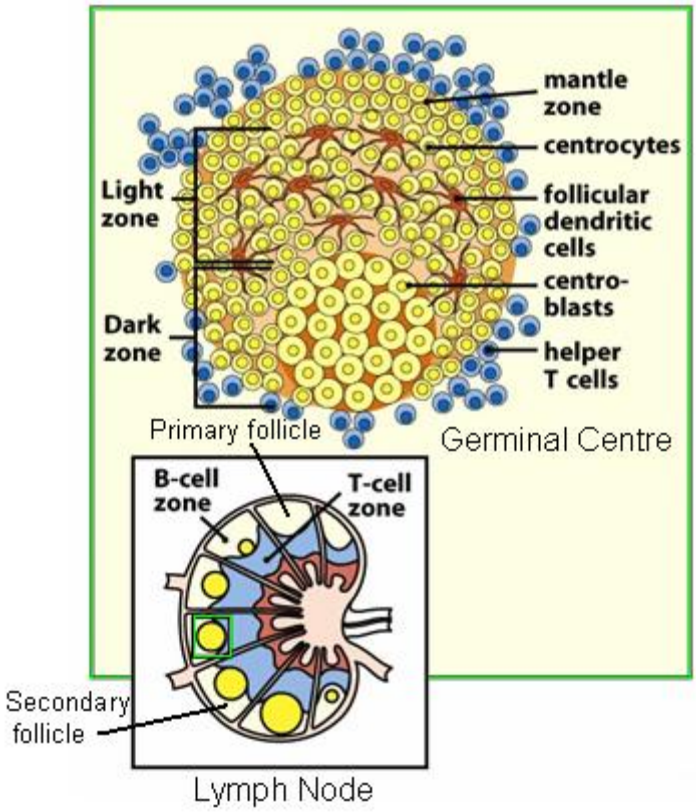


Figure 3.2: Schematic Representation of a Germinal Centre, and its Location within the Lymph Node. Note; Helper T cells (T_{FH}) play a role in antibody class switching and affinity maturation. Centroblasts are proliferating B cells which express CXCR4 and undergo SHM; centrocytes are non-dividing B cells which express CXCR5. A primary follicle is a structure composed of resting B cells within a network of FDCs. Within primary follicles, GC-precursor B cells start to rapidly proliferate and push the resting B cells aside to form the mantle zone around the GC, creating a structure known as the secondary follicle. Murphy *et al.,* 2008; reprinted with permission.

GCs contain many cell types including macrophages, follicular dendritic cells (FDCs) and a subset of recently characterised $CD4^+$ T cells known as follicular helper T cells (T_{FH}), (King *et al.,* 2008; Elgueta *et al.,* 2009). These cells are the true helper cells for antibody responses and have recently been shown to be the dominant source of IL-4 (which is crucial for antibody class switching), within the lymph node (King *et al.,* 2009b). They are distinguishable from other $CD4^+$ T helper subsets due to their expression of the chemokine receptor CXCR5 and the costimulatory molecule ICOS (Inducible costimulator), along with the production of the cytokine IL-21, and the chemokine CXCL13. In addition these cells are classified by their migration to B cell follicles and their function in B cell

help (King *et al.,* 2008). CXCR5 promotes the co-localization of T and B cells in lymphoid follicles, through recognition of its ligand, the chemokine CXCL13, which is also produced by FDCs. The cytokine IL-21 is one of the most important stimulators of B cell differentiation, proliferation and class switching. ICOS engaging with its ligand (ICOS-L; located on B cells), induces the production of helper cytokines such as IL-4, (see section 3.1.1.2.2 for details of class switching), (King *et al.,* 2008).

After initial contact with antigen-experienced DC, antigen-specific effector T_{FH} cells migrate to both the follicular regions of lymphoid organs to form stable contacts with antigen-primed B cells and also to germinal centres, to form stable contacts with GC B cells. Memory B cells are long lived and carry antibody genes that have undergone somatic hypermutation in response to antigen encounter. Memory B cells can participate in secondary immune responses by developing into plasma cells or returning to the germinal centre for further clonal expansion and refinement of antibody affinity. Memory T_{FH} cells persist within the priming environment to regulate the antigen-specific memory B cell response to re-challenge (King *et al.,* 2008).

Within GCs, cells are organized into two major zones; the dark and light zones. In the classical model, the dark zone contains centroblasts, which are large, rapidly proliferating B cells which undergo SHM of their antibody variable region genes (discussed in section 3.1.1.2.1). Centroblasts are suggested to give rise to smaller non-proliferating centrocytes, which (along with follicular dendritic cells; FDCs), populate the light zone. FDCs are stromal cells with the ability to capture large amounts of antigen in the form of immune complexes (antigen:antibody:complement). Immune complexes are not internalised by FDCs but remain on the surface of the cell in highly ordered units termed iccosomes, where they can be recognised by B cell receptors, (Murphy *et al.,* 2008).

3.1.1.2.1 Affinity Maturation of Antibodies:

Somatic hypermutation (SHM) is a process that introduces single nucleotide substitutions (point mutations) throughout the rearranged V region exon at a very high rate. This process is initiated by an enzyme (activation-induced deaminase; AID), which is only expressed in activated B cells and results in mutant B cell receptors expression on the surface of the B cell. All four bases can be targeted for mutation, and in humans C:G pairs and A:T pairs are targeted with approximately equal frequency. AID catalyzes targeted deamination of deoxycytidine residues in DNA (and also underpins isotype class switching). Deamination changes deoxycytidine into deoxyuridine and consequently transforms C:G pairs into U:G mispairs. This can ultimately lead to base excision via base-excision repair enzymes, introducing a single-strand nick in the DNA at the site of the original cytosine, (Murphy *et al.,* 2008).

Some mutant B cell receptors bind antigen with higher affinity than the original clone. In the GC, B cell clones (centrocytes) compete with each other for interaction with antigen on FDCs and T cell help, (Fazilleau *et al.,* 2009). This results in a selective, positive expansion of B cells which have surface immunoglobulin that binds antigen with higher affinity and it is these clones that are selected to differentiate into antibody secreting plasma cells. This process is known as antibody affinity maturation. Follicular helper T (T_{FH}) cells are now recognized as the class of helper T cells that regulate this process, (McHeyzer-Williams *et al.,* 2009). Following contact with antigen-experienced DC, antigen-specific effector T_{FH} cells migrate to the appropriate area of the lymphoid organs to form stable contacts with both antigen-primed B cells and GC B cells. The strength of antigen receptor binding and duration of cellular contact with T_{FH} cells is now thought to play a role in the differentiation and survival of B cell clones (and memory B cells), (McHeyzer-Williams *et al.,* 2009).

3.1.1.2.2 Antibody Class Switching:

The isotype or class of an antibody is determined by the structure of its heavy chain, and thus the Fc fragment and hinge region of each isotype of antibody differs. There are five main heavy chain isotypes, denoted by lower case Greek letters. The corresponding immunoglobulins are known as IgA, IgD, IgE, IgG and IgM. Thus, it is the class of the heavy chain that determines the functional properties of the antibody. Different heavy chains are produced in a given clone of B cell by linking different heavy chain regions (C_H) to the rearranged V_H gene. During the course of an antibody response the activated B cells can switch the expression of C_H genes by a type of somatic mutation known as class switching.

3.1.1.3. Sources of Antibody Following Primary Antigen Challenge:

Ultimately, B cells can either differentiate into plasma cells that survive in the bone marrow, and continuously secrete antibody or into memory cells that can subsequently differentiate into plasma cells upon secondary antigenic challenge. Long-lived plasma cells are germinal centre–experienced cells that survive for months to years in the absence of antigen or cell division. The survival of long lived plasma cells is not fully understood, but the bone marrow is an environment which provides survival signals to these cells, (Wrammet and Ahmed, 2008).

3.1.1.4 Kinetics of Antibody Responses:

A primary response occurs the first time an individual is exposed to an antigen; a secondary response occurs upon subsequent exposure to that antigen. Following a primary antigenic challenge there is an initial lag phase when no antibody can be detected. This is followed by phases in which the antibody titre rises logarithmically to a plateau and finally declines again as the antibodies are naturally catabolised or bind to the antigen and are cleared from circulation. The secondary response has a shorter lag phase and an extended plateau and decline. The plateau in a secondary response is typically 10 times greater than in primary response (Murphy, 2006). Primary antibody responses are mediated by IgM and as

the immune response continues (and if a secondary response is induced) a class and subclass switch in the antibodies produced occurs.

3.1.1.5 Biological Functions of Antibodies:

IgA, IgG and IgM antibodies have diverse biological activity, (mediated by their Fc region), comprising of complement fixation, opsonisation, neutralisation and antibody dependent cellular cytotoxicity (ADCC). The Fc fragment of an antibody can bind to Fc receptors (FcRs) expressed on various cell types; specifically FcγRs bind to IgG antibodies, whilst FcαRs bind to IgA antibodies and so on. There are three isoforms of FcγRs; FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) which bind with differing affinities. Various combinations of these receptors are expressed by a range of cells including neutrophils, monocytes and macrophages, NK cells, platelets, eosinophils, basophils mast cells and B cells (Gessner *et al.,* 1998). The biological consequences of activating Fc receptors depend on the cell type which expresses them. For example, NK cells express FcγRIII (CD16), which plays a crucial role in antibody dependent cellular cytotoxicity (ADCC). This is a process whereby target cells are coated in antibody and NK cells are subsequently able to bind to this coating IgG antibody via their FcRs. Upon binding, the NK cells degranulate releasing perforin and granzymes, which cause the target cell to lyse. During ADCC the virus does not need to be phagocytosed to be destroyed.

Neutralizing antibodies exhibit their biological effect by inhibiting viral infectivity. This is achieved by antibodies binding to the viral surface structures that interact with the cellular receptors involved in viral entry. Opsonising antibodies bind to pathogens and facilitate their destruction by phagocytic cells. For instance, the F'ab portion of an opsonising antibody binds to the viral antigen, whilst the Fc portion of the antibody binds to an FcR on the phagocyte, enabling phagocytosis. Complement activating antibodies bind to a target and form a receptor for the first protein of the complement system. This can either lead to direct lysis via complement or an increase in uptake by phagocytic cells.

3.1.1.5.1 Biological Activity of Different Subclasses of IgG:

IgG is the most abundant immunoglobulin and has several subclasses (IgG1, IgG2, IgG3 and IgG4), which are named according to their respective abundance in human serum. The half lives of the different subclasses vary; IgG3 has a half life of seven days, compared to 21 days for the other three subclasses. IgG1 functions are complement fixation, neutralisation, opsonisation and ADCC. The IgG3 antibody functions in neutralization, opsonisation, and to a lesser extent than IgG1, in ADCC. This subclass also has the greatest capacity of all IgG subclasses for complement fixation. IgG2 and IgG4 do not activate complement very efficiently or bind well to most FcγRs and are therefore thought to function primarily as neutralising antibodies. The IgG2 subclass is a major reactant to bacterial polysaccharide, and does not act as an opsonin. Likewise, IgG4 does not fix complement, participate in ADCC or opsonisation.

3.1.2: Antibodies to VZV:

The presence of antibodies to VZV in serum has been shown to correlate both with a past history of varicella and with protection against subsequent infection (Saiman *et al.,* 2001; Williams *et al.,* 1974). During the course of varicella IgM antibodies are produced within seven days (Arvin and Koropchak, 1980a; Gerna *et al.,* 1979; Van Loon *et al.,* 1992), and have been demonstrated to peak during the second week of disease, and then progressively decrease in titre until they disappear, usually within two months (Gerna *et al.,* 1979). Primary infection with VZV is also associated with production of IgA antibodies within one to five days after onset of exanthem and is also usually undetectable by two months post infection (Wittek *et al*., 1983; Van Loon *et al.,* 1992). The roles of these different classes of antibodies are discussed in detail in section 3.1.2.2.

During cases of zoster, detection of IgM varies and appears to be dependent on the methodology used (Gerna *et al.,* 1979; Namazue *et al.,* 1986; Arvin and Koropchak, 1980; Van Loon *et al.,* 1992; Doerr *et al.,* 1987). However IgG and IgA are seen at increased levels compared with

those seen during acute varicella (Wittek *et al.,* 1983; Van Loon *et al.,* 1992; Namazue *et al.,* 1986), and are detectable between one and four weeks after onset of zoster, and in some cases are still seen two months after the onset of disease (Wittek *et al.,* 1983).

During varicella, IgG, IgM and IgA respond to a range of structural viral proteins including capsomers and the viral glycoproteins (Van Loon *et al.,* 1992; Terada *et al.,* 2002; Bogger-Goren *et al.,* 1984; Arvin and Koropchack, 1980; Dubey *et al.,* 1988; Echevarria *et al.,* 1990; Palumbo *et al.,* 1984; Schmidt and Gallo, 1984). Known targets of VZV antibodies are discussed in more depth in section 3.1.3. The IgG antibody titres to VZV antigens are similar in both acutely and latently infected patients. However, in a report where IgM was detected during zoster, antibodies to early antigens, membrane antigens and thymidine kinase were detected later in zoster than in varicella (Namazue *et al.,* 1986). This suggests that the kinetics of antibody responses are different between varicella and zoster.

The role of antibodies in these two diseases differs considerably. No correlation between VZV-specific antibody titres and the prevention of zoster has been demonstrated (Uduman *et al.,* 1975; Brunell *et al.,* 1975), but antibodies are known to play an important role in modifying the response to varicella. A passive antibody preparation containing VZV IgG antibodies (VZIG) which is prepared from high-titre immune human serum (Brunell *et al.,* 1975) is used as a post-exposure-prophylactic treatment. The preparation is administered to susceptible individuals who have been exposed to varicella or zoster and are at risk of serious complications of chickenpox (Gershon *et al.,* 1974). VZIG has been used successfully to prevent or modify disease in immunocompromised children, including transplant recipients and leukaemia patients (Kavaliotis *et al.,* 1998; Pandya *et al.,* 2001; Zaia *et al.,* 1983; Styczynski *et al.,* 2008), otherwise healthy adults, including pregnant women (Chapman, 1998; Enders, 1985; Enders *et al.,* 1994) and HIV infected individuals (Vafai and Berger, 2001). Transplacentally acquired VZV specific IgG antibodies are also known to

prevent or modify the severity of varicella during the first six months of life (Brunell, 1992).

3.1.2.1 The Role of VZV-Specific Antibodies in the Prevention of Re-Infection:

In most individuals, one attack of varicella confers lifelong protection against exogenous re-infection and subsequent clinical disease. However, despite the presence of detectable serum VZV antibody, clinical and subclinical re-infection can occur, as indicated by a rise in antibody titre after exposure (Weigle and Grose, 1984). This is particularly common in adults who have had varicella but who subsequently have close household contact with varicella. A study of 25 immune adults demonstrated that 64% of individuals who had had a household exposure experienced a four-fold increase in IgG and 70% had significant increases in IgA levels (Arvin *et al.,* 1983a). The first population based surveillance study of the frequency of re-infections revealed that 13% of the 1,472 mostly healthy children who presented with chickenpox (in California, USA between 1995 and 1999) had a previous history of varicella infection (Hall *et al.,* 2002). In a smaller study of 21 pregnant women who were exposed to varicella, four developed varicella, despite testing seropositive for VZV antibodies prior to exposure (Martin *et al.,* 1994). The largest reported series of cases of symptomatic, recurrent varicella due to re-infection included 14 apparently immunocompetent children who had intact humoral (and cell mediated) immune responses to VZV, each of whom experienced between two and five bouts of varicella over a period of years, (Junker *et al.,* 1991). It has been suggested that since the magnitude of VZV replication and degree of viraemia is reflected in severity of disease expression (Weigle and Grose, 1984; Malavige *et al.,* 2008b), clinical infection of VZV in individuals who have specific humoral (and cell mediated immunity) to VZV may occur if a virus load is high enough to overwhelm immune defences (Martin *et al.,* 1994). In addition, a case report documenting two separate cases of zoster (four years apart) within one immunocompetent individual, found that in each episode of zoster, a separate genotype of virus was reactivated (Taha *et al.,* 2006). A study which examined the avidity of IgG

VZV-specifc antibodies (see section 3.1.4 for more details on avidity studies) during an outbreak at a geriatric home, also demonstrated that one resident who developed varicella (from an index case of zoster), had high avidity antibodies during acute infection; signalling existing humoral immunity; and therefore re-infection, (Lopez *et al.,* 2008).

3.1.2.2. The Roles of Different Classes and Subclasses of Antibodies in VZV Infection:

3.1.2.2.1 Immunoglobulin G:

The long lasting humoral immunity to VZV resides in the IgG of the sera (Asano *et al.,* 1983; Asano *et al.,* 1985a; Asano *et al.,* 1985b). Following primary and recurrent VZV infections IgG1 and IgG3 predominate while the occurrence and importance of IgG2 and IgG4 subclasses in the serum is reported to differ (Doerr *et al.,* 1987, Sundqvist *et al.,* 1984, Asano *et al.,* 1987). IgG3 is indicative of recent infection or reactivation of VZV (Doerr *et al.,* 1987) and IgG3 levels have been shown to be related to recovery from VZV and fall dramatically during convalescence (Asano *et al.,* 1987). A study by Echevarria (*et al.,* 1990) confirmed the dominance of the IgG1 and IgG3 antibodies in both primary and recurrent VZV infections, and Leonard (*et al.,* 1970) demonstrated the presence of IgG1 antibodies in the case of natural VZV infection. Doerr's study concluded that IgG2 antibodies are of no significance for humoral immune responses after VZV infection, and Leonard's study found that IgG4 had very little neutralizing activity against the virus. In contrast to the findings of Doerr and Leonard, Echevarria (*et al.,* 1990), found an IgG2 response in a significant number of zoster patients, along with IgG4 responses in nearly all zoster cases, and 45% of varicella cases.

Only IgG crosses the placenta (IgA, IgD, IgE and IgM are excluded), (Kohler and Farr, 1966). Infants under six months of age may acquire varicella more frequently than other viral infections such as measles, leading to the suggestion that the VZV antibody transfer across the placenta does not provide complete protection against the disease. Thus; the subclasses of IgG that are transferred, may shed light onto the role of

each subclass in VZV infection. In a study of 46 mothers who were seropositive for VZV, all transferred IgG1 antibodies to their infants (Asano *et al.,* 1988). In contrast to Doerr's findings all of the mothers tested positive for VZV-specific IgG4, but all had no, or very low antibody activity in IgG2 and IgG3. As IgG2 is not predicted to be protective against VZV and IgG3 antibody levels fall rapidly after the initial infection (Asano *et al.,* 1988) this finding is somewhat expected, but IgG4 is usually present in very low concentrations in serum.

3.1.2.2.2 Immunoglobulin A:

Production of secretory-IgA (s-IgA) is induced in various mucosal surfaces including nasal-pharyngeal associated lymphoid tissue and bronchus-associated lymphoid tissue, upon stimulation with antigen and plays an important role in preventing viruses from invading the body; by means of viral neutralization (Brown, 1996) and can participate in ADCC. A substantial VZV IgA antibody response is elicited during both primary and recurrent VZV infections (Bogger-Goren, *et al.,* 1982; Doerr *et al.,* 1987).

With natural infection, virus has been recovered from throat swabs upon the onset of disease (Ozaki *et al.,* 1986; Ozaki *et al.,* 1994), but vaccination by-passes replication in the regional lymph nodes around the nasopharynx because it is subcutaneously injected. Throat swabs from vaccinees in the Ozaki's 1994 study were negative for VZV DNA. In a study by Tereda (*et al.,* 2000), VZV-s-IgA values were significantly higher in matched children after natural chicken pox than in those who had received the vaccine, although there was not much difference in total IgA. Two of the vaccinees in this study lacked s-IgA altogether. The elderly and immunocompromised children in the study who had been naturally infected had the same level of s-IgA as the naturally infected children. Those who had recently had zoster, or were HCWs (in regular contact with VZV) had higher levels of s-IgA. Doerr's study also confirmed a boost in IgA antibodies following zoster. It has been hypothesized that low or no s-IgA may be a cause of breakthrough (Tereda *et al.,* 2000).

3.1.3 Targets of Humoral Immunity to VZV:

Research into targets of humoral immunity has primarily focused on the glycoproteins which play important roles in fusion, entry and spread of the virus (see chapter 1, section 1.5 for details). Tegument proteins (IE62 and IE63) that invoke good cell mediated immunity and are transcribed early in the viral replication process have also been investigated (Arvin *et al.,* 1986a; Englund *et al.,* 1990, Sadzot Delvaux *et al.,* 1998), whilst during zoster antibodies to thymidine kinase have been documented (Kallander *et al.,* 1982). Many of the studies examining targets of adaptive humoral immunity have been carried out in small animal models (rabbits, guinea pigs and mice) or have used monoclonal antibodies raised in these models. Some studies have been carried out using human serum, and these are noted in the text where relevant. Table 3.1, summaries the VZV proteins (and their function) that are known to induce antibody responses. Several glycoproteins (gB, gE and gI) have had antibody epitopes identified (see figure 3.3 and table 3.2) and are discussed in more detail.

3.*1.3.1 Glycoproteins:*

Antibodies against gB are produced following natural infection and vaccination (Harper *et al.,* 1990b), and can neutralize virus (Forghani *et al.,* 1990b). Both complement dependent (Edson *et al.,* 1985b) and complement independent (Edson *et al.,* 1985b) neutralising antibodies against gB have been reported in animals. A study by Giller (*et al.,* 1989b) revealed that this protein served as a potent B cell antigen during both acute infection and convalescence. A more in depth study by Kjartansdottir (*et al.,* 1996) revealed that a 31-mer-peptide corresponding to a hydrophilic segment of the gB reacted with 2 out of 4 varicella and 5 out of 9 zoster sera, respectively (see table 3.2 for epitope details). One of the predominant antibody responses in sera from the elderly is to gB (Vafai *et al.,* 1988a).

Gene	Function	Reference
31	Glycoprotein B	Harper *et al.,* 1990b; Forghani *et al.,* 1990b; Edson *et al.,* 1985b*, Vafai *et al.,* 1988a; Giller *et al.,* 1989b; Kutinova *et al.,* 2001* Kjartansdottir *et al.,* 1996; Haumont *et al.,* 1997;
37	Glycoprotein H	Němečková *et al.,* 1996*; Akahori *et al.,* 2009; Forghani *et al.,* 1990b; Arvin *et al.,* 1986a; Harper *et al.,* 1990b; Kutinova *et al.,* 2001*; Montalvo and Grose, 1986a; Ito *et al.,* 1993; Vafai *et al.,* 1988a
40	Major Nuclear Capsid Protein	Weigle and Grose 1984; Vafai *et al.,* 1988a; Vafai *et al.,* 1990
60	Glycoprotein L (in Complex with gH)	Němečková *et al.,* 1996*; Kutinova *et al.,* 2001*
62	Transcriptional Activator and Tegument Protein	Arvin *et al*., 1986a; Englund *et al.,* 1990; Lowry *et al.,* 1992*; Forghani *et al.,* 1990a
63	Transcriptional Activator and Tegument Protein	Sadzot-Delvaux *et al.,* 1997;
67	Glycoprotein I	Vafai *et al.,* 1987*; Vafai *et al.,* 1989*; Vafai *et al.,* 1988d*; Vafai *et al.,* 1988a; Vafai *et al.,* 1991*
68	Glycoprotein E	Ludvikova *et al.,* 1991*; Fowler *et al.,* 1995; Kutinova *et al.,* 1996*; Kutinova *et al.,* 2001*; Vafai *et al.,* 1988d*; Vafai *et al.,* 1988a; Vafai *et al.,* 1989*; Vafai *et al.,* 1991*; Vafai, 1993*; Vafai *et al.,* 1987*; Vafai, 1994; Vafai, 1995; Harper *et al.,* 1990b; Lowry *et al.,* 1992*; Forghani *et al.,* 1990b, Arvin *et al.,* 1986a; Wu and Forghani, 1997; Hatfield *et al.,* 1997

Table 3.1: Function of VZV Proteins which Induce Antibody Responses. Key: *Study carried out in animal models, or using mAbs raised in animals; all other studies were carried out using human sera.

A comparison of the antibody responses to gB after natural VZV infection or after vaccination with live attenuated Oka vaccine determined that there was the same order of magnitude of antibody responses in sera from individuals with a history of varicella and in vaccinated children (Haumont *et al.,* 1997). Antibody levels were comparably higher in individuals given booster vaccination. The ratio of anti-gE to anti-gB antibody is highly variable from one individual to another but relatively stable over a long

period of time for a particular individual, even after a zoster episode (Haumont *et al.,* 1997). With respect to neutralizating antibodies, anti-gE and anti-gB are equally prevalent in vaccinated children and anti-gE is generally, but not always, more abundant than anti-gB in VZV infected individuals (Haumont *et al.,* 1997). Antibodies to these two glycoproteins appear to predominate amongst the neutralizing antibody response to VZV antigens (Haumont *et al.,* 1997). This study also suggested that the contribution of anti gB-antibodies to neutralisation may be more important than those of gE in the case of vaccination.

Glycoprotein	aa Number	Sequence	Reference
gB	417-447	HSPQKHPTRNTRSRRSVPVELRANRTITTTS	Kjartansdottir *et al.,* 1996
gI	55-69	LVFIGEQLPTGTNYS	Vafai *et al.,* 1988d
gI	245-259	VKEGIENHVYPTDMS	Vafai *et al.,* 1988d

Table 3.2: Known Antibody Epitopes of gB and gI. The gB epitope was identified using sera from varicella and zoster patients, whilst the gI epitopes were mapped using mouse mAbs.

Anti-gE antibodies have been shown to mediate ADCC (Ito *et al.,* 1985). Ludovikova (*et al.,* 1991) and Kutinova (*et al.,* 1996) have shown that recombinant vaccinia viruses expressing gE elicit an antibody response in mice which is capable of neutralising VZV infectivity in the presence of complement. These findings were supported by the findings of Vafai (1993), who demonstrated that a purified, truncated version of gE (511 aas), expressed in a vaccinia virus vector, was capable of inducing complement-dependent neutralising antibodies in rabbits. Further work by the author in 1994, again using the recombinant vaccinia virus expression system identified new epitopes (see figure 3.3 for details) which were recognised by antibodies within human serum (Vafai, 1994). The author went on to deliver this as a booster vaccine to a seropositive human subject, and demonstrated that neutralising antibodies were produced

(Vafai, 1995). An increase in gE antibodies was seen as early as six days post immunisation and increased over a 40 day period.

Harper *et al.,* (1990) demonstrated that live attenuated Oka vaccination also induces gE antibodies, which are abundant after vaccination and natural infection (Harper *et al.,* 1990), and can neutralize virus (Forghani *et al.,* 1990b, Arvin *et al.,* 1986a). Wu and Forghani, (1997) later characterised the neutralising domains of gE by use of a monoclonal antibody (mAb) library and plasmid vectors containing either the whole gE, the N-terminal (212 aas) or the Carboxy-terminal (160 aas). Monoclonal antibodies with high titre complement-dependent neutralising activities bound the N-terminal truncated infected cells, whilst non-neutralising mAbs stained the C-terminal truncated infected cells. However the complement enhanced neutralising mAbs bound both truncated forms indicating that they were reacting with the overlapping region of the constructs.

Fowler and colleagues (1995) mapped gE epitopes by inserting overlapping fragments of the gE gene into Virus Like Particles (VLPs) of a yeast expression system, and tested these against seropositive human sera. Pepscan analysis was then used to determine the position of the B cell linear epitopes, see figure 3.3 for a schematic of all gE epitopes which have been mapped. Using sera from mice and guinea pigs immunized with gE-VLPs and pepscan analysis, the authors mapped the dominant epitopes, listed as epitopes 5-10 in figure 3.3. These sera also showed neutralizing activity against VZV *in vitro*. These authors also mapped the epitopes recognized by neutralizing mAbs 3B3 and IFB9 used for diagnostics, as residues 141-161 (3B3) and 71-90 (IFB9). Simultaneously, Hatfield and colleagues (*et al.,* 1997) mapped the gE 3B3 epitope to 11 residues (151-161).

Naturally occurring VZV escape mutants which are not recognised by the gE 3B3 mAb have been identified in north America and Europe. The first to be discovered was VZV-MSP, which arose through a single aa change (aspartic acid into an asparagine (D150N)), which abolished the epitope. More importantly, this loss resulted in more virulent virus phenotype; capable of accelerated cell-to-cell spread *in vitro* (Santos *et al.,* 2000; Santos *et al.,* 1998) and *in vivo* in skin implants grafted onto the SCID-Hu mouse model (Santos, *et al.,* 2000). The authors went on to suggest that the escape mutation arose due to immunological pressure (Padilla and Grose, 2001). In 2002, another variant strain of VZV was isolated from an elderly man with herpes zoster in Canada (Tipples *et al.,* 2002), and was named VZ-BC. It is likely that these gE mutant viruses arose through independent mutation as phylogenetically, they were not closely related. A third isolate, identified in Sweden, arose through a single aa change at a different position (R152S), (Wirgart *et al.,* 2006), supporting the notion that each isolate mutated independently.

Vafai *et al.,* 1987 demonstrated that antibodies against gE and gI could neutralize VZV infectivity *in vitro*. Antigenic cross reactivity between gE and gI (determined by the binding of a mAb) mapped to residues 109-123 on gE and within the first 153 residues on gI (Vafai *et al.,* 1989). See table 3.2 for the sequences of gI epitopes. Residues 107-121 of that epitope had a degree of similarity to the epitopes found on gI: residues 55-69 and 245-259) (Vafai *et al.,* 1988d). A history of zoster in the elderly has been demonstrated to correlate significantly with the presence of antibody to gI (Vafai *et al.,* 1988a).

Anti-gH antibodies have been shown to mediate ADCC (Ihara *et al.,* 1991). Antibodies against gH are produced after natural infection and vaccination (Harper *et al.,* 1990b), and can neutralize virus (Forghani *et al.,* 1990b, Arvin *et al.,* 1986a; Akahori *et al.,* 2009). Immunoblotting experiments indicated that the conformational neutralisation epitope was located within the tertiary structure of this glycoprotein (Montalvo and Grose, 1986a). The epitope elicits a complement independent neutralising antibody response

(IgG1) of high magnitude (Forghani *et al.,* 1984, Forghani *et al.,* 1982, Grose *et al.,* 1983a, Keller *et al.,* 1984), and is still present when the gH:gL complex is formed (Forghani *et al.,* 1994). The epitope is highly conserved as the structure is recognised within the vaccine strain vOka, wild type strains and laboratory strains of VZV.

Competition blocking experiments using mouse monoclonal antibodies indicated that four neutralising antibodies were directed against the same or closely related epitope on gH indicating that this is an immunodominant neutralisation epitope (Montalvo and Grose, 1986a). The authors also identified one non-neutralising antibody that reacted with a different antigenic site. More recent experiments using human monoclonal antibodies and immunohistochemistry found that the neutralization domain of gH comprised of a cluster of seven conformational-neutralization-epitopes which prevented cell-to-cell infection *in vitro* (Akahori *et al.,* 2009).

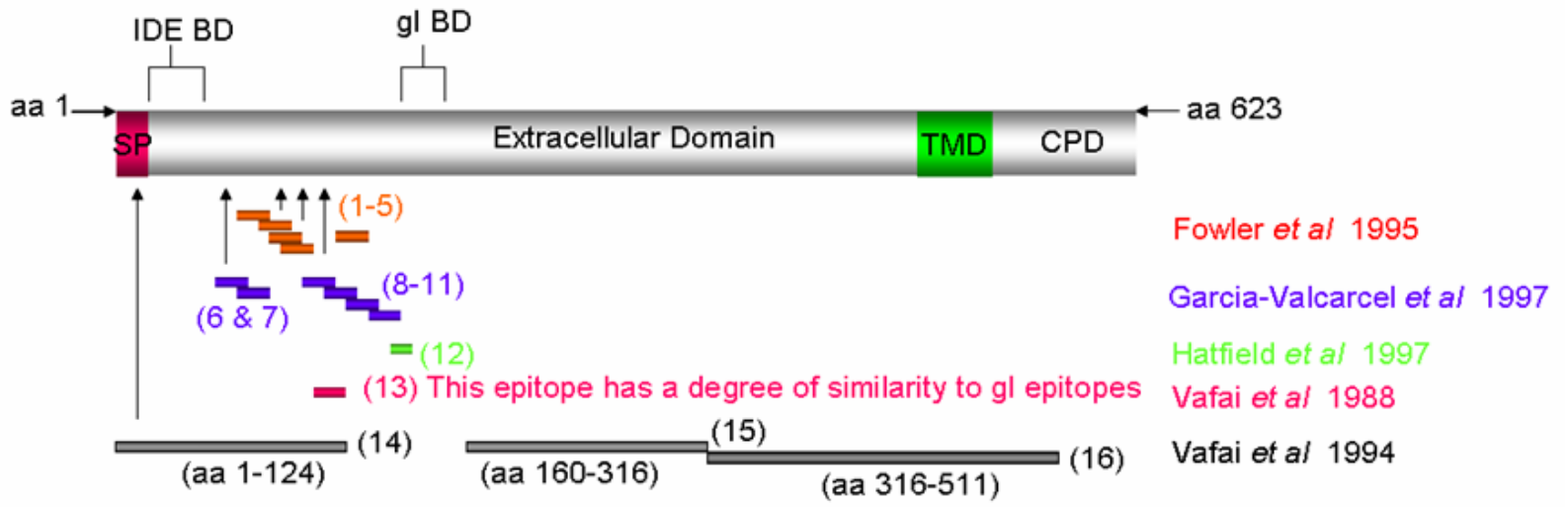
In a study carried out in mice and guinea pigs using vaccinia virus vectors expressing various glycoproteins (Kutinova *et al.,* 2001), immunisations with double recombinants rather than single was more effecitive in raising gE, gB and gH neutralising antibodies. Indeed, Němečková (*et al.,* 1996) showed that anti-gH neutralizing antibodies could not be induced in mice unless gH was expressed with gL in the same cell. This data suggests that gH either forms a compound epitope with gL or that co-expression of gL is a requirement for surface gH expression.

Figure 3.3: The Structure of Glycoprotein E and the Position of Known Antibody Epitopes.

Linear epitopes **1-5** were identified by use of VLPs expressing fragments of gE, peptides from the most immunogenic fragment, western blotting, ELISA and pepscan analysis, tested against seropositive sera from varicella and zoster patients within 4 months of disease (Fowler *et al*., 1995). Epitope **1**: Recognised by 3/5 zoster and 2/5 chickenpox sera. Epitope **2**: All varicella sera reacted strongly, but no zoster sera reacted. Epitope **3**: One zoster patient reacted very strongly, but residues 71-80 of this epitope were recognised by all chickenpox sera but not by any of the zoster sera. A neutralizing mouse monoclonal antibody (IFB9) reacted with sequence 71-90. Epitope **4**: All varicella and zoster sera tested reacted with this epitope. Epitope **5**: All varicella sera and 4/5 zoster sera reacted. Epitopes **6-11** were mapped by inserting overlapping fragments of the gE gene into Virus Like Particles (VLPs) of a yeast expression system, and tested against seropositive human sera. Pepscan analysis was then used to determine the position of the B-cell linear epitopes. Dominant epitopes were confirmed using sera from mice and guinea pigs immunized with gE-VLPs. These sera also showed neutralizing activity against VZV *in vitro*. In addition the epitopes recognized by neutralizing mAbs 3B3 and IFB9 used for diagnostics, were mapped to residues 141-161 (3B3) and 71-90 (IFB9), (Garcia-Valcarcel *et al.,* 1997). Epitope **12:** recognised by the mAb 3B3 was determined by recombinant PCR mutagenesis and is found at aa 151-161 (Hatfield *et al.,* 1997). Epitope **13**: Identified by use of mAb, cloned gene products and immunoprecipitation (Vafai *et al.,* 1988). Monoclonal antibodies against this epitope also bind to gI within the first 153 aa (Vafai *et al.,* 1988). Note, residues 107-121 of this epitope have a degree of similarity to the epitopes found on gI: residues 55-69 and 245-259, (Vafai *et al.,* 1988). Fragments **14-16** were identified as immunogenic, using human sera, recombinant vaccinia viruses expressing truncated parts of gE and immunoprecipitation. These fragments contained epitopes for several mAbs (Vafai *et al.,* 1994).

Key: IDE BD, Insulin Degrading Enzyme Binding Domain (aa 24-71); gI BD, gI Binding Domain (aa 163-208); SP, Signal Peptide; TMD, Transmembrane Domain; CPD, Cytoplasmic Domain.

NB: Epitopes (1 and 7), (5 and 9) are identical.



Epitope Number	aa Number	Sequence
1	56-75	HSDHAESSWVNRGESSRKAY
2	66-85	NRGESSRKAYDHNSPYIWPR
	71-80	SRKAYDHNSP
3	71-90	SRKAYDHNSPYIWPRNDYDG
	81-90	YIWPRNDYDG*
4	86-105	NDYDGFLENAHEHHGVYNQG
5	116-135	QPTQMSAQEDLGDDTGIHVI
6	41-60	DEDKLDTNSVYEPYYHSDHA

* Recognised by the IFB9 monoclonal antibody

Epitope Number	aa Number	Sequence
7	56-75	HSDHAESSWVNRGESSRKAY
8	101-120	VYNQGRGIDSGERLMQPTQM
9	116-135	QPTQMSAQEDLGDDTGIHVI
10	131-150	GIHVIPTLNGDDRHKIVNVD
11	141-161	DDRHKIVNVDQRQYGDVFKGD£
12	151-161	QRQYGDVFKGD£
13	109-125	DSGERLMQPTQMSAQ

£ Recognised by the 3B3 monoclonal antibody

3.1.3.2 Capsid and Tegument Proteins:

Antibodies to the major nuclear capsid protein (NCP; encoded by ORF40) are induced soon after the onset of varicella rash (Weigle and Grose 1984) and are consistently present in VZV seropositive individuals throughout life (Vafai *et al.,* 1988d). One of the predominant antibody responses in sera from the elderly is to this protein (Vafai *et al.,* 1988d) and mAbs to VZV NCP are cross reactive with HSV-1 NCP (Vafai *et al.,* 1990). This may suggest that infection with one virus may help to confer humoral immunity to the other.

Arvin (*et al*., 1986) demonstrated that IgG antibodies to IE62 were detectable in human sera from healthy immune donors, and those in the convalescent phase of both varicella and zoster. A study of healthy children who were naturally infected with VZV demonstrated that antibodies against IE62 were detectable at one month and one year post infection (Englund *et al.,* 1990). Antibody responses to IE63 (from serum of naturally immune adults with a history of chickenpox) have been demonstrated using the protein expressed in bacteria and fused to glutathione-S-transferase (GST) as a stimulating agent. Although anti-IE63 specific antibodies were demonstrated by Western blot analysis banding in the blots was deemed to be weak (Sadzot-Delvaux *et al.,* 1997). Whether any of these antibodies are neutralising has not been investigated, but based on the fact that neutralising antibodies target viral proteins involved in host cell entry, anti-IE63 antibodies would not be expected to have a neutralising function.

3.1.4 Avidity Studies:

Antibody affinity describes the relative strength of interaction between an antibody combining site and antigenic determinant. Avidity or functional affinity indicates the overall reaction between a population of antibodies and a complex antigen (Griswold, 1987). Avidity testing for the differentiation between a primary and an enduring antibody response to infectious disease was introduced in 1984 when a simple and reliable method was developed by

using enzyme-linked immunosorbent assay (ELISA) and a mild protein-denaturing agent (Inouye *et al.,* 1984).

IgG avidity is low after primary antigenic challenge but increases progressively during subsequent weeks and months due to affinity maturation and antigen-driven B cell selection. Low avidity IgG antibodies predominate early after primary infection, but avidity increases markedly during convalescence (Kangro *et al.,* 1991). In cases of recurrent infection high avidity antibodies were found to predominate at all times. An interesting finding of this study is that changes in antibody avidity are dependent on the peptide to which the antibody is raised. Kangro (*et al.,* 1991) found that nuclear assembly proteins (p32 and p36, also known as the nucleoprotein complex) had consistently lower avidity responses than glycoprotein antibodies.

A case in a South African geriatric home of three residents who were believed to have developed varicella following exogenous reinfection revealed some interesting results about the role of avidity. Serology samples tested one day after each patient was admitted to hospital with a disseminated vesicular rash revealed that the avidity of the VZV-IgG antibodies was lower than those of primary chickenpox controls, which were in turn expectedly lower than those of the zoster controls. Elderly individuals' susceptibility to varicella reinfection may well be associated with the decay of specific humoral immunity and by the presence of low avidity antibodies (Schoub *et al.,* 1992). Another study was carried out in immunocompetent children who experienced recurrent varicella (each patient had had between two and five episodes of varicella), but all had comparable VZV cellular immune responses and total antibody levels comparable to controls. However the mean antibody avidity was significantly lower than in controls (Junker and Tilley 1994). These patients were able to mount high avidity antibodies to CMV and Rubella, and did not experience repeat infection with other agents (Junker and Tilley, 1994).

3.1.5 Differences in the Magnitude of the Humoral Immune Response to Varicella Vaccine:

3.1.5.1 Genetic Factors:

The influence of host factors on the magnitude of the humoral response is supported by data showing a significantly greater concordance of VZV antibody levels following immunisation with Oka vaccine, between siblings than between unrelated individuals (Klein *et al.,* 2007). A heritable effect on antibody levels has also been reported following infection with EBV (Yasui *et al.,* 2008), as well as following vaccination against MMR (Ovsyannikova *et al.,* 2006), hepatitis B (Hennig *et al*., 2008), influenza (Lambkin *et al.,* 2004) and Polio (Paul *et al.,* 2009).

3.1.5.2 Age of Infection:

Studies in vaccinated children have shown high seroconversion rates of 97%, (Watson *et al.,* 1990), with seroconversion rates four to six years later of 95-100%, (Watson *et al.,* 1994; Watson *et al.,* 1995b). Adult studies suggest that antibody responses are not retained at such a high rate (Gershon *et al.,* 1988; Lugwig *et al*., 2006; Ampofo *et al.,* 2002, Saiman *et al.,* 2001; Ndumbe *et al.,* 1985a). In a study of 34 VZV seronegative nurses who were vaccinated with Varilrix and followed for periods of up to 36 months, it was revealed that at 5 and 12 months post vaccination, 94% of the nurses had seroconverted but at 3 years, only 64% retained antibody seropositivity (Ndumbe *et al.,* 1985b). In a separate study of 187 vaccinated adults, although seroconversion rates were 94% after two doses of vaccine, 25% were seronegative at follow-up (Gershon *et al*., 1988).

3.1.5.3 Vaccination Versus Natural Infection:

Studies have indicated that there are differences in the humoral immune response induced by vaccination compared to that of naturally induced immunity. A vaccine study (in children) carried out in the 1980s, revealed that although natural infection, subcutaneous immunization, and respiratory

inhalation of large doses of vaccine consistently resulted in the development of VZV-specific IgG antibodies, which persisted for at least eight to 12 months (the length of the study) after either form of infection, the mean IgG antibody titres were four- to eight-fold higher after natural infection than after immunization. As mentioned earlier, natural infection but not VZV vaccine, was associated with the development of serum and nasopharyngeal IgA responses to VZV in most subjects (Bogger-Goren *et al.,* 1982).

3.1.5.4 Factors Affecting Vaccine Seroconversion Rates:

Seroconversion is defined as the change of a serological test result from negative to positive, indicating the development of antibodies in response to natural infection or immunisation. Rates of seroconversion in subjects immunized with the Oka vaccine are dependent on viral titre per dose, relative antigen content and the number of vaccine doses administered. In short, the higher the viral titre, the higher the response. Weibel (Weibel *et al.,* 1984) reported that children who were immunized with 435 plaque forming units (pfu) developed an antibody response quicker than those who received only 42pfu. In several studies, the minimal effective dose required for seroconversion rates of 95% in healthy children ranged from 200-500pfu (Takahashi *et al.,* 1974; Arbeter *et al.,* 1984; Ozaki *et al.,* 1984a; Horiuchi 1984). Children receiving 2,650 pfu or more had higher geometric mean antibody titres (GMTs) six weeks after immunization compared with those who received 950pfu (Johnson *et al.,* 1989). A randomised placebo-controlled study in over 500 Finnish children vaccinated between the ages of 10 to 30 months with either a high-titre (10,000-15,850 pfu) or low titre (1,260-630 pfu, partially heat-inactivated) vaccine found that the protective efficacy of the live attenuated varicella vaccine was dependent on the vaccine titre administered (Varis and Veskari, 1996). Although comparable seroconversion rates of 100% and 99% were initially recorded in children immunized with either vaccine the GMT of post-vaccination varicella antibodies was higher in the high titre recipients compared to the low titre recipients. In addition at follow-

up (a mean of 29 months post vaccination), incidence of varicella was significantly higher (p=0.005) in the low-titre vaccine group (11% n=19/166) compared to the high-titre vaccine group (3% n=5/166). The crude efficacy of the high titre vaccine was 88% (CI 72-96%) versus 55% (CI 31%-72%) for the low titre vaccine.

As well as live replicating vaccine virus, the vaccine preparations also contain incomplete virus particles and dead virus, both of which can stimulate an immune response. Reducing the amount of live virus from 1,770 to 80-160pfu, while maintaining the relative antigen content only reduced seroconversion rates from 97 to 92% (Watson *et al.,* 1993). When the pfu content was maintained at 1,000pfu, and the antigen content was reduced from 96 to 87%, antibody titres produced were equivalent, (Bergen *et al.,* 1990).

The representative viral titre in a single dose of the commercially available varicella vaccines is a minimum of 1,350pfus for VARIVAX® and a minimum of 1,995pfus for VARILRIX™. Ninety five percent of healthy children seroconvert after one vaccine dose (Kuter *et al.,* 1991; White *et al.,* 1991a; Takahashi *et al.,* 1974).

Healthy children given two doses of Oka/Merck varicella vaccine 3 months apart, have significantly stronger humoral immune responses 1 year later, than children given a single dose (Watson *et al.,* 1995a) and adolescents and healthy adults require two doses for 99% seroconversion (Kuter *et al.,* 1995). Leukaemic children and children with chronic renal insufficiency require two doses for 90% and 98% seroconversion respectively (Hardy *et al.,* 1991; LaRussa *et al.,* 1996; Furth *et al.,* 2003). A second dose of varicella vaccine has been demonstrated to increase seroconversion rates to 99% (Kuter *et al.,* 2004; Ngai *et al.,* 1996; Reisinger *et al.,* 2006).

3.1.6 Antibodies as a Measure of VZV Immunity and Breakthrough Infection:

One of the shortcomings of the current vaccine is the incidence of breakthrough varicella. Breakthrough varicella is generally defined as wild type varicella occurring in a vaccine recipient 42 days or more after vaccination (Sharrar *et al.,* 2000), and this places the sufferer at risk of wild type zoster in the future (Takahashi, 2001). Although mild breakthrough is less transmissible than wild type varicella, moderate or severe breakthrough disease (more than 50 skin lesions) has been found to be as contagious as varicella in the unvaccinated controls (Seward *et al.,* 2004). Thus breakthrough also represents a transmission risk within the community.

The clinical symptoms of breakthrough varicella are generally considerably milder than those of natural infection. The number of lesions is reduced, in both adults and children, usually to no more than 50 (Ampofo *et al.,* 2002, Ozaki *et al.,* 2000, Sharrar *et al.,* 2000) and in leukaemic children to generally <100 (LaRussa *et al.,* 1996), compared to 250-300 normally seen following natural disease (Ross *et al.,* 1962). The incidence and severity of fever is also reduced (White *et al.,* 1992; Asano and Takahashi 1977; Bernstein *et al.,* 1993) as is the overall duration of illness (Bernstein *et al.,* 1993).

Protection of immunized individuals from breakthrough varicella infection correlates with the level of VZV antibodies six weeks post-vaccination. Vaccine efficacy among children with a six-week post-vaccination antibody titre of five glycoprotein-based enzyme-linked immunosorbent assay (gpELISA) units or greater was 95.5% compared with 83.5% for subjects with a titre of <5 gpELISA units. The latter group were 3.5 times more likely to develop breakthrough varicella compared to the former group (Li *et al.,* 2002). White and colleges made similar observations and reported that the number of varicella lesions in these cases tended to decrease as the six-week gpELISA titre increased (White *et al.,* 1992).

The most cited risk factors for breakthrough varicella include; three-five-year interval since immunization and immunization at the youngest ages, especially 12 months (Lee *et al.,* 2001; Tugwell *et al.,* 2004). The data from these studies contrast with studies of vaccine efficacy that were initiated prior to widespread vaccination against VZV, which in general were not suggestive of declining immunity over long-term follow-up. This discrepancy is most likely due to the higher levels of exogenous boosting that existed prior to widespread vaccination. However, a study reported by Kuter *et al.,* (2004) suggests that the administration of a single dose of varicella vaccine to children is associated with higher rates of breakthrough varicella over long-term follow-up than is a two-dose schedule.

A recent study in the United States (Chaves *et al.,* 2007) which examined 10 years (1995-2004) of active surveillance data from a sentinel population of 350,000 subjects suggested that vaccine-induced immunity wanes over time, a condition that may result in increased susceptibility later in life, when the risk of serious complications may be greater than in childhood. Children between the ages of eight to 12 years who had been vaccinated at least five years previously and developed breakthrough infection were significantly more likely to have moderate or severe disease than were those who had been vaccinated less than five years previously (risk ratio, 2.6; 95% confidence interval (CI), 1.2 to 5.8). The annual rate of breakthrough varicella significantly increased with the time since vaccination, from 1.6 cases per 1000 person-years (95% CI, 1.2 to 2.0) within one year after vaccination to 9.0 per 1000 person-years (95% CI, 6.9 to 11.7) at five years and 58.2 per 1000 person-years (95% CI, 36.0 to 94.0) at nine years.

3.1.7 Methodologies for Serology Testing:

3.1.7.1 Cell and Bead Based Assays:

The fluorescent antibody to membrane antigen (FAMA) assay measures antibodies to viral glycoproteins (Williams *et al.,* 1974) and is the most extensively validated assay and correlates best with susceptibility to and protection against, clinical varicella infection (Saiman *et al.,* 2001; Williams *et al.,* 1974; Iltis *et al.,* 1982; Grose *et al.,* 1979a; Gershon *et al.,* 1994a; Gershon *et al.,* 1988; Michalik *et al.,* 2008). Importantly, the presence of neutralizing antibody is associated with seropositive FAMA results (≥ 1:4) (Grose *et al.,* 1979a). This assay has also been shown to correlate with protection from varicella following household exposure; both in naturally infected and in vaccinated individuals (Gershon *et al.,* 1994a). For this reason, FAMA is widely regarded as the gold standard against which other serological tests are measured.

The assay can either be performed using fixed or unfixed VZV infected human fibroblast diploid lung cells (HFDL) or the MeWo melanoma cell line (Zaia *et al.,* 1977; Grose *et al.,* 1979a). The increased sensitivity of this method is assumed to arise from the preservation of the conformational structure of the glycoprotein antigens on the surface of the cells. In a study of 86 healthy indviduals with household contact with varicella, 58 who had a positive FAMA titre before exposure, did not become ill, whereas the 28 subjects who were FAMA negative (<1:2 prior to exposure) all developed varicella (Gershon *et al.,* 1994a). Throughout the history of the FAMA assay only two cases have been reported where individuals with a positive titre subsequently developed varicella, but both were mild cases (Saiman *et al.,* 2001; Steinberg and Gershon, 1991). Positive FAMA titres have also been shown to correlate with protection in immunized children with leukaemia (Gershon *et al.,* 1984b).

However this assay is semi-quantitative, not suited for testing large numbers of sera, is laborious, cannot be automated, and its interpretation can be subjective. Although the test has been established in several laboratories, due to these limitations, it is difficult to know if the assay results are comparable between laboratories.

The latex agglutination (LA) assay, which uses antigen-coated latex particles, has been shown to compare closely with the FAMA assay in a study of naturally immune and vaccinated HCWs who had been exposed to household varicella (Saiman *et al.,* 2001, Gershon *et al.,* 1994a, Steinberg and Gershon, 1991). However, false-negative results due to prozone formation may occur (Landry and Ferguson, 1993; Unadkat *et al,*. 1995), whilst false-positive LA results have led to failures to vaccinate. In some cases this has resulted in the subsequent development of varicella in HCWs with unrecognized susceptibility (Saiman *et al.,* 2001; Behrman *et al.,* 2003; Martins *et al.,* 1994).

3.1.7.2 Enzyme-Linked Immuno Sorbent Assays (ELISAs):

In general there has been a strong correlation between VZV neutralizing antibodies and ELISA antibody titre, as observed in studies on sera from vaccinated recipients (Provost *et al.,* 1991; Krah *et al.,* 1997). There are at least 15 Enzyme-Linked Immuno Sorbent Assays (ELISAs) available for VZV testing (Maple *et al.,* 2009c). Most use VZV infected cell lysate as a coating antigen, but some use highly purified glycoproteins (gpELISA). Many laboratories use non-commercials ELISAs that have whole viral lysate as a coating antigen (Behrman *et al.,* 2003; de Ory *et al.,* 2006; Wasmuth and Miller, 1990; Maple *et al.,* 2006).

Using the manufacturer's cut-offs a range of sensitivities and specificities (discussed overleaf) have been reported for VZV ELISAs, but as there have been few comparisons with FAMA it is difficult to determine the clinical relevance of these cut-offs (Maple *et al.* 2009a; Maple *et al.* 2009c). In brief,

the results of a diagnostic assay can be classified according to four outcomes. These are true positives (individuals who have experienced disease and yield a positive assay result); false positives (individuals who have not experienced disease yielding a positive result); true negatives (individuals who have not experienced disease yielding a negative result) or false negative (individuals who have experienced disease yeilding a negative result). The sensitivity of an assay is defined as the proportion of individuals who have experienced disease that have a positive test result (true positives), whilst the specificity is defined as the proportion of individuals who have not experienced disease that have a negative test result (true negatives). Therefore, sensitivity is the ability of the assay to ascertain what it *is* testing for and specificity is the ability of the test to reject what it is *not* testing for. Whether an assay is required to have a higher sensitivity or specificity depends on the consequences of a false reading.

For example, assays used to screen potential blood donors for transmissible infectious diseases require a high sensitivity, as false negative result would create a transmission risk. However, if a disease was ‘mild’, but the treatment had severe side effects, an assay would be required which identified as few false positives as possible (thus an assay with a high specificity). If an assay used to identify healthcare workers who require varicella vaccination, produced false positive readings this would lead to a failure to vaccinate susceptible subjects and a failure to potentially eradicate a transmission risk to immunocompromised patients. If the assay produced false negatives, this would lead to unnecessary vaccination of previously immune individuals and create an unnecessary economic burden. Therefore the optimal cut-off for such an assay is one that identifies as few false positives as possible (highest specificty), with a correspondingly low false negative rate (high sensitivity).

A non-commercial ELISA which uses highly purified glycoproteins has been developed by Merck and tested extensively in immunised children (Wasmuth and Miller, 1990), has proved to be highly sensitive, but has not been tested in relation to naturally acquired infection, (Gershon and Krugman, 1975) and is not widely available. The Merck group presented evidence that the gpELISA cut-off level of 5 ELISA Units (EU)/mL correlates with protection (Li *et al.,* 2002). The study on breakthrough infection in children vaccinated with a single dose, showed that 96% of those with an antibody titre of ≥5EUs were protected, whereas only 83% who had <5EUs were protected against disease. Children with a lower gpELISA reading were 3.5 times more likely to develop breakthrough chickenpox. However a recent study of primary vaccine failure, (using a FAMA cut-off of ≥1:4), demonstrated that 76% of children had not seroconverted after one dose of vaccine (Michalik *et al.,* 2008). As the gpELISA cut-off has demonstrated that 95% of children seroconvert after one dose, this cut-off is now thought to be too sensitive; leading to an over estimation of seroconversion following vaccination, (Michalik *et al.,* 2008). Commercial ELISA tests are considerably less sensitive than either gpELISAs or FAMA for the detection of seroconversion after vaccination (Saiman *et al.,* 2001, Watson *et al.,* 1994). This is likely to be due to the measurement of antibodies directed against viral glycoproteins in the case of FAMA (Williams *et al.,* 1974) and as a consequence of the more purified and concentrated nature of the capture antigen used in the gpELISA, compared to the VZV-infected cell extracts used in other EIAs, (LaRussa *et al.,* 1987; Provost *et al.,* 1991).

FAMA, LA and three commercial ELISAs have been validated in clinical studies of protection following natural infection (Gershon *et al.,* 1994a, LaRussa *et al.,* 1987). Of 19 VZV naïve subjects who developed varicella infection following household exposure, all had a FAMA titre of <1:2 prior to infection and all developed FAMA titres of ≥ 1:2 post infection (Gershon *et al.,* 1994). The ELISAs tested were less sensitive than FAMA, detecting only

between 43-76% of naturally infected subjects with positive FAMA results (LaRussa *et al.,* 1987). The LA results correlated better with FAMA than that of the commercial ELISAs (Gershon *et al.,* 1994a). The subjective nature of LA and the occurrence of both false-positive and false-negative results implies this test is not suitable for routine screening, (Behrman *et al.,* 2003; Martins *et al.,* 1994).

3.1.7.3 Time Resolved Fluorescence Immuno Assay (TRFIA):

The variation and relative insensitivity of most commercially available EIAs led to the development of the in house VZV time resolved fluorescence immunoassay (TRFIA), (HPA; London). A significant advantage of TRFIA is that it uses purified whole-cell antigen extract that is much more readily obtained than glycoprotein antigen. The loss in sensitivity due to use of cell extract antigen is compensated for by the inherent gains in sensitivity achieved through use of fluorescence decay measurement of an Europium labelled IgG conjugate (Maple *et al.,* 2006). In the final stages of the assay an enhancement solution is added which contains a Europium ligand. This causes the Europium bound to IgG to dissociate. Subsequent excitation by UV light causes the Europium-ligand complexes to fluorescence (see figure 2.1; materials and methods). The half life of this fluorescence is of the order of several hundredths of a micro-second compared to the background fluorescence which is of the order of nanoseconds. Once background fluorescence has decayed, a time resolved fluorimeter can measure the fluorescence of the Europium-ligand complex, enabling highly sensitive detection. Initial evaluation of the TRFIA assay on serum from healthy adults and children derived a cut-off of 93mIU/mL, via mixture modelling of non-immune and naturally immune populations within that cohort, (Maple *et al.,* 2006). Using this cut-off, TRFIA was shown to be more sensitive (but less specific) than most commercial antibody assays, including the Merck gpELISA (Maple *et al.,* 2006), see table 3.3.

Assay	Sensitivity (%)	Specificity (%)	Specificity + Sensitivity
Merck gpELISA	97.8	93.5	191.3
Behring	98.4	80.7	179.1
Diamedix (Eq=Neg)	76.4	97.1	173.5

Table 3.3: Sensitivity and Specificity of Merck gpELISA, Behring and Diamedix Assays in Comparison to TRFIA (Cut-Off: 93mIU/mL; Maple *et al.,* 2006). Key; Eq; Equivocal, Neg; Negative.

However, in this particular analysis, the concentration of antibodies to Merck VZV glycoprotein was determined by interpolation from a standard curve of British standard VZV antibody, (which is also used for TRFIA assay). In addition, a standard curve of the Merck reference serum was also plotted and used for calibration purposes. Using these two methods to standardize units between the Merck and TRFIA assays, a negative cut-off ≤10mIU/mL was used for the Merck ELISA. More recently, repeated testing of eight seropositive and two seronegative donors and analysis via receiver operating characteristic (ROC) curves calculated a cut-off of 150mIU/mL in naturally infected individuals (Maple *et al.,* 2009a). The specificity and sensitivity of this cut-off and a cut-off of 100mIU/mL in comparison to the Merck gpELISA (using a cut-off of 5EU/mL) is presented in table 3.4. Regardless of the cut-off, this assay has not been validated against protection from infection, and has not been validated in a vaccinated population.

Cut-Off (mIU/mL)	Sensitivity (%)			Specificity (%)			Specificity + Sensitivity
	%	95%CI	95%CI Range	%	95%CI	95%CI Range	
100	98.8	95.8–99.9	4.1	88.7	81.1–94.0	12.9	187.5
150	87.5	81.5–92.1	10.6	94.3	88.1–97.9	9.8	181.8

Table 3.4: Sensitivity and Specificity of the Two TRFIA Cut-Offs in Naturally Immune Adults. A Merck gpELISA cut-off 5EU/mL was used for comparison, (Maple *et al.,* 2009a).

In summary, there are several methodologies which have been developed to measure VZV-specific antibodies, but few assays have been validated against protection from infection, and the accuracy of several tests is questionable. Targeted immunisation programmes such as those utilised in the UK, require a robust test which generates as few false negative and false positive values as possible, in order to ensure that HCWs are no longer susceptible to infection, whilst the economic burden of vaccination is kept to a minimum.

3. 2 AIMS AND HYPOTHESIS:

Aims:

1. Evaluate the UK reference assay (time resolved immunofluorescence assay: TRFIA) for the detection of low level naturally induced, and vaccine induced VZV-specific antibodies in an adult population.
2. To use this assay and other methodologies to characterise humoral immune responses to vaccination.
3. To investigate the relationship between ethnicity and antibody titre following vaccination.

Hypothesis:

The UK reference assay (TRFIA) will have a higher sensitivity than the Diamedix assay when assessing VZV-specific IgG titres in this study population.

3.3 RESULTS:

3.3.1 Characteristics of the ROVE Study Population:

The methods and study design of healthcare worker (HCW) recruitment are described in the materials and methods and appendix 7.1. In brief, 110 HCWs who tested negative or equivocal by the standard commercial VZV ELISA (Diamedix) used at Bart's and the London NHS, were recruited. Two doses of vaccine were administered approximately six weeks apart and blood samples were taken at baseline, six and 12 weeks post first vaccination.

Seventy five study participants were re-recruited approximately 18 months post first vaccination, for a follow-up study. Demographic data (gender, age, ethnicity, country of birth, years resided in the UK) for each study participant are presented in appendix 7.5.

3.3.1.1 Summary of ROVE Study Participants Demographics:

Age at baseline is presented in figure 3.4.

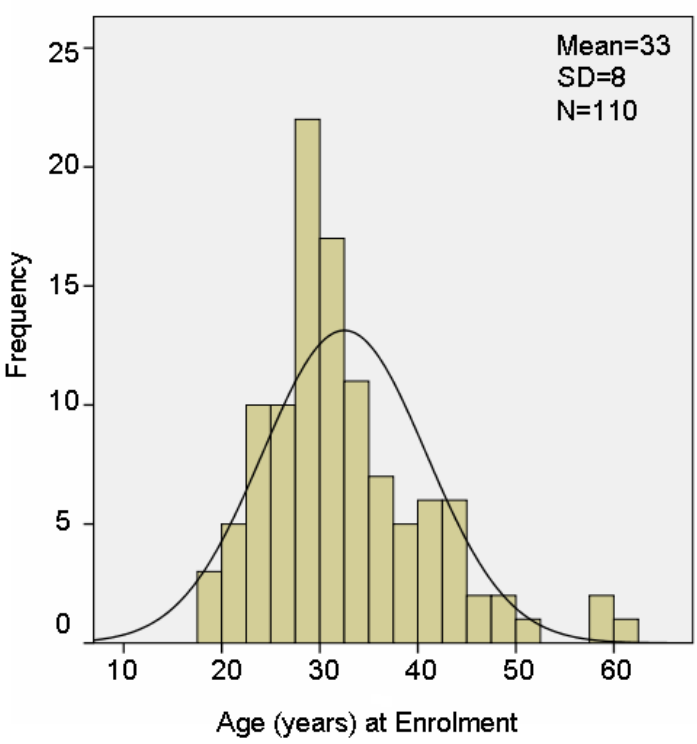


Figure 3.4: Age at Baseline (Enrolment) for ROVE Study Participants. Age has been taken as age at last birthday.

This distribution is skewed slightly towards the younger participants and is representative of the local working population. Seventy four percent of the participants were female, and the country of origin for the study population was diverse. Figure 3.5 shows the country of origin grouped into continents or geographical areas, rather than as separate countries, for ease of analysis. The Philippines has been kept as a separate country of origin as the frequency of study participants from here was quite high, and previous

studies have shown the genetic background of Filipinos is quite distinct from other Oriental or Asian races (Keegan *et al*., 2007).

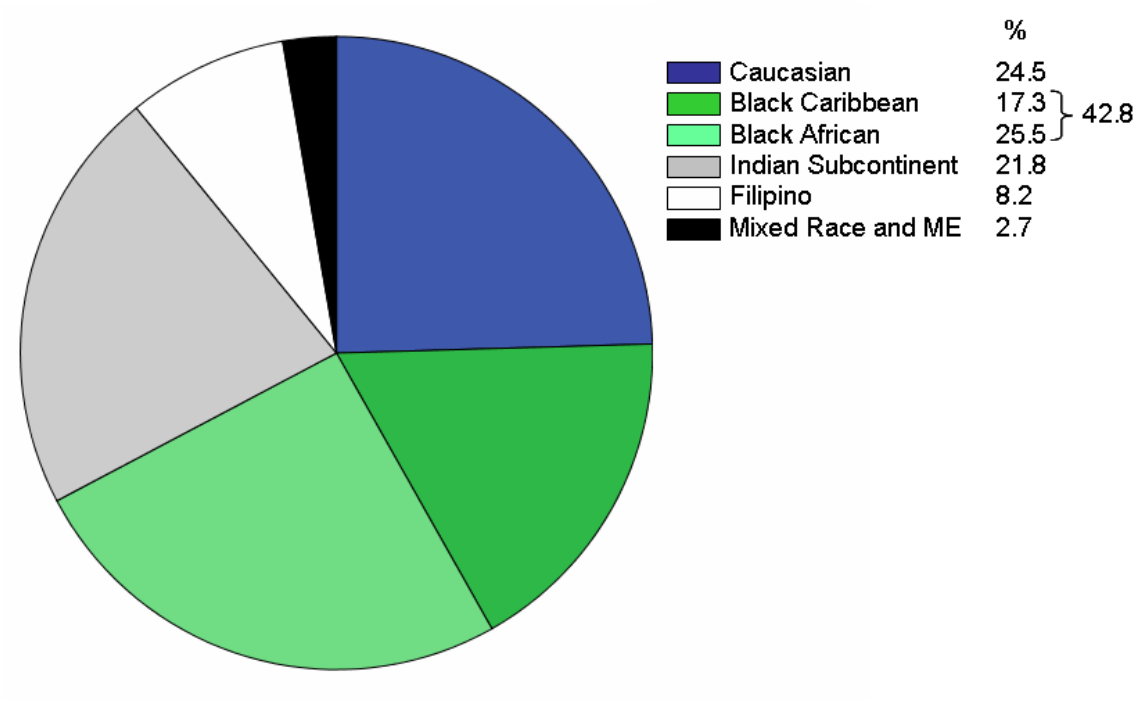


Figure 3.5: Ethnicity of ROVE Study Participants. Black African and Black Caribbean are displayed adjacently in similar shades to illustrate that almost 43% of the study population were black. Key: ME; Middle Eastern, mixed race; Black with Caucasian, Indian subcontinent; Asian. The Caucasian population where of European and Australasian origin.

Sixty seven percent (n=74) of study participants were born in the tropics; table 3.5 summaries data on the country of birth, age of migration to the UK, history of varicella prior to enrolment and any previous vaccination for Caucasian and non-Caucasian study participants. Study participants who tested equivocal (15-19EU/mL) by Diamedix are also listed.

Caucasians were significantly more likely to report a history of varicella at baseline than non-Caucasians (χ^2 test p = 0.02). Of the study participants born outside the UK, 72% (n=79), migrated after the age of 15. Four migrated at or under the age of five, and seven migrated between the ages of 6-15

years old. Subjects with equivocal Diamedix results (n=12) had significantly higher baseline titres than those with negative Diamedix results (n=96) (independent 2 tailed t-test, p=<0.0001).

	Birth Country					
	UK (n=21)		Non-UK (n=89)			
	Cauc	Non Cauc	Temperate		Tropical	
			Cauc	Non Cauc	Cauc	Non Cauc
N	17	4	5	7	2	72
Age (Years) Range (Mean)	33 (21-59)	28 (20-35)	36 (24-50)	29 (23-36)	25 (19-31)	33 (19-61)
Age Migrated to the UK; Range (Mean)	Not Applicable		25 (21-39)	19 (2-30)	2 and 5	26 (1-56)
Years in UK			10 (3-21)	10 (4-26)	14 and 29	7 (0-27)
Diamedix Equivocal	3	0	0	1	1	7
History of Varicella	6 (1 DNK)	1	2	1	1	7 (1 DNK)
Previous Vaccination	1	0	0	1	0	1

Table 3.5: Summary of the Country of Birth, Migration to the UK, History and Previous Vaccination for Caucasian and Non-Caucasian Study Participants. Key: Cauc; Caucasian, DNK; did not know if they had a history of varicella, Equivocal Diamedix values; 15-19EU/mL. Tropical countries of birth were defined as lying between the tropics of Cancer and Capricorn (study participants born in Australia were omitted from that particular analysis; n=3).

3.3.1.2 Characteristics of Humoral Immune Responses to Vaccination:

Within this study, serology results were obtained using the VZV reference antibody test (time resolved immunofluorescence assay: TRFIA). Data for each HCWs TRFIA titres at each study visit are presented in appendix 7.6. The TRFIA readings at each visit for the study population are summarised in figure 3.6.

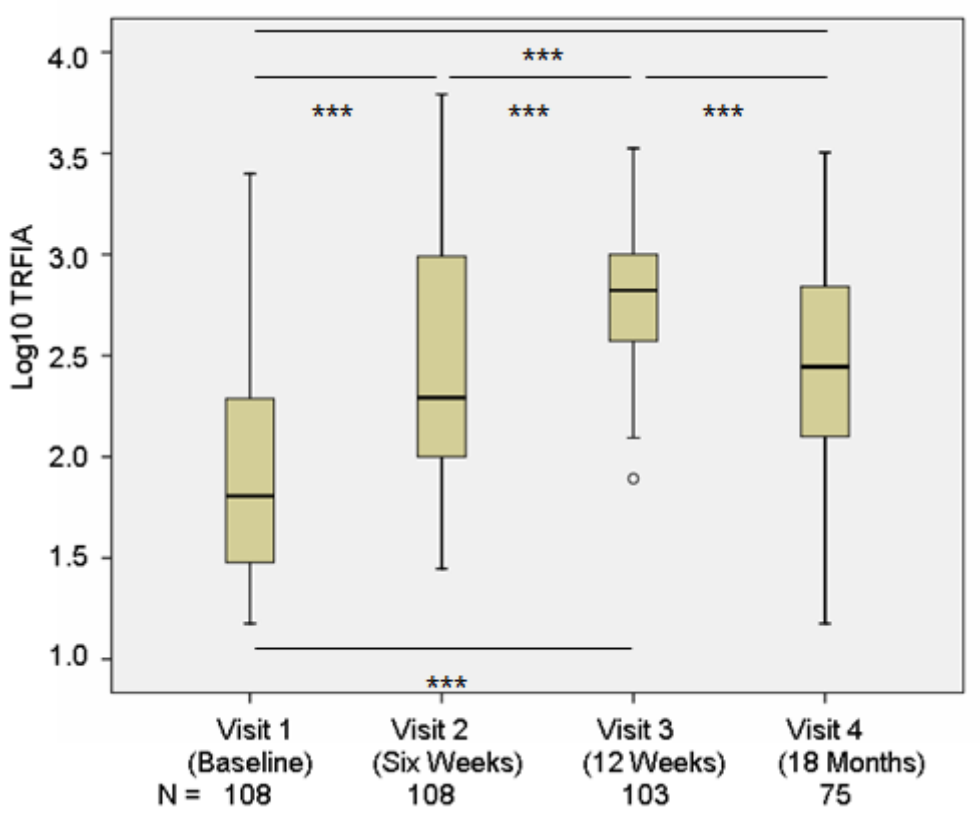


Figure 3.6 Comparison of TRFIA VZV-Specific IgG Antibody Titres at Each Study Visit. Baseline (visit 1), induced after the first dose of vaccine (six weeks; visit 2), the second dose of vaccine (12 weeks; visit 3) and the 18 month follow-up visit (visit 4). Horizontal bars within box plots represent the median value; whiskers represent the lowest data point within 1.5 IQR (interquartile range) of the lower quartile and the highest data point within 1.5 IQR of the upper quartile respectively. Data points falling outside the whiskers (outliers) are represented as circles. *** p=<0.0001, (independent 2 tailed t test). Descriptive statistics for each visit are presented in appendix 7.7.

The mean TRFIA titre for each visit was significantly different from that of the other visits p=<0.0001 (one sample, 2 tailed t-test), apart from between visit 2 and visit 4 (p=0.88). The mean TRFIA value at the 18 month follow-up (visit 4), was 4-fold above that seen at baseline (visit 1) (72mIU/mL: 295mIU/mL, (log_{10}1.86: 2.47)).

3.3.2 Evaluation of UK Reference VZV IgG Antibody Assay for Detection of Vaccine Induced Antibody and Low Level Antibody:

As outlined in the chapter introduction, long term follow-up studies allow for a correlation between a positive assay result and clinical protection. As long term follow-up studies have not been carried out using TRFIA, we made use of antibody avidity (measured using the commercially available EUROIMMUN assay) and TRFIA titres at six weeks post first vaccination to determine humoral immune status. Antibody avidity can be classed as low, equivocal/intermediate or high, which with the EUROIMMUN assay is set at ≤40%, 41-59% and ≥60% respectively. As avidity responses mature over several months, six weeks is not a sufficient time frame to allow for the maturation of high avidity antibodies, thus study participants with high avidity antibodies after the first dose of vaccine, were classified as having pre-existing humoral immunity. In addition, subjects with pre-existing immunity were identifiable by a six week TRFIA titre above a certain cut-off (discussed in section 3.3.2.2). Developing an avidity assay based on the TRFIA assay was not carried out as part of this study, because TRFIA was under evaluation.

3.3.2.1 Assay Characteristics for TRFIA and Avidity (EUROIMMUN):

The intra-assay reproducibility for both EUROIMMUN and TRFIA were assessed separately. In each case nine samples with values representing the range of results were retested. In both cases the first and second test results were significant correlated (paired t-test p=<0.001). For further analysis the original result, (not the mean of the two results), was used because the number of subjects with repeated results was so small.

To evaluate the relationship between the more sensitive in house assay (TRFIA) and the commercial avidity assay (EUROIMMUN), we compared results for VZV-specific IgG antibody in the two assays. Figure 3.7 shows the relationship between values generated by the TRFIA assay against the value generated from the EUROIMMUN assay (PBS wash), (R^2 = 0.70). The results indicate a linear relationship which was significantly correlated (paired sample t-test; p=<0.0001). According to the manufacturer's instructions, an accurate avidity assay cannot be carried out if the concentration of VZV-specific IgG is too low. As can be seen from figure 3.7, TRFIA values below 32mIU/mL ($\log_{10}$1.50) did not correlate as strongly with EUROIMMUM assay values (p = 0.81). Indeed, for samples with TRFIA values below $\log_{10}$2.0 (100mIU/mL), the corresponding avidity readings did not correlate significantly (p = 0.25). When the values generated by the two assays were re-compared; excluding samples with TRFIA values below $\log_{10}$2.0 (100mIU/mL), a stronger correlation was seen (R^2=0.72). Therefore avidity assays were not carried out on serum samples with a TRFIA titre of less than 100mIU/mL ($\log_{10}$2.0).

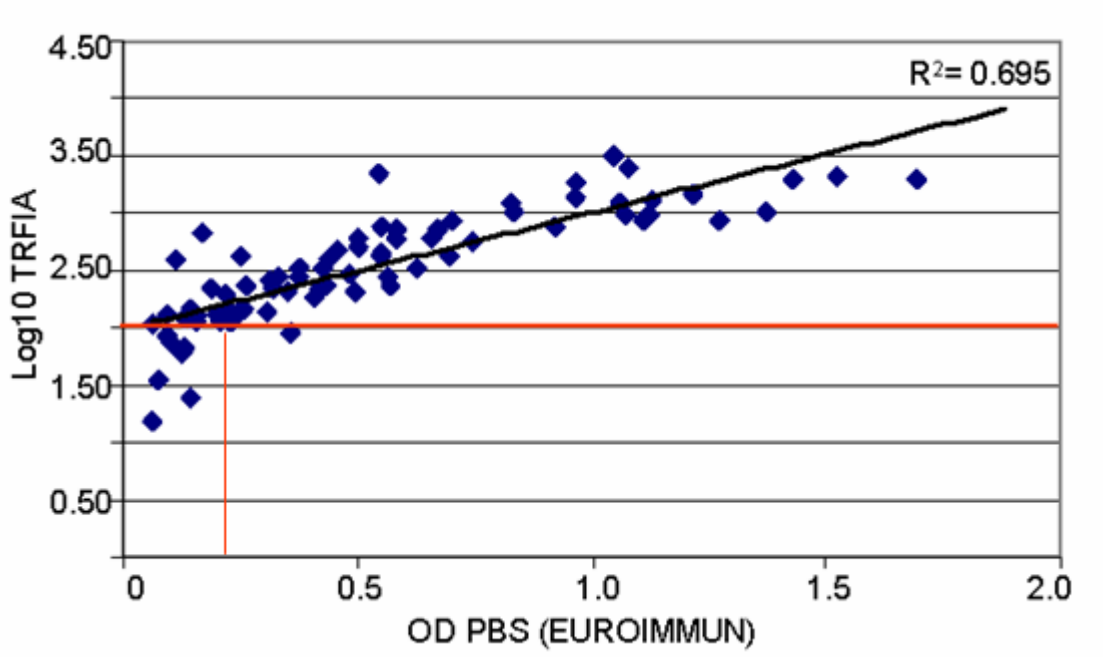


Figure 3.7: Correlation between VZV-Specific IgG Measured by TRFIA and EUROIMMUN Assays. Log_{10}TRFIA values plotted against EUROIMMUN (PBS wash) values. The horizontal red line represents the TRFIA cut-off value of $log_{10}2.0$ (100mIU/mL) above which an accurate avidity assay can be carried out, the vertical red line represents the EUROIMMUN cut-off value 0.2, above which an accurate avidity assay can be carried out.

3.3.2.2 Correlation of TRFIA and Avidity Values Six Weeks Post Vaccination:

Figure 3.8A summarises antibody avidity seen after the first and second dose of vaccine and at the 18 month follow-up visit in subjects who had no pre-existing humoral immunity to VZV (defined by a six week avidity reading ≤60%); (see appendix 7.8 for data of each HCWs avidity reading, at each study visit). Figure 3.8B summaries data on individual study participant's avidity maturation during the study. Data is presented on the 36 subjects who had avidity readings following each dose of vaccine and at the 18 month follow-up. There was an overall maturation of avidity at each time point, and this was significant between all visits (independent 2 tailed t-test, p=<0.0001). Baseline avidity readings are not presented, as few samples contained high enough concentrations of VZV-specific IgG (as determined by TRFIA) to allow an accurate avidity assay to be carried out.

Figure 3.8: Avidity Maturation Seen After Each Dose of Vaccine and at the Follow-up Visit for Study Participants with Six Week Avidity <60%. Note; baseline data (visit 1) has been excluded as few samples had high enough TRFIA values to warrant an accurate avidity reading. Part **A**: Horizontal bars within box plots represent the median value; whiskers represent the lowest data point within 1.5 IQR of the lower quartile and the highest data point within 1.5 IQR of the upper quartile respectively. Part **B**: Individual study participant's avidity maturation during the study. Data is presented on the 36 subjects who had avidity readings following each dose of vaccine and at the 18 month follow-up (n=36). *** p=<0.0001, (independent 2 tailed t test). Descriptive statistics for each visit are presented in appendix 7.9.

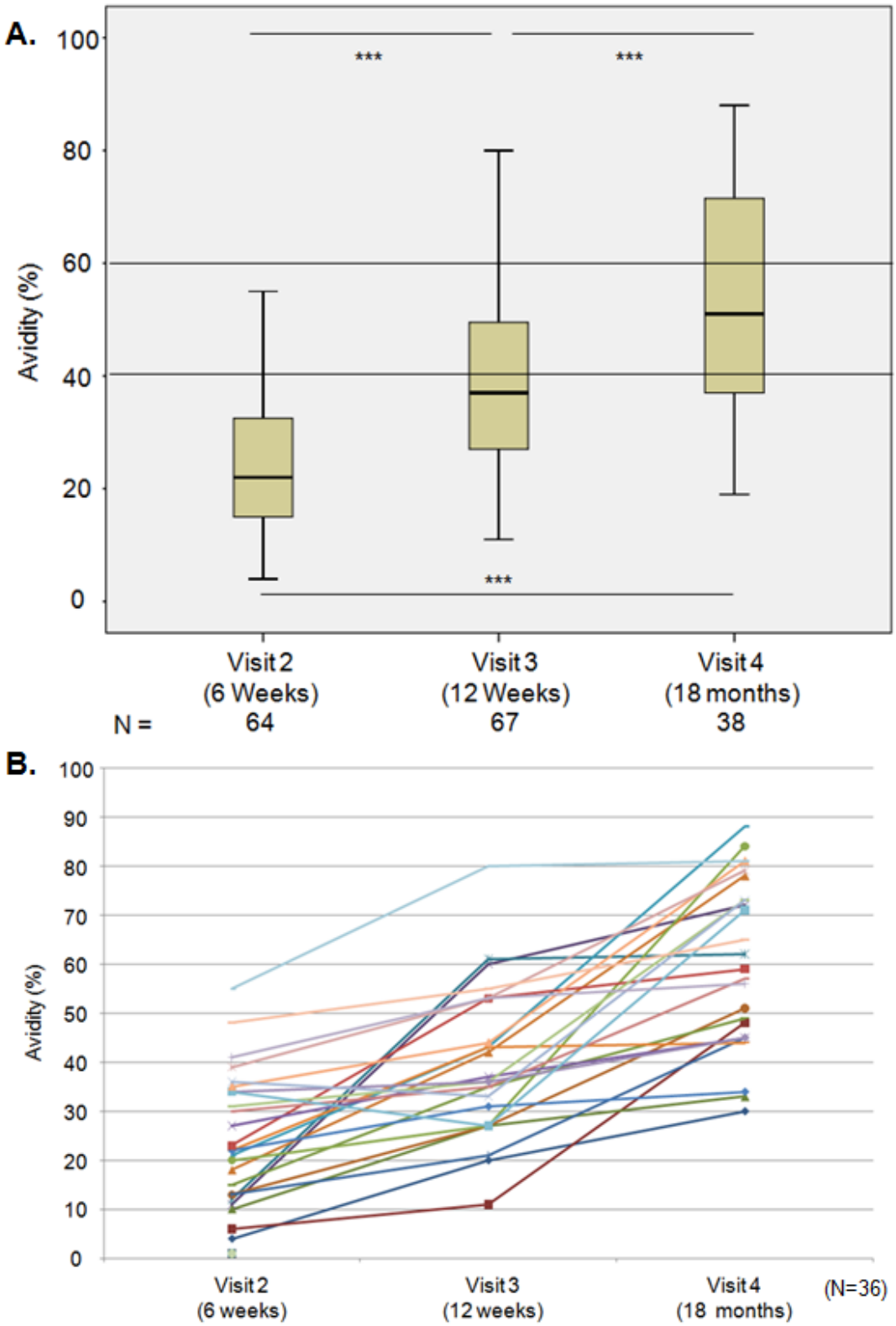


Figure 3.8: Avidity Maturation Seen After Each Dose of Vaccine and at the Follow-up Visit for Study Participants with Six Week Avidity <60%. (Figure legend on opposite page).

Six weeks after the first dose of vaccine was administered (visit 2), sera from 98 patients had a high enough concentration of VZV-specific IgG antibodies to carry out a valid avidity assay. These results are plotted against the corresponding log_{10}TRFIA value at the same time point in figure 3.9.

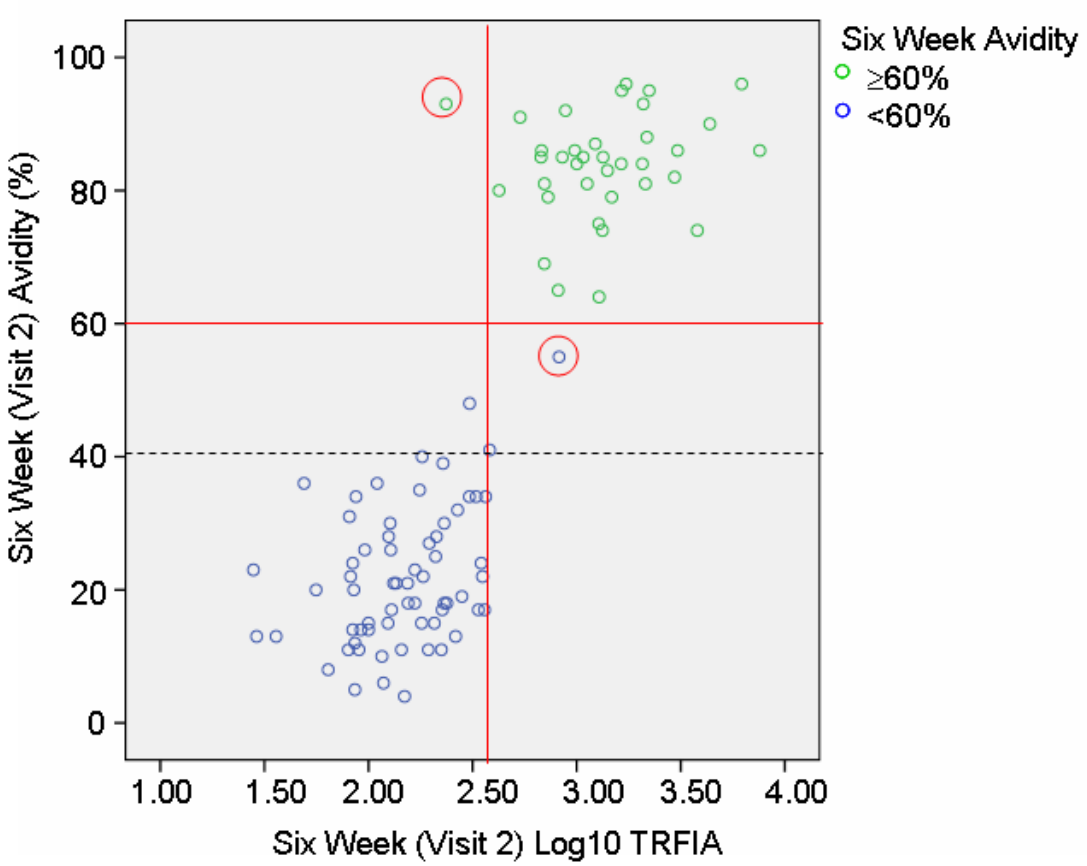


Figure 3.9 Scatter Plot to Show the Relationship Between TRFIA Titres and Avidity Levels at Six Weeks After the First Vaccination (Visit 2). The red lines represent the avidity and TRFIA cut-offs (60% and log_{10}2.60; 400mIU/mL respectively) used to classify the data into two distinct populations. Two outliers which do not cluster within the two populations are ringed in red. The dashed horizontal line represents the low avidity antibody cut-off of 40%. N = 98; R^2 = 0.86.

Figure 3.9 shows that there was a strong correlation between antibody titres and avidity values (R^2 = 0.86), and a bimodal distribution in avidity values and TRFIA titres six weeks after the first vaccination can be seen. One population had avidity values below 60% and TRFIA titres below log_{10}2.60 (400mIU/mL)

and were classified as primary responders (n=59). The second population had avidity and TRFIA readings above 60% and 400mIU/mL respectively and were classified as secondary responders (n=37). Demographic and serological data for the two outliers, ringed in red in figure 3.9 (study participants 1032; high TRFIA, low avidity and 1089; low TRFIA, high avidity) are presented in appendices 7.20 and 7.21. TRFIA titres and avidity values for the two populations were significantly different from one another, regardless of whether the outliers and the three data points with six week avidity values between 41-59% (between the horizontal dashed black and red lines) were included or excluded, (independent 2 tailed test; p =<0.0001).

3.3.2.3 Extrapolating a VZV-Specific IgG TRFIA Cut-Off from an Adult Population, Using Vaccination as a Tool to Assess Naturally Derived Antibody:

To extrapolate an assay cut-off, the baseline (visit 1) $\log_{10}$ TRFIA titres for those classified as primary or secondary responders were plotted (see figure 3.10). The negatively distributed samples (primary responders) had a mean of $\log_{10}1.65$ (SD $\log_{10}0.31$), whilst the positively distributed samples (secondary responders) had a mean of $\log_{10}2.36$ (SD $\log_{10}0.43$). A cut-off of $\log_{10}2.11$ was taken where the two fitted populations intercepted each other, (as indicated by the red arrow). This cut-off gave a sensitivity of 90% (95% CI 80-96), and a specificity of 78% (95% CI 61-90).

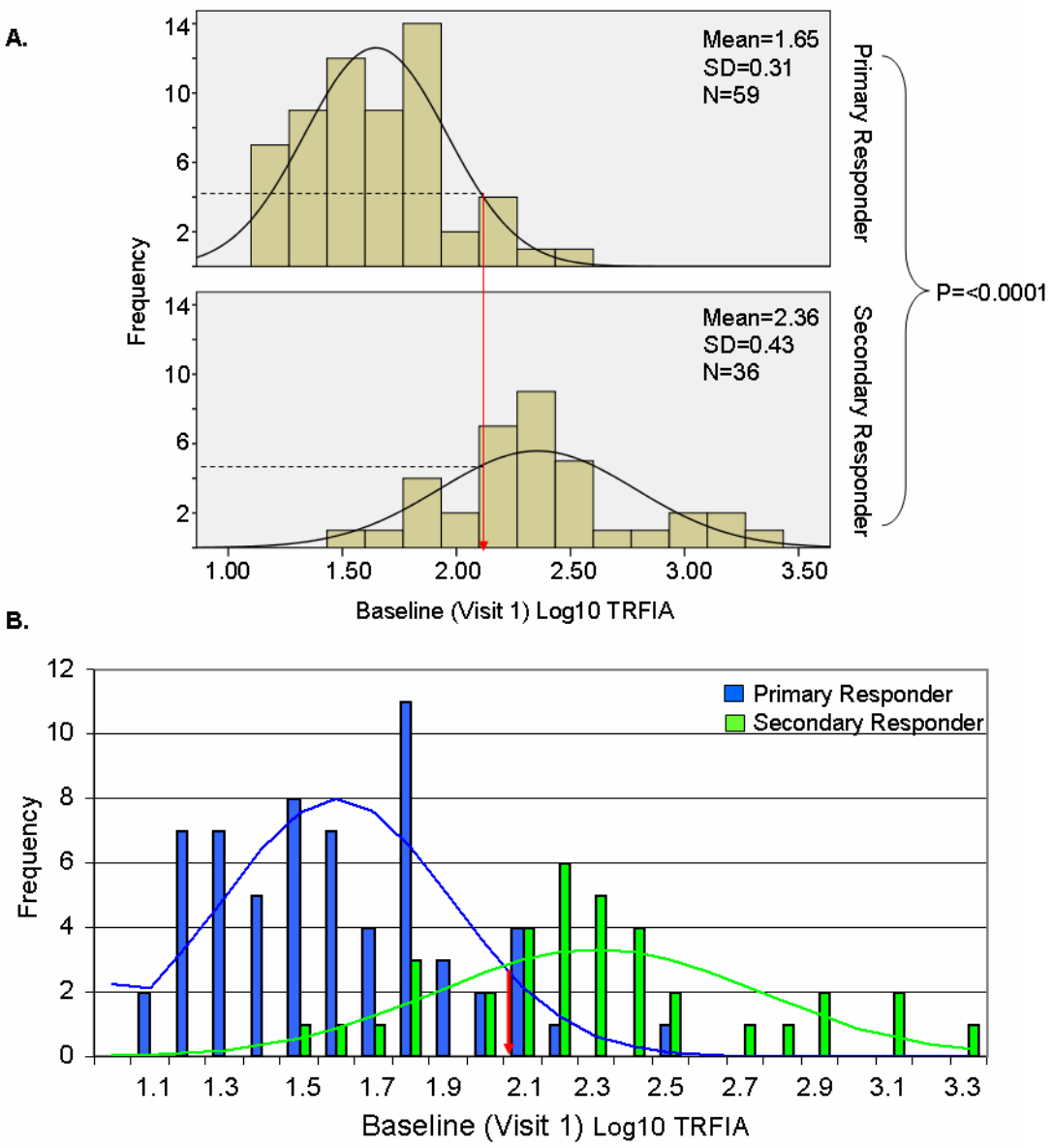


Figure 3.10: Observed and Fitted Positive and Negative Distributions of Baseline Samples Classified by Avidity Readings and TRFIA Titres After the First Dose of Vaccine (Six Weeks). Three samples with six week avidities of 41-59% were excluded from this analysis, (n=95). Negative samples were classified as samples with avidity readings of ≤40% and TRFIA titres <400mIU/mL ($log_{10}2.60$) after the first dose of vaccine. Positive samples were classified as samples with avidity readings of ≥60% and TRFIA titres of >400mIU/mL, after the first dose of vaccine. The red arrow indicates the cut-off of $log_{10}2.11$ (130mIU/mL); the point where the two fitted populations intercept. Figure A shows the two populations plotted separately and figure B shows the two populations plotted on the same histogram (using Excel Solver).

3.3.2.3.1 Defining a Cut-off Using Mixture Modelling:

Mixture modelling is a methodology that is used to identify sub-groups within a heterogeneous population. This approach can therefore be used to estimate a cut-off of humoral immunity, by defining sub-populations within an antibody profile; (i.e.: seronegative and seropositive). The mixture model (in this case; a mixture of two normal distributions) was fitted to the baseline data by first creating a histogram with appropriate bin (classification) sizes. The solver function in Excel was then used to find the optimal fitted distribution (via maximum likelihood), by adjusting (in this case), five parameters (the mean and SD of the primary and secondary responder populations, and the proportion which fall into these two distributions). Maximum likelihood gives the set of parameter values from which the observed data is most likely to have been produced and is thus the best fitting mixture (of two normal distributions) to the histogram. This process facilitates the identification of sub-populations within the data set, allowing an estimation of an optimal cut-off value where the fitted sub-populations intercept each other. Mixture modelling was applied to the baseline (visit 1) TRFIA VZV-specific IgG titres. Data was available for all but two of the participants (study numbers 1061 and 1110); n=108.

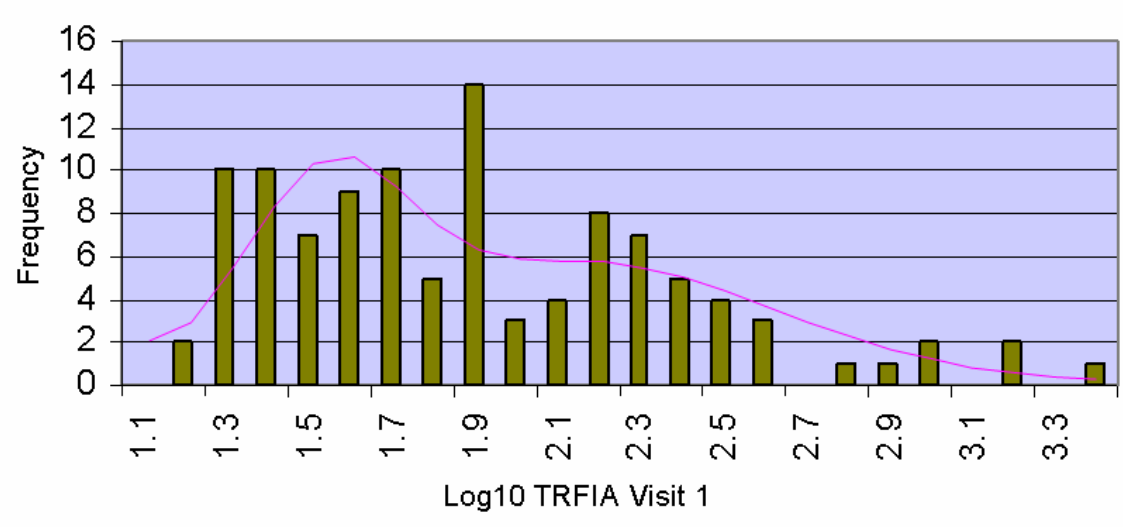


Figure 3.11: Observed and Fitted Counts of ROVE Baseline (Visit 1) Log_{10} TRFIA Readings Using Mixture Modelling. N = 108

Two peaks can be seen in figure 3.11. The negatively distributed samples had a mean of $\log_{10}1.48$ (30mIU/mL) (SD $\log_{10}0.20$), whilst the positively distributed samples had a mean of $\log_{10}2.11$ (130mIU/mL), (SD $\log_{10}0.45$). Using this model it was estimated that 40% of samples were negative at baseline and a cut off of approximately $\log_{10}1.8$ would be suggested. Assigning 95% confidence intervals based on the mixture model was attempted using profile likelihood by shifting the negative/positive mean up and down and re-fitting until the deviance was significantly different from the best fitting model (with a constraint that the proportion in the negative distribution was between 20 and 80%). This gave a confidence interval around the estimate of 100% specificity of (75%-100%) and around the estimate of 51% sensitivity of (29%-95%).

3.3.2.3.2 Comparison between the Six Week Post Vaccination and the Mixture Modelling Defined Cut-offs, Using Probability Density Curves:

Probability density curves plot the fitted distribution (based on the observed data) against probability density, rather than the frequency of observed data. The probability density is therefore the underlying continuous distribution of the fitted data and is independent of sample size. The probability densities of the fitted distributions based on both the mixture modelling and on the six-week post vaccination status (defined using avidity/TRFIA cut-offs), were plotted; see figure 3.12.

The mean value of the positive and negative populations in the probability density curves based on the six week avidity/TRFIA data were $\log_{10}1.75$ and $\log_{10}2.45$ (56 and 282mIU/mL), whilst those based upon mixture modelling were $\log_{10}1.95$ and 2.4 (89 and 251mIU/mL) respectively. Cut-offs of approximately $\log_{10}2.1$ (six week avidity/TRFIA) and $\log_{10}2.3$; 200mIU/mL (mixture modelling) would be predicted from the probability density curves in figure 3.12. The discrepancy in the mixture model cut-offs derived by plotting probability densities or frequencies, demonstrates that applying mixture

modelling to this data set is not an appropriate method of extrapolating a cut-off.

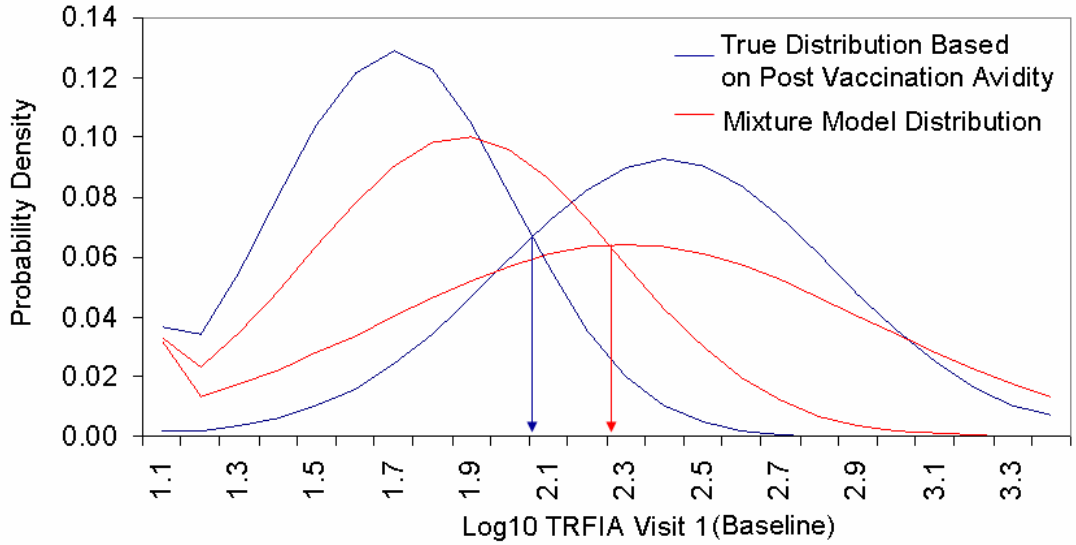


Figure 3.12: Comparison of Probability Density Curves for Positive and Negative Distributions of Baseline (Visit 1) Samples Using Either Mixture Modelling, or Avidity Cohorts Defined After the First Dose of Vaccine (Six Weeks).

3.3.2.3.3 Using ROC Analysis to Evaluate an Optimum Cut-Off:

ROC analysis was used to investigate an optimal cut-off. A receiver (or relative) operating characteristic (ROC) curve is a graphical plot of the sensitivity versus (1 - specificity) for an assay. The ROC can be represented by plotting the fraction of true positives versus the fraction of false positives (as determined by a reference assay; in this case, high or low avidity antibodies at six weeks, after the first dose of vaccine). Log_{10} baseline antibody titres were ordered and successive cut-offs (increasing by $\log_{10}0.02$) were applied to the data, for which the sensitivity and specificity was calculated. The ROC curve was produced by plotting the true and false positives rates for each successive cut-off (see figure 3.13). This process allows optimal cut-offs to be selected and suboptimal ones to be rejected.

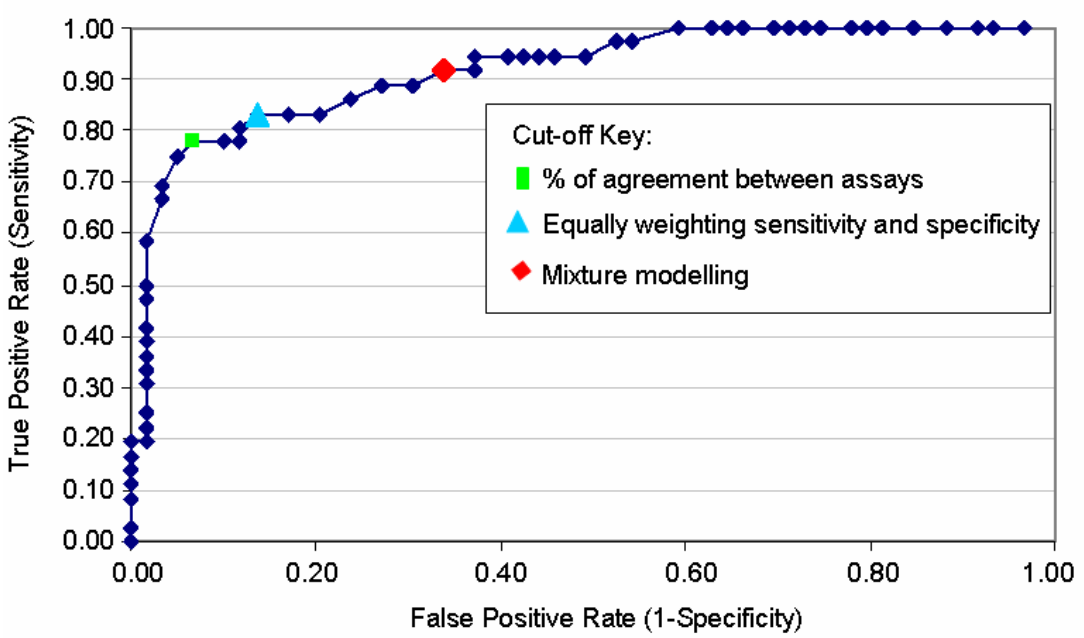


Figure 3.13: ROC Analysis of Baseline TRFIA Readings Classified by Immune Status According to Corresponding Six Week Avidity Cut-Off (≥60%). Various Cut-offs are represented on the plot: corresponding $\log_{10}$TRFIA readings to the '% of agreement between assays' were values 2.12 (132mIU/mL) or 2.14 (138mIU/mL); 'Equally weighting sensitivity and specificity' were 1.94, 1.96, 1.98 or 2, (87-100mIU/mL) and the 'mixture modelling' cut-off was 1.8 (63mIU/mL). The area under the curve is 0.92.

As discussed earlier, for an assay which is used to identify HCWs who require varicella vaccination, equal weighting of sensitivity and specificity is not appropriate (blue triangle in figure 3.13). Instead, the cut-off with the highest sensitivity and a high specificity is required (the highest true positive rate with a low false negative rate). Therefore the cut-offs which fulfil this criteria are marked on the ROC curve by the green square in figure 3.13, which correspond to the highest agreement between assays ($\log_{10}$2.12; 132mIU/mL, or $\log_{10}$2.14; 138mIU/mL). The mixture model cut-off however gives a higher sensitivity (true positive rate) but at the expense of a higher false positive rate, thus this cut-off of $\log_{10}$1.8 is not appropriate.

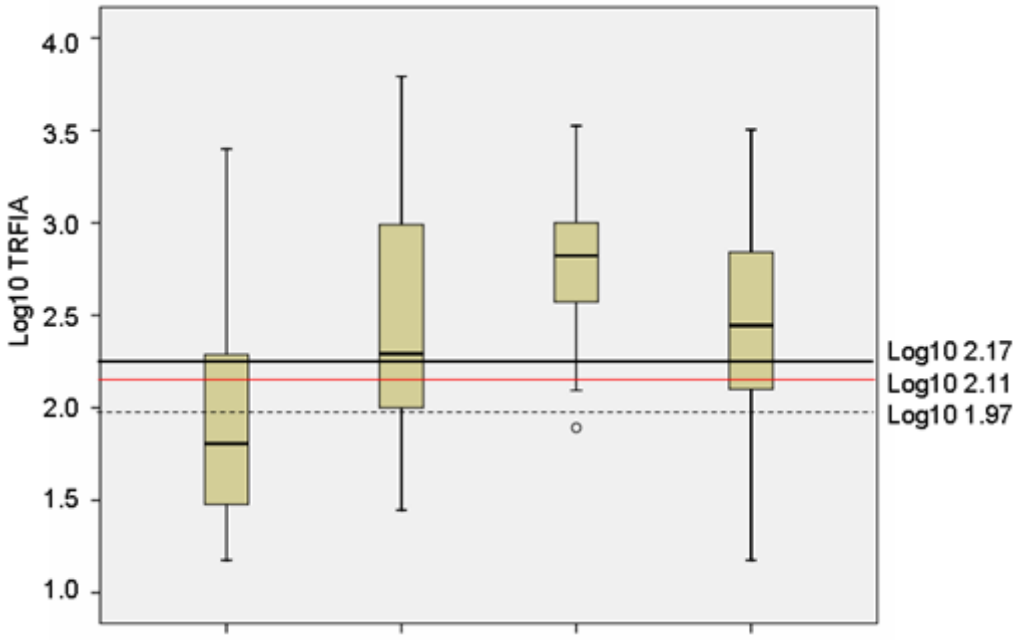
Both the probability density curve and the ROC analysis indicated that mixture modelling was not an appropriate methodology for selecting an optimal cut-off using this data set. Thus the mixture model cut-off was

Figure 3.14 Comparison between TRFIA Negative (%) at Each Study Visit, Classified by Three TRFIA Cut-Offs. Key: NIA; Cut-off derived from analysis of naturally immune adults, VA; Cut-off derived from analysis of vaccinated adults. Black horizontal bars within box plots represent the median values, whiskers represent the lowest data point within 1.5 IQR of the lower quartile and the highest data point within 1.5 IQR of the upper quartile respectively. Data points falling outside the whiskers (outliers) are represented as circles. Horizontal dashed-black bar; $\log_{10}1.97$; 93mIU/mL (Maple *et al.,* 2006), Horizontal red bar; $\log_{10}2.11$; 130mIU/mL, horizontal black bar; $\log_{10}2.17$; 150mIU/mL (Maple *et al.,* 2009). Descriptive statistics and the number of subjects who were negative at each visit according to the vaccinated adult cut-off (130mIU/mL)**,** are presented in appendix 7.25

rejected and the six week avidity/TRFIA assigned cut-off of $\log_{10}2.11$ (130mIU/mL) was accepted as the appropriate TRFIA cut-off for vaccinated adults. In the following text, titres below and above 130mIU/mL are referred to as TRFIA negative and positive respectively.

3.3.3 Assessing Humoral Immune Responses to Vaccination Using the TRFIA Cut-Off of 130mIU/mL:

Figure 3.14 below shows the percentage of TRFIA negative at each study visit, classified by the $\log_{10}$ 2.11 (130mIU/mL) cut-off and the two published TRFIA cut-offs.



V1	V2	V3	V4	Visit Number
108	108	103	75	N
				Cut-Off
66	24	2	13	Log10 1.97 (93mIU/mL) Maple *et al.*, 2006
69	30	4	25	Log10 2.11 (130mIU/mL)
76	37	5	32	Log10 2.17 (150mIU/mL) Maple *et al.*, 2009a

3.3.3.1 Characteristics of the Humoral Immune Response Following Vaccination and at Follow-Up:

Study participants were classified as either negative (**N**) or positive (**P**) according to the baseline TRFIA cut-off of $\log_{10}2.11$ (130mIU/mL). Sixty seven percent (72/106) of individuals were identified as TRFIA negative and 33% (34/106) as TRFIA positive. Humoral subsets were classified as either primary or secondary responders based on TRFIA/avidity cut-offs of $\geq\log_{10}2.60$ (400mIU/mL) and ≥60% respectively after the first dose of vaccine (six weeks). Figure 3.15 summarises the divison of study participants into primary and secondary responders and subsequently into each subset whilst table 3.6 summarises the cut-offs used to define each subset. Of the TRFIA negative cohort, 10% (7/72) were identified as negative secondary responders (acronym **N**egative '**I**mmune'; **NI**). The negative primary responder cohort (65/72) were subdivided into those that seroconverted (**SC**) following one dose of vaccine (TRFIA ≥130mIU/mL); (**NSC1**; 32/65), seroconverted following two doses of vaccine (**NSC2;** 29/65), or failed to seroconvert in response to vaccine. This latter group was classified as **L**ow level **R**esponders to vaccine (**NLR**; 4/65). Those with a positive TRFIA at baseline were subdivided into those that exhibited a secondary response to the first dose of vaccine (with the acronym **P**ositive '**I**mmune' **(PI)**; 29/35) and those who made a **L**ow level **R**esponse to vaccine (**PLR**; 6/35). Thus, six humoral subsets were identified. Figure 3.16 highlights the clustering of each humoral subset after the first dose of vaccine, based on TRFIA and avidity readings. Appendices 7.11-7.15 summarise the descriptive statistics on TRFIA and avidity data at each study visit, for each humoral subset.

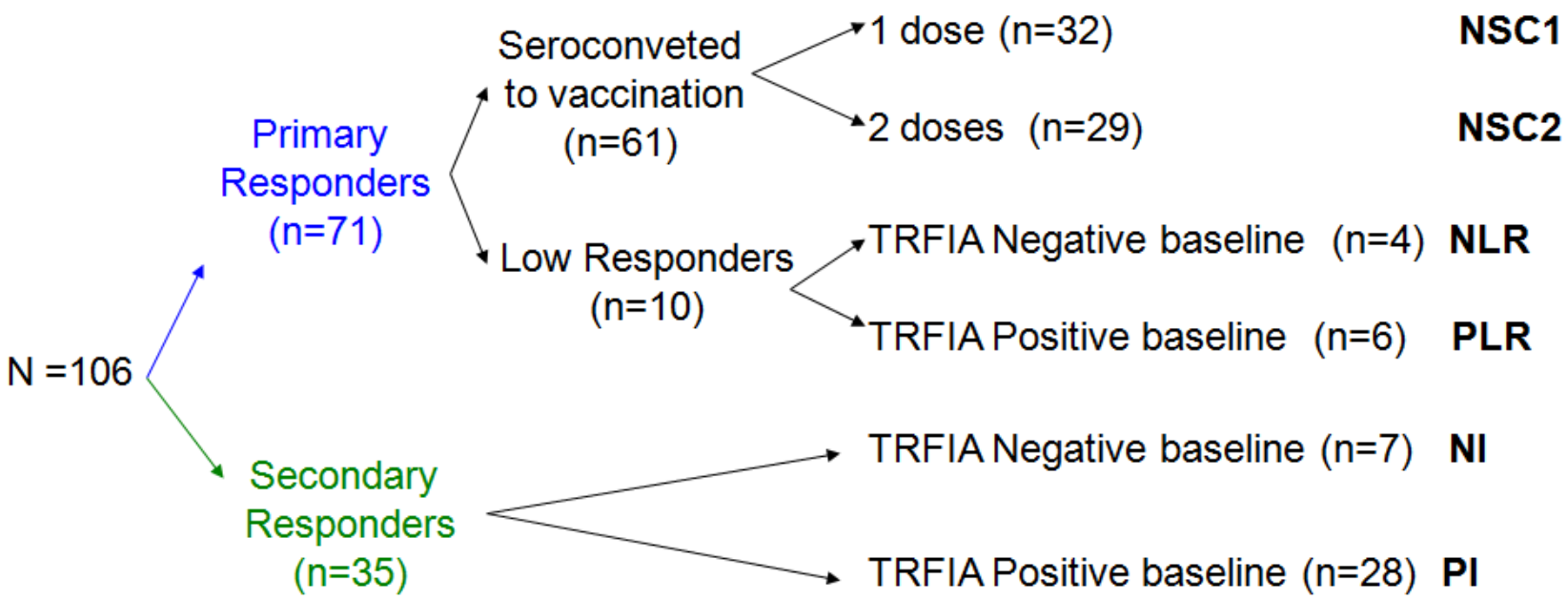


Figure 3.15 Schematic of Humoral Subset Classification. Key: **NSC1**; Negative at baseline, Seroconverted 1 dose**, NSC2**; Negative at baseline, Seroconverted 2 Doses, **NLR**; Negative at baseline, Low primary Responder, failed to seroconvert following two doses (vaccine humoral non-responder), **PLR**; Positive at baseline, Low Responder, **NI**; Negative at baseline, secondary responder ('Immune'), **PI**; Positive at baseline, secondary responder ('Immune'). Four individuals were excluded from analysis; two individuals withdrew from the study after the baseline visit, and two outliers (1032 and 1089).

				Baseline (Visit 1)		Six weeks (Visit 2)		12 weeks (Visit 3)	
N	Subset			TRFIA (mIU/mL)	Avidity (%) (if applicable)	TRFIA (mIU/mL)	Avidity (%)	TRFIA (mIU/mL)	Avidity (%)
32	Primary Responders	Seroconverted to Vaccination	NSC1	<130	<60	≥130	<60	≥130	
29			NSC2			<130			
4		Low Responders	NLR	<130	<40	<130	<40	<130	<40
6			PLR	≥130	<60	<400	<60		
7	Secondary Responders		NI	<130	≥60	≥400	≥60		
28			PI	≥130	≥60*				
106									

Table 3.6: Summary of Humoral Response Subset Classification. Key: **NSC1**; Negative at baseline, Seroconverted 1 dose**, NSC2**; Negative at baseline, Seroconverted 2 Doses, **NLR**; Negative at baseline, Low primary Responder, failed to seroconvert following two doses (vaccine humoral non-responder), **PLR**; Positive at baseline, Low Responder, **NI**; Negative at baseline, secondary responder ('Immune'), **PI**; Positive at baseline, secondary responder ('Immune'), *all subjects in this subset had baseline avidities above 40%, but n=6 were <60%, however at 12 weeks these 6 subjects had a minimum avidity of 64%. Four individuals were excluded from analysis; two individuals withdrew from the study after the baseline visit, and two outliers (1032 and 1089).

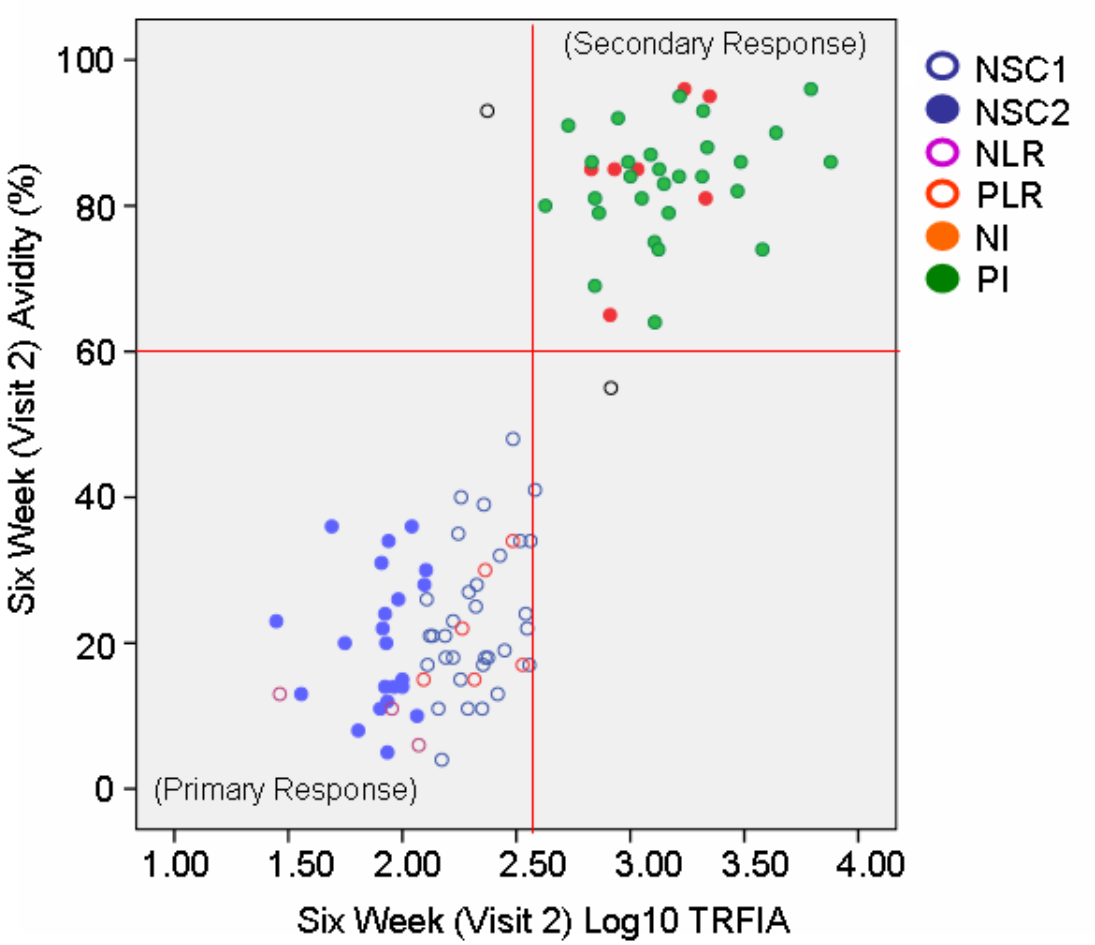


Figure 3.16: Clustering of each Humoral Subset After the First Dose of Vaccine. Key: **NSC1**; Negative at baseline - Seroconverted to 1st dose, **NSC2**; Negative at baseline - Seroconverted to 2nd dose, **NLR**; Negative at baseline - Low Responder, (failed to seroconvert following two doses: vaccine humoral non-responder), **PLR**; Positive at baseline - Low Responder, **NI**; Negative at baseline, secondary responder ('Immune'), **PI**; Positive at baseline, secondary responder ('Immune'),

Study participants 1032 and 1089 were outliers according to the six week TRFIA/avidity cut-offs of 400mIU/mL ($\log_{10}2.60$) and 60% respectively, and were excluded from analysis. According to the baseline TRFIA cut-off of $\log_{10}2.11$ (130mIU/mL), both would be classified as negative at baseline and seroconverting following the first dose of vaccine. However, 1089 could only be classified as a secondary responder based on their six week avidity, whilst 1032 could only be classified into this subset based on their TRFIA titre.

Figure 3.17 summaries TRFIA titres at baseline and following each dose of vaccine for each subset. The results from the longer term follow-up visit 4 which occurred approximately 18 months post vaccination, are discussed separately in section 3.3.3.4

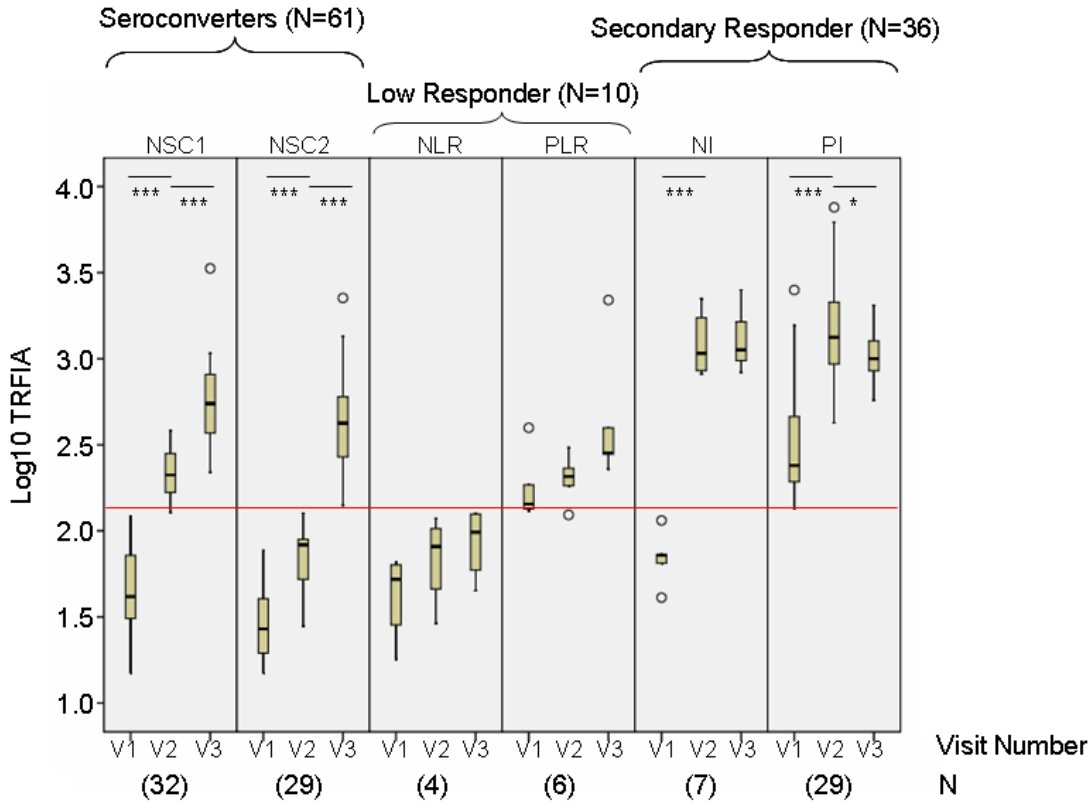


Figure 3.17: Summary of Humoral Immune Responses Following Two Doses of Vaccine, for Each Subset. The horizontal red line represents the TRFIA cut-off of $\log_{10}2.11$ (130mIU/mL), horizontal black lines within boxes represents the median value; whiskers represent the lowest data point within 1.5 IQR of the lower quartile and the highest data point within 1.5 IQR of the upper quartile respectively. Data points falling outside the whiskers (outliers) are represented as circles. A total of 106 study participants are represented. Key: NSC1; Negative Seroconverted, 1 dose, NSC2; Negative Seroconverted, 2 doses, NLR; Negative Low Responder (humoral non-responder to vaccine) , PLR; Positive Low Responder, NI; Negative 'Immune', PI; Positive 'Immune', V1; Visit 1 (baseline), V2; Visit 2 (six weeks), Visit 3 (12 weeks), *** p=<0.0001, * p=<0.05, (independent 2 tailed t test).

As can been seen from figure 3.17, for those who were classified as seroconverting following vaccination (NSC1 and NSC2), there was a highly

significant (p=<0.0001; 2 tailed independent t test) increase in antibody titre after each dose of vaccine. Therefore even in study participants who seroconverted following one dose of vaccine, a highly significant increase in antibody titre was obtained following a second dose of vaccine. Following the first dose of vaccine (visit 2; six weeks) the difference in antibody titre between these two subsets was highly significant (p=<0.0001), but was no longer significant following the second dose of vaccine (visit 3; 12 weeks) (p=0.1). For secondary responders (NI and PI) there was a highly significant increase following the first dose of vaccine (p=<0.0001), but following the second dose, either no significant difference (NI), or a significant decrease (p=0.024) (PI) was observed in mean titre. However for low responders (NLR and PLR), although the mean increased after each dose of vaccine, this was not significant. The log_{10} TRFIA titres for each low responder at baseline and after each dose of vaccine are presented in figure 3.18

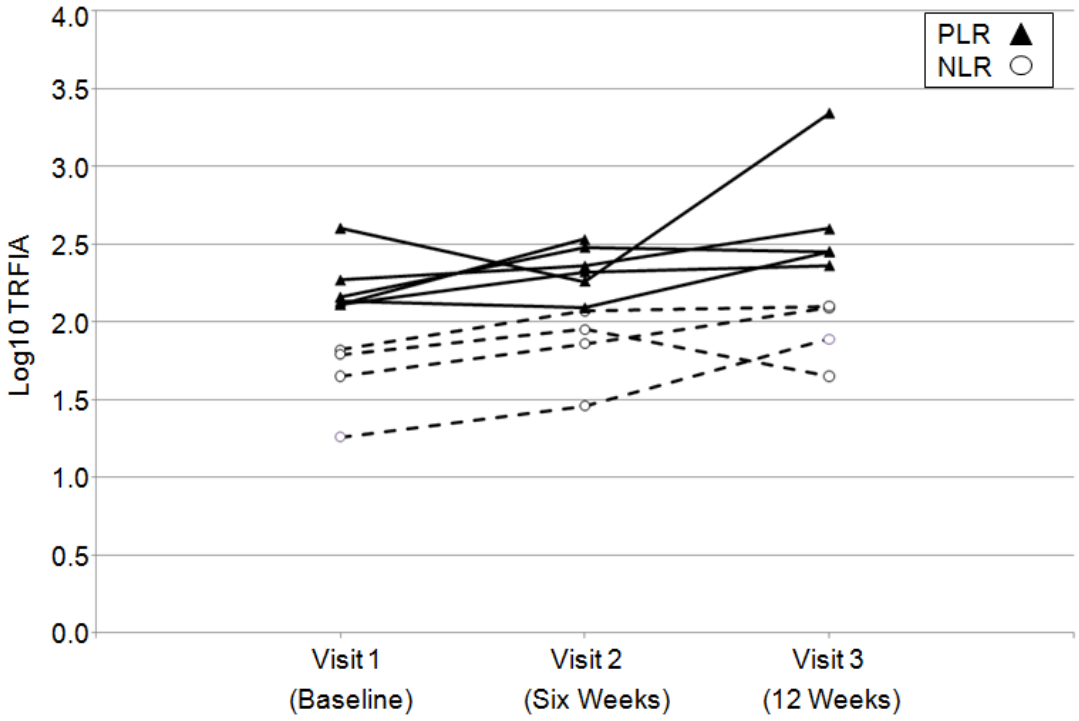
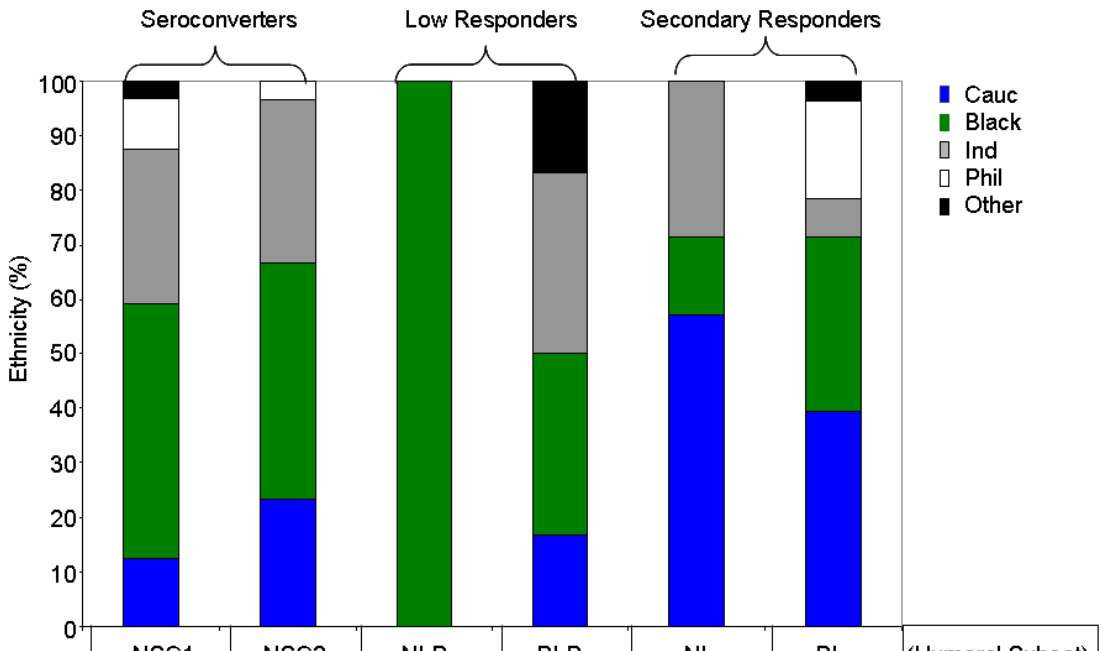


Figure 3.18: Log_{10} TRFIA titres at Baseline and after Each Dose of Vaccine for Study Participants Classified as Low Responders. (NLR (n=4), PLR (n=6)).

3.3.3.1.1 Demographic Characteristics for each Subset:

Demographic data for each humoral subset are summarised in figure 3.19. There was no significant difference between the mean age or gender between any of the humoral subsets.



NSC1	NSC2	NLR	PLR	NI	PI	(Humoral Subset)
32	29	4	6	7	29	(N)
75	83	50	77	75	76	(% Female)
31	30	37	34	28	35	(Mean Age; Years)

Figure 3.19: Summary of Ethnicity, Gender and Age for Each Humoral Subset. Key: NSC1; Negative Seroconverted, 1 dose, NSC2; Negative Seroconverted, 2 doses, NLR; Negative Low Responder, failed to seroconvert following two doses (vaccine humoral non-responder), PLR; Positive Low Responder, NI; Negative 'Immune', PI; Positive 'Immune', Cauc; Caucasian (the Caucasian population where of European and Australasian origin), Ind; Indian subcontinent, Phil; Filipino, Other; Middle Eastern or mixed race (Black with Caucasian). Two subjects withdrew from the study, and two subjects were classed as outliers and were excluded from this analysis (n=106). There was no significant difference between the mean age or gender between cohorts and that of the total study population.

Figure 3.20 Summary of Ethnicity, Gender and Age for Subsets which were Classified as Seroconverters, Low Responders or Secondary Responders.
Key: NSC1; Negative Seroconverted, 1st dose, NSC2; Negative Seroconverted 2nd doses, NLR; Negative Low Responder, PLR; Positive Low Responder, NI; Negative 'Immune', PI; Positive 'Immune', Cauc; Caucasian (the Caucasian population where of European and Australasian origin), Ind; Indian subcontinent, Phil; Filipino, Other; Middle Eastern or mixed race (Black with Caucasian).

Data was combined for analysis into those that seroconverted (NSC1 and NSC2), were low responders (NLR and PLR) or were secondary responders (NI and PI), see figure 3.20. Caucasians were significantly more likely to be classified as secondary responders (PI and NI), (χ^2 p=0.003) than non-Caucasians. Although not significant, there was a suggestion that Black (versus non-Black) and Indian subcontinent (versus non-Indian subcontinent) were less likely to be classified as secondary responders (χ^2 p=0.066 and p=0.057 respectively), whilst Indian subcontinent (versus non-Indian subcontinent) were more likely to be classified as seroconverting (χ^2 p=0.055).

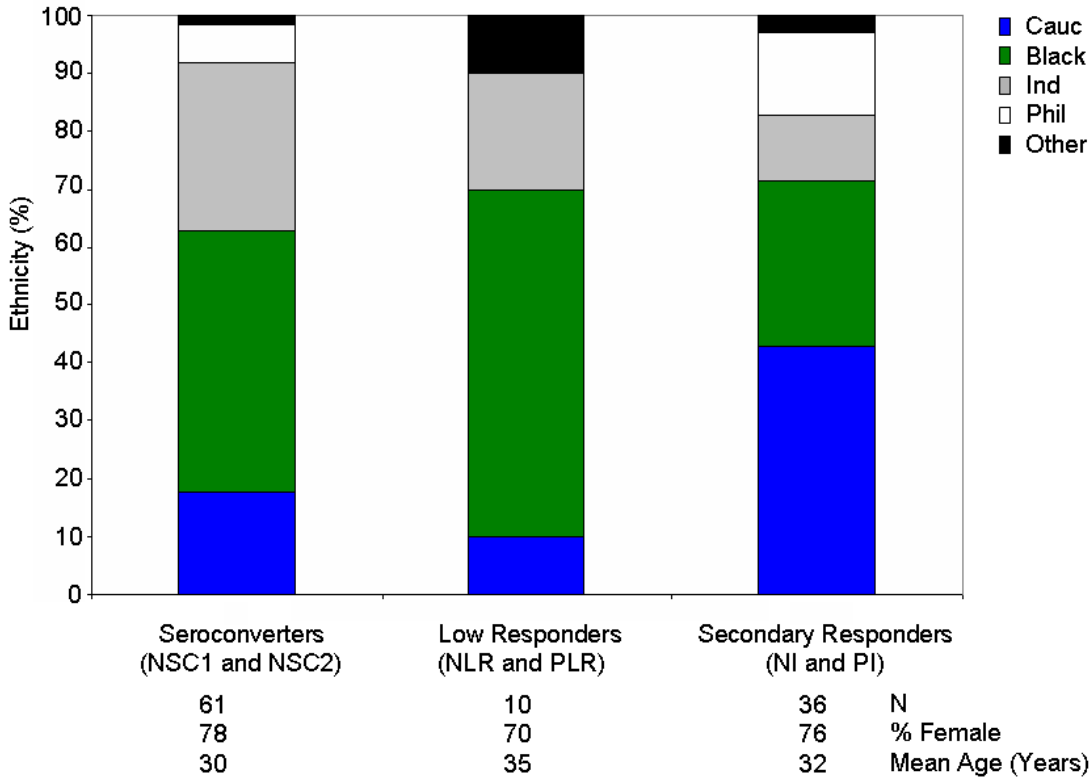


Figure 3.20 Summary of Ethnicity, Gender and Age for Subsets which were Classified as Seroconverters, Low Responders or Secondary Responders. (Figure legend on opposite page).

3.3.3.1.2 Characteristics of Primary Responders:

3.3.3.1.2.1 Characteristics of Primary Responders who Seroconverted Following Vaccination (NSC1 and NSC2):

As mentioned earlier, of the 72 subjects who were TRFIA negative at baseline, 65 (90%) were defined as primary responders by avidity and TRFIA readings at six weeks. Thirty two (49%) seroconverted after one dose of vaccine (NSC1), and 29 (45%) seroconverted after two doses (NSC2). NSC1 had significantly higher mean TRFIA titres at both baseline (independent 2 tailed t test, p=0.008) and after the first dose of vaccine (six weeks, p=<0.0001) than NSC2. There was no difference in mean TRFIA titre between these two subsets following the second dose of vaccine (12 weeks), but avidity readings at this time point were significantly higher for the NSC1 group, (p=0.005). The antibody titre seen at the 18 month follow-up was also found to differ between these two groups, and is discussed in more detail in section 3.3.3.4.

3.3.3.1.2.2 Characteristics of Primary Responders who Made Low Level Responses to Vaccine (NLR and PLR):

Study participants who did not have a significant difference between mean TRFIA titres after each dose of vaccine were classified as low responders. Of the 72 subjects who were TRFIA negative at baseline, four failed to seroconvert following two doses of vaccine (NLR). The TRFIA values for these four vaccine humoral non-responders were 45, 78, 123 and 126mIU/mL, (log_{10}1.65, 1.89, 2.09 and 2.10), and for those samples tested, all were low avidity, (11-27%). All four subjects were Black. A further six subjects, who although positive for TRFIA antibodies at baseline, responded poorly to each dose of vaccine and were classified as PLR. In all six individuals, baseline antibody avidity was low (<40%) and antibody avidity remained low at 12 weeks, (26-41%). Five of the six were non-Caucasian (two black, one mixed race, two Indian subcontinent). Although the difference in mean TRFIA titre at 12 weeks was significantly different between NLR and

PLR (p=0.016) the difference between this time point and those of the NSC1 and NSC2 groups was highly significant (p=<0.0001).

To further explore an association between ethnicity and antibody titre, TRFIA titres after two doses of vaccine, (12 weeks; visit 3) were compared for all primary responders who were TRFIA negative at baseline (NSC1, NSC2 and NLR; n=65), see figure 3.21. Black study participants had significantly lower TRFIA titres than non-black at this time point (independent 2 tailed test, p=0.009). This was further supported by the finding that after ranking TRFIA titres at this time point, black subjects were significantly more likely to fall into the bottom quartile than non-blacks (χ^2 p=0.003), and this was still significant if NLRs were removed from analysis (χ^2 p=0.017).

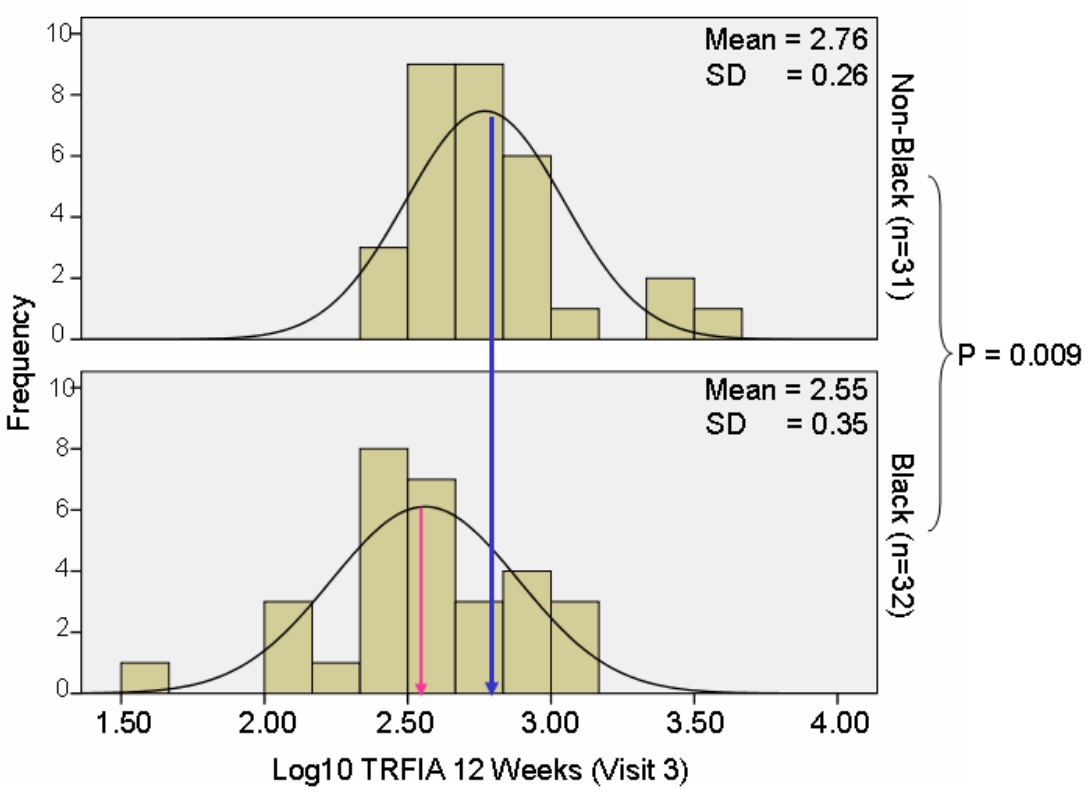


Figure 3.21: Log_{10} VZV-Specific IgG TRFIA Titres Following Two Doses of Vaccine for Primary Humoral Responders who were TRFIA Negative at Baseline. Vertical arrows represent the mean value for each cohort.

3.3.3.1.2.3 NLRs: Response to a Third Dose of Vaccine:

Two study participants who failed to seroconvert following two doses of vaccine (study numbers 1002 and 1027; NLRs) received a third dose. Study number 1002 (African male; 28 years old; no history of varicella prior to enrolment), failed to seroconvert with a third dose achieving a final TRFIA of just 62mIU/mL. This HCW was not recruited back for the follow-up visit. In the case of 1027 (African female; 49 years old; no history of varicella prior to enrolment), the HCW experienced what was classified in our study as a 'vaccine related event' (VRE) 12 days after the third dose was administered, and was visited by the research nurse. In this case, the VRE was a headache and an itchy (but not painful) rash on the left shoulder. Swabs taken from the rash site (which did not resemble that of a varicella infection) were negative for VZV DNA by PCR analysis. A full set of blood samples was taken at this additional time point, a TRFIA of $\log_{10}3.03$ (1,074mIU/mL) was recorded. No exposure to either varicella or zoster was reported by the HCW prior to their third visit, or between administration of the third dose and the VRE. The VZV-specific IgG level fell to $\log_{10}2.43$ (270mIU/mL) two months after the third dose was administered. At the follow-up visit (in this case, only 13 months post vaccination) their TRFIA level had dropped back down to $\log_{10}2.09$ (123mIU/mL). A third HCW did not return for a third dose of vaccine and remained negative at their follow-up visit ($\log_{10}1.77$).

3.3.3.1.2.4 NLRs: *Responses to Natural Infection:*

Study number 1050 (African male; 36 years old, who did not know if he had had varicella before or not, born in Nigeria and migrated to the UK 17 years prior to enrolment) also failed to seroconvert after two doses of vaccine, but was exposed to varicella via a household contact (his daughter) two days prior to his third visit (12 weeks). At this time point, a TRFIA reading of just 78mIU/mL was obtained. No symptoms were reported following the household exposure and a full set of bloods was taken five weeks post household contact. At this time point, the HCW had seroconverted (TRFIA

2,118mIU/mL; $\log_{10}$3.33; avidity 31%). This study participant was recruited back for the follow up visit (17 months post vaccination) and had a TRFIA reading of $\log_{10}$2.45 (282mIU/mL), with an avidity of 51%.

3.3.3.1.2.5 Secondary Humoral Immune Responses to Vaccination (PI and NI):

Secondary responders were classified as having a six week TRFIA of >400mIU/mL and an avidity of >60%, and were subdivided into two further subsets based on baseline TRFIA titres. Twenty eight subjects had positive baseline TRFIA≥130mIU/mL (PI), while 7 were negative at baseline (NI). In both cases a highly significant increase in the mean TRFIA titre following the first dose of vaccine was observed (independent 2 tailed t test, p=<0.0001), and secondary responders had significantly higher mean antibody avidity at each time point compared to all primary responder subsets (p=<0.0001). Fifty percent of Caucasians enrolling in the study were classed as secondary responders and this was significant (χ^2 p=0.039). In addition, secondary responders were significantly more likely to have grown up in a temperate rather than a tropical climate (χ^2 p=0.003).

3.3.3.2 Correlation Between FAMA and TRFIA in Vaccinated Adults; Benchmarking Humoral Subsets:

A positive FAMA score (≥2) has been shown to correlate with clinical protection, and the presence of neutralising antibodies (Gershon *et al.,* 1994a and Grose *et al.,* 1979). Sixty four ROVE serology samples, (16 subjects, 4 visits each), were tested by FAMA (with thanks to Sharon Steinberg and Anne Gershon, Columbia University College of Physicians and Surgeons). FAMA and corresponding TRFIA and avidity values for each sample are presented in appendix 7.22. The FAMA assays were repeated on five samples, and in one case, the FAMA readings did not agree on immune status (<2 first assay, 2 second assay). This sample was therefore omitted from analysis (n=63). There was an 81% (51/63) agreement on assay positive/negative status

between the two assays. In comparison to FAMA, TRFIA had a sensitivity of 81% (39/48) and a specificity of 80% (12/15), see table 3.7. The positive and negative predictive values were 93% (39/42) and 57% (12/21) respectively (see materials and methods, for formulas).

		FAMA		
		Positive >2	Negative <2	Total
TRFIA (mIU/mL)	Positive ≥130	39	3	42
	Negative <130	9	12	21
	Total	48	15	63

Table 3.7: Comparison of Positive and Negative Status between FAMA and TRFIA.

There was a significant difference (independent 2 tailed t-test, p=<0.0001) between the mean $\log_{10}$TRFIA titre for samples with positive FAMA scores, compared to the mean of samples with negative FAMA scores, see figure 3.22. The mean $\log_{10}$ TRFIA titre differed significantly for each FAMA score (4, 8, >8) (see figure 3.23). For samples with a FAMA of >8 all the corresponding TRFIA values (bar one) were above the TRFIA cut-off of $\log_{10}$ 2.11. There was no significant difference between the mean of the negative FAMA group (<2) and the lowest of the FAMA positive groups (4), however the sample size for the later group was small (n=7), see figure 3.23.

Figure 3.22: Correlation between FAMA Assay Negative and Positive Status and Log_{10} TRFIA Titres. Horizontal black lines within boxes represents the median value; whiskers represent the lowest data point within 1.5 IQR of the lower quartile and the highest data point within 1.5 IQR of the upper quartile respectively. The horizontal red line indicates the TRFIA cut-off of $log_{10}2.11$ (130mIU/mL).

Figure 3.23: Correlation between FAMA Scores and Log_{10} TRFIA Readings. Horizontal black lines within boxes represents the median value; whiskers represent the lowest data point within 1.5 IQR of the lower quartile and the highest data point within 1.5 IQR of the upper quartile respectively. Data points falling outside the whiskers (outliers) are represented as circles. The horizontal red line indicates the TRFIA cut-off of $log_{10}2.11$ (130mIU/mL). The difference between the mean of the FAMA <2 and FAMA 4 groups was not significant.

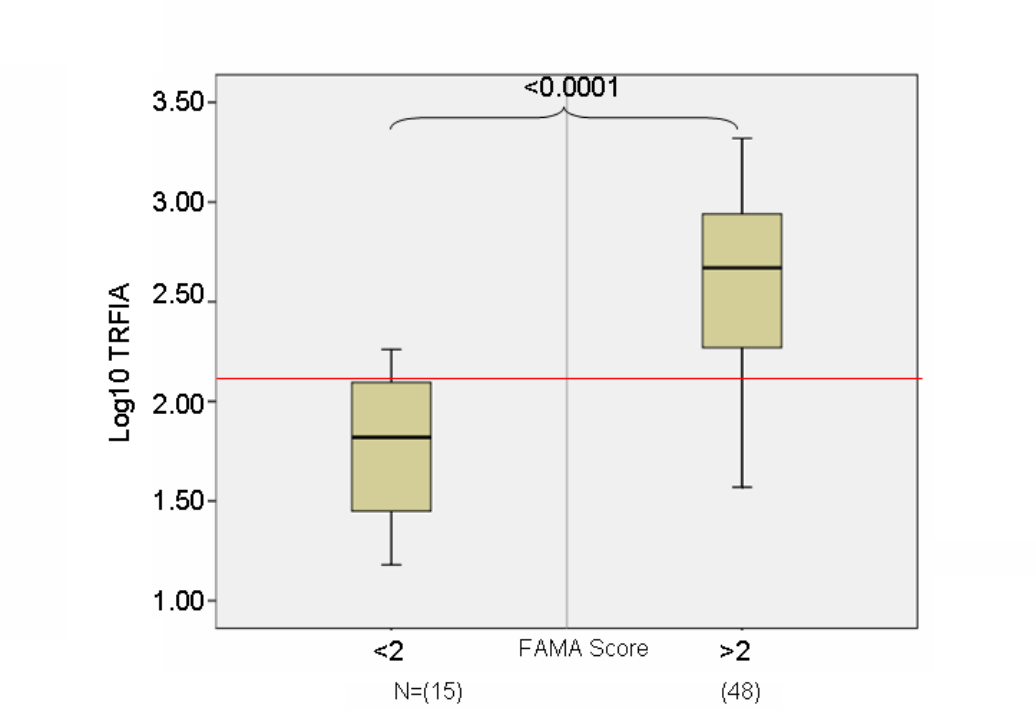


Figure 3.22: Correlation between FAMA Assay Negative and Positive Status and Log_{10} TRFIA Titres. (Figure legend on opposite page).

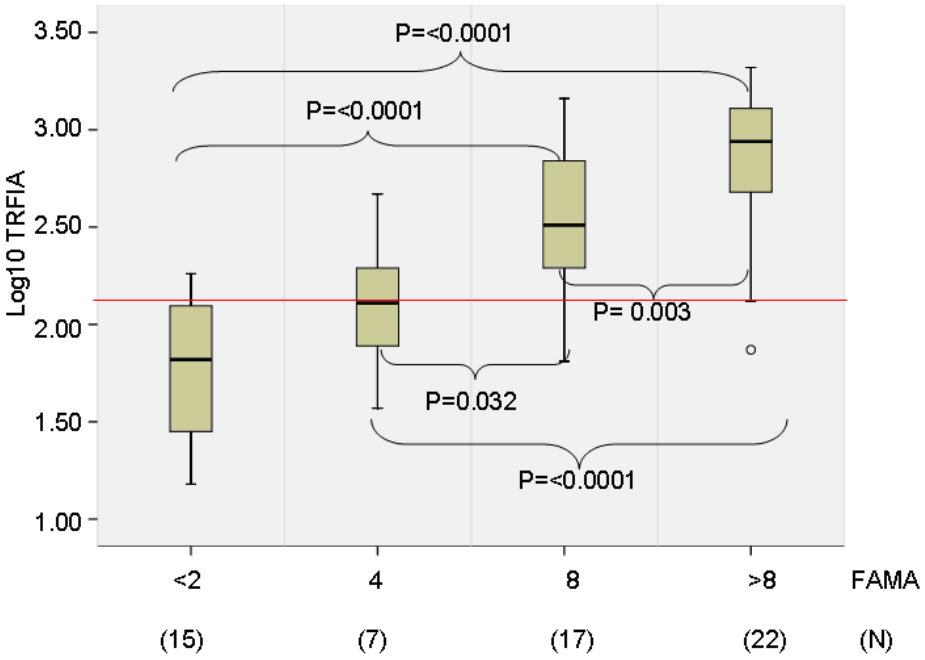


Figure 3.23: Correlation between FAMA Scores and Log_{10} TRFIA Readings. (Figure legend on opposite page).

Representative samples chosen randomly from the humoral subsets were tested by FAMA and consisted of secondary responders (PI; n=6 and NI; n=1), primary responders who seroconverted (NSC1, n=2, NSC2, n=5) and low responder groups (PLR, n=1 and NLR, n=1). In summary, correlation between FAMA and TRFIA was good, with correlation at all time points for 9/16 study participants. Both assays agreed on negative/positive status at visit 3 (12 weeks, response to second dose of vaccine) for all study participants. Seven individuals; (1001 (PLR), 1013 (PI), 1015 (NSC2), 1017 (NSC2), 1019 (NSC2), 1026 (NSC2) and 1032 (NI)) showed discrepancies between FAMA and TRFIA assay status at one or more time points, see table 3.8.

Three discrepant results were classified as TRFIA positive/FAMA negative, whilst eight samples were classified as TRFIA negative/FAMA positive. For two samples, (1032 and 1013), the cause of the discrepancies could be explained by assay failure (TRFIA for 1032 and FAMA for 1013) as results for these individuals at other visits correlated well. At three time points (1001, visit 1, 1017 visit 2, and 1019 visit 2) discrepant results may be explained by borderline TRFIA titres (125, 128, 130mIU/mL). For study participant 1001, discrepant assay results were also seen at follow-up (visit 4), and it is not possible to predict which assay status is correct. Strikingly, four out of five NSC2 samples tested were discordant at one or more time points, with FAMA indicating that each individual was seropositive at six weeks (visit 2). In addition, FAMA classified two of these study participants as seropositive at baseline, however, for all four NSC2s listed above, only low avidity antibodies were seen at any time point, indicating that these subjects did not have pre-existing humoral immunity at baseline, and therefore that the TRFIA status was more likely to be correct at this timepoint. The two NSC2 individuals that were classed as seropositive by FAMA at baseline, had discordant assay status at three time points, however both assays agreed that all four NSC2 were seropositive at 12 weeks (visit 3).

Study Number	Visit	TRFIA (mIU/mL)	TRFIA Status	Avidity (%)	FAMA Score	FAMA Status	History or contacts	Agreement on Assay Status
1001 (PLR)	V1	**130**	**P**	**28**	**<2**	**N**	None	**No**
	V2	207	P	15	8	P		Yes
	V3	229	P	38	8	P		Yes
	V4	**87**	**N**	**X**	**8**	**P**		**No**
1013 (PI)	V1	**183**	**P**	**70**	**<2**	**N**	None	**No**
	V2	700	P	81	8	P		Yes
	V3	778	P	85	8	P		Yes
	V4	463	P	72	4	P		Yes
1015 (NSC2)	V1	**37**	**N**	**X**	**4**	**P**	None	**No**
	V2	**96**	**N**	**26**	**4**	**P**		**No**
	V3	429	P	33	8	P		Yes
	V4	**74**	**N**	**X**	**>8**	**P**		**No**
1017 (NSC2)	V1	**64**	**N**	**X**	**4**	**P**	None	**No**
	V2	**125**	**N**	**28**	**8**	**P**		**No**
	V3	374	P	25	>8	P		Yes
	V4	**148**	**P**	**29**	**<2**	**N**		**No**
1019 (NSC2)	V1	21	N	X	<2	N	None	Yes
	V2	**64**	**N**	**X**	**8**	**P**		**No**
	V3	326	P	30	8	P		Yes
	V4	15	N	X	<2	N		Yes
1026 (NSC2)	V1	41	N	X	<2	N	None	Yes
	V2	**128**	**N**	**26**	**4**	**P**		**No**
	V3	481	P	34	>8	P		Yes
	V4	**116**	**N**	**28**	**8**	**P**		**No**
1032 (outlier: NSC1/NI)	V1	**94**	**N**	**X**	**4**	**P**	None	**No**
	V2	823	P	55	8	P		Yes
	V3	464	P	80	>8	P		Yes
	V4	194	P	81	8	P		Yes

Table 3.8: FAMA, TRFIA and Avidity Readings for Study Participants with Multiple Assay Discrepancies Throughout the Study. Key: V1; Visit 1 (baseline), V2; Visit 2 (six weeks), V3; Visit 3 (12 weeks), V4; Visit 4 (18 month follow-up), P; positive, N; negative. Assay discrepancies are highlighted in **Red bold**.

3.3.3.3 Benchmarking the Subsets Against a History of Varicella at Enrolment:

Eighteen HCWs gave a history of varicella infection prior to enrolment, of whom two recalled having the disease twice, two were unable to recall if they had chickenpox or not and three had been previously vaccinated; see table 3.9 and appendix 7.16, for details of which participants reported a history. The previously vaccinated individuals have been discussed in the text where appropriate, but only one of these three participants completed the study (see appendix 7.17 and 7.18 for demographic and serological data on these subjects).

Subset	N	History of Chickenpox	Did Not Know	Previous Vaccination
NSC1	32	0	0	1
NSC2	29	2 (x1 twice) (7%)	0	0
NLR	4	0	1	0
PLR	6	0	0	0
NI	7	3 (43%)	0	1
PI	28	12 (x1 twice) (43%)	1	1
Total	**106**	**17**	**2**	**3**

Table 3.9: Summary of Subjects who Reported a History of Chickenpox, or a Previous Varicella Vaccination, at Enrolment.
Four subjects were excluded from analysis, (two withdrew from study (1010 and 1084), and two were humoral subset outliers (1032 and 1089, the latter of which reported a history).

Excluding those that did not know if they had previously experienced varicella (n=2) and those that had been previously vaccinated (n=3), reporting a history at enrolment gave a PPV of 20% and NPV of 83% respectively against baseline TRFIA. Those enrolling with a history were significantly more likely to have a positive baseline TRFIA titre than those without a history (χ^2 test p= 0.001). In addition, those with a history were significantly more likely to have a positive TRFIA titre at the 18 month follow-up visit (independent 2 tailed t-test p = <0.0001) than those that enrolled without a

history. The two patients with a positive history of chickenpox who were TRFIA negative at baseline were in the NSC2 group. Neither had been tested by FAMA. Both were Caucasian and one had a history of recurrent chickenpox. Another patient with a history of recurrent chickenpox was classified as a positive baseline secondary responder (PI).

3.3.3.4 Antibody Boosting and Waning Immune Responses at 18 Months Post Vaccination:

At the 18 month follow-up visit, (visit 4) no cases of varicella (or herpes zoster) were reported. Seventy five subjects participated in the follow-up study and were classified according to their 12 week and 18 month TRFIA titres. Of these individuals, 27% (20/75) had a negative TRFIA titre and 55/75 had a positive TRFIA titre at 18 months. The negative cohort was further subdivided into those who were TRFIA seropositive at 12 weeks and had thus lost seropositive status at follow-up (lost; n=18/20) and those who were seronegative at 12 weeks (n=2/20). The cohort which was seropositive at 18 months was also further subdivided into those that had higher TRFIA titre at 18 months than 12 weeks (boosted; n=15/55) and those who had a lower TRFIA titre at 18 months than 12 weeks (retained; n=40/55). See appendix 7.19, for which study participants were classified into which follow-up cohorts.

Figure 3.24 is a plot of TRFIA titres at 12 weeks versus 18 months for those who lost or retained antibody at 18 months (those with boosted responses have been excluded). This figure illustrates that the majority of study participants who had a negative TRFIA at follow-up seroconverted after two doses of vaccine (NSC2).

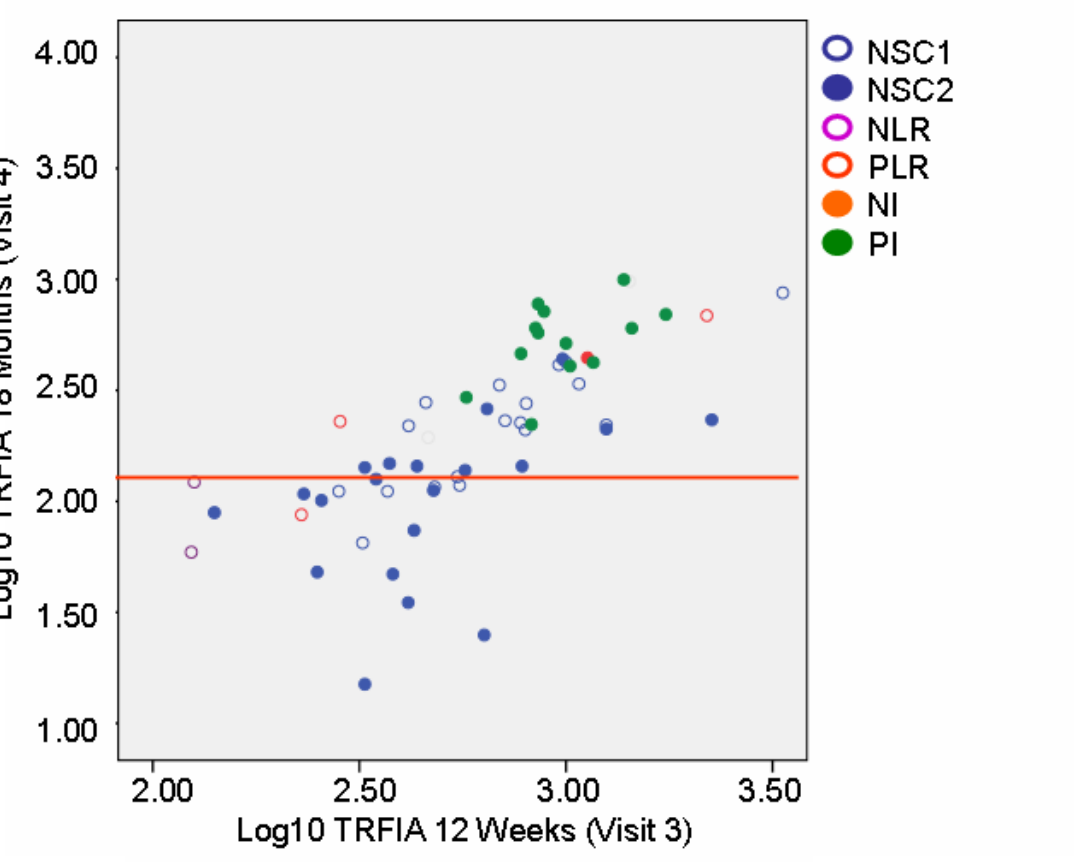


Figure 3.24: Correlation between TRFIA Titres at 12 Weeks and 18 Months for Study Participants who Lost or Retained Seropositive Status. Data are coded by humoral subset. Individuals who received a boost in antibody titres at follow-up were excluded from analysis. The correlation between 12 week and 18 month TRFIA titres was R^2 0.52. The horizontal red line represents the vaccinated-adult TRFIA cut-off of $\log_{10}2.11$ (130mIU/mL).

TRFIA titre at 12 weeks (visit 3) and follow-up (visit 4) for each of the six humoral subsets that were identified based on their responses to vaccination, are summarised in figure 3.25.

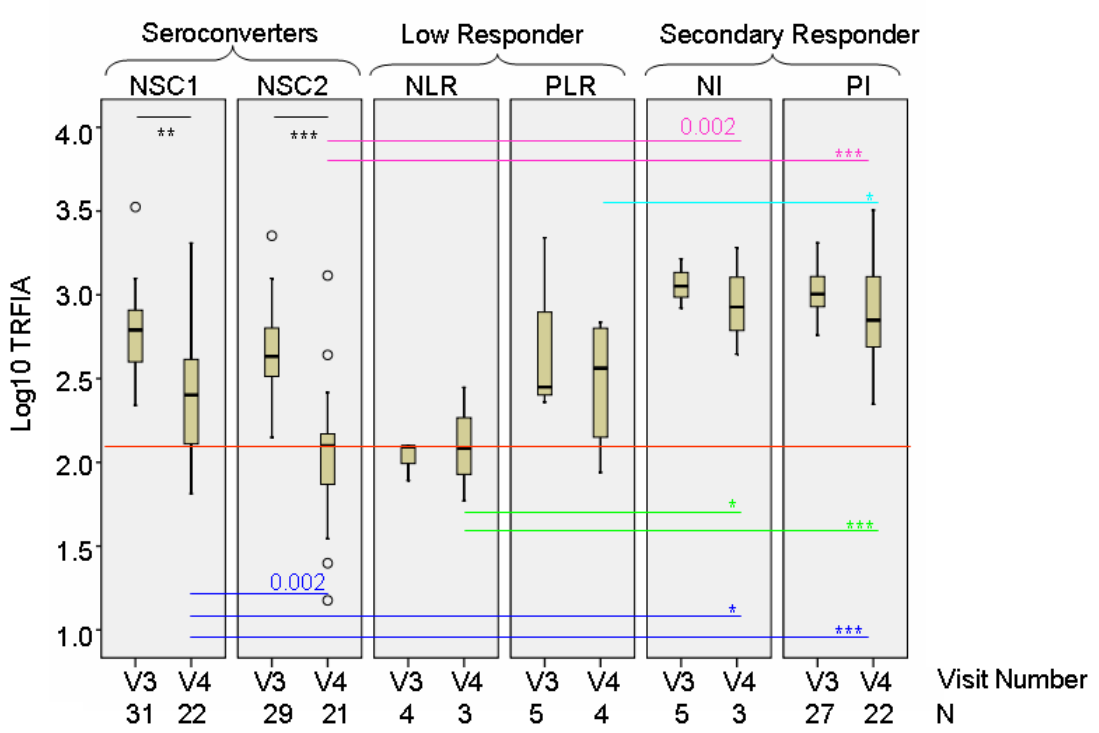


Figure 3.25; Comparison between Log_{10}TRFIA Antibody Titres Following Two Doses of Vaccine (12 Weeks) and 18 Months Post Vaccination for Each Humoral Subset. Key: NSC1; Negative Seroconvert 1st dose, NSC2; Negative Seroconvert 2nd doses, NLR; Negative Low Responder, PLR; Positive Low Responder, NI; Negative secondary responder ('Immune'), PI; Positive secondary responder ('Immune'), V3; Visit 3 (12 weeks), V4; Visit 4 (18 month follow-up), *** p=<0.0001, ** p=0.001 or below, * p=0.05 or below (independent 2 tailed t test). The horizontal red line represents the vaccinated-adult TRFIA cut-off of log_{10}2.11 (130mIU/mL), horizontal black lines within boxes represents the median value; whiskers represent the lowest data point within 1.5 IQR of the lower quartile and the highest data point within 1.5 IQR of the upper quartile respectively. Data points falling outside the whiskers (outliers) are represented as circles.

As can be seen from figure 3.25**,** all individuals who were classified as secondary responders, were TRFIA positive at follow-up, as were the majority of PLR (3/4) and 77% (17/22) of those that seroconverted following one dose of vaccine. Subjects who seroconverted after one dose had significantly higher mean TRFIA titre at follow-up than those who seroconverted after two doses. Secondary responders had significantly higher follow-up titres than primary responders (seroconverters and low responders).

3.3.3.4.1 Characteristics of Subjects who Retained Seropositive TRFIA Status at 18 Months Post Vaccination:

Of the study participants who retained a positive TRFIA titre at follow-up, nine had a history of varicella at enrolment and one had been previously vaccinated. Thirty five percent (14/40) had been classified as secondary responders, (NI; 1, PI; 13) and of those classified as primary responders, 52% (12/23) had seroconverted after the first dose of vaccine. As can be seen from figure 3.24 and 3.25 all study participants who were secondary responders (NI and PI) remained seropositive at follow-up (some of whom experienced a boost in antibody response; see section 3.3.3.4.3).

3.3.3.4.2 Waning Humoral Immunity:

Of the 20 subjects who had antibody titres below the TRFIA cut-off, 90% (18/20), had been positive by TRFIA following two doses of vaccine (12 weeks), whilst two had been classified as NLR (vaccine humoral non-responders). For the following analysis the two NLR subjects were excluded, so that only subjects who had lost positive antibody titre between 12 weeks and 18 months could be examined. Such subjects accounted for 25% of the follow-up cohort (18/73).

As illustrated in figure 3.26, the majority (65%; 11/17) were classified as seroconverting after two doses of vaccine (NSC2); whilst 29% (n=5) were classified as seroconverting after one dose (NSC1). One individual (6%) was classified as a PLR, but it is noteworthy that the baseline titre for this individual was on the cut-off (130mIU/mL) and this individual still had low avidity antibodies at 12 weeks. Thus based on six week avidity and TRFIA readings all individuals who had antibody titres below the cut-off at 18 months had been a primary responder at enrolment, and those that seroconverted after two does were significantly more likely to be TRFIA negative at follow-up (χ^2 p=0.003). No study participants in this follow-up cohort reported a history prior to enrolment or had received previous vaccination.

TRFIA titres at follow-up ranged from log_{10}1.18–2.10 (15-126mIU/mL) with a mean value of log_{10}1.85 (71mIU/mL) S.D: log_{10}0.26 and median of log_{10}1.95 (89mIU/mL). Seven subjects had adequate IgG levels to allow an accurate avidity assay to be conducted. These readings ranged from 28–73%, with a mean of 44% (S.D: 16%) and a median of 41%. Only one of these readings was above the 60% cut-off. For seven subjects, corresponding FAMA readings were available, three of which were positive (8, 8, <8). The discrepancy in assay results between these assays is discussed in more detail in section 3.3.3.2. Neither the mean age, nor the ethnicity of this 'lost seropositive status' cohort, were significantly different from that of the total study population; (26% Caucasian, 42% Black, 21% Indian and 11% Filipino, with a mean age of 32 years).

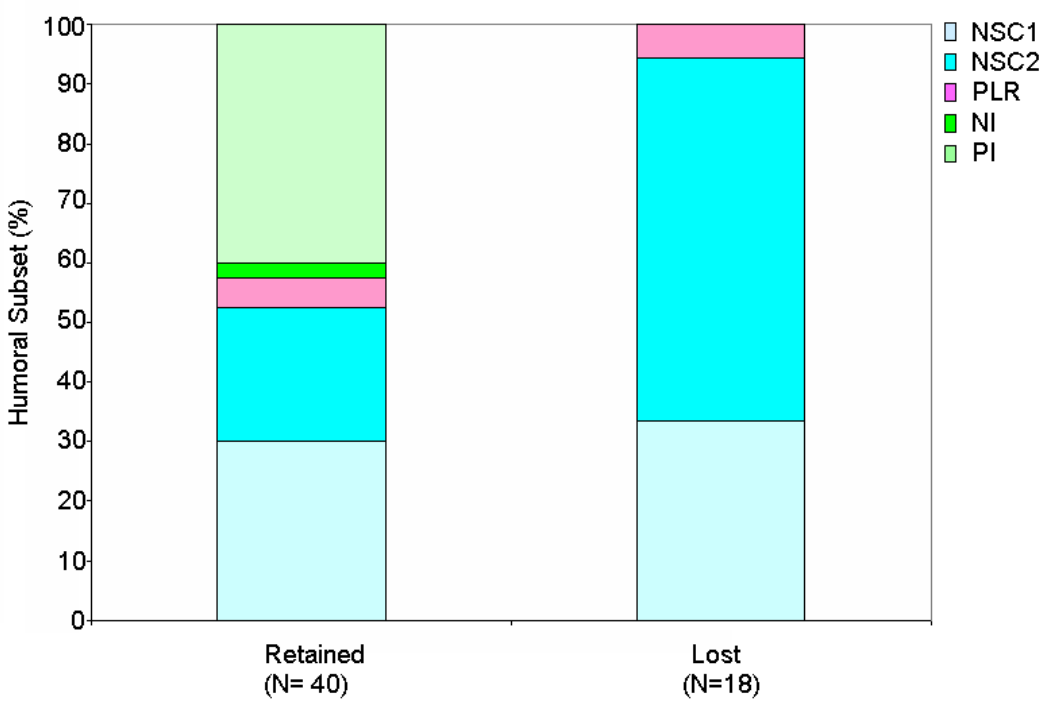


Figure 3.26 Comparison between Study Participants who Retained or Lost TRFIA Seropositive Status at 18 Months Post Vaccination. Key: NSC1; Negative Seroconvert 1st dose, NSC2; Negative Seroconvert 2nd doses, PLR; Positive Low Responder, NI; Negative secondary responder ('Immune'), PI; Positive secondary responder ('Immune'), V3; Visit 3 (12 weeks), V4; Visit 4 (18 month follow-up).

The naturally immune adult cut-off of 150mIU/mL ($log_{10}2.18$) would have classified an additional five study participants as negative at follow-up, all of whom seroconverted after two doses. As the majority of those that had lost seropositive status at follow-up were classified as seroconverting after two doses of vaccine, a comparison was made between the individuals in that subset who lost or retained antibody titres at follow-up. During the study, there were 29 HCWs who seroconverted after two doses, and 21 were available at follow-up. As mentioned earlier, 11 were classified as having lost antibody at follow up, whilst nine retained antibody above 130mIU/mL, (one subject received a boost to their antibody titre and was excluded from this analysis), see figure 3.27 part B. The same analysis was conducted for those who seroconverted after one dose of vaccine, although the sample size for the TRFIA negative at follow-up cohort was small (n=5), see figure 3.25A.

Figure 3.27: Comparison in TRFIA Titres at Each Visit for Subjects who were Seropositive or Seronegative 18 Months Post Vaccination who were Either Classified as Seroconverting After One or Two Dose(s) of Vaccine.
Part A; Seroconverted after one dose, *Part B;* seroconverted after two doses. The adult vaccine cut-off of $\log_{10}2.11$ (130mIU/mL) is represented by the horizontal red line; black horizontal bars within box plots represent the median value; whiskers represent the lowest data point within 1.5 IQR of the lower quartile and the highest data point within 1.5 IQR of the upper quartile respectively. Data points falling outside the whiskers (outliers) are represented as circles. Key: V1; Visit 1 (baseline); V2; visit 2 (six weeks), V3; Visit 3 (12 weeks), V4; visit 4 (18 month follow-up), *** p=<0.0001, * p=0.05 or below (independent 2 tailed t test).

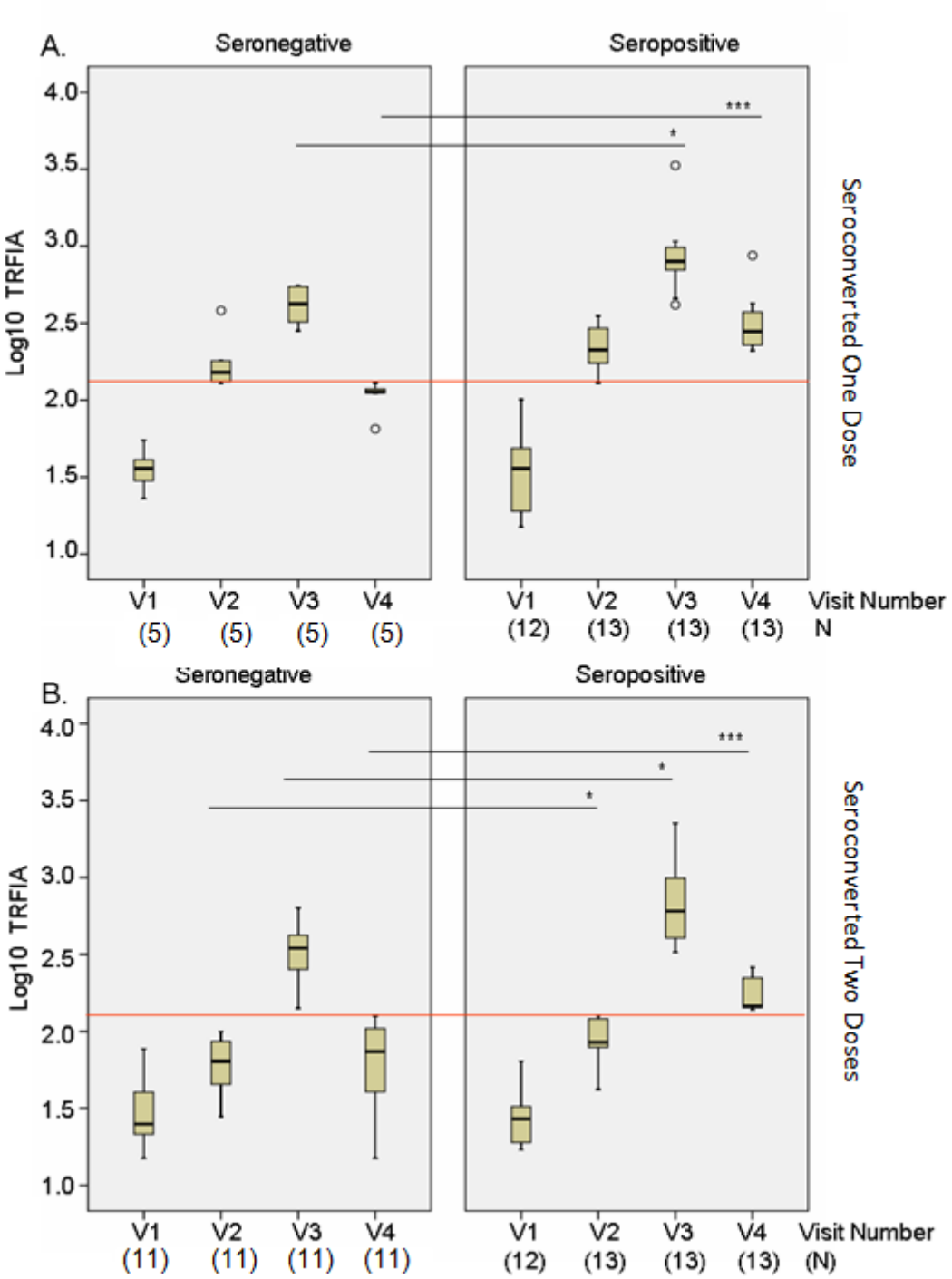


Figure 3.27: Comparison in TRFIA Titres at Each Visit for Subjects who were Seropositive or Seronegative 18 Months Post Vaccination who were Either Classified as Seroconverting After One or Two Dose(s) of Vaccine. (Figure legend on opposite page).

At baseline, there was no significant difference between the two sub-cohorts of the 'seroconverted after two doses' cohort (independent 2 tailed t test, p=0.74). However those that would later be classified as retained at follow-up had significantly higher TRFIA titres at both six and 12 weeks (both p=0.04).

For those seroconverted after one dose of vaccine (see figure 3.27 part B), again, at baseline, there was no significant difference (independent 2 tailed t test, p=0.86). Unlike the 'two doses' cohort, no significant difference was seen at six weeks (p=0.29), but those that would later be classified as retained at follow-up had significantly higher TRFIA titres at 12 weeks (p=0.007). At follow-up the difference was highly significant (p=<0.0001).

Although no distinct populations could be identified when plotting 12 week and 18 month titres (see figure 3.24), data was combined for the two subsets that seroconverted in response to vaccination and TRFIA titres at 12 weeks for those who and lost or retained antibody at follow-up were plotted (see figure 3.28). For the combined data, individuals who lost antibody at follow-up had significantly lower 12 week titres (p=<0.0001). Primary vaccine responders with a 12 week TRFIA titre above 500mIU/mL ($\log_{10}2.70$) were significantly more likely to have an 18 month TRFIA titre above 130mIU/mL (χ^2 p=<0.0001).

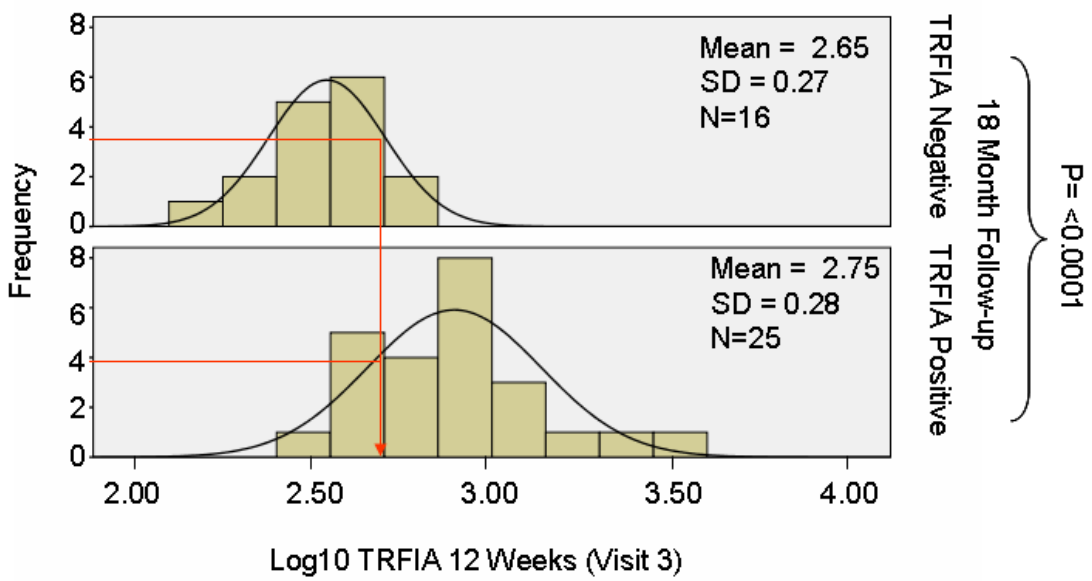


Figure 3.28: Comparison between 12 week TRFIA (Visit 3) Titres for Study Participants who Serconverted after One or Two Doses and were Either TRFIA Positive or Negative at Follow-Up (Visit 4). The horizontal red lines represent the same point on the y axis, whilst the vertical red arrow indicates where these two populations intercept: 500mIU/mL ($log_{10}2.70$).

3.3.3.4.3 Antibody Boosting:

Fifteen study participants had received a boost to their antibody titres between their 12 week visit and their 18 month follow-up visit, with antibody increases ranging from 111mIU/mL to 1,642mIU/mL. However, only 47% (n=7/15) reported contacts occurring between these time points, all of which were occupational exposures. Four subjects recalled multiple exposures, three of which were to varicella (ranging from two to six exposures), and one subject was exposed to two cases of zoster.

Avidity readings taken at 18 months ranged from 45-95% (with a mean of 80%, (SD: 15%), and a median of 85%). All but one was seropositive following two doses of vaccine (12 weeks); study participant 1050 discussed in detail in section 3.3.3.1.2.4. Fifty seven percent (n=9) of those that experienced an antibody boost had been classified as secondary responders, (PI; n=7 and NI; n=2). Twenty five percent (n=4) had seroconverted after one dose, one had seroconverted after two doses and one was a PLR.

3.3.4 Failure to Undergo IgG Antibody Affinity Maturation, 18 Months Post Vaccination:

Of subjects who had low or intermediate avidity antibodies at 12 weeks, 18 were not reclassified into a higher avidity classification at 18 months (ie: they had not matured from low to intermediate, or intermediate to high). These subjects were all primary responders, (NSC1=11/18 (61%), NSC2=5/18 (28%), PLR=2/18 (11%)). Fifty two percent (11/21) of those who seroconverted after one dose of vaccine and were recruited back at follow-up had failed to develop high avidity antibodies. Likewise, 45% (5/11) of those that seroconverted after two doses had also failed to develop high avidity antibodies. Two out of three PLRs had still failed to develop high avidity antibodies, but one had an avidity reading of 71%. The two NLRs (vaccine humoral non-responders) who were recruited at follow-up had undergone antibody affinity maturation, but the antibodies were classed as intermediate (12 week avidity readings were 11 and 27%, whilst 18 month readings were 48 and 51% respectively).

Figure 3.29 illustrates avidity values (part A) and corresponding TRFIA titres (part B) at baseline (where applicable), and the six week, 12 week and 18 month time points for each humoral subset. Table 3.10 summarises the percentage of each humoral subset which was classified as low, intermediate or high avidity at each study time point. As can be seen from figure 3.29A one subject who was classified as PI (based on six week serology readings) had a baseline avidity of 48%. At six weeks antibodies had undergone affinity maturation and antibodies were classed as high (64%). However, at 12 weeks and 18 months, the antibodies had not matured their antibody affinity further (64% and 65% respectively).

As can be seen from figure 3.29A, secondary responders (NI and PI) had higher mean antibody avidity readings than primary responders (NSC1, NSC2, NLR and PLR) at six weeks, 12 weeks and 18 months (independent 2

Figure 3.29 Comparisons of Avidity Readings and Log_{10} TRFIA Titres at Each Time Point for Each Humoral Subset. The Avidity Cut-offs of 40% and 60% and the TRFIA cut-off of Log_{10} 2.11 are represented by the horizontal red lines; black horizontal bars within box plots represent the median value; whiskers represent the lowest data point within 1.5 IQR of the lower quartile and the highest data point within 1.5 IQR of the upper quartile respectively. Data points falling outside the whiskers (outliers) are represented as circles. Key: V1; Visit 1 (baseline); V2; visit 2 (six weeks), V3; Visit 3 (12 weeks), V4; visit 4 (18 month follow-up), *** p=<0.0001, ** p=0.001 or below, * p=0.05 or below (independent 2 tailed t test).

tailed t test p=<0.0001). At six weeks (visit 2), NLR had significantly lower avidity readings than NSC1 (p=0.032). This was seen at 12 weeks (p=0.001) and was also seen for NSC2 at this time point (p=0.009). At 12 weeks there was also a significant difference in avidity readings between NSC1 and NSC2 (p=0.007), but the difference at six weeks and 18 months was not significant.

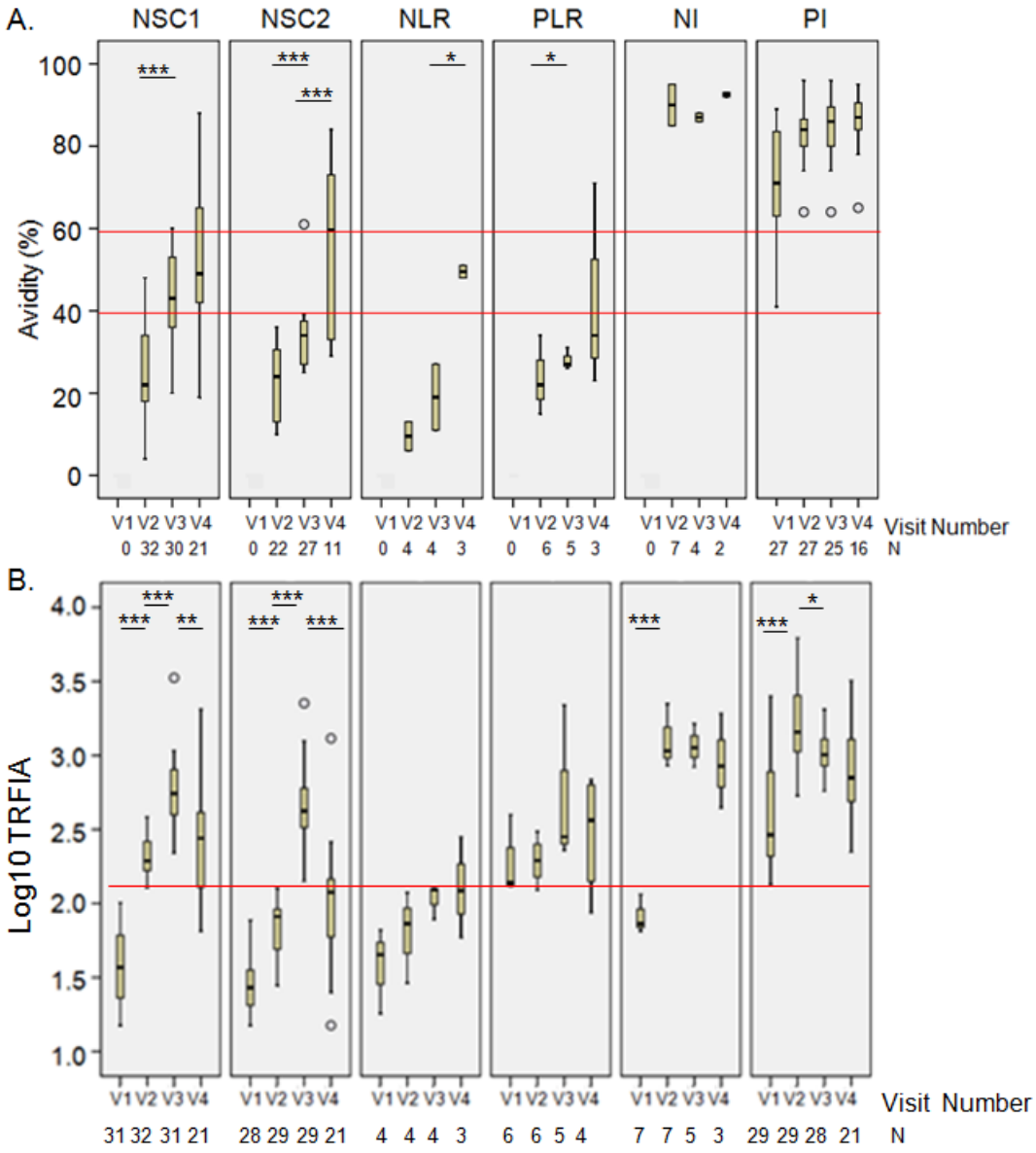


Figure 3.29 Comparison of Log_{10} TRFIA Titres and Avidity Readings at Each Time Point for Each Humoral Subset. (Figure on opposite page).

Subset	NSC1			NSC2			NLR			PLR			NI			PI		
	% in Each Avidity Classification (n)																	
	Low	Intermediate	High	Low	Intermediate	High	Low	Intermediate	High	Low	Intermediate	High	Low	Intermediate	High	Low	Intermediate	High
Visit 1	50 (1)	50 (1)	0	0	0	0	0	0	0	100 (5)	0	0	0	0	100 (1)	0	21 (6)	85 (22)
Visit 2	94 (30)	6 (2)	0	100 (22)	0	0	100 (4)	0	0	100 (6)	0	0	0	0	100 (7)	0	0	100 (27)
Visit 3	47 (14)	50 (15)	3 (1)	85 (23)	11 (3)	4 (1)	100 (4)	0	0	80 (4)	20 (1)	0	0	0	100 (4)	0	0	100 (25)
Visit 4	24 (5)	48 (10)	28 (6)	36 (4)	27 (3)	36 (4)	0	67 (2)	33 (1)	67 (2)	0	33 (1)	0	0	100 (2)	0	0	100 (16)

Table 3.10 Percentage of Each Subset with Low, Intermediate or High Avidity Antibodies at Each Visit.
Key; NSC1; Negative Seroconvert 1st dose, NSC2; Negative Seroconvert 2nd doses, NLR; Negative Low Responder, PLR; Positive Low Responder, NI; Negative secondary responder ('Immune'), PI; Positive secondary responder ('Immune')

3.3.5 Western Blotting of Serum from Varicella Vaccinees:

To analyse the profile of antibodies present in serum from HCWs 18 months post immunisation, western blotting of sera was carried out. The concentration of a commercial VZV-infected cell lysate, and the corresponding uninfected cell lysate was established using a Bradford assay and identical amounts of protein were used for western blot analysis. Figure 3.30 is an example of a coomassie stained gel, which demonstrates that same amount of each lysate was loaded and there were multiple protein bands in the uninfected lysate control.

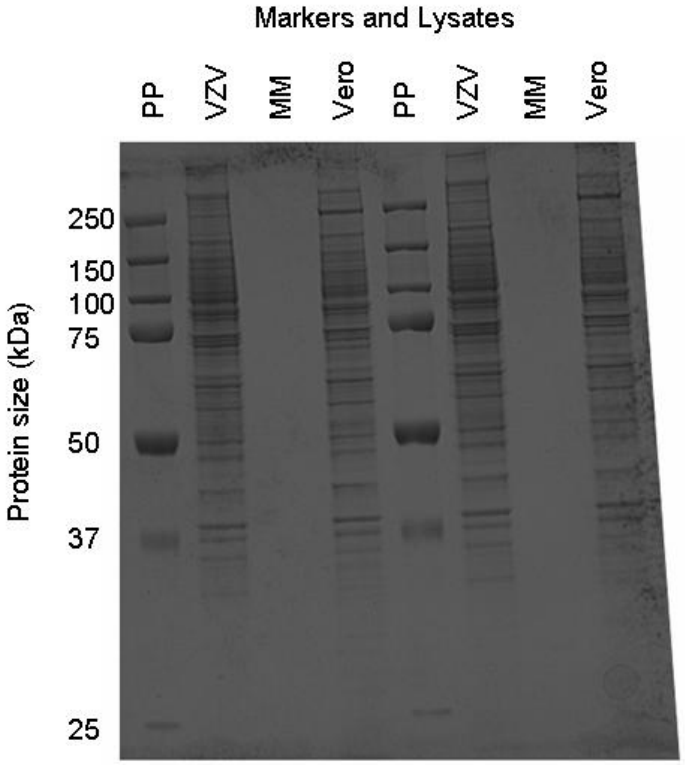


Figure 3.30: Coomassie Staining of Lystaes and Markers used for Western Blotting. Key: PP; Precision plus proteins standard, VZV; VZV infected vero cell lysate, MM; MagicMark™XP standard, Vero; uninfected vero cell lysate. 7μg of each lysate was loaded, and gels were stained with EZBlue Coomassie stain. Images were visualised on a G:Box™ gel documentation machine (Synegene, UK), using GeneSnap acquisition software (Synegene, UK).

In addition to the size of protein bands, (see figure 3.31) the locations of VZV proteins within the blot were confirmed using a commercial monoclonal antibody, containing a mixture of antibodies specific for seven VZV epitopes (located within the nuclear capsid protein (155kDa), IE62 (175kDa), gB (60-70kDa), a precursor product within gE, as well as fully glycosylated gE (82-95kDa), gH (100-118kDa), and gI (45-60kDa). Various concentrations of this primary antibody were used (see figure 3.32), in order to visualise the different proteins.

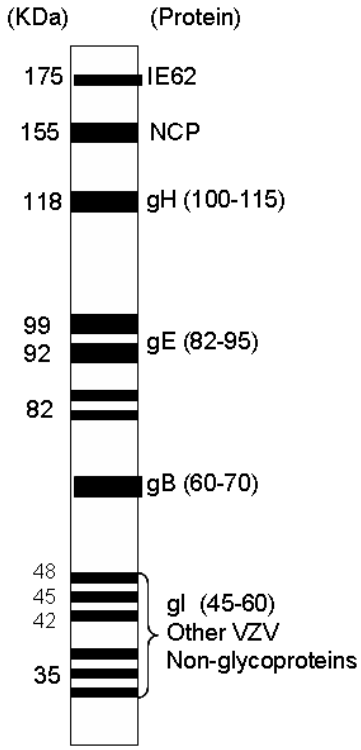


Figure 3.31: Schematic of VZV-Specific Bands Identified in Sera from Subjects with a History of Varicella under Reducing Conditions. Key; NCP; nuclear capsid protein, g; glycoprotein, (referenced from Harper *et al.,* 1988; Dubey *et al.,* 1988 and Zweeink *et al.,* 1981).

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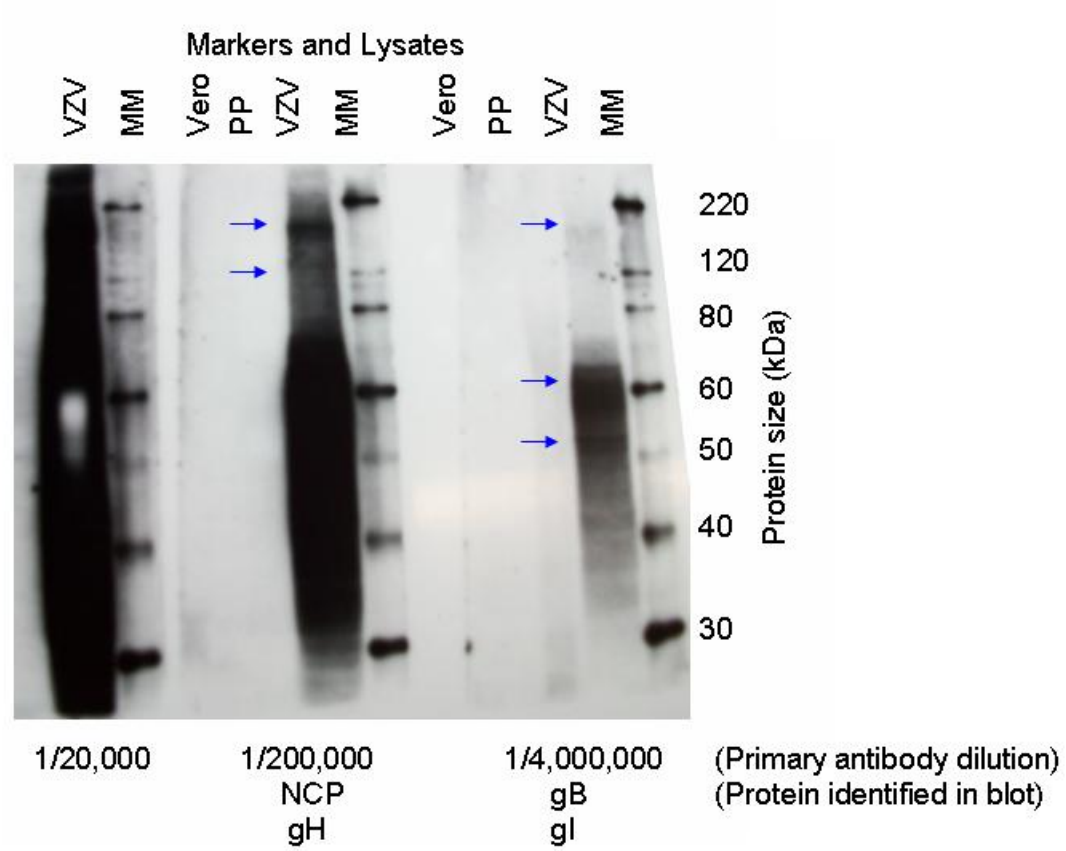


Figure 3.32: Western Blot of VZV-Infected Cell and Uninfected Cell Lysate with Multiple VZV-Specific Monoclonal Antibodies. The monoclonal antibody mix was used at three concentrations to identify various proteins. Key: PP; Precision plus proteins standard, VZV; VZV infected vero cell lysate, MM; MagicMark™XP standard, Vero; uninfected vero cell lysate, NCP; nuclear capsid protein, g; glycoprotein. Arrows indicate the presumed position of NCP (155kDa), gH (100-115kDa), gB (60-70kDa) and gI (45-60kDa). Images were generated with a digital Kodak M863 camera.

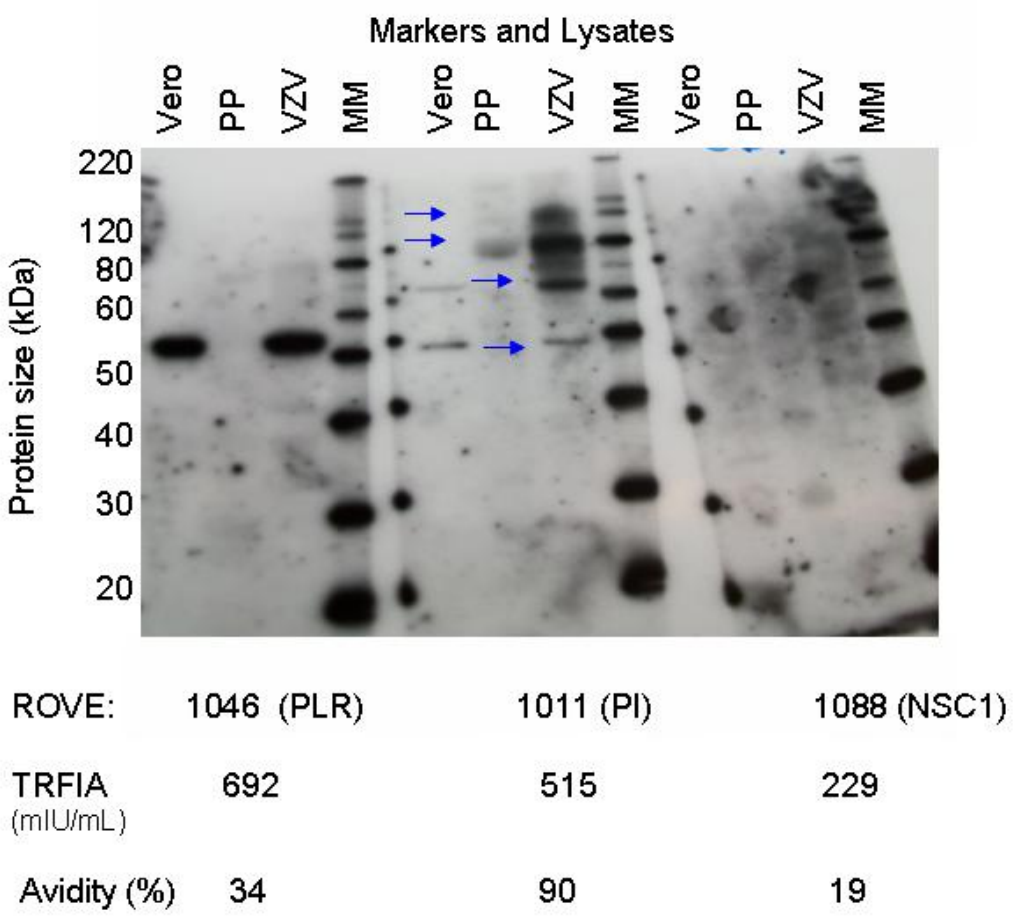


Figure 3.33 Examples of Antibody Detection Using ROVE Follow-Up Serum Samples of Varying Avidity. Key: PP; Precision plus proteins standard, VZV; VZV-infected vero cell lysate, MM; MagicMark™XP standard, Vero; uninfected vero cell lysate. Arrows indicate the multiple protein bands within one sample, which are discussed further in the text. The top arrow is VZV-specific and is presumed to be gH (100-115kDa). Images were generated with a digital Kodak M863 camera.

Serum samples of varying avidity were tested, and results indicated that for samples with low avidity antibodies banding could not be detected in seropositive samples, as demonstrated in figure 3.33 with serum samples 1046 and 1088 which had an avidity of less than 40%. Although 1046 had a band (≈55kDa), this cross reacted with the uninfected cell lysate. Multiple dilutions of serum samples were used with less stringent washing conditions (Tween 20 reduced, wash times reduced), but this had no effect on yielding bands in samples with low avidity. Samples with high avidity antibodies yielded bands, but the majority were non-specific, reacting with the uninfected

control lysate and/or the protein marker, as demonstrated with sample 1011 in figure 3.33. In this sample only one specific band was seen, located between 100-120 kDa markers, and thus presumed to be to gH. Other bands at ≈80kDa, ≈65kDa and ≈55kDa, which could have been presumed to be gE, gB and gI cross reacted with the uninfected control lysate/protein marker.

To assess the optimum TRFIA titre which could be used to detect VZV-specific proteins in serum with high avidity antibodies in this system, a serial two fold dilution of a study participant's serum sample was carried out prior to the 1/20,000 dilution (see figure 3.34). This revealed that certain VZV-specific proteins could be visualised at a higher antibody concentrations, whilst others could be visualised at lower concentrations.

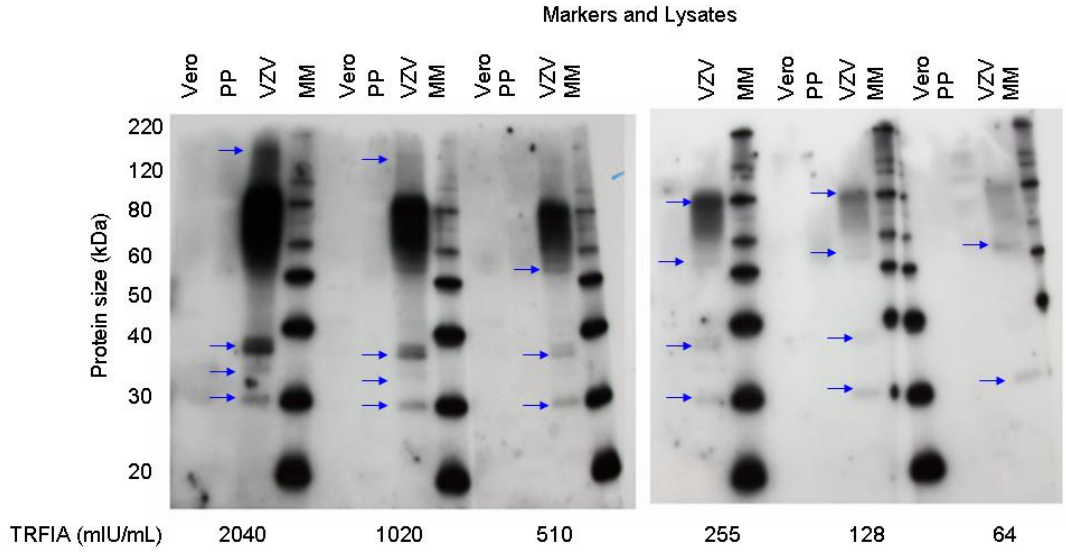


Figure 3.34 Serial Two Fold Dilution of Vaccinees Serum Sample to Deduce the Limit of VZV-Specific IgG that this Western Blotting System could be used to Detect. Key: PP; Precision plus proteins standard, VZV; VZV infected vero cell lysate, MM; MagicMark™XP standard, Vero; uninfected vero cell lysate. Black Horizontal arrows indicate VZV-specific proteins. Images were generated with a digital Kodak M863 camera.

As can be seen from figure 3.34, at a concentration of 2,040mIU/mL multiple banding could be seen, including a protein presumed to be to NCP (155kDa), multiple banding (or very intense luminescence) between 120-50kDa and

bands at ≈37, ≈35 and ≈30kDa (thought to be non-glycosylated proteins). However at a concentration of 510mIU/mL the NCP and the 35kDa protein was no longer detectable, whilst at a concentration of 128mIU/mL a protein at ≈80kDa (thought to be gE) was better defined than at higher antibody concentrations.

As this system of detection was not suitable on low avidity samples, and multiple dilutions would have to be carried out for each high avidity sample in order examine the repertoire of VZV-specific bands, this methodology was not pursued further.

3.4 DISCUSSION:

3.4.1 Summary of Main Findings:

In an effort to provide insight into factors which may influence antibody responses and subsequently possible protection against re-infection, the differences in antibody titre and avidity maturation were used to group individuals by the pattern of their humoral immune response to VZV vaccine. Distinct groups of humoral responders were identifiable and certain characteristics were found to be associated with different subsets. Caucasians (raised in temperate climates) were significantly more likely to report a history and be classified as secondary responders than study participants raised in the tropics. For those who were identified as primary responders, black ethnicity was associated with a significantly lower TRFIA titre following two doses of vaccine. Delayed seroconversion (NSC2) was associated with an increased risk of developing seronegative status 18 months post vaccination, as was a 12 week TRFIA titre below 500mIU/mL. This study also illustrated that primary responders can take longer than 18 months to develop high avidity antibody following varicella vaccination and that overall there was a good correlation between seronegative/positive assay status for TRFIA and FAMA.

3.4.2 Defining a TRFIA Cut-Off in an Adult Population Using Vaccination as a Tool to Assess Naturally Derived VZV-Specific Antibodies:

One of the aims of this study was to validate a highly sensitive immunoassay for measuring low levels of naturally induced VZV-specific IgG levels in an adult population. In adults, VZV population immunity is typically in excess of 90% (Lerman *et al.,* 2004), making it difficult to accurately assess an assay cut-off in a positively skewed antibody profile. This particular problem was overcome in our study, by enrolling subjects who were either negative or equivocal by Diamedix. In addition to this, two markers of pre-existing humoral immunity were used to define those who were truly negative (primary responders) at baseline; avidity readings below 60% and TRFIA titres below 400mIU/mL ($\log_{10}2.60$) at six weeks post first vaccination. This enabled us to identify a negative population (defined on the basis of titres obtained following natural infection) with greater accuracy, than using the mixture modelling methodology. Wide confidence intervals for the mixture model demonstrated that the sample size was insufficient to fit a reliable mixture model to this data set. As there was a lot of overlap between the two distributions in the model, a sample size of double that which was used would probably be needed.

Because there was a strong correlation between IgG antibody titres measured by TRFIA and the EUROIMMUN avidity assays, this was indicative that a comparable antibody population was being measured in both assays. To our knowledge, this is the first time the properties of antibody avidity and titre following vaccine antigen challenge have been used to determine an assay cut-off in the baseline population. Moreover, this method was more robust for determining a cut-off in a smaller population size than the mixture modelling methodology, which was confirmed by probability density curve and ROC analysis.

The TRFIA cut-off determined in this thesis (130mIU/mL; $\log_{10}2.11$) is higher than that which was originally derived for this assay (93.3mIU/mL; $\log_{10}1.97$), using the mixture modelling technique, in healthy adults, (Maple *et al*., 2006). However, more recently ROC analysis and comparison with the Merck gpELISA suggested this cut-off was too low and a cut-off of 150mIU/mL ($\log_{10}2.18$) (Maple *et al.,* 2009a), was suggested. This level also accommodated the high (20%) intra assay variation observed with this assay.

As FAMA is thought to measure neutralising antibody and TRFIA is thought to measure total IgG (regardless of antibody function), this would account for discrepancies where a high TRFIA titre was obtained for a sample, but the corresponding FAMA was low. The discrepancies seen between TRFIA and FAMA suggest that TRFIA suffers from the same drawbacks as other EIAs in comparison to FAMA, namely poor discrimination between low positives and true negatives around the cut-off.

3.4.3 Study Population Demographics:

The vaccine study consisted of an ethnically diverse population of healthcare workers, of which fewer than 25% were Caucasian. In tropical climates, seropositivity in adults is lower than in temperate climates, (Vyse *et al.,* 2004; Khoshnood *et al.,* 2006; Garnett *et al.,* 1993; Lokeshwar *et al.,* 2000). Thus, when recruiting adults who where seronegative (or equivocal) by the Diamedix assay, a skew towards those born and raised in the tropics, was expected in our recruited population.

3.4.4 Failure to Seroconvert Following Two Doses of Vaccine in Primary Responders:

The vaccination study examined in this thesis reported a vaccine humoral non-responder rate (defined as a failure to seroconvert after two doses of vaccine), of 4% (4/103) of the population that completed the study, and 6% of those who were primary humoral responders (defined as a six week avidity of

<60% and a baseline TRFIA of <130mIU/mL; n=65). Rates of failure to seroconvert in children and adults following two doses of varicella vaccine have ranged between 4-14% (Gershon *et al.,* 1988; Kuter *et al.,* 2004; Li *et al.,* 2002), however a more recent study examining primary vaccine failure (*i.e.* no measurable immune response following vaccination and remaining susceptibility) found that in healthy children vaccinated with one dose of vaccine found the rate to be higher at 24% (Michalik *et al.,* 2008). The rate of failure to seroconvert found in our study is similar to published literature on other vaccines. For HBV a rate of 5-10% has been reported and has been linked to advanced age (Hollinger, 1989), obesity (Alper *et al.,* 1989, Weber *et al.,* 1985), smoking (Shaw *et al.,* 1989) and alcohol consumption (Nalpas *et al.,* 1993). In addition an association between HBV primary vaccine failure and various HLA types has been reported (DRB1*03, DRB1*07, DQB1*02, DPB1*1101), (Milich and Leroux-Roels, 2003; Thursz, 2001). The HLA-DRB1*0701 allele was also found to be over represented in healthy adults who were classified as humoral non-responders following influenza vaccination, compared to those who seroconverted (Lambkin *et al.,* 2004). Likewise, a high degree of heritability and an HLA association (MHC-I and MHC-II) was found amongst primary vaccine failure following measles vaccination (Jacobson and Poland, 2004).

Interestingly two of the vaccine humoral non-responders (NLRs) in this study seroconverted either following a third dose of vaccine or a household exposure to chickenpox. However the HCW who seroconverted following a third dose was seronegative at follow-up. The response to the third dose was of the magnitude of those seen for secondary responses (although avidity was low) suggesting that this was a memory B cell response. However as the HCW was seronegative at follow-up this indicates that they lacked long lived plasma cells specific for varicella vaccine antigen. Vaccine humoral non-responders to HBV vaccine have been shown to seroconvert with a booster

immunisation (Van der Sande *et al.,* 2007), although in these cases, antibody responses were of the same magnitude as primary responses.

With the second HCW (whose CMI response is discussed in chapter 4), this subject was able to seroconvert asymptomatically to a household exposure, and produce an antibody titre that was of a magnitude seen following primary infection (i.e. 10 fold higher), (Bogger-Goren *et al.,* 1982). In addition, this subject retained a seropositive status at follow-up. See chapter 4 discussion for suggestions as to why this exposure was asymptomatic.

3.4.5 Humoral Immune Responses and an Association with Ethnicity

In our vaccination study, no difference in ethnicity was found between those who seroconverted following one dose and those who seroconverted following two doses. However, as our data indicated that low humoral responses to varicella vaccination were associated with ethnicity, it is possible that a difference in ethnicity would have been seen with those who seroconvert after one dose or two if a larger population size had been examined. In addition, there may be differences between the individuals that were classified in these two groups which were not measured as part of this study. These could possibly be innate immune responses or other genetic markers. Being able to identify these differences would be useful as individuals who did not seroconvert following primary antigen challenge (one dose) were significantly more likely to be TRFIA negative at 18 months.

The data presented in this thesis demonstrated that following varicella vaccination, black ethnicity was associated with lower TRFIA titres in primary responders. Data from our laboratory has found that there is an association between VZV-specific IgG antibody titres and ethnicity following natural infection (Ayres, McDonald, Talkuder and Breuer, unpublished). This study examined antibody titres in Caucasian women born and raised in the UK with titres from Bangladeshi women who were either born and raised in the UK, or

born and raised in Bangladesh and had migrated to the UK. Irrespective of country of birth and residence, Bangladeshi women had significantly lower VZV-specific IgG titres than Caucasian women. This study demonstrated that the difference in titre was associated with ethnicity rather than geographical factors such as age of infection or seroprevalence, as Bangladeshi women born and raised in the UK had comparable seroprevalence rates and age of infection to Caucasian women. However, a lower antibody titre following vaccination in Asian study participants was not seen in the study presented in this thesis. This could be attributable to either the small sample size or a less pronounced difference in titre following vaccination compared to that seen in black study participants.

To our knowledge, an association with lower antibody titre following varicella vaccination or natural infection and ethnicity has not been reported previously. However the influence of host factors on the magnitude of the humoral immune response is supported by a study which showed a significantly greater concordance of VZV antibody titres following varicella vaccination, between siblings than between unrelated individuals (Klein *et al.,* 2007). A heritable effect on antibody levels has also been reported following infection with Epstein Barr Virus (Yasui *et al.,* 2008) and Herpes Simplex Virus (Lio *et al.,* 1994), as well as following vaccination against measles, mumps, rubella (MMR) (Ovsyannikova *et al.,* 2006), hepatitis B (Henning *et al.,* 2008), and influenza (Lambkin *et al.,* 2004).

3.4.6 Negative immune (NI) Humoral Responders:

Seven secondary responders (n=7) had antibody titres which classified them as negative at baseline, despite three reporting previous varicella and one reporting prior vaccination. As these individuals had high avidity antibodies and a significant increase in VZV-specific IgG titre at six weeks after the first antigen challenge, this indicates that these individuals had memory B cells specific for VZV that had undergone affinity maturation. However, as these subjects were seronegative at baseline this suggests that these individuals

either lacked long lived plasma cells which were specific for VZV, or that their VZV-specific plasma cells were producing very low titres of antibody.

3.4.7 Failure to Undergo Antibody Affinity Maturation at 18 Months Post-Vaccination:

Within the study population were 18 subjects who had failed to undergo affinity maturation at 18 months post vaccination (i.e. they had not been reclassified into a higher maturation group at this time point). The time period attributed to maturation of high avidity antibodies in immunocompetent individuals is reported to be 4 months to CMV infection (Lazzarotto *et al.,* 1998) and has been reported to occur between 5-6 months in response to the mumps vaccine and natural infection (Narita *et al.,* 1998). A report for EBV also demonstrated that high avidity antibodies were seen at seven months post infection, but this study did not examine antibody avidity in between a six week time point (when low avidity antibodies were seen) and this latter time point (Robertson *et al.,* 2003).

However there have been several reports which document delayed maturation of antibody and the absence of high avidity antibodies to a variety of pathogens, in some cases, years post infection. Several reports document that CMV-specific antibody affinity maturation (induced following vaccination or natural infection) takes place over a much longer period of time than previously thought, (24 months in the case of vaccination and up to 23 months to natural infection) in immunocompromised patients (Marshall and Alder, 2003; Lazzarotto *et al.,* 1998). One of the studies reported that gB-CMV specific antibody affinity maturation was slower following vaccination with an attenuated CMV vaccine than with a recombinant-gB vaccine, implicating the antigen source as the reason for slow affinity maturation (Marshall and Alder, 2003). Another study carried out in immunocompromised subjects (systemic lupus erythematosus and rheumatoid arthritis patients) showed that these individuals produced low avidity antibodies and restricted

class switching to tetanus toxoid compared to healthy controls (Devey *et al.,* 1987). However a study in healthy subjects in Japan showed that some patients immunised with the mumps vaccine failed to produce high avidity antibody responses 6 months post immunisation (Narita *et al.,* 1998). In addition, many studies have been carried out on the avidity of antibodies to the parasite *toxoplama gondii* in pregnant women (reviewed in Lefevre-Pettazzoni *et al.,* 2006) and in one study, low avidity antibodies were still detected in some patients six years post infection. The authors suggested that anti-parasitic drug treatment may have been responsible for this phenomenon (Sensini *et al.,* 1996).

As explained in this chapter introduction, our understanding of events which take place in the germinal centre is rapidly evolving, and one could speculate as to which of these process is hampering antibody affinity maturation to varicella vaccination, although it is known that humans and mice with deficiencies in AID, CD40-CD40L or ICOS have no ability to class switch or undergo affinity maturation (King *et al.,* 2008; Elgueta *et al.,* 2009). A study carried out on affinity maturation of measles-specific IgG demonstrated that age of vaccination affected both antibody avidity and class switching (Nair *et al.,* 2007). Recently a study in dizygotic and monozygotic twins in the Gambia demonstrated that environmental rather than genetic factors predominantly controlled affinity maturation and the production of high-avidity antibodies to tetanus toxoid (Marchant *et al.,* 2006).

Recently, the lack of antibody affinity maturation induced following formalin inactivated RSV vaccine administration in mice has been shown to be due to poor TLR stimulation (Delgado *et al.,* 2009). TLRs are known to play a crucial role in antibody production as TLR signalling is necessary for both DC maturation and T-helper and B cell activation (Pasare and Medzhitov, 2005). In addition this paper demonstrated that the poor immunogenicity of this vaccine was due to the lack of affinity maturation of the antibodies produced and not due to the disruption of immunodominant epitopes by formalin, as the

quantity of antibodies produced to an immunodominant epitope were shown to be the same in mice immunised with WT RSV and the formalin inactivated vaccine.

However, numerous publications have previously reported that IgG antibody avidity is a suitable tool for distinguishing between current and persistent infection for a variety of pathogens (Heldman *et al.,* 1993), including rubella virus (Polanec *et al.,* 1994), HIV (Suligoi *et al.,* 2002), West Nile virus (WNV), (Levett *et al.,* 2005), dengue virus (DeSouza *et al.,* 2004; Matheus *et al.,* 2005), as well as various other herpes viruses, including CMV (Blackburn *et al.,* 1991; Lazzarotto *et al.,* 2008), EBV (Weissbrich, 1998) and HHV-6 (Pietiläinen *et al.,* 2009). Our data and that of others suggest that low avidity antibodies can still predominate many months after infection/vaccination. However, when study participants were classified according to serological readings six weeks post first vaccination, a dichotomous population was clearly seen, based on both avidity and TRFIA values, indicating that secondary responders had high avidity antibodies following natural infection, which in those who reported a history occurred a minimum of three years previously.

3.4.8 Loss of Seropositive Status 18 Months Post Vaccination and the Risk of Breakthrough Infection:

This study found that 25% of follow-up study participants had converted from seropositive to seronegative at this time point. Loss of seropositive status at follow-up in 31% and 25% of subjects have been reported in other studies (Saiman *et al.,* 2001; Gershon *et al.,* 1998); however the latter study found that even those who were seronegative were partially protected.

Breakthrough infections have been correlated with low levels of VZV antibodies at six weeks post immunisation and with loss of antibodies over time (Li *et al.,* 2002; Ampofo, 2002). Although no breakthrough infections

were reported at the 18 month follow-up visit, we believe subjects with TRFIAs below 130mIU/mL at this time point are possibly at risk of infection. Our data support the notion that low antibody titres at six weeks correlate with breakthrough infection as the subset of humoral responders who required two doses of vaccine to seroconvert were more likely to seronegative at 18 months. In addition this study also found that a TRFIA titre below 500mIU/mL following two doses of vaccine (12 weeks) was more likely to result in seronegative status at follow-up. A similar finding in long term follow-up studies of HBV vaccination, demonstrated that peak vaccine-induced IgG titres in primary responders were shown to directly correlate with antibody decay and subsequent risk of infection (Inskip *et al.,* 1991; Van der Sande *et al.,* 2007).

At follow-up, study participant 1019 was seronegative by FAMA, TRFIA (and IFN-γ ELISPOT; see chapter 4 for more details), and thus negative by every parameter measured at this time point. It is therefore likely that this subject would be susceptible to infection. However, this individual reported an occupational shingles contact 84 days (12 weeks) prior to follow-up, which in this particular case was at 16 months post vaccination. As there is no indication of antibody boosting or CMI, one possibility is that the contact was insufficient to allow transmission. DIAMEDIX, FAMA, and TRFIA readings agreed this HCW was seronegative at baseline, with a 12 week avidity reading of 30%. This HCW was classified as seroconverting after two doses during the study. Although (as discussed in chapter 4) seronegative HCWs have been shown to be protected from infection, a CMI response has been seen in these subjects.

3.4.9 Antibody Boosting at Follow-Up:

Continued immunity to varicella is thought to be maintained by exogenous boosting, where individuals are asymptomatically reinfected throughout their lifetime and evidence for this is growing. Repeated cases of zoster within an

individual, which were caused by different genotypes of virus is strong evidence for this theory (Taha *et al.,* 2006). In a study of vaccinated HCWs who lost positive antibody titre following vaccination, re-infection was higher following household exposure (18%; 4/22) than occupational exposure (6%; 8/72), (Saiman *et al.,* 2001). However in this study, all subjects who had a boosted antibody titre at follow-up and reported a contact, reported an occupational exposure. As 53% of those with boosted antibody titres did not report a contact between their 12 week visit and follow-up visit, this means that these individuals were either exposed without their knowledge, or they had experienced endogenous boosting. Endogenous boosting of immunity has been observed in latently infected patients who have undergone bone marrow transplantation (Redman *et al.,* 1997).

3.4.10 Future work:

It could be hypothesized that there would be a difference in IgG subtypes produced by HCWs in different humoral subsets. As outlined in the chapter introduction, there is conflicting data on the subtypes which predominate after natural infection, and antibody subtype would also be indicative of function. Although it was not possible to accurately determine which VZV proteins vaccinees were raising antibodies to in this study by Western blotting, cloning known immunodominant epitopes into a vaccinia virus expression system could facilitate a more precise methodology for examining this. Although VZV is a difficult virus to work with *in vitro* (as it is a highly cell associated and generates low titres), complement dependent neutralisation assays would be useful in determining the correlation between antibody function, avidity and FAMA scores. The presence of neutralising antibodies has been shown to correlate with a positive FAMA score (Grose et al., 1979) and studies with flavivirus vaccines (yellow fever, Japanese encephalitis and tick-borne encephalitis) have also demonstrated that neutralizing antibody correlates with protection (Green *et al.,* 2006).

CHAPTER 4: CELL MEDIATED IMMUNE RESPONSES TO VACCINATION

Chapter Summary:

In this chapter VZV-specific IFN-γ responses were investigated 18 months post vaccination, in an attempt to deduce if HCWs were likely to be susceptible to VZV infection. In addition, a highly specific methodology (using the only existing MHC-II tetramers available for VZV epitopes) was used to examine $CD4^+$ T cell responses to vaccination in a subset of our study population.

4.1 INTRODUCTION:

4.1.1 An Introduction to Adaptive Cell Mediate Immunity (CMI):

T cells are lymphocytes that mature within the thymus. These cells express membrane bound receptors (T cell receptors; TCRs) which are composed of either αβ or γδ chains. These chains are responsible for the specificity of the receptor. αβTCRs recognize peptide antigens presented by major histocompatibility complex (MHC) proteins. MHC-I, which is expressed on virtually all nucleated cells of the body, presents endogenous peptides, whilst MHC-II, expressed on professional antigen presenting cells (DCs, B cells and macrophages) presents exogenous peptides. αβT cells generally express either CD4 or CD8; $CD8^+$ T cells recognize peptide in the context of MHC-I, whilst $CD4^+$ T cells recognize peptide in the context of MHC-II. T cells with an αβTCR are classified into numerous subsets, defined by several criteria, including chemokine receptor expression, location/migration, and function. Subsets include cytotoxic $CD8^+$ T cells (Tc) which have the ability to lyse target cells; and a range of T helper $CD4^+$ cells, which when activated can express various phenotypes including a Th1 (to be discussed later) or Th2 phenotype, (which amongst other functions produce cytokines that help mediate antibody isotype switching), T follicular helper cells (T_{FH} cells), (which provide a helper function to B cells and are discussed in more detail in

chapter 3, section 3.1.1.2) and T regulatory cells (T_{regs}) which are generally characterized as $CD25^+$, $FOXP3^+$ and suppress immune responses.

Cytotoxic $CD8^+$ T lymphocyte cells (CTLs) have cytoplasmic granules which contain perforin and granzymes. These cells are typically involved with immune responses to both virally infected and cancer cells. Upon binding to a target cell, the contents of the granules are released; perforin creates pores in the target cell membrane, causing lysis, and allowing granzymes to enter the target cell and induce apoptosis. Cytotoxic $CD4^+$ T cell clones with the ability to lyse target cells *in vitro* were first described in the early 1980s (Fleischer, 1984). They have since been found *in vitro* to several viral infections including influenza, HIV and polio virus (Lukacher *et al.,* 1985; Littaua *et al.,* 1992; Norris *et al.,* 2001; Mahon *et al.,* 1995), and have been detected directly from peripheral blood *ex vivo* in HIV, CMV and EBV patients, (Appay *et al.,* 2002; Zaunders *et al.,* 2004). Studies of clones and cell lines have indicated that $CD4^+$ CTLs use the perforin-dependent cytotoxic mechanism, rather than the Fas-dependent pathway (Norris *et al.,* 2001; Williams and Engelhard, 1996; Echchakir *et al.,* 2000; Yasukawa *et al.,* 2000). *Ex vivo* analysis of cytotoxic $CD4^+$ T cells demonstrated that they have lytic granules containing cytotoxic factors such as granzymes and perforin, and that their lytic activity is HLA class-II restricted (Appay *et al.,* 2002; Zaunders *et al.,* 2004; Porakishvili *et al.,* 2004).

Naïve T cells that have been activated and have differentiated into a Th1 phenotype are associated with cell-mediated inflammatory reactions and are potent producers of IFN-γ. This cytokine causes upregulation of MHC-II on macrophages and DCs as well as promoting macrophage activation and DC maturation. IFN-γ also leads to the expression of inflammatory cytokines including IL-1β, IL-6, and TNFα. The cytokines produced by Th1 cells promote the production of IgG1 opsonising and complement fixing antibodies, macrophage activation, and antibody dependent cell mediated cytotoxicity. T

cells which have differentiated into a Th2 phenotype produce cytokines such as IL-4, IL-5, IL-13 and IL-10 and provide optimal help for humoral immune responses including IgG4 and IgE switching (see chapter 3, section 3.1.1.2.2 for more details), mucosal immunity and IgA synthesis. Th2 cells are associated with strong antibody and allergic responses.

The kinetics of primary and secondary immune responses are discussed in chapter 3, section 3.1.1.4. Naïve T cells traffic through peripheral lymphoid organs such as lymph nodes, where they encounter antigen expressing mature dendritic cells. When a T cell encounters its specific antigen it becomes activated via TCR signalling and co-stimulation provided by membrane bound molecules. Activated T cells undergo clonal expansion and differentiation into effector cells. Upregulation of appropriate homing receptors allows the effector cells to migrate out of the lymph node and back into the blood stream, subsequently trafficking to the site of antigen challenge. Following the acute phase of the immune response, the majority of the vastly expanded population of effector T cells will apoptose; the remainder will become memory cells. Memory T cells are divided into effector memory (T_{EM}) and central memory (T_{CM}). T_{EM} are capable of immediate effector function upon re-exposure to antigen and are found in peripheral tissues particularly epithelia of skin and gut, whilst T_{CM} proliferate to give rise to more effector cells and recirculate through secondary lymphoid organs. Thus, these two subsets are distinguished by their migratory capacity, state of differentiation and ability to proliferate.

4.1.2 The Role of Adaptive Cell Mediated Immunity in Varicella and Herpes Zoster:

VZV-specific T cells are believed to be important in the control of viral replication, both during primary infection and in the maintenance of latency (Asano *et al.,* 1985a; Arvin *et al.,* 1986b; Park *et al.,* 2004; Saibara *et al.,* 1993). Several studies have documented that proliferative T cell responses,

(unlike the early production of VZV-specific IgM and IgG antibodies) correlate inversely with the severity of clinical varicella infection (Arvin *et al.,* 1986b; Asano *et al.,* 1985a; Patel *et al.,* 1979; Gershon and Steinberg, 1979; Malavige *et al.,* 2008b), and a study of healthy and immunocompromised patients with primary infection found that milder illness was associated with the detection of T cell proliferation *ex vivo* to VZV antigen within three days after the appearance of exanthem (Arvin *et al.,* 1986b). The rapid host response to primary VZV infection is also associated with rapid termination of viraemia in healthy subjects (Arvin *et al.,* 1986b).

With zoster, immunosenescence of the cell mediated immune (CMI) response appears to be more important than a waning antibody response in preventing virus reactivation, as evidenced by studies investigating the increased risk of herpes zoster with ageing (Guess *et al.,* 1985; Opstelten *et al.,* 2002; Thomas and Hall 2004). Numerous studies that have investigated the potential decline in immunity to VZV in the elderly have found no reduction in VZV-specific antibody titres in elderly subjects compared to young adult controls (Burke *et al.,* 1982; Hayward and Herberger, 1987; Miller, 1980; Berger *et al.,* 1981). In contrast, the incidence and severity of herpes zoster and postherpetic neuralgia among older adults are closely linked to a progressive age-related decline in T cell responses (Burgoon *et al.,* 1957; Ragozzino *et al.,* 1982; Galil *et al.,* 1997; Wilson *et al.,* 1992; Buchbinder *et al.,* 1992; Miller, 1980; Berger *et al.,* 1981; Burke *et al.,* 1982; Levin *et al.,* 1992).

Strong associations have been observed between the occurrence of herpes zoster and low $CD4^+$ T cell count in HIV positive children (Von Seidlein *et al.,* 1996). Similarly, in breast cancer patients, an inverse correlation between VZV re-activation and the total number of T cells transferred with the stem cell graft was observed (Zambelli *et al.* 2002). High incidence of herpes zoster in patients with systemic lupus erythematosus (SLE) has been shown to be associated with a lower frequency of VZV specific, IFN-γ^+ $CD4^+$ memory T

cells (Park *et al.,* 2004), and reduced $CD8^+$ and $CD4^+$ T cell counts have been shown to be significantly associated with the onset of zoster in patients having undergone autologous peripheral blood progenitor cell transplantation (Offidani *et al.,* 2001). In addition, there is no indication that passive antibody prophylaxis reduces the risk of VZV reactivation in high-risk populations, and passive antibody administration does not alter the clinical course of herpes zoster (Stevens and Merigan, 1980; Brunell *et al.,* 1975).

Reactivation of the virus results in VZV-specific T cell proliferation (Ruckdeschel *et al.,* 1977). The number of circulating VZV-specific T cells increases immediately as a consequence of the re-exposure to viral antigens although the response is decreased in immunocompromised compared to otherwise healthy individuals with zoster (Hayward *et al.,* 1991b). Enhanced cell mediated immunity persists for a prolonged period after reactivation and may explain why second episodes of shingles are very rare (Arvin, 1996a). Boosting of VZV-specific $CD4^+$ T cells has been observed in immune adults after re-exposure via household contact with children experiencing varicella, (Vossen *et al.,* 2004), and zoster cases are lower in those frequently exposed to varicella (Thomas *et al.,* 2002).

4.1.3 Variation in T Cell Responses and Factors that Affect T Cell Responses in Individuals:

4.1.3.1 Age:

Notable differences in VZV-specific T cell responses occur between children and adults in response to varicella vaccination. One year after vaccination, adults who received two doses had significantly lower VZV-specific T cell responses than children who had received just one dose of vaccine, (Nader *et al.,* 1995). As mentioned above, the incidence and severity of herpes zoster also increase with age (and thus the subsequent immunosenescence of the CMI response); more than half of all persons in whom herpes zoster develops are older than 60 years (Hope-Simpson, 1965; Levin *et al.,* 2003b).

4.1.3.2 Gender:

Some epidemiological studies have noted gender differences in the incidence of zoster. Recently, Fleming (*et al.,* 2004) evaluated 14,000 cases of zoster over eight years and found a consistent female excess of 28% in the rate of zoster. A separate, smaller study reported a female prevalence among an elderly population of 105 patients with zoster admitted to a study hospital (Torrens *et al.* 1998). However two separate studies which examined zoster in 1,075 and 957 cases did not identify gender differences in the incidence of zoster (Donahue *et al.,* 1995; Oxman *et al.,* 2005). Given the importance of memory T cells in zoster development, a recent study compared the frequencies of VZV-specific memory T cells between 17 males and eight females (Klein *et al.,* 2006). Although asymptomatic adults typically had minimal fluctuations in their VZV-specific memory T cells over the course of a year, the $CD4^+$ T cells responses differed over time between men and women. The results suggest the possibility that males maintain higher levels of VZV-specific memory T cells than women, even though women exhibited episodes of detectable response during the course of the study. The authors did acknowledge that the differences observed here could in part, be due to the smaller sample size for women. However, gender differences in the cytokine responses of memory T cells have been noted for other herpes viruses; memory T cells from women have been found to secrete higher levels of IFN-γ and IL-2 in response to both HCMV and HSV as compared to men, which might be expected to be associated with higher responder cell frequency (RCF) against these viruses and subsequently lower rates of reactivation in women (Villacres *et al.,* 2004).

4.1.3.3 Immunosuppression:

Suppression of the immune system can be caused by multiple factors such as anti-cancer therapy, immunosuppressive drug treatment required for organ and bone marrow transplantation, and diseases such as lymphoma and human immunodeficiency virus (HIV) infection. The incidence and severity of

varicella along with the frequency of complications in immunocompromised patients with suppressed CMI is higher compared with varicella in healthy subjects (Rowland *et al.*, 1995; Rogers *et al.*, 1995; Grant *et al.*, 2002, McCluggage *et al.*, 1994; Kim and Haycox, 1999). In contrast no evidence exists of more severe varicella in those with abnormal humoral immunity (Locksley *et al*, 1985). Failure of cellular responses to halt the spread of VZV, which can often occur in immunocompromised patients, can result in VZV visceral dissemination to the lungs, liver, kidneys, and spleen and in progressive life-threatening infection (Rowland *et al.,* 1995; Rogers *et al.,* 1995; Grant *et al.,* 2002).

After cardiac transplantation, herpes zoster infections are more frequent than in healthy subjects and depressed or absent cellular responses to VZV parallel with that susceptibility (Rand *et al.,* 1977). These findings were supported by a later study, in which, immune responses and infections with herpes viruses were examined in 36 cardiac transplant recipients. Herpes zoster occurred in 22% of patients during the first year after transplantation, and in those cases, VZV-specific T cell responses returned thereafter (Pollard *et al.,* 1982). Several studies have demonstrated that intensive anti-cancer therapy using bone marrow transplantation (BMT) puts patients at increased risk for zoster, increasing both morbidity and mortality of the disease. Reports indicate that 14-41% of BMT recipients developed zoster (or subclincal VZV-viraemia), within a year of transplantation (Schuchter *et al.*, 1989; Locksley *et al.*, 1985; Koc *et al.*, 2000; Hogewoning *et al.*, 2001; Wilson *et al.,* 1992). One of these studies found that BMT recipients had significantly fewer circulating cytotoxic T cells that recognized VZV IE62 or gE than healthy VZV immune controls (Wilson *et al.,* 1992).

Herpes zoster is also a frequent complication of lymphoreticular malignancy. In a study of normal subjects with recent and remote VZV infection and patients with lymphoma, T cell responses were measured before treatment

and during long-term remission and then compared with those of normal subjects (Arvin *et al.,* 1978b). Despite levels of antibody to VZV that were equivalent to those in normal subjects, 44% of the untreated lymphoma patients showed a lower T cell transformation response to VZV antigen, than the normal patients. Twenty-two percent of lymphoma patients in long-term remission continued to have diminished cellular immune responses to VZV antigen. Observations in these patient populations and in normal subjects with acute herpes zoster suggest that deficiencies in lymphocyte responses seen *in vitro* may correlate with increased susceptibility to clinical infection with VZV (Arvin *et al.,* 1978b).

VZV causes severe and/or recurrent varicella or herpes zoster in untreated HIV-infected patients (Glesby *et al.,* 1993; Engels *et al.,* 1999). A longitudinal study of VZV infections in HIV-infected children found that in 70% of patients who had low $CD4^+$ T cells counts at the time of varicella infection went on to develop zoster during the course of the study (Gershon *et al.,* 1997). The approximate incidence of zoster in HIV-infected individuals, compared to uninfected individuals, is approximately ten times greater (Buchbinder *et al*, 1992; Veenstra *et al*, 1995; Morgan *et al.,* 2001), and zoster has been found to be a strong predictor of HIV-1 infection in children and young adults in Sub-Saharan Africa (Lindan *et al.*, 1992; Tyndall *et al.*, 1995; Colebunders *et al.*, 1988; Naburi and Leppard, 2000). In addition several studies have found the incidence of herpes zoster increases as $CD4^+$ T cell counts decrease and HIV induced immunosuppression progresses, (Veenstra *et al.*, 1995; Engels *et al.*, 1999).

4.1.3.4 Genetic Factors:

Evidence for a heritable contribution to varicella zoster virus disease comes from a number of sources, although the role of T cells in these studies was not examined *per se*. In age and sex matched individuals, zoster is four times more common in Caucasian than in African American subjects (Schmader *et*

al., 1995), and these findings were supported by later work by the same authors when studying multiple elderly cohorts in the same area (Schmader *et al.,* 1998a; Schmader *et al.,* 1998b). In a retrospective study it was found that black people were at less than half the risk of zoster than Caucasian people after adjusting for age and sex, (Thomas and Hall, 2004). However a recent report of individuals with incident zoster participating in antiviral trials showed that being non-Caucasian and resident in tropical countries were independently associated with significantly younger age at zoster, although this tendency could simply indicate participation bias or undiagnosed HIV infection (Nagasako *et al.,* 2003).

Reports of racial differences in HLA genes and susceptibility to infectious diseases such as malaria, tuberculosis and herpes labialis (Tiwari and Terasaki, 1981; Hill *et al.,* 1991, Stead *et al.,* 1990, Embil *et al.,* 1975) may provide indirect evidence consistent with racial differences in the VZV-specific immune response and therefore subsequent development of zoster (Schmader *et al.,* 1998a). However studies which have directly examined VZV infection and HLA prevalence have been conflicting. An early population-based study of herpes virus infection and HLA phenotype, conducted during the late 1970s revealed that HLA-Bw16 was found less often and HLA-Cw2 more often, in individuals with histories of herpes labialis than in other individuals. However, no strong association was found between HLA and a history of herpes zoster or to HSV-2, or HCMV (Blackwelder *et al.,* 1982). Similarly, a study carried out in a large and diverse group of individuals to examine the relationship between HLA-A, HLA–B and antibodies to a wide range of viruses, including the measles virus and VZV revealed that although there was a relationship between the presence of the HLA-B15 allele and a lack of circulating measles antibodies, there was no association between significantly elevated titres to HCMV and VZV and the presence or absence of any particular HLA-A or -B antigen (Cuthbertson *et al.,* 1982).

An association between HLA and PHN has been suggested. Results from a Japanese study on 32 patients with PHN (and 136 healthy controls) found a positive association in the susceptibility to VZV and of the development of PHN with the HLA class I antigens HLA-A33 and -B44, (the HLA-A33-B44 haplotype), (Ozawa *et al.,* 1999). Following on from the work of Ozawa, Sato (*et al*., 2002c) found that in addition to HLA-A*3303 and HLA -B*4403, that the HLA-DRB1*1302 allele was also significantly associated with PHN. No significant association between PHN and HLA class II alleles (Ozawa *et al*., 1999), or between genes in the HLA class III region; (tumour necrosis factor alpha (TNFA) promoter, and polymorphisms in the natural killer cell activating receptor, NKp30) were demonstrated in these studies (Sato *et al.,* 2002c).

4.1.3.5 Relative Antigen Content of Varicella Vaccine:

The representative viral titre in a single dose of the commercially available varicella vaccines is a minimum of 1,350 pfus for VARIVAX® and a minimum of 1,995 pfus for VARILRIX™. As well as live replicating vaccine virus, the vaccine preparations also contain incomplete virus particles and dead virus, both of which can stimulate an immune response. Studies have been carried out to examine the immunogenicity of vaccine preparations with varying ratios of live virus and total antigen content, (Watson *et al.,* 1993; Sperber *et al.,* 1992; Rothstein *et al.,* 1997 and Bergen *et al.,* 1990), but the majority of these studies focused on serological responses. In one study of healthy children, when plaque forming unit (pfu) content was maintained at 1000 pfu, but the relative antigen content was reduced from 96 to 87%, the frequency and strength of cell mediated immune responses were drastically reduced, although antibody titres were equivalent, (Bergen *et al.,* 1990).

4.1.4 Nature of the T Cell Response to VZV:

Both $CD8^+$ and $CD4^+$ T cells are primed during primary VZV infection, but in contrast to classic antiviral T cell responses, (including to other herpes viruses such as EBV and CMV (Callan *et al.,* 1998; Maini *et al.,* 2000;

Sylwester *et al.,* 2005), the response to wild-type and vaccine VZV appears to be $CD4^+$ rather than $CD8^+$ dominated (Hayward, 1990; Hayward *et al.,* 1992; Huang *et al.,* 1992; Sharp *et al.,* 1992; Vossen *et al.,* 2004; Jones *et al.,* 2006b; Jones *et al.,* 2007; Malavige *et al.,* 2007; Malavige *et al.,* 2008a; Milikan *et al.,* 2006; Milikan *et al.,* 2007; Milikan *et al.,* 2009). VZV-specific $CD4^+$ and $CD8^+$ T cell responses are discussed in more detail in sections 4.1.4.1 and 4.1.4.2. Memory T cells specific for VZV have been found at lower frequencies, compared with those of other herpes viruses, such as HCMV, HSV, and EBV, in healthy immune individuals (Asanuma *et al.,* 2000; Khan *et al.,* 2004). Recent *ex vivo* analysis of IE63 and gE -specific $CD4^+$ T cells from naturally immune adults (with no history of reactivation), demonstrated evidence of recent cell activation and a mixed central and effector memory differentiation phenotype (Jones *et al.,* 2007; Malavige *et al.,* 2008a).

VZV-specific $CD4^+$ T cells that are induced during primary infection are predominantly of the Th1 phenotype and produce high levels of IFN-γ and TNF-α, (Zhang *et al.,* 1994; Zhang *et al.,* 1995; Milikan *et al.,* 2007; Jenkins *et al.,* 1998a; Asanuma *et al.,* 2000). Such T cell clones have been shown to lyse infected cells *in vitro* (Hayward *et al.,* 1989; Hayward *et al.,* 1986b; Huang *et al.,* 1992; Sharp *et al.,* 1992; Milikan *et al.,* 2007). T cell antigen specificity has been addressed in a number of studies which have documented reactivity to several VZV proteins in healthy naturally immune and vaccinated adults, including the regulatory and structural proteins encoded by ORF4, ORF10, ORF62, and ORF63 and glycoproteins gB, gC, gE, gI and gH (for references and summary see table 4.1).

Gene	Function	Reference
4	Transcriptional activator, Tegument protein	Arvin *et al.,* 2002; Jones *et al.,* 2006b; Milikan *et al.,* 2007[$]
10	Transcriptional activator, Tegument protein	Arvin *et al.,* 2002; Milikan *et al.,* 2007[$]
14	Glycoprotein C	Sharp *et al.,* 1992; Lowry *et al.,* 1992*; Milikan *et al.,* 2007[$]
18	Ribonucleotide reductase (small subunit)	Milikan *et al.,* 2007[$]
29	Single stranded DNA binding protein	Arvin *et al.,* 2002; Milikan *et al.,* 2007[$]
31	Glycoprotein B	Giller *et al.,* 1989b; Hayward, 1990; Watson *et al.*, 1990; Milikan *et al.,* 2007[$]
33	Assembly protein	Welsh *et al.,* 1999; Garcia-Valcarcel *et al.,* 1997b*
37	Glycoprotein H	Arvin *et al.,* 1986a; Giller *et al.,* 1989b; Watson *et al.*, 1990
61	Transcriptional regulator	Milikan *et al.,* 2007[$]
62	Transcriptional activator, Tegument protein	Arvin *et al.,* 1986a; Arvin *et al.,* 1991; Frey *et al.,* 2003; van der Heiden *et al.,* 2009; Arvin *et al.,* 2002; Sharp *et al.,* 1992; Lowry *et al.,* 1992;* Sabella *et al.,* 1993*`; Milikan *et al.,* 2007[$]; Arvin *et al.,* 1987*
63	Transcriptional activator, Tegument protein,	Jones *et al.,* 2007; Milikan *et al.,* 2007[$] Sadzot-Delvaux *et al.,* 1997
67	Glycoprotein I	Malavige *et al.,* 2007; Hayward, 1990; Sharp *et al.,* 1992; Lowry *et al.,* 1992*; Huang *et al.,* 1992; Milikan *et al.,* 2007[$]
68	Glycoprotein E	Malavige *et al.,* 2008a; Arvin *et al.,* 1986a; Arvin *et al.,* 1991; Giller *et al.,* 1989b; Sharp *et al.,* 1992; Lowry *et al.,* 1992*; Huang *et al.,* 1992; Welsh *et al.,* 1999; Watson *et al.*, 1990; Milikan *et al.,* 2007[$] Arvin *et al.,* 1987*; Garcia- Valcarcel *et al.,* 1997a*

Table 4.1 VZV Proteins which are Targets of T Cell Immune Responses. Key: *Study carried out in Guinea pigs or mice (all other studies were carried out using human PBMCs, (or T cells recovered from intraocular fluid[$]).

4.1.4.1 The Role of $CD8^+$ T Cells in VZV Infection:

Several studies have demonstrated that MHC-I restricted $CD8^+$ T cells were capable of responding to VZV antigens (expressed by VZV-infected fibroblasts and lymphoblastoid cells), (Hickling *et al.,* 1987; Arvin *et al.,* 1991; Sadzot-Delvaux *et al.,* 1997; Milikan *et al.,* 2007). $CD8^+$ T cells have been shown to play a role in the response gE as well as IE62 and IE63, and in one

study, quantitive analysis of VZV cytotoxicity by limiting dilution assays demonstrated that the numbers of circulating virus-specific $CD8^+$ and $CD4^+$ CTL precursors were equivalent in naturally immune subjects at least 20 years after infection (Arvin, *et al.,* 1991). However in another study, VZV-specific $CD8^+$ T cells were shown to be present in study subjects PBMCs at relatively low frequencies after primary infection, but at much higher frequencies in zoster patients (Hickling *et al.,* 1987).

More recently, a study from the Arvin laboratory supported the idea that virus-specific $CD8^+$ T cells may circulate at a relatively low frequency after primary infection, as IE62 peptide-specific memory $CD8^+$ T cells were detected only after *in vitro* expansion (Frey *et al.,* 2003). There has been very little work on mapping $CD8^+$ T cells epitopes to date; one study has identified HLA-A*02 restricted IE62 epitopes in naturally immune subjects, (see table 4.2). In brief, synthetic 12-mer overlapping peptides were produced to correspond to the complete IE62 protein. Single IE62 peptides were combined to prepare peptide pools (PPs). The study found that after secondary *in vitro* stimulation, two peptides within a particular pool induced a response in latently infected subjects. Furthermore, upon booster vaccination in three individuals, increased $CD8^+$ T cell frequencies specific for those peptides resulted, (Frey *et al.,* 2003).

Recently, another HLA-A2 restricted IE62 epitope has been mapped (van der Heiden *et al.,* 2009), see table 4.2 for details. This was carried out via the use of HLA-A2 pentamers and *ex vivo* staining of PBMCs from T cell depleted allogeneic stem cell transplantation patients experiencing VZV reactivation. The epitope was recognized by $CD8^+$ T cells in 42% of patients *ex vivo*, and in a total of 63% (n=12) patients after *in vitro* expansion.

Protein	aa number	Sequence	HLA Restriction if Known	Reference
IE62	445-453	SLPRSRTPI	HLA*0201	Frey *et al.,* 2003
IE62	472-480	SAPLPSNRV	HLA*0201	Frey *et al.,* 2003
IE62	593-601	ALWALPHAA	HLA*0201	van der Heiden *et al.,* 2009

Table 4.2: HLA-A*0201 Restricted $CD8^+$T Cell IE62 Epitopes.

4.1.4.2 The Role of $CD4^+$ T Cells in VZV Infection:

VZV-specific $CD4^+$ T cells synthesize Th1-like cytokines, such as interleukin-2 (IL-2) and interferon-γ (IFN-γ) (Jenkins *et al.,* 1998a; Zhang *et al.,* 1994), and some are capable of MHC-II-restricted cytotoxicity *in vitro* (Arvin *et al.,* 1991; Cooper *et al.,* 1988; Diaz *et al.,* 1989; Hayward *et al.,* 1989; Hayward, 1990). EBV transformed human B cells expressing cell surface VZV antigens have been shown to be lysed by HLA-DR3 restricted $CD4^+$ T cells *in vitro* (Hayward *et al.,* 1986b). This added support to an earlier finding by Pontesilli (*et al.,* 1987) that most T cells responding to VZV antigens in culture were $CD4^+$ T cells. In addition, a study that was the first to document the infiltration of VZV-specific T-cells in VZV-induced ocular lesions, found that $CD4^+$ T cells predominated over $CD8^+$ T cells (Milikan *et al.,* 2006). Later work by the same authors (Milikan *et al.,* 2009) indicated that human retinal pigment epithelial cells may play a role as the retina-resident antigen-presenting cells in the $CD4^+$ T cell–mediated inflammatory response of VZV-induced uveitis.

4.1.4.3 Targets of $CD4^+$ T Cells

4.1.4.3.1 Glycoproteins:

The report by Arvin (*et al.,* 1986a) demonstrated that $CD4^+$ T cells in healthy subjects with a diverse HLA background (with naturally acquired VZV immunity), could respond to purified gE and gH proteins. In a similar study by Giller (*et al.,* 1989b) it was found that purified gH protein did not induce T cell responses of the same magnitude to those seen for gE and gB. Giller's study also revealed for the first time that gB was a potent inducer of T cell immunity,

and in the case of acute infection of one adult during the study, gB was the predominant T cell inducer. Hayward (1990) demonstrated that $CD4^+$ T cells can recognize peptides of gB (and gI; discussed later), but only mount weak proliferative responses (see table 4.3). $CD4^+$ cytotoxic T cells from both naturally immune and vaccinated healthy adults have been shown to lyse autologous lymphoblastoid cell lines infected with vaccinia virus expressing either recombinant gC, gE or gI (Sharp *et al.,* 1992).

Diaz (*et al.,* 1989) also showed that $CD4^+$ T cells lysed VZV-infected targets after *in vitro* expansion with gE antigen, and these cells were found to circulate in individuals with naturally acquired immunity to VZV. In a second study examining $CD4^+$ T cell response to gE peptides, T cells from the majority of healthy immune donors recognised carboxy-terminal residues that constitute the transmembrane (and thus intracellular anchor segments) of the protein. The peptides were recognised from donors with very different MHC-II backgrounds. Indeed, two donors with distinct HLA-DR, -DQ, and –DP phenotypes responded to all of the gE peptides, although only the HLA-DR alleles were published (DRB1 and DRB5 for one individual, and DRB9 and DRB6 for the other), (Bergen *et al.,* 1991). More recently three gE epitopes were mapped using either PBMCs from naturally immune donors and *ex vivo* and cultured IFN-γ ELISPOT analysis against successive peptides (Malavige *et al.,* 2008a), whilst another was mapped by use of T cell lines derived from intraocular fluid samples from patients with VZV-uveitis (Milikan *et al.,* 2007). In both cases, HLA restriction was deduced using HLA-specific antibodies followed by peptide pulsing of various APC lines of known HLA type. See figure 4.1, for more details of identified epitopes.

Huang (*et al.,* 1992), were the first to look at the role of $CD4^+$ T cells in gI recognition. Cold target inhibition studies were used to map gI epitopes (see figure 4.2), and identified an epitope that lies between position 212-354, and that residues 245-259 are the likely candidate peptide for the gI epitope. More

recently a study by Malavige (*et al.,* 2007) confirmed that gI is a $CD4^+$ T cell target, and demonstrated that VZV gI-specific rapid effector functional immune responses are observed in all healthy VZV-seropositive individuals with a history of VZV infection. An epitope restricted by HLA-DRB4*01 was mapped in this study (see figure 4.2). Glycoprotein I was shown to account for 9–56.5% (mean, 23.2%) of the total $CD4^+$ $IFN\gamma^+$ responses to VZV lysate *ex vivo* (Malavige *et al.,* 2007).

Further data from Huang (*et al.,* 1992) generated using MHC-II restricted VZV-specific T cell clones from four naturally immune donors, demonstrated that 30 out of a total of 68 clones examined were able to lyse gE expressing target cells. Of these 30 clones, 50% were also able to lyse gI expressing target cells, demonstrating that gI and gE share a $CD4^+$ T cell epitope. The study also found that gE-specific $CD4^+$ cytotoxic T cells outnumber gI-specific $CD4^+$ T cells circulating in the peripheral blood of naturally immune donors.

4.1.4.3.2 Tegument Proteins:

IE62 is one of the key targets of T cell immunity. The report by Arvin (*et al.,* 1986) demonstrated that T cells (in healthy subjects with a diverse HLA background and who had naturally acquired VZV immunity), could respond to the IE62 protein. A later study by Bergen (*et al.,* 1991) revealed that IE62 could elicit potent $CD4^+$ T cell responses to a wide variety of peptides (see figure 4.3), which span the length of the protein. Residues from the amino terminal half of the IE62 protein tended to be more immunogenic, and were reactive in more donors, who were from a diverse genetic background. Using the peptides identified as immunogenic by Bergen, Jenkins (*et al.,* 1998a and Jenkins *et al.,* 1999) demonstrated that these epitopes could induce $CD4^+$T cell responses in naïve donors when presented using autologous DCs. More recently two epitopes were mapped by use of T cell lines derived from intraocular fluid samples from patients with VZV-uveitis (Milikan *et al.,* 2007),

with one of these epitopes being recognised by both HLA DRB1*1404 and HLA DRB1*0703 restricted T cell lines (Milikan *et al.,* 2007).

Studies carried out in naturally immune adult donors demonstrated that IE63 is highly immunogenic and elicits a long-term immune response (Sadzot-Delvaux *et al.,* 1997). In this study, $CD4^+$ Th1 cytotoxic T cells were shown to respond to IE63 at frequencies which are equivalent of those seen for IE62 (Sadzot-Delvaux *et al.,* 1997). More recently, high frequencies of IE63 protein-specific T cells were detected *ex vivo* in naturally immune donors and a DRB1*1501 restricted $CD4^+$ T cell epitope was mapped (Jones *et al.,* 2007), see table 4.3.

High frequencies of ORF4 protein-specific T cells have also been detected *ex vivo* in individuals up to 52 years after primary infection and identified immunogenic regions of the ORF4 protein, including a commonly recognized epitope (see table 4.3) which was restricted through HLA-DRB1*07 (Jones *et al.,* 2006b). Like the IE63 epitope, the ORF4 epitope was processed and presented by keratinocyte cell lines after stimulation with infected cell lysate or the live VZV vaccine. The total ORF4 protein responses comprised approximately 20% of the total lysate or vaccine stimulated responses, suggesting that the ORF4 protein is an immunodominant target antigen. This raises the possibility that such T cells have a role in the control of viral reactivation. In a study of healthy immune donors, mean RCFs (75% $CD4^+$ T cells, 10% $CD8^+$ T cells) to ORF10 protein were found to be equivalent to those invoked by IE62 (Arvin *et al.,* 2002).

4.1.4.3.3 Other Targets:

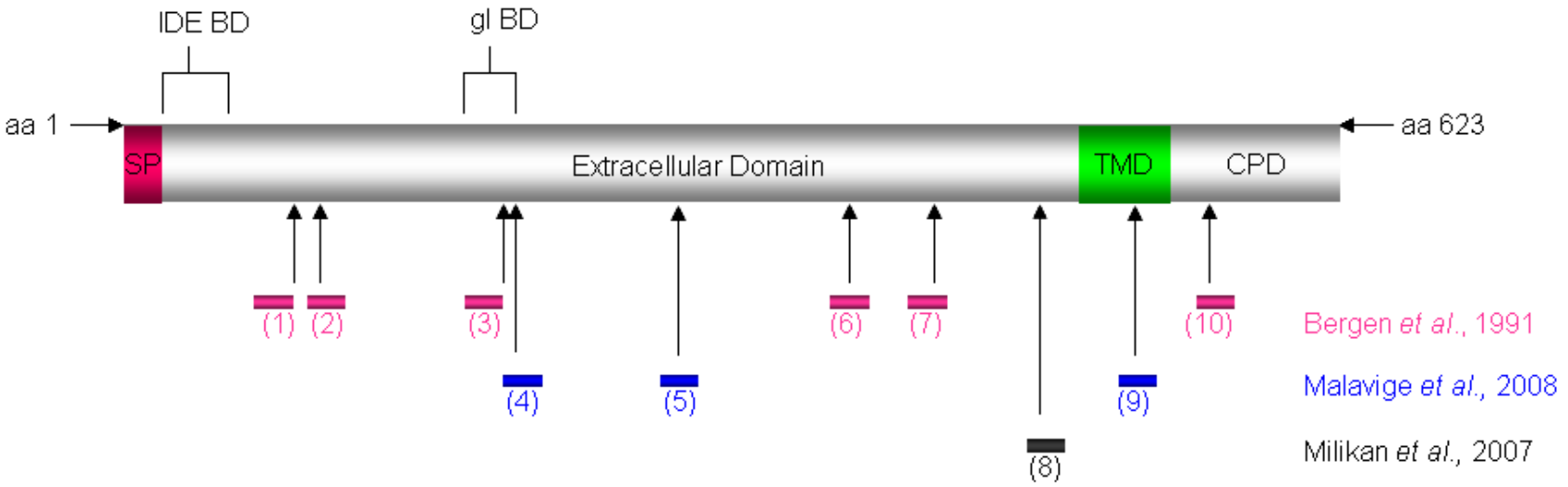
In the same study as mentioned previously, the mean RCF to ORF29 protein was found to be lower than those of IE62, ORF4 and ORF10 (Arvin *et al.,* 2002). In a separate study in guinea pigs, immunization with the ORF29 protein did not prime animals for an enhanced T cell response upon

challenge with infectious virus. Thus in this study the ORF29 gene product did not elicit protection (Sabella *et al.,* 1993).

A study in mice found that three out of four inbred strains of mice immunised with (ORF33) AP-VLPs produced a VZV-specific T cell response (Garcia-Valcarcel *et al.,* 1997b). A later study of varicella and zoster patients using lymphocyte proliferation assays (LPA) against VLPs demonstrated that a VZV-specific lymphocyte response to the assembly protein (AP) was measurable in some patients (Welsh *et al.,* 1999), but in comparison to the response induced by gE fragments (which were recognised by 85% of varicella and 75% of zoster patients in this study), AP could not be considered a dominant T cell antigen (Welsh *et al.,* 1999).

Glycoprotein/ Protein	aa number	Sequence	HLA Restriction (if Known)	Reference
gB	139 -149	EITDTIDKFGK		Hayward, 1990
gB	769 -779	LPEGMDPFAEK		Hayward, 1990
IE4	251 - 270	MLYGHEL<u>Y</u>RTFESYK<u>M</u>DSRI	HLA-DRB1*07	Jones *et al.,* 2006b
IE63	229 -243	QRA<u>I</u>ERYAGAETA<u>E</u>Y	DRB1*1501	Jones *et al.,* 2007

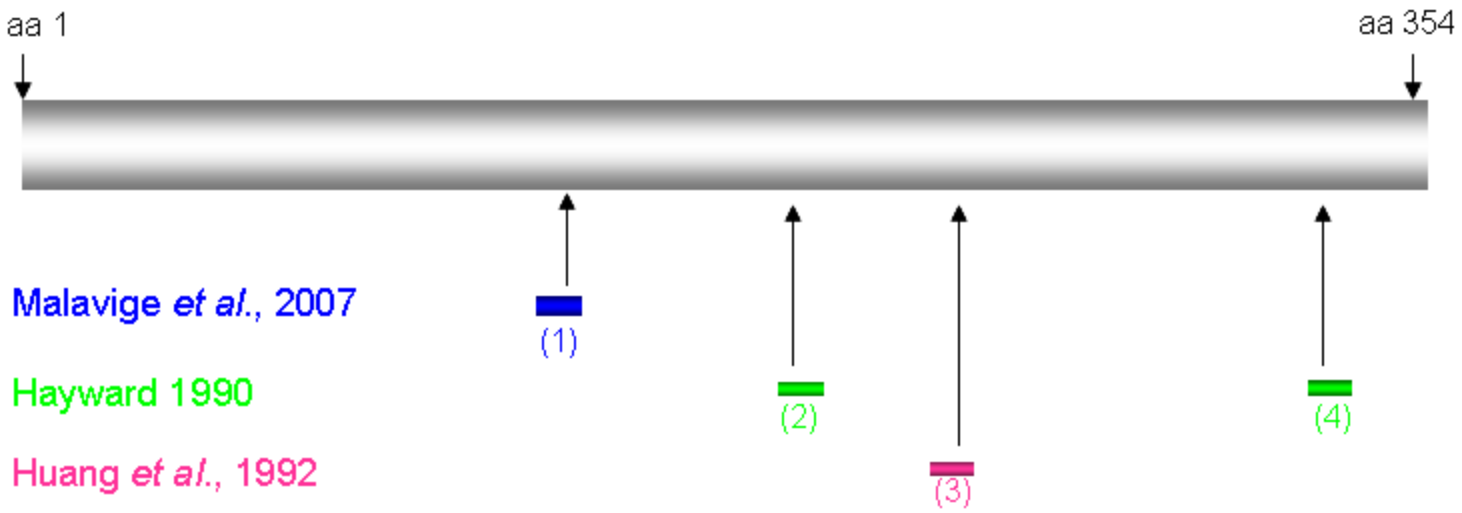
Table 4.3: Glycoprotein B, ORF4 and IE63 $CD4^+$ T Cell Epitopes Identified in Naturally Immune Donors. IE4 and IE63 epitopes were identified *ex vivo*, (underlined amino acids are critical for presentation); gB epitopes were identified using proliferation and cytotoxicity assays.



Epitope Number	aa Numbers	Sequence	HLA Restriction if Known
1	87-101	DYDGFLENAHEHHGV	
2	103-119	NQGRGIDSGERLMQPTQ	
3	181-194	PFTLRAPIQRIYGV	
4	193-206	GVRYTETWSFLPSL	DRB1*07
5	281-300	IEPGVLKVLRTEKQYLGVYI	DRB4*01

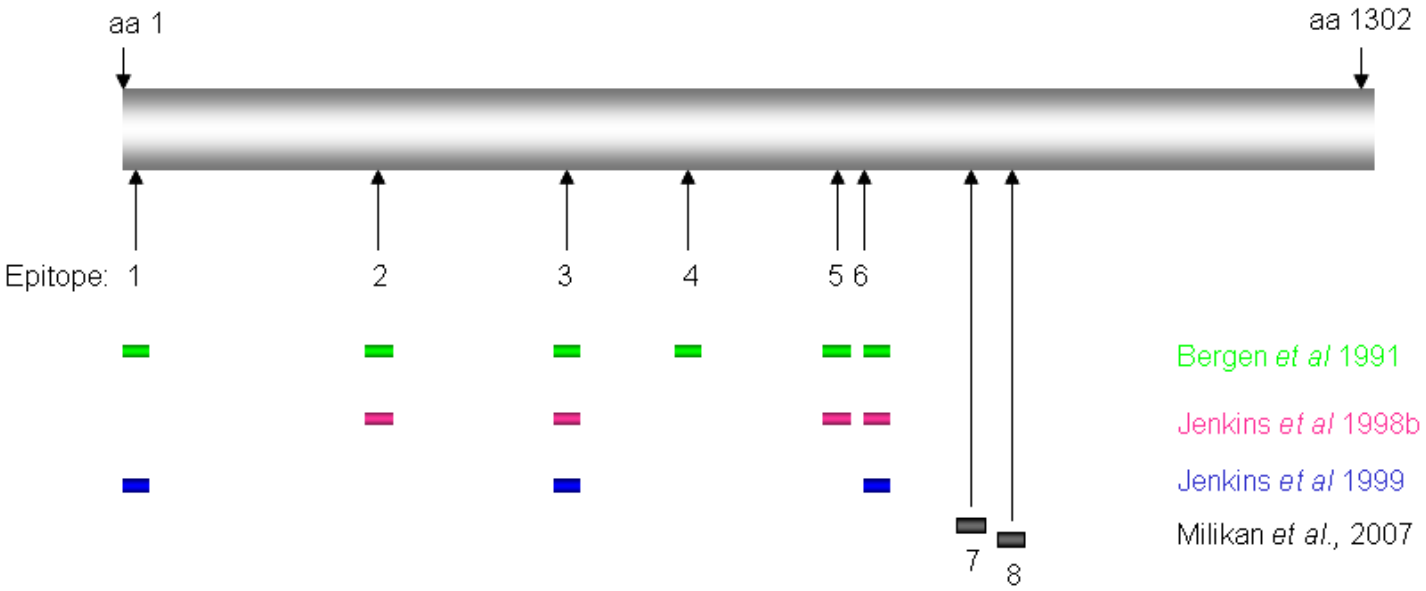
Epitope Number	aa Numbers	Sequence	HLA Restriction if Known
6	370-381	APFDLLLEWLYV	
7	420-440	LAQRVASTVYQNCEHADNYTA	
8	474-489	LYVFVVYFNGHVEAV	DRB1*1501
9	532-546	TSPLLRYAAWTGGLA	DRB1*1501
10	562-574	KRMRVKAYRVDKS	

Figure 4.1: Glycoprotein E $CD4^+$ T Cell Epitopes and their HLA Restriction, Identified in Naturally Immune, Adult Donors. Epitopes 1-3, 6, 7 and 10 were recognised by $CD4^+$ T cells from 67-92% of donors in lymphocyte proliferation assays. Epitopes 4, 5 and 9 were identified *ex vivo* and following *in vitro* stimulation. Epitope 8 was mapped using T cell clones from patients with VZV-induced uveitis; HLA restrictions are noted above. Key: IDE BD, Insulin Degrading Enzyme Binding Domain (aa 24-71); gI BD, gI Binding Domain (aa 163-208); SP, Signal Peptide; TMD, Transmembrane Domain; CPD, Cytoplasmic Domain.



Epitope Number	aa Numbers	Sequence
1	144-155	YVLLVRLDHSRS
2	197-207	ARLCDLPATPK
3	245-249	VKEGIENHVYPTDMS
4	334-344	AQLATIREESP

Figure 4.2: Glycoprotein I $CD4^+$ T Cell Epitopes in Naturally Immune Adult Donors.
Epitopes 2 and 4 were identified as weakly immunogenic (Hayward, 1990); Huang (*et al.,* 1992) suggest that the shared epitope between gE and gI lies between 212-354, and predict the epitope (3) to be at position 245-259. An optimum HLA DRB4*01 restricted epitope (1) was identified *ex vivo* and following *in vitro* stimulation.



Epitope Number	aa Numbers	Sequence	HLA Restriction If Known
1	18-28	DTLELMDLLDA	
2	266-281	VPGPVEQLYHVLSDSV	
3	519-530	LGDPARQYRALI	
4	620-631	QSLRRAFASMAY	

Epitope Number	aa Numbers	Sequence	HLA Restriction If Known
5	750-764	GGFRRIPRGALHTPV	
6	774-786	CTPETIARLVDDP	
7	911-930	ALNARGVLLLSTRDLAFAGA	DRB1*1404 DRB1*0703
8	941-960	ARRRLLVLDAVALERWPRDG	DQB1*0202

Figure 4.3: IE62 $CD4^+$ T Cell Epitopes Identified in Either Healthy Naturally Immune, or Vaccinated Donors, or in Patients with VZV-Induced Uveitis. Epitopes 1-5 were identified as immunogenic (Bergen *et al.,* 1991) and stimulated $CD4^+$ T cells from 75-83% of naturally immune donors. Epitopes 2, 3 and 5 also induced an immune response in naïve donors whose $CD4^+$ T cells were cultured with peptides and autologous DCs (Jenkins *et al.,* 1998). The authors also found that epitope 6 induced an immune response in 2 out of 3 donors using this methodology, and in a separate study confirmed the importance of epitope 1, 3 and 6 as these stimulated $CD4^+$ T cell responses in 71-100% of naturally immune and vaccinated donors (Jenkins *et al.,* 1999). Epitpes 7 and 8 were identified using T cell clones from patients with VZV-induced uveitis; HLA restriction is noted.

4.1.5 Measuring CMI Responses to Vaccination and Naturally Acquired Infection:

There are various methodologies that can be used to measure CMI responses, dependent on which T cell subset is to be studied. Lymphoproliferation assay (LPA), ELISPOT, Intracellular cytokine flow cytometry (ICFC), MHC tetramer analysis, or ELISA for T cell cytokines are all methodologies which have been used in the studies discussed above. In addition skin testing can also be used to measure CMI (Takahashi *et al.,* 2001; Asano *et al.,* 1981b; LaRussa *et al.,* 1985; Hata, 1980) and has been shown to correlate with IFN-γ ELISPOTs carried out in the same patients (Sadaoka *et al.,* 2008).

In this study both MHC-II tetramer analysis and IFN-γ ELISPOTs were used to measure CMI responses to VOka vaccination. MHC-II (and MHC-I) tetramers facilitate the detection and quantification of epitope specific $CD4^+$ (and $CD8^+$) T cells respectively. Tetramers are comprised of four recombinant, biotinylated, peptide-loaded MHC molecules, bound by a fluorescently labelled streptavidin molecule. Incorporating tetramer staining into multicolour flow cytometric analysis of whole blood or PBMC samples allows for a sophisticated phenotypic analysis of epitope specific T cells (Voller and Stern *et al.,* 2008). IFN-γ ELISPOTs provide a tool to quantitate the number of cells producing IFN-γ in response to an antigen.

4.2. AIMS:

The aim of this chapter was to measure cell mediated immune (CMI) responses to varicella (Oka) vaccination and to determine the relationship between CMI, antibody responses and protection from varicella following varicella (Oka) vaccination.

4.3 RESULTS:

In this study of vaccinated HCWs, T cell responses were measured *ex vivo* using either a commercial, partially purified, whole VZV lysate as a stimulus in overnight IFN-γ ELISPOT assays, or (in study participants expressing HLA-DRB1*1501) by use of HLA-DRB1*1501 tetramers complexed with either gE or IE63 epitopes (see chapter 2, section 2.5.3.1 for details). In some cases, where a second aliquot was available, samples which had tetramer-specific T cells *ex vivo*, were cultured with the corresponding peptide for 10 days and the proliferation of tetramer-specific cells was examined.

4.3.1 Ex Vivo VZV-Specific IFN-γ ELISPOT Optimization;

The number of PBMCs/well was restricted by the availability of resuscitated live PBMCs. Thus, assays were optimized using cell concentrations of $2x10^5$ PBMCs/well; the lowest concentration of cells previously shown to yield reproducible and consistent results (Smith *et al.,* 2001; Jones *et al.,* 2006). Concentrations of mitogen and lysates were titrated against resuscitated PBMCs from three healthy seropositive donors with a history of varicella or zoster >20 years previously (see figure 4.4). The concentration of VZV lysate that gave the highest SPFs/10^6PBMCs was selected (a final concentration of 10μg/mL/well) and the same concentration of uninfected cell lysate was used as a negative control.

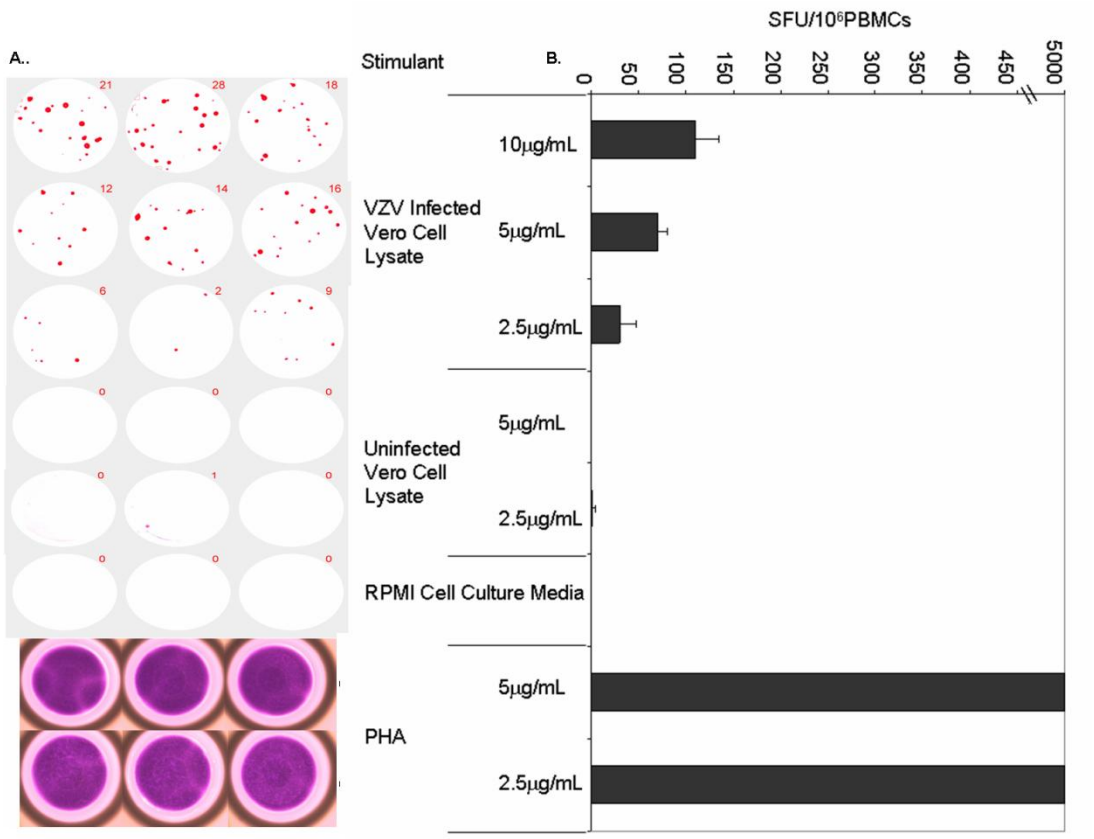


Figure 4.4: Optimisation of IFN-γ ELISPOT Using PBMCs from Naturally Immune Healthy Adults. Key: PHA; phytohaemoglutinin mitogen. PBMCs from healthy seropositive donors ($2x10^5$/well) were stimulated with various concentrations of either whole VZV-infected Vero cell lysate; uninfected Vero cell lysate; PHA mitogen or RPMI media alone. The result is representative of three donors. *Part A:* Processed images (Biosystem 5000 ELISPOT reader) of all wells (apart from those stimulated with PHA, where live images are presented as there were too many SFUs to count; *Part B:* Corresponding bar graph representing the SFU/10^6PBMCs. PHA stimulated wells were given an arbitrary count of 1000 SFU/10^6PBMCs as the magnitude of the response was uncountable. 10μg/mL of VZV lysate generated 112 SFU/10^6PBMCs; (5μg/mL = 70SFU/10^6PBMCs; 2.5μg/mL = 28SFU/10^6 PBMCs).

4.3.1.1 Optimizing the Number of PBMCs Available for ELISPOT Assays After Thawing:

As can be seen from table 4.4, (see chapter 2, section 2.5.1.2 for details), incubating cells at 4^{o}C was optimal for maximizing the number of live PBMCs available. For subsequent ELISPOT assays, cells were incubated on ice whilst the plate was prepared for cell addition.

Temperature	Number of Live PMBCs at 0min	Number of Live PMBCs at 30min	Total Loss of Live PBMCs Due to Cell Aggregation (% of original)
4^{o}C	1.4x10^{6}	1.4x10^{6}	0 (100%)
RT	1.4x10^{6}	8x10^{5}	6x10^{5} (43%)
37^{o}C	1.4x10^{6}	8x10^{5}	6x10^{5} (43%)

Table 4.4: Loss of Live PBMCs Due to Cell Aggregation, after 30 Minutes Incubation at Various Temperatures. PBMCs from a healthy donor were resuscitated, counted and split into three equal volumes and incubated at various temperatures. Cells were recounted after 30min.

4.3.2 *Ex Vivo* VZV-Specific IFN-γ ELISPOT Responses at 18 Months Post Vaccination:

Ex vivo ELISPOT assays were carried out on 18 month follow-up PBMC samples (n=75). The number of live PBMCs recovered was not sufficient to allow corresponding 10 day cultures to be established. Only 33% (25/75) of results were informative with no or low numbers of spot forming units (SFUs) in negative control wells, and high levels of IFN-γ production in response to PHA mitogen. A representative image of a PBMC sample which had responsive PBMCs and produced a VZV-specific IFN-γ response is presented in figure 4.5. Nineteen of these subjects (76%) had positive VZV-specific IFN-γ responses (referred to as positive ELISPOT) at this time point, which are presented in table 4.5, whilst six were classified as not having a VZV-specific IFN-γ response (table 4.7, discussed later in the text). See chapter 2 section 2.5.1 for the definition of a positive response.

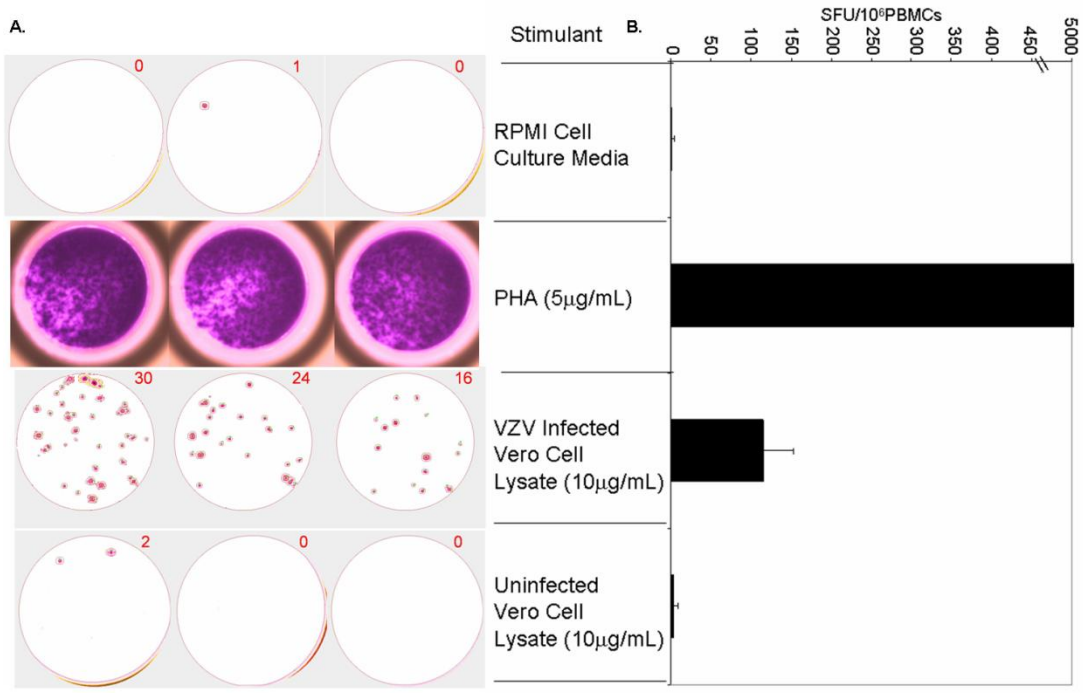


Figure 4.5: Representative Image of an *Ex Vivo* IFN-γ ELISPOT Generated Using Responsive PBMCs Which Produced a VZV-Specific IFN-γ Response Following Overnight Stimulation. PBMCs from ROVE study participant 1004 (18 month follow-up visit), are presented (112SFU/10^6PBMC). PBMCs were either stimulated with PHA mitogen (5μg/mL/well), VZV or uninfected lysate (10μg/mL/well) or RPMI alone. *Part A:* Processed images (Biosystem 5000, ELISPOT reader) of all wells (apart from those stimulated with PHA, where live images are presented as there were too many SFUs to count). *Part B:* Corresponding bar graph representing the SFU/10^6PBMCs. PHA stimulated wells were given an arbitrary count of 1000 SFU/10^6PBMCs as the magnitude of the response was uncountable. The total number of SFUs in the six negative control wells were subtracted from the total of SFUs in the three VZV stimulated wells and the mean was calculated. Samples were classified as having a positive VZV-specific IFN-γ response if the number of SFUs in the VZV lysate stimulated wells was greater than the (mean+3SD) of the negative control wells. In this example the mean of the negative control wells was 0.5, and the SD was 0.83, making a cut-off of 3SFU.

	Humoral Subset		Serology (18 Months)			CMI	Demographics			
Study Number	**Follow-Up**	**During Study**	**TRFIA (mIU/mL)**	**Avidity (%)**	**FAMA**	**SFU/10^6 PBMC**	**History**	**Ethnicity**	**Gender**	**Age**
1025	**N(L)**	**NSC1**	**65**		**<2**	**35**	**No**	**Ind**	**M**	**38**
1026	**N(L)**	**NSC1**	**116**	**28**	**8**	**100**	**No**	**Phil**	**F**	**27**
1036	**N**	**NLR**	**59**			**10**	**No**	**Black**	**F**	**33**
1047	**N(L)**	**NSC1**	**118**	**56**		**75**	**No**	**Ind**	**F**	**25**
1027[$]	**N**	**NLR**	**122**	**48**	**<2**	**10**	**No**	**Black**	**F**	**50**
1008	R	NSC2	144	73		45	No	Ind	F	41
1009	R	PI	717	65	2; 8	35	No	Black	F	34
1017	R	NSC2	148	29	<2	15	No	Black	F	29
1029	R	PI	574	82	<2	15	No	Black	M	20
1034	R	NSC2	138	33		20	No	Black	F	38
1049	R	NSC1	334	44		10*	No	Black	F	29
1060	R	NSC1	210	39		10*	No	Cauc	F	30
1062	R	PI	407	82		30	No	Black	F	33
1090	R	PI	422	93		132	x1	Cauc	F	19
1004	Boost	PI	1,376	85		112	No	Phil	F	30
1020	Boost	PI	1,447	87	8	40	DNK	Cauc	F	24
1021	Boost	NSC2	1,303	84		27	No	Ind	F	31
1031	Boost	PI	2,434	90		13	x1	Cauc	F	27
1050	Boost	NLR	280	51		42	DNK	Black	M	36

Table 4.5: HCWs who had VZV-Specific IFN-γ Responses at the 18 Month Follow-Up Visit. Key: NSC1; Negative Seroconverted to one dose, NLR; Negative Low Responder; NSC2; Negative seroconverted to two doses, PI; Positive 'Immune', N; negative at 18 months, N(L); negative, had lost TRFIA titre below 130mIU/mL at 18 months, R; retained antibody (TRFIA ≥130mIU/mL) at 18 months, Boost; 18 month TRFIA> 12 week TRFIA, SFU; spot forming units, History; History of chickenpox at enrolment, DNK; did not know, Phil; Filipino, Cauc; Caucasian; Ind; Indian subcontinent, M; male, F; female, *PHA responses for these individuals were low, [$]HCW received a third dose of vaccine. HCWs with values in **bold** had TRFIA values below 130mIU/mL. Mean; 40 SFU/10^6PBMCs, median; 30 SFU/10^6PBMCs, SD 37 SFU/10^6PBMCs. See table 4.7 for ELISPOT negative data.

Positive ELISPOT readings ranged from 10-132 SFU/10^6 PBMCs, (mean; 40, median; 30 SD 37 SFU/10^6 PBMCs), but two individuals with counts of 10 SFU/10^6PBMCs had low PHA responses in comparison to all other individuals (who had mitogen responses classified as 'too many to count.') Thus, in these two cases it is likely that 10 SFU/10^6PBMCs is an under estimate of the true number of VZV-specific IFN-γ producing cells in the total cell population. Thus, excluding these two samples from analysis gave a range of 10-132 SFU/10^6 PBMCs, (mean; 44, median; 35 SD 38 SFU/10^6 PBMCs).

4. 3.2.1 Comparison Between VZV-Specific IFN-γ Responses and VZV-Specific IgG Titres at 18 Months Post Vaccination:

There was no correlation between study participants $\log_{10}$ TRFIA titres and the number of SFU/10^6PBMCs at 18 months post vaccination, (R^2=0.0097). Table 4.6 summarises the ELISPOT and TRFIA assay status at this time point.

ELISPOT	N	TRFIA	N
Positive	19	Positive	14
		Negative	5
Negative	6	Positive	1
		Negative	5
		Total	25

Table 4.6 Summary of the ELISPOT and TRFIA Assay Status at 18 Months Post Vaccination.

4. 3.2.1.1 Positive ELISPOT:

Of the 19 individuals classified as having a positive ELISPOT reading, 14 (74%) had corresponding positive TRFIA VZV-specific IgG titres (≥130mIU/mL). For these 14 individuals, ELISPOT readings ranged from 10-132 SFU/10^6PBMC, with a median of 29 SFU/10^6PBMC whilst TRFIA readings ranged from 138-2,434mIU/mL with a median of 415mIU/mL. Fifty percent of this cohort had been classified as secondary humoral responders

after the first dose of vaccine, with a positive baseline TRFIA titre (positive 'immune' (PI), n=7), whilst one individual failed to seroconvert following vaccination (negative low responder; NLR = 7%), 29% were negative seroconverted to two doses (NSC2; n=4), and 14% were NSC1 (n=2).

Three of the 14 reported exposure to VZV in between their 12 week visit and their 18 month follow-up visit, in all cases, to two cases of chickenpox. Two were household (1034, 1049), and one was occupational (1050). This last individual was of particular interest as this was the HCW who was classified as a negative low responder, failing to seroconvert following two doses of vaccine, but asymptomatically seroconverted when he had a household contact with chickenpox. VZV-specific IFN-γ responses for this participant were un-interpretable at 12 weeks (visit 3) and at an additional time point taken two months after the household contact. This subject had a VZV-specific IFN-γ response of 42 SFUs/10^6PBMCs, which was above the mean value of positive responses, and a TRFIA of 282mIU/ml (log10 2.45) at the 18 month time point. (Their humoral immune response is discussed at length in chapter 3).

Five study participants with positive ELISPOT readings had corresponding TRFIA titres which were negative (<130mIU/mL), (highlighted in **bold**; table 4.5). Their ELISPOT readings ranged from 10-100 SFU/10^6PBMCs with a median of 35 SFU/10^6PBMCs. TRFIA readings ranged from 59-122mIU/mL with a median of 116mIU/mL. During the study 60% of these participants were classified as NSC1 (n=3), whilst 40% were NLR (n=2). Three of the five had corresponding FAMA readings and in two of these cases, FAMA scores were also negative (<2). Both cases are particularly interesting as study number 1025 seroconverted following a natural varicella infection which occurred between baseline and their six week visit and study number 1027 failed to seroconvert despite receiving a third dose of vaccine. The third of the five subjects with a FAMA reading, was classified as positive (FAMA

score; 8). It is noteworthy that this individual had one of the highest VZV-specific IFN-γ responses of all study participants (100SFU/10^6PBMC); comparable to that seen in latently infected naturally immune donors (see figure 4.4). Within this sub-group of positive ELISPOT/negative TRFIA, the two study participants with the lowest scoring positive ELISPOT responses were classified as NLR during the study. The remaining three subjects within this subgroup seroconverted following the first dose of vaccine.

4.3.2.1.2 Negative ELISPOT:

Six of the 25 18 month follow up samples with interpretable results were classified as not having a VZV-specific IFN-γ response (negative ELISPOT), (see table 4.7 and figure 4.6 for a representative image of a negative ELISPOT).

	Humoral Subset		Serology (18 Months)			Demographics			
Study Number	During Study	Follow-Up	TRFIA (mIU/mL)	Avidity (%)	FAMA Score	Gender	Ethnicity	Age	History
1007	NSC2	N (L)	25	-		F	Phil	32	No
1015	NSC2	N (L)	74	-	>8	F	Black	28	No
1019*	NSC2	N (L)	15	-	<2	F	Cauc	40	No
1024	NSC1	N (L)	111	30		M	Black	39	No
1041	NSC2	N (L)	108	73		F	Ind	27	No
1063	PLR	R	229	23		M	Black	29	No

Table 4.7: Study Participants who had No Measurable VZV-Specific IFN-γ Response at the 18 Month Follow-Up Visit. Key: History; History of chickenpox prior to enrolment, NSC1 and NSC2; Negative, Seroconverted to one/two doses; PLR; Positive Low Responder (baseline TRFIA >130mIU/mL, six week avidity <60% and six week TRFIA <400mIU/mL), N; Negative (TRFIA <130mIU/mL at 18 months) N(L); lost TRFIA titre of ≥130mIU/mL between 12 weeks and 18 months, R; retained (TRFIA >130mIU/mL at 18 months), F; female, M; male, Phil; Filipino, Cauc; Caucasian, Ind; Indian subcontinent, *All subjects were born and raised in the tropics apart from 1019, who was born and raised in the UK.

Of the six participants who had negative ELISPOT readings at 18 months post vaccination, five (83%) had negative TRFIA titres (<130mIU/mL). Two of the five with negative TRFIA titres had a FAMA reading, one of which was negative (<2). Thus, this individual (1019) was negative by ELISPOT, TRFIA and FAMA at 18 months. Although an ELISPOT reading at 12 weeks was not available for this subject, they were positive by both TRFIA and FAMA at that time point (326mIU/mL and 8 respectively). Four of the six individuals with a negative ELISPOT required two doses of vaccine to seroconvert (NSC2). One study participant had a corresponding TRFIA titre which was positive (>130mIU/mL), and was classified as a PLR during the study. All study participants with a negative ELISPOT had a negative history, but study participant 1019 reported an occupational exposure to zoster 84 days prior to their follow-up visit, whilst 1063 reported a brief non-household, non-occupational exposure to chickenpox 119 days prior to follow-up.

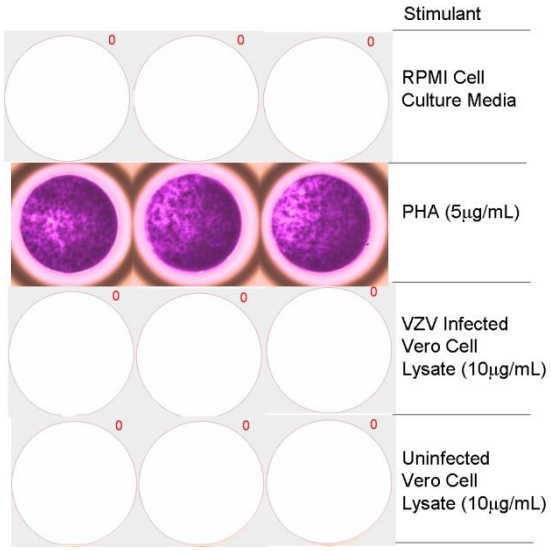


Figure 4.6 Representative Image of an *Ex Vivo* IFN-γ Negative ELISPOT Response After Overnight Stimulation. PBMCs from ROVE study participant 1015 (18 month follow-up visit), are presented (0 SFU/10^6PBMC). PBMCs were either stimulated with PHA mitogen (5μg/mL/well), VZV or uninfected lysate (10μg/mL/well) or RPMI alone. Processed images are presented for all wells, apart from those stimulated with PHA, where live images are presented as there were too many SFUs to count (Biosystem 5000, ELISPOT reader).

4.3.3 *Ex Vivo* and Cultured IE63 and Glycoprotein E Epitope Tetramer Specific $CD4^+$ T Cell Responses:

HLA-DRB1*1501^+ tetramers coupled to either gE or IE63 epitopes have been used previously to examine the frequencies of $CD4^+$ T cells specific for these epitopes in naturally immune adults (Jones *et al.,* 2007; Malavige *et al.,* 2008a). For seven ROVE study participants who were HLA-DRB1*1501^+, *ex vivo* IE63 and gE tetramer specific $CD4^+$ T cell responses were quantified at various time points throughout the study, and phenotypic antibody staining was carried out for each sample (n=52, with 6 samples stained twice for each tetramer, using antibodies to different cell surface markers). The tetramers used in this study (with thanks), were designed and optimized by collaborators in the laboratory of Dr. Graham Ogg, University of Oxford.

Represenative images of negative control IE63- and gE-tetramer staining, carried out in two healthy adult donors, are presented in figure 4.7 (with thanks to Dr. Louise Jones and Dr. Graham Ogg (University of Oxford) for permission to reproduce these figures). Images generated from the HLA-DRB1*1501 negative and VZV-seropositive donor are presented in figures 4.7A and 4.7B, and images for the HLA-DRB1*1501 positive and VZV-seronegative donor are presented in figure 4.7C and 4.7D. An example of the gating used to select live $CD14^-$, $CD19^-$, tetramer specific $CD4^+$ T cells from the PBMC population is shown in appendix 7.26. In figure 4.7 A, B and C no tetramer-specific $CD4^+$ T cells are present in the gated population of live, $CD14^-$, $CD19^-$ T lymphocytes. In figure 4.7D a very low percentage (0.00023%) of gE-tetramer-specific $CD4^+$ T cells are seen, but these cells are not brightly stained.

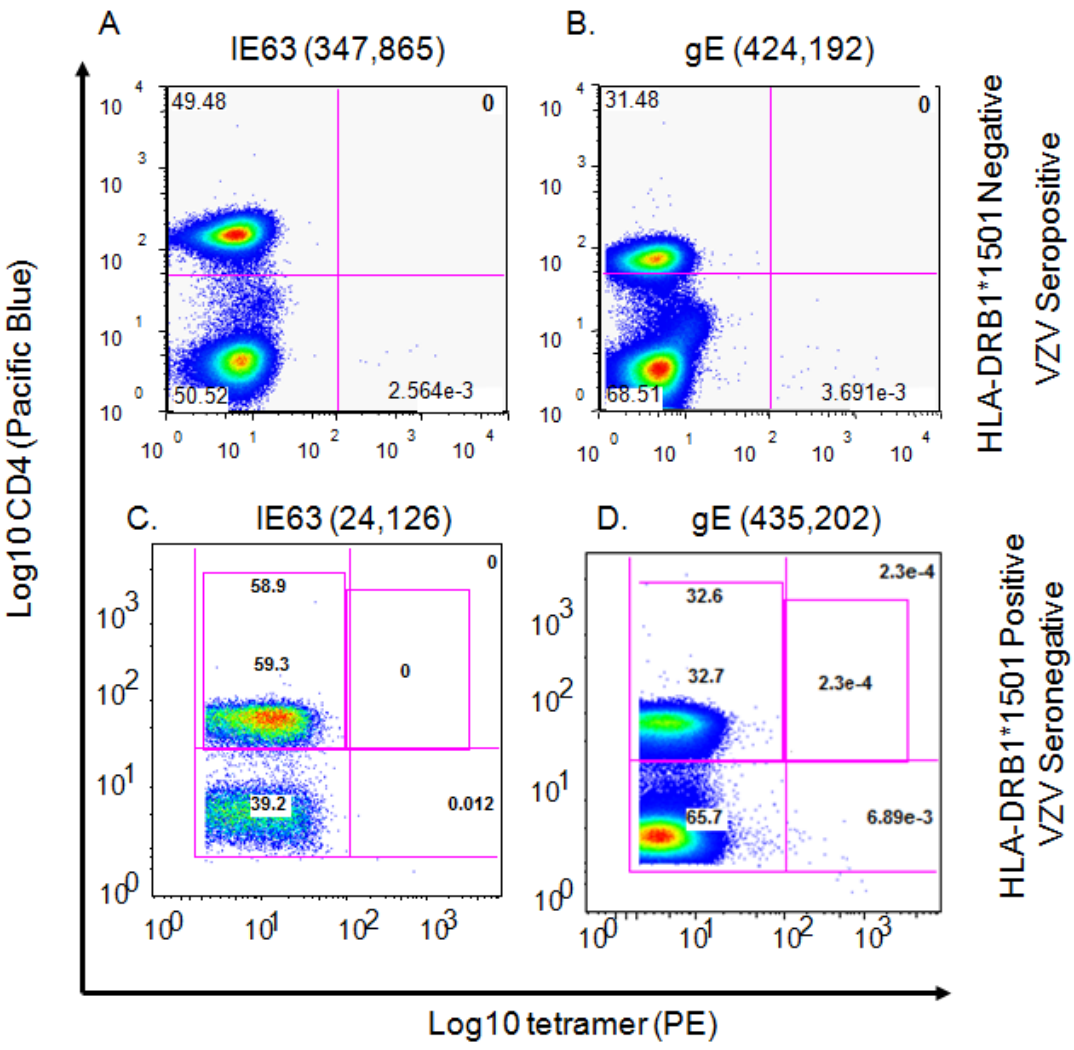


Figure 4.7: Representative Images of *Ex vivo* IE63- and gE-Tetramer-Specific $CD4^+$ T Cells in Populations of Live, $CD14^-$, $CD19^-$ T Lymphocytes in a Healthy Adult HLA-DRB1*1501 Negative/VZV-Seropositive Donor and a Healthy Adult HLA-DRB1*1501Positive/VZV-Seronegative Donor. Event counts (given in parentheses) represent the total number of live T lymophocytes for each sample of the four donors. With thanks to Dr. Louise Jones and Dr. Graham Ogg (University of Oxford) for permission to reproduce these figures.

4.3.3.1 Comparison between IFN-γ Responses and Tetramer Staining:

One of the healthy seropositive donors whose PBMCs were used for ELISPOT optimization assays was DRB1*1502^{+}, and although the tetramers were a different subtype (DRB1*1501), CD4^{+} T cells from DRB1*1502 subjects are also able to bind to the IE63 and gE epitopes presented on the DRB1*1501 tetramers. Thus, a comparison between *ex vivo* VZV-specific IFN-γ responses measured using VZV-infected cell lysate ELISPOT and *ex vivo* frequencies of CD4^{+} T cells specific for gE and IE63 epitopes measured using tetramers and flow cytometry was carried out in this healthy, naturally immune, adult donor. As can be seen from figure 4.8 a dose dependent response to VZV-infected vero cell lysate was observed, with the number of SFU/10^{6}PBMCs increasing from 28 (2.5μg/mL); 70 (5μg/mL) to 112 (10μg/mL). This donor also had tetramer^{+} CD4^{+} T cells which accounted for 0.023% (gE) and 0.057% (IE63) of the live lymphocytes examined.

4.3.3.2: Examining the Frequency of CD4^{+} Tetramer^{+} Cells in Low Numbers of PBMCs:

The total numbers of PBMCs/sample that were examined during *ex vivo* tetramer staining was small and ranged from approximately 0.24x10^{6} - 0.84x10^{6} per 'staining set' (tetramer + antibodies to cell surface markers). Frequencies of tetramer specific CD4^{+} T cells for each subject at each visit are summarised in appendix 7.28. An abbreviated version is presented in table 4.8, in the context of corresponding TRFIA titres. As the populations of live, CD14^{-}, CD19^{-} lymphocytes examined were so small, for 45% (n=24) of samples it was not possible to conclude that the absence of tetramer specific CD4^{+} T cells represented a true absence of such cells from the subject's peripheral blood. Again due to small sample size, for cases where tetramer specific CD4^{+} T cells were identified, phenotypic analysis was not conducted as there were so few cells. However, despite the fact that very small populations were analysed, brightly stained, tetramer specific CD4^{+} T cells were visualized in 28 samples, albeit at very low frequencies (see figure 4.9,

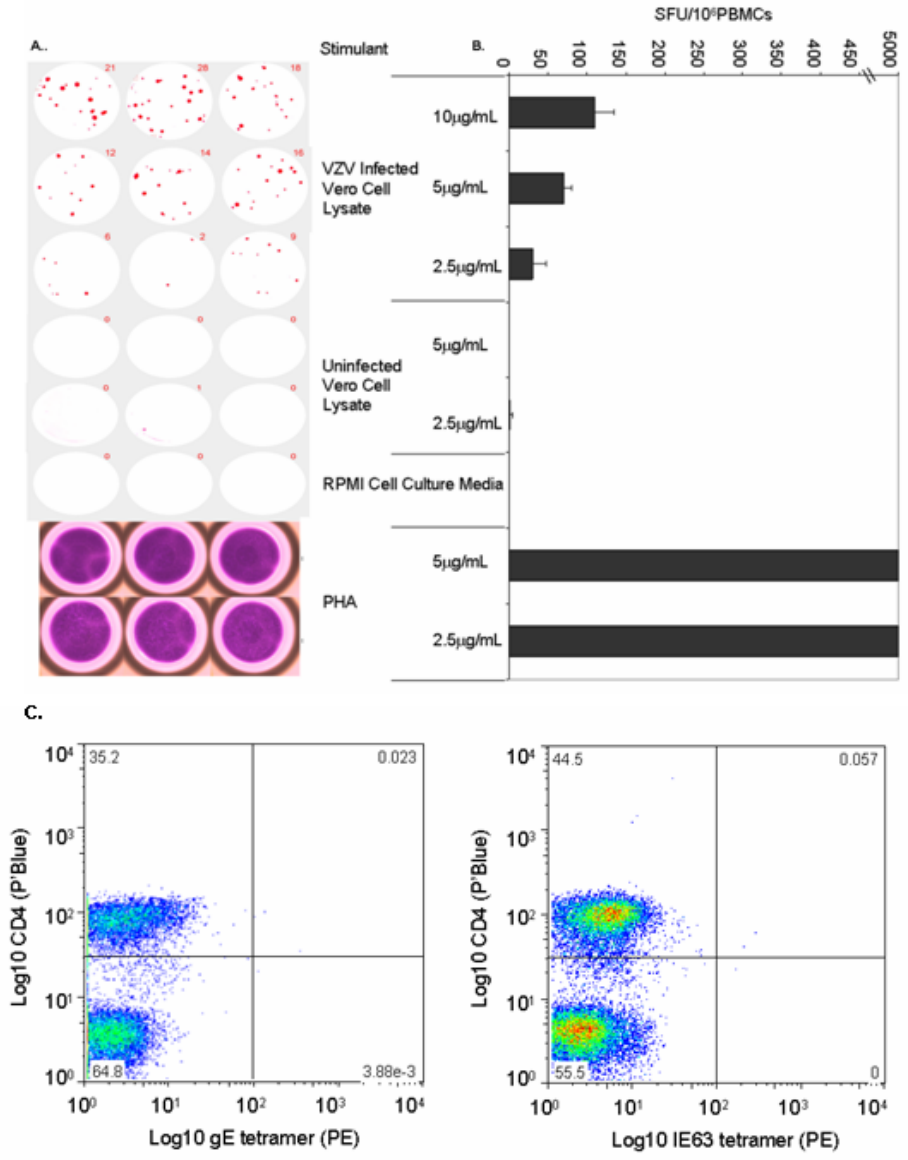


Figure 4.8: Comparison between *Ex Vivo* VZV-Specific IFNγ Responses (ELISPOT) and Frequencies of Tetramer-Specific $CD4^+$ T Cells Using PBMCs from a DRB1*1502 Naturally Immune Donor. PBMCs from a naturally immune, healthy, seropositive, $DRB1*1502^+$ donor were either stimulated overnight with commercial VZV lysate prior to ELISPOT (part A and B) or *ex vivo* gE and IE63 tetramer staining was performed (part C). *Part A*: Processed ELISPOT images; *Part B:* 10μg/mL of VZV lysate generated 112 SFU/10^6PBMCs; *Part C*: 0.023% and 0.057% of live lymphocytes were gE $tetramer^+$ and $IE63^+$ tetramer $CD4^+$ T cells respectively. Processed images are presented in part A for all wells apart from those stimulated with PHA, where live images are presented as there were too many SFUs to count. Key: PE; Phycoerythrin, P'Blue; Pacific Blue.

for examples of bright tetramer staining gated from populations of approximately 2,000 and 5,000 live T lymphocytes).

Samples which contained gE tetramer specific $CD4^+$ T cells had a mean of 0.022%; (range 0.0006 to 0.042%; median; 0.021, SD 0.012%) of the (live, $CD14^-$, $CD19^-$) lymphocyte population, whilst for IE63 the mean was significantly higher (independent 2 tailed t test p= 0.049) at 0.057 (range 0.014-0.212; median; 0.041, SD 0.053).

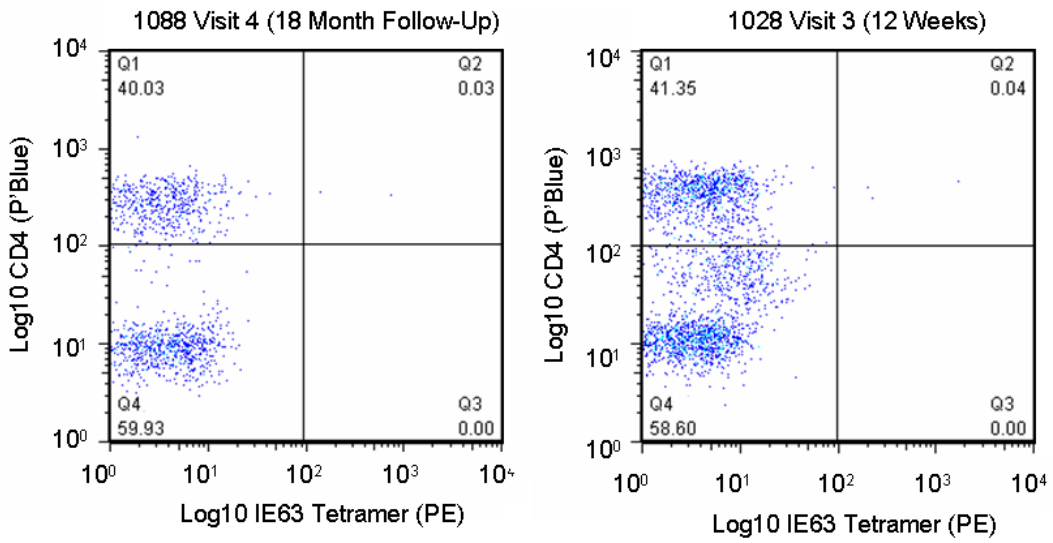


Figure 4.9: Representative Images of Low Frequency, Brightly Stained IE63 Tetramer-Specific $CD4^+$ T Cells Identified in Small Populations of Live, $CD14^-$, $CD19^-$ T Lymphocytes. Samples from study participant 1088 (follow-up) and 1028 (12 weeks) are shown, which had populations sizes of approximately 2,000 and 5,000 respectively.

				Glycoprotein E			IE63		
Number		**TRFIA**		**Ex Vivo**			**Ex Vivo**		
Study (Subset)	**Visit**	**Titre (mIU/mL)**	**Assay Status**	**Tetramer Present**	**N (%) of Live T Lymphocyte**	**Lines (10 Day Culture)**	**Tetramer Present**	**N (%) of Live T Lymphocyte**	**Lines (10 Day Culture)**
1001 (PLR/NL)	V1	130	Pos	SSSCA	(0/4)	X	SSSCA	(0/4)	X
	V2	207	Pos	Sample Not Available					
	V3	229	Pos	Yes	2/20,000 (0.010)	X	SSSCA	(0/2000)	X
	V4	87	Neg	SSSCA	0/6000 (0)	Non Proliferative	**Yes**	3/21,000 (0.014)	Proliferative
1018 (NSC1/Boost)	V1	72	Neg	SSSCA	(0/<100)	X	SSSCA	(0/<100)	X
	V2	176	Pos	Yes	2/6000 (0.033)	Non Proliferative	Yes	4/8000 (0.051)	Non Proliferative
	V3	398	Pos	**Yes**	2/31000 (0.0006)	Proliferative	**Yes**	9/27,000 (0.01)	Proliferative
	V4	2,040	Pos	Yes	13/38000 (0.029)	X	Yes	17/108,000 (0.016)	X

Table 4.8 (continued overleaf)

				Glycoprotein E			IE63		
Number		TRFIA		Ex Vivo		Lines (10 Day Culture)	Ex Vivo		Lines (10 Day Culture)
Study (Subset)	Visit	Titre (mIU/mL)	Assay Status	Tetramer Present	N (%) of Live T Lymphocyte		Tetramer Present	N (%) of Live T Lymphocyte	
1028 (NSC1)	V1	68	Neg	SSSCA	(0/<1000)	X	SSSCA	(0/<1000)	X
	V2	226	Pos	SSSCA	(0/<2000)	Non Proliferative	Yes	3/1000 (0.212)	Non Proliferative
	V3	603	Pos	Yes	1/2000 (0.042)	X	Yes	3/2000 (0.04)	X
	V4			Sample Not Available					
1058 (NSC1/R)	V1	23	Neg	SSSCA	(0/<100)	X	SSSCA	(0/100)	X
	V2	262	Pos	SSSCA	(0/2000)	X	Yes	2/2000 (0.089)	X
	V3	776	Pos	SSSCA	(0/<1000)	X	SSSCA	(0/<500)	X
	V4	226	Pos	Yes	5/17000 (0.034)	Non Proliferative	Yes	4/7000 (0.055)	Non Proliferative

Table 4.8 (continued overleaf)

				Glycoprotein E			IE63		
Number		TRFIA		Ex Vivo		Lines (10 Day Culture)	Ex Vivo		Lines (10 Day Culture)
Study (Subset)	Visit	Titre (mIU/mL)	Assay Status	Tetramer Present	N (%) of Live T Lymphocyte		Tetramer Present	N (%) of Live Lymphocyte	
1067 (NSC1/R)	V1a	55	Neg	**(Yes)**	3/53,000 (0.001)	(Proliferative)	Yes	3/57,000 (0.001)	Non-Proliferative
	V1b				4/34,000 (0.002)	X		5/38,000 (0.002)	
	V2	180	Pos	Yes	1/5,000 (0.0188)	Non-Proliferative	Yes	4/8,000 (0.05)	Non-Proliferative
	V3	546	Pos	Yes	2/7,000 (0.030)	X	SSSCA	0/3,000	X
	V4	130	Pos	Yes	2/34,000 (0.0001)	(Proliferative)	Yes	11/21,000 (0.005)	Non-Proliferative
1088 (NSC1/R)	V1	39	Neg	SSSCA	0/<1,000	X	SSSCA	0/<500	X
	V2a	130	Pos	SSSCA	0/<1,000	X	SSSCA	0/<1,000	X
	V2b			SSSCA	0/<1,000		X	X	
	V3	712	Pos	Yes	1/15,000 (0.007)	X	Yes	2/9,000 (0.022)	Non-Proliferative
	V4	231	Pos	SSSCA	0/1000	X	Yes	2/1,000 (0.03)	X

Table 4.8 (continued overleaf)

				Glycoprotein E			IE63		
Number		**TRFIA**		**Ex Vivo**		**Lines (10 Day Culture)**	**Ex Vivo**		**Lines (10 Day Culture)**
Study (Subset)	**Visit**	**Titre (mIU/mL)**	**Assay Status**	**Tetramer Present**	**N (%) of Live T Lymphocyte**		**Tetramer Present**	**N (%) of Live T Lymphocyte**	
1106 (NSC1/R)	V1	101	Neg	SSSCA	0/22,000	Non-Proliferative	SSSCA	0/12,000	Non-Proliferative
	V2	194	Pos	Yes	1/11,000 (0.009)	X	SSSCA	0/6,000	X
	V3a	3,346	Pos	Yes	3/9,000 (0.034)	Non-Proliferative	Yes	5/8,000 (0.065)	Non-Proliferative
	V3b				1/5,000 (0.02)			1/7,000 (0.014)	
	V4	870	Pos	SSSCA	0/<500	X	SSSCA	0/<100	X

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Percentage of IE63 and gE Tetramer$^+$, CD4$^+$ T Cells Quantified *Ex Vivo* and Following Short Term (10 Day) Culture with the Corresponding Peptide.

Key: PLR; Positive Low Responder, NSC1; Negative, Seroconverted after One dose, NL; Negative at follow-up, had Lost TRFIA titre (≥130mIU/mL), R; Retained TRFIA titre (≥130mIU/mL) at follow-up, V1; visit 1 (baseline), V2; visit 2 (six weeks post vaccination), V3; visit 3 (12 weeks post first, six weeks post second vaccination), V4; visit 4 (18 month follow-up), a and b; two sets of surface markers were used on these samples, Neg; Negative (<130mIU/mL), Pos; positive (≥130mIU/mL), SSSCA; Sample Size too Small to Conclude tetramer specific CD4$^+$ T cells were Absent from Periphery, **Yes**; tetramer specific CD4$^+$ T cells identified *ex vivo* were able to proliferate following short term culture, X; T cell lines were not set up for these samples, or a second set of surface stains was not used, Live Lymphocytes; total number of live, CD14$^-$ CD19$^-$ lymphocytes, from which tetramer specific CD4$^+$ T cells were gated. Cell numbers have been rounded off to the nearest 1,000 (or in some cases 100 cells), true values are presented in appendix 7.28.

For 6/7 individuals, tetramer specific $CD4^+$ T cells were not visualised *ex vivo* at baseline, however, in these cases sample sizes were too small to conclude that tetramer specific $CD4^+$ T cells were actually absent from the peripheral blood. One subject (1067) had both gE and IE63 tetramer specific $CD4^+$ T cells *ex vivo* at baseline, and is discussed in more detail later in the text. In samples with positive TRFIA titres both IE63 and gE tetramer specific $CD4^+$ T cells were visualised in 12 samples, whilst in three samples gE tetramer specific $CD4^+$ T cells alone were visualised and in four samples IE63 tetramer specific $CD4^+$ T cells were visualised. In one case, IE63 tetramer specific $CD4^+$ T cells were visualised *ex vivo* in a sample with a negative TRFIA titre at this time point (1001, follow-up visit), and is discussed in more detail later in the text.

In addition to tetramer specific $CD4^+$ T cells staining brightly when examined *ex vivo*, in three cases these cells were able to proliferate in response to short term culture with the corresponding peptide, the results of which are summarised in table 4.8. An example of a comparison between *ex vivo* staining and 10 day culture results (for both gE and IE63 epitope specific $CD4^+$ T cells) is shown for ROVE study participant 1018 at the 12 week time point (response to second dose of vaccine, visit 3) in figure 4.10. (An example of gating used to select tetramer-specific $CD4^+$ T cells following short term culture is presented in appendix 7.27). Due to the numbers of live PBMCs following resuscitation (counts ranged from approximately 0.17-$2.16x10^6$/well), corresponding mitogen or VZV lysate stimulation was not established.

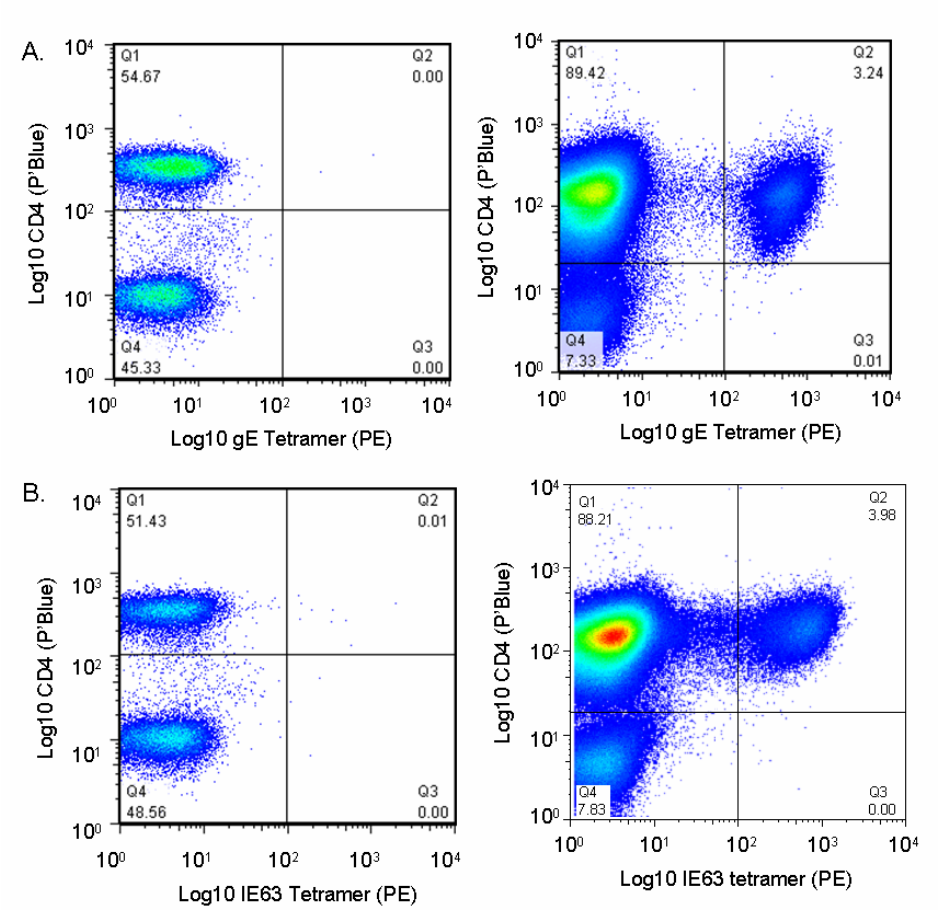

Figure 4.10: Glycoprotein E and IE63 Tetramer-Specific CD4$^+$ T Cell Staining at 12 weeks (visit 3) for ROVE 1018. Part A; gE tetramer, Part B; IE63 tetramer. Both *ex vivo* (left plots) and 10 day culture (right plots) are shown. Two brightly stained gE tetramer specific CD4$^+$ T cells were identified *ex vivo* (0.0006%). Live lymphocytes were plotted by CD4 and tetramer staining. Key: PE; Phycoerythrin, P'Blue; Pacific Blue.

From the results presented in figure 4.10, both brightly stained gE and IE63 tetramer specific CD4$^+$ T cells can be seen *ex vivo* (although at very low frequency) and these corresponded with extensive proliferation of tetramer specific CD4$^+$ T cells after 10 days of culture with the corresponding peptide. In another case, (1001; follow-up visit; IE63 tetramer), the number of PBMCs available for staining after 10 day cultures was very small, but expanded brightly stained tetramer specific CD4$^+$ T cells were clearly visible, see figure 4.11 part A. In this example, CD4$^+$ T cells were able to proliferate in response to the IE63 peptide, but not the gE peptide (figure 4.11, part B).

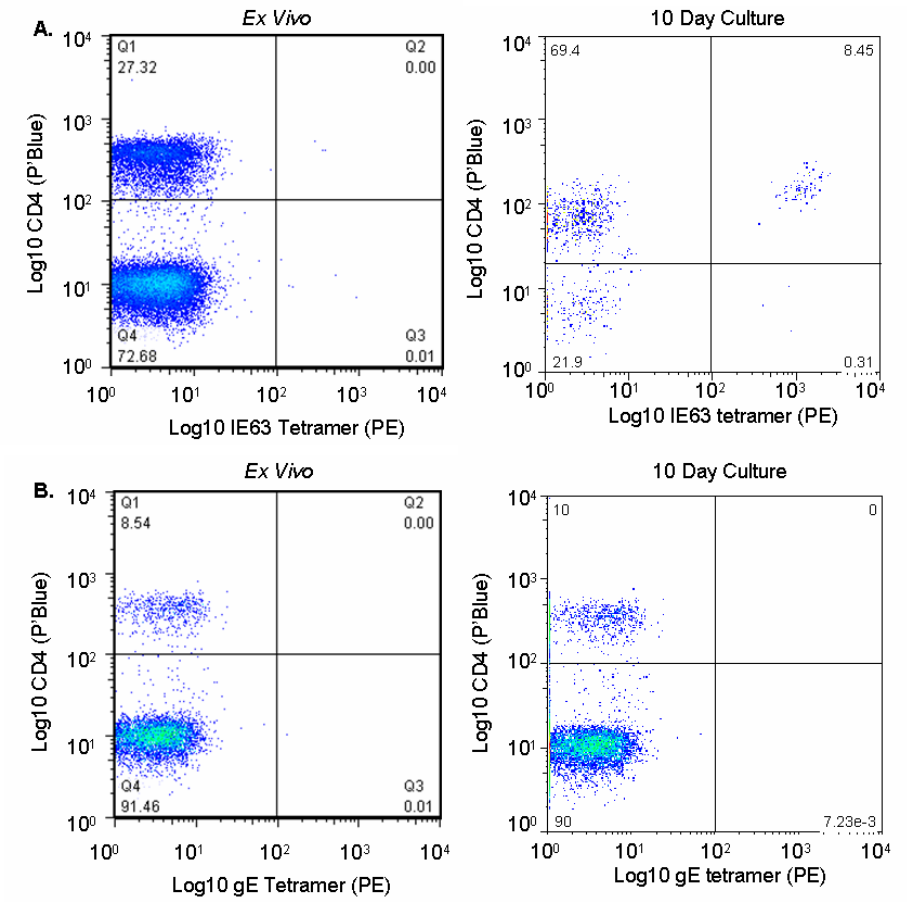

Figure 4.11: IE63 and gE Tetramer Specific $CD4^+$ T Cell Staining at 18 Months Post Vaccination for ROVE Study Participant 1001. Both *ex vivo* and 10 day culture results are shown. Live lymphocytes were plotted by CD4 and tetramer staining. Key: PE; Phycoerythrin, P'Blue; Pacific Blue.

For study participant 1067, their baseline TRFIA was negative, and they responded as a primary responder during the study (i.e. six week TRFIA <400mIU/mL, avidity <60%). However as mentioned earlier, both IE63 and gE tetramer specific $CD4^+$ T cells were identifiable in the baseline sample, (see figure 4.12). A higher percentage of IE63 tetramer specific $CD4^+$ T cells, than gE tetramer specific cells were seen at baseline (0.01% (IE63) and 0.004% (gE) of the live lymphocyte population, gated from populations of approximately 55,000). However this study participant gave a negative history, reported no contacts prior to enrolment and was asymptomatic prior

to enrolment, and at all visits throughout the study. This subject was a 27 years old Indian female, who was born in India and moved to the UK the same year as enrolling in the study, and was heterozygous for DRB1*1501; (second allele: DRB1*1101).

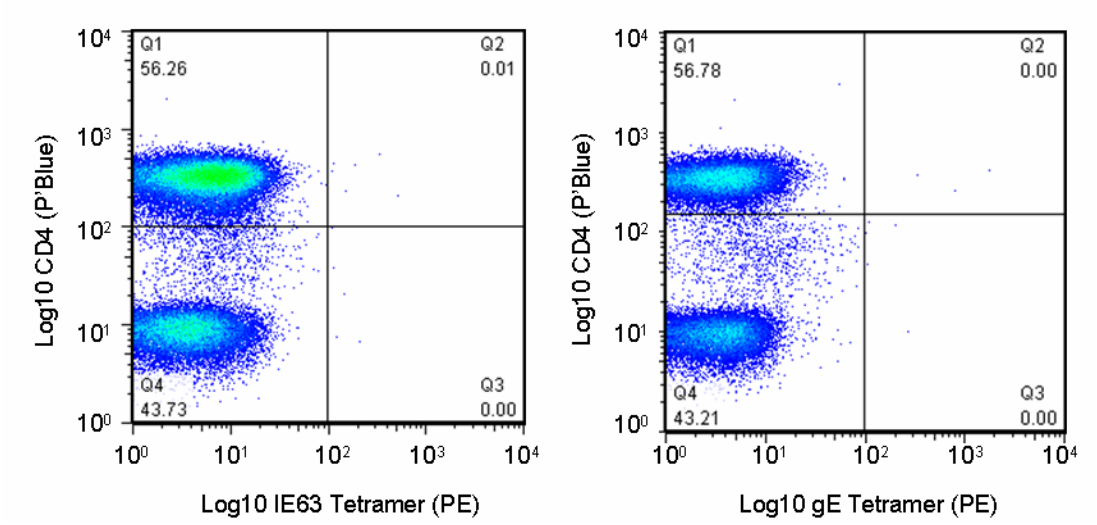

Figure 4.12: IE63 and Glycoprotein E Tetramer Specific $CD4^+$ T Cell Staining at Baseline for ROVE Study Participant 1067. Key: PE; Phycoerythrin, P'Blue; Pacific Blue.

Cell numbers examined at six and 12 weeks were very low, but at the follow-up visit (16 months), cell numbers for the sample stained with gE tetramer were comparable to the sample size examined at baseline. Tetramer-specific $CD4^+$ T cells were identified, but at a lower frequency than at baseline (0.001% for each). Glycoprotein E tetramer specific $CD4^+$ T cells were able to proliferate following short term culture with the gE peptide for the baseline and follow-up sample (figure 4.13), although this was not seen with the IE63 peptide. For both baseline and follow-up staining of gE cell lines, antibody staining was not of the same intensity as other lines analysed during the same experiment.

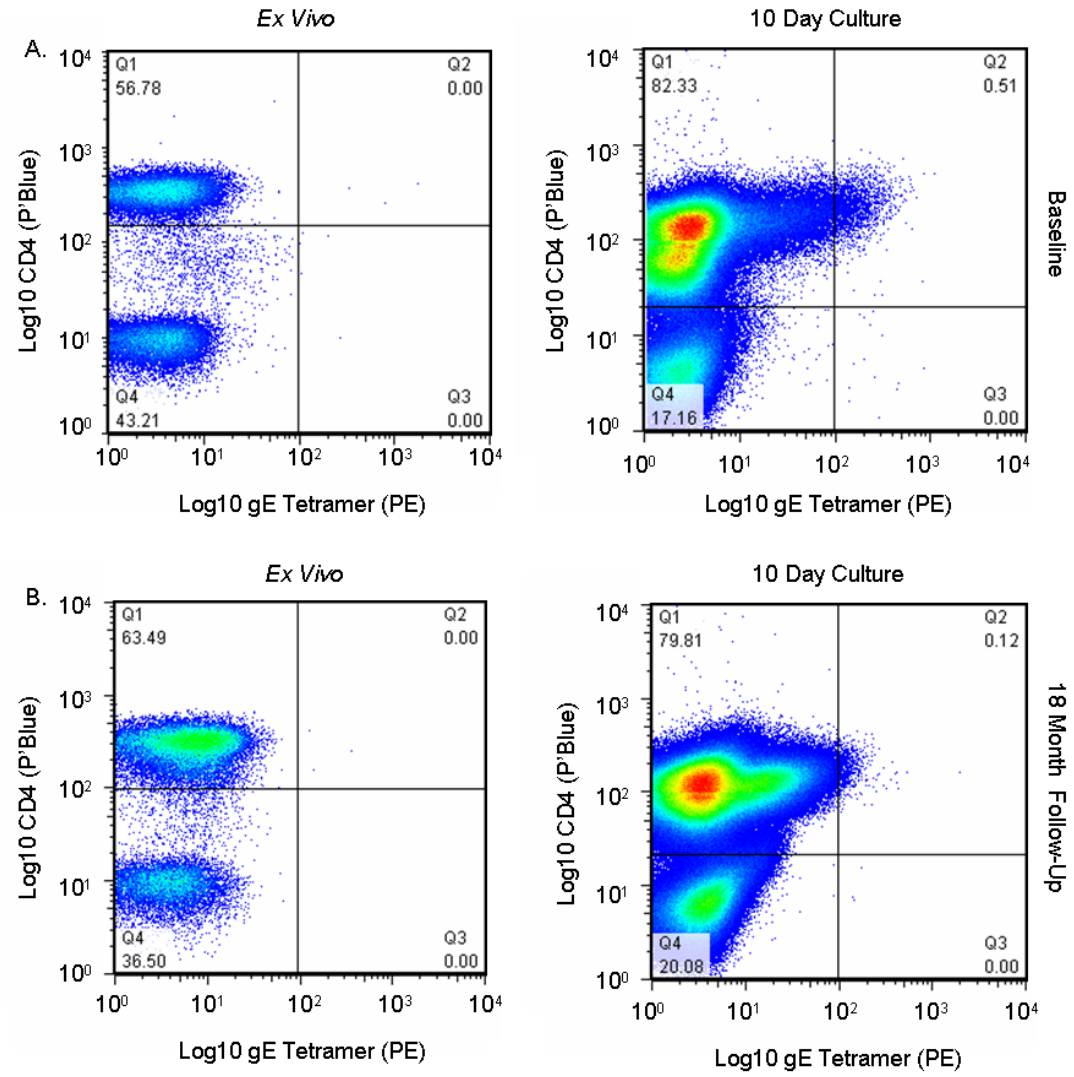

Figure 4.13: Glycoprotein E Tetramer Specific $CD4^+$ T Cell Staining at Baseline and Follow-up for ROVE Study Participant 1067. A: Baseline; B: Follow-up. Both *ex vivo* and 10 day culture results are presented. Live lymphocytes were plotted by CD4 and tetramer staining. Key: PE; Phycoerythrin, P'Blue; Pacific Blue.

4.4 DISCUSSION:

4.4.1 Summary of Results:

Although baseline VZV-specific IFN-γ responses were not measured in this study and baseline data on gE/IE63 epitope-specific $CD4^+$ T cells was severely limited, the results presented in this chapter are consistent with the possibility that that the live attenuated varicella vaccination is capable of generating VZV-specific IFN-γ responses and gE/IE63 epitope-specific $CD4^+$

T cell responses in the absence of clinical disease, which are detectable 18 months post vaccination. Although there is no published literature on epitope specific responses following vaccination, several studies have shown that VZV-specific CMI is detectable in adults and children between 1-20 years post vaccination (Sharp *et al.,* 2002; Levin *et al.,* 2006; Hayward *et al.,* 1992; Asano *et al.,* 1994). The data presented here demonstrate that 76% (19/25) of vaccinated HCWs had positive IFN-γ ELISPOTS at 18 months post vaccination, whereas a study in HIV positive children vaccinated with two doses found that 83% of study participants had positive CMI at one year (Levin *et al.,* 2006).

4.4.2 VZV-Specific IFN-γ ELISPOT Responses and Corresponding TRFIA Titres at 18 Months Post Vaccination:

In agreement with the findings in this study, several reports have found that there is a lack of correlation between antibody titre and corresponding measurements of cell mediated immunity in long term studies, both following natural varicella infection (Sadaoka *et al.,* 2008) and vaccination, (Trannoy *et al.,* 2000; Levin *et al.,* 2008b; Hayward *et al.,* 1992). However, in this study it was found that there was a 76% agreement on assay status (i.e. positive or negative by both assays). Twenty percent (5/25) of those with interpretable ELISPOT results at follow-up were negative by both assays. This suggests that these subjects would be at risk of infection, however, one of these individuals had a positive FAMA titre at this time point, which has been shown to correlate with clinical protection (Gershon *et al.,* 1994a). Therefore, in subjects who were not tested by FAMA it was not possible to predict if they would be at risk of re-infection. However one subject (1019) was found to be negative by all three assays at this time point, indicating that they would be at risk of re-infection; this subject is discussed in more detail in chapter 3.

As IFN-γ responses at earlier study time points were not available, it is not possible to deduce if these subjects failed to mount a VZV-specific IFN-γ response to vaccination (and is therefore possibly indicative of a primary vaccine failure), or whether they had lost the response in the time which had elapsed since vaccination (and is thus possibly indicative of a secondary vaccine failure). In addition, it was not possible to examine a threshold of IFN-γ responses to vaccination in this study, which was indicative of a positive or negative CMI response at 18 months post vaccination.

Interestingly, of those that were ELISPOT negative, all were born and raised in the tropics, bar one, and 67% (4/6) were classified as NSC2 during the study, a humoral subset that we identified as being more likely to have lost a positive TRFIA titre at 18 months. However, we were unable to identify factors such as age, ethnicity and gender which would predict who would have a positive or negative VZV-specific IFN-γ ELISPOT at 18 months due to small sample sizes.

There was a subset of five individuals who were positive by ELISPOT, but negative by TRFIA. Two of these subjects, were classified as NLR, and failed to seroconvert following two or three doses of vaccine, yet they had measurable CMI at 18 months post vaccination, demonstrating that a failure to seroconvert to vaccination did not necessarily correlate with a negative CMI at follow-up. Some previous studies on varicella vaccine demonstrated that seronegative vaccinees were protected from disease, indicating that CMI was playing a role in protection (Levin *et al.,* 2003). In addition, two separate case reports have documented failure to seroconvert following two or three doses of vaccine, but a correspondingly positive CMI in immunocompetent HCW (Katial *et al.,* 1999; Ludwig *et al.,* 2006). In one report the HCW was negative by commercial VZV IgG ELISA (Wampole) following three dose of Varivax vaccine, but had a VZV-specific T cell response (determined by LPA) comparable to that of other vaccinees, and in addition had positive FAMA and

LA scores (both of which were 8), (Katial *et al.,* 1999). In the second report Dade Behring VZV IgG ELISA and ICFC were used to assess humoral and cellular responses respectively (Ludwig *et al.,* 2006). The subject seroconverted to the third dose of Varilrix vaccine but had a positive $CD4^+$ T cell response after the second dose. This individual was found to be seronegative at 18 months, but had retained a positive CMI. At this time point, the HCW had an occupational contact with a varicella patient, which included a physical examination and daily visitations over a one week period without any special protective clothing such as gloves or face mask. Following this close contact exposure the HCW remained asymptomatic, but still failed to seroconvert.

This last case was in contrast to a case seen during this study, where an individual (1050), who failed to seroconvert following two doses of vaccine, seroconverted shortly afterwards following a household exposure to varicella, (remaining asymptomatic throughout). Although this subject's CMI response was unknown during the course of the vaccination study, in light of the two published case reports, and the fact that this individual's antibody response was negative by TRFIA at the time of exposure, it is plausible to suggest that this subject was protected by a cell mediated-immune response (in combination with an innate immune response). This individual was positive by both assays 18 months post vaccination.

It is interesting to note that two further subjects who failed to seroconvert during the vaccination study (NLR subset) had positive ELISPOT readings at 18 months post vaccination, whilst those with negative ELISPOTs at this time point, were seropositive on completion of the vaccination study (NSC1, NSC2 and PLR). All study participants who were classified as PI during the study had positive ELISPOTs at 18 months, indicating that secondary responders were more likely to have positive CMI at follow-up, but failing to seroconvert was not predictive of a negative ELISPOT at follow-up.

4.4.3 Problems Encountered with the Measurement of CMI after VOka Vaccination:

*4.4.3.1 Low Frequencies of IE63 and gE DRB1*1501 Tetramer Specific CD4$^+$ T Cells Identified in this Study:*

IE63 and gE tetramer specific CD4$^+$ T cells have been observed in latently infected, naturally immune, adult donors *ex vivo* by others, (Jones *et al.,* 2007 and Malavige *et al.,* 2008a). In these donors the mean percentage of gE and IE63 tetramer positive cells (of the live, CD19$^-$, CD14$^-$, CD4$^+$ lymphocyte population) was 0.003% and 0.006% respectively, (Jones *et al.,* 2007; Malavige *et al.,* 2008a). This equates to 3 and 6 tetramer positive cells per 100,000 cells respectively. During acute infection, the median percentages of tetramer positive cells identified in adult patients hospitalized with varicella were 0.0157% (gE) and 0.007% (IE63), (Malavige *et al.,* 2008b). Thus, even during acute infection a minimum of 100,000 live, CD19$^-$ CD14$^-$ CD4$^+$ lymphocytes would need to be examined in order for tetramer specific cells to be identified. Therefore when *ex vivo* analysis was conducted on sample sizes smaller than this during this study, in the absence of tetramer specific CD4$^+$ T cells, it was not possible to conclude that the individual actually lacked CD4$^+$ T cells specific for that epitope in their peripheral blood, merely that they were absent from that particular sample. However, had acceptable sample sizes been examined, as the quantification of CMI is highly specific to an individual epitope level, the lack of a response to these two particular epitopes is not indicative that the individual is unable to mount responses to other VZV epitopes (within these, and other VZV proteins).

It is also plausible that the absence of proliferation following short term culture with the corresponding peptide is attributable to the low cell numbers examined. In addition as mitogen and VZV lysate controls could not be set up in parallel, again due to low cell numbers, it was not possible to distinguish if these cells were unable to proliferate specifically in response to the peptide, or if these cells would be unable to proliferate in response to any stimulus (for

example; a non-proliferative PBMC sample, due to sample degradation in long term storage). As the proportion of samples which were found to be unresponsive to mitogen stimulation during IFN-γ ELISPOT analysis was high, this is a likely scenario (especially with earlier time points) in cases where tetramer specific cells were identified *ex vivo*, but no proliferative response was seen following short term culture.

4.4.3.2 Specificity of Tetramer Staining:

In many of the FACS plots presented in this chapter as well as in those published by collaborators, a small population of tetramer$^+$ CD4$^-$ cells can sometimes be seen. One explanation for this could be that these cells are phagocytic cells that are in the process of engulfing an apoptotic CD4$^+$ T cell that is specific for that epitope. However at this stage of flow cytometry analysis, dead and phagocytic cells have been gated out of the population and reanalysis using 'tighter' gating on the lymphocyte population and the viaprobe negative populations did not eliminate this subset of cells. It could be argued that this phenomenon sheds doubt on the specificity this tetramer staining, however proliferative responses in this study and in others (Jones *et al.,* 2007; Malavige *et al.,* 2008a) have demonstrated that low frequency CD4$^+$ tetramer$^+$ cells identified *ex vivo* have the ability to proliferate following short term culture with the corresponding peptide. In addition, experiments by collaborators (Jones *et al.,* 2007; Malavige *et al.,* 2008a) demonstrated that *ex vivo* frequencies of tetramer$^+$ CD4$^+$ T cells were significantly higher in HLA-DRB1*1501 individuals than the frequencies seen in non-HLA-DRB1*1501 individuals ($p=0.0003$; IE63 and $p=<0.0001$ gE), and this difference was also seen after short term culture ($p=0.0094$; IE63), (Jones *et al.,* 2007; Malavige *et al.,* 2008a).

In agreement with the finding of Jones *et al.,* 2007 and Malavige *et al.,* 2008a, the mean frequency of IE63 tetramers specific $CD4^+$ T cells was approximately double that of gE. However, the mean frequencies found in this study were higher than those reported in latently infected individuals and those hospitalised with acute varicella (Malavige *et al.,* 2008a; Malavige *et al.,* 2008b and Jones *et al.,* 2007). The discrepancies in the 10 fold higher means between this study and those of others, is likely to be due to the inaccuracy incurred when examining 5-10 fold smaller sample sizes examined in this study. An addition explanation could be that the results presented in this thesis examined responses following vaccination as opposed to latently or acutely infected individuals.

4.4.4 Discrepant TRFIA and Tetramer Assay Results:

In this study, it was possible to conclude that proliferative tetramer-specific $CD4^+$T cells were present in some samples. Study participant 1001 had a population of brightly stained IE63 tetramer-specific $CD4^+$T cells following short term culture, but was negative at this time point by TRFIA. Thus as was seen with IFN-γ ELISPOTs responses, there was a discrepancy between a negative TRFIA titre and in this case, the presence of epitope specific VZV $CD4^+$ T cells at 18 months post vaccination. A discrepancy was also seen with study participant 1067 at baseline. This subject was classified as a primary humoral responder who seroconverted following one dose of vaccine. Glycoprotein E tetramer-specific $CD4^+$ T cells were able to proliferate following short term culture. However, gE tetramer-specific $CD4^+$ T cells did not stain as brightly as for other individuals whose cell lines were analysed during the same experiment. The fact that this phenomenon was seen in lines established at baseline and 18 months for the same individual suggests that this staining pattern is specific to this individual (discussed further below). As cells from the same aliquot were used to establish IE63 cell lines, a lack of proliferation suggests that the cells identified *ex vivo* were not specific for this epitope. However, tetramer specific $CD4^+$ T cell responses could not be

accurately assessed at six weeks and 12 weeks as the total number of live lymphocytes examined were several fold lower at these time points in comparison to those seen at baseline; (5 and 11 fold lower for IE63 at six weeks and 12 weeks respectively and 10 and 8 fold lower at these time points for gE).

It was confirmed that the baseline sample was not mislabelled during initial sample processing as the other sample processed at the same time, was not HLA-DRB1*15^{+}. One possibility that may explain why staining was not as bright in this individual, is that this gE epitope may represent a shared CD4^{+} T cell epitope between VZV and HSV, (however the HSV status of this subject was not known). Recently DRB1*15^{+} CD4^{+} T cell clones developed from a patient experiencing acute dengue infection, were shown to be able to cross react with both West Nile Virus, Japanese Encephalitis Virus, and to a lesser extent to Tick Borne Encephalitis virus and Yellow Fever virus epitopes (Moran *et al.,* 2008). If study participant 1067 had HSV-gE specific CD4^{+} T cells that were able to bind to this VZV-gE epitope, (but at a lower affinity than CD4^{+} T cells that were specific for the VZV epitope) this could possibly explain why tetramer staining was seen, but at a lower magnitude than for other samples in this experiment. However, although cross reactive gB antibody epitopes have been identified between VZV and HSV (Kitamaru *et al.,* 1986; Edson *et al.,* 1985), no cross reactive epitopes have been reported for VZV T cell epitopes.

Assays carried out in naturally immune adult donors as part of this research and by others has demonstrated that the presence of low frequency tetramer specific CD4^{+} T cells also corresponds with a positive IFN-γ ELISPOT response, (Jones *et al.,* 2007; Malavige *et al.,* 2008a). In addition, the results presented in this chapter and by others (Jones *et al.,* 2007 and Malavige *et al.,* 2008a) also demonstrate that low frequencies of tetramer positive cells are capable of extensive proliferation, as seen with corresponding short term

day cultures. In one case (1018) both proliferative gE and IE63 tetramer-specific $CD4^+$ T cells, were identified following two doses of vaccine, and in another case, proliferative IE63 tetramer-specific $CD4^+$ T cells were identified 18 months post vaccination, despite this subject being seronegative at this time point.

4.4.5 Correlation Between Clinical Protection and CMI:

Although VZV-specific IFN-γ ELISPOT has been used to measure CMI in naturally immune and vaccinated subjects (Smith *et al.,* 2001; Levin *et al.,* 2003; Levin *et al.,* 2008; Jones *et al.,* 2006; Jones *et al.,* 2007; Malavige *et al.,* 2007; Malavige *et al.,* 2008a; Malavige *et al.,* 2008b) a threshold level of protection has not been identified which correlates with clinical protection. CMI data has been (or is being) obtained in the course of the vaccine approval process for a variety of pathogens including VZV, HIV, HPV, influenza A, smallpox, hepatitis C virus, as well as malaria, leishmania and tuberculosis (reviewed in Thomas *et al.,* 2009). However in a recent HIV vaccine trial carried out in high risk male and female populations, it was demonstrated that despite this vaccine being immunogenic and generating CMI in the vast majority of study participants this did not correlate with either protection from infection, or a reduction in viral load when study participants were infected, (Buchbinder *et al.,* 2008; McElrath *et al.,* 2008). CMI was measured using numerous methodologies, all of which indicated the vaccine induced a strong CMI responses in the majority of study participants; 77% (n=258) had *ex vivo* IFN-γ responses (with responses ranging from 163-686 SFUs/10^6PBMCs to each of the three proteins used in the vaccine); 41% (n=58) had HIV-specific $CD4^+$ T cells identified by intracellular cytokine staining, (with the median percentage of 88% expressing IL-2, with 72% co-expressing IFN-γ or TNF-α, or both). In addition, HIV-specific $CD8^+$ T cells were identified in 73% (n=117) participants, which expressed either IFN-γ alone or with TNF-α. This study indicated that although CMI responses of a

high magnitude may be measured *in vitro* and *ex vivo*, this does not necessarily correlate with clinical protection.

4.4.6 Concluding Remarks:

The results of this study and of others have confirmed that there are often discrepancies between correlates of cell mediated and humoral immunity, indicating that both arms of the immune system need to be examined in order to gain insight into the possible risk of infection. Despite the fact that CMI is important in host defence against varicella, no clinical correlate of CMI and protection from infection exists. However, the serology based FAMA assay has been shown to correlate with clinical protection (Gershon *et al.,* 1994a) and the presence of neutralizing antibodies (Grose *et al.,* 1979). In addition to this, separation of PBMCs from blood is laborious and highly technical in comparison to processing serum (or plasma) samples. In addition PBMCs, (unlike serology samples) are susceptible to degradation in long term or sub-optimal storage (Smith *et al.,* 2007). Therefore screening HCWs for susceptibility to infection is more practically suited to serology based assays.

CHAPTER 5: SUMMARY OF KEY FINDINGS AND FUTURE STUDIES:

A serology based assay to assess immune status in heathcare workers is preferable to a cell mediated based assay as early studies with Varivax demonstrated that antibody titres were a better correlate of efficacy than CMI, (Levin *et al.,* 2003b). In addition a positive FAMA score >2 has been shown to correlate with clinical protection (Gershon *et al.,* 1994a), and with the presence of neutralising antibody (Grose *et al.,* 1979), and as discussed in chapter 4, no clinical correlates have been shown for any T cell based assays. As discussed in chapter 3, the only serological assays which have been shown to correlate with clinical protection are either not suitable for high through put screening (FAMA) or are not widely available (gpELISA). There is a need to develop a widely available assay with a correlate of clinical protection as VZV immunity wanes over time and exposure to VZV may be more likely than for other viruses which do not establish latency, and thus a reservoir in the host population. A highly sensitive assay is required to screen HCWs to identify those who truly require vaccination, as vaccinating 'false negatives' creates an unnecessary economic burden. However, a high specificity is also required to ensure that 'false positives' are not excluded from vaccination; to eliminate the transmission risk to immunocompromised or naïve adult patients. Therefore, a larger, long term follow-up study of healthcare workers is needed to assess if a TRFIA cut-off of 130mIU/mL correlates with clinical protection. This study could also be used to assess is a TRFIA cut-off of 500mIU/mL after the second dose of vaccine (12 weeks) if a useful tool to identify subjects who require booster vaccination. A detailed reporting system of contacts would also be useful in assessing protection from infection.

This study identified different phenotypes of humoral immune responses to vaccination, and an association with ethnicity suggests genetic differences may underlie these phenotypic differences. In order to investigate this further, a large scale genetic study would need to be undertaken as part of the long

term follow-up study. As part of this genetic study, it would be useful to investigate innate immune response to vaccination, and whether such responses correlate with particular humoral subsets. As discussed in chapter 3, a lack of affinity maturation may be associated with innate immune responses, such as signalling through Toll like receptors. It would therefore be of use to attempt to identify any correlates of innate immunity and a lack of affinity maturation. Innate immune systems contribute significantly to the ability of vaccines to generate adaptive immune responses against pathogens and licensed vaccines (such as the live attenuated yellow fever vaccine; YF-17D) and adjuvants have been shown to activate innate immune signalling pathways and receptors, (Querec *et al.,* 2006). In subjects who were unable to produce high avidity antibodies many months/years post varicella vaccination, investigating whether they could produce high avidity antibodies to other pathogens would indicate that this phenomenon was varicella vaccine specific. If a lack of affinity maturation was shown to correlate with impaired immune responses, alternative adjuvants could be investigated for the vOka vaccine in an attempt to increase immunogenicity.

Carrying out a large scale study may also highlight differences that were not seen between subsets in this study, (such as age and gender, or a difference in ethnicity between primary responders who seroconvert following one or two doses of vaccine). Investigating a larger population of Asian HCWs could possibly identify if there was an association with lower antibody titre following vaccination, as we have shown following natural infection. In order to confirm some of the findings of this study, a larger Black population would need to be investigated, to examine lower antibody titre in primary responders and likelihood of being vaccine humoral non-responders (NLRs).

In addition, a large scale study could be used to phenotype T cell responses to vaccination, which was not possible as part of this study due to poor cell recovery. As discussed in chapter 4, larger volumes of blood for PBMC

analysis and shorter storage times would yield larger numbers of cells for tetramer and IFN-γ ELISPOT analysis.

Recently a 'systems biology' approach was used to examine innate immune response to the yellow fever YF-17D vaccine and identify markers which predicted the magnitude of the subsequent adaptive immune response (Querec *et al.,* 2009). Multiple techniques (such as gene expression profiling; multiplex analysis of cytokines and chemokines and multiparameter flow cytometry), in combination with computational modelling were used to identify the genes which correlated with adaptive immune responses. Several genes that regulate glucose transport and glycolysis in mammalian cells were found to be positive predictors of $CD8^+$ T cell response, (suggesting a possible role for glucose metabolism in regulating the $CD8^+$ T cell response), whilst a gene encoding for a B cell growth factor receptor was predictive of antibody responses to vaccine. However, although this approach facilitated the prediction of adaptive immune responses soon after vaccination, these were correlates of immunogenicity, and not a correlate of vaccine efficacy. However, if such an approach was undertaken in a long term follow-up study, it may be possible to correlate markers with clinical protection, rather than just immunogenicity.

CHAPTER 6: BIBLIOGRAPHY

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CHAPTER 7: APPENDICES

Appendix 7.1: ROVE Study Protocol

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STUDY PROTOCOL

Responses to Oka Vaccine Evaluation studies 1 and 2 (ROVEs 1 and 2)

Aims:

1. To determine whether a subset of viruses in the vaccine are responsible for complications (rash).
2. To evaluate the process whereby single vaccine viruses are found in rash vesicles despite a mixture of viruses being inoculated in the vaccine itself
3. To evaluate the relationship between development of rash and the immune response in blood and saliva
4. To evaluate the host factors, including HLA which might contribute to poor immune response or complications

Background:

Since December 2003 a programme of immunisation of seronegative healthcare workers has been introduced in the UK. The main aims of the programme were to prevent staff infecting seronegative patients and to reduce the cost to trusts associated with prevention of chickenpox in staff and patients. However, since chickenpox even in healthy adults, is a serious disease, the programme is directly of benefit to the healthcare workers themselves. Almost without exception, seronegative adults acquire

chickenpox naturally over time, either from household contact or in the case of healthcare workers occasionally from occupational exposure[1]. Complications from natural chickenpox, including pneumonitis and encephalitis, are over 100 times more common than in children and mortality is at least 20 times higher[1]. By contrast the Oka vaccine is very safe for adults. Serious complications requiring hospitalisation have been reported in fewer than one in 10,000 subjects as compared to 1 in 400 in wild type infection[1]. No vaccine related deaths in adults and children have been recorded in 40 million doses administered in the USA as compared with a mortality of 1 in 4,000 in adults infected with wild type chickenpox. Inadvertent immunisation of 362 pregnant women has resulted in no congenital infections or abnormalities[1].

The main side effect of the Oka vaccine is a rash, which occurs in 5-10% of adults[1]. Although vaccine rashes are mild (a median of 60 lesions versus 300 in wild type infection) they can be inconvenient for the health care worker, who may need to be off work, and costly for their trust.

Preliminary evidence suggests that the viruses that cause rashes may not be representative of the mixture of viruses in the original vaccine, suggesting the possibility that a selective biological process is occurring. Some of the rash viruses my have a biological advantage for growth in skin, while others appear to have acquired mutations, which have enabled them to escape from the immune system. Understanding the basis for rash formation after vaccination may enable us to improve the safety and efficacy of the vaccine formulation. Such improvements are likely to improve vaccine uptake among health care workers that will be of benefit for them and their patients.

Reference:

1. Gershon A, Takahashi M, Seward J.Varicella vaccine. In: Plotkin S, Orenstein W, eds. Vaccines. 4th ed. Philadelphia: Saunders, **2003**:783–823.

Inclusion Criteria:

1. All seronegative Healthcare workers receiving Oka vaccine

[ROVES 1]

2. All Health care workers who develop fever, local blister, disseminated rash, or other complication following vaccination. **ROVES 1 and ROVES 2** (patients not enrolled at baseline but who agree to participate if a vaccine related event occurs).

Baseline Visit:

All healthcare workers are given Oka vaccine dose 1 as usual

All health care workers are given the Oka information sheet PLS as usual.

ROVE 1 Only:

1. Consent healthcare worker for inclusion in the study
2. Consent healthcare worker for informing their GP of the study
3. Compete questionnaire V1
4. Obtain baseline samples (three blood samples totaling 20mLs will be taken, see below)
   - Serum for baseline VZV antibody testing
   - CPT blood (bottles available from HPA Varicella Ref Lab*) for baseline cellular immunity to varicella
   - EDTA for VZV viral load if necessary
   - Salivary swab
5. Send blood and questionnaire in pack by courier to HPA VZV reference laboratory*
6. Send letter to GP

GIVE FIRST DOSE VACCINE

- Note Batch Manufacturer.
- Retain vaccine vial until final visit.
- Issue HCW with ROVE study card (overleaf).

ROVE STUDY CARD

Name _____ Hospital _____ROVEs No_____

If you have any questions about the study please contact:
_____in _____
OR
Fiona Scott, ROVE study nurse _____
OR
Professor Judy Breuer principal investigator_____

If you develop any of the following symptoms in the 6 weeks following your chickenpox vaccine:

1. Temperature/flu like symptoms
2. Local blister
3. Rash
4. Any other symptoms

Please telephone Fiona Scott ROVE study nurse_____
OR
Occupational health--_____

Vaccine Related Event (VRE)[1] occurring during weeks 1-6:

ROVEs 1 (Patients Already Enrolled):

1. Administer Vaccine Related Event questionnaire
2. Obtain Samples :

   Three blood samples totalling 20mls will be taken(see below)

   a. Fluid/crusts from at least 3 vesicles in separate saline pots EDTA blood
   b. CPT sodium heparin blood
   c. Serum for baseline VZV antibody testing
   d. CPT blood (bottles available from HPA Varicella Ref Lab*)for baseline cellular immunity to varicella
   e. EDTA for VZV viral load if necessary
   f. Salivary swab
3. Send blood and questionnaire in pack by courier to HPA VZV reference laboratory*

ROVEs 2 (For Patients Who Were Not Enrolled at Baseline):

1. Consent healthcare worker for inclusion in the study
2. Consent healthcare worker for informing their GP of the study
3. Complete questionnaire V1
4. Complete questionnaire for Vaccine Related Events
5. Obtain samples (three samples totalling 20mls will be taken, see below)
    a. Fluid/crusts from at least 3 vesicles in separate saline pots EDTA blood
    b. CPT sodium heparin blood
    c. Serum for VZV antibody testing
    d. CPT blood (bottles available from HPA Varicella Ref Lab*)for cellular immunity to varicella
    e. EDTA for VZV viral load
    f. Salivary swab
6. Send blood and questionnaire in pack by courier to HPA VZV reference laboratory*
7. Send letter to GP

Patients whose rashes are found to be due to wild type virus can be counselled that they have had ordinary chickenpox and now have natural protection. In these cases, there will be no need to administer the second dose of vaccine. Please note, this advice should be given to all patients with post vaccine rashes due to wild type virus irrespective of whether they are in the study or not.

Visit 2 Week 6: GIVE SECOND DOSE VACCINE

- Note Batch Manufacturer.
- Retain vaccine vial until final visit.
- Issue HCW with ROVE study card (below).

1. Complete questionnaire V2
2. Obtain samples (three blood samples totalling 20 mls will be taken, see below)
    a. Serum for VZV antibody testing
    b. CPT blood (bottles available from HPA Varicella Ref Lab*) for cellular immunity to varicella
    c. EDTA for VZV viral load
    d. Salivary swab
3. Send blood and questionnaire in pack by courier to HPA VZV reference laboratory*
4. Ensure HCW has ROVE card

Vaccine Related Event (VRE)[1] Occurring During Weeks 6-12:

ROVEs 1 (Patients Already Enrolled):

1. Administer Vaccine Related Event questionnaire
2. Obtain Samples : (three blood samples totalling 20mls will be taken, see below)
    a. Fluid/crusts from at least 3 vesicles in separate saline pots EDTA blood
    b. CPT sodium heparin blood
    c. Serum for baseline VZV antibody testing
    d. CPT blood (bottles available from HPA Varicella Ref Lab*)for baseline cellular immunity to varicella
    e. EDTA for VZV viral load if necessary
    f. Salivary swab
3. Send blood and questionnaire in pack by courier to HPA VZV reference laboratory*

ROVEs 2 (For Patients Who Were Not Enrolled At Baseline):

1. Consent healthcare worker for inclusion in the study
2. Consent healthcare worker for informing their GP of the study
4. Complete questionnaire V1
5. Complete questionnaire for Vaccine Related Events
6. Obtain samples (three blood samples totalling 20mls will be taken, see below)
    a. Fluid/crusts from at least 3 vesicles in separate saline pots EDTA blood
    b. CPT sodium heparin blood
    c. Serum for VZV antibody testing
    d. CPT blood (bottles available from HPA Varicella Ref Lab*)for cellular immunity to varicella
    e. EDTA for VZV viral load
    f. Salivary swab
7. Send blood and questionnaire in pack by courier to HPA VZV reference laboratory*
8. Send letter to GP

Week 12-18: all HCWs in ROVES 1 and 2:

1. Complete questionnaire V2
2. Obtain samples (three blood samples totalling 20mls will be taken (see below)
    a. Serum for VZV antibody testing
    b. CPT blood (bottles available from HPA Varicella Ref Lab*) for cellular immunity to varicella
    c. EDTA for VZV viral load
    d. Salivary swab
3. Send blood and questionnaire in pack by courier to HPA VZV reference laboratory*

Extra work for OHD:

1. Consenting HCW at baseline
2. Extra visit at 12-18 weeks to check seroconversion
3. Blood samples at 0,6,12 weeks and if rash appears

Advantages for HCW and OHD:

1. Confirmation of seroconversion to VZV
2. Investigation of vaccine related events

1. Definition of vaccine related event
   Pyrexia/Flu like symptoms
   Inoculation blister
   Rash (localised or generalised)
   Other including pneumonitis, encephalitis, hepatitis requiring admission to hospital.

Appendix 7.2: ROVE Study Questionnaires and VRE Form:

Responses to Oka Vaccine Evaluation Study (ROVE)
(V1) Baseline Questionnaire (1st Dose of Vaccine)

Study No:_____

Date __/___/____ Hospital: _____ Surname_____

First Name_____Ethnicity:_____ Sex: _____

Country of Birth:_____ Age of entry to UK:_____

Contact Phone No:_____ DOB___/___/_____

VZV antibody negative pre-vaccination: Yes ☐ Equivocal ☐

Have you had Chickenpox? Yes ☐ Age:_____ No ☐ Don't Know ☐

Name and manufacturer of screening assay: _____

Vaccine 1st dose

Manufacturer: GlaxoSmithKline / Aventis Pasteur (please delete one)

Batch /lot Number of _____ *Date of vaccination* ___/___/____

Recent Chickenpox or Shingles contacts (last 4 weeks) Yes ☐ No ☐

If yes:

Chickenpox Household? Yes ☐ Number of days since contact[1] _____

Occupational? Yes ☐ Number of days since contact[1] _____

Other (describe)_____ Number of days since contact[1] _____

Shingles Household? Yes ☐ Number of days since contact[1] _____

Occupational? Yes ☐ Number of days since contact[1] _____

Other (describe) _____ Number of days since contact[1]_____

Samples: 2 Sodium Heparin ☐ 1 Serum ☐ 1 EDTA ☐ 1 Saliva ☐

---

1. Days since first contact

Responses to Oka Vaccine Evaluation Study (ROVE)
Vaccine Related Events Form

VRE No: _____

Study No:_____ *Date __/___/____ Hospital:*

Date of last vaccination ___/___/____

Manufacturer: GlaxoSmithKline / Aventis Pasteur (please delete one)

Batch /lot Number of _____

Date of onset of symptoms ___/_____/___

Nature of symptoms

Rash at injection site: ☐ No. of Lesions: ___

Localised rash **NOT** inj. site: ☐ No. of Lesions: ___

Generalised rash: ☐ No. of lesions _____

Pyrexia/flu like symptoms:_____ Temp:_____° C

Other :_____

Swab of inoculation site X 1 ☐

Swab of lesion 1 in saline X 1 ☐ [2]Swab of lesion 2 in separate saline pot X 1 ☐

Swab of lesion 3 X ☐ in separate saline pot

Samples: 2 Sodium Heparin ☐ 1 Serum ☐ 1 EDTA ☐ 1 Saliva ☐

Date of recovery__/___/_____

Responses to Oka Vaccine Evaluation Study (ROVE)
(V2) Six Week Visit Questionnaire (2nd dose of Vaccine)

Study No:_____ *Date* __/___/____ *Hospital:* _____

No. of weeks since previous vaccination? _____

Manufacturer: GlaxoSmithKline / Aventis Pasteur (please delete one)

2nd Batch /lot Number of _____*Date of 2nd vaccination* ___/___/____

Any Chickenpox or Shingles contacts since last dose? Yes ☐ No ☐

Chickenpox Household? Yes ☐ Number of days since contact[1] _____

Occupational? Yes ☐ Number of days since contact[1] _____

Other (describe)_____ Number of days since contact[1] _____

Shingles Household? Yes ☐ Number of days since contact[1] _____

Occupational? Yes ☐ Number of days since contact[1] _____

Other (describe) _____ Number of days since contact[1]_____

Post vaccination complications? Yes ☐ No ☐

If Yes: Was vaccine related event form filled in ? Yes ☐ No ☐

V2 Samples: 2 Sodium Heparin ☐ 1 EDTA ☐ 1 Serum ☐ 1 Saliva ☐

1.Days since first contact

Responses to Oka Vaccine Evaluation Study (ROVE)
(V3) 12-18 Week Visit Questionnaire (Final Visit)

Study No:_____ *Date __/___/____ Hospital:*_____

No. of weeks since previous vaccination? _____

Any Chickenpox or Shingles contacts since last dose? Yes ☐ No ☐

Chickenpox Household? Yes ☐ Number of days since contact[1] _____

Occupational? Yes ☐ Number of days since contact[1] _____

Other (describe)_____ Number of days since contact[1] _____

Shingles Household? Yes ☐ Number of days since contact[1] _____

Occupational? Yes ☐ Number of days since contact[1] _____

Other (describe) _____ Number of days since contact[1]_____

Post vaccination complications? Yes ☐ No ☐

If Yes: Was vaccine related event form filled in ? Yes ☐ No ☐

V3 Samples: 2 Sodium Heparin ☐ 1 Serum ☐ 1 EDTA ☐ 1 Saliva ☐

---

1. Days since first contact

Responses to Oka Vaccine Evaluation Study (ROVE)
(V4) 18 Month Visit Questionnaire (Follow-up Visit)

Study No:_____ Date __/___/____ Hospital: _____

Surname_____ First Name_____

Contact Phone No:_____

Number of months since 2nd vaccination _____

Have you had any Chickenpox or Shingles <u>contacts</u> since vaccination?
Yes ☐ No ☐

If yes:

Chickenpox Number of contacts ___

Household? Yes ☐ Number of weeks since last contact ___

Occupational? Yes ☐ Number of weeks since last contact ___

Other (describe)_____
Number of weeks since last contact ___

Shingles Number of contacts ___

Household? Yes ☐ Number of weeks since last contact ___

Occupational? Yes ☐ Number of weeks since last contact ___

Other (describe)_____
Number of weeks since last contact ___

Have you <u>had</u> Chickenpox or Shingles since your last visit?
Yes ☐ No ☐

If yes:

Chickenpox Yes ☐ Number of weeks since illness ___

Severity of rash _____

Complications _____

Number of days lost from work ___

Shingles Yes ☐ Number of weeks since illness ___

Severity of rash _____

Location of Rash _____

Complications _____

Number of days lost from work ___

Did you transmit the virus to anyone else? **Yes** ☐ **No** ☐

If yes:

Member of household **1.**_____ **Age**___

Number of days after your infection ___

2._____ **Age**__

Number of days after your infection ___

3._____ **Age**___

Number of days after your infection ___

Patient **1.**_____ **Age**___

Number of days after your infection ___

2._____ **Age**__

Number of days after your infection ___

Other **1.**_____ **Age**___

Number of days after your infection ___

2._____ **Age**__

Number of days after your infection ___

Other relevant details_____

Samples: 2 Sodium Heparin ☐ 2 Serum ☐ 1 EDTA ☐ 1 Saliva ☐

Apendix 7.3: Vaccine Brand Administered to Each Study Participant:

Study Number	Brand of vaccine	Vaccine Batch Number First Dose	Vaccine Batch Number Second Dose
1001	Merck	HV50280	HW04720
1002	Merck	HV50280	HW04720
1003	GSK	AD06A0431	AD06A0531
1004	GSK	AD06A0431	AD06A0531
1005	GSK	AD06A0431	AD06A0531
1006	GSK	AD06A0531	AD0680531
1007	GSK	AD06A0531	AD06A0531
1008	GSK	AD06A0531	AD06A0531
1009	GSK	AD06A0531	AD06A0531
1010	Merck	HV50280	Withdrew from study
1011	Merck	HW04720	HW04720
1012	Merck	HW04720	HW04720
1013	Merck	HV50280	HW04720
1014	Merck	HV40280	HW04720
1015	Merck	HV50280	HW04720
1016	Merck	HV50280	HW04720
1017	Merck	HV50280	HW04720
1018	Merck	HV50280	NA29040
1019	Merck	HV50280	HW04720
1020	Merck	HV04720	HW04720
1021	Merck	HV50280	HW04720
1022	Merck	HV50280	HW04720
1023	Merck	HV50280	HW04720
1024	Merck	HW04720	NA29040
1025	Merck	HW04720	HW04720
1026	Merck	HW04720	HW04720
1027	Merck	HW04720	HW04720
1028	Merck	HW04720	NA29040
1029	Merck	HW04720	NA29040
1030	Merck	HW04720	NA29040
1031	Merck	HW04720	NA29040
1032	Merck	HW04720	NA29040

Appendix 7.3 continued 1 of 4

Study Number	Brand of vaccine	Vaccine Batch Number First Dose	Vaccine Batch Number Second Dose
1033	Merck	HW04720	NA29040
1034	Merck	HW04720	NA29040
1035	Merck	HW04720	HW04720
1036	Merck	HW04720	HW04720
1037	Merck	HW04720	NA29040
1038	Merck	HW04720	HW04720
1039	Merck	HW04720	NA29040
1040	Merck	HW04720	NA29040
1041	Merck	HW04720	NA29040
1042	Merck	HV50280	NA29040
1043	Merck	HW04720	NA29040
1044	Merck	HW04720	NA29040
1045	Merck	NA29040	NA29040
1046	Merck	HW04720	NA29040
1047	Merck	HW04720	NA29040
1048	Merck	HW04720	NA29040
1049	Merck	HW04720	NA29040
1050	Merck	NA29040	NA29040
1051	Merck	NA29040	NA29040
1052	Merck	NA29040	Withdrew from study
1053	Merck	NA29040	Withdrew from study
1054	Merck	NA29040	NA29040
1055	Merck	NA29040	NA29040
1056	Merck	NA29040	NA29040
1057	Merck	NA29040	NA29040
1058	Merck	NA29040	NA29040
1059	Merck	NA29040	NA29040
1060	Merck	NA29040	NA29040
1061	Merck	NA29040	NA29040
1062	Merck	NA29040	NA29040
1063	Merck	NA29040	NA29040
1064	Merck	NA29040	NA29040
1065	Merck	NA29040	NA29040

Study Number	Brand of vaccine	Vaccine Batch Number First Dose	Vaccine Batch Number Second Dose
1066	Merck	NA29040	NA29040
1067	Merck	NA29040	NA29040
1068	Merck	NA29040	NA29040
1069	Merck	NA29040	NC38010
1070	Merck	NA29040	NC38010
1071	Merck	NA29040	NC38010
1072	Merck	NA29040	NC38010
1073	Merck	NA29040	NC38010
1074	Merck	NA29040	NC38010
1075	Merck	NA29040	NC38010
1076	Merck	NA29040	NC38010
1077	Merck	NA29040	NC38010
1078	Merck	NA29040	NC38010
1079	Merck	NA29040	NC38010
1080	Merck	NA29040	NC38010
1081	Merck	NA29040	NC38010
1082	Merck	NA29040	NC38010
1083	Merck	NA29040	NC38010
1084	Merck	NC38010	Withdrew from study
1085	Merck	NC38010	NC38010
1086	Merck	NC38010	NC38010
1087	Merck	NC38010	NC38010
1088	Merck	NC38010	NC38010
1089	Merck	NC38010	NC38010
1090	Merck	NC38010	NC38010
1091	Merck	NC38010	NC38010
1092	Merck	NC38010	NC38010
1093	Merck	NC38010	NC38010
1094	Merck	NC38010	NC38010
1095	Merck	NC38010	NC38010
1096	Merck	NC38010	NC38010
1097	Merck	NC38010	NC38010
1098	Merck	NC38010	NC38010

Appendix 7.3 continued 3 of 4

Study Number	Brand of vaccine	Vaccine Batch Number First Dose	Vaccine Batch Number Second Dose
1099	Merck	NC38010	NC38010
1100	Merck	NC38010	NC38010
1101	Merck	NC38010	NC38010
1102	Merck	NC38010	NC38010
1103	Merck	NC38010	NC38010
1104	Merck	NC38010	NC38010
1105	Merck	NC38010	NC38010
1106	Merck	NC38010	NC38010
1107	Merck	NC38010	NC38010
1108	Merck	NC38010	NC38010
1109	Merck	NC38010	NC38010
1110	Merck	NC38010	NC38010

Appendix 7.3: Manufacture Brand of Vaccine Received by Each Study Number Enrolled in the ROVE Study Between July 2005–November 2006. Key: GSK; GlaxoSmithKline.

Appendix 7.4: Length of Time between Visits for Each Study Participant:

	Visit 2	Visit 3	Visit 4
Study Number	Weeks Elapsed Since First Dose	Weeks Elapsed Since Second Dose	Months Elapsed Since Second Dose
1001	7	6	18
1002	7	7	-
1003	6	6	27
1004	6	7	21
1005	8	6	18
1006	6	6	-
1007	7	6	18
1008	6	8	21
1009	7	8	18
1010	Withdrew from study	Withdrew from study	-
1011	6	8	21
1012	7	7	-
1013	7	6	16
1014	8	Withdrew from study	-
1015	6	7	17
1016	6	6	25
1017	6	6	17
1018	7	6	16
1019	6	6	16
1020	8	8	16
1021	6	6	18
1022	6	6	16
1023	5	9	17
1024	6	8	18
1025	8	7	17
1026	6	11	17
1027	6	9	13
1028	7	8	-
1029	6	6	17
1030	6	6	-
1031	6	10	18
1032	6	7	17

Appendix 7.4 continued 1 of 4

	Visit 2	Visit 3	Visit 4
Study Number	Weeks Elapsed Since First Dose	Weeks Elapsed Since Second Dose	Months Elapsed Since Second Dose
1033	6	7	-
1034	6	7	19
1035	7	7	19
1036	6	9	17
1037	6	6	-
1038	6	7	-
1039	9	7	19
1040	9	7	-
1041	8	7	18
1042	6	6	-
1043	6	6	-
1044	6	7	-
1045	7	7	-
1046	7	9	18
1047	7	7	20
1048	6	7	18
1049	6	6	17
1050	6	8	17
1051	6	6	-
1052	7	Withdrew from study	-
1053	7	Withdrew from study	-
1054	7	7	16
1055	6	7	-
1056	7	12	18
1057	7	10	16
1058	7	8	16
1059	6	6	-
1060	7	6	16
1061	7	6	16
1062	6	6	16
1063	7	9	16
1064	7	7	16
1065	6	7	19

Appendix 7.4 continued 2 of 4

	Visit 2	Visit 3	Visit 4
Study Number	Weeks Elapsed Since First Dose	Weeks Elapsed Since Second Dose	Months Elapsed Since Second Dose
1066	8	Withdrew from study	-
1067	6	7	16
1068	6	7	-
1069	8	7	15
1070	6	7	15
1071	6	6	16
1072	6	8	17
1073	6	12	-
1074	10	6	14
1075	6	7	16
1076	7	7	14
1077	9	13	-
1078	6	6	-
1079	6	6	-
1080	6	6	-
1081	8	13	15
1082	6	6	-
1083	6	7	17
1084	Withdrew from study	Withdrew from study	16
1085	7	13	16
1086	7	15	-
1087	6	9	15
1088	6	10	15
1089	6	8	16
1090	6	11	16
1091	6	9	14
1092	6	7	14
1093	8	6	-
1094	7	6	14
1095	6	6	15
1096	6	7	-
1097	8	9	14
1098	7	10	14

Appendix 7.4 continued

	Visit 2	Visit 3	Visit 4
Study Number	Weeks Elapsed Since First Dose	Weeks Elapsed Since Second Dose	Months Elapsed Since Second Dose
1099	8	10	14
1100	6	7	14
1101	6	7	-
1102	6	7	14
1103	6	7	-
1104	6	7	-
1105	6	6	12
1106	7	10	14
1107	7	7	12
1108	9	9	12
1109	6	8	13
1110	10	8	12

Appendix 7.4: Length of Time between Visits for ROVE Study Participants.
Key: - Did not participate at Visit 4 (18 month follow up).

Appendix 7.5: Demographic Data for Each Study Participant:

ROVE Study Number	Gender	Age at Enrolment£	Ethnicity	Birth Country	Age Relocated to UK	Years Resided in UK
1001	M	35	Cauc	Australia	21	14
1002	M	28	Black Af	Nigeria	25	3
1003	F	23	Black Cbn	Trinidad	18	6
1004	F	30	Phil	Philippines	28	2
1005	F	31	Phil	Philippines	29	2
1006	F	44	Black Cbn	Jamaica	40	5
1007	F	32	Phil	Philippines	30	2
1008	F	41	Ind Sub	Trinidad	26	15
1009	F	34	Black Cbn	UK	0	35
1010	M	29	Ind Sub	India	25	4
1011	F	35	Phil	Philippines	8	27
1012	M	40	Black Cbn	Trinidad	20	21
1013	F	46	Cauc	UK	0	46
1014	F	21	Cauc	UK	0	21
1015	F	28	Black Af	Zimbabwe	23	5
1016	F	37	Black Cbn	Grenada	33	4
1017	F	29	Black Cbn	Jamaica	28	1
1018	F	33	Phil	Philippines	31	3
1019	F	40	Cauc	UK	0	40
1020	F	24	Cauc	UK	0	25
1021	F	31	Ind Sub	India	29	3
1022	F	31	Cauc	Hong Kong	2	29
1023	F	42	Black Af	Nigeria	21	22
1024	M	39	Black Af	Uganda	28	11
1025	M	38	Ind Sub	India	29	9
1026	F	27	Phil	Philippines	27	0
1027	F	49	Black Af	Ghana	46	4
1028	M	20	Black Cbn	Montserrat	10	10
1029	F	38	Black Af	Ghana	27	11
1030	M	47	Black Af	Nigeria	44	4
1031	F	27	Cauc	UK	0	28
1032	M	35	Black Af	Uganda	28	8

Appendix 7.5 continued

ROVE Study Number	Gender	Age at Enrolment£	Ethnicity	Birth Country	Age Relocated to UK	Years Resided in UK
1033	F	28	Black Cbn	Trinidad	21	7
1034	F	38	Black Af	Ghana	31	7
1035	F	24	Black Af	Nigeria	14	10
1036	F	33	Black Cbn	Trinidad	19	14
1037	F	34	Black Af	Ghana	24	11
1038	F	44	Ind Sub	Mauritius	39	5
1039	F	29	Black Cbn	Jamaica	24	5
1040	F	28	Other	Iran	2	26
1041	F	27	Ind Sub	India	24	3
1042	F	30	Black Cbn	Jamaica	26	5
1043	F	21	Black Af	Ghana	12	9
1044	F	32	Other	South Africa	26	6
1045	F	30	Phil	Philippines	24	6
1046	F	28	Other	South Africa	21	8
1047	F	25	Ind Sub	India	25	1
1048	M	44	Black Af	Nigeria	41	3
1049	F	29	Black Cbn	Tobago	24	6
1050	M	36	Black Af	Nigeria	19	17
1051	M	36	Ind Sub	Mauritius	31	5
1052	F	31	Ind Sub	India	31	1
1053	F	29	Cauc	UK	0	29
1054	F	28	Ind Sub	Bangladesh	1	27
1055	M	34	Black Af	Nigeria	32	2
1056	F	25	Phil	Philippines	25	1
1057	M	58	Phil	Philippines	29	29
1058	F	39	Cauc	UK	0	39
1059	F	43	Black Af	Ghana	39	5
1060	F	30	Cauc	Germany	20	11
1061	M	61	Black Cbn	Trinidad	56	5
1062	F	33	Black Af	Nigeria	29	4
1063	M	29	Black Af	Ghana	20	9
1064	F	24	Cauc	UK	0	24
1065	F	24	Ind Sub	India	23	1

Appendix 7.5 continued 2 of 4

ROVE Study Number	Gender	Age at Enrolment£	Ethnicity	Birth Country	Age Relocated to UK	Years Resided in UK
1066	M	35	Ind Sub	India	30	6
1067	F	27	Ind Sub	India	27	1
1068	M	34	Ind Sub	Sri Lanka	34	1
1069	F	29	Black Af	Sierra Leione	25	4
1070	M	29	Cauc	UK	0	30
1071	F	25	Ind Sub	India	24	2
1072	F	27	Cauc	UK	0	27
1073	F	30	Black Af	Ghana	25	5
1074	F	34	Black Cbn	Jamaica	27	7
1075	F	32	Black Af	Burundi	26	6
1076	F	29	Cauc	UK	0	30
1077	F	30	Black Cbn	Jamaica	24	7
1078	F	44	Cauc	Eire	24	21
1079	M	40	Black Af	Nigeria	35	6
1080	M	32	Black Af	Ghana	26	6
1081	F	19	Black Af	Nigeria	11	8
1082	F	23	Ind Sub	Switzerland	12	12
1083	M	28	Cauc	UK	0	29
1084	F	22	Black Af	Sierra Leione	17	6
1085	F	40	Ind Sub	India	36	4
1086	F	22	Black Af	Germany	18	5
1087	F	31	Ind Sub	Bangladesh	26	5
1088	M	21	Cauc	UK	0	22
1089	F	25	Black Cbn	Jamaica	20	6
1090	F	19	Cauc	Ecuador	5	14
1091	F	26	Black Cbn	Montserrat	17	9
1092	M	45	Cauc	UK	0	45
1093	F	41	Black Af	Ghana	37	4
1094	M	22	Ind Sub	UK	0	23
1095	F	26	Cauc	UK	0	27
1096	M	33	Ind Sub	UK	0	33
1097	F	27	Ind Sub	India	27	1
1098	F	19	Ind Sub	UK	0	20

Appendix 7.5 continued 3 of 4

ROVE Study Number	Gender	Age at Enrolment£	Ethnicity	Birth Country	Age Relocated to UK	Years Resided in UK
1099	F	25	Ind Sub	India	24	2
1100	F	31	Black Cbn	Trinidad	26	5
1101	F	32	Black Af	Ghana	20	13
1102	M	23	Cauc	Spain	21	3
1103	F	33	Cauc	Australia	28	6
1104	F	29	Cauc	Russia	23	6
1105	M	59	Cauc	UK	0	59
1106	F	28	Cauc	Australia	26	2
1107	M	29	Ind Sub	India	28	1
1108	F	31	Cauc	UK	0	32
1109	F	50	Cauc	Portugal	39	11
1110	F	20	Black Cbn	Jamaica	15	6

Appendix 7.5: Demographic Data for Each ROVE Study Participant.
Key: F; Female, M; Male, £ Age has been taken as the age at their last birthday rather than decimalised (e.g. 26 rather than 26.86), Cauc; Caucasian, Black Af; Black African, Black Cbn, Black Caribbean, Phil; Pilipino, Ind sub; Indian subcontinent.

Appendix 7.6: TRFIA Titre (mIU/mL and log_{10}) for Each Study Participant at Each Visit

	Baseline (Visit 1)		Six Weeks (Visit 2) Immune Response to First Dose of Vaccine		12 weeks (Visit 3) Immune Response to Second Dose of Vaccine		18 Month Follow-Up (Visit 4)	
ROVE Study Number	Log_{10} TRFIA	TRFIA mIU/mL	Log_{10} TRFIA	TRFIA mIU/mL	Log_{10} TRFIA	TRFIA mIU/mL	Log_{10} TRFIA	TRFIA mIU/mL
1001	2.11	130	2.32	209	2.36	229	1.94	87
1002	1.79	62	1.95	89	1.65	45	-	-
1003	1.68	48	1.94	87	2.41	257	2	100
1004	2.33	214	3.09	1230	3.02	1023	3.14	1380
1005	2.33	214	3.32	2089	3.24	1738	2.84	692
1006	1.74	55	2.54	347	2.65	447	-	-
1007	1.4	23	1.93	85	2.8	631	1.4	23
1008	1.3	20	1.91	81	2.64	437	2.16	145
1009	3	1000	3.11	1288	2.95	891	2.86	724
1010	1.34	22	WFS	WFS	WFS	WFS	WFS	WFS
1011	3.19	1549	3.58	3802	3	1000	2.71	513
1012	2.09	123	2.32	209	3	1000	-	-
1013	2.26	182	2.85	708	2.89	776	2.67	468
1014	2.16	145	3.13	1349	WFS	WFS	-	-
1015	1.57	37	1.98	95	2.63	427	1.87	74
1016	1.88	76	2.49	309	3	1000	2.63	427
1017	1.81	65	2.1	126	2.57	372	2.17	148
1018	1.86	72	2.25	178	2.6	398	3.31	2042
1019	1.32	21	1.81	65	2.51	324	1.18	15
1020	2.81	646	3.15	1413	3	1000	3.16	1445
1021	1.51	32	1.75	56	2.67	468	3.11	1288
1022	1.18	15	1.45	28	2.62	417	1.54	35
1023	2.42	263	3.22	1660	2.93	851	2.78	603
1024	1.54	35	2.17	148	2.45	282	2.05	112
1025	1.36	23	2.12	132	2.51	324	1.81	65
1026	1.61	41	2.11	130	2.68	479	2.06	115
1027	1.82	66	2.07	117	2.1	126	2.09	123
1028	1.83	68	2.35	224	2.78	603	-	-
1029	2.97	933	3.05	1122	2.93	851	2.76	575
1030	2.26	182	3.11	1288	3	1000	-	-
1031	3.18	1513	3.48	3020	3.31	2042	3.39	2455
1032	1.97	93	2.92	832	2.67	468	2.29	195

Appendix 7.6 continued 1 of 4

	Baseline (Visit 1)		6 Weeks (Visit 2) Immune Response to First Dose of Vaccine		12 weeks (Visit 3) Immune Response to Second Dose of Vaccine		18 Month Follow-Up (Visit 4)	
ROVE Study Number	Log_{10} TRFIA	TRFIA mIU/mL	Log_{10} TRFIA	TRFIA mIU/mL	Log_{10} TRFIA	TRFIA mIU/mL	Log_{10} TRFIA	TRFIA mIU/mL
1033	1.86	72	2	100	2.26	182	-	-
1034	1.45	28	2.06	115	2.76	575	2.14	138
1035	1.57	37	2.19	155	2.57	372	2.05	112
1036	1.65	45	1.86	72	2.09	123	1.77	59
1037	1.84	69	1.93	85	3.13	1349	-	-
1038	2.36	229	2.83	676	3.13	1349	-	-
1039	1.89	78	1.91	81	2.15	141	1.95	89
1040	1.62	42	2.35	224	3	1000	-	-
1041	1.64	44	1.69	49	2.37	234	2.03	107
1042	1.86	72	2.56	363	2.61	407	-	-
1043	1.48	30	1.56	36	2.38	240	-	-
1044	2.28	191	2.95	891	2.86	724	-	-
1045	2.16	145	2.84	692	3.27	1862	-	-
1046	2.6	398	2.26	182	3.34	2188	2.84	692
1047	1.48	30	2.58	380	2.74	550	2.07	117
1048	1.49	31	2.36	229	2.34	219	3.27	1862
1049	1.79	62	2.55	355	2.84	692	2.52	331
1050	1.26	18	1.46	29	1.89	78	2.45	282
1051	2.26	182	2.63	427	2.8	631	-	-
1052	1.28	19	2.43	269	WFS	WFS	-	-
1053	1.58	38	2.83	676	WFS	WFS	-	-
1054	1.53	34	2.1	126	2.51	324	2.15	141
1055	2.27	186	2.36	229	2.6	398	-	-
1056	1.93	85	2.29	195	2.48	302	2.51	324
1057	2.47	295	3	1000	3.16	1445	2.78	603
1058	1.36	23	2.42	263	2.89	776	2.35	224
1059	1.86	72	2.16	145	2.47	295	-	-
1060	1.56	36	2.26	182	2.9	794	2.32	209
1061	*	*	2.13	135	3.1	1259	2.34	219
1062	2.29	195	2.86	724	3.01	1023	2.61	407
1063	2.13	135	2.09	123	2.45	282	2.36	229
1064	2.56	363	3.17	1479	2.93	851	2.89	776
1065	1.3	20	2.45	282	3.03	1072	2.53	339

Appendix 7.6 continued

	Baseline (Visit 1)		6 Weeks (Visit 2) Immune Response to First Dose of Vaccine		12 weeks (Visit 3) Immune Response to Second Dose of Vaccine		18 Month Follow-Up (Visit 4)	
ROVE Study Number	Log_{10} TRFIA	TRFIA mIU/mL	Log_{10} TRFIA	TRFIA mIU/mL	Log_{10} TRFIA	TRFIA mIU/mL	Log_{10} TRFIA	TRFIA mIU/mL
1066	2.11	130	2.53	339	WFS	WFS	-	-
1067	1.74	55	2.26	182	2.74	550	2.11	130
1068	1.2	16	1.92	83	2.83	676	-	-
1069	1.41	26	1.62	42	2.89	776	2.16	145
1070	1.49	31	1.93	85	3.1	1259	2.33	214
1071	1.2	16	2.22	166	2.66	457	2.45	282
1072	2.76	575	3.47	2951	2.82	661	2.89	776
1073	1.28	19	1.96	91	2.35	224	-	-
1074	2.29	195	3.12	1318	2.92	832	2.35	224
1075	2.45	282	3.64	4365	3.14	1380	3	1000
1076	2.06	115	3.35	2239	3.21	1622	3.28	1905
1077	1.61	41	2.38	240	2.34	219	-	-
1078	2.2	158	3.88	7586	2.94	871	-	-
1079	1.89	78	2.56	363	2.94	871	-	-
1080	2.52	331	3.32	2089	3.04	1096	-	-
1081	3.4	2512	3.21	1622	3.06	1148	3.28	1905
1082	1.23	17	1.93	85	2.69	490	-	-
1083	2.47	295	3.79	6166	3.3	1995	3.5	3162
1084	1.65	45	WFS	WFS	WFS	WFS	-	-
1085	2.16	145	2.48	302	2.45	282	2.76	575
1086	1.62	42	2.19	155	2.48	302	-	-
1087	1.93	85	2.36	229	2.91	813	3.01	1023
1088	1.59	39	2.11	130	2.85	708	2.36	229
1089	2	100	2.37	234	3.15	1413	2.99	977
1090	2.31	204	2.99	977	3.07	1175	2.63	427
1091	1.58	38	2.52	331	2.62	417	2.34	219
1092	1.4	25	1.69	49	2.4	251	1.68	48
1093	1.61	41	2.91	813	2.99	977	-	-
1094	1.81	65	2.93	851	3.05	1122	2.65	447
1095	1.26	18	1.89	78	2.81	646	2.42	263
1096	1.86	72	3.24	1738	3.4	2512	-	-
1097	1.26	18	2.33	214	2.9	794	2.44	275
1098	1.34	22	2	100	2.58	380	1.67	47

Appendix 7.6 continued 3 of 4

	Baseline (Visit 1)		Six Weeks (Visit 2) Immune Response to First Dose of Vaccine		12 weeks (Visit 3) Immune Response to Second Dose of Vaccine		18 Month Follow-Up (Visit 4)	
ROVE Study Number	**Log_{10} TRFIA**	**TRFIA mIU/mL**	**Log_{10} TRFIA**	**TRFIA mIU/mL**	**Log_{10} TRFIA**	**TRFIA mIU/mL**	**Log_{10} TRFIA**	**TRFIA mIU/mL**
1099	1.18	15	2.22	166	2.98	955	2.61	407
1100	1.48	30	1.62	42	2.68	479	2.05	112
1101	1.67	47	1.9	79	2.45	282	-	-
1102	1.23	17	1.58	38	2.54	347	2.1	126
1103	1.77	59	3.33	2138	WFS	WFS	-	-
1104	1.34	22	1.77	59	2.71	513	-	-
1105	2.13	135	2.73	537	2.76	575	2.47	295
1106	2	100	2.29	195	3.52	3311	2.94	871
1107	1.23	17	1.92	83	3.35	2239	2.37	234
1108	2.38	240	3.34	2188	3.08	1202	3.08	1202
1109	1.86	72	3.03	1072	2.92	832	2.93	851
1110	*	*	2.04	110	2.99	977	2.64	437

Appendix 7.6: TRFIA Titre (mIU/mL and log_{10}) for Each Study Participant at Each Visit
Key; * sample not available for testing (study participant difficult to bleed),
- Did not participate in follow up visit, WFS: withdrew from study.

Visit Number	N	Range Log_{10} (mIU/mL)		Mean Log_{10} (mIU/mL)	SD Log_{10} (mIU/mL)
		Minimum	Maximum		
Visit 1 (Baseline)	108	1.18 (15)	3.40 (2,512)	1.86 (72)	0.49 (3)
Visit 2 (Six weeks)	108	1.45 (28)	3.88 (7,586)	2.46 (288)	0.57 (4)
Visit 3 (12 weeks)	103	1.65 (45)	3.52 (3,311)	2.78 (603)	0.33 (2)
Visit 4 (18 months)	75	1.18 (15)	3.50 (3,162)	2.47 (295)	0.49 (3)

Appendix 7.7 Summary of TRFIA Readings for Each Visit During the Study. Data is presented as Log_{10} values, with corresponding mIU/mL units shown in parentheses. (See corresponding figure 3.6).

Appendix 7.8: Avidity Readings (with Corresponding TRFIA) Titre for Each Study Participant at Each Visit

	Baseline (Visit 1)		Six Weeks (Visit 2) Immune Response to First Dose of Vaccine		12 weeks (Visit 3) Immune Response to Second Dose of Vaccine		18 Month Follow-Up (Visit 4)	
ROVE Study Number	TRFIA mIU/mL	Avidity (%)	TRFIA mIU/mL	Avidity (%)	TRFIA mIU/mL	Avidity (%)	TRFIA mIU/mL	Avidity (%)
1001	130		209	15	229	38	87	X
1002	62	X	89	11	45	19	-	-
1003	48	X	87	34	257	46	100	X
1004	214	41	1230	87	1023	88	1380	85
1005	214	59	2089	84	1738	79	692	84
1006	55	X	347	24	447	50	-	-
1007	23	X	85	20	631	33	23	X
1008	20	X	81	31	437	36	145	73
1009	1000	48	1288	64	891	51	724	65
1010	22	X	WFS	WFS	WFS	WFS	-	-
1011	1549	89	3802	74	1000	87	513	90
1012	123	33	209	25	1000	35	-	-
1013	182	70	708	81	776	85	468	87
1014	145	64	1349	85	WFS	WFS	-	-
1015	37	X	95	26	427	33	74	X
1016	76	X	309	48	1000	55	427	65
1017	65	X	126	28	372	25	148	29
1018	72	X	178	35	398	44	2042	81
1019	21	X	65	8	324	30	15	X
1020	646	84	1413	83	1000	91	1445	87
1021	32	X	56	20	468	27	1288	84
1022	15	X	28	23	417	19	-	-
1023	263	83	1660	95	851	96	603	95
1024	35	X	148	4	282	20	112	30
1025	23	X	132	21	324	31	65	X
1026	41	X	130	26	479	34	115	28
1027	66	X	117	6	126	11	123	48
1028	68	X	224	17	603	55	-	-
1029	933	86	1122	81	851	80	575	82
1030	182	68	1288	75	1000	80	-	-
1031	1513	89	3020	86	2042	92	2455	90
1032	93	X	832	55	468	80	195	81

Appendix 7.8 continued

	Baseline (Visit 1)		6 Weeks (Visit 2) Immune Response to First Dose of Vaccine		12 weeks (Visit 3) Immune Response to Second Dose of Vaccine		18 Month Follow-Up (Visit 4)	
ROVE Study Number	TRFIA mIU/mL	Avidity (%)	TRFIA mIU/mL	Avidity (%)	TRFIA mIU/mL	Avidity (%)	TRFIA mIU/mL	Avidity (%)
1033	72	X	100	15	182	25	-	-
1034	28	X	115	10	575	27	138	33
1035	37	X	155	21	372	40	112	35
1036	45	X	72	X	123	12	59	X
1037	69	X	85	5	1349	30	-	-
1038	229	73	676	86	1349	99	-	-
1039	78	X	81	22	141	27	89	X
1040	42	X	224	11	1000	43	-	-
1041	44	X	49	36	234	33	107	73
1042	72	X	363	34	407		-	-
1043	30	X	36	13	240	32	-	-
1044	191	53	891	92	724	89	-	-
1045	145	54	692	69	1862	87	-	-
1046	398	42	182	22	2188	31	692	34
1047	30	X	380	41	550	53	117	56
1048	31	X	229	39	219	53	1862	79
1049	62	X	355	22	692	43	331	44
1050	18	X	29	13	78	27	282	51
1051	182	73	427	80	631	94	-	-
1052	19	X	269	32	WFS	WFS	-	-
1053	38	X	676	85	WFS	WFS	-	-
1054	34	X	126	30	324	35	141	57
1055	186	35	229	30	398	41	-	-
1056	85	X	195	27	302	37	324	45
1057	295	71	1000	84	1445	87	603	90
1058	23	X	263	13	776	21	224	45
1059	72	X	145	11	295	21	-	-
1060	36	X	182	40	794	43	209	39
1061	*	*	135	21	1259	43	219	88
1062	195	65	724	79	1023	80	407	82
1063	135	10	123	15	282	26	229	23
1064	363	78	1479	79	851	86	776	78
1065	20	X	282	19	1072	59	339	55

Appendix 7.8 continued 2 of 4

	Baseline (Visit 1)		6 Weeks (Visit 2) Immune Response to First Dose of Vaccine		12 weeks (Visit 3) Immune Response to Second Dose of Vaccine		18 Month Follow-Up (Visit 4)	
ROVE Study Number	TRFIA mIU/mL	Avidity (%)	TRFIA mIU/mL	Avidity (%)	TRFIA mIU/mL	Avidity (%)	TRFIA mIU/mL	Avidity (%)
1066	130	42	339	17	WFS	WFS	-	-
1067	55	X	182	15	550	35	130	49
1068	16	X	83	24	676	48	-	-
1069	26	X	42	X	776	35	145	51
1070	31	X	85	12	1259	61	214	62
1071	16	X	166	18	457	47	282	42
1072	575	78	2951	82	661	74	776	87
1073	19	X	91	14	224	37	-	-
1074	195	52	1318	74	832	80	224	84
1075	282	75	4365	90	1380	91	1000	93
1076	115	79	2239	95	1622	88	1905	92
1077	41	X	240	18	219	23	-	-
1078	158	72	7586	86	871	83	-	-
1079	78	X	363	17	871	29	-	-
1080	331	68	2089	93	1096	88	-	-
1081	2512	69	1622	84	1148	88	1905	90
1082	17	X	85	X	490	40	-	-
1083	295	85	6166	96	1995	93	3162	95
1084	45	X	WFS	WFS	WFS	WFS	-	-
1085	145	44	302	34	282	27	575	71
1086	42	X	155	18	302	35	-	-
1087	85	X	229	18	813	42	1023	78
1088	39	X	130	17	708	27	229	19
1089	100	49	234	93	1413	93	977	94
1090	204	61	977	86	1175	76	427	93
1091	38	X	331	34	417	36	219	45
1092	25	X	49	X	251	31	48	X
1093	41	X	813	65	977	70	-	-
1094	65	X	851	85	1122	86	447	93
1095	18	X	78	X	646	34	263	37
1096	72	X	1738	96	2512	95	-	-
1097	18	X	214	28	794	52	275	57
1098	22	X	100	14	380	24	47	X

	Baseline (Visit 1)		6 Weeks (Visit 2) Immune Response to First Dose of Vaccine		12 weeks (Visit 3) Immune Response to Second Dose of Vaccine		18 Month Follow-Up (Visit 4)	
ROVE Study Number	**TRFIA mIU/mL**	**Avidity (%)**	**TRFIA mIU/mL**	**Avidity (%)**	**TRFIA mIU/mL**	**Avidity (%)**	**TRFIA mIU/mL**	**Avidity (%)**
1099	15	X	166	23	955	53	407	59
1100	30	X	42	X	479	42	112	41
1101	47	X	79	11	282	15	-	-
1102	17	X	38	X	347		126	40
1103	59	X	2138	81	WFS	WFS	-	-
1104	22	X	59	X	513	9	-	-
1105	135	69	537	91	575	84	295	91
1106	100	42	195	11	3311	60	871	72
1107	17	X	83	14	2239	39	234	33
1108	240	82	2188	88	1202		1202	74
1109	72	X	1072	85	832		851	74
1110	*	*	110	36	977		437	48

Appendix 7.8: TRFIA Data (mIU/mL) and the Corresponding Avidity Readings (%) for Each ROVE Study Participant, at Each Study Visit.
Key; X: TRFIA too low to allow accurate avidity to be carried out, *sample not available for testing (study participant difficult to bleed), - Did not participate in follow up visit, WFS: withdrew from study.

Visit Number	N	Range (%)		Mean (%)	Median (%)	SD (%)
		Minimum	Maximum			
Visit 2 (Six weeks)	64	4	55	20	21	11
Visit 3 (12 weeks)	67	9	80	36	35	13
Visit 4 (18 months)	38	19	88	52	50	19

Appendix 7.9: Summary of Avidity Readings for Each Visit during the Study. (See corresponding figure 3.8).

Appendix 7.10: Classification of Each Study Participant, Number of Doses Required to Seroconvert and Diamedix Screening Results.

ROVE Study Number	Six Week Avidity ≥60%	Dose Required for Seroconversion If Seronegative at Enrolment	Humoral Subset	Diamedix from Occupational Health
1001	No	Positive B'line	PLR	Neg
1002	No	NLR	NLR	Neg
1003	No	Two	NSC2	Neg
1004	Yes	Positive B'line	PI	Neg
1005	Yes	Positive B'line	PI	Neg
1006	No	One	NSC1	Neg
1007	No	Two	NSC2	Eq
1008	No	Two	NSC2	Neg
1009	Yes	Positive B'line	PI	Neg
1010	X	WFS	WFS	Neg
1011	Yes	Positive B'line	PI	Neg
1012	No	One	NSC1	Neg
1013	Yes	Positive B'line	PI	Eq
1014	Yes	Positive B'line	PI	Neg
1015	No	Two	NSC2	Neg
1016	No*	One	NSC1	Neg
1017	No	Two	NSC2	Neg
1018	No	One	NSC1	Neg
1019	No	Two	NSC2	Neg
1020	Yes	Positive B'line	PI	Neg
1021	No	Two	NSC2	Neg
1022	No	Two	NSC2	Neg
1023	Yes	Positive B'line	PI	Neg
1024	No	One	NSC1	Neg
1025	No	One	NSC1	Neg
1026	No	One	NSC1	Neg
1027	No	NLR	NLR	Neg
1028	No	One	NSC1	Neg
1029	Yes	Positive B'line	PI	Eq
1030	Yes	Positive B'line	PI	Neg
1031	Yes	Positive B'line	PI	Eq
1032	No*	One	Outlier NSC1/NI♣	Neg

Appendix 7.10 continued

ROVE Study Number	Six Week Avidity ≥60%	Dose Required for Seroconversion If Seronegative at Enrolment	Humoral Subset	Diamedix from Occupational Health
1033	No	Two	NSC2	Neg
1034	No	Two	NSC2	Neg
1035	No	One	NSC1	Neg
1036	X	NLR	NLR	Neg
1037	No	Two	NSC2	Neg
1038	Yes	Positive B'line	PI	Eq
1039	No	Two	NSC2	Neg
1040	No	One	NSC1	Neg
1041	No	Two	NSC2	Neg
1042	No	One	NSC1	Neg
1043	No	Two	NSC2	Neg
1044	Yes	Positive B'line	PI	Neg
1045	Yes	Positive B'line	PI	Eq
1046	No	Positive B'line	PLR	Neg
1047	No*	One	NSC1	Neg
1048	No	One	NSC1	Neg
1049	No	One	NSC1	Neg
1050	No	NLR	NLR	Neg
1051	Yes	Positive B'line	PI	Neg
1052	No	One	NSC1	Neg
1053	Yes	One	NI	Neg
1054	No	Two	NSC2	Neg
1055	No	Positive B'line	PLR	Neg
1056	No	One	NSC1	Neg
1057	Yes	Positive B'line	PI	Eq
1058	No	One	NSC1	Neg
1059	No	One	NSC1	Neg
1060	No	One	NSC1	Neg
1061	No	Two"	WFS	Neg
1062	Yes	Positive B'line	PI	Neg
1063	No	Two[&]	PLR	Neg
1064	Yes	Positive B'line	PI	Neg
1065	No	One	NSC1	Neg

Appendix 7.10 continued 2 of 4

ROVE Study Number	Six Week Avidity ≥60%	Dose Required for Seroconversion If Seronegative at Enrolment	Humoral Subset	Diamedix from Occupational Health
1066	No	Positive B'line	PLR	Neg
1067	No	One	NSC1	Neg
1068	No	Two	NSC2	Neg
1069	X	Two	NSC2	Neg
1070	No	Two	NSC2	Neg
1071	No	One	NSC1	Neg
1072	Yes	Positive B'line	PI	Eq
1073	No	Two	NSC2	Neg
1074	Yes	Positive B'line	PI	Eq
1075	Yes	Positive B'line	PI	Neg
1076	Yes	Positive B'line	NI	Neg
1077	No	One	NSC1	Neg
1078	Yes	Positive B'line	PI	Neg
1079	No	One	NSC1	Neg
1080	Yes	Positive B'line	PI	Eq
1081	Yes	Positive B'line	PI	Neg
1082	X	Two	NSC2	Neg
1083	Yes	Positive B'line	PI	Neg
1084	X	WFS	WFS	Neg
1085	No	Positive B'line	PLR	Neg
1086	No	One	NSC1	Eq
1087	No	One	NSC1	Neg
1088	No	One	NSC1	Neg
1089	Yes	One	Outlier (NSC1/NI)♣	Neg
1090	Yes	Positive B'line	PI	Eq
1091	No	One	NSC1	Neg
1092	X	Two	NSC2	Neg
1093	Yes	One	NI	Neg
1094	Yes	One	NI	Neg
1095	X	Two	NSC2	Neg
1096	Yes	One	NI	Neg
1097	No	One	NSC1	Neg
1098	No	Two	NSC2	Neg

Appendix 7.10 continued

ROVE Study Number	Six Week Avidity ≥60%	Dose Required for Seroconversion If Seronegative at Enrolment	Humoral Subset	Diamedix from Occupational Health
1099	No	One	NSC1	Neg
1100	X	Two	NSC2	Neg
1101	No	Two	NSC2	Neg
1102	X	Two	NSC2	Neg
1103	Yes	One	NI	Neg
1104	X	Two	NSC2	Neg
1105	Yes	Positive B'line	PI	Neg
1106	No	One	NSC1	Neg
1107	No	Two	NSC2	Neg
1108	Yes	Positive B'line	PI	Neg
1109	Yes	One	NI	Neg
1110	No	Two"	NSC2	Neg

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Appendix 7.10: Classification of Each Study Participant, Number of Doses Required to Seroconvert and Diamedix Screening Results.

Seroconversion was classified as a TRFIA reading of 130mIU/mL (log_{10}2.11). Subset classification was based on baseline TRFIA of ≥130mIU/mL, Six week avidity readings of ≥60% and TRFIA of ≥400mIU/mL (log_{10}2.60). Diamedix screening results (assayed when the HCW joined BLT NHS trust) were obtained from the occupational health database.

Key: X: Visit 1 and 2 TRFIA readings were too low to allow accurate avidity readings to be taken, WFS; withdrew/withdrawn from study, Positive b'line; positive TRFIA at baseline, SC1; Seroconverted following one dose, SC2; Seroconverted following two doses, 'Immune'; True secondary responder, Neg; negative (<15EU/mL) Eq; equivocal (15-19EU/mL), *Avidity readings were below 60%, but they were above low cut-off of 40%, "baseline (visit 1) serology was not available, but were classified according to subsequent serology readings, [&] this HCWs had log_{10} TRFIA readings of 2.13 at baseline, 2.09 at six weeks, and 2.45 at 12 weeks, and was classified as seroconverting after 2 doses, [*]these two individuals were classified as outliers and did not group with the two populations seen at six weeks (primary responders = avidity <60%, TRFIA < log_{10}2.60; 400mIU/mL; secondary responders = avidity >60%, TRFIA > log_{10}2.60; 400mIU/mL), and were excluded from analysis. (1032 had a six week avidity reading was below 60% (55%), but experienced a nine fold increase in antibody titre following the first dose of vaccine (log_{10}2.92; 832mIU/mL), suggesting this individual was actually a false negative; 1089 had a baseline TRFIA of log_{10}2.00; 100mIU/mL and an intermediate avidity of 49%, at six weeks avidity was at 93% although the corresponding TRFIA reading was below the log_{10}2.60 cut-off at log_{10}2.37; 234mIU/mL.

	Log_{10} TRFIA (mIU/ml)						Avidity (%)					
	n	Range		Mean	SD	Median	n	Range		Mean	SD	Median
		Minimum	Maximum					Minimum	Maximum			
Baseline (Visit 1)	32	1.18 (15)	2.09 (123)	1.6 (40)	0.25	1.62 (42)	2	33	42			
Six Weeks (Visit 2)	32	2.11 (130)	2.92 (832)	2.35 (224)	0.18	2.34 (219)	32	4	55	24	12	22
12 Weeks (Visit 3)	31	2.34 (219)	3.52 (3,311)	2.75 (562)	0.25	2.74 (550)	30	20	80	42	14	43
18 Months (Visit 4)	22	1.81 (65)	3.31 (2,042)	2.46 (288)	0.39	2.4 (251)	21	30	81	49	19	45

Appendix 7.11: Descriptive Statistics for TRFIA and Avidity Values for the NSC1 Subset. Study participants were classified into this subset if their baseline TRFIA readings were below $log_{10}2.11$; 130mIU/mL, (negative) and (if applicable) their corresponding avidity value was <60%. Their six week TRFIA was ≥130mIU/mL value with a corresponding avidity below 60% (seroconverted following one dose of vaccine).

	Log_{10}TRFIA (mIU/ml)						Avidity (%)					
	n	Range		Mean	SD	Median	n	Range		Mean	SD	Median
		Minimum	Maximum					Minimum	Maximum			
Baseline (Visit 1)	29	1.18 (15)	1.89 (78)	1.47 (30)	0.21	1.43 (27)	0					
Six Weeks (Visit 2)	29	1.45 (28)	2.11 (130)	1.89 (78)	0.17	1.92 (83)	22	5	36	20	9	20
12 Weeks (Visit 3)	29	2.15 (141)	3.35 (2,239)	2.65 (447)	0.27	2.63 (427)	27	9	61	32	10	33
18 Months (Visit 4)	21	1.18 (15)	3.11 (1,288)	2.05 (112)	0.43	2.1 (126)	13	62	84	51	18	48

Appendix 7.12: Descriptive Statistics for TRFIA and Avidity Values for the NSC2 Subset. Study participants were classified into this subset if their baseline TRFIA readings were below $log_{10}2.11$; 130mIU/mL (negative), and (if applicable) their corresponding avidity value was <60%. Their six week TRFIA was <130mIU/mLvalue with a corresponding avidity below 60%, with 12 week TRFIA readings ≥130mIU/mL (seroconverted following two doses of vaccine).

	Log_{10}TRFIA (mIU/ml)						Avidity (%)					
	n	Range		Mean	SD	Median	n	Range		Mean	SD	Median
		Minimum	Maximum					Minimum	Maximum			
Baseline (Visit 1)	6	2.11 (130)	2.6 (397)	2.24 (174)	0.21	2.13 (135)	5	10	44	37	14	42
Six Weeks (Visit 2)	6	2.09 (123)	2.53 (337)	2.31 (204)	0.16	2.32 (209)	6	15	34	20	6	17
12 Weeks (Visit 3)	5	2.36 (229)	3.34 (2,192)	2.69 (490)	0.45	2.53 (339)	5	26	41	36	10	35
18 Months (Visit 4)	4	1.94 (87)	2.84 (687)	2.38 (240)	0.45	2.36 (229)	3	23	71	41	35	21

Appendix 7.13: Descriptive Statistics for TRFIA and Avidity Values for the PLR Subset. Study participants were classified into this subset if their baseline TRFIA readings ≥ log_{10}2.11; 130mIU/mL (positive) with a corresponding avidity of <60%. In addition, their six week TRFIA was below log_{10}2.60; 400mIU/mL (low responder) with a corresponding avidity below 60%. The six HCWs in this subset were 1001, 1046, 1055, 1063, 1066, 1085.

	Log_{10}TRFIA (mIU/ml)						Avidity (%)					
	n	Range		Mean	SD	Median	n	Range		Mean	SD	Median
		Minimum	Maximum					Minimum	Maximum			
Baseline (Visit 1)	7	1.58 (38)	2.06 (115)	1.82 (66)	0.17	1.84 (68)	1	79				
Six Weeks (Visit 2)	7	2.83 (676)	3.35 (2,239)	3.09 (1,230)	0.21	3.03 (1,072)	7	65	96	85	10	85
12 Weeks (Visit 3)	5	2.92 (832)	3.4 (2,512)	3.12 (1,318)	0.17	3.1 (1,259)	4	70	95	85	11	87
18 Months (Visit 4)	3	2.65 (447)	3.28 (1,905)	2.96 (912)	0.26	2.96 (912)	2	92	93			

Appendix 7.14: Descriptive Statistics for TRFIA and Avidity Values for the NI Subset. Study participants were classified into this subset if their baseline TRFIA readings were below $log_{10}2.11$; 130mIU/mL (negative), and their six week TRFIA was above $log_{10}2.60$; 400mIU/mL with a corresponding avidity above 60% (secondary responder; immune). The seven HCWs in this subset were 1053, 1076, 1093, 1094, 1096, 1103 and 1109.

	Log_{10} TRFIA (mIU/ml)						Avidity (%)					
	n	Range		Mean	SD	Median	n	Range		Mean	SD	Median
		Minimum	Maximum					Minimum	Maximum			
Baseline (Visit 1)	28	2.13 (135)	3.4 (2,512)	2.49 (309)	0.34	2.36 (229)	28	41	89	71	12	71
Six Weeks (Visit 2)	28	2.63 (427)	3.88 (7,586)	3.18 (1,514)	0.32	3.13 (1,349)	28	64	96	84	7	84
12 Weeks (Visit 3)	27	2.76 (575)	3.31 (2,042)	3.02 (1,047)	0.15	3.01 (1,023)	26	51	99	86	6	87
18 Months (Visit 4)	20	2.35 (224)	3.5 (3,162)	2.89 (776)	0.3	2.84 (692)	20	65	95	73	8	72

Appendix 7.15: Descriptive Statistics for TRFIA and Avidity Values for the PI Subset. Study participants were classified into this subset if their baseline TRFIA readings were above $log_{10}2.11$; 130mIU/mL (positive), with a corresponding avidity of >60%. Their six week TRFIA values were above $log_{10}2.60$; 400mIU/mL with a corresponding avidity above 60% (secondary responders; immune).

Appendix 7.16: History of Chickenpox or Varicella Vaccination Prior to Study Enrolment for Each Study Participant

	History of Chickenpox			
ROVE Study Number	History of Chickenpox	Age of Chickenpox*	Number of Years Since Chickenpox[$]	Additional Vaccination
1001	No			
1002	No			Third dose
1003	No			
1004	No			
1005	Once	12	19	
1006	No			
1007	No			
1008	No			
1009	No			
1010	No			1 Year Previously
1011	No			
1012	No			
1013	No			
1014	No			
1015	No			
1016	No			
1017	No			
1018	No			
1019	No			
1020	Unknown			
1021	No			
1022	No			
1023	Once	16	26	
1024	No			
1025	No			
1026	No			
1027	No			Third dose
1028	No			
1029	No			
1030	No			
1031	Once	<1 year	26	
1032	No			

Appendix 7.16 continued

	History of Chickenpox			
ROVE Study Number	**History of Chickenpox**	**Age of Chickenpox***	**Number of Years Since Chickenpox$**	**Additional Vaccination**
1033	No			
1034	No			
1035	No			
1036	No			
1037	No			
1038	No			
1039	No			
1040	No			
1041	No			
1042	No			
1043	No			
1044	Once	< 1 year	31	
1045	No			
1046	No			
1047	No			
1048	No			
1049	No			
1050	Unknown			
1051	Once	< 1 year	35	
1052	No			
1053	No			1 Year Previously
1054	No			
1055	No			
1056	No			
1057	Once	40	18	
1058	No			
1059	No			
1060	No			
1061	No			
1062	No			
1063	No			
1064	Once	3	21	
1065	No			

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	History of Chickenpox			
ROVE Study Number	**History of Chickenpox**	**Age of Chickenpox***	**Number of Years Since Chickenpox[$]**	**Additional Vaccination**
1066	No			
1067	No			
1068	No			
1069	No			
1070	Twice	15	14	
1071	No			
1072	No			
1073	No			
1074	No			1 year Previously
1075	Twice	8	24	
1076	Once	6	23	
1077	No			
1078	Once	<1 year	43	
1079	No			
1080	No			
1081	No			
1082	No			
1083	Once	5	23	
1084	No			
1085	No			
1086	No			
1087	No			
1088	No			
1089	Once	22	3	
1090	Once	3		
1091	No			
1092	No			
1093	No			
1094	Once	9	13	
1095	No			
1096	No			
1097	No			
1098	No			

Appendix 7.16 continued

	History of Chickenpox			
ROVE Study Number	**History of Chickenpox**	**Age of Chickenpox***	**Number of Years Since Chickenpox[$]**	**Additional Vaccination**
1099	No			
1100	No			
1101	Once	< 1 year	31	
1102	No			
1103	No			
1104	No			
1105	No			
1106	No			
1107	No			
1108	Once	3	28	
1109	Once	6	44	
1110	No			

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Appendix 7.16: History of Chickenpox or Varicella Vaccination Prior to Study Enrolment for Each Study Participant. Data on additional vaccination within the ROVE study is also given.
Key: *In cases where study participants experienced varicella twice, the age is given of the last episode, <1 year; chickenpox under the age of 1 year old, [$] In cases where study participants experienced varicella twice, the age is given since the last episode, Previous; Previously vaccinated, see appendix 8.17 and 8.19 for details, Third dose; third dose administered during the ROVE study.

Study Number	Gender	Age	Ethnicity	Birth Country	Years UK
1010	Male	29	Indian subcontinent	India	4
1053	Female	29	Caucasian	UK	29
1074	Female	34	Black	Jamaica	7

Appendix 7.17: Demographic Data of HCWs who were Previously Vaccinated Prior to Enrolment in the ROVE Study.

	Previous vaccination		ROVE Vaccination		Serology								
Study Number	Manufacturer	Number of Doses	Manufacturer	Number of Doses	V1 Log_{10} TRFIA (mIU/mL)	V1 Avidity	V2 Log_{10} TRFIA (mIU/mL)	V2 Avidity	V3 Log_{10} TRFIA (mIU/mL)	V3 Avidity	V4 Log_{10} TRFIA (mIU/mL)	V4 Avidity	Comments
1010	GSK	1 dose	Merck	1 dose	1.34 (22)	NA	WFS	WFS	WFS	WFS	WFS	WFS	Developed fever and general malaise after 1st dose
1053	GSK	2 doses	Merck	1 dose	1.58 (38)	NA	2.83 (676)	85%	WFS	WFS	WFS	WFS	Seroconveted after 1st dose
1074	GSK	2 doses	Merck	2 doses	2.29 (195)	52%	3.12 (1,318)	74%	2.92 (832)	80%	2.92 (832)	66%	Requested 2nd dose as different brand of vaccine to previous course

Appendix 7.18: HCWs who were Previously Vaccinated Prior to Enrolment in the ROVE Study. Key: WFS; withdrew from study, NA; Not applicable. Three HCWs were vaccinated one year prior to enrollment into the ROVE study, whilst employed at other NHS trusts.

Appendix 7.19: Classification of Each Study Participant at Follow-up with Corresponding Humoral Subset Classification During the Study:

ROVE Study Number	Humoral Subset	Classification at 18 Month Follow-Up
1001	PLR	Negative
1002	NLR	-
1003	NSC2	Negative
1004	PI	Boost
1005	PI	Retained
1006	NSC1	-
1007	NSC2	Negative
1008	NSC2	Retained
1009	PI	Retained
1010	WFS	-
1011	PI	Retained
1012	NSC1	-
1013	PI	Retained
1014	PI	-
1015	NSC2	Negative
1016	NSC1	Retained
1017	NSC2	Retained
1018	NSC1	Boost
1019	NSC2	Negative
1020	PI	Boost
1021	NSC2	Boost
1022	NSC2	Negative
1023	PI	Retained
1024	NSC1	Negative
1025	NSC1	Negative
1026	NSC1	Negative
1027	NLR	Negative
1028	NSC1	-
1029	PI	Retained
1030	PI	-
1031	PI	Boost
1032	Outlier NSC1/NI♣	Retained

Appendix 7.19 continued

ROVE Study Number	Humoral Subset	Classification at 18 Month Follow-Up
1033	NSC2	-
1034	NSC2	Retained
1035	NSC1	Negative
1036	NLR	Negative
1037	NSC2	-
1038	PI	-
1039	NSC2	Negative
1040	NSC1	-
1041	NSC2	Negative
1042	NSC1	-
1043	NSC2	-
1044	PI	-
1045	PI	-
1046	PLR	Retained
1047	NSC1	Negative
1048	NSC1	Boost
1049	NSC1	Retained
1050	NLR	Boost
1051	PI	-
1052	NSC1	-
1053	NI	-
1054	NSC2	Retained
1055	PLR	-
1056	NSC1	Boost
1057	PI	Retained
1058	NSC1	Retained
1059	NSC1	-
1060	NSC1	Retained
1061	WFS	Retained
1062	PI	Retained
1063	PLR	Retained
1064	PI	Retained
1065	NSC1	Retained

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ROVE Study Number	Humoral Subset	Classification at 18 Month Follow-Up
1066	PLR	-
1067	NSC1	Retained
1068	NSC2	-
1069	NSC2	Retained
1070	NSC2	Retained
1071	NSC1	Retained
1072	PI	Boost
1073	NSC2	-
1074	PI	Retained
1075	PI	Retained
1076	NI	Boost
1077	NSC1	-
1078	PI	-
1079	NSC1	-
1080	PI	-
1081	PI	Boost
1082	NSC2	-
1083	PI	Boost
1084	WFS	-
1085	PLR	Boost
1086	NSC1	-
1087	NSC1	Boost
1088	NSC1	Retained
1089	Outlier (NSC1/NI)♣	Retained
1090	PI	Retained
1091	NSC1	Retained
1092	NSC2	Negative
1093	NI	-
1094	NI	Retained
1095	NSC2	Retained
1096	NI	-
1097	NSC1	Retained
1098	NSC2	Negative

ROVE Study Number	Humoral Subset	Classification at 18 Month Follow-Up
1099	NSC1	Retained
1100	NSC2	Negative
1101	NSC2	-
1102	NSC2	Negative
1103	NI	-
1104	NSC2	-
1105	PI	Retained
1106	NSC1	Retained
1107	NSC2	Retained
1108	PI	Boost
1109	NI	Boost
1110	NSC2	Retained

Appendix 7.19: Classification of Each Study Participant at Follow-up with Corresponding Humoral Subset Classification During the Study.
Key: WFS; withdrew/withdrawn from study, NSC1; TRFIA negative at baseline, seroconverted following one dose, NSC2; TRFIA negative at baseline seroconverted following two doses, PI; TRFIA positive at baseline, 'immune' (secondary responder), - HCW did not participate in follow-up visit, Negative; HCW had a TRFIA titre below $\log_{10}2.11$ (130mIU/mL) at follow-up, Retained; HCW had retained an TRFIA titre at or above 130mIU/mL; Boost; HCWs had TRFIA titres above 130mIU/mL at 12 weeks, and TRFIA titres had increased at 18 months, ♣these two individuals were classified as outliers and did not group with the two populations seen at six weeks (primary responders = avidity <60%, TRFIA < $\log_{10}2.60$; 400mIU/mL; secondary responders = avidity >60%, TRFIA >$\log_{10}2.60$; 400mIU/mL), and were excluded from analysis.

		Study Number	
		1032	**1089**
Baseline (Visit 1)	**Log_{10}TRFIA (mIU/mL)**	1.97 (94)	2.00 (101)
	Avidity (%)	N/A	49
	FAMA	4	N/A
Six Weeks (Visit 2)	**Log_{10}TRFIA (mIU/mL)**	2.92 (823)	2.37 (236)
	Avidity (%)	55	92
	FAMA	8	N/A
12 Weeks (Visit 3)	**Log_{10}TRFIA (mIU/mL)**	2.67 (464)	3.15 (1,424)
	Avidity (%)	80	93
	FAMA	>8	N/A
18 Month Follow-Up (Visit 4)	**Log_{10}TRFIA (mIU/mL)**	2.29 (194)	2.99 (980)
	Avidity (%)	81	93
	FAMA	8	N/A

Appendix 7.20: Summary of Serological Data (TRFIA Titres, Avidity and FAMA Scores) for Humoral Subset Outliers.

Key: N/A; not applicable as corresponding TRFIA <100mIU/mL, or FAMA not performed on these samples. (1032 had a six week avidity reading was below 60% (55%), but experienced a nine fold increase in antibody titre following the first dose of vaccine (log_{10}2.92; 832mIU/mL), suggesting this individual was actually a false negative; 1089 had a baseline TRFIA of log_{10}2.00; 100mIU/mL and an intermediate avidity of 49%, at six weeks avidity was at 93% although the corresponding TRFIA reading was below the log_{10}2.60 cut-off at log_{10}2.37; 234mIU/mL.

		Study Number	
		1032	**1089**
Immune Status	Baseline Diamedix	Negative	Negative
	Baseline TRFIA	Negative	Negative
	Baseline FAMA	Positive	N/A
	Six Week Avidity	Unclassified	Previously Immune
Classification During Study	Doses Required to Seroconvert	One	One
	Humoral Subset	SC1/False Negative	SC1/False Negative
	18 Month Classification	Immune (Retained)	Immune (Retained)
Demographics	Gender	Male	Female
	Ethnicity	Black	Black
	Country of Birth	Uganda	Jamaica
	Age (Years)	35	25
	Years Resided in the UK	8	6
History	History of C'Pox	No	Yes
	Age C'pox (Years)	N/A	22
	Previous Vaccination	No	No
Exposure During Study	Basline (Visit1)	None	Occuptational HZ; 3 Days Prior
	Six Weeks (Visit 2)	Occuptational HZ; 28 Days Prior	None
	12 Weeks (Visit 3)	None	None
	18 Month Follow-Up (Visit 4)	X4 Occupational HZ; Last Exposure 35 Days Prior	None

Appendix 7.21: Summary of Demographics, Assay Status during Study and Exposure Data, for the Two Humoral Subset Outliers.

Key: HZ; herpes zoster, C'Pox; chickenpox, Diamedix negative; <15EU/mL, TRFIA negative; <130mIU/mL ($log_{10}2.11$), FAMA positive; >2, N/A; not applicable, SC1; seroconverted after one dose.

Appendix 7.22: FAMA with Corresponding TRFIA (mIU/mL) and Avidity (%) Values for 16 ROVE Study Participants at Four Time Points

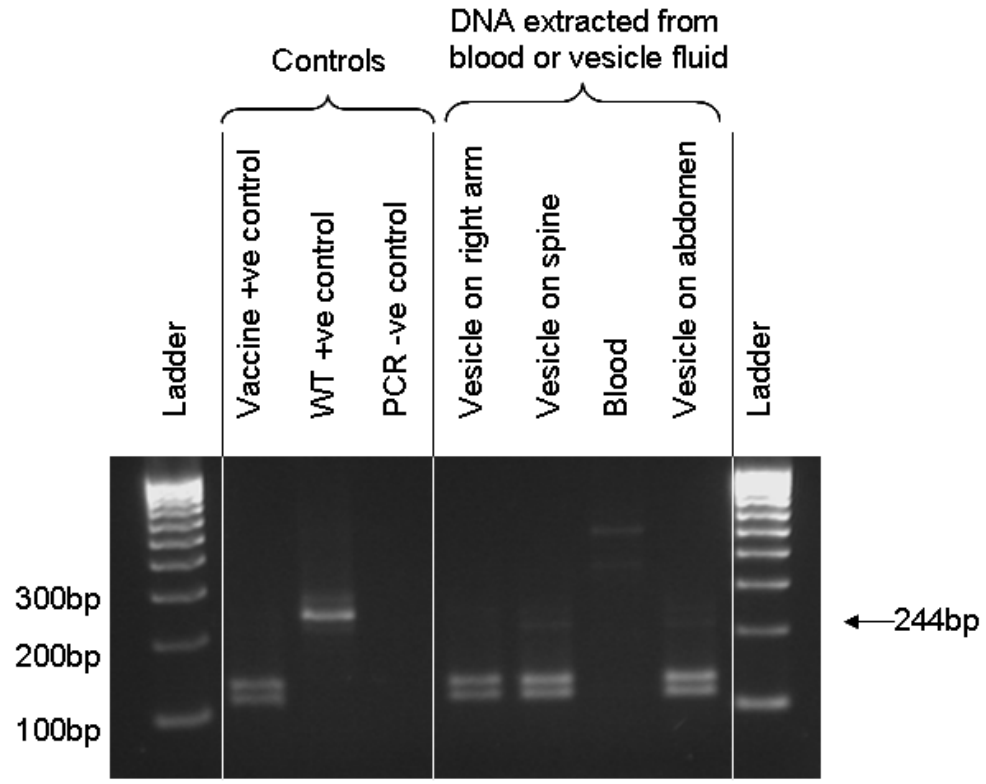
Study Number and Humoral Subset	VISIT	TRIFIA	AVID	FAMA
1001 (PLR)	V1	130	28	<2
	V2	207	15	8
	V3	229	38	8
	V4	87	X	8
1005 (PI)	V1	215	59	4;4
	V2	2068	84	>8
	V3	1744	79	>8
	V4	695	70	8
1009 (PI)	V1	1000	48	>8
	V2	1282	64	>8
	V3	885	51	>8
	V4	717	36	2;8
1013 (PI)	V1	183	70	<2
	V2	700	81	8
	V3	778	85	8
	V4	463	72	4
1015 (NSC2)	V1	37	X	4
	V2	96	26	2;4
	V3	429	33	8
	V4	74	X	>8
1017 (NSC2)	V1	64	X	4
	V2	125	28	8
	V3	374	25	>8
	V4	148	29	<2
1018 (NSC1)	V1	72	X	<2
	V2	176	35	4
	V3	398	44	>8
	V4	2040	81	>8
1019 (NSC2)	V1	21	X	<2
	V2	64	X	8
	V3	326	30	8
	V4	15	X	<2
1020 (PI)	V1	639	84	>8
	V2	1406	83	>8
	V3	1000	91	>8
	V4	1447	87	8
1022 (NSC2)	V1	15	X	<2
	V2	28	23	2;(<2)
	V3	415	19	8
	V4	35	X	<2
1023 (PI)	V1	263	83	>8; 8
	V2	1647	95	>8
	V3	844	96	>8
	V4	604	77	>8
1025 (NSC1)	V1	23	X	<2
	V2	132	21	>8
	V3	322	31	8
	V4	65	X	<2
1026 (NSC2)	V1	41	X	<2
	V2	128	26	4
	V3	481	34	>8
	V4	116	28	8
1027 (NLR)	V1	66	X	<2
	V2	118	6	<2
	V3	126	11	<2
	V4	122	48	<2
1029 (PI)	V1	942	86	>8
	V2	1123	81	>8
	V3	856	80	>8
	V4	574	72	>8
1032 Outlier (NI/NSC1)	V1	94	X	4
	V2	823	55	8
	V3	464	80	>8
	V4	194	81	8

Key; Visit: V1 – baseline, V2: 6 weeks, V3: 12 weeks; V4 18 month Follow up, NSC1/2; Negative-seroconverted after one/two dose(s), NLR; Negative low responder (humoral non-responder to vaccine), NI; Negative immune (secondary responder), PLR; positive low responder PI; positive immune (secondary responder), FAMA: <2 = negative, X: Avidity was not carried out as TRFIA reading was too low to allow accurate avidity assay to be carried out. Values in red represent discordant negative/positive status between assays.

Appendix 7.23: Vaccine Related Rash:

One study participant developed a vaccine related rash 13 days after receiving one dose of Merck vaccine (lot NA29040). The HCW was a 31 year old female of Indian ethnicity, who was born and raised in India and had lived in the UK for one year prior to the study. At enrolment this individual gave a negative history of varicella and was deemed Negative at enrolment with a TRFIA reading of $\log_{10}1.28$; (19mIU/ml).

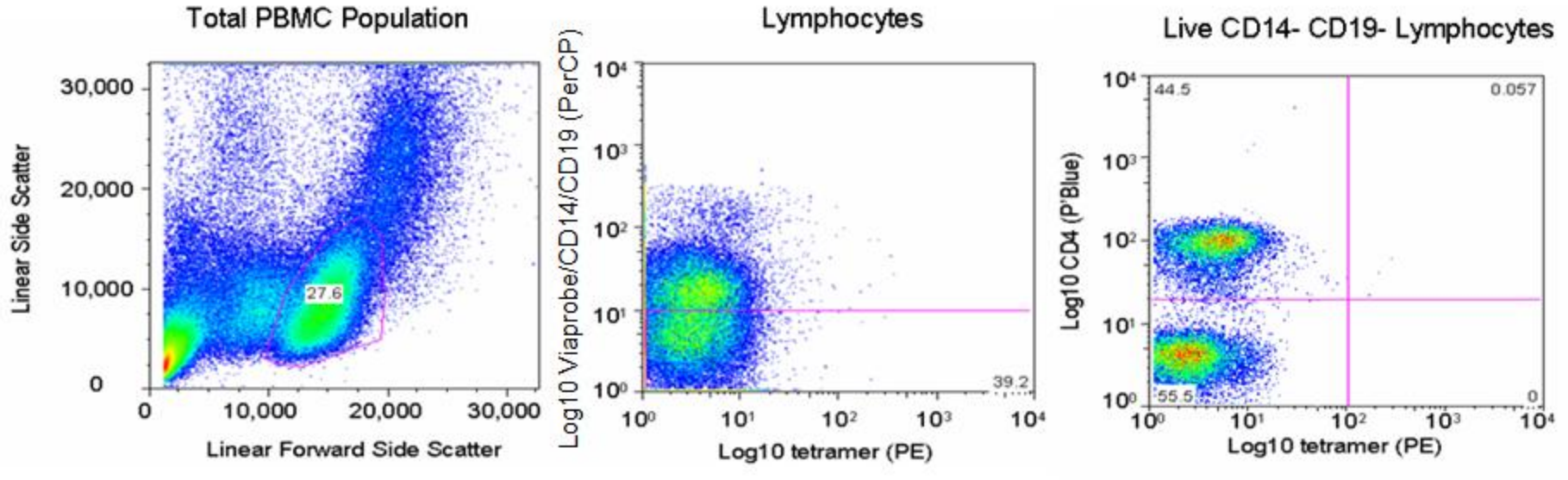
The individual was visited by the ROVE research nurse three days after the onset of symptoms. The HCW had a mild rash consisting of five lesions; one on the forehead, one on each arm, and one on both the spine and abdomen and also reported feeling tired at the onset of symptoms. The research nurse recorded a body temperature of 37.8^{o}C; the lesions were examined and swabbed and a full set of blood samples were taken. Vesicle swabs were transported to the laboratory in saline and DNA was extracted within 2h of collection. As can be seen from appendix 7.24 vaccine virus DNA was obtained from all vesicle fluids. The date of recovery was noted as 10 days after the onset of symptoms. The TRFIA reading obtained from serum drawn three days after the rash developed was 30mIU/ml and VZV specific IgM was also detected. Six weeks after the subject was first vaccinated, their antibody titre had risen $\log_{10}2.43$ (269mIU/ml) with an avidity of 32%. The HCW was subsequently withdrawn from the study and the second dose of vaccine was not administered.



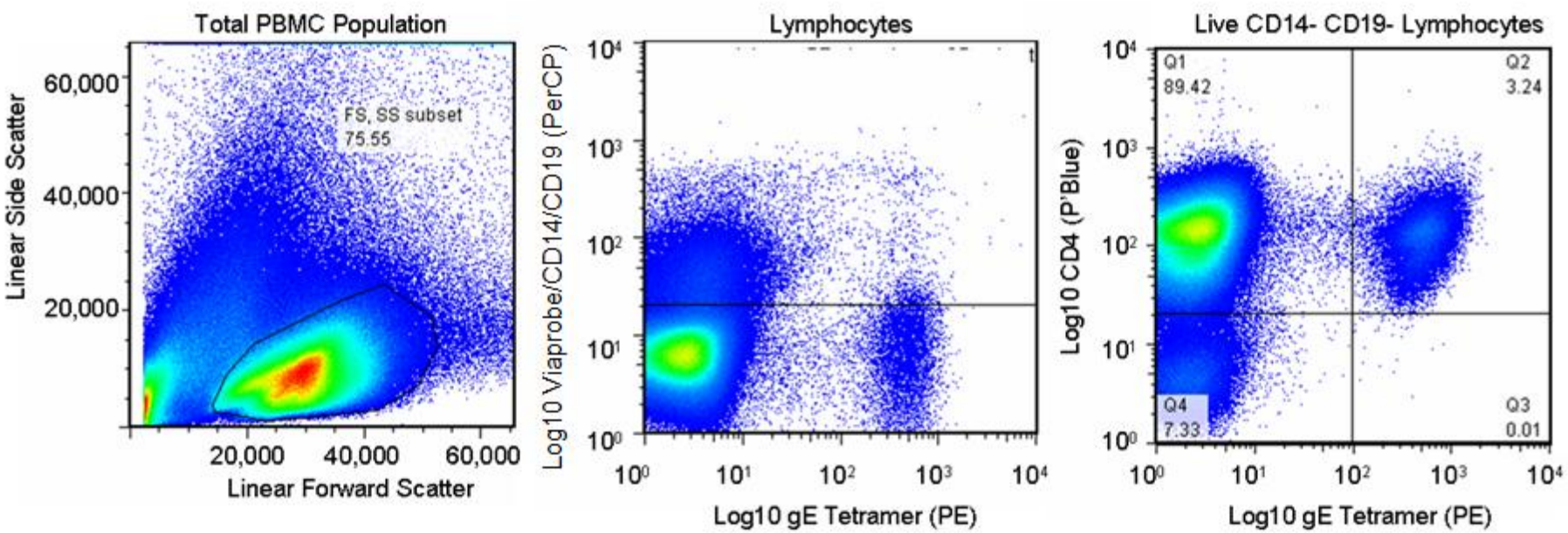
Appendix 7.24: Confirmation of a Vaccine Related Rash in ROVE Study Participant 1052. Agarose Gel of *sma1* PCR carried out on DNA extracted from blood and fluid of vaccine related rash vesicles from ROVE study participant 1052. Key: WT +ve; wild type positive, -ve; negative, bp; base pair.

	Baseline (Visit 1)		Six weeks (Visit 2)		12 weeks (Visit 3)		18 Month Follow-Up (Visit 4)	
	Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive
%	69	31	30	70	4	96	25	75
n	74	34	32	76	4	99	19	56

Appendix 7.25: Summary of Positive TRFIA Status at each Study Time Point, Classified According to the TRFIA cut-off of $log_{10}2.11$ (130mIU/ml). (See corresponding figure 3.14).



Appendix 7.26: An Example of Gating Used to Identify Tetramer$^+$ CD4$^+$ T Cells *Ex Vivo*. Lymphocytes were identified by size and granularity (using forward and side scatter); live lymphocytes (T cells) were identified as Viaprobe, CD14 and CD19 (PerCP) negative and subsequently plotted by CD4 (P' Blue) against tetramer (PE). Key: PerCP; Peridinin chlorophyll protein, PE; Phycoerythrin, P'Blue; Pacific Blue.



Appendix 7.27: An Example of Gating Used to Identify Tetramer$^+$ CD4$^+$ T Cells Following Short Term Culture. Lymphocytes were identified by size and granularity (using forward and side scatter); live lymphocytes (T cells) were identified as Viaprobe, CD14 and CD19 (PerCP) negative and subsequently plotted by CD4 (P' Blue) against tetramer (PE). Key: PerCP; Peridinin chlorophyll protein, PE; Phycoerythrin, P'Blue; Pacific Blue. (1018, visit 3, gE).

Number		Glycoprotein E				IE63			
		Ex Vivo		Lines (10 Day Culture)		Ex Vivo		Lines (10 Day Culture)	
Study (Subset)	Visit	Population Size	% Tet$^+$ (n)	Population Size	% Tet$^+$ (n)	Population Size	% Tet$^+$ (n)	Population Size	% Tet$^+$ (n)
1001 (PLR/NL)	V1	4	0 (0)	X	X	4	0 (0)	X	X
	V2	Sample Not Available							
	V3	19,199	0.010 (2)	X	X	2,325	0 (0)	X	X
	V4	5,946	0 (0)	13,931	0	21,721	0.022 (5)	968	4.545
1018 (NSC1/Boost)	V1	96	0 (0)	X	X	45	0 (0)	X	X
	V2	6,082	0.033 (2)	15,766	0	7,778	0.051 (4)	4,853	0
	V3	31,160	0.0006 (2)	573,134	3.24	27,431	0.01 (9)	807,500	3.98
	V4	37,671	0.029 (13)	X	X	107,873	0.016 (17)	X	X
1028 (NSC1)	V1	571	0 (0)	X	X	733	0 (0)	X	X
	V2	1,758	0 (0)	9,114	0.0219	1,412	0.212 (3)	4,860	0.103
	V3	2,269	0.042 (1)	X		2,090	0.144 (3)	X	
	V4	Sample Not Available							
1058 (NSC1/R)	V1	81	0 (0)	X	X	111	0 (0)	X	X
	V2	2,771	0 (0)	X		2,249	0.089 (2)	X	
	V3	348	0 (0)	X	X	354	0 (0)	X	X
	V4	17,213	0.034 (5)	725,763	0.004271	7,225	0.055 (4)	463,239	0.002375

Appendix 7.28 (1 of 2)

Number		Glycoprotein E				IE63			
		Ex Vivo		Lines (10 Day Culture)		Ex Vivo		Lines (10 Day Culture)	
Study (Subset)	Visit	Population Size	% Tet$^+$ (n)	Population Size	% Tet$^+$ (n)	Population Size	% Tet$^+$ (n)	Population Size	% Tet$^+$ (n)
1067 (NSC1/R)	V1a	52,632	0.01 (6)	569,995	0.51	56,546	0.004 (3)	73,892	0.0298
	V1b	34,838	0.021 (7)	X	X	38,090	0.024 (9)	X	X
	V2	5,308	0.0188 (1)	9,640	0.0519	7,712	0.05 (4)	7,416	0.027
	V3	6,771	0.030 (2)	X	X	3,389	0 (0)	X	X
	V4	34,390	0.003 (3)	860,968	0.12	20,804	0.001 (3)	265,168	0.0211
1088 (NSC1/R)	V1	884	0	X	X	477	0 (0)	X	X
	V2a	987	0 (0)	X	X	881	0 (1)	X	X
	V2b	691	0 (0)			X	X		
	V3	14,736	0.007(1)	X	X	9,299	0.022 (2)	25,537	0.117
	V4	1,000	0 (0)	X	X	1,199	0.03 (2)	X	
1106 (NSC1/R)	V1	21,602	0 (0)	12,783	0.0548	11,635	0 (0)	216,890	0.012
	V2	11,248	0.009 (1)	X	X	5,544	0 (0)	X	X
	V3a	8,779	0.034 (3)	8,403	0	7,689	0.065 (5)	11,348	0
	V3b	4,972	0.02 (1)			6,913	0.014 (1)		
	V4	209	0 (0)	X	X	95	0 (0)	X	X

Appendix 7.28 Percentage of Tetramer$^+$, CD4$^+$ T Cells Quantified *Ex Vivo* and Following 10 Days of Culture with the Corresponding Peptide.

Key: PLR; Positive Low Responder, NSR1; Negative, 'Susceptible' Responded to One dose, V1; visit 1 (baseline), V2; visit 2 (six weeks post vaccination), V3; visit 3 (12 weeks post first, six weeks post second vaccination), V4; visit 4 (18 month follow-up), a and b; two sets of surface markers were used on these samples, X; T cell lines were not set up for these samples, or a second set of surface stains was not used. The number of tetramer$^+$ cells are expressed as a percentage of the population size (total number of live, CD14$^-$ CD19$^-$ lymphocytes).