Investigation of Varicella Zoster virus Glycoprotein-specific T cell responses

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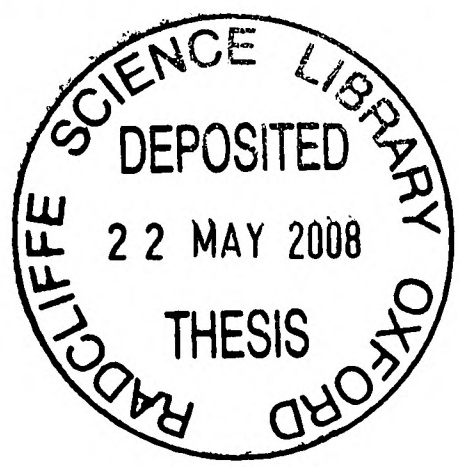

Abstract

T cells are believed to be important in the control of varicella zoster virus (VZV) replication but little is known of T cell epitopes and the relationships between T cell responses, viral load and clinical disease during primary infection.

I initially set to investigate the immune responses to two of the main VZV glycoproteins (gE and gI) using *ex vivo* and cultured IFNγ ELISpot assays. I identified several novel CD4+ T cell epitopes within gE and gI and characterized the phenotype of gE DRB1*1501 tetramer specific responses in healthy immune donors. I then set out to investigate the function and phenotype of VZV specific T cells in primary infection and their relationship to viral loads and clinical disease severity by using glycoprotein E/DRB1*1501 specific MHC class II tetramers, *ex vivo* IFNγ ELISpot assays and quantitative real time PCR assays. I compared the frequency and phenotype of specific T cells with virological and clinical outcomes in 32 adult individuals with primary VZV infection.

In healthy immune donors, the gE specific T cells showed a early intermediate stage of differentiation with evidence of recent activation. Patients with acute primary infection had higher VZV/DRB1*1501 tetramer specific T cell responses and expressed markers of activation and effector differentiation. Viral loads were found to be significantly higher in patients with moderate to severe infection compared to those with mild infection ($p<0.001$). A significant inverse correlation was seen between the viral loads and the *ex vivo* IFNγ ELISpot responses of the patients ($p<0.05$, $r=-0.64$).

These data would be compatible with a role for gE and gI-specific T cells in the control of viral replication during both primary infection and re-activation.

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Publications arising from this thesis:

1. **Malavige GN**, Jones L, Black AP, Ogg GS. Rapid effector function of varicella-zoster virus glycoprotein I-specific CD4+ T cells many decades after primary infection. J Infect Dis. 2007 Mar 1;195(5):660-4. Epub 2007 Jan 22
2. **Malavige GN**, Jones L, Black AP, Ogg GS. Varicella zoster virus glycoprotein E-specific CD4+ T cells show evidence of recent activation and effector differentiation, consistent with frequent exposure to replicative cycle antigens in healthy immune donors. Clinical and Experimental Immunology: In press.
3. Jones L, Black AP, **Malavige GN**, Ogg GS. Persistent high frequencies of varicella-zoster virus ORF4 protein-specific CD4+ T cells after primary infection. J Virol. 2006 Oct;80(19):9772-8.
4. Jones L, Black AP, **Malavige GN**, Ogg GS. Phenotypic analysis of human CD4(+) T cells specific for immediate early 63 protein of varicella-zoster virus. Eur J Immunol. 2007 Dec;37(12):3393-403

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List of abbreviations

aa	Amino acid
CMV	Cytomegalovirus
CD	Cluster of differentiation
DNA	Deoxyribonucleic acid
EBV	Epstein Barr virus
ELISpot	Enzyme-linked immunospot assay
FACS	Flurorescence-activated cell sorter
FITC	Fluorescein isothicyanate
FCS	Foetal calf serum
HCV	Hepatitis C virus
HIV	Human Immune deficiency virus
HLA	Human leukocyte antigen
HSV	Herpes Simplex virus
HZ	Herpes zoster
ICS	Intracellular Cytokine Assay
IFNγ	Interferon gamma
IL	Interleukin
MHC	Major histocompatibility complex
NK	Natural killer
NKT	Natural killer T cells
PBMC	Peripheral blood mononuclear cells
PBS	Phosphare buffer saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PHA	Phytohaemagglutinin
ORF	Open reading frame
RNA	Ribonucleic acid
SEM	Standard error of mean
SFU	Spot forming units
VZV	Varicella Zoster virus

Chapter 1. Introduction

1.1. Varicella zoster virus: historical aspects

The Varicella Zoster virus (VZV) causes chickenpox during primary infection and herpes zoster during reactivation. The virus is thought to have emerged 70 million years ago (Nogueira and Traynor, 2004). Both chickenpox and herpes zoster have been described from ancient times and the name 'herpes' is thought to be attributed to Hippocrates which originates from a Greek word meaning 'creeping'(Wood, 2000). The word 'zoster' is thought to also originate from Greek which means 'girdle' and 'Shingles' is derived from Latin which means 'belt'(Nogueira and Traynor, 2004).

Initially chickenpox was thought to be a milder form of small pox and thus the name chickenpox which was thought to imply 'weaker' pox. The fact that chickenpox was different to small pox was first described by William Heberden in 1818 who also discovered that it elicits life long immunity(Wood, 2000). However, the relationship between chickenpox and herpes zoster was not discovered until much later and was first suggested in 1888 by a Viennese physician Janos von Bókay when he observed that household exposure to herpes zoster gave rise to chickenpox in young children. Later in 1925, Kundratitz confirmed his observations by showing that vesicular fluid of either chickenpox or herpes zoster could induce a varicella like rash in healthy unexposed children (Nogueira and Traynor, 2004). The virus was isolated in 1954 by Thomas H. Weller, who showed conclusively that both were caused by the same virus (Weller and Coons, 1954) which he named as VZV (Nogueira and Traynor, 2004). Later with the development of molecular genetic techniques it could be confirmed that although there were differences between individual VZV strains, the strain isolated from individuals with herpes zoster was the same strain isolated from their chickenpox lesions (Straus et al., 1984).

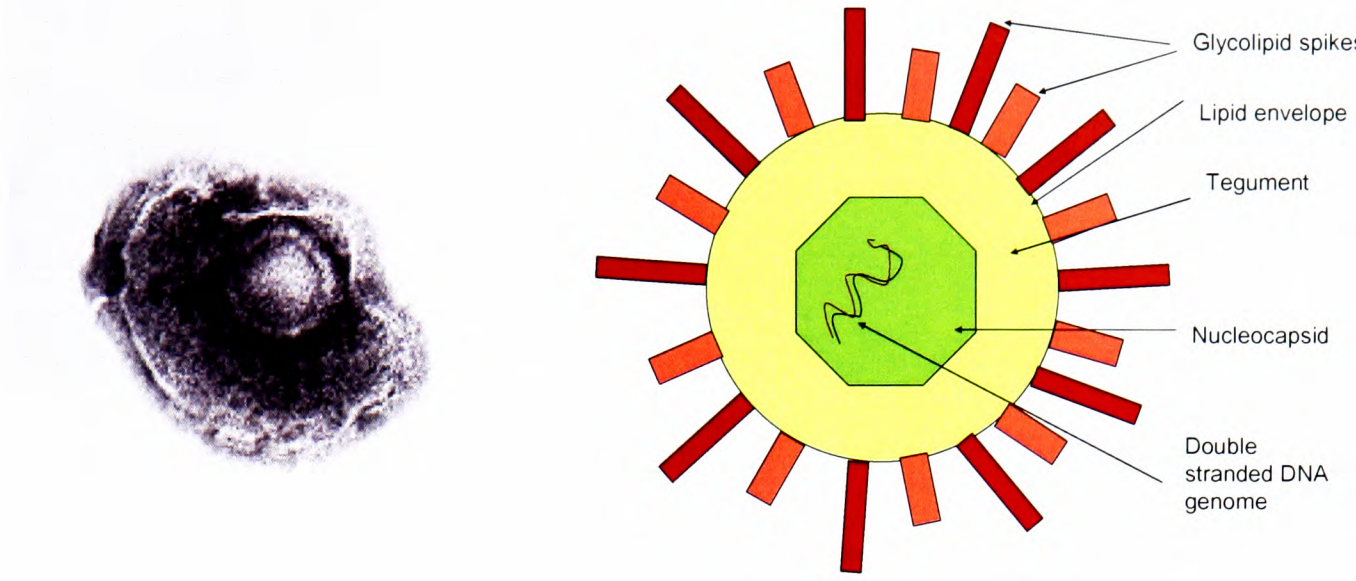
Date	Event
1818	Chickenpox was caused by a different infective agent than small pox and infection resulted in life long immunity(Wood, 2000).
1888	Relationship between chickenpox and herpes zoster was first suggested(Wood, 2000).
1925	Kundratitz first showed that the VZV could be transmitted from infectious vesicular fluid from one person to another and also that vesicular fluid from zoster could also give rise to chickenpox in a non immune host.
1906	Description of multinucleated giant cells in skin lesions in chickenpox
1943	The possibility that herpes zoster occurs due to reactivation of VZV was first suggested.
1948	Visualization of the virus by electron microscopy
1952	Weller and Stoddard isolated the virus from both chickenpox and herpes zoster lesions(Weller and Coons, 1954)
1958	Confirmation and naming the infective agent of both chickenpox and herpes zoster as the varicella zoster virus(Weller and Witton, 1958; Weller et al., 1958)
1972	It was first demonstrated that VZV established latency in the sensory ganglion (Esiri and Tomlinson, 1972)
1986	Sequencing of the entire VZV(Davison and Scott, 1986) by Davison and Scott

Table 1: A brief history of the VZV infections

1.2. The virus

VZV is a member of the *Herpesviridae* family and because of its rapid replication and neurotropism it is classified as an alpha herpes virus. It has a very narrow host range with infection exclusively limited to humans and cells of simian origin(Arvin, 2000). Following primary infections as characteristic to all alpha herpes viruses, it then lies dormant in dorsal sensory root ganglia. It has many other features common with the Herpes Simplex virus which belongs to the same group.

VZV is an enveloped double stranded DNA virus consisting of around 125,000 base pairs (Arvin, 1996). The viral glycoproteins make up the outer layer and the tegument is sandwiched between the nucleocapsid and the glycoprotein layer (Quinlivan and Breuer, 2006). VZV has the smallest genome among the herpes viruses with approximately 71 open reading frames (ORFs). VZV genes are transcribed in a controlled manner and accordingly are called immediate early (IE), early (E) and late proteins, depending on the time in which they are transcribed during the virus replicative cycle (Abendroth and Arvin, 2001b). IE and E genes encode proteins important for replication of viral DNA, whereas the late genes encode the structural components of the virion such as the glycoproteins. However, as the virus is the smallest of the group of alpha herpes viruses it lacks genes encoding several proteins in HSV(Arvin, 1996).



1-1 Electron microscopic image of the VZV (left) and the structure of the Varicella zoster virus (right)

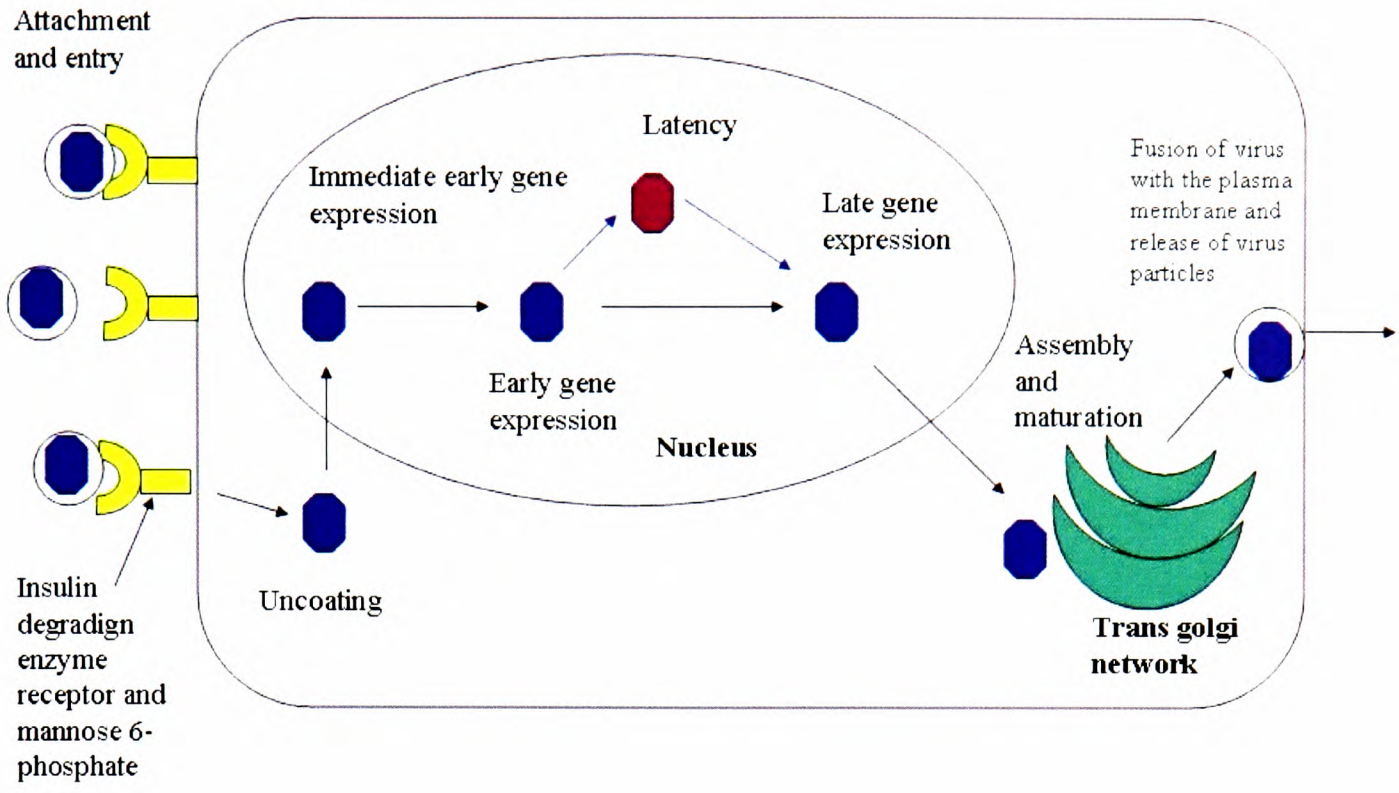
The complete nucleotide sequence of the VZV Dumas strain was determined by Davidson and Scott in 1986 (Davison and Scott, 1986). It is thought to be the most stable human herpes virus with 0.05% to 0.06% nucleotide diversity (Quinlivan and Breuer, 2006). Molecular epidemiological studies have identified four major clades of the virus which have a varied geographical distribution (Peters et al., 2006). Virus isolates from Japan/Singapore regions segregate into clade B and C whereas, those of Western Europe and North American origin were segregate into clade A and D (Quinlivan and Breuer, 2006). Accordingly, the Dumas strain is classified under clade A and the Oka virus strain (vaccine strain) comes under clade B (Peters et al., 2006). However, others have classified the genotypes of VZV in to 3 major genotypes E (European), J (Japanese) and Mosaic (M) (Loparev et al., 2004). According to this classification M1 and M2 strains were commoner in the tropics (Loparev et al., 2004). Although, most of these strains do not have significant differences in viral virulence, the mutation in a wild type virus in the immune dominant gE B cell epitope (VZV-MSP), is thought to result in accelerated cell

to cell spread (Santos et al., 2000). In addition, the VZV-MSP virus is shown to progress more extensively in the skin implants in the SCID-hu mouse model (Santos et al., 2000).

1.3. Virus life cycle

The virus uses the insulin degrading enzyme as the receptor for cell to cell spread of the virus as well as in the spread of cell free virus (Li et al., 2006). It has been shown that the main VZV glycoprotein gE binds to this receptor through its extracellular domain. It is thought that the mannose 6-phosphate receptor also facilitates entry of the virus into the cell. However, current evidence shows that the insulin degrading enzyme receptor may play a greater role (Li et al., 2006).

Once the virus enters the cell, VZV proteins encoded by ORF4, ORF10 and ORF62 are thought to be released and enter the nucleus where they initiate transcription of viral genes. ORF62 is thought to be the most important transcription regulator (Quinlivan and Breuer, 2006). These viral gene transcripts are translated in the cytoplasm and the IE proteins then regulate expression of early genes. The early genes express enzymes for viral DNA replication (Gershon and Gershon, 1999). The virus particles initially assemble in the cell nucleus and enter the endoplasmic reticulum. However, the assembly of the mature virion occurs in the trans-golgi network. Vacuoles containing the virus are then thought to fuse with the plasma membrane and sequentially release the virus (Quinlivan and Breuer, 2006). Virus replication is highly cell associated and the virus is not released at any phase of the replicative cycle in cell culture (Rentier et al., 1996).



1-2 The life cycle of the Varicella zoster virus

Viral proteins are expressed within 4 to 6 hours following initiation of replication and spreads to neighboring cells as early as 8 to 16 hours after infection(Arvin, 2000). Formation of multinucleated giant cells and other cytopathic changes usually take place within the next 2-7 days (Arvin, 2000). By using immunohistochemical methods, VZV infected cells have been shown to be visible 4 to 10 hours following inoculation (Arvin, 2000). Syncytia formation is the hallmark of VZV cytopathic effects in cell culture.

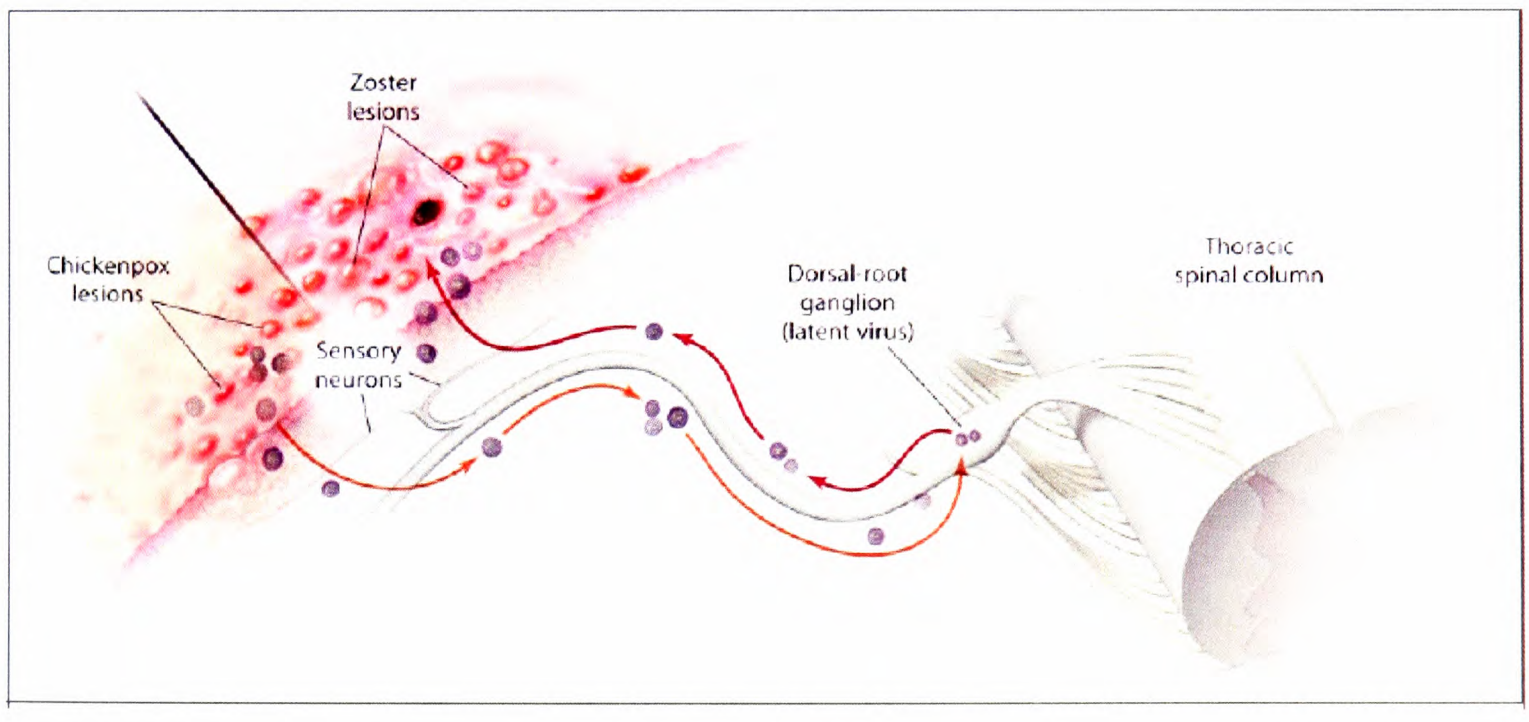
1.4. Varicella zoster virus latency

The VZV, as common to all alpha herpes viruses becomes latent in the dorsal root sensory ganglia, the trigeminal ganglia and the autonomic ganglia in humans. However, the mechanisms of VZV latency are different to that of other human alpha herpes virus such as herpes simplex virus (Sadzot-Delvaux et al., 1999). Early studies showed that VZV RNA was seen in the cytoplasm of neuronal cells (Hyman et al., 1983) which was later confirmed by many (Dueland, 1996; Kennedy et al., 1999; Lungu et al., 1995). However, some have found viral RNA in non neuronal cells such as satellite cells (Lungu et al., 1995; Meier et al., 1993). Therefore, although the site of VZV latency is a much debated issue, it is generally believed that its site of latency is predominantly the neuronal cells while some virus may be present in non neuronal satellite cells (Kennedy, 2002; Lungu et al., 1995).

In contrast to HSV where there is complete inactivation of viral gene transcription during latency, several VZV immediate early and early genes transcripts have been shown in human dorsal root and trigeminal ganglia during latency (Lungu et al., 1998). Analysis of these gene transcripts in human trigeminal ganglia by quantitative real time PCR showed that gene 63 was the most abundantly transcribed (Cohrs and Gilden, 2007). Experiments carried out by using ORF 63 deletion mutant viruses showed that ORF 63 was essential for establishing viral latency (Cohen et al., 2004). Transcripts of genes 21, 29, 62 and 66 were also found in some ganglia (Cohrs and Gilden, 2007; Cohrs et al., 2000). Other studies that used in situ hybridization for the detection of viral RNA and DNA also found the above transcripts and in addition had detected transcripts of ORF 4 (Kennedy et al.,

1999). So far transcripts of ORF 10, 40, 51 or 61 have not been detected (Cohrs and Gilden, 2007; Kennedy et al., 1999; Meier et al., 1993).

The percentage of cells harboring the VZV has reported to be in the range of 0.01% to 30% (Lungu et al., 1998) with an average of 565 ±639 viral copies per 10^5 trigeminal ganglion cells (Verjans et al., 2007). Analysis of viral gene copy numbers in ganglia showed that the viral loads were not significantly different in the left and the right ganglia. However, there was a wide range of the number of copies of viral genes seen among different individuals (Cohrs et al., 2000). Although, the differences in viral copy numbers in ganglia could reflect the initial viral load during acute primary infections this has still not been investigated.



1-3 Establishment of VZV Latency in Sensory-Nerve Ganglia.

After a primary VZV infection (chickenpox), latent VZV infection is established in the dorsal-root ganglia, and zoster occurs with subsequent reactivation of the virus. (Kimberlin DW, Whitley RJ. Varicella-zoster vaccine for the prevention of herpes zoster. N Engl J Med. 2007 Mar 29;356(13):1338-43).

Recently experiments on trigeminal ganglia resident T cells showed that many were specific for the herpes simplex virus. Interestingly, VZV specific T cells were not detected in the these ganglia despite expression of VZV protein (Hufner et al., 2006; Verjans et al., 2007). However, VZV specific T cell responses in these T cells were studied by infecting antigen presenting cells (EBV transformed lymphoblastoid lines) with recombinant vaccinia viruses expressing VZV ORF 4, 29, 62 and 63 only (Verjans et al., 2007). Therefore, this study does not rule out the presence of T cells specific to other VZV viral proteins.

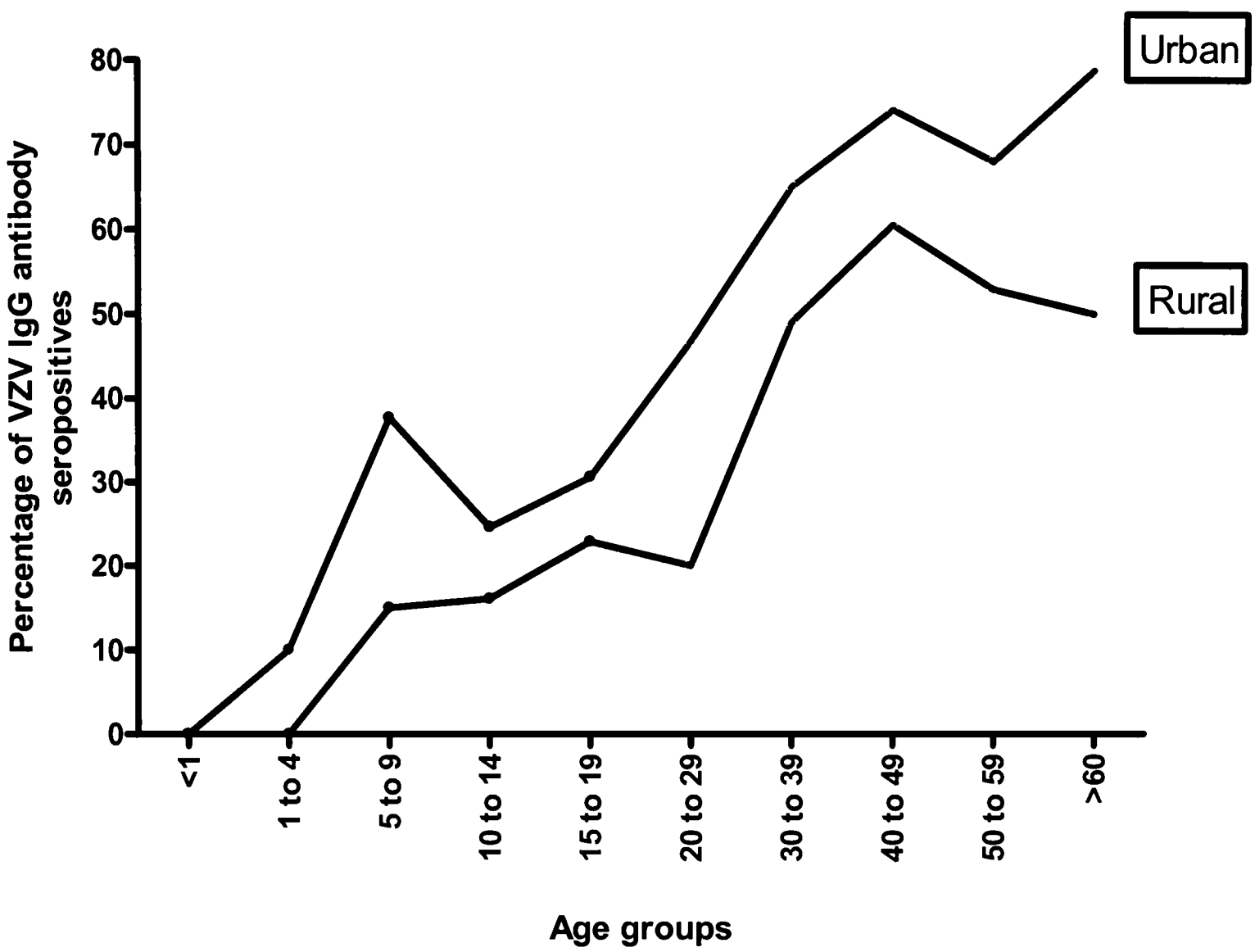
1.5. Epidemiology of Primary VZV infections

VZV infections occur worldwide and infections are mainly seen among children below the age of 5 years in the temperate climates (Nardone et al., 2007). Annual epidemics in these countries are seen during late winter and spring. VZV seropositivity rates are reported to be between 53% to 100% in 5 year olds in these countries (de Melker et al., 2006; Khoshnood et al., 2006; Thiry et al., 2002; Vyse et al., 2004) and VZV transmission rates have shown to be quite high (Nardone et al., 2007). Netherlands is one of the countries that reports one of the highest sero prevalance rate at 5 years of age and also a very high transmission rate. Interestingly, it has the lowest rate of varicella associated complications (Nardone et al., 2007). Therefore, it is possible that high circulation rates of VZV in the community is somehow protective against development of VZV associated complications, probably due to continued boosting of VZV-specific immunity.

In contrast to temperate climates, the epidemiology in of VZV infections is remarkably different in tropical climates. In the tropics, infection mainly occurs among young adults resulting in significant morbidity, higher hospital admission rates and mortality. The seroprevalance rates of VZV among 5 year olds in India was 29% (Lokeshwar et al., 2000) while in Sri Lanka it was 10% (Liyanage et al., 2007). In Singapore only 20% of 15 year olds had detectable anti VZV antibodies (Ooi et al., 1992). In Sri Lanka, only 50% of those living in the rural areas had chickenpox even at the age of 60 years (Fig1-4). Most importantly, 56.2% of females in both rural and urban populations of child bearing age (15- 45 years), were non immune to chickenpox. Similar seroprevalence rates are seen in other tropical countries where primary varicella is mainly a disease in young adults (Garnett et al., 1993; Venkitaraman and John, 1984). VZV associated complications and mortality rates are much higher in Sri Lanka when compared to temperate climates(Bovill and Bannister, 1998; Marchetto et al., 2007) possibly due to the older age of acquiring the infection. For instance, primary VZV infection was associated with a mortality rate of 3.7% in the infectious diseases hospital in Sri Lanka which is geared to treat adult patients with infectious diseases (Welgama et al., 2003). Although, the reasons for the differences in epidemiology in tropical and temperate climates are incompletely understood, some have suggested that the differences in temperature and humidity could affect virus transmission (Garnett et al., 1993; Lolekha et al., 2001).

The introduction of the live attenuated VZV vaccine has had a significant impact on the epidemiology of primary VZV infections in countries where universal childhood VZV

immunization is carried out. The vaccine efficacy is reported to be between 71% to 100% for the occurrence of infections and more than 90% against moderate to severe infection (Miron et al., 2005). The VZV vaccine is discussed in more detail later in this chapter.



1-4 Age specific VZV IgG antibody seroprevalance rate in the urban and rural populations in the Colombo district, Sri Lanka(Liyanage et al., 2007).

1.5.1. Epidemiology of herpes zoster

The lifetime risk of zoster is estimated to be 10–30% and incidence increases markedly with age affecting up to 50% of people who are 85 years (Thomas and Hall, 2004). The incidence of HZ is around 0.74 per 1000 person-years in children aged <10 years, while it is around 7.8 per 1000 person-years in those aged >60 years. However, the incidence of

HZ is thought to be less in countries where primary varicella occurs at a later age (Sri Lanka, India, Pakistan etc) (Thomas and Hall, 2004). As the virus is believed to be kept in its latent form due to VZV specific cell mediated immunity, diseases such as malignancy, HIV infection and the use of immune suppressant drugs are risk factors for reactivation (Schmader, 2001). In addition, primary varicella infection in the first year of life is also thought to be a risk factor for herpes zoster in childhood (Takayama et al., 2000; Terada et al., 1993).

With the introduction of the live VZV vaccine many have voiced concerns over a possible increase in the incidence of HZ due to the reduced exposure to the wild type virus (Brisson et al., 2003). In fact one study reported a rise in the incidence of herpes zoster in the USA (Yih et al., 2005). However, more recent studies which examined if exposure to chickenpox actually prevented the development of HZ found no such relationship (Chaves et al., 2007b). Therefore, currently there is no evidence to suggest that universal VZV vaccination increases the incidence of HZ and suggests that it does not have an impact on the epidemiology of HZ. However, this issue is still a 'hotly' debated topic.

Recently a 'zoster vaccine' was licensed for the protection against HZ (Mitka, 2006). This is a high potent form of the live attenuated VZV vaccine which could have a dramatic impact in the incidence of HZ and its complications (Holcomb and Weinberg, 2006).

1.6. Clinical features of primary VZV infections

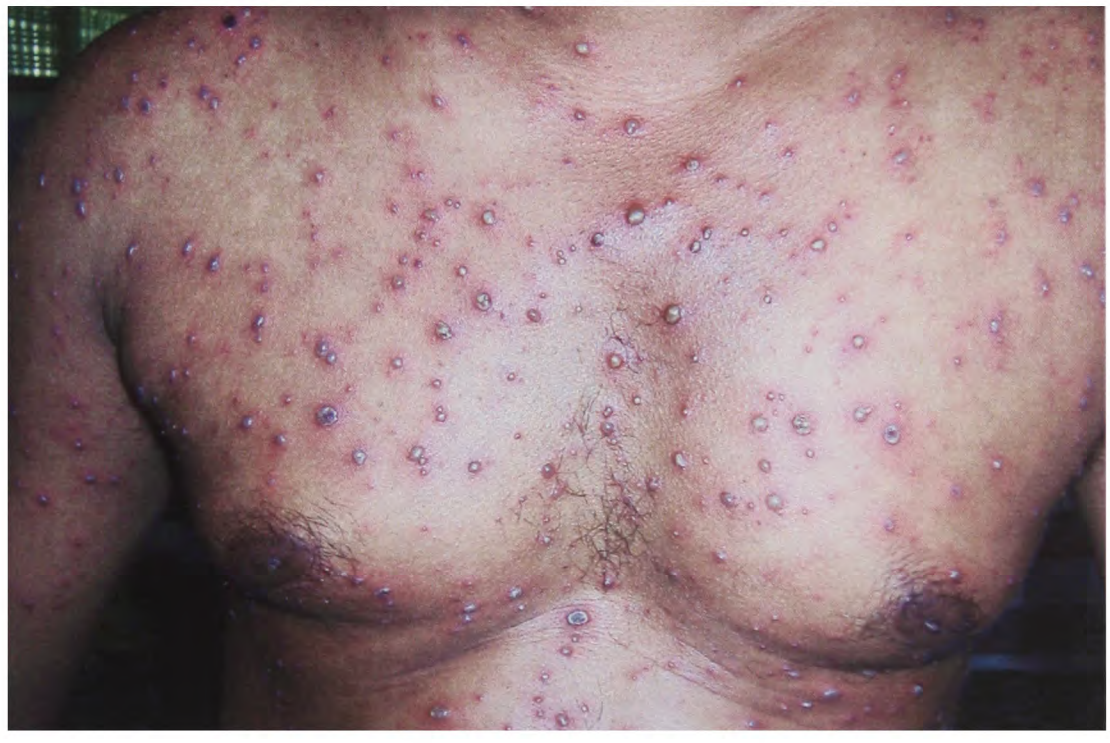
Chickenpox is a common childhood illness, characterized by fever and a pruritic vesicular rash of generalized distribution. Prodomal symptoms such as headache, malaise and fever usually occur 24 to 48 hours before the onset of the rash and are more severe in adults and older children. The initial vesicles arise on the trunk and face and then spread gradually to involve the whole body. The lesions which arise in crops, are macular papular first and then gradually evolve in to vesicles (Arvin, 1996). Painful vesicles are sometime also seen on mucous membranes such as the oropharynx, conjuctiva and vagina (Arvin, 1996). However, the number of lesions and disease severity vary widely among individuals with children usually having more milder disease than adults (Danovaro-Holliday et al., 2004; Meyer et al., 2000b).

Chickenpox is thought to often cause symptomatic disease even in children with a wide range of clinical disease (less than 10 lesions in some and over 1000 lesions in others) (Arvin, 1996). Although chickenpox is usually a mild illness in the majority it can give rise to serious complications such as skin and soft tissue infections, pneumonia, otitis media, endocarditis and neurological complications (cerebellar ataxia and encephalitis) (Jackson et al., 1992; Losurdo et al., 2005; Peterson et al., 1996). Skin and soft tissue infections were the commonest complication in children while pneumonitis was the commonest in adults (Galil et al., 2002a).

Neurological complications in hospitalized children have been shown to vary between 11.5% (Somekh et al., 2000) to 38% (Tseng et al., 2000) (Koturoglu et al., 2005). Acute

cerebellar ataxia is reported to occur in approximately 1 in 4000 patients with VZV, usually occurring 1 week after the onset of the rash and has a good prognosis (Gnann, 2002). However, encephalitis the most serious neurological complication of VZV, is reported in about 4 cases per 10,000 cases and may have a fatal outcome (Tarlow and Walters, 1998).

As with many other viral infections, adults are more likely to suffer from more severe disease during chickenpox. Adults are 9 to 15 more likely to be hospitalized (Galil et al., 2002a) and 25 times more likely than children to die from varicella (Meyer et al., 2000b). They are also 25 times more likely to get pneumonia than children (Mohsen et al., 2001). VZV infection associated pneumonia has an incidence of 0.3% to 50% and a reported mortality of 2.15% to 20% in adults (Frangides and Pneumatikos, 2004).



1-5 Primary VZV infection in an adult patient on day 5 of illness

Primary varicella infection during pregnancy may in result serious sequelae in both mother and the baby. Varicella pneumonia is more frequent and severe in pregnant women and they have a 10% risk of developing severe varicella pneumonitis (Gnann, 2002). Primary varicella in the first or second trimester may adversely affect the fetus resulting in congenital varicalla. This is characterized by limb hypoplasia, ocular and neurological abnormalities and cicatricial scarring of the skin (Corbeel, 2004).

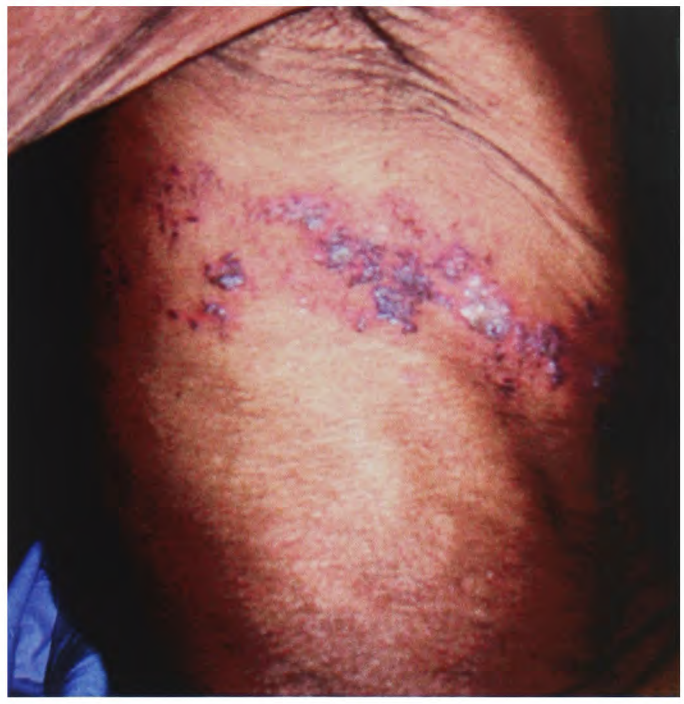
1.6.1. Primary varicella in the immunocompromised

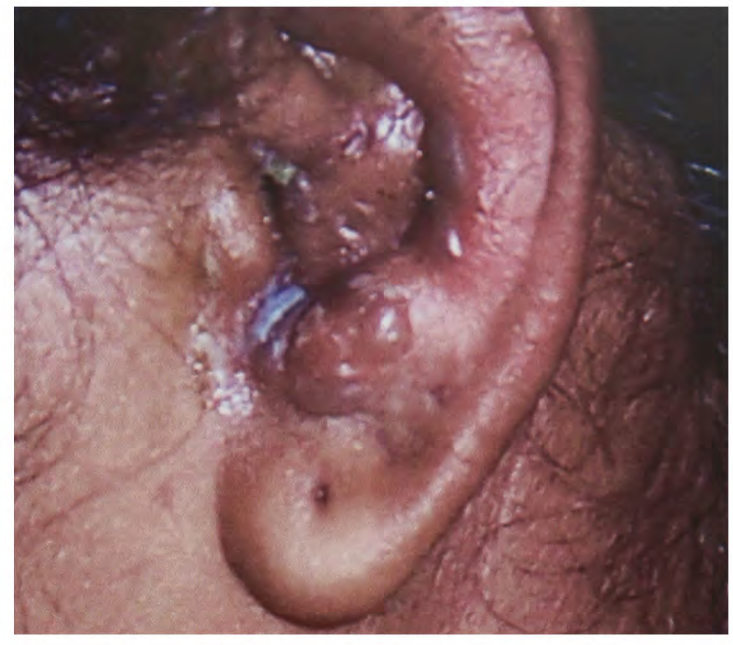
Primary VZV infection is prolonged (more than 10 days) in the immune suppressed and many suffer from complications (Feldhoff et al., 1981; Leibovitz et al., 1993; von Seidlein et al., 1996). Visceral dissemination and multi organ failure subsequently leading to death has been reported in many (Feldman et al., 1975). The initial clinical presentation of primary VZV infections is thought to be different in the immunocompromised. For instance, abdominal pain and back ache have been reported to precede the onset of the typical rash (Milone et al., 1992; Vadoud-Seyedi et al., 1993). In some, skin lesions are absent throughout the disease and thus delaying the diagnosis (Pishvaian et al., 2006). Severe abdominal and back pain is thought to be due to hepatitis and/or pancreatitis indicating visceral dissemination. Fulminant hepatic failure leading to encephalopathy, disseminated intravascular coagulation and thus multi organ failure is thought to be the cause death in such patients (Pishvaian et al., 2006).

1.6.2. Clinical features of herpes zoster

Following primary infection with the VZV, it establishes latency in the dorsal root and the cranial nerve ganglia. After a variable period of time, the virus then reactivates to form herpes zoster which is also known as 'shingles'. The rash is typically seen in the skin segment supplied by that particular cutaneous nerve and therefore, is describes as having a dermatomal distribution. Reactivation is most commonly seen in the thoracic and cranial dermatomes and is preceded by symptoms of malaise, fever and headache.

When viral reactivation is not controlled, viral replication occurs within the ganglion resulting in ganglionitis and extensive infection and destruction of the neurons and surrounding tissue (Dworkin et al., 2007). This is thought to be responsible for the prodromal pain occurring in the dermatome before the rash erupts. The prodromal pain lasts for 2-3 days and is described as excruciating by many. The rash then appears over 3 to 4 days and crusting is usually complete within a week (Dworkin et al., 2007). Viral reactivation may also in the geniculate ganglion resulting in facial nerve palsy and lesions in the ear and side of the tongue (Ramsay Hunt syndrome).





1-6 Herpers zoster and Ramsey Hunt syndrome
Herpes zoster in a thoracic dermatome in an adult male patient (left). VZV reactivation in the distribution of the facial nerve is associated with facial nerve palsy (Ramsey Hunt Syndrome). Lesions are only seen in the inner auricle (right).

Although most patients do not complain of any muscle weakness, it has shown that muscle involvement may occur in 5% to 15% of patients. Electromygraphy during acute herpes zoster has shown that muscles may be involved in half of the cases (Haanpaa et al., 1997). Reactivation causing dermatomal pain in the absence of skin lesions has also been reported (Gilden et al., 1992) and is known as 'zoster sine herpete' (Dworkin et al., 2007). Sometimes reactivation may occur with predominant central nervous system manifestation such as cerebellitis (Ratzka et al., 2006), cranial nerve palsies, myelitis and VZV related unifocal vasculopathy (Gilden, 2004) in the absence of skin lesions. The disease may be more severe in the immune suppressed with multi dermatomal involvement, prolonged disease and visceral dissemination which can be lethal

(Gallagher and Merigan, 1979; Graue et al., 2006; Hackanson et al., 2005; Onunu and Uhunmwangho, 2004).

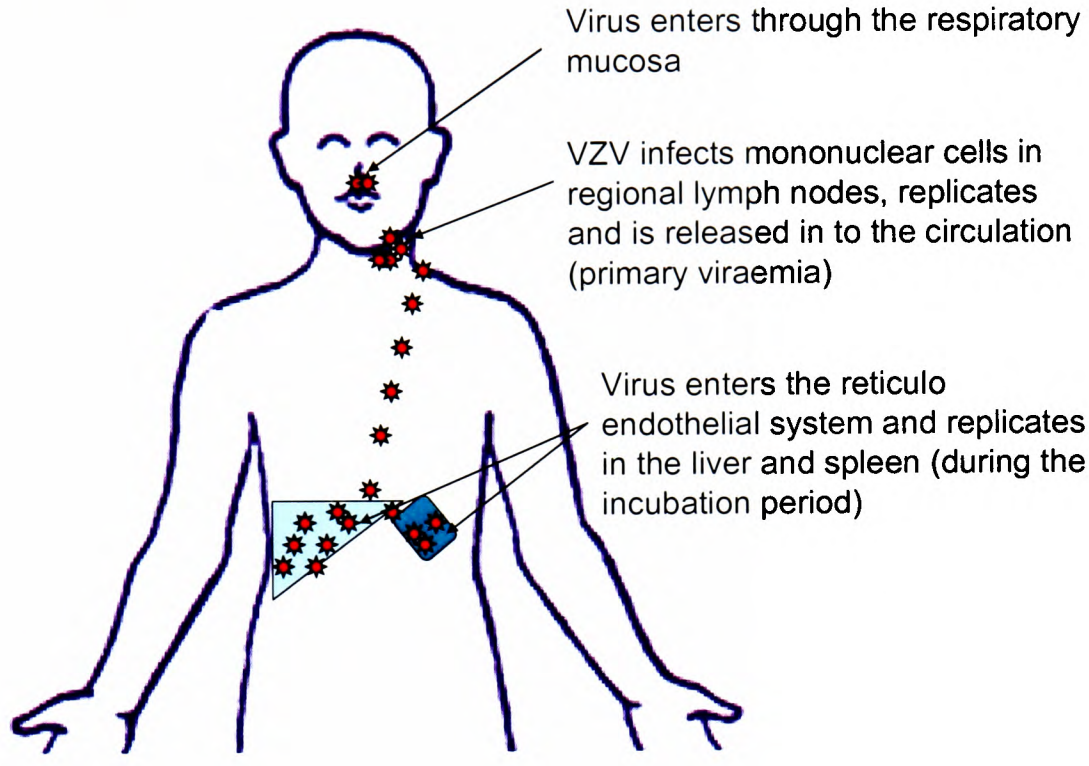
The commonest complication is post herpetic neuralgia (PHN) where a severe and burning pain is seen in affected dermatome that lasts for more than 3 months (Johnson, 2003; Mounsey et al., 2005). It is thought to affect 20% to 30% of individuals with herpes zoster who do not receive antiviral drugs (Wood et al., 1996). 5% to 10% of patients may still have pain even after 1 year (Bowsher, 1999). Greater age, rash severity, peripheral blood viraemia and adverse psychological factors are thought to be predisposing factors of PHN (Bowsher, 1999; Jung et al., 2004). Acute VZV encephalitis, herpes zoster ophthalmicus, hemiparesis, transverse myelitis and retinitis are other rare but serious complications that occur in immune competent adults (Dworkin et al., 2007).

1.7. Pathogenesis

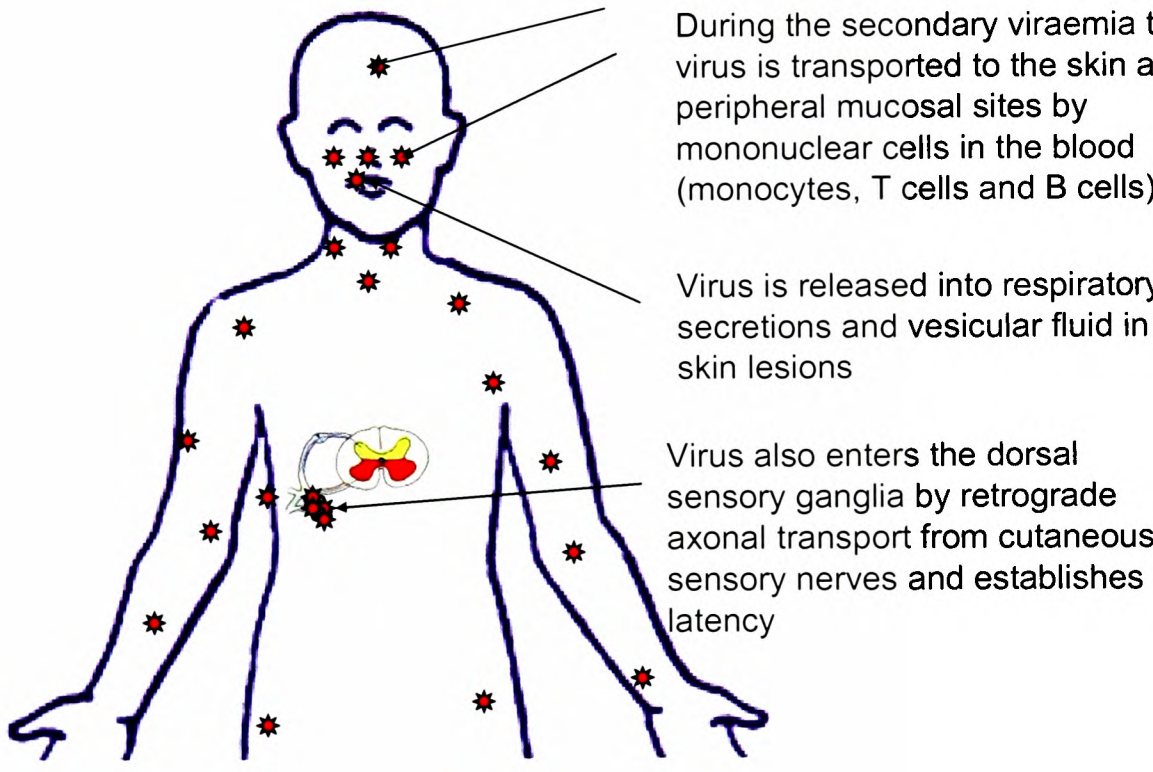
VZV is transmitted by respiratory secretions from the pharynx of patients with chickenpox or from skin lesions from patients with chickenpox or herpes zoster. After the virus comes into contact with the respiratory mucosal epithelium of the 'new' host, following an incubation period of 10 to 21 days, primary varicella is manifested as a diffuse cutaneous skin rash commonly known as chickenpox (Arvin, 1996). Viral DNA has been detected in respiratory mucosal sites prior to the appearance of the rash (Sawyer et al., 1992) which supports epidemiological evidence that the virus is transmissible before the appearance of the rash.

The widely accepted model of the events that take place following infection with VZV is based on the sequence of event in mousepox due to lack of animal models to study VZV infections (Arvin, 1996). Accordingly, it is believed that the VZV infects 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. This is then thought to be followed by a secondary viremia in the late incubation period that results in VZV transport to skin resulting in the typical chickenpox rash (Arvin, 1996).

Incubation period



Acute primary varicella infection

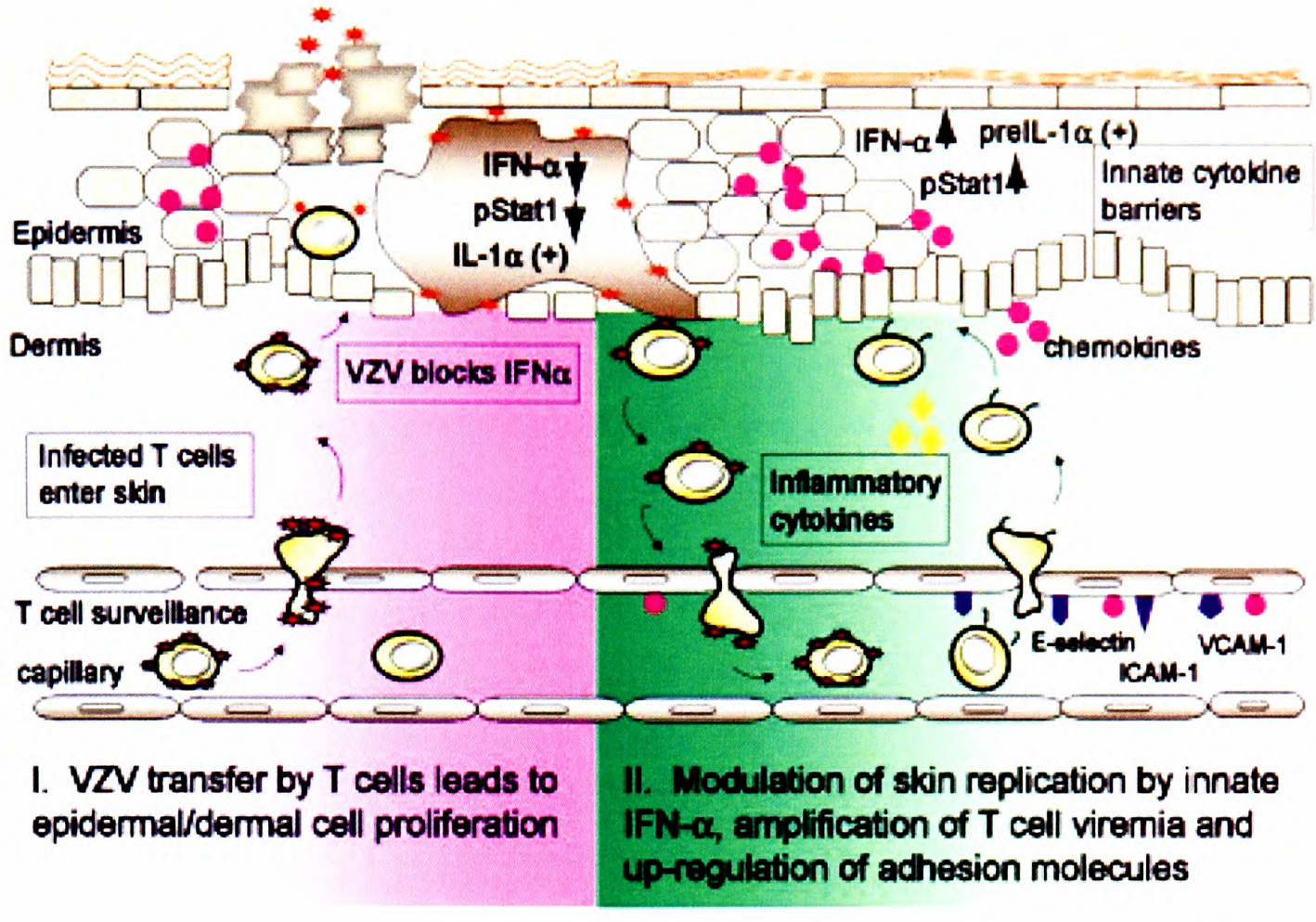


1-7 Pathogenesis of primary VZV infection based on sequence of event in mousepox infection

However, this concept has been criticized by some who believe that the virus is transmitted directly to the skin by infected T cells immediately following infection during primary viraemia (Ku et al., 2005). This has been suggested by Ku C.C. *et al.* based on their experiments in the SCIDHu mouse model. They suggest that the prolonged interval between exposure and the appearance of varicella skin lesions reflects the time required for VZV to overcome previously unrecognized but potent innate immune barriers, such as IFN-α production by epidermal cells in vivo (Ku et al., 2005; Ku et al., 2004). Severe combined immunodeficiency mice (SCID) with human skin xenografts (SCIDhu) were used in these experiments and they were inoculated with VZV infected tonsillar T cells and observed for the development of the skin rash. Inoculated T cells were seen in the skin lesions within 24 hours following inoculation and the VZV was recovered from the skin after 7 days in the mice. IL-1 and IFNα were also shown to be present in VZV uninfected epidermal cells.

Based on these findings Ku C.C *et el* have suggested that once tonsillar T cells are infected with VZV, the virus is directly transmitted to the skin by T cells and accordingly have proposed their model for VZV pathogenesis. In this model they propose that virus infected T cells enter the skin through usual immune surveillance of the skin and in this process infect keratinocytes and other cells in the skin. Subsequently, non infected T cells that are involved in the immune surveillance are infected in this process and disseminate the virus throughout the body resulting in a secondary viraemia.

However, in their model 15 to 30% of the T cells in the inoculum contained the virus and it is doubtful whether such high numbers of tonsillar T cells are infected in 'natural' infection. Although the virus was detected in the skin in mice in 7 days, the relatively shorter incubation period in these mice when compared to natural infection in humans could also be due to higher infective dose of the virus. It has been shown by many that the length of the incubation period is related to the intensity of exposure (Poulsen et al., 2005) which suggests that a higher infective dose of the virus shortens the incubation period by overcoming the innate immune barriers. Therefore, currently the sequences of events that occur prior to appearance of the chickenpox rash during the incubation period are still unclear.



1-8 Model proposed by Ku C.C at el for 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 transport the virus to the skin immediately and release infectious VZV. The remainder of the 10–21-d 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–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 viremia. Intact host immune responses appear to be required to trigger up-regulation of adhesion molecules, facilitating the clearance of VZV by adaptive immunity (Ku et al., 2004).

VZV is thought to cause a viraemia by infecting predominantly T cells (Ku et al., 2004; Moffat et al., 1995) which is highly cell associated (Ku et al., 2005). In acute primary VZV infection viral loads have been reported in the range of 1 to 5000 viral copies per 10^5 PBMCs (Kimura et al., 2000; Mainka et al., 1998) and 100 to 10000 per ml of blood(de Jong et al., 2000). Interestingly, although VZV is highly cell associated VZV DNA has been detected in equal frequency in whole blood, plasma and serum of patients with acute primary VZV and herpes zoster (de Jong et al., 2000).

The virus has shown to infect T cells, B cells and monocytes resulting in apopotosis *in vitro* (Konig et al., 2003). Similar findings have also been observed during acute primary infection, where viral DNA has been found in equal frequency in both $CD8^+$ and $CD4^+$ T cells, B cells and in monocytes (Ito et al., 2001). VZV ORF 66 protein kinase is thought to be and essential protein for T cell tropism (Schaap-Nutt et al., 2006). Studies in SCIDhu mice by Moffatt *et el* have also shown that VZV infects $CD8^+$ T cells and $CD4^+$ T cells at an equal frequency (Moffat et al., 1995). Ku C.C. *et al* who had focused on VZV infection of $CD4^+$ T cells found that the VZV preferentially infected $CD4^+$ T cells that express skin homing markers which they suggest might facilitate the spread of virus to the skin (Ku et al., 2002). Their experiments also show that the virus preferentially infected activated $CD4^+$ T cells expressing the memory phenotype ($CD45RA^-$). Dendritic cells have also been shown to be infected with the virus *in vitro* and were also able to transfer the virus to T cells suggesting that they could have a role in virus dissemination in acute infection (Abendroth et al., 2001b).

The events that occur during acute infection to control the virus are not well understood although, both virus specific T cells and NK cells are thought to play an important role. This will be discussed in greater detail later in this chapter under cellular immune responses to the virus. During acute infection the virus is transported from the skin along sensory nerve fibers to the peripheral sensory ganglia where it establishes latency. The virus then resides in the ganglia till it reactivates later to cause herpes zoster. More details about viral latency are described earlier in this chapter.

Although, VZV is thought to reactivate very infrequently giving rise to herpes zoster, there have been reports of sub clinical reactivation of the virus in both immune competent and immune suppressed individuals (Schunemann et al., 1998). Subclinical viral reactivation was detected by PCR in 19% (Wilson et al., 1992) to 26% (Ljungman et al., 1986) patients who underwent bone marrow transplantation. The virus has also been detected in healthy immune adults over 60 years of age with no evidence of reactivation while it was not detected in younger adults (Devlin et al., 1992). However, a retrospective study investigating viral reactivation in solid organ recipients found no evidence of viral reactivation in the patients' plasma (Kronenberg et al., 2005). As the VZV is highly cell associated and possibly due to lower viral loads in sub clinical reactivation, it might not be possible to rule out viral reactivation solely by performing quantitative PCR on plasma. Indeed, recently VZV viraemia was detected in 9% of asymptomatic blood donors in the UK by using quantitative real time PCR, providing strong evidence for frequent sub clinical reactivation(Quinlivan et al., 2007a).

1.8. Re infection with VZV

It is thought that infection with VZV results in life long immunity to the virus and prevents further re-infection with the virus. However, there are many reports which document re-infection with VZV resulting in sub clinical infection or a second or third episode of chickenpox (Hall et al., 2002; Quinlivan and Breuer, 2006) . For instance, there was a recent report of an individual having 2 episodes of herpes zoster due to reactivation of 2 different VZV strains (Taha et al., 2006) which suggests that indeed re-infection with VZV can occur. There are also reports of acute primary infection in pregnant women who had detectable VZV specific IgG antibodies. VZV specific antibodies in these patients prior to re-infection were confirmed by 4 different assays (Martin et al., 1994).

Although re-infection with VZV currently is not a significant problem, there is a possibility of it being so due to universal use of the live attenuated VZV vaccine which contains the Japanese strain of the virus. There is concern among some that by giving this vaccine strain to those who live in countries which have different VZV strains might not prevent infection with the wild type virus strain in these countries (Loparev et al., 2007).

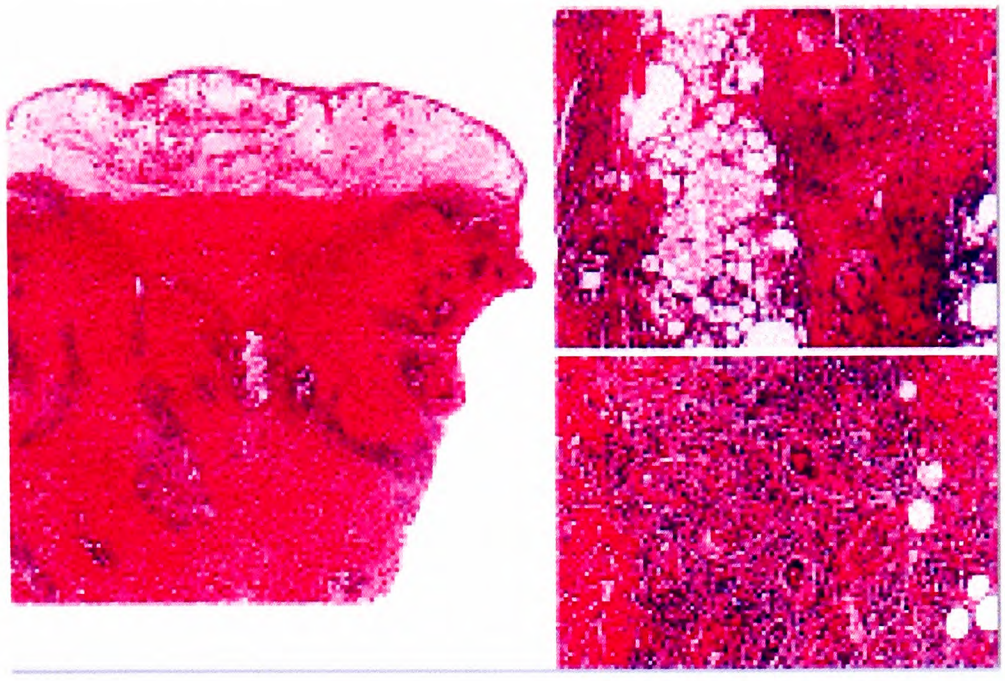
1.9. Characteristics skin lesions due to VZV

The skin lesions due to VZV have a similar histological appearance in both primary infection and in herpes zoster. They are characterized by ballooning of keratinocytes, multinucleated or necrotic cells and eosinophilic inclusions of nuclei (Fig 1-9). Dense lymphoid infiltrates predominantly consisting of T cells (both CD4+ and CD8+) are seen

in the majority of individuals. Atypical lymphocytes with large hyperchromatic nuclei are also commonly seen (Leinweber et al., 2006; Morizane, 2005). Of the infiltrating T cells, CD4/CD8 ratios are thought to vary from 1·0 to 2·0 and CD20+ cells and CD56+ cells rarely detected in the early infiltrates (Morizane, 2005; Nikkels et al., 2004). This is in contrast to cellular infiltrates in HSV lesions where the CD4+ T cells are 3 to 6 times more frequent than CD8+ T cells (Cunningham et al., 1985). However, as seen during infection with the HSV, during the later stages of lesions, CD56+ NK cells were detected at a higher frequency indicating a possible role of NK cells in viral clearance (Nikkels et al., 2004). In HSV-2 infections, infiltration of virus specific CD8+ T cells were associated with virus clearance and CD8+ T cells were seen in post lesional skin possibly preventing further viral reactivation (Zhu et al., 2007). However, the factors associated with VZV clearance from chickenpox and herpes zoster lesions are currently unknown.

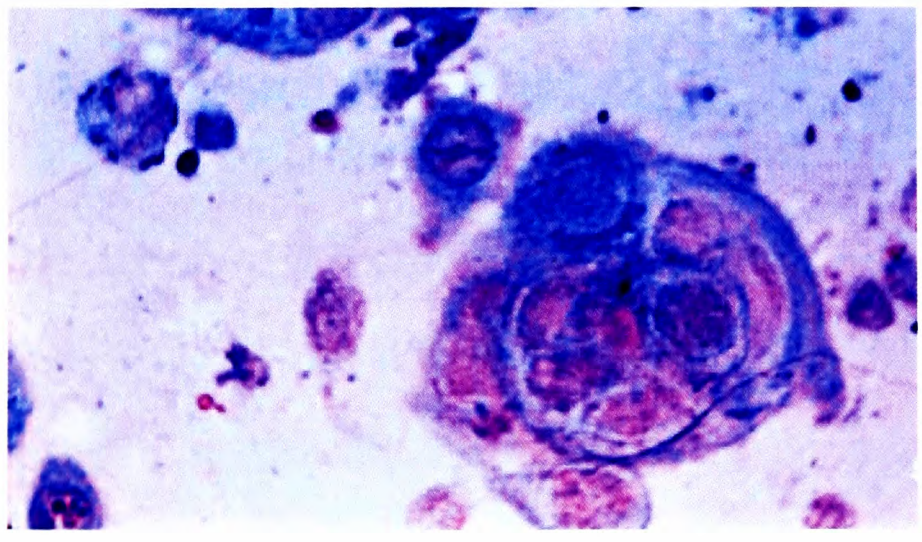
Plasmacytoid dendritic cells (pDC) appear to be important in controlling virus spread in initial stages of VZV lesions. Large numbers of pDCs have been observed in the dermis in acute chickenpox lesions and these cells have been shown to secrete IFNα and IFNβ which are important in preventing infection of non infected cells (Gerlini et al., 2006). Skin biopsies taken from patients with chickenpox showed that viral proteins (ORF63, gE and gB) were detected in keratinocytes, sebaceous glands, langerhans cells, dermal dendrocytes, monocytes and macrophages and endothelial cells (Nikkels et al., 1995). Reduced expression of ICAM-1 and HLA-DR was seen in infected keratinocytes in VZV lesions when compared to non infected keratinocytes which indicate that the virus is possibly down regulating these receptors on keratinocytes to escape immune recognition

(Nikkels et al., 2004). Acute skin lesions of patients with chickenpox and herpes zoster have also demonstrated a reduced level of class II expression in VZV infected cells (Arvin, 2000).



1-9 Histological characteristics of VZV skin lesions.

Marked involvement of surface epidermis in varicella eruption, vesicle with reticular alteration, necrosis of follicles, sebaceous glands and eccrine glands (Image from Böer, A et al. Herpes folliculitis: clinical, histopathological, and molecular pathologic observations. (Image from British Journal of Dermatology **154** (4), 743-746)



1-10 Multinucleated giant cells seen in a skin lesion from a patient with varicella

(Image from Koranda F.C. Images in clinical medicine. Use of multinucleated giant cells to diagnose a viral eruption. N Engl J Med. 2004 Feb 12;350(7):e6

1.10. Prevention of primary VZV infection

Although primary varicella is usually a benign self limiting illness in children, it can cause serious complications and even death in adults, neonates and immune suppressed individuals. Therefore, prevention of primary varicella in such individuals would have a significant impact on varicella associated disease. Primary varicella could either be prevented post exposure by administering immunoglobulins or could be prevented by inducing life long immunity by the VZV live attenuated vaccine.

1.10.1. Varicella-zoster immune globulin

Currently varicella zoster immune globulin (VZIG) is the most effective treatment in preventing primary varicella following exposure. It has shown to be effective in preventing disseminated and progressive varicella in immune compromised patients if given within 96 hours after exposure (Ogilvie, 1998). However, it has no effect on the disease course in established clinical varicella or herpes zoster. Due to its high cost, it is only given to individuals belonging to high risk groups and to neonates of pregnant mothers who contracted primary varicella a few days prior to or after delivery.

1.10.2. The live attenuated VZV vaccine

This vaccine was developed in 1974 by Michiaki Takahashi, by isolating the virus from a 3 year old boy in Japan. The virus strain known as 'Oka' was named after the child it was isolated from. The virus was serially passaged 11 times through human embryonic lung cells then 12 times through guinea pig embryo cells and finally propagated in human diploid cells (Takahashi et al., 1974). The vaccine was first licensed in the USA 1995 for

universal childhood vaccination and has shown to have an efficacy of 70% to 85% against the development of any form of varicella and 85% to 100% effective against moderate to severe disease (Galil et al., 2002b; Miron et al., 2005). It is thought that an antibody titre for VZV glycoprotein antigen of ≥5 units per milliliter was indicative of protection against varicella (Li et al., 2002). The VZV antibody titre at 6 weeks post vaccination (measured by gpELISA) is thought to correlate with neutralizing antibody concentration as well as with lymphocyte proliferative responses and the risk of break through varicella infection (Chan et al., 2002; Li et al., 2002). It was recently shown that with one dose of the VZV vaccine, protective immunity to the vaccine decreased over time especially 5 years after receiving the vaccination (Chaves et al., 2007a). Therefore, a second dose of the vaccine is recommended by some as a preventive measure to halt the decline of protective immunity (Kuter and Schodel, 2007; Lopez et al., 2006).

The attenuation process of the Oka virus has lead to several differences in nucleotide and amino acid sequences when compared to the parent virus. Most of these changes have been shown to be localized to the ORF62 gene which is also known as the major transactivation factor of the VZV (Cohen, 2007). Single nuclear polymorphisms of ORF62 are used to distinguish the vaccine strain from the wild strain. However, as the majority of the single nucleotide polymorphisms are variable, the vaccine preparation is thought to show genetic diversity (Quinlivan et al., 2007b). Vaccination is associated with development of a rash in 5% of vaccines (Sharrar et al., 2000) and it has been shown that the genotype of the virus found within such lesions was different to the genotype of the virus within the vaccine. In addition, it was also shown that within a lesion, the

viruses had an identical genotype but the genotypes of the viruses were different within different lesions in the same individual. However, as the 4 genotypes predominated over others in the lesions, it is thought that the virus genotypes influence rash formation following vaccination and may thus affect virulence of the virus(Quinlivan et al., 2007b).

The attenuated virus has impaired ability to replicate in human skin but not in T cells (Moffat et al., 1998) . Therefore, even when vesicles are formed due to the vaccine virus the number of viruses in the vesicle have been shown to be lower possibly due to lower infective ability of keratinocytes (Quinlivan et al., 2007b). However, the vaccine virus is still able to establish latency in the vaccinated host (Quinlivan et al., 2007b) and can be transmitted to non immune individuals from skin lesions of individuals with vaccine varicella resulting in primary varicella (Grossberg et al., 2006; LaRussa et al., 1997; Salzman et al., 1997). Although very rare, severe disseminated primary varicella with a fatal outcome has been reported in several immune suppressed individuals following vaccination. Some of these individuals had primary immune deficiencies such as natural killer T cell (NKT) cell deficiency (Levy et al., 2003), adenosine deaminase deficiency (Ghaffar et al., 2000) and others were immune suppressed due to HIV infection (Kramer et al., 2001), transplants or cancers (Christensen et al., 1999; Kraft and Shaw, 2006; Schrauder et al., 2007).

Reactivation of the vaccine virus has been reported in both immune competent and immune suppressed individuals (Chan et al., 2007; LaRussa et al., 2000; Sauerbrei et al., 2003; Uebe et al., 2002). Such reactivation in an immune suppressed child has resulted in

herpes zoster which later progressed into chronic verrucous lesions. In this child, the vaccine virus was found to have developed a mutation that made it resistant to acyclovir (Levin et al., 2003a). Therefore, it appears that although attenuated, the vaccine virus can still cause significant infection in immune suppressed individuals.

1.10.3. Zoster vaccine

A zoster vaccine was first licensed in the USA in 2006 for the use in patients over 60 years who are not immune compromised (Mitka, 2006). This vaccine also consists of the Oka live attenuated strain but has a higher number of plaque-forming units per dose (1350 units in 'chickenpox vaccine' and 19,400 units in the zoster vaccine) (Kimberlin and Whitley, 2007). The zoster vaccine was designed to contain a higher dose of the virus as initial studies had shown that to in order to stimulate significant and long lasting immunity in older individuals a higher dose would be needed (Levin, 2001). The vaccine was shown to reduce the overall incidence of HZ from 11.12 per 1000 person years to 5.42 per 1000 person years and reduced the incidence of post herpatic neuralgia by 66.5% (Oxman et al., 2005).

1.11. Immune responses to the Varicella Zoster virus

Cell mediated immune responses are thought to be important in controlling the virus during primary VZV infection and in the prevention of virus reactivation. The importance of T cell responses in VZV infections was initially shown by observing that patients with agammaglobulinaemia recovered normally from both primary varicella (Gershon and Steinberg, 1979b) and herpes zoster, while those with impaired cell mediated immune responses tend to get complications (Ruckdeschel et al., 1977; Timar et al., 1981). Recently it was reported that primary VZV infection in a child with idiopathic CD4+ T cell lymphopenia resulted in death (Hochauf et al., 2005). In addition, severe and prolonged primary VZV infection with visceral dissemination in a child who had a liver transplant was successfully treated with autologous transfusion of VZV specific T cells (Melzi et al., 2006). VZV specific T cells were obtained by stimulating the T cells of the patient with VZV vaccine virus pulsed, irradiated dendritic cells. The specificity of the patients T cells for VZV stimulated in this manner were confirmed by the lysis of VZV-pulsed target cells by the chromium release assay. These cells were then re-infused in to the patient which achieved a cure of prolonged (>7 months) primary VZV infection(Melzi et al., 2006).

A study done on a limited number of patients showed that primary VZV infections in immune suppressed patients were associated with normal humoral immune responses but markedly low T cell responses. The T cell responses were shown to be lowest in those who died despite having antibody levels similar to those of healthy donors (Gershon and Steinberg, 1979b). Furthermore, VZV specific proliferative T cell responses within the

first 72 hours of symptoms were associated milder disease, whereas no correlation was seen between disease severity and the presence of VZV specific IgM and IgG antibodies. The early development of T cell responses was associated with disappearance of viraemia in the healthy individuals (Arvin et al., 1986). Collectively, these data suggest that T cell responses play an important role in control of the virus during acute primary VZV infection.

1.11.1. Innate immune responses to VZV

Once the virus infects the tonsillar lymphocytes and prior to appearance of the rash the virus replicates within the host, evading recognition by the host immune response. The virus disseminates throughout the body during the viraemia that occurs immediately prior to appearance of the chickenpox vesicles. Although, T cells are likely to play a major role in controlling the virus, natural killer (NK) cells are also thought to be important at clearance of herpes virus infections (Sirianni et al., 2007; Williams et al., 2005). Severe and recurrent VZV infection have been observed in children with absent or significantly reduced level of NK cells (Etzioni et al., 2005; Notarangelo and Mazzolari, 2006). In addition, in a study involving 5 children with severe disease, reduced levels of circulating NK cells were seen in acute infection which were normalized during convalescence (Vossen et al., 2005a). Similar finding have been observed in acute EBV infection which showed that patients with infectious mononucleosis who had lower viral loads had significantly higher NK cells (Williams et al., 2005). Therefore, NK cells appear to be important in reducing disease severity in primary VZV infection and in controlling the

virus. However, the exact role of NK cells and the relative importance of them in primary VZV infection and during reactivation is currently unknown.

The VZV virus infects keratinocytes and other cells in the skin during primary infection and during reactivation. However, unlike in *in vitro* epidermal cell cultures, viral replication and subsequent cytolysis of infected cells was observed at a slower speed in the SCIDhu mouse model. It was shown that in this mouse model (described earlier in the chapter), the IL-1α and IFNα produced by adjacent uninfected cells retarded cell to cell spread of the virus. Therefore, the skin innate immune system appears to be important in reducing viral replication and subsequent spread of the virus to uninfected keratinocytes in the skin (Ku et al., 2004).

1.12. Cellular Immune responses to VZV

The adaptive immune response plays a critical role in overcoming primary VZV infection, prevention of virus reactivation and in the prevention of re-infection with the virus. Although, humoral immune mechanisms probably are more important in the prevention of re-infection with the virus, T cell responses are thought to be crucial in viral clearance in primary infection and prevention of reactivation. During primary infection, virus specific T cell responses develop which result in virus clearance and long lasting virus specific T cell memory responses are established.

1.12.1. T cell responses in primary VZV infection

T cell responses in primary VZV were first investigated by Patel *et al* and Gershon *et al* in 1979 by using *in vitro* proliferative assays to detect virus specific T cells (Gershon and Steinberg, 1979b; Patel et al., 1979). Patel *et al* showed that in immune competent individuals, an intense proliferative response of VZV specific T cells were seen 8 to 10 days after the onset of illness. Virus specific proliferative T cell responses were found to be lower in immune suppressed individuals with primary varicella (Patel et al., 1979). Similar results were observed by Gershon *et al* who used 'VZV plaque reduction assay' to detect VZV specific T cells. In this assay they stimulated PBMCs with the VZV antigen in vitro for 3 days and then added them to human embryonic lung monolayers. The number of VZV plaques in this monolayer was counted after another 3 days. A reduction in VZV plaques were seen the cultures which contained antigen stimulated PBMCs from immune persons. By using this method to detect VZV specific T cells in primary varicella in 8 patients, they showed immune suppressed patients with varicella had lower VZV specific immune responses. The lowest responses were detected in those who died. However, no significant differences in VZV specific antibody titres were observed in immunocompetent patients when compared to those who were immune suppressed (Gershon and Steinberg, 1979b).

A larger study done by Arvin *et al* in 1986 showed similar results. They observed that development of VZV specific proliferative T cell responses within the first 72 hours after appearance of the rash was associated with milder disease. They also showed that VZV specific lymphocyte transformation occurred at a higher rate in patients with fewer

lesions (<100) than those with more lesions (>400). Interestingly, they too found no association with VZV specific antibody titres and disease severity (Arvin et al., 1986). Another study involving 5 children, using intracellular cytokine assays for IFNγ, VZV specific CD4+ T cell were shown to be <0.25% during the acute phase of infection in patients with mild to moderate infection. However, a very high percentage virus specific CD4+ T cells (1.55%) was seen in a child who had severe varicella. This is contrary to previous reports which show that high numbers of virus specific T cells were associated with milder disease. This child, who was not immune suppressed, also had very high initial viral loads and continued to have high viral loads for 1.5 years despite the presence of virus specific T cells. Virus specific CD8+ T cell responses were not investigated in this study (Vossen et al., 2005b).

However, despite the above described studies, the kinetics and dynamics of T cell responses in primary varicella is not well documented. In addition, differences in the viral loads with disease severity and the role of NK cells in the amelioration of infection are not known.

1.12.2. Memory T cell responses in healthy immune individuals

VZV specific T cell responses are observed in healthy immune individuals following primary infection and vaccination. When VZV infected autologous lymphoblastoid cells were used as targets to evaluate the cytotoxic T cell responses in individuals with acute varicella, healthy immune donors following natural infection and in vaccinnees, lysis of target cells were observed in all groups. Interestingly, the CD4 +T cell subset was predominantly responsible for target cell lysis (Diaz et al., 1989).

Proliferative responses and cytotoxic T cell responses specific to several viral glycoproteins and tegument proteins have also been demonstrated by others following natural infection and vaccination (Arvin et al., 2002; Arvin et al., 1991; Bergen et al., 1991; Diaz et al., 1989; Sadzot-Delvaux et al., 1997; Sharp et al., 1992; Watson et al., 1990). However, memory T cells specific for VZV were found to be at a lower frequency when compared to other herpes viruses in healthy immune individuals (Asanuma et al., 2000). For instance, the mean frequency of VZV specific CD4+ T cell was 0.11%, HSV specific CD4 + T cells 0.22% and CMV specific CD4+ T cells 1.21%. In addition, consistent with previous studies, VZV specific CD8 + T cells were not detected (Asanuma et al., 2000). Recently, a VZV specific CD8 T cells epitope was identified but responses to this epitope was only detected after *in vitro* stimulation of PBMCs with the specific peptide and no responses were detected *ex vivo* (Frey et al., 2003). Another study which investigated the changes of VZV specific memory T cells over a period of 1 year also showed that VZV specific memory CD8+ T cell were seen at a lower frequency than virus specific CD4+ T cells. This study which measured virus specific T cells by

intracellular cytokine assays showed that virus specific memory T cell responses remained more or less the same over a period of 1 year. However, this study which included 17 males and 8 females also shows that median VZV CD4+ T cell responder frequencies were lower in females than in males (Klein et al., 2006).

There are several reports highlighting the importance of CD4+ T cells in the control of VZV. For instance, fatal infection has been reported in individuals with idiopathic CD4+ T-cell lymphocytopenia, in which there are normal CD8+ T cell counts but markedly low CD4+ T cells (Hochauf et al., 2005). In addition, it has been shown that VZV specific CD4+ T cells (measured by intracellular cytokine assay) increase in healthy immune individuals following re exposure to the virus (Vossen et al., 2004). Furthermore, VZV specific CD4+ T cell have shown to be lower in patients with systemic lupus erythematosus and was shown to correlate with severity of the disease. Based on these findings it was suggested that lower VZV specific CD4+ T cells in SLE were associated with a higher risk of developing HZ (Park et al., 2004).

VZV specific cellular immune responses are also believed to be vital in preventing virus reactivation. VZV specific T cell responses have been shown to be significantly lower in the elderly when compared to young adults (Levin et al., 2003b; Miller, 1980). Both VZV specific lymphoproliferative responses and *in vitro* lymphocyte stimulation responses to VZV in the elderly were shown to be significantly lower than younger individuals. However, VZV specific antibody levels remained high in the elderly and were similar to levels seen in younger individuals. Furthermore, VZV specific IFNγ producing CD4+ T

cells, measured by intracellular cytokine stimulation, were also significantly lower in the elderly when compared to younger individuals.

A study which investigated the presence of VZV DNA in the peripheral blood by PCR in individuals of different age groups, revealed that in the absence of any clinical features of viral reactivation, VZV DNA was not detected in individuals below the age of 60 years, whereas it was present in a few individuals who were more than 60 years old (Devlin et al., 1992). However, by using quantitative real time PCR, VZV viraemia was detected in 9% of healthy UK blood donors who had no clinical evidence of reactivation (Quinlivan et al., 2007a). This group comprised individuals of different age groups. Therefore, it appears that VZV associated viraemia can be detected in individuals who are <60 years of age if more sensitive techniques are used. However, the above evidence taken together suggests that a decline in VZV specific T cell responses occur in the elderly and is likely to be associated with viral reactivation.

1.12.3. Immune responses in herpes zoster

The incidence of HZ rises with age and affects 50% of individuals over 85 years of age (Thomas and Hall, 2004). The higher incidence of HZ is believed to be due to the decline in VZV specific memory T cell responses with age. Virus specific T cell responses (measured by lymphocyte reactivity) were barely detectable in individuals with HZ in the first 4 days of illness whereas high titres of antibody responses were seen (Berger et al., 1985). However, the occurrence of HZ was associated with an increase in the frequency of virus specific T cells. These high levels of VZV-specific T cells were maintained for

approximately 2 years following an episode of HZ(Hayward et al., 1991). Apart from these studies, few data are available regarding the frequency, functional and phenotypic characteristics of virus specific T cells during HZ.

1.12.4. Immune responses to the live attenuated varicella zoster vaccine

The live attenuated VZV vaccine is an immunogenic and a relatively safe vaccine which has been shown by some to induce virus specific memory T cell responses comparable to those seen following natural infection (Diaz et al., 1989; Sharp et al., 1992). Vaccination in children was associated with 95% seroconversion and development of virus specific T cell responses measured by lymphocyte proliferation assays (Clements et al., 1995; Watson et al., 1990). Also, T cells in the vaccinated children were shown to respond to several purified VZV glycoproteins (Watson et al., 1990). Antibody titres to two main VZV glycoproteins (gE and gB) were also found to be similar in both vaccines and following natural infection (Haumont et al., 1997).

In contrast, others have shown that both antibody and T cell responses were much lower in vaccinees than in individuals who had natural infection (Gershon and Steinberg, 1990). The antibody responses following vaccination was shown to be four to eight fold lower when compared to those after natural infection. In addition, virus specific IgA responses that were seen in serum and the naso pharynx following natural infection were not present following vaccination (Bogger-Goren et al., 1982). Moreover, T cell responses to the virus although seen was still lower 1 year after vaccination when compared with responses after natural infection (Austgulen, 1985). However, some have shown that

VZV specific antibody titres and T cell responses increase over time and were higher at 5 years following vaccination than at 1 year post vaccination (Zerboni et al., 1998). Collectively, it appears that immune responses to the vaccine may vary in individuals and the longitudinal T cell responses to the virus need to be investigated further.

Immune responses to the vaccine are thought to be influenced by the number of doses received, the immune status of the individual and also the age of the individual when they receive the vaccine (Leung et al., 2004; Nader et al., 1995). Adult individuals even after receiving 2 doses of the vaccine had significantly lower T cell responses (measured by T cell proliferation to VZV antigen) when compared to children (Nader et al., 1995). Seroconversion rates have been lower in adult vaccinees with 4 to 23%. having no detectable antibodies following vaccination predisposing them to breakthrough varicella (Ampofo et al., 2002; Saiman et al., 2001). In addition, 31% of adults lost detectable antibodies 1 to 11 years post vaccination (Saiman et al., 2001). Seroconversion rates were also lower in children with malignancies after one dose of the vaccine with only 19% showing evidence of detectable VZV specific antibodies (Austgulen, 1985; Chaves Tdo et al., 2005; Leung et al., 2004). Recently the persistence of virus specific CD4+ T cells in a vaccine recipient despite the loss of detectable antibodies was reported (Ludwig et al., 2006).

In summary, longitudinal immune responses to the VZV vaccine have not been well documented and the similarities or differences in the frequency, functionality and differentiation of virus specific T cells following vaccination and natural infection are

unknown. In addition, the dynamics of virus and T cell responses during acute primary infection and reactivation has not been investigated. Such data would enable us to better understand disease pathogenesis and T cell and virus interactions. This in turn would facilitate development of more efficient vaccine to prevent acute primary VZV infection and also to prevent the development of HZ.

1.13. Immune evasion strategies of the VZV

The VZV uses many strategies to evade the host immune response during primary infection and during latency. After the virus establishes infection in the tonsillar epithelium and regional lymph nodes the virus disseminates around the body to where infection in other lymph nodes and the reticuloendothelial system occurs. During a period of 10 to 21 days virus amplification occurs and the secondary viraemia occurs (Arvin, 2000). Therefore, the virus very successfully evades immune recognition during this relatively long period.

The VZV is thought to use many mechanisms to evade host immunity during primary infection such as down regulation of MHC class I and class II expression and down regulation of ICAM-1. Down regulation of MHC class I has been observed in VZV infected human fibroblasts and T cells *in vitro* (Abendroth et al., 2001a; Cohen, 1998). Abendroth *et al* demonstrated 75% reduction in MHC class I expression in fibroblasts 24 hours post infection. They also showed that retention of class I molecules in the golgi complex was not due to products encoded by the late genes of the VZV, but possibly due to the ORF 66 gene (Abendroth et al., 2001a). Evidence of involvement of ORF 66 gene

in class I down regulation has also been observed more recently by others (Eisfeld et al., 2007). However, the exact mechanism by which the VZV retains MHC class I molecules in the golgi complex is not well understood. Interestingly, immunohistochemistry of skin lesions from patients with herpes zoster showed no reduction in MHC Class I expression. In contrast, a reduction of MHC class II expression was seen in VZV keratinocytes (Nikkels et al., 2004). Therefore, the *in vitro* findings of MHC class I downregulation should be carefully investigated in VZV lesions. However, many viruses down regulate MHC class I expression and interfere with MHC class I antigen processing pathways to evade recognition by CD8+ T cells (Andrieu et al., 2001; Ehrlich, 1997; Zeidler et al., 1997). Therefore, it is very likely that the VZV could adopt similar strategies to evade detection by the immune system.

In addition to down regulating class I expression, many have shown that the VZV down regulates class II expression in human fibroblasts and keratinocytes present in VZV lesions (Abendroth et al., 2000; Nikkels et al., 2004). Abendroth *et al* showed that VZV infected fibroblasts had reduced expression of MHC class II following treatment with IFNγ. The virus was shown to reduce IFNγ induced expression of MHC class II mRNA in infected cells (Abendroth et al., 2000). In addition, they showed that both Jak2 and Stat 1α protein expression was reduced in infected cells. Therefore, it appears that the virus interferes with IFNγ induced expression of MHC class II in APCs by interfering with the Jak/Stat signal transduction pathway. However, infection of the virus in mature dendritic cells resulted in reduced MHC class I, CD80, CD84 and CD86 but class II expression was unaltered (Morrow et al., 2003). Therefore, the virus also appears to impair the

antigen presenting ability of dendritic cells by altering expression of costimulatory molecules.

Reduction of MHC class II expression in VZV infected cells *in vivo* was shown by Abdendroth *et al* and Nikkels *et al* in both primary varicella and herpes zoster but their expression was enhanced in adjacent non infected cells and also in the lymphocytic infiltrates in lesion (Abendroth et al., 2000; Nikkels et al., 2004). Nikkels *et al* also showed absence of ICAM-1expression of VZV infected keratinocytes within lesions (Nikkels et al., 2004). Therefore, reduced expression of ICAM-1 in infected keratinocytes would impair antigen presentation to infiltrating T cells.

Aims of the thesis

- To characterize immune responses to overlapping gI and gE peptides in healthy immune donors.
- To identify immunodominant epitopes within these 2 VZV glycoproteins.
- To phenotype gI and/or gE specific T cells using HLA tetramers in healthy immune donors.
- To investigate VZV-specific T cell responses using IFNγ ELISpot and ICS assays in patients with acute primary VZV infection and if possible identify the associations of severe clinical disease.
- To investigate the viral loads and clinical disease severity in patients with acute primary varicella and to determine whether a relationship is observed between viral loads and VZV-specific T cell responses.

Chapter 2. Materials and Methods

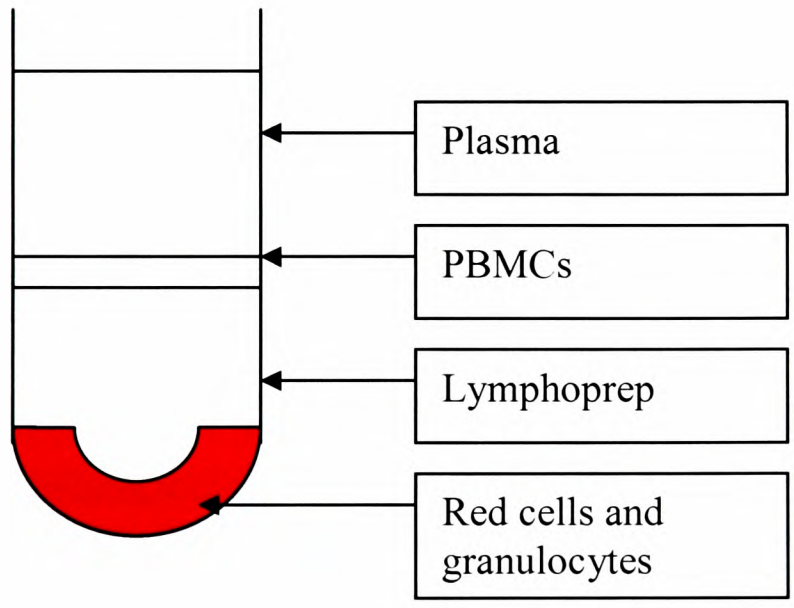
2.1. Subjects

2.1.1. Healthy immune donors

The study participants consisted of 20 healthy seropositive adult individuals living in the UK, with a history of primary VZV infection but no clinical reactivation. Mean age of the donors was 34 years (range 25-62 years) with an average age of primary infection at 5.6 years (range 2-15 years). Negative controls consisted of healthy seronegative volunteers living in the UK and also health care workers employed by the Oxford Radcliffe NHS trust. The studies were done under local ethical approval.

2.2. Separation of PBMCs

Fresh heparinized blood was initially diluted 1:1 with RPMI 1640 supplemented with 2mM L-glutamine, 100IU/ml penicillin. 15 to 25ml diluted blood was layered on top of 14-15ml of lymphoprep. The sample was then centrifuged at 2000 r.p.m. for 20 minutes without brakes. The PBMC layer was then seen between the lymphoprep and the plasma layer (Fig. 2.1).



2-1 Separation of layers following centrifugation of blood layered on lymphoprep

The PBMC layer was then pipetted out and the cells were washed in an equal volume of RPMI 1640 at 1600 r.p.m. for 10 minutes. The cell pellet was then resuspended in RPMI 1640 supplemented with 2mM L-glutamine, 100 IU/ml penicillin and 100µg/ml plus 10% fetal calf serum (R10) or human serum (hR10). Cells were resuspended in R10 for use in *ex vivo* ELISpot assays while hR10 was used for other purposes.

2.2.1. Counting of cells

A plastic calibrated counting chamber was used for counting cells. 10µl of the cell suspension was diluted with an equal volume of trypan blue and 10 µl of this was taken for counting. Cells that were alive were seen as bright while dead cells were seen as black spots. 3 small squares were counted and then the total number of cells was calculated as:

Total number of cells = No. of cells in 3 squares×3×2× volume of fluid in mls

2.3. Freezing and thawing cells

2.3.1. Freezing cells

The cells were centrifuged at 4°C at 1600 r.p.m. for 10 minutes and then resuspended in freeze mix (10% DMSO with 90% FCS). Approximately 1ml of freeze mix was used for 10 million PMBCs.The cryovials, Mr. Frostie and freeze mix were kept at 4°C prior to use for cell freezing. Once the freeze mix was added to the cells it was transferred to cryovials in a Mr.Frostie and then stored in -80°C for a few days before transferring them to liquid nitrogen. The aim was to lower the temperature approximately 1°C per minute.

2.3.2. Thawing cells

PBMCs or cell lines stored in liquid nitrogen or in -80°C was warmed rapidly to 37°C. The cryovials were warmed by hand or immersed in a water bath until they were beginning to thaw and then the contents dropped in the R10 at 37°C. Cells were washed twice at 1600 r.p.m. for 10 minutes in R10 and the finally resuspended in R10.

2.4. Peptides

2.4.1. Synthetic overlapping 20mer peptides

Synthetic 20 mer peptides overlapping by 10 amino acids which spanned the whole length of the gI protein were purchased from Sigma and also synthesized in house in an automated synthesizer using F-MOC chemistry by Ms. Katie de Gleria. All synthetic overlapping 20mer peptides for gE protein were synthesized in house. The purity of the peptides was determined to be greater than 90% by high-pressure liquid chromatography analysis and mass spectrometry.

2.4.2. Dilution of peptides

Peptides were weighed and initially diluted with 75 to 100µl of DMSO and then with R0. When making 1mM concentration of 20 mer peptides; peptides were diluted in half the volume of R0 for the weight of the peptide (e.g. for a peptide weighing 1mg, it was diluted in 500mls of R0). When making 1mM concentration of 10mer or 12 mer peptides the peptides was diluted in an equal volume of R0 as the weight of the peptide (1mg of peptide was diluted in 1ml of peptide). The peptides were further diluted to a final concentration of 40µl for use in ELISpots after filtering to ensure sterility.

2.4.3. Truncations of the 20mer peptides

Truncations of the 20mer peptide were made by taking off one amino acid at a time from either end (Fig. 2.2).

1-20 YYAGLPVDDFEDSESTDTEE
1-19 YYAGLPVDDFEDSESTDTE
1-18 YYAGLPVDDFEDSESTDT
1-17 YYAGLPVDDFEDSESTD
1-16 YYAGLPVDDFEDSEST
1-15 YYAGLPVDDFEDSES
1-14 YYAGLPVDDFEDSE
1-13 YYAGLPVDDFEDS
2-20 YAGLPVDDFEDSESTDTEE
3-20 AGLPVDDFEDSESTDTEE
4-20 GLPVDDFEDSESTDTEE
5-20 LPVDDFEDSESTDTEE
6-20 PVDDFEDSESTDTEE

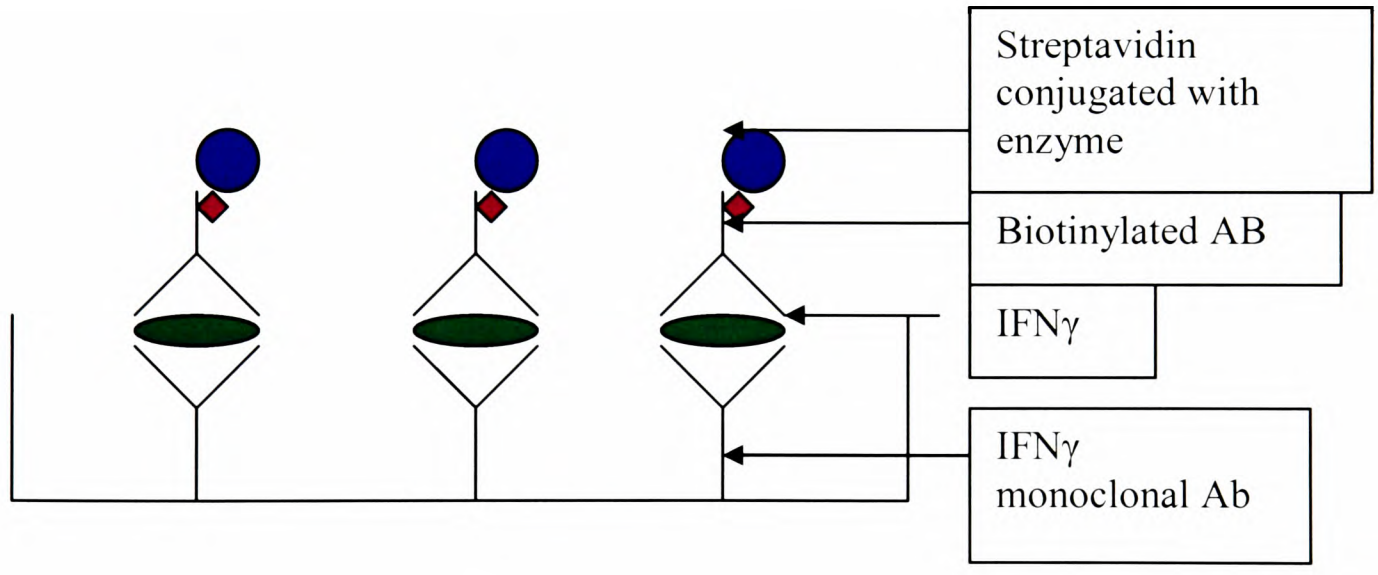
Fig.: Truncations of gE peptide 59 20mer

2.5. Varicella zoster viral lysate and Vaccine

The VZV lysate obtained by inactivated VZV ROD strain (ABI, 10-514-001) was used in all experiments. For ELISpots 2µl of the lysate and/or VZV live attenuated vaccine was used in each well. For short term cell cultures 20µl of each were added to 5×10^6 cells in a 24 well plate. When pulsing keratinocytes for antigen presentation, keratinocytes were pulsed with 10µl of the VZV lysate and/or VZV live attenuated vaccine for 1 hour at 37°C, in 5% CO_2. The uninfected lysate used was Vero uninfected cell extract (ABI, 10-508-001) and the live attenuated VZV vaccine used was Varilirix (GlaxoSmithKline).

2.6. Ex vivo ELISpot assays

ELISpot plates (Millipore Corp., Bedford, Massachusetts, USA) were coated with anti-human IFNγ antibody overnight (Mabtech AB, Nacka, Sweden) and incubated at 4°C. Briefly, IFN γ was used at 15µl per 1ml of PBS and 50µl of this was added to each ELISpot well. Parafilm was wrapped around the plates to prevent evaporation of antibody solution. The following day, plates were washed six times with RPMI 1640 and incubated for 1 hour with RPMI-1640 and 10% FCS. 0.1×10^6 PBMCs were added to a final volume of 200 µl (150µl of cells and 50µl of 40µM peptide). Peptide was at a final concentration of 10 µM. Responses were either done in duplicates or in triplicates. However, the number of responses for each test was a constant (i.e. all ex vivo gI responses were done in duplicates while all gE responses and patient responses done in duplicates). PHA (20µl of PHA at 40µg/l) was always included as a positive control.



2-2 Schematic representation of an IFNγ ELISpot

The plates were incubated overnight at 37°C in 5% CO_2. The cells were removed and the plates developed with the second biotinylated Ab to human IFNγ and washed six times

with PBSTween (500ml of Tween to 1L of PBS). The plates were then incubated for 2 to 4 hours at room temperature and washed again 6 times with PBSTween before putting the 3rd antibody (Fig.). Both the secondary and the third antibody were added at a concentration of 1µl per 1ml of PBS (100µl were added to each well). The plates were then incubated for a further 1-2 hours at room temperature and then developed with streptavidin-alkaline phosphatase (Mabtech AB) and colorimetric substrate. Briefly, for a 96 well plate, 400µl of the buffer was added to 9.6ml of milli Q water of distilled water. 100µl of the calorimetric substance A and B were added to this and 100 µl of this solution was added to each well. Once the spots had developed (5 to 20 minutes) the development solution was tipped off and the plates washed with normal water under a running tap.

Once the plates were dry the spots were enumerated using an automated ELISpot reader. Peptide specific responses were calculated by subtracting the number of spots, in wells which contained only media (RPMI 1640) and cells. ELISpot results were expressed as spot forming units (SFU)/10^6 PBMC. All peptides that induced an IFN-γ response of more than mean+2 standard deviations of the irrelevant peptide were considered positive.

2.7. Cultured ELISpot assays

T cells from short term T cell cultures were used in cultured ELISpot assays. Cells from 24 plate wells were then washed on day 11 with RPMI 1640 or sterile PBS and resuspended in hR10. The cells were rested for 1 to 2 days before testing. The ELISpot method followed is similar to that of ex vivo techniques except instead of 100,000

PBMCs, 40,000 T cells were used in each well. T cells of short term T cell cultures were tested with individual peptides, antigen presenting cells or peptide truncations on a 96 ELISpot plate. All peptides that induced an IFN-γ response of more than mean+ 2 standard deviations of the irrelevant peptide were considered positive.

2.7.1. Perforin ELISpot assays

Anti-human perforin antibody precoated ELISpot plates (Mabtech AB, Nacka, Sweden) were used in a similar way as described above.

2.8. Generation and maintenance of cell cultures

2.8.1. Short term T cell cultures

4-5 million PBMCs of each donor were incubated in a 24 well plate with 200µl of 40µmol peptide for 10 days. The peptides were peptide pools (such as gE consisting of 62 20mers), individual 20mer peptides, defined epitopes or the VZV lysate or vaccine. When the vaccine or VZV lysate were used the PBMCs were incubated with 20µl of either the reconstituted vaccine or the VZV lysate. IL-2 was added on day 3 and 7 at a concentration of 100units/L (1ml of media was taken off and 1ml of hR10 with IL-2 was added). All cell lines were routinely maintained in RPMI 1640 supplemented with 2mM L-glutamine, 100IU/ml penicillin and 100µg/ml plus 10% human serum at 37°C, in 5% CO_2. If the wells turned deep yellow or the cells appeared too crowded the cells were split in to another well.

2.8.2. EBV transformed lymphoblastoid B cell lines

EBV transformed B cell lines were obtained by transformation of the PBMC's of healthy donors by incubating them for 1 hour at 37°C in 1ml of the supernatant from an EBV producing marmoset cell line B958. The cells were then diluted into 9mls of RMPI 1640 plus 10% foetal calf serum containing 1μg/ml of Cyclosporin. All B cell lines were routinely maintained in RPMI 1640 supplemented with 2mM L-glutamine, 100IU/ml penicillin and 100μg/ml plus 10% fetal calf serum at 37°C, in 5% CO_2.

2.8.3. Keratinocyte cell lines

Three established keratinocyte lines were used: HaCat cells (a gift from Dr. N. Fusenig) were spontaneously developed from adult epidermal keratinocytes, NK and NFK are HPV-16 immortalised keratinocytes (a gift from Dr. E.O'Toole). All three lines were HLA-typed as described below. Keratinocyte cell lines were maintained in Dulbecco modified Eagle medium (Gibco, Grand Island, NY) supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin (D10). They were checked regularly for confluent growth and were split when over growth was seen. Prior to splitting or using the keratinocytes as antigen presenting cells they were trypsinized. Briefly, once the growth media was discarded, 2.5ml of trypsin EDTA was added to the flasks so that any excess growth media would be removed. Once this trypsin EDTA was removed, another 2ml of trypsin EDTA at 37°C was added and the cells incubated for 2 to 5 minutes in 37°C. Then 12mls of D10 was added to neutralize the effects of trypsin EDTA and the cells were then removed and centrifuged at 1500 r.p.m. for 5 minutes before pulsing with peptide.

2.8.4. L cell lines

Murine L cell lines transfected with DRB1*1501 (kindly supplied by Prof. Lars Fugger) were maintained as used in the same as keratinocytes. However, they were not incubated with IFNγ prior to use as antigen presenting cells.

2.9. Intracellular cytokine secreting assay for IFNγ (ICS)

E*x vivo*- PBMC or T-cell lines were stimulated at 1 x 10^6 to 2 x 10^6/ml in RPMI 1640 plus 10% human serum with the relevant peptides (20µl of 1mM peptide), VZV viral lysate (20µl) or vaccine (20µl) for 16 hours according to manufacturers instructions. Briefly, following stimulation of the cells with the peptide for 1-2 hours at 37°C at 5% CO_2, 1µl of Brefeldin (BD GolgiStop^{TM}) was added. The cells were then incubated overnight. Cells were washed and stained with antiCD3 (FITC), anti CD4 (PerCP) (BD Biosciences), anti CD8 (PE) and IFNγ APC for four color ICS assays. The wave lengths and excitation of the flurochromes used are shown in table 2.

After adding cells for the compensation tubes (nil, CD8 FITC, CD8 PE, CD8 PerCP and IFNγ APC), cells were washed twice with 1 ml of PBA at 1500 r.p.m. for 5 minutes. Then the above mentioned surface stains were added (5µl of FITC, 2µl of PE, 5µl of PerCP) and the tubes were wrapped in foil and incubated for 20 minutes in 4°C. Then following another wash with PBA the surface stains were fixed with 300µl of Cytofix/Cytoperm (BD Biosciences) for 20 minutes at 4°C. 1ml of perm wash (1:10 of perm wash solution to distilled water) was added to the cells and then incubated for a further 10 minutes at 4° C. Following this the samples were centrifuged at 1500 r.p.m. for

5 min and 300µl of IFN γ APC in perm wash (1µl of IFN γ APC per 300µl of perm wash) was added to all the sample tubes and to the IFN γ APC compensation tube and incubated at 4°C for 45 minutes. Then 1ml of perm wash was added to each tube and the cells washed and the cells fixed in 350µl of 2% formaldehyde.

2.9.1. Flurochromes used in ICS

Dye	Laser excitation line (nm)	Maximal absorbance (nm)	Maximal emission (nm)	Fluorescence colour
FITC (FL1)	488	490	525	Green
Phycoerythrin (FL2)	488	490,565	578	Yellow
PerCP (FL3)	488	490	675	red
APC (FL4)	633, 635, 647	650	661	red

Table 2: Flurochromes used in ICS

The samples were then analyzed using a FACScalibur (Becton Dickinson Immunocytometry Systems, San Jose, California, USA) with CellQuest software (Becton Dickinson) or FlowJo software.

2.10. Detection of CD107a expression

In detection of CD107a expression by T cells a similar method as above was used. However, cells were incubated with CD107a FITC (BD Biosciences) for 30 minutes prior to stimulation with peptide. In addition, in contrast to IFNγ ICS assays, in CD107a assays cells were only stimulated for 4 hours and cells were not permeabilized. When CD107a expression along with other cytokines (IFNγ, TNFα) was studied the cells were permeabilized .

2.11. MHC class II restriction

2.11.1. Use of anti DR, anti DQ and anti DP antibodies

Cells from short term cultures were incubated with 10µl of monoclonal antibodies at 0.2mg/ml specific for HLA-DR (L243), HLA-DQ (SPV-L3) and HLA-DP (B7.21) at 37°C for 1 hour before addition of peptides (kindly supplied by Prof. Lars Fugger).

2.11.2. Use of B cells and keratinocytes in defining HLA restriction

B cells partially matched or mismatched to the testing HLA molecule were initially pulsed with 25µl of 40µM peptide for 1 hour at 37°C, in 5% CO_2. They were then washed three times in RPMI plus 10% fetal calf serum and used as antigen presenting cells to washed T cells harvested from cell cultures.

Following overnight incubation (or sometimes 48 hour incubation) with IFNγ, keratinocytes were initially pulsed with 100 µl of 100 µM peptide for 1 hour at 37°C, in 5% CO_2. Once pulsed they were washed three times in RPMI plus 10% fetal calf serum and used as antigen presenting cells to washed T cells harvested from cell cultures.

2.12. Tetramer and phenotypic staining and flow cytometry

DRB1*1501 iTAg MHCII tetramer was purchased from Beckman Coulter. DRB1*1501 tetramer was complexed to VZV gE peptide 54 (aa531-545; TSPLLRYAAWTGGLA). Unless stated otherwise, cell lines and PBMC were incubated with 2μg/ml HLA class II tetramer for 60 min at 37°C in RPMI-1640 and 10% human serum. I analyzed the tetramer expression within the $CD4^+$T cell subset by gating on the lymphocytes and excluding B cells, monocytes and dead cells (via probe positive population).

The cell surface marker Abs CD4-pacific blue (Biolegend), CD14-PerCP, C19-PerCP and 7-aminoactinomycin D (7-AAD) (all BD Pharmingen) were added for 20 minutes at room temperature. For phenotypic analysis of tetramer-positive $CD4^+$ cells, antibodies to CD27 (FITC); CD28 (allophycocyanin, APC); CD38 (APC); CD45RO (APC); CD62L (APC); CD56 (Pe-Cy7); CCR4 (Pe-Cy7); CCR7 (Pe-Cy7); CLA (FITC); PD1 (FITC); and Perforin (fitc) were added with the other surface antibodies. Stained cells were washed with PBS, and fixed in 0.5% PBS/formaldehyde. Cells were acquired on a CyAn™ (DakoCytomation) and analysed using FlowJo software.

2.13. Serology

Serum was analysed for VZV IgG antibodies using the VIDAS Varicella-Zoster IgG Assay (bioMérieux). Serology was performed by Dr. Katie Jeffery, Department of Microbiology, John Radcliffe Hospital, Oxford.

2.14. DNA extraction

DNA was extracted from EDTA blood using Gentra PureGene Kit (D5000) according to the manufactures instructions. Briefly, 2 to 3ml of EDTA blood was added to a 15ml conical falcon tube containing 6 to 9ml (3 times the volume of blood) RBC lysis solution. The tubes were inverted several times and left at room temperature for 10 minutes. Then they were centrifuged at 2500 r.p.m. for 10 minutes and the supernatant was taken off. The pellets were vigorously vortexed and then 2 to 3 ml of cell lysis was added. The solution was pipetted up and down to lyse the cells. If any clumping occurred the tubes were incubated in 37°C till the clumps were gone. 15µl of RNAse was then added and the tubes were incubated at 37°C for 15 minutes. Following this the tubes were cooled at 4°C for 3 minutes till they reached room temperature. 1ml of protein precipitation solution was added to the tubes and vortexed for 20s. The tubes were then centrifuged at 3000 r.p.m. for 10 minutes to pellet the protein. The supernatant was then carefully aspirated with pasture pipettes in to tubes containing 2 to 3 ml of 100% isopropanol. The tubes were inverted several times to form threads of DNA. Following this the tubes were again centrifuged at 3000 r.p.m. for 3 minutes to pellet DNA. The isopropanol was carefully discarded and 1 to 2mls of 70% ethanol was added and the sample transferred to 2ml tubes. These tubes were then centrifuged in a Microfuge for 1 min at maximum speed and the ethanol was aspirated off as much as possible. The DNA in the tubes was air dried for about 30 minutes prior to addition of 150 to 200µl of DNA hydration buffer. The pellets were rehydrated overnight at room temperature.

2.15. HLA typing

HLA typing was performed by Mr. Tim Rostron and I extracted the DNA in most of the patient samples. HLA class I and class II alleles were typed as previously described(Bunce et al., 1995). Briefly, DNA was extracted from EDTA blood using Gentra PureGene Kit (D5000). Extracted DNA was amplified and DNA typing was done using a 144 sequence-specific primer (SSP) reactions to simultaneously detect all known HLA-A, B, C, DRB1, DRB3, DRB4, DRB5 and DQB1 specificities in an allele specific or group specific manner using the same method, reagents, PCR parameters and protocols for all loci. The PCR products were electrophoresed in 1.0% agarose gels using Orange G as an electrophoresis marker. The gels were run for 15 minutes (or until the Orange G had traveled 3cm) at 15V/cm in the 0.5×TBE buffer and visualized using UV illumination. A Phototype was considered to be successful when the control amplifications are positive and at least one allele or group was present in each locus.

Chapter 3. Investigation of VZV Glycoprotein I specific T cell responses

3.1. Introduction

3.1.1. Varicella zoster virus glycoproteins

The VZV genome encodes 7 glycoproteins gB, gC, gE, gI, gK and gH which are expressed by the late genes (Cole and Grose, 2003). The VZV glycoproteins forms the viral envelope which not only encloses the viral tegument, nucleocapsid and the viral genome, but also provides many other functions such as cellular attachment, penetration and cell to cell spread of the virus (Gershon and Gershon, 1999). It is believed that unlike other related alpha herpes viruses, VZV infection is not associated with spontaneous release of virus particles from infected cells and that cell to cell spread of the virus is the main mechanism of spread of infection (Cole and Grose, 2003). The glycoproteins thus appear to be vital for infectivity as they are thought to facilitate cell to cell spread of the virus by promoting the cell membranes of infected cells to fuse with uninfected cells and thereby passage of virus particles in to these cells (viral super highways) (Gershon and Gershon, 1999). As antibodies specific for gE, gI, gB and gH inhibit infection of cells these glycoproteins are thought to have a role in cellular attachment and penetration.

The glycoproteins are displayed on the surface of infected cells and many of them have shown to be immune dominant T cell targets. Experimental data in animal models and human subjects have shown that gI and gE are particularly important T cells targets and that immune responses targeted to these proteins are important in overcoming infection in animal models.

3.1.2. Varicella zoster virus glycoprotein I

gI is a 58 to 62kd glycoprotein encoded by the open reading frame 67 (Arvin, 2000; Rahaus and Wolff, 2003). After synthesis, gI non-covalently links with gE to form a heterodimer and this complex is expressed on the surface of infected cells (Mallory et al., 1997). Both ORFs encoding gI and gE are located in the unique short segment (Us) of the virus genome which is thought to be a relatively recent addition to the genome. The Us segment is thought provide the virus with a biological advantage for its survival (Rahaus and Wolff, 2000). gI plays a chaperone function in gE trafficking in infected cells. In gI delected viruses, post translational modification and surface expression of gE was reduced and virion egress to cell surface was impaired (Mallory et al., 1997; Olson and Grose, 1998; Wang et al., 2001).

Experiments using VZV cosmids showed that gI was not essential for virus replication in tissue culture (Mallory et al., 1997). However, the mutant viruses partially or completely deficient in gI were associated with lower viral yields and abnormal syncytia formation (Mallory et al., 1997; Moffat et al., 2002). Confocal microscopy images showed that gI deleted viruses failed to create 'viral super highways' and therefore, cell to cell spread of the virus was impaired (Mallory et al., 1997). Normal cell-to-cell spread and replication kinetics were restored only by insertion of the ORF67 into the virus (Mallory et al., 1997; Mallory et al., 1998). This is perhaps not surprising given the important role that gI plays in envelopment of the virus in the trans-golgi network (TGN). The VZV nucleocapsids, tegument and the glycoproteins are brought together in the TGN where post translational modification of viral protein and envelopment of the virus occurs (Gershon and Gershon,

1999). gI is required for proper envelopment of the virus and in its absence the transport of virions beyond the TGN is blocked and the virus tegument forms bizarre structures along with the TGN (Gershon and Gershon, 1999).

Experiments carried out in SCIDHu mouse models have shown that gI is essential for VZV infectivity of the skin and T cells (Moffat et al., 2002). In these experiments SCIDhu mice received thymus or liver implants that contained human cells and also skin implants and then gI mutant virus was inoculated on to skin implants. In these experiments, the researchers looked for the presence of infectious VZV virions in these mice at different points following inoculation to determine if gI mutant viruses were slow in replication. However, they were unable to detect any infectious virions even after 28 days following inoculation in these mice (Moffat et al., 2002). Therefore, these experiments suggest that gI is essential for skin and T cell infectivity *in vivo* although it is not essential for viral replication and infectivity in cell cultures.

The requirement of gI for viral latency has been much debated. Studies in rat models have revealed that it is not needed for establishing latency in dorsal root ganglia of rats (Grinfeld et al., 2004). However, these data cannot be directly transferred to human model of infection as rats are not permissive to infection. Recently, requirement of gI for VZV latency was studied using the SCIDhu mouse model which has dorsal root ganglia (DRG) xenografts which showed that gI was required for infection of DRG *in vivo* (Zerboni et al., 2007). They also showed that gI deleted mutants could not establish persistence in the DRG even 70 days following infection. In addition, the DRG

inoculated with the gI mutant viruses had lower viral yields which was thought to be a result of altered intracellular trafficking of gE. Therefore, gI appears to be important for neurotropism of the virus and also possibly in establishing latency.

3.1.3. Immune responses to gI

The viral glycoproteins that elicited the most immunogenic humonal immune responses were first identified by experiments carried out in guinea pig and rabbit models in 1981. These data showed that glycoproteins with molecular weight of 62kd (gI), 98kd and 118kd were the most immunogenic (Grose et al., 1981). Proliferative T cell responses to several viral glycoproteins (including gI) and tegument proteins have been demonstrated following natural infection and vaccination (Diaz et al., 1989; Sharp et al., 1992; Watson et al., 1990) and the decline in VZV specific proliferative responses has been shown to be associated with viral reactivation (Park et al., 2004; Saibara et al., 1993). Furthermore, cytotoxic memory T cell responses specific for viral tegument proteins viral glycoproteins (including glycoprotein I) and regulatory proteins have also been demonstrated in healthy immune adults (Arvin et al., 2002; Arvin et al., 1991; Bergen et al., 1991; Sadzot-Delvaux et al., 1997).

Of vaccinia virus recombinants expressing certain glycoproteins (gE, gI and gV), the gE recombinant induced the highest proliferative T cell responses in guinea pigs (Lowry et al., 1992). Furthermore, guinea pigs immunized with recombinant VZV gE and gI, developed protective antibody responses and were able to clear the virus efficiently (Kimura et al., 1998). As guinea pigs do not develop chickenpox but do develop uvetitis

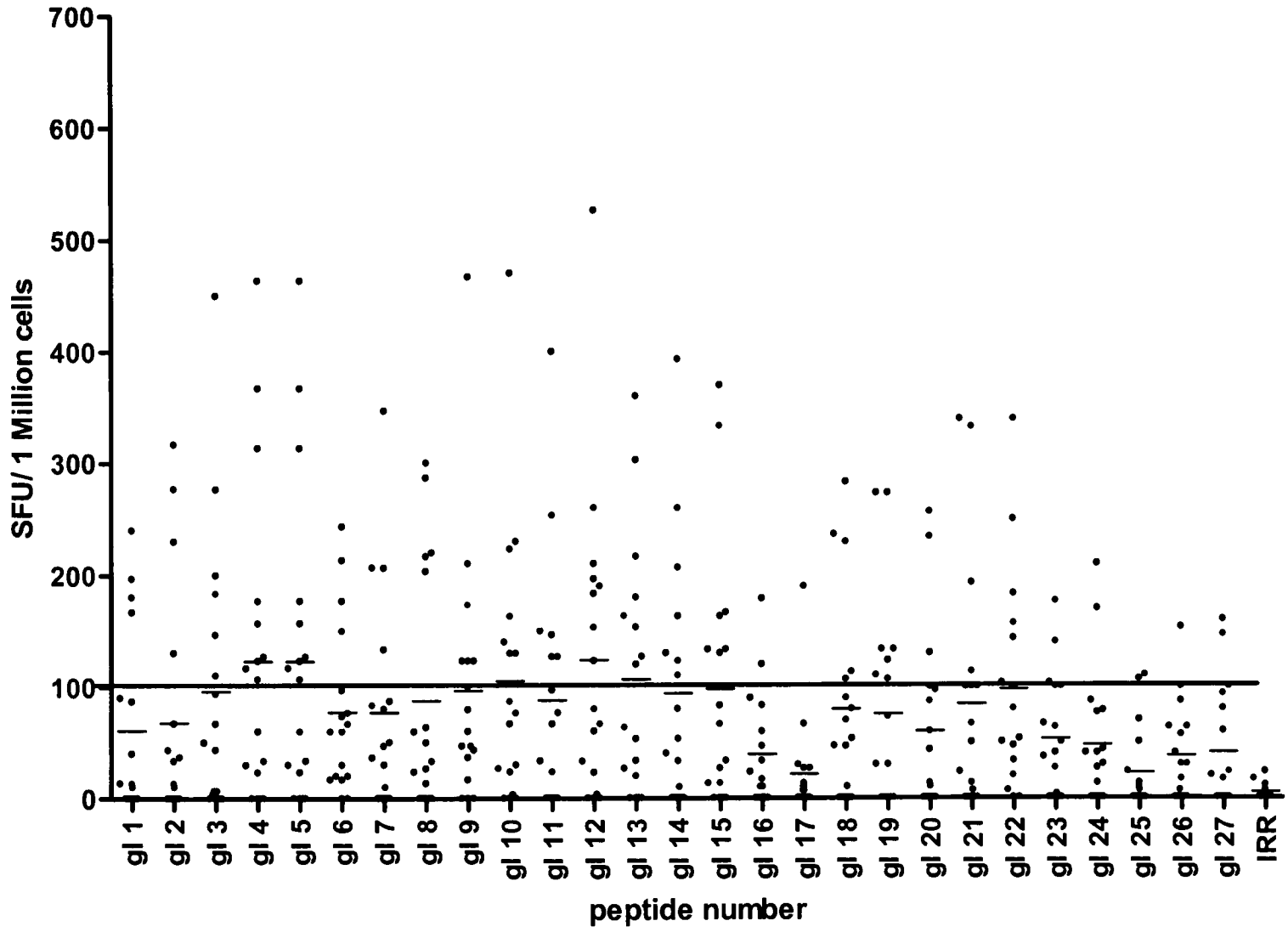
when inoculated the virus, clearance of infectious virus particles from these animals' eyes was used as a marker of clearance of infection. The guinea pigs who have received the gE-gI recombinant vaccine were shown to clear the virus whereas the virus could be isolated from the other guinea pigs suggesting that both gI and gE specific immune responses are important in controlling the virus.

In summary, these data suggest that gI plays an important role in the virus life cycle and gI specific immune responses appear to be important in the control of viral replication and viral clearance. However, T cell responses to this viral protein have not been characterized in detail and T cell targets within this protein have not been mapped. Such data would enable us to understand the kinetics of virus and T cell interactions in acute infection, in healthy immune donors and the role (if any) of gI specific immune responses in the prevention of viral reactivation. Therefore, my initial objective was to characterize T cell responses to this glycoprotein in healthy seropositive donors and also to identify immune dominant CD8+ and CD4+ T cell epitopes within this protein.

3.2. Results

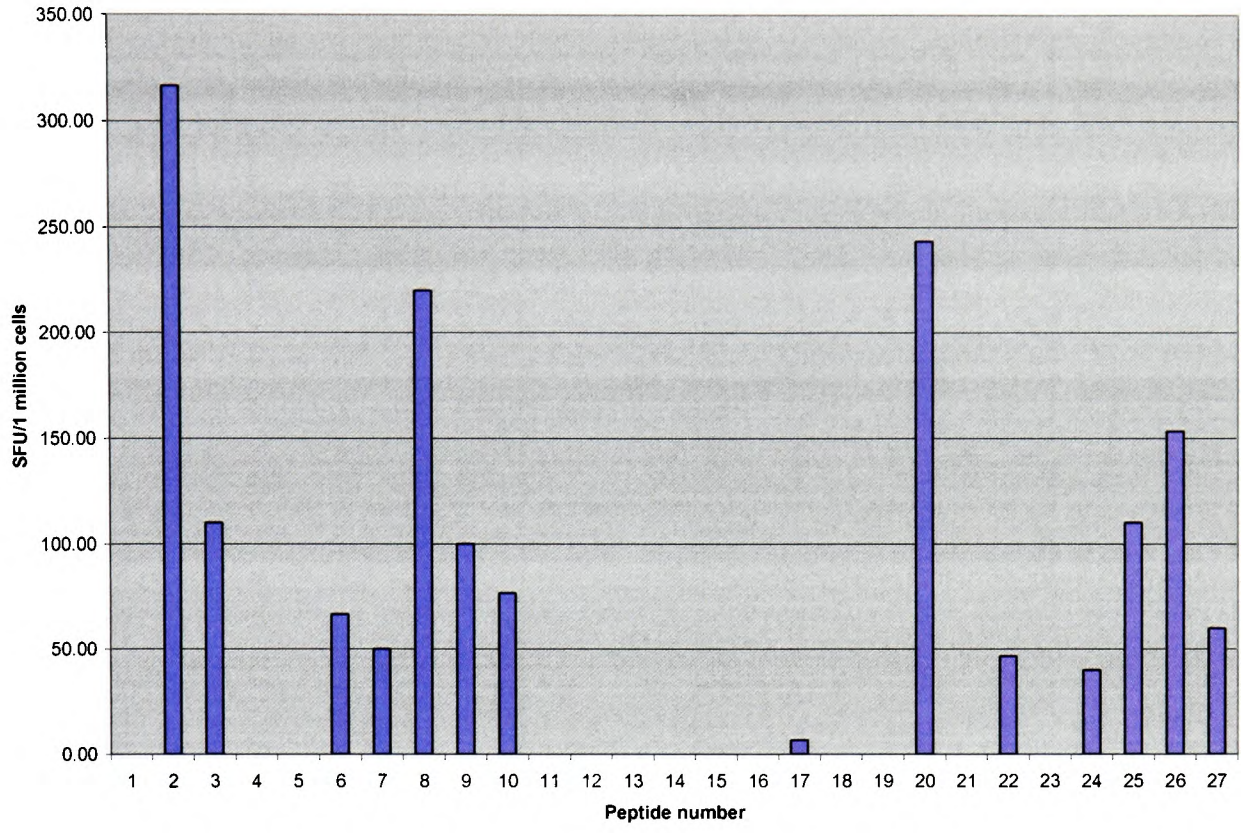
3.2.1. Mapping immune dominant epitopes

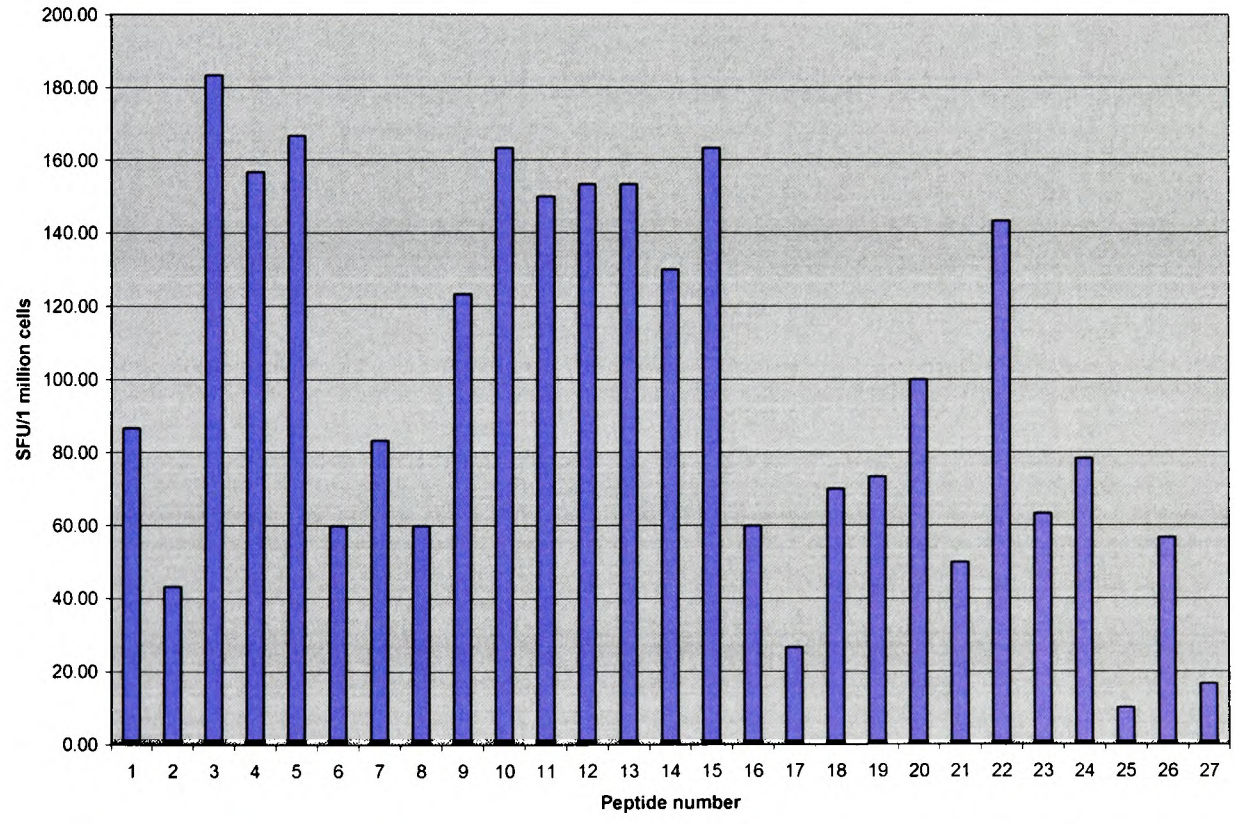
Ex vivo ELISpot responses based on overlapping peptides from gI revealed that healthy donors with a history of VZV infection and a positive serology produced IFN-γ responses to many of the 20mer synthetic peptides (Fig 3-1). The *ex vivo* PBMC responses specific for these peptides ranged from 0-526 SFU/million and were predominantly for the peptides representing the early part of the gI protein.



***3-1 Ex vivo* IFNγ ELISpot responses to overlapping gI peptides in cohort of healthy immune donors.**
The horizontal bar shows the mean+2SD of the irrelevant peptide control.

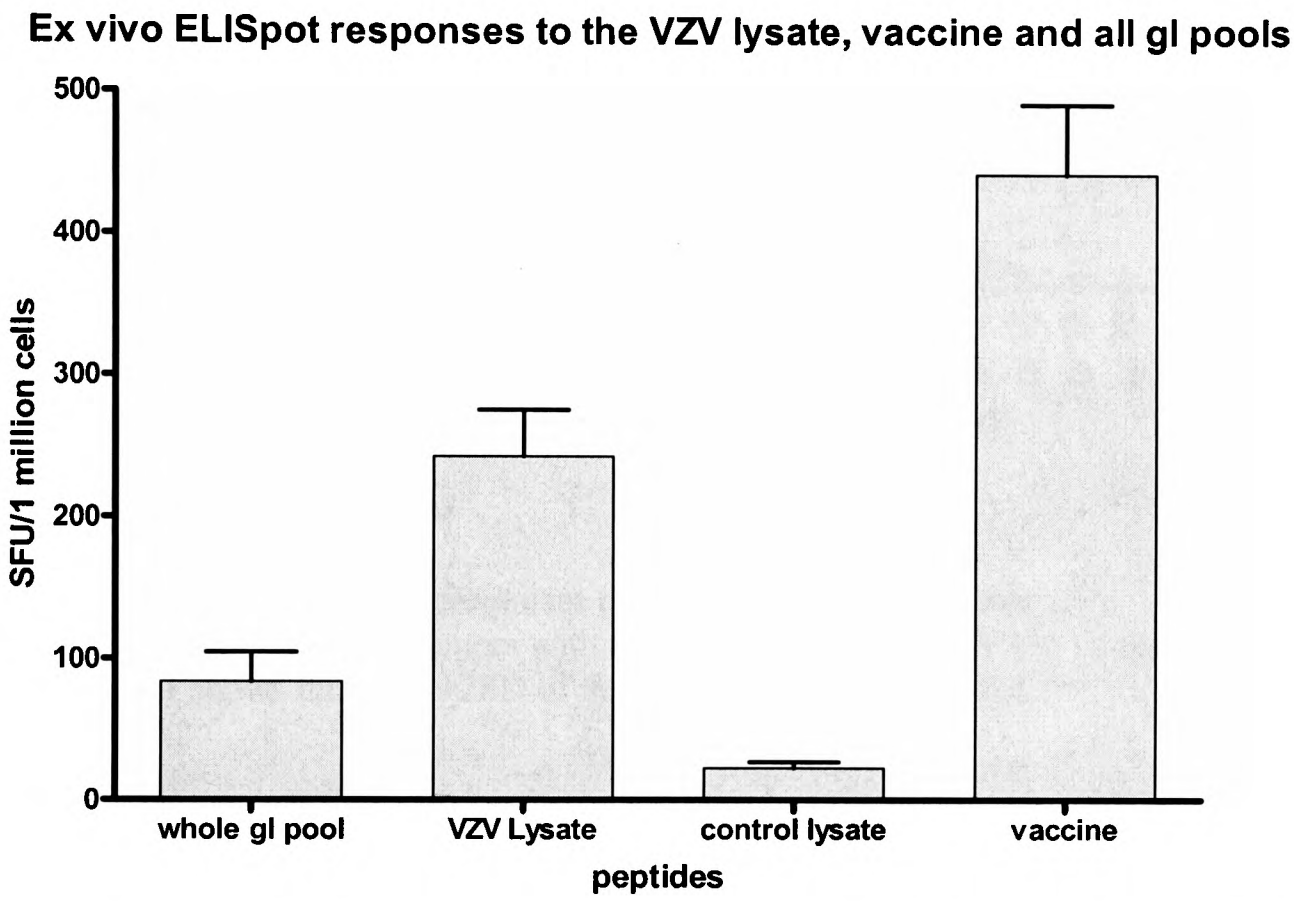
The IFN-γ responses for individual 20mers varied considerably among donors with some donors responding to 24/28 peptides and some responding to only a few peptides.





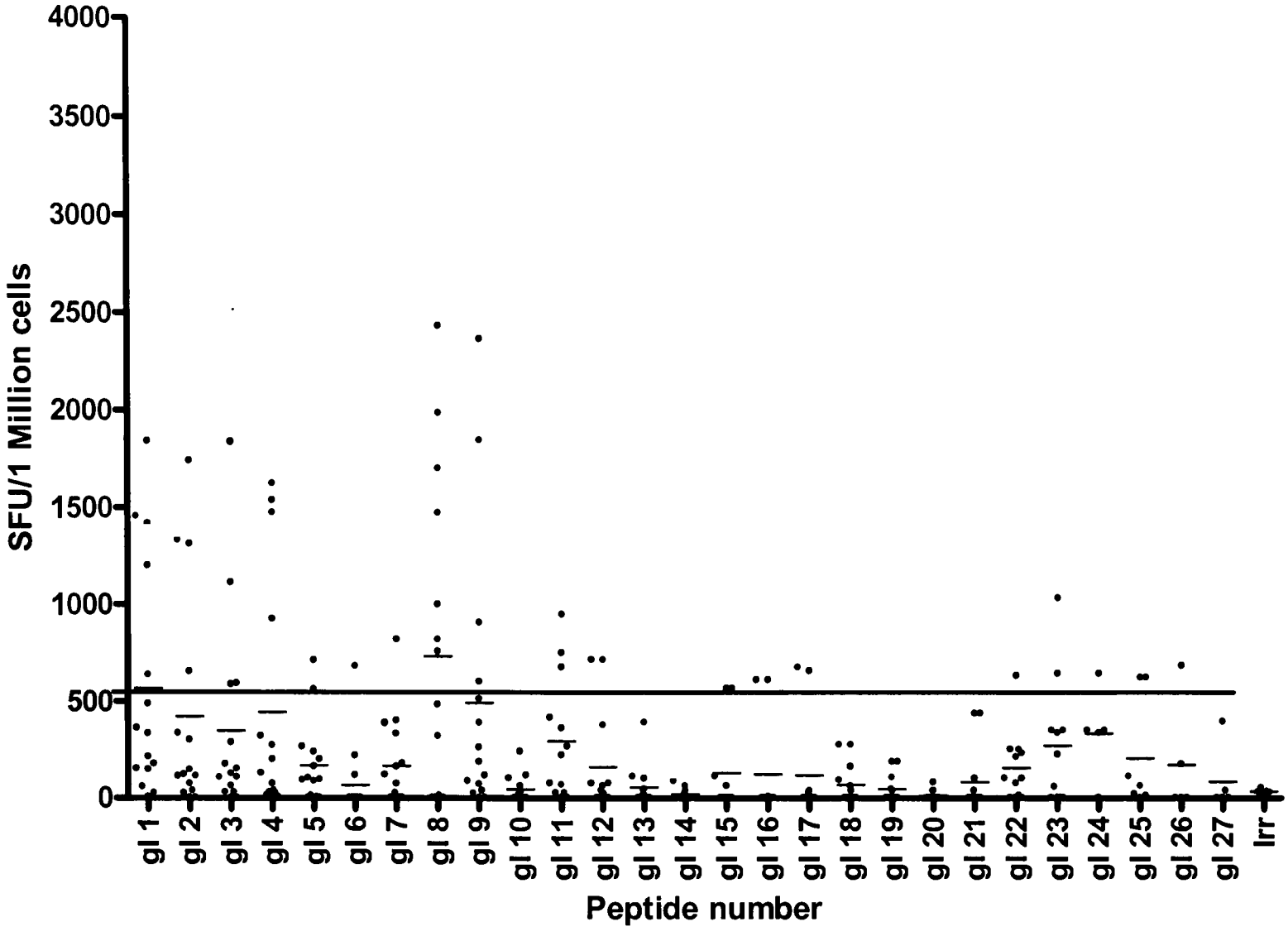
***3-2 Ex vivo* IFNγ ELISpot responses in 2 individuals**

Ex vivo responses to the overlapping gI peptides were then compared to the responses to that of VZV lysate and VZV live vaccine. The responses to the pooled gI peptides ranged from 5.8% to 92.8% (mean 32.3, SD±31.4) of the VZV lysate and from 2.9 % to 33.33% (mean 16.7, SD±12.5) to the vaccine (Fig.3-3). Unless otherwise stated the horizontal bar indicates the mean. gI specific IFNγ responses or IFNγ responses to the vaccine or lysate could not be identified from seronegative donors (n=8).



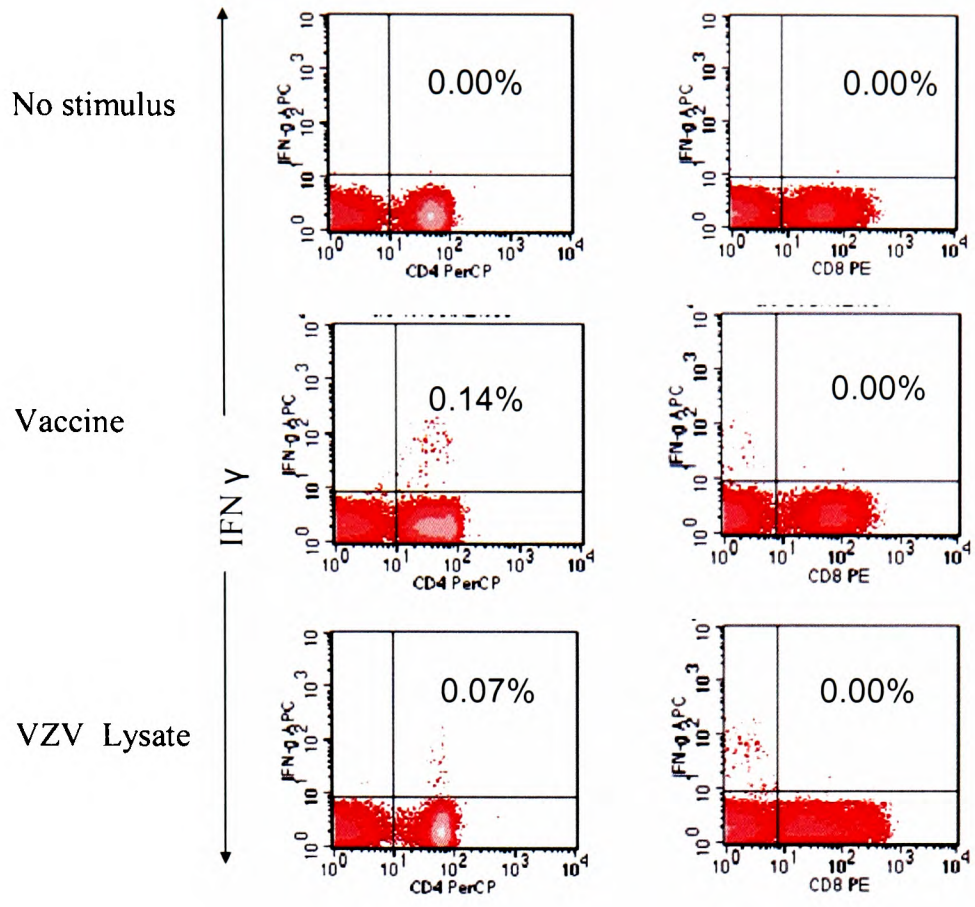
***3-3 Ex vivo* IFNγ ELISpot responses to all gI overlapping peptides, VZV lysate, control lysate and the vaccine in a cohort of healthy immune donors with a history of primary VZV but no reactivation. Error bars represent the SEM.**

I then proceeded to confirm the responses detected *ex vivo* by expanding peptide specific cells *in vitro*. Such cultured ELISpot assays again showed that most responses were to the early part of the gI protein (pool 1 and 2). The cultured ELISpot responses showed that 9/15 individuals responded predominantly to peptide gI 8, 4/15 responded to the adjacent 20mer peptide gI 9 and 6/15 responded to peptide gI 1 (Fig 3-4).



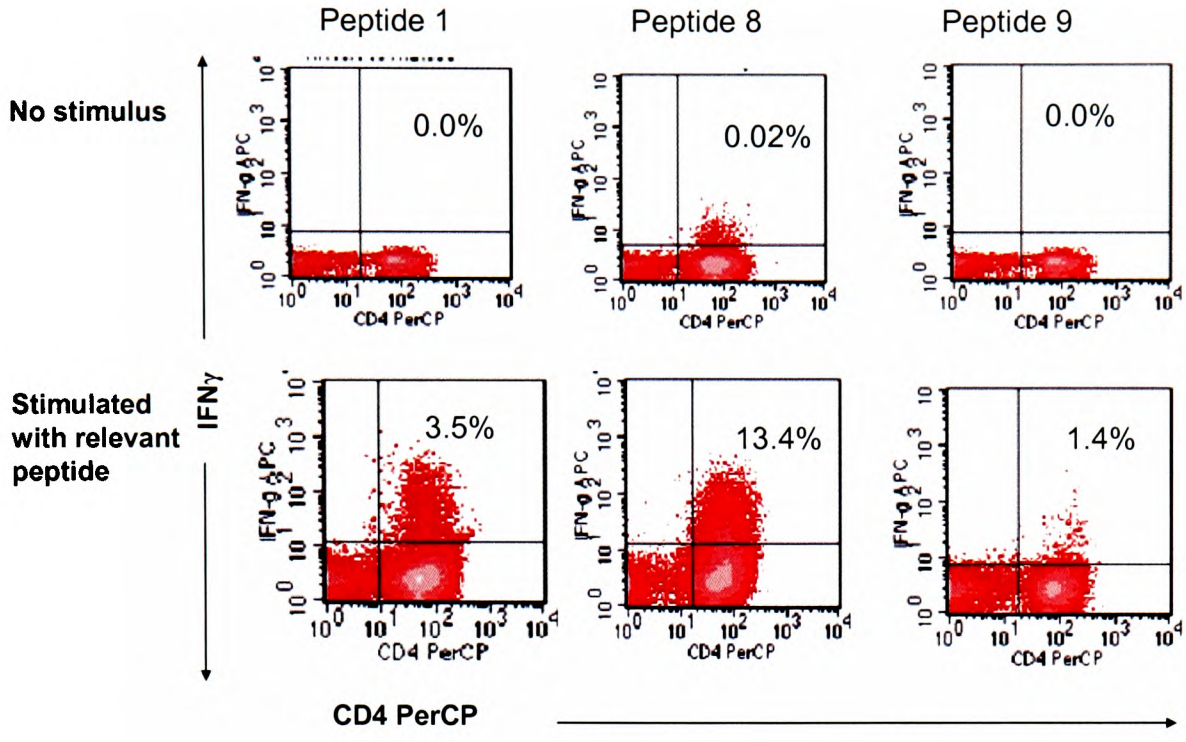
3-4 Cultured IFNγ ELISpot responses to overlapping gI peptides
in cohort of healthy immune donors with a history of primary VZV but no reactivation. The horizontal bar shows the mean+2SD of the irrelevant peptide control.

Intracellular cytokine staining was used to investigate the T cell subsets responding to gI. The assays showed that responses to gI peptides were predominantly mediated by CD4+ T cells. I then undertook a similar analysis using the viral lysate and the vaccine as stimulating antigens and showed a similar CD4+ T cell dominance both *ex vivo* and after short term cultures (Fig. 3-5).



3-5 Ex vivo IFNγ responses to the VZV lysate and vaccine by ICS. (The PBMCs have been gated on CD3.)

I observed strong responses in a majority of donors to peptide 8 and went on to investigate this response in more detail. I found that responses to peptide 8 were predominantly mediated by CD4+ T cells and furthermore, a CD4+ T cell dominance was also observed for the other 2 immune dominant peptides (peptide 1 and 9) in short term cultures and indeed also for all the gI 20mer overlapping peptides (Fig 3-6).

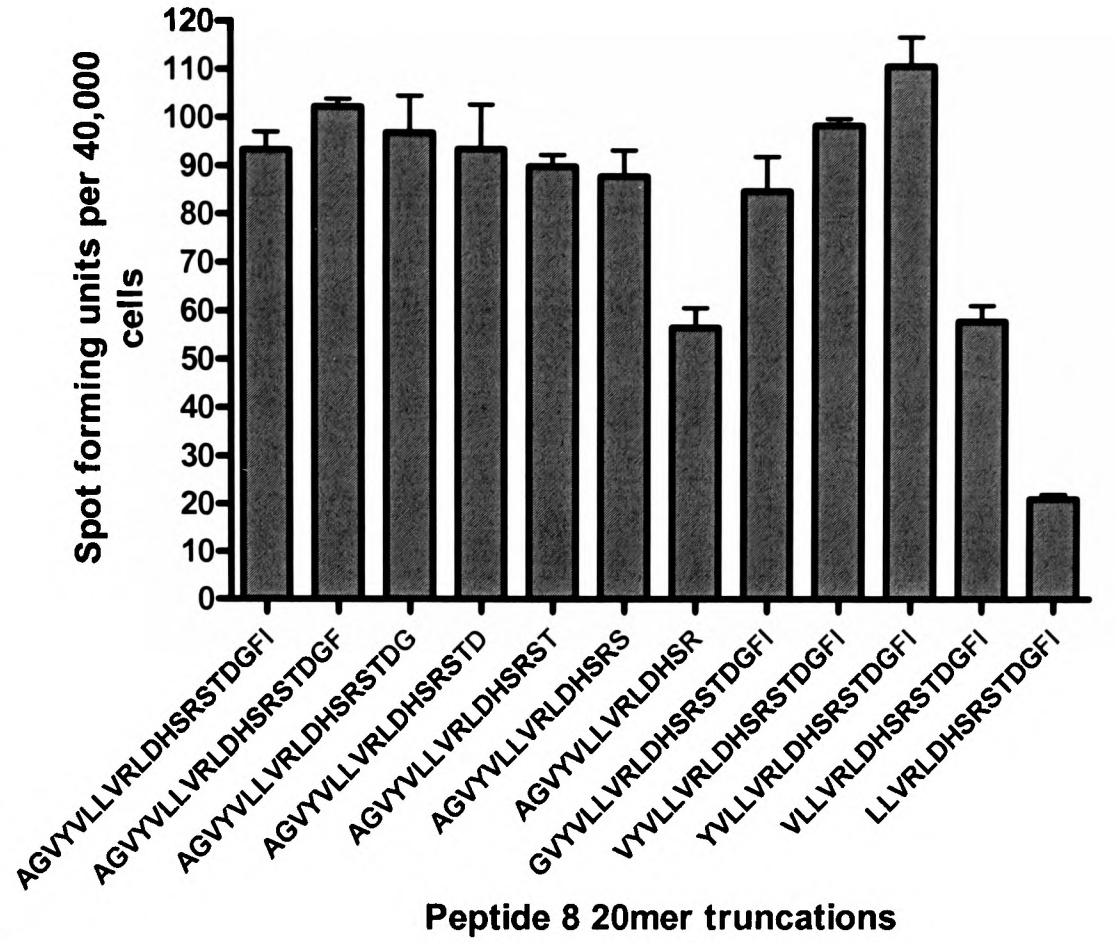


3-6 IFNγ responses to peptide 1,8 and 9 by intracellular cytokine assays.

The short term T cell cultures specific for the peptide were restimulated with the same 20mer peptide. The cells have been gated on CD3.

3.2.2. Detailed characterization of responses to gI peptide 8

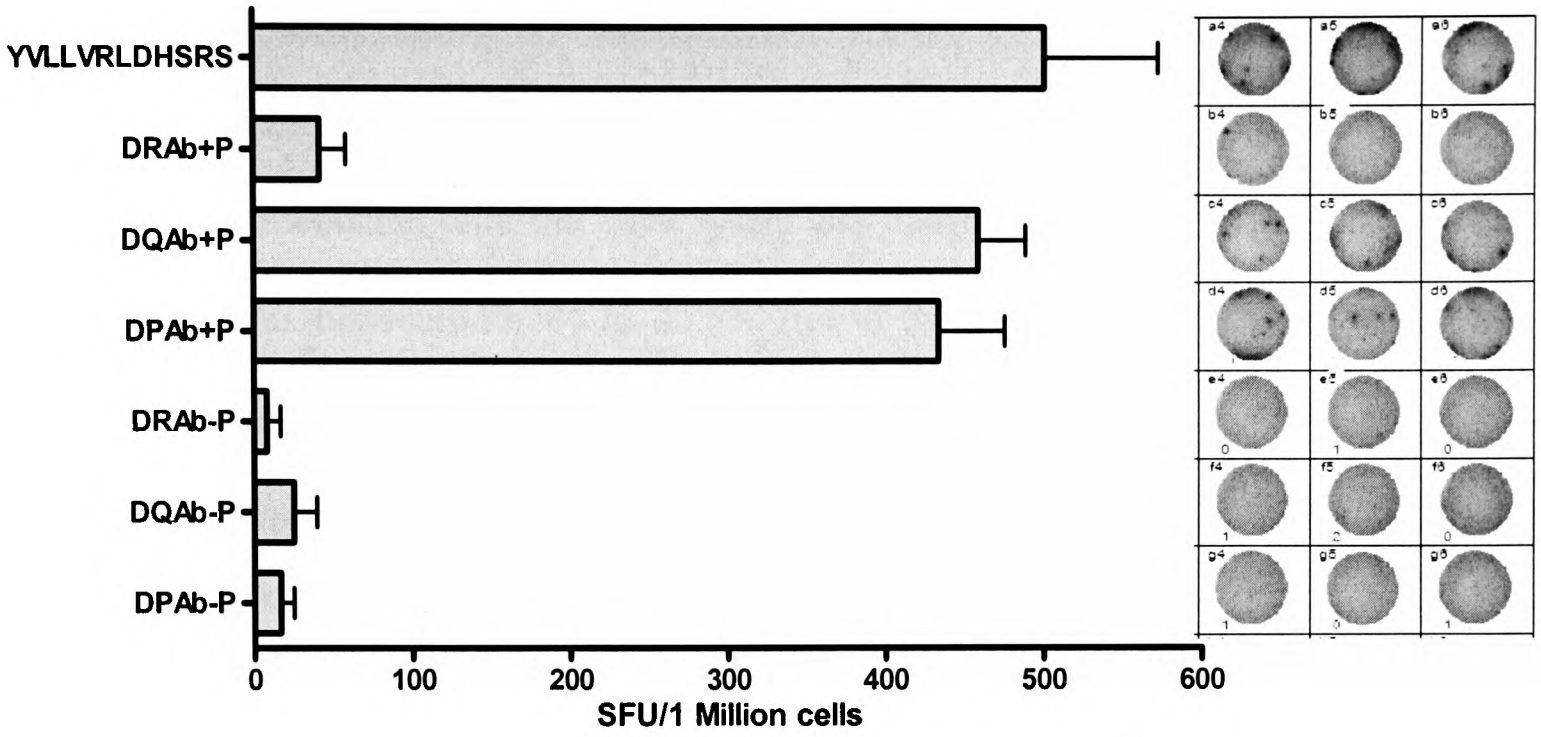
To determine the minimum length of the epitope, T cells cultured with the 20mer peptide were tested by ELISpot assays using peptide truncations of the 20mer index peptide. By testing the individuals who predominantly responded to peptide gI 8 I found that the optimum epitope to be a 12mer (YVLLVRLDHSRS) (Fig. 3-7).



3-7 IFNγ ELISpot responses to truncated peptides from the 20mer peptide 8 of glycoprotein I. Error bars represent the SEM.

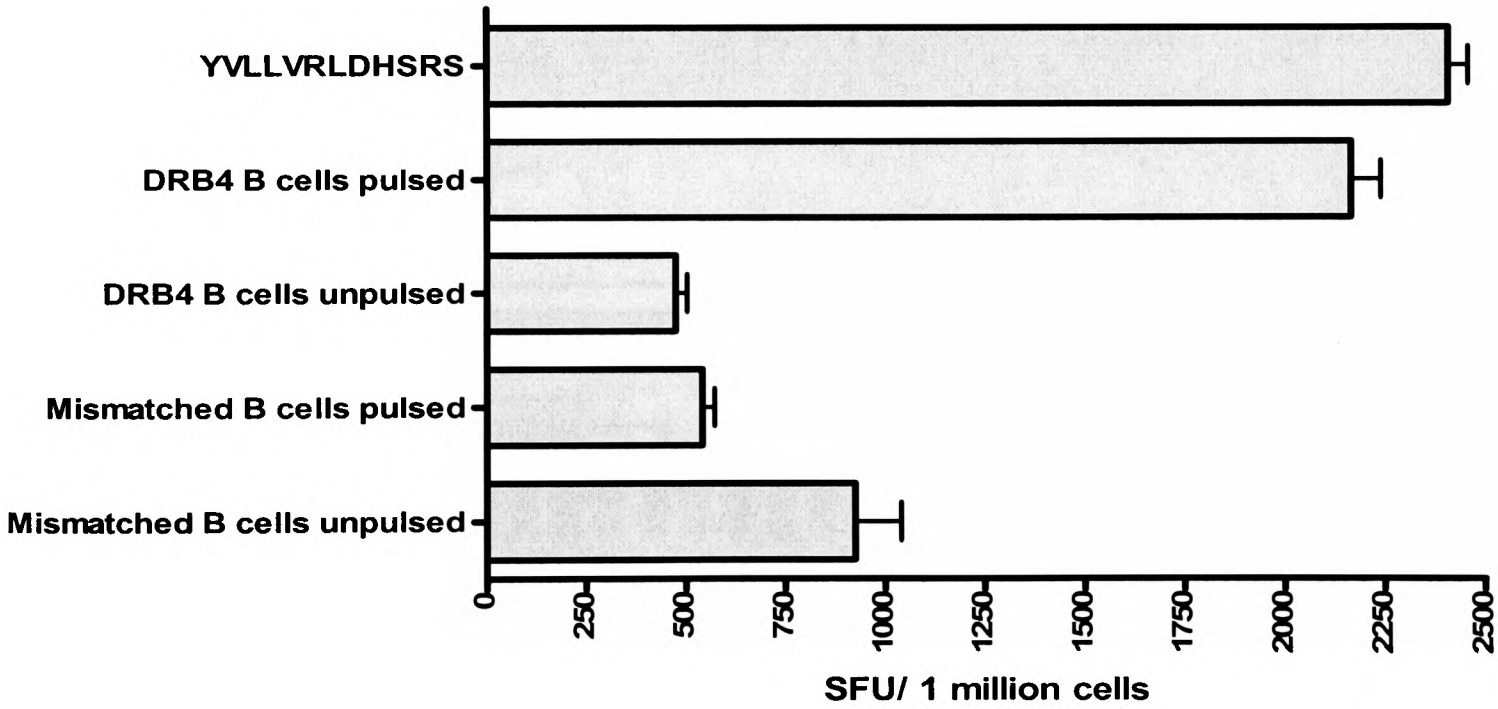
I then proceeded to determine the HLA restriction of this epitope. I initially used DR, DQ and DP blocking antibodies to determine which MHC class II molecules were involved in presentation of the epitope and showed that the DR molecules were involved in antigen presentation (Fig.3-8).

To determine which of the DR molecules presented the epitope, EBV transformed B cells lines (both matched and mismatched to the testing HLA molecule) were used as antigen presenting cells to T cells cultured with the epitope. This showed that the YVLLVRLDHSRS epitope was presented by the DRB4*01 molecule (Fig.3-9).



3-8 HLA restriction of peptide 8 by using class II blocking antibodies

IFNγ ELISpot responses using peptide specific line incubated with peptide, anti-DR+peptide, anti-DQ+peptide, anti DP+ peptide, anti-DR alone, anti-DQ alone, anti-DP alone. Error bars represent the SEM.



3-9 HLA restriction of peptide 8 by using EBV transformed B cell lines.

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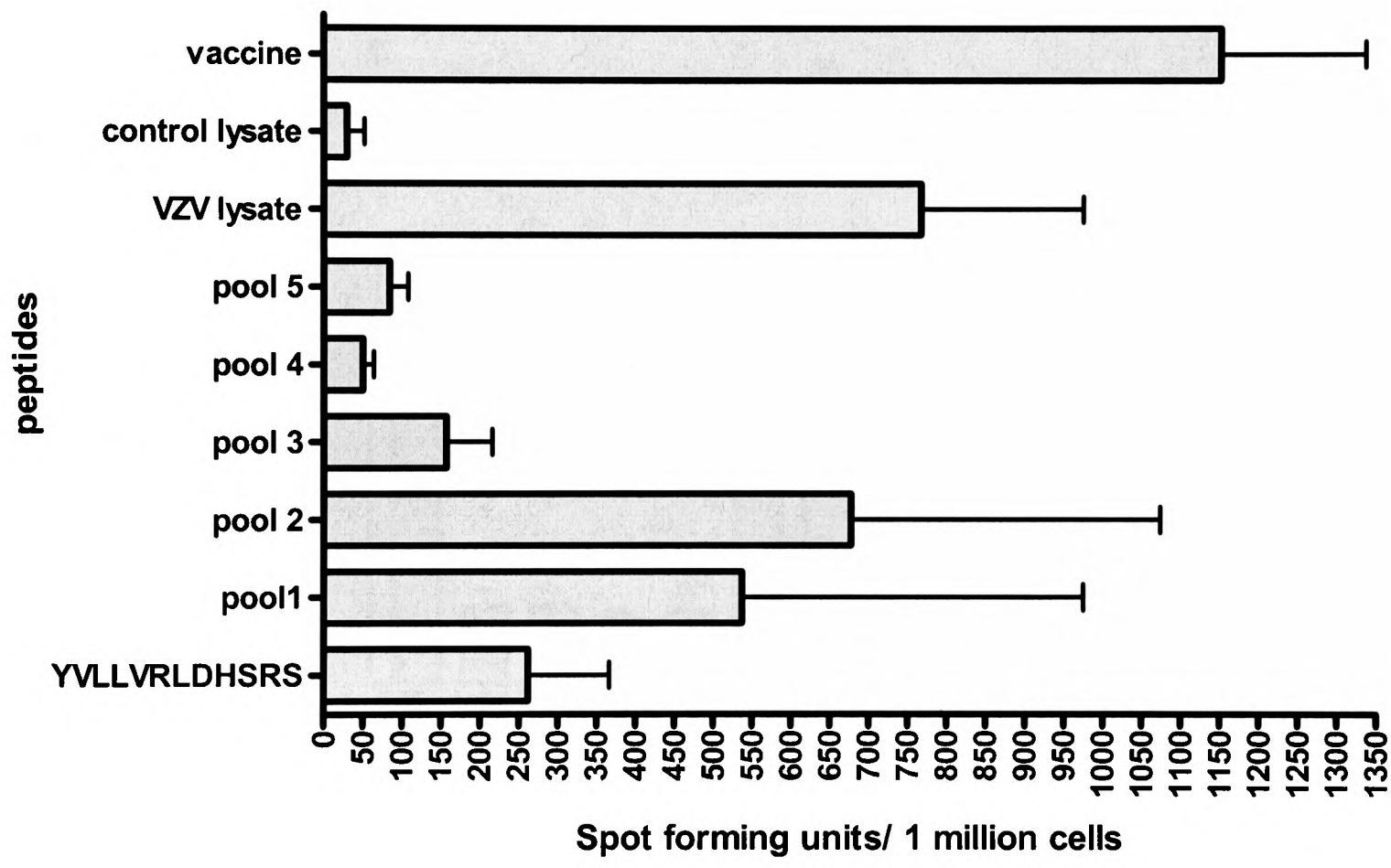
IFNγ ELISpot responses using peptide specific line incubated with peptide directly added to T cells or on DRB4-matched peptide-pulsed or -unpulsed and DRB4 mis-matched B cells peptide-pulsed or –unpulsed. Error bars represent the SEM

Ex vivo ELISpot assays revealed that the responses to this epitope ranged from 9.4% to 56.6% (mean 23.2, SD±19.5) of the VZV lysate in healthy immune volunteers comparable to those seen with *ex vivo* ICS assays. Cultured ELISpot responses in which the cells were incubated with the VZV lysate and vaccine revealed that the responses to this epitope was between 13.5% to 43.5% (mean 31.2, SD±13.9) of the spot forming units (SFU) that were obtained with the lysate and between 5.3% to 52.3% (mean 24.2, SD±18.9) SFU obtaibed with the vaccine again comparable to the *ex vivo* ELISpot data (Fig 3-10).



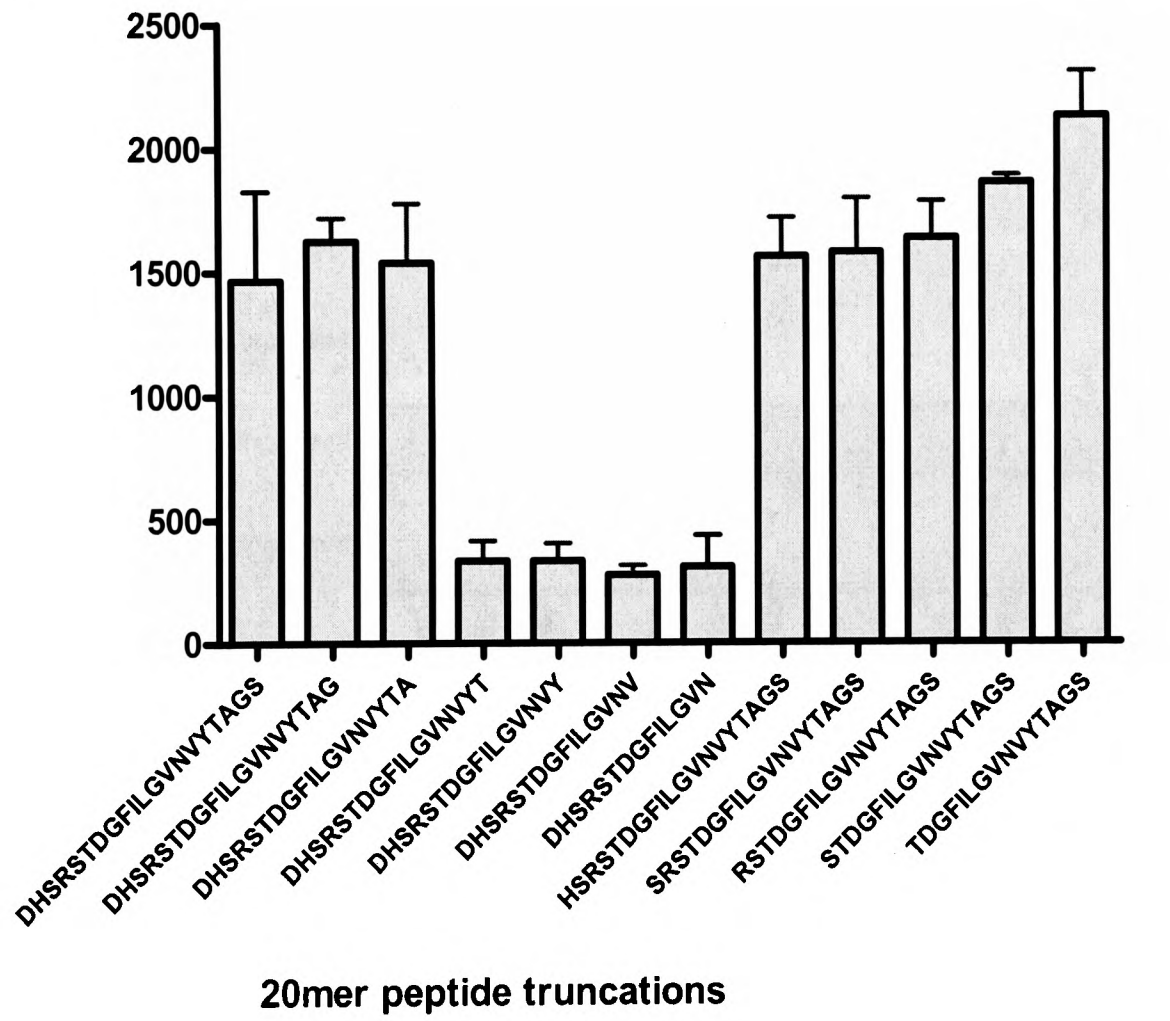
3-10 Comparing IFNγ responses to peptide 8

PBMCs stimulated with viral lysate were tested at day 10 with vaccine, control lysate, VZV lysate, gI pools, optimum peptide 8 (which is contained within pool 2). Error bars represent SEM.

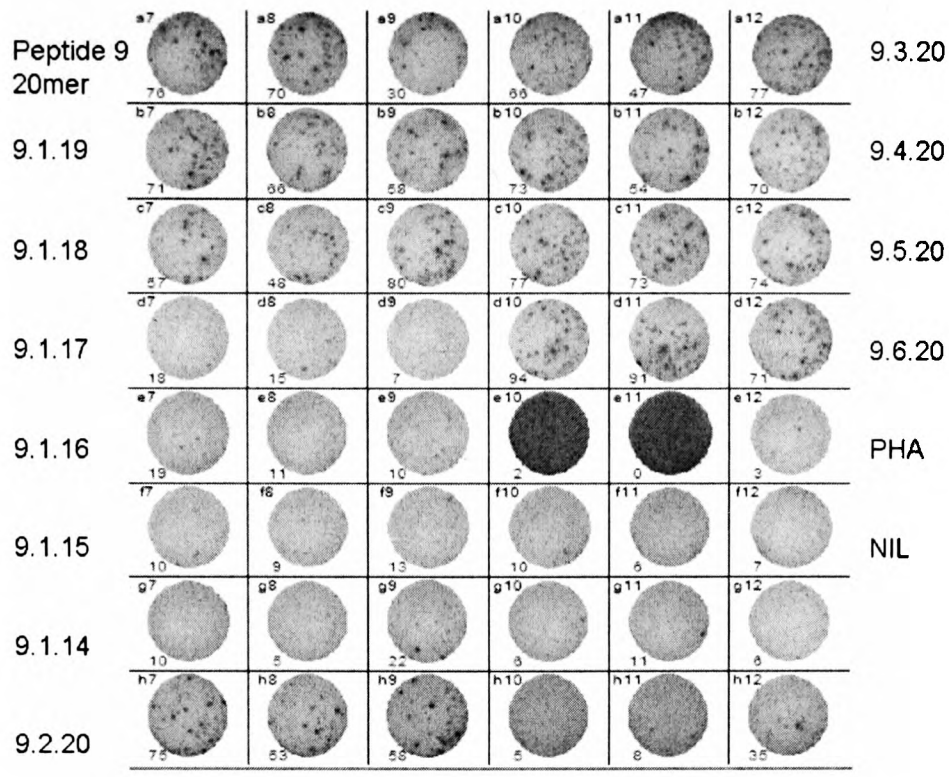
3.2.3. Detailed characterization of responses to peptide 9

To determine the minimum length of the epitope, T cells cultured with the 20mer peptide were tested by ELISpot assays using peptide truncations of the 20mer index peptide. By testing the individuals who predominantly responded to peptide gI 9 we found the optimum epitope to be a 13mer (TDGFILGVNVYTA) (Fig. 3-11).

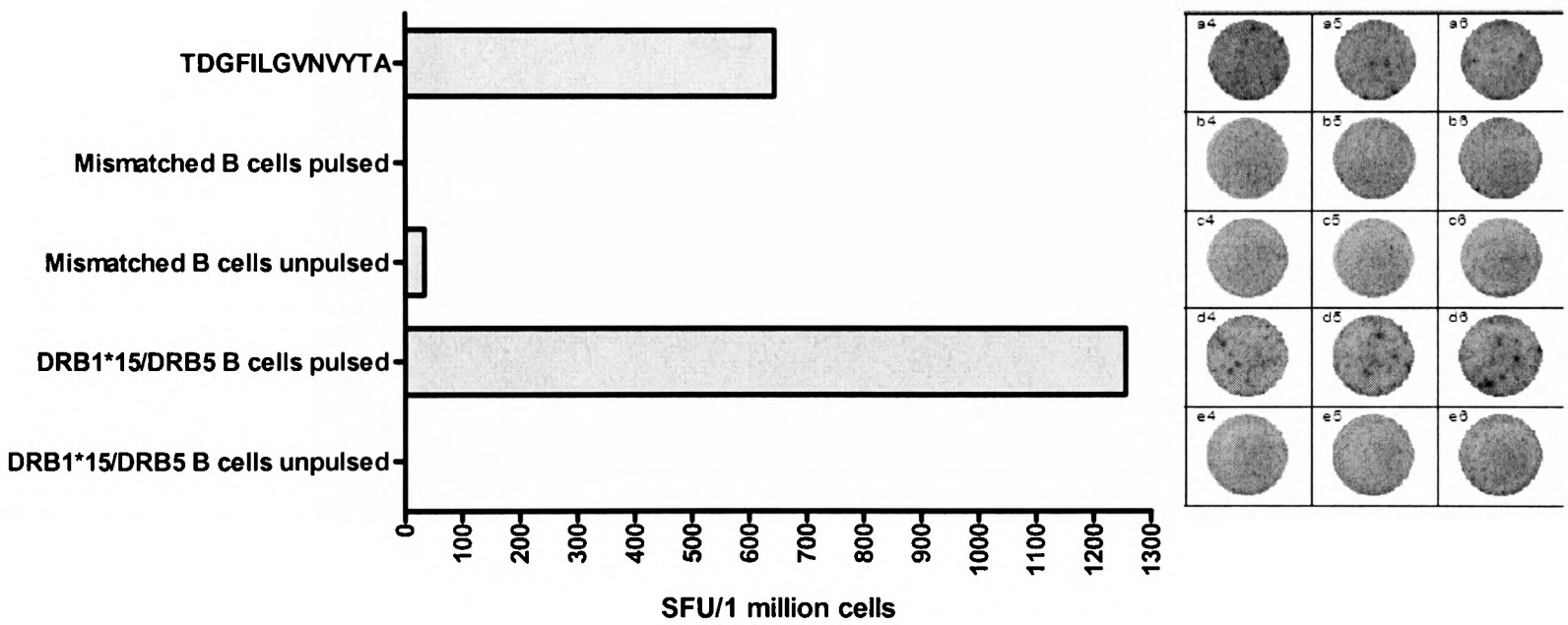
I then proceeded to determine the HLA restriction of this epitope by using EBV transformed lymphoblastoid matched and mismatched to the HLA molecules of the testing T cell line. I found that DRB1*1501/DRB5 B cell lines present this epitope (Fig.3-12)



3-11a IFNγ ELISpot responses to truncated peptides from the 20mer peptide 9 of glycoprotein I. Error bars represent SEM.



3-11b IFNγ ELISpot responses to truncated peptides from the 20mer peptide 9 of glycoprotein I is shown in triplicates. PHA is not added to the outer well.



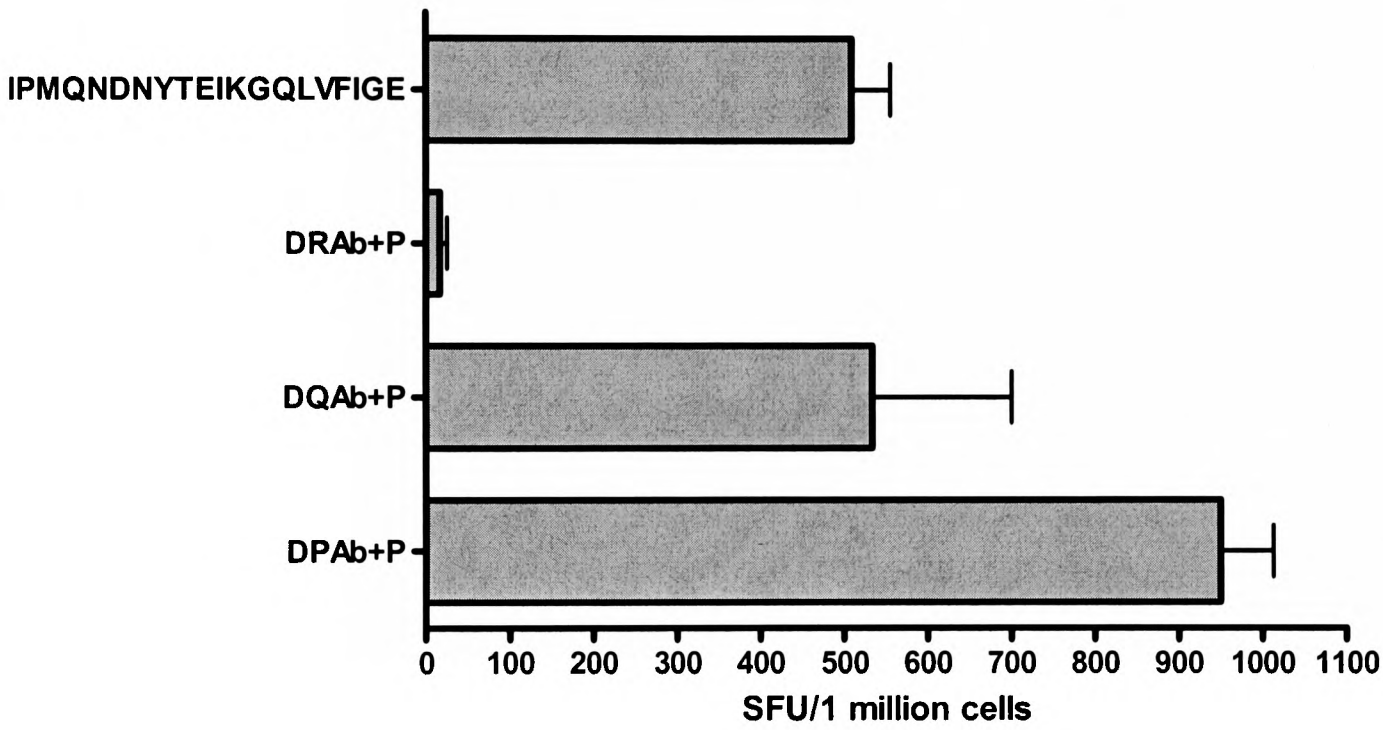
3-12 HLA restriction of peptide 9

IFNγ ELISpot responses using peptide specific line incubated with peptide directly added to T cells or on DRB1*1501/DRB5-matched peptide-pulsed or -unpulsed and DRB1*1501/DRB5*01 mis-matched B cells peptide-pulsed or –unpulsed.

As this epitope could be restricted through either DRB1*1501 or DRB5*01, it could be possible to investigate the restriction in more detail by using murine L cells transfected with DRB1*1501 or DRB5*01.

3.2.4. Detailed characterization of responses to peptide 1

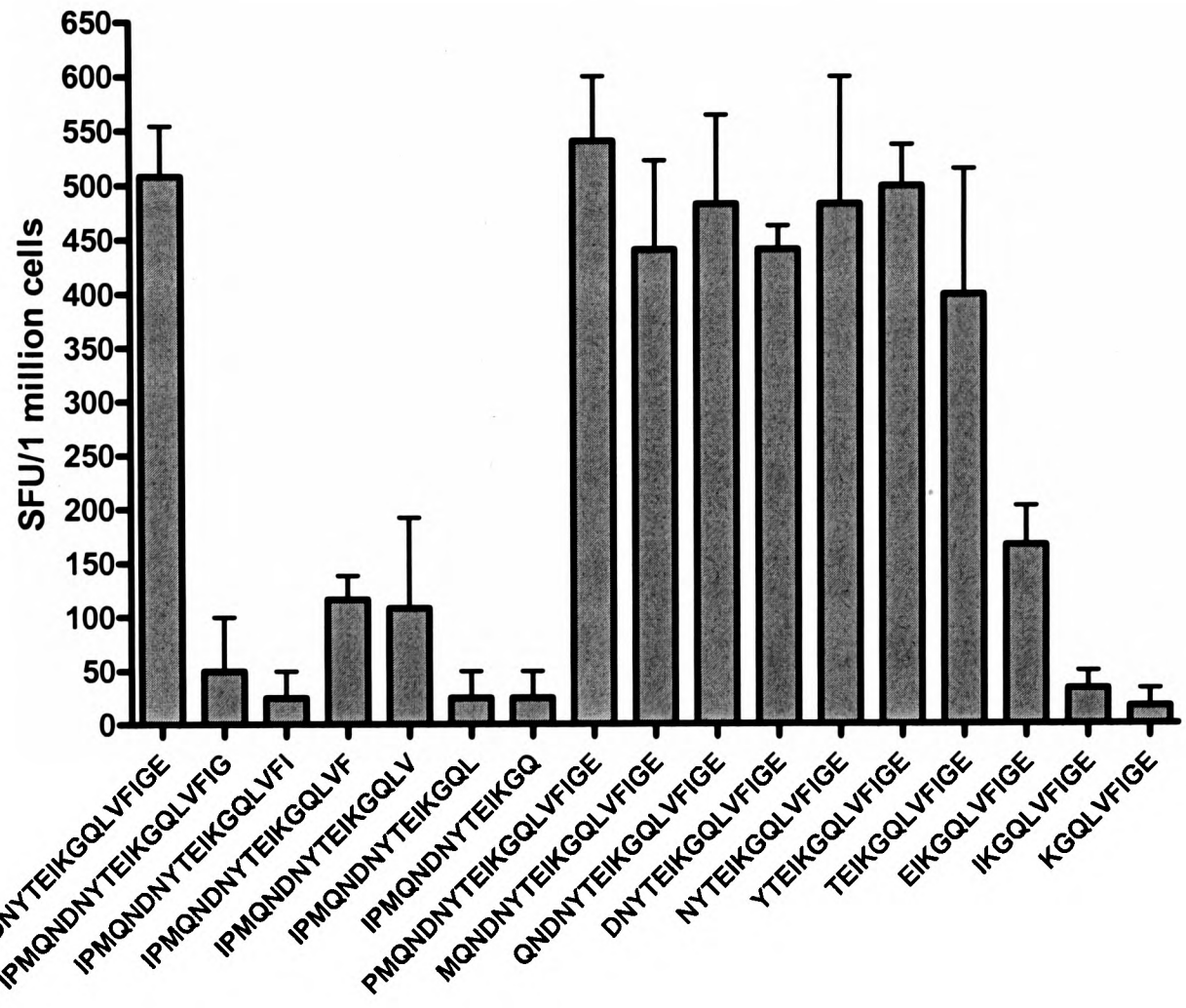
As with the other two peptides I found that peptide 1 specific T cell responses were also predominantly from the CD4+ subset of T cells. Therefore I proceeded to determine the HLA restriction of the epitope by using MHC class II blocking antibodies which showed that the peptide was presented by HLA-DR molecules (Fig. 3-13).



3-13 HLA restriction of peptide 1 using class II blocking antibodies

IFNγ ELISpot responses using peptide specific line incubated with peptide, anti-DR+peptide, anti-DQ+peptide, anti DP+ peptide. Error bars represent SEM.

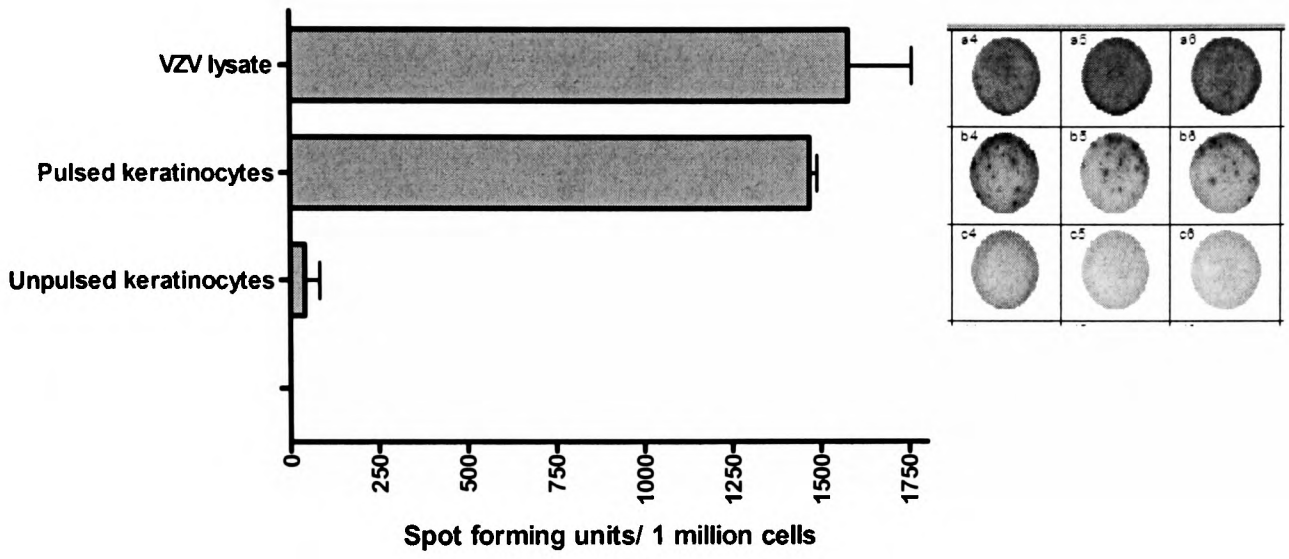
To determine the minimum length of the epitope, T cells cultured with the 20mer peptide were tested by ELISpot assays using peptide truncations of the 20mer index peptide. By testing the individuals who predominantly responded to peptide gI 1, we found the minimum epitope to be a 12mer (Fig 3-14).

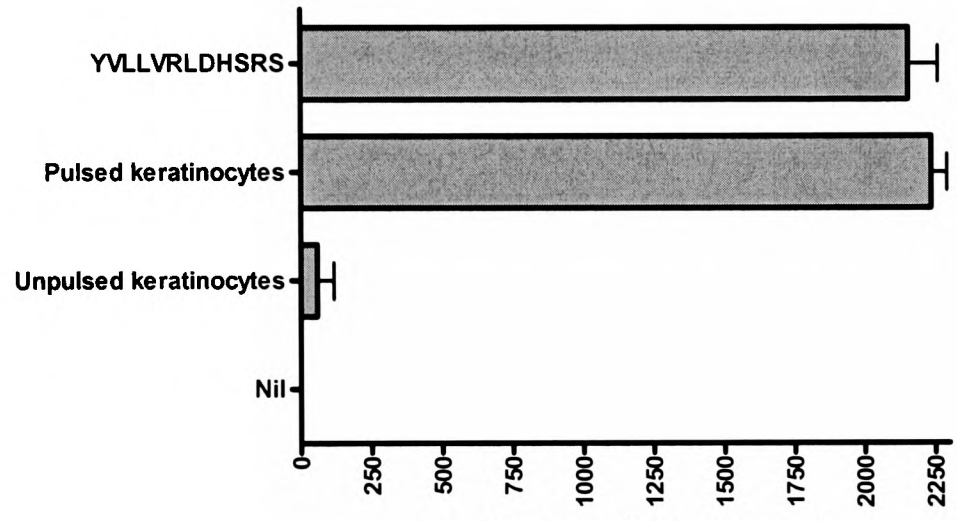


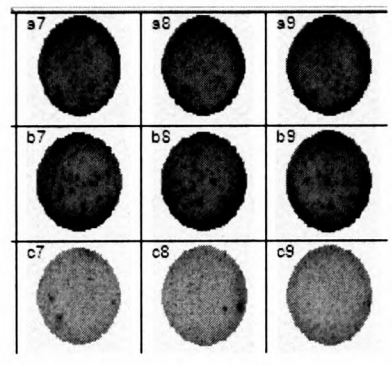
3-14 IFNγ ELISpot responses to truncated peptides from the 20mer peptide 1 of glycoprotein I
Error bars represent SEM.

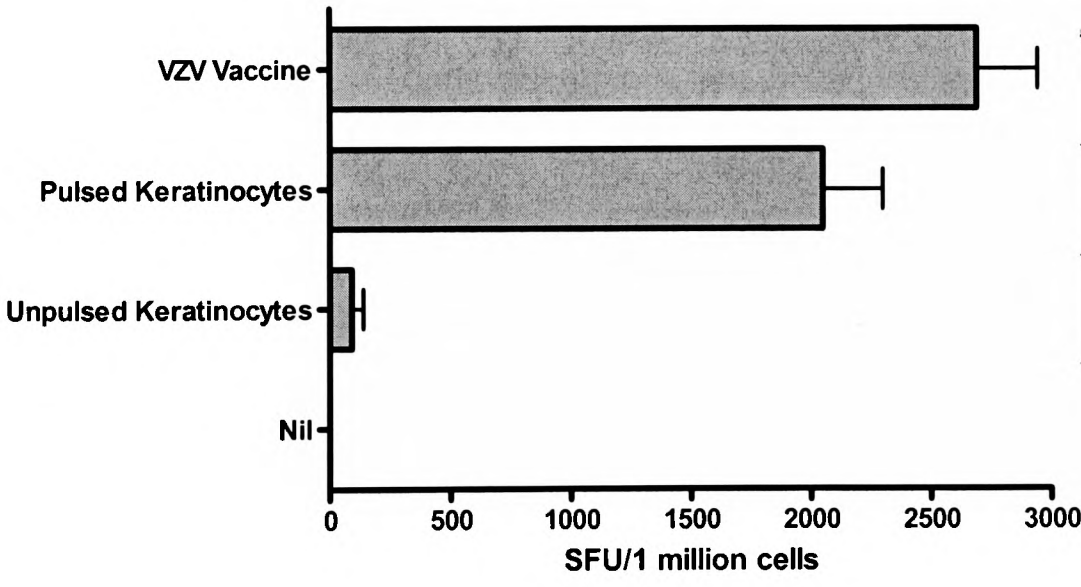
3.2.5. Epitope presentation by keratinocytes

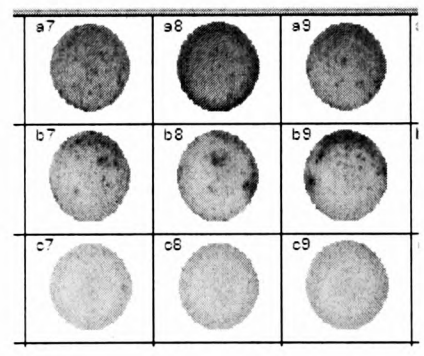
CD4+ and CD8+ T cells infiltrate acute cutaneous lesions (Morizane, 2005) and keratinocyte death is a prominent histological feature of acute VZV infection. However, there are few data that have examined the ability of keratinocytes to present antigens to T cells (Black et al., 2007). Having identified the HLA restriction of the epitope within peptide 8, I then proceeded to investigate whether this epitope could be presented by keratinocytes. I observed that IFNγ treated DRB4*01 keratinocytes efficiently presented this epitope to T cell lines when incubated with specific peptide, viral lysate or vaccine (Figure 3-15). These data show that keratinocytes are able to efficiently process and present class II restricted peptides to CD4+ VZV specific T cells and induce rapid functional responses.











3-15 gI, VZV lysate and VZV vaccine responses using keratinocytes.
The keratinocytes are pulsed with VZV lysate (top), gI peptide 8, VZV vaccine and then used as antigen presenting cells to stimulate a VZV lysate, peptide 8 and VZV vaccine specific T cell line. Error bars represent SEM.

3.3. Discussion

These results show that VZV gI specific rapid effector functional immune responses are observed in all healthy VZV seropositive individuals with a history of VZV infection and that gI is a dominant CD4+ T cell target. I have proceeded to fine map an epitope restricted by HLA-DRB4*01 and showed that IFNγ responses represented 9% to 56.5% (mean 23.2%) of those seen with the VZV lysate *ex vivo*. However, as the concentration of gI peptides within the VZV lysate is difficult to determine, the comparison of the IFNγ responses to this epitope with that for the whole VZV lysate may not be appropriate. *Ex vivo* and cultured glycoprotein I specific T cell responses in these donors were predominantly mediated by $CD4^+$ T cells.

3.3.1. Importance of virus specific CD4+ T cell in herpes virus infection

Glycoproteins of other herpes viruses have also been shown to be preferentially recognized by $CD4^+$ T cells (Bellner et al., 2005; Elkington et al., 2004; Hegde et al., 2005; Sylwester et al., 2005). $CD4^+$ T cells have been shown to be important in controlling many viral infections (Appay et al., 2002b) and there is evidence that they may especially be important in controlling herpes virus infections (Hegde et al., 2005; Landais et al., 2005). For instance, the occurrence of clinical symptoms of cytomegalovirus reactivation in renal transplant patients were preceded by a marked decrease in CMV specific CD4+ T cell frequencies (Sester et al., 2001; Sester et al., 2002a). VZV specific CD4+ T cell responses are also shown to be crucial in preventing virus reactivation in patients with systemic lupus erythematosus (Park et al., 2004). Recent reports have implied that CD4+ T cells are also important for recovery from

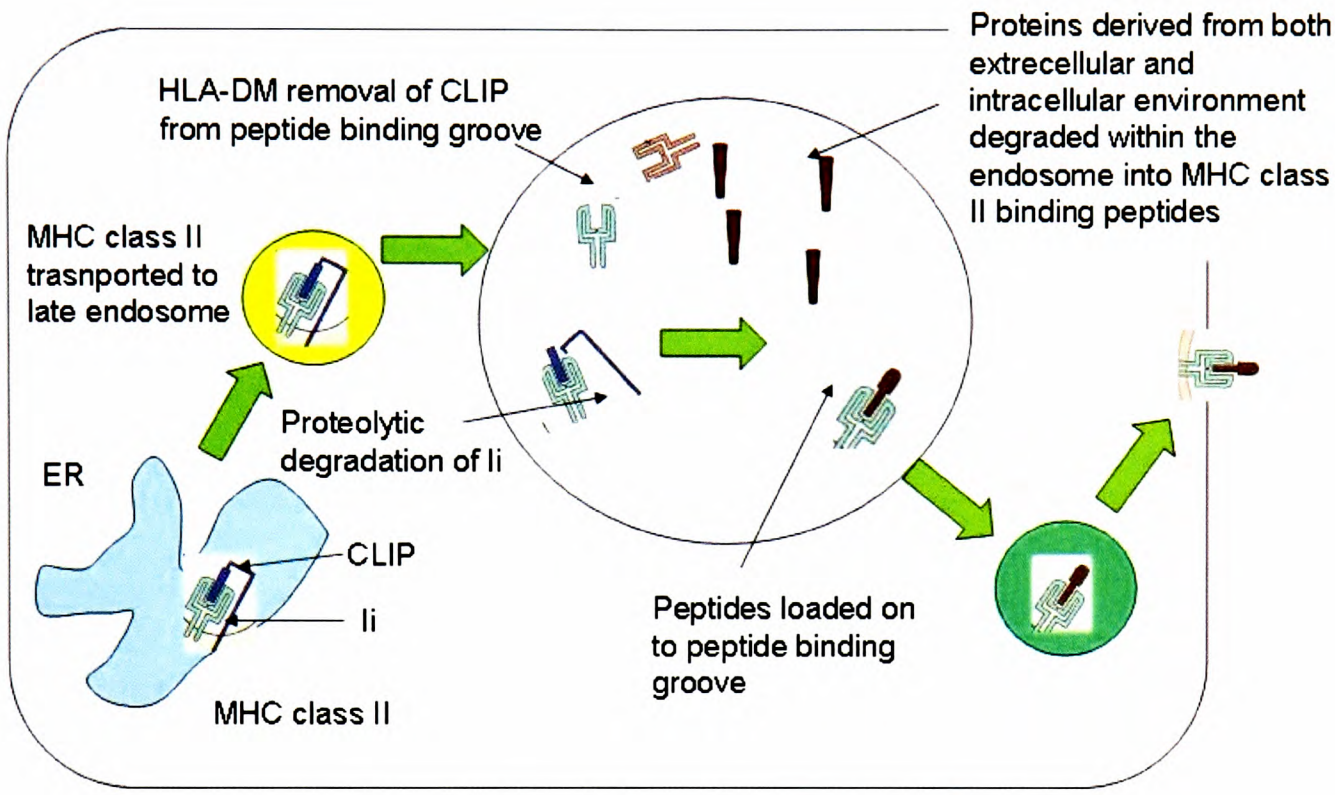
primary VZV infection as a fatal infection in a child was associated with an idiopathic CD4+ T cell lymphopenia (Hochauf et al., 2005). Nevertheless I had anticipated that gI-specific CD8+ T cell responses would have been detectable at low levels and I was surprised by the absence of gI-specific CD8+ T cells even after culture (with peptides, lysate or vaccine).

CD8+ T cell epitopes have been documented within the IE62 protein of VZV(Frey et al., 2003) but these IE62 responses were at a relatively low frequency, only being detectable after *in vitro* expansion (Frey et al., 2003). Such relative paucity of VZV specific CD8+ T cell responses may implicate forms of CD8+ T cell escape in the mechanism of viral persistence, such as the previously documented class I down regulation (Abendroth and Arvin, 2001a; Abendroth et al., 2001a; Morrow et al., 2003).

3.3.2. Pathways in MHC class II antigen processing and presentation

Although earlier it was believed that only exogenous protein antigens entered the lysosomes or endosomes to be processed into peptides that could be loaded into MHC class II molecules, is has since been demonstrated that endogenous proteins derived from the cytoplasm, nucleus, intra cellular organelles and viral products also enter the endosomes by micro or macroautophagy. Autophagy is thought to be a process that cells use to degrade intracellular products for amino acid recycling and in this process many intracellular proteins are transported into the endosomes for degradation(Strawbridge and Blum, 2007). Some viral proteins are thought to be predominantly processed by this method. Autophagy is thought to be the main process by which Epstein-Barr virus

nuclear antigen-1 is presented on MHC class II molecules, as blocking this pathway led to a significant reduction of MHC class II presentation of this antigen (Paludan et al., 2005).



3-16 Diagrammatic representation of MHC class II pathways in antigen processing

As with all herpes viruses, VZV viral assembly occurs in the trans golgi network. It is believed that in Cytomegalovirus (CMV) infection, during viral assembly, structural proteins are delivered to the endosomal compartment where class II antigen presentation occurs. Class II presentation of endogenous viral glycoproteins of CMV is thought to be an important mechanism where the host is able to respond to the virus despite MHC class I down regulation by the virus (Hegde et al., 2005). Therefore, it is likely that a similar mechanism occurs in the processing of VZV glycoproteins which would explain the predominance of VZV gI specic CD4+ response. Alternatively, autophagy of VZV

infected cells may lead to class II processing and presentation of gI peptides as with the Epstein Barr virus nuclear antigen 1 which is a dominant CD4+ T cell target (Paludan et al., 2005).

3.3.3. Presentation of gI CD4+ T cell epitopes by keratinocytes

CD4+ T cells have been shown to be able to induce target cell .death in a number of infections, including human immune deficiency viral infection, and keratinocyte death is a histological feature of cutaneous lesions (Appay et al., 2002b). Keratinocytes are a main target of VZV infection and it was shown that DRB1*04 epitope was naturally processed by HLA matched keratinocytes and efficiently presented to an epitope specific T cell line. Therefore, it is possible that the live VZV vaccine was taken up from the exogenous environment and processed into the DRB1*04 epitope or alternatively class II presentation by infected cells occurred via an endogenous pathway such as autophagy. Nevertheless, the presentation of the DRB1*04 epitope by DRB4*01 positive keratinocytes is consistent with the possibility of keratinocyte presentation within lesional tissue.

3.3.4. Future directions

I observed restriction through HLA-DRB4, which has a frequency of 42%- 46.5% in different populations (Machulla et al., 2001; Oguz et al., 2003; Rihs et al., 2002). Therefore, as we have documented in our cohort this epitope is likely to be recognized by a large proportion of seropositive individuals. These data are consistent with a role for gI-specific CD4+ T cells in the control of VZV reactivation and identify the first gI epitope

for use in future studies of VZV pathogenicity and vaccination strategies. However, in order to define this in more detail, it would now be important to investigate the frequency and phenotype of gI specific cells in different populations, such as during acute primary infection and reactivation in both cross-sectional and longitudinal studies. Having characterized gI CD4+ T cell epitopes, these studies are now possible at the epitope-specific level.

Chapter 4. Investigation of VZV glycoprotein E specific T cells

4.1. Introduction

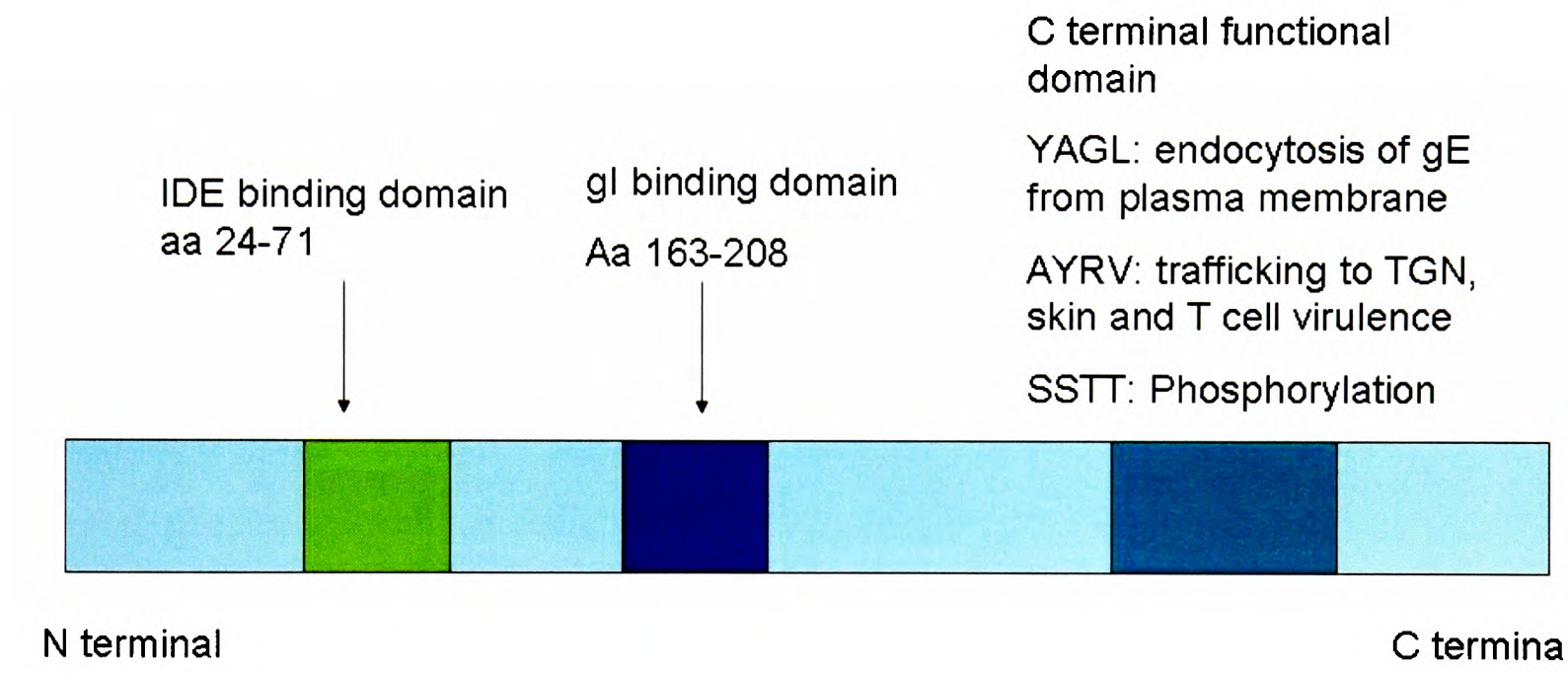
gE is a type I membrane protein consisting of 623 amino acids and is the most abundant glycoprotein of VZV. It is encoded by open reading frame (ORF) 68 (Rahaus and Wolff, 2003). As all other VZV glycoproteins it is encoded by late genes and therefore, is only expressed during active viral replication. It is expressed on the plasma membrane and in the cytoplasm of infected cells and is thought to have multiple functions essential in the formation of infectious virions (Mo et al., 2002). Although glycoprotein E in other alpha herpes viruses is not important for viral replication, VZV gE is thought to play a unique role in VZV as it is an essential glycoprotein for the virus and also a major T cell and B cell target. So far attempts to generate gE deletion mutants have been unsuccessful (Mo et al., 2002). In addition, unlike in other alpha herpes viruses, gE and gI are the only VZV glycoproteins in VZV that are encoded by the unique short region which is thought provide the virus with a biological advantage for its survival (Rahaus and Wolff, 2000).

As described in the previous chapter gE forms a heterodimer with gI in infected cells and together forms a Fc receptor for human IgG (Litwin et al., 1992). gE is believed to be essential for cell entry, cell to cell spread of the virus, viral replication, secondary envelopment of the virus and skin tropism (Berarducci et al., 2006; Li et al., 2007). In addition to gI, gE also forms complexes with another VZV glycoprotein gH which is thought to be important in secondary envelopment of the virus (Maresova et al., 2001). The regions within gE which are important for most of the above functions have been mapped and the amino acids 24 to 71 have been shown to be critical for binding of gE to the insulin-degradation enzyme which was identified as the cellular receptor for both cell

free and cell associated VZV (Li et al., 2006; Li et al., 2007). In addition, binding to gI required the amino acids from 163 to 208 of the gE protein (Li et al., 2007).

VZV gE has shown to have a unique region in the N terminal region (amino acid 1 to 188) of the protein which is not conserved in other alpha herpes viruses. This N terminal region of gE is vital for viral replication and syncytium formation and has also shown to determine skin virulence (Berarducci et al., 2006). A gE mutant virus in which the aspartic acid (D) at position 150 is replaced with asparagine (N) is thought to have an increased ability to cell to cell spread *in vitro* and in the SCIDhu mouse model (Grose et al., 2004; Santos et al., 2000). This first VZV mutant strain was initially identified in 1998 and was named as VZV-MSP (Santos et al., 2000). This mutation has resulted in the loss of one of the major B cell epitopes within gE (Santos et al., 1998). The most immune dominant B cell epitope within gE was identified in the region spanned by amino acids 101 to 161 within the region where the mutation lies (Fowler et al., 1995; Santos et al., 1998). Subsequently a monoclonal antibody named 3B3 was developed to this region (Hatfield et al., 1997) and was used in immunofluorescence and western blot assays to identify VZV. This amino acid substitution in VZV-MSP has resulted in impaired binding of the virus to the monoclonal antibody 3B3 which in turn results in loss of detection of the virus by immunofluorescence and western blots (Santos et al., 2000; Santos et al., 1998). The enhanced ability of cell to cell spread of the VZV-MSP strain of virus in cell culture has shown to be clearly distinguishable from the other virus (Santos et al., 2000) and therefore, the mutated virus possibly is more virulent by enhancing spreading of the virus in the skin.

The C terminal of gE is also shown to have multiple functions and is thought to be particularly important in viral replication in the skin. The C terminal contains 3 major functional domains that mediate: endocytosis of gE from the plasma membrane (YAGL); trafficking of gE into the trans golgi network (AYRV) where it is involved in virion assembly; and phosphorylation (SSTT) (Moffat et al., 2004). The AYRV region (amino acids 568 to 571) has also been implicated in skin virulence and in T cell virulence to some extent in the SCIDhu mouse model (Moffat et al., 2004).



4-1 Map of regions within gE that are important in binding to IDE, gI and also involved with viral replication, assembly and virulence.

4.1.1. Immune responses to gE

VZV gE is thought to be a major T cell and B cell target. Experiments carried out by Grose *et al* in 1981 first identified that gE was one of the main immunogenic glycoproteins within VZV (Grose et al., 1981). Among the VZV antigens gE and gB have been shown to elicit the highest neutralizing antibody titres and gE specific antibody

were highest among individuals following natural infection(Haumont et al., 1997). The 3 most immunogenic B cell epitopes within gE have been characterized (Fowler et al., 1995) and one of the monoclonal antibodies (3B3) is currently used in identifying the virus as mentioned previously in this chapter.

Experiments carried out by several groups in the early 1990s have shown that gE specific cytotoxic and proliferative T cell responses are seen in healthy immune donors following natural infection and vaccination (Arvin et al., 1991; Bergen et al., 1991; Diaz et al., 1989; Sharp et al., 1992; Watson et al., 1990). However, most of these groups used the limiting dilution cultures to determine T cell responses to antigens which are much less sensitive than the more recent assays such as ELISpot and intra cellular cytokine assays in detecting antigen specific T cell responses (Smith et al., 2001). More recent work done by Milikan *et al* using these methods have shown that intra ocular T cells in patients with VZV induced uveitis recognize many VZV proteins including gE and were of the effector memory phenotype (Milikan et al., 2007).

4.1.2. Experimental data on animals

Although typical VZV infection does not occur in animal models, vaccination of guinea pigs with gE recombinant vaccines have provided evidence of the immunogenicity of this protein. Of vaccinia virus recombinants expressing certain glycoproteins (gE, gI and gV), the gE recombinant induced the highest proliferative T cell responses in guinea pigs (Lowry et al., 1992). Furthermore, guinea pigs immunized with a recombinant VZV gE and gI, developed protective antibody responses and were able to clear the virus

efficiently (Kimura et al., 1998). Vaccination of mice with a DNA expression vector encoding gE both intramuscularly and subcutaneously was shown to generate gE specific antibodies of the IgG2 subclass (Hasan et al., 2000). Jacquet *et al* who investigated the immunogenicity of a recombinant VZV gE-IE63 vaccine in guinea pigs showed that while antibodies to both gE and IE63 were detected in high concentration in the guinea pigs, only gE specific antibodies were able to neutralize VZV(Jacquet et al., 2002).

Despite these few studies which clearly show that gE is a major T cell target, T cell responses to gE have not been characterized in healthy immune donors and T cell epitopes within this protein have not been mapped. As gE is expressed only during the lytic cycle of the virus investigating gE specific T cell responses and comparing them to other VZV proteins which are expressed even during latency would enable us to understand the dynamics of VZV specific T cell responses following primary infection. Moreover, mapping of immune dominant epitopes within gE would useful in studying virus specific T cell responses in detail and also to investigate the phenotype of virus specific T cells in different populations.

4.2. Methods

4.2.1. Peptides

As gE consists of 623 amino acids, 62 20mer overlapping peptides were synthesized in house in an automated synthesizer using F-MOC chemistry. The 62 20mer over lapping peptides were made in to 20 pools with each containing 3 20mer peptides (the last pool consisted of 5 20mer peptides). Therefore, PBMCs from each individual donor were tested with 20 peptide pools.

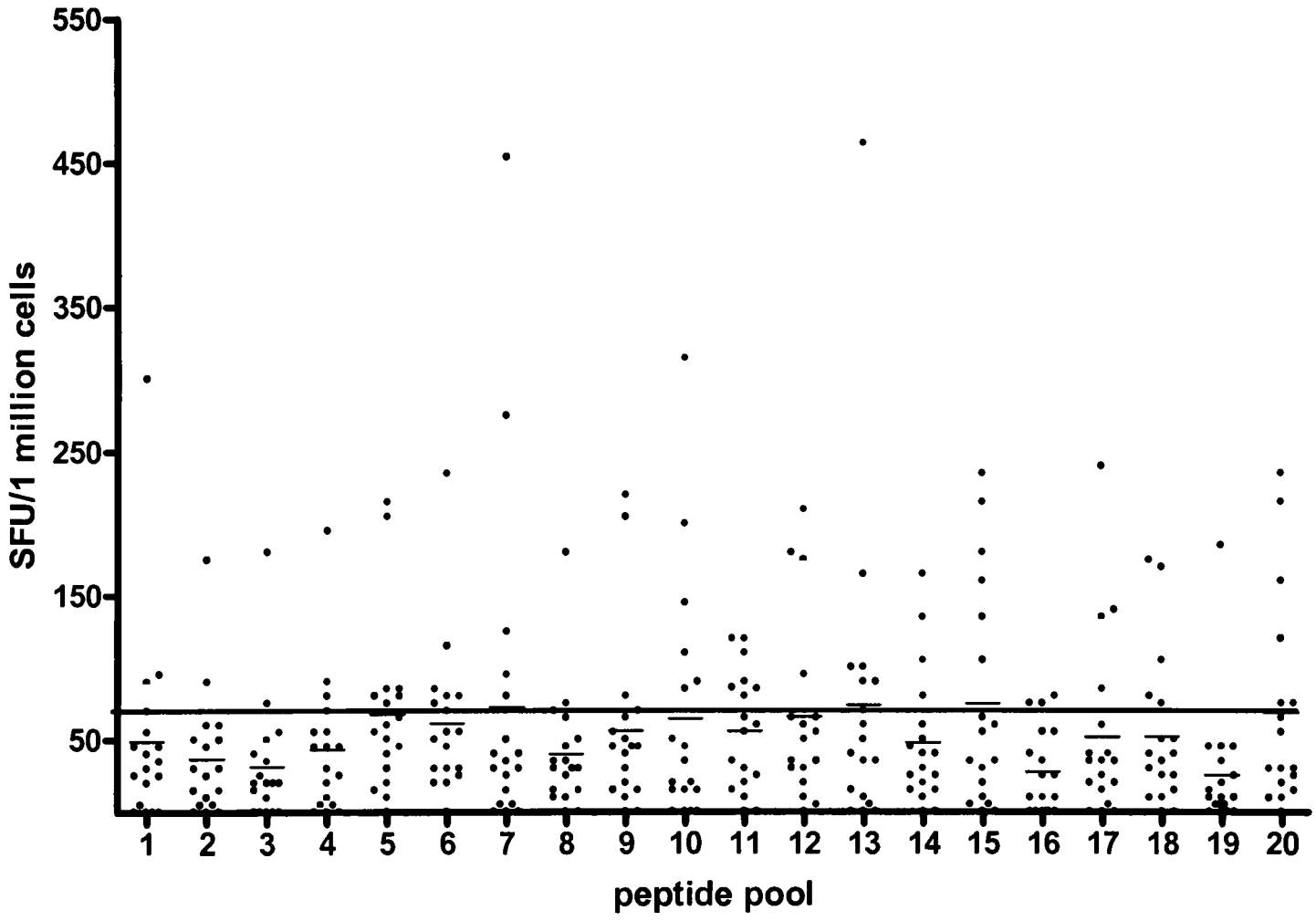
4.2.2. Cultured ELISpot assays

PBMCs from each donor were incubated with the gE peptide pools (consisting of all overlapping peptides). T cell lines were tested after 10 days culture for the relevant peptide pool specific responses.

4.3. Results

4.3.1. Overall gE-specific T cell responses ex vivo

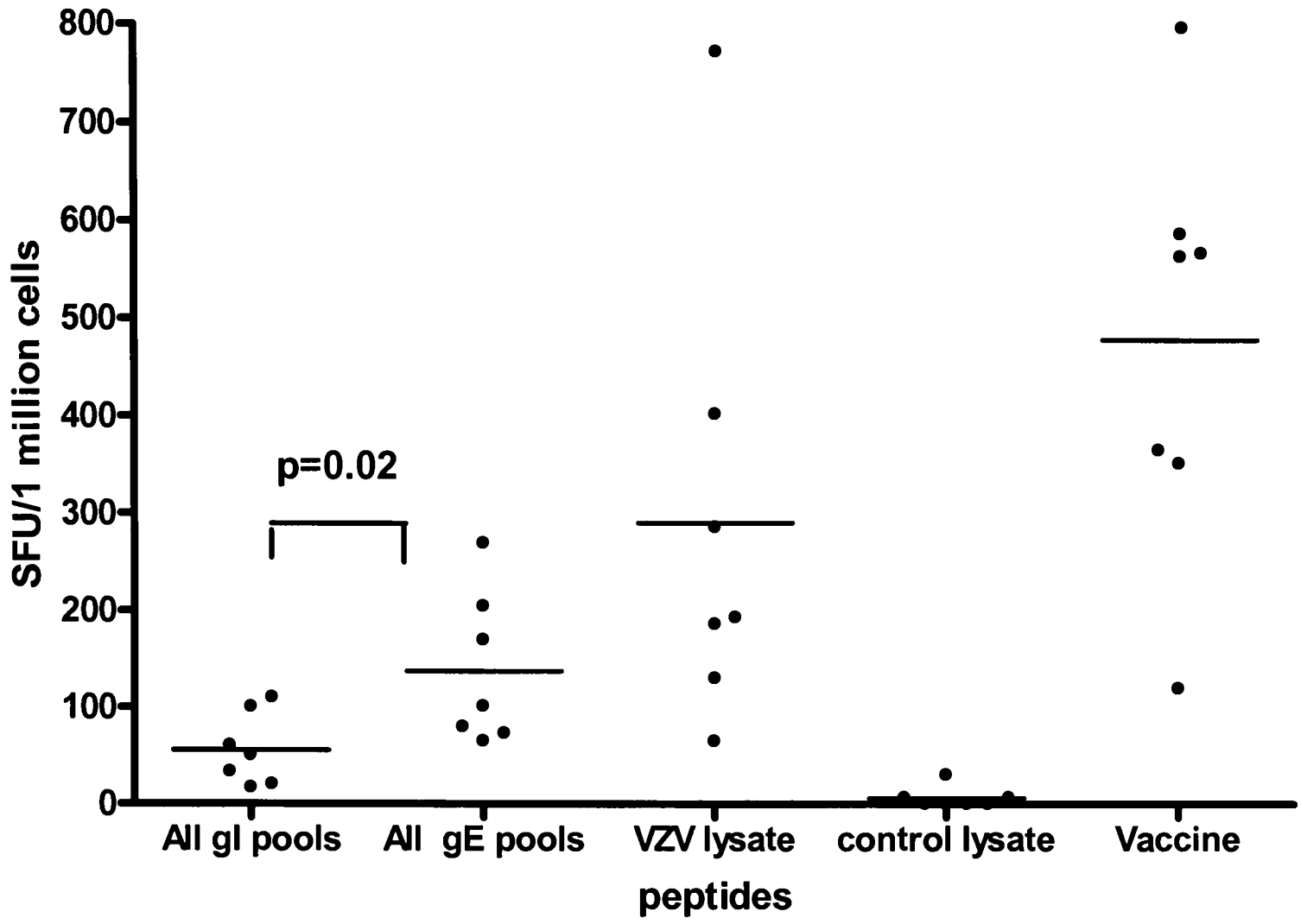
Using the 20 peptide pools (each consisting of 3 20mer peptides) encompassing the gE protein, I screened the *ex vivo* ELISpot responses with PBMC derived from healthy immune donors with a history of VZV infection and positive VZV serology (Fig 4-2). The *ex vivo* PBMC responses specific for these peptide pools ranged from 0-465 Spot forming units/million. Seronegative individuals (n=8) made no responses to any of the gE peptide pools *ex vivo* (data not shown). There was no correlation with age of the donors and time since primary infection.



***4-2 Ex vivo* IFNγ ELISpot responses to overlapping gE peptide pools.**

Ex vivo IFNγ ELISpot responses to overlapping gE peptide pools in cohort of healthy immune donors with a history of primary VZV but no reactivation. The horizontal bar shows the mean+3SD of the irrelevant peptide control.

As I had previously studied ex vivo responses of gI protein I then went on to compare *ex vivo* immune responses of overlapping gI peptides to gE peptides. I found that gE specific IFNγ responses were present at a significantly higher level than gI specific immune responses ($p=0.02$). Furthermore, the responses to the pooled gE peptides ranged from 0% to 100% (mean 41.05%, SD±38.2) of the VZV lysate response and from 0 % to 56.5% (mean 22.05, SD±16.3) of the vaccine response (Fig.4-3). Whilst these data raise the possibility that gE-specific immune responses are dominant, it is not possible to be definite as we do not know the concentration of peptides within the lysate, nor the comparative level of antigens generated by the vaccine. However, overall these data showed that gE-specific T cell responses could be identified *ex vivo* and are present at a higher level than those of gI.



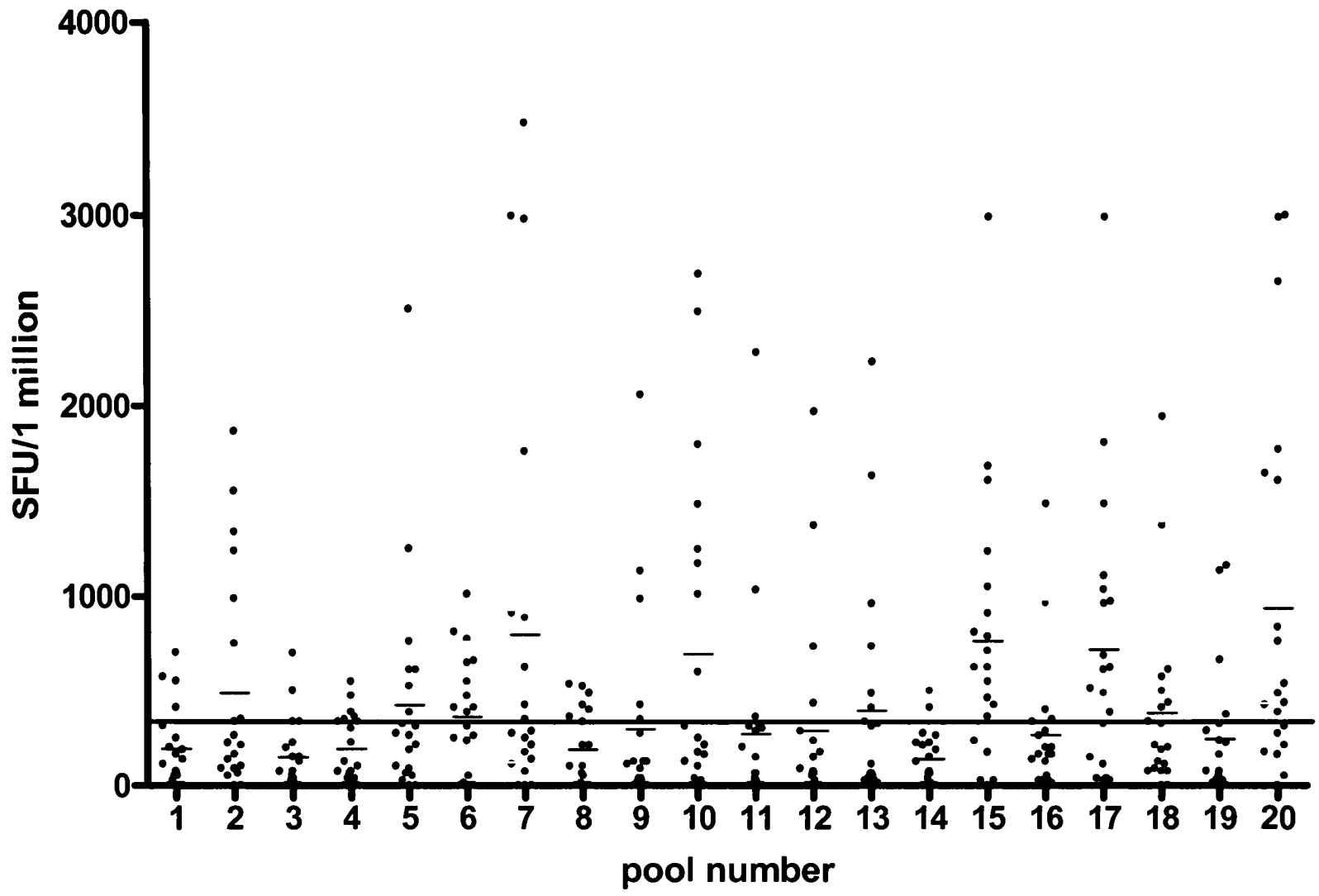
4-3 Comparison of IFNγ responses to gE with gI, VZV vaccine and the lysate

Ex vivo IFNγ ELISpot responses to overlapping gE and gI peptide pool, the VZV lyaste, control lysate and the live attenuated vaccine in cohort of healthy immune donors with a history of primary VZV but no reactivation.

4.3.2. Cultured T cell responses to gE

I then proceeded to confirm the responses detected *ex vivo* by expanding peptide specific cells *in vitro*. I observed high levels of gE-specific T cells after 10 days of culture, reaching a maximum of 3488 IFNγ-producing cells per million total cells (Fig 4-4). The cultured ELISpot responses showed that 14/20 individuals responded to pool 20, 15/20 responded to pool 15, 14/20 responded to pool 17, 9/20 to pool 10 and 9/20 responded to pool 7. After further testing the individuals for each 20mer peptide included in these

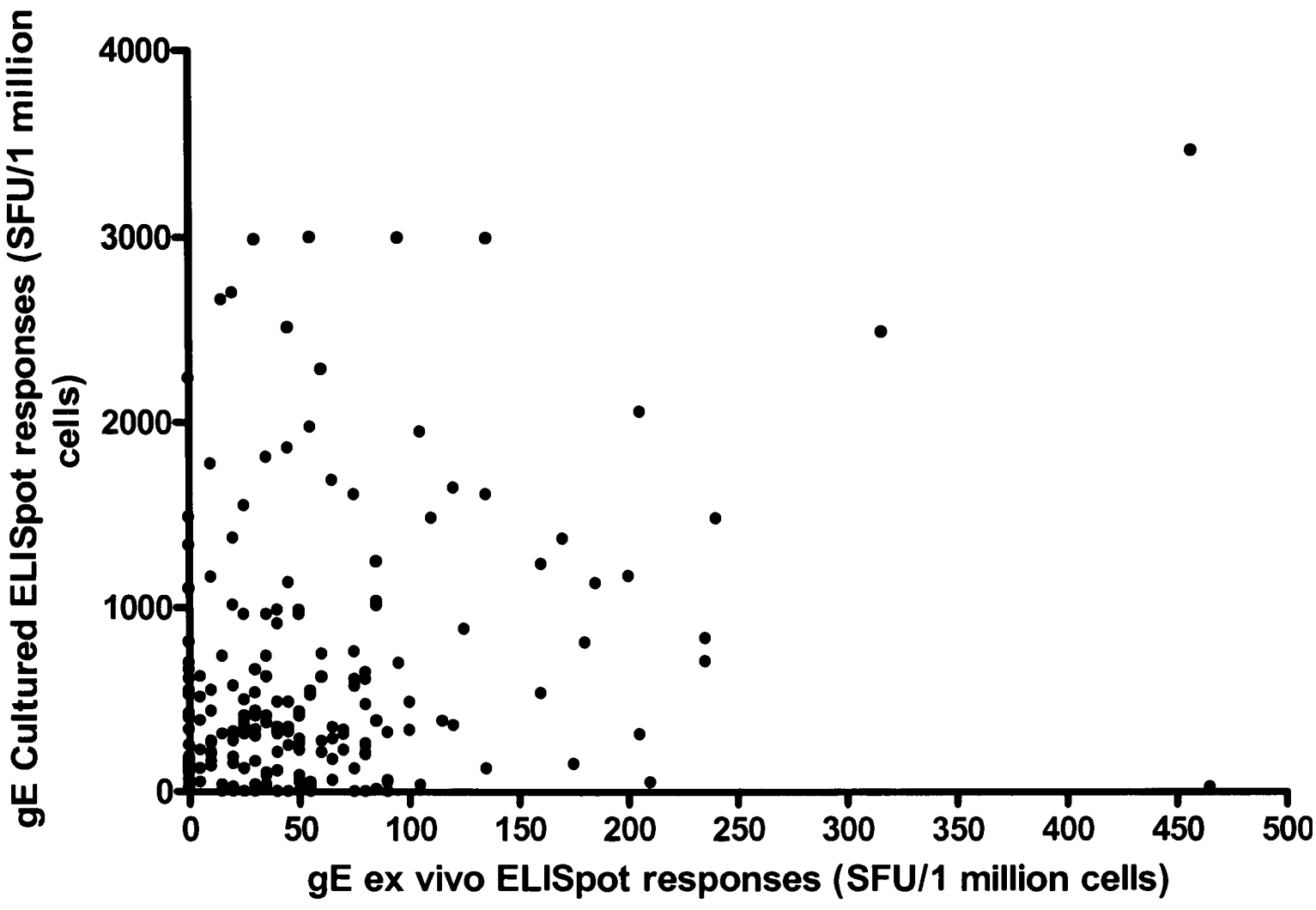
peptide pools, we found that the majority of the responding individuals reacted to peptide 20 in pool 7, peptide 58 and 59 in pool 20 and peptide 29 in pool 10 (data not shown).



4-4 Cultured IFNγ ELISpot responses to overlapping gE peptide

Cultured IFNγ ELISpot responses to overlapping gE peptide pools in cohort of healthy immune donors with a history of primary VZV but no reactivation. The horizontal bar shows the mean+3SD of the irrelevant peptide control.

I observed that there was a significant positive correlation between the *ex vivo* and cultured gE peptide pool-specific responses, confirming that the cultured approach was expanding cells carrying an overlapping specificity to those detected *ex vivo* ($p<0.0001$, r, 0.3016).

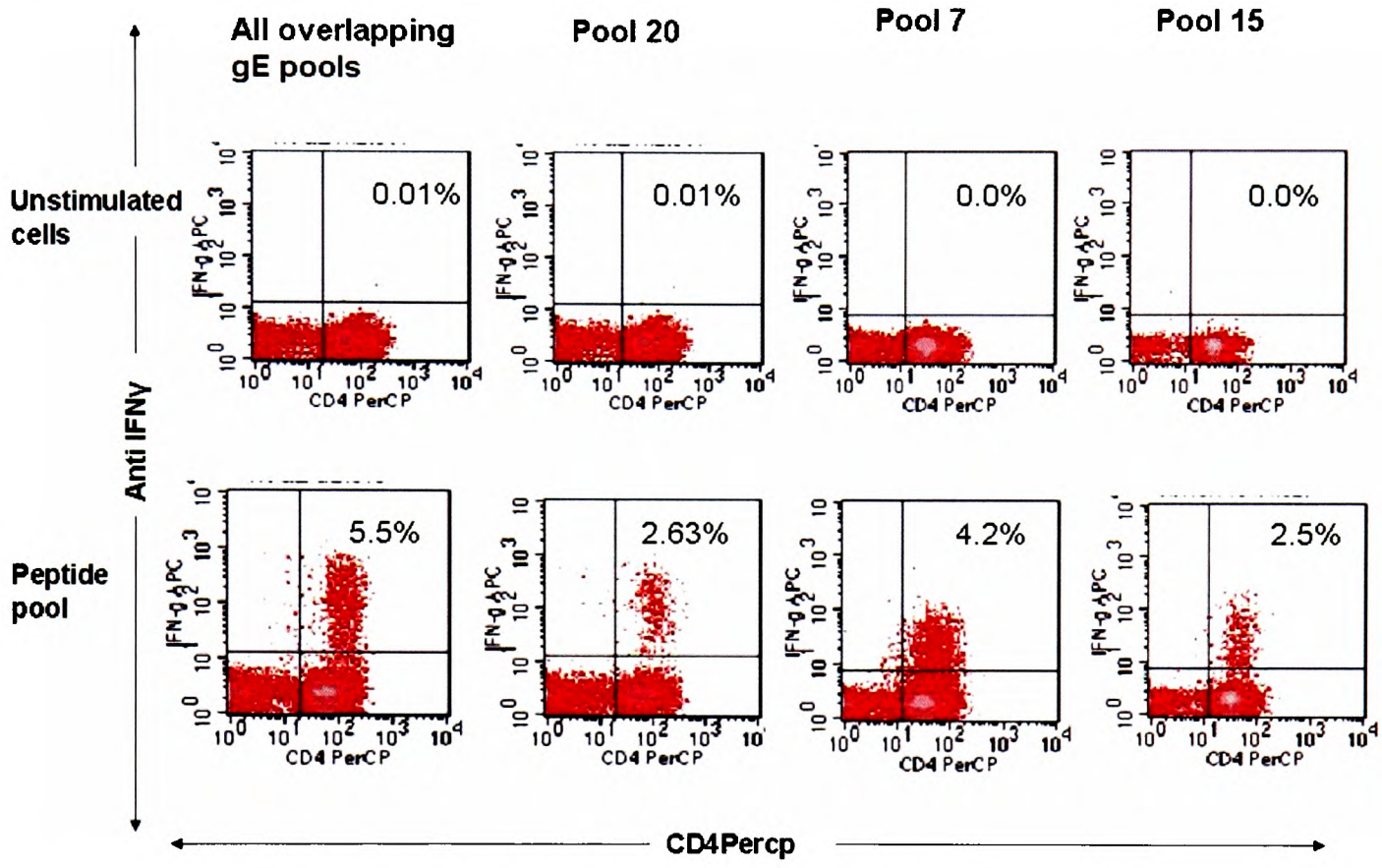


4-5 Correlation between ex vivo and cultured IFNγ ELISpot responses for overlapping gE peptide pools in a cohort of healthy immune donors.

4.3.3. Determining the responding T cell subsets to immune dominant peptides

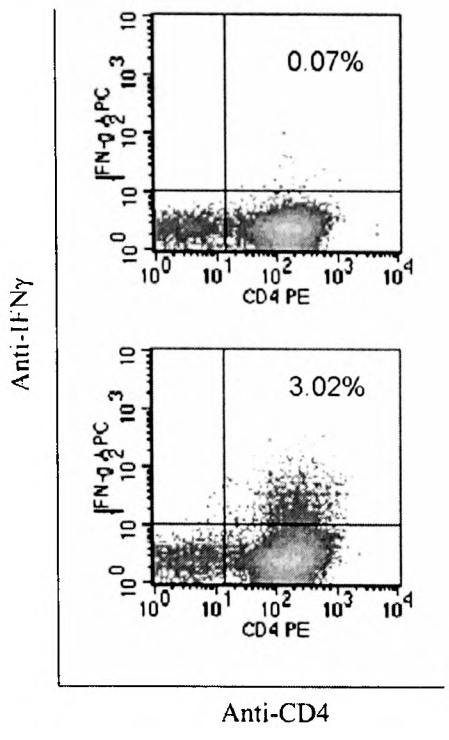
Intracellular cytokine staining (ICS) was used to investigate the responding T cell subsets and showed that responses to gE peptide pools were predominantly mediated by CD4+ T cells (Fig 4-6). I tested all immune dominant pools and found that responses to all immune dominant peptide pools and 20mer peptides were largely mediated by CD4+ T cells. My earlier results in chapter 3 shows that using the viral lysate or vaccine as stimulatory antigen, I observed a similar CD4+ T cell dominance of responding T cells both *ex vivo* and cultured (Jones et al., 2006). In these experiments as I considered that the use of the peptides and lysate might bias the assay towards stimulation of CD4+ T

cells. Therefore, I repeated the assay using live viral vaccine as stimulation. However, I found that as seen with gI, the live attenuated vaccine induces a gE specific cellular immune response which is predominantly mediated by CD4+ T cells. Overall these data show that it is possible to expand gE-specific T cells *in vitro* and that such responses are dominated by CD4+ T cells.



4-6 Predominance of CD4+ T cell responses to gE peptide pools

PBMCs cultured with the overlapping gE 20mer pools for 10 days and tested with overlapping gE pools of peptides. Cells are gated on CD3. The top panel shows the unstimulated control cells and in the lower panel the cells are stimulated with overlapping gE peptides.



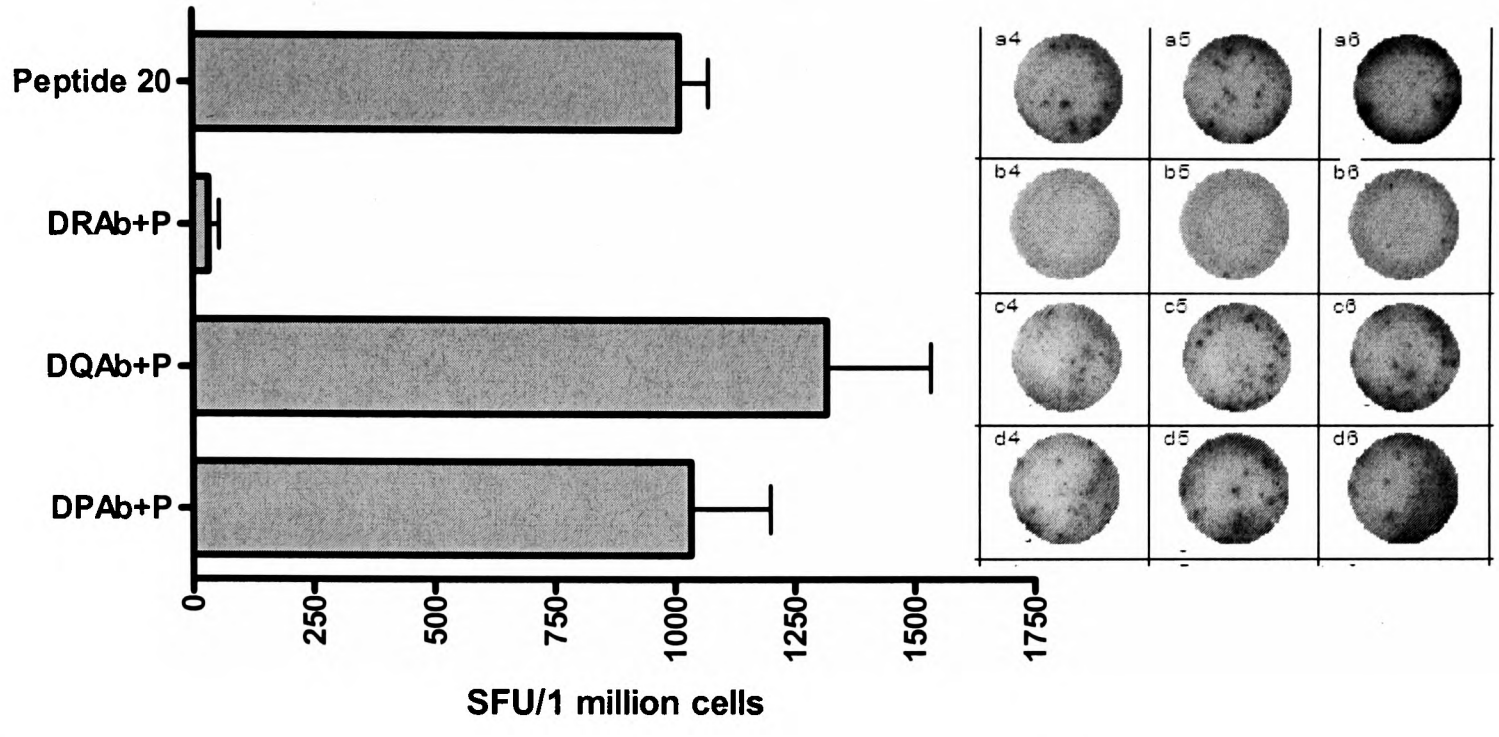
4-7 Predominance of CD4+ T cell responses to overlapping gE peptides

PBMCs cultured with the live VZV vaccine for 10 days and tested with overlapping gE pools of peptides. Cells are gated on CD3. The top panel shows the unstimulated control cells and in the lower panel the cells are stimulated with overlapping gE peptides

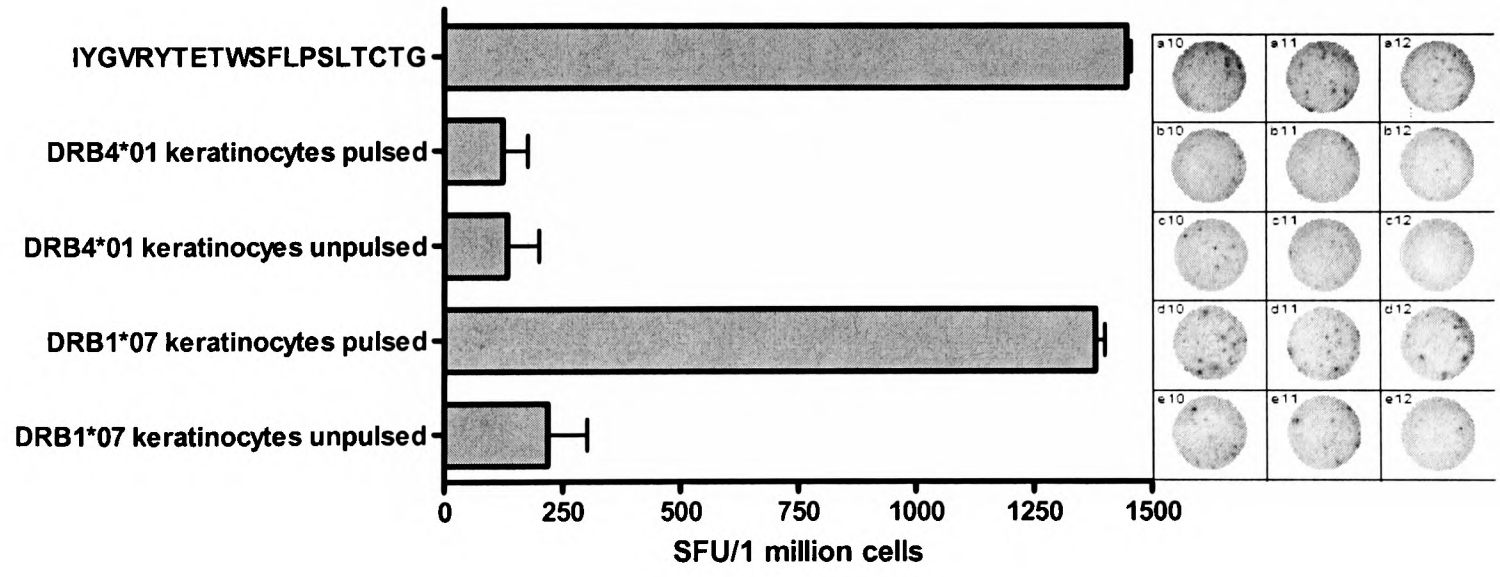
4.3.4. Detailed characterization of responses to gE peptide 20

As I found T cell responses to peptide 20 (gE pool 7) were predominantly from the CD4+ T cells I proceeded to determine the HLA restriction of this epitope. I initially used DR, DQ and DP blocking antibodies to determine which MHC class II molecules were involved in presentation of the epitope and showed that the DR molecules were involved in antigen presentation. Since these experiments were carried out in a donor whose MHC class II alleles were homozygous, the epitope was presented by either DRB1*07 or DRB4*01 molecules (Fig 4-8). To determine which of the DR molecules presented the epitope, keratinocyte lines (both matched and mismatched to the testing HLA molecule)

were used as antigen presenting cells to T cells cultured with the epitope. This showed that the epitope in peptide 20 was presented by the DRB1*07 molecule (Fig 4.9).



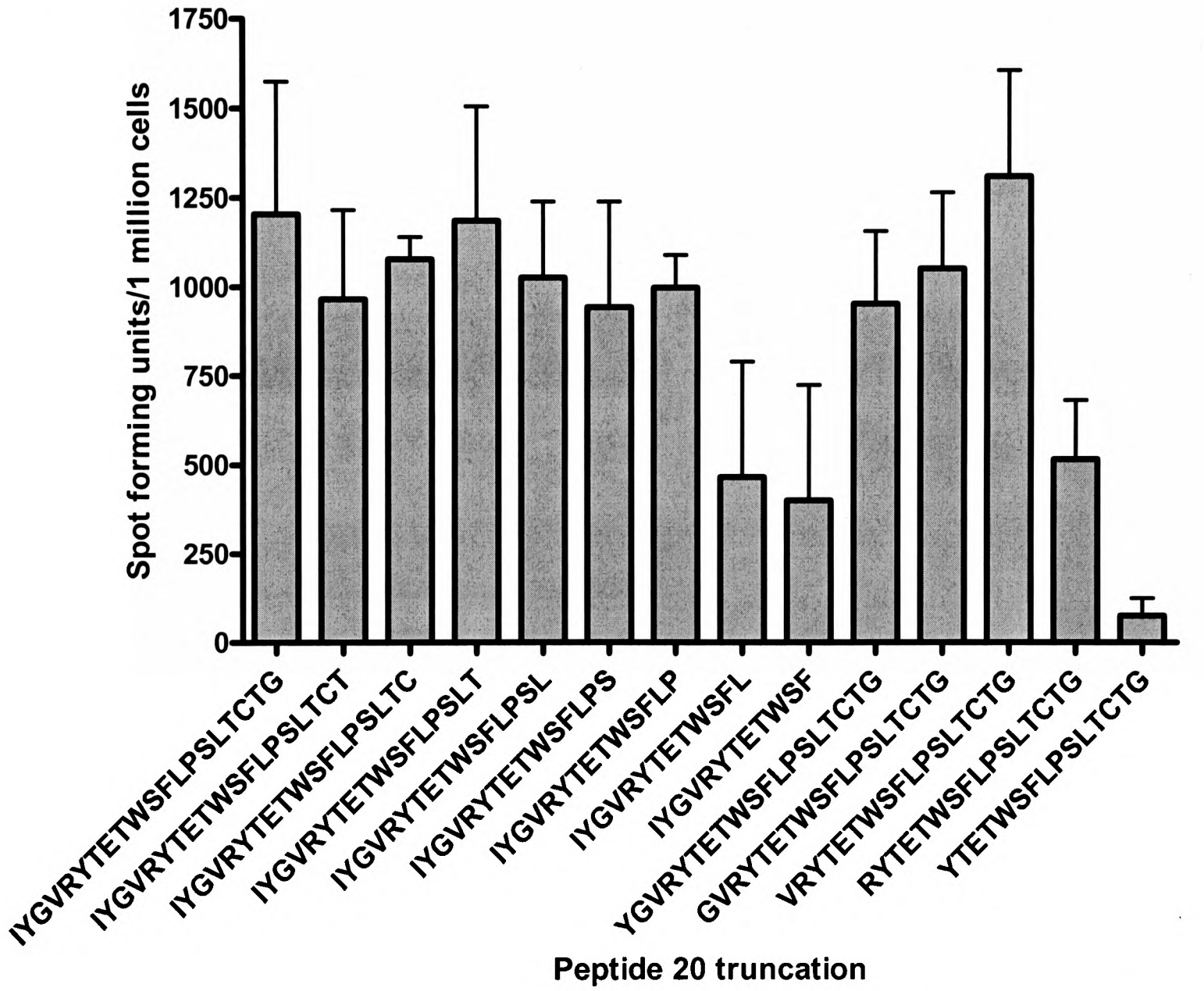
4-8 IFNγ ELISpot responses using peptide 20 specific line incubated with peptide, anti-DR+peptide, anti-DQ+peptide and anti DP+ peptide. Error bars represent SEM.



4-9 HLA restriction of peptide 20

IFNγ ELISpot responses using a peptide specific line incubated with peptide directly added to T cells or on DRB4*01 matched peptide-pulsed or -unpulsed and DR1*07 matched keratinoctes peptide-pulsed or –unpulsed. Error bars represent SEM.

To determine the minimum length of the epitope, T cells cultured with the 20mer peptide were tested by ELISpot assays using peptide truncations of the 20mer index peptide. By testing the individuals who responded to gE peptide 20, I found that the minimum peptide to be a 9mer (Fig.4-10). I further proceeded to characterize the optimum epitope by determining the peptide truncations responding at lower peptide titrations. By this method I found that the optimum epitope was a 15 mer GVRYTETWSFLPSLTC (Data not shown).

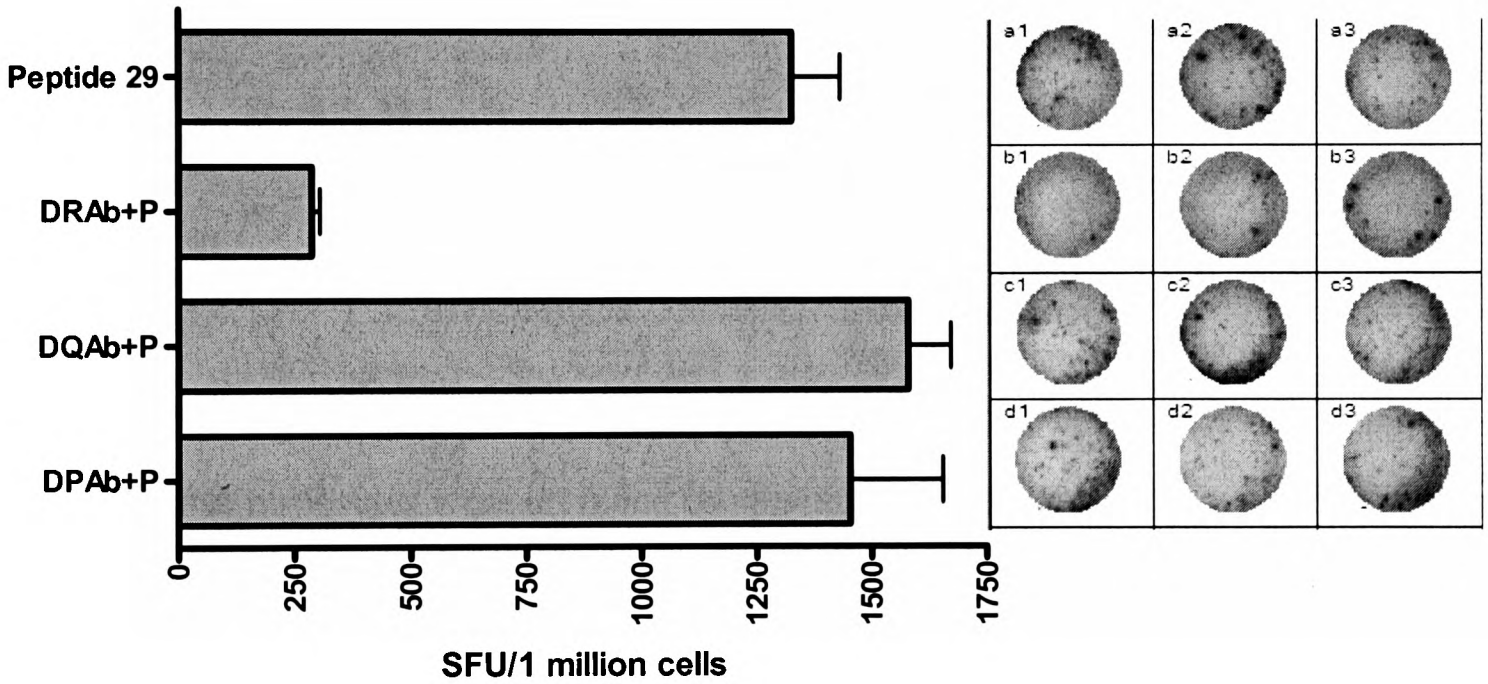


4-10 IFNγ ELISpot responses to truncated peptides from the 20mer peptide 20 of glycoprotein E

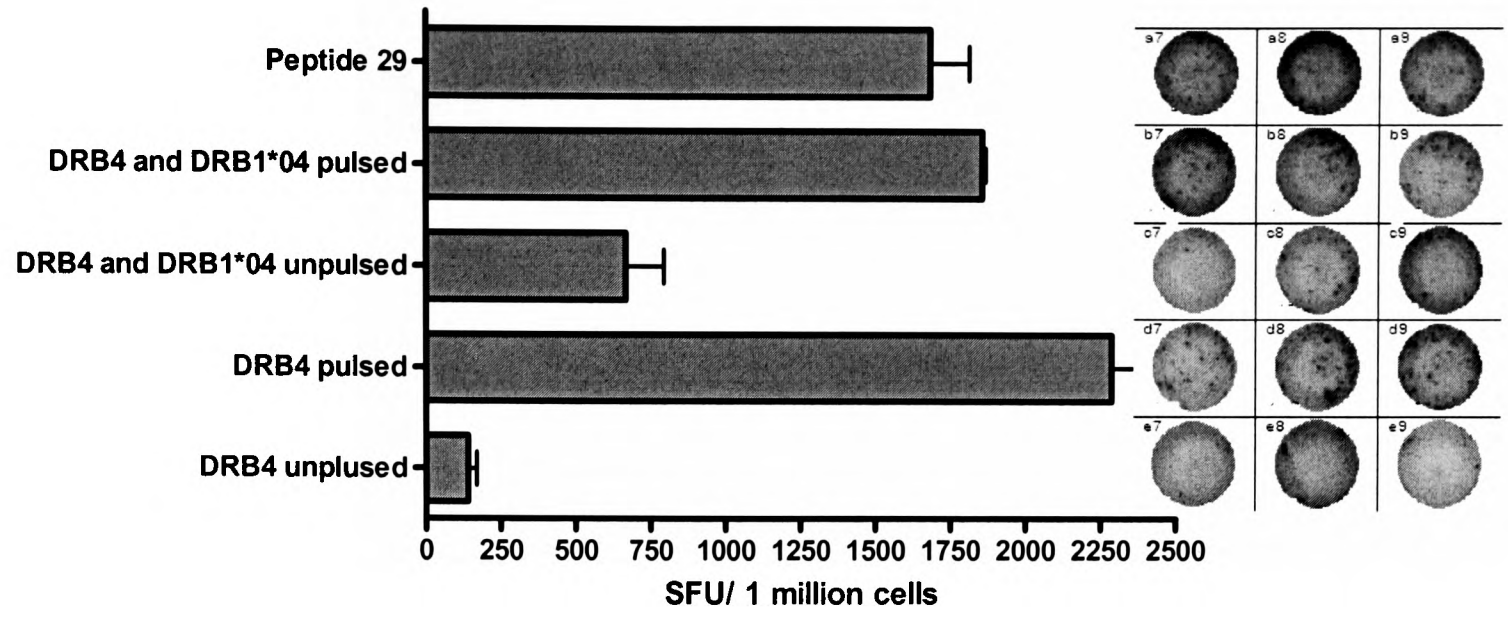
Ex vivo ELISpot responses to this epitope was found to be 65 to 925 (mean 292, SD±356.6) SFU/million cells among healthy seropositive donors. Error bars represent SEM.

4.3.5. Detailed characterization of responses to gE peptide 29

T cell responses to gE peptide 29 (pool 10) were also found to be predominantly mediated by CD4+ T cells (Fig.4-11). Again by using DR, DQ and DP blocking antibodies to determine which MHC class II molecules were involved in presentation of the epitope I found that DR molecules were involved in antigen presentation. To determine which of the DR molecules presented the epitope, EBV transformed lymphoblastoid B cell lines both matched and mismatched for the presenting HLA molecule were used. By this method I found that the epitope was presented by HLA DRB4*01 molecules (Fig.4-12). As further truncation of the 20mer peptide did not yield an enhanced response, I used the 20mer in subsequent experiments.



4-11 IFNγ ELISpot responses using peptide 29 specific line incubated with peptide, anti-DR+peptide, anti-DQ+peptide and anti DP+ peptide
Error bars represent SEM.

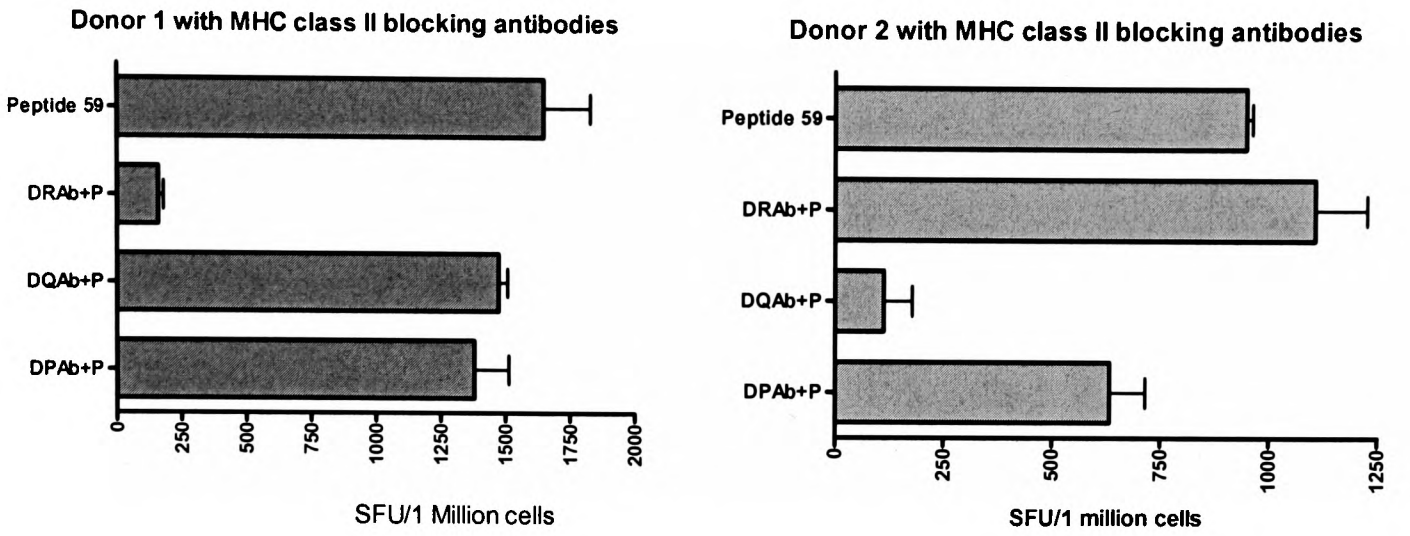


4-12 HLA restriction of peptide 29

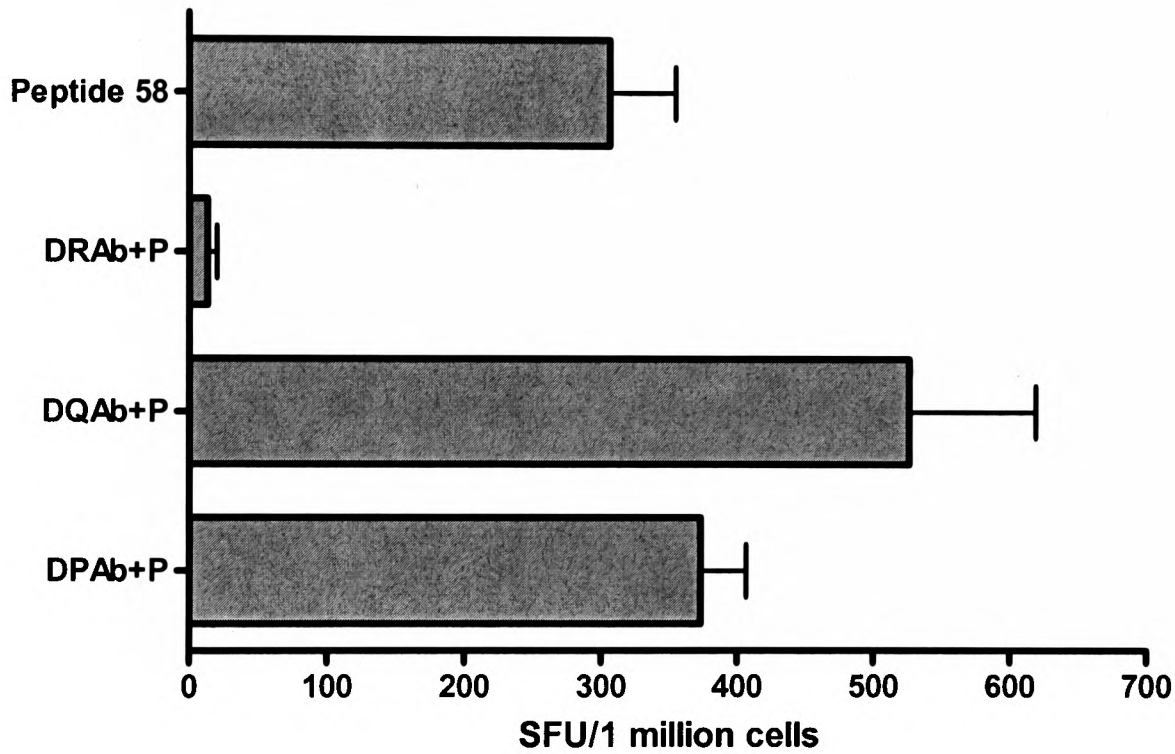
IFNγ ELISpot responses using a peptide specific line incubated with peptide directly added to T cells or on DRB4*01 and DRB1*04 matched peptide-pulsed or -unpulsed and DRB*01 only matched keratinoctes peptide-pulsed or unpulsed. Error bars represent SEM.

4.3.6. Detailed characterization of responses to gE peptide 58 and 59

T cell responses to gE peptide 58 and 59 (pool 20) were also found to be predominantly mediated by CD4+ T cells. However, individuals who responded to peptide 58 and 59 were of different HLA types. By using MHC class II DR and DQ blocking antibodies I found that DR molecules were involved in presenting peptide 59 in some individuals and DQ molecules to be presenting the antigen in some (Fig. 4-13) suggesting that there were two epitopes within the 20mer peptide. I also found that responses to peptide 58 which overlapped peptide 59 by 10 amino acids was blocked by MHC class II DR antibodies (Fig.4-14).



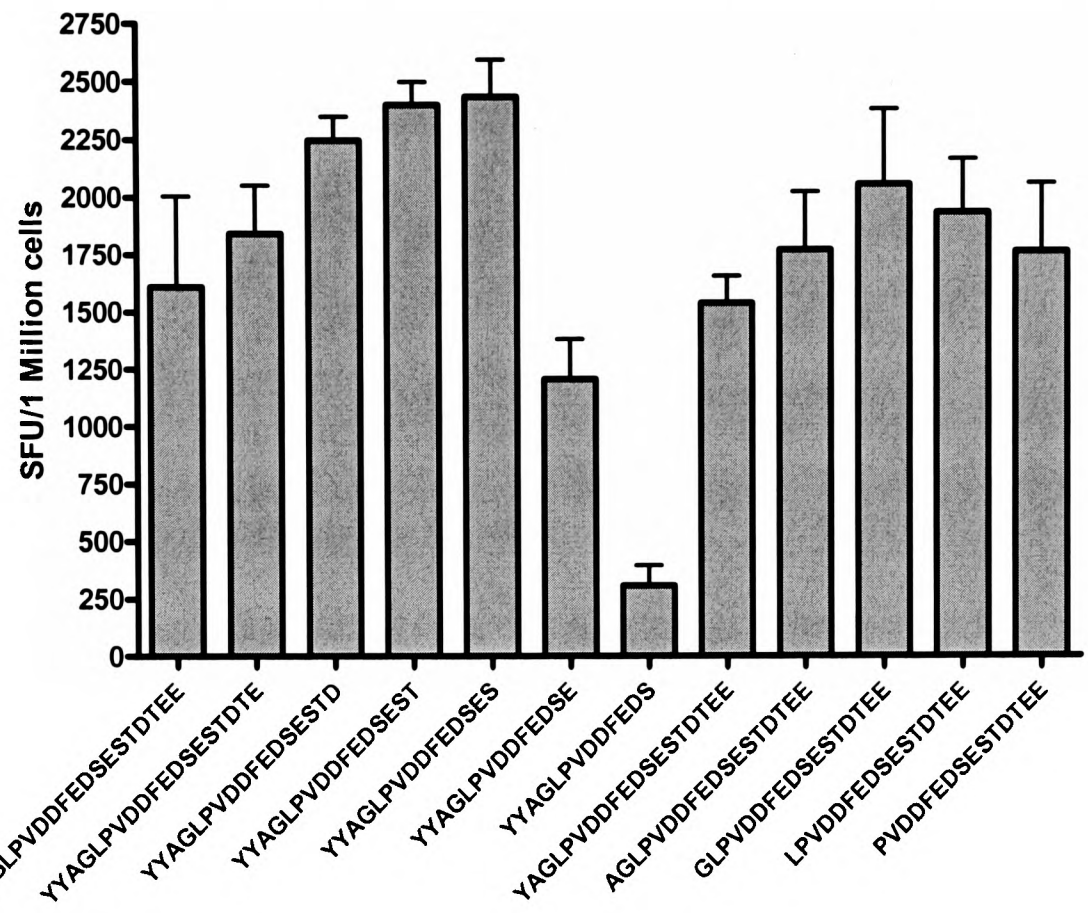
4-13 IFNγ ELISpot responses using a peptide 59 specific line incubated with peptide, anti-DR+peptide, anti-DQ+peptide and anti DP+ peptide. Error bars represent SEM.



4-14 IFNγ ELISpot responses using a peptide 58 specific line incubated with peptide, anti-DR+peptide, anti-DQ+peptide and anti DP+ peptide. Error bars represent SEM.

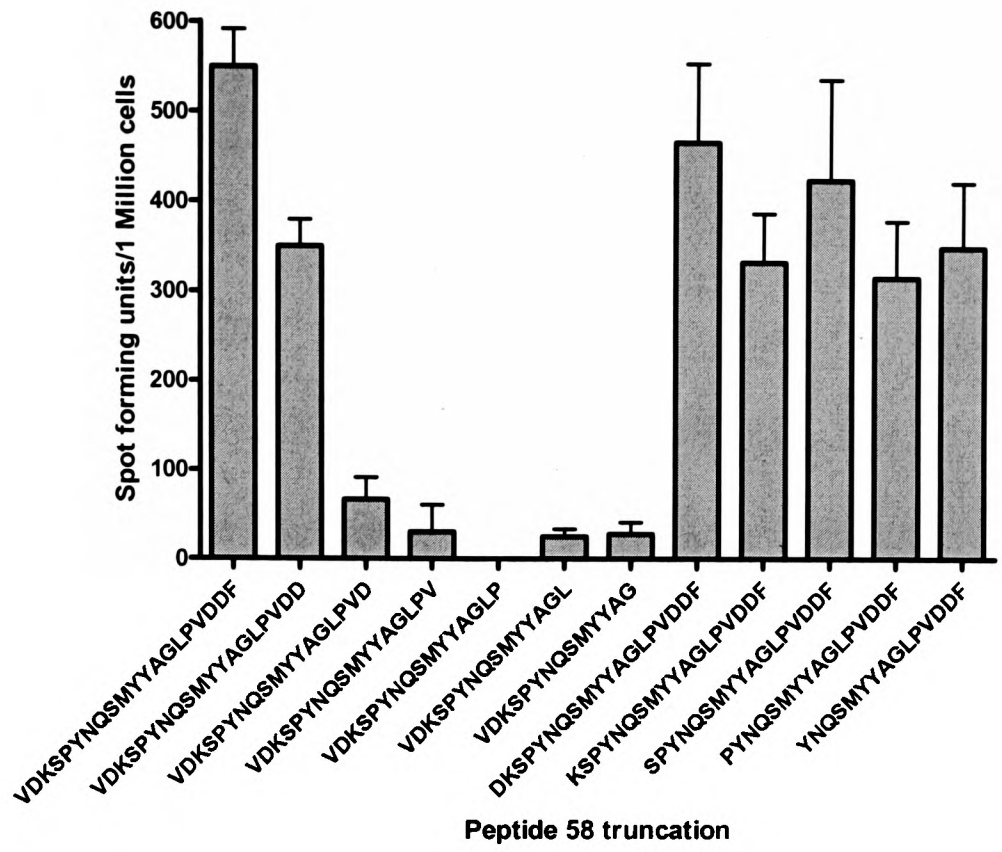
I then proceeded to determine the minimum length of the epitope and the optimum epitope as descried of these 2 peptides. T cells cultured with the 20mer peptide were tested by ELISpot assays using peptide truncations of the 20mer index peptide. By testing the individuals who responded to gE peptide 59, we found the minimum peptide to be a

10mer (Fig.4-15) (LPVDDFEDSE) and the optimum epitope to be a 15mer (GLPVDDFEDSESTDT) (Data not shown). *Ex vivo* responses to this epitope were between 0 to 100 (mean 28 ±30.12) SFU/1 million cells. In addition, 0.0% to 0.02% of the CD4+ T cells in healthy seropositive donors responded to this epitope *ex vivo* by ICS.



4-15 IFNγ ELISpot responses to truncated peptides from the 20mer peptide 59 of glycoprotein E.
Error bars represent SEM.

By testing the individuals who responded to gE peptide 58, we found that the minimum peptide to be a 12 mer (SMYYAGLPVDDF) (Fig.4-16). Therefore, I found that 14 (70%) of our healthy immune donors recognized the region YNQSMYYAGLPVDDFEDSESTDT. However, we could not determine the HLA restriction of the epitopes within these 2 20mer peptides, possibly due to the presence of 2 or 3 epitopes within the region spanned by 30 amino acids.



4-16 IFNγ ELISpot responses to truncated peptides from the 20mer peptide 58 of glycoprotein E. Error bars represent SEM.

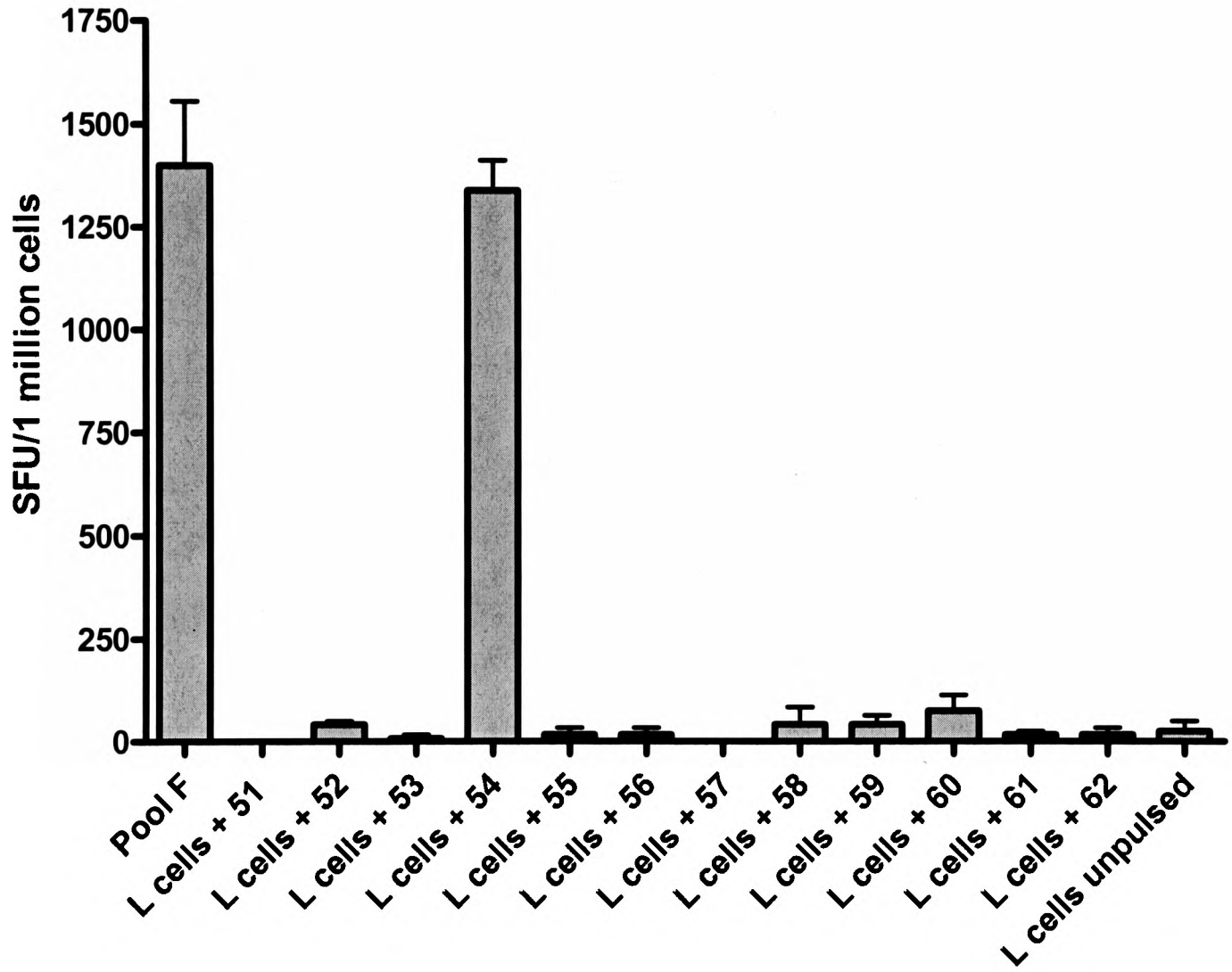
4.3.7. Identification of DRB1*15 epitopes within gE

The availability of tetramer technology has revolutionized the investigation of the frequency and phenotype of virus specific T cell responses. Although, I was able to identify many epitopes within gE and gI as described above, class II tetrameric complexes are currently only synthesized for certain class II molecules only, which do not include the epitopes above. Therefore, in order to investigate the phenotype of gE specific T cells in healthy immune donors and in patients with acute primary infection I set to identify a DRB1*1501 specific epitope within gE.

PBMCs were cultured with pools of gE 20mer peptide (10 20mer peptides in each pool named A to F) and then tested for IFNγ production using peptide-pulsed DRB1*1501 expressing transfected L cells for antigen presentation (we are grateful for the gift for

Prof. Lars Fugger). By this approach I identified two potential DRB1*1501 specific epitopes in pool E and pool F (Data not shown).

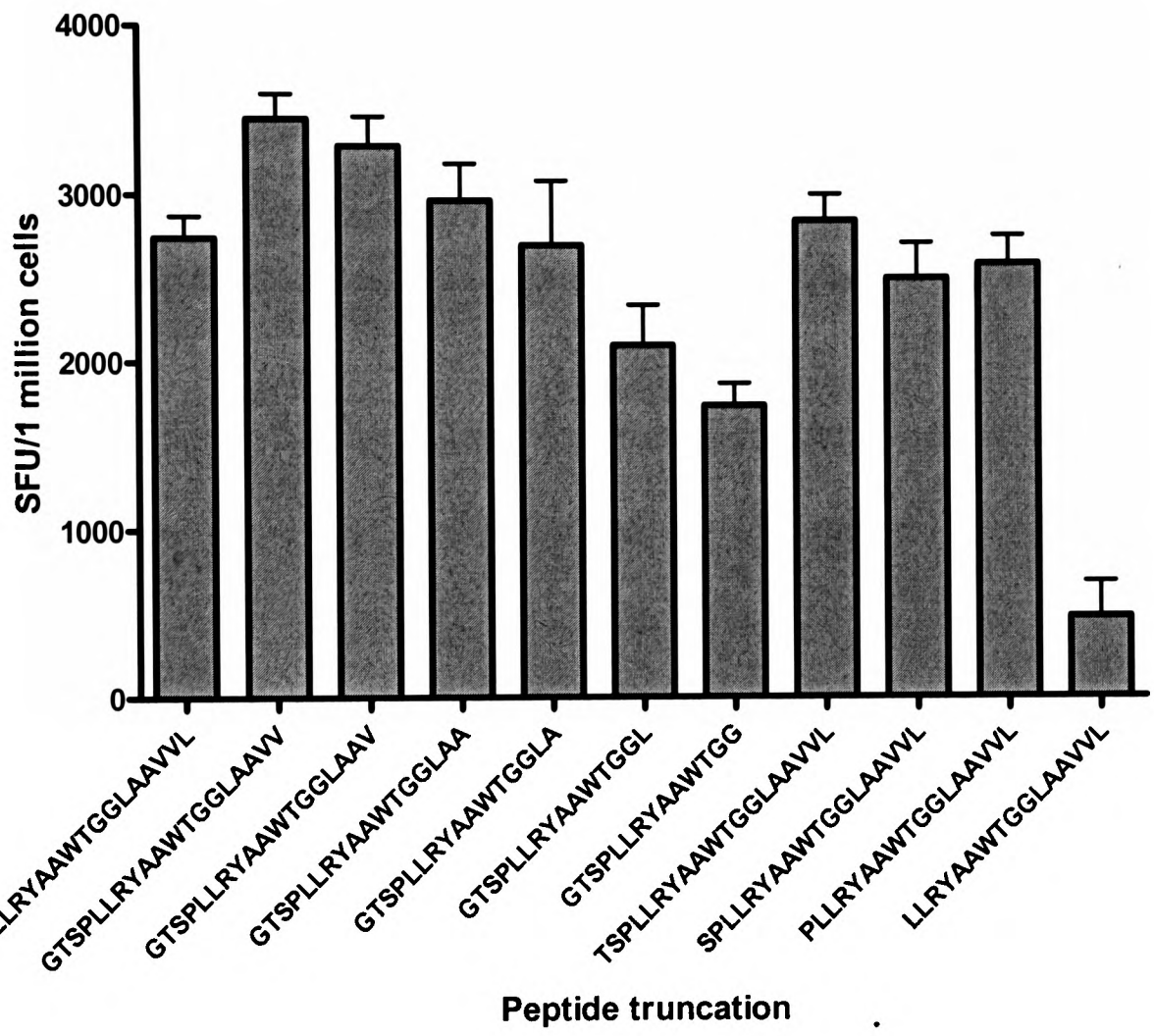
I then proceeded to investigate pool F in greater detail as the majority of *ex vivo* responses in healthy immune donors were directed to this pool. The L cells were pulsed with the 20mer peptides in pool F (peptide 51 to 62) and were used as antigen presenting cells to a pool F specific T cell line. Using this approach I identified that peptide 54 gave a positive responses with the L cells (Fig.4-17).



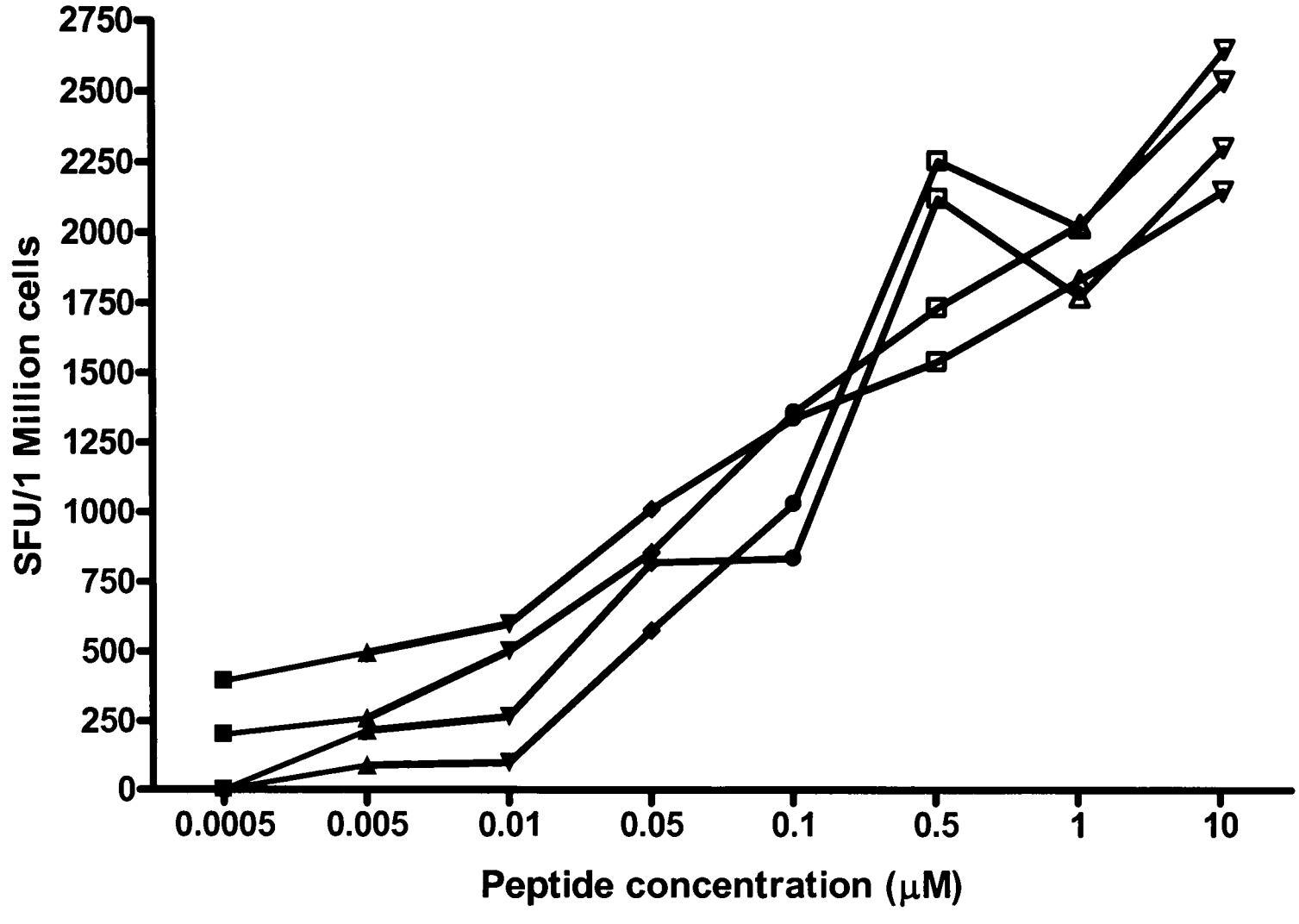
4-17 Identifying DR1*1501 epitopes within gE

IFNγ ELISpot responses using gE pool F specific line, incubated with pool F peptide directly added to T cells and DRB1*1501 expressing L cells pulsed with each of the 20 mer peptides in pool F and used as antigen presenting cells to the T cell line. Error bars represent SEM.

To determine the minimum length of the epitope, T cells cultured with the 20mer peptide were tested by ELISpot assays using peptide truncations of the 20mer index peptide. I found the minimum peptide to be a 12 mer (Fig.4-18) the optimum peptide to be a 15mer (TSPLLRYAAWTGGLA) (Data not shown). Peptide specific IFNγ responses were seen even at peptide concentrations of 0.0005μM (Fig. 4-19).



4-18 IFNγ ELISpot responses to truncated peptides from the 20mer peptide 54 of glycoprotein E
Error bars represent SEM.



4-19 IFNγ ELISpot responses of T cell lines from 4 different individuals to the optimum peptide 54.2.16

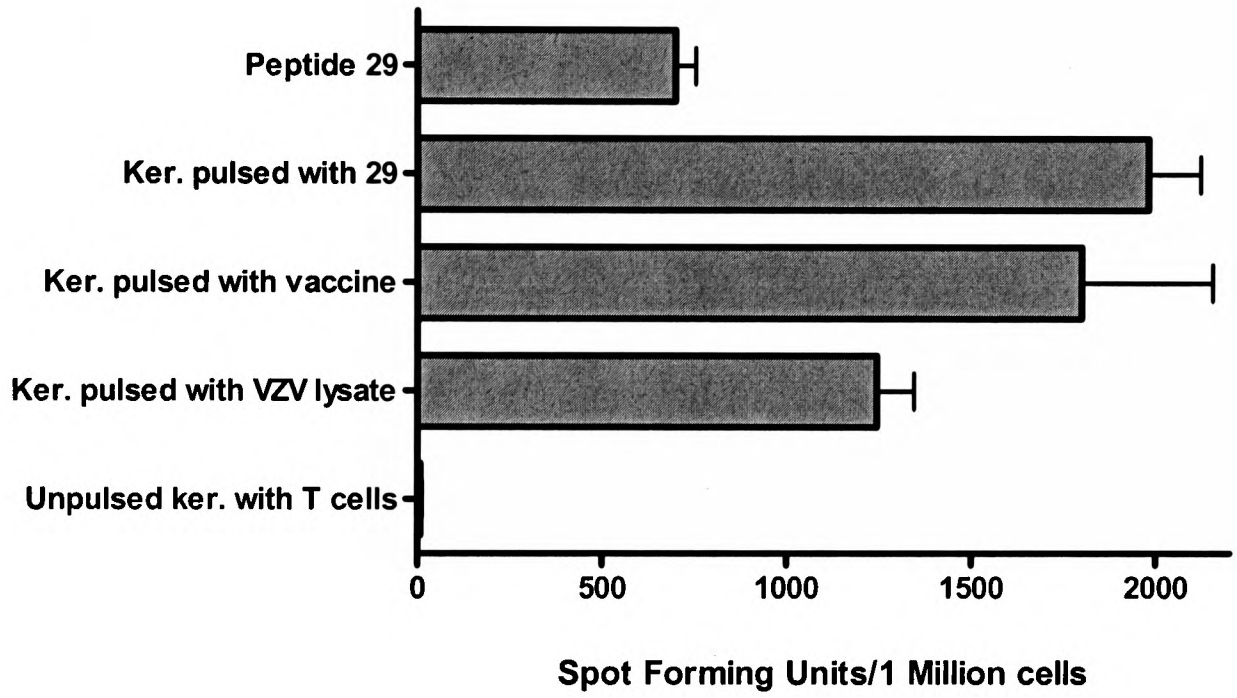
A list of the identified epitopes within gE and their HLA restriction is given in the table below.

Epitope	**HLA restriction**
GVRYTETWSFLPSLTC	DRB1*07
TSPLLRYAAWTGGLA	DRB1*1501
IEPGVLKVLRTEKQYLGVYI	DRB4*01
GLPVDDFEDSESTDT	DRB5? DRB1*1501? DQ5?
SPYNQSMYYAGLPVDDF	DRB4*01? DRB1*07? DRB1*04?

Table 3: A list of epitopes identified within VZV glycoprotein E.

4.3.8. Presentation of gE epitopes by keratinocytes

Keratinocyes are major targets for VZV replication (Taylor and Moffat, 2005). Therefore, we proceeded to investigate if the gE epitopes were naturally processed by keratinocytes and presented to T cells. Following pulsing of DRB4*01 restricted keratinocytes with the VZV vaccine or VZV lysate pulsed we used them as antigen presenting cells to T cell lines specific to gE DRB4*01 peptide 29 (IEPGVLKVLRTEKQYLGVYI). I found that after IFNγ incubation the keratinocyes were very efficient in presenting the DRB4*01 epitope to epitope specific T cell lines which suggests that this epitope was processed naturally by keratinocytes (Fig 4-20).

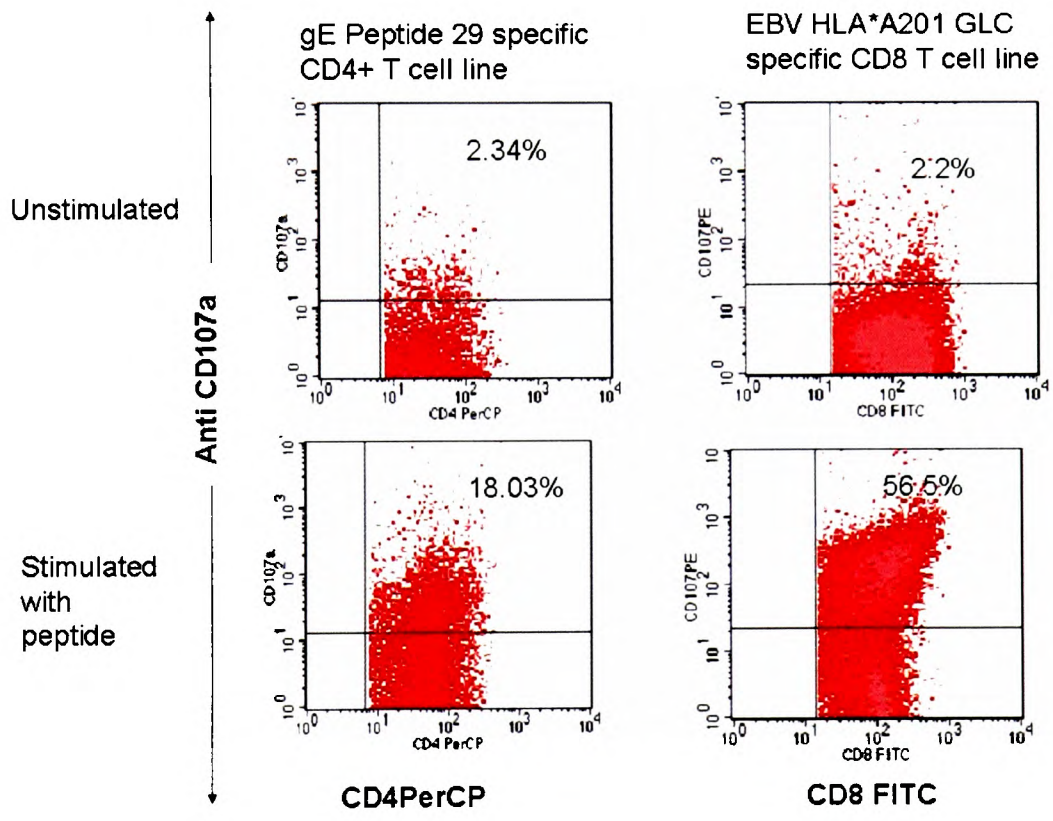


4-20 Presentation of epitopes within gE by keratinocytes

IFNγ ELISpot responses using a peptide 29 specific line incubated with peptide directly added to T cells or on DRB4*01 matched peptide-pulsed, live VZV vaccine pulsed, VZV lysate pulsed and unpulsed keratinocytes. Error bars represent SEM.

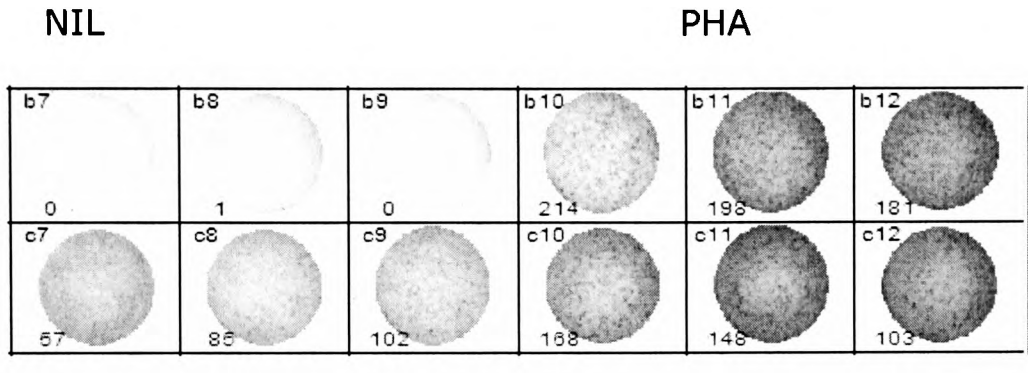
4.3.9. CD107a and perforin expression of epitope specific T cells

CD107a (lysosomal-associated membrane protein-1) is a vesicle membrane protein that becomes transiently mobilized to the cell surface during degranulation which involves the release of cytolytic mediators such as perforin and granzyme. The ability of CD8+ T cells to degranulate is thought to directly correlate with their cytolytic ability (Rubio et al., 2003). Therefore, I proceeded to investigate if the identified CD4+ T cell epitope specific T cell lines expressed CD107a. When stimulated with the relevant peptide, all characterized epitope specific short term T cell lines expressed CD107a to varying levels. However, expression of CD107a was far less than that expressed by a CD8+ Epstein Barr virus HLA-A*02 GLC peptide specific T cell line (Fig.4-21).

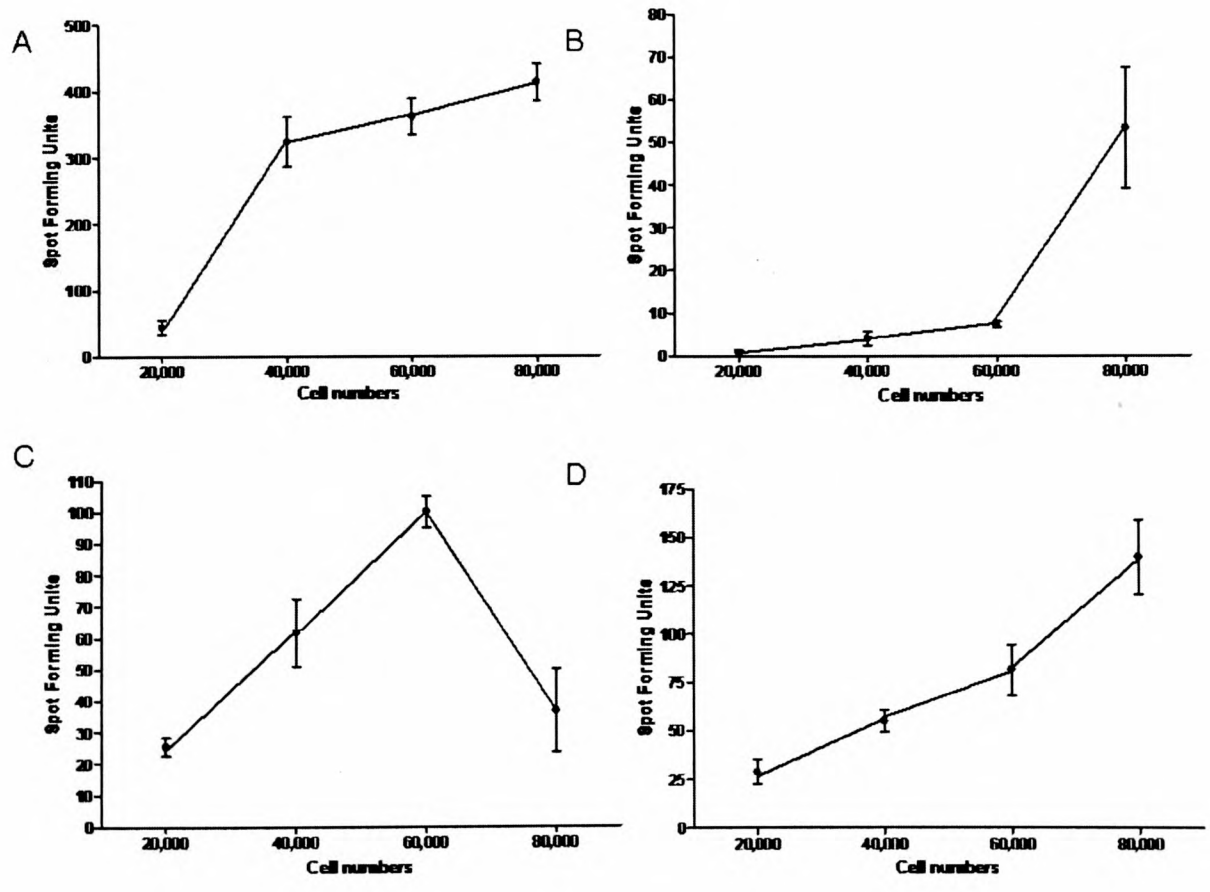


4-21 CD107a responses for gE peptide 29 and EBV GLC peptide
PBMCs cultured with the overlapping gE peptide 29 (left) and EBV GLC peptide (right) for 10 days and tested with the relevant peptide. Cells are gated on CD4 (left) and CD8 (right). The top panel shows the unstimulated control cells and in the lower panel the cells are stimulated with peptides.

As it is thought that CD107a expression correlates with the cytolytic ability of cells, by using cultured and *ex vivo* perforin ELISpot assays, I proceeded to investigate perforin expression by CD4+ epitope specific short term T cell lines. I found that the CD4+ epitope specific T cell lines express perforin although to a lesser degree than those produced by CD8+ Epstein Barr virus HLA-A*02 GLC peptide specific T cell lines (Fig. 4.23).



4-22 Perforin ELISpot responses to gE Peptide 59 specific T cell line stimulated with the peptide



4-23 Perforin ELISpot responses of short term T cell lines specific EBV GLC specific T cell line (A), peptide 20 (B), peptide 29 (C), peptide 59 (D) Error bars represent SEM.

4.4. Discussion

These results show that CD4+ T cells specific for gE peptides circulate at high frequencies in healthy donors with a history of previous infection. These findings are compatible with the fact that gE is the most abundant viral protein. Neutralizing antibodies to gE have been shown to be predominant among neutralizing antibodies directed to VZV viral antigens and also gE elicits the highest levels of antibody responses in individuals following natural infection and immunization (Haumont et al., 1997). In addition, gE specific T cell responses were higher than responses to gI. Therefore, perhaps it is not surprising that gE is also an immune dominant T cell target.

Glycoprotein E specific T cell responses in our donors were predominantly mediated by $CD4^+$ T cells. This was consistent with our previous experiments that glycoprotein gI and IE4-specific T cell responses were also predominantly mediated by CD4+ T cells (Jones et al., 2006; Malavige et al., 2007) and fits with other findings for VZV (Asanuma et al., 2000). Although, we expected some CD8+ T cell responses to this protein, our findings are also compatible with others who have shown that T cell immune responses directed at glycoproteins derived from other viruses are predominantly from CD4+ T cells (BenMohamed et al., 2003; Elkington et al., 2004). The importance of CD4+ T cells in herpes virus infections has been discussed in the previous chapter. As gE specific T cell responses appear to be predominantly mediated by CD4+ T cells in healthy immune donors, it would now be important to investigate responses to this protein along with VZV proteins expressed during latency (IE62, IE63 and ORF4) in patients with acute

infection and reactivation to see the kinetics and dynamics of T cell responses to these proteins.

It was discussed in the previous chapter the different pathways of MHC class II antigen presentation and how endogenous viral proteins may enter this process by a process known as autophagy. It has been shown that in both EBV and CMV infection structural proteins are delivered to the endosomal compartment where class II antigen presentation occurs. This has been shown to occur with the Epstein Barr virus nuclear antigen 1 which is a dominant CD4+ T cell target (Paludan et al., 2005) and also in CMV infection (Hegde et al., 2005). Therefore, it is likely that a similar mechanism occurs along with autophagy in the processing of VZV glycoproteins which would explain the predominance of VZV gE and gI specific CD4+ response.

4.4.1. Immune dominant epitopes within gE

I have characterized several epitopes within gE. Of these DRB1*07 (GVRYTETWSFLPSL) and DRB4*01 epitope (IEPGVLKVLRTEKQYLGVYI) were found to be recognized by 40% of healthy immune individuals. The levels of epitope specific IFNγ responses seen here were comparable to the magnitude of CD4+ T cell responses seen during other persistent viral infections (Godkin et al., 2001; Leen et al., 2001). However, the most immune dominant region within gE (YNQSMYYAGLPVDDFEDSESTDT) was recognized by 70% of our donors. This region which is in the C terminal of the gE protein contains the sequence YAGL which is essential for replication of the virus and mediates endocytosis of gE from the plasma

membrane (Moffat et al., 2004). Therefore, perhaps it is not surprising that it this region contains 2 or 3 CD4+ T cell epitopes. As MHC class II tetrameric complexes are available for DRB1*1501 restricted epitopes, the frequency and phenotype of the epitope TSPLLRYAAWTGGLA will be described in detail in the next chapter.

B cell epitopes within the gE protein have previously been identified (Fowler et al., 1995), the major B cell epitope is located between amino acids 151 to 161 of the gE protein (Hatfield et al., 1997). However, we did not observe any responses *ex vivo* or under cultured conditions to this segment of the gE protein.

4.4.2. Cytolytic properties of gE epitope specific CD4+ T cells

Although less efficient than CD8+ T cells, viral specific CD4+ T cells especially in chronic viral infections such as EBV, CMV and HIV have been shown to possess cytolytic abilities and lyse target cells (Appay et al., 2002b; Landais et al., 2004). CMV specific CD4+ T cells have been shown to express CD107a and contain granzyme A and B and perforin as they mature. In addition, direct *ex vivo* killing of CMV epitope specific target cells has been demonstrated (Casazza et al., 2006). The CD4+ T cells that have such a cytotoxic potential are thought to be in a late differentiation stage (Appay et al., 2002b). Although I could not characterize epitope specific perforin secretion or degranulation in *ex vivo* due to the relatively lower numbers of VZV specific T cells when compared to those of CMV (Casazza et al., 2006), EBV and HIV (Appay et al., 2002b), this was seen in epitope specific T cells in short term T cell cultures. In fact, all epitope specific short term T cell lines produced perforin when stimulated with the

relevant antigen. In addition, they also degranulated in response to antigens. By using the Chromium^{51} release assay, VZV IE63 specific short term T cells lines have been shown to be capable of killing peptide pulsed keratinocytes (Louise Jones unpublished data). As, it could be possible that the phenotype of these epitope specific T cells could have changed during *in vitro* conditions, it would be now important to investigate the possible cytotoxic ability of VZV gE specific cells and possible mechanisms of cytotoxicity by cloning epitope specific T cells.

4.4.3. Future directions

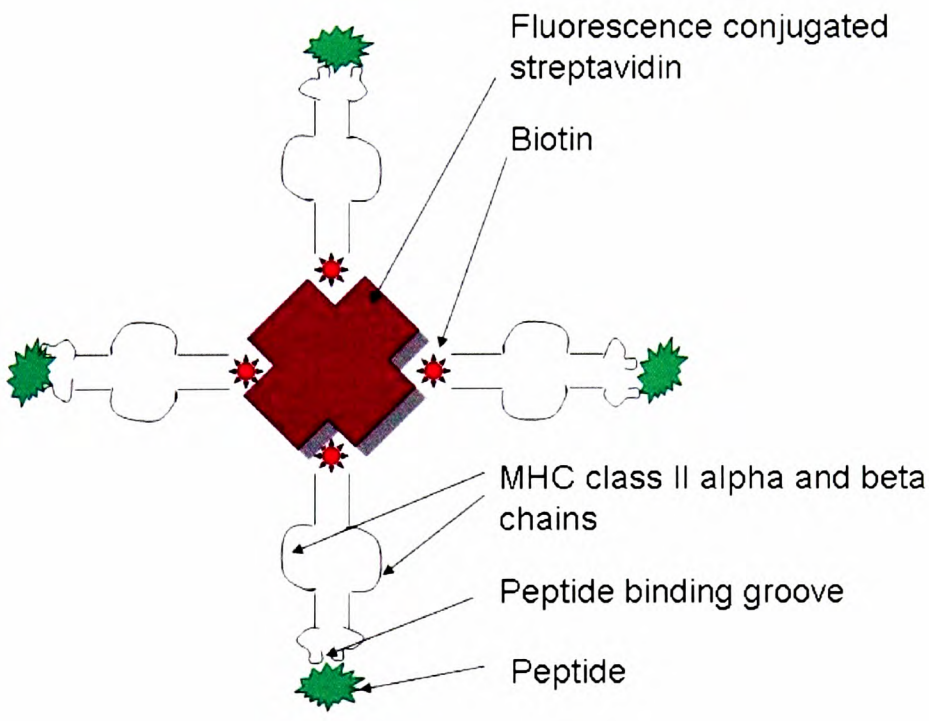
VZV infections can be largely prevented by the highly effective live attenuated varicella zoster virus vaccine. However, although rare, it has caused serious illness and even death in some immune suppressed individuals (Ghaffar et al., 2000; Kraft and Shaw, 2006; Kramer et al., 2001; Levy et al., 2003). Furthermore, the vaccine virus establishes latency and may later reactivate causing severe disease in some individuals (Kramer et al., 2001). Therefore, it will be important to continue the development of new alternative vaccination strategies for use in such individuals who are arguably amongst those with most to gain from protection. The epitope IEPGVLKVLRTEKQYLGVYI which the majority of donors responded to was found to be restricted through HLA-DRB4*01 which has a frequency of 42% - 46.5% in different populations (Machulla et al., 2001; Oguz et al., 2003; Rihs et al., 2002). In addition, the region YNQSMYYAGLPVDDFEDSESTDT within gE was also recognized by 70% of the individuals. Therefore, these epitopes will be of possible use in the future development of safer subunit or recombinant vaccines.

Chapter 5. Analysis of glycoprotein E DRB1*1501 specific CD4+ T cell responses in healthy immune donors

5.1. Introduction

The use of MHC class I tetramers in the tracking anti viral CD8+ T cell responses have significantly contributed to our understanding of the role of CD8+ T cell in acute and persistent viral infection. However, due to the lower frequency of virus specific CD4+ T cells and also due to the relative difficulty in producing MHC Class II tetramers, such data regarding virus specific CD4+ T cells are more limited. Phenotypic analysis of epitope specific T cell responses using intra cellular cytokine assays is also limited as certain phenotypic markers are altered upon T cell stimulation. However, recent developments in MHC Class II tetramer technology have enabled detection, phenotypic and functional analysis of virus specific CD4+ T cells.

5.1.1. MHC Class II tetrameric complexes



5-1 The structure of a MHC class II tetrameric complex

MHC class I tetramers were first developed in 1996 by Altman *et al (*Altman et al., 1996*)* to overcome the limitations in the detection of antigen specific T cells by functional assays such as limited dilutional assays, intracellular cytokine staining and ELISpot assays; identification which depend on proliferation or cytokine secretion by antigen specific T cells. As the tetrameric MHC-peptide complexes are able to bind to more than one T cell receptor at a given time, they are thought have a slower dissociation rate than monomers and therefore, bind more strongly and for longer periods (Altman et al., 1996). In addition, as they do not rely on functional capability of T cells, they are also not restricted to the measurement of one or more of the functional activities (van Baarle et al., 2001). MHC class II tetrameric complexes came into use several years later after class I tetramers (Crawford et al., 1998) largely due to technical challenges with synthesis.

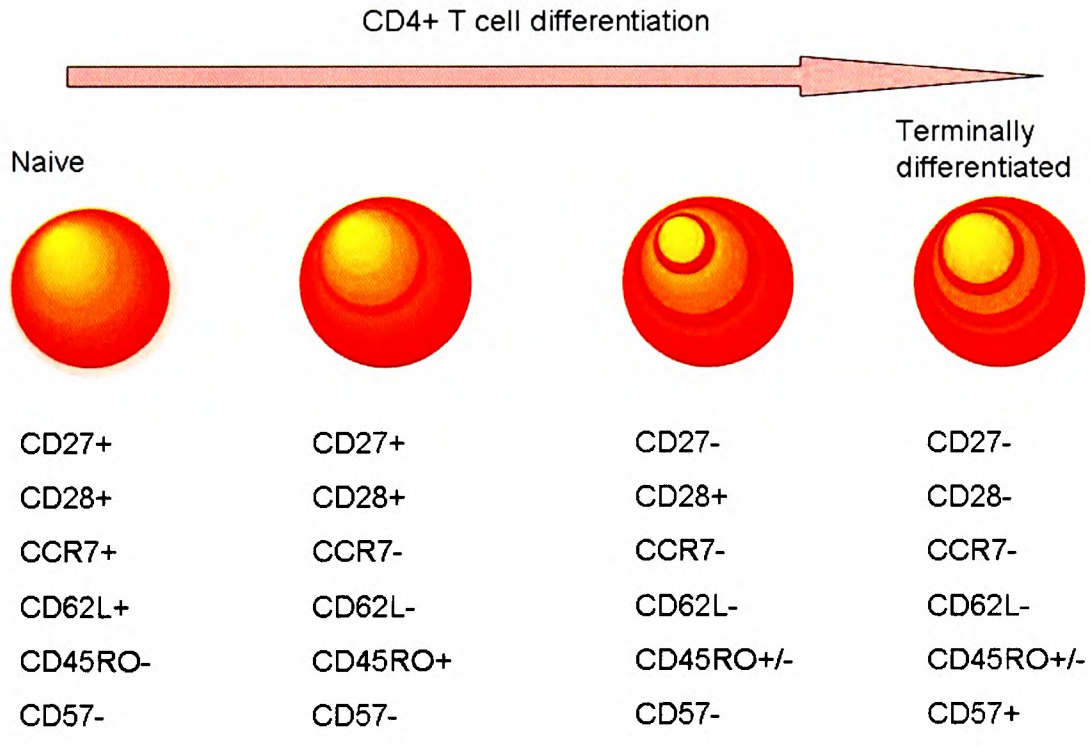
5.1.2. Differentiation and functional characteristics of virus specific CD4+ T cells

Expression of different co stimulatory molecules on virus specific T cells is thought to correlate with differentiation and functional characteristics of these cells (Appay et al., 2002a). Therefore, the expression of co stimulatory molecules (CD27 and CD28), lymph node homing markers (CD62L and CCR7), memory markers (CD45RO) and cytolytic ability (expression of perforin) of virus specific T cells have been used to characterize different stages of differentiation. The table below describes in detail the function and the binding ligands of each of these molecules.

	Expression	Binding ligand
CD27	Member of TNF super family. Expressed on CD4+ and CD8+ T cells, B cells and NK cells. Interact with CD70 expressing cells, which in turn activate T cells.	CD70 (CD27L)
CD28	Constitutively expressed on most T cells and plasma cells. Activation of T cells leads to enhanced expression. Binds to CD80 and CD86 and provides co stimulation.	CD80 and CD86
CCR7	Lost rapidly upon antigenic stimulation(Sallusto et al., 2004). Essential for lymphocytes to traverse high endothelial venules (HEV).	chemokine (C-C motif) ligand 19 (CCL19/ECL) and CCL21
CD62L (L-selectin)	Expressed on naïve T and B cells, some mature T cells and NK cells. It is rapidly lost upon activation. Important of homing of T cells to lymph nodes and payers patches (by traversing through HEV.	MAdCAM-1, Glycam-1
CD45RO	Once naïve T cells encounter antigen the CD45RA isoform is changed to the CD45RO isoform. Essential for normal signaling through the TCR and also regulate signaling through the cytokine receptors(Tchilian and Beverley, 2006).	

Table 4: Expression, function and the ligands of memory and homing markers expressed on T cells

Memory T cells are broadly classified into effector memory and central memory phenotype based on the expression of memory T cell markers (CD45RO) and lymph node homing markers (CD62L and CCR7). Effector memory T cells (CD45RO-) are capable of rapid effector function (cytokine secretion and cytolytic capablities) but poor proliferative capacity and express homing molecules which direct them to inflamed sites. In contrast, central memory T cells (CD45RO+) express lymph node homing markers and proliferate efficiently upon encounter with the specific antigen (Sallusto et al., 2004). Therefore, based on the expression of the above memory and lymph node homing markers, and co-stimulatory molecules (CD27 and CD28), virus specific T cells have been classified as early, intermediate, late and in the terminal stage of differentiation(Appay and Rowland-Jones, 2004; Harari et al., 2006).



5-2 Differentiation of anti viral CD4+ T cells in chronic viral infections (Appay, 2004; Harari et al., 2006)

Virus specific CD4+ T cells in chronic viral infections have shown to be of varying phenotypes and thus at different stages of differentiation. CMV specific CD4+ T cells are predominantly of the terminally differentiated phenotype, while HIV specific CD4+ T cells are of early intermediate stage of differentiation (Yue et al., 2004). The differentiation stage of VZV IE63 specific T cells was similar to those of EBV specific CD4+ T cells also showing an early intermediate stage of differentiation (Jones, 2007). It is believed that the antiviral potential (cytokine secretion ability and cytotoxicity) of T cells varies according to the stage of differentiation (Appay, 2004). Therefore, it is possible that the differentiation stage of a particular virus specific CD4+ T cell is best suited to control that particular virus. On the other hand, it is possible that viruses such as HIV and EBV which persist within T cells and B cells, influence the expression of the phenotypic markers of virus specific CD4+ T cells as an immune evasion strategy (Yue et al., 2004).

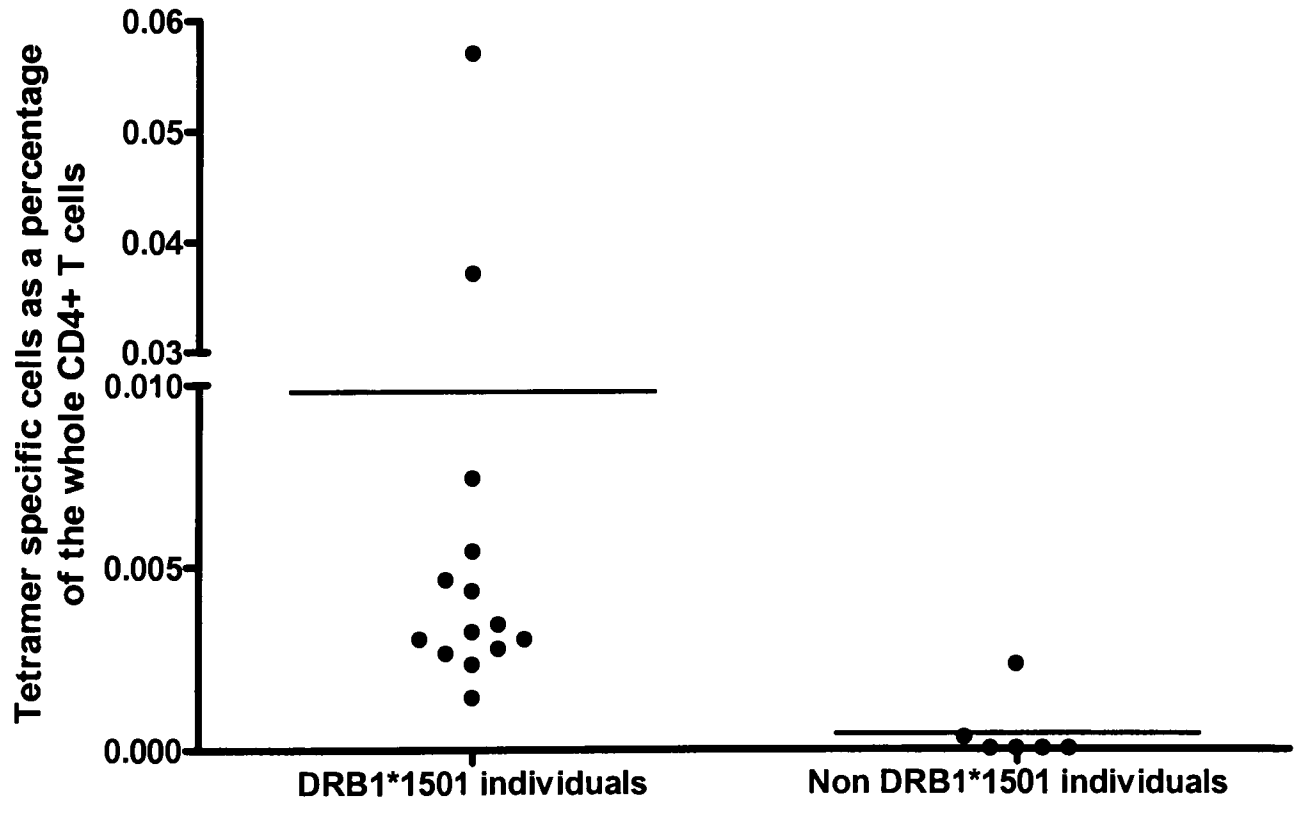
As mentioned above, the phenotype of VZV IE63-specific CD4+ T cells were recently characterized and were shown to express markers of early-intermediate differentiation and up to 30% showed evidence of recent activation (Jones, 2007). This could be explained by frequent re-exposure or re-activation of the virus, or alternatively that as IE63 is expressed during latency, it could reflect T cell exposure to latently expressed protein. However, as VZV gE is only expressed during the replicative cycle of the virus, the presence of activated gE-specific CD4+ T cells would argue in favor of frequent re-exposure or reactivation. Longitudinal responses to an immediate early and a late protein in cytomegalovirus in healthy donors have shown that while T cell responses to the IE

protein increased over time, responses to the late protein do not (Khan et al., 2007). Therefore, it would be important to distinguish these possibilities in order to understand disease pathogenesis and the critical components of immune control of VZV latency.

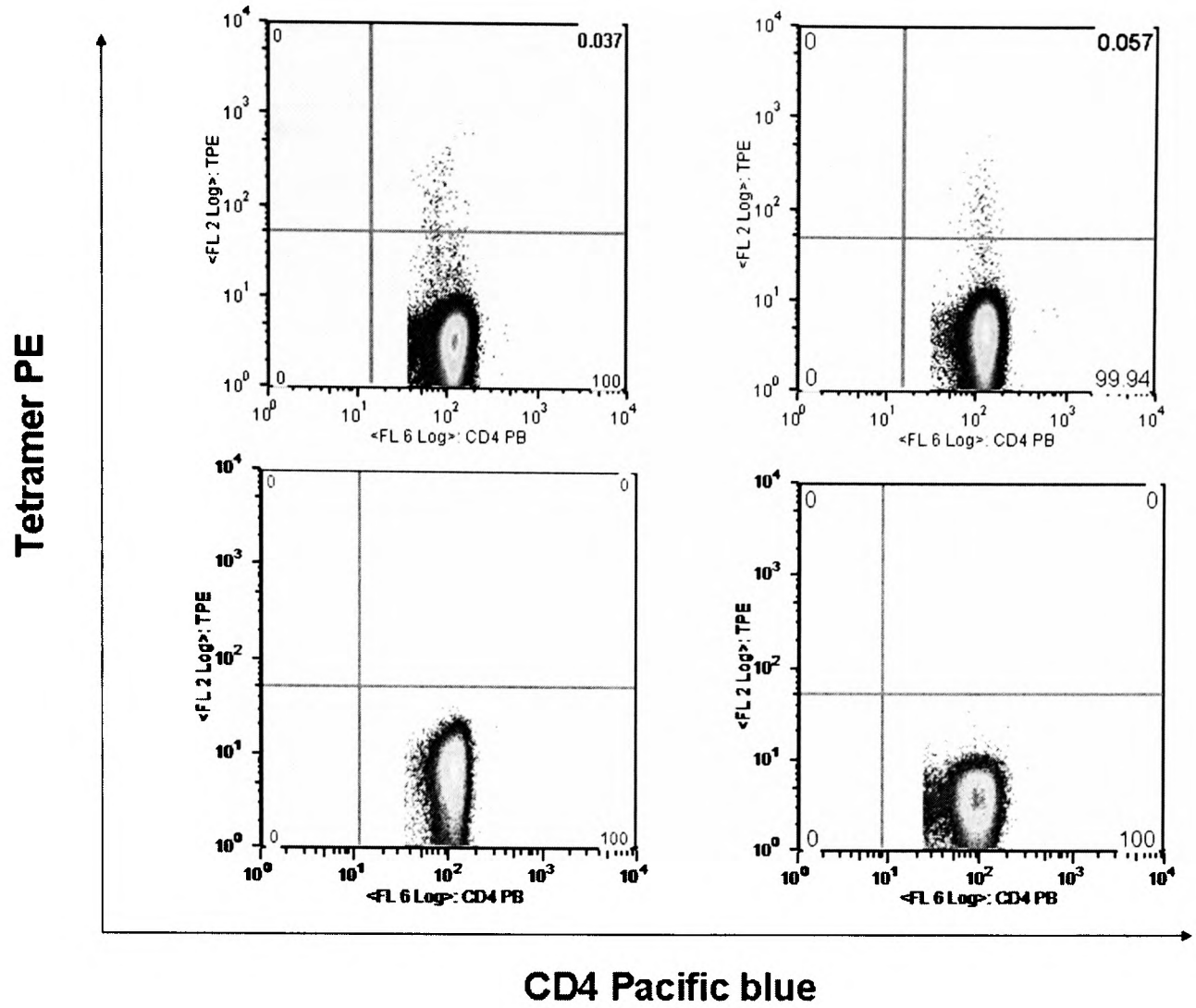
5.2. Results

5.2.1. Frequency of DRB1*1501 tetramer specific T cells in healthy immune donors

I initially proceeded to investigate the *ex vivo* frequency of tetramer specific T cells in VZV immune healthy immune donors. I also analyzed *ex vivo* tetramer specific responses in 6 non DRB1*1501 individuals to exclude any non specific binding and to set a cut off value for the tetramer. As shown in Fig. 5-3 tetramer positive CD4+ T cell responses were seen at a relatively low frequency in DRB1*1501 individuals. Although 2 donors (who had never had clinical reactivation) had tetramer positive responses >0.03% of CD4+ T cells, the *ex vivo* tetramer positive responses in other donors were in the range of 0.0014% to 0.007% (0.009±0.016) (Fig. 5-3). However, all DRB1*1501 individuals had significantly higher tetramer positive T cells when compared to non DRB1*1501 individuals ($p<0.0001$).



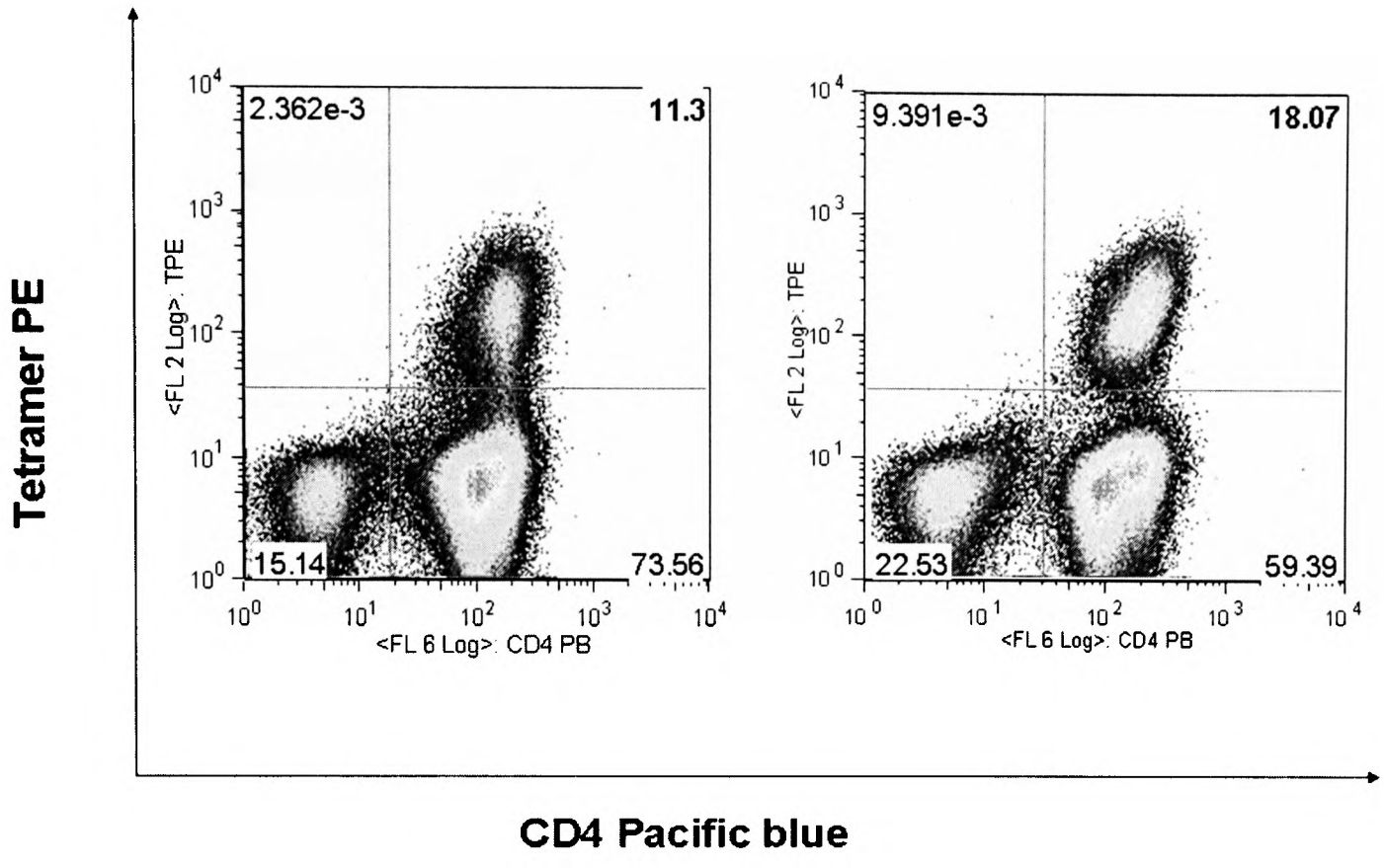
5-3 % $CD4^+$ $tetramer^+$ cells ex vivo in HLA-DRB1*1501 positive and negative individuals.



5-4 CD4$^+$ tetramer$^+$ cells in HLA-DRB1*1501 positive (top panels) and negative individuals (bottom panels) *ex vivo*

Similar frequencies found with VZV IE63- tetramer specific CD4+ T cells and parvovirus DRB1*1501 tetramer-specific CD4+ T cells were confirmed after magnetic bead-based tetramer enrichment technique (enriching for tetramer specific cells) (Jones, 2007; Kasprowicz et al., 2006).

Having identifying tetramer positive T cells *ex vivo*, I then went on to investigate the frequency of tetramer specific CD4+ T cells in short term T cell cultures specific for peptide 54. Tetramer positive T cells were seen at a high frequency in short term T cell cultures of PBMCs from DRB1*1501 individuals (Fig 5-5).



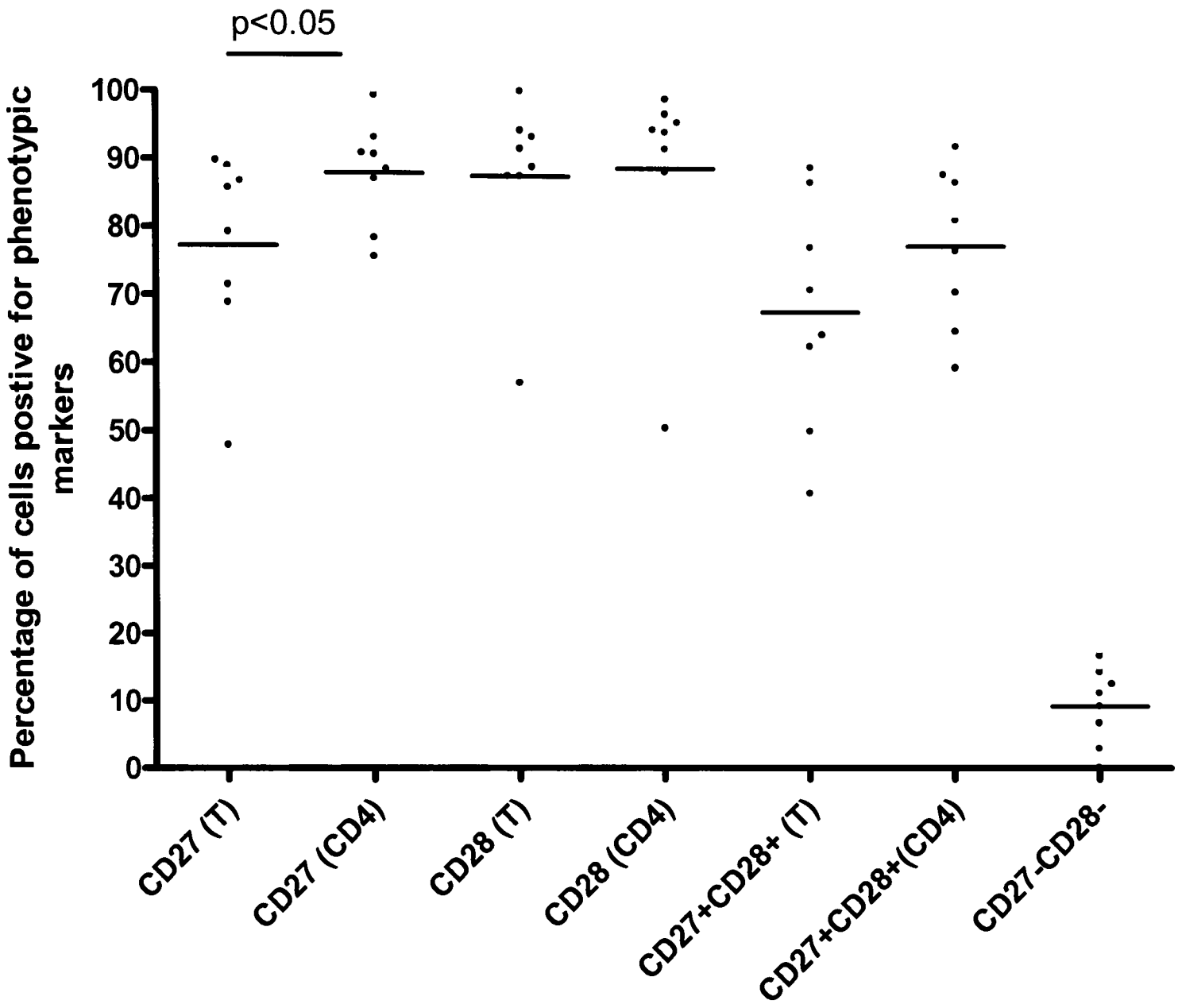
5-5 % CD4$^+$ tetramer$^+$ cells in HLA-DRB1*1501 positive individuals after short term culture

5.2.2. Analysis of the phenotype of DRB1*1501 tetramer specific CD4 + T cells

Expression of co-stimulatory molecules by tetramer specific T cells

I then went on to investigate the phenotype of VZV gE DRB1*1501 tetramer positive T cells. For this purpose I analyzed the phenotype of tetramer positive cells of 8 VZV immune individuals (Fig.5.6). The majority of the tetramer positive cells expressed CD28 (87.53%,±12.9) and CD27 (77.28%,±14.3) (Fig.5-6). Although not statistically significant, CD27 expression of tetramer positive cells was lower when compared to CD28. The expression of CD27 on tetramer specific T cell was significantly lower (<0.05) than expression of this molecule on the whole CD4+ T cell population (Fig. 5-6). However, the expression of CD28 was similar on both the tetramer specific T cell population (87.5%, ±12.9) and the whole CD4 + T cell population (88.67±15.8). In

addition, 67.6% of tetramer positive cells expressed both CD27 and CD28 while 9.2% of cells did not express either co stimulatory molecules.



5-6 Expression of co stimulatory molecules on tetramer specific T cells

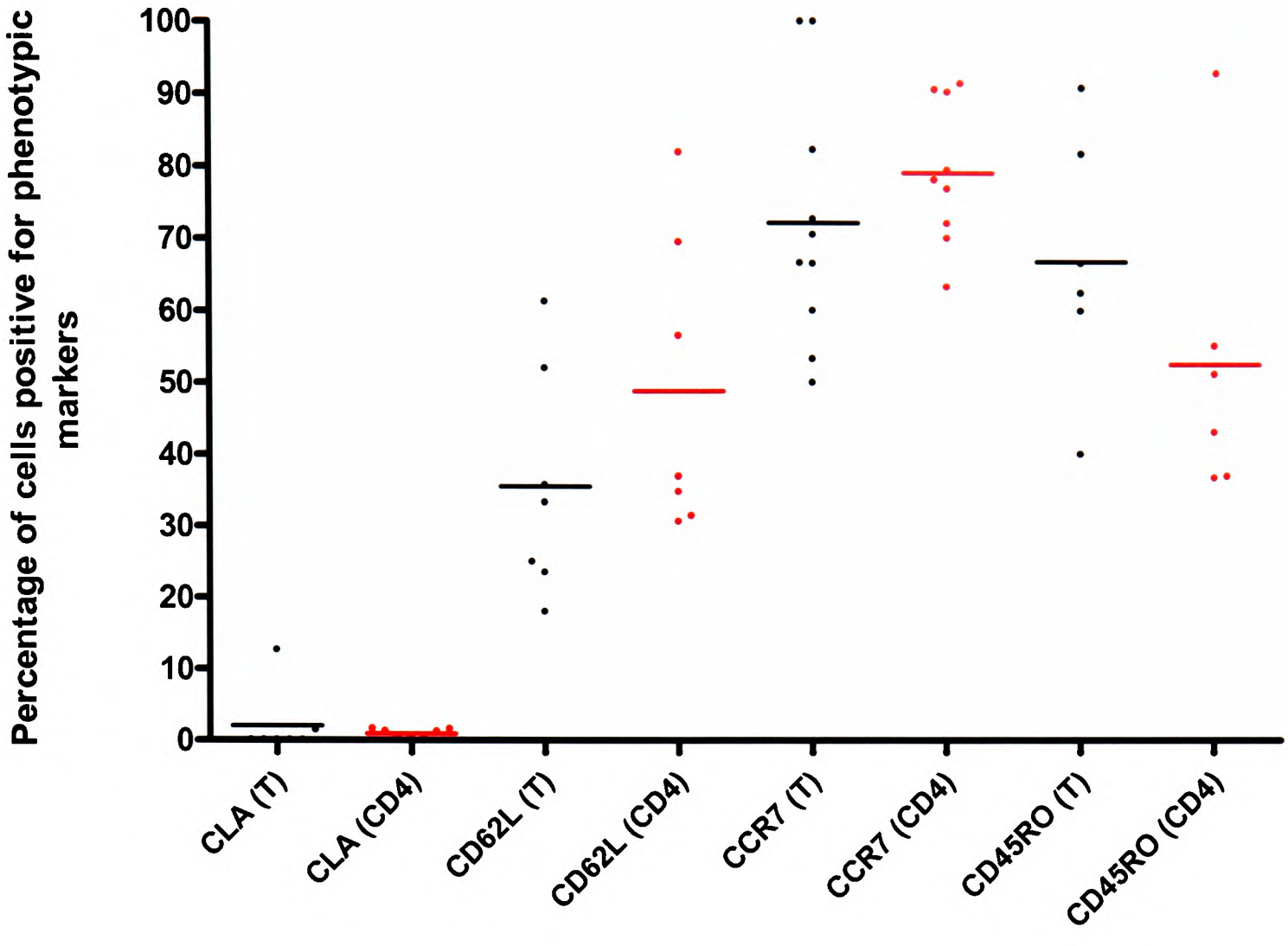
Expression of CD27 and CD28 in the tetramer specific T cell population (T) and the whole CD4+ T cell population (CD4). Cells are gated on CD4+ Tetramer+ and $CD14^-$, $CD19^-$ cells.

Expression of lymph node homing markers

I then proceeded to investigate the expression of lymph node homing markers by tetramer positive cells. 72.23% (SD±17.4) of the cells expressed CCR7 while the expression of CD62L was more variable (mean 35.5, SD±15.8) (Fig. 5-7). However, the expression of both CD62L and CCR7 was similar to that of the total CD4+ T cell population of the individual. CD45RO expression (52.6, ±21.08) but again was similar to CD45RO expression by the total CD4+ T cell population. Interestingly, CD62L and CD45RO expression was lower in the individuals who also had low expression of CD27 and CD28.

Expression of skin homing markers and activation markers

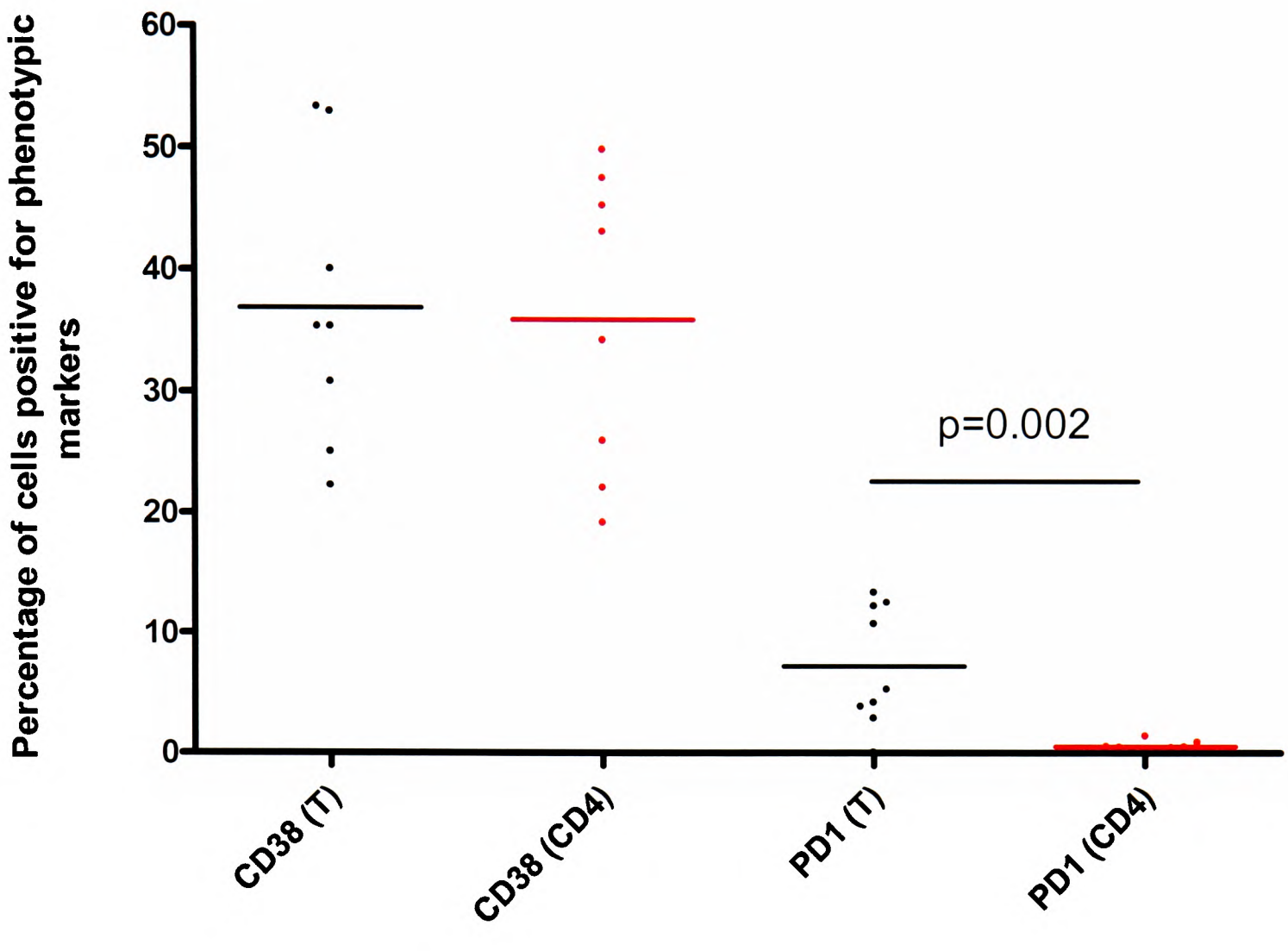
CLA which is considered to be expressed on T cell homing to the skin was only present on 2.0% (SD±4.7) of tetramer positive cells suggesting that in healthy immune donors, the gE-specific CD4+ T cells might not provide rapid control of cutaneous antigen. 5 out of the 8 individuals had absent CLA expression in their tetramer positive CD4 T cells (Fig 5-7). Although the overall expression of CLA on tetramer positive cells was higher than the total CD4+ T cell population (0.9, ±0.61), this difference was not significant. Therefore, it will be important to examine expression of other markers linked to skin homing such as CCR10.



5-7 Expression of homing and memory markers on tetramer specific T cells

Comparison of the expression of skin and lymph node homing molecules and the memory phenotype in the tetramer specific T cell population (T) and the whole CD4+ T cell population (CD4). Cells are gated on CD4+ Tetramer+ and $CD14^-$, $CD19^-$ cells

CD38 was expressed in 36.86% (SD±11.57) tetramer positive T cells and PD1 on 7.2% (SD±4.9) tetramer positive cells. PD1 expression was significantly higher in tetramer positive cells than the CD4+ population (0.51%, SD±0.4).



5-8 Expression of CD38 and PD-1 on tetramer specific T cells

Comparison of the expression of activation and PD-1 phenotypic markers in the tetramer specific T cell population (T) and the whole CD4+ T cell population (CD4). Cells are gated on CD4+ Tetramer+ and $CD14^-$, $CD19^-$, cells

Although the frequency of tetramer specific cells were low *ex vivo*, phenotypic analysis repeated with tetramer enrichment using anti PE beads with both VZV-IE63 tetramer and parvovirus DRB1*1501 tetramer showed that a similar phenotype of tetramer specific cells were seen by direct *ex vivo* analysis of tetramer specific cells and tetramer enrichment (Jones, 2007; Kasprowicz et al., 2006).

5.3. Discussion

These results show that CD4+ T cells specific for gE DRB1*1501 circulate at high frequencies in healthy VZV immune donors. The frequency of viral-specific CD4+ T cells were found to be similar to antigen specific CD4+ T cell responses observed with other infections and allergens (Bateman et al., 2006; Day et al., 2003; Lucas et al., 2004; Meyer et al., 2000a). The frequencies were also similar to the frequency of VZV IE63 (Jones, 2007) which is surprising given that gE is expressed only during the replicative cycle of the virus, whereas IE63 is expressed during latency as well. Therefore, the presence of a similar frequency of tetramer positive T cells to these two proteins could suggest recent re-exposure to the virus or viral reactivation.

5.3.1. Differentiation stage of gE specific CD4+ T cells

As described previously virus specific memory T cells vary in the expression of phenotypic markers which are thought to reflect different functional properties required by these cells in order to control viral replication (Appay and Rowland-Jones, 2004) (Appay, 2004; Appay et al., 2002a). According to this classification, gE specific T cells appear to be largely at the early/intermediate stage of differentiation. Although, 77.28% and 87.88% of tetramer positive cells expressed CD27 and CD28 respectively, 9.2% did not express either marker. However, whilst 72.23% of the cells expressed CCR7, the expression of CD62L was lower (mean 35.5%) suggesting that a significant subset of gE-specific CD4+ T cells had progressed along the differentiation pathway towards effector memory status. CD45RO expression was also seen in only 66.9% of tetramer specific

cells, which also supports the possibility that that a significant proportion of T cells could in fact be late effector T cells.

CMV specific CD4+ T cells were shown to acquire greater effector and cytotoxic potential as they differentiate from CD27+ memory to terminally differentiated effector memory phenotype and were shown to be less capable of producing IFNγ, TNFα and IL-2(Casazza et al., 2006). The frequency of CMV specific CD4+ T cells has been shown to increase with age and become more differentiated. However, although elderly individuals had more CMV specific CD4+ T cells, they were less capable of producing IL-2 and expressed phenotypic markers indicative of immune senescence (Pourgheysari et al., 2007). Herpes zoster which occurs due to reactivation of the virus affects up to 50% of people who are over 85 (Thomas and Hall, 2004) and is believed to be due to the decline in VZV specific memory T cell responses with age (Berger et al., 1985). Therefore, it would be important to investigate if VZV specific CD4+ T cells follow a similar fate. The donor population included in this study consisted of individuals below the age of 40 years and it would now be interesting to investigate if the phenotypes, functional abilities and frequency of VZV specific CD4+ cells change with age.

5.3.2. Expression of activation markers by tetramer specific T cell

CD38 is associated with the CD3/TCR complex on the surface of T cells and signals through MAP kinases which in turn results in T activation, cytokine production and proliferation of T cells (Deaglio et al., 2001). On antigen withdrawal, CD38 expression by antigen-specific T cells is lost with a half-life of several weeks (Ogg et al., 1998).

CD38 was expressed by 36.86% of gE tetramer binding T cells. This is therefore suggestive of activation of gE specific T cells within the preceding few weeks. Overall the differentiation and activation marker expression would support a mixed central and effector memory pattern with evidence of recent activation in approximately one third of the gE-specific CD4+ T cells. These would be compatible with ongoing replicative cycle antigen exposure and frequent re-activation or re-exposure. Boosts of glycoprotein-specific antibody titres have been observed in those who received the live attenuated VZV vaccine (Krause and Klinman, 2000). Although boosting of antibody responses in healthy immune donors following natural infection has not been investigated, the significant expression of CD38 and PD-1 by gE-specific T cells argues that exposure to VZV replicative cycle antigens occurs very frequently within a population not covered by universal vaccination. Indeed the recent detection of viraemia within 9% of healthy asymptomatic UK blood donors, would be compatible with these immunological findings (Quinlivan et al., 2007a) .

Programmed cell death-1 (PD-1) is a type 1 transmembrane glycoprotein that is expressed on double negative T cells in the thymus and is believed to be induced on T and B cells in the periphery upon activation(Okazaki and Honjo, 2007). Although, PD-1 specific T cells are thought to play an important role in controlling viruses in acute infection, it is has shown to have a negative impact on anti viral immune responses in chronic viral infections (Keir et al., 2007). PD-1 has been shown to be expressed at moderately high levels in chronic viral infections such as CMV, EBV, HIV and chronic hepatitis C infection and its expression is thought to be associated with T cell dysfunction

due to exhaustion (D'Souza et al., 2007; Freeman et al., 2006; Golden-Mason et al., 2007). In addition, blockage of PD-1 was shown to revive the proliferative and cytokine secretion potential of dysfunctional T cells in chronic hepatitis C, chronic LCMV and in HIV (Keir et al., 2007; Yao et al., 2007). However, recently it was shown that expression of PD-1 on viral specific CD8+ T cell was related to activation and an earlier stage of differentiation, with its expression having no effect on the functional capacity of HIV specific CD8+ T cells. These authors showed that CD8+ T cells that were in the early/intermediate stage of differentiation expressed PD-1 while expression was not seen in naïve T cells or CD8+ T cells in later stages of differentiation. In addition, activation of T cells was further shown to up regulate expression of PD-1 (Sauce et al., 2007).

PD-1 expression was significantly higher among tetramer positive cells than the whole CD4+ T cell population. Therefore, based on the observations made by others, the moderately high expression of PD-1 on VZV gE tetramer specific T cells could be due to recent encounter with the virus rather than exhaustion of tetramer specific T cells. However, it would be worth investigating the relative expression of this molecule in longitudinal studies of elderly and younger individuals to determine if PD-1 expression was solely a marker of activation and differentiation or if it indeed reflected viral specific CD4+ T cell dysfunction.

5.3.3. Expression of skin homing markers

Cutaneous leucocyte antigen (CLA) is expressed by T cells homing to the skin. Its ligands are both E-selectin and P-selectin which are constitutively expressed on dermal microvessels, but the expression is markedly up regulated in inflamed skin (Agace, 2006). CLA is expressed on less than 5% of circulating CD4+ T cells from healthy individuals (Bateman et al., 2006) but in 85% of T cells in inflammatory skin lesions (Hollo et al., 2005).

Interestingly, only 2.0% of gE tetramer specific T cells expressed CLA. The highest expression of CLA (12.6% of tetramer positive cells) was seen in the individual who had the highest frequency of tetramer positive T cells (0.057% of the whole CD4+ T cells). The level of expression of CLA in gE tetramer specific T cells was similar to levels seen in VZV IE63 tetramer specific T cells (Jones, 2007). Although, CLA expression by tetramer specific T cells was significantly higher than expression in the total CD4+ T cell population, it was significantly less when compared to expression in herpes simplex (HSV) virus specific T cells. For instance, 50-70% of HSV specific CD8+ T cells and 15-20% CD4+ T cells (Koelle et al., 2005) have been shown to express CLA. However, CLA expression in these studies were determined following antigen stimulation which could over estimate the expression of CLA by HSV specific CD4+ T cells as CLA expression has been shown to be up regulated with stimulation (Reddy et al., 2005).

CCR4 and CCR10 are two chemokine receptors that are thought regulate T cell tropism to the skin are expressed on all CD4+CLA+ T cells (Agace, 2006). However, CCR4

expression on gE and IE63 tetramer specific T cells in a limited number of individuals showed that the level of expression of this was similar to the CLA expression (unpublished data). Therefore, collectively these data suggest that in healthy VZV immune donors, the majority of circulating antigen specific T cells do not home efficiently to the skin.

5.3.4. Comparison of the phenotype of VZV gE and IE63 tetramer-specific CD4+ T cells

Similarities and differences have been observed for the phenotype of memory T cell responses to different proteins in persistent virus infections. Therefore, I compared the phenotype of gE tetramer specific T cells with those of IE63 specific T cells (work of Louise Jones). Although, the phenotypes of tetramer specific T cells appeared to be similar overall, there were some differences. For instance, although CD27 and CD28 expression IE63 specific T cells were similar, IE63 specific T cells appeared to lose CD28 expression before CD27 expression which was in contrast to what was seen with gE. In addition, the expression of CD45RO was higher in IE63 specific T cells.

IE63-specific CD4+ T cells expressed mixed early and intermediate levels of differentiation in the context of moderate activation(Jones, 2007). As IE63 is expressed during latency, it is not possible to interpret whether such IE63-specific T cell activation was related to latent expression or to frequent re-activation or re-exposure (Cohrs and Gilden, 2007). In contrast, gE is expressed only during the replicative cycle and therefore the presence of activated T cells with effector memory differentiation would argue in favour of frequent VZV re-activation or re-exposure. Comparison of longitudinal

responses to an immediate early and a late protein in cytomegalovirus in healthy donors have shown that while T cell responses to the IE protein increased over time, responses to the late protein do not (Khan et al., 2007).. Therefore, it will clearly be important to extend these findings to different epitopes within different proteins and to examine longitudinal changes during the course of natural infection in individuals of different age and clinical groups.

Chapter 6. Analysis of T cell responses in patients with acute varicella zoster virus infection

6.1. Introduction

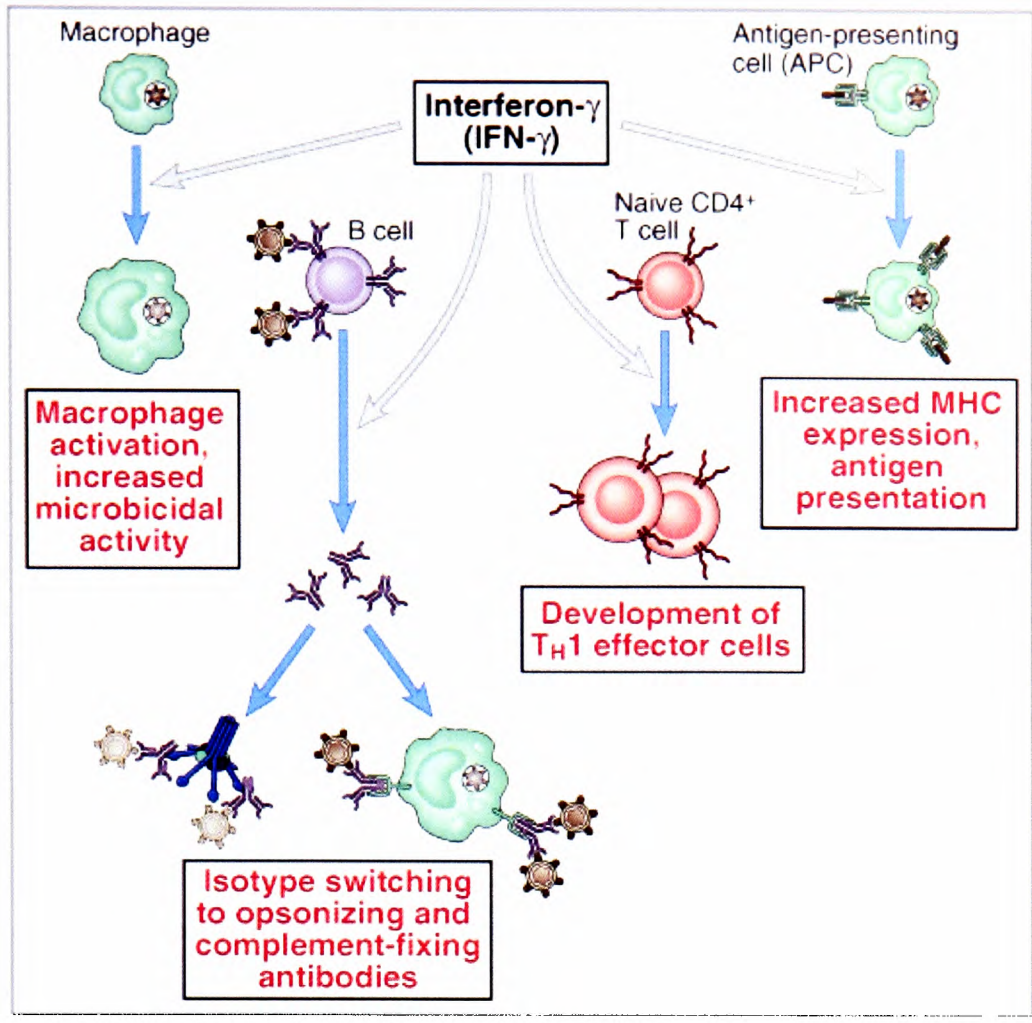
One of the main roles of the immune system is to eliminate any invading pathogen. The innate immune system provides the first line of defense against viruses with the help of many cell types and their antiviral cytokines. Virus infected cells produce many antiviral cytokines (e.g. IFNα/β) and also secrete many chemokines and other cytokines which attract NK cells, NKT cells and granulocytes (Guidotti and Chisari, 2001). Although the role played by these cells and other antiviral mechanisms in the innate immune system should not be undervalued, sometimes innate immune defense mechanisms alone are inadequate to clear a virus. The adaptive immune system consists of T cells and B cells that not only have superior antiviral mechanisms, but also subsequently develop specific memory which reduces the chance of further infection with the same pathogen on re-exposure.

6.1.1. T cell responses in acute viral infections

T cell responses in acute viral infections are aimed at eradicating the virus and in the development of effective life long memory T cell responses. Once T cells encounter their specific antigen, a marked expansion of antigen specific T cells occur, and these activated T cells up regulate the expression of growth factor receptors, alter the expression of chemokine receptors and cell adhesion molecules. They also acquire the ability to produce many anti viral cytokines and may develop intracellular granules and are capable of cytotoxicity (Welsh et al., 2004).

CD4+ T cells are thought to play many important roles during acute infection such as activation of antigen presenting cells following interactions with CD40, priming of antigen specific CD8+ T cells thus enabling to become effective killers and also stimulating naïve B cells to differentiate in to plasma cells that secrete larger amounts of antigen specific antibodies throughout life. IL-2 secreted by CD4+ T cells have shown to be essential for the clonal expansion and activation of CD8+ T cells (La Gruta et al., 2004). Although anti viral CD4+ T cells have shown to have a cytotoxic potential (Appay et al., 2002b; Landais et al., 2004) especially in chronic viral infections (Aslan et al., 2006), their role in killing virally infected cells in acute viral infection of humans has not yet been defined. Other than those mentioned above, CD4+ T cells exert their main anti viral effects by secreting cytokines such as IFNγ and TNFα which have many important roles such as activation of phagocytes, stimulation of antigen presentation and regulation of several cellular functions that are illustrated in Fig. 6-1.

CD8+ T cell eliminate virus infected cells by granule or Fas mediated or other pathways which ultimately lead to death of infected cells by triggering their inherent apoptotic mechanisms and also by secreting anti viral cytokines (Barry and Bleackley, 2002).



6-1 Main functions of IFNγ

Image from Abbas and Lichtman: Cellular and Molecular Immunology, 5th Edition.

Although both naïve CD4+ and CD8+ T cells have shown to have a similar precursor frequency, the frequency of antigen specific CD8+ T cells are typically higher during acute infection when compared to CD4+ T cells (Whitmire et al., 2006). Antigen specific naïve CD8+ T cells are thought to undergo 14 to 15 cycles of cell division during the acute immune responses(Blattman et al., 2002), while the number of cell divisions by antigen specific CD4+ T cells are thought to be lower. This in turn is thought to alter CD4+ and CD8+ T cell percentages in acute infection (Foulds et al., 2002). Possibly due to this lower proliferative capacity, it has been difficult to detect antigen specific CD4+ T cells until at least 5 days post infection (Whitmire et al., 2006). Although CD4+ T cells

play a crucial role in eliminating the virus in acute infection, because of their cytotoxic ability and because of the larger clonal expansion, CD8+ T cells are thought to be vital in effectively clearing the virus. Indeed, it has been suggested that failure of maturation and impaired anti viral effects by the CD8+ T cells result in failure to clear the viraemia in acute infection such as in HIV (Appay et al., 2000; Champagne et al., 2001). However, CD4+ T cell responses appear to be particulary important in acute herpes virus infections as reported in primary CMV (Gamadia et al., 2003). Early development of IFNγ secreting CD4+ T cell responses was associated with asymptomatic primary CMV infection (Gamadia et al., 2003). Patients with symptomatic infection were shown to have delayed functional CD4+ T cell responses and appearance of these cells were associated with clearance of the virus (Gamadia et al., 2003).

6.1.2. Primary VZV infection

Primary infection with VZV results in chickenpox, which is usually a benign self limiting illness characterized by fever and a generalized pruritic rash. However, in certain groups of individuals such as neonates, adults, pregnant women and immune suppressed individuals, it may cause severe infection which can sometime be fatal. Adults are 9 to 15 times more likely to be hospitalized (Galil et al., 2002a) and 25 times more likely than children to die from varicella (Meyer et al., 2000b). Varicella associated complications such as pneumonia are much commoner among adults than children (Mohsen et al., 2001). Primary varicella in immune suppressed individuals may result in visceral dissemination, multi organ failure and death.

T cell responses are believed to be important in controlling the virus and preventing viral reactivation. Virus specific proliferative T cell responses, which were characterized using *in vitro* proliferative assays, were found to be impaired in immune suppressed individuals with severe disease (Gershon and Steinberg, 1979a; Patel et al., 1979). Arvin *et al*, showed that proliferative T cell responses in the first 72 hours since symptoms were associated with milder disease and also that the VZV specific lymphocyte transformation occurred at a higher rate in patients with fewer skin lesions (Arvin et al., 1986). In both studies, VZV specific antibody titres did not seem to correlate with clinical disease severity (Arvin et al., 1986; Gershon and Steinberg, 1979b). However, contrary to these finding, a study done by Vossen *et al*, in 5 children with acute chickenpox showed that the highest VZV specific CD4+ T cell response was observed in the child with most severe infection and who also had a very high viral load (Vossen et al., 2005b).

The majority of above studies suggest that a strong VZV-specific immune response early in infection may protect the individual from severe disease and also possibly that severe disease in immune suppressed individuals was due to the lack of this 'strong' immune response. However, this possibility and the associations between the frequency and functional T cell responses with clinical disease severity in acute primary VZV infection have not been investigated in detail. Such data would provide us with insight regarding the dynamic interactions between host and the pathogen in primary VZV infection which would in turn enable us to better understand disease pathogenesis.

6.2. Patients

Fresh heparinized venous blood samples were obtained from 34 individuals with acute primary infection who were admitted to the Infectious Diseases Hospital in Sri Lanka under ethical approval. Mean age of the donors was 32.8 (SD±1.7) years. Acute samples were collected during the first 7 days of the onset of symptoms and the post acute samples were obtained approximately 2 weeks after the collection of the first blood sample. 2 samples were collected also from 2 patients 5 days after collecting the first sample. Mean duration of symptoms at the time of taking the first blood sample 4.5 days (SD±1.7). (Median 4.5, range 3 to 7 days).

6.2.1. Clinical characteristics of the patients

The scale shown below (table 5) was used to define the severity of disease in our patient cohort(Vazquez et al., 2001). Based on the number and character of the lesions, presence or absence of fever, systemic signs and also the subjective assessment of the patient, each patient was given a score and accordingly categorized as having mild, moderate to severe and severe disease. I performed the subjective assessment in all these patients.

According this classification clinical disease severity of our patients is as follows:

- Mild infections : 12
- Moderate to severe : 20
- Severe : 2

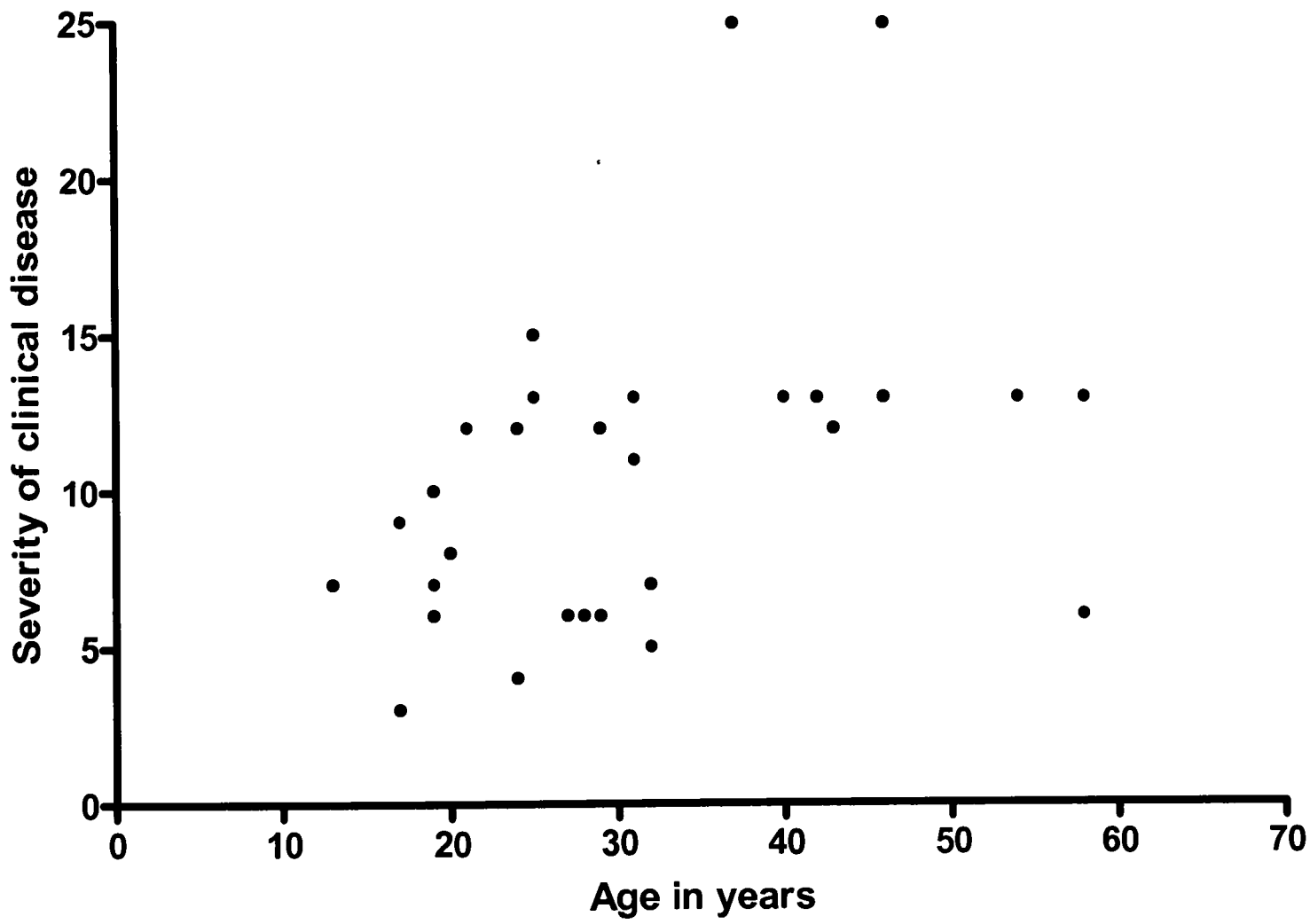
Scale used to assess the severity of illness	
Variable	**No. of points**
Rash	
Number of lesions	
1-50	1
51-100	2
101-500	4
>500	6
Character of lesions	
Macular popular	2
Mostly vesicular	4
Mostly haemorrhagic	4
Fever	
Temperature 38.8 to 39.9° C	1
Temperature >40° C	3
Systemic signs	
Pain in the back or abdomen	4
Interestial pneumonia	5
Encepahlitis	5
Subjective assessment	
Does not appear ill	0
Appears moderately ill	2
Appears severely ill	5
Severity (Total score)	
Mild disease	≤7
Moderately severe disease	8-15
Severe disease	≥16

Table 5: Taken from: Vazquez M et al. The effectiveness of the varicella vaccine in clinical practice. N Engl J Med; 2001;344(13):955-60

The majority of our patients (13/34) had more >500 skin lesions and 7 had <50 skin lesions. The remaining 14 patients had skin lesions in the range of 50 to 500. 4 patients had other co morbid conditions. 2 of them (one had nephrotic syndrome and the other systemic lupus erythematosus) were on prednisolone 30mg/day, 1 patient had melanoma and another had acute myeloblastic leukemia. Of the 34 patients, 2 patients developed varicella pneumonitis and 1 developed secondary bacterial infection of the skin lesions. Another patient who presented with clinical features suggestive of a menigoencephalitis was later diagnosed as having co-existing bacterial meningitis, based on his cerebrospinal fluid analysis.

6.3. Results

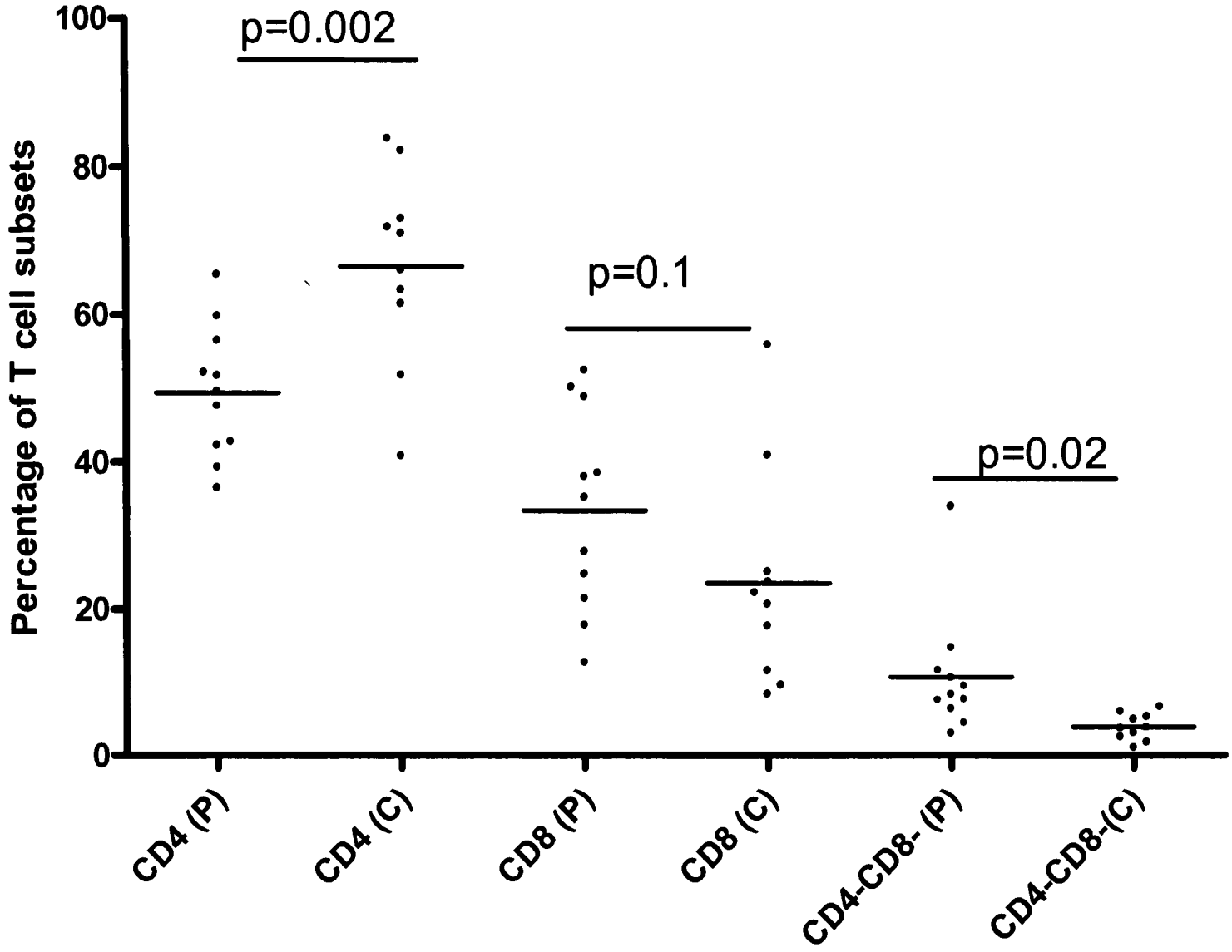
Adult patients with VZV infection are more likely to suffer from severe disease and are 9 to 15 times more likely to be hospitalized (Galil et al., 2002a) and 25 times more likely than children to die from varicella (Meyer et al., 2000b). Therefore, I first investigated if a similar observation was seen with our patient cohort. As expected a significant correlation (p=0.0067) was seen between the age of the patients and clinical disease severity score (Spearmans r= 0.47).



6-2 Association of severity of infection with age of the patients

I then went on to characterize the CD4+ and CD8+ percentages and the CD4+, CD8+ T cell ratios in these patients. As shown below (Fig 6-3) the CD4+ T cells in patients with acute VZV infection were significantly lower (p=0.002) when compared to healthy

donors. In addition, a significantly large population of CD3+CD8-CD4- T cell population was seen in the patients.

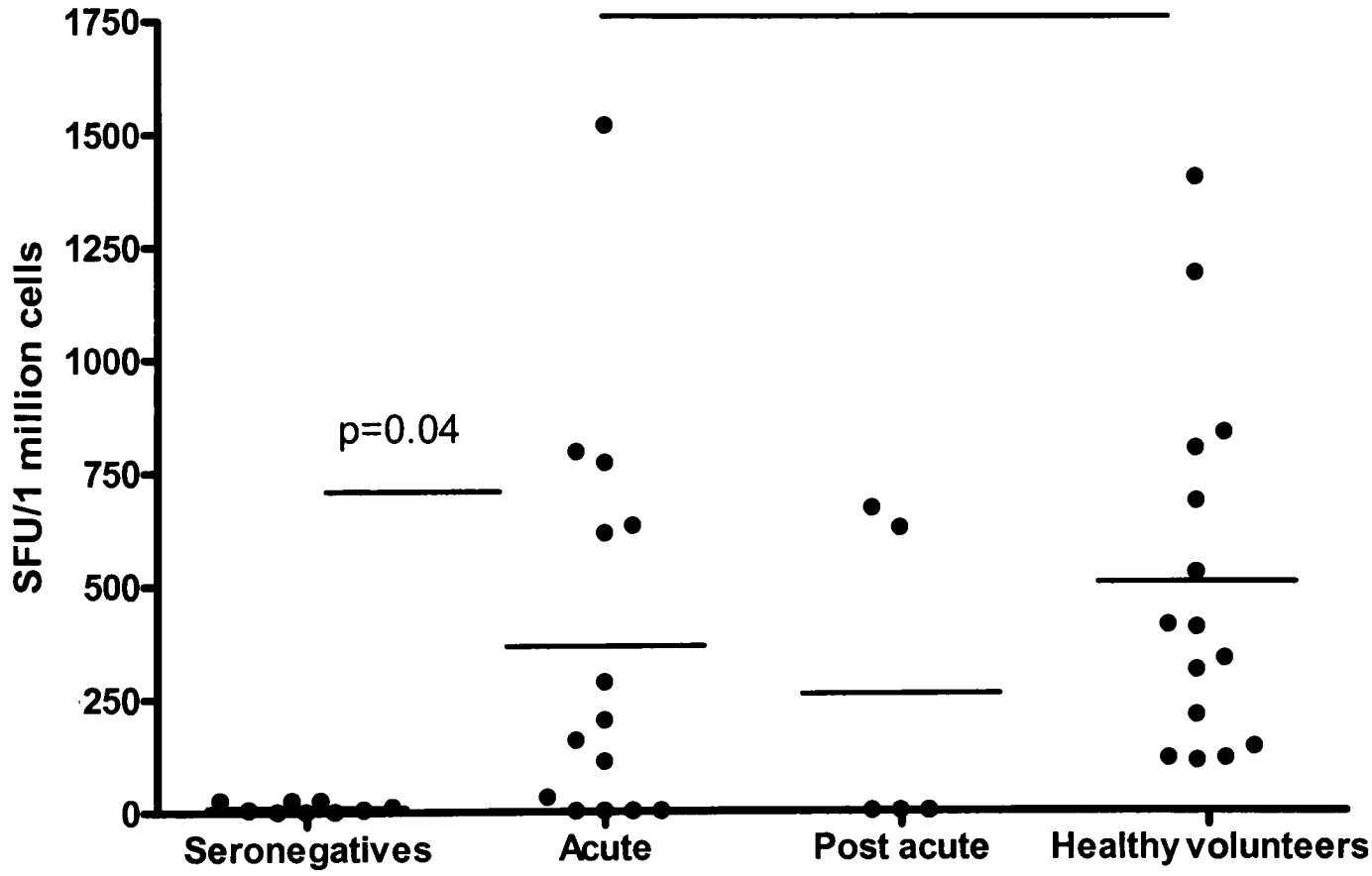


6-3 Percentages of CD8+, CD4+ T cells in patients with acute VZV infection

Cells were gated on CD3+ T cells. (C) indicates percentage of T cell subsets in healthy donors and (P) indicates the percentage in patients with acute infection. p values were determined by the paired t test.

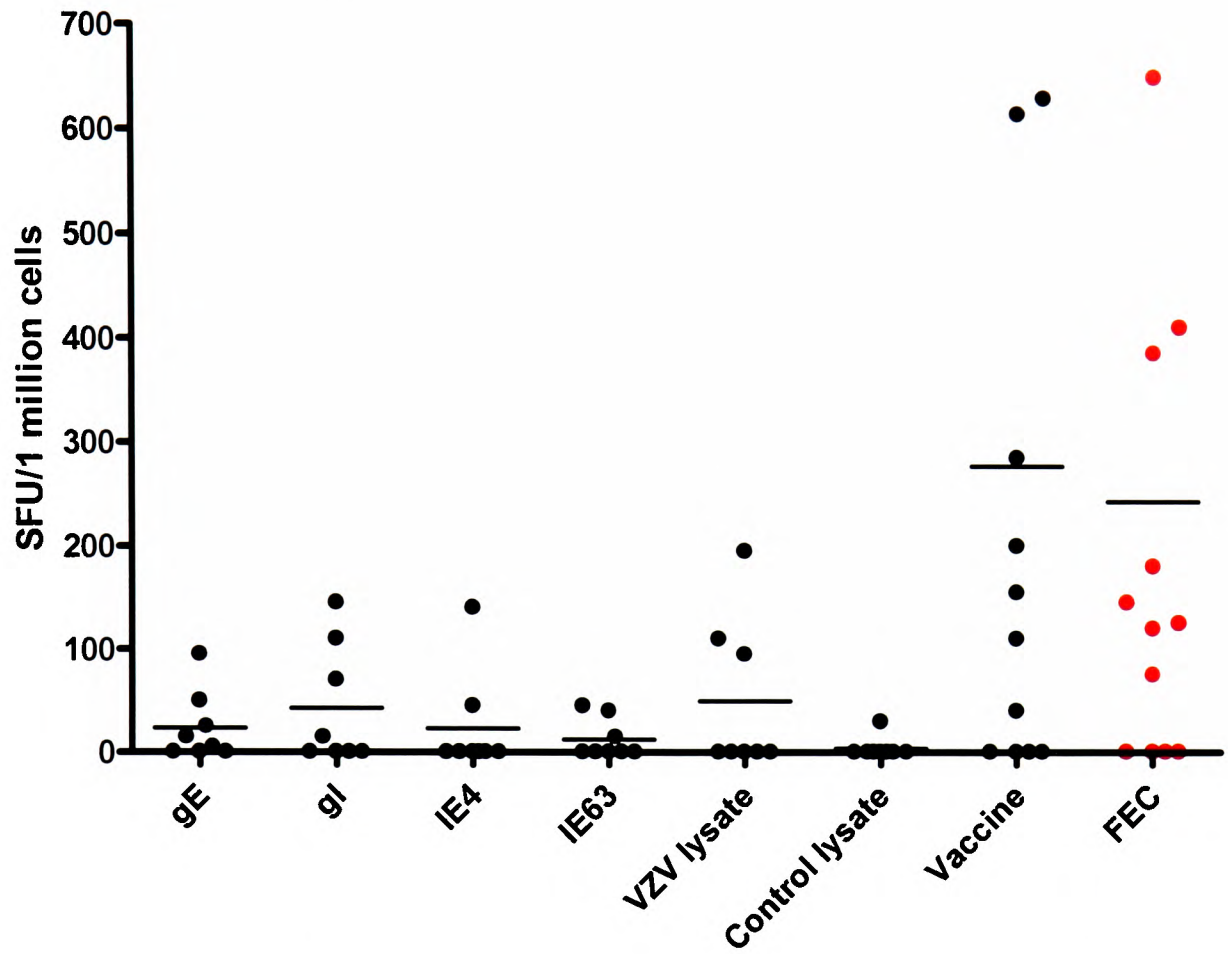
As my main aim was to investigate the functional and frequency of antigen specific T cell responses in patients with acute chickenpox, I initially proceeded to investigate the functional T cell responses by using *ex vivo* IFNγ ELISpot assays using the VZV live

attenuated vaccine as the stimulus. PHA (phytohemagglutinin) and FEC (peptide pool corresponding to T cell epitopes of influenza, EBV and CMV) were used as positive controls and media with cells alone was used as a negative control. As shown below a varied *ex vivo* IFNγ response to the VZV live vaccine were seen in the patients. 4 patients had no detectable VZV-specific IFNγ production despite presence of symptoms for 3 to 5 days. Although, the IFNγ responses to the vaccine were significantly higher in patients with acute infection when compared to seronegative individuals (p=0.04), they were lower than healthy seropositive donors who had primary varicella many years previously (Fig. 6-4). However, this difference was not statistically significant. Interestingly, no vaccine specific IFNγ production was seen in 3 patients with moderate to severe infection even between 16-21 days following onset of symptoms.



***6-4 Ex vivo* IFNγ ELISpot responses in patients with acute VZV infection.**
The VZV live attenuated vaccine is used as the stimulus. Unpaired t test was used to determine the p value.

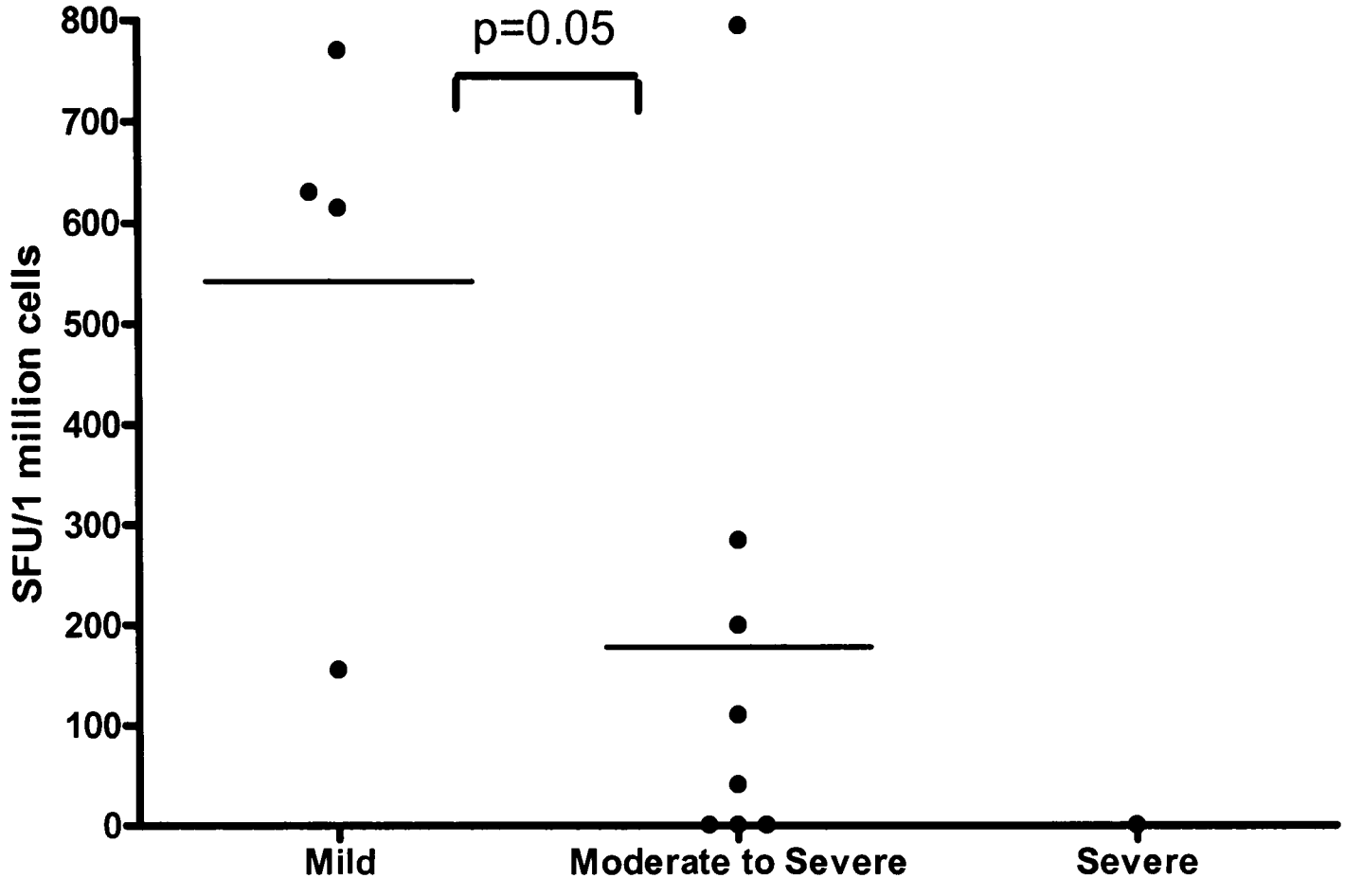
Most patients who had vaccine specific IFNγ responses also showed responses to FEC, the VZV lysate and peptide pools comprising of VZV overlapping gE, gI, IE63 and IE4 20mer peptides (Fig. 6-5).



***6-5 Ex vivo* IFNγ ELISpot responses to different VZV overlapping pools of 20mer peptide, the live attenuated vaccine, the VZV lysate and FEC in patients with acute VZV infection.**

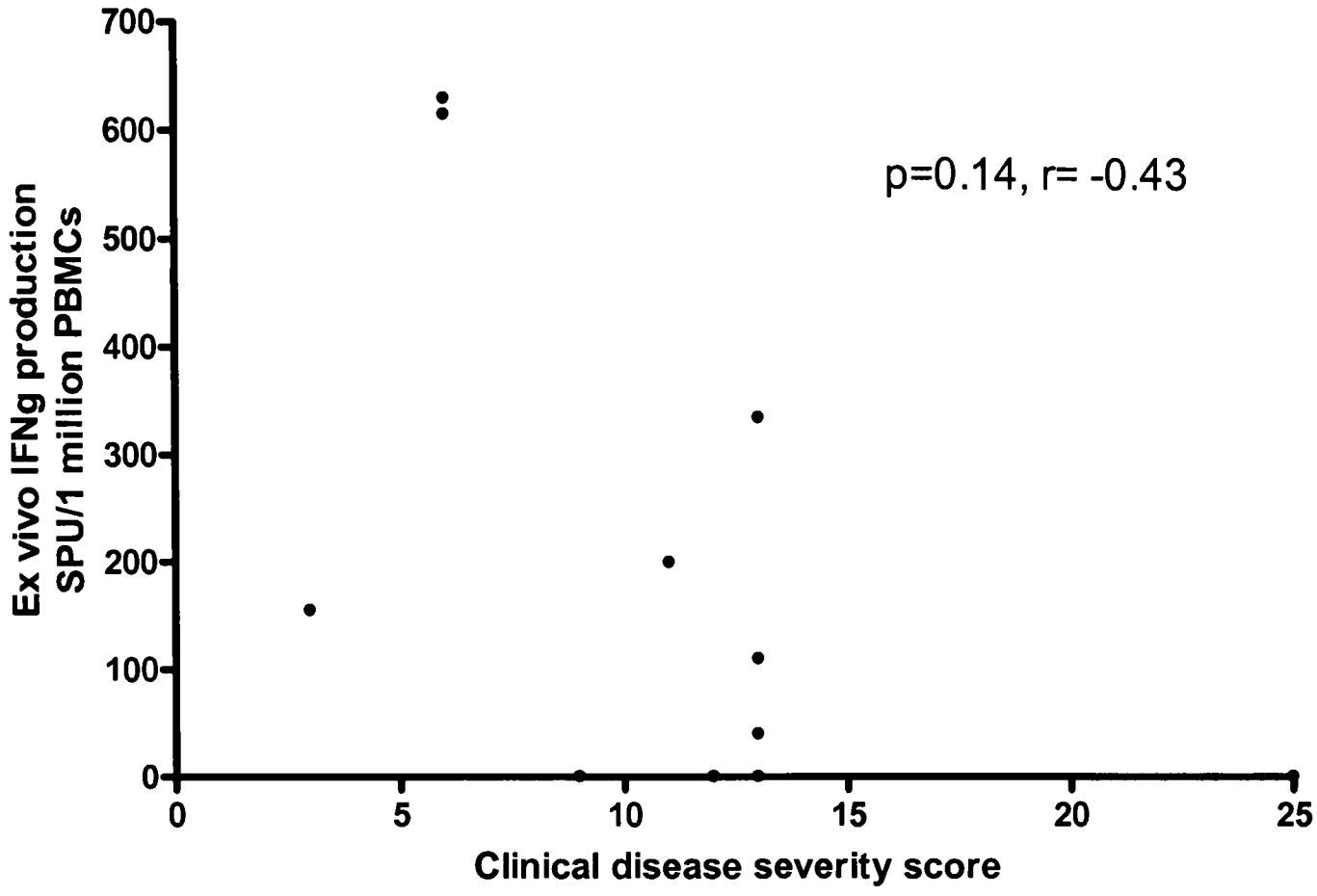
I then went on to investigate whether if VZV vaccine specific IFN γ production was correlated to severity of infection. Patients with mild infection had significantly higher vaccine specific IFNγ responses than those with moderate to severe infection (p=0.05) or severe infection (Fig.6-6). I also went on to determine whether there was any association between clinical disease severity and *ex vivo* IFNγ production. A negative correlation was seen between the clinical disease severity score and VZV vaccine specific IFNγ production. However, this was not statistically significant (p=0.14, pearsons r= -0.47).

However, no correlation was seen between the FEC and VZV clinical disease severity or FEC responses and VZV-specific T cell responses in these patients.



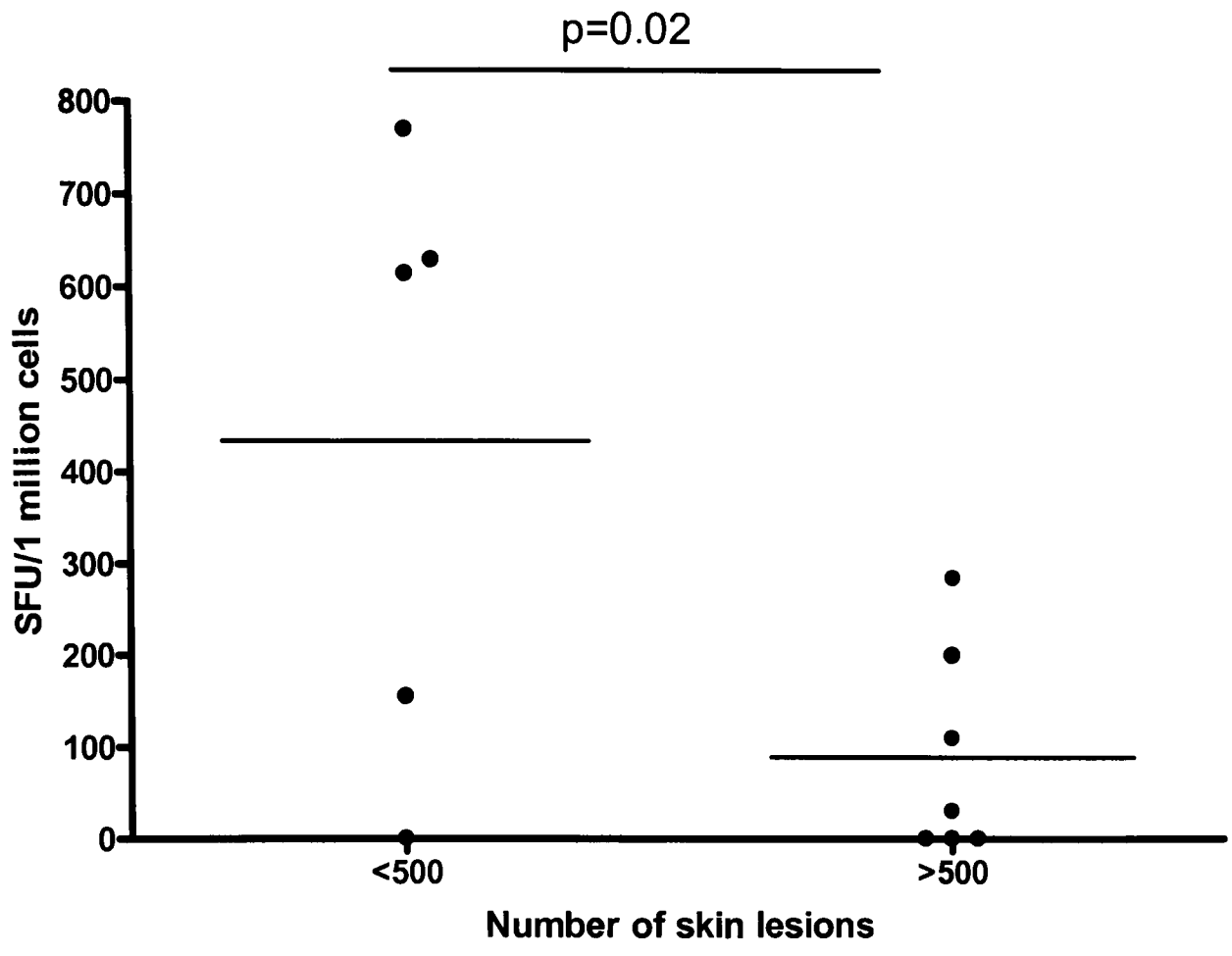
6-6 Ex vivo IFNγ ELISpot responses in patients with varying severity of primary VZV infection

VZV live attenuated vaccine is used as the stimulus. Unpaired t test was used in p value calculation.



6-7 Association between clinical disease severity score and ex vivo IFNγ production in patients with acute primary varicella infection.
Pearsons r value was used to determine correlation.

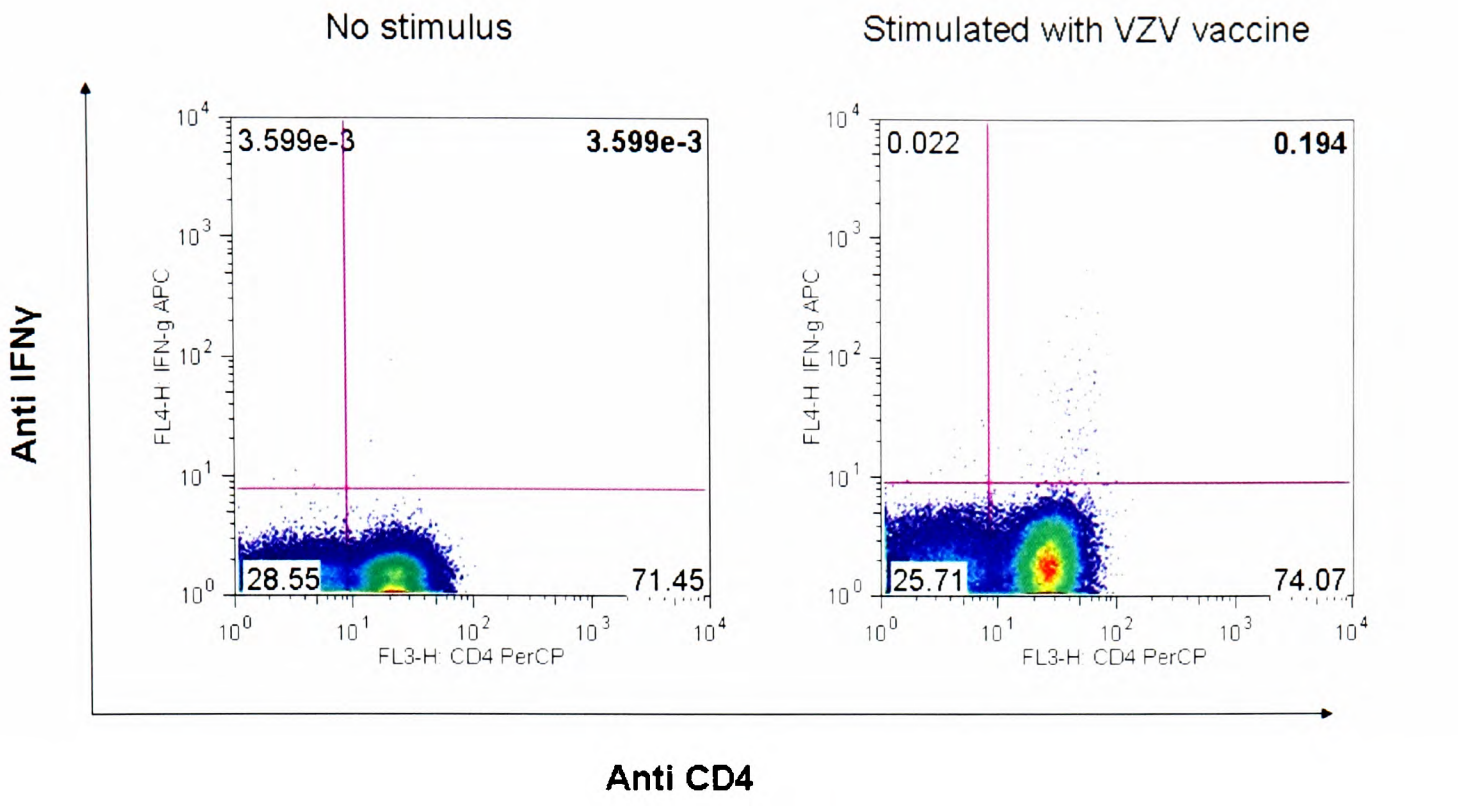
As the number of skin lesions is an important determinant of clinical disease severity according to the scale used, I proceeded to investigate if there was any difference in T cell function in patients with varying number of skin lesions. As shown below, patients with <500 skin lesions had significantly higher (p=0.02) VZV vaccine specific IFNγ responses than patients with >500 lesions.



6-8 Ex vivo IFNγ ELISpot responses in patients with varying numbers of skin lesions.

The live attenuated VZV vaccine is used as the stimulus. P value was calculated by the use of unpaired t test.

As shown in previous chapters, VZV specific T cells were predominantly from the CD4+ T cell subset. Intracellular cytokine assays performed both *ex vivo* and on T cells from short term cultures using different stimuli showed that IFNγ production was exclusively from the CD4+ T cell subset. As I observed these results in healthy immune donors who had primary varicella many years previously, I went on to investigate the subset of T cells in acute infection that were responsible for functional immune responses. Using *ex vivo* ICS assays and using the VZV vaccine as the stimulus, I was surprised to find that even in acute primary varicella infection IFNγ was predominantly from the CD4+ subset of T cells (Fig 6-9).



6-9 Ex vivo ICS assay of PBMCs of a patient with acute varicella infection.

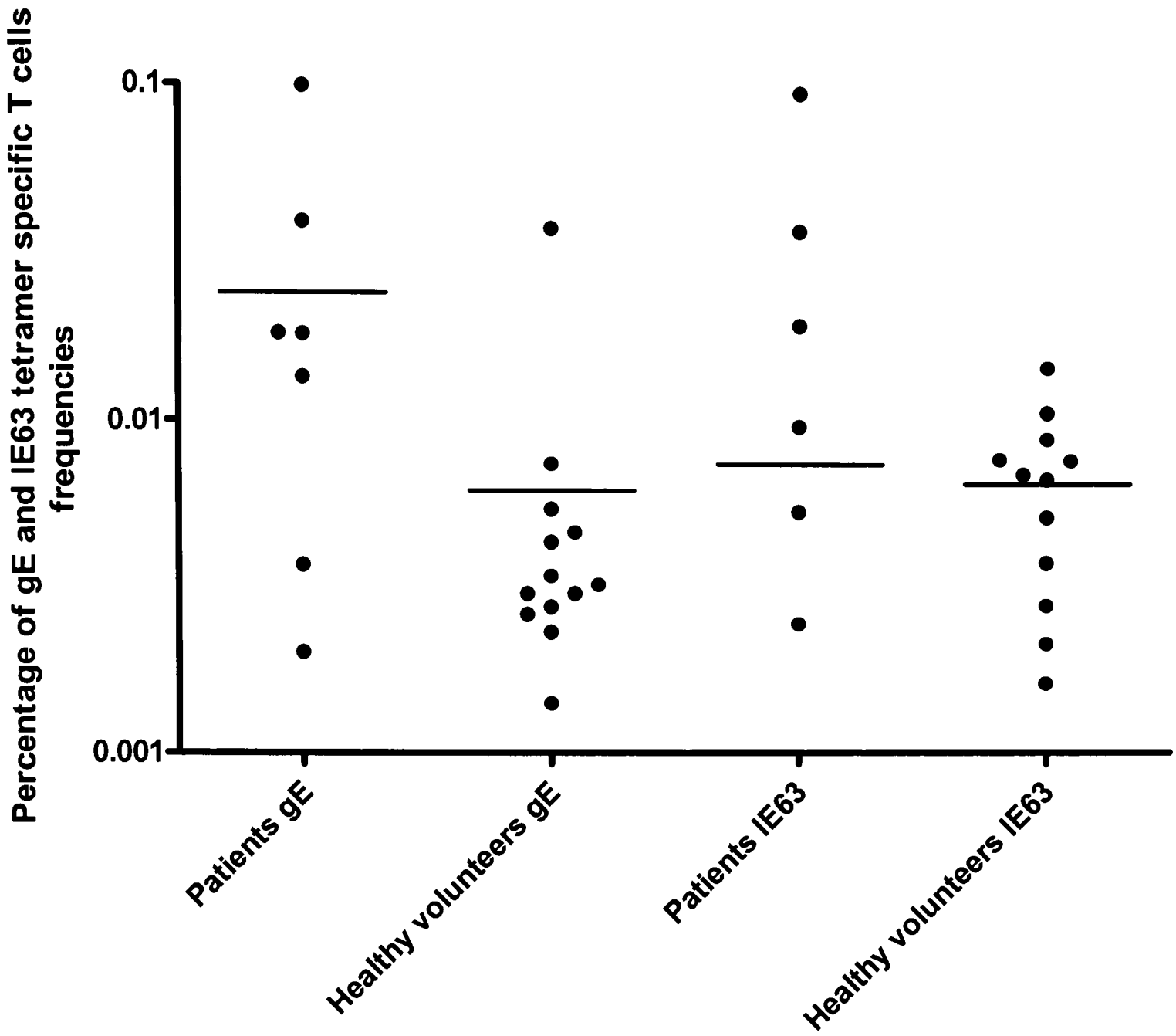
The VZV vaccine was used as the stimulus. Cells are gated on CD3+.

6.3.1. Analysis of the frequency and phenotype of gE and IE63 DRB1*1501 tetramer specific responses in patients with acute VZV infection

Following analysis of the functional responses of the T cells in patients with acute VZV infection, I then proceeded to investigate the frequency and phenotype of gE and IE63 DRB1*1501 tetramer specific CD4+ T cell responses in these patients. In our cohort, 8 patients were of the DRB1*1501 HLA phenotype and I went on to characterize the tetramer specific responses in these patients.

In contrast, to what was seen with the ELISpot responses, I found that both gE and IE63 DRB1*1501 tetramer specific responses were higher in the patients with acute infection ($p=0.07$ for gE and $p=0.1$ for IE63) than the frequencies observed in healthy immune

donors (Fig 6-10). However, a wide range of frequencies for both gE and IE63 DRB1*1501 tetramers were seen in these patients. For instance, the responses for the gE DRB1*1501 tetramer ranged from 0 to 0.097% (mean 0.023, SD±0.03) while the responses to the IE63 DRB1*1501 ranged from 0 to 0.092% (mean 0.02, SD±0.03). Therefore, the frequencies of both gE and IE63 tetramer specific T cells were similar in patients with acute infection.



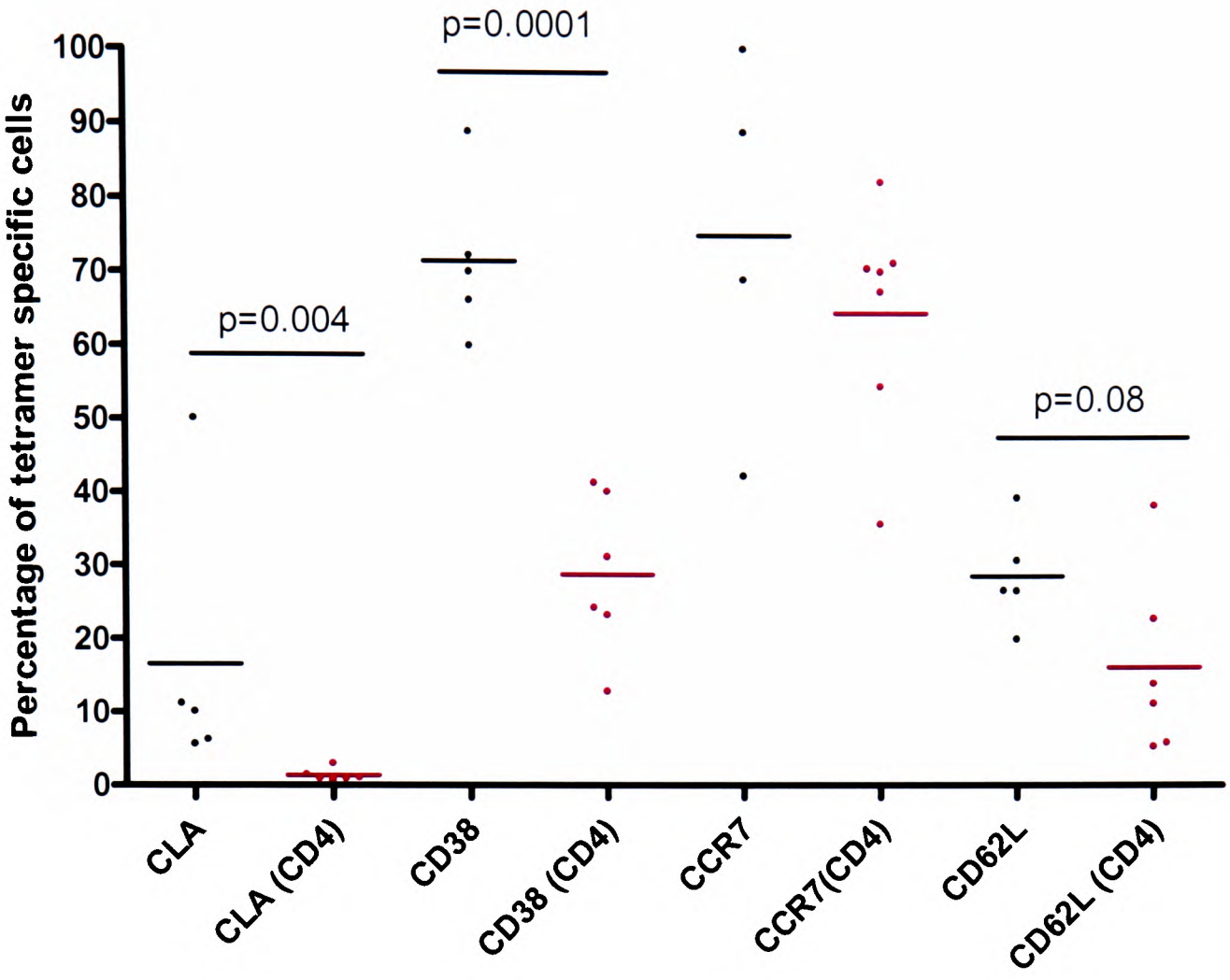
6-10 Comparison of ex vivo tetramer responses in healthy donors and patients with acute chickenpox.

Percentage of ex vivo gE and IE63 DRB1*1501 tetramer specific T cell responses in VZV healthy immune donors and patients with acute primary varicella infection. Cells are gated on CD4+ Viability$^-$CD14$^-$CD19$^-$ cells. The horizontal bar denotes the median.

6.3.2. Phenotypic analysis of tetramer specific CD4+ T cells

As 5 of the 8 patients had tetramer specific frequencies above 0.01% (of the whole CD4+ T cell population) I went on determine of the phenotype of the tetramer specific T cells in these individuals.

The CLA expression on tetramer specific T cell (mean, 16.5;SD±18.8) was significantly higher (p=0.004) than CLA expression in the total CD4+ T cell population (mean ,1.25;SD ±0.8) of these patients (Fig. 6-11) CLA expression in the tetramer specific T cells in the patients was also significantly higher (p=0.03) than its expression in healthy immune donors (mean, 2.0; SD ±4.7) (Fig. 6-12), suggesting that a significant proportion of antigen specific CD4+ T cells were homing to the skin in patients with acute infection.

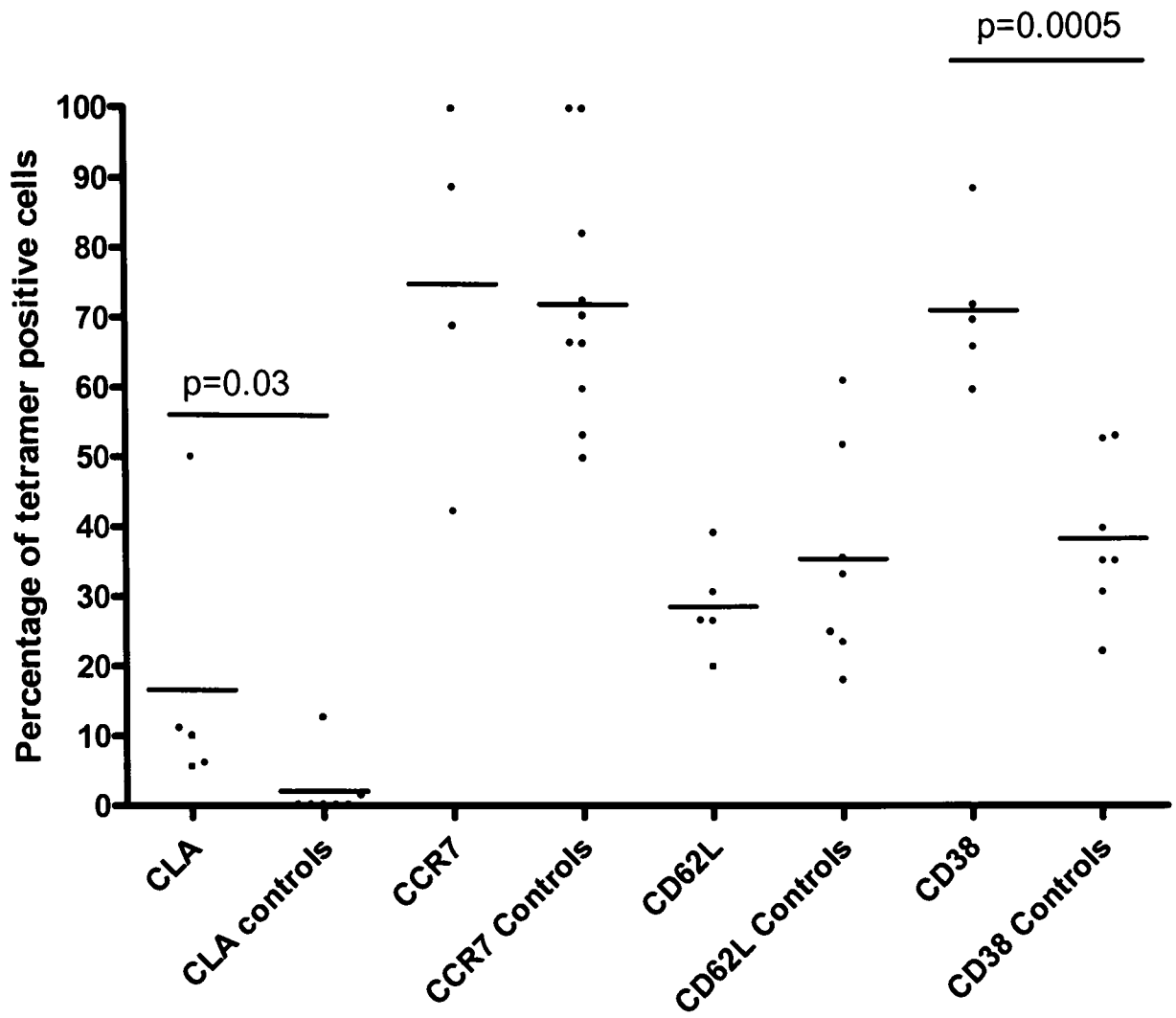


6-11 Phenotypic markers on tetramer specific T cells in patients with acute chickenpox

Comparison of the expression of CLA, lymph node homing and activation markers on tetramer specific T cells (black) and in the total CD4+ T cell population (red) in patients with acute varicella infection. Paired t test was used in p value calculation.

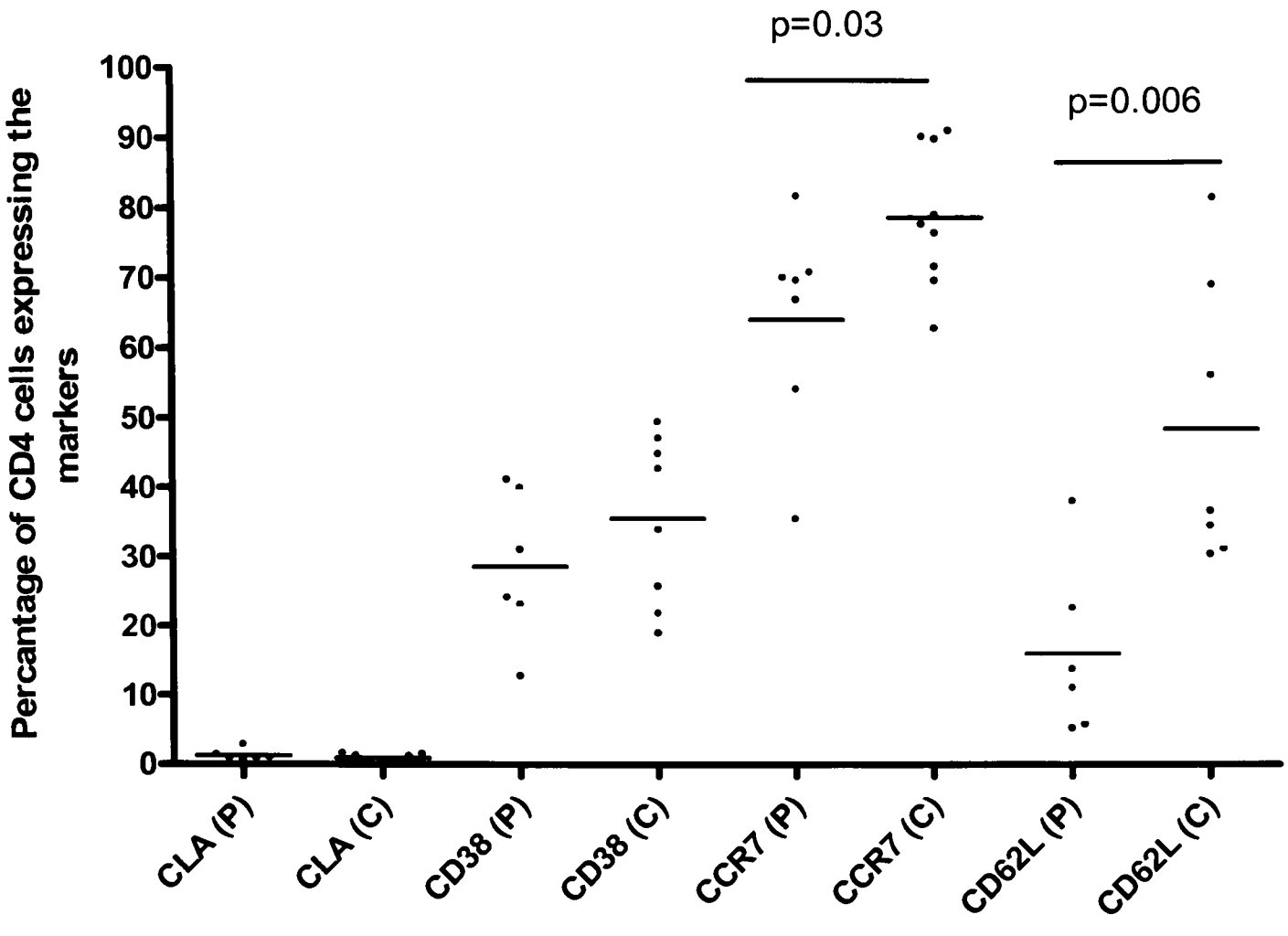
The majority of the tetramer specific cells expressed the lymph node homing marker CCR7 (mean 75.05; SD± 25.32), whereas the expression of CD62L was much lower (mean 28.68; SD± 7.09). Expression of CCR7 by the tetramer specific T cells were similar to its expression in the whole CD4+ T cell population, while the expression of CD62L in the whole CD4+ T cell population was even lower than its expression on tetramer specific T cells (mean 16.17; SD±12.59). However, expression of both lymph

node homing markers CCR7 and CD62L in the tetramer specific T cell in patients and healthy immune donors was similar.



6-12 Phenotypic differences in tetramer specific T cells in patients and healthy donors

Comparison of the expression of CLA, lymph node homing markers and activation markers on the tetramer specific T cells in healthy immune donors (purple) and patients with acute infection (black). Unpaired t test was used in p value calculation.

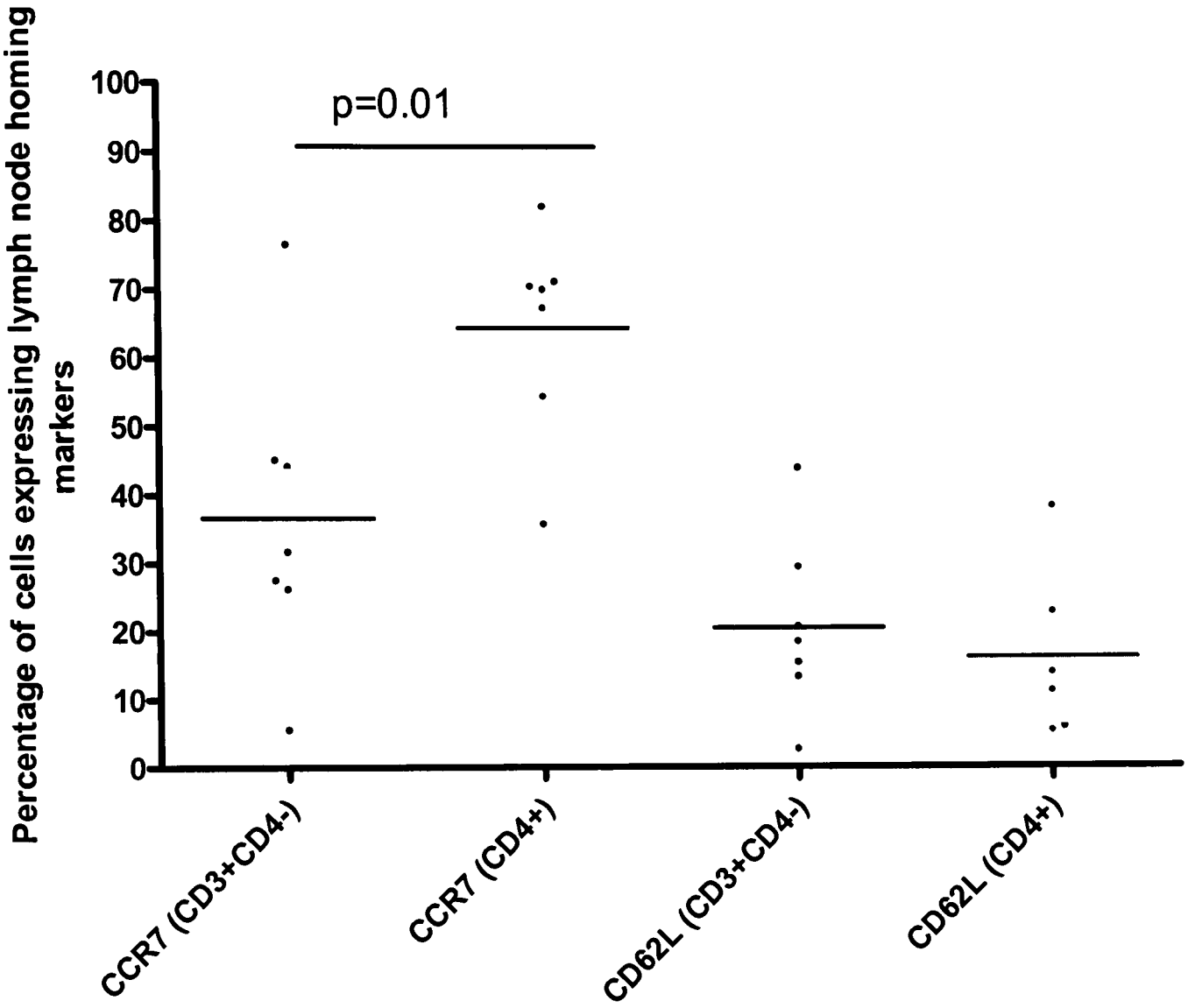


6-13 Differences in the phenotype in the whole CD4+ T cell population in patients and healthy donors

Comparison of the expression of CLA, lymph node homing markers and activation markers on the tetramer specific T cells in healthy immune donors (green) and tetramer specific cells patients with acute infection (black). Unpaired t test was used in p value calculation.

The expression of both CCR7 and CD62L in the whole CD4+ T cell population was significantly lower in the patients with acute infection, when compared to their expression in healthy immune donors (Fig6-13). For instance, the CD62L was expressed in 48.78% (SD±20.65) of the whole CD4+ T cell population in healthy volunteers and in 16.17% (SD±12.59) in the patients. CCR7 expression was also significantly higher ($p=0.03$) in the healthy volunteers (mean 79.18; SD±10) when compared to patients (mean 64.52; SD±15.07). In addition, expression of CCR7 in the CD3+CD4- population

was even lower (mean 36.64; SD±22.03) than the whole CD4+ population in the patients (p=0.01). No difference was seen in the expression of CD62L in the CD4+ T cell population and CD3+CD4- T cell population.



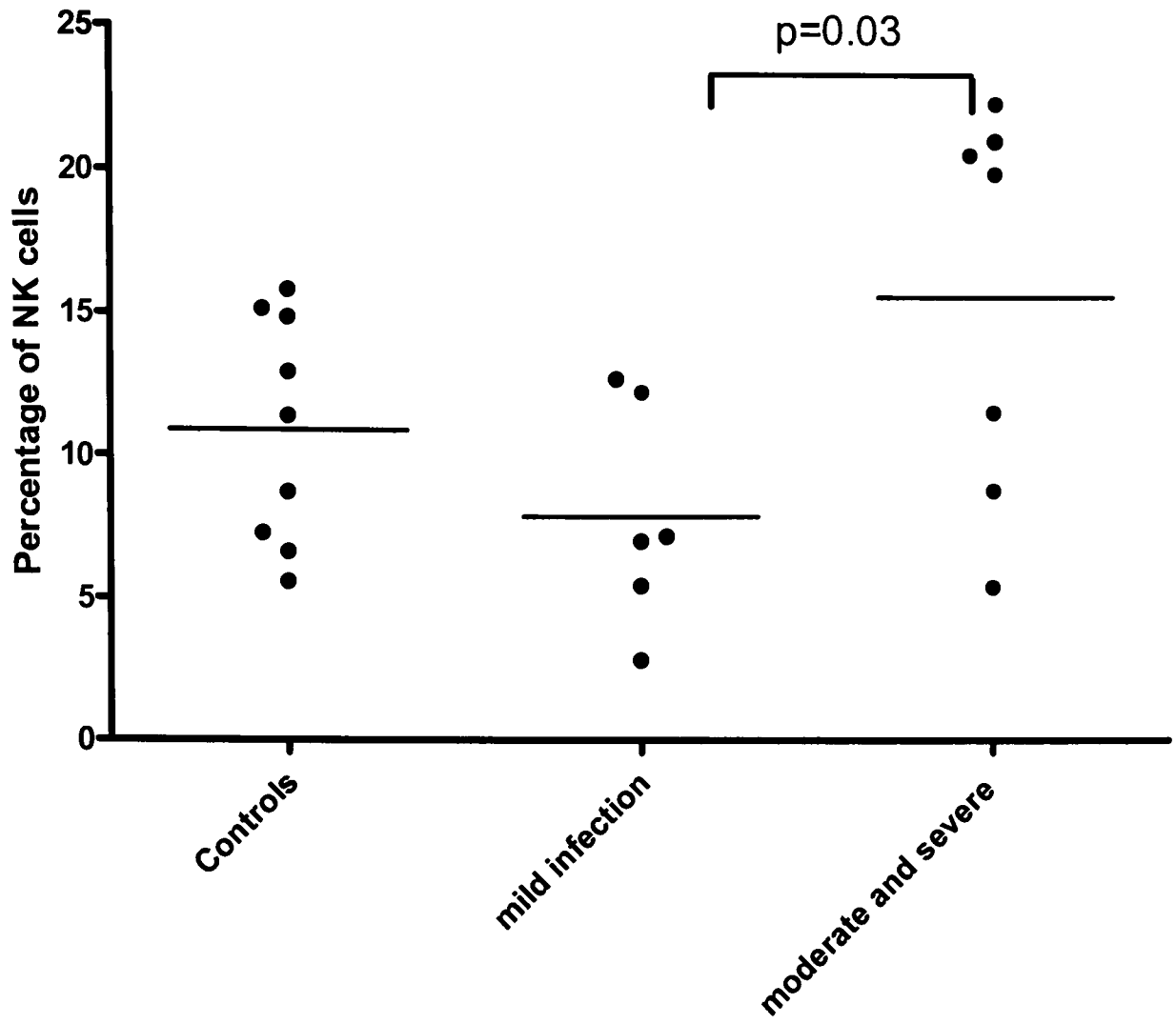
6-14 Expression of lymph node homing markers in the CD3+CD4+ T cell population and CD3+CD4- T cell population in patients with acute primary VZV infection
Paired t test was used in p value calculation.

CD38 was expressed on 71.45% (SD±10.8) of tetramer specific T cells, which was significantly higher (p=0.0001) than its expression on the whole CD4+ T cell population (mean 28.8; SD±10.9). In addition, the expression of CD38 on tetramer specific T cells was also significantly higher (p=0.0005) in the patients when compared to healthy

immune donors (mean 38.55; SD±11.37). However, I did not see any difference in the expression of CD38 in the whole CD4+ T cell population in patients and healthy immune donors. Therefore, it appears that the expression of CD38 by tetramer specific cells in patients with acute infection is consistent with them being recently activated by antigen.

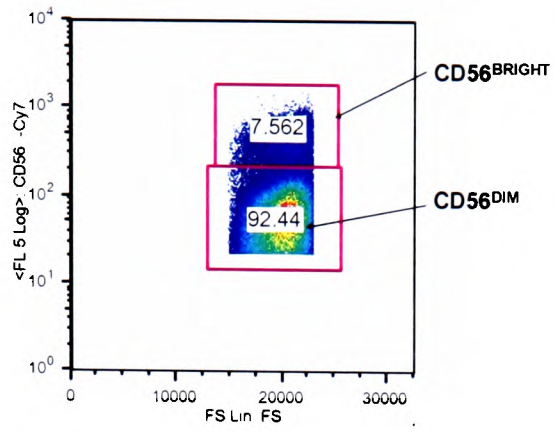
6.3.3. Preliminary analysis of the role of natural killer cells in patients with acute VZV infection

As shown above, the functional T cell responses in patients with moderate and severe infection was impaired. In addition, although not significant VZV specific IFNγ responses in patients with acute infection were lower than healthy VZV immune donors. Therefore, as T cell function appeared to be impaired or delayed in patients with acute infection, I went on to investigate if innate immune mechanisms such as natural killer (NK) cells could be playing a role to control the virus in these patients. As shown below, patients with moderate to severe infection (mean 15.5, SD±6.8) had a significantly higher ($p=0.03$) percentage of NK cells (defined as CD3-CD56+) than patients with mild infection (mean 7.8, SD±3.9) and healthy donors (mean 10.9, SD±3.9) (Fig 6-15).

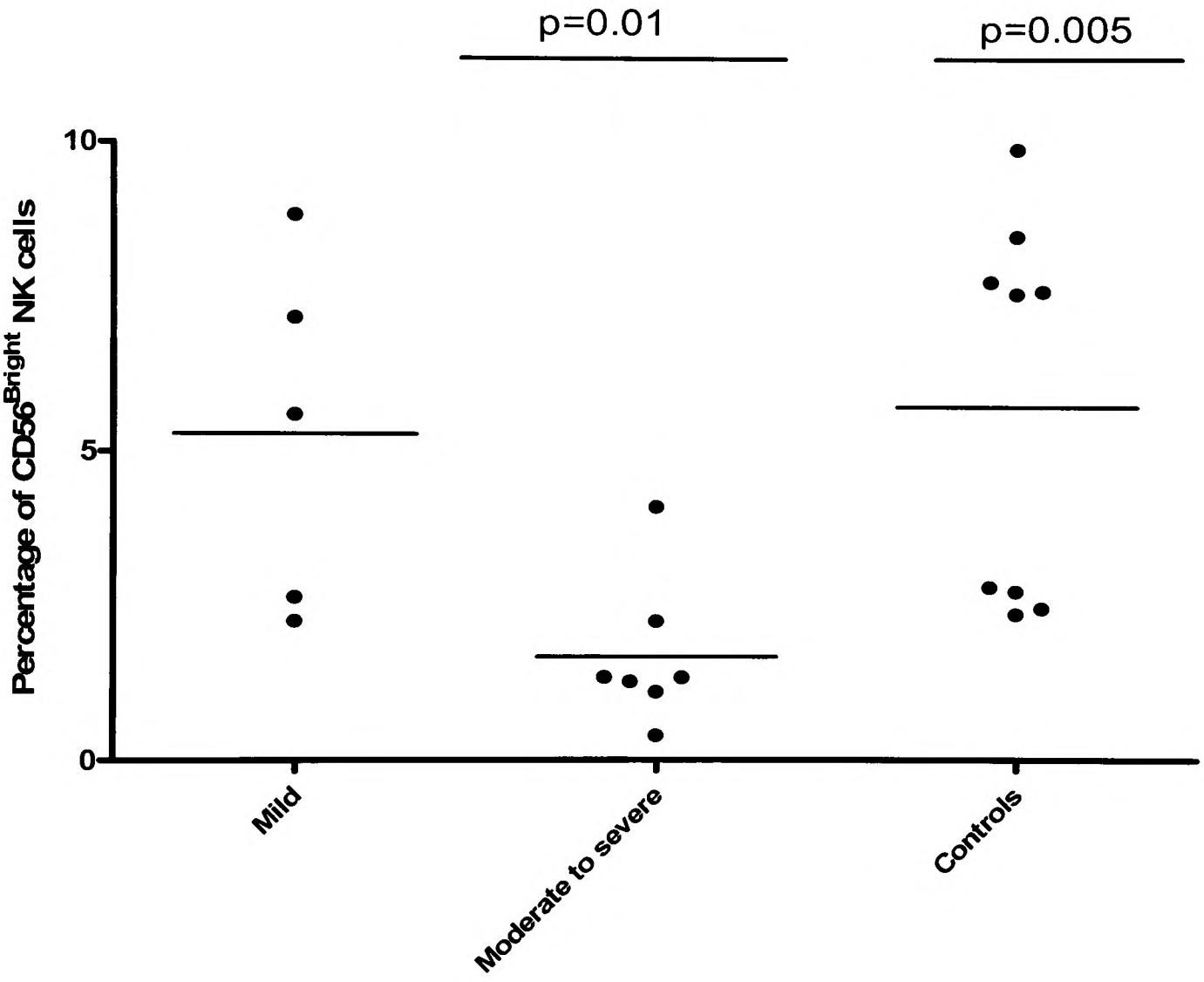


6-15 Percentages of NK cells in patients with moderate to severe infection, mild infection and healthy immune donors.
Unpaired t test was used in p value calculation.

NK cells (CD56+) can be further classified as $CD56^{BRIGHT}$ and $CD56^{DIM}$ as shown below (Fig 6-16). NK cells have shown to comprise of various subsets that differ in function, phenotype and tissue localization (Romagnani 2007). $CD56^{DIM}$ NK cells are thought to express homing markers for inflamed peripheral sites, contain lytic granules for rapid cytotoxicity. In contrast, $CD56^{BRIGHT}$ NK cells express low levels of lytic granules, secrete larger amounts of IFNγ and TNF and proliferate more vigorously upon activation (Romagnani 2007). Patients with moderate to severe disease (mean 1.6, SD±1.2) had significantly less $CD56^{BRIGHT}$ NK cells than patients with mild infection (mean 5.3, SD±2.8) and healthy donors (mean 5.7, SD±3.1).



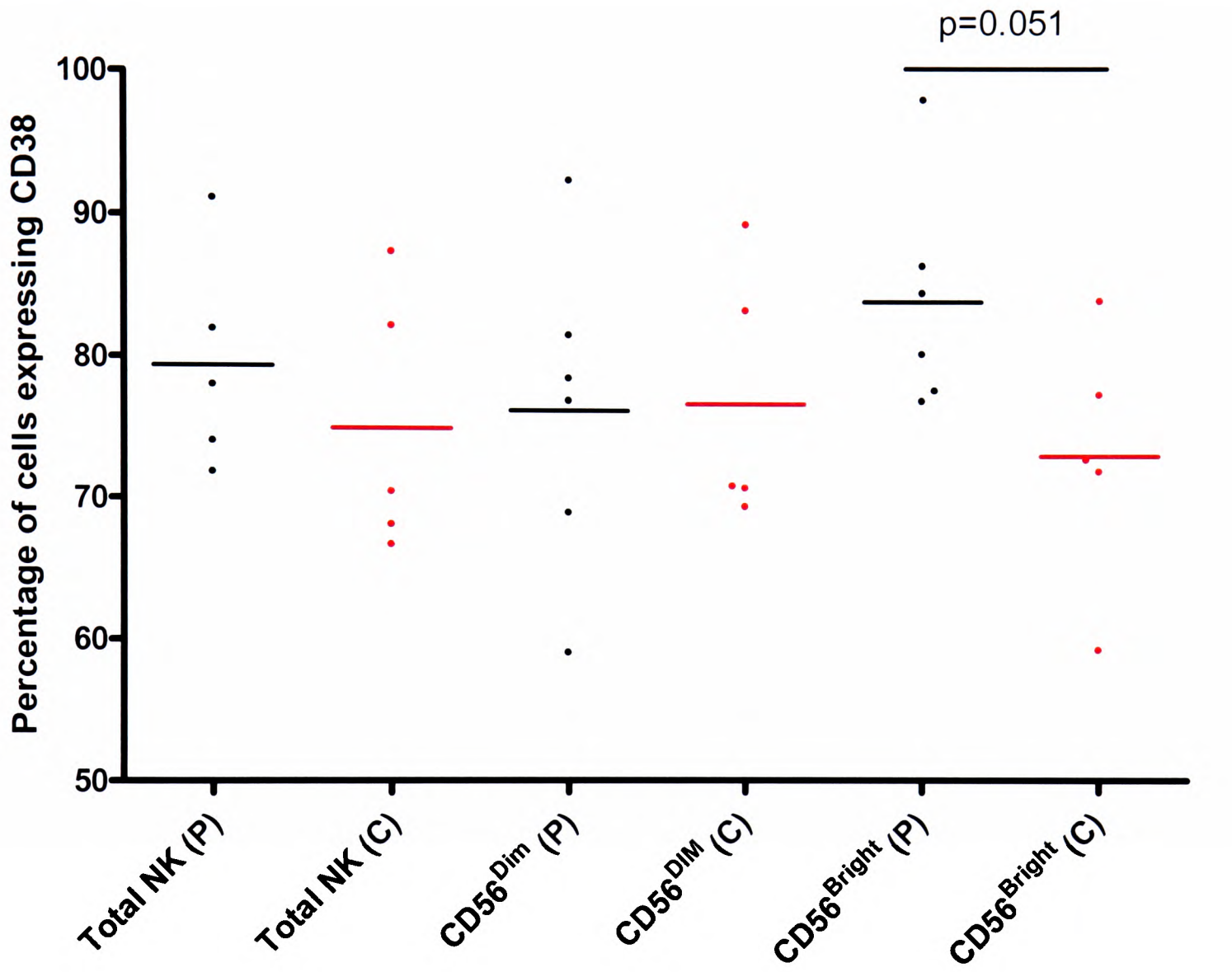
6-16 CD56^{BRIGHT} and CD56^{DIM} populations in a healthy donor



6-17 CD56^{BRIGHT} NK cell percentages in patients with moderate to severe, mild infection and healthy donors.

Unpaired t test was used in p value calculation.

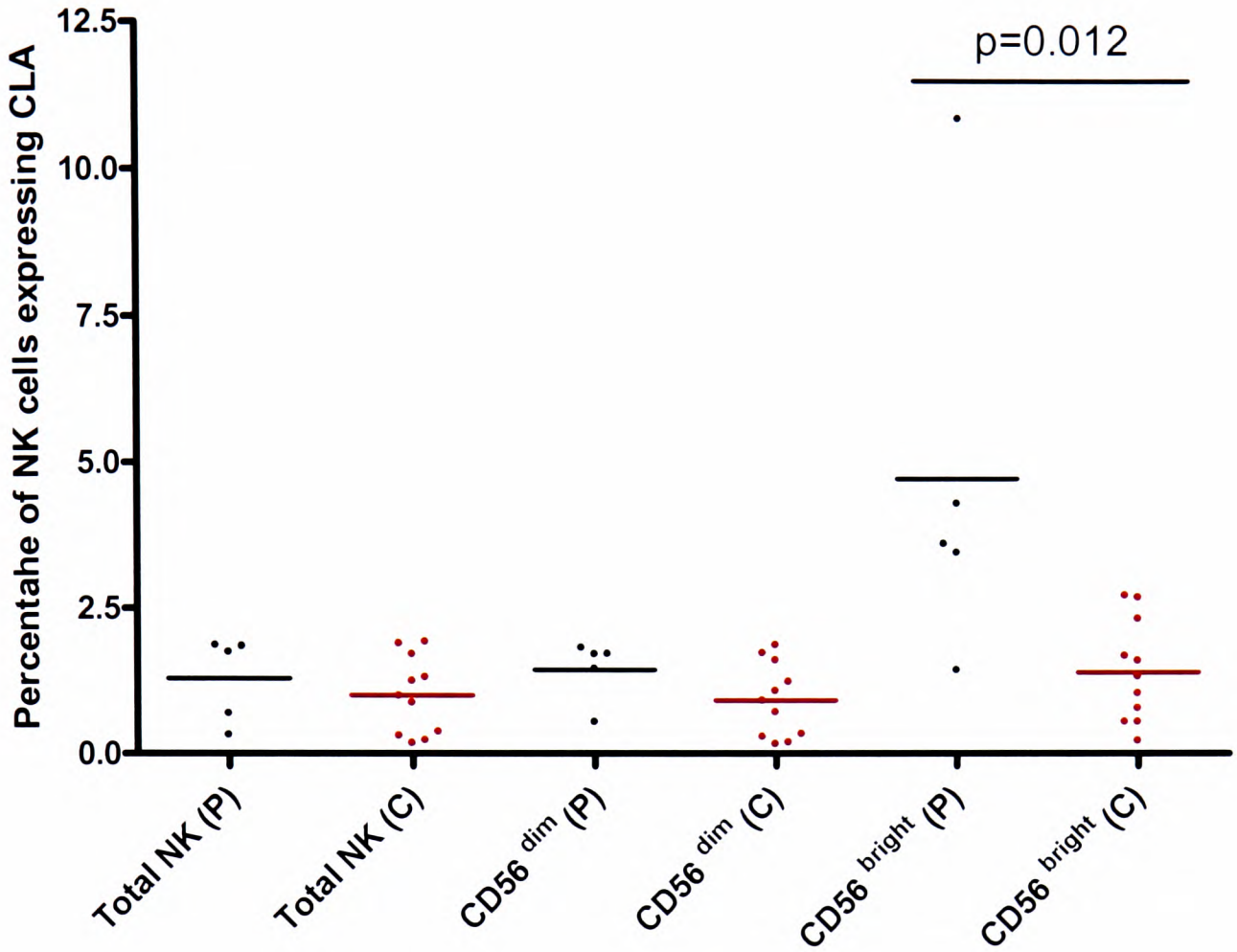
I then went on to investigate the phenotype of the NK cells in a limited number of patients with acute infection. Interestingly as shown below, the $CD56^{BRIGHT}$ NK cell population in patients with acute infection was more activated (expressed CD38) than the $CD56^{DIM}$ population and the total NK cell population (Fig. 6-18).



6-18 Expression of CD38 by the $CD56^{BRIGHT}$, $CD56^{DIM}$ and the whole CD56 cell population in patients with acute infection (P) and healthy donors

As VZV replicates in the skin in acute infection, I then went on to investigate if the NK cells in patients with acute infection expressed cutaneous lymphocyte antigen (CLA) and were thus able to home to the skin. As seen with CD38 expression, the $CD56^{BRIGHT}$

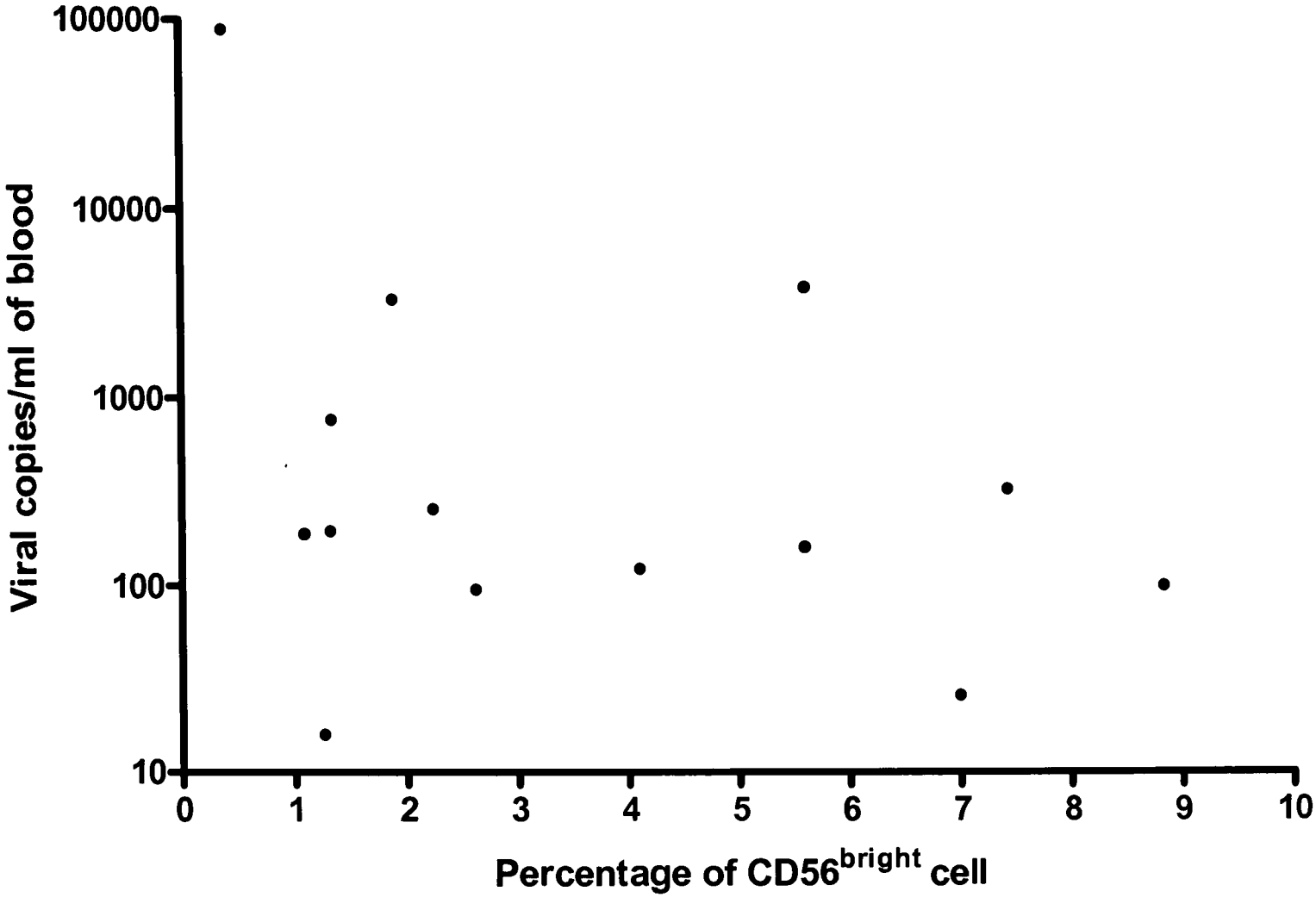
population of patients with acute infection had a significantly higher expression of CLA (p=0.012) than the $CD56^{DIM}$ population and the whole CD56+ cell population.



6-19 Expression skin homing markers by the $CD56^{bright}$ and $CD56^{dim}$ NK cells

Comparison of CLA expression in different populations of NK cells in patients (black) and in healthy donors (red). Unpaired t test was used in p value calculation.

As I found a significant association with the viral loads and VZV specific IFNγ production, I then proceeded to determine if there was any relationship between the NK percentages and the viral loads (see chapter 7). However, no association was seen with the total NK cell percentages of with the $CD56^{BRIGHT}$ percentage of cells in patients with acute VZV infection (Fig 6-20).



6-20 Correlation of viral loads with the percentage of $CD56^{BRIGHT}$ population in patients with acute VZV infection

Correlation was determined by the use of Spearmans r value.

6.4. Discussion

These data show the association of T cell responses with clinical disease severity in adult patients with primary VZV infection. It is well established that adults are more likely to develop more severe disease and complications (Galil et al., 2002a; Meyer et al., 2000b) in primary VZV infection and indeed these results show that the clinical disease severity score increases significantly with advancing age.

Functional T cell responses in acute VZV infection

A huge variation was observed in VZV-specific IFNγ responses in patients with acute infection. Responses were also observed for the some VZV glycoproteins (gE and gI) and for two of the immediate early proteins (IE4 and IE63) that were tested. However, 4 patients did not have any detectable VZV-specific IFNγ responses in acute infection, and specific IFNγ responses were not seen in 2 of these patients even at day 16 post infection. Patients with milder disease had more VZV-specific IFNγ responses than those with more severe disease ($p=0.05$). Moreover, patients with a >500 skin lesions also had lower VZV-specific T cells than those with a lesser number of lesions. However, even in patients with mild disease, the virus specific IFNγ responses were similar to those observed in healthy VZV immune donors. Therefore, a vigorous expansion of virus specific T cell responses as seen in many other acute viral infections (Callan et al., 1998; Jaye et al., 2003) was not observed in patients with acute primary VZV infection. However, it is also possible that VZV-specific T cells are in the skin or other peripheral sites and therefore, lower numbers of antigen specific T cells would be circulating in the peripheral blood.

Although patients with acute infection had comparatively less VZV-specific T cells as determined by IFNγ secretion, they had a higher frequency of gE and IE63 DRB1*1501 tetramer- specific T cell responses. Therefore, it is possible that although a high frequency of antigen specific T cell responses were present in these patients these were non-functional. On the other hand, it is possible that VZV-specific T cells were predominantly secreting other cytokines other than IFNγ, and therefore, were not detected by the IFNγ ELISpot assays. As tetrameric complexes do not rely on functional capability of T cells, they are also not restricted to the measurement of one or more of the functional activitities (van Baarle et al., 2001). Therefore, the use of tetrameric complexes to identify antigen specific T cells could result in higher detection rates than those identified by functional T cell assays. However, the possibility that VZV could preferentially infect antigen specific T cells and therefore alter their immune functions is currently being explored by our laboratory.

Collectively these data suggest that patients with severe disease had impaired functional VZV-specific T cell responses and also in acute VZV infection VZV-specific T cell responses are generally delayed. This would indicate that either milder disease is associated with the development of a stronger VZV-specific immune response which results in elimination of the virus and reduced clinical disease severity. On the other hand, it is possible that in patients with more severe clinical disease had a higher viral load and therefore, possibly more infection of their T cells thus making them dysfunctional. A third possibility would be that as patients with more severe clinical disease had a higher number of skin lesions, virus specific T cells could be localized in

the skin thereby reflecting a lower number of specific T cells in the peripheral blood. If this is the case, individuals with more severe disease might be expected to have higher numbers of antigen specific T cells once infection resolves due to return of these cells into the peripheral blood. We are planning longitudinal studies of blood and skin vesicle scrapings for my return to Sri Lanka.

Although patients with acute VZV infection seemed to have a higher percentage of CD8+ T cells than healthy individuals this was not significant and the CD4:CD8 ratios were similar to those seen during health. However, a significant reduction was seen in the percentages of CD4+ T cell and a significant increase in the CD3+CD4-CD8- population of T cells. Despite the apparent reduction in CD4+ T cells in the patients, surprisingly, VZV-specific IFNγ responses were predominantly mediated by CD4+ T cells as seen in healthy immune donors. As VZV-specific immune responses were only evaluated by IFNγ secretion in these patients, it is possible that the virus specific CD8+ T cells if present, were producing different cytokines or that they only exerted their antiviral effects by lysis of infected cells. Although, CD8+ T cell are thought to play a major role in eliminating the virus in acute infection, virus specific CD4+ T cells have been shown to possibly play a more 'dominant' role in some herpes virus infections. For instance, it was shown that IFNγ secreting CD4+ T cell responses developed earlier in patients with asymptomatic CMV infection than in patients with symptomatic infection and was associated with clearance of the virus. Moreover, despite the presence of specific antibody and CMV-specific CD8+ T cells, clearance of virus in symptomatic patients

only occurred after emergence of IFNγ secreting CD4+ T cells (Gamadia et al., 2003; Rentenaar et al., 2000).

As mentioned above patients with acute VZV appear to have delayed VZV-specific T cell responses. Similar findings have been observed in other primary herpes viral infections. Delayed CMV-specific proliferative CD4+ and CD8+ T cell responses were observed in both pregnant and non pregnant women with primary CMV infection and these responses were lower than CMV seropositive individuals even at day 235 post infection (Lilleri et al., 2007). However, others have shown that while frequency of CMV-specific CD4+ T cell responses during primary CMV infection was similar to healthy CMV seropositive individuals, there was a significant CMV-specific CD8+ T cell expansion during acute primary CMV(Sester et al., 2002b). Therefore, investigation of longitudinal CD4+ and CD8+ T cells response and their relationship with viral clearance in patients with acute infections, would enable us to define the relative role of each of these T cell subsets in patients with acute infection.

Phenotype of antigen specific T cells in patients with acute primary VZV infection

During acute viral infections, a rapid and substantial expansion of antigen specific CD8+ and CD4+ T cells occurs (Whitmire et al., 2006) in order to exert their antiviral effects (Barry and Bleackley, 2002; Whitmire et al., 2006). For instance, in EBV infection CD8+ T cell frequencies to a single EBV epitope has been shown to range from 5.6% to 6.6% of the whole CD8+ T cell population in the majority of individuals, whereas some individuals responses were between 40% to 44% (Callan et al., 1998). However, as

mentioned above and as found by others, VZV-specific T cell responses have been shown to be predominantly mediated by CD4+ T cells (Asanuma et al., 2000; Jones et al., 2006; Jones, 2007; Milikan et al., 2007; Park et al., 2004; Vossen et al., 2004). Possibly due to the paucity of VZV-specific CD8+ T cells, currently there has been only one VZV-specific CD8+ T cell epitope defined. However, this CD8+ epitope specific response was only seen following *in vitro* expansion with the peptide, and even so only at a very low frequency (Frey et al., 2003). Therefore, the phenotypic analysis of tetramer specific T cell responses in these patients had to be limited to the investigation of CD4+ tetramer specific responses.

Although the frequency of gE and IE63 DRB1*1501 tetramer specific T cell responses were higher in patients with acute infection when compared to healthy immune donors, the frequencies were still lower than in seen in most other viral infections. However, interestingly, equal tetramer specific T cell frequencies were seen for both gE which is a glycoprotein expressed during later stages of viral replication and for IE63 which is an immediate early protein. In acute EBV infection, CD4+ T cell responses specific for the lytic proteins were found to be higher than those for the latent proteins (Precopio et al., 2003).

CD38 is a cell surface molecule which is a marker of T cell activation. It is associated with the CD3/TCR complex on the surface of T cells and signals through MAP kinases which results in T activation, cytokine production and proliferation of T cells (Deaglio et al., 2001). CD38 was expressed on 71.45% of tetramer specific T cells in patients with

acute infection and were significantly more activated than the whole CD4+ T cell population (28.8%) suggesting that only antigen specific T cells showed evidence of activation.

Naïve T cells constitutively express CD62L and CCR7 which bind to their specific ligands (e.g. CCL19 and CCL21) in high endothelial venules, which enable them to continuously re-circulate through blood and the lymph nodes until they encounter their specific antigen. However, during acute infection with EBV,CMV and HIV, antigen specific T cells have been shown to lose the expression of CCR7 and CD62L once activated which possibly enables them to home to peripheral sites and remain the blood where their in on going infection (Chen et al., 2001). However, in HIV infection the lack of CCR7 and CD62L expression by T cells may contribute to the failure of the CD8+ T cells to clear the virus as viral replication mostly occurs in lymph nodes(Chen et al., 2001). In addition to being one of the main chemokine receptors that allow T cells to enter high endothelial venules, CCR7 is also thought to be important in regulating T cell movement within lymph nodes and facilitate entry in to the efferent lymphatics. The overall expression of CCR7 by the whole CD8+ T population has shown to be around 15% in individuals with HIV compared to 60% of CD8+ T cells in non HIV positive healthy volunteers (Chen et al., 2001).

In patients with acute VZV infection, 75.05% of the tetramer specific T cells expressed CCR7 and 28.68 % expressed CD62L. Although expression of CCR7 was similar to the rest of the CD4+ population, CD62L expression of tetramer specific cells was higher than

the whole CD4+ T cell population. In addition, the expression of these 2 lymph node homing molecules on tetramer specific T cells was similar to expression levels seen in VZV immune healthy individuals (Jones, 2007). However, the expression of both CCR7 and CD62L was significantly less in the whole CD4+ T cell population in the patients when compared to controls. In addition, expression of CCR7 by CD3+CD4- T cells (36.64%) was even less than its expression in the whole CD3+CD4+ T cell population (64.52%) in these patients. Therefore, it appears that in acute VZV infection, the majority of tetramer specific T cells identified by the 2 tetramers were capable of continued homing to the lymph nodes. This could be appropriate in VZV infection, as like in HIV, viral replication is thought to occur in peripheral lymph nodes at least during the early phase of infection. On the other hand, it is possible that antigen specific T cells defined by these 2 tetramers were not the most immune dominant and important in controlling the virus in acute infection.

Cutaneous leucocyte antigen (CLA) is expressed by T cells homing to the skin(Agace, 2006) and was expressed by 16.56% (SD±18.8) tetramer specific cells in patients with acute infection. This expression was significantly higher than the whole CD4+ T cell population in patients with acute infections and also higher than expression levels seen in healthy VZV immune donors (Jones, 2007). This suggests that antigen specific T cells in patients with acute infection probably home to the skin where there is active viral replication. However, there was no difference in the CLA expression in the whole CD4+ T cell population in patients with acute infection and healthy VZV immune donors. Therefore, based on CD62L and CCR7 expression although the majority of CD4+ and

CD3+CD4- T cells appeared to be homing to peripheral sites in patients with acute infection they do not appear to be dominated by cells homing to the skin. However, it will be important to examine other markers that are putatively linked to skin homing such as CCR4 and CCR10.

Natural killer cells in primary VZV infection

NK cells are cells of the innate immune system that provide early defense against invading micro organisms. They eliminate intracellular microorganisms by killing mechanisms similar to those of CD8+ T cells and also secrete many cytokines such as IFNγ and TNFα (Bryceson et al., 2006). NK cells are defined by the expression of CD56, CD16 and by lack of expression of CD3+ (Chidrawar et al., 2006). Of these molecules, expression of CD16 has shown to vary while CD56 expression is more stable (Romagnani et al., 2007). Based on the brightness of the expression of the CD56 molecule, NK cells can be broadly classified as CD56bright and CD56 dim. CD56 dim cells are thought to have a more cytolytic potential (Jacobs et al., 2001) and are thought to express homing molecules that enable them to preferentially home to peripheral sites (Campbell et al., 2001). In contrast, CD56 bright NK cells have shown to express less cytolytic granules but are able to proliferate more vigorously and secrete anti viral cytokines such as IFNγ and TNFα (Romagnani et al., 2007). Although there has been much debate regarding the differentiation of CD56 bright and CD56 dim NK cells, it was recently shown that in fact CD56 bright cells were precursors of CD56 dim cells and that following activation, CD56 bright cells acquire the characteristics of CD56 dim NK cells (Romagnani et al., 2007).

As mentioned above, VZV-specific functional T cell responses were delayed especially in patients with severe clinical disease. In addition, a significant increase in the CD3+CD4-CD8- population of T cells was seen in patients with acute infection. Therefore, I have started to explore the possibility if other innate cell types such as NK cells that could be controlling the virus during early infection, especially in those with severe disease. As shown, the percentage of NK cells was significantly increased in patients with more severe forms of infection when compared to those with mild infection. Furthermore, the population of CD56 bright cells was significantly lower in those with more severe disease. Therefore, this suggests that NK cells could be important, especially in patients with severe disease. In addition, the fact that the CD56 bright NK cell population was significantly less in those with severe infection, could be suggestive of rapid proliferation and 'conversion' of this population to CD56 dim cells which have greater cytotoxic abilities. Indeed, these results indicate that the CD56bright population in the patients was far more activated than in healthy individuals and also expressed CLA which indicates that these preferentially home to the skin. Therefore, it is quite possible that the NK cells play a significant role in primary VZV infection by controlling viral replication until the development of virus specific T cells.

NK cells deficiencies are thought to result in severe herpes virus infections, including VZV, EBV, CMV and HSV(Orange, 2002). In addition, occurrence of human herpes virus-8 associated disease in patients with HIV is thought to be associated with NK cell dysfunction (Sirianni et al., 2007). The importance of NK cells in HSV infection and their role in controlling the disease and the viraemia has also been demonstrated in

several mouse models (Mossman and Ashkar, 2005; Reading et al., 2006). The role of NK cells in these infections is not surprising, as most herpes viruses like VZV down regulates MHC class I as an immune evasion strategy (Abendroth et al., 2001a; Eisfeld et al., 2007). This is in turn removes the inhibitory signals for the some of the NK cells and thereby results in NK cell activation.

In acute primary EBV infection, NK cell numbers were shown to inversely correlate with the viral load (Williams et al., 2005). As we did not know the total white cell count in patients with acute primary VZV infection, the relationship between viral loads was only analyzed with NK cell percentages which were not significant. Therefore, given their potential role in controlling the virus in acute VZV infection and also due to their expansion which was especially seen in patients with more severe grades of infection, the role of NK cells in acute VZV infection needs to be investigated in greater detail. We plan to incorporate more detailed NK and indeed NKT marker analysis when we analyze the samples from future longitudinal studies.

Chapter 7. Viral loads in patients with acute primary varicella infection

7.1. Introduction

VZV infects many cell types in the host during acute infection including T cells, B cells, monocytes and dendritic cells(Abendroth et al., 2001b; Ito et al., 2001; Konig et al., 2003). Infection of T cells by the virus is thought to be one of the main mechanisms by which the virus disseminates around the body subsequently infecting keratinocytes and other cells(Abendroth et al., 2001b; Ku et al., 2002). During the viraemic phase, which is highly cell associated (Ku et al., 2005), VZV is thought to predominantly infect T cells (Ku et al., 2004; Moffat et al., 1995). In addition, Ku CC *et al* have suggested that the virus preferentially infects CD4+ T cells (by *in vitro* studies in the SCIDhu mouse model). However, Ito *et al* who investigated the viral loads in children with acute primary VZV infection showed that all lymphocyte sub populations were infected by the virus and the viral loads in T cells, B cells and the monocytes were similar(Ito et al., 2001). In fact the mean viral loads in CD8 + T cells (2.1, SD±0.3) and CD14+ cells (2.3, SD±0.4) were higher than the mean viral loads in CD4+ cells (1.5, SD±0.4). However, this difference was not statistically significant (Ito et al., 2001). Similar results were seen by Mainka *et al* who also showed that T cells, B cells as well as monocytes expressed gE on their cell surface by confocal microscopy. However, his results showed that two thirds of the PBMCs infected were the T cells, whereas the B cells and monocytes consisted of the remaining 1/3 of infected PBMCs (Mainka et al., 1998).

In acute primary VZV infection viral loads have been reported in the range of 1 to 5000 viral copies per 10^5 PBMCs (Kimura et al., 2000; Mainka et al., 1998) and 100 to 10000

per ml of blood (de Jong et al., 2000) by using quantitative real time PCR assays. However, only a very small percentage of PBMCs have been shown to be actually infected during acute chickenpox. Confocal microscopy studies in which PBMCs were stained anti VZV gE antibody showed that only 0.01% to 0.001% of the PBMC were infected (Mainka et al., 1998).

The virus has also been detected in patients with herpes zoster (HZ) and the viral loads observed have been several fold lower than in patients with acute infection(Kimura et al., 2000; Mainka et al., 1998). In addition, although the VZV virus has been recovered from the peripheral blood in patients with acute chickenpox, not all patients with HZ have a detectable viraemia(Kimura et al., 2000; Quinlivan et al., 2007a). By using quantitative real time PCR a detectable viraemia was only observed in 16% to 78% (Kimura et al., 2000; Mainka et al., 1998; Quinlivan et al., 2007a) of patients with acute HZ. The differences in the range of detection of the virus in acute HZ could be due to differences in methods of viral DNA extraction and also the sensitivity of the quantitative real time PCR assay. In these studies the mean viral loads of these patients were 1160 copies/10^5 PBMCs(Quinlivan et al., 2007a). Higher viral loads have been observed in patients with disseminated HZ and in immunocompromised patients with HZ by some (Aberle et al., 2005; de Jong et al., 2000). In contrast, Quinlivan *et al* who investigated the viral loads and severity of HZ in 130 patients, reported that they did not find a significant differences in the viral loads in patients with uni dermatomal HZ when compared to those who had multi dermatomal HZ. Instead, they reported that viral loads correlated with older age and poor immune status of the patients. They suggest that the higher viral loads in older

patients and immunocompromised patients are probably due to inability of the immune system to control the virus. In addition, they have showed that the baseline viral loads did not correlate with the occurrence of post herpetic neuralgia but was associated with prodromal pain prior to the onset of the rash.

Vesicles in patients with chickenpox and HZ are thought to be equally infectious. Viral loads in the range of 10^8 and 10^{10} per ml of vesicular fluid have been observed in the skin lesions in acute primary varicella and HZ (Kimura et al., 2000). Therefore, although the viral loads in the PBMCs were far less in patients with uncomplicated HZ they appear to be similar in the skin lesions.

In many infectious diseases, viraemia is thought to be associated with severity of clinical disease(Lavreys et al., 2002; Screaton and Mongkolsapaya, 2006; Simpson et al., 2002). However, in some infections such as chronic hepatitis C infection, it has been shown that the magnitude of the viraemia has no correlation with disease severity (Puoti et al., 1999). As clinical disease due to VZV is not thought to be due to immune mediated mechanisms but rather due to the cytopathic effects of the virus, it is likely that individuals with higher viral load could have more severe disease. For instance, in patients with neurological disease due to VZV reactivation, the CSF viral loads were shown to correlate with clinical disease severity and also were found to be higher in patients with encephalitis when compared to those with meningitis. The viral loads were shown to be highest in patients with most severe encephalitis (Aberle et al., 2005).

Although, a few studies have investigated the viral loads in acute primary infection, the degree of correlation of viraemia with clinical disease severity is unknown. In addition, as mentioned earlier Quinlivan *et al* showed that the viral loads were not significantly different in patients with more severe HZ when compared to those with milder disease. Although they suggested that the viral load is more likely to correlate with the ability of the immune system to control the virus they have not investigated this. Therefore, no data are currently available regarding the association of viraemia with the function and the magnitude of the VZV specific T cell response in acute primary VZV infection. In addition, whether or not the viraemia correlates with clinical disease severity in acute infection is unknown. Therefore, I set out to investigate the association of viraemia with clinical disease severity and with the T cell response during acute primary VZV infection. As quantitative real time PCR is currently the most sensitive method for detecting viral loads I started by developing a quantitative PCR assay to detect VZV viral loads.

7.2. Methods

7.2.1. Viral DNA extraction from the live VZV vaccine

QIAamp DNA blood Mini Kit (51306) was used to extract viral DNA from the live VZV vaccine according to the manufacturer's instructions. As the live attenuated vaccine contained free virus particles, 1µl of poly dT was added to 200µl of the VZV vaccine prior to DNA extraction. The DNA was eluted in 200µl of buffer AE and the DNA concentration and purity measured with the nanodrop. Absorbance was measured at 260nm. Purity of the DNA was calculated by the A_{260}/ A_{280} ratio.

7.2.2. PCR

PCR was performed to obtain the PCR product of the ORF 29 gene to use as an insert in a plasmid. Therefore, the PCR was carried out using a forward and a reverse primer of VZV ORF 29. The primers used were:

Forward primer: 5'-CGTACACGTATTTTCAGTCCTCTTC-3'

Reverse primer: 5'-GGCTTAGACGTGGAGTTGACA-3'

The PCR product was predicted to be 85 base pairs:

cgtacacgtattttcagtcctcttcaagtggaaccactaccgcccgtggagcgcgtcgaaacgatgtcaactccacgtctaagcc

The PCR reaction was set as follows:

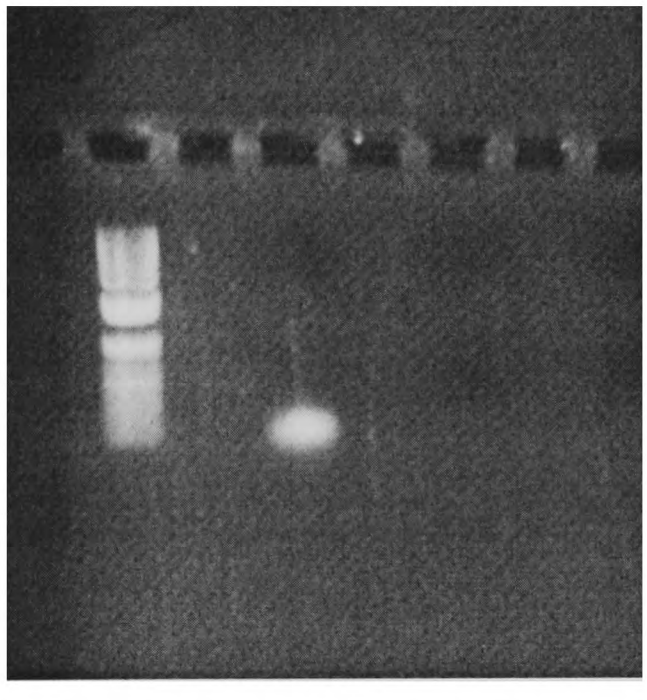
Buffer	5µl
dNTP	1µl
Forward primer	3µl (30pmols)
Reverse primer	3µl (30pmols)
$MgCl_2$	3µl (25mmol/L)
Taq polymerase	0.5µl
DNA	2.5µl (50ng)
Water	32µl
Total	50µl

The $MgCl_2$ concentrations and DNA concentrations were titrated to determine the optimum conditions. Taq polymerase was used as the enzyme because it has a non template dependant terminal transferase activity that adds a single deoxyadenosine to the 3' ends of the PCR product. This extension is required for successful topo cloning.

The PCR was set as follows:

95°C	5 minutes	
95°C	1 minute	35 cycles
50°C	1 minute	
72°C	1 minute	
72°C	10 minutes	

6µl of the product was run on a 1% agarose gel (0.5g of agarose, 50ml of TAE and 2µl of Ethidium bromide at 5µg/ml) and run at 50mV for 25 minutes before visualizing under UV illumination.



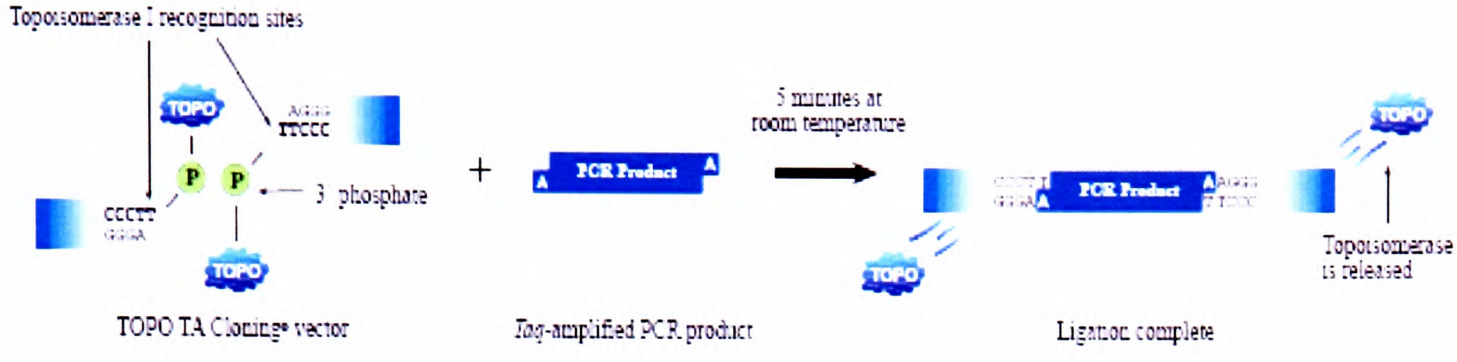
7-1 The 100kb PCR product obtained following PCR with the ORF29 primers

7.2.3. Purification of PCR products

QIAquick PCR purification kit was used for purification of the PCR product and purification was carried out according to manufacturers' instructions.

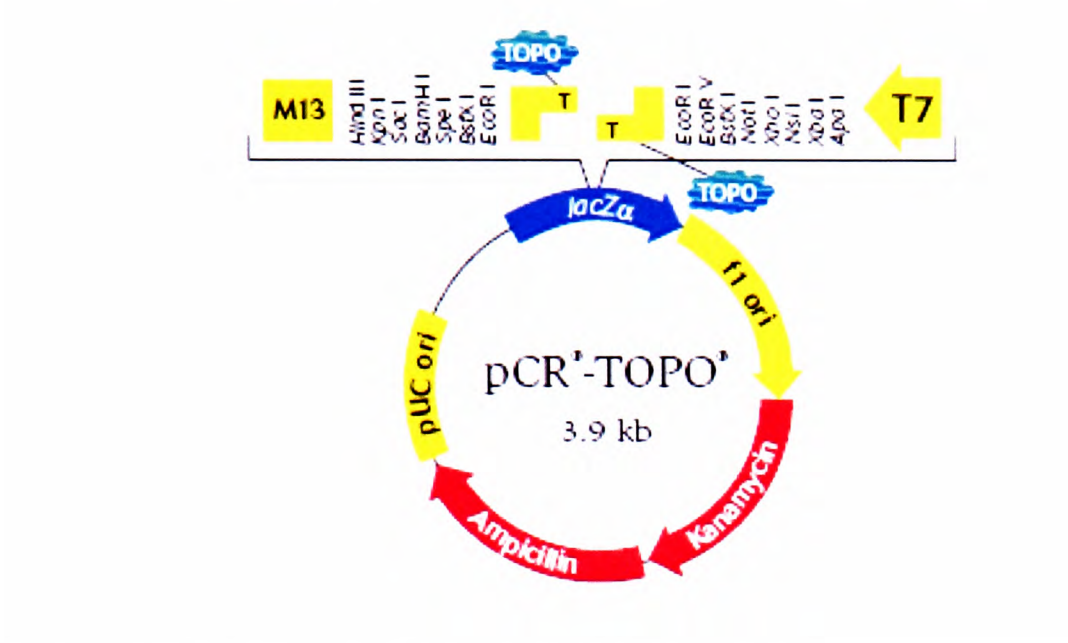
7.2.4. Topo cloning

Cloning of the PCR into the vector was done using the TOPO® cloning technique which uses an enzyme DNA topoisomerase I which functions both as a restriction enzyme and a ligase.



7-2 Diagrammatic representation of the Topo cloning reaction
Image from Qiagen TOPO cloning brochure

The PCR product was inserted to the vector pCR 2.1-TOPO® which is a 3.9kb vector with an ampicillin resistant gene. It also has linearalised 3'T overhangs to accept the PCR products containing 3'A overhangs. It also has EcoR1 sites flanking the PCR product insertion site for easy removal of the PCR product.



7-3 A diagrammatic representation of the TOPO vector
Image from Qiagen TOPO cloning brochure.

7.2.5. Setting up of the TOPO® cloning reaction

1µl of the salt solution (1.2M of NaCl and 0.06M of $MgCl_2$) was added to a 1.5ml microfuge tube. 1µl of the vector and 4µl of the PCR product was added to this and the sample was kept at room temperature for 20 minutes.

7.2.6. Transforming competent cells

2µl of the TOPO® cloning reaction was added to a vial of One Shot DH5α™-T1 chemically competent *E.coli* and was mixed gently. It was incubated on ice for 15 minutes and the cells were then heat shocked for 30s at 42°C without shaking. The sample was then immediately transferred to ice. 250µl of the S.O.C. medium was added to the sample and the tube tightly capped and shacked horizontally for 1 hour at 37°C. 60µl and 80µl of the transformed competent *E. coli* were plated on to 2 pre-warmed LB

agar plates (with ampicillin) which had 40µl of X-gal plated (40mg/ml). The plates were then incubated for 16 hours at 37°C.

X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) was used in blue/white colony screening to detect recombinants (white) from non-recombinants (blue). X-gal indicates whether the E.coli expresses the Beta-galactosidase enzyme, which is encoded by the lacZ gene. The vectors used contain the lacZ gene. However, acquisition of foreign DNA disrupts the lacZ gene, thus making the bacteria unable to make beta-galactosidase, and no blue colour will appear.

The following day the plates were observed for the appearance of blue and white/light blue colonies. The white colonies were selected and subcultured on ampicillin selective LB agar plates which had X-gal plated (near a flame). At the same time the same colonies were inoculated into universal tubes containing 5mls of LB media. These tubes were then incubated at 37°C on shakers for 18 hours. The following day the subcultured agar plates were observed for the colour of the colonies picked for growth in liquid media. These colonies for selected for plasmid DNA extraction.

7.2.7. Extraction of Plasmid DNA

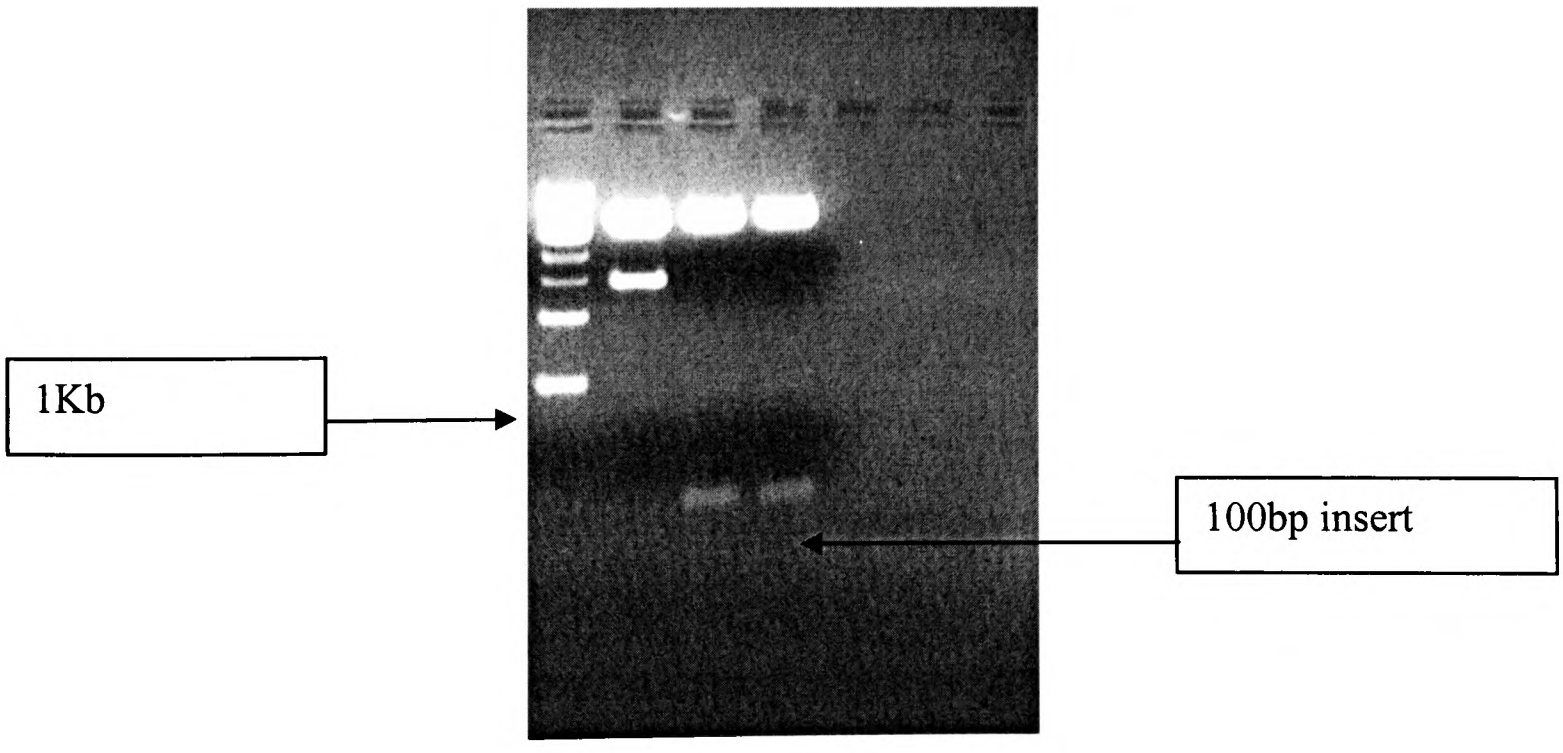
Plasmid DNA was extracted from the competent E. coli using the QIAprep Spin Miniprep Kit according to the manufacturers instructions.

7.2.8. Digestion of plasmid DNA

Digestion of plasmid DNA was performed to determine the molecular weight of the DNA fragment which was inserted into the vector. EcoR1 enzyme was used to cut the vector. The EcoR1 sites flank the sites where the PCR product is inserted into the vector. The reaction used is as follows:

Water	12.5µl
EcoR1 buffer	2µl
EcoR1 enzyme	0.5µl
DNA	5µl
Total reaction	20µl

The reaction was carried out in a 0.5ml microfuge tube and the reaction was incubated at 37°C for 2 hours. Following this 10µl of the sample was run on a 1% agarose gel at 50mV for 30 minutes.



7-4 Visualization of the 100kb PCR product that was inserted in to the plasmid

The DNA concentration was then measured by the nanodrop and the product sent for sequencing which was found to be the same as the expected PCR product.

7.2.9. Sequence of the cloned product:

CGTACACGTATTTTCAGTCCTCTTCAAGTGGAACCACTACCGCCCGTGGAGCG

CGTCGAAACGATGTCAACTCCACGTCTAAGCC.

7.2.10. Making the standard curve for performing quantitative PCR

The number of copies in 1µl of plasmid was calculated by the following equation.

1kb ds DNA (Na^+) = 6.6×10^5 Da

Molecular weight of 1 DNA base pair: 660 Da

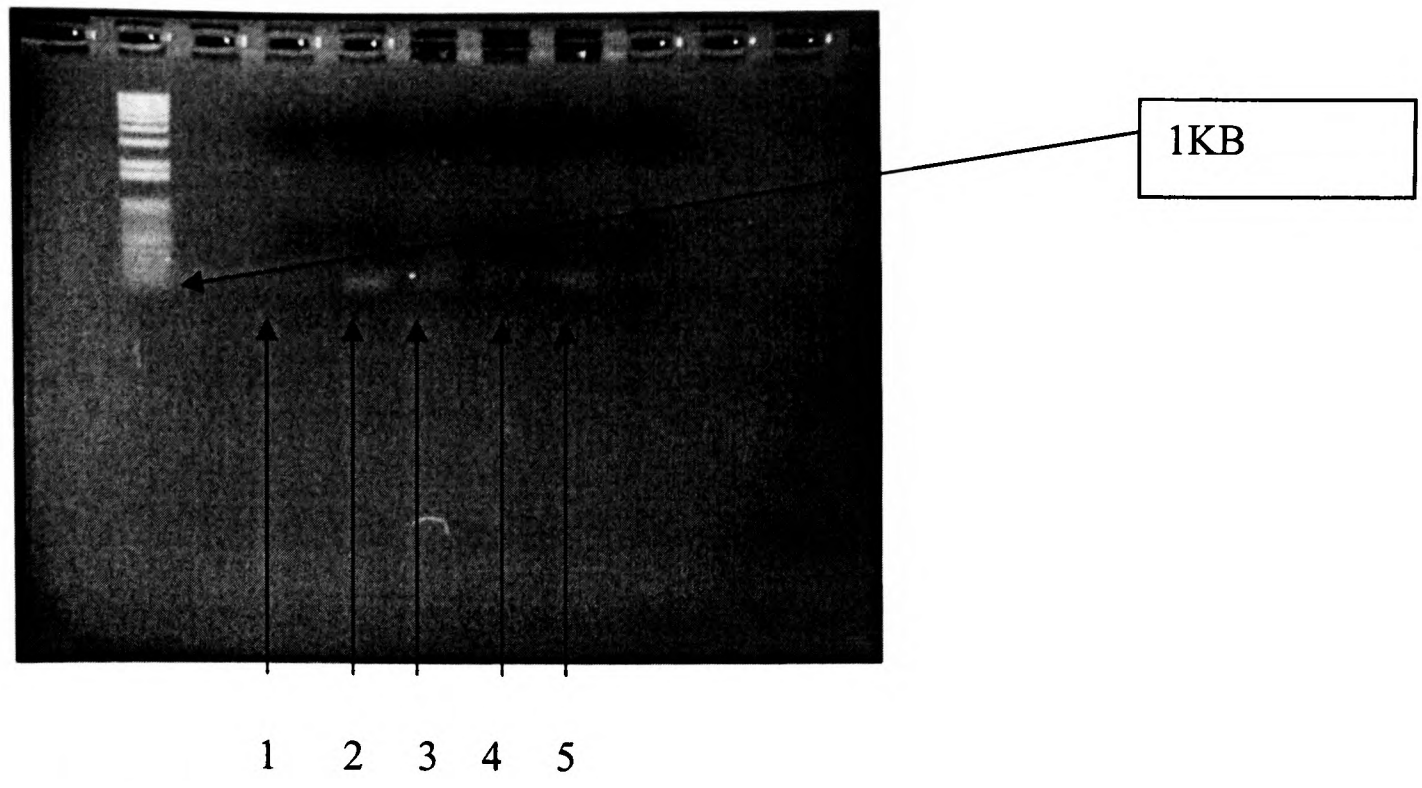
Therefore the molecular weight of the plasmid: 26.4×10^5 Da

Concentration of plasmid DNA in clone 7: 361.7 ng/µl

Therefore, the copies of plasmid in 1µl = 8.2×10^{10} /µl

This plasmid was then diluted to make 1×10^6/ 5µl stock solution

The 10^6 plasmid stock solution was diluted in salmon sperm DNA (0.3pg/µl) to make concentrations of 10^5 copies/5µl, 10^4 copies/5µl, 10^3 copies/5µl, 10^2 copies/5µl, 50 copies/5µl, 10 copies/5µl, 5 copies/5µl and 1 copy/5µl. The accuracy of the dilution was tested by a PCR on 1 copy/5µl which showed the presence of a PCR product in 2 to 3 of the 5 PCR runs.



7-5 PCR with 1 copy of the plasmid

The 100kb PCR product is only visible in 3 of the 5 PCR reactions as expected confirming that the correct dilution of the plasmid was achieved. PCR product can be seen in row 2, 3 and 5.

7.2.11. Quantitative PCR reaction (QPCR)

Quantitative real time PCR is based on the detection of a fluorescent signal produced proportionally during the amplification of a PCR product. The amount of fluorescence released during the amplification cycle is proportional to the amount of product generated in each cycle.

Double-dye oligonucleotide probes are used by many in QPCR which consists of a single stranded probe sequence that is complementary to one of the strands of the amplified product. The strand has a flurophore (FAM) attached to one end and a quencher (TAMRA) attached to the other. During the reaction the florophore is excited and passes its energy by FRET to the quencher TAMRA. Once the TAMRA is excited by the energy from FAM is fluoresces which is detected by the machine.

The quantitative PCR was performed using the ABI Prism 7700 sequence detector system. ABI MicroAmp® Optical Tubes, ABI optical lids and MicroAmp™ 96-Well Support Base were used to set up the PCR reaction. The primers were used at a concentration of 100ng/µl and the probe was used at a concentration of 5pmol/µl. The DNTP set was mixed together (6.25µMol concentration) and was diluted in 2400µl of water for the working concentration (1mMol). To quantify the amount of viral copies in each sample, an exogenous control (the plasmid with the ORF 29 PCR product insert) was used to form the standard curve.

The sequence of the probe is as follows:

5'-(FAM)CCCGTGGAGCGCGTCGAAA(TAMRA)-3'

The PCR mix was set as follows for 96 tubes:

Distilled water	1300µl
10× Hot star Taq buffer	250µl
25mM $MgCl_2$	50µl
DNTP's	75µl
Forward primer	100µl
Reverse primer	100µl
Probe	100µl
Hot Star Taq	25µl
Total	2000µl

20µl was aliquoted per well of the plate. For each tube 5µl of the standard curve was added and for the test samples 5µl of DNA was added. Salmon sperm DNA and water were used as negative controls. All samples were run in triplicate and after determining the number of viral copies in each well, the average of the number of viral copies was taken for each sample. The following template was used to run the samples:

Std 1	1	9	17
Std 5	2	10	18
Std 10	3	11	19
Std 50	4	12	20
Std 100	5	13	21
Std 1000	6	14	NTC
Std 10,000	7	15	Water
Std 100,000	8	16	Positive Control

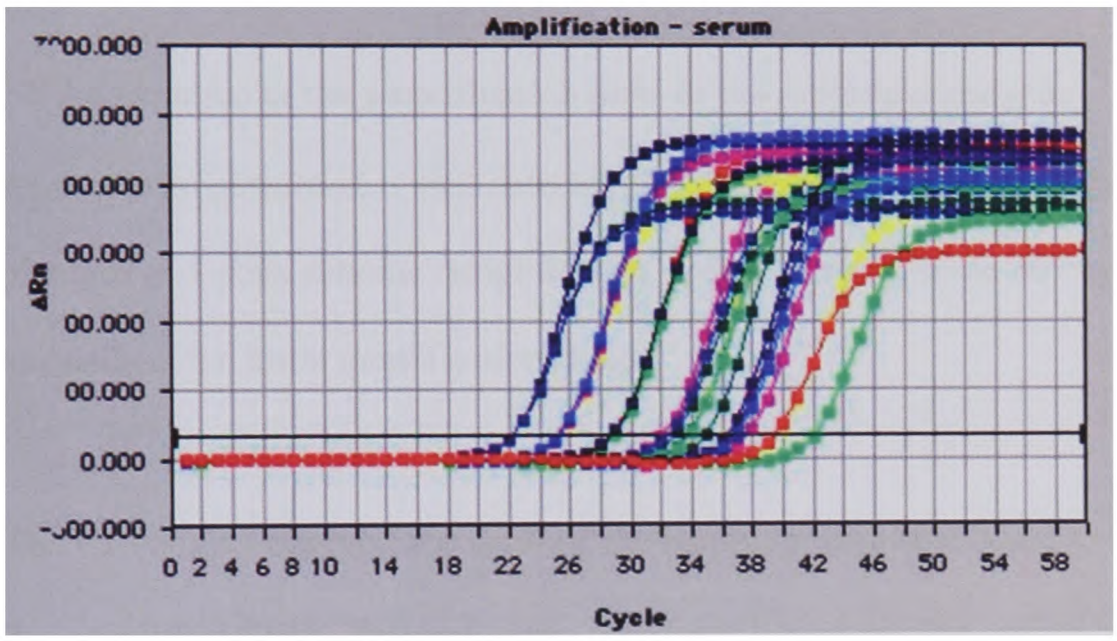
7-6 A display of the worksheet for the quantitative PCR reaction

The PCR conditions were as follows:

Stage 1	50°C	2 min	
Stage 2	95°C	10 min	
Stage 3	95°C	15 sec	60 cycles
	60°C	60 sec	

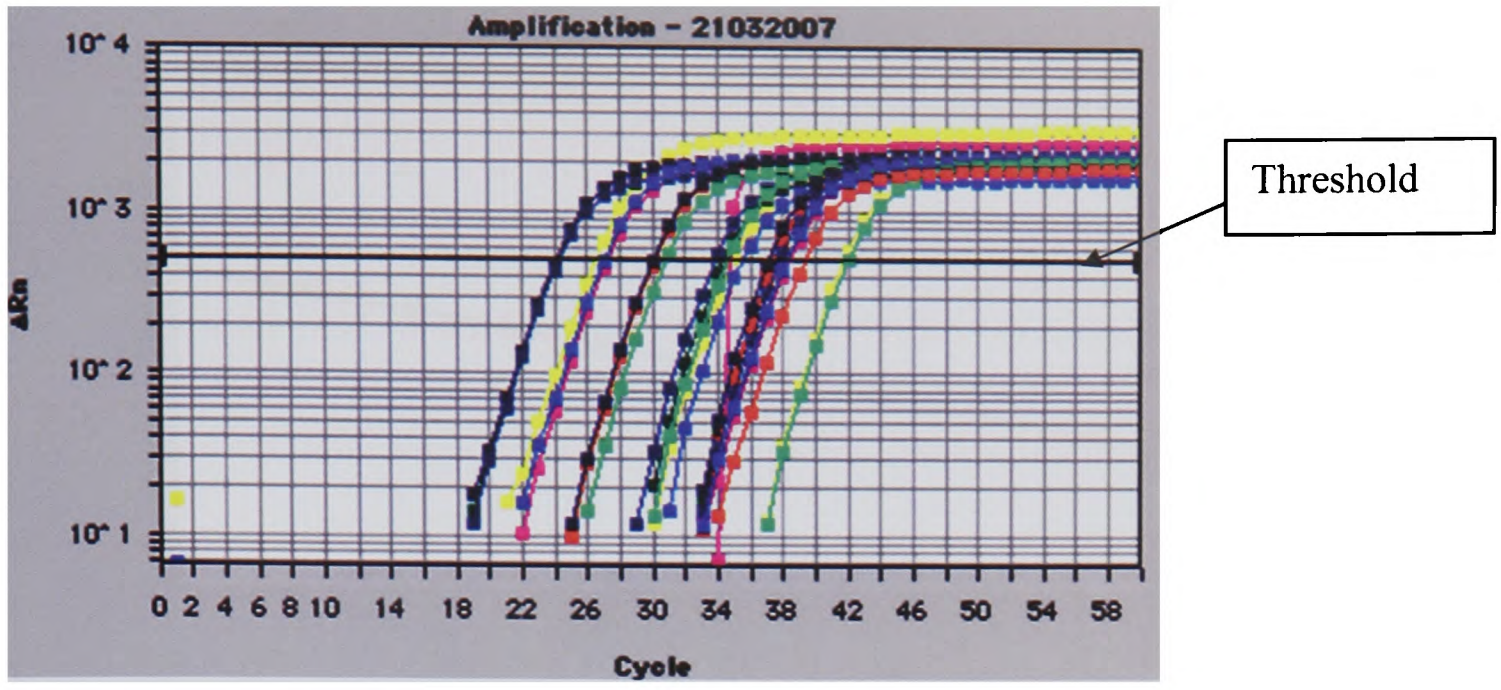
7.2.12. Analysis of Data

Data was analyzed using the PE Applied Biosystems- sequence detection systems 1.5. The real-time fluorescence values were measured by the quantity of a reporter dye FAM released during amplification. However, as the level of background fluorescence may fluctuate depending on the reaction medium the baseline was decided by viewing the linear amplification plots. This represents the cycle in which the amplification in the samples is truly above the background amplification value.



7-7 An example of a linear view of the amplification plots. Amplification is first detectable at 18th cycle.

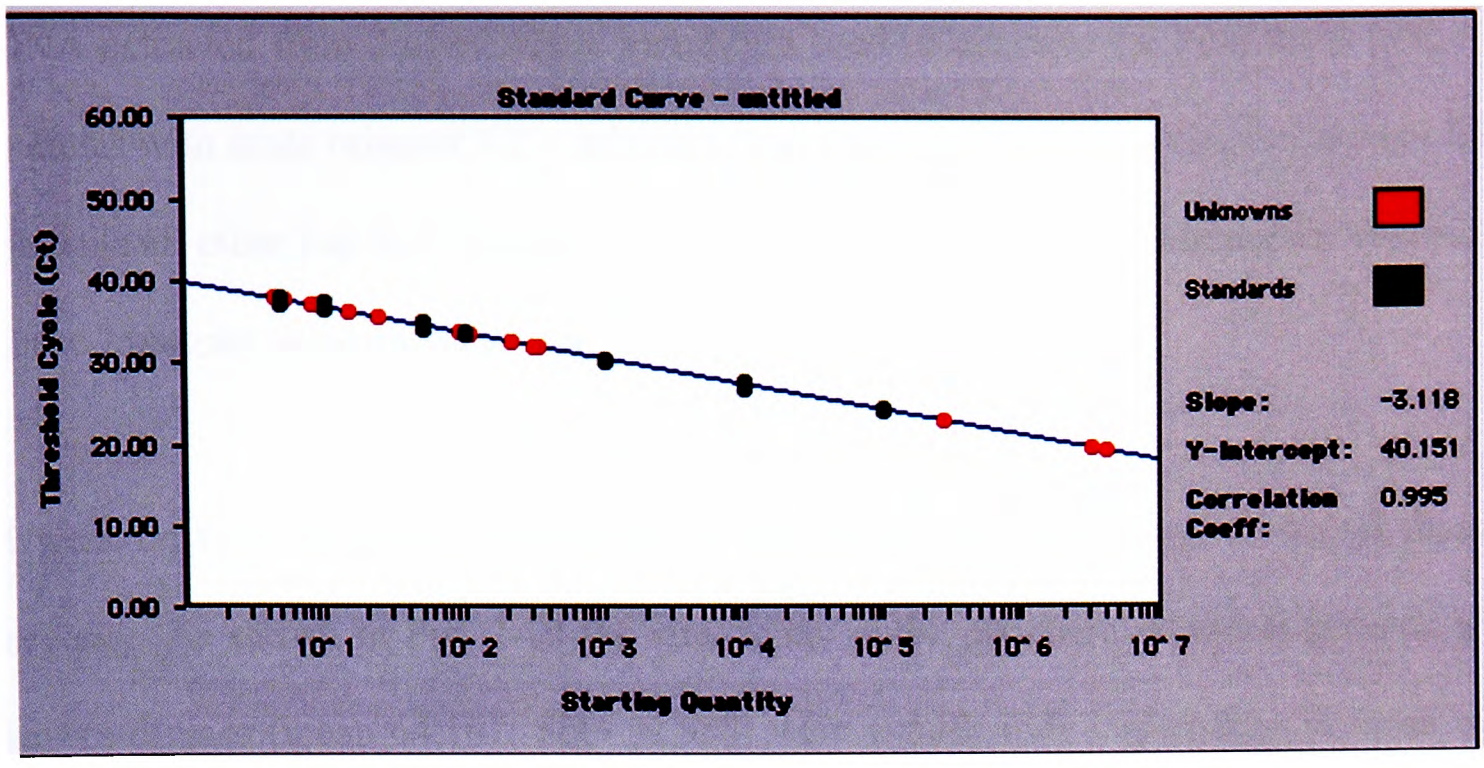
The threshold cycle value (Ct) for each reaction was determined by manually setting the threshold limit. Therefore, the Ct value reflects the cycle number in which the fluorescence exceeds the threshold. The threshold limit was set in the linear phase of exponential amplification after viewing the log linear view of the amplification plot.



7-8 An example of the amplification plots of the plasmid standards

As shown amplification is first detected for the plasmids at the 10^5 at the 20^{th} cycle and for the plasmids at 1 copy dilution amplification is first detected at the 40^{th} cycle. The threshold is set manually at the linear amplification phase.

The Ct values observed for each of the samples were then used to calculate the copies of the amplified ORF 29 gene copy numbers based on the standard curves generated by using sequential dilutions of plasmids with the ORF29 gene insert. A sample was considered for analysis if 2 Ct values of the triplicate were within 1 Ct of each other and was discarded as non-reproducible if this criterion was not met. Samples were considered negative if all 3 triplicates were negative at 45 Ct. The patient was considered to be viraemic on the basis of a postitive PCR result by this method.



7-9 A typical example of a standard curve of the plasmids which was used as the external reference for the quantitative PCR reaction

The standard curves used for data analysis all had a correlation coefficient exceeding 0.985 and a slope between -3.1 and -3.8 (an indicator of the efficiency of the PCR). The Y intercept which is when 1 copy of the plasmid was amplified was always 40.5 (±0.2).

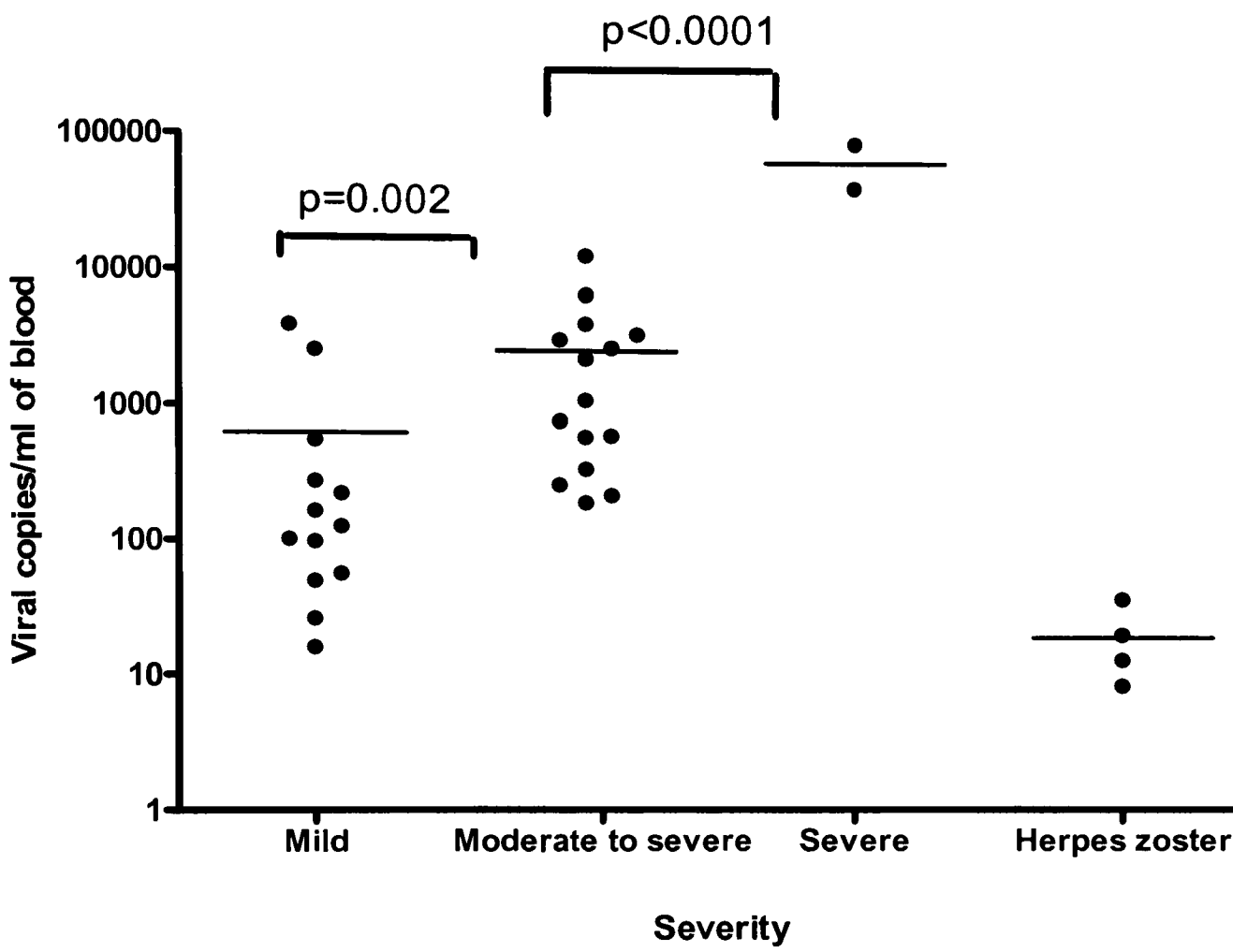
7.3. Results

DNA extracted from 2ml of whole blood was used to measure the viral loads in the 34 patients with acute primary VZV infection described in the previous chapter. Except for 2 patients all other had been started on acyclovir on the same day of admission. Therefore, these 2 patients were excluded from the analysis.

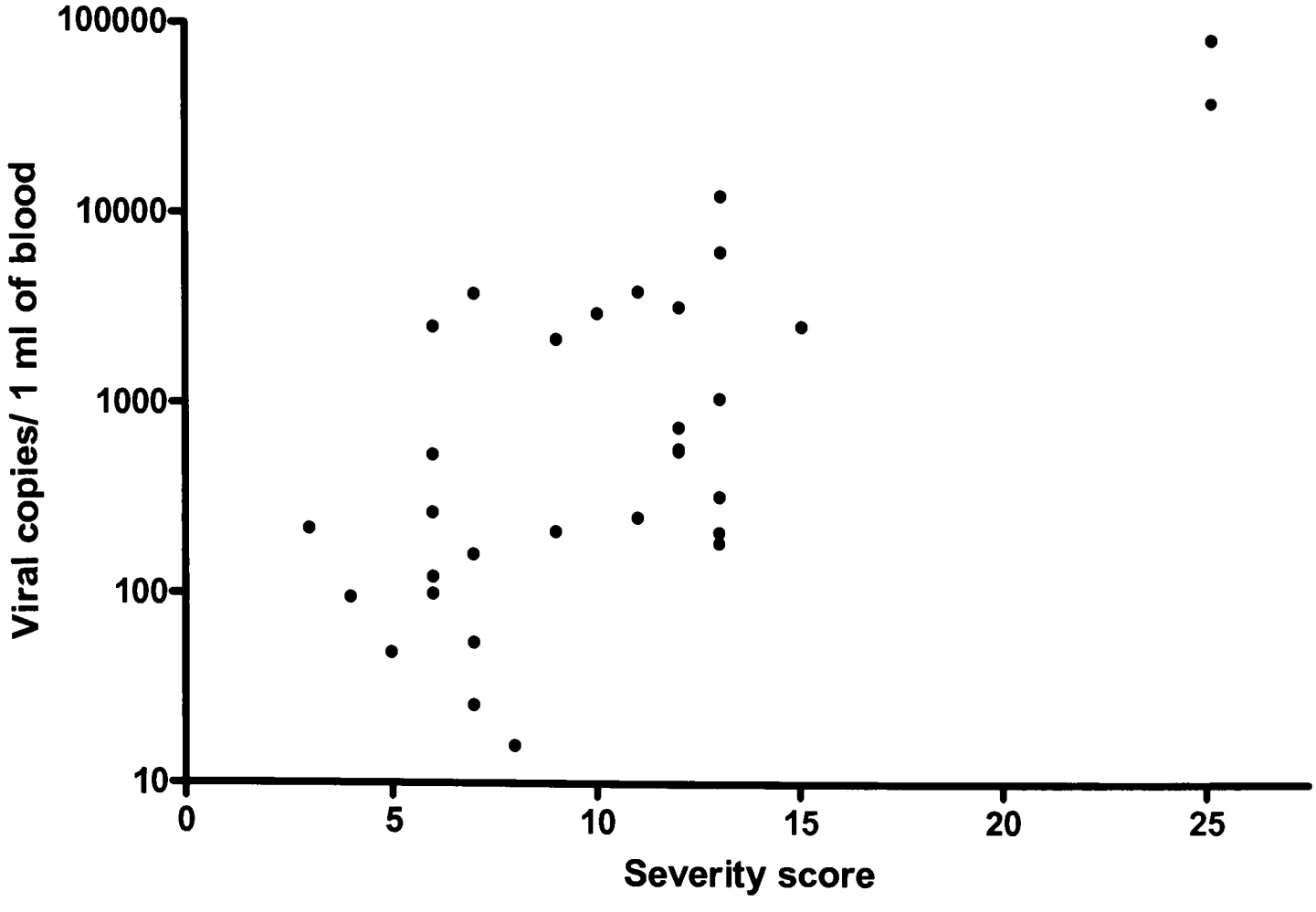
I went on to compare the viral loads of the 32 patients according to clinical disease severity. As shown in Fig. 7-10 the viral loads in the peripheral blood in patients with severe disease (mean 64,167; SD± 32,988) were significantly higher than in those with moderate to severe disease (mean 2568, SD ±3348). In addition, patients with moderate to severe disease had significantly higher viral loads in the peripheral blood, than patients with mild disease (mean 620.2; SD ±1184). Except for the 2 patients with severe VZV infection, all other patients had viral loads less than 12,000 viral copies. One of these patients was known to be immune suppressed due to high dose steroid therapy. The 32 patients included in the analysis had symptoms for 3 to 7 days when blood was taken for analysis of viral loads. However, one of the excluded patient who was admitted due to VZV associated cerebellitis on day 14 after the onset of symptoms had detectable viraemia in her blood. As I did not have more blood samples at this time point, it is difficult to ascertain whether the viraemia in this patient was due to the presence of her cerebellitis.

The virus was still detected in the peripheral blood of 4 patients with herpes zoster even on day 3-6 of illness. However, the number of viral copies was significantly less than in

the peripheral blood of patients with acute primary infection (mean 19.26, SD±12.34). No virus was detected in healthy lab volunteers. A significant correlation (p=0.0005, Spearmans r=0.56) was also seen between the clinical diseases severity and the number of viral copies in these patients (Fig 7-11).

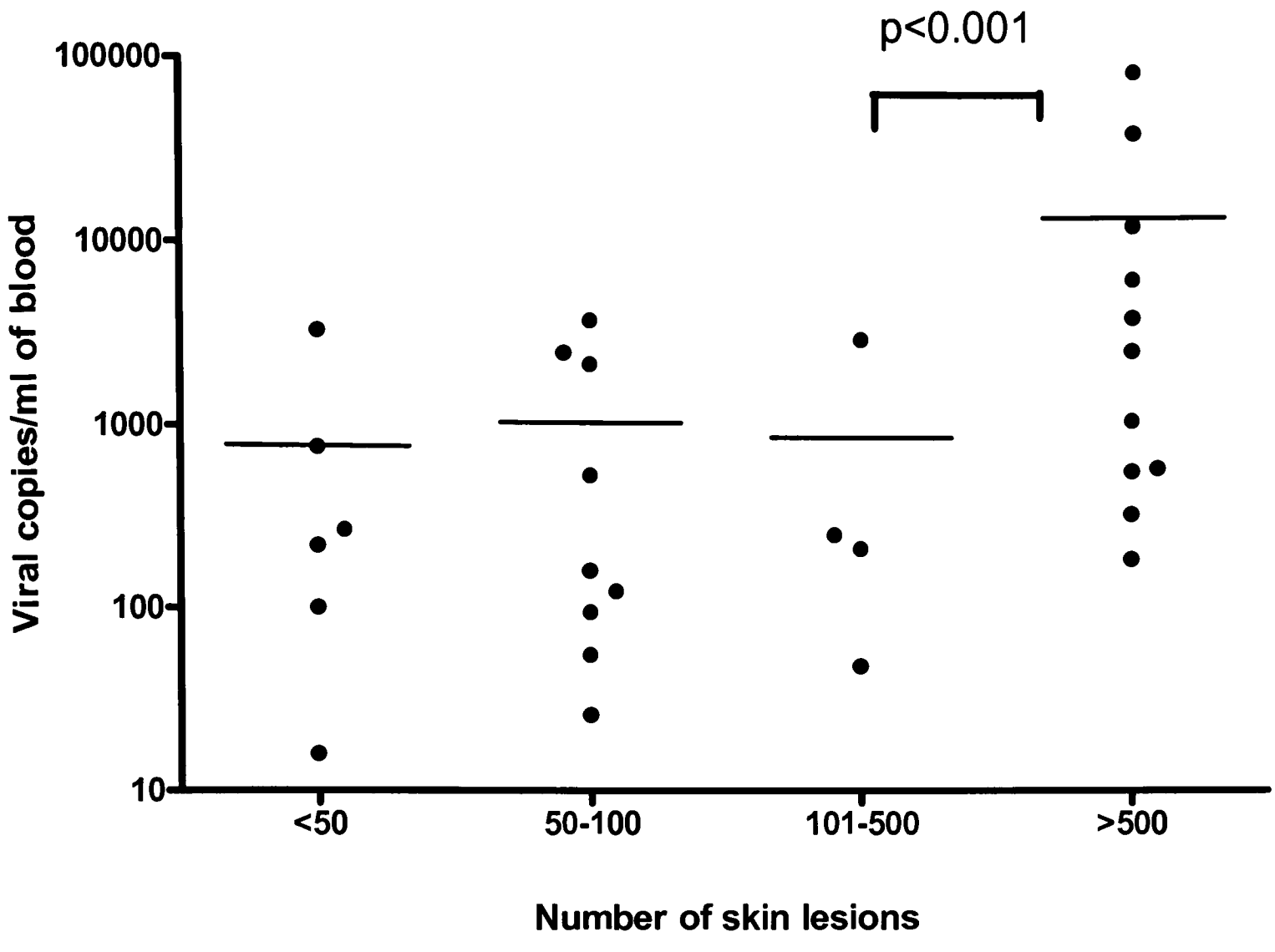


7-10 Quantity of viral copies in patients with varying severity of primary VZV infection
Unparied t test was used to determine p value.



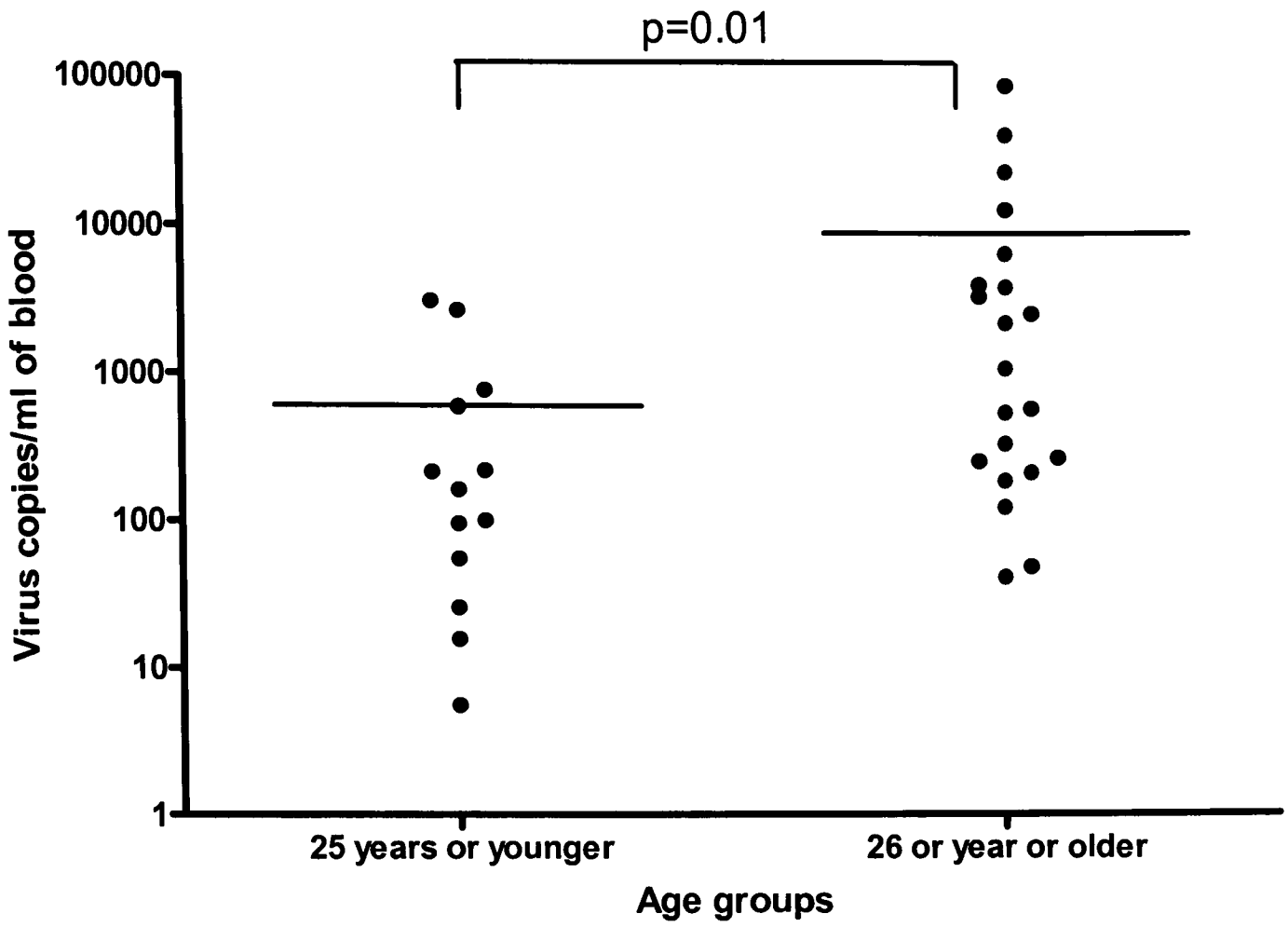
7-11 Relationship between the viral loads and clinical disease severity in the patients
Correlation was determined by using Spearmans correlation.

According to the scale that classifies clinical disease severity, a significant emphasis is given to the number of skin lesions in the patients as an indicator for clinical disease severity. Therefore, after establishing that the viral loads correlated well with clinical disease severity, I then went on to compare the viral load in patients with different numbers of skin lesions. As shown in fig. 7-12 the number of viral copies was significantly higher in patients with >500 skin lesions, when compared to those with <500 skin lesions. Therefore, >500 skin lesions was an indicator of the presence of a higher viral load in patients with acute infection.

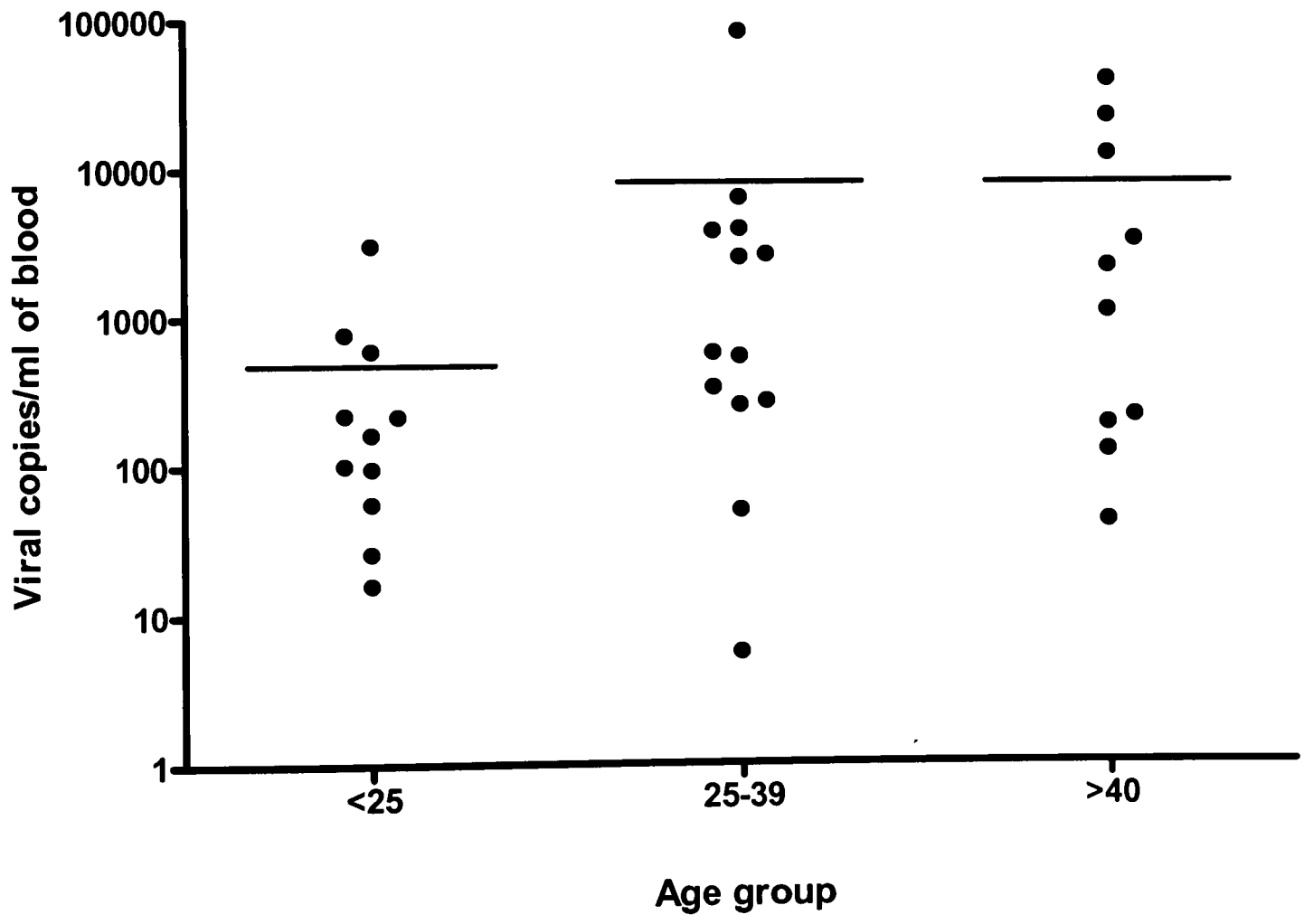


7-12 Quantity of viral copies in patients with different numbers of skin lesions
Unpaired t test was used in p value calculation.

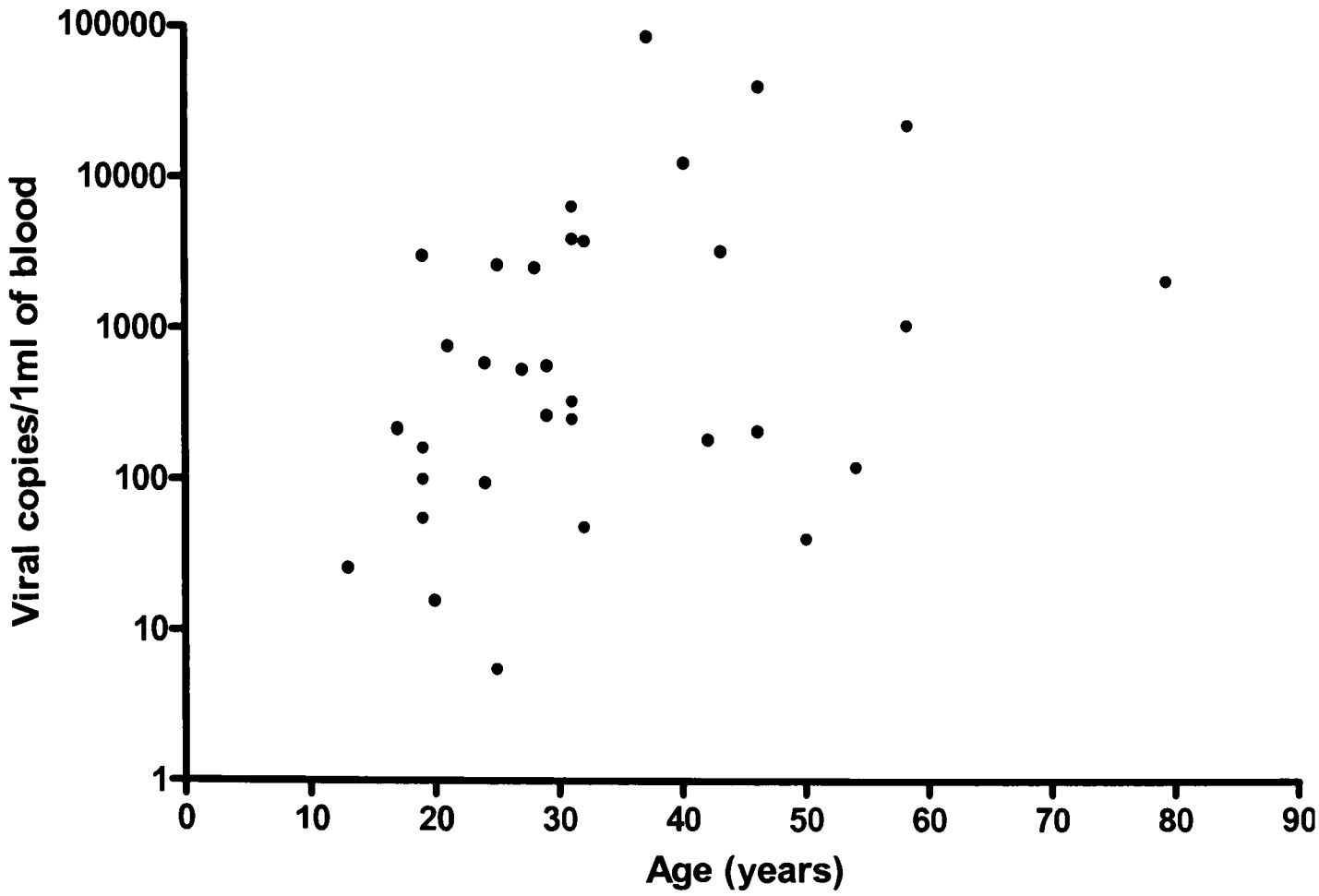
Adults tend to have more severe disease when infected with VZV. Therefore, I compared the viral loads in patients who were 25 years or younger and who were more than 25 years old. Interestingly, the viral loads were significantly higher in those aged more than 25 years ($p=0.01$) (Fig 7-13). I then went on to investigate if individuals who were aged >40 years had higher viral loads than 25 to 39 year olds. As shown in fig. the viral loads in individuals of 25 to 39 years of age were similar to those aged >40 years. However, a significant correlation was observed between the age of the patients and the viral loads ($p=0.02$, Spearman's $r=0.39$).



7-13 Viral loads in patients who were younger and older than 25 years of age.

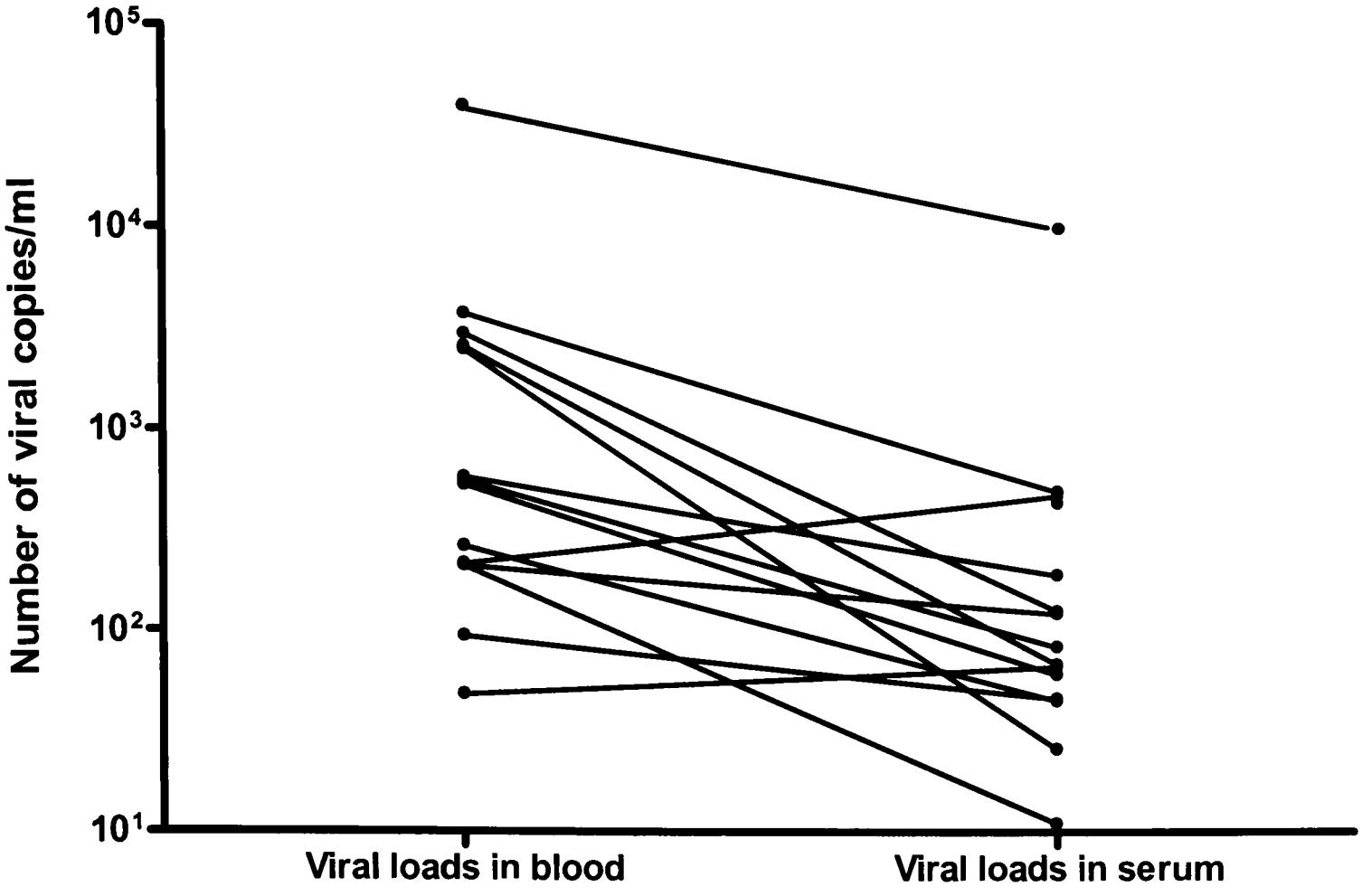


7-14 Viral loads in patients in different age groups

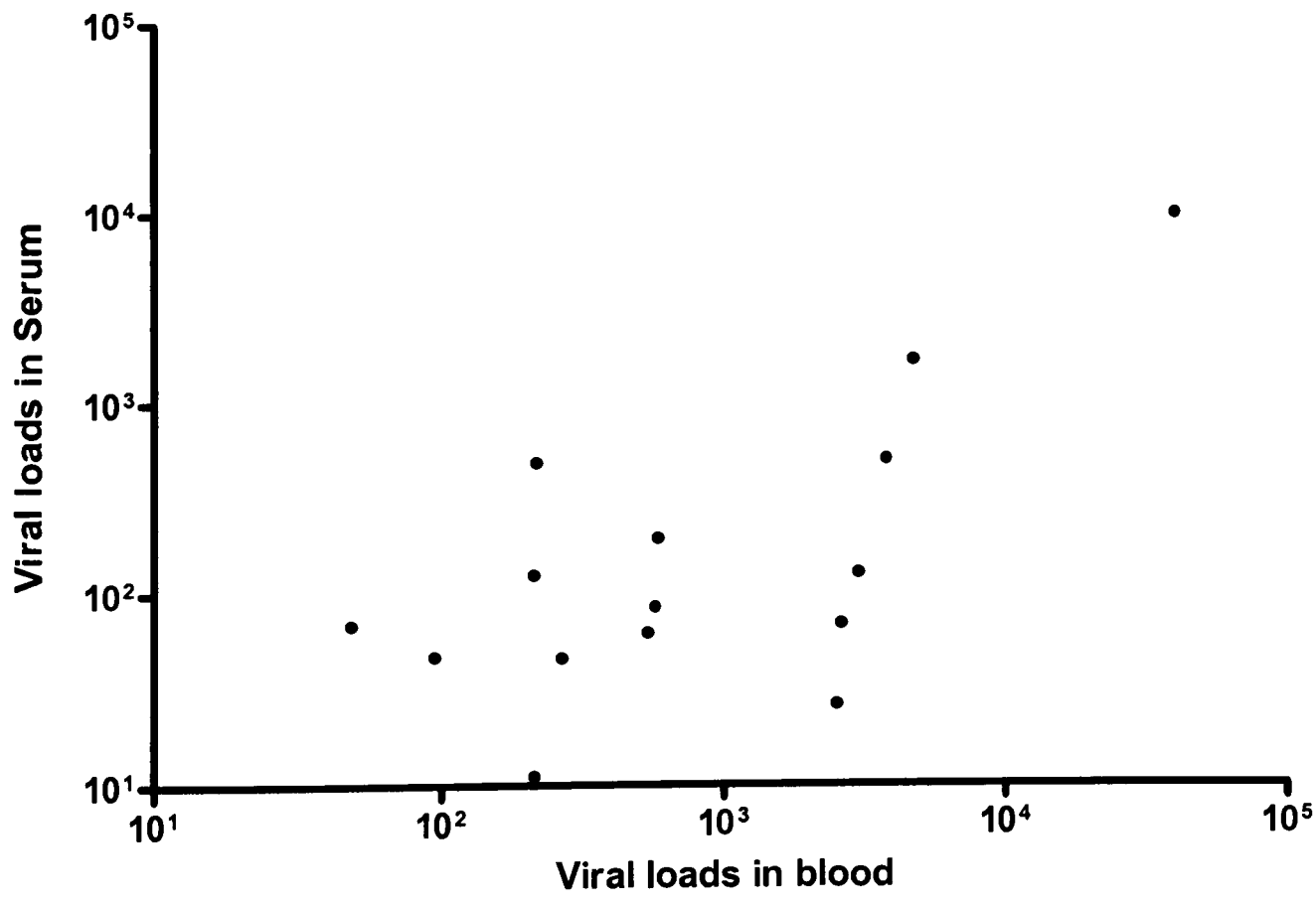


7-15 Correlation of viral loads with age of individuals
Correlation was determined by the use of Spearmans test.

Although VZV is thought to be a highly cell associated virus, some have shown that it can be isolated from serum and plasma (de Jong et al., 2000; Ito et al., 2001). Therefore, I proceeded to quantify the viral loads in the serum in these patients and also to determine the relationship between the viral loads in whole blood and serum. We too were able to isolate the virus from the serum of patients with acute primary varicella (Fig. 7-16). However, the viral loads were several folds lower that the viral loads in whole blood. Interestingly, a very good correlation ($r=0.99$, $p<0.0001$) was seen between the viral loads in whole blood and serum.

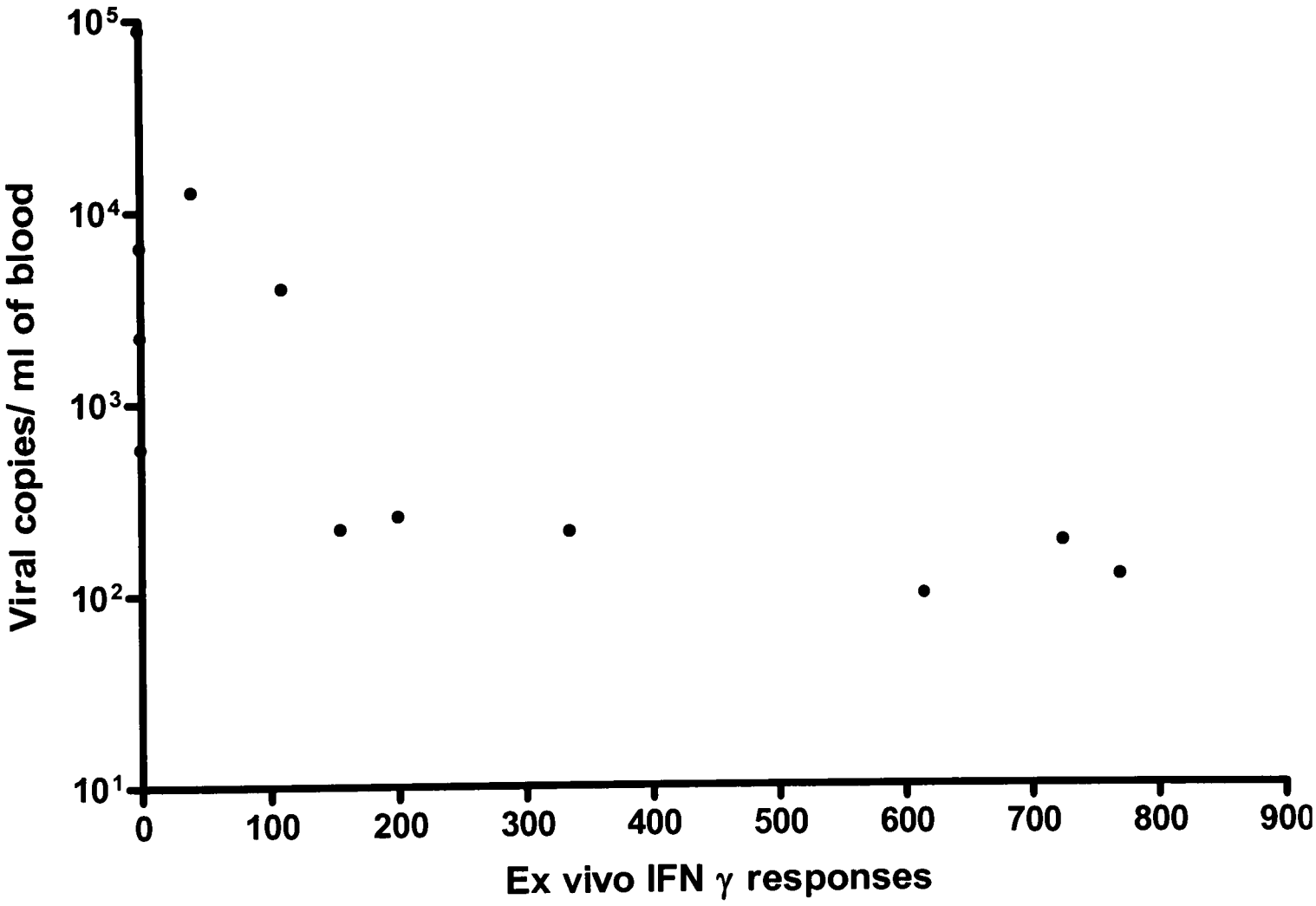


7-16 Comparison of viral loads in blood and serum



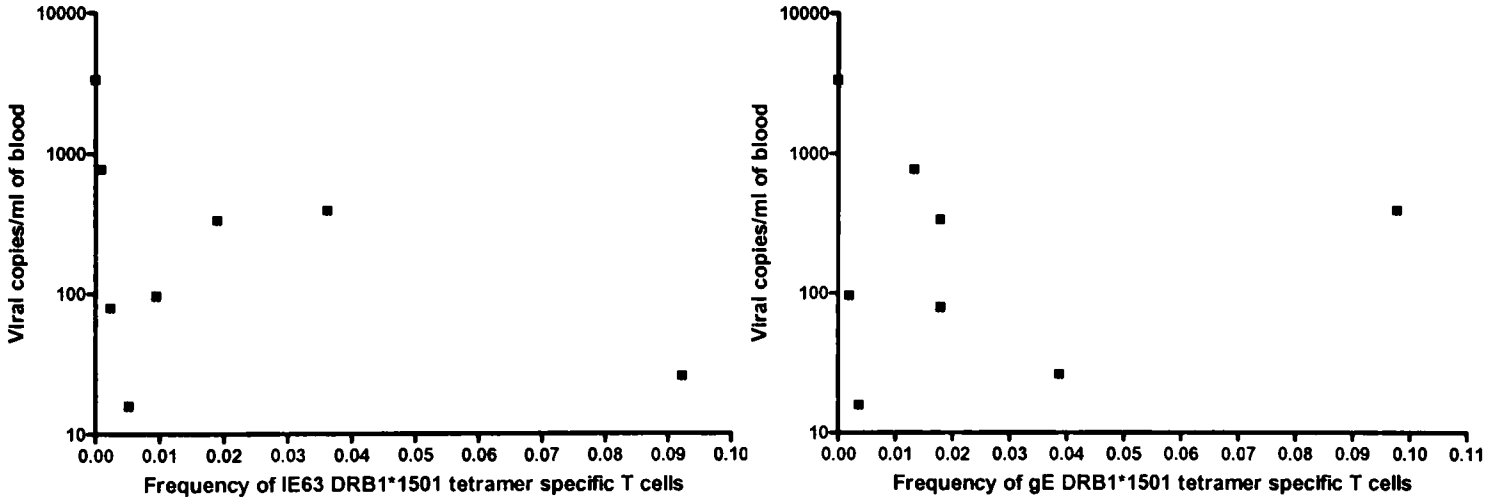
7-17 Correlation of viral loads in whole blood and serum
Correlation was determined by the use of Pearsons r.

As it was suggested by Quinlivan *et al* that a higher viraemia was probably due to the inability of the immune system to control the virus (Quinlivan et al., 2007a), I then went on to investigate the relationship of viral loads with T cell function. T cell function of these patients was assessed by *ex vivo* IFNγ ELISpot assays by using the VZV live attenuated vaccine as the stimulus. As shown below (Fig. 7-18), a significant (p=0.0005) inverse correlation (Spearmans r -0.85) was seen between the viral loads and the VZV vaccine specific *ex vivo* IFNγ ELISpot responses of the patients.



7-18 Correlation of viral loads with ex vivo IFNγ ELISpot responses of patients with acute varicella infection.
Correlation was determined by the use of Spearmans test.

I then went on to investigate if a similar association could be seen between the viral loads and the frequency of tetramer specific T cells in patients with acute varicella. Using DRB1*1501 gE and IE63 specific tetrameric complexes, I investigated the association of the frequencies of gE and IE63 (Jones, 2007) tetramer specific cells and the viral loads in 8 patients who were of the DRB1*1501 HLA type haplotype (tetramer data of these patients are described in detail in the previous chapter). Interestingly, although a significant negative correlation was observed with the viral load and functional T cell responses, no such correlation was seen with the frequency of gE or IE63 tetramer specific T cells in these patients. However, it will be important to study more DRB1*1501 patients to address this question definitively.



7-19 Correlation of viral loads of patients with acute varicella with both IE63 DRB1*1501 tetramer specific T cells and gE DRB1*1501 tetramer specific T cells
Correlation was determind by te use of Spearmans test.

7.4. Discussion

These data show that the viral loads in patients with acute primary varicella correlated well with clinical disease severity and rash severity. This is in contrary to what was seen in patients with HZ where it was shown that the extent of viraemia did not correlate with the severity of clinical disease (Quinlivan et al., 2007a).

The viral loads in this patient cohort (15.75 to 87500 copies/ml of blood) were similar to the range observed in previous studies. Similar to the observations made by others, the mean viral loads in our patient cohort with acute varicella infection (5829, SD±16,492) were significantly higher than the mean viral loads in patients with HZ (19.26, SD±12.34) (de Jong et al., 2000). Except for the 2 patients with severe VZV infection, all other patients had viral loads less than 12,000 viral copies. Others who had investigated viral loads in children with acute infection had reported viral copies between the ranges of 10 to 10,000 in blood of these patients (Kimura et al., 2000; Vossen et al., 2005b). Although the severity of infection was not reported in these studies they do not mention that the patients had severe infection. Vossen *et al* reported an initial viral load of >340,000/ml of whole blood in a child with severe varicella who went on to develop persistant viraemia (Vossen et al., 2005b). De Jong *et al* also report viral loads more than 1 million copies in immunocompromised patients (de Jong et al., 2000). Taken together with our data which are the first to compare the level of viraemia with VZV disease severity, it appears that severe VZV infection is associated with a viral load of >10,000 copies of virus/ml of blood.

Patients with > 500 skin lesions had significantly higher viral loads than patients with less numbers of lesions. Therefore, the presence of >500 skin lesions appears to be an indicator of higher viral loads in whole blood and a good indicator of disease severity during primary infection. This was again in contrast to the observations made by Quinlivan *et al* in HZ, where they reported that rash severity did not correlate with the extent of viraemia in acute HZ. Rather the viral load in HZ appeared to be associated with age and immune status of the individual (Quinlivan et al., 2007a). In primary VZV infection too, it is well recognized that adults are likely to have more severe disease and are 9 to 15 more likely to be hospitalized (Galil et al., 2002a) and 25 times more likely than children to die from varicella (Meyer et al., 2000b). Therefore, it is not surprising that in acute chickenpox individuals aged >25 years had significantly higher viral loads than younger individuals. However, due to small sample size only 2 individuals were immune suppressed. Therefore, I could not determine the effect of immune suppression on the viraemia in acute infection.

The VZV specific IFNγ production showed a significant negative correlation with the viral loads in the patients. This suggests either that T cell contribute to the control of viraemia or that antigen specific T cells in individuals with higher viral loads had impaired functional capability. As the VZV is known to infect T cells (Ku et al., 2002; Moffat et al., 1995), it is possible that in patients with severe disease more T cells are infected thereby reducing their capacity to exert antiviral effects such as cytokine production. Alternatively, as discussed in the previous chapter, it is possible that antigen specific T cells in individuals with severe infection could be secreting different cytokines

such as TNFα and IL-10. Therefore, it would be worth investigating these possibilities to understand the host pathogen interactions in acute primary VZV infections.

Although a negative correlation was seen with the functional effects of antigen specific T cells in acute chickenpox, no correlation was observed between the frequencies of either DRB1*1501 gE or IE63 tetramer specific T cells. As responses to these epitopes represent only a fraction of the responses to the whole VZV virus, it is possible that responses to these 2 epitopes are not the most important ones in controlling the virus in acute infection. Alternatively, it could be possible that the virus preferentially infects antigen specific T cells thereby rendering them dysfunctional despite these cells circulating in significantly high numbers. It will be important to increase the DRB1*1501 patient numbers to address this question in more detail. The laboratory is currently investigating whether VZV-specific T cells are preferentially infected and their relative anti-viral activity.

Although, VZV is thought to be a highly cell associated virus, we and others (de Jong et al., 2000; Ito et al., 2001) were able to detect the virus in the serum of these patients. De Jong *et al* reported that the isolation rate of virus and viral copy numbers from serum and plasma was comparable to that of whole blood (de Jong et al., 2000). However, we found that although the virus was detected in the serum in all patients tested, the viral loads were several fold lower when compared to copy numbers in whole blood. Therefore, these results highlight the possibility of a cell free viraemia as cell free virus exists in varicella skin vesicles. On the other hand, it could be possible that there was

contamination of the serum samples with PBMCs and therefore, the viruses detected in serum were not truly 'cell free' virus.

In summary, these findings show that the extent of viraemia was significantly associated with clinical disease severity and rash severity and that it negatively correlated with functional T cell responses. However, as these data represent a cross sectional sample, it will be now important to investigate the kinetics of viraemia and functional T cell responses longitudinally including other cytokines. In addition, it would be important to determine if the viraemia persists for a longer duration in individuals with more severe disease and also the effect of antiviral drugs on controlling the virus in patients with varying disease severity. Such data would thus enable us to understand the mechanisms of virus T cell interactions and to develop more effective antiviral drugs to curb severe disease.

Chapter 8. Preliminary analysis of Varicella zoster virus infection of T cells

8.1. Introduction

The VZV has been shown to infect many cell types such as T cells, B cells, monocytes, dendritic cells, fibroblasts, melanoma cells and human skin cells (Desloges et al., 2007) (Konig et al., 2003). Preferential infection of T cells by the virus is thought to result in a highly cell associated viraemia in which the T cells carry the virus to peripheral sites where subsequent infection of other cells occur (Ku et al., 2004; Moffat et al., 1995) (Ku et al., 2005). In the SCIDhu mouse model, it was shown that VZV infected T cells did infect human skin Xenografts in these mice resulting in typical VZV like skin lesions (Ku et al., 2004). Infection of T cells results in alterations in MHC class I expression(Abendroth et al., 2001a) and also gene transcription patterns. Micro array analysis of changes in gene transcription patterns in VZV infected T cells revealed that the majority of genes that up regulated or down regulated by VZV were those involved in immune and stress responses, signal transduction and apopotosis (Jones and Arvin, 2003). Most importantly, down regulation of caspase-8 which is a main regulator of apoptotic pathways was seen in VZV infected T cells which would suggest that VZV infected T cells would survive longer, facilitating spread to peripheral sites(Jones and Arvin, 2003).

In acute primary infection in children, viral DNA has been found in equal frequency in both $CD8^+$ and $CD4^+$ T cells, B cells and in monocytes (Ito et al., 2001). However, viral loads as detected by quantitative PCR using primers for both VZV glycoprotein B and IE62 showed that the viral loads were in fact higher in CD8+ T cells though it was not statistically significant. Experiments carried out on the SCIDhu mouse model by Moffatt

et el have also shown that both the wild type and the live attenuated vaccine virus infects $CD8^+$ T cells and $CD4^+$ T cells at an equal frequency (Moffat et al., 1995). However, later experiments by Ku *et al* who also used the SCIDhu mouse model showed that CD4+ T cells expressing skin homing markers and of CD45RO+ T cell memory phenotype were preferentially infected by the virus (Ku et al., 2002). These authors suggested that the occurrence of more severe infection in adults could be due to preferential infection of T cells expressing T cell memory markers which were higher in adults resulting in a higher viral load(Ku et al., 2002). In these experiments, these investigators have initially infected tonsil T cell (which consisted of 60% of CD4+ T cells and 30% of CD8+ T cells) with the virus and show that 20.5% of CD4+ T cells and 13% of CD8+ T cell were infected in 4 different experiments. In their subsequent experiments they have only focused on CD4+ T cells and have not done any further experiments on infection of CD8+ T cells. Therefore, based on the fact that 20.5% of tonsil CD4+ T cells were infected when compared to 13% of CD8+ T cells they have claimed that the VZV preferentially infects CD4+ T cells.

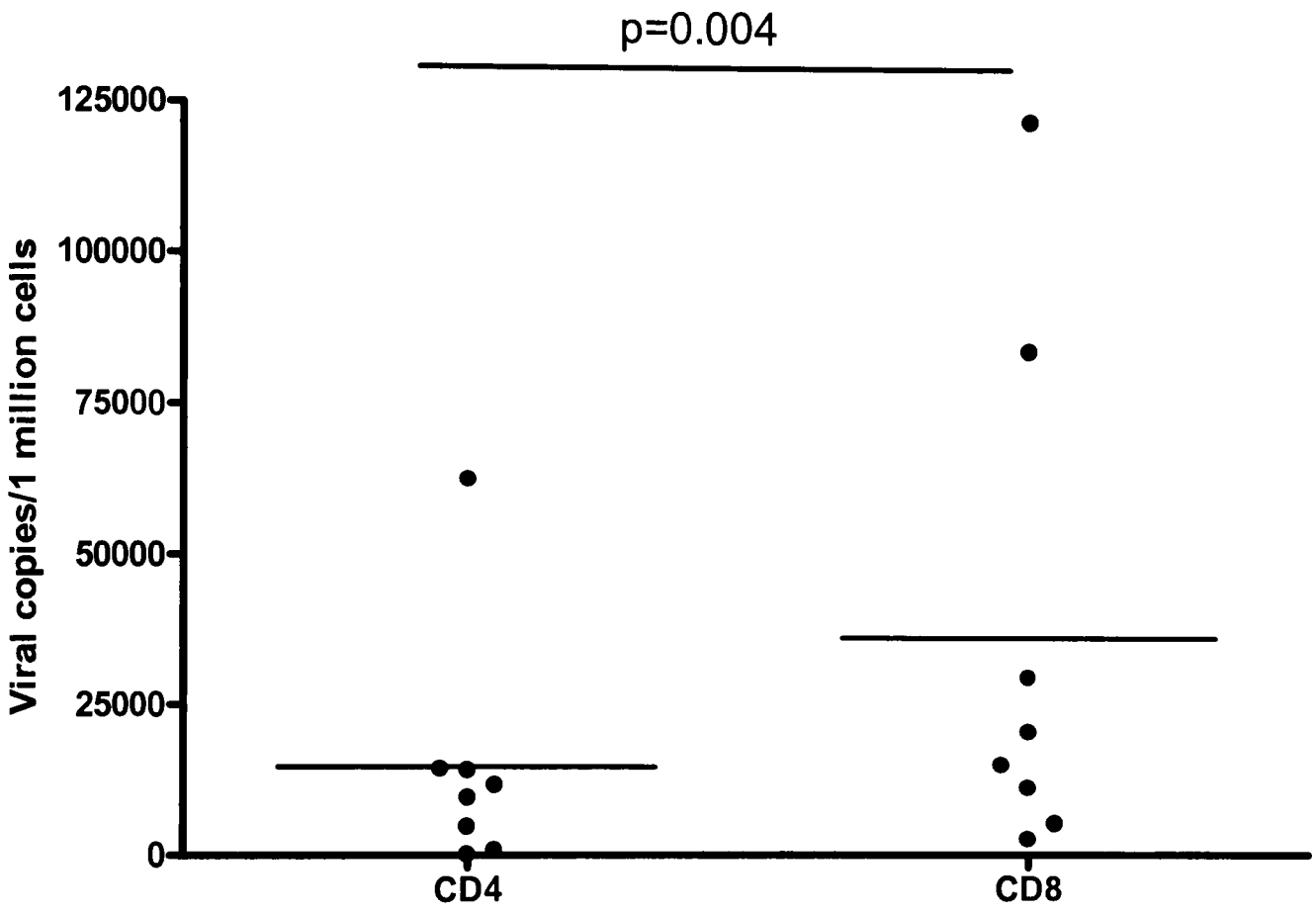
As infection of T cells with VZV has shown to alter transcription patterns of many genes(Jones and Arvin, 2003), this could result in altered function of infected T cells. In HIV infection, the virus is known to preferentially infect CD4+ T cells and especially HIV specific memory CD4+ T cells thereby altering their immune function (Douek et al., 2002; McCune, 2001; McMichael and Rowland-Jones, 2001; Yue et al., 2005). A larger proportion of HIV infected CD4+ T cells were shown to undergo apoptosis which directly correlated to the viral load and disease progression (Yue et al., 2005). Therefore,

in HIV infection, preferential infection of CD4+ T cells by the virus appears to be a very successful strategy adopted by the virus to evade elimination by the immune system enabling it to persist in the host efficiently (McMichael and Rowland-Jones, 2001). As shown in the previous chapters, VZV specific functional T cell responses appeared to be diminished especially in acutely infected individuals with a higher number of skin lesions. These individuals were also shown to have higher viral loads than those with a fewer number of skin lesions. Therefore, it is possible that VZV infection of T cell during acute primary varicella alters their immune function or proliferative capacity which would enable the virus to cause a larger number of skin lesions resulting in higher infectivity and virus dissemination. Therefore, in order to address these issues and to determine if a particular subset of T cells were more susceptible to infection, I began to investigate the viral loads in CD4+ and CD8+ T cells in patients with acute primary VZV infection and also *in vitro* infection of T cells by the virus.

8.2. Results

8.2.1. Viral loads in CD8+ and CD4+ T cells in acute infection

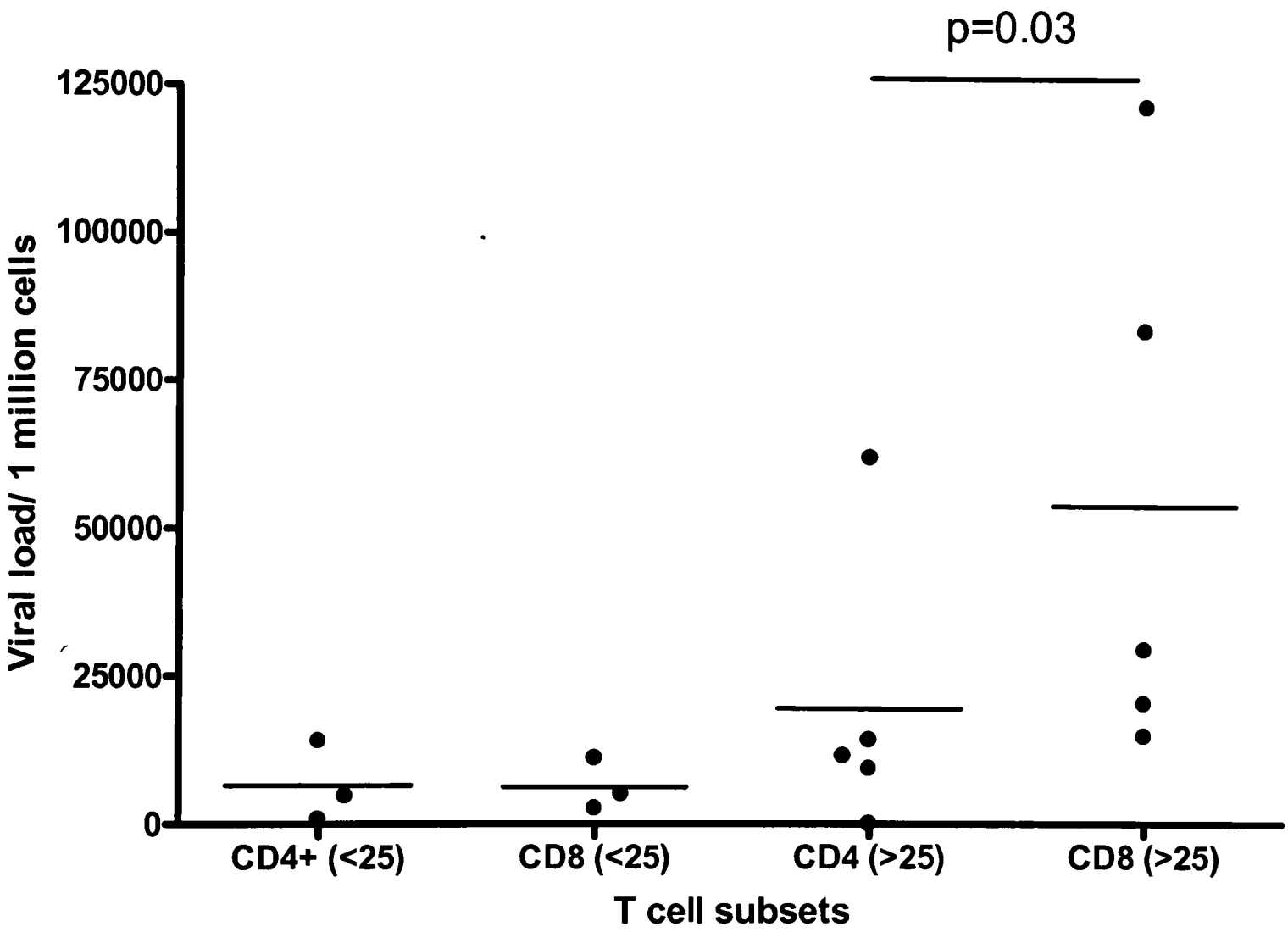
Using quantitative real time PCR as described in the previous chapter, I initially sought to investigate the viral loads in CD8+ and CD4+ T cells in patients with acute infection. As shown in Fig. 8-1 the viral loads in CD8+ T cells (mean 36,258 viral copies/ 1 million cells; SD±43,399) in patients with acute infection were significantly higher (p=0.004) than in the CD4+ T cells (mean 14,671 viral copies/ 1 million cells; SD±20,124).



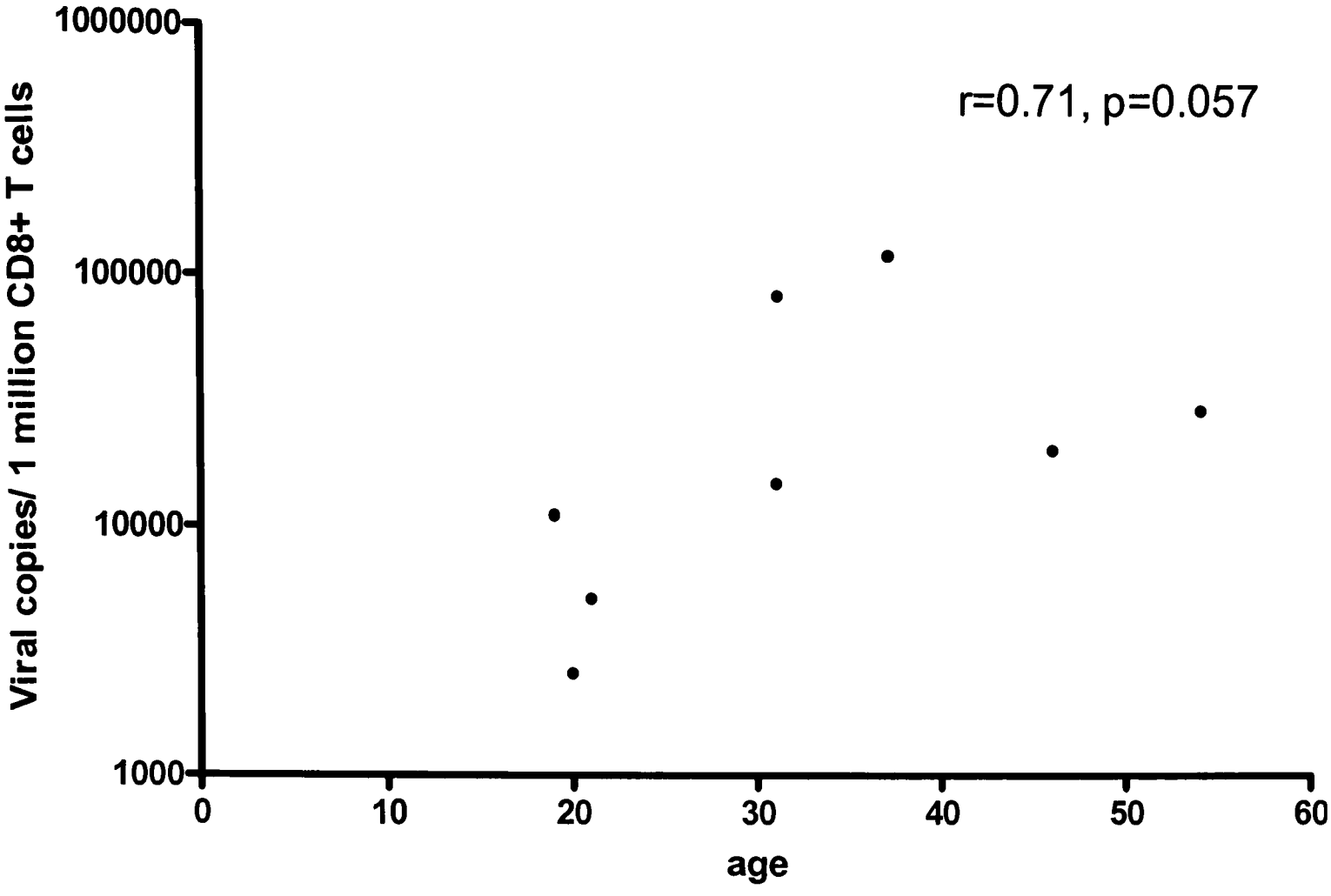
8-1 Viral loads in CD4+ and CD8+ T cells in patients with acute primary varicella infection. P values were calculated by the use of paired t test.

As in the previous chapter it was shown that the viral loads were significantly higher in individuals aged >25 years when compared to younger individuals, I then went on to compare the viral loads in CD4+ and CD8+ T cells in these 2 age groups. Surprisingly, it was seen that there was no difference in the viral loads in CD4+ T cells (mean 6,500, SD±6,779) and CD8+ T cells (mean 6,261, SD±4,378) in patients <25 years of age

whereas a significant difference (p=0.03) was seen between then viral loads in CD4+ T cells (mean 19,574, SD±24,609) and CD8+ T cells (mean 54,256, SD±46,976) in patients >25 years of age (Fig8-2). Interestingly, a positive correlation was seen between the viral loads in CD8+ T cells and the age of the individual (Spearmans's r=0.71) although this was not statistically significant (p=0.057). No such correlation was seen with age and CD4+ T cells.



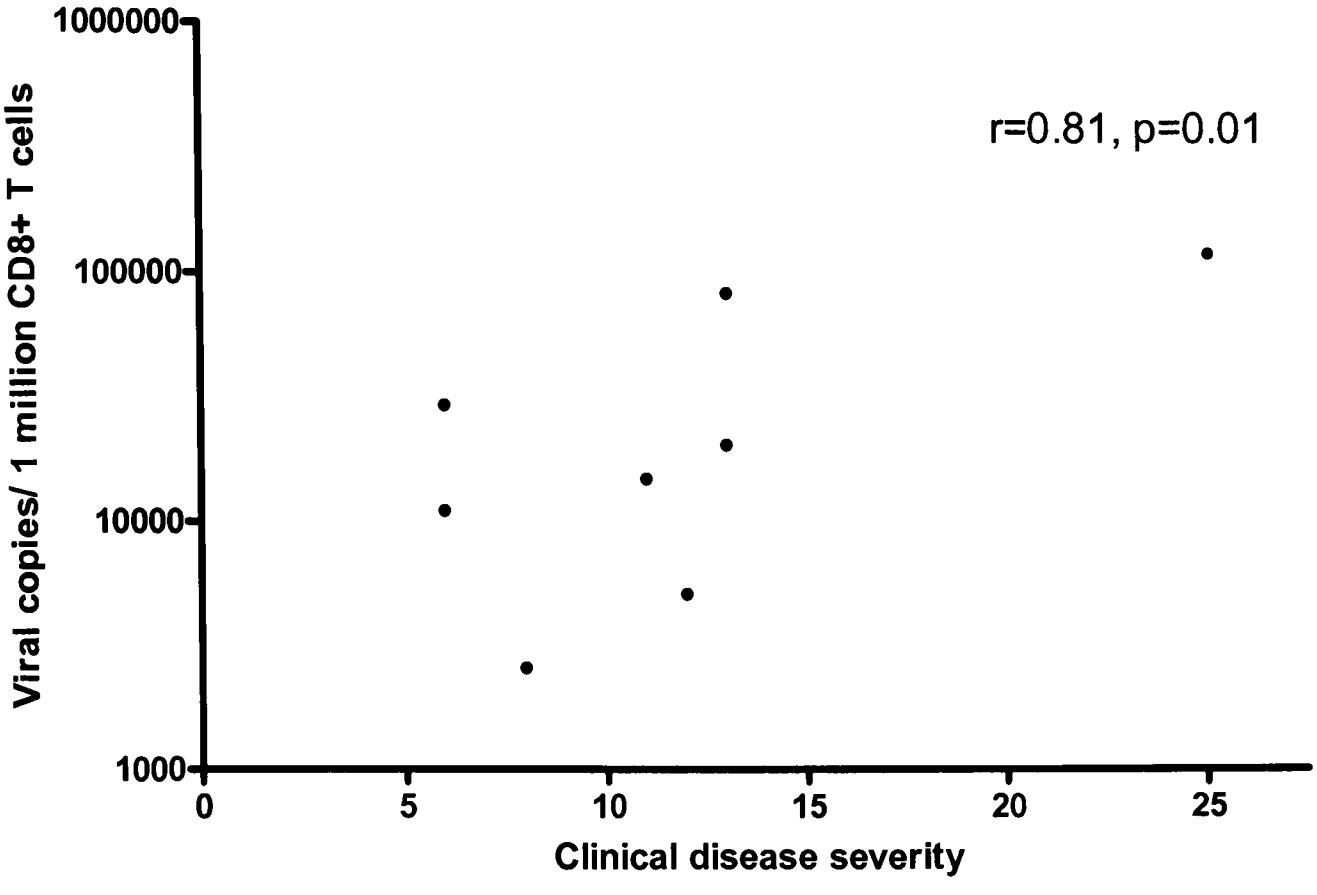
8-2 Viral loads in CD4+ and CD8+ T cells in patients with acute infection who are aged <25 (left hand 2 columns) and aged >25 years (right hand 2 columns)
P values were calculated by the use of unpaired t test.



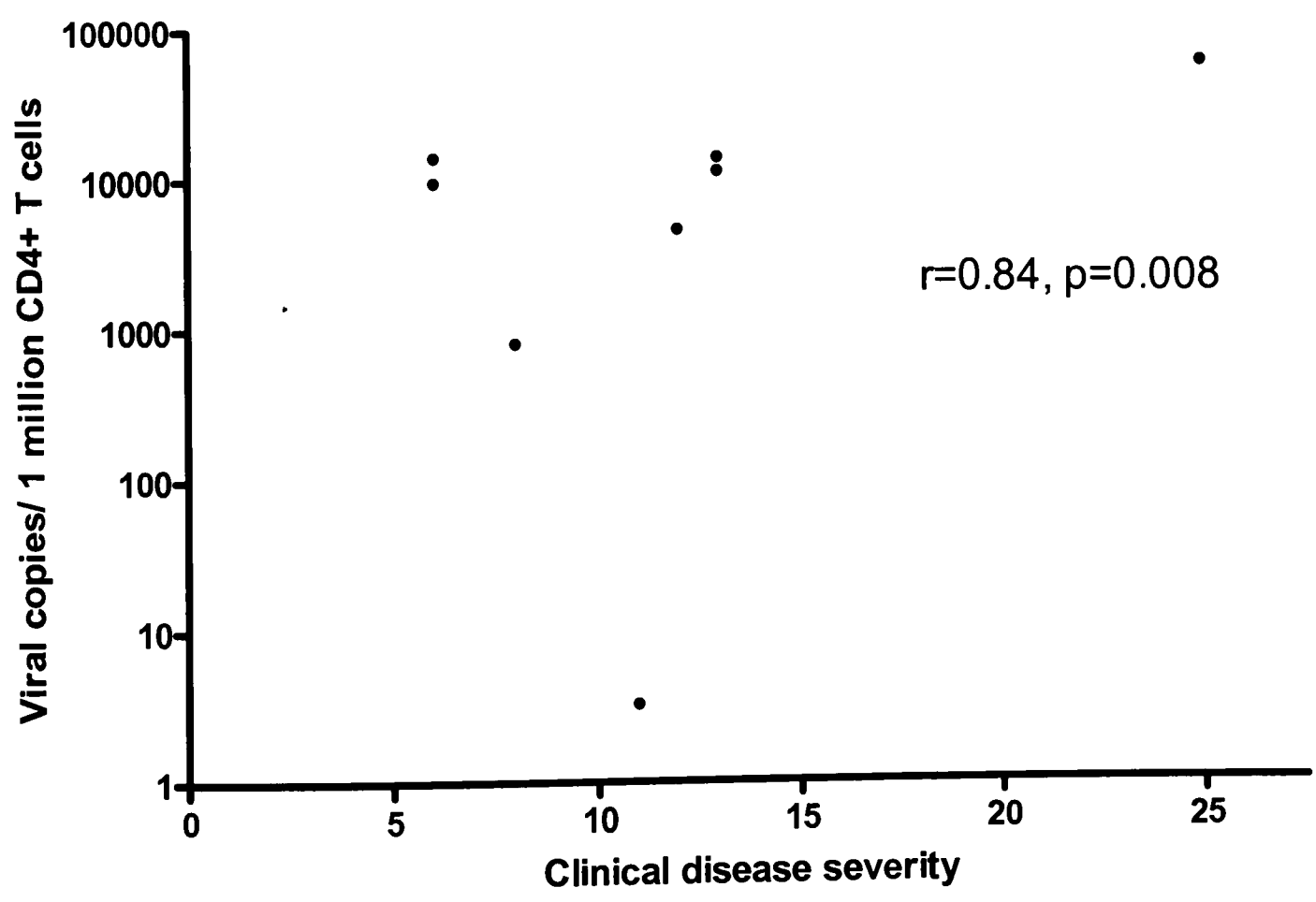
8-3 Correlation of viral loads in CD8+ T cells of patients with acute infection with the age of the individual

Correlation was determined by the use of Spearmans test.

Establishing that viral loads in CD8+ T cells were higher than in the CD4+ T cells and also that the viral loads in CD8+ T cell were significantly higher in patients aged >25 years, I then went on to investigate if there was a relationship between the viral loads in CD8+ T cells and the clinical disease severity score. Indeed a significant positive correlation ($r=0.81$, $p=0.01$) was seen with the viral loads in CD8+ T cells and clinical disease score and for the viral loads in CD4+ T cells ($r=0.84$, $p=0.008$). Correlation was determined by the use of Spearmans test.

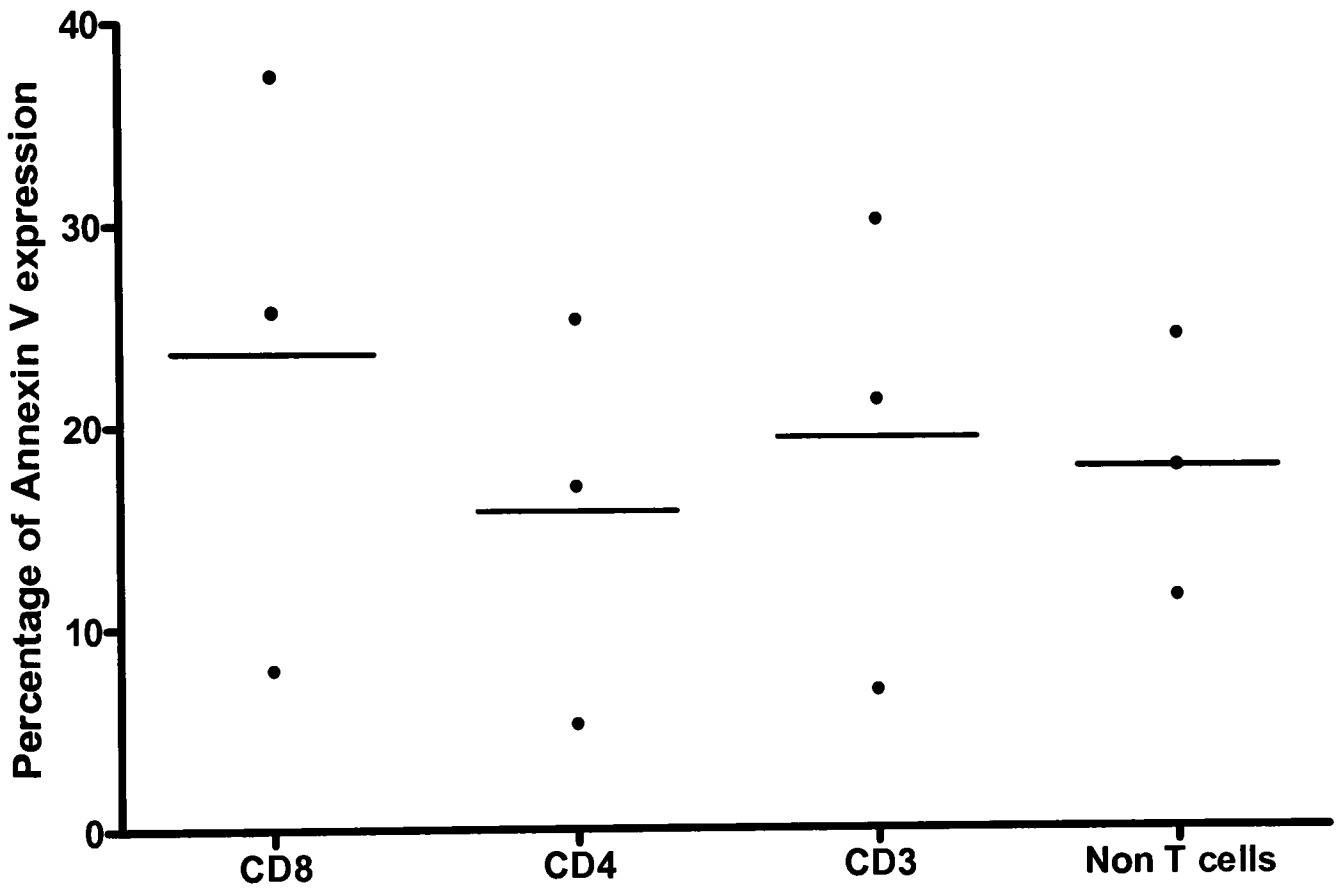


8-4 Correlation of clinical disease severity with viral loads in CD8+ T cells



8-5 Correlation of clinical disease severity with viral loads in CD4+ T cells

After establishing that CD8+ T cells were preferentially infected in patients with acute VZV infection, I then went on to investigate which T cell subsets were more prone to apoptosis in the patients by looking at the level of Annexin V expression by these cells. However, due to limited samples level of Annexin V expression was only determined in 3 patients. As shown below, Annexin V expression was higher in CD8+ T cells in all three patients tested. However, clearly more patients will be required to address this question definitively.

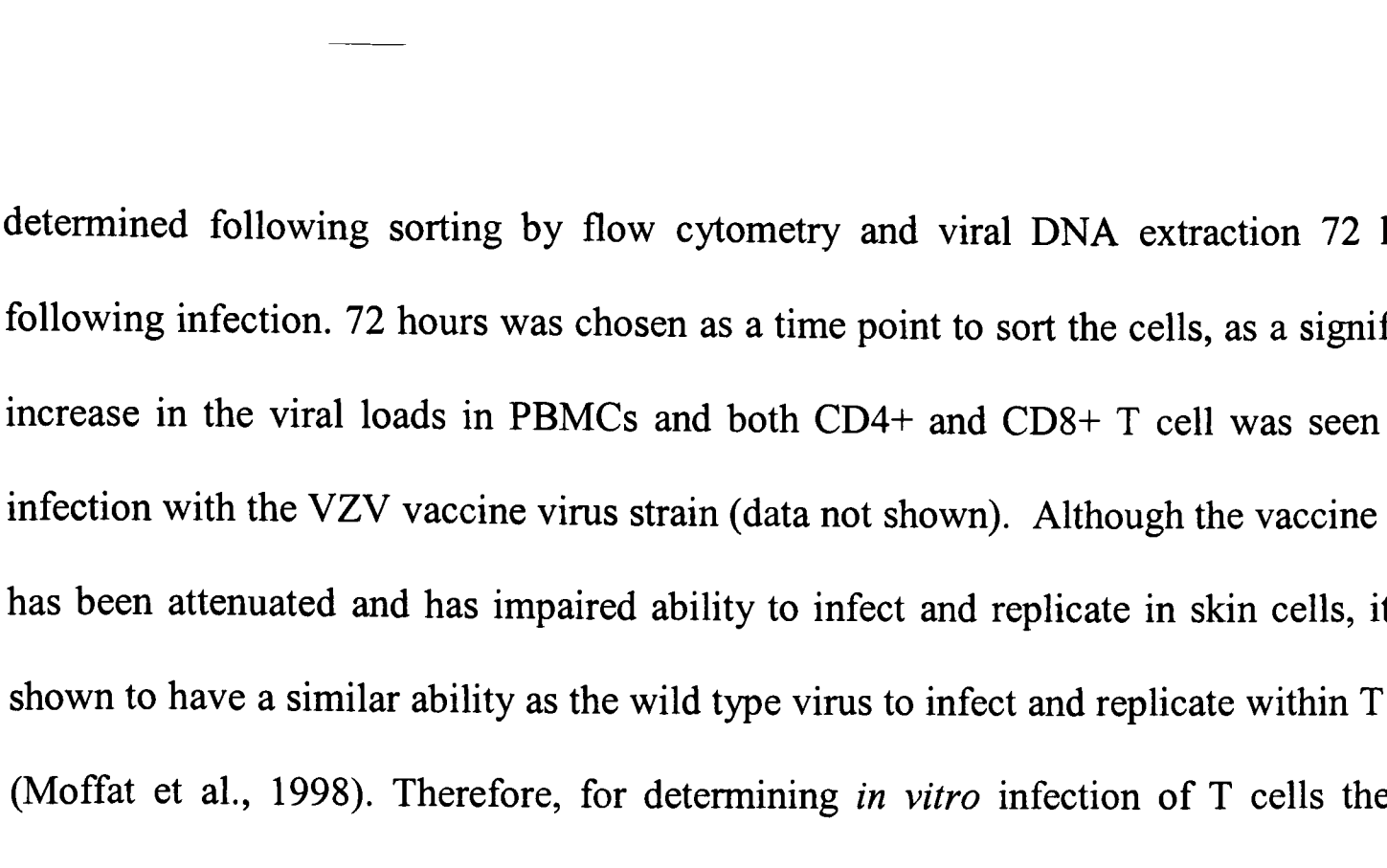


8-6 Expression of Annexin V in 3 patients with acute primary VZV infection

8.2.2. Infectivity of T cell subsets by VZV *in vitro*

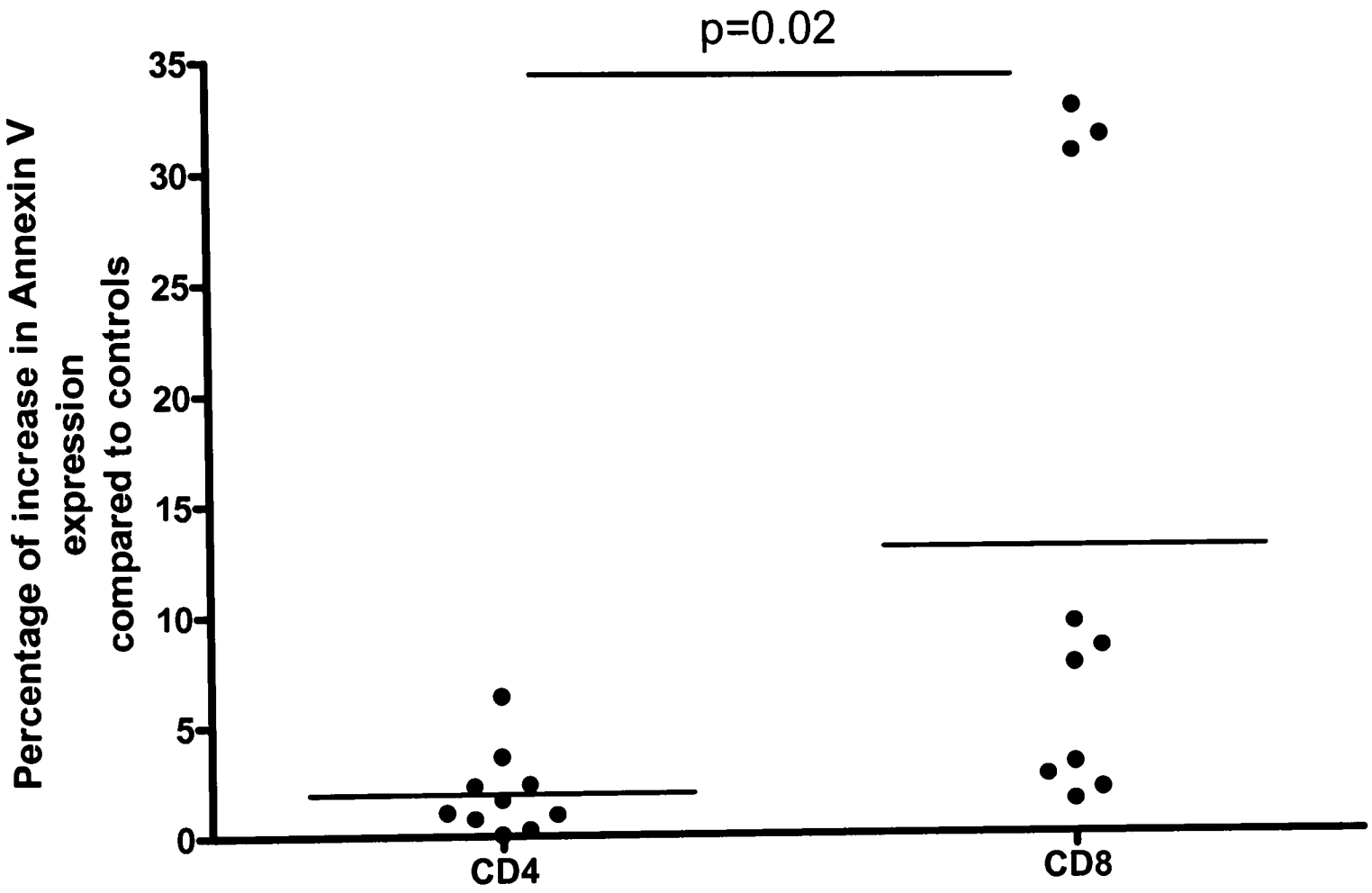
As the above data suggested that CD8+ T cells were preferentially infected than CD4+ T cells and may be more prone to apoptosis, I then went on to investigate in this more detail *in vitro*. For these experiments, PBMCs from donors were infected with the live attenuated VZV vaccine virus and the viral loads in CD4+ and CD8+ T cells were

determined following sorting by flow cytometry and viral DNA extraction 72 hours following infection. 72 hours was chosen as a time point to sort the cells, as a significant increase in the viral loads in PBMCs and both CD4+ and CD8+ T cell was seen after infection with the VZV vaccine virus strain (data not shown). Although the vaccine virus has been attenuated and has impaired ability to infect and replicate in skin cells, it was shown to have a similar ability as the wild type virus to infect and replicate within T cells (Moffat et al., 1998). Therefore, for determining *in vitro* infection of T cells the live attenuated vaccine virus was used. However, current experiments in the lab are investigating infection with the wild type virus. Following *in vitro* infection with the attenuated VZV, viral loads in CD8+ T cells (mean 8545, SD±10,724) were significantly higher (p=0.02) than those in CD4+ T cells (mean 4752, SD±3348).



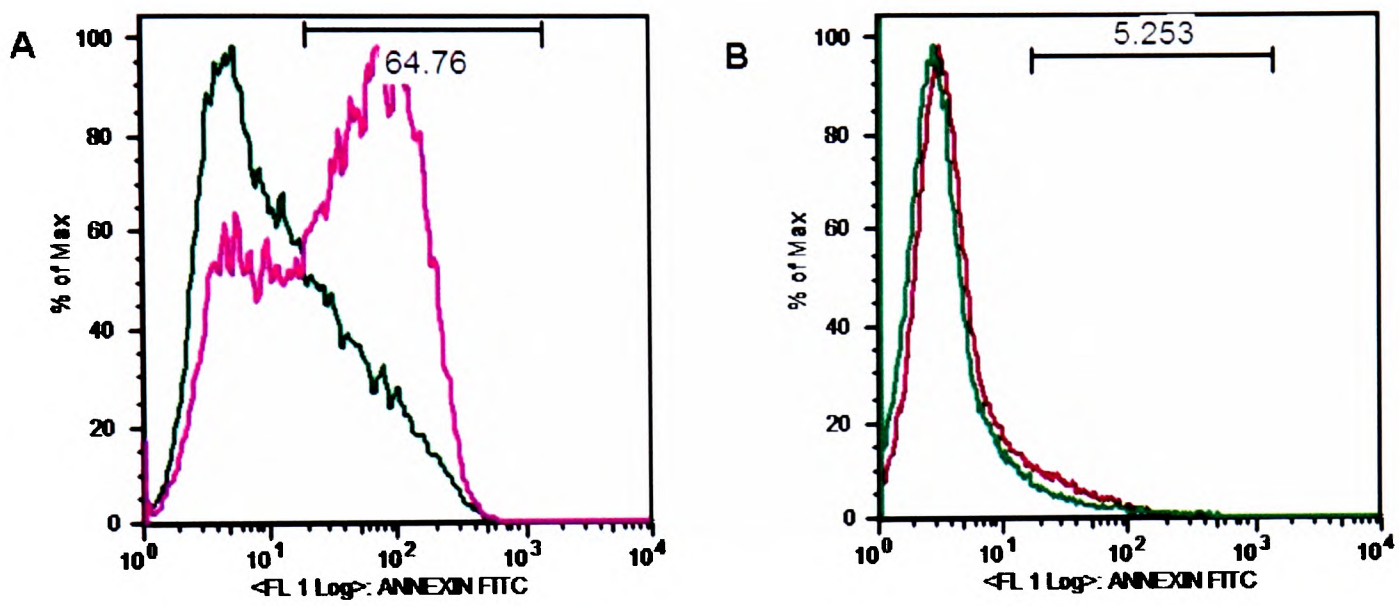
8-7 Viral loads in CD8+ and CD4+ T cells after 72 hours of in vitro infection with the live attenuated VZV vaccine virus

After establishing that CD8+ T cells were preferentially more infected when compared to CD4+ T cells *in vitro* conditions, as seen with patients with acute infection, I then went on to investigate if CD8+ T cells were more prone to apoptosis. Again using Annexin V as a marker of early apoptosis, even following *in vitro* infection with the virus the Annexin V expression by CD8+ T cells were significantly higher (p=0.02) than that seen in the CD4+ T cells.



8-8 Annexin V expression by CD4+ and CD8+ T cells following in vitro infection with the live attenuated VZV vaccine virus
P value was calculated by the use of paired t test.

However, as it was possible that more CD8+ T cell apopotosis could occur during *in vitro* conditions, I went on to compare the level of Annexin V expression *in vitro* in PBMCs infected by the live attenuated VZV virus and also uninfected PBMCs. Interestingly, as shown below, Annexin V expression was significantly higher in CD8+ T cells infected with the virus, than in uninfected CD8+ T cells; while there was no difference in Annexin V expression in infected and uninfected CD4+ T cells (Fig.8-9).



8-9 Annexin V expression by infected and uninfected CD8+ (A) and CD4+ (B) T cells

The green line indicates the level of expression in uninfected cells. Pink line indicated Annexin V expression in infected CD8+ T cells and red line indicates Annexin V expression in infected CD4+ T cells.

8.3. Discussion

The above results show that CD8+ T cells are preferentially infected when compared to CD4+ T cells in patients with acute infection and also during *in vitro* infection of T cells with the live attenuated VZV vaccine virus. Ito *et al* who also investigated viral loads in different subpopulations of T cells and in B cells in children with acute infection showed that CD8+ T cell had higher viral loads although it was not statistically significant(Ito et al., 2001). I also found that there was no difference in the viral loads CD4+ and CD8+ T cells in patients who were aged <25 years and that the viral loads in CD8+ T cells showed a positive correlation with the age of the individual. Therefore, the higher viral loads in CD8+ T cells seen in this study when compared to those seen by Ito *et al* could be due to the age of the patients that were included in the study.

It is possible that the expression of T cell memory (CD45 RO) and activation markers (CD38, CD69) on CD8+ T cells alters with age and that VZV possibly preferentially infects cells bearing these markers. If this is the case, we would then be able to explain the occurrence of more severe infection in adults when compared to children. We clearly will also investigate whether CD45RO subsets differ between CD4+ and CD8+ T cells in our cohort.

In the previous chapters it was shown that in VZV-specific immune responses were largely mediated by the CD4+ T cell subset in healthy VZV immune donors whatever the stimulus used (gI, gE, VZV lysate and live attenuated vaccine). Investigation of VZV-specific immune responses in healthy immune donors by others has also shown similar

results(Asanuma et al., 2000; Jones et al., 2006; Jones, 2007). Furthermore, although there are several VZV-specific CD4+ T cell epitopes defined, only one CD8+ T cell epitope has been characterized so far(Frey et al., 2003). This CD8+ T cell epitope was also identified by *in vitro* expansion of T cells and CD8+ T cells specific for this epitope was not detected *ex vivo* (Frey et al., 2003). However, we have not been able to reproduce the published findings for the CD8+ epitopes. In the previous chapters it was also described that in patients with acute primary VZV infection, VZV-specific T cell responses were largely mediated by the CD4+ T cell subset. Indeed the lack or paucity of VZV-specific CD8+ T cell responses in healthy immune donors and patients with acute infection is quite surprising. As these data suggest that VZV preferentially infects CD8+ T cells, it would now be important to confirm the above data by extending these experiments to larger patient number and also to determine if VZV preferentially infects T cells expressing certain surface markers, including cell surface receptors for VZV (preliminary analysis suggests that IDE is not more commonly expressed in CD8+ T cells or in infected T cells *in vitro*).

HIV is known to preferentially infect CD4+ T cells and alter their immune function (Douek et al., 2002; McCune, 2001; McMichael and Rowland-Jones, 2001; Yue et al., 2005). Therefore, it is possible that preferential infection of CD8+ T cells could alter their anti viral functions and thereby, allow more severe infection of the skin and other organs such as the lungs. This would result in more severe skin infection and a higher number of skin lesions and also higher number of viral copies in respiratory secretions in the event of a VZV associated pneumonitis. As expected the viral loads in both CD4+ and CD8+ T

cells did correlate significantly with the clinical disease severity in these few patients. Therefore, it would be now important to examine the effects of viral infection on the function of both CD4+ and CD8+ T cells in order to understand how the virus evades host defenses in early primary VZV infection.

Future directions

As this chapter only highlights some preliminary data on infection of T cells, it gives rise to more questions regarding possible events occurring during acute primary VZV infection. Although the above data suggest that there is preferential infection of CD8+ T cells when compared to CD4+ T cells by VZV and also that CD8+ T cells are possibly more susceptible to apoptosis, these findings need to be clarified by experiments on more donors.

The insulin degrading enzyme (IDE) was recently identified as a key receptor for VZV(Li et al., 2006; Li et al., 2007). However, Li *et al* also showed that along with IDE, mannose-6- phosphatase was also a receptor for VZV and possibly that more receptors could exist (Li et al., 2006). Therefore, we also aim to investigate if the level of expression of IDE varies in patients with acute VZV infection and also if its expression has any association with clinical disease severity. Furthermore, other phenotypic subsets such as CD45RO/RA, CD38, and CLA could also possibly act as VZV co-receptors. Indeed, investigations on SCIDhu mice have indicated that T cells expressing CD45RO and CLA could be preferentially infected by VZV (Ku et al., 2002). Therefore, we aim to investigate the viral loads in T cells expressing different phenotypes in patients with

acute infection, in order to determine which phenotypic markers could be implicated. Hopefully, such data would help us to understand the pathogenesis of VZV during acute infection and also during reactivation.

Chapter 9. Discussion and Conclusions

The main aims of this thesis were to determine T cell responses to 2 of the important VZV glycoproteins, to identify immune dominant T cell epitopes within them, to determine phenotypic markers expressed by glycoprotein specific T cells in healthy VZV immune donors by using class I and/or class II tetramers and finally to determine the immune responses in primary VZV infection and determine the factors that contribute to severe clinical disease. The initial aims of the thesis which were to identify immune responses to gI and gE and to identify T cell epitopes have been achieved by this thesis. I was able to identify several CD4+T cell epitopes within both glycoproteins and these epitopes were found to circulate at a high frequency and were shown to have rapid effector function in VZV immune donors many years following primary infection. I was also able to define the frequency and phenotype of gE DRB1*1501 tetramer specific T cells by using class II tetramers. However, as hardly any CD8+ responses were detected *ex vivo* and after short term T cell cultures for both gI and gE, I was not able to identify any CD8+ T cell responses and thus was not able to generate any class I tetramers.

One of the main aims of the thesis was to investigate the immune responses in primary VZV infection and to determine associations of severe clinical disease. T cell responses in acute primary infection were investigated by using IFNγ ELISpots, ICS and by MHC class II tetramers and it was shown that functional T cell responses were impaired in patients with severe clinical disease. Viral loads were also determined by quantitative real time PCR which showed that patients with higher viral loads tend to have more severe clinical disease and also that viral loads negatively correlated with functional T cell responses. Although significant amount of new data was generated by the investigation of

immune responses in primary VZV, the results have raised many questions. I have summarized the main findings of this thesis and the questions it has answered and also what we intend to do in the future.

9.1. Are VZV glycoproteins immune dominant T cell targets?

The VZV genome encodes 7 glycoproteins gB, gC, gE, gI, gK and gH (Gershon and Gershon, 1999) that are displayed on the surface of infected cells, many which have been shown to be immune dominant T cell targets. Among these both gI and gE play a vital role in viral replication, T and skin cell infectivity along with many other functions. They were also shown to induce the strongest T cell responses in animal models (Kimura et al., 1998; Lowry et al., 1992). The results illustrated in this thesis demonstrate that T cells specific for both gI and gE circulate at high frequencies in healthy donors with a history of previous infection and that they are important CD4+ T cell targets. However, gE specific T cell responses were higher than responses to gI possibly as gE is a more abundant viral glycoprotein. The responses were sufficiently robust to map the first T cell epitopes and to generate glycoprotein-specific T cell lines. Thus the glycoprotein specific T cells had a good proliferative capacity and rapid effector function.

9.2. Why were T cell responses to these 2 glycoproteins largely mediated by the CD4+ subset of T cells?

Both gE and gI specific T cell responses were predominantly mediated by $CD4^+$ T cells. In addition, VZV-specific immune responses were largely mediated by the CD4+ T cell subset in healthy VZV immune donors whatever the stimulus used (gI, gE, VZV lysate and live attenuated vaccine). Investigation of VZV-specific immune responses in healthy immune donors by others has also shown similar results(Asanuma et al., 2000; Jones et al., 2006; Jones, 2007). Although the lack of CD8+ T cells was surprising, glycoproteins of other herpes viruses have also been shown to be preferentially recognized by $CD4^+$ T cells (Bellner et al., 2005; Elkington et al., 2004; Hegde et al., 2005; Sylwester et al., 2005). In addition, there is evidence that CD4+ T cells

may especially be important in controlling many herpes virus infections (Hegde et al., 2005; Landais et al., 2005; Sester et al., 2001; Sester et al., 2002a). Furthermore, it is thought that cellular mechanisms such as autophagy may contribute to MHC class II presentation of viral glycoproteins (Paludan et al., 2005). Furthermore, it has been shown that processing of endogenous viral glycoproteins occurs in the endosomal compartment resulting in class II antigen presentation (Hegde et al., 2005). Therefore, it is likely that all these mechanisms could have contributed to a predominance of CD4+ T cell responses to VZV glycoproteins. However, these mechanisms do not explain the lack of CD8+ T cell responses to other immediate early VZV proteins (Jones et al., 2006; Jones, 2007) and to the live attenuated VZV. As the general lack of CD8+ T cell responses specific for VZV is intriguing, it would be important to continue to detect the presence of such responses by using other functional assays. However, whilst it is known that VZV infection leads to down regulation of MHC class I molecules (Abendroth et al., 2001a), we have also started to investigate the possibility of preferential CD8+ T cell infection by VZV as discussed later.

9.3. What role do VZV glycoprotein specific T cells play in healthy immune donors?

Both gE and gI specific T cells showed a rapid effector function by releasing IFNγ upon encounter with the specific antigen. Furthermore, when compared to the total IFNγ responses observed for the VZV live attenuated vaccine, both gE and gI specific T cells were shown to circulate at a comparatively high frequency. These T cells were also capable of *in vitro* proliferation when stimulated with the respective overlapping peptides, the VZV lysate and the VZV vaccine. All identified gE epitope specific T cells (following *in vitro* expansion) were also shown to be capable of degranulation and release perforin when stimulated with the respective

antigens. The presence of circulating gE and gI specific T cells many decades following primary infection would argue in favor of a role of such T cells in control of viral replication. It would be clearly important to investigate the frequencies and functional activity in other groups such as he elderly with and without VZV reactivation, and to determine the *in vitro* antiviral activity of the specific T cells.

9.4. What does the frequency and phenotype of VZV glycoprotein E specific responses tell us about VZV latency?

The results show that CD4+ T cells specific for gE DRB1*1501 circulate at high frequencies in healthy VZV immune donors. Most importantly the frequency of gE tetramer specific T cells were similar to the frequency of VZV IE63 (Jones, 2007) which is surprising given that gE is expressed only during the replicative cycle of the virus, whereas IE63 is expressed during latency as well. Furthermore, CD38 was expressed by 36.86% of gE tetramer binding T cells which is suggestive of activation of gE specific T cells within the preceding few weeks. Therefore, the significant expression of CD38 and PD-1 by gE-specific T cells argues that exposure to VZV replicative cycle antigens occurs very frequently possibly due to frequent re-activation or re-exposure.

During acute primary infection the virus is transported from the skin along sensory nerve fibres to the peripheral sensory ganglia where it establishes latency. The virus then resides in the ganglia till it reactivates later to cause herpes zoster(Kennedy, 2002). However, recently the presence of VZV vireamia was detected in 9% of healthy asymptomatic UK blood donors (Quinlivan et al., 2007a). Therefore, collectively the immunological and virological data suggest

that VZV reactivation or re-exposure occurs more frequently than previously thought in healthy immune donors and that most of these episodes do not result in clinical disease. Indeed, relatively low expression of CLA in gE and IE-63 tetramer specific T cells (Jones, 2007) in healthy immune donors indicate that the majority of these cells do not preferentially home to the skin, which would be compatible with the requirement for these T cells to circulate within the lymph nodes where there are most likely to encounter their antigen.

PD1 was expressed on 7.2% tetramer positive cells which was a significantly higher frequency in tetramer positive cells than the CD4+ population (0.51%). PD-1 has been shown to be expressed at moderately high levels in chronic viral infections and its expression is thought to be associated with T cell dysfunction due to exhaustion (D'Souza et al., 2007; Freeman et al., 2006; Golden-Mason et al., 2007). However, it was recent suggested that expression of PD-1 on viral specific CD8+ T cell was related to activation and an earlier stage of differentiation, with its expression having no effect on the functional capacity of HIV specific CD8+ T cells (Sauce et al., 2007). Therefore, at present it is not clear whether increased PD-1 expression on tetramer specific T cells indicates recent activation or represents T cells in the stage of functional exhaustion. In order to understand the implications of PD-1 expression on VZV-specific T cells, it would now be important to investigate the phenotypic expression of these markers in different groups of individuals with varying susceptibility to VZV reactivation such as the elderly and the immune suppressed.

9.5. What factors contribute to severe disease in primary varicella infection?

Although primary VZV infection is usually a benign illness it can cause severe disease and even death in certain groups of individuals such as neonates, immune suppressed individuals and adults. In this thesis factors contributing to severe VZV infection were investigated in adult patients with acute primary varicella infection and it was shown that clinical disease severity score increases significantly with advancing age and with immunosuppression. Furthermore, it was also shown that the viral loads were significantly higher in those aged >25 years when compared to those aged <25 years and a significant positive correlation was observed between the age of the patients and the viral loads. Interestingly, a positive correlation was seen between the viral loads in CD8+ T cells and the age of the individual (Spearman's r=0.71), although this was not statistically significant (p=0.057). Therefore, these results suggest that age alone is a risk factor for severe clinical disease possibly due to higher viraemia and also a higher viral load in CD8+ T cells.

It was previously suggested that immune suppressed patients who had severe disease had impaired VZV-specific proliferative T cell responses (Gershon and Steinberg, 1979a; Patel et al., 1979) indicating that T cell responses are probably important in controlling the virus during acute infection. In our study, higher functional T cell responses were observed in patients with milder clinical disease when compared to those with more severe clinical disease (p=0.05). Furthermore, patients with >500 skin lesions also had lower VZV-specific T cells than those with a lesser number of lesions. A similar relationship was also seen with regard to the viral loads as patients with more severe disease had significantly higher viral loads than those with milder disease and patients with >500 skin lesions also having higher viral loads. These data

indicate two broad possibilities. One possibility is that patients with more severe disease have higher viral loads because of impaired development of VZV-specific T cell responses in these patients, which in turn leads to poor control of the virus resulting in higher viral replication and higher viral loads. On the other hand it is possible that these patients had higher initial viral loads at the onset of symptoms which results in more infected T cells which leads to dysfunction of infected T cells. Dysfunction of T cells would then lead to less control of viral replication and more infection of T cells. Indeed some patients did not have any detectable VZV-specific functional T cell responses even 14-16 days after onset of symptoms.

A disparity was seen between the frequencies of functional T cell responses in patients with acute infection which were lower than healthy immune donors, and gE and IE63 tetramer specific T cell responses, which were higher than seen in healthy immune donors. This would indicate the possibility of the presence of antigen specific T cells in acute primary VZV infection which are dysfunctional and therefore, are underestimated by functional T cell assays such as ELISpots. It is also possible that these antigen specific T cells were secreting other cytokines other than IFNγ which was the only cytokine measured by the ELISpot responses. A third possibility is whether, VZV preferentially infects antigen specific T cells thus rendering them dysfunctional. Furthermore, although a significant negative correlation was seen with the functional T cell responses and the viral loads, no correlation was seen between the frequencies of gE and IE63 tetramer specific T cells and viral loads. Our laboratory is currently investigating these possibilities in order to better understand the events that occur in acute primary VZV infection.

As the functional T cell responses are impaired in acute VZV, especially in those with more severe disease, it appears that NK cells could be playing a significant role until the appearance of T cell responses. The percentage of NK cells was significantly increased in patients with more severe forms of infection and the population of CD56 bright cells was significantly lower. Furthermore, as the CD56bright NK population in the patients was far more activated than in healthy individuals and also expressed CLA indicates that these preferentially home to the skin. These data would argue in favour of NK cells playing a significant role in primary VZV infection until the development of virus specific T cells. Therefore, I intend to study the function of NK cells in patients with acute VZV on collection of future sample sets.

9.6. What does the phenotype of antigen specific T cells and the overall phenotype changes in T cell subsets tell us about the events that occur in acute primary VZV infection?

gE and IE63 specific T cells in patients with acute infection showed an activated phenotype and expressed markers which suggested that they were preferentially homing to the skin. However, the tetramer specific T cells showed a mixed expression of lymph node homing markers as 75.05% of the tetramer specific T cells expressed CCR7 and 28.68 % expressed CD62L. Furthermore, CLA was expressed by 16.6% of T cells which also suggestsed that a significant proportion of tetramer specific T cells also home to the skin. Therefore, it appears that in acute VZV infection, the majority of tetramer specific T cells identified by the 2 tetramers were capable of continued homing to the lymph nodes and also to the skin. This could be best suited to control the virus in VZV infection, as like in HIV, viral replication is thought to occur in peripheral lymph nodes at least during the early phase of infection and also in the skin.

9.7. What do viral loads in CD4+ and CD8+ T cell tell us about VZV pathogenesis and clinical disease severity?

It was shown that CD8+ T cells are preferentially infected when compared to CD4+ T cells in patients with acute infection and also during *in vitro* infection of T cells with the live attenuated VZV vaccine virus. Interestingly, viral loads in CD8+ T cell increased with the age of the individual and the viral loads were significantly higher in those aged >25 years when compared to younger individuals. However, as these are only preliminary data, I could not determine which factors led to increase viral loads in CD8+ T cells. It is possible that as the expression of T cell memory (CD45 RO) and activation markers (CD38, CD69) on CD8+ T cells alter with age and that VZV possibly preferentially infects cells bearing these markers. These data actually give rise to more questions than answers. One of the other main questions would be whether CD8+ T cell infection then leads to dysfunction of CD8+ T cells which would explain the paucity of functional CD8+ T cell responses in patients with acute infection. It would also be important to find out if VZV preferentially infects antigen specific T cells thereby rendering them inactive which would partly explain the higher number of tetramer specific T cells in patients with acute infection but comparatively lower frequencies of functional T cells. As I only investigated the frequencies and phenotype of tetramer specific T cells in acute infection, it would be worthwhile looking to see if these T cells were able to secrete anti viral cytokines or if they were indeed dysfunctional.

9.8. Future plans

9.8.1. What are the kinetics of VZV-specific T cell response and viraemia in acute primary VZV infection?

Although certain important questions were answered in this thesis, most experimental data have posed further questions regarding immune responses to the VZV. First of all, although absence of T cell responses and a high viral load were associated with severe clinical disease in primary VZV infection, I could not ascertain the sequence of events relative to time since infection. It would be important to know the kinetics of development of VZV-specific T cell responses and viral loads in these patients, in order to understand when 'strong' T cell responses do appear and when the viraemia subsides. In addition, although it was shown for the first time that the extent of viraemia correlated well with clinical disease severity, it would also be important to know if patients with more severe disease have more prolonged viraemia and a delayed VZV-specific T cell response when compared to those with milder forms of clinical disease.

It was also shown in this thesis that in acute VZV infection, VZV-specific IFNγ responses were predominantly mediated by the CD4+ subset of T cells. This was especially interesting since it was also observed that in acute VZV, CD8+ T cells were preferentially infected by the virus. However, the patients tested were in the very acute stage of illness (between days 3 to 6). As mentioned above, Gamadia *et al.* showed that in primary CMV infection, CD8+ T cell responses appeared somewhat later than CMV-specific CD4+ responses in patients with asymptomatic infection. Therefore, it would be important to carry out similar longitudinal studies in patients with acute primary VZV infection, to determine if VZV-specific CD8+ T cell responses do appear at a later time point and also which correlates well with the control of viraemia.

9.8.2. Does VZV infection of T cells alter their immune function?

Whether VZV preferentially infects antigen specific T cells are currently been investigated by our laboratory. Along with exploring this possibility, it is also important to find out if VZV-infection results in any T cell dysfunction. As the frequencies of tetramer specific T cells to a single epitope in patients with acute primary VZV were considerably higher than IFNγ responses to the whole virus, it is worth investigating if the T cells that do not secrete IFNγ, secrete other cytokines by methods such as luminex. It would also be worth investigating the functional capabilities of tetramer specific T cells in patients with acute infection and determine which percentages of cells are functional.

9.8.3. What is the role of NKT and NK cells in acute primary VZV infection?

Preliminary investigation of NK cells in patients with acute primary infection pointed towards the possibility that they could indeed be playing a significant role especially in those with severe clinical disease. I aim to expand these findings in a larger group of patients and also to determine the role of NK cells in acute VZV in more detail using more complete phenotypic and functional analysis.

A significant expansion in the CD3+CD4-CD8- T cell subset was also seen in patients with acute infection. Due to the limited patients samples I could not further determine what this subset of T cell were. However, given the role of NKT cells in other herpes virus infections(Ashkar and Rosenthal, 2003; Grubor-Bauk et al., 2003; Raftery et al., 2006) and also the report of fatal VZV infection in a child deficient in NKT cells(Levy et al., 2003), it is possible that NKT cells could be important in primary VZV infection. In addition, a significant expansion of NKT cells was

seen in a very limited number of patient samples (data now shown). Therefore, I hope to explore the role of NKT cells in these patients in more detail by investigating their expansion in longitudinal samples and also to determine their phenotype. Furthermore, I also hope to investigate their possible antiviral role by *in vitro* infection of dendritic cells.

9.8.4. Could the relatively low frequency of VZV-specific T cells in patients with acute infection possibly be due to sequestration in the skin?

The overall frequencies of tetramer specific T cells and IFNγ secreting T cells were considerably lower in patients with acute infection, when compared to other acute infections (Barry and Bleackley, 2002; Callan et al., 1998; Jaye et al., 2003; Whitmire et al., 2006). However, as VZV lesions predominantly occur in the skin, it is possible that most of the antigen specific T cells were sequestrated to the skin which would result in lower number in the peripheral blood. Therefore,I hope to investigate the T cell subsets, their antigen specificity and function within lesional tissue and vesicle fluid from patients with acute infection.

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