Analysis of the Immune Evasion Mechanisms of Varicella Zoster Virus

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Declaration

I declare that the content of this thesis entitled ‘Analysis of the immune evasion mechanisms of varicella zoster virus’ is entirely my own work and where indicated includes the contributions of my collaborators who have been duly acknowledged. No part of this work has been presented or submitted for any other degree or qualification in this university or institution elsewhere.

Signed:

Agnes A. Gwela

Abstract

Analysis of the immune evasion mechanisms of varicella zoster virus

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Submitted for the degree of DPhil. at the University of Oxford, Michaelmas 2013.

Varicella zoster virus (VZV) is an alpha herpes virus that causes primary infection with varicella (chicken pox), establishes latency in ganglia and may later reactivate as herpes zoster (shingles). Innate immune effectors are thought to control initial viral replication, but it is the adaptive immune system, involving T cells that mediates eventual control of viraemia and the associated clinical disease. Although both $CD4^+$ and $CD8^+$ T cells mediate viral clearance during acute illness, memory responses are dominated by $CD4^+$ T cells.

We tested the hypothesis that the paucity in memory $CD8^+$ T cell effectors is partly attributed to immune evasion mechanisms that are mounted by VZV. We confirmed that VZV readily down regulates cell surface HLA-A and HLA-C but spares HLA-B onVZV infected keratinocytes and VZV infected Mewo cells. Analysis of intracellular HLA protein expression and gene transcription showed global down regulation of all HLA subtypes. Further analysis showed that VZV inhibits IFN-γ mediated up regulation of HLA expression and augments IFN-γ mediated up regulation of HLA-E and CD71 expression.

Furthermore, we show that acute VZV infection lowers the frequency of circulating peripheral blood myeloid dendritic cells (mDC), reduces the expression of the DC activation marker HLA-DR and impairs inflammatory cytokine secretion in blood DC populations. Inhibition of DC cytokine secretion was found to be dependent on viral replication as irradiated virus resulted only in mild inhibition of IFN-α and TNF-α secretion. Lastly, we observed that VZV infection results in increased expression of host peptides, including MHC derived leader sequences that potentially bind to HLA-E. Cell surface HLA-E is known to be a ligand for the natural killer (NK) cell inhibitory receptor CD94/ NKG2A identifying a novel mechanism of viral immune escape from NK cell surveillance.

In conclusion, our data reiterates the fact that VZV targets different aspects of antigen presentation to evade the immune system with implications for pathogenesis and approaches to improved vaccination and treatment.

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Abbreviations

aa	Amino Acid
APC	Antigen Presenting cell
APC (fluorochrome)	Allophycocyanin
BHV	Bovine Herpes Virus
CCR4	Chemokine receptor 4
CCR7	Chemokine receptor 7
CD	Cluster of Differentiation
CLA	Cutaneous Lymphoid Antigen
CMV	Cytomegalovirus
CTL	Cytotoxic T Lympocyte
DC	Dendritic Cell
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing Non-integrin
DMSO	Dimethyl Sulfoxide
E	Early VZV protein
ELISA	Enzyme Linked Immunosorbent Assay
ELISPOT	Enzyme Linked Immunosorbent Spot
FACS	Fluorescence Activated Cell Sorting
FCS	Fetal Calf Serum
FITC	Fluorescein Isothiocyanate

HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HSV	Herpes Simplex Virus
HZ	Herpes Zoster
ICS	Intracellular cytokine stain
IE	Immediate early VZV Protein
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IU	International Units
L	Late VZV protein
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
MDDC	Monocyte derived Dendritic cell
NK	Natural Killer Cell
ORF	Open Reading Frame
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate buffered Saline
PE	Phycoerythrin
PE-Cy5	Phycoerythrin and a cyanine 5 dye
PE-Cy7	Phycoerythrin and a cyanine 7 dye

PerCP	Peridinin-chlorophyll-protein complex
PHA	Phytohemagglutinin
PMA	Phorbol 12-myristate 13-acetate
PO	Pacific Orange
SEM	Standard Error of Mean
SFU	Spot Forming Units
SILAC	Stable isotope labelling by amino acids in culture
TAP	Transporter associated with antigen presentation
TCR	T cell Receptor
Th	T helper
TNF	Tumor Necrosis Factor
VZV	Varicella Zoster Virus

CHAPTER 1: Introduction

1.0 Herpesviruses

Herpesviridae are a large family of DNA viruses that cause diseases in humans and animals. They are extremely infectious with a seroprevalence of between 50-100% worldwide and have in common the ability to cause acute disease then remain latent in the host for years with subsequent bursts of reactivation. The name herpes is derived from the Greek word '*herpein*'- 'to creep' describing the chronic, latent or recurring nature of infections. Also common among herpes viruses is the structural organization of virion particles. Herpes viruses possess relatively short linear dsDNA genomes ranging from 100-200 kilobase pairs enclosed in a capsid. The nucleocapsid is surrounded by a layer of protein called the tegument. The whole particle is encircled by a lipid bilayer envelope with glycoprotein spikes commonly used for cell attachment, fusion and cell spread.

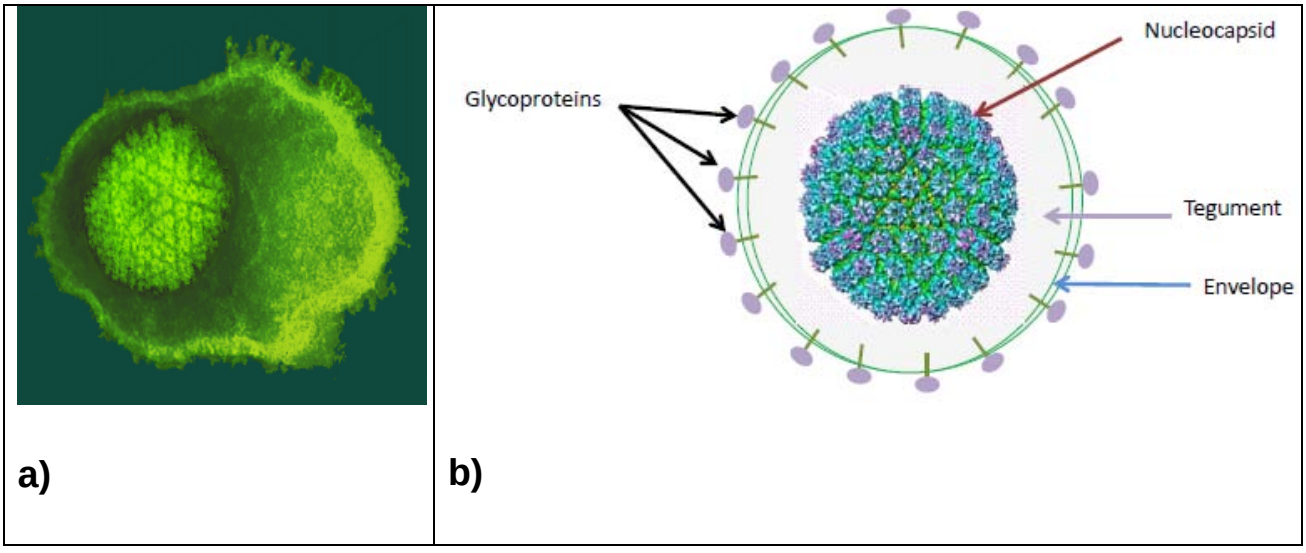


Figure 1: a.) Typical 'fried-egg' appearance of a herpes virion after a collapsed envelope seen under an electron microscope after negative staining. **b.)** A cartoon representation of a herpes virion outlining the general structural organisation. Viral nucleic acid (dsDNA) is

packed inside an icosahedral nucleocapsid surrounded by an amorphous layer of protein called the tegument which also contains mRNA. The protein layer is further enclosed by a lipid bilayer envelope which contains glycoprotein spikes.

1.1 Human Herpes viruses

There are about 25 known herpes viruses and eight of these are known to infect humans. Human herpes viruses generally fall into three distinct families based on the length of reproductive cycle, cellular tropism and site of latency. Consequently we have alpha herpes viruses which have a short reproductive cycle, variable host range and establish latency primarily in sensory ganglia. Beta herpes viruses have a long reproductive cycle, a restricted host range and establish latency in leukocytes, kidneys, secretory glands and other tissues. Gamma herpes viruses are specific for either T or B lymphocytes and establish latency exclusively in lymphoid tissue.

Properties of Human Herpes viruses					
Human herpes type	Name	Sub Family	Target cell type	Latency	Transmission
1	Herpes simplex-1 (HSV-1)	Alphaherpesvirinae	Mucoepithelia	Neuron	Close contact
2	Herpes simplex-2 (HSV-2)	Alphaherpesvirinae	Mucoepithelia	Neuron	Close contact usually sexual
3	Varicella Zoster virus (VSV)	Alphaherpesvirinae	Mucoepithelia	Neuron	Contact or respiratory route
4	Epstein-Barr Virus (EBV)	Gammaherpesvirinae	B lymphocyte, epithelia	B lymphocytes	Saliva
5	Cytomegalovirus	Betaherpesvirinae	Epithelia,	Monocytes,	Contact, blood

	(CMV)		monocytes, lymphocytes	lymphocytes and possibly others	transfusions, transplantation, congenital
6	Herpes lymphotropic virus	Betaherpesvirinae	T lymphocytes and others	T lymphocytes and others	Contact, respiratory route
7	Human herpes virus-7 (HHV-7)	Betaherpesvirinae	T lymphocytes and others	T lymphocytes and others	Unknown
8	Human herpes virus-8 (HHV-8) Kaposi's sarcoma-associated herpes virus (KSHV)	Gammaherpesvirinae	Endothelial cells	Unknown	Exchange of body fluids?

Table 1: Herpes viruses that infect humans.

Adapted from http://pathmicro.med.sc.edu/virol/herpes.htm

1.2 Varicella zoster virus (VZV)

Varicella zoster virus (VZV) is an alpha-herpes virus that exclusively infects humans resulting in the clinical manifestations of varicella/ chicken pox during primary infection. This is usually followed by establishment of latency in sensory ganglia and dorsal route ganglia from where the virus may later reactivate as a secondary infection known as herpes zoster or shingles. The nucleic acid message is organized into a double stranded DNA of 125kb encoding approximately 70 Open Reading Frames (ORFs) (Cohen ; Arvin 1996; Cohen 1999) surrounded by a tegument and an outer envelope. The virus envelope is important for viral entry and attachment with

several glycoproteins playing a role in cell attachment, fusion, entry or cell to cell spread. As with many other viruses, cell tropism is determined by the availability of the correct receptor on the surface of the target cell. VZV enters the host cell through fusion of the viral envelope with the cell membrane or by endocytosis. Initial attachment of VZV particles to cells is thought to occur non-specifically through binding of viral glycoproteins gB and gC to cell surface heparan sulfate proteoglycans (Zhu, Gershon et al. 1995). To date, only three VZV entry receptors have been identified; Mannose-6-phosphate receptor (MPR^{ci}), insulin degrading enzyme (IDE) and myelin-associated glycoprotein (MAG) (Chen, Zhu et al. 2004; Li, Ali et al. 2006; Suenaga, Satoh et al. 2010). Several VZV glycoproteins contain mannose-6-phosphate groups and may bind to MPR^{ci} (Zhu, Gershon et al. 1995; Chen, Zhu et al. 2004). Consequently, dephosphorylation of VZV by exposure to alkaline phosphatase rapidly destroys infectivity. IDE binds VZV gE; inducing a conformational change in the glycoprotein that enhances infectivity (Li, Ali et al. 2006; Li, Krogmann et al. 2007; Li, Ali et al. 2010) while gB engages MAG (Suenaga, Satoh et al. 2010). MPR^{ci} and IDE show low level surface and endosomal expression in various cells but MAG is predominantly expressed on glial cells in nervous tissue and is absent on both neurons and epithelial cells (Suenaga, Satoh et al. 2010). Binding of VZV to its entry receptor(s) is followed by fusion of the viral envelope to the plasma or endosomal membrane leading to host cell penetration. This process is thought to be mediated by the fusion machinery composed of glycoproteins gB, gH and gL (Connolly, Jackson et al. 2011; Vleck, Oliver et al. 2011; Oliver, Brady et al. 2013). Following fusion, viral tegument proteins and the nucleocapsid are released into the cytoplasm. The nucleocapsid then localises to the nuclear membrane and eventually releases the viral genome into the nucleus where

the viral DNA circularizes and host cell RNA polymerase II commences transcription of viral genes in parallel to those of the host (Cohen JI, Straus SE et al. 2007).

Transcription of VZV genes follows a pattern of three temporal phases during the viral replicative cycle giving rise to immediate early (IE), early (E) and late (L) gene products (Cohen ; Cohen 1999). Other members of the alpha-herpesviridae sub-family include HSV-1 and HSV-2, with which VZV shares structural similarity and gene sequence homology. Consequently, the functions of many VZV proteins have been inferred from those of their corresponding homologues in HSV. Other important alpha herpes viruses include Pseudo-rabies (PRV) virus and Bovine herpes virus (BHV) which infect swine and cows respectively.

Although chicken pox is often considered a relatively common childhood illness, the high risk of potential complications such as secondary infection with staphylococcus aureus, otitis media, endocarditis, encephalitis and pneumonia make it an important cause of concern (Losurdo, Bertoluzzo et al. 2005). Pneumonia is the most common complication seen in both adults and children with a fatality rate of 9-50% (McCoy, Sorvillo et al. 2004). Generally, complications such as pneumonia are more common in adults (Mohsen, Peck et al. 2001; Danovaro-Holliday, Gordon et al. 2004) or immune-suppressed individuals, a factor that underscores the importance of cell mediated immunity in the control of VZV infection, replication and disease severity.

1.3 VZV Infection and Pathogenesis

Infectious VZV particles are transmitted to susceptible individuals from aerosolized lesional fluid and respiratory droplets or through direct contact with skin lesions which contain infectious virus. Infection occurs following attachment of the virus to epithelial cells of the upper respiratory tract (URT) or conjunctivae. In the absence of experimental data, early theories of VZV pathogenesis were based on conclusions drawn using the mouse pox model described in 1948 by Fenner (Fenner 1948; Grose 1981). According to this model, pathogenesis follows two viremic phases. First, virus begins to replicate in the nasopharynx and the local area lymph nodes of the URT 4-6 days after infection. Infection of the blood stream soon follows where virus is disseminated by tissue infiltrating hematopoietic cells including antigen presenting cells (APC) such as Dendritic cells which have been shown to be permissive to VZV infection (Abendroth, Morrow et al. 2001; Morrow, Slobedman et al. 2003; Huch, Cunningham et al. 2010) resulting in primary viraemia. 14-16 days after infection a second wave of viral replication occurs in the body's internal organs including the liver and the spleen resulting in a secondary viraemia. This widespread secondary vireamia was thought to be accompanied by infection of capillary endothelial cells and cutaneous cells resulting in the characteristic varicella rash on the skin. This earlier model has since been replaced by new insights from studies conducted in the severe combined immunodeficient (SCID-hu) mouse model. Current understanding suggests that the 14-21 day long incubation period between VZV infection and eruption of the skin rash is due to the effects of innate immunity. Following cutaneous infection, type I interferon and other innate immune cytokines from intact/ uninfected cutaneous tissue and uninfected epidermal cells are thought to inhibit viral

replication in the skin (Ku, Zerboni et al. 2004; Ku, Besser et al. 2005). Also, after infection via the upper respiratory tract route, virus is believed to be disseminated mainly by T cells present at sites of early replication such as the tonsils and local area lymph nodes. Infection of migratory tonsillar $CD4^+$ T cells expressing skin homing markers is thought to enhance virus transport to the skin where cutaneous infection eventually results in a vescicular rash (Ku, Padilla et al. 2002; Schaap, Fortin et al. 2005; Arvin, Moffat et al. 2010).

It is difficult to be certain of the timing of the first detectable adaptive immune response to VZV as patients tend to present after the appearance of the rash. Adaptive immune responses are however usually detectable following the onset of the rash. After the adaptive immune system sets in, the acute infection is partly controlled. The virus travels along sensory nerve axons that innervate replication sites on the skin and establishes latency in cranial nerve ganglia, dorsal root ganglia, and autonomic ganglia along the entire neuraxis (Wang, Lau et al. 2005).

VZV is thought to remain relatively quiescent during latency with minimal expression of a restricted number of genes including the products of ORF4, ORF21, ORF29, ORF62, ORF63 and ORF66 (Meier, Holman et al. 1993; Cohrs, Barbour et al. 1996; Lungu, Panagiotidis et al. 1998; Cohrs, Gilden et al. 2003; Zerboni, Sobel et al. 2010) all of which are also expressed during lytic infection. Although concerted experimental efforts have confirmed the expression of these transcripts during latency, there is much debate on the number and abundance of VZV transcripts expressed during latency owing to disparity in many study samples and results. Most early studies that identified many of these transcripts employed the use of ascites derived monoclonal antibody preparations. Recent studies have however shown that

ascites-derived VZV IE62- and IE63-specific mouse monoclonal antibodies contain trace levels of endogenous anti-human blood group A1 antibodies which may result in false detection of IE62 and IE63 antigens in blood group A1 positive donors as has been reported in various tissues where ascites derived or rabbit polyclonal antibody preparations have been used (Oriol, Mollicone et al. 1985; Mollicone, Davies et al. 1986; Spicer, Spivey et al. 1994; Ouwendijk, Flowerdew et al. 2012; Zerboni, Sobel et al. 2012). Development of better monoclonal antibodies will prove whether these previously reported VZV transcripts are expressed during latency or not. The fact that unlike in HSV, VZV neuronal latency sites rarely have VZV specific T cell infiltrates also suggests that perhaps virus protein expression is not a characteristic of VZV latency (Verjans, Hintzen et al. 2007).

The controversies surrounding latent VZV gene products are further underscored by the fact that studies are restricted to using human ganglia obtained at autopsy. A recent study, though reporting abundant expression of IE63, witnessed a global increase in the level of transcripts (IE4, IE62, IE63, E29, L11, L40, L41, L43, L57 and L68) that positively correlated with increasing post mortem intervals; suggesting that perhaps detection was related to VZV reactivation occurring postmortem, even though there was no accompanying increase in viral DNA load (Ouwendijk, Choe et al. 2012). Thus it seems that the level and number of detectable VZV transcripts during latency can be attributed to the post mortem interval and may not represent the true status of the latent VZV transcriptome. Additional studies will continue to shed light on this important yet controversial subject.

The exact role of these identified gene products during latency is also yet to be established but some studies suggest that the product of ORF63 which is abundantly

expressed may play a role in immune evasion by inhibiting the activities of type I interferon (Ambagala and Cohen 2007) and maintaining cell survival through inhibition of apoptosis (Hood, Cunningham et al. 2003; Hood, Cunningham et al. 2006). It is also not clear how latency is maintained in VZV though this has been compared to the epigenetic control seen in HSV. Cellular gene expression is regulated by epigenetic chromatin modifications, the availability of transcription factors and intranuclear genome location (Sexton, Schober et al. 2007). Some HSV genomes have been shown to localize to specific nuclear regions that restrict viral gene expression, (Catez, Picard et al. 2012). Presumably, latent virus genomes are bound to histones in the nuclei of infected neurons and are therefore subject to epigenetic chromatin modifications that determine gene expression. Histone acetylation and methylation determine the accessibility of genes to transcription factors. Consequently, open chromatin (euchromatin) is associated with histone H3 acetylation on lysines 9 and 14 (H3K9ac/14ac) and dimethylation on lysine 4 (H3K4me2). Closed conformation chromatin (heterochromatin) is associated with multiple methylations (H3K9me2, H3K9me3 and H3K27me3) (Jenuwein and Allis 2001). During HSV latency, most genes are found to be enriched for heterochromatic marks except at the latency associated transcript (LAT) locus which is enriched for euchromatin resulting only in expression of LATs. (Bloom, Giordani et al. 2010). Analysis of the epigenetic configuration of the latent VZV genome in ganglia 24 hours post mortem revealed heterochromatic ORFs 14 and 36 and euchromatic ORFs 62 and 63 (Gary, Gilden et al. 2006) which correlates well with the abundant expression of IE63. It is possible that the widespread ‘reactivation’ seen in VZV infected ganglia post-mortem could result from a change in chromatin conformation due to cell stress possibly induced by hypoxia after death (Ouwendijk, Choe et al.

2012) hence the increase in the number of detectable transcripts. Alternatively, VZV latency may solely be controlled by the pre-existing local innate and adaptive immune response at neuronal sites. The latter response is debatable because VZV latency is not associated with the retention of virus-specific T cells in human ganglia (Verjans, Hintzen et al. 2007). However, VZV has been shown to be capable of establishing latency in the absence of an adaptive immune system in the SCID-hu mouse model (Zerboni, Ku et al. 2005) suggesting that ganglionic innate immunity can restrict viral replication and force VZV into latency.

During latency, periodic sub-clinical reactivations are thought to occur but these are well contained by the pre-established anti-viral immune response. Anytime the immune threshold needed to contain the virus is breached following a decline in cell mediated immunity, the virus travels along nerve axons back to sites on the skin causing herpes zoster (shingles) with a restricted dermatomal distribution.

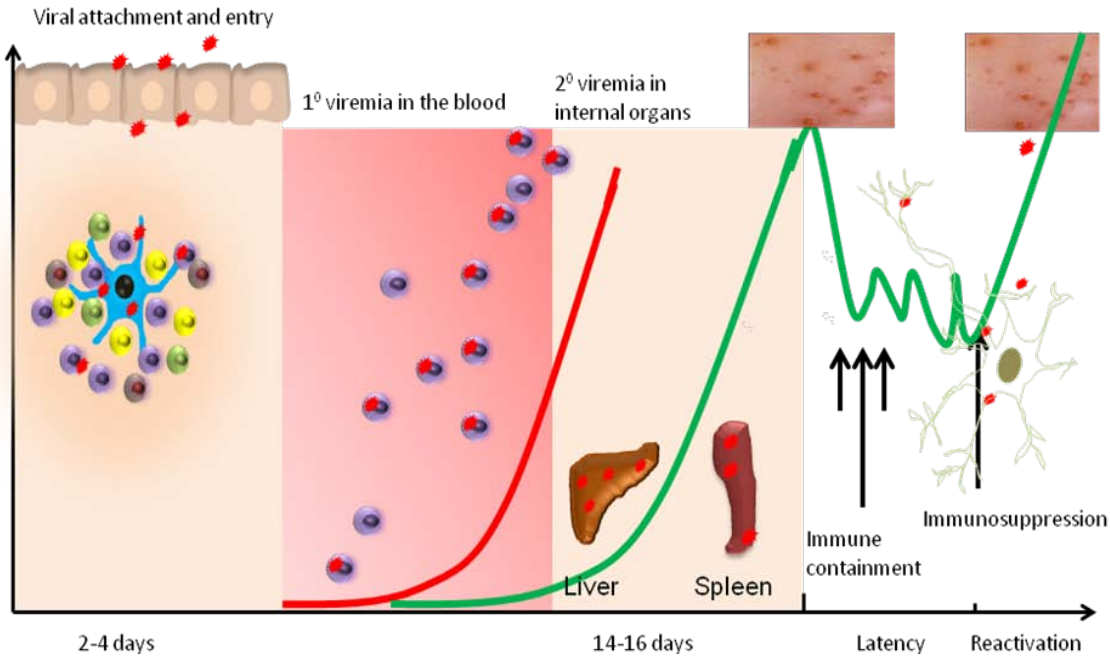


Figure 2: Summary of the 6 stages of VZV Infection and Pathogenesis showing. Viral attachment and **entry**, **primary viraemia** in the blood, **secondary viraemia** characterised by viral replication in internal organs including the liver and spleen, cutaneous lesions

characteristic of **skin infection** and **latency** in sensory ganglia from where viral **reactivation** occurs to cause shingles.

1.4 Herpes Zoster Pathogenesis and Complications

VZV reactivation causes herpes zoster. Unlike varicella, herpes zoster is predominantly associated with waning cell mediated immunity (CMI). T helper 1 (Th1) and cytotoxic T cell responses have been shown to be essential for recovery form herpesvirus infections (Arvin, Koropchak et al. 1986; Manickan, Francotte et al. 1995) and are also believed to be responsible for the maintenance of virus in the latent state (Volpi 2005). Consequently, conditions which depreciate CMI such as pregnancy (Paryani and Arvin 1986), immunosuppressive therapy in patients undergoing solid organ transplants or chemotherapy (Feldman, Hughes et al. 1973; Dolin, Reichman et al. 1978; Wilson, Sharp et al. 1992), increasing age (Miller 1980; Berger, Florent et al. 1981) and immunodeficiencies such as HIV (Colebunders, Mann et al. 1988; Derryck, LaRussa et al. 1998) are associated with an increased risk of developing zoster. Another peculiar risk of developing herpes zoster is the incidence of varicella in the first year of life (Terada, Kawano et al. 1993; Takayama, Yamada et al. 2000) possibly influenced by improper development of the immune system. Maternal varicella has also been associated with the development of shingles early in life. Herpes zoster is characterized by a painful vesicular rash usually confined to a single dermatome (defined as an area on the skin that is mainly supplied by a single spinal nerve) compared to the disseminated rash seen in varicella. Reactivation most commonly involves the thoracic and cranial dermatomes. Symptoms of zoster typically mirror a fever including high temperature, chills and headache together with symptoms associated with the rash

which include pain, itching and tingling sensations under the skin in affected areas. These initial symptoms are often followed by a breakout of a patchy or clustered rash, usually on one side of the body. This can often be around the waistline or on one side of the face or torso. The rash eventually develops into fluid filled painful blisters which eventually begin to dry out within a few days or weeks. The painful sensations on the skin may last long after the clearance of infection, a complication known as post-herpetic neuralgia (PHN) which can significantly reduce an individual's quality of life (Bowsher 1999; Mounsey, Matthew et al. 2005). If the virus affects ophthalmic nerves around the eyes, inflammation of the cornea or serious eye infection may occur. Rarely, such a viral infection of a facial nerve may cause paralysis or weakness of one side of the face a condition known as herpes zoster ophthalmicus (Volpi 2007). Other complications may arise following VZV reactivation including vasculopathy, myelopathy, retinal necrosis, cerebellitis and zoster sine herpete (Mueller, Gilden et al. 2008).

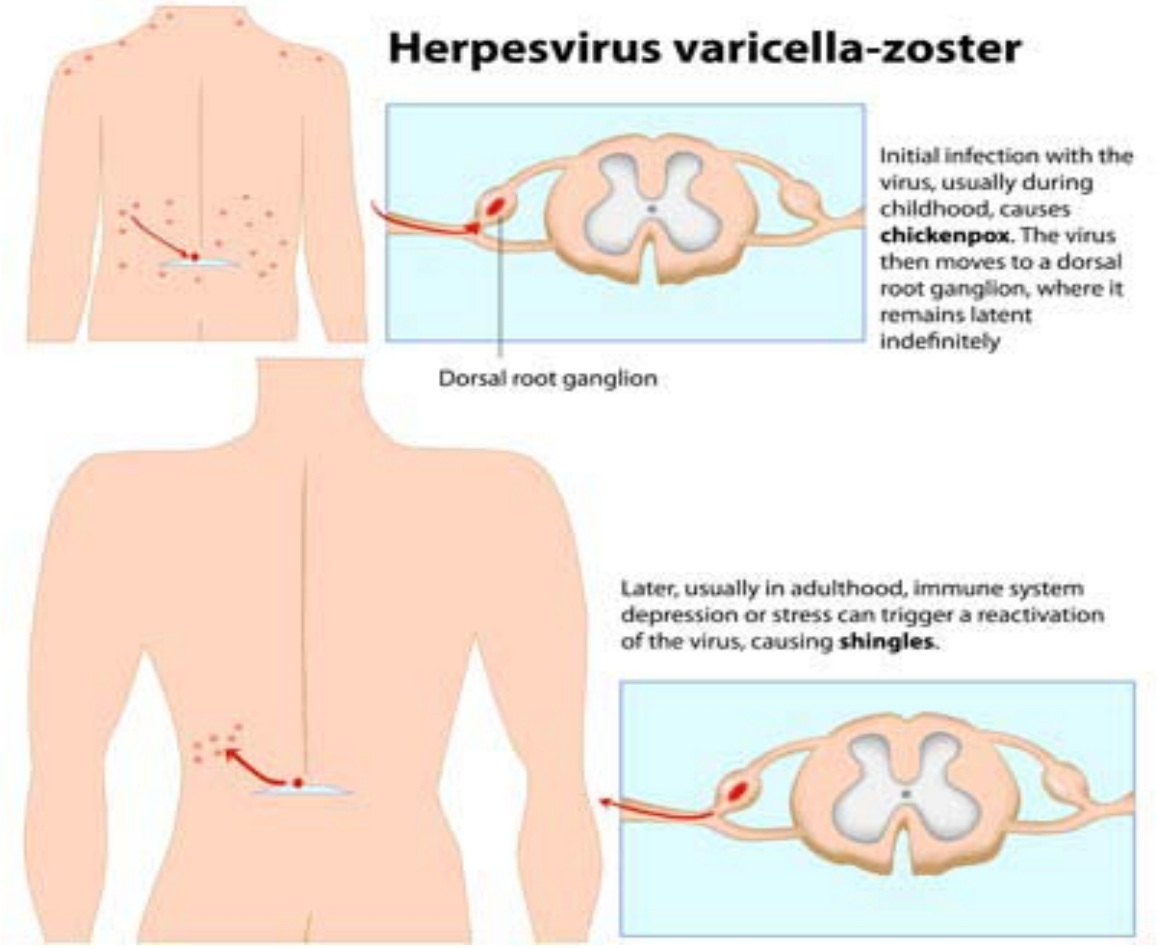


Figure 3: Diagramatic representation of the pathologies associated with varicella and zoster infections. Notice the distinguishingly unique distribution of the rash associated with the two infections.

Adapted from http://www.drhealth.md/infectious-diseases/viral-diseases/herpes/

1.5 Epidemiology of varicella and herpes zoster

Varicella and zoster infections occur worldwide. VZV is a highly infectious pathogen with a seroprevalence of over 90% in the US alone. The epidemiology of varicella differs quite significantly between temperate and tropical regions with annual epidemics in temperate regions typically occurring in late winter and spring. In the United States, the highest incidence is recorded between March and May and

the lowest between September and November. Less seasonality is reported in tropical areas but has been described in some countries including Guatemala, Nigeria, Sri-Lanka and parts of India with high incidences occurring in the driest coolest months (Maretic and Cooray 1963; Salomon, Gordon et al. 1966; Sinha 1976; White 1978; Iyun 1984; Venkitaraman and John 1984; Balraj and John 1994). In both temperate and tropical regions, epidemic years are followed by years with lower incidence (Gordon 1962; Salomon, Gordon et al. 1966; Sinha 1976; Joseph and Noah 1988; Balraj and John 1994). No seasonal variation has been reported for Herpes zoster with incidences reported throughout the year; probably because incidence is linked to immunological status.

Also, in temperate regions infection spreads and is common among children of school going age (Thiry, Beutels et al. 2002; Vyse, Gay et al. 2004; de Melker, Berbers et al. 2006; Khoshnood, Debruyne et al. 2006) whereas in most tropical climates, VZV cases are more common in adults (Venkitaraman and John 1984; Garnett, Cox et al. 1993) in whom severe disease is more likely (Wharton 1996). This difference in age distribution has not yet been attributed to any known factor, but may be related to lack of childhood varicella infection in rural populations, different social practices on infection, effects of climate on viral replication and different host or viral genetics.

In the pre-vaccine era, varicella was endemic in the USA with a high sero-prevalence and incidence rates approximately equivalent to the birth rate. However, in the face of changing intervention such as the adoption of standard vaccination of healthy children and susceptible adults against varicella and zoster; a new change in disease epidemiology is emerging. The Varicella vaccine (Varivax, Merck) was approval by

the Food and Drug Administration for use in the United States in 1995. Since then, there is strong evidence suggesting that vaccination against varicella and zoster is highly effective at reducing the incidences of varicella in children (Takahash.M, Otsuka et al. 1974; Seward, Marin et al. 2008) and zoster in adults (Weir 2005). Consequently, incidence rates have greatly reduced. Several sero-epidemiological investigations are currently being conducted to elucidate the long term impact and/or benefits of vaccination on herd immunity. This is especially important in evaluating vaccination policies or assessing the rationale for existing vaccination campaigns. As more countries adopt vaccination as a preventive measure, the epidemiology of varicella and zoster driven by mass herd immunizations are likely to change even further.

1.6 Treatment and management of varicella and Zoster

1.6.1 Chemotherapy

There is currently no cure for varicella and zoster infections. As varicella is not a serious disease in immunocompetent individuals, treatment mainly consists of easing the symptoms of the condition. Some treatments are however available for relieving symptoms while the immune system suppresses the virus. The condition usually resolves by itself within a couple of weeks after the onset of the rash when the immune system kicks in. Treatment of chickenpox in children is aimed at alleviating symptoms while the immune system deals with the virus. The use of acetaminophen (paracetamol) is recommended for relief of fever symptoms. People at risk of developing severe complications who have had significant exposure to the virus may be given intra-muscular varicella zoster immune globulin (VZIG), a preparation

containing high titres of antibodies to varicella zoster virus, to reduce the risk of disease (Parmet, Lynm et al. 2004). If oral acyclovir is started within 24 hours of onset of rash, in children, it decreases the severity of chickenpox and duration of symptoms but has little effect on complication rates (Dunkle, Arvin et al. 1991; Kay 2001; Kay 2001). Use of acyclovir is therefore not recommended for immunocompetent individuals or children under 12 years of age. Varicella infection in otherwise healthy adults tends to be more severe (Tunbridge, Breuer et al. 2008). Remedies to ease the symptoms of chickenpox in adults are basically the same as those used in children including the use of acetaminophen for the relief of itching and other symptoms such as fever or pains. Adults are more often prescribed antiviral medication as it is effective in reducing the severity of the condition and the likelihood of developing complications. Treatment with antiviral drugs such as acyclovir (Whitley and Gnann 1992) or valacyclovir (Acosta and Fletcher 1997) is generally advised, as long as it is started within 24–48 hours from the onset of the rash. Antiviral medicines do not kill the virus, but stop it from multiplying by interfering with viral DNA replication. Antihistamines may also be used to relieve itches in cases where itching prevents sleep, as they are also sedative. As with children, antiviral medication is considered more useful for those adults who are more susceptible to complications such as pregnant women and immunocompromised individuals. A nucleoside analogue, Sorivudine has been reported to be effective in the treatment of primary varicella in healthy adults (case reports only), but large-scale clinical trials are still needed to demonstrate its efficacy (Tunbridge, Breuer et al. 2008). Advanced therapies are still being considered for treatment of varicella in high risk and susceptible groups.

1.6.2 Vaccination

A live attenuated varicella vaccine was developed by Takahashi and his colleagues in the early 70's in Japan (Takahash.M, Otsuka et al. 1974) in response to a varicella epidemic in a children's ward. The origin of the vaccine can be traced back to 1952, when Weller and Stoddard recovered varicella–zoster virus by cell culture and reported the first successful propagation of a herpes virus *in vitro*. In 1974, Takahashi and colleagues attenuated the wild-type Oka strain by serial passage in guinea-pig and human cells, while preserving its immunogenicity after subcutaneous inoculation. The vaccine was first licensed in 1989 in Japan for use in healthy children. Although first received with skepticism around the world, clinical trials in subsequent years would prove that the vaccine was safe and effective not only in children but also in certain immunocompromised patients who were at high risk of developing severe varicella (Gershon, Steinberg et al. 1988). In the USA, vaccine studies were first conducted in immunocompromised children with underlying leukemia with much success. Following this successive trial, large clinical trials were carried out in the 1980s (White 1997) with healthy children as the target group (Krause and Klinman 1995). The vaccine was eventually approved by the Food and Drug Administration and licensed for general use in children over the age of 1 year and varicella susceptible adults in 1995. Oka-derived vaccines are now used in many developed countries. To date, the live attenuated varicella vaccine is the only vaccine licensed for the prevention of a disease caused by a human herpes virus.

1.6.3 Vaccination against varicella

Two live, attenuated varicella-zoster virus-vaccines are licensed in the United States for the prevention of chickenpox, a single-antigen varicella vaccine (VARIVAX, Merck) and a combination measles, mumps, rubella, and varicella vaccine formulation (ProQuad, Merck) although the latter is currently unavailable due to production problems (Marin, Guris et al. 2007). In the UK, the use of (Vairilix, GSK) is common. The live attenuated varicella vaccine has had a remarkable record since its licensure in the U.S.A. The vaccine elicits persistent B and T cell immune responses and reduces the incidence of varicella in vaccinated children and of zoster in elderly vaccinees (Takahash.M, Otsuka et al. 1974; Asano, Nagai et al. 1985; Kamiya, Asano et al. 2001; Oxman, Levin et al. 2005; Levin, Oxman et al. 2008). It is currently being administered to children in a 2 dose regimen with a reported efficacy of upto 98% in vaccinated individuals (Shapiro, Vazquez et al. 2011). The use of live attenuated vOka is also reassuring especially because there is currently very little concern that the vaccine strain may mutate to a virulent form. Even though studies have reported single nucleotide polymorphisms (SNPs) in the vaccine strain, these did not result in increased virulence (Quinlivan, Gershon et al. 2007). It is thus postulated that the use of this vaccine will become widespread as additional countries adopt a vaccination policy against varicella possibly in children and other vulnerable groups.

Although generally hailed for reducing the incidence of disease, there are still concerns surrounding the use of a live varicella vaccine due to potential pathogenic outcomes. The vaccine (vOka) strain though significantly attenuated from the parent strain (pOka) could still bear some of the virulence markers of the wild type strain.

The specific determinants of attenuation of the vaccine strain are still uncertain and though this is still an area of on-going research, some studies have already reported possible mechanisms. Moffat and colleagues were the first to report a basis of attenuation following experiments in the SCID-hu mouse model which revealed impaired infectivity and replication of the vaccine strain in human skin but not in T cells. They found that vOka infectivity was further diminished in the absence of glycoprotein C (gC) expression (Moffat, Zerboni et al. 1998). Other studies have attempted to provide a molecular basis for vOka attenuation related to genetic changes. DNA sequence analysis of pOka and vOka revealed that the two strains differ at 42 nucleotides and 20 amino acids. Majority of the differences were concentrated on the ORF62 region encoding the regulatory immediate early protein 62 (IE62); bearing 15 nucleotide and 8 amino acid changes in vOka compared to pOka. Furthermore, these changes were only present in vOka and not in 9 clinical isolates tested in parallel. The study also reported a functional basis of vOka attenuation where changes in IE62 resulted in reduced transactivational activity of vOka IE62 compared to that of wild type. Viruses with the IE62 amino acid mutations also formed smaller plaques and exhibited a reduced ability to spread in human embryonic lung cells (Gomi, Sunamachi et al. 2002). Against this basis however, another study comparing gene expression patterns of vOka and pOka failed to conclude that mutations in IE62 alone accounted for the vaccine virus attenuation, but they only tested IE62 transactivational activity on 6 VZV gene promoters (Cohrs, Gilden et al. 2006). Further studies are still needed to determine the specific single nucleotide polymorphisms (SNPs) in IE62 that are associated with attenuation. Another basis of molecular attenuation has since emerged from gene sequence comparisons between a highly attenuated laboratory VZV strain (Ellen) and other

complete VZV genomic sequences. Bioinformatic comparisons revealed no polymorphisms in all the ORFs in Ellen that are also known to be mutated in vOka except 2 SNPs in IE62 and a STOP codon mutation in ORF0. The product of this ORF may be involved in intracellular virus trafficking like its HSV homolog (UL56) (Koshizuka, Goshima et al. 2002) and in replication (Kaufer, Smejkal et al. 2010) which would explain the reduced replicative abilities of both Ellen and vOka observed in human skin xenografts in the SCID-hu mouse. Since the two strains are not ancestrally related, it is believed that the mutation in ORF0 could not have occurred by chance and is indeed one of the determinants of attenuation in vOka (Peters, Tyler et al. 2012). Despite evidence of attenuation and reduced ability to replicate in the skin, the vOka strain can still establish latency and thus carries the ever present risk of reactivation and dissemination (Gershon and Gershon 2010). It is feared that mass vaccination may increase the incidence of zoster among vaccinated children following reactivation of latent vaccine virus. Other concerns about development of zoster in vaccinated children follow the belief that mass vaccination of children will reduce the amount of circulating virus needed to boost established anti-VZV immunity, posing another risk of zoster incidences (Leung, Harpaz et al. 2011; Goldman and King 2013).

1.6.4 Vaccination against herpes zoster

As earlier described, reactivation of VZV from latently infected ganglia is limited by T cell mediated immunity. Whenever cell mediated immune responses fall below a given threshold, reactivated virus is able to replicate and propagate resulting in antegrade transport to nerve endings on the skin and the subsequent dermatomal

eruption that is herpes zoster/ shingles. The rationale for prevention of herpes zoster follows a hypothesis that enhancement of cell mediated immunity in high risk groups will prevent disease. One of the more susceptible groups is the elderly in whom the incidence and severity of HZ increases with increasing age and correlates directly with a decline in VZV specific cell mediated immunity (Berger, Florent et al. 1981; Donahue, Choo et al. 1995). Currently, the only vaccine licensed against shingles in the USA is Zostavax which is recommended for use in persons aged 60 years or older. In one clinical trial, vaccination with Zostavax reduced the risk of developing shingles by 51% and the risk of post-herpetic neuralgia by 67% in individuals over 60 years of age followed over a period of 5 years (Oxman, Levin et al. 2005). Although the vaccine was most effective in the 60-69 years age group, it also provided some protection for older groups. A vaccination plan against herpes zoster in older people is scheduled to start in the UK in September 2013. Like varicella vaccines, successful use of Zostavax will certainly determine the extent of usage in subsequent years.

1.7 Immune responses to VZV infection

Both the innate and adaptive arms of the immune system play a role in the control of VZV infection (Abendroth and Arvin 1999). Innate immune responses are responsible for limiting viral replication and spread (Arvin, Koropchak et al. 1986; Abendroth and Arvin 2001) as well as shaping the initiation of the adaptive response. Early innate surveillance after VZV infection is mediated by type I and type II interferons as well as activated NK and NKT cells. Type I interferons (IFN-α and IFN-β) stimulate the production of a variety of interferon stimulated genes which act

on both infected and uninfected cells to induce an 'anti-viral' state. Both types of interferon have been shown to inhibit VZV replication *in vitro* (Balachandra, Thawaranantha et al. 1994; Desloges, Rahaus et al. 2005). Furthermore, a possible *in vivo* role has been highlighted by the finding that treatment of immunocompromised individuals with IFN-α can reduce the severity of varicella (Arvin, Schmidt et al. 1982). Apart from their ability to express IFN-γ, NK cells have been shown to mediate lysis of VZV infected fibroblasts *in vitro* (Bowden, Levin et al. 1985; Ito, Watanabe et al. 1996) and to secrete granulysin which enhances the death of target cells *in vitro* (Arvin, Hata et al. 2001). Absence of NK cells has also been linked to fatal outcomes in case studies of VZV infection pointing to an important role of NK cells in mediating responses against varicella infection (Etzioni, Eidenschenk et al. 2005; Notarangelo and Mazzolari 2006). NKT cell deficiencies in the blood have been associated with outcomes of fatal varicella (Levy, Orange et al. 2003) suggesting their role in the control of varicella disease severity.

The adaptive arm of the immune response to VZV consists of both humoral and Cell Mediated Immune (CMI) responses. Although antibodies are generated against some VZV glycoproteins and structural proteins, their role in the control of disease seems far less important compared to that of the CMI during natural infection. The incidence and severity of herpes zoster correlates with CMI levels but not antibody levels and individuals with inherited antibody deficiencies do not have significantly worse primary or secondary VZV disease. However, if antibodies are given early after exposure to varicella they can reduce disease. Infection with VZV elicits somewhat delayed but potent T cell responses that correlate with viral clearance and resolution of disease which means that antibodies probably appear too late to control

the disease. The frequency of VZV-specific T cells is inversely associated with the risk of viral reactivation (Malavige, Rohanachandra et al. 2010). Both $CD4^+$ and $CD8^+$ T cells appear in circulation soon after the outbreak of cutaneous lesions (Arvin, Koropchak et al. 1986). Majority of these T cell responses are targeted against surface glycoproteins gB, gC, gE/I, regulatory proteins IE62 and IE63 as well as the gene products of ORF4, ORF10 and ORF29 (Arvin, Koropchak et al. 1990; Sharp, Terada et al. 1992; Jones, Black et al. 2006; Jones, Black et al. 2007). Furthermore, both $CD4^+$ and $CD8^+$ cells seem to efficiently recognize some common epitopes derived from the regulatory protein IE62 and the major VZV glycoprotein gE (Arvin, Sharp et al. 1991; Sharp, Terada et al. 1992). $CD8^+$ T cells act via classical cytotoxicity, targeting and killing virus infected cells whereas circulating $CD4^+$ T cells that are predominantly of the Th1 subset secrete IFN-γ and IL-2 (Zhang, Cosyns et al. 1994). VZV also manages to elicit some $CD4^+$ T cells that display cytotoxic activity in addition to secreting IFN-γ and IL-2 (Hayward, Pontesilli et al. 1986; Diaz, Smith et al. 1989; Milikan, Baarsma et al. 2009).

Although there is clear evidence of the induction of both VZV specific $CD4^+$ and $CD8^+$ T cells, some studies have reported higher frequencies of circulating $CD4^+$ than $CD8^+$ T cells in VZV seropositive individuals (Asanuma, Sharp et al. 2000; Jones, Black et al. 2006; Malavige, Jones et al. 2008). It is not understood why there seems to be a paucity of $CD8^+$ T cell responses during VZV infection but there is a possibility that VZV fails to establish an effective memory $CD8^+$ CTL response. This is corroborated by several studies which have shown that VZV-specific $CD8^+$ T cells also circulate at low frequencies during convalescence (Asanuma, Sharp et al. 2000; Jones, Black et al. 2006; Malavige, Jones et al. 2008; van der Heiden, de Boer

et al. 2009; Malavige, Rohanachandra et al. 2010). Other work has shown that $CD8^+$ T cells are prone to cell death after isolation and are refractory to *in vitro* culture alone or in the presence of antigen-presenting cells (APCs) bearing VZV antigens (Jones, unpublished). It is also possible that VZV specific memory $CD8^+$ T cells become sequestered away from the blood, perhaps at neuronal sites of latency as has recently been reported by Gowrishankar et., al (Gowrishankar, Steain et al. 2010). Notably, they also observed that the large immune infiltrate at these neuronal sites consisted of non-cytolytic $CD8^+$ T cells suggesting that $CD8^+$ T cells elicited during varicella infection are either functionally impaired or lose effector function after the resolution of primary infection. In retrospect, this paucity in $CD8^+$ T cells could be attributed to aspects of immune evasion employed by VZV.

1.8 Immune evasion mechanisms of VZV

VZV has developed various means of avoiding detection by both the innate and adaptive arms of the immune system. It is thought that VZV evades host responses in the long 10-21 day incubation period following inoculation and also during latency which lasts throughout the lifetime of the infected host. These events underscore the immune evasion potency of VZV; targeting both innate immune responses mediated by type I interferons and adaptive responses mediated by T cells.

Histochemical examination of cells in skin xenografts from SCID-hu mice has revealed that while IFN-α is expressed in bystander uninfected cells, it is down regulated in VZV positive cells (Ku, Zerboni et al. 2004). Further studies using viral mutants showed that the product of VZV ORF63 inhibited IFN-α induced antiviral

responses *in vitro* (Ambagala and Cohen 2007). IFN-α regulates signaling by the double stranded RNA sensor PKR. Activation of PKR initiates a signaling cascade which eventually leads to inhibition of initiation and translation. Ambagala and Cohen demonstrated that VZV interferes with this signaling cascade and the activity of the sensor by targeting a molecule downstream of PKR known as the eukaryotic initiation factor 2 (eIF-2α).

Another VZV protein encoded by ORF66 has been shown to interfere with the activity of Type II interferons by blocking the induction of IFN signaling pathway in T cells following exposure to IFN-γ (Schaap, Fortin et al. 2005). It is not yet known how the ORF66 protein mediates this function but it is postulated that it may act in a similar manner to the related HSV-1 gene product US3 which phosphorylates the IFN-γ receptor and blocks downstream signaling. VZV also targets the NF-kB signaling cascade. NF-kB is a potent transcription factor whose nuclear translocation stimulates expression of a large range of genes including those involved in the immune response (Ghosh and Hayden 2008; Hayden and Ghosh 2008). VZV interferes with this pathway by sequestering NF-kB proteins p50 and p56 in the cytoplasm of infected cells (Jones and Arvin 2006). No VZV gene product has been identified as responsible for modulating NF-kB signaling.

Antigen processing and presentation plays a key role in initiating and maintaining the specificity of adaptive immune responses. VZV interferes with various components of the antigen processing and presentation pathway and with key cells that are involved in antigen presentation. Dendritic cells (DCs) are the most potent antigen presenting cells (APC) of the immune system. They express activation and co-stimulatory molecules that govern their interaction with immune effector cells. One

such receptor family includes members of the Major Histocompatibility Complex (MHC) or the Human leukocyte antigens (HLA). They are divided into three classes, MHC class I, MHC class II and MHC class III.

MHC class I molecules are expressed on the cell surface as heterotrimeric complexes of heavy chain, β_2-microglobulin (β_2m) light chain and antigenic peptide. These complexes are required for target cell recognition by $CD8^+$ T cells (Hansen and Bouvier 2009). There are three classical HLA class I subtypes namely HLA-A, HLA-B and HLA-C and three non-classical subtypes namely HLA-E, HLA-F and HLA-G. Apart from presenting antigens, HLA molecules also modulate NK cell function by binding inhibitory and activatory receptors on the surface of NK cells and influencing lysis of normal cells (Braud, Allan et al. 1998; Brooks, Borrego et al. 1999). In general, cells expressing low levels of MHC class I are more susceptible to NK cell lysis (Borrego, Ulbrecht et al. 1998). Expression of MHC class II molecules is restricted to APCs but can be induced on non APCs by IFN-γ treatment (Pober, Gimbrone et al. 1983; Collins, Korman et al. 1984).

DCs process and present endogenous antigenic peptides on MHC class I molecules to naive $CD8^+$ T cells and exogenous antigenic peptides on MHC-II molecules to naive $CD4^+$ T cells. This leads to cell activation, differentiation (Banchereau, Briere et al. 2000) and eventual killing of infected cells. Several viruses including herpes viruses target the antigen presentation pathway by DCs in order to evade detection by the host.VZV productively infects human DCs, inhibits their maturation and interferes with their function through down regulation of various surface molecules including MHC class I (Abendroth, Morrow et al. 2001; Morrow, Slobedman et al.

2003). This finding could help explain the paucity in $CD8^+$ T cell responses in VZV seropositive individuals observed in some studies.

Over a decade ago Cohen first pointed out that VZV could possibly down regulate MHC class I to evade host T cell responses. He reported that both a wild type strain and the vaccine strain of VZV (vOka) could down regulate MHC class I molecules from the surface of infected human fibroblasts (HFs) (Cohen 1998) and that this was a post translational event. Similar reports on MHC down regulation soon followed from experiments using HFs and T cells (Abendroth, Lin et al. 2001; Eisfeld, Yee et al. 2007) also revealing that VZV interferes with MHC class I transport from the *cis*-golgi compartment. This revealed a mechanism unique to VZV unlike other alpha herpes viruses (HSV-1 and Bovine Herpes Virus) which interfere with the transporter associated with antigen presentation (TAP) and prevent the transport of antigenic peptides from the cytoplasm into the ER lumen where class I assembly occurs (Tomazin, Hill et al. 1996; Ahn, Gruhler et al. 1997; Koppers-Lalic, Reits et al. 2005; Koppers-Lalic, Verweij et al. 2008; Verweij, Koppers-Lalic et al. 2008) via their proteins HSV-1 ICP47 and BHV UL49.5. Other studies following these and using different cells have reported similar results where MHC class I molecules on the surface of VZV infected keratinocytes have been observed to be reduced but not absent (Black, Jones et al. 2009).

VZV infection has also been shown to inhibit IFN-γ mediated up regulation of MHC class II molecules with the effect being abrogated if cells are treated with IFN-γ prior to VZV infection (Abendroth, Slobedman et al. 2000). Modulation of IFN-γ signaling also inhibits expression of co-stimulatory molecules such as ICAM-1 on keratinocytes *in vitro* and within lesional tissue (Nikkels, Sadzot-Delvaux et al. 2004;

Black, Jones et al. 2009). The study by Black and colleagues was also able to demonstrate that VZV infected keratinocytes treated with IFN-γ are impaired in their ability to stimulate antigen specific $CD4^+$ and $CD8^+$ T cells (Black, Jones et al. 2009).

One VZV gene product responsible for MHC Class I down regulation has been identified. Transfection of cells with plasmids encoding IE and E proteins identified the E gene product of ORF66 as a mediator of MHC class I down regulation in the absence of influencing MHC class I synthesis, degradation and association with β_2m. (Abendroth, Lin et al. 2001; Eisfeld, Yee et al. 2007). However, a VZV mutant lacking ORF66 kinase activity still down regulated class I expression to a lesser extent suggesting that other proteins may be involved. With these proven modes of immune evasion and other potentially unidentified means, the underlying effects of VZV infection on the immune response warrant further investigation if effective measures to combat VZV infection are to be developed.

1.9 Consequences of VZV mediated immune evasion

Although the effect of VZV on MHC expression is relatively well defined, the functional implications are not. Surface MHC class I down regulation makes VZV infected cells targets for NK lysis (Farrell, gt et al. 1998; Tortorella, Gewurz et al. 2000). In this respect, VZV infected Human Fibroblasts are susceptible to NK mediated lysis *in vitro* (Ihara, Starr et al. 1984; Bowden, Levin et al. 1985; Ito, Watanabe et al. 1996). Whether this susceptibility of infected cells to NK cell lysis also occurs *in vivo* remains to be determined.

In vivo susceptibility to NK cell lysis would enable the innate immune system to potently clear VZV infection. However, this is not the case suggesting that VZV may employ certain mechanisms to escape NK cell lysis. Human cytomegalovirus (HCMV) and murine cytomegalovirus (MCMV) have evolved strategies to evade both $CD8^+$ CTL and NK cell recognition (Wilkinson, Tomasec et al. 2008; Miller-Kittrell and Sparer 2009) through expression of MHC class I homologues. In contrast, VZV has not been shown to encode any MHC class I homologues making this an unlikely mechanism. Human immunodeficiency virus (HIV) selectively down regulates certain HLA alleles while sparing others (Cohen, Gandhi et al. 1999) to maintain NK cell inhibition and escape from $CD8^+$ CTLs. We hypothesize that VZV might employ a similar mechanism to evade both innate and adaptive immunity. We aim to investigate whether VZV down regulates MHC class I molecules in an allele specific manner. Abendroth *et al*. observed an unusual truncated form of class I after immunoprecipitation with W6/32 raising the possibility that VZV alters the forms of class I molecules on the surface of infected cells but this is yet to be proven (Abendroth and Arvin 2001). We will conduct experiments on human keratinocytes to determine how VZV infection regulates the expression of MHC class I molecules, HLA-A, HLA-B and HLA-C. In addition, we have set out to try and define the MHC class I associated antigenic peptide repertoire of VZV infected cells compared to mock infected cells by a novel technique involving mass spectrometry.

The paucity of $CD8^+$ T cell memory responses during VZV infection is unexplained. We aim to understand why VZV fails to mount an effective circulating memory $CD8^+$ CTL response. We suspect that this is closely tied to the ability of VZV to infect antigen presenting cells (APCs) such as dendritic cells (DC) and down regulate

important co-stimulatory and accessory molecules such as CD83, ICAM-I and MHC class I. This potentially limits DC's potential to prime naive $CD8^+$ T cells and to establish effective CTL memory responses to VZV. Using samples from acutely infected VZV patients and VZV seronegative controls, we will try and understand how acute VZV infection alters the frequency, phenotype and function of peripheral blood DCs, a factor which probably contributes to the poor induction of varicella specific immune responses.

We hope that our findings on the mechanisms of VZV mediated immune evasion will have relevance in the understanding of how varicella manages to evade immune surveillance. We also hope that they will have significant implications in the design of new interventions to combat VZV by counteracting immune evasion mechanisms that may be mounted by the virus; especially those targeting the MHC antigen presentation pathway and antigen presenting cells such as DC.

1.10 Overall Thesis Aims

1. To assess the impact of acute varicella zoster virus infection on the phenotype and function of peripheral blood dendritic cells
2. To analyse the expression profiles of HLA class I molecules on the surface of mock and VZV infected keratinocytes
3. To analyse the repertoire of HLA class I bound peptides expressed on the surface of VZV infected keratinocytes compared to mock infected cells

CHAPTER 2: Materials and Methods

2.1 Samples

Patient Samples: PBMC Samples were obtained from consenting acutely infected VZV patients attending Infectious Diseases Hospital, Colombo, Sri Lanka (Dr. Neelika Malavige). A total of 39 acute VZV infected patients were recruited into the study. The age range was 14-80 with an average age of 35.29 and a male to female ratio of 1.9. A clinical disease severity scale based on number and character of the lesions, presence of fever, systemic signs and subjective patient assessment was previously developed to classify VZV cases. According to this scale emphasis is put on the number of lesions with mild disease described by <100 lesions, moderate to severe disease by 101-500 lesions and >500 lesions representing severe disease (Vazquez, LaRussa et al. 2001). A study defining disease severity based on number of lesions and viral loads managed to find a positive correlation where patients with >500 lesions had severe disease and higher viral loads compared to those with <500 lesions who had lower viral loads and mild disease (Malavige, Jones et al. 2008). In our study and where possible, patients were subjectively classified according to the number of lesions (inferred by eye) into mild <100 lesions, moderate 100-500 or severe disease >500 lesions. This information was however not available for all patients and in most cases was not used in subsequent analyses.

Healthy Donors**:** ‘‘Normal Control’’ PBMC samples were also obtained from consenting donors in Sri-Lanka. ‘Normal controls’ were defined as VZV negative adults, from the same population as the acute VZV patienst who also tested negative for VZV antibodies by serological assays. A total of 25 control PBMC and sera were used for this study. The age range for the controls was 21-63 with an average age of

37. The male to female ratio was 1.3. For other experiments, venous blood for PBMC separation was obtained from consenting adults (*n=6*) working at the Weatherall Institute of Molecular Medicine with an average age of 27.

Although the patients and controls were not age matched, there was no significant difference in age range or male to female ratios. All controls were drawn from the same population as the acutes, were VZV negative at the time of sampling and had had no previous exposure to VZV according to serological tests for VZV antibodies.

2.2 PBMC Separation: Venous blood from consenting donors was collected in vacutainor tubes containing the anti-coagulant sodium heparin (>1IU/ml). Heparinized blood was diluted in R0 (RPMI, no serum) at 1:1 ratio. Diluted blood was layered onto Ficoll-Hypaque medium (Lymphoprep: Nycomed Pharma, Norway) in a 50ml falcon tube. After centrifugation at 2000 rpm for 20 minutes with brake off, the PBMC interface was aspirated into a clean falcon tube and rinsed twice with R0 centrifuging at 1400 rpm for 7 minutes each time. The resulting pellet was resuspended in appropriate volume of medium. Cells were counted in 0.4% Trypan blue solution (Sigma) to exclude dead cells using a hemocytometer. Cells were then resuspended in at 1 X 10^6 cells/ ml in R10 medium for immediate assays or in freeze medium (FCS + 10% DMSO) for storage in liquid nitrogen.

2.3 Cell lines: HaCat cells (a gift from Dr, N Fussenig) were spontaneously developed from adult epidermal keratinocytes. MeWo cells are derivatives of

malignant human melanoma cells. Both cells lines were maintained in Dulbelco's Modified Eagle's medium (Gibco DMEM medium supplemented with 10% fetal calf serum (FCS; PAA Laboratories, Linz, Austria), Penicillin Streptomycin and Glutamine.

2.4 Viruses: The VZV vaccine strain vOka was used to infect keratinocytes for 72 hours. Consequently virus was propagated in infected keratinocytes, by adding uninfected keratinocytes at a ratio of 1:1. The parental strain from which the vaccine was derived, designated pOka was passaged in Mewo cells and infected cells were maintained by periodic addition of uninfected Mewo cells at a ratio of 1:5 (infected: uninfected).

2.5 Infection of cells: Cells were infected by co-cultivation of infected and uninfected cells. Uninfected Mewo cells were counted and mixed with pOka infected Mewo cells at a Multiplicity of infection (MOI) of 0.2. Keratinocytes (both transduced and non-transduced) were infected by co-culture with infected keratinocytes at an MOI of 1. Cultures were maintained in DMEM (FCS) at 37^0C and 5% CO_2 for defined experimental durations.

2.6 In vitro stimulation of DC: To stimulate DC responses *in vitro*, freshly thawed or freshly isolated PBMC was seeded at a cell concentration of 0.5 X 10^6 per

well in a round bottomed 96-well plate. Cells were either mock stimulated (R10 only) or stimulated with VZV strain vOka (replication competent or irradiated) at a MOI of 0.005 or with the TLR7/8 ligand, CL097 (Invivogen) at a final concentration of 10μg/ml. After 1 hour, 0.5 of Golgi-Plug (BD Biosciences) was added per well to stop intracellular protein transport. For enumeration of pDC cytokines, PBMC were incubated for a total of 3 hours and for enumeration of cytokine secretion from mDC cells were incubated for a total of 20 hours. Alternatively, in some experiments, cells were incubated for 10 hours.

2.7 Intracellular Cytokine Staining: In order to measure secreted cytokines after cell stimulation, stimulated cells were treated with protein transport inhibitors, either Golgi-plug (0.5μl per well in a 200μl culture) for DC cytokine assays or for T cell cytokine assays Brefeldin A at 10μg per well. In respective T cell assays, a positive control stimulated with phorbol myristate acetate (PMA) at 50ng/ml and the clcium ionophore ionomycin at 250ng/ml was included as a positive control. Accordingly, DC were incubated for 3, 10 or 20 hours (as described above) and T cell cultures were stimulated for 6 hours. Cells were washed twice in FACS Buffer containing (PBS + 1% BSA + 0.1% Sodium azide). First cells were stained for expression of surface markers with respective surface marker antibodies diluted in 100μl of FACS buffer for 25 minutes at 4^0C. Cells were washed twice and permeabilized using 150μl of permeabilization buffer (BD Biosciences) for 20 minutes at 4^0C in the dark. Next cells were rinsed once with permwash solution (BD Biosciences) before being incubated with antibodies to intracellular cytokines diluted

in permwash solution. Cells were stained for a further 30 minutes in the dark then rinsed twice with permwash and resuspended in FIX buffer (2% formaldehyde solution) ready for analysis by flow cytometry. Typically, cells were analysed within a day or two of staining to avoid loss of fluorescence. Cells were acquired on a Cyan ADP machine and data analysis was conducted using Flowjo Software.

2.8 Flow cytometry: The following HLA antibodies were used for surface immunostaining. W6/32 (WIMM) is a mouse monoclonal antibody that recognises all HLA-I trimeric complexes, Anti-HLA-A antibody I-AP produced in rabbit (Proteintech), anti-HLA-B antibody H-300 (Santa-Cruz Biotechnologies) and a mouse anti-HLA-C antibody DT-9 (WIMM). A mouse monoclonal antibody against HLA-B27, ME1 (WIMM) was used to stain cells expressing HLA-B27 after transduction. After labelling cells, primary antibodies were detected using PE conjugated goat anti-mouse IgG $F(ab')_2$ (Dako) and PE conjugated goat anti-rabbit IgG PE (eBioscience). After washing, cells were stained with a monoclonal antibody against VZV (Millipore) washed and fixed in FIX buffer (2% paraformaldehyde). Samples were run on a Cyan ADP machine. Analysis of flow cytometer acquired data was carried out using Flowjo while statistical analysis was performed on Summit Software.

2.9 IFN-γ ELISPOT Assays

2.9.1 Ex-vivo ELISPOT

Assays were conducted using IP lined ninety six well multi-screen plates (Millipore UK, Ltd). Briefly, plates were coated with IFN-γ capture antibody clone mAb 1.D1K (Mabtech) diluted in sterile PBS or bicarbonate buffer (1 capsule per 100 ml dH_20, Sigma Aldrich) to a final concentration of 15μg/ml. Plates were incubated overnight at 4^0C. Before the assay plates were washed 5X with sterile PBS and blocked with 200 μl per well of R10 medium (RPMI + 10% FCS) for 1hour at 37^0C. PBMC were resuspended in R10 to a final concentration of 1 X 10^6 cells per ml and 100 μl was added to each well. Peptides were added to the test wells to a final concentration of 4 μg/ml, PHA was used as a positive control at a concentration of 5 μg/ml and R10 alone was used as negative control. Plates were incubated for 16-20 hours. After incubation, plates were washed then developed first by adding biotinylated anti- IFN-γ clone mAb 7-B6-1 (Mabtech) to detect secreted IFN-γ. Next the detection antibody was probed with Streptavidin conjugated to alkaline phosphatase (Mabtech). Spots were developed for visualization using an AP conjugate substrate kit (Biorad, 170-643) according to the manufacturer's instructions. Spots were read on a AID ELISpot reader. Background spots (R10 only wells) were subtracted from test wells and data expressed as number of spot-forming units (SFU) per 10^6 PBMC.

2.9.2 Cultured ELISPOT

For cultured ELISpot, 2 X 10^6 PBMC were first cultured with the test peptides at a concentration of 4 μg/ml, for 10 days. On day 10 cells were washed twice with warm R0 and allowed to rest overnight in R10. On day 11, cells were counted and seeded

at 4 or 5 X 10^4 cells per well with the test peptides before proceeding with the rest of the assay as described (above).

2.10 Transduction: Two plasmids encoding HLA-B2705 and HLA-B2709 alleles were a kind gift from the Bowness-Kollnberger lab (BOTNAR institute). Lentiviruses were generated by transfecting each plasmid into semi-confluent 293 T cells. Viable lentivirus was harvested from culture supernatants and used to transduce keratinocytes. Successful transduction was confirmed by flow cytometry staining of HaCaT cells with the HLA-B27 specific antibody ME-1.

2.11 Protein extraction and Western Blot: To obtain whole cell lysates, uninfected or VZV-infected cells were washed with ice-cold PBS and lysed in buffer containing (50mM Tris-HCl pH8.0, 25% glycerol, 0.5% IGEPAL CA-630 and 0.2M NaCl). Cells were agitated for 2 hours or overnight at 4°C and centrifuged at 10,000 × *g* for 10 min at 4°C to obtain the supernatant. Sub-cellular protein fractions including cytoplasmic, membrane and nuclear extracts were isolated using a sub-cellular protein extraction kit (Thermo-Scientific). Protein supernatants obtained after extraction were quantified using the Bradford assay. Protein concentrations ranging from 13 µg - 15 µg were loaded per well onto SDS-PAGE gels. Proteins were transferred onto nitrocellulose membranes which were then probed with various antibodies specific for proteins of interest. The following antibodies were used for western blot detection at a dilution of 1:1000: Rabbit anti-human HLA-A (Proteintech, Europe), rabbit anti-human HLA-B (Santa Cruz Biotechnologies)

mouse anti-human HLA-C (Santa Cruz Biotechnologies) and mouse anti-human β-actin (Sigma, UK). Primary antibodies were detected using HRP conjugated goat anti-moue IgG (Dako) at 1:3000 dilution and mouse anti-rabbit HRP (MaxDiscovery) at a dilution of 1:100. Membranes were developed using Pierce ECL western blotting substrate (Thermo Scientific) followed by exposure to Kodak X-OMAT film (Fisher Scientific, Pittsburgh, PA) and development using a Kodak X OMAT 1000 processor.

2.12 RT-PCR*:* RNA was prepared using a kit (RNAeasy, Qiagen, Hilden, Germany) which isolates RNA molecules longer than 200 nucleotides following cell lysis in guanidinium isothiocyanate salts and purification over a silica gel column. cDNA was generated using the Stratagene Reverse Transcription Kit (Agilent Technologies). The target genes were amplified by PCR using locus specific primers specific to genes HLA-A, -B, and –C. The following primers were used for HLA amplification according to an established protocol (Zemmour, Little et al. 1992) **common foward** primer 5'GCCCGTCGACGGACTCAGAATCTCCCCAGACGCCGG 3' **HLA-A** reverse primer 5'CCGCAAGCTTTTGGGGAGGGAGCACAGGTCAGCGTGG GAAG **3',** **HLA-B** reverse 5’CCGCAAGCTTCTGGGGAGGAAACACAGGTCAGCA TGGGAAC3’ and **HLA-C** reverse 5’ CCGCAAGC'TTTCGGGGAGGGAACACAGG TCAGTGTGGGGAC 3’. The following primer pair was used to amplify β-actin **F:** 5’ ATG GGT CAG AAG GAT TCC TA 3’ and β-actin **R:** 5’ TCC ATG TCG TCC CAG TT 3’. PCR products were visualized on 1% agarose gels under UV illumination.

2.13 Mass Spectrometry

2.13.1 LC-MS/MS of acid eluted cell surface peptides: For cell surface elution of peptides, keratinocytes were infected with VZV strain vOka by co-culturing VZV infected keratinocytes with uninfected keratinocytes at a ratio of 1:1. Infected and Mock infected cells were maintained in DMEM medium supplemented with 10% FCS (D10) or DMEM without FCS (D0). Cells were maintained at 37^0C in a 5% CO_2 incubator. After 72hours, cells were washed twice with PBS. Cells were then scraped into a falcon tube and rinsed further in 5 ml of PBS. After washing, cells were resuspended in 5ml citric acid buffer (0.263 M $Na_3C_6H5O_7$ citric acid + 0.123 M Na_2HPO_4, pH3) and incubated for 25 min at 37^0C. Following incubation, cells were centrifuged and the supernatant filtered through a 0.45 μm filter. Tri-fluoro acetic acid (TFA) was then added to the supernatant to a final conc. of 1% before freezing down the samples.

For mass spectrometry analysis, samples were desalted using SepPac SPE columns from (Waters). Purified peptides were resuspended in 0.1% formic acid and injected into a LC-MS/MS workflow. Peptides were chromatographically separated on a C18 column (25 cm, particle size 1.7um) using acetonitrile gradient from 3 to 40% in 60 minutes and injected into a LTQ Orbitrap Velos (Thermo). MS-spectra were acquired with a resolution of 30000 and the 20 most abundant signals were selected for a MS/MS experiment (Top 20 CID). Samples were injected in triplicates and data analysed by Progenesis LCMS (nonlinear Dynamics) and

Mascot (matrixscience). The parent proteins of the isolated peptides were identified using MASCOT and a VZV protein database.

2.13.2 SILAC (Stable Isotope Labeling by amino acids in Culture):

Keratinocytes were passaged in SILAC medium (Sigma Ltd) reconstituted in two ways. For uninfected cells SILAC medium was supplemented with filtered FCS lacking arginine and lysine (Kessler lab) and 1 mg/ml of modified Arginine (R) and Lysine (K) ($R^{10}K^{8}$). For infected cells SILAC medium was supplemented with arginine and lysine free filtered FCS (Kessler lab) and 1mg/ml of label free Arginine (R) and Lysine (K) ($R^{0}K^{0}$). Cells were first grown for at least 15 rounds of division to incorporate the modified amino acids. For infection, the HaCaT cells maintained in label free amino acid SILAC medium R0K0 were inoculated by adding infected HaCaT cells at a ratio of 1:1 and propagated for 72 hours for the infection to take effect. Infected and Mock infected cells were maintained in the respective SILAC media to the end of the experiment. In total 10^8 vOka infected and 10^8 uninfected HaCaT cells were obtained. After 72hours of infection, VZV and Mock infected cells were washed twice in ice-cold PBS and lysed in buffer containing (50mM Tris-HCl pH8.0, 25% glycerol, 0.5% IGEPAL CA-630 and 0.2M NaCl) overnight. Cell lysates were then subjected to centrifugation at 10,000 rpm and the supernatants collected for analysis.

2.13.3 HPLC Separation of SILAC samples: IP-elutions were dried and re-suspended in 100 µl buffer A (0.1% Formic Acid). Samples were loaded onto on a 4.6 x 50 mm ProSwift^{TM} RP-1S column (ThermoFisher) and eluted using a 500 µl/min flowrate over 15 minutes from 2 % buffer A to 35 % buffer B (0.1% Formic

Acid in acetonitrile) using a Ultimate 3000 HPLC system (ThermoFisher). 0.5 ml fractions were collected from 2-15 minutes. Detection was performed using a variable wavelength detector at 280 nm. Fractions up to 12 minutes that did not contain the beta b2-microglobulin peak were combined, dried and further analyzed by LC-MS/MS.

2.13.4 LC-MS/MS of SILAC Samples: Samples were resuspended in 20 µl buffer A and analysed on an orbitrap velos (ThermoFisher) as described previously (Ternette et al. 2013, Cell Reports). Peptides were separated on an Acquity nano UPLC system (Waters) supplemented with a 25 cm BEH130 C18 column, 1.7 mm particle size (Waters) using a linear gradient from 8 % buffer A (0.1% formic acid in water) to 35% buffer B (0.1% formic acid in acetonitrile) at a flow rate of 250 nl/min (approx. 7000 psi) from 0 to 60 min. Peptides were introduced to an LTQ Orbitrap Velos tandem mass spectrometer (Thermo Scientific) using an electrospray ionization (ESI) source. Subsequent isolation and collision induced dissociation (CID) was induced on the twenty most abundant ions per full MS scan using an isolation width of 1.5 Da. All fragmented precursor ions were actively excluded from repeated MS/MS analysis for 15 s. Raw data was converted to Mascot generic files using msconvert (Kessner, Chambers et al. 2008). Sequence interpretation of MS/MS spectra were performed using a database containing all annotated human SwissProt entries and all SwissProt VZV virus entries (02/2013, 20,322 entries) with Peaks (Bioinformatics Solutions Inc.) and Mascot (Perkins, Pappin et al. 1999) with the following settings: Variable modifications: Label:13C(6)15N(2) (K) for SILAC K^8 label (8.0142 Da), Label:13C(6)15N(4) for SILAC R^{10} label (+10.008 Da) and

oxidation on methionine, (+15.995 Da), peptide tolerance: ± 5 ppm, fragment tolerance: ± 0.5 Da. Relative quantitation of SILAC labeled peptides was performed using PEAKS.

2.14 Data Analyses

Flow cytometry data acquired on a Cyan ADP cytometer and was analysed using Flowjo software. In each experiment where patient samples were used for DC assays, a total of 200, 000 events were acquired on the machine. To control for inter-experimental variation, the same machine was used each time to analyse different patient samples and the same voltage and gating settings were applied. Where Mean Fluorescence Intensities (MFI) are reported, the same gating strategies were employed and the same control sample (PBMC from the same individual) was used to set the gates so as to ensure minimal variation.

Statistical analyses: Where necessary statistical significance between two groups was determined using unpaired student t-test. Where differences were determined between multiple groups, Ordinary One way ANOVA for Multiple Comparisons using Tukey's test was employed. In all cases a $p<0.05$ was considered significant. Differences in p-value are represented by *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$. For SILAC generated data, analysis was performed using the software; PEAKS which compares values and generates p-values. A cutoff $p<0.001$ was selected to define statistically significant alterations.

CHAPTER 3:

Analysis of the Effects of Acute Varicella Infection on Peripheral Blood Dendritic Cells

3.1Background

3.1.1 Dendritic Cells

Dendritic Cells (DC) were first described in mice as a special subset of hematopoietic cells functionally capable to initiating a primary T cell response (Steinman and Witmer 1978). Later on they were found to be abundant in several human tissues and in rats (Williams, Hart et al. 1980; Hart and Fabre 1981; Daar, Fuggle et al. 1983; Hart and Mckenzie 1988). DC are often referred to as the sentinels of the immune system because they bridge the gap between innate and adaptive immunity and are the only Antigen Presenting Cells of the immune system that are capable of activating naïve T cells.

3.1.2 Human Blood Dendritic cell Subsets

Two main populations of DC precursors exist in human blood: immature plasmacytoid DC (pDC) precursors and mature myeloid DC precursors (mDC) also known as classical or conventional DC (cDC) (O'Doherty, Peng et al. 1994; Robinson, Patterson et al. 1999). pDC precursors originate from CD34+ precursors in the bone marrow. They undergo maturation in response to IL-3 plus CD40 ligand (CD40L) or IL-3 alone. Bone marrow myeloid progenitor derived mDC precursors and monocytes ($CD11c^{+}$ $CD14^{+}$) depend on GM-CSF and IL-4 for their

differentiation into immature DC which can then be prompted to undergo further maturation under inflammatory conditions in the presence of TNF-α and CD40L (Liu 2001). These 2 subsets have been described in humans based on the lack of expression of lineage markers (CD3, CD14, CD19, CD20, CD56), high expression of HLA-DR and differential expression of CD123 and CD11c. Accordingly, mDC have been defined as Lin^- $HLAD\text{-}R^+$ $CD11c^{high}$ $CD123^{int}$, and pDC as Lin^- $HLAD\text{-}R^+$ $CD11c^-$ $CD123^{high}$. Effective identification of multiple/other DC subsets in humans have largely been hampered by technical challenges in isolation of sufficient numbers as peripheral blood DCs typically range anywhere between 0.5-1% of the total lymphocyte population. Recent advances in detection methods such as multicolor flow cytometry and identification of new DC specific markers have however allowed human blood DC sub-populations to be further characterized. Consequently, experimental analysis of human DC has resulted into three populations defined by the heterogenous expression of the lectins/ Blood Dendritic Cell antigens: BDCA-1/CD1c, BDCA-2/DLEC, BDCA-3/Thrombomodulin and BDCA-4 together with CD123 and CD11c (Dzionek, Fuchs et al. 2000; MacDonald, Munster et al. 2002). This has now resulted in the definition of 4 distinct peripheral blood DC subsets namely $CD303^+$ (BDCA-2), $CD123^+$ (IL-3Rα) $CD304^+$ (BDCA-4) pDC and $CD11c^+$ mDC. In blood, mDCs are further subdivided into three additional subsets, namely $CD1c^+$ (BDCA-1) DC, $CD141^+$ (BDCA-3) DC and $CD16^+$ DC (Mittag, Proietto et al. 2011). pDC have also sub-divided into two functionally distinct groups based on the expression of CD2, with $CD2^{high}$ pDC being more potent at naïve T cell stimulation and cytokine secretion (Matsui, Connolly et al. 2009).

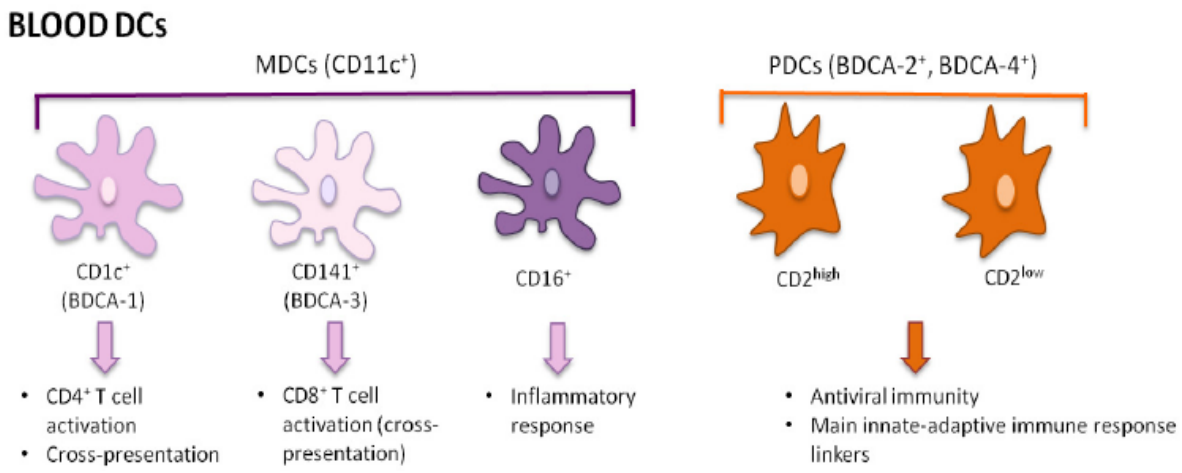


Figure 3.1: Human Blood Dendritic cell subsets. *Adapted from (Salvador, Igartua et al. 2012)*

3.1.3 DC induction of anti-viral immune responses

A special and distinguishing feature of DC is their ability to initiate innate responses and orchestrate adaptive immunity by activating naïve T cells, priming memory responses and inducing immunological tolerance. They direct naïve T cell differentiation to either the Th1 or Th2 effector subsets and are also pivotal in inducing tolerance (Mahnke, Schmitt et al. 2002; Lebre, Burwell et al. 2005).

Among the APCs of the immune system, including monocytes, macrophages, B cells and Dendritic cells, DC are the only ones armed with the necessary machinery needed to induce both innate immunity responses and naïve T cell activation and differentiation. DC express a variety of unique cell surface and intracellular proteins known as pattern recognition receptors (PRR). Extensive studies have identified multiple receptor classes involved in the control of innate immunity. They include cell surface and intracellular Toll like receptors (TLRs), membrane bound C-type Lectin receptors (CLRs), cytosolic proteins such as NOD-like receptors (NLRs) and

RNA helicases such as RIG-I like receptors (RLRs) among others (Loo and Gale ; Osorio and Reis e Sousa ; Kawai and Akira 2010; Elinav, Strowig et al. 2011). Among the PRRs, TLRs are the most extensively studied as they were the first to be identified. 10 functional TLRs recognising a range of PAMPs have been identified and characterized in humans. Lipoproteins are recognised by (TLR1, TLR2 and TLR6), ds RNA by TLR3, lipopolysacharride (LPS) by TLR4, flagellin by TLR5, ss RNA by TLR7 & TLR8 and DNA by TLR9 (Akira 2006). Various PRRs work independently and in concert enabling DC to detect microbes and microbial products collectively known as pathogen-associated molecular patterns (PAMPs) or components of damaged cells known as damage-associated molecular patterns (DAMPs) (Kawai and Akira 2011).

DC are located in almost all peripheral tissues in the body and in blood but are mostly concentrated in antigen rich areas such as the skin and at mucosal sites where they are constantly sampling their environment for foreign antigens. Under steady state, DC generally express an immature phenotype and are referred to as ‘’immature DC’’ (imDC). imDC are generally quiescent but still possess optimal antigen capturing ability and antimicrobial activity. PRR ligation by PAMPs and DAMPs initiates intracellular signaling cascades that activate immunological pathways leading to clearance of particular microbes expressing the respective PAMP/ DAMP. Signaling via all TLR with the exception of TLR3 specifically recruits the TIR-domain-containing adaptor molecule MyD88 (myeloid-differentiation primary-response gene 88), downstream signaling through which leads to the activation of NF-κB and MAP kinase (mitogen-activated protein kinase) and the induction of pro-inflammatory cytokines such as IL-6 and TNF-α. TLR2, TLR4 and TLR7-9/MyD88 signaling leads to activation of IRAK-1 (IL-1R associated kinase 1) and IFN-β.

Alternatively, TLR3 and TLR4 use the adaptor protein TRIF (TIR-domain containing adaptor protein inducing IFN-β) to activate another pathway leading to NF-κB and IRF3 activation culminating in the induction of Type I interferons and inflammatory cytokines. Also, TLR2 and TLR4 use another adaptor molecule TIRAP to recruit MyD88. Virus recognition via PRRs and the subsequent production of Type I interferon and inflammatory cytokines constitutes the first line of defence against invading pathogens. pDC and mDC have been shown to possess a distinct distribution of TLRs. Immature mDC express many TLRs including TLR 1, 2, 3, 4, 5, 6, 7, 8 and 10 (Jarrossay, Napolitani et al. 2001; Kadowaki, Ho et al. 2001) but lack mRNA for TLR 9 (Mittag, Proietto et al. 2011). pDC express TLR 1, 6, 7, 9, and 10 but TLR7 which recognises ssRNA and TLR9 which recognises unmethylated CpG DNA are abundant on pDC and are the key inducers of type I IFN secretion.

Antigenic stimulation via PRR or infection with viruses ultimately stimulates ‘‘immature DC’’ (imDC) to undergo maturation which is accompanied by decreased ability to uptake antigen and enhanced ability to present antigen. Consequently, there is alteration in morphology and phenotype to ‘’mature DC’’ (maDC) which migrate to secondary lymphoid organs where they present antigens to naïve T cells. DC maturation is accompanied by up-regulation of various maturation, co-stimulatory and antigen presentation markers such as CD1a, CD40, HLA-DR, CD80, CD86, ICAM-1 and the classical DC maturation marker CD83. Mature DC also express chemokine receptors such as CCR7 which direct homing to secondary lymphoid organs. pDC and mDC precursors differ in their distribution and functions a factor that is critical in defining their functions in antiviral immunity. While pDC tend to be

distributed in T cell rich areas of secondary lymphoid tissues and mucosal associated lymphoid tissues, mDC precursors are mainly found in non-lymphoid tissues and in T cell rich zones of draining lymph nodes. Also mDC tend to exist in a mature state in peripheral blood, so that freshly isolated mDC can promptly acquire a typical DC morphology on *in vitro* culture and can capture process and present antigens to T cells (Markey, Banovic et al. 2009) thus linking innate and adaptive immunity. pDC on the other hand have poor phagocytic potential and are less efficient antigen presenters, capable only of minimal presentation of exogenous antigens (Villadangos and Young 2008). Recently however, some studies have demonstrated the ability of pDC to efficiently phagocytose and present exogenous antigens (Tel, Lambeck et al.) even cross-presenting in the context of viral infections (Hoeffel, Ripoche et al. 2007; Di Pucchio, Chatterjee et al. 2008; Lui, Manches et al. 2009). Recently, a study by Jurjen et al. further emphasized the antigen presenting ability of pDC by demonstrating that pDC bear antigen cross presenting potential; even stimulating tumor antigen specific $CD4^+$ and $CD8^+$ T cell responses (Tel, Schreibelt et al. 2013). It is therefore plausible to postulate that human pDC may participate in antiviral immunity through antigen presentation to naïve T cells just like their mDC counterparts. pDC are capable of migrating into infected/ inflamed tissues and producing large amounts of Type I interferon, being the highest interferon producers in the body in response to viral or bacterial pathogens (Fitzgerald-Bocarsly 1993; Cella, Jarrossay et al. 1999; Siegal, Kadowaki et al. 1999). Consequently they play an important role in antiviral immunity through their interferon secreting property (Zhang and Wang 2005) even more importantly because they don't require endogenous virus replication for IFN-I production (Siegal, Kadowaki et al. 1999; Rasmussen, Sorensen et al. 2007); though this latter observation is debatable. Type I

interferons have potent direct and indirect antiviral activities (Isaacs and Lindenmann 1957; Theofilopoulos, Baccala et al. 2005). They play a crucial role in inhibiting viral replication (Garcia-Sastre and Biron 2006) and are also important in linking antiviral innate and adaptive immune responses. Type I interferon can directly potentiate the cytotoxic abilities of NK cell an $CD8^+$ T cells by enhancing their activation, clonal expansion and memory formation (Kolumam, Thomas et al. 2005; Aichele, Unsoeld et al. 2006; Le Bon, Durand et al. 2006; Martinez, Huang et al. 2008) or indirectly by enhancing the activity of accessory cells such as conventional cDC/ mDC (Nguyen, Salazar-Mather et al. 2002; Lucas, Schachterle et al. 2007; Le Bon and Tough 2008) . Finally, pDC participate in antiviral immunity by secreting immunomodulatory cytokines and chemokines such as IP-10, IL-6 and TNF-α that can activate and recruit other cell types such as NK cells, NKT cells, B cells and T cells to the site of inflammation/ infection (Colonna, Trinchieri et al. 2004; Palucka, Blanck et al. 2005).

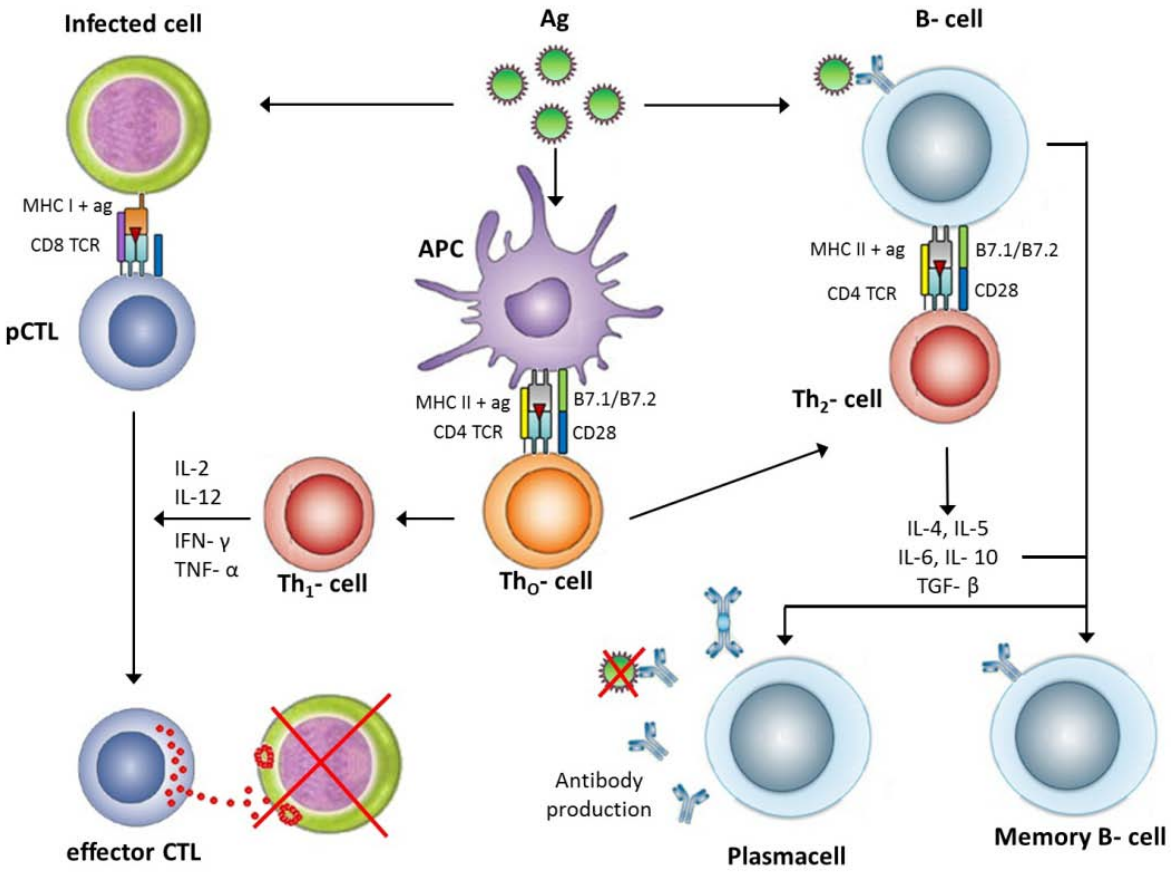


Figure 3.2: Diagrammatic overview of DC function in initiating innate and adaptive immune responses

Adapted from (Haes, Pollard et al. 2012) *http://dx.doi.org/10.5772/51583*

3.1.4 Virus interference with DC Function

Owing to the key role of DC in inducing adaptive immunity and orchestrating adaptive responses, they are often targeted by viruses to evade the immune system(Cunningham, Donaghy et al. 2010). Viruses exploit various facets of DC function including interference with key proteins that mediate signaling via PRRs leading to type I interferon production, inhibition of DC maturation, interference with antigen presentation and T cell activation (Bonjardim, Ferreira et al. 2009).

The human metapneumovirus (HMPV) has been shown to block RIG-I mediated signaling in mDC by preventing nuclear translocation of IRF3 and IRF7 consequently blocking the production of type I interferons (Goutagny, Jiang et al. 2010). Other viruses interfering with IRF3 signaling include hepatitis B virus via its polymerase protein and dengue virus both of which lead to blockade of type I interferon production (Rodriguez-Madoz, Bernal-Rubio et al. 2010; Yu, Chen et al. 2010). The V protein expressed by measles virus and the ML protein of Thogoto virus have both been shown to interfere with IRF7 activity preventing its phosphorylation, transcriptional activity and interaction with the adaptor molecule TRAF6 (Abt, Gassert et al. 2009; Buettner, Vogt et al. 2009). Some herpes viruses have also been shown to block PRR signaling. The vhs protein of HSV-1 has been shown to block TLR independent production of inflammatory cytokines in human and murine cDCs (Cotter, Nguyen et al. 2010).

Other viruses directly target DC by inhibiting their survival and promoting cell death. This evasion mechanism is particularly detrimental if DC are permissive to infection by the specific virus. *In vitro* HSV-1 and HSV-2 infection of human monocyte derived DCs (MDDC) has been shown to induce DC apoptosis in a replication dependent manner. In this study apoptotic DC were found to be a good source of antigen as they were readily phagocytosed and cross-presented to $CD8^+$ T cell clones by bystander uninfected DC (Bosnjak, Miranda-Saksena et al. 2005). In a related study HSV-1 infection of immature human MDDC was accompanied by onset of cytopathic effects leading to a loss of 20-45% of cells within 24 hours post infection (Mikloska, Bosnjak et al. 2001). EBV has also been shown to prevent GM-CSF and IL-4 induced DC development by infecting and promoting apoptosis of *in vitro*

cultured monocytes in a replication independent manner (Li, Liu et al. 2002). In a clinical study of dengue virus infection, a decrease in the frequency of circulating pDC and mDC was correlated to an increase in disease severity though the mechanism leading to DC loss was not determined (Pichyangkul, Endy et al. 2003). These findings highlight the fact that a reduction in the frequency of circulating DC prompted by virus induced cell death may lead to impaired virus specific adaptive immunity and uncontrolled viral replication.

A common mechanism of viral interference with DC function is through inhibition of DC maturation, antigen presentation and cytokine secretion. This results in poor induction of adaptive immune responses thus allowing viral replication, survival and transmission. Viruses have been known to interfere with the ability of DC to present antigens to T cells thus curtailing virus specific adaptive immune responses. DC maturation is a critical step in the DC antigen presentation process as mature DC up-regulate co-stimulatory and accessory molecules necessary to prime naïve T cells such as CD40, CD80, CD83 and CD86 as well as antigen presenting molecules in the form of MHC class I and II (Young and Steinman 1990; Young, Koulova et al. 1992; Cella, Engering et al. 1997; Banchereau and Steinman 1998). Consequently, any virus that subverts DC maturation blocks the ability of such DC to efficiently present antigen to naïve T cells leading to impaired antigen specific T cell responses. Immature MDDC have been shown to be permissive to productive HSV-1 infection resulting in inhibition of DC maturation characterized by down-regulation of CD1a, CD40, ICAM-1, CD80 and CD86. Surprisingly, this was not accompanied by loss of CD11c or MHC class I and II (Salio, Cella et al. 1999; Mikloska, Bosnjak et al. 2001). Other studies have shown that HSV-I infected mature MDDC have reduced

allogeneic T cell stimulatory capacity coupled with down regulation/ lysosomal degradation of the DC maturation marker CD83 (Kruse, Rosorius et al. 2000).

A classical example of viral interference with antigen presentation is the IE cytosolic HSV protein ICP47 which conveniently blocks transporter associated with antigen presentation (TAP) in human keratinocytes and fibroblasts by binding to its peptide binding site; blocking transfer of peptide antigens from the cytosol to the ER for MHC class I assembly (Hill, Jugovic et al. 1995; Ahn, Meyer et al. 1996; Tomazin, Hill et al. 1996). This affects cell surface MHC class I trafficking and consequently prevents antigen presentation to CD8+ T cells (York, Roop et al. 1994) . A similar mechanism has been shown for HCMV through its US6 protein. HCMV US6 doesn't bind directly to TAP but potentially targets it within the ER lumen thus resulting in defective peptide translocation within the ER and impairment of intracellular MHC class I trafficking (Ahn, Gruhler et al. 1997). The overall impact of viral inhibition of DC formation, survival, maturation and antigen presenting function is lack of establishment of innate antiviral responses and induction of virus specific adaptive immunity.

3.1.5 Impact of VZV infection on human dendritic cells

Most immune studies looking at responses elicited by acute varicella infection have focused on adaptive T cell responses; defining the various T cell populations that mediate viral clearance and maintenance of latency. However, few studies have defined the effect of acute varicella infection on the innate immune system and the role that innate cells such as dendritic cells (DC) play in controlling VZV infection. VZV spread occurs through inoculation of mucoepithelial cells of the upper

respiratory tract and the conjuctiva. It is postulated that DCs of the URT mucosa are among the first to become infected by varicella particles. Infected DC then migrate and transfer virus to T cells in draining lymph nodes and secondary lymphoid organs as they present antigen (Abendroth, Morrow et al. 2001; Morrow, Slobedman et al. 2003). T cells are then thought to complete these cycle of transfer by spreading virus to reticuloendothelial organs such as the liver and spleen where another wave of replication occurs and virus is transported to peripheral tissues such as the skin where the varicella associated vesicular/pruritic rash eventually breaks out (Grose 1981; Arvin, Moffat et al. 1996). Some *in vitro* studies using monocyte derived DCs (MDDC) have reported the susceptibility of both immature (Abendroth, Morrow et al. 2001) and mature DC (Morrow, Slobedman et al. 2003) to VZV infection. Both studies cited adverse effects to DC function resulting from down regulation of cell surface immune molecules such as MHC-I, CD80, CD83 and CD86 after VZV infection. Even though such studies using *in vitro* derived DC have contributed substantially to our current understanding of some aspects of human DC biology, they do not offer conclusive evidence of the exact impact of varicella on DC during natural infection. Futhermore, there is evidence that MDDC differ substantially from their *in vivo* counterparts both phenotypically and functionally (Ardavin, del Hoyo et al. 2001). Evidence that VZV affects *in vitro* generated DC does however suggest that VZV infection may interfere with the ability of DC to induce sufficient virus specific T cell responses during natural infection. This theory is particularly supported by the observed paucity in varicella specific memory $CD8^+$ T cell responses. Although, many studies have been conducted to determine the effects of other herpes virus infections on DC biology, VZV has not received much focus so

that the impact of acute varicella infection on peripheral blood DC function is currently undetermined.

Herpesviruses		natural host	Impact on pDC biology: IFN-I production	Infection	Maturation[$]
Herpes Simplex virus 1	HSV-1	human	Yes* [2]	ND	ND
Herpes Simplex virus 2	HSV-2	human	Yes*# [3]	No [4]	ND
Varicella-zooster virus	VZV	human	ND	ND	ND
Human cytomegalovirus	HCMV	human	Yes [5,6,7]	No (blood pDCs) [6] Yes (tonsil pDCs) [7]	Partial [8]
Human Herpesvirus 6	HHV-6	human	ND	Yes [9]	ND
Human Herpesvirus 7	HHV-7	human	ND	Yes [10]	ND
Epstein-Barr virus	EBV	human	Yes [11]	ND	Yes [11]
Human Herpesvirus 8	HHV-8	human	ND	ND	ND
Murine cytomegalovirus	MCMV	mouse	Yes*# [12]	No# [13]	Yes# [13]

Table 3.1: A summary of current understanding of the impact of different herpes virus infections on Dendritic Cell biology and function. *Adapted from (Baranek, Zucchini et al. 2009)*

A recent *in vivo* study looked at the impact of VZV infection on skin dendritic cell subsets and observed an influx of DC in VZV infected skin tissue. They extended these to *in vitro* studies and found that pDC were permissive to VZV infection which impaired IFN-α production (Huch, Cunningham et al. 2010). Similar studies to elucidate the impact of VZV on peripheral blood DCs have not yet been reported. A clear understanding of the mechanisms leading to poor VZV specific adaptive responses is needed in order to find ways of averting VZV immune evasion mechanisms that target DC. In this study we sought to elucidate the consequences of acute varicella infection on the frequency, phenotype and function of peripheral blood mDC and pDC compared to healthy controls.

3.2 Aims and Objectives

- To analyse the effect of VZV infection on the frequency and phenotype of peripheral blood dendritic cells

- To assess the impact of acute VZV infection on the homing capacity of peripheral blood dendritic cells

- To determine the consequences of acute VZV infection on peripheral blood dendritic cell function

3.3 Results

3.3.1 Flow cytometry gating to identify DC Populations. pDC and mDC both comprise about 0.5% of human PBMC. The two populations express distinctive phenotypic markers that can enable their effective identification. To identify DC subsets in acute VZV patients and healthy controls, PBMC were stained with a cocktail of antibodies against lineage markers (anti-CD3, CD14, CD16, CD19, CD20 and CD56) and against DC markers (anti-HLA-DR, CD11c and CD123). Dead cells were excluded from analysis by staining cells with a viability marker. DC were identified as lineage marker negative cells characterized by the putative expression of the activation marker HLA-DR. mDC and pDC were further identified from the DC population as $CD11c^{bright}$ $CD123^{int}$ and $CD11c^{-}$ $CD123^{bright}$ cells respectively. In most normal controls the frequency of mDC was found to always exceed that of pDC. We noted however that in a significant number of patient samples there was decreased frequency of mDC relative to pDC. (**Fig 3.3 panel C**) shows 32.1% mDC in the control subject compared to only 24.5% in the patient.

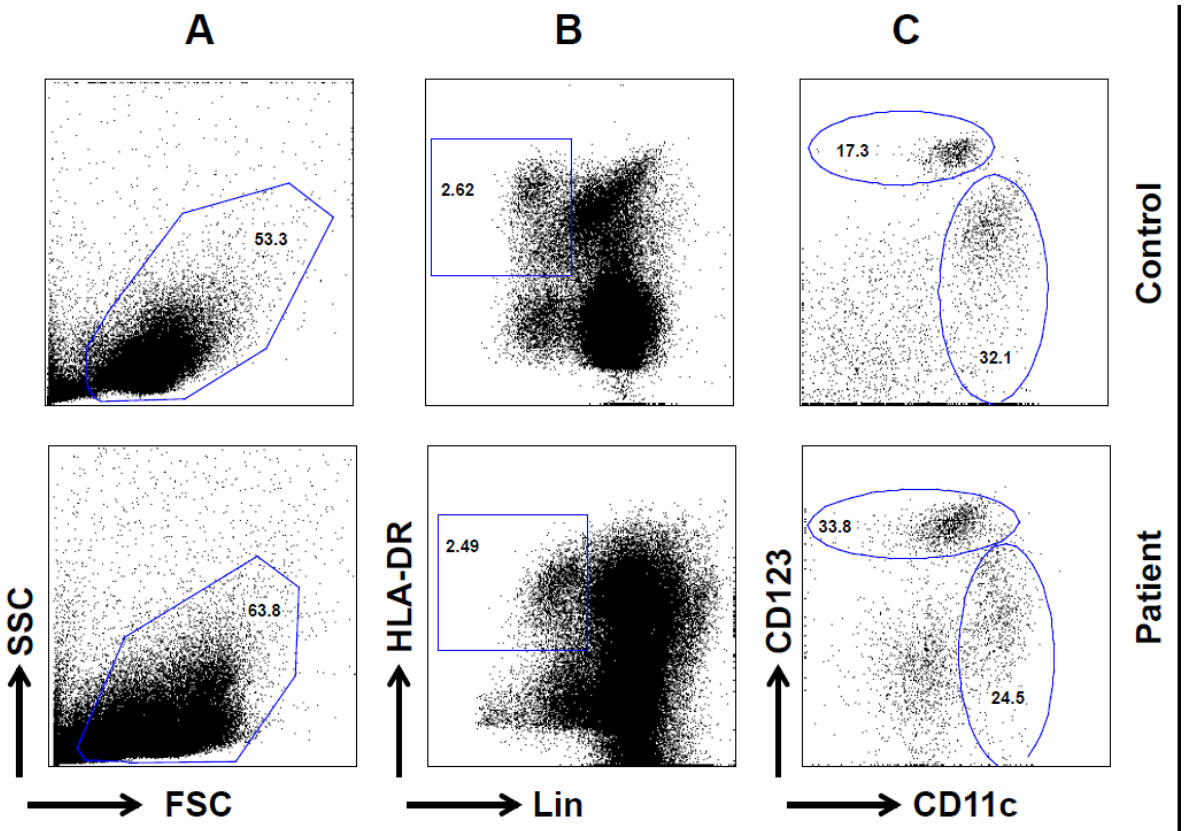


Figure 3.3: Identification of blood dendritic cells from PBMC. Representative flow cytometry plots from one control and one patient. Panel A.) Gating in forward scatter (FSC) and side scatter (SSC) modes to exclude dead cells and debris and to select lymphocytes. Panel B.) Blood DCs were identified from the lymphocyte gate as Lin^{-} $HLA\text{-}DR^{+}$. Panel C.) $HLA\text{-}DR^{+}$ $CD11c^{bright}$ $CD123^{int}$ mDC and $HLA\text{-}DR^{+}$ $CD123^{bright}$ $CD11c^{-}$ cells pDC subsets were further gated from the DC population.

3.3.2 Acute VZV infection leads to decreased frequency of peripheral blood myeloid dendritic cells. We quantified the frequency of peripheral blood DCs in PBMC from healthy controls (*n=25*) and from patients with acute varicella infection (*n=39*) by flow cytometry. We first analysed the frequency of total DC in the lymphocyte gate. No significant change was observed between patients and controls.

We then looked at the fraction of mDC and pDC populations in the DC gate. Analysis of means between the two groups (unpaired t-test) identified a significant decrease in the level of circulating mDC in acute infection compared to healthy controls (**Fig 3.4 B**). Despite not reaching significance, there was also a trend towards decreased frequency of circulating pDC in acute VZV.

It is possible that the observed differences in the levels of circulating mDC could be attributed to a general alteration in blood cell numbers that occurs during acute infection. Unfortunately due to lack of patient data, the observed differences could not be linked to absolute changes. However, it is possible to conclude that acute varicella infection leads to decreased mDC frequency without significantly altering total DC numbers.

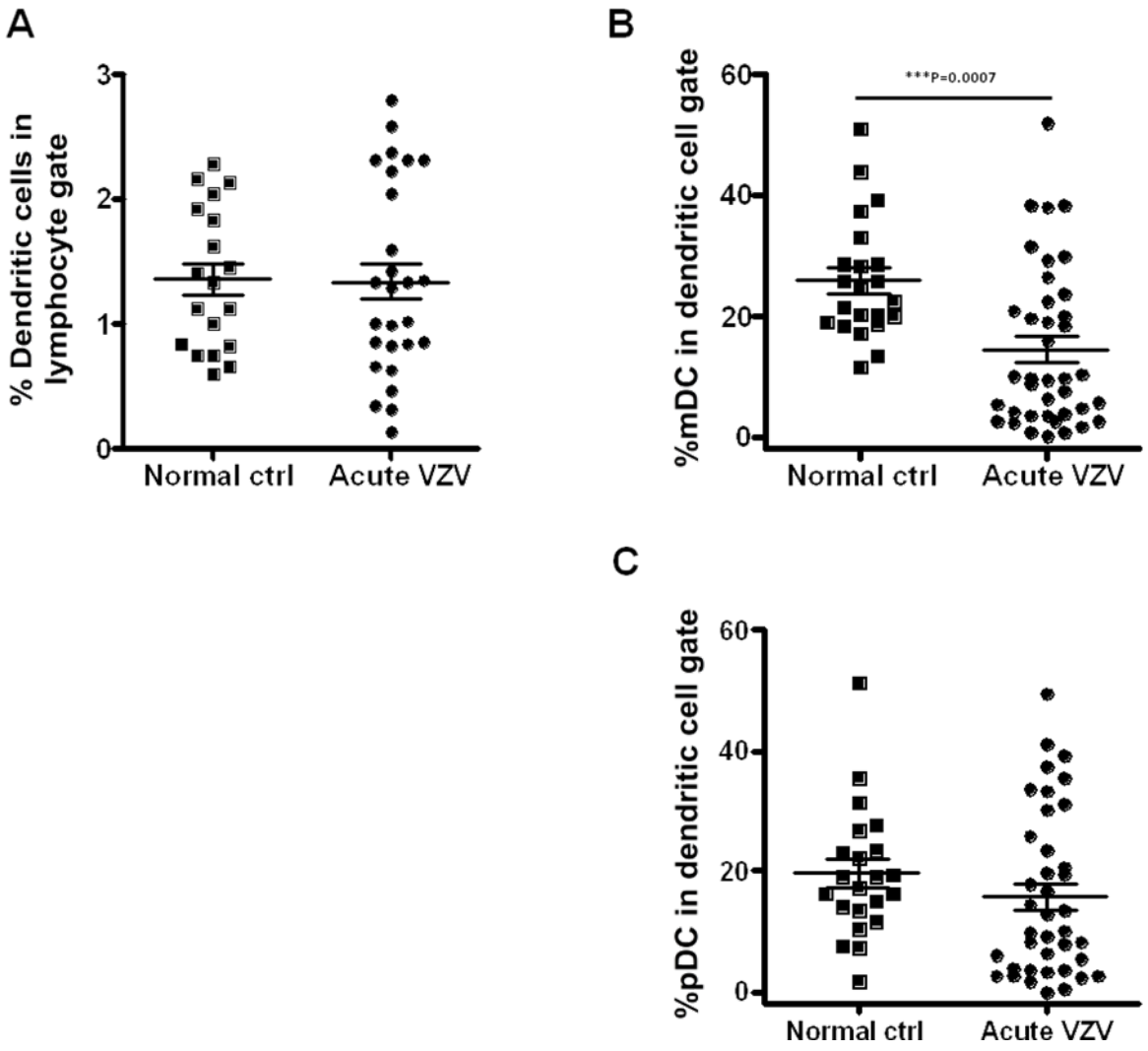
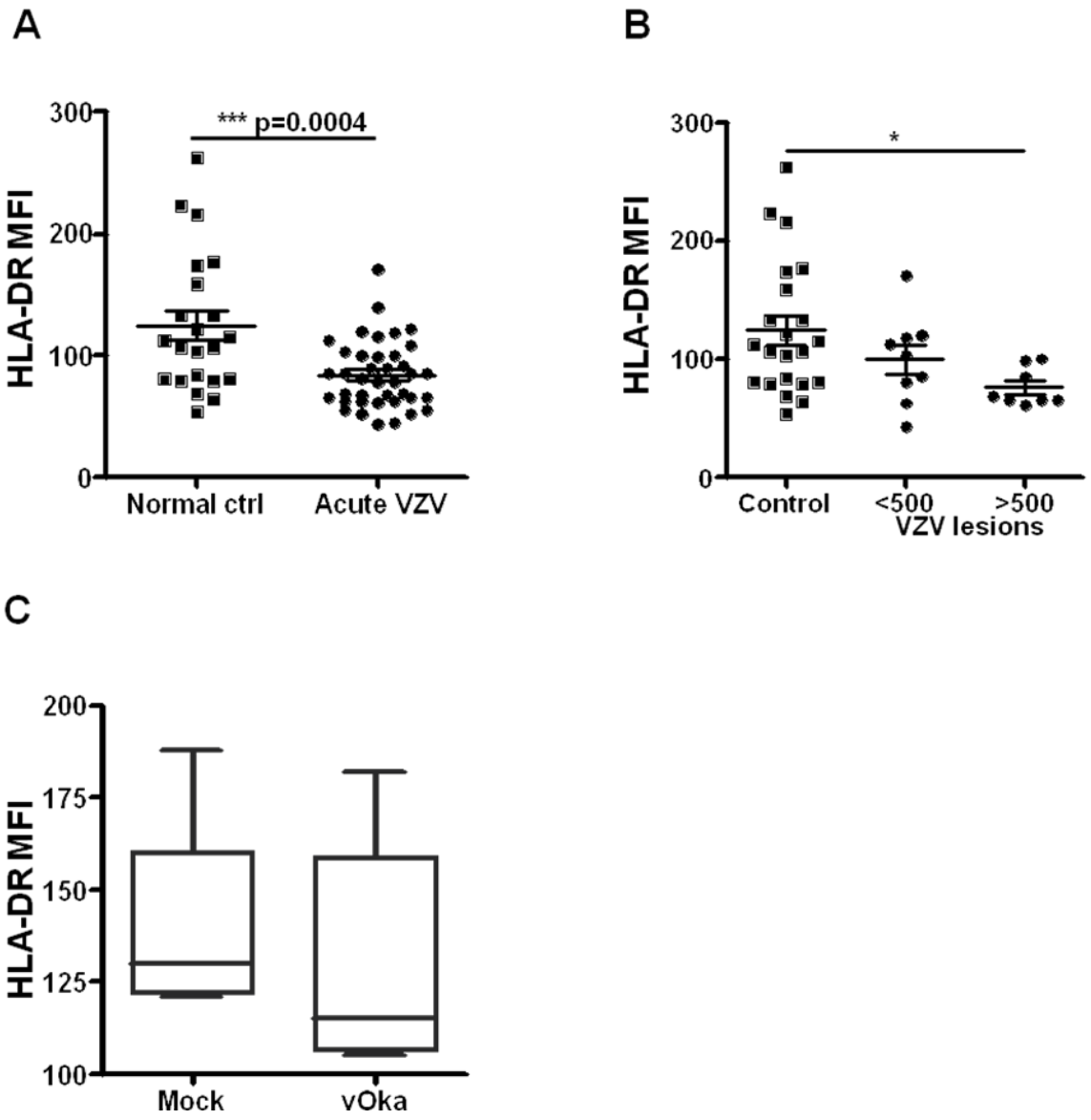


Figure 3.4 Quantification of peripheral blood DC from PMBC. A) Flow cytometry analysis of DC frequency in the lymphocyte gate and the proportion of mDC and pDC subsets. The frequency of DC in circulation was expressed as a fraction of total lymphocytes. The frequency of **(B)** mDC and **(C)** pDC was expressed as a fraction of total DC. Frequencies were measured from frozen PBMC of acute VZV patients *(n=39)* and healthy controls *(n=25)* by flow cytometry. Each point represents a single donor and horizontal bars represent the mean with Standard Error of Mean. Statistical differences between groups are indicated as *p<0.05 and ***p<0.001(using unpaired student t-test).

3.3.3 Phenotypic characterization of peripheral blood DC during acute varicella infection. Following stimulation and/or antigen uptake, immature, tissue resident DC alter their phenotype to facilitate antigen presentation and T cell stimulation. This is characterized by increased expression of antigen presenting machinery in the form of HLA class I, HLA class II, co-stimulatory molecules and accessory proteins. We examined whether acute varicella infection induces phenotypic changes in DC consistent with activation and maturation. We analysed the expression of HLA-DR and co-stimulatory molecules CD40 and CD86. These findings indicate that acute VZV infection leads to a significant down regulation of HLA-DR. This decrease was further shown to correlate with disease severity as DC from patients exhibiting advanced symptoms characterized by increased number of skin VZV lesions expressed even less HLA-DR (**Fig. 3.5 B**). When HLA-DR expression was assessed on DC from *in vitro* Mock or vOka stimulated PBMC, no significant change was observed in HLA-DR levels although a trend towards a decrease was evident in the vOka stimulated cells (**Fig. 3.5 C**). A possible explanation is the shorter time of exposure to virus in *in vitro* cultures which were only maintained for a total of 10 hours before analysis. On examination of CD40 and CD86 expression, both molecules were found to be significantly increased on pDC in acute infection (**Fig 3.5 E & G**) respectively. There was also notably increased expression of CD40 and CD86 on mDC though this did not reach statistical significance (**Fig 3.5 D & F**). This observed increase in the expression of co-stimulatory molecules shows that peripheral blood DC from acute VZV patients are mature or in an 'activated state' compared to controls but there is impaired late phase maturation though no enhanced HLA-DR expression. Such down regulation of HLA-DR suggests that these DC may not be able to efficiently present to naïve T cells despite being activated.


A
*** p=0.0004
HLA-DR MFI
300
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Normal ctrl
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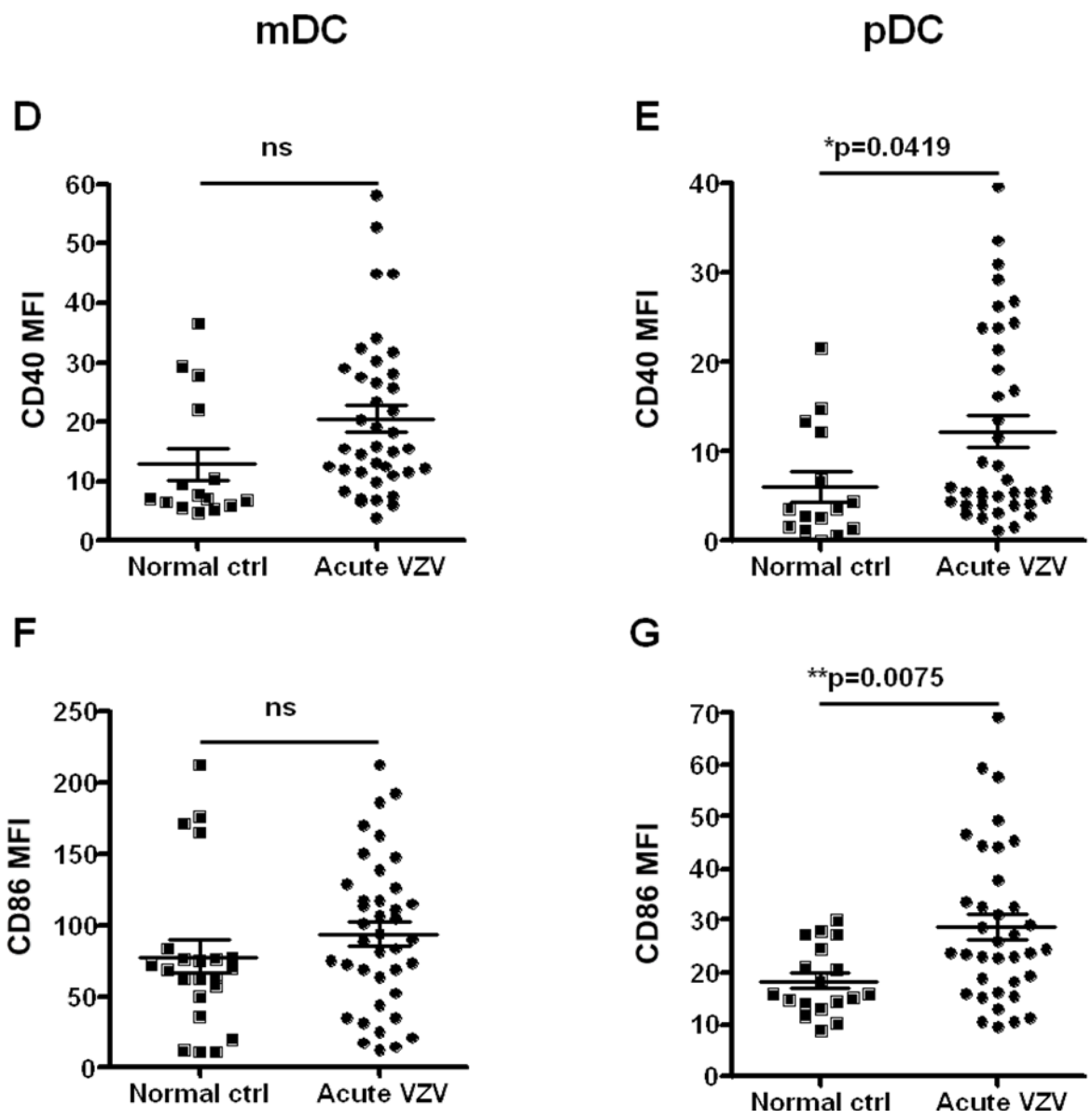


Fig 3.5 Acute VZV infection leads to down regulation of HLA-DR. PBMC from acute VZV infected patients (*n=39*) and healthy controls (*n=25*) were surface stained for the expression of the activation marker HLA-DR, CD40 and CD86. **(A)** HLA-DR Mean Fluorescence Intensity showing (MFI) decreased levels of expression in VZV patients compared to controls. **(B)** HLA-DR MFI in controls versus patients with varying degrees of disease severity defined by the number of VZV lesions on the skin. **(C)** HLA-DR MFI on DC from *in vitro* stimulated PBMC of healthy donors (*n=6*) either Mock infected or stimulated with VZV vaccine strain vOka at a MOI of 0.005 for 10 hours. **D-G)** CD40 and

CD86 MFI on mDC (left panel) and pDC (right panel) from acute VZV patients *(n=39)* and controls *(n=29)* showing increased surface expression. Each dot represents a single donor; horizontal bars are means with Standard Error of Mean (SEM). Statistical tests: Unpaired t test (Fig A, D, C, E, F, G) or Ordinary One Way ANOVA, Multiple Comparisons using Tukeys test (Fig B). Significant p values are represented by asterisks as *p<0.05, **p<0.01. Gating strategy is shown in **Appendix A: Supplememntary fig. S2. A& B**

3.3.4 Expression of DC homing markers during acute VZV Infection. DC activation via PRR is often accompanied by maturation, a change in phenotype and morphology, increase in antigen presenting capacity and migration to T cell areas in adjacent lymph nodes. Migrating DC are known to express CCR7 which directs homing to lymph nodes. It is not known which other homing markers potentially mediate DC homing during acute varicella infection. CLA expression directs T cell homing to the skin and CCR4 while mediates homing to the liver. We assessed whether peripheral blood DC expressed any phenotype that was consistent with homing to particular tissues during acute VZV infection. PBMC were co-stained with DC phenotypic markers and markers of DC migration/ homing. The proportion of mDC expressing CCR4 was found to be significantly increased in acute infection unlike that of pDC which showed a trend towards a decrease (**Fig. 3.6 A & B**). CCR4 mediates homing to the liver. It is not clear why there would be an increased frequency of blood mDC bearing CCR4 but it is possible that this population present in peripheral blood at the time of sample collection could be en route to the liver. The proportion of CCR7 expressing DC was generally low (<10%) although a few individuals showed increased numbers of pDC and mDC expressing this marker (>10%) (**Fig. 3.6 C & D**). There was a slight trend towards an increased number of CCR7 expressing pDC compared to $CCR7^{+}$ mDC whose proportion was slightly

decreased though both observations were statistically insignificant. Since mDC are the main antigen presenting blood DC subset, a reduction in blood $CCR7^+$ mDC could mean that this population has migrated to local area lymph nodes. The proportion of DC expressing CLA was generally high in both patients and controls. After acute infection, the proportion of CLA^+ mDC was only slightly decreased while the number of CLA^+ pDC in circulation was significantly reduced (**Fig. 3.6 E & F**) showing different skin homing capacities in the two DC subsets. Indeed, pDC have been shown to bear the capacity to migrate to sites of inflammation for the purposes of type I interferon secretion (Cella, Jarrossay et al. 1999; Siegal, Kadowaki et al. 1999) . Since VZV causes a severe cutaneous infection, we postulate that CLA^+ pDC migrate to sites of skin inflammation to secrete IFN-α for local control of viral replication. Whether the slight reduction in the number of peripheral blood CLA^+ mDC could also be due to migration to sites of VZV replication in the skin is not clear.

We conclude that acute varicella infection influences the distribution of blood DC subsets in both the skin and secondary lymphoid organs. Our results indicate a trend towards mDC migration to lymph nodes compared to pDC as assessed by CCR7 expression. It is also possible that the increased number of mDC expressing CCR4 in circulation migrate to the liver accounting for the observed decrease in the frequency of circulating mDC (**Fig. 3.4 B**). Even though CLA strongly governs the homing and effector function of skin infiltrating lymphocytes (Teraki, Hotta et al. 2000; van den Wijngaard, Wankowicz-Kalinska et al. 2000), it is not known whether CLA expression influences DC migration. Our results point to high numbers of CLA expressing DC in both patients and controls. A number of CLA^+ pDC however, potentially migrate to sites of inflammation on the skin during acute VZV infection

thus accounting for reduced blood levels of CLA^+ pDC. This is in line with the findings of Huch and colleagues who observed an influx of pDC into VZV^+ skin (Huch, Cunningham et al. 2010).

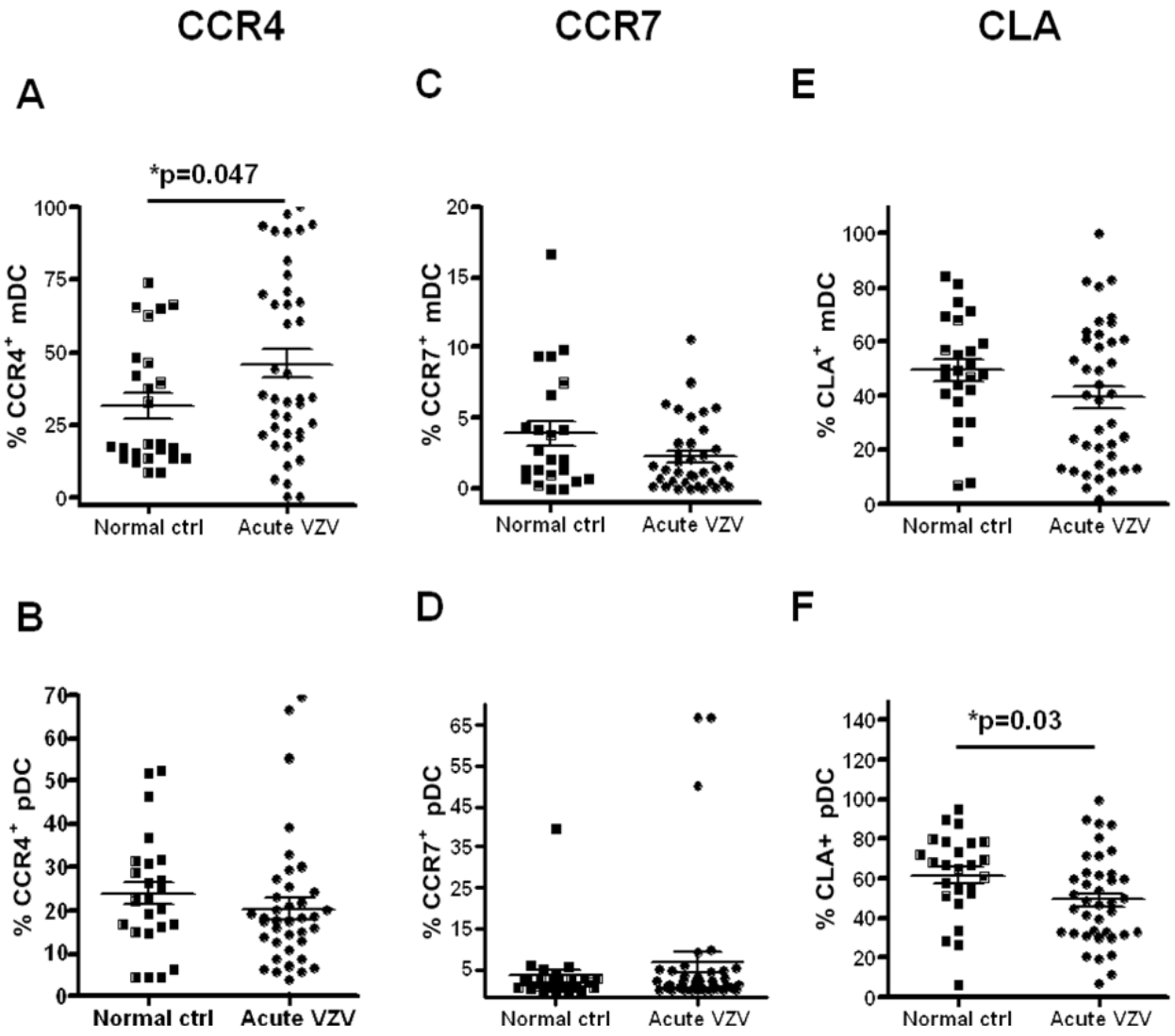


Fig 3.6: **Expression of homing markers on DC during acute VZV infection.** The proportion of mDC (panel **A, C, E**) and pDC (panel **B, D, F**) expressing homing markers CCR4, CCR7 and CLA was assessed using PBMC of healthy controls (*n=25*) and VZV patients (*n=39*). Each dot represents an individual sample. Horizontal bars are mean and standard error of mean (SEM). Statistical significance between groups was determined using unpaired t-test. A $p<0.05$ was considered significant. Where significant, calculated *p

values are shown for each plot. Gating strategy is shown in **Appendix A: Supplememntary fig. S3. A& B**

3.3.5 Acute VZV infection interferes with the ability of DC to secrete cytokines.

To determine whether VZV infection, alters the capacity of freshly isolated DC to secrete cytokines, we analysed the potential of patient pDC and mDC to secrete IFN-α, IL-12p70 and TNF-α. After *in vitro* TLR7/8 stimulation of patient and control PBMC with the TLR ligand CL097, the ability of pDC to produce IFN-α and TNF-α (**Fig 3.7 A & B**) and of mDC to produce TNF-α (**Fig 3.7 B**) and IL-12p70 was measured by intracellular cytokine staining (ICS). pDC and mDC from acute VZV patients were found to be severely impaired in their cytokine secretion capacity compared to DC from healthy controls. There were no detectable levels of IL-12p70 measured by ICS in mDC after TLR7/8 stimulation. VZV infection clearly impairs the ability of DC to secrete type I interferons and pro-inflammatory cytokines corroborating the study of Huch et al., (Huch, Cunningham *et al.* 2010) who found that induction of IFN-α secretion in VZV infected pDC cultures was severely impaired.

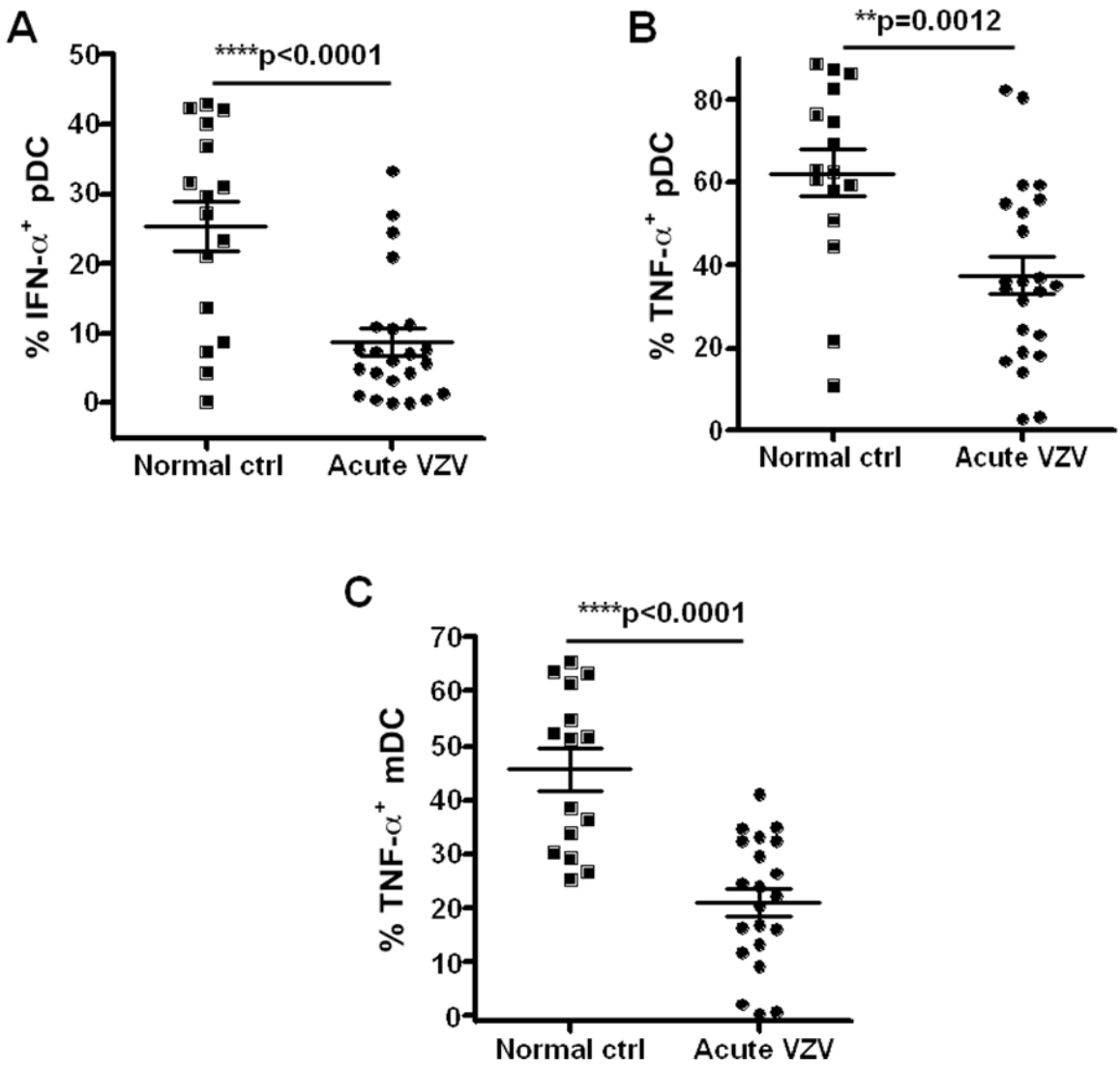


Fig 3.7: Cytokine secretion by mDC and pDC from acute varicella patients. PBMC form healthy controls (*n=16*) or acute VZV patients (*n=23*) were seeded at 5 X 10^5 cells per well and stimulated with the TLR 7/8 ligand CL097. IFN-α, TNF-α and IL-12p70 secretion was measured after 3 hours (pDC) or 20 hours (mDC) by intracellular cytokine staining. Data are expressed as percentage of each DC subtype staining positive for a given cytokine. Horizontal bars represent mean and standard error of mean (SEM). Statistical significance between groups was determined using unpaired t-test and a $p<0.05$ was considered significant. Calculated *p values are shown on each plot. **p<0.01 and ****P<0.0001. Gating strategy is shown in **Appendix A: Supplememntary fig. S4. A& B**

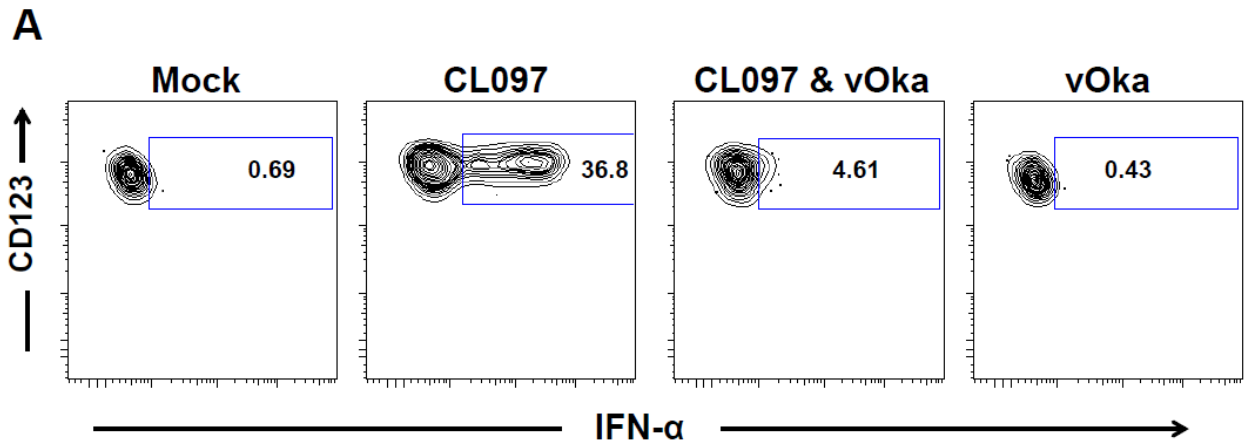
3.3.6 vOka infection interferes with the ability of *in vitro* stimulated DC to secrete cytokines. To determine whether VZV infection, alters the capacity of freshly isolated DCs to secrete cytokines, we analysed the potency of *in vitro* stimulated healthy donor (*n=6*) pDC and mDC to secrete IFN-α and TNF-α after TLR7/8 stimulation using CL097. PBMC were either Mock stimulated or stimulated with CL097 alone or in the presence of vOka or with vOka alone for 10 hours. The ability of pDC to produce IFN-α and TNF-α (**Fig. 3.8a**) and of mDC to produce TNF-α (**Fig. 3.8b**) was measured by intracellular cytokine staining (ICS). The TLR7/8 ligand CL097 induces significant secretion of type I interferon from pDC and TNF-α secretion from both pDC and mDC. No detectable level of IFN-α was measured in the Mock or vOka stimulated samples although a small amount of TNF-α could be induced by vOka in both pDC and mDC and in Mock infected mDC. The presence of vOka in PBMC cultures almost completely abrogated CL097 mediated release of IFN-α from pDC and significantly decreased TNF-α secretion (**Fig. 3.8a A & C**). To determine whether this property of the virus to inhibit cytokine secretion in pDC was dependent on its ability to replicate, the experimental set up was repeated using gamma irradiated vOka. Interestingly, irradiated vOKa only caused a slight but insignificant decrease in the levels TNF-α and IFN-α from pDC (**Fig. 3.8a B & D**) showing that the cytokine secretion inhibition capability of vOka is replication dependent. We next determined how vOka would affect cytokine secretion from mDC. Again healthy donor PBMC (*n=6*) were stimulated with CL097 in the presence or absence of vOka or with vOka alone. A slight amount of TNF-α is spontaneously produced by Mock infected mDC compared to pDC (**Fig 3.8b A)**. There was some inhibition of TNF-α secretion from CL097 stimulated mDC by vOka though this was not significant. Since varicella is highly cell associated,

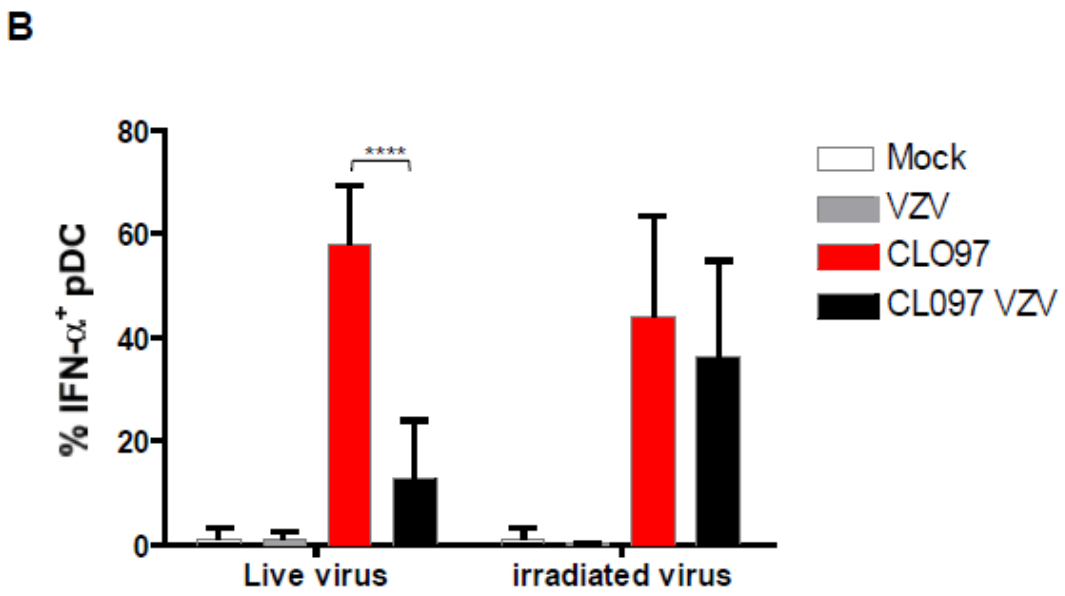
propagation of the vOka vaccine strain occurs in Vero cells. Consequently, lyophilized preparations of virus contain cellular debris and nucleic acid fragments. To completely eliminate the possibility that the observed DC activation seen after vOka stimulation resulted from the presence of cell debris, we performed parallel experiments using HaCaT cells to stimulate DC. 20 hour stimulation of DC with Mock or vOka infected HaCaT cells or cell lysates did not induce IFN-α or TNF-α secretion (data not shown). This reiterates the fact that vOka alone is needed to induce TNF-α secretion from DC.

The observed difference in IFN-α and TNF-α inhibition by vOka suggests that perhaps the two DC subsets respond to VZV differently, or that VZV targets distinct pathways in mDC and pDC with mDC being more resilient to virus mediated cytokine inhibition than pDC, or that the pathway targeted in pDC is absent in mDC. CL097 stimulates both TLR7 and TLR8. TLR7 is present on pDC while TLR8 is present on mDC. IFN-α production in pDC is tied to their ability to express TLR7 which signals via IRF7. VZV potentially targets the TLR7 pathway which would explain the difference in cytokine inhibition patterns between pDC and mDC.

In conclusion, the attenuated VZV vaccine strain vOka, clearly bears the cytokine secretion inhibitory properties of the pathogenic strain in infected patients; being capable of inhibiting IFN-α production by pDC and reducing TNF-α secretion by both pDC and mDC. Neither the replication competent nor the irradiated form of vOka was able to elicit any cytokine production suggesting that vOka is capable of subverting host virus detection pathways that lead to type I interferon and inflammatory cytokine production. Irradiation however seemed to abrogate the cytokine inhibitory effect of the virus suggesting that the gene products involved in this inhibition require viral replication to be activated. Finally vOka appears to target

different pathways to inhibit IFN-α and TNF-α secretion with the former being affected more severely compared to the latter.





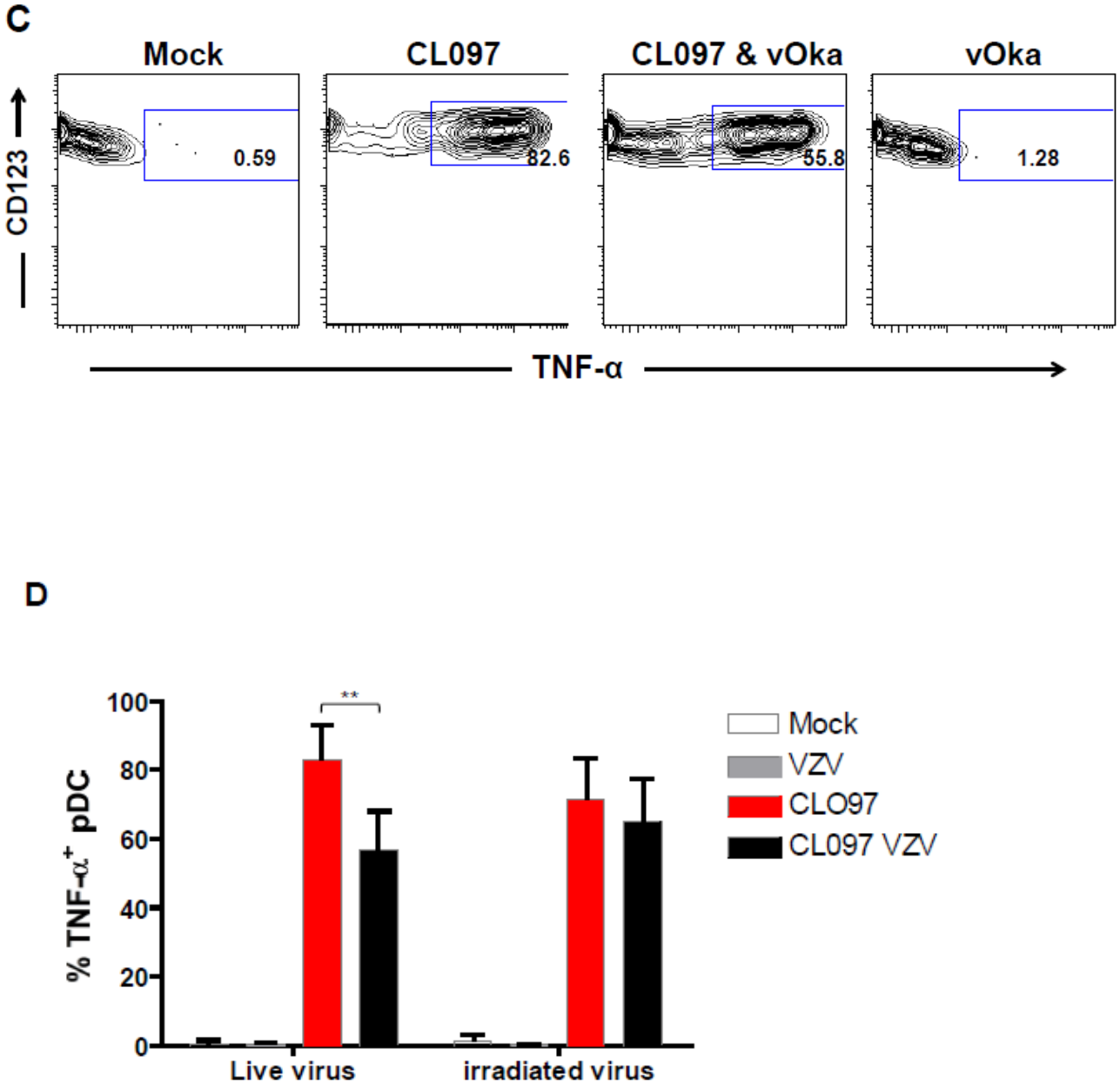


Fig 3.8a: pDC cytokine secretion after *in vitro* stimulation with varicella and a TLR ligand. PBMC from healthy donors (*n=6*) was stimulated at a density of (0.5 X 10^6/ well) under 4 conditions, Mock, varicella vaccine strain vOKa at a MOI of 0.005 or with the TLR7/8 ligand CL097 in the absence or presence of vOka or γ irradiated vOka. Cells were incubated for a total of 10 hours after which cytokine secretion was measured by ICS. **A)** Flow cytometry plots from a single donor showing IFN-α secretion by $CD11c^-$ $CD123^{bright}$ pDC. **B)** Summary plot showing IFN-α secretion after *in vitro* stimulation using replication competent vOka or irradiated vOka. **C)** Single donor flow cytometry plots showing

intracellular TNF-α secretion by pDC under the stated conditions. **D)** Summary graph showing TNF-α secretion in the presence of vOka and irradiated vOka. Statistical differences between groups is represented by *p<0.05, **p<0.01 and ****p< 0.0001 (Ordinary One way ANOVA, Multiple Comparisons using Tukeys test).

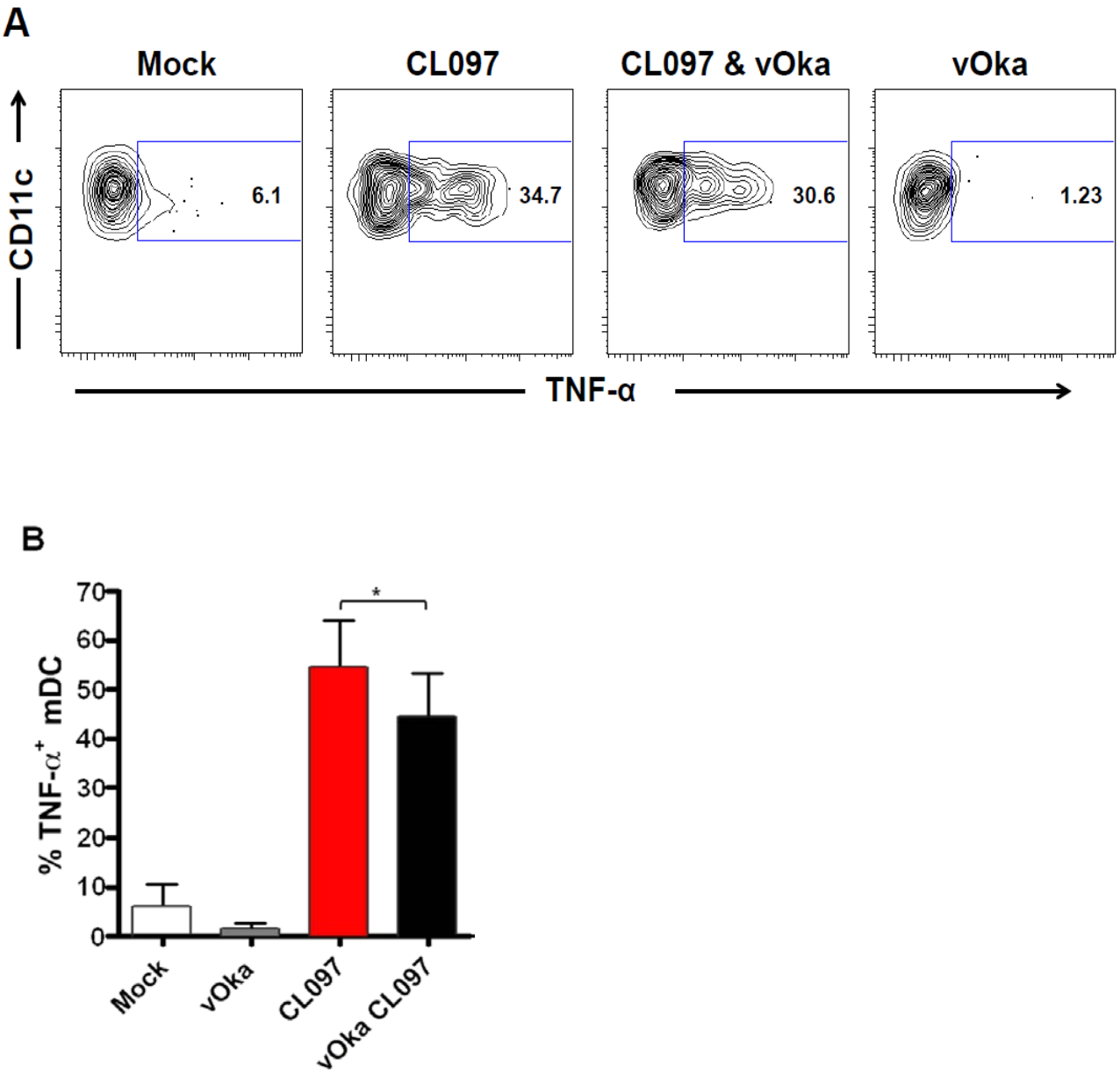
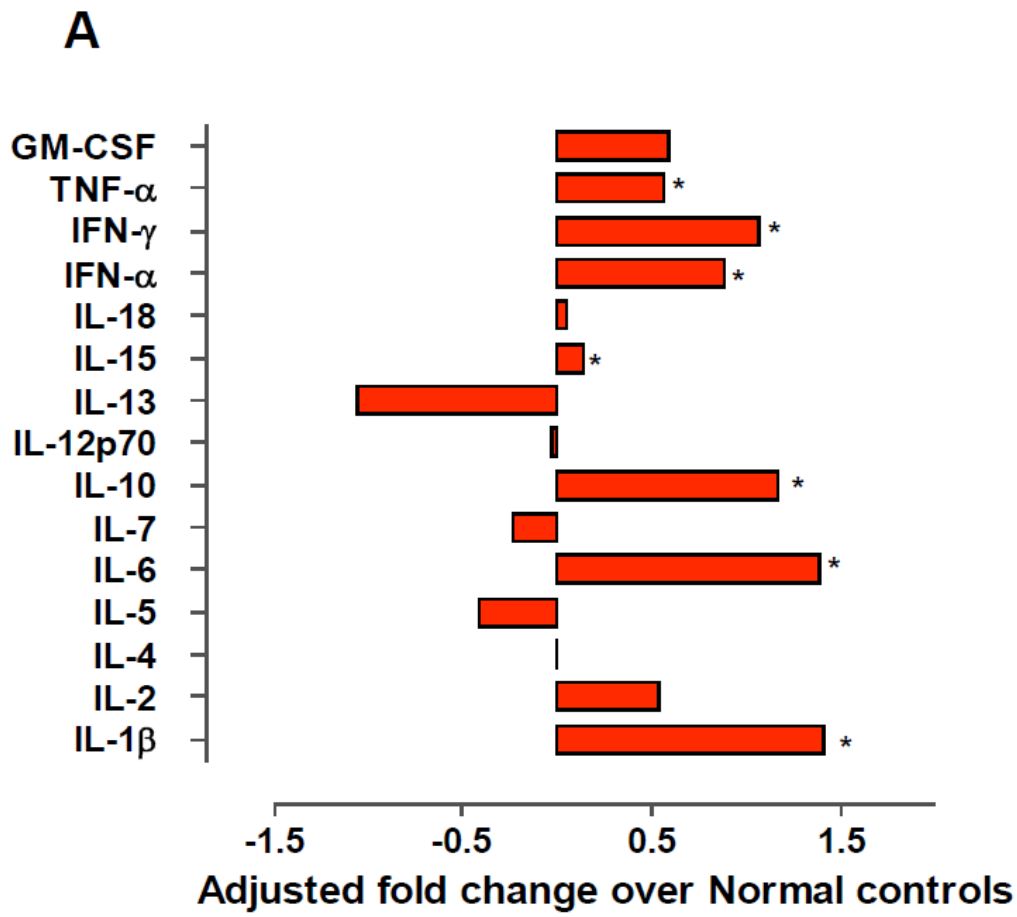


Fig 3.8b: C**ytokine secretion by mDC after *in vitro* stimulation with varicella and a TLR ligand**. PBMC from healthy donors (*n=6*) were stimulated at a density of (0.5 X 10^6/ well)

under 4 conditions, with or without (Mock) varicella vaccine strain vOKa at a MOI of 0.005 or with TLR7/8 ligand CL097 in the absence or presence of vOka. Cells were incubated for a total of 10 hours after which cytokine secretion was measured by ICS. **A)** Flow cytometry plots from a single donor's PBMC showing secretion of TNF-α by $CD11c^{+}$ $CD123^{int}$ mDC under different stimuli. **B)** Summary plot showing TNF-a secretion after *in vitro* stimulation. Statistical differences between groups is represented as *$p<0.05$ (using Ordinary One way ANOVA, Multiple Comparisons using Tukeys test).

3.3.7 Acute VZV infection induces an inflammatory cytokine and chemokine milieu. In order to understand how acute varicella infection alters the systemic cytokine milieu, we used Quantikine ELISA kits and fluorescent bead-based (Luminex) immunoassay kits to simultaneously measure interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12p70, IL-13, IL-15, IL-18, IFN-α, IFNγ, tumor necrosis factor-α, granulocyte colony-stimulating factor and 6 chemokines: IP-10, MIP-1, MIP-1β, MCP-1, RANTES and IL-8. We tested identical aliquots of serum from 19 healthy individuals and acute VZV patient sera from 14 donors. Out of the 15 cytokines measured, 6 inflammatory cytokines (TNF-α, IFN-α, IFN-γ, IL-15, IL-6 and IL-1β) were found to be significantly elevated in acute VZV infection compared to controls. The levels of anti-inflammatory cytokines IL-4 and IL-5 were decreased in patient sera while the level of IL-10 was significantly increased (**Fig 3.9 A**). Furthermore, the levels of all the chemokines analysed were found to be significantly altered in acute patient sera compared to healthy controls (**Fig 3.9 D**). Chemokine levels were elevated in sera with the exception of RANTES which was significantly decreased in acute VZV patients and this decrease appeared to be consistent with the extent of disease severity. Consequently, RANTES levels though not reaching statistical significance were lowest in the sera of VZV patients with the highest number of varicella skin lesions (**Fig 3.9 E**). Overall, the blood cytokine-chemokine

profile in acute VZV infection is generally consistent with on-going inflammation. The observation that the levels of serum IFN-α and TNF-α are increased in acute infection despite reduced synthesis by pDC and mDC as determined by ICS (**Fig 3.7 A, B, C**) points to the fact that other cells other than DC such as epithelial cells and NK cells produce cytokines that account for these elevated serum levels. We conclude that acute VZV infection induces a general inflammatory state with elevation of most inflammatory cytokines and chemokines and the anti-inflammatory cytokine IL-10. The innate inflammatory serum cytokine profile appears not to correlate with the inhibited cytokine secretion capacity of innate immune cells such as DC, suggesting that the serum cytokine levels are a sum total of cytokines secreted by other cell types other than DC.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GM-CSF
TNF-α
IFN-γ
IFN-α
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IL-15
IL-13
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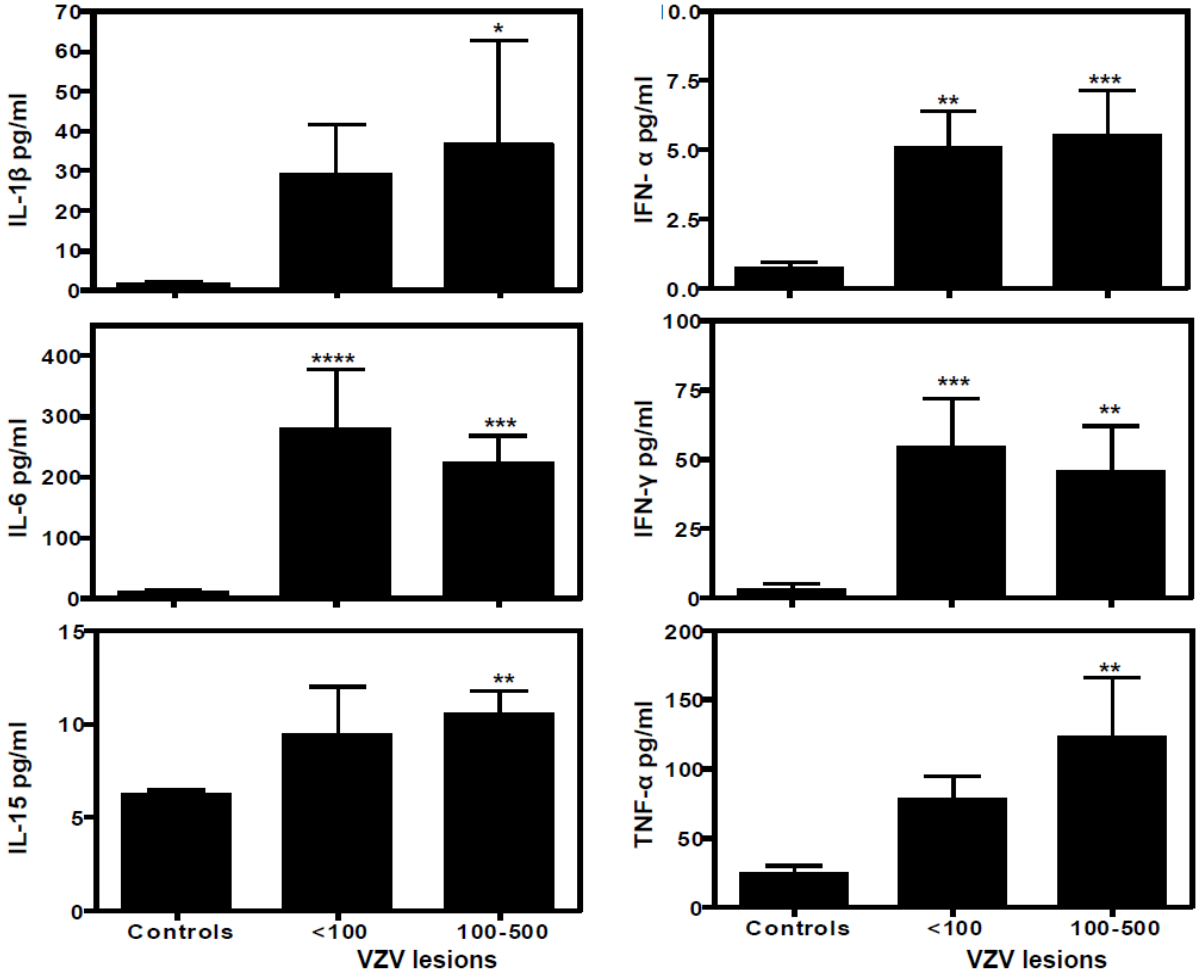
B


IL-1β pg/ml
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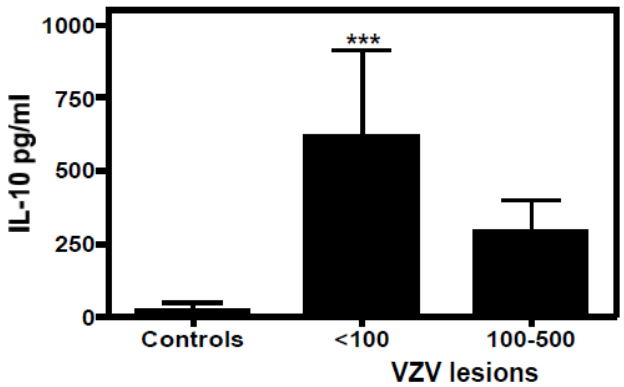
C


IL-10 pg/ml
Controls
<100
100-500
VZV lesions

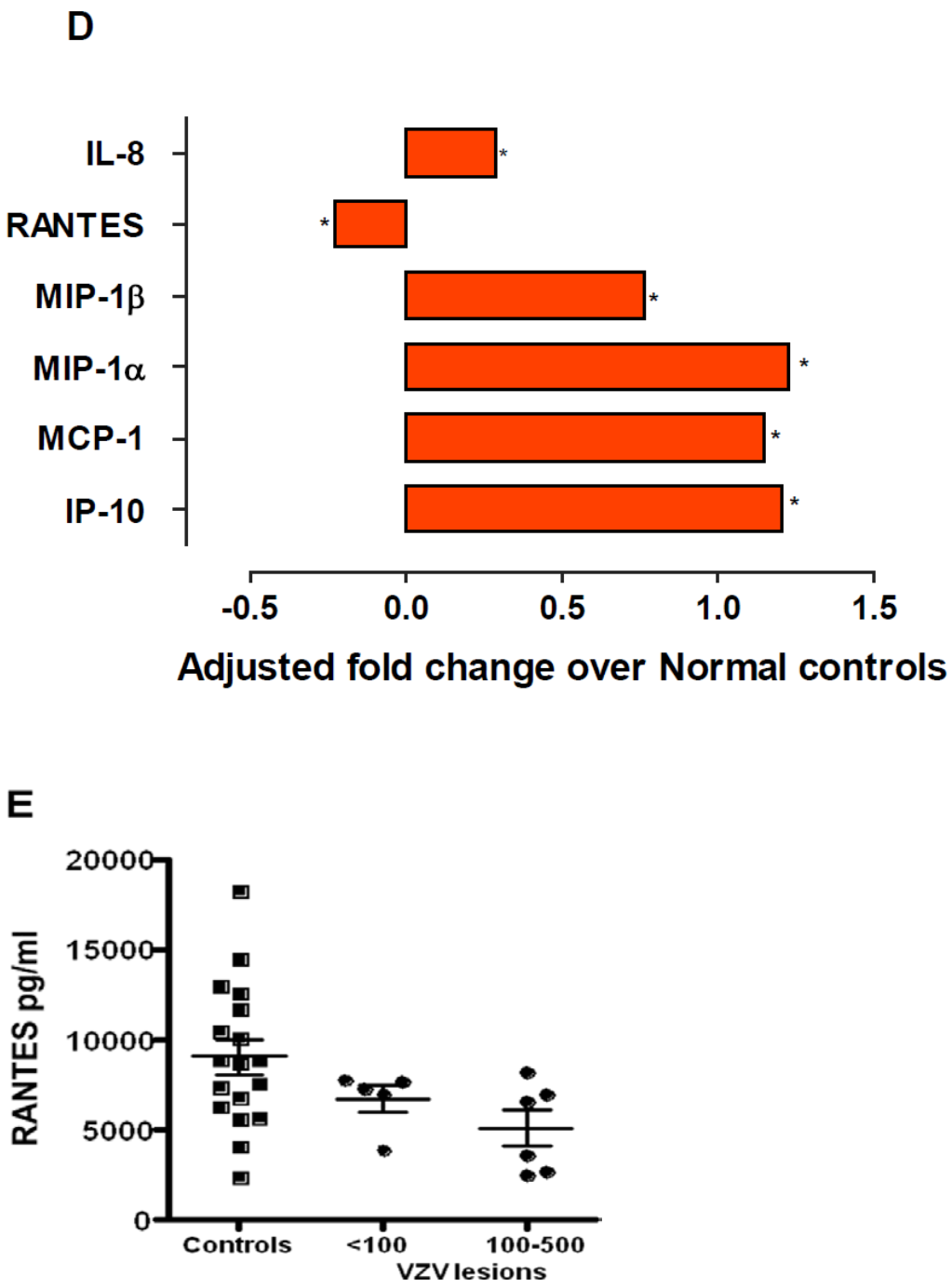


Figure 3.9: **Serum cytokine/chemokine levels in acute varicella infection.** Serum concentration of 21 different cytokines was determined by multiplex LUMINEX and ELISA. **A)** Cytokine and chemokine concentrations **(D)** are expressed as adjusted fold change over the average level of the corresponding cytokine in uninfected healthy controls. Cytokines whose levels were significantly altered compared to healthy controls are marked using

asterisks (*p<0.05 using unpaired t-test). **B)** Individual plots showing inflammatory cytokines (IL-1β, IL-6, IFN-α, IFN-γ, TNF-α and IL-15) and **(C)** Anti-inflammatory cytokine (IL-10) whose levels were significantly increased in acute VZV patients compared to controls. Plots show controls and patients with varying levels of VZV severity. **E)** Graph showing levels of serum RANTES levels between controls and patients. Statistical significance was determined using (Ordinary One Way ANOVA, Multiple Comparisons using Tukeys test).

3.4 Discussion

Dendritic cells are the most potent antigen presenting cells of the immune system, bridging the gap between innate and adaptive immunity. They are key players in the initiation of effective immune responses during pathogen infections including infection with herpes viruses. Varicella zoster virus is a alpha herpesvirus that causes chicken pox during primary infection and herpes zoster following reactivation of latent virus. Human blood contains two main populations of DC, the mDC subset and the pDC subset with distinguishing phenotypic and functional properties. mDC mainly act as potent antigen presenting cells, bridging the gap between innate and adaptive immunity while pDC are potent interferon producers with minimal but significant antigen presenting potential (Liu 2005). Needless to say the distinct properties of these two DC subsets make them capable of initiating successful antiviral immune responses.

Viral infections including those caused by most herpes viruses like VZV are commonly associated with reduced functional abilities in dendritic cells and it is believed that these pathogens deliberately target DC to evade the immune system. Many viruses subvert a range of dendritic cell properties including maturation, antigen presentation and cytokine secretion in order to promote their propagation and survival in the host (Pichyangkul, Endy et al. 2003; Huang, Yang et al. 2011). VZV has been shown to be capable of infecting both immature and mature *in vitro* monocyte derived dendritic cells (MDDC) (Abendroth, Morrow et al. 2001; Morrow, Slobedman et al. 2003) resulting in selective down regulation of important immune molecules including major histocompatibility complex (MHC) class I, CD80, CD83, CD86 and reduced ability of DC to stimulate naive T cells. One *in vivo* study looking

at skin DC subsets reported that VZV can productively infect skin infiltrating pDC and potentially alter their cytokine secretion function (Huch, Cunningham et al. 2010). It is clear that VZV may target DC to evade immunity and promote pathogenesis but the extent of VZV involvement in the modulation of blood DC function is yet been determined.

In this study, we analyzed the potential effects of acute VZV infection on DC frequency, phenotype and function. We focused on the two human blood DC precursors: mDC and pDC; using PBMC from acutely infected VZV patients compared to healthy controls. mDC and pDC express distinct surface markers which can allow effective identification and characterization. Consequently, pDC were defined as Lin^- HLA-DR^+ $CD11c^-CD123^{high}$ and mDC as Lin^- HLA-DR^+ $CD11c^+$ $CD123^{int}$ cells.

First, we determined whether acute VZV infection had any effect on the frequency of circulating DC. Although there was no notable difference in the overall frequency of circulating DC, there was a marked reduction in the frequency of circulating mDC in acute infection. pDC numbers remained largely similar between patients and controls with only a slight trend towards reduction in acute infection. This differential variation in mDC and pDC frequency could be explained by the fact that mDC are evidently more susceptible to viral infections compared to pDC. Though not measured in this study, mDC have been found to express a glycan protein of C-type lectin known as DC-specific ICAM3-grabbing non-integrin (DC–SIGN) which interacts with viral surface glycoproteins and acts as a receptor for many viruses aiding viral entry, transmission and possible death of infected DC (Geijtenbeek and van Kooyk 2003; Navarro-Sanchez, Altmeyer et al. 2003; Tassaneetrithep, Burgess et al. 2003). The expression of this receptor has been commonly associated with

reduced mDC numbers compared to pDC during acute viral infections. Dengue virus infection of mDC has previously showed a positive correlation with the level of expression of DC-SIGN (Sun, Fernandez et al. 2009). Despite strong evidence of DC-SIGN as a factor promoting preferential infection of mDC by several viruses, it is not clear whether mDC are more permissive to VZV infection compared to pDC. Furthermore, a recent study analysing the effects of varicella infection on skin DC subsets found no significant difference in the expression of DC-SIGN between VZV+ skin compared to uninfected control skin (Huch, Cunningham et al. 2010). A solid conclusion on VZV dependence on DC-SIGN for infection of mDC will warrant further investigation.

It is also possible that other factors such as migration of mDC to lymph nodes and other secondary lymphoid organs for the purposes of antigen presentation may account for the reduced frequencies seen in blood of acute VZV patients. Indeed, analysis of homing molecules on DC revealed that there was significantly increased proportion of mDC expressing CCR4, a chemokine receptor that mediates homing to the liver. Recently CCR4 expression on mDC has been linked to the development of experimental autoimmune encephalomyelitis in mice by regulating GM-CSF and IL-23 production in dendritic cells and Th17 recruitment (Poppensieker, Otte et al.). Whether the increased number of mDC expressing CCR4 in acute VZV infection is linked to their recruitment to inflammatory sites remains to be elucidated. Analysis of the expression of other homing markers such as the key lymphocyte skin homing marker CLA and CCR7 which directs DC homing to the lymph nodes did not reveal any significant regulation in acute VZV infection. There was a slight but insignificant decrease in the proportion of $CCR7^{+}$ mDC suggestive of mDC migration to the lymph nodes. There was also a significant decrease in the proportion

of blood CLA^+ pDC suggesting that these DC may have actually migrated to inflammatory sites on the skin. This is supported by a recent study that has shown that pDC infiltrate VZV infected skin (Huch, Cunningham et al. 2010). Although these findings cannot conclusively address key questions about DC homing during acute varicella infection, they point to a potential role of CLA and CCR4 in regulating pDC and mDC trafficking perhaps to the skin and the liver respectively. It is also possible that the observed alteration in chemokine receptor expression could just be a result of VZV infection reducing the level of cell surface proteins or causing increased cell turnover. Additional studies are still required to comprehensively elucidate the main chemokines that govern DC trafficking to VZV infected skin.

Analysis of DC phenotype during acute varicella infection, revealed increased expression of the maturation markers CD40 and CD86 suggesting that VZV infection induces activation and maturation of both mDC and pDC. In contrast, acute infection also resulted in decreased surface expression of HLA-DR in patients; suggesting that infection differentially regulates DC activation marker expression. HLA-DR is one of the crucial markers of antigen presentation on DC and it is possible that VZV alters its expression as a means to impair their antigen presenting ability. VZV has previously been shown to inhibit IFN-γ induced up regulation of HLA-DR (Abendroth, Slobedman et al. 2000) a property it shares with Hepatitis C virus which also inhibits DC antigen presentation by a similar mechanism (Saito, Ait-Goughoulte et al. 2008). A similar observation has been made with human herpes virus 6 (HHV-6), a beta herpes virus which up-regulates maturation markers on infected DC, but inhibits their capacity to present alloantigens and exogenous virus antigens to T lymphocytes compared to uninfected DC (Kakimoto, Hasegawa et al. 2002). In other studies, VZV has been shown to be capable of down regulating MHC class I

molecule expression by shutting off MHC trafficking (Abendroth, Lin et al. 2001). Taken together, these data suggest that VZV is capable of inducing DC maturation while impairing antigen presentation by either targeting the MHC class II and or the MHC class I antigen presentation pathway.

Analysis of acute VZV patient DC to secrete cytokines was measured after *in vitro* re-stimulation with a TLR ligand CL097. pDC are known to produce type I IFNs in response to viral infections while mDC mainly secrete IL-12 (Banchereau, Pulendran et al. 2000). We observed a significant reduction in the proportion of IFN-α and TNF-α secreting DCs in acutely infected patients compared to healthy controls in response to TLR ligation. It is noteworthy that a similar observation has been made in acute herpes simplex virus (HSV) infection (Bjorck 2004) and human immunodeficiency virus (HIV) infection (Tilton, Manion et al. 2008) where the resulting high viraemia and heightened inflammatory cytokine environment is thought to induce a state of DC unresponsiveness during *in vitro* re-stimulation. Considering that the cytokine/chemokine profile in the serum of VZV patients mirrors on-going inflammation, it is possible that the same factors of high viremia and high inflammatory cytokine exposure reported for HSV and HIV could be operating during acute VZV infection leading to *ex vivo* pDC and mDC's unresponsiveness to re-stimulation. When these DC functional assays were extended to *in vitro* studies, we observed inhibition of pDC and mDC cytokine secretion by the attenuated VZV vaccine strain vOka when added to TLR7/8 stimulated PBMC cultures. This inhibition was abrogated when the virus was irradiated indicating that the ability of vOka to inhibit cytokine secretion is dependent on its ability to replicate. We therefore postulate that cytokine secretion is inhibited by a viral protein that is only present during replication. Another peculiar observation was that vOka

affects IFN-α secretion more severely compared to TNF-α pointing to virus targeting of different pathways to abrogate DC cytokine secretion. Since the TLR7 pathway is the dominant pathway in inducing type I IFN production, it is possible that VZV targets this pathway to evade innate immunity. What is surprising is that these observed functional changes in mDC and pDC were not associated with alterations in the surface expression of maturation molecules CD40 and CD86 suggesting that cytokine inhibition by VZV affects mature DC. Although we draw conclusions on DC cytokine inhibition based on observations made using vOka stimulated cultures, it is possible that wild type virus may have similar or totally different effects on DC function. We did not test the effect of any wild type VZV isolates and are cautious to conclude that the observed effects of vOka on *in vitro* stimulated cultures mirror natural infection caused by wild type virus.

Finally, analysis of serum cytokines during acute infection revealed a general increase in the level of inflammatory cytokines and chemokines consistent with on-going inflammation. Surprisingly, the anti-inflammatory cytokine IL-10 was elevated in patient sera. Induction of IL-10 secretion is an immune evasion tactic employed by some viruses to dampen effector cell responses during acute viral infections (Saito, Ait-Goughoulte et al. 2008) though this has not been shown in the case of varicella infection. Despite elevated serum levels of all the inflammatory chemokines measured, the level of the chemokine RANTES was decreased in acute infection and this reduction was positively correlated with disease severity. Certain viruses have been shown to express chemokine receptor homologs that sequester inflammatory chemokines; making them unavailable to effect their immune functions. Cytomegalovirus (CMV) encodes a chemokine receptor homolog US28 which was shown to bind endogenous and exogenously secreted RANTES and MCP-1 thus

hampering immune cell trafficking. (Randolph-Habecker, Rahill et al. 2002). VZV has not been shown to express any chemokine receptor homologs but the reduction in RANTES levels could point to this possibility. This decrease in RANTES levels during acute varicella infection could particularly have a severe impact on the induction of adaptive T cell responses as RANTES has been shown to be particularly important in the promotion of $CD8^+$ T cell cytolytic activty and the establishment of $CD8^+$ T cell memory in a murine model of systemic chronic lymphocytic choriomeningitis virus infection (LCMV) (Crawford, Angelosanto et al. 2011).

3.5 Conclusions

In summary, the results of this study show that acute varicella infection alters the frequency and function of blood dendritic cells while maintaining an activated mature phenotype. Our analysis revealed a reduced frequency of circulating mDC in acute infection despite having fairly constant numbers of total DC. We also observed that DC generally exhibit an activated phenotype characterized by increased expression of co-stimulatory molecules CD40 and CD86 albeit with reduced antigen presentation capacity signified by the down regulation of HLA-DR. We observed possible induction of mDC homing to the liver and lymph nodes via CCR4 governed by CCR7 expression and possible pDC homing to skin sites mediated by CLA expression. Finally we find that acute varicella infection severely impairs the cytokine secretion capacity of peripheral blood DC, inhibiting type I interferon and TNF-α secretion from both pDC and mDC possibly by targeting the TLR7 pathway.

These findings may help explain the paucity in VZV specific memory $CD8^+$ T cell responses as a consequence of virus mediated inhibition of DC function which promotes VZV pathogenesis and latency. We propose that VZV potentially employs immune evasion mechanisms that target DC and affect the two arms of the immune system. First, VZV inhibits innate anti-viral immunity by subverting type I interferon and inflammatory cytokine secretion from DC allowing virus incubation and spread to peripheral sites such as the skin. Next, the virus inhibits the initiation of adaptive immunity by reducing antigen presentation indicated by reduced surface HLA-DR expression on activated DC and the reduction of adaptive immune cell promoting chemokines such as RANTES. The combined effect of reduced antigen presentation

and cytokine secretion means DC are unable to effectively prime naïve T cells, promote their differentiation and commitment to memory.

Despite observed per cell inhibition of DC cytokine secretion by VZV, serum cytokine levels were elevated; pointing to the fact that VZV infection still manages to trigger an inflammatory response, with cytokines possibly emanating from other sources such as epithelial cells, NK cells and tissue resident DC which also participate in the clearance of primary varicella infection. Nevertheless, the induction of IL-10 could have a severe impact in dampening any effector cell responses elicited by acute infection thus negating the effects of inflammation and promoting virus persistence. It is important to note that this is a descriptive study based on the fact that patient samples were collected at a single time point only, with many patients reporting different durations of illness. We are cautious to draw conclusions that the observations of this study truly reflect the sum total of changes that may occur in DC numbers or functions following acute VZV infection. Answers to questions such as how the immune status before infection may influence DC function during and after infection are crucial to determining the exact impact of VZV on peripheral blood DC. Nevertheless, we hope that these findings will provide insights into the design of prophylactic and therapeutic interventions such as vaccines and new strategies to curb viral immune evasion mechanisms that target dendritic cells. We also hope that these preliminary observations on the potential effects of VZV on DC function will influence the design of more comprehensive studies in future to elucidate the interplay between VZV and DC.

CHAPTER 4

Regulation of MHC class I Expression during Varicella Zoster Virus Infection

4.0 Background

4.1 Antigen Processing and Presentation

One of the unique features of the vertebrate adaptive immune response is the ability to recognise and respond to specific pathogens and to remember ‘past’ antigens. Immunological memory allows a quicker and stronger response upon subsequent re-exposure to a pathogen while specificity allows antigenic targeting. Adaptive immunity relies on the capacity of immune cells to distinguish between the body's own cells and foreign invaders. This is made possible by the surface expression of αβ T cell receptors (TCRs) which recognise antigenic peptides in complex with major histocompatibility complex proteins (MHC). This is a central event in the cellular adaptive immune response and mediates leukocyte interaction. MHC class I and class II molecules are encoded by genes within the major histocompatibility complex and play a central role in regulating immune responses through their ability to bind and display small peptides derived from self and foreign proteins. Antigenic peptides are derived from extracellular or intracellular proteins, processed through designated pathways and presented in complex with MHC molecules and β-2 microglobulin to T cells.

4.2 The Major Histocompatibility Complex

The major histocompatibility complex (MHC) is a cluster of genes found on the short arm of human chromosome 6 that encode cell surface proteins which regulate the adaptive immune response. It is also the most polymorphic locus in the human genome. It is commonly referred to as H2 in mice and HLA (human lymphocyte antigen) in humans. Class I MHC contains three major genes called HLA-A, HLA-B, and HLA-C. These are designated as class Ia. This region also encodes minor class I molecules, HLA-E, HLA-F, HLA-G and HLA-H which are designated as MHC class Ib. Class II MHC genes are located within the D region of the gene complex and encode HLA-DR, DQ, and DP whose proteins are expressed on antigen-presenting cells including macrophages, dendritic cells and B cells. The function of all these proteins is to present fragments of antigens to T cells. The receptor of T cells can only recognise antigen fragments in complex with MHC proteins. The MHC molecules' ability to present a wide range of antigenic peptides for T cell recognition requires a compromise between broad specificity and high affinity. Three-dimensional structures of both class I and class II MHC molecules show a unique structural solution to this problem. The peptide main chain is tightly bound whilst peptide side chains show less restrictive interactions. It is primarily the peptide side-chain contacts and conformational variability that ensures that the peptide-MHC complex presents an antigenically unique surface to T cell receptors. MHC class I and class II molecules form membrane-distal structures that comprise a cleft in which the antigenic peptide ligands reside (Madden 1995; Garcia and Teyton 1998; Scott, Peterson et al. 1998). The T-cell receptor (TCR) on $CD4^+$ or $CD8^+$ T cells

recognizes MHC molecules in the context of both the class I or class II molecule and the peptide antigen presented in the antigen binding groove of these molecules.

4.2.1 Major Histocompatibility Complex Class I

A detailed structure of MHC class-1 molecule is shown in the picture below. The MHC Class I molecules is a 350 amino acid, 45 kDa transmembrane glycoprotein composed of two proteins. The intracellular domain is 75 amino acids long and forms the membrane spanning and carboxylic end of the protein. The remaining 270 amino acids from the extracellular portion consists of three globular domains/ alpha chains designated α1, α2 and α3. Alpha-1 (*blue-green*) is closest to the amino terminus while Alpha-3 prime (*magenta*) alpha-3 is closest to the membrane. The α1 and α2 domains are structurally important in the formation of the peptide binding groove. The groove is made of eight anti-parallel β-pleated sheets flanked by anti-parallel α-helices. The α3 domain mediates interaction with CD8 during antigen presentation and is more conserved than α1 and α2. The second portion of the molecule is a small globular protein called beta-2 microglobulin (*green*). It associates primarily with the alpha-3 prime domain and is necessary for MHC stability. The bound peptide (*cream*) sits within the peptide binding groove.

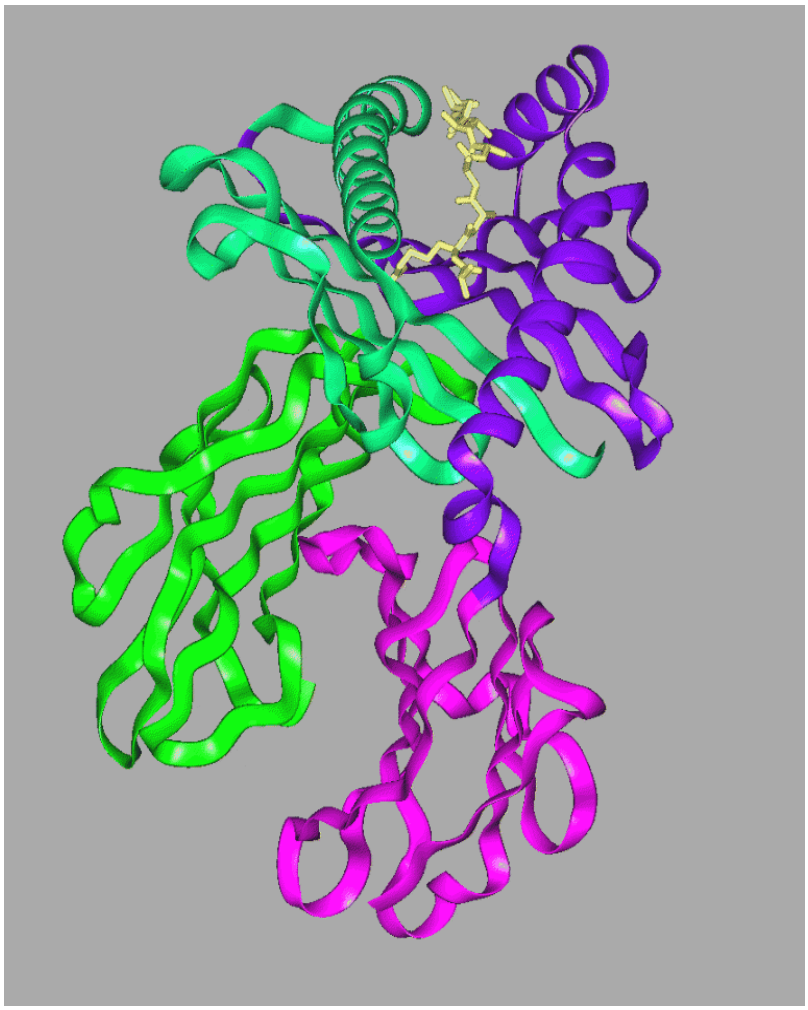


Figure 4.1: Structure of MHC class I molecule bearing antigenic peptide
Adapted from *http://www.cryst.bbk.ac.uk/pps97/assignments/projects/coadwell/004.htm*

Examination of the peptide binding groove shows that the solvent-accessible amino acids along the centre and widest end of the cleft are the most polymorphic in mouse and human alleles. Conserved side-chains are clustered at the narrower ends of the groove. Six pockets in the antigen-binding groove, of variable shape and composition, bind side-chains from antigenic peptides.

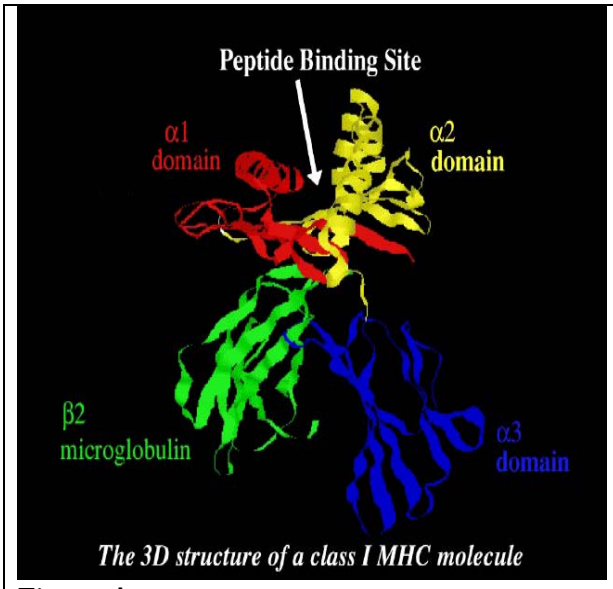


Figure A

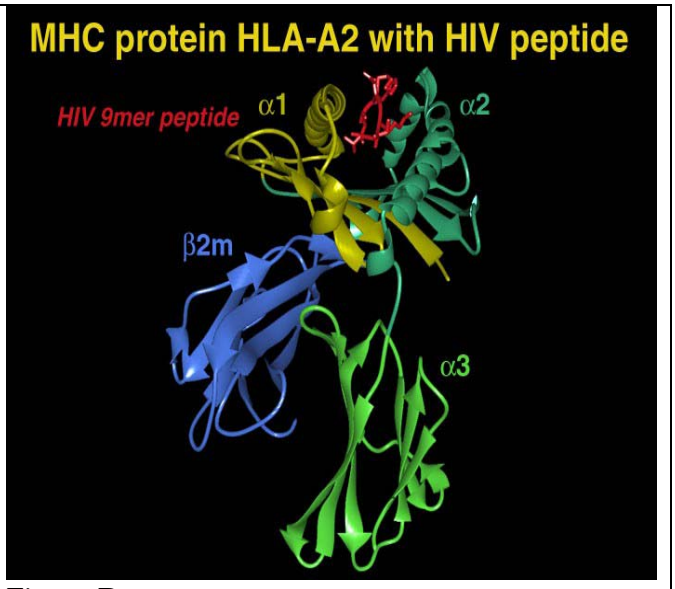


Figure B

Figure 4.2: A) Diagrammatic representation of MHC class I molecule showing various domains. **B)** Crystal **s**tructure of the MHC class I molecule, HLA-A2 bearing antigenic peptide from a HIV protein

Figure A: Adapted from http://www.imtech.res.in/raghava/mmbpred/supl/glos.html

Figure B: Adapted from

http://www.personal.psu.edu/faculty/s/x/sxt30/gallery/mhc_ribbon1.jpg

HLA class I molecules are expressed on all nucleated cells and associate with short peptides (8–11 amino acids in length) derived from both self and foreign antigens. These peptides usually possess conserved residues known as 'anchor residues' located in various positions within the chain. Anchor residues ensure specificity and stability of bound peptides. These peptide ligands are primarily generated in or transported into the cytoplasm and subsequently translocated into the endoplasmic reticulum (ER) by the transporter associated with antigen presentation (TAP) where they assemble with nascent MHC class I molecules. Mature, peptide-loaded complexes are then transported to the cell surface where they are recognised by $CD8^+$ cytotoxic T lymphocytes (CTL). Should the peptide ligand be derived from a

pathogen and be recognised as foreign in an immunocompetent host, the cell is killed via the cytotoxic potential of the CTL.

4.2.1 Major Histocompatibility Complex Class II

Although similar to Class I, the MHC Class II molecule is composed of two membrane spanning glycoproteins. Each chain is approximately 30 kDa in size, and made up of two globular domains as shown in the ribbon diagram. The domains are named Alpha-1 *(blue-green),* Alpha-2 (*green*), Beta-1 (*purple*) and Beta-2 (*magenta*). The two regions farthest from the membrane are α-1 and β-1. The two chains associate without covalent bonds to form a peptide binding groove. The bound peptide (*cream*) sits within the groove.

Class II molecules are dimers consisting of an alpha and beta polypeptide chain. Each chain contains an immunoglobulin like region, next to the cell membrane. The antigen binding cleft, composed of two alpha-helices above a beta-pleated sheet, specifically binds long peptides, about 15 to 24 residues long. This is the main difference between class I and class II molecules. While MHC class I molecules are restricted to bind short peptides, MHC class II molecules are more robust. This is because the ends of the antigen binding cleft of Class I molecules taper and are blocked by bulky tyrosines that bind the N terminus of the peptide unlike in Class II molecules where smaller residues (glycine or valine) replace the larger tyrosines.

Broad specificity is accomplished through five cavities that accommodate the residue side chains of the bound peptide. The residues that line these pockets are highly polymorphic and this contributes to the specificity of MHC Class II alleles. The

pockets differ in the size and hydrophobicity of the side chain they are able to accommodate. The binding pockets of MHC class II molecules are more permissive in their accommodation of different amino acid side chains making the definition of anchor residues in the class particularly difficult.

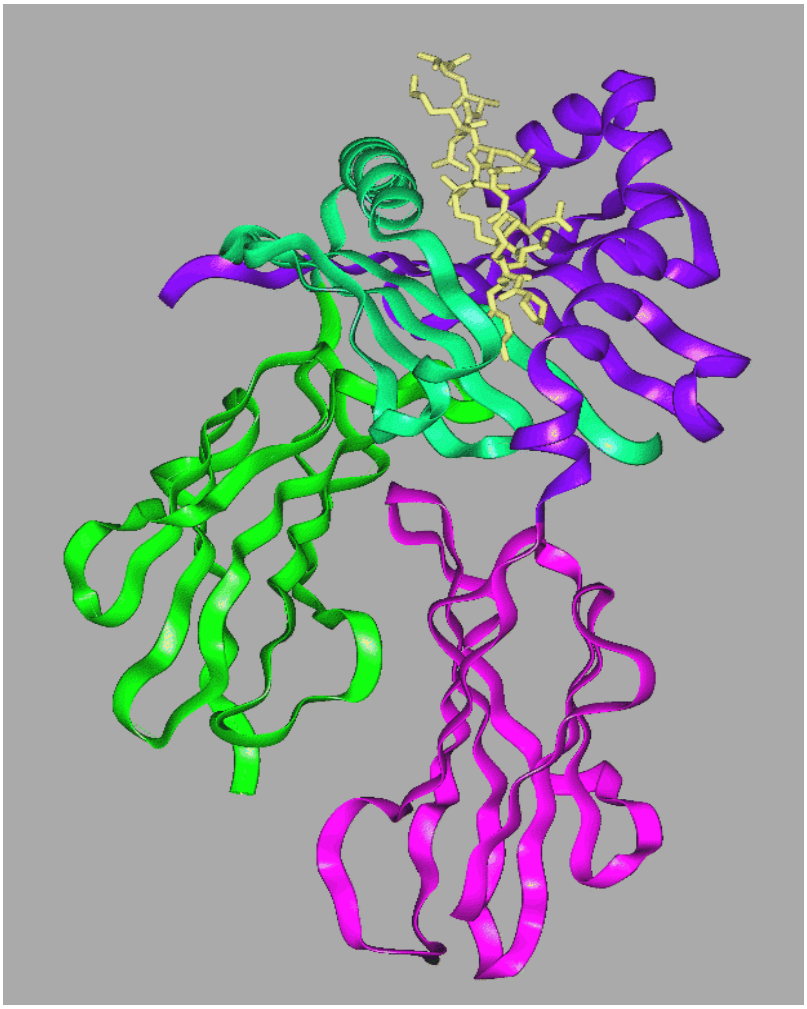


Figure 4.3: Crystal **s**tructure of the MHC class II molecule, bearing antigenic peptide. *Adapted from* *http://www.cryst.bbk.ac.uk/pps97/assignments/projects/coadwell/006.htm*

The expression of HLA class II molecules is confined to a small subset of highly specialized cells called antigen-presenting cells (APCs). The class II molecules

associate with longer peptides (9–24 amino acids in length) than class I molecules and this association occurs in late endosomal compartments, a distinct and separate cellular compartment to the ER-Golgi route inhabited by assembling MHC class I molecules. Class II molecules are recognized by $CD4^+$ T helper cells and functional recognition of these complexes is intimately involved in both the humoral and cellular immune response.

4.3 Regulation of MHC expression

4.3.1 IFN-γ mediated control of MHC expression

Although MHC class I is ubiquitously expressed on all nucleated cells, constitutive expression of MHC class II is restricted to a set of special immune cells collectively known as antigen presenting cells (APC) including Dendritic cells, monocytes, macrophages and B cells. At steady state this expression pattern is maintained; but can be altered by immunologically relevant events such as infection or exposure to certain immune cytokines. Infections by various pathogens can affect MHC gene and protein expression directly by regulating complex synthesis and or degradation or indirectly by inducing the expression of immune cytokines and chemokines which alter the expression of MHC related genes. The interferons have emerged as key cytokines that can significantly regulate the expression of both MHC class I and II molecules.

One of the first findings was the role of IFN-γ in up regulating MHC class I gene expression. This was related to the already established antiviral role of type I interferons such as IFN-α in increasing antigen presentation through increased MHC class I gene and protein expression. Increased MHC expression during virus

infection ultimately enhances the recognition of infected cells leading to virus clearance. IFN-γ can also increase the transcription of other genes involved in antigen processing and class I assembly such as those encoding for proteosomal subunits, MHC alpha chains, tapasin and TAP. This IFN-γ mediated up-regulation of MHC class I expression has been correlated to the induction of proteosomal subunits *[Lmp]-2* and *Lmp-7*) indicating that IFN-γ treatment increases proteosomal generation of peptide antigens thereby increasing peptide cargo for MHC loading and consequently leading to increased trafficking of peptide loaded MHC complexes to the cell surface.

IFN-γ also plays a unique role in regulating MHC class II gene expression. Besides enhancing the levels of class II molecules expressed on the surface of APC, IFN-γ can induce expression of MHC class II genes in non-APC by inducing expression of the MHC class II transactivator gene (CIITA). CIITA has been shown to be a general regulator of both inducible and constitutive MHC class II expression. A functional CIITA gene is necessary for class II induction, and transfection of CIITA has been shown to be sufficient in activating expression of MHC class II genes in class II-negative cells even in the absence of IFN-γ (Steimle, Siegrist et al. 1994).

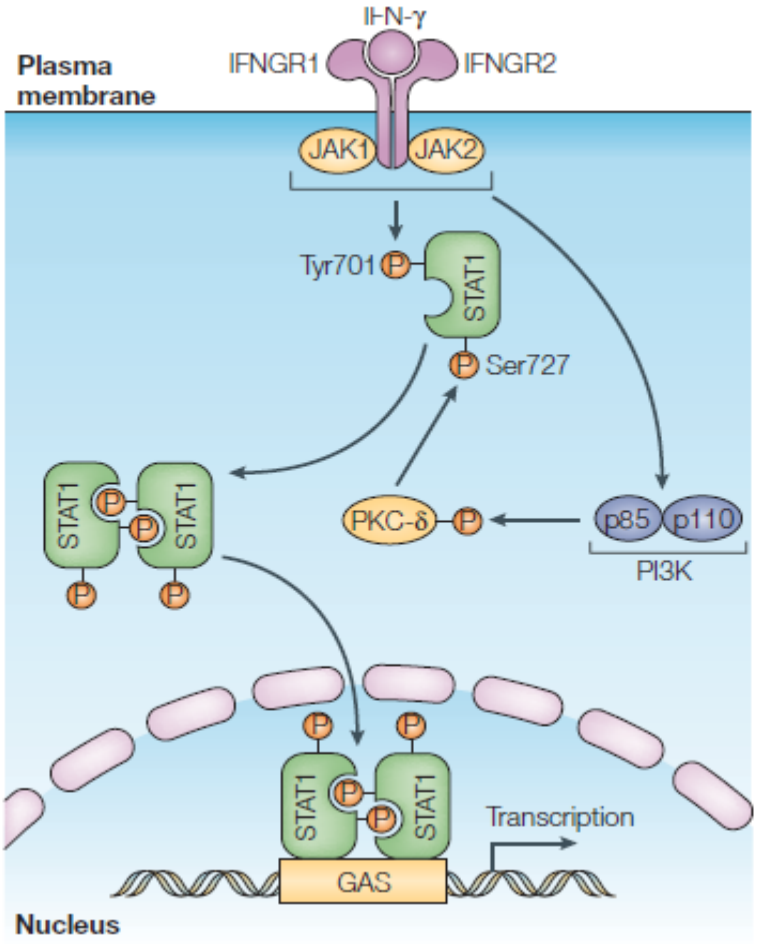


Figure 4.4: IFN-γ signaling pathway. *Adapted from (Platanias 2005)*

The mechanism of IFN-γ mediated MHC class I and class II up regulation depends on signalling via the JAK-STAT pathway (Platanias 2005). Due to its importance in regulating antigen presentation, this pathway forms a key target for viruses to subvert immune responses leading to reduced antigen presentation.

4.3.2 MHC as a viral target for immune evasion

The processing and presentation of antigens through the class I major histocompatibility complex (MHC) pathway is the initial specific response to viral

infection. This recognition of peptide MHC complexes is important for immune responses against herpes viruses, limiting viral replication by promoting both $CD4^+$ and $CD8^+$ CTL recognition (Yasukawa, Inatsuki et al. 1989; Tigges, Koelle et al. 1992; Koelle, Frank et al. 1998; Koelle, Chen et al. 2001). Like many other pathways in the cell, the MHC pathway is routinely targeted and exploited by viruses and virus derived products as a means to evade detection by both $CD4^+$ and $CD8^+$ T cells. Several viral pathogens including herpes viruses express immunoevasins that either down regulate cell surface MHC expression, target MHC molecules for degradation, interfere with intracellular MHC trafficking, or curtail MHC assembly by interfering with various antigen processing and presentation machinery.

Herpes viruses may differentially regulate MHC class I and MHC class II expression though some mechanisms can be common to both pathways. Human cytomegalovirus (HCMV) US2 and US11 mediate MHC degradation whereas herpes simplex virus (HSV) ICP47, bovine herpes virus (BHV) UL49.5 and another HCMV protein US6 all bind to the transporter associated with antigen presentation (TAP) and interfere with its peptide transport function.

Some viruses have also been shown to impede cell surface MHC molecule expression by interfering with intracellular MHC trafficking. HCMV was shown to alter the distribution of cell surface HLA-DR molecules by promoting their intracellular retention in langerhan cells (Lee, Hertel et al. 2006). The ultimate consequence of MHC down modulation by viruses is reduced antigen presentation and escape from T cell surveillance. Disruption of the class I MHC pathway by these virus encoded proteins also results in decreased $CD8^+$ cytotoxic T lymphocyte (CTL) responses, poor T cell memory and promotes survival of infected cells in the host

(Jugovic, Hill et al. 1998; Berger, Xuereb et al. 2000). Studies have shown that these negative effects of viruses on antigen presentation can be reversed by cytokine treatment such as limited exposure to IFN-γ which is known to enhance antigen presentation by inducing or up regulating MHC expression (Tigges, Leng et al. 1996; Radosevich, Seregina et al. 2003). However, in many cases viruses target both MHC molecules and the IFN signaling pathway to promote their survival.

4.3.3 VZV targets MHC to evade the immune system

Over a decade ago Cohen first pointed out that VZV could possibly down regulate MHC-I to evade host T cell responses. He reported that both a wild type strain and the vaccine strain of VZV (vOka) could down regulate MHC-I molecules from the surface of infected human fibroblasts (HFs) (Cohen 1998) and that this was a post translation event. Similar reports on MHC-I down regulation soon followed using HFs and T cells (Abendroth, Lin et al. 2001; Eisfeld, Yee et al. 2007) also revealing that VZV interferes with MHC-I transport from the golgi compartment. This revealed a mechanism unique to VZV unlike other alpha herpes viruses (HSV-1 and BHV) which interfere with the transporter associated with antigen presentation (TAP) and prevent the transport of antigenic peptides from the cytoplasm into the ER lumen where class I assembly occurs (Tomazin, Hill et al. 1996; Ahn, Gruhler et al. 1997; Koppers-Lalic, Reits et al. 2005; Koppers-Lalic, Verweij et al. 2008; Verweij, Koppers-Lalic et al. 2008) via their proteins HSV-1 ICP47 and BHV UL49.5. Other studies following these and using different cells have reported similar results. In one study, MHC-I molecules on the surface of VZV infected keratinocytes were

observed to be reduced but not absent (Black, Jones et al. 2009) indicating that MHC down regulation occurs at varying degrees.

VZV infection has also been shown to inhibit IFN-γ mediated up regulation of MHC-II molecules with the effect being abrogated if cells are treated with IFN-γ prior to VZV infection (Abendroth, Slobedman et al. 2000). Modulation of IFN-γ signaling also inhibits expression of co-stimulatory molecules such as ICAM-1 on keratinocytes *in vitro* and within lesional tissue (Nikkels, Sadzot-Delvaux et al. 2004; Black, Jones et al. 2009). Ultimately, VZV down regulating MHC class I and other co-stimulatory molecules such as ICAM-1 leads to decreased recognition of varicella infected cells and escape from T cell surveillance. Indeed, the study by Black and colleagues was able to demonstrate that VZV infected keratinocytes are impaired in their ability to stimulate antigen specific $CD4^+$ and $CD8^+$ T cells; even following IFN-γ treatment (Black, Jones et al. 2009).

One VZV E gene product possibly responsible for MHC class I down regulation has been identified (Abendroth, Lin et al. 2001). Detailed studies involving transfection of cells with plasmids encoding IE and E proteins identified the E gene product of open reading frame 66/ ORF66, a serine/threoninne kinase as a potential mediator of MHC class I down regulation. This study showed that the product of ORF66 neither affected MHC class I synthesis and degradation nor its association with $\beta_2$microglobulin; indicating that it acts via an otherwise unidentified mechanism (Abendroth, Lin et al. 2001; Eisfeld, Yee et al. 2007). Despite these findings, a VZV mutant lacking ORF66 kinase activity was still able to down regulate class I expression to a lesser extent suggesting that other proteins may be involved.

It is clear that VZV regulates MHC expression possibly to escape CTL lysis. A reduction of cell surface HLA expression is however a common cue for NK cell lysis. NK cell responses are mediated by the balance between activating and inhibitory receptors found on the NK cell surface. If the activating signals received by a NK cell exceed inhibitory signals, the cell is lysed and vice versa. Cell surface HLA class I molecules act as ligands for inhibitory NK receptors. Consequently, missing self in the form of HLA molecules result in activating signals leading to NK cell lysis of target cells. Even though viruses target MHC class I molecules resulting in reduced surface expression they must strike a balance between evading CTL lysis and avoiding NK cell recognition. Viruses such as HIV have been shown to balance this by down regulating only some sub-types of MHC class I while retaining cell surface expression of others. In a famous study by Cohen and colleagues HIV viral protein nef was shown to bind MHC class I molecules leading to down regulation. This was however only restricted to HLA-A and HLA-B leaving HLA-C and HLA-E whose presence would prevent NK mediated lysis (Cohen, Gandhi et al. 1999).

We hypothesised that VZV potentially exerts a similar pattern of MHC down regulation to escape both $CD8^+$ T cell surveillance and maintain NK cell inhibition.

4.2 Aims and Objectives

- To analyse the expression patterns of cell surface HLA Class I molecules after VZV infection of keratinocytes

- To analyse the expression patterns of MHC class I genes and proteins in different cell compartments following VZV infection

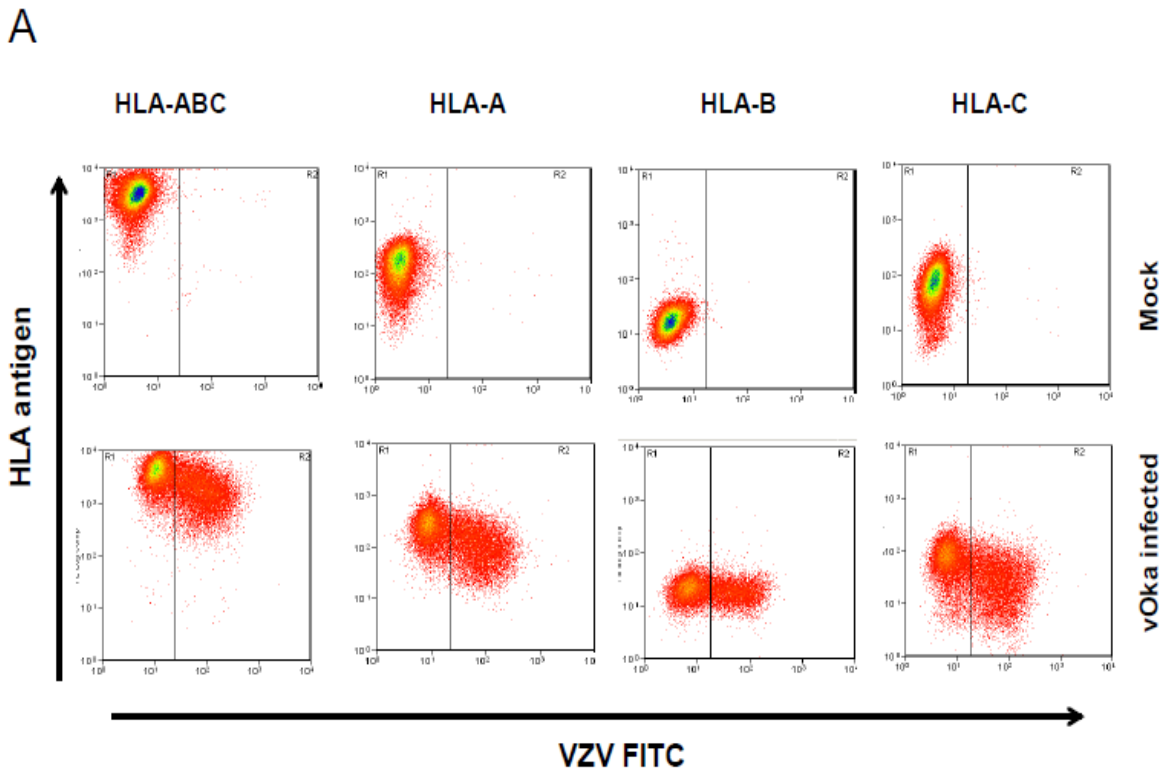
- To characterize the impact of IFN-γ treatment on the expression patterns of cell surface MHC class I molecules

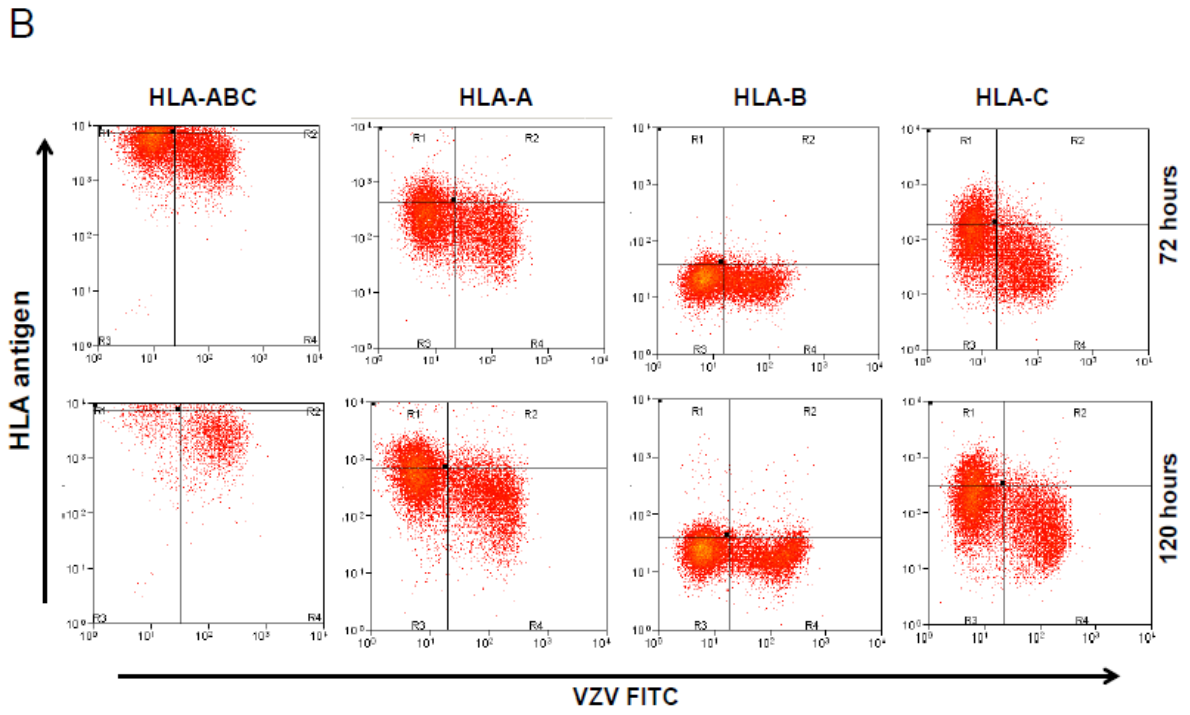
- To assess the impact of VZV infection on IFN-γ mediated regulation of HLA expression

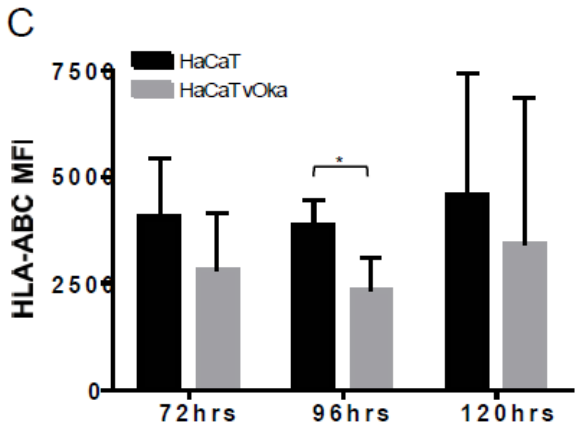
4.3 Results

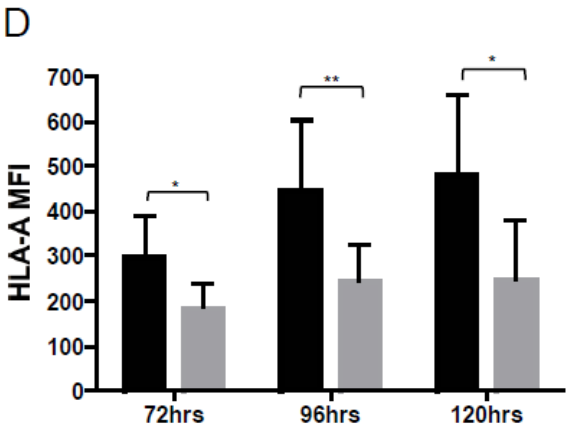
4.3.1 vOka infection of keratinocytes readily down-regulates cell surface HLA-A and HLA-C expression while seemingly sparing HLA-B. The HLA expression profile on Mock and vOka infected keratinocytes was analysed over a period of three time points at 72, 96 and 120 hours. Cells were co-stained with HLA antibodies and a monoclonal antibody specific for a VZV glycoprotein to designate infected cells. No VZV staining was present in uninfected cells while infection was readily detected in VZV infected cells at 3 time points (**(Fig 4.5 A)**. Flow cytometry analysis revealed that the total amount of MHC class I was reduced as evidenced by W6/32 simultaneous staining of HLA-A, HLA-B and HLA-C. Further analysis of specific HLA subtypes however revealed a allotype-specific pattern of cell surface HLA down regulation with HLA-A and HLA-C being readily down modulated from the cell surface compared to HLA-B which showed relatively stable expression **(Fig 4.5 C-F)**. Temporal examination of HLA profiles revealed increased levels of MHC protein expression at 120 hours possibly elevated by secreted type I interferons from bystander uninfected cells. There was however no change in the expression pattern of the different HLA allotypes at 120 hours compared to 72 or 96 hours **(Fig 4.5 B)** proving that prolonged viral replication though increasing MHC protein levels does not lead to HLA-B down regulation. Consequently, the relative fluorescence intensity (RFI) of VZV infected cells was calculated as (MFI infected/MFI uninfected) x 100. This conversion confirmed that HLA-C is the most severely down regulated HLA subtype in keratinocytes while the RFI of HLA-B though slightly decreased in infected cells is relatively comparable to Mock infected cells **(Fig 4.5**

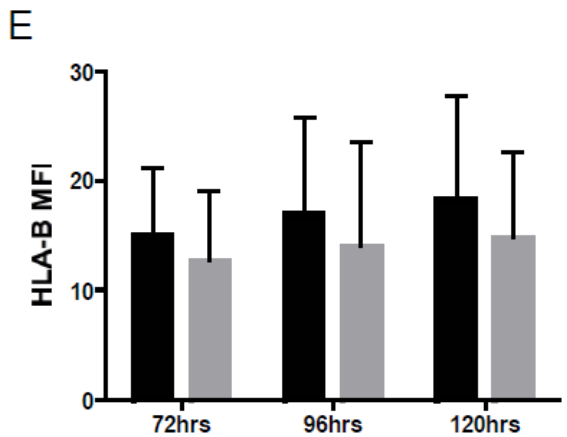
G). We conclude that VZV selectively down regulates MHC class I A and C while possibly sparing MHC class I B.

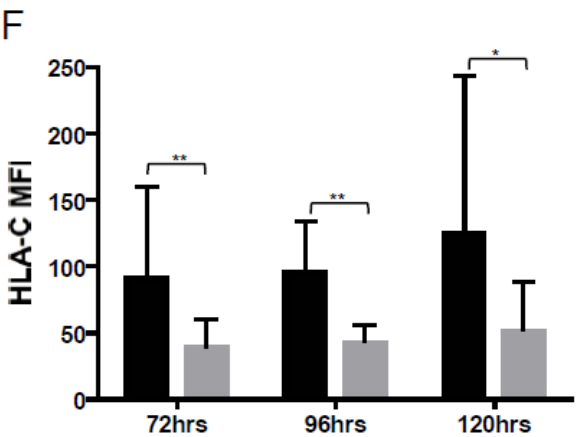



B
HLA-ABC
HLA-A
HLA-B
HLA-C
HLA antigen
72 hours
120 hours
VZV FITC


C
HaCaT
HaCaTvOka
HLA-ABC MFI
7500
5000
2500
0
72hrs
96hrs
120hrs


D
HLA-A MFI
700
600
500
400
300
200
100
0
72hrs
96hrs
120hrs


E
HLA-B MFI
30
20
10
0
72hrs
96hrs
120hrs


F
HLA-C MFI
250
200
150
100
50
0
72hrs
96hrs
120hrs

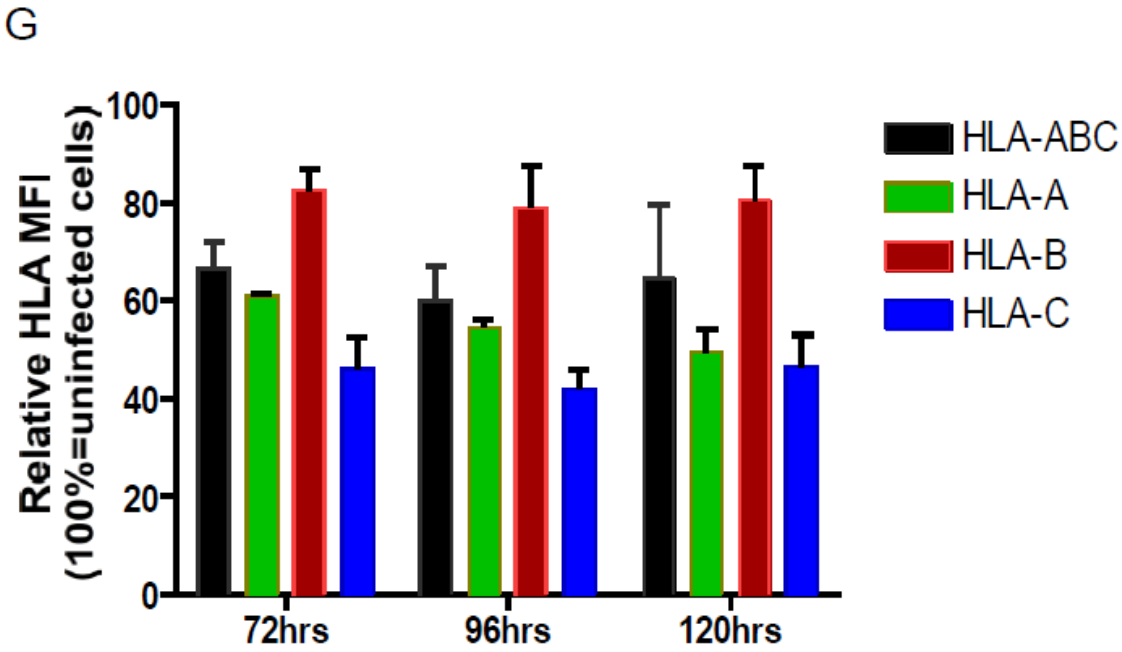
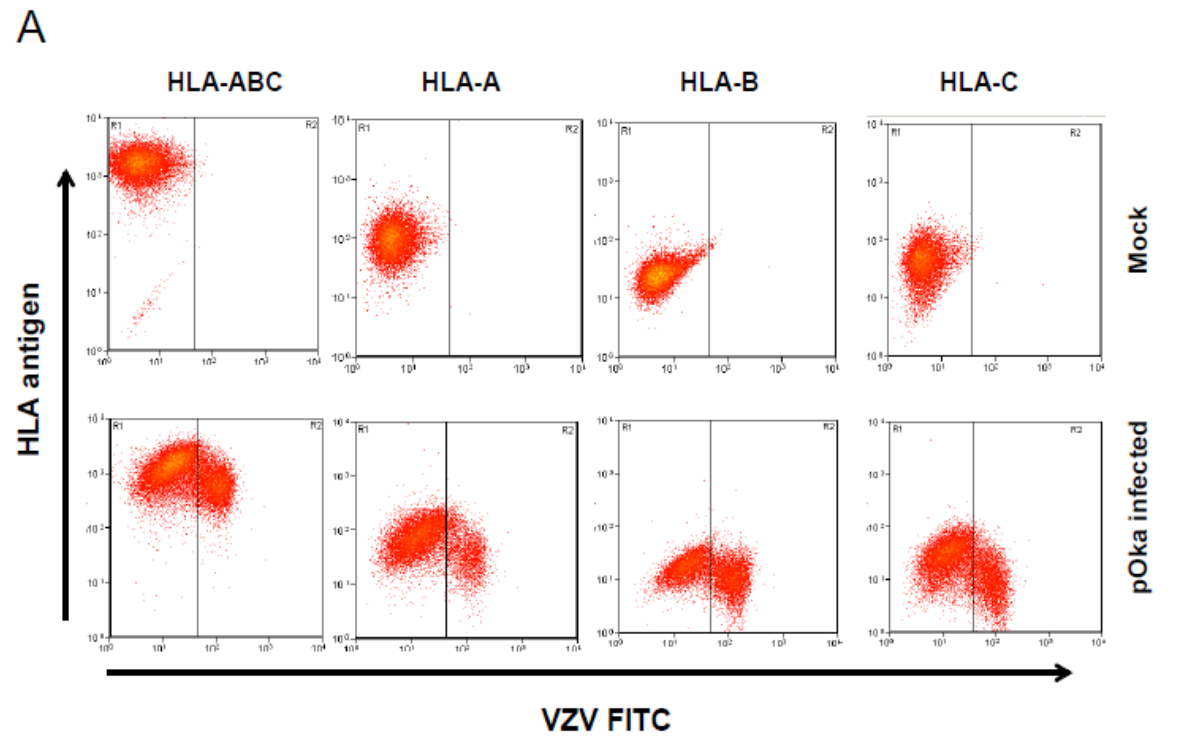


Figure 4.5: Regulation of HLA expression after vOka infection of keratinocytes. HaCaT cells were either mock infected or infected with VZV strain vOka at am MOI of 1. At 72, 96 and 120 hours, cells were stained for surface MHC class I proteins and analysed by flow cytometry. **A)** Comparison of HLA expression between Mock and vOka infected cells at 72 hours. **B)** MHC down modulation profile in 72 hour and 120 hour infected HaCaTs. **C-F)** Summary figures from 3 independent experiments showing the mean fluorescence intensity profiles for HLA-A, B and C at 72, 96 and 120 hours. Statistical differences were determined using Ordinary One Way ANOVA for multiple comparisons using Tukeys test. A *$p<0.05$ was considered significant. **G)** Relative fluorescence intensities comparing the extent of HLA down modulation in HaCaT cells.

4.3.2 pOka infection of MeWo cells down-regulates cell surface HLA-A and HLA-C expression while seemingly sparing HLA-B. Next we assessed the extent of MHC class I down modulation after infection with the parental Oka strain, pOka. HLA expression pattern was analysed on a human melanoma cell line (MeWo) after infection with pOka for 48, 72 and 96 hours. Just like vOka in keratinocytes, the

pOka strain was found to reduce the cell surface expression of MHC class I molecules on MeWo cells compared to mock infected cells **(Fig 4.6 A)**. Similarly, HLA-A and HLA-C were readily down regulated while HLA-B though showing some extent of down regulation but showed relatively stable expression on infected cells to a level comparable to mock infected cells. Simultaneous staining of HLA-A, HLA-B and HLA-C with W6/32 also revealed that the total MHC class I surface proteome is negatively affected after pOka infection at all three designated time points **(Fig 4.6 B)**. Furthermore, it was clear that each HLA molecule showed reduced expression in a time dependent manner, a trend that was not apparent after vOka infection of HaCaTs where the opposite effect was observed. Calculated relative fluorescence intensities revealed a profile similar to that found on keratinocytes with HLA-A and HLA-C down modulation being readily apparent compared to HLA-B **(Fig 4.6 F)**. In conclusion, vOka and pOka strains of VZV seem to exert a similar effect on human HLA class Ia expression; readily mediating the down regulation of cell surface HLA-A and HLA-C molecules while only mildly affecting HLA-B. Whether this finding is significant in viral evasion of NK cell surveillance remains to be conclusively determined.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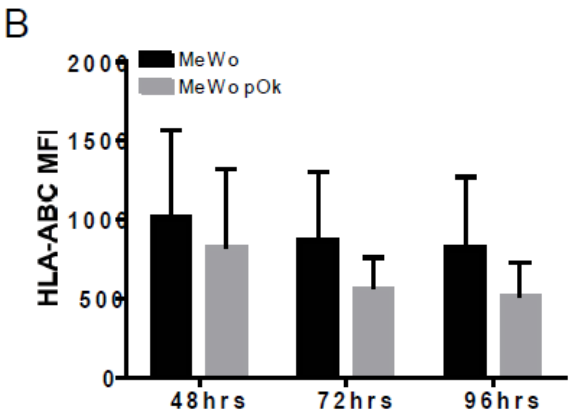
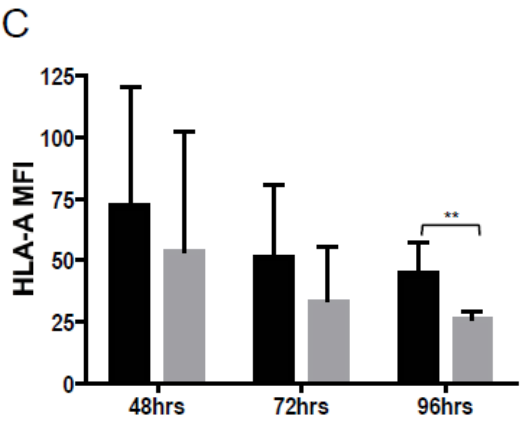
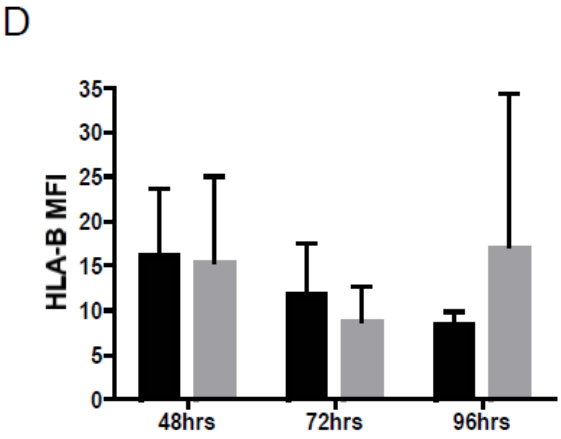
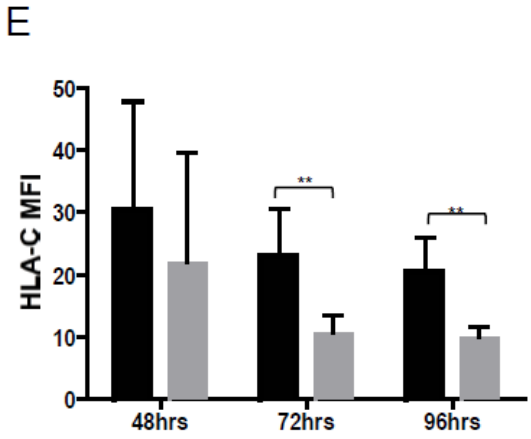
HLA-ABC
HLA-A
HLA-B
HLA-C
HLA antigen
Mock
pOka infected
VZV FITC


B
MeWo
MeWo pOk
HLA-ABC MFI
2000
1500
1000
500
0
48hrs
72hrs
96hrs


C
HLA-A MFI
125
100
75
50
25
0
**
48hrs
72hrs
96hrs


D
HLA-B MFI
35
30
25
20
15
10
5
0
48hrs
72hrs
96hrs


E
HLA-C MFI
50
40
30
20
10
0
**
**
48hrs
72hrs
96hrs

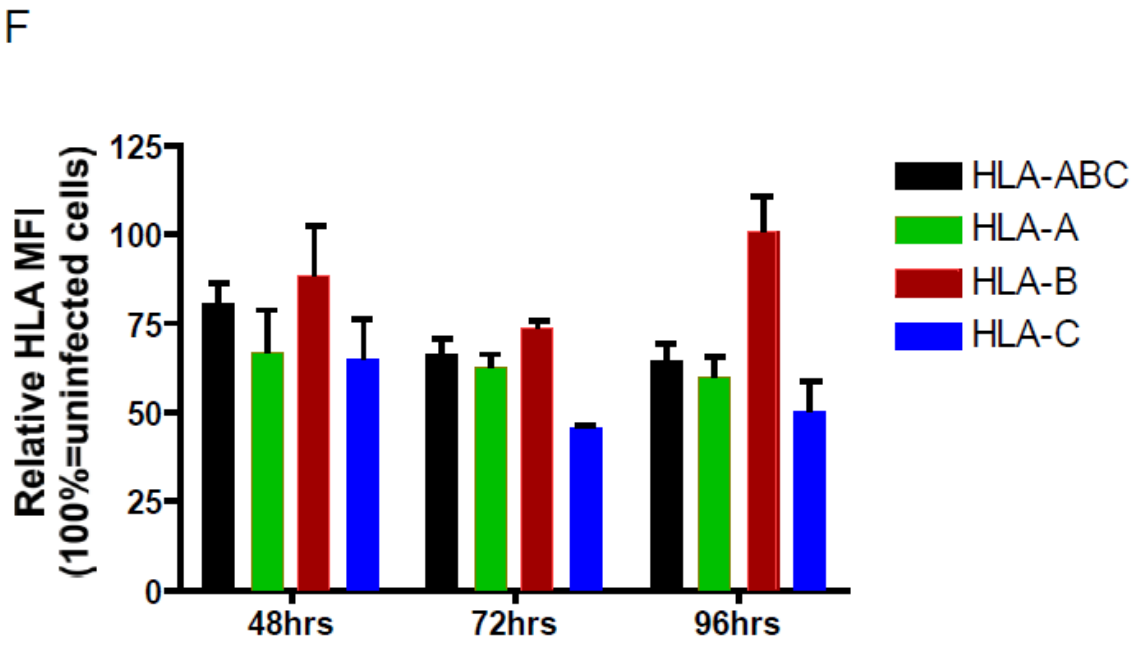
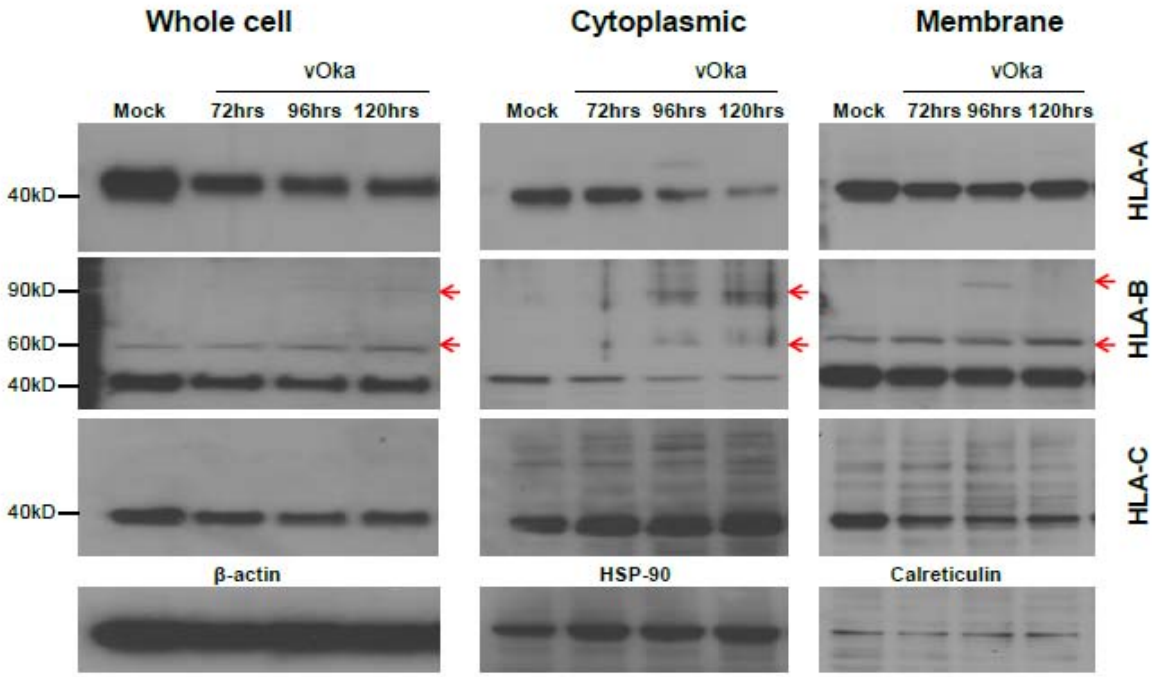


Figure 4.6: pOka infection of MeWo cells selectively down regulates HLA-A and HLA-C proteins but spares HLA-B. The effect of VZV infection on the expression of HLA class Ia surface molecules was assessed by FACS analysis on mock and pOKa infected melanoma cells. Cell surface immunostaining was carried out at 48, 72 and 96 hours followed by flow cytometry analysis. **A)** A representative experiment showing HLA class I expression on the surface of Mock infected (first row) and pOka infected (second row) cells after 72 hours. The y axes show the MFI of individual HLA antigens indicated above the dot plots while the x-axes represent the MFI of the VZV specific monoclonal antibody. **B-E)** MFI summary plots from 3 independent experiments showing allele specific HLA expression profiles. Statistical differences were determined using Ordinary One Way ANOVA for multiple comparisons using Tukeys test. A *$p<0.05$ was considered significant. **F)** The (RFI) of surface HLA molecules on VZV infected versus VZV negative MeWo cells. Results are representative of 3 independent experiments.

4.3.3 VZV infection leads to a general decrease in intracellular MHC class I protein.

The observation of a unique pattern of HLA class I down regulation from the cell surface led to analysis of intracellular HLA class I protein expression in whole cell lysates and sub-cellular protein fractions from mock and VZV infected keratinocytes and Mewo cells. Proteins from cytoplasmic, membrane and whole cell extracts were analysed by SDS-PAGE and Western blot. Results show that all three HLA allotypes A, B and C were down regulated in all the cellular fractions analysed following infection with vOka or pOka. Even though cell surface HLA-B seemed stable after flow cytometry analysis, membrane protein extracts showed decreased HLA-B expression after VZV infection. Of peculiar interest was the observation of high molecular weight protein bands at 60kD and 90kD **(Fig 4.7 A and 4.7 B red arrows)** for HLA-B and in one case HLA-C in the Mewo cell extracts (**Fig 4.7 B middle panel)**. In most cases, these bands were present only after VZV infection and in some fractions seemed to intensify in a time dependent manner after infection suggesting that VZV infection might induce HLA multimerization. Certain HLA-B alleles such as HLA-B27 are known to form dimers and other multimers. However, both of the cell lines used in this assay do not express endogenous HLA-B27 thus the multimers cannot be explained by the presence of HLA-B27. It is not yet certain whether other HLA-B alleles or all HLA-B alleles have a general tendency to form multimers especially following viral infection. It is possible that these protein bands could represent HLA molecules interacting with viral proteins although standard immune-precipitation and SDS analysis failed to reveal any observable interactions (data not shown). We conclude that VZV infection leads to a general decrease in cytoplasmic MHC protein regardless of the cell surface HLA expression profile.

A.


Whole cell
Cytoplasmic
Membrane
vOka
Mock
72hrs
96hrs
120hrs
40kD
90kD
60kD
40kD
40kD
HLA-A
HLA-B
HLA-C
β-actin
HSP-90
Calreticulin

B.

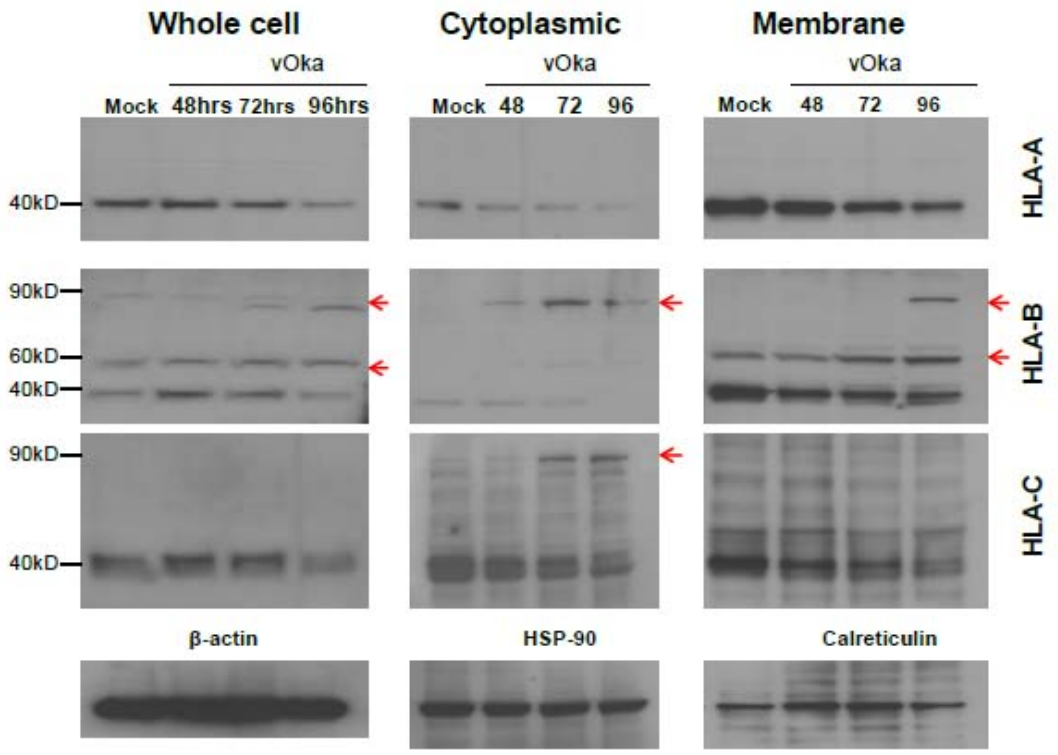


Figure 4.7: Expression of Intracellular HLA class I proteins is decreased after VZV infection.

Keratinocytes and Mewo cells were infected with vOka or pOka at an MOI of 1 and 0.2 respectively. At different time intervals after infection, cells were harvested and lysed. Equal concentrations of reduced protein was loaded onto SDS-PAGE gels and analysed by western blot. **A**) Representative diagrams showing down regulation of all HLA proteins in cell lysates from infected keratinocytes. The red arrows indicate high molecular weight bands present when membranes were probed with HLA-B antibodies. **B**) Representative diagrams showing down regulation of all HLA proteins in infected Mewo cell extracts. The red arrows indicate high molecular weight bands present when membranes were probed with HLA-B (all fractions) and HLA-C antibodies (cytoplasmic fraction).

4.3.4 HLA-B2709 and HLA-B2705 are down regulated after VZV infection of HaCaTs.

To further assess the effect of VZV on the expression of HLA-B, we obtained lentiviral vectors expressing two HLA-B27 alleles HLA-B2705 and HLA-B2709 (we are most grateful to Prof. Paul Bowness and Dr. Simon Kollnberger). HaCaT cells do not express endogenous HLA-B27 but express HLA-B40 and HLA-B51. HaCaT cells were transduced to express HLA-B27 with lentiviral vectors expressing the two HLA-B2705 and HLA-B2709 alleles followed by infection with VZV at an MOI of 1 for 72 hours. Flow cytometry analysis was conducted after cell surface immunostaining using ME-1, a monoclonal antibody against HLA-B27. Results indicate that both HLA-B27 alleles are down regulated following VZV infection. Out of two populations expressing HLA-B27, down regulation of either HLA-B allele appeared to be present in the high HLA-B27 expressing HaCaT cells but not the low expressors. Whether this is a significantly important observation will remain to be confirmed. This observed down regulation of HLA-B27 is inconsistent with the earlier findings looking at the expression profiles of all HLA-B alleles on the surface of infected keratinocytes. VZV infection seems to down regulate the expression of HLA-B27 on keratinocytes suggesting that the effect VZV has on HLA-B expression may be allele specific; not affecting endogenous HLA-B*4001 and HLA-B*5101 alleles that are expressed by the HaCaT cell line but affecting HLA-B27 alleles. Further experiments using other HLA-B alleles are needed to confirm this theory. It is also possible that down regulation of HLA-A and HLA-C could be allele specific. HaCaT cells express HLA-A*3101, HLA-C*0304 and HLA-C*1502 so if the observed down regulatory effects of VZV could be solely directed against these alleles. Unfortunately, we were not able to test the effect of VZV infection on other

HLA-A or HLA-C alleles; thus the exact effect of varicella on different HLA alleles is a question that warrants further investigation.

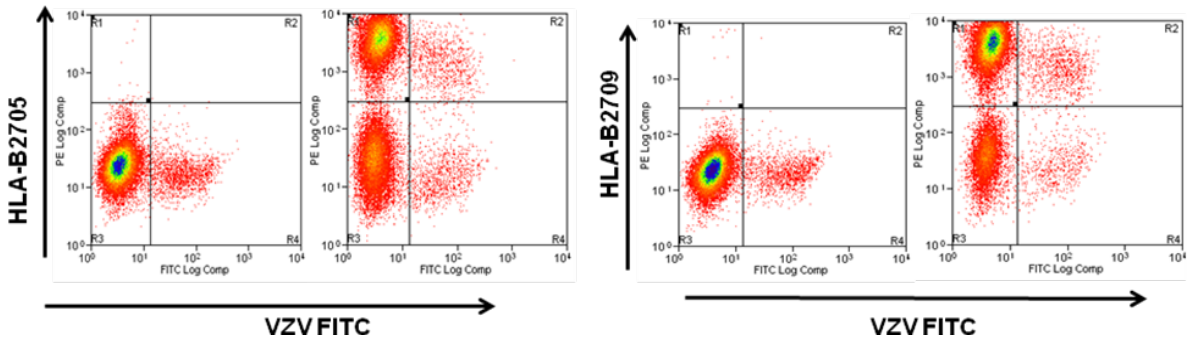
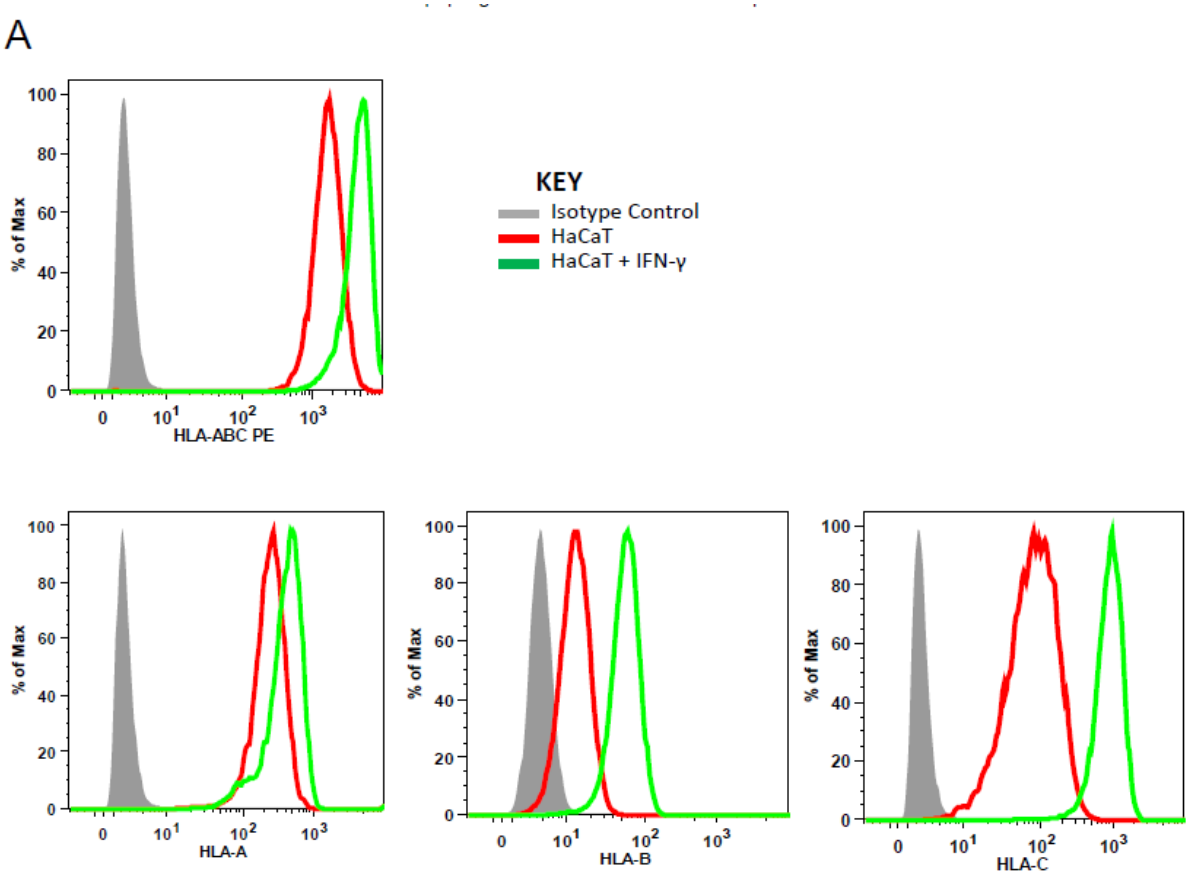


Figure 4.8: HLA-B2705 and HLA-B2709 alleles are down regulated on HaCaT cells expressing high levels of HLA-B27. Keratinocytes (HaCaT cells) were transduced with lentiviral vectors expressing two HLA-B27 alleles before being co-cultured with VZV infected keratinocytes at a MOI of 1. Cells were harvested and expression of HLA-B27 assessed in VZV^+ and VZV^- cells by flow cytometry. **Note:** These are results from a single experiment.

4.3.5 IFN-γ treatment up-regulates HLA class Ia expression on HaCaT cells.

To determine the effect of IFN-γ treatment on HLA expression, keratinocytes were treated with 300IU of IFN-γ for 24 hours and surface stained for HLA class Ia expression. Generally, all the MHC molecules were increasingly expressed following IFN-γ treatment **(Fig 4.9 A)**. However, IFN-γ treatment did not increase the expression of two minor MHC class 1b molecules HLA-F and HLA-G despite slightly increasing the expression of HLA-E **(Fig 4.9 B)**. We also assessed the expression of ICAM-1 and CD71 as a control and the expression of both molecules were found to be increased after IFN-γ treatment **(Fig 4.9 C)**. These findings are

consistent with previous findings of Black et al., showing that the effect of IFN-γ treatment of keratinocytes is increased cell surface HLA class I and ICAM-1 expression (Black, Jones et al. 2009). It is not clear why class 1b molecules HLA-F and HLA-G are not increased, but we cannot guarantee that the antibodies used to stain these molecules were optimal. Additional work will have to be done to ascertain the true nature of minor class I molecule expression on keratinocytes both at steady state and following IFN-γ treatment.



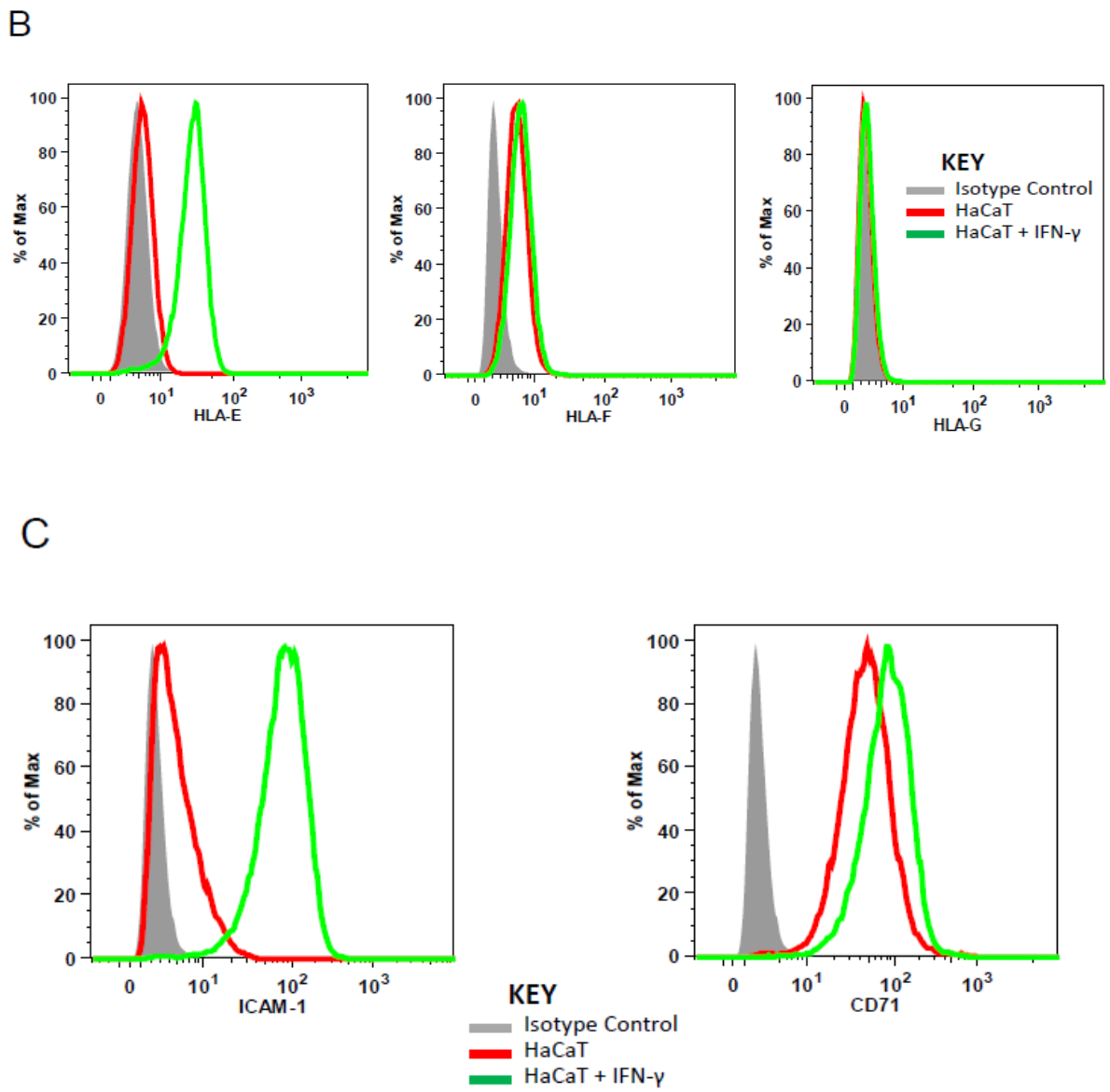
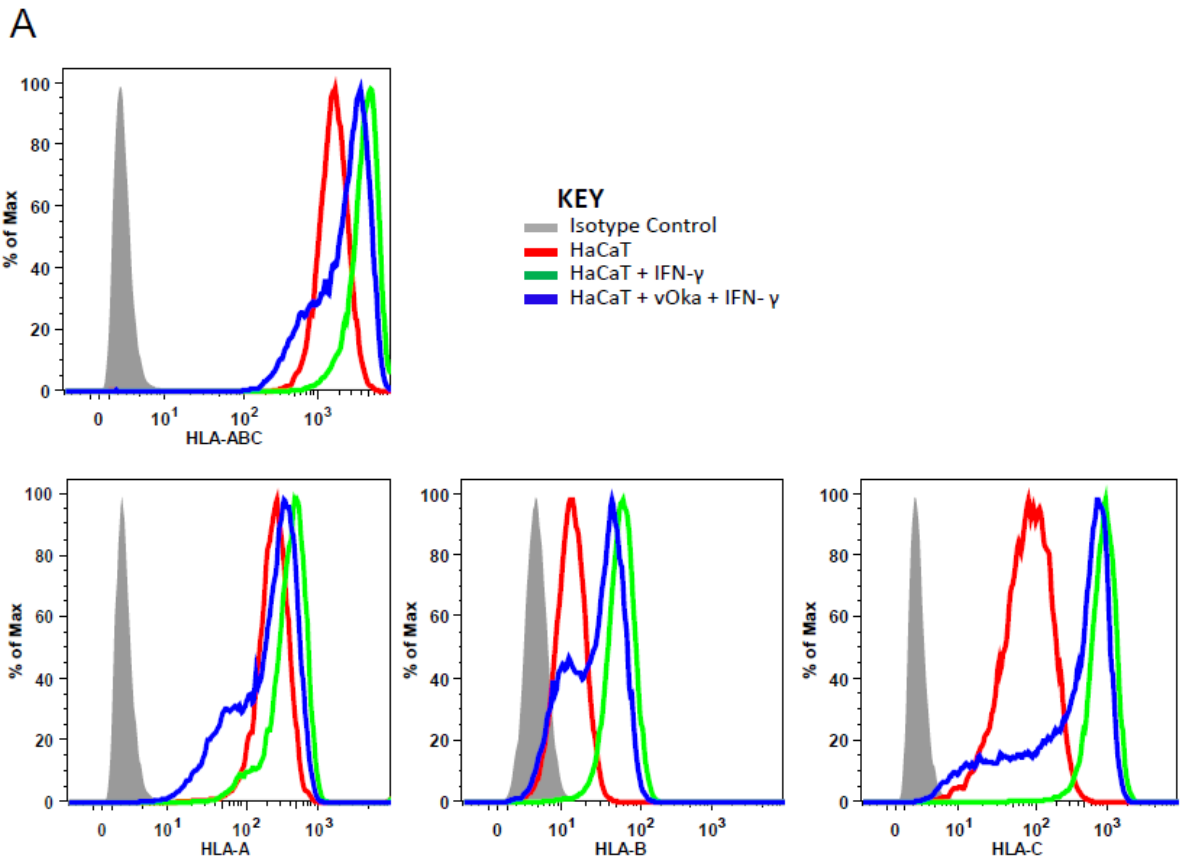


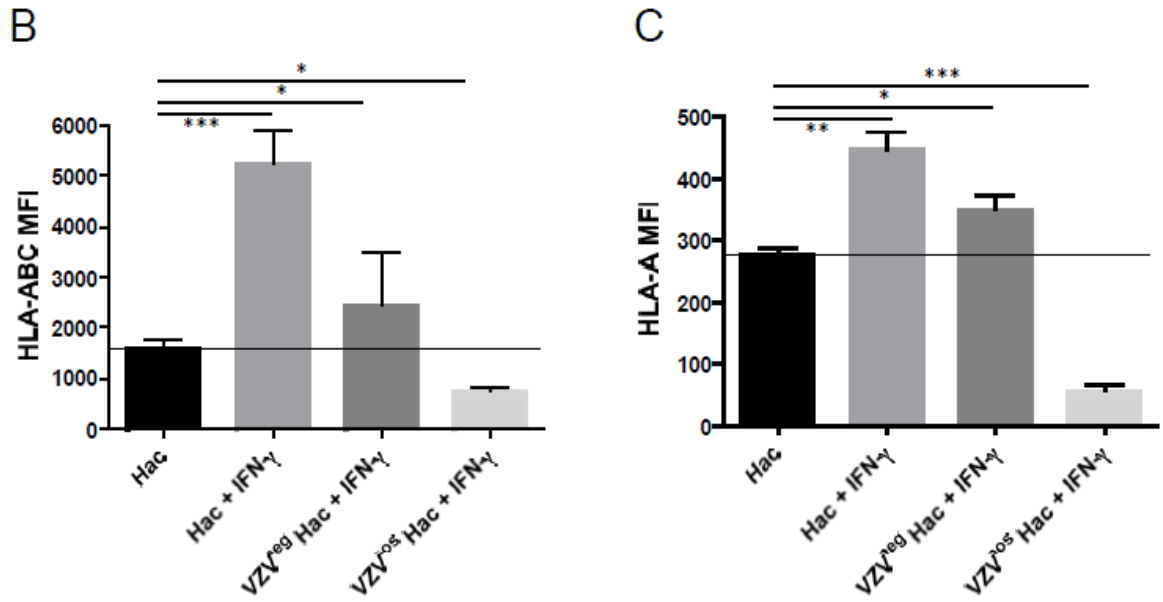
Figure 4.9: IFN-γ treatment up regulates the expression of MHC class Ia molecules on keratinocytes. Keratinocytes (HaCaT cells) were treated with IFN-g for 24hours followed by surface staining for MHC Class I expression. **A)** Expression of all HLA sub-types is increased following IFN-γ treatment. **B)** Expression of MHC class I b molecules after IFN-γ treatment showing up regulation of HLA-E but not HLA-F or HLA-G. **C)** IFN-γ up regulates surface expression of both ICAM-1 and CD71 on keratinocytes.

4.3.6 IFN-γ treatment of vOka infected HaCaT cells up-regulates HLA expression on VZV negative but not VZV positive cells. We next examined whether IFN-γ mediated up regulation of HLA class I expression was affected by VZV infection. Keratinocytes were first infected with VZV vaccine strain vOka at a MOI of 1 for 72 hours. Cells were then treated with IFN-γ for 24 hours before being immunostained for cell surface HLA class I expression. We observed dual regulation of HLA expression on infected cells following IFN-γ treatment. Cells that were positively infected with VZV as determined by VZV surface glycoprotein staining did not exhibit increased HLA **(Fig 4.10 B, C, D, E)**, ICAM-1 **(Fig 4.10 G)** or CD71 **(Fig 4.10 I)** expression after IFN-γ treatment while VZV negative fractions readily up regulated HLA expression. This is consistent with studies which have demonstrated a similar effect of inflammatory cytokines on VZV negative but not VZV positive cells. Both studies looked at MHC class II and ICAM-1 expression which were found to be up regulated on VZV negative but not VZV positive cells. (Nikkels, Sadzot-Delvaux et al. 2004; Black, Jones et al. 2009). Abendroth et al., also reported that VZV blocks IFN-γ mediated up regulation of MHC class II molecules on human fibroblasts (Abendroth, Slobedman et al. 2000). Even though VZV negative fractions up regulated HLA expression, the levels were significantly less than levels on mock infected cells treated with a similar amount of IFN-γ. We hypothesize that VZV infection may lead to production of viral gene products that interfere with IFN-γ mediated up regulation of HLA protein expression in bystander uninfected cells. Opposite observations were made for ICAM-1 and CD71 expression; with VZV infection seeming to augment IFN-γ mediated up regulation of protein expression on bystander VZV negative cells **(Fig 4.10 G and I)**. This points to a selective regulation of protein expression on bystander uninfected cells where VZV infection targets MHC but does not interfere with ICAM-1 and CD71 protein up regulation on bystander cells.

However, MHC class I protein and ICAM-1 down regulation in VZV infected IFN-γ treated cultures was similar to levels observed in VZV infected IFN-γ untreated cultures, with protein levels dipping below the baseline expression levels seen in mock infected cells. This was true for the HLA subtypes, HLA-ABC, HLA-A and HLA-C **(Fig 4.10 B, C, E)**, but not HLA-B whose levels on VZV positive cells in IFN-γ treated cultures were not significantly reduced compared to baseline levels in

mock infected cells, again confirming the earlier observation that VZV doesn't seem to target HLA-B as it does HLA-A and HLA-C **(Fig 4.10 D)**. We conclude that VZV infection of keratinocytes interferes with IFN-γ mediated up regulation of HLA, ICAM-1 and CD71 expression on infected cells by inhibiting protein expression. Also VZV differentially regulates IFN-γ signaling in bystander uninfected cells, subverting HLA up regulation but seemingly augmenting ICAM-1 and CD71 protein expression.




B
HLA-ABC MFI
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Hac
Hac + IFN-γ
VZV^neg Hac + IFN-γ
VZV^pos Hac + IFN-γ
C
HLA-A MFI
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200
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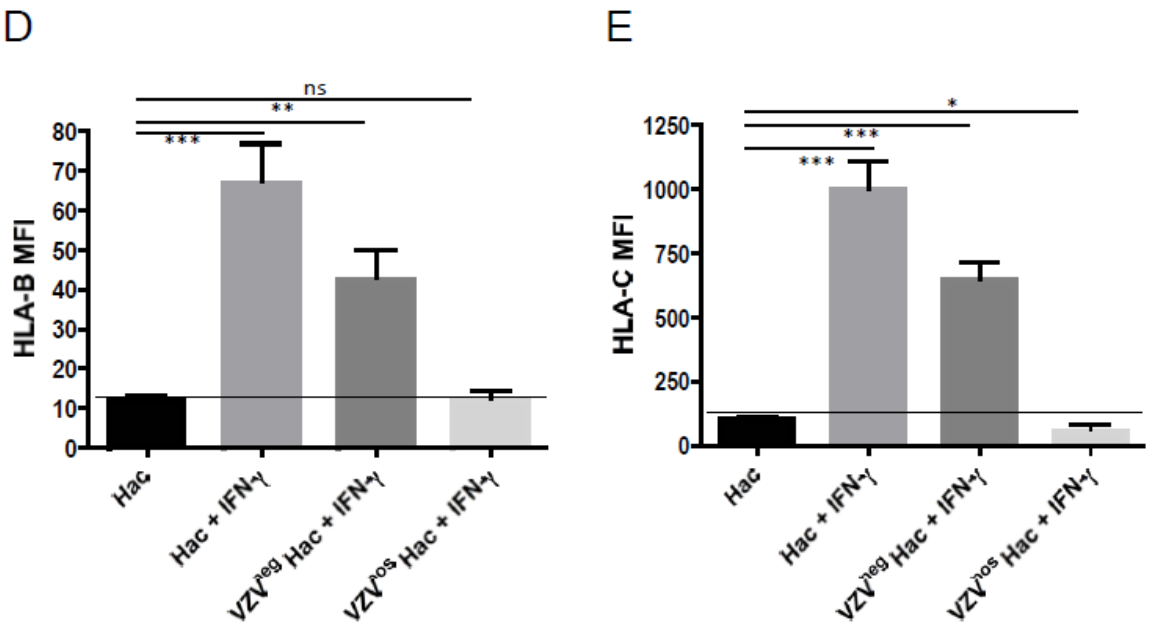
Hac
Hac + IFN-γ
VZV^neg Hac + IFN-γ
VZV^pos Hac + IFN-γ


D
HLA-B MFI
80
70
60
50
40
30
20
10
0
ns
**
***
Hac
Hac + IFN-γ
VZV^neg Hac + IFN-γ
VZV^pos Hac + IFN-γ
E
HLA-C MFI
1250
1000
750
500
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*
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***
Hac
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VZV^neg Hac + IFN-γ
VZV^pos Hac + IFN-γ

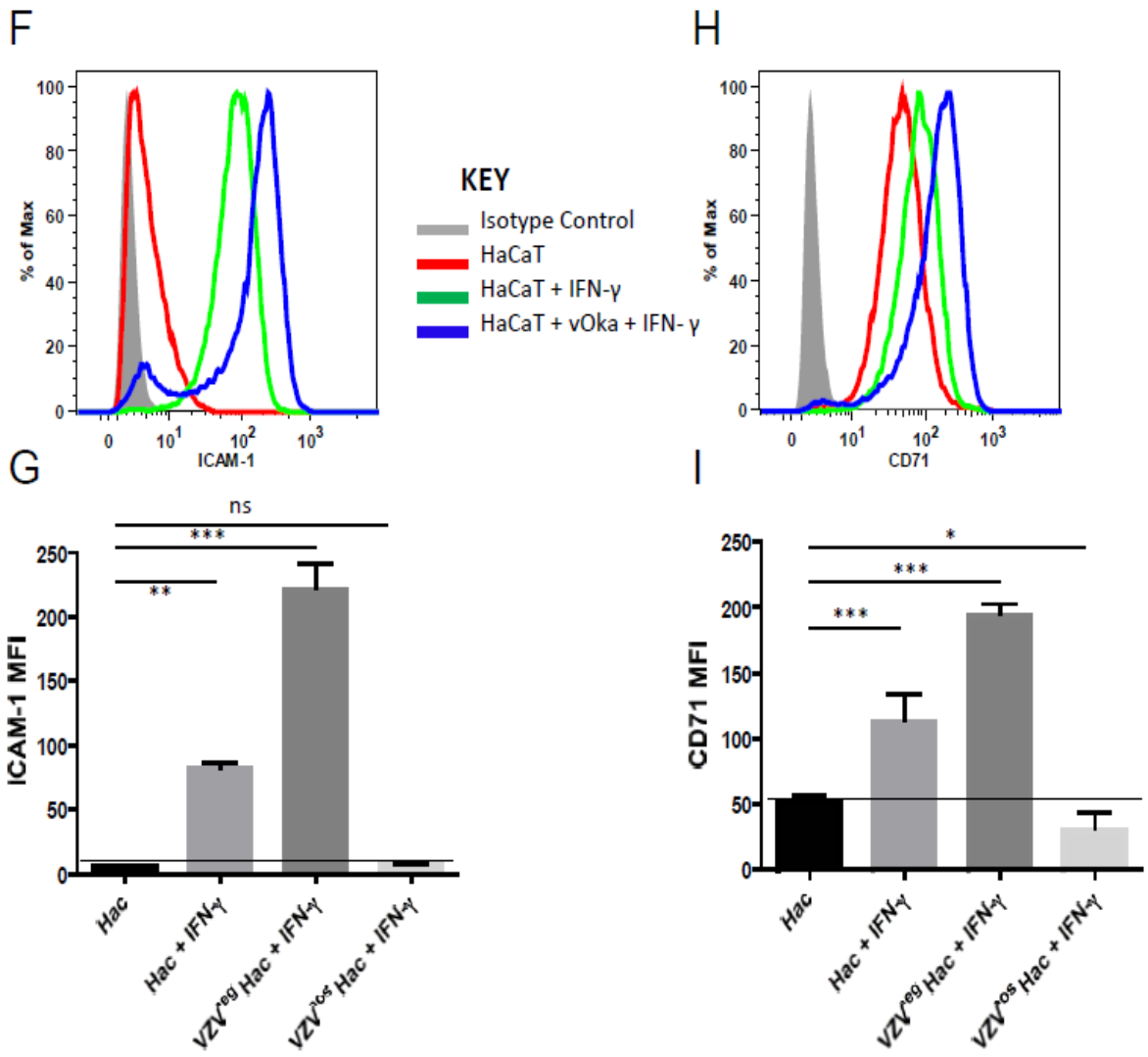


Figure 4.10: IFN-γ treatment up regulates the expression of MHC class Ia molecules on VZV negative keratinocytes but not on infected cells. vOka infected Keratinocytes (HaCaT cells) were treated with IFN-γ for 24 hours followed by surface staining for MHC Class Ia, ICAM-1 and CD71 expression. **A, F, H)** Expression profiles of HLA, ICAM-1 and CD71 molecules on mock (red) IFN-γ treated Mock infected (green) and IFN-γ treated vOka infected cells (blue). **B-E, G, I)** Summary graphs comparing levels of protein expression of VZV^{neg} and VZV^{pos} cells in IFN-γ treated mock and vOka infected cultures. Horizontal bars indicate baseline expression levels on mock infected cells. Statistical significance between groups was determined by (Ordinary one Way ANOVA for multiple comparisons using Tukey's test). A *$p<0.05$ was considered to be significant.

4.3.7 IFN-γ treatment of vOka infected keratinocytes synergistically up-regulates HLA-E expression on VZV negative cells. We assessed the expression patterns of the minor HLA class Ib molecules HLA-E, HLA-F and HLA-G following IFN-γ treatment after VZV infection. vOka infected HaCaT cells were treated with IFN-γ for 24 hours, surface stained for MHC class Ib proteins and analysed by flow cytometry. Our results indicate that neither VZV infection nor IFN-γ treatment induces HLA-F and HLA-G expression on keratinocytes **(Fig 4.11 C and D)**. However, just like HLA-A, B and C IFN-γ treatment of VZV infected cultures up regulated HLA-E expression on VZV negative cells but not VZV infected cells. Furthermore, just as shown for ICAM-1 and CD71, VZV infection seemed to augment IFN-γ mediated up regulation of HLA-E to levels above those on IFN-γ treated mock infected cells **(Fig 4.11 A)**. This observation suggests that VZV infection may differentially target different MHC class I molecules, or that IFN-γ mediated HLA-E up regulation on bystander cells is resistant to the inhibitory effects of the virus unlike HLA-A, B and C. Noteworthy is the fact that VZV infection alone is insufficient to induce HLA-E expression on keratinocytes but synergizes with IFN-γ to up regulate HLA-E expression.

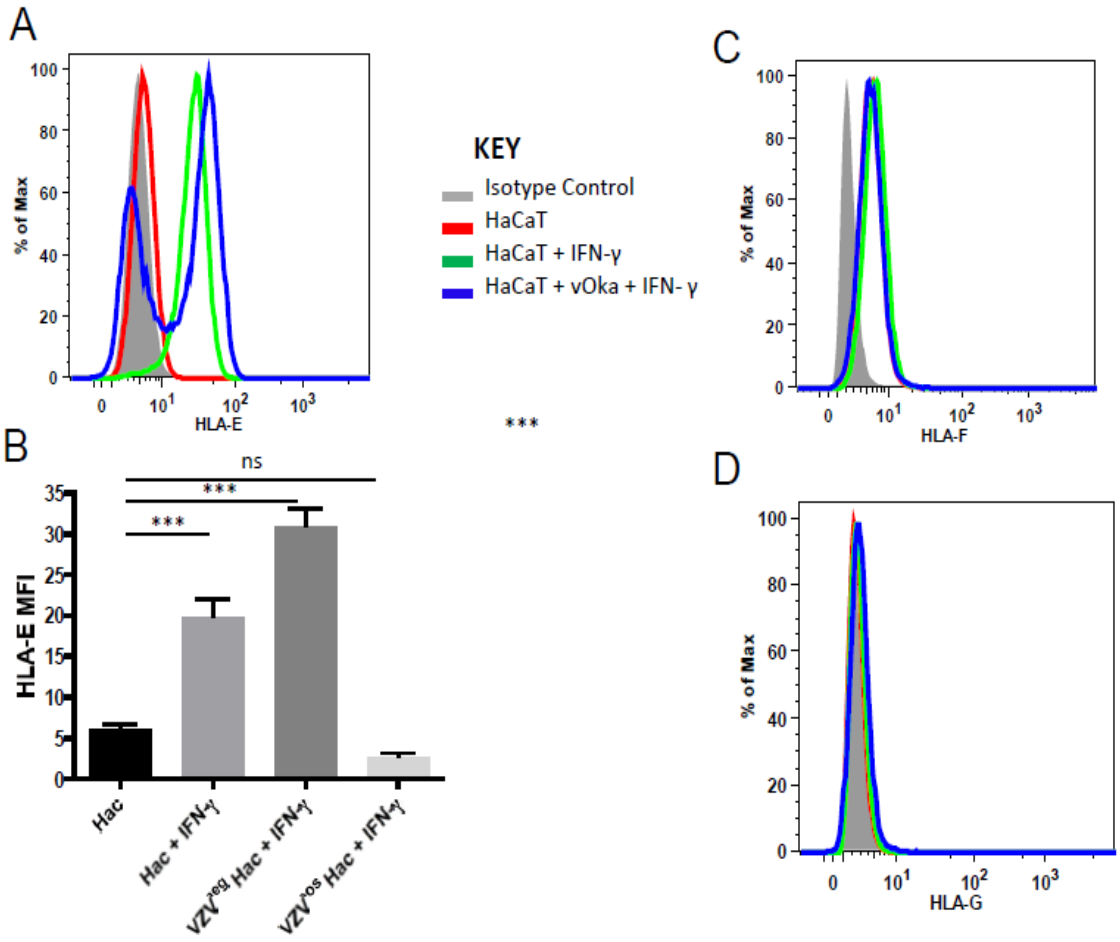


Figure 4.11: IFN-γ treatment up regulates the expression of HLA-E on VZV negative keratinocytes. vOka infected Keratinocytes (HaCaT cells) were treated with IFN-γ for 24 hours followed by surface staining for HLA-E, F and G expression. **A, C, D)** Expression profiles of MHC class Ib molecules on mock (red) IFN-γ treated Mock infected (green) and IFN-γ treated vOka infected cells (blue). **B)** Summary graph comparing levels of HLA-E expression of VZV^{neg} and VZV^{pos} cells in IFN-γ treated mock and vOka infected cultures. Horizontal bar indicates baseline expression levels on mock infected cells. Statistical significance between groups was determined by (Ordinary one Way ANOVA for multiple comparisons using Tukeys test). A *$p<0.05$ was considered to be significant.

4.4 Discussion

A central event in adaptive immunity is immune cell recognition through leukocyte interaction that is mediated by proteins encoded by the major histocompatibility complex (MHC). MHC encoded proteins are broadly classified as MHC class I, MHC class II and MHC class III molecules. These molecules are encoded within the cell, assembled in the endoplasmic reticulum (ER) in an elaborate process and eventually expressed on the cell surface as heterotrimeric complexes consisting of a membrane bound heavy chain (αchain), a light chain of $\beta_2$microglobulin (β_2m)and an antigenic peptide. Unlike their MHC class II counterparts, MHC class I molecules are expressed on the surface of all nucleated cells and serve to alert the immune system on whether a cell is infected or not (Hansen and Bouvier 2009). Antigenic peptides are crucial to MHC protein expression as they help stabilize the complexes. At steady state, these antigenic peptides are derived from proteosome-degraded intracellular proteins that form part of 'self'. In the context of a virus infection, virus derived proteins are degraded by the proteosome, forming part of the MHC peptide cargo which can then be recognised as 'non-self' by effector $CD8^+$ T cells leading to prompt elimination of the infected target cell through killing. Some MHC molecules also act as ligands for specific natural killer cell (NK) receptors, regulating their killing activity (Brooks, Borrego et al. 1999). The activity of any NK cell is controlled by the balance of inhibitory versus activating signals it receives. If activating signals override inhibitory signals, the target cell is killed. Conversely, if the cell receives inhibitory signals say by interacting with MHC class I ligands that bind to NK inhibitory receptors, the target cell is spared.

VZV down regulates cell surface MHC class I expression on T cells, keratinocytes and human fibroblasts (Cohen 1998; Abendroth, Lin et al. 2001; Black, Jones et al. 2009) possibly to escape $CD8^+$ T cell lysis. Conversely, HLA down regulation makes infected cells susceptible to NK cell lysis. It is not clear how VZV manages to escape NK cell lysis after MHC class I down modulation. We hypothesized that VZV might employ the dual $CD8^+$ T cell and NK escape mechanism employed by viruses such as HIV which down regulates only cell surface HLA-A and HLA-B but does not affect HLA-C and HLA-E (Cohen, Gandhi et al. 1999). Cell surface HLA-E is particularly important as it has been shown to bind the NK cell inhibitory receptors CD94/NKG2A, B and C (Borrego, Ulbrecht et al. 1998; Braud, Allan et al. 1998). The HLA-E-NKG2A interaction ultimately leads to inhibition of NK cell mediated killing, a mechanism that is exploited by HIV to escape NK cell lysis of virus infected cells.

Analysis of cell surface HLA expression after vOka infection of keratinocytes and pOka infection of MeWo cells reveals that VZV down regulates cell surface HLA-A and HLA-C but does not significantly affect HLA-B confirming that VZV might use the same mechanisms employed by HIV, targeting some MHC molecules while sparing others. Notable is the fact that HLA down regulation appeared to be restricted to the cells staining positive for VZV glycoproteins suggesting that intracellular virus replication is crucial for MHC class I down regulation.

To confirm whether this effect of VZV on HLA-B was universal, we analysed the expression of two HLA-B27 alleles on the surface of keratinocytes transduced with replication deficient lentiviral vectors. Results showed decreased HLA-B27 expression on VZV infected cells. This down regulation was limited to populations of cells expressing high levels of HLA-B27 suggesting that VZV's effect on HLA-B

may be allele specific so that non-transduced cells expressing only endogenous HLA-B*4001 and HLA-B*5101 alleles are unaffected while HLA-B27 alleles are down regulated. The allele specific effect of virus on HLA expression is not a new concept as the differential activities of HIV protein nef on HLA-A and HLA-B versus HLA-E and HLA-F have previously been attributed to differences in their cytoplasmic domains (Schwartz, Marechal et al. 1996; Cohen, Gandhi et al. 1999). As a consequence of this differential down regulation, the possession of certain HLA-B alleles such as HLA-B27, HLAB-51 HLA-B57, HLA-B58 and HLA-B81 is associated with slower progression to AIDS in HIV infected individuals possibly because of better cytotoxic T cell responses to HLA-B peptides (Kiepiela, Leslie et al. 2004; Stephens 2005; Goulder and Watkins 2008). A recent study has exploited the differences in cytoplasmic tails of HLA-A and HLA-B and reported that HLA-B may be more resistant to nef down regulation compared to HLA-A; accounting for better HLA-B restricted CTL responses and hence protection (Rajapaksa, Li et al. 2012). So far, there has been no association of HLA-B allotype/ genotype with disease outcomes in varicella or zoster infections. It is however possible that just like HIV, inability of VZV to down regulate HLA-B may skew T cell responses towards HLA-B derived peptides.

Analysis of intracellular MHC protein expression in sub-cellular fractions showed diffuse down regulation of all HLA alleles. HLA-B showed down regulation in membrane protein extracts, a finding that is inconsistent with HLA-B retention on the cell surface. A possible explanation for this discrepancy is that the membrane protein extract contained in addition to cell surface molecules, other HLA-B proteins from intracellular membranes such as those of the endoplasmic reticulum which may already be decreased. High molecular weight bands were also observed in HLA-B

profiles from VZV infected extracts analysed by western blot and seemed to intensify with increasing duration of infection **(Fig 4.7 A& B red arrows)**. We hypothesized that these bands represent HLA-B molecules in complex with VZV proteins. Further analysis to enumerate them revealed no viral proteins after conventional co-immunoprecipitation assays with W6/32 (data not shown). Analysis of silver-stained gel-extracted bands by mass spectrometry also failed to identify any proteins (data not shown). It is possible that the high molecular weight bands could also represent forms of HLA-B multimers that probably protect it from VZV down regulation. HLA-B allelic forms such as HLA-B27 have been shown to form dimers due to the presence of an unpaired cysteine within the peptide-binding groove, a factor that is associated with the pathogenesis of inflammatory conditions such as arthritis (Lenart, Guiliano et al. 2011; Makhadiyeva, Lam et al. 2011). Whether HLA-B27 dimerisation or the dimerisation of other HLA-B alleles can be induced by viral infections remains to be determined. Although our data point to possible HLA-B surface retention following VZV infection, further studies using other HLA-B alleles are still needed to shed light on the exact effect of varicella infection on cell surface HLA-B expression.

Cohen first reported that MHC down regulation only occurred post translation (Cohen 1998). Analysis of HLA mRNA expression in Mock and VZV infected keratinocytes revealed that VZV down regulates MHC class I gene transcription. Our findings contradict the findings of Cohen but may be explained by the fact that our experiments used allele specific primers to specifically amplify HLA-A, HLA-B and HLA-C genes from nascent RNA while Cohen's studies looked at global HLA-ABC protein expression using radioactive labeling. These findings suggest that VZV employs several mechanisms to down regulate MHC class I protein expression, first

targeting gene transcription and then MHC protein possibly through targeting into intracellular degradation compartments. Other studies have revealed that VZV interferes with MHC transport to the cell surface, leading to intracellular retention in the golgi compartment (Abendroth, Lin et al. 2001). The mechanism employed by VZV differs significantly from others identified for its alpha herpes virus counterpart HSV. HSV encodes a protein ICP47 which binds to and interferes with the transporter associated with antigen presentation (TAP) thus blocking transfer of degraded peptides from the cytoplasm to the ER for MHC assembly (Tomazin, Hill et al. 1996). Another human herpes virus HCMV, a gamma herpes virus also encodes a protein US6 which inhibits the function of TAP (Ahn, Gruhler et al. 1997). A clear understanding of the VZV gene products responsible for MHC down modulation may help shed light into the exact mechanisms involved. The VZV ORF66 protein kinase has been identified as one of the proteins mediating MHC down regulation, but others are also thought to be involved (Abendroth, Lin et al. 2001; Eisfeld, Yee et al. 2007). Other VZV gene products expressed in the Immediate Early and Early phases of gene expression are thought to be involved but still remain to be identified.

VZV infection has previously been shown to interfere with IFN-γ induced up regulation of MHC Class II expression on human fibroblasts and keratinocytes possibly to escape $CD4^+$ T cell surveillance (Abendroth, Slobedman et al. 2000; Black, Jones et al. 2009). We examined whether VZV infection or IFN-γ treatment would influence the expression of MHC class I molecules on keratinocytes. IFN-γ treatment was shown to increase the level of expression of all MHC class Ia molecules and ICAM-1. However, the expression of MHC class 1b molecules HLA-F, and HLA-G was not induced by IFN-γ despite increased expression of HLA-E.

When cells were first infected with the VZV vaccine strain vOka before IFN-γ treatment, up regulation of MHC and ICAM-1 occurred only on the VZV negative fraction of cells. This observation has previously been reported for ICAM-1 on infected keratinocytes by Black et al,. (Black, Jones et al. 2009). Next we analysed what effect IFN-γ would have on MHC and ICAM-1 expression on bystander uninfected cells in IFN-γ treated cultures. Up regulation of MHC class 1a expression on bystander cells by IFN-γ was significantly impaired but not absent. However, VZV infection did not affect ICAM-1 and HLA-E expression on bystander cells suggesting that VZV probably targets specific molecules on the MHC pathway. In fact, VZV infection seemed to augment IFN-γ up regulation of ICAM-1 and HLA-E expression to levels above those on mock infected IFN-γ treated cells. It is still not clear why VZV infection alone is insufficient to induce HLA-E expression but augments IFN-γ mediated up regulation of this molecule. Just as in MHC Class I protein down regulation, additional studies are needed to determine the mechanisms by which VZV interferes with IFN up regulation of MHC Class I expression. The product of ORF66 has been shown to block IFN signaling in human T cells (Schaap, Fortin et al. 2005) but whether it is involved in blocking IFN signaling that leads to HLA expression will remain to be determined. Whatever viral gene products are involved, it is clear that the ability to directly inhibit MHC expression as well as block IFN-γ signaling in human keratinocytes may provide VZV with a immune evasion advantage during viral replication in the skin. It is also likely that the selective up regulation of HLA-E and possibly cell surface retention of HLA-B could add to VZV's strategy to escape NK cell killing.

4.5 Conclusions

Our findings clearly suggest that VZV infection of keratinocytes down regulates cell surface MHC class I expression. Furthermore, it is possible that VZV targets MHC molecules, in an allele specific manner, targeting HLA-A and HLA-C but sparing HLA-B to escape from $CD8^+$ T cell and maintain NK cell inhibition. We observe that cell surface down modulation of HLA expression is consistent with decreased MHC gene expression and a reduction in intracellular MHC protein suggesting that VZV employs multiple mechanisms to subvert cell surface MHC expression. Analysis of the effects of IFN-γ on MHC expression reveals evidence of IFN-γ mediated MHC class 1a and HLA-E up regulation but not of HLA-F and HLA-G. Conversely, VZV infection interferes with MHC up regulation induced by IFN-γ treatment on infected cells but not on bystander VZV negative cells. A comparison with ICAM-1 expression reveals differential regulation as VZV infection seems to augment ICAM-1, CD71 and HLA-E up regulation by IFN-γ.

The findings discussed here not only identify new mechanisms of VZV mediated regulation of MHC expression, but open up new questions into the mechanisms underlying virus host interaction in the presence of inflammation when the pre-existing cytokine milieu such as high IFN-γ levels may influence the immune response. Our observations also have implications for vaccine design considering that the attenuated VZV vaccine strain vOka affects antigen presentation in a manner similar to the pathogenic wild type pOka strain, a factor that could account for poor vaccine responses in some individuals. We hope that these findings will reveal new insights on strategies to counter mechanisms of VZV immune evasion that interfere

with the antigen presentation pathway and stimulate the design of new prophylactic interventions that are resistant to virus subversion.

CHAPTER 5:

Isolation and characterization of VZV derived peptides bound to MHC class I molecules by Mass Spectrometry

5.0 Introduction

Antigens recognised by T cells are expressed as peptides bound to major histocompatibility complex (MHC) molecules. Both $CD8^+$ and $CD4^+$ T lymphocytes, which normally function as cytotoxic T lymphocytes (CTLs) and T helper (Th) cells, respectively, recognise pathogenic antigens through interactions between the T-cell receptor and pathogen derived peptides displayed by host human leukocyte antigen (HLA) class I and II molecules. In turn, these molecules are expressed on antigen-presenting cells (APCs). $CD8^+$ CTLs recognize HLA class I-presented peptides derived mainly from intracellular proteins and processed primarily by proteases in the cytosol, whereas $CD4^+$ T cells recognize HLA class II-presented peptides that are preferentially derived from the degradation of proteins accessing the endocytic pathway. Identification of HLA-associated, pathogen-derived, and potentially antigenic peptides recognized by T cells is a key to the development of peptide based vaccines. Technologies that allow the direct isolation and identification of peptide antigens associated with class I or II molecules have highlighted the ligand specificity of different MHC molecules and allowed direct identification of naturally processed and presented antigens derived from infectious micro-organisms as well as self-peptides associated with autoimmune disorders and cancers.

5.1 Isolation of MHC bound peptides

Several different approaches have been used to isolate MHC-bound peptides from cells, these include analysis of acidified cell lysates (Rotzschke, Falk et al. 1990; Falk, Rotzschke et al. 1991; Sijts, Neisig et al. 1996), elution of peptides from the cell surface (Storkus, Zeh et al. 1993; Storkus, Zeh et al. 1993), and immunoaffinity purification of the MHC-peptide complexes from detergent solubilized cell lysates (Falk, Rotzschke et al. 1991; Rammensee, Falk et al. 1993). Each approach has advantages, with the latter providing the best chance of epitope identification owing to the additional specificity of the immunoaffinity chromatography step and subsequent simplification of the range of cellular peptides isolated. However, they all share common features: (i) that upon cell lysis, peptides bound to MHC molecules (and other chaperones/receptors) are protected from intracellular and extracellular proteolysis; and (ii) acid treatment dissociates bound peptides from their MHC complexes. In the first approach, peptides are extracted from whole cell lysates following treatment with trifluoroacetic acid (TFA). The presence of TFA also aids in the precipitation of larger proteins leaving a complex mixture of intracellular and extracellular polypeptides, a proportion of which were bound to and protected from proteolysis by MHC molecules. Typically, these preparations are fractionated by reversed-phase high-pressure liquid chromatography (RP-HPLC) and screened with an immunological readout, such a T-cell assay to confirm the presence of a particular T-cell epitope or to quantitate known T-cell epitopes in different cell types (Rotzschke, Falk et al. 1990; Falk, Rotzschke et al. 1991; Sijts, Neisig et al. 1996).

An alternative to this method utilizes a non-lytic approach for recovering cell surface-associated peptides. The cells are washed in an isotonic buffer containing

citrate at pH 3.3, the acidic nature of this buffer facilitates dissociation of MHC-bound peptides from the cell surface without affecting cell viability (Storkus, Zeh et al. 1993). The great advantage of this technique is that the same cells may be harvested daily in an approach to obtain MHC-bound material over a time scale. Although the specificity of this process is a little better for MHC-bound material than whole cell lysates, some form of biological readout is generally necessary to locate peptides of interest prior to attempts at sequencing and characterization. The use of immunoaffinity chromatography dramatically improves the specificity of the peptide extraction process. The use of appropriate monoclonal antibodies can select a single MHC allele and some antibodies can even select a subpopulation of MHC molecules with defined molecular or functional properties (Urban, Chicz et al. 1994). The use of immunoaffinity chromatography to isolate specific MHC molecules provides the most effective approach for identifying individual peptide ligands restricted by a known MHC allele. Furthermore, in all the approaches discussed, the complexity of the eluates/lysates can be reduced by using cell lines that express reduced numbers of HLA alleles such as C1R cells which express very low levels of endogenous class I molecules, but support high-level expression of transfected class I molecules. This property makes these cells very attractive for examining endogenous peptides presented by individual class I alleles under normal physiological conditions or during infection (van Els, Herberts et al. 2000). This chapter explores methods for the direct isolation of MHC class I molecules and focuses on the use of immunoaffinity chromatography and the subsequent separation and identification of the bound peptide antigens by mass spectrometry.

5.2 Mass Spectrometry

Mass spectrometry is an analytical tool used for measuring the molecular mass of a sample. For large samples such as biomolecules, molecular masses can be measured to within an accuracy of 0.01% of the total molecular mass of the sample *i.e.* within a 4 Daltons (Da) or atomic mass units (amu) error for a sample of 40,000 Da. This is sufficient to allow minor mass changes to be detected, *e.g.* the substitution of one amino acid for another, or a post-translational modification. For small organic molecules, the molecular mass can be measured to within an accuracy of 5 ppm or less, which is often sufficient to confirm the molecular formula of a compound. Structural information can be generated using certain types of mass spectrometers, usually those with multiple analysers which are known as tandem mass spectrometers. This is achieved by fragmenting the sample inside the instrument and analysing the products generated. This procedure is useful for the structural elucidation of organic compounds and for peptide or oligonucleotide sequencing.

In this regard, mass spectrometry (MS) can play a central role in direct sequencing of the repertoire of HLA-associated peptides displayed by HLA molecules for recognition by T lymphocytes and hence represent vaccine candidates (Poland, Ovsyannikova et al. 2001). Microcapillary high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry was used to fractionate and sequence sub-picomolar amounts of peptides isolated from the MHC class I molecules, HLA.A, HLA-B and HLA-C. The sensitivity and speed of this approach should enhance the analysis of peptides from small quantities of virally infected and transformed cells as well as those associated with autoimmune disease states.

5.3 Mass Spectrometry Applications

5.3.1Epitope Identification to promote vaccine development

The idea of using an epitope-based approach for vaccine development is not new. $CD8^+$ and $CD4^+$ T-cell epitopes from defined pathogens may be predicted using computer generated HLA peptide binding algorithms and T-cell epitope mapping tools (Sidney, del Guercio et al. 1995; Sette and Fikes 2003; De Groot and Moise 2007). However, the extensive clinical implementation of epitope-based vaccines targeting the induction of specific B and T-cell-mediated immunity is hampered because naturally processed and HLA-presented pathogen derived peptides are currently known for only a limited number of pathogens (Tsomides, Aldovini et al. 1994; Tsai, Chen et al. 1996; Herr, Ranieri et al. 1999; van Els, Herberts et al. 2000; Johnson, Ovsyannikova et al. 2005; Meiring, Kuipers et al. 2005). Limitations in identifying HLA presented peptides include the difficulty in detecting pathogen-derived peptides eluted from HLA–peptide complexes. Of many viral peptides that can be displayed in the context of HLA molecules at the surface of infected cells, only some elicit measurable T-lymphocyte immune responses, a phenomenon known as immunodominance (Yewdell and Del Val 2004). A study by Tsai et al. (Tsai, Chen et al. 1996) identified a naturally occurring HBV peptide P2 isolated from the HLA class I molecules of HBV-infected liver cell membrane. This HBV-derived peptide, YVNVNMGLK, exhibited the activity of sensitizing target cells for lysis by HLA-A*11-restricted $CD8^+$ CTL. Several HLA class I epitopes eluted from immunoaffinity-purified HLA-A*0201 molecules have been identified by MS from Mycobacterium tuberculosis-infected cells (Flyer, Ramakrishna et al. 2002). More complex pathogens lead to an immune response that is more diverse, responding to a

large number of targets (Moutaftsi, Peters et al. 2006). Specifically, reports have pointed out how the identification of immunogenic T-cell epitopes, restricted by multiple HLA alleles, can be beneficial in the context of designing efficacious epitope-based vaccines. Studies of peptide binding capacity to HLA molecules have demonstrated the structural similarities between class I and II molecules (Perkins, Lai et al. 1989; Hickling, Fenton et al. 1990). Promiscuous peptides capable of binding both class I A2.1 and II DRB1 (DR1, DR5, and DR7) alleles have been identified that can elicit both class I and class II-restricted immune responses (Sette, Vitiello et al. 1991) by this approach. This is therefore a field of on-going research that holds hope for future vaccine developments.

5.3.2 Isotope labelling of epitopes

One of the approaches that has been developed to augment identification of molecules between two or more different conditions is labelling. Weinzierl and colleagues (Weinzierl, Lemmel et al. 2007) were the first to utilize labeled and unlabeled HLA associated peptides to show that there is no correlation between mRNA expression levels and epitope presentation on the cell surface. This work was performed on tumor associated antigens and to our knowledge, has not been performed in virus-infected cells. In an effort to elucidate HLA class I peptides derived from self-proteins and measles or respiratory syncytial virus infection, Meiring et al. (Meiring, Soethout et al. 2006) enriched cell media with isotopically labeled amino acids corresponding to the known major anchor residues at positions 2 and 9 of HLAA* 0201 and HLA-B*7 in a technique called SITE (stable isotope tagging of epitopes). The labeling scheme allowed discrimination of unaltered self-peptides from up regulated self-peptides in addition to the identification of HLA-

presented viral peptides. As an alternative form of isotopic labelling, other studies employed the use of isotope depletion of pathogen-derived proteins as a means of distinguishing pathogenic peptides from self peptides. Isotopic depletion has previously been described as an aid in determining the monoisotopic mass of larger biopolymers and simplifying the spectra from mixtures of intact proteins (Shi, Hendrickson et al. 1998; Jensen, Pasa-Tolic et al. 1999; Zubarev, Horn et al. 2000; Stump, Jones et al. 2003; Xiong, Schroeder et al. 2004). Peptides originating from any viral isotope-depleted proteins can be identified by their depleted isotope pattern. The down side to isotope depletion is that within 24 hours after infection, viral replication completely restores the naturally occurring isotope distribution. The SITE methodology utilized by Meiring et al. (Meiring, Soethout et al. 2006) overcomes this phenomenon by inserting the label at the time of infection. The approach of Meiring et al. involves direct incorporation of isotopically labeled amino acids in the media and subsequently the HLA-associated peptides themselves.

In contrast, Lemmel et al. (Lemmel, Weik et al. 2004) isotopically labeled HLA peptides after their isolation by first derivatizing lysine side chains with O-methylisourea hemisulfate. This uniformly derivatized all of the e-amino groups but left the N-terminal amino group intact. The N-terminal group was then nicotinylated with nicotinoyloxy succinimide. This derivatization scheme was shown to perform better than acetylation or a combination of guanidination and acetylation, as it retained the charge state of the peptides and improved recovery. Additionally, the derivatization was performed on a C18 microcolumn, thus avoiding loss of peptide that can occur when manipulating peptides in solution. The isotopic labeling was used by Lemmel et al. to compare the relative expression of peptides between two cell states i.e control versus infected.

Another important study added to our understanding of the identification of naturally processed HLA class II-presented meningococcal peptides recognized by CD4+ T lymphocytes (Meiring, Kuipers et al. 2005). The labelling strategy involved growing Neisseria meningitidis serogroup B in a medium containing either $^{14}NH_4Cl$ or $^{15}NH_4Cl$ as a nitrogen source to obtain uniformly labelled outer membrane vesicles. The authors then pulsed HLA-DRB1-positive immature dendritic cells with a 1:1 protein mixture of the 14N- and 15N-labeled outer membrane vesicles. After peptide isolation, the HLA class II meningococcal peptides appeared as doublets upon MS analysis. The outer membrane vesicle DRB1*0101-associated bound peptides were assigned to four neisserial porin A regions and their relative expression was quantified. These earlier labelling techniques set precedence to the development of more sophisticated labelling techniques to ensure efficient sample handling with minimal loss.

5.3.3 SILAC-Stable Isotope Labeling by amino acids in cell culture

SILAC was developed in CEBI by Ong and colleagues (Ong, Blagoev et al. 2002) as a simple and accurate approach for mass spectrometric (MS)-based quantitative proteomics. The method relies on the incorporation of amino acids with substituted stable isotopic nuclei (in this case heavy Lysines and Arginines (^{10}R and ^{8}K).

In SILAC, two groups of cells are grown in culture media that are identical except in one respect: the first media contains the ‘light’ and the other a ‘heavy’ form of a particular amino acid (for e.g. L-leucine or deuterated L-leucine). Through the use of essential amino acids (those not synthesizable by the cell-type) the cells are forced

to use the particular labelled or unlabeled form. Thus, with each cell doubling the cell population replaces at least half of the original form of the amino acid, eventually incorporating 100% of a given ‘light’ or ‘heavy’ form of the amino acid.

SILAC relies on metabolic incorporation of the quantitative label. Cells are encoded with the label as they grow in cell culture. All SILAC labeled proteins are therefore fully labeled, in contrast to chemical modification methods which may not have 100% labeling efficiency. This also means that SILAC-labeling of tissue samples is not possible. Primary culture of cells harvested from tissue in SILAC is possible. Alternatively, the use of the tissue sample as the natural stable isotope abundance sample (LIGHT) is a valid comparison with sample labeled with SILAC (HEAVY).

The SILAC approach neither requires additional purifications to remove excess labelling reagent, nor does it involve multi-step labelling protocols. Hence the labelling process is straightforward and highly efficient – 100% of the sample is available for analysis. Cells are cultivated in labelling media under typical cell culture conditions. Unlabeled and labelled samples can be combined prior to lysis of the cells and treated as a single sample in subsequent steps. This allows the experimenter to use any method of protein or even peptide purification (after enzymatic digestion) without introducing error into the final quantitative analysis. The amount of labelled protein in SILAC required for quantitative analyses is far less than in the case of chemical incorporation where a large excess of labelling reagent and sample is required to ensure an unbiased labeling reaction.

The comparison of the various MS-quantitation methods indicates the stage at which the two labeled states exist in the experiment. With labeled cells in SILAC, one can

proceed to do sub-cellular purification of organellar structures or multi-protein complexes whilst they exist in their native forms. The two samples can be combined as whole cells and a single subcellular preparation of nuclei, mitochondria etc. be performed. Any sample preparation bias introduced by the comparison of two separate preparation steps (as would be the case in a chemical modification method) would thus be avoided.

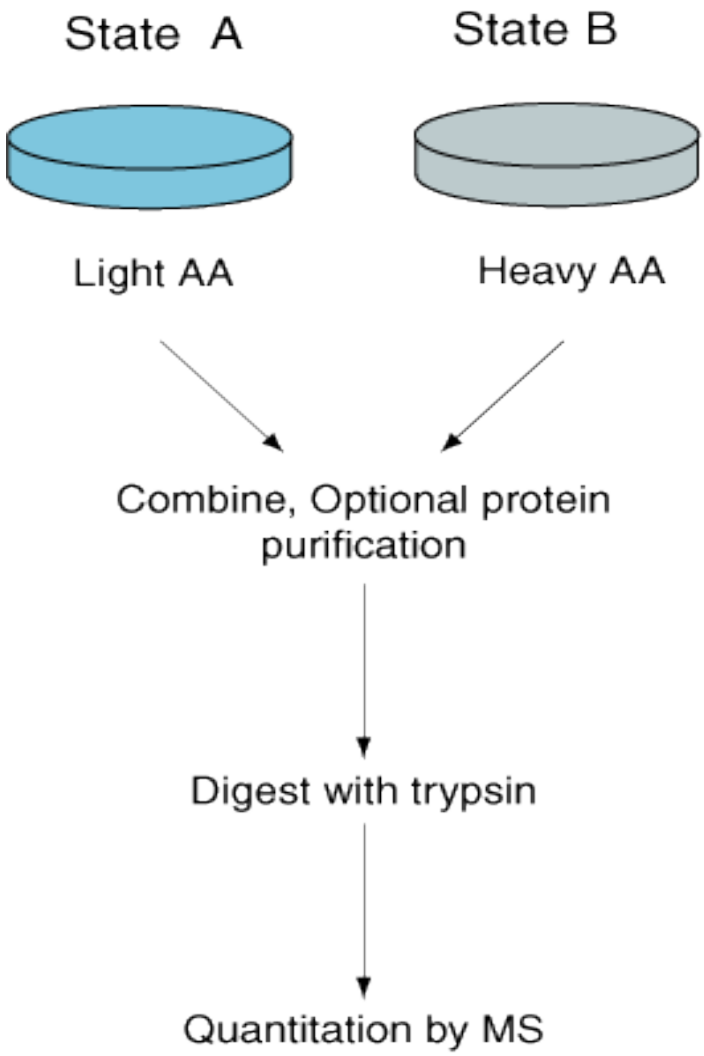


Figure 5.1: Diagramic representation of a simple typical SILAC experimental set up.

Adapted from [*http://www.cebi.sdu.dk/silac_intro.htm*](http://www.cebi.sdu.dk/silac_intro.htm)

5.4 Aims and Objectives

- To generate keratinocytes expressing differentially labeled Arginine and Lysine residues by SILAC

- To isolate MHC class I bound peptides from SILAC labeled vOka infected and uninfected keratinocytes by immunoprecipitaion using W6/32

- To identify and characterize MHC class I bound peptides by Mass Spectrometry

- To elucidate the proportion of VZV derived and host derived peptides presented during natural varicella infection of keratinocytes

5.5 Results

5.5.1 VZV infection increases the frequency of cell surface presented peptides.

We conducted an experiment to compare the abundance of presented peptides after 72 hour vOka infection of HaCaT cells compared to mock infected cells. Peptides were isolated from the cell surface by mild acid elution and filtered to exclude unwanted protein. Pure peptide fractions were then analysed on a mass spectrophotometer to sequence and identify individual peptides. Principal component analysis of the relative abundance of peptides when cells were grown in serum rich or serum free media in the absence or presence of infection revealed that VZV infection generally increases the frequency of cell surface peptides. Peptides from infected cells cluster to the right showing increased abundance compared to peptides from mock infected cells **(Fig 5.2 A)**. Furthermore, infection appeared to induce the presentation of some peptides which were absent in fractions from mock infected cells **(Fig 5.2 B)**. Also, more peptides were isolated when cells were grown in medium supplemented with serum than in serum free medium though the difference was only slight. We conclude that serum doesn't significantly influence the abundance of peptide presentation while VZV infection significantly increases the frequency of cell surface presented peptides.

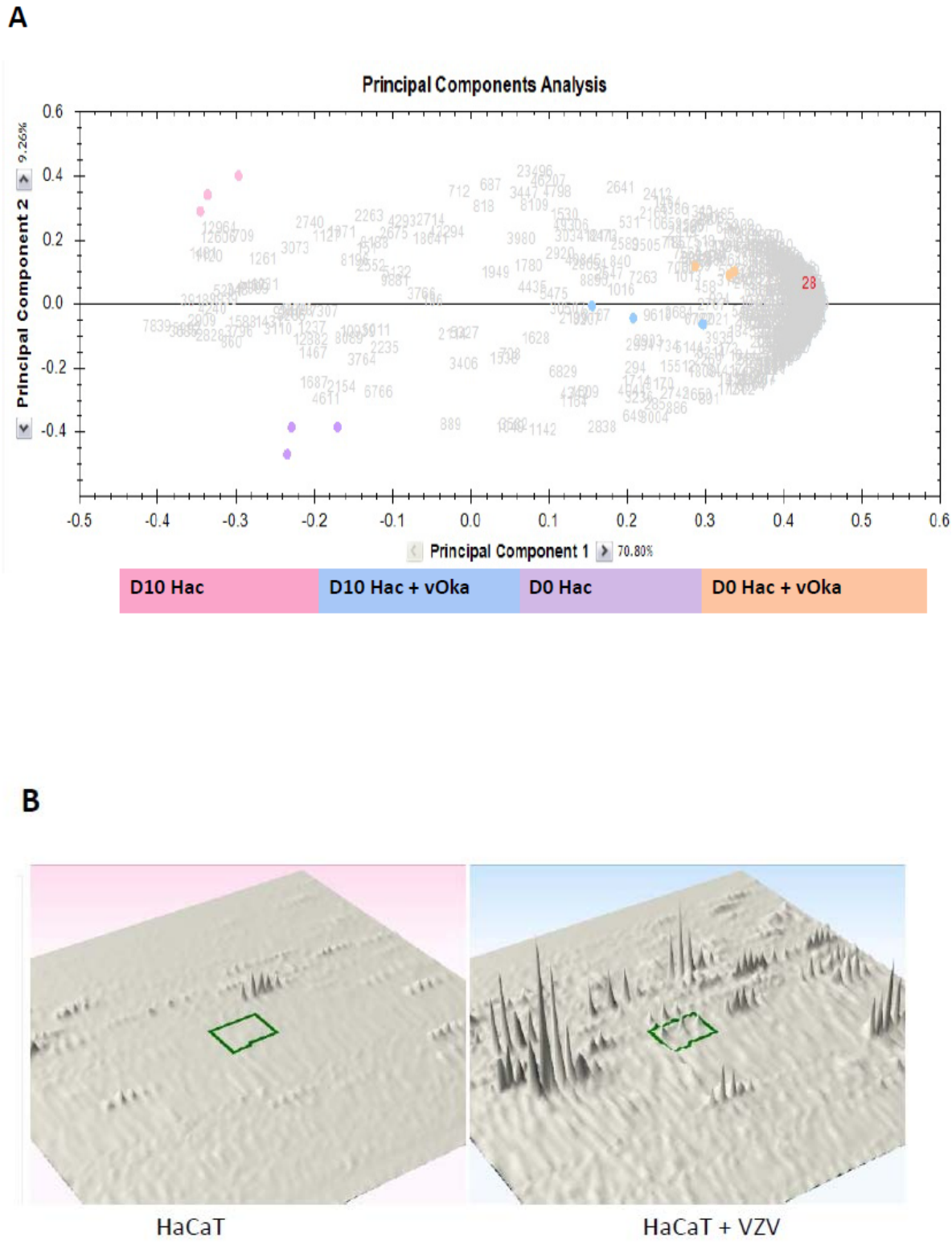
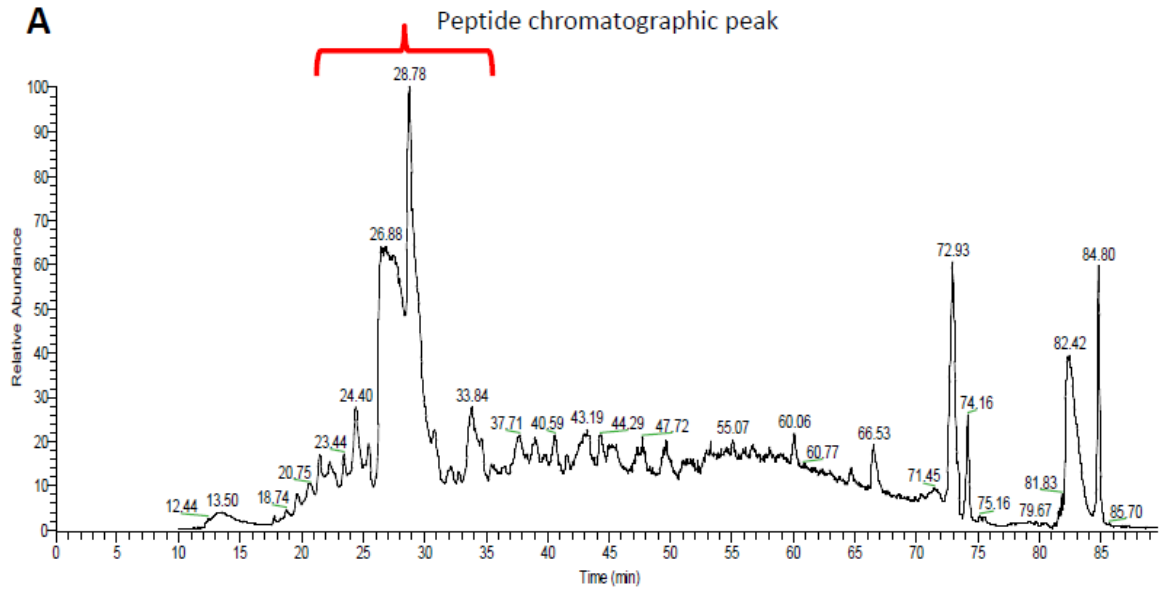


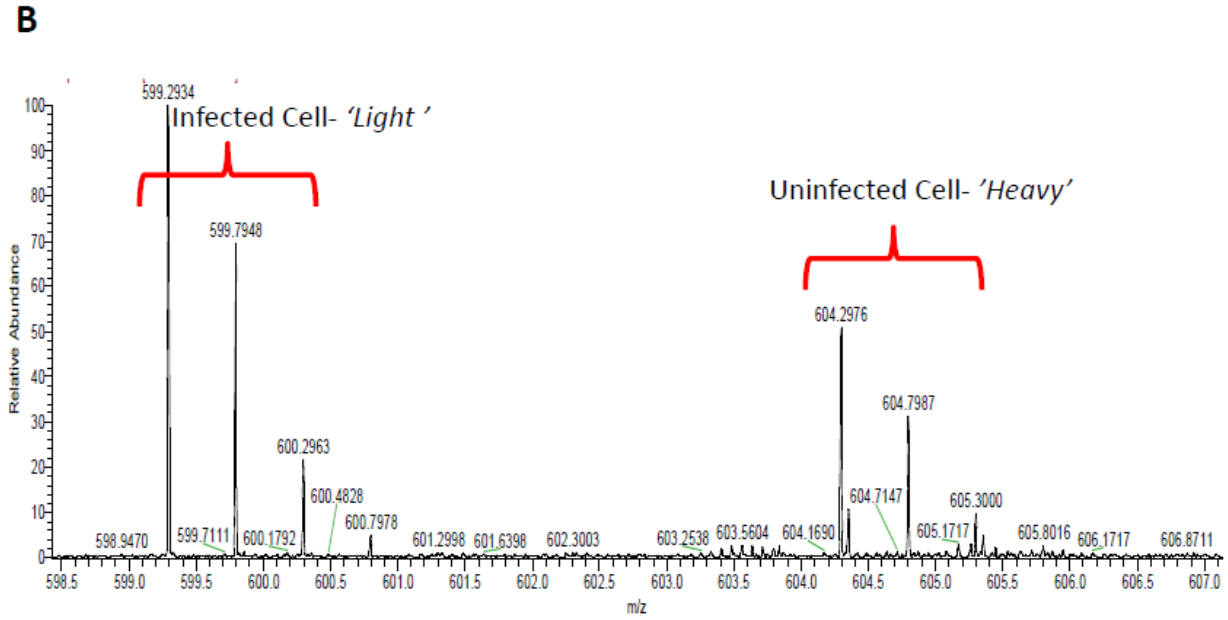
Figure 5.2: Increased presentation of host peptides during VZV infection. We analysed the abundance of cell surface presented peptides eluted from the surface of vOka infected and uninfected keratinocytes. **A)** Principal component analysis showing the distribution of

peptides from cells grown in the presence of serum (D10 Hac and D10 Hac + vOka) or in the absence of serum (D0 Hac and D0 Hac + vOka). **B)** Contour plot showing elevated frequency of presented peptides after VZV infection. Note induced presentation of peptides in vOka infected HaCaTs that are virtually absent in mock infected cells (green spot).

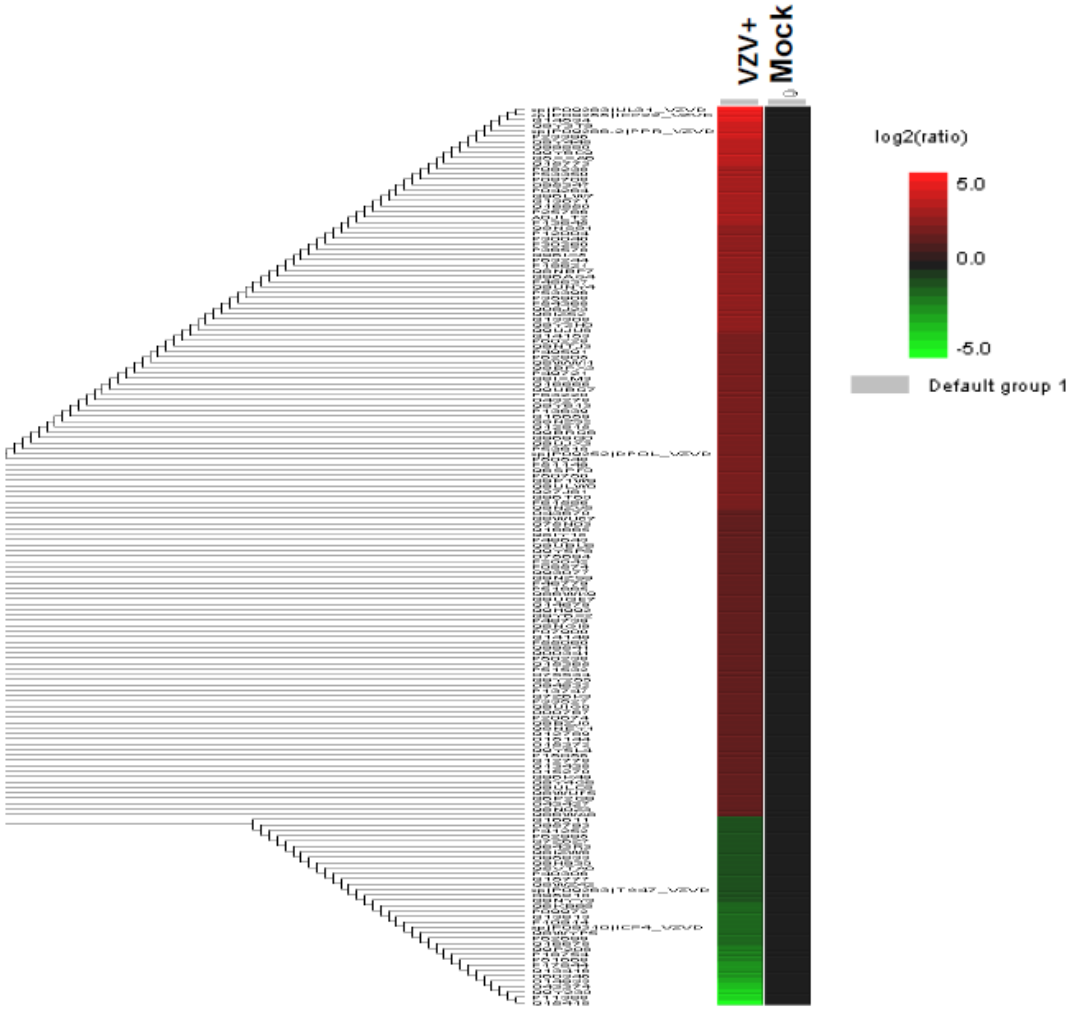
5.5.2 VZV infection increases the frequency of MHC class I bound peptides. In order to quantify the amount of peptides presented after VZV infection, we set up experiments to label mock infected cells by SILAC (Stable Isotope labeling by amino acids in culture). HaCaT cells were differentially labeled by growing them in 'light' medium containing normal Arginine and Lysine or in 'heavy' serum free medium supplemented with dialysed serum devoid of normal Lysine and Arginine. The two amino acids were then replaced with 'heavy' forms modified by addition of carbon atoms giving Arginine (R^{10}) and Lysine residues (K^{8}) residues which are 10 and 8 times heavier than the normal forms respectively. Cells were grown in these media for at least 9 rounds of division to allow incorporation of the modified amino acids into all their proteins. The cells grown in the 'light' medium were then infected with VZV strain vOka for 72 hours while the cells grown in 'heavy' medium were mock infected. After 72 hours both sets of cells were harvested and lysed separately. Cell lysates were then mixed at a ratio of 1:1 after which MHC class I molecules were immunoprecipitated using the pan HLA antibody W6/32. Peptides were then eluted from MHC molecules, sequenced and analysed by mass spectrometry. Because the samples were run against the same background, this method allowed effective quantitation of paired 'light' and 'heavy' peptides while avoiding confounding factors. **Fig 5.3A & B** show the presentation of a single peptide in two forms. The 'heavy' (uninfected cells) form appears at a later time in the chromatogram because it is heavier than the normal 'light' (infected cell) form and shows lower relative abundance compared to the 'light' form consistent with up regulation after VZV infection. Pairwise comparison of 'heavy' and 'light' peptides was performed using the analysis software PEAKS which compares identical

peptides and returns a value relative to the amount of specific peptide **(Fig 5.3C)** plus a p-value indicating whether the comparison was significant or not.(a PEAKS generated p-value < 0.001 was considered significant). SILAC labeling of 'heavy' fractions was validated by generating a heat map showing that presentation of 'light' peptides from 'VZV infected' cells was either increased or decreased when presentation of 'heavy' peptides was kept at a constant during analysis. VZV derived peptides segregated solely in the 'light' compartment further validating the labeling experiment **(Fig 5.3C)**. Data was converted into ratios of 'light':'heavy' and log transformed to assess the change in peptide presentation occurring after virus infection; that is whether presentation of individual peptides is increased or decreased. Over 4000 peptides were initially identified prior to analysis. Upon PEAKS analysis, peptides that could not be paired with sister peptides in 'labeled' and 'unlabelled' fractions to allow fro quantitation were excluded from further analysis. Furthermore, peptides that were not significantly altered were excluded from analysis. An examination of the regulation profile of peptides (~2500) whose presentation was significantly altered after VZV infection of HaCaTs, indicated that presentation of most host peptides is generally increased after VZV infection (~1800) while presentation of some (~700) is decreased **(Fig 5.3D)**. A list of all the peptides whose presentation was either significantly increased or decreased can be found in the Appendix **(Appendix B supplementary figures S1 & S2 respectively)**.


A
Peptide chromatographic peak
Relative Abundance
Time (min)
28.78
26.88
72.93
84.80
82.42
24.40
33.84
74.16
37.71
40.59
43.19
44.29
47.72
55.07
60.06
66.53
23.44
20.75
60.77
71.45
81.83
18.74
13.50
12.44
75.16
79.67
85.70


B
Infected Cell- 'Light '
Uninfected Cell- 'Heavy'
Relative Abundance
m/z
599.2934
599.7948
604.2976
604.7987
600.2963
600.4828
604.7147
605.3000
600.7978
598.9470
599.7111
600.1792
601.2998
601.6398
602.3003
603.2538
603.5604
604.1690
605.1717
605.8016
606.1717
606.8711

C



D

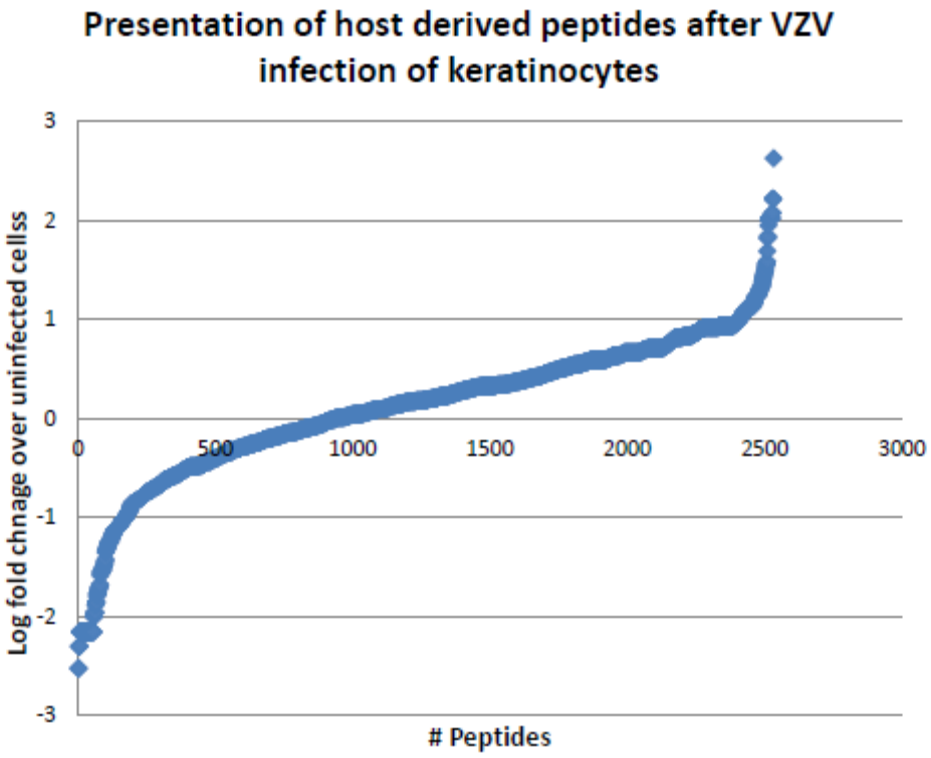
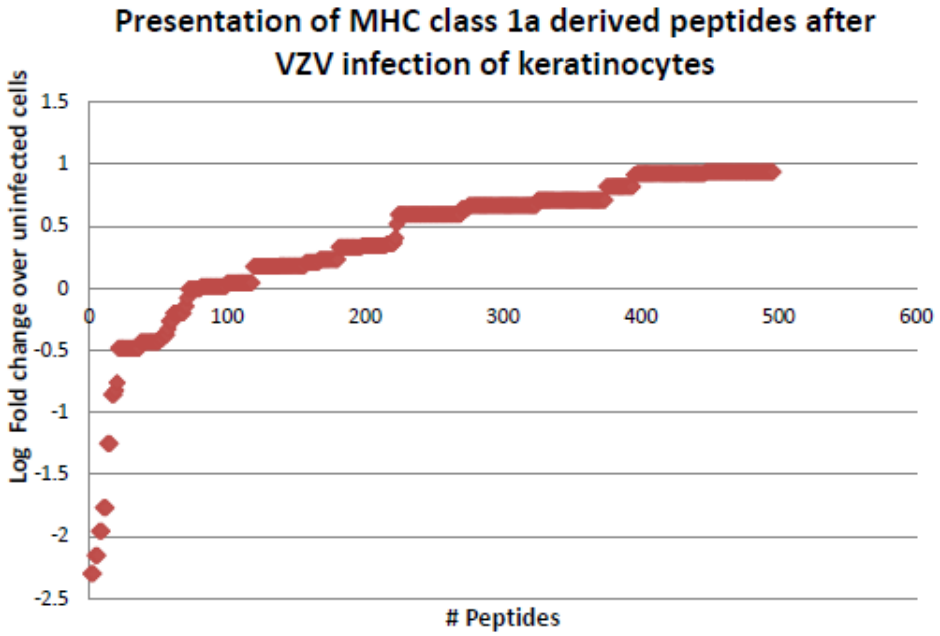


Figure 5.3: Presentation profile of peptides from SILAC fractions

A) A representative spectrum from mass spectrometry analysis of peptides showing a typical chromatographic peak **B)** Mass spectrum at a single time point showing elucidation of a peptide present in 'heavy' and 'light' forms and seems to be upregualted in infected cells. **C)** Heat Map diagram showing the distribution of VZV and host derived peptides in 'light' (VZV+) and 'heavy' (Mock infected) fractions after analysis of SILAC samples using PEAKS software. Analysis was performed to show how peptides containing 'heavy' or 'light' residues segregate in the mock versus vOka infected cells. **D)** Log transformed (Light/Heavy) ratios showing how host peptide presentation is regulated after vOka infection.

5.5.3 A significant number of MHC class Ia bound host peptides are derived from MHC proteins. After determining that VZV infection increases the frequency of MHC class I bound peptides, we examined the protein sources of the sequenced peptides and discovered that a significant number of peptides are derived from MHC class 1a proteins. Out of over 4000 peptides initially identified by mass analysis, about 500 were derived from MHC class Ia and class Ib proteins. Furthermore, the frequency of these MHC derived peptides was significantly increased in the 'light' fraction, suggesting that VZV infection increases the presentation of MHC class 1a derived peptides (~400) **(Fig 5.4 A)** while decreasing presentation of a small number (<100). In line with earlier observations that VZV decreases the amount of intracellular MHC protein and reduces expression of cell surface MHC class I molecules **(Chapter 4)**, it is possible to speculate that vOka infection of cells down modulates MHC expression by promoting the intracellular degradation of MHC proteins. When we assessed the amount of MHC protein using Ingenuity Pathway Analysis (IPA), we found that the level of cellular MHC protein was generally decreased after VZV infection compared to other host proteins stressing the possibility of a decreased intracellular protein reservoir. There was differential levels of MHC allelic proteins with high levels of HLA-B, HLA-C, HLA-E and HLA-G (pink) and low levels of HLA-A and HLA-F (green) **(Fig 5.4 B)**. In summary, VZV infection seems to decrease the presentation of MHC derived peptides compared to other host peptides. These observations match earlier findings that VZV reduces intracellular MHC protein possibly due to reduced gene expression and or MHC protein degradation.

A


Presentation of MHC class 1a derived peptides after VZV infection of keratinocytes
Log Fold change over uninfected cells
1.5
1
0.5
0
-0.5
-1
-1.5
-2
-2.5
0
100
200
300
400
500
600
Peptides

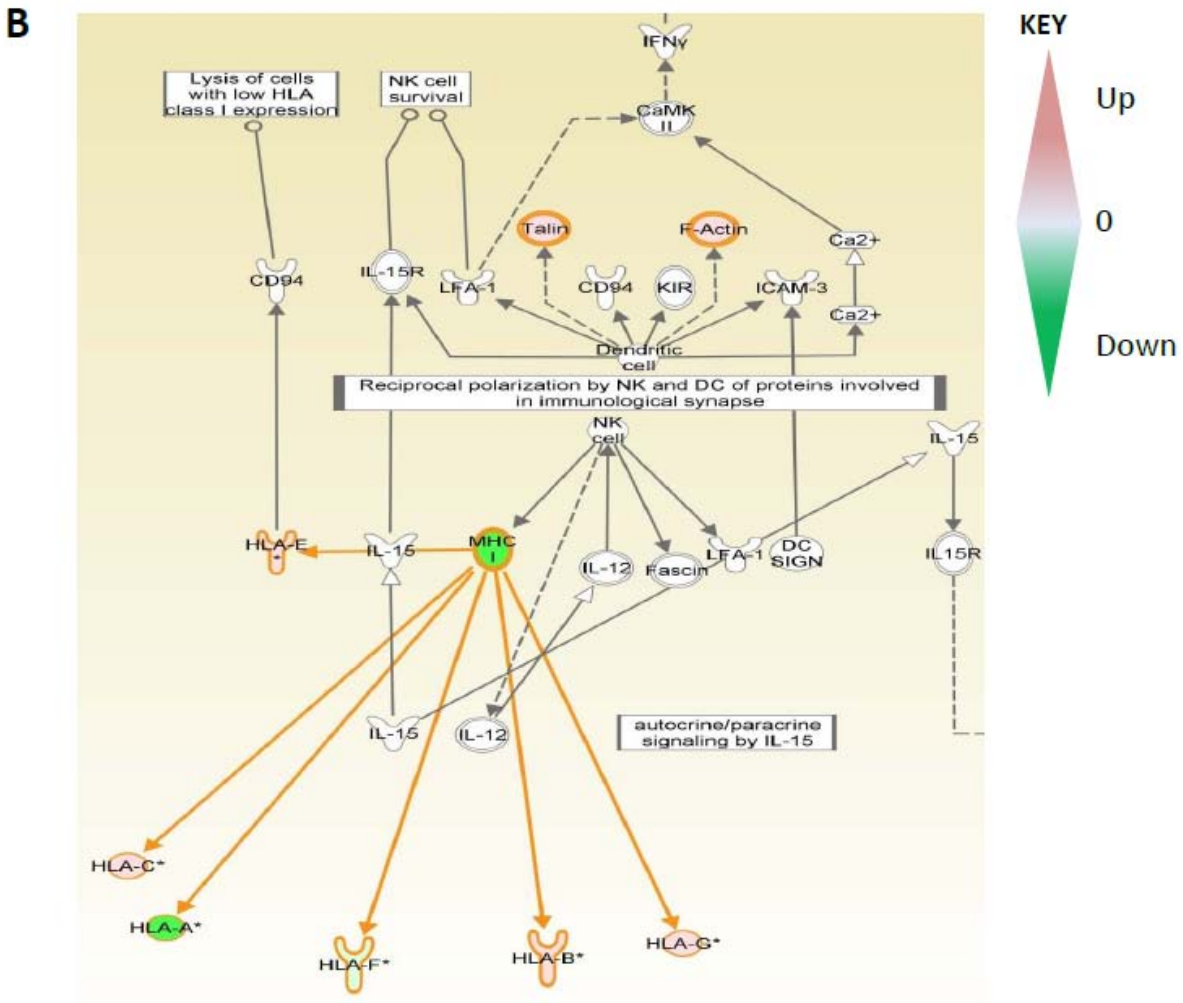


Figure 5.4: Presentation of MHC protein derived peptides after VZV infection.

A) Log adjusted level of peptide presentation over mock infected cells showing increased presentation of most MHC derived peptides. **B)** Ingenuity Pathway Analysis showing the intracellular levels of MHC derived proteins/peptides compared to peptides from other host proteins. The level of proteins/ peptides from MHC molecules was generally low compared to other host proteins. Proteins/peptides from HLA-A and HLA-F were less abundant (green) while proteins/peptides from HLA-B, HLA-C, HLA-G and HLA-E were increased (pink) after vOka infection.

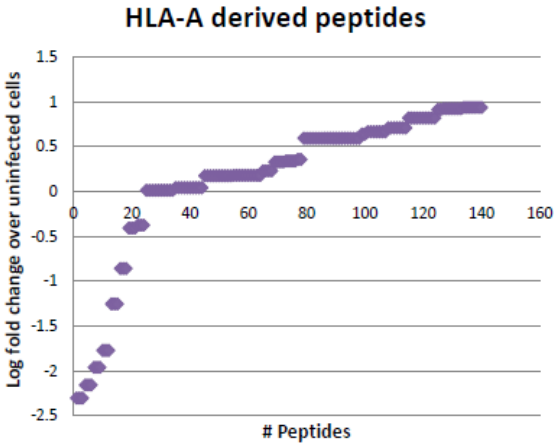
5.5.4 A small number of MHC peptides are derived from leader sequences.

Next we analysed the (~400) peptide sequences derived from MHC proteins. About 140 of these were derived from HLA-A, 210 from HLA-B and 160 from HLA-C. **Figures 5.5 A, C, E** show regulation of peptide presentation from the three alleles respectively. Quantitative analysis of the (~400) peptides derived from these alleles found that only 50 were significantly altered based on a PEAKS generated p-value of 0.001 cut off. **Fig 5.5 Tables B, D & F** show the sequences from HLA-A, HLA-B and HLA-C that were significantly down regulated (lighter section of the table) or significantly up-regulated (darker section of table) after VZV infection. Sequence alignment of the significantly altered peptides revealed that some of them were derived from MHC leader/ signal sequences; these are marked red on **Tables B, D & F**. Many of the identified signal sequence peptides generally showed increased presentation after vOka infection except for one leader sequence from HLA-B; **VTAPRTVLLLL** whose presentation was decreased. Also, there was significant sequence similarity between the signal sequence derived peptides among the HLA allelic forms sometimes differing by just a single amino acid or two. The identification of these leader sequences is of particular interest since they have been shown to bind to the minor MHC class Ib molecules HLA-E with hydrophobic anchor residues at positions 2 and 9 and secondary anchor residues at position 3 and 7 (Braud, Yvonne Jones et al. 1997). Braud et al. in their study tested the HLA-E binding capacities of VMAPRTLVL and VMAPRTVLL derived from the HLA-A*0201 and HLA-B*0801 signal sequences respectively. Our findings show the presence of signal 9mer peptides with a similar backbone: VMAPRTLLL from HLA-A, VMAPRTLIL and VTAPRTVLLLL from HLA-B and VMAPRTLVL and

VMAPRTVLL from HLA-C bearing the right hydrophobic anchor residues at positions 2 (Methionine) and 9 (Leucine) suggesting that these peptides are likely to bind HLA-E. Not all leader sequences can bind and stabilize HLA-E; binding to which is sensitive to variations in the leader sequences (Brooks, Borrego et al. 1999). It is not clear whether the signal sequence from HLA-B VTAPRTVLLLL with only an anchor residue at pos 9 and none at pos 2 can also bind HLA-E. In the light of earlier finding that VZV augments IFN-γ mediated up regulation of HLA-E, it is likely that this effect of VZV in the context of an inflammatory response would increase cell surface expression of HLA-E and promote generation of HLA-E binding leader sequences. The combined effects of cytokine and virus could therefore promote escape from NK Cell lysis by increasing leader sequences that stabilize cell surface HLA-E ligands for NK inhibitory receptor CD94/NKG2A (Braud, Allan et al. 1998). This would promote viral immune evsion by avoiding both $CD8^+$ and NK cell lysis.

Surprisingly, most MHC derived peptides came from HLA-B which is inconsistent with the earlier finding that cell surface HLA-B expression seemed to be spared from VZV mediated down regulation. Increased presentation means increased degradation and so would mean reduction in the level of HLA-B. Since this is not the case and following that VZV globally reduced intracellular protein, cell surface retention of HLA-B may be independent of intracellular protein processing.

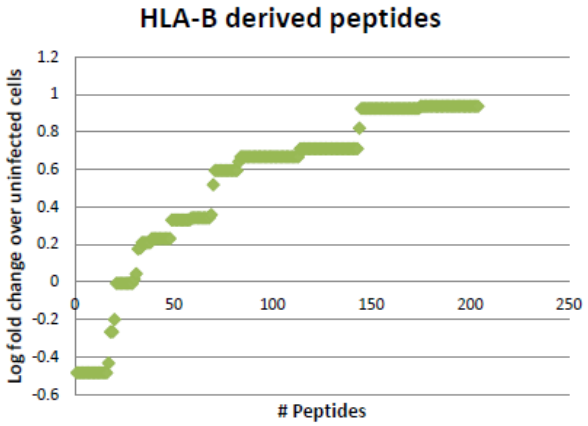
A



B

Significantly regulated HLA-A derived peptides		
#	Peptide sequence	Peptide length
1	GSHSMRYFTTSVSRPGR	17mer
2	- -HSMRYFTTSVSRPGR	15mer
1	VMAPRTLLL	9mer
2	-MAPRTLLL	8mer
3	AAVVVPSGQEQRYT	14mer
4	AAVVVPSGQEQR- -	12mer
5	AAVVVPSGEEQRYT	14mer
6	AAVVVPSGEEQRY-	13mer
7	AAVVVPSGEEQR- -	12mer
8	GAMFAGAVVAAVR	13mer
9	EAARVAEQLRA	11mer
10	YAYDGKDYIAL	11mer
11	- - - - - - - - - - -VSRPGR	6mer

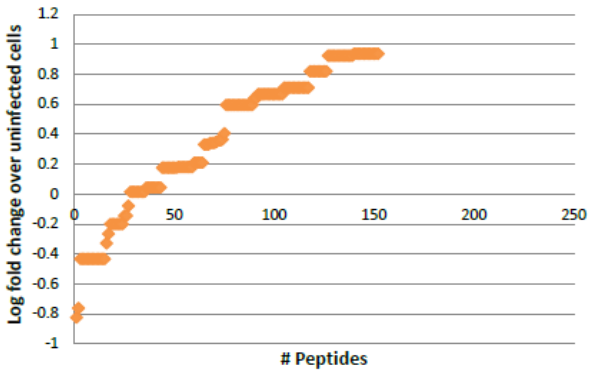
C



D

Significantly regulated HLA-B derived peptides		
#	Peptide sequence	Peptide length
1	GSHSMRYFYTAMSRPGR	17mer
2	SGGKGGSCSQAA	12mer
3	TAADTAAQISQRK	13mer
4	RYFYTAVSR	9mer
5	VTAPRTVLLLL	11mer
1	VMAPRTLIL	9mer
2	- MAPRTLIL	8mer
3	AAVVVPSGQEQRYT	14mer
4	AAVVVPSGQEQR- -	12mer
5	AAVVVPSGEEQRYT	14mer
6	AAVVVPSGEEQRY -	13mer
7	AAVVVPSGEEQR - -	12mer
8	ADKAAQITQR	10mer
9	EAARVAEQLRA	11mer 11mer
10	YAYDGKDYIAL	9mer
11	FAYDGKDYI - -	9mer
12	- - - - - - - - - - -VSRPGR	6mer

E



F

Significantly regulated HLA-C derived peptides		
#	Peptide sequence	Peptide length
1	GSHSMRYFYTAVSRPGR	17mer
2	- - - - - --RYFYTAVSR - - -	9mer
3	GSHSLRYFSTAVSRPGR	9mer
4	- - - - --RYFSTAVSR - - -	13mer
5	TAADTAAQISQRK	13mer
6	SGGKGGSCSQAA	12mer
1	VMAPRTLIL	9mer
2	- MAPRTLIL	8mer
3	VMAPRTLLL	9mer
3	AAVVVPSGQEQRYT	14mer
4	AAVVVPSGQEQR- -	12mer
5	AAVVVPSGEEQRYT	14mer
6	AAVVVPSGEEQRY -	13mer
7	AAVVVPSGEEQR - -	12mer
8	YAYDGKDYIAL	10mer
9	FAYDGKDYI - -	11mer
10	FAYDGKDYLTL	11mer
11	TAADTAAQISHRK	13mer
12	- - - - - - - - - - -VSRPGR	6mer

Figure 5.5: Some MHC protein derived peptides originate from leader sequences.

The sequences of the identified MHC peptides were mapped to the proteins through a BLAST search and linked to source proteins. Most of the MHC peptides were derived from signal sequences. **A, C, E)** Regulation of MHC class I presented peptides derived from HLA-A, HLA-B and HLA-C respectively by VZV infection. Infection generally increases presentation of MHC derived peptides. **Tables B, D, F)** HLA-A, HLA-B and HLA-C derived peptide sequences whose presentation was significantly increased (darker sections on the tables) or decreased (lighter sections of the tables) after VZV infection. Signal sequences on the tables are marked **red.** Presentation of the leader sequence peptides was generally increased after infection.

5.5.5 Most varicella derived MHC class I associated peptides are derived from structural proteins. Differential labeling of HaCaT cells before mass spectrometry using SILAC allowed us to isolate upto 21 VZV derived peptides **(Fig 5.6 C)** unlike cell surface acid elution which returned only 3 VZV peptides **(Fig 5.6 A)** and W6/32 immunoprecipitaion which returned 6 peptides **(Fig 5.6 B)**. When these VZV derived peptides were subjected to sequence to protein matching, most were found to be derived from VZV structural proteins. There were also peptides from proteins involved in virion assembly and nuclear egress as well as some Immediate Early (IE) proteins. **Fig 5.6 E** shows some key host proteins whose peptides were significantly regulated after VZV infection.

A

Cell Surface Acid Elution	
Peptide Sequence	**Homologous Protein**
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18	
R A G K S L E D N P W L H E	Envelope glycoprotein I/ORF67
A T I R E E S P P H S V V N P F V K	Envelope glycoprotein I/ORF67
- - I R E E S P P H S V V N P F V K	Envelope glycoprotein I/ORF67

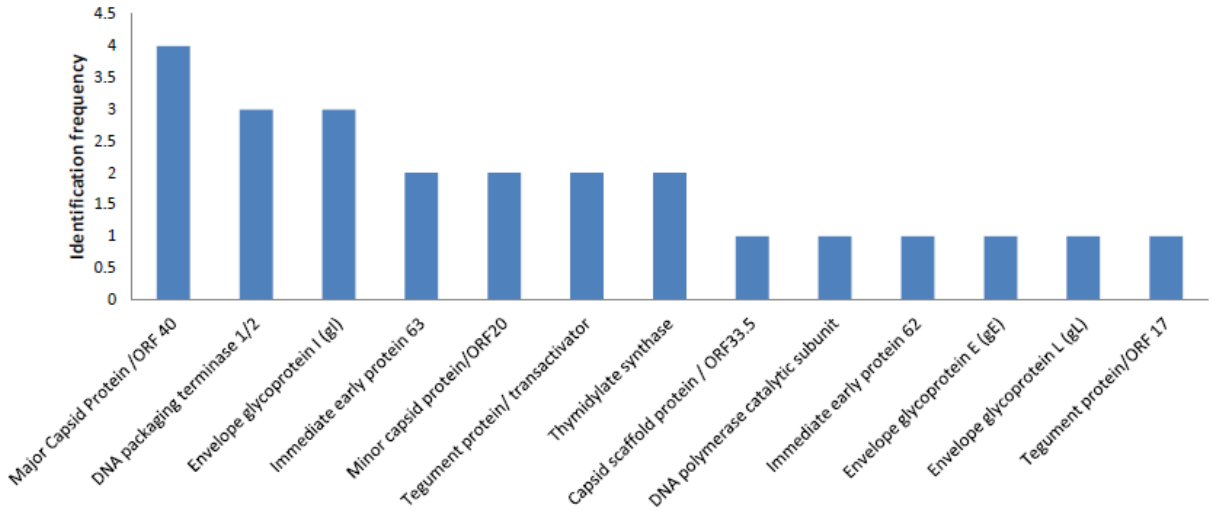
B

W6/32 Immunoprecipitation										
Peptide Sequence										Homologous Protein
1	2	3	4	5	6	7	8	9	10	
G	E	T	G	A	D	E	V	H	L	Major Capsid Protein
Y	A	A	P	P	V	S	N	L	-	Envelope glycoprotein L
V	A	M	P	V	S	T	D	L	-	Major Capsid Protein
V	E	D	I	N	R	V	F	L	-	Immediate early protein 63
L	E	K	A	P	P	L	-	-	-	Major Capsid Protein
A	R	A	A	L	L	-	-	-	-	Major Capsid Protein

C

W6/32 Immunoprecipitation after SILAC													
Peptide Sequence													Homologous Protein
1	2	3	4	5	6	7	8	9	10	11	12	13	
R	T	L	G	E	E	S	V	R	Y	F	E	R	DNA packaging terminase subunit 1
K	Q	K	S	T	V	F	L	V	P	R	-	-	DNA packaging terminase subunit 1
L	S	R	P	P	S	A	Y	I	N	R	-	-	DNA polymerase catalytic subunit
L	P	Y	N	A	G	K	M	Y	V	-	-	-	Tegument protein and transactivator
G	E	T	G	A	D	E	V	H	L	-	-	-	Major Capsid Protein
Y	A	A	P	P	V	S	N	L	-	-	-	-	Envelope glycoprotein L
V	A	M	P	V	S	T	D	L	-	-	-	-	Major Capsid Protein/ORF 40
V	E	D	I	N	R	V	F	L	-	-	-	-	Immediate early protein 63
D	A	A	P	A	I	Q	H	I	-	-	-	-	Envelope glycoprotein E
D	P	Y	M	V	I	N	T	L	-	-	-	-	Tegument protein/ ORF 17
F	A	Q	P	P	N	T	A	L	-	-	-	-	DNA packaging terminase subunit 2
G	E	L	Q	Y	L	K	Q	V	-	-	-	-	Thymidylate synthase
H	D	I	P	P	P	H	G	V	-	-	-	-	Immediate early protein 63
K	V	K	V	P	L	P	A	R	-	-	-	-	Immediate early protein 62
N	A	S	E	H	H	P	E	L	-	-	-	-	DNA packaging protein
R	V	S	P	S	Y	W	Q	R	-	-	-	-	Capsid scaffold protein
T	A	I	W	H	H	M	I	L	-	-	-	-	Tegument protein and transactivator
Y	V	I	F	P	G	K	S	V	-	-	-	-	Nuclear egress lamina protein
I	E	H	A	G	S	I	V	L	-	-	-	-	Capsid protein/ ORF 20
D	A	N	R	T	A	I	V	-	-	-	-	-	Capsid protein/ ORF 20

D



E

Peptide	**Function**	**Relative abundance on VZV infected cells**
Anti-microbial peptides	Potent against bacteria and other pathogens and mediate protection from pathogens at epithelial sites	Increased
Psoriasin	A chemotactic inflammatory protein for CD4+ lymphocytes and neutrophils	Increased
Macrophage migration inhibitory factor	Inhibits macrophage migration to allow surveillance	Increased
Translation initiation factor 2	Inhibits initiation and translation	Increased

Figure 5.6: Identification of protein sources of VZV derived MHC class I associated peptides.

The sequences of the identified MHC peptides were mapped to the proteins through a BLAST search and linked to source proteins. Most of the VZV derived peptides were derived from structural proteins. **A)** VZV derived peptide sequences from cell surface acid elution were derived from glycoproteins. **B)** VZV peptides from W6/32 immunoprecipitation were mainly derived from capsid proteins. **C)** VZV peptides from SILAC labeled lysates were mainly derived from structural proteins and proteins associated with virion assembly. **D)** Summary of the frequency of identified peptides and their protein sources. **E**) A table showing some key identified host peptides that have a role in the immune response. The relative abundance of each peptide after VZV infection is indicated on the third column.

5.5.6 Global cellular protein levels after VZV infection correlate with the presentation of MHC class I bound peptides. After the observation that VZV infection increases the frequency of presentation of host peptides on MHC class I molecules, we hypothesized that the most presented peptides were probably derived from abundant proteins. In an attempt to determine whether the level of intracellular protein governs peptide presentation, we sequenced and quantified proteins from SILAC labeled cell lysates. We successfully quantified just over 1300 proteins whose levels were either decreased (~300) or increased (~1000) after VZV infection **(Fig 5.7 A)**. There was a general increase in intracellular protein level, possibly induced by inflammatory cytokine signaling **(Fig 5.7 B)**. However, we failed to identify any real correlation between the frequencies of selected VZV or host derived peptides with their intracellular levels. This observation implies that the factors that govern MHC class I peptide presentation are probably tied to MHC and antigen processing than intracellular protein availability.

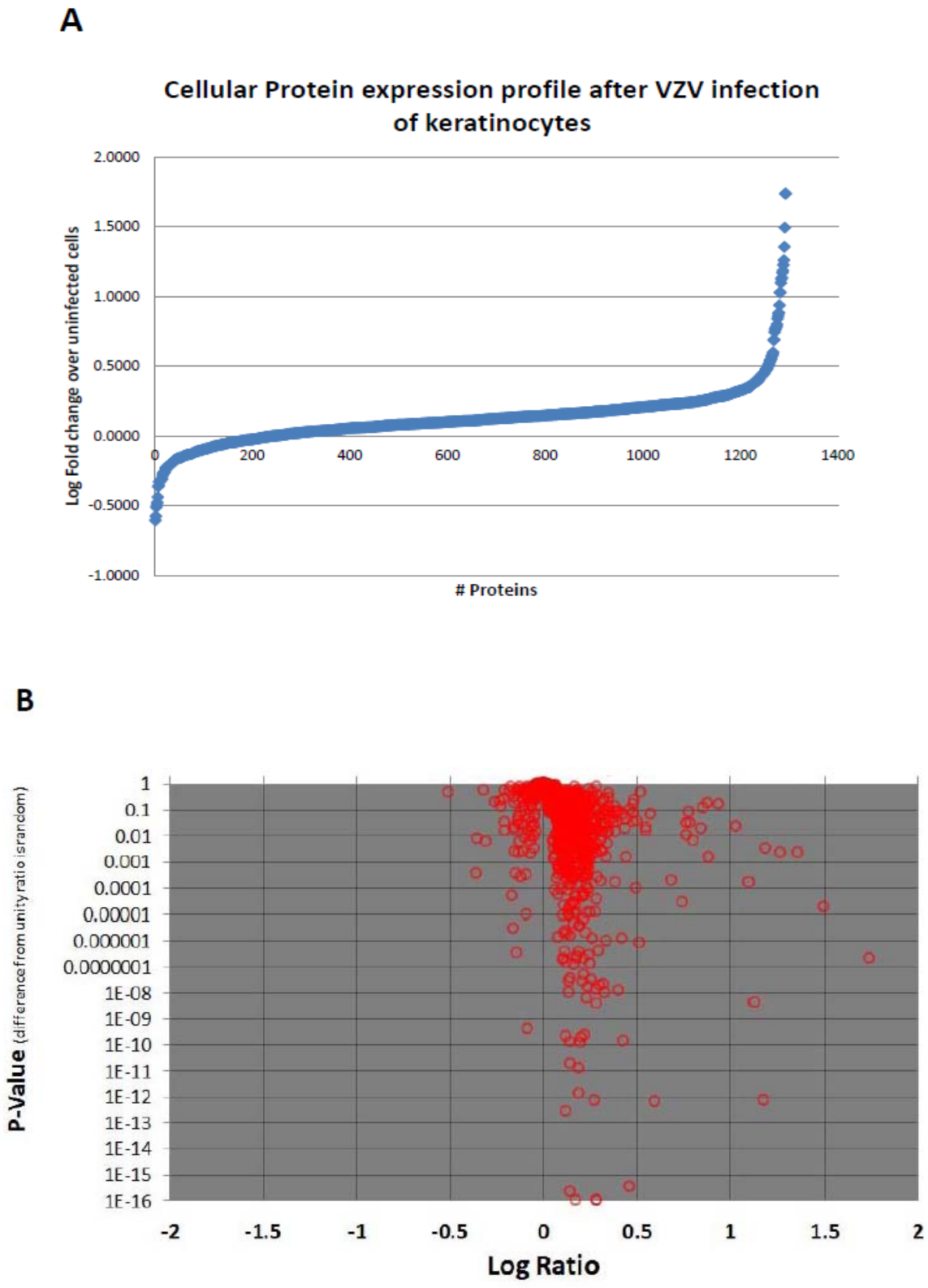


Figure 5.7: Most intracellular proteins show increased expression after VZV infection.
A) Regulation of protein expression after VZV infection. Most proteins are up regulated after infection **B)** Volcano plot showing the segregation of cellular proteins from mock and

VZV infected cells expressed as log ratios of levels in infected cells over mock infected cells. Most proteins cluster on the positive scale showing that their levels are increased in infected cells.

5.6 Discussion

Adaptive immune responses especially the T cell mediated response is largely reliant on the quality and quantity of the peptide pool presented on the cell surface. This is the cornerstone of immune recognition, bridging leukocyte interactions that drive immune responses and graft rejection. Host/'self' or antigenic/ 'non-self' derived peptides complexed to either MHC class I or MHC class II molecules traffick to the cell surface where they are sampled by $CD8^+$ and $CD4^+$ effector T cells respectively. The nature of the presented epitope determines the fate of the immunological response i.e the presenting cell is spared if a 'self' antigen is detected or killed if a 'foreign' antigen is seen. Advances in the development of vaccines, have become heavily reliant on the identification of specific T cell targets presented on MHC molecules. In the context of viral infections, knowledge of the MHC peptide repertoire can greatly accelerate the development of key interventions, such as induction of antigen specific T cell responses in infected hosts to subvert viral replication and disease pathogenesis. Although the characterisation of viral derived peptides bound to MHC molecules is still a developing field, several advances in microchemical analysis such as HPLC and mass analysis have now made it possible isolate, detect and characterise these low abundance MHC bound peptides. Indeed, similar studies have already yielded important clues regarding tumor specific CTL recognition (Henderson, Cox et al. 1993; Cox, Skipper et al. 1994; Huang, Gulden et al. 1996).

In this study we focused on the quantitative sequencing, identification and characterisation of varicella derived peptides presented on MHC class I molecules by mass spectrometry. We hypothesised that varicella infection might potentially alter

the origin, amount and repertoire of peptides presented on the cell surface during infection thus affecting T cell priming and differentiation.

Firstly, analysis of growth conditions on peptide presentation showed that cell growth in serum free medium does not significantly reduce the amount of presented peptides. Secondly we observed that virus infection greatly increases the amount of cell surface presented peptides. An unexpected observation was the finding that VZV infection generally increases the presentation of MHC derived peptides. This mirrors previous findings and our own observations that VZV infection generally down regulates cell surface MHC class I protein and gene expression (Cohen 1998; Abendroth and Arvin 2001) (previous chapter) by retaining them in the cis Golgi compartment (Abendroth, Lin et al. 2001). It is possible that MHC down regulation is also tied to intracellular targeting to the ubiquitin proteosomal degradation pathway leading to presentation of MHC derived peptides. When we examined the repertoire of peptides emanating from each MHC subtype, it was clear that most peptides derived from the HLA-B molecule (210 peptides) whereas HLA-A peptides were the least presented (140 peptides) and presentation from HLA-C was moderate (160 peptides). On examination of the presentation profiles, we observed a similar presentation pattern from all three molecules, with presentation of most peptides being up regulated after varicella infection.

Interestingly, on examination of the identified peptide sequences we observed several MHC molecule-derived leader sequences whose presentation was markedly increased after VZV infection. This is of particular interest in relation to the non classical class Ib molecule, HLA-E expression which bears significant importance in regulating Natural Killer cell (NK Cell) mediated immune responses. Treatment of

cells with IFN-γ did up-regulate HLA-E expression and this was synergised by VZV infection in the proportion of uninfected cells. The expression of several HLA-E stabilising MHC-derived leader sequences could have severe implications in the regulation of innate immune responses to VZV. HLA-E molecules exhibit limited polymorphism compared to their classical MHC 1a counterparts and are ubiquitously expressed in several tissues albeit at much lower levels (Koller, Geraghty et al. 1988; Wei and Orr 1990; Ulbrecht, Honka et al. 1992). MHC derived leader sequences and in particular VMAPRTLIL have previously been shown to bind and stabilise human HLA-E molecules with anchor residues at positions 2 and 9 (Braud, Yvonne Jones et al. 1997; Braud, Allan et al. 1998; Lee, Goodlett et al. 1998). Several studies have demonstrated that HLA-E but not the classical class I HLA molecules is the exclusive ligand for heterodimeric CD94/NKG2A, B and C receptors found on NK cells and a subset of T cells (Braud, Allan et al. 1998; Lee, Llano et al. 1998; Posch, Borrego et al. 1998; Brooks, Borrego et al. 1999). A later study by Miller et. al investigated the HLA-E peptide binding motif and revealed that the recognition of these HLA-E-leader sequence complexes by NK CD94/NKG2A is dependent on a key non-anchor residue at position 5 and another possibly at position 8 of the leader sequence. Whereas a substitution of the non-anchor amino acid residue at position 5 completely abrogated CD94/NKG2A binding, a substitution at position 8 only resulted in partial inhibition (Miller, Weber et al. 2003). This observed HLA-E binding motif was largely similar to that of its murine counterpart QA-1 which has also been shown to bind class 1a leader sequence derived peptides (Aldrich, DeCloux et al. 1994; Kraft, Vance et al. 2000). Although HLA-E leader sequence complexes have the capacity to stimulate adaptive immune responses in humans (Pacasova, Martinozzi et al. 1999; Li, Goldstein et al. 2001; Pietra, Romagnani et al. 2001;

Garcia, Llano et al. 2002; Heinzel, Grotzke et al. 2002; Romagnani, Pietra et al. 2002) and mice (Martinozzi, Pacasova et al. 1999), complex binding to NK cell inhibitory receptor ligands ultimately confers protection from lysis by NK cells (Borrego, Ulbrecht et al. 1998).

Analysis of the host peptide repertoire presented during VZV infection shows that VZV alters antigen processing pathways. This is exemplified by a high abundance of certain host peptides combined with decreased presentation of others following infection. Alteration of antigen presentation could be an immune evasion strategy that VZV uses to target proteins that mediate important immune function for degradation. One of the proteins whose presentation was enhanced is the eukaryotic initiation factor eIF-2α which acts downstream of the double stranded RNA sensor PKR. PKR signaling is regulated by IFNα. Activation of eIf-2α leads to inhibition of initiation and translation and ultimately viral replication. Ambagala and colleagues have demonstrated that VZV ORF 63 interferes with IFNα and PKR signaling by mediating the phosphorylation and inactivation of eIf-2α (Ambagala and Cohen 2007). Targeting this protein for activation and degradation at the same time suggests stringency on the part of the virus. Perhaps VZV targets eIf-2α inactivated for degradation pathways that process antigens for presentation. Overall, these findings suggest that VZV targets some immune effector proteins for degradation to evade immune detection. It will be interesting to correlate these alterations in protein degradation pathways to gene expression profiles in VZV infected cells.

On examination of virus derived peptides, most were found to emanate from structural proteins and proteins involved in virion assembly and egress. The functional consequence of structural proteins on immune responses is not known.

However, most cell mediated immune responses are known to be directed against VZV glycoproteins (Arvin, Koropchak et al. 1990; Arvin, Sharp et al. 1991) and this could indicate a tendency towards presentation of structural proteins. What remains is to test the immunogenicity of the identified VZV derived peptides on their ability to induce varicella specific immunity. Further studies are required to assess whether varicella infection alters the nature and repertoire of MHC presented peptides to evade host immunity, potentially promoting the presentation of non-immunogenic peptides while masking presentation of immunogenic ones.

5.7 Conclusions

In summary, our findings indicate that VZV infection generally increases cellular protein levels and presentation of host protein derived peptides associated with MHC Class I molecules. Despite the observation that levels of intracellular proteins increase after VZV infection, we could not conclusively determine whether the level of intracellular protein influences the rate of peptide presentation. We also discover that VZV causes an increase in the presentation of MHC derived peptides especially MHC signal sequences, which can bind to and stabilize HLA-E, a ligand for NK inhibitory receptor CD94/NKG2A. This finding may explain how VZV potentially avoids NK cell lysis after MHC class I down regulation. This phenomenon may be aided by the possibility of surface retention of HLA-B molecules which may also promote NK inhibition. We hope that the findings of this study will aid advances towards development of peptide based VZV vaccines and in better understanding and enhancement of varicella specific T cell responses based on MHC peptide recognition.

CHAPTER 6: Overall Discussion and Future Directions

6.0 Discussion

Primary infection with Varicella zoster virus (VZV) occurs through inoculation of epithelial cells of the conjunctiva and the upper respiratory tract (URT). Virus is transmitted by direct contact or through uptake from respiratory droplets. Infection may be followed by direct establishment of primary viremia resulting from infection of tonsillar $CD4^+$ T cells that express skin homing markers and are thought to transport virus from the lymph nodes to the skin where further replication occurs (Ku, Padilla et al. 2002; Ku, Zerboni et al. 2004). Alternatively, VZV is thought to infect mucosal dendrtitic cells of the URT which act as reservoirs for virus transport to the draining lymph nodes, where as they present antigens to naïve T cells also transfer infectious particles leading to primary viraemia through T cell infection (Abendroth, Morrow et al. 2001; Morrow, Slobedman et al. 2003). Virus is subsequently transported to the reticuloendothelial organs such as the liver and spleen where another round of replication occurs resulting in secondary cell associated viremia and virus transport to the skin by infected T cells (Arvin, Moffat et al. 1996). VZV replication in the skin marks the final round of pathogenesis accompanied by cutaneous infection and an outbreak of vesiculopustular lesions on the skin surface. Following resolution of primary infection, the virus travels via local nerve axons on the skin and establishes latency in trigeminal ganglia and dorsal root ganglia where virus persists throughout the lifetime of the infected host. Latent virus periodically reactivates but is contained by the established cell mediated adaptive

immune response leading only to sub-clinical disease. In cases where adaptive immunity is compromised such as in severe immunosuppression, VZV reactivates from the ganglia and travels back via retrograde transport to skin sites served by individual nerve axons known as dermatomes causing characteristic pustular lesions with a characteristic clustered distribution. The time span between VZV exposure and cutaneous infection is about 10-21 days. During this long incubation period and during latency, VZV is thought to conveniently hide from the immune system and remain undetected.

Both innate and adaptive immune responses are induced during acute VZV infection. Innate immune effectors such as type I interferons and NK cells are thought to control initial viral replication (Abendroth and Arvin 2001). After the outbreak of cutaneous infection, adaptive immunity involving both $CD4^+$ and $CD8^+$ T cells sets in and the infection is cleared (Arvin, Koropchak et al. 1986). $CD8^+$ T cells act via classical cytotoxicity, targeting and killing virus infected cells whereas circulating $CD4^+$ T cells that are predominantly of the Th1 subset and secrete IFN-γ and IL-2 (Zhang, Cosyns et al. 1994). Although humoral immunity is also induced as part of the adaptive response, it is the cell mediated immune response (CMI) governed by T cells that mediates eventual control of viraemia and the associated clinical disease.

Against this evidence of the induction of both VZV specific $CD4^+$ and $CD8^+$ T cells during acute varicella infection, some studies have reported higher frequencies of circulating memory $CD4^+$ than $CD8^+$ T cells in VZV seropositive individuals (Asanuma, Sharp et al. 2000; Jones, Black et al. 2006; Malavige, Jones et al. 2008). This is corroborated by other studies which have also shown that VZV-specific $CD8^+$ T cells circulate at low frequencies during convalescence (Asanuma, Sharp et al.

2000; Jones, Black et al. 2006; Malavige, Jones et al. 2008; van der Heiden, de Boer et al. 2009; Malavige, Rohanachandra et al. 2010). Other work has shown that $CD8^+$ T cells are prone to cell death after isolation and are refractory to *in vitro* culture alone or in the presence of antigen-presenting cells (APCs) bearing VZV antigen (Jones, unpublished). It is not understood why there seems to be a paucity of memory $CD8^+$ T cell responses but it is thought that VZV fails to establish an effective $CD8^+$ CTL response. It could be that $CD8^+$ T cells are sequestered away from the blood, perhaps at neuronal sites of latency as has recently been reported by Gowrishankar et., al (Gowrishankar, Steain et al. 2010). Notably they also observed that the neuronal lymphocyte infiltrate consisted of non-cytolytic $CD8^+$ T cells suggesting that the $CD8^+$ T cells elicited during acute varicella infection are either functionally impaired or lose effector function after the resolution of primary disease. The fact that VZV manages to remain virtually undetected during a long incubation period, establishes lifelong latency and induces poor CD8+ T cell responses suggests that VZV probably evades the immune system to promote its persistence in the host. In this study we, tested the hypothesis that VZV targets the antigen processing and presentation pathway to evade immune detection.

First, we examined the effect of acute VZV infection on two peripheral blood dendritic cell subsets. Dendritic cells (DCs) are a key component of innate immunity and play a critical role in activating naive T cells to initiate the adaptive immune response (Steinman and Witmer 1978; Banchereau and Steinman 1998; Mahnke, Schmitt et al. 2002; Lebre, Burwell et al. 2005). It is postulated that DCs of the upper respiratory tract are the first to encounter VZV during natural infection and are thought to transfer virus to T cells in draining lymph nodes (Abendroth, Morrow et

al. 2001). Since DCs are the most potent antigen presenting cells of the immune system, this observation has prompted a lot of investigation into the impact of varicella infection on DC biology. Several studies have shown that DCs are usually targeted by several herpes viruses as a means to evade immunity (Baranek, Zucchini et al. 2009; Cunningham, Donaghy et al. 2010). Likewise, VZV potentially targets DCs to evade immune detection. VZV can productively infect immature and mature DCs leading to down regulation of key markers of co-stimulation and maturation such as CD80, CD83, CD86 and ICAM-1 and poor stimulation of allogeneic T cells (Morrow, Slobedman et al. 2003). We analysed the consequences of acute varicella infection on plasmacytoid (pDC) and myeloid dendritic cells (mDC) frequency, phenotype, homing and function. Our results show that acute VZV infection significantly lowers the frequency of circulating mDC while the overall DC frequency and the frequency of pDC was the same between patients and controls. We suspect that this differential down regulation of mDC could be attributed to the fact that mDC probably express higher levels of DC-SIGN which could interact with virus glycoproteins, promote DC infection and possible death (Navarro-Sanchez, Altmeyer et al. 2003; Tassaneetrithep, Burgess et al. 2003; Sun, Fernandez et al. 2009). However, there is currently no evidence that VZV, preferentially target mDC, causes DC cell death or that infected DC express higher levels of DC-SIGN (Huch, Cunningham et al. 2010). Analysis of homing markers revealed a significant increase in the proportion of mDC expressing CCR4 and a significant decrease in CLA^+ pDC. There was also a trend towards a decrease in circulating CCR7 mDC in the blood, suggesting possible migration to local area lymph nodes where mDC present antigens. This could also explain the observed decreased mDC frequency in peripheral blood. Cutaneous Lymhocyte-associated antigen (CLA) mediates

lymphocyte homing to the skin. The decrease in CLA expressing pDC points to possible migration to skin sites of VZV replication. Indeed pDC have been shown to infiltrate sites of inflammation for purposes of secreting IFN-α (Huch, Cunningham et al. 2010). Analysis of DC phenotype showed that although VZV infection induced a general activation phenotype characterized by increased expression of CD40 and CD86, the expression of HLA-DR was significantly low in DCs from acutely infected patients. HLA-DR is an antigen presenting molecule of the MHC Class II family. It is possible that VZV causes DC activation but also inhibits antigen presentation possibly through a previously identified mechanism of inhibiting IFN-γ induced up regulation of HLA-DR (Abendroth, Slobedman et al. 2000). Another human herpes virus, HHV-6 and Hepatitis C virus have also been shown to induce DC maturation while down modulating antigen presentation (Kakimoto, Hasegawa et al. 2002; Saito, Ait-Goughoulte et al. 2008) suggesting that this seems to be a feature of many viral infections. Apart from naïve T cell activation, DCs secrete cytokines that shape the immune response. Accordingly, pDC are known to produce type I IFNs in response to viral infections while mDC mainly secrete IL-12 (Banchereau, Pulendran et al. 2000). We investigated the ability of DCs from acute VZV patients to secrete various inflammatory cytokines upon Toll Like Receptor 7/8 (TLR7/8) stimulation. VZV infection was shown to impair inflammatory cytokine secretion in both pDC and mDC subsets. This property was confirmed using *in vitro* viral stimulation of fresh DCs also confirming that DC cytokine inhibition was dependent on virus replication as gamma irradiated virus failed to significantly inhibit cytokine secretion. Type I and II interferons are thought to control initial virus replication during natural infection and in *in vitro* experiments. The fact that VZV inhibits this pathway supports our hypothesis the virus has devised mechanisms to evade innate

immunity. Despite reduced DC cytokine synthesis during acute VZV infection, the serum levels of most inflammatory cytokines were increased consistent with ongoing inflammation. However, the level of the inflammatory chemokine RANTES/ CCL5 was significantly reduced in correlation with disease severity. RANTES has been shown to be critical for the development of efficient $CD8^+$ T cell responses in a murine model of chronic viral infection (Crawford, Angelosanto et al. 2011). The observation that VZV infection decreases serum RANTES levels could help explain why VZV establishes poor memory $CD8^+$ T cell responses. Cytomegalovirus (CMV) encodes a chemokine receptor homolog US28 which was shown to bind endogenous and exogenously secreted RANTES and MCP-1 thus hampering immune cell trafficking (Randolph-Habecker, Rahill et al. 2002). VZV has not been shown to express any chemokine receptor homologs but the reduction in RANTES levels points to this possibility.

One aspect of VZV mediated immune evasion mechanisms is directed towards the antigen presenting proteins of the major histocompatibility complex. $CD8^+$ cytotoxic T cells recognise peptides from viral antigens bound to MHC class I/ HLA class I molecules and kill infected cells by the activity involving granzymes and perforins. VZV infection has been shown to down-regulate the expression of MHC class I molecules in order to escape cytotoxic T cell lysis (Cohen 1998; Abendroth, Lin et al. 2001; Morrow, Slobedman et al. 2003). MHC class I molecules are known to modulate NK cell function by interacting with inhibitory and activatory receptors on the surface of NK cells to influence cell killing(Borrego, Ulbrecht et al. 1998; Braud, Allan et al. 1998). Consequently, cells expressing reduced or low levels of surface class I molecules may be more susceptible to NK cell lysis (Borrego, Ulbrecht et al.

1998). Some viruses such as HIV selectively down regulate certain HLA subtypes and spare others in order to escape CTL lysis and maintain NK inhibition (Cohen, Gandhi et al. 1999). It is unclear how MHC class I modulation by VZV ultimately affects CD8+ T cell and NK cell recognition, but it could potentially lead to poor T cell activation resulting in the observed poor memory $CD8^+$ T cell responses as has been shown with *in vitro* VZV infected keratinocytes (Black, Jones et al. 2009). We tested the hypothesis that VZV infection selectively down-regulates specific HLA class I subtypes to avoid T cell recognition and maintain NK cell inhibition.

HLA-I expression was assessed on *in vitro* VZV infected Keratinocytes and Mewo cells. We observed differential HLA class subtype down-regulation in different cell types with the virus severely down regulating cell surface HLA-A and HLA-C but sparing HLA-B and augmenting IFN-γ induced expression of HLA-E. The observation of MHC class I down regulation was consistent with previous findings (Cohen 1998; Abendroth, Lin et al. 2001; Black, Jones et al. 2009) but also pointed to the fact that VZV can down modulate HLA class I expression in an allele specific manner. Analysis of intracellular HLA protein expression and gene transcription showed global down regulation of all HLA genes and proteins. This differs from reports by Cohen and colleagues who did not observe MHC inhibition at the level of gene expression a fact that can be explained by use of different experimental methods employed by the former study (Cohen 1998). The fact that VZV simultaneously reduces cell surface and intracellular MHC class I protein as well as gene expression suggests that the virus might employ multiple mechanisms to to target the MHC pathway as a means of immune evasion. A key finding was also that VZV alone though not capable of inducing HLA-E expression on keratinocytes,

augments IFN-γ mediated up regulation of HLA-E. Since HLA-E is one of the key ligands for NK inhibitory receptors; and since HIV has been shown to use it to evade NK surveillance, it is possible that VZV employs a similar mechanism as HIV to evade both T cell and NK surveillance. Taken together, our data point to multiple mechanisms employed by VZV to evade CTL and NK cell lysis including differential MHC class I down regulation, inhibition of HLA gene transcription and up regulation of HLA-E expression. What remain to be determined are the exact molecular mechanisms by which VZV targets MHC molecules and the virus proteins that may be involved in this process. The VZV ORF66 protein kinase has been identified as one of the proteins mediating MHC down regulation, but others are also thought to be involved (Abendroth, Lin et al. 2001; Eisfeld, Yee et al. 2007) warranting future studies to propel their identification. It is of significance to not ethat MHC class I down regulation was equally induced by both vaccine strain vOka and parental strain pOka infection. This has severe implication for vaccination strategies that are aimed at inducing optimal protective T cell responses as immune evasion may limit vaccine efficacy. Potentially, there is a need to develop better live attenuated virus based vaccines that do not employ immune evasion, in order to achieve vaccination success.

A key advancement in the study of immune responses to viruses has been the ability to identify and synthesise specific viral peptide epitopes. These are normally used to generate tetrameric complexes which are routinely used to identify and isolate peptide specific T cells and also offer hope for the development of peptide based vaccines (Henderson, Cox et al. 1993; Cox, Skipper et al. 1994; Huang, Gulden et al. 1996). Due to the refractory nature of VZV, attempts to identify immunogenic viral

peptides that are recognised by T cells have been fruitless. We took advantage of recent advances in mass spectrometry techniques and proteomic data analysis to isolate and characterize VZV derived peptides presented on MHC class I molecules from keratinocytes (HaCaTs) infected with the live attenuated varicella vaccine strain (vOka). Stable Isotope Labelling by Amino acids in Culture (SILAC) is a technique that when combined with mass spectrometry allows quantitative isolation of host peptides that are altered during infection. By combining SILAC with mass spectrometry, we were able to successfully isolate and quantify 21 varicella derived peptides with MHC class I binding properties. These peptides will be tested for their ability to stimulate VZV specific T cell responses. A general observation was the increase in host peptide presentation following VZV infection. This is probably attributable to the action of type I interferons which are known to augment antigen processing during infection. It was evident that VZV infection results in reduced expression of some host peptides while increasing the expression of others. It is not clear what factors influence this differential regulation. A possible explanation would be intracellular levels of source proteins. We thus compared the levels of presented peptides with endogenous protein levels. Even though we observed increased protein levels after VZV infection, it was not clear whether intracellular protein levels of individual peptides correlated with peptide presentation on MHC class I molecules. Interestingly, on examination of the identified peptide sequences, we observed several MHC molecule-derived leader sequences whose presentation was markedly increased after VZV infection. This is of particular interest in relation to the non classical class Ib molecule, HLA-E expression which bears significant importance in regulating Natural Killer cell (NK Cell) mediated immune responses. We think that the expression of several HLA-E stabilising MHC-derived leader sequences could

have severe implications in the regulation of innate immune responses to VZV. MHC derived leader sequences and in particular VMAPRTLIL have previously been shown to bind and stabilise human HLA-E molecules with anchor residues at positions 2 and 9 (Braud, Yvonne Jones et al. 1997; Braud, Allan et al. 1998; Lee, Goodlett et al. 1998). Several studies have demonstrated that HLA-E but not the classical class I HLA molecules is the exclusive ligand for heterodimeric CD94/NKG2A, B and C receptors found on NK cells and a subset of T cells (Braud, Allan et al. 1998; Lee, Llano et al. 1998; Posch, Borrego et al. 1998; Brooks, Borrego et al. 1999). A later study by Miller et. al investigated the HLA-E peptide binding motif and revealed that the recognition of these HLA-E-leader sequence complexes by NK CD94NKG2A is dependent on a key non-anchor residue at position 5 and another possibly at position 8 of the leader sequence. Whereas a substitution of the non-anchor amino acid residue at position 5 completely abrogated CD94/NKG2A binding, a substitution at position 8 only resulted in partial inhibition (Miller, Weber et al. 2003). This observed HLA-E binding motif was largely similar to that of its murine counterpart QA-1 which has also been shown to bind class 1a leader sequence derived peptides (Aldrich, DeCloux et al. 1994; Kraft, Vance et al. 2000). A few of the identified MHC leader sequences were shown to possess a similar motif, suggesting that they are likely to be bind HLA-E. We will still need to conduct experiments to prove this. Although HLA-E leader sequence complexes have the capacity to stimulate adaptive immune responses in humans (Pacasova, Martinozzi et al. 1999; Li, Goldstein et al. 2001; Pietra, Romagnani et al. 2001; Garcia, Llano et al. 2002; Heinzel, Grotzke et al. 2002; Romagnani, Pietra et al. 2002) and mice (Martinozzi, Pacasova et al. 1999), complex binding to NK cell inhibitory receptor ligands ultimately confers protection from lysis by NK cells

(Borrego, Ulbrecht et al. 1998). We will wait to see whether some of these leader sequences stimulate T cell responses. The combined effect of VZV augmenting HLA-E expression and up regulating generation of HLA-E stabilising leader sequences underscores the possibility that VZV infection uses HLA-E to evade NK cell surveillance.

We have thus identified a powerful tool with which to effectively isolate viral derived peptides and assess alterations in host peptide levels on MHC class I molecules. We hope that these data will aid in the search for T cell reactive VZV peptide epitopes with adequate immunogenicity for potential use in vaccination regimens.

In conclusion, our data reiterates the fact that VZV targets different aspects of antigen presentation to evade the immune system. First, acute infection reduces the proportion of circulating mDC and inhibits inflammatory cytokine secretion from both pDC and mDC. We also show that VZV potentially down modulates MHC class I gene and protein expression in an allotype specific pattern that targets HLA-A and HLA-C but seemingly spares HLA-B and up-regulates HLA-E, a mechanism that could protect infected cells from both $CD8^+$ T cell activity and NK cell mediated lysis. Lastly we are able to show that despite reduced cell surface HLA expression, MHC class I complexes are still present during infection and bear VZV derived antigenic peptides that could potentially be immunogenic. The findings of this study have implications on strategies to combat VZV infection using attenuated virus. Most of the immune evasion mechanisms we observed were induced by the VZV vaccine strain vOka. There is therefore a need to develop a vaccine that is significantly attenuated to avoid the negative effects of MHC down regulation and

immune evasion. We hope that these findings will not only add to our understanding of host virus interactions during acute varicella infection but also aid in the development of useful strategies to counteract immune evasion by varicella which leads to viral persistence and pathogenesis in vulnerable groups. Finally, identification of VZV derived MHC associated peptides offers new hope in the development of immunogenic peptide vaccines and isolation of VZV specific T cells.

6.1 Future Directions

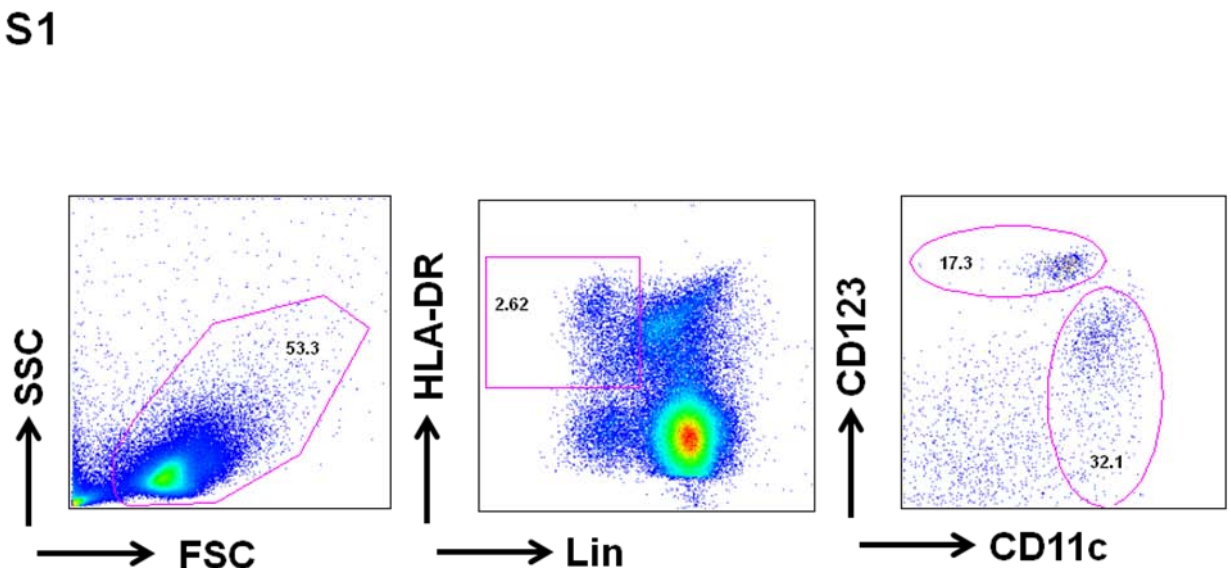
1. Identify viral gene products that target dendritic cells and inhibit cytokine production. Also further studies are needed to determine blood DC function and gene expression profile after VZV infection.

2. Identify viral gene products that associate with HLA molecules and possibly cause HLA-I down regulation. Conventional immune precipitations have so far failed to identify any candidate genes. In addition, the molecular mechanisms of MHC down regulation by VZV are yet to be determined.

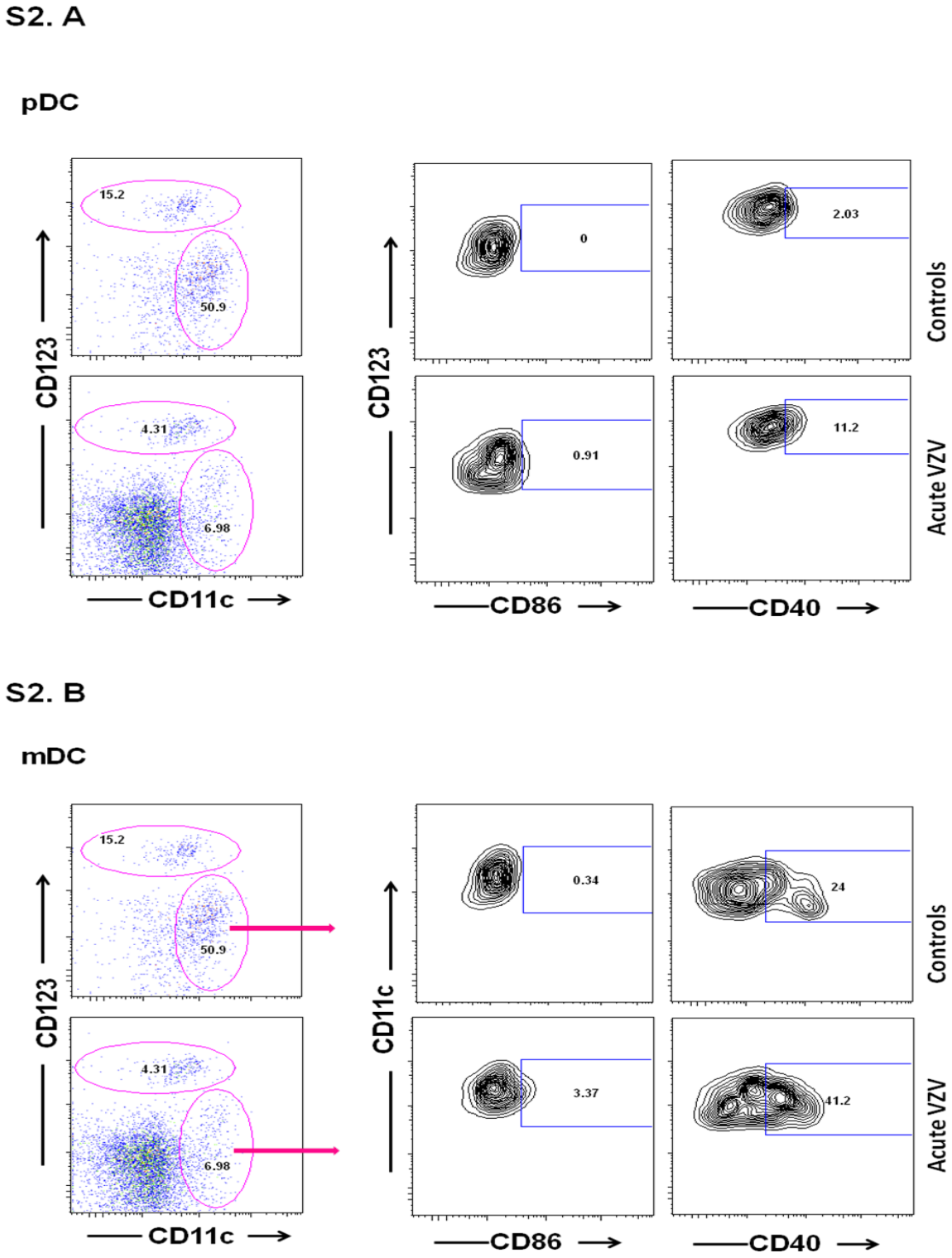
3. Assess the immunogenicity of VZV derived peptides identified by mass spectrometry.

Appendix A

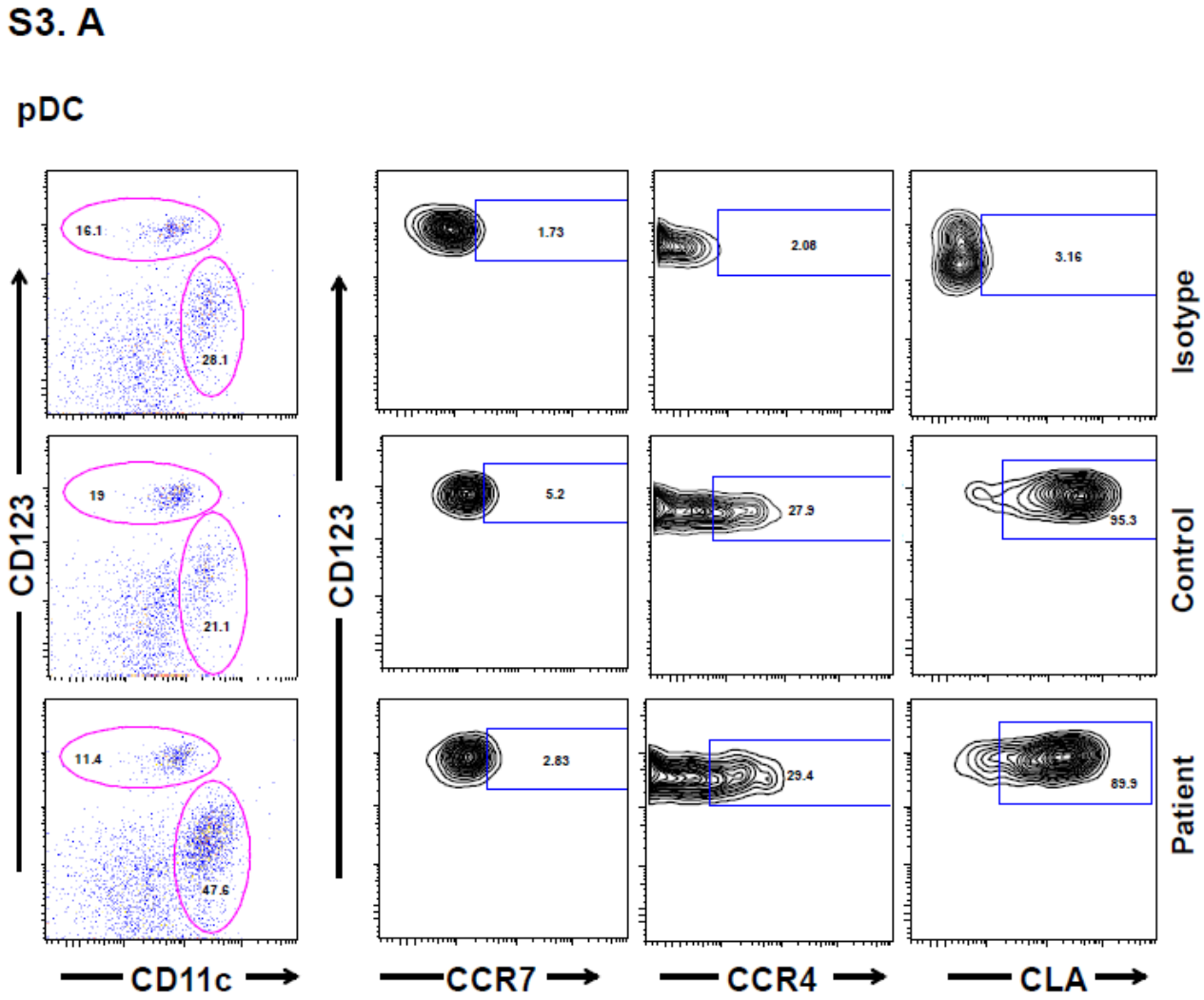
Supplementary Figure 1: General gating strategy to enumerate Dendritic cells. Cells were gated on the forward scatter (FSC) and side scatter (SSC) and lymphocytes were selected. DCs were defined from the lymphocyte gate as HLA-DR+ LIN- cells. Next pDC and mDC populations were defined as $CD11c^{int}$ $CD123^{hi}$ and $CD11Ccd123^{+/-}$ cells respectively.



Supplementary Figure 2A&B: General gating strategy to elucidate the expression of activation markers on CD123^{+} pDC and CD11c^{+} mDC. Representative flow diagrams from one patient and one control showing the proportion of cells expressing either CD40 or CD86.

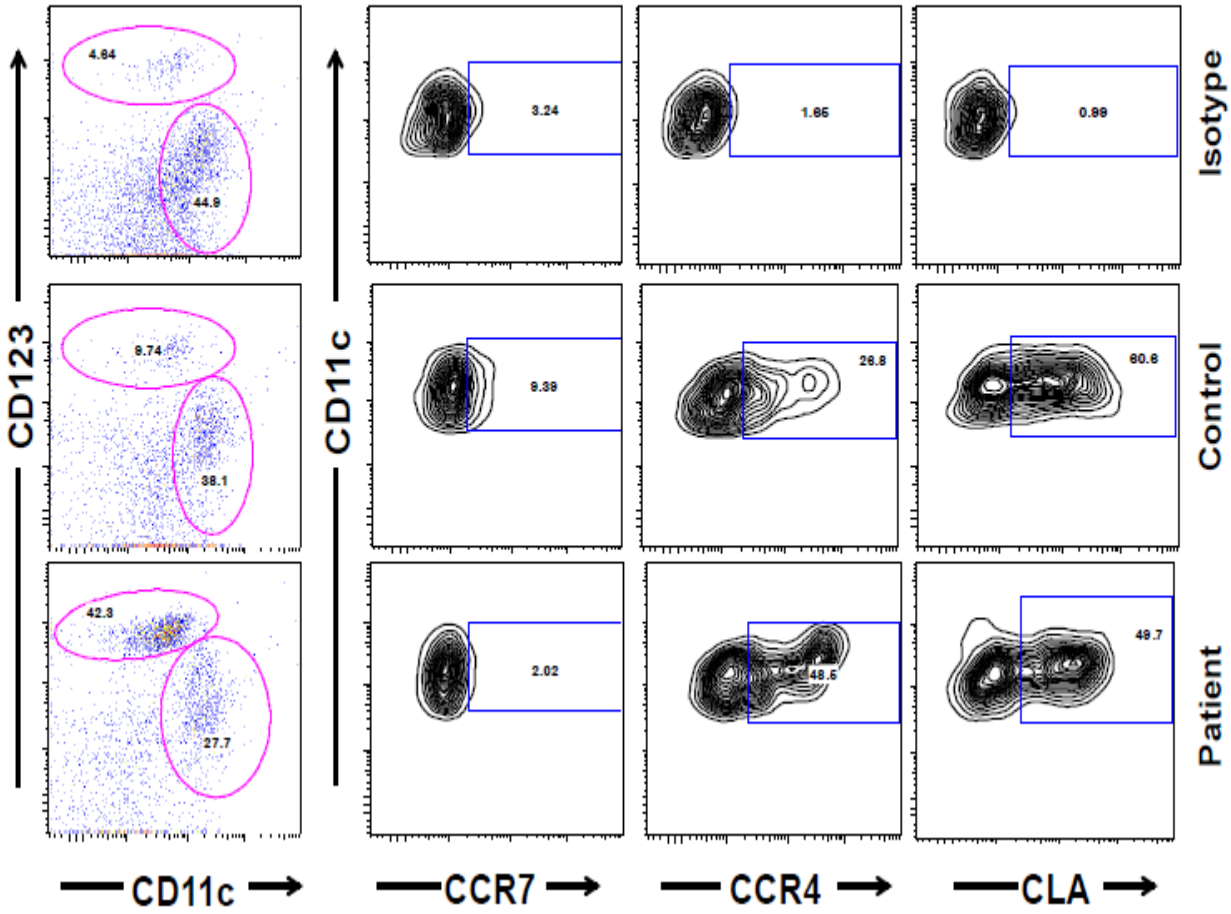


Supplementary Figure 3A&B: General gating strategy to elucidate Homing marker expression on CD123^{+} pDC and CD11c^{+} mDC. Representative flow diagrams from one patient and one control showing the proportion of cells expressing CCR7, CCR4 and CLA.

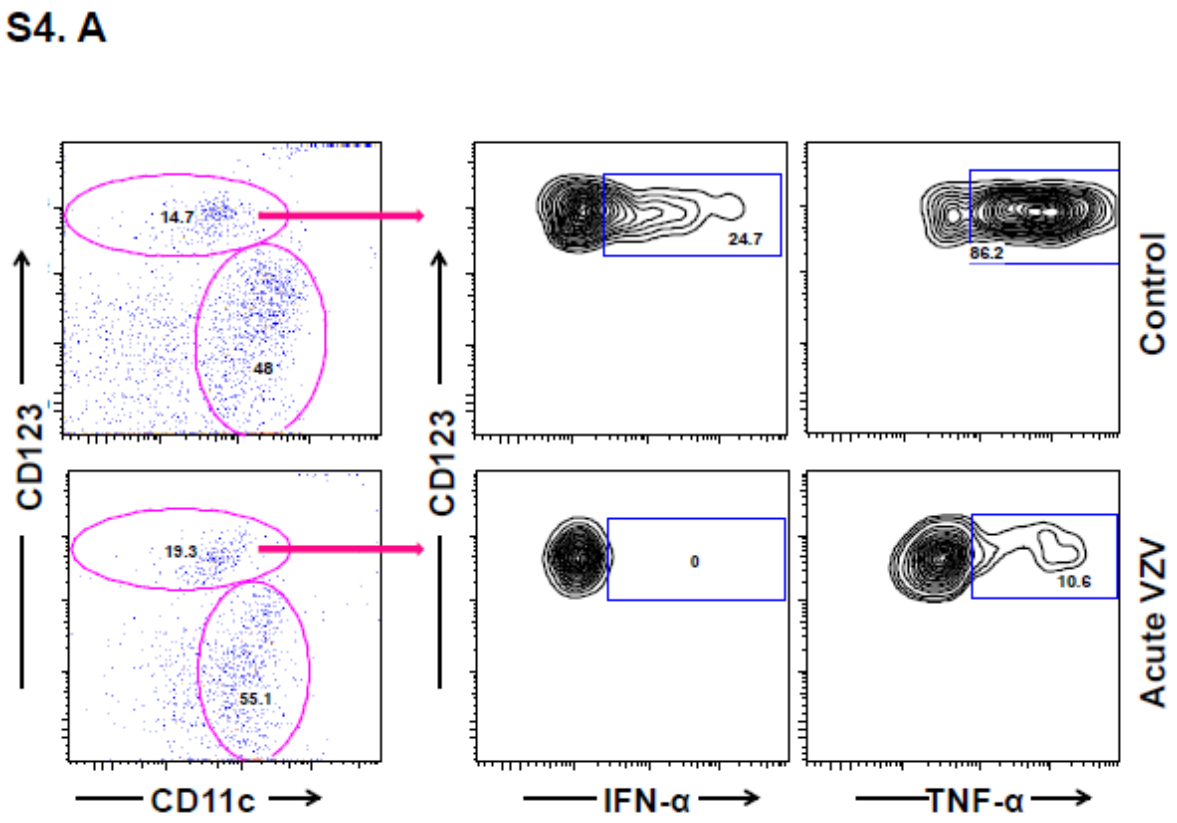


S3. B

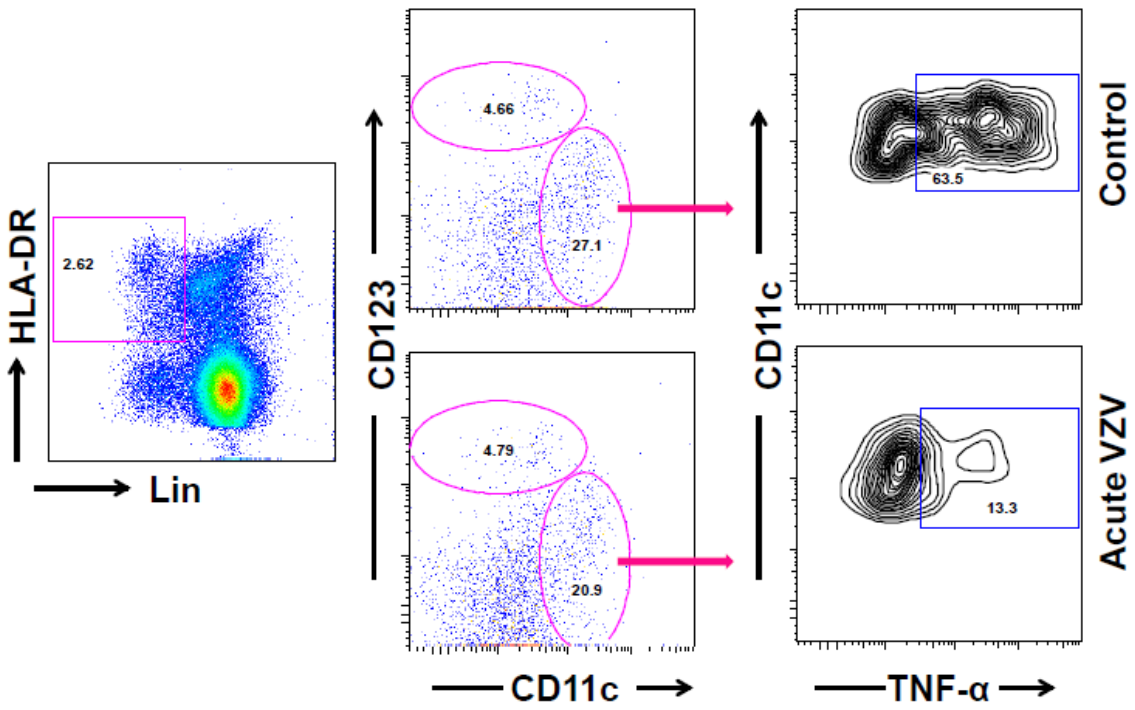
mDC


4.84
44.9
9.74
38.1
42.3
27.7
3.24
1.65
0.99
8.38
26.8
80.8
2.02
48.6
49.7
CD123
CD11c
CD11c
CCR7
CCR4
CLA
Isotype
Control
Patient

Supplementary Figure 4A&B: General gating strategy to determine the proportion of CD123^{+} pDC and CD11c^{+} mDC secreting TNF- α and IFN-α after *in vitro* stimulation with a TLR ligand, CL097 or vOka.



S4. B


HLA-DR
Lin
2.62
CD123
CD11c
4.66
27.1
4.79
20.9
CD11c
TNF-α
63.5
13.3
Control
Acute VZV

Appendix B

S1: List of peptides whose presentation was up-regulated after VZV infection as determined by pairwise comparison and quantitation of SILAC labelled peptides from 'light' (infected keratinocytes) and 'heavy' (uninfected keratinocytes) using mass spec analysis software PEAKS. Each mass spec identified peptide sequence is flanked by 2 amino acids in the parent protein at either end separated by a dot.

Parent Protein	Peptide Sequence
neuroligin 2	Q.RGGGGPGGGAPGGPGLGLG.S
zinc finger, CCHC domain containing 17	D.KVWVKLIGR.E
protein tyrosine phosphatase, receptor type, N	R.PGGLGGSGGLR.L
talin 1	A.KNLGTALAEL.R
Usher syndrome 2A (autosomal recessive, mild)	I.PRSGTPVSIR.S
endothelin converting enzyme 2	P.GAGRAPPELPERN.C
collagen, type I, alpha 2	F.KGIRGHN.G
glycogen synthase kinase 3 alpha	G.PGGSASGPGGTGGGKASVGAMGGGVGASS.S
protein tyrosine phosphatase-like A domain containing 1	T.LPYPVKIKV.R
major histocompatibility complex, class I, C	R.VMAPRTLIL.L
major histocompatibility complex, class I, B	R.VMAPRTLIL.L
major histocompatibility complex, class I, C	R.VMAPRTLLL.L
RUN and SH3 domain containing 2	E.GPAAMAGPGSPPRR.V
titin	D.MCSAQLSVKEP.P
integrator complex subunit 1	R.LSDVRGGLLR.L
PDZ domain containing 2	E.KGKGLGFSIAGGR.D
protocadherin 20	I.KLFSKIGGSVL.E
collagen, type III, alpha 1	R.GRPGLPGAAGAR.G
titin	E.KYAISVKGT.I
transcription termination factor, RNA polymerase II	N.LQFPDRSVQR.K
zinc finger protein 687	T.GGQKVNGASVVMV.Q
chromosome 6 open reading frame 226	S.LPPPTLRI.G
complement component (3d/Epstein Barr virus) receptor 2	I.VPGGYKIRGST.P
WD repeat domain 12	T.RTPIVTLSGH.M
scavenger receptor class F, member 1	C.HPVSGSCQPGSGSR.D
phosphoglycolate phosphatase	E.TAVPGAPEALR.A
polymerase (DNA directed), gamma	Q.VVTAGTITRR.A
APC membrane recruitment protein 2	E.AAEGRAPGGGLI.L
KIAA0556	L.RLSAVPTSMGDMPSAPATSP.P
metastasis associated 1 family, member 3	D.RQIDQFLVV.A
keratin 75	D.AEQRGELAL.K
keratin 79	E.AEQRGELAL.K

protein tyrosine phosphatase-like A domain containing 1	T.LPYPVKIKV.R
lamin B receptor	N.AVYDFFIGR.E
histamine receptor H3	R.SLKRGSKPSASSA.S
Rho/Rac guanine nucleotide exchange factor (GEF) 18	V.TVGTNILPSRPAASANTARE.D
UPF1 regulator of nonsense transcripts homolog (yeast)	L.VVLGIRPIR.L
zinc finger protein 132	S.NLGQLPEVCTTQK.L
NLR family, pyrin domain containing 5	K.KESSVTEFI.S
BPI fold containing family B, member 4	R.VAINGKSLIG.F
strawberry notch homolog 1 (Drosophila)	A.KKARKVGGLTGSS.S
keratin 72	V.DAAYMNKV.E
keratin 75	V.DAAYMNKV.E
heat shock protein 90kDa beta (Grp94), member 1	S.DALDKIRLI.S
fibronectin type III domain containing 3B	C.SESLPVRTL.S
cullin 7	T.ATQSFSTFR
uncharacterized LOC100128265	G.SRAALACVLR.P
seizure related 6 homolog (mouse)	P.MLRITAP.L
alpha-kinase 2	T.IDSLVGRPV.D
sal-like 3 (Drosophila)	A.RAGDAPVGAQASAAPTSVDG.A
Sp1 transcription factor	Q.NKKGGPGVALS.V
chromodomain helicase DNA binding protein 4	Y.GIKPEWMMIHR.I
chromodomain helicase DNA binding protein 5	Y.GIKPEWMMIHR.I
collagen, type IV, alpha 6	G.NPGPVGIPSPR.R
alanyl-tRNA synthetase	D.STLTASEIRQR.F
ankyrin repeat domain 28	E.LLGKGASVLAVD.E
frizzled family receptor 8	H.RGGGRGGGGGDAAAP.P
osteosarcoma amplified 9, endoplasmic reticulum lectin	Q.RQKELESNYR.R
collagen, type I, alpha 2	G.NKGEPGVVGAVG.T
Y box binding protein 1	L.GTVKWFNVR.N
Y box binding protein 2	L.GTVKWFNVR.N
cold shock domain protein A	L.GTVKWFNVR.N
fibrillin 2	C.PPGLAVGMDGR.V
cell cycle associated protein 1	V.AQADPLVRRQR.V
coronin, actin binding protein, 1A	C.AVNPKFVAL.I
phosphatidylinositol binding clathrin assembly protein	F.LKVAEQVGI.D
sema domain,Ig domain, transmembrane domain and short cytoplasmic domain	T.ATAVPGPSLRR.P
collagen, type V, alpha 3	T.GIDGAPGAKGNVGPPG.E
Sp8 transcription factor	G.GGGGGGSAGSGSGGK.K
solute carrier family 26, member 5 (prestin)	A.VRLVPDDIV.I
death associated protein 3	S.FAYPAIRYL.L
rhomboid 5 homolog 2 (Drosophila)	T.SVRSGYSHLPR.R

cleavage and polyadenylation specific factor 1, 160kDa	P.PSKKKRVDATAGWSAAGKSVP.Q
transmembrane protein 200C	A.GSRRAAAATAAAAASSCSSPAP.C
glutathione reductase	L.VIGGGSGGLASAR.R
coiled-coil domain containing 166	S.RPGSLAAPI.S
MAP7 domain containing 3	M.ADGAAAGAGGSPSLR.E
elastin	P.RVPGAL.A
nyctalopin	R.RVPGAL.R
desmocollin 3	G.MKNGGQETIE.M
fer-1-like 4 (C. elegans) pseudogene	R.KPGPGACAQLKQA.L
DnaJ (Hsp40) homolog, subfamily C, member 13	V.RASGNRDVCVK.M
short stature homeobox 2	V.RAAGGGGGGGGGGG.G
tetratricopeptide repeat domain 40	K.GRKGSIPR.T
Bloom syndrome, RecQ helicase-like	S.ATRKNLFER.P
mitogen-activated protein kinase 4	R.RLSASPPGRP.A
perilipin 4	K.QMVSGAKDLV.C
Treacher Collins-Franceschetti syndrome 1	F.VDPNRSPAGPAATPA.Q
ajuba LIM protein	A.RMREPEAR.E
GRB2 associated, regulator of MAPK1-like	G.AAGGTGGGGARPVK.G
metastasis associated 1	N.SAAIKAECTAR.L
mortality factor 4 like 1	T.PLDEKSLALL.L
GTPase activating protein (SH3 domain) binding protein 1	L.RGPGGPRGGLGGGM.R
metadherin	S.TSDPAEVLVK.N
nuclear receptor corepressor 2	L.SYEGGMSVTQCSKEDGRSSS.G
KH-type splicing regulatory protein	D.RGGGGPGGGGPGGGSAGG.P
microtubule-associated protein 1A	L.TGLGPACPTRE.P
A kinase (PRKA) anchor protein 11	T.VLRSQCDAAS.Q
Rho GTPase activating protein 21	G.RGVGSVSQFKK.I
calpain 9	A.RVIPQ.D
fibroblast growth factor receptor-like 1	F.RVLPQ.G
solute carrier family 12 (potassium/chloride transporters), member 9	L.RVLPQ.G
calcium binding protein 1	Q.RVLPQ.A
cyclic nucleotide gated channel beta 1	Q.RVLPQ.P
codanin 1	S.RVLPQ.G
putative protein FAM90A7	C.WKAALV.P
coatomer protein complex, subunit beta 1	A.TTLTKIALR.Y
desmocollin 3	D.LPLPLPIRV.E
ribosomal protein S3	I.RELTAVVQKR.F
MDN1, midasin homolog (yeast)	Y.VTSIAKAPAV.Q
multiple endocrine neoplasia I	G.REEPDLVLL.S
discs, large (Drosophila) homolog-associated protein 4	S.STRPPSVTR.G
histone cluster 1, H2bm	S.REIQTAVRL.L

heterogeneous nuclear ribonucleoprotein R	L.REFNEEGALSVL.Q
Sp8 transcription factor	S.AGPSAPLGGSPRS.S
alkaline phosphatase, placental	L.VQEWLAKR.Q
transmembrane protein 200C	D.SSPLAKAASPSP.P
myosin VI	S.HLFDHVVNR.V
leucine rich repeat neuronal 4	G.TTVAPSAAPATRPAG.D
family with sequence similarity 46, member A	K.EKITPLTLKEA.Y
lipoyl(octanoyl) transferase 2 (putative)	G.GLTPEETARLR.A
spectrin repeat containing, nuclear envelope 1	I.NEIITVLER.E
butyrophilin, subfamily 2, member A2	P.RSAFTVPVR.P
tenascin N	V.AEVTVPKSSDP.K
serine/arginine-rich splicing factor 5	G.RYSDRFSSR.R
SWI/SNF related, matrix associated, actin dependent regulator of chromatin	R.RAFVPQLR.S
hydroxysteroid (17-beta) dehydrogenase 14	T.RYAGKVVVVTGGGR.G
nuclear receptor subfamily 2, group F, member 6	G.RFGAGGGAAGAVLGI.D
THAP domain containing 11	R.RLWLKNVSR.A
atlastin GTPase 2	L.LPHPGLKV.A
ubiquitin-conjugating enzyme E2D 1	D.YPFKPPKI.A
ubiquitin-conjugating enzyme E2L 3	E.YPFKPPKI.T
alanyl-tRNA synthetase	A.RTITVALADGGRP.D
diacylglycerol kinase, alpha 80kDa	E.KYPEKFNSR.M
pleckstrin homology domain containing, familyG (with RhoGef domain) member 1	P.HKPVSDKL.S
BCL2-like 10 (apoptosis facilitator)	P.EAAVLRSAAAR.L
eukaryotic translation elongation factor 2	Y.RETVSEESNVL.C
histone deacetylase 9	R.QQKLLVAGGVP.L
tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	V.AYKNVVGAR.R
tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta polypeptide	V.AYKNVVGAR.R
tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide	V.AYKNVVGAR.R
FAT tumor suppressor homolog 3 (Drosophila)	N.VAAGTKVIHV.R
ribosomal protein L10	R.RAKFKFPGR.Q
G protein-coupled receptor 179	G.RRMTQGLGER.K
paired box 1	A.AGPGAGGSALRCR.A
filamin A, alpha	K.GEITGEVRM.P
filamin C, gamma	K.GELTGEVRM.P
tubulin tyrosine ligase-like family, member 11	R.VTWAASPMRSLG.L
transmembrane protein 64	D.RLPRGGGASAAAAA.A
glyoxylate reductase/hydroxypyruvate reductase	R.RLKPFGVQR.F
microtubule-actin crosslinking factor 1	K.NTISVKAV.C
Ets2 repressor factor	MKTPADTGFAF.P
ribosomal protein L17	N.AELKGLDVDSL.V

apoptotic chromatin condensation inducer 1	L.PKMPEAVGTDPS.T
thioredoxin interacting protein	Y.LANGQTKVL.T
thioredoxin interacting protein	Y.LANGQTKVL.T
additional sex combs like 3 (Drosophila)	P.GKLLVEPDVKG.V
SET domain containing 5	S.IPKVLR.S
TAF1 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 210kDa-like	M.QVGMATKIK.N
TAF1 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 250kDa	M.QVGMATKIK.N
heat shock 70kDa protein 1-like	N.TVFDAKRLIGR.K
heat shock 70kDa protein 7 (HSP70B)	N.TVFDAKRLIGR.K
heat shock 70kDa protein 6 (HSP70B')	N.TVFDAKRLIGR.K
heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	N.TVFDAKRLIGR.T
glucokinase (hexokinase 4) regulator	R.CIESLLRAI.H
chromodomain helicase DNA binding protein 7	E.IARAAAAAAAVAST.S
desmoplakin	L.RVLLQEEGTRKR.E
ryanodine receptor 1 (skeletal)	P.ALRGEGGSGLLA.A
tetratricopeptide repeat domain 3	Q.PVPPGRAAR.S
NACC family member 2, BEN and BTB (POZ) domain containing	E.LPPAGPGLAPK.R
keratin 18	L.REVEARYAL.Q
AHNAK nucleoprotein 2	E.GELSLADKDV.T
mitochondrial carrier 1	W.KYLSVQGQLFR.G
mucin 19, oligomeric	S.AGVTVTSGQSVR.K
forkhead box K2	E.IFTPPGGGGHGGAAPELPPAQPRPD.A
collagen, type XI, alpha 2	G.PAGPKGAKGATGP.G
collagen, type XIII, alpha 1	P.GTVALVAARA.E
lon peptidase 2, peroxisomal	R.RTYVGSMPGR.I
signal transducer and activator of transcription 3 (acute-phase response factor)	L.STKPPGTFLLR.F
DEAH (Asp-Glu-Ala-Asp/His) box polypeptide 57	S.HGSGGGGGGGGGGGGNRKASSRI.W
collagen, type IV, alpha 3 (Goodpasture antigen)	Q.GVPGAPGPPGEAGPR.G
transcription elongation factor A (SII), 1	A.KSLIKSWKK.L
myosin IA	A.RYLGLLENVRVR.R
cyclin-dependent kinase 13	E.KEGFPITAI.R
oxysterol binding protein	G.GRGDAGPGSGAASGTVVAAAAGGPG.P
unc-80 homolog (C. elegans)	L.DLEAPVVAR.A
collagen, type XVI, alpha 1	S.PGVKGATGPVGP.P
autophagy/beclin-1 regulator 1	E.DALSRIQRLM.A
phosphoribosylformylglycinamidine synthase	I.NILGEGRLALEK.A
cat eye syndrome chromosome region, candidate 6	A.RGGAGGAGGGLGAA.A
PDS5, regulator of cohesion maintenance, homolog A (S. cerevisiae)	S.DLATQNRPLW.Q
F-box protein 27	W.RALVDGQALW.L
ladinin 1	S.LAPGMALGSGRRL.V

mucin 6, oligomeric mucus/gel-forming	L.SLVVDISIPGR.Y
mediator complex subunit 13	R.RPLPTSTNVKT.L
G protein-coupled receptor 98	V.GWRAASVFIR.V
titin	T.FVAGKATSTAT.L
major histocompatibility complex, class I, A	A.VMAPRTLLL.L
ubiquitin specific peptidase 5 (isopeptidase T)	M.DAALNKEEL.L
WNK lysine deficient protein kinase 1	L.HTRTPPIIHR.D
WNK lysine deficient protein kinase 2	L.HTRTPPIIHR.D
WD repeat domain 82	I.RYFPGHSKR.V
EPH receptor B3	C.KVSDFGLSR.F
EPH receptor B2	C.KVSDFGLSR.F
EPH receptor A5	C.KVSDFGLSR.V
EPH receptor A2	C.KVSDFGLSR.V
EPH receptor A6	C.KVSDFGLSR.V
tankyrase 1 binding protein 1, 182kDa	L.FPGLSPSALKAK.L
HECT and RLD domain containing E3 ubiquitin protein ligase 2	L.AVIGGIDGRLR.L
PRP8 pre-mRNA processing factor 8 homolog (S. cerevisiae)	F.REAVVNTQELL.D
protein kinase, DNA-activated, catalytic polypeptide	L.KSKDFVQVMR.H
chromosome 2 open reading frame 72	A.RAAGAAGAAAAAAR.A
polyhomeotic homolog 1 (Drosophila)	R.KGTGVVQPLPAA.Q
polo-like kinase 1	V.FAGKIVP.K
transcription termination factor, RNA polymerase II	P.AAPGLSLGEGREAATSS.D
lipoyl(octanoyl) transferase 2 (putative)	L.RALGAEVR.V
pannexin 2	Q.DDKAGALAALL.L
endothelial PAS domain protein 1	S.KETEVFYEL.A
Rap guanine nucleotide exchange factor (GEF) 6	V.SVTPEGVIKQR.R
chromosome 16 open reading frame 58	W.LVKDSTGMLGR.I
poly(A) binding protein, cytoplasmic 4 (inducible form)	R.KVFVGRFKSR.K
dachsous 1 (Drosophila)	G.PGAGGPYPRGGSLD.P
splicing factor 3b, subunit 1, 155kDa	L.KIKNGTPPMR.K
centromere protein K	F.SESRIFNEL.K
chaperonin containing TCP1, subunit 6A (zeta 1)	A.DALLIIPKV.L
chaperonin containing TCP1, subunit 6B (zeta 2)	A.DALLIIPKV.L
spastic ataxia of Charlevoix-Saguenay (sacsin)	G.KQIAKVPGNVDA.A
SET nuclear oncogene	I.KWKSGKDLTKR.S
REST corepressor 2	E.KPSAGSGILSR.S
ornithine decarboxylase antizyme 1	S.DAPHPPLKI.P
multiple EGF-like-domains 8	Y.ISGGFGGVALGRL.L
interleukin 17 receptor C	F.HPPGTPAPGRGV.G
perilipin 4	A.MSMAKGAVQGGLD.T
cat eye syndrome chromosome region, candidate 6	R.GARGGAGGAGGGLGA.A
Ets2 repressor factor	D.LGAFRGPPLAR.L

major histocompatibility complex, class I, C	Q.FAYDGKDYI.A
major histocompatibility complex, class I, B	Q.FAYDGKDYI.A
major histocompatibility complex, class I, E	Q.FAYDGKDYL.T
plastin 1	Y.AISVARKIGAR.I
progesterone receptor	C.KAPGASGCLL.P
interferon-induced protein with tetratricopeptide repeats 5	L.RYAAKFYRR.K
family with sequence similarity 186, member A	A.EAEKLGIIKA.K
G protein-coupled receptor 37 (endothelin receptor type B-like)	K.VSASSALGVAPASR.N
lon peptidase 2, peroxisomal	R.RTYVGSMPGR.I
coiled-coil domain containing 74A	S.GAGVAAGTRPPSS.P
HECT and RLD domain containing E3 ubiquitin protein ligase family member 1	D.RLIGLPEGRAR.N
titin	E.PHVVPKA.V
ribosomal protein L28	R.SQKPVMVKR.K
tetra-peptide repeat homeobox 1	P.SPAQIPGPGRL.Q
loricrin	V.KTSGGGGGGGSGGG.G
AT hook containing transcription factor 1	S.SMKSPLYLVSR.S
SPEG complex locus	G.TRAPPSPGVPP.K
Ras association (RalGDS/AF-6) domain family (N-terminal) member 7	R.PDRGPPGTQGP.L
casein kinase 1, alpha 1	L.RQLFRILFR.T
nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4	S.VRLGGPGGGAGGAG.G
Y box binding protein 1	G.GPGGLTSAAPAGGDKKVI.A
heat shock protein 90kDa alpha (cytosolic), class B member 1	L.KEFEGKTLV.S
crooked neck pre-mRNA splicing factor-like 1 (Drosophila)	L.LPPPPQQKI.T
leucine rich repeat containing 33	S.LSLHSCHLER.I
sterile alpha motif domain containing 1	V.SYKGSISYR.N
oxysterol binding protein	F.KVQPVIGENGGDARQR.G
heat shock protein 90kDa alpha (cytosolic), class B member 1	I.KEKYIDQEEL.N
heat shock protein 90kDa alpha (cytosolic), class A member 1	I.KEKYIDQEEL.N
heat shock protein 86, partial	T.KEKYIDQEEL.N
mediator of DNA-damage checkpoint 1	N.GVVPAGVILER.S
major histocompatibility complex, class I, B	W.EAARVAEQLRA.Y
major histocompatibility complex, class I, A	W.EAARVAEQLRA.Y
ribosomal protein L28	A.IRRASAILR.S
smg-5 homolog, nonsense mediated mRNA decay factor (C. elegans)	I.RSFGHFIARL.Q
keratin 15	S.RSISASSARFVS.S
asparagine-linked glycosylation 3, alpha-1,3-mannosyltransferase homolog (S. cerevisiae)	S.LAVSVKMNVL.L
ribosomal protein L28	F.RYNGLIHRK.T

PRP8 pre-mRNA processing factor 8 homolog (S. cerevisiae)	E.RLKEAYSVKSR.L
lipin 1	S.DGLRDLPSI.A
vacuolar protein sorting 13 homolog C (S. cerevisiae)	V.SSIPIISGGTK.G
TEK tyrosine kinase, endothelial	P.ADLGGGKMLLI.A
ADAMTS-like 2	S.LVTHIPAGAR.D
tumor protein p53 binding protein 1	I.RQGGKAPVT.P
Rap guanine nucleotide exchange factor (GEF) 2	V.SVTPEGVIKQR.R
Ca++-dependent secretion activator 2	P.GRAGGGGAARSVSP.S
KN motif and ankyrin repeat domains 4	P.KEPPASSSSP.P
solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3	E.KGSSASLVL.K
proline rich 14-like	H.CAPARLALGEA.L
PRP8 pre-mRNA processing factor 8 homolog (S. cerevisiae)	F.ASYKWNVSR.P
nucleoporin 210kDa-like	N.LPRSVDVEL.L
UPF1 regulator of nonsense transcripts homolog (yeast)	R.YKGDLAPLWK.G
uncharacterized LOC100271840	P.SRASLIGTPSR.A
intercellular adhesion molecule 5, telencephalin	E.LAASPPGGVR.P
biorientation of chromosomes in cell division 1-like 1	R.KREVSPPGART.R
ribosomal protein L4	A.ATKKPAPEK.K
ribosomal protein L4	D.KAAAAAAALQAKS.D
SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4	A.GMFDQKSSSHER.R
SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 2	A.GMFDQKSSSHER.R
fructosamine 3 kinase related protein	D.LFRDLEII.P
keratin 9	S.YSRFSSSGGGGGGGR.F
zinc finger and BTB domain containing 32	C.PPRPHPPPAPPARSR.P
catenin (cadherin-associated protein), alpha 1, 102kDa	L.LAYLQRIAL.Y
diacylglycerol kinase, iota	A.RAPAAAAAAAASP.P
KIAA0319-like	E.LSGGPKNVSVQ.P
DEAH (Asp-Glu-Ala-His) box polypeptide 33	D.VLFGVVKAAQ.K
piezo-type mechanosensitive ion channel component 2	N.MITTAGKVVVT.I
ribosomal protein L4	K.GESSGKNVTL.P
CCR4-NOT transcription complex, subunit 1	I.DLAALASRRE.Y
dachsous 1 (Drosophila)	Y.RLASGGDGHFR.L
PHD finger protein 20-like 1	L.VKAAAAAAAKNKTG.S
E1A binding protein p400	Q.NVRAGAPGPGLG.L
EP400 N-terminal like	Q.NVRAGAPGPGLG.L
acetylcholinesterase	P.ARLREALSDVVG.D
transmembrane protein 102	K.VARKGGGLAGVGG.G
proteasome (prosome, macropain) subunit, beta type, 2	F.KMSEKILLL.C

cytochrome c-1	C.HSMDFVAYR.H
cytochrome P450, family 51, subfamily A, polypeptide 1	Q.RLKDSWVER.L
mindbomb E3 ubiquitin protein ligase 2	R.ERQAGGGAAPGP.R
H6 family homeobox 1	L.LAAEAKGAGRAT.Q
ribosomal protein S6 kinase, 90kDa, polypeptide 5	D.DLAAKKVPA.P
transmembrane protein 102	A.GTLPHYFLNGR.Q
cubilin (intrinsic factor-cobalamin receptor)	I.LTGPYGSIKS.P
Ras association (RalGDS/AF-6) domain family (N-terminal) member 7	L.VSRALEAAER.A
EFR3 homolog B (S. cerevisiae)	A.VIKTVGSFAS.T
collagen, type V, alpha 3	G.KKGPPGEDGAKGSVGPTGLPG.D
leucine rich repeat containing 8 family, member A	L.RYFADTQPAYR.I
endothelial PAS domain protein 1	S.KETEVFYEL.A
thyroid hormone receptor interactor 12	A.SLNPKTWGR.L
solute carrier family 27 (fatty acid transporter), member 4	Q.VRMALGNGLR.Q
eukaryotic translation initiation factor 3, subunit H	S.TYYGSFVTR.A
zw10 kinetochore protein	A.FSVLGELHSKL.K
eukaryotic translation initiation factor 3, subunit H	S.TYYGSFVTR.A
K(lysine) acetyltransferase 7	L.RYKEKVAELR.K
family with sequence similarity 21, member C	S.ASNLKGASLL.P
mitochondrial carrier 2	L.RSMVQFIGR.E
keratin 17	S.SFGGVDGLLAGGEK.A
Ras association and DIL domains	P.GTFGQLLLKI.A
ornithine decarboxylase antizyme 1	S.DAPHPPLKI.P
vacuolar protein sorting 33 homolog B (yeast)	K.LVTDKAAGKI.T
periplakin	E.YVVKEVL.R
valyl-tRNA synthetase 2, mitochondrial	A.REVAAELTGR.P
KIAA1147	G.RGGGGGARPAAEPPR.R
collagen, type II, alpha 1	Q.GASGPAGPSGPRG.P
SP100 nuclear antigen	C.RNLVPVQR.V
DEAD (Asp-Glu-Ala-Asp) box polypeptide 52	A.LIISPTREL.A
DEAD (Asp-Glu-Ala-Asp) box polypeptide 10	V.LIISPTREL.A
immunoglobulin mu binding protein 2	S.KRAPRPRAALG.P
Ras and Rab interactor 3	Y.RPLDGGGGGGGGSPP.C
DNA (cytosine-5-)-methyltransferase 1	D.SSKPLYLAR.V
collagen, type XXVII, alpha 1	S.RPVPARVSRP.A
scribbled homolog (Drosophila)	S.DAPPSRVSV.I
inositol-trisphosphate 3-kinase A	F.KAAGTSGLILK.R
transforming, acidic coiled-coil containing protein 3	Q.KELSKAEIQ.K
purine-rich element binding protein B	L.RVSEVKPSYR.N
F-box protein 10	N.RGSGLQLLPR.S
Fc fragment of IgG, low affinity IIa, receptor (CD32)	E.PPGRQMIAI.R
Treacher Collins-Franceschetti syndrome 1	E.SPRKGAAPAPPGK.T

FYVE, RhoGEF and PH domain containing 5	L.LSLEGKPL.E
TBC1 domain family, member 2B	L.DARLISI.S
polymerase (RNA) II (DNA directed) polypeptide A, 220kDa	V.RILPWSTFR.L
progestin and adipoQ receptor family member VII	N.KYIQKPGLLGR.T
	G.ISTPRPKIV.S
transcription elongation factor B polypeptide 3C (elongin A3)	MAAGSTTLR.A
laminin, gamma 1 (formerly LAMB2)	T.LASARPGPGVPA.T
midnolin	A.AAAAAAARGDPSI.A
ADP-ribosylation-like factor 6 interacting protein 4	R.TKGSGAAL.P
cell cycle associated protein 1	Y.KVLKEIVER.V
keratin 9	S.RGGSGGSHGGGSGFG.G
keratin 9	S.GGGGGGGLGSGGSIR.S
solute carrier family 16, member 1 (monocarboxylic acid transporter 1)	S.YAFPKSITV.F
hook homolog 2 (Drosophila)	E.GTPGLTAK.K
SET domain containing 1A	P.PEPPAGPPAPAPR.P
sorting nexin family member 30	L.GTIDRIAQR.I
staufen, RNA binding protein, homolog 1 (Drosophila)	K.KEKEPEYTLL.T
structural maintenance of chromosomes 2	A.YKDPEKNWNR.N
cytochrome c-1	C.HSMDFVAYR.H
eukaryotic translation elongation factor 2	P.EGKKLPRTFC.Q
immediate early response 3	R.RVLYPRVVRR.Q
ring finger protein 40, E3 ubiquitin protein ligase	D.ATLLIVNR.Y
neuron navigator 1	L.RKQKSLTN.L
nucleoporin 188kDa	C.PPLLHR.A
5'-nucleotidase, cytosolic III-like	G.RVQEIVGALR.K
cyclin G associated kinase	K.PPIIHR.D
myosin XVI	S.PPLLHR.A
WNK lysine deficient protein kinase 1	T.PPIIHR.D
WNK lysine deficient protein kinase 2	T.PPIIHR.D
WD repeat domain 87	A.GEKLS.P
transcription factor AP-4 (activating enhancer binding protein 4)	D.GEKLS.K
titin	E.GEKLS.I
microtubule-associated protein 1B	E.GEKLS.P
KIAA1199	G.GEKIS.D
echinoderm microtubule associated protein like 5	K.GEKLS.I
transient receptor potential cation channel, subfamily C, member 6	L.GEKLS.M
NGFI-A binding protein 2 (EGR1 binding protein 2)	L.GEKLS.P
zinc finger, FYVE domain containing 26	L.GEKLS.W
dystonin	N.GEKLS.Q
zinc finger protein 687	Q.RVSVFKCPSCPL.L

bassoon (presynaptic cytomatrix protein)	S.GEKLS.S
defensin, beta 128	S.GEKLS.V
neurocan	G.KPAVPPGTPTA.A
eukaryotic elongation factor, selenocysteine-tRNA-specific	P.VAAKPGGPEAPET.E
teneurin transmembrane protein 2	Q.ARQRALGTAWAK.E
major histocompatibility complex, class I, A	Q.YAYDGKDYIAL.K
major histocompatibility complex, class I, B	Q.YAYDGKDYIAL.N
major histocompatibility complex, class I, C	Q.YAYDGKDYIAL.N
major histocompatibility complex, class I, G	Q.YAYDGKDYLAL.N
collagen, type I, alpha 2	V.AGAVGEPGPLGIAGPPGARGPPGAVGSPGV.N
dolichyl-phosphate (UDP-N-acetylglucosamine) N-acetylglucosaminephosphotransferase 1 (GlcNAc-1-P transferase)	L.KVLGPIHER.N
cleavage stimulation factor, 3' pre-RNA, subunit 2, 64kDa, tau variant	L.LSVTGEVEPR.G
ribosomal protein S17	T.VGMNFKTPR.G
polymerase (RNA) II (DNA directed) polypeptide A, 220kDa	V.RILPWSTFR.L
hemicentin 1	Q.RVLSSGSLQIA.F
stearoyl-CoA desaturase (delta-9-desaturase)	T.ITAPPSRVL.Q
trio Rho guanine nucleotide exchange factor	F.ERDAIDII.S
germ cell associated 2 (haspin)	A.ASLPGPGSRLF.R
stearoyl-CoA desaturase (delta-9-desaturase)	T.ITAPPSRVL.Q
insulin-like growth factor 2 mRNA binding protein 2	G.KGGKTVNELQ.N
ABI family, member 3 (NESH) binding protein	E.RTTSAGTITPKI.S
cysteine-rich protein 1 (intestinal)	A.AMFGPKGFGR.G
anaphase promoting complex subunit 1	Q.RTMALPVGR.G
poly(rC) binding protein 1	A.ASRPPVTLR.L
poly(rC) binding protein 2	A.ASRPPVTLR.L
heat shock protein 90kDa alpha (cytosolic), class B member 1	N.KEIFLRELI.S
heat shock protein 90kDa alpha (cytosolic), class A member 1	N.KEIFLRELI.S
heat shock protein 90kDa beta (Grp94), member 1	N.KEIFLRELI.S
phosphatidylinositol transfer protein, beta	L.KSKVPAFVR.M
pericentriolar material 1	T.NKSKDASTSPPNRETIG.S
transmembrane protease, serine 4	C.RQMGYSSKPTFR.A
UPF1 regulator of nonsense transcripts homolog (yeast)	V.IIVGNPKAL.S
major histocompatibility complex, class I, E	Q.FAYDGKDYLTL.N
diacylglycerol kinase, gamma 90kDa	F.AKHPPVAVL.P
keratin 9	S.SGAGKIGLGGRGGSGGS.Y
zinc finger protein 516	E.SKAGIAASVSI.L
thioredoxin-related transmembrane protein 3	A.AWKSWTALR.L
zinc finger protein 574	R.RTHGVGGVPLPTT.P
protocadherin gamma subfamily A, 6	I.VKDLGLEPQEL.A

leucine rich repeat containing 26	A.PGTFAPLR.A
structural maintenance of chromosomes 5	L.AGKPAFMGR.A
adenomatosis polyposis coli 2	K.VASALVPGRR.A
Y box binding protein 1	T.KPGTTGSGAGSGGPGGLTS.A
major histocompatibility complex, class I, C	W.AAVVVPSGQEQR.Y
major histocompatibility complex, class I, A	W.AAVVVPSGQEQR.Y
major histocompatibility complex, class I, B	W.AAVVVPSGQEQR.Y
macrophage receptor with collagenous structure	P.KGAPGQAGQK.G
glyoxylate reductase/hydroxypyruvate reductase	R.RLKPFGVQR.F
chromosome 2 open reading frame 78	P.IQGAPKAKI.Q
ribosomal protein S3	T.RTQNVLGEKGR.R
hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	S.KESEVFYEL.A
Treacher Collins-Franceschetti syndrome 1	E.KAGKTGN.S
Snf2-related CREBBP activator protein	S.LVPPKDLL.P
Kallmann syndrome 1 sequence	C.LAAGPGAAAARR.L
DENN/MADD domain containing 6A	F.HTVLKQIAP.E
aminopeptidase-like 1	A.RAFPLFTHR.S
selenoprotein N, 1	F.RLFVPNHR.S
ribosomal protein L28	R.SQKPVMVKR.K
transforming growth factor beta 1 induced transcript 1	F.SSSSGVLGTGLCELDR.L
v-rel reticuloendotheliosis viral oncogene homolog A (avian)	Q.AVAPPAPKPTQAGEG.T
huntingtin	Y.WLVRTELL.E
trinucleotide repeat containing 6C	S.NNKAPSGPGVWG.D
serine peptidase inhibitor, Kunitz type 1	R.REIPIPSTGSVEM.A
ADP-ribosylation factor guanine nucleotide-exchange factor 2 (brefeldin A-inhibited)	L.GNLVSGGVDKR.Q
collagen, type XI, alpha 2	G.PKGAKGATGPGGPKG.E
hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	S.KESEVFYEL.A
microtubule associated serine/threonine kinase 3	R.KNVAKGRMAR.R
frizzled family receptor 8	G.GDAAAPPARGGGGGGK.A
ankyrin 2, neuronal	T.AILTTDVSDK.A
cysteine-rich protein 1 (intestinal)	M.FGPKGFGR.G
heat shock protein 90kDa alpha (cytosolic), class A member 1	L.KEFEGKTLV.S
SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4	R.RAFVPQLR.S
kinesin family member 26B	Q.RSHSPVPAAAP.A
collagen, type XXVII, alpha 1	E.MGPKGPPGAV.G
ADP-ribosylation factor 5	S.RIFGKKQMR.I
ADP-ribosylation factor 4	S.RLFGKKQMR.I
bromodomain adjacent to zinc finger domain, 1B	K.HSVIPPAAR.S
keratin 15	I.AIREAASGGGGSSS.N

RUN and SH3 domain containing 1	L.RVITTVDEDWLR.C
CDK5 regulatory subunit associated protein 2	K.NGRHVLGL.I
SERPINE1 mRNA binding protein 1	K.KEETQPPVAL.K
pyridoxamine 5'-phosphate oxidase	N.RLHDRIVFR.R
nuclear transcription factor, X-box binding-like 1	G.TSPGGVATTAAAGSRHSPAGSQ.A
tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein	K.KEMQPTHPIRL.G
fidgetin-like 2	G.RCLATQLGATL.L
megakaryocyte potentiating factor-like CRA isoform	N.SLLAGGKTSLGP.P
cell cycle associated protein 1	Y.KVLKEIVER.V
Y box binding protein 1	G.TTGSGAGSGGPGGLTSAAPAGGDKKVI.A
keratin 75	N.RGLGGSGSSVK.F
pim-2 oncogene	T.RVYSPPEWISR.H
Sin3A-associated protein, 130kDa	D.YPAERSSLIP.I
Ets2 repressor factor	F.KLQPPPLGR.R
epiplakin 1	G.FVIDPVRNL.R
eukaryotic translation initiation factor 4 gamma, 1	L.PRGPAGLGPR.R
autoimmune regulator	A.RLAPGPA.K
transmembrane protein 87B	C.RSVAGLLPR.R
SET domain containing 1B	S.IPAQPHASTR.A
agrin	W.RYLKGKDLVAR.E
serine peptidase inhibitor, Kunitz type 1	R.REIPIPSTGSVEM.A
keratin 9	L.SRSGGGGGGGLGSGGSIR.S
chaperonin containing TCP1, subunit 6A (zeta 1)	MAAVKTLNPK.A
coatomer protein complex, subunit beta 1	H.AIKSVKIYR.G
major histocompatibility complex, class I, C	W.TAADTAAQISHRK.L
collagen, type IV, alpha 1	P.PGARGPPGGQGPPGL.S
SH3 and multiple ankyrin repeat domains 1	F.DIRGSPTGGAGGSA.D
cation channel, sperm associated 4	D.RMGFGGAVAAL.R
fumarate hydratase	A.NDIRFLGSGPR.S
transcription termination factor, RNA polymerase II	Q.KSLPQGHFQER.P
proline-rich basic protein 1	Q.RSPVGPAGVR.S
chromosome 2 open reading frame 71	G.SSPCSPELQGGTR.R
BRF1 homolog, subunit of RNA polym III transcription initiation factor IIIB	R.PKAKGGLASLAKD.G
eukaryotic translation initiation factor 3, subunit E	D.FAMDVYKNL.Y
suppressor of glucose, autophagy associated 1	N.RVLLSNLQR.C
zinc finger protein 207	R.RAQLPKYQR.N
KH domain containing, RNA binding, signal transduction associated 1	L.QEETGAKISVL.G
ras homolog family member B	D.IEVDGKQVEL.A
ras homolog family member A	D.IEVDGKQVEL.A
ras homolog family member C	D.IEVDGKQVEL.A
solute carrier family 4, sodium bicarbonate	F.ILLGPSGRAKS.Y

cotransporter, member 5	
CUB domain containing protein 1	T.MLSIKSGERI.V
keratin 2	A.FGGSGGRGSSSGGG.Y
coatomer protein complex, subunit beta 1	G.PDAAVTGHIRIR.A
human immunodeficiency virus type I enhancer binding protein 1	Q.ISSAAQDKIEL.Q
chemokine (C-C motif) receptor 2	V.KVTTQGLL.D
lethal giant larvae homolog 1 (Drosophila)	E.VPAAAVGGEKR.P
cadherin, EGF LAG seven-pass G-type receptor 2 homolog (Drosophila)	N.ITVGGIPGPAGGVAR.G
chaperonin containing TCP1, subunit 5 (epsilon)	A.DGYEQAARV.A
proline-rich coiled-coil 2C	E.RPSILSASEL.K
mucin 19, oligomeric	K.TGLSAGVTGKTG.L
chromodomain helicase DNA binding protein 4	N.AIRGGKKASR.M
CDC-like kinase 1	S.RSINEKDYHSR.R
myosin, heavy chain 13, skeletal muscle	T.IAVTGDKK.K
zinc finger protein 516	G.WGVSGPGLEHR.G
APEX nuclease (apurinic/apyrimidinic endonuclease) 2	N.SYFSFSRNR.S
zinc finger CCCH-type containing 14	L.RVLSGHLMQTR.D
collagen, type V, alpha 2	G.PKGEVGAPGSKGEAGP.T
protein-O-mannosyltransferase 2	G.PQAARAAGRDVAA.E
calcium channel, voltage-dependent, T type, alpha 1I subunit	I.SSLKPIGNIVL.I
phosphatidylinositol binding clathrin assembly protein	S.RYLNEKAVSYR.Q
mitochondrial ribosomal protein L14	T.RIKTPIPTSLR.K
cyclin G associated kinase	H.RQKPPIIHR.D
ribosomal protein S19	A.ASTARHLYL.R
MLX, MAX dimerization protein	V.TALKIMKV.N
DEAH (Asp-Glu-Ala-His) box polypeptide 15	S.RIMDRFNLPR.R
keratin 9	Y.SRFSSSGGGGGGGR.F
dynein, axonemal, heavy chain 7	E.KIRNASTAAEGLC.K
deoxynucleotidyltransferase, terminal, interacting protein 2	D.PGLSIKQLGGLY.I
chromosome 1 open reading frame 127	V.RPAALLTPE.A
KH-type splicing regulatory protein	I.PAGKAGLVIGKGG.E
serine/arginine repetitive matrix 2	A.GLAARMSQVPA.P
REST corepressor 2	P.TSLSQPPPLLR.P
poly(rC) binding protein 1	A.SRPPVTLR.L
poly(rC) binding protein 2	A.SRPPVTLR.L
pim-2 oncogene	T.RVYSPPEWISR.H
poly(rC) binding protein 4	V.SRPPVTLR.L
ZFP64 zinc finger protein	A.KFKIS.S
solute carrier organic anion transporter family, member 1B7	K.KFKLS.L

FERM and PDZ domain containing 3	L.KFKIS.P
Rho guanine nucleotide exchange factor (GEF) 1	S.LKVPAPASR.P
eukaryotic translation initiation factor 2, subunit 2 beta, 38kDa	F.VMKPPQVVR.V
isocitrate dehydrogenase 3 (NAD+) gamma	C.KSLPGVVTR.H
proteasome (prosome, macropain) 26S subunit, non-ATPase, 7	N.LINNKIANR.D
structural maintenance of chromosomes 5	L.AGKPAFMGR.A
PDS5, regulator of cohesion maintenance, homolog A (S. cerevisiae)	T.ALCGVVSADGK.I
forkhead box D1	K.AGGPGASALAR.S
structural maintenance of chromosomes 5	L.AGKPAFMGR.A
myeloid leukemia factor 2	F.RQQRPLEFR.R
collagen, type V, alpha 2	G.DRGDPGPAGLPGSQG.A
Rho family GTPase 1	E.RRAPQPVVAR.C
discs, large (Drosophila) homolog-associated protein 3	R.RCSSADGLDGP.A
collagen, type XXVII, alpha 1	G.LPGFPGARGKPGPL.G
ATPase family, AAA domain containing 5	S.GELKAAAEALS.F
mortality factor 4 like 1	L.KYVDTNLQKQR.E
catenin (cadherin-associated protein), delta 2	P.LAAPQGGSPTKL.Q
inositol 1,4,5-trisphosphate receptor, type 2	H.NRELQNFLR.N
RAB7A, member RAS oncogene family	Y.NEFPEPIKL.D
ribosomal protein L28	R.SQKPVMVKR.K
SET binding protein 1	F.LPVSSAKPPA.A
cysteine-rich protein 1 (intestinal)	A.MFGPKGFGR.G
anaphase promoting complex subunit 1	D.FVKPEFLL.L
desmoplakin	E.TGMRLLEAQI.A
sosondowah ankyrin repeat domain family member B	Q.RPPVPAAAAAGAQ.A
keratin 9	S.GGGGGGGLGSGGSIRSS.Y
keratin 9	K.KGPAA.I
ribosomal protein S3	L.RYKLLGGLAVR.R
tubulin tyrosine ligase-like family, member 4	W.IVKPPASAR.G
keratin 17	S.IKGSSGLGGGSSR.T
anaphase promoting complex subunit 7	A.KTLLDKALTQR.P
collagen, type V, alpha 2	A.GRVGPPGPAGAPGPAG.P
cadherin-related 23	I.ITAIDQDKGR.P
KIAA0368	G.KAWPRNAETQR.C
pelota homolog (Drosophila)	T.RAKVEVNIPR.K
FAT tumor suppressor homolog 3 (Drosophila)	V.VTLKVFLDD.V
anoctamin 8	A.DEGGGGGSGGGGRR.C
heparan sulfate proteoglycan 2	P.IQPGGKCRPV.N
desmoplakin	F.LRGAGSIAGASA.S
patatin-like phospholipase domain containing 2	S.STNIHELRV.T
teneurin transmembrane protein 4	N.VSLGKAALVGIYG.R

ribosomal protein L36a	MVNVPKTR.R
TCDD-inducible poly(ADP-ribose) polymerase	R.KMFGRDRIINER.H
tripartite motif containing 67	C.KSPGGAGAGATGGSTA.R
heat shock protein 90kDa alpha (cytosolic), class B member 1	E.KYIDQEELNKTK.P
heat shock protein 90kDa alpha (cytosolic), class A member 1	E.KYIDQEELNKTK.P
SET binding protein 1	G.KGIPVGGER.M
basic leucine zipper and W2 domains 1	T.VEWNKKEEL.V
mitogen-activated protein kinase 8 interacting protein 2	A.PAAPRPGPAQP.G
YY1 transcription factor	L.VTVAAAGKSGGGGSSSSGGG.R
up-regulated during skeletal muscle growth 5 homolog (mouse)	K.KYFNSYTLTGR.M
collagen, type V, alpha 2	R.GRPGPAGPPGSQG.P
Treacher Collins-Franceschetti syndrome 1	A.KGTISAPGKVVTA.A
interferon, gamma-inducible protein 16	K.TVAKC.Q
dishevelled, dsh homolog 1 (Drosophila)	L.TVAKC.W
dishevelled, dsh homolog 2 (Drosophila)	L.TVAKC.W
KIAA0947	S.TVAKC.D
NOP2/Sun RNA methyltransferase family, member 2	I.RTFVPKNER.L
sal-like 3 (Drosophila)	L.LGGPPLTKAEP.V
major histocompatibility complex, class I, B	A.ADKAAQITQR.K
ATPase, H+ transporting, lysosomal V0 subunit a2	E.EGSRESGATI.P
regulator of chromosome condensation 2	R.KRGGPAGR.K
NLR family, pyrin domain containing 4	V.DADIPALLGTK.I
golgi brefeldin A resistant guanine nucleotide exchange factor 1	F.RLPGEAPVIQR.L
MORC family CW-type zinc finger 3	Q.RILEMNDKYVK.K
SIX homeobox 5	Q.RDRTGAGGGAPCK.S
trophoblast glycoprotein	L.AVLPAGAFARR.P
SON DNA binding protein	L.RYKPDLKEGSR.K
heat shock protein 90kDa beta (Grp94), member 1	V.KNLGTIAKSGT.S
TAF4 RNA polymerase II, TATA box binding protein-associated factor,135kDa	Q.QPPKPGALIR.P
collagen, type VII, alpha 1	P.PGLKGAKGEPG.S
attractin	C.FGNKAAVL.S
titin	V.FAENLAGLSK.P
nuclear export mediator factor	A.RAAEPLLTL.E
collagen, type XIII, alpha 1	A.RGPGELGAPGTVAL.V
glucosamine (N-acetyl)-6-sulfatase	M.RLLPLAPGRLR.R
leucine rich repeat containing 37, member A3	N.ITVKPADVEVT.I
ArfGAP with FG repeats 2	F.GDLGSAKLGQR.P
interferon, gamma-inducible protein 16	L.KVKGPALSR.K
eukaryotic translation initiation factor 3, subunit E	V.KEIYNEKELL.Q
structural maintenance of chromosomes 3	E.KTFMPKQR.S

GLIS family zinc finger 3	S.RSGTAAGAVPPPHP.V
zinc finger, MIZ-type containing 2	H.GGPRGPSVPAGM.N
RBBP8 N-terminal like	A.ALAAAGLSGGRHT.Q
sushi, von Willebrand factor type A, EGF & pentraxin domain containing 1	C.GKPPPI.Q
uncharacterized LOC402483	A.PSSAK.A
zinc finger, DBF-type containing 2	K.PSSAK.A
neural precursor cell expressed, developmentally down-regulated 9	P.PSSAK.G
formin 1	P.PSSAK.V
thyroid hormone receptor interactor 12	S.PSSAK.K
exportin 6	S.PSSAK.L
VGF nerve growth factor inducible	S.PSSAK.R
microtubule-associated protein tau	S.PSSAK.S
non-SMC condensin II complex, subunit D3	T.PSSAK.E
mitochondrial ribosomal protein L41	F.QLYPRNFLR
zinc finger protein 207	R.RAQLPKYQR.N
AT-hook transcription factor	T.LAGRSSSNAPK.Y
family with sequence similarity 53, member B	S.KVWTPVEKR.R
desmoplakin	K.LLSAERAVTG.Y
transient receptor potential cation channel, subfamily C, member 6	I.VEAILSHPAFAEGK.R
non-SMC condensin I complex, subunit G	A.KTLPKIVGR.T
nuclear protein, ataxia-telangiectasia locus	L.MILSRAAISR.T
seryl-tRNA synthetase	L.KEFMPPGLQEL.I
leucine-rich repeats and calponin homology (CH) domain containing 4	L.RAVVGGAAAVS.T
LIM domain kinase 2	R.FAKGIAS.G
suppressor of cytokine signaling 7	Q.PAGPGVKTVGGG.C
discs, large homolog 1 (Drosophila)	L.KEAGSIVRL.Y
discs, large homolog 4 (Drosophila)	L.KEAGSIVRL.Y
discs, large homolog 2 (Drosophila)	L.KEAGSIVRL.Y
Rap guanine nucleotide exchange factor (GEF) 6	S.SQALAVPESTGALEKTEHAS.G
B-cell CLL/lymphoma 6	P.PNAPLNRKGLVS.P
zinc finger and BTB domain containing 4	A.KPVGGIGGGGGPP.T
SOGA family member 2	L.RVLHSPPAVR.R
bromodomain adjacent to zinc finger domain, 2A	V.LEKALLSTPNGAPE.G
protein tyrosine phosphatase-like	A.AAKGNGGGGGRAGAG.D
origin recognition complex, subunit 1	E.RTLTPISGGQR.S
lemur tyrosine kinase 3	D.PEGRGAGETLAGDPA.E
family with sequence similarity 53, member B	S.KVWTPVEKR.R
galactokinase 1	E.LAVSAPGRVN.L
ets variant 4	A.RLWGIQKNR.P
DEAH (Asp-Glu-Ala-His) box polypeptide 33	L.RVFQGAPKGYR.K
lon peptidase 2, peroxisomal	K.RVIAIRPIR.R

keratin 17	S.SIKGSGGLGGGSSR.T
testis expressed 2	T.APSSPLTSPSDTRS.F
collagen, type V, alpha 1	P.GPDGLRGIPGPVG.E
uracil-DNA glycosylase	Q.RNKAAALLRLAA.R
anoctamin 1, calcium activated chloride channel	V.AFFKGRFVGR.P
anoctamin 2	V.AFFKGRFVGR.P
PTK7 protein tyrosine kinase 7	D.RILDPTKLGPR.M
platelet-derived growth factor receptor, alpha polypeptide	S.GAFGKVVEG.T
proteasome (prosome, macropain) subunit, beta type, 2	D.RYYTPTISR.E
LIM domain 7	I.SAVEPKTAL.P
ribosomal protein S21	M.GESDDSILRL.A
collagen, type V, alpha 1	G.PKGNSGGDGPAGPPGERG.P
coiled-coil domain containing 124	L.KGGKAPRVA.T
SET domain containing 1A	Q.GKGLIAASAGPPG.G
synergin, gamma	K.RVELGIKATAVC.S
ets variant 3	I.KLQPPPVGR.K
ATP synthase mitochondrial F1 complex assembly factor 1	L.RVFPVRPGSGR.P
frizzled family receptor 8	A.SKGAAVGGGAGATA.A
zinc finger protein 281	T.RVKTPTSQSYR
keratin 17	C.RLSGGLGAGSCR.L
major histocompatibility complex, class I, C	A.VSRPGR.G
major histocompatibility complex, class I, G	A.VSRPGR.G
major histocompatibility complex, class I, B	A.VSRPGR.G
major histocompatibility complex, class I, F	A.VSRPGR.G
mucin 5AC, oligomeric mucus/gel-forming	D.VSRPGR.G
major histocompatibility complex, class I, C	S.VSRPGR.G
major histocompatibility complex, class I, E	S.VSRPGR.G
major histocompatibility complex, class I, B	S.VSRPGR.G
major histocompatibility complex, class I, A	S.VSRPGR.G
adaptor-related protein complex 5, beta 1 subunit	V.VSRPGR.D
RUN and SH3 domain containing 1	S.VVEASVKPGSS.T
general transcription factor IIIC, polypeptide 1, alpha 220kDa	M.RYIPPIPVHR.D
REX1, RNA exonuclease 1 homolog (S. cerevisiae)	G.LSGLTTLFPGQKRR.I
microtubule-associated protein 7	P.DPPPVLRV.D
collagen, type III, alpha 1	P.AGANGAPGLR.G
chaperonin containing TCP1, subunit 6A (zeta 1)	G.TTSNVLIIGELLKQ.A
chaperonin containing TCP1, subunit 6B (zeta 2)	G.TTSNVLIIGELLKQ.A
filamin C, gamma	K.VFTKGAGSGEL.K
hydroxysteroid (11-beta) dehydrogenase 1	S.RVNVSITLCVL.G
chromosome 2 open reading frame 78	W.DSTASTVKKSSSL.R
spectrin, alpha, non-erythrocytic 1	D.KEAIVTSEEL.G

KIAA1430	E.AVKPTVGMKR.S
zinc finger, C2HC-type containing 1A	V.SSSSSSLGNKLQ.T
glyoxylate reductase/hydroxypyruvate reductase	R.RLKPFGVQR.F
transmembrane protein 108	S.LSTGPAPAAMATTSSKPEGR.P
ribosomal protein L4	L.NPYAKTMR.R
zinc and ring finger 1, E3 ubiquitin protein ligase	Y.LGSRASLADAL.P
myosin XIX	L.TVVSKFKASLEQ.L
huntingtin	L.QSVLALGHKR.N
adaptor-related protein complex 5, beta 1 subunit	S.RVGAGLGGLLTDK.V
eukaryotic translation elongation factor 2	D.RYFDPANGKFSK.S
DEAH (Asp-Glu-Ala-Asp/His) box polypeptide 57	Q.EEAFALKSI.C
transducer of ERBB2, 2	M.KKGGGAAS.G
inositol-trisphosphate 3-kinase B	L.PLRKLSSSSA.S
	I.RTQMRVGISSLR.T
transcription factor 20 (AR1)	E.QSKSQASFNNKK.S
golgin A6 family-like 4	E.PRGSESAAAARPVAGAPV.P
potassium voltage-gated channel, Shal-related subfamily, member 2	F.RVFRI.F
DENN/MADD domain containing 2D	G.RVFRL.F
MYC binding protein 2, E3 ubiquitin protein ligase	L.RVFRL.I
dihydroorotate dehydrogenase (quinone)	P.RVFRL.P
zinc finger protein 646	P.RVFRL.P
mitogen-activated protein kinase kinase kinase kinase 5	T.RVFRL.L
potassium voltage-gated channel, shaker-related subfamily, member 10	V.RVFRI.F
potassium voltage-gated channel, shaker-related subfamily, member 5	V.RVFRI.F
potassium voltage-gated channel, shaker-related subfamily, member 4	V.RVFRI.F
SWI/SNF related-matrix associated-actin dependent regulator of chromatin	V.RVFRL.I
ornithine decarboxylase antizyme 1	S.RTMPLLSLHSR.G
gamma-glutamyltransferase 7	C.GTYLALGANGAAR.G
heat shock protein 90kDa alpha (cytosolic), class B member 1	L.KEFDGKSLV.S
met proto-oncogene (hepatocyte growth factor receptor)	M.KYLASKKFVHR.D
plastin 1	L.KSKDISKTFR.K
absent in melanoma 1	E.LGRAAGAPGAS.D
major histocompatibility complex, class I, C	W.AAVVVPSGQEQRYT.C
major histocompatibility complex, class I, A	W.AAVVVPSGQEQRYT.C
major histocompatibility complex, class I, B	W.AAVVVPSGQEQRYT.C
myeloid/lymphoid or mixed-lineage leukemia 4	F.IMPVVSARSSR.V
protocadherin alpha 3	S.ADILSKFHL.D
thrombospondin, type I, domain containing 7B	K.KADVKNLSGKNR.P
ornithine decarboxylase antizyme 1	S.RTMPLLSLHSR.G

aspartyl-tRNA synthetase 2, mitochondrial	V.VQVSGTVISR.P
A kinase (PRKA) anchor protein 11	F.RNNHMKTKASVR.K
proprotein convertase subtilisin/kexin type 9	L.GTHKPPVLR.P
G protein-coupled receptor 116	K.GVTVTGFKSGSVVV.T
ribosomal protein L4	L.KLNPYAKTMR.R
actin-related protein 10 homolog (S. cerevisiae)	G.KTQPPLMKR.A
ribosomal protein L4	G.RMFAPTKTWRR.W
NAC alpha domain containing	E.IAEALSRPGR.E
major histocompatibility complex, class I, C	W.AAVVVPSGEEQRYT.C
major histocompatibility complex, class I, B	W.AAVVVPSGEEQRYT.C
major histocompatibility complex, class I, A	W.AAVVVPSGEEQRYT.C
major histocompatibility complex, class I, H (pseudogene)	W.AAVVVPSGEEQRYT.C
major histocompatibility complex, class I, G	W.AAVVVPSGEEQRYT.C
major histocompatibility complex, class I, E	W.AAVVVPSGEEQRYT.C
formin homology 2 domain containing 3	A.VTAVDTKRGV.K
ribosomal protein L28	L.RSQKPVMVKR.K
Ras and Rab interactor 1	L.RTYSPSAQVKR.L
keratin 9	S.GAGKIGLGGR.G
KIAA1324-like	N.PTKSGAGVISVPSK.C
regulator of G-protein signaling 12	D.VDLVTGSAPGR.D
FAT tumor suppressor homolog 3 (Drosophila)	I.VTARPLNTV.K
GRAM domain containing 4	L.RDPAGPGTLIMA.T
olfactory receptor, family 5, subfamily T, member 1	T.VVTPKMLVNFLAKN.K
olfactory receptor, family 5, subfamily T, member 3	T.VVTPKMLVNFLAKN.K
Sp8 transcription factor	A.ERLGPAGASLR.R
Sp9 transcription factor	A.ERLGPAGASLR.R
ankyrin repeat domain 34C	L.IAPSVLAASTRQ.D
PIF1 5'-to-3' DNA helicase homolog (S. cerevisiae)	L.KLAAAPGPGPASAR.A
WNK lysine deficient protein kinase 1	T.TAVAPSKL.L
ribosomal protein L13a	N.KLKYLAFLR.K
PHD finger protein 8	C.WYVGKHIL.D
KIAA1467	S.EGHPAALVVSK.L
MORN repeat containing 1	Y.PVSSPAAGVPGPR.A
ornithine decarboxylase antizyme 1	D.RAALLRTF.S
collagen, type II, alpha 1	Q.GPMGPSGPAGARGI.Q
collagen, type XXVII, alpha 1	P.PFTALSSSPAPTPGSTR.S
proliferating cell nuclear antigen	S.GELGNGNIKL.S
l(3)mbt-like 3 (Drosophila)	F.KVKPPHGFQK.K
chromodomain helicase DNA binding protein 5	S.SKGGNLAASAKK.K
heat shock protein 90kDa alpha (cytosolic), class B member 1	L.KEFDGKSLVSV.T
Rho GTPase activating protein 6	D.LPSVLAGPASSR.S
interferon, gamma-inducible protein 16	D.RNMEIPKGLIR.S

bromodomain, testis-specific	P.SVFPKTSISPLN.V
major histocompatibility complex, class I, C	W.AAVVVPSGEEQR.Y
major histocompatibility complex, class I, B	W.AAVVVPSGEEQR.Y
major histocompatibility complex, class I, A	W.AAVVVPSGEEQR.Y
major histocompatibility complex, class I, H (pseudogene)	W.AAVVVPSGEEQR.Y
major histocompatibility complex, class I, C	W.AAVVVPSGEEQR.Y
major histocompatibility complex, class I, G	W.AAVVVPSGEEQR.Y
major histocompatibility complex, class I, C	W.AAVVVPSGEEQR.Y
major histocompatibility complex, class I, E	W.AAVVVPSGEEQR.Y
HEG homolog 1 (zebrafish)	L.LSPSAVESRR.N
polycystic kidney disease 1 (autosomal dominant)	E.QQLHSLQGRR.S
ribosomal protein L4	G.RMFAPTKTWR.R
bromodomain containing 3	K.KGGKQAS.A
ribosomal protein L10	A.KFKFPGRQK.I
structural maintenance of chromosomes 2	F.AYKDPEKNWNR.N
N-acetylglucosaminidase, alpha	I.IGSLFLRELI.K
DDHD domain containing 1	S.ITNIGKASILGAASI.G
plexin B1	R.HKPGSVFSVE.G
DTW domain containing 2	A.RTLQEPVAR.P
cat eye syndrome chromosome region, candidate 2	E.KVKAVEGMC.S
asparagine-linked glycosylation3, alpha-1,3-mannosyltransferase homolog	Y.RVHSIFVLR.L
YdjC homolog (bacterial)	L.RVLTAPTLR.A
RGP1 retrograde golgi transport homolog (S. cerevisiae)	E.RVQPEYQRR.R
RAD51 associated protein 1	D.DVGGVQGKR.K
cytochrome P450, family 51, subfamily A, polypeptide 1	F.AYVPFGAGRHR.C
signal peptide, CUB domain, EGF-like 3	K.KGYKL.L
basic helix-loop-helix family, member e41	E.KGKGAGASRV.T
smoothelin	P.ARLLGPSLTSTT.P
spermatogenesis associated 5-like 1	C.RLGPAALHALG.A
FERM and PDZ domain containing 2	R.TAVSLVTALPGR.P
catenin (cadherin-associated protein), beta 1, 88kDa	M.LKHAVVNLI.N
amnion associated transmembrane protein	R.VGLGPGASPVRV.R
keratin 9	S.RSGGGGGGGLGSGGSI.R
dishevelled associated activator of morphogenesis 1	S.RLKLSNDEIKR.A
RUN and SH3 domain containing 2	L.SRAESLARGGGEGSMATRPS.N
germ cell associated 2 (haspin)	L.RARPSLTVTPR.R
polymerase (RNA) II (DNA directed) polypeptide B, 140kDa	A.RGGQGAK.K
drebrin-like	Q.GLCARALY.D
tectonic family member 1	E.RTILISTAVTF.V
C2 calcium-dependent domain containing 3	I.RACGLQAAAKA.L
	H.IFNGTELARLR.D

uncharacterized LOC100505530	T.FPGRAGGGEAGSRPP.R
general transcription factor IIIC, polypeptide 1, alpha 220kDa	T.PLGVVRCPR.V
drebrin-like	C.RVKDPNSGLPK.F
RAD18 homolog (S. cerevisiae)	V.KVYTPVASR.Q
sterol regulatory element binding transcription factor 1	M.LMRLGGGTTV.T
ryanodine receptor 1 (skeletal)	K.ALRMLGEAVR.D
cadherin, EGF LAG seven-pass G-type receptor 3 homolog (Drosophila)	Q.EPGPRSATVR.V
cardiolipin synthase 1	Q.RSALGSALDPL.A
inner centromere protein antigens 135/155kDa	D.PKGQGVGTGRS.A
zinc finger homeobox 2	A.AIDPSAPAR.G
ubiquitin specific peptidase 19	A.EDPGSAGEAAR.A
2-hydroxyacyl-CoA lyase 1	C.RLYTKFSAR.P
collagen, type XIX, alpha 1	G.LPGLEGFPGVKGDRGPAGPPGIAGM.S
cysteine-rich protein 1 (intestinal)	A.MFGPKGFGR.G
myeloid leukemia factor 2	M.RSAPGGIRETR.R
cancer susceptibility candidate 5	E.KQNVKIWGR.K
major histocompatibility complex, class I, H (pseudogene)	L.MAPRTLLL.L
major histocompatibility complex, class I, C	V.MAPRTLIL.L
major histocompatibility complex, class I, B	V.MAPRTLIL.L
major histocompatibility complex, class I, C	V.MAPRTLLL.L
major histocompatibility complex, class I, A	V.MAPRTLLL.L
myosin binding protein C, cardiac	V.YAVNAIGMSRPS.P
immunoglobulin superfamily containing leucine-rich repeat 2	R.WGPGPGGAGGAPRP.G
dynein, axonemal, heavy chain 5	I.RIAMAALKEI.R
CAP-GLY domain containing linker protein family, member 4	K.PNHGVLVRPSR.V
sal-like 3 (Drosophila)	S.PQSLLGGPPLTKAEP.V
WD repeat containing planar cell polarity effector	C.IRNKI.Q
keratin 12	D.LRNKI.I
keratin 16	D.LRNKI.I
KIAA1731	E.IRNKI.H
centromere protein F, 350/400kDa (mitosin)	E.LRNKI.N
B-box and SPRY domain containing	E.LRNKI.V
polyamine modulated factor 1 binding protein 1	E.LRNKL.A
RAD50 homolog (S. cerevisiae)	E.LRNKL.Q
exportin 6	F.IRNKL.C
dedicator of cytokinesis 5	F.LRNKI.F
centrosomal protein 290kDa	G.IRNKL.K
low density lipoprotein receptor-related protein 4	I.IRNKL.H
intraflagellar transport 172 homolog (Chlamydomonas)	I.LRNKI.E

paired box 9	I.LRNKI.G
paired box 1	I.LRNKI.G
ribosomal protein S17	K.LRNKI.A
protein kinase, AMP-activated, gamma 1 non-catalytic subunit	L.IRNKI.H
chromosome 2 open reading frame 29	L.IRNKI.I
signal transducer and activator of transcription 2, 113kDa	M.LRNKL.F
zinc finger E-box binding homeobox 2	Q.LRNKL.E
tumor protein p53 binding protein, 2	Q.LRNKL.N
coiled-coil domain containing 27	R.LRNKI.I
smg-5 homolog, nonsense mediated mRNA decay factor (C. elegans)	S.LRNKL.R
tudor domain containing 12	V.LRNKI.K
Fanconi anemia, complementation group D2	V.LRNKI.R
mitogen-activated protein kinase kinase kinase kinase 4	W.LRNKI.L
misshapen-like kinase 1	W.LRNKI.L
striatin, calmodulin binding protein	A.AGDGAAAAGAARA.Q
transcription factor 24	R.SGSGRPAAANAA.R
collagen, type XVI, alpha 1	S.EGLPGPPGPAGPRGER.G
citron (rho-interacting, serine/threonine kinase 21)	K.KVKQSLAQSH.L
chromosome 9 open reading frame 89	N.RYYPQILTNK.E
nucleoporin 214kDa	S.SPNKTGGFGAAPVF.G
DEAH (Asp-Glu-Ala-His) box polypeptide 33	R.RVAAISLATR.V
AT rich interactive domain 2 (ARID, RFX-like)	K.LARGGILTSTG.F
keratin 9	L.SRSGGGGGGGLGSGGSIRSS.Y
E4F transcription factor 1	D.ARAGSGAGAAGLGTA.T
heparan sulfate (glucosamine) 3-O-sulfotransferase 6	A.PGLPLASGPGRR.R
RAD18 homolog (S. cerevisiae)	V.KVYTPVASR.Q
huntingtin interacting protein 1 related	R.SELEKIKL.E
lon peptidase 2, peroxisomal	D.KLGKGLQGDPAAA.L
fucokinase	L.AGTALAALQR.A
immediate early response 3	R.VLYPRVVRR.Q
eukaryotic translation initiation factor 1A, Y-linked	P.KNKGKGGKN.R
eukaryotic translation initiation factor 1A, X-linked	P.KNKGKGGKN.R
Aly/REF export factor	A.GGFGGGGGTRRGTRG.G
chromosome 5 open reading frame 20	L.PAGVLR.E
attractin	P.RLLSPPLRPR.L
mindbomb E3 ubiquitin protein ligase 1	D.NGAKNLYRVG.F
ZFP64 zinc finger protein	S.SAAALRV.H
kelch-like 29 (Drosophila)	V.FILGGAYAR.A
dynein, axonemal, heavy chain 1	D.QLGEVCRGPR.M
proprotein convertase subtilisin/kexin type 9	L.LPLAGGYSRVL.N
succinate-CoA ligase, alpha subunit	A.PPGRRMGHAGAIIAG.G

chromosome 9 open reading frame 89	N.RYYPQILTNK.E
ribosomal protein S17	I.IEVDPDTKEML.K
homeobox D8	S.KYKAAAAAAAAAGEA.I
microtubule-actin crosslinking factor 1	D.VDRVTKTYKR.K
epilepsy, progressive myoclonus type 2A, Lafora disease (laforin)	P.APPTGASASGRPRR.P
non-SMC condensin I complex, subunit G	M.FSGLLVSSR.I
Rap guanine nucleotide exchange factor (GEF) 6	L.LSGSVLVKGSMV.L
G protein-coupled receptor 98	V.SRIIIAKSDS.P
major histocompatibility complex, class I, A	F.GAMFAGAVVAAVR.W
DnaJ (Hsp40) homolog, subfamily C, member 11	G.VPQGVSLKV.K
methyl-CpG binding domain protein 6	A.RAAVPLPPRAR.P
G protein-coupled receptor 98	T.VVRSPGGKGT.V
glyoxylate reductase/hydroxypyruvate reductase	V.RLMKVFVTR.R
retinitis pigmentosa 1-like 1	A.GCRVS.L
integrin, alpha 11	K.TPKQRITAS.E
major histocompatibility complex, class I, C	W.AAVVVPSGEEQRY.T
major histocompatibility complex, class I, B	W.AAVVVPSGEEQRY.T
major histocompatibility complex, class I, A	W.AAVVVPSGEEQRY.T
major histocompatibility complex, class I, H (pseudogene)	W.AAVVVPSGEEQRY.T
major histocompatibility complex, class I, G	W.AAVVVPSGEEQRY.T
G protein-coupled receptor 62	C.RAARFLSAAL.L
cystic fibrosis transmembrane conductance regulator	R.GQLLAVAGSTGAGKTSLL.M
bicaudal D homolog 1 (Drosophila)	E.LKQSRAVVT.N
mucin 12, cell surface associated	T.TALPGSTTKP.G
methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1-like	Q.RVLDTNDRFLR.K
minichromosome maintenance complex component 5	I.RSSYIRVL.G
microtubule associated serine/threonine kinase family member 4	F.RRAPAPGT.L
myosin binding protein C, fast type	P.KGKDAPKGAPKE.A
sushi domain containing 2	T.ATRVAHQLHQR.R
neogenin 1	R.DVVASLVSTRFI.K
proline-rich transmembrane protein 3	A.LAAVAAARPRPP.T
tumor necrosis factor, alpha-induced protein 3	P.EIRAVPL.V
myosin, heavy chain 1, skeletal muscle, adult	E.AGGGKKGG.K
Wiskott-Aldrich syndrome	V.PAGGLAPGGGRGALLD.Q
ribosomal protein L17	A.KQWGWTQGRWPK.K
ubiquitin specific peptidase 9, X-linked	Q.REKLLELI.R
squalene epoxidase	L.KEPDRIVGEFL.Q
low density lipoprotein receptor-related protein 3	P.VSLEAAQGRL.T
chromosome 1 open reading frame 167	A.HGSALLLALKG.H
collagen, type XVII, alpha 1	D.LKTVSTKGKTT.T
microtubule associated serine/threonine kinase	R.SLSPGRSPACC.D

family member 4	
Snf2-related CREBBP activator protein	R.QLAVGQPRPLQR.N
chaperonin containing TCP1, subunit 6B (zeta 2)	E.HVESKQLVGV.D
anaplastic lymphoma receptor tyrosine kinase	G.REGRLSAAIR.A
TBC1 domain family, member 25	Q.EVGSPKDPGKSL.P
hydroxyacid oxidase (glycolate oxidase) 1	A.LALGAKAVFVGR.P
solute carrier family 16, member 11 (monocarboxylic acid transporter 11)	P.RALPSCGPASPPATP.P
complement component 4B (Chido blood group)	L.SLQKPRL.L
regulating synaptic membrane exocytosis 1	MSSAVGPRGPR.P
RAD21 homolog (S. pombe)	I.SLLELCRNTN.R
family with sequence similarity 8, member A1	A.SVRATPVTRVG.S
immediate early response 3	R.RVLYPRVVR.R
germ cell associated 2 (haspin)	L.PCSQEEATGGAK.D
antigen identified by monoclonal antibody Ki-67	L.KASLGKVGVK.E
sterile alpha motif domain containing 14	P.RSAPSSDSSPS.F
centrosomal protein 290kDa	D.IAKKNQSITD.L
WD repeat containing planar cell polarity effector	E.HGNLEKKQKLA.E
cytochrome b-561	L.QGNALLVYRVF.R
F-box and leucine-rich repeat protein 6	W.LPKPPGRGV.A
ryanodine receptor 3	L.VHFIKGEKGDT.D
SPATA31 subfamily A, member 3	E.KVSSLSLTQL.A
gamma-glutamyltransferase 6	L.LCHADGTPLGAGARA.T
DDB1 and CUL4 associated factor 13	I.RIFPVDKSRSR.E
endoplasmic reticulum protein 29	A.AAVPRAAFL.S
Wolf-Hirschhorn syndrome candidate 1	S.RYQGVRGIGR.V
nuclear export mediator factor	V.RSALAN.Q
NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 11, 17.3kDa	A.AGLFGLSARRL.L
keratin 17	S.SSSIKGSSGLGGGSS.R
RAB GTPase activating protein 1	D.SLYKI.C
G protein-coupled receptor 125	D.SLYKL.Q
transmembrane protein 131	D.SLYKL.S
smg-1 homolog, phosphatidylinositol 3-kinase-related kinase (C. elegans)	F.SLYKL.S
bestrophin 2	G.SIYKL.L
formin 2	K.SLYKI.K
component of oligomeric golgi complex 5	N.SLYKL.H
FAT tumor suppressor homolog 4 (Drosophila)	Q.SLYKL.N
chromosome 5 open reading frame 42	R.SIYKI.Q
collagen, type XIX, alpha 1	S.SLYKI.K
olfactory receptor, family 2, subfamily T, member 10	T.SLYKI.F
myosin, heavy chain 1, skeletal muscle, adult	V.SIYKL.T
myosin, heavy chain 8, skeletal muscle, perinatal	V.SIYKL.T
myosin, heavy chain 2, skeletal muscle, adult	V.SIYKL.T

THAP domain containing 9	V.SLYKI.P
dipeptidyl-peptidase 8	V.SLYKL.S
asp (abnormal spindle) homolog, microcephaly associated (Drosophila)	Y.SLYKL.T
serine/threonine kinase 36	S.KVAPGTAPLPR.L
chromosome 16 open reading frame 88	D.RNASKSVKLE.D
ATPase, H+ transporting, lysosomal 70kDa, V1 subunit A	H.KIMLPPRNR.G
keratin 75	A.RSAAGSGGLGRISSA.G
serine/arginine repetitive matrix 2	P.RSAHATAPVNIAGSRT.A
RAN binding protein 9	G.KTPKDAASVR.A
ets variant 4	P.RAEQRNFLR.S
non-SMC condensin I complex, subunit G	H.QNQAKLVVALSR.T
kelch-like 17 (Drosophila)	R.ARVGVAAVGN.R
malic enzyme 2, NAD(+)-dependent, mitochondrial	D.RGHVR.S
macrophage stimulating 1-like	H.RGHVR.E
regulator of chromosome condensation 1	S.RGHVR.F
calmodulin binding transcription activator 2	S.RGHVR.L
ribosomal protein S25	H.RAQVIYTR.N
forkhead box D4-like 4	Q.VARWGGVALPRE.H
forkhead box D4-like 3	Q.VARWGGVALPRE.H
dynein, cytoplasmic 2, heavy chain 1	L.IRGLGGNLN.M
PTK7 protein tyrosine kinase 7	T.QVRPRNAGIYR.C
methyltransferase like 24	G.RRAPPGGG.G
laminin, alpha 4	I.GDSIRGAPQFCQ.P
protein phosphatase 1, regulatory subunit 26	C.PPKLVPGSGGG.P
trophoblast glycoprotein-like	MAPRAGQPGL.Q
thyroid adenoma associated	A.ASGLGEKGVPPL.L
alkaline phosphatase, intestinal	Q.DSKAYTSIL.Y
spectrin repeat containing, nuclear envelope 1	F.HPMLR.Y
ryanodine receptor 1 (skeletal)	T.HPMLR.P
polo-like kinase 1	A.RTMVDKLLSSR.S
SRY (sex determining region Y)-box 11	L.RVSGSGGGGAGKTV.K
myeloid/lymphoid or mixed-lineage leukemia 4	V.VTTPVKAEV.S
cadherin-related family member 4	L.ELARTSGTAVSR.L
anaphase promoting complex subunit 1	L.EGSGNLVLYTGVVR.V
MGA, MAX dimerization protein	A.PIAAKVGSV.G
hemicentin 1	L.KGVVYTT.R
potassium voltage-gated channel, Shaw-related subfamily, member 3	T.RLAGLTEPEAAAR.F
desmoplakin	A.LYKAISVPR.V
collagen, type VI, alpha 1	D.IAPRGVKGAKG.Y
methyl-CpG binding domain protein 5	P.AITKTT.S
CXXC finger protein 11	T.PGDDLGKGGVVIAI.P
MAP/microtubule affinity-regulating kinase 1	V.MATYILLGR.K

POM121 transmembrane nucleoporin	V.RIAPPDRRFSR.S
elastin	V.APGIGPGGVAAAAKSA.A
AE binding protein 2	P.LPPGSPGSAARGR.A
ubiquitin specific peptidase 13 (isopeptidase T-3)	D.ISPPIVIPDDSK.D
myeloid/lymphoid or mixed-lineage leukemia 2	R.KVPAADKAPYLQ.K
G protein regulated inducer of neurite outgrowth 1	F.RALLQSVRR.P
matrix-remodelling associated 8	V.LAVARGAPALL.T
collagen, type VII, alpha 1	A.VILGPPGPRGAK.G
arginine vasopressin-induced 1	H.RVAEALKRLR.R
protease, serine, 16 (thymus)	Q.TPGANKVLFV.N
family with sequence similarity 81, member B	D.KSHAFLPI.I
DEAH (Asp-Glu-Ala-His) box polypeptide 37	P.AVFIPVNR.S
immediate early response 3	R.RVLYPRVVR.R
solute carrier family 52, riboflavin transporter, member 1	A.FRGLL.L
titin	D.FRGLL.Q
immunoglobulin superfamily, member 22	D.FRGLL.R
schlafen family member 11	F.FRGIL.I
Fanconi anemia, complementation group A	F.FRGLL.N
zinc finger protein 449	H.FRGLI
chordin	L.FRGLL.E
proline-rich coiled-coil 2C	P.FRGLI.P
TBC1 domain family, member 20	R.FRGLL.R
tripartite motif containing 46	S.FRGLL.E
chromosome 7 open reading frame 55	T.FRGLL.R
tubulin, gamma complex associated protein 6	V.FRGLL.T
myosin XVA	S.REAVALVKPVT.S
anoctamin 9	P.ASIFSARS.T
jumonji domain containing 1C	E.FLVDK.Q
vacuolar protein sorting 13 homolog D (S. cerevisiae)	I.FLVDK.K
diacylglycerol kinase, zeta	R.FIVDK.V
poly (ADP-ribose) polymerase family, member 10	L.RGPGHVL.L

S2: List of peptides whose presentation was down-regulated after VZV infection as determined by pairwise comparison and quantitation of SILAC labelled peptides from 'light' (infected keratinocytes) and 'heavy' (uninfected keratinocytes) using mass spec analysis software PEAKS. Each mass spec identified peptide sequence is flanked by 2 amino acids in the parent protein at either end separated by a dot.

Parent Protein	Peptide
sterol regulatory element binding transcription factor 1	L.RGFQRDLSSLR.R
piezo-type mechanosensitive ion channel component 2	K.KEPGELAS.K
suppressor of Ty 6 homolog (S. cerevisiae)	A.IERAL.Q
phosphodiesterase 4D interacting protein	A.LERAI.D
sterile alpha and TIR motif containing 1	A.LERAL.P
ornithine aminotransferase	A.LERAL.Q
thyroglobulin	D.IERAL.V
envoplakin	D.LERAI.S
MORC family CW-type zinc finger 4	D.LERAL.A
transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)	D.LERAL.Y
glutamate receptor, ionotropic, AMPA 2	E.IERAL.K
calcium binding and coiled-coil domain 1	E.LERAL.A
golgin A6 family, member C	E.LERAL.C
INO80 complex subunit D	E.LERAL.Q
golgin A8 family, member C, pseudogene	E.LERAL.S
golgin A6 family, member C	E.LERAL.S
FK506 binding protein 4, 59kDa	G.LERAI.Q
leucine-rich repeats and calponin homology (CH) domain containing 1	G.LERAL.E
ATP-binding cassette, sub-family B (MDR/TAP), member 6	G.LERAL.N
family with sequence similarity 110, member D	G.LERAL.V
NODAL modulator 1	H.IERAL.P
myosin, heavy chain 7B, cardiac muscle, beta	H.LERAL.E
armadillo repeat containing 10	I.IERAL.I
NRDE-2, necessary for RNA interference, domain containing	I.LERAI.E
angiopoietin 4	K.LERAI.K
neurobeachin	K.LERAL.E
3-oxoacid CoA transferase 2	L.LERAI.R
sterol regulatory element binding transcription factor 1	L.LERAL.N
abhydrolase domain containing 14A	L.LERAL.R
ATP-binding cassette, sub-family A (ABC1), member 13	M.IERAI.I
SH3 and multiple ankyrin repeat domains 3	N.IERAL.R

ubiquitin specific peptidase 13 (isopeptidase T-3)	N.LERAL.D
translational activator of mitochondrially encoded cytochrome c oxidase I	N.LERAL.E
	P.LERAL.Y
biorientation of chromosomes in cell division 1	Q.IERAI.H
coiled-coil domain containing 151	Q.LERAL.R
enhancer of mRNA decapping 4	R.LERAL.A
transforming, acidic coiled-coil containing protein 1	S.LERAL.Q
ADNP homeobox 2	S.RTEGPIVKDEALQ.I
HECT and RLD domain containing E3 ubiquitin protein ligase family member 1	T.LERAL.Q
protocadherin gamma subfamily A, 4	V.LERAL.D
protocadherin gamma subfamily A, 3	V.LERAL.D
INO80 homolog (S. cerevisiae)	Y.LERAL.R
ubiquitin-conjugating enzyme E2D 1	D.YPFKPPKI.A
ubiquitin-conjugating enzyme E2L 3	E.YPFKPPKI.T
lon peptidase 2, peroxisomal	K.RVIAIRPIR.R
major histocompatibility complex, class I, A	A.GSHSMRYFTTSVSRPGR.G
neuron navigator 1	A.KAPGGGGGMAKA.S
KIAA1551	E.SSTKGMPAKS.D
vacuolar protein sorting 13 homolog A (S. cerevisiae)	L.IVPSSR.I
adenomatosis polyposis coli 2	P.RDQPGGPAGRQR.P
ring finger protein 40, E3 ubiquitin protein ligase	V.RTNERLKVALR.S
leucine-rich repeats and death domain containing 1	L.KELDISNNAIR.E
ATPase, H+/K+ exchanging, alpha polypeptide	A.KMSKKKKAGGGGG.K
unc-51-like kinase 2 (C. elegans)	L.SPSTAVKQVVKN.L
sterol regulatory element binding transcription factor 1	L.RGFQRDLSSLR.R
mediator complex subunit 13	D.PFPKSGVI.S
AU RNA binding protein/enoyl-CoA hydratase	G.RRAGPAIWA.Q
hemicentin 1	D.NVLLANLLGKYT.A
integrator complex subunit 3	G.GGAGAGAPGGGRLL.L
family with sequence similarity 120C	R.VLVDAGSALPR.L
immunoglobulin superfamily, member 9B	P.ATVVKWN.K
microtubule associated monoxygenase, calponin and LIM domain containing 1	L.RALGAKKFYGR.F
slingshot homolog 2 (Drosophila)	A.ISVKEI.V
RAD51 associated protein 1	A.LSVKEL.P
eukaryotic translation initiation factor 3, subunit E	F.LSVKEI.Y
KIAA1210	H.ISVKEL.K
sec1 family domain containing 1	L.LSVKEL.R
bromodomain and WD repeat domain containing 1	Y.SMTLRLSAL.F
zinc finger, MYM-type 6	P.QNKVNISKAKT.A
keratin 9	S.SSYLSRSGGGGGGGLGSGGSIR.S
lon peptidase 2, peroxisomal	K.RVIAIRPIR.R

ryanodine receptor 1 (skeletal)	K.SGPEIVKAGLRS.F
twist basic helix-loop-helix transcription factor 1	S.RRSAGGGAGPGGAAGGGV.G
serine/threonine kinase 36	T.MVLTSIKGT.P
cytochrome P450, family 27, subfamily A, polypeptide 1	K.ATGAPGAGPGVRR.R
WNK lysine deficient protein kinase 1	P.PPARSGSGGGSAKE.P
growth arrest-specific 2 like 3	Q.KSKDKNIVSATK.K
neurofascin	E.SPPTTTSGTKIH.E
N-myristoyltransferase 1	V.YTAGVVLPKPVGT.C
FH2 domain containing 1	A.KAPGITRTVSQR.Q
ribosomal protein L4	R.IQRRGPCII.Y
calcium channel, voltage-dependent, L type, alpha 1F subunit	L.KLYGLGPSA.Y
gametogenetin	A.KASLGGGGGGGLFAAS.G
ribosomal protein L4	G.RMFAPTKTWRR.W
myeloid leukemia factor 2	M.RSAPGGIRETR.R
signal-induced proliferation-associated 1 like 3	L.RASLRDLR.S
2'-5'-oligoadenylate synthetase 3, 100kDa	T.VKGGSSGRGTALKG.G
ribosomal protein L28	N.SFRYNGLIHR.K
praja ring finger 1, E3 ubiquitin protein ligase	V.SFRPPTSQRER.I
suppressor of Ty 6 homolog (S. cerevisiae)	N.GVTGFIPTKFLS.D
adaptor-related protein complex 3, beta 1 subunit	K.RIVGMIAKGKN.A
ALX homeobox 1	Y.SKASAGKCVQAF.G
collagen, type IV, alpha 4	G.PGCKGEPGLDGRR.G
ornithine decarboxylase antizyme 1	S.RTMPLLSLHSR.G
glucosamine (N-acetyl)-6-sulfatase	M.RLLPLAPGRLR.R
suppressor of variegation 4-20 homolog 1 (Drosophila)	E.PEGPAQAAVASGCLTR.H
collagen, type V, alpha 1	P.RGITGKPGPKG.N
interferon, gamma-inducible protein 16	F.TPKKIIAIANYVC.R
ABI family, member 3 (NESH) binding protein	Y.TTPAPKDVLL.P
cadherin, EGF LAG seven-pass G-type receptor 1 (flamingo homolog)	V.RSVVVGGASEDK.V
heterogeneous nuclear ribonucleoprotein R	R.GGGRGGRGAPPPPR.G
BCL6 corepressor	S.EKPSGKRLCKT.K
serine/arginine repetitive matrix 2	V.PSPTPAPKEAV.R
heat shock 70kDa protein 6 (HSP70B')	N.TVFDAKRLIGR.K
heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	N.TVFDAKRLIGR.T
zinc finger, SWIM-type containing 8	C.SASGIRAGGEAGRGM.P
SET domain containing 1A	A.PTGSGEPGATRE.S
WNK lysine deficient protein kinase 1	L.HTRTPPIIHR.D
WNK lysine deficient protein kinase 2	L.HTRTPPIIHR.D
solute carrier family 4, sodium bicarbonate cotransporter, member 4	L.KPLISPAAERI.R
MAPKAPK5 antisense RNA 1	R.RGGPRPGAPCKGLA.G

tenascin N	L.RPSAVT.Q
PR domain containing 8	L.SPDGIATGGGKGK.R
three prime repair exonuclease 1	P.RPSAVT.T
solute carrier family 35, member C2	V.LGASFIGGIR.W
zinc finger protein 804B	Q.ISCTGSSKKPP.N
protocadherin 7	I.LIVVMARY.C
transformation/transcription domain-associated protein	F.RTATGAISAVFGR.S
ankyrin repeat domain 13C	L.QLKAAPASSNPPG.A
MLX, MAX dimerization protein	R.RGASLLSPKSPT.L
chloride intracellular channel 3	A.AYRPAVHPR
desmoplakin	G.KTVSVSEAIKKN.L
titin	I.RGIPPKIEA.L
collagen, type I, alpha 2	Q.PGAPGVKGEPGAP.G
RuvB-like 2 (E. coli)	E.DAYTVLTRI.G
POM121 transmembrane nucleoporin	S.PPAKSTANGNL.L
non-SMC condensin I complex, subunit G	A.KTLPKIVGR.T
heat shock 70kDa protein 1-like	N.TVFDAKRLIGR.K
heat shock 70kDa protein 7 (HSP70B)	N.TVFDAKRLIGR.K
heat shock 70kDa protein 6 (HSP70B')	N.TVFDAKRLIGR.K
heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	N.TVFDAKRLIGR.T
centriolar coiled coil protein 110kDa	G.TPKTSVKGVVQN.R
SKI family transcriptional corepressor 2	P.PLAPQKASGGGS.S
ribosomal protein S6 kinase, 90kDa, polypeptide 1	S.DGYVVKETI.G
patatin-like phospholipase domain containing 2	C.LGEAGAKFI.E
NOBOX oogenesis homeobox	G.VTPQRIMV.K
ryanodine receptor 1 (skeletal)	A.LTEDQGLVVVDASK.A
chromosome 11 open reading frame 96	A.PPPGPADAGGAMAAKP.G
calcium channel, voltage-dependent, R type, alpha 1E subunit	R.RRRGGPGPGMMC.G
cytochrome P450, family 1, subfamily B, polypeptide 1	H.SMMRNFFTR.Q
mannosidase, alpha, class 1A, member 1	R.KGSGPAALRLT.E
collagen, type XI, alpha 1	S.RGARGPTGKPGPKG.T
DDB1 and CUL4 associated factor 13	I.RIFPVDKSRSR.E
histone cluster 1, H1c	A.KKAGGTPRKASGPPV.S
mortality factor 4 like 1	T.KKNKQKTPGN.G
G protein-coupled receptor 124	A.LRGGAAGALELL.S
forkhead box E1 (thyroid transcription factor 2)	V.FGLVPERPL.S
unc-13 homolog C (C. elegans)	T.RTNGSSLLS.S
ubiquilin 1	L.SAMSNPRAM.Q
ubiquilin 2	L.SAMSNPRAM.Q
Y box binding protein 1	L.GTVKWFNVR.N
endogenous retrovirus group 3, member 1	C.GKFNLTNCCL.E

F-box protein 41	S.ASLGRGGGGGGAGPNARGPGRM.R
forkhead box D1	A.KAGGPGASALARS.P
SPEG complex locus	E.RIARKPTVC.E
integrator complex subunit 3	L.RDKVLQLQK.G
protein inhibitor of activated STAT, 3	Y.GELIRPTTL.A
major histocompatibility complex, class I, A	S.HSMRYFTTSVSRPGR.G
NHS-like 1	H.SVINVFVGR.A
CDK5 regulatory subunit associated protein 2	R.KTVSLEHLQR.E
serine/threonine kinase 35	G.NKSSQLYLR.L
polymerase (DNA directed), theta	K.NKSNTIFSDS.Y
cell cycle associated protein 1	Y.KVLKEIVER.V
loricrin	G.GGGIGGCGGGSGGSVKY.S
collagen, type IV, alpha 3 (Goodpasture antigen)	P.PGPIGPKGPPG.V
GLI family zinc finger 3	C.ASFGGSRR.Q
FYN binding protein	F.GQKPPLSTENS.H
poly(rC) binding protein 1	A.ASRPPVTLR.L
poly(rC) binding protein 2	A.ASRPPVTLR.L
TBC1 domain family, member 28	V.CKVIPLAVR.G
KH and NYN domain containing	W.KRGARGGN.L
protein tyrosine phosphatase, receptor type, f polypeptide, interacting protein	E.DGRRGSALGPDEAGGELER.L
heparan sulfate (glucosamine) 3-O-sulfotransferase 4	P.LAAPPPPGASAKG.P
collagen, type XII, alpha 1	P.PGRPGNSGIRG.P
dispatched homolog 2 (Drosophila)	W.RALQALTGPRK.L
major histocompatibility complex, class I, F	A.GSHSLRYFSTAVSRPGR.G
methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1-like	N.IAHGNSSVLADK.I
protocadherin alpha 8	P.RVGGTGGAASKLV.P
5'-3' exoribonuclease 1	S.RFTGSIFIGR.G
zinc finger and BTB domain containing 12	T.SGLVEAAAVAMAARG.A
contactin 4	C.EVKGNPKPHIR.W
bassoon (presynaptic cytomatrix protein)	G.PLPAKASPL.S
zinc finger, SWIM-type containing 8	N.SPTGGGGGGGSGGTRMRDGLVI.P
transforming, acidic coiled-coil containing protein 2	A.LATPGEKAGAGRSAVG.K
prune homolog 2 (Drosophila)	R.IKLTAPNIN.L
family with sequence similarity 173, member A	Q.PVTAVGEGLDR.V
ribosomal protein L13	V.ARTIGISVDPR.R
coiled-coil domain containing 85C	E.ERAALAATGAASGGGGGGGGAGSR.S
otolin 1	R.GFKGSKGELA.R
l(3)mbt-like 2 (Drosophila)	A.AEPATPLKAK.E
ribosomal protein L13	A.RVITEEEKNFK.A
SET binding protein 1	E.PLLSTPGPGK.G
glyoxylate reductase/hydroxypyruvate reductase	R.RLKPFGVQR.F
collagen, type I, alpha 1	P.PGPAGKEGGKGPR.G

striatin interacting protein 2	Q.LAPPPSKLRG.R
zinc finger CCCH-type containing 15	D.FKAGKALVIS.G
phosphatidylinositol transfer protein, beta	L.KSKVPAFVR.M
DnaJ (Hsp40) homolog, subfamily C, member 1	K.KKTGSKSVD.V
sterol regulatory element binding transcription factor 1	G.ATVKAAGLSPL.V
kinesin family member 13B	I.LSVGIGCVKVR.P
heparan sulfate proteoglycan 2	C.VCPAGFTGSR.C
methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1-like	E.IYGKSKAKVR.L
paraneoplastic Ma antigen family member 6C	R.PMGHFTVLGKV.F
titin	G.KEVKASDRLTMKN.D
myeloid/lymphoid or mixed-lineage leukemia translocated to, 4	L.SIVAAKGAGQDKL.G
MORC family CW-type zinc finger 3	Q.RILEMNDKYVK.K
G protein-coupled receptor 112	L.KQKSTNTGALPI.S
POU class 3 homeobox 1	Q.YLPRGPGGGA.G
OTU domain containing 1	P.DPVPGAAGSAAAPRG.R
breast cancer anti-estrogen resistance 1	P.LAPGRTGGLGPSDR.Q
dachsous 1 (Drosophila)	A.PLASDSLQKL.G
Rho guanine nucleotide exchange factor (GEF) 26	Y.RAASPRLRR.P
cysteine-rich protein 1 (intestinal)	A.AMFGPKGFGR.G
GS homeobox 2	G.AGGSGVAGAAGALPLLK.G
piezo-type mechanosensitive ion channel component 1	G.EVHRGVL.D
ribosomal protein S3	L.RYKLLGGLAVR.R
inter-alpha-trypsin inhibitor heavy chain family, member 6	P.TYPKAK.I
CDC42 binding protein kinase gamma (DMPK-like)	G.RGPSVR.L
spectrin repeat containing, nuclear envelope 1	I.QGNAKKAL.L
strawberry notch homolog 1 (Drosophila)	Y.AEYMPIKL.K
protein tyrosine phosphatase, receptor type, Q	H.ARSGIVVKDVSI.R
scavenger receptor class F, member 2	N.QRKGVMGAGAL.L
FERM domain containing 4A	Y.IEGGATPVVVR.S
DEAD (Asp-Glu-Ala-Asp) box helicase 1	I.IGGVAAR.D
mutS homolog 2, colon cancer, nonpolyposis type 1 (E. coli)	M.SEENITIKL.K
elastin	P.LGGVAAR.P
C2 calcium-dependent domain containing 3	S.ADSFKKLP.L
trinucleotide repeat containing 6C	L.LVKQPPPPPPP.P
collagen, type XI, alpha 2	L.PGPAGPKGAKGATGPGGPKG.E
collagen, type V, alpha 1	G.FDGLAGLPGEKG.H
interferon, gamma-inducible protein 16	L.TTKSEDTISKM.N
homeobox B3	G.GSGGGGGGGGGDKSPP.G
phosphatidylinositol-4-phosphate 5-kinase, type I, gamma	Y.KKTTSSTLKGAIQ.L

HECT domain containing E3 ubiquitin protein ligase 4	V.YGLGHKV.K
FAT tumor suppressor homolog 2 (Drosophila)	K.TGVLTQFTKTI.L
nuclear pore associated protein 1	P.RAAAAPLGVLPAVG.W
laminin, alpha 4	I.KKGEFSGDDSLL.D
ELK1, member of ETS oncogene family	T.VSGKPGTPKGAG.M
chromosome 1 open reading frame 167	V.SPKPAALPK.P
cannabinoid receptor 1 (brain)	E.SCIKSTVKI.A
T-cell leukemia homeobox 2	S.SGVGPGGVIRVPA.H
nucleoporin 205kDa	Y.RHLPSRGIT.Q
plexin D1	MAPRAAGGAPL.S
B-cell CLL/lymphoma 9	P.GEGPLGRPSNL.P
collagen, type XXVII, alpha 1	K.RGNPGVAGLPGA.Q
tetratricopeptide repeat domain 22	A.KNQPPILNR.L
SH2B adaptor protein 1	D.RPSASI.S
obscurin, cytoskeletal calmodulin and titin-interacting RhoGEF	L.RPSASL.P
polycystic kidney disease 1 (autosomal dominant)	Y.SPAVGLHAAVTLR.L
shroom family member 2	E.EGSKAAAVDKLLAG.D
anaphase promoting complex subunit 7	A.KTLLDKALTQR.P
sidekick cell adhesion molecule 1	D.SRMARLEVI.E
ATP-binding cassette, sub-family C (CFTR/MRP), member 4	Y.EKVIKACALKK.D
lactate dehydrogenase A	E.RLGVHPL.S
chromosome 15 open reading frame 55	A.ACLGKVSSSGK.R
periplakin	R.RQYENFINR.N
PRP8 pre-mRNA processing factor 8 homolog (S. cerevisiae)	I.PGGPKFEPLVR.D
ankyrin repeat domain 34C	Q.VLKIPVSSAPASW.K
collagen, type XIII, alpha 1	L.GLPGASGLDGRPG.P
lon peptidase 2, peroxisomal	R.RTYVGSMPGR.I
forkhead box O3	E.SSSLGSAKHQQQ.S
CD109 molecule	N.VKASGSSRR.R
heparan sulfate proteoglycan 2	I.EASVIPGPIPPVR.I
phosphatidylinositol transfer protein, beta	L.KSKVPAFVR.M
inositol 1,4,5-trisphosphate receptor, type 2	N.IIEKLQDVV.A
codanin 1	S.GESLPGAGGRR.L
RELT tumor necrosis factor receptor	P.VSKLPPAPPNV.P
heat shock protein 90kDa alpha (cytosolic), class B member 1	L.KEFDGKSLV.S
interferon regulatory factor 2 binding protein 1	P.KDPGGGGGPVRAGG.A
huntingtin	P.KKGSEASAASR.Q
SH3 and multiple ankyrin repeat domains 1	P.RGTRGQGSGAPGSL.A
attractin	P.RLLSPPLRPR.L
Y box binding protein 1	L.GTVKWFNVR.N
Y box binding protein 2	L.GTVKWFNVR.N

cold shock domain protein A	L.GTVKWFNVR.N
collagen, type XIII, alpha 1	G.PPGPTGRPGLPGDKGAIGMP.G
sterol regulatory element binding transcription factor 1	T.RTHQLLDRSLR.R
Rap guanine nucleotide exchange factor (GEF) 6	G.ARQALRKKPP.E
cytochrome P450, family 1, subfamily B, polypeptide 1	R.RYGDVFQIR.L
nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4	G.GPGGGAGGAGGGR).V
notch 1	C.ESVINGCKGKP.C
hemicentin 1	S.CKATGIPLPKL.T
aminopeptidase-like 1	A.RAFPLFTHR.S
serine/arginine repetitive matrix 2	S.RRSRSLSSPR.S
insulin receptor substrate 1	T.ATSPASMVGGKPGSFRV.R
keratin 15	F.RLKYENELALR.Q
obscurin, cytoskeletal calmodulin and titin-interacting RhoGEF	L.KNAAVRAGAQACF.T
HYDIN, axonemal central pair apparatus protein	P.GEQGPVLVAKGR.S
collagen, type I, alpha 2	G.ARGSDGSVGPVGPAGP.I
histone cluster 1, H2bm	S.REIQTAVRL.L
alkaline phosphatase, placental	S.SIFGLAPGKARD.R
exocyst complex component 3-like 4	I.KVPSAMAVLIT.C
KIAA0100	K.RGVPTSASAPPR.V
coatomer protein complex, subunit beta 1	H.AIKSVKIYR.G
midnolin	P.MSLAIHSTTGTRY.D
RNA binding motif protein 15	S.LEPRVGAGAGAAPF.R
xin actin-binding repeat containing 1	H.KISVTVSSSARP.S
chromosome 11 open reading frame 96	P.SGAGWRGAGGAG.E
collagen, type IV, alpha 6	V.HGKPGLLGPKGER.G
microtubule associated serine/threonine kinase family member 4	S.GKGEGTEKSS.Q
mucin 5AC, oligomeric mucus/gel-forming	V.PSSSTVGTTRTPAVL.P
ubiquitin specific peptidase 54	H.RRTVGEGFLFV.L
collagen, type I, alpha 1	S.GNAGPPGPPGPAGKEGG.K
titin	A.SDGGAKIRN.Y
solute carrier family 38, member 10	L.AVGGGEKAKGGPP.P
protein kinase N3	P.RTPTTLREA.S
sterile alpha motif domain containing 1	A.PRGAPAAAAAAAP.P
transmembrane protein 80	E.RPLAAS.L
family with sequence similarity 98, member A	H.GDRGGGRGGR.G
sushi, von Willebrand factor type A, EGF and pentraxin domain containing 1	P.RPIAAS.L
histocompatibility (minor) 13	R.DAARFPII.A
elastin	Q.PGAGVKPGKVPG.V
zinc finger protein 687	L.SPATPTSEGPKVV.S
coronin 7	G.PGPKGGRGARI.V

SET domain containing 1A	E.EVRTSPRPASPA.R
jun proto-oncogene	G.FAEGFVRAL.A
DEAH (Asp-Glu-Ala-His) box polypeptide 40	V.PISKSEALQR.S
RUN and FYVE domain containing 2	E.LAIAKNNII.K
hemicentin 2	C.IAKNSAGSAMGKTR.L
cylicin, basic protein of sperm head cytoskeleton 1	C.EPSLPSPKVR.R
codanin 1	E.SLPGAGGRRLR.G
syntabulin (syntaxin-interacting)	G.ADRELLVGDSIAN.S
catenin (cadherin-associated protein), beta 1, 88kDa	W.TTSRVLKV.L
proline rich 18	A.RAPATCAPPR.P
synapse defective 1, Rho GTPase, homolog 2 (C. elegans)	Q.QVSPPRSPQR.E
glutamate receptor, metabotropic 5	S.RGLGAGAGAGGSAGGVGATGGAGCAGA.G
family with sequence similarity 217, member B	K.KLKTNGVKQ.N
doublesex and mab-3 related transcription factor 1	G.KASGALVGAASGSSAGGSSRGGGS.G
epilepsy, progressive myoclonus type 2A, Lafora disease (laforin)	A.RGGGAGGRGGGAG.R
ribosomal protein L4	D.KAAAAAAALQAK.S
proteasome (prosome, macropain) subunit, beta type, 10	E.RVLPGLKVPHAR.K
agrin	L.VLWSGKAT.E
hemochromatosis type 2 (juvenile)	A.LRGGGGGGRGGGVG.S
collagen, type IV, alpha 2	P.GPRGLPGDAGR.E
neurocan	W.PSVNRNV.A
mucin 5AC, oligomeric mucus/gel-forming	Q.ESAAPTLSRV.L
interferon regulatory factor 2 binding protein 1	E.AVAEGARRALL.G
SKI family transcriptional corepressor 2	G.GAGAGGAGAAPKAGLSGL.F
contactin associated protein-like 3	P.MARCAAGAASGSP.A
Rap guanine nucleotide exchange factor (GEF) 2	R.NGAPHLPKIGDIKKAS.R
leucine rich repeat and fibronectin type III domain containing 1	P.PTLALVPGGAAARPR.P
nuclear autoantigenic sperm protein (histone-binding)	Q.FSKSIEVIE.N
serine/arginine repetitive matrix 3	P.ARGKESPSPRSAPS.S
lon peptidase 2, peroxisomal	K.RVIAIRPIRR.I
basic helix-loop-helix family, member e41	D.SRGGGSGGGPGGGAAAAAAALLGPD.P
major histocompatibility complex, class I, B	A.GSHSMRYFYTAMSRPGR.G
CCAAT/enhancer binding protein (C/EBP), beta	E.RAIDFSPYL.E
crumbs homolog 3 (Drosophila)	K.LPPEERLI
SOGA family member 2	L.LAAPLAAGACPGGR.S
ubiquitin protein ligase E3 component n-recognin 5	S.SGVGGSGGGSSGR.S
fidgetin-like 2	S.YKDLEAALAKVG.P
alkaline phosphatase, placental	T.RGNEVISVMNR.A
SPEG complex locus	F.RVLSTTVK.S
growth factor independent 1 transcription repressor	P.KRAAGGAGAGAPGSC.S
trafficking protein particle complex 12	R.GSMVPFSMR.I

protocadherin alpha 7	C.KFRGDLLEV.N
chaperonin containing TCP1, subunit 4 (delta)	N.ISAAKAVADAIR.T
protocadherin alpha 3	H.VPFKLVSTF.K
protocadherin alpha 5	H.VPFKLVSTF.K
protocadherin alpha 4	H.VPFKLVSTF.K
protocadherin alpha 11	H.VPFKLVSTF.K
protocadherin alpha 1	H.VPFKLVSTF.K
protocadherin alpha 2	H.VPFKLVSTF.K
protocadherin alpha 6	H.VPFKLVSTF.K
protocadherin alpha 8	H.VPFKLVSTF.K
exonuclease 5	L.EKPLGPSVLR.H
protocadherin alpha 7	R.VPFKLVSTF.K
collagen, type XXIII, alpha 1	G.KGNAAGGGGGGRSAT.T
catenin (cadherin-associated protein), alpha 1, 102kDa	L.KVVEDGILKLR.N
keratin 15	S.LSGGGGSRSIS.A
endoplasmic reticulum aminopeptidase 1	N.MPLVKSVTV.A
DiGeorge syndrome critical region gene 2	P.APGDGGSEGALLR.R
SOGA family member 2	S.PRDSDAESDAGK.K
SOGA family member 3	S.PRDSDAESDAGK.K
BTB (POZ) domain containing 6	K.AEYSVKIEL.K
F-box protein 41	A.SLGRGGGGGGAGPNARGPG.R
PR domain containing 6	G.PLKGSGAAGLL.S
Epstein-Barr virus induced 3	P.EGVRLSPL.A
transmembrane protein 214	R.PGVGAGAGGRGGGRNR.R
centrosomal protein 104kDa	L.QPGKSSAVAAS.G
nuclear receptor binding SET domain protein 1	L.LARGRSSAQNKQ.V
rabphilin 3A-like (without C2 domains)	F.RPLPTEPAE.R
insulin receptor substrate 2	S.SPASSLGSGTPGTSSDSRQR.S
neuron navigator 2	A.RVSAAGSEAKTRGGSTTANN.R
NHL repeat containing 1	C.KVCFEKFGHR.Q
chromatin licensing and DNA replication factor 1	F.RSMDTIVGMLHNR.S
nuclear receptor corepressor 2	G.GSIARGAPVIVP.E
major histocompatibility complex, class I, C	S.SGGKGGSCSQAA.C
major histocompatibility complex, class I, C	S.SGGKGGSCSQAA.S
nuclear receptor binding SET domain protein 1	G.SPLASISKSGKV.D
nuclear receptor binding SET domain protein 1	D.KSIGAASPRP.Q
ribosomal protein S19	A.STARHLYLR.G
Aly/REF export factor	L.GTADVHFER.K
ladinin 1	L.RLSGVVTSR.L
retinoblastoma binding protein 7	S.EFILATGSADKT.V
molybdenum cofactor synthesis 3	P.TRYLVNDACVLAGRPL.V
protein tyrosine phosphatase, receptor type, Q	K.PVTVFGDIVITK.L
dynein, axonemal, heavy chain 10	E.YIKRLASL.S

SH3 and multiple ankyrin repeat domains 1	G.RPSSSGTPRE.G
DNA methyltransferase 1 associated protein 1	E.LRSDLVL.L
cullin-associated and neddylation-dissociated 2 (putative)	G.PRVPTSPTAIR.T
mucin 19, oligomeric	P.GVTRTTRSSAGL.T
mex-3 homolog D (C. elegans)	S.RRGAPDPVGALS.W
THUMP domain containing 3	K.LPWSNPLKV.W
RAB32, member RAS oncogene family	Y.YKEAVGAFV.V
discs, large (Drosophila) homolog-associated protein 3	A.RAPYLLGSR.E
ribosomal protein L4	L.KLNPYAKTMR.R
mitochondrial intermediate peptidase	E.ACRSIGTMVEK.L
ubiquitin specific peptidase 43	A.RPPGAQGLKN.H
DEAD (Asp-Glu-Ala-Asp) box helicase 5	Q.AINPKLLQL.V
	A.RILGSVLNR.L
TNF receptor-associated protein 1	T.DAPLNIRSI.F
ribosomal protein L4	L.KLNPYAKTMR.R
elastin	G.LGALGGGALGPGGKP.L
AT hook containing transcription factor 1	N.DLKVSTVTSPSRMI.R
ZXD family zinc finger C	R.PFGCPVGGCGKKFT.T
excision repair cross-complementing rodent repair deficiency, complementation group 5	Y.ESKFDSSLLSS.D
G protein-coupled receptor 125	R.PRGAGRAAGAAEGK.V
zinc finger protein 687	T.AGKGAGGALLTPK.T
T-cell leukemia homeobox 2	V.GPGGVIRVPA.H
keratin 9	Y.LSRSGGGGGGGLGSGGSIRSSY.S
desmoplakin	F.SGKTVSVSEAIK.K
arrestin domain containing 1	P.GEPLAGTVRV.R
mortality factor 4 like 1	L.KYVDTNLQKQR.E
structural maintenance of chromosomes 2	F.AYKDPEKNWNR.N
microtubule-associated protein tau	K.SPSSAKSRLQT.A
chromodomain helicase DNA binding protein 3	K.RSSRASSPTKT.S
importin 5	A.LPLLVRVI.Q
proline dehydrogenase (oxidase) 2	V.RRPGASLEL.S
trafficking protein particle complex 1	V.RSLPFFSAR.A
chromodomain helicase DNA binding protein 8	Q.NHQAGAPAPSLSR.C
selenoprotein N, 1	F.RLFVPNHR.S
ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 2	N.KALCAAVVKP.D
acyl-CoA oxidase-like	A.LTPSRL.A
SP100 nuclear antigen	C.RNLVPVQR.V
neuron navigator 1	G.LTPSRL.K
interferon regulatory factor 1	L.TPVRLPSI.Q
myosin XVIIIB	V.RLPAGGGAQDARGL.F
collagen, type V, alpha 2	P.VGPPGLAGERGEQGP.P

SPEG complex locus	R.AKVGAGGGAPVA.V
elastin microfibril interfacer 1	Q.RHLAGLAVGRR.P
alkaline phosphatase, placental	G.EDVAVFARGPQAHLVH.G
alkaline phosphatase, intestinal	G.EDVAVFARGPQAHLVH.G
DENN/MADD domain containing 4C	Y.MNLKSPLGSK.S
tetratricopeptide repeat and ankyrin repeat containing 1	N.DPGPILRI.I
Y box binding protein 1	T.KPGTTGSGAGSGGPGGLTSAAPAGG.D
periplakin	G.KYSPTVQTR.S
CREB binding protein	G.MAKMGITGNTSPF.G
mediator complex subunit 14	L.MVLTDLLPR.K
elastin	G.VVGAGPAAAAAAAKA.A
sosondowah ankyrin repeat domain family member A	L.PRSASAPGAPP.L
G protein-coupled receptor 98	V.GWRAASVFIR.V
zinc finger protein 687	P.LALPALGKGEGA.I
zinc finger protein 318	G.RSSGSSSGPARR.S
forkhead box K2	G.GGAGGGGAGGGGSPPGGWAVARLE.G
coiled-coil domain containing 132	L.EKLPPVLNL.Q
chromosome 11 open reading frame 34	K.LREKLSTLAL.A
collagen, type V, alpha 2	K.GDRGGIGEKGAEGTAGNDGAR.G
mitochondrial translation optimization 1 homolog (S. cerevisiae)	G.GHAGTEAATAAAR.C
collagen, type VII, alpha 1	H.VRAHVAGVDGP.P
Uncharacterized protein FLJ37310	S.QIGGGGGGGGGRR.N
Treacher Collins-Franceschetti syndrome 1	P.RKGAAPAPPGKT.G
SPEG complex locus	R.VWVTMPRR.P
sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3F	S.RATDGALR.P
collagen, type I, alpha 2	E.RGRVGAPGPAGAR.G
keratin 9	S.YLSRSGGGGGGGLGSGGSIRSS.Y
cyclin A1	G.KKALPDCGVQEPPKQGF.D
histone cluster 1, H2bm	E.RIAGEASR.L
collagen, type XIV, alpha 1	N.LPYKGGNTL.T
collagen, type XII, alpha 1	N.LPYKGGNTL.T
MORC family CW-type zinc finger 3	Q.RILEMNDKYVK.K
immunoglobulin superfamily, member 9B	I.ASVIGTRGLAAE.G
myeloid leukemia factor 2	M.RSAPGGIRETR.R
immunoglobulin superfamily, member 9B	E.MEPSLKSR.R
far upstream element (FUSE) binding protein 3	D.KMVGFIIGR.G
zinc finger and BTB domain containing 12	E.ASLVSSISATKSLLPP.A
apoptosis, caspase activation inhibitor	E.RPGAAAAVARGGG.G
death domain containing 1	F.QVREGEQLLL.R
mucin 19, oligomeric	S.ARVAGVTGTSAEVSGRIEPSAT.E
poly(rC) binding protein 1	A.SRPPVTLR.L

poly(rC) binding protein 2	A.SRPPVTLR.L
poly(rC) binding protein 4	V.SRPPVTLR.L
tenascin XB	H.GVPPGGKPSDPI.I
laminin, beta 2 (laminin S)	K.TFRPAAMLVER.S
laminin, beta 4	K.TFRPAAMLVER.S
Ras and Rab interactor 1	L.RTYSPSAQVKR.L
cancer/testis antigen 1B	G.APRGPHGGAASAQ.D
ADP-ribosylation factor-like 6 interacting protein 1	L.VPILAPRI.F
golgi-associated, gamma adaptin ear containing, ARF binding protein 1	L.STAPQPIR.N
unc-93 homolog B1 (C. elegans)	W.LPRPVPLV.A
titin	D.RVHIVIDKQ.S
copine IV	F.GFGARI.P
BCL2-associated athanogene 6	S.GFGARL.L
pericentrin	S.GFGARL.S
keratin 15	S.STFGGGSTR.G
ladinin 1	L.RLSGVVTSR.L
transmembrane protein 131	S.ILSSPRHL.K
N-acyl phosphatidylethanolamine phospholipase D	S.WSVLGPWNR.F
adaptor-related protein complex 3, delta 1 subunit	Y.KVFLKYPESLR.P
perilipin 4	S.ALTGTKEVVSSGVTGA.M
solute carrier family 16, member 11 (monocarboxylic acid transporter 11)	P.LPRLLAVFGALT.G
eukaryotic translation initiation factor 2B, subunit 1 alpha, 26kDa	S.VYVTESQPDLSGK.K
desmoplakin	L.KVQEQELTRLR.I
major histocompatibility complex, class I, C	W.TAADTAAQISQRK.L
major histocompatibility complex, class I, B	W.TAADTAAQISQRK.L
collagen, type IV, alpha 6	G.IGIGAPGKPGLR.G
integrator complex subunit 3	K.GKGAAAAAAASGAAG.G
methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1	L.IKETGVPIAGR.H
heat shock 70kDa protein 1-like	P.TAAAIAYGLDK.G
heat shock 70kDa protein 2	P.TAAAIAYGLDK.K
heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	P.TAAAIAYGLDK.R
eukaryotic translation initiation factor 4 gamma, 3	G.KGSSGGAKASETD.A
DMRT-like family A2	E.AAPAPGLGGGSGPR.Q
thioredoxin interacting protein	R.IVVPKAAIVAR.H
transcription factor-like 5 (basic helix-loop-helix)	A.RADGAAKEGAGAAA.A
SOGA family member 2	P.AVPSSGRAPAPAAPR.S
transmembrane protein 52	Q.KPSPLLGASGL.E
WAS/WASL interacting protein family, member 3	R.LPNKTISGPL.I
v-rel reticuloendotheliosis viral oncogene homolog A (avian)	E.YPEAITRLV.T
ectonucleoside triphosphate diphosphohydrolase 6	D.LAAGVGLIDAEKGGS.L

(putative)	
syntrophin, beta 1 (dystrophin-associated protein A1, 59kD basic component 1)	K.MPILISKI.F
BRCA1 associated RING domain 1	S.MKSLLLLP.E
transmembrane protein 87B	C.RSVAGLLPR.R
protein tyrosine phosphatase, non-receptor type 23	E.IFSGKSVAHEDI.K
solute carrier family 26, member 8	L.IAGCHSSIVR.A
K(lysine) acetyltransferase 6A	E.RPMPRL.E
plexin B2	G.PLGGGIRITI.L
benzodiazapine receptor (peripheral) associated protein 1	P.APATLTGVPRR.T
DNA (cytosine-5-)-methyltransferase 3 alpha	Q.RPMPRL.T
coiled-coil domain containing 43	E.KLDALQGIL.S
small nuclear ribonucleoprotein 70kDa (U1)	G.DAFKTLFV.A
plexin B1	G.EAQGVPVKVL.D
dynein, axonemal, heavy chain 5	L.KTYSVLQDST.I
kinesin family member 21B	L.LSACRAGVIKV.W
nuclear export mediator factor	I.KNPTGEPIPPR.T
zona pellucida-like domain containing 1	R.ERRDAGRRTTWSP.Q
glyoxylate reductase/hydroxypyruvate reductase	A.NNLLAGLRGEPM.P
GC-rich sequence DNA-binding factor 1	G.PGGGDRAPGGESLL.G
SWI/SNF related, matrix associated, actin dependent regulator of chromatin	V.RVWLGKHYK.K
collagen, type IV, alpha 6	G.KKGSKGEPGPKGF.P
collagen, type III, alpha 1	G.RDGSPGGKGDRGENGSPGAPGA.P
lysine (K)-specific demethylase 2B	A.SAVKLAANRTTAGA.R
titin	L.KNVAGTRSVA.V
titin	L.SISKDSVTL.Q
GINS complex subunit 1 (Psf1 homolog)	E.KAMELIREL.H
PTK7 protein tyrosine kinase 7	R.VVLAPQDVVVAR.Y
keratin 16	C.GIGGGIGGGSSRI.S
cadherin-related family member 1	N.PMKAVGVLAGTMA.T
chromosome 2 open reading frame 72	Q.RAARAAGAAGAAAAAA.R
collagen, type I, alpha 1	K.GLTGSPGSPGPDGKTGPPGPAG.Q
WAS/WASL interacting protein family, member 1	P.KGAGAGGGGGGFG.G
proteasome (prosome, macropain) subunit, beta type, 10	A.DAEMTTRMV.A
keratin 17	F.RTKFETEQALR.L
deltex homolog 4 (Drosophila)	M.SAAGLPVCLTR.P
dynein heavy chain domain 1	N.WLAVTKQAL.D
RUN domain containing 3B	G.LSGIRGGGGGGGKKS.L
ribosomal protein L13a	L.KYLAFLRKR.M
desmoplakin	F.LRGAGSIAVAS.A
scavenger receptor cysteine rich domain containing, group B (4 domains)	G.GEPRLAACQSLG.W
elastin	P.GGVVGAGPAAAAAAAAKA.A

phosphatidylinositol binding clathrin assembly protein	T.DRITAAQHSVTG.S
hexokinase domain containing 1	G.GTNFRVLLV.K
trafficking protein particle complex 1	V.RSLPFFSAR.A
SRY (sex determining region Y)-box 18	L.RTAPPAAPLAGL.Y
vacuolar protein sorting 8 homolog (S. cerevisiae)	K.RDESGAIHVTK.Q
splicing factor 3a, subunit 1, 120kDa	I.YPPPEVRNI.V
polo-like kinase 1	A.PEVLSKKGHS.F
trinucleotide repeat containing 18	V.GLPALVAEAGRG.G
major histocompatibility complex, class I, C	M.RYFYTAVSR.P
major histocompatibility complex, class I, B	M.RYFYTAVSR.P
desmoplakin	A.LYKAISVPR.V
titin	R.ISCGGAIRSQKGVS.V
ArfGAP with GTPase domain, ankyrin repeat and PH domain 2	G.KGAGSRL.S
natriuretic peptide receptor B/guanylate cyclase B	H.KGAGSRL.T
keratin 75	K.AQYEDIANR.S
protein tyrosine phosphatase, receptor type, D	I.MPYESTRV.C
protein tyrosine phosphatase, receptor type, S	I.MPYESTRV.C
titin	T.SGKLTVAGGAIS.K
GRB2 associated, regulator of MAPK1	Q.FRGSVR.S
cholinergic receptor, nicotinic, alpha 4 (neuronal)	MELGGPGAPRL.L
protein phosphatase 6, regulatory subunit 2	V.TRKAPLLASDSSSSGGSHSE.D
lon peptidase 2, peroxisomal	V.TKDGPSAGVTI.V
nuclear receptor corepressor 2	A.KSPAPGLASGDR.P
dystonin	S.RPGSRAGSKAGSR.A
serine/arginine repetitive matrix 2	S.RCLIAQTTPVAGSQ.S
BAH domain and coiled-coil containing 1	D.GPEALKTPGKK.S
Treacher Collins-Franceschetti syndrome 1	E.SPRKGAAPAPPGK.T
desmoplakin	Q.ISNNRTLEL.Q
mitochondrial ribosomal protein L1	P.YPFASEINK.V
ubiquitin-conjugating enzyme E2S	G.RAEAGR.A
sterile alpha motif domain containing 9	I.MPRGKFLVV.F
potassium voltage-gated channel, shaker-related subfamily, member 5	G.KIVGSLCAIAGV.L
potassium voltage-gated channel, shaker-related subfamily, member 4	G.KIVGSLCAIAGV.L
dachsous 1 (Drosophila)	P.AALHVVARDPD.L
ribosomal protein S3	R.YKLLGGLAVR.R
bromodomain PHD finger transcription factor	G.GRTGGGGGGGHLA.R
discs, large (Drosophila) homolog-associated protein 4	P.RTTSKPFISVTVQSSTESAQ.D
ubiquitin specific peptidase 19	E.GDTGLPRV.W
WD repeat domain 87	G.PSGSLLALCETVVR.V
calmodulin-like 5	L.RRAMAGLGQP.L
syntrophin, beta 2 (dystrophin-associated protein	R.MPILISKI.F

A1, 59kD, basic component 2)	
one cut homeobox 3	L.LSPGHARSAAAQ.H
zinc finger protein 469	R.GTFHKGSATKPAGCQSSSKDR.S
Y box binding protein 1	T.GSGAGSGGPGGLTSAAPAGGDKKVIAT.K
catenin (cadherin-associated protein), alpha 1, 102kDa	G.ILQKNVPIL.Y
proline rich 25	P.VGRSGGGASARSSR.P
transmembrane protein 214	R.RPGVGAGAGGRGG.G
dynein, axonemal, heavy chain 3	A.QDLKLKDLL.K
myosin IIIB	G.RLFSWIVNR.I
F-box protein 46	G.LPSGGGGRPGCAYPGSPGPGAR.A
eukaryotic translation initiation factor 3, subunit C	E.RVFDHKQGTYGGYFR.D
mucin 5AC, oligomeric mucus/gel-forming	E.DAPGVPLR.A
autophagy related 2A	L.GSLHLLLTPR.Q
NCK interacting protein with SH3 domain	R.RGPSASSVAVMT.S
DEAH (Asp-Glu-Ala-His) box polypeptide 9	D.PSVPVALNIGK.L
intraflagellar transport 172 homolog (Chlamydomonas)	L.YLKAGLPAK.A
dihydroxyacetone kinase 2 homolog (S. cerevisiae)	V.AKAMGTLGVSL.S
mitogen-activated protein kinase kinase kinase 1, E3 ubiquitin protein ligase	G.RTVKSESPGV.R
ubiquitin protein ligase E3 component n-recognin 4	I.SIMKPVR.K
Cas scaffolding protein family member 4	Y.LEANIDAIHR.S
NFAT, cytoplasmic, calcineurin-dependent 2 interacting protein	K.RGRWSGGSGAGR.G
titin	I.AKNAAGAISKPSD.S
	D.RPSAGATVGGEGQASQIGGGGGGGGGRR.N
collagen, type II, alpha 1	G.IAGAPGFPGPR.G
calcium channel, voltage-dependent, T type, alpha 1H subunit	T.DTPGPGPGSPQR.R
F-box protein 41	G.RGGGGGGAGPNA.R
E1A binding protein p400	S.RTAVPPGLSSL.P
EP400 N-terminal like	S.RTAVPPGLSSL.P
major histocompatibility complex, class I, C	A.GSHSMRYFYTAVSRPGR.G
protein tyrosine phosphatase, receptor type, N	N.RAEGPPEPSR.V
collagen, type XXVII, alpha 1	G.EMGPKGPPGAV.G
keratin 15	I.KTRLEQEIATYR.S
teneurin transmembrane protein 1	V.ISTIMGNGHQRS.V
keratin 17	V.KTRLEQEIATYR.R
keratin 16	V.KTRLEQEIATYR.R
Polymerase	T.TTSAEQHLTGKK.N
periplakin	G.KYSPTVQTR.S
cAMP responsive element binding protein 1	S.DAPGVPRI.E
spectrin, beta, non-erythrocytic 5	L.STKEALLVWCQR.K
F-box protein 41	T.PSASLGRGGGGGGAGPNARGPG.R
keratin 3	N.KVLETKWNL.L

bassoon (presynaptic cytomatrix protein)	P.AGGGQLPAAGAAR.S
fibroblast growth factor receptor-like 1	G.AFPPAAAAR.G
DNA (cytosine-5-)-methyltransferase 3 alpha	G.HKGTYGLLR.R
titin	L.TVAGGAISKPLT.D
keratin 17	N.REVATNSEL.V
mediator complex subunit 12-like	G.DLSVTASTR.P
serine/arginine repetitive matrix 2	R.SRSRTSPVSR.R
collagen, type III, alpha 1	E.RGPPGPQGLPGLA.G
polyamine oxidase (exo-N4-amino)	G.EAPGGPRVL.V
nucleotide-binding oligomerization domain containing 2	R.AKGVVPGSTAP.L
KIAA1614	F.HRLVGSLDRR.G
ubiquitin specific peptidase 19	L.EGYARYSVS.V
selenophosphate synthetase 2	P.DVRPPLGRGL.V
retinoic acid induced 1	E.DGGEEAAPADK.G
ArfGAP with coiled-coil, ankyrin repeat and PH domains 3	K.KLKDALTVVVDD.L
myosin, heavy chain 14, non-muscle	R.VGQSKIFFR.A
ATP-binding cassette, sub-family F (GCN20), member 1	G.RRYGLVGPNGKG.K
ADAM metallopeptidase with thrombospondin type 1 motif, 20	N.VVVKIPAGATNV.D
purine-rich element binding protein B	L.RVSEVKPSYR.N
desmoplakin	L.KVQEQELTRLR.I
keratin 18	F.RVKYETELAMR.Q
AT hook, DNA binding motif, containing 1	S.RGAKASPVAVGSSGA.G
ATP-binding cassette, sub-family F (GCN20), member 1	E.LRATGAAAAEAK.A
paired-like homeobox 2a	E.RAASAKGAAG.A
myosin IC	G.HLAVVAPR.L
catenin (cadherin-associated protein), beta 1, 88kDa	E.LATRAIPEL.T
zinc finger CCCH-type containing 12D	G.ALEHPAAPRL.V
dedicator of cytokinesis 6	M.AIAGGPLAPGSR.A
filamin A, alpha	A.LEFLDRESI.K
URB1 ribosome biogenesis 1 homolog (S. cerevisiae)	Q.SVLKELQNR.R
collagen, type I, alpha 2	N.KGEPGVVGAVG.T
ceroid-lipofuscinosis, neuronal 3	W.KNAVGF.W
titin	L.EASKIVKAGDSSR.L
acyl-CoA synthetase short-chain family member 3	A.LVVPGPRGGLG.G
WDFY family member 4	L.DVSIALTGKPSKTSPAV.T
butyrophilin, subfamily 2, member A2	P.RSAFTVPVR.P
septin 2	I.VPVIAKADTL.T
myosin IXA	L.RQASVIIQR.F
coiled-coil domain containing 85B	G.PSGAGGSGAGPAPELALPPCGPR.D
GRAM domain containing 4	N.LFGKMADIL.E
G protein-coupled receptor 98	R.GRTNAAEIPLIL.Y

paired-like homeobox 2b	G.GEPGKGGAAAAAAAAAAAAAAAAA.A
methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1	D.LISRLSREHGA.F
heat shock protein 90kDa alpha (cytosolic), class B member 1	L.KEFDGKSLVSV.T
homeobox A3	K.RYTAAGAGAGGT.P
centrosomal protein 164kDa	S.SLSSAVGKGRQG.S
pleckstrin homology domain containing, family H	G.SIAREGGGGAGTAAA.V
titin	R.VAARNAVGVSLPR.E
desmoplakin	D.DPFSGKTV.S
leucine-rich repeat containing G protein-coupled receptor 6	L.RPRAGDSGPLA.Y
collagen, type VII, alpha 1	G.RAGNPGTPGAPGLK.G
heterogeneous nuclear ribonucleoprotein L	G.RYYGGGSEGGRAPK.R
ubiquitin protein ligase E3 component n-recognin 4	R.LPYQIKKI.T
solute carrier family 27 (fatty acid transporter), member 3	D.GAARGGGAAAPLSPGAT.V
tubulin tyrosine ligase-like family, member 3	L.KFDDLDGTHALMVGLCLN.L
chromodomain helicase DNA binding protein 2	E.RENGQEILL.S
chromodomain helicase DNA binding protein 4	K.KYYKYILTR.N
chromodomain helicase DNA binding protein 3	K.KYYKYILTR.N
5'-nucleotidase, cytosolic III-like	G.RVQEIVGALR.K
alanyl-tRNA synthetase 2, mitochondrial	S.RVVAQGTGSTTDL.E
CTF8, chromosome transmission fidelity factor 8 homolog (S. cerevisiae)	L.ASNPATFQR.S
collagen, type XXV, alpha 1	K.GDPGSSAAGIK.G
keratin 9	K.IGLGGRGGSGGSYGRGSRGGSGGSY.G
major histocompatibility complex, class I, F	L.RYFSTAVSR.P
BAH domain and coiled-coil containing 1	T.ARQGRAAPAFKG.G
myeloid/lymphoid or mixed-lineage leukemia 4	V.ALRRGGGATGPGGA.E
ribosomal protein L4	G.RMFAPTKTWR.R
asparagine-linked glycosylation 3, alpha-1,3-mannosyltransferase homolog	Y.RVHSIFVLR.L
formin-like 3	F.IALTVKEKYPD.L
protein Z, vitamin K-dependent plasma glycoprotein	F.LPASKANDVL.V
splicing factor, suppressor of white-apricot homolog (Drosophila)	E.VGARAGSGGK.K
keratin 75	D.AEQRGELAL.K
neurturin	R.WKAAALASVL.C
TBC1 domain family, member 15	S.DAIPGLKI.N
activating signal cointegrator 1 complex subunit 3	A.YVAQNAARI.V
zinc finger protein 687	K.TSCGNITRTV.T
glyceraldehyde-3-phosphate dehydrogenase, spermatogenic	C.LAPLAKVI.H
microtubule-associated protein 1A	Y.LEVTKAPSLD.S
leucine-rich repeats and calponin homology (CH) domain containing 4	Q.LPLRVLIV.S

cat eye syndrome chromosome region, candidate 2	H.RLQPPPVPAPSSL.F
FERM domain containing 4A	N.LAARGGAGGAGGAGG.G
serine/threonine kinase 36	L.LTEQGKASLI.R
keratin 17	R.LSGGLGAGSCRLGSAGGLGSTL.G
collagen, type IV, alpha 4	I.KGKVGPPGGRGPKG.E
3-oxoacid CoA transferase 1	G.SVAIASKPR.E
adaptor-related protein complex 1, gamma 2 subunit	Q.AAVPKSLQL.Q
pinin, desmosome associated protein	A.KQRDLEGAVSR.L
5-hydroxytryptamine (serotonin) receptor 3B, ionotropic	R.VEPRAQRAVVT.E
cytochrome P450, family 51, subfamily A, polypeptide 1	F.AYVPFGAGRHR.C
chromodomain helicase DNA binding protein 5	G.GITGGLRQEAI.D
myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 6	G.AEGSGGGPKGGTAD.K
son of sevenless homolog 1 (Drosophila)	S.LAKRRLSESACR.F
UDP-N-acetyl-alpha-D-galactosamine: N-acetylgalactosaminyltransferase 2	G.SALAGGAGGGAGR.K
Y box binding protein 1	G.AEAANVTGPGGVPVQGSKYAA.D
S100 calcium binding protein P	H.KYFEKAGLK
BTB (POZ) domain containing 6	N.AGAAVGRKAGPR.S
desmoplakin	Y.RSNKPIILR.A
solute carrier family 12 (potassium/chloride transporters), member 9	G.VALPANGAGGPGGASAR.K
smg-5 homolog, nonsense mediated mRNA decay factor (C. elegans)	A.EGLLPAVK.V
Y box binding protein 1	A.PALSAADTKPGTTGSGAGSGGPG.G
plexin B2	A.TVGLVSSTGPGGDR.V
progesterone receptor	P.RALGGAAAGGGAAAVPPG.A
growth hormone inducible transmembrane protein	A.IAISRTPVL.M
myosin XIX	L.TVVSKFKAS.L
non-SMC condensin I complex, subunit G	A.KTLPKIVGR.T
S100 calcium binding protein P	H.KYFEKAGLK
scavenger receptor class F, member 1	P.GATGPMAVRPE.E
kinesin family member 26A	P.RAAPRAGPSVGAKA.G
periplakin	K.AEVKEKVVL.S
kinesin family member 26B	G.RISELLQGGAGAR.G
discs, large homolog 1 (Drosophila)	I.SSGSGSLRTSQK.R
KIAA1468	G.VPGAAGVGGAGGR.E
myosin binding protein C, cardiac	N.SPTDTILFIR.A
mortality factor 4 like 1	Y.AEGKMRGAAPGKKT.S
praja ring finger 2, E3 ubiquitin protein ligase	L.DPPYSRVI.T
erbb2 interacting protein	L.KMLPKTMNR.L
kinesin family member 26A	D.LGSCEAAAGRAGEAAGGPLCLS.L
DEAD (Asp-Glu-Ala-Asp) box helicase 1	L.DDLVSTGKLNL.S
major histocompatibility complex, class I, B	R.VTAPRTVLLLL.S

major histocompatibility complex, class I, B	R.VTAPRTVLLLL.W
DIP2 disco-interacting protein 2 homolog B (Drosophila)	I.LPGVKVVI.V
GH3 domain containing	L.AGAIAAGNPGAPLR.E
carbohydrate (N-acetylglucosamine-6-O) sulfotransferase 2	D.VAVLAPLLRD.P
E74-like factor 3 (ets domain transcription factor, epithelial-specific)	S.RAMRYYYKR.E

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