Investigating T cell immunity against the oncogenic Merkel cell polyomavirus

By

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Abstract

Merkel cell polyomavirus (MCV) is a causative factor in Merkel cell cancer (MCC). This aggressive skin malignancy is associated with UV-light exposure, ageing or immunosuppression, implying immune constraint of MCC development. We examined immune control over MCV in MCC patients by comparing immune parameters with donor groups who share risk factors alongside healthy controls. This showed MCC patients had frequent and strong MCV antibody responses but no differences in responses to other polyomaviruses suggesting no general defect in humoral immunity to these viruses. MCC patients had lower frequencies of B-cells while T-cells from patients with active disease proliferated relatively poorly. Quantifying peripheral T-cell responses to the large- and small-T-antigens in patient groups and healthy donors by ELISpot showed that like with other polyomaviruses, responses were weak. Novel epitopes were identified by establishing T-antigen-specific CD4 and CD8 T-cell clones from healthy donors which recognised antigen-expressing cells. However MCC tumours and lines were found to have low levels of surface HLA Class I and Class II and could poorly process and present epitope to T-cells. Consistent with this, preliminary experiments showed that small-T inhibited epitope presentation suggesting that small-T function must be inhibited for efficient T cell targeting of infected cells.

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Abbreviations

AID	Activation-induced cytidine deaminase
APC	Antigen presenting cell
BCC	Basal cell cancer/carcinoma
BCR	B cell receptor
BKV	BK polyomavirus
cDNA	complementary DNA
CEF	CMV, EBV, Influenza
CK	Cytokeratin
CLA	Cutaneous Lymphocyte Antigen
CLIP	Class II-associated invariant chain peptide
CLL	Chronic Lymphocytic leukaemia
CMV	Cytomegalovirus
CR1	Conserved Region 1
CT	Common T
CTACK	Cutaneous T cell attracting chemokine
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
DRiPS	Defective ribosomal products
DTS	Digital Transcriptome Subtraction
EBV	Epstein-Barr virus
ER	Endoplasmic reticulum
ERAAP	Endoplasmic reticulum aminopeptidase associated with antigen processing
GM-CSF	Granulocyte Macrophage Colony Stimulating factor
HIV	Human Immunodeficiency Virus
HLA	Human Leucocyte antigen
HPyV	Human polyomavirus

HSV	Herpes Simplex Virus
IFN	Interferon
Ig	Immunoglobulin
Ii	Invariant chain
JCV	John Cunningham polyomavirus
KI	Karolinska Institut polyomavirus
KSHV	Kaposi Sarcoma Herpes Virus
LT	Large T antigen
MCV	Merkel cell polyomavirus
MCC	Merkel cell cancer/carcinoma
MHC	Major Histocompatibility Complex
MIIC	MHC Class II Compartment
MJS	MelJuSo cells
mTOR	mammalian target of rapamycin
MUR	MCV Unique Region
MW	Malawi polyomavirus
MX	Mexico polyomavirus
NCCR	non-coding control region
NK	Natural killer
NLS	Nuclear Localisation Signal
NMSC	Non melanoma skin cancer
OBD	Origin Binding Domain
PAMPS	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PD-1	Programmed cell Death protein-1
PDL-1	Programmed cell Death protein ligand-1
PHA	Phytohaemagglutanin

PML	Progressive multifocal leukoencephalopathy
PP2A	Protein Phosphatase 2A
PRR	Pattern recognition receptors
RB	Retinoblastoma
SCC	Squamous cell cancer
SFC	Spot forming cells
sT	Small T antigen
SV40	Simian virus 40
T-antigen	Tumour antigen
TCR	T cell receptor
TEMRA	T Effector Memory CD45RA+
TGF-β	Transforming growth factor beta
TNFα	Tumour necrosis factor alpha
TLR	Toll like receptor
TRAIL	TNF-related apoptosis-inducing ligand
Tregs	Regulatory T cells
Trm	Tissue resident memory
TS(V)	Trichodysplasia Spinulosa (polyomavirus)
UV	Ultraviolet
VLP	Virus-like particle
VP-1/2/3	Viral capsid protein 1/2/3
VZV	Varicella Zoster virus
WHIM	Warts with Hypoglobulinaemia, Infections, and Myeloathexis

Chapter 1

Introduction

1.1 Merkel Cell Cancer (MCC)

1.1.1 Merkel cells

In 1875, Merkel first described small, oval basophilic cells within the skin, predominantly in the basal layer of the epidermis [1]. They are present all over the skin but are found in higher density in hairless areas, such as the soles and palms [2]. They can be found in distinct populations and in certain conditions do undergo hyperplasia which occurs independently of epidermal cell proliferation [3]. Initially termed "Tastzellen" (touch cells), they were assumed to have a sensory function from being bound to terminal ends of sensory nerves. There is some evidence that they are important for light touch determination [4], however, their exact function remains unclear, even today. Histologically, these cells are identified by the presence of secretory granules and through commonly expressing keratin containing filaments termed cytokeratins (CK). Of this family of proteins that form the cytoskeleton of epithelial cells, the members most commonly associated with Merkel cells are CK8, CK18, CK19 and CK20 [5, 6]. Merkel cells can undergo a malignant transformation forming Merkel cell cancer (MCC).

1.1.2 MCC clinical features

MCC tends to develop as a firm, pink-violet, non-pigmented nodule and it is often difficult to diagnosis by clinical examination alone, often being mistaken for a simple cyst [7]. The majority of cases are found on sun-exposed skin, particularly the head and face [8]. The

median age of diagnosis is 74 (range 8-101) and it is commoner in Caucasians and females [8, 9]. Definitive diagnosis is established by positive staining for chromogranin, synaptophysin, neuron-specific enolase and CD56 (also known as the neural cell adhesion molecule), which represent both the epithelial and neuroendocrine antigens present in normal Merkel cells [10, 11]. Additionally, expression of cytokeratins can aid in diagnosis with CK20 para-nuclear dot like staining and seen in 97-100% of MCC [10, 12, 13]. Another cytokeratin, CK19, is expressed in 33% of all cases and can be useful in CK20 negative cases [10].

1.1.3 MCC staging, prognosis and treatment

MCC tends to be an aggressive tumour that grows rapidly and metastasises early. In a large retrospective cohort of 5823 MCC patients from the USA, 66% presented with local disease, 27% with nodal involvement and 7% with metastatic disease [14]. Disease stage classification is shown in Table 1.1.

Table 1.1. MCC disease staging classification. Adapted from [14].

Stage	Definition	Treatment
1 A B	Local disease, tumour < 2cm Nodes negative by pathological examination Nodes negative by clinical examination only	Surgical +/- Radiotherapy
2 A B C	Local disease, tumour > 4cm Nodes negative by pathological examination Nodes negative by clinical examination only Tumour invades deeper structures e.g muscle, bone	Surgery + Radiotherapy
3 A B	Regional Lymph node disease Micrometastasis Macrometastases (clinically detectable)	Surgery + Radiotherapy if resectable Systemic agents if unresectable
4	Distant metastatic disease	Systemic agents

Stages 1 and 2 (Local disease)

When primary tumour is the only area of disease, treatment is through surgical resection. Unless the tumour is very small, adjuvant radiotherapy to the tumour bed would also be recommended. This is because recurrence rate can be as high as 30-43% and even patients with margin-negative resected disease have a recurrence rate of 8% [15, 16]. There is some evidence that radiotherapy reduces rates of local recurrence [17]. The size of primary tumour is also important prognostically with tumours less than 2cm (stage 1) having survival rates of 66-81% compared with 51-67% in tumours greater than 2cm (stage 2) [14, 15]. The thickness of the primary tumour has also been identified as having a prognostic impact, where patients with tumours over 10 mm thick having a significantly worse five year disease free survival of 18 % compared with 69 % for patients with tumors less than or equal to 10 mm [18]. Other investigators have found that, even thin tumours had the potential to have spread to other areas [19], perhaps explained by the early ability of MCC to invade lympho-vascular vessels, with two-thirds of MCC less than 2 cm in diameter showing histological evidence for this [20].

Stage 3 (Regional lymph node involvement)

Patients with confirmed Stage 3 disease are treated with a combination of surgery and radiotherapy with the documented 5 year survival for these patients being 39% [14]. A role for adjuvant chemotherapy or radiotherapy in this patient group is not clear with no evidence of survival benefit [21]. However, it has the potential to be helpful in well selected cases [15]. The number of lymph nodes involved by MCC metastatic spread is a strong predictor of overall survival (0 nodes involved, 76% 5-year survival, more than or equal to 6 nodes involved, 24% 5 year overall survival) [22].

Stage 4 (Metastatic disease)

At this advanced stage, patients have a very poor outcome with the majority of patients living less than 12 months and 5 year survival rate of 18% [14]. Treatment is limited to palliative surgery and radiotherapy for particularly symptomatic areas of disease, delivered alongside systemic platinum-based chemotherapy. Although the initial response rate to chemotherapy is good (60-76%), these effects are not durable and patients tend to progress quickly [23, 24]. There are no approved second line treatments. Importantly, due to the mostly elderly patient population, cytotoxic chemotherapy can be extremely difficult to tolerate and be associated with significant morbidity.

1.1.4 Epidemiology, risk factors and clinical associations

MCC is rare in the general population but appears to be on the increase [25] with age-standardised incidence rate per million doubling in ten years from 1.7 in 1993-1997 to 3.5 in 2003-2007 [26].

There is a dramatic increase in incidence of MCC seen over the age of 50 [8]. Although MCC is more commonly seen in females, male MCC patients have poorer outcomes, with a 5-year relative survival ratio amongst men of 36% (95% confidence interval 20-54%) compared with 69% (56-82%) in women [27]. There appears to be a relationship between UV radiation exposure and MCC [28] with the majority of cases occurring on areas of sun-exposed skin (36% occur on the face), disease rates correlating with proximity to the equator [8] and also with the side of the body more exposed to the sun during driving [29, 30].

Immunocompromise is another associated risk factor for MCC, with HIV-positive patients having a 13-fold increase in risk of developing MCC compared to the general population [31].

Patients who are on immunosuppressive medication post-organ transplant have at least a ten times increased risk relative compared to their peers [32, 33]. Given the rarity of Merkel cell cancer, it has been difficult to make associations with other diseases, however, the review of large cancer registry datasets has repeatedly shown two interesting points. Firstly, the chance of developing a second cancer after diagnosis with MCC is slightly but significantly increased [34]. Secondly, a number of cancers, particularly skin (Squamous cell carcinoma, Melanoma) or haematological malignancies (Chronic Lymphocytic Leukaemia (CLL), Multiple Myeloma, Non-Hodgkin Lymphoma) are associated with higher than expected risk of subsequent MCC development [34-36]. One study found a standardised incidence ratio of 15.7 (3.2-46) of developing MCC after CLL [37]. MCC patients who are immunosuppressed have a poorer outcome in terms of MCC specific survival [38, 39]. For example, MCC patients who have received solid organ transplants have significantly reduced MCC-specific survival compared with immunocompetent patients, independent of disease stage at presentation (56.3% versus 95.2% survival at 1 year) [40]. Additionally, CLL patients with MCC are 3.8 times more likely to die from MCC compared to MCC patients with no history of CLL [35]. In a single centre retrospective study, a low absolute lymphocyte count at diagnosis of MCC has been associated with poorer overall survival, an effect which is independent of other tumour, patient or treatment factors [41].

1.2 Merkel cell polyomavirus (MCV)

1.2.1 Discovery of MCV

The link between immunocompromise and MCC led to the hypothesis that an infectious agent may link the two. In 2008, the laboratory of Moore and Chang applied digital transcriptome subtraction (DTS) technology on MCC specimens [42]. By creating two cDNA libraries, one from a single tumour and the other from pooled tissue from three other patients, pyrosequencing of these two libraries generated 382,747 sequences to use as a high fidelity data set. Of these sequences, 99.4% aligned to known human RNA, mitochondrial, assembled chromosomes or immunoglobulin sequences. Of the remaining sequences, one (DTS 1) from the single source cDNA library revealed a transcript highly homologous to African green monkey lymphotropic polyomavirus and to human BK polyomavirus T-antigen sequence. The second DTS transcript had no homology to deposited polyomavirus sequences. By viral genome walking, the complete 5387 base pair (bp) sequence of viral DNA was defined from tumour specimen MCV350 and used to create a closed circular form of the genome typical of polyomaviruses (see Figure 1.1). Using primers derived from this sequence, a second 5201 bp sequence was identified from another tumour MCV339. Of ten tumours tested, viral DNA was discovered in 80%, as compared to 16% of skin from control skin samples.

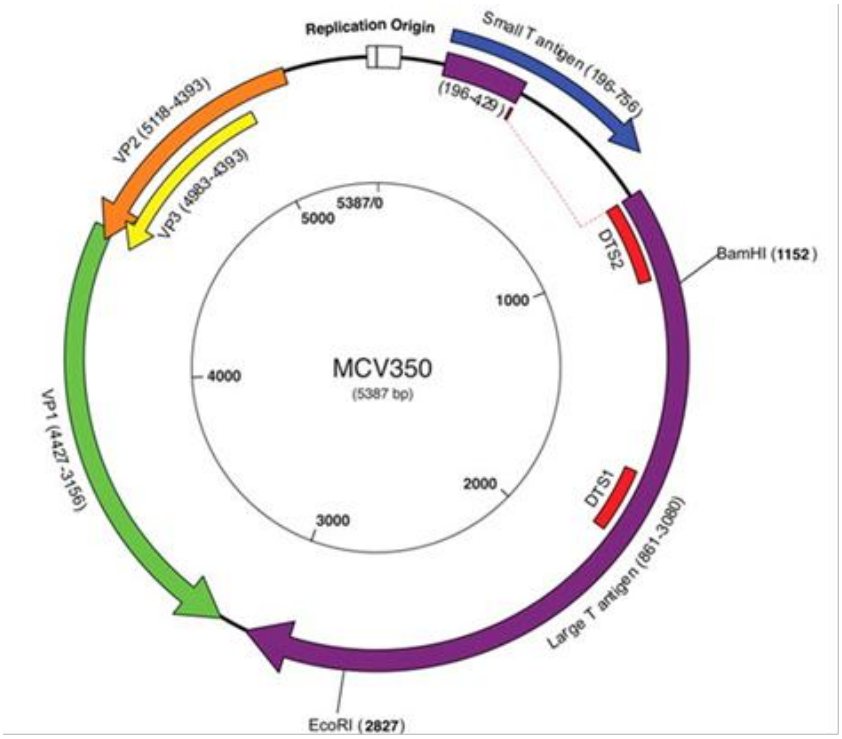


Figure 1.1. A full map of the MCV350 isolate genome identified by viral genome walking following discovery of viral DNA sequences (DTS1 and DTS2) within a MCC tumour. The position of early (Large and small T-antigens) and late (VP-1, VP-2 and VP-3) genes are shown. Figure adapted from [42].

1.2.2 Introduction to polyomaviruses

MCV was the fifth polyomavirus to be discovered in humans although other polyomaviruses have been detected in many other vertebrate hosts including birds, rodents and cattle [43]. The polyomavirus family has a very highly conserved genome sequence (between 50-80% shared amino acid), but each virus has a very narrow species and cell specificity [44]. This is likely due to both the presence or absence of cell surface receptors that allow cell entry and also the presence or absence of cell signalling factors within host that allow viral DNA replication [45]. All polyomaviruses consist of non-enveloped, icosahedral virions with small (around 5000 base pairs) super-coiled double stranded DNA genome which can be divided into three sections, encoding early and late genes as well as a non-coding control region.

The early genes encode two major proteins through alternative splicing of the same transcript. These proteins, Large T-antigen (LT) and Small T-antigen (sT) facilitate viral genome replication and transformation by disrupting cell cycle control, the mechanisms of which are discussed in sections 1.2.3 and 1.2.4. The late coding region contains a primary transcript encoding the structural capsid proteins. The non-coding control region (NCCR) contains the viral promoters that mediate both early and late gene transcription and origins of replication.

1.2.3 Large T -antigen (LT)

LT is a protein expressed early in infection and is essential for viral replication. There are a number of well conserved regions throughout the entire polyomavirus family that appear to be integral to function (see Figure 1.2).

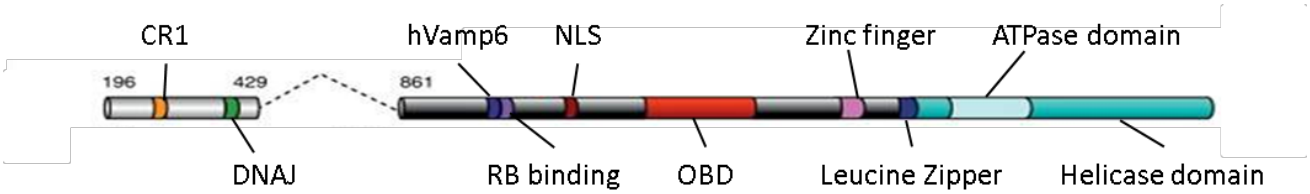


Figure 1.2. Structure of the MCV Large T-antigen (LT) gene showing the position of functional domains within both exon 1 (bp196-429) and exon 2 (bp861-3080) of MCV350 genome. CR1, Constant Region 1; NLS, Nuclear Localisation Signal; OBD, Origin Binding Domain. Adapted from[44].

Conserved Region 1 (CR1), DNAJ and Retinoblastoma (RB) binding domains

The CR1 and DNAJ domains exist within exon 1 of the T-antigen, a region common between both LT and sT. The DNAJ domain functions as a molecular chaperone, interacting with a

heat shock protein Hsc70 regulating ATPase activity. Early in infection, LT accumulates in the cell nucleus, driven by factors including the nuclear localisation signal (NLS) [46]. There, ATP generated from the J domain and Hsc70 interaction provides energy to disrupt the important Retinoblastoma (RB)-E2F complex [47]. The E2F family are a tightly regulated group of transcription factors that when activated allow gene transcription and entry into cell cycle [48]. E2Fs are usually held in a repressive complex with a member of the RB family of proteins (RB, p106, p130), inhibiting transcription in the quiescent cell. When required, a chromatin remodelling factor can bind to RB using an LXCXE domain, releasing these factors.

LT contains a LXCXE RB binding domain that binds and inactivates all three RB proteins after disruption of the RBRB-E2F complex, temporarily displace RBRB and releasing activated E2F leading to up-regulation of E2F gene targets and the induction of unregulated cell entry into S phase. Large T-antigen mutants lacking either a functional LXCXE motif or the J domain fail to increase E2F-dependent transcription and do not stimulate proliferation, indicating both domains are essential for viral replication [49]. The CR1 domain is so-called as it is homologous to a region conserved between the 12S and 13S splice variants of the adenovirus E1A viral protein. Its function has not been as well characterised as the other regions although it is also thought to be involved in binding to the RB family of proteins [50, 51].

Origin Binding Domain (OBD)

DNA replication is initiated in polyomaviruses by the binding of LT to the replicative origin, found in the non-coding control region (NCCR) located between the early and late genes in the viral sequence. The replicative origin contains multiple copies of the pentameric DNA

sequence GAGGC, each separated by a one base space to which the LT OBD is able to bind [52]. This allows full-length LT molecules to assemble and form a dodecameric structure wherein two head-to-head hexameric LT rings encircle the DNA and from this position bi-directional DNA replication can commence [53, 54].

Zinc binding and ATPase Domains

The zinc binding and ATPase domains form the enzymatic core of T-antigen's DNA helicase activity. The zinc-binding domain is responsible primarily for the formation of T-antigen hexamers, the active helicase form which can then be positioned on the OBD as described above. The ATPase domain provides the energy needed for helicase activity. For full helicase activity, co-ordinated actions of OBD, Zinc binding and ATPase domains are all required [55].

The ATPase domain also contains a site capable of binding P53, an important transcription factor which activates pathways associated with tumour suppressor activity. Under normal circumstances, P53 levels are low, but in response to stress, DNA damage or active oncogenes, the half-life of P53 rises dramatically, leading to its accumulation within the cell leading to the up-regulation of genes such as P21 capable of promoting cell cycle arrest, inducing apoptosis and thereby preventing further viral replication [56, 57]. The binding of polyomavirus LT to P53 can lead to P53 inactivation and thereby inhibit the cellular anti-viral protective process [58]. Although full length MCV LT has a putative P53 binding domain and is able to modulate this pathway, it has been shown that this does not occur through direct interaction [59, 60]. Even the case of SV40, in which P53/LT binding does occur, this is not independently sufficient to block P53 function. It is, therefore, likely that other indirect

mechanisms perhaps involving sT, the DNAJ and RB binding domains on LT are also important in functional suppression of P53 [61, 62].

Helicase Domain

Found at the carboxyl end of LT, the helicase domain is important in allowing viral DNA replication by initiating bi-directional double-stranded DNA unwinding once LT has bound to the OBD. This domain also recruits cellular proteins involved in DNA synthesis such as DNA polymerase thereby enabling viral replication [63]. The T-antigen is unique amongst viruses by being able to initiate DNA winding without need for a separate initiator molecule [55].

Nuclear Localisation Signal (NLS)

All polyomavirus LT proteins, with the exception of avian polyomavirus, contains a nuclear localization signal (RKRK) which results in the typical nuclear LT localisation pattern seen in most LT-expressing cells [50].

MCV Unique Regions (MUR)

MCV LT has two unique sequences, starting after the J Domain and separated by the RB binding site, known as MUR 1 and 2. Although these regions do not appear essential to LTs growth promoting function, they may be important in stabilising the protein and thereby allowing high level expression in the cells [64]. The MUR1 contains a binding site for the cytosolic human protein Vam6p, resulting in sequestration of this protein into the nucleus. This may have a role in regulating MCV replication as inhibition of this binding results in enhanced replication through an as yet unknown mechanism potentially involving lysosomal trafficking or an impact on pathways such as TGF-β and mTOR [65, 66].

1.2.4 Small T-antigen (sT)

The Small T-antigen shares the amino-terminal 78 amino acids that encode exon 1 with Large T and 57kT containing the DNAJ domain binding sites, but lacks the Large T Origin Binding Domain (OBD) that is required for DNA recognition (Figure 1.3).

Figure 1.3. Small T-antigen (sT) gene structure showing position of functional domains in single exon (bp196-756) of MCV350 genome. CR1, Constant Region 1; PP2A, Protein Phosphatase 2A. Adapted from [67].

The sT does encode for a Protein Phosphatase 2A (PP2A) binding domain (CXCXXC) that is spliced out of the other T-antigen variants. PP2A binding by SV40 sT results in inhibition of Akt dephosphorylation which causes constitutive activation of this molecule and stimulation of numerous cell signalling pathways causing up-regulation of transcription [68].

In vitro, infection with sT-deficient SV40 virus results in defective viral replication which is reversed if sT DNA is re-introduced into the cell. The binding of sT to PP2A may be important here by leading to dephosphorylation of serine residues adjacent to the DNA binding domain on LT that increase the binding affinity of LT for the viral DNA-binding site with a consequent increase in DNA replication efficiency [69]. Similarly, MCV sT co-expressed with LT is able to increase viral replication above that seen with LT alone. This increase is abrogated if the PP2A binding site of sT is mutated, again indicating that sT mediated sequestration of PP2A may be important to efficient replication [52, 65, 67].

MCV sT is able to stimulate cellular proliferation through the constitutive phosphorylation of 4E-BP1, a translation initiation factor important downstream of Akt and mTOR in the Akt

signalling pathway known to be deregulated in many cancers [70]. Although the mechanism for this is unknown, it is not thought to be due to sT binding to PP2A or heat shock proteins, both mechanisms utilised by SV40 sT to induce proliferation. The sT-antigen is also able to inhibit an ubiquitin ligase complex, Fbw7, that promotes the degradation of several important proto-oncogenic proteins including c-myc and cyclin E by binding to them and consequently activating related transcriptional pathways [71]. Alongside this, inhibition of Fbw7 also reduces the degradation of MCV LT, leading to increased cellular levels of this protein [71].

Other than mediating viral replication and cellular proliferation, sT may also have other roles. The binding of sT to the protein phosphatase PP2C, leads to high cellular levels of unphosphorylated stathmin, a protein thought to be important in destabilising cellular microtubules, resulting in cells with a more motile and migratory phenotype [72].

1.2.5 57kT and Alternate frame of the Large T Open reading frame (ALTO)

The MCV early region mRNA can also splice to form two alternate open reading frames producing unique proteins. 57kT is identical to full-length LT but lacks an origin-binding domain and seems to share similarity with the 17kT protein found in SV40 and whose function remains mostly unknown [42]. The second alternate protein, termed ALTO conserves only the region around the RB binding site. This protein shares similarities with the Middle T in murine polyomavirus and again its functional role is unknown although it is not thought to be essential to viral replication [73].

1.2.6 MCV Late genes

The MCV late region encodes open reading frames for three capsid proteins by alternative splicing, VP-1, VP-2 and VP-3. The capsid is made up primarily of VP1 molecules (around 90% of the total). VP-1 and VP-2, when expressed in uninfected cells, self-assemble into a 55-nm diameter icosahedral viral particles that can be harvested as antigen for serological assays [74, 75]. The MCV VP-3 sequence contains aberrations not seen in other polyomaviruses and attempts to synthesise this protein have been unsuccessful, suggesting VP-3 has no functional role in MCV [74].

1.2.7 MCV DNA integration within MCC genome

In the same study that discovered MCV [42] the previously mentioned DTS1 viral transcript described was extended using 3'RACE (Rapid amplification of cDNA ends) to three other cDNAs. Two of these showed different length fusions of viral DNA with intron 1 of the human receptor tyrosine phosphatase type 2 (PTPRG) gene on chromosome 3p14.2. The integration of viral DNA into the cellular genome was subsequently confirmed through sequencing of PCR products using viral DNA and PTPRG sequences primers [42]. To confirm whether viral genome integration was a generalised feature of MCC, southern blot analysis was performed on DNA from eight enzymatically digested tumours which were then probed with MCV-specific primers. These results showed that five of eight cases tested contained MCV DNA, that virus was indeed integrated to the human genome and that the integration pattern was identical for all cells within the tumour and also between the primary tumour and distant metastases, suggesting viral integration is an early event in cancer development [42]. Since the seminal discovery of MCV within the MCC tumour genome,

numerous laboratories have confirmed this finding and throughout the world, 64-86% of cases express LT and so are defined as MCV positive (MCV+) [76-79]. More recently, assessment of sT expression alongside LT, the use of novel LT antibodies and improved PCR techniques have shown that up to 97% of cases may be MCV+ [80, 81]. All mutations lead to truncations of LT which retain the RBRB protein binding domain whilst deleting the helicase domain and P53 binding site within the ATPase domain [82-84]. The loss of the helicase domain makes the virus incompetent to replicate proving its presence within the tumour cells is not simply as a "passenger" [85]. The mutations can be substitutions, frameshift, missense, insertions or deletions and the majority result in a truncation secondary to a stop codon within exon 2. There does not appear to be specific sites within the host cell genome that are associated with the integration of LT [82, 84].

The clinical significance of MCV+ compared to MCV negative MCC has been investigated retrospectively in a number of studies. These virus positive primary tumours tend to occur on the limbs and may be slower to metastasise than virus negative disease [86, 87]. There is some discordance in the published literature regarding the effect on disease outcome, with some studies finding improvement while others finding no significant difference in overall survival [87-91].

The viral load found within tumours may be a more sensitive predictor of outcome and various studies have shown that the numbers of viral copies per cell in MCV+ disease is extremely heterogeneous (0.119-42.8) and perhaps this is useful as a clinical biomarker, with values over 1 copy/cell (40-60% of MCV+ MCC) appearing to have some clinical benefit [83, 87, 91].

1.2.8 Role of MCV LT in MCC oncogenesis

The oncogenic effects of MCV LT-antigen have been demonstrated in vitro as it has with other polyomaviruses. In MCC, the LT protein is truncated by mutation to leave the constant region 1 (CR1), J domain and RB binding regions (see Figure 1.4) [85]. While these domains play a role in oncogenesis, it is likely that transformation occurs in concert with cellular mutations and with the support of other factors, such as cellular heat shock protein 70 (hsc70) as inhibition of this protein is able to limit proliferation and survival in LT expressing MCV+MCC cell lines [64, 92].

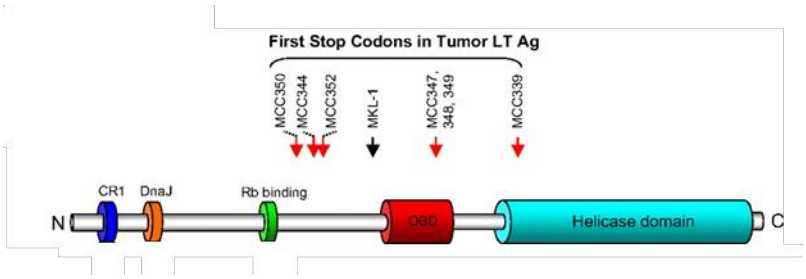


Figure 1.4. The sites of truncation mutations seen in a variety of published MCC tumours. All mutations occur in the region between the RB binding domain and the helicase domain. Adapted from [85]

Large T-antigen expression is essential for the growth of MCV+ MCC cell lines and this is dependent on ability of LT to bind to RB [93]. Knockdown of Large T with shRNA in these same cell lines used in a xenograft model leads to tumour regression [93, 94]. The truncation of the LT appears to be an essential part of cancer development as transfection of truncated LT into a MCV negative MCC cell line, unlike wild type LT, 57KT or Small T, results in a cell type that has a defective response to UV light with decreased levels of DNA repair and cell cycle arrest [95].

Truncated LT is more effective at growth promotion than its full length counterpart perhaps as the 100 residues of the C-terminal portion of LT that are lost as part of the truncation have been shown to have growth inhibitory function [60]. This may be due to the fact that the LT helicase domain initiates a DNA damage response that leads to p53 phosphorylation and activation of downstream p53 target genes and consequent inhibition of cell proliferation, a function that is lost after the oncogenic truncation [96, 97].

Alongside this, truncated LT binds RB with a stronger affinity than the full length protein and is also able to relocalise RB to the cytoplasm, which may also increase its tumourigenic potential [59]. In tumours, high levels of RB expression are mostly limited to MCV+ cases, defined either by evidence of MCV DNA in the tumour, or through demonstrable presence of Large T-antigen expression [79, 98], although this has been controversial [99]. However, not all MCV+ MCC express RB and one study has shown that these patients have significantly poorer outcomes [79, 88, 100].

A truncation mutation affecting the nuclear localization signal can result in more diffuse nuclear or cytoplasmic distribution [101], however this motif is not required for the accumulation of truncated LT-antigen within the nucleus nor is it required to promote proliferation of MCC cells [64].

The integration of MCV expressing LT with an intact RB binding domain in MCC leads to the of up regulation of transcription of Survivin mRNA by up to 7 fold as compared to virus negative cases. This protein, also known as BIRC5a (baculoviral inhibitor of apoptosis repeat-containing 5), is anti-apoptotic, through its ability to inhibit caspase activation. It is commonly up-regulated in cancer and it is an attractive potential therapeutic target due to its absence from terminally differentiated cells. In vitro tests with a small molecule inhibitor of

Survivin, YM155, leads to the irreversible, non-apoptotic death of MCV positive cell lines. When MCV+ MCC xenograft models are treated with YM155, the effect is cytostatic while treatment is given, but tumour growth continues after treatment is withdrawn [102, 103].

1.2.9 Role of MCV sT in MCC oncogenesis

MCV sT expression is detected in the majority of MCV+ MCC and no truncation mutations have been found to impact on its structure or function [70, 104]. sT expression is likely to have an effect on cell growth. In vitro, this has been demonstrated by ectopic expression of sT in fibroblasts that leads to cellular proliferation [70, 105]. However, the importance of continued sT expression in established MCC cells is less clear, with two groups performing shRNA knockdown experiments in MCV+ MCC cell lines. Shuda et al found that growth inhibition is specific to MCV+ cell lines only and occurs on a similar scale to pan T-antigen knockdown [70]. Angermeyer et al found a non-specific growth retardation in both MCV+ and MCV- cell lines on sT shRNA knockdown and also that pan-T-antigen knockdown could be completely rescued with re-expression of LT alone, suggesting sT is not essential in this process [105].

The strongest evidence for oncogenesis mediated by sT comes from recently developed transgenic mouse models. The postnatal induction of sT using an inducible transgene leads to epithelial neoplastic lesions that resemble squamous cell carcinoma. This is accompanied by the elevated expression of proteins such as Cyclin E usually targeted for degradation by Fbw7, suggesting impairment of this pathway by sT is important to cellular transformation [106]. In another transgenic mouse model, induction of sT at an embryonal stage led to the proliferation of Merkel cells in the touch domes of late embryos but these normalised post-

natally. Here, high level of sT expression in mice was universally fatal after a few days while low level expression led to some dermal hyperkeratosis but no tumour growth. Tumours did develop, however, when p53-null mice were used, suggesting that dysregulation of this pathway may be required in order for sT to be oncogenic [107].

Lastly, it is important to note that expression levels of MCV sT may be important to the oncogenic process as that these can be altered by external factors. MCV sT is likely to have a UV-inducible gene promoter which leads to increased mRNA transcription after stimulation of MCV-infected skin with UV light and this may be important to MCC given the link between the cancer and sunlight exposure [108].

1.3 Pathogenesis of MCV-negative MCC

The recurrent finding of a proportion of MCC that do not contain detectable MCV DNA or stain positively for LT/sT by immunohistochemistry [109] suggests that there are at least two pathogenic pathways. This may be reflected phenotypically, with the suggestion that the classical neuroendocrine disease tends to be virally associated (63% of such cases), whereas tumours deficient in expression of endocrine markers are virus negative [110]. In Australia, a larger proportion of MCCs are MCV negative and this may be due to an inverse relationship in MCV positivity and the prevalence of sun damaged skin [111, 112]. Further evidence for this relationship has recently become available with the finding that MCV- tumours have a significantly higher rate of somatic single nucleotide mutations than MCV+ tumours and a high number of these are C>T amino acid substitutions which are a signature of UV-mediated mutagenesis [113].

The genes most commonly affected by mutation in MCV- MCC are RB1 and P53. In MCV-MCC, loss of function RB1 gene mutations are prevalent and may be a driver of oncogenesis in these cases [113, 114]. Mutations of P53, leading to a loss of function, are much more common in the virus negative subgroup, with between 50 and 100% harbouring mutations in this gene [79, 80, 100, 115, 116].

1.4 The polyomavirus family

The first polyomavirus to be discovered was murine polyomavirus, first described in the 1950s and which although ubiquitous is able to cause tumours in experimentally infected new born or immunosuppressed mice [117]. Much of our knowledge on polyomavirus biology is based on Simian Virus 40 (SV40), the first identified primate polyomavirus, discovered in 1960 within cultures of African green monkey kidney cells used to produce the polio vaccine [118]. The finding that this virus is capable of inducing tumours in rodents through expression of Large and Small T-antigens, in other experimental models has led to major discoveries of important oncogenic pathways as described in section 1.2 [119].

Until recently, polyomaviruses were split into 3 clades; SV40, avian, and murine; with the human viruses BK and JC belonging to the SV40 clade. However due to improved molecular techniques, a number of new human polyomaviruses have been discovered leading to a reclassification by the International Committee on Taxonomy of Viruses (ICTV) recommending the division of the family of Polyomaviridae into three genera: Genus Orthopolyomavirus, Genus Wukipolyomavirus and Genus Avipolyomavirus [120].

1.5 Merkel cell polyomavirus (MCV)

According to the most recent taxonomical classification, MCV is placed in the orthopolyomavirus genus (mammalian viruses with high nucleotide similarity) [43]. Phylogenetically MCV is most similar to the murine polyomavirus, unlike the other human polyomaviruses which are more closely-related to SV40. Although many domains of MCV share homology with the other human polyomaviruses, the closest homology is actually with the African Green Monkey Lymphotropic virus.

1.5.1 MCV Epidemiology

Following the initial discovery of MCV in MCC specimens, studies into the prevalence of MCV in the wider population were initiated. A difficulty with this investigation is the fact that primary MCV infection appears to be asymptomatic and so data relies on examination of serological evidence of infection only. The seroprevalence of MCV in the general adult population is high, between 64 and 88% [75, 121] and this finding is consistent throughout North America and Europe. Infection appears to start from early childhood and increases steadily with age as shown by the prevalence of IgG antibodies against MCV VP-1 capsid protein which are present in 9% of 1-4 year olds, increasing to 35% by age 13, [122, 123].

1.5.2 MCV Tropism and Transmission

MCV appears to be a persistent infection and viral DNA has been isolated from a number of sites. Most commonly, it can be found in skin (up to 40% of healthy control subjects) suggesting on-going infection here [124]. In these cases, viral load correlates positively with

antibody titre [125]. Recent work infecting different skin cell types with MCV pseudovirus shows that dermal fibroblasts were the most capable of being infected and so are likely to be a host cell for MCV [126]. MCV DNA has been uncommonly found in numerous other sites such as respiratory secretions and urine, and digestive tract [127-129]. Lymphotropism has been investigated and revealed inconclusive results. Buffy coats from 22% of healthy donors have low level expression of MCV DNA (10-100 molecules per 100000 cells) using nested PCR amplifying a sequence of Large T-antigen [130]. In a cohort of patients being investigated for a coagulopathy, none had detectable levels of MCV in their peripheral blood. However, in MCC- HIV+ subjects, viral DNA was isolated in between 9.5%-39.1% [101, 131]. Furthermore, a small study using peripheral blood from immunocompromised renal transplant patients showed the presence of MCV in the peripheral blood and furthermore, for DNA to be specifically found within inflammatory monocytes [132]. In MCC patients, viral DNA is more readily assessable within the peripheral blood, particularly, when disease is active [83]. Whether this is due to presence of circulating tumour cells, viral reactivation or actual lymphotropism remains unknown. The exact mode of transmission of MCV remains to be elucidated but is likely to involve skin to skin contact, given the apparent tropism, continued shedding and common finding of MCV DNA on environmental surfaces [133]. However, it is possible that other routes such as faecal-oral, mucosal, or respiratory may also be involved.

1.5.3 MCV and other disease associations

The relationship between MCV infection and other diseases have been investigated. In Chronic Lymphocytic Leukaemia (CLL), a haematological malignancy associated with higher

than expected numbers of cases of MCC, the search for a role of MCV in pathogenesis has been controversial. In one study, 27.1% of CLL cases studied showed evidence of viral DNA in purified CD19+ leukaemic cells. Within this group, they also found evidence of LT truncation mutations, similar to those found in MCC, in some cases, hypothesising some similarity in pathogenesis between MCC and a sub-group of CLL [134]. However, other groups have not been able to corroborate these findings. In these studies, MCV DNA was found in only 2% of cases and in these only at a low level [135] or found in the T cell rather than B cell population [136]. The largest study also investigated tumour cell tissue microarrays from 173 cases of lymphoid malignancies, most commonly CLL, for expression of LT protein and was unable to detect it in any cases [109].

As MCV appears to be most commonly resident in the skin, an association with other skin conditions has also been investigated. MCV DNA has been isolated from squamous cell carcinoma (11-38% of cases) [78, 137, 138], Actinic keratosis (6-15%) [137, 139, 140], and Basal cell carcinoma (0-14%) [137, 139]. Despite cumulative testing of a large number of non-MCC cutaneous tumours, expression of the oncoprotein, T-antigen has been seen only very sporadically and not always specifically in tumour cells [140-142]. Therefore while it is possible that a tiny minority of these cancers are driven by MCV, it is much more likely that it is a passenger virus and perhaps tissue sampling at a time of active viral replication may account for the rare expression of T-antigen.

There are also no strong links to MCV mediated oncogenesis in other non-cutaneous cancers [110, 143-146]. However there has been a suggestion from the Far East of MCV Large T expression in a subset of non-small cell lung cancer that is currently being further investigated [147, 148].

1.6 Other human polyomaviruses

1.6.1 BK and JC polyomaviruses:

In 1971, BK virus was isolated from the urine of a renal transplant patient with these initials[149]. In the same year, the JC virus (again named after the patient suffering from Progressive Multifocal Leukoencephalopathy (PML) [150] from whom the virus was first isolated) was discovered. Both viruses are often acquired in childhood and are seroprevalent. BK infects over 70% of the healthy adult population [151-154] whilst JC infects around 50% [151, 152, 154]. Primary infection is asymptomatic, making identification of route of transmission difficult. Both viruses, however, are known to reside latently in the urinary tract [155], occasionally becoming detectable in the urine of healthy asymptomatic people [151]. Both viruses only become pathogenic in times of immunocompromise, probably secondary to viral reactivation. BK virus is responsible for a nephropathy and haemorrhagic cystitis in renal transplant and stem cell transplant patients respectively [156, 157]. JC virus, although present in the renal tract is very infrequently associated with nephropathy. Instead, it is associated with a serious neurological condition, progressive multifocal leukoencephalopathy (PML) in patients with HIV-AIDs and also patients with Multiple Sclerosis treated with the anti-α4-integrin antibody Natalizumab that inhibits lymphocyte trafficking through the blood-brain barrier [158, 159].

Both BK and JC viruses can induce tumour formation in new-born rodents and so are putatively oncogenic [160, 161]. Although BK and JC viral DNA has been recovered from a wide range of tumour types, the evidence of a role in oncogenesis is currently controversial, made particularly difficult by the high seroprevalence of both viruses throughout the general population.

1.6.2 KI and WU polyomaviruses

The 3rd and 4th human polyomaviruses were discovered in 2007 [162, 163]. Although isolated from different areas of the world, KI (Karolinska Institut) and WU (Washington University) were both found using similar techniques, isolated from respiratory secretions of children suffering with respiratory tract infections. By concentrating virus using filtration and ultra-centrifugation, then performing nuclease digestion and PCR using random primers, residual nucleic acid was amplified and the products cloned and subjected to high throughput DNA sequencing and analysed bioinformatically. Both viral genomes bear some homology to BK, JC and SV40, though they lack Middle T and agnoprotein regions, thus forming their own phylogenetic branch within the virus family.

Although, both viruses seem to reside in the lung and were discovered in patients with respiratory symptoms, it is not known whether either is pathogenic. In a study comparing adults with and without respiratory symptoms, the number with evidence of viral DNA in the nasal aspirates was low and not significantly different between the groups [164]. Seroprevalence studies show that most (55-98%) of the healthy adult population have been exposed [152, 154]. No formal link has been made with either of these viruses and cancer.

1.6.3 Human polyomavirus 6 and 7 (HPyV6 and HPyV7)

In 2010, while looking for presence of MCV DNA in skin swabs from healthy volunteers, using rolling circle DNA amplification, Schowalter et al, discovered two novel double-stranded circular genome sequences [124]. These share close homology with the late regions of KI and WU viruses but very divergent early regions, resulting in sharing a common phylogenetic branch but separate clade. Seroprevalence of both is high, with 69-83.4% and

35%-63.6% of healthy people having evidence of HPyV6 and HpyV7 VP-1 antibodies respectively [124, 165]. While HPyV6 DNA is found in 30% of swabs taken from normal skin, HPyV7 appears less frequent (20%) [166-169]. Again, neither virus has any clear pathogenic association although HPyV6 has been found in the majority of epithelial proliferations in skin of patients treated with BRAF inhibitors [170] as well as the CSF of a HIV+ patient with leukoencephalopathy [171]. HPyV7 has been found in two cases of pruritic epidermal hyperplasia in patients who had undergone lung transplantation [172]. Neither virus has been associated with any skin malignancy including MCC [173, 174].

1.6.4 Trichodysplasia Spinulosa polyomavirus (TSV)

Trichodysplasia Spinulosa (TS), a skin condition associated with immunocompromise associated with friable follicular spinous process formation. In 1999, electron microscopy of thin tissue sections from a patient revealed the presence of intracellular small icosahedral viral particles [175]. In 2010, using DNA extracted from a spinous process from a patient and performing rolling circle amplification, another novel DNA sequence was discovered [176]. Although encoding LT, sT and VP1, 2 and 3 like the other recently discovered polyomaviruses, phylogenetically this was more closely related to Borneo Orang-utan polyomavirus and has been classed as such. It is seroprevalent with 70%-80% of healthy adults showing detectable TSV VP-1 seroresponse [165, 177-179]. It is rarely detected within the skin or hair follicles, with a 4% DNA detection rate in immunosuppressed patients not suffering with TS and <1% of skin of healthy donors [169, 176]. It is not known to have a role in any other disease processes.

1.6.5 Human polyomavirus 9 (HPyV9)

This was detected in the serum of a renal transplant patient as part of a general screen for novel polyomaviruses capable of causing opportunistic infections in immunocompromised populations by screening DNA extracted from various tissues and performing PCR with primers targeting well conserved regions of the VP-1 gene. 84 of 514 samples contained polyomavirus and after sequencing, 1 of these did not belong to a previously identified virus and was named HPyV9 [180]. This virus was noteworthy for its close relationship to Lymphotropic polyomavirus (LPV) found in African green monkeys as this may explain why around 30% of adult human sera contain antibodies to LPV. The seroprevalence of HPyV9 in healthy adults is roughly 10–40% [165, 178, 181]. Alongside serum, HPyV9 has also infrequently found to reside in skin. There are no current links of HPyV9 with disease.

1.6.6 Human polyomavirus 10 (also known as MW or MX)

Isolated from a healthy stool sample of a 15 month old child from Malawi, through purification of virus-like particles and shotgun pyrosequencing, this virus was identified as part of a global gut microbiome survey. This sequence was then used to develop primers to screen stool from a sample of American children, with a detection rate of 2.3% from 514 samples [182]. This virus was originally termed Malawi (MW) polyomavirus.

Concurrently, polyomaviruses isolated from a stool sample in Mexico (originally named MX) [183] and from chondyloma specimens from a patient with Warts with Hypoglobulinaemia, Infections, and Myeloathexis (WHIM) syndrome (originally termed HPyV10) [184], although originally thought to be novel, were seen to have 95-99% similarity between to MW virus making them likely to be strains of the same virus. This virus has an extremely high

seroprevalence (>99%) in healthy adults but no known pathogenic associations [181]. It has also been rarely detected from skin swabs of healthy donors at a frequency of 3.5% [169].

1.6.7 Human polyomavirus 11 (HPyV11, also known as St Louis polyomavirus)

This polyomavirus was isolated from stool samples in Malawi, Gambia and the USA [185]. Although sharing an ancestral recombinant origin with MW virus, it is distinct in its ability to form a unique T-antigen splice variant, termed 229T, alongside the usual Large and Small T-antigens. Very little is known about this virus and the role of 229T. The seroprevalence of this virus is thought to be approximately 70% of healthy adults [186].

1.6.8 Human polyomavirus 12 (HPyV12)

Most recently, this virus was isolated from resected liver tissue and was subsequently found in other GI tract organs such as the caecum as well as in stool samples. The seroprevalence is 23% in healthy adults [187]. Again, there are no known associations with this virus and any diseases.

1.7 Viral Immunity

Viruses are small pathogenic intracellular organisms that utilise cell machinery hijacked from infected host cells in order to replicate. The nature and length of infection is dependent on the virus in question. Some viruses, such as influenza A, cause acute lytic infections that impact seriously on the normal physiology of the host cell leading to cell death, the release of infectious virions and the forward transmission of infection to other cells and other hosts.

Other viruses, such as the polyomavirus and herpes virus families, cause latent, persistent infections which are usually silent and asymptomatic but do occasionally reactivate and undergo lytic replication. Individuals with functioning immune systems are capable of mounting immune responses against both types of infection. However, viruses have developed mechanisms to evade the immune system and thus allow them to persist within the host.

There are two major arms to the normal host immune response to viruses, the innate response and the adaptive response.

1.7.1 Innate immune response

After microorganisms breach the physical and chemical barriers protecting the skin and mucosal surfaces of the body, they contact tissue resident cells that are able to initiate an immediate immune response. Microorganisms, including viruses, display simple invariant molecules, termed pathogen-associated molecular patterns (PAMPS) on their surface. These include peptidoglycans, lipopolysaccharides and unmethylated CpG DNA that are not present on the host cells. These "non-self" molecules can be recognised through interaction with pattern recognition receptors (PRRs) on the surface of innate immune cells such as macrophages, neutrophils and dendritic cells. There are a number of different PRR types with different functions. Engagement of microorganism with a phagocytic receptor stimulates the innate immune cell to bind, engulf and kill it. Engagement with signalling receptors, such as the Toll-like Receptors (TLRs), which result in production of pro-inflammatory cytokines and chemokines directed at damaging the microorganism and attracting other immune cells to the site of infection. Each TLR is specific for a particular ligand and are found on certain cell types, for example, TLR-7 engages with single stranded RNA and is found on plasmacytoid

dendritic cells, NK cells, eosinophils and B cells. TLR-9 interacts with DNA with unmethylated CpG sites, found on many DNA viruses, and is expressed by plasmacytoid dendritic cells, eosinophils, basophils and B cells.

Type 1 interferons

Interferon-α (IFNα) and interferon-β (IFNβ) are the two cytokines that make up the Type 1 interferons (IFN). Upon TLR activation, several downstream signalling pathways are initiated including the NFκB and the interferon regulatory factor (IRF) transcription pathways. Almost all cells are able to produce Type 1 interferons when they are infected with a virus. Some cell types, particularly, plasmacytoid dendritic cells, which express TLRs 7 and 9 that recognise viral RNA or DNA, are particularly specialised at this. These cells then accumulate in peripheral lymphoid tissue at times of viral infection and produce large amounts of interferon-α and interferon-β. Production of interferon-β induces surrounding cells to secrete interferon-α and so is able amplify the size and length of response. Interferon- α and β both bind to interferon receptors on the infected and surrounding cells stimulating the JAK-STAT transcriptional pathway leading to synthesis of proteins, such as the enzyme oligoadenylate synthetase, that are capable of inhibiting viral replication [188]. Type 1 interferons are also responsible for the activation of cytotoxic Natural Killer (NK) cells both directly and through activating macrophages to produce stimulatory cytokines IL-12 and IL-18. Type 1 interferons stimulate production of chemokines capable of recruiting lymphocytes (CXCL9, CXCL10, CXCL11) and also increase expression of MHC Class 1 molecules on the surface of all cells, enabling enhanced recognition as well as protecting them from NK cell mediated killing [189]. Interferon-β promotes the differentiation of monocytes into mature dendritic cells allowing them to function as antigen presenting cells and initiate an adaptive immune response.

1.7.2 Adaptive immune response

The innate response provides rapid protection against invading pathogens, however this can do so against only those that display molecular patterns that are capable of inducing a response. In order to provide protection against a more focused response to a range of pathogens, an adaptive immune system is required. This system is able to recognise a variety of molecules, termed "antigens". The two major types of the adaptive immune response are humoral and cell mediated.

1.7.3 Humoral immunity

B cells contain highly specialised antigen-recognition molecules termed as immunoglobulins, which are bound to the cell membrane and also known as the B cell receptor (BCR). Naïve B cells travel into secondary lymphoid tissue and interact with follicular dendritic cells displaying antigen. Upon recognition of its cognate antigen by the BCR, the B cell is stimulated. It is then further stimulated by antigen specific CD4 T follicular-helper ($T_{fh)}$ cells that express CD40L and IL-4 which drive B cell clonal expansion and a proportion of these cells differentiate into IgM antibody secreting plasmablasts [190]. Remaining activated B cells form germinal centres within the lymphoid tissue and upon further interaction with CD4 T cells, undergo somatic hypermutation of immunoglobulin gene and class switching, leading to the production of B cells with higher affinity BCR [191]. These B cells will then either differentiate into plasma cells secreting high affinity antibody or long-lived "memory" B cells, capable of providing a secondary response if the same antigen is re-encountered in the future. Secreted antibodies have a number of functions that help protect against infections. Firstly, they are able to prevent or "neutralize" binding of bacterial toxins or virus particles to

cells. Secondly, binding of antibody to pathogens causes "opsonisation" through either the activation of the classical complement cascade or by engagement of the Fc portion of antibody by Fc Receptors displayed by macrophages, neutrophils and dendritic cells, leading to phagocytosis of the antibody bound pathogen.

1.7.4 Cell mediated immunity

Unlike immunoglobulins which can recognise pathogenic material in the extracellular space, T cells interact with fragments of the antigen when displayed on the surface of a cell. There are two distinct T cells subsets, αβ T cells, which are the majority and γδ T cells, a minority population that differ in antigen recognition properties and function. As αβ T cells are most associated with an anti-viral role, we will concentrate on describing their development and function. Infected cells display protein fragments of intracellular pathogen as peptides which are delivered to the cell surface by specialised glycoprotein structures, named major histocompatibility complex (MHC) molecules and also called Human Leucocyte antigen (HLA) in humans. The peptide:MHC complex is recognised by the T cell through direct interaction with the surface T cell receptor (TCR). TCRs are made up of an α and β polypeptide chain, linked by a disulphide bond with each chain spanning the cell membrane and ending in a short cytoplasmic tail. The αβ TCR has homology with the BCR in that both have an amino-terminal variable (V) region, a constant (C) region and a short cysteine containing segment that forms a disulphide bond between the two chains. There are two major classes of αβ T cell, CD8 and CD4, which are able to recognise antigen presented in MHC Class I and Class II molecules respectively.

1.7.4.1 The development of αβ T cells

T cell progenitors are released from the bone marrow and mature in the thymus by passing through a number of selective stages. During these stages, the T cells begin to express TCR that has undergone VDJ rearrangement of both α and β loci, CD3 and initially both CD4 and CD8. These double-positive T cells express only low levels of TCR and are stimulated to mature if able to recognise self-peptide:self-MHC complexes; a process known as positive selection [192]. These selected cells then begin to express high levels of TCR and only one of CD4 or CD8. These T cells then go through negative selection, whereby they are eliminated if they recognise self-antigens [193]. The remaining cells are then exported from the thymus into the periphery as mature CD4 or CD8 T cells.

1.7.4.2 T cell priming

In order for a naïve T cell to be stimulated to form effector T cells, it must recognise their cognate peptide in the context of the correct MHC molecule usually presented by a professional antigen presenting cell, such as a dendritic cell. Conventional dendritic cells are found under most surface epithelia and in most solid organs in an immature state. These cells can ingest extracellular pathogens either by endocytosis or macropinocytosis and present antigen in the context of MHC molecules (described in further detail below). Immature dendritic cells also express a variety of pathogen recognition receptors, such as TLRs and the lectin DC-SIGN. When these receptors are stimulated at a time of infection, it leads to a process of cell maturation, up-regulating expression of the lymphoid homing receptor CCR7, T cell co-stimulatory molecules CD80 and CD86 and also levels of both MHC Class I and II [194]. Mature dendritic cells then migrate to the peripheral lymphoid tissue with a phenotype capable of inducing strong T cell responses.

In order to meet antigen presenting cells displaying cognate antigen, naive T cells must also home towards lymphoid tissue. To do this, they express receptors such as the adhesion molecule CD62L and chemokine receptor CCR7, whose ligands, GlyCAM-1 and CCL19 and CCL21 respectively, are expressed by stromal and endothelial cells resident within the lymphoid tissue. Naïve T cells also express other molecules such as CD28, CD27 and the CD45 isoform, CD45RA on their cell surface. Alongside recognition of presented antigen via TCR and CD4 or CD8 co-receptor, further co-stimulatory signals are required to activate the T cell. These include ligation of CD28 on the naïve T cell with CD80 or CD86, found on antigen presenting cells or CD27 ligating with CD70 on the surface of activated T and B cells [195, 196]. Following adequate stimulus, the T cell undergoes clonal expansion forming effector and memory T cells which then migrate from the lymphoid tissue back into the circulation. The process of clonal expansion is mostly driven by secretion of the cytokine IL-2 by the activated T cells alongside an alteration in the IL-2 receptor on the T cell such that the affinity for IL-2 is significantly increased. After 4-5 days of rapid proliferation, the activated T cells differentiate into effector T cells that down regulate CD62L and CCR7 expression and thus can leave the lymphoid tissue and independently respond to cells displaying their cognate epitope. These activated T cells can maintain expression of CD27 and CD28 but due to alternate splicing of the CD45 extracellular domain, express the CD45RO isotype rather than CD45RA [197].

1.7.4.3 T cell memory

Upon resolution of infection, the majority of effector T cells will die through apoptosis due to the lack of on-going stimulatory signalling. In the presence of IL-7 and IL-15, some effector cells are retained and go on to form memory T cell populations which can enable a rapid secondary response to a previously experienced pathogen even after many years [198]. There

are two populations of memory T cells termed the effector memory and central memory subsets which differ in surface expression of various molecules as well as in function [199]. The CCR7-negative effector memory T cells are able to circulate throughout the periphery and may lose expression of one or both of CD27 and CD28. These cells are able to rapidly produce cytotoxic molecules such as perforin, as well as inflammatory cytokines such as IFNɣ, IL-4 and IL-5 upon recognition of a previously encountered cognate antigen. The central memory T cells retain expression of CCR7 and so are able to recirculate back to secondary lymphoid tissues. They may also retain expression of CD27, CD28 and CD45RO and produce cytokines such as Interferon-ɣ (IFNɣ) and tumour necrosis factor-α (TNFα) but no cytotoxic molecules [200].

1.7.4.4 CD4 T cell response

Upon priming, CD4 T cells can differentiate into a number of subtypes, each with a distinct function. This is not pre-determined and depends on the co-stimulatory cytokine profile which drives specific transcription factor usage at the time of T cell activation. T_h1 cell development is induced by the cytokines IL-12 and IFNɣ and driven by the up-regulation of transcription factors Signal Transducer and Activator of Transcription (STAT)-1, STAT-4 and T-bet [201]. T_h1 cells are also termed inflammatory T cells and assist in the killing of intracellular pathogens such as viruses and mycobacteria through the activation of IL-12 producing macrophages. T_h1 cells stimulate phagocytosis by macrophages through first engaging with antigen held within the MHC Class II surface molecule leading to the production of IFNɣ. Macrophages responding to this stimulation then bind to the T_h1 cells through interaction of CD40, found on the macrophage, and CD40L, found on the T_h1 cell. The combination of these signals causes the macrophage to up-regulate its antimicrobial properties and phagocytose the infected cell. T_h1 cells additionally produce the stimulatory cytokine IL-2,

leading to further recruitment and proliferation of T cells to the site of infection, as well as GM-CSF which activates release and maturation of macrophages from the bone marrow [202]. There is increasing evidence that Th1 cells can also have a direct cytotoxic effector function on virally infected cells through a perforin-dependent mechanism [203, 204].

The T_h2 cells are also termed T helper cells as they are integral to B cell activation and the production of high affinity antibody. They can also control parasitic infections by stimulating responses by mast cells and eosinophils. Development towards this phenotype is driven by the cytokine IL-4 that up-regulates the transcription factors STAT6 and GATA-3 [205, 206]. T_h2 cells produce the cytokines IL-4 and IL-13 following interaction with its cognate antigen on the surface of the B cell and this, in addition to CD40-CD40L interaction, results in B cell activation and clonal expansion within the germinal centre. T_h2 cells also produce the cytokines IL-5 and IL-6 which are able to help direct the differentiation of B cells into IgE-producing plasmablasts which are predominantly active in parasitic infection.

T_h17 cells are induced early on in the adaptive immune response and act to stimulate neutrophils to kill extracellular fungi and bacteria [207]. These cells do not produce IFNɣ, instead secreting cytokines of the IL-17 family which act on local stromal and epithelial cells stimulating production of the chemokine IL-8 which recruits innate immune cells. These cells may also have a role in provoking auto-immunity by inducing experimental autoimmune encephalomyelitis in mice [208]. They develop in the presence of cytokines IL-6 or IL-21 and TGF-β and in the absence of IL-4 and IL-12 through up-regulation of the transcription factors RORɣt or RORα in co-operation with STAT3 [209].

There is thought to be a reciprocal relationship between Th17 cells and another CD4 T cell subset, termed regulatory T cells (T_{regs}) that develop from naïve cells in the presence of TGF-

β but in the absence of IL-6/IL-21 and other pro-stimulatory cytokines [210]. The development of T_{regs} is dependent on the activation of the transcription factor FoxP3 and these cells are defined by expression co-expressing cell surface molecules CD4 and CD25 [211, 212]. T_{regs} have important roles in both health and disease through their ability to suppress the immune response through the production of cytokines TGF-β and IL-10. They are able to terminate immune responses following clearance of pathogen as well as preventing inappropriate auto-immune responses [213].

T follicular helper cells (T_{fh}) cells migrate into the lymphoid follicle through Bcl-6-mediated CXCR5 surface up-regulation and loss of CCR7. Through the expression of cell surface receptors such as ICOS and OX40 and production of the cytokine IL-21, T_{fh} cells are able to interact with cognate B cells leading to their activation and differentiation to form plasma cells capable of producing high affinity antibody responses [214].

The stimulation by TGF-β alongside IL-4 causing up-regulation of transcription factor STAT6 and SMADs leads to the differentiation of another T cell subset, T_h9 cells, so-called due to their secretion of the cytokine IL-9. Although the exact role these cells play in the immune response is not known they have been linked with causing inflammatory cell infiltrate and antibody production within the airways and may be important in conditions such as asthma [215].

1.7.4.5 CD8 T cell response

CD8 T cells typically differentiate into cytotoxic T cells which amongst other functions are able to kill their target cells. These cells can be activated by mature dendritic cells, which can sometimes lead to clonal expansion of CD8 T cells without need of co-stimulation from other cell types. More usually, a co-stimulatory signal from the dendritic cells is provided by

interactions with CD4 T cells which, when they engage with the antigen presenting cell, produce IL-2 and CD40L. CD40L binds to CD40 on the dendritic cell, macrophage or B cell providing a further activation signal, increasing expression of CD80/86 and 4-1BBL on the dendritic cell. These provide further activation signals to the CD8 T cell leading to cell proliferation.

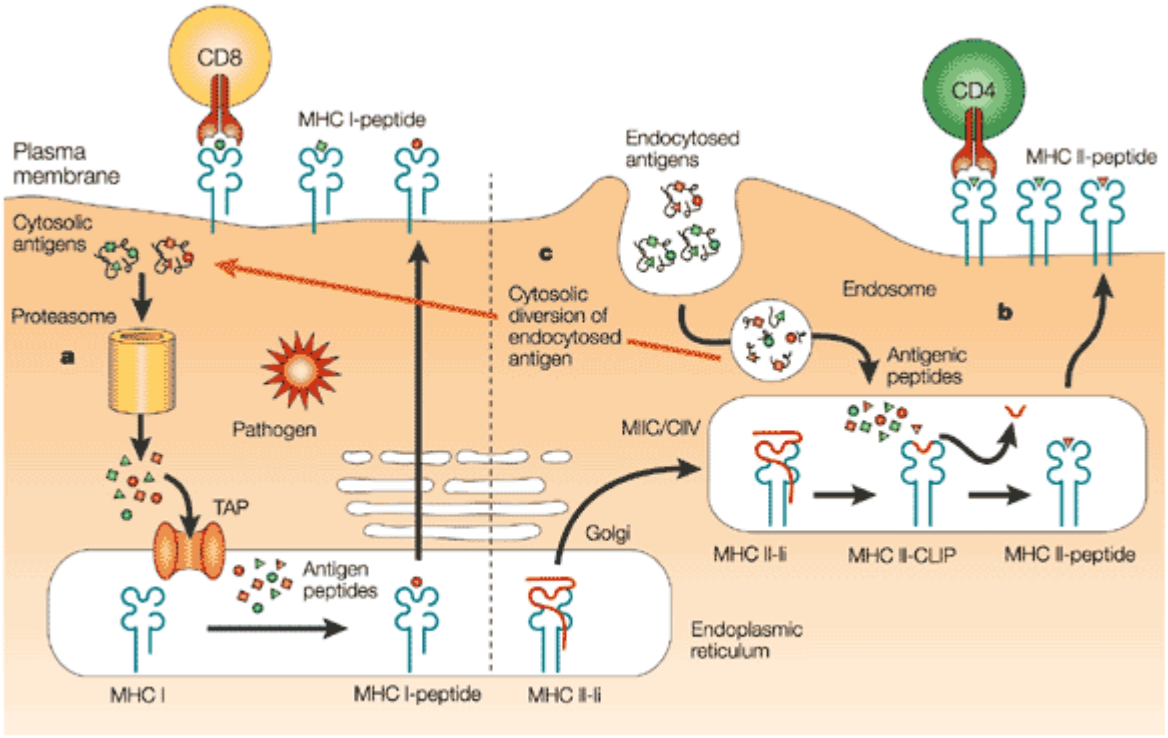
The main effector molecules released by CD8 T cells are perforin, granzyme and granulysin which are contained within cytotoxic granules and released upon conjugation with the target cell at the immunological synapse. Perforin is a protein capable of disrupting the cell membrane and so enables delivery of the granule contents to the target cell. Granzymes are a family of cytotoxic proteases, of which granzyme A and B are most abundant in humans, with the ability to cleave cellular proteins. Within the highly acidic granules in which they are stored, perforin and granzymes are inactive, however when released into the neutral pH extracellular space in a calcium-dependent process, they become activated and cause apoptosis of the infected cell. Both granzymes and perforin are needed for cell killing [216, 217]. Granulysin is a cytotoxic protein that can penetrate into the cell through pores caused by perforin and leads to the apoptosis of intracellular bacteria by causing lysis of their membranes [218]. CD8 T cells can also release the effector cytokines IFNɣ, TNFα and TNFβ. IFNɣ is particularly important through blocking viral replication and increasing antigen presentation by the infected cell through activation of the MHC Class I and II antigen processing pathways. Alongside IFNɣ, TNFα and TNFβ are able to activate and recruit macrophages to sites of infection. The activated CD8 T cells also express the membrane-associated protein Fas ligand (FasL), which interacts with its receptor Fas, usually present on the cell surface of other activated lymphocytes as well as virally infected cells and leads to caspase-mediated apoptosis even when perforin is not functional [219]. This mechanism,

therefore, limits lymphocyte proliferation after infection has been cleared and prevents an auto-immune reaction. TNF-related apoptosis-inducing ligand (TRAIL) is expressed on CD8 T cells following stimulation with IFNα and IFNβ and when this protein interacts with cells such as those that have been virally infected or transformed bearing TRAIL receptors such as TRAIL-R or TRAIL-R2, this leads to caspase-dependent apoptosis of the target cell [220, 221].

1.7.5 Antigen presentation and processing

T cells are only capable of responding to antigen when it is held in the form of a processed peptide within an MHC molecule. Although there are hundreds of different versions of MHC molecules, termed alleles, an individual will only express a small number of these and each product of the allele is capable of binding a range of peptides. MHC molecules have highly polymorphic peptide binding grooves that leads to alterations in the specificity of the peptide amino acid sequence that it will bind to. There are two classes of MHC, Class I and Class II, which present to CD8 and CD4 T cells respectively (see Figure 1.5). Although both molecules are similar in overall structure there are important differences in the route through which antigens are usually processed and loaded onto the MHC molecules and how they are presented on the cell surface. The expression of MHC Class I and II is regulated by cytokines, particularly interferons released by both innate and adaptive immune responses. Interferon-α and –β increase Class I expression on all cell types. Interferon-ɣ can also increase expression of MHC Class I and II as well as inducing Class II expression in certain cell types, such as fibroblasts, that do not constitutively express it.

Figure 1.5. Schematic diagram of MHC Class I and MHC Class II antigen processing pathways as described in sections 1.7.5.1 and 1.7.5.2. TAP (transporter associated with antigen-processing), Ii (invariant chain), CLIP (class II-associated invariant-chain peptide). Taken from [222]



1.7.5.1 MHC Class I pathway

MHC Class I is expressed by all nucleated cells and consists of an α polypeptide chain that spans the cell membrane alongside a non-polymorphic β_2-microglobulin polypeptide chain. The complete molecule folds such that it forms a peptide binding groove on the most distal region of the complex at the cell surface. The peptides that go onto fit into this groove are derived in a number of ways from protein antigens. Pathogen-derived protein can directly enter the cytosol following, for example, a viral infection. It is also possible that antigens from endocytosed viral particles or phagocytosed viral infected cells can be translocated into the cytosol in a process called cross presentation. The peptide source can also come from the

rapid degradation of newly synthesised proteins known as defective ribosomal products (DRiPS) including peptides translated from mRNA that has undergone frameshift mutation or from proteins that have been folded incorrectly [223]. The pathogen-derived or self-proteins are then degraded by a large protease complex named the proteasome. Cytosolic peptides are then transported into the endoplasmic reticulum (ER) by the ATP-dependent proteins TAP (Transporter associated with Antigen Processing)-1 and TAP-2 [224]. The TAP-1 and TAP-2 genes are up regulated by interferons which are produced by innate immune cells as a response to viral infection [225]. Peptides too long to bind to Class I MHC can be shortened within the endoplasmic reticulum by the aminopeptidase, endoplasmic reticulum aminopeptidase associated with antigen processing (ERAAP), which can be up regulated by IFNɣ [226].

MHC Class I molecules are assembled in the ER through interactions with the chaperone proteins, calnexin, calreticulin, ERp57 and then binds to TAP via the associated protein tapasin [227]. Peptide transported in the ER via TAP is then loaded onto the MHC Class I molecule forming a complex that can be transported via the Golgi apparatus, where it is glycosylated, to the cell surface for display to CD8 T cells. The stability of MHC molecules is markedly increased when they are bound to peptide leading to sustained expression on the cell surface. Peptides held within Class I molecules are usually 8-10 amino acids long and held by contacts usually between atoms in the free amino and carboxy termini of the peptide [228]. Class I MHC can bind longer peptide if binding at the carboxy terminus is possible, these are then subsequently cleaved by exopeptidases in the endoplasmic reticulum [229]. Each MHC allele binds to peptides that exhibit a certain amino acid signature, consisting of two or three residues in particular positions within the sequence that allow stable anchoring to the MHC

molecule. This means that an individual MHC allele can bind to a range of peptides that share these features.

1.7.5.2 MHC Class II pathway

MHC Class II is expressed by a more limited number of cell types involved in the immune response such as B cells, macrophages and dendritic cells which are termed professional antigen presenting cells. MHC Class II molecules consists of a non-covalent complex of α and β chains, folded together much like the MHC Class I molecule. MHC Class II molecules present peptides that have mostly been derived from exogenous proteins and generally these are taken up by cells through endocytosis into vesicles which enter the endosomal pathway [230]. These vesicles fuse with others that have an increasingly acidic content and also contain proteases called cathepsins that degrade the protein into peptide fragments [231]. These peptides are then transferred into the late endosomal compartment known as the MHC Class II compartment (MIIC) where it can interact with MHC Class II molecules [232]. MHC Class II is synthesised in the ER and its stable assembly is aided by binding to peptides with a membrane protein called the MHC Class II-associated invariant chain (MHC II-Ii) which acts as a scaffold. This interaction between invariant chain (Ii) and MHC Class II also prevents premature binding to peptides that have been transported to the ER or that are encountered while travelling to the endosomal compartment [233]. The invariant chain plays a further important role by targeting MHC Class II molecules to the endocytosed vesicles containing the degraded antigen ready for peptide loading. Upon reaching the acidified endosome, the invariant chain is cleaved, leaving a short peptide fragment, termed class II-associated invariant chain peptide (CLIP) still bound to the Class II binding pocket [234]. CLIP is removed when a MHC Class II like molecule, HLA-DM binds to Class II, leaving the binding pocket groove for the exogenously-derived peptide fragments to be loaded [235]. The Class II

peptide binding cleft has a more open conformation than that of the Class I and as such, peptide binding to MHC Class II is not constrained by peptide length and tend to be between 13 and 17 amino acids [236]. Here the peptide is held by peptide side chains that protrude into pockets within the peptide binding cleft and by interactions between peptide backbone and side chains of conserved amino acids within the binding cleft itself. The Class II molecule:peptide complex can be released from the endosome and travel to the cell surface to enable recognition by CD4 T cells.

1.8 Immunity and ageing

The ageing process has been well documented to affect the adaptive immune system. Although absolute lymphocyte counts tend to stay stable with age, there are changes in the proportions of the cell types with a decline in T and B cell number alongside an increase in NK cells. The number of naïve T and B cells decrease with age as a result of a shift towards myeloid rather than lymphoid precursor production from the bone marrow [237]. Alongside this, the thymus involutes in most adults over the age of 40, limiting production to that of peripheral differentiation only. This reduced pool of naïve lymphocytes also becomes increasingly dysfunctional, with impairment of production of cytokines, such as IL-2, following stimulation [238, 239].

Through exposure to higher numbers of lifetime pathogens, the memory cell compartment is increased with ageing. However, the function of T cell memory deteriorates with age as seen through the diminished response to vaccines such as influenza, as well as the common reactivation of infections such as varicella zoster virus (VZV) [240-242]. A reason for this, particularly affecting CD8 T cells, is through replicative senescence, driven by the chronic

stimulation of T cells by persistent infections, such as CMV, EBV and VZV [243, 244]. This leads to the loss of the co-stimulatory molecules CD27 and CD28, the shortening of telomeres and terminal differentiation to CD45RA expressing T Effector Memory RA+ (TEMRA) cells. TEMRA are resistant to normal apoptotic mechanisms that usually control the size of memory T cell clonal populations that respond to a particular antigen resulting in the oligoclonal expansion of CD8 T cells that fill immunological space and reduce the diversity of the T cell repertoire from 10^8 Vβ TCR in young people to 10^6 in the elderly [245].

Expansion of CD28-CD8+ T cells appears to correlate with frailty and poor responses to vaccines such as influenza and pneumococcus [242]. Indeed the combination of CMV seropositivity, an inversed CD4:CD8 ratio and expansion of the CD28-CD8+ T cell has been termed the "immune risk profile" and is associated with higher mortality rates in the elderly population [246].

Age-associated changes to the CD4 cell compartment are much less well defined with apparent preservation of both number and function in the majority of elderly people [237, 247]. However, changes in the ability of CD4 T cells to provide adequate help may be reduced in older people through mechanisms such as dysfunction within the CD40-CD40L system which may limit B cell and CD8 T cell responses to infection or vaccination [248].

As the production of naïve B cells decreases in the elderly, there is a consequent expansion of antigen experienced B cells leading to a reduced repertoire of B cells in older humans [249]. Alterations in immunoglobulin generation, class switching and affinity maturation is seen in old humans, potentially as a result of reduced AID expression [250]. This may all have a contributory role in the decline of quality of humoral response in the elderly as seen, for example, following vaccinations for influenza and pneumococcus [240, 251].

Healthy elderly people can generate large numbers of dendritic cells with a phenotype and antigen presentation capacity similar to those from younger people. However, there are reports of an impaired capacity to cross tissue barriers and a relatively poor cytokine response following TLR stimulation [252]. Also the ability to trigger IFNɣ or IL-10 production from virus-specific T cells is reduced, suggesting the interaction with T cells may be impaired with aging or chronic illness, perhaps secondary to reduced GM-CSF levels [253].

Age related changes also occur in the innate immune system. Older adults appear to have a dysregulated inflammatory response, often leading to lingering low level inflammation, termed "inflamm-aging" [254]. In comparison to younger people, the elderly have higher levels of basal pro-inflammatory cytokines such as IL-6, which has been associated with an increase in mortality [255]. This may be due to higher levels of non-cell associated DNA released from senescent or damaged cells that may contain DAMPs that activate innate immunity and inflammation.

This increase in cytokine production does not seem to be simply due to increased production of innate cell populations, although some studies have reported increased numbers of CD14+CD16+ monocytes in the elderly. Despite higher levels of basal inflammatory cytokine production, production of cytokines such as IL-6 and TNFα, following stimulation from monocytes are impaired in older people. Although changes in neutrophil number are not commonly seen with aging, neutrophilia has been associated with increased mortality in the elderly. There is also some evidence that neutrophils from older adults have functional impairments, particularly related to chemotaxis and phagocytosis [253, 256].

1.9 Cutaneous Immunity

The skin provides a physical barrier to invading pathogens but needs mechanisms to combat infection should this barrier be breached. To this end, the skin contains a large number of resident immune cells capable of dealing with invading pathogens and these are shown schematically in Figure 1.5. The majority of these cells function as part of the innate immune system or as antigen presenting cells (APCs) and include keratinocytes and Langerhan's cells (LCs) which are found in the dermis and with mast cells, neutrophils, innate lymphoid cells (ILCs), dendritic cells (DC) and macrophages found in the epidermis.

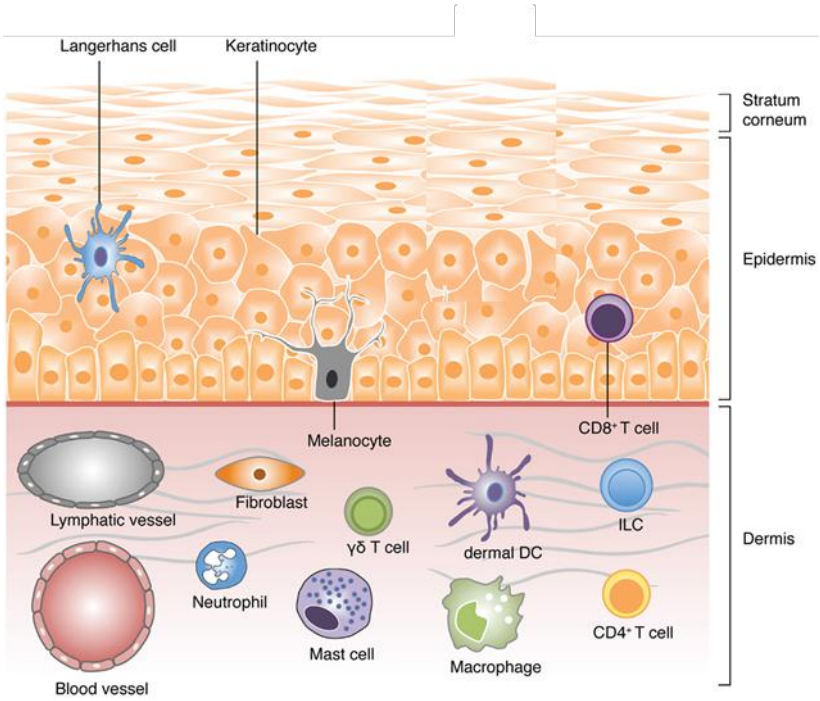


Figure 1.6. A schematic diagram of the different immune cell types populating human skin. The stratum corneum composed of dead keratinocytes acts as a physical barrier. The epidermis is a dense and poorly vascularized region that comprises mainly of keratinocytes, few melanocytes and Langerhans cells (LCs) and CD8 T-cells. The dermis contains fibroblasts, T-cells (CD4 αβ, and γδ), innate lymphoid cells (ILCs), dendritic cells (DCs), macrophages, mast cells, and neutrophils amongst others. Taken from [257]

Keratinocytes produce IL-1α and TNF in response to stimulation of TLRs they express e.g. TLR-9 after recognising PAMPs. This leads to the activation of the NF-κβ pathway which induces local inflammation and increases the expression of cell adhesion molecules such as E- and P-selectin, Intracellular Adhesion Molecule-1 (ICAM-1) and Vascular Cell Adhesion Molecule-1 (VCAM-1) [258]. Langerhan's cells resident in the epidermis and dendritic cells from the dermis are both activated by the innate immune response and take up antigen and undergo maturation before travelling to the local lymph nodes to engage with and activate T cells. Activated effector T cells express integrins such as Lymphocyte Function-associated Antigen 1 (LFA-1) and Very Late Antigen-4 (VLA-4) which can bind to ICAM-1 and VCAM-1, found on inflamed tissue, and allows migration into these sites. More specifically to T cells homing to the skin, CD3 stimulation in the presence of IL-12 induces expression of cutaneous lymphocyte antigen (CLA) which binds to E-selectin on the vasculature. The vast majority of T cells found in both inflamed and non-inflamed skin express CLA [259]. Further chemokine receptors are also associated with skin-homing T cells including CCR4, CCR6 and CCR10. CCR4 is able to bind to its ligand CCL17 which is expressed in high levels on the endothelium of cutaneous blood vessels as well as on keratinocytes and fibroblasts [260]. CCR10 is seen on a subset of CLA+ cells and its ligand is CCL27, also known as cutaneous T cell attracting chemokine (CTACK) is expressed on cell types such as keratinocytes [261]. CCR10 up-regulation has been shown to occur in the presence of $1,25(OH)_2$ Vitamin D_3 which can be produced in the skin as a result of sunlight exposure and this may be a mechanism which works synergistically with IL-12 by which some antigen specific T cells are retained in the skin [262, 263]. These antigen-specific CD4 and CD8 T cells are termed tissue resident memory (Trm) T cells are thought to up-regulate expression of the integrin CD103 upon exposure to TGF-β in the skin which can interact with its ligand E-Cadherin

found on keratinocytes [264]. The TCR of these T cells can be activated at a lower threshold than that seen with circulating T cells and so can lead to rapid clearance of pathogen upon re-exposure [265, 266]. A normal cutaneous immune response can be abrogated by a number of factors. Exposure to ultraviolet radiation (UVR) has been implicated in recruitment of regulatory T cells and in inhibition of antigen presentation via direct damage to antigen presentation cells (APCs) or via functional inhibition of APCs by cytokines (IL-10, tumour necrosis factor-α) released by keratinocytes and mast cells [267]. Ageing is also associated with reduced cutaneous immunity through the accumulation of immunosuppressive T_{regs} [268], the up regulation of inhibitory molecules such as PD-1 on cutaneous T cells [269] and reduction in TNFα production by macrophages which in turn reduces T cell migration into tissue [270]. Both UV light exposure and ageing factors are particularly relevant when considering MCC as shown below.

1.10 The importance of immunity in MCV+ MCC

The three major risk factors associated with MCC are ageing, UV light exposure and immunosuppression, all of which are associated with either local or systemic immune dysfunction. There also appears to be a prognostic impact of immune dysfunction in MCC patients, with immunosuppressed patients [39] and those with low absolute lymphocyte counts [41] associated with poorer clinical outcomes.

MCV cutaneous viral loads are increased in MCC patients compared to patients with other skin disorders [271]. In HIV+ patients, in whom risk of MCC is higher than expected, MCV viral DNA is more commonly seen in skin swabs compared with HIV- controls. Furthermore, HIV+ patients with poorly controlled disease (defined using CD4 count and HIV-1 viral load)

have significantly higher MCV viral loads in the skin compared with HIV+ patients with well controlled disease [272]. These findings suggest a loss of MCV viral immune control may be important early in the pathogenic process of MCV+ MCC development. Interestingly, the recovery of immune function, for example with withdrawal of immunosuppressive medications in solid organ transplant patients with MCC, has led to tumour regression [273]. This suggests that the anti-viral immune response may also have an anti-tumour effect and as such, investigations into potential mechanisms through which the loss of MCV of viral control occurs and the impact this may have both on the development and treatment of MCC are underway and are discussed below.

1.10.1 Alterations and potential therapeutic avenues in innate immunity

As previously described, the innate response to viruses includes recognition of viral PAMPS by PRRS such as TLR3, 7 and 9. The expression of TLR2, 4, 5, 7 and 9 has been investigated by immunohistochemistry in a panel of 128 MCC tumours and retained expression was noted in most specimens. A decrease in TLR7 expression was associated with older age and a decrease in TLR9 expression was predominantly seen in MCV+ tumours [274]. The down-regulation of the TLR9 surface expression is thought to occur through inhibition of the transcription factor C/EBPβ by MCV LT, potentially in co-operation with sT, although the exact mechanism is unknown [275]. A small numbers of cases of MCC have been treated successfully with Imiquimod, a synthetic TLR7 agonist usually used to treat squamous cell skin cancer (SCC) [276, 277]. This topical treatment results in increased infiltration by effector T cells producing IFNɣ, perforin and granzyme as well as reducing production of immunosuppressive cytokines IL-10 and TGF-β although the mechanism for this remains

unproven [278]. Upon TLR stimulation several downstream transcriptional signalling pathways are activated including the NFκB pathway. NFκB is usually held in its inactive cytoplasmic form via binding to the inhibitor of κB (IκB) proteins. As part of the NFκB signalling pathway, IκB is phosphorylated by the inhibitor of κB kinase (IKK) complex leading to its rapid degradation and the consequent activation of NFκB which can translocate to the nucleus and up-regulate downstream transcriptional activity. This leads to the production of pro-inflammatory cytokines and chemokines as well as the up-regulation of co-stimulatory molecules on DCs that activate T-cells. MCV sT-antigen is able to inhibit this pathway by recruiting the cellular phosphatase PP2C and directly interacting with the NFκB essential modulator (NEMO) protein which prevents the phosphorylation of the IKK sub-units IκB kinase α (IKKα) and IκB kinase β (IKKβ), which in turn prevents phosphorylation of IκB and limiting the translocation of NFκB to the nucleus [279].

Although not proven to be virally-induced, MCC can commonly evade NK-mediated cell killing through the hypoacetylation of promoters of the MHC class I chain-related protein (MIC) -A and –B genes, leading to non-production of these stress-induced NKG2D receptor ligands. This process can be reversed, both in vitro and in vivo, using a epigenetic modifying HDAC inhibitor resulting in the re-introduction of NK-mediated cytotoxicity [280]. In MCV+ MCC cell lines and an in vivo xenograft MCV+ model, exposure to type 1 interferon's led to an increase in apoptosis and reduction in cell proliferation [281]. This has led to the use of type 1 interferons to treat cases of MCC, however, patient numbers are small and results are inconsistent [282, 283].

1.10.2 Humoral Immunity

Published data suggests all patients with MCV positive MCC have IgG antibodies to MCV VP-1 in their serum. In over 60% of these cases, antibody titres were high (>10,000). In comparison, healthy people have a mean titre around 60-fold lower than this [74, 75, 121]. The fact that VP-1 capsid protein is not expressed by MCV+ MCC implies this may be driven by viral reactivation in the setting of immune suppression. Anti-VP-1 antibodies have a neutralising function and this is stronger in MCC patients compared with healthy control patients with comparable VP-1 antibody levels [74, 137]. Interestingly, patients with higher anti-VP-1 antibody titres have a significantly longer progression free survival and overall survival compared with those patients with low titres, perhaps reflecting a role in mediating some control over MCC progression [121, 284].

Serological response against T-Antigen (specifically a portion of the common region shared between large and small T-antigen), which is expressed in tumours, is less prevalent, seen in less than 1% of the general population. However, in MCC patients, it is seen in around 40% [284, 285]. In this case antibody titres are potentially driven by tumour-associated antigen load as longitudinal studies have shown that titres follow disease burden with a fall following treatment and return with disease recurrence [285]. In patients who do relapse, the rise in antibody titre predates clinical evidence of disease and so may be a useful monitoring test in certain patients.

1.10.3 Cellular Immunity

Given its recent discovery, detailed information regarding the cellular immune response against MCV is limited. However, such responses have been studied for other members of the

polyomavirus family. In wild-type mice infected with murine polyomavirus, viral DNA is detected in most organs for the first months before becoming rapidly undetectable a pattern repeated in CD4 -/- and CD8-/- single knockout mice. However, in CD4 and CD8 double-knockout or mice that have undergone chemo/radiotherapy inducing immune suppression, polyomavirus was still detectable after 2 months suggesting that both CD4 and CD8 T lymphocytes are required for initial viral control [286]. The CD4 subset plays an important role in providing help to maintaining both a polyomavirus-specific CD8 T cell pool as well as humoral antibodies which are both reduced in MHC Class II deficient mice persistently infected with murine polyomavirus [287].

In humans, the frequency of BKV and JCV virus-specific T cells in the peripheral blood have been measured through ex-vivo stimulation by peptides corresponding with the virus proteins. A repeated finding in healthy seropositive donors is that the total frequency of T cells in the peripheral blood is low and these responses tend to be CD4 predominant [288-290]. For example, one study using ELISpot recognition assays to assess frequency of BK-specific T cells responding following stimulation showed healthy donors had a median response of 24(15-95) responding cells/million peripheral blood mononuclear cells (PBMC). The number of VP-1 specific cell was equally low with a median of 25 (7-113) responding cells/million [291].

Through extended culturing of T cell responses, CD8 T cell responses to distinct JC and BK VP-1 and T-antigen epitopes have been defined and the ex-vivo frequency of T cells measured using MHC class I tetrameric complexes to identify epitope-specific cells. Although responses are frequently detected in healthy patients, these have always been of low frequency, ranging from 0.01% to a maximum of 0.53% of total CD8 cells directed at a single epitope [292-294]. In patients suffering with polyomavirus-mediated diseases, such as PML,

an increased T cell frequency appears to be associated with an improved prognosis, particularly if the response develops early [295-297]. Recently it has been shown that in humans combating PML, efficient CD4 T cell JCV recognition is important to allow CD8 T cell infiltration into brain lesions and that this CD8 infiltration is hindered if CD4 epitopes are altered by mutation which leads to delayed viral clearance [298]. Given the homology in VP-1 and T-antigen seen throughout the polyomavirus family, a number of potentially cross-reactive BK and JC CD8 T cell epitopes have been discovered and these may be important in providing control for both viruses [299-301]. Whether this same phenomenon occurs within the other more recently discovered members of the polyomavirus family and the potential importance it may have to general polyomavirus immune control has not been fully addressed.

Assessments focusing on MCV-specific T cell responses have been undertaken and appear to show similarities with those seen with other polyomaviruses. In vitro, MCV VP-1 expressing virus-like-particles (VLPs) are able to induce T cell proliferation in MCV seropositive healthy donors to a greater extent than seronegative donors. These proliferating T cells produce IFNγ, IL-10 and IL-13 but the response disappeared if anti-MHC class II antibodies or CD4 cells depletion was used, indicating it to be CD4-mediated response [302]. This group did attempt to assess potential cross-reactivity between MCV and TSV specific T cell responses in healthy donors by comparing IFNɣ and IL-10 secretion from CD4 T cells following stimulation with either MCV or TSV VP-1 expressing VLP. They found that donors who were MCV seropositive and TSV seronegative made stronger responses to MCV VLP and vice versa with TSV VLP, indicating that at least some of the T cell response appears to be virus-specific [303].

Efforts to identify MCV-specific T cells by using peripheral blood from MCC patients and healthy controls in recognition assays against peptides spanning MCV VP-1 as well as the truncated LT and sT have been carried out in one study. Overall T cell responses were small and were more readily identified in patients than in healthy donors (37% v 8%) although the difference was not considered statistically significant [304]. This identified 26 peptides (five from the common T (CT) region shared by LT and sT, nine from the remainder of LT, ten from sT and two from VP-1) able to induce T cell responses from both healthy donors and patients. Although the Class II HLA allele presenting the epitope was not identified, eight of these responses were shown to be CD4-mediated and only one was CD8-mediated. In the majority of cases (sixteen) the subtype of the responding T cell was not identified [304]. In one case, CD4 T cells specific for a LT (aa57-69) peptide were isolated from the tumour of an MCC patient and these were found to secrete both T_h1 and T_h2 cytokines, IFNγ, IL-5 and IL-10 after ex vivo expansion and challenge with this peptide [304].

The single CD8 T cell response identified in the aforementioned study came as a result of performing T cell cloning on T lymphocytes extracted from an MCC tumour and was then found to be directed against a HLA:A24 restricted LT epitope (aa92-101). These cells were found at very low frequency in the peripheral blood of the patient, but enriched within the tumour (0.14% of total CD8 T cells in blood versus 4% in tumour). Using an HLA:A24 tetramer specific for this epitope, it has been possible to quantify frequency of MCV-specific T cells in a wider spectrum of patients and healthy donors. This has confirmed that these epitope-specific responses are seen in the peripheral blood of the majority of HLA:A24 MCV+ MCC patients but at low frequency (0.01-0.24% of total CD8 T cells). These MCV-specific T cells are not seen in HLA:A24 healthy controls or in a MCV-MCC patient [305].

Due to the difficulty in discovering MCV-specific CD8 epitopes using screening recognition assays, a second study by Lyngaa et al searched for MCV-specific T cells using a high throughput epitope discovery platform [306]. In particular, they looked in MCC patients and healthy donors for T cell responses specific for HLA-A1, -A2, -A3, -A11 and -B7 epitopes from MCV proteins VP-1, truncated LT and sT. VP-1 responses were found in both MCC patients and healthy donors and 23 novel VP-1 epitopes were discovered. In addition, LT (9 epitopes found) and sT (3 epitopes found) responses were exclusively seen in MCC patients and again were of low frequency (0.05-0.1% CD8 T cells) in peripheral blood ex vivo [306].

The role of T cell-mediated immunity in polyomavirus-associated cancer has been investigated since it was known that the SV40 virus was capable of promoting tumour growth in rodents. It is thought that both CD4 and CD8 T cells are important to a successful anti-tumour response [307]. In a series of experiments, mice were immunised with recombinant SV40 T-antigen protein, subjected to CD4 and/or CD8 T cell depletion immediately before and after challenge with the mKSA, SV40-transformed, tumour cell line. Tumours were seen to develop in immunised mice in which CD8 but not in the CD4 T cells were depleted, causing the authors to suggest that CD8 immunity is key to preventing tumour development in this model [308]. In a follow-on study, when CD4 and CD8 depletion occurred around the time of immunisation, tumours only developed in the CD4 depleted mice. In the CD8 depleted mice, a significantly larger anti-T-antigen antibody response was seen leading the authors to suggest that an important role of CD4 cells may be to activate B cells to produce anti-T-antigen antibodies which bind to tumour cells and lead to killing of tumour by antibody directed cell killing [309]. Interestingly in this same study, when CD8 T cells were depleted around time of injection with mKSA tumour cell line, there was no tumour growth but tumours did develop in CD4 depleted mice. This challenges the original hypothesis that CD8

T cells are essential for clearing SV40 mediated tumourigenesis and a potential explanation offered for this is that the CD4 T cells may act directly as effectors through secretion of cytokines such as IFNɣ [309].

Since the discovery of a polyomavirus-associated malignancy in humans, MCC, investigators have begun to focus attention on whether T cell immunity can have an anti-tumour role. Some strong evidence in support of this idea emerged when retrospective unbiased gene expression analyses of MCC patients were data stratified into groups with both favourable and poor prognosis. This revealed an over representation of immune response genes in tumours with favourable prognoses, including genes that encode components of cytotoxic granules (granzymes), chemokines (CCL19), lymphocyte-activation molecules, and CD8 receptor molecules [310]. This suggested that the T cell response to tumour may be important in determining outcome and now a number of studies have examined the degree of T cell infiltration into MCC tumours in comparison with clinical outcome (summarised in Table 2). The earliest studies conducted, limited by small sample size, found that heavy infiltration was associated with a poor prognosis. However, recently, two large studies looking at over 100 cases each have suggested that the opposite is true. The first, by Paulson et al, found that in significant number of cases, T cells appeared to be "stalled" at the tumour periphery, a finding that has been replicated by others [311, 312]. However, in tumours with CD8 T cell infiltration there was a significant and independent association with an improvement in overall survival [310]. The second study, by Sihto et al, found that increased numbers of CD3, CD8 or FoxP3 T cells were all associated with improved survival rates as was high CD8/CD4 and FoxP3/CD4 ratios [313].

Table 1.2. Studies investigating prognostic impact of tumour infiltrating lymphocytes (TIL) in MCC. N=number of patients in study. Adapted from [51].

Study	N	TIL characterisation	Survival	Comment
Mott et al. 2004 [314]	25	Minimal/moderate=17(68%) Heavy=8(32%)	Heavy infiltration was associated with poor prognosis in multivariate analysis.	Depth of invasion was associated with poor prognosis in multivariate analysis
Llombart et al. 2005 [315]	20	Absent=8(40%) Discontinuous=12(60%) Continuous=0	Absent is an independent prognostic factor of DFI in multivariate analysis.	Tumour size>30 mm, stage II, and >50% Ki67+ tumor cells prognostic indicators of disease free interval in univariate analysis.
Andea et al. 2008 [316]	156	Absent=81(53%) Present non-dense=55(36%) Dense=17(11%)	Present non-dense and Dense associated with longer survival in univariate analysis.	Nodular growth pattern, low tumour depth, and absence of lymphovascular invasion associated with longer survival on multivariate analysis.
Paulson et al. 2011 [311]	130	Not identified=44(34%) Non-brisk/Brisk=86(66%)	Intratumoural CD8+ was independently associated with improved survival in multivariate analysis.	Overall TIL infiltration was associated with better prognosis on univariate but not multivariate analyses.
Sihto et al. 2012 [313]	116	Numbers of intratumoural CD3+, CD8+, CD16+, FoxP3+, and CD68+ cells (IHC) per 1 high power field	High CD3+, CD8+, FoxP3+ cells, high CD8+/CD4+ or FoxP3+/CD4+ ratios, significantly associated with favourable overall survival.	Numbers of T cells are generally higher in MCV+ than in MCV-ve MCC, high intratumoural T cell counts also associated with favourable survival in MCV-ve MCC.
Paulson et al. 2014 [317]	137	Intratumoural CD8+ lymphocytes analysed. Absent (n=46), low (n=85), moderate/strong (n=6)	3 year MCC-specific survival: Moderate/strong 100% Low 72% Absent 56%	Increased intratumoural CD8+ infiltration associated with improved MCC specific survival independent of stage, age and sex.

The finding that T cells can often be "stalled" at the periphery of MCC tumours suggests that there may be active inhibitory mechanisms at play, of which there are a number of possible mechanisms. MCC tumour cells may establish a local immunosuppressive microenvironment in order to thrive by produce immunosuppressive cytokines, such as TGF-β, molecules such as Fas-L, or inhibiting T-cell responses through production of galectin-1 and indoleamine 2,3-dioxygenase (IDO) [318, 319].

MHC Class I expression is down-regulated alongside other components of cellular antigen–presenting machinery such as the TAP, in a large number of MCV+ MCC tumours through an as yet unidentified mechanism. This leads to the inability of CD8 T cell to identify tumour cells through lack of presented antigen and as such they can evade immune control [320].

T cells can also be inhibited from infiltrating the tumour through down-regulation of appropriate tissue-specific T-cell homing signals. In MCC cases where intra-tumoural T cell infiltration is poor, this may be due to the down-regulation of vascular E-selectin through production of nitric oxide by the tumour [321]. This means that T cells expressing CLA, a skin-homing adhesion molecule which binds E-Selectin, will not adhere to the endothelium and so not be able to enter the skin to infiltrate the tumour. Lastly, other suppressive T cell populations, such as T_{regs} may inhibit CD8 anti-tumour responses. The role of these in MCC is not known, but both positive and negative outcomes have been associated with their presence and so the situation remains unclear [313, 322].

Since it has been hypothesized that CD8 T cells may be important as anti-tumour effectors, some work on the potential epitopes against which these responses may be directed has been conducted, focusing on the MCV T-antigen, commonly expressed in a truncated form by MCC tumours. In the previously described paper by Iyer et al in which the original CD8

HLA A:24 LT epitope (EWWRSGGFSF) was found, transfection of LT and HLA:A24 into the Cos7 cell line proved that this epitope was capable of being processed and presented by a model cell line. Additionally it was shown that Cos7 cells expressing LT could be killed by the LT-epitope specific T cells derived from TIL [304]. Furthermore, T cells specific for the HLA-A2 LT epitope KLLEIAPNC (aa 15-23), after in vitro expansion, were able to lyse cells from the HLA-A2 MCV+ MCC cell lines MKL-2 and WaGa, importantly indicating that at least some MCC tumour cells are likely to be able to process and present antigen to CD8 T cells thus increasing the evidence that T-antigen specific T cells may exert an anti-tumour effect [306].

The functionality of CD8 T cells in vitro does not always mirror what the in vivo situation. Indeed, in the HLA:A24 patient from whose TIL the EWWRSGGFSF-specific T cells were discovered, 20% of HLA:A24 tetramer binding tumour infiltrating cells produced IFNɣ in comparison with only 2% of cells from the peripheral blood. In four other HLA:A24 patients, two did not have a tetramer positive cell population, while from the other two in whom a tetramer binding CD8 response was detected, these cells failed to produce IFNɣ following peptide stimulation [304].

Recently, it has been confirmed that MCV epitope specific CD8+ T cells in MCC patients express inhibitory markers PD-1 and TIM-3 [305]. The interaction of programmed death (PD)-1 expressed on T cells with its ligand or PD-L1 is an potentially important mechanism of T-cell inhibition that could be mediating this problem [322]. In one study, PD-L1 expression by MCV+ MCC tumour cells or by tumour infiltrating lymphocytes was seen in 49% and 55% of patients respectively with MCV negative tumours appearing to be uniformly negative for PD-L1 expression. All tumours with moderate or strong T cell infiltration were associated with PD-L1 expression particularly concentrated around areas of lymphocyte

infiltration [323]. It is known that chronic viral antigen exposure often leads to the development of an inhibitory T cell phenotype with poor effector function and sustained expression of inhibitory receptors [324, 325]. Whether MCV infection is capable of driving this process in MCC is not yet known although the observed higher MCV viral load on the skin of MCC patients as compared to the general population may suggest MCC patients are exposed to increased levels of viral antigens [271]. The targeting of the PD-1/PDL-1 axis therapeutically to overcome T cell inhibition has been widely tested recently, most successfully in melanoma [326]. Very recently a phase 2 clinical trial utilising a PD-1 antibody, pembrolizumab, to treat 26 MCC patients with advanced disease was associated with a response rate of 62% in patients with MCV+ MCC [327]. This strongly suggests that immune-modulating therapy that enables improved T cell anti-tumour responses are of benefit to MCC patients and underlines the importance of T cell immunity in this cancer.

1.11 Project Aims

As we have described, MCV is a common skin-associated virus that leads to asymptomatic infection in the majority of healthy adults. The MCV-associated cancer, MCC, is rare and appears to be strongly linked with immunosuppression and the loss of MCV-directed immune control.

The first aim of this project is to further investigate the clinical finding that although immunosuppression is a risk factor for development of MCC, the majority of MCC patients have no known immune dysfunction. MCC patients are known to have higher levels of MCV antibodies than healthy controls. However, an assessment of levels of antibodies against other polyomaviruses, particularly those that have been more recently discovered, has not been

conducted. There has also been little work looking at these same antibody responses in healthy donors or in patients at a higher risk of developing MCC. To address this issue and with help from our collaborators in Germany, we will firstly assess the serum of a cohort of MCC patients for the presence and size of antibody responses against a number of members of the polyomavirus family. We will then directly compare these with antibody responses from a healthy control group, an elderly but otherwise healthy group, patients with a history of NMSC and patients with CLL. These results will potentially help clarify two important issues. Firstly, whether the increase in MCV antibody levels seen in MCC patients is a phenomenon generalizable to other polyomaviruses suggesting that the loss of MCV viral control seen may also be occurring with other similar viruses and reflects an underlying problem with polyomavirus-directed immunity in these patients. By assessing the same antibody responses in the other donor cohorts mentioned, we can see if those donors who have risk factors for developing MCC, such as ageing, sunlight exposure and immune dysfunction, share any similarity in terms of antibody responses with MCC patients that may help further explain how these risk factors are linked with the development of MCC.

Next, using blood samples from MCC patients and NMSC patients with no history of immunosuppression, we will perform a broader analysis of their peripheral immune function to see if there are differences in the MCC cohort that will explain why these patients have developed MCC. To do this we will measure a number of immune parameters including the frequency of T and B cell subsets, assess the proliferation response to a mitogenic stimulus and look at antibody levels both in general and also at levels specific for various vaccine antigens.

The second aim of this project focuses on T cell immunity against MCV, driven by increasing evidence that T cells specific for the MCV T-antigen may have an important role not only in controlling viral infection but also as a potential therapeutic agent in the treatment of MCC.

Currently, our knowledge of the T cell response against MCV in healthy patients is very limited and in particular, to our knowledge, no attempt has been made to characterise the T cell response against epitopes on the entire breadth of the T-antigen expressed by the wild type replicating virus.

We intend to characterise the T cell response, in healthy donors of known MCV serostatus, against the MCV T-antigen to assess the frequency of specific cells as well as the regions on the T-antigen they are specific for. This can then be compared with a similar characterisation of MCV T-antigen responses in both MCC patients alongside an age-appropriate control cohort of NMSC patients. Differences in either size or targets of responses between the cohorts can then be examined to see if there are any clear changes in the MCC group that help explain why viral control was lost.

After identifying MCV-specific T cells in our donors from these initial screening experiments, we will define a repertoire of T-antigen epitopes and characterise the epitope-specific T cells by establishing T cell clones from these same donors. Following the discovery of any CD8 epitopes, we will use tetramer analysis to allow us to characterise the phenotype of ex-vivo MCV-specific cells, particularly focusing on the differentiation status and tissue-homing profile of these cells. We will also assess whether these MCV-specific T cells are capable of recognising antigen presenting cells expressing the T-antigen, the first step in determining whether such cells will have any therapeutic utility.

Lastly, both viruses and cancer utilise mechanisms to evade T cell immunity and the presence of these may be important in determining whether T cell directed treatments would have clinical beneficial. Of particular interest is the potential role that the T-antigen viral proteins that are expressed by MCC may play in this. To investigate this we will focus on assessing the effects that these proteins may have on T cell recognition through the individual transfection of wild type LT, tumour-associated forms of LT and sT into model cell lines as well as looking at any changes these proteins may have on MHC Class I and II expression. This introductory work may help uncover mechanisms that the virus uses to evade T-cell recognition which, through the expression of these same proteins by MCC, may also have an importance within the tumour environment.

Chapter 2

Materials and Methods

2.1 Patients and blood/tissue preparation

Patients: Peripheral blood was donated by patients with Merkel cell cancer (MCC) or non-melanoma skin cancers (NMSC) recruited from skin oncology clinics at Queen Elizabeth Hospital, Birmingham, UK. Clinical information was obtained through a combination of patient interview and retrospective case note review. Healthy donors were recruited from University employees. Written informed consent was obtained before venepuncture All experiments were undertaken with ethical approval from the Trent Local Research Ethics Committee (Reference number: 08/H0405/59).The serum from MCC patients collected prior to 2012 was carried out by Prof David Blackbourn and Dr Neil Steven within the currently held ethical approvals. The serum samples from Chronic Lymphocytic Leukaemia (CLL) and elderly healthy donors were collected by Dr Helen Parry and Prof Paul Moss as part of her PhD thesis and were approved by the South East Wales Research Ethics Committee.

Peripheral blood mononuclear cell (PBMC) preparation: PBMCs were obtained from peripheral blood collected either by venepuncture into heparinised vacutainer tubes, or from buffy coat preparations (National Blood Service). Blood donations were diluted 1:1 in RPMI-1640 media and then layered onto Lymphocyte Separation Media (PAA) before centrifugation at 1600 rpm for 30 minutes, with no brake applied. PBMCs were aspirated from the density gradient interface with a Pasteur pipette and then washed thrice with RPMI-1640. The PBMCs were then counted with a haemocytometer and were either used fresh or if being

stored were divided into $5x10^6$ cell aliquots, pelleted and then re-suspended in freezing medium and cryopreserved.

HLA typing: The HLA status of the majority of healthy donors was already known. In cases where further typing was necessary, this was confirmed through PCR-based DNA typing conducted at the Anthony Nolan Trust, Hampstead, London.

Serum collection: Serum was collected by allowing heparinised or EDTA blood tubes to stand for 20 minutes at room temperature and then aspirating the resulting layer of straw coloured fluid, centrifuging this at 2000rpm for ten minutes and then transferring the resultant supernatant to a fresh 1.5ml eppendorf tube for storage at -20^{o}C.

Cryopreservation of cells and recovery of cryopreserved cells: Cells to be stored were pelleted by centrifugation at 1800rpm for 7 minutes before the supernatant was tipped off and the cells were re-suspended in 1ml of freezing medium (RPMI-1640/20% FCS/10% DMSO). The cells were then immediately transferred into sterile 1.5ml cryovials (Nunc) and stored in a Mr Frosty (Nalgene), containing isopropanol. Initially this was placed in -80^{o}C freezer but for longer-term storage, cells were transferred into liquid nitrogen tanks. Cells were thawed from storage by placing cryovial in a 37^{o}C water bath and then transferring the cells into a 15ml tube (Corning) followed by the slow addition of 10mls of media. The cells were then pelleted by centrifugation at 1800rpm for 7 minutes and re-suspended in the media for onward use.

Tumour lymphocyte extraction: Freshly resected MCC tumour was taken directly from operating theatre and excess tissue not required for diagnostic purposes was placed into cold standard cell culture media and put on ice. The sample and media was then placed in a sterile petri dish within a tissue culture hood and diced into small pieces using disposable scalpels. The petri dish contents was then aspirated using a Pasteur pipette ensuring all cells removed and contents put into a MACS C-Tube (Miltenyi). This was then placed on a gentleMACS cell dissociator (Miltenyi) and spun on programme "mouse-spleen 4". The homogenised tissue and media were then placed through a fine sterile mesh and washed through with cold RPMI-1640. For Class I/Class II assessment, cells were stained for flow cytometry at this point as described in relevant section. For lymphocyte isolation, the collected filtrate (approximately 50mls) was added to a 50ml tube and centrifuged for 7 minutes at 1600rpm, room temperature. During this stage, a layered Percoll (GE Healthcare) gradient was set up in a 15ml tube with 4mls of 30% Percoll (diluted with sterile 10x PBS) carefully layered on top of 4mls of 70% Percoll. The pelleted cells were re-suspended in 4mls of cold RPMI-1640 and layered on top of the Percoll and centrifuged at 1600 rpm for 30 minutes with no brake applied. The cell layer at the 70%:30% interface was carefully aspirated as the lymphocytes and a portion were frozen down for storage while the rest was plated in 24 well plates and stimulated with MCV-specific peptides using the same method described below to generate polyclonal T cell cultures. The remaining cells at the other interfaces in the tube were also either stored or cultured as "tumour cells".

2.2 Serological assays

Polyomavirus VP-1 and T-antigen seroresponses: This was possible only through collaboration with Dr Tim Waterboer, German Cancer Research Center (DKFZ), Heidelberg, Germany. Serum samples from MCC patients, NMSC patients, CLL patients, Healthy elderly and healthy young donors were all sent for analysis. Samples were anonymised and no clinical data including diagnosis was sent. VP-1 and T-antigen seroresponses were quantified for ten polyomaviruses using a previously described fluorescent bead-based multiplex assay [181]. Raw assay data was returned, matched to correct patient/donor and then analysed statistically using Prism 6.0 software (Graphpad).

CMV serology: An in-house CMV IgG ELISA assay was used in order to assess CMV serostatus, by the presence or absence of CMV-specific antibodies, as previously described [328]. All reagents were generously donated by Prof. Paul Moss. Mock and UV-inactivated CMV-infected cell lysates were coated onto 96-well MaxiSorp plates (Nunc) at 1/4000 dilution and incubated overnight at 4^{o}C. The following day the plates were thoroughly washed before serum samples were diluted to 1:600 and added in duplicate wells to both the CMV-lysate and mock-lysate coated plates for 1 hour. Standards made from a mixture plasma samples from 3 CMV-positive donors were also plated in parallel. After three washes with PBS/0.05% Tween 20, the anti-human IgG-horseradish peroxidase secondary antibody (HRP) was then added to the wells for one hour. After further washing, TMB (3, 3′, 5, 5′-tetramethylbenzidine) substrate was added and the plate incubated in the dark for 10 minutes before adding 1 M HCl. The plate was read using an ELISA plate reader at 450 nm (Bio-rad). To determine CMV antibody levels, mock values were first subtracted from lysate values. The data were then analysed using statistical software Prism Version 6 (GraphPad), and CMV

titres were calculated with reference to the standard curve. Donor serum with a calculated titre greater than ten were considered to be seropositive.

Serum Immunoglobulin G,A,M and functional antibody measurements: Serum Ig G,IgA and IgM were measured by the staff at the Clinical Immunology Service, University of Birmingham using a Roche HITACHI Cobas®6000, with Roche IgG, IgA and IgM reagent kits as per manufacturer's instructions. Antibody measurement against a variety of common bacterial antigens (pneumococcal (serotypes 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F and 23F), tetanus, diphtheria, haemophilus influenza B) was undertaken by a previously described multiplexed bead based assay [329]. The definitions of protective antibody concentrations used have been previously published and are the standards used in the laboratory [330-332].

2.3 Tissue culture reagents

Cell lines: Lymphoblastoid cell lines (LCLs), MelJuSo (MJS) cells and MCC tumour cell lines (MKL-1 and MCC13) were used in this work. MCC tumour cell lines were a kind gift from Prof Adrian Whitehouse, University of Leeds. In addition MJS cells stably transfected to express HLA-DR51 were also used in some experiments (kind gift from Dr Jianmin Zuo, University of Birmingham)

RPMI-1640 supplemented with 2mM L-glutamine (Sigma) was stored at 4°C and used for washing cells and as part of the media used in the culture of all cells used in this thesis.

Foetal calf serum (FCS, PAA Laboratories) was stored at -20°C in 50ml aliquots until use. Once an aliqout was thawed, it was kept at 4°C.

Penicillin-streptomycin solution (P/S, Invitrogen) contained 5000IU/ml penicillin and 5000 µg/ml streptomycin and was used at a final dilution of 1/100 in culture medium.

Opti-MEM1 (Invitrogen) was stored at 4°C and used for cell transfections.

Phosphate buffered saline (PBS) was made by dissolving one Dulbecco A tablet (Oxoid) per 100ml of distilled water; and was autoclaved if required.

Recombinant interleukin-2 (IL-2, Peprotech) stored at -20°C and used at 50 IU per ml. Lymphocyte Separation Media (PAA) was stored at 4°C.

Human serum (HuS, PAA) was virus and mycoplasma free and stored in 50ml aliquots at -20C until use, after which it was stored at 4°C.

MLA-144 supernatant (MLA) The gibbon cell line MLA-144 is an established IL-2 secreting cell line used to stimulate T cell growth. These non-adherent cells were maintained in standard culture media for 2 weeks without feeding, after which supernatant was harvested after centrifugation at 1600rpm for 10mins and then sterile-filtered through steritop 0.22µm filter (Millipore) by vacuum suction. The filtered MLA was stored at -20°C in 60ml aliquots.

Synthetic peptides: Peptides that spanned the full length of MCV LT and sT-antigens (sequence from Merkel cell polyomavirus isolate 206, Accession number: FJ173812) were synthesised (Alta Bioscience). Each peptide was 15 amino acids in length and overlapped each other by 10 amino acids. These were each dissolved in dimethyl sulfoxide (DMSO, Sigma) for 30mins at RT and then stored at -20^{o}C.

The resulting 185 peptides were split into five pools of 35-40 peptides (for NMSC/MCC patient ELISpots) or 16 pools of 10-12 peptides (healthy donor ELISpots) and used at a final concentration of 5µg/ml. Further individual peptides were ordered as necessary from Alta Bioscience and treated using these same methods.

Biuret assay: This was used to determine the concentration of dissolved peptides. Twenty µl of each peptide solution was added to 100µl biuret reagent (Sigma) in 96-well V-bottom plates (Nunc). In order to allow calculation of peptide concentrations, duplicate wells of bovine serum albumin (BSA, Sigma) standards ranging from 0 to 40mg/ml were also included. The plates were incubated for 30 minutes at room temperature to allow colour development before being centrifuged at 1300rpm for 5 minutes. One hundred µl of supernatant from each well was then transferred into 96-well flat bottom plate (Nunc), and the absorbance was measured at wavelength of 540nm using an automatic microplate reader (Bio-Rad).

2.4 Culture media recipes

Standard culture medium: was used as standard media for culturing LCLs and MJS cells. It was made up of RPMI-1640 + 10% FCS + 50IU/ml penicillin + 50ug/ml streptomycin. Non-adherent cells were split once or twice a week by removing half of the culture and replacing with fresh media. Adherent MJS and MCC13 cells were split by careful removing of media so as not to disturb cells, twice washing with 5ml sterile PBS which was removed before careful addition of approximately 1ml of 1x Trypsin-Express (Gibco). Flask was then gently tapped until become non-adherent and then re-suspended in standard culture media of which 1/10 was transferred to a new flask which was then topped up with fresh media as appropriate.

T cell cloning medium: was used during routine T cell feeding and T cell cloning experiments. As standard it was made up of RPMI, 30%MLA-144 filtered supernatant, 10%FCS, 1%HuS, 1%Pen/Strep and 10IU/ml IL-2. When used for T-cell cloning, the media was supplanted with anti-CD3 antibody (30ng/ml, eBioscience).

Mycoplasma Testing: Testing for presence of mycoplasma infection in cell cultures was kindly performed by Mr Gordon Ryan, University of Birmingham on a regular basis using the MycoAlert Mycoplasma detection assay kit (Cambrex) according to the manufacturer's instructions.

2.5 Immunological techniques

Thymidine Incorporation Proliferation assay: Lithium-heparinised whole blood was diluted 1:5 with RPMI-1640 and added in triplicate to wells of a 96-well plate containing media with phytohaemagglutinin (PHA) to give a final concentration 25µg/ml, or media alone. Plates were placed in a humidified incubator at 37^{o}C, 5% CO_2 for 72 hours before addition of 0.5µCi 3^{H}–Thymidine/well. After 6 hours, plates were stored frozen at -20^{o}C. Following this, cells were thawed to room temperature and harvested onto glass fibre filter paper using a Skatron cell harvester and read using Beta scintillation counter with results given as counts per minute. The mean count was calculated from the triplicate values of PHA-stimulated and un-stimulated counts and fold change assessed by dividing mean PHA-stimulated value by mean media alone values.

IFNɣ Enzyme-linked Immunospot (ELISpots): These assays were conducted using PBMCs freshly isolated from healthy donors, MCC patients and NMSC patients. The day prior to venepuncture, 20µl of 70% ethanol was added to each well of the ninety-six well plates (EMD Millipore) with Immobilon-P polyvinylidene difluoride membranes. The ethanol was left for one minute, flicked off and then the plate washed twice with 150µl filtered PBS. The PBS was flicked off and the plate coated with 50µl of monoclonal antibody 1-D1K (Mabtech) diluted to 7.5ug/ml in filtered PBS, ensuring the bottom of each well was fully covered. The plate was left overnight at 4^{o}C. The following day, after PBMCs had been prepared, the 1-D1K antibody was flicked off and the plate washed six times with 150µl RPMI. After the final wash, the plate was then blocked with 150µl of filtered RPMI-1640/10% FCS added to each well and left at room temperature for two to four hours. PBMCs were then made to a density of 5 x 10^{6} cells/ml with filtered RPMI-1640/10% FCS. The RPMI-1640/10% FCS

block was flicked off the plate and PBMCs plated out at 100µl/well (so achieving $0.5x10^6$ cells/well). Then 10ul of peptide pools (made up as described above) were added to the appropriate wells alongside the negative control dimethyl sulfoxide (DMSO) and the positive controls, 5µg/ml Phytohaemagglutinin (PHA) and also in the MCC/NMSC patient assays, 2µg/ml of the pre-made CMV, EBV, Flu (CEF) peptide mix (Mabtech). The plates were incubated overnight at 37^oC 5% CO_2. The following day, the cells from the plate were flicked into virkon and the plate washed with 150µl filtered PBS/0.05% Tween 20. To each well, 50µl of 1µg/ml biotinylated monoclonal antibody 7-B6-1 (Mabtech) were added before leaving the plate at room temperature for 2-4 hours. The plate was then washed six times with PBS/0.05% Tween 20 again before addition of 1:1000 dilution of Streptavidin-Alkaline Phosphatase (Mabtech) and further incubation at room temperature for 1-2 hours. After a further six washes, the chromogenic substrate 5-bromo-4chloro-3-indolyl phosphate and nitro blue tetrazolium (BioRad) was added to the wells and the plate incubated in the dark for 15 minutes to 1 hour. After the appearance of spots representing individual IFNɣ secreting T cells the reaction was stopped by washing the plate with tap water. When dry, the number of spots was quantified automatically with the AID automated ELISpot reader and data transformed to give the number of spot forming cells 10^6 PBMC.

Generation of polyclonal T cell cultures: In order to expand MCV T-antigen-specific T cells from the blood of healthy donors, a previously developed protocol [333] was used in which polyclonal PBMC cultures were established by pelleting $1\text{-}10^6$ PBMCs and then stimulating them through the addition of the MCV T-antigen peptide pools (5µg/ml) for ninety minutes at $37^oC/5\%CO_2$, re-suspending cells every twenty minutes. The cells were then washed in RPMI-140 before culturing for 7 days in cell culture media containing

25ng/ml of IL-7 (Peprotech) in a 24-well plate (Iwaki). On days 3 and 6 of culture, recombinant IL-2 was added to a final concentration of 10 IU/ml.

IFNɣ capture enrichment of MCV-specific T cells On day 7 of culture, the polyclonal T cells were re-stimulated with the MCV T-antigen peptides (5µg/ml) for one hour (re-suspending every 15 minutes), washed and then incubated at 37^{o}C/5%CO_2 for three hours in RPMI-1640/5% human serum. The IFNγ Secretion Assay–Cell Enrichment and Detection Kit (Miltenyi) was then used to increase yield of MCV T-antigen specific T cells. In brief, the cells were initially washed in MACS buffer (PBS, 1% BSA and 2mM EDTA) before re-suspension in 80µl of RPMI/10% FCS with 20µl of IFNɣ-catch reagent. The cells were incubated on ice for 5mins, and then 10mls of warm RPMI-1640/10% FCS was added and incubated at 37^{o}C for 45mins on a cell roller. After a further wash in MACS buffer, the cells were re-suspended in 80µl of MACS buffer with 20µl of IFNɣ-detection antibody added. After ten minute incubation on ice, the cells were washed and re-suspended in 80µl of MACS buffer with 20µl of magnetic microbeads. The cells were then kept at 4^{o}C for 15mins, shaken every five minutes). Finally, the cells were washed once more and re-suspended in 500µl of MACS buffer before being applied to a MACS MS column (Miltenyi) within a magnetic field. After washing as per manufacturer's instructions, the column was removed from the magnetic field and the retained labelled cells collected by flushing the column with 1ml of MACS buffer. The cells were then counted using a haemocytometer ready for use in limiting dilution cloning.

Limiting dilution cloning The T cells collected following IFNɣ enrichment were seeded at 0.3, 3 and 30 cells per well in 96-well round-bottom tissue culture plates (Iwaki). This was

done in a total volume of 100µl per well of T cell cloning media containing 10^5 cells/well irradiated (4000 rads) mixed allogeneic buffy coat feeder cells that had been activated overnight with 10µg/ml phytohaemaglutanin (PHA). Alongside this the anti-CD3 monoclonal antibody OKT3 (eBioscience) was also added at 30 ng/mL. After one week, a further 100µl of standard T cell medium was added to each well.

Identification of peptide specific clones: After two to three weeks, visual assessment of proliferating microcultures was conducted and a small aliquot of cells (approx. 20µl) was taken from wells in which the cell pellet had enlarged. These cells were then screened for peptide specificity using T cell recognition assay and IFNɣ ELISA as described below. Wells containing T cell clones with confirmed MCV peptide specificity were then transferred into 24 well plates (Nunc) using 1ml/well T cell media and irradiated (4000 rads) buffy coat feeder cells (10^6 cells/well) for expansion. These T cell clones were then maintained in culture by feeding twice weekly with T cell media by removing 1ml and replacing this with 1ml of fresh media.

T cell recognition assays: To test the ability of T cell clones to recognise targets, autologous LCLs were either sensitised with appropriate peptide or with DMSO (negative control) by pelleting cells, re-suspending with 5µg/ml peptide or equivalent amount DMSO and incubating at $37^oC/5\%CO_2$ for 1 hour, shaking every 15 minutes. LCLs were then washed three times with RPMI-1640 and suspended in standard culture medium at a cell density of $1x10^6$ cells/ml. Then 50µl of these cells were added in duplicate to appropriate wells of V-

bottom 96-well plates. T cells were then counted and washed in standard culture media to give $1x10^5$ cells/ml and 50µl of T cells were added to the LCLs. As a further control, T cells were also plated in the absence of LCLs to ensure response was specific. The plates were then incubated overnight at 37ºC/5% CO_2 ready for IFNɣ ELISA the following day.

When transfected MJS cells were used as target cells to assess T cell recognition, these experiments followed the same above protocol with transfected MJS cells being treated exactly as peptide sensitised LCLs. In such experiments, peptide-sensitised MJS cells (5µg/ml) were used as the positive control.

IFNɣ Enzyme-Linked Immunosorbent Assay (ELISA): IFNɣ ELISA was commonly used to quantify T cell recognition. The day before the assay, 96-well MaxiSorp plates (Nunc) were coated with 50µl/well of anti-human IFNɣ antibody (0.75ng/ml) (Thermo Scientific) in coating buffer (0.1M Na_2HPO_4, pH 9). The plates were then incubated overnight at 4ºC. On the day of the assay, the plates were washed with wash buffer (PBS/0.05% Tween 20) and then blocked with 200µl of blocking buffer (PBS/0.05% Tween 20/10% BSA) for two hours at room temperature. The plates were then washed five times with wash buffer and then 100ul of cell supernatant from the T cell assay was harvested and added to appropriate wells on the MaxiSorp plates. To enable quantification of results, doubling dilutions of recombinant IFNɣ standard was also plated in triplicate at 100µl/well (Peprotech, 2000pg/ml – 31.25pg/ml). Plates were then incubated at room temperature for three to four hours and then washed five times with wash buffer. Then 50µl of biotinylated anti-human IFNɣ (3.75µg/ml, Thermo Scientific), diluted in blocking buffer, was added to each well and the plates incubated for a further hour at room temperature. After a further five washes, 50µl of streptavidin-peroxidase

(ExtraAvidin-Peroxidase, Sigma), diluted 1/1000 in blocking buffer, was added to each well and plates incubated at room temperature for 30 minutes. The plates were washed eight times in wash buffer and then 100µl of the peroxidase substrate (3, 3', 5, 5'-tetramethylbenzidine (TMB) solution, Life Technologies) was added and the plates incubated for up to ten minutes to allow for colour development. The reaction was stopped by the addition of 100ul of 1M hydrochloric acid, resulting in a soluble yellow product. The plates were then read on ELISA plate reader (Bio-rad) using dual wavelengths of 450nm and 695nm. The amount of IFNɣ release could be calculated after constructing a standard curve using the values derived from the recombinant IFNɣ standards.

Table 2.1. List of antibodies used in thesis

Target	Fluorochrome	Clone	Origin	Dilution	Manufacturer
TCRα/β	Vio-blue	BW242/412	mouse	1/40	Miltenyi
CCR4	PE-Cy7	IG1	mouse	1/20	BD
CCR6	PerCP-Cy5.5	11A9	mouse	1/20	BD
CLA	APC	HECA-452	rat	1/10	Miltenyi
CD4	APC-Cy7	RPA-24	mouse	1/40	BioLegend
CD8	AmCyan	SK1	mouse	1/33	BD
CD45RA	AF700	A100	mouse	1/100	Cambridge Biosciences
CCR7	FITC	150503	mouse	1/10	R&D Systems
Propidium Iodide	Texas Red	N/A	N/A	1/20	Miltenyi
CD4	PE	RPA-T4	mouse	1/50	BD Pharmingen
CD8	PE	RPA-t8	mouse	1/50	BD Pharmingen
HLA-B7	PE	BB7.1	mouse	1/20	Merck Millipore
HLA DR (Class II)	APC/PE	L243	mouse	1/50	Biolegend
HLA A,B,C (Class I)	APC/PE	w6/32	mouse	1/50	Biolegend
CD45	V500	30-F11	mouse	1/33	BD Horizon
IgD	V450	IA6-2	mouse	1/33	BD Horizon
CD45RA	V450	HI100	mouse	1/33	BD Horizon
CD3	APC-Cy7	SK7	mouse	1/33	BD
CD8	PerCP	SK1	mouse	1/33	BD
CD25	APC	2A3	mouse	1/33	BD
CD19	APC-Cy7	SJ25C1	mouse	1/33	BD
CD4	PE	L120	mouse	1/33	BD
CD127	PE-Cy7	HIL-7R-M21	mouse	1/33	BD Pharmingen
CD27	PerCP-Cy5.5	L128	mouse	1/33	BD Pharmingen
Mouse IgG1	PE	MOPC-21	mouse	1/50	BD Pharmingen
Mouse IgG2a	PE	G155-178	mouse	1/50	BD Pharmingen
Rat IgM	APC	ES26-13D3.4	rat	1/20	Miltenyi
anti-HLA-DR	n/a	HB-55	mouse	10µg/ml	ATCC
anti-HLA-DQ	n/a	SPV-L3	mouse	10µg/ml	Serotec

Determining T cell clone functional avidity: This was calculated by sensitising the T cells clone's LCL with cognate peptide at 10-fold dilutions ranging from 10^{-5}M to 10^{-11}M for 1hr at 37^{o}C, mixing every 15 minutes. The LCLs were then washed thoroughly before incubating with the T cell clone overnight as per standard T cell assay. The amount of IFNɣ secreted by the T cells was quantified by ELISA as previously described and the functional avidity was defined as the concentration of peptide capable of eliciting 50% maximal IFNɣ secretion.

Determining epitope HLA restriction: This was assessed by collating a panel of LCLs which shared one or two HLA-alleles with the donor from whom T cell in question was derived. For CD8 epitopes, each LCL was pulsed with the clone's specific peptide at 5μM for 1 hour, agitating every 15 minutes. Sensitised LCLs were then washed 4 times with RPMI-1640, re-suspended in RPMI-1640 medium/10% FCS and incubated overnight with the T cell clone in a standard T cell assay. IFNɣ release from the T cells was measured by ELISA. LCLs expressing the correct HLA restriction molecule would induce IFNɣ secretion and thus this could be identified through a process of elimination.

For CD4 epitopes, the autologous LCL was sensitised with peptide as above, washed three times and then pre-incubated with either anti-HLA-DR, anti-HLA-DQ or no antibody at 10μg/ml for 1hr (see Table 2.1). T cells were then added to the LCLs without washing antibody off and left overnight in incubator at 37^{o}C/5%CO_2. The following day, IFNɣ ELISA was performed and if IFNɣ secretion had been inhibited by a blocking antibody, only partially-matched LCLs for that particular family of Class II molecule were used to determine the exact restricting allele.

Protein feeding assay: MKL-1 and MCC13 whole cell lysates were prepared from $2x10^6$ cells which were pelleted and then applied to a sonicator on ice. Protein concentration was measured using a spectrophotometer (Nanodrop). $2x10^6$ LCLs from donors from whom MCV-specific CD4 clones had been previously isolated were spun in a centrifuge to form a cell pellet before washing and re-suspending in 500 µl of AIM-V media (serum free, Gibco) in a 48 well plate (Iwaki). Whole cell lysate was then added to the cells so that final concentrations of 400µg/ml and 200µg/ml. These cells were then placed in incubation overnight at 37^{o}C and 5% CO_2. The next day the cells were washed in RPMI-1640 and then used in T cell recognition assay followed by IFNɣ ELISA (as described above) standard T cell assays with the appropriate CD4 T cells.

In cell fixation experiments, LCLs were pelleted and then re-suspended in 1ml 1% paraformaldehyde for 10 min followed by quenching with 1ml 0.2 M glycine for 10 min. Cells were then washed with PBS twice before being re-suspended in media. For cells fixed immediately following feeding with lysate, re-suspension was in AIM-V media. For cells fixed 18 hours following feeding with lysate, re-suspension was with standard cell culture media.

Modified vaccinia Ankara (MVA) infection assays: MCC tumour cell line cells (both HLA-A11+) were infected with a recombinant MVA virus expressing the EBNA3B reporter gene or PSC-11 control MVA at a multiplicity of infection (MOI) of 10 for 90 minutes. These were then washed three times prior to incubation with EBV antigen specific T cells in T cell recognition assay described above. In experiments when cells were exposed to irradiation, this was done immediately following MVA infection.

Standard flow Cytometry: For determining CD4/CD8 status of T cell clones and Class I/II expression levels, flow cytometry was based on the same protocol. 2-3x10^5 cells were placed in 5ml FACS tubes (Falcon) and washed with 2ml of PBS/2%FCS. Tubes were then pelleted at 2000rpm for 5 minutes and supernatant tipped off. The cells were then re-suspended in 50-100µl of PBS/2%FCS containing appropriate concentrations of flurochrome-conjugated antibodies (See Table 2.1). The tubes were then incubated in the dark on ice for 30mins before washing again in 2.5ml of PBS/2%FCS and re-suspended in 200µl of PBS/2%FCS and, if to be analysed at a later time, also 200µl of 4% paraformaldehyde (Sigma) in PBS. Analysis was conducted using the LSRII flow cytometer (BD Biosciences).

Multi-colour flow cytometric characterisation of peripheral immune cells: EDTA-treated whole blood from MCC and NMSC patients underwent immediate erythrocyte lysis with ammonium chloride. Cells were then washed in RPMI and stained with a pre-defined panel of multi-colour antibodies used routinely within the Clinical Immunology Service for investigation of immunodeficiency (See Table 2.1). The cells were incubated, fixed with FACS lyse buffer and then washed with CellWash buffer (BD) before being analysed on the FACSCanto II flow cytometer (BD) using DIVA software.

Absolute lymphocyte subset cell quantification: This work was carried out by the biomedical scientists in the Clinical Immunology Service, University of Birmingham. In brief, whole blood in EDTA was added to tubes containing Multi-test 6-color TBNK antibody reagent and counting beads (BD) as per manufacturer's instructions. After incubation with the antibodies and beads, erythrocytes were lysed with 1x FACS Lysing solution (BD) and

samples analysed on FACSCanto II flow cytometer (BD) using DIVA software. The absolute lymphocyte count was defined automatically by size appropriate CD45+ cells. Subset counts were defined by presence of appropriate markers: T cell (CD3), CD4+ T cell (CD4), CD8+ T cell (CD8), B cell (CD19), NK cell (CD16/CD56).

Absolute neutrophil count: Results from MCC patients were obtained from clinical records from blood tests undertaken by hospital clinical haematology laboratory as part of routine full blood count that includes a white cell differential measurement.

Tetramer synthesis: Biotinylated monomers were synthesised from appropriate synthetic peptides by the Protein Expression Facility, University of Birmingham. For use in flow cytometry, monomers needed to be conjugated to the flurochrome, phycoerythrin (PE). To this end, calculated volumes of Streptavidin, R-Phycoerythrin Conjugate (SAPE, ThermoFisher) were added in a slow, step-wise manner over approximately 8 hours such that the final molar ratio of monomer to streptavidin was 4:1.

Tetramer flow-cytometry staining: In order to characterise tetramer-specific cells, a two-step staining procedure was conducted. Initially, $5x10^5$ PBMCs from appropriate donors were washed with PBS/2%FCS and pelleted with centrifugation at 1600rpm for 5 minutes. Supernatant was then tipped off completely and the cells were re-suspended in 50µl PBS/2% FCS containing 1/50 dilution of fluorochrome-conjugated tetramer. These tubes were then placed in an incubator at $37^oC/5\%$ CO_2 for 15 minutes. Cells were then immediately washed

with 2mls of ice-cold PBS/2%FCS, centrifuged as before and supernatant tipped off. While the tubes remained on ice, further multi-colour antibodies were added as previously mentioned and left for 30 minutes on ice in the dark. The cells were then washed once more and re-suspended in cold PBS/2%FCS and kept on ice until analysed on the LSRII flow cytometer (BD Biosciences).

Cell surface MHC Class I and Class II levels following IFNɣ or irradiation: To assess the impact that IFNɣ had on up-regulating MHC Class I and Class II expression on the cell surface of MKL-1 and MCC13 tumour cell lines, $0.3x10^6$ cells were plated in standard culture media in duplicate wells of a 24 well plate (Iwaki). Additionally, healthy donor-derived fibroblasts (kind gift from Andrew Hislop) were also used to act as positive control. For each tested cell line, to one well was added 200ng/ml recombinant IFNɣ (Peprotech) and to the other media alone. The plates were incubated at 37^oC/5%CO2 for 96 hours before harvesting. MKL-1 cells were non-adherent and so just aspirated after mixing while MCC13 cells required trypsinisation. Harvested cells were then washed twice with PBS/2%FCS before being split into three aliquots, one for staining with fluorochrome-conjugated Class I antibody, another with fluorochrome-conjugated Class II antibody and the third for staining with isotype control (See Table 2.1). Cells were stained and analysed on LSRII flow cytometer as previously described.

To assess the impact of irradiation on the same surface markers, MKL-1, MCC13 and MJS cells were split into three $25cm^2$ flasks in standard culture media each and incubated 37^oC/5%CO2. After cell populations had grown to near confluence, flasks from each cell line were exposed to 0Gy (not irradiated), 4Gy or 10Gy irradiation using the ɣ-irradiator (BMSU,

University of Birmingham). Following appropriate irradiation, cells were harvested and stained for Class I, Class II and isotype control as before.

2.6 Molecular biology reagents and techniques

LB Media LB (Luria Broth) was prepared by dissolving 20g/L of LB powder (Invitrogen) in sterile distilled water and then then sterilised by autoclaving at 121ºC for 20 minutes.

LB Agar was prepared by dissolving 20g/L of LB agar powder (Invitrogen) in sterile distilled water and sterilised by autoclaving.

Ampicillin (Roche) was used at a final concentration of 100µg/ml in LB agar and LB media for use in molecular gene cloning techniques

MCV gene expression plasmid construction: A codon-optimised MCV wild-type LT (WTLT) DNA sequence (from MCV344 strain, Accession number: FJ173806) was synthesised and cloned into entry vector plasmid pDONR221 (Geneart, Invitrogen). DNA was extracted from $2x10^6$ MKL-1 cultured cell line cells using a Nucleospin Tissue DNA extraction kit as per manufacturer's guidelines (Machery-Nagel). MCV350 DNA was derived from the pcDNA3.MCV350 plasmid originally synthesised by Moore and Chang [42] and made available via the AIDS reagents programme. sT DNA was originally extracted from the pcDNA3.MCV350 plasmid but was found to have an C>T substitution mutation at aa 898 leading to amino acid change from leucine to proline compared to the original deposited

sequence. Subsequently, sT-GFP plasmid DNA was used (kind gift from Professor Adrian Whitehouse, University of Leeds) which was found not to contain this mutation.

Between 50-100ng DNA was used from each source in polymerase chain reactions (PCR) in order to amplify WTLT, MCV350 LT, MKL-1 LT and Small T (sT). The primers used in reactions (Sigma-Aldrich) are documented in Table 2.2. To facilitate later ligation into vector plasmid, appropriate restriction sites were engineered to the 5' and 3' DNA fragements as shown in the table. As both MCV350 LT and MKL-1 LT DNA were derived from genomic DNA and are encoded by 2 separate exons, each exon was amplified individually and then being joined together by a further PCR step.

Table 2.2 Primer sequences used in molecular gene cloning

DNA fragment amplified	5' primer (additional restriction site)	3' primer (additional restriction site)	Fragment size	Template DNA source
WT LT	ttgcggccgcttgccgccatgga tttagtcctaaata (Not1)	gctgcggccgcctttattgagaaa aagtaccagaat (Not1)	2482	pDONR221.LT
MCV350LT/MKL-1 LT exon 1	gaagaattcgccgccatggattt agtcctaaatag (EcoR1)	tatataggggcctcgtcaacctca tcaaacatagagaagt (N/A)	261	pcDNA3.MCV350
MCV350LT exon 2	gttgacgaggcccctatatatgg gaccactaaattcaaag (EcoR1)	cgtgcggccgcgtactaatctgta aactgagatg (Not1)	569	pcDNA3.MCV350
MKL-1 LT exon 2	gttgacgaggcccctatatatgg gaccactaaattcaaag (EcoR1)	cgtgcggccgcgtactatagctta tatacagcat (Not1)	785	MKL-1 cell line DNA
MCV350 LT	gaagaattcgccgccatggattt agtcctaaatag (EcoR1)	cgtgcggccgcgtactaatctgta aactgagatg (Not1)	774	MCV350LT exon1+2
MKL-1 LT	gaagaattcgccgccatggattt agtcctaaatag (EcoR1)	cgtgcggccgcgtactatagctta tatacagcat (Not1)	990	MKL-1 LT exon 1+2
sT	gaagaattcgccgccatggattt agtcctaaatag (EcoR1)	gaggcggccgcagtctagaaaag gtgcagatg (Not1)	590	sT-GFP

PCR recipe was made as follows using Pfx DNA polymerase kit (Invitrogen) into PCR reaction tubes.

Component	Volume	Final concentration
10x PFX amplification buffer	5µl	1x
10mM dNTP	1.5µl	0.3mM each
50mM $MgSO_4$	1	1mM
5' and 3' primers (10mM each)	1.5µl	0.3µM each
Template DNA (50-100ng)	>1µl	As required
Pfx DNA polymerase	0.4µl	1 unit
Distilled water	To 50µl total volume	

The sample was denatured at 94^{o}C for 5 minutes before undergoing 30 cycles of PCR amplification (Denaturing: 94^{o}C, 15s→ Annealing: 65^{o}C reducing to 60^{o}C by 1^{o}C per cycle for 5 cycles, 30s each→ Extension 68^{o}C, 1min per kilobase of DNA).

DNA ligation: MCV genes were to be inserted into the plasmid vector pcDNA3-IRES-NLS-GFP (kind gift from Eric Reits, Netherlands Cancer Institute) through the use of EcoR1 and Not1 restriction sites. The MKL-LT, MCV350 LT and sT contained neither of these sites and so EcoR1 (5') and Not1 (3') were added as described above. The WTLT contained an EcoR1 site within the gene sequence and so Not1 sites were used at both 5' and 3' ends. The MCV DNA fragments and the pcDNA3-IRES-NLS-GFP were cut in a large scale restriction digest using both EcoR1 (Roche) and Not1 (Roche) or just Not1 in the case of WT LT (Mix: 10µl DNA, 20U each restriction enzyme, 5µl Buffer H (Roche), 10µl Tris-HCl, made to 50µl with nuclease-free water). After 2 hours digestion in water bath at 37^{o}C, 5µl loading buffer was

added to each sample and these were run on a 1% agarose gel for 1 hour. Appropriate size fragments were cut from the gel using a scalpel and then DNA extracted using a gel extraction kit as per manufacturer's guidelines (Machery-Nagel). The insert and vector DNA were then mixed in a 3:1 molecular ratio with Quick T4 DNA Ligase and 2x Ligase buffer as per manufacturer's instructions (New England BioLabs). After five minutes incubation at room temperature, ligated DNA was used to transform competent bacteria as detailed below.

Transformation of competent bacteria: In order to amplify ligated plasmids, competent bacteria (XL-1 blue) were transformed by mixing 50-100ng of plasmid DNA gently with 200µl of competent bacteria in an eppendorf tube that was then incubated on ice for half an hour. The bacteria where then shocked for 90 seconds in a 42ºC water bath before being rested briefly on ice. 800µl of LB broth was then added to the bacteria and the samples were left in a shaking incubator at 37ºC for one hour. Bacteria transformed with each vector/insert combination were individually spread onto two ampicillin-containing agar plates, one at high concentration (200µl) and one at a low concentration (20 µl). These plates were incubated overnight at 37ºC.

Bulk preparation of plasmid DNA: From the incubated agar plated, individual bacterial colonies were picked with a pipette tip touched to a numbered area on a new ampicillin agar plate (placed back into 37^{o}C incubator) before mixing into a 15ml falcon tube that contained 3ml LB broth (and ampicillin). Usually four to six colonies were picked per transformation and these were placed in a 37ºC shaking incubator overnight. The next day plasmid DNA was extracted from the bacteria using a Nucleospin Plasmid DNA extraction mini-prep kit (Machery-Nagel) as per instructions and successful ligation assessed by repeat restriction

digest by the presence of both insert and vector fragments post digest. This was then confirmed through DNA sequencing which was conducted by the facility within the School of Biosciences, University of Birmingham). These colonies were then picked the following day from the numbered agar plate and inoculated into 200mls of ampicillin-containing LB media in a 1L conical flask which was incubated overnight in a 37ºC shaking incubator. The next day the entire bacterial culture was used for large-scale DNA extraction using a Maxi Prep plasmid DNA extraction kit (QIAgen), as per manufacturer's guidelines. Recovered DNA was eluted in 50µl of nuclease free water and concentration quantified using a Nanodrop spectrophotometer (Thermo Scientific). DNA was subsequently stored long-term at -20ºC.

Transfection of plasmid DNA into MJS cells: Plasmids containing MCV DNA were transfected into the melanoma cell line MelJuSo (MJS) to assess impact on MHC Class I and II levels as well as for use in T cell recognition assays. MJS cells were plated at a density of $2x10^5$ cells per well into a 24-well plate (Iwaki) and then incubated overnight in 500µl of RPMI-1640/10% FCS with no antibiotics at 37^{o}C and 5% CO2. The following morning, 1.2µg of each plasmid DNA were mixed separately with 50µl of Opti-MEM1 (Invitrogen). For co-transfection experiments, total DNA for both plasmids added up to 1.2 µg. For each planned transfection, a further tube containing 2µl of transfection reagent Lipofectamine 2000 (Invitrogen) was mixed with 50µl of Opti-MEM1. The tubes were incubated for 5 minutes at room temperature and then plasmid DNA and Lipofectamine 2000 were mixed together and left for 20mins at room temperature before a further 50µl of Opti-MEM1 was added to each plasmid transfection mixture. The media was then carefully pipetted from the MJS cells without disturbing them and replaced with 150µl of the DNA and Lipofectamine 2000 mixture. Once all transfections were completed, the plates were returned to the incubator at

37^{o}C and 5% CO2. After six hours, 500µl of RPMI-1640/10% FCS without antibiotics was added carefully to each well and then incubated for 24-48 hours prior to use.

Chapter 3

Prevalence of multiple polyomavirus antibody seroresponses in Merkel cell cancer patients compared with other donor groups

3.1 Introduction

The majority of polyomaviruses cause asymptomatic infections and prevalence rates are based on serological assays measuring antibodies against viral antigens. Epidemiological studies measuring VP-1 antibody levels have shown that antibodies for the majority of the polyomavirus family are highly seroprevalent within the healthy adult population. Studies comparing Merkel cell polyomavirus (MCV) VP-1 antibody levels with the amount of viral DNA in the normal skin of Merkel cell cancer (MCC) patients and healthy controls have shown a positive correlation between the two [125, 137]. Similar correlative findings are seen with BKV and JCV viruses in healthy donors and other patient groups with an increase in VP-1 antibody levels associated with detectable viruria or viraemia [151, 334, 335]. Importantly, higher VP-1 antibody responses are seen in individuals who develop polyomavirus-associated diseases. MCV VP-1 antibody levels are higher in MCC patients than healthy controls [74, 75, 152, 336] and this increase can predate tumour onset by a number of years [337]. JCV VP-1 antibody levels are higher in patients with multiple sclerosis treated with natalizumab and who go on to develop progressive multifocal leukoencephalopathy (PML) [338-340]. These data lead to the suggestion that higher polyomavirus VP-1 serum antibody levels reflect increased viral replication and that this may be important to the development of polyomavirus-mediated disease.

The cause of increased polyomavirus viral replication may be due to the loss of viral immune control. This is evidenced by the fact that most examples of polyomavirus-mediated disease

are strongly linked with immune dysfunction. BKV-associated nephropathy occurs commonly in patients who are iatrogenically immunosuppressed following renal transplant and BKV viral loads are higher in these patients with nephropathy compared to those without [341]. The TSV-associated skin disease, Trichodysplasia Spinulosa, occurs exclusively in immunosuppression and high TSV viral loads are seen in all TS lesions while TSV DNA is only infrequently found in skin from healthy donors only ever at very low viral loads [342]. In JCV-associated PML, JCV VP-1 serum antibodies are higher than in patients without PML [343] and JCV DNA is found more commonly in the serum and bone marrow of HIV+ patients compared with HIV negative donors [344, 345]. Systemic immunosuppression is also a recognised risk factor for MCV-associated MCC with a higher incidence in patients who are at risk of other polyomavirus-mediated disease such as those who have received organ transplants or who suffer with HIV. Indeed, patients with poorly controlled HIV (defined by higher viral loads and reduced CD4 counts) have significantly higher MCV viral DNA loads detectable from skin swabs compared to those with good disease control [272].

It may be expected that when viral control for one polyomavirus is lost, this may also be seen with the other members of the virus family. There is some evidence for this in HIV+ patients where lymphoid tissue taken from autopsy patients is more commonly found to contain detectable levels of BKV, JCV, KI and WU viral DNA compared with controls [346]. Additionally, skin swabs assessing for the presence of HPyV6, HPyV7, TSV, HPyV9 and HPyV10 viral DNA showed significantly higher frequencies of DNA detection for each virus in HIV+ patients compared with healthy controls [169]. Whether all MCC patients exhibit a similar generalised loss of polyomavirus control is not fully understood. VP-1 antibody levels for BK, JC KI and WU polyomaviruses have been compared between MCC patients and healthy controls with no evidence of any difference seen between the groups for all four

viruses [74, 152]. This suggests that the loss of MCV viral control in most MCC patients may not extend to all polyomaviruses and may reflect the finding that although there is a clear link between immune suppression and MCC, the majority of MCC patients do not exhibit any obvious immune dysfunction [7]. However, a comparative assessment between MCC patients and other donors involving the control of more recently discovered polyomaviruses, particularly those that are associated with the skin (such as HPyV6, HPyV7, TSV, HPyV9 and HPyV10) has not been conducted to our knowledge.

Another patient group that is at increased risk of developing MCC are patients with Chronic Lymphocytic Leukaemia (CLL) [347, 348] but the exact link between the two diseases is unclear. Although not a group that commonly suffers with other polyomavirus-mediated diseases, CLL is associated with immune dysfunction including hypogammaglobulinaemia that may impact on humoral polyomavirus control [349]. Indeed, a Spanish study assessing polyomavirus antibodies showed reduced prevalence of most polyomavirus VP-1 responses in CLL patients compared with controls [350]. It is possible that this finding of a general pattern of reduced polyomavirus-specific antibodies may contribute to the loss of viral polyomavirus control and lead to the increased risk of MCC. It is important that as these are the results of a single study, such findings be firstly confirmed on an independent CLL cohort. They can also be directly compared with results from MCC patients and healthy donors to assess whether there are patterns of antibody responses that may help us to understand whether the link between CLL and MCC is through the loss of polyomavirus immune control.

Although the majority of MCC patients have no known immune dysfunction, MCCs are more common in elderly patients and in geographical areas with higher sun exposure as measured by UVB solar index [8]. Ageing is known to reduce cutaneous anti-viral immunity through the accumulation of regulatory and PD-1 expressing T cells [269]. UV light exposure leads to

a loss of antigen presenting Langerhan's cells [267], an accumulation of regulatory T cells [351] and the stimulation to produce the immunosuppressive cytokine IL-10 [352]. As MCV is a skin-associated virus, the cause for reduced MCV immune control may be due to localised cutaneous rather than systemic immunosuppression. It may then be expected that in MCC patients a loss of viral control may be seen not only for MCV but also the other skin-associated polyomaviruses (such as HPyV6, HPyV7 and TSV) while sparing viruses not thought to be skin associated (such as BK, JC, KI and WU). An investigation of the control of these other skin-associated viruses in MCC patients in direct comparison to non-skin associated viruses has never been conducted and will be useful to help answer these questions.

If ageing and UV light exposure are able to contribute to a specific loss of skin-associated polyomavirus control in MCC patients, it may be expected that this same phenomenon occur with other conditions that share these risk factors. Non-melanoma skin cancers (NMSCs) are much more prevalent in the elderly and occur most commonly on areas of sun-exposed skin. However, unlike MCC, they are not known to be associated with any viral infection [140, 141, 353]. The question of whether the risk factors associated with a loss of cutaneous viral immunity can lead to similar patterns of skin-associated and non-skin associated polyomavirus antibodies detected in the serum is an interesting one that should be further explored.

The majority of polyomavirus serological studies have focused on VP-1-specific antibodies and T-antigen seroresponses analysis has been much more limited. It may be expected that as T-antigen is a nuclear antigen rather than a capsid protein, antibodies may only be detectable at times of active viral replication. As MCC patients are thought to be undergoing increased MCV viral replication through having higher viral DNA loads in the skin than healthy controls, it may be expected that they would also have higher frequencies of MCV T-antigen

responses. Indeed, MCV T-antigen antibodies have been shown to have a prevalence of 40% in MCC patients compared with <1% in age and sex matched control subjects [354]. However, MCV T-antigen antibody levels, unlike VP-1 antibodies, do not appear to correlate well with MCV DNA in the skin, with levels instead tracking disease burden and often becoming undetectable at times of disease remission. This suggests increased T-antigen antibody prevalence may be mostly provoked by tumour cells expressing T-antigen rather than as a result of MCV viral replication. Some recent studies have suggested that a proportion of prostate cancer may be linked with BKV infection and in such patients there is a higher prevalence of BK T-antigen antibody seroresponses compared to patients with BKV negative tumours [355, 356]. It has been therefore been postulated that polyomavirus T-antigen antibody levels may be a useful biomarker to predict recurrence or assess treatment efficacy in patients with polyomavirus-associated tumours. To date, there has been no data from UK MCC patients that confirm this finding and give an indication of whether T-antigen antibody levels could be a useful clinical biomarker. The investigation into other polyomavirus T-antigen antibody responses is more limited than with MCV. In healthy controls and MCC patients, seroresponses to BKV T-antigen were of low frequency with no significant difference between the two groups [354]. A number of gaps remain in our knowledge regarding polyomavirus T –antigen seroresponses, such as the overall prevalence of MCV T-antigen responses in groups other than MCC patients and young, healthy donors as well as the prevalence of T-antigen seroresponses against the other non-tumour associated polyomaviruses in any donor group. These data will help clarify whether these responses do have link with active viral replication or are only specific to detect the presence of tumour.

The work in this chapter uses serological assessment of VP-1 and T-antigen antibody responses against a variety of polyomaviruses in a number of donor groups to increase

available knowledge on a number of topics. Firstly, it will allow us to confirm previous serological findings seen in MCC patients in other geographical locations in the USA and Europe, but not to our knowledge in a UK population. In particular the finding that MCC patients have higher serum MCV VP-1 antibody levels in comparison to other donor groups and that MCV T-antigen antibody prevalence is positively correlated with MCC disease activity.

Additionally, it will help to show whether MCC patients have an increased susceptibility to infection by other polyomaviruses other than MCV. This will be assessed through comparison of frequency and size of VP-1 seroresponses in healthy donors and also in NMSC and CLL patients, both of whom may share similar patterns of polyomavirus infection and antibody responses to MCC patients as discussed above. Lastly, we can assess the prevalence of a variety of polyomavirus T-antigen responses in the MCC, CLL, NMSC and healthy donor cohorts to see if these are detectable and if so does this provide evidence for a possible link between T-antigen responses and viral reactivation.

3.2 Results

3.2.1 Donor populations

In order to assess polyomavirus antibody responses from the serum of appropriate donor populations, MCC patients, CLL patients, NMSC patients and healthy donors (elderly and young) were recruited from the local area.

3.2.2 MCC patients

Between 2009 and 2015, forty-eight MCC patients were recruited from Queen Elizabeth Hospital Birmingham (QEHB). Samples prior to 2012 were collected by David Blackbourn and Neil Steven during an earlier phase of this project. The mean age of this patient population was 74 (range 41-91) with 56% being female. There were eight patients (17%) also suffering with non-melanoma skin cancers, one (2%) had chronic lymphocytic leukaemia and three were immunosuppressed (1 renal transplant, 1 systemic sclerosis, 1 sarcoidosis). When considering other relevant co-morbidities, three (6%) had other cancers (2 locally advanced breast cancer on hormone treatment and 1 renal cancer on no treatment). There were five (10%) with other illnesses that may impact on immunity including two with multiple sclerosis not on any immunomodulatory therapy, two with rheumatoid arthritis; one was on sulfasalazine and the other on methotrexate, and one with chronic obstructive pulmonary disease that has required numerous courses of steroids over a number of years. Serum was collected from patients with both active disease and those in remission, with eleven patients having multiple samples taken. At first blood draw, 26 patients had active MCC, thirteen had been in remission for less than six months and nine had been in remission for over six months. The detailed demographics of these sub-groups are found in Table 3.1.

3.2.3 Other donor groups

Serum from a number of control groups was collected to be used as comparators to the MCC group. We recruited twenty-three patients with either active or history of non melanoma skin cancer (NMSC) from the dermatology clinic at QEHB. The mean age of these patients was 75 (range 49-87) and 39% were female. Of these, seventeen patients had basal cell cancer (BCC) and six had squamous cell cancer (SCC). None of these patients had any history of immunosuppression. Serum from twenty patients with CLL was kindly provided by Dr Helen Parry (University of Birmingham). Of these, ten had Stage A disease, one Stage B, eight Stage C and one Richter's transformation. The mean age of this group was 71 (range 57-101) and 35% were female. Serum from twenty local healthy elderly participants of the "1000 elders" programme, run by the University of Birmingham, was also provided by Dr Helen Parry. The mean age of this group was 81 (range 74-90) with 80% females. None of these patients were immunosuppressed. Serum from 23 healthy members of staff from the University of Birmingham was also taken. The mean age of this group was 43 (range 22-55) and 35% were female.

Table 3.1. MCC patient demographics.

		Active MCC N=26	Remission < 6 months N=13	Remission >6 months N=9	Total N=48
Age		73	75	74	74
Sex	Female	11 (42%)	8 (62%)	8 (89%)	27 (56%)
	Male	15 (58%)	5 (38%)	1 (11%)	21 (44%)
Stage	1	1 (4%)	8 (62%)	5 (56%)	14 (29%)
	2	6 (23%)	4 (31%)	3 (33%)	13 (27%)
	3	13 (50%)	1 (8%)	1 (11%)	15 (31%)
	4	6 (23%)	0 (0%)	0 (0%)	6 (13%)
Co-morbidity	NMSC	4 (15%)	3 (23%)	1 (11%)	8 (17%)
	CLL	1 (4%)	0 (0%)	0 (0%)	1 (2%)
	Other cancer	1 (4%)	2 (15%)	0 (0%)	3 (6%)
	Immunosuppression	1 (4%)	2 (15%)	0 (0%)	3 (6%)
	Other	3 (12%)	2 (15%)	0 (0%)	5 (10%)

MCC patients were stratified by diseases state at time of serum sampling. If multiple samples had been taken, disease state at time of first sample was used. NMSC=Non melanoma skin cancer, CLL=Chronic Lymphocytic Leukaemia

3.2.4 Comparison of frequency of polyomavirus VP-1 seroresponses amongst the MCC and other donor cohorts

Initially, we were interested to know whether MCC patients were likely to be infected with more polyomaviruses in comparison to other donor groups. To assess this, serum from MCC patients, NMSC patients, CLL patients and healthy donors was taken and polyomavirus VP-1 seropositivity was determined using an established multiplexed bead based luminex assay testing for responses to ten of the twelve known human polyomaviruses [181]. Firstly we looked at the presence or absence of individual polyomavirus seroresponses in all the donors using an established cut-off value (MFI >250) to define seropositivity for each VP-1 antigen. In a recent large American epidemiological study using this same assay, there was no evidence of significant cross-reactivity between the various polyomavirus-specific responses [181]. When our donor seroresponses were analysed, there was no difference in the total frequency of polyomavirus-specific VP-1 antibody responses seen in individuals from the different cohorts, with median values of between six and seven responses found in all groups (Figure 3.1).

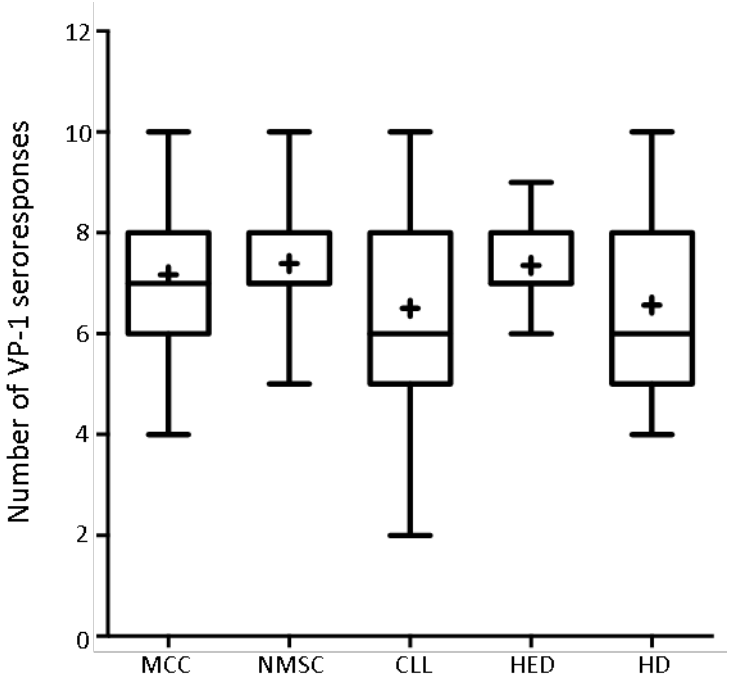


Figure 3.1. Number of polyomavirus VP-1 seroresponses present in MCC, NMSC, CLL, HED and HD groups. The number of positive seroresponses (defined as MFI>250) were identified for individual donors within each group. The box and whisker plots show the interquartile ranges (boxes) and minimum and maximum values (whiskers) for each cohort. Horizontal lines and crosses represent median and mean numbers of responses respectively. MCC=Merkel cell cancer, NMSC=Non-melanoma skin cancer, CLL=Chronic lymphocytic leukaemia, HED=Healthy elderly donors, HD=healthy donors.

Next, we considered whether there were any differences in the patterns of individual virus seropositivity between the different cohorts (Table 3.2). MCV seropositivity was more common in MCC patients (85%), compared with the other populations, although there was only a significant difference when compared with CLL patients (45%, p=0.0017, Fisher's Exact Test). VP-1 antibody responses against BK, KI, WU, TSV and HPyV10 were almost ubiquitous (70-100% positive) in all cohorts. JC VP-1 seroprevalence was similar in the MCC, NMSC, CLL and healthy elderly groups (43-45% positive) but appeared lower in the younger healthy donors (22%). This pattern was repeated for HPyV6 infections but no significant differences were seen between any of the groups. When assessing HPyV7, there

was no difference between any of the elderly cohorts and although a large difference was seen between the cohort with the highest and lowest response rates, NMSC (87%) and young healthy donors (35%) this was not statistically significant.

	MCC (n=48)	NMSC (n=23)	CLL (n=20)	HED (n=20)	HD (n=23)
Mean age (range)	74 (41-91)	75 (49-87)	71 (57-101)	81 (74-90)	43 (22-55)
Sex (M:F) (%)	44%:56%	61%:39%	65%:35%	20%:80%	65%:35%
BK VP-1 seroprevalence (%)	88%	96%	75%	75%	96%
JC VP-1 seroprevalence (%)	44%	43%	45%	45%	22%
KI VP-1 seroprevalence (%)	79%	96%	80%	90%	78%
WU VP-1 seroprevalence (%)	92%	100%	85%	100%	100%
MCV VP-1 seroprevalence (%)	85%*	52%	45%*	60%	74%
HPyV6 VP-1 seroprevalence (%)	77%	74%	65%	85%	57%
HPyV7 VP-1 seroprevalence (%)	63%	87%	65%	65%	35%
TSV VP-1 seroprevalence (%)	79%	87%	80%	85%	70%
HPyV9 VP-1 seroprevalence (%)	15%	13%	15%	15%	17%
HPyV10 VP-1 seroprevalence (%)	88%	96%	90%	100%	96%

Table 3.2. VP-1 antibody seroprevalence in each patient cohort. Age and sex characteristics are shown for each individual cohort. The percentage of donors considered VP-1 seropositive (defined as MFI>250) has been calculated for each polyomavirus. Difference between groups analysed using Fisher's Exact test and statistically significant result highlighted, * p=0.0017.

Overall, the frequency of polyomavirus VP-1-specific antibody responses suggests that MCC patients are no more susceptible to polyomavirus infections than the other donor cohorts assessed. MCV alone appears more prevalent in the MCC patient population as compared with the other control populations, particularly when compared with CLL patients. Rates of infections of the other polyomaviruses as judged by antibody response are broadly similar throughout the elderly donor cohorts, but occasionally lower in the younger healthy donors.

3.2.5 Comparison of size of polyomavirus VP-1 seroresponses amongst MCC and other donor groups

MCC patients have comparable frequency of polyomavirus VP-1 seroresponses when compared to the other tested donor groups. As the size of the VP-1 response is thought to be a correlate for the degree of viral replication, higher VP-1 antibody values may be seen in donors in whom there are higher levels of replication. We were therefore interested to compare the sizes of VP-1 antibody responses between MCC patients, NMSC patients, CLL patients and healthy donors to see if any groups had higher values and against which viruses this occurred. Here MFI values of antibody bound to antigen coated beads were assessed.

A significant difference was seen in MCV VP-1 antibody levels with the 48 MCC patients having higher MFI values compared with the 86 other donors (mean MFI 4247 v 1526, $p<0.0001$, Mann-Whitney U Test) (Figure 3.2). There was no significant difference in the other polyomavirus VP-1 antibody MFI values tested when comparing MCC patients with all other donors (Figure 3.2). The reduction of MCV MFI seen in the non-MCC donor groups was shared by all comparator cohorts, with NMSC, CLL, healthy elderly and young healthy donors having significantly lower MFI values when compared individually against the MCC patients (Figure 3.3A). This pattern was maintained when only MCV seropositive donors were included in analysis (Figure 3.3B). When sizes of all VP-1 polyomavirus responses were compared for each individual donor cohorts, a significant difference was seen when HpyV7 VP-1 levels from NMSC patients and healthy donors were compared (Table 3.3, $p=0.006$, Kruskall-Wallis test with Dunn's multiple comparison correction). However, given the very small sample sizes of the cohorts involved, this difference must be interpreted with caution.

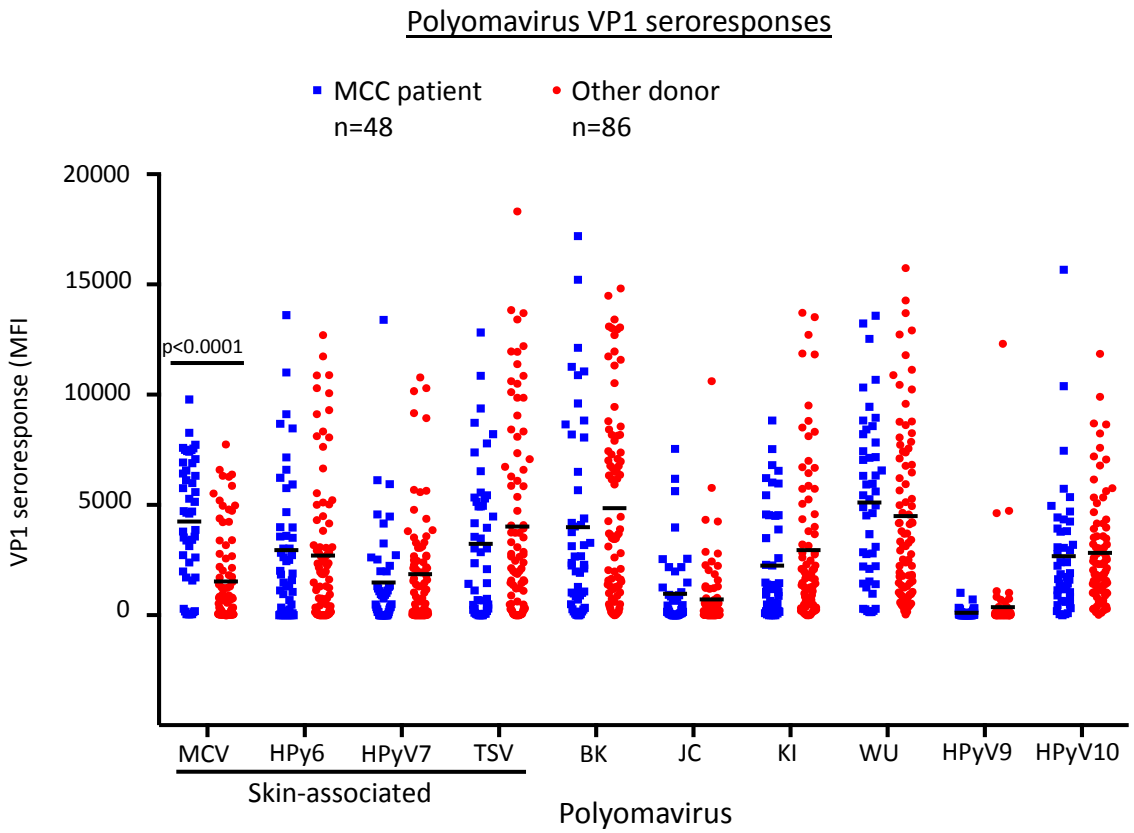


Figure 3.2. Polyomavirus VP-1 seroreactive responses from MCC (blue, n=48) patients were compared with all other donor groups pooled together (red, n=86). Each point represents an individual donor, horizontal line represents mean value for each group. A significant difference between MCC patients and other donors was only seen for MCV VP-1 seroresponse (mean MFI 4622 v 539, $p<0.0001$, Mann-Whitney U Test.)

As MCC patients appeared to have higher levels of MCV VP-1 antibodies compared to other donors, we wanted to clarify if this effect was limited to patients at a certain disease stage. We therefore split the MCC cohort into three categories dependent on disease stage at time of blood draw (active disease, remission of less than six months and remission over six months). Comparing MCV VP-1 antibodies between these groups, there was no significant difference between the disease stages (Figure 3.4). In addition, when comparing VP-1 antibody levels of the other tested polyomaviruses there was no significant differences between any of the disease stages (Figure 3.4). Overall this data suggests that MCV VP-1 antibody responses are

higher in MCC patients and this is independent of stage of disease. The levels of VP-1 response for the other polyomaviruses, including those that are skin-associated, are not significantly different between any of the donor cohorts assessed.

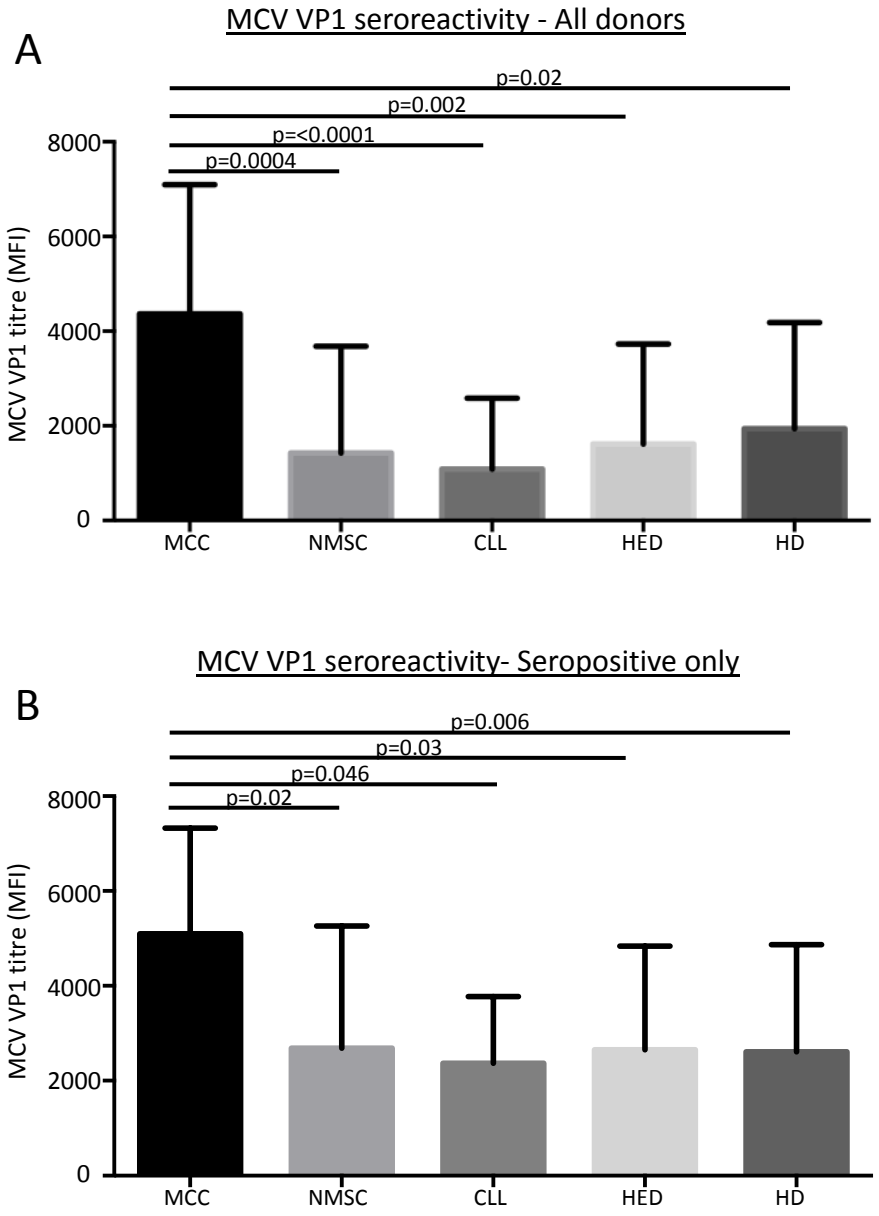


Figure 3.3. Comparison of MCV VP1 seroresponses for all individual cohorts. (A) shows mean and standard error MFI values from the serum of all donors. (B) shows mean and standard error when only MCV seropositive donors are considered. Statistical comparison made with Kruskal-Wallis Test corrected for multiple comparisons using Dunn's Test.

	BK			JC			KI			WU			HPyV6			HPyV7			TSV			MCV			HPyV9			HPyV10		
	Mean	Med	Max	Mean	Med	Max	Mean	Med	Max	Mean	Med	Max	Mean	Med	Max	Mean	Med	Max	Mean	Med	Max	Mean	Med	Max	Mean	Med	Max	Mean	Med	Max
MCC	4029	2327	17194	931	151	7538	2270	1359	8837	4985	4935	13579	2941	1929	13606	1504	626	13405	3270	2604	12824	4368	4672	9777	106	30	1008	2616	1688	15664
NMSC	3911	3468	11584	501	73	2789	4215	2612	13712	5550	4570	14269	3970	1891	11740	2695	1959	10164	4466	2466	18320	1424	267	6587	149	63	1088	3153	2038	9902
CLL	4171	2046	14820	1004	226	4324	1556	1035	5863	4386	3518	13700	2004	1547	10077	1724	1382	5645	3826	2205	13696	1084	114	5204	545	46	4732	2081	1765	5335
HED	5194	3045	13411	754	232	5770	3606	2014	13524	4569	3726	15741	2657	1862	10304	2040	958	10775	3929	2386	13839	1613	772	6274	131	48	831	2543	1730	8240
HD	6086	6495	12965	619	41	10611	2308	1638	9507	3456	1832	12913	2121	1294	12696	967	27	10305	3828	2138	13420	1940	1256	7745	623	13	12311	3414	2443	11854

Table 3.3. Sizes of VP-1 antibody responses for MCC, NMSC, CLL, HED and HD donor groups. Mean, Median (Med) and maximum MFI values are shown from results of multiplexed, bead based assay measuring seroreactivity against various polyomavirus VP-1 antigens. Statistical associations were analysed used Kruskal-Wallis test. Significant difference between groups were noted for MCV VP-1 which was higher in MCC patients as compared to NMSC ($p=0.0004$), CLL ($p<0.0001$), HED ($p=0.0023$), HD ($p=0.015$). HPyV7 seroreactivity was significantly higher in the NMSC group compared with the HD group ($p=0.006$), but not when compared with the other groups.

MCC= Merkel cell cancer, NMSC=Non-melanoma skin cancer, CLL=Chronic lymphocytic leukaemia, HED= Healthy elderly donors, HD=healthy donors.

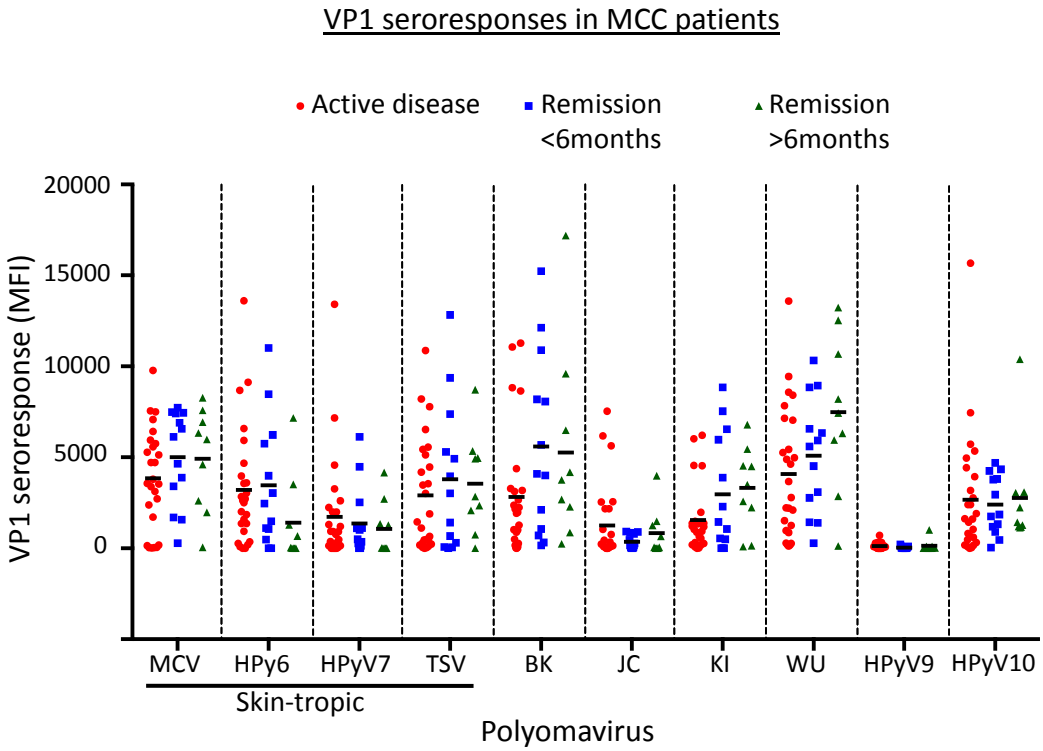


Figure 3.4. VP-1 antibody seroresponses for all studied polyomaviruses in MCC patients at different disease stages. MCC patients who had serum analysed for VP-1 seroresponses were categorised into groups depending on disease state at time of blood draw, active disease (red circles), in remission for period of less than 6 months (blue squares) and in remission for over 6 months (green triangles). In patients who had multiple samples, only the first available sample was included. Horizontal lines represent mean values. No significant differences were seen between any of the MCC disease stages (Kruskal-Wallis test).

3.2.6 Polyomavirus VP-1 antibody levels in MCC patients throughout disease course

Our previous data showed that MCC disease status did not appear to affect size of polyomavirus VP-1 antibody responses when the entire cohort was considered. However, we were also interested to assess and confirm this in individual patients. Multiple serum samples from different time points were available in eleven MCC patients to enable longitudinal comparison of VP-1 antibody titres. All cases were MCV seropositive. In four cases, samples

were taken at only at times of active disease and in one patient, samples were taken both during active disease and when in remission (Figure 3.5). In six patients samples were taken while in remission only (Figure 3.6).

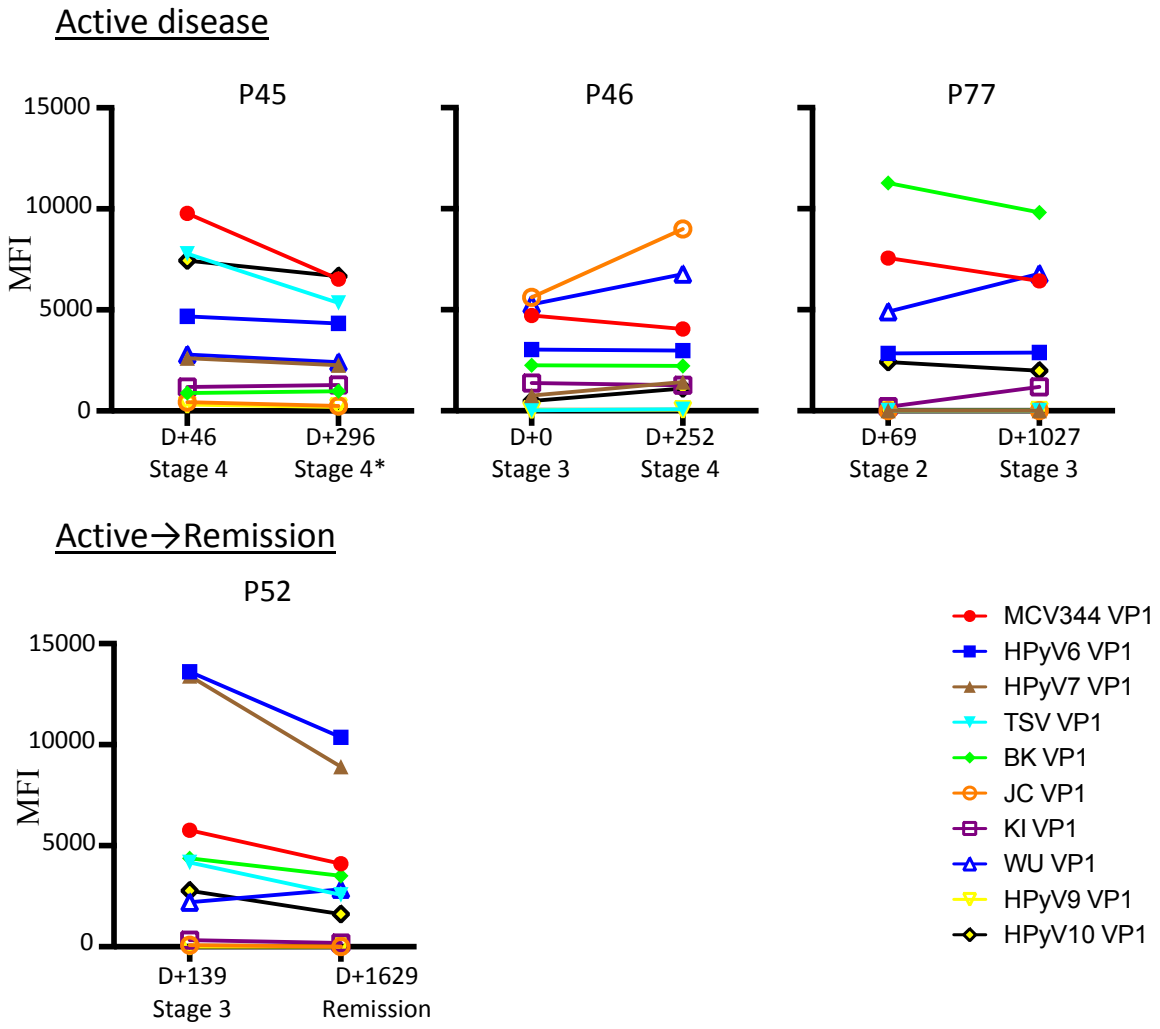


Figure 3.5. Serial VP-1 seroresponses in MCC patients with active disease. Four patients with active MCC had multiple serum samples taken and each were analysed for VP-1 seroresponses against multiple polyomaviruses. The number of days after initial diagnosis that sample was taken is shown. P45, P46 and P47 had both samples taken at time of active disease. P45 received recent chemotherapy prior to second sample (*). P52 had an initial sample taken while in active disease and second sample taken while in remission.

In the majority of cases, MCV VP-1 antibody levels remained grossly stable over time. This was the case even in the two patients (P46 and P77) who suffered disease progression and in the single patient (P52) who gained disease control (Figure 3.5). One patient, P45, who had initial sample taken during metastatic disease and second sample taken following a period of chemotherapy, did show a reduction of VP1 antibody titre (9777 to 6516 MFI), but this was unfortunately not reflected by a clinical response to treatment and the patient died soon afterwards.

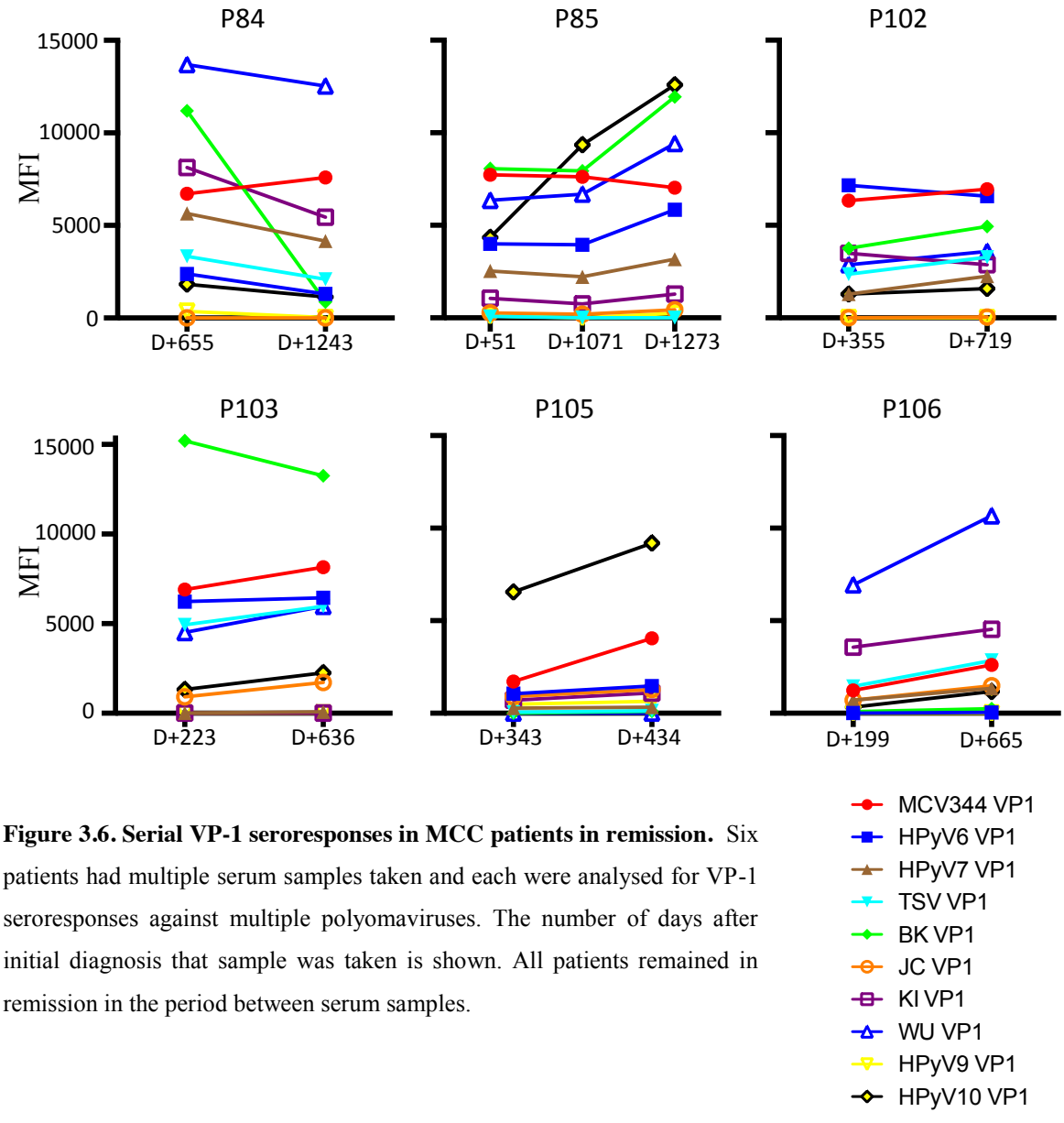


Figure 3.6. Serial VP-1 seroresponses in MCC patients in remission. Six patients had multiple serum samples taken and each were analysed for VP-1 seroresponses against multiple polyomaviruses. The number of days after initial diagnosis that sample was taken is shown. All patients remained in remission in the period between serum samples.

Other than MCV VP-1 antibody responses, we also assessed VP-1 antibodies against the other polyomaviruses. In all but one patient (P45), at least one other VP-1 antibody response was higher than MCV, but the viruses responsible differed between patients, the most common being WU and BK (3 patients each) (Figure 3.5&3.6). In most cases, as with MCV, VP-1 antibody levels remained stable over time with some notable exceptions, for example, P46, had an increase in JCV VP1 antibody titre (Figure 3.5), while from the patients in remission, P85 had an increase in HPyV10 VP1 antibody titre and P106 and P107 had increases in WU titre (Figure 3.6). The most striking change seen, however, is the dramatic fall in BKV VP1 antibody titre seen in P84 from over 11000 MFI to 866MFI. This data shows that in the vast majority of individual MCC cases, VP-1 antibody levels against both MCV and the other polyomaviruses remain stable over time, independently of the burden of disease. In almost all cases another polyomavirus VP-1 antibody level was greater than that of MCV although the candidate virus was not consistent and only skin-associated in a minority of cases.

3.2.7 The potential for cross-reactivity between individual polyomavirus T-antigen antibodies

Having assessed VP-1 antibody responses, we next turned attention to T-antigen antibody seroresponses which have been much less investigated. As the polyomavirus family share 36-80% identical amino acid sequence of the T-antigen (Table 3.4) there is a concern of possible cross-reactivity between tested antigens. In order to see if this could have an effect on our results, we looked for a correlative association between MCV T-antigen levels and levels of the other individual viral LT-antigens in all of our donors. The strength of these correlations were then tested statistically using the non-parametric Spearman's test (Figure 3.7).

	BKV	JCV	KI	WU	MCV	HPyV6	HPyV7	TSV	HPyV9	HPyV10	STL	HPyV12
BK		83	53	55	49	43	44	44	44	48	47	38
JC			51	53	48	44	43	46	42	47	47	38
KI				70	46	41	44	47	44	46	47	39
WU					47	41	43	46	42	48	49	46
MCV						46	47	47	46	47	48	49
HPyV6							69	42	42	47	46	41
HPyV7								45	45	48	48	36
TSV									50	49	47	45
HPyV9										47	46	42
HPyV10											61	43
STL												40
HPyV12												

Table 3.4. Percentage of shared LT homology between members of the polyomavirus family. BLAST analysis comparing amino acid similarity between pairs of polyomaviruses. The following sequence ascension codes were used: BKV NC_001538, JCV NC_001699, KI NC_009238, WU NC_009539, MCV NC_010277, HPyV6 NC_014406, HPyV7 NC_014407, TSV NC_014361, HPyV9 NC_015150, HPyV10 JX262162 STL JX463183, HPyV12 JX308829.

There were correlations seen between MCV T-antigen antibody responses and those against other viral T-antigens, most significantly between MCV LT exon 1 antigen and the LT exon 1 antigen of HPyV6 ($r=0.42$, $p=<0.0001$), HpyV7 ($r=0.38$, $p=<0.0001$) and TSV ($r=0.46$, $p<0.0001$). This data suggests that there may be relationship between MCV T-antigen antibody responses and T-antigen antibody responses against other polyomaviruses, particularly those that are skin-associated. Unfortunately, determining whether this correlation is due to multiple co-infections occurring with MCV or simply a product of cross-reactivity is

impossible with the data available. This means that although the assay used is the best that is currently available for this work, our results must be interpreted with caution and any findings will need further validation before firm conclusions can be drawn.

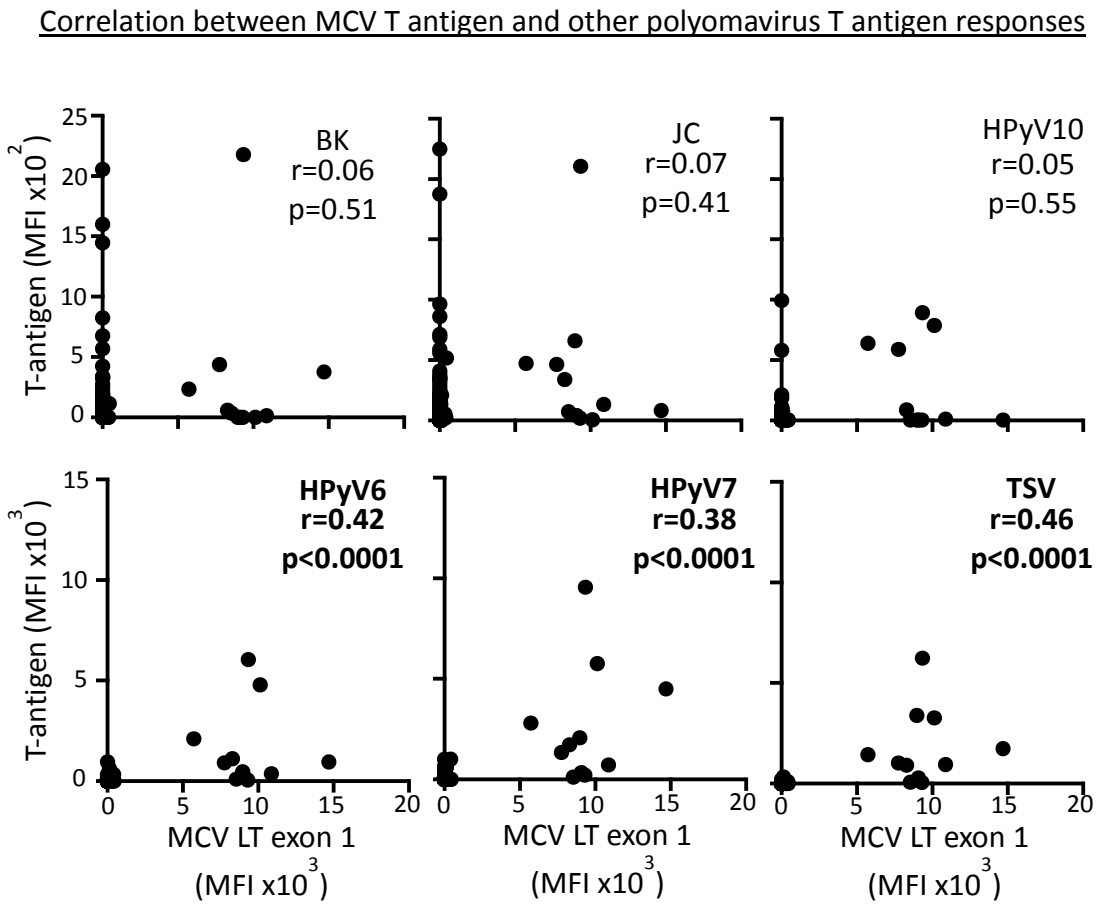


Figure 3.7. Non parametric Spearman correlation analysis between MCV T-antigen (Large T exon 1) MFI values compared with other polyomavirus T-antigen responses. Analysis was conducted using non-parametric Spearman correlation calculation comparing MCV VP-1 responses (x-axis) with other polyomaviruses individually (y-axis) using data from all donors (n=134).Each point represents a single donor. Significant correlations are highlighted in bold type.

3.2.8 Comparison of polyomavirus T-antigen seroresponses amongst MCC and other donor groups

Previous studies have shown that MCV T-antigen antibody responses are seen at much higher frequency in MCC patients compared with healthy donors, but little is known of the prevalence in CLL patients, who have an increased risk of MCC and NMSC patients, who share MCC-associated risk factors. Data on the frequency and size of T-antigen antibody responses for other polyomaviruses is limited, even in healthy donors. In order to increase our understanding of the prevalence and level of T-antigen antibodies, we assayed serum collected from MCC patients, NMSC patients, CLL patients and healthy donors for polyomavirus-specific T-antigen seroresponses. Polyomavirus T-antigen antibodies were measured using a multiplex bead based assay and seropositive responses were determined using defined MFI cut-off values. For MCV, seroreactivity to four different T-antigen epitopes were measured (LT exon 1, LT exon 2, Large T and sT). To simplify comparison between the groups, donors with MCV T-antigen responses were classified as either having T-antigen response (if one or more MCV T-antigen positive) or not having response (if no MCV T-antigen positive).For BKV, JCV, HPyV6, HPyV7, TSV and HPyV10, a single LT exon 1 antigen response was measured. When focusing only on MCV T-antigen antibody seroresponses (Figure 3.8A), these are limited only to MCC patients and seen in 50% of patients with active disease, 31% of those in remission for less than 6 months and 22%, of those in remission for over 6 months.

We next looked at the individual donors in each cohort and quantified the detectable T-antigen seroresponses against any of the measured polyomaviruses seen. When conducting this analysis, it was clear that a number of donors had multiple detectable T-antigen responses. Overall, there was a clear trend showing that MCC patients with active disease had

an increased number of T-antigen responses compared with the other cohorts, in whom detection of any T-antigen responses was uncommon (Figure 3.8B). This was statistically significant when compared with the CLL ($p=0.002$) and the young healthy donor ($p=0.02$) populations but not with the MCC patients in remission ($p>0.99$), the NMSC patients ($p=0.21$) and healthy elderly patients ($p=0.11$). In these groups, although infrequent, there were individuals whose serum contained detectable levels of T-antigen antibodies specific for polyomaviruses other than MCV. In some NMSC patients and healthy donors, multiple T-antigen responses could be seen. This data suggests that MCV T-antigen antibodies are specific to MCC patients and more prevalent in those with active disease at time of blood draw. MCC patients with MCV T-antigen responses were also frequently seen to have detectable responses against other polyomavirus T-antigens. Although, detection of any T-antigen seroresponses was rare in the non-MCC donor cohorts, there were instances of this, including a small number with multiple T-antigen responses.

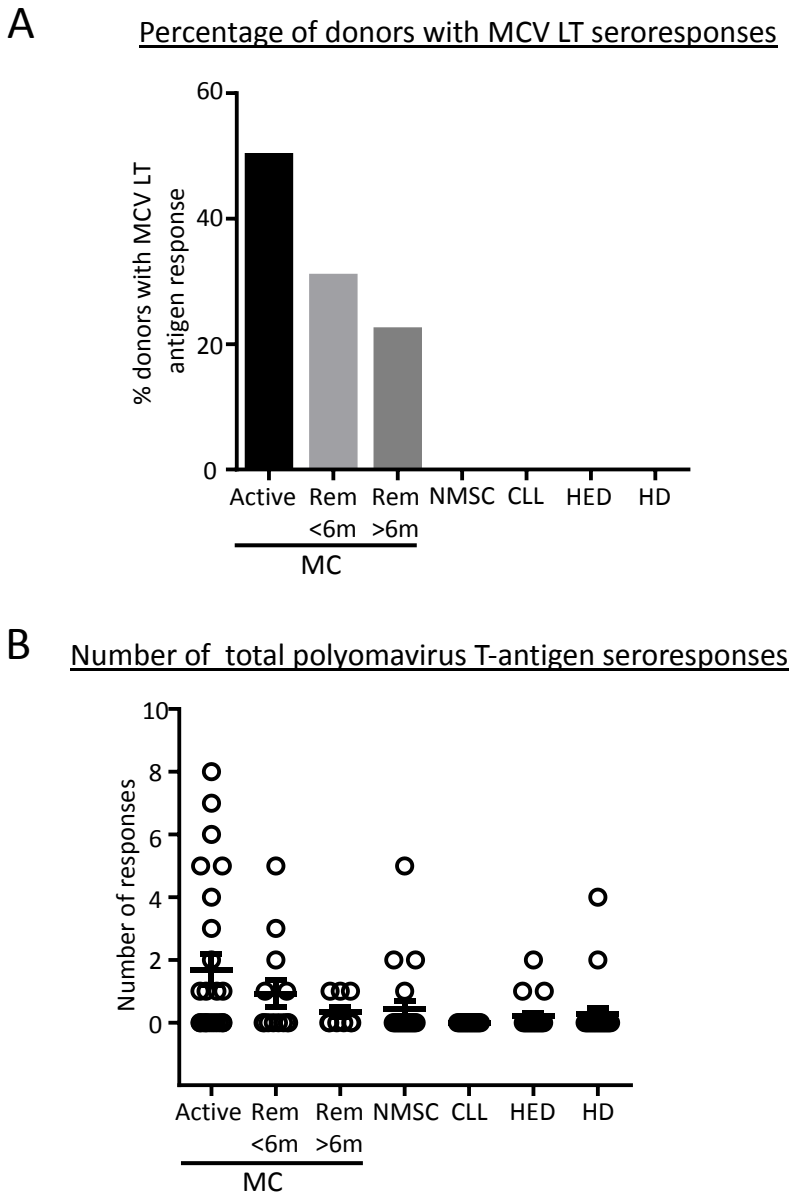


Figure 3.8. T-antigen seroresponses in MCC, NMSC, CLL, HED and HD groups. (A) The percentage of donors from each cohort with a positive (MFI>400) MCV T-antigen seroresponse. (B) The total numbers of T-antigen seroresponses for donors in each studied cohort. Circles represent individual donors, horizontal and vertical lines represent mean and standard error of mean. Active=Active disease, Rem<6m=MCC Remission for less than 6 months, Rem>6m=MCC Remission for over 6 months, NMSC= Non melanoma skin cancer, CLL=Chronic Lymphocytic Leukaemia, HED=Healthy Elderly Donors, HD=Healthy younger donors

3.2.9 Polyomavirus T-antigen antibody levels in MCC patients throughout disease course

Data from other studies has shown that serum levels of MCV T-antigen antibody correlate with disease burden in MCC patients. To examine this and other co-resident polyomavirus T-antigen antibody seroresponses, we used serum from the MCC patients for whom multiple samples were available (as described previously) and first tested for MCV T-antigen antibody levels. As a number of MCC patients had been shown to have multiple viral T-antigen antibodies detected in the serum, we were also interested to see how levels of other non-MCV T-antigen antibodies changed with time (Figure 3.9). Three patients with active and progressing disease (P45, P46 and P77) had high MCV LT exon 1 and sT-antigen antibody values that appeared relatively stable over time. P52, who was successfully treated after donating the first serum sample, which contained high MCV LT and sT antibodies, saw a large drop in levels by the time of the second sample, taken over 3 years later. The same decrease was also seen for the other elevated T-antigen responses seen at first sample. The patients who had multiple samples taken whilst in remission (e.g. P102, P106) mostly showed T-antigen responses below the levels of detection. P84 whose first sample was taken two years after initial diagnosis, had levels of MCV T-antigen antibodies just above the limit of detection and these both decreased by time of second sample collection, 18 months later. Interestingly, P85 also had detectable T-antigen antibodies against MCV and JC at a low level 2 months after receiving curative treatment for MCC. By the time of the next sample, 3 years later, the MCV T-antigen antibody levels had decreased below the limits of detection, however, JC T-antigen antibody levels remained stable and at the time of a third sample, 6 months later, JC T-antigen antibody levels had increased further. Overall, the longitudinal assessment of MCV T-antigen antibodies in MCC patients does show antibodies are more detectable at times of active disease. Also, although changes in T-antigen antibody levels

against other polyomaviruses in patients with multiple responses can follow the same pattern as seen with the MCV T-antigen antibody, this may not always be the case.

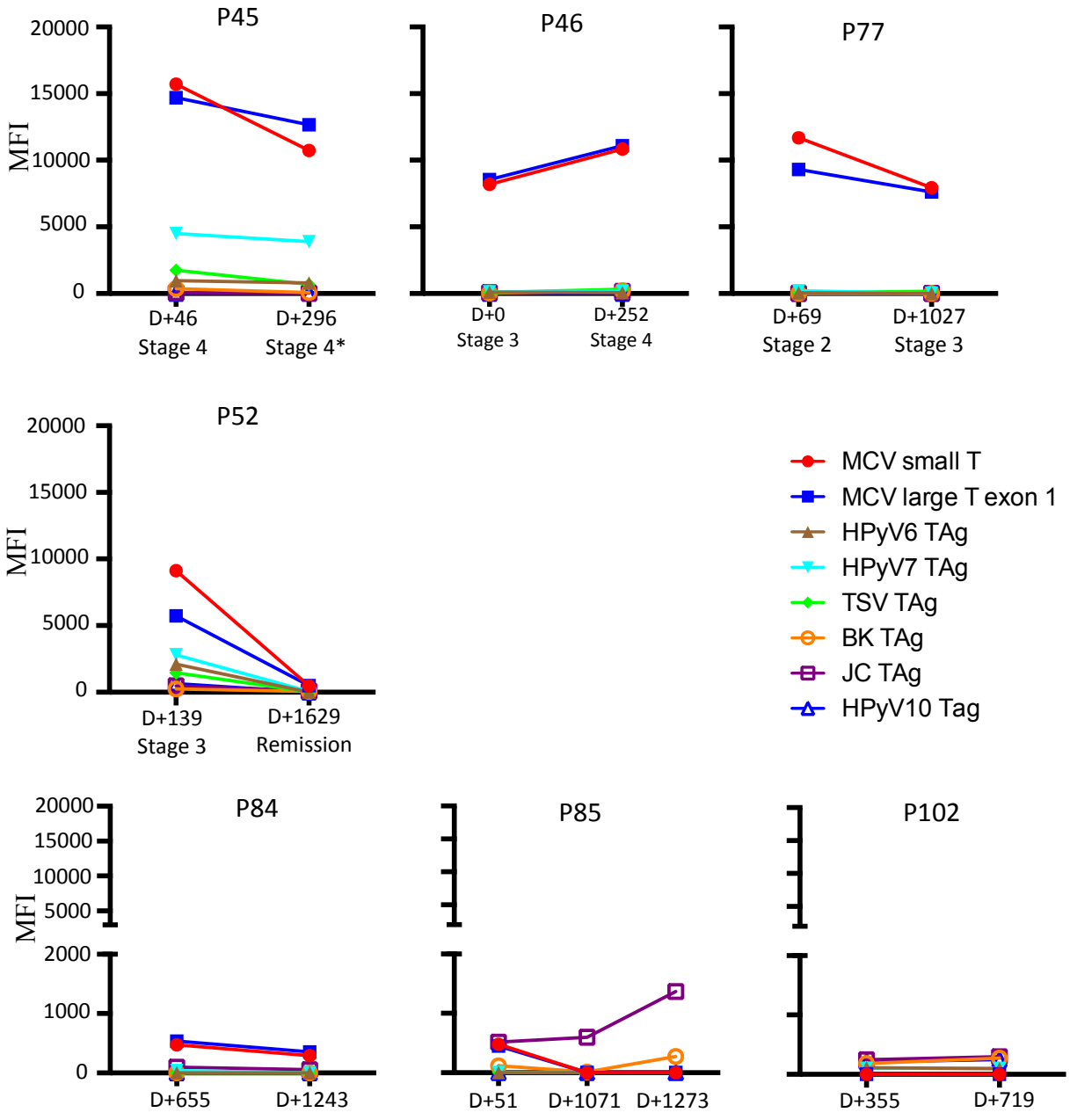


Figure 3.9. Serial T-antigen seroreactivity in MCC patients. Serum samples from patients who had donated at least twice were analysed for T-antigen seroresponses against multiple polyomaviruses. The number of days after initial diagnosis and disease state at time of sampling is shown. P45, P46 and P47 had both samples taken at time of active disease. P52 had an initial sample taken while in active disease and second sample taken while in remission. P84, P85 and P102 all had samples taken when in remission only.

3.3 Discussion

This study assessed the size and frequency of polyomavirus VP-1 and T-antigen antibody responses in the serum of a number of patients and volunteer groups. It demonstrated that all donor groups tested showed similar median numbers of total viral VP-1 responses indicating that multiple infections are common. As no difference in the number of serum antibody responses was seen when the healthy controls and the patient groups at higher risk of immune dysfunction (MCC or CLL patients) were compared, this suggests that these groups are not at increased risk of accumulating polyomavirus infections. The size and frequencies of anti VP-1 antibody responses are similar for the majority of polyomaviruses in the donor groups of similar age profile (MCC, NMSC, CLL and HED). In the younger healthy control group, prevalence of some VP-1 antibody responses were lower (JC, HPyV6 and 7) which may be expected as rates of polyomavirus infection increase with age [165, 178]. As expected given the strong link between MCV and MCC, the prevalence of MCV VP-1 antibodies was highest in the MCC group [75, 152]. The CLL patient group had the lowest frequency of MCV seroresponses while responses to other polyomaviruses appeared broadly maintained. A previous study assessing serum from a large group of CLL patients using methodology identical to that in our present work has shown that the frequency of multiple polyomavirus responses are lower in CLL patients compared to healthy control donors [350]. It is therefore possible that the reduced frequency of MCV VP-1 responses in the CLL group may reflect a decreased ability of these patients to mount MCV-specific humoral immune responses that may be related to the hypogammaglobulinaemia commonly associated with CLL [349]. This may allow for uncontrolled viral replication and increase the chance of CLL patients subsequently going on to develop MCC. However given the small size of the cohorts

involved, it is not possible to say that these findings are not due to chance alone and further confirmatory work is required.

As the sizes of VP-1 seroresponses are thought to correlate with the degree of on-going viral replication, this study also compared the sizes of response to individual virus VP-1 antigens between the donor groups. Only MCV VP-1 antibody levels showed any statistical difference, with significantly higher levels in serum of MCC patients when compared to all other cohorts, as seen in previous studies [74, 121]. There was no significant difference in MCV VP-1 antibody MFI values between healthy donors and both NMSC and CLL patient groups although CLL patients had the lowest mean MCV VP-1 antibody MFI values even when analysis is limited to seropositive individuals only. This suggests that although CLL patients are able to mount polyomavirus-specific antibody responses, the size of these may be limited when compared to other patient groups. MCV VP-1 specific antibodies are believed to have neutralising function [74, 337] and so a loss of these could also have a negative impact on MCV immune control and increase risk of MCC. Although a lack of MCV-specific antibodies may provide a possible reason for the increased incidence of MCC in CLL patients, the finding that the majority of MCC patients with documented immune compromise still mount high MCV-specific VP-1 antibody responses [121]. This suggests that a lack of specific antibodies is not the only underlying link between immunosuppression and MCC and that this is likely to be multi-factorial.

The significantly higher MCV VP-1 seroreactivity seen in MCC patients compared with NMSC patients suggests that it is not simply the exposure to shared risk factors such as age or increased UV light exposure that is responsible for a loss of MCV viral control. A large study assessing MCV VP-1 seroreactivity in patients with squamous cell skin cancers (SCC) compared with matched healthy controls found that mean VP-1 MFI levels were higher in the

SCC group possibly due to reduced MCV immune control [138]. In our study, we did not see any difference in MCV VP-1 MFI results in the NMSC cohort when compared to either the healthy elderly or the younger healthy control groups. This may be due to important differences between the studied cohorts with for example, immunosuppressed SCC patients being included in the published study while being excluded in our work. Additionally, the small cohorts used in the present study may make it difficult to identify small differences between groups. In our study, when non-MCV VP-1 antibody seroreactivities are compared, there are no significant differences seen in MFI values for either skin-associated or non-skin-associated viruses between any individual donor cohorts. This would appear to indicate that using the current assay, there is no specific loss of viral immune control of skin-associated polyomaviruses in MCC and NMSC patients suggesting that whatever the mechanism for loss of viral control of MCV in MCC patients, it may be specific for MCV and not impact upon responses against the other tested polyomaviruses.

In serial serum samples from MCC patients, MCV VP-1 seroreactivity remained stable independently of disease burden, confirming the findings of a previously published study [354]. VP-1 antibody responses against other polyomaviruses also mostly appear stable over time. This pattern is similar to what is seen with JCV VP-1 antibodies in serial samples taken from seropositive multiple sclerosis patients on treatment with natalizumab [357]. Similarly, in renal transplant patients suffering with BKV-associated nephropathy, VP-1 antibody levels appear to rise after disease onset and this increase is maintained at least 6 months following successful treatment [358]. In another study assessing both humoral and cellular BKV immune response kinetics in HLA-A2+ kidney-transplant patients suffering with BKV-associated nephropathy, increases in BKV VP-1 antibody levels occurred soon after disease onset. Interestingly, patients with higher frequency of BK-specific CD8 T-cells exhibited

lower peak antibody levels which declined rapidly. Conversely, patients with low frequency CD8 responses had high level VP-1 antibody responses that were long-lasting [359]. The authors of this study suggest that the cause of chronically high antibody levels may be due to persistently active virus that is not being controlled due to defective cellular immunity. Whether this could possibly be the reason that high MCV VP-1 antibody levels are seen in MCC patients is and interesting question yet to be answered. However despite this, it is unlikely that cellular immunity alone controls MCV and that the VP-1 antibody response is still likely to have an important role exemplified by the finding that high MCV VP-1 antibody levels in MCC patients correlate with a better prognosis [121].

Unlike VP-1, polyomavirus T-antigen antibody seroresponses were an overall infrequent occurrence although MCV T-antigen antibody responses were seen commonly in MCC patients with active or recently treated disease, in agreement with published data [354]. This suggests that this assay may have potential utility as a disease biomarker in MCC patients from the UK. A significant proportion of patients with MCV T-antigen antibody responses had responses against other T-antigens. The role of T-antigen antibody responses in polyomavirus infections is not clear and data is extremely limited. In young kidney transplant patients, BKV LT antibodies were less frequent, smaller and arose later than the VP-1 antibody response [360]. In this group, LT antibody levels were highest in patients with sustained viraemia indicating the antibody response may occur as a consequence of increased antigen exposure. Interestingly, the rise of BKV-specific LT antibodies coincided with the decline of viral load, suggesting this may be a marker of emerging immune control [360].

A number of MCC patients showed multiple viral LT antibody responses aside from MCV with moderate positive correlations ($r = 0.38$-0.46) seen between MFI values for MCV those of HPyV6, 7 and TSV LT antibodies. To definitively determine whether these correlations are

due to multiple co-infections or assay cross-reactivity is not easy. Cross-reactivity of polyomavirus LT antibodies have not been intensively investigated particularly with regard to the more recently discovered family members but it has been seen previously, particularly between SV40 and BKV [361, 362]. With regard to the particular assay used in our study, it is possible that there is some unavoidable cross reactivity (T. Waterboer, personal communication) which means that our results must be interpreted with caution. However, in some non-MCC patients multiple T -antigen antibody responses occurred in the absence of an MCV response and not all MCC patients with MCV LT seroresponses displayed multiple LT responses. These findings may argue against simple cross-reaction as a cause for multiple T-antigen-specific responses and may suggest that in some circumstances (e.g. a period of reduced immune surveillance) multiple viruses are allowed to reactivate at the same time, hence increasing the prevalence and accessibility to the immune system to T-antigens resulting in the antibody responses seen. If a generalised reduction in cutaneous immunity was present in MCC patients, it may be expected to result in the predominant reactivation of skin-associated viruses, particularly MCV, HPyV6, HPyV7 and TSV. Perhaps it is unsurprising then that these are the four viruses for which T-antigen antibody responses appear to correlate most strongly, as described above. As our earlier work using VP-1 antibody levels did not demonstrate any obvious signs of a reduction in control of these skin-associated viruses, investigations into whether the use of T-antigen antibodies is a more sensitive method to assess this are warranted.

Overall, this work broadens our current knowledge of the seroprevalence and seroreactivity of VP-1 and T-antigen antibody responses in a large group of MCC patients and provides comparison with responses from both relevant patient and healthy donor cohorts. It provides confirmatory evidence that the majority of MCC patients exhibit features suggesting a loss of

MCV viral control that is not explained solely by increasing age or exposure to UV light exposure. It appears that the increased risk of MCC in CLL patients may have in part due to a lower prevalence and level of MCV-specific antibodies, a mechanism not shared with MCC patients. Lastly, results from T-antigen antibody responses may suggest that in MCC patients multiple and predominantly skin-associated polyomaviruses may be reactivated at the same time indicating that a generalised loss of cutaneous anti-viral immune control may be prevalent in this group.

Chapter 4

A comparative analysis of immunological markers in peripheral blood of Merkel cell cancer (MCC) and non-melanoma skin cancer (NMSC) patients

4.1 Introduction

Merkel cell cancer (MCC), an aggressive cutaneous cancer of neuroendocrine origin, is extremely rare with an age-standardised incidence of 3.5 cases/million in a European population [26]. Conversely non-melanoma skin cancers (NMSCs), primarily basal cell (BCC) and squamous cell cancers (SCC), are extremely common, making up approximately 20% of all new cancer diagnoses. NMSCs and MCC share a number of risk factors, both being strongly associated with UV light exposure [30, 363] and a marked increased incidence in patients over the age of 50 [8, 364] . There also appears to be an important role of the immune system in the development of MCC and NMSC given the increased incidence of both cancer types in immunosuppressed patients with, for example, haematological malignancies [37, 365]or solid organ transplants [33, 366]. However, the mechanisms underlying this remain unclear.

An important difference between MCC and NMSC stems from the recent discovery of Merkel cell polyomavirus (MCV) DNA clonally integrated within the genome of the vast majority of MCC tumours [42]. MCV has subsequently been identified as a common pathogen, thought to infect the skin asymptomatically and persist latently in the majority of the healthy adult population [75]. It is hypothesised that in immunosuppressive states, normal immunosurveillance is disturbed, allowing viral reactivation and an increased likelihood of viral DNA integration into the host cell genome and the consequent development of cancer.

Despite intensive investigation, a strong link between NMSCs and any viral aetiology, including MCV, has not been found [142, 367].

While the above model of MCC development is attractive, the vast majority of MCC patients have no history of immunosuppression [7]. The widely held belief is that these patients are instead affected by changes mediated by the well-recognised phenomenon of immunosenescence [237]. This age related decline in the functioning of the adaptive immune system involves a progressive impairment in response to new antigens through reduced naïve T and B lymphocyte output from the thymus and bone marrow respectively [238, 239]. Also in elderly populations chronic infection with viruses such as cytomegalovirus and Epstein-Barr virus is associated with the inflation of terminally differentiated CD8+ T cells, specific for a small range of viral targets [243, 246, 368]. These cells, termed T effector memory CD45RA+ (TEMRA) cells, are themselves dysfunctional and potentially decrease the ability to make other functional memory responses [369, 370]. These changes in adaptive immunity may negatively affect the ability to recognise tumour-associated antigens and thus eliminate or control tumour development [371]. The potential importance of functional T cell immunity has been demonstrated in MCC through the finding that patients with MCC tumours with significant CD8 T cell infiltration have a significantly improved prognosis compared to those with no or little infiltration [317]. Alongside this, MCC patients with higher absolute lymphocyte counts have an improved overall survival compared with those with low counts, independent of factors such as immunosuppression and stage of disease [41].

Older patients are also at an increased risk of developing autoimmune diseases and also underlying low grade inflammation, the latter thought to be mediated through the relative preservation of the innate immune system. Collectively these can impact on cell-mediated responses and additionally create an environment capable of cell damage and potential

oncogenic transformation through the release of inflammatory mediators [254, 256]. Increased numbers of both circulating and tumour associated neutrophils, inflammatory effectors that may play a role in this context, correlate with a poor prognosis in a number of cancers [372, 373]. In MCC, a single study showed increased tumour associated neutrophils associated with a trend towards poorer prognosis [374], but little is known regarding the importance of circulating neutrophil levels.

This investigation aimed to formally characterise the immune markers present in peripheral blood samples from patients with MCC and NMSC but no history of immunosuppressive conditions in order to identify any deficits. In particular, given the similar risk factors and demographic profiles of patients affected by both cancers, we were interested to look at features associated with immunosenescence and whether there were differences that would favour the development of a virally-associated cancer in individuals with MCC compared to those with non-virally associated NMSC.

4.2 Results

4.2.1 Patient Characteristics

To investigate the immunophenotype peripheral blood samples from patients with a history of MCC or NMSC were recruited from a single UK tertiary referral centre. The detailed patient demographics of 21 patients with active or past history of MCC are shown in Table 4.1. No patients with a history of overt immunosuppression, as defined by HIV infection, haematological malignancy or history of organ transplantation were included. A patient with locally advanced breast cancer treated with endocrine therapy only and a patient with rheumatoid arthritis treated with sulphasalazine and never requiring long term steroids were included. Within this group, 4 patients had a past history of multiple NMSCs. The mean age of the cohort was 75 years (range 53-88), 67% were female and 86% were MCV seropositive. At the time of blood draw 6 patients had active disease while in patients who were disease free, remission lengths ranged from 2-40 months.

As a comparator group, 25 NMSC patients with active or past history of basal cell cancer (BCC) or squamous cell cancer (SCC) but not MCC or overt immunosuppression were recruited (see Table 4.2). The mean age of the group was 73 (range 48-87) with 40% female and 13 patients had active disease at the time of blood draw. From 23 evaluable patients 52% were found to be MCV seropositive.

	Sex	Age	Co-morbidity	Stage at diagnosis	Primary resected	Adjuvant RT	Recurrence	Further treatment	MCC clinical state at time of research bloods	MCV serostatus	CMV serostatus
P109	F	85	Nil	2	Yes		Reg	RT	Remission (3m)	Pos	Pos
P104	F	67	Multiple Sclerosis	1b	Yes	Yes	Nil	N/A	Remission (12m)	Pos	Pos
P92	F	70	Nil	2b	Yes		Nil	N/A	Remission (32m)	Pos	Pos
P105	F	88	Breast cancer	2	Yes		Reg	RT	Remission (4m)	Pos	Pos
P107	M	77	Nil	3	No		Met	Chemo	Metastatic	Neg	Pos
P103	M	69	Nil	1b	Yes	Yes	Nil	N/A	Remission (11m)	Pos	Neg
P95	F	85	Nil	1b	Yes		Nil	N/A	Remission (40m)	Pos	Pos
P85	F	73	Nil	1b	Yes		Nil	N/A	Remission (33m)	Pos	Pos
P113	M	86	Cardiovascular disease	2	Yes		Met	RT	Metastatic	Pos	Pos
P87	F	74	Nil	1b	Yes	Yes	Nil	N/A	Remission (26m)	Pos	Pos
P125	F	64	Multiple Sclerosis, Multiple NMSC	1b	Yes		Nil	N/A	Remission (2m)	Pos	Neg
P130	F	74	Multiple NMSC, COPD	3	Yes		Nil	N/A	Active	Pos	Pos
P124	M	83	Bronchiectasis	2	No	Yes*	Met	Surg	Active	Pos	Neg
P132	F	81	Rheumatoid Arthritis	2	Yes		Met	Surg	Metastatic	Pos	Neg
P84	M	80	Colon cancer 2010	3	Yes**		Nil	N/A	Remission (40m)	Pos	Pos
P102	F	53	Nil	2b	Yes		Nil	N/A	Remission (23m)	Pos	Neg
P106	F	78	Nil	2b	Yes		Nil	N/A	Remission (20m)	Pos	Neg
P139	M	69	Nil	1b	Yes		Nil	N/A	Remission (5m)	Pos	Pos
P140	F	79	Cardiovascular disease, Diabetes, NMSC	1b	Yes		Nil	N/A	Remission (11m)	Pos	Pos
P141	M	57	Depression	2b	Yes		Reg	Surg	Active	Neg	Neg
P114	F	78	Hypertension, NMSC	1b	Yes		Nil	N/A	Remission (6m)	Neg	Neg

Table 4.1. Merkel cell cancer (MCC) patient cohort demographics.

F=Female, M=male,, COPD=chronic obstructive pulmonary disease, NMSC=non melanoma skin cancer, *Primary RT given instead of surgery, ** No primary tumour identified, Reg=Regional, Met=Metastatic, RT=Radiotherapy, Chemo=Chemotherapy, Surg=Surgery, N/A=Not applicable, Pos=Positive, Neg=Negative

	Diagnosis	Sex	Age	Co-morbidity	Primary resected	Clinical state at time of research bloods	MCV VP-1 Serostatus	CMV serostatus
P122	BCC	F	68	Nil	Yes	Remission	Neg	Pos
P118	BCC	M	86	Nil	No	Active	Neg	Neg
P120	BCC	F	49	multiple NMSC	Yes	Remission	Pos	Neg
P123	BCC	M	83	Nil	No	Active	Neg	Pos
P128	BCC	F	71	Nil	Yes	Remission	Not Done	Not Done
P129	BCC	M	48	Nil	Yes	Remission	Not Done	Not Done
P119	SCC	M	87	Nil	Yes	Remission	Pos	Pos
P121	SCC	M	83	Cardiovascular disease, Asthma	Yes	Remission	Neg	Pos
P127	SCC	M	65	Nil	Yes	Remission	Pos	Neg
P131	SCC	F	64	NMSC	Yes	Remission	Neg	Pos
P133	BCC	F	84	Peripheral vascular disease	Yes	Remission	Pos	Neg
P134	SCC/BCC	M	80	Cardiovascular Disease	Yes*	Remission	Neg	Pos
P136	BCC	M	80	Cardiovascular Disease	No	Active	Pos	Pos
P135	BCC	M	67	Nil	No	Active	Neg	Pos
P142	BCC	M	72	Hypertension	Yes	Remission	Pos	Neg
P143	BCC	F	55	Nil	No	Active	Pos	Pos
P144	BCC	M	82	Diabetes	No	Active	Neg	Neg
P145	BCC	F	68	Osteoarthritis, High Cholesterol	No	Active	Pos	Pos
P146	BCC	F	71	Hypertension	No	Active	Neg	Pos
P147	BCC	F	79	Hypertension	No	Active	Pos	Pos
P148	BCC	M	69	Nil	No	Active	Neg	Pos
P154	BCC	M	78	Osteoarthritis	No	Active	Pos	Neg
P155	BCC	F	80	Hypertension	No	Active	Pos	Neg
P156	BCC	M	80	Nil	No	Active	Pos	Neg
P138	SCC	M	87	Nil	Yes	Remission	Neg	Neg

Table 4.2. Non melanoma skin cancer (NMSC) patient cohort demographics.

F=Female, M=Male, NMSC= non-melanoma skin cancer, BCC= Basal cell cancer, SCC= Squamous cell cancer,

*Adjuvant Radiotherapy, Pos=Positive, Neg=negative.

4.2.2 Absolute Lymphocyte counts

Initially to assess general adaptive immune status of MCC patients, absolute lymphocyte subset cell counts were determined from whole blood taken from 21 patients and compared to blood collected from 22 NMSC patients (Figure 4.1A). The analysis showed that MCC patients had a non-significant reduction in median absolute CD45+ lymphocyte count (NMSC:1.53 $x10^9$/L, MCC:1.18 $x10^9$/L , p=0.07). There was a reduction in all lymphocyte subsets with reduced median counts of CD3+ T cells (NMSC:1.09 $x10^9$/L, MCC:0.83 $x10^9$/L, p=0.16) and reduced median counts of NK (CD56+, CD16+) cells (NMSC:0.27 $x10^9$/L, MCC:0.19 $x10^9$/L, p=0.13). However, a significant reduction was seen within the CD19+ B cell fraction (NMSC:0.21 $x10^9$/L, MCC:0.08 $x10^9$/L, p=0.0008) . A similar trend occurred when considering frequencies of the different cell types as a proportion of total lymphocytes, with the only significant difference between NMSC and MCC patients being in B cell subset frequencies (NMSC:11.1% v MCC:7.1% , p=0.006) (Figure 4.1B).Within the CD3+ T cell population, there was no significant difference between CD4 and CD8 populations between the two cohorts both in absolute (Figure 4.1C) or percentage (Figure 4.1D) terms. An increasing CD4/8 ratio has been described as a marker of an aging immune system [237], but there was no significant difference between the values from NMSC and MCC patients (Figure 4.1E). Overall, MCC patients NK and T cell subsets showed similar frequencies and numbers while B cell numbers and frequencies were significantly reduced.

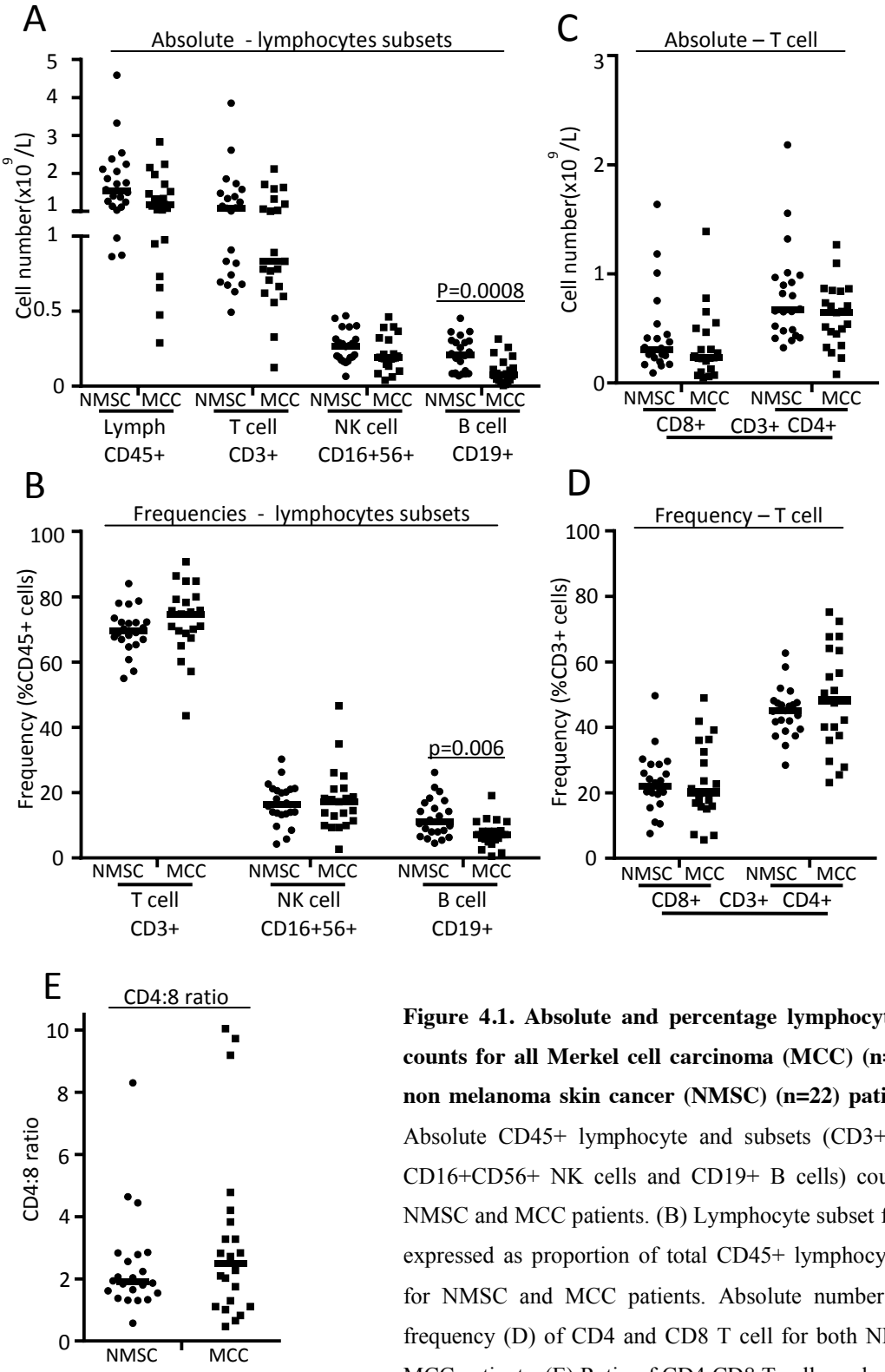


Figure 4.1. Absolute and percentage lymphocyte subset counts for all Merkel cell carcinoma (MCC) (n=21) and non melanoma skin cancer (NMSC) (n=22) patients. (A) Absolute CD45+ lymphocyte and subsets (CD3+ T cells, CD16+CD56+ NK cells and CD19+ B cells) counts from NMSC and MCC patients. (B) Lymphocyte subset frequency expressed as proportion of total CD45+ lymphocyte counts for NMSC and MCC patients. Absolute number (C) and frequency (D) of CD4 and CD8 T cell for both NMSC and MCC patients. (E) Ratio of CD4:CD8 T cell number for both cohorts. Horizontal lines represent median values. Statistical analysis used Mann-Whitney U Test. Where no p value is given, result was not significant.

4.2.3 B cell differentiation and antibody production

Given the unexpected finding of reduced total CD19+ B cell numbers in the MCC cohort, we further investigated this by examining the differentiation phenotypes of these B cells to assess whether this was a subset specific or generalised phenomenon. B cell subsets were defined using the markers IgD and CD27 which identify naïve B cells (N, IgD+ CD27-), non-switched memory cells (NSM, IgD+ CD27+) and switched memory B cells (SM, IgD-CD27+). Representative plots from a NMSC and MCC patient are presented in Figure 4.2A. Comparing the frequency of B cell subsets in the NMSC to MCC cohorts showed the MCC patients had reduced proportions of naïve B cells, equivalent frequencies of non-switched memory and increased switched memory populations (p=ns, Figure 4.2B). When considering absolute B cell numbers, reductions were seen in all MCC patients B cell populations but particularly notable in the naïve subset (p=0.005, Figure 4.2C).

A reduction in B cell number may be associated with changes in humoral immunity, in particular, antibody production. We assessed patient serum immunoglobulin A, G and M concentrations in serum samples from 17 of the NMSC patients and 16 of the MCC patients. There was no significant difference in mean serum immunoglobulin concentrations between the cohorts (Figure 4.3A, Median IgM 0.68g/L NMSC v 0.62g/L MCC , Median IgG 8.90g/L NMSC v 9.85g/L MCC , Median IgA 2.81g/L NMSC v 1.91 g/L MCC).

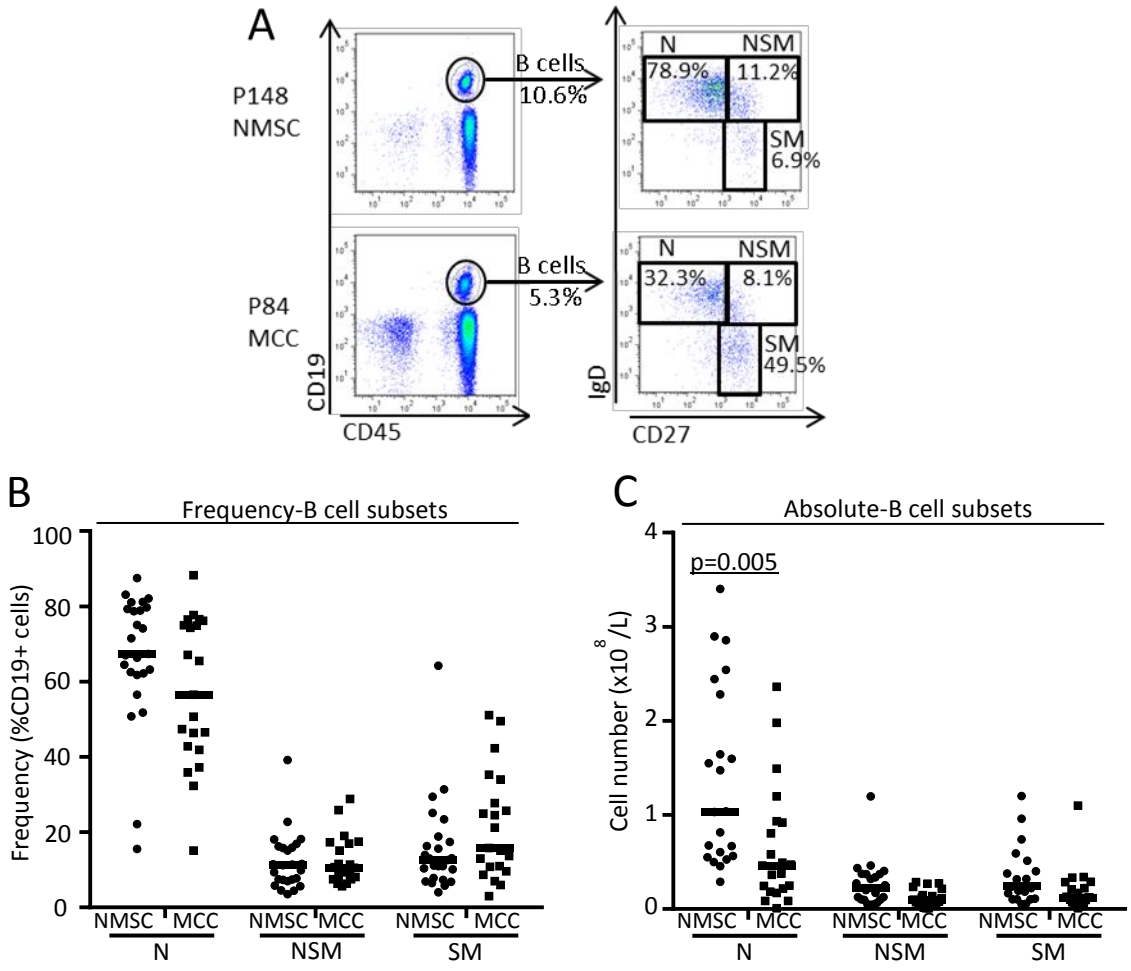


Figure 4.2. B cell peripheral blood phenotype of NMSC (n=25) and MCC (n=21) patients. (A) Flow cytometry was used to determine frequencies of B cell phenotype subsets. The strategy and plots from a representative NMSC patient (P148) and MCC patient (P84) are shown. CD45+CD19+ B cells were divided into naïve (N;IgD+CD27-), non-switched memory (NSM; IgD+CD27+) and switched memory (SM; IgD-CD27+). (B) Frequencies of B cell subsets as a percentage of total CD19+ B cells for NMSC and MCC patients. (C) Absolute numbers of B cell subset cells were determined using patient-specific absolute B cell counts from Figure 4.1.

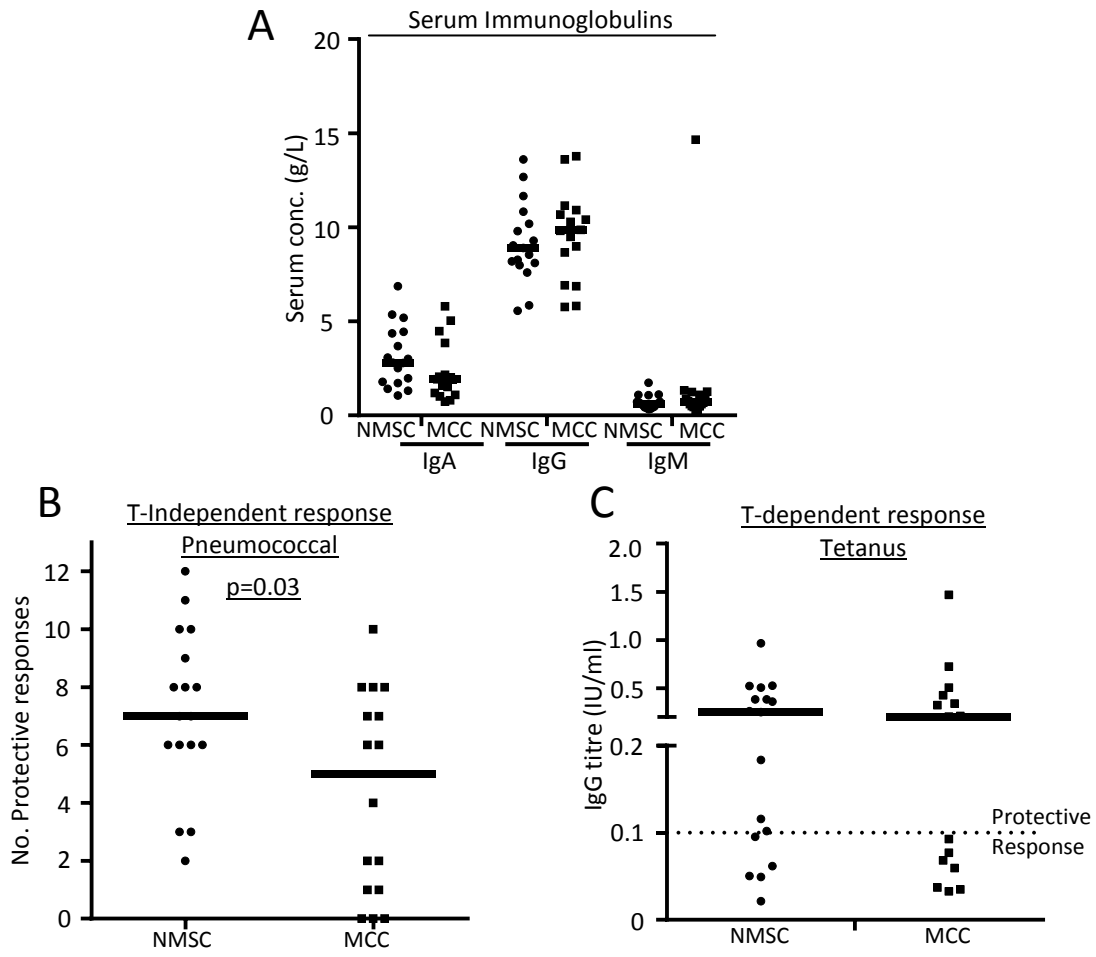


Figure 4.3. Assessment of humoral immune response in NMSC (n=25) and MCC (n=21) patients. (A) Serum immunoglobulin (IgG, IgA, IgM) concentrations from NMSC (n=17) and MCC (n=16) patients. (B) Numbers of responses considered protective (serum concentration >0.35μg/ml, for all 12 antigens tested) against T-independent pneumococcal antigens in NMSC (n=17) and MCC (n=16) patients. Pneumococcal vaccination rates were 76% in NMSC group and 75% in MCC group. (C) Number of same NMSC and MCC patients with protective T-dependent antibody response against tetanus toxoid antigen (defined as serum concentration >0.1μg/ml). Horizontal lines represent median values. Statistical analysis by Mann-Whitney U test.

As total serum antibody levels were unchanged between groups, we next assessed whether there were differences in specific antibody responses to vaccine antigens which were either independent or dependent on T cell help. To quantify responses against T-independent (TI) antigens, anti-polysaccharide antibodies against 12 pneumococcal strains were measured. NMSC patients had a mean number of 7/12 protective responses, defined as serum antibody level >0.35μg/ml for each antigen, compared with 5/12 in MCC patients (p=0.03) (Figure 4.3B). Both groups had nearly identical pneumococcal vaccination rates, 76% NMSC v 75% MCC, with a median time since vaccination of 10 years in both groups. Specific antibody responses were also measured for a number of T-dependent antigens including tetanus and diphtheria toxoids as well as conjugated haemophilius influenzae B polysaccharide. There was no significant difference in numbers of patients with protective responses with 8/15 MCC patients compared with 12/17 NMSC patients having serum level >0.1 μg/ml, p=0.84 (Figure 4.3C). Other TD responses (haemophilius influenzae B and diptheria, data not shown) also showed no difference between cohorts.

4.2.4 CD8 T cell differentiation phenotype

Memory and effector CD8 T cells have an important anti-viral role through killing infected cells and suppressing virus replication [200]. However, chronic virus infections particularly in the elderly, can lead to the global expression of a highly differentiated TEMRA phenotype on these cells which may be associated with dysfunction [368, 370, 375]. As MCV is thought to be maintained as a chronic infection, we next sought to compare the CD8 T cell memory differentiation phenotype of the two patient groups to determine whether MCC or MCV infection is associated with changes in these populations. Using an established phenotyping

strategy (Figure 4.4A) [269], we analysed for cell surface marker expression of CD27 and CD45RA on CD8 T cells from all donors and found variable frequencies of different memory cell populations (Figure 4.4B).

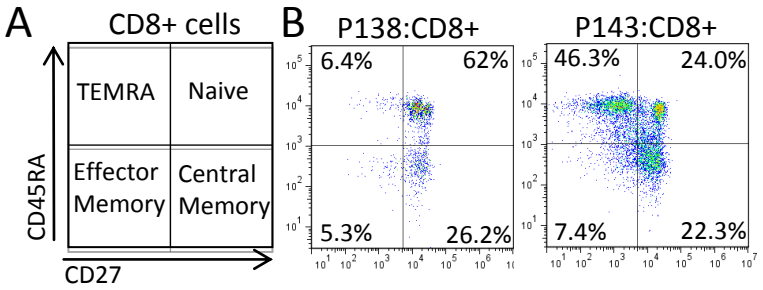


Figure 4.4. Definition and examples of CD8+ T cell phenotype subsets by flow-cytometric analysis. (A) Flow cytometry strategy to define CD8+ T cell phenotype subsets, Naïve-like (N; CD27+CD45RA+), Central memory (CM; CD27+CD45RA-), Effector memory (EM; CD27-CD45RA-), T Effector memory CD45RA (TEMRA;CD27-CD45RA+). Representative flow plots from patients with differing subset frequency patterns; with either small (P138) or large (P143) TEMRA populations.

Examining whether MCV could affect the frequency of the TEMRA population showed no evidence for this comparing MCV seropositive and seronegative patients from both disease groups (Figure 4.5A). CMV infection is known to drive the excessive accumulation of TEMRA cells and influence the overall CD8 T cell repertoire [368]. To remove this potentially confounding factor from our analysis we analysed NMSC and MCC patients for CMV serostatus and found similar rates of seropositivity between the groups (56% NMSC and 62% MCC patients). As expected CD8 T cells from CMV positive patients more frequently showed a highly differentiated CD8 phenotype in terms of frequency and absolute cell numbers [375] (Figure 4.5B). When considering CMV seronegative donors, both groups

shared a pattern of increased naïve and central memory T cell frequency and reduced effector memory and TEMRA populations. However here there were differences between the two groups of CMV negative patients, with CD8 populations from MCC patients having a significantly increased median frequency of naïve T cells (21.9% NMSC v 42.3% MCC, p=0.01) and reductions in both effector memory (7.7% NMSC v 4.4% MCC, p=0.003) and TEMRA (11.6% NMSC v 3.0% MCC, p=0.02) population compared with NMSC patients. When considering absolute cell numbers (Figure 4.5C), the increased naïve cell frequency seen in MCC patients becomes insignificant. However, the significant reductions in effector memory (median 0.02 v 0.007 $x10^9$cells/L, p=0.01) and TEMRA populations are maintained (median 0.025 v 0.0065$x10^9$cells/L, p=0.046) suggesting that in the absence of CMV co-infection, MCC is associated with reduced numbers of these subsets.

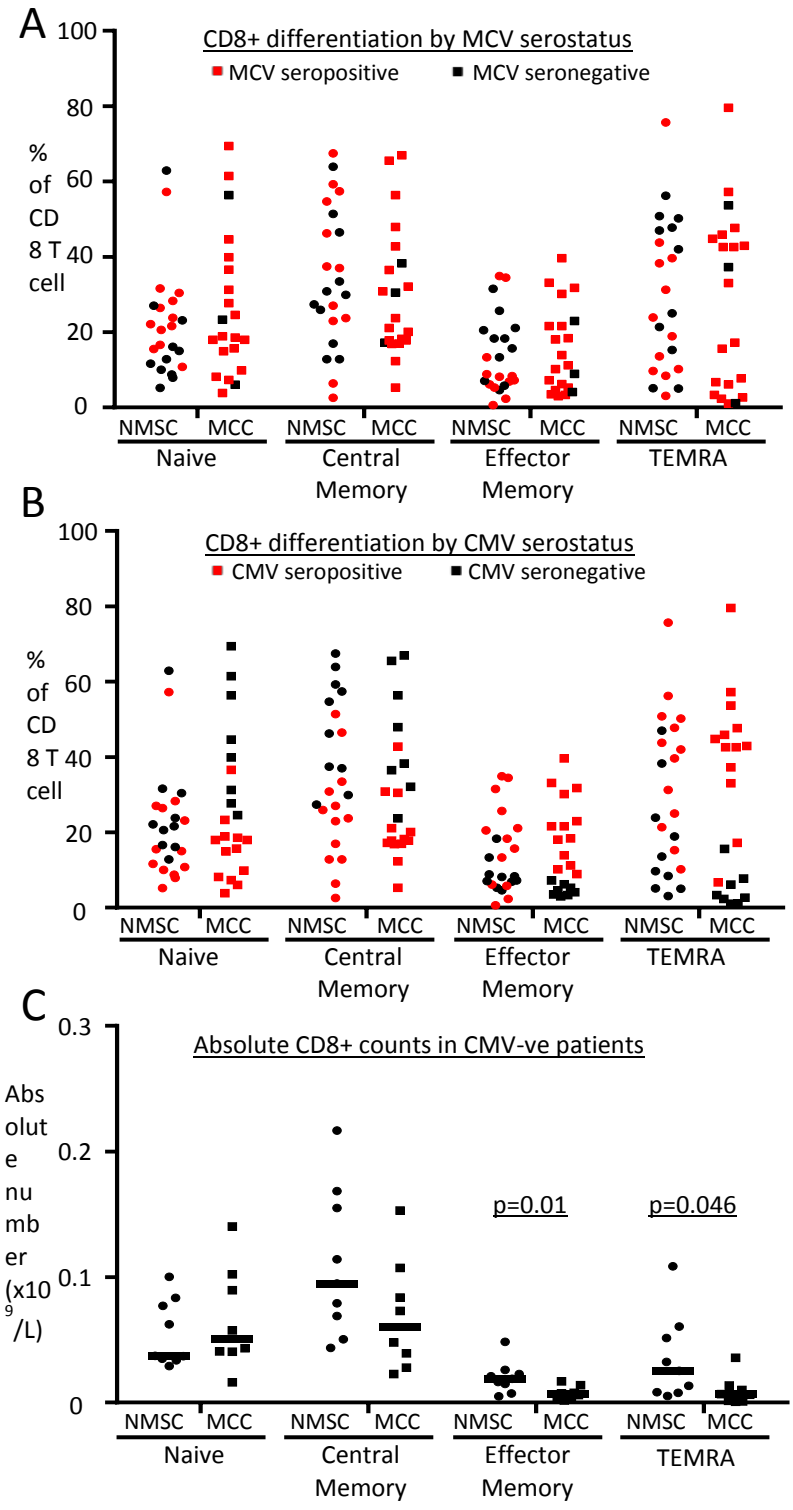


Figure 4.5. Frequencies of each CD8 phenotype subset expressed as proportion of total CD8+ T cells for NMSC (n=23) and MCC (n=21) patients. Of the NMSC patients 2 were excluded as serological investigation not possible. MCV seropositive and CMV seropositive patients are show in red in (B) and (C) respectively. (D) Absolute CD8+ T cell subset numbers in CMV seronegative NMSC (n=9) and MCC (n=8) patients. Horizontal lines represent median values. Statistical analysis by Mann-Whitney U test.

4.2.5 Lymphocyte proliferation following mitogenic stimulation

We next turned to study the functional capacity of the lymphocytes from these patients by assessing their ability to proliferate in response to the mitogen phytohaemagglutanin. Comparison of lymphocytes from NMSC patients and MCC patients revealed a reduction in lymphocyte proliferation from MCC patients (573 (NMSC) v 299 (MCC) fold increase over non-stimulated cells, data not shown). To determine if reduced proliferation was associated with disease state, patients were stratified depending on presence or absence of active disease at time of blood draw. Comparing fold proliferation over background, NMSC and MCC patients in remission both had similar responses to stimulation (326 (NMSC) v 303 (MCC) fold increase). However, when considering patients with active disease, there was a marked difference between the two groups: NMSC patients showed high proliferation over background while MCC patients had very low proliferative response to stimulation (734 fold (NMSC) v 18 fold (MCC) increase, p=0.0002) (Figure 4.6A).

A potential cause for reduced proliferation of lymphocytes in MCC patients with active disease may be an increase in circulating regulatory T cells (T_{regs}) and neutrophils which have been reported to suppress T cell function [376, 377]. We assessed then numbers of T_{regs}, defined as CD4+CD127lo/CD25high) and neutrophils in all MCC patients comparing those with active disease to those in remission. Although there was no difference in T_{regs} number between the MCC patients with active or past disease, there was a trend of increasing absolute neutrophil count seen in MCC patients with active disease ($4.25x10^9$cells/L NMSC v 5.95 $x10^9$cells/L MCC , p=0.08) (Figure 4.6B and 4.6C). These results suggested that T_{regs} or neutrophil numbers were unlikely to account for the decrease in proliferation seen in lymphocytes taken from MCC patients with active disease.

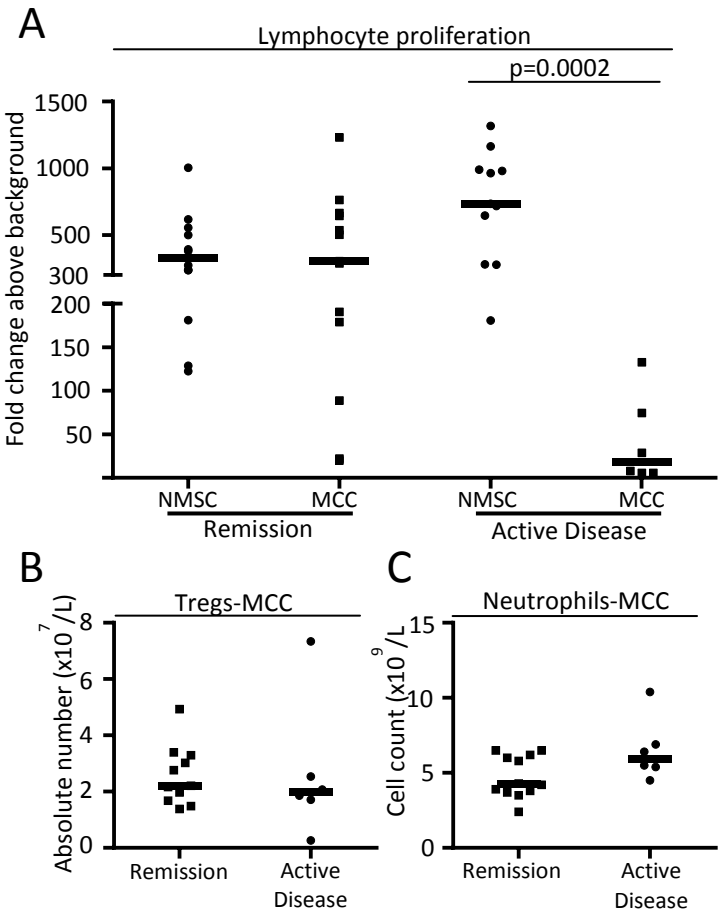


Figure 4.6. Lymphocyte proliferation after 72 hours PHA stimulation of whole blood as determined by ^{3}H Thymidine uptake. (A) Whole blood from NMSC (n=23) and MCC (n=19) patients with either active disease or disease remission were analysed. Results are expressed as fold change in scintillation count between PHA-stimulation and background (media only). Absolute number of regulatory T cells (B) and absolute neutrophil count (C) for MCC patients in remission or with active disease. Regulatory T cell counts were determined by flow cytometric assessment of frequency of CD4+CD127lo/CD25high cells and converted to absolute numbers using patient specific counts as shown in Figure 1. Horizontal lines represent median values. Statistical analysis by Mann-Whitney U test.

4.3 Discussion

This study was prompted by the observation that although immunosuppression is a strong risk factor for the development of MCC, the majority of patients, who are elderly, exhibit no obvious underlying immunodeficiency. The rarity of this tumour and the potential confounding from often multiple co-morbidities makes identifying distinct aberrations with the immunity of MCC patients extremely challenging. Analysis of peripheral lymphocyte subsets showed most were similar between the cohorts although MCC patients showed decreases in the B cell compartment mostly from a decrease in the naïve subset and also a decreased frequency of T-independent antibody responses. Lymphocytes from both patients groups in remission showed similar proliferative potential, however compared to this group and NMSC patients with active disease, lymphocytes from MCC patients proliferated poorly. There were a disproportionate number of females in the MCC group, which was difficult to avoid given the known increased incidence of MCC in females, which was not present in the NMSC cohort [27, 364]. However, as female gender is associated with a reduced incidence of an immunosenescent phenotype [378], this makes the changes that we have observed in the MCC cohort more striking.

Although, absolute lymphocyte count was not significantly different between the cohorts, B cell number was reduced in both percentage and absolute terms in the MCC group, with median values below the 5^{th} percentile of the reference adult population [379]. A reduction in B cells is a recognised feature of immunosenescence likely due to preferential differentiation of haematopoietic cells to a myeloid rather than lymphoid fate. The most reduced B cell phenotype in the MCC cohort was the naïve IgD+CD27- phenotype, with relative sparing on the CD27+ memory cells, which mirrors results from other studies investigating immunity in the elderly [380, 381]. The prognostic impact of absolute lymphocyte count has been

investigated in a retrospective study finding that patients with lower absolute lymphocyte count had a worse overall survival independently of factors such as the presence of immunosuppression [41]. In this previous study, it was not possible to elucidate whether reduction in lymphocyte count was global or limited to a single subset. The present study suggests that in non-immunosuppressed patients, the reduction may preferentially involve the B cell subset.

The reduction of circulating B cells seen within the MCC group did not translate into a reduction in serum IgG, IgA or IgM concentration when compared with the NMSC patients. This echoes the findings of a previous study [251], examining serum immunoglobulin levels of elderly donors. We were also able to test frequency and titre of specific antibody responses against a variety of T-independent and T-dependent bacterial antigens. The ability to mount protective T-dependent antibody responses post-vaccination are not thought to decline with age [382], however a significant proportion of elderly people do not make protective responses to the T-independent pneumococcal vaccine [383]. All measured T-dependent antibody responses (tetanus, diphtheria, haemophilus influenza) were similar between the two cohorts while T-independent responses against pneumococcal antigens showed that NMSC patients had more protective responses than MCC patients despite almost identical vaccination rates. The reason for this difference is unclear but may be related to the decreased numbers and frequency of peripheral naive B cells seen in MCC patients.

Immunosenescent changes in the T lymphocyte population have been mainly attributed to the CD8 T subset [384]. This is thought to be through the expansion of highly differentiated memory T cell populations specific for chronic viral infections. We wanted to investigate the role that MCV may play in this process, however in the MCC cohort, it was difficult to compare seropositive to seronegative groups as there were few seronegative patients. By

contrast, in the NMSC group, MCV seropositivity (52%) did not appear to correlate with differentiated memory T cell expansion. This is perhaps unsurprising as the size of ex-vivo T cell responses for polyomaviruses, such as JCV, BKV and MCV has consistently been found to be difficult to detect [289, 293, 385]. However, CMV seropositivity, which was similar between both cohorts, was associated with expansions of CD8 TEMRA cell population expansion as has been described previously [246]. Although, CMV co-infection and memory inflation is not a pre-requisite for the development of MCC, it potentially may influence MCV control or MCC development as CMV infection is accompanied by a reduction of TCR diversity which negatively impacts on other anti-viral immune responses [386, 387]. It is also interesting to note, when removing the dominant confounding effect of CMV seropositivity on lymphocyte subsets, by looking exclusively at CMV negative patients, the MCC cohort had lower numbers of effector memory cells than their NMSC peers. Potentially then there may be less T cell mediated immune surveillance in this population leading to poorer control of viral driven disease. There is some evidence for the loss of viral control being important for MCC pathogenesis with one study showing increasing serum MCV antibody titres, a known surrogate for rising viral load in the skin, can be seen prospectively in patients later diagnosed with MCC, in some cases years in advance [137, 337]. After development of MCC, there is evidence that patients whose tumours have increased CD8+ T cell infiltration within them have improved outcomes [317]. However, the differentiation phenotype of these infiltrative cells has not been well characterised and whether they belong to an effector memory subset would be interesting question to answer.

Studying the functional capacity of lymphocytes from the different groups in terms of proliferation it was clear that in remission, both MCC and NMSC patients have similar proliferative capacity. This suggests that it is unlikely that MCC patients have an underlying

abnormality preventing proliferative lymphocyte responses upon challenge. However, active MCC is associated with a marked reduction in proliferative capacity. Cancer-related immunosuppression is widely reported and may be due to the effect of one or a combination of influences including increased concentrations of suppressive cytokines such as IL-10, T cell exhaustion or accumulation of cell types with suppressive function such as T_{regs} or myeloid derived suppressor cells (MDSCs) [371]. Investigating T_{regs} counts, we found that there was no difference seen between the patient groups. Neutrophils, especially from the elderly, have been shown to produce more anti-inflammatory cytokines than those from young patients [388] and tumour associated neutrophils are capable of inhibiting anti-tumour immune response [389]. Investigating neutrophil counts showed a borderline significant increase between MCC patients with active disease compared to those without. Although this increased neutrophil count may represent increased inflammatory activity related to the tumour these may be inhibiting lymphocyte proliferative function in active disease cases. It has recently been shown that a raised neutrophil:lymphocyte ratio is a poor prognostic marker in MCC associated with worse survival [390]. The impact of circulating MDSCs or expression of T cell exhaustion markers was not examined in this study, however it has been shown that circulating MCV-specific T cells express high levels of exhaustion markers such as PD-1 in patients with active disease [305]. Conceivably targeting this expression may have clinical usefulness in future therapies.

Recent advances in measuring human cutaneous immune responses have shown that the function of skin-infiltrating T cells can be quantified and that this immunity wanes with ageing [269]. Furthermore in the elderly population at risk of MCC and NMSC tumours, localised cutaneous mechanisms of immune suppression such as increases in T_{regs} or expression of exhaustion markers have been identified that are also likely to contribute to

cancer development and progression [323, 391, 392]. The question of whether the normal skin of patients who have MCC or NMSC display any of these changes or whether they only occur following tumour onset is as yet unanswered but should be the focus of on-going research.

The observations made in this study provide new information that suggests there are differences in immune phenotype between MCC and NMSC patients, although this cannot be attributable to a single mechanism of immune dysfunction. The subtle differences involving T cell, B cell and humoral immunity in MCC patients suggest an on-going multi-factorial process that consistent with immunosenescence. The reasons that MCC patients appear more greatly affected when compared with NMSC patients with exposure to broadly similar risk factors is not known and warrants further investigation. It does, however, fit with the currently held hypothesis that patients who develop MCCs may be less able to control viral infections, such as MCV, that initiates tumourigenesis. MCC patients may also be less able to control other types of infections (e.g. pneumococcal), particularly at times of active disease, which may have clinical importance when treating these patients with agents such as chemotherapy. Lastly, given the breadth of changes seen in MCC patients, the use of immunomodulatory therapies for MCC may need to be multi-targeted in order to exert maximal benefit.

Chapter 5

T cell Immunity Against MCV Large T and Small T-antigens

5.1 Introduction

The cellular immune response is integral to establishing control and limiting the pathogenic consequences of viral infections. Polyomaviruses usually cause asymptomatic chronic infections in their hosts however disruptions to the virus host balance, such as immunosuppression for bone marrow or organ transplant, can result in BK virus-associated nephropathy and JC virus-associated progressive multifocal leukoencephalopathy [149, 150]. This suggests T cell immunity has an important role in the control of polyomavirus infections and prevention of disease in humans. Recently, another polyomavirus, Merkel cell polyomavirus, has been strongly associated with Merkel cell carcinoma, which can be seen in apparently immunocompetent individuals but exaggerated disease may be seen in patients whose T cell responses are suppressed, also implying a role for these effectors in control of disease caused by this virus [42, 337].

The nature of T cell immunity directed to MCV in controlling MCV viral reactivation and the role it plays in controlling development of MCC is poorly understood. Data presented in the previous chapter has shown that the reduced T cell frequency by itself is unlikely to explain tumour occurrence in MCC patients compared to a relevant control group of NMSC patients. However, it is not clear whether any differences exist in the frequency or function of MCV-specific T cells between these groups. Similarly existing knowledge on the quantity of circulating functional MCV-specific T cells and how this varies between healthy people and MCC patients is limited. Of the studies undertaken, T cell responses to the truncated T-antigen fragments retained in MCC tumours showed a trend toward increased Common T

(CT, the first 78 amino acids shared by both LT and sT proteins), LT- and sT-specific T cell frequencies in MCC patients compared to controls. However there was no assessment of responses to the terminal portion of LT which is present in intact MCV infections and no stratification of healthy donors based on MCV seropositivity. [385].

In order to better understand how T cell control of MCV is mediated, it is necessary to identify specific T cell epitopes presented by MCV infected cells that are capable of stimulating immune responses. So far the number of currently identified MCV LT and sT T cell epitopes is limited, however an intensive search using MHC multimers loaded with potential epitope-peptides identified nine LT and three sT MHC Class I epitopes, again focusing on the truncated LT-antigen [306]. On examining peripheral blood of MCC patients and healthy donors, only MCC patients displayed evidence of T cell responses against these T-antigen epitopes. Additionally, a number of MCV peptides capable of inducing functional responses from either CD8 and CD4 T cells have been mapped, however their counterpart MHC molecules have mostly not been identified [385]. The only formally identified epitope using this screening method is an HLA:A24 LT sequence (aa92-101), recognised by T cells isolated from tumour and blood of an MCC patient. Using MHC class I tetramer analysis, T cells recognising this epitope were enriched in tumours as compared to the peripheral blood. Also in two other HLA:A24 MCC patients, tetramer staining cells were present at a low frequency in the peripheral blood, however these did not appear functional, as defined by lack of production of IFNɣ after ex vivo stimulation with the peptide.

Alongside CD8 T cells, there is increasing evidence of the importance of CD4 T cells with regard to controlling both polyomavirus infection [298, 324] as well as polyomavirus mediated tumours [309]. There have been no formally MHC restricted CD4 epitopes identified to date, although a number of peptides from truncated LT and sT have been shown

to induce CD4 T cell responses in PBMCs from both MCC patients and healthy controls [385]. Similarly CD4 T cells specific for a CT (aa57-69) peptide isolated from the tumour of an MCC patient have been found to secrete the Th1 and Th2 cytokines, IFNɣ, IL-5 and IL-10 after ex vivo expansion and challenge with this peptide potentially raising a role for these effectors in MCC [385].

In terms of understanding the role of MCV-specific T cells in vivo, studies have been performed examining the ex vivo frequency and cell surface phenotype of CD8 T cells specific for the HLA:A24 presented peptide from LT [305]. Here the frequency of these MCV-specific T cells positively correlated with disease burden, and these T cells expressed high levels of checkpoint inhibitor receptors such as PD-1 and Tim-3. This MCV-specificity in MCC patients also expressed the skin homing receptor Cutaneous Lymphocyte Antigen (CLA) on 39% of tetramer positive cells (mean value, n=4), which was significantly more frequent than that seen on T cells specific for non-skin-tropic viruses EBV and CMV [321]. This finding may be expected given that MCV DNA is found commonly on the skin, which is thought to represent shed viral particles [125]. So far ex-vivo analyses of MCV-specific T cell responses have only been conducted on material from MCC patients and so the phenotype of such cells in MCV-seropositive healthy donors, who are apparently controlling their MCV infection, is largely unexplored.

The aims of this work were to firstly quantify MCV LT- and sT-specific T cells in the peripheral blood of MCC patients and compare these to two other groups namely patients with non-melanoma skin cancers and healthy volunteers. Secondly using blood from suitable donors and primary MCC tumours, establish MCV-specific T cell clones specific for LT and sT epitopes. These T cell clones were then used to map responsive LT and sT peptides, their restricting elements identified and the clones used to examine antigen processing and

presentation of these epitopes by antigen expressing cells. Lastly, I aimed to characterise the MCV-specific T cells ex vivo phenotype using tetramer based flow cytometric analyses.

5.2 Results:

5.2.1 Detection of MCV-specific T cell responses in different patient groups

In this first section of the study, experiments were conducted to identify MCV-specific T cell responses which would allow estimation of the size and epitope identity of LT- and sT-reactivities. Initially, it was planned that tumours which had been resected from patients would have the lymphocyte component stimulated with a panel of overlapping peptides which spanned the LT and sT sequences (Figure 5.1), to amplify MCV-specific T cells and these used to identify epitopes. However, of the tumours collected (n=3) diagnostic pathology analysis indicated that none of these expressed LT and were considered MCV negative (P. Taniere personal communication). Consistent with this, when lymphocytes were stimulated with the LT and sT peptides and assessed for IFNɣ production, no MCV-specific responses were detected in lines grown from these tumours (data not shown).

As a second source of MCV-specific T cells, PBMC samples were accessed from MCC patients and initially the size of the MCV LT- and sT-specific response estimated by IFNɣ ELISpot in response to stimulation with the overlapping T-antigen peptides. As the MCC patients were typically frail and PBMC recoveries were generally low, the overlapping peptides were grouped into pools of 30-40 peptides and these pools used to stimulate responses. Additionally a second comparator patient group was included; patients with non-melanoma-skin cancer (NMSC). These patients have a similar demographic profile, with similar risk factors and also included a more equal proportion of MCV seropositive and seronegative donors.

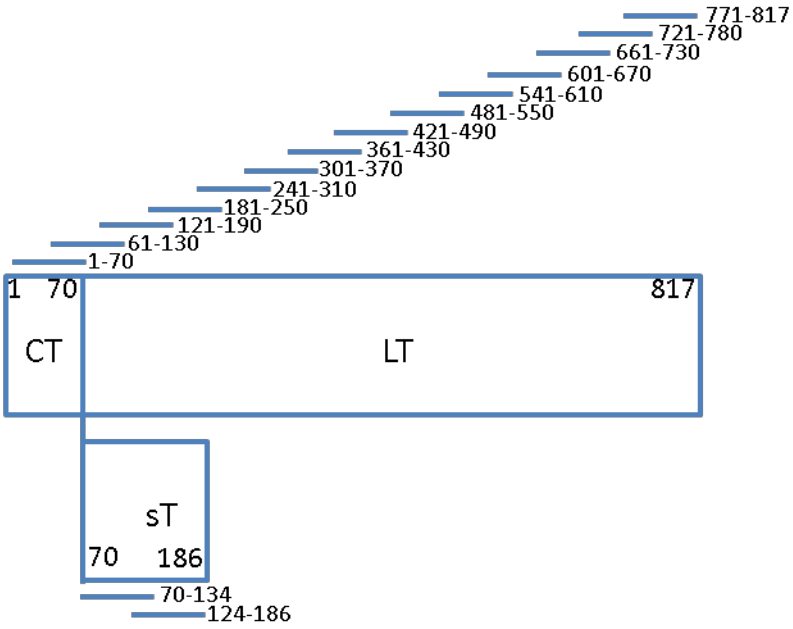


Figure 5.1. Schematic representation of overlapping peptide pools used in T cell recognition assays. Each pool was made up of 15mer peptides spanning Common T (CT), Large T (LT) and Small T (sT) regions of the MCV T-antigen protein.

PBMC were isolated from MCC and NMSC patients, who had been previously assessed for MCV serostatus (see Chapter 3), and these challenged with the overlapping panels of LT and sT peptides in IFNɣ ELISpot assays. As controls, in these assays the PBMC samples were assessed for their response to two positive controls: first a mitogenic stimulus phytohaemagglutinin (PHA) and second, a mixture 23 peptides corresponding to common MHC Class I CMV, EBV and influenza epitopes (CEF) which induces IFNɣ responses in 90% of Caucasian people. The negative control was the peptide solvent dimethyl sulfoxide (DMSO).

Using this protocol, ELISpots were performed on blood from 18 MCC patients and 17 NMSC patients. Of the MCC patients, 15 of 18 were MCV seropositive (83%) while 10 of 17 NMSC patients were MCV seropositive (59%). The overall results of the ELISpots expressed as spot

forming cells (SFC) per million PBMC are shown in Figure 5.2A-D, with MCC and NMSC patients split depending by MCV serostatus. Responses to PHA appeared to be similar in all four groups and there was no significant difference when analysed using the Kruskall-Wallis test. Lymphocytes from the majority of donors made responses to CEF peptides although a number of MCV seropositive donors in both MCC and NMSC groups showed stronger responses to these peptides compared to the MCV seronegative groups. No significant difference in SFC numbers was seen between the groups using Kruskall-Wallis test. The responses to these control antigens indicated that the lymphocytes within the PBMC preparations were functional.

When considering responses to MCV T-antigen peptide pools, these were of low frequency in all four groups with no donors having greater than 100 IFNɣ SFC per million PBMC. Of the weak responses observed these were seen after stimulation with the pool containing peptides corresponding to the LT aa721-816 and sT aa70-186 in MCV seropositive groups (both MCC and NMSC) compared with seronegative groups. However, statistical analysis using the Kruskall-Wallace test indicated that there was no difference in frequencies of responses between any of the 4 donor groups. Overall, these assays showed that there are low frequencies of IFNɣ secreting MCV T-antigen-specific T cells in the peripheral blood of MCC and NMSC patients.

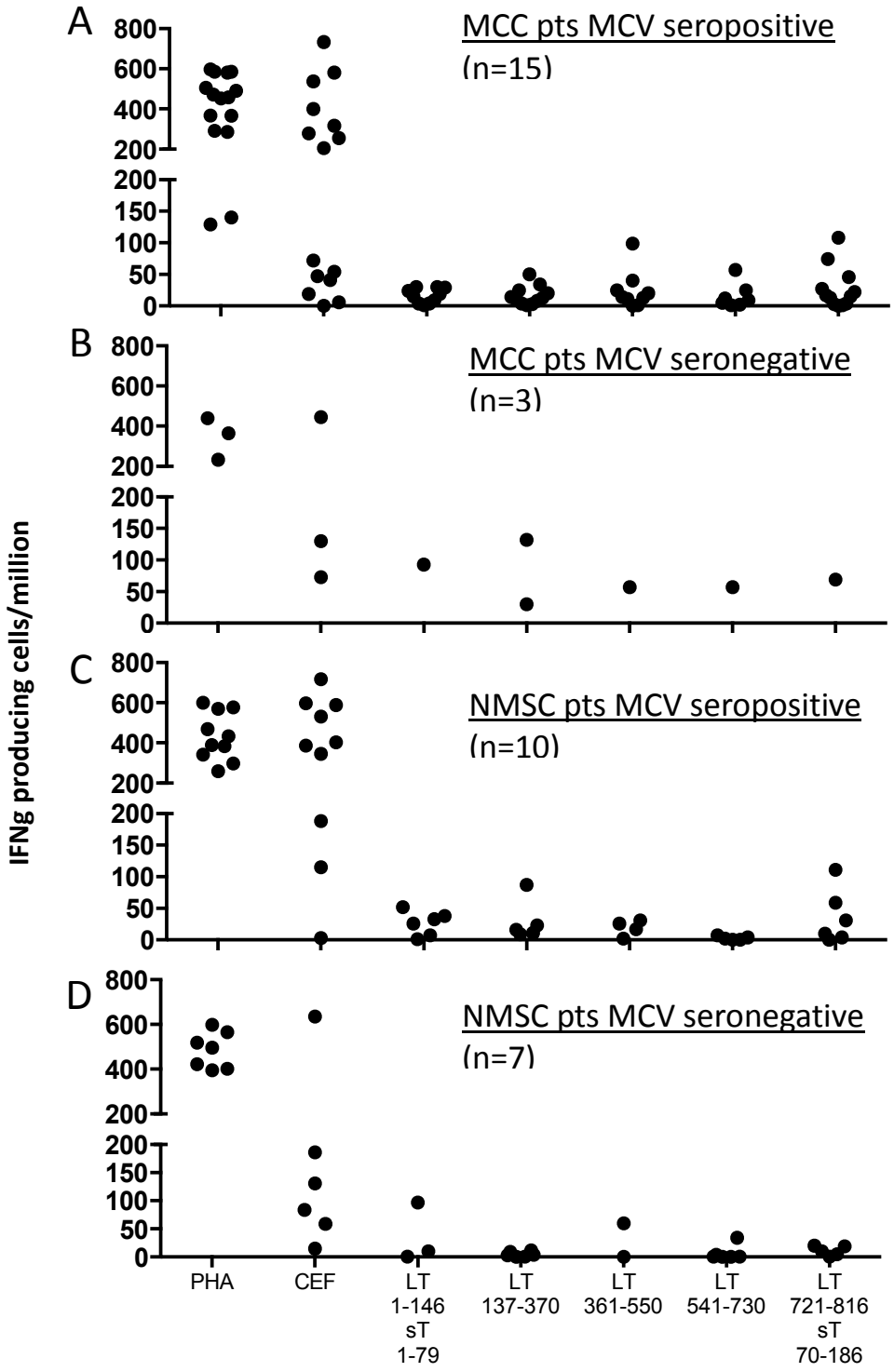


Figure 5.2. IFNγ ELISpot results using peripheral blood mononuclear cells from MCC and NMSC patients stimulated with MCV T-antigen peptides. PBMCs from MCV seropositive and seronegative MCC (A+B) and NMSC (C+D) patients were used in IFNγ ELISpot assays following stimulation with MCV T-antigen peptides, Phytohaemagglutinin (PHA) and CMV, EBV and Flu (CEF) peptides (both positive controls) and DMSO (negative control). The magnitude of response was calculated from the mean value of duplicate wells – background (DMSO) and adjusted to no.IFNγ producing cells/million PBMCs. Each point represents an indidual donor response to each pool of peptides.

5.2.2 Quantification of MCV-specific T cell frequency in the peripheral blood of healthy donors

Both MCC and NMSC patients in the studied cohorts were elderly with median age over 70 in both groups. Although patients with known immunosuppression were not included in this study, it was conceivable that the anti-viral immune response in the elderly may not be as robust as in younger healthy donors. Therefore, a further cohort of 19 healthy volunteers was recruited of whom 14 (74%) were MCV seropositive and had a mean age of 44 years old (range 22-60). This allows for further comparison with a group of donors that have been exposed to MCV, but do not fall into the characteristic demographic of patients that develop MCC. However, when IFNɣ ELISpots were conducted on PBMCs from these donors using the MCV T-antigen peptides, the majority again showed low frequencies of MCV specific IFNɣ secreting T cells in both the MCV-seropositive (Fig 5.3A) and -seronegative (Fig 5.3B) populations. There did appear to be an increased number of MCV seropositive donors making detectable responses against sT aa125-186 compared with MCV seronegative donors, but no statistically significant association was seen. From this cohort, two donors made larger responses to a particular pool of peptides. One of these, MCV seropositive donor 14464, made a response to the pool of peptides corresponding to LT aa 541-610. The second donor, MCV seronegative 14461, made a response against peptides corresponding with LT aa 601-670. Overall, MCV-specific cells which secrete IFN-g after challenge with peptides are found in a low frequency in peripheral blood of healthy MCV seropositive donors as well as seronegative donors.

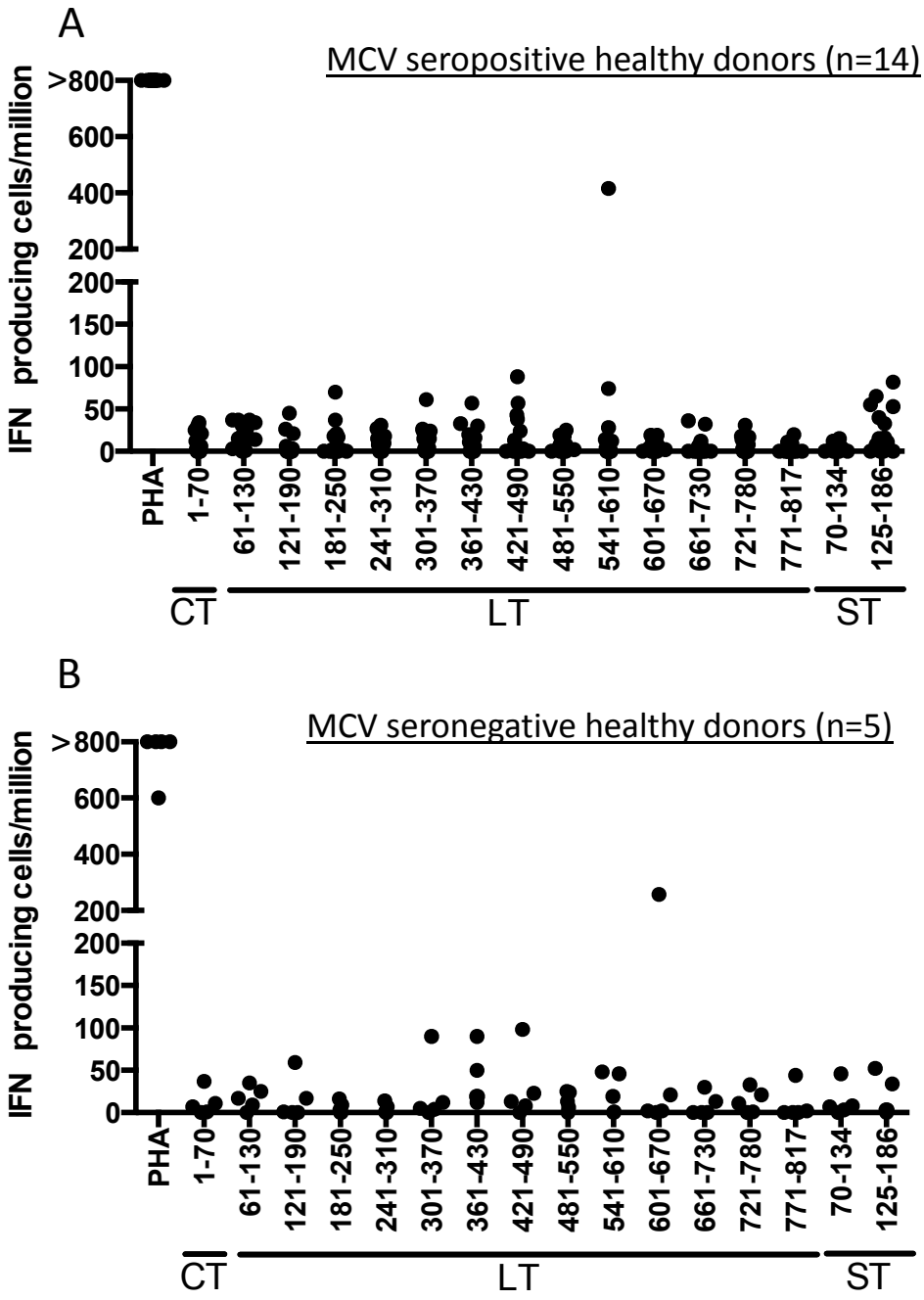


Figure 5.3. IFNγ ELISpot results using peripheral blood mononuclear cells from MCV+ and MCV negative healthy volunteers stimulated by MCV T-antigen peptides. PBMCs from 14 MCV seropositive (A) and 5 seronegative (B) healthy donors were used in IFNγ ELISpot assays following stimulation with MCV T-antigen peptides, Phytohaemagglutinin (PHA, positive control) and DMSO (negative control). The magnitude of response was calculated from the mean value of duplicate wells – background (DMSO) and adjusted to no. IFNγ producing cells/million PBMCs. Each point represents an individual donor response to each pool of peptides.

5.2.3 Generation of MCV-specific T cell clones using blood from healthy donors

The results of the previous section quantifying IFN-g secreting PBMC in response to challenge with the T-antigen peptides indicated that weak responses were mostly found in all donor groups and this would make epitope mapping difficult. To identify T-antigen epitopes, an alternative strategy was used through the generation of MCV-specific T cell clones which could be used to map epitopes and restricting elements as well as be used as tools to study antigen processing of T-antigens. This work focussed on generating T cell clones from PBMCs of healthy donors due to the increased ability to perform larger volume blood donations as well as being able to access the two donors who made strong ELISpot responses to specific peptide pools.

A panel of MCV seropositive healthy donors as well as seronegative donor 14461, who made a significant response to LT 601-670 peptide pool, were selected to generate T cell clones. PBMCs from these donors were expanded into polyclonal cultures enriched for MCV specificities by stimulating these with the T-antigen peptides and allowing them to grow for one week. T-antigen-specific cells were then enriched from these cultures by re-challenging with the peptides and magnetically sorting IFN-g secreting these cells. These were then subjected to limiting dilution cloning and after further expansion the specificity, restriction and functional avidity of the clones determined.

Figures 5.4 and 5.5 show how this characterisation process was undertaken. Figure 5.4 shows data from analysis of clone 98 (c98) from healthy donor 14496 which when stimulated with the pools of the T-antigen peptides recognised a peptide from within the CT 1-70 pool as determined by secretion of IFNɣ in ELISA assay (Fig 5.4A). Repeating recognition assays using the individual peptides that made up pool CT 1-70 indicated this clone was specific to peptide, NIP (Fig 5.4B). Clones were confirmed to be either CD4 or CD8 by staining with

antibodies specific for these markers and analysing by flow cytometry, with c98 being CD4 (Fig5.4D). To determine the MHC Class II molecule which presents this peptide, the ability of antibodies specific to MHC-DR or MHC-DQ to prevent recognition of EBV transformed B cells (LCL) sensitised with this peptide was initially assessed. Here, autologous LCLs were loaded with peptide, washed and then incubated with either anti-DR, anti-DQ antibody or without antibody. Peptide-specific T cells were then added and recognition assessed by measuring IFNɣ from the T cells by ELISA. In the example using NIP peptide, it was confirmed that T cell c98 peptide recognition was reduced when presentation was blocked using anti-DR antibody but unaffected with anti-DQ antibody, indicating the peptide is likely presented by MHC-DR molecules (Fig 5.4E). To identify which DR type presented the NIP peptide, c98 T cells were challenged with peptide loaded, partially DR-matched LCLs and recognition assessed by IFNɣ ELISA assays. Recognition, as demonstrated by secretion of IFNɣ was only seen in HLA-DR13 matched LCLs, indicating the NIP peptide is presented by HLA-DR13 molecule (Fig 5.4F). The functional avidity of c98 was then determined by repeating IFNɣ ELISAs assessing the clone's response to target cells sensitised with 10-fold dilutions of peptide (Fig 5.4C). In the case of c98, functional avidity as measured as the concentration which gives 50% maximal IFN-g production was 10^{-8}M of peptide.

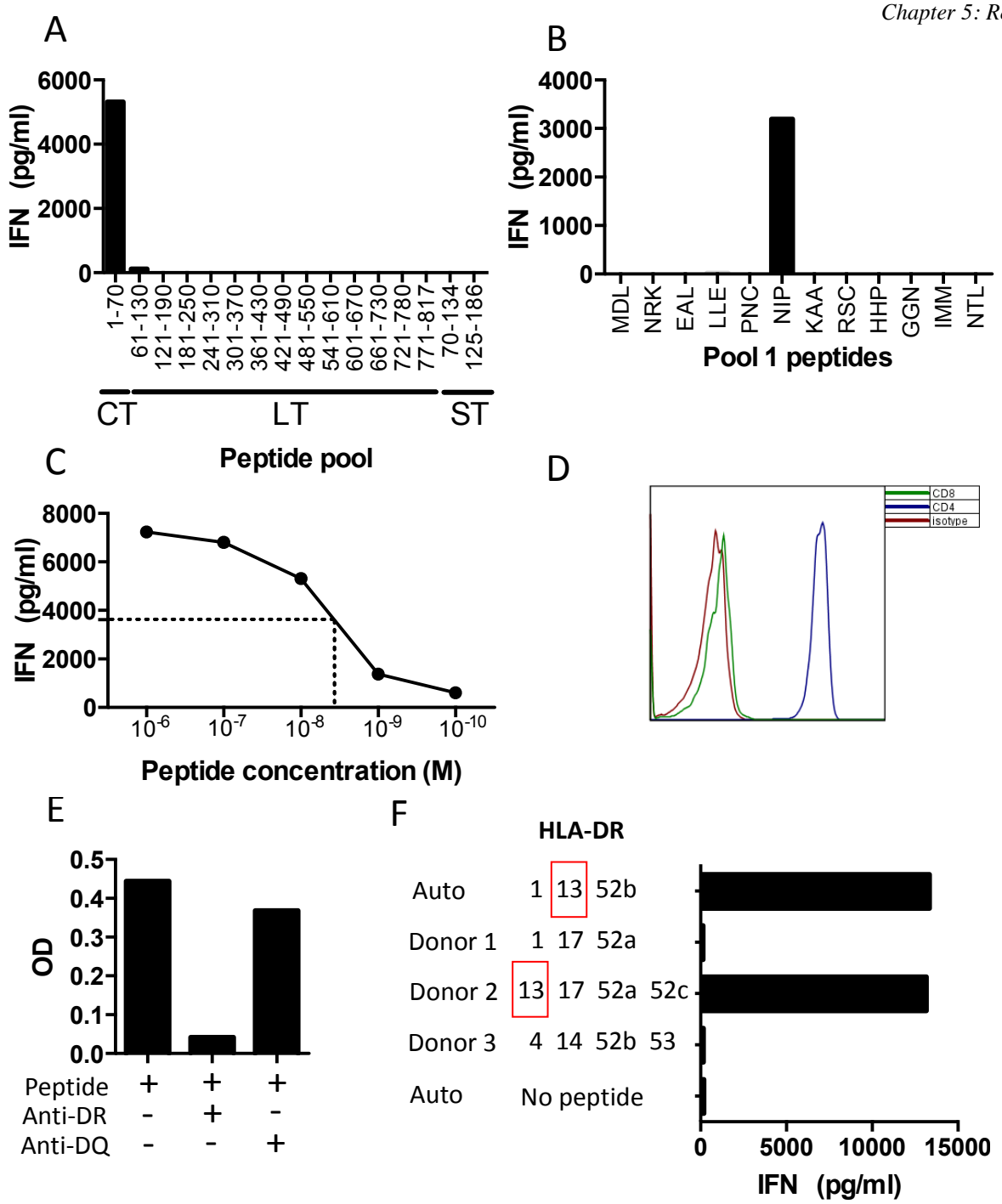


Figure 5.4. Identification of a CD4 T cell clone specific for HLA-DR13 MCV LT epitope, NIP. T cell clone c98 isolated from healthy donor 14496 was screened for IFNγ secretion by ELISA after stimulation with T-antigen peptide pools (A), followed by individual peptides from the reactive pool (B). The functional avidity (peptide conc. required for 50% max IFNγ production) was calculated by incubating T cells with autologous LCL sensitised with 10-fold dilutions of NIP peptide. (C). Using flow cytometry, the clone was assessed for surface CD4 or CD8 expression (D). MHC Class II subtype presenting NIP peptide was assessed by ability of T cell clone to recognise peptide-sensitised autologous LCL in the presence or absence of anti-DR and anti-DQ antibodies (E). MHC restriction of clone was assessed by sensitising partially HLA-DR matched LCLs with NIP peptide, incubating with T cell clone and measuring IFNγ release (F).

Figure 5.5 shows the characterisation of T cell clone, c190, isolated from healthy donor 14464 PBMCs. This clone was able to recognise peptide FKG within pool LT 541-610 (Fig 5.5A and B). Flow cytometric analysis showed this clone was CD8 (Figure 5.5C). Using FKG peptide loaded partially HLA-matched LCLs, recognition only occurred with LCLs matched for HLA-B7, indicating that the peptide FKG is presented through the HLA-B7 molecule. (Figure 5.5D). Unlike Class II epitopes, MHC Class I peptides generally have a defined length of 8-10 amino acids. As such, the 15mer FKG peptide (FKGPINSGKTSFAAA) likely contains the minimal HLA-B7 epitope sequence. It is well recognised that HLA-B7 epitopes share common features including proline at position two and a hydrophobic or aromatic residue at the C-terminal anchor position [393]. This aided the prediction of a likely minimal epitope GPINSGKTSF, with a proline at position two and phenylalanine (a large aromatic, hydrophobic amino acid) at position 10. A series of overlapping peptides encompassing this region was synthesised and used to sensitise MHC-B7 matched LCLs at ten-fold concentrations ranging from 10^{-6}M to 10^{-9}M. The recognition of c190 T cells at these varying peptide concentrations was assessed in IFNɣ ELISA (Fig 5.6). From this experiment, it could be seen that the sequence GPINSGKTSF was recognised by the T cell clone even at low concentrations (functional avidity 10^{-8}-10^{-9}M) and this recognition was greater than that of the 15mer FKG peptide. The other candidate minimal epitopes were comparatively poorly recognised by the T cell clone. Therefore, the minimal HLA-B7 epitope was concluded as being the GPINSGKTSF sequence and will henceforth be referred to as GPI.

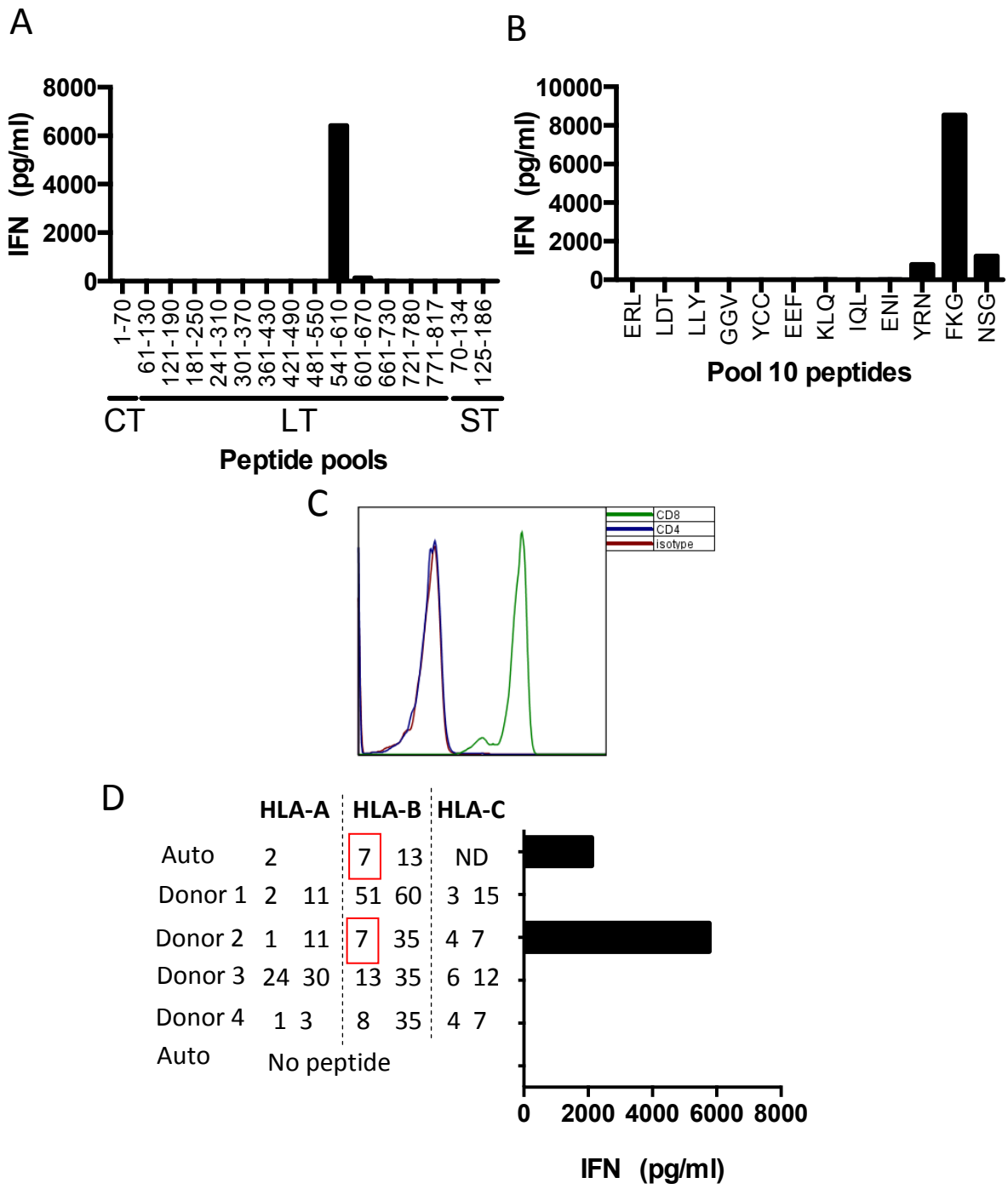


Figure 5.5. Identification of a CD8 T cell clone specific for HLA-B7 MCV LT epitope, FKG. T cell clone c190 isolated from peripheral blood of healthy donor 14464was screened for IFNγ secretion by ELISA after stimulation with T-antigen peptide pools (A), followed by individual peptides from the reactive pool (B). Using flow cytometry, the clone was assessed for surface CD4 or CD8 expression (C). MHC restriction of clone was assessed by sensitising partially MHC Class I matched LCLs with FKG peptide, incubating with T cell clone and measuring IFNγ release (D).

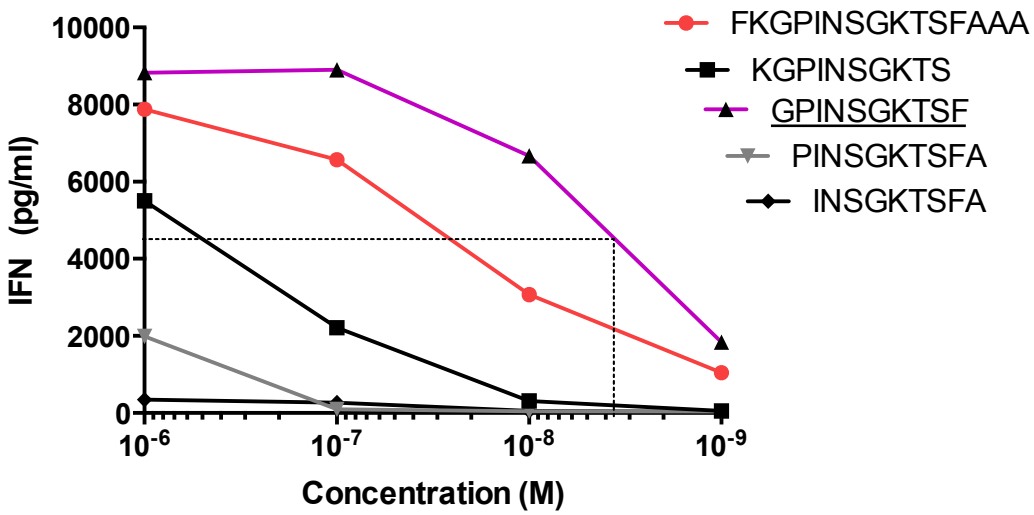


Figure 5.6. Identification of minimal HLA-B7 CD8 T cell epitope, GPI. Using the original 15mer FKG peptide, four candidate 10mer peptides were synthesised and all were used at used to sensitise autologous LCL at 10-fold serial dilutions to stimulate T cell clone c190 in IFNγ ELISA. The functional avidity (peptide concentration required for production of 50% maximal IFNγ production) was calculated for each candidate peptide.

Overall, successful T cell cloning experiments using four donors resulted in the identification of eight novel Class II restricted and one novel Class I restricted MCV T-antigen-epitopes (summarised in Table 5.1). Of the Class II epitopes, 2 (LLE and NIP) were found in the CT region and the remainder found in the LT region. MCC specific LT mutations are individual and result in truncated proteins varying in length between 258 amino acids in tumour MCC350 (GenBank: JN038583.1) to 469 amino acids in tumour MCC339 (GenBank: EU375804.1). Therefore, two Class II epitopes (ALD, QSE) are found outside this region and are only likely to be retained in the full length T-antigen. The frequency of T cell clones isolated against the majority of Class II targets was low, with the exception of ALD (LT 631-646) specific response against which 85 clones were generated from blood of an MCV-seronegative donor 14461. This correlates with the significant ELISpot response seen using

cells from the same donor in response to peptide pool LT 601-670 (Fig 5.3B), suggesting this response was likely to be specific for the ALD peptide. There were 2 donors (14457 and 14464) who made low frequency responses against the same HLA-DR15 epitope PID. The HLA-B7 Class I epitope was discovered after peripheral blood from donor 14464 was found by ELISpot to contain a high frequency of CD8 T cells directed against a pool of peptides, subsequently identified as being specific for the GPI epitope, found outside the truncated region of LT.

Table 5.1. Identified CD4 and CD8 MCV Large T-antigen epitopes.

	Donor	Peptide	Amino acid	HLA restriction	Functional avidity	No of clones
CD4	14461	LLEIAPNCYGNIPLM	16-30 (CT)	Unknown	10^{-8}	4
	14461	ALDKFMVVFEDVKGQ	631-646 (LT)	DR 4 or 53	10^{-9}	85
	14464	KEWWRSGGFSFGKAY	90-105 (LT)	DR 7	10^{-8}	6
	14464 AND 14457	PIDLSDYLSHAVYSN	315-330 (LT)	DR 15	10-^{8}	4+2
	14464	QSEISYGKFCQMIEN	770-785 (LT)	Unknown	10^{-8}	1
	14496	PYKLLQENKPLLNYE	415-430 (LT)	DR 52b	10^{-8}	15
	14496	NIPLMKAAFKRSCLK	25-40 (CT)	DR 13		1
CD8	14496	TPPPFSRKRKFGGSR	270-285 (LT)	DR 13		
	14464	GPINSGKTSF	593-603 (LT)	B7	10^{-8}	2

5.2.4 Recognition of antigen presenting cells fed MCC tumour cell lysate by MCV specific CD4 T cell clones

The previous set of experiments provided a number of CD4 clones capable of recognising 15mer peptides corresponding with the MCV T-antigen. To validate that these clones could recognise processed and presented antigen, a series of experiments was undertaken where MCV proteins were loaded on cells and these assessed for their ability to process and present peptide-epitopes from these proteins to the T cell clones. Here autologous LCLs were fed different concentrations of lysates from either the MCC line MKL-1, which expresses a truncated form of LT (amino acids 1-330) or lysates from the MCV negative tumour line MCC13, and these incubated with the T-antigen-specific CD4 T cells. The ability of the T cell clones to recognise the lysate fed LCLs was assessed by IFNɣ ELISA. As shown in Figure 5.7, this experiment showed that T cell clones specific for epitopes LLE, NIP, KEW, TPP and PID were all able to recognise antigen presenting cells fed with the MKL-1 (MCV+) lysate and recognition decreased as lysate concentration reduced. These epitopes are all retained by the truncated T-antigen expressed by the MKL-1 cell line. However the T cell clone specific for the LT-antigen epitope, ALD, which is lost by the MKL-1 truncation, did not recognise LCLs fed with MKL-1 lysate. None of the T cell clones recognised LCLs fed with MCV negative MCC13 lysate. These studies indicate that peptides identified within the truncated T-antigen are valid epitopes.

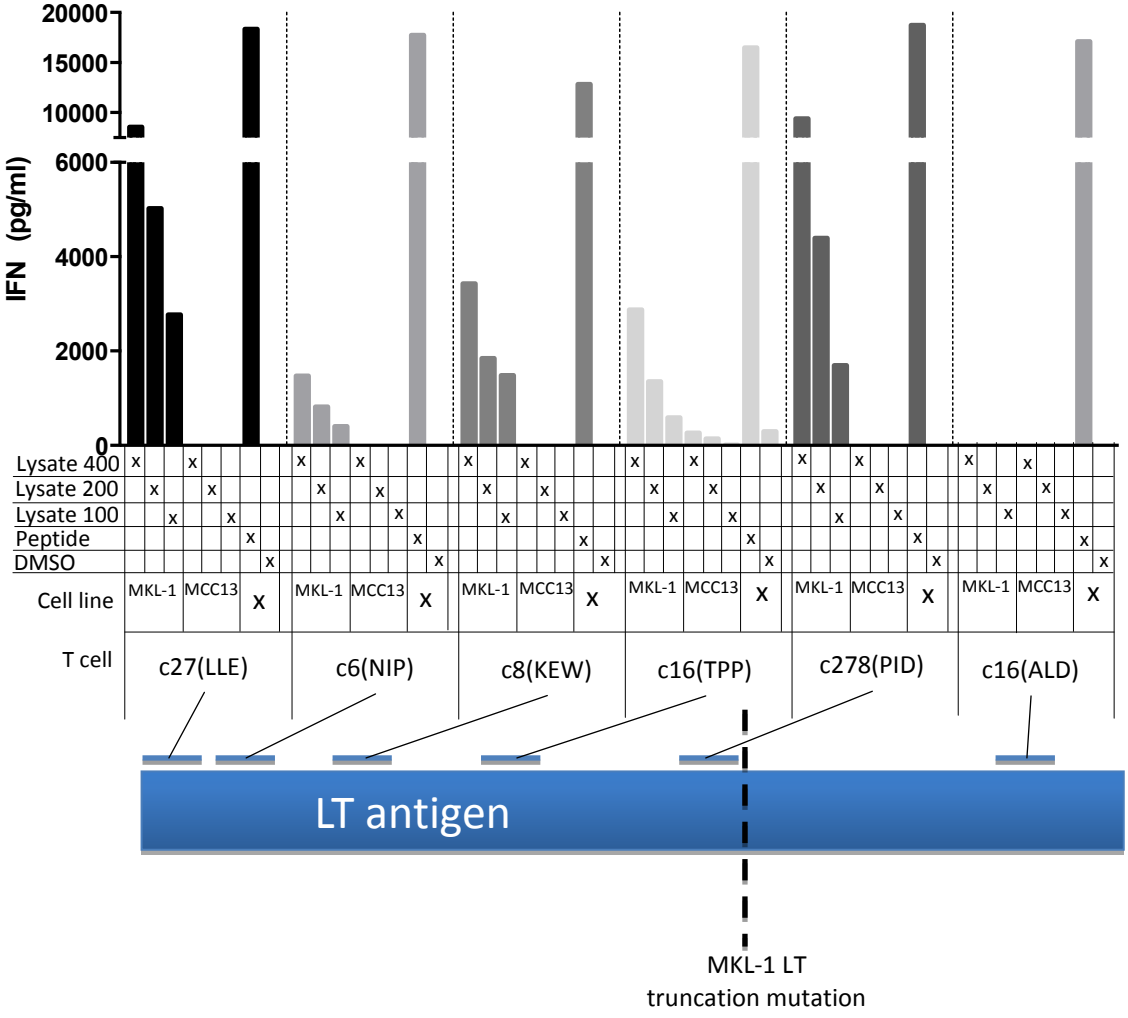


Figure 5.7. CD4 T cell clone recognition of autologous LCL fed with MCC tumour cell line lysates, MKL-1 (MCV+) and MCC13 (MCV negative). CD4 T cell clones specific for MCV T-antigen peptides were incubated independently with autologous LCLs fed either MKL-1 lysate, MCC13 lysate or loaded with cognate peptide (positive control) or DMSO (negative control). IFNγ secretion was measured by ELISA. The position of each epitope on the T-antigen is represented schematically along with position of truncation of T-antigen in MKL-1 cell line.

It is conceivable that through the lysate production process, short fragments of the T-antigen could be created through degradation of the protein and that these are able to bind directly to surface MHC Class II. To confirm whether the lysate was being processed and presented through the MHC Class II pathway LCLs were either fixed immediately upon feeding to with lysate to preventing processing but not presentation, or fixed after 18 hours of processing with 1% paraformaldehyde. Positive and negative controls were LCLs loaded with peptide or DMSO respectively. These LCLs were then used in recognition assays with clones corresponding to two of the MCV T-antigen-epitopes, PID and KEW. As shown in Figure 5.8, the ability for the LCL to bind peptide to the surface MHC Class II molecules is not affected by the fixation process for either of the two clones. T cell recognition did not occur if cell fixation took place at time of feeding, indicating that presence of fragments corresponding to the epitope peptide within the lysate preparation was unlikely. Overall, this work confirms that the CD4 MCV T-antigen-epitopes discovered through T cell cloning are processed and presented by cells with functional antigen presenting machinery. Due to our finding, discussed in chapter 6, that MCC tumours and cell lines do not express surface MHC Class II, the role of these CD4 T cells was not likely to be as cytotoxic effectors and so further analysis of these clones was not conducted as part of this project.

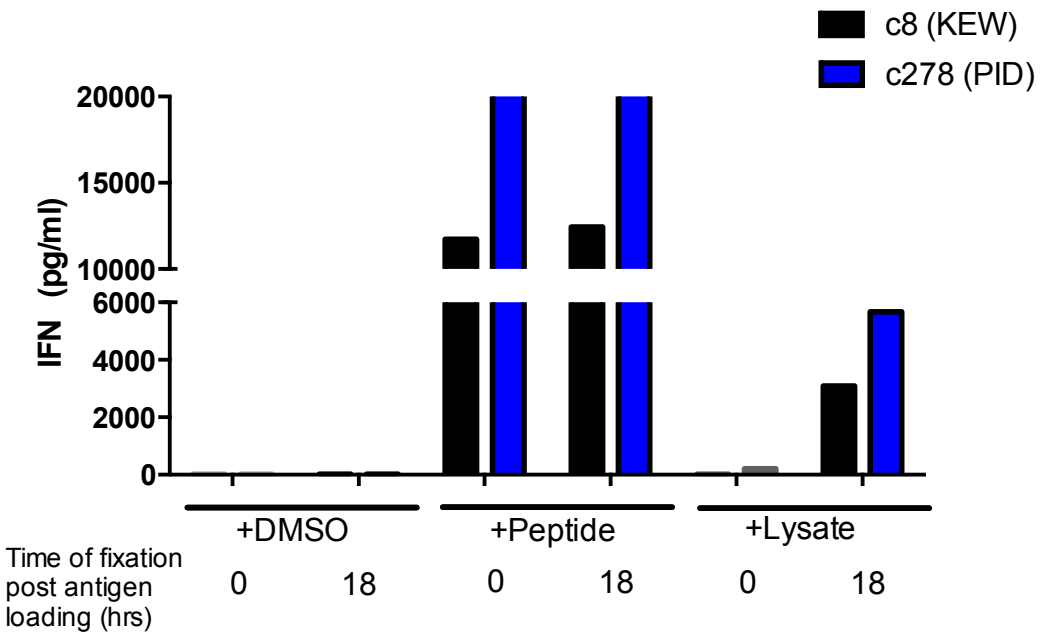


Figure 5.8. Impact of cell fixation on CD4 T cell clones recognition of autologous LCLs fed MKL-1 lysate. CD4 T cell clones, c8 (specific for KEW) and c278 (specific for PID), were incubated with autologous LCLs fixed in 1% paraformaldehyde either immediately or 18 hours after feeding with MKL-1 lysate. LCLs sensitised with cognate peptide or DMSO were used as positive and negative controls respectively.

5.2.5 Ex-vivo phenotypic analysis of MCV specific CD8 response using tetramer flow-cytometric analysis.

Very little is known about the characteristics of MCV-specific T cells. The majority of work that has been done is limited by the low frequency of circulating MCV specific T cells and so have often relied on cultured expansion of these cells which is likely to alter their phenotype. The purpose of this next portion of work was to use the identified HLA-B7 epitope, GPI, to create an MHC class I tetramer for use in flow cytometric phenotyping analysis of ex-vivo PBMC samples to better understand how these cells may behave in vivo.

As a first step, it was essential to validate that the GPI epitope was capable of being processed and presented on the cell surface. To confirm this, the HLA-B7 melanoma cell line MelJuSo

(MJS), was transfected with the pCDNA3-IRES-nls-GFP plasmid expressing MCV wild type LT (WTLT) or the control pCDNA3-IRES-nls-GFP empty vector. Using IFNɣ ELISA, we could show that the MCV GPI-specific T cell clone, c190, was able to recognise WTLT expressing MJS cells but not MJS cells expressing the control empty vector (Figure 5.9). This experiment did confirm that the MCV GPI LT epitope is capable of being processed and presented by a model cell line.

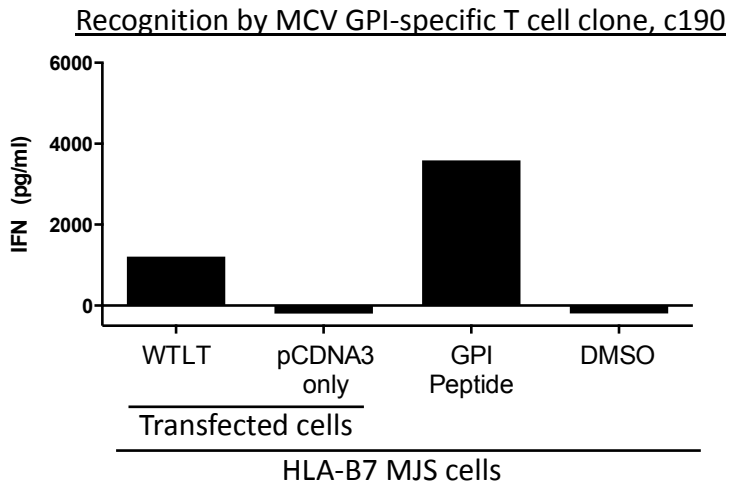


Figure 5.9. Recognition of WTLT MCV GPI epitope by T cell clone c190 following processing and presentation by MJS cells. MJS cells (HLA-B7+) were transfected with pCDNA3-IRES-nls-GFP plasmid expressing wild type LT (WTLT) or the plasmid backbone alone (pCDNA3 only). MJS cells loaded with 5µg/ml GPI peptide or DMSO were used as positive and negative controls respectively. After 24 hours, GPI-specific T cells were added and recognition assessed by IFNɣ ELISA.

From initial ELISpot screening, donor 14464, from whom GPI specific CD8 T cells had been cloned, showed a readily detectable frequency of GPI-specific T cells in the peripheral blood. PBMCs from this donor where therefore used as a positive control to test the tetramer was able to stain GPI-specific CD8 T cells. As shown in Figure 5.10A, using blood from donor

14464, a population of CD8 cells stained with tetramer with a frequency measuring 0.204%. No detectable CD8 tetramer staining was seen in PBMCs from an HLA-B7 MCV seronegative healthy donor, 14495 (Figure 5.10B) showing the specificity of this reagent.

In order to understand whether the detectable tetramer response seen in donor 14464 was generalizable to other HLA-B7 MCV seropositive donors, PBMCs from relevant donors were stained with tetramer and sizes of responses are shown in Figure 5.10C. Alongside healthy donors, PBMCs from a single HLA-B7 MCC patient, P84, were also used in the tetramer staining assay. The frequency of tetramer positive cells in all screened donors was very low, ranging from 0.002-0.019% of CD8 cells. Interestingly, the tetramer staining cells in these donors did not stain to the same intensity as seen in donor 14464. However, the fact that there are MCV seropositive donors who do not have any detectable tetramer binding cells (e.g 14498, 14501), suggests that this is may not be simply due to non-specific binding. Additionally, donor 14463, in whom tetramer binding CD8 cells were seen at a frequency of 0.017%, did make a small but reproducible response to the pool containing GPI peptide in the ELISpot screening experiments described in Figure 5.3A. The discordant results between tetramer and ELISpot assays are further discussed in section 5.2.6. The MCV patient, P84, included in these assays also had a similar low frequency of CD8 tetramer binding cells in the peripheral blood (0.019%). However, in this case, no detectable IFNɣ response was detected in ELISpot screening, possibly suggesting these cells do not secrete IFNɣ. Overall, these tetramer assays showed that although a significant MCV GPI-specific tetramer response was detectable in one healthy donor, the majority of healthy donors and one MCC patient had very low frequencies of tetramer binding CD8 T cells.

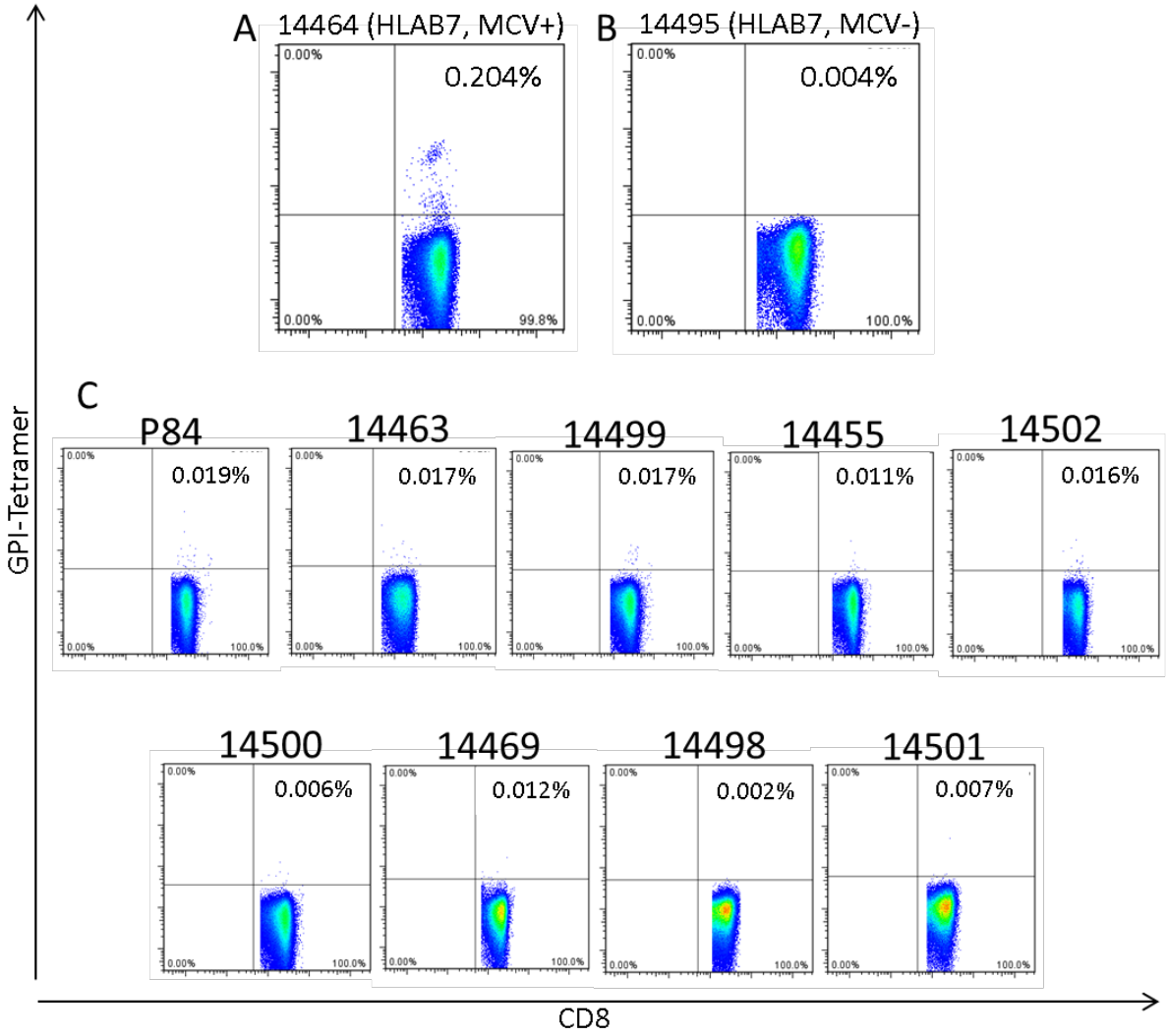


Figure 5.10. Screening of HLA-B7 healthy donors for populations of MCV GPI-specific T cells. PBMCs from HLA-B7+ healthy donor 14464, from whom GPI specific CD8 T cells were isolated, were stained with GPI-tetramer and CD8 (A). PBMCs from healthy donor 14495 who was HLA-B7 but MCV seronegative were also stained with tetramer and CD8 to act as negative control (B). PBMCs from a panel of HLA-B7 healthy donors alongside one MCC patient (P84) were stained with GPI tetramer and CD8.

The GPI tetramer was next used to characterise the cognate T cells in PBMC from donor 14464. We were interested in in the differentiation status of these cells (using CD45RA and CCR7 surface expression), as well as whether they exhibit a skin homing phenotype (using CLA, CCR4 and CCR6 surface expression) as may be expected by cells specific for the skin-associated MCV. This CD8 T cell response could then be compared with that of CD8 T cells specific for other viruses, namely the skin-tropic Herpes Simplex Virus (HSV) and lymphotropic Epstein-Barr Virus (EBV). In donor 14464, the majority of MCV GPI-tetramer positive cells displayed an effector memory type phenotype being CCR7-CD45RA-. There were some apparent CCR7+ CD45RA+ tetramer positive cells; a phenotype that has been seen in other polyomavirus LT specific CD8 T cells (Ester Remmerswaal, Personal communication). With regard to expression of skin homing markers, there was only a slight increase in the frequency of tetramer positive cells expressing CLA (10%) when compared with tetramer negative CD8 cells (4%) (Figure 5.11A).

Although the GPI-tetramer response was very small in HLA-B7 MCC patient P84, an attempt was made to perform some characterisation of tetramer positive cells however the low frequency of tetramer binding cells means these results must be interpreted with much caution (Figure 5.11B). Again the majority of cells were CCR7-CD45RA-, however in this case, although cell numbers were small, an increased frequency of expression of skin homing markers was seen in tetramer positive cells as compared with tetramer negative cells. Of the tetramer positive cells, 50% expressed CLA and 92.9% expressed CCR4. This was in comparison to tetramer negative cells, of which 4.2% were CLA positive and 1.25% were CCR4+. There was negligible CCR6 expression seen in either tetramer binding or tetramer non-binding CD8 T cells.

As a comparator viral response with expected skin-tropism, the Herpes Simplex Virus 1 (HSV 1) glycoprotein gD HLA-A2 specific response against the VLL epitope (gD70-78) was used. Following tetramer screening of multiple donors HLA-A2 healthy donors (data not shown), donor 14457 was shown to have the most detectable tetramer response and so was used in further characterisation assays. In these, VLL tetramer binding CD8 T cells did show an increased frequency of expression of skin homing markers (CLA: 45%, CCR6:51.5%, CCR4: 98.5%) when compared with tetramer negative CD8 cells (Figure 5.11C).

As a control response from a virus not thought to be skin-associated and against which virus-specific CD8 T cells do not express high levels of CLA [289, 321], responses against EBV lytic and latent HLA A2 and HLA B7 epitopes were measured and characterised using healthy donors 14464, 14463 and 14457 (Figure 5.12). In our assays, using tetramers specific for HLA-A2 BMLF1 epitope GLC, and HLA-B7 EBNA 3A epitope RPP, it was seen that, as expected, tetramer binding cells showed a low frequency of expression of skin homing markers (4-10% CLA expressing) as compared with tetramer negative cells.

Overall, characterisation of MCV-specific response in healthy donor 14464 shows that the majority of these cells are of effector memory type (CCR7-CD45RA-) and do not show any evidence for increased expression of markers of skin-homing in comparison to EBV specific T cells. This is in contrast to the MCV-specific response seen in MCC patient P84 in whom, although the size of response is small, there is an increased frequency of MCV-tetramer binding cells expressing skin homing markers to a similar level seen in the skin tropic HSV response.

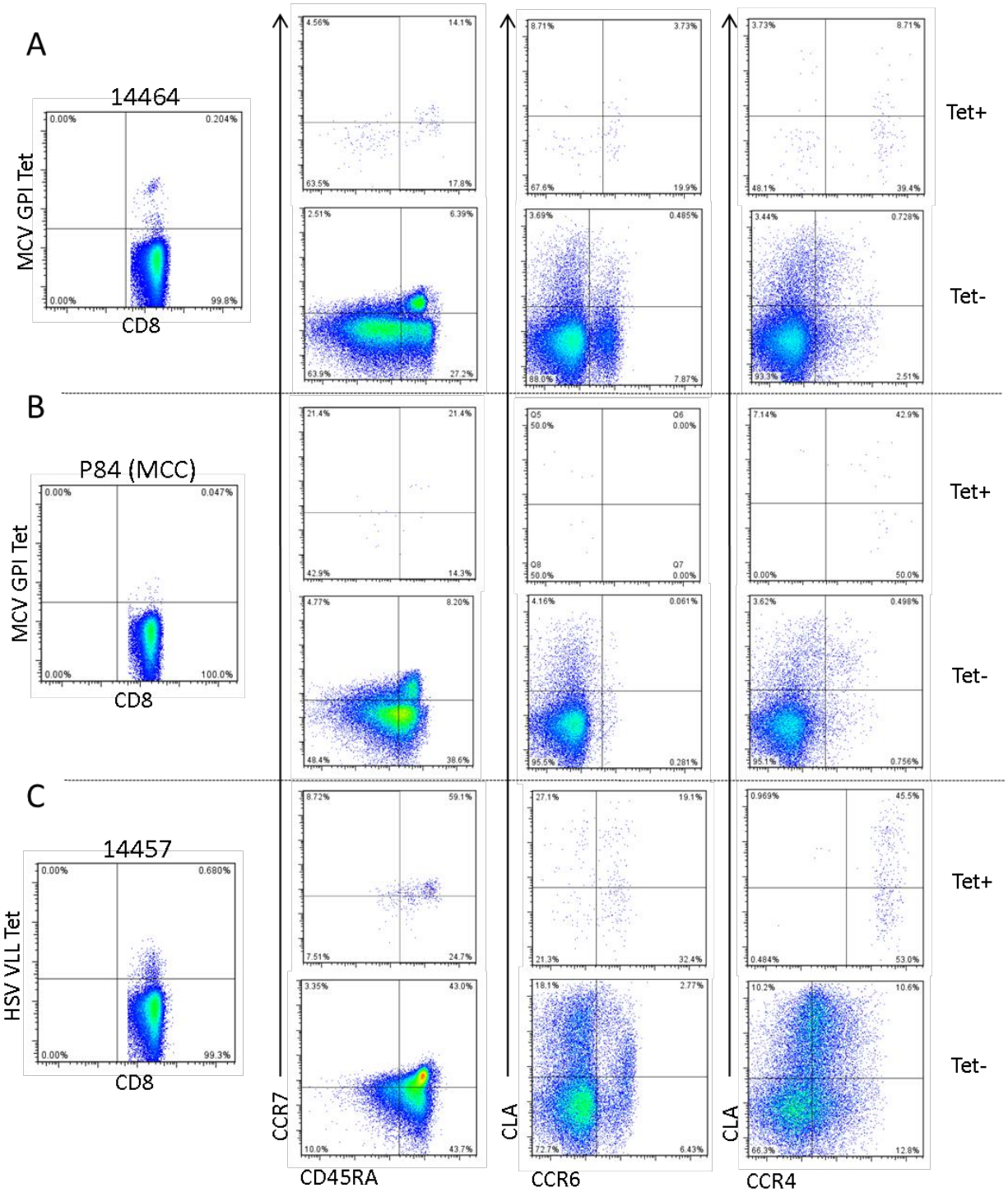


Figure 5.11. Characterisation of MCV GPI specific CD8 T cell response and comparison with HSV VLL CD8 response. PBMCs from healthy donor 14464 (A) and P84 (B) were stained with MCV GPI HLA-B7 tetramer. PBMCs from healthy donor 14457 were stained with HSV VLL HLA-A2 tetramer. Tet+ and Tet- CD8 T cells from were analysed for expression of CCR7, CD45RA, CLA, CCR6 and CCR4.

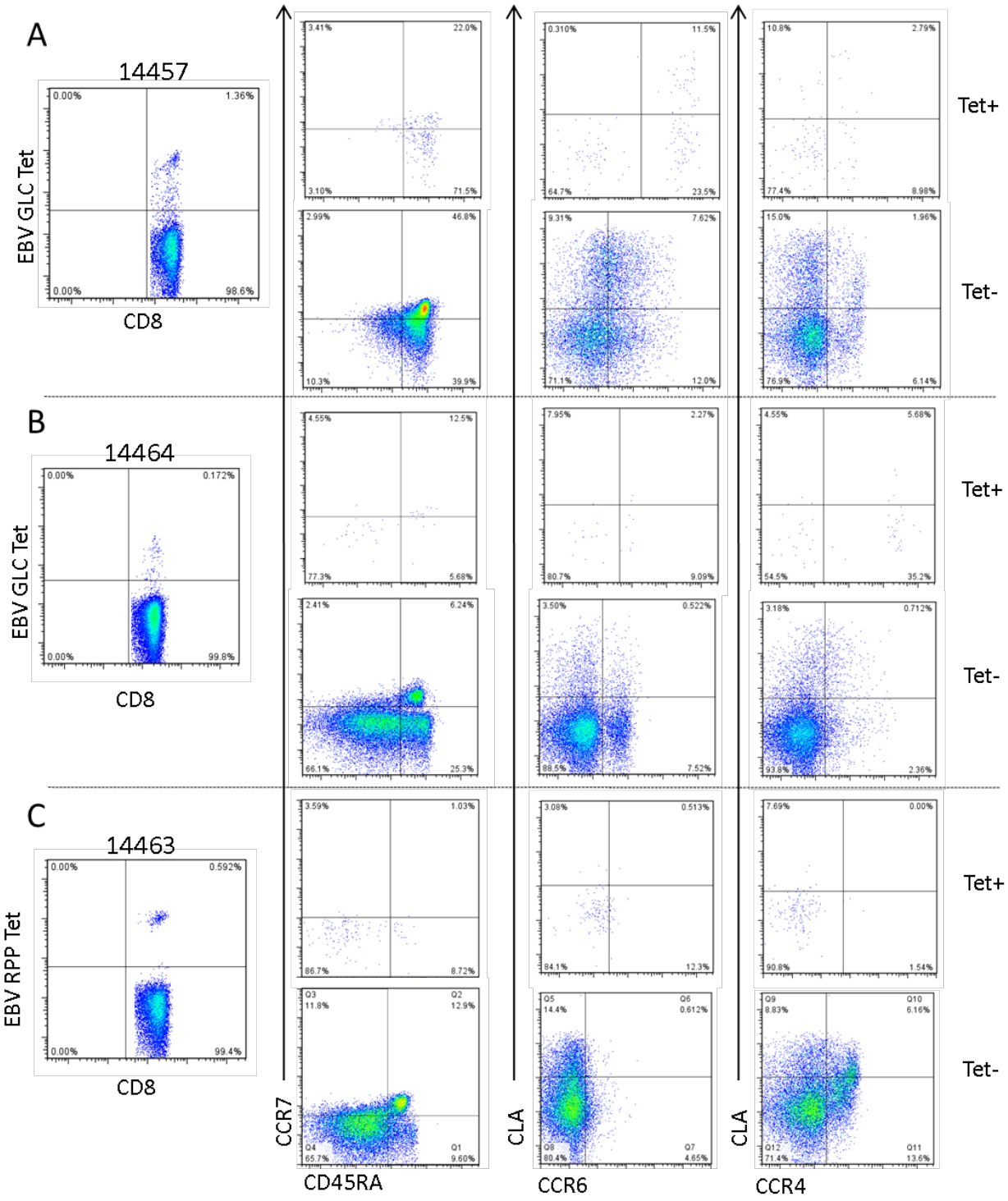


Figure 5.12. Characterisation of EBV specific CD8 T cell responses against GLC and RPP epitopes. PBMCs from HLA-A2+ donors 14457 (A) and 14464 (B) were stained with GLC tetramer. PBMCS from donor 14463 was stained with RPP tetramer (C). Tet+ and Tet- staining CD8+ T cells were analysed for expression of CCR7, CD45RA, CLA, CCR6 and CCR4.

5.2.6 Investigating cross reactivity of CD8+ T cell clones specific for MCV GPI epitope

As stated earlier, ELISpot screening assays had suggested that alongside donor 14464, donor 14463 would also be expected to have a detectable response in MCV GPI tetramer assays. Both donors 14464 and 14463 show serological evidence of infection by multiple polyomaviruses with 14464 having serum antibody VP-1 responses against BKV, KI, WU, HPyV6, HPyV7, TSV and HPyV10. Donor 14463 has serum antibody responses against BKV, JCV, KI, WU, HPyV6, HPyV7, TSV, HPyV9 and HPyV10. Given the homology of the MCV T cell epitope, GPI (see Table 5.2), amongst the other members of the polyomavirus family we were interested to know whether the IFNɣ secreting PBMCs from donor 14463, detected by ELISpot assay but not through tetramer staining, were specific for one of the homologous epitopes. In addition to this, we wanted to see whether T cells specific for the other polyomavirus homologs could be detected alongside MCV specific tetramer binding cells in PBMCs from donor 14464. In order to do this, polyclonal cultures using PBMCs from both donors were set up by stimulating with a mix of five synthetic peptides, each corresponding to a GPI epitope homolog from the known human polyomaviruses (MCV and HPyV12: GPINSGKTSF, JCV and BKV: GPIDSGKTTL, KI, HPyV6,7,9: GPINSGKTTL, WUV, HPyV10: GPINSGKTTV, TSV: GPINSGKTTF). T cell clones were established as before and an aliquot of each proliferating clonal culture was screened in IFNɣ secretion ELISA following stimulation with the mix of five peptides at micromolar concentration. Specific clones were then expanded in culture and then re-screened with IFNɣ ELISA as before. Of nine expanded CD8 clones initially identified from donor 14464, six were confirmed to be specific for the peptides following repeat ELISA. From donor 14463, of 64 CD8 clones selected for bulk expansion, 55 had confirmed specificity for the peptides. Ten

randomly selected clones from donor 14463 and all from donor 14463 were then used in experiments to allow further characterisation.

Virus	CD4 Epitopes			CD8 Epitope
MCV	LLEIAPNCYGNIPLM	ALDKFMVVFEDVKGQ	NIPLMKAAFKRSCLK	GPINSGKTSF
JCV	LLGLDRSAWGNIPVM	GIDQFMVVFEDVKGT	NIPVMRKAYLKKCKE	GPIDSGKTTL
BKV	LLGLERAAWGNLPLM	AIDQYMVVFEDVKGT	NLPLMRKAYLRKCKE	GPIDSGKTTL
KI	LLCLDMSCWGNLPLM	ALDQYMVVFEDVKGQ	NLPLMRRQYLVKCKE	GPINSGKTTL
WU	LLGLDMTCWGNLPLM	AIDQFTVVFEDVKGQ	NLPLMRTKYLSKCKE	GPINSGKTTV
6	LIGLSMACWGNLPLM	AIDKFMCVIEDVKGT	NLPLMQQKIRLACKK	GPINSGKTTL
7	LIGLNMACWGNLPLI	AIDKYMVVIEDVKGT	NLPLIQHKVRLASKK	GPINSGKTTL
TSV	LLQIPRHCYGNFALM	AIDQFVVIFEDVKGQ	NFALMKINHKKMSLK	GPINSGKTTF
9	LLQLTRAAWGNLSLM	AIDQFCVLLDDVKGQ	NLSLMKKAYKTVSKI	GPINSGKTTL
10	LLSLNTAAWGNIPLM	AIDEYMVVFEDVKGQ	NIPLMQYKYRQTCLK	GPINSGKTTV
STL	LLGLPEDSWGNVPLI	AIDEFMVVFEDVKGQ	NVPLITYRFRQKSKI	GPINSGKTTV
12	LLKISGDTFGNVPAM	ALDQFAVVFEDVKGQ	NVPAMARAYKLAAKR	GPINSGKTSF

Table 5.2 Amino acid sequence homology of selected MCV CD4 and CD8 epitopes amongst other members of the human polyomavirus family. Letters highlighted in red represent amino acids changes compared with MCV sequence.

Firstly, the CD8 clones identified from donors 14463 and 14464 were stained with the GPI-tetramer. All six clones from donor 14464 bound with high intensity to the tetramer (see example in Figure 5.13A, clone 19), with no difference in strength of binding seen between the clones. Of the ten clones from donor 14463, nine bound to tetramer with lower MFI, suggesting a lower avidity (see example in Figure 5.13A, clone 111) and one, clone 44 did not bind to tetramer at all (see Figure 5.13C). These results appear to replicate those of the tetramer staining of PBMCs from these two donors in which a population of strongly tetramer binding CD8 T cells was seen in donor 14464 but not with donor 14463.

We were next interested to assess which GPI homolog peptide was responsible for inducing IFNɣ secretion from the T cell clones. This was done through IFNɣ ELISA using titrating concentrations of individual peptides in parallel. It was clear that no clones from either donor were stimulated to secrete IFNɣ by the BK/JC and WUV/HPyv10 peptides (data not shown). All six clones from donor 14464 recognised the three remaining peptides, most strongly to the MCV peptide with a functional avidity of 10^{-8}-10^{-9}M of peptide and this being around ten-fold more sensitive than the TSV or KI/HPyV6/HPyV7/HPyV9 peptides (see Figure 5.13, c19). Of the clones from donor 14463, the nine that exhibited intermediate tetramer binding showed broadly similar recognition of the three peptides with a functional avidity of approximately 10^{-8}M (see Figure 5.13, c84).The clone from donor 14463 that did not bind tetramer showed specific recognition of the KI/HPyV6/HPyV7/HPyV9 peptide, with a functional avidity of 10^{-7}-10^{-8}M (see figure 5.13, c44). There was no recognition of either MCV or TSV peptides seen. This data suggests that the strongly tetramer-binding clones from donor 14464 are able to recognise MCV peptide more strongly than the KI/HPyV6/HPyV7/HPyV9 and TSV peptides, although recognition of these peptides does still occur. Intermediate tetramer-binding clones from donor 14463 recognise MCV, KI/HPyV6/HPyV7/HPyV9 and TSV peptides to a broadly similar level with no obvious specificity for any single peptide. The non-tetramer binding clone from donor 14463 is specific for the KI/HPyV6/HPyV7/HPyV9 peptide.

Lastly, we wanted to see whether these T cell clones were capable of recognising the GPI epitope following processing and presentation from protein in cells. Here, HLA-B7 expressing cell line, MelJuSo (MJS), was transfected with plasmids expressing either wild-type Large T-antigen (WTLT) or truncated MCC350 Large T-antigen (MCC350LT); The WTLT construct contains the GPI epitope while the MCC350LT does not. MJS cells sensitised with MCV peptide or DMSO were used as positive and negative controls

respectively and these cells were then used in IFNɣ recognition assay with the T cell clones. All six clones from donor 14464 recognised MJS cells expressing WTLT but not MCC350LT (see Figure 5.13C, c19). Nine of the ten clones from donor 14463 were also able to recognise WTLT expressing cells (see figure 5.13C, c84), albeit to a lesser extent. The tenth clone, clone 44, did not recognise WTLT expressing MJS cells at all (see Figure 5.13C, c44). This experiment shows that T cell clones from donor 14464 which bind tetramer and have high avidity for MCV peptide are able to recognise MJS cells expressing the WTLT. Alongside this, T cell clones from donor 14463 which bind to tetramer less well and show less avidity for the MCV peptide are still able to recognise WTLT expressing cells. The T cell clone, c44, that does not bind tetramer and does not recognise MCV peptide, also does not recognise WTLT expressing MJS cells.

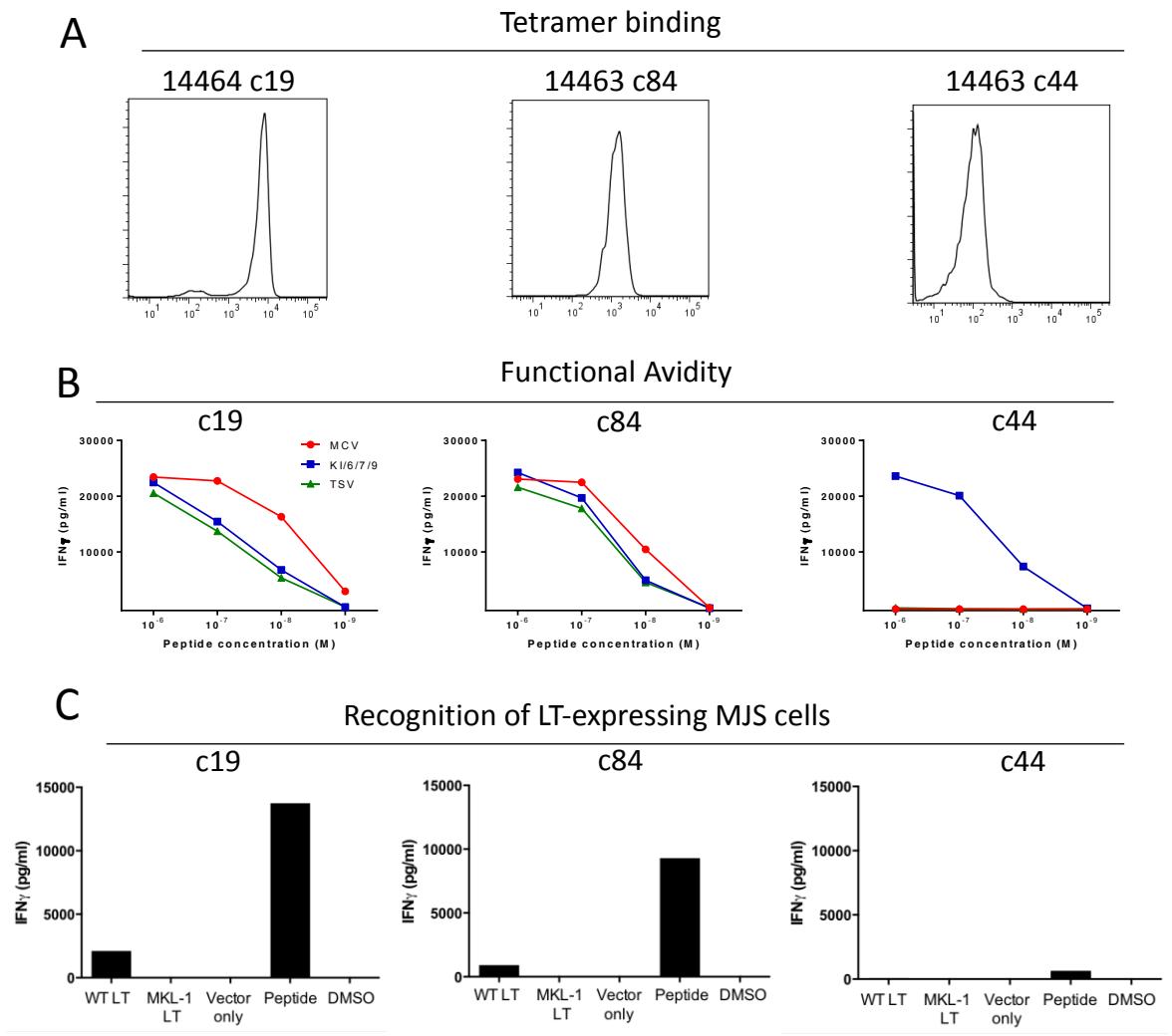


Figure 5.13. Assessment of GPI tetramer staining, recognition of GPI-peptide homologs and recognition of MCV LT expressing MJS cells using GPI-specific T cell clones from healthy donors 14463 and 14464. T cell cloning was conducted using PBMCs from healthy donors 14463 and 14464 following stimulation with GPI peptide homologs. Isolated T cell clones specific for GPI peptide(s) were then further characterised by assessment of MCV GPI-tetramer staining by flow cytometry. Data from three representative clones is shown (A). The functional avidity of T cell clones was assessed by IFNγ ELISA upon stimulation with autologous LCLs stimulated with 10-fold dilutions of individual peptides (B). The recognition of MJS cell transfected with plasmid expressing wild type MCV LT-antigen (WTLT), truncated MKL-1 LT-antigen (MKL-1 LT) and plasmid vector backbone only by T cell clones was assessed by IFNγ ELISA. Peptide and DMSO sensitised MJS cells were used as positive and negative controls respectively.

5.3 Discussion

The first part of this chapter showed the results of IFNɣ ELISpot assays looking at the frequency of circulating PBMCs specific for peptides spanning the length of the MCV Large T (LT) and Small T (sT) antigen proteins. It compared the frequencies of IFNɣ secreting cells between donor cohorts of healthy volunteers, non-immunocompromised patients with non-melanoma skin cancer (NMSC) and non-immunocompromised Merkel cell cancer (MCC) patients. A previous study assessing frequency of MCV specific PBMCs in healthy donors and MCC patients using peptides corresponding with truncated LT and sT-antigens showed that the frequency of these cells was low in both groups but there was a trend towards increased numbers of IFNɣ producing T cells in the MCC cohort [385]. In our study, we also found that the frequency of MCV specific T cells was low and broadly similar between healthy donors, NMSC patients and MCC patients. This suggests that as measured in the periphery, a low frequency of circulating cells is sufficient to control MCV infection in health and that there does not appear to be a significant decline of total circulating IFNɣ secreting MCV specific T cells in older NMSC or MCC patients. Our work extended that of the previous study by assessing frequency of MCV specific T cells for the entire LT and not just the tumour-associated truncated form but found no differences amongst the donor groups tested. We were also able to identify MCV seropositive and seronegative donors based on the presence or absence of MCV VP-1 antibodies in the serum. There was no significant difference between the frequency of MCV specific T cells in any of the donor groups when MCV seropositive and seronegative donors are compared separately. This work has concentrated on IFNɣ secreting T cells that has been shown to be integral to the control of polyomavirus control in vivo [394]. However, it is possible that variations in frequency of CD8 and CD4 cells producing stimulatory cytokines such as IL-2, or other effector molecules

such as perforin and granzyme differ between the groups investigated and this may be an avenue for further work.

During the ELISpot screening experiments mostly weak responses to the MCV peptides were observed except for when two healthy donors exhibited high frequency responses to particular pools of peptides which were then mapped to specific LT epitopes using T cell cloning. Donor 14461 made a CD4 T cell response to peptide ALD (LT aa631-646) despite being MCV seronegative. It is possible that the response to the peptide may be due to cross reactive recognition from CD4 T cells specific for another polyomavirus to which the donor has been exposed. Indeed, there is a significant amount of sequence homology of the ALD peptide within the polyomavirus family as shown in Table 5.2. Alternatively, it is possible that this T cell response is MCV specific but the donor is seronegative either through primary failure to produce detectable levels of antibodies or the loss of detectable antibodies through the complete clearance of virus following a robust cellular immune response. Whether such a phenomenon can occur following MCV infection is not known, however the finding of T cell clones for the less homologous CD4 epitope LLE (CT 16-30, Table 5.2) from the same donor increases the likelihood that prior infection with MCV may have occurred. Interestingly, it has been shown for JCV that the absence of polyomavirus-specific antibodies is linked with certain HLA Class II alleles leading the authors to suggest that in these cases, the lack of detectable serum antibodies may be a result of a more robust CD4 anti-viral response [395]. In addition to the aforementioned two, a further six novel Class II epitopes were discovered through this work from three MCV seropositive healthy donors.

Importantly, most of the CD4 epitopes identified are present in the region of LT remaining after the truncation mutation seen in the MKL-1 cell line, established from a primary MCC tumour. In the described experiments, we show that CD4 T cells specific for five LT epitopes

are able to recognise and secrete IFNɣ in response to antigen presenting cells fed with lysates made from the MKL-1 cell line. This suggests that these discovered MCV-specific CD4 T cells have the potential to act directly against MHC Class II expressing virally-infected cells as has been shown in other viral infections [396-398]. There is no current strong evidence that MCV has tropism for Class II expressing cells but studies are limited. Alternatively, CD4 cells may act indirectly following uptake of viral antigens by professional antigen presenting cells resulting in anti-viral cytokine production [399], as well as having a role in the maintenance of CD8 memory cell populations during persistent viral infections [287]. As the LT Class II epitopes are shared and potentially expressed by MCC tumour cells as well as virally infected cells, it is also possible that MCV specific CD4 T cells may be able to similarly exert immune control over tumour cells as has been shown with SV40 polyomavirus mediated tumourigenesis in vivo [309].

The second large ELISpot response, seen in MCV seropositive donor 14464, was attributable to CD8+ T cells specific for the novel class I epitope, GPI (LT 593-603). This epitope is found in the full length T-antigen but not the truncated tumour associated T-antigen and was the only Class I epitope discovered during this study. This pattern is similar to the previous published study assessing MCV VP-1 and truncated LT-antigen specific responses in peripheral blood that also found only one CD8 T cell epitope as compared with numerous CD4 responses [385]. Together, these results may suggest that CD4 mediated cellular immunity against MCV may be more dominant than CD8. Indeed, the majority of IFNɣ secretion in response to MCV VP-1 virus like particles is attributable to the CD4 subset [302]. The high frequency of CD4 responses in comparison to CD8 has also been noted in cellular immunity against other members of the polyomavirus family, BKV and JCV, suggesting this may not be a feature exclusive to MCV [288-290, 400].

Using the newly-identified HLA-B7, CD8 epitope, GPI, we investigated the phenotypic characterisation of MCV specific CD8 T cells ex vivo. Although MCV DNA has been isolated from many tissues at low frequency, it has been by far most commonly found on the skin, leading to the assumption that it is a skin-tropic virus. Alongside this, immunohistochemical analysis of MCC tumours has found that the majority of CD8 T cells found within tumours express Cutaneous Lymphocyte Antigen (CLA) [321], a marker found on the majority of T cells recovered from the skin (alongside the chemokine receptors CCR4, CCR6 and CCR10) as compared to 5-10% of circulating PBMCs [259, 401, 402]. Whether this up regulation of CLA expression occurs in the blood or in the skin is not completely clear. Koelle et al assessed circulating CD8 lymphocytes specific for the HLA B7 epitope VP22 from the HSV-2 UL49 open reading frame, finding that 50-80% expressed CLA in comparison to 2-10% of EBV and CMV specific CD8 T cells [403]. This suggests that specialisation towards skin-homing occurs within the circulation. In a small number of MCC patients, using the defined HLA-A24 MCV LT-antigen epitope, 20-80% of tetramer binding cells were CLA expressing, again an increased proportion when compared to CD8 lymphocytes specific for other non-skin tropic viruses CMV and EBV [321].

In our study, one healthy HLA-B7+ donor, 14464, had a sizable GPI-specific CD8 T cell population suitable for phenotypic assessment using tetramer assay. These cells had an effector memory phenotype but, in contrast to HSV-specific cells, did not exhibit the phenotype of skin-homing lymphocytes based on expression levels of CLA. In comparison we saw that in the MCC patient, P84, the tetramer-binding CD8 response exhibited more skin homing characteristics in keeping with that seen in MCC patients in a previous study [321]. It must be noted that the frequency of cells in this donor was very small and so results must be interpreted with caution. As MCC patients tend to have high levels of viral DNA in the skin it

is likely that MCV antigens would have been presented at this site and so memory lymphocytes may continue to maintain this skin-tropic phenotype. As the GPI epitope is not one found in LT-expressing MCC cancers, the CD8 response could not have developed as a result of an anti-tumour immune response. In the healthy donor, the mechanism and site of antigen presentation to CD8 T cells is less certain and may not have occurred via the skin, possibly explaining the lack of skin-homing phenotype seen. It is also possible that the majority of skin-homing lymphocytes are sequestered within the skin and so are not found in examinations of the peripheral blood. This could be further assessed by looking at the phenotype of MCV specific skin infiltrating lymphocytes, however, it is likely that this process would be very complicated by low cell frequency and difficulty obtaining appropriate samples. The examination of virus-specific T cells has been conducted using skin infiltrating lymphocytes, however, this has been preceded by antigenic stimulation in the form of vaccination in order to maximise and localise the numbers of T cells present to enable detailed analysis [269].

Lastly, we saw that PBMCs from two MCV seropositive healthy donors, 14463 and 14464, both made IFNɣ secreting T cell responses following stimulation with GPI peptide in ELISpot assays. However, unlike PBMCs from donor 14464, PBMCs from donor 14463 displayed no detectable tetramer binding. Similar findings of non-tetramer binding, virus specific CD8 T cells have been seen in mice with influenza infections [404] and human donors with chronic hepatitis B [405]. It has been suggested that this occurs through alterations in lipid raft integrity that affects the arrangement of the TCR on the lymphocyte surface [406]. Rather than reflecting low avidity to peptide, this “partial tolerance” to viral antigen may act as a protective mechanism to prevent T cell exhaustion and subsequent deletion in the face of high prolonged antigen exposure as seen in chronic infections. It is therefore possible that a similar

situation is present in donor 14463. Importantly, these virus-specific, non-tetramer binding cells have been shown to remain functional with regard to cytokine production. A second explanation for the lack of tetramer binding is that the response seen in donor 14463 is cross reactive response, particularly as there is such close homology between polyomaviruses at the GPI epitope (Table 5.2). We further investigated this possibility by isolating GPI specific T cell clones from donor 14463 and 14464. CD8 T cell clones from donor 14464 clearly showed increased specificity for the MCV GPI peptide and a single clone from donor 14463 showed specificity for the novel KI/HPyV6/HPyV7/HPyV9 GPI epitope. However, the majority of clones from donor 14463 showed broadly similar responses to the MCV, TSV and KI/HPyV6/HPyV7/HPyV9 peptides. These clones were also able to recognise antigen presenting cells expressing MCV LT. This indicates that these CD8+ T cells do appear to have the ability to respond to homologous GPI epitopes from a number of the polyomavirus family. Cross reactive CD8 T cells against polyomavirus epitopes have been widely described, particularly for shared BK and JC epitopes [291, 294, 299-301] and this work extends this and highlights what may be an important mechanism of cellular immune control of this closely related and growing family of viruses.

Chapter 6

Investigation of immune evasion properties of MCV LT and sT-antigens

6.1 Introduction

The data presented in the previous chapter, confirms and extends our knowledge that there are epitopes on the MCV LT and sT-antigens that can be recognised by CD4 and CD8 T cells. This suggests that, as MCV LT (truncated) and sT are expressed by the majority of MCC cells, tumours can be potentially recognised and killed through T cell mediated mechanisms. Indeed, this is supported by clinical data that positively correlates CD8 T cell infiltration into MCC tumours with improved survival [311]. However, the avoidance of destruction by the immune system is a recognised hallmark of cancer, as defined by Hanahan and Weinberg [407]. In virus-associated cancers, viral proteins may be involved in this process of immune evasion through a number of mechanisms involving both innate and cellular immune pathways. The impact of immune evasion in MCC and the role of viral proteins integrated within the cancer cell genome is not well understood.

All nucleated cells express MHC Class I on the cell surface enabling T cell recognition. The down-regulation of MHC Class I alongside other molecules involved in Class I antigen processing such as TAP-1 and -2 has been described and associated with poorer prognosis in numerous cancers, such as melanoma [408, 409]. In MCC tumours, surface MHC Class I expression assessed by immunohistochemistry is low or undetectable in over 50% of cases and this is seen significantly more frequently in MCV+ as compared with MCV negative tumours. Alongside this, in MCV+ MCC tumour cell lines (MKL-1and WaGa), the expression of MHC Class I is also reduced when compared with MCV negative MCC tumour cell lines (MCC13, MCC26) [320]. The surface expression of MHC Class I in MCV+ cell

lines can be up-regulated through the use of treatment modalities included IFN-β, etoposide chemotherapy and ionizing radiation [320]. Conducting mRNA analysis of a subgroup of MCC tumours showed that reduced expression of MHC Class I was strongly correlated with the reduced expression of β2 microglobulin and TAP -1 and TAP-2, suggesting dysregulation of multiple molecules from the Class I antigen processing pathway. The role, if any, that the tumour expressed MCV LT and sT play in regulation of MHC Class I expression of infected cells is not known and may represent an important mechanism of virally mediated immune evasion, similar to other immune evasion proteins expressed by viruses such as EBV [410, 411]. It is also not known whether the up regulation of MHC Class I is by itself sufficient to allow recognition by CD8 T cells or if other co-existing mechanisms of evasion will prevent this.

CD4 T cell recognition is mediated though interaction with MHC Class II molecules. Unlike Class I, constitutive Class II expression is classically limited to a small number of cell types, namely professional antigen presenting cells; dendritic cells, macrophages and B cells. Other cell types, such as fibroblasts, endothelial and epithelial cells can be induced to express Class II under certain conditions, through activation of the Class II transactivator (CIITA) gene (reviewed in [412]). As a number of CD4 epitopes have been defined for MCV LT and sT, it is important to know whether Merkel cells fall into either of these categories. There is no published data to our knowledge that describes the level of Class II expression on Merkel cells and given the low frequency of these cells in the skin, this may be difficult to characterise. The expression of Class II in MCC primary tumours and cell lines has also not been described to our knowledge. Immune evasion through modulation of Class II has been seen with other viral proteins such as the KSHV latent protein vIRF3 [413] but any similar function of MCV T-antigens are not clearly defined.

The aims of this chapter are initially to confirm using primary tumour cells and MCC cell lines, that MHC Class I expression on the cell surface is down-regulated as well as determining MHC Class II surface expression status on these same cell types.

Secondly, using MCC cell lines, we will assess the effect of IFNɣ and ionising radiation on MHC Class I and Class II expression in addition to the effect ionising radiation has on the ability of CD8 T cells to recognise MCC cell lines.

Lastly, we will use a model antigen presenting system to see whether the MCV LT and ST-antigens have any role in MHC Class I and II regulation and/or the ability of LT/ST expressing cells to be recognised by CD4 and CD8 T cells.

6.2 Results

6.2.1 MHC Class I and II expression on primary MCC tumour cells

A wide range of tumours, including MCC, down-regulate expression of MHC Class I in order to evade detection by CD8 T cells. There is little known of the expression of MHC Class II on MCC tumours. Therefore we evaluated MHC Class I and II expression on surface of MCC tumour cells. Due to the scarce availability of MCV+ clinical samples, this was only possible on a single tumour specimen from patient P164 (MCV+). A small portion of tumour was minced into a cell suspension, filtered and stained with CD3, CD56, CD45, MHC Class I and Class II fluorochrome-conjugated antibodies and analysed on flow cytometer. Figure 6.1 shows that tumour associated lymphocytes (CD45+) cells expressed Class I and Class II, however, both Class I and Class II expression is low on tumour cells (CD56+, CD45-). Although limited to a single tumour specimen, this experiment confirms previous work that shows MCC tumours express little surface MHC Class I. It also suggests that MCC tumours do not express high levels of surface MHC Class II.

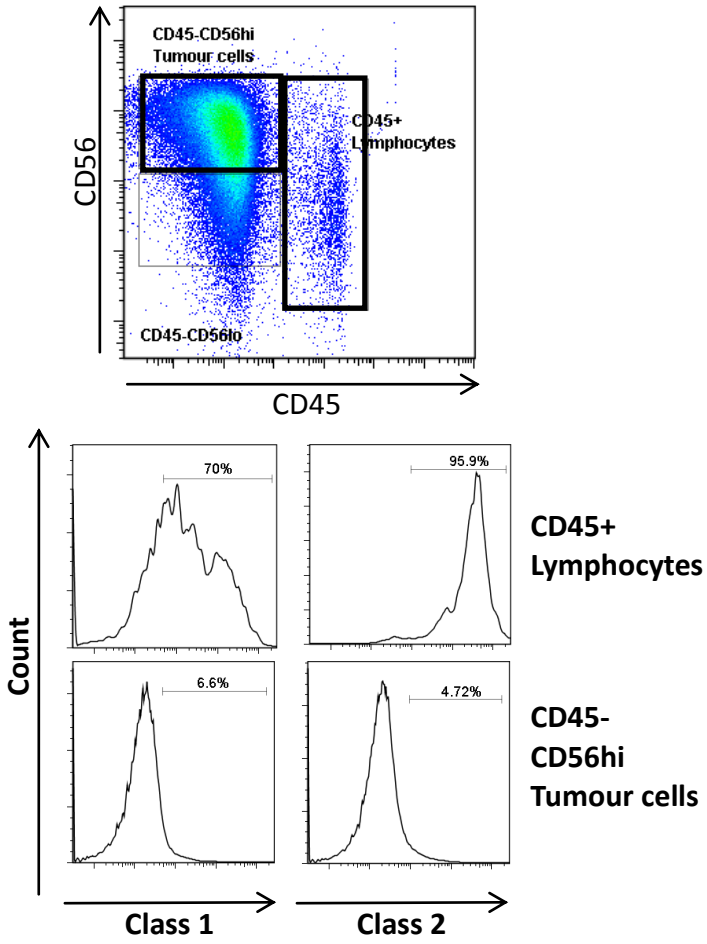


Figure 6.1. Surface MHC Class 1 and Class 2 expression on P164 MCC tumour cells and tumour associated lymphocytes. Tumour was minced and stained with fluorochrome-conjugated markers to differentiate lymphocytes (CD45+) from tumour cells (CD56+, CD45). Surface MHC Class 1 and 2 levels were compared between the two populations.

6.2.2 MHC Class I levels on MCC tumour cell lines and the impact of IFNɣ

A previous study has shown that, like the majority of primary tumours, MCV+ MCC cell lines express very low levels of MHC Class I compared when compared with MCV negative cell lines but that this could be up-regulated through stimulation with IFNs. We sought to confirm this using the MKL-1 (MCV+) and MCC13 (MCV negative) cell lines and figure 2 shows staining both cell types for MHC Class I cell surface expression. This confirms that MKL-1 cells had barely detectable levels of MHC Class I, while there was clear expression of in MCC13 cells. Incubation in the presence of IFNγ for 72 hours, led to surface MHC Class I up-regulation on both cell types. This confirms that the MCV+ cell line, MKL-1 expresses low levels of MHC Class I when compared to the MCV negative cell line, MCC13. In both cell types, Class I levels can be up-regulated through incubation with IFNɣ.

6.2.3 MHC Class II levels on MCC tumour cell lines and the impact of IFNɣ

Although only a subset of cells express MHC Class II on the surface, other cell types, such as fibroblasts, can be induced to express this through different mechanisms including stimulation with IFNs. We were interested to see if MCC tumour cell lines express MHC Class II and whether expression was altered by IFNɣ. We assessed surface MHC Class II expression on MKL-1 and MCC13 cell lines as well as fibroblasts, finding that MHC Class II expression was not seen (Figure 6.2). Following exposure to IFNɣ for 72 hours this was not altered in MKL-1 or MCC13 cell types, however, up-regulation was seen in the fibroblast cells. This suggests that both MCV+ and MCV negative MCC cell lines do not natively express MHC Class II, nor can they be induced to do so with IFNɣ.

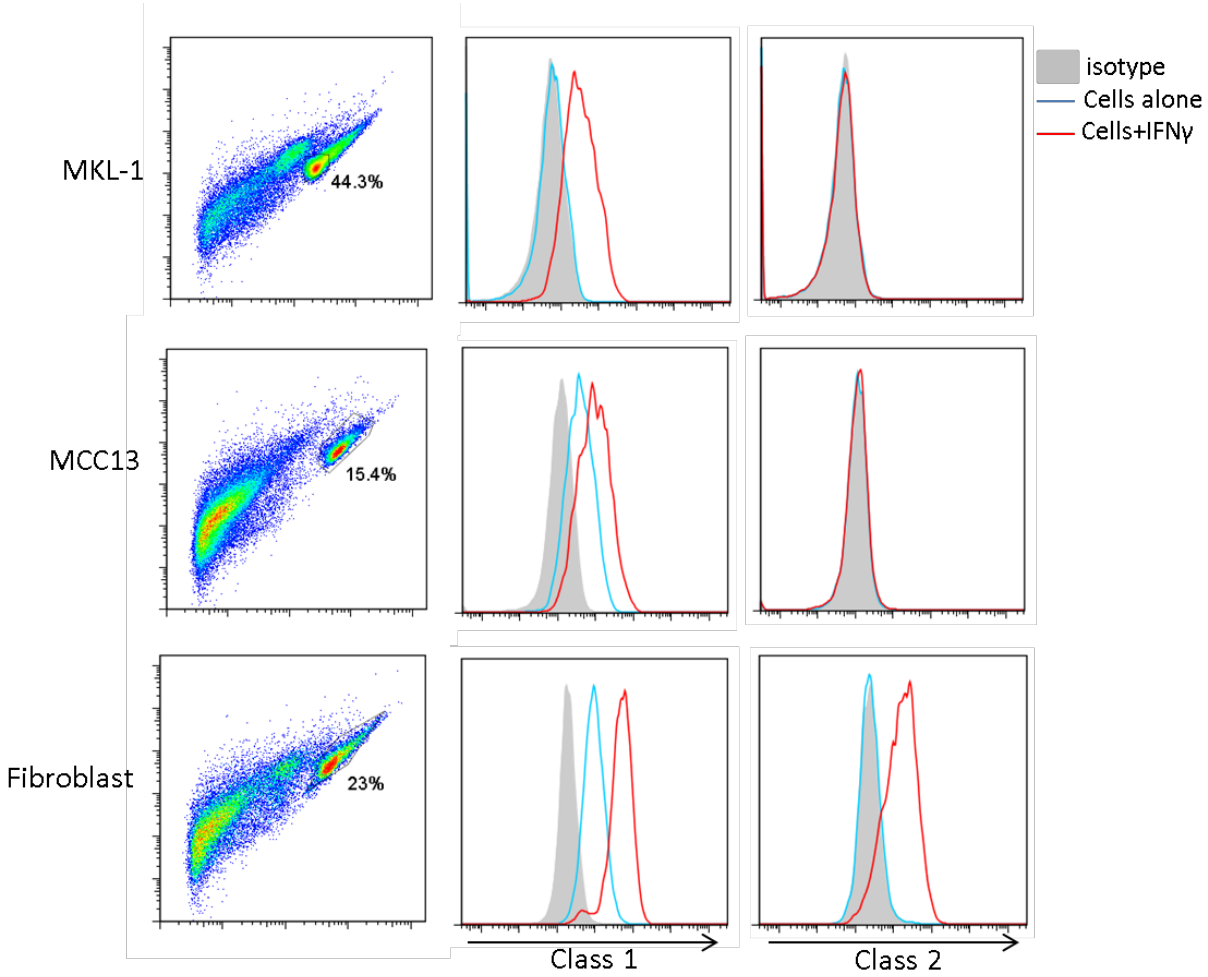


Figure 6.2. Surface Class I and Class II expression on MCC cell lines and fibroblasts with and without stimulation by IFNγ. MCC cell lines, MKL-1 (MCV+) and MCC13 (MCV-) were assessed for expression of Surface MHC Class I and Class II by flow cytometry after 72 hours incubation with 50IU/ml IFNγ (red line) or media alone (blue line). Fibroblast cells, known to up-regulate Class I and Class II upon stimulation with IFNγ were assayed in parallel to act as experimental control. Isotype controls are shown as shaded regions.

6.2.4 Effect of ionising radiation on MHC Class I and Class II on MCC cell lines

Ionising radiation is a well-recognised mode of anti-cancer therapy. Alongside direct tumour cell killing, it has also been shown to have immunogenic properties such as up-regulation of MHC Class I and II in some cancer cell lines [414, 415]. Using our MCC cancer cell lines, we investigated the effect of different doses of ionizing radiation on MHC Class I and II surface expression levels (Figure 6.3). Cells were treated with varying doses of radiation (0 Gy, 4 Gy or 10Gy). We also analysed the impact of radiation on MelJuSo (MJS) cells that constitutively express Class I and Class II as a control cell line. In MJS cells, there was minor up-regulation with Class I and Class II following exposure to 4 and 10Gy. The MCV negative MCC13 cell line showed MHC Class I expression level similar at baseline to that seen with MJS cells and there was no obvious increase upon exposure to radiation. In MCC13 cells, MHC Class II was seen neither at baseline nor following radiation. The low baseline levels of MHC Class I expression on the MCV+ MKL-1 cell line could be up-regulated with exposure to ionising radiation, although there was no obvious difference between the two radiation doses. As with MCC13, there was no appreciable expression of MHC Class II at baseline and no change with exposure to ionising radiation. In summary, these experiments show that in MCC cell lines, MKL-1 cells can be induced to up-regulate MHC Class I upon exposure to relatively low doses of radiation. However, irradiation of MCC13 and MJS cell lines that already express Class I on their surface does not significantly alter expression. With regard to MHC Class II expression, in natively expressing Class II MJS cells there is some increase in levels associated with radiation exposure. Irradiating MCC cell lines that do not natively express Class II, has no impact on Class II levels.

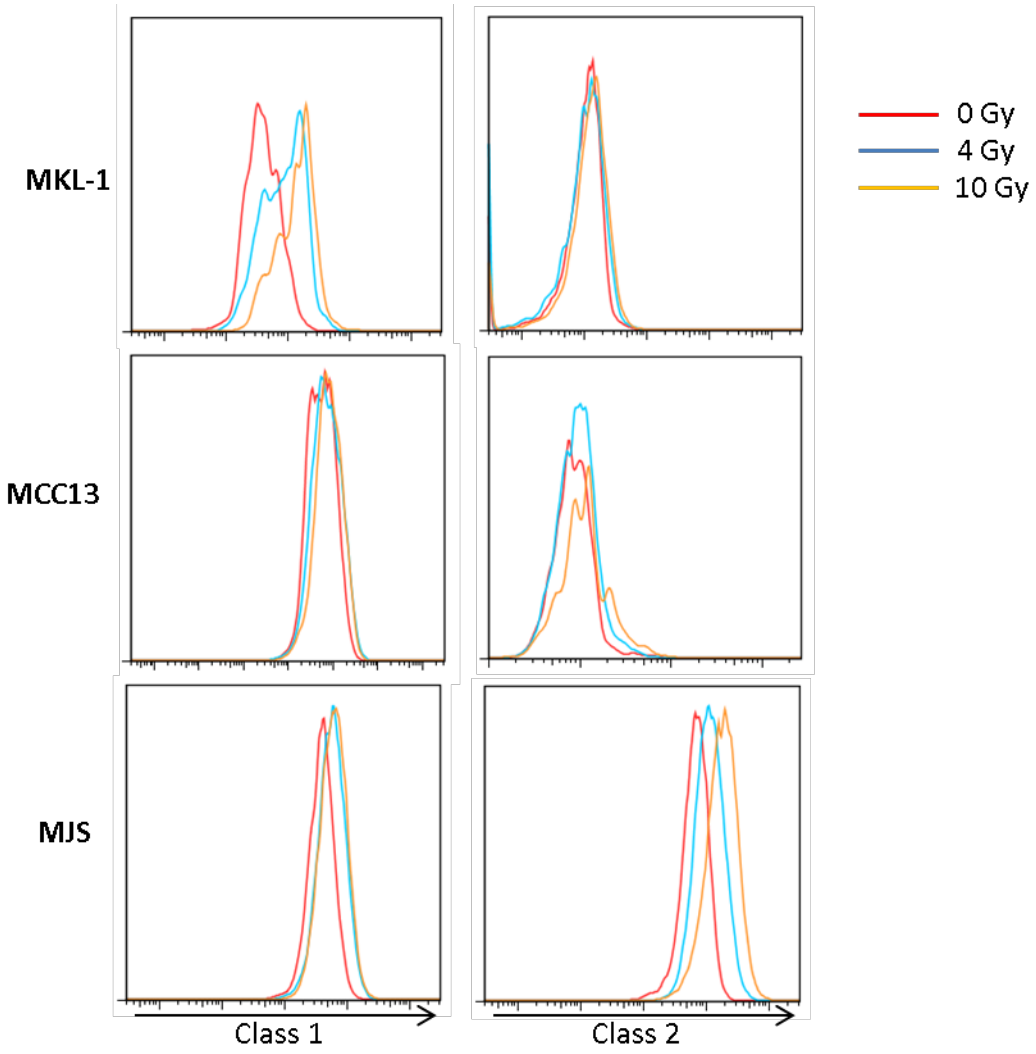


Figure 6.3. The impact of radiation on surface MHC class I and II levels on MCC cell lines. MKL-1 and MCC13 cell lines were exposed to 0 (red), 4 (blue) or 10 (orange) Gy doses of radiation and surface MHC class I and II levels were assessed by flow cytometry. MelJuSo (MJS) cells known to express both Class I and Class II naturally were also exposed to the same radiation doses and acted as positive control in experiment.

6.2.5 Impact of ionising radiation on recognition of MCC Cell lines by CD8 T cells

The previous set of experiments showed that the MCV+ MKL-1 cell line expresses very low levels of MHC Class I, but that this can be up-regulated with exposure to irradiation. The MCV negative MCC13 cell line, does express MHC Class I and this is maintained with exposure to ionising radiation. We were next interested to see whether MKL-1 and MCC13 cells were capable of processing and presenting antigen on the cell surface and the effect ionising radiation had on this. HLA typing analysis showed that both cell lines expressed the Class I HLA-A11 allele. Using a modified vaccinia virus Ankara construct expressing the EBV EBNA3B gene (MVA-EBNA3B, kind gift from Dr G Taylor) we infected these cell lines to induce expression of this reporter antigen. This antigen contains the HLA-A11 CD8 epitope, IVT, for which specific T cell clones were available to be used in T cell recognition experiments. MKL-1 and MCC13 cells were irradiated (0, 4 and 10Gy) and these were then infected for two hours at a multiplicity of infection (MOI) of 10 by either the MVA-EBNA3B, a control MVA-PSC11 (MVA-PSC) that did not express EBNA3B, or loaded with 5µg/ml IVT peptide. In parallel, HLA-A11 expressing EBV transformed LCL cells, known to be capable of antigen processing and presenting were also similarly treated to act as a positive control for the experiment. After infection/peptide loading, cells were washed thoroughly and then $1x10^5$ cells/well were added, in duplicate, to a 96 well plate with $5x10^3$ cells/well of IVT-specific T cell clone. T cell recognition was then assessed after 18 hours using IFNɣ ELISA assay and these results are summarised in Figure 6.4.

As expected LCL cells, both when infected with MVA-EBNA3B or when peptide loaded, elicited high levels of IFNɣ release from T cells. It was noted that there was low level recognition of LCL infected with the control MVA due to the presence of endogenous EBNA3B in these cells. The MCC13 cells not exposed to radiation elicited similar levels of T

cell recognition after both MVA-EBNA3B infection and peptide loading (~15000pg/ml). The amount of IFNɣ produced following MVA-EBNA3B infection was similar to that seen with the LCL. Exposure of MCC13 cells to 4 or 10Gy radiation did not appear to affect IFNɣ production compared with non-irradiated cells. MKL-1 cells loaded with peptide or infected with EBNA-3B MVA did provoke low level IFNɣ secretion from IVT specific T cells (~300pg/ml). This was not seen after infection with the control MVA. There was no change in IFNɣ secretion from T cells after irradiation of peptide loaded MKL-13 cells at either 4 or 10Gy doses. There was also no dramatic change in IFNɣ secretion after irradiation of EBNA-3B MVA infected MKL-1 cells, although there was a suggestion of an increased production of IFNɣ at the 4Gy dose which was not maintained at the 10Gy dose.

Overall, this experiment shows that MCC13 cells are capable of processing and presenting EBNA-3B to IVT specific T cells. Although MKL-1 cells are also able to process and present antigen, the amount of IFNɣ secreted by T cells following peptide loading or EBNA-3B MVA infection is approximately 100 fold less than seen with MCC13 cells. In this assay, irradiation does not appear to alter the ability of either MCC13 or MKL-1 cells to process and present antigen.

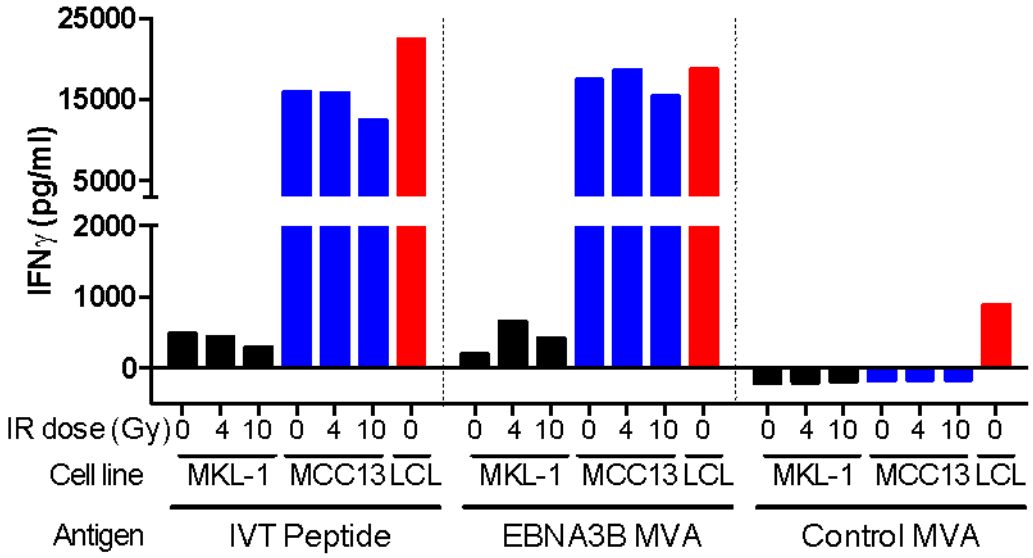


Figure 6.4. CD8 T cell recognition pre and post radiation using MCC cell lines infected with MVA expressing reporter antigen EBNA3B. MKL-1 (black bars) and MCC13 cells (blue bars) were irradiated with 0, 4 or 10Gy and were then either loaded with 5µg/ml IVT peptide or infected with the EBNA3B MVA or empty pSC11 (control) MVA at an MOI of 10. To act as positive control, non-irradiated HLA-A11 LCL cells with functional antigen processing and presentation machinery were also infected with peptide or MVA (red bars). $2.5x10^4$ of each antigen presenting cells were then plated in duplicate with $5x10^3$IVT specific CD8+ T cells in 96 well plates and T cell recognition quantified by IFNγ ELISA after 24 hours.

6.2.6 The impact of MCV LT and sT on Surface MHC Class I expression

We, and others, have shown that the MCV+ MCC cell line, MKL-1 expresses lower levels of MHC Class I at the cell surface when compared with MCV negative cell line, MCC13. We were, therefore, interested to see whether the MCV tumour associated proteins, LT or sT, are involved in the down regulation of surface Class I levels. To test this, DNA constructs corresponding with wild-type LT, two tumour-derived truncated LT (MCC350 and MKL-1) and sT were cloned into the pCDNA3-IRES-NLS-GFP plasmid. This is a bicistronic vector that allows simultaneous expression of both protein of interest alongside GFP using an Internal Ribosomal Entry Site (IRES) from the same RNA transcript (see Figure 6.5). These

constructs were then individually transfected into MJS cells and then surface MHC Class I expression was analysed after 24 and 48 hours by flow cytometry through comparison of live, transfected (GFP+) and non-transfected (GFP-) cell subsets. The EBV genes BNLF2a and BDLF3 which are both known to down-regulate MHC Class I were also cloned into the same vector and used as positive controls (kind gifts from Dr A Hislop and Dr L Quinn respectively). The empty pCDNA3-IRES-NLS-GFP plasmid (henceforth called pCDNA3) was used as a negative control.

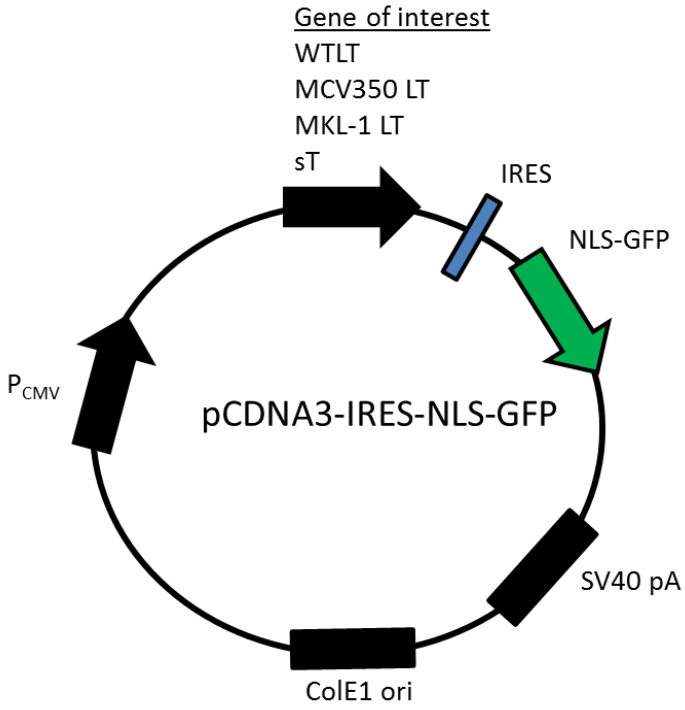


Figure 6.5. Schematic diagram of MCV T-antigen expression constructs. Expression of wild-type Large T (WTLT), two tumour-associated truncated Large T (MCV350, MKL-1) and Small T (sT) was achieved through individual insertion of genes into the pCDNA3-IRES-GFP vector using a CMV promoter (P_{CMV}). The construct also contained an internal ribosomal site (IRES) GFP allowing for identification of transfected cells by flow cytometry.

As shown in Figure 6.6, cells transfected with the pCDNA3 showed no change in Class I expression compared with non-transfected cells. As expected, there was a down-regulation of Class I expression in both BNLF2a and BDLF3 transfected cells compared with non-transfected cells at 24 hours, but this became less pronounced at 48 hours. Transfection with any of the MCV genes (WT LT, MCV 350 LT, MKL-1 LT and sT) had no impact on surface Class I levels at either 24 or 48 hours. Overall, this experiment suggests that transient transfection with MCV LT, truncated LT or sT is not capable of down-regulating surface Class I levels on MJS cells.

6.2.7 The impact of MCV LT and sT on Surface MHC Class II expression

As our earlier experiments had found that surface MHC Class II expression was low in both MCC primary tumour cells and MCC tumour cell lines, we were interested in uncovering whether MCV T-antigens had any role to play in this. Using the same model system as described in the above section, GFP-tagged plasmids containing MCV LT, truncated LT and sT genes were transfected into MJS cells. Again, the empty pCDNA3 vector was used as a negative control. Transfection with genes known to down-regulate surface Class II, the EBV gene BDLF3 and the KSHV gene vIRF3, were used as experimental positive controls. In Figure 6.7, it is shown that transfection with the empty control pCDNA3 vector has no impact on the Class II level. Transfection with BDLF3 shows down-regulation of Class II at 24 hours but this is lost at 48 hours. In contrast, transfection with vIRF3, shows no change in Class II levels at 24 hours, but some slight reduction at 48 hours. MCV WT LT, truncated LT and sT show no impact on surface class II expression at either 24 or 48 hours. These experiments suggest that transient transfection with MCV T-antigens are not capable of affecting surface Class II levels.

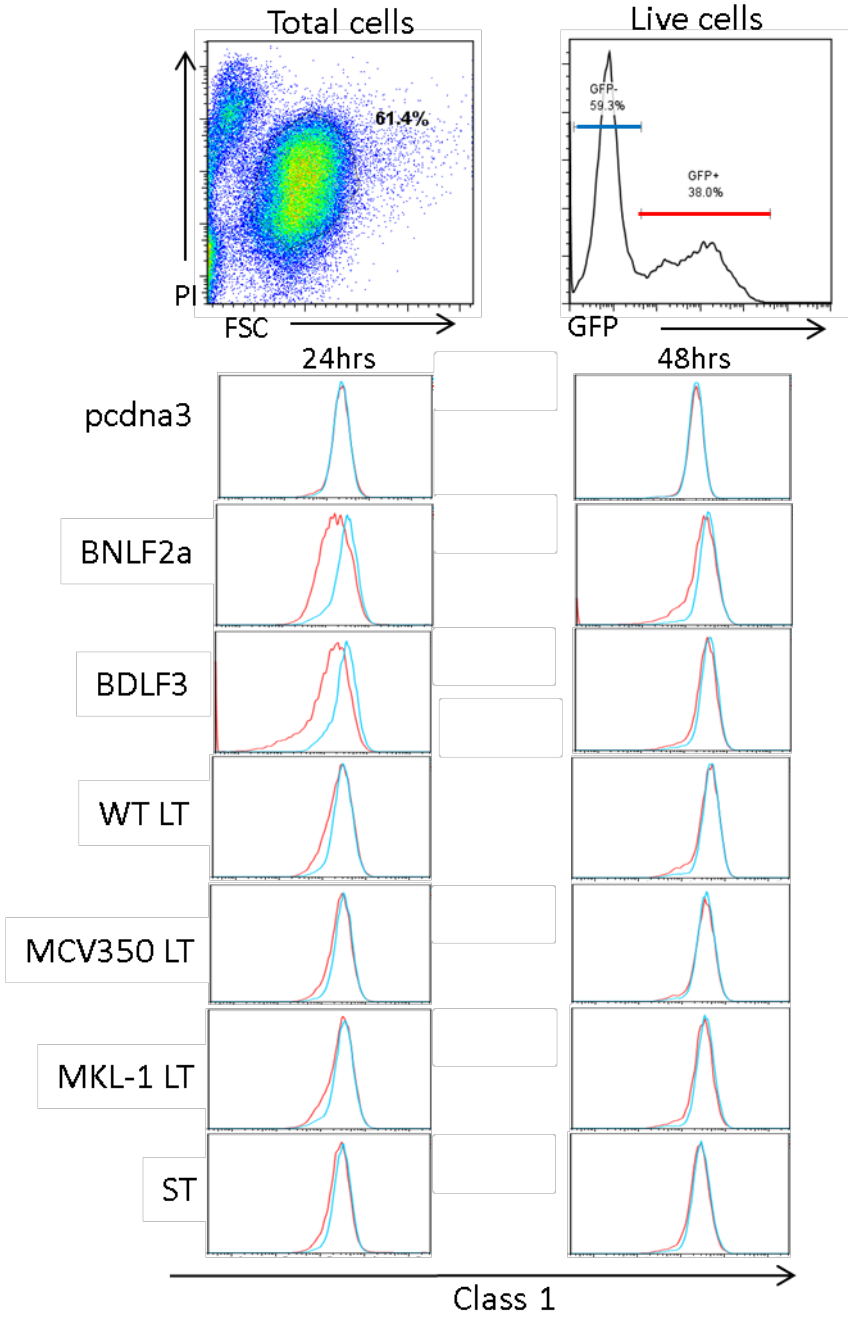


Figure 6.6. Effect of MCV T-antigen genes on surface Class I expression of MJS cells. MelJuSo (MJS) cells were transfected with GFP-tagged pcdna3 plasmid constructs containing genes of interest. Surface Class I was assessed at both 24 and 48 hours post transfection comparing transfected, GFP+ cells (red) with non-transfected GFP- cells (blue). Plasmid containing no gene (pcdna3) was used as negative control. EBV genes BNLF2a and BDLF3 known to down-regulate Class I were used as positive controls.

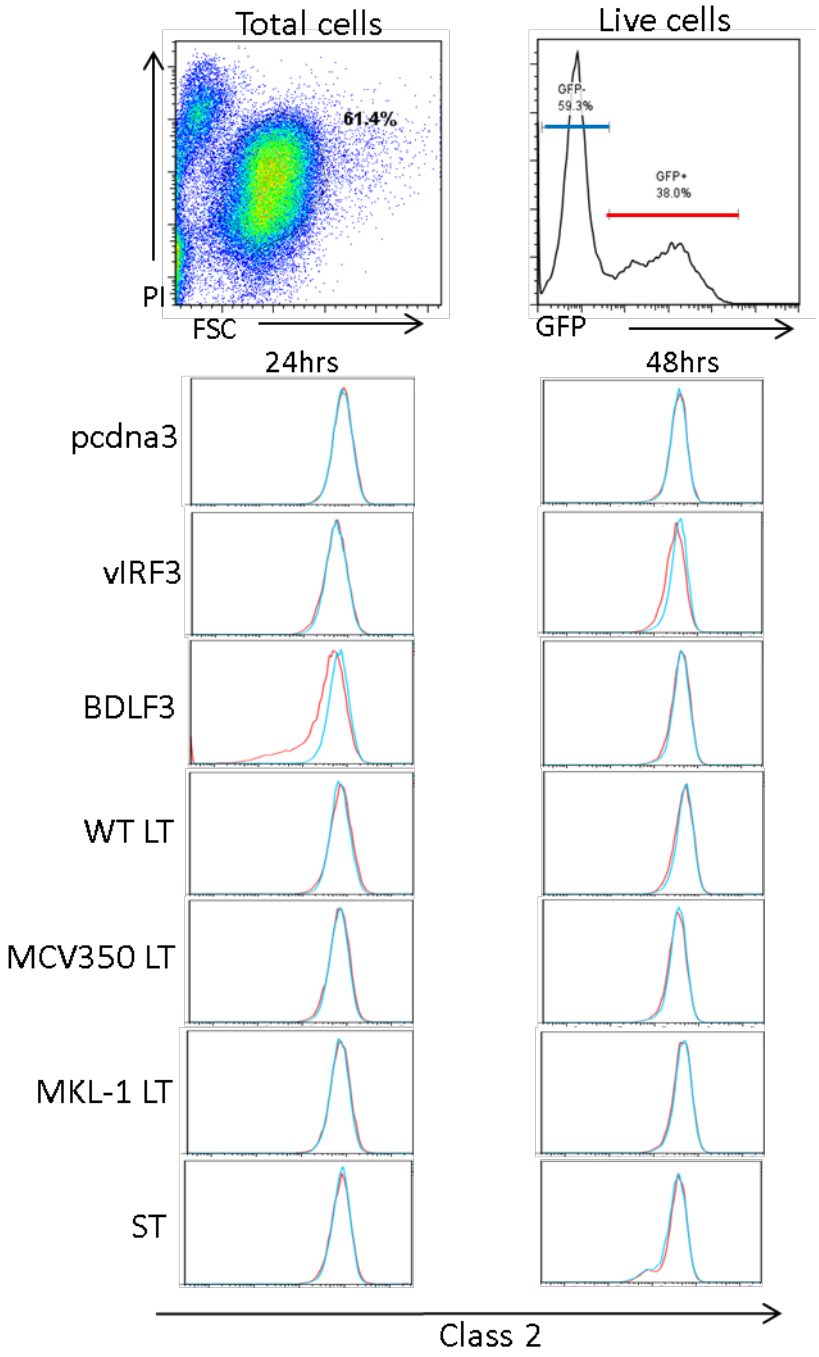


Figure 6.7. Effect of MCV T-antigen genes on surface Class II expression of MJS cells. MelJuSo (MJS) cells were transfected with GFP-tagged pCDNA3-IRES-nls-GFP plasmid constructs containing genes of interest. Surface Class II was assessed at both 24 and 48 hours post transfection comparing transfected, GFP+ cells (red) with non-transfected GFP- cells (blue). Plasmid containing no gene (pcdna3) was used as negative control. KSHV gene vIRF3 and EBV gene BDLF3 known to down-regulate Class II were used as positive controls.

6.2.8 The effect of MCV T-antigens on recognition of reporter antigens by CD8 and CD4 T cells

The previous experiments suggested that MCV T-antigen proteins did not appear to have a role in immune evasion through direct down-regulation of surface Class I or II in the model system used. However, it remains possible that MCV proteins may affect the recognition of virally-infected or MCC tumour cells by either CD8 or CD4 T cells through other mechanisms. In order to assess the impact of MCV LT and sT proteins on epitope recognition by T cells, a model system utilising EBV reporter antigens and appropriate CD4 and CD8 T cell clones was used. For the CD8 T cell recognition work, MJS cells were co-transfected with individual plasmids encoding the MCV T-antigen genes described above (Figure 5) together with a plasmid encoding the EBV gene BZLF1 which encodes the HLA-B8 restricted CD8 peptide, RAK, as a target reporter antigen (kind gift from Mr Calum Forrest). For the CD4 T cell recognition assays, plasmid encoding EBNA1, which encodes the HLA-DR51 restricted CD4 peptide, SNP (kind gift from Dr Graham Taylor) were co-transfected with the MCV T-antigen gene constructs. As MJS cells do not naturally express the DR-51 allele, MJS cells engineered to stably express this allele though retroviral transduction were used (kind gift from Dr Jianmin Zuo [416]). After 24 hours, co-transfected cells were harvested and percentage of live, transfected cells (as defined using flow cytometry staining gating on propidium iodide negative and GFP positive cells) were quantified. Duplicate wells of a 96 well plate were then filled with $1x10^4$ live transfected cells and equalised with the addition of non-transfected MJS cells, such that the total number of cells in each well was the same. Finally, $5x10^3$ T cell clones specific for either RAK epitope (CD8 assay) or SNP epitope (CD4 assay) were added to each well and T cell recognition measured the following day using IFNɣ ELISA.

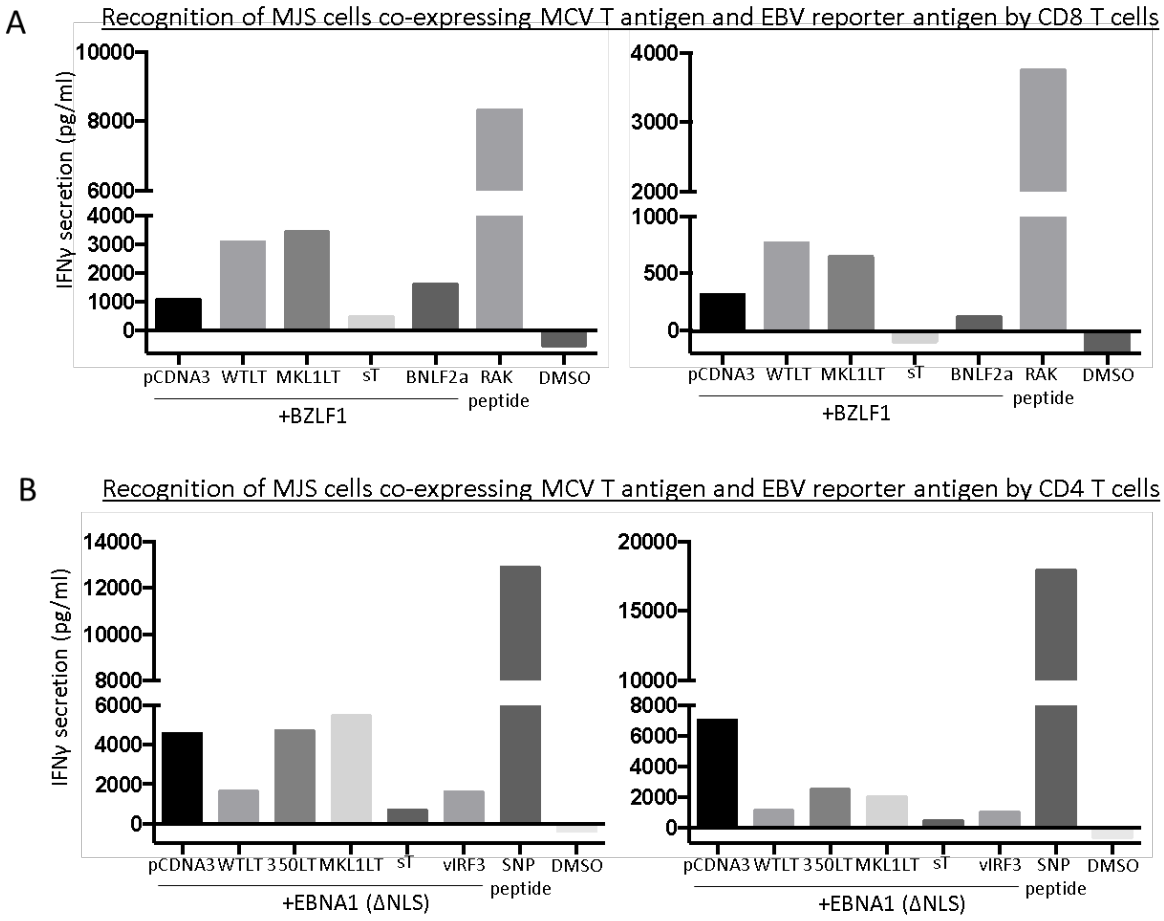


Figure 6.8. CD4 and CD8 T cell recognition of transfected MJS cells co-expressing MCV LT, truncated LT or sT alongside an EBV reporter antigen. (A) MJS cells were transfected with pCDNA3-IRES-nls-GFP plasmids expressing individual MCV T-antigens, wild type LT (WTLT), two tumour-associated truncated LT (350LT and MKL1LT) and Small T (sT). The known EBV CD8 immune evasion protein, BNLF2a expressed in the same vector acted as positive control. Plasmid expressing EBV BZLF1 gene was co-transfected as reporter antigen. 24 hours post transfection, IFNγ secretion measured by ELISA following stimulation of transfected MJS cells with RAK specific CD8 T cells. (B) MJS cells were transfected with the same pCDNA3-IRES-nls-GFP plasmids expressing individual MCV T-antigens as mentioned above. The known KSHV CD4 immune evasion protein, vIRF3 expressed within the same vector as a positive control. Plasmid expressing the EBV gene with a deletion of nuclear localisation signal, EBNA1 (ΔNLS) was co-transfected to act as reporter antigen. 24 hours post transfection, IFNγ secretion measured by ELISA following stimulation of transfected MJS cells with SNP specific CD4 T cells.

Figure 6.8A shows results from duplicate experiments assessing the effect of viral gene transfection on the recognition of MJS cell expressing the EBV BZLF1 epitope by RAK-specific T cells. In one assay, there appeared to be a reduction in T cell recognition with MCV sT to a similar extent to that seen with the known CD8 immune evasion EBV gene, BNLF2a, used as a positive control. However, in the duplicate experiment, although the reduction of recognition by sT expressing cells was maintained, there was no change in recognition of the BNLF2a expressing cells. In both experiments, the WT and the MKL-1 truncated LT appeared to have a positive effect on T cell recognition with increased IFNɣ secretion seen when compared with the transfection of the control pCDNA3 vector. Unfortunately due to time constraints, it was not possible to perform further replicate experiments to confirm these findings.

Figure 6.8B shows duplicate experiments assessing the effect of viral gene transfection on the recognition of MJS cells expressing the EBV EBNA1 gene by a SNP epitope-specific CD4 T cell clone. In these assays, both MCV WT LT and MCV sT appeared to impair recognition by CD4 T cells to a similar level seen with the known KSHV immune evasion gene, vIRF3. However, there were inconsistent results with the truncated forms of MCV LT, MCV350 and MKL-1 which in one assay appeared to have an effect on reducing CD4 T cell recognition though not to the same extent as the WT LT or sT while in the other appearing to have no effect compared with transfection with the control pCDNA3 vector. Again, unfortunately it was not possible to perform further replicates of this experiment to confirm the findings.

These experiments suggest that MCV T-antigens may have an impact on recognition by both CD8 and CD4 T cells. The MCV sT gene appears to impair recognition of both CD4 and CD8 T cells, whilst MCV WT LT has a negative impact on CD4 recognition only. Both WT and truncated forms of MCV LT seem to increase recognition by CD8 T cells while the effect of

truncated LT on CD4 recognition remains uncertain. It must be noted that there were some inconsistencies demonstrated in these assay replicates and so further work is needed to confirm findings before definitive conclusions can be made.

6.3 Discussion

In the work described, we have investigated the Class I and Class II expression of MCC tumour cells and cell lines as well as the antigen processing and presentation ability of MCC cell lines. We have also assessed whether the MCV LT or sT-antigens have properties that may affect recognition of cells expressing these antigens by T cells.

All nucleated cells can express MHC Class I and thus virally infected cells should present antigen directly to CD8 T cells which can then go on to kill the cell. In order to avoid recognition, viruses have evolved mechanisms to minimise their impact, such as inhibiting surface MHC Class I expression and this feature is retained in many virally-induced cancers [417]. Recently, it was shown that MHC Class I down-regulation is prevalent in MCV+ MCC tumours but not as common in MCV negative tumours. [320]. We have confirmed that MCV+ MCC tumour cells and the MCV+ MCC cell line, MKL-1, express low levels of MHC Class I compared with the MCV negative MCC13 cell line. The exact mechanism for the down-regulation of Class I is unknown and likely to be multi-factorial. In the previously mentioned study, the Class I-low MCC tumours all commonly down-regulated mRNA levels for the class I heavy chain, β2 microglobulin and TAP suggesting multiple proteins of the Class I pathway are being simultaneously affected [320]. Given the finding that MHC Class I levels are lower in MCV+ MCC compared with MCV negative, potentially the MCV genes expressed by MCC, namely LT and sT, may play a role in Class I down-regulation. In our assays the transient transfection of LT (both the WT and truncated forms) or sT had no impact on surface Class I levels at 24 or 48 hours, suggesting that these MCV proteins are not solely responsible for changes in Class I expression. It is also possible the effects on Class I take longer than 48 hours to become apparent or that the high-level, short-term expression of these proteins in our experiment does not replicate the effects of longer-term, low-level expression

that may be seen in an MCV infected or MCC tumour cell. Developing stably-transfected LT and sT expressing cell lines in which levels of expression can be regulated through inducible gene promoters could further address this question.

As tumours that have down-regulated Class I will likely have reduced susceptibility to CD8 T cell mediated cytotoxicity, interventions aimed at reversing this process may be useful to re-instate a anti-tumour cell immune response. Interferons are able to induce MHC Class I up-regulation through the stimulation of Interferon Regulatory Factors (IRFs) which can directly activate Class I genes [418-420]. Radiation is also able to up-regulate Class I by causing degradation of cellular proteins resulting in the production of increased peptides which leads to higher levels of TAP activity [421]. Both IFNɣ and irradiation led to an increase in MHC Class I levels in MCV+ MCC cell lines in our experiments, in agreement with results from a previous study [320]. This change in Class I levels may have been expected to also lead to an improvement in recognition of the MCC MKL-1 cell line by CD8 T cells. In our assay using an EBV reporter antigen, radiation appeared to have little impact on the ability of T cells to recognise the MKL-1 cells as measured by IFNɣ secretion. Previously published work, using chromium release assays have shown that CD8 T cell mediated cytotoxicity increases following radiation of cancer cell lines [422]. However, rather than being IFN-mediated, it appears that this increased cytotoxicity is driven by Fas-Fas ligand interaction between T cell and tumour cell [423]. This may explain why we did not see an increase in IFNɣ secretion from CD8 T cells following irradiation of MCC cell lines. Additionally, our assay measured the recognition of one specific T cell clone and its cognate peptide epitope. As radiation driven Class I up-regulation is thought to occur through the creation of novel peptides, it is possible that although in our assay recognition of a single epitope remains unchanged, the same stimulation in vivo would increase the overall breadth of T cell response and lead to an

overall increased amount of T cell recognition. Interestingly, Chapuis et al, performed an in vivo experiment using a patient with metastatic MCC treated with a combination of irradiation or intralesional interferon-β as well as infusion of polyclonal MCV T-antigen specific T cells [424]. This resulted in an up-regulation of MHC Class I on the surface of tumour cells, an accumulation of tumour-specific T cells within metastatic deposits and tumour regression. It is unlikely that changes in Class I levels is solely responsible for causing T cell infiltration into MCC tumours as when such tumours have been examined histologically, there is no direct correlation between Class I levels and T cell infiltration [320]. Overall, the use of radiotherapy or IFN treatments is likely to provide clinical benefit in MCC through modulating tumour immunogenicity. However, the underlying mechanism behind this is likely to be multi-factorial and not just governed by changes in MHC Class I levels.

Evasion of the immune system by viruses and virally-induced tumours can also affect the MHC Class II pathway. To our knowledge, investigation of the Class II surface expression on Merkel cells or MCC has not been conducted and so the potential importance of Class II in the context of MCV and MCC has been thus far undefined. As Merkel cells are thought to have a sensory rather than professional antigen presenting function, native surface Class II expression would not be expected. However, given these cells are found only in the skin and also at low frequency, to confirm this experimentally would be very difficult. The use of MCC tumours and cell lines provides an opportunity to investigate Class II expression in cells that are more readily available. In our assays, primary tumour cells and MCC cell lines did not appear to express detectable levels of MHC Class II on the cell surface. As with our Class I results, the transient transfection of MCV LT or sT did not appear to have any impact on Class II surface levels suggesting these proteins are not responsible for the failure to express

Class II. However, the same cautions must be taken when interpreting these results as documented above with our Class I data.

Some non- professional antigen presenting cells such as fibroblasts and endothelial cells that do not ordinarily express Class II can be induced to express it commonly as a response to IFNɣ stimulation. This activates downstream signalling pathways such as JAK/STAT and activates the pIV promoter of the CIITA gene leading to the up-regulation of Class II associated antigen presentation machinery [412, 425]. Although less clearly defined, irradiation has also been shown to increase Class II expression in some tumour cell lines [414]. In our described assays, IFNɣ or irradiation could not induce Class II expression on MCV+ or MCV negative MCC cell lines. The reasons for this are unknown but may be related to these cells having reduced responsiveness to IFNɣ stimulation through a block in the JAK/STAT pathway preventing activation of the CIITA promoter [426]. Whether the MCV T-antigen has a any role in this process is not known, although there is evidence that murine polyomavirus LT can block activity of this pathway through direct binding to and inactivation of JAK1 [427]. It has also been shown that TGF-β is able to inhibit Class II up-regulation by IFNɣ [428] and this is potentially a further pathway that may be important as TGF-β can be up-regulated within tumours [429]. The potential benefit of MCV inhibiting Class II expression is not clear, due to a lack of knowledge of the wider cellular tropism of the virus. There is some recent evidence that dermal fibroblasts are a candidate host cell for MCV infection and as mentioned above, such cells can express Class II under certain conditions [126]. Additionally, MCV DNA has also been previously isolated from peripheral blood monocytes [132] and the gene expression signature of MCC tumour cells bears greater similarity to a pro-B cell than the epidermal Merkel cell [430] suggesting that MCV may have a tropism for MHC Class II expressing cells and consequently may require mechanisms to

evade detection. Overall, the effect of MCV on the Class II pathway remains unclear but our results do suggest that MCC tumour cells are incapable of presenting antigen through the Class II pathway and so cannot be directly targeted by CD4 T cells.

Although they did not have any obvious impact on Class I and Class II surface expression, our preliminary experiments showed both MCV LT and sT independently appeared to affect the ability of antigen-specific CD4 and CD8 T cells to recognise cells expressing their cognate antigen. To our knowledge, no immune evasion function of the MCV T-antigen has previously been identified. MCV sT appeared to reduce CD8 T cell recognition of a reporter antigen; an effect not seen with either the WTLT or the MCC derived truncated LT (MCC350 and MKL-1) which surprisingly appear to increase recognition. The potential mechanism of action for this is not known but a number of other viral proteins have been shown to reduce CD8 T cell recognition through a variety of means. The EBV protein BNLF2a blocks both ATP and peptide binding to the TAP transporter protein, inhibiting the formation of MHC Class I complexes bound to peptide and limiting antigen display on the surface [410]. The KSHV proteins, K3 and K5, are E3 ubiquitin ligases that promote the endocytosis of Class I chains from the cell surface leading to their subsequent degradation within an intracellular endolysosomal compartment [431]. These mechanisms of action are usually associated with a reduction of surface Class I, which was not seen with the transfection of MCV sT suggesting that it may not operate in this fashion. Further work to confirm these early results and investigate possible mechanisms of action are required. Given the opposite effects on CD8 T cell recognition caused by expression of LT and sT when expressed individually, the effect of co-expressing both genes as occurs in infected or MCC tumour cells is not clear. Interestingly, exposure to UV light has been shown to increase mRNA expression of sT while not affecting LT levels [108]. As MCC is more commonly found in areas of sun exposed skin, it is possible

that this increased sT transcription may favour a reduction in CD8 T cell recognition and provide a mechanism of immune evasion for the developing tumour.

In our preliminary experiments, WT LT and sT both appear to impair CD4 T cell recognition of a reporter antigen while truncated forms of LT do not. The mechanism through which this may occur is unclear as with CD8 recognition. Again, other viral proteins have been shown to have effects on recognition by affecting the Class II pathway. Viral proteins from EBV (BZLF1) and KSHV (vIRF3) have been shown to reduce CD4 recognition by inhibiting transcription of CIITA resulting in reduced synthesis of Class II molecules [413, 432]. This is associated with reduction in surface Class II expression, which we did not see with MCV WT LT or sT. However, as the kinetics of this down-regulation can be very slow, as exemplified by vIRF3, it is possible that such changes were simply not identified by our assay. Another immune evasion protein from EBV, BZLF2, is able to sterically hinder the interaction between Class II and the TCR, consequently reducing CD4 T cell recognition [433]. This does not alter surface expression of MHC Class II and so provides a potential alternative mechanism through which MCV LT and sT may affect recognition of CD4 T cells.

Although preliminary, our results suggest that both MCV LT and sT may exhibit some immune evasion function by reducing T cell recognition. MCV sT in particular appears to affect both CD4 and CD8 T cell recognition although how this occurs is not known. Further confirmatory work must follow as well as investigation to assess if this property of sT is specific to MCV or shared more generally by other polyomaviruses.

Chapter 7

Final Discussion

It has long been known that the rare and aggressive cutaneous malignancy, Merkel cell cancer (MCC), is associated with risk factors such as ageing, UV light exposure and immunosuppression [7]. A more recent finding is that an important causal factor in the majority of cases of MCC is the integration of DNA from the Merkel cell polyomavirus (MCV) into the cellular genome [42]. This viral DNA encodes truncated Large T (LT) and Small T (sT) antigens, proteins that that are able to dysregulate cell cycle control through a number of mechanisms and lead to uncontrolled cellular proliferation and tumour formation. It has subsequently been hypothesised that the link between immunosuppression and this virally-associated cancer stems from a loss of anti-viral immunity against MCV. However, unlike other polyomavirus-mediated diseases such as BKV-associated nephropathy (BKVAN) or JCV-associated progressive multifocal leukoencephalopathy (PML), in which affected patients are almost universally immunosuppressed, the majority of MCC patients have no history of clinical immunosuppression [39]. This meant that a detailed investigation into both the general immune function and specific anti-MCV viral immunity in MCC patients in comparison with other control groups was warranted in order to further our understanding of the link in this context.

Multiple studies have shown that MCC patients have higher MCV VP-1 serum antibody levels in comparison to healthy controls and that this appears to correlate with higher levels of MCV DNA isolated from skin swabs [125, 137]. This suggests that there has been a loss of MCV-specific viral control in these patients and this may have contributed to the development of MCC, particularly as the increase in serum VP-1 antibodies can predate the onset of cancer

by a period of years [337]. However, studies assessing antibody levels of the other members of the polyomavirus family as well as directly comparing MCV VP-1 serum responses of MCC patients with other donor populations has not been previously conducted. We aimed to improve our knowledge in this area through collaboration with Dr Tim Waterboer (Heidelberg, Germany), who had developed a multiplexed-bead binding luminex assay capable of identifying and quantifying VP-1 seroresponses against ten of the twelve known human polyomaviruses [181]. Serum from a cohort of patients either with an active or past history of MCC attending clinic at Queen Elizabeth Hospital Birmingham was collected alongside serum from young and elderly healthy controls as well as other patient cohorts. This included non-melanoma skin cancer (NMSC) patients, chosen due to shared risk factors with MCC such as ageing and UV light exposure but no known link with viral infection, and a cohort of chronic lymphocytic leukaemia (CLL) patients who are known to be at increased risk of MCC development and are commonly immunosuppressed. Comparative analysis between these cohorts assessing for total numbers of polyomavirus-specific seroresponses showed that evidence of multiple infections was commonly seen in all groups and median numbers of responses did not differ suggesting that none of the studied groups are more at risk of acquiring polyomavirus infections than the others. It was clear, in agreement with the published literature, that MCV VP-1 seroresponses were more commonly seen in the MCC patients [75], however, this finding was not replicated for any of the other viruses tested and leads us to suggest that any loss of immune control in MCC patients may be specific for MCV rather than being a generalised polyomavirus-wide phenomenon. This is in agreement with previous studies that have assessed for responses against BKV, JC, KI and WU in MCC patients [74, 152].

Our work importantly extends this through the inclusion of responses for the skin-associated polyomaviruses such as HPyV6, HPyV7 and TSV. As responses against these viruses also appear unchanged in MCC patients compared with the control groups, this suggests that the underlying cause for a loss of MCV control is not mediated simply by dysfunctional cutaneous immunity occurring in MCC patients. We were also able to make an assessment of serum T-antigen antibody responses against the same panel of polyomaviruses utilising the same methodology. Here, we saw that MCV T-antigen seroresponses were seen specifically in MCC patients with active or recently treated disease, the majority of whom had no history of immunosuppression. This mirrors a previous report that described MCV T antigen responses are found extremely rarely in the serum of non-MCC patients, become elevated in active disease and then fall following treatment, thus having potential utility as a biomarker of tumour burden in MCC [354]. The assessment of the other polyomavirus T-antigen responses has been much more limited and from our data, detectable responses are generally rare. However, of MCC patients in whom MCV T-antigen seroresponses were detectable, a large proportion also had detectable responses for multiple other viral T-antigens, particularly the skin-associated HPyV6, HPy7 and TSV. Unlike MCV T-antigen antibody responses, which can be driven by tumour-associated antigen load, other polyomavirus T-antigen responses may only be expected at times of active viral replication. An interpretation of these results could be that multiple skin-associated polyomaviruses may be actively replicating at the same time as a result of cutaneous immunosuppression. This is inconsistent with the VP-1 seroresponse data discussed above and may mean that T-antigen antibody measurement is the more sensitive serological marker for detecting viral replication. An alternative possibility that cannot be excluded based on this data alone is that, given the high degree of homology seen between T-antigens of the polyomavirus family, these results are a product of cross-

reactivity between the seroresponses for the different antigens. In order to clarify this, further work could include the recovery of DNA from skin swabs from MCC patients and use of real-time quantitative PCR using multiple primers specific for distinct sequences from each individual polyomavirus in order to see whether there are sequences from just one or many viruses present.

Although this serology data provides some evidence suggesting that the anti-viral control of MCV may be dysfunctional in MCC patients including those with no history of immunosuppression, it provides no underlying mechanism for why this may be the case. In order to further understand this we performed a detailed analysis assessing general immune markers of peripheral blood samples in non-immunosuppressed MCC patients and compared this with the same markers in a group of NMSC patients of a similar age profile. No such similar investigation has been completed previously although some indication as to the potential importance of cellular subsets in MCC have been intimated through the recent findings that non-immunosuppressed MCC patients with lower absolute lymphocyte counts and higher neutrophil:lymphocyte ratio at diagnosis have a poor prognosis [41, 390].

In our dataset, MCC patients did have lower median lymphocyte counts (although not statistically significant) compared with NMSC patients, with the B cell subset being most significantly affected. Potentially related to this was the finding that numbers of T cell-independent antibody responses against 12 vaccination polysaccharide antigens from different serotypes of pneumococcus were significantly reduced in MCC patients compared with NMSC patients while T cell-dependent antibody responses such as those against tetanus toxoid and diphtheria were retained. Although an interesting finding, the functional relevance of this in the context of MCV directed immunity is uncertain as MCC patients are known to have high levels of MCV-antibodies compared to healthy individuals and although not

demonstrated clearly with MCV itself, other polyomavirus-specific antibody responses appear to be T-cell dependent [121, 336, 434].

In the elderly, anti-viral cellular immune responses may be affected by the accumulation of large populations of terminally differentiated effector memory CD45RA+ (TEMRA) CD8 T cells directed against a small number of epitopes taking up immunological space infection and preventing adequate cellular responses against other viral infections [246, 387]. This process is most commonly driven by co-infection by cytomegalovirus (CMV) infection and may be a potential mechanism contributing to the loss of control of MCV infection seen in MCC patients. Our data did not support this as evidence of CMV infection and the prevalence of expanded TEMRA populations are both split relatively equally between MCC and NMSC patients. A further cause for loss of viral control may relate to impaired functionality of responding lymphocytes and so this was assessed by measuring proliferative response of lymphocytes following mitogenic stimulation. This showed there was no difference in proliferative response between MCC patients currently in remission with NMSC patients. However, MCC patients with active disease did have a significantly lower functional response when compared with other MCC patients indicating the likely presence of cancer-induced immunosuppression that has been widely documented in the context of other cancers [435]. Overall, our assessment of peripheral immunity of MCC patients did not uncover any clearly definable differences that would account for a loss of MCV-directed immune response leading us to believe that this occurs as part of a multi-factorial process. Further work could include the assessment of other immune cell subsets such as myeloid derived suppressor cells (MDSCs) in MCC patients compared with other patient groups to assess their importance in immunosuppression in this context.

In general, cellular immunity is integral to the control of viral infections and in the context of MCC, increased infiltration by CD8 T cells into tumours is associated with a good prognosis and when examined these infiltrating T cells can be specific for MCV T-antigen epitopes that can be utilised therapeutically [311, 385, 424]. Taken together this suggests that MCV-directed T cell immunity may be important not only for the control of MCV, but also have a role in determining clinical outcomes of MCC and thus we began to focus attention on the investigating the T cell response to MCV in greater detail.

We aimed to assess whether there were differences in MCV-specific T cell immunity in MCC patients in comparison with a similarly aged cohort of NMSC patients through the quantification of circulating IFNɣ secreting T cells in response to stimulation with peptides spanning the length of the LT and sT proteins in ELISpot assays. These experiments showed that in general, the total numbers of responding T cells was low, a pattern that has been demonstrated previously in MCV as well as the other well-studied polyomaviruses, BKV and JCV [288, 294, 385]. In our study, the sizes of responses were not significantly different when MCC patients and NMSC patients were compared even after stratification on the basis of MCV seropositivity. In a previous study using similar assays to quantify numbers of responding T cells against MCV VP-1, truncated LT and sT proteins, MCC patients were shown to have (non-significantly) higher numbers of MCV-specific T cells compared with healthy donors [385]. This would appear to broadly agree with our findings and certainly suggest that MCC patients do not have a peripheral deficiency of MCV-specific T cells when compared to control groups. Although not directly compared with the MCC and NMSC patients due to differences in assay design, the majority of younger healthy volunteer donors recruited as part of this study also showed low numbers of responding cells suggesting that even in healthy individuals, the number of circulating T cells required to control MCV

infection is low. What remains unknown and is an interesting avenue for further research lies in understanding whether the peripheral MCV-specific T cell response is representative of the MCV-specific response in the skin. It is possible that in MCC patients, although circulating MCV-specific T cells are present at normal frequency, these are not able to infiltrate the skin due to abnormal functional or homing phenotype. This may be possible by performing biopsies from normal skin of MCC patients, NMSC patients and healthy donors to see if there are differences in the frequency of MCV-specific T cells. However, as biopsy samples are small, this type of characterisation will be extremely difficult unless we have a greater understanding of what viral epitopes the T cells are targeting. This led on to the next part of this investigation in which we focused on trying to address this question.

Defining the epitopes of T cell response against MCV LT and sT will help in our understanding of how such an anti-viral response is directed as well as potentially providing therapeutic targets for intervention against MCC. Due to the low numbers of detectable circulating virus-specific T cells this was attempted through limiting dilution T cell cloning from healthy donors rather than with direct ex vivo assessment of peripheral blood. Using this method, we identified a number of T cell clones from donors of different HLA types that were specific for novel epitopes on the T-antigen. The majority of these were CD4 T cells recognising MHC Class II targets along the length of the LT protein. This is consistent with previous studies which have shown that most detectable MCV-specific responses in healthy donors are due to CD4 T cells [302, 304]. This is also a similar scenario to that seen in responses against both BKV and JCV and suggests that a diverse range of CD4 T cell responses is seen in polyomavirus infections [289-291]. Whether the diversity of CD4 T cell responses diminishes in MCC patients and this contributes to the loss of viral control is not known and would be an avenue for further work.

We were able to isolate one CD8 T cell response specific for a HLA-B7 restricted epitope contained within the portion of MCV LT usually lost by truncation mutation during tumourigenesis. To our knowledge, this is the first described CD8 response to the MCV T-antigen in an individual not suffering with MCC. Although, the response was readily detectable using tetramer analysis in one healthy donor, responses in a panel of further HLA-B7 donors were not easily seen ex vivo. This corresponds with existing data in which the majority of CD8 T cell responses against MCV T-antigen have been identified only after extended culture in the presence of the relevant peptide rather than directly ex vivo [306]. It would be interesting to perform such extended culture using peripheral blood lymphocytes from the panel of HLA-B7 healthy donors used in this study in order to better confirm the wider frequency of this response. In the donor with a detectable ex-vivo response, the MCV-specific CD8 T cells did not appear to express markers typical of a skin-homing phenotype, particularly with regard to a lack of CLA expression. This is in contrast to what is seen with T cells specific for the skin-associated virus, HSV as well as a previous study looking at CD8 T cells from MCC patients specific for a single MCV LT epitope [321, 403]. Ex vivo CD8 T cells were available from a single HLA-B7 MCC and were stained with our tetramer and although response was very small, making interpretation difficult, those cells that did bind tetramer appeared to show increased expression of skin-homing markers such as CLA. The reason that the MCV-specific T cells isolated from our healthy donor do not have a classical skin-homing phenotype is unclear. It may be that in health, such cells are usually retained within the skin and those that are recirculating within the peripheral blood do not share this phenotype. In our healthy donor, it would be very interesting to perform comparative studies of the phenotype of the circulating MCV-specific T cell with those that are found in the skin. Such experiments have been conducted to investigate responses to the VZV virus through

skin biopsy after delivery of the VZV vaccination in order to concentrate specific cells to the area to be examined [269]. In the case of MCV, the lack of an available vaccination in order to stimulate congregation of virus specific T cells to one area in the skin will mean it is unlikely a biopsy would contain significant numbers of MCV-specific T cells and so be difficult to interpret. It is also possible that, in this donor, the site of MCV infection is in a tissue other than the skin as although most commonly detected from cutaneous swabs, MCV DNA has also been found at low frequency in a number of other tissues [127, 129].

Although, epitope-specific CD8 T cells were only readily detectable in a single donor using tetramer analysis, we did find evidence in another healthy donor of the presence of CD8 T cell clones that despite not binding tetramer with a high avidity were able to recognise the same MCV LT peptide as well as cells transfected with the MCV LT. These CD8 T cells appeared to be equally sensitive to peptides corresponding the homologs of this epitope seen in other polyomaviruses, KI, HPyV6, HPyV7, TSV and HPyV9 as shown by similar functional avidities. This leads to the suggestion that there may be some cross-reactivity between polyomavirus-specific CD8 T cells where there is a degree of homology of the epitope. In order to confirm this, it would be necessary to assess if the CD8 T cell clones recognise cells expressing the other candidate polyomavirus LT protein as well as MCV. Cross-reactivity of CD8 T cells in the context of BK and JC polyomavirus infection have been the subject of previous studies and a number of cells that can recognise homologous epitopes between these two viruses have been identified, although to our knowledge such studies with any of the other members of the human polyomavirus family have not taken place [299, 301]. Given the high degree of homology seen between VP-1, LT and sT of the different polyomaviruses, it is possible that so–called “heterologous immunity”, in which memory T cells raised following infection with a certain pathogen can be re-activated following infection with a separate

pathogen sharing a homologous epitope, may be an important protective mechanism of the anti-polyomavirus immune response [436]. It is also possible that cross-reactivity could be detrimental to a viral response if such cross-reactive T cells have a lower avidity for the newly infecting pathogen than would ordinarily have been produced in the absence of pre-existing cells. This concept, known as "original antigenic sin" is thought to be important in delaying effective CD8 responses to Dengue virus, in which during acute haemorrhagic infection, the majority of virus-specific CD8 T cells show higher affinity for other, probably previously encountered, viral serotypes [437]. Investigating the order in which polyomavirus infections are acquired in MCC patients compared with other elderly donors may be useful to help understand whether such a process is important to the development of MCC, however, as MCC is so rare and polyomavirus infections tend to be asymptomatic, conducting such a project would be extremely difficult.

The MCV peptides capable of inducing IFNɣ T cell responses identified through T cell cloning were all capable of being processed and presented as Class I and Class II epitopes by model antigen presenting cells. This is particularly important as although all these newly discovered epitopes are likely to be expressed by virally-infected cells, the majority of the CD4 T cell epitopes reside in the portion of the T-antigen that becomes integrated within the MCC tumour genome and so would also be expressed by MCV+ MCC tumour cells. Although our data suggests that MCC tumour cells do not themselves express MHC Class II, there is potential for tumour cell debris to be taken up by professional antigen presenting cells. This would lead to the presentation of tumour-associated epitopes displayed and consequently cause activation of CD4 T cells that could stimulate other effector immune cells including CD8 T cells leading to further tumour cell killing. This "indirect" mechanism of anti-tumour immune response has been described previously in models of cancer that do not

express MHC Class II [438-440]. As little is known regarding the anti-tumour effect of CD4 T cells in MCC, this may be an interesting avenue to explore in future work, particularly focusing on whether such responses can be therapeutically manipulated to improve clinical outcomes.

Our finding and that of other groups, of multiple T cell epitopes on the MCV T-antigen means that there is potential for T cell recognition and killing of both virally-infected cells and tumour cells. To protect from such attacks, both virus-infected cells and tumour cells can evolve mechanisms of immune evasion and in cases of virally-associated cancers, such mechanisms can be shared. In the case of MCV, the best candidate viral proteins to have an immune evasion function would be LT and sT proteins as these are at least partially expressed by MCV+ MCC tumour cells. No such function affecting cellular immunity has been previously described although both LT and sT can negatively impact on the innate immune response through TLR9 down-regulation and inhibition of NF-κB pathway activation [275, 279]. We saw, as has been previously described [320], that in MCC primary tumour cells and MCV+ MCC cell lines, MHC Class I was low and that this was reversible with either exposure to IFNɣ or irradiation. In our in vitro assays, up-regulation of Class I did not result in a significant alteration in the ability of T cells to recognise tumour cells and so it is difficult to know how much effect such a treatment may have. It is possible that an effect may be more readily detectable in vivo as in a single patient with metastatic MCC treated with a combination of IFN, radiation and infusions of an expanded population of CD8 T cells specific for a MCV LT epitope, there was a clinical response to treatment with an increase in both tumour-infiltrating lymphocytes and MHC Class I expression [424]. Although, MHC Class I is down-regulated in MCV+ MCC, the mechanism behind this remains unknown with our studies showing no obvious reduction in surface expression levels seen in cells transiently

transfected with either MCV LT or sT. In a previously published paper in which MHC Class I down-regulation was found to be commonly prevalent in MCV+ MCC, there were changes in expression of multiple proteins within the MHC Class I pathway leading the authors to suggest that a multi-factorial process was occurring [320]. How expression levels of these proteins, such as TAP-1 and -2, change following transfection of MCV T-antigen proteins would be useful to assess what role, if any, is played by LT and/or sT in this process. Additionally, in a provisional set of experiments assessing the recognition of T cells specific for a reporter antigen in cells co-expressing MCV LT or sT, sT appeared to reduce the ability of CD8 T cells to recognise the antigen presenting cell. This finding requires further confirmatory experiments that should then lead onto investigations into the potential mechanisms underpinning these changes in T cell recognition.

Our data suggests that MHC Class II is not expressed by MCC primary tumour cells or MCC tumour cell lines and expression of Class II cannot be induced with exposure to IFNɣ or irradiation. As previously mentioned, this means that direct cytotoxicity against tumour cells by CD4 T cells is unlikely. However, as recent evidence suggests that dermal fibroblasts may be the preferential host cell for MCV infection within the skin, manipulation of the MHC Class II pathway by MCV may be important to evade immune detection [126]. As was seen with Class I, the transfection of MCV LT or sT into model antigen presenting cells did not appear to affect Class II expression levels. However, in provisional experiments, cells transfected with a reporter antigen alongside either MCV LT or sT were poorly recognised by CD4 T cells when compared with cells not expressing either T-antigen. These results also require further confirmation but may uncover a potential viral immune evasion for the T-antigens. Whether this attribute is specific to MCV or shared with the homologous T-antigen proteins from other members of the human polyomavirus family is not known and would be a

good direction of further study. If fibroblasts are potentially the primary host cell for MCV infection, the impact of expression of MCV T-antigens on Class I and Class II regulation would be very interesting. In particular, being a cell type in which Class II expression is not constitutive but can be up-regulated in response to stimuli such as IFNɣ, one would want to assess whether LT or sT proteins are capable of preventing up-regulation of Class II upon stimulation with IFNɣ to give an indication of how such a potential immune evasion function may help protect the virus during infection.

In a period where cancer immunotherapy is undergoing huge expansion, understanding how this may be best delivered in the context of MCV+ MCC is important. Although, clinical studies with immunotherapeutic agents such as anti-PD-1 antibodies have begun with encouraging early results, it is clear that some patients will not respond for unknown reasons [327]. The role of T cell control, particularly by CD4 T cells, in MCC has not been well studied and the discovery of a number of CD4 T cell clones specific for novel MCV T-antigen epitopes provides tools with which this can be further probed. The finding that both MCV LT and sT may have T cell immune evasion properties needs confirmation but may occur through a mechanism which if reversible may improve responses to immunotherapy. The characterisation of peripheral immunity in MCC patients suggests there are a number of subtle but significant differences seen when compared with an age-similar cohort of NMSC patients. Whether such differences could be used as biomarkers to predict the likelihood of response or resistance to immunotherapy is a further avenue of investigation aimed at improving the future effective management of MCC patients.

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