

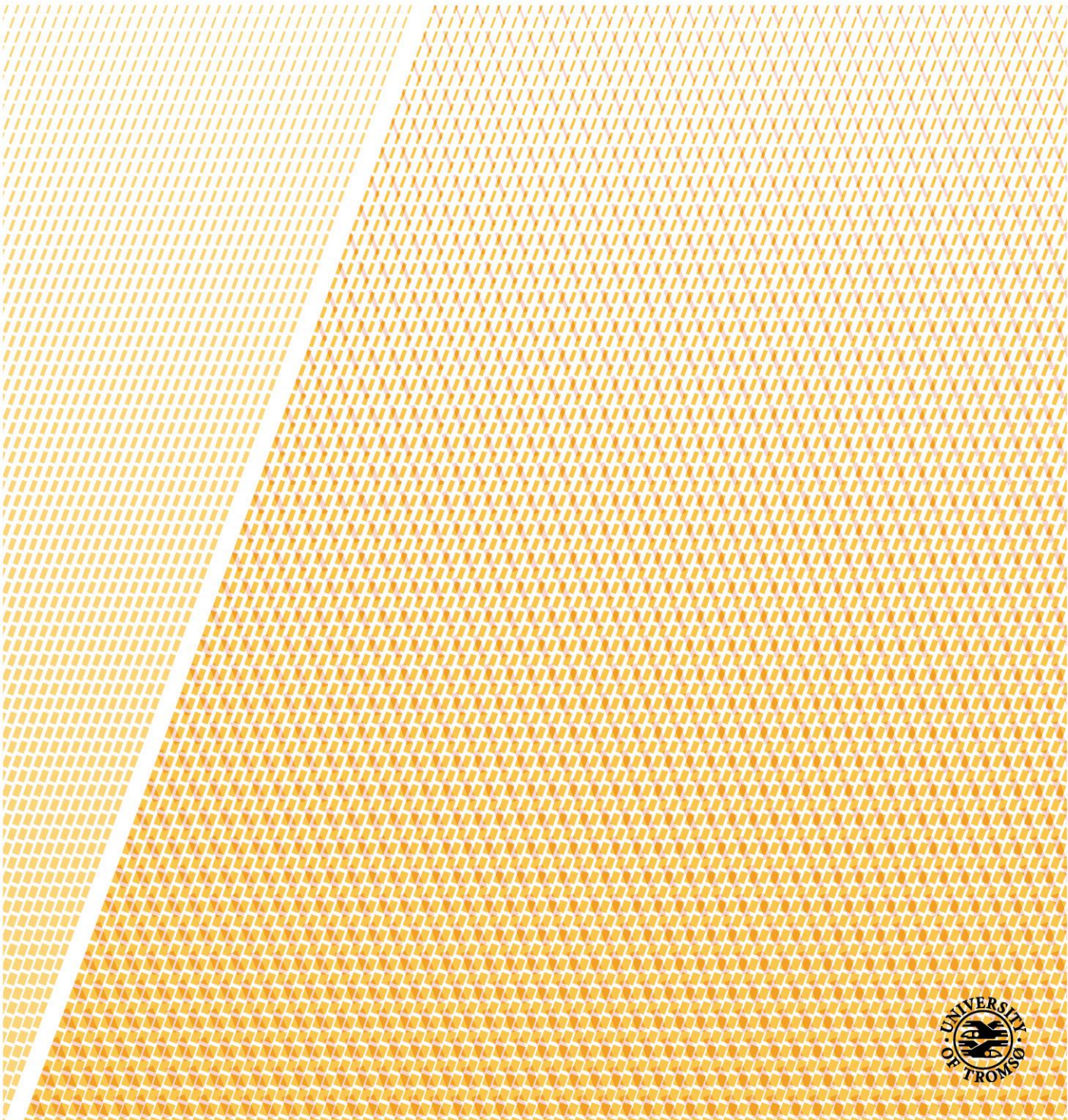


Faculty of Health Sciences
Department of Medical Biology

Merkel Cell Polyomavirus and Merkel Cell Carcinoma

—
Kashif Rasheed

A dissertation for the degree of Philosophiae Doctor – September 2020



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Molecular Inflammation research Group (MIRG)

Department of Medical Biology

Faculty of Health Science

UiT- The Arctic University of Norway

September 2020

With faith, discipline and selfless devotion to duty, there is nothing worthwhile that you cannot achieve.

Founder of Pakistan - Muhammad Ali Jinnah (1876-1948)

Science is part of the reality of living; it is the what, the how, and the why of everything in our experience.

Rachel Carson (2011).

“Lost Woods: The Discovered Writing of Rachel Carson”, p.91, Beacon Press

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LIST OF PAPERS

Paper I

Abdulsalam I, Rasheed K, Sveinbjörnsson B, Ehlers B and Moens U. **Promoter activity of Merkel cell Polyomavirus variants in human dermal fibroblasts and a Merkel cell carcinoma cell line.** Virology Journal (2020) 17(54). <https://doi.org/10.1186/s12985-020-01317-x>

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Paper II

Rasheed K, Abdulsalam I, Fismen S, Grimstad Ø, Sveinbjörnsson B and Moens U. **CCL17/TARC and CCR4 expression in Merkel cell carcinoma.** Oncotarget. (2018) 9:31432-31447. <https://doi.org/10.18632/oncotarget.25836>

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Paper III

Rasheed K, Shi H, Tummler C, Policastro B, Fismen S, Johnsen JI, Lui WO, Moens U and Sveinbjörnsson B. **The Merkel cell polyomavirus T-antigens and IL-33/ST2-IL1RAcP axis: Role in Merkel cell carcinoma.**

Manuscript

Paper IV

Rasheed K, Sveinbjörnsson B and Moens U. **Merkel cell polyomavirus large T antigen and small t antigen increase the expression of high-risk human papillomaviruses 16 and 18 E6 and E7 in cervical cancer cells.**

Manuscript

Additional Manuscripts Published During Ph.D But Not Included In The Thesis

Paper S1

Fan K, Gravemeyer J, Ritter C, Rasheed K, Gambichler T, Moens U, Shuda M, Schrama D, Becker JC. **MCPyV Large T antigen induced atonal homolog 1 (ATOH1) is a lineage-dependent oncogene in Merkel cell carcinoma.** Journal of Investigative Dermatology (2019) 140(1). <https://doi.org/10.1016/j.jid.2019.06.135>

Paper S2

Moens U, Rasheed K, Abdulsalam I, Sveinbjörnsson B. **The role of Merkel cell polyomavirus and other human polyomaviruses in emerging hallmarks of cancer.** Viruses (2015) Volume 7(4). <https://doi.org/10.3390/v7041871>

Paper S3

Csoboz B, Rasheed K, Sveinbjörnsson B, Moens U. **Merkel cell polyomavirus and non-Merkel cell carcinomas: Guilty or circumstantial evidence.** Journal of Pathology Microbiology and Immunology (2020) 128. <https://doi.org/10.1111/apm.13019>

ABBREVIATIONS

ALTO	Alternative large Tumorigenic antigen open reading frame
APC	Antigen presentation cell
B2M	β 2-microglobulin
BKPyV	BK polyomavirus
C/EBP	CCAAT/enhancer-binding protein
CCL17	Chemokine ligand-17
CD	Cluster of differentiation
CI	Cancer incidence
CLA	Cutaneous lymphocyte antigen
CTLA-4	Cytotoxic T-lymphocyte antigen-4
CR1	Conserved region 1
DAMP	damage associated molecular pattern
DDR	DNA damage repair
EM	Electron microscopy
ERE	Estrogen responsive element
Fbw7	F-box and WD repeat domain-containing 7
FLT	MCPyV full-length large T-antigen
GATA1/2	GATA-binding factor 1/2
H2A	Histone 2 A
H2B	Histone 2 B
HDAC	Histone deacetylase-3
HPyV12	Human polyomavirus 12
HPyV6	Human polyomavirus 6
HPyV7	Human polyomavirus 7
HPyV9	Human polyomavirus 9
HR-HPV	High risk human papilloma virus
hTERT	Human telomerase reverse transcriptase
ICI	Immune checkpoint inhibitor
IgG	Immunoglobuline
IL	Interleukine
IL1RAcP	IL-1 receptor accessory protein
IL1RL1	IL-1 receptor ligand 1
IL-33R	IL-33 receptors
INF	Interferon
iNKT	Invariant natural killer t cells
JCPyV	JC polyomavirus
KIPyV	Karolinska Institute polyomavirus
KNSTRN	Kinetochores Localized Astrin (SPAG5) Binding Protein
LIPyV	IARC-Lyon polyomavirus
LSD	Large T-antigen stabilization domain
LT	Large tumor antigen

MAPK	mitogen-activated protein kinase
MCC	Merkel cell carcinoma
MCPyV	Merkel cell polyomavirus
MHC	Major histocompatibility complex
MICA	MHC class I chain-related protein A
MICB	MHC class I chain-related protein B
miRNA	micro RNA
mTOR	Mammalian target of the rapamycin
MUR-1	MCPyV unique region 1
MUR-2	MCPyV unique region 2
MWPyV	Malawi polyomavirus
NCCR	Non-coding control region
NEMO	NFkB-essential modulator
NF-HEV	Nuclear factors from high endothelial venule
NJPyV	New Jersey polyomavirus
NK	Natural killer cells
NKG2D	Natural killer group 2D
NKT	Natural killer T cells
NLS	Nuclear localization signals
NOTCH1	Notch (Drosophila) Homolog 1
PD-1	Programmed death receptor 1
PD-L1	PD ligand 1
PI3K	phosphatidyl-3-kinase
PML	Progressive multifocal leukoencephalopathy
PP1	Protein phosphate 1
PP2A	Protein phosphate 2A
PP2C α	Protein phosphate catalytic subunit alpha
PP2C β	Protein phosphate catalytic subunit beta
PP4C	Protein phosphatase 4C
PP4R1	Protein phosphatase 4 regulatory subunit 1
PRUNE2	Prune Homolog 2 With BCH Domain
PyV	Polyomavirus
QPyV	Quebec polyomavirus
RB1	Retinoblastoma protein 1
RNOS	Reactive nitrogen oxygen species
SCFFbw7	Skp1-Cul1-F-box protein
SEER	Serveillance, epidemiology, and end results
SIR	Standardized incidence rate ratio
sST2	soluble ST2
sT	Small tumor antigen
ST2	Suppression of tumoerigenicity 2
ST2L	ST2 ligand (membrane bound)
ST2LV	ST2 ligand variant
ST2V	ST2 variant

STLPyV	St Louis polyomavirus
TAAAs	Tumor associated antigens
T-ag	Tumor antigen
TARC	Thymus and activation-regulated chemokine
TCR	T cell receptor
TGF	Tumor growth factor
Th2	T helper cells 2
TIM-3	T-cell immunoglobulin and mucin-domain containing-3
TLR9	Toll-like receptor 9
TME	Tumor microenvironment
TNF	Tumor necrotic factor
TP53	Tumor suppressor 53
TRAE	Treatment related adverse event
Treg	Regulatory T cells
tLT	MCPyV truncated Large T-antigen
TSPyV	Trichodysplasia spinuolsa associated polyomavirus
UV	Ultra violet
VN	Virus negative
VP	Merkel cell polyomavirus positive
VP	Viral capsid protein
WHO	World health organization
WUPyV	Washington University polyomavirus
4E-BP1	4E-binding protein 1

ABSTRACT

Merkel cell carcinoma (MCC) is a rare, highly aggressive neuroendocrine skin cancer. Merkel cell polyomavirus (MCPyV) is the major aetiology with almost 80% of the examined MCC tumors contain integrated viral DNA in their genome, while the remaining 20% of the MCC tumors are virus negative. MCC is particularly linked to immune suppression as compared to other tumors but can be immunogenic. Immunotherapy is rapidly becoming a preferred systemic therapy in several cancer types, especially because responses to immunotherapy (when they occur) are generally long-lasting.

This thesis aims to identify novel inflammatory mediators and pathways in MCC to contribute to a better understanding of MCC biology, a prerequisite for novel therapeutic approaches. Several MCPyV variants with polymorphism in their promoter region have been isolated, but it is not known whether these differences affect the biological properties of the virus. In first study, we have found that full-length large T-antigen (FLT) inhibited early and late promoter activities while truncated large T-antigen (tLT), which is expressed in MCPyV-positive (VP) MCCs, stimulated the activity of its cognate promoter in both MCC-13 and human dermal fibroblast cell lines.

Previous studies have shown altered cytokine expression in MCC. In the second and third study, by performing RT² profile PCR array for inflammatory cytokines and receptors, we compared the cytokine expression pattern in VP with MCPyV-negative (VN) MCC cells and examined the role of the viral oncoproteins, LT and sT on cytokine expression. The second and third studies demonstrated an increased expression of CCL17/TARC and IL-33 in the VP cell lines compared to the VN cell lines. Furthermore, recombinant CCL17/TARC and IL-33 proteins activated both the mitogen-activated protein (MAP) kinase and the nuclear factor- κ B (NF- κ B) pathways. Finally, immunohistochemical staining on human MCC tissues showed a strong staining of CCL17/TARC, CCR4, IL-33, ST2/IL1RL1 and IL1RAcP in both VP- and VN-MCC. So, targeting CCL17/CCR4 and/or IL-33/ST2 complex could be an option to treat MCC. Recent findings reported the co-detection of MCPyV DNA in high-risk human papilloma virus (HR-HPV) -positive cervical cancers, though a role for MCPyV in cervical carcinogenesis has not been proven. The fourth study demonstrated that MCPyV LT and sT stimulated the promoter activity of the HR-HPVs HPV16 and HPV18, and induced the expression of their E6 and E7 oncoproteins. These results indicate that the co-infection of MCPyV may act as a co-factor in the initiation and/or progression of HPV-induced cervical cancer, or in other HPV-associated cancers.

1. INTRODUCTION

Cancer is a group of diseases that involves abnormal cell division and growth, where cells have the potential to invade and spread to other parts of the body [1]. It can develop almost anywhere in the body [2]. Cancer is the second leading cause of death worldwide after cardiovascular diseases with 16.23%. The World Health Organization (WHO) reported that in 2018 worldwide more than 18 million new cases of cancer occurred and that approximately 9.6 million people died of cancer [3]. However, in high-income countries, deaths by cancer are leading among other causes [4].

Merkel cell carcinoma (MCC) is rare, highly aggressive neuroendocrine skin cancer [5]. MCC is a relatively recently described entity, although the Merkel cell was identified more than 100 years ago. In 1875, human Merkel cells were first described by Friedrich S. Merkel (1845-1919). He named these cells Tastzellen (touch cells) assuming that they had a sensory touch function within the skin because of their association with nerves [6]. MCC was first described as “trabecular carcinoma of the skin” in 1972 by Cyril Toker [7]. Six years later, in 1978, Tang and Toker found dense granules in tumors by electron microscopy (EM) [8]. Merkel cells are the only cells in the skin that have dense granules. This fact led to a hypothesis that trabecular carcinoma of the skin arises from Merkel cells, hence named as MCC [9].

1.1. Epidemiology

1.1.1. Incidence

The most common cancer is skin cancer, including melanoma and non-melanoma skin cancers such as basal cell and squamous cell carcinoma (Figure 1; [10]). The incidence of skin cancer is increasing among newly diagnosed cancers with one out of three cases being skin cancer [11]. Melanoma is the 19th most common cancer in men and women and WHO estimates around 3 million new cases [12] and almost 1 million of non-melanoma cases as 5th most commonly occurring worldwide in 2018 [1]. The incidence of both melanoma and non-melanoma skin cancers may exceed 4.6 and 2 million respectively by 2040 [13]. Basal cell carcinoma and squamous cell carcinoma have an incidence of ~70% and ~17% respectively, whereas melanoma accounts for ~10% of the skin cancers. MCC is relatively rare with <2% of all skin cancers (Figure 1). Basal cell and squamous cell skin cancers grow more slowly than

melanomas and are easier to treat, whereas melanoma and MCC are highly aggressive and metastatic and are responsible for ~75% of all deaths caused by skin cancer. The 5-year survival rate for melanoma ranges from 95% (localized) to 25% (distant spreading), while it is ~50% for MCC with distal spreading [14, 15].

In the US, the standardized incidence rate of all solid cancers decreased from 2000 to 2013, but the rate of aggressive skin cancer increased significantly. Surveillance, Epidemiology, and End Results 18 (SEER18) database reported a decline from 429 cases/100,000 to 379 cases/100,000 during 2000-2013 respectively. In contrast, for the most aggressive skin cancers, MCC, incidence rates significantly increased from 0.5 cases per 100,000 in 2000 (95% CI 0.4–0.5) to 0.7 per 100,000 in 2013 (95% CI 0.7–0.8). The total number of MCC cases reported annually to the SEER-18 database also increased almost 95.2% (from 334 cases captured by SEER in 2000 to 652 in 2013). Furthermore, SEER also predicted that the incidence of MCC increased from 2835 cases per year in 2020 to 3284 cases in 2025 [16]. In other populations, the incidence rate also increased over time. The incidences were higher among some populations (men: Australia, New Zealand, and Israel; women: New Zealand, Australia, Ireland, and the Netherlands) while the number of cases remained relatively stable among some of the populations (men: U.S. Black population, Japan, Norway, Denmark; women: Denmark, Norway, Sweden) [17]. SEER-18 registry also reported a 10-fold increase in incidence in MCC between the ages 40-44, 60-64 and > 84 with 0.1 to 1 and 9.8 cases per 100,000 persons per year [16].

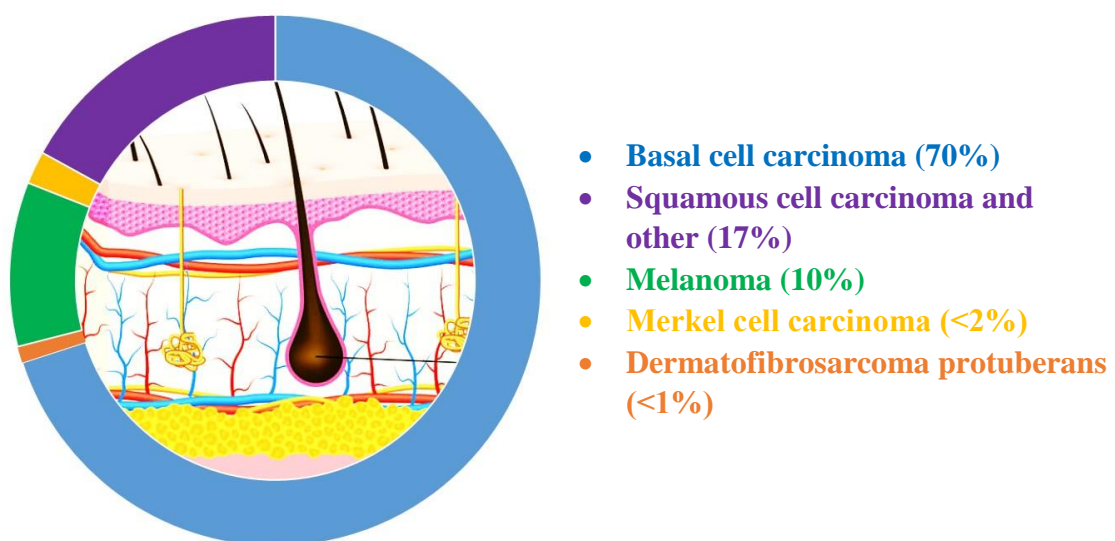


Figure 1: Incidence of different types of skin cancer [18].

1.1.2. Risk Factors

There are several risk factors associated with MCC, including heavily exposure to UV/ sunlight (Figure 2), light skin, history of other cancers, advanced age, weakened immune system and immunosuppression (Figure 3) [19].

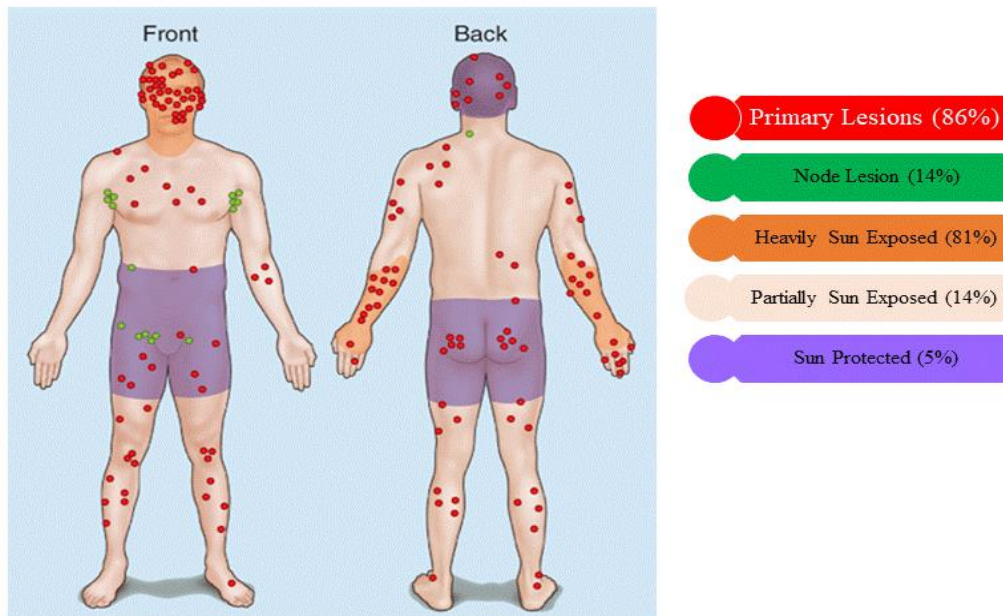


Figure 2: Most MCCs occur at sun-exposed sites with 81% on heavily, 14% on partially and less than 5% on protected areas from sun exposure. For almost 86% of MCC patients, the primary site of the tumor is the skin while 14% have shown nodal metastasis with unknown primary lesion [5]. Modified from [20] copyrights © The McGraw-Hill Companies, Inc. All rights reserved.

MCC is more common in white population as compared to non-white populations with 94.9% versus 4.1% [21]. Similarly, heavily exposure to sunlight and UV light cause DNA mutations (C[C>T]N and N[C>T]C), which also increases the incidence of MCC in VN-MCC [7]. Another risk factor for MCC is the human polyomavirus Merkel cell polyomavirus (MCPyV). Most of the MCC patients have genomic integration of MCPyV in the tumor. However, this percentage varies according to region, as in Australia and New Zealand, there is a higher incidence of VN- as compared to VP-MCC with 18-24% (Australia) and 23% (New Zealand) VP-MCC compared to 80% in northern Europe [22, 23]. The risk of getting MCC augments as people get old. The mean age of diagnosis is 74 years for men and 76 years for women [5]. MCC has been diagnosed in patients at young age but this is frequently related to immunosuppression due to organ transplant, HIV-infection or B cell malignancies [24-30]. The

United States Scientific Registry of Transplant Recipients database and 15 population-based cancer registries reveal that transplant recipients have a 10-fold higher risk of MCC than immunocompetent patients [27]. Moreover, males are affected more commonly as compared to females (61% male vs. 39% female) [31]. The risk of MCC is significantly increased in patients with other malignancies. SEER database study of over 2 million patients showed that the risk of developing MCC significantly increased with multiple myeloma, chronic lymphocytic leukemia and malignant melanoma with standardized incidence ratio (SIR) of 3.7, 6.9, and 3.1, respectively [32]. Likewise, after the first year of MCC diagnosis, the incidence of other cancer of salivary gland, biliary tract, and non-Hodgkin lymphoma also increase (SIR 11.6, 7.2, and 2.6, respectively) [26]. Data from the Danish National Health and Population Register show that the MCC incidence rate more than 1 year after the diagnosis of another cancer was 2.6 times higher (women: SIR = 1.8, and in men SIR = 4.0) than expected (2.2 cases/ million) based on the MCC incidence in the general Danish population. There was significant elevated risk of being diagnosed with MCC more than 1 year after a diagnosis of any skin cancer (SIR = 2.6), basal cell carcinoma (SIR = 4.3), squamous cell carcinoma of the skin (SIR = 14.6), cutaneous malignant melanoma (SIR = 3.3), chronic lymphocytic leukemia (SIR = 12.0), Hodgkin lymphoma (SIR = 17.6), or non-Hodgkin lymphoma (SIR = 5.6) [33].

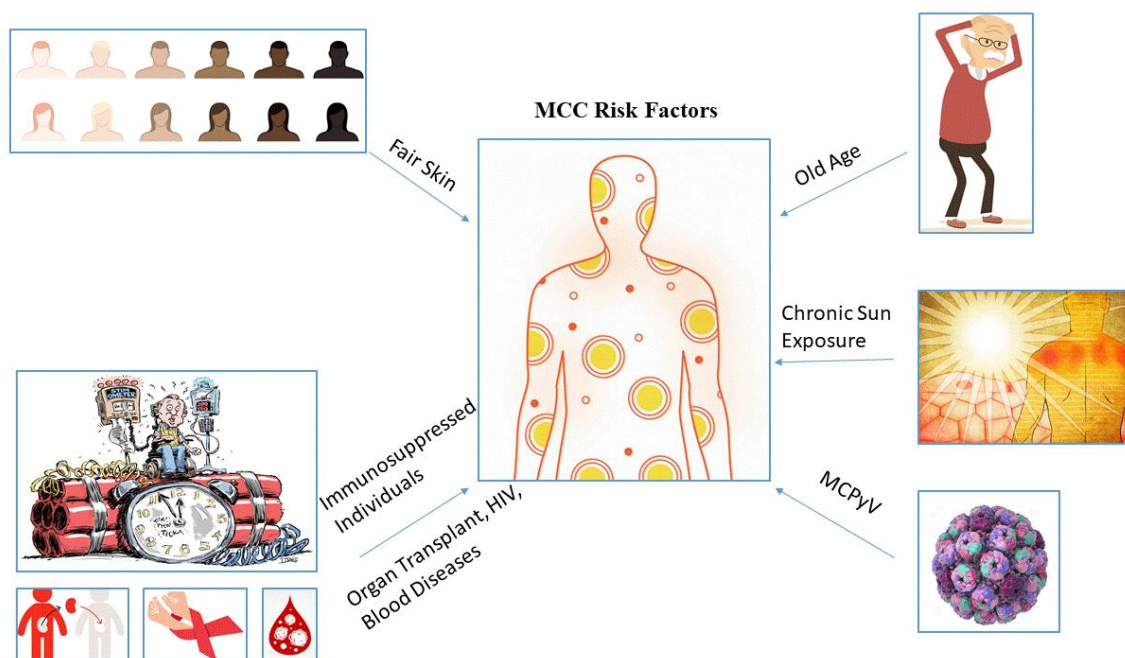


Figure 3: Factors that may increase risk of Merkel cell carcinoma.

1.2. Merkel Cell Carcinoma (MCC) Pathogenesis

MCC tumors can be roughly classified as VP- and VN-MCC (Figure 4).

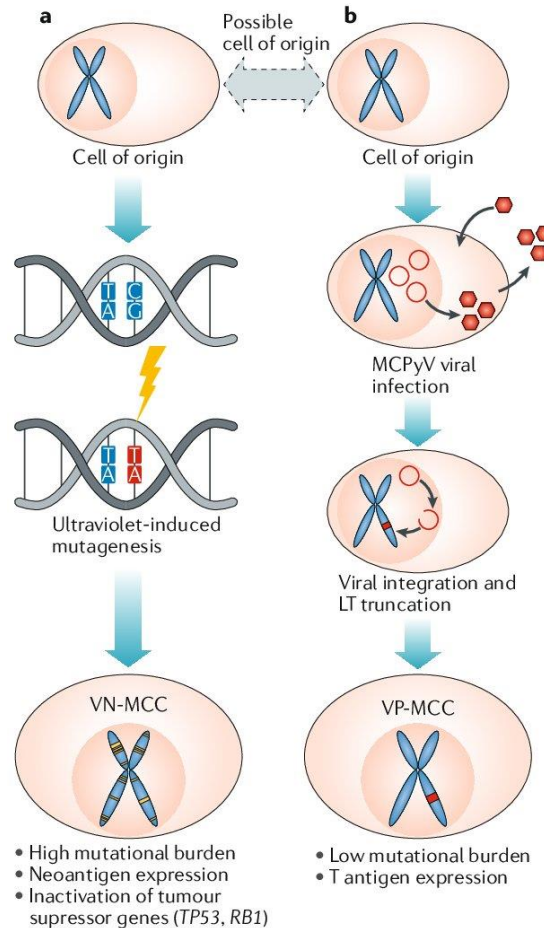


Figure 4. Proposed MCC tumorigenesis in the presence or absence of MCPyV. (a) In VN-MCCs, the cell of origin undergoes ultraviolet-mediated DNA damage, resulting in a high tumor mutational burden and inactivation of tumor suppressor genes, including RB1 and TP53. The high mutational burden might result in the expression of tumor neoantigens that represent potential targets for antitumor immunity. (b) In VP-MCCs, the cell of origin is infected by wild-type MCPyV, which undergoes episomal replication. Rarely, MCPyV can become integrated into the host cell genome and further acquires a truncating mutation of the large T antigen (LT), resulting in deficient viral replication with continued production of viral oncoproteins. The resulting tumor has a low burden of cellular genomic mutations. Hence, for patients with VP-MCCs, T antigen proteins might be better targets for treatments designed to promote antitumor immunity [34]. Adopted with permission from Springer Nature.

1.2.1. Merkel Cell Polyomavirus positive (VP) MCC

1.2.1.1. Merkel Cell Polyomavirus

For nearly 40 years, BK polyomavirus (BKPyV) and JC polyomavirus (JCPyV) have been the only known human polyomaviruses (PyVs). During the last decade, 11 new human PyVs, including Karolinska Institute polyomavirus (KIPyV), Washington University polyomavirus (WUPyV), Merkel cell polyomavirus (MCPyV), Human polyomavirus 6 (HPyV6), Human polyomavirus 7 (HPyV7), Human polyomavirus 9 (HPyV9), New Jersey Polyomavirus (NJPyV), Trichodysplasia spinuolsa associated polyomavirus (TSPyV), Malawi polyomavirus (MWPyV), HPyV12, and St Louis polyomavirus (STLPyV) have been discovered [35]. To add to this list, a putative human PyV named IARC-Lyon PyV (LIPyV) was recently isolated from human skin [36] and Quebec polyomavirus (QPyV) isolated from 85-years old male in Canada [37]. Although there is little information known about the pathogenesis of these novel human PyVs, some of them have been linked to human diseases: BKPyV-associated nephropathy, JCPyV-associated progressive multifocal leukoencephalopathy (PML), WU-PyV-associated bronchitis, HPyV6/HPyV7-associated dermatosis and TSPyV-associated trichodysplasia spinulosa [38, 39].

HPyV infection is common in the human population. Serological studies have shown a seroprevalence ranging from ~5% for HPyV12, NJPyV, and LIPyV, ~20% for HPyV9 and $\geq 60\%$ for the other HPyV in the healthy adult population. Moreover, each individual is infected with several HPyVs [40, 41]. Primary infection occurs in early childhood, after which the virus establishes a life-long and sub-clinical co-existence with its host [42]. The molecular detection of HPyV genomes has been complemented by important serological evidence of infection using HPyV VP1 capsid-specific IgG antibodies. The results indicated that HPyV infections frequently occur during childhood, reaching high seroprevalence rates of 40% to 90% in the general adult population, with average coexposure rates of 6 to 7 HPyVs [41, 43]. Despite this high rate, clinical symptoms or signs of primary HPyV infection have not been identified. In fact, only 5 HPyVs have been consistently linked to disease.

Evidence for persistent infection by a specific polyomavirus is reflected in serum antibodies against the corresponding polyomavirus coat protein VP1 [44]. Based on the VP1 serology assay, different studies have investigated prevalence of seven most important human polyomavirus in health individuals with different age groups [41].

MCC is associated with the MCPyV, with studies showing up to 80% presence in MCC tumors [45-47]. Although evidence suggests a causative link between MCPyV and MCC [48], further research is needed to evaluate the absolute risk of cancer. Infection with this virus is common during childhood and is usually asymptomatic. MCPyV antibodies are present in 35% of 13-year-olds [49] and increase to 80% at age 50 years [50].

HPyV diseases occur almost exclusively in patients with inherited, acquired, or therapeutic immunodeficiency states such as transplantation, HIV-AIDS, autoimmune disease, and cancer/chemotherapy [51, 52]. Evidence of HPyV disease is emerging for KIPyV [53], WUPyV [54, 55], HPyV6 [56, 57], HPyV10 [58], and NJPyV-2013 [59] due to dedicated studies correlating histopathology and virus infection by specific immunohistochemistry.

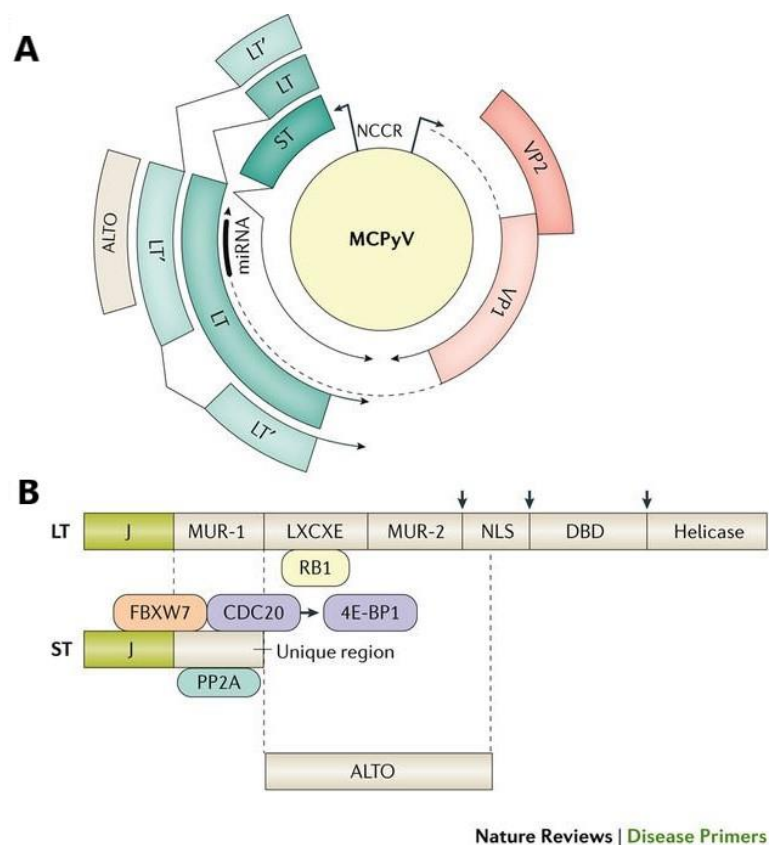


Figure 5: (A) Circular map and (B) linear maps of the MCPyV early genes [7]. MCPyV is a non-enveloped, double-stranded DNA (dsDNA) virus with genome of ~5kb and belongs to the family *Polyomaviridae* [36]. The viral genome is divided into three major regions: the non-coding control region (NCCR), early region and late region. The NCCR contains the origin of replication and transcriptional regulatory elements. The early region also called functional region encodes large T (LT) antigen, small T (sT) antigen, 57kT antigen and a protein called

alternative LT open reading frame (ALTO) [60-63]. The late region also called structural region encodes the viral capsid proteins VP1 and VP2 [64]. The polyomavirus genome is maintained episomal during normal life cycle. MCPyV has been found to encode a single microRNA (miRNA) precursor, which can produce a mature miRNAs, termed mcv-miR-M1. mcv-miR-M1 have the ability to negatively regulate expression of viral gene products required for viral DNA replication [65]. Adopted with permission from Springer Nature.

The original report from the Moore and Chang's lab outlined several important features about VP-MCC. Approximately, 80% of all MCC contain clonally integrated copies of the virus [45]. A vital question in VP-MCC is that how do MCPyV-encoded proteins contribute to viral activities and MCC development. Most of our knowledge of MCPyV oncogenic mechanisms was gained from studies of the viral oncogenes, LT and sT antigens, both of which have been implicated in MCPyV-induced tumorigenesis, at least in cell cultures and animal models [66]. An important feature of VP-MCC is that the tumor maintains expression of LT and sT antigen [45]. The functions of the 57 kT and ALTO proteins remain unknown, but mutant MCPyV virus lacking expressing of ALTO replicated similar to wild-type virus [63].

1.2.1.1A. small T antigen (sT antigen)

The primary MCPyV oncogenes are thought to be the sT and LT antigens. The sT antigen is expressed from two of four alternative spliced mRNA of the MCPyV virome [65]. MCPyV LT and sT antigen share exon 1 of T antigen locus with the DnaJ, CR1, and Hsc70 domains [67] (Figure 5). MCPyV sT was found to bind the catalytic subunit of PP1 [68]. The inhibition of PP1 prevents dephosphorylation of retinoblastoma and ensures cell cycle progression [69]. It is known that HPyV drive cells into the S phase that may be (partially) achieved by sT-mediated inhibition of PP1 thus facilitate viral genome replication [70]. This may be (partially) achieved by sT-mediated inhibition of PP1, which results in hyperphosphorylation of retinoblastoma [71].

In many PyVs, sT antigen binds protein phosphatase 2A (PP2A) and this interaction is mediated by the N-terminal J domain and the C-terminal zinc binding motif of sT [72]. PP2A is a phosphoserine/threonine phosphatase that exists as a heterotrimer composed of a structural subunit A, a regulatory subunit B, and a catalytic C subunit [73]. Different studies showed the interaction between MCPyV sT and PP2A A β , and weakly with PP2A A α , but also with the catalytic subunits PP2C α and C β . The binding of sT to PP2A reduced the catalytic activity of

the enzyme [68, 74, 75]. The biological implication of the sT:PP2A interaction is not known because mutations that abrogate PP2A binding had no effect on sT's transforming activity [76].

Interestingly, MCPyV sT targets the NF- κ B regulator NEMO by binding of a complex formed by protein phosphatase 4C (PP4C) and protein phosphatase 4 regulatory subunit 1 (PP4R1) leading to reduced NF- κ B translocation and transcriptional activity. Regulation of PP4C and PP4R1 by the sT could be a mechanism by which MCPyV modulates host anti-viral response or autoimmunity [74].

sT also has the large T stabilization domain (LSD) located between amino acids 91–95 that helps in transformation. The LSD binds and inhibits E3 ubiquitin ligase (SCFFbw7). MCPyV LT is a target of Fbw7-mediated ubiquitination to induce proteasomal degradation; therefore sT increases the half-life of LT protein. Furthermore, inhibition of Fbw7 also increases the levels of other Fbw7 target proteins of the cell cycle such as c-Myc and cyclin E [77]. The MCPyV sT LSD also increases hyperphosphorylated 4E-binding protein 1 (4E-BP1), a regulator of cap-dependent translation, thereby increasing protein translation [76].

Taken together, MCPyV sT increases protein translation, reduces proteasomal degradation, and thereby promotes tumor cell survival via increased levels of c-Myc, cyclin E, and MCPyV LT antigen.

1.2.1.1B. Large T antigen (LT antigen)

In all cases reported to date, VP-MCCs express a truncated form of LT (tLT) lacking the C-terminal of the protein due to premature stop codon. The tLT, however, preserves the N-terminal J domain and LXCXE motif but lacks the DNA binding and helicase domains. In addition, the C-terminal domain also contains around 100 amino acid residues in exon 3 with growth inhibitory activity that is not expressed in VP-MCC. In some of VP-MCC, LTs retained the nuclear localizations signal (NLS) while others lost it [78, 79].

MCPyV LT also plays specific fundamental roles in oncogenesis. LT contains a helicase domain at the C-terminal that helps in initiating viral DNA replication [67, 80]. Importantly, mutations in the C-terminal domain are essential for preventing initiation of replication within integrated virus genome. LT helps to prevent host cell death from DNA damage response to host cells [62, 67, 77, 80-82]. Studies have shown that when FLT was expressed in VP-MCC cell lines, a specific DNA-damage response was observed [62, 82].

MCPyV LT has different domains that inhibit tumor suppressors and activate oncoproteins. Most VP-MCC express a tLT with an intact retinoblastoma suppressor gene (RB1) binding domain (LxCxE motif; amino acids 211–217) [83, 84]. LT binding inhibits Rb activity thus, preventing Rb-mediated suppression of transcriptional activity of E2F. Release of E2F repression allows transcription of genes involved in cell cycle G1 to S-phase transitions, thus promoting tumor growth [78, 83]. Rb binding to LT domain also helps in upregulation of the anti-apoptotic oncoprotein, survivin [85]. Tumor suppressor p53 is another target of LT. Although MCPyV LT is not known to bind p53 directly, but higher LT expression leads to reduced p53 transactivation activity [86]. Therefore, in short, the preservation of N-terminal and mutation of C-terminal LT in tumors suggests importance for host cell transformation. LT also interacts with hVam6p, a protein involved in lysosomal trafficking, but is unlikely to contribute to cellular transformation and tumorigenesis [87]. LT has two unique domains, MUR-1 and MUR-2, with minimal contributions to transformation [88].

Taken together, integrated MCPyV employs multiple sT and LT-mediated mechanisms to promote tumor development and growth.

1.2.1.2. Merkel Cell Polyomavirus negative (VN) MCC

About 20% of MCC are VN-MCC and occurs due to excessive sun exposure (Figure 4) [45]. The mechanisms of oncogenesis leading to MCC is not completely understood. Next-generation sequencing analysis of both VN- and VP-MCC showed an overall higher mutational burden in VN-tumors compared with VP-tumors [89].

VN-MCCs contain an exceptionally high somatic mutational burden (50 mutations/Mb in VN- as compared to <1 mutation/Mb in VP-MCC) [90], enriched for ultraviolet signature C > T transitions throughout the genome [84, 91, 92]. *RB1* and *TP53* genes are two most commonly mutated genes in VN-MCC. Interestingly both of tumor suppressor proteins (p53 indirect) are also target for MCPyV LT [84-86]. Most of *RB1* mutations result in genome deletion or epigenetic hypermethylation [84, 89, 91, 93], which ultimately leads to increased E2F activity and thus increased tumor growth [78]. Likewise, *TP53* mutations in VN-MCC results in inactivation of p53. These p53 inactivating mutations result in downregulation of p53 targets, thereby preventing tumor cell senescence, cell cycle arrest, DNA damage repair, and apoptosis [84, 85, 91].

Different studies have shown activating oncogenic mutations in *HRAS*, *PIK3CA*, *KNSTRN*, *PREX2* and *RAC1* in the majority of VN- (6 out of 8) as compared to VP-MCC (2 out of 8). In addition to that, recurrent mutations in other tumor suppressor genes, including *NOTCH1*, and *PRUNE2*, were found in VN-MCC. All of these mutations suggest that genetic aberrations independent of MCPyV infection are involved in the pathogenesis of VN-MCCs [84, 89, 91].

Viruses are intracellular pathogens that not only replicate in host cell but also transform infected cell into a tumor cell. This is done by hijacking the host machinery and reprogram it through interfering signaling pathways [71]. Previous studies have shown different signaling pathways targeted in MCC. The main targets include phosphatidylinositol-3-kinase/AKT/mammalian target of the rapamycin (PI3K/AKT/mTOR) pathway [94-96], Mitogen-Activated Protein Kinase (MAPK) Pathways [97], Notch Signaling Pathway [98, 99] hedgehog signaling pathway [100, 101], DNA damage repair (DDR) pathways [82, 102, 103], Retinoblastoma-E2F Pathway [62, 86, 104], tumor suppressor p53 [104], Programmed cell death or apoptosis pathways [105, 106] and ubiquitination-proteasomal degradation pathway [77, 107-109].

Taken together for MCC pathogenesis, loss of tumor suppressive function of both Rb and p53 seems to be a consistent feature of both VP- and VN-MCC. In addition to that, additional oncogenic processes are consistently activated by viral oncogenes in VP-MCC.

1.2.2. Merkel cell polyomavirus and non-MCC tumors

Many studies have detected MCPyV DNA in various non-cancerous tissues of the body like the skin, adrenal gland, spleen, bone marrow, stomach, gallbladder, pancreas, heart, and aorta, although with a relatively low viral load between 0.00026 and 0.22 copies per cell [110]. But, the copy number of MCPyV genome was 60 times higher in MCC tissues as compared to healthy tissues. A higher presence of MCPyV among different tissues are digestive system, saliva, and in the upper digestive tract [111]. Since the role of MCPyV in the development of MCC is critical, so widespread prevalence of the virus encouraged researchers to explore a possible role of MCPyV in non-MCC cancers. Different studies have found MCPyV DNA, transcripts, and proteins presence in malignant tissues, but the integration state and the truncations of LT have rarely been examined. Moreover, the viral copy numbers are <1 copy/cell, adjacent non-malignant tissue was not always examined and the number of samples was often low (reviewed in [112]). Therefore, a possible role of MCPyV in other tumors remains elusive.

There are few reports in which MCPyV DNA was found in reproductive system-related tumors; for example in prostate cancer [111, 113], breast cancer [114], and cervical cancer [115]. In one study with testicular cancer, a relatively high viral load with 0.934 copies per cell was detected, but LT protein expression was not assessed [111]. Other studies of MCPyV-positive prostate cancers and cervical cancer showed that these samples exhibited a lower viral copy number with 0.002 [111] and 0.00003055 and 0.0015 [115, 116] copies per cell respectively. A small subset of breast cancer were MCPyV DNA positive [114, 117], but viral DNA could not be detected in ovarian and vulva cancer [48, 117]. Even though, MCPyV may not be the perpetrator, its oncoproteins LT and sT may have an effect on the expression of oncoproteins expressed by other co-infecting oncovirus. One example is high-risk human papilloma virus (HR-HPV) associated cervical cancer. Different studies have shown the presence of MCPyV in HR-HPV associated cervical cancer [118-120].

Papillomaviridae is a family of nonenveloped, double-stranded DNA viruses that can cause cancer in humans [121]. HR-HPV are mainly associated with cervical cancer, but also with penile, anal, vulvar, vaginal and oropharyngeal cancers [122, 123]. Among different HR-HPV strains, HPV-16 and HPV-18 are responsible for almost 70% of cervical cancers worldwide. There is viral genome integration into host genome and HR-HPV is considered one of the most important risk factors for cervical cancer development [124-126]. The oncogenic potentials of HR-HPV is mainly associated with the viral proteins E6 and E7. These proteins can bind p53, mTOR, hTERT and pRb family members, respectively. Additionally both E6 and E7 can also interfere with other hallmarks of viral oncogenesis [127] as seen by MCPyV LT in MCC. So co-expression of LT, sT, E6 and E7 may collaborate in the neoplastic processes by these viruses. Co-infection with MCPyV and the oncoviruses Epstein-Barr virus and human herpesvirus-8 in respectively chronic lymphocytic leukemia, Kaposi's sarcoma has been described [35], but whether MCPyV plays a causal role in these tumors has not been established.

1.3. Inflammation

Inflammation is described as a series of events induced by the body in response to stimuli that are potentially harmful to the body, such as infection or injury. The inflammatory response is characterized by the rapid accumulation of neutrophils, macrophages, immune cells and later on production of soluble mediators with the ultimate aim of protecting the organism from foreign invaders and initiating healing processes [128]. The process of inflammatory response depends on the nature of the initial stimulus and the site in the body. They all share common

mechanisms with: 1) detrimental stimuli recognition by cell surface pattern receptors; 2) activation of inflammatory pathways; 3) release of inflammatory mediators; and finally 4) recruitment of inflammatory cells [129].

1.3.1. Immune System

The human immune system categorized as the innate immune response that is fast but not specific and the adaptive immune response that is slow but involving specificity and memory. The innate immune system provides the first barrier against any type of infections and consists of granulocytes, macrophages, mast cells, dendritic cells (DCs), natural killer cells and humoral part such as mucosal barrier, complement system, lysozyme. In the adaptive immune response, immune cells (B- and T-cells) specifically target invaders in an antigen-antibody specific manner. There are also some other cells that function at the interface between the innate and adaptive immune response and consists of Natural killer T cells (NKT) cells and $\gamma\delta$ T cells. The immune system not only protects host against pathogens like viruses and bacteria, but also plays an important role in the surveillance of cancer [130].

1.3.2. Cancer Immunology

A role for inflammation in cancer development was proposed in 1893, when Rudolf Virchow observed the presence of immune cells in neoplastic tissues [131]. In 2011, after more than 100 years of its original proposal by Virchow, it was formally acknowledged with the inclusion of immune system evasion and inflammation as additional hallmarks of cancer [132, 133].

Cancer immunology is the study of interactions between the immune system and cancer cells. In cancer immunology, the immune response is of particular interest with most important work as cancer-specific antigens recognition known as tumor-associated antigens (TAAs). The immune system can recognize the antigenic changes and further develop specific T-cells and antibodies to recognized neo-antigen [134-136]. These anti-TAAs that are associated with cancer, might be considered as reporters from immune system to identify antigenic cellular proteins changes involved in transformation process [137]. These anti-TAAs autoantibodies have advantage over antigens with persistence and stability in serum samples of cancer patients [138]. In recent years, anti-TAAs autoantibody have been explored as early cancer biomarker as well as indicators of disease prognosis [139].

1.3.3. Inflammatory Mediators

Various intrinsic (reactive nitrogen oxygen species RNOS, oncogenic events) and extrinsic (infection and other inflammatory conditions) factors trigger the recruitment of the inflammatory cells to the site of inflammation [140]. This results in the activation of several molecular signaling cascades. These signaling cascades are associated with increased production of inflammatory cytokines and hence the establishment of inflammation [140].

Cytokines are central mediators between cells in the inflammatory tumor microenvironment (TME) [141]. They also have normal function in body as messengers between the cells. They are released in response to a diverse range of cellular stresses, including carcinogen-induced injury, infection and inflammation. In these settings, cytokines function to stimulate a host response that is aimed at controlling the cellular stress and minimizing cellular damage [130]. They are usually classified into two classes: anti-inflammatory cytokines, such as IL-4, IL-10, IL-13, IFN- α , and TGF- β , and pro-inflammatory cytokines, such as IL-1 β , IL-6, IL-15, IL-17, IL-23, and TNF- α [140]. The anti- and pro-tumorigenic function depends on cellular contents and concentrations in TME. Various therapeutic approaches have been developed to either target tumor-supporting inflammatory mediators or promote anti-tumorigenic inflammation and immune responses, with response to reeducate the TME and promote anti-cancer function [142-144].

1.3.3.1. CCL17/TARC

The human cytokines system consists of 50 distinct proteins that act via distinct receptors of almost 20 different types [145, 146]. Chemokines are a family of small (8-12KDa), structurally related, secreted proteins that regulate leukocyte trafficking in immunity and inflammation [147]. Based on the first two of the four conserved cysteine positions, chemokines are divided into four subfamilies: CXC, CC, XC, and CX3C [148, 149]. Based on physiological features, chemokines are divided into two categories, inflammatory and homeostatic [150]. Upon stimulation by pro-inflammatory cytokines, inflammatory chemokines are expressed in inflamed tissues. This type of chemokines is specialized for recruitment of effector cells, including granulocytes, monocytes, and effector T cells. Homeostatic chemokines, on the other hand, are produced in discrete microenvironments within lymphoid and non-lymphoid tissues such as the skin and mucosa. These chemokines maintain physiological traffic and positioning of cells during immune surveillance [151].

TARC (thymus and activation-regulated chemokine/CCL17) is a member of CC chemokine group expressed in the thymus and also expressed by keratinocytes, endothelial cells, dendritic cells, bronchial epithelial cells and fibroblasts (Figure 6). It is assumed that TARC/CCL17 works both as a inflammatory and homeostatic chemokine [148].

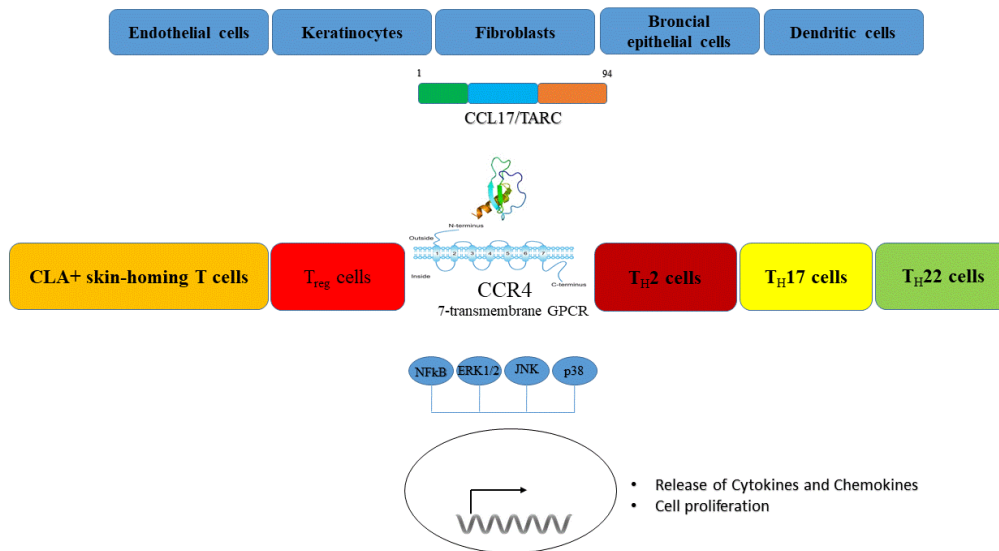


Figure 6: CCL17/TARC expressing, target cells and signaling pathway.

1.3.3.2. CCR4

All chemokine receptors are class A G-protein-coupled seven-transmembrane receptors that induce signal transduction via G proteins [152]. Based on the chemokine subfamily they bind, chemokines receptors are classified as CXCR1-5, CCR1-9, CXCR1 and CX3CR1. The specific functional high affinity receptor for TARC/CCL17 is CC chemokine receptor 4 (CCR4). CCR4 is predominantly expressed on CD4⁺ T cells [153], but also on a variety of functionally distinct thymocytes including skin-homing T cells, CD25⁺ T suppressor cells and T helper (Th) 2 cells. Since their discoveries, numerous studies have explored the expression and function of CCR4 and its ligands in skin diseases, such as atopic dermatitis [154], bullous pemphigoid [155], mycosis fungoides [156] and several other allergic diseases including allergic asthma [157], allergic rhinitis [158] and allergic contact dermatitis [159]. The chemokine receptor CCR4 is expressed in the TME of several cancers and is associated with poor prognosis. New findings show that CCR4 is highly expressed in human renal cell carcinoma (RCC) [160], lung cancer [161, 162], melanoma brain metastasis [163] many hematologic malignancies such as Adult T-

cell leukemia (ATL) [164, 165] relapsed peripheral T cell lymphoma (PTLC), and Cutaneous T-cell lymphoma (CTL) [156].

1.3.3.3. IL-33

The IL-1 family (IL-1 α , IL-1 β , IL-18) of cytokines plays a major role in inflammatory, infectious, and autoimmune diseases [166, 167]. Interleukin-33 (IL-33), previously known as nuclear factor from high endothelial venules or NF-HEV also belongs to IL-1 cytokine family [168]. This cytokine is produced by various types of immune cells, such as mast cells, macrophages, and dendritic cells, and also by nonimmune cells, such as endothelial cells, epithelial cells, smooth muscle cells, and fibroblasts [169]. IL-33 is a 30-kDa protein that functions both as a transcription factor and a cytokine. Full length IL-33 (FL-IL-33) is divided into three major domains, a nuclear domain (N terminal), an activation domain and an IL-1 like cytokine (C terminal) domain (Figure 7). The nuclear domain contains a nuclear localization signal, a DNA-binding homeodomain-like helix-turn-helix motif, and a chromatin-binding domain [168, 170]. Upon IL-33 synthesis, it is targeted to the nucleus, where it binds to chromatin and is thought to regulate gene expression. It can bind to histones H2A and H2B [168, 171] and can activate histone deacetylase-3 (HDAC) activity [172] thereby affecting gene expression by remodeling chromatin structure and by epigenetic mechanisms. It has been shown to interact with the N-terminal domain of the p65 subunit of nuclear factor κ B (NF- κ B) and to repress the expression of NF- κ B-regulated genes that are necessary for pro-inflammatory signaling [173]. In response to cellular damage, tissue injury or viral infection, IL-33 is quickly released from the nucleus of necrotic cells and secreted into extracellular space where it can bind to the membrane-bound suppression of tumorigenicity 2 receptor (ST2) through its cytokine domain [174, 175]. Binding to its receptor triggers an inflammatory cascade, thus IL-33 acts as an “alarmin” and is considered a damage-associated molecular pattern (DAMP) [176]. The nuclear and cytokine functions of IL-33 are tightly regulated through its localization [177].

1.3.3.4. IL-33 receptors

The IL-33 receptor (IL-33R) is a heterodimeric complex of ST2 and IL-1 receptor accessory protein (IL-1RAcP) [178]. The ST2 receptor had been extensively studied and was known as an orphan receptor for >10 years before the discovery of IL-33 [179]. ST2 was first identified in murine fibroblasts as an oncogene-induced gene [180, 181]. It is encoded by *IL1RL1* that produces four isoforms through alternative splicing: ST2L (ligand), sST2 (soluble), ST2V

(variant), and ST2LV (ligand variant). ST2L is a membrane-embedded receptor that is highly homologous to IL-1 type-1 receptors and harbors three Ig-like extracellular domains, a transmembrane spanning region, and an ILI-R1-like intracellular domain [182, 183].

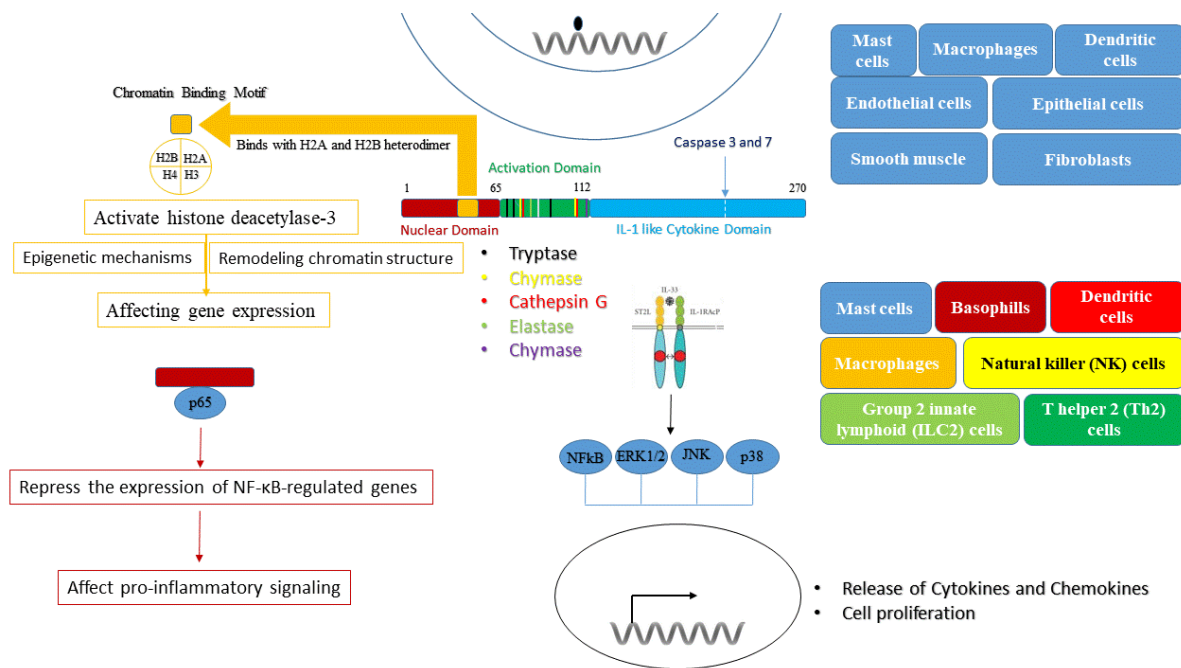


Figure 7: IL-33 producing cells, functional domains and signaling.

Of the four isoforms, ST2L and its decoy receptor sST2 have been most studied, while not much is known about ST2V and ST2LV [184]. Expression of the *IL1RL1* gene is regulated by GATA1/2, and estrogen-response elements (EREs) that are found on the distal and proximal promoters that regulate ST2L and sST2 expression [185-187]. While both isoforms can be transcribed from both promoters, the cell type appears to govern gene expression from either proximal or distal promoters [186]. ST2L is expressed in fibroblasts, mast cells, eosinophils, Th2 lymphocytes, dendritic cells, basophils, invariant natural killer cells (iNKT) cells, and macrophages, while sST2 is predominantly expressed by fibroblasts and epithelial cells [188].

IL-1RAcP is a co-receptor for IL-33 signaling, and this molecule acts as a shared co-receptor for other IL-1 family members [178, 189, 190]. ST2L creates a heterodimeric transmembrane receptor complex with the IL1-RAcP [191], and hetero-dimerization brings together the intracellular domains of the two transmembrane proteins, and its assembly initiates the recruitment of adaptor molecules through which the IL-33 signal is transduced [170, 192].

1.3.4. Immunogenicity of MCC

Immunogenicity is an important inherent feature of tumor cells. This feature is determined by the tumor cell itself, and is also influenced by the TME [193]. Fundamental determinants of tumor immunogenicity include tumor antigenicity, and antigen processing and presenting efficiency [194].

MCC is particularly linked to immune suppression as compared to other tumors. Indeed, almost 8 % of MCC patients are immune suppressed [5]. There are different groups of individuals who are at high risk of getting MCC. Among those, are HIV infected patients with an 8-fold higher risk [30], solid organ transplant recipients with a 24-fold increased risk [28], chronic lymphocytic leukemia and other hematologic malignancies with a 34 to 48-fold increased risk [5] and also patients with auto-immune diseases, treated with immunosuppressive medication are at higher risk to develop a MCC compared to immunocompetent individuals [195]. This suggests that defective cellular immunity predisposes individuals to not only developing MCC, but also to poorly controlling their disease.

In MCC, both VP- and VN-MCC can be immunogenic, as revealed by the finding of robust intratumoral infiltration of CD8 T cells into MCC tumors with 100% survival, independent of the stage of the cancer [196]. Among VP-MCCs, immunogenicity can be assessed with MCPyV oncoprotein-specific antibodies and T-cell responses, which are quite prevalent. Whereas VN-MCCs do not express viral oncoproteins, these tumors often express extremely high numbers of tumor neoantigens which may provide tumor-specific targets for immune recognition [197].

There are also different findings about MCCs regression following withdrawal of immune suppressive treatment [198, 199]. MCCs with either partial regression (PR), complete spontaneous regression (CSR) or regression after recurrence and/or metastasis have been reported in the literature. CSR in MCC is much more common (1.3 per 1,000 cases) than in other malignancies (1 in 60,000-100,000 cases) [200]. Until 2016, a total of 32 cases with CSR have been reported. The exact mechanism is still unknown. However previous literature revealed a female tendency (24F:6M), with the most common site being on the head and neck region (26/30, 87%), with regression occurring most often after the initial biopsy. This supports the hypothesis that the biopsy procedure may induce an antitumoral, T-cell-mediated immune response [200, 201]. Among the 32 CSR patients, MCPyV status was evaluated in only four studies with three were VP and only one was VN MCC patient [202]. The CSR has also been

reported after local recurrence and/or metastasis in total of 16 MCC cases. In this cohort of MCC patients, there was slight male predominance (7F:9M), with the most common site of the primary MCC on the head and neck region (14/15, 93%). The average time interval from the detection of the recurrence and/or metastatic disease to regression was 6.02 months (11 days–30 months) [202]. Additional mechanisms that attribute to the elevated proportion of regression include a higher rate of apoptotic activity [203], dense lymphocytic infiltration around the tumor cells [204] and antiretroviral therapy [205].

1.3.5. Immune Evasion in MCC

In 2000, Hanahan and Weinberg initially postulated the six hallmarks of cancer [133], but in their update in 2011, inflammation and immune evasion was added as an important feature of cancer cells [132]. Despite of immune surveillance, it had become apparent that tumors are still able to develop, even in hosts with fully functioning immune system [206].

During the last two decades, conceptual developments have demonstrated that the immune system can paradoxically constrain and promote tumor development and progression. This process is referred to as cancer immunoediting and proceeds through three phases, termed elimination, equilibrium and escape [207]. During first phase of (1) eliminations, also called immunesurveillance, the immune system reacts to tumor and tumor cells are eliminated; and in (2) equilibrium phase, the immune system does react, but is not able to eliminate the tumor cells to a full extent. In the last phase of (3) escape, surviving immune resistant cancer cells can form tumors that are evading the immune response (Figure 8) [206]. For VP-MCC, immune evasion is difficult to accomplish despite continuous expression of viral T-antigens. While MCPyV specific T cells are present in the majority of MCC patients, they rarely infiltrate into MCC tumors [208]. This fact suggests that MCC cells and cells of the MCC TME create an immunosuppressive environment that inactivates MCC specific T cells and prevents them from infiltrating, thus facilitating the immune escape of MCCs.

Indeed, during the last years several studies accumulated evidences that MCC cells employ a variety of immune escape strategies. During viral infection and/or cellular transformation, natural killer group 2D (NKG2D) ligands, major histocompatibility complex (MHC) class I chain-related protein A and B (MICA/MICB) undergo upregulations. The NKG2D and MICA/MICB interaction stimulates proliferation and cytotoxic potential of NK cells [209]. In a study of MCC tumors and cell lines, MICA and MICB mRNA levels were low and protein

levels were rarely observed. These observations indicate that MCC mediate immune evasion by downregulating MICA and MICB through epigenetic mechanism [210].

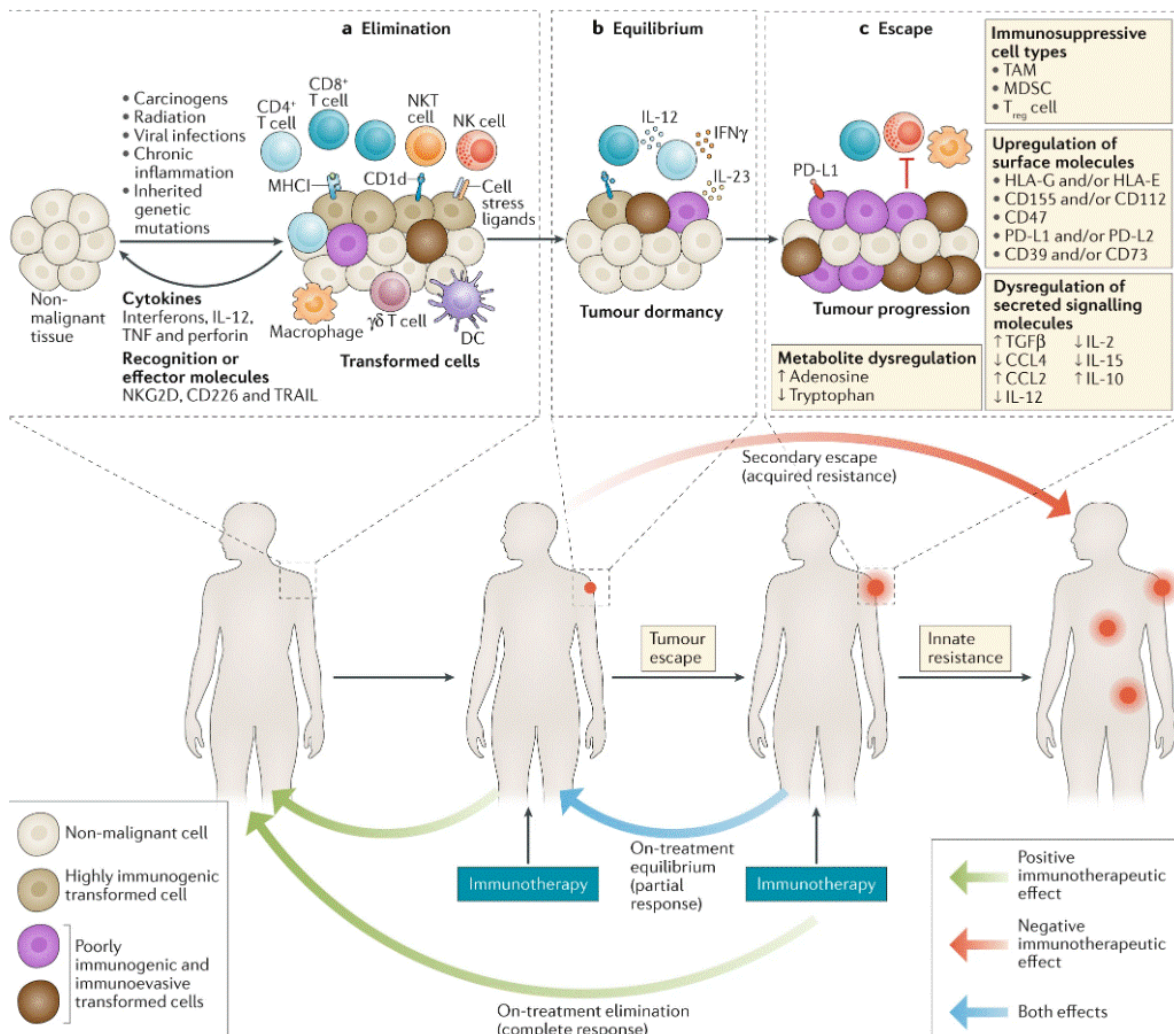


Figure 8: Cancer immunoeediting is a host intrinsic cellular mechanism that proceeds through three phases: elimination, equilibrium and escape. (a) During the elimination phase, the innate and adaptive immune systems recognize transformed cells that have escaped intrinsic tumor suppression and to eliminate them before tumors become clinically detectable. (b) Tumors developing cells that survived from the elimination phase can progress into the equilibrium phase, in which net growth is limited and cellular immunogenicity is edited by the adaptive immune system. (c) Edited tumors can then enter into the escape phase, in which their growth is unrestricted. Escaped tumors are those clinically detectable as visible tumors [207]. Adopted with permission from Springer Nature.

During active immune system, CCAAT/enhancer-binding protein (C/EBP) transcription factor, a positive regulator of the Toll-like receptor 9 (TLR9) promoter, acts as an important mediator of pro-inflammatory immune responses. TLR9 activation results in activation of NF- κ B-mediated transcription, which produces pro-inflammatory cytokines and type 1 interferons (IFNs) that are important for clearing virus-infected cells and promoting further immune activation [211]. In VP-MCC, T-antigen mediated inhibition of C/EBP leads to reduced TLR9 expression thus inhibiting NF- κ B-mediated transcription that ultimately results in reduced expression of proinflammatory cytokines and IFNs [212, 213]. However, TLR's involvement is not well understood in VN-MCC.

Previous studies also have shown macrophage infiltration in MCC and the level of infiltrate was higher in VP- as compared to VN-MCC [214]. Surprisingly, a portion of the macrophage infiltrate was found to express CD163, a marker of M2 phenotype. M2 phenotype is linked to tumor growth and survival through secretion of immune suppressive cytokines, rather than M1 that is pro-inflammatory phenotype [214]. However, the level of CD163⁺ macrophages was not associated with the status of MCPyV [214]. Taken together, NKG2D and MICA/MICB inhibition interaction, downregulation of TLR9 and M2 macrophage infiltrate in tumor contributes to innate immunity evasion of MCC.

The effectiveness of immune responses may decrease due to inability of all activated T cells to properly home tumor tissue in MCC. For T cells to migrate to the area of inflammation, interactions must occur between T cells and endothelium. E-selectin, a receptor present on endothelium, has been proposed to be critical for T lymphocyte migration [215]. A subset of T cells expresses a ligand for E-selectin, the cutaneous lymphocyte antigen (CLA) [215, 216]. In a study of 56 MCC samples, 52% of MCC samples displayed downregulation of E-selectin expression in intratumoral vasculature [217]. A study by Dowlatshahi et al., demonstrated correlation between CLA expression and T cell infiltration into tumors. The authors found that 3/3 MCC tumors with high CLA expression were infiltrated by an increased number of T cells present within tumor nests. They also found 4/4 MCC tumors with decreased T cells CLA expression, peritumoral pattern of T cells without penetration into tumor nest [218]. These above two events, downregulation of E-selectin and lacking CLA contributes to the lack of T cell migration into sites of MCC tumors.

Previous studies have shown that optimal T cell activation requires two signals: an antigen-specific signal (mediated by antigen presentation) through the T cell receptor (TCR) and a

costimulatory signal [219, 220]. Antigen presentation is essential for identification and successful eradication of tumor cells by CD8⁺ T cells. Dysregulation of antigen presentation, by loss of MHC-I and β 2-microglobulin (B2M) is a common mechanism of immune evasion by various types of cancers [221]. In a study of 114 MCC patient samples, 84% showed reduced MHC-I expression as compared with surrounding tissues with 51% had poor or undetectable MHC-I expression. The authors of this study also found that VP-MCC showed lower MHC-I expression as compared to VN-MCC [222].

In addition to the downregulation of immune cell recognition receptors, inhibitory receptors are upregulated on tumor-targeted immune cells. Negative regulatory pathways in the immune system help in successful clearing pathogens and malignant cells thus limiting immunopathology [223]. Programmed death receptor 1 (PD1) has been identified as critical negative regulator of T cell activity [224]. PD1-mediated T cell inhibition is an important mechanism to prevent autoimmunity, cancer and chronic infectious diseases [225]. Chronic antigen stimulation from viral infections and tumor cells upregulates inhibitory receptors on active T cells that results in T cells functionality loss over time and leads to T cell exhaustion [226, 227]. Increased expression of T cell receptors, PD-1 and TIM-3 results in immune dysfunction and prevents CD8⁺ T-cell mediated clearance of virus infected and malignant cells [228]. MCPyV-specific T-cells in blood and MCC tumors show simultaneous PD-1 and TIM-3 expression [229]. Another study of effector T-cells isolated from VP-MCC have shown lower levels of activation markers (CD25 and CD69) and higher levels of PD-1 compared with normal skin T cells [218]. As tumor-specific epitopes are necessary for T-cells isolation, VN-MCC-specific T-cells have not been identified. So, it is still unclear whether T-cell exhaustion markers are present on tumor-specific effector T-cells present in TME of VN-MCC [230]. In case of melanoma, tumor-specific CD8⁺ T –cells also upregulate PD-1 and TIM-3, indicating that somatic mutations induced by UV exposure contribute to T-cell dysfunction; this may also occur in UV exposure–mediated MCC [231]. Additionally MCC can also evade the immune system by overexpressing PD-L1 receptor present on tumor cells, thus inactivating active CD8⁺ T cells [232]. A study of 49 MCC patient tissues analyzed PD-L1 expression on tumor cells and infiltrating lymphocytes. This study showed that 50% of the VP-MCC samples showed PD-L1 expression and had a moderate-severe immune infiltrate, as compared to 0% of the VN-MCC tissue samples [232]. However, another study demonstrated that PD-L1 overexpression in VN-MCC correlated with increased mutational burden [91].

Additionally, Tregs cells recruitment to the area of inflammation also results in immune suppression. Tregs can inactivate CD8⁺ T-cells and antigen presenting cells (APCs), thus leading to disease progression in response to UV radiation exposure and viral infection [233]. Different studies have shown high Tregs infiltration in MCC as compared to normal skin (reviewed in [218]). While comparing the role of Tregs in MCC as compared to other cancers, a study of 116 MCC patients with Tregs presence is associated with longer survival. This possibly indicating that association between MCC and viral infection results in distinct profile of T-cell response [214]. In another study of MCC patients, CD4⁺ and CD8⁺ Tregs were found within MCC tumors, but their presence was not associated with overall survival [218]. Thus, role of Tregs in establishment and progression of MCC is unclear.

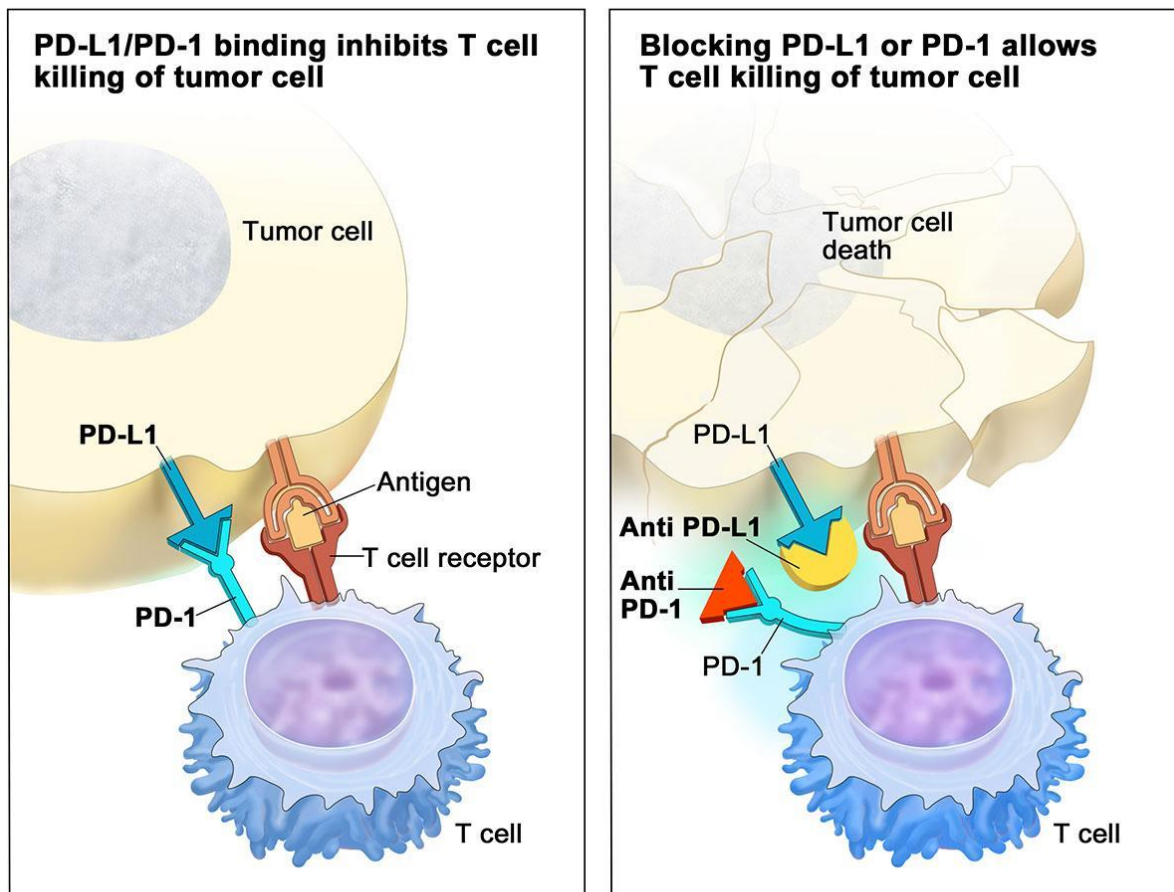
1.4. Current treatment options for MCC

For MCC, there are different options for treatment including standard (currently used options) to some therapies that are being tested in clinical trials. There are four different types of standard treatment options being used in clinics. These standard options include surgery, radiation therapy, chemotherapy and immunotherapy. All of these options are used alone or in combinations for better recovery. The local recurrence chances after surgical treatment are still higher as compared to other most common skin cancers (basal cell carcinoma, squamous cell carcinoma and even melanoma) [234, 235]. Radiation therapy can markedly lower the risk of recurrence of MCC in irradiated areas. Most of time radiation therapy is used in combination with local surgical excision. Radiotherapy decreases local recurrence about 3.7 time when used in combination [236]. Chemotherapy is also another line for treating MCC that leads to significant shrinkage of tumors, but chemotherapy is not very effective as tumors often get resistant and recur within 90 days. The reason for this failure is either immune suppression by chemotherapy and/or MCC tumor cells acquire resistance towards chemotherapy [237]. A study of standard chemotherapy in metastatic MCC patients showed initial shrinkage of the tumors in the majority of patients, but no durable effect. It was found that half of the patients after 3 months and more than 90% of the patients after 10 months had recurrent growth of their tumors [238]. For patients who do not have problems with their immune system for example, no autoimmune disease, it is typically recommended to first try an immune stimulating therapy also called immunotherapy. The main idea of using cancer immunotherapy is to stimulate the host immune defense system against specific type of diseases. This can be done by using

monoclonal antibodies [239], vaccines [240], cytokines [241], oncolytic viruses [242], chimeric antigen receptors (CAR) T-cell therapy [243] and immune checkpoint inhibitors (ICIs) [244].

Immunotherapy is rapidly becoming a preferred systemic therapy in several cancer types, especially because responses to immunotherapy (when they occur) are generally long-lasting. The durability of immunotherapy responses places this approach in stark contrast to chemotherapy, which was previously considered the standard option for patients with metastatic MCC [238]. Immune cells, such as T cells, and some cancer cells have certain proteins, called checkpoint proteins, on their surface that keep immune responses in check. The development of ICIs is a revolutionary milestone in the field of immuno-oncology. Tumor cells evade immunosurveillance and progress through different mechanisms, including activation of immune checkpoint signaling that suppress antitumor immune responses.

Two types of ICIs therapies are currently being used, targeting programmed cell death-1 (PD-1) (Figure 9) and cytotoxic T-lymphocyte antigen-4 (CTLA-4) (Figure 10). PD-1 is a protein expressed on the T-cells. When PD-1 attaches to another protein called PD-L1 on a cancer cell or myeloid cells (DCs, TAM), it stops the T cell from killing the cancer cell. PD-1 inhibitors attach to PD-L1 and allow the T cells to kill cancer cells. Avelumab (antibody against PD-L1) [245] and pembrolizumab (antibody against PD-1) [246] are used to treat advanced Merkel cell carcinoma. Nivolumab (targets PD-1) [247] is being studied to treat advanced Merkel cell carcinoma. CTLA-4 is another immune checkpoint protein expressed on the surface of Tregs cells that helps keep the body's immune responses in check. When CTLA-4 attaches to another protein called B7 on a APCs. Ipilimumab is a type of CTLA-4 inhibitor being used to treat advanced Merkel cell carcinoma [248, 249]. There are still a number of clinical trials are going on different phases to target ICIs.



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Figure 9: Immune checkpoint inhibition. Checkpoint proteins, such as PD-L1 on tumor cells and PD-1 on T cells, help keep immune responses in check. The binding of PD-L1 to PD-1 keeps T cells from killing tumor cells in the body (left panel). Blocking the binding of PD-L1 to PD-1 with an immune checkpoint inhibitor (anti-PD-L1 or anti-PD-1) allows the T cells to kill tumor cells (right panel) [250]. The figure is adopted with permission from the National Cancer Institute © (2015) Terese Winslow LLC, U.S. Govt. has certain rights.

Despite the success of anti-CTLA-4 and anti-PD-1/PD-L1 therapies, only a fraction of patients benefit from ICIs. Antitumor immunity, regulated through complex factors in the TME, could create variable immune responses [244]. Immunotherapeutic approaches other than targeting ICIs are also being investigated in clinical studies (table). These approaches involve either stimulations of NK cells, macrophages, T-cells (NCT02054884), infusion of activated NK-92 cells (NCT02465957), IL-12 gene therapy (NCT01440816), targeting CD47 (NCT02890368) and TLR4 agonist (NCT02035657).

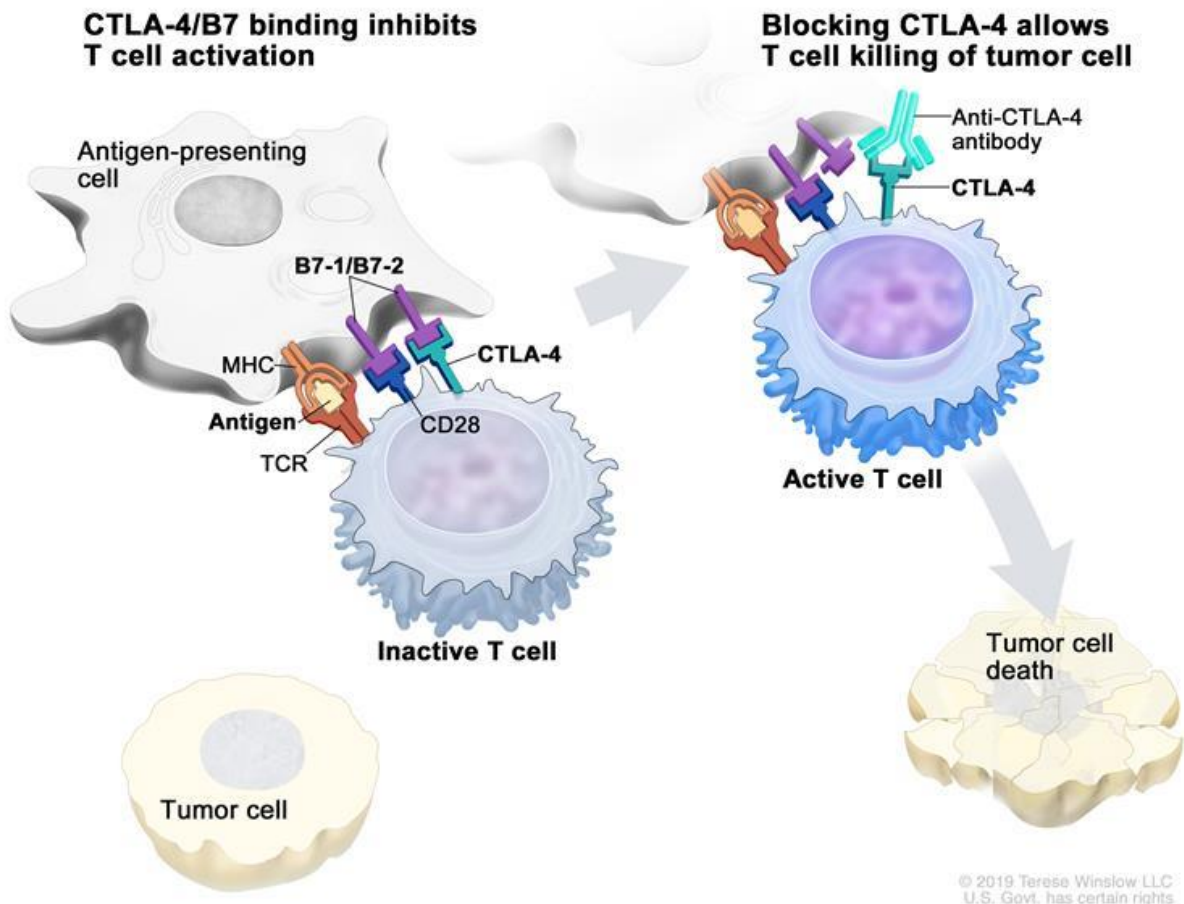


Figure 10: Immune checkpoint inhibition. Checkpoint proteins, such as B7-1/B7-2 on APCs and CTLA-4 on T cells, help keep the body’s immune responses in check. When the TCR binds to antigen and MHC proteins on the APC and CD28 binds to B7-1/B7-2 on the APC, the T cell can be activated. However, the binding of B7-1/B7-2 to CTLA-4 keeps the T cells in the inactive state so they are not able to kill tumor cells in the body (left panel). Blocking the binding of B7-1/B7-2 to CTLA-4 with an immune checkpoint inhibitor (anti-CTLA-4 antibody) allows the T cells to be active and to kill tumor cells (right panel) [250]. The figure is adopted with permission from the National Cancer Institute © (2019) Terese Winslow LLC, U.S. Govt. has certain rights.

1.5. Limitations of Immunotherapy

However, not all patients experience durable response to immune-based therapies. Furthermore, patients requiring immunosuppression in the setting of solid organ transplants or those with autoimmune disease may not be optimal candidates for immune-based therapy. Therefore,

predicting and improving response to immunotherapy, and identifying alternative therapies for cases in which immunotherapy is contraindicated or ineffective, represent current research priorities in MCC [34].

A clinical trials with MCC patients using pembrolizumab had shown 56% objective response with 16% complete and 40% shown partial response with a median follow-up of 33 weeks (7-53 weeks). The response duration ranged from 2.2 months to at least 9.7 months. The progression-free survival rate was 67% in 6 months. The response rate in VP- and VN-MCC was 62% and 44% respectively. In the same study, the authors observed treatment-related adverse event (TRAE) of any stage including adrenal insufficiency, colitis, hepatitis, myocarditis, nephritis, pneumonitis, thyroiditis, and transaminitis [246]. In another clinical trial with avelumab, the investigators found objective response in 33% of patients with 65.5% of whom had partial response. Among patients who have shown objective response, 93% had durable response ≥ 6 months, 71% had ≥ 12 months and 29% had at 18 months [251]. Considering evaluable safety, 71.8% had TRAE with 15.4% grade-1 immune-related adverse events and only 20.5% grade 3 TRAE but no grade 4 TRAE or treatment related deaths occurred. The patients also had shown some adverse effects i.e. cholangitis, elevated aspartate aminotransferase and alanine aminotransferase levels, paraneoplastic syndrome, gait disturbance, paraneoplastic encephalomyelitis and polyneuropathy and infusion-related reaction [252]. It became apparent that cancer patients respond very different to immunotherapy and so far, there are almost no predictive markers, which patient will benefit from which immunotherapy. In addition, it became obvious that combination of different drugs will be the key to success for immunotherapy.

2. AIMS OF THE THESIS

The overall aim of this thesis was to investigate the effect of Merkel cell polyomavirus effect in merkel cell carcinoma and other cancers.

The specific aims were:

Paper I: To compare the transcriptional activity of NCCR of different MCPyV variants isolated from virus-positive MCC and non-MCC samples in a MCC cell line, and in human dermal fibroblasts.

Paper II: To study effect of MCPyV LT on CCL17/TARC expression and impact on viral expression.

Paper III: To study effect of MCPyV T-ag on IL-33 expression and role in viral expression.

Paper IV: To study effect of MCPyV oncoproteins LT and sT on expression of HR-HPV oncoproteins E6 and E7, thereby accelerating the initiation and/or progression of cervical cancer.

3. METHODOLOGICAL CONSIDERATIONS

3.1. Biological material

Cancer cell lines are an extremely useful tool to study diverse biological processes and the efficacy of therapeutic agents in basic science research. A broad variety of MCC cell lines are available from different patients and represent various aspects of MCC biology, such as presence or absence of MCPyV. MCC cell lines are a convenient tool as their handling is not complicated, allowing extensive experimental set ups. However, we should keep in mind unavoidably changes in the cells due to passages of cell line. Therefore, we should considered a useful model that can however not replace patient material and *in vivo* studies.

3.1.1. Cell lines

To confirm the identity of the cell lines used in this work STR (short tandem repeat) profiling was performed at the Centre of Forensic Genetics, University of Tromsø. The cell lines were routinely grown without antibiotics as it has been demonstrated that antibiotics can affect the proliferation and gene regulation of cultured cells [253, 254]. Furthermore, the use of antibiotics can hide low level contaminations and mycoplasma infections [255]. Mycoplasma tests were performed regularly.

3.1.2. Human tissue and plasma samples.

MCC tumor tissues were obtained in accordance with the ethical approval during 2000-2015 from the St. Olavs University Hospital Trondheim, Norway according to the ethical approval from the Regional Ethical Committee (REK NORD application number 2016/988). MCC plasma samples were obtained in accordance with approved by the Ethics Committee of Karolinska Institutet (2010/1092-31/3).

3.2. Promoter luciferase assay

This method makes use of *luciferase* as a reporter gene to test and compare promoter(s) activities. One of the commonly used reporter genes used in monitoring promoter strength of interest is the *luciferase* gene of the firefly *Photinus pyralis*. The firefly *luciferase* gene placed under the control of the promoter of interest was used in this thesis. Through transient transfection, the plasmid containing the promoter of interest controlling the transcription of the *luciferase* gene is introduced into the desired cells. Luciferase protein is then expressed by the

transfected cells and the amount of luciferase produced is proportional to the strength of the promoter. The strengths of the various promoters can be determined by measuring the amount of luciferase produced. This indicates that a strong promoter produces more luciferase mRNA and therefore synthesizes more luciferase protein while weak promoter produces less luciferase mRNA and therefore expresses less luciferase protein.

Luciferase buffer used contains luciferin, a substrate of the produced luciferase protein (enzyme) which it oxidizes with the use of ATP to oxyluciferin with a concomitant emission of light, detectable in the blue range of the visible spectrum, 440-479nm [256]. The intensity of light emitted corresponds to the amount of oxidized luciferin and hence the promoter's strength [256]. A luminometer, which is a special instrument is used for a fast and effective measurement of the emitted light. This method is an excellent method because it is very sensitive, highly reproducible, fast, quantitative, and relatively cheap.

Although luciferase assay is a reproducible, quantitative, high-throughput, nonradioactive, nontoxic, relatively low cost, and easily adaptable to automation method. The primary disadvantages of luciferase are the requirement for exogenous substrates and the 4–6 h delay from stimulus to response to allow transcription to occur. The method measures product (=post-transcriptional) and not transcripts (= promoter activity). A major disadvantage of this method is that the promoter is not studied in its natural context. Moreover, transfection can introduce >>2copies of the plasmid and thus the promoter, which more than a normal diploid cell. Transcriptional activator or repressors present in low concentration may therefore become limited for the tested promoter. These limitations may result in a measured promoter activity that does not necessary reflects its bona fide activity.

3.3. Gene expression studies

3.3.1. RT² Profiler PCR array

RT² Profiler PCR Array is a reliable tool for analyzing the expression of a focused panel of genes. Each 96-well plate includes SYBR® Green-optimized primer assays for a thoroughly researched panel of relevant, pathway- or disease-focused genes. The high-quality primer design and RT² SYBR® Green qPCR Mastermix formulation enable the PCR array to amplify different gene-specific products simultaneously under uniform cycling conditions. This combination provides the RT² Profiler PCR Array with the specificity and the high amplification efficiencies required for accurate real-time SYBR® Green results. PCR arrays

are easy to use in any research laboratory. RT² Profiler PCR Arrays are sensitive enough for use with RNA prepared from regular samples (0.1–5 µg RNA), FFPE samples, and small samples (1–100 ng RNA). Each RT² Profiler PCR Array also includes control elements for Data normalization, Genomic DNA contamination detection, RNA sample quality and General PCR performance.

Data can be analyzed using an easy-to-use Excel-based data analysis template or Web-based software. Data analysis is based on the $\Delta\Delta$ CT method with normalization of the raw data to either housekeeping genes.

3.3.2. RT-qPCR

Quantitative PCR (qPCR) is used to detect, characterize and quantify nucleic acids for numerous applications. Commonly, in RT-qPCR, RNA transcripts are quantified by reverse transcribing them into cDNA first and then qPCR is subsequently carried out. As in standard PCR, DNA is amplified by 3 repeating steps: denaturation, annealing and elongation. However, in qPCR, fluorescent labeling enables the collection of data as PCR progresses. This technique has many benefits due to a range of methods and chemistries available.

In dye-based qPCR (typically SYBR green), fluorescent labeling allows the quantification of the amplified DNA molecules by employing the use of a dsDNA binding dye. During each cycle, the fluorescence is measured. The fluorescence signal increases proportionally to the amount of replicated DNA and hence the DNA is quantified in “real time”. The disadvantages to dye-based qPCR are that only one target can be examined at a time and that the dye will bind to any ds-DNA present in the sample.

In probe-based qPCR, many targets can be detected simultaneously in each sample but this requires optimization and design of a target specific probe(s), used in addition to primers. There are several types of probe designs available, but the most common type is a hydrolysis probe, which incorporates the use of a fluorophore and quencher. Fluorescence resonance energy transfer (FRET) prevents the emission of the fluorophore via the quencher while the probe is intact. However, during the PCR reaction, the probe is hydrolyzed during primer extension and amplification of the specific sequence it is bound to. The cleavage of the probe separates the fluorophore from the quencher and results in an amplification-dependent increase in fluorescence. Thus, the fluorescence signal from a probe-based qPCR reaction is proportional

to the amount of the probe target sequence present in the sample. Because probe-based qPCR is more specific than dye-based qPCR, it is often the technology used in qPCR diagnostic assays. Different from classical northern blot, which allows detects of full length transcripts (and possible splice variants), qPCR amplifies only a short fragment of the transcript.

3.4. Protein detection

3.4.1. Western blot

Western blotting (immunoblotting, protein blotting) is a sensitive immunological method for detecting electrophoretically separated proteins. The technique was developed independently by several groups in 1979 and was later termed ‘western blotting’ because of its analogy to Southern (to detect DNA; named after its inventor Edwin Southern) and northern blotting (to detect RNA). Its characteristic feature is the transfer of a pattern resolved by slab to a membrane, which serves as a carrier for all subsequent immunochemical reactions. Major fields of application in basic research are determination of molecular weights of protein antigens, detection of antigens in crude mixtures of proteins.

Western blot is a common method used to detect specific proteins in cell or tissue lysates. Briefly, protein lysates are separated according to their size by SDS PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) and transferred to a membrane. Unspecific binding sites are subsequently blocked and the membranes are incubated in the primary antibody solution, with the primary antibody being directed against the protein of interest. Following washing steps to remove unbound antibodies, the membranes are incubated in a secondary antibody solution containing antibodies directed against the species of the primary antibody. The secondary antibody is conjugated with an enzyme, for example horseradish peroxidase (HRP), or a fluorescent dye. If an enzyme-conjugated secondary antibody is used, the addition of, for example a chemoluminescent substrate, leads to substrate conversion and a chemoluminescent signal that can be detected. A marker containing proteins of a known size allows for size estimation of the obtained signal/protein band. This is a major advantage of western blotting because information can be gained concerning antibody specificity based on the number of visible bands and size estimations. Western blotting is a semi-quantitative method allowing comparisons of band strength, corresponding to protein amounts, between samples on the same gel/membrane. The amount of the protein of interest should be normalized to the amount of a housekeeping protein (ideally in the same size range) to ensure equal sample

input and even protein transfer and blocking Western blot analyses were used in this work to determine the presence of different proteins in Merkel cell lysates and to evaluate the specificity of antibodies. A disadvantage of western blotting (immunoblot) is that it is time-consuming (compared to ELISA) and has a high demand in terms of experience of the experimenter. Additionally, it requires optimizing the experimental conditions (i.e. protein isolation, buffers, type of separation, gel concentration, etc.), it is not as sensitive as qPCR and requires highly specific antibodies [257, 258].

3.4.2. Immunohistochemistry

Immunohistochemistry (IHC) is a method that determines the localization of proteins in tissues. The use of different fluorescent dyes or chromogens allows for multiplexing and colocalization studies. Either primary (direct detection) or secondary antibodies (indirect detection) can be conjugated to a fluorescent dye or chromogen. Indirect detection has the advantage of greater sensitivity since multiple secondary antibodies can bind to a primary antibody leading to an amplification of the signal. The use of isotype control antibodies (same species as the primary antibody) instead of primary antibodies is important to control for unspecific binding. A crucial step in IHC is the use of the optimal antigen retrieval approach (heat induced or enzymatic) to unmask epitopes, as cross-linking of proteins during fixation can mask antigens. ICC and IHC are commonly used to determine the subcellular location of a specific protein and to study protein expression patterns in different cell types and areas of tissues (IHC). A prerequisite for successful ICC and IHC is the availability of high quality antibodies to avoid false positive staining (lack of specificity) or false negative staining (lack of sensitivity). In this work, ICC and IHC were used to determine the presence/absence and localization of specific proteins in Merkel cell lines and tissues. An alternative method to detect the subcellular localization of a protein in a cell is by making an expression vector that encodes a fusion protein consisting of the protein of interest and a fluorescent protein (e.g. GFP, RFP, etc). Confocal microscopy of cells transfected with this plasmid can then be used to detect the subcellular localization of the fusion protein. However, the results should be interpreted with care as the GFP tag may alter the subcellular localization or interfere with expression of the protein of interest.

3.4.3. ELISA

Enzyme-linked immunosorbent assay (ELISA) is a method to quantify proteins in cell supernatants, plasma, serum, and other body fluids, and tissue lysates. In this work,

commercially available, validated sandwich ELISAs were used to determine the concentration of IL-33, ST2 and IL1RAcP in plasma of blood. In typical sandwich ELISAs, plates are coated with an antibody directed against the protein of interest. Unspecific binding sites are blocked followed by addition of the samples. An additional antibody specific to the protein of interest is added that is either directly enzyme-conjugated (e.g. HRP) or tagged (e.g. biotin). If a tagged antibody is used, an enzyme-conjugated antibody targeting the tag is added. Thorough washing between each step is essential to remove unbound sample and antibodies. Finally, the appropriate substrate, such as TMB (3,3',5,5'-tetramethylbenzidine), for HPR is added and after sufficient color development, the reaction is stopped. Absorbance measurements at the appropriate wavelength (e.g. 450nm for TMB) are performed to quantify the amounts of the reaction product. A dilution series of known protein concentrations is included in each run to draw a standard curve, allowing for the extrapolation of the concentration of the protein of interest in the samples. One major drawback of ELISA is high possibility of false positive or negative results because of insufficient blocking of the surface of microtiter plate immobilized with antigen [259].

3.5. Regulation of cell signaling pathways

3.5.1 Phosphospecific western blots

Phospho-specific western blots were used in paper I and II to determine the activation or inhibition of specific cell signaling pathways in response to specific stimuli or inhibitors. Many components of signaling pathways are often activated after phosphorylation. Hence examining the phosphorylated form of such a component reflects activation [260]. Normalization to the corresponding total protein and/or a house keeping protein are important to ensure equal loading and consequently that the differences observed are in fact due to stimulation or inhibition and not a result of unequal protein input. During our work in paper II, we experienced difficulties to completely remove phospho-antibodies prior to re-probing with the total-protein antibodies. This can lead to signals from the remaining phospho-antibodies or can impair the binding of the total protein antibodies. Incomplete antibody removal is a well-recognized problem for relative phospho-protein quantification [261]. We, therefore, chose an approach to circumvent this problem, which was running separate gels for phospho and total protein and to normalize each to its loading control before calculating phospho/total protein ratios.

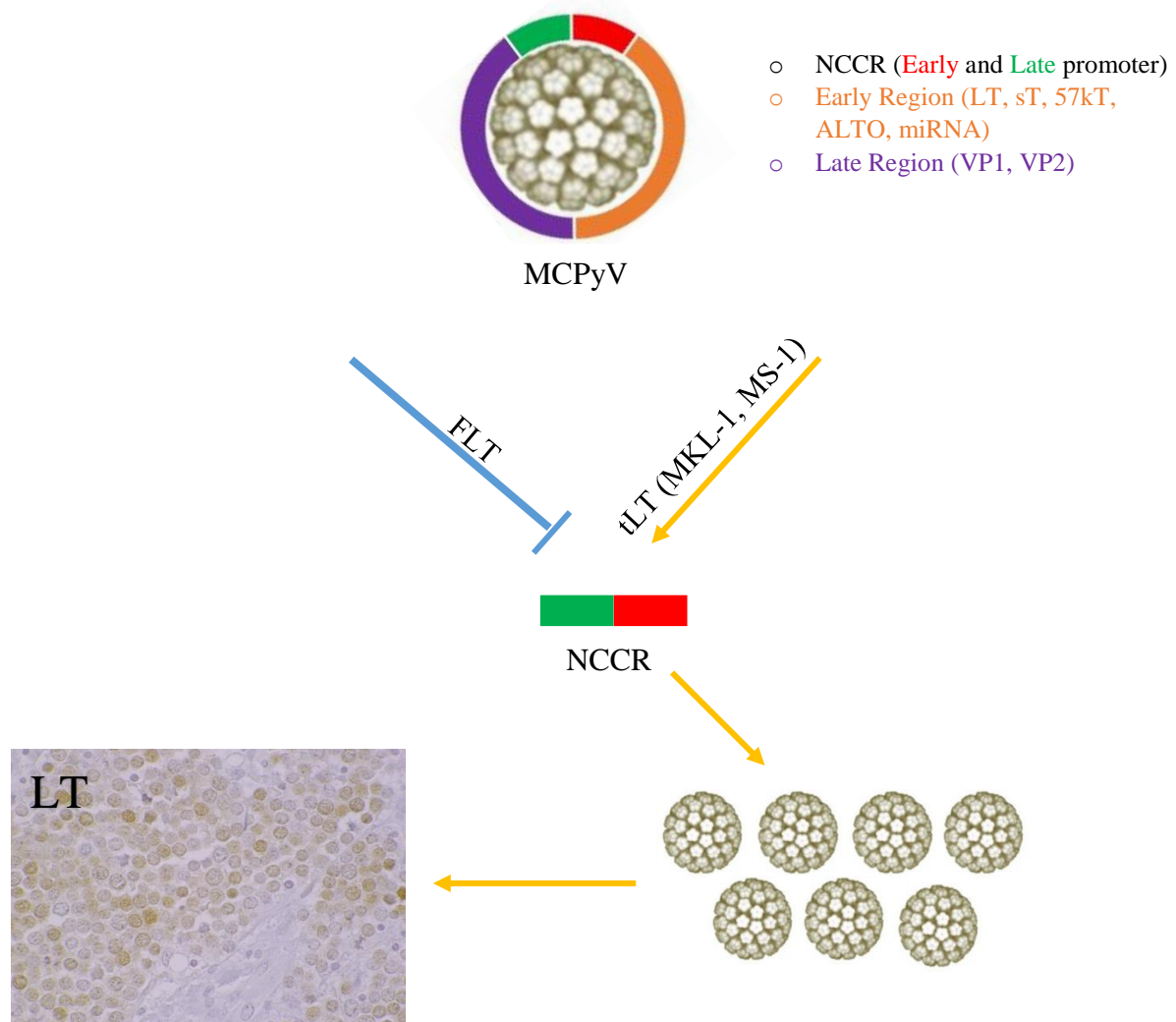
3.6. MTT cell viability assay

The MTT assay was used to determine cell viability in response to stimuli, inhibitors or drugs. The cell viability was determined in comparison to control cells that were set as 100% viable cells. There are a variety of colorimetric or fluorescence based cell viability assays. We chose the MTT assay because it is inexpensive, widely used, and a well-established method in our lab. MTT is tetrazolium dye that is reduced to a purple insoluble formazan by nicotinamide adenine dinucleotide phosphate (NADPH)-dependent cellular oxidoreductase enzymes (mainly mitochondrial dehydrogenases present in living cells) [262]. Therefore, the production of formazan is a measure of metabolic activity/cell viability. The MTT assay can also be used as a proliferation assay as an increase in cell numbers correlates with an overall increase in cellular activity. However, it is important to note that more specific assays are available to determine proliferation directly, such as the BrdU assay or the 3H-thymidine incorporation assay, whose measures of DNA synthesis are more specific to determining proliferation.

4. SUMMARY OF MAIN RESULTS

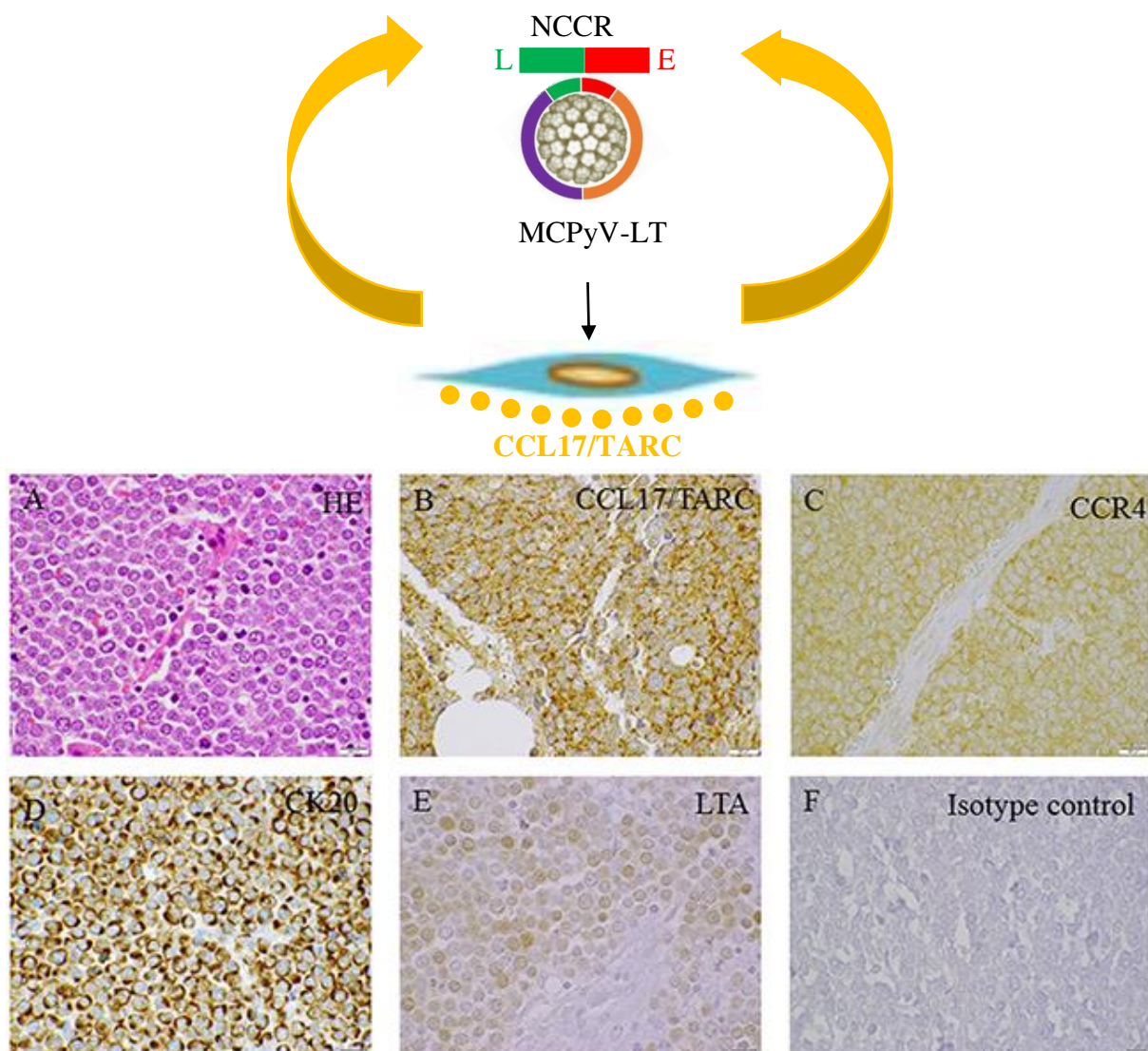
4.1. PAPER I: Promoter activity of Merkel cell Polyomavirus variants in human dermal fibroblasts and a Merkel cell carcinoma cell line

The promoter activities of all MCPyV variants tested was stronger in human dermal fibroblasts, a cell line that supports viral replication better than MCC13 cells, which are not permissive for MCPyV. tLT, but not FLT stimulated viral promoter activity. Whether, the difference in promoter strength and regulation by large T-antigen may affect the replication and tumorigenic properties of the virus remains to be determined.



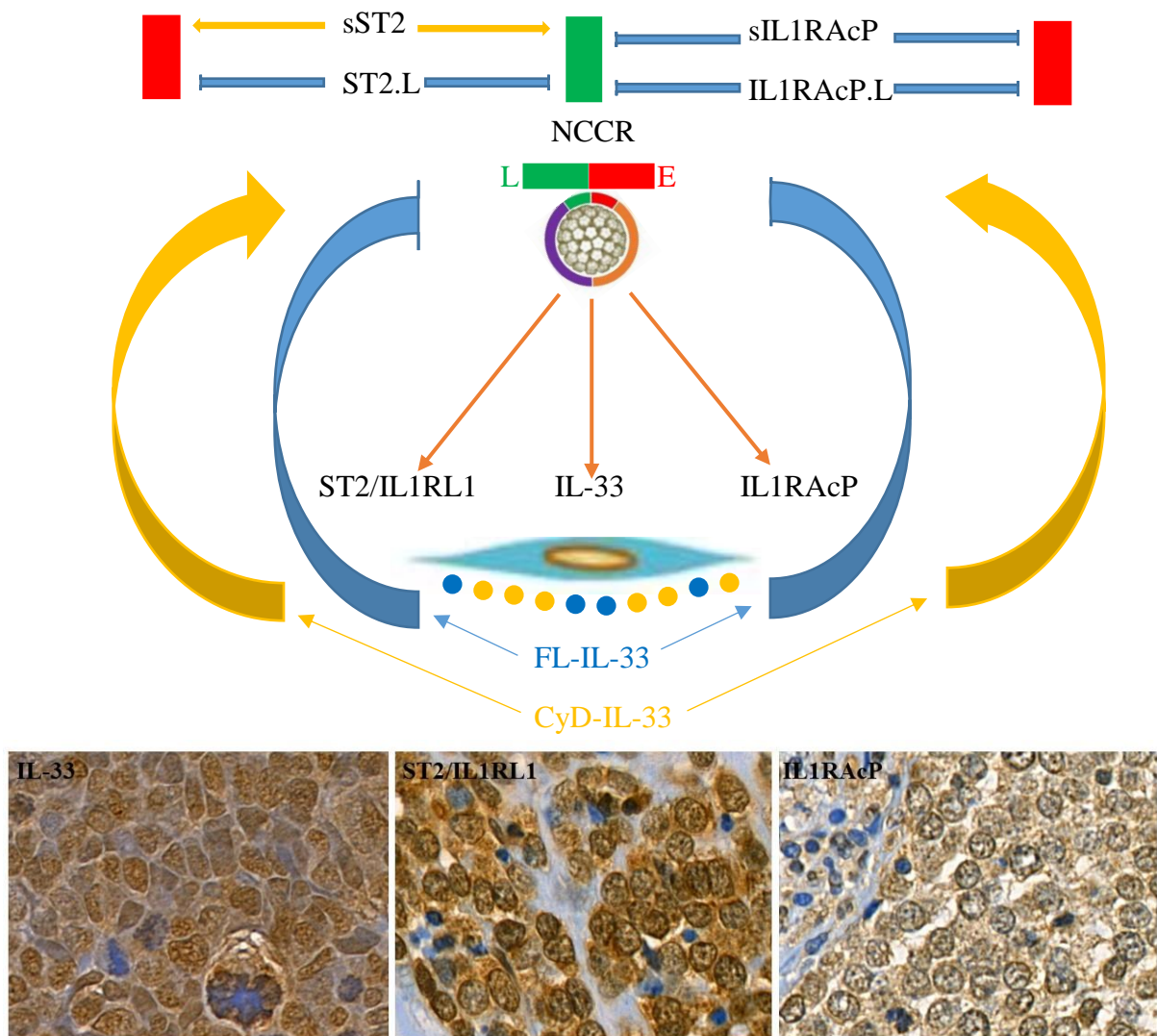
4.2. PAPER II: CCL17/TARC and CCR4 expression in Merkel cell carcinoma

We demonstrate that MCPyV LT is linked with an altered expression of CCL17/TARC. The ectopic expression of CCL17/TARC upregulated MCPyV early and late promoter activities in MCC13 cells. The exogenous stimulation of MCC-13 cells with recombinant CCL17/TARC activated both the mitogen-activated protein kinase and the NF- κ B pathways. Finally, immunohistochemical staining on human MCC tissues showed a strong staining of CCL17/TARC and its receptor CCR4 in MCC tissue samples. The expression of CCL17/TARC and CCR4 may constitute an autocrine or paracrine survival loop which contributes to the growth and survival of the tumor, and also mediates immune suppression through the recruitment of regulatory T cells. Thus, strategies based on the selective targeting of the CCL17/CCR4 axis, either by monoclonal antibodies or specific receptor antagonists, could be a therapeutic interventions for patients with MCC.



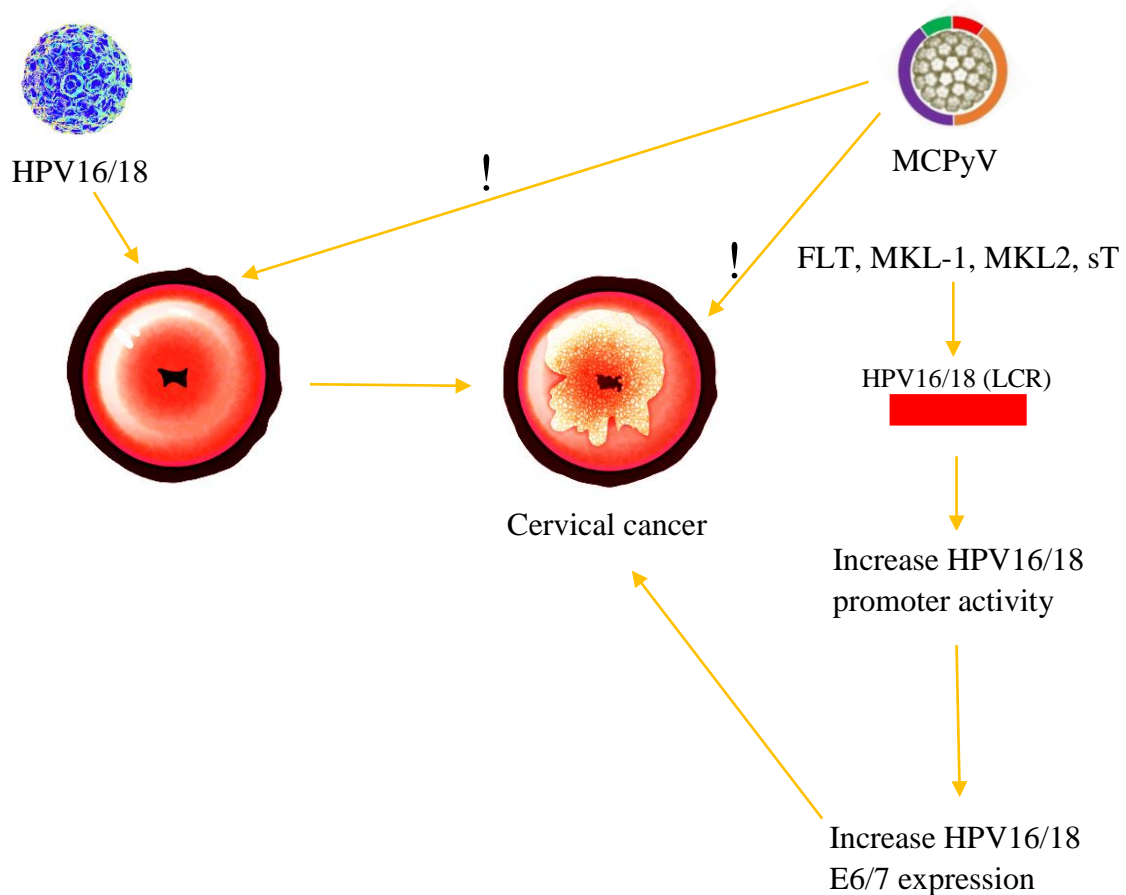
4.3. PAPER III: The Merkel cell polyomavirus T-antigens and IL33/ST2-IL1RAcP axis: Role in Merkel cell carcinoma.

We demonstrated that MCPyV T-ag is linked with an altered expression of IL-33 and its receptors, ST2/IL1RL1 and IL1RAcP. The FL-IL-33 showed inhibitory while CyD-IL-33 showed stimulatory effect on both early and late promoter activity. The soluble form of ST2/IL1RL1 also activate both early and late promoter activity while soluble form of IL1RAcP and membrane bound forms of both ST2/IL1RL1 and IL1RAcP have inhibitory effect. Finally, immunohistochemical staining on human MCC tissues showed a strong staining of IL-33 and its receptors, ST2/IL1RL1 and IL1RAcP in MCC tissue samples. However, either patient- or disease-specific therapy based on the selective targeting of the IL-33/ST2-IL1RAcP axis, by monoclonal antibodies or specific receptor antagonists could be therapeutic interventions for patients with MCC.



4.4. PAPER IV: Merkel cell polyomavirus large T antigen and small t antigen increase the expression of high-risk human papillomaviruses 16 and 18 E6 and E7 in cervical cancer cells

The tumorigenic human polyomavirus MCPyV can occasionally be found in cervical cancers. We found that its oncoproteins LT and sT can stimulate the activity of the HR-HPV16 and 18 promoters, and therefore increase the transcript and protein levels of the oncoproteins E6 and E7 in cervical cancer cell lines. This suggests that MCPyV may collaborate to transform cervical tissue. Further studies are required to prove a possible role of MCPyV in HR-HPV-induced cervical carcinoma.



5. GENERAL DISCUSSION

5.1. Transcriptional activity of NCCR of different MCPyV variants

Human PyV DNA genomes contain three functional regions denoted the early region, encoding the regulatory T-antigens and one microRNA (only BKPyV, JCPyV and MCPyV), the late region, encoding the structural capsid proteins (VP), and the noncoding control region (NCCR). The NCCR harbors the origin of viral genome replication and bidirectional promoter/enhancer functions governing early and late region expression on opposite DNA strands. Despite principal similarities, HPyV NCCRs differ in length, sequence, and architecture [263]. Mutations in the non-coding control region (NCCR) of human polyomaviruses like BKPyV, JCPyV, KIPyV, HPyV7, HPyV9 and HPyV12 have an impact on the transcriptional activity of the promoter, and may affect the virulence of the virus [263-272]. A previous study by Moens et al. compared promoter (both early and late) activity of at that time 13 known HPyVs in 10 different human cells lines derived from brain, colon, kidney, liver, lung, the oral cavity and skin. They found that the BKPyV, MCPyV, TSPyV and HPyV12 early and late promoters displayed the strongest activity in most cell lines tested as compared to other HPyVs. These findings suggest which cell lines may be suitable for virus propagation and may give an indication of the cell tropism of the HPyVs [264].

A vital question in MCPyV research has been how MCPyV-encoded proteins contribute to viral activities and MCC development. Most of our knowledge of MCPyV oncogenic mechanisms was gained from studies of the viral oncogenes, LT and sT antigens, both of which have been implicated in MCPyV-induced tumorigenesis (See [66, 104, 273] for more detailed discussion on this subject). Sequence analysis of the MCPyV NCCR of different virus isolates revealed genetic variability, but the biological implications in the viral life cycle and the development of MCC have not been studied.

In paper I, we studied whether changes in NCCR of MCPyV had effect on promoter activity, as this may have pathogenic consequences during MCC because the promoter strength determines the expression levels of the oncoproteins LT and sT. We used promoters from MCPyVs isolated from MCC patients and non-MCC samples. We classified MCPyVs NCCR into seven different groups based on changes in NCCRs either point mutations, larger deletion or insertions. The basal activity of both early (almost 4-folds) and late (almost 3-folds) promoter from each seven groups was higher in human dermal fibroblast (HDF) as compared to MCC-

13 cells. This may indicate that the MCPyV promoter is more adaptive to HDF and suggests this cell type as a natural host cell for the virus, while the infection of Merkel cells and subsequent transformation of these cells could be seen as an accidental and unfortunate event.

In paper I, we also checked the effect of LT on early and late promoter variants from MCPyVs. We found that LT has an inhibitory effect on the activity of the early and late promoter of all variants we tested in both MCC-13 and HDF cell lines. A dose-dependent inhibitory effect was observed for all variants in MCC-13 and HDF except the late promoter from both MS-1 and HUN MCPyV variants in MCC-13 cell line. Both these variants were isolated from VP-MCCs [274, 275]. This effect was also observed by Kwun *et al.* by using MCPyV isolate MCC339 in HEK293 cells, but they did not study the effect of LT in MCC-13 or HDF [108]. There is also one study by Ajuh *et al.* that showed results contrary to both our and Kwun's work. This study reported that LT trans-activated MCPyV R17b (consensus) early and late promoter activity in HEK293T cells [263]. This discrepancy could be because they used HEK293T cells constitutively expressing SV40 LT and sT but not MCPyV LT. Moreover, no dose-dependent studies were performed. Additionally, possible co-stimulatory effect of MCPyV LT and sT was not studied in our work. Ajuh *et al.* also used bidirectional reporter plasmid, thus allowing simultaneous monitoring of both early and late promoter activities. While, in our study we used early and late promoters independently that better reflects the situation during infection, because during viral infection, both early and late promoters are activated in time-dependent manner with the early promoter active in the initial phase of infection, while the late promoter is active after viral DNA genome replication has initiated [70].

Because VP-MCC express tLT, we also tested the effect of tLTs in initiating MCC and further development. We found that tLT, MKL-1 and MS-1 stimulated both their cognate early and late promoter activity in MCC-13 cells. We also found that both tLTs also upregulate late promoter activity in HDF but showed inhibitory effect on early promoter activity. This cell specific effect of tLT is unknown but MCPyV LT interacts with different cellular factors, so this interaction pattern may determine activity in different cells. The observation that tLT stimulates the early promoter suggest a positive feedback loop in which LT promotes expression of LT and sT and this may accelerate MCC tumorigenesis.

A previous study by Velásquez *et al.* showed that even though both CVG-1 and MKL-1 contains seven copies of integrated genome per diploid cell. Although these promoters differ by only one nucleotide change, early mRNA expression is almost 2.5X higher in CVG-1 as

compared to MKL-1 [276]. An explanation is that the chromatin structure or/and DNA methylation pattern may affect the activity of the integrated viral promoter.

Taken together, our studies showed that MCPyV NCCR variants possess different promoter activity, and the promoter is much stronger in HDF as compared to MCC-13. tLT also stimulate viral promoter activity. The question whether difference in promoter strength and regulation by LT may affect the viral replication and tumorigenic properties of virus is still undetermined.

5.2. Merkel cell polyomavirus T antigens alter inflammatory cytokine gene expression

Cancer and inflammation are connected by the intrinsic and extrinsic pathway. The intrinsic pathway is activated by genetic events for example, the activation of various types of oncogene by mutation, chromosomal rearrangement or amplification, and the inactivation of tumor-suppressor genes. The transformed cells produce inflammatory mediators, thereby generating an inflammatory microenvironment in tumors. On the other side, in the extrinsic pathway is driven by inflammatory conditions that increase cancer risk, such as inflammatory bowel disease increase risk for the development of colorectal cancer [277].

Inflammation has a dual role in activating either pro- or anti-tumor effect. This dual role solely depends on plasticity of mostly tumor and stromal cells, their phenotype in TME and their secretory factors such as cytokines, chemokines, growth factors and proteolytic enzymes [278]. These cells produce inflammatory mediators that activate transcriptional factors and downstream signaling pathways. This immune-mediated activation leads to tumor invasion, migration and vascularization [279].

The anti-tumor effect of immune cells requires not only the generation of tumor-specific T cells but additionally, also the ability of these T cells to penetrate within the tumor as well [280]. The T cells are primed with the expression of tissue-specific adhesion molecules (addressins) and specially migrate to the peripheral tissue in which they first encountered antigen [281, 282].

Similarly, skin-specific T cells should express the skin-homing addressin CLA. MCC are cutaneous tumors and skin-draining lymph nodes are the first place where T cells first encounter antigen. Previous studies have shown decreased numbers of skin-homing CLA⁺ T cells in a subset of MCCs. In a number of human cancers, including malignant melanoma, cutaneous squamous cell carcinoma, breast, gastric, cervical, and lung cancers impaired T-cell homing was demonstrated because of decrease in vascular addressin expression [283-286]. However, a

study with MCC tumors that showed CLA⁺ skin-homing T cells infiltration did not have a markedly improved survival, suggests that other immunosuppressive mechanisms are also at work [218].

MCC is a neuroendocrine skin cancer with old age, immunosuppression, polyomavirus infection, and exposure to UV radiation as identified risk factors [45, 287, 288]. Although 80% of MCCs have genomic integration of MCPyV and produce viral proteins including sT and LT antigens which are targets for the immune system, these tumors are still highly malignant in immunocompetent individuals [45, 47, 78, 289, 290]. Approximately 92% of MCC patients are immunocompetent individuals, and mortality is 30%, making MCC a more fatal cancer than malignant melanoma [5]. The highly malignant nature of this virally mediated cancer suggests that MCC has potent strategies for evading immune response and that neutralization of these strategies may enhance anti-tumor immunity.

The TME has lately emerged as a major contributor to tumorigenesis, by providing either proliferative or inhibitory signals to the malignant cells [291]. This is especially true for cancers whose progression is substantially affected by cytokines and inflammatory mediators released in the TME [277, 292].

As with other virally associated cancers, T cell immunity plays a critical role in the susceptibility and immune responses to MCC. Incidence is markedly increased in immunosuppressed individuals and withdrawal of iatrogenic immunosuppression or biopsy itself has induced regression of MCC [5, 198, 199, 293, 294]. T cells specific for MCPyV oncoproteins are present in the blood and tumors of patients with MCC and these patients have levels of circulating antibodies specific for MCPyV oncoproteins that fluctuate with disease activity [295, 296]. However, these immune responses are insufficient in most cases to control growth of the cancer, suggesting that MCC tumors have potent immune evasion strategies [218].

In view of the limited number of treatment options for MCC patients, there is an urgent need for the discovery of tumor-specific pathways and possible therapeutic targets [31, 297]. It has been shown that virus-driven human cancers have developed immune escape strategies, and therapeutic intervention of immune checkpoint pathways has gained considerable interest in MCC [211].

Paper II and III are results from an initial study where we examined a panel of 84 inflammatory cytokines and receptors for differential expression between VP-MCC and VC-MCC cell lines.

Previous studies have shown expression of different inflammatory mediators including, CCL20, CCL24, CXCL1, CXCL5, CXCR1, CXCR2, IL1A, IL1R2, IL1RL1, IL1RL2, IL20RA, IL24, IL26G, IL36RN [298] in both VP- and VN-MCC.

As LT (FLT and tLT) and sT are the two main oncoproteins expressed in VP infection and MCC development, we also investigated how differential regulation of inflammatory cytokines and receptors is regulated by MCPyV oncoproteins in paper II and III. Previous studies have reported that MCPyV sT downregulates IL2, IL-8, CCL20 and CXCL9 expression in MCC-13 cells [75], while MCPyV FLT, tLT (LT339) and sT upregulate IL-1 β , IL-6, IL-8, CXCL1 and CXCL6 in hTERT-immortalized BJ human foreskin fibroblasts [299].

In paper II and III, we demonstrate that among 84 different inflammatory cytokines and receptors, Chemokine (C-C motif) ligand 17/thymus and activation-regulated (CCL17/TARC) and Interleukine-33 (IL-33) expression were upregulated ≥ 2 -fold in VP-MCC cell lines as compared to VN-MCC cell lines.

CCL17/TARC is constitutively expressed in thymus and by other cells including dendritic cells, endothelial cells, keratinocytes and fibroblasts [300]. In paper II, we investigated the effect of MCPyV LT and sT on CCL17/TARC expression. We found that MCPyV FLT and tLTs, but not sT stimulated CCL17/TARC promoter activity in transient transfection studies with a luciferase reporter plasmid. CCL17/TARC promoter sequence analysis showed that there are no predicative binding sites of LT (GRGGC), indication an indirect effect of LT. This is underscored by the finding that tLT lacking its DNA binding domain, still enhanced CCL17/TARC promoter activity. MCPyV LT can interact with different transcription factors including, Brd4, the E2F family members 2 and 3, MED14/CRSP2, PRTF1, SALL2 and DP1 [71, 301]. The FLT and tLT (MKL-1, MKL-2 and MS-1) contain the pRb binding motif LxCxE and usurping pRb by LT leads to release of pRb-mediated inhibition of E2F. The CCL17/TARC promoter contains the putative GGCGGCA E2F binding site at position -1085/-1079 [302]. In paper II, we used three different CCL17/TARC promoter variants, -2535/+40, -1084/+40 and -375/+40. Thus two of our large promoter fragments, -2535/+40 and -1084/+40 contain, whereas the shortest fragment -375/+40 lack E2F putative binding site. In paper II, all of CCL17/TARC promoter fragments showed LT-mediated promoter activation. So, LT-mediated CCL17/TARC17 promoter activation seems to be E2F-independent. The exact mechanism by which LT transactivates the CCL17/TARC promoter remains to be determined. We found, however, that sT had no effect on the CCL17/TARC promoter in MCC13 cells.

We next investigated the effect of CCL17/TARC on both early and late promoter activity. Our results showed that this autocrine release of CCL17/TARC results in an increase of both early and late promoter activity of MCPyV. These results indicate that CCL17/TARC not only helps in MCPyV replication but also promotes MCC development. So, this MCPyV LT-mediated increased expression of CCL17/TARC helps in positive feedback mechanism. Previous studies in different cell types also reported increased expression of CCL17/TARC by the latent membrane protein-1 and the nuclear antigen-leader protein of Epstein-Barr virus in B-cells [303, 304], after infection of lung cells by respiratory syncytial virus [305] or human bocavirus [306], and hepatitis C virus infection of Huh7 cells [307]. Hence, induced expression of CCL17/TARC may be a general strategy used by viruses, probably as a mechanism to evade the immune system by recruiting Treg cells at the site of inflammation.

We further checked expression of both CCL17/TARC and its receptor CCR4 in MCC tumor tissues by immunohistochemistry. A total of 23 tissues were stained with CK-20 (a MCC marker), LT, CCL17/TARC and CCR4. We did not observe any difference in expression of both CCL17/TARC and CCR4 expression between VP- and -VN MCC tumor tissues. As immunohistochemistry is more qualitative analysis tool, difference in expression between both groups may not be visible.

The CCR4-CCL17/TARC axis has been shown to activate the MAP kinase and NF- κ B signaling pathways [298]. Additionally, CCR4-mediated MMP13 activity in colorectal cancer cells requires NF- κ B [308]. Chemokine-like factor (CKLF1), which also uses the CCR4 receptor, can activate the NF- κ B pathway [309], and NF- κ B signaling is significantly downregulated in CCR4^{-/-} macrophages [310]. We therefore monitored the effect of CCL17/TARC on these pathways in MCC-13 cells. Stimulation of MCC-13 cells with exogenous rhCCL17/TARC showed increase in phosphorylation of both MAP kinases ERK1/2 and NF- κ B p65 and p105 proteins, indicating activation of these signaling pathways. This CCL17/TARC-mediated induction of these pathways was confirmed by blocking the CCR4 receptor with the CCR4 receptor antagonist C021. Activation of the NF- κ B pathway by CCL17/TARC was further confirmed by using a luciferase reporter plasmid containing a NF- κ B responsive promoter. Recombinant CCL17 stimulated the NF- κ B responsive promoter in a CCR4-dependent manner.

CCL17/TARC acts as a chemoattractant, which helps in the recruitment of CD4⁺ Treg cells, Th2 and Th17 cells [311, 312]. CCL17/TARC exerts its effect by CCR4 receptor [313, 314]. Treg cells are attracted by TMEs created by cancer cells. The natural function of Treg cells is

suppression of excessive activity of the immune system that is supposedly linked to an increased risk of cancer. It is clearly shown that Treg cells are associated with acceleration of existing tumors [315]. The Treg cells suppress the activity of the main subsets of immune cells involved in immune surveillance against tumors and this regulation is very much localized and precise. A local mode of the suppression exerted by Treg cells is of special importance in immune regulation. The studies on graft rejection in animals showed that these cells mainly accumulate and activate in the graft and in the local lymphoid tissues [316]. In addition, Treg cells regulate cytotoxic subsets, such as CD8⁺ T cells and NK cells, mainly through direct cell-to-cell contacts. Both CD8⁺ T cells and NK cells show reduced cytotoxicity and lower production of IFN- γ and perforin when co-cultured with Treg cells [317]. Interestingly, some studies also observed protective effects of Treg cells against some types of malignancy. Increased levels of Treg cells infiltrates in affected lymphoid tissues can be a positive predictor of patients survival in follicular lymphoma and aggressive forms of diffuse large B-cell lymphoma (DLBCL) have shown reduction in Treg cells [318]. There has been also reported that large number of Treg cells are associated with lower metastasis in colorectal cancer (CRC) [319]. This effect probably depended on the inhibition of Th17 response by Treg cells. *Ex vivo* studies showed that Treg cells have the ability to inhibit the secretion of pro-inflammatory cytokines (IL-17 and IL-22) by Th17 cells in this environment [320]. To be fair, this may be a unique feature of this particular kind of tumor, in which the inflammation promotes metastases. CD4⁺ helper T (Th) cells mainly participate in tumor immunology and are functionally divided into different subsets, Th1, Th2, and Th17 cells [321]. Th1 cells secrete IL-1 β , IL-2, IL-12, TNF- α , and IFN- γ cytokines and are associated with good prognosis, whereas Th2 cells produce IL-4, IL-5, and IL-10 cytokines and are related to tumor growth or metastasis [277, 322]. Although there is uncertainty about the role of Th17 cells secreting IL-17, IL-21, and IL-22, there is evidence of Th17 cells contributing to HCC progression [323, 324].

Chemokines play pleiotropic roles in promoting tumor invasion, migration, and vascularization [279, 325]. Because CCR4 is expressed by Tregs and recently we demonstrated high CCL17 expression by MCC, this chemokine is thus thought to play a role in immune evasion by attracting Tregs to the TME [326]. So targeting either CCL17 or CCR4 with monoclonal antibodies or antagonist could be a novel strategy for MCC, especially for the treatment of VP tumors because the virus enhances CCL17/TARC expression. CCR4 is under consideration as a target molecule for the therapy of some hematologic malignancies that express CCR4. In particular, a humanized anti-CCR4 mAb, mogamulizumab, is undergoing clinical testing

(NCT00355472) in Adult T-cell leukemia/lymphoma (ATLL) patients, and these trials have been showing favorable results [327]. In our study, we also found CCR4 expression by MCC cells. Thus, it is possible that an anti-CCR4 mAb therapy could help by not only kill the CCR4-expressing MCC cells directly, but also inhibit the suppressive function of CCR4-expressing Tregs in the TME. Additionally, combinational therapy along with targeting ICIs could be an option for treating MCC. There are different clinical trials undergoing by targeting CCR4 receptor along with other inhibitors in different types of malignancies. We believe that molecular-targeted therapy and the blockade of CCL17/CCR4 pathways using anti-CCR4 mAb may be a potential approach for adjuvant MCC treatment.

Interleukin-33 (IL-33) is member of IL-1 family cytokines and considered as an “alarmin” released after cellular damage [141]. IL-33 is expressed in multiple organs and cell types in humans and mice and is the ligand for the suppression of tumorigenicity 2 L receptor (ST2) and IL-1 receptor accessory protein (IL1RAcP) [184]. It was initially believed that IL-33 gets mature by caspase-1 [170] but later on, different studies have shown that FL-IL-33 is biologically active and processing by caspases-1 resulted in IL-33 inactivation [175, 328-330]. Upon cell stress and damage, IL-33 is released and cleaved by different inflammatory proteases from neutrophils (proteinase 3, elastase, and cathepsin G) [331] and mast cells (chymase, tryptase, and granzyme B) [332] into shorter mature forms (18–21 kDa) with 10- to 30-fold higher activity [333].

IL-33 was first described as a potent initiator of type 2 immune responses through the activation of many cell types, including the Th2 cells, type 2 innate lymphoid cells (ILC2s), mast cells, basophils, eosinophils, and myeloid cells such as myeloid-derived antigen-presenting cells including macrophages and dendritic cells (DCs) [170, 178, 334-340]. Moreover, IL-33 was shown to play either important pro- or anti-tumorigenic functions in several cancers [341]. Currently, IL-33 is a possible inducer and prognostic marker of cancer development with a direct effect on tumor cells promoting tumorigenesis, proliferation, survival, and metastasis [341]. IL-33 helps in remodeling of TME thus promotes tumor growth, metastasis and inducing angiogenesis [342]. Induction of M2 macrophage polarization, tumor infiltration and activation of immunosuppressive cells such as myeloid-derived suppressor cells (MDSC) or Treg cells by IL-33 favors tumor progression [343, 344].

Alongside its pro-tumorigenic role, IL-33 can also behave as a tumor suppressor by *in vitro* inhibition of proliferation and induction of apoptosis of MIA PaCa-2, a pancreatic cancer cell line [345]. IL-33 has a significant role in cancer immune-surveillance in primary prostate and

lung tumors, which can be lost during the metastatic transition inducing immune escape. The down-regulation of IL-33 during the metastatic process ultimately decreases the functionality of HLA-I and reduces immune-surveillance favoring tumor development [346]. It has been shown that IFN- γ -producing cells present in tumors associated with an IL-33 antitumor effect, were CD8⁺ T cells and NK cells. Indeed, IL-33 expression in several cancers affects the number of CD8⁺ T cells and NK cells in tumor tissues and the production of IFN- γ /TNF- α , thereby favoring tumor eradication through tumor cell cytolysis [347, 348]. Finally, the ILC2s which support type 2 immune responses by producing IL-5 and IL-13 in response to IL-33 could also have an antitumor function. Indeed, their tissue-repair function can induce cholangiocarcinoma and liver metastasis [349]. ILC2s can also be mobilized from the lung and other tissues thanks to IL-33, to penetrate tumors, mediate immune-surveillance with DC, and promote adaptive cytolytic T cell responses and attraction [350, 351]. The anti-tumorigenic function of IL-33 is carried out by recruiting and activating CD8⁺T lymphocytes, natural killer (NK) cells and by promoting second type immune response by the type 2 innate lymphoid cells (ILC2) [348, 351-353].

Paper III described upregulated expression of IL-33 in VP-MCC cell lines compared to VN-MCC cell lines. We demonstrated that MCPyV both sT and LT (FLT and tLT) significantly upregulate IL-33 promoter activity. IL-33 promoter sequence analysis predicts two putative binding sites for LT (-821/-817 and -199/-195). We could not find any altered effect of MCPyV T-ag by mutating these binding sites. Additionally MKL-2 LT lack nuclear localization signals among FLT and tLT variants, MKL-1 and MS-1. We still see IL-33 promoter activation by MKL-2 LT. This suggest that MCPyV T-ag may have indirect effect by interacting with other transcription factors or bind with some other protein that not only helps in nuclear localization but also activation of the IL-33 promoter. This induction of promoter activity was also confirmed by checking IL-33 protein expression after transiently transfecting MCC-13 cells with MCPyV T-ag. An increased expression of IL-33 was observed in different malignancies including, breast cancer [347, 354], colorectal cancer [355-357], gastric cancer [358], hepatocellular carcinoma [359, 360], pancreatic cancer [361, 362], lung cancer [363, 364], prostate and kidney cancer [346], skin cancer different hematological malignancies [365], as well as in inflammatory diseases [366].

We also found that MCPyV upregulates promoter activity of IL-33 heterodimeric receptors, ST2/IL1RL1 and IL1RAcP. Previously it has been reported that the human and mouse *ST2/IL1RL1* gene have two alternative promoter regions, the distal and proximal promoters,

followed by noncoding first exons, E1a and E1b [186]. Transcription from the distal promoter generates the mRNA for the membrane bound ST2L/IL1RL1L receptor protein, while the proximal promoter is used to produce the transcript for the soluble form of sST2/sIL1RL1 receptor [185].

Further, we checked the ectopic effect of IL-33 on both MCPyV early and late promoters activity. We found that FL-IL-33 downregulates while CyD-IL-33 upregulates both MCPyV early and late promoter activity in a dose-dependent manner. IL-33 has three domain: nuclear, central and cytokine domain. IL-33 expressing the nuclear domain localizes in nucleus while IL-33 expressing only the cytokine domain resides in the cytoplasm. We have found this inhibitory effect by only FL-IL-33. This suggests that the nuclear domain of FL-IL-33 has either direct or indirect inhibitory effect on early and late promoter activity. The mechanism by which FL-IL-33 represses the MCPyV promoter remains elusive.

As both of receptors for IL-33 are also found in soluble forms, we investigated effect of both the membrane-bound and the soluble forms of both ST2/IL1RL1 and IL1RAcP receptors on MCPyV both early and late promoter activity. We demonstrated that sST2 increase both early and late promoter activity, indicating that during disease, sST2 acts as positive feedback mechanism by upregulating MCPyV T-ag expression and contributes in MCPyV induced development of MCC. Additionally, we also found that IL1RAcP has inhibitory effect on MCPyV both early and late promoter activity.

We also studied the effect of IL-33, ST2/IL1RL1 and IL1RAcP proteins on IL-33/ST2-IL1RAcP complex promoter activity. We found that sIL1RAcP upregulated sST2/IL1RL1 promoter activity. We also observed increased IL1RAcP promoter activity by both FL-IL-33 and CyD-IL-33, and increased IL1RAcP promoter activation by sST2/IL1RL1. Furthermore, immunohistochemistry confirmed that MCC tumors expressed IL-33 and its receptors ST2/IL1RL1 and IL1RAcP.

Given the fact that MCC produces IL-33 and expresses ST2/IL1RL1 and IL1RAcP, in paper III, we found that exogenously added recombinant hIL-33 (CyD-rhIL-33) resulted in ERK1/2, p38 and JNK phosphorylation in MCC-13 cells. Previous studies have shown that IL-33 induced phosphorylation of p38 MAPK in breast cancer positive cells [367], ERK1/2 and JNK in gastric cancer cells [368, 369] and JNK in renal cell carcinoma cells [370]. We also demonstrated that IL-33 triggered NF- κ B pathway activation in MCC-13 cells. This is in agreement with a previous study that reported IL-33/ST2 dependent NF- κ B activation in

different cancers including, glioblastoma [371] and colorectal cancer [371]. Additionally, the nuclear domain of IL-33 also interacts with the p65 subunit of NF- κ B and represses expression of NF- κ B regulated genes necessary for pro-inflammatory signaling [177, 372].

Given their central roles in the regulation of immune responses, cytokines are appealing targets for therapeutic intervention. In the past 2 decades, scientists have made remarkable progress in the development of IL-33/ST2 blocking tools. IL-33 and ST2 have been drug targets in preclinical studies and pharmaceutical pipelines. There are 3 major therapeutic strategies for directly blocking the binding of IL-33 to ST2: (1) IL-33 neutralizing antibodies; (2) soluble decoy receptors; and (3) anti-ST2 receptor antibodies. IL-33/ST2 signaling has diverse cellular targets and functions and its functions both in physiological and pathological conditions are not fully understood and needs additional research exploration. Using genetically modified IL-33 mice will allow us to better understand its role and mechanisms involving IL-33/ST2 signaling. This will facilitate us to develop novel therapeutic strategies targeting IL-33/ST2 pathway. Monoclonal antibodies against IL-33 or ST2 are under development by pharmaceutical companies and in phase I–II clinical trials for the treatment of allergic diseases (NCT03546907, NCT03615040). The combination therapy targeting IL-33 with other therapeutics could be an option for the efficacious treatment. However, the key to best therapy is to determine the various components of combination. It is currently too early to answer this question because the scientific evidence is insufficient. Further studies comparing the efficacies of different therapeutic combinations will help answer these questions.

5.3. Role of Merkel cell polyomavirus in non-Merkel cell carcinomas

MCPyV is the major causative factor of MCC with characteristics of host genome integration and expression of sT and tLT proteins, viral oncoproteins. This strong association urged scientists to explore the role of MCPyV in other cancers. Previous studies have shown presence of MCPyV in non-MCC cancers (reviewed [112]). On average, the MCPyV viral genome copy number was 60 times lower in healthy tissues as compared to MCC samples across the body [111]. However, these studies have not investigated a possible role in those non-MCC tumors.

The route of MCPyV infection is still unknown, though sexual transmission has been suggested as one of possible route of transmission. Previous studies have detected MCPyV DNA among 31% (37/120) of HIV positive men and 72% (86/120) of HR-HPV positive angiogenital mucosal samples [373]. Also another study of 120 Iranian cervical carcinoma positive women

showed presence of HR-HPV in 40% (45/120) of cervical samples and 35% (14/45) were also positive for MCPyV infection [115]. In another study of cervical samples with HR-HPV detected, 124/140 (88.6%) HIV positive African women and 24/50 (48%) French women showed coexistence of MCPyV in 81/148 (55%) samples [374]. These studies have shown coexistence of MCPyV and HR-HPV in genital tissues from both benign and malignant cervical tissues but the relationship have not been classified yet. The frequent concomitant occurrence of MCPyV and HR-HPV in cervical cancer and the transactivating properties of LT and sT suggest that MCPyV may promoter HPV-associated cervical cancer. So, all of previous findings prompted us to investigate the effect of MCPyV oncoproteins LT and sT on HR-HPV. Since more than 70% of cervical carcinoma are associated with two types of HPV, HPV16 and HPV18, we used them in our studies.

In paper IV, we found that the basal activity of HPV18 long control region (LCR) promoter is 30-50X stronger as compared to HPV16 in C33A cells, a non-HPV cervical carcinoma cell line. Previous studies also showed higher HPV18 LCR basal activity in Hela cells (3X times higher) and T47D breast cancer cells (3.2X) [375], C33A (20X) and HeLa cells (7X) [376].

As both MCPyV and HR-HPV exist as coinfection in angiogenital mucosal tissues, we investigated the effect of MCPyV oncoproteins, both FLT and tLT (isolated from MKL-1 and MKL-2 VP-MCC cells) and sT independently or expressed together on both HPV16 and HPV18 LCR promoter activity. We found that both FLT, tLT and sT, independently and together, upregulate both HPV16 and HPV18 LCR promoter activity. Interestingly, we also found that higher concentrations of both FLT and sT have inhibitory effect on both HPV16 and HPV18 LCR promoter activity.

E6 and E7 are the two major oncoproteins involved in tumorigenesis by HR-HPV [375, 377-379]. In paper IV, transcriptional activity regulated by LCR region of both E6 and E7 HPV16/HPV18 genes also confirmed both at mRNA and protein levels. We found HPV16 E6 and E7 mRNA stimulatory effect with above 1.5 fold changes by MCPyV FLT and tLT (MKL-1 and MKL-2) alone or in combination with sT in HPV16 positive SiHa cells transfected for 48 hrs. Similarly, increased HPV18 E6 and E7 mRNA levels were also observed in HVP18 positive HeLa cells transiently transfected with MCPyV LT or/and sT oncoproteins. Furthermore, we found above 3-fold stimulatory effect of HPV18 E6 and E7 by MCPyV FLT and tLT (MKL-1 and MKL-2) alone or in combination with sT. Interestingly, sT alone had no or an inhibitory effect (30% reduced) on HPV18 E6 and E7 expression, respectively. We have also observed that sT is able to stimulate HPV18 LCR activity but did not showed same results

while studying HPV18 E6 and E7 at mRNA level. We do not know the exact reason but this could be either use of different methods used to study. C33A cells were transiently cotransfected with HPV18 E6 and E7 and HPV18 LCR reporter plasmid, while in HeLa cells, the HPV18 genome is integrated and the chromatin structure or/and DNA methylation of LCR could affect transcriptional activity.

Both HR-HPV E6 and E7 are synthesized from a bicistronic mRNA with proximal and distal ORF for E6 and E7 respectively. During translation, the ribosomal read-through of E7 ORF is 25-30% of E6 protein level [380]. So, because of difference in translation efficiency of the E6 and E7 ORFs, we examined E6 and E7 protein levels in both SiHa (HPV16) and HeLa (HPV18) cells.

We found that sT, FLT and tLT (MKL-1 and MKL-2) increased protein levels of HPV18 E6 ~1.5-fold both after 24 and 48 hrs, while co-expression of sT along with FLT and tLT did not showed any further effect. Similarly, we also observed 2- to 3.5-fold increase in HPV18 E7 expression in HeLa cells 24 hrs after transfected with sT, FLT and tLT (MKL-1 and MKL-2). We did not find any additive effect of sT co-expression with LT on HPV18 E7 expression. We observed that sT, FLT and tLT (MKL-1 and MKL-2) up-regulated HPV16 E6 protein expression 1.5-3 fold after 24 and 48 hrs. tLT was more potent than FLT on HPV16 E6 expression after 24 hrs. We also observed almost 2-fold increase in HPV16 E7 protein expression when SiHa cells were transfected with sT, FLT and tLT (MKL-1 and MKL-2), whereas co-expression of sT and FLT and tLT (MKL-1 and MKL2) did not showed any additional effect.

The biological significance and hence the indirect role of MCPyV in HPV-induced cervical cancer can be discussed. However, all human oncoviruses have a very long incubation time [122]. Therefore, the long-term moderate MCPyV-induced increased expression of E6 and E7, two potent oncogenes that can transform cells *in vitro* and induce tumors in animal models [381, 382], may enhance the tumorigenic process by these HPV oncoproteins in human cervical cancer. HR-HPV or MCPyV have also been detected in oropharyngeal cancers [383, 384], and concomitant infections have been described [118, 385, 386]. Whether MCPyV is a co-factor in HPV-induced cancers may be worth investigating.

6. CONCLUSION

The fact that MCC is either VP- or VN-MCC presents both unique opportunities and a therapeutic conundrum. At this time, MCC viral status does not help stratify patients into those who are more or less likely to respond to any specific therapy, given the present state of our knowledge and the available therapeutic options.

Although there is a long road ahead to achieving successful treatment of MCCs, the past decade has marked a turning point in the understanding, care, and prognosis of this rare skin cancer. Multiple clinical trials on MCC are ongoing or enrolling at the present time and many of them focus on novel or combination treatments. We will likely witness changes to the treatment guidelines as the literature continues to grow. Nonetheless, when compared with the dismal outlook that accompanied a diagnosis of MCC 10 years ago, the future is looking brighter for patients with MCC. With this work, I hope to contribute some small pieces to the puzzle of MCC biology and in the development of potential novel and improved treatment.

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PAPER I

RESEARCH

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Promoter activity of Merkel cell Polyomavirus variants in human dermal fibroblasts and a Merkel cell carcinoma cell line

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Abstract

Background: Merkel cell polyomavirus (MCPyV) is a human polyomavirus that establishes a life-long harmless infection in most individuals, with dermal fibroblasts believed to be the natural host cell. However, this virus is the major cause of Merkel cell carcinoma (MCC), an aggressive skin cancer. Several MCPyV variants with polymorphism in their promoter region have been isolated, but it is not known whether these differences affect the biological properties of the virus.

Methods: Using transient transfection studies in human dermal fibroblasts and the MCC cell line MCC13, we compared the transcription activity of the early and late promoters of the most commonly described non-coding control region MCPyV variant and six other isolates containing specific mutation patterns.

Results: Both the early and late promoters were significantly stronger in human dermal fibroblasts compared with MCC13 cells, and a different promoter strength between the MCPyV variants was observed. The expression of full-length large T-antigen, a viral protein that regulates early and late promoter activity, inhibited early and late promoter activities in both cell lines. Nonetheless, a truncated large T-antigen, which is expressed in virus-positive MCCs, stimulated the activity of its cognate promoter.

Conclusion: The promoter activities of all MCPyV variants tested was stronger in human dermal fibroblasts, a cell line that supports viral replication, than in MCC13 cells, which are not permissive for MCPyV. Truncated large T-antigen, but not full-length large T-antigen stimulated viral promoter activity. Whether, the difference in promoter strength and regulation by large T-antigen may affect the replication and tumorigenic properties of the virus remains to be determined.

Keywords: Non-coding control region, Large T-antigen, Luciferase assay, MCC13 cells, MCPyV, Mutations

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Background

In 2008, a new human polyomavirus was isolated, which rekindled the field of polyomavirus research [1]. This virus was isolated from Merkel cell carcinoma (MCC), a rare but aggressive skin cancer. Accordingly, this virus was named Merkel cell polyomavirus (MCPyV). The original study showed that 8 out of the 10 examined MCC samples contained MCPyV DNA [1]. Numerous studies by different groups worldwide have confirmed that approximately 80% of MCCs are positive for this virus [2–5]. Because cell culture and transgenic mice studies have shown that MCPyV has an oncogenic potential that can be attributed to its viral proteins large T-antigen (LT) and small t-antigen (sT) ([6–9]), and the association of the virus with MCC, MCPyV is considered an etiological factor in MCC and is classified as probably carcinogenic to humans [10]. Two hallmarks of MCPyV-positive MCCs are the integration of the viral genome in the host chromosome and expression of a truncated version of LT [5, 11]. Integration disrupts the late region so that no infectious particles are generated in MCCs, while the truncation of LT results in a non-DNA binding variant that retains the ability to bind the tumor suppressor retinoblastoma protein, but not p53 [12].

Serological studies demonstrated that seroprevalence against MCPyV increases with age, and reaches up to ~80% in healthy individuals [13–18]. Little is known about the route of infection, transmission and the cell tropism of MCPyV. Dermal fibroblasts are a genuine host cell for MCPyV [19], and the virus seems to persist in the skin [20–22]. However, PCR-based analyses detected MCPyV DNA in other sites in the body, both in healthy individuals and patients (Supplementary Table S1), as well as in sewage water and environmental surfaces (Supplementary Table S2). The implication of MCPyV in cancers other than MCC remains unknown, although viral DNA, RNA and proteins can be detected in some cases of other malignancies [23]. Sequence analysis of the MCPyV LT, sT and VP1 genes of different virus isolates revealed genetic variability, but the biological implications in the viral life cycle and the development of MCC have not been studied.

Mutations in the non-coding control region (NCCR) of human polyomaviruses like BKPyV, JCPyV, KIPyV, HPyV7, HPyV9 and HPyV12 have an impact on the transcriptional activity of the promoter, and may affect the virulence of the virus [24–33]. Whether changes in the NCCR of MCPyV have an effect on the promoter activity, and have pathogenic consequences, has not been investigated. Here, we compare the transcriptional activity of NCCR of different MCPyV variants isolated from virus-positive MCC and non-MCC samples in a MCC cell line, and in human dermal fibroblasts.

Methods

Cells

The MCPyV-negative MCC13 cell line was kindly provided by Dr. Baki Akgül (University of Cologne, Germany)

and was grown in RPMI-1640 (Sigma Life Science, St. Louis, MO, USA; cat. no. R8758) with 10% fetal bovine serum (Gibco, Life Technologies Limited, Pailey, UK) in the presence of 100 µg/ml streptomycin and 100 units/ml penicillin. Immortalized human dermal fibroblasts fHDF/TERT166 were purchased from Evercyte (Vienna, Austria) and kept in DMEM/Ham's F12 (1:1) (Biochrom, Berlin, Germany; cat. no. F4815), 10% fetal bovine serum, 2 mM GlutaMax™-I (Gibco; cat. no. 35050–038) and 100 µg/ml G418 (Santa Cruz Biotechnology, Dallas, TX, USA; cat. no. sc-29,065). Cells were kept in a humidified CO₂ incubator at 37 °C.

Plasmids

The luciferase reporter plasmids with the consensus NCCR MCPyV in early (pGL3-cons-E) or late (pGL3-cons-L) orientation have been previously described [24]. The luciferase reporter plasmids containing the NCCR of the variants 10b, 15a, 16b, HUN, MKL-1, MS-1 were generated by GenScript (Piscataway, NJ, USA). Each NCCR was cloned in both early (NCCR-E) and late (NCCR-L) orientation, respectively. The luciferase plasmids with the NCCR containing the 25 bp duplication described by Hasida et al. [34] was generated by site-directed mutagenesis using the plasmid pGL3-cons-E (pGL3-cons-L, respectively) containing the consensus NCCR and the complementary primers 5'-GGCCGGAGGCTTTTTTCTCTTACAAAGGGAG-GAGGACATTTCTCTTACAAAGGG-3' and 5'-CCCTTTGTAAAGAGAAAAGCCTCCGGCC-3'. The empty expression vector pcDNA3.1(+) was purchased from Invitrogen (ThermoFisher Scientific, Oslo, Norway). The MCPyV expression vectors for full-length and truncated LT have been previously described [35], and all plasmids were verified by sequencing. Expression of full-length and truncated LT was confirmed by western blotting using antibody CM2B4 from Santa Cruz Biotechnology (Dallas, TX, USA; cat. no. sc-136,172; results not shown).

Transfection and luciferase assay

Cells were seeded out in 12-well culture plates. At the time of transfection, the cells were approximately 70% confluent, with a total of 1 µg luciferase reporter plasmid DNA used per well and polyethylenimine (PEI linear MW25000; transfection grade, cat. no. 23966–1, Polysciences, Warrington, PA, USA). DNA was mixed with 150 mM NaCl, and a mixture of PEI:150 mM NaCl was then added to the DNA. The ratio DNA:PEI used was 1:2. This mixture was incubated for 15 min at room temperature, and then carefully added to the cells. The medium containing the transfection mixture was replaced 4 h later. Cells were harvested 24 h after transfection in a 100 µl Tropix lysis buffer per well with 0.5 mM DTT freshly added. Cells were centrifuged for 3 min at

12,000 g, and the supernatant was then transferred to a fresh tube. As previously described, 20 µl of supernatant was used in the luciferase assay [35]. Each experiment was repeated at least 3 times, with three independent parallels for each experiment. Luciferase values for each sample were corrected for total protein concentration as determined with the MN protein quantification assay described by the producer (Macherey-Nagel GmbH, Düren, Germany). We corrected luciferase values by measuring the protein concentration in the corresponding sample rather than co-transfection with a Renilla reporter plasmid to avoid promoter interference between the MCPyV NCCR directing expression of the firefly luciferase gene and a promoter controlling expression of the Renilla luciferase gene. In addition, many of our transfection studies include co-transfection with LT expression plasmids, containing the strong competing CMV promoter. Moreover, LT of polyomaviruses have shown activate many promoters, including the SV40 promoter or the herpes simplex virus thymidine kinase promoter [36], which are commonly used in Renilla reporter plasmids.

Statistics

A two-tailed Student’s *t*-test was used to determine statistical differences between the MCPyV promoter variants.

Results

The known MCPyV NCCRs can be classified in six different groups

A comparison of all available complete NCCR sequences of MCPyV variants revealed a predominant sequence, which is hereafter referred to as the consensus sequence shown in Fig. 1. Based on this consensus sequence, we classified the different NCCR variants in seven groups (Fig. 1 and Table 1). Group 1 contains the MCPyV strains with a consensus or quasi consensus (i.e. one or few point mutations). Group 2 contains NCCR variants with an insertion of the AAC or AACTC sequence at nucleotide 369 (numbering according to the consensus sequence). Group 3 NCCR has an insertion of the TCAAT sequence at nucleotide 372, while group 4 has deletion of the CCTTAGAT sequence (nucleotides 105–112). Group 5 has both an insertion (ACAA or ACAAC at nucleotide 372) and a deletion of nucleotides 381–387 (AACAAGG). The NCCR in group 6 has three insertions: CAAC after nucleotide 373, T after nucleotide 379 and AA after nucleotide 383. Lastly, group 7 variants have a 25 bp duplication.

The biological source of each NCCR variant is given in Table 1 and Supplementary Table S1. Consensus NCCRs are found in strains present in non-diseased and diseases tissue/individuals. Likewise, NCCR variants circulate in healthy individuals and patients. MCPyV variants with consensus and mutated NCCRs have been isolated from

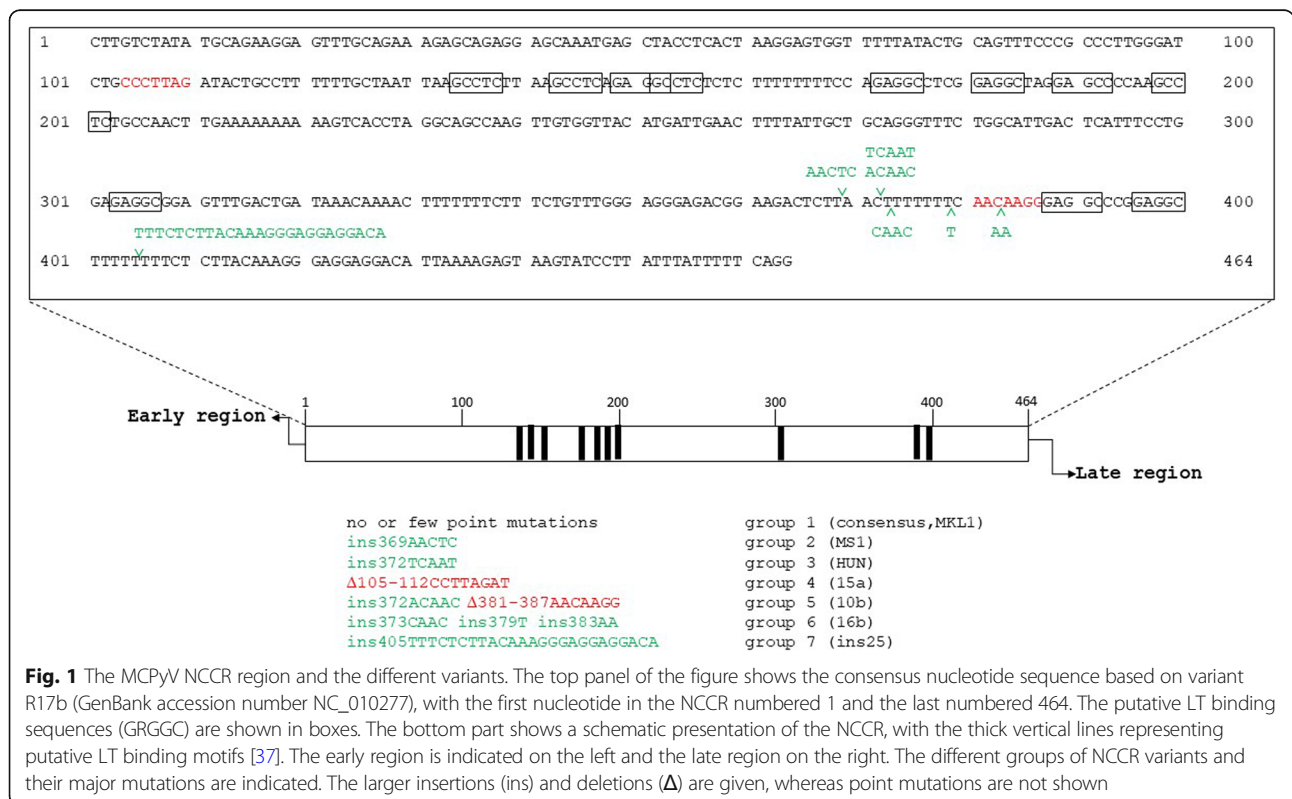


Fig. 1 The MCPyV NCCR region and the different variants. The top panel of the figure shows the consensus nucleotide sequence based on variant R17b (GenBank accession number NC_010277), with the first nucleotide in the NCCR numbered 1 and the last numbered 464. The putative LT binding sequences (GRGGC) are shown in boxes. The bottom part shows a schematic presentation of the NCCR, with the thick vertical lines representing putative LT binding motifs [37]. The early region is indicated on the left and the late region on the right. The different groups of NCCR variants and their major mutations are indicated. The larger insertions (ins) and deletions (Δ) are given, whereas point mutations are not shown

Table 1 MCPyV NCCR variants examined in this study

Group	NCCR variant	Referred to in this paper	Source	Reference
1	R17b	cons-E and cons-L	healthy skin	[20]
	MKL-1	MKL1-E and MKL1-L	MCC	[38]
2	MS-1	MS1-E and MS1-L	MCC	[1]
3	7673/2011/HUN	HUN-E and HUN-L	metastatic cervical lymph node	[39]
4	R15a	15a-E and 15a-L	healthy skin	[20]
5	R10b	10b-E and 10b-L	healthy skin	[20]
6	R16b	16b-E and 16b-L	healthy skin	[20]
7	Subtype I	Ins25-E and ins25-L	healthy skin	[34]

sewage water (see Table 1 for references). The mutations for each NCCR variant are presented in Supplementary Table S3.

Basal early and late promoter activities in MCC13 and human dermal fibroblasts cells

Because MCPyV-positive Merkel cell carcinoma derives from virus-transformed Merkel cells [1], and human dermal fibroblasts (HDF) have been shown to be permissive for this virus and considered as genuine host cells for the virus [19], we examined the basal early and late promoter activity of MCPyV variants in these two cell lines. To study the effect of mutations in the NCCR on basal early and late promoter activity, one NCCR variant was selected from each group, with the exception of group 1, in which both the consensus sequence and a variant with few point mutations were tested (Table 1). The NCCRs were cloned in both orientations, and the basal early and late promoter activities were monitored in the MCPyV-negative MCC cell line MCC13, in addition to primary dermal fibroblasts.

Comparing the relative basal early and late promoter activities showed that both promoters were stronger in HDF cells compared to MCC13 cells (Supplementary Fig. S1). The cons-E promoter was ~4-fold stronger, while the cons-L was ~3x stronger. The difference is probably even more because the transfection efficiency in MCC13 cells was approximately 60–70%, whereas in HDF the transfection efficiency was estimated to be ~30% (results not shown). Comparing the cons-E and cons-L in MCC13 revealed that the late promoter was approximately 8x stronger than the early promoter. The cons-L was approximately 6x stronger in HDF than in MCC13 cells (Supplementary Fig. S1).

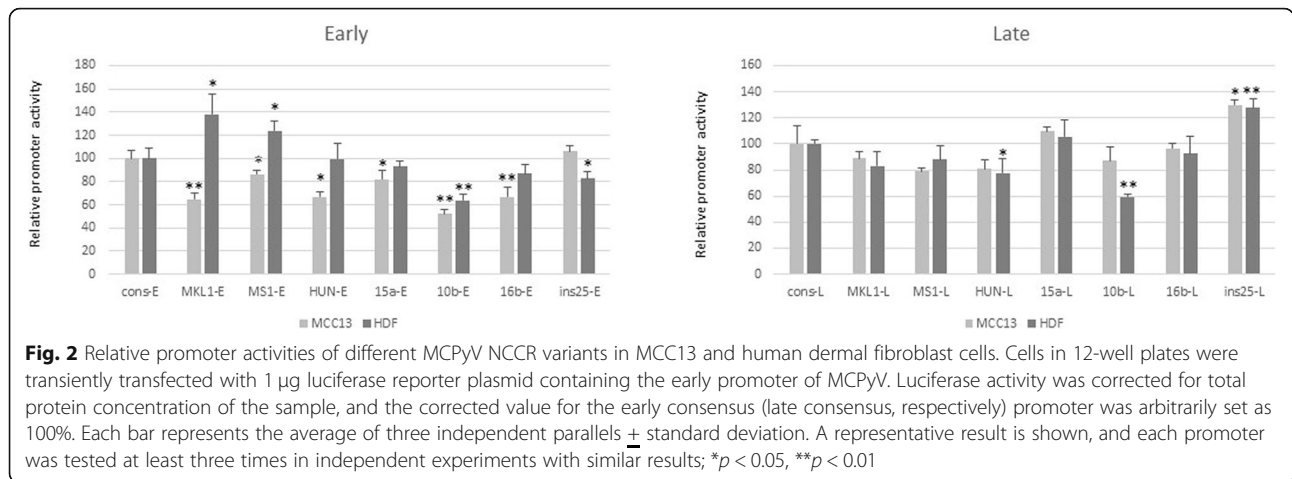
Effect of large T-antigen on early and late promoter activities

Large T-antigen of polyomaviruses has been shown to affect the early and late promoter activities. To examine the effect of LT on the MCPyV promoters, we co-transfected MCC13 cells with 1 µg luciferase reporter

plasmid with the cons-E promoter, with increasing amounts (0, 100, 200, 400, 500, 800 and 1000 ng) of empty pcDNA3.1 vector or LT expression vector. All concentrations reduced cons-E promoter activity (results not shown), but high concentrations (800 and 1000 ng) of empty vector almost completely inhibited MCPyV promoter activity. Both the empty vector and the LT expression plasmid contain the strong CMV immediate early promoter. We found this promoter to be >5-fold stronger than the cons-E promoter in MCC13 cells and ~40x stronger in HDF cells (Supplementary Fig. S2). We decided to test the effect of two different concentrations (100 and 500 ng) of LT expression plasmid on all variant NCCRs. Both concentrations of LT expression plasmid (100 ng and 500 ng) significantly reduced the activity of all early promoters in MCC13 cells (Fig. 3a and b). A similar effect was observed for the late promoters, with the exception of the MS1 and HUN promoters, which were induced when cells were co-transfected with 500 ng of LT expression plasmid (Fig. 3c and d).

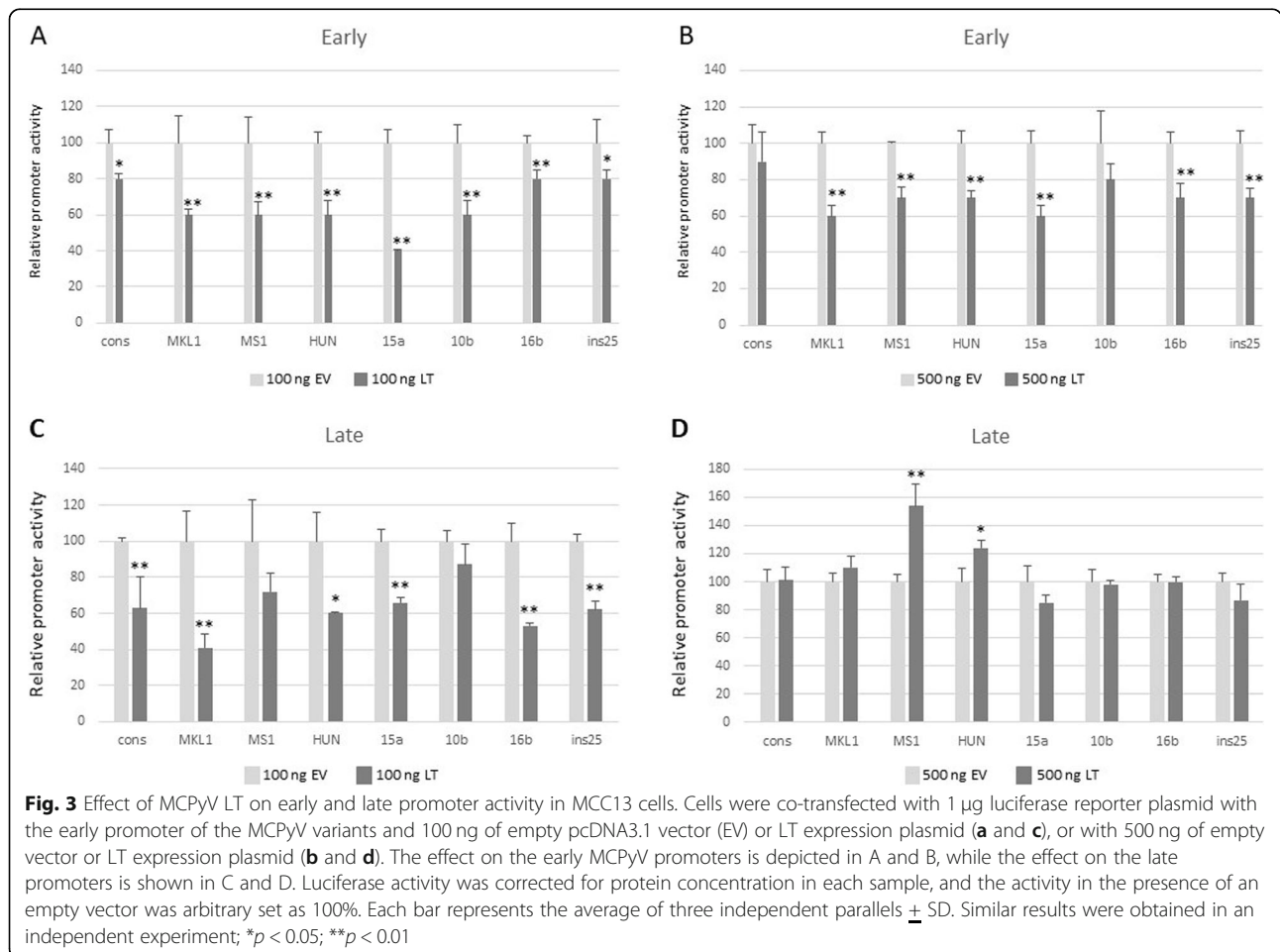
We also examined the effect of LT on the MCPyV promoters in HDF cells. Both concentrations of LT expression plasmid (100 ng and 500 ng) significantly repressed the early promoter activity (Fig. 4a and b) and the late promoter activity (Fig. 4c and d) of all variant NCCRs tested. However, a somewhat stronger inhibition was observed with the lowest concentration of LT expression plasmid, while at higher concentrations promoter interference becomes more pronounced.

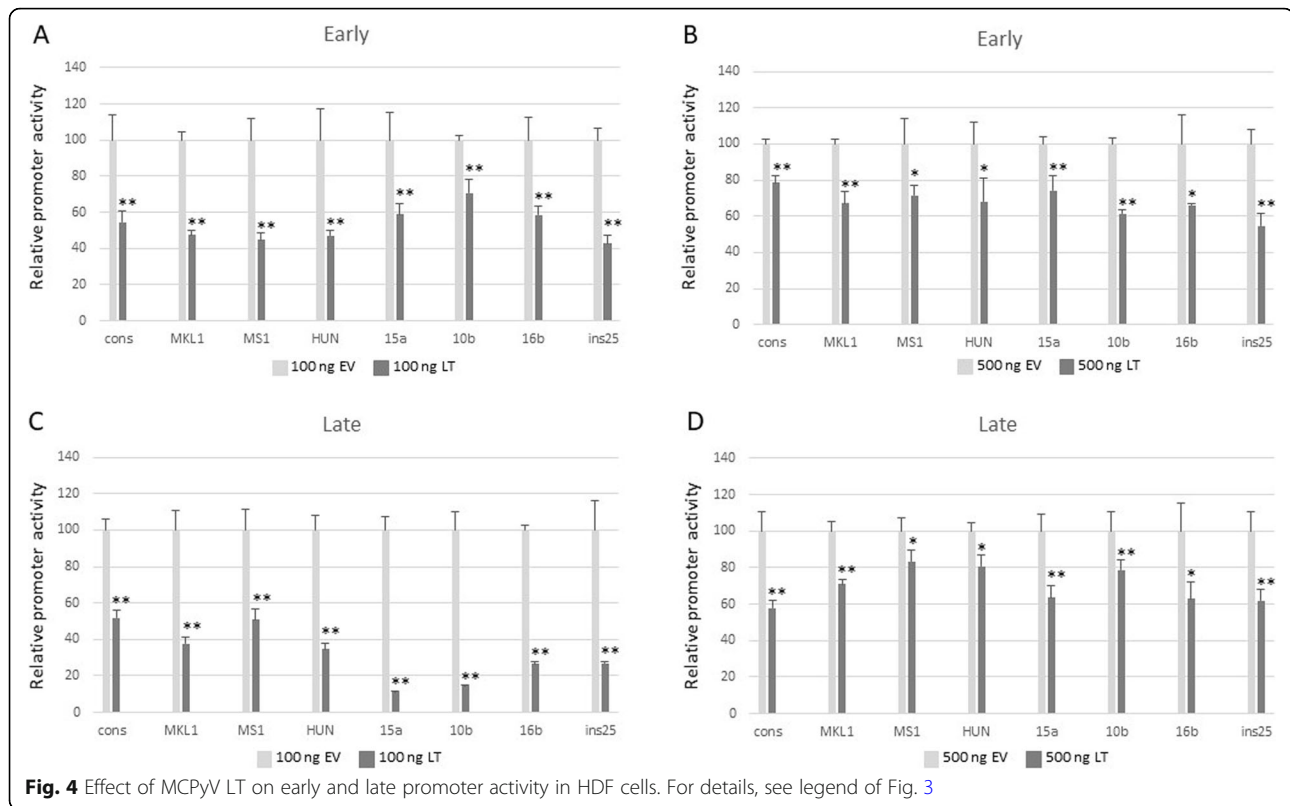
MCPyV-positive MCCs contain integrated viral DNA, and are further characterized by the expression of a C-terminal truncated LT [Feng, 2008]. Thus, we examined the effect of truncated LT on the early and late promoter. Of the seven different strains examined in this work, only MKL-1 and MS-1 have been isolated from MCC [40, 41], whereas the HUN strain was obtained from a metastatic cervical lymph node from a Hungarian patient, though no further information is available [39]. We therefore decided to test the impact of MKL-1 (MS-1, respectively) truncated LT on their cognate promoter. The luciferase reporter plasmids with the early or late



promoter of MKL-1 (MS-1, respectively) were co-transfected with an expression plasmid for MKL-1 LT (MS-1 LT, respectively) in MCC13 and in HDF cells, and the promoter activity was monitored. Truncated MKL-1 LT stimulated the MKL-1 early and late promoter activities in both MCC13 and HDF cells (Fig. 5a and b). The only exception was when 500 ng of MKL-1

LT expression plasmid was used, with a significant inhibition of the early promoter observed in HDF cells (Fig. 5b). Truncated MS-1 LT also stimulated the early and late MS-1 promoter in MCC13 cells and the late promoter in HDF cells (Fig. 5a and b), but inhibited the early promoter in HDF cells (Fig. 5b). With 100 ng of truncated MS-1 LT expression plasmid, a reduction in





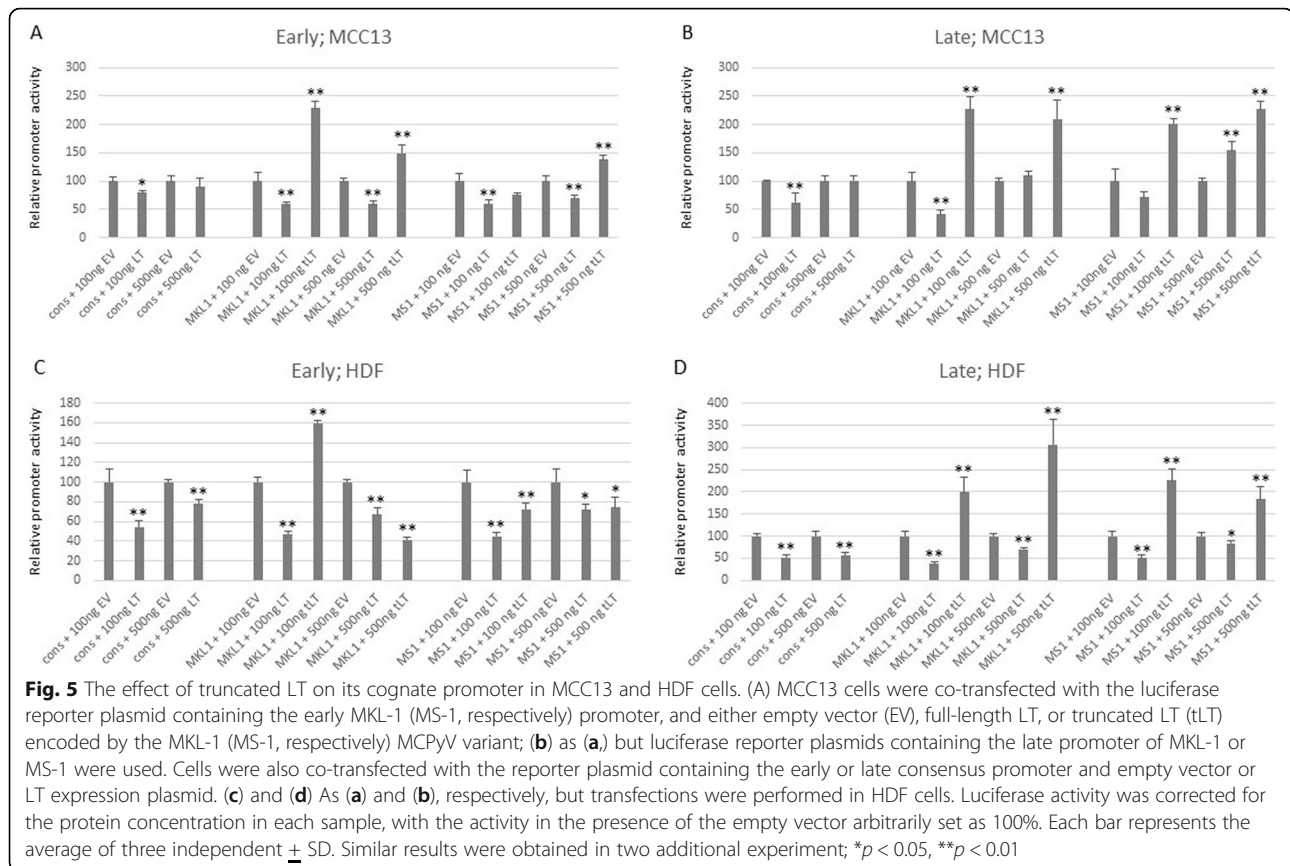
promoter activity of 15–24% compared to the control was observed. However, this decrease was not significant (p -values between 0.0553 and 0.0632 in the different experiments).

Discussion

MCPyV has a seroprevalence of approximately 80% in the healthy, adult population [13–18]. MCPyV is chronically shed from healthy skin [20], but can also cause an aggressive skin cancer known as Merkel cell carcinoma [1]. Several MCPyV variants have been described with mutations in their NCCR (Supplementary Table S3). These variants have been isolated from both healthy tissue and tumors, but so far, no typical strain seems to be associated with MCC (Supplementary Table S3). The mutations described in the known MCPyV variants could be classified in seven groups with group 1 containing the most common NCCR, which was referred to as the consensus sequence in our study and variants with one or a few point mutations. Groups 2–7 contain insertions and/or deletions in their NCCR. Significant differences in basal early and late promoter activities in HDF and MCC13 cells were observed. The basal early-, as well as late promoter activity, of all variants tested was higher in HDF cells than in MCC13 cells despite a lower transfection efficiency (Fig. 6). This may indicate that the MCPyV promoter is more adapted to the former cell

type. HDF have been suggested as natural host cells for the virus and are permissive for the virus, while the infection of Merkel cells and the subsequent transformation of these cells could be seen as an accidental and unfortunate event [19].

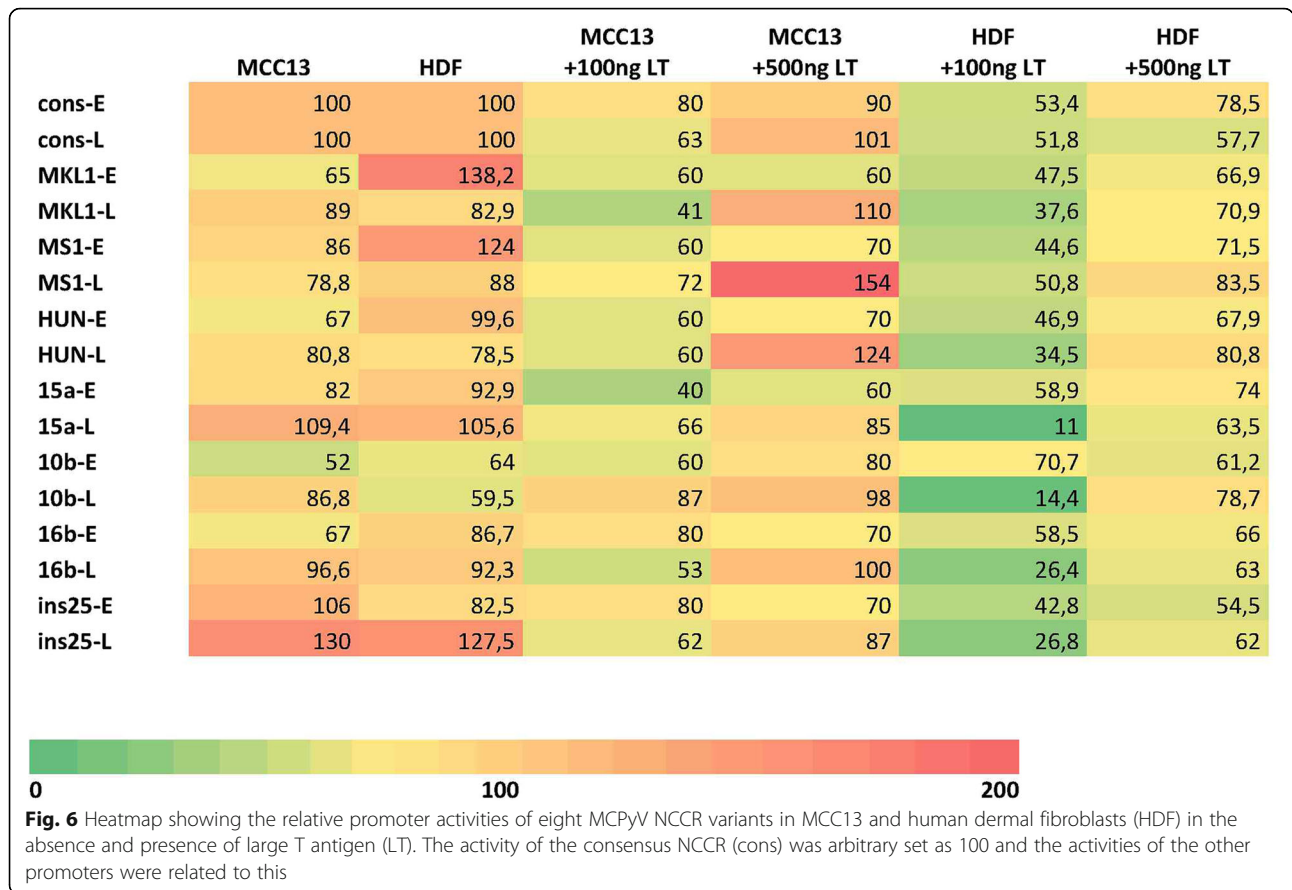
Co-transfection with a 100 ng of full-length LT expression plasmid resulted in reduced early and late promoter activity of all variants in both MCC13 and HDF cells (Fig. 6). However, 500 ng of LT expression plasmid reduced early and late promoter activities in HDF and early promoter activity in MCC13 cells, but had only a slight or no effect, but significantly stimulated the HUN and MS1 (a 20 and 40% increase, respectively) late promoters. Kwun et al. found that MCPyV LT repressed early and late promoter activity of MCPyV isolate MCC339 in HEK293 cells, but they did not examine the effect of LT in MCC13 or HDF cells [42]. Yet, in another study by Ajuh and co-workers, the authors showed that LT trans-activated the MCPyV R17b (= consensus) early and late promoter in HEK293T cells [29]. The discrepancy between their results and the findings by Kwun et al. and us can be explained by the use of different LT. HEK293T cells express both SV40 LT and sT, but not MCPyV LT [29]. Moreover, the possible contribution of sT in the trans-activation of the early and late MCPyV promoters in these cells was not investigated. Furthermore, Ajuh et al. studied the



effect of LT using a bidirectional reporter vector, thereby allowing for the simultaneous monitoring of the early and late promoter activity, whereas both we and Kwun et al. examined early and late promoter independently, which better reflects the situation in infected cells. Indeed, during the polyomavirus life cycle, early and late promoters are activated in a time-dependent fashion. The early promoter is active early during infection, and the expression of LT will result in the autorepression of the early promoter and a switch to activation of the late promoter [43]. Another experimental difference was that we measured promoter activities 24 h after transfection, whereas Ajuh and colleagues determined promoter activities 48 h post transfection. Lastly, we used dose-dependent studies with LT, while Ajuh and co-workers used cells constitutively expressing LT. The authors also examined the effect of MCPyV LT on early and late promoter activity of the consensus MCPyV variant and MCVw156 (consensus with one substitution and one deletion; Supplementary Table S3) in HEK293MCT cells (i.e. HEK293 cells stably expressing MCPyV LT). While early promoters of both MCPyV variants were significantly stimulated by MCPyV LT, no effect was observed on their late promoter. A possible explanation for the different

effects of SV40 LT and MCPyV LT on the MCPyV late promoter was not provided by the authors.

Because the viral genome in MCC expresses a truncated LT, we examined the effect of truncated LT on its cognate promoter. Truncated MKL-1 (MS-1, respectively) LT stimulated its cognate early and late promoter in MCC13 cells, whereas stimulation was only observed for the late promoter in HDF cells. In these cells, truncated MKL-1 and MS-1 LT inhibited the corresponding early promoter. The reason for the cell-specific effect of truncated LT on the MCPyV promoter is not known, but MCPyV LT has been shown to interact with several cellular factors [42]. Different interaction partners in distinct cell types may determine the effect of LT on the MCPyV promoter activity. The fact that truncated LT stimulates their cognate early promoter in MCC13 may indicate a positive feedback loop that results in higher expression levels of the early proteins, including the oncoproteins sT and LT. Our preliminary results shows that also truncated MKL-2 LT (which differs from HUN truncated LT by replacement of Ala20 into Ser and Ser263 into Phe, and the lack of the three C-terminal amino acids Ser-Arg-Lys) was able to stimulate the HUN-E promoter approximately 2-fold in MCC13 cells (our unpublished results), suggesting that it may be a



common feature of truncated LT to autostimulate its expression. This positive autoregulation of LT and sT could be potentially important for tumorigenesis. Since full-length LT had an inhibitory effect on the promoters, it would be interested to test whether full-length LT reverses the activity of truncated LT. However, to be of biological relevance, both full-length and truncated LT must be co-expressed in MCPyV infected cells. To our best knowledge, only truncated LT is expressed in virus-positive MCCs. We are aware of only two studies where co-expression of full-length and truncated LT was observed. One case of non small cell lung cancer (a squamous cell carcinoma) with both episomal and integrated viral DNA and both full-length and truncated LT protein was described by Hashida and co-workers [44]. In another study, mRNAs for truncated and full-length LT were confirmed by highly sensitive qRT-PCR in two cases of chronic lymphocytic leukemia, but expression of truncated and full-length LT at protein level was not investigated [45]. The genome copy per chronic lymphocytic leukemia cell was 3 to 4 logs lower than MCPyV-positive MCCs, suggesting that very low levels of LT/truncated LT are present in these cells. It remains to be determined whether such low levels have any biological relevance for the viral life cycle or tumorigenesis. Because integration of the

MCPyV genome interrupts the late region, no late proteins are expressed and no viral particles are produced. The biological implication of enhanced late promoter activity by truncated LT in MCC remains elusive.

Our transient transfection studies showed that the MCPyV NCCR variants possess different promoter activity, and can lead to different expression levels of the viral proteins. This has been confirmed by in situ studies. The CVG-1 and MKL-1 MCC cell lines both contain seven copies of the integrated virus genome per diploid cell [46]. The CVG-1 cell line contains the consensus NCCR sequence, while MKL-1 contains one single point mutation (T52C; Supplementary Table S2). Quantitative real-time PCR demonstrated that the total LT and sT mRNA expression levels were approximately 2.5 times higher in CVG-1 cells compared to MKL-1 cells, thus indicating that the former promoter is 2.5x stronger than the latter. Our transient transfection study in MCC13 cells confirmed that the MKL-1 early promoter was weaker than the consensus early promoter (Fig. 2). The expression levels of early proteins in MCC not only depend on the strength of the early promoter, but the number of integrated viral genomes and the integration site (hetero- versus euchromatin) may also influence the promoter strength. Velásquez et al. determined that MKL-2 and

MS-1 MCC cells contain two and four genome copies per diploid cells, respectively [46]. The LT transcript levels were approximately 2-fold higher in MS-1 cells compared to the MKL-2 cells, while the ST mRNA levels were 4x higher in MS-1 cells than in MKL-2 cells. The complete NCCR sequence of the MKL-2 variant has not been determined, but the 240 nucleotides downstream of the LT start codon are identical with the consensus sequence.

The MCPyV NCCR contains a plethora of putative transcription factor binding motifs (see Supplementary Fig. 3 and Supplementary Table S4), but the binding of the corresponding transcription factor has not been confirmed. Whether the mutations found in these sites in the NCCR variants we investigated abolished binding of the transcription factor, has not been tested. The 25 bp insertion generates putative binding motifs for the transcription factors FOXO3a, SRY, Elk-1 and p300, but their possible role in regulating the promoter activity remains to be investigated.

Conclusions

Our study shows that the promoters of different MCPyV isolates possess unlike transcriptional activity, and that full-length LT and MCC-associated truncated LT have a distinct impact on the promoter. Whether these differences in promoter activity contribute to the replication and transformation properties of the virus remains to be determined.

Supplementary information

Supplementary information accompanies this paper at <https://doi.org/10.1186/s12985-020-01317-x>.

Additional file 1 Supplementary Fig. S1. Early and late promoter activity of seven MCPyV NCCR variants in MCC13 and human dermal fibroblasts. **Supplementary Fig. S2.** Relative promoter activity of the immediately early cytomegalovirus and the consensus early MCPyV promoter in MCC13 and human dermal fibroblasts. **Supplementary Fig. S3.** Putative transcription factor binding sites in the consensus MCPyV NCCR. **Supplementary Table S1.** Detection of MCPyV in benign and diseased human tissues other than Merkel cell carcinoma. **Supplementary Table S2.** MCPyV isolated in sewage water and environmental surfaces. **Supplementary Table S3.** MCPyV NCCR variants and mutations compared to the consensus sequence. **Supplementary Table S4.** Putative transcription factor binding sites in the consensus MCPyV NCCR.

Abbreviations

HDF: Human dermal fibroblasts; LT: Large T antigen; MCC: Merkel cell carcinoma; MCPyV: Merkel cell polyomavirus; NCCR: Non-coding control region; sT: Small T antigen; tLT: Truncated large T antigen

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Authors' contributions

Conceptualization, U.M. and B.E.; experiments, I.A., K.R., and U.M.; validation, B.E., U.M.; writing—original draft preparation, U.M. and B.E.; writing—review and editing, I.A., K. R, B.S., E. B, and U.M.; supervision, U.M. and B.S.; project administration, U.M.; funding acquisition, U.M. and B.S. The author (s) read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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PAPER II

CCL17/TARC and CCR4 expression in Merkel cell carcinoma

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ABSTRACT

Merkel cell carcinoma (MCC) is a rare, highly aggressive neuroendocrine skin cancer. In more than 80% of the cases, Merkel cell polyomavirus (MCPyV) is a causal factor. The oncogenic potential of MCPyV is mediated through its viral oncoproteins, large T antigen (LT) and small t antigen (sT). To investigate the role of cytokines in MCC, a PCR array analysis for genes encoding inflammatory cytokines and receptors was performed on MCPyV-negative and MCPyV-positive MCC cell lines, respectively. We detected an increased expression of CCL17/TARC in the MCPyV-positive MKL2 cell line compared to the MCPyV-negative MCC13 cell line. Transfection studies in MCC13 cells with LT expression plasmid, and a luciferase reporter plasmid containing the CCL17/TARC promoter, exhibited stimulated promoter activity. Interestingly, the ectopic expression of CCL17/TARC upregulated MCPyV early and late promoter activities in MCC13 cells. Furthermore, recombinant CCL17/TARC activated both the mitogen-activated protein kinase and the NF- κ B pathways. Finally, immunohistochemical staining on human MCC tissues showed a strong staining of CCL17/TARC and its receptor CCR4 in both LT-positive and -negative MCC. Taken together, CCL17/TARC and CCR4 may be a potential target in MCC therapy providing MCC patients with a better overall survival outcome.

INTRODUCTION

Merkel cell carcinoma (MCC) is a rare, highly aggressive neuroendocrine skin cancer [1, 2]. In 2008, using digital transcriptome subtraction, a new virus belonging to the family of Polyomaviruses, and hence named Merkel cell polyomavirus (MCPyV), was identified in MCC. Worldwide studies have shown that approximately 80% of all examined MCC contain clonal integrated MCPyV DNA [3], thus indicating that MCPyV is associated with the etiology of MCC [4, 5]. MCPyV has a circular, double-stranded DNA genome of approximately 5.4 kb [6]. The viral genome possesses the typical polyomavirus tripartite organization, with

an early region encoding the regulatory proteins large T (LT) antigen and small T (sT) antigen [7], the late region encoding the capsid proteins [8], and a non-coding control region encompassing the origin of replication and transcription regulatory elements [9]. In addition, the early region encodes the 57kT antigen and a protein called alternative LT ORF (ALTO), although their function remains unknown [7]. All MCPyV-positive (MCPyV+) MCC express a C-terminal truncated form of LT antigen that has lost its DNA binding and p53 interaction domains, but retains the ability to interact with retinoblastoma protein pRb [10]. Both sT and full-length LT, as well as truncated LT, have been shown to possess oncogenic potential in both cell culture and

animal models [11-14]. The proliferation of MCC cell lines depends on the expression of LT [15], while the role of sT has been disputed [16, 17].

Inflammation has long been associated with tumor progression [18]. Many cancers arise from sites of infection, chronic irritation and inflammation, and inflammatory signaling pathways are often activated by oncogenic mutations [19]. Chemokines are a family of cytokines that regulate leukocyte trafficking in immunity and inflammation, playing a significant role in processes attributed to tumorigenesis, such as tumor cell survival, senescence, angiogenesis, metastasis and immune escape. The aberrant expression of chemokines and chemokine receptors in tumors may regulate the trafficking of leukocytes into the tumor microenvironment [20]. Chemokines secreted within the tumor microenvironment may also act in an autocrine manner to promote the proliferation and migration of the tumor cells. Chemokine (C-C motif) ligand 17/thymus and activation-regulated (CCL17/TARC) is a member of the CC chemokine family, and is highly expressed by thymus and other cells, including keratinocytes, endothelial cells, dendritic cells, bronchial epithelial cells and fibroblasts [21]. CCL17/TARC acts as a chemoattractant, which primarily aids the recruitment of CD4⁺ T regulatory cells and Th2, in addition to Th17 cells [22, 23]. The effect of CCL17/TARC is mediated by the chemokine receptor CCR4 [24, 25].

In this study we compared the cytokine expression pattern in MCPyV-positive with MCPyV-negative MCC cells and examined the role of the viral protein LT on cytokine expression. In addition, we investigated whether cytokines have an impact on viral expression.

RESULTS

Differential expression profile of inflammatory modulators in MCPyV-negative and MCPyV-positive MCC cell lines

Inflammatory mediators such as cytokines are known to play a role in cancer. This prompted us to compare the expression of 84 inflammatory cytokines and cytokine receptors in a MCPyV-negative (MCC13) and a MCPyV-positive MCC cell line (MKL-2), respectively. All 84 human inflammatory cytokines and receptor transcripts were detectable (Cq < 35) by RT profiler PCR array. Out of 84 transcripts, with the fold changes critical value set ≥ 2 fold differentiated, the expression of 11 (13.09%) were upregulated and the expression of 18 (21.42%) were downregulated in MKL-2 cells compared with MCC13 cells, while 55 (65.47%) of the genes had comparable transcription levels in both cell lines (Figure 1A) (data not shown).

Expression analysis of cytokines and their receptor in MCPyV-negative MCC13 cells and MCC13 cells transiently expressing exogenous LT

Because the LT of polyomaviruses is known to affect viral and cellular gene expression (30), we wanted to examine whether LT may be responsible for the differential expression of cytokines and their receptors in these MCC cell lines [10, 26]. Therefore, the eukaryotic expression vectors for MKL-2 LT, but also for the LT of two other virus-positive MCC cell lines (MKL-1 and MS-1), were generated. These virus-positive expression plasmids containing full-length LT and truncated LT (MKL-1, MKL-2 and MS-1) were then confirmed by sequencing (Supplementary Figure 1). A Western blot of lysates from MCC13 cells transfected with these expression plasmids confirmed the presence of LT with a correct predicted molecular mass (Supplementary Figure 2). Next, we transfected MCC13 cells with pcDNA3-full-length LT, pcDNA3-MKL-2, or empty vector pcDNA3 as a control, and measured the transcript levels of the 84 inflammatory cytokines and receptors by qPCR. For cells transfected with full-length LT expression plasmid, 71 out of 84 human inflammatory cytokines and receptor transcripts were detectable (Cq < 35), whereas the transcript for the remaining 13 genes was undetectable or had Cq values ≥ 35 by RT profiler PCR array. Out of 84 transcripts, with the fold changes critical value set ≥ 2 , 8 (9.52%) genes were upregulated, while 76 (90.48%) genes did not show any effect or undetectable compared with empty vector transfected cells (Figure 1B) (data not shown). For cells transfected with MKL-2 truncated LT expression plasmid, 70 out of 84 human inflammatory cytokines and receptor transcripts were detectable (Cq < 35), while of the remaining, 14 were undetectable or had Cq values ≥ 35 by RT profiler PCR array. Out of 84 transcripts, with the fold changes critical value set ≥ 2 , 32 (38.09%) genes were upregulated and 4 (4.76%) genes were downregulated, while 48 (57.14%) genes did not show any effect or undetectable compared with the control cells (data not shown) compared to MCC13 cells transfected with an empty vector (Figure 1C). One of the genes whose transcript levels were consistently upregulated in MKL-2 cells, and in MCC13 cells transiently expressing full-length or truncated MKL-2 LT compared to MCC13 cells, was CCL17/TARC.

MCPyV large T antigen induces CCL17/TARC promoter activity

Because CCL17/TARC expression was shown to be upregulated in cells that were either MCPyV-positive or that expressed a full-length or truncated LT, we tested whether MCPyV LT could induce CCL17/TARC promoter

activity. For this purpose, MCC13 cells were transiently co-transfected with a plasmid encoding a full-length or truncated LT (MKL-1, MKL-2 and MS-1), and with a luciferase reporter driven by different fragments of the CCL17/TARC promoter (fragment -2535/+40, -1084/+40 or -378/+40, respectively). The empty vector pcDNA3 was used as a control. Full-length, as well as the truncated LT versions MKL-1 and MS-1 significantly stimulated the CCL17/TARC promoter activity. The largest CCL17/TARC promoter fragment (-2535/+40) was more potently activated than the shorter promoter fragments (-1084/+40 and -378/+40, respectively) by LT MKL-1 and MS-1,

while comparable full-length LT-mediated transactivation the three different CCL17/TARC promoter fragments. MKL-2 LT significantly stimulated the activity of the CCL17/TARC promoter encompassing nucleotides -2535/+40 and -378/+40, but induced only slightly, but statistically insignificant the CCL17/TARC -1080/+40 promoter sequence (Figure 2). These results confirm the qPCR data showing that CCL17/TARC expression is higher in LT expressing MCC13 compared to MCC13 cells. The enhanced CCL17/TARC transcript levels in MKL-2 cells, compared to the virus-negative MCC13 cells, may at least be partially triggered by LT. In contrast,

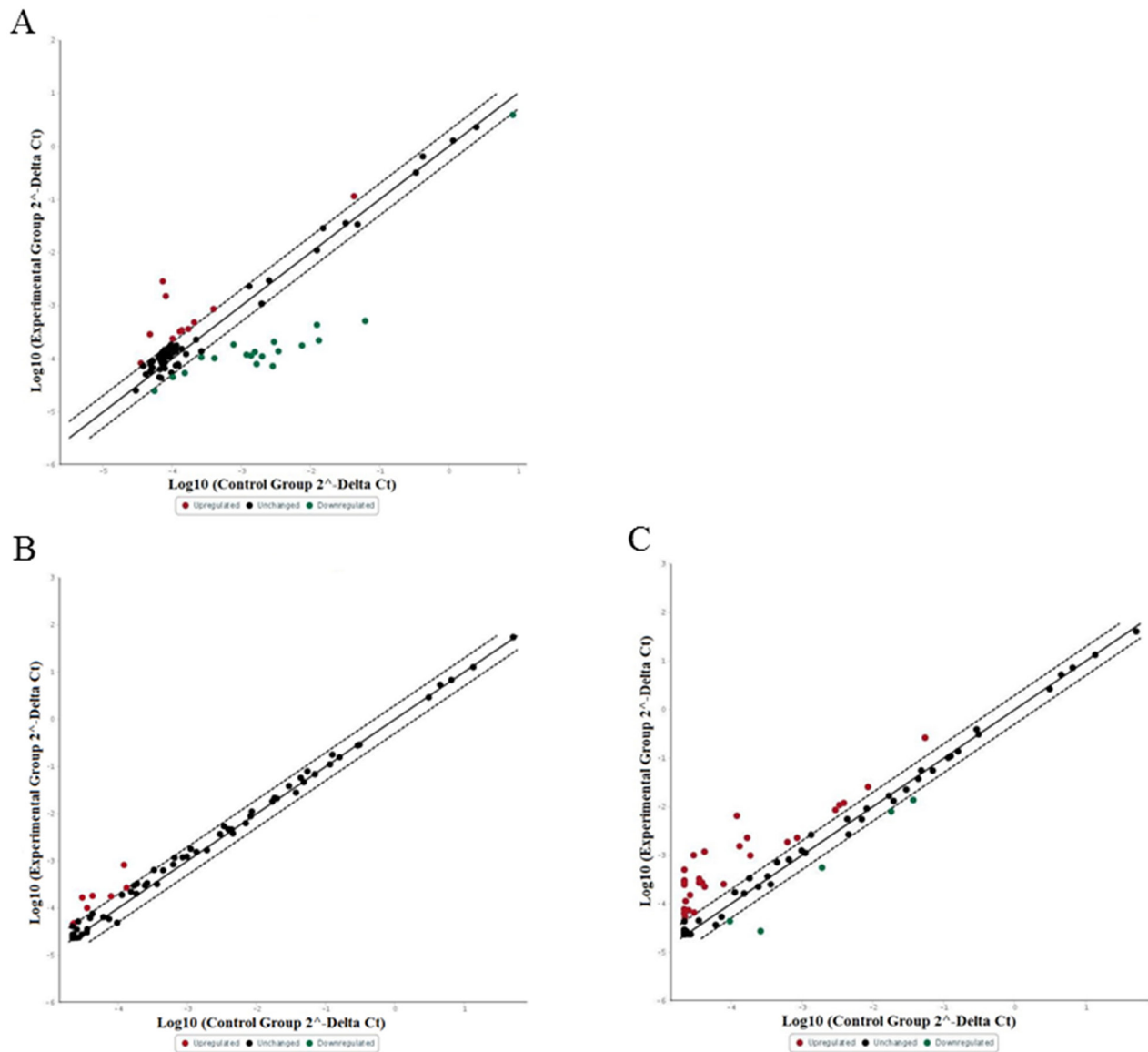


Figure 1: Relative expression comparison of 84 inflammatory cytokines and receptors genes between MCPyV-associated and non-associated Merkel cell carcinoma. The figures depict a log transformation plot of the relative expression level of each gene ($2^{-\Delta Ct}$) between (A) MCC13 cells vs. MKL-2 cells, (B) full-length LT vs. empty vector transfection in MCC13 cell line and (C) MKL-2 truncated LT vs. empty vector transfection in MCC13 cell line. The dotted lines indicate a two-fold change in gene expression threshold.

sT did not stimulate the activity of any of the CCL17 promoter fragments or increase sT protein levels when expressed in MCC13 cells (results not shown).

CCL17/TARC ectopic effect on MCPyV early and promoter activity

Cytokines such as IL-1 β , TGF β and TNF- α have been shown to stimulate the activity of human polyomavirus promoters [27-30]. Therefore, we investigated whether CCL17/TARC could exert an effect on the MCPyV early and late promoter activity. MCC13 cells were transfected with a luciferase reporter plasmid containing, either the early or late MCPyV promoter, and cells were either co-transfected with a CCL17/TARC expression plasmid or treated with recombinant human CCL17/TARC protein. Both an ectopic expression of CCL17/TARC and administering of recombinant CCL17/TARC to cells resulted in a significant upregulation of both the early and late MCPyV promoter activity (Figure 3).

Expression of CCL17/TARC and CCR4 in MCC cells

To confirm the stimulating effect of full-length and truncated LT on CCL17/TARC promoter activity, we first evaluated the mRNA expression of CCL17/TARC by qPCR in MCC13 cells transfected with expression vector for full-length LT or truncated LT variants. Full-length and MKL-1 LT significantly increased CCL17/TARC expression in the MCC13 cell line ($P < 0.01$), while MKL-2 and MS-1 LT variants, modestly, but significantly increased CCL17/TARC mRNA levels (Figure 4A). Western blot analysis with anti-CCL17/TARC antibodies confirmed that CCL17/TARC protein levels were increased in MCC13 cells expressing either full-length or truncated LT compared to MCC13 cells (Figure 4B and 4D). In our screening experiments, we also found a slight upregulation of CCR4 mRNA by exogenous expression of both pcDNA3-FLTA and pcDNA3-MKL-2 plasmids. So, to check at the protein level, we conducted a Western blot.

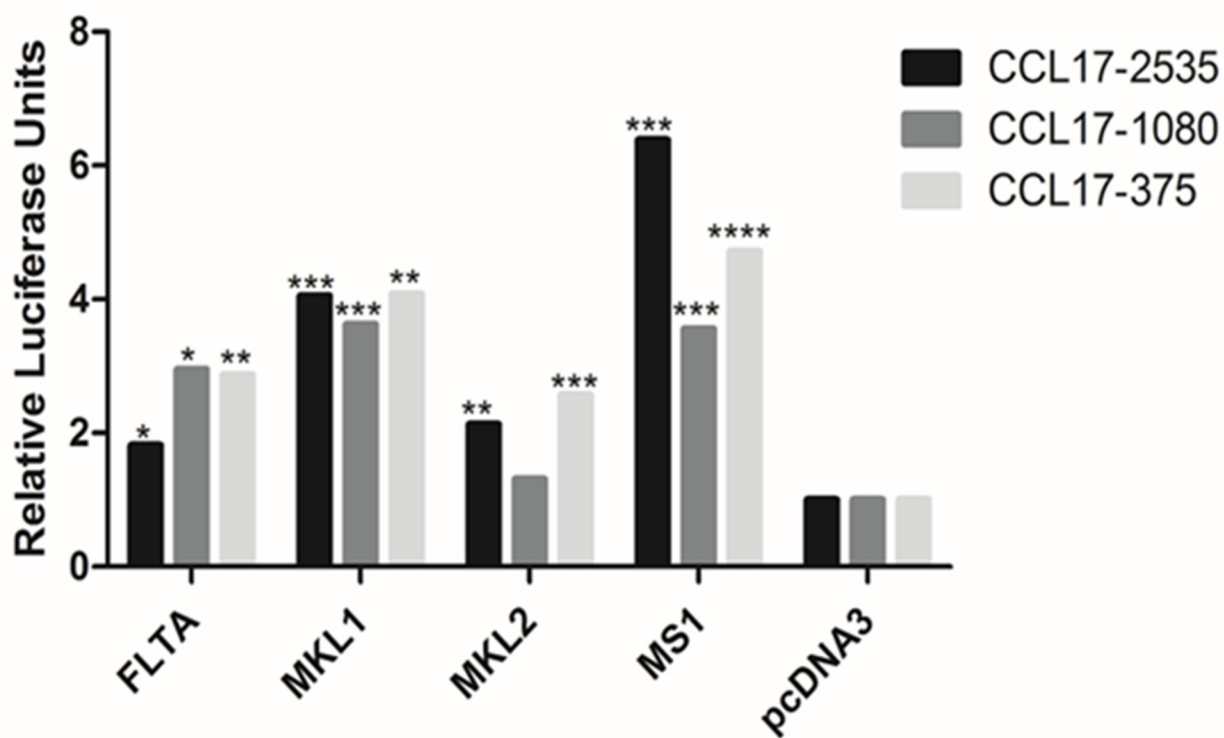


Figure 2: Effect of full-length and truncated (MKL-1, MKL-2 and MS-1) LT on CCL17/TARC promoter activity in MCC13 cells. Cells were transfected with a luciferase reporter vector driven by CCL17/TARC promoter fragments spanning nucleotides -2535/+ 40, -1084/+ 40 or -375/+40, respectively. Expression plasmid for full-length LT (FLTA), MKL-1, MKL-2 or MS-1 LT, or pcDNA3 control, were co-transfected. Luciferase activity was assessed after an overnight cultivation of transfected cells. The promoter activity in presence of pcDNA3 was arbitrary set as 1.0 and the promoter activity measured in the presence of LT was related to this. Each bar represents the average of three independent parallels \pm SD. The experiment was repeated two more times, and similar results were obtained. Luciferase values were normalized with a total protein in each sample. $P^* \leq 0.05$, $P^{**} \leq 0.01$, $P^{***} \leq 0.001$ and $P^{****} \leq 0.0001$.

We did not find expression at a significant level, but only a slight upregulation of CCR4 in MCC13 cells with an exogenous expression of MCPyV LT (Figure 4C and 4D).

CCL17/TARC activates the mitogen-activated protein kinase (MAP kinase) and NF- κ B pathways in MCC cells

Both CCL17/TARC and ERK1/2 have been shown to be involved in skin inflammation [31-33], thereby suggesting that CCL17/TARC may activate the MEK1/2-ERK1/2 mitogen-activated protein kinase pathway. To help investigate the effect of CCL17/TARC on MEK1/2-ERK1/2 activation in MCPyV-associated MCC, we stimulated MCC13 cells with recombinant human CCL17/TARC. The cells were stimulated with different concentrations (2.5 ng/ml to 15 ng/ml) and time periods (5 min to 60 min) and we monitored the phosphorylation of ERK1/2 using phospho-specific antibodies and western blotting. Activation of ERK1/2 by rhCCL17/TARC was shown to be concentration dependent (Figure 5). The ERK1/2 phosphorylation activity was inhibited by using a specific CCR4 antagonist (C021 dihydrochloride) (Figure 6).

Previous studies have indicated that NF- κ B is a target downstream of CCL17/TARC. We found that CCL17/TARC increased the phosphorylation of NF- κ B/p65 activity in a concentration-dependent manner (Figure 7A, 7B), which was inhibited by using a specific CCR4 antagonist (Figure 7D, 7E). Furthermore, NF- κ B promoter assay confirmed an increased luciferase activity of NF- κ B when cells were co-transfected with pCMV2-CCL17 and NF- κ B-luc reporter plasmids (Figure 7C).

CCL17/TARC stimulates cell proliferation of MCC13 cells

Next, we examined the effect of rhCCL17/TARC on MCC13 proliferation. A dose-dependent increase in cell proliferation was observed (Figure 7F). The CCR4 antagonist C021 inhibited CCL17/TARC-induced cell proliferation (Figure 7G).

CCL17/TARC and its receptor are expressed in MCC tissue samples

A total of 23 primary cutaneous MCCs were immunohistochemically stained for LT, CK20, CCL17/TARC and CCR4, respectively. Fifteen out of 23 (65.2%) demonstrated an intranuclear positivity consistent with a positivity for LT. All of the 15 LT-positive tumors demonstrated a uniform positivity for CK20 (dot-like cytoplasmic) and CCL17/TARC (dot-like cytoplasmic). With regard to CCR4, 12 out of 15 (80%) of the LT-positive tumors demonstrated cytoplasmic, membranous positivity for CCR4. Of the remaining eight (34.8%) LT-negative tumors, all of these demonstrated a uniform positivity for CK20, CCL17/TARC and CCR4, respectively (Figure 8).

DISCUSSION

There is increasing evidence that inflammatory mediators such as chemokines and chemokine receptors are involved in promoting tumor invasion, migration and vascularization [34]. Previous studies have demonstrated the involvement of chemokines, such as CXCL1, CXCL5,

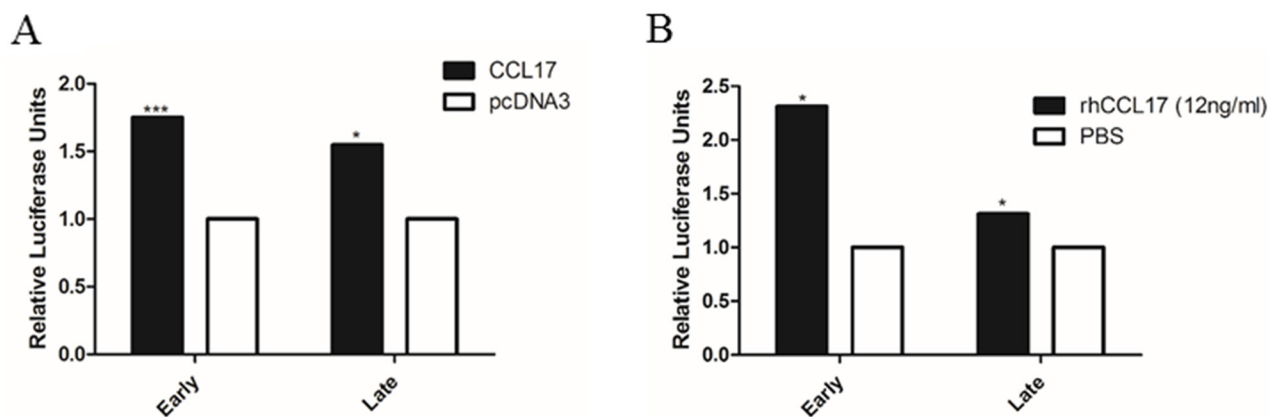


Figure 3: CCL17/TARC stimulates MCPyV early and late promoter activity. (A) MCC13 cells were seeded, and after 24 hrs the cells were serum starved. After serum starvation for 24 hrs, the cells were co-transfected with either CCL17/TARC expression plasmid or empty expression vector pcDNA3.1, and luciferase reporter plasmid containing either the MCPyV early or the late promoter. Luciferase activity was measured 24 hrs after transfection. The activity of the MCPyV early (respectively late) promoter in the presence of pcDNA3 was arbitrary set as 1.0 and the activity in the presence of CCL17 was related to this. (B) Cells were transfected as in (A), and were then exposed to rhCCL17/TARC (12ng/ml) or vehicle (PBS) for 4 hrs with a subsequent analysis of luciferase activity. Each bar represents the average of three independent parallels \pm SD* $P < 0.05$, *** $P < 0.001$.

CXXC4 and IL20RA in MCPyV-associated MCC [35]. In the current study, we performed the screening of different inflammatory cytokines and receptors, and compared their expression in MCPyV LT-positive and -negative MCC, respectively. Other groups have previously reported that LT and sT have an impact on cytokine expression. Ectopic sT expression in MCC13 cells resulted in decreased IL2, IL-8, CCL20 and CXCL9 expression [36]. Also, expression of full-length LT, truncated LT339, and truncated LT339 plus sT in hTERT-immortalized BJ human foreskin fibroblasts increased the expression of IL-1 β , IL-6, IL-8, CXCL1 and CXCL6 [37].

Here we demonstrate that one of the cytokine member whose expression was shown to be significantly enhanced in MCPyV LT-positive cells was CCL17/TARC. CCL17/TARC is a member of CC-motif chemokine family, and is constitutively expressed in thymus and by dendritic cells, endothelial cells, keratinocytes and fibroblasts [38]. An enhanced expression of CCL17/

TARC has been reported in several human malignancies, such as Hodgkin's and B cell lymphoma [39, 40]. Previous studies have revealed that CCL17/TARC possesses several important effects attributed to tumor growth, such as the proliferation [41-43], migration and recruitment of regulatory T-cells [44-46].

We detected a higher expression of CCL17/TARC in MCPyV-positive MCC compared to MCPyV-negative MCC cells at both the RNA and protein level. Furthermore, by co-transfecting MCC cells with MCPyV full LT, MKL-1 LT, MKL-2 LT and MS-1 LT expression plasmids, as well as CCL17/TARC promoter, resulted in an upregulation of CCL17/TARC promoter activity (Figure 2), which in turn increased the expression of CCL17/TARC (Figure 4). At present, we do not know why the different LT variants activate the CCL17 promoter at different levels. MCPyV full-length LT can interact with several transcription factors including Brd4, the E2F family members 2 and 3, MED14/CRSP2, PRTF1,

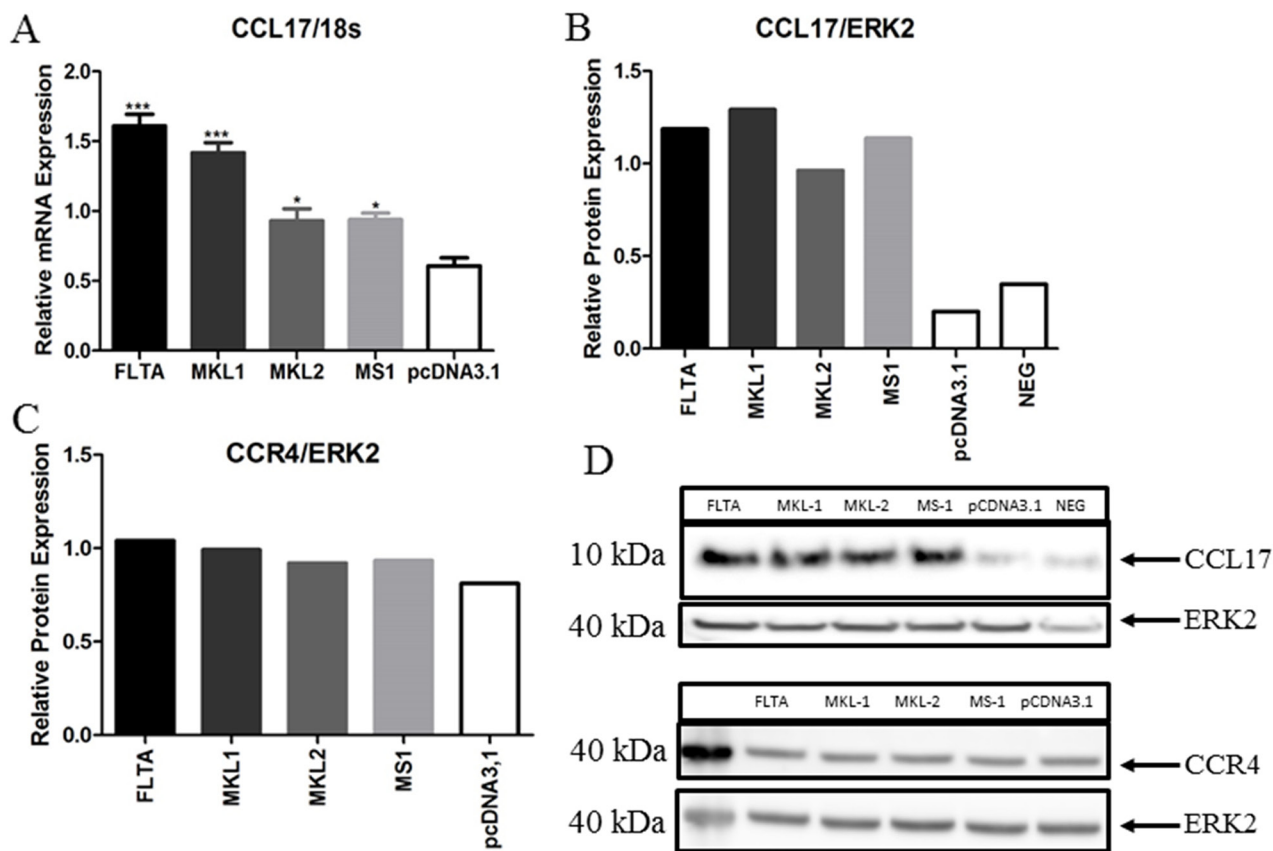


Figure 4: Transient expression of full-length or truncated MCPyV LT increases the transcript and protein levels of CCL17/TARC. MCC13 cells were transfected with an empty vector or expression plasmid for MCPyV full-length LT (FLTA), or truncated MKL-1, MKL-2, or MS-1 LT. (A) qRT-PCR analysis shows CCL17/TARC mRNA levels normalized with eukaryotic 18S rRNA levels. (B) CCL17/TARC protein levels analyzed by Western blotting. The uttermost right lane represents baseline expression of CCL17/TARC by MCC13 cells. (C) CCR4 protein levels analyzed by Western blotting. A Western blot with ERK2 antibodies was used as a loading control. (D) Representative figures of CCL17/TARC (B) and CCR4 (C) Western blots. The lane most to the left in the lower part contains the protein molecular mass marker (in kDa). Bars in (B) and (C) shows a densitometric scanning of the Western blot signals. $P^* \leq 0.05$ and $P^{***} \leq 0.001$.

SALL2 and DP1 (see supplementary data in Moens et al. [47]). However, no putative binding sites are present in the CCL17 promoter fragments used in our study. The truncated MCPyV LT variants MKL-1, MKL-2 and MS-1 retain the pRb binding motif and may thus relieve the pRb-mediated inhibition of E2F by using pRb. The CCL17/TARC promoter contains the putative GGCGGCA E2F binding site at position -1085/-1079 [48]. This means that our luciferase reporter plasmids containing

the CCL17/TARC promoter fragments -2535/+40 and -1084/+40 contain this putative E2F binding site. LT may thus activate the CCL17/TARC promoter fragments with an E2F motif by usurping pRb and releasing the repressive effect on the E2F site. However, the shorter promoter fragment (-375/+40) which lacks the putative E2F binding site is still trans-activated by LT, indicating that LT-mediated activation seems to be independently of the E2F site. The autocrine release of CCL17/TARC

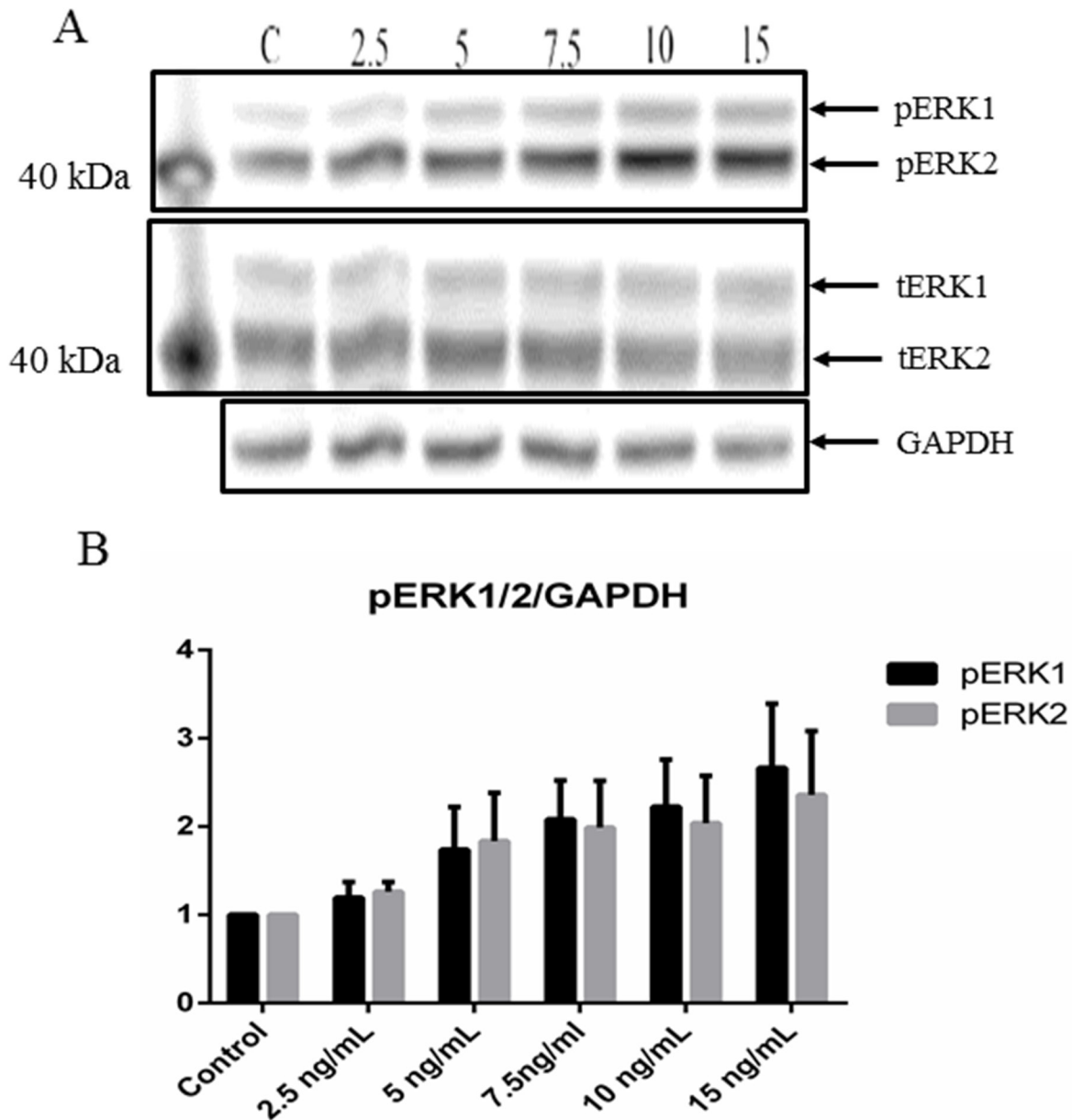


Figure 5: CCL17/TARC upregulates ERK1/2 activity. (A) PhosphoERK1/2 (pERK1/2) expression levels were determined by Western blot analysis using a phospho-specific antibody detecting Thr202/Tyr204 phosphorylation and compared to total ERK1/2 (tERK1/2) levels using an anti-pan-ERK1/2 antibody. MCC13 (1×10^5) cells were seeded in a 6-well plate. Cells were then serum-starved for 24 hrs, and thereafter stimulated with either PBS (control or C) or with 2.5, 5, 7.5, 10 and 15 ng/ml of rhCCL17/TARC for 45 min. (B) Densitometry scanning represent the expression of pERK1 (respectively pERK2) protein relative to GAPDH.

results in an increase of MCPyV, both in early and late promoter activity, as shown by the overexpression of CCL17 with pCMV2-CCL17-flag plasmid and stimulation with recombinant CCL17/TARC (Figure 3). These data above indicated that MCPyV LT increases CCL17/TARC expression, which results in the replication of MCPyV and the release of viral oncoproteins, which in turn promotes MCC development. In line with our data, the virus-mediated expression of CCL17/TARC has been reported in different types of cells. The Epstein-Barr infection of B cells induces the expression of CCL17/TARC [49], whereas the respiratory syncytial virus infection of Balb/c mice results in an increased CCL17/TARC production in the lung [50]. Hence, the virulent properties of viruses may depend on their ability to stimulate the expression of CCL17/TARC.

The role of CCR4 in tumor growth and survival has previously been reported. CCR4 is expressed in T-cell leukemia [51], non-lymphoid solid tumors, such as breast cancer, lung cancer, colorectal cancer, gastric and hepatocellular carcinoma [42, 43, 52-56], where it may contribute to the proliferation of tumor cells and chemotaxis of regulatory T cells [43, 46, 53, 57]. Interestingly, an elevated expression of CCR4 in different types of human cancers has been related to a poor prognosis [52, 58-61].

By immunohistochemistry, we detected CCL17/TARC and its receptor CCR4 in the tumor cells of all MCC tissue samples analyzed. The normal epidermis of the skin was also shown to express CCL17/TARC and CCR4, and has been reported earlier [62-65]. We did not observe any difference in the protein expression

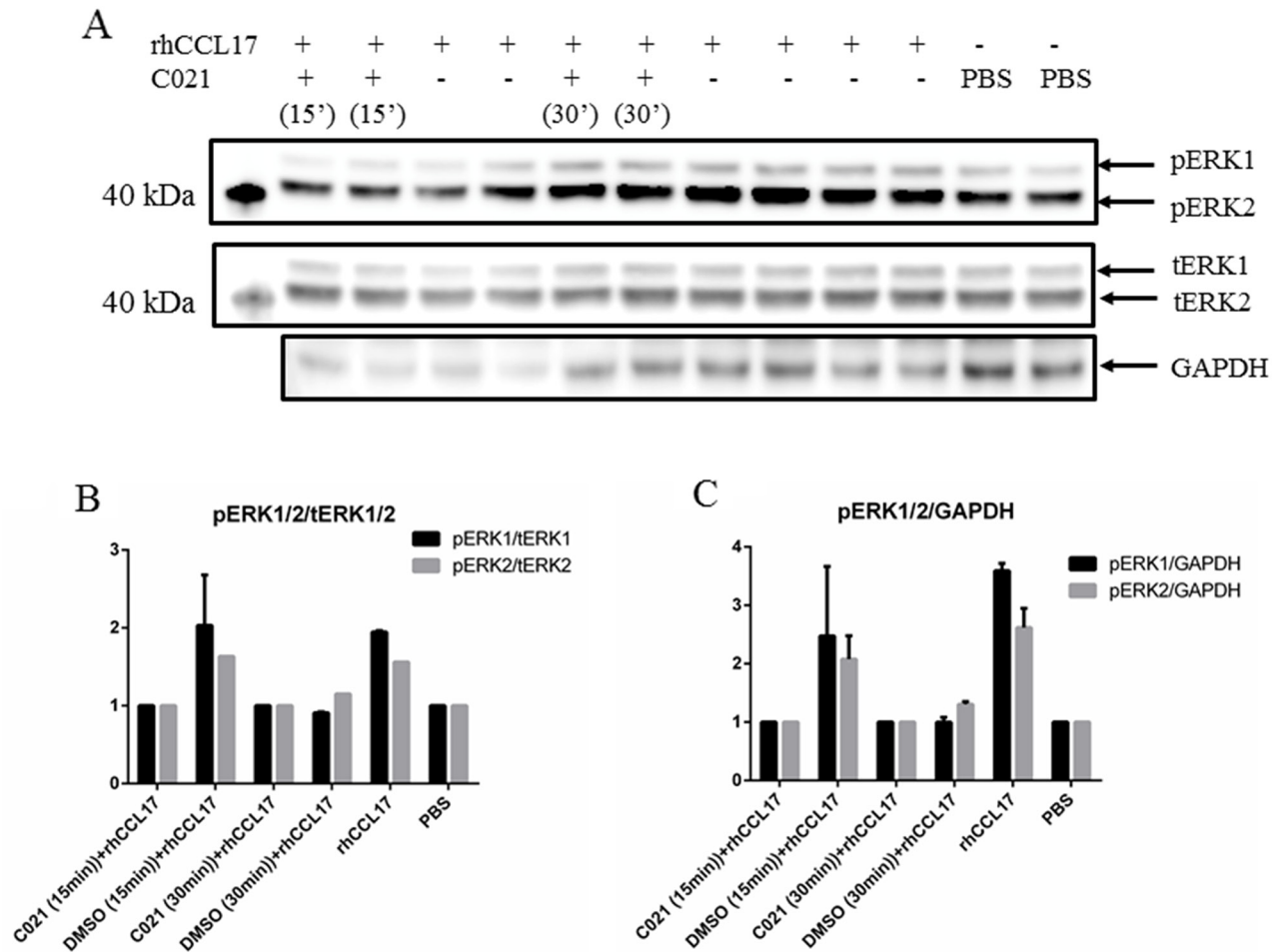


Figure 6: The CCR4 receptor antagonist C021 reduces CCL17/TARC-induced phosphorylation of ERK1/2. (A) MCC13 (1×10^5) cells were seeded in a 6-well plate and were serum starved for 24 hrs. The cells were either pre-exposed to PBS, C021 dihydrochloride (+), a specific CCR4 receptor antagonist, or left untreated (-) for 15 min (15') or 30 min (30') as indicated. The cells were subsequently incubated with rhCCL17/TARC (15ng/ml) or left untreated (-) for another 45 min. Cell lysates were prepared and phosphoERK1/2 and total ERK1/2 levels were monitored. GAPDH levels were used as loading control. Densitometry represents the expression of pERK1 (respectively pERK2) protein relative to (B) total ERK (tERK1/2) and (C) GAPDH.

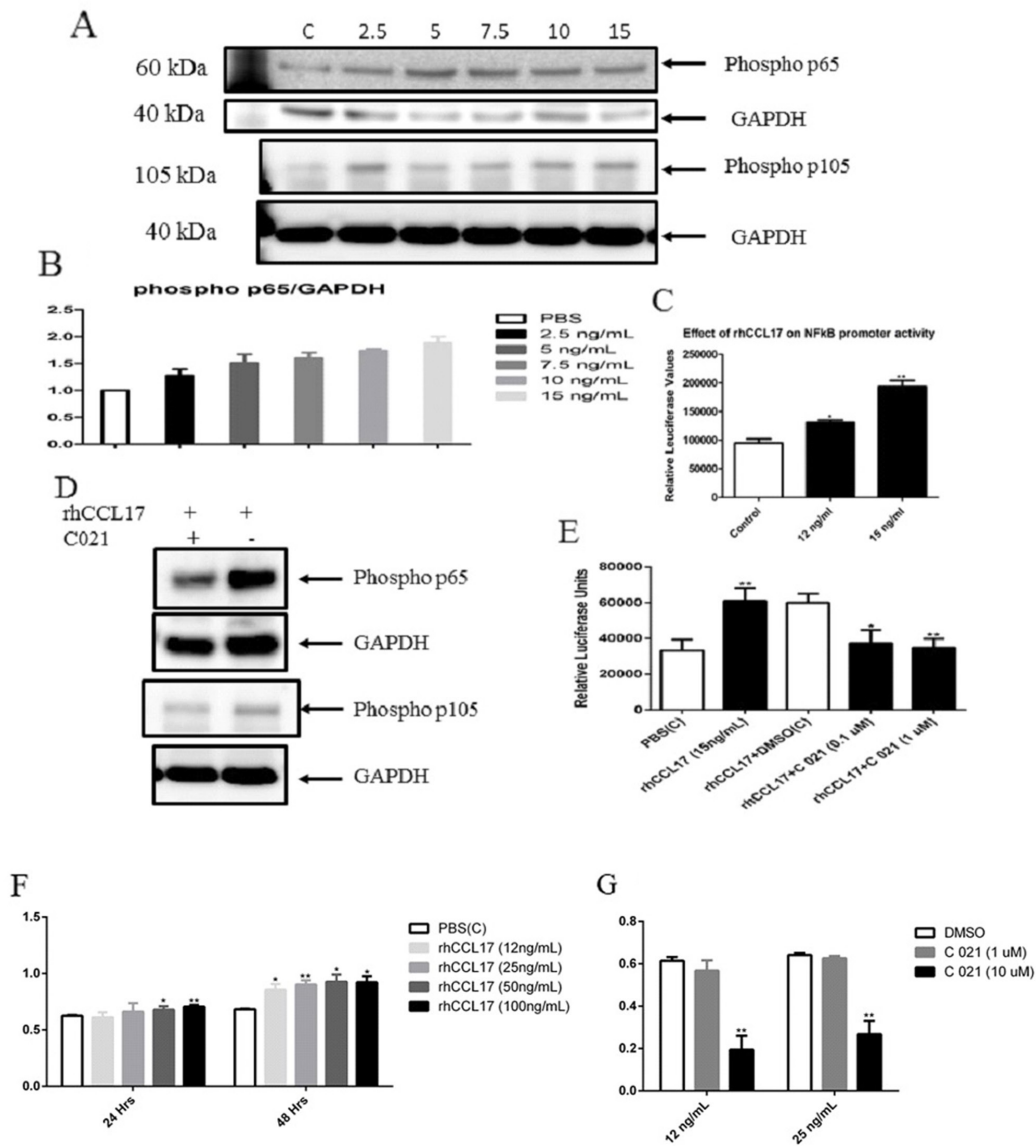


Figure 7: CCL17/TARC upregulated NFκB activity. (A) NFκB-p65 activation was determined by monitoring phosphorylation of p65 and p105. MCC13 cells (1×10^5) were seeded in a 6-well plate. Cells were serum starved for 24 hrs, and then stimulated with 2.5, 5, 7.5, 10 and 15 ng/ml of rhCCL17/TARC for 45 min. PBS was used as a control. Relative phospho p65 and phospho p105 were determined by western blot with phosphospecific antibodies. (B) Shows densitometry bars of western blot. (C) NFκB activity was measured by using a luciferase reporter plasmid containing a NFκB-responsive promoter. Cells were stimulated with 12ng/ml or 15ng/ml of rhCCL17/TARC for 4 hrs. Luciferase values were normalized with total protein. (D) The CCR4 receptor antagonist interferes with CCL17/TARC-induced activation of NFκB. Phosphop65 and phosphor p105 levels were determined by western blot. MCC13 (1×10^5) cells were seeded in a 6-well plate and were serum starved for 24 hrs. The cells were pre-incubated with C021 dihydrochloride ($0.3 \mu\text{M}$) for 30 min. The cells were then stimulated with rhCCL17/TARC (15ng/ml) in the presence or without CCR4 receptor antagonist for 45 min. DMSO was used as a control. (E) C021 ablates CCL17/TARC-induced activation of an NFκB-responsive promoter. Cells were transfected with the luciferase reporter plasmid with an NFκB responsive promoter and exposed to rhCCL17/TARC (15ng/ml) in the presence or without CCR4 receptor antagonist C012 (0.1 or $1 \mu\text{M}$) for 45 min. DMSO was used as a control. Each bar represent the average of three independent parallels. Luciferase values were corrected for protein concentration of the samples. $P^* \leq 0.05$ and $P^{**} \leq 0.01$. (F) CCL17/TARC stimulates proliferation of MCC13 cells. Cell were exposed to PBS or increasing concentrations of rhCCL17/TARC /12-100 ng/ml) and cell proliferation was measured after 24 and 48 hrs. Each bar represents the average of three independent parallels. (G) Cells were incubated for 45 min with 0.1 or $1 \mu\text{M}$ C021 and rhCCL17/TARC (12 or 25 ng/ml) was subsequently added. Proliferation was monitored 24 hrs later. $P^* \leq 0.05$ and $P^{**} \leq 0.01$.

of CCR4 and CCL17/TARC between MCPyV-negative and MCPyV-positive primary cutaneous MCCs. Since immunohistochemistry is more qualitative rather than a quantitative analysis therefore, increase in CCL17/TARC levels between MCPyV-positive and -negative samples may not be visible by IHC.

Given the fact that MCC produces CCL17/TARC and expresses CCR4, we decided to investigate the effect of exogenously added CCL17/TARC on intracellular signaling pathways. Recombinant hCCL17/TARC induced the proliferation in MCPyV-negative MCC cells, which was abolished in the presence of the CCR4 receptor antagonist. The addition of CCL17/TARC resulted in ERK1/2 phosphorylation in MCC13 cells (Figure 6). Previous studies have shown that CCL17/TARC induced chemotaxis of the mouse T-cell lymphoma cell line EL4 in a MEK1/2-ERK1/2-dependent manner [66].

We also demonstrate that CCL17/TARC activates the NF- κ B pathway in MCC13 cells. Previous studies have shown that CCL17/TARC is a NF- κ B target gene [67, 68], but it has not been shown that CCL17/TARC can itself activate NF- κ B. CCR4-mediated MMP13 activity in colorectal cancer cells requires NF- κ B [52]. Chemokine-like factor (CKLF1), which also uses the CCR4 receptor, can activate the NF- κ B pathway [69], and NF- κ B signaling is significantly downregulated in CCR4^{-/-} macrophages [70].

In the tumor microenvironment, a high expression of CCL17/TARC and CCR4 by MCC cells may contribute to the activation of inflammatory pathways and the

promotion of tumor growth and immune suppression. We found that CCL17/TARC stimulated proliferation of MCC13 cells, whereas several studies have reported that CCL17/TARC or CCL22-associated CD4⁺CD25⁺Foxp3⁺ increases the population in tumor-infiltrating lymphocytes (TILs), with peripheral blood lymphocytes (PBLs) being one of the reasons for impaired anti-tumor immunity in both gastric and esophageal squamous cell carcinoma [44, 45]. It is postulated that the preferential attraction of CCR4-bearing Th2 lymphocytes may cause a shift towards a Th2-dominated cytokine microenvironment, thereby hampering the cytotoxic immune response and providing a mechanism by which neoplastic cells are able to escape from the immune system [71]. Targeting CCR4 is an emerging strategy for immunotherapy for cancer [57, 72]. Gain-of-function mutations in adult T cell lymphoma have been reported [73] and recently, Mogalizumab, a monoclonal antibody targeting CCR4 receptor has shown promising results in the treatment of relapsed adult T cell lymphoma patients [74-78].

Taken together, we demonstrate that MCPyV LT is linked with an altered expression of CCL17/CCR4. The expression of CCL17/TARC and CCR4 may constitute an autocrine or paracrine survival loop which contributes to the growth and survival of the tumor, and also mediates immune suppression through the recruitment of regulatory T cells. Thus, strategies based on the selective targeting of the CCL17/CCR4 axis, either by monoclonal antibodies or specific receptor antagonists, could be a therapeutic interventions for patients with MCC.

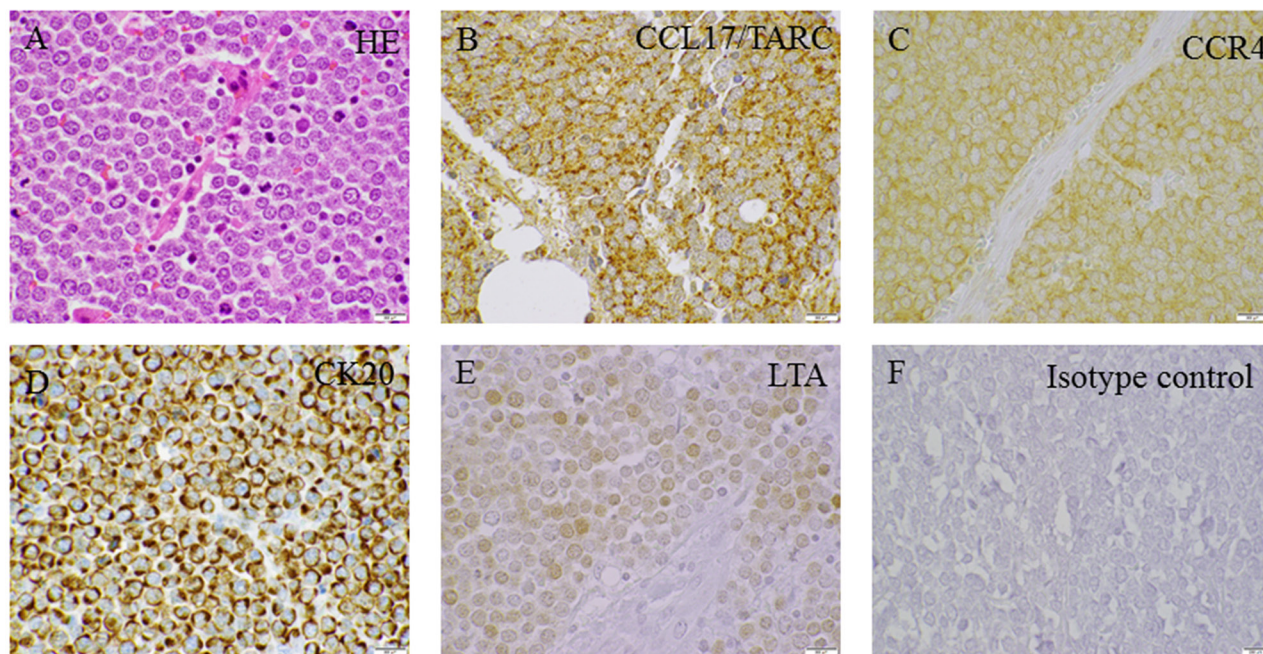


Figure 8: Immunoperoxidase staining of MCPyV-associated MCC primary tumors. (A) HE, (B) CCL17/TARC, (C) CCR4, (D) CK20, (E) LTA and (F) Isotype control. The displayed images are representative stainings from a panel of MCC primary tumors (Scale bar= 500 μ m).

Table 1: Primer sequences for full-length LT and generating truncated Large Tag transcripts

MCPyV full-length LT sequence primers	
Full_LT.F	5'-TACAAGCACTCCACCAAAGC-3'
Full_LT.R	5'-TCCAATTACAGCTGGCCTCT-3'
Site-directed mutagenesis primers to make MKL-1 LT	
MKL-1_LTstop.F	5'-GCCATGCTGTGTACAAGTTTTAAACAGTCTCCTGTTTTGC-3'
MKL-1_LTstop.R	5'-GCAAAACAGGAGACTGTTTAAACTTGTACACAGCATGGC-3'
Site-directed mutagenesis primers to make MKL-2 LT	
MKL-2_LTstop.F	5'-GAAGACCCCTCCTCCATAGTCAAGAAAGCG-3'
MKL-2_LTstop.R	5'-CGCTTCTTGACTATGGAGGAGGGGTCTTC-3'
Site-directed mutagenesis primers to make MS-1 LT	
MS-1_LTstop.F	5'-GCCACTGCTAAATTAGGAATTTCAAGAAAAAG-3'
MS-1_LTstop.R	5'-CTTTTTCTTGAAATTCCTAATTTAGCAGTGGC-3'

MATERIALS AND METHODS

Materials

In our study, the following primary antibodies were used: i.e. monoclonal mouse MCPyV LTA (Sc-136172, Santa Cruz Biotechnologies), polyclonal rabbit CCL17/TARC (Ab-182793, Abcam), polyclonal rabbit anti-CCR4 (cat.#PA1516, Boster, USA), polyclonal rabbit ERK2 (Sc-136172, Santa Cruz Biotechnologies, Dallas, TX, USA), monoclonal rabbit keratin 20 (cat.# 13063, Cell Signaling, Danvers, MA, USA), monoclonal rabbit Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (cat.#4370 Cell Signaling), monoclonal rabbit p44/42 MAPK (Erk1/2) (cat.#4695S, Cell Signaling), Phospho-NF- κ B p105 (Ser933) (18E6) (cat.#4806S, Cell Signaling) rabbit anti-GAPDH (cat.# G9545, Sigma Aldrich, St. Louis, MO, USA) and CCR4 chemokine receptor antagonist (C 021 dihydrochloride) (cat. #3581, Tocris Bioscience Minneapolis, MN, USA)).

Plasmids

The empty expression plasmid pcDNA3.1(+) was purchased from Invitrogen. pcDNA3-full large T-antigen (pcDNA6.MCV.cLT206.V5_CM2B4) was purchased from Addgene (Cambridge, MA, USA), while pcDNA3-MKL-1 large T-antigen, pcDNA3-MKL-2 large T-antigen and pcDNA3-MS-1 large T-antigen were constructed by site-directed mutagenesis using the original pcDNA6.MCV.cLT206.V5_CM2B4 plasmid. pCMV2-CCL17-flag (cat.# HG10233-M-F) expression plasmid was purchased from SinoBiological (Beijing, China). The plasmids pCCL17-2532+40-LUC, pCCL17-1080/+40-LUC, and pCCL17-375/+40-LUC containing CCL17 promoter fragments in

the luciferase reporter plasmid pGL3-basic (Promega) were a kind gift from Dr. Daniel Hebenstreit [79].

Cell lines and human tissue samples

MCC13 and MKL-2 cell lines were kindly provided by Dr. Baki Akgül (University of Cologne, Germany). MCC13 is a MCPyV-negative MCC cell line, whereas MKL-2 is a MCPyV-positive MCC cell line [58, 59]. MCC13 cells were grown in RPMI-1640 with 10% FBS in the presence of 100 μ g/ml streptomycin and 100 units/ml of penicillin, while MKL-2 cells were grown in RPMI-1640 with 20% FBS in the presence of 100 μ g/ml streptomycin and 100 units/ml penicillin. Cells were kept in a humidified CO₂ incubator at 37°C. Human MCC tissue were obtained during 2000-2015 from the St. Olavs University Hospital Trondheim, Norway according to the ethical approval from the Regional Ethical Committee (REK NORD application number 2016/988).

PCR-based site-directed mutagenesis of MCPyV truncated LT encoding plasmid

To generate expression plasmids encoding truncated variants of MCPyV LT expressed in the virus-positive MKL-1, MKL-2 and MS-1 MCC cell lines, an oligonucleotide-directed mutagenesis was performed using the QuickChange site-directed mutagenesis kit from Stratagene (cat. no. 200518; Stratagene La Jolla, CA, USA). Plasmid pcDNA6.MCV.cLT206.V5 was used as a template to generate the plasmids pcDNA3-MKL-1 LT, pcDNA3-MKL-2 LT, pcDNA3-MS-1 LT. Table 1 shows different primers to generate truncated MKL-1, MKL-2 and MS-1 sequences from full-length LT.

Transfection

Cells were seeded out in 6- and 12-well cell culture plates with a total number of 1.5×10^5 and 2×10^5 , respectively. At the time of transfections, the cells were approximately 60-70% confluent. jetPRIME (Polyplus-transfection SA, Illkirch, France) was used to transfect all plasmids according to the manufacturer's instructions. A total of 2- μ g per well in a 6-well plate and 800 ng per well in a 12-well plate DNA was used to transfect cells. All experiments were performed 24 hrs after transfection.

RNA extraction

RNA extraction was done by using RNeasy® Plus Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. For RNA yield and quality, A_{260} , A_{280} and $A_{260/A230}$ ratios were analyzed with Nano-Drop® ND-2000 spectrophotometer (NanoDrop Technologies, ThermoFisher Scientific, Waltham, MA, USA).

cDNA construction and quality control

iScript™ cDNA Synthesis Kit (BioRad, Hercules, CA, USA) was used to make cDNA. A total of 1-2 μ g RNA was used to generate cDNA according to the manufacturer's instructions. PCR with the housekeeping APRT primers (5'-CCCGAGGCTTCCTCTTTGGC-3' and 5'-CTCCCTGCCCTTAAGCGAGG-3') were used to check for genomic DNA contamination in the cDNA prep. An 800 bp fragment was obtained with genomic DNA as a template, while a 300 bp amplicon was obtained with cDNA (76). PCR products were visualized on a 1% agarose gel stained with Gelred™ Nucleic Acid Gel Stain gel red stain (Biotium, Cambridge Bioscience, Bar Hill, UK).

RT² Profiler PCR array

A human cytokines and receptor genes transcription was measured using the human RT² Profiler PCR Inflammatory Cytokines and Receptors Array (PAHS-011ZA, SABiosciences, Qiagen). Twenty μ l cDNA were diluted to 111 μ l by adding 91 μ l of RNase-free water. One hundred and two μ l were added in a 1350 μ l 2x RT² SYBR Green Mastermix according to the manufacturer's protocol. Twenty-five μ l PCR components mix was added to each well of a 96-well plate. A two-step real-time PCR was initiated at 95°C (10 min) for one cycle, and followed by alteration of 95°C (15 sec) and 60°C (1 min) for 45 cycles by using Light Cycler 96 (Roche Diagnostics, Indianapolis, IN, USA). All data was collected from the PCR machine by Light Cycler 96 SW 1.1 software (Provided by manufacturer), and analyzed by SA Bioscience's Gene Glob PCR Array Data Analysis Web Portal. For considering a gene differentially expressed, we used a differential cut-off of 2-fold (up- or downregulated).

Luciferase assays

For luciferase assays, approximately 24 hrs after transfection, cells were lysed in a 100 μ l Luciferase Assay Tropix Lysis solution (ThermoFisher Scientific), with 0.5 mM DTT. Cells were scraped, transferred to Eppendorf tubes and then centrifuged for 3 minutes at 12,000 g. Twenty μ l of the supernatant was used in a 96-well microtiter plate, and a 50 μ l luciferase buffer (Promega, Madison, WI, USA) was added. A Luminometer (Labsystem, Luminoscan RT) used to measure lights units. Each experiment was repeated three times with three independent parallels for each experiment, and luciferase values were corrected for protein content in each sample. The total protein concentration was measured using the MN protein quantification assay (Macherey-Nagel GmbH, Düren, Germany).

Quantitative real-time PCR

The gene expression level of CCL17/TARC was measured by real-time quantitative RT-PCR using an ABI PRISM® 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The expression level was measured by using a FAM-labeled TaqMan gene expression assay CCL17/TARC probe/primer (Cat. # Hs00171074_m1), and the expression level was normalized by using a VIC/MGB probe/primer Eukaryotic 18S rRNA Endogenous Control (Cat. #4319413E, Applied Biosystems). PCR reactions were prepared in a total volume of 25 μ l, with a final concentration of 1X TaqMan® Universal Master Mix and cDNA from 1 μ g total RNA.

Immunoblotting

Western blot was done by running samples in 4-12% of NuPAGE Bis-Tris Mini Gels (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol and blotted onto a 0.45 μ m PVDF membrane (Millipore, Billerica, MA, USA). Membrane blocking was performed by using TBS-T (TBS with 0.1% Tween-20; Sigma Aldrich) containing 5% (w/v) dried skimmed milk for 1 hour. The protein was probed by using an appropriate primary antibody overnight at 4°C. After washing the membrane 3 times with TBS-T, an appropriate secondary antibody was added for 1 hour at room temperature. After 2 washes with TBS-T and 2 washings with washing buffer, antigen-antibody complex was visualized by using SuperSignal™ West Pico Chemiluminescent Substrate (Cat.#34080 Thermo Fisher Scientific, Rockford, IL, USA). Magic-Mark™ Western standard from Invitrogen Life Technologies was used to estimate the molecular mass of the detected proteins.

Immunohistochemistry

Formalin-fixed and paraffin-embedded tissue sections were deparaffinized in xylene and graded alcohols, hydrated and washed in PBS. After antigen retrieval in

a sodium citrate buffer (pH 6) in a microwave oven, the endogenous peroxidase was blocked by 0.3% H₂O₂ for 15 min. Sections were incubated overnight at 4°C with the primary antibody CCR4 (Abcam Cat.#ab1699, Cambridge, UK), CCL17/TARC (Abcam Cat.#ab182793), MCPyV-LT (Santa Cruz Biotechnology Cat.#sc-136172) and CK20 (Roche Cat.#790-4431). As a secondary antibody, the anti-rabbit-HRP SuperPicTure Polymer detection kit (87-9663, Zymed-Invitrogen, San Francisco, CA, USA) or anti-mouse EnVision-HRP (Dako, Agilent Technologies, Inc., Santa Clara, CA, USA) was used. A matched isotype control was used as a control for nonspecific background staining.

MTT assay

To measure cell proliferation, the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)-assay was used [80].

Statistical analysis

The RT2 Profiler PCR Array data analysis version 3.5 (<http://dataanalysis.sabiosciences.com/pcr/arrayanalysis.php>) was used for inflammatory cytokines and receptor data analysis. For the analysis, the significant values were considered with a fold change/fold regulation ≥ 2 and a p-value less than 0.05. GraphPad software was used for the statistical analysis and graphs. The sample *t*-test was used to compare differences between the experimental and control group and a p-value set < 0.05 . The densitometry analysis of Western blot was done by using imageJ.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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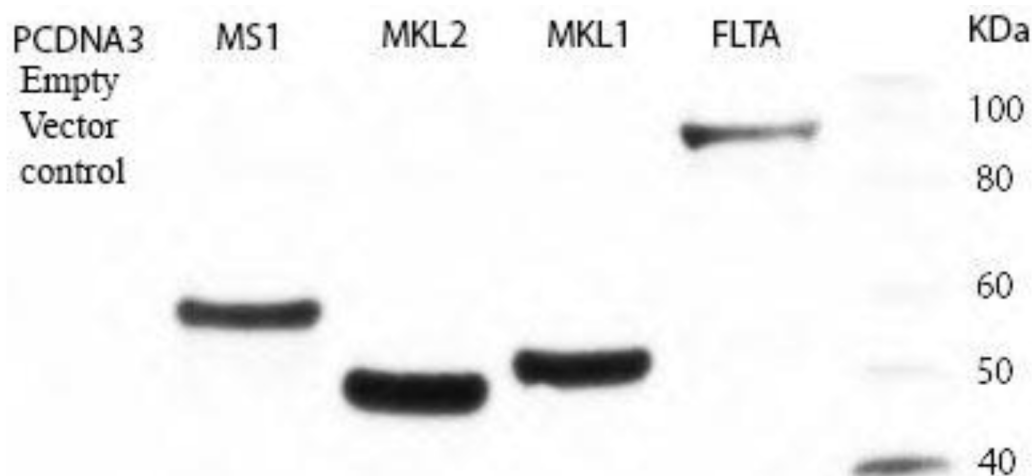
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CCL17/TARC and CCR4 expression in Merkel cell carcinoma

SUPPLEMENTARY MATERIALS

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>gi|531990549|dbj|BAN78688.1| large T antigen [Merkel cell polyomavirus]
MDLVLNRKEREALCKLLEIAPNICYGNIPLMKAAFKRSCLKHHPDKGNPVMIMELNTLWSKF
QQNIHKLRSDFSMFDEVDEAPIYGTTFKKEWWRSGGF'SFGKAYEYGNPHGTNSRSRKPSN
ASRGAPSGSSPPHSQSSSSGYGSFSASQTSDSQSRGPDIPPEHHHEEPTSSSGSSSREETTNS
GRESSTPNGTSVPRNSSRTDGTWEDLFCDESLSSPEPPSSSEPEEPPSSRSRSPRQPPSSA
EEASSSQFTDEECRSSSFSTTPKTPPPEFSRKRKFGGSRSSASSASSASFTSTPPKPKKNRETP
VPTDFPIDLSDYLSHAVYSNKTVSCFAIYTTSDKAIELYDKIEKFKVDFKSRHACELGCILL
FITLSKHRVSAIKNFCSTFCTISFLICKGVNKMPEMYNNLCKPPYKLLQENKPLLNYEFQEK
EKEASCNWNLVAEFACEYELDDHFII LAHYLDFAKPFCQKCENRSRLKPKHAHEAHSNAK
LFYESKSQKTICQQAADTVLAKRRLEMLEMTRTEMLCKKFKKHLERLRDLDTIDLLYYMGV
AWYCCLFEEFEKQLQKI IQLLTENIPKHRNIWFKGP INSGKTSFAAALIDLLEGKALNINCP
SDKLPFELGCALDKFMVVFEDVKGQNSLNKDLQPGQGINNLDNLRDHLDGAVAVSLEKKHVN
KKHQIFPPCIVTANDYFIPKTLIARFSYTLHFSPKANLRDSDLQDQMEIRKRRILQSGTTLLL
CLIWCLPDTTFKPCLOEEIKNWKQILQSEISYGFQMIENVEAGQDPLLNLVVEEGPEET
EETQDSGTF SQ
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Supplementary Figure 1: Amino acids sequence of full-length MCPyV LT. The amino acids marked in red represent the last amino acid of the truncated LT in MS-1 (N), MKL-1 (Y) and MKL-2 (F). Green amino acid are residues that differ in MKL-2 LT compared to MKL-1 and MS-1 LT.



Supplementary Figure 2: Western blot detection of MCPyV full-length and truncated (MKL-1, MKL-2 and MS-1) LT in MCC13 cells. Cells were transfected with an empty control vector (pcDNA3) or expression plasmid for full-length (FLTA) and truncated (MKL-1, MKL-2 and MS-1) LT. Lysate from full-length LT showed a band at approximately 100kDa, and lysates from cells transfected with an expression plasmid for truncated MKL-1, MKL-2 and MS-1 LT showed a band at approximately 50kDa, 45kDa and 60kDa, respectively. The molecular mass marker is shown in the utmost right lane.

PAPER III

The Merkel cell polyomavirus T-antigens and IL33/ST2-IL1RAcP axis: Role in Merkel cell carcinoma

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Abstract

Merkel Cell polyomavirus (MCPyV) is a causal factor in Merkel cell carcinoma (MCC). The oncogenic potential is mediated through its viral oncoproteins large T antigen (LT) and small T antigen (sT). Using the human RT² Profiler PCR Inflammatory Cytokines and Receptors Array, we performed a PCR array analysis of 84 different human cytokine and receptor genes in virus positive (VP) and virus negative (VN) MCC cell lines. We observed an increased expression of IL-33 in VP MKL-1, MKL-2, MS-1 and WaGa cell lines compared with VN-MCC-13 cells. Transfection studies demonstrated that MCPyV LT and sT stimulate IL-33, ST2/IL1RL1 and IL1RAcP promoter activity in MCC-13 cells. Furthermore, promoter luciferase assays were used to study the effect of IL-33, ST2/IL1RL1 and IL1RAcP on MCPyV early and late promoter activity. We determined that the cytokine domain of IL-33 and the membrane-bound and soluble forms of ST2/IL1RL1 - stimulated early and late promoter activity while full-length IL-33 and IL1RAcP (membrane and soluble form) inhibited early and late promoter activity in MCC-13 cells. An addition, induction of IL-33 expression was confirmed by transfecting MCC-13 cells with MCPyV LT. Furthermore, recombinant human cytokine domain IL-33 activated both MAP kinase and NF-κB signaling pathways and blockade of the ST2 receptor abolished IL-33-induced MAP kinase and NF-κB activation. Moreover, significantly higher IL-33 and IL1RAcP protein levels were observed in MCC patient plasma compared to plasma from healthy controls. Immunohistochemical analysis demonstrated a significantly stronger IL-33, ST2 and IL1RAcP expression in MCC tissues compared to normal skin.

Our study revealed a T-antigens-dependent IL-33 function in MCC. Therefore, neutralizing the IL-33/ST2 axis may present a novel therapeutic approach for MCC patients.

Keywords: Merkel cell carcinoma; inflammation; cytokines; IL-33; ST2/IL1RL1; IL1RAcP

Introduction

Merkel cell carcinoma (MCC) is a rare, highly aggressive neuroendocrine skin cancer [1]. MCC is a relatively recently described entity, although the Merkel cells were identified more than 100 years ago by Friedrich S. Merkel (1845-1919) in 1875 [2]. MCC was originally described as “trabecular carcinoma of the skin” in 1972 by Cyril Toker [3]. Six years later, in 1978, Tang and Toker found dense granules in tumors using electron microscopy (EM) with similarities to Merkel cells [4], hence renaming this tumor as Merkel cell carcinoma [5]. Despite common phenotypic features, MCC is not believed to originate directly from Merkel cells in the skin. Dermal, epidermal, and pre/pro- B cell markers, such as CK14, SOX2, CD56, PAX5, and TdT are commonly expressed in MCC [6, 7]. Consequently, various other candidates, including epithelial, fibroblastic and neuronal precursors or pre/pro-B-cells have been suggested as the cell(s) of origin [6-9].

MCPyV is a non-enveloped, double-stranded DNA virus with a circular genome of ~5kb [10]. The viral genome is divided into three major regions: the non-coding control region (NCCR), the early region and the late region. The NCCR contains the origin of replication and transcriptional regulatory elements. The early region encodes large T antigen (LT), small T antigen (sT), 57 kT and a protein called alternative LT open reading frame (ALTO) [11-14]. While the oncogenic potential of LT and sT are well documented, the roles of 57 kT and ALTO in the viral life cycle and MCC tumorigenesis remain elusive [15]. The late region encodes for the viral capsid proteins VP1 and VP2 [16]. The MCPyV genome is maintained as episomal circular dsDNA during the normal life cycle but is integrated in virus positive (VP) MCCs [17]. Characteristic for VP-MCC is the expression of a C-terminal truncated LT (tLT) resulting from mutations or reading frame shift which introduce premature start codons [18].

Cytokines are central mediators in an inflammatory tumor microenvironment. Interleukin-33 (IL-33) is considered as an “alarmin” (endogenous danger signal) released following cellular damage [19] and belongs to the IL-1 family of cytokines that are expressed in multiple organs and cell types in both humans and mice. It is the ligand for the “suppression of tumorigenicity 2 L receptor” (ST2) and “IL-1 receptor accessory protein” (IL1RAcP) [20].

IL-33 induces cytokine and chemokine expression in various immune cells, including mast cells, basophils, eosinophils, Th2 lymphocytes, invariant natural killer T and natural killer cells [21]. In cancer, IL-33 function have been linked to tumor growth, metastasis, neo-angiogenesis and evasion of programmed cell death [19]. Furthermore, IL-33 affects the tumor microenvironment (TME) through immune cells, such as myeloid-derived suppressor cells (MDSCs), dendritic cells (DCs), and regulatory T cells (Tregs) [19].

Despite important advances in understanding the biological role of IL-33, very little is known about mechanisms regulating its activity. Full-length IL-33 is a 270aa protein localized in the nucleus of blood vessel endothelial cells and epithelial barrier tissues [22, 23]. It binds chromatin [23] and histones through a short chromatin binding motif located in the N-terminal part (40-58 amino acids) [24]. Upon cellular damage and necrotic cell death, IL-33 is released in the extracellular space [25, 26], thus acting as an alarmin, alerting the immune system to tissue injury following infection or trauma [25-27]. Proteases secreted by different inflammatory cells regulate IL-33 activity [26]. Full-length IL-33 is biologically active and these proteases process IL-33 into its mature form containing an intact IL-1-like cytokine

domain with increased biological activity [28]. In the current study, we examined the effect of MCPyV T-antigens (T-ags) on IL-33 expression and its pro- or anti-tumorigenic role in MCC.

Material and Methods

Materials

Primary antibodies used in this study are displayed in Supplementary Table 1. The recombinant proteins include recombinant human full-length IL-33 (FL-rhIL-33) (cat.#TP760633, Origen) and recombinant human cytokine-domain IL-33 (CyD-rhIL-33) (cat.# 3625-IL, R&D systems).

Plasmids

The empty expression plasmid pcDNA3.1(+) was purchased from Invitrogen. pcDNA3-full length LT (pcDNA6.MCV.cLT206.V5_CM2B4) was purchased from Addgene (Cambridge, MA, USA), while pcDNA3-MKL-1 LT, pcDNA3-MKL-2 LT and pcDNA3-MS-1 LT were constructed by site-directed mutagenesis using the original pcDNA6.MCV.cLT206.V5_CM2B4 plasmid and have been previously described [29]. pNF- κ BLUC plasmid was obtained from Clontech (Takara Bio, Mountain View, CA, USA) and MCPyV sT-FLAG was a kind gift from Andrew Macdonald [30]. IL-33, ST2 (IL1RL1) and IL1RAcP expression plasmids were generated by cloning RT-PCR amplified cDNA into pCMV3_His(N)_FLAG(C). Supplementary Table 2 displays the primers used to construct expression plasmids. The plasmids pGL3_IL33(-1050/+50), ST2/IL1RL1 distal promoter pGL3_ST2L(-100/+82), ST2/IL1RL1 proximal promoter pGL3_sST2(-499/+100), pGL3_IL1RAP(-1397/+182) and pGL3_IL1RAP(-517/+182) were amplified by PCR of promoter sequence from genomic DNA purified from blood and cloned into the luciferase reporter plasmid pGL3-basic (Promega). Luciferase reporter plasmids containing truncated versions of the IL-33 promoter were generated by site directed mutagenesis using complementary primers (see Supplementary table 3) containing a *KpnI* restriction enzyme motif, and additional *KpnI* site was introduced in the pGL_IL33(-1050/+50) vector. The plasmid was then digested with *KpnI* and re-ligated. Site-directed mutagenesis was also applied to destroy each of the two putative LT motifs in the IL-33 promoter. A double IL-33 promoter mutant in which both LT motifs were destroyed was also generated. The different primers used are shown in supplementary Table 3. All constructs were sequenced to verify the presence of the mutations.

Cell lines and human tissue samples

MCC-13, MCC-26, UISO, MKL-1, MKL-2 and MS-1 cell lines were kindly provided by Dr. Baki Akgül (University of Cologne, Germany). The WaGa cell line was a kind gift from Dr. Weng-Onn Lui (Karolinska Institute, Sweden). MCC-13, MCC-26 [31] and UISO [32] are VN MCC cell lines, whereas MKL-1 [33], MKL-2, WaGa [34, 35] and MS-1 [36] are VP MCC cell lines. Both VP and VN cells were grown in RPMI-1640 containing 10% FBS, streptomycin (100 μ g/ml) and penicillin (100 U/ml). The cells were kept in a humidified CO₂ incubator at 37°C. Human MCC tissues were obtained during 2000-2015 from the St. Olavs University Hospital Trondheim, Norway in accordance with the ethical approval from the Regional Ethical Committee (REK NORD application number 2016/988).

Transfection

Cells were seeded out in 6- and 12-well cell culture plates with a total number of 2×10^5 and 1.0×10^5 , respectively. At the time of transfections, the cells were approximately 80-90% confluent and jetPRIME (Polyplus-transfection SA, Illkirch, France) was used to transfect all plasmids for RNA and protein analysis according to the manufacturer's instructions. For gene promoter analysis, the cells were transfected with polyethylenimine (PEI linear MW25000; transfection grade, cat.# 3966-1, Polysciences, Warrington, PA, USA). DNA was mixed with 150mM NaCl, and a mixture of PEI:150mM NaCl was then added to the DNA at a ratio DNA:PEI of 1:4. This mixture was incubated for 15 min at room temperature and then carefully added to the cells. The medium containing the transfection mixture was replaced 4 hrs later. A total of 2- μ g DNA per well in a 6-well plate and 1- μ g DNA per well in a 12-well plate was used to transfect cells. All experiments were performed 24 hrs after transfection.

RNA extraction

RNA extraction was performed using the RNeasy® Plus Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. For RNA yield and quality, A260/A280 and A260/A230 ratios were analyzed with the Nano-Drop® ND-2000 spectrophotometer (NanoDrop Technologies, ThermoFisher Scientific, Waltham, MA, USA).

cDNA construction and quality control

The iScript™ cDNA Synthesis Kit (BioRad, Hercules, CA, USA) was used to generate cDNA. A total of 1-2 μ g RNA was converted to cDNA according to the manufacturer's instructions. To determine the presence of genomic DNA contamination PCR with the housekeeping APRT primers (5'- CCCGAGGCTTCCTCTTTGGC-3' and 5'-CTCCCTGCCCTTAAGCGAGG-3') was performed. While an 800 bp fragment is obtained with genomic DNA as a template, a cDNA template results in an 300 bp amplicon [37]. PCR products were visualized on a 1% agarose gel stained with GelRed™ Nucleic Acid Gel Stain (Biotium, Cambridge Bioscience, Bar Hill, UK).

RT² Profile PCR array

Human cytokine and receptor gene transcription was measured using the human RT² Profiler PCR Inflammatory Cytokines and Receptors Array (PAHS-011ZA, SABiosciences, Qiagen). The cDNA was diluted by adding 20 μ l cDNA to 91 μ l of RNase-free water and 102 μ l of the diluted cDNA were added into 1350 μ l 2x RT² SYBR Green Mastermix according to the manufacturer's protocol. Afterwards, 25 μ l PCR mix were added to each well of a 96-well plate. A two-step real-time PCR was initiated at 95°C (10 min) for one cycle, followed by alteration of 95°C (15 sec) and 60°C (1 min) for 45 cycles using the Light Cycler 96 (Roche Diagnostics, Indianapolis, IN, USA). All data was collected from the PCR machine by Light Cycler 96 SW 1.1 software and analyzed by SA Bioscience's Gene Glob PCR Array Data Analysis Web Portal. For considering a gene differentially expressed, we used a differential cut-off of 2-fold (up- or downregulated).

Luciferase assays

For luciferase assays, approximately 24 hrs after transfection, cells were lysed in 100 μ l Luciferase Assay Tropix Lysis solution (ThermoFisher Scientific) with freshly added 0.5 mM DTT. Cells were scraped, transferred to Eppendorf tubes and then centrifuged for 3 min at

12,000xg. Twenty μ l of the supernatant were added to a 96-well microtiter plate containing 50 μ l luciferase buffer (Promega, Madison, WI, USA). The CLARIOstar Plus Microplate reader (BMG Labtech, Ortenberg, Germany) was used to measure relative luciferase units (RLU). Each experiment was repeated three times with three parallel samples for each experiment. Luciferase values were corrected for the total protein concentration, which was measured using the MN protein quantification assay (Macherey-Nagel GmbH, Düren, Germany).

Immunoblotting

Western blot was performed by separating protein samples on 4-12% NuPAGE Bis-Tris Mini Gels (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. Proteins were blotted onto a 0.45 μ m PVDF membrane (Millipore, Billerica, MA, USA) and blocking was performed using TBS-T (TBS with 0.1% Tween-20; Sigma Aldrich) containing 5% (w/v) dried skimmed milk for 1 hour. The protein was probed by incubating the membrane with the primary antibody overnight at 4°C. After washing the membrane 3 times with TBS-T, an appropriate secondary antibody was added for 1 hour at room temperature. After 2 washes with TBS-T and 2 washes with washing buffer, antigen-antibody complex was visualized using SuperSignal™ West Pico Chemiluminescent Substrate (Cat.#34080 Thermo Fisher Scientific, Rockford, IL, USA). The Magic-Mark™ Western standard from Invitrogen Life Technologies was used to estimate the molecular mass of the detected proteins.

Immunohistochemistry

Formalin-fixed and paraffin-embedded tissue sections were deparaffinized in xylene and graded alcohols, hydrated and washed in PBS. After antigen retrieval in a sodium citrate buffer (pH 6) in a microwave oven, the endogenous peroxidase was blocked by 0.3% H₂O₂ for 15 min. Sections were incubated overnight at 4°C with the primary antibodies against MCPyV-LT, IL-33, ST2/IL1RL1, IL1RAcP and CK20 (Supplementary Table 1). As a secondary antibody, the anti-rabbit-HRP Signalstain (R) DAB Substrate kit (Cat.# 8059S cell signaling) or anti-mouse EnVision-HRP (Dako, Agilent Technologies, Inc., Santa Clara, CA, USA) was used. A matched isotype control was used as a control for nonspecific background staining.

Plasma levels of IL33, ST2/IL1RL1 and IL1RAcP in MCC patients and control

Patient plasma samples were obtained with consent and in accordance with the ethical approval from the Ethics Committee of Karolinska Institutet, Sweden (2010/1092-31/3). Plasma concentrations of IL-33, ST2/IL1RL1 and IL1RAcP were measured by enzyme-linked immunosorbent assay (ELISA) using Human ST2/IL-33R DuoSet ELISA, Human IL-1RAcP/IL-1 R3 DuoSet ELISA and Human IL-33 Quantikine ELISA Kit (R&D Systems Minneapolis, MN, USA) according to manufacturer's protocol. The assay ranges for IL-33, ST2/IL1RL1 and IL1RAcP were 3.1 - 200 pg/mL, 31.2 - 2,000 pg/mL and 31.2 - 2,000 pg/mL, respectively.

Statistical analysis

The RT² Profiler PCR Array data analysis version 3.5 (<http://dataanalysis.sabiosciences.com/pcr/arrayanalysis.php>) was used for inflammatory cytokines and receptor data analysis. For the analysis, values with a fold change/fold regulation ≥ 2 and a p-value less than 0.05 were considered significant. GraphPad Prism 6 software was used for statistical analysis and graph design. The sample t-test was used to compare differences

between the experimental and control group and a p-value <0.05 was considered statistically significant. Densitometric analysis of Western blots was performed by using imageJ.

Results

Differential Expression of Inflammatory cytokines and receptors in MCC cell lines

RT² profiler PCR array was used to access expression pattern of different inflammatory cytokines and receptors in VP (MKL-1, MKL-2, MS-1 and WaGa) and VN (MCC-13) MCC cell lines. Gene expression of 84 different genes was determined and a gene was considered as differentially expressed when the expression level exceeded two-fold in all of the cell lines examined. Most of the genes examined were detectable (MCC-13 82/84, MKL-1 83/84, MKL-2 83/84, MS-1 80/84, WaGa 81/84) with Cq values <35.

The inflammatory cytokines and receptor genes regulation were compared among VP cell lines (MKL-1, MKL-2, MS-1 and WaGa cells) and the VN MCC-13 cell line. Compared to MCC-13 cells the number of differentially expressed genes were 65 for MKL-1 (13 upregulated, 52 downregulated), 71 for MKL-2 (60 upregulated, 11 downregulated), 63 for MS-1 (18 upregulated, 45 downregulated) and 70 for WaGa (19 upregulated, 51 downregulated) (Figure 1A-D). Overall the data demonstrated that 22 different inflammatory cytokines and receptors were differentially expressed (12 upregulated, 10 downregulated) between all VP cell lines and the VN MCC-13 cell line (figure 1E).

IL-33 expression in MCC cell lines

IL-33 was among genes that were differentially expressed in VP cell lines compared to the VN cell line. To confirm this, western blot was performed on lysates from VP and VN cell lines using a IL-33-specific antibody. Western blot analysis shows that two- to three-fold higher IL-33 levels were present in the VP cell lines (MKL-1, MKL-2, MS-1 and WaGa) in comparison to the VN cell lines (MCC-13, MCC-26 and UISO) and hTERT-immortalized human dermal fibroblasts (Figure 2).

MCPyV T-ag stimulates IL-33 promoter activity and hence increases *IL-33* expression

Since VP-MCC cell lines express sT and tLT, we investigated if these proteins could induce the expression of *IL-33*. Therefore, MCC-13 cells were transiently co-transfected with a luciferase reporter plasmid driven by the IL-33 promoter and an expression plasmid for either MCPyV full-length LT (FLT), MKL-1 LT, MKL-2 LT, MS-1 LT, or sT. The pcDNA3 empty vector (EV) was used as a control plasmid. MCPyV sT, FLT, truncated MKL-1, MKL-2 and MS-1 LT significantly upregulated IL-33 promoter activity (Figure 3A). Analysis of the IL-33 promoter sequence demonstrated that there are two predicted binding sites for MCPyV LT at position -821/-817 and -199/-195. Consequently we deleted one or both LT motifs in the IL-33 promoter. MCC-13 cells were transiently co-transfected with pGL3_IL-33 (-1050/+50), pGL3_IL-33 (-460/+50) or pGL3_IL-33 (-160/+50) luciferase plasmids with either of FLT, MKL-1 or sT expression plasmids. The truncated promoter (-160/+50) had a significantly higher basal activity than the (-1050/+50) and (-460/+50) IL-33 promoter sequences. However, the activity of all three promoter fragments was still induced by LT, MKL-1 LT and sT (Figure 4A-C). Mutation of either LT motif or both did not abrogate stimulation of the IL-33 promoter activity by LT or tLT (Figure 4D-E).

Furthermore, the stimulatory effect of FLT, tLT (MKL-1, MKL-2 and MS-1) and sT on IL33 promoter was evaluated at the IL-33 protein level. The MCC-13 cells were transfected with MCPyV T-ag and pcDNA3.1 as control. MCPyV T-ag upregulated IL-33 protein in MCC-13 cells 24 hrs post transfection (Figure 3B).

MCPyV T-ag stimulate ST2/IL1RL1 and IL1RAcP promoter activity

Human and mouse *ST2/IL1RL1* genes have two alternative promoter regions, the distal and proximal promoters, followed by the noncoding first exons, E1a and E1b [38]. Transcription from the distal promoter generates the mRNA for the membrane bound ST2L/IL1RL1L receptor protein, while the proximal promoter produces the transcript for the soluble form of sST2/sIL1RL1 receptor [39]. To study the effect of the MCPyV T-ag on ST2/IL1RL1 promoter regulation, we cloned the distal (pGL3_ST2L-100/+82) and the proximal promoter (pGL3_sST2-499/+100) into pGL3-basic plasmid. MCC-13 cells were co-transfected with MCPyV FLT, tLT, or sT and luciferase reporter plasmid with either the distal or the proximal promoter. The results demonstrate a moderate, but significant upregulation of the distal promoter activity. While a more pronounced stimulation of the proximal promoter activity was observed for all LT, a slight, but not significant, increase was detected for sT. Interestingly, truncated variants of LT more potently stimulated the distal ST2 promoter than full-length LT (Figure 5A).

We also evaluated the effect of MCPyV T-ag on the IL1RAcP promoter activity. MCC-13 cells were transiently co-transfected with FLT, MKL-1, MKL-2, or MS-1 tLT, or sT and either of pGL3_IL1RAcP (-1397/+184) and pGL3_IL1RAcP (-517/+184) luciferase reporter plasmids. MCPyV T-ag significantly upregulated the IL1RAcP promoter activity (Figure 5B) and, moreover, MKL-1 and MS-1 LT-ag provoked higher IL1RAcP promoter activity compared to FLT, MKL-2 and sT.

Effect of IL-33, ST2 and IL1RAcP on activity of the MCPyV early and late promoter

Previous studies have demonstrated that different cytokines can modulate human polyomavirus promoter activity [29, 40-43]. Therefore, we examined the effect of IL-33, ST2/IL1RL1 and IL1RAcP (both the membrane-bound and soluble forms of the receptors) on MCPyV early and late promoter activity. Co-transfection with increased concentrations of plasmid encoding full-length IL-33_{1-270aa} (FL-IL-33) increased both early and late promoter activity. Interestingly, we also found that increased plasmid concentrations showed an inhibitory effect on both early and late promoter activity (Figure 6A). Stimulation of transfected cells with recombinant cytokine domain of hIL-33 (CyD-IL-33) alone increased both early and late promoter activity in a dose-dependent manner (Figure 6A). Recombinant human FL-IL-33 and CyD-IL33 expressing protein also stimulated the MCPyV late promoter activity, whereas no significant effect was observed on the early promoter, except for 12.5 ng/ml full-length rhIL-33 (Figure 6B). To characterize which domain(s) of IL-33 mediated induction of the MCPyV promoter activity, we generated expression plasmids for separate or a combination of IL-33 domains. We determined that IL-33 expression plasmids containing the nuclear domain either alone (aa1-65) or in combination with the activation domain (aa1-111) and/or the IL-1-like cytokine domain aa1-65,112-270) downregulated both early and late MCPyV promoter activity while IL-1-like domain without the nuclear domain (aa112-270) activated both MCPyV early and late promoter activity (Figure 6 C-D).

Furthermore, we evaluated the effect of ST2, both the membrane-bound (ST2L) and the soluble form (sST2) of the protein, on MCPyV early and late promoter activity. We demonstrated that ST2L decreased both MCPyV early and late promoter activity, while sST2 increased the activity of both promoters in a dose-dependent manner (Figure 7A). Furthermore, we observed that sST2 had a more robust effect on late promoter compared to early promoter activity.

Moreover, we investigated the effect of both the membrane-bound and the soluble form of IL1RAcP on the MCPyV early and late promoter activity. Interestingly, we found that low concentrations of both membrane-bound and soluble forms of IL1RAcP resulted in both early and late promoter activation while an inhibition was observed with an increase in expression plasmid concentration (Figure 7B).

Effect of IL-33, ST2/IL1RL1 and IL1RAcP on IL-33/ST2-IL1RAcP complex promoters

Next, we investigated the effect of IL-33, ST2/IL1RL1 or IL1RAcP on IL-33/ST2-IL1RAcP complex promoter activity. We observed that both FL-IL-33 and CyD-IL-33 activated IL1RAcP promoter activity (Figure 8A). We also demonstrated IL1RAcP promoter activation or inhibition by sST2/IL1RL1 and ST2.L, respectively (Figure 8B). Moreover, sIL1RAcP upregulated sST2/IL1RL1 promoter activity without displaying an effect on the ST2.L promoter (Figure 8C). We did not observe an effect on IL-33 promoter activity by either ST2/IL1RL1 or IL1RAcP and no significant effect of FL-IL-33 or CyD-IL-33 on the membrane bound nor soluble forms of ST2 receptor was seen.

IL-33 activates mitogen-activated protein kinase (MAP kinase) and NF- κ B signaling pathways in MCC cells

IL-33 activates multiple signaling pathways in different cellular systems, including NF- κ B and MAPK signaling cascades (ERK, JNK and p38) (Figure 8) [44-49]. The effect of recombinant IL-33 was investigated by stimulating VN MCC-13 cells with CyD-IL-33 (1 ng/ml) for different time periods (5-60 min). Phosphorylation of ERK1/2, JNK and p38 was assessed by western blotting using different phospho-specific antibodies (Figure 10A). An increase in of ERK1/2, p38 and JNK phosphorylation was observed, although with somewhat different kinetics. ERK1/2 phosphorylation was already seen 5 min after stimulation, while p38 and JNK phosphorylation was noticed after 45 min and 30 min, respectively. Pre-blocking the IL-33 receptor with ST2L receptor specific antibody abrogated rhIL-33-induced phosphorylation of ERK1/2 (Figure 10B).

NF- κ B is an inducible transcription factor that respond to different inflammatory mediators and is regulated through several mechanisms both in the nucleus and cytoplasm [50]. To evaluate the effect of IL-33 on NF- κ B activation, we stimulated MCC-13 cells with 1 ng/ml CyD-rhIL-33 protein determining the phosphorylation status of p65 and p105 at different time points (5-60 min). An increase in p65 and p-105 phosphorylation was observed following stimulation with CyD-rh-IL33 (Figure 11A). Furthermore, pre-incubation with an ST2L receptor-specific antibody inhibited IL-33-induced phosphorylation of p65 (Figure 11C).

To support our western blot findings, we performed a NF- κ B promoter reporter assay. The MCC-13 cells were co-transfected with the CyD-IL-33 expression plasmid and a luciferase reporter plasmid with an NF- κ B responsive promoter. We determined that CyD-rhIL-33 activates p65 promoter activity with increasing CyD-IL-33 plasmid concentration (figure 11B) and is inhibited by blocking ST2l receptor using an anti-ST2L antibody (Figure 11D).

IL-33, sST2 and sIL1RAcP measurement in human patient plasma samples

A total of 24 plasma samples were examined for IL-33, sST2/IL1RL1 and sIL1RAcP with 12 samples being obtained from healthy controls and 12 samples from MCC patients. The minimum level of 3.1 pg/mL, 31.3 pg/mL and 31.2 pg/mL was set as detectable for IL-33, sST2 and sIL1RAcP respectively. The mean IL-33 plasma values were 26.14 ± 5.53 pg/mL and 37.81 ± 4.64 pg/mL for control and MCC patients, respectively (Figure 12A). The mean sST2/IL1RL1 plasma values were 39.36 ± 14.32 pg/mL and 41.92 ± 14.96 pg/mL for control and MCC patients respectively (Figure 12B). Similarly, the mean sIL1RAcP plasma values were 189.20 ± 139.288 pg/mL and 463.45 ± 110.28 pg/mL for healthy controls and MCC patients, respectively (Figure 12C).

IL-33 and its receptors, ST2/IL1RL1 and IL1RAcP are expressed in MCC tissue samples

A total of 23 primary cutaneous MCCs were immunohistochemically stained for LT, CK20, IL-33, ST2/IL1RL1 and IL1RAcP. (Figure A-J). Fifteen of 23 (65.2%) demonstrated an intranuclear positivity for LT. All of 23 MCC tissue samples displayed a uniform positivity for CK20 (dot-like cytoplasmic), IL-33 (strong nuclear and weaker cytoplasmic), ST2/IL1RL1 (membranous positivity) and IL1RAcP (membranous positivity) (13A-J).

Discussion

In the present study, we investigated differential expression of 84 inflammatory mediators between VP and VN MCC cell lines. Furthermore, we also examined if MCPyV T-ag affected the regulation of those differentially expressed inflammatory mediators. Previous studies reported that MCPyV sT downregulates IL2, IL-8, CCL20 and CXCL9 expression in MCC-13 cells [51], while MCPyV FLT, tLT (LT339) and sT upregulate IL-1 β , IL-6, IL-8, CXCL1 and CXCL6 in hTERT-immortalized BJ human foreskin fibroblasts [52]. Additionally, a previous study by our group found that MCPyV FLT and tLT upregulates CCL17/TARC expression in a VP MCC (MKL-2) cell line as compared to a VN MCC (MCC-13) cell line [29].

In this work, we demonstrated that expression of *IL-33* is upregulated in the VP MCC cell lines MKL-1, MKL-2, MS-1 and WaGa cells compared to VN MCC cell lines MCC-13, MCC-26 and UISO. IL-33 is a chromatin-associated nuclear cytokine that binds to the acidic pocket formed by the histone heterodimer H2A–H2B on the surface of the nucleosome. The IL-33 nuclear domain contains nuclear localization signals and was originally described as a nuclear protein designated 'nuclear factor from high endothelial venules' (NF-HEV). Furthermore, the nuclear domain also interacts with the p65 subunit of NF κ B and represses the expression of NF κ B-regulated genes that are necessary for pro-inflammatory signaling. The activation domain of IL-33 has different protease cleavage site and the IL-1-like cytokine domain exerts its cytokine activity [53, 54].

Furthermore, we found that MCPyV T-ag stimulated the promoter activity of the IL-33 receptors, ST2/IL1RL1 and IL1RAcP and IL-33 protein expression was increased in MCC-13 cells transiently transfected with MCPyV T-ag. Previous studies have demonstrated enhanced expression of IL-33 in different malignancies including, breast cancer [55, 56], colorectal cancer [57-59], gastric cancer [60], hepatocellular carcinoma [61, 62], pancreatic cancer [63, 64], lung cancer [65, 66], prostate and kidney cancer [67]. The functions of IL-33 in cancer

include involvement in Th2 immune responses [68-70], regulatory T cell (Treg) development in intestinal tissue [71], and viral-specific CD8+ T cell functions [72].

It was initially believed that IL-33 matures by caspase-1 cleavage, as has been described for IL-1 β and IL-18 [68]. However, different studies have shown FL-IL-33 as the biologically active form and that processing by caspases-1 results in IL-33 inactivation [25, 26, 73, 74]. IL-33 is released in response to cell stress or damage and inflammatory proteases from neutrophils (proteinase 3, elastase, and cathepsin G) [28] and mast cells (chymase, tryptase, and granzyme B) [21] can process full-length IL-33 into shorter mature forms (18–21 kDa) with 10- to 30-fold higher activity [75, 76]. Based on the diverse activity of IL-33, we studied a possible autocrine effect on MCPyV early and late promoter activity. We observed that while FL-IL-33 downregulated both MCPyV early and late promoter activity in a dose-dependent manner CyD-IL-33 upregulated promoter activity. We also found that the inhibitory effect of FL-IL-33 was mediated by its nuclear domain.

Since both IL-33 receptors occur in soluble form, we examined if these affected MCPyV promoter activity. We observed that sST2 increased both early and late promoter activity, indicating that during disease, sST2 also may upregulate MCPyV T-ag expression and contribute to MCPyV induced development of MCC. Additionally, we found an inhibitory effect of sIL1RAcP on MCPyV early and late promoter activity.

Using immunohistochemistry, we detected the presence of IL-33 and its receptors ST2/IL1RL1 and IL1RAcP in the tumor cells of all analyzed MCC tissues. Moreover, IL-33 was also present in the healthy epidermis of the skin. Unfortunately, quantification of IL-33 protein levels in VP and VN tumor samples was not possible with the IHC protocol used in this study.

Our work demonstrates that MCC produces IL-33 and expresses ST2/IL1RL1 and IL1RAcP, therefore, we decided to investigate the effect of exogenous IL-33 on intracellular signaling pathways. Stimulation with CyD-rhIL-33 resulted in phosphorylation of ERK1/2, p38 and JNK in MCC-13 cells (Figure 9). Previous studies have demonstrated that IL-33 induced phosphorylation of p38 MAPK in breast cancer cells [77], ERK1/2 and JNK in gastric cancer cells [44, 78] and JNK in renal cell carcinoma [47]. Furthermore, we observed that IL-33 triggered NF- κ B pathway activation in MCC-13 cells (Figure 10). IL-33/ST2 dependent NF- κ B activation has previously been described in other cancers including, glioblastoma [79] and colorectal cancer [80]. Additionally, it has been demonstrated that the nuclear domain of IL-33 can interact with the p65 subunit of NF- κ B and repressing the expression of NF- κ B regulated genes related to pro-inflammatory signaling [53, 54].

The TME serves as a central operating system for tumor progression, local invasion, and metastasis [81]. Various studies using patient samples, in vitro experiments, and in vivo mouse models describe a versatile role of the IL-33-ST2 pathway in the tumor microenvironment with respect to tumor initiation, development and resistance to therapy. In different cancers pro-tumorigenic functions of IL-33 have been demonstrated, however, anti-tumorigenic functions have also been described [82]. As an alarmin, IL-33 amplifies innate immune responses that can contribute to different types of inflammatory disorders as well as to the modulation of tumorigenesis [53, 83, 84]. A xenograft model using the breast cancer cell line 4T1 showed significantly reduced metastasis following injection with *ST2*^{-/-} compared to *ST2*^{+/+} cells. Furthermore, in breast cancer patients IL-33 expression correlated to cancer progression. Moreover, patients with estrogen receptor-positive breast cancer showed increased IL-33 and

sST2 serum levels that correlated with elevated levels of the angiogenic factors [85], matrix metalloproteinase-11 (MMP-11) and platelet derived growth factor-c (PDGF-C) [86]. In addition, overexpression of IL-33 in SW620 human colon cancer cells increased tumor growth, migration, and colony formation in vitro and enhanced tumor growth and lung metastasis in vivo, while inhibition of IL-33 had the opposite effect [58].

Taken together, we demonstrated that MCPyV T-ag is linked to an altered expression of IL-33 and its receptors, ST2/IL1RL1 and IL1RAcP. The expression of IL-33 and its receptor ST2/IL1RL1 may constitute an autocrine or paracrine survival loop, which contributes to the growth and survival of the tumor. Further research, including in vivo studies, is necessary to fully understand the functions of IL-33 and its receptors in MCC TME. However, either patient- or disease-specific therapy based on the selective targeting of the IL-33/ST2-IL1RAcP axis, by monoclonal antibodies or specific receptor antagonists may be a therapeutic approach for patients with MCC.

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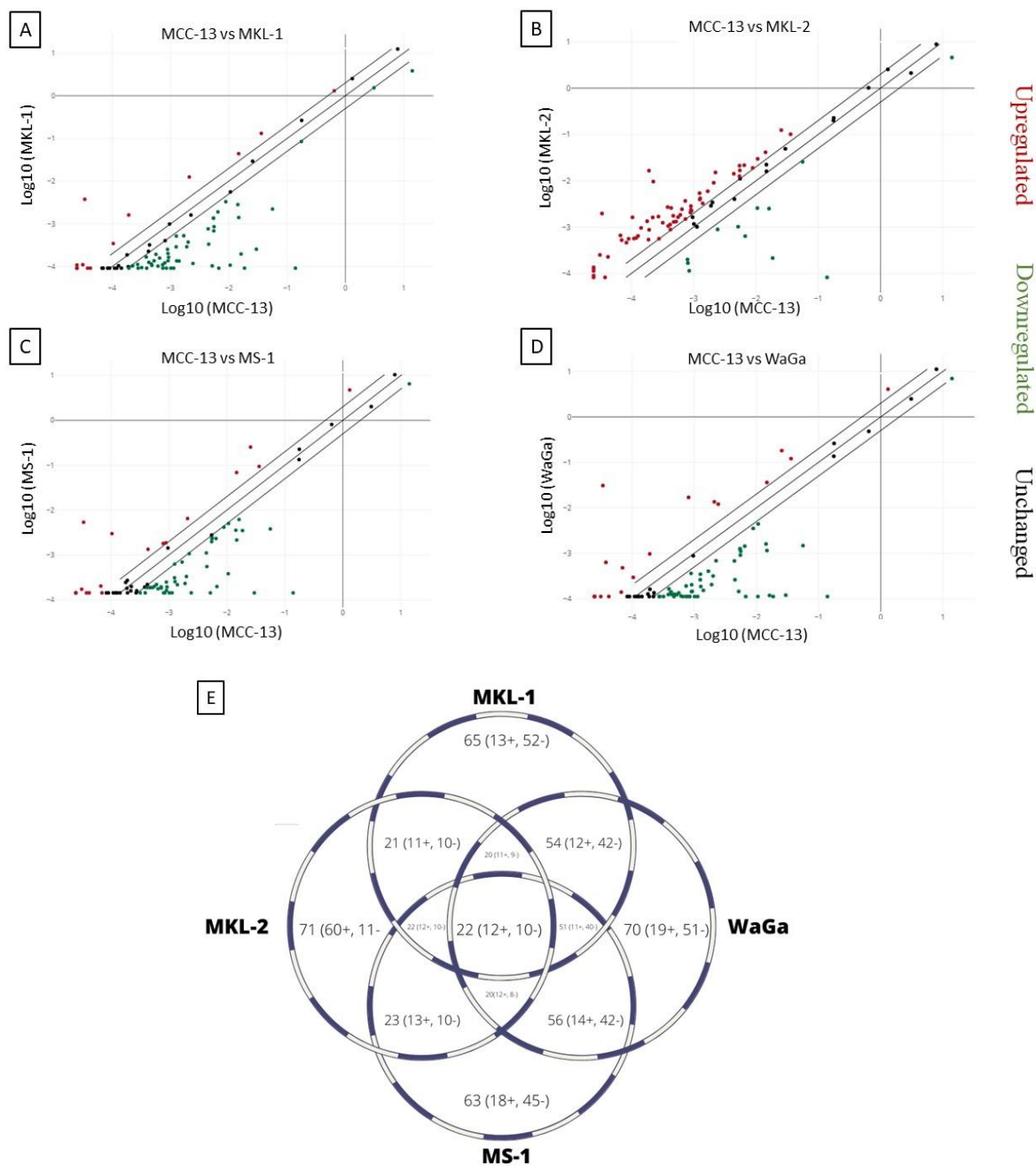


Figure 1: Relative expression comparison of 84 inflammatory cytokines and receptors genes between MCPyV-positive and negative Merkel cell carcinoma cell lines

The figure depicts a log transformation plot of the relative expression level differences for each gene ($2^{-\Delta C_t}$) between (A) MCC13 cells vs. MKL-1 cells, (B) MCC13 cells vs. MKL-2 cells, (C) MCC13 cells vs. MS-1 cells, (D) MCC13 cells vs. WaGa cells. The lines indicate a two-fold change in gene expression threshold. (E) Venn diagram showing the number of differentially expressed cytokines and receptors between the groups A-D with (+) indicating upregulated and (-) indicating downregulated genes.

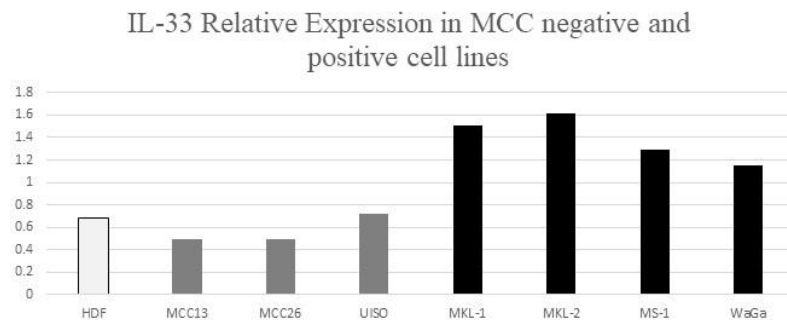
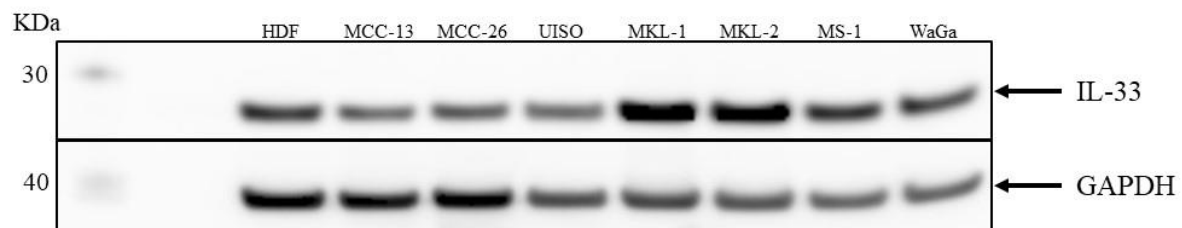


Figure 2: IL-33 expression in different MCC cell lines.

Top panel: Western blot analysis of IL-33 expression in MCPyV-positive and negative cell lines. Immortalized human dermal fibroblasts (HDF) were used as control cells and GAPDH was used as a loading control. Bottom panel: densitometry of the signals obtained with IL-33 and GAPDH antibodies.

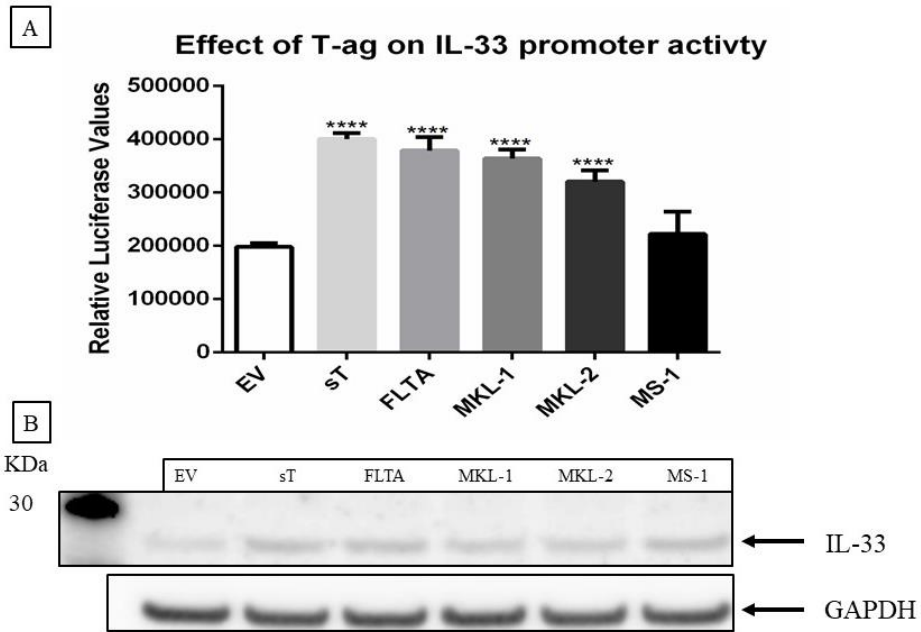


Figure 3: Effect of MCPyV T-ag on IL-33 regulation in MCC-13 cells.

(A) Cells were transfected with a luciferase reporter vector driven by the IL-33 promoter fragment spanning nucleotides $-1050/+50$. Expression plasmid for full-length LT (FLTA), MKL-1, MKL-2 or MS-1 LT, sT or pcDNA3 (empty vector EV) control, were co-transfected. Luciferase activity was assessed after overnight cultivation of transfected cells. Each bar represents the average of three independent parallels \pm SD. Luciferase values were normalized for total protein in each sample. Comparable results were obtained for three independent experiments. (B) MCC13 cells were transfected with expression plasmids for full-length or truncated MCPyV LT-ag, sT or empty vector and protein expression was measured 24 hrs after transfection. IL-33 expression was normalized with GAPDH. The image is representative for three independent experiments. $p^{**} \leq 0.01$ and $p^{***} \leq 0.001$.

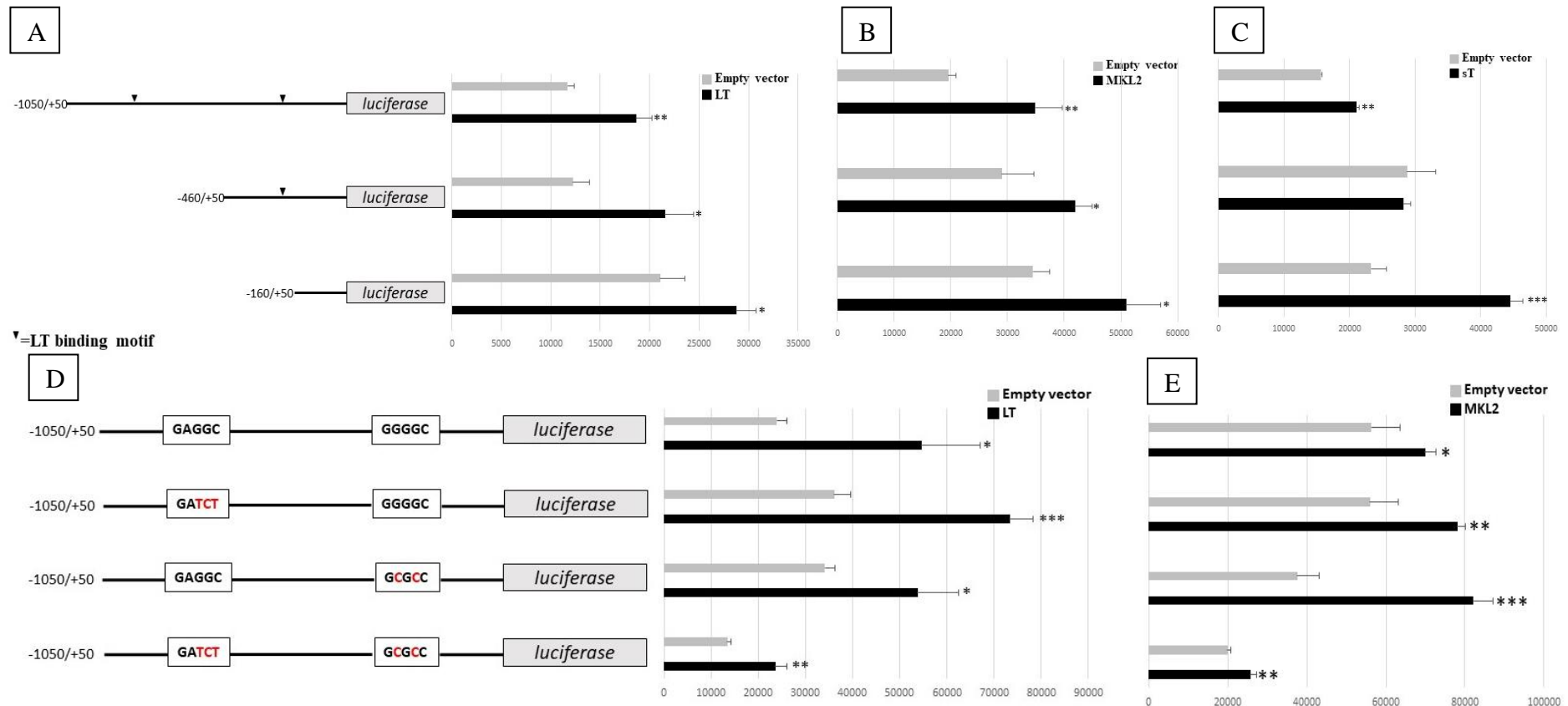


Figure 4. MCPyV large and small T-antigens stimulate IL-33 promoter activity. (A) MCC 13 cells were co-transfected with a luciferase reporter plasmid containing the human IL-33 promoter (sequences -1050/+50 or truncated version) and either an empty expression vector pCDNA3.1 or an expression plasmid encoding MCPyV full-length LT. (B) as in (A) but co-expression of MKL2 truncated LT. (C) as in (A) but co-transfection with an expression plasmid for MCPyV sT. (D) MCC13 cells were co-transfected with a luciferase reporter plasmid containing IL-33 promoter sequences -1050/+50 without mutations of the putative LT binding motifs or with mutation of either one or both potential LT binding sites and with empty vector or LT expression vector. (E) as in (D) but co-transfection with MKL2 truncated LT. Luciferase values were normalized the total protein concentration in the sample. Each bar represents the average \pm SD of three independent parallels. Each experiment was performed 2 to 4 times and similar results were obtained. * $p < 0.05$, ** $p < 0.01$; *** $p < 0.005$.

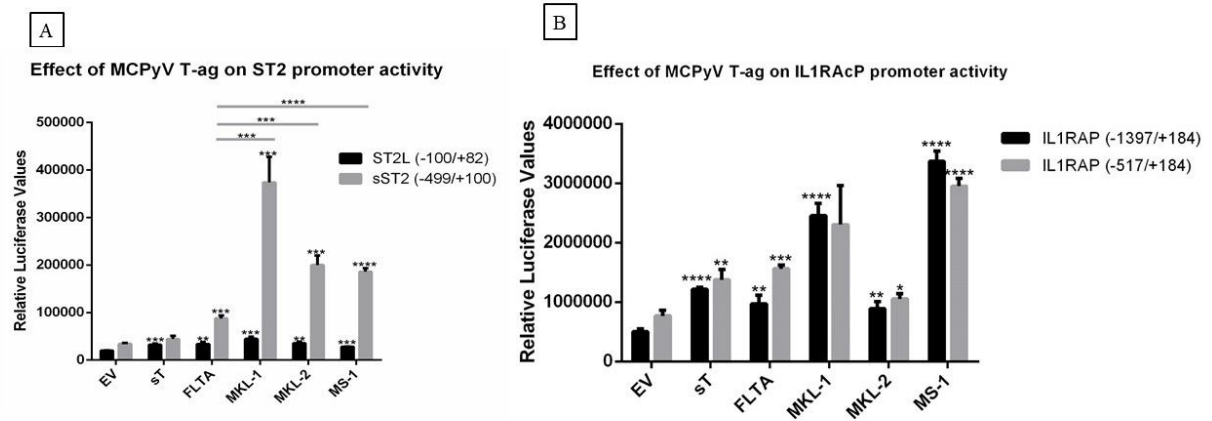


Figure 5: Effect of MCPyV T-ag on ST2/IL1RL1 and IL1RAcP promoter activity in MCC-13 cells.

Cells were transfected with a luciferase reporter vector driven by ST2/IL1RL1 and IL1RAcP promoter sequences. The promoter fragments spanning nucleotides for (A) ST2/IL1RL1 and (B) IL1RAcP were -100/+82, -499/+100, and -1397/+184, -517/+184, respectively. Expression plasmid for full-length LT (FLTA), MKL-1, MKL-2 or MS-1 LT, sT or empty vector (EV) control, were co-transfected. Luciferase activity was assessed following overnight cultivation of transfected cells. Each bar represents the average of three independent parallels +SD. The experiment was repeated 3 times, and similar results were obtained. Luciferase values were normalized to the total protein in each sample. $p^* \leq 0.05$, $p^{**} \leq 0.01$, $p^{***} \leq 0.001$ and $p^{****} \leq 0.0001$.

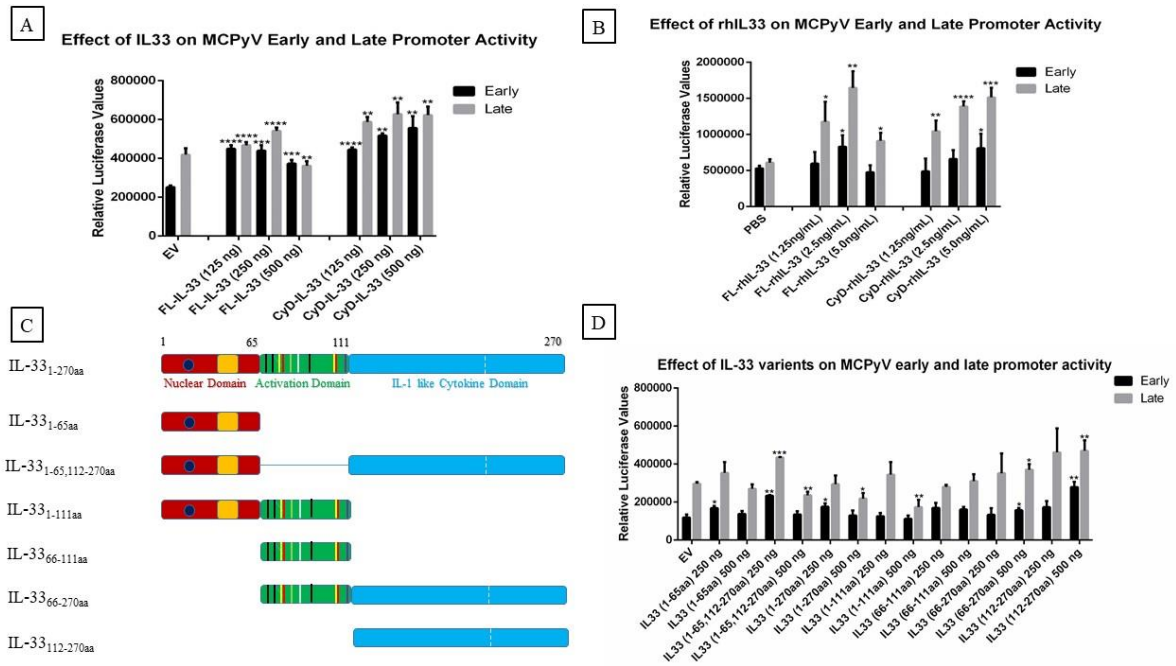
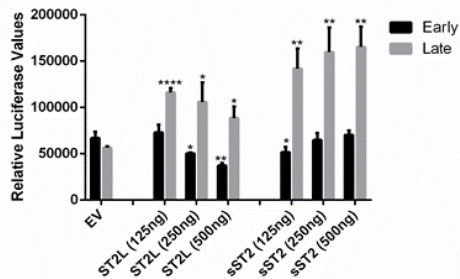


Figure 6: Effect of IL-33 on MCPyV promoter activity in MCC-13 cells.

MCC-13 cells were transiently co-transfected with either IL-33 expression plasmid or empty expression vector pcDNA3.1, and luciferase reporter plasmid containing either the MCPyV early or the late promoter. Luciferase activity was measured 24 hrs after transfection. (B) MCC-13 cells were seeded and serum starved for 24 hrs. Cells were transfected with luciferase reporter plasmid containing either the MCPyV early or the late promoter and were then exposed to rhIL-33 (1.25-5.0 ng/ml) or vehicle (PBS) for 4 hrs with a subsequent analysis of luciferase activity. (C) Schematic presentation of the different forms of the IL-33 protein. Human *IL33* mRNA (2.7 kb) encodes a protein of 270 residues with three functional domains: a nuclear, a central and a IL-1-like cytokine domain. (D) Sequences of the different IL-33 domains were cloned into pCMV_His(N)_FLAG(C) plasmid and MCC-13 cells were transiently co-transfected with either MCPyV early and late promoter and with plasmids expressing different domains of IL-33, IL-33_{1-270aa}, IL-33_{1-65aa}, IL-33_{1-65,112-270aa}, IL-33_{1-111aa}, IL-33_{66-111aa}, IL-33_{66-270aa} and IL-33_{112-270aa}. Abbreviations: FL-IL33 (full length IL-33), CyD-IL-33 (IL-33 with only cytokine domain). Each bar represents the average of three independent parallels +SD* $p \leq 0.05$, $p^{**} \leq 0.01$, $p^{***} \leq 0.001$ and $p^{****} \leq 0.0001$.

A

Effect of ST2 on MCPyV Early and Late Promoter Activity



B

Effect of IL1RAP on MCPyV Early and Late Promoter Activity

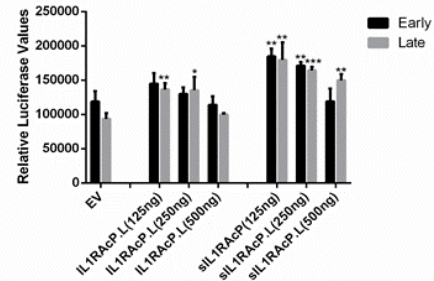


Figure 7. Effect of ST2/IL1RL1 and IL1RAcP on MCPyV promoter activity in MCC-13 cells.

MCC-13 cells were transiently co-transfected with luciferase reporter plasmid and either the early or the late luciferase promoter of MCPyV and (A) ST2/IL1RL1 and (B) IL1RAcP expression plasmids, respectively. pcDNA3.1 was used as empty expression vector (EV). Luciferase activity was measured 24 hrs after transfection. Abbreviations: ST2L (membrane bound ST2/IL1RL1), sST2 (soluble form of ST2/IL1RL1), IL1RAcP.L (membrane bound IL1RAcP), s IL1RAcP (soluble form of IL1RAcP). Each bar represents the average of three independent parallels \pm SD* $p^* \leq 0.05$, $p^{**} \leq 0.01$, $p^{***} \leq 0.001$ and $p^{****} \leq 0.0001$.

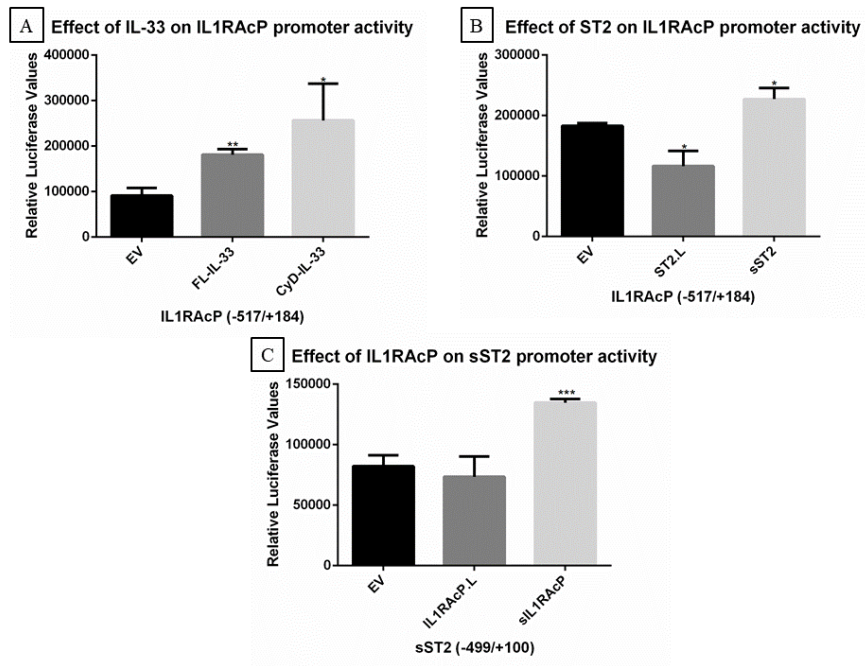


Figure 8: Effect of IL-33, ST2/IL1RL1 and IL1RAcP on IL-33/ST2-IL1RAcP complex promoters

Cells were transiently co-transfected with IL-33, ST2/IL1RL1, IL1RAcP or empty vector (EV) as control expression plasmids. The promoter fragments spanning nucleotides for (A), (B) IL1RAcP and (C) sST2/sIL1RL1 were -517/+100, and -499/+100, respectively. Each bar represents the average of three independent parallels +SD. The experiment was repeated total 3 times, and similar results were obtained. Luciferase values were normalized with a total protein in each sample. $p^* \leq 0.05$, $p^{**} \leq 0.01$ and $p^{***} \leq 0.001$.

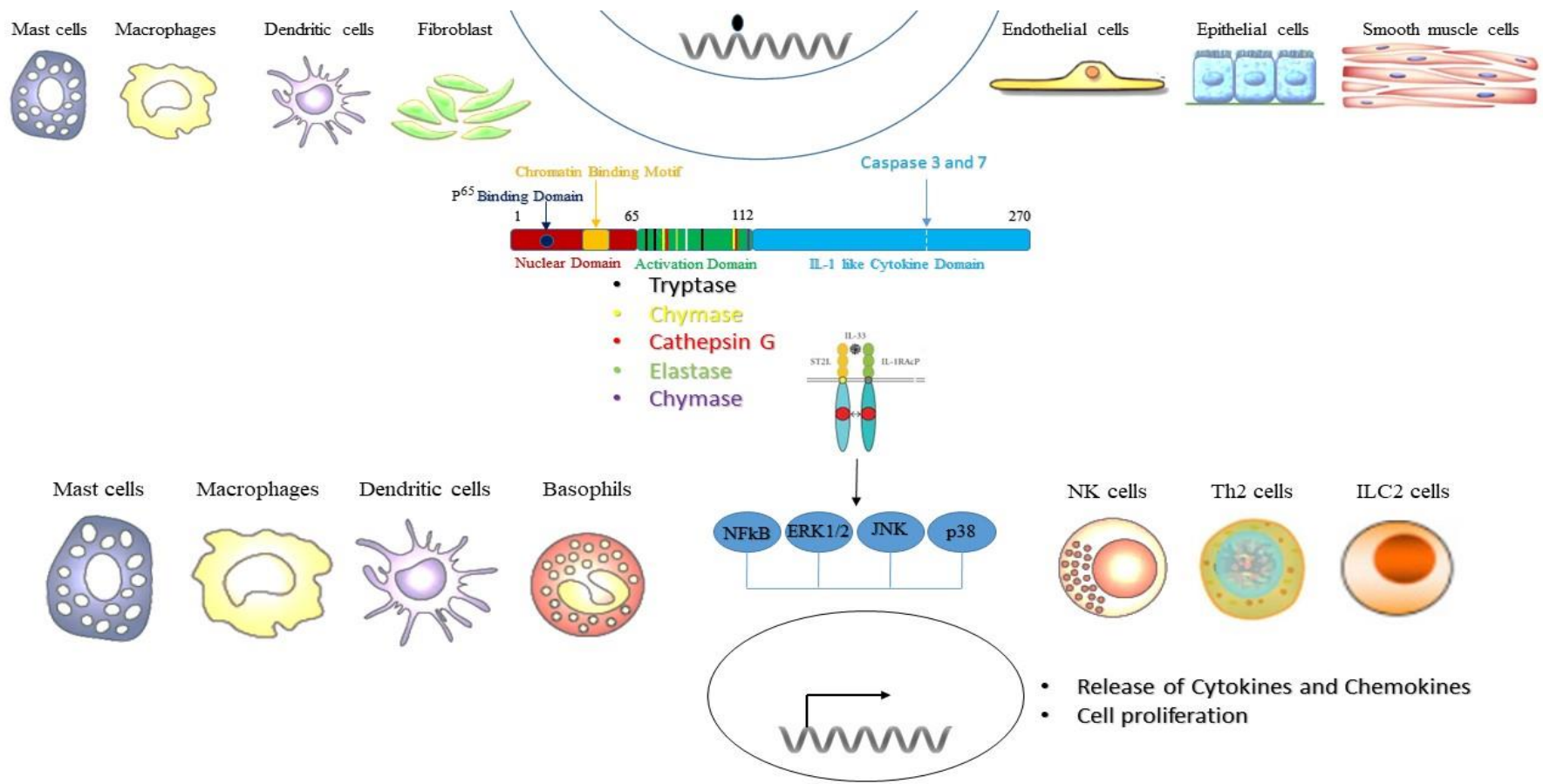


Figure 9: IL-33 signaling pathway

IL-33 binds to ST2L causing a conformational change that leads to recruitment of IL1-RAcP. Heterodimerization of the transmembrane proteins results in interaction between their intracellular C-terminal domains that facilitates recruitment of adaptor molecules including MyD88, IRAK1, IRAK4, and TRAF6. Subsequent activation of signaling pathways NF-κB, JNK, ERK, and p38 leads to expression of genes encoding cytokines, chemokines, and growth factors.

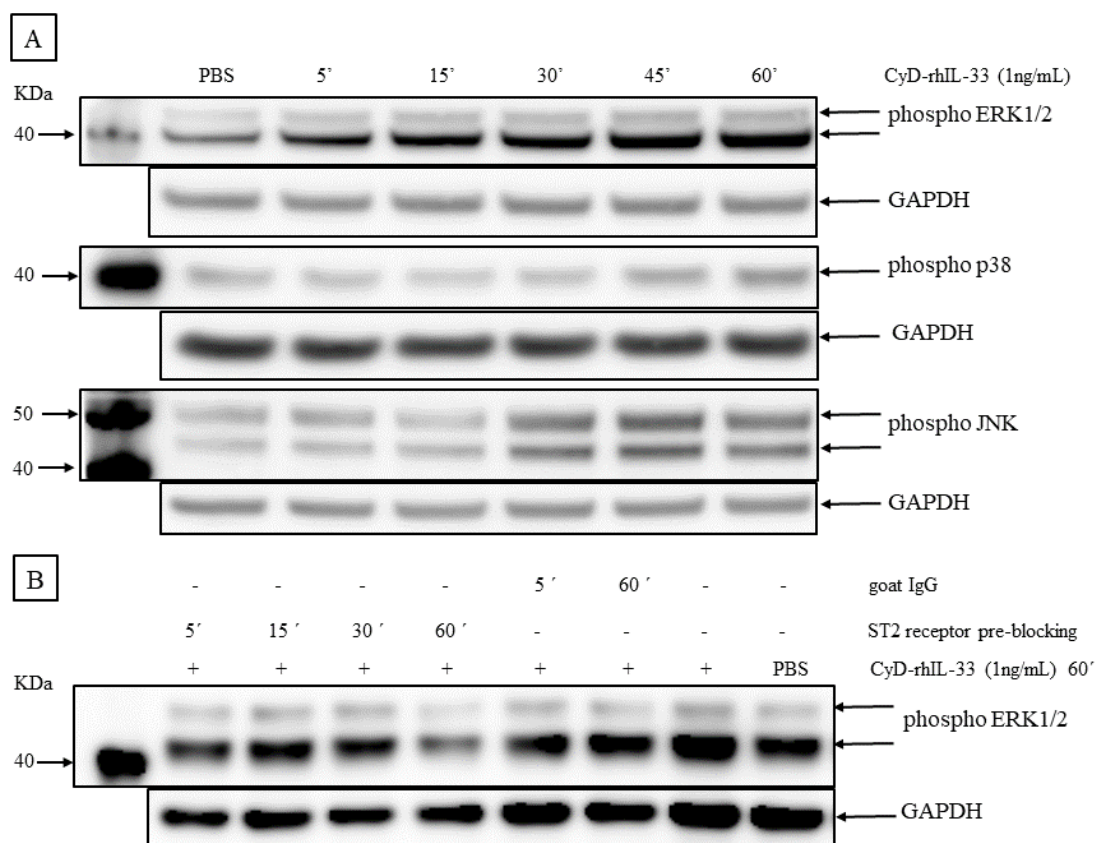


Figure 10: IL-33 upregulates MAP kinase activity

(A) Phosphorylation levels of ERK1/2, p38 and JNK were determined by Western blot analysis using phospho-specific antibody detecting Thr202/Tyr204 (ERK1/2), Thr183/Tyr185 (JNK) and Thr180/Tyr182 (p38) phosphorylation and compared to GAPDH. MCC13 cells were serum-starved for 24 hrs, and thereafter stimulated with either vehicle (PBS) or 1 ng/ml of CyD-rhIL-33 for different time points (5', 15', 30', 45' and 60'). (B) The blockade of the ST2 receptor interferes with IL-33-induced activation of ERK1/2 activity. Phospho ERK1/2 levels were determined by western blot. MCC13 cells were serum starved for 24 hrs. The cells were pre-blocked with goat anti-ST2 antibody (2 ng/mL) for varying time duration 5-60 min or with polyclonal normal goat IgG for 5 or 60 min. The cells were then stimulated with CyD-rhIL-33 (1 ng/ml) in the presence or without ST2 receptor antibody for 60 min. PBS was used as a vehicle control.

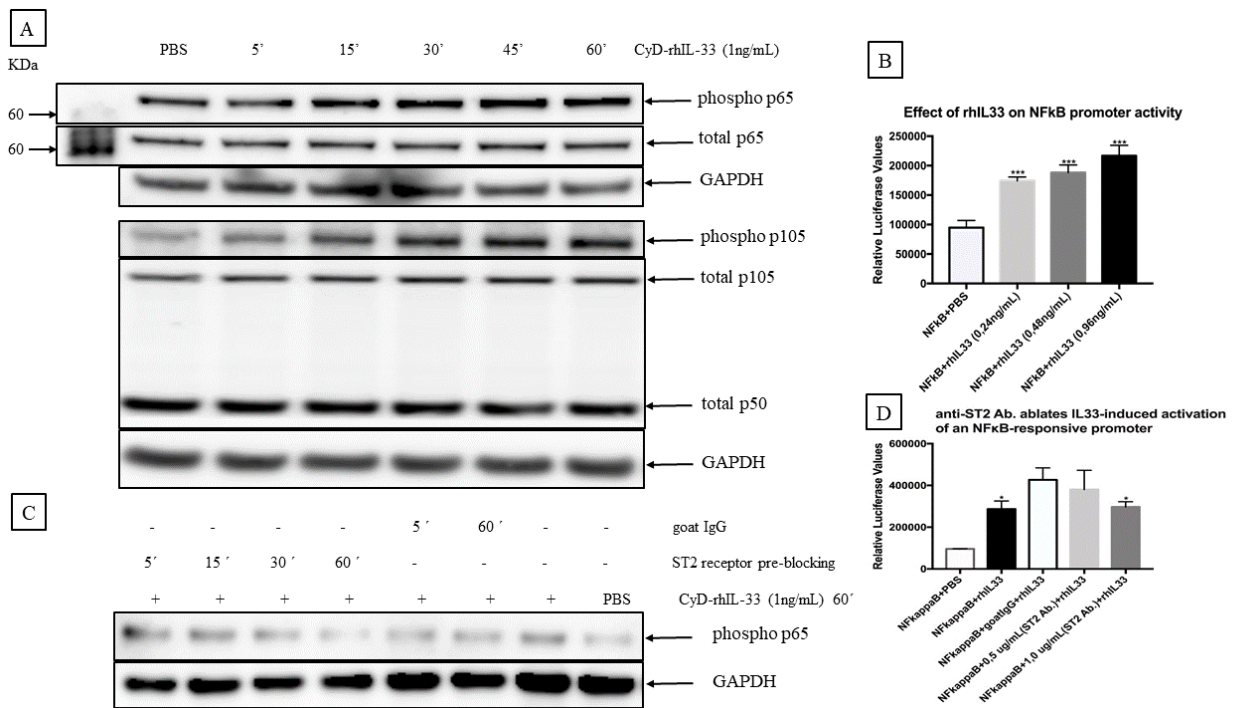


Figure 11: IL-33 upregulates NF-κB activity

(A) NFκB-p65 activation was determined by monitoring phosphorylation of p65 and p105. MCC-13 cells were serum starved for 24 hrs, and then stimulated with 1 ng/ml of CyD-rhIL-33 for varying times 5-60 min. PBS was used as a vehicle control. Relative phospho-p65 and phospho-p105 were determined by western blot using phospho-specific antibodies. (B) NF-κB activity was measured by using a luciferase reporter plasmid containing a NF-κB-responsive promoter. Cells were stimulated with 0.24, 0.48, 0.96 ng/ml for 4 hrs. Luciferase values were normalized to total protein. (C) The blocking of the ST2 receptor interferes with IL-33-induced activation of NF-κB. Phospho p65 levels were determined by western blot. MCC13 were serum starved for 24 hrs and then pre-blocked with goat anti-ST2 antibody (2 ng/mL) or normal goat IgG for varying time duration (5-60 min). The cells were then stimulated with CyD-rhIL-33 (1 ng/ml) in the presence or absence of ST2 receptor antibody for 60 min. PBS was used as a control. (D) anti-ST2 antibody ablates IL-33-induced activation of an NF-κB-responsive promoter. Cells were transfected with the luciferase reporter plasmid with an NF-κB responsive promoter and exposed to CyD-rhIL-33 (1 ng/ml) in the presence or without anti-ST2 antibody (0.5 or 1 μg/mL) for 4 hrs. Goat IgG was used as a control. Each bar represents the average of three independent parallels. Luciferase values were corrected for protein concentration of the samples. $P^* \leq 0.05$ $P^{***} \leq 0.001$.

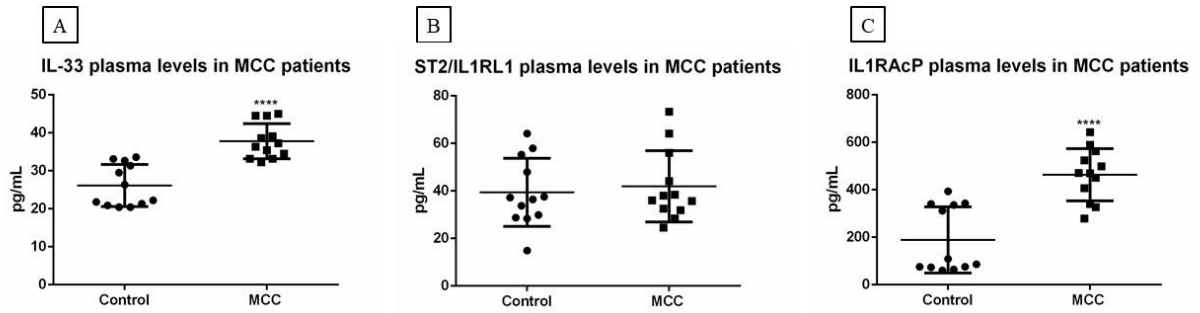


Figure 12: Correlation between IL-33, ST2/IL1RL1 and IL1RAcP levels among MCC patients and control plasma samples.

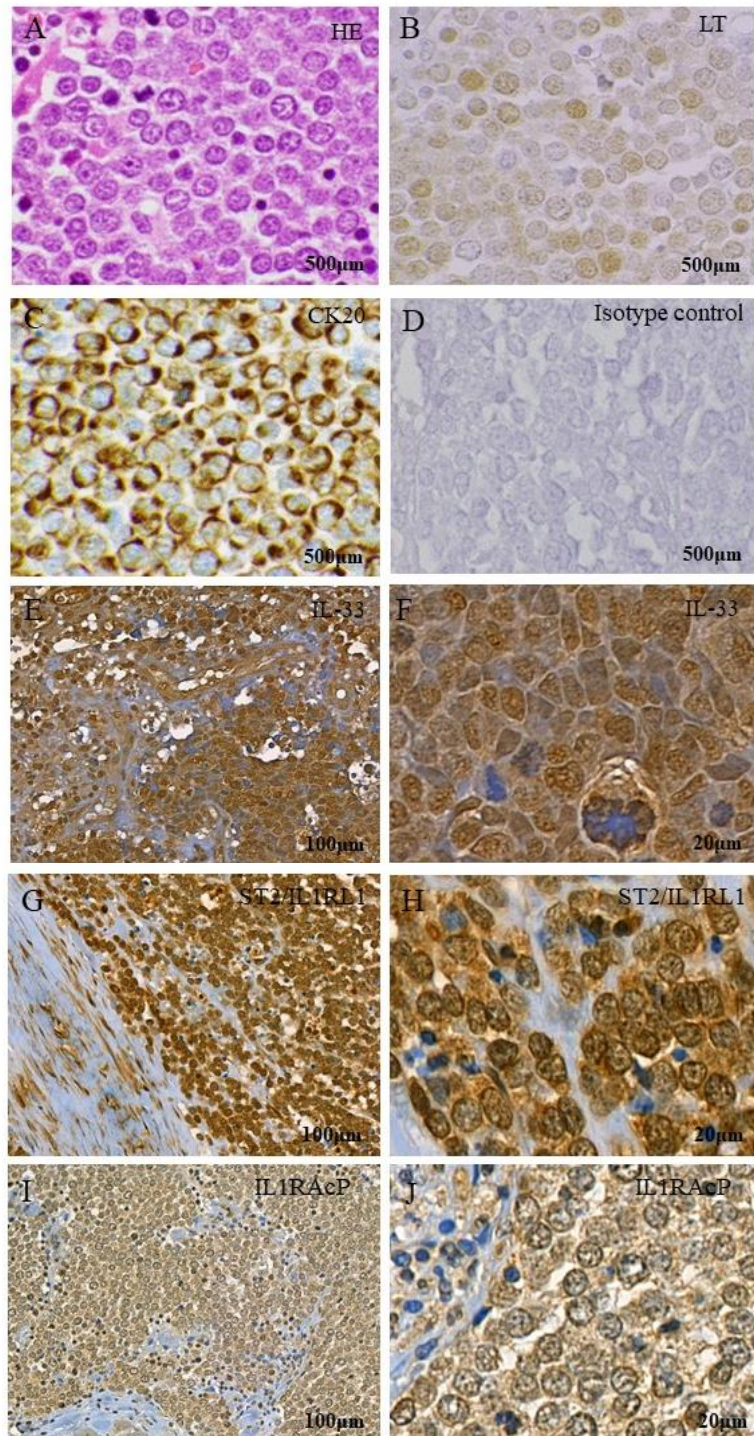


Figure 13: Immunoperoxidase staining of MCPyV-associated MCC primary tumors

(A) HE, (B) LT, (C) CK20, (D) Isotype, (E,F) IL-33, (G,H) ST2/IL1RL1 and (I,J) IL1RAcP. The displayed images are representative stainings from a panel of MCC primary tumors (Scale bar= 500 µm for A, B, C, D; 100 µm for E, G, I and 20 µm for F, G, J).

PAPER IV

Merkel cell polyomavirus large T antigen and small t antigen increase the expression of high-risk human papillomaviruses 16 and 18 E6 and E7 in cervical cancer cells

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Abstract

A causal role of high-risk papillomaviruses (HR-HPV) in anogenital cancers is well-established, while Merkel cell polyomavirus (MCPyV) seems to be an etiological factor in Merkel cell carcinoma. Recent findings reported the co-detection of MCPyV DNA in HR-HPV-positive cervical cancers, though a role for MCPyV in cervical carcinogenesis has not been proven. We investigated whether the MCPyV early proteins large T-antigen (LT) and small antigen (sT) could increase the expression of the HR-HPV E6 and E7 oncoproteins. Transactivation of the HPV16 and the HPV 18 promoters by MCPyV LT and sT was monitored by transient transfection studies in the HPV-negative cervical cancer cell line C33A. The effect of these MCPyV proteins on HPV16 and HPV18 E6 and E7 expression was examined by real-time PCR and western blot in SiHa and HeLa cells, respectively. MCPyV LT and sT stimulated the transcriptional activity of the HPV16 and HPV18 promoters, and induced the expression of HPV E6 and E7 oncoproteins. These results indicate that the co-infection of MCPyV may act as a co-factor in the initiation and/or progression of HPV-induced cervical cancer, or in other HPV-associated cancers.

Keywords: co-infection; promoter; tumor, luciferase assay, cervical cancer

Introduction

Papillomaviridae and *Polyomaviridae* are two families of naked, double-stranded DNA viruses that can cause cancer in humans [1]. High-risk human papillomaviruses (HR-HPV) are predominantly associated with cervical cancer, but also with penile, anal, vulvar, vaginal and oropharyngeal cancers [1, 2]. HPV-16 and HPV-18 are responsible for almost 70% of cervical cancers worldwide, and integration of the viral genome is considered one of the most important risk factors for cervical cancer development [3-5]. The oncogenic potentials of HR-HPV mainly depend on the viral proteins E6 and E7. These proteins can bind p53 and pRb family members, respectively, but can also interfere with other hallmarks of viral oncogenesis (reviewed in [6]). Merkel cell polyomavirus is an etiological factor for Merkel cell carcinoma (MCC), which is an aggressive skin cancer [7]. Approximately 80% of all MCC are positive for MCPyV DNA [8-10], and similarly to HR-HPV in cervical cancer, the MCPyV genome is integrated into all virus-positive MCC examined thus far [3]. The viral oncoproteins of MCPyV are denoted as large tumor antigen (LT) and small tumor antigen (sT) [11]. Equivalently, as seen in HPV, LTag can interfere with the activity of p53 and pRb proteins [12]. This similar effect also suggests that E6, E7 and LT can act together to augment the transformative properties of HR-HPV.

The route of MCPyV infection is not known, though sexual transmission has been suggested since viral DNA has been detected in 37/120 (31%) anogenital mucosa samples of HIV-positive men [13]. Interestingly, 86/120 (72%) of the anogenital mucosa samples were also positive for HR-HPV. These results indicate that MCPyV and HR-HPV may coexist in genital tissue. The co-presence of HR-HPV and MCPyV DNA in both benign and malignant cervical tissue has also been confirmed by other studies. For instance, MCPyV DNA could be amplified in 9/48 (19%) of cervical squamous cell carcinomas, and in 4/16 (25%) of cervical adenocarcinomas. All MCPyV positive specimens were also positive for HR-HPV DNA [14]. Furthermore, a study on 112 cervical samples of Iranian women showed that 40% of the samples were HR-HPV positive. Of these, 14 (35%) were also MCPyV positive [15]. Even so, the authors did not find a significant association between concomitant infection and the grade of cervical lesions, with less than one copy number/cell of the MCPyV genome found. Normal cervical tissue was not examined for the presence of MCPyV. The coexistence of HR-HPV and MCPyV in precancerous and cancerous cervical lesions of women with or without HIV has also been reported [16]. The authors detected HR-HPV in 124 out of 140 (88.6%) cervical samples from HIV-positive African women, as well as in 24 out of 50 (48%) samples from HIV-negative

French women. Of the 148 HR-HPV samples, 81 (55%) were also positive for MCPyV, but there was no significant difference with HR-HPV negative samples, of which 24/42 (57%) were MCPyV positive.

Although the relationship between MCPyV and cervical cancer has not yet been clarified, the frequent detection of MCPyV DNA in HR-HPV positive cervical tumor specimens suggests that MCPyV may promote HPV-induced cervical cancer. This prompted us to investigate whether the MCPyV oncoproteins LT and sT could stimulate the expression of the major HR-HPV oncoproteins E6 and E7, thereby accelerating the initiation and progression of HR-HPV-induced oncogenesis.

Materials and methods

Cell lines

The human cell lines C33A (human papilloma virus-negative cervical cancer) and HeLa (HR-HPV18-positive cervical cancer) were obtained from the American Type Culture Collection (cat. nos. CCL-2 and HTB-31, respectively). SiHa cells (HPV16-positive) were a kind gift from Moe Bjørn Thorvald Greve (University Hospital of Northern Norway). HeLa cells were maintained in Dulbecco's Modified Eagle's Medium (Sigma D5796) with 10% foetal bovine serum (Life Technologies, cat. no. 10500-064), and SiHa cells were cultivated in RPMI1604 medium with 10% foetal bovine serum. Cells were kept at 37°C in a humidified CO₂ incubator.

Plasmids

The HPV16 luciferase reporter plasmid (pGL-LCR-HPV16-LCR (nt 7000-100-luciferase) and the HPV18 luciferase reporter plasmid (pGL4.20 HPV18-LCR luciferase) were obtained from Addgene (cat. no. #32888 and #22859, respectively). The luciferase reporter plasmid with the HPV16 long control region (LCR) contains nucleotides 7000 to 100 (GenBank accession NC_001526.4; [17]), whereas the luciferase reporter plasmid with the HPV18 LCR encompasses nucleotides 6943 to 105 (GenBank accession X05015; [18]). Using these plasmids, we analyzed luciferase activity driven by these LCR. The empty expression vector pcDNA3.1(+) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). The MCPyV LT expression vector was obtained from Addgene, while the MCPyV sT expression plasmid was a kind gift of Dr. Andrew Macdonald [19]. The expression vectors for MKL1 and MKL2 LT have been previously described [20].

Transfection

Cells were seeded out in 12-well cell culture plates so that they were approximately 70% confluent the next day when they were transfected. JetPrime (Polyplus-transfection, Illkirch, France) was used as the transfection reagent, with cells transfected with 400 ng luciferase plasmid according to the manufacturer's instructions. In the experiments in which the effect of LT or sT on promoter activity was monitored, co-transfection was performed with increasing amounts of LT and/or sT expression plasmid being used. The amount of DNA in the different experiments was kept constant by adding the appropriate amount of empty vector pcDNA3.1(+).

Firefly luciferase assay

Cells were lysed 24 h post-transfection in 100 μ l of Luciferase Assay Tropix Lysis solution (Applied Biosystems, Foster City, CA, USA), with 0.5 mM DTT (Sigma-Aldrich Norway AS) freshly added. Cells were scraped and transferred to Eppendorf tubes, followed by 3 min of centrifugation at 12,000 g. Twenty μ l of supernatant were transferred to a 96-well microtiter plate, and luciferase buffer (Promega, Madison, WI, USA) was added. Light units were measured using the CLARIONstar (BMG Labtech GmbH, Ortenberg, Germany).

RNA isolation and cDNA synthesis

RNA extraction was done by using an RNeasy® Plus Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. For RNA yield and quality, A260/A280 and A260/A230 ratios were analyzed with a Nano-Drop® ND-2000 spectrophotometer (NanoDrop Technologies, ThermoFisher Scientific, Waltham, MA, USA). An iScript™ cDNA Synthesis Kit (BioRad, Hercules, CA, USA) was used to make cDNA. A total of 100 ng of RNA was used to generate cDNA according to the manufacturer's instructions. PCR with the housekeeping APRT primers (5'-CCCGAGGCTTCCTCTTTGGC-3' and 5'-CTCCCTGCCCTTAAGCGAGG-3') were used to check for genomic DNA contamination in the cDNA prep. An 800 base-pair fragment was obtained with genomic DNA as a template, while a 300 base-pair amplicon was obtained with cDNA [21]. PCR products were visualized on a 1% agarose gel stained with a GelRed® Nucleic Acid Gel Stain (Biotium, Cambridge Bioscience, Bar Hill, UK).

Real-time quantitative PCR

HeLa (SiHa, respectively) cells were transfected with 500 ng LT or/and sT expression plasmid. The total amount of transfected DNA was kept constant at 1 µg by adding the appropriate amount of empty vector DNA. The transcript levels of HPV18E6/E7 and HPV16E6/E7 were measured by real-time quantitative RT-PCR, using an ABI PRISM® 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The expression level was measured by using a JOE/FAM-labeled TaqMan gene expression assay HPV18E6/E7 and HPV16E6/E7 probe/primer (Table 1), and the expression level was normalized by using a VIC/MGB probe/primer eukaryotic 18S rRNA endogenous control (cat. no. #4319413E, Applied Biosystems). PCR reactions were prepared in a total volume of 25 µl, with a final concentration of 1X TaqMan™ Fast Advanced Master Mix (cat. no. #4444557, Applied Biosystems) and cDNA from 100 ng total RNA.

Protein concentration assay

The luciferase values of each sample were corrected for protein concentration, as measured by the Protein Quantification Assay from Macherey-Nagel (Düren, Germany) according to the instructions of the manufacturer. OD570 was measured using the CLARIOstar Plus Microplate Reader (Biogen Cientifica Madrid Spain). We corrected luciferase values by measuring the protein concentration in the corresponding sample rather than co-transfection with a Renilla reporter plasmid to avoid promoter interference between the HPV promoter directing expression of the firefly *luciferase* gene and a promoter controlling expression of the Renilla *luciferase* gene. In addition, many of our transfection studies include co-transfection with LT and/or sT expression plasmids, containing the strong competing CMV promoter. Moreover, LT of polyomaviruses have shown activate many promoters, including the SV40 and the herpes simplex virus thymidine kinase promoters, which are used in Renilla reporter plasmids [22].

Western blotting

Cells were plated out in 6-well plates and transfected the next day with 2 µg of DNA (either empty vector pcDNA3.1 or LT and/or sT expression plasmid) using JetPrime. Twenty-four hours after transfection, the cells were briefly washed with phosphate-buffered saline (PBS, Biochrom GmbH), and harvested in NuPage LDS sample buffer (Invitrogen, Carlsbad, CA, USA) with 100mM of DTT. The samples were then sonicated and heated for 10 min at 70°C. Proteins were separated on NuPAGE™ Novex™ 4-12% Bis-Tris Protein Gels (Thermo Fisher

Scientific Inc.), and transferred onto a 0.45µm PVDF Membrane (Merck Life Science AS, Oslo, Norway). The membrane was blocked in TBST (Tris-buffered saline with 0.1% Tween-20; Sigma-Aldrich Norway AS) containing 5% (w/v) skimmed milk powder. Incubation with the primary HPV16 E6 antibody (Santa Cruz Biotechnology Inc., Dallas TX, USA; cat. no. sc-460), HPV18 E6 antibody (Santa Cruz; cat. no. sc-365089), HPV16 E7 (Santa Cruz; cat. no. sc-65711) and HPV18 E7 antibody (Santa Cruz; cat. no. sc-365035) was done overnight at 4°C in a blocking buffer. Following three washes in TBST, the membrane was incubated with the polyclonal swine anti-rabbit secondary antibody conjugated with an alkaline phosphatase (D0306, Dako, Santa Clara, CA, USA) solution for 1h at room temperature. After four washes, detection and visualization were performed using CDP-Star chemiluminiscent (C0712, Sigma-Aldrich) and the ImageQuant LAS 4000 imager (GE Healthcare, Oslo, Norway). MagicMark™ XP Western Protein Standard (Thermo Fisher Scientific Inc.) was used to estimate the molecular mass of the detected proteins, with ERK2 levels serving as loading control. Subsequently, a western blot was performed with anti-ERK2 antibody (SC-154) as described above.

Statistical analysis

The *t*-test was employed to determine statistical differences between the promoter activities.

Results

MCPyV LT and sT increase the HPV16 and HPV18 promoter activity

MCPyV DNA can be co-detected in HR-HPV-positive cervical carcinoma samples [14-16], and the LT and sT of polyomaviruses can affect heterologous promoters [23]. Hence, we wanted to investigate whether these polyomavirus proteins had an effect on the activity of HR-HPV promoters. First, the relative promoter strength of HPV16 and HPV18, the two most common HPVs in cervical cancer [24], was compared in the HPV-negative cell line C33A. The HPV18 promoter was 30-50x stronger than the HPV16 promoter in these cells (Figure 1).

Next, we tested the effect of MCPyV LT and/or sT on the HPV16 and HPV18 promoter activity in this cell line. LT as well as sT were able to *trans*-activate the HPV18 promoter (Figure 2A). A moderate (~2-fold) but highly significant induction of the promoters was measured. The simultaneous expression of LT and sT did not result in an additive effect. High concentrations (1200 ng of expression plasmid per transfection) of LT or sT resulted in a reduction of the HPV18 promoter activity. A similar increase (~2-fold) in HPV16 promoter activity as with the HPV18 promoter was measured in the presence of LT or of sT (Figure 2B). Higher LT or sT concentrations also inhibited the HPV16 promoter activity.

All examined MCPyV-positive MCCs express a truncated version of LT due to a nonsense mutation in the gene [25-31]. We therefore tested the effect of two C-terminal truncated LT versions identified in the MCC cell line MKL1 and MKL2 on the HPV16 and HPV18 promoter. The truncated LT variants lack their DNA binding domain [25, 32]. Similar to full-length LT, truncated MKL-1 and MKL-2 LT variants stimulated the HPV16 and HPV18 promoters with comparable levels. While no significant differences were observed for full-length LT, MKL-1, and MKL-2 on the HPV16 promoter, full-length LT was a stronger trans-activator ($p < 0.05$) than MKL-1 and MKL-2 of the HPV18 promoter (Figure 3).

MCPyV LT and sT increase the protein levels of E6 and E7

E6 and E7 are the two major oncoproteins involved in tumorigenesis by HR-HPV [33-36]. They are translated from a bicistronic mRNA with the E6 open reading frame (ORF) proximal and the E7 ORF distal. Ribosomal readthrough of the E6 ORF allows the E7 protein to be synthesized at a rate of approximately 25-30% of the E6 protein level [37]. Forty-eight hrs after transfection of SiHa cells with expression plasmids encoding sT, full-length LT, truncated MKL-1 or MKL-2 LT, or a combination of sT and LT, HPV16 E6 and E7 mRNA levels

significantly increased 1.5- to 2-fold (except for MKL2 LT plus sT where a 4- to 6-fold increase was measured) compared with empty vector. HPV18 E6 and E7 mRNA levels in HeLa cells were also significantly induced (3- to 5-fold) by all three LT variants alone or in combination with sT, except for sT alone, which had no effect on E6 mRNA levels and reduced E7 mRNA levels by 30% (Figure 4).

Because of the different translation efficiency of the E6 and E7 ORFs and because the oncogenic activities are attributed to the proteins rather than to their mRNA, we examined the E6 and E7 protein levels in non-transfected SiHa (HPV16-positive) and HeLa (HPV18-positive) cells, and in cells transiently transfected with an expression plasmid for MCPyV LT and/or sT. Because MCPyV expresses C-terminal truncated LT in MCC [8], we also tested the effect of the truncated LT variants MKL1 and MKL2 on E6 and E7 levels in HeLa and SiHa cells. Western blot analysis showed that the expression of full-length LT or truncated versions of LT increased HPV18 E6 levels ~1.5-fold 24 and 48 h after transfection, whereas sT stimulated HPV E6 levels approximately 1.5-fold 48 h after transfection (Figure 5A). Co-expression of LT (truncated or full length) plus sT did not cause a further increase in E6 levels compared to LT or sT alone. For HPV18 E7 levels, a 2- to 3.5-fold increase was observed in the presence of the LT (full-length and MKL-1 and MKL-2 truncated variants) and/or sT but only 24 h after transfection. No increase in E7 levels were detected 48 h after transfection (Figure 5B). Again, co-expression of LT and sT did not further enhance HPV18 E7 protein levels. The expression of full-length LT (resp. sT) in SiHA cells resulted in a 1.5-fold (resp. 2-fold increase in HPV16 E6 protein levels 24 h post transfection) and a 3-fold (resp. 2-fold) increase 48 h after transfection (Figure 5C). Again, no additional stimulation was observed when LT and sT were co-expressed. The truncated LTs were more potent than full length LT to stimulate the protein levels of HPV16 E6 (Figure 5C). The levels of HPV16 E6 in the presence of MCPyV early proteins were lower 48 h after transfection compared to 24 h post transfection, but still higher than the control cells transfected with empty vector. An approximately 2-fold increase in HPV16 E7 levels was measured in the presence of LT (full length and truncated) or sT 24h and 48 h after transfection. Co-expression of LT and sT did not further enhance the E7 protein levels (Figure 5D).

Discussion

HR-HPV16 and HPV18 are the two most common HPVs in cervical cancer [24]. Because MCPyV DNA is frequently found in samples of cervical cancers [14-16], we investigated whether MCPyV could affect the promoter activity of HPV16 and HPV18 in cervical cancer cell lines. Comparing the basal transcriptional activity of the HPV16 and HPV18 LCR in the HPV-negative cervical cancer cell line C33A showed that the activity of the HPV18 promoter was 30-50 times stronger than the HPV16 promoter, which is in agreement with other studies. Chen *et al.* also found that the basal transcriptional activity of the HPV18 LCR was 3x higher than that of HPV16 LCR in HeLa cells, and 3.2x in the breast cancer T47D cells [38]. Ottinger *et al.* compared the basal activities of the HPV18 and HPV16 LCR in six different cell lines, including C33A and HeLa and demonstrated that the HPV18 LCR activity was 20-fold stronger in C33A and 7-fold stronger in HeLa cells [17]. Moreover, SiHa cells have 1-2 copy numbers of HPV16, whereas HeLa cells contain 17-35 copy numbers of HPV18 [39]. Transient expression of MCPyV ST, full-length LT, truncated MKL-1 or MKL-2 LT, or a combination of ST and LT resulted in a significant increase in HPV16 and HPV18 E6 and E7 mRNA levels, which is in accordance with the ability of these proteins to stimulate the transcriptional activity of the HPV16 and HPV18 LCR. In the luciferase reporter assay, sT was able to potentiate the HPV18 LCR activity, yet sT had no or minor effect on HPV18 E6 and E7 mRNA level (Figure 4). We do not know the reason for this discrepancy, but could be due to the different method. In the luciferase assay, enzyme activity in form of light units produced by the conversion of luciferin into oxyluciferin are measured, while the qRT-PCR determines E6/E7 mRNA copy numbers. Although the E6 and E7 ORFs are on the same mRNA, differences in relative E6 and E7 mRNA levels were measured. This is in accordance with other studies (e.g. [40-42]).

Despite the weaker promoter activity and the lower number of HPV16 genome copies, we found comparable E6 and E7 proteins levels in the HR-HPV18-positive HeLa cells and the HR-HPV16-positive SiHa cells. This has also been confirmed in other studies [39, 43-48]. The reason for this may be that the comparison of the HPV16 and HPV18 LCR transcriptional activity was done with an isolated promoter in a transient transfection assay in C33A cells, while in HeLa and SiHa cells, respectively, the promoter is integrated and the conformation of the chromatin (tightly packed or more open) and methylation pattern may have affected the transcriptional activity of the promoter and hence the expression of the *E6* and *E7* genes. Nevertheless, we cannot exclude that the use of different antibodies may have had an effect on

the sensitivity of detecting target proteins (i.e. HPV16 E6 and E7 and HPV18 E6 and E7), a well-known phenomenon [49-51].

Our results demonstrate that both MCPyV LT and MCPyV sT stimulated the transcriptional activity of the HPV16 and HPV18 promoter in a dose-dependent manner. A moderate increase (2- to 3.5-fold) was observed. Previous studies with the HPV16 and HPV18 promoter in other cell lines showed a variable stimulating effect of LT and sT of the polyomavirus SV40. SV40 LT increased HPV18 promoter activity 13-fold in HeLa cells, ~3-fold in SW13 (human adrenocortical carcinoma) and in 3T6 (mouse fibroblasts) cells, but had no effect in monkey kidney CV-1 cells [52]. In human keratinocytes, a 9-fold increase in HPV18 promoter strength was observed in the simultaneous presence of LT and sT [53]. In human embryonal fibroblasts, the HPV16 promoter activity was stimulated 20- to 30-fold by SV40 sT, while the effect of LT was 5- to 6-fold weaker than sT [54]. These experiments with SV40 LT and sT lacked dose-dependent studies. We found that increasing the concentration of the LT and sT expression plasmids eventually resulted in the inhibition of HPV16 and HPV18 promoter activity. This reduction in promoter activity occurred at lower concentrations of LT and sT expression plasmids for the HPV16 promoter compared to the HPV18 promoter (Figure 2). The difference in HPV16 and HPV18 promoter strength in C33A cells depends on the transcription factors that regulate the promoter activity. The HPV16 and HPV18 promoters do not show significant sequence homology [55] and they possess exclusive binding sites for different transcription factors [56]. One possible explanation could be that the CMV promoter, which directs the expression of LT and sT (see Materials and Methods) and has a stronger activity than the HPV16 and HPV18 promoters [57], usurps mutual transcription factors that regulate the activity of the HPV16 (respectively HPV18) and CMV promoters. The CMV promoter may sequester more transcription factors from the HPV16 promoter compared to the HPV18 promoter, resulting in an inhibition of the HPV16 promoter activity at lower concentrations of LT/sT expression plasmids.

MCPyV early proteins moderately (~2-fold) induced the E6 and E7 protein levels of HR-HPVs 16 and 18 (Figure 4). It should be noticed that, although both proteins are translated from the same transcript, differences in E6 and E7 protein levels were monitored both in the absence and presence of LT and/or sT. This has also been observed in other studies [48, 58-62]. The difference could have a technical explanation because the antibodies directed against respectively E6 and E7 may have different affinity. Another explanation may be the differences in half-life of the E6 and E7 proteins [63-65]. Another possibility is that LT and/or sT have a different impact on the stability of E6 and E7 proteins. It is not known whether LT or sT have an impact on the

stability of E6 and E7 proteins, but sT of MCPyV has been shown to prevent degradation of MCPyV LT through inhibition of the ubiquitin ligase complex SCF^{Fbw7} [66]. HPV16 E7 has been shown to be a substrate of the ubiquitin ligase complex SCF [67].

The biological importance of this restricted increase in E6 and E7 levels, and hence the indirect role of MCPyV in HPV-induced cervical cancer can be discussed. However, all cancers induced by human oncoviruses have a very long incubation time [2]. Therefore, the long-term moderate MCPyV-induced increased expression of E6 and E7, two potent oncogenes that can transform cells *in vitro* and induce tumors in animal models [68, 69], may enhance the tumorigenic process by these HPV oncoproteins in human cervical cancer. The co-infection of cervical cancer with HPV and other viruses has been reported [70, 71], but the effect of viral proteins on HPV E6 and E7 has not always been studied. Infection of the SiHa or CaSki cells (both HPV16 positive) with Kaposi's sarcoma herpesvirus led to a 50-60% reduction in HPV16 E6 and E7 levels [72, 73], while HIV Tat protein increased HPV16 E6 protein levels 1.35-fold in SiHa cells, but did not upregulate E7 protein levels [74]. A 3-fold increase in E6 mRNA levels was observed in HSV-2 infected CaSki (HPV16 positive) cells compared to uninfected cells [75], whereas the infection of HeLa cells with HSV-1 resulted in a 50-fold reduction of E6 mRNA [76].

The mechanism by which MCPyV LT and sT stimulates the expression of HPV18 E6 and E7 is unknown. LT can activate its own promoter and heterogeneous promoters by binding to adjacently repeated G^A/GGGC motifs [23]. Yet, a sequence analysis of the HPV16 and HPV18 promoters did not reveal repeated LT binding motifs. Moreover, the C-terminal truncated LT variants MKL1 and MKL2, which lack the DNA binding domain still activated the HPV16 and HPV18 promoters (Figure 3) and enhanced the E6 and E7 protein levels (Figure 4). LT and sT of MCPyV have been shown to modulate the activity of transcription factors (see supplementary Tables S2 and S3 in [23]), and may hence stimulate the HPV16 and HPV18 promoter activity indirectly without binding to DNA.

The co-infection of MCPyV and HR-HPV has been detected in other malignancies, including squamous cell carcinoma patients [77-81], tonsillar carcinoma [82], epidermodysplasia verruciformis [83], epithelial proliferations [84] and actinic cheilitis [85]. Whether MCPyV affects the expression of HPV oncoproteins and plays a role in these cancers, remains unknown. To unambiguously establish a role of MCPyV in HPV-induced cervical and other cancers, the state and sequence of genomes of MCPyV, and LT and sT expression in these tumors should be determined. MCPyV DNA is found to be integrated and a truncated LT is expressed in MCCs [7, 8], but it is not known whether MCPyV DNA is integrated and C-terminal truncated LTA_g

is expressed in HPV-positive cancers. It should also be examined whether more pathogenic MCPyV variants with e.g. changes in the promoter region, LT or ST are present in HPV positive cancers. Furthermore, the absence of MCPyV in the majority of cervical and other cancers examined so far does not rule out the involvement of this virus as a co-factor in HPV-positive cancers because MCPyV may be required for the initiation of the tumorigenic process. Indeed, a hit-and-run mechanism for MCPyV triggered tumorigenesis has been suggested [86].

Two other human polyomaviruses have been linked to cancer. JCPyV has been associated with colorectal cancer and the viral genome was integrated in the genome of the tumor cells and LT, but not late proteins were expressed [87]. An oncogenic role for BKPyV in renal carcinomas and cancers of the urogenital tract has been suggested [88-90]. For BKPyV-positive tumors, integrated, episomal viral DNA or both were observed and LT was expressed. The virus was replication deficient because late proteins were absent, dysfunctional or incomplete expressed. Expression of the late genes encoding the capsid proteins was impaired due to deletions in the non-coding control region [91]. Whether MCPyV on its own can induce cervical cancer remains to be proven. In analogy with JCPyV and BKPyV-induced tumors, the expression of LT and late proteins should be examined, the state of the viral genome should be determined and the non-coding region should be sequenced. In the few cases of cervical cancer where MCPyV DNA was present in a few, LT protein was not always detected and late gene expression was been monitored (10.1186/1743-422X-9-154; 10.1007/s00705-015-2368-4; 10.1136/sextrans-2015-052430). Moreover, the viral genome copy number per tumor cell (0.00008 to 0.0021) was several logs lower than the viral genome copy number in MCCs, which have an average of 5.2 (range 0.8-14.3) copies per cell [92]. The low copy number may suggest that the viral genome is not integrated in MCPyV-positive cervical cancers, but this has not been investigated. The non-coding region of MCPyV in virus-positive cervical tumors has not been sequenced. Finally, PCR was used to detect the co-presence of MCPyV DNA in HPV-positive cervical tumors, but it has not been examined whether the viruses actually co-infect the tumor cells or whether MCPyV DNA was present in other cells of the tumor microenvironment.

In conclusion, the tumorigenic human polyomavirus MCPyV can occasionally be found in cervical cancers. Its oncoproteins LT and sT can stimulate the activity of the HR-HPV16 and 18 promoters, and therefore increase the transcript and protein levels of the oncoproteins E6 and E7 in cervical cancer cell lines. This suggests that MCPyV may collaborate to transform cervical tissue. Further studies are warranted to prove a possible role of MCPyV in HR-HPV-induced cervical carcinoma.

Authors and contributions

Conceptualization, B.S. and U.M.; methodology, K.R. and U.M.; validation, K.R., B.S. and U.M.; investigation, K.R. and U.M.; data curation, K.R., B.S. and U.M.; writing—original draft preparation, U.M.; writing—review and editing, K.R., B.S. and U.M.; visualization, K.R. and U.M.; supervision, B.S. and U.M.; project administration, U.M.; funding acquisition, B.S. and U.M.

Conflicts of interest

The authors declare that there are no conflict of interest.

Ethical approval

Not applicable

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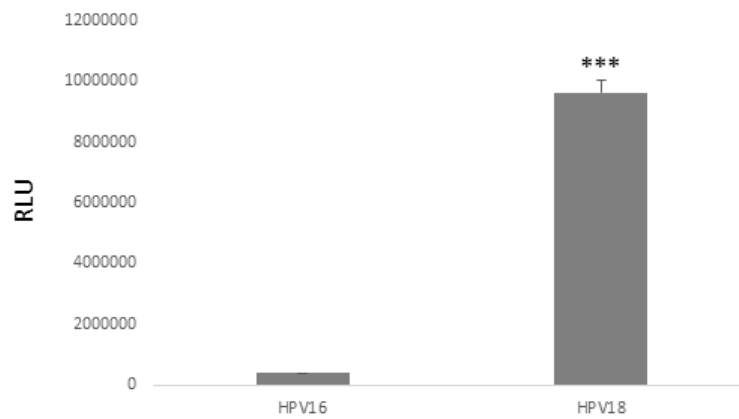


Figure 1. The basal HPV18 promoter is stronger than the HPV16 promoter in C33A cells.

HPV-negative C33A cells were transiently transfected with 400 ng/well of a luciferase reporter plasmid containing the long control region (LCR) of HPV16 or HPV18. Cells were harvested 24 h after transfection and luciferase activity was determined. The luciferase values were corrected for the protein content in each sample, and are expressed as relative luciferase units (RLU). The bars represent the average of three independent parallel \pm SD (** $p < 0.001$). Similar results were obtained in two additional experiments. RLU = relative luciferase units; EV= empty vector (pcDNA3.1).

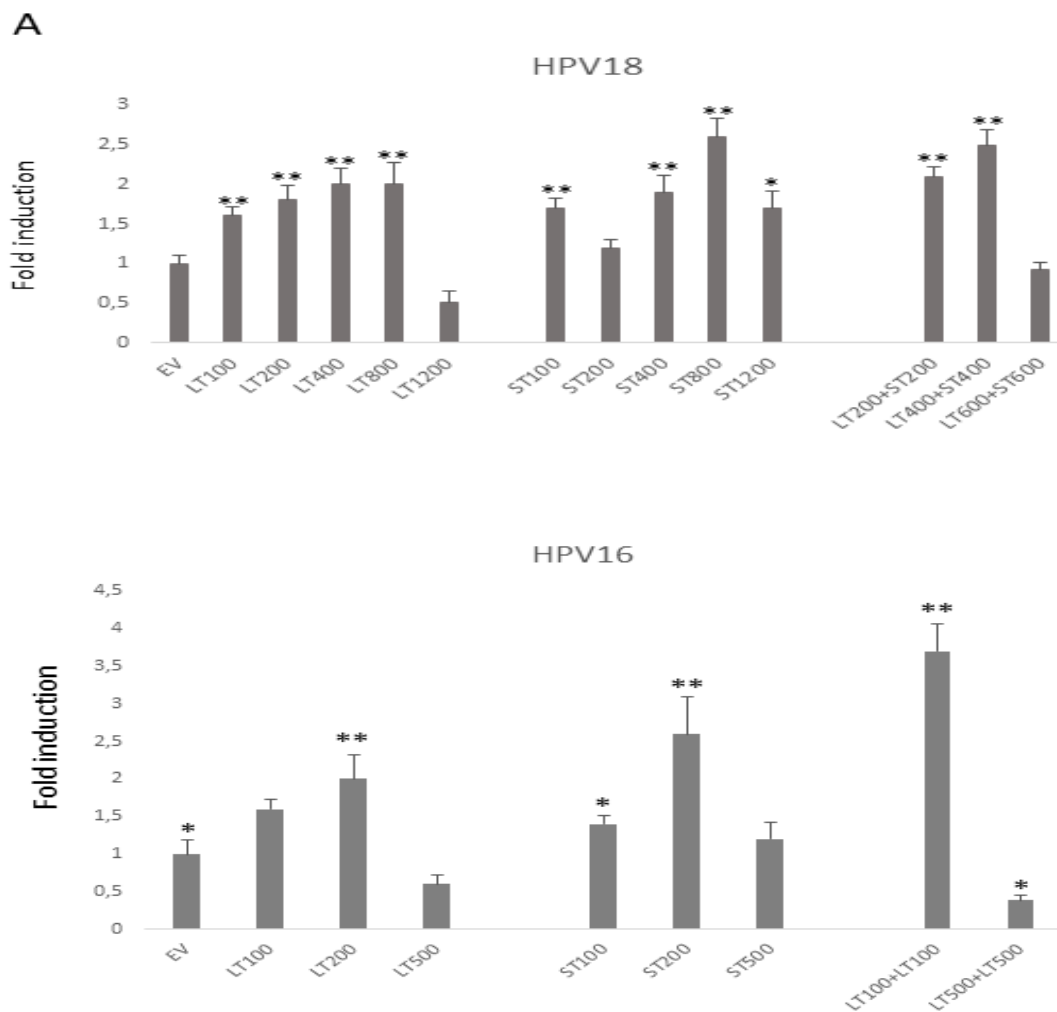


Figure 2. MCPyV LT and ST stimulate the HPV16 and HPV18 promoter activity. (A) C33A cells were co-transfected with a reporter plasmid containing the luciferase gene under control of the HPV18 promoter and increasing amounts of MCPyV LTA_g and/or STA_g expression plasmid. The amount of DNA in each sample was kept constant by adding an appropriate concentration of empty vector pcDNA3.1(+). Each bar represents the average+SD of 3-5 separate experiments performed with three independent parallels. Luciferase activity was corrected for the protein content in each sample. The corrected luciferase activity in the presence of an empty vector was arbitrarily set as 1.0, with the promoter activity in the presence of LTA_g and/or STA_g shown as fold induction. (B) as in (A) but the effect of MCPyV LTA_g and STA_g was tested on the HPV16 promoter. For each well, cells were co-transfected with 400 ng of luciferase plasmid and different concentrations of expression plasmid of LTA_g or/and STA_g. The total amount of DNA in each sample was kept constant by adding empty vector DNA. EV= empty vector (pcDNA3.1). * $p < 0.05$; ** $p < 0.01$.

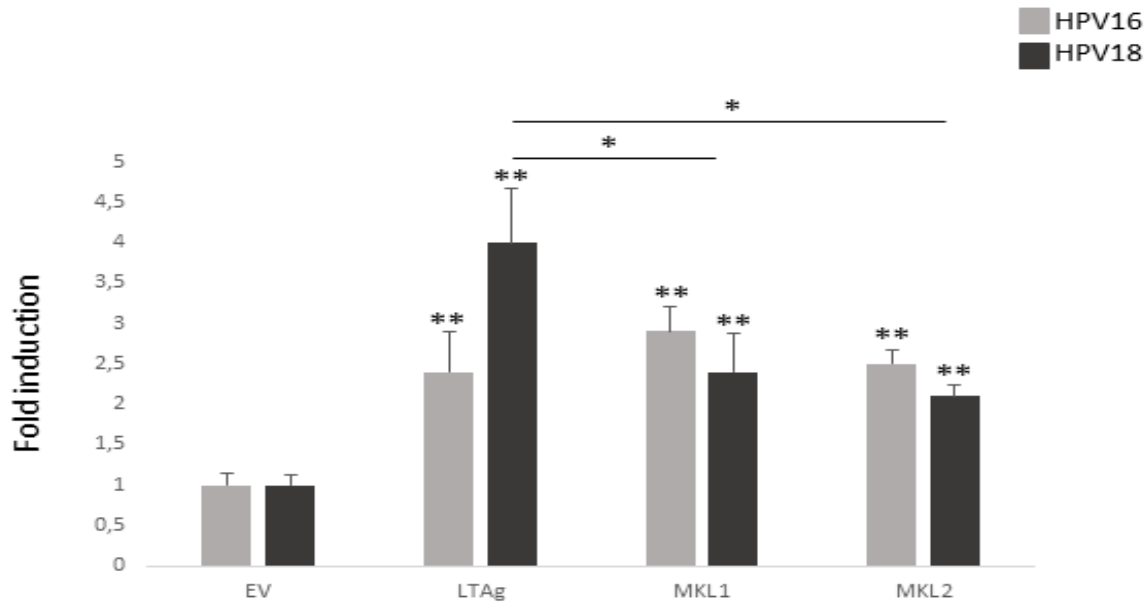


Figure 3. Full-length and truncated LTA activate the HPV16 and HPV18 promoters.

Each well with C33A cells was co-transfected with 400 ng of luciferase reporter plasmid with either the HPV16 or the HPV18 promoter and with 200 ng of empty vector (EV) or expression plasmid for full-length LTA (LTA) or truncated versions MKL1 or MKL2. Luciferase activities were standardized to the protein content in the sample and the promoter activity in the presence of empty vector was then arbitrarily set as 1.0. Each bar represents the average of three independent parallels \pm SD (*p<0.05; **p<0.01).

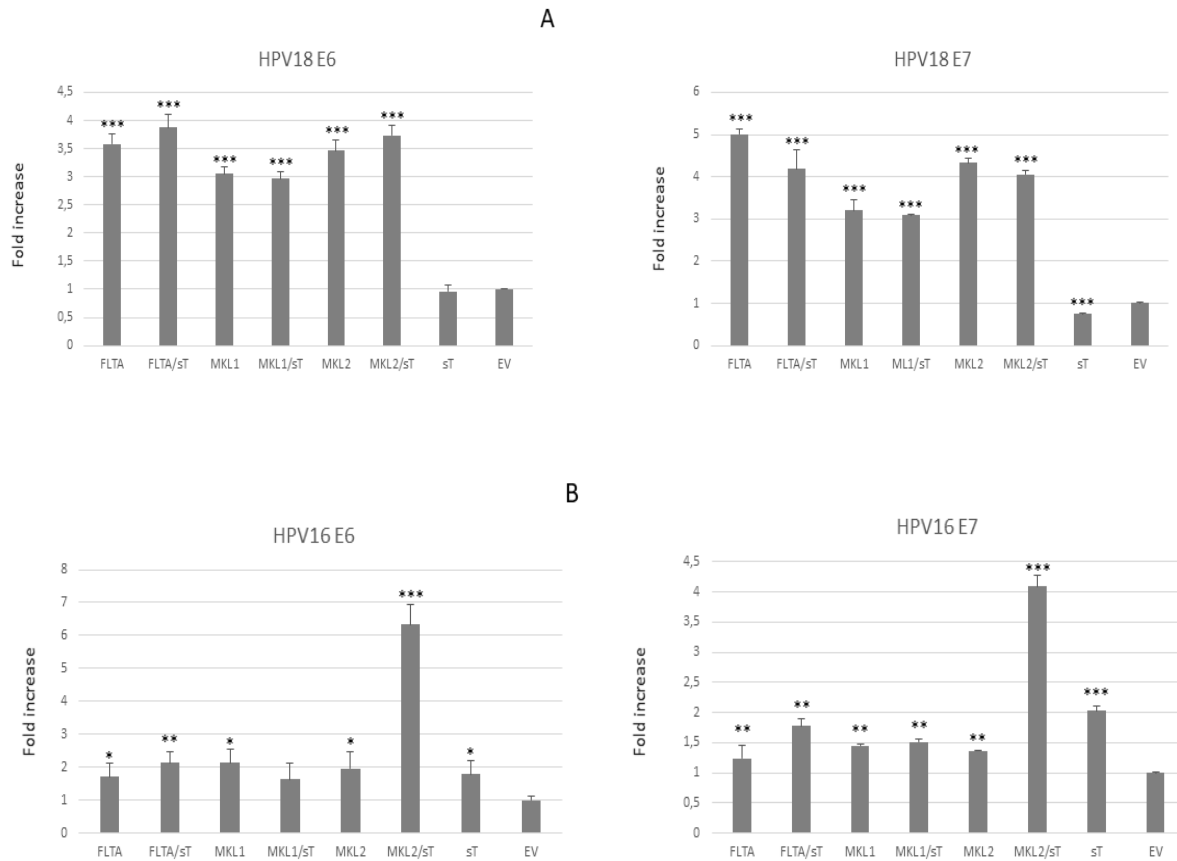


Figure 4. MCPyV LT and ST increase HPV16 and HPV18 E6 and E7 mRNA. (A) HeLa cells were transfected with expression plasmids for full-length LT (FLTA), sT, truncated LT MKL1 or MKL2 or empty expression vector pcDNA3.1 (EV). Total RNA was isolated 48 hrs after transfection and cDNA was generated. qPCR was performed with specific primers for E6 and E7 and 18S rRNA was used to standardize. Relative mRNA levels in cells transfected with empty vector was arbitrarily set as 1. Each bar represent the average of three parallels \pm SD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. (B) as in (A) but SiHa cells were transfected and relative HPV16 E6 and E7 mRNA levels were determined.

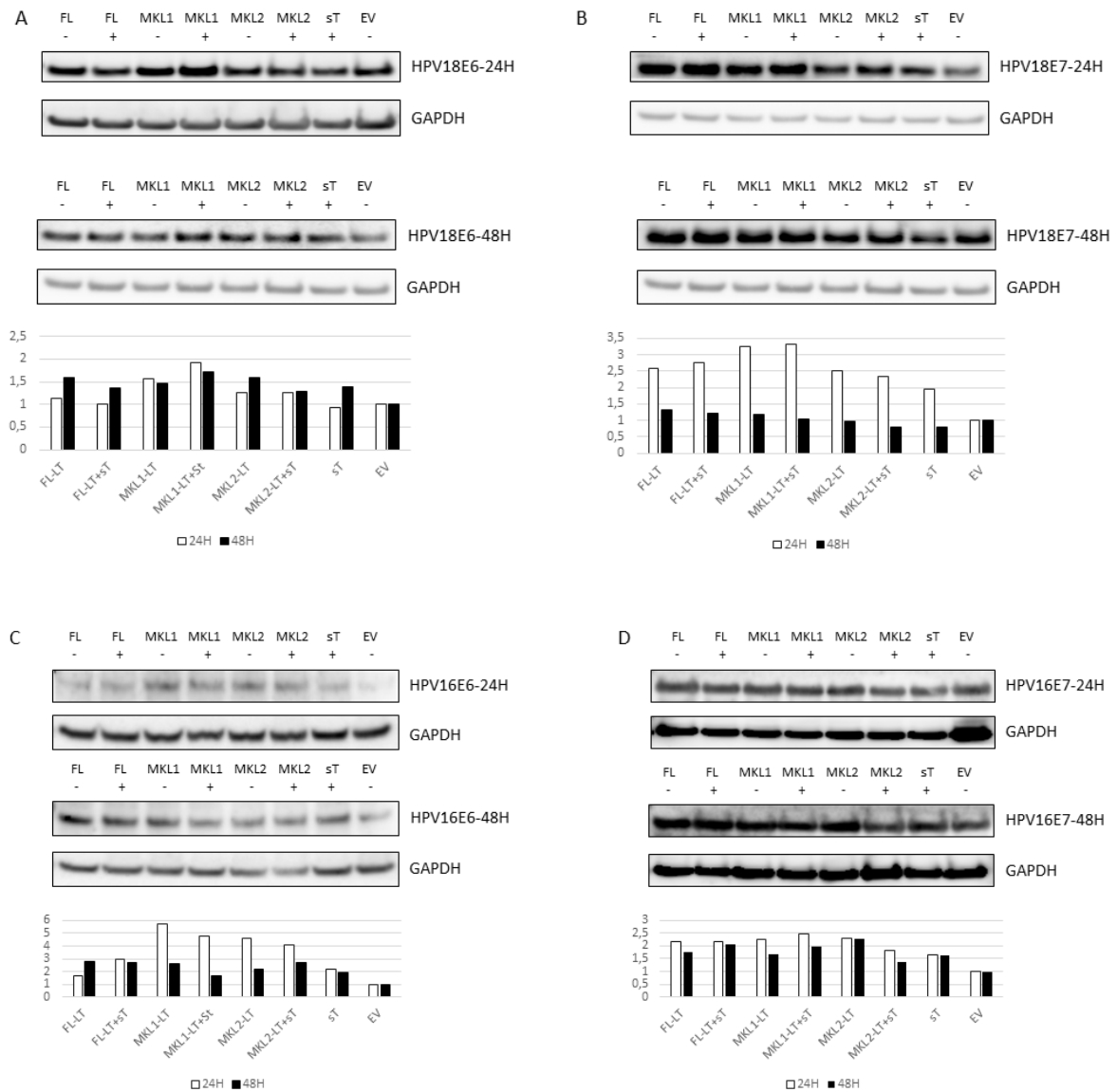


Figure 5. Expression of MCPyV LT and ST augment HPV16 and HPV18 E6 and E7 protein levels. HeLa cells (panels A and B) or SiHa cells (panels C and D) were transfected with the empty expression vector (EV) pcDNA3.1(+) or with an expression plasmid for full-length LT (FL-LT), truncated MKL-1 LT, truncated MKL-2 LT, sT, or a combination of LT plus sT. Five hundred ng of DNA of the expression plasmids for LTag and sTag were used per well. The total amount of plasmid DNA in each well was kept constant at 1 μ g by adjusting with EV DNA. Levels of E6 (panels A and C) and E7 (panels B and D) were monitored by western blot 24 h and 48 h after transfection. Densitometry was used to determine the relative amounts of E6 and E7, with GAPDH levels used for standardization. The relative expression level of E6 (resp. E7) in the presence of empty vector was arbitrarily set as 1. Similar results were obtained in an independent experiment

Table 1: HPV18E6/E7 primer/probe sequence labelled with specific fluorophore

ORF	Sense Primer		Anti-Sense Primer		Labeled Probe	
	Sequence (5'-3')	Conc (nM)	Sequence (5'-3')	Conc (nM)	Fluorophore - Sequence (5'-3')	Conc (nM)
HPV18 E6	AGCTGGGCACTAT AGAGGC	50	TGTGTTTCTCTGCGTCGT TGG	200	JOE-CCATTCGTGCTGCAACCGAGCACGAC- BHQ1	50
HPV18 E7	GAACCACAACGTC ACACAATG	100	CAGAAACAGCTGCTGGA ATG	50	FAM- AGTAGAAAGCTCAGCAGACGACCTTCG- BHQ1	50
HPV16 E6	GCGACCCAGAAAG TTACCACAG	100	CCATCTCTATATACTAT GCATAAATCCCG	100	JOE- TACCTCACGTCGCAGTAACTGTTGCTTG	175
HPV16 E7	AGAACCGGACAGA GCCCATTAC	100	GCCCATTAACAGGTCTT CCAAAG	100	FAM- CGCACAACCGAAGCGTAGAGTCACACTT	50