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Junchao Miao

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The roles of MCPyV early region in IL-17A-mediated inflammation and autophagy

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RESUME

Le carcinome à cellules de Merkel (MCC) est une tumeur maligne cutanée agressive et rare qui touche principalement les patients âgés et immunodéprimés. Le Polyomavirus à cellules de Merkel (MCPyV) est l'agent étiologique de 80 % des MCC. Le génome de MCPyV est intégré de manière clonale dans les cellules MCC. Il est constitué d'une chaîne d'ADN circulaire avec un promoteur bidirectionnel qui dirige la transcription de la région précoce (ER) et de la région tardive. ER code des protéines fonctionnelles comprenant le petit antigène T (sT), le grand antigène T (LT) et deux autres protéines 57kT et ALTO. Les protéines sT et LT sont cruciales pour la réplication virale et présentent un potentiel oncogène impliqué dans la carcinogenèse du MCC, alors que les fonctions de 57kT et ALTO restent méconnues. Dans les cellules MCC, la protéine sT est exprimée sous sa forme sauvage, tandis la protéine LT est déletée de sa séquence C-terminale ce qui donne naissance à une forme tronquée de LT, appelée trLT ou LT de type onco (otLT). Cette troncature C-terminale supprime le domaine hélicase de la LT, empêchant la réplication virale et améliorant son effet de stimulation de la croissance cellulaire. L'expression d'otLT est caractéristique du MCC induit par MCPyV et est essentielle pour le développement de la forme viro-induite du MCC.

L'IL-17A est une cytokine pro-inflammatoire aux fonctions pléiotropes essentielles à l'homéostasie de la peau et des muqueuses. Elle est particulièrement importante pour la défense contre les agents pathogènes de la peau et des muqueuses, qu'il s'agisse de champignons, bactéries ou virus. Elle joue également un rôle essentiel dans la réparation et le remodelage des tissus. Cependant, la dérégulation de l'inflammation qui produit l'IL-17A de façon chronique est délétère, car elle peut conduire à des maladies inflammatoires et favoriser l'oncogenèse. Nous avons montré une induction variable d'IL-17A par les protéines codées par ER avec une forte induction quand LT est sous sa forme sauvage et moins d'induction comparativement, avec otLT. La transition d'une inflammation aiguë d'IL-17A de haut niveau face au LT sauvage à une inflammation chronique d'IL-17A de faible niveau induite par l'otLT pourrait être un événement important qui contribue au développement du MCC.

Au cours de l'infection, la réponse inflammatoire est contrôlée négativement par de multiples mécanismes pour éviter l'inflammation pathologique. L'autophagie est un processus de dégradation conservé dans les cellules Eucaryotes qui joue un rôle essentiel dans le recyclage des matériaux et l'homéostasie cellulaire. Elle est également impliquée dans la régulation de la réponse immunitaire et des maladies, inclus le cancer. Nous avons révélé que MCPyV LT améliorait l'autophagie cellulaire, contrairement à otLT qui ne l'induisait pas. Enfin, nous avons démontré que l'inflammation médiée par l'IL-17A augmentait l'autophagie. À l'inverse, l'activation de l'autophagie inhibe l'inflammation médiée par l'IL-17A, suggérant un rétrocontrôle négatif de l'autophagie pour prévenir une inflammation trop exacerbée. Notre étude suggère que le faible niveau d'IL-17A induit par otLT ne déclenche pas le contrôle négatif médié par l'autophagie, conduisant à une inflammation persistante de l'IL-17A de faible niveau. Cette nouvelle piste physiopathologique du MCC ouvre de nouvelles perspectives de recherche et d'applications cliniques.

ABSTRACT

Merkel cell carcinoma (MCC) is a rare aggressive skin malignancy that affects predominantly elderly and immunocompromised patients. Merkel cell polyomavirus (MCPyV) is the etiologic agent of 80% MCC, the genome of which is clonally integrated into MCC cells. The genome of MCPyV consists of a circular DNA chain divided by a bidirectional promoter that directs the transcription of the early region (ER) and the late region. The MCPyV ER encodes functional proteins including the small T antigen (sT), the large T antigen (LT), the 57kT, and a protein called alternate frame of the large T open reading frame (ALTO). The sT and LT are crucial for viral replication and exhibit oncogenic potential involved in MCC carcinogenesis, whereas the function of 57kT and ALTO remains elusive. In MCC cells, the sT is expressed in an intact form, while the C-terminal sequence of LT is deleted, giving rise to a truncated form of LT, referred to as trLT or onco-type LT (otLT). The C-terminal truncation removes the helicase domain of LT, preventing viral replication and enhancing its cell growth-promoting effect. The expression of otLT is the hallmark of MCPyV-induced MCC and is pivotal for MCC development.

IL-17A is a proinflammatory cytokine with pleiotropic functions essential for the skin and mucous homeostasis. It is specifically crucial for the first-line defense against skin and mucosal pathogens, including fungi, bacteria, as well as viruses, and plays an essential role in tissue repair and remodeling. However, dysregulated and mild chronic IL-17A inflammation is deleterious that can lead to inflammatory diseases and cancer. We showed variable IL-17A induction by MCPyV ER-induced proteins. We observed high induction of IL-17A by LT. Interestingly, otLT induced significantly less IL-17A compared to LT. The transition from the acute high-level IL-17A to the low-grade IL-17A inflammation caused by otLT could be an important event that contributes to MCC development.

During infection, the inflammatory response is negatively controlled by multiple mechanisms to avoid pathological inflammation, including autophagy. Autophagy is a conserved degradative process in eukaryotic cells that plays a critical role in biomacromolecules recycling and cellular homeostasis. It is also involved in the regulation of immune responses and some diseases, such as cancer. We revealed that MCPyV LT enhanced cellular autophagy while otLT did not. Finally, we demonstrated that High level of IL-17A-mediated inflammation, but not low level, increased autophagy. Conversely, autophagy activation inhibits IL-17A-mediated inflammation, suggesting negative feedback of autophagy to prevent over-exuberant inflammation. Our study suggests that low IL-17A level induced by otLT doesn't trigger the autophagy-mediated negative control, leading to persistent low-grade IL-17A inflammation.

ABBREVIATIONS

| | |
|--------------------------------|--|
| ALTO | Alternative frame of the large T open reading frame |
| 3-MA | 3-methyladenine |
| 4E-BP1 | 4E-binding protein 1 |
| AD | Alzheimer's disease |
| ADCC | Antibody-dependent cell cytotoxicity |
| APC | Antigen-presenting cell |
| ATG | Autophagy-related gene |
| Bag3 | BCL2-associated athanogene 3 |
| C/EBPβ | CCAAT/enhancer-binding protein |
| cGAS | Cyclic-GMP-AMP synthase |
| CK | Cytokeratin |
| CNS | Central nervous system |
| CTL | Cytotoxic T lymphocyte |
| CTLA-4 | Cytotoxic T-lymphocyte-associated protein 4 |
| DC | Dendritic cell |
| EBV | Epstein-Barr virus |
| ER | Endoplasmic reticulum |
| FasL | Fas ligand |
| GM-CSF | Granulocyte-macrophage colony-stimulating factor |
| HCMV | Human cytomegalovirus |
| HLA | Human leucocyte antigen |
| HPV | Human papillomavirus |
| HPyV | Human polyomavirus |
| HSV | Herpes simplex virus |
| IDO | Indoleamine 2,3 dioxygenase |
| IFN | Interferon |
| IHC | Immunohistochemistry |
| IKK | Phosphorylates I κ B kinase |
| IL | Interleukin |
| iNKT | Invariant NKT cell |
| IRAK | IL-1 receptor-associated kinase |
| IRF | Interferon regulatory factor |
| JAK | Janus kinase |
| KSHV | Kaposi sarcoma herpesvirus |
| LAG3 | Lymphocyte-activation gene 3 |
| LAP | LC3-associated phagocytosis |
| LC3 | microtubule-associated protein 1 light chain 3 β |
| LSD | LT stabilization domain |
| LT | Large T antigen |
| MAPK | Mitogen-activated protein kinases |
| MCC | Merkel cell carcinoma |
| MCPyV | Merkel cell polyomavirus |

| | |
|--------------------------------|---|
| MCPyV ER | MCPyV early region |
| MDSC | Myeloid-derived suppressor cell |
| MHC | Major histocompatibility complex |
| MMP | metalloproteinase |
| MPyV | Murin polyomavirus |
| mTOR | Mammalian target of rapamycin |
| MUR | MCPyV unique region |
| MyD88 | Myeloid differentiation primary response 88 |
| NCCR | Non-coding regulatory region |
| NEMO | NF- κ B essential modulator |
| NFP | Neurofilament protein |
| NF-κB | Nuclear factor κ B |
| NK | Natural killer cell |
| NKT | Natural killer T cell |
| NLS | Nuclear localization signal |
| OBD | Origin binding domain |
| otLT | Onco-type LT |
| PAMP | Pathogen-associated molecular pattern |
| PD-1 | Programmed cell-death 1 |
| PD-L1 | Programmed cell-death ligand 1 |
| PI3K | Phosphoinositide 3-kinase |
| PP2A | Protein phosphatase 2A |
| PRR | Pattern-recognition receptor |
| PyV | Polyomavirus |
| RIG-I | Retinoic acid-inducible gene I |
| RORγt | RAR-related orphan receptor gamma |
| ROS | Reactive oxygen and reactive nitrogen species |
| SCC | Squamous cell carcinoma |
| SCLC | Small-cell lung carcinoma |
| sT | Small T antigen |
| STAT | Signal transducer and activator of transcription |
| STING | Stimulator of IFN gens |
| SV40 | Simian virus 40 |
| TAK | TGF- β activated kinase |
| TAP | Transporter associated with antigen processing |
| TAM | Tumor-associated macrophage |
| TBK | TANK-binding kinase |
| TCR | T cell receptor |
| TGF-β | Transforming growth factors β |
| Th | T helper cell |
| TIM-3 | T-cell immunoglobulin and mucin-domain containing-3 |
| TLR | Toll-like receptor |
| TME | Tumor microenvironment |
| TNF | Tumor-necrosis factor |

| | |
|-------------|--|
| TRAF | TNF receptor associated factor |
| Treg | Regulatory T cell |
| TRIF | TIR-domain-containing adapter-inducing interferon- β |
| trLT | Truncated LT |
| TTFI | Thyroid transcription factor 1 |
| VEGF | Vascular Endothelial Growth Factor |
| VP | Viral protein |
| Znf | Zinc finger |

SUMMARY

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INTRODUCTION

CHAPTER I. Merkel cell carcinoma (MCC)

1. Merkel cell and Merkel cell carcinoma

Merkel Cell Carcinomas (MCC) is a rare, neuroendocrine, cutaneous malignancy that is highly metastatic. The first description for MCC can be traced back to the 1970s, called “trabecular carcinoma of the skin” at the time (Toker, 1972). Later, the name was changed to Merkel cell carcinoma because the tumor was initially believed to originate from the transformed Merkel cells, which are located in the basal layer of the epidermis and around the hair follicles (Gould et al., 1985). Most often, Merkel cells are associated with the terminal ends of the afferent sensory nerve and serve as mechanoreceptors for gentle touch stimuli transduction (Halata et al., 2003; Maricich et al., 2009). The identification of Merkel cells is based on some neuroendocrine markers such as chromogranin-A, synaptophysin, cytokeratin 20 (CK20), and neuro-specific enolase. Among these markers, CK20 is considered the most sensitive and specific markers of Merkel cells and is abundantly expressed in MCC (Moll et al., 2005a). MCC is greatly aggressive with a mortality rate of more than 30% which is higher than that of melanoma (Becker et al., 2009). Almost one-third of patients present at first diagnosis with loco-regional metastases, for example, in-transit metastases (a tumor distinct from the primary lesion and located either between the primary lesion and the draining regional lymph nodes or distal to the primary lesion) or lymph node metastases (Allen et al., 2005a). Despite the mounting understanding of MCC biology and the development of novel anti-cancer drugs in recent years, the incidence rate of MCC is still increasing and the 5-years overall survival of advanced MCC remains very low (0 – 18%) (Lemos et al., 2010a; Santamaria-Barria et al., 2013a).

2. Epidemiology of MCC

2.2 Worldwide Incidence

The accurate incidence rate of MCC is difficult to determine because of its rarity and the inability to perform large international retrospective analyses. The general incidence rate of MCC ranges from 0.13 to 1.6 per 100,000 persons across different geographic regions of the globe. In United States (US), the incidence rates reported in the Surveillance, Epidemiology, and End Results (SEER) program increased from 0.15 to 0.44 per 100,000 from 1986 to 2004

(Hodgson, 2005). A recent analysis of SEER showed the incidence rate in the US was 0.79 per 100,000 in 2011 (Fitzgerald et al., 2015). The Surveillance of Rare Cancers in Europe (RARECARE) database reported an incidence rate of 0.13 per 100,000 between 1995 and 2002 (van der Zwan et al., 2013). In United Kingdom (UK), France, Sweden, the overall incidence rate is about 0.15 per 100,000 (Schadendorf et al., 2017a), whereas in other European countries like Germany, Denmark, Netherlands, the incidence rate is about 3 times higher (Eisemann et al., 2014; Reichgelt and Visser, 2011). From early 1990 to 2010, the incidence rate has been increased by 3-fold in most European countries except in Norway, Sweden, and Denmark (Stang et al., 2018). In the southern hemisphere countries, the incidence rates of MCC are the highest. Similar to other UV-related skin cancers, incidence rates in Queensland, Western Australia, and New Zealand are higher than those in the rest of the world and are still increasing (age-adjusted incidence of 1.6, 0.82, and 0.88 per 100,000, respectively) (Girschik et al., 2011; Robertson et al., 2015; Youlden et al., 2014). Another recent study showed that the overall incidence rates in New Zealand from 2000 to 2015 is about 0.96 per 100,000 (Lee et al., 2019). In the Asian-pacific region, the incidence rates remain very low (Chun et al., 2013; Hattori et al., 2013, 2013). In Japan, the overall incidence rate is approximately 0.2 per million and it has been stable since 1990 (Stang et al., 2018). In mainland China, only 22 cases were reported from 1970 to 2009 (Song et al., 2012). MCC cases were reported in Africa and Latin America but the incidence rate is unclear (Neto et al., 2019; Tenea et al., 2019). The lack of data in these continents is presumably due to insufficient medical conditions and the difficulty of data collection (Schmerling et al., 2018). The comparisons of incidence across countries are complicated because different studies use different measures (for example, crude rates or age-standardized rates with different age standards) and calendar periods. Although the improvements in cancer registration and immunohistochemical characterization (particularly the use of CK20 staining) may contribute to the increased incidence, the global prevalence of MCC has been raising since the last decades (Hodgson, 2005).

2.2 Age, gender, and ethnicity (demographics)

Consistent with the increased risk associated with advanced age, like many other cancers, the incidence of MCC augments with age. First primary MCC is a disease of elderly people. It is only sporadically reported before age 50 (Agelli and Clegg, 2003a). 75% of MCC patients are over the age of 65 years at the time of diagnosis, with the average age for developing MCC

being 74(Fitzgerald et al., 2015). It is exceedingly rare in children (Lemos et al., 2010b), few cases were reported (Köksal et al., 2009, 2009; Marzban et al., 2011). In New Zealand, the age-specific incidence increased with age at diagnosis, from 0.03 per 100,000 person-years in those aged 35-39 years to 17.56 per 100,000 person-years in those aged over 85 years. In a US study, the age-specific incidence in 2001 ranged from 0 to 0.50 per 100,000 in the group aged 40-64 years to 1.71 to 5.62 per 100,000 in the group older than 65 years (Schadendorf et al., 2017a). The incidence of MCC is generally higher in men than in women especially in the US, Australia, and New Zealand. The overall incidence ranges for men and women are 0.4-2.5 and 0.18-0.9 per 100,000, respectively. According to the SEER database, MCC is 8 times more prevalent in white Caucasian people than in other populations. In the US, 94.9% of patients were white, 1.0% were black and 4.1% were of another race (Lemos et al., 2010b).

3. Clinical feature of MCC

3.1 Tumor morphology and staging

MCC is characterized as a rapidly growing red or violaceous, solitary, cutaneous or subcutaneous nodule that is located mostly on sun-exposed areas, particularly the head and neck and also, less frequently, the extremities and buttocks (Becker et al., 2008; Llombart et al., 2005) (Figure 1). However, it is unclear whether MCPyV⁺MCC and UV-associated MCPyV⁻MCC is different in terms of morphological appearance and localization. Microscopic evaluation revealed that MCPyV⁻MCC more frequently displayed a "large-cell neuroendocrine carcinoma" phenotype with larger cell size and prominent nucleoli (Kervarrec et al., 2019). In addition, CD99 staining with a dot-like pattern is strongly associated with the presence of MCPyV in MCC tumors, suggesting CD99 as a marker of MCPyV⁺MCC (Kervarrec et al., 2019). MCC is dramatically aggressive with high metastatic capacity. Most MCC recurrences occur within 2 years of the first diagnosis (Allen et al., 2005b; Bichakjian et al., 2007a). Up to 15% of patients with MCC present with a tumor-positive lymph node without an identifiable cutaneous tumor (Foote et al., 2012; Vandeven et al., 2018). Regional lymph nodes are the first site of metastasis. Besides, metastases are commonly found in the skin, lungs, adrenal glands, liver, brain, and bones (Becker et al., 2017). The most common features of MCC are recapitulated by the acronym "AEIOU" which stands for Asymptomatic, Expanding rapidly, Immunosuppressed, Older than age 50, and UV-exposed site on the fair-skinned individuals (Heath et al., 2008). According to the American Joint Committee on Cancer (AJCC)

consensus staging system, four main stages of MCC have been recognized base on tumor size, lymph node implication, and the presence of distant metastasis (Edge and Compton, 2010; Lemos et al., 2010c). Stage I MCC is a localized disease with a primary tumor dimension less than 2 cm. There is no spread to regional lymph nodes neither distant metastasis. Stage II MCC is also localized but with a tumor size greater than 2 cm. At stage III, tumor cells become invasive and spread to regional lymph nodes. At stage IV, MCC can be any size and distant metastasis occurs. The overall survival depends on the stage at diagnosis. 5-year survival is 62.8% in patients with stage I MCC, 34.8–54.6% in stage II, 26.8–40.3% in stage III and 13.5% in stage IV (Schadendorf et al., 2017a).

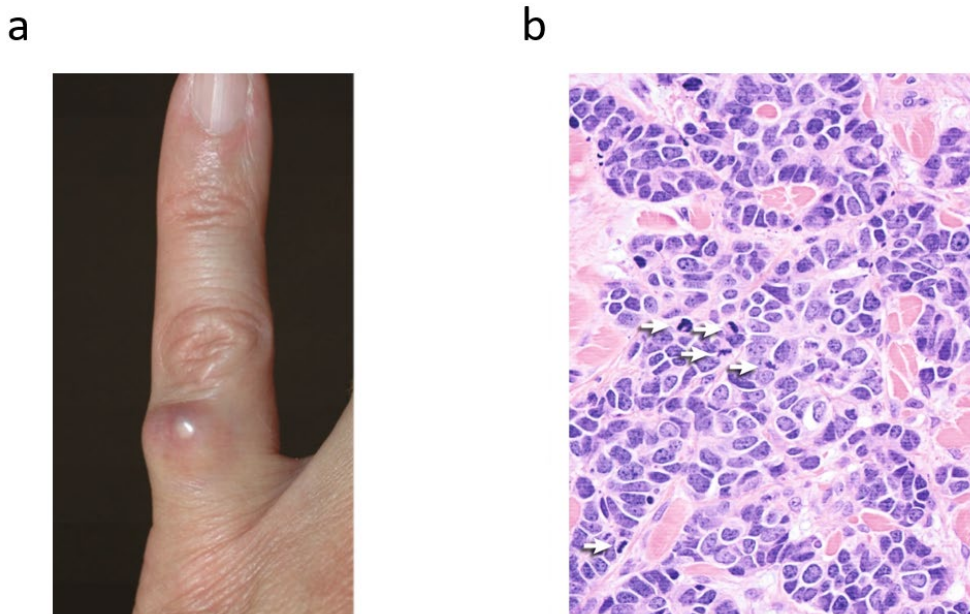


Figure 1. Clinical and histological appearance of MCC. (a) Photograph depicting the clinical appearance of Merkel cell carcinoma (MCC), presenting as a rapidly growing nodule on an extremity; such lesions can also be commonly observed on a patient’s head or neck. **(b)** Light micrograph (×400) of a sample stained with haematoxylin and eosin depicting the histological appearance of MCC, demonstrating the presence of round cells with scant cytoplasm, neuroendocrine chromatin, and numerous mitotic figures (arrows). Trabecular patterning might also be prominent. **Adapted from (Harms et al., 2018).**

3.2 Immunohistological marker and diagnosis of MCC

Histologically, MCC appears as a dermal tumor nodule, which frequently extends into the subcutaneous fatty tissue. The tumor cells are small-blue-round cells with basophilic nuclei and minimal cytoplasm (Jaeger et al., 2012a) (Figure 1). Mitoses are frequent and the apoptosis index is high (McCardle et al., 2010). However, histopathological characteristics are not sufficient for a confirmative diagnosis of MCC. The “small-round-blue cell” histologic pattern of MCC can be confused with several other tumors including small-cell lung carcinoma (SCLC), carcinoid tumor, malignant lymphoma, and small-cell melanoma (Jaeger et al., 2012b). MCC can be also misdiagnosed as basal-cell carcinoma (Ball and Tanhuanco-Kho, 2007). Therefore, immunohistochemical investigations are required. MCC cells express a panel of immunohistological markers, including cytokeratin 8, CK18, CK19, and CK20, and the neuroendocrine markers chromogranin A, synaptophysin, and neuron-specific enolase. Of these markers, CK20 is the most sensitive and specific marker for routine MCC diagnosis. Anti-CK20 staining can be observed in over 90% MCC in a paranuclear dot-like pattern (Dancey et al., 2006). This highly sensitive staining feature makes CK20 the key marker for routine histopathology to distinguish MCCs from other small round blue cell tumors, in particular SCLC (Acebo et al., 2005; Bobos et al., 2006). However, a small number of MCC do not express CK20. Thus, other markers are necessary for robust distinguish of CK20-negative MCC from SCLC. Thyroid transcription factor 1 (TTF1) and CK7, usually expressed in SCLC, is consistently absent in MCC (Dancey et al., 2006). Neurofilament protein (NFP) is another useful marker for the distinction between MCC and SCLC. MCC is NFP positive while SCLC is NFP negative (Bobos et al., 2006). In most cases, morphological features, positive staining for CK20 and neuroendocrine markers, and negative staining for TTF1, CK7, and lymphoid markers are sufficient to confirm the diagnosis of MCC. Importantly, few metastatic SCLC, parotid, and uterine cervical primary tumors can be CK20 positive, making them more difficult to differentiate from MCC, especially in the case of metastatic MCC from an unknown primary site (Harms, 2017). In such circumstances, NGS-based analysis of mutational signature and the presence of Merkel cell polyomavirus (MCPyV) might be combined with immunohistochemistry (IHC) test to confirm the diagnosis of MCC (Harms et al., 2016). Currently, no markers have been reliably associated selectively with either MCPyV⁺MCC or MCPyV⁻MCC. Therefore, there is no differential diagnosis between the two MCC subtypes on the basis of IHC test alone. Whereas MCPyV large T antigen (LT) staining is recommended for

the diagnosis of MCPyV⁺MCC (Bichakjian et al., 2018) because of its constant expression in this MCC subtype (Houben et al., 2010a).

4. Etiology

4.1 Age, UV radiation, immunodeficiency, and MCPyV as Risk factors

Advanced age is considered as one risk factor for MCC because of the increased incidence rate in old people. As people with fair skin are considerably more susceptible to develop MCC, skin pigmentation seems to protect against MCC. UV radiation from sun exposure may play a crucial role in the development of MCC. Indeed, the solar UV index is positively correlated with the incidence of MCC (Agelli and Clegg, 2003b; Miller and Rabkin, 1999). Other evidence is that primary MCC lesions typically develop on sun-exposed skin and chronic sun exposure contributes to the increased incidence in elderly people (Howard et al., 2006). Patients with other UV-linked skin cancers also have a greater risk of MCC (Howard et al., 2006; Miller and Rabkin, 1999). Another critical factor associated with increased risk is immunodeficiency. MCC is more prevalent in immune-compromised individuals suffering hematological malignancies, organ transplantation (Clarke et al., 2015; Garrett et al., 2017; Penn and First, 1999), chronic inflammatory diseases, or HIV infection. Patients with leukemia, especially chronic B cell lymphocytic leukemia, have an increased risk of developing MCC (Ziprin et al., 2000). Chronic inflammatory disorders such as rheumatoid arthritis are associated with a higher incidence of MCC (Sahi et al., 2017). Given that immunosuppression is linked to a higher incidence rate, an infectious cause had been suspected. In 2008, a novel polyomavirus named Merkel Cell Polyomavirus (MCPyV) was first identified in MCC patients using digital transcriptome subtraction (Feng et al., 2008). The genome of MCPyV is clonally integrated into MCC cells and it is the causative agent of about 80% of MCC patients worldwide (Feng et al., 2008).

4.2 Two MCC subtypes

In terms of etiology, MCC is currently divided into two different subtypes: MCPyV associated MCPyV-positive MCC (MCPyV⁺MCC) and UV induced MCPyV-negative MCC (MCPyV⁻MCC) (Figure 2). MCPyV⁺MCC represents approximately 80% of total MCC while MCPyV⁻MCC represents only 20% (Harms et al., 2018).

4.3 Different pathophysiology and mutational landscape in MCC subtypes

Although the exact mechanism is not fully understood, evidence proves that MCC carcinogenesis can be initiated either by the clonal integration of the MCPyV genome or by the UV-mediated DNA damage caused by chronic exposure to sunlight. MCPyV⁻MCC harbors a high mutational burden (10.09 ± 2.32 mutations/Mb) and is characterized by a prominent UV-signature pattern with C > T transitions comprising 85% of mutations (Harms et al., 2015). Similar to other UV-mediated cutaneous malignancies, these mutations affect mostly genes implicated in DNA repair and cell cycle regulation including *TP53* and *RB1* which are known as tumor suppressor genes (Cimino et al., 2014; Goh et al., 2016; Wong et al., 2015b). The mutations or deletions of p53 and pRb result in loss of their cell cycle arrest function, hence promote cell division and facilitate carcinogenesis (Fischer et al., 2016). In addition to UV-induced signatures, genomic aberrations including chromosomal copy number variation and other mutations are also identified (Harms et al., 2018). For instance, a focal amplification of chromosome 1p34, containing the *MYCL* oncogene, is present in approximately 39% MCC and can be observed in both MCPyV⁻MCC and MCPyV⁺MCC (Paulson et al., 2009). Most MCPyV⁻MCCs have detectable activation of at least one known proto-oncogene. Hotspot activating mutations of several different oncogenes are observed in MCPyV⁻MCC, including mutations in *HRAS*, *KRAS*, *PIK3CA*, *KNSTRN*, *RAC1*, *AKT1*, *CTNNB1*, and *EZH2* (Hafner et al., 2012; Harms et al., 2015; Nardi et al., 2012a; Veija et al., 2016). *HRAS* hotspot mutations in MCC have been associated with increased ERK phosphorylation but not with sensitivity to MEK inhibitors in an analysis involving MCPyV⁻MCC cell lines (Wong et al., 2015b). Epigenetic deregulation, including modifications of both DNA (especially promoter silencing by CpG island hypermethylation) and histones have been also described in MCC. Promoter hypermethylation in MCC can affect several genes, including *DUSP2*, *CDKN2A* and members of the *RASSF* tumor suppressor family (Helmbold et al., 2009; Lassacher et al., 2008; Richter et al., 2013). Epigenetic silencing of the human leucocyte antigen (HLA) genes by histone deacetylases is also observed, which might contribute to immune evasion in the development of MCC (Paulson et al., 2014a; Ritter et al., 2017).

In contrast, MCPyV⁺MCC displays much fewer mutational burden (0.40 ± 0.09 mutations/Mb) (Goh et al., 2016). Hotspot mutations like *HRAS*, *KRAS*, and *PIK3CA* are reported in a few MCPyV⁺MCC cases (Hafner et al., 2012; Nardi et al., 2012a). However, there's no definitive

mutational signature and the typical *TP53* and *RB1* mutations are absent (Goh et al., 2016; Harms et al., 2016; Wong et al., 2015b). The oncogenesis of MCPyV⁺MCC is mainly driven by viral antigens expression in host cells (Grundhoff and Fischer, 2015), although UV radiation may conduce to MCPyV⁺MCC development by immune suppression while aging. The oncogenic potential of viral antigens and their involvement in MCC progression will be detailed and discussed in subsequent chapters.

4.4 Unclear cells of origin of MCC

Identifying the cell type from which a neoplasm arises has implications for ascertaining the mechanisms of tumor initiation, experimental modeling and possibly uncovering therapeutic susceptibilities. There is substantial debate in the literature about the cells of origin of MCC which is still a mystery to date (Figure 2). MCC was originally believed to arise from the transformation of Merkel cells because it has a similar immunophenotype and ultrastructure to Merkel cells (Harms, 2017). Nevertheless, most investigators do not consider mature Merkel cells to be a likely candidate for the MCC cell of origin because of emerging evidence (Sunshine et al., 2018). First of all, Merkel cells are terminally differentiated and postmitotic with limited proliferative potential. Merkel cell turnover during adult homeostasis is ensured by differentiated pluripotent epidermal stem cells but not by their own proliferation (Van Keymeulen et al., 2009). Second, the pattern of expression of CK20, a common neuroendocrine marker shared by MCC and Merkel cell, differs sharply. (Moll et al., 2005b). CK20 is loosely arranged and diffused in the cytoplasm in Merkel cell whereas it is organized in plaque-like arrangement and displays dot-like staining pattern on IHC in MCC (Moll et al., 2005c). Furthermore, MCCs express several markers such as c-kit, CD171, and CD24 that are not expressed in Merkel cells (Tilling and Moll, 2012). In addition, studies using Merkel cell-specific Cre drivers to generate mouse models of MCPyV⁻MCC have failed (Shuda et al., 2015a; Spurgeon et al., 2015; Verhaegen et al., 2015a, 2017). Based on these observations, it is unlikely that Merkel cells are the cells of origin of MCC. Instead, MCCs are more likely to be derived from other population that recapitulates the Merkel cell differentiation pathway either before or during neoplastic transformation. Tumor phenotype is not always a reliable predictor of the cell of origin (Visvader, 2011).

Given that MCPyV⁻MCC and MCPyV⁺MCC have a different mutational landscape, these tumor subtypes might arise from distinct cells of origin (Sunshine et al., 2018). The mutational landscape of MCC suggests that the epidermal cells, especially keratinocytes may be the cells of origin for MCPyV⁻MCC. UV mutational signature found in MCPyV⁻MCC is characteristic of epidermal-derived cancers. The same signature is identified only in proliferations of epidermal cells, including normal keratinocyte, actinic keratosis, squamous cell carcinoma (SCC), melanoma, etc. (Chitsazzadeh et al., 2016; Hodis et al., 2012; Martincorena et al., 2015). In addition to the overall mutational burden being compatible with a keratinocyte origin, MCPyV⁻MCC harbors mutations in *NOTCH1*, *HRAS*, and *FAT1* that are also frequently mutated in SCCs (South et al., 2014).

On the other hand, the fact that MCPyV⁺MCC harbors a low mutation burden and no UV signature implies a dermal origin for this subtype. Recent studies suggest that dermal fibroblast is a putative cell of origin for MCPyV⁺MCC. Liu and colleagues demonstrate that MCPyV could not infect CK20-positive Merkel cells and only dermal cells expressing fibroblast markers were capable of expressing the MCPyV viral antigens. Moreover, dermal fibroblasts are the only cell types that can support the full viral life cycle *ex vivo* (Liu et al., 2016a). Other possible candidates for the MCPyV⁺MCC cell of origin located in the dermal compartment including pro-B cells or pre-B cells and dermal mesenchymal stem cells are also proposed (Becker and Zur Hausen, 2014; Murakami et al., 2014a; Zur Hausen et al., 2013). Some other virus-associated malignancies might support the possibility that the cells of origin for MCC undergo transdifferentiation to become tumor cells. For patients with Kaposi sarcoma herpesvirus (KSHV)-associated primary lymphoma, studies suggest that mesothelial cells might be the cell of origin via mesenchymal-to-lymphoid transformation (Sanchez-Martin et al., 2017). In cervical squamous cell carcinomas, the activity of viral oncogenes in intraepithelial precursor lesions that progress to epithelial tumors supports human papillomavirus (HPV)-infected epithelial stem cells as the cells of origin (Sell, 2010). However, no consensus has been reached nowadays regarding the most likely candidate for the MCC cell of origin.

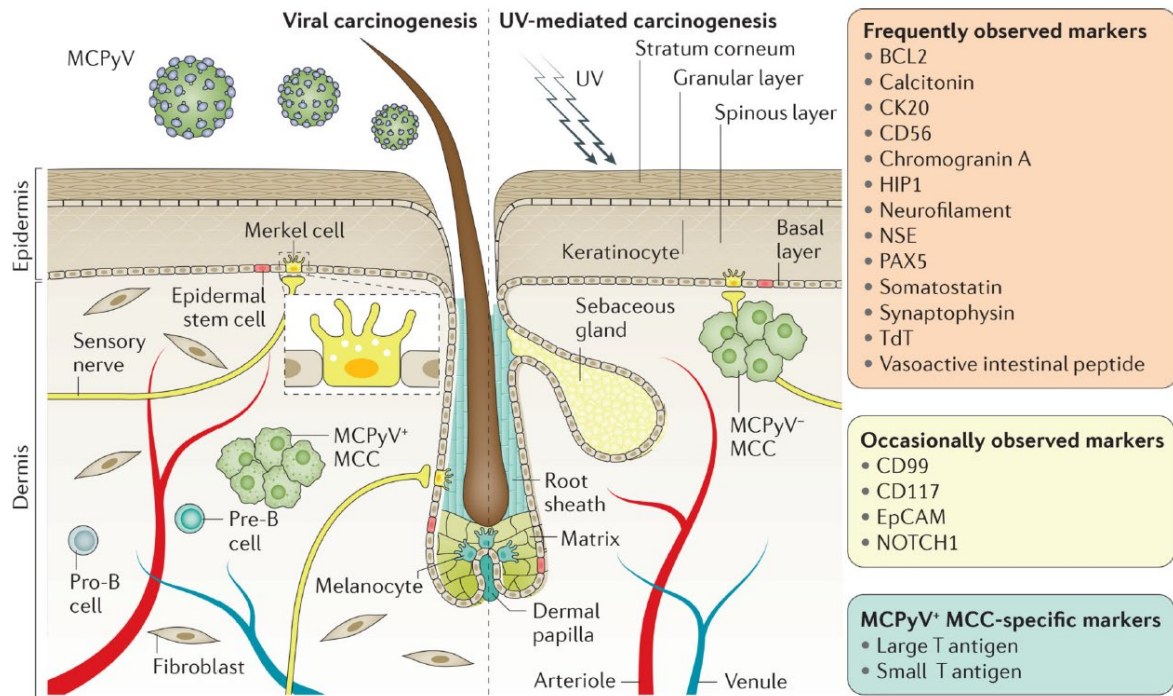


Figure 2. Hypothetical cells of origin, causal events, and tissue markers of MCC. The cell of origin of Merkel cell carcinoma (MCC) has not been identified. Possible candidates include epidermal stem cells, keratinocytes (the predominant cells in all the epidermal cell layers), dermal fibroblasts, pro-B cells, or pre-B cells. Fibroblasts, pro-B cells, and pre-B cells are localized in the dermal compartment, which is not exposed to relevant amounts of ultraviolet light (UV), and, therefore, are probably not cells of origin in UV-mediated carcinogenesis. Merkel cells are postmitotic cells and, therefore, are probably not the cell of origin of MCC. Merkel cells are found in the basal layer of the epidermis and are probably derived from epidermal or hair follicle stem cells. Merkel cells function as mechanoreceptors to detect gentle touch and are associated with sensory nerves. Merkel cell polyomavirus (MCPyV) is a common component of the commensal skin microbiota. However, it is not known what cell type MCPyV preferentially infects. In countries with low UV exposure, the majority of MCCs are positive for MCPyV (MCPyV+ MCC), whereas in countries with high UV exposure, MCPyV is less frequently associated with MCC; these MCPyV- MCCs are characterized by DNA mutations bearing a UV signature. The two MCC types have similar phenotypes. Tissue markers that can be frequently or occasionally observed in both MCPyV+ MCC and MCPyV- MCC, as well as MCPyV+ MCC-specific markers, are listed. BCL2, apoptosis regulator BCL2; CK20, cytokeratin 20; CD56, neural cell adhesion molecule 1; CD99, CD99 antigen; CD117, mast/stem cell growth factor receptor Kit; EpCAM, epithelial cell adhesion molecule; HIP1, huntingtin-interacting protein 1; NSE, neuron-specific enolase, also known as γ -enolase; NOTCH1, neurogenic locus notch homologue protein 1; PAX5, paired box protein Pax-5; TdT, DNA nucleotidylexotransferase. **Adapted from (Becker et al., 2017).**

5. Management of MCC

5.1 Surgery and radiotherapy as the first line of treatment for early MCC

The option of treatment for MCC depends on disease characteristics, including tumor staging, regional lymph node involvement, comorbidities, and the performance status of the patient (Bichakjian et al., 2018; Lebbe et al., 2015). While guidelines are established for the management of loco-regional MCC, no consensus has been reached on the most effective treatment strategy for advanced-stage MCC (Cassler et al., 2016a). Local surgical excision is the standard of care for patients with loco-regional primary tumors. As MCC usually spread to lymph nodes first and the risk of occult nodal micro-metastasis, sentinel lymph node biopsy is widely recommended for clinical node-negative patients (Servy et al., 2016; Vázquez Doval et al., 2019). Another conventional treatment is radiotherapy, which is applied at different stages of the disease, either as an adjuvant treatment to surgery or as palliative treatment for inoperable cases of MCC (Lewis et al., 2006; Mortier et al., 2003; Poulsen et al., 2003). For patients ineligible for surgical interventions due to poor performance status or large tumor burden, radiotherapy may also be considered and can lead to long-term tumor control (Mortier et al., 2003). MCC is very responsive to radiotherapy. Surgery and/or radiotherapy may be curative for most patients with local and regional MCC, but recurrences are still possible even if combined (Allen et al., 2005c; Bichakjian et al., 2007b; Santamaria-Barria et al., 2013b). Retrospective study showed improved overall survival for patients with stage I/II MCC receiving adjuvant radiotherapy compared to those receiving only surgery. However, neither radiotherapy nor chemotherapy was associated with survival benefit in stage III MCC (Bhatia et al., 2016). Before the introduction of emerging novel therapies, standard chemotherapy for other small-cell carcinoma, including platinum-based drugs, taxanes, anthracyclines, and etoposide was used for the treatment of metastatic MCC not amenable to surgery. Nevertheless, Recurrences develop within months of chemotherapy, and few studies to date show a survival benefit (Poulsen et al., 2003, 2006; Sharma et al., 1991). Owing to a lack of durable responses, no established effects on survival, and decreased quality of life, chemotherapy is currently not recommended in clinical guidelines (Nghiem et al., 2017).

5.2 Emerging treatment

5.2.1 Immune checkpoint inhibitors, a milestone for the management of advanced-MCC

Since the last decade, immunotherapy has revolutionized the traditional management of several types of cancer and become the most promising anti-tumor approach. MCC is considered as immunogenic cancer based on the poorer prognosis in immunosuppressed individuals (Paulson et al., 2013) and the presence of immune response to MCPyV antigen in MCPyV⁺MCC (Lyngaa et al., 2014a). Furthermore, spontaneous regression of MCC is an uncommon but well-documented event, presumably indicating an anti-tumor immune response (O'Rourke and Bell, 1986; Pang et al., 2015). T-cell infiltrated MCC is associated with improved prognosis and outcome (Paulson et al., 2011a; Sihto and Joensuu, 2012). Increasing evidence of the role of the immune system in controlling MCC has paved the way for immune-based therapies. Of these approaches, the blockade of immune checkpoint programmed cell-death 1/programmed cell-death ligand 1 (PD-1/PD-L1) axis has shown safety and efficacy for the treatment of advanced-MCC (Terheyden and Becker, 2017). Clinical trials involving anti-PD-1 or anti-PD-L1 antibodies have demonstrated durable responses to these agents in a subset of patients with MCC. In a study involving 26 patients, treatment with the anti-PD-1 antibody pembrolizumab resulted in a 56% ORR, including complete responses in 15.4% of patients, as first-line systemic therapy (Nghiem et al., 2016). In a larger study of 88 patients with stage IV MCC failing chemotherapy previously, treatment with the anti-PD-L1 antibody avelumab resulted in over 30% objective response rate and an improvement in quality of life (Kaufman et al., 2016, 2018). The anti-PD-1 antibody nivolumab has also shown some positive results (Mantripragada and Birnbaum, 2015). In the avelumab trial, a trend toward a higher response rate was observed in patients with fewer lines of prior treatment, which along with the pembrolizumab data strongly suggest that immunotherapy should be considered for first-line treatment in patients with advanced MCC. Currently, avelumab is the only FDA and European Medicines Agency-approved treatment of metastatic MCC (Baker et al., 2018). Several clinical trials of immune checkpoint inhibitors administered as monotherapy or in combination with other agents are currently ongoing (Emge and Cardones, 2019). Recent studies have highlighted the importance of other immune checkpoints, including the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and T-cell immunoglobulin and mucin-domain containing-3 (TIM-3). Anti-CTLA-4 antibody utomilumab and anti-TIM3 are currently in phase I study to determine their preliminary efficacy (Kwiatkowska and Reich, 2020; Segal et al.,

2018). Lymphocyte-activation gene 3 (LAG3) is another immune checkpoint receptor that could act synergistically with PD-1 to inhibit immune response. Targeting LAG3 is also being evaluated in clinical trials, as well as its combination with PD-1.

5.2.2 Other immunotherapies, combined therapies, and targeted therapy

The anti-tumor activity can also be provoked and enhanced by immunostimulatory cytokines including certain types of interleukins and type I interferons (IFNs). Targeted intratumoral delivery of IL-12 cDNA using electroporation was investigated in a phase II trial and led to tumor regression (Bhatia et al., 2020). Adoptive cell transfer using in vitro expanded autologous MCPyV-specific T cells in combination with IFN- β and radiotherapy resulted in tumor regression in one patient with metastatic MCPyV⁺MCC (Chapuis et al., 2014; Davies et al., 2020). A phase I/II trial combining adoptive cell transfer with IL-2 and avelumab after conditioning the tumor with radiotherapy or IFN β is currently underway (Villani et al., 2019). Combinations of immunotherapies with traditional treatments might also be effective in patients with MCC. Investigating the safety and efficacy of avelumab in combination with major histocompatibility complex class I (MHC I) upregulation via IFN- β administration and radiotherapy has also been implemented (Paulson et al., 2014b). Radiotherapy also has the potential to induce the release of tumor-associated antigens and increase inflammation, thus further synergizing with immunotherapy (Cassler et al., 2016b). A toll-like receptor 4 (TLR4) agonist that stimulates dendritic cells and promotes Th1-type response has been tested in clinical trials for patients with MCC. Preliminary results demonstrate acceptable safety and show encouraging clinical activity (Bhatia et al., 2019). Modified oncolytic herpes simplex virus I (HSV1), Talimogene Laherparepvec, is being evaluated in clinical trials for their safety and efficacy after intra-tumoral administration (Kwiatkowska and Reich, 2020). Since MCPyV T antigens specific intra-tumoral T cell responses are identified in MCPyV⁺MCC, and ongoing T-Ag expression is required to drive MCC cell proliferation, therapeutic vaccine using T-Ags is a rational strategy to treat MCPyV⁺MCC patients and is currently being explored (Jing et al., 2020; Tabachnick-Cherny et al., 2020).

MCCs express somatostatin receptors; hence, somatostatin analogues are being investigated for both molecular imaging and treatment of patients with MCC (Buder et al., 2014; Kwekkeboom et al., 1992). Although tumor suppressors and oncogenes are not commonly

mutated in MCC, targeted therapy using specific inhibitors has been explored. Clinical remission following treatment with imatinib, a tyrosine kinase commonly used in targeted therapy for certain types of cancer, has been reported in one patient (Loader et al., 2013), although a phase II trial was suspended due to non-evident benefit (Samlowski et al., 2010). Multitargeted tyrosine kinase inhibitor, pazopanib, showed limited clinical benefit in advanced-MCC (Davids et al., 2009). Phosphoinositide 3-kinase (PI3K) activating mutations have been detected in MCC tumors, and the PI3K inhibitor idelalisib provided a complete clinical response in a single patient with stage IV MCC (Nardi et al., 2012b; Shiver et al., 2015). Mammalian target of rapamycin (mTOR), a regulator of the PI3K and Mitogen-activated protein kinases (MAPK) pathways, is activated in MCC cell lines and tumor tissues (Lin et al., 2014b). Several clinical trials are investigating the safety and efficacy of mTOR inhibition in patients with MCC (Harms et al., 2018).

CHAPTER II. Merkel cell polyomavirus (MCPyV)

1. The virology of MCPyV

1.1 Human polyomavirus and the discovery of MCPyV

1.1.1 human polyomavirus and diseases

Polyomaviridae was newly classified as a novel family of double-stranded DNA (dsDNA) virus after the split of the obsolete family Papovaviridae (Moens et al., 2017). Polyomavirus (PyV) infections were accidentally discovered in the 1950s when characterizing a transmissible agent causing multiple tumors in rodents, thus the name murine polyomavirus (MPyV) (Gross, 1953; Stewart et al., 1958). The Greek roots “polyoma-” refers to the viruses' ability to produce multiple (poly-) tumors (-oma). The first primate PyV, simian virus 40 (SV40), was identified in African green monkey kidney cells used for the production of polio- and adenovirus vaccine. SV40 is oncogenic in mice and rodents leading to the concern of oncogenic potential in humans following the distribution of SV40-contaminated vaccines by the early 1960s (Young, 1971). PyVs are widely abundant but have a narrow host and specific cell tropism. Despite their highly conserved structural organization, they have been detected in different vertebrates host including avian, mammal, and human (Krumbholz et al., 2009). Thus far, according to the recent update of the taxonomy for the family of *Polyomaviridae* from the International Committee on Taxonomy of Viruses (ICTV), 14 human polyomavirus (HPyV) have been yet identified (Moens et al., 2017) (Table 1). HPyV infections are prevalent in the general population, but cause clinically apparent disease or pathology only in certain circumstances, such as in immunocompromised individuals. Thus, they are considered as opportunistic agents. The first two HPyVs BKPyV and JCPyV were isolated in a patient with ureteral stenosis and an immunosuppressed patient with progressive multifocal leukoencephalopathy, respectively (Gardner et al., 1971; Padgett et al., 1971). The names of both viruses derive from the initials of the respective patient. Subsequent studies revealed both viruses are ubiquitous and widely spread (seropositivity rates vary from 72 to 98%), with subclinical primary infection occurring in early childhood and normally leading to a lifelong persistence. Viral reactivation may occur as a result of immunosuppression but is also sometimes observed in immunocompetent hosts. In bone marrow recipients, BKPyV reactivation is linked to hemorrhagic cystitis (Hirsch, 2005). JCV is the etiologic agent of multifocal leukoencephalopathy, a fatal demyelinating disease of the central nervous system

caused by lytic infection of oligodendrocytes. In recent years, high-throughput molecular screening technology has led to the identification of several new HPyV species. Polyomaviruses KI (KIPyV) and WU (WUPyV) were detected in the nasopharyngeal aspirates from patients presenting acute respiratory tract infections (Allander et al., 2007; Gaynor et al., 2007). MCPyV was detected from patients with MCC (Feng et al., 2008). Likewise, TS polyomavirus (TSPyV), was also named according to its identification in trichodysplasia spinulosa, a rare skin disease (van der Meijden et al., 2010). HPyV6 and HPyV7 were also identified in the skin and are associated with pruritic and dyskeratotic dermatosis and epithelial hyperplasia (Ho et al., 2015; Nguyen et al., 2017; Schowalter et al., 2010a). The remaining HPyVs include HPyV9, MWPyV, HPyV12, STLPyV, NJPyV, and LIPyV. They are currently not reported to be associated with any human diseases.

| Species | Virus name | abbreviation | year of discovery | Clinical correlate |
|-----------------------|--|--------------|-------------------|--|
| Human polyomavirus 1 | BK polyomavirus | BKPyV | 1971 | Polyomavirus-associated nephropathy; haemorrhagic cystitis |
| Human polyomavirus 2 | JC polyomavirus | JCPyV | 1971 | Progressive multifocal leukoencephalopathy |
| Human polyomavirus 3 | KI polyomavirus | KIPyV | 2007 | None known |
| Human polyomavirus 4 | WU polyomavirus | WUPyV | 2007 | None known |
| Human polyomavirus 5 | Merkel cell polyomavirus | MCPyV | 2008 | Merkel cell cancer |
| Human polyomavirus 6 | Human polyomavirus 6 | HPyV6 | 2010 | HPyV6 associated pruritic and dyskeratotic dermatosis (H6PD) |
| Human polyomavirus 7 | Human polyomavirus 7 | HPyV7 | 2010 | HPyV7-related epithelial hyperplasia |
| Human polyomavirus 8 | Trichodysplasia spinulosa polyomavirus | TSPyV | 2010 | Trichodysplasia spinulosa |
| Human polyomavirus 9 | Human polyomavirus 9 | HPyV9 | 2011 | None known |
| Human polyomavirus 10 | MW polyomavirus | MWPyV | 2012 | None known |
| Human polyomavirus 11 | STL polyomavirus | STLPyV | 2013 | None known |
| Human polyomavirus 12 | Human polyomavirus 12 | HPyV12 | 2013 | None known |
| Human polyomavirus 13 | New Jersey polyomavirus | NJPyV | 2014 | None known |
| Human polyomavirus 14 | Lyon IARC polyomavirus | LIPyV | 2017 | None known |

Table 1. List of human polyomavirus (HPyV). Currently, 14 HPyVs have been identified. 6 of them are associated with human diseases (highlighted in grey). Only Merkel cell polyomavirus (MCPyV) or HPyV5 is clearly related to malignancy (highlighted in orange). Sources from the International Committee on Taxonomy of Viruses (ICTV).

1.1.2 MCPyV, the etiologic agent of 80% MCC

PyVs have been suspected as potential etiologic agents in human cancer since the discovery of MPyV and SV40. However, no HPyVs were reported to be clearly associated with human malignancies until 2008. MCC is a rare neuroendocrine skin cancer with an increased incidence rate in patients suffering immunosuppression caused either by organ transplantation or other diseases, such as chronic immune disorders and HIV infection (Becker et al., 2017). Intriguingly, another rare skin cancer, Kaposi's carcinoma, is also an immunosuppression-related cancer and occurs especially in AIDS patients. Subsequent studies revealed that the etiologic agent of Kaposi's carcinoma is human herpesvirus 8 (HHV8) (Mesri et al., 2010). Given the common features shared between Kaposi's carcinoma and MCC, an infectious viral origin for MCC had been proposed. To search for the putative viral sequence in MCC, Feng and colleagues used digital transcriptome subtraction (DTS) they developed. DTS uses a combination of high throughput cDNA sequencing and *in silico* methods to sequentially exclude human sequences from a tissue sample transcriptome. For each normal or diseased sample, cDNA libraries are created and then subjected to sequencing, and the results are compared to human sequence databases. In 2008, the DTS technique was applied to MCC tumors and revealed previously unidentified viral transcripts with high sequence-identity to archival polyomavirus tumor antigen (T antigen) sequences. Ultimately, a full-length, 5387 base-pair (bp) viral genome (prototype MCV350) was sequenced from MCC tumor and was aptly named Merkel cell polyomavirus (MCPyV). MCPyV DNA sequences are detected in 80% MCC and are clonally integrated within the tumor genome, suggesting a prior MCPyV infection (Feng et al., 2008). Increasing evidence has now unveiled the oncogenic capacity of MCPyV, thus strongly suggest that MCPyV is the causative agent of most MCC or MCPyV⁺MCC (Liu et al., 2016b). Up to now, MCPyV is the only HPyV known to be tightly linked to human malignancy. Similar to other HPyVs, subsequent studies have revealed that MCPyV is widespread in the general population and is continuously shed from healthy skin (Liu et al., 2016b). Overall, MCPyV infection is ubiquitous and causes MCC only in the case of immunosuppression. Asymptomatic and persistent infection of MCPyV in healthy people suggest an immune tolerance process.

1.2 Structure and genome organization of MCPyV

1.2.1 MCPyV harbors conserved structure and genome organization with PyVs

The morphology and structural organization of polyomavirus are highly conserved and share similarities. The virions are small non-enveloped icosahedral particles of 40-50 nm diameter, with a circular dsDNA genome of approximately 5 kilobases (kb) wrapped around host cell-derived histones. The genome of PyVs consists of the early region (ER) and late region (LR) divided by a non-coding regulatory region (NCCR) containing a bidirectional promoter and the origin of replication (Dalianis and Hirsch, 2013). The prototype MCPyV MCV350 has a circular, dsDNA genome of 5387 bp and the genomes of all subsequent MCPyV strains sequenced average 5.4 kb (Figure 3). The segregation of the early and late transcriptional regions promotes a temporally regulated cascade of gene expression distinguished by the onset of viral DNA replication, a replication scheme consistent with other DNA viruses (Feng et al., 2011a). The ER genes that encode T antigens are transcriptionally activated immediately upon viral entry and are generally involved in viral genome replication. Following DNA replication, the LR genes express structural viral proteins (VP) that form the viral capsid and thus then contribute to progeny virion production. Despite these common features, the MCPyV viral genome has several unique genetic characteristics compared to other HPyVs.

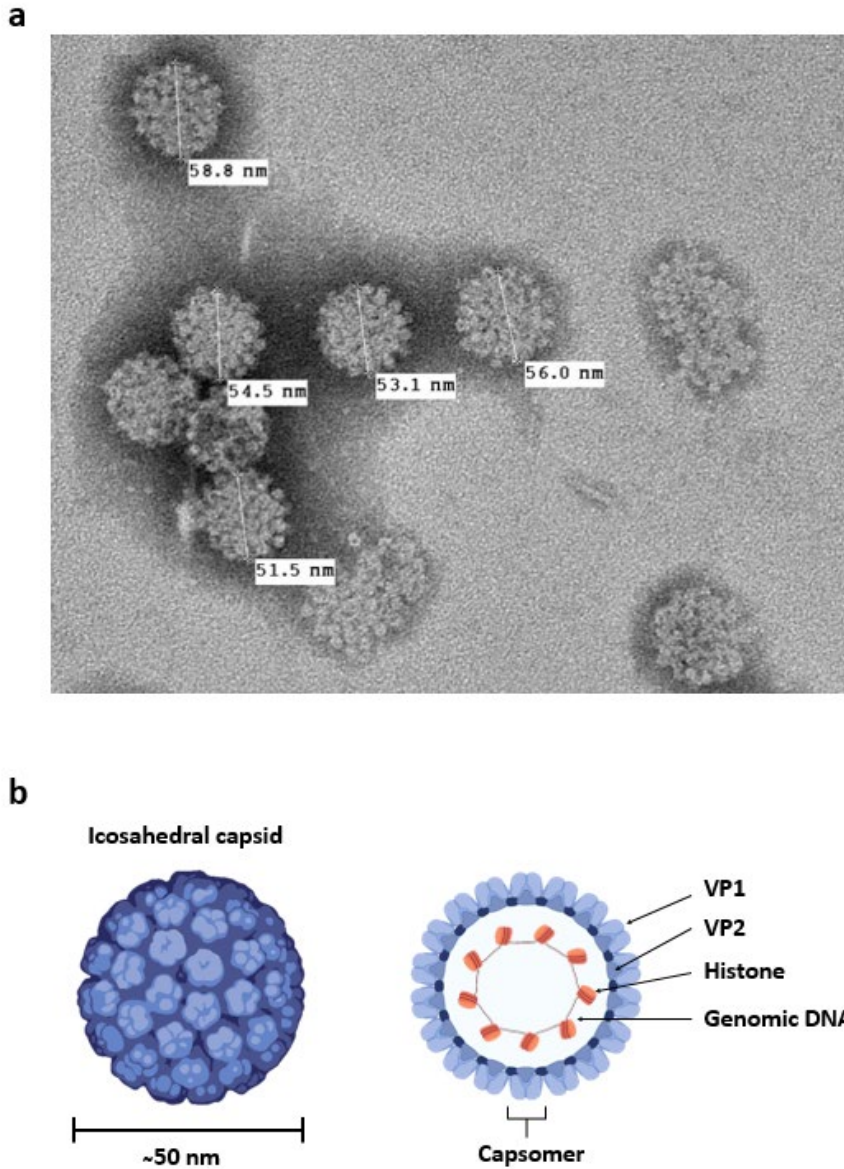


Figure 3. The structure of MCPyV. (a) Transmission electron microscopy (x50,000) of MCPyV virus-like-particle (VLP). The viral capsid has typical icosahedral symmetry with around 50 nm in diameter (Spurgeon et al., 2013). (b) Schematic presentation of MCPyV structure. MCPyV is a non-enveloped DNA virus. The viral genome consists of a circular double-stranded DNA chain swathed with host cell-derived histones. The genomic DNA is englobed in the capsid assembled by 72 capsomeres made of structural proteins, viral proteins 1 (VP1) and VP2.

1.2.2 MCPyV Early Region (ER) and Late Region (LR) gene products

Historically, PyVs ER genes products target cellular proteins that are often cell cycle regulators and tumor suppressors (DeCaprio et al., 1988; Pallas et al., 1990). The ER is frequently referred to as the “Tumor antigen locus” or “T antigen (T-Ag) locus” because of the tumorigenic potential of ER genes products. The MCPyV ER is a 2885 bp sequence. Upon alternative splicing, the MCPyV express 4 T antigens: large T antigen (LT), small T antigen (sT), 57kT antigen (57kT) and a protein called alternative LT Open reading frame (ALTO) (Carter et al., 2013; Shuda et al., 2008a) (Figure 4). LT, sT, and 57kT share a short, common N-terminus, but alternative splicing downstream of the first exon gives rise to three unique proteins. The ALTO protein is generated by an alternative T antigen open reading frame (ORF) present in the second exon of LT. ALTO is expressed during, but not required for, replication of the MCPyV genome. Phylogenetic analysis reveals that ALTO is evolutionarily related to the middle T antigen of MPyV despite almost no sequence similarity (Carter et al., 2013). The MCPyV LT is a protein of 816 aa encoded by two exons (Figure 5). The MCPyV LT has conserved domains presented in well-documented LT functions of other PyVs, including CR1 (LXXLL) and DnaJ (HPDKGG) domains, a retinoblastoma protein (pRb)-binding domain (LXCXE), an origin-binding domain (OBD), and a helicase domain (Pipas, 1992). It is reasonable that MCPyV LT shares some common functions with other PyVs LT. A nuclear localization signal (NLS) is located between the pRb-binding LXCXE motif and the OBD (Nakamura et al., 2010a). The carboxyl-terminus part of LT contains the OBD and ATPase/helicase domain that are necessary for viral DNA replication. The c-terminal region is also the zone of tumor-specific mutations that are always truncated in MCC. Of note, MCPyV LT is distinct from other HPyVs and shares only 30% aa identity with other HPyVs counterparts (Johnson, 2010). Between the beginning of exon 2 and the OBD lies a stretch of unique sequence of about 200 amino acids. This so-called MCPyV unique region (MUR) is specific to MCPyV and contains a binding motif for the cellular vacuolar sorting protein Vam6p just proximal to the LXCXE motif (Liu et al., 2011b). The MCPyV sT, as aforementioned, shares exon 1 of the T antigen locus and therefore has conserved CR1 and DnaJ motif. sT contains in addition a sequence of 106 aa at c-terminus in the continuity of exon 1 that makes the entire sT a 186 aa protein. The unique c-terminus part of MCPyV sT harbors a protein phosphatase 2A (PP2A) site and a LT stabilization domain (LSD) (Wendzicki et al., 2015). It also contains a protein phosphatase 4C (PP4C) binding site at a region (95–111 aa) directly adjacent to the LSD at 91–95 aa. The 57 kT protein is the product of a transcript

that undergoes alternative splicing to join three exons encoding for 432 aa, and this protein shares many of the same motifs present in LT and sT. The 57 kT protein has the same amino terminus as both LT and sT, including the CR1 and DnaJ domains, and also contains the MUR, pRb-binding LXCXE motif, and the most C-terminal 100 amino acids that are present in LT. Information on the role of 57 kT in the viral life cycle or MCPyV-induced tumorigenesis is scarce. In addition to aforementioned proteins products, the MCPyV ER genes encode a micro-RNA sequence of 22 nucleotides, MCV-miR-M1 (Seo et al., 2009). This miRNA is reverse complementary to a sequence in LT adjacent to the LXCXE motif, implying its role in the regulation of ER gene expression. The T antigens in the biology of MCPyV and host cellular transformation will be detailed in subsequent paragraph.

The LR of MCPyV contains 3 ORF that encodes the major viral capsid protein 1 (VP1) and the minor capsid protein VP2 and VP3. However, MCPyV lacks the conserved Met-Ala-Leu motif that normally forms the amino-terminus of all previously described polyomavirus VP3 proteins. This anomaly, taken together with failed attempts to express correctly VP3 *in vitro*, suggests that MCPyV either expresses non-functional VP3 or does not express it (Schowalter and Buck, 2013).

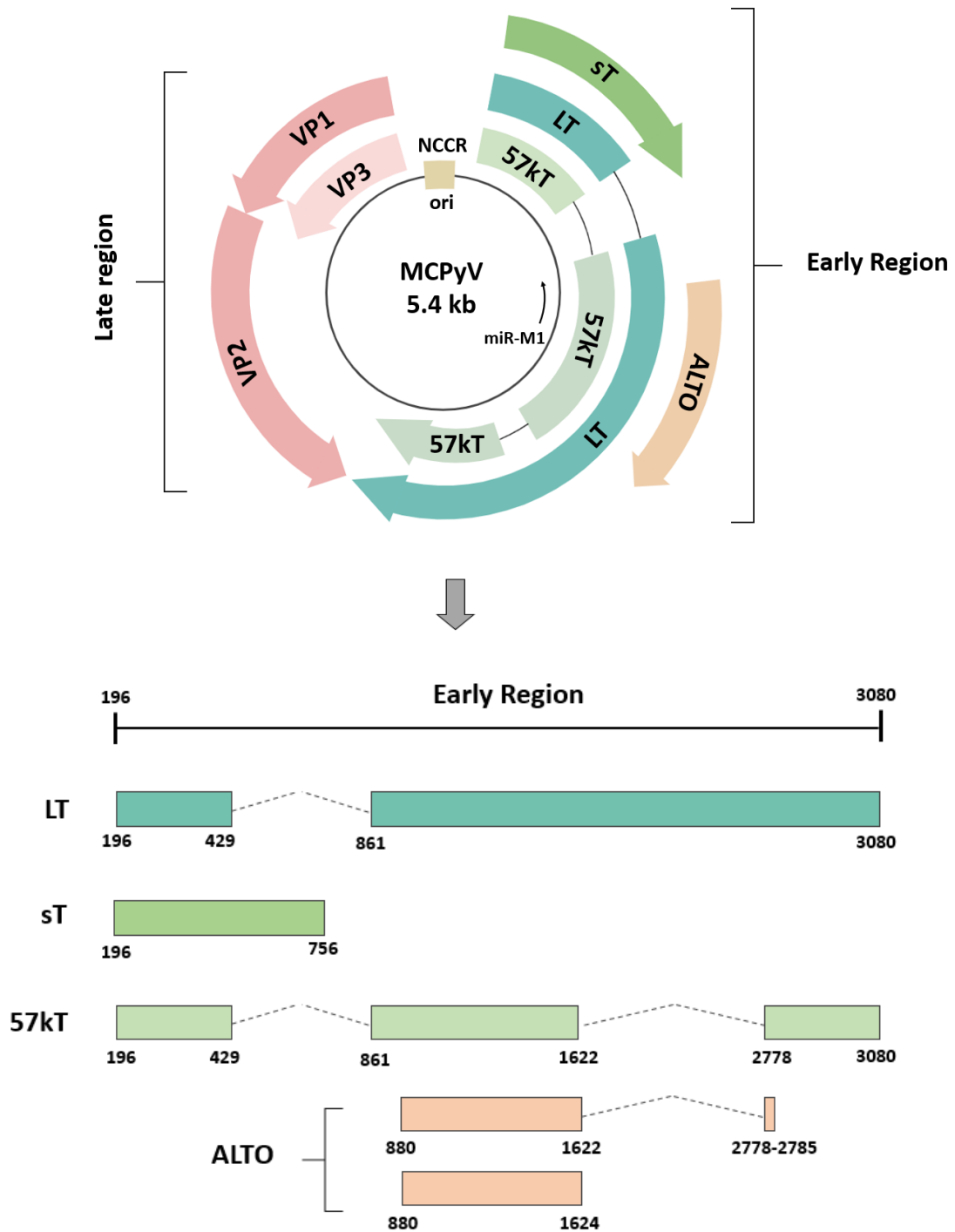


Figure 4. Genome organization of MCPyV. MCPyV genome consists of a 5.4 kb circular double-stranded DNA molecule separated into an early region and a late region by the non-coding regulatory region (NCCR) containing a bidirectional promoter and the origin of replication (ori). The late region encodes theoretically 3 transcripts for viral proteins (VP), VP1, VP2, and VP3. VP3 is not observed in MCPyV VLP. The early region generates multiple transcripts through alternative splicing, including small T (sT), large T (LT), 57kT, alternative frame of the large T open reading frame (ALTO), and microRNA (miR-M1).

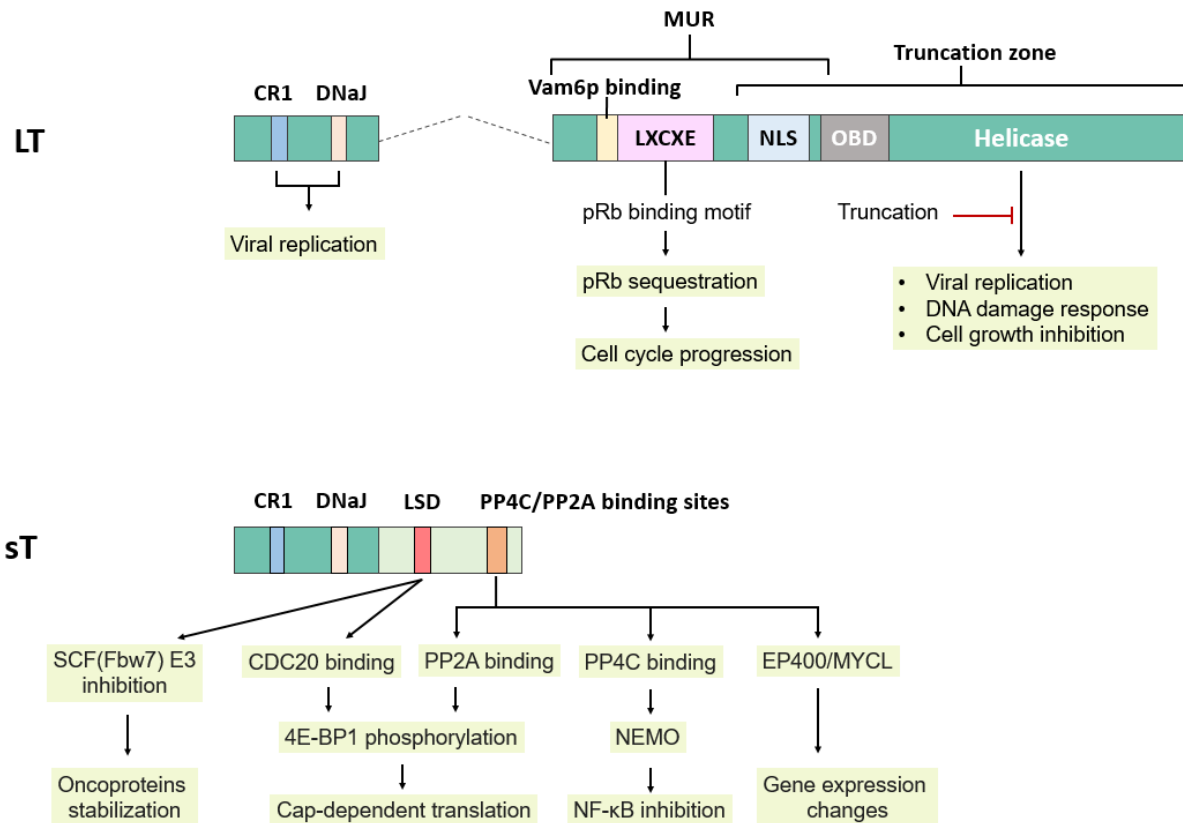


Figure 5. Structure and function of MCPyV LT and sT. The CR1 and DNaj domains are indispensable for viral DNA replication. The MCPyV unique region (MUR) includes the retinoblastoma-associated protein (pRb) binding motif responsible for direct sequestration of RB, thus leading to cell cycle progression to the S phase. The MUR mediates binding to the vacuolar sorting protein Vam6p. The nuclear localization signal (NLS), origin binding domain (OBD), and the helicase domain are essential elements for viral replication. The integrated MCPyV genome present in MCPyV⁺MCCs harbors truncating mutations in LT that disrupt the helicase domain, resulting in a replication-incompetent truncated LT that nonetheless retains the ability to promote cell cycle progression. sT shares a common N-terminal sequence with LT that contains the CR1 and DNaj motifs. In addition, sT has the LT stabilization domain (LSD) that inhibits E3 ubiquitin ligase activity via interactions with SCFFbw7 and cell division cycle protein 20 homologue (CDC20), resulting in increased oncoprotein stability and cap-dependent translation, respectively. sT binds to protein phosphatase 2 A (PP2A) via its PP2A binding motif, resulting in hyperphosphorylation of the eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), thus also leading to enhanced cap-dependent translation. sT also interacts with NF-κB essential modulator (NEMO) and EP400/MYCL to inhibit NF-κB signaling and induce gene expression change, respectively. LT and sT downregulate Toll-like receptor 9 (TLR9) expression via the inhibition of the CCAAT/enhancer-binding protein (C/EBPβ) transcription factor (not shown).

1.3 Natural life cycle and cell tropism of MCPyV

1.3.1 Attachment and infectious entry of MCPyV

The natural life cycle of MCPyV is not fully elucidated (Figure 6). Viral infections are initiated by the specific attachment of a virus particle to receptors at the surface of the host cell. Initial study found that MCPyV pentameric capsomeres composed of recombinant VP1 proteins bound sialic acid components of the ganglioside Gt1b (Erickson et al., 2009). The result suggested that MCPyV abides by the typical attachment and uptake processes used by other PyVs, which primarily utilize sialylated oligosaccharides as primary receptors (Neu et al., 2011). However, a subsequent study using MCPyV pseudovirions (composed of VP1, VP2, and a luciferase reporter plasmid) demonstrated that MCPyV requires sulfated glycosaminoglycan, particularly heparan sulfate, for infectious entry. Interestingly, in the same study, the authors also found that MCPyV pseudovirions efficiently bound to cells that lack sialylated glycans, but the particles were deficient in gene transduction. These results implied that sialic acids may form an essential element of a possible co-receptor for viral entry that is engaged after the initial attachment of MCPyV to the cell via glycosaminoglycan (Schowalter et al., 2011). Consistently, another study identified sialic acid binding sites on VP1 that are required for a post-attachment step in MCPyV infectious entry; however, mutation of these sites did not affect the initial cellular attachment of MCPyV pseudovirions (Neu et al., 2012). Taken together, these reports suggest a novel infectious entry process that requires glycosaminoglycan for initial attachment and subsequent association with sialic acid for gene transduction. The use of sialic acid as a secondary receptor by MCPyV differs from the current consensus for other PyVs (Neu et al., 2011). After binding to the cell surface, PyVs capsid undergoes endocytosis and is transported to the nucleus where the viral DNA is uncoated and transcription of the early region begins. Host cell entry of most PyVs occurs by caveolar/lipid raft-mediated endocytosis (Anderson et al., 1996; Norkin et al., 2002; Parton and Richards, 2003), whereas JCPyV, for example, uses clathrin-mediated endocytosis (Pho et al., 2000). After endocytosis, virions are routed through the endosomal pathway to be delivered to the endoplasmic reticulum (ER) (Dupzyk and Tsai, 2016). There, a chaperone- and disulfide isomerase-mediated uncoating step occurs, whereupon the partial disassembled viral particles are retrotranslocated into the cytosol by the endoplasmic reticulum-associated degradation machinery (Inoue and Tsai, 2015; Schelhaas et al., 2007; Walczak and Tsai, 2011). To explore the infectious entry process and cytoplasmic trafficking of MCPyV, Becker and colleagues

analyzed the cell biological determinants of MCPyV entry into A549 cells, a highly transducible lung carcinoma cell line (Becker et al., 2019). They found that MCPyV enters cells via caveolar/lipid raft-mediated endocytosis but not clathrin-mediated endocytosis. The pseudovirions were internalized in small endocytic pits that led the virus to endosomes and from there to the endoplasmic reticulum. Similar to other PyVs, trafficking required microtubule transport, acidification of endosomes, and a functional redox environment. Consistently and precisely, potassium and calcium ion channels on the endosome are important for MCPyV endosomal trafficking (Dobson et al., 2020a). Interestingly, during the trafficking, the MCPyV pseudovirion particles were found to acquire a membrane envelope within endosomes, a phenomenon not reported for other viruses. And only minor amounts of viruses reached the ER, while the majority was retained in endosomal compartments, suggesting an innate antiviral defense during endosome-to-ER trafficking. The mechanism of translocation of the MCPyV genome into the nucleus remains elusive. PyVs including SV40, BKPyV and JCPyV enter the host cell nucleus via the nuclear pore complex (NPC) and require the importin α/β heterodimer (Bhattacharjee and Chattaraj, 2017). Whether nuclear entry of MCPyV is also mediated by this mechanism has not been yet investigated.

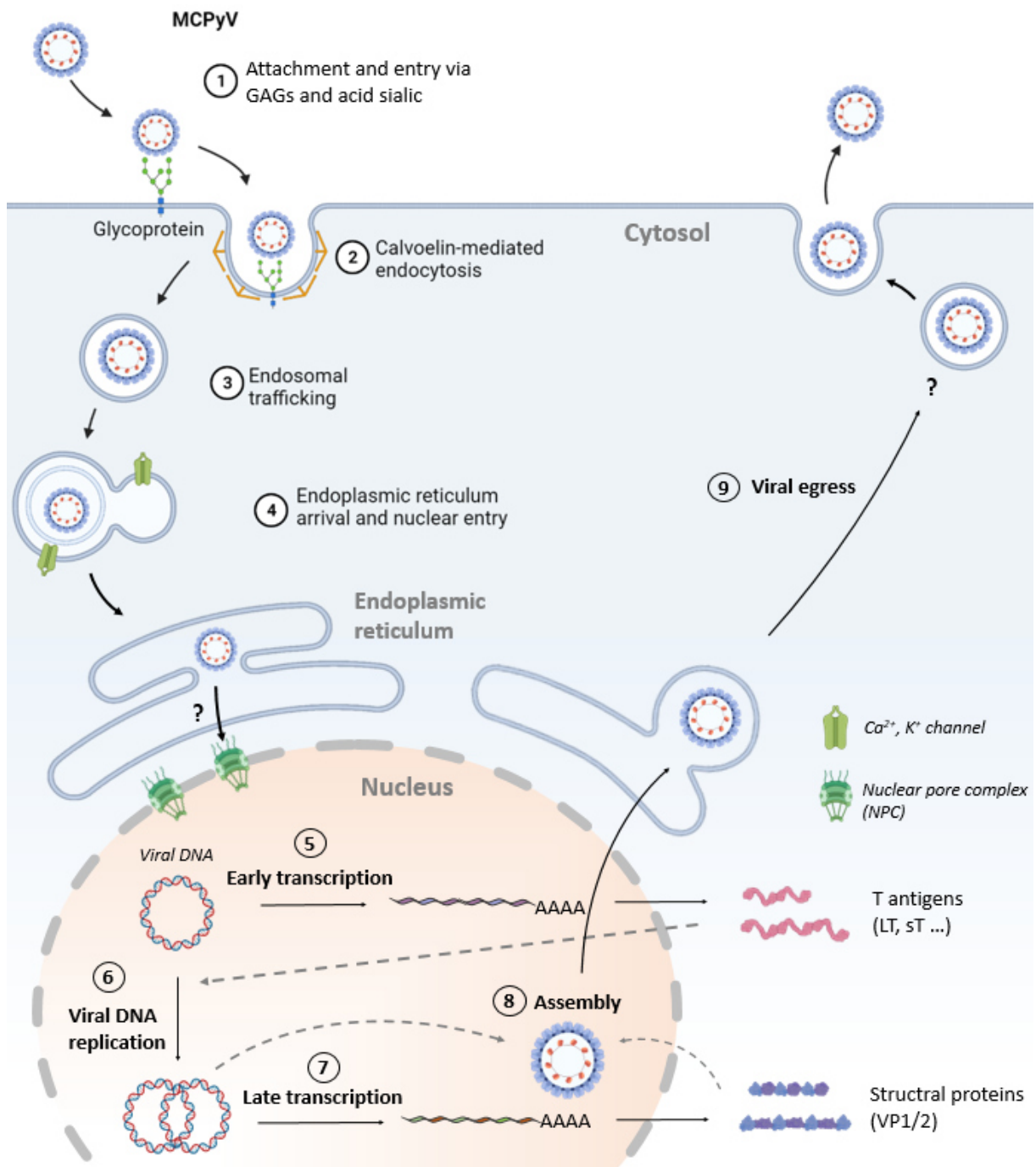


Figure 6. Schematic presentation of MCPyV life cycle. The initial attachment of MCPyV to the target cell's surface is engaged by the interaction between glycosaminoglycans (GAGs) and viral capsid. The interaction between viral capsid protein and acid sialic facilitates further viral entry. MCPyV enters the cytosol via caveolin-mediated endocytosis. The virus migrates to the endoplasmic reticulum (ER) lumen through the endosomal pathway. During trafficking, MCPyV acquires a membrane envelope within endosomes. A functional redox environment involving ion channels is required for trafficking. Following the deposition within the ER, the virus enters into the nucleus and releases its genome upon decapsidation. The early region encodes T antigens that are necessary for viral DNA replication,

whereas the late region encodes structural proteins required for viral assembly. New virions may exit the cell via exocytosis.

1.3.2 Viral replication and the involvement of MCPyV ER products

Although human primary dermal fibroblasts support the full MCPyV life cycle (Liu et al., 2016a), cell culture systems to produce sufficient quantities of infectious MCPyV are not readily available. *In vitro* study of MCPyV replication is conducted basically by using recombinant MCPyV genome and/or MCPyV pseudovirion. Multiple reports were published attempting to recapitulate the virus life cycle in cultured cells using consensus MCPyV genomes that share high homology to DNA found within encapsidated virions shed from healthy human skin (Feng et al., 2011b; Neumann et al., 2011; Schowalter et al., 2010b, 2011). In these studies, viral DNA replication and expression of T antigens and VP1 were observed following the transfection of the circularized MCPyV genome. Acknowledging widely variable transfection efficiencies between cell lines, only 3 out of 22 lines tested supported early and late gene expression: human embryonic kidney cells 293 (HEK293), human non-small cell lung carcinoma cells (H1299), and a human neuroectodermal cell line (PFSK-1) (Neumann et al., 2011). Gene expression kinetics was consistent with a systematic early to late cascade of gene expression. And progeny pseudo viral particles were observed. Encouragingly, Liu and colleagues successfully produced infectious progeny MCPyV in human dermal fibroblast, thus identified human dermal fibroblast as the natural host cell that supports the full life cycle of MCPyV (Liu et al., 2016a). Interestingly, the progeny MCPyV can also infect fibroblast cell lines such as WI-38 and IMR-90 derived from lung tissues and express LT and VP1. The replication of MCPyV is tightly regulated by ER gene products, in particular LT and sT.

Upon the nuclear entry of MCPyV, the genome is released and the ER genes are immediately expressed to support viral DNA replication. The HPDKGG hexapeptide sequence in DnaJ domain of sT and LT interact with Hsc 70. Disrupting the interaction between HPDKGG motif and Hsc 70 with a point mutation dramatically decreases MCPyV replication *in vitro* (Kwun et al., 2009a). The LT-Vam6p interaction, which can be ablated by mutation of a single tryptophan residue at position 209, results in the nuclear sequestration of this cytosolic protein and disrupts lysosomal clustering. This function appears to be unique to MCPyV LT (Liu et al., 2011b). In an *in vitro* replication assay using an infectious molecular clone of MCV mutated at position 209, Feng *et al.* demonstrated that loss of LT-Vam6p binding leads to

enhanced viral replication compared to wild-type control (Feng et al., 2011c). It is possible that in the natural life cycle of MCV, LT-Vam6p interaction inhibits or minimizes viral reactivation, a potential mechanism of 'viral latency'. The OBD domain in the center portion of LT binds to a serial of pentanucleotide consensus sequences present in the origin of replication within NCCR. The ATPase/helicase domain in the C-terminus of LT is crucial for LT oligomerization and directs the initiation of viral DNA replication (Fanning and Zhao, 2009; Harrison et al., 2011; Theiss et al., 2015). Phosphorylation of LT upstream of the OBD at residues T297 and T299 decreases the origin-binding affinity of LT and negatively affects replication initiation (Diaz et al., 2014). Interestingly, a novel cellular factor has been recently identified to interact with both N- and C-terminus of LT to modulate viral replication. The direct interaction of Ubiquitin-specific protease Usp7 to MCPyV LT increases the binding affinity of LT to the origin of replication. In the opposite of previous studies, this increased binding regulates negatively viral replication (Czech-Sioli et al., 2020). A possible explanation could be that the binding of Usp7 to the C-terminal part of LT prevents its oligomerization and helicase activity. Studies have also shown that components of the ATM- and ATR-mediated DNA damage response pathways were found to accumulate in the MCPyV LT-positive nuclear foci. This implies an important role of DNA damage response in MCPyV replication (Tsang et al., 2014).

Although MCPyV sT share exon 1 of the T antigen locus, and the DnaJ domain of LT is required for replication, the functional significance of this domain in sT is unknown. It has been shown that mutating Hsc70 binding on sT does not interfere with its effect on MCV viral replication (Kwun et al., 2009b). However, sT prevents LT degradation by inhibiting SCFFbw7 E3 ubiquitin ligase. Since LT is a target of Fbw7, co-expression of sT prevents LT degradation, increases steady-state LT levels target, and has been shown to enhance viral replication (Kwun et al., 2013a). The precise function of the micro-RNA MCV-miR-M1 is not fully elucidated. *Thesis et al.* showed that similar to other PyVs miRNAs, mcv-miR-M1 is capable to negatively regulate the expression of viral gene products required for viral DNA replication. They also observed that mcv-miR-M1 augments long-term episomal persistence of MCPyV genomes (Theiss et al., 2015). Given that MCPyV establishes chronic infections in the majority of healthy populations, these results suggest a potential mechanism of a lifelong viral persistence.

1.3.3 Viral assembly and egress

Assembly of virions occurs either in the cytosol or in the nucleus of the host cell and involves polymerization of the capsid protein-like viral minichromosomes (Garber et al., 1980). The release of non-enveloped virus usually occurs through cell lysis, but in some cases, they may escape either by some secretory mechanisms or use cellular autophagy pathways for exit (Altenburg et al., 1980; Jackson et al., 2005). Studies aiming to decipher the mechanism of MCPyV assembly and egress are scarce probably due to the lack of an appropriate routine cell culture system that supports high infectious MCPyV yield. Research on viral assembly and egress of other PyVs, such as SV40, BKPyV, and JCPyV may provide some inspiration. Viral structural proteins are gradually added to the viral DNA–histone complex to form immature virions, which subsequently give rise to extracellular mature virions (Fanning and Baumgartner, 1980). In vitro studies with expressed VP1 protein of JCPyV and BKPyV revealed that it has the capability of self-assembly into virus-like particles (VLP) that are composed of 72 capsomers (Chang et al., 1997; Li et al., 2003; Montross et al., 1991). These VLPs had the same antigenicity as that of natural viral empty capsids. Likewise, MCPyV VP1 can auto-assemble into MCPyV-like particle of icosahedral form with about 50nm in diameter that is composed of 72 capsomers. However, the MCPyV-LPs did not share antigenic determinants with BKPyV and JCPyV (Li et al., 2015b; Touzé et al., 2010). Disulfide bonds are found to have an important role in maintaining the integrity of the human PyV VP1 structure by protecting calcium ions from chelation (Chen et al., 2001). In a study of the DNA-binding capacity of JCPyV VP2, it was shown that the last 13 amino acids, most importantly Lys (332) and Lys (336), within the DNA binding domain of VP2 were essential for the binding (Huang et al., 2003). The host chaperone system, including Hsp70, interacts with C-terminal domain of VP1 to help VP1 capsomeres assembly (Chromy et al., 2003). Some of the viruses assemble at specific intracellular locations, often termed “virus factories” that represent subcellular scaffolds, where genomes and capsid proteins are brought together for assembly into virion particles (Erickson et al., 2012). The site for nuclear virus assembly factories is promyelocytic leukemia nuclear bodies (PML-NBs). For JCPyV, the major and minor capsid proteins cooperatively accumulate in PML-NBs where they assemble into virion particles (Shishido-Hara et al., 2004). Non-enveloped viral egress is generally thought to be associated with host cell lysis. However, the process of release of PyV has been reported not to cause significant cytopathic effects in the host cell. For instance, SV40 can be unleashed from the apical surface of infected cells without causing cell lysis,

including human mesothelial, epithelial, fibroblast, and HEK cells (Bocchetta et al., 2000; Clayson et al., 1989; O'Neill et al., 1990). Similarly, a non-lytic release mechanism has recently been observed in BKPyV (Evans et al., 2015). Little is known about the egress of MCPyV. The mechanism by which MCPyV progeny virions exit the host cell to infect other cells remains unknown.

1.3.4 Natural cell tropism of MCPyV

Despite the similarity in structure and genome organization shared with other PyVs, the natural life cycle of MCPyV is not fully elucidated. Owing to the lack of knowledge of the natural host cell tropism of MCPyV and the technical difficulties of propagating the virus in cell culture mechanistic studies to fully understand the molecular biology of MCPyV have been hindered. Thus far, only human dermal fibroblast has been identified as the natural host cell that supports productive MCPyV infection and the induction of matrix metalloproteinase (MMP) genes by the WNT/b-catenin signaling pathway, along with other growth factors are crucial for MCPyV infection (Liu et al., 2016a). As MCPyV is constantly shed from the skin, how the virions produced in the dermis reach the surface of the skin remains unclear. Identification of human dermal fibroblasts as the target cells of MCPyV infection presents new opportunities to understand the natural life cycle of MCPyV and cellular transformation in MCC. This finding is consistent with the fact that most MCC tumors develop in the human dermis (Calder and Smoller, 2010).

Of note, as multiple cells could be the origin of cells of MCC, dermal fibroblast might not be the only target cells of MCPyV. Such hypothesis is also supported by the evidence that MCPyV DNA was also detected in a wide range of anatomical locations (Spurgeon and Lambert, 2013), including respiratory tract (Babakir-Mina et al., 2010a), saliva (Foulongne et al., 2010), lymphoid tissues (Toracchio et al., 2010), gastrointestinal tract (Campello et al., 2011a), urine (Bofill-Mas et al., 2010a), peripheral blood and inflammatory monocytes in MCC patients (Mertz et al., 2010a). The presence of MCPyV sequence in other organs raises the possibility that MCPyV could persist in different cell types and spreads along the migration routes of immune cells, such as monocyte.

1.4 Epidemiology of MCPyV

Since its discovery in 2008, epidemiological studies have established MCPyV as a common virus infecting the human population. MCPyV is considered as part of normal skin microflora, as viral genome and encapsidated virion can be detected from healthy human skin (Schowalter et al., 2010b). Similar to other HPyVs, MCPyV is highly prevalent in the healthy population, with up to 60–80% of adults showing serum reactivity against the VP1 protein (Carter et al., 2009a; Kean et al., 2009a; Pastrana et al., 2009a). Seropositivity is not detected in prenatal blood (Gustafsson et al., 2012a), yet becomes widely prevalent in children in their first decade of life and increases with age, suggesting primary infection occurs in early childhood and is clinically inapparent (Chen et al., 2011b; Tolstov et al., 2009; Viscidi et al., 2011). Patients with MCPyV⁺MCC exhibit higher antibody titers than those with MCPyV⁻MCC (Pastrana et al., 2012a). In contrast to VP1 specific antibodies, seroreactivity against ER gene products can only be detected in rare cases and at very low levels in the general population. However, patients with MCPyV⁺MCC demonstrate high antibody titers against T antigens and correlate with tumor burden (Paulson et al., 2010a). The quantity of DNA shed from the skin correlates with serum antibody responsiveness suggesting the skin as a primary site of MCPyV replication (Pastrana et al., 2012a). Nevertheless, MCPyV can be detected in various other tissues as aforementioned. The transmission mode of MCPyV is still an enigma, several possible routes have been proposed. For instance, the presence of viral DNA in the gastrointestinal tract and urban sewage suggests a fecal-oral mode of transmission (Bofill-Mas et al., 2010b; Campello et al., 2011b; Loyo et al., 2010), while present in the respiratory tract supports an airborne route (Babakir-Mina et al., 2010b; Kantola et al., 2009). As encapsidated viruses continuously shed from human skin and viral DNA can be detected on environmental surfaces (Foulongne et al., 2011), direct contact between human to human or contaminated surfaces might be one of the transmission modes.

2. Biological significance of MCPyV in MCC

2.1 The association between MCPyV and MCC

The establishment of the causal relationship between MCPyV and MCC had been proposed since the discovery of the virus and clonal integration of the viral genome into MCC cells. MCPyV integrates as head-to-tail concatemers at different locations randomly distributed

throughout the cellular genome, and no hot-spot of viral integration has been identified yet (Laude et al., 2010; Martel-Jantin et al., 2012; Sastre-Garau et al., 2009). The viral integration event appears to take place before the clonal expansion of tumor cell proliferation (de Biase et al., 2012; Laude et al., 2010; Sastre-Garau et al., 2009). MCC tumor metastases are also MCPyV- positive and share the same integration pattern as the original tumor (Feng et al., 2008; Laude et al., 2010). These findings strongly suggest MCPyV integration facilitates the initiation of tumorigenesis. Other evidence supporting the causality is that MCPyV T antigens are consistently expressed in MCC tumor cells. Up to 80% MCC express LT antigen and it is restricted to tumor cells and cells within the tumor but not the surrounding tissues (Shuda et al., 2009). Almost all MCC cells express sT antigen (Shuda et al., 2011a). The direct evidence for MCPyV-induced carcinogenesis is strongly reinforced by the fact that T antigens expression is required for the growth of MCC cell lines. T antigens knockdown in MCC cell lines results in impaired cell survival and proliferation (Houben et al., 2010b). Another convincing evidence and a hallmark of MCC is that the C-terminal domain of LT antigen of MCPyV is consistently truncated in MCC cells. The mutation area is located downstream of the LXCXE motif (Fischer et al., 2010; Shuda et al., 2008b). This tumor-specific mutation gives rise to truncated LT (trLT) or onco-type LT (otLT) antigens in which the CR1, DnaJ, MUR, NLS, and pRb-binding motifs are preserved, yet domains associated with replication are lost. This renders the virus incapable of replication, yet still proficient in the induction of cellular proliferation. The C-terminal truncation of LT helps the virus to maintain a replication-deficient status that is crucial for viral-induced oncogenesis. Taken together, these findings strongly suggest that MCPyV is the etiologic agent for MCPyV⁺MCC. Nevertheless, the precise mechanisms that direct integration of the MCPyV genome and C-terminal truncation of LT are not understood. One reasonable hypothesis is that UV-induced DNA break may facilitate MCPyV integration and C-terminal truncation of LT, and DNA damage responses might be involved.

2.2 Oncogenic potential of ER gene product and cellular transformation

Though the mechanism of carcinogenesis of MCC is not fully elucidated, evidence showed that the T antigens of MCPyV have oncogenic potential and contribute to the development and progression of MCPyV⁺MCC (Figure 5). Much of the oncogenic function of LT antigen has been attributed to its high binding affinity for pRb. The pRb is known as a tumor suppressor that negatively controls cell proliferation. The binding of pRb to MCPyV LT via LXCXE motif causes

the sequestration and inactivation of pRb, thus upregulates subsequently E2F target genes involved in cell growth, such as cyclin E (Borchert et al., 2014). The pRb-binding function of MCPyV LT is required for sustained growth of MCPyV⁺MCC tumor both *in vitro* and in xenograft models (Houben et al., 2012). The C-terminal truncation of LT implies a selective pressure for the elimination of viral replicative elements in tumor development. Because full-length LT could initiate unlicensed replication at the site of integration in the host genome leading to a DNA damage response and cell death (Nakamura et al., 2010b). Moreover, the expression of OBD and helicase domain of LT in the C-terminal part causes DNA damage and stimulates host DNA damage response. This leads to p53 activation and inhibition of cell proliferation, which functions as a barrier to tumor growth (Li et al., 2013). The inhibitory function of cell growth of full-length LT is also reported by Cheng and colleagues, who mapped this function to the C-terminal 100 residues (Cheng et al., 2013). The phosphorylation of the MCPyV LT C-terminus at S816 by Ataxia telangiectasia mutated (ATM) kinase induces apoptosis and inhibits host proliferation, contributing to the antitumorigenic properties of LT (Li et al., 2015a).

Unlike LT, expression of sT alone is sufficient to transform immortalized rodent fibroblasts and also has transforming activity *in vivo* (Verhaegen et al., 2015b). MCPyV sT shares an N-terminal region with LT and carries a unique C-terminal domain that contains LT-stabilization domain (LSD) and phosphatase PP2A binding domain. The binding of PP2A leads to hyperphosphorylation and inactivation state of the eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), resulting in dysregulation of cap-dependent translation that accelerates cell proliferation and malignant transformation (Shuda et al., 2011b). Likewise, sT binds through its LSD region to the cell division cycle protein 20 (Cdc20) and cdc20 homolog 1 (Cdh1) E3 ligase adapters. This activates cyclin-dependent kinase 1/cyclin B1 (CDK1/CYCB1) to directly hyperphosphorylate 4E-BP1 at authentic sites, leading to increased cell mitogenesis (Shuda et al., 2015b). sT also targets the cellular ubiquitin ligase SCFFbw7 to inhibit proteasomal degradation of MCPyV LT and other cell-cycle regulators such as c-Myc and cyclin E, thus contributing to viral replication and host cell transformation (Kwun et al., 2013b). The LSD domain is also responsible for inducing genome instability that might contribute to MCC carcinogenesis (Kwun et al., 2017). In MCC, no truncation or mutation has been found in sT, further confirming that the intact MCPyV sT is needed to drive MCPyV-induced carcinogenesis.

It has been shown that sT can bind specifically to the MYC homolog MYCL and recruits it to the EP400 complex, a histone acetyltransferase complex involved in transcriptional activation. The sT-MYCL-EP400 complex results in specific gene promoter activation, contributing further cellular transformation and reprogramming of MCC (Cheng et al., 2017).

2.3 The role of ER gene products in metastasis of MCC

Viral oncogene promotes human malignancy either by initiating tumorigenesis or by inducing cell mobility. Recent studies demonstrated that sT and LT contribute to the high metastatic capacity of MCC by inducing cell mobility. MCPyV sT affects the remodeling of the actin cytoskeleton to promote the formation of filopodia via the activation Rho-GTPase cdc42 and RhoA (Stakaitytė et al., 2018). And the enhanced cell mobility requires the activation of p38 MAPK signaling which also relies on MCPyV sT (Dobson et al., 2020b). Another study showed that sT expression disrupts the integrity of cell-cell junctions and enhances cell dissociation by inducing the cellular sheddases, A disintegrin, and metalloproteinase (ADAM)(Nwogu et al., 2018).

CHAPTER III. Immune response against MCPyV

1. Innate antiviral immunity

1.1 Sensing of dsDNA virus

The sensing of the virus by the immune system serves as the first line of host anti-viral defense by deploying numerous germline-encoded receptors called pattern-recognition receptors (PRRs) that detect viral infection. PRRs recognize pathogen-associated molecular patterns (PAMPs) that are conserved molecular structures important for the life cycle of pathogens (Kawai and Akira, 2010). Several PRR families have been identified so far, including toll-like receptors (TLRs), RIG-I-like receptors (RLRs), NOD-like receptors (NLRs), and the cytosolic DNA sensors. The engagement of PRRs by viral PAMPs triggers different signaling pathways that lead to the production of type I interferon IFN and pro-inflammatory cytokines, thus, initiating the innate anti-viral response (Figure 7). The innate sensing of non-enveloped dsDNA virus is generally mediated by TLR9 located on the membrane of the endosomal compartment of antigen-presenting cells (APC), notably dendritic cells (DC) and plasmacytoid DC (pDC). TLR9, the only DNA sensor among all other identified TLRs, recognizes hypo/unmethylated CpG-rich DNA (CpG DNA) motif that is frequently present in viral genome but is rare in mammalian (Latz et al., 2004). Following the interaction with the cell membrane, non-enveloped dsDNA viruses enter the host cell primarily via endocytosis (Mercer et al., 2010). As the internalized virus traffics in the endosomal compartment, it undergoes a capsid uncoating process and releases its genome that contains CpG DNA motif. The detection of viral DNA by the upstream DNA sensors triggers a series of signaling cascades that leads to the expression of Type I IFNs and pro-inflammatory cytokine, thus initiating the inflammatory response. The binding of viral DNA to TLR9 triggers its dimerization and activates the recruitment of the adaptor protein MyD88 to the cytosolic Toll/IL-1 Receptor (TIR) domain of TLR9. MyD88 recruits IL-1 receptor-associated kinase-4 (IRAK-4) to TLRs through the interaction of the death domains of both molecules. IRAK4 phosphorylates IRAK1 and IRAK2 that activate the E3 ubiquitin ligase TRAF6. TRAF6 then activates TGF- β activated kinase 1 (TAK1) that, in turn, phosphorylates I κ B kinase (IKK) complex containing a regulatory subunit, NF- κ B essential modulator (NEMO) or IKK γ , as well as two catalytic kinase subunits IKK α /IKK β , leading to subsequent I κ B phosphorylation and degradation and release of NF- κ B. NF- κ B, a key transcription factor that regulates immune-related gene expression, translocates into the nucleus and activates the expression

of pro-inflammatory cytokines. The IRAK-4/TRAF6 complex also phosphorylates the interferon regulatory factor 7 (IRF7) and IRF3. Phosphorylation of IRF7/3 induces their dimerization and translocation to the nucleus, thus leading to the expression of IFN- α and IFN- β respectively. The TLR9/MyD88 signaling also activates MAPK pathway through TAK1, resulting in cell proliferation and survival.

The virus' DNA can also be detected by cytosolic DNA sensors. The stimulator of IFN genes (STING), a transmembrane protein expressed on the endoplasmic reticulum membranes and the outer mitochondrial membrane of antigen-presenting cells such as macrophage and DC, recognizes non-CpG intracellular DNA produced by various DNA pathogens (Ishikawa and Barber, 2008). Cyclic-GMP-AMP (cGAMP) synthase (cGAS), another cytosolic DNA sensor, catalyzes the production of cGAMP from ATP and GTP upon DNA binding. cGAMP serves as a second messenger, which binds and activates STING. Activated STING translocates to the Golgi, where it recruits and activates TANK-binding kinase 1 (TBK1) and I κ B kinase (IKK) leading to activation of IRF3 and NF- κ B, resulting in type I IFNs and proinflammatory cytokines expression (Ablasser et al., 2013; Motwani et al., 2019). STING is essential for type I IFN induction in response to certain non-enveloped DNA viruses, such as adenovirus and human papillomavirus (HPV) (Ma and Damania, 2016). Since the discovery of STING, several putative DNA sensors have been reported to detect cytosolic DNA and activate type I IFN production dependent on STING. IFN γ -inducible protein 16 (IFI16) is a member of the PYHIN (pyrin and HIN domain-containing protein) protein family, that binds to intracellular dsDNA via HIN domain and mediates type I IFN response (Unterholzner et al., 2010). Other cytosolic DNA sensors, including IFN γ -inducible protein 16 (IFI16), DNA-dependent activator of IFNs (DAI), and DEAD box helicase 41 (DDX41) can initiate anti-viral response upon viral DNA stimulation (Ma et al., 2018). RNA polymerase III has also been shown to play a role in sensing non-enveloped DNA viruses. RNA polymerase III present in the cytoplasm recognizes AT-rich DNA and transcribes it into uncapped 5' triphosphate group RNA (5'-ppp RNA) that will activate RIG-I pathway to initiate the innate response. Adenovirus-induced IFN- β expression in murine cells is dependent on RNA polymerase III (Chiu et al., 2009).

In addition to these DNA sensors mentioned above, a multi-protein complex named inflammasome plays an important role in the detection of viral infection and in the innate anti-

viral defense. The inflammasome is generally composed of PYHIN proteins, such as Absent in melanoma 2 (AIM2) and NLR pyrin domain-containing 3 (NLRP3), and an adaptor apoptosis-associated speck-like protein (ASC) containing a caspase recruit domain (CARD). After binding viral DNA, the PYHIN protein recruits ASC that further recruits and activates caspase-1. Caspase-1 in turn causes proteolytic maturation of some pro-inflammatory cytokines, including IL-1 β and IL-18 to initiate the antiviral response (Fernandes-Alnemri et al., 2009). NLRP3 mediated inflammasome is activated upon adenovirus infection in the mouse model (Muruve et al., 2008).

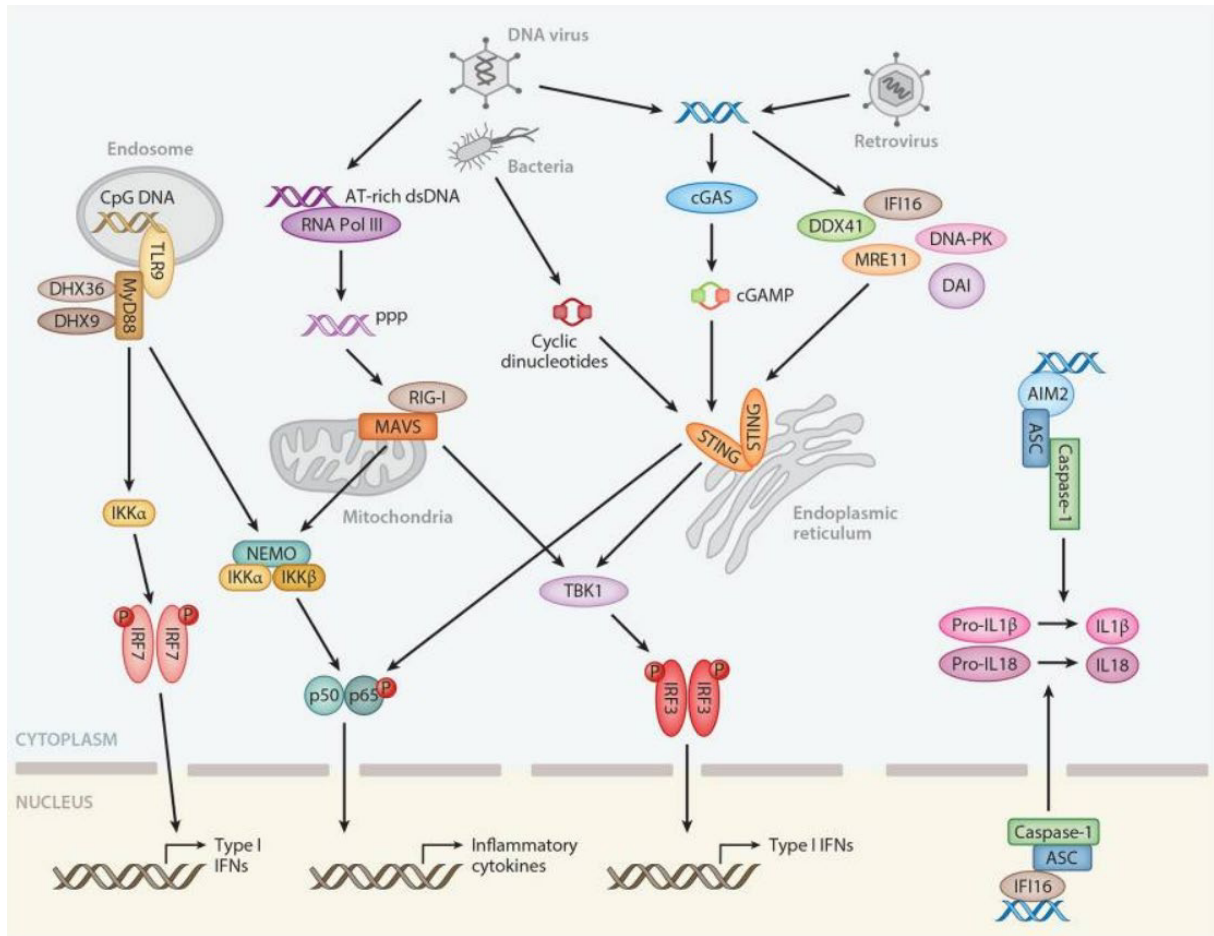


Figure 7. DNA sensors and related signaling pathways. TLR9 recognizes CpG DNA in the endosome and recruits MyD88 to activate IRF7 and NF- κ B, leading to induction of type I interferons (IFNs) and inflammatory cytokines. AT-rich dsDNA is transcribed by RNA Pol III into 5'-triphosphate double-stranded RNA (5'-ppp-dsRNA), which in turn activates the RIG-I–MAVS pathway. DNA from viruses or bacteria could be detected by cGAS and potentially also by other putative DNA sensors, which are all proposed to activate the endoplasmic reticulum–resident adaptor protein STING. STING translocates from the endoplasmic reticulum to the Golgi to activate TBK1–IRF3 and NF- κ B, resulting in robust type I IFN induction and inflammatory cytokine production. Detection of DNA by IFI16 and AIM2 in the nucleus and cytoplasm, respectively, activates the inflammasome via recruitment of ASC and caspase-1, leading to proteolytic cleavage of pro-IL1 β and pro-IL18. **Adapted from (Ma et al., 2018).**

The innate detection of MCPyV

The innate response against MCPyV is scarcely studied, probably due to the difficulty of having sufficient wild-type infectious MCPyV virions. So far, the PRRs that directly sense the virus has not yet been identified. One study reported that MCPyV LT downregulates TLR9 expression in epithelial and MCC-derived cells (Shahzad et al., 2013a). It has been demonstrated that TLR9 is capable to detect other non-enveloped dsDNA tumor viruses, such as human papillomavirus (HPV). The CpG motif derived from the HPV E6 DNA sequence activates TLR9 and conversely, the oncoprotein E6 and E7 downregulates TLR9 transcripts to counteract the innate sensing (Hasan et al., 2007). Since MCPyV LT also inhibits TLR9 expression, TLR9 may likely serve as the PRR that recognizes MCPyV DNA to initiate the innate response. Apart from TLR9, there is evidence to support a role for the cGAS-STING pathway in the detection of DNA tumor virus, including HPV and adenovirus (Lau et al., 2015). The oncoprotein E7 from HPV and E1A from adenovirus antagonize the cGAS-STING pathway. LXCXE motif present in these oncoproteins, which is essential for blockade of the retinoblastoma tumor suppressor, is important for inhibiting this DNA sensing. E1A and E7 inhibit viral DNA-sensing through direct binding of STING via the LXCXE motif, and silencing of these oncogenes in human tumor cells restores the cGAS-STING pathway (Lau et al., 2015). In the same study, the authors also found that expression of LT from SV40 strongly reduce the innate response by inhibiting both cGAS-STING and RIG-I pathway. Interestingly, the large T antigen of MCPyV LT contains the LXCXE motif in its N-terminus, suggesting that the cGAS-STING may be involved in the innate recognition of MCPyV. Additionally, a recent study showed that in MCC cell lines STING is completely silenced (Liu et al., 2020). These data reinforce the possibility of cGAS-STING as an innate detection pathway of MCPyV. Indeed, MCPyV infection induces the innate antiviral inflammatory response in human dermal fibroblast via cGAS-STING/NF- κ B axis (Krump et al., 2021). The inhibition of cGAS-STING signaling by LXCXE motif-containing oncoprotein and the downregulation of TLR9 by MCPyV LT could serve as an immune evasion mechanism that contributes to the immune tolerance of early MCPyV infection. Importantly, although MCPyV is a DNA virus, its innate sensing may not be restricted to DNA sensors, as other PRR members also play a role in the detection of DNA virus infections. For example, Epstein-Barr virus (EBV)-encoded RNAs, which may be released from infected cells in exosomes, also activate TLR7 to promote IFN α production (Quan et al., 2010). TLR2 was identified as a host factor that detects the human cytomegalovirus (HCMV) glycoproteins and initiates inflammatory cytokine

secretion in response to HCMV infection (Boehme et al., 2006). Retinoic acid-inducible gene I (RIG-I), predominantly recognizes RNA virus infection, is also activated during infection with several DNA viruses, including HSV-1, EBV, and KSHV (Rasmussen et al., 2009; Samanta et al., 2006; West et al., 2014). Whether MCPyV encoded RNAs or proteins can be detected and activate other PRRs is also interesting to investigate.

1.2 Interferons response

Type I interferons

Type I IFNs response is the frontline anti-viral defense strategy and it is one of the main outcomes of the cytosolic sensing of DNA. Type I IFNs family comprises multiple IFN- α isotypes, IFN- β and several other subtypes including IFN- ϵ , IFN- κ , IFN- ω and IFN- δ , of which IFN- α and IFN- β are the most studied members. Innate immune cells, especially pDCs, are the main cellular sources of Type I IFNs, although they can also be produced by other cell types (Liu, 2005). Type I IFNs signal through a common IFNAR1/IFNAR2 receptor heterodimer that leads to recruitment and activation of Janus kinase (JAK1) and tyrosine kinase 2 (TYK2). The JAK1/TYK2 induces the phosphorylation and dimerization of the ISG1 and STAT2 proteins and subsequent translocation, along with IRF9, to the nucleus. The complex of STATs dimer and IRF9, known as IFN-stimulated gene factor 3 (ISGF-3), triggers the transcription of a wide range of IFN-stimulated genes (ISG), which in turn inhibit the viral life cycle by various mechanisms (Schoggins et al., 2011; Teijaro, 2016). For example, among the ISGs implicated in the direct antiviral actions, the Mx protein GTPases, the 2', 5'-oligoadenylate synthetase (OAS), and RNase L, the RNA-specific adenosine deaminase (ADAR), and the protein kinase R (PKR), are the first characterized ISGs. The Mx family GTPases, which comprise MxA and MxB in humans, inhibit a broad range of viruses by blocking endocytic traffic of incoming viral particles and uncoating of ribonucleocapsids. The OAS family in the combination of RNase L nuclease inhibits the viral life cycle by inducing RNA degradation. The ADAR affects the viral life cycle by editing double-stranded RNA by deamination of adenosine to yield inosine. The PKR suppresses viral genes expression by inhibition of translation initiation through the phosphorylation of protein synthesis initiation factor eIF-2 (Samuel, 2001). So far, hundreds and thousands of antiviral ISGs have been uncovered, such as TRIM5, ZAP, ISG15, IFITM, etc., that prevent viral infection by targeting nearly every step of the viral life cycle (Schoggins and

Rice, 2011; Schoggins et al., 2011). Apart from the induction of direct antiviral ISGs effectors, type I IFNs are key regulators of the innate immune responses and bridge the innate and adaptive antiviral immunity. Type I IFNs regulate Natural killer (NK) cells and cytotoxic T lymphocytes (CTLs) whose role is to eliminate infected cells. The mechanisms whereby type I IFNs contribute to NK and CTL responses are diverse and include the production of chemokines that recruit these cells to the site of infection and induction of cytokines that positively regulate cytotoxic cell counts and activities. Type I IFNs induce pro-inflammatory cytokines production and promote the development and maturation of APCs, such as macrophage and dendritic DCs. Type I IFNs promote antigen presentation by upregulating both major histocompatibility complex class I and class II (MHC I and MHC II) (Gessani et al., 2014). Importantly, although type I IFNs are the key drivers that promote antiviral immunity, prolonged type I IFNs response during chronic viral infection can induce immunosuppression that facilitates viral chronicity and persistence (Snell et al., 2017). In the chronic lymphocytic choriomeningitis virus (LCMV) infection model, type I IFNs contribute to viral persistence by inducing immuno-regulatory cytokine IL-10 and immune checkpoint PD-L1 (Cunningham et al., 2016).

Type II interferon

Apart from type I IFNs, type II IFN or IFN γ also plays a significant role in both innate and adaptive immune responses against viral infections. IFN γ is produced predominantly by NK and NKT cells as part of the innate immune response, and by CD4 Th1 and CTL cells once antigen-specific immunity develops (Billiau and Matthys, 2009). Similar to type I IFNs, IFN γ can exert direct antiviral functions by targeting the viral life cycle (Kang et al., 2018). For example, inhibition of viral gene expression is an effective antiviral strategy used by IFN γ against many viruses, including HPyVs. IFN γ treatment effectively inhibits the lytic infection of BKPyV regardless of its strains (Abend et al., 2007). The transcription of ER genes and subsequent expression of T-Ag proteins is suppressed by IFN γ . Consequently, the expression of late genes and virion production is reduced by IFN γ . Treatment of IFN γ before or after viral infection leads to a similar inhibition in viral protein expression and virion production, indicating that the antiviral mechanism of IFN γ against BKV is independent of viral entry (Abend et al., 2007). IFN γ also plays an important regulatory role in regulating JCPyV infection and reactivation by limiting viral gene expression and replication (De-Simone et al., 2015).

IFN γ cannot inhibit viral DNA replication directly but can inhibit it indirectly via post-transcriptional suppression of TAg expression. IFN γ controls the gene expression of JCPyV by downregulating T-Ag proteins in human glial cells (De-Simone et al., 2015). Given the role of IFN γ in the control of BKPyV and JCPyV infection, IFN γ may possibly regulate MCPyV infection. IFN γ also inhibits indirectly viral infection by enhancing various innate and adaptive immune responses. The indirect antiviral functions of IFN γ include but are not limited to the enhancement of NK and macrophage activity, augmentation of class I MHC as well as class II MHC expression on APCs, and induction of IgG production of activated plasma B cells (Ivashkiv, 2018).

Type III interferons

Type III IFNs (IFN- λ s), a relatively recent discovery, are distinct antiviral cytokines with many features in common with type I IFNs (Kotenko et al., 2003; Sheppard et al., 2003). IFN- λ 1, - λ 2, and - λ 3 are induced by virus PAMPs and normally signal through the JAK/STAT cascade, but use a separate receptor. They are able to activate the expression of ISGs and have been shown to inhibit the replication of viruses. However, whereas all nucleated cells express the receptor for type I IFNs, the type III IFN receptor is limited to epithelial cells on mucosal barriers. IFN- λ s are therefore important for limiting the infection and transmission by respiratory and gastrointestinal viruses. Moreover, while type I IFN signaling activates ISG expression in a fast, strong, and transient manner, type III IFN signaling is characterized by a weaker, delayed, but longer-lasting ISG response. Besides the direct antiviral effect mediated by ISGs, IFN- λ s also stimulate adaptive immune responses (Mesev et al., 2019; Wack et al., 2015; Weber, 2020; Zhou et al., 2018).

1.3 Innate cellular immunity

Myeloid cells

Innate immune cells, such as monocyte, macrophage, and neutrophils, etc, play unquestionably a vital role in host defense. Monocytes are circulating mononuclear cells in the bloodstream that are differentiated from myeloid lineage. In human peripheral blood, three functionally different subsets of monocytes have been identified and characterized based on their expression of surface markers CD14 and CD16. The major subset of monocyte,

representing 90% of the total monocyte population, expresses a high level of CD14 and no CD16 (CD14⁺⁺ CD16⁻). These cells are referred to as classical monocytes or inflammatory monocytes. Two other subpopulations are intermediated monocytes expressing CD14 and a low level of CD16 (CD14⁺⁺ CD16⁺), and non-classical monocytes expressing a low level of CD14 and a high level of CD16 (CD14⁺ CD16⁺⁺) (Wong et al., 2012). Upon injury or infection, inflammatory monocytes are immediately recruited to the inflamed tissue and contribute to immunological responses, such as recognizing and removing pathogens and dying cells. Intermediate monocytes are recruited later and are mainly associated with antigen presentation, high secretion of pro-inflammatory cytokines and chemokines, and wound healing. Nonclassical monocytes display a patrolling behavior and constantly survey the endothelium as part of the innate local surveillance (Guilliams et al., 2018). Monocytes circulate in the bloodstream and can differentiate into macrophages that are effector cells of the innate immune system important for viral elimination. Monocyte-derived macrophages, as well as tissue-resident macrophages, are potent phagocytic cells capable to eliminate viral particles and apoptotic bodies from viral-infected dying cells. Activated macrophages also secrete pro-inflammatory cytokines and present antigens to T cells, thus triggering the adaptive immune response. Of note, macrophages are a heterogeneous population with different phenotypes. Macrophage polarization, a dynamic phenotype switching process, shapes macrophage responses and clusters them according to the stimuli perceived. During viral infection and depending on the stage of the infection, a mixed phenotype of macrophages from classical pro-inflammatory macrophages (M1-like) to regulatory anti-inflammatory macrophages (M2-like) can occur (Atri et al., 2018). Granulocytes, such as neutrophils, are also able to remove viral particles and apoptotic bodies by phagocytosis. They are rapidly attracted to inflammatory sites and enter the tissue by diapedesis migration. Their most fascinating ability is however the release of neutrophil extracellular traps (NETs), which are networks of proteins and chromatin that immobilize and inactivate extracellular pathogens (Amulic et al., 2012).

Natural killer (NK) cells

Natural killer (NK) cells are a group of the innate lymphoid cell (ILC) that display, in an antigen-independent manner, cytotoxic activity against cells under stress such as tumor cells and virus-infected cells (Chiossone et al., 2018). NK cell's activity is regulated by a fine balance between

stimulatory and inhibitory receptors. When stimulatory signaling is predominant, NK cells will be activated. Conversely, if inhibitory receptor signaling is dominant, the activity of NK cells is inhibited. Normal healthy cells express MHC I molecules on their surface which act as ligands for inhibitory receptors and contribute to the self-tolerance of NK cells. However, virus-infected cells or tumor cells often lose surface MHC I expression, leading to lower inhibitory signals in NK cells. Simultaneously, cellular stress associated with viral infection or tumor development such as DNA damage response, senescence program, or tumor suppressor genes upregulates ligands for activating receptors in these cells. As a result, the signal from activating receptors in NK cells shifts the balance toward NK cell activation and elimination of target cells directly through NK cell-mediated cytotoxicity or indirectly through secretion of pro-inflammatory cytokines. NK cells-mediated cell lysis can be induced by delivering granules containing cytotoxic molecules, including perforin and granzymes, into target cells through the immunological synapse. Perforin forms pores in the cell membrane of the target cell, creating an aqueous channel through which the granzymes and associated molecules enter, inducing either apoptosis or osmotic cell lysis. NK cells can also induce the death receptor-induced target cell apoptosis. NK cells express Fas ligand (FasL), tumor-necrosis factor (TNF), and TNF-related apoptosis inducing ligand (TRAIL) which binds to their corresponding receptor on target cells (Paul and Lal, 2017). Engagement of the death receptor with its cognate ligand induces a conformational change in the receptor and recruitment of adaptor protein leading to caspase activation, thus apoptosis of target cells (Thorburn, 2004). Another important process by which NK cells mediate target cell killing is antibody-dependent cell cytotoxicity (ADCC). NK cells express Fc γ RIIIA and/or Fc γ RIIC, which can bind to the Fc portion of immunoglobulins. The engagement of Fc γ R with the Fc portion of antigen-specific antibodies on the surface of infected cells or tumor cells activates NK cytotoxicity, resulting in target cell death (Wang et al., 2015a). A great number of studies have shown that NK cells are important and involved in immune defense against various virus infections, including polyomavirus (Waggoner et al., 2016).

$\gamma\delta$ T cells

$\gamma\delta$ T cells are T cells that have a distinctive T-cell receptor (TCR) on their surface. Most T cells are $\alpha\beta$ T cells with TCR composed of two glycoprotein chains α and β TCR chains. In contrast, $\gamma\delta$ T cells have a TCR that is made up of one γ chain and one δ chain. This group of T cells is

usually less common than $\alpha\beta$ T cells but plays a crucial role in the early defense mechanism and tissue homeostasis. $\gamma\delta$ T cells, which mature in the thymus, do not require further peripheral maturation or extensive clonal expansion to initiate terminal effector functions. Human $\gamma\delta$ T cells can be classified into two main populations, V δ 1 and V δ 2, according to their TCR expression, which is determined early in the thymus (Jin et al., 2010b). V δ 1 preferentially resides in the skin, gut mucosa, lungs, and genitourinary; in contrast, V δ 2 cells are abundant in peripheral blood (Chennupati et al., 2010). In addition to their diversified and localized distribution, they are well suited to provide the first line of defense and immunosurveillance in many tissues. $\gamma\delta$ TCRs are not restricted to the recognition of peptides bound to MHC molecules, which distinguishes them from the great majority of $\alpha\beta$ T cells. The capacity to recognize a wide range of antigens that are rapidly displayed following infection or other forms of stress, and to respond in large numbers without requiring extensive clonal expansion, permits $\gamma\delta$ T cells to participate in the early stages of the immune response. The functions of $\gamma\delta$ T cells in defending the host against a broad range of infectious are well documented and achieved through different mechanisms. $\gamma\delta$ T cells can directly lyse and eliminate infected or stressed cells through the production of granzyme. They produce a diversified set of cytokines and chemokines to coordinate other immune or non-immune cells. They are also APCs that are capable to present antigens for $\alpha\beta$ T cell priming and to trigger dendritic cell maturation. $\gamma\delta$ T cells also provide help for B cells and promote the production of immunoglobulins (Gaya et al., 2018; Vantourout and Hayday, 2013).

NKT cells

NKT cells are a heterogeneous group of unconventional T cells that share properties of both T cells and NK cells. They were first described as CD3⁺ T cells co-expressing NK cell-associated marker NK1.1 (CD161), a classic marker used to identify mouse NK cells (Lantz and Bendelac, 1994). NKT cells present in both mice and humans that express semi-invariant TCR and NK cell marker and specifically recognize lipid antigens presented by the nonpolymorphic MHC class I-like molecule, CD1d (Godfrey et al., 2004). CD1d-restricted NKT cells are further divided into two broad categories: type I NKT cells, referred to as invariant NKT cells (iNKT), and type II NKT. iNKT cells express an invariant TCR α chain and a limited repertoire of TCR β chain, whereas type II NKT expresses more diverse TCR chains compared to the iNKTs. Although NKT cells constitute only approximately 1% of the total T cell population, they play important roles in

bridging innate and adaptive immune responses against infections. The best-known CD1d-restricted cells are iNKT cells. The major outcome of iNKT cells activation is the rapid production of different sets of cytokines. Based on this property, they are subdivided into iNKT1, iNKT2, and iNKT17 that mirror T helper (Th) cell subsets in cytokine production. iNKT cells are notable for their ability to interact with other immune cells and to respond rapidly to danger signals and pro-inflammatory cytokines. iNKT cells induce NK transactivation, DC activation, and cross-presentation activities. iNKT cells promote macrophage activation and regulate macrophage polarization. iNKT cells promote the recruitment and regulate the activities of neutrophils. iNKT cells are also capable to promote directly or indirectly B cell activation and the differentiation and activation of T cells (Brennan et al., 2013).

1.4 Innate immune response in early MCPyV infection

So far, only human dermal fibroblast can serve as a cell culture model supporting the full life cycle of MCPyV (Liu et al., 2016a). Because of the limitation of the study model, the research on the role of the immune system against MCPyV infection is hampered. However, in-vitro studies in cell lines that express MCPyV antigens may provide insights into the understanding of the anti-MCPyV immune response. Immortalized human foreskin fibroblasts that stably expressing tumor-derived trLT₃₃₉ display an increased expression of genes involved in the inflammatory response and it is fortified further by sT (Richards et al., 2015). The expression of genes such as *OSA1*, *OSA2*, and *ISG20* is upregulated by trLT₃₃₉ and trLT₃₃₉ plus sT. In addition, the expression of chemokines and pro-inflammatory cytokines, such as CXCL1, CXCL6, IL-1 β , IL-6, and IL-8 is also largely upregulated by trLT₃₃₉ and is further enhanced by adding sT (Richards et al., 2015). These data suggest that MCPyV trLT may elicit an antiviral inflammatory response. However, given that the C-terminal truncation of LT occurs in a late-stage and is MCC specific these data are not pertinent to explain the innate inflammatory response against the early infection of MCPyV. Therefore, investigating the impact of wild-type MCPyV ER genes on the expression of inflammatory response-related genes would be more appropriate. It is shown that ectopic expression of only MCPyV sT impedes the expression of genes associated with innate response, especially NF- κ B-targeted genes such as CCL20 and IL-8. Mechanistically, MCPyV sT binds NEMO to prevents IKK α /IKK β phosphorylation and thus subsequent I κ B degradation, rendering NF- κ B sequestration in the cytoplasm and unable to translocate into the nucleus and activate its transcriptional targets. The inhibition of the phosphorylation of

IKK complex requires the recruitment of cellular phosphatase PP2A A β and/or PP4C (Griffiths et al., 2013). Together, these findings suggest a potential role of MCPyV sT to subvert the innate immune response, contributing to the immune tolerance of early MCPyV infection and helping the establishment of persistent infection. This study is based on *in-vitro* expression of only one MCPyV gene in epithelial and MCC cell lines, which is far from physiological conditions. To understand the innate immune response against MCPyV, further studies with infectious wild-type MCPyV should be considered, if possible.

Because of the limitation of knowledge about MCPyV biology and the difficulty of obtaining infectious viral particles, little data have been yet reported regarding the function of innate cellular response against MCPyV infection. As innate immune cells serve as the front line to pathogens insult, they probably play a crucial role in the defense against MCPyV. But somehow, MCPyV succeeds in evading host immune response and becomes a part of skin commensal microflora. In one study of detection of MCPyV in MCC patients, the DNA sequence of MCPyV LT and VP1 was found exclusively in CD14⁺ CD16⁻ inflammatory monocytes but not in other cells from PBMCs, suggesting CD14⁺ CD16⁻ monocytes as a potential reservoir for MCPyV (Mertz et al., 2010b). This raises the possibility that monocytes are elicited in inflammatory response against MCPyV infection and may serve as a cellular carrier to help the systemic dissemination of the virus. However, this finding is obtained from one isolated case, additional investigation in a large size patient cohort needs to be done. To date, no studies have been published regarding other innate cellular responses against MCPyV infection. Of interest, it is reported that BKPyV and JCPyV can escape NK cell-mediated cytotoxicity in order to set up viral latency. A miRNA encoded by both BKPyV and JCPyV targets 3'UTR of UL16 binding protein 3 (ULBP3) transcript and downregulates its expression. ULBP3 is a cell surface glycoprotein that functions as a ligand for the activating NK receptor, the 2 natural killer group 2D (NKG2D) (Cosman et al., 2001). Thus, the reduction of ULBP3 induced by viral miRNA dampens NKG2D-mediated cell killing and may contribute to viral persistence (Bauman et al., 2011). It is intriguing to investigate whether the miR-M1 of MCPyV could impact NK cell activities.

Due to the wide dispersed anatomical localization, $\gamma\delta$ T cells serve as the body guardian that provides the first line of defense facing pathogens challenge. The involvement of $\gamma\delta$ T cells, as

well as NKT cells in the immunity against viral infection, is well documented (Poccia et al., 2005; Tessmer et al., 2009). Given the nature of $\gamma\delta$ T cells and NKT cells in controlling viral infection they probably play important roles in MCPyV infection, although no evidence has shown their implications in anti-MCPyV innate response.

2. Autophagy

Autophagy is a fundamental self-degradative cellular process that allows degradation of cytoplasmic content, such as misfolded or aggregated proteins, damaged organelle, as well as intracellular pathogens in the context of infection. The primary function of autophagy serves as a recycling system that brings materials and energy for cellular renovation and homeostasis (Mizushima and Komatsu, 2011). In the most common form, autophagy involves the engulfment of cytosolic material within a double-membrane-bound vesicle, termed the autophagosome, and the subsequent fusion of the autophagosome with lysosomal vesicles leads to degradation and recycling of the sequestered substrates. In response to stressors, in particular nutrient deprivation, autophagy is substantially induced by inhibiting the mechanistic target of rapamycin complex 1 (mTORC1) (Figure 8). The initiation of canonical autophagy consists of the formation of the autophagosome that is mediated by the activation of ULK1 complex comprising ULK1, FIP200, ATG13, and ATG101. The inhibition of mTORC1 activates ULK1 complex which in turn leads to phospho-activation of the phosphoinositide 3-kinase catalytic subunit type III complex (PI3KC3), encompassing beclin 1, vacuolar protein sorting 34 (VPS34), VPS15, and ATG14L, necessary for the formation of phosphatidylinositol 3-phosphate (PI3P)-rich domains on the intracellular membrane-associated with the endoplasmic reticulum, Golgi apparatus or mitochondria. The PI3P-binding protein WIPI2 then recruits ATG16L1 complex to the autophagosome precursor. ATG16L1 recruits cleaved microtubule-associated protein 1 light chain 3beta protein (LC3)-I protein to the lipid bilayer by conjugating them to phosphatidylethanolamine (PE) to form the so-called LC3-II. Subsequently, LC3-II complex induces membrane expansion and fusion to give rise to the double-membrane autophagosome vesicle. Finally, the fusion of the autophagosome with endolysosomal vesicles leads to the degradation and recycling of the sequestered substrates. In contrast to conventional or canonical autophagy, the non-canonical autophagy can occur in a beclin 1 or PI3KC3-independent way (Scarlati et al., 2008a, 2008b; Yano et al., 2007). This specific type of autophagy is neither blocked by the knockdown of BECN1 or its binding

partner PI3KC, nor inhibited by pharmacological inhibitors wortmannin (WM) or 3-methyladenine (3-MA), which interfere with PI3KC3.

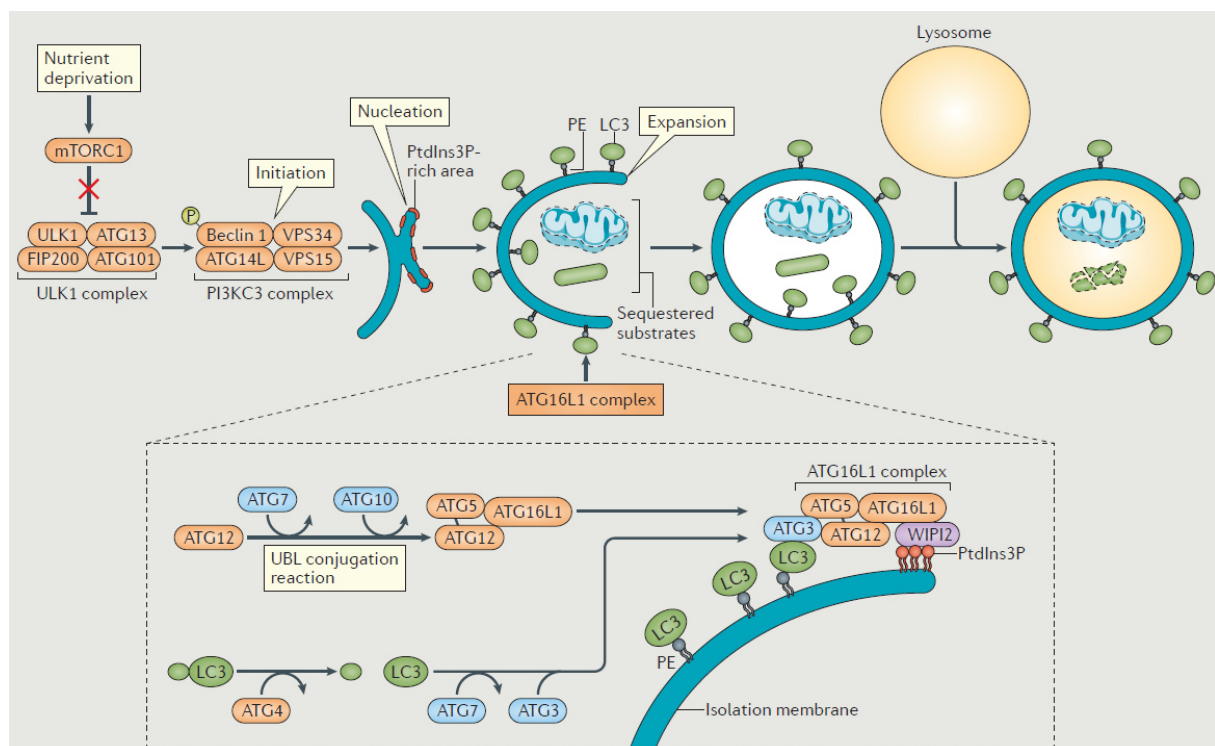


Figure 8. Canonical autophagy pathway. The generation of the autophagosome is mediated by the sequential activities of three key protein complexes: the ULK1 complex (comprising ULK1, FIP200, ATG13, and ATG101), the phosphoinositide 3-kinase catalytic subunit type III (PI3KC3) complex (comprising beclin-1, vacuolar protein sorting 34 (VPS34), VPS15 and ATG14L), and the ATG16L1 complex (comprising ATG16L1, ATG5, and ATG12). Inhibition of mechanistic targets of rapamycin complex 1 (mTORC1), such as through nutrient deprivation, allows the ULK1 complex to activate the PI3KC3 complex. Following the creation of phosphatidylinositol-3-phosphate (PtdIns3P)-rich regions on the surface of the endoplasmic reticulum (ER)–Golgi intermediate compartment or other membranes, the ATG16L1 complex is recruited by the PtdIns3P-binding protein WIPI2 to the autophagosome precursor structure (that is, the isolation membrane). The ATG16L1 complex is generated through an ubiquitin-like (UBL) conjugation reaction in which the UBL molecule ATG12 is conjugated to ATG5 by the sequential action of ATG7 and ATG10. ATG16L1 non-covalently binds the ATG5–ATG12 conjugate to form a multimeric complex. In parallel, another UBL molecule, LC3, is processed by the ATG4 protease and subjected to a second UBL conjugation reaction involving ATG7 and ATG3. The ATG16L1 complex conjugates LC3 and phosphatidylethanolamine (PE) to complete the reaction. LC3–PE mediates membrane tethering and fusion to extend the isolation membrane by recruiting membranes from multiple sources, eventually leading to self-fusion and the formation of

the autophagosome. Subsequent fusion with the lysosome, mediated by class C vacuolar protein sorting (VPS) and SNARE-like proteins (not shown), leads to the degradation of the sequestered substrates. **Adapted from (Cadwell et al., 2016).**

2.1 Autophagy as a cell-intrinsic defense mechanism

In addition to its central role in cellular homeostasis, autophagy participates in various functions of the host immune system. It plays a role in inflammation regulation, antigen presentation, and elimination of intracellular pathogens and infected cells that can be cancer cells, in the context of an oncopathogen. Intracellular pathogens such as parasites, bacteria, fungi, and viruses can be selectively degraded by a cell-intrinsic defense mechanism involving autophagy-related process, referred to as xenophagy (Cadwell, 2016). Xenophagy depends on adaptor molecules that crosslink pathogens with the autophagy machinery. These binding events allow the isolation membrane to grow around the pathogens, resulting in its sequestration within the autophagosome. In the case of viral infection, studies have shown that autophagy displays an anti-viral effect either by degrading directly the virus or by assisting in innate and adaptive anti-viral immunity (Chiramel et al., 2013).

Autophagic degradation of virus

The cytoplasm offers a diverse range of autophagic targets that vary in size and complexity, ranging from protein aggregates up to complete organelles and intracellular pathogens, which can be selectively recognized and sequestered by proteins that function as autophagy adaptors. Adaptors involved in xenophagy include sequestome 1 (also known as p62), nuclear dot protein 52kDa (NPD52), optineurin (OPTN), and TAX1-binding protein 1 (TAX1BP1). Adaptors proteins contain cargo recognition and capture domains, LC3-interacting region to target captured intracellular pathogens to the autophagosome compartment. In addition of the role of autophagy in virus sensing, autophagy can selectively target the virus via adaptor proteins, therefore inducing viral degradation. Autophagy protects against Sindbis virus (SIN) infection by degrading the virus in a p62-dependent manner (Orvedahl et al., 2010). SIN capsid protein interacts with p62 that binds to LC-3 present in the inner membrane of the autophagosome, thus triggering the xenophagy and degradation of the virus. The precise signal for capsid recognition by p62 is unknown but is independent of ubiquitin association³³³. Another example showing that virus can be selectively degraded by autophagy is from the

study of the identification of selective autophagy factors in HSV infection. Using a genome-wide RNA interference screen, authors identified SMURF1, an E3 ubiquitin ligase, is required for autophagic targeting of viral nucleocapsids (Orvedahl et al., 2011).

2.2 Interplay between autophagy and virus sensing

As an organelle gathering cytosolic content into a double-membrane vesicle that fuses with endosomal compartments, the autophagosome can specifically deliver intracellular viral PAMPs to endosomal PRRs to initiate innate immune responses. The first demonstration of the involvement of autophagy in virus sensing comes from the study of vesicular stomatitis virus (VSV)-infected pDCs (Lee et al., 2007). The authors showed that the production of IFN- α by VSV-infected pDCs depends on the autophagic delivery of viral RNA to TLR7 present on the late endosomes. By using Vps34 inhibitors, such as 3-MA, that block PI3KC3 activities, authors showed that inhibition of autophagosome formation suppresses IFN- α expression in VSV-infected pDCs. In addition, pDCs from infected Atg5 knockout mice exhibited a similar phenotype and these mice carried higher viral loads, suggesting that autophagy is important in controlling VSV replication in vivo. However, the exact mechanism by which viral RNA is selectively targeted by autophagosomes to the TLR-resident late endosomal compartment remains to be determined. In non-immune cells, the involvement of autophagy in delivering a viral nucleic acid to PRRs is also conserved. In Coxsackie virus B3 (CVB3) infected fibroblasts, endosomes that harbor TLR3, a double-stranded RNA (dsRNA) PRR, colocalized with LC3-positive structures (Gorbea et al., 2010). The activation of TLR3 via poly (I:C) or CVB3 was suppressed by inhibitors of the autophagy pathway and by reduced expression of Beclin-1, Atg5, or LC3, suggesting that the activation of TLR3 requires autophagy (Gorbea et al., 2010). The activation of certain TLRs can in turn induce autophagy in a MyD88 and/or TRIF-dependent manner. Upon TLR activation, MyD88 and TRIF directly interact with Beclin-1 leading to the formation of Beclin-1/Vsp34 and PI3KC3 complexes that induce autophagy (Delgado and Deretic, 2009; Shi and Kehrl, 2008).

Whereas autophagy proteins typically promote virus sensing in the presence of vesicle-bound nucleic acid, autophagy also negatively controls TLR signaling to avoid excessive and pathological inflammation. Autophagy proteins mediate mitophagy to inhibit signaling downstream of cytosolic sensors of viral nucleic acid. Mitophagy is the selective degradation

of mitochondria by autophagy. The RIG-I and melanoma differentiation-associated gene 5 (MDA5) sense dsRNA in the cytosol and signal through mitochondrial antiviral signaling protein (MAVS), which oligomerizes on the mitochondrial outer-membrane to induce type I IFN expression by activating IRF3 and IRF7. Upon stimulation, RIG-I also induces mitochondrial reactive oxygen species (ROS). When MAVS signaling is triggered, the ATG16L1 complex is recruited to mitochondria by NLR family member X1 (NLRX1), elongation factor Tu, mitochondria (TUFM), and cytochrome c oxidase subunit 5B (COX5B). This promotes mitophagy, thus suppressing ROS production and inhibiting type I IFN production by removing the MAVS (Lei et al., 2012; Zhao et al., 2012). Autophagy proteins are also involved in controlling cGAS-STING mediated response. Cyclic dinucleotides induce the activation of AMP-activated protein kinase (AMPK), which leads to phosphorylation and activation of ULK1. ULK1 in turn phosphorylates STING to inhibit IRF3 and aberrant type I IFN hyperexpression (Konno et al., 2013). Moreover, during HSV1 infection, beclin 1 binds and inhibits cGAS, and activate PI3KC3-dependent autophagy that target cytosolic viral DNA, thus reducing type I IFN production (Liang et al., 2014).

2.3 Autophagy in viral antigen presentation

The role of autophagy is not limited to the innate immune response but also coordinates a multicellular adaptive immune response by processing and delivering antigens for presentation in complex with MHC-I and MHC-II molecules. Typically, MHC-I molecules present endogenous antigens that are degraded by the proteasome and transported into the endoplasmic reticulum through the transporter associated with antigen processing (TAP1/2) proteins, where they are loaded onto MHC-I molecules. After transport to the cell surface, the complex MHC-I/peptide stimulates a CD8⁺ T-cell response for controlling infections. MHC-II molecules present exogenous antigenic peptides that are processed by lysosomal degradation. Peptides are loaded onto MHC-II through the vesicular pathway and transported to the cell surface to stimulate a CD4⁺ T- cell response. Of note, in professional APCs, such as DCs, macrophages, exogenous antigens can also be presented on MHC-I molecules to mediate CD8⁺ T-cell response by a process termed cross-presentation (Joffre et al., 2012). Numerous studies have convincingly demonstrated the implication of autophagy in the presentation of viral antigens. Autophagy targets Epstein-Barr virus protein EBNA1 and deliver it to MHC-II enriched endolysosome for antigen presentation (Paludan et al., 2005). Inhibition of

autolysosomal acidification resulted in accumulation of EBNA1-positive autophagosomes, and knock-down of Atg12 rendered the cells unable to stimulate MHC-II-dependent activation of T-cells (Paludan et al., 2005). Autophagy has been shown to be crucial for the processing of exogenous antigens in DCs by mediating the fusion of phagosomes containing TLR ligands or apoptotic corpses with lysosomes (Brooks et al., 2015; Martinez et al., 2011; Romao et al., 2013). This process is necessary for the MHC class II presentation that drives the antiviral TH1 cell response during HSV2 infection *in vivo* (Lee et al., 2010). Viral encoded proteins could inhibit the autophagy pathway to counteract recognition by CD4⁺ T cells. For example, the HSV1 protein ICP34.5 binds beclin 1 to inhibit autophagy-mediated MHC class II antigen presentation in DCs (Gobeil and Leib, 2012). Autophagy is also involved in cross-presentation. Vaccination of humans with the yellow fever virus vaccine YF-17D induces autophagy in DCs through induction of the amino acid starvation sensor GCN2. Inhibition of either autophagy proteins or GCN2 impedes the ability of DCs to mediate proliferation of CD8⁺ T cells when co-cultured with infected cells (Ravindran et al., 2014). This study provides a clinically relevant example of autophagy-dependent MHC-I cross-presentation of viral antigen.

2.4 Intersection between autophagy and cytokines

As an important cell-intrinsic process involved in host immune defense, autophagy can also interact with the cytokine signaling pathway. T helper 1 (Th1) cytokines such as IFN- γ and TNF generally promote xenophagy in target cells, which is consistent with the role of autophagy in supporting cellular immunity (Gutierrez et al., 2004; Harris et al., 2007; Mostowy et al., 2011). IFN- γ induces autophagy through JAK1/JAK2 and p38 MAPK pathway, independently of STAT1 (Matsuzawa et al., 2012). In turn, autophagy promotes IFN- γ signaling by reducing mitochondrial ROS production, which activates the JAK/STAT inhibitory phosphatase SHP2 (Chang et al., 2010). In addition to conventional autophagy, IFN- γ also induces an incompletely understood non-canonic autophagy to restrict norovirus infection (Hwang et al., 2012). Interestingly, cytokines that signal through other STAT molecules can inhibit autophagy. The binding of protein kinase R (PKR) by STAT3 prevents PKR-mediated phosphorylation of eIF2 α , a stress-responsive regulator of translation that promotes autophagy (Shen et al., 2012). Therefore, cytokines that activate STAT3 can theoretically inhibit autophagy, as seen with the regulatory cytokine IL-10 (Van Grol et al., 2010). Th2 cytokine signaling, such as IL-4 and IL-13, suppress IFN- γ -induced autophagy in a STAT6 dependent manner in macrophages (Harris et

al., 2007). However, DCs that differentiate in the presence of IL-4 display increased expression of RUFY4, a positive regulator of autophagosome formation and fusion with lysosomes, suggesting a positive role of IL-4 in autophagy induction (Terawaki et al., 2015). The amount of time of Th2 cytokine stimulation and the presence of other cytokines such as IFN- γ and GM-CSF may explain the contradictory effect of Th2 cytokines on autophagy. These studies establish an interaction between autophagy and Th2 and regulatory cytokines, but the impact of this interaction on host defense requires further exploration.

Autophagy inhibits the production of other cytokines. For instance, autophagy inhibits IL-1 β production by repressing inflammasome activation through the sequestration of damaged mitochondria and NLRP3-binding protein ASC (Nakahira et al., 2011; Saitoh et al., 2008; Zhong et al., 2016). Interestingly and conversely, even though autophagy suppresses IL-1 β production, it facilitates the secretion of this cytokine (Dupont et al., 2011). IL-1 β does not undergo conventional secretion owing to the absence of a signal peptide, but it can pass through a permeabilized plasma membrane (Cullen et al., 2015). Alternatively, when membrane integrity is intact, IL-1 β can be incorporated into the space between the inner and outer membrane of the autophagosome and is released from the cell following autophagosome fusion with the plasma membrane or multivesicular body (Zhang et al., 2015). Once secreted, IL-1 β continues to influence autophagy in the target cell through TBK1, which interacts with RAB8B, to promote autophagosome maturation (Pilli et al., 2012). Autophagy also inhibits the production of other IL-1 family members, specifically IL-1 α and migration inhibitory factors (MIF). The secretion of these cytokines is increased as a downstream consequence of mitochondrial ROS accumulation in autophagy-deficient macrophages (Castillo et al., 2012; Lee et al., 2016). Autophagy may also prevent Th17 response because the cytokines that are suppressed by autophagy are important for IL-17A induction. Excess IL-1 α released by autophagy-deficient macrophages induces Th17 cell differentiation and exacerbates lung inflammation during *M. tuberculosis* infection (Castillo et al., 2012). Similarly, IL-1 β produced following autophagy inhibition in DCs acts in an autocrine manner to mediate IL-23 production, leading to IL-17 secretion by $\gamma\delta$ T cells (Peral de Castro et al., 2012). However, whether there is an interaction between autophagy and the signaling downstream of IL-17 receptors remains elusive. In mice ischemia model, IL-17A can induce autophagy through src-PP2B-mTOR pathway (Liu et al., 2019). In this model, IL-17A signaling induces the

phosphorylation of Src kinase that activate PP2B phosphatase, which in turn inhibit mTOR complex, thus enhancing autophagy.

2.5 Autophagy and polyomavirus

The interaction between autophagy and polyomavirus was firstly observed in SV40 infected cells. The study showed that cells expressing the small T (sT) antigen of SV40 were more resistant to glucose deprivation-induced cell death than cells lacking the sT. Mechanistically, the sT antigen of SV40 activates the AMP-activated protein kinase (AMPK), a nutrient-sensing kinase that inhibits mTORC, thus leading to increased autophagy. And enhanced autophagy contributes to the resistance of nutrient depletion-induced death (Kumar and Rangarajan, 2009). The interplay between autophagy and HPyVs is also reported later. In JCPyV infection, the interaction between autophagy and the virus is established through an intermediate protein, Bcl-2-related athanogene 3 (Bag3) that promotes both canonical and non-canonical autophagy (Bq et al., 2013; Carra et al., 2008; Gamedinger et al., 2009, 2011). Ectopic expression of Bag3 induces autophagic degradation of T antigens of JCPyV, thus inhibiting JCPyV infection (Sariyer et al., 2012). Interestingly, to counteract this Bag3-mediated viral inhibition, the T antigens of JCPyV inhibit autophagy by downregulating Bag3 expression. Gel shift and chromatin immunoprecipitation assay demonstrated that JCPyV T antigens prevent the binding of the AP2 transcription factor to its site on Bag3 promoter, resulting in impaired Bag3 expression (Basile et al., 2009). Importantly, autophagy does not always prevent viral infection. Instead, autophagy seems to be propitious for BKPyV infection. The addition of excess amino acids during viral infection reduced BKPyV infection. Perturbing autophagy levels using inhibitors, 3-MA, bafilomycin A1, and spautin-1, also reduced infection, while rapamycin treatment of host cells increased infection. Moreover, siRNA knockdown of autophagy genes, such as *ATG7* and *BECN1*, led to decreased infection. BKPyV infection not only correlated with autophagosome formation but also virus particles localized to autophagy-specific compartments early in infection. These data support a role for autophagy in the promotion of BKPyV infection (Bouley et al., 2014).

As autophagy plays an important role in viral oncogenesis, the interplay between MCPyV and autophagy has been recently attracting attention. *Kumar* and colleagues showed that T-antigens of MCPyV induce cellular microRNAs that target and suppress multiple autophagy-

related genes (Kumar et al., 2020). Ectopic expression of either wild-type LT, trLT, or sT increases *miR-375*, *miR-30a-3p*, and *miR-30a-5p* expression. And consistently, shRNA silencing of T-antigens downregulates these three microRNAs. Furthermore, *miR-375*, *miR-30a-3p*, and *miR-30a-5p* inhibit autophagy by targeting either the untranslated region (UTR) or the coding sequence (CDS) of several autophagy-related genes, including *ATG7*, *BECN1*, and *SQSTM1* (p62). In addition, the authors show that MCPyV⁺MCC display reduced levels of ATG7 and p62 compared to MCPyV⁻MCC, suggesting a new perspective in understanding how MCPyV promotes tumorigenesis (Kumar et al., 2020). Ectopic expression in MCPyV⁻MCC cell lines of MCPyV sT and trLT, but not the wild-type LT, resulted in autophagy suppression, suggesting the importance of autophagy evasion in MCPyV-mediated tumorigenesis. This is the first study showing the T-antigens of MCPyV are involved in the regulation of autophagy. Further studies are required to confirm the findings and to unveil other regulatory mechanisms. For instance, it is not yet clear whether T-antigens of MCPyV directly interfere with autophagy-related proteins. All polyomaviruses encode the sT antigens that share a conserved protein phosphatase PP2A binding domain and it is experimentally well demonstrated that sT antigen of several polyomaviruses, including MCPyV, indeed interacts with PP2A (Kwun et al., 2015; Moens et al., 2015). Given that PP2A coordinates with mTORC1 to dephosphorylate ULK1 complex to promote autophagy (Wong et al., 2015a), MCPyV sT may likely influence autophagy flux by interacting with PP2A.

3. Adaptive immunity against MCPyV

3.1 Principal actors of adaptive immunity

Adaptive immunity is triggered when the innate defense cannot efficiently eliminate the invading pathogens. While innate immunity provides an immediate and non-specific response, the adaptive response is pathogen-specific that requires antigen presentation, and involves T and B lymphocytes.

T cells

Conventional or $\alpha\beta$ T cells are divided into 2 major subsets according to their principal functions and selective surface marker, CD4 and CD8. CD8⁺ T cells or cytotoxic T lymphocytes (CTLs) are activated by infected cells or tumor cells that present MHC I/antigen complex to TCR. Once activated, CTLs kill the target cell by three major mechanisms. CTLs secrete TNF and

IFN- γ that have anti-tumor and anti-viral microbial effects. The second major function is the perforin and granzymes-induced cell death. The third mechanism of CTLs cytotoxicity is via Fas/FasL interaction. Activated CD8⁺ T cells express FasL on the cell surface, which binds to its receptor, Fas, on the surface of the target cell, resulting in apoptosis of target cells. In addition to the cytotoxicity, CTLs also produce cytokines to influence the functions of other cells (Wong and Pamer, 2003). In the contrast to CD8⁺ cells, CD4⁺ T cells do not possess cytotoxic activities but are crucial for assisting other immune cells to ensure the efficacy and homeostasis of host immunity. Naïve CD4⁺ cells are activated by professional APCs, including primarily DCs, macrophages, and B cells, that present MHC II/antigen complex. Effector CD4⁺ cells can be divided into several subpopulations, such as T helper type I cells (Th1), Th2, follicular B helper T cell (Tfh), Th17, and regulatory T cells (Treg), according to their main functions and cytokines produced. Th1 cells produce mainly IL-2 and IFN- γ and predominantly lead to enhanced cell-mediated response against intracellular pathogens. IL-2 promotes the proliferation and cytotoxic effect of CTLs and IFN- γ increases the phagocytic activity of macrophages. Th2 and Tfh cells lead to a humoral response that can activate B cell differentiation and production of antigen-specific immunoglobulins. The key effector cytokines produced by Th2 and Tfh include IL-4, IL-9, IL-10, IL-13, and amphiregulin. Th17 cells are important to mount an immune response against extracellular pathogens and are involved in chronic inflammation and tissue homeostasis. They produce mainly IL-17, IL-21, IL-22 and IL-23. Treg and induced Treg (iTreg) play important role in the maintenance of immune tolerance to self and foreign antigens. After clearance of pathogens, they negatively regulate the immune response, thereby protecting against excessive inflammation and immunopathology. Their main effector cytokines include IL10, TGF- β , and IL-35, which are potent inhibitory cytokines that in general suppress the immune response (Luckheeram et al., 2012). It is worthy to note that CD4⁺ T cells are a heterogeneous population that exhibits plasticity. Depending on the pattern of cytokines received and under certain circumstances, each subpopulation is able to switch phenotype towards another CD4⁺ lineage among them, especially Th17 and Treg cells (Geginat et al., 2014). This plasticity is important for the dynamic and fine functioning of the immune system.

B cells

B lymphocytes are the main effector cells of humoral immunity. B cells can be either activated by antigens alone or with the help of Th cells, referred to as T cell-independent (TI) and T cell-

dependent (TD) B cells, respectively. TI B cells are generally activated by bacterial lipopolysaccharide, repetitive surface antigens, or unmethylated CpGDNA, and differentiate into short-lived plasmablasts that produce early and low-affinity IgM. Instead, TD B cells internalize antigens through B cell receptor (BCR)-mediated endocytosis and degrade them in order to present onto MHC II. Th cells, typically Th2 and Tfh recognize and bind these MHC II/antigen complex to activate TD B cells (Crotty, 2015). Once activated, B cells participate in a two-step differentiation process that yields both short-lived plasmablasts for immediate protection and long-lived plasma cells and memory B cells for persistent protection. The first step, known as the extrafollicular response, occurs outside lymphoid follicles in the secondary lymphoid organs, such as the spleen and lymph node. During this step activated B cells proliferate, may undergo immunoglobulin class switching, and differentiate into plasmablasts that produce early and low affinity of IgM. The second step consists of activated B cells entering a lymphoid follicle and forming a germinal center, where B cells differentiate into plasma cells and undergo extensive proliferation, Ig isotype switching, and affinity maturation directed by somatic hypermutation. Resultant plasma cells secrete large amounts of antigen-specific antibodies (Nutt et al., 2015). One of the key features that define adaptive immunity is immunological memory, albeit some innate immune cells may exhibit memory activity. After the first contact of pathogens, a small population of T and B lymphocytes differentiates into long-lived memory cells that allow a quicker and stronger response when encountering the same pathogens again (Ratajczak et al., 2018).

3.2 Humoral and cellular responses against MCPyV

The humoral anti-viral response is mediated by natural antibodies and viral-specific antibodies. Natural antibodies, which serve as the first line of humoral defense, are low-affinity and polyreactive that are present at low titers in the blood of naïve individuals. The polyreactive specificity of natural antibodies allows them to bind to a broad range of antigens, including proteins, nucleotides, polysaccharides, and lipids at low affinity (Palma et al., 2018). The viral antigen-specific antibody response is often T cell-dependent and requires the cooperation of B cells and Th CD4⁺ cells, as mentioned above. The functions of anti-viral antibodies include direct virion neutralization, activation of complement-mediated response, and ADCC of infected cells (Hangartner et al., 2006). The first infection of MCPyV is thought to occur in early childhood since the viral DNA is not present in neonatal blood but then becomes detectable

in children (Gustafsson et al., 2012b; Kean et al., 2009b). MCPyV persists in children and becomes a common infectious virus that is highly prevalent in the healthy adult population (Grundhoff and Fischer, 2015). Around 70% of the population possess specific antibodies IgG targeting viral capsid proteins VP1 and VP2 (Carter et al., 2009b; Kean et al., 2009c; Pastrana et al., 2009b). And there is a positive correlation between capsid-specific antibody titer and viral burden (Pastrana et al., 2012b). The titers of antibodies to viral capsid are much higher in both virus-positive and negative MCC patients than those in the general population (Touzé et al., 2011). In contrast, the IgG antibodies that specifically recognize LT and sT are only detected in the serum of MCPyV⁺MCC. The titers of these antibodies diminished rapidly in patients whose cancer did not recur, whereas they rose rapidly in those with progressive disease, suggesting T-Ag specific antibodies are relatively associated with MCC and do not protect against disease progression (Paulson et al., 2010b). The presence of MCPyV-specific antibodies indicates a strong and specific anti-MCPyV Th response. High production of various adaptive cytokines has been observed when human PBMC are stimulated with MCPyV-like particles, especially IL-13, IFN- γ , and IL-10. HLA class II blocking and CD4⁺ T cells depletion reduce dramatically the production of these cytokines, suggesting strongly a MCPyV-specific Th2, Th1, and Treg response (Kumar et al., 2011). Indeed, T cells recognizing MCPyV-VP1 are identified in both patients with MCPyV⁺MCC and MCPyV⁻MCC and healthy donors, although this is only found in some individuals of the cohort (Lyngaa et al., 2014b). Nonetheless, neither MCPyV-VP specific antibodies nor T-Ag specific antibodies are efficient to lead to virus clearance. It is known that most virus-specific antibodies don't have neutralizing activity because they are elicited by virion fragments, or by viral proteins that are released from dying, infected cells. Antibodies that are directed against such antigens are often specific for particular proteins: internal proteins that are not accessible on intact virions or infected cells; proteins that have been denatured, degraded, or incompletely translated or processed (in terms of cleavage or glycosylation); or proteins that are not oligomerized (which is required for TI activation of B cells) (Hangartner et al., 2006). Therefore, it is likely that T-Ag-specific antibodies cannot recognize MCPyV since T-Ag oncoproteins are not expressed on the surface of MCPyV virion but in infected cells. Alternatively, MCPyV capsid-specific antibodies may not have neutralizing activity because they are directed against epitopes for which antibody binding does not interfere with viral attachment or entry.

The cell-mediated response against MCPyV is further detailed together with anti-MCC response in the next chapter.

CHAPTER IV. Immunobiology of MCC

1. General of cancer immunology

1.1 Cancer immunoediting

The immune system plays a vital role in controlling tumor development and progression. Cancer immunoediting is a dynamic process that consists of immunosurveillance and tumor growth (Figure 9). It is characterized by the dual role of the immune system in suppressing or promoting tumors through three phases termed elimination, equilibrium, and escape (Schreiber et al., 2011). The elimination phase is the period where pre-tumoral or tumoral cells are constantly being killed and controlled by innate and adaptive immunity before they become clinically apparent. During this period, multiple lineages of both innate and adaptive immune cells are involved in the anti-tumor response, especially NK cells, $\gamma\delta$ T cells, NKT cells, M1 macrophages, and CD8⁺ T cells (Gajewski et al., 2013; Mittal et al., 2014; Silva-Santos et al., 2015; Smyth et al., 2000; Street et al., 2001). NK cells, $\gamma\delta$ T cells, and NKT cells provide the first line of defense against tumor cells by exerting direct cytotoxic effects and secreting high levels of IFN- γ to promote tumor cell destruction. Under these conditions, macrophage polarization is skewed toward the M1 phenotype, which is associated with a high level of phagocytosis and secretion of pro-inflammatory cytokines that contribute to tumor cell destruction. Control of tumor cell growth is further achieved by activation of the adaptive immune response. APCs, such as DCs, take up antigens from tumor cells and present these antigens in the presence of co-stimulatory signaling molecules and secreted cytokines to naïve CD8⁺ and CD4⁺ T cells to prime T cell activation. Activated CD8⁺ T cells have direct cytotoxic activity against tumor cells and both these cells and Th1 cells secrete high levels of IFN- γ to drive tumor elimination (Ponomarev and Shubina, 2019). In addition, CD4⁺ T cells promote B cell differentiation and activation, leading to the production of tumor-specific antibodies initiating subsequently tumor cell lysis via ADCC (Lo Nigro et al., 2019). The continuous killing of tumor cells exerts a selective pressure which eventually results in the emergence of less immunogenic and immune-resistant variants. These rare tumor cell variants capable of surviving the elimination phase progress into the equilibrium phase, in which net tumor growth is limited and cellular immunogenicity is edited by the adaptive immune system. In this phase, tumors can stay dormant for a long period until the tumor microenvironment (TME) becomes immunosuppressive thus propitious for tumor growth. The mechanism by which

TME becomes adaptive resistant is a complex process that remains to be well defined. However, chronic exposure to certain inflammatory cytokines, such as IFN- γ and TNF- α , is unequivocally a key event that drives T cell exhaustion through upregulation of immune checkpoints (Baxter and Kaufmann, 2016; Sun et al., 2018). Immunosuppressive microenvironment and low immunogenicity tumor cells prompt tumor enter the escape phase, wherein tumor growth is poorly constrained and becomes clinically detectable (Almog, 2010). Tumor cell escape occurs through three different major mechanisms (Teng et al., 2015). Firstly, cancer cells display increased resistance against the cytotoxicity of anti-tumor response and enhanced expressions of anti-apoptotic and pro-survival genes, such as *BCL2*, *STAT3*, and *HER2*. Secondly, tumor cells evade immune recognition through the downregulation or loss of tumor antigens and antigen-presenting machinery, such as MHC-I, or lack of costimulatory molecules. Lastly and most importantly, edited TME is highly immunosuppressive, a hallmark of established tumors in the escape phase.

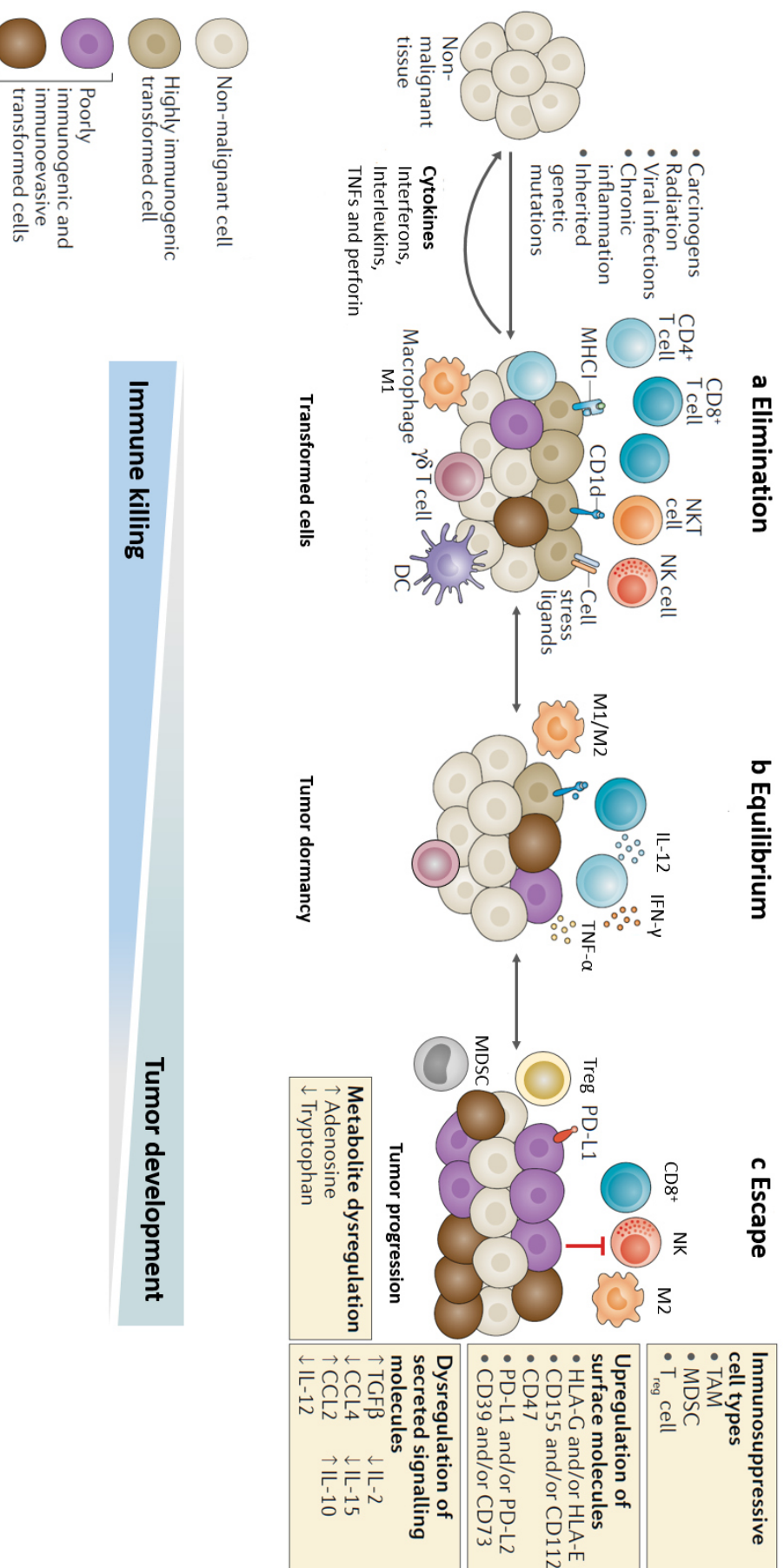


Figure 9. Concept of immunoeediting. Cancer immunoeediting proceeds through three phases: elimination, equilibrium, and escape. **(a)** During the elimination phase, the innate and adaptive immune systems cooperate to recognize transformed cells that have escaped intrinsic tumor suppression and to eliminate them before tumors become clinically detectable. If tumor cells are destroyed, elimination constitutes the totality of the immunoeediting process. In this phase, immune killing capacity is predominant **(b)** Tumors capable of surviving 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)** Tumor progression becomes superior. Edited tumors enter into the escape phase, in which their growth is unrestrained largely owing to the activation of immunosuppressive pathways. Escaped tumors are those clinically detectable as visible tumors. **Modified from (O'Donnell et al., 2019).**

1.2 Immune evasion in tumor microenvironment

During the escape phase, the immune system fails to restrain the tumor outgrowth owing to the immunosuppressive microenvironment. It can be attributed to the presence of potent immunosuppressive cytokines or mediators, such as IL-10, TGF- β , PEG2, indoleamine 2,3 dioxygenase (IDO), inducible nitric oxide synthase (iNOS), and adenosine. These immunoregulatory molecules produced either by tumor cells or tumor stromal cells or immune cells suppress the anti-tumor immunity through multiple different mechanisms, including the inhibition of antigen presentation, DCs maturation, T cell activation, cytotoxic activities, and proliferation of both innate and adaptive immune cells (Beavis et al., 2012; Platten et al., 2014; Tormoen et al., 2018). The tumor microenvironment is also shaped by residents and the ongoing recruitment of different immune cell subsets.

CD8⁺ T cell exhaustion

CD8⁺ T cells become exhausted or dysfunctional in tumors due to persistent antigen exposure and chronic TCR signaling. Characteristics of exhausted T cells include up-regulated and sustained expression of multiple inhibitory immune checkpoint receptors, reduced proliferation, decreased production of effector cytokines, and reduced cytotoxicity, which limits the anti-tumor immune response (Jiang et al., 2020). Inhibitory receptors that are up-regulated on exhausted CD8⁺ T cells include PD-1, CTLA-4, TIM-3, LAG-3, 2B4, BTLA, CD160, VISTA, and TIGIT. Once engaged with their ligands expressed on APCs, suppressive immune cells, or tumor cells, these immune checkpoint receptors deliver negative signals that suppress T cell functions (He and Xu, 2020). Other membrane-bound enzymes or receptors such as CD39, CD73, and adenosine receptors also contribute to immunosuppression. CD39 and CD73 are ectonucleotidase expressed by tumor cells, suppressive immune subsets, endothelial cells, and stromal cells that convert ATP and AMP to adenosine, which has an immunosuppressive effect (Beavis et al., 2012; Moesta et al., 2020).

NK cell exhaustion

NK cells are part of the first line of defense against tumor cells and their activation depends on the balance of activating and inhibitory signals received from cell surface receptors. Under normal physiological conditions, NK cell activation is inhibited by ligands expressed on healthy cells that bind to inhibitory receptors on NK cells. A reduction in the expression of these

ligands or an up-regulation of stress-induced ligands that can occur in tumor cells leads to NK cell activation and results in the production of IFN-gamma and direct cytotoxic activity against these cells. However, similar to CD8⁺ T cells, NK cells can be exhausted in the TME. NK cells exhaustion is attributed to the reduced expression of specific activating receptors such as NKG2D, CD16/FcγRIII, DNAM-1/CD226, CD94-NKG2C, NKp30, NKp44, NKp46, and NKp80, increased expression of inhibitory receptors such as PD-1, CD94-NKG2A, TIM-3, and TIGIT, decreased production of effector cytokines such as IFN-gamma, and reduced cytolytic activity associated with the reduced expression of perforin and granzymes (Bi and Tian, 2017).

Regulatory T cell-mediated immunosuppression

Regulatory T cells (Tregs) are a unique subset of T cells with immunosuppressive properties that are important for maintaining immune homeostasis and self-tolerance, limiting excessive inflammation, and preventing autoimmunity. Tregs function by inhibiting the activities of multiple immune cell types including CD8⁺ and CD4⁺ effector T cells, NK cells, and dendritic cells to restore homeostasis following inflammation. In cancer, Tregs are recruited to the TME by chemokines expressed by tumor cells or tumor-associated macrophages (TAMs), and these and other immunosuppressive cells in the TME promote their expansion. Tregs inhibit the anti-tumor immune response through multiple mechanisms and are considered as a major obstacle for cancer immunotherapy (Plitas and Rudensky, 2020).

Tumor-associated macrophage

Tumor-associated macrophages (TAMs) are M2-like macrophages with immunosuppressive properties that are abundant in the TME. TAMs inhibit anti-tumor immunity through multiple mechanisms including the expression of non-classical MHC-I molecules (HLA-E and HLA-G), the expression of ligands for T cell inhibitory and apoptotic receptors such as PD-L1, TRAIL, Fas L, and B7-H4, and the expression of SIRP-α, which binds to CD47 on tumor cells and inhibits macrophage-mediated tumor cell detection and phagocytosis. Additionally, TAMs produce immunosuppressive factors including IL-10, TGF-β, IDO, and PGE₂, and secrete chemokines that promote the recruitment of Tregs to the TME. Besides their abilities to suppress anti-tumor immune responses, TAMs further drive tumor progression by promoting angiogenesis and tumor cell migration and invasion (Zhou et al., 2020).

Myeloid-derived suppressor cell (MDSC)

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature myeloid cells with immunosuppressive activities that accumulate in different disease states including cancer. MDSCs produce high levels of IL-10, TGF- β , IDO, PGE₂, arginase 1, adenosine, and both reactive oxygen and reactive nitrogen species (ROS and RNS), which together inhibit the activities of natural killer cells, and CD8⁺ and CD4⁺ T cells, and promote the expansion of regulatory T cells. As a result, MDSCs are thought to play a critical role in the suppression of the anti-tumor immune response in cancer patients. Further supporting their involvement in tumor progression is the observation that MDSCs are abundant in the bone marrow, blood, and secondary lymphoid organs of cancer patients and their accumulation correlates with clinical stage, metastatic burden, and chemoresistance (Yang et al., 2020).

Tumor-derived exosome

Tumor-derived exosomes are small, membrane-bound vesicles released by tumor cells that contain tumor cell-associated lipids, proteins, and nucleic acids including double-stranded DNA, mRNAs, and miRNAs. Additionally, they express immunosuppressive molecules such as PD-L1, Fas L, TRAIL, TNF- α , IL-10, TGF- β , and PGE₂, as well as decoy ligands for receptors involved in natural killer cell and CD8⁺ T cell activation, which are thought to reduce tumor cell recognition by these cells. Tumor-derived exosomes are capable of delivering signals to target cells and influencing their functions through either receptor-ligand interactions, membrane fusion followed by the release of the exosomal cargo into the cytoplasm of the recipient cell, or endocytosis-mediated exosome uptake by the recipient cell. They have been shown to inhibit immune cell functions either directly by delivering suppressive or apoptosis-inducing signals or indirectly by promoting the development and accumulation of Tregs and MDSC (Olejarczyk et al., 2020).

1.3 Cancer-immunity cycle

The effective anti-tumor immune response is a series of stepwise events that must be initiated and allowed to proceed and expand iteratively, referred to as the cancer-immunity cycle (Chen and Mellman, 2013) (Figure 10). The first step consists of the release of tumor-specific antigens that will be captured by DCs for processing. Tumor-specific antigens can be derived

from either oncoviral antigens, neoantigens resulted from somatic mutations, or cancer-germline antigens, against which immune tolerance has not been established (Coulie et al., 2014; Lee et al., 2018; Linnemann et al., 2016; Matsushita et al., 2012). In order for the first step to yield a specific anticancer T cell response, it must be accompanied by signals that specify immunity lest peripheral tolerance to the tumor antigens be induced. Such immunogenic signals might include proinflammatory cytokines and factors released by dying tumor cells or by the gut microbiota (Lippitz, 2013). The next step, DCs present captured antigens on MHC-I and MHC-II to naïve T cells, leading to the priming and activation of effector T cells responses in lymph nodes (Mempel et al., 2004). The nature of the immune response is determined at this stage, with a critical balance representing the ratio of T effector cells versus Tregs being key to the final outcome. Activated effector T cells, especially CD8⁺ cells, migrate and infiltrate through extravasation into the tumor bed, wherein they specifically recognize and bind to cancer cells via the interaction between TCR and MHC-I/antigen complex. This triggers target tumor cell death. The killing of the cancer cell releases additional tumor-associated antigens to increase the breadth and strength of the anti-tumor response in subsequent revolutions of the cycle (Chen and Mellman, 2013).

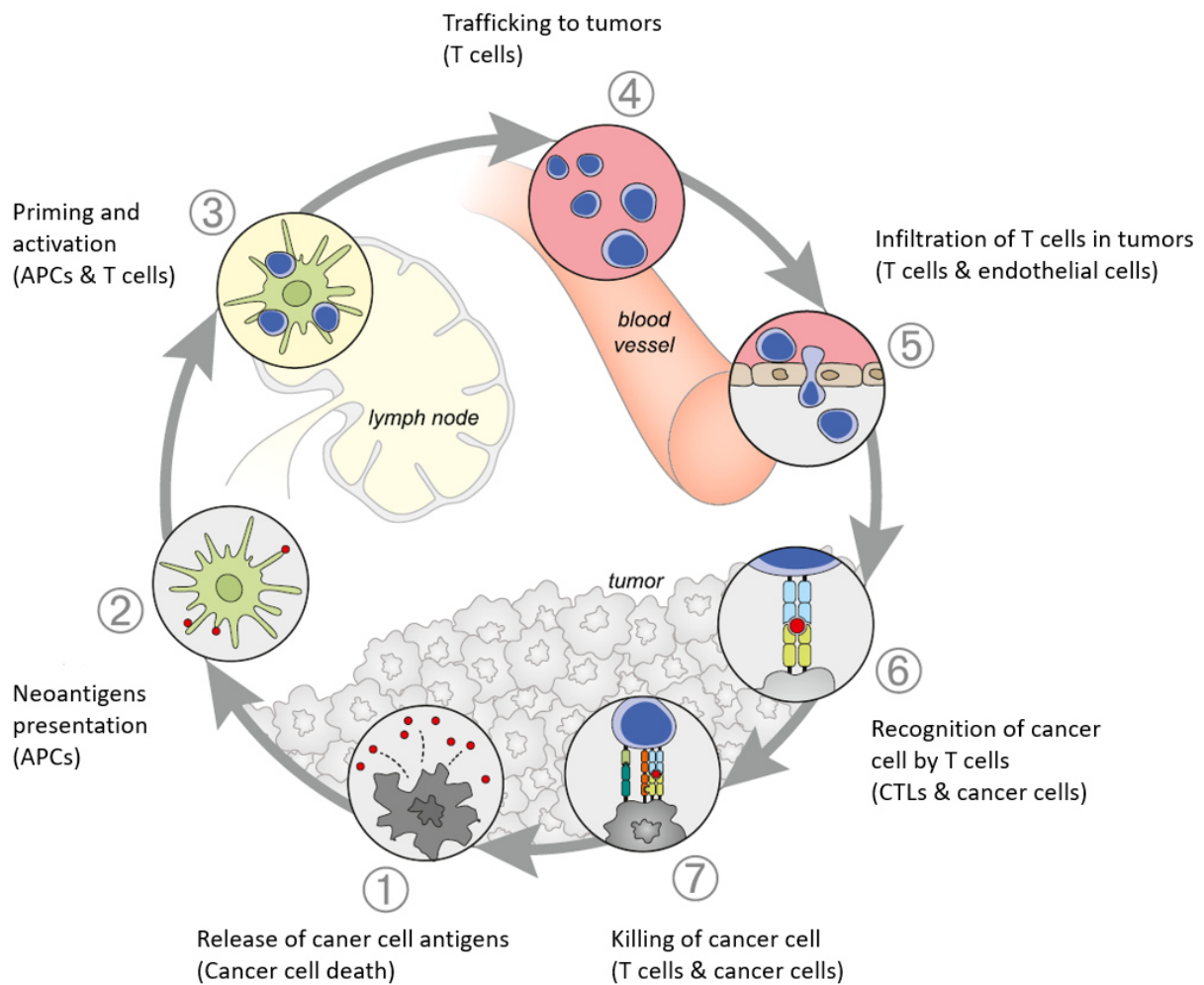


Figure 10. The Cancer-Immunity cycle. The generation of immunity to cancer is a cyclic process that can be self-propagating, leading to an accumulation of immune-stimulatory factors that in principle should amplify and broaden T cell responses. The cycle is also characterized by inhibitory factors that lead to immune regulatory feedback mechanisms, which can halt the development or limit the immunity. This cycle can be divided into seven major steps, starting with the release of antigens from the cancer cell and ending with the killing of cancer cells. Each step is described above, with the primary cell types involved and the anatomic location of the activity listed. **Adapted from (Chen et al., 2013).**

1.4 Definition of hot and cold tumor

The density, diversity, and location of tumor-infiltrating immune cells are closely related to the prognosis and prediction of cancer treatment efficacy. In order to better understand the tumor immune microenvironment and set rationally guided stratification of patients and therapeutic strategies, a simplified but powerful classification for the TME has been proposed (Galon and Bruni, 2019). This classification is implemented according to the immunoscore of the TME, which is a robust consensus, standardized scoring system based on the quantification of two lymphocyte populations (CD3 and CD8) both at the tumor center and the invasive margin (Angell and Galon, 2013; Galon et al., 2014). The Immunoscore ranges from Immunoscore 0 (I0, for low densities, absence of both cell types in both regions) to I4 (high immune cell densities in both locations). By assorting cancers according to their immune infiltration, the system proposed an immune-based classification of cancer, introducing the notion of hot and cold tumor (Galon and Bruni, 2019; Galon et al., 2014). The so-called hot tumors or inflamed tumors (highly infiltrated, Immunoscore I4) are characterized by T cell infiltration and molecular signatures of immune activation, including pro-inflammatory cytokines and costimulatory molecules, whereas cold tumors or non-inflamed tumors (non-infiltrated, Immunoscore I0) are devoid of infiltrated T cells. Tumors with intermediate Immunoscore are defined as altered or mild tumors which can be further divided into two distinct subgroups, excluded and immunosuppressed tumors. In some cases, T cells are found at the edge of tumor sites (invasive margin) without being able to infiltrate them. This 'excluded' phenotype reflects the intrinsic ability of the host immune system to effectively mount a T cell-mediated immune response and the ability of the tumor to escape such response by physically hindering T cell infiltration. For the 'immunosuppressed' phenotype, tumor sites display a low degree of immune infiltration suggesting the absence of physical barriers and the presence of an immunosuppressive environment that limits further recruitment (Gajewski, 2015; Galon and Bruni, 2019). In general, higher Immunoscore is associated with better prognosis and present higher response rate to immunotherapy, such as immune checkpoint blockade .

2. Immune response in MCC

2.1 Innate response against MCC

Innate cells are key players in the antitumor response frontline. NK cells, histochemically characterized as CD16⁺ small cells with dense cytoplasm, are found infiltrated in MCC tumor site, and their cell number is higher in MCPyV⁺MCC versus MCPyV⁻MCC (Sihto et al., 2012a). This suggests that the presence of MCPyV may prompt NK cell proliferation or infiltration. In addition, higher number of infiltrated NK cells is associated with better prognosis in MCPyV⁺MCC, implying a critical role of NK cell-mediated killing in MCC anti-tumor response (Laniosz et al., 2017). Interestingly, NK cells isolated from healthy donors are capable to kill directly two MCPyV⁻MCC cell lines, MCC13 and MCC26 (Ollier et al., 2018a), suggesting also a role of NK cell killing in MCPyV⁻MCC. Moreover, ADCC screening reveals CD326 (EpCAM) as a potent target inducing a NK cell line-mediated ADCC against MCC cell lines (Ollier et al., 2018b). Little is known about the role of NKT cells in anti-MCC response. In one recent study, CD4/CD8 double negative and CD1d- α -GalCer positive T cells are not detected in fresh tumor samples from a cohort of MCC patients, suggesting the absence of NKT cells in MCC tumor (Gherardin et al., 2021). In contrast, interestingly, $\gamma\delta$ T cells with proinflammatory profiles are present in the tumor site. Single-cell RNAseq analysis reveals that these cells display highly innate immunity signature (*NKG7*, *GLNY*, *IFNG*, and *FCGR3A*, which encodes CD16); markers of cytotoxic function, including granzymes (*GZMA*, *GZMH*, and *GZMB*), perforin (*PRF1*); and markers of T-cell exhaustion (*PDCD1*, which encodes PD-1, *LAG3*, and *HAVCR2*, which encodes TIM3), suggesting that $\gamma\delta$ T cells infiltration may serve as a prognostic biomarker and should be explored for therapeutic interventions for MCC patients (Gherardin et al., 2021). However, these $\gamma\delta$ T cells are not correlated with MCPyV status, suggesting common antigens on both MCPyV⁺MCC and MCPyV⁻MCC may be recognized by $\gamma\delta$ T cells. Tumor-infiltrating macrophages (CD68⁺) have also been identified in MCC tumor and their number is higher in MCPyV⁺MCC than in MCPyV⁻MCC. The infiltrated macrophages are not associated with better survival probably due to the presence of M2 phenotype expressing CD163 (Gaiser et al., 2018; Sihto et al., 2012a).

2.2 Adaptive response against MCC

Cellular response

An active, adaptive immune response is crucial for clearing virus-infected cells and cancer cells. Following stimulation, CD8⁺ T cells target cells expressing viral or tumor antigens and induce cell death, which eliminates the infected or malignant cells and further activates the immune response. The presence of activated T cells within the tumor microenvironment is a positive prognostic factor in multiple cancers. In MCC, T cell infiltration into tumors has been observed in multiple studies and has consistently been associated with a survival benefit (Schadendorf et al., 2017b). In a population-based study, MCPyV T-Ag specific CD4⁺ or CD8⁺ T cells are identified in the blood and tumor site of some MCPyV⁺MCC patients but are depleted in MCC patients who don't express T-Ag. Virus-specific CD8⁺ T cells were significantly enriched among tumor-infiltrating lymphocytes as compared with blood, implying intact T-cell trafficking into the tumor (Iyer et al., 2011). These findings indicate that MCPyV T-Ag can serve as tumor neoantigens prompting a specific adaptive anti-MCC tumor response. Although T cells infiltration is observed in most MCC patients, MCC is not always considered as hot tumor. Depending on the patients, the tumor size, and the tumor stage, the immune cells infiltration profile varies. In altered MCC tumors, CD3⁺ and CD8⁺ T cells are detected at the tumor periphery and higher density of these T cells are associated with improved overall survival which is even more significant in MCPyV⁺MCC compared to MCPyV⁻MCC (Feldmeyer et al., 2016). Consistent with the findings of *Iyer et al.*, TCR sequencing of these T cells reveals a higher clonal overlap among the MCPyV⁺ MCC samples compared with the MCPyV⁻MCC patient samples, suggesting an MCPyV antigen-specific response in these tumors (Feldmeyer et al., 2016). Transcriptomic studies of MCC gene-expression profile reveal an immune response gene signature indicative of high levels of intratumoral CD8⁺ T-cell infiltration that correlates with a better prognosis (Paulson et al., 2011b). CD8⁺ T cells isolated from MCC tumors are capable to kill autologous tumor cells after in-vitro expanding with IL-2/IL-15. (Dowlatshahi et al., 2013). In one study of 38 patients with MCC and 30 healthy donors, both populations had MCPyV-VP1 specific CD8⁺ T cells. However, MCPyV-LT and sT specific CD8⁺ cells with effective cytotoxic potential are only found in patients with MCC (Lyngaa et al., 2014b). Some studies report no association between CD8⁺ T cell infiltration of MCC tumors and MCPyV status (Lyngaa et al., 2014b; Paulson et al., 2011b), possibly because MCPyV⁻MCC

present other tumor-specific neoantigens that elicit an adaptive anti-MCC response. CD4⁺ T cell responses, which are both essential for mediating humoral responses and for dendritic cell “licensing” (promoting the capacity of dendritic cells to stimulate cytotoxic T cells) and subsequent CD8⁺ T cell activation, have also been identified within tumor infiltrated lymphocytes (TILs) from MCPyV⁺MCC. Indeed, a total of 6 distinct CD4⁺ T cell epitopes were detected within a limited region of LT. One epitope (LT209-228) was highly prevalent among MCC patients, as tetramer-positive cells were detected in 14 of 18 patients (Longino et al., 2019). In recent years, T cells recognizing different T-Ag-derived epitopes have been identified, suggesting vaccination as a rational strategy of immunotherapy (Jing et al., 2020; Tabachnick-Cherny et al., 2020). Overall, MCPyV T-Ag specific T cell response is found in MCPyV⁺MCC and is associated with tumor burden. Of interest, L-selectin/CCR7⁺ central memory T cells (T_{CM}) are significantly enhanced in MCPyV⁺MCC compared to normal skin (Dowlatshahi et al., 2013), although their specificity is not clear.

Humoral response

MCPyV infection is ubiquitous, with 70% of the population possessing MCPyV-specific antibodies that are generally targeted to viral capsid proteins VP1 and VP2. MCPyV-capsid antibodies are detected in all patients with MCC and 85% of control subjects. However, the titers of antibodies to viral capsid are much higher in both MCPyV⁺MCC and MCPyV⁻MCC patients than in the general population, and high antibody titers correlated with longer progression-free survival among patients (Touzé et al., 2011). Patients with MCPyV⁺MCC also produce IgG antibodies that recognize a portion of T-Ag shared between small and large T-Ags. These antibodies are however rarely detectable and at very low level in healthy people (Paulson et al., 2010c). The titers of these antibodies decrease dramatically in patients whose cancer did not recur, whereas they rose rapidly in those with progressive disease, suggesting T-Ag specific antibodies are relatively associated with MCC burden and do not protect against disease progression (Paulson et al., 2010c).

3. Immune evasion of MCC

As described previously, a large amount of evidence shows that both humoral and cell-mediated immune responses, including tumor-specific T cells, are generated in the majority

of patients with MCC but are unable to control tumor growth in patients presenting with active disease. Failure of immune responses despite the presence of tumor-specific T cells has been widely observed in other tumor types, such as colorectal, breast, and ovarian cancers, hepatocellular carcinoma, and head and neck squamous cell carcinoma (Denkert et al., 2010; Galon et al., 2006; Gao et al., 2007; Sato et al., 2005). This may be related to the functional status of the tumor-infiltrating lymphocytes or the influence of tumor- and stroma-derived suppressive mechanisms. In MCC, multiple evasion mechanisms have been identified in different steps of the cancer-immunity cycle (Figure 11).

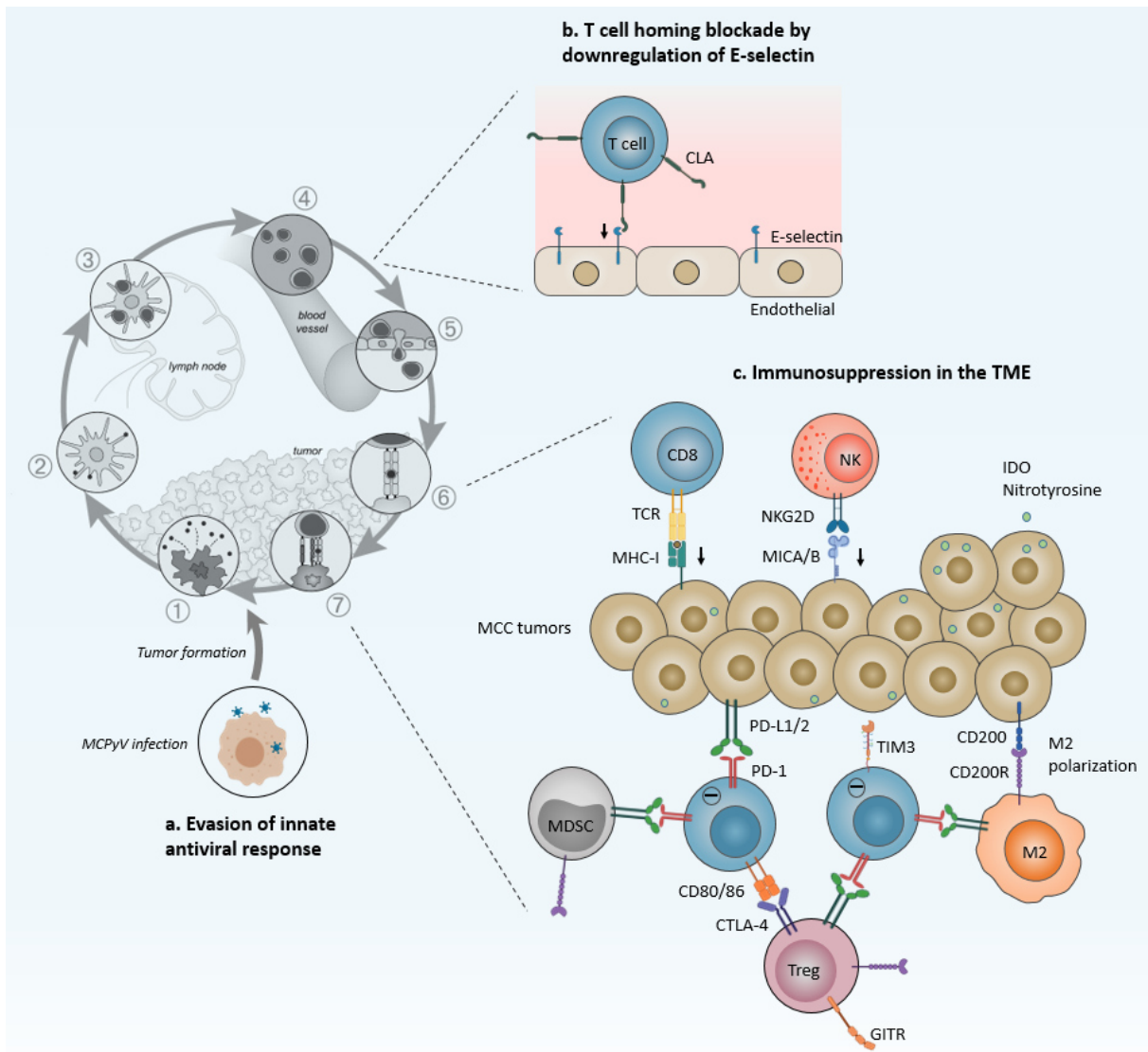


Figure 11. Immune evasion mechanisms of MCC. (a) MCPyV evades the innate antiviral immunity, which helps the establishment of chronic infection and contributes further to tumorigenesis. (b) The migration and infiltration of tumor-specific T cells via extravasation to the tumor site are hindered in the vasculature. This is achieved by decreased expression of E-selectin on the endothelial cells. (c) The TME of MCC is immunosuppressive. MCC tumor cells display low levels of MHC-I and MICA/B, which are recognized and targeted by CD8 T cell and NK cell respectively for cytotoxic killing. Immunosuppressive cells are induced and infiltrated into the tumor nest. Tumor cells express CD200 that binds to CD200R on the macrophage to promote the pro-tumoral phenotype M2 polarization. Tumor cells, M2 macrophages, MDSCs, and Tregs express immune checkpoint molecules, including PD-L1/2, CTLA-4, GITR, which interact with their receptors on T cells to transduce inhibitory signals causing T cell exhaustion. The immunosuppressive mediator, such as IDO-1, nitrotyrosine are also present in the tumor.

3.1 Innate immune evasion of MCC

Immune evasion mechanism is a common strategy deployed by oncoviruses to set up a chronic infection and promote cancer (White et al., 2014). Similarly, MCPyV presents some evasion strategies that protect the virus from the host innate defense and may contribute further to viral persistence and oncogenesis. MCPyV LT and sT impede TLR9 expression to avoid innate recognition and inflammatory response. This is achieved by T-Ag-mediated inhibition of the CCAAT/enhancer-binding protein (C/EBP β) transcription factor, a positive regulator of the TLR9 promoter (Shahzad et al., 2013b). Likewise, it has been shown that MCPyV sT is capable to repress the NF- κ B-mediated inflammatory gene expression, especially CCL20 and IL-8. This is due to the interaction between MCPyV sT and NEMO, which hinders the phosphorylation and degradation of I κ B, preventing thus NF- κ B translocation to the nucleus (Griffiths et al., 2013). These findings suggest a role of MCPyV T-Ag in innate anti-viral response subversion that may contribute to successful persistent infection and tumorigenesis. Properly, MCC tumors can evade NK cell killing. The ligands of the NK activating receptor NKG2D, such as the major histocompatibility complex (MHC) class I chain-related protein A and B (MICA/MICB) are present at low level in normal cells but are highly upregulated in cells undergoing stress responses, such as viral infection and neoplastic transformation (Marcus et al., 2014). The engagement between NKG2D and MICA/MICB stimulates the proliferation and cytotoxic activities of NK cells (Bauer et al., 2018; Pende et al., 2002). However, despite transformation and the continued presence of MCPyV T-Ag, MICA and MICB are only expressed in a minority of MCC tumors in situ and are completely absent on MCC cell lines in vitro. This lack of MICA/MICB expression in MCC cell lines is due to epigenetic silencing of MIC promoter via hypo-acetylation (Ritter et al., 2016). Interestingly, MICA/MICB expression can be restored by pharmacological inhibition of histone deacetylases (HDACs) both in MCC cell lines and in MCC xenotransplantation model in mice. Restored MICA/MICB expression renders MCC cells more sensitive to NKG2D-mediated cytotoxic lysis (Ritter et al., 2016).

3.2 Adaptive immune evasion of MCC

T cell infiltration and cancer cell recognition

The ability of activated T-cell to properly home to tumor site is a prerequisite for an effective anti-tumor T-cell response. Loss or dysregulation of cell homing may decrease the

effectiveness of immune response against MCC, even in the presence of functioning T cell response. E-selectin, also known as endothelial-leucocyte adhesion molecule (ELAM) is a cell adhesion molecule expressed only on endothelial cells. It binds to T cells via the cutaneous leucocyte antigen (CLA), a homing receptor that directs T cells to areas of inflammation, and enables migration to the tumor microenvironments (Clark et al., 2008). In one study, intratumoral vasculature E-selectin is downregulated in half of MCC samples from 56 patients (Afanasiev et al., 2013a). The downregulation of E-selectin may be attributed to the high levels of nitrotyrosine expression in MCC tumors, suggesting also iNOS/arginase pathway as a tumor escape mechanism. Increased number of E-selectin-positive vessels in the tumor are associated with greater intratumoral CD8⁺ lymphocyte infiltration and improved MCC-specific outcome (Afanasiev et al., 2013a). Consistently, it has been reported that a subset of MCC tumors lacks CLA⁺ T cells, which correlate with a lower number of tumor-infiltrating T cells (Dowlatshahi et al., 2013). These data show that disruption of T cell migration into the TME is an evasion strategy for some MCC. When activated T cells successfully enter the tumor site, the recognition between TCR and MHC-I/peptide complex is required for an effective CD8⁺ T cell-mediated killing. Dysregulation of antigen presentation, including the loss of MHC-I and β 2-microglobulin (B2M), is a common mechanism of immune escape by various cancers and is observed in both MCPyV⁺ and MCPyV⁻MCC (Paulson et al., 2014c). In 80% MCC tumors samples and cell lines, MHC-I expression is dramatically reduced compared to normal tissues, and lower MHC-I level is observed in virus-positive than virus-negative MCC, implying a role of MCPyV antigens in the regulation of MHC expression. Of interest, the low level of MHC-I in both MCC cell lines and patients can be reversed by IFNs treatment (Paulson et al., 2014c).

Immune checkpoints expression

In addition to the downregulation of cell surface molecules necessary for immune cell migration and recognition, immune checkpoints are often upregulated on tumor-targeted immune cells. Inhibitory regulation pathways provide the immune system with a mechanism for successfully clearing pathogens and malignant cells while limiting excessive immune response. One such receptor, PD-1, involved in the attenuation of the immune response to infections or tumorigenesis is also essential for the induction and maintenance of self-tolerance, as evidenced by the development of autoimmune disorders in PD-1 null mice (Nishimura et al., 1999). Chronic antigen stimulation from viral infections and tumor cells

upregulates inhibitory receptors on active T cells, causing T cells to lose functionality over time. Increased expression of immune checkpoints, such as PD-1 and TIM3, occurs following persistent viral infection or tumor antigen exposure, resulting in T cell exhaustion (Jin et al., 2010a; Wherry and Kurachi, 2015). T-cell exhaustion correlates with immune dysfunction and prevents CD8⁺ T cell-mediated clearance of virus-infected and malignant cells (Sakuishi et al., 2010; Wherry et al., 2007). MCPyV T-Ag specific CD8⁺ cells isolated from MCC patients' blood and tumor samples express high levels of PD-1 and TIM-3 (Afanasiev et al., 2013b). Upon interaction with their ligands on tumor cells or other immune cells, these receptors deliver inhibitory signals to suppress T cell functions and proliferation (Dong et al., 2002). One best-studied immune checkpoint ligand is PD-L1. Following the activation of the adaptive immune response to foreign antigens, the expression of PD-L1 serves to reinstate immune homeostasis and protect tissue from extravagant cytotoxicity. Interestingly, immunohistochemistry staining shows PD-L1 expression on both MCC tumor cells and TILs, and there is a geographic colocalization between PD-L1⁺ tumor cells and TILs. The expression of PD-L1 on tumor cells is strongly associated with the presence of MCPyV, with almost no PD-L1 expression in MCPyV⁻ MCC tumor cells (Lipson et al., 2013; Mitteldorf et al., 2017). In contrast, the PD-1 expression in the TME seems to be variable between 5% and 60% and independent of the MCPyV status (Mitteldorf et al., 2017). In another study, a subset of MCPyV⁻ MCC tumors is identified to exhibit TILs and PD-L1 expression, although the level of TILs and PD-L1 expression is less important than in MCPyV⁺ MCC (Wong et al., 2015c). Furthermore, a subset of APCs, including CDc11⁺ DC and CD163⁺ macrophages are found with MCC tumor and express another PD-1 ligand, PD-L2 (Dowlatshahi et al., 2013). Collectively, these findings suggest that a local tumor-specific and potentially MCPyV-specific immune response drives PD-1/PD-L1/L2 axis-mediated T cell exhaustion in MCC microenvironment, leading to MCC tumor escape. Whether TIM-3 ligands, such as galectin-9, are expressed in MCC tumor site is currently unknown. In addition to immune checkpoints, immunohistochemistry staining shows the presence of IDO-1 expression in MCC at the tumor-stromal interface (Aung et al., 2019). IDO is an immunoregulatory enzyme catabolizing tryptophan that is necessary for sustainable immune response. Increased expression of IDO is known to be immunosuppressive that dampens T and NK cells, induce Tregs and MDSCs in the microenvironment of various human cancer (Prendergast et al., 2014).

Tregs and other myeloid cells

The recruitment of diverse immune suppressive cells in the TME is often observed in human cancers that contribute to tumor progression (Labani-Motlagh et al., 2020). The presence of FoxP3⁺ Tregs in the areas of inflammation is a mechanism to suppress an overactive immune response and help reestablish peripheral immune homeostasis. Tregs inactivate CD8⁺ T cells and APCs, and may contribute to disease progression in response to UV radiation exposure and viral infection (Beissert et al., 2006). A higher number of FoxP3⁺ Tregs have been identified in MCC tumors than in normal skin but it is not correlated with poor survival (Dowlatshahi et al., 2013; Gaiser et al., 2018). The non-correlation between Tregs and decreased survival may be due to the absence of PD-L1 expression on half of these cells (Mitteldorf et al., 2017). In contrast, another study reports that the presence of FoxP3⁺ Tregs is associated with longer survival (Sihto et al., 2012b), suggesting the complexity of Treg functions in shaping the distinct profile of T cell response. Classical FoxP3⁺ Tregs are CD4⁺, but a small population of CD8⁺/FoxP3⁺ Tregs has been also described in human transplant recipients, mouse models of autoimmune diseases, and the microenvironments of many tumors (Yu et al., 2018). Interestingly, CD8⁺/FoxP3⁺ Tregs expressing immunoregulatory molecules, such as CTLA-4 and glucocorticoid-induced tumor necrosis factor receptor (GITR), are also identified in MCC. The presence of these Tregs may be induced by frequently observed CD123⁺ pDC in MCC (Dowlatshahi et al., 2013), which have been shown to induce the formation of CD8⁺/FoxP3⁺ in the TME of ovarian carcinoma (Wei et al., 2005). Whether these Tregs are MCPyV specific is unclarified. Tumor-infiltrating myeloid cells, including TAMs M2 phenotype macrophages and MDSCs are also found in MCC tumor microenvironment (Dowlatshahi et al., 2013; Gaiser et al., 2018; Kervarrec et al., 2018; Sihto et al., 2012b). These myeloid cells affect tumor development by modulating immune responses and enhancing vascular density (Elliott et al., 2017). In MCC, macrophages (CD68⁺) with M2 immunosuppressive phenotype (CD163⁺) are found both at the tumor periphery and infiltrated in the tumor nests, and they are an important source of PD-L1 (Lipson et al., 2013; Sihto et al., 2012b; Walsh et al., 2016). In addition, CD68⁺ CD163⁺ macrophages, CD11b⁺ myeloid cells, and FoxP3⁺ Tregs express CD200 receptor that is recognized by CD200, another immune checkpoint molecule expressed on MCC tumor cell (Gaiser et al., 2018). CD200 signaling inhibits classic macrophages activation (M1 polarization) and supports an immunosuppressive M2 polarized state that secretes high levels of IL-10, thereby inducing Tregs and promoting tumor growth (Zhang et al., 2004).

Human MDSCs can be defined as CD33⁺, HLA-DR^{low} myeloid cells, which are observed only in a minority of MCC. Most MCC, in contrast, display CD33⁺, HLA-DR^{high} cells that are associated with improved outcomes (Kervarrec et al., 2018).

CHAPTER V. IL-17A

1. The cytokine interleukin-17A (IL-17A)

1.1 Th17 and IL-17A induction

IL-17A, often referred to as IL-17, is the most studied cytokine in the IL-17A family that consists of 6 cytokines, IL-17A to IL-17AF. IL-17A was discovered in the early 1990s from a rodent T-cell hybridoma and was cloned a few years later (Rouvier et al., 1993; Yao et al., 1995a, 1995b). In humans, IL-17A was originally found to be expressed in peripheral blood CD4⁺ T cell upon phorbol-myristate-acetate (PMA) and ionomycine stimulation (Yao et al., 1995b). Research on IL-17A remained superficial from its discovery until the early 2000s with several studies showing its role in inflammation and autoimmune diseases. The critical insight into this field was the identification of the main cellular source of IL-17A, Th17 cells, a lineage of effector CD4⁺ T cells distinct from Th1/Th2 paradigm (Harrington et al., 2005; Langrish et al., 2005; Park et al., 2005). Th17 cells were established as an independent subset of T helper cells by the identification of differentiation factors and transcription factors that are unique to Th17 cells. The differentiation of T helper cells is initiated by the combined signals mediated downstream of the T cell receptor (TCR) and cytokine receptors. Those signals then induce and activate specific transcription factors responsible for the expression of lineage-specific genes such as cytokines. The differentiation of Th17 from naïve CD4⁺ cells requires antigens presentation by APC in the presence of multiple cytokines including transforming growth factor- β (TGF- β), IL-1, IL-6, IL-21, and IL-23 (Bettelli et al., 2006). This initial engagement activates JAK2/STAT3 pathway that leads to the upregulation of a retinoic acid related-orphan nuclear receptor (ROR γ t) (Yang et al., 2008). ROR γ t is a lineage-specific transcription factor that binds directly to the promoter to activate IL-17A expression (Ivanov et al., 2006). Another member of the retinoid nuclear receptor family, ROR α , is also selectively expressed in Th17 cells and dictates IL-17A expression (Yang et al., 2008). Besides ROR γ t, other transcription factors can participate also the upregulation of IL-17A expression. For instance, Runt-related transcription factor1 (Runx1) regulates Th17 differentiation by upregulating ROR γ t expression and by direct binding to ROR γ t (Liu et al., 2015; Zhang et al., 2008). The transcriptional activity of ROR γ t and IL-17A induction can be enhanced by Sirtuin 1 (SIRT1), a protein deacetylase (Lim et al., 2015). It has been shown that RelA (p65) and c-Rel of NF- κ B family bind to ROR γ t promoter to activate ROR γ t expression, increasing indirectly IL-17A induction (Huang et al., 2008; Ruan et al., 2011).

Interferon regulatory factor 4 (IRF4) is also critical for IL-17A and IL-21 induction (Ciofani et al., 2012; Huber and Lohoff, 2014). TGF β -mediated activation of Rho- ROCK pathway, promotes phosphorylation of IRF4 by ROCK kinase. Phosphorylated IRF4 translocates to nuclei and binds to IL-17A and IL-21 promoters (Biswas et al., 2010; Mudter et al., 2011). The aryl-hydrocarbon receptor (AhR) regulates Th17 differentiation and development (Quintana et al., 2008; Veldhoen et al., 2009). Kruppel-like factor 4 (KLF4), a transcription factor that plays a pivotal role in the pluripotency of stem cells, has been shown to regulate Th17 differentiation by binding to the IL-17A promoter directly without altering ROR γ t expression (An et al., 2011; Lebson et al., 2010). Basic leucine zipper transcription factor, ATF-like (BATF), synergizes with ROR γ t to induce IL-17A by direct interaction with conserved intergenic elements of *IL17A/IL17F* loci (Jordan-Williams et al., 2013). Additionally, other signaling pathways Rho-associated kinase, ROCK2, Ets-family transcription factor, Sphingosine 1-phosphate- /type 1 S1P receptors, and AhR have also shown their importance in IL-17A induction (Khan and Ansar Ahmed, 2015).

1.2 Cellular sources of IL-17A

IL-17A is a pro-inflammatory cytokine that plays important role in the development of inflammatory and autoimmune diseases, in host defense as well as in cancer (Gu et al., 2013). Although IL-17A was originally found in Th17 cells, cytotoxic CD8⁺ T cells from the lung following the Influenza challenge also produce IL-17A and are named as Tc17 (Hamada et al., 2009a). The Th17 cell pathway cannot explain the early IL-17A-mediated immune responses that occur during tissue damage and host defense. The IL-17-mediated immune pathway is induced within hours following epithelial cell injury or activation of PRR (Ferretti et al., 2003; Happel et al., 2003a, 2005). The rapidity of this early IL-17A response is not coherent with the development of Th17 cells which requires a few days. Therefore, there must be other IL-17A producing cell types that direct IL-17A-dependent innate immune responses. Studies have shown that $\gamma\delta$ T cells are important innate IL-17A-producing cells during autoimmune inflammation and infectious diseases. $\gamma\delta$ T cell is a subset of unconventional T cell that expresses a TCR consisting of a γ -chain and a δ -chain. These T cells are present in the intestinal epithelium as intraepithelial lymphocytes. $\gamma\delta$ T cells are also present in the lungs, skin, liver, and other tissue sites during inflammation (Chien et al., 2014). CD27⁻ $\gamma\delta$ T cells were shown

to constitutively express ROR γ t and Runx1 and can be stimulated with PMA and ionomycin to produce IL-17A (Ribot et al., 2009). Invariant natural killer T (iNKT) cells, another subset of unconventional T cells that play a key role in immune surveillance, have been shown to produce IL-17A (Michel et al., 2008). Consistent with other IL-17A producing T-cells, IL-17A expression in these iNKT cells is induced by ROR γ t. The innate lymphoid cells (ILCs) are other considerable cellular sources of IL-17A. The ILCs is a family of small innate immune cells residing in both lymphoid tissues and non-lymphoid tissues that play a key role in mucosal immunity and homeostasis (Panda and Colonna, 2019). Characteristics allowing their differentiation from other immune cells include the absence of recombination activating gene (RAG)-dependent rearranged antigen receptors (B cell and T cell receptors), the lack of myeloid cell and dendritic cell phenotypical markers and their lymphoid morphology (Spits et al., 2013). IL-17A producing ILCs include some NK cells, the group 3 ILCs (ILC3s), and lymphoid tissue inducer (LTi) cell. Peritoneal NK1.1⁺ NK cells isolated from *Toxoplasma gondii*-infected mice were shown to produce IL-17A in an IL-6- and IL-23-dependent manner (Passos et al., 2010). ILC3s, often found in lamina propria of the intestine, are involved in innate response against intestinal bacteria and fungi (Colonna, 2018). Natural cytotoxicity triggering receptor negative (NCR⁻) ILC3s are an innate source of IL-17A (Takatori et al., 2009). LTi cells reside in peripheral lymph nodes, spleen, and gut lamina propria are also ROR γ t-dependent IL-17A producing cells (Meier et al., 2007; Yoshida et al., 1999). Myeloid cells, such as neutrophils, have also been reported to produce IL-17A (Li et al., 2010; Taylor et al., 2014). Dendritic cells from Langerhans cell histiocytosis (LCH), a rare inflammatory neoplasm of unknown etiology, express a high level of IL-17A (Coury et al., 2008a). Of note, IL-17A is not restricted to immune cells, other non-immune cells have also been reported to produce IL-17A, such as Paneth cells (Takahashi et al., 2008). Paneth cells are specialized epithelial cells present in the intestine epithelium. Known for their production of antimicrobial peptides, they are crucial for homeostatic balance with colonizing microbiota and innate immune protection from enteric pathogens (Vaishnava et al., 2008; Wilson et al., 1999). Paneth cells activated in a model of TNF-induced shock have been shown to rapidly secrete IL-17A and are responsible for increased chemokine production and recruitment of neutrophils (Takahashi et al., 2008).

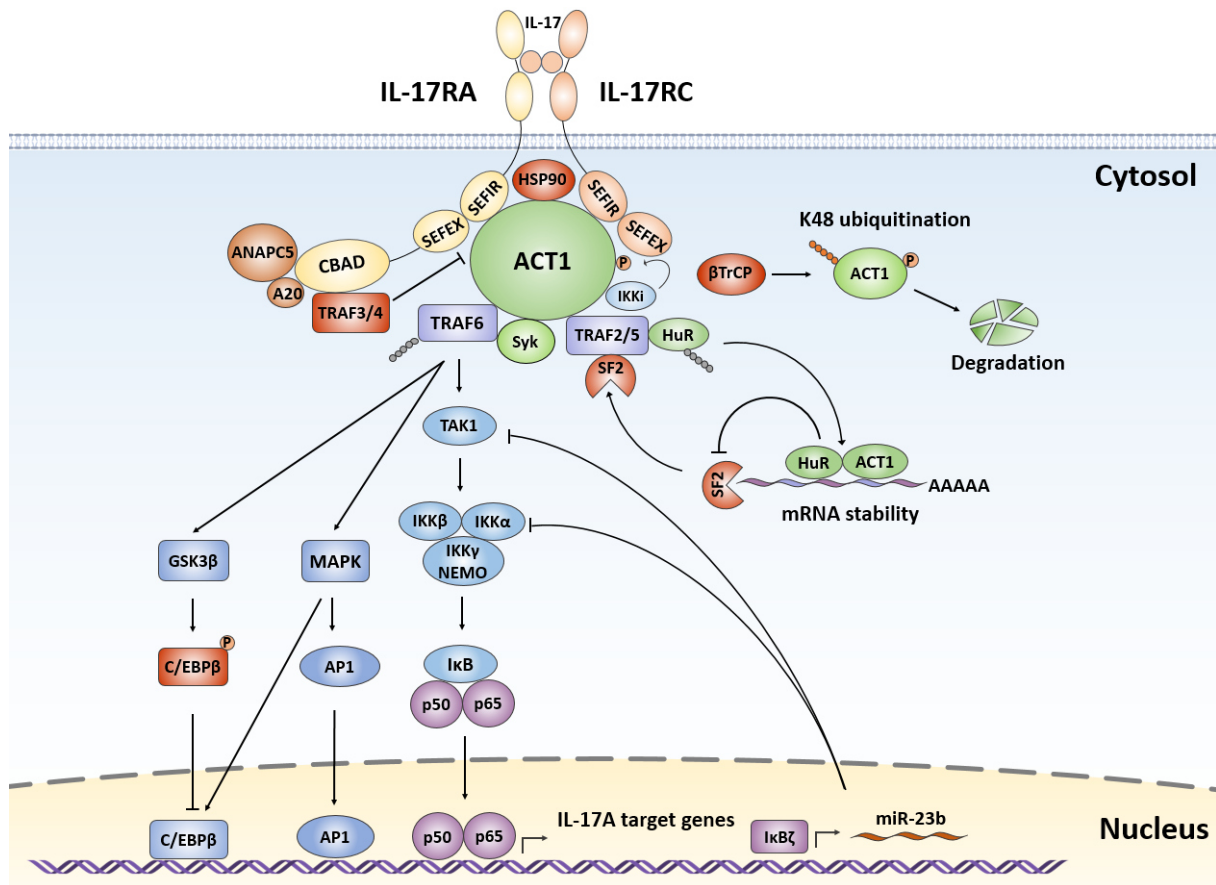


Figure 12. IL-17R signaling pathway. IL-17 signaling is activated upon binding of IL-17A/A, IL-17F/F, or IL-17A/F dimer to a dimeric receptor complex, IL-17RA/IL-17RC. The cytoplasmic part of both IL-17RA and IL-17RC contains SEFIR and SEFEX domain, which are essential for the activation of signaling. Both IL-17R subunits recruit, via SEFIR, the protein ACT1, which serves as an adaptor and an E3 ubiquitin ligase. ACT1 mediates the K63-linked ubiquitination of TRAF6, which results in the downstream activation of TAK1, IKK complex, and canonical NF- κ B signaling. TRAF6 also activates MAPK pathways, leading to the activation of AP1 and C/EBP β -mediated transcription. Syk binds to ACT1 and TRAF6 to initiate MAPK and NF- κ B pathways. In addition, IL-17 signaling induces the phosphorylation of the C/EBP β by GSK3 β -mediated pathways, inhibiting C/EBP β -mediated gene expression. In an alternative signaling pathway, ACT1 is phosphorylated by IKKi. Phosphorylated ACT1, together with TRAF2 and TRAF5, sequesters the RNA-binding protein SF2 and recruits HuR. ACT1 alone, or with HuR prevents SF2-mediated mRNA decay and stabilizes target mRNA transcripts. The HSP90 controls stability of ACT1, promoting signal transduction. The deubiquitinating enzyme A20 is induced by IL-17 and interacts with IL-17RA through the CBAD. A20 mediates negative feedback by targeting TRAF6 to negatively regulate NF- κ B and MAPK signaling. The ANAPC5, another negative regulator of IL-17 signaling, associates with IL-17RA through the CBAD. The microRNA miR-23b also acts as an inhibitor of IL-17 signaling by targeting IKK α and TAK1 complex. The E3 ubiquitin-protein ligase complex β TrCP facilitates

proteasomal degradation of ACT1 via K48-linked ubiquitination. TRAF3 and TRAF4 inhibit IL-17 by competing with ACT1 for binding to IL-17RA and TRAF6, respectively.

1.3 Molecular features of IL-17A signaling

1.3.1 IL-17A and IL-17A receptors

IL-17A is originally termed as cytotoxic T-lymphocyte-associated protein 8 (CTLA8) that presents unexpected sequence homology with an open reading frame in Herpesvirus saimiri, a T cell tropic γ -herpesvirus, but not with other known cytokine families (Yao et al., 1995a). Screens for homologous genes led to the discovery of IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F. These cytokines adopt an unusual cysteine-knot fold architecture, analogous to nerve growth factor (NGF) and platelet-derived growth factor (PDGF) which are unique structural features distinct from other immune cytokine subclasses (Hymowitz et al., 2001). IL-17A and IL-17F are the most closely related and are co-expressed on linked genes and are usually co-produced by IL-17A producing cells (Akimzhanov et al., 2007). Similarly, the IL-17 receptor (IL-17AR) family consists of five receptor subunits, IL-17RA, IL-17RB, IL-17RC, IL-17RD, and IL-17RE. IL-17RA is the founding member of the IL-17R family and is a co-receptor used by several other IL-17 family ligands (Gaffen, 2009). IL-17A and IL-17F form either homodimers or heterodimers that induce signals through an obligate dimeric IL-17RA and IL-17RC receptor complex. The IL-17A signaling is summarized in (Figure 12). As a distinct cytokine family, the IL-17R family contains a conserved cytoplasmic motif termed as SEF/IL-17A receptor (SEFIR) domain, which is distantly related to the TIR domain found in toll-like receptors (TLRs) and IL-1 receptors (Novatchkova et al., 2003). Additionally, an extended 100 residues beyond the SEFIR, called “SEFIR-Extension” SEFIRX, is necessary for the IL-17R-mediated pathway (Onishi et al., 2010). The cytoplasmic tail of IL-17RA additionally contains a distal domain distinct from the SEFIR/SEFEX, named C/EBP β activation domain (CBAD) (Shen et al., 2009). The expression of IL-17A receptors is ubiquitous. IL-17A mainly induces signaling in cells of non-hematopoietic origin, including epithelial and mesenchymal cell types (Chen and Kolls, 2017).

1.3.2 Transcription factors in IL-17A pathway

IL-17A upregulates target gene expression either by inducing de novo gene transcription or by stabilizing target mRNA transcripts. The initial event in IL-17R signaling is the recruitment of

Act1, also known as Connection to I κ B kinase and Stress-activated protein kinases (CIKs), a unique cytosolic adaptor protein necessary for all IL-17A-dependant signal transduction (Qian et al., 2007). Act1 contains also a SEFIR domain that allows its homotypic interactions with IL-17RA and IL-17RC. Upon IL-17A signaling, Act1 rapidly recruits and ubiquitinates tumor necrosis factor receptor-associated factor 6 (TRAF6) via its E3 Ubiquitin ligase activity (Schwandner et al., 2000). Ubiquitination of TRAF6 activates the transforming growth factor β -activated kinase (TAK) 1 that subsequently activates the canonical nuclear factor κ B (NF- κ B) pathway. TAK1 activates the inhibitor of NF- κ B kinase (IKK) complex composed of IKK α , IKK β , and IKK γ (NEMO) which then phosphorylates the I κ B subunit of the NF- κ B. Phosphorylated I κ B undergoes proteasomal degradation, thus releasing NF- κ B into the nucleus to activate inflammatory gene transcription (Napetschnig and Wu, 2013). NF- κ B also upregulates the expression of I κ B ζ and B cell lymphoma 3-encoded protein (Bcl3), noncanonical members of the NF- κ B family that in turn promote expression of various IL-17A-NF- κ B-mediated pro-inflammatory and anti-microbial genes (Karlsen et al., 2010; Tohyama et al., 2018). Additionally, I κ B ζ facilitates IL-17A-induced gene expression by inhibiting the microRNA miR-23b, an inhibitor of IL-17A signaling (Zhu et al., 2012a). TRAF6 also activates the mitogen-activated protein kinase (MAPK) pathway including extracellular signal-regulated kinase (ERK), p38 and JUN N-terminal kinase (JNK) that promote activator protein 1 (AP1)-mediated gene expression (Maitra et al., 2007). Although the IL-17A signaling is primarily driven by serine/threonine kinases, studies have shown the involvement of one tyrosine kinase in the activation of this pathway. In keratinocytes, the spleen tyrosine kinase (Syk) is activated upon IL-17A stimulation and binds to Act1 and TRAF6 to initiate the NF- κ B pathway (Wu et al., 2015). The CCAAT/enhancer-binding protein (C/EBP) family of transcription factors is also activated by IL-17A signal transduction. The expression of some target genes requires the involvement of both C/EBP and NF- κ B. For example, the *Il6* and *Lcn2* promoters require both NF- κ B and C/EBP elements, as deletion of either element renders their promoters insensitive to IL-17A-mediated signals (Ruddy et al., 2004; Shen et al., 2006). C/EBP δ is induced at mRNA level and protein level upon IL-17A signaling while C/EBP β is regulated post-transcriptionally and post-translationally, including alternative translation and phosphorylation. The CBAD domain of IL-17R induces phosphorylation of C/EBP β through glycogen synthase kinase 3 β (GSK3 β)- and ERK pathway. Subsequently, phosphorylated C/EBP β inhibits IL-17A-dependant gene expression (Shen et al., 2009).

1.3.3 RNA controlling in IL-17A pathway

In addition to transcriptional activation of target gene expression, IL-17A regulates a number of gene expressions by controlling mRNA turnover. Cytokine and chemokine mRNA are very unstable and have short half-lives due to conserved cis-element, such as AU-rich elements (AREs) and stem-loop structures in their 3' UTRs (Leppek et al., 2013; Stoecklin et al., 2006). To ensure the rapid production of cytokines upon environment stimuli during inflammation, the transcripts of some cytokines are stabilized and stored in cytoplasmic granules (Anderson, 2008). IL-17A-mediated mRNA stabilization is also granted by Act1, but is independent of TRAF6. The inducible I κ B kinase (IKKi) is recruited by IL-17R/Act1 complex and phosphorylates Act1 on Ser 311. Phosphorylated Act1 interacts with TRAF2 and TRAF5, which sequester the mRNA splicing regulator factor 2 (SF2), a RNA decay factor, thus preventing mRNA degradation (Bulek et al., 2011; Sun et al., 2011). The Act1/TRAF2/TRAF5 complex recruits also the RNA binding protein Human Antigen R (HuR) and activates it through Act1-dependant ubiquitination. Activated HuR binds to target mRNA and sterically competes with SF2 and prevents SF2-mediated RNA decay (Herjan et al., 2013). A recent study showed the Act1 SEFIR domain binds directly a stem-loop structure, SBE (SEFIR-binding element), in the 3' UTR of target genes mRNA. The binding of Act1 to the SBE drives the formation of three distinct protein-RNA complexes (RNPs) that regulate three disparate events in inflammatory mRNA metabolism: preventing mRNA decay in the nucleus, inhibiting mRNA decapping in P-bodies, and promoting translation (Herjan et al., 2018). Although enhanced inflammation due to IL-17A-mediated mRNA stabilization is beneficial during host defense, restriction of this pathway is critical for prevention of severe inflammation conditions. Depending on the site of phosphorylation of Act1, it acts either as an activator or as a suppressor of IL-17A signaling. Phosphorylation of Act1 on three other Ser sites by IKKi and the IKK-related kinase, TANK binding kinase 1 (TBK1) prevents NF- κ B activation, thus suppressing IL-17A-mediated gene expression (Qu et al., 2012). The endoribonuclease MCPIP1 (MCP-1-induced protein 1) negatively regulates IL-17A signaling by degrading IL-17A-induced mRNA through their 3' UTR. MCPIP1 also indirectly regulates the expression of other I κ B ζ -dependent IL-17A target genes by controlling I κ B ζ mRNA stability (Garg et al., 2015). MCPIP1 also degrades the mRNA transcripts encoding IL-17A receptor subunits Il17ra and Il17rc. Another RNA binding protein, Roquin, has been shown to cooperate with MCPIP1 to recognize stem-loop structural motifs

in the 3'UTR of their target transcripts, dampening IL-17A signaling (Mino et al., 2015). MicroRNAs are gene expression regulators that induce mRNA decay or suppress translation. In the IL-17A signaling pathway, miR-23b was found to target signaling intermediates such as TAB2, TAB3, and IKK- α , thus impeding NF- κ B activation. IL-17A downregulates miR-23b transcription, resulting in feedback activation of IL-17A signaling (Zhu et al., 2012b). The miR-30a dampens IL-17A-mediated gene expression by inducing the degradation of Act1 mRNA (Wan et al., 2015).

1.3.4 Negative control and ubiquitination in IL-17A signaling

As important as phosphorylation, ubiquitin-related post-translational modifications plays a pivotal role in IL-17A signaling. Deubiquitinating enzymes (DUBs) reduce IL-17A-mediated signal transduction by targeting TRAF proteins. The ubiquitin C-terminal hydrolase 25 (USP25) removes K63-linked ubiquitin chains on both TRAF5 and TRAF6, reducing target gene expression as well as mRNA stability (Zhong et al., 2012). A20, encoded by *TNFAIP3*, is a feedback inhibitor of IL-17A signaling. IL-17A induces A20, which triggers the deubiquitination of TRAF6 and downregulates the activation of NF- κ B and MAPK. A20 directly associates with IL-17ARA through the CBAD domain of the IL-17ARA, explaining why deletion of this receptor region leads to exacerbated IL-17A-dependent gene induction (Garg et al., 2013). A20 associates with anaphase-promoting complex subunit 5 (ANAPC5), which is best known as a subunit of a multiprotein E3 ubiquitin ligase complex involved in cell cycle progression. Deletion of ANAPC5 enhances IL-17A signaling (Ho et al., 2013). TRAF proteins are not always activators of IL-17A signaling. For instance, TRAF3 binds directly to the CBAD domain of IL-17ARA and interferes with IL-17ARA/Act1, thus dampening IL-17A-dependant signaling. Similarly, TRAF4 directly binds Act1 upon IL-17A stimulation and competes with TRAF6 for Act1 (Zepp et al., 2012; Zhu et al., 2010). association. As Act1 is a E3 ubiquitin ligase that plays a central role in IL-17A signaling, post-translational modification of Act1 is another important way to control inflammatory signaling. IL-17AR signaling can be impaired by the E3 ubiquitin-protein ligase complex β -transducin repeat-containing protein (β TrCP), which induces the K48 ubiquitination of phosphorylated forms of ACT1 that in turn leads to its proteasomal degradation (Shi et al., 2011). Moreover, heat shock protein 90 (Hsp90) involved in protein assembly and folding maintain the integrity of Act1 protein. Inhibiting of Hsp90 leads to proteasomal degradation of Act1 and thus downregulates IL-17A activity (Wang et al., 2013a).

2. IL-17A in host-defense and tissue homeostasis

2.1 IL-17A-mediated response against microorganisms

IL-17A signals predominantly in non-hematopoietic cells to induce both innate and adaptive immune defenses. The primary function of IL-17A in healthy individuals is control of homeostasis of skin and mucosal barrier, as well as the host defense against foreign pathogens' infection including bacteria, fungi, parasites, and viruses (Figure 13). IL-17A signaling induces chemokines and pro-inflammatory cytokines that promote myeloid-driven innate inflammation in the infected or injured tissue (McGeachy et al., 2019). Although IL-17A plays a pivotal role in inflammation, it is not considered as a potent inducer of inflammation on its own. The strong effects during inflammation are attributed to its synergistic action with other cytokines, such as TNF, IL-22, IL-23, IFN- γ and etc. During inflammation, IL-17A, acting in synergy with other cytokines, is a powerful inflammatory signal that results in the rapid recruitment and sustained presence of neutrophils. This is achieved by PGE2-mediated vasodilation and the induction of chemokines such as CXCL1, CXCL2, and CXCL8, which enables immune cell trafficking and extravasation; MMP expression, which induces accessibility to tissues; and the enhanced survival and activity of phagocytic cells through the induction of G-CSF and GM-CSF (Veldhoen, 2017). The requirements of IL-17A signaling in host defense were well documented and appreciated before the identification of Th17 cells (Ye et al., 2001). In mouse models of pneumonia, knockout of IL-17A or IL-17ARA increased the susceptibility to bacterial infections, such as *Klebsiella pneumonia* and *Mycoplasma pneumonia* (Wu et al., 2007; Ye et al., 2001). Upon *K. pneumonia* infection IL-17ARA knockout mice showed impaired G-CSF production and neutrophil recruitment in the lungs and, as a consequence, the IL-17ARA knockout mice had higher bacterial burdens in the lung as well as increased systemic dissemination to the spleen when compared to their littermate controls (Ye et al., 2001). The induction of G-CSF and chemokines in lung tissue is due to the combined effects of IL-17A and IL-22, with IL-17A providing the initial critical signal. *K. pneumonia* infection induces IL-17A production via TLR4 signaling (Happel et al., 2003b). In addition to innate IL-17A-mediated responses, antigen-specific Th17 is also involved and can provide immunological memory response upon re-encountering the same pathogen. Antigen-specific Th17 has been shown to recognize conserved antigens among different *K. pneumonia* strains and confer protection by

providing serotype-independent antibodies (Chen et al., 2011a). In the nasopharyngeal and oral cavity, IL-17A-producing cells offer crucial anti-fungal protection. IL-17A produced by $\gamma\delta$ T cell and Th17 control opportunistic *Candida albicans* infections. Pro-inflammatory cytokines and chemokines are induced in the mucosa upon IL-17A stimulation of the epithelia (Conti et al., 2014; Huang et al., 2004). IL-17A is also important for the defense against *Aspergillus fumigatus* and *Pneumocystis carinii* infection (Puel et al., 2011; Rudner et al., 2007). Acting with TNF, IL-17A can induce the expression of the receptor activator of NF- κ B ligand (RANKL), which is involved in tissue remodeling and dendritic cell maturation (Hashizume et al., 2008; Lin et al., 2014a). RANKL increases osteoclast differentiation, thereby enhancing bone resorption. In synergy with RANKL, IL-17A in the gingiva prevent bone destruction that is initiated by pathogens, such as *Porphyromonas gingivalis* (Lin et al., 2014a; Yu et al., 2007, 2008).

In the intestinal epithelium, IL-17A-producing cells, such as $\gamma\delta$ T cells, ILCs, NKT cells, resident Th17, and Tc17, are vital for the maintenance of barrier integrity and defense. Upon microbial invasion, in synergy with IL-22 produced by ILC3s, IL-17A induces the expression of antimicrobial peptides (AMPs) such as β -defensin and lipocalin 2, which are capable to directly inhibit or even kill invading microorganisms (Liang et al., 2006; Liévin-Le Moal and Servin, 2006). Upon barrier breakthrough, IL-17A combined with TNF activates endothelial cells and induces the expression of selectin as well as chemokines CXCL1, CXCL2, and CXCL5 that are crucial for sustaining phagocytic cells recruitment to prevent the spread of infection (Griffin et al., 2012). For instance, in synergy with TNF and IL-22, IL-17A controls *Citrobacter rodentium* infection in mice (Basu et al., 2012). IL-17A produced by $\gamma\delta$ T cells protects against *Clostridium difficile* infection (Chen et al., 2020). In addition, IL-17A produced by Th17 in Payer's patches, in synergy with BAFF, is important for the generation of IgA, which offers lifelong protection against invading microorganisms (Hirota et al., 2013). Although not crucial, IL-17A is involved in the protection against intracellular bacteria and parasites. Synergizing with IFN- γ , IL-17A can control an intracellular bacterial infection, such as *Listeria monocytogenes* or *Chlamydia muridarum* (Hamada et al., 2008; Zhang et al., 2012). Similar results have been observed in infection with intracellular parasites such as *Toxoplasma gondii* and *E. falciformis*. Although IL-17A signaling is not critical for controlling *Toxoplasma*, it is important for optimal recruitment of granulocytes that are vital for parasites elimination (Kelly et al., 2005). A balanced response among IL-17A, IFN- γ , and IL-22 is required to limit immunopathology

during *E. falciformis* infection (Stange et al., 2012). In *trypanosoma cruzi* infection, IL-17A was found to be crucial for sustaining the CTL response against infected cells. It has been shown that IL-17A promoted parasite-specific CD8⁺ T cell survival by dampening the pro-apoptotic protein BAD (Tosello Boari et al., 2018).

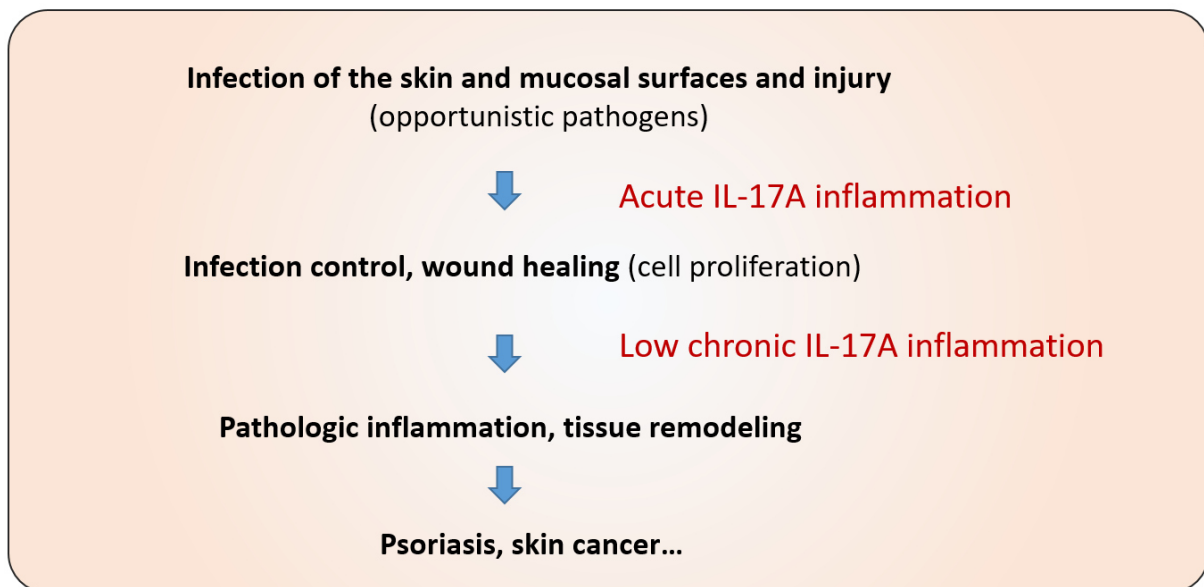


Figure 13. The roles of IL-17A in the skin and mucosal surfaces. The primary role of IL-17A is the induction of an acute inflammatory response against invading pathogens on the skin and the mucosal barriers, especially opportunistic pathogens. The infections or injuries of the barrier surfaces induce an acute IL-17A inflammation helping to control the infection and repair tissues damages. When infection and injury persist, IL-17A response becomes low and chronic, which could lead to the development of inflammatory diseases, such as psoriasis, and cancers.

2.2 IL-17A in viral infection

2.2.1 IL-17A enhances anti-viral responses

While the function of IL-17A in host defense against bacteria and fungi is extensively explored, its role in viral infection is less understood. It has been reported that numerous different viruses can induce an IL-17A response. However, unlike its general protective role in host defense against microorganisms, whether IL-17A-mediated response upon viral infection is beneficial or detrimental is context-dependent and varies among viruses. Similar to its protective role in host defense against bacteria, IL-17A has been shown to fight against influenza virus infection by enhancing inflammatory response. In response to being

challenged with lethal doses of influenza infection, the number of Tc17 cells in the lung increased sharply and was shown to play a beneficial role, since the neutralization of IL-17A resulted in higher morbidity and mortality of the infected mice. And the protection conferred by Tc17 cells coincided with an influx of neutrophils into the lung (Hamada et al., 2009b). Consistently, upon respiratory syncytial virus (RSV) infection in mice, IL-17A induced neutrophils migration into the lung that partly contributes to reduced viral load and increased survival rate (Zhang et al., 2016). Following vaccinia virus infection, adoptively transferred Tc17 cells can acquire strong cytotoxic potential *in vivo*, as shown by their increased FasL expression levels that correlate with effective viral clearance (Yeh et al., 2010). In another model, IL-17A is important not only for generating an efficient Th1 immune response following intravaginal HSV-2 challenge but also for enhancing memory attack upon re-exposure with the same virus (Anipindi et al., 2016; Bagri et al., 2017). IL-17A-deficient mice failed to mount a protective Th1 immune response and were more susceptible to HSV-2 infection (Anipindi et al., 2016). Upon rechallenging, the lack of IL-17A production resulted in higher viral shedding, more severe morbidity and mortality, and significantly compromised Th1 responses, with fewer IFN- γ -expressing CD4⁺ tissue-resident memory T cells observed in the female genital tract (Bagri et al., 2017). However, the mechanism by which IL-17A upregulates Th1 response and memory T cells is unclear. IL-17A also plays a role in promoting cytotoxic CD8⁺ T-cell activity during certain viral infections. In adenovirus infected mouse model, IL-17A was crucial for hepatic CD8⁺ T cells activity since IL-17A neutralization or IL-17ARA-knockout resulted in significantly decreased CTL counts and compromised CTL effector functions (Hou et al., 2013). Consistently, combined with IL-23, IL-17A was shown to participate in the clearance of vaccinia virus infection in mice by promoting the activation and clustering of CD8⁺ T cells in the spleen (Kohyama et al., 2007). Furthermore, in the West Nile virus infection model, IL-17A-deficient mice exhibited a higher viral burden and a lower survival rate. Both of these outcomes were associated with reduced CD8⁺ T-cell cytotoxicity characterized by a lower expression of cytotoxic-mediator genes, including Granzymes, Perforin, and FasL (Acharya et al., 2017). The expression of cytotoxic-mediator genes in sorted splenic CD8⁺ T cells could be directly induced by the addition of recombinant IL-17A *in vitro*, suggesting a direct effect of IL-17A on CD8⁺ T cell activity (Acharya et al., 2017). Accumulating evidence reveals that B cells are critically involved in the host's control of multiple viruses. The protective role of B cells during viral infection is largely mediated through an effective humoral immune response that includes

both the production of natural antibodies at infection onset and isotype-switched virus-specific antibodies at a later phase of infection (Crotty, 2018). IL-17A promotes B-cell activity to hinder viral infection. For example, H5N1-infected IL-17A knockout mice exhibit markedly increased weight loss, more pronounced lung immunopathology, and significantly reduced survival rates compared to infected wild-type controls. This phenomenon was attributed to impaired B-cell recruitment in the lung, which correlated with reduced CXCR5 expression in B cells and decreased chemoattractant CXCL13 production (Wang et al., 2011). Interestingly, IL-17A also promotes the migration and differentiation of B-1a cells, a subset of B-cells that are the major source of natural immunoglobulin IgM. Deficiency of IL-17A led to severely impaired B-1a-derived antibody production in the respiratory tract, leading to a defective viral clearance. Mechanistically, IL-17A induced the expression of B lymphocyte-induced maturation protein (Blimp)-1 and NF- κ B in B-1a cells that lead to the plasmacytic differentiation of these cells and eventually the production of natural IgM (Wang et al., 2016).

2.2.2 Protective role of IL-17A in viral-induced inflammation

Abnormal and excessive inflammatory responses can be detrimental and are involved in a number of inflammatory and autoimmune diseases. Although IL-17A is well recognized as an active inducer of inflammation, studies also indicate that it is potentially involved in the suppression of detrimental tissue inflammations during viral infections. Using a neonatal mouse model of RSV, neonates failed to develop an IL-17A response during RSV infection and exhibited more severe lung inflammation and pathology than adult mice. And exogenous IL-17A administration to RSV-challenged neonatal mice decreased lung inflammation, whereas IL-17A neutralization in adults caused increased inflammation and airway mucus (Huang et al., 2015). In SIV-infected rhesus macaques, the ratio of intestinal Th17 cells is negatively correlated with plasma SIV levels, with Th1 cell number predominating over that of Th17 cells in highly viraemic animals. Moreover, during and after antiretroviral therapy, Th17 cell function and frequency were negatively associated with both systemic inflammation and virus persistence (Ryan et al., 2016). Similar findings were observed in HIV patients receiving antiretroviral therapies. In these patients, persistent immune activation was still evident, despite effective viral suppression, and was associated with a low frequency of Tc17 cells exhibiting severely compromised IL-17A secretory ability (Perdomo-Celis et al., 2018). Intriguingly, in children suffering influenza infection, IL-17A orchestrates a protective immune response that is essential for lung homeostasis (Guo et al., 2018). In influenza infected mice,

IL-17A-producing $\gamma\delta$ T cells were not implicated in viral clearance or IFN- γ production but functioned as crucial immune regulators. IL-17A secreted by $\gamma\delta$ T cells stimulated IL-33 production by lung epithelial cells. In turn, IL-33 production further facilitated lung infiltration of ILC2s and Treg cells, both of which are important sources of amphiregulin that is necessary for tissue repair. Neonates lacking IL-17A-producing $\gamma\delta$ T cells or IL-33 production showed more serious morbidity and mortality. This response is of particular interest due to previously observed influenza-affected children profiles. In these children, IL-17A, IL-33, and amphiregulin expression were correlated and increased IL-17A levels in nasal aspirates were associated with better clinical outcomes (Guo et al., 2018). It is not surprising that IL-17A is involved in lung homeostasis during viral infection since it has long been recognized that IL-17A plays a central role in the maintenance of tissue integrity, repair, and regeneration. IL-17A may also provide a protective role against viral infection by maintaining tissues integrity. During chronic SIV or HIV infection, there is a substantial insult to the gut mucosa, characterized by a progressive depletion of CD4⁺ T cells and a concomitant breakdown of the epithelial barrier of the gastrointestinal tract (Pastor et al., 2017; Vidya Vijayan et al., 2017). These changes result in increased microbial translocation from the gastrointestinal tract, further contributing to the systemic dissemination of microbial constituents, pathological immune activation, and progressive disease development (Estes et al., 2010). In the SIV infection model, colonic epithelial barrier damage was associated with markedly reduced IL-17A production which is due to the loss of multiple lineages of IL-17A-producing lymphocytes (Klatt et al., 2012). The loss of IL-17A-producing lymphocytes is caused by the depletion of CD103⁺ DCs, which have been shown to both express genes favorable to IL-17A production and induce the differentiation of IL-17A and ROR γ t-expressing cells upon coculture with naive T cells *in vitro* (Klatt et al., 2012).

2.2.3 Detrimental role of IL-17A in viral infection

Despite the protective role of IL-17A in viral infection defense, it has also been associated with enhanced viral infections and viral infection-induced tissue pathology. IL-17A can antagonize antiviral Th1 and CTL responses that are essential for eliminating invading viruses. For example, in mice infected with Theiler's murine encephalomyelitis virus, IL-17A inhibits directly CTL response to promote viral infection (Hou et al., 2009, 2014). Following RSV or coxsackievirus infection, increased IL-17A level was found to be associated with impaired Th1 and virus-

specific CD8⁺ T cell responses and neutralization of IL-17A restored these antiviral responses (Mukherjee et al., 2011; Yuan et al., 2010). The suppression of antiviral responses mediated by IL-17A was attributed to the downregulation of IFN- γ , T-bet, eomesodermin, and granzyme B in CD8⁺ cytotoxic T cells (Hou et al., 2009, 2014). Theiler's murine encephalomyelitis virus-induced chronic demyelinating disease displays symptoms similar to those of progressive multiple sclerosis in humans. In this murine model, persistent viral infection was always accompanied by vigorous production of IL-17A. IL-17A prevented virus-induced apoptosis by inducing the expression of pro-survival proteins, such as Bcl-2 and Bcl-xL (Hou et al., 2009, 2014). This is consistent with the reported growth-promoting effect of IL-17A in other studies (Olsson Åkefeldt et al., 2013a; Prabhala et al., 2010). During viral infection, uncontrolled and excessive production of IL-17A is pathogenic and induces relevant tissue pathology. Increased IL-17A stimulates the production of chemokines (i.e. IL-8 and CXCL1) by epithelial cells that lead to the excessive accumulation of neutrophils, thereby generating a strong inflammatory response and thus leading to tissue damage. During RSV infection, it has been observed that increased IL-17A level is usually associated with more severe tissue pathology and higher mortality after infection (Ryzhakov et al., 2011). A significant increase in IL-17A level induced by human influenza infection could elicit acute lung injury (Li et al., 2012). Systemic infection of HSV in aged mice induced rapid increases of IL-17A in serum that further led to increased morbidity and mortality. In this model, the hepatic NKT cells are the main producer of IL-17A that stimulated the activation of hepatic neutrophils that subsequently contributed to hepatocyte necrosis and virus-induced death (Stout-Delgado et al., 2009). In Dengue virus-infected patients, serum IL-17A level was correlated with the severity of virus-induced illness (Jain et al., 2013). This finding was corroborated by an independent investigation, which confirmed that the increased levels of IL-17A, mostly produced by hepatic $\gamma\delta$ T cells, were accompanied by neutrophil accumulation in the liver that contributed to disease progression during dengue virus infection of mice (Guabiraba et al., 2013). Consistent with these findings, increased IL-17A-associated tissue damage has also been highlighted in patients with HIV infection. In these patients, the percentage of IL-17A-producing cells is significantly higher than in non-infected subjects, with IL-17A level positively correlating with markers of neutrophil activation, another typical feature of HIV pathology (Campillo-Gimenez et al., 2014). Intriguingly, IL-17A and Th17 responses were positively associated with the severity of the recent global pandemic coronavirus diseases-2019 (COVID-19) (Pacha et al., 2020; Wu and

Yang, 2020). COVID-19 was caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). COVID-19 patients developed acute respiratory distress syndrome (ARDS), which leads to pulmonary edema and lung failure and have liver, heart, and kidney damages. These symptoms were associated with a cytokine storm, manifesting elevated serum levels of a panel of pro-inflammatory cytokines and cytokines involved in Th17 responses (Huang et al., 2020). Therefore, targeting IL-17A may be a plausible strategy to control ARDS caused by SARS-CoV-2 (Pacha et al., 2020; Wu and Yang, 2020).

IL-17A has also been shown to promote fibrosis development in chronic HBV and hepatitis C virus (HCV) infection (Cachem et al., 2017; Cho et al., 2017; Yang et al., 2013). Fibrosis is a consequence of chronic inflammation or repeated injuries of a target tissue characterized by excessive deposition of extracellular matrix (ECM) proteins during wound healing and a typical feature of many chronic diseases (Jun and Lau, 2018). In HBV-infected patients, IL-23 production by DCs and macrophages stimulates the differentiation of naive CD4⁺ T cells into Th17 cells, the primary source of IL-17A in HBV-infected livers. The production of IL-17A activates hepatic stellate cells which are the major ECM producing cells in the liver (Wang et al., 2013b).

3. IL-17A in human immunopathology

3.1 IL-17A in neurodegenerative disorders

The pro-inflammatory activities of IL-17A are key in anti-microbial protection of the host, but uncontrolled and chronic IL-17A activity is associated with different immunopathological inflammations, autoimmune diseases, and cancer progression (Chen and Kolls, 2017). For example, IL-17A is involved in a various number of chronic neurological diseases including multiple sclerosis (MS), Alzheimer's disease (AD), and ischemic brain injury (Milovanovic et al., 2020). Multiple sclerosis is a chronic inflammatory disease of the central nervous system (CNS) caused by immune cells, which attack and destroy the myelin sheath that insulates neurons in the brain and spinal cord. The murine model of multiple sclerosis is the experimental autoimmune encephalomyelitis which can be induced by chronic infection of Theiler's murine encephalomyelitis virus. The inflammatory process in MS is initiated by the binding of PAMPs from pathogens or commensal bacteria and DAMPs from dead or dying cells to PRRs. This

leads to the activation of innate immune cells and production of IL-1, IL-6, IL-12, IL-18, and IL-23, cytokines that promote the differentiation and expansion of encephalitogenic Th1 and Th17 cells (McGinley et al., 2018). Although the precise mechanism and etiology of MS are unknown, cytokines play critical roles in the pathogenesis of MS in the processes of inducing oligodendrocyte cell death, neuronal dysfunction, and axonal degeneration. Th17 cells are considered to be one of the key effectors of MS. Increased expression of IL-17A- and Th17-associated transcripts have been demonstrated in MS plaques collected at autopsy (Lock et al., 2002). Likewise, IL-17A can induce a strong inflammatory response in the brain that may contribute to the development of AD and ischemic brain injury. AD is the most common neurodegenerative disorder causing cognitive impairment in old people. The histopathological hallmarks of AD are amyloid plaques in the brain, mainly consisting of fibrillary forms of amyloid β peptide (Gouras et al., 2015). Increased insoluble amyloid β peptide activates the complement pathway and stimulates microglia to produce the pro-inflammatory cytokines and chemokines and thus induce accumulation of inflammatory cells into the CNS. This pro-inflammatory process mediated by microglia leads to neurodegeneration (Wang et al., 2015b). Amyloid β peptides also increase the production of reactive nitrogen and oxygen species by microglial cells, leading to oxidative stress development, stimulation of Th17 cells, and IL-17A production. It appears that the main roles of IL-17A in AD pathogenesis are the attraction of neutrophils and the stimulation of their function. It has been shown that A β aggregates mediate the chemotaxis and the recruitment of neutrophils in the CNS of mice overexpressing human mutant amyloid precursor protein, which produce IL-17A and thus amplify neutrophil migration into the CNS (Zenaro et al., 2015). Neutrophils are the main targets of IL-17A in the CNS but are also important sources of this cytokine. By promoting inflammation and CNS tissue damage, neutrophils could have an important role in the development of AD pathology. Results from *in vitro* experiments indicate that IL-17A might promote autophagy in neurons and thus induce neurodegeneration (Wang et al., 2019). Similarly, in brain ischemia, damaged CNS tissue release DAMPS that stimulate resident innate immune cells and microglia in the CNS (Shekhar et al., 2018). These cells have both beneficial and detrimental roles. They play a beneficial role by phagocytosis of damaged tissue but also release inflammatory mediators TNF- α , IL-1 β , IL-6, and IL-17A, which enhance inflammation and tissue damage (Kigerl et al., 2014).

3.2 IL-17A in other inflammatory diseases

It has been long recognized that IL-17A is involved in a number of autoimmune diseases. For instance, one autoimmune disease tightly associated with Th17 cells is rheumatoid arthritis (RA), a chronic disorder characterized by long-term joint inflammation, auto-antibody production, which lead to the destruction of cartilage and bone (McInnes and Schett, 2011). The level of IL-17A in the synovium correlates with tissue damage (Kirkham et al., 2006). Rheumatoid synovium is characterized by hyperplasia, neo-angiogenesis, and excessive inflammation. IL-17A mediates cartilage and bone destruction that occurs in RA via activation of synoviocytes and osteoblasts. *In vitro* experiments show that IL-17A induces synoviocyte activation, migration, and invasion that promote cartilage destruction. Through the induction of matrix metalloproteinase (MMP), IL-17A induces matrix destruction. In addition, IL-17A promotes receptor activator of NF- κ B ligand (RANKL) expression on osteoblasts that bind to RANK, activates osteoclastogenesis, and finally triggers bone erosion by osteoclasts (Robert and Miossec, 2019). Systemic lupus erythematosus, commonly referred to as lupus, is a complex autoimmune disorder that affects the skin, joints, kidneys, and brain. Although the physio-pathogenesis of lupus is not fully understood, it has been reported that IL-17A and Th17 cells are involved (Garrett-Sinha et al., 2008). Serum IL-17A levels in lupus patients are higher than in healthy population (Shen et al., 2019). IL-17A and IL-17A producing cells were also detected in kidney tissue and skin biopsies from lupus patients (Crispín et al., 2008; Oh et al., 2011; Yang et al., 2009). Th17 cells and IL-17A have also been linked to inflammatory bowel diseases (IBD), such as Crohn's disease and ulcerative colitis although the exact role of IL-17A in the pathogenesis of these diseases is elusive. In the healthy intestine, IL-17A producing cells are abundantly present. The commensal microflora stimulates the expression of IL-17A that acts on local epithelium to promote anti-microbial responses that are necessary and sufficient to maintain a homeostatic balance without causing overt inflammation (Ivanov et al., 2009; Kumar et al., 2016). In IBD patients, Th17 cells infiltrate massively to the inflamed tissue and both *in vitro* and *in vivo* studies have shown that Th17-related cytokines may initiate and amplify multiple pro-inflammatory pathways (Monteleone et al., 2012).

Another autoimmune disease strongly associated with IL-17A is psoriasis. Psoriasis is an auto-inflammatory skin disease characterized by excessive growth and aberrant differentiation of keratinocytes that lead to circumscribed, crimson red, silver-scaled, plaque-like inflammatory

lesions. Numerous types of immune cells can be found in these lesions, including IL-17A producing cells, infiltrating CD4⁺ T cells and dendritic cells in the dermis as well as cytotoxic T cells and neutrophils in the epidermis. These immune cells trigger rapid keratinocyte proliferation, abnormal keratinocyte differentiation, and dermal angiogenesis (Lowe et al., 2007). In normal skin, IL-17A is constantly induced by commensal bacteria such as *Staphylococcus epidermidis*. Skin-localized IL-17A is produced by non-classical MHC Ib-activated microbiota-responsive Tc17 cells without causing overt inflammation. Commensal-induced IL-17A, in turn, regulates microbiota and protects from other infectious agents (Linehan et al., 2018; Naik et al., 2015). As well as mediating microbial protection at barrier surfaces, IL-17A also contributes to tissue repair after injury. When skin injury breaches the epithelial barrier, IL-17A promotes epithelial-cell proliferation. In addition, Tc17 produces tissue repair and immunoregulatory molecules (e.g., amphiregulin, VEGF, IL-10) to promote wound healing. Skin healing events are also mediated through IL-17A induction of anti-microbial protein regenerating family member 3 alpha (REG3A) in keratinocytes to promote their proliferation after injury (Lai et al., 2012). If pathogens become invasive, epithelial injury leads to the production of pro-inflammatory cytokines to activate and expand IL-17A-producing Th17, Tc17, and $\gamma\delta$ T cells. In synergy with TNF, IL-17A induces enhanced production of chemokines to recruit neutrophils and antimicrobial peptides to combat the infection. The chronic pro-inflammatory and pro-proliferative roles of IL-17A likely contribute to pathogenic hyper-proliferation of keratinocytes and skin inflammation that finally lead to psoriasis or even skin cancer (Chen et al., 2019; Ha et al., 2014). In fact, FDA-approved anti-IL-17A drugs are currently used for clinical treatment of psoriasis, which might be the most compelling evidence of IL-17A as a causal driver of chronic inflammation (Beringer et al., 2016).

IL-17A is also strongly linked with a rare inflammatory neoplasm called Langerhans cells histiocytosis (LCH). LCH is a rare cancer of unknown etiology involving the clonal proliferation of Langerhans cells and formation of chronic granuloma consisting of pale histiocytic cells with unfolded nuclei, eosinophils, and multinucleated giant cells (MGC). Clinical manifestations range from isolated organ lesions, such as skin or bone lesions to multisystem disease. Although the cause of LCH is unclear, IL-17A plays a critical role in the pathogenesis of this disease (Delprat and Aricò, 2014). In LCH patients' serum, high levels of IL-17A have been detected, which is associated with LCH disease activity. The serum IL-17A in LCH patients is

mainly produced by monocytes and monocyte-derived DCs present in peripheral blood (Lourda et al., 2014). In addition, in LCH skin and bone lesions, DCs express a large amount of IL-17A that in turn act autocrine on these cells, leading to the fusion of DCs and thus contributing to the formation of MGC and granuloma (Coury et al., 2008b). The accumulation of pathogenic DCs is one important characteristic in LCH and is attributed to the abnormal proliferation and extended life span of these DCs. It has been demonstrated that the proliferation and the long-term survival of LCH DCs are partly regulated by IL-17A signaling. DCs survival is basically regulated by the exogenous concentration of GM-CSF on CSF2R. Low GM-CSF concentration stimulates the selective activation of Ser585/14-3-3/PI-3 kinase (PI3K), the “survival only” pathway, and high GM-CSF concentration can give rise to assembling of CSF2R chains in dodecamer, thus involving the Jak/STAT, MAPK, and PI3K pathways (Hercus et al., 2009). In the presence of IL-17A, the survival of monocyte-derived DCs can be prolonged by weeks via the activation of TRAF6-dependent IL-17AR transduction that activates both PI3K/Akt and NF- κ B pathways (Olsson Åkefeldt et al., 2013b). In LCH patients, although the low concentration of GM-CSF will normally induce PI3K/Akt, other molecular specificities turn DC survival and phenotype into aggressive myeloid cells: the presence of IL-17A activates both PI3K/Akt and NF- κ B pathways, leading to increased survival, inflammation, and DC fusion (Coury et al., 2008b). The pathogenesis of LCH is currently unknown. Since LCH displays an excess of spontaneous chromosomal breaks that resemble the genomic instability induced by several viruses (Kamranvar et al., 2007; Scappaticci et al., 2000), such as RSV, HCV, and EBV, a viral etiology of this disease has also been suspected. Interestingly, Murakami and colleagues have found MCPyV DNA sequences in peripheral blood and tissues from LCH patients with high-risk organ involvement, suggesting that MCPyV infection may be involved in LCH development (Murakami et al., 2014b).

4. IL-17A in cancer immunology

Similar to its role in host defense, IL-17A is a double-edged sword in controlling cancers. Tumor-infiltrating Th17 cells, as well as innate IL-17A producing cells, have been observed in both mouse and human cancers, including melanoma, breast, colon, hepatocellular, ovarian, pancreatic, prostate, and renal tumors (Zou and Restifo, 2010). Besides IL-17A producing cells from the immune system, other non-immune cells, including endothelial cells, stromal cells as well as cancer cells can also be the sources of IL-17A in the TME. Whether IL-17A plays

beneficial or detrimental roles in the development and progression of cancer is context-dependent and multifactorial. The wide diversity, the plasticity of the IL-17A producing cells in the TME, and the pleiotropic nature of IL-17A, along with other related cytokines (i.e. IL-1 β , IL-22, IL-23, TNF- α , IFN- γ) that are often secreted in conjunction with IL-17A, can bring out drastically different outcomes in a complex and dynamic disease such as cancer (Guéry and Hugues, 2015).

4.1 Protumoral role of IL-17A

The apparent evidence of the pro-tumorigenic role of IL-17A is its growth-promoting and survival effect. IL-17A signaling promotes the early stages of cancer cell growth via the activation of the proliferation pathway. In addition, the survival pathway with anti-apoptotic proteins such as Akt, mTOR, Bcl-2, and Bax is also stimulated in a number of cancers (Yang et al., 2014). For example, IL-17A stimulates the proliferation and self-renewal of ovarian cancer stem cells in a dose-dependent fashion via the NF- κ B and MAPK pathways (Xiang et al., 2015). IL-17A promotes tumor growth through the IL-6/Stat3 pathway in a model of melanoma and bladder cancer (Wang et al., 2009). IL-17A has been shown to directly prompt colonic tumorigenesis by activating the proliferation of transformed enterocytes via NF- κ B and MAPK signaling (Wang et al., 2014). It has been also demonstrated that IL-17A induces angiogenesis, one of the cancer hallmarks that influence the tumor grade and metastasis. IL-17A is often correlated with high vascular density and VEGF production within tumors. It has been shown that IL-17A induces the expression of VEGF and other angiogenic factors by tumors cells and fibroblasts (Liu et al., 2011a; Numasaki et al., 2003; Pan et al., 2015). In humans, IL-17A has been associated with increased angiogenesis in gastric, colorectal, hepatocellular, breast, lung, and pancreatic tumors (He et al., 2011; Iida et al., 2011; Liu et al., 2011a; Ma et al., 2017; Zhang et al., 2009; Zhu et al., 2008). In patients with gastric cancer, high level of tumor-infiltrating Th17 cells and IL-17A are associated with increased lymph-vascular invasion (Iida et al., 2011). Increased expression of VEGF correlating with IL-17A production was found to be an independent factor worsening the prognosis of patients with colorectal cancer (Liu et al., 2011a). IL-17A is also capable to aggravate certain types of cancer by enhancing metastasis. For instance, IL-17A promotes hepatocellular carcinoma (HCC) metastasis by fostering cell motility through the upregulation of MMP2 and MMP9 expression via the NF- κ B pathway (Li et al., 2011). Likewise, IL-17A promotes prostate cancer via MMP7-induced epithelial-to-

mesenchymal transition (Zhang et al., 2017). IL-17A can also enhance tumor progression by suppressing the anti-tumor immune response in the TME. In mouse models, IL-17A promotes tumor development through the induction of MDSCs. Administration of IL-17A inhibits CD8⁺ cell infiltration and increases MDSCs in tumors. And IL-17A is required for the development and tumor-promoting activity of MDSCs in tumor-bearing mice (He et al., 2010). Th17 cells and IL-17A induce G-CSF expression in tumor-associated fibroblast through NF- κ B and ERK signaling, leading to the mobilization and recruitment of MDSCs into the TME that promote tumor resistance to anti-angiogenic therapy (Chung et al., 2013). In recent years, the relationship between IL-17A and immune checkpoint has been also explored. It has been reported that IL-17A alone or with other cytokines, such as TNF- α , may contribute to T cell exhaustion and tumorigenesis by upregulating PD-L1 expression in numerous cancers, including liver, breast, prostate and colon cancer (Ma et al., 2017; Wang et al., 2017; Wei et al., 2019).

4.2 Antitumor effects of IL-17A

While the substantial evidence supporting the pro-tumor role of IL-17A, conflicting results showing antitumor roles have been also reported. In ovarian cancer patients, tumor-infiltrating Th17 cells exhibit a polyfunctional effector T-cell phenotype, are positively associated with effector cells and are negatively associated with tumor-infiltrating Tregs. Through synergistic action between IL-17A and IFN- γ , Th17 cells stimulate CXCL9 and CXCL10 production to recruit effector T cells to the TME (Kryczek et al., 2009). Transfer of *in vitro* polarized Th17 cells induced established tumor regression or reduced number of tumor foci in B16 melanoma model (Ankathatti Munegowda et al., 2011; Martin-Orozco et al., 2009). It has been also reported that tumor-infiltrating Th17 cells induced CCL20 production, promoting DC recruitment within the tumor and subsequent migration to draining lymph nodes of tumor material containing DCs, leading to potential activation of CD8⁺ T cells (Martin-Orozco et al., 2009).

RESEARCH PROJECTS

1. MCPyV and IL-17A inflammation

MCPyV is the etiologic pathogen that causes 80% MCC, a rare aggressive skin cancer affecting elderly and immunosuppressive patients. Functional viral proteins encoded by the early region are key components for MCC carcinogenesis. IL-17A is a proinflammatory cytokine with pleiotropic effects playing crucial roles in skin and mucosal defense, tissue homeostasis, as well as cancers. The first objective of my thesis consists of studying the role of MCPyV early region in the induction of IL-17A. As MCPyV *in vitro* culture is still a challenge to date, we addressed this question by transfection with plasmids expressing different viral proteins from the early region. The first part of my work consists of generating plasmids expressing the full-length early region, the small T antigen, the large T antigen, the 57kT, and the truncated LT, using the Gateway cloning technology. Then we studied the IL-17A expression profile in cells transfected with the above-mentioned plasmids using intracellular staining and flow cytometry.

2. Crosstalk between IL-17A-mediated inflammation and autophagy

Autophagy is an evolutionarily conserved process in eukaryotic cells that plays an essential role in macromolecules recycling and cellular homeostasis. It participates in both innate and adaptive immune responses against foreign pathogens and is involved in multiple pathologies, such as cancer. This part of my thesis aimed to study the interplay between IL-17A-dependent inflammation and autophagy in the context of MCPyV infection. We first studied the role of different viral proteins in the regulation of autophagy. Using an IL-17A receptors-expressing cell line, we investigated the IL-17A-induced inflammation in the regulation of autophagy. Reciprocally, we studied the impact of autophagy activity on IL-17A-dependent inflammation by modulating autophagy with either autophagy activator or inhibitor.

RESULTS

Article 1

Variable IL-17A induction by onco-type versus wild-type Merkel cell Polyomavirus

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Variable IL-17A induction by onco-type versus wild-type Merkel cell Polyomavirus

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KEYWORDS: Merkel cell carcinoma, MCPyV early region, IL-17A, inflammation, host-pathogen interactions, oncovirus

ABSTRACT

Merkel cell carcinoma (MCC) is a rare aggressive skin malignancy characterized in aged or immunosuppressed patients. MCPyV is the causative agent associated to 80% of MCC. The C-terminal truncation of the MCPyV large T antigen (LT) gives rise to onco-type large T antigens (otLT), which is a hallmark of cancer cells in MCPyV+ MCC. In this study, we built five plasmids encoding length early region sequence (ER), and four sub-products: small T antigen (sT), 57kT protein (57kT), wild-type LT (wtLT) and otLT. We transfected HEK293T cells with these plasmids to study IL-17A induction by MCPyV ER products. Transfection efficiency and IL-17A induction were assessed by western-blot and flow cytometry analysis. This is the first demonstration that ER products highly induce IL-17A expression. sT alone did not induce IL-17A contrary to 57kT, wtLT and otLT. Interestingly, C-terminal truncation of LT decreased IL-17A induction, compared to ER or to wtLT. This study suggests that, in addition to its role in

cell immortalization, integration of otLT may be involved in the transition from a high-grade acute to a low-grade chronic IL-17A-mediated inflammation in the skin.

ABBREVIATIONS

ER, early region; MCC, Merkel cell carcinoma; MCPyV, Merkel cell Polyomavirus; NLS, nuclear localization signal; OBD, origin binding domain; otLT, onco-type form of LT; pX, plasmid encoding the sequence of X; sT, small T antigen; wtLT, wild-type large T antigen.

INTRODUCTION

Merkel cell carcinoma (MCC) is an aggressive neuroendocrine malignancy of the skin that primarily affects immunocompromised individuals and the elderly, increasing with the population aging (1). With an incidence in the US of 0.2-0.45 case per 100,000 per year, MCC is classified in rare cancers. However, due to its 5-year relative survival rate, which is 18%, MCC is the second leading cause of skin-cancer death after melanoma (2–4). Based on the etiology and mutational landscape, MCC is categorized into two subtypes. A minority of MCC harbors a high rate of mutations compatible with long-term UV-exposure (5,6), while 80% of MCC were triggered by the latest discovered human oncovirus, named Merkel cell Polyomavirus (MCPyV) or human Polyomavirus 5 (HPyV5) (7–9).

MCPyV is an opportunistic oncovirus discovered as clonally integrated into the genome of MCC cells (7). MCPyV is considered as part of the skin microflora that persistently and commonly infects the human population (10–12). MCPyV DNA genome is divided into early region (ER) and late region encoding structural proteins of the viral capsid (7). ER gives rise to several functional proteins, including two tumor antigens, involved in viral replication and oncogenic activities: the large T antigen (LT) and the small T antigen (sT) (13–15). ER also encodes 57kT in the same reading frame (16). The LT protein contains five domains: an N-terminal DnaJ domain, an LXCXE domain binding the retinoblastoma protein, a nuclear localization signal sequence (NLS), an origin binding domain (OBD) and a C-terminal Helicase/ATPase domain, required for viral replication (14,17). LXCXE inhibits the tumor suppression function of the retinoblastoma protein, thus promoting cell growth (18). In viral-induced MCC, the C-terminal helicase domain of LT is always either truncated or deleted or mutated, resulting in an onco-type form of LT (otLT) (19). The loss of functional helicase domain prevents viral replication and enhances the growth-promoting activity of LT (15,20,21). An alternatively spliced form of the LT sequence includes the three domains DnaJ, LXCXE and NLS to encode the 57kT protein, which is not expressed in most MCC tumor cells. The function of 57kT remains elusive, although it has growth-promoting activity (19,20). Unlike otLT, the sT sequence is intact in MCC, with a DnaJ domain and a single LT-stabilization domain (LSD) at the C-terminus. The sT protein displays a strong oncogenic potential. Alone, sT can induce cell transformation and MCC-like tumor in transgenic mice model (22–24). LSD inhibits SCF^{Fbw7} E3 ligase, thus protecting its substrates from protease degradation, encompassing LT and cell cycle regulators (25). In parallel, sT activates cell proliferation by keeping the hyperphosphorylation of the eukaryotic translation initiation factor 4E-binding protein 1 (26) and contributes to transformation through the activation of EP400, a histone

acetyltransferase complex (27). Moreover, sT enhances cell mobility (28–30) and induces cell dissociation (31), thus promoting MCC metastasis. Consequently, otLT and sT together play crucial roles in MCC development and progression.

A prominent cytokine of the skin, the developmental site of MCC, is IL-17A (32). IL-17A is a pro-inflammatory cytokine involved in skin host defense, but is also characterized in chronic inflammations, autoimmune diseases, and some cancers. The primary function of IL-17A in healthy individuals is to control the homeostasis of the skin and mucosal barriers and to protect them from pathogen insults (32). The induction of IL-17A and IL-17A-mediated response upon viral infections is well documented, albeit the outcomes can be both beneficial or detrimental, depending on the context (33). IL-17A-mediated chronic inflammation can promote cancer via its growth-promoting, tissue remodeling, angiogenic, and immune suppressive effects (34). The role of IL-17A in MCPyV infection has not yet been investigated.

In the present study, we show that the full-length ER induces high level of IL-17A, similar to that induced by LT (or wild-type LT or wtLT) alone. Compared to ER or wtLT, otLT induces less IL-17A. Overall, our work demonstrates a novel role of MCPyV LT in regulating the level of IL-17A inflammation, which opens new understanding of MCC pathogenesis.

RESULTS

Constructs from oncovirus MCPyV genome to express wild-type or truncated early region products

In order to investigate the functions of MCPyV ER and its encoded products, different constructs were generated using the Gateway[®] system. First, we cloned the full-length wild-type ER sequence to generate the ER-expressing plasmid (pER). A Flag tag was added to the 5' of the ER sequence to allow the subsequent detection of ER-encoded proteins (Figure 1A). The sequence of pER construct was confirmed by PCR (Figure 1B) and sequencing (data not shown). We then cultured HEK293T cells non-transfected or transfected with the control plasmid or pER to validate the pER construct. Transfection efficiency, assessed by flow cytometry after anti-Flag staining, raised up to 63% (Figure 1C). Using the anti-MCPyV LT antibody, we checked the expression of both wtLT and 57kT (Figure 1D, left). This antibody recognizes a peptide of the LXCXE domain in the LT or 57kT exon 2, with no cross-reaction on sT protein. Using the anti-MCPyV sT antibody, we verified the expression of sT (Figure 1D, right). The anti-MCPyV sT antibody recognizes a peptide of the LSD domain only present in the C-terminal of sT. Western-blot analysis showed no expressions of wtLT, 57kT and sT in

non-transfected cells or in cells transfected with the control plasmid while wtLT, 57kT and sT were expressed in pER-transfected cells. These data validate the functionality of pER construct to express its encoded products.

To further investigate the role of ER-encoded antigens from wild-type MCPyV versus the role of otLT in the induction of IL-17A, cDNA sequences of either sT or wtLT or 57kT versus otLT were cloned to generate the related expression plasmids using the Gateway[®] system (Figure 1E). We used the truncated LT sequence of WaGa cells to construct the otLT plasmid of this study. WaGa cell line is a MCPyV⁺ MCC cell line, which express a truncated onco-type form of LT. The truncation eliminates the 3' part of the NLS motif, OBD and helicase/ATPase domains. Different tags were added to the sequences of sT, wtLT, 57kT and otLT to allow their detection: V5/His tag to the 3' downstream of sT and Flag tag to the 5' upstream of wtLT, 57kT and otLT. We then cultured HEK293T cells non-transfected or transfected with control or psT, pwtLT, p57kT versus potLT plasmids. Using anti-V5 antibody, we detected the expression of sT by western-blot in psT-transfected cells, but not in non-transfected cells or cells transfected with the control plasmid (Figure 1F, left). Western-blotting with the anti-MCPyV LT antibody also confirmed the expression of wtLT, 57kT and otLT in transfected cells (Figure 1F, right). Together, these results validate the constructs used for subsequent investigations.

High IL-17A induction by MCPyV early region

We investigated whether MCPyV ER induces IL-17A expression. We cultured HEK293T cells non-transfected or transfected with the control or pER plasmids. We used intracellular staining and flow cytometry analysis to assess the transfection efficiency with anti-Flag antibody and the IL-17A production with anti-IL-17A antibody (Figure 2A). At 24h post-transfection, non-transfected cells or cells transfected with the control plasmid did not express IL-17A. In the same conditions, the transfection efficiency with pER raised up to 64%, as assessed by Flag staining. Surprisingly, 47% of the cells transfected with pER expressed IL-17A. Below, double staining allowed to study if the pER-transfected cells express IL-17A. Flag-negative cells expressed less than 1% of IL-17A. The double positive cells (IL-17A⁺ Flag⁺) represented 41% of the total viable cells. In the pER-transfected cells (Flag⁺), about 20% expressed no or low IL-17A. Thus, MCPyV ER products induced high IL-17A expression in 60% of the transfected cells. Dose-response (Figure 2B) and kinetic (Figure 2C) studies were performed to confirm the results. The cells were non-transfected or transfected with four doses of the control or pER plasmids and cultured for 24h. The transfection efficiency ranged from 55 to 70% (Figure 2B, top). The two low doses (0.25 and 0.5 μ g) of pER induced 3-32% of IL-17A-expressing cells

while the two highest doses (1 and 1.5 μg) induced 39-50% of IL-17A-expressing cells (Figure 2B, middle). The double positive cells (IL-17A⁺ Flag⁺) dose-dependently increased from 3-43% along the four doses of pER used (Figure 2B, down). Concerning the kinetic studies, the Flag intracellular staining, measured at 24h, demonstrated pER efficient transfection in about 70% of the cells (Figure 2C, top). IL-17A intracellular staining, performed at four different time-points from 0 to 36h, showed an increased IL-17A expression over time with an optimum at 24h post-transfection (Figure 2C, middle). Consistently, the double positive cells (IL-17A⁺ Flag⁺) increased from 0-45% over time after transfection (Figure 2C, down). Thus, dose-response and kinetic studies strengthened the conclusion that MCPyV ER products induce high level of IL-17A in HEK293T cells. To our knowledge, this is the first demonstration that MCPyV ER products are able to induce IL-17A.

No IL-17A induction by small T antigen

Given that the full length ER encodes several proteins, the next step was to address which viral protein(s) are responsible for IL-17A induction. We first examined whether sT is able to induce IL-17A. HEK293T cells were either non-transfected or transfected with control or psT plasmids. We checked the expression of sT at transcriptional level by RT-PCR 24h post-transfection (Figure 3A). The sT mRNA was detected in psT-transfected cells, but neither in non-transfected cells nor in cells transfected with the control plasmid. We then confirmed the expression of sT by western-blot (Figure 3B). sT protein was detected either using anti-V5 antibody or anti-MCPyV sT antibody. The first antibody is specific of the tag present in sT construct but not in WaGa cells. Anti-V5 antibody detected sT protein solely in psT-transfected cells (Figure 3B, left) while anti-MCPyV sT antibody revealed sT protein expression also in WaGa cells, as expected (Figure 3B, right). The molecular weight of the tagged-sT protein is slightly higher than native sT as observed on the western-blot. Together, these data confirmed sT expression in psT-transfected cells. In order to investigate whether sT induces IL-17A, cells were either non-transfected or transfected with control or psT plasmids. Harvested cells were subjected to intracellular staining with anti-IL-17A antibody at indicated time-points. Flow cytometry analysis showed no IL-17A induction in any cell culture conditions (Figure 3C). Increased amounts of psT to perform the cell transfection did not change the previous result. psT-transfected cells did not express IL-17A regardless the quantity of psT used for the transfection (Figure 3D). Collectively, these results demonstrate that the MCPyV sT antigen alone does not induce IL-17A.

Low IL-17A induction by 57kT

The role of MCPyV 57kT in the carcinogenesis of MCC is not yet understood, if any. Here, we investigated whether 57kT induces IL-17A. As a reminder, ER region encodes LT and 57kT in the same reading frame thanks to an alternative splicing. HEK293T cells were non-transfected or transfected with either control or pER or p57kT plasmids. After 24h of culture, we used intracellular staining and flow cytometry analysis to assess the transfection efficiency with anti-Flag antibody and the IL-17A production with anti-IL-17A antibody (Figure 4). No IL-17A was detected in either non-transfected cells or cells transfected with the control plasmid. Representative experiment shows that the transfection efficiency was quite similar with pER (33%) or p57kT (32%) (Figure 4A, top), while IL-17A was highly induced with pER (25%) compared to p57kT (14%) (Figure 4A, down). The expression of 57kT after transfection was confirmed by western-blot analysis as in Figure 1F (data not shown). Repeated experiments consolidated that despite a similar transfection efficiency (Figure 4B, top), the IL-17A induction is significantly reproducibly twice lower in p57kT-transfected cells than in pER-transfected cells (Figure 4B, down).

To our knowledge, this is the first time a function can be attributed to the 57kT protein of MCPyV. Although 57kT is a weaker inducer of IL-17A, it can contribute to the total IL-17A induction previously observed in pER-transfected cells.

High IL-17A induction by wild-type LT

The most studied MCPyV protein is LT with established functions in viral replication, oncogenesis, and disruption of TLR9-mediated viral detection. We investigated whether wtLT induces IL-17A by comparing intracellular IL-17A expression in pER- versus pwtLT-transfected cells. HEK293T cells were non-transfected or transfected with either control or pER or pwtLT plasmids. After 24h of culture, we used intracellular staining and flow cytometry analysis to assess the transfection efficiency with anti-Flag antibody and the IL-17A production with anti-IL-17A antibody (Figure 5). No IL-17A was detected in either non-transfected cells or cells transfected with the control plasmid. Representative experiment shows that the transfection efficiency with pER was slightly lower (31%) than with pwtLT (39%) (Figure 5A, top). Similarly, IL-17A expression was slightly lower with pER (21%) compared to pwtLT (25%) (Figure 5A, down). The expression of wtLT after transfection was confirmed by western-blot analysis as in Figure 1F (data not shown). Repeated experiments consolidated that the difference between the two conditions was not significant: both transfection efficiency and IL-

IL-17A induction were similar after pER- or pwtLT- transfection (Figure 5B). Thus, wtLT and ER sequences induce high and comparable IL-17A expression.

Truncation of LT decreases IL-17A induction by onco-type MCPyV

The C-terminal truncation of LT was characterized as a hallmark for the MCPyV+ MCC, giving rise to the presence of onco-type LT (otLT) in cancer cells. otLT contributes to the development of MCC due to its growth-promoting and transforming activities. Cancer promotion by low-grade chronic inflammation has been established, but otLT role in inflammation has not yet been studied. It is therefore important to investigate whether otLT regulates IL-17A induction. We investigated whether otLT induces IL-17A by comparing intracellular IL-17A expression in pER- versus potLT- transfected cells. HEK293T cells were non-transfected or transfected with control or pER or potLT plasmids. After 24h of culture, we used intracellular staining and flow cytometry analysis to assess the transfection efficiency with anti-Flag antibody and the IL-17A production with anti-IL-17A antibody. No IL-17A was detected in either non-transfected cells or cells transfected with the control plasmid. Representative experiment shows that the transfection efficiency with pER (39%) was slightly lower than with potLT (44%) (Figure 6A, top). Conversely, IL-17A expression was much higher with pER (23%) than with potLT (9%) (Figure 6A, down). The expression of otLT after transfection was confirmed by western-blot analysis as in Figure 1F (data not shown). Repeated experiments consolidated that the transfection efficiency showed no significant difference between the two conditions (Figure 6B, top), whereas potLT showed a reproducible reduced ability to induce IL-17A expression compared to pER (25%) (Figure 6B, down).

Finally, we directly compared the IL-17A expression level induced by wtLT or otLT with the same techniques. HEK293T cells were non-transfected or transfected with control or pwtLT or potLT plasmids. After 24h of culture, no IL-17A was detected in either non-transfected cells or cells transfected with the control plasmid. Representative then repeated experiments showed that the transfection efficiency with pwtLT (50%) or potLT (48%) were close and not significantly different (Figure 6C-D, top). However, IL-17A expression was reproducibly significantly higher after transfection with pwtLT (26%) compared to potLT (13%) (Figure 6C-D, down). These data demonstrate that the C-terminal truncation of LT decreases IL-17A induction.

DISCUSSION

Despite recent advances in cancer immunotherapy, MCC is the second most lethal skin malignancy affecting immunocompromised individuals and the elderly. MCPyV, the only Polyomavirus known to induce cancer in humans, is the causative agent of most MCC (7). The oncogenic and metastatic activities of MCPyV T antigens are reported in numerous studies investigating the role of MCPyV in the MCC carcinogenesis and progression. Immunosurveillance plays a critical role in initiating antiviral and anti-tumor immune responses to kill infected cells and cancer cells. However, the functions of MCPyV antigens in host-oncopathogen interactions remain poorly understood.

A common strategy used by oncoviruses to set up chronic infections and promote cancer relies on the immune evasion activities of their viral proteins, such as impairment of infected-cell recognition by danger receptors. As previously demonstrated for human Papillomavirus (HPV) and Epstein-Barr virus (EBV) (35–37), MCPyV sT and wtLT limit recognition of MCPyV-infected cells by the innate immune system (38). MCPyV sT and wtLT repress Toll-like receptor 9 (TLR9) expression thanks to the inhibition of the CCAAT/enhancer-binding protein (C/EBP), a transactivator that binds to TLR9 promoter to activate its expression (38). In addition, as it occurs with HPV E7, adenovirus E1A and polyomavirus SV40 LT proteins (39), the LXCXE motif of MCPyV LT may bind STING to antagonize cGAS-STING pathway thus preventing viral DNA detection by innate immunity receptors.

Herein, we reveal a novel function of MCPyV LT in the positive regulation of IL-17A, a pro-inflammatory cytokine with pleiotropic effects involved in both innate and adaptive immunity to fight against certain infections or cancers (32). The relative high-grade IL-17A inflammation we observed upon wild-type MCPyV ER challenge may be reminiscent of an acute and intense IL-17A response, possibly protective against acute MCPyV replication, *in vivo*. As part of the commensal microbiota shed from the human skin (12), MCPyV may concomitantly participate in immune system stimulation to support skin homeostasis since IL-17A is known to be protective against opportunistic pathogens of the skin and mucosal surfaces, such as *Candida albicans* and *Staphylococcus aureus* (40–42).

Compared to MCPyV ER and wtLT, we observed no and lower inductions of IL-17A by sT and otLT, respectively. We therefore propose that the truncation or deletion of the C-term LT may install a chronic low-grade and pro-tumoral IL-17A inflammation. Previous observations are supporting this hypothesis (43): (i) chronic IL-17A production upon repeated tissue repair instigates skin cancer by triggering the proliferation of premalignant cells (44,45). (ii) Persistent dysbiosis installs chronic IL-17A expression from $\gamma\delta$ T cells then promoting lung

adenocarcinoma development (46). In the context of MCC tumors, we should first look for IL-17A to confirm the sustainable presence of low and chronic IL-17A production. The potential cell source(s) of IL-17A in MCC lesion should be identified, thanks to immunohistofluorescence studies. It may be either the cancer cells whose origin is still a matter of debate (47) or monocytes, which were demonstrated to be a cell reservoir for MCPyV (48). Whether a shift in high-grade to low-grade IL-17A-dependent inflammation may participate in the MCC oncogenesis remains to be addressed, *in vivo*.

We also observed a weak induction of IL-17A with 57kT alone, further confirming that the intact C-term of LT is required for a high induction of IL-17A. Future molecular studies such as site-directed mutagenesis and interactome studies are required to reveal the specific LT sequence involved in cell-host interaction, leading to decreased induction of IL-17A.

MCPyV sT alone has no effect on IL-17A induction. However, sT targets NF- κ B essential modulator NEMO complex and inhibits I κ B phosphorylation, thus limiting NF- κ B translocation and related inflammatory cascade (49). sT expression resulted in a marked downregulation of genes associated with innate immunity, such as CCL20, CXCL-9, IL-8 and TANK (50). These results highlight that, wild-type MCPyV antigens limit viral DNA detection by TLR9 and NF- κ B-related inflammation in infected cells, as a function of sT, while efficiently stimulating high-grade IL-17A inflammation, as a function of LT, contributing to viral clearance by the immune system. Onco-type MCPyV antigens decrease immunogenicity by preventing MCPyV replication and may shift to a low-grade IL-17A inflammation, according to our results. Overall, our data complement previous studies to understand better, how the immune response is stimulated by the wild-type ER while limited by the onco-type ER, thus leading to the persistence of oncogenic and poorly immunogenic viral sequences in MCC.

A low-grade chronic production of IL-17A may participate in MCC development according to its previously described pro-tumoral activities: matrix metalloproteinase (MMP) upregulation promoting cell mobility and metastasis formation (51,52), myeloid-derived suppressor cells recruitment (53–55) and PD-L1 immune checkpoint upregulation limiting the anti-tumor response (56–58). In MCC, enhancement of cell migration is to date attributed to sT, which induces cytoskeleton turnover and cell-cell junction disruption; the contribution of otLT via IL-17A induction should be addressed. In MCPyV+ MCC, PD-L1 expression is higher than in MCPyV- MCC and blocking the interaction between PD-L1 and PD-1 is helpful to overcome tolerance of cancer cell (59). First-line treatment with avelumab (anti-PD-L1) led to responses in 40% and durable responses in 30%, and was associated with a low rate of grade 3/4 treatment-related adverse events (60). It is possible that, by inducing low-grade chronic IL-17A, otLT

dampens the anti-tumoral response via immune suppressive effects, leading to advanced MCC, rapidly metastasizing and deadly, due to impaired immunosurveillance.

Of note, our results are obtained from the *in vitro* expression of MCPyV genes, which is far from physiological conditions. To investigate whether IL-17A is indeed involved in MCPyV+ MCC, studies on serum and biopsies from MCC patients are further needed.

In summary, our study unveils a new role of MCPyV T-antigens in the induction of IL-17A, which may have important biological significance in MCC pathogenesis and progression. From the immunological aspect, our findings suggest a novel function of MCPyV T-antigens in promoting MCC.

METHODS

Antibodies

Anti-Flag® M2-FITC (Sigma-Aldrich) used at 2 µg/mL. Mouse Ig G1-FITC (Beckman Coulter) used at 2 µg/mL. Anti-IL-17A (eBio64CAP17, eBioscience) anti-mouse IgG1 used at 2µg/mL. Mouse Ig G1-PE (Beckman Coulter) used at 2 µg/mL. Goat Fab'2 anti-Mouse IgG (H+L)-PE (Beckman Coulter and Abcam) used at 2 µg/mL. Anti-MCPyV large T-antigen mouse IgG (CM2B4) (Santa Cruz) is highly specific for MCPyV large T and 57kT isoforms, but does not detect MCPyV small T antigen. used at 1 µg/mL. Mouse anti-V5 (Thermo Fischer Scientific) used at 1.1 µg/mL. Mouse anti-MCPyV-sT (CM5E1) used at 5 µg/mL. Goat Anti-Mouse IgG HRP conjugate used at (Promega) 1 µg/mL. Rabbit anti-human β-actin (Cell Signaling) used at 1 µg/mL. Goat Anti-Rabbit IgG HRP conjugate (Promega) used at 1 µg/mL.

Plasmids and constructs

The expression plasmids pER, psT, p57kT, pwtLT and potLT were generated using the Gateway® system kit (Invitrogen™, life technologies). The reactions were performed by following the provided user guide of Gateway® system. Briefly, the full-length MCPyV ER and sT sequences were initially obtained and amplified by PCR from the wild-type MCPyVw156 clone using attB-ER/LT primers and attB-sT primers. The PCR products contain the sequences of interest flanked by two attB sites. The entry plasmids (pENTR) were created by performing the BP recombination reaction between the donor vector and the PCR products. 57kT cDNA sequence was obtained via RT-PCR of total RNA from pER-transfected HEK293T cells using attB-ER/LT as primers. After migration of amplified cDNA, 57kT cDNA was collect from the gel using the QIAquick Gel Extraction Kit (Qiagen) according to the sequence

size. The cDNA sequences of wtLT (MCPyVw156) and otLT (LT278 WaGa cell) were synthesized by Eurogentec in pcDNA3.1(+) at demand and then cloned into Gateway® system. The expression plasmids pER, p57kT, pwtLT and potLT were generated by using LR recombination reaction between the related pENTR-X (with X being ER or 57T or LT or otLT) and the 3xFlag N-terminal fusion destination vector (pDEST-CIneo-3xFlag). The expression plasmid psT was generated by performing LR recombination reaction between the pENTR-sT and the V5 C-terminal fusion destination vector (pcDNA-DEST40, purchased from Thermo Fisher Scientific). All MCPyV sequences were validated by sequencing using the M13 priming sites flanking the sequences on the pENTR. All the expression plasmids were subsequently cloned in One Shot® TOP10 (E. coli). Bacteria were transformed by heat shock and then selected in LB Agar medium with appropriate antibiotics. The selected colonies were amplified in liquid LB medium with appropriate antibiotics. All plasmids were extracted using QIAprep® Spin Miniprep or Hispeed Midiprep (Qiagen).

Cell culture and transfection

The HEK293T cell line (generously provided by NEGRE Didier, Centre International de Recherche en Infectiologie, Lyon, France) were cultured in DMEM medium (Invitrogen Life Technologies) supplemented with 10% fetal bovine serum, 10mM HEPES (Gibco, Thermofischer) and GlutaMAX 1X (Gibco, Thermofischer). WaGa cells (from the lab of Prof Antoine TOUZE, INRA ISP 1282, Tours, France) were grown in RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% fetal bovine serum, 10mM HEPES (Gibco, Thermofischer) and GlutaMAX 1X (Gibco, Thermofischer). All cells were cultured at 37°C with 5% CO₂. All cells were transfected in 12 wells plate with 0.4 x 10⁶ cells per well seeded 24h before transfection. The transfection was performed using JetPRIME® according to the manufacturer's protocols. The cells were harvested and stained at 12h, 24h and 36h post-transfection.

PCR, RT-PCR

Total RNA were extracted using TRIzol reagent (Invitrogene) according to the manufacturer's instructions. 2 µg of total RNA was used for reverse transcription using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) following the user guider with a 3-step programme (25°C for 10 minutes, 37°C for 120 minutes and 85°C for 5 minutes). For PCR of MCPyV sT, amplification was performed using attB-sT primers (Forward: 5'GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGGATTTAGTCCTAAATA

GGAA 3', Reverse: 5'
 GGGGACCACTTTGTACAAGAAAGCTGGGTTCGAAAAGGTGCAGATGCAGTAA 3')
 with first denaturation step at 98°C for 30 seconds, followed by 30 cycles of denaturation at 98°C for 10s, primer annealing at 64°C for 30s, and extension at 72°C for 1min. Upon completion of the cycling steps, a final extension at 72°C for 10min was done and then the reaction was stored at 16°C. For PCR of MCPyV ER/wtLT/57kT, the PCR was performed using attB-ER/LT primers (Forward: 5'
 GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGATTTAGTCCTAAATAGGAA
 AGAAAGA 3', Reverse: 5'
 GGGGACCACTTTGTACAAGAAAGCTGGGTCTTATTGAGAAAAAGTACCAGAATCT
 TG 3') with the same amplification process of sT except that the elongation was at 72°C for 1min 30s. For PCR of MCPyV otLT, amplification was performed using the attB-ER/LT forward primer and the attB-otLT reverse primer (5'
 GGGGACCACTTTGTACAAGAAAGCTGGGTCTCACTTTCTTGAGAATGGAGGAGGG
 GTC 3') with the same amplification process of MCPyV ER/wtLT/57kT. The PCR product sizes are: 2946 bp for ER detection, 1360 bp for 57kT detection, 2515 bp for 57kT detection and 910 bp for otLT detection.

Intracellular staining and flow cytometry

The intracellular immunostaining is performed in a 96W plate V-bottom with 0.2×10^6 cells per well. Cells were firstly fixed by a fixation buffer (BD, Bioscience) containing formaldehyde and then permeabilized by 1X Cytoperm Wash (BD, Bioscience) buffer. For Flag -ER staining, the cells were incubated directly with Abs anti-Flag M2-FITC (2 μ g/mL). For IL-17A staining, the cells were incubated firstly with mouse anti-human Abs anti-IL-17A used at 2 μ g/mL (eBioscience) and then with the secondary goat anti-mouse Abs anti-Fab'2-PE. Each incubation of antibody was performed for 30min at 4°C then washed twice with 1X Cytoperm Wash. Cells were resuspended into staining buffer (PBS 1X, 3% human serum) and then analyzed by flow cytometry (FACSCalibur; BD Biosciences) and the data analyzed using CellQuest software.

Immunoblot analysis

Cells were lysed in NP-40 lysis buffer (150mM sodium chloride, 1% NP-40, 50mM Tris pH 8) supplemented with 10% cocktail inhibitors (Roche). Total proteins concentration of cell lysate was determined using BCA Protein Assay Reagent (Pierce BCA, Thermo Fisher Scientific). 40 μ g of the protein were separated on a Mini Protean TGX Stain Free 4-15% Precast Gel (Biorad)

and then transferred onto a TransBlot Turbo™ Midi-size Nitrocellulose membrane (Biorad). Membranes were treated with the blocking buffer (TBS 1X/tween 0.05%, 5% non-fat dry milk) for 1h at room temperature and then incubated with primary antibodies Anti-V5, Anti-MCPyV large T-antigen (CM2B4), Anti-MCPyV sT (CM5E1) at 4°C overnight. Membranes were washed with TBS/Tween 0.05% 4 times and then probed with HRP-conjugated Goat Anti-Mouse IgG or Goat Anti-Rabbit for 1h at room temperature. Proteins bands were revealed using ECL SuperSignal West Femto (Thermo Fisher Scientific).

DISCLOSURE OF COMPETING INTEREST

The authors declare no conflicts of interest.

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FIGURE LEGENDS

Figure 1. MCPyV constructs and expression. (A) The entire ER DNA sequence (2885 bp) was cloned into the expression plasmid pER that contains a Flag tag sequence (Fla) in 5', in frame with the ER sequence. The 5'-3' wtLT mRNA encodes five Nterm-Cterm protein domains: DnaJ (Dn), LXCXE, a nuclear localization signal sequence (NL), an origin binding domain (OBD) and a C-terminal Helicase/ATPase domain. Alternative splicing produces 57kT and sT proteins, which lack the OBD and Helicase/ATPase domains. The LT-stabilization domain (LSD) uniquely characterizes sT. (B) mRNA detection by RT-PCR in HEK293T cells either non-transfected (NT) or transfected with 1.5 µg of pCtrl or pER or p57kT or pwtLT or potLT for 24h. Purified total RNA was reverse transcribed and then amplified by PCR to detect ER, wtLT, 57kT and otLT mRNAs. (C, D) HEK293T cells were either non-transfected (NT) or transfected with 1.5 µg of control (pCtrl) or with expression (pER) plasmids. (C) Transfection validation by flow cytometry analysis of pER-transfected cells stained intracellularly with anti-Flag antibody or related isotype control. (D) ER protein expression validation by western-blot analysis. 24h post-transfection, cell lysate protein contents were stained with either (left) anti-MCPyV LT antibody (CM2B4 clone recognizes LT and 57kT, but does not detect sT), or (right) anti-MCPyV sT antibody. β-actin was used as a control of equal loading. Data shown are representative of n>3 independent experiments. (E) cDNA sequences of sT, wtLT, 57kT versus otLT were cloned with a 3'-V5tag for sT sequence and a 5'-Flag for wtLT, 57kT or otLT sequences. (F) Western-blot validation of all the constructs. HEK293T cells were either non-transfected (NT) or transfected with 1.5 µg of pCtrl or psT or pwtLT or p57kT versus potLT and cultured for 24h. The WaGa cell line was used as otLT expression control. Harvested cell lysates were stained with anti-V5 antibody (left) or anti-MCPyV LT antibody (right). Results shown are representative of 3 independent experiments.

Figure 2. Intracellular IL-17A detection in pER-transfected cells. (A) Raw data from flow cytometry analysis quantifying transfection efficiency and IL-17A intracellular expression. HEK293T cells were either non-transfected (NT) or transfected with 1.5 µg of control (pCtrl) or expression (pER) plasmids. Cells were harvested 24h post-transfection and stained with anti-Flag or/and anti-IL-17A antibodies, or related IgG isotype controls. Data shown are representative of 10 independent experiments. (B) Dose-response study. HEK293T cells were either non-transfected (NT) or transfected with 1.5 µg of pCtrl or with the indicated amount of pER. Cells were then either (top, middle) single-stained or (down) double-stained with the

indicated antibodies. Data shown are the mean \pm SD of 3 experiments. (C) Kinetic study. HEK293T cells were either non-transfected (NT) or transfected with 1.5 μ g of pCtrl or pER. Cells were harvested at indicated time-points and either (top, middle) single-stained or (down) double-stained with the indicated antibodies. Data shown are the mean \pm SD of 3 independent experiments.

Figure 3. IL-17A detection in psT-transfected cells. (A) RT-PCR of sT mRNA. HEK293T cells were either non-transfected (NT) or transfected with 1.5 μ g of pCtrl or psT for 24h. Total RNA was extracted by TRIzol. cDNA was obtained by reverse transcription reaction and then amplified by PCR using attB-sT primers. (B) Immunoblot of MCPyV sT. HEK293T cells were either non-transfected (NT) or transfected with 1.5 μ g of pCtrl or psT for 24h. WaGa cell lysates (WaGa) was used as sT-expressing control cells. Cell lysates were then stained with anti-V5 antibody or anti-MCPyV sT antibody. β -actin was used as a measure of equal loading. (C) Kinetic study. HEK293T cells were either non-transfected (NT) or transfected with 1.5 μ g of pCtrl or psT, then cultured for 36 hours, with harvest points 12 hours apart. (D) Dose-response study. HEK293T cells were either non-transfected (NT) or transfected with indicated amount of pCtrl or psT for 24h. (C, D) Harvested cells were stained intracellularly with anti-IL-17A antibody and analyzed by flow cytometry. Data are representative of 3 independent experiments.

Figure 4. IL-17A detection in 57kT-transfected cells.

(A) Raw data from flow cytometry analysis quantifying transfection efficiency and IL-17A intracellular expression. HEK293T cells were either non-transfected (NT) or transfected with 1.5 μ g of the following plasmids: pCtrl or pER or p57kT plasmids. Cells were harvested 24h post-transfection and stained with (top) anti-Flag or (down) anti-IL-17A antibodies. Vertical line delineates area of cells stained with isotype controls. Data shown are representative of 4 independent experiments. (B) Cumulative data of the 4 independent experiments whose mean \pm SD are reported; the significance is evaluated by paired Student t-test. ** $p < 0.01$, ns = not significant.

Figure 5. Detection of IL-17A in wtLT expressing cells.

(A) Raw data from flow cytometry analysis quantifying transfection efficiency and IL-17A intracellular expression. HEK293T cells were either non-transfected (NT) or transfected with 1.5 μ g of the following plasmids: pCtrl or pER or pwLT plasmids. Cells were harvested 24h post-transfection and stained with (top) anti-Flag or (down) anti-IL-17A antibodies. Vertical

line delineates area of cells stained with isotype controls. Data shown are representative of 4 independent experiments. (B) Cumulative data of the 4 independent experiments whose mean \pm SD are reported; the significance is evaluated by paired Student t-test. ns = not significant.

Figure 6. Detection of IL-17A in potLT transfected cells.

(A,C) Raw data from flow cytometry analysis quantifying transfection efficiency and IL-17A intracellular expression. HEK293T cells were either non-transfected (NT) or transfected with 1.5 μ g of the following plasmids: pCtrl or pER or potLT or pwLT plasmids, as indicated. Cells were harvested 24h post-transfection and stained with (top) anti-Flag or (down) anti-IL-17A antibodies. Vertical line delineates area of cells stained with isotype controls. Data shown are representative of 4 independent experiments. (B, D) Cumulative data of the 4 independent experiments whose mean \pm SD are reported; the significance is evaluated by paired Student t-test. * $p < 0.05$, ** $p < 0.01$, ns = not significant.

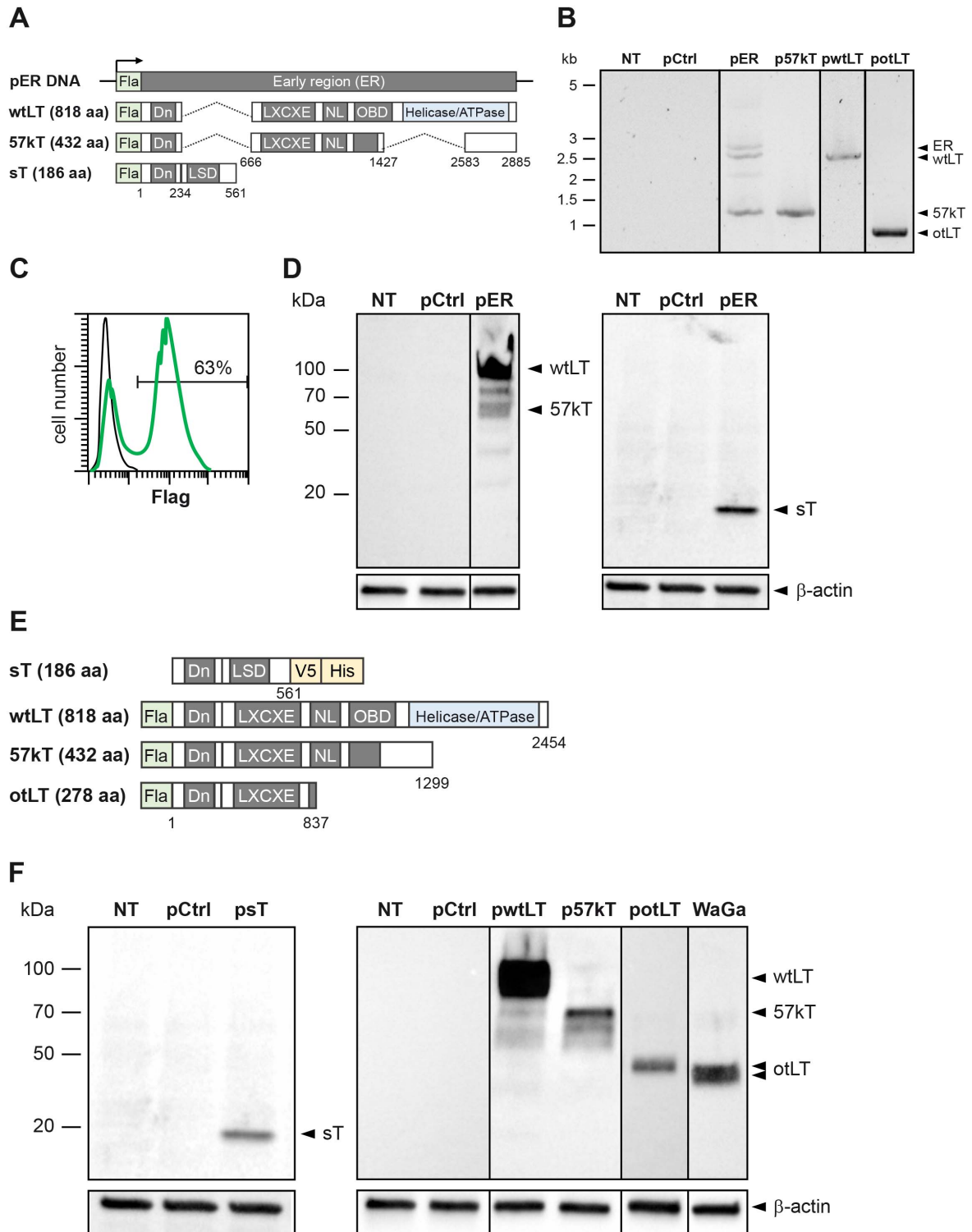


Figure 1 Junchao Miao *et al.*

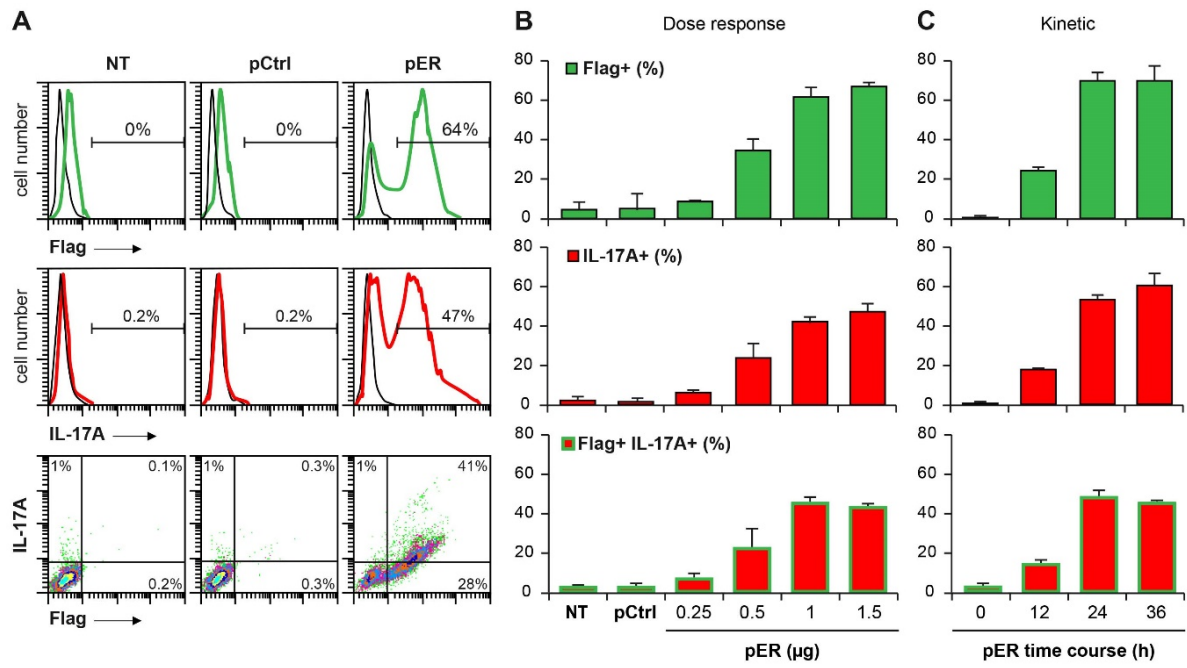


Figure 2 Junchao Miao *et al.*

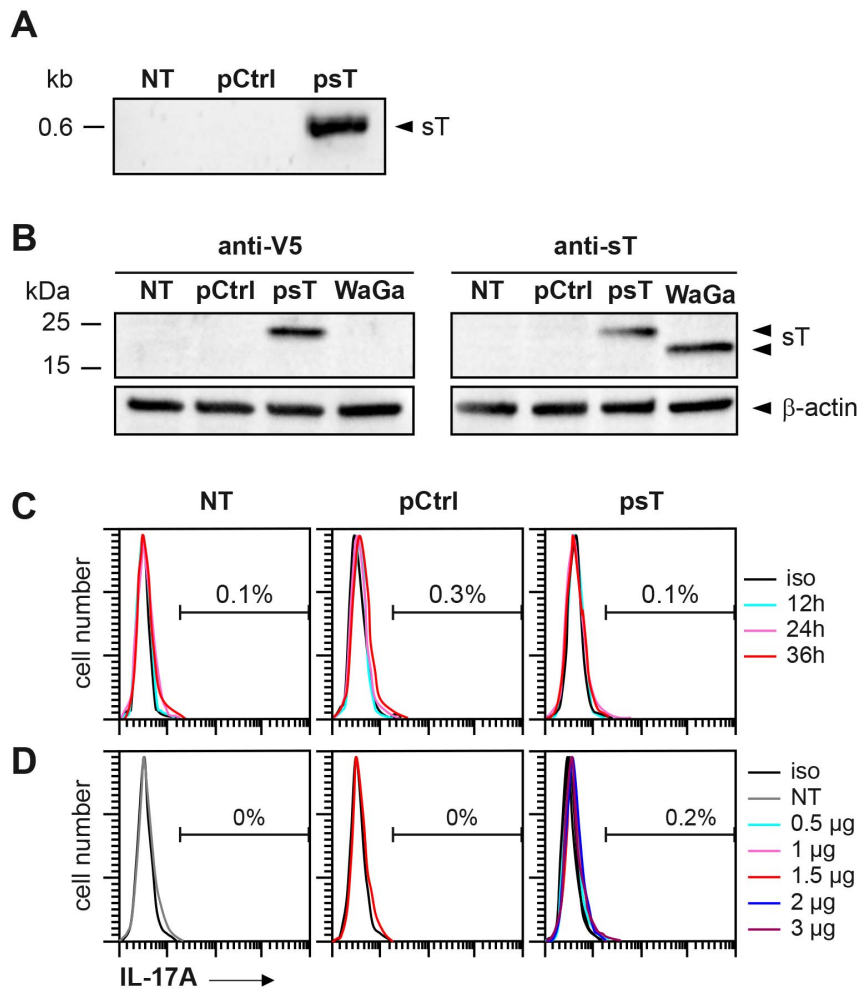


Figure 3 Junchao Miao *et al.*

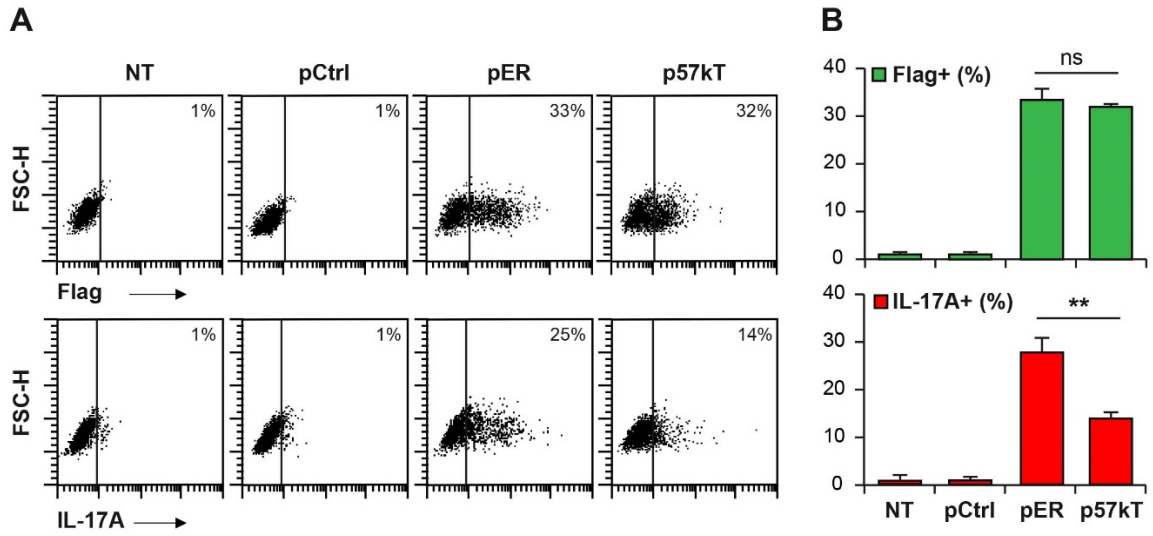


Figure 4 Junchao Miao *et al.*

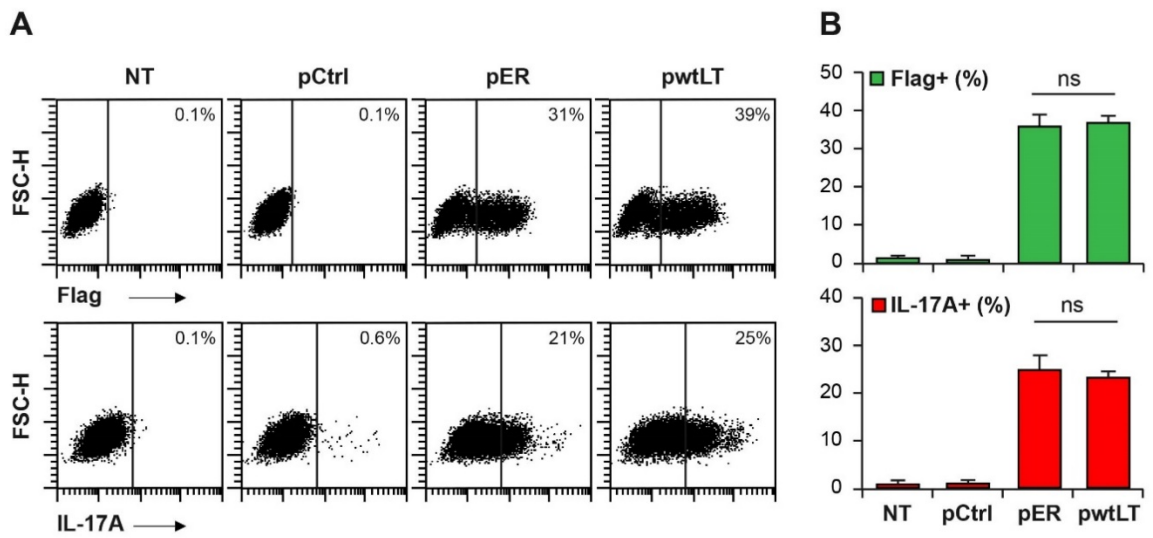


Figure 5 Junchao Miao *et al.*

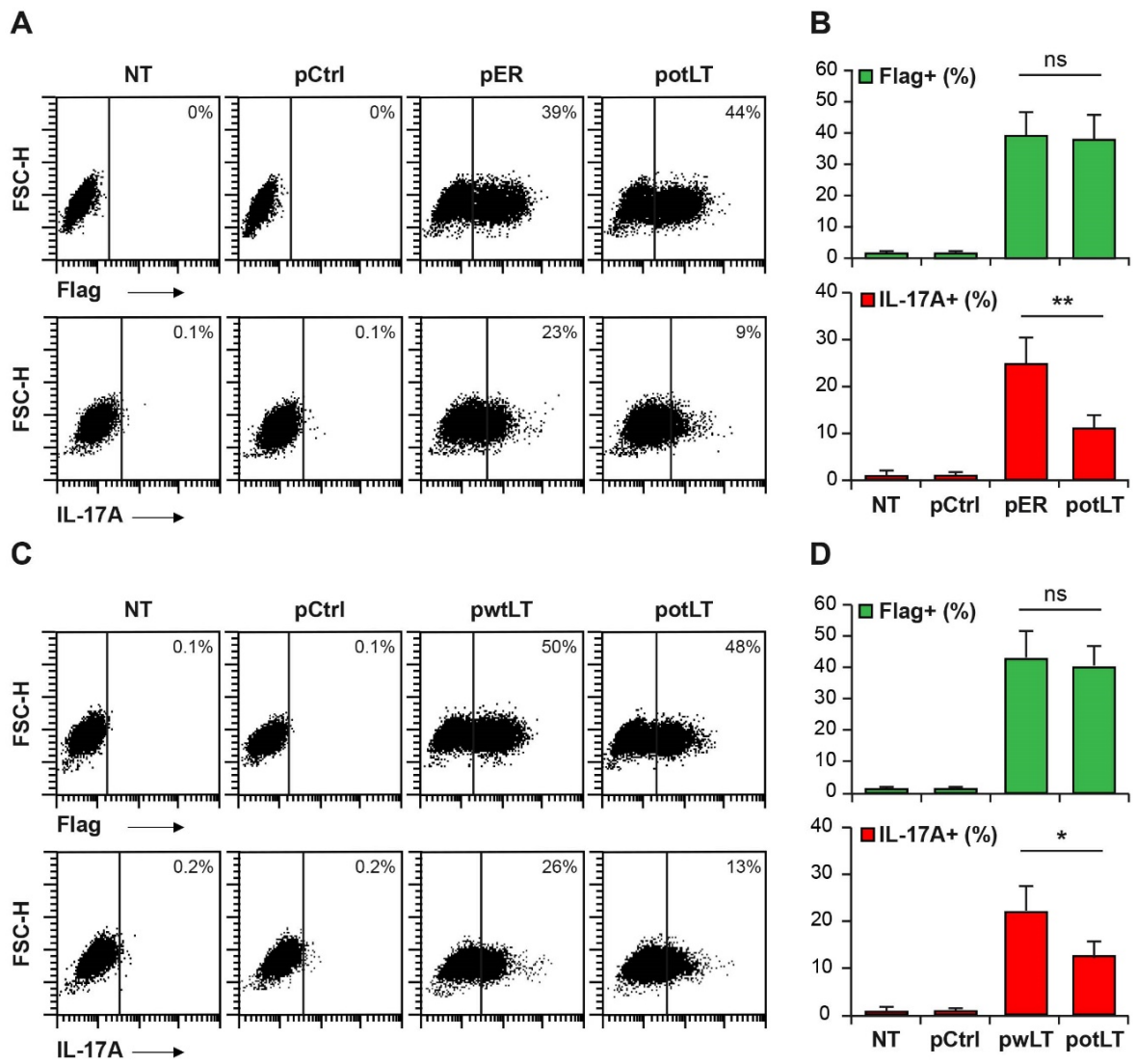


Figure 6 Junchao Miao *et al.*

Article 2

**Autophagy and IL-17A-dependent inflammation crosstalk
in MCPyV infection**

Primary results

Autophagy and IL-17A-dependent inflammation crosstalk in MCPyV infection

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KEYWORDS: Merkel cell Polyomavirus, MCPyV early region, large T antigen, IL-17A, inflammation, autophagy

ABSTRACT

Merkel cell polyomavirus (MCPyV) is the etiologic pathogen that causes most Merkel cell carcinoma (MCC). The C-terminal truncation of MCPyV large T antigen (LT), giving rise to onco-type LT (otLT), is characteristic of MCPyV⁺ MCC cells. Autophagy, an intracellular degradative process, harmonizes with inflammatory signaling to participate in the innate antiviral response. In this study, we show increased autophagy after LT transfection by using the autophagy flux assay. However, no autophagy increase was observed after otLT transfection, suggesting a critical role of the C-terminal end of LT in the induction of autophagy. Using an IL-17 receptor-expressing cell line and a reporter gene system, we observed that IL-17A-dependent NF- B signaling induced autophagy. Reversely, autophagy activation upon rapamycin treatment inhibited IL-17A-mediated inflammation, implying a negative feedback

of autophagy to restrain over-exuberant and chronic IL-17A inflammation. Our findings suggest that the crosstalk between autophagy and IL-17A signaling plays an important role in the anti-MCPyV response and MCC development.

ABBREVIATIONS

3-MA, 3-methyladenine; ER, early region; HEK-17R, HEK-Blue™ IL-17 cells; MCC, Merkel cell carcinoma; MCPyV, Merkel cell Polyomavirus; NLS, nuclear localization signal; OBD, origin binding domain; otLT, onco-type form of LT; pX, plasmid encoding the sequence of X; Rapa, rapamycin; sT, small T antigen; wtLT, wild-type large T antigen.

INTRODUCTION

Merkel cell Polyomavirus (MCPyV) is the causative agent of 80% of Merkel cell carcinoma (MCC), a rare aggressive skin malignancy affecting the elder and immunocompromised patients (1,2). The early region (ER) of the viral genome encodes the small T antigen (sT), the large T antigen (LT) also called wild-type LT (wtLT) in this study and the 57kT, in the same reading frame (3–7). The sT and LT are the main factors that drive MCC oncogenesis. The LT contains an LXCXE domain restraining retinoblastoma protein (pRb) to promote cell growth and a helicase domain in the C-terminal ends required for effective viral replication (5,8). The constitutive expression of sT and of a C-terminal truncated form of LT, called onco-type LT (otLT), in tumor cells are key features of MCPyV⁺ MCC cells (9). The C-terminal truncation of LT results in the loss of functional helicase, which blocks viral replication and enhances the growth-promoting activity of LT (10,11). Clearance of MCPyV prevents the MCPyV-induced MCC. It is therefore important to understand the immune mechanisms able to control MCPyV. Human cells are equipped with danger receptors, which sense viral antigens and initiate innate immune mechanisms including activation of inflammation and autophagy (12).

Basically, autophagy was characterized as a conserved intracellular degradative process dedicated to digest various cellular components ranging from biomacromolecules, via molecular aggregates to organelles (13). Canonical autophagy is monitored by the complex mTOR, which is inhibited by Rapamycin. mTORC activates ULK1 complex (14), which in turn, activates PI3KC3, a molecular complex including the Beclin-1 protein. PI3KC3 activation produces PI3P (phosphatidylinositol-3-phosphate) on the membranes derived from double-membrane organelles (15). PI3P then recruits ATG16L1 complex that conjugates LC3-I to PE (phosphatidyl-ethanolamine) to form the so-called LC3-II, firmly bound on the surface of the

membranes. LC3-II recruits additional membranes to form autophagosomes that engulf some cytoplasmic content (16).

Beyond this role in cellular homeostasis, autophagy is also involved in defense against intracellular pathogens, thanks to a selective autophagy response achieved by specific autophagy receptors (17), including the proteins p62, NDP52, OPTN, NBR1 and TAX1BP1. These receptors simultaneously interact with the protein LC3-II and cargos in the nascent autophagosome (18). Whereas various studies have documented the role of autophagy in human viral infections such as HSV-I, EBV and HIV (20,21,22), little is known regarding autophagy facing human Polyomavirus infections. Bag3 is a co-chaperone promoting the association of Hsc70/Hsp70 machinery with p62, thus driving the targeted proteins to selective autophagy for degradation (21–23). LT antigen of JCPyV, an opportunistic human Polyomavirus, can be degraded by Bag3-induced autophagy (24). This autophagic degradation of LT requires the interaction between the zinc finger domain of JCPyV LT and the proline-rich repeat of Bag3 (25). During infection, the autophagy pathway coordinates with inflammation signals to build an effective response against intracellular pathogens (26).

Downstream of danger receptors, viral antigen detection leads to the production of proinflammatory cytokines and type-I interferons (IFN-I). In 2014, the crosstalk between anti-viral inflammation and autophagy was revealed thanks to the HSV-I model of infection (27,28). Detection of DNA viral sequence by the cytosolic danger receptor cGAS primarily produces the second messenger cGAMP to initiate the STING pathway and subsequent IFN-I production. cGAS also binds Beclin-1 and activates the Beclin-1-PI3KC3 complex, thus promoting autophagy. Then, enhanced autophagy removes viral antigens as well as the cGAMP and STING from the cytosol, which down-regulates inflammation, while destroying viral products. Overall, the cGAS-Beclin-1 interaction shapes innate immune responses and autophagy to orchestrate a well-balanced anti-viral response. *In vitro*, infection of fibroblasts with MCPyV induces an inflammatory response mediated by IL-1 β , IL-6 and TNF- α , while IFN-I is weakly produced (29). IL-18 and IL-17A were not evaluated in the study. Recently, we demonstrated that human cell transfection with MCPyV-ER or wtLT alone induces IL-17A (Miao *et al*, submitted), a proinflammatory cytokine with pleiotropic effects and critical roles in skin and mucous homeostasis (30). Interestingly, otLT was less potent than wtLT and therefore limited IL-17A-dependent inflammation to a low-grade. The crosstalk between inflammation and autophagy in the context of MCPyV infection has to be studied.

Here, we studied the crosstalk between MCPyV-related IL-17A-dependent inflammation and autophagy. We showed that wtLT, high-grade inducer of IL-17A, induces LC3-II flux, while

otLT, low-grade inducer of IL-17A, does not. We therefore studied the crosstalk between IL-17A-mediated inflammation and the autophagy pathway to understand that IL-17A-dependent inflammation enhances autophagy, while activated autophagy down-regulates IL-17A-dependent inflammation, as a negative feedback. We discuss if the C-terminal truncation of LT may reshape the innate immune response and autophagy crosstalk to an imbalanced immune response supporting MCC development.

RESULTS

MCPyV wild-type LT increases autophagy flux, contrary to onco-type LT

The autophagy flux assay is a standardized technique coupling culture with Bafilomycin A1 (BafA1) or its diluent, dimethyl sulfoxide (DMSO), to the LC3-II quantification by western blot. During active autophagy, LC3-II primarily bound on membrane of autophagosomes, is digested after autophagosome-lysosome fusion. BafA1 is an inhibitor of the vacuolar H⁺-ATPase present on lysosomes, which blocks the lysosome acidification and fusion with autophagosome, thus preventing the digestion of LC3-II. In the presence of BafA1 and when autophagy is active, LC3-II accumulates. Western blot is used to quantify LC3-II. The autophagy flux is the difference of LC3-II amount in one culture condition performed with DMSO versus BafA1. In order to investigate the impact of MCPyV T antigens on autophagy, we performed the autophagy flux assay of different culture conditions. HEK293T cells were transfected with either control plasmid (pCtrl) or plasmids expressing full-length ER (pER), sT (psT), 57kT (p57kT), wild-type LT (pwtLT) and onco-type LT (potLT). 24h post-transfection, we treated cells with DMSO (Figure 1A, left) or BafA1 (Figure 1A, right) and then performed western blot analysis of LC3-II proteins, as shown on the representative experiment (Figure 1A). LC3-II protein level increased in cells transfected with pwtLT, suggesting an upregulation of autophagy flux in the presence of MCPyV wtLT. We also observed a reduced LC3-II level in potLT-transfected cells compared to wtLT. Autophagy flux quantification in cumulative experiments confirmed that (i) the LC3-II flux in pwtLT-transfected cells is significantly higher than that in control cells ; (ii) cells transfected with potLT displayed a lower autophagy flux than pwtLT-transfected cells; (iii) the LC3-II flux observed with otLT is close to the control (Figure 1B). Statistical analysis demonstrated no significant differences in the autophagy flux of the following culture conditions: pCtrl-, pER-, psT- , p57kT and potLT-transfected cells. Taken together, our data suggest that the C-terminal part of wtLT is a key domain to support

the upregulation of the autophagy flux and the onco-type form of LT from WaGa cells has lost this autophagy stimulating activity.

IL-17A signaling upregulates autophagy

Viral detection induces inflammation that can regulate autophagy (27). We previously demonstrated that MCPyV wtLT induces the expression of the pro-inflammatory cytokine IL-17A (Miao et al, submitted). It would be interesting to investigate whether IL-17A signaling can regulate autophagy. We used a biological assay set up with HEK-BlueTM IL-17 cells (abbreviated HEK-17R), which quantifies the intensity of the IL-17A signaling. HEK-17R cells were generated by stable transfection of the human genes encoding the IL-17 receptor (IL-17RA/IL-17RC) into the human embryonic kidney HEK293 cell line. HEK-17R cells constitutively express NF- κ B. HEK-17R cells also express an NF- κ B-inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene. Following the binding of IL-17A, downstream of the IL-17 receptor, NF- κ B transcription factor is activated and translocated into the nucleus. Then, NF- κ B induces SEAP transcription leading to the secretion of the phosphatase in the cell supernatant. Addition of the phosphatase substrate gives rise to a blue product quantified by spectrometry. Overall, the blue (630 nm) optical density reflects the NF- κ B activity. We first validated this biological assay by incubating overnight, at 37°C with 5% CO₂, HEK-17R cells with a range (0-10 ng/mL) of IL-17A concentrations (Figure 2A). In the absence of IL-17A, HEK-17R cells showed constitutive NF- κ B activity (OD~0.3, data not shown) that we removed from total NF- κ B activity to access NF- κ B activity specifically induced by IL-17A. Addition of IL-17A dose-dependently increased NF- κ B activity. Thus, the biological assay nicely quantifies the intensity of IL-17A signaling and can be used in a range of IL-17A concentrations from 0.1 to 10 ng/mL. In parallel, we studied if IL-17A signaling regulates the autophagy flux in HEK-17R cells. We measured the autophagy flux in HEK-17R cells treated with increasing concentrations of IL-17A. Representative western blot detecting LC3-II versus β -actin proteins showed higher LC3-II levels from 3.3 ng/mL of IL-17A (Figure 2B). Autophagy flux quantification in cumulative experiments confirmed this result (Figure 1B). Therefore, in HEK-17R cells, we demonstrated the concomitant actions of IL-17A on NF- κ B and autophagy activation. However, the IL-17A threshold to activate NF- κ B (0.1 ng/mL) was lower than the threshold to upregulate LC3-II flux (3.3 ng/mL), thus suggesting that intense IL-17A signaling may be required to upregulate autophagy.

Autophagy activation impairs IL-17A signaling

Conversely, autophagy can destroy key elements required for signal transduction and affect related inflammatory grade (28). We therefore studied whether autophagy interferes with IL-17A signaling. We used autophagy activator or inhibitor to modulate autophagy in HEK-17R cells while quantifying IL-17A-mediated NF- κ B activity. We first validated the effect of rapamycin (Rapa), a known inhibitor of mTOR, expected to activate autophagy (Figure 3A, 3B). Western blot analysis showed that LC3-II level increased upon treatment with 50 and 100 nM of Rapa in a dose-dependent manner (Figure 3A). Consistently, autophagy flux quantification confirmed that Rapa increased autophagy at 50 and 100 nM (Figure 3B). We stimulated HEK-17R cells overnight with the same range of IL-17A doses as used in the previous experiment (0-10 ng/mL) in the presence of 50 or 100 nM of Rapa or DMSO as control. NF- κ B activity was measured. To ensure that regulation of NF- κ B activity was not due to any cytotoxic effect of Rapa, we checked the cell viability after the treatment using MTT assay. No toxicity was detected upon Rapa treatment (Figure 3C). In DMSO control condition, constitutive NF- κ B activity induces 0.3 OD while addition of Rapa lowered constitutive NF- κ B activity to 0.1 OD (data not shown). We then plotted the IL-17A-induced NF- κ B activity after removal of the constitutive NF- κ B activity (Figure 3D). The 17A-induced NF- κ B activity was consistently lowered by Rapa. However, the percentage of inhibition decreased when IL-17A increased: 73-40-31-7% of inhibition by Rapa for 0.3-1.1-3.3-10 ng/mL of IL-17A, respectively. It was therefore clear that Rapa, which activates autophagy, limits NF- κ B activity, whatever it is constitutive or induced by IL-17A.

Taken together, our data indicate that autophagy activation by Rapa reduced NF- κ B activity downstream of the IL-17 receptor, thus impairing IL-17A transduction, with no cell cytotoxicity.

Autophagy inhibition potentiates IL-17A signaling

We next investigated whether autophagy inhibition increased IL-17A signaling. We first determined the working doses of a class III PI3K inhibitor that blocks autophagy, 3-Methyladenine (3-MA). Western blot analysis showed that 3-MA reduced cellular LC3-II levels at 1 and 5 mM (Figure 4A). Autophagy flux analysis confirmed that 3-MA is a potent autophagy inhibitor when used at 1 mM and beyond (Figure 4B). HEK-17R cells were cultured for 18 hours with the same doses of IL-17A ranging from 0 to 10 ng/mL in the presence of 1 or 5 mM of 3-MA or DMEM as control. NF- κ B activity was measured. MTT assay showed that 1 and 5 mM 3-MA presented cytotoxicity that killed till 20% of cells (Figure 4C). In DMEM control condition, constitutive NF- κ B activity induces 0.3 OD while addition of 3-MA

enhanced constitutive NF- κ B activity to 0.5 OD (data not shown). We then plotted the IL-17A-induced NF- κ B activity after removal of the constitutive NF- κ B activity and taking into account the cell death (Figure 4D). The IL-17A-induced NF- κ B activity was consistently enhanced by 3-MA and stabilized at 30% of activation compared to the DMEM control condition. It was therefore clear that 3-MA, which inhibits autophagy, enhances NF- κ B activity, whatever it is constitutive or induced by IL-17A.

In conclusion, autophagy inhibition by 3-MA exerted a weak cytotoxicity while enhancing NF- κ B activity downstream of the IL-17 receptor, thus potentiating IL-17A transduction.

DISCUSSION

In this work, we revealed that the C-terminal sequence of MCPyV LT is important for LC3-II upregulation, suggesting its role in autophagy activation. Since (i) we have previously demonstrated that C-terminal sequence of LT MCPyV LT is also essential for IL-17A induction, and (ii) crosstalk between inflammation and autophagy has been documented (12), we decided to determine if a crosstalk between IL-17A transduction and autophagy is existing. If applicable, this crosstalk may be regulated during the oncogenic process of MCPyV+ MCC, thanks to the truncation of the C-terminal part of LT.

First, our data demonstrate that IL-17A recapitulates the role of wtLT by upregulating LC3-II flux. In ischemia and rheumatoid arthritis, IL-17A was already involved as autophagy inducer in specific cell types including neurons, macrophages, and synoviocytes (31–33). The crosstalk between autophagy and IL-17A transduction was investigated thanks to activator (Rapa) and inhibitor (3-MA) of autophagy. We discovered, for the first time, the existence of a feedback control since IL-17A may upregulate autophagy, which in turn, downregulates NF- κ B activity, an essential transcription factor of the IL-17A-mediated inflammation. The mechanisms have not been elucidated in our model. However, IL-17A transduction is initiated via ACT1 and TRAF6 (34). Interestingly, it has been shown that TRAF6 promotes the assembly of PI3KC3 complex through TRAF6-mediated K63 ubiquitylation of Beclin-1 to free it from the inhibitory sequestration with BCL-2 (35). Thus, IL-17A transduction may upregulate autophagy via stimulating the assembly of the PI3KC3 complex. For the feedback, autophagy may lower the amount of NF- κ B and subsequently diminish NF- κ B activity thanks to its ability to engulf and digest cytoplasm content. Chronic activation of IL-17A causes tissue remodeling leading to autoimmune diseases and cancer (36). It is known that mild sustained IL-17A inflammation can result in skin cancer by promoting the proliferation of premalignant cells (37,38). The negative

feedback of autophagy can therefore be vital to control IL-17A inflammation and tissue homeostasis.

We have documented that wtLT induces IL-17A (Miao et al, submitted). To date, we ignore if the upregulation of autophagy induced by wtLT is only mediated by IL-17A or also by other mechanisms. However, several articles are in favor of this latest hypothesis. MCPyV is a small, non-enveloped virus with a double-stranded DNA genome. Intracellular endosomal Toll-like receptors (TLRs) are primary sensors that recognize viral antigen to induce inflammatory responses. TLR9 is a DNA sensor. The interaction between MCPyV and the innate immune system is poorly documented. However, TLR9 may play a role in the detection of MCPyV infection given that wtLT downregulates TLR9 expression (39). More recently, in primary fibroblast replicating MCPyV, the viral DNA sensor cGAS activates the STING pathway, in coordination with NF- κ B and mediates induction of innate immune gene expression program (29). These TLR9 and cGAS pathways may participate in autophagy activation. First, TLR9 transduction uses the MYD88 adaptor, like TLR7 / 8 whose stimulation induces the binding of MYD88 to Beclin-1, thereby dissociating Beclin-1 from BCL-2 and ultimately inducing autophagy in mouse macrophages (40). Second, as demonstrated in infection with HSV-I, the cGAS competes with the inhibitory complex Rubicon for Beclin-1 binding, thus triggering autophagy and promoting the autophagic degradation of cytosolic viral DNA to prevent over-exuberant inflammation (41). In HSV-I-infected fibroblast cells, negative feedback of innate inflammation was ensured by autophagy since Beclin-1 suppresses cGAMP production and activates ULK1 to phosphorylate STING for its degradation (42). Furthermore, ATG9 inhibits the translocation of STING on Golgi apparatus-derived compartments to suppress the pathway (43). In general, upon viral infection, the danger receptor signaling tends to activate autophagy to improve viral clearance and inflammatory responses, while in a later stage, autophagy provides negative regulation to restrain the inflammatory signaling.

We wondered whether the crosstalk between IL-17A and autophagy may play some roles in cancerogenesis. We have built plasmid expressing the otLT form found in WaGa MCC cell line to address this question. Our results showed a significantly reduced LC3-II flux with otLT compared to wtLT. Previously, we had found that otLT is also a weaker inducer of IL-17A compared to wtLT. Therefore, the C-terminal truncation, which deletes the Zinc-finger domain, not only restricts viral replication, but also decreases IL-17A and eventually autophagy. Interestingly, this Zinc-finger domain is a conserved motif of all HPyV LT, including MCPyV, suggesting that MCPyV LT also induces the chaperone-mediated selective autophagy through Bag3.

Of note, although we previously demonstrated that the full-length ER was able to induce IL-17A as efficiently as wtLT, our results did not show increased autophagy in cells transfected with full-length ER. The difference between ER and wtLT is the presence of other products encoded by ER such as sT. sT antigen was presented in a recent paper as an inducer of miR-375, which suppresses several autophagy genes, but further experiments and evidences are required to support the conclusion (kumar, *int j cancer* 2020). sT is also known to bind to and disrupts NF- κ B essential modulator, thereby inhibiting NF- κ B activation and signaling (45), which is to date not directly linked to autophagy inhibition. Thus, while the role of each MCPyV protein can be studied separately, the intracellular physiological outcome of autophagy in cells infected with MCPyV is still a matter of debate.

Conceptually, our study suggest an important role of autophagy-IL-17A inflammation crosstalk in MCPyV infection and MCC carcinogenesis. Especially, the oncogenic form of MCPyV producing otLT instead of wt LT protein may lower autophagy while maintaining a low-grade IL-17A inflammation with limited viral antigen presentation to prevent cancer cell targeting and immune-mediated cell death. These data contribute to open new avenues to understand MCPyV-mediated tumorigenesis and potential MCC treatment.

MATERIALS AND METHODS

Antibodies and reagents

Polyclonal Rabbit IgG1 Clone Anti-LC3 antibody, Sigma Cat#L7543, 1mg/mL, used at 1/1000. Rabbit anti-human β -actin, Cell Signaling - Cat#4967S, used at 1/1000. Goat anti-Rabbit IgG HRP conjugate, Promega, Cat# W4018, used at 1 μ g/ml. Recombinant human (rh) IL-17A lyophilized powder was purchased from InvivoGen then dissolved into endotoxin-free water (InvivoGen) to obtain the stock rhIL-17A solution at 100 μ g/mL. All tested concentrations were made by diluting the stock solution into DMEM medium (Invitrogen Life Technologies). The stock solution of 50mM 3-MA (#M9281, Sigma) was prepared in DMEM medium and solubilized by vortexing and heating at 50°C. The solution was then filtered (0.2 μ m filter). Dimethyl sulfoxide DMSO (Sigma) was used as solvent to prepare BafA1 (Santa Cruz, SC-201550) solution. Rapamycin (Ready Made Solution), 2.5 mg/mL in DMSO (2.74 mM) was purchased from Sigma.

Plasmids and constructs

Plasmids were generated using the Gateway® system kit (Invitrogen™, life technologies) as previously described. Briefly, the full-length MCPyV ER and sT sequences were initially obtained and amplified by PCR from the wild-type MCPyVw156 clone using attB-ER/LT primers and attB-sT primers. The PCR products contain the sequences of interest flanked by two attB sites. The entry plasmids (pENTR) were created by performing the BP recombination reaction between the donor vector and the PCR products. 57kT cDNA sequence was obtained via RT-PCR of total RNA from pER-transfected HEK293T cells using attB-ER/LT as primers. After migration of amplified cDNA, 57kT cDNA was collected from the gel using the QIAquick Gel Extraction Kit (Qiagen) according to the sequence size. The cDNA sequences of wtLT (MCPyVw156) and otLT (LT278 WaGa cell) were synthesized by Eurogentec in pcDNA3.1(+) at demand and then cloned into Gateway® system. The pER, p57kT, pwtLT and potLT were generated by using LR recombination reaction between the pENTR-ER and the 3xFlag N-terminal fusion destination vector (pDEST-CIneo-3xFlag). The psT was generated by performing LR recombination reaction between the pENTR-sT and the V5 C-terminal fusion destination vector (pcDNA-DEST40, purchased from Thermo Fisher Scientific). All MCPyV sequences were validated by sequencing using the M13 priming sites flanking the sequences on the pENTR. All the expression plasmids were subsequently cloned in One Shot® TOP10 (E. coli). Bacteria were transformed by heat choc and then selected in LB Agar medium with appropriate antibiotics. The selected colony was amplified in liquid LB medium with appropriate antibiotics. All plasmids were extracted using QIAprep® Spin Miniprep or Hispeed Midiprep (Qiagen).

Cell culture and transfection

The HEK293T cell line (generously provided by NEGRE Didier, Centre International de Recherche en Infectiologie, Lyon, France) were cultured in DMEM medium (Invitrogen Life Technologies) supplemented with 10% fetal bovine serum, 10mM HEPES (Gibco, Thermofischer) and GlutaMAX 1X (Gibco, Thermofischer). HEK-Blue™ IL-17 cells (InvivoGen), called HEK-17R in the paper, were cultured and maintained in DMEM medium (Invitrogen Life Technologies) supplemented with 10% fetal bovine serum, 10mM HEPES (Gibco, Thermofischer), GlutaMAX 1X (Gibco, Thermofischer), and selection antibiotics (InvivoGen) according to the provided user guide. All cells were cultured at 37°C with 5% CO₂. HEK293T cells were transfected in 12 wells plate with 0.4 x 10⁶ cells per well seeded 24h before transfection. The transfection was performed using JetPRIME® according to the

manufacturer's protocols. The cells were harvested and subjected to western blot at 24h post-transfection.

Western blot for LC3-II quantification

Cells were lysed with the help of RIPA lysis buffer, containing protease and phosphate inhibitors, and the supernatant was collected after centrifugation at 12000 RPM for 10 min à 4°C. Pierce™ BCA Protein Assay (ThermoFisher) was performed to determine the protein concentrations before loading the samples on SDS-PAGE gels. 30 µg of the protein were loaded on a Mini Protean TGX Stain-Free 4-15% Precast Gel (Biorad). After migration gels were activated by UV radiation to visualize and quantify the total proteins in the gel. Gel contents were then transferred onto a TransBlot Turbo™ Midi-size Nitrocellulose membrane (Bio-rad). Membranes were saturated with the blocking buffer (TBS 1X/tween 0.05%, 5% non-fat dry milk) for 1h at room temperature and then incubated with primary antibodies anti-LC3 and anti-β-actin at 4°C overnight. Membranes were washed the next day with TBS/Tween 0.05% 4 times and then probed with HRP-conjugated secondary Goat Anti-Rabbit antibody for 1h at room temperature. Immunostaining was detected with SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific). Membranes were visualized with a ChemiDoc™ Touch Imaging System (BioRad). Integrated densities of Western blot bands were measured using Image Lab™ software (Bio-rad). Densitometry measurements of LC3-II were normalized with either Actin (LC3-II/Actin ratio) or the total proteins in the gel (LC3-II/Total ratio).

Autophagy flux assay

For autophagy flux assay, before the LC3-II quantification by western blot, the same culture condition is treated for 2 hours at 37°C with 5% CO₂ with either DMSO (1:1,000 dilution; Sigma-Aldrich) used as the diluent control, or BafA1 (100 nM; Santa Cruz, SC-201550). Then LC3-II quantification is performed by western blot with either DMSO or BafA1. For each culture condition, the autophagy flux is calculated by subtracting the LC3-II quantity in the presence of BafA1 versus DMSO, the diluent. When LC3-II is normalized with Actin, the autophagy flux is calculated as follow: LC3-II/Actin (BafA1) - LC3-II/Actin (DMSO). Similarly, when LC3-II is normalized with the total proteins in the gel the formula becomes LC3-II/Total proteins (BafA1) - LC3-II/Total proteins (DMSO).

Dose-response and autophagy modulator assay

HEK-17R cells (70-90% confluent) were collected and 50 000 cells were plated in each well in a 96 well plate in triplicates for each condition for all the dose-response curves and autophagy modulator assays. For the dose-response curves, wells were supplemented with 20 μ L of varying concentrations of rhIL17A to obtain the final concentration range (0-0.1-0.33-1.1-3.3-10 ng/mL). 20 μ L DMEM was used as control (0 ng/mL). Then 180 μ L of cell suspension was added and incubated at 37°C, 5% CO₂ overnight before performing the colorimetric assay (QUANTI-Blue assay). Autophagy modulator assays were performed in 96 well plates with 50 μ L of autophagy modulators (rapamycin and 3-MA) at each concentration to be tested as a treatment condition in triplicate. The same volume in triplicate of DMSO alone (1:1,000 dilution; Sigma-Aldrich) and DMEM was used as control condition for Rapamycin and 3-MA, respectively. Varying concentrations of IL17A were added at 50 μ L to all wells to have the final concentration range at 0-0.1-0.33-1.1-3.3-10 ng/mL. Finally, 100 μ L of cell suspension was added to all treatment conditions. The plates were incubated at 37°C, 5% CO₂ overnight and the NF- κ B activity and cell viability were analyzed by spectrophotometry (QUANTI-Blue and MTT assays, respectively).

Measurement of NF- κ B activity in HEK-17R cells

HEK-17R cells are incubated without or with IL-17A. After 18 h of culture, NF- κ B activity of HEK-17R cells is quantified by a colorimetric enzyme assay because HEK-17R cells secrete alkaline phosphatase in the supernatant under the control of NF- κ B activation, as explained in the results. We used the QUANTI-Blue™ Solution (InvivoGen) as substrate to reveal alkaline phosphatase in the cell supernatant. QUANTI-Blue solution was reconstituted in sterile H₂O at a 1:1 QUANTI-Blue reagent to buffer ratio and stored in dark at 4 °C when not in use. 20 μ L of cell supernatant possibly containing secreted alkaline phosphatase was added to 180 μ L of QUANTI-Blue reagent in 96 well plates. The plates were incubated in dark at 37°C for 30 min to 60 min before recording absorbance with the help of IDK microplate reader at 630 nm. The optical density reflects the total NF- κ B activity, which is composed of constitutive NF- κ B activity and IL-17A-induced NF- κ B activity. We plotted the IL-17A-induced NF- κ B activity. For this purpose, constitutive NF- κ B activity of HEK-17R cells, measured in absence of IL-17A, was removed from each OD measurements.

Cell viability assessed with the MTT assay

6mM MTT (3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide; thiazolyl blue; Thermofisher) solution was prepared in PBS (Gibco 14190-094) devoid of Ca²⁺ and Mg²⁺, and filtered on a 0.22 µM membrane after solubilization. 10 µl of MTT solution was added to wells containing 80 µl of media and cells and the plates incubated for 3 hrs at 37°C, 5% CO₂. Then 200 µl of DMSO was added and the plate was left undisturbed for 20 min at 37°C. The MTT formazan crystals were solubilized with the help of a pipette before measuring OD at 540nm. MTT is a colorimetric assay based on the reduction of a yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT) to purple formazan crystals by cellular activity. The viable cell mitochondria contain active NAD(P)H-dependent oxidoreductase enzymes which reduce the MTT to formazan (46).

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FIGURE LEGENDS

Figure 1. Autophagy flux quantification in cells transfected with full length MCPyV early region or products (A, B) HEK293T cells were transfected with either pCtrl or pER or psT or p57kT or pwtLT or potLT for 24 h. Each culture condition was treated with either DMSO or BafA1 for 2 hours before cell lysis. Lysates were analyzed by western blot performed with either anti-LC3 or anti- β -actin antibodies. **(A)** Western blot of one representative experiment, after treatment with DMSO (left) or BafA1 (right). **(B)** Autophagy flux quantification of 3 independent normalized experiments. Autophagy flux calculation was performed as described in the methods. β -actin was used for normalization of LC3-II. Then, each experiment was normalized with the control culture condition sets at the value 1, which is HEK293T cells transfected with pCtrl (pCtrl). Shown is the mean \pm SD of 3 independent normalized experiments. Subsequent one-way ANOVA statistical analysis was performed to compare the autophagy flux of the different culture conditions. * $p < 0.05$, ns = not significant.

Figure 2. Assessment of NF- κ B activation and autophagy flux in IL-17A-stimulated HEK-17R cells. (A-C) Dose-response study of HEK-17R cells cultured in DMEM for 18 h without or with increased doses of IL-17A. **(A)** NF- κ B activation was quantified in HEK-17R cells cultured with 0.1, 0.33, 1.1, 3.3, 10 ng/mL of IL-17A. The optical density reflects NF- κ B activation. Data shown are representative of $n > 5$ experiments. **(B)** Western blots of LC3 protein levels in HEK-17R cells treated with the indicating concentrations of IL-17A. Each culture condition was treated with either DMSO (left) or BafA1 (right) for 2 hours before cell lysis. Lysates were analysed by western blot performed with either anti-LC3 or anti- β -actin antibodies. Representative experiment of 2 independent experiments is shown. **(C)** Autophagy flux quantification of 2 independent normalized experiments. Autophagy flux calculation was performed as described in the methods. Total protein was used for normalization of LC3-II. Then, each experiment was normalized with the control culture condition sets at the value 1, which is HEK-17R cells cultured in DMEM (Ctrl). Shown is the mean \pm SD of 2 independent normalized experiments.

Figure 3. Effect of rapamycin on NF- κ B activation in IL-17A-stimulated HEK-17R cells. (A) Western blots of LC3 protein levels in HEK-17R cells treated with increasing concentrations of Rapa (0, 25, 50, 100 nM) for 18 h. Lysates were analyzed by western blot performed with either anti-LC3 or anti- β -actin antibodies. Representative result of 2

independent experiments is shown. **(B)** Autophagy flux quantification of 2 independent normalized experiments. Autophagy flux calculation was performed as described in the methods. β -actin was used for normalization of LC3-II. Then, each experiment was normalized with the control culture condition sets at the value 1, which is HEK-17R cells cultured in the presence of DMSO, the diluent of Rapa (Ctrl). Shown is the mean \pm SD of 2 independent normalized experiments. **(C)** MTT assay to assess cell viability after treatment with indicated concentrations of Rapa and IL-17A. Data shown are representative of 2 independent experiments. **(D)** IL-17A-dependent NF- κ B activity in the presence of Rapa. NF- κ B activation was quantified in HEK-17R cells cultured with 0.1, 0.33, 1.1, 3.3, 10 ng/mL of IL-17A and Rapa (50 and 100 nM) or DMSO (diluent control) for 18 h. The optical density reflects NF- κ B activation. Data shown are representative of 2 experiments.

Figure 4. The effect of 3MA in IL-17A-dependent inflammation. **(A)** Western blots of LC3 protein levels in HEK-17R cells treated with increasing concentrations of 3MA (0, 0.5, 1, 5 mM) for 18 h. Lysates were analyzed by western blot performed with either anti-LC3 or anti- β -actin antibodies. Representative result of 2 independent experiments is shown. **(B)** Autophagy flux quantification of 2 independent normalized experiments. Autophagy flux calculation was performed as described in the methods. β -actin was used for normalization of LC3-II. Then, each experiment was normalized with the control culture condition sets at the value 1, which is HEK-17R cells cultured in DMEM (Ctrl). Shown is the mean \pm SD of 2 independent normalized experiments. **(C)** MTT assay to assess cell viability after treatment with indicated concentrations of 3-MA and IL-17A. Data shown are representative of 2 independent experiments. **(D)** IL-17A-dependent NF- κ B activity in the presence of 3-MA. NF- κ B activation was quantified in HEK-17R cells cultured with 0.1, 0.33, 1.1, 3.3, 10 ng/mL of IL-17A and 3-MA (1 and 5 mM) or DMEM (control) for 18 h. The optical density reflects NF- κ B activation. Data shown are representative of 2 experiments.

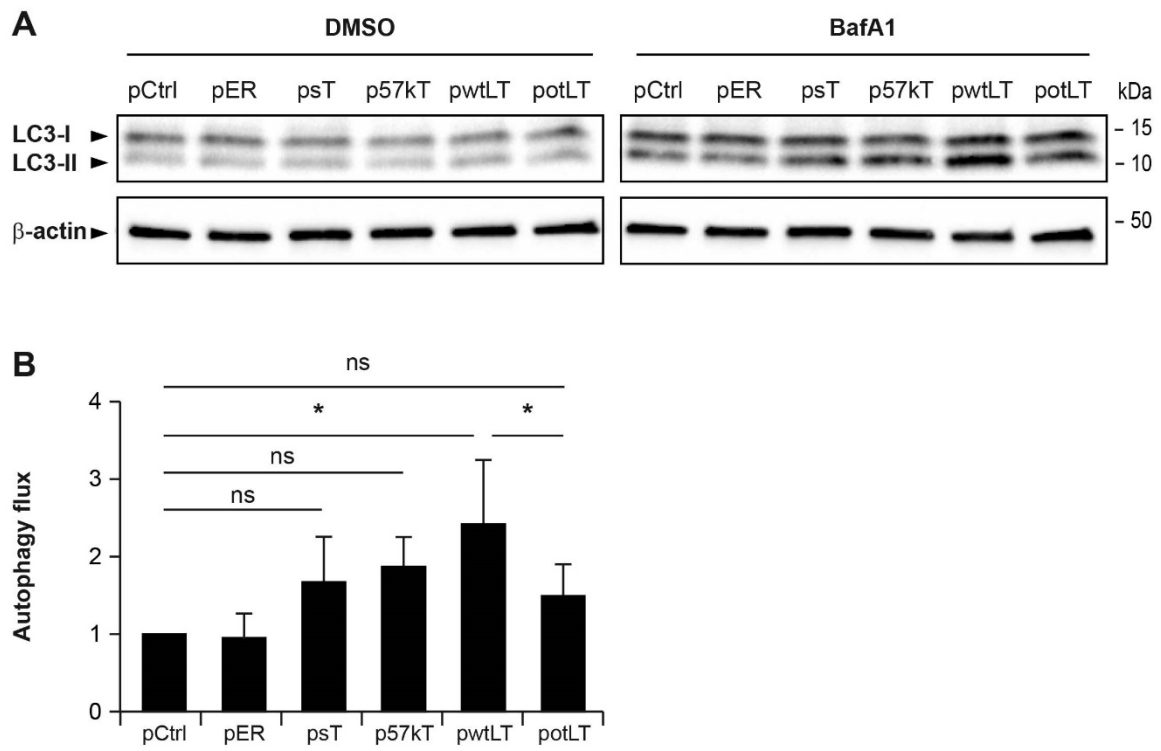


Figure 1 Junchao Miao *et al.*

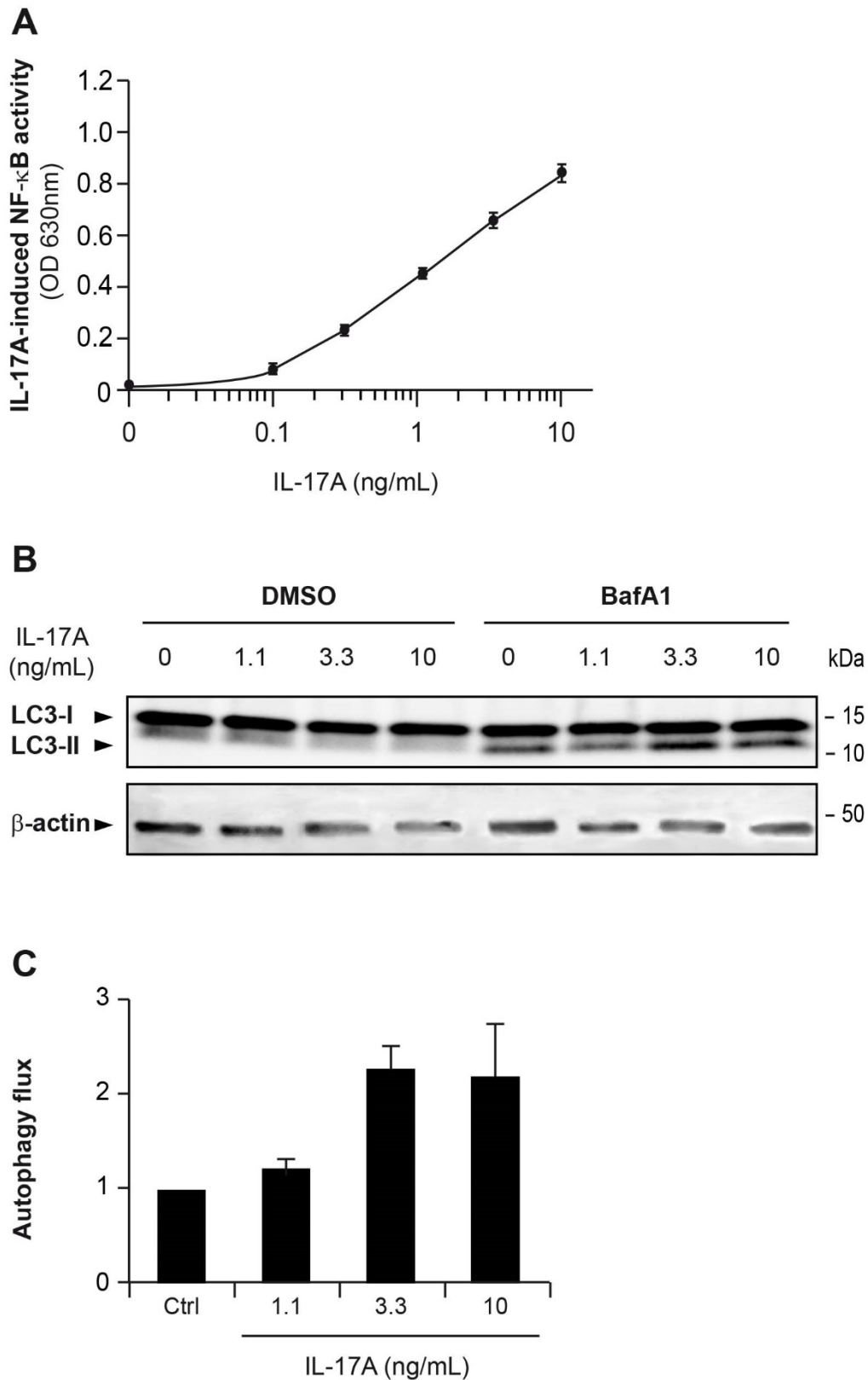


Figure 2 Junchao Miao *et al.*

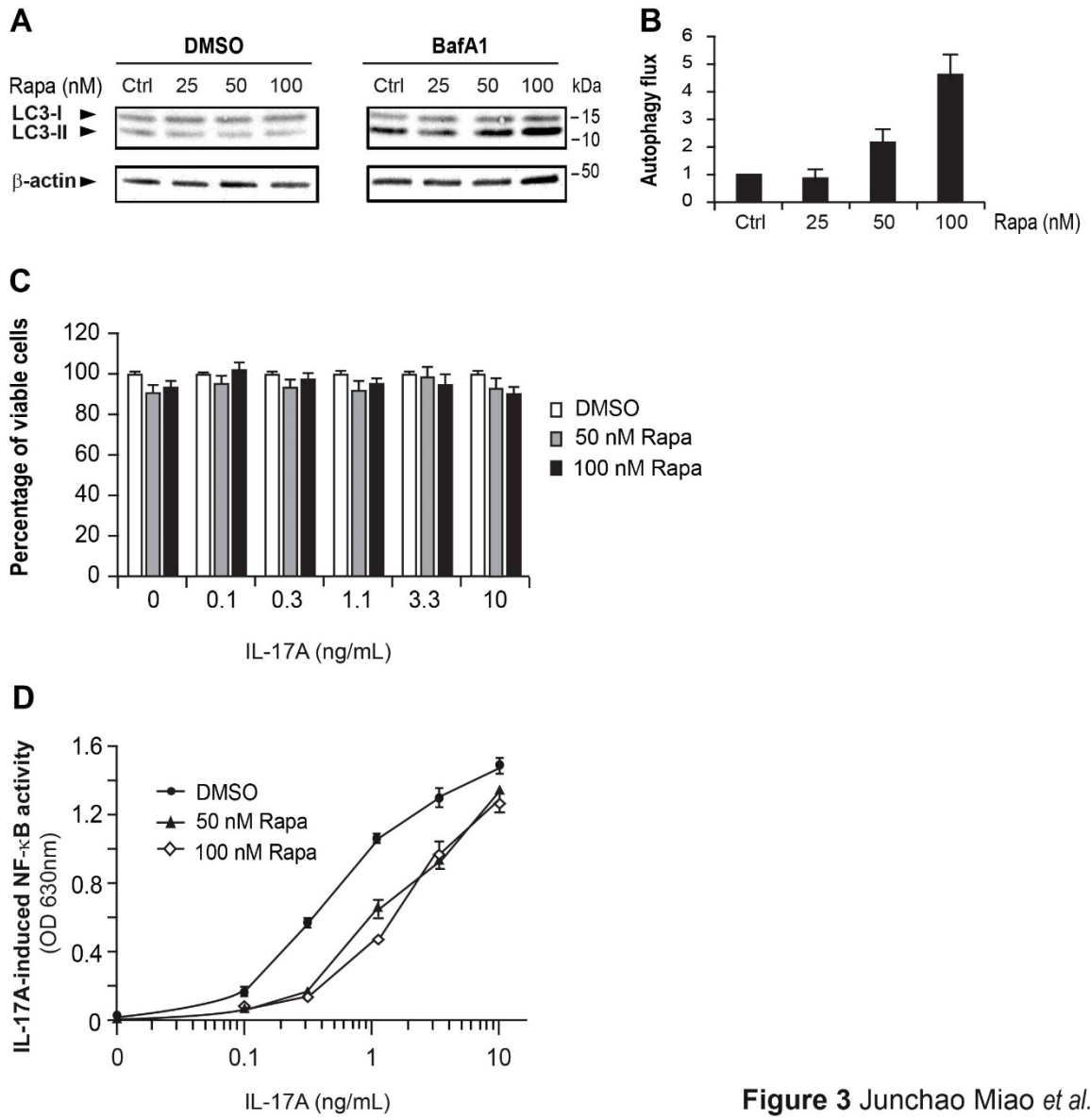


Figure 3 Junchao Miao *et al.*

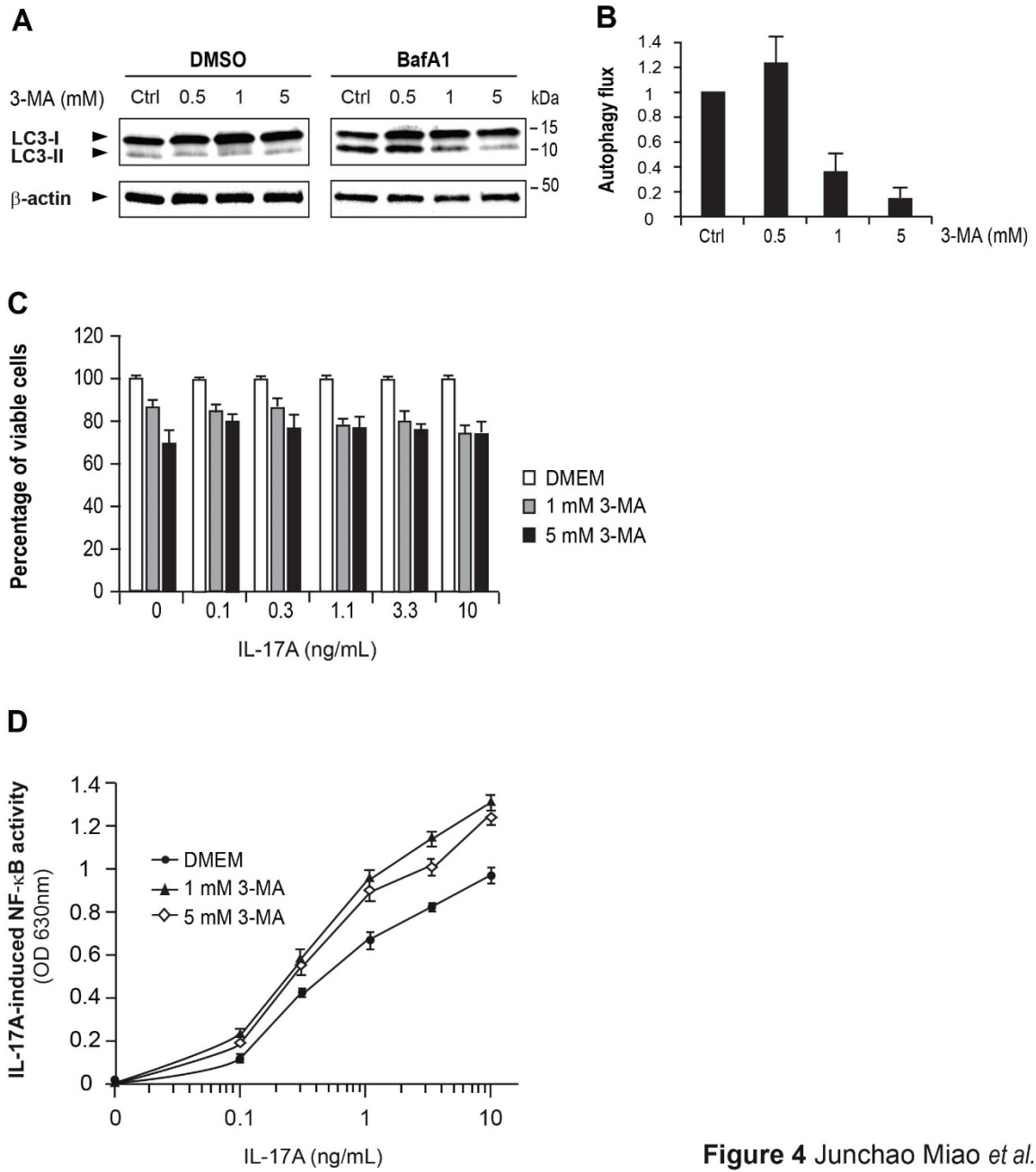


Figure 4 Junchao Miao *et al.*

DISCUSSION AND PERSPECTIVES

1. Induction of IL-17A by MCPyV ER

Using the intracellular staining and facs analysis, our study demonstrated the induction of IL-17A by MCPyV ER in HEK293T cells. However, secreted IL-17A was not detectable in the cell supernatant by standard HRP-based colorimetric ELISA test, of which the detection limit is around 100 pg mL^{-1} (Zhang et al., 2014). As the signal of intracellular IL-17A is amplified by the indirect intracellular staining, the real amount of IL-17A induced by MCPyV ER in the cell may remain low. Consistent with the fact that human embryonic kidney cells are not known as natural IL-17A producers, the capacity to express and secrete this cytokine can be moderate. In addition, IL-17A predominantly signals non-hematopoietic to induce innate-like immune defense, and IL-17A receptors are expressed in epithelial cells, including kidneys (Ho and Gaffen, 2010; McGeachy et al., 2019). Thus, secreted IL-17A induced by MCPyV ER may be quickly uptaken by its receptor present on the HEK293T cell surface, reducing the IL-17A level in the cell supernatant. Together, these factors render a very low concentration of IL-17A that may not be detected by colorimetric HRP-based ELISA test. To tackle this issue, more sensitive ELISA tests using a chemiluminescent or fluorescent substrate with a detection limit of a few picograms per mL would be helpful. To go further, a nanoparticle-enhanced ELISA test allowing to detect femtogram per mL of captured protein of interest can be attempted. To check the intracellular expression of IL-17A in transfected cells, western-blot analysis of cell lysate was also performed. However, we did not find a significant increase in IL-17A in MCPyV-ER-transfected cells compared to control plasmid-transfected cells.

As the concentration of IL-17A in the cell might be too low to easily detect, we sought the expression of IL-17A at the transcriptional level. Unexpectedly, the mRNA of IL-17A was not found in transfected cells by performing classic RT-PCR or qRT-PCR. The proinflammatory cytokine-encoding mRNA molecules usually have a short half-life and a quick turnover to prevent tissue damage caused by excessive cytokine production. One characteristic of short-lived mRNA is the presence of the adenine and uridine-rich elements (AREs) located in the 3' UTR of the mRNA that prompt the ARE-mediated decay (Anderson, 2008). The initial finding of IL-17A showed that the transcript of IL-17A bears the AU-rich repeats on its 3' UTR, making it an unstable and rapidly-decayed mRNA (Rouvier et al., 1993). Thus, a low level of IL-17A

transcripts and its instability nature may render it hard to detect by classic PCR. To this end, more sensitive and precise techniques, such as droplet digital PCR, should be considered.

The fact that IL-17A cannot be detected by classic PCR also raises the possibility of a false-positive result in intracellular staining. Instead of inducing IL-17A, MCPyV-ER may induce another molecule presenting epitope homology that is recognized by the anti-IL-17A antibody (eBio64CAP17) used for the intracellular staining. It is known that some viruses are capable to encode proteins that imitate cytokines to evade the immune response. For instance, EBV produces an IL-10-like molecule that inhibits the synthesis of IL-12 and thus the generation of Th1 immune responses (Hsu et al., 1990). Similarly, the UL111A open reading frame of HCMV has sequence homology with IL-10 (Kotenko et al., 2000). UL146 and 147 of HCMV encode an IL-8-like molecule that may affect lymphocyte trafficking (Penfold et al., 1999). Interestingly, Herpesvirus saimiri (HVS), a T cell tropic γ -herpesvirus that causes a lymphoproliferative syndrome, encodes an IL-17A-like molecule from the viral ORF13. This viral IL-17A-like protein shares 57% identity with human IL-17A (Rouvier et al., 1993). Hence, we analyzed sequence homology between human IL-17A and all MCPyV ORF-encoded proteins. No significant sequence homology was identified between IL-17A and MCPyV viral proteins, suggesting that the anti-IL-17A antibody recognized molecule is probably from the cell rather than from the virus itself. To identify this putative protein, mass spectrometry analysis upon affinity chromatography purification is required.

2. Inflammatory response upon MCPyV infection

IL-17A is a pro-inflammatory cytokine that can be induced in response to different viral infections, although the outcome can be protective or pathologic according to the context (Ma et al., 2019). Our kinetic study showed an immediate expression of IL-17A upon full-length MCPyV ER transfection, suggesting an IL-17A-mediated inflammatory response upon MCPyV infection. Among wild-type (wt) MCPyV ER genes, our data showed that wt-LT and 57kT were capable to induce IL-17A but not sT. Since sT is encoded by only one exon and bears the same exon 1 sequence as LT, the exon 2 of LT is responsible for the induction of IL-17A. We also observed a decreased IL-17A induction by 57kT and trLT compared to wt-LT. The 57kT is an alternatively spliced form of LT, which shares the same N-terminal of LT but lacks a sequence in the exon 2 encoded peptide. In line with the reduced IL-17A induction by truncated LT (trLT),

these data suggest that the intact exon 2 of LT is needed for a maximum IL-17A induction. The upstream signaling by which the exon 2 of LT induces IL-17A deserves further investigation. Using primary human dermal fibroblasts, the only cell model to date that supports the infectious MCPyV life cycle, *Krump* and colleagues reveal that MCPyV infection triggers innate antiviral response via cGAS-STING pathway. MCPyV infection activates cGAS-STING-IRF3 axis and NF- κ B pathway, inducing proinflammatory cytokine expressions, such as IL-6, IL-1 β , and TNF- α (Krump et al., 2021). However, the expression of IL-17A was not assessed in this study. It would be interesting to investigate whether MCPyV infection induces IL-17A in fibroblasts and the impact of IL-17A in viral replication. In addition, to better understand the cellular inflammatory response and draw an overall landscape of gene expression profile upon MCPyV infection, multiplex ELISA test, transcriptomic and mass spectrometry-based proteomic analysis can be considered.

The essential role of IL-17A in healthy tissue is the control of skin and mucosal homeostasis and the protection of pathogens challenge. One important cellular source of IL-17A in barrier surfaces is the resident $\gamma\delta$ T cell that plays a vital role in the innate immunity against infection (Papotto et al., 2017). Although the mechanisms underlying $\gamma\delta$ -T cell-mediated immune responses against viruses are still incompletely understood, their implications have been confirmed in several acute and chronic viral infections, including DNA oncoviruses (Poccia et al., 2005; Sciammas and Bluestone, 1999). For example, human synovial tissue- and peripheral blood-derived V δ 1 T-cell clones proliferate in response to stimulation with autologous and allogeneic EBV-transformed B-lymphoblastoid cell lines. This proliferative response requires the interaction between $\gamma\delta$ -T cells and stimulator cells since the blockade of the lymphocyte function-associated antigen 1 (LFA-1) and $\gamma\delta$ TCR/CD3 complex disrupt the response (Orsini et al., 1993). Similarly, an increase in $\gamma\delta$ T cell number during HBV seroconversion has been described. It is conceivable that $\gamma\delta$ T cells may be involved in HBV immunosurveillance and maintaining low virus levels during seroconversion (Sing et al., 1998). Together, these findings raise the possibility that $\gamma\delta$ T cells could be activated by MCPyV infection and express IL-17A to induce inflammatory responses. However, challenging $\gamma\delta$ T cells with MCPyV is tricky. On the one hand, the culture of MCPyV is still a major hurdle although the finding of human primary dermal fibroblast as the permissive cell type supporting the full MCPyV life cycle. On the other hand, despite the active roles of $\gamma\delta$ T cells in diverse human viral infections, the

mechanisms used by these cells to sense viruses and initiate rapid responses remain elusive. Human $\gamma\delta$ -T cells are capable to recognize danger signals derived directly from pathogens. TLRs play critical roles in the antiviral activities of $\gamma\delta$ T cells (Wesch et al., 2011). The production of pro-inflammatory cytokines and cytolytic activity of $\gamma\delta$ T cells can be induced by stimulation with the CpG oligonucleotide sequence via TLR9 (Rothenfusser et al., 2001). In addition to TLRs, $\gamma\delta$ TCRs can induce the antiviral activity of $\gamma\delta$ T cells by recognizing viral peptide, such as HSV glycoprotein I (Johnson et al., 1992). Therefore, it is plausible that $\gamma\delta$ T cells can directly sense MCPyV without the help of APCs. Additionally, $\gamma\delta$ T cells can be sensitized by phosphoantigens presented by infected cells or APCs (Rigau et al., 2020). Whether MCPyV-infected fibroblasts activate $\gamma\delta$ T cells is worthy of investigation. Another alternative to study the impact of MCPyV on $\gamma\delta$ T cells is transduction with lentiviral vectors expressing MCPyV genes, despite the difficulty.

3. IL-17A and skin commensal microflora

MCPyV is a commensal microbiota since the infection of MCPyV is clinically inapparent and it is chronically shed from healthy human skin (Schowalter et al., 2010). Interestingly, IL-17A is commonly induced by commensal bacteria, including *Staphylococcus epidermidis*, on the skin tissue in non-human mammals and humans under steady-state conditions (Naik et al., 2015a). This skin-localized IL-17A is produced by non-classical MHC Ib activated commensal-specific CD8⁺ Tc17 cells which also express an immunoregulatory and tissue repair signature (Linehan et al., 2018). Microbiota-driven IL-17A acts on local epithelium to promote anti-microbial responses that are necessary and sufficient to maintain a homeostatic balance but without causing overt inflammation in the normal tissue (Ivanov et al., 2009; Kumar et al., 2016; Linehan et al., 2018). As a commensal microbiota, MCPyV may also elicit a localized MCPyV-specific Tc17 response without provoking a noticeable inflammation.

4. IL-17A and MCC

The initial induction of transient and acute IL-17A inflammation is protective against opportunistic pathogens present on the skin and mucosal surface, such as *Candida albicans* and *Staphylococcus aureus* (Bär et al., 2014; Naik et al., 2015b; Sparber et al., 2018). However,

substantial evidence proves that chronic and dysregulated IL-17A inflammation can drive tumor development and progression. For instance, persistent dysbiosis prompts lung adenocarcinoma by inducing sustained IL-17A expression from $\gamma\delta$ T cells (Jin et al., 2019). IL-17A induced by chronic viral infections, including HBV and HCV promotes liver fibrosis, a major risk factor of HCC (Cachem et al., 2017; Cho et al., 2017; Yang et al., 2013). Furthermore, commensal-induced IL-17A is known to facilitate tumor development. It has been demonstrated that in the mouse gastrointestinal tract, IL-17A elicited by commensal flora is propitious for commensal-induced colon carcinogenesis and IL-17A blockade significantly attenuated Th17-driven colon tumorigenesis (Housseau et al., 2016; Sethi et al., 2018). In pancreatic cancer, the intestinal microbiome was shown to induce IL-17A expression from Th17 cells and promote pancreatic oncogenesis. Chronic inflammation and overexpression of IL-17A accelerate the initiation and progression of pancreatic intraepithelial neoplasia, while inhibition of IL-17A signaling prevents tumor formation (McAllister et al., 2014; Sethi et al., 2018). The pro-tumoral role of IL-17A is mainly attributed to its growth-promoting and survival signals that are essential for tissue remodeling and repair. Sustained IL-17A production upon repeated tissue repair induces skin cancer by triggering the proliferation of premalignant cells (Chen et al., 2019a; Wu et al., 2015a). Together with the human skin commensal feature of MCPyV, our data suggest strongly MCPyV may induce chronic IL-17A inflammation that acts synergistically with the long-term UV-exposure-mediated skin injury repair to drive MCC tumorigenesis. The relatively high level of IL-17A we observed upon full-length MCPyV ER transfection may be reminiscent of an acute and transient IL-17A response that exhibits a protective role against the very first MCPyV infection. Compared to full-length MCPyV ER and wtLT, we observe nonproduction and lower induction of IL-17A by sT and trLT, respectively. As MCC tumors express both intact sT and trLT, it is possible that, in the contrast to MCPyV ER and wtLT, these two oncoproteins provoke a mild chronic IL-17A inflammation that induces the hyperproliferation of MCC premalignant cells, although the cells of origin of MCC is still unclear.

The idea that MCPyV-induced IL-17A promotes MCC raises further interesting points that deserve in-depth exploration. Such a hypothesis suggests that MCPyV also contributes to the development of MCPyV⁻MCC by inducing chronic IL-17A without integrating its genome into MCC cells. In this circumstance, the major IL-17A sources might come from the cells

persistently infected by MCPyV. These cells could be immune cells sensing and responding to MCPyV infection such as DCs, $\gamma\delta$ T cells, Tc17 cells, and Th17 cells, or other MCPyV permissive cells. Due to the lack of knowledge about the cells of origin of MCC, the effects of IL-17A on MCC stem cells or premalignant cells cannot be assessed. Nevertheless, it would be interesting to examine the role of IL-17A in the proliferation and survival of both MCPyV⁺ and MCPyV⁻ MCC cell lines. As the majority of MCC bears MCPyV and expresses trLT, IL-17A might be induced directly by MCPyV in MCC cells and act in an autocrine way to foster the proliferation of these cancer cells. To investigate whether integrated MCPyV persistently induces IL-17A, cells stably expressing MCPyV genes established by using lentiviral vectors would be an appropriate model. Another simple alternative is the use of MCPyV⁺MCC cell lines bearing naturally MCPyV genes. We tested IL-17A expression in WaGa cells, one MCPyV⁺MCC cell line that persistently expresses sT and trLT. Unexpectedly, IL-17A was not found in this cell line. Therefore, other MCPyV⁺ cell lines, such as MKL-1, Peta, and MS-1, should be examined. To explore further the role of IL-17A in MCC progression, it is crucial to evaluate the expression of both systemic and intratumoral IL-17A in the physiological condition in patients with MCC.

4.1 IL-17A signaling and MCC carcinogenesis

It is well documented that IL-17 receptors are ubiquitous and IL-17A signals preferentially non-hematopoietic cells and can transduce signaling leading to cell proliferation and survival. Owing to the absence of knowledge about MCC stem cells and appropriate animal models, whether MCPyV-induced IL-17A contributes to MCC tumorigenesis and the underlying mechanisms are arduous to investigate. However, emerging studies concerning the role of IL-17A in tumorigenesis may provide important insight. IL-17A is emerging as an important cytokine in the initiation and progression of skin cancer (He et al., 2012; Li et al., 2019). Normal tissue homeostasis at mucosal surfaces such as the skin and intestine is maintained by a steady turnover of epithelial cells generated by adult tissue stem cells (Ge and Fuchs, 2018). The dynamic turnover is finely controlled to prevent abnormal tissue growth. The action of mitogenic factors (growth factors and morphogens) required for tissue stem cell self-renewal is often anatomically restricted in a niche, such as the crypt in the intestine and hair follicle in the skin, to limit the proliferative activity to a confined compartment (Yang et al., 2017a). In addition, adult tissue stem cells also express negative regulators that restrict the activity of potent mitogenic stimuli (Powell et al., 2012). In contrast, infection and tissue injury can

readily accelerate the proliferation of tissue stem cells to rapidly supply new epithelial cells that migrate to and repair a breached barrier (Karin and Clevers, 2016). Interestingly, IL-17 was recently identified as a critical mediator that activates a group of Lrig1⁺ stem cells residing in the hair follicle to participate in wound healing and tumorigenesis in the skin (Chen et al., 2019). This is achieved by the integration of IL-17 signaling with epidermal growth factor receptors (EGFR) through Act1-TRAF4-ERK5 axis. In Lrig1⁺ stem cells in the skin, stimulation with IL-17 leads to recruitment of EGFR to the IL-17 receptor complex by TRAF4. The close proximity of IL-17R and EGFR allows Act1 to recruit Src for IL-17-induced phosphorylation of EGFR and subsequent activation of the MEKK3–MEK5–ERK5 pathway. Activation of this axis causes the Lrig1⁺ cells to produce progeny that migrates out of the hair follicle and participate in wound healing and tumorigenesis (Chen et al., 2019). Similarly, IL-17A signaling promotes keratinocyte proliferation and tumorigenesis via the IL-17R-Act1-TRAF4-MEKK3-ERK5 cascade (Wu et al., 2015). Although this mechanism is demonstrated in a mouse model, the TRAF4-ERK5 is also hyperactivated in human skin SCC (Wu et al., 2015b). Since keratinocytes and epidermal stem cells are possible candidates for the cells of origin of MCC, it is rational to infer that MCPyV-induced IL-17A may promote the formation of premalignant MCC from these cells using the same mechanism.

4.2 IL-17A and MCC progression

Angiogenesis and metastasis

Apart from its direct mitogenic effect, IL-17A creates a pro-tumoral microenvironment to promote cancer progression. This effect is dependent on its ability to induce cancer cell mobility and angiogenesis, reshaping the phenotype of stromal cells, and establishing the immunosuppressive microenvironment. IL-17A promotes angiogenesis via the induction of VEGF by tumor cells and cancer-associated fibroblast (Liu et al., 2011; Numasaki et al., 2003; Pan et al., 2015). Thus, it is interesting to examine the expression of IL-17A and VEGF in MCC tumor biopsies and scrutinize whether IL-17A induces VEGF in MCC cells and MCPyV-infected fibroblast. IL-17A also accelerates cancer metastasis by stimulating cell mobility potential. This is achieved via the upregulation of MMPs (Li et al., 2011). Interestingly, MCPyV T-Ags promote cell mobility and disrupt cell-cell junction by remodeling the cytoskeleton turnover and inducing a disintegrin and metalloproteinase, respectively (Nwogu et al., 2018; Stakaitytė et

al., 2018), suggesting that MCPyV T-Ags and T-Ags-induced IL-17A act synergistically to promote MCC metastasis.

MDSCs

IL-17A is also known to promote cancer progression by introducing immunosuppressive properties. IL-17A causes the mobilization and recruitment of MDSCs into the tumor site by inducing myeloid-mobilizing cytokines, such as G-CSF, in tumor-associated fibroblast through NF- κ B and ERK signaling (Chung et al., 2013). IL-17A also enhances MDSCs infiltration by inducing the expression of chemokines CXCL1 and CXCL5 via tumor cells (Novitskiy et al., 2011). In humans, MDSCs are generally divided into three distinct subsets: monocytic-MDSCs phenotypically defined as CD11b⁺CD33⁺CD15⁻CD14⁺HLA-DR^{low} and granulocytic-MDSCs defined as CD11b⁺CD33⁺CD15⁺CD14⁻HLA-DR⁻, and an immature state lacking expression of both granulocytic and monocytic markers (Bronte et al., 2016). In a small proportion of MCC, CD11b⁺ CD33⁺ HLA-DR^{low} myeloid cells are present in the tumor nest and around the tumor periphery (Gaiser et al., 2018; Kervarrec et al., 2018). Further investigation should be considered to examine whether these MDSCs can be elicited by IL-17A. Owing to the lack of animal models for MCC, the hypothesis of MDSCs induction via IL-17A and the underlying mechanism are strenuous to study. Nonetheless, multicolor immunohistochemistry or immunofluorescence staining of crucial markers, such as IL-17A, CD11b, CD33, HLA-DR, and G-CSF can be performed using MCC and normal skin biopsies.

5. Th17 in MCPyV infection and MCC

Another critical cellular source of IL-17A is Th17 cells. Whether MCPyV infection evokes Th17 response is unclear. However, Th17 responses are observed in multiple viral infections, including oncoviruses, and contribute to cancer development and progression. Persistent infection with the high-risk HPVs, a small family of non-enveloped dsDNA viruses, is a major cause of uterine cervical cancer (Schiffman et al., 2016). In the patients with uterine cervical cancer, high level of Th17 related cytokines, such as IL-17A, IL-22, and IL-23, are observed in cervical mucosa and the number of Th17 cells is directly associated with the levels of IL-6, IL-23, and IL-17 (Alves et al., 2018; Christensen-Quick et al., 2016). These cytokines, specifically IL-17A, increases the expression and activity of MMP2 and 9 due to their ability to reduce the

expression of tissue inhibitors of matrix metalloproteinase (TIMP) 1 and 2 through the p38/NF- κ B signaling pathway. This then ultimately leads to an increased ability of the tumor cells to migrate and invade (Feng et al., 2014). This event shows the potential roles of IL-17 in tumor formation and metastasis by destroying the extracellular matrix (ECM), which results in the invasion of the tumor cells from the ECM to the blood or lymph vessels reaching other tissues and organs and eventually in the production of a new tumor. Studies have shown that women with cervical cancer have higher levels of Th17 and Treg lymphocytes than the other lymphocytes in tumor tissues, compared to non-infected women with no history of cervical lesions (Alves et al., 2018; Christensen-Quick et al., 2016). More interestingly, BKPyV infection could induce Th17 responses that subsequently promote BKPyV-associated cancer. Similar to MCPyV, BKPyV is a member of hPyV that can cause persistent infections in the kidneys and the urinary tracts. It is an opportunistic virus that provokes several morbidities in immunocompromised individuals, including nephritis, hemorrhagic cystitis, urethral stenosis, and cancers, although the latter is less frequent (Ambalathingal et al., 2017; Levican et al., 2018). The main mechanisms used by BKPyV to induce human cancers are yet to be clarified but it has been proposed that BKPyV may use several strategies to promote cancers in humans including attenuation of immune responses via up-regulation of immunosuppressor molecules, such as TGF- β (Kariminik and Kheirkhah, 2017). TGF- β is a vital pleiotropic cytokine that modulates the attenuation of the immune responses and favors tumor formation and promotion (Batlle and Massagué, 2019). The major role of TGF- β has been shown in the development of Th17 and Treg subtypes, induction of the inflammatory responses by promoting the differentiation of Th17 cells, prevention of the immune responses through the activation of Treg cells, suppression of the Th cells, and cytotoxic T cell functions, and ultimately involvement in angiogenesis and tumor progression (Batlle and Massagué, 2019). Taken together, the abovementioned findings strongly suggest that Th17 responses can occur upon MCPyV infection and contribute to MCC formation.

6. Immune tolerance in MCPyV-induced MCC

TGF- β

TGF- β is an immunosuppressive mediator that restrains the anti-tumor immunity (Batlle and Massagué, 2019). Interestingly, it has been shown that murine polyomavirus middle T antigen

induces breast cancer in mice via the upregulation of TGF- β type III receptor (T β RIII) in the mammary cells (Criswell et al., 2008). Similarly, the growth of polyoma middle T antigen-induced tumor can be hindered by inhibiting the TGF- β intracellular signaling pathway (Yee et al., 2006). In addition, upregulation of TGF- β in the polyomavirus middle T antigen-expressing tumor cells is associated with increased metastasis in animal models (Muraoka-Cook et al., 2004). In the human case, TGF- β is a main factor for the regulation of BKPyV gene expression. TGF- β regulates BKPyV gene expression via modulation of viral early promoter activity in a strain-dependent manner (Abend and Imperiale, 2008). Similar effects have been also observed in JCPyV (Ravichandran et al., 2007). The roles of TGF- β receptor downstream signaling molecules, SMADs, on the regulation of expression of the early and late antigens of JCPyV have also been documented (Stettner et al., 2009). Together, these studies imply that TGF- β could play important role in MCPyV infection and MCC progression. Although MCPyV doesn't have the middle T antigen, the ALTO which is evolutionarily related to middle T of murine polyomavirus is expressed during MCPyV replication (Carter et al., 2013). The function of MCPyV ALTO remains elusive. The expression of ALTO is not essential for MCPyV replication in cell culture but could play an accessory role in MCPyV life cycle and MCC tumorigenesis. It would be interesting to study whether MCPyV infection induces TGF- β and how TGF- β , in turn, influences MCPyV life cycle and its role in MCC development and progression.

Treg

High numbers of Treg cells are identified in MCC tumor site. Natural Treg (nTreg), induced by self-antigens in the thymus, home to sites of tumors, while iTreg, induced by antigens in the periphery, are created as a result of a specific cytokine milieu of the TME. In terms of cellular markers, both Treg cells are characterized by the expression of the master transcription factor, Foxp3. iTreg cells are derived from peripheral naive T cells induced by tumor microenvironmental signals, including tumor antigens, cytokines such as TGF- β and IL-10, and other soluble molecules (Wang et al., 2019). The TCR repertoires of tumor-resident iTregs vary yet display significant overlap with circulating Tregs. TCRs isolated from Tregs display specific reactivity against autologous tumors and mutated neoantigens, suggesting that intratumoral Tregs act in a tumor antigen-selective manner, leading to their activation and expansion in the TME. Tumor antigen-specific Treg-derived TCRs reside in the tumor and in the circulation, implying that both Tregs types serve as sources for tumor-specific TCRs (Ahmadzadeh et al.,

2019). Whether Tregs isolated from MCC are MCPyV specific requires further investigations. Interestingly, Th17 cells are another important source of tumor-induced Foxp3⁺ cells. In addition to nTregs and iTregs that develop from naive precursors, Th17 cells can switch their phenotype toward a suppressive IL-17A⁺Foxp3⁺ and ex-Th17 IL-17⁻Foxp3⁺ phenotypes which are sources of tumor-associated iTregs (Downs-Canner et al., 2017). The transdifferentiation of Th17 cells to Treg phenotype can be triggered by the presence of TGF- β and PEG2 (Downs-Canner et al., 2017). Based on these facts, it is likely that MCPyV-specific Treg cells may be induced during the infection and contribute to viral persistence and MCC formation. Whether MCPyV-specific Th17 cells, if exist, could be the origin of a part of Treg cells in MCC require more investigation. To unravel these puzzles, identification, and isolation of circulating and tumor-infiltrating Th17 (CD4⁺IL-17A⁺FoxP3⁻) and Treg cells (CD3⁺FoxP3⁺) using blood samples and tumor biopsies from patients with MCC are required. As T cell-specific MCPyV epitopes are being identified (Jing et al., 2020; Tabachnick-Cherny et al., 2020) (Figure), using HLA-MCPyV epitope tetramer to obtain MCPyV-specific Th17 and Treg cells is possible. Finally, using *in vitro* cell culture model with different cytokine cocktails, such as TGF- β and PEG2, the plasticity between MCPyV-specific Th17/Treg cells can be investigated.

Immune checkpoints

Immune checkpoint expression that results in T cell exhaustion is often observed in human tumor microenvironments. Chronic viral infection and tumor antigen stimulation can cause T cell exhaustion via upregulation of immune checkpoints. In MCPyV⁺MCC tumor, a high level of PD-L1 expression is identified, whereas PD-L1 is absent in MCPyV⁻MCC, suggesting that MCPyV infection induces PD-L1 expression (Lipson et al., 2013; Mitteldorf et al., 2017). It has been shown that in some human cancers, including liver, prostate, and colon cancers, PD-L1 is upregulated by IL-17A. In human prostate and colon cancer cells, IL-17A treatment increase PD-L1 expression through AKT/ERK axis and NF- κ B signaling (Wang et al., 2017). In HCC, IL-17A induces PD-L1 upregulation in hepatoma stem cells (Wei et al., 2019). Hepatoma stem cells interact with lymphatic endothelial cells through the engagement between the high-mannose type N-glycans and the mannose receptor, which activates IL-17A expression in lymphatic endothelial cells. Induced IL-17A promotes the self-renewal of hepatoma stem cells and upregulates PD-L1 expression via STAT3 phosphorylation (Wei et al., 2019). Taken together with our data showing IL-17A induction by MCPyV, we speculate that MCPyV induces PD-L1

expression in MCPyV⁺MCC tumors through the action of IL-17A. We examined the PD-L1 expression in MCPyV-ER transfected HEK293T cells, as well as IL-17A treated HEK293T cells by performing cell surface staining using an anti-PD-L1 antibody. After the transfection and treatment, cells barely expressed PD-L1 on their surface, and the level of PD-L1 was not changed regardless of the transfection and treatment. This may be due to two main reasons. Firstly, HEK293T cells are not known as the natural cell tropism for MCPyV. It would be more adequate to use human dermal fibroblast as a cell model to study whether MCPyV infection upregulates PD-L1. Secondly, the induction of immune checkpoints can be a result of chronic inflammation mediated by persistent viral infection. In this case, using cells stably expressing MCPyV genes generated by transduction with lentiviral vectors would be more appropriate. Whether IL-17A treatment upregulates PD-L1 expression in MCPyV⁺MCC cells is also interesting to study.

7. Autophagy and MCC

7.1 Limitations of our study on the autophagy

Importantly, the conclusion on the role of MCPyV ER products and IL-17A in the regulation of autophagy is drawn from western blot analysis of LC3-II flux. Further experiments are indispensable to confirm our findings. First, it is crucial to examine in transfected cells the protein level of each MCPyV antigens which can be potentially targeted by autophagy. Secondly, the increase of LC3-II flux can also result from the activation of non-canonical autophagy, such as LC3-associated phagocytosis (LAP). LAP combines the molecular machinery of phagocytosis and autophagy, resulting in the conjugation of LC3 to phosphatidylethanolamine (LC3-II) on the phagosome membrane, generating so-called "LAPosomes" that undergo fusion with lysosomes (Heckmann et al., 2017; Herb et al., 2020). Canonical autophagy targets cytoplasmic components into double-membrane autophagosome whereas LAPosomes retain the single-membrane nature of phagosomes (Huang et al., 2009). LAP process requires overlapping but non-identical genes compared to canonical autophagy. The maturation of LAPosome is independent of ULK1 complex but requires the association of rubicon with the PI3KC3 complex (Martinez et al., 2015). Therefore, the transfection of MCPyV gene expressing plasmid might also activate the LAP leading to increased LC3-II flux. To affirm the involvement of the autophagy process in our experiments

images of autophagosomes or autolysosomes from transmission electron microscopy are needed. Moreover, p62 is frequently used as another marker for autophagy in addition to LC3-II, which can bind to LC3 and be degraded by autophagy (Pankiv et al., 2007). The observation of p62 level in cell lysate by western blot is helpful to confirm our results.

It is worthy to note that our analysis of LC3-II flux was only carried out at 24h after transfection. Furthermore, most transfection reagent contains lysosome inhibitor to protect the plasmid from degradation. Therefore, the analysis of LC3-II flux at an early time point is not robust enough as an argument. To consolidate the results and draw a dynamic picture of the autophagy process, a kinetic study of LC3-II flux at different time points is necessary.

Our studies on the crosstalk between IL-17A signaling and autophagy were based on pharmacologic drugs that can modulate autophagy, such as BafA1 and 3-MA. However, these drugs are not specific to autophagy. For instance, the 3-MA inhibits autophagy process by blocking the PI3CK3, which is also required for LAP. Therefore, to obtain convincing results, genetic approaches altering autophagy-specific genes should be applied.

7.2 Autophagy and MCPyV infection

Our data show that ectopic expression of MCPyV wtLT increases significantly the LC3-dependent autophagy flux in HEK293T cells, suggesting activation of autophagy upon the infection of wild-type MCPyV. This raises several interesting questions. First, whether the increased autophagy leads to the degradation of wtLT and ultimately prevents MCPyV replication needs to be attentionally investigated. Because increased autophagy is not always associated with reduced viral replication but in contrast, promotes viral infection (Hussein et al., 2012; Meng et al., 2012; O'Donnell et al., 2011; Sir and Ou, 2010). Autophagy orchestrates the vesicles trafficking to new locations within the cell thereby providing membranous structures for viral replication. RNA viruses require intracellular membranes to serve as scaffolds for viral replication within the cytoplasm (Salonen et al., 2005). For instance, HCV hijacks autophagy pathways in a PI3K III independent manner to favor the expression of viral proteins as well as viral replication (Sir et al., 2012). DNA viruses, such as adenovirus and HPV induce autophagy to promote viral replication (Rodriguez-Rocha et al., 2011; Zhou and Münger, 2009). BKPyV, another widespread opportunistic HPyV causing nephropathy in immunosuppressed patients, requires the autophagy pathway for optimal viral replication

(Bouley et al., 2014). BKPyV activates autophagy and localizes to the autophagosome upon early infection, leading to enhanced viral replication. The interaction between BKPyV and autophagosome may facilitate trafficking the virus towards the endoplasmic reticulum (ER) for viral disassembly. The autophagosome-lysosome fusion-induced acidification is required for BKPyV infection (Eash et al., 2004) probably because the acid environment induces viral capsid degradation or the modification of capsid protein structure leading to the release of the viral genome (Bouley et al., 2014). Of interest, in the study of infectious entry of MCPyV, *Becker*, and colleagues found that MCPyV VLP is encircled by a lipid envelope in endosomal and endolysosomal compartments (Becker et al., 2019). This suggests that autophagy may be involved during the cytoplasmic trafficking of MCPyV from endosome to ER. However, this putative involvement of autophagy seems to be protective for host cells since only a minor number of viral particles were found in the lumen of ER (Becker et al., 2019).

Autophagy can directly degrade virus or viral components to restricts viral replication via the selective pathway which involves autophagy adaptor proteins, such as p62, NPD52, OPTN, and TAX1BP1 (Choi et al., 2018). The adaptor proteins recognize ubiquitinated cargo proteins through their C-terminal ubiquitin-associated domain (Kraft et al., 2010; Stolz et al., 2014). Whether MCPyV wtLT is targeted by adaptor proteins and thus degraded via selective autophagy pathway requires further investigations. It is known that the LT antigen of JCPyV, another member of HPyVs that opportunistically cause leukoencephalopathy in immunocompromised patients, can be degraded by Bag3-induced autophagy (Sariyer et al., 2012). Bag3 is a co-chaperone that participates in the chaperone-assisted selective autophagy leading to the engulfment and degradation of target proteins (Kraft et al., 2010; Rosati et al., 2011). This is achieved by inducing the association of the Hsc70/Hsp70 chaperone machinery with p62, thus directing the cargo proteins to the selective autophagy pathway for lysosomal degradation (Arndt et al., 2010). The interaction between JCPyV LT and Bag3 requiring the zinc finger (Znf) domain of LT and proline-rich repeat of Bag3 induces autophagic degradation of LT (Sariyer et al., 2012). Interestingly, the Znf domain is a conserved sequence also presented at the N-terminus of the helicase domain in MCPyV wtLT, suggesting strongly that MCPyV wtLT can be targeted by Bag3-mediated selective autophagy. Consistently, our data show a decreased autophagy flux in truncated LT-transfected cells compared to wtLT, which may be probably due to the lack of Znf domain on the otLT because of the truncation.

7.3 Autophagy and MCPyV⁺MCC

The adaptive immune response is initiated by the T-cell priming, which consists of the MHC/antigen complex presentation by APCs to the TCR. The presence of MCPyV T-Ag specific CD4 and CD8 cells within the MCC tumor indicates effective T-cell priming. However, these MCPyV T-Ag-specific responses are not efficient enough to restraint MCC progression because of multiple immune evasion mechanisms. In APCs, endogenous antigens can be degraded either by canonical autophagy or by chaperone-assisted autophagy and then are presented onto MHC class II via the lysosome MHC II compartment (Deffit and Blum, 2015; Dengjel et al., 2005). Autophagy also contributes to the presentation of endogenous viral antigens on MHC class I, when TAP proteins are blocked by viral proteins (Verweij et al., 2015). For instance, despite the conventional MHC class I presentation pathway during the initial infection of HSV1 in macrophages, a second pathway involving a vacuolar compartment was triggered later during infection. Specifically, a previously unknown type of autophagosome originated from the nuclear envelope and mediated the presentation of HSV-1 antigens on MHC class I molecules (English et al., 2009). Likewise, during latent infection, the HCMV protein UL138 is primed to CD8⁺ T cells by MHC class I in an autophagy-dependent pathway and colocalizes with LAMP2-positive late endosomal structures (Tey and Khanna, 2012; Tey et al., 2010). Therefore, autophagy is crucial for both MHC class II and I presentation of cytoplasmic viral antigens.

MCPyV wtLT-induced autophagy may not only result in LT degradation that prevents viral replication but also enhance antigen presentation to activate specific anti-MCPyV immune responses. This hypothesis needs to be further verified in APCs harboring the LT cocultured with naïve T cells. However, when transfected with the full-length wild-type MCPyV ER, which is more relevant to mimic MCPyV infection, the cellular autophagy flux was not affected compared to control plasmid-transfected cells. This suggests that MCPyV may establish mechanisms to hamper autophagy pathways to evade the autophagy-mediated antigen presentation, explaining partially the absence of T-Ag-specific responses in MCPyV-infected healthy population. The putative autophagy evasion mechanisms of MCPyV remain elusive but may not be mediated by MCPyV sT or 57kT, since the expression of either protein did not downregulate the autophagy flux in our HEK293T cell model. Our results are not in line with the recent study showing that ectopic expression of MCPyV sT in MCPyV⁻MCC cell lines

increase microRNAs that suppress autophagy by targeting multiple autophagy-related genes (Kumar et al., 2020). In the study, the authors showed that transfection of MCPyV⁻MCC cell lines with sT expressing vector decreased LC3-II level. However, the LC3-II protein level at an indicated time point does not reflect the number of newly synthesized autophagosomes and cannot represent the autophagic activity. In other words, decreased amount of LC3-II upon MCPyV sT expression does not necessarily indicate a reduced autophagy activity. Because LC3-II can ectopically localize on non-autophagosome structures that are not turned over in the lysosome (Mizushima et al., 2010; Tanida et al., 2005). Therefore, it is essential to determine the autophagy flux that is the amount of LC3-II degraded in a lysosome-dependent manner during a certain time period. The level of autophagy flux can be measured by the difference in the amount of LC3-II between cells treated with and without lysosome inhibitors, such as bafilomycin A1 (BafA1), E64D, or pepstatin A. Thus, to investigate the impact of MCPyV sT on autophagy activity, it is certainly more appropriate to compare the autophagy flux in MCPyV sT expressing cells to control cells. In the study of *Kumar et al.*, the differences between normalized LC3-II levels in cells treated with and without BafA1 were not assessed. Further analysis are needed to confirm the results. In addition, according to the provided results of LC3-II western-blotting, MCPyV LT transfection seemed to enhance autophagy activity which is consistent with our findings. The controversial results may be explained by the use of different cell models because autophagy is a complex process that is often cell- and context-dependent.

Compared to wtLT, the expression of otLT did not influence the LC3-II flux, suggesting an evasion mechanism of otLT to avoid autophagic degradation. This may contribute to the sustainable presence of otLT, ultimately promoting MCC. In addition, in MCC cells, mTOR signaling is chronically activated, leading to suppressed autophagy, thus preventing the tumor cells from autophagic cell death (Lin et al., 2014). Taken together, these findings suggest that enhancing autophagy can be a considerable therapeutic strategy for patients with MCPyV⁺MCC. Of note, autophagy is a double-edged sword in cancer that can be deleterious depending on the context (Yun and Lee, 2018). The inhibitors of autophagosome/lysosome fusion and lysosomal degradation, including chloroquine (CQ) and hydroxychloroquine (HCQ), have been used in clinical trials for the treatment of certain types of solid tumors, and have shown safety and efficacy (Mulcahy Levy and Thorburn, 2020). Tumors are exposed to

stressful conditions, including hypoxia and nutrient deprivation. Autophagy helps cells overcome these stresses. Autophagy fulfills the high metabolic and energetic demands of proliferating tumors by recycling intracellular components to supply metabolic substrates (Rabinowitz and White, 2010). Therefore, autophagy contributes to tumor-cell survival by enhancing stress tolerance and supplying nutrients to meet the metabolic demands of tumors. The inhibition of autophagy or knockdown of autophagy genes can result in tumor-cell death (Mulcahy Levy and Thorburn, 2020). Furthermore, autophagy promotes the growth of high mutational burden liver cancer by inducing immune tolerance, including enhancing Treg cells and T cell exhaustion and suppressing IFNs responses (Poillet-Perez et al., 2020). Moreover, autophagy can promote immune evasion of cancer cells by degrading MHC-I, such as in pancreatic cancer (Yamamoto et al., 2020).

8. Interplay between autophagy and IL-17A signaling

The crosstalk between inflammatory response upon PRR activation and autophagy play important role in the innate defense against pathogens. The adaptor proteins of certain PRRs initiating the inflammatory signaling, such as MYD88 and TRIF, bind to Beclin-1, unleashing it from its inhibitory complex, activating thus autophagy (Shi and Kehrl, 2008). Signaling through MYD88 and NF- κ B further increases autophagosome maturation by inducing the expression of DNA damage-regulated autophagy modulator protein 1 (DRAM1), a lysosomal transmembrane protein involved in lysosomal acidification (Meijer and van der Vaart, 2014). Moreover, TLR4-mediated TRAF6 activation induces K63 ubiquitylation of beclin 1 to release it from the inhibitory interaction with BCL-2, thus activating autophagy (Shi and Kehrl, 2010). Inflammatory signaling also induces selective autophagy. TRAF3 mediates K63 ubiquitylation of TBK1, which phosphorylates optineurin to increase its binding affinity for LC3, thereby promoting xenophagy by crosslinking LC3 and bacteria bound by OPTN (Wild et al., 2011). The inflammation-mediated autophagy has also been validated *in vivo*. For instance, Rift Valley fever virus triggers autophagy downstream of TLR7 signaling to restrict viral replication in *Drosophila melanogaster* (Moy et al., 2014). In mice, MYD88-dependent induction of autophagy in the intestinal epithelium prevents the dissemination of *Salmonella enterica* (Benjamin et al., 2013). Interestingly, however, autophagy can negatively regulate inflammatory signaling which is believed to avoid over-exuberant inflammation. Autophagy

inhibits TLR signaling by promoting selective degradation of TRIF through the tripartite motif-containing protein 32 (TRIM32)–TAX1-binding protein 1 (TAX1BP1) complex (Yang et al., 2017b). Beclin 1 interacts with and impairs the nucleotidyl transferase activity of cGAS, decreasing the amount of cGAMP available to stimulate STING (Liang et al., 2014). During the late phase of HSV-1 infection, STING is also targeted by CMA-mediated degradation to terminate IFN signaling (Hu et al., 2016). Therefore, it is generally accepted that inflammation-induced autophagy reinforces the innate host defense at the beginning of the inflammatory responses. Reversely, autophagy offers negative feedback that inhibits inflammatory signaling to prevent excessive and long-term inflammation.

Our study revealed that IL-17A-mediated inflammation induces LC3-II flux, suggesting an increased autophagy flux. It would be interesting to further investigate whether the induction of autophagy is triggered by TRAF6-mediated K63 ubiquitylation of Beclin-1, as TRAF6 is responsible for canonical IL-17A signaling transduction. Although our data showed IL-17A-mediated inflammation induces autophagy, it does not necessarily indicate IL-17A-specific autophagy. The induction of autophagy may be simply as a result of TRAF6 activation which also occurs upon the stimulation of other PRRs. Thus, it is necessary to examine whether ACT1 activation is sufficient to induce autophagy. ACT1 is the specific adaptor protein and E3 ubiquitin ligase that initiate IL-17A signaling. In addition to the activation of TRAF6-dependent inflammation, ACT1 prevents the degradation of target mRNA to stabilize target gene expression (Herjan et al., 2018). It is intriguing to study whether ACT1 induces the stabilization of autophagy-related genes transcripts to ultimately activate autophagy. The inhibition of IL-17A-mediated inflammation upon autophagy activation that we observed suggests strongly regulatory feedback of autophagy to avoid prolonged inflammation. Whether autophagy activation results in the degradation of proteins involved in ACT1-mediated signaling to inhibit the inflammation requires further investigations. To confirm whether MCPyV LT-induced autophagy is able to dampen IL-17A inflammation, Quanti-Blue assay after MCPyV LT transfection in HEK-Blue™ IL-17 cells should be carried out.

In the context of MCPyV infection, we showed that MCPyV LT induced a higher level of IL-17A than otLT did. Coincidentally, our results suggest that the LT induces a high level of autophagy while otLT barely activates autophagy. Thus, it is also possible that LT induces IL-17A that subsequently signals the cell in an autocrine manner to indirectly activate autophagy. To test

this hypothesis, IL-17A neutralizing antibody could be used after the LT transfection in HEK-Blue™ IL-17 cells. Of note, the tested IL-17A doses that induced autophagy (1.1-3.3-10 ng/mL) in our study are much higher than the physiologic level in healthy human serum which is in the range of picogramme per mL. Only patients with IL-17A-related inflammatory diseases, such as psoriasis and Langerhans cell histiocytosis can reach such a high level of seral IL-17A concentration (Ismail et al., 2020; de Oliveira et al., 2015). According to our *in-vitro* results, low concentration of IL-17A (< 1.1 ng/mL) did not activate autophagy. Therefore, the otLT-induced mild IL-17A may not strong enough to trigger the autophagy-mediated negative feedback. This leads to persistent and low-grade IL-17A inflammation that contributes to MCC development. Therefore, it is crucial to study the IL-17A profile in the serum and biopsies of patients with MCC.

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