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Neuroimmune regulation of JCV by Immune mediators in glial cells

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ABSTRACT

The human polyomavirus JC (JCV) is a small DNA virus responsible for the initiation of progressive multifocal leukoencephalopathy (PML), an often lethal disease of the brain characterized by lytic infection of oligodendrocytes in the central nervous system (CNS). Patients undergoing immune modulatory therapies for the treatment of autoimmune diseases such as multiple sclerosis, and individuals with an impaired-immune system, most notably AIDS patients, are in the high risk group of developing PML. Previous studies suggested that soluble immune mediators secreted from PBMCs inhibited viral genomic replication. However little is known regarding the molecular mechanism of this regulation. Here we investigated the impact of conditioned media (CM) from activated PBMCs on viral replication and gene expression by molecular virology techniques. Our data showed that viral gene expression as well as viral replication was suppressed by the CM. Further studies revealed that soluble immune mediators from PBMCs possessed a dual control on T-antigen expression at transcription and post-transcription level. These observations demonstrate a novel role of immune mediators in regulation of JCV gene expression, and provide a new avenue of research to understand molecular mechanism of viral reactivation in patients who are at risk of developing PML.

CHAPTER 1

INTRODUCTION

1.1 Polyomaviruses

Polyomaviruses were originally classified as *Papovaviridae*, which included both polyomaviruses and papillomaviruses. In 2000, the International Committee on the Taxonomy of Viruses split the family into *Polyomaviridae* and *Papillomaviridae*. *Polyoma* stems from the Greek words "poly," meaning many, and "oma," meaning tumors, as many of these viruses have been found to cause tumors in non-native host species.

There are 15 known polyomaviruses. They can infect a diverse array of species from humans to non-human primates, murinae, bovinae, and aves (Table 1). The most commonly studied polyomaviruses are the simian virus 40 (SV40), mouse polyoma, and human polyomaviruses JCV and BKV. Over the past few years, new human polyomaviruses have been discovered after the screening of infected human respiratory secretions and tumor tissues. The first among these new human polyomaviruses was discovered by sequencing DNA in respiratory secretions and analyzing its sequences using GenBank. The analysis results suggested that the discovered DNA molecule presented a circular structure homologous to polyomaviruses. Furthermore, its

genes were found to be closely related to the early genes of polyomaviruses, such as JCV, BKV and SV40, while the late genes were found to be quite divergent.

This first new human polyomavirus has been named "KIPyV" (or "KI") after its discovery at the Karolinska Institute. Its infectivity and effect on humans has not been characterized.

Simultaneous to the KI discovery, another polyomavirus was identified in patients with respiratory disorders. This virus was isolated using patient nasal secretions and it was named "WU" because it was discovered in a research lab at Washington University. WU viral genes were found to be similar to those of SV40, BKV and JCV, although a low homology, ranging between 30% and 40%, to these already known polyomaviruses was found. However, when WU was compared to the newly discovered KI, investigators found a much higher homology with it, about 65%. Interestingly, the WU virus is ubiquitous across human races and populations. In fact it has been isolated in most continents and in patients whose ages range between 3 and 53 years old. Similar to the KI virus, WU does not seem to be contagious or capable of replicating in respiratory cells. In some studies, it has been hypothesized that WU and KI viruses belong to a separate branch of the human polyomavirus family and that they share common characteristics with murine or simian families.

The third newly discovered human polyomavirus has been called Merkel Cell Polyomavirus (MCP), because it was isolated from the analysis of Merkel Cell Carcinoma (MCC). In addition, MCP has been hypothesized to be the cause of the tumorigenesis seen in MCC. MCC is an aggressive form of skin cancer, commonly found in elderly people and immunodepressed patients. As this cancer's phenotype was similar to that of Kaposis' sarcoma (tumors caused by herpesviruses); MCC viral components were investigated. MCC samples were

analyzed using a digital transcriptome subtraction approach, which is a technique developed to identify foreign transcripts using cDNA-sequencing data. When this technique was used on MCC, it was found that MCC had a genome with homology to other polyomaviruses and that its genome was integrated into the tumor DNA. Further, there were sequences resembling large-T, the viral capsid proteins and the viral origin.

Host	Virus	Characteristics
Human	JC virus (JCV)	• Infects kidney epithelium in healthy patients • Infects oligodendrocytes in immuno-compromised patients, causing PML
	BK virus (BKV)	• Infects kidney epithelium in healthy patients • Causes PVAN in transplant recipients
	WU	• Found in patients with respiratory syndromes
	KIPyV	• Found in respiratory secretions
	Merkel Cell polyomavirus	• Found in Merkel cell tumors
Monkey	simian virus 40 (SV40)	• Naturally occurring in kidneys of macaques • Causes PML-like illness in immuno-compromised macaques
	simian agent 12 (SA12)	• Found naturally in baboons
	lymphotrophicpapovavirus (LPV)	• Found in lymphoblasts of African green monkeys
Cattle	bovine polyoma virus	• Common in cattle
Rabbit	rabbit kidney vacuolating virus	• Found in wild rabbits
Mouse	mousepolyoma virus	• Naturally occurring in kidneys of mice
	K virus	• Naturally occurring in lung epithelium of mice
Hamster	hamsterpapovavirus	• Found to produce tumors in hamsters
Rat	ratpolyomavirus	• Found in parotid gland of athymic rat
Parakeet	Budgerigar Fledgling Disease virus (BFDV)	• Causes fatal illness in avian species

Table 1:*Polyomaviridae*. All 15 known polyomavirus family members and their associated diseases.*(Adapted from Fields Virology, Fifth Edition Knipe and Howley, 2007)*
Legend**.** PML: Progressive Multifocal Leukoencephalopathy
PVAN**:** Polyomavirus Associated Nephropathy

1.2 JCV

JC virus (JCV) is a member of Polyiomaviridae family, characterized by icosahedral capsids, circular and double-stranded DNA. Polyomaviruses (PyVs) are renowned for their ability to infect a very broad spectrum of species, including humans, other primates, rabbits, rodents and birds. (M.J. Imperiale 2001). BL Padgett discovered JCV in brain tissue of a patient (John Cunningham) while treating his Hodgkin's lymphoma. Eventually the patient died of progressive multifocal leukoencephalopathy (PML), a lytic infection of the myelin-producing oligodendrocytes in the Central Nervous System (CNS) (Padgett et al., 1971).

JC viral genome is characterized by a specific bipartite organization that is composed of two regions, called early and late transcription units, which, despite being similar in size, are transcribed in opposite directions. A common hypervariable non-coding control region (NCCR), also called a Regulatory Region (RR), divides them. This region contains the origin of replication (ORI) as well as promoter and enhancer elements, as shown in Figure1. (Frisque et al., 1984).
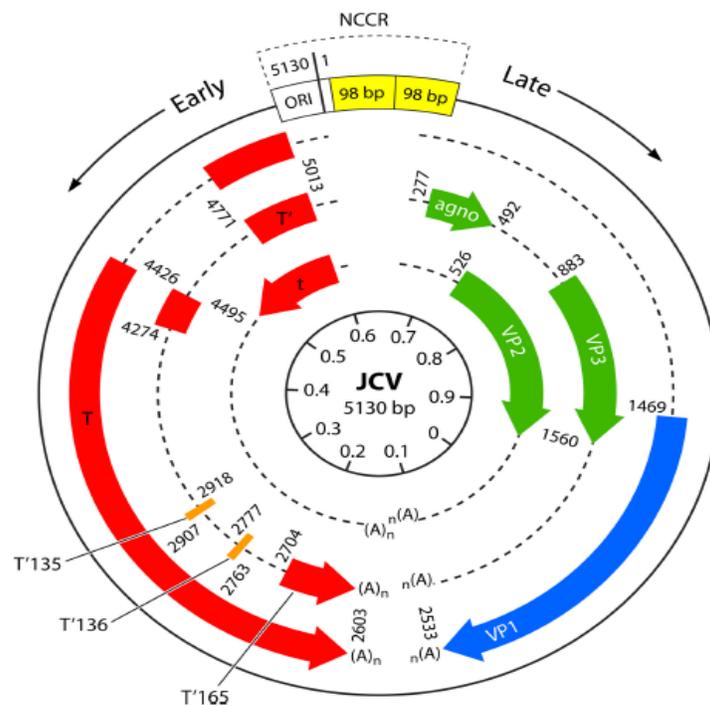
Figure1: *Reprinted with permission (Ferenczy et al., 2012)*

The early region starts transcription before DNA replication begins. This early transcription unit is able to encode for early regulatory proteins, such as small-t, Large-T, T'135 and t'165. These are produced by alternative splicing of the viral early mRNA. (Frisque et al., 1984; Saribas et al., 2010).

The LT-Ag is the JCV main regulatory protein and is necessary for the viral genome replication, for the genome late promoter transactivation and the autoregulation of its own early promoter as well. (Saribas. et al., 2010). Although the small-t-Ag function not being completely clear, it appears to be responsible for regulating the viral cycle replication and**,** together with Large-T**,** it pushes the cell into S-phase of cell cycle, where all DNA viruses replicate their DNA

(Sariyer IK et al., 2008; TK et al., 2008; Frisque RJ 2001). Large-T antigen and their variants are multifunctional, interacting with both host and viral proteins and DNA (Ferenczy et al., 2012).

The late side of the viral genome is transcribed concomitant with DNA replication and it encodes for all structural capsidic proteins, VP1, VP2 and VP3, and a small regulatory protein Agno, which accumulates mostly around the perinuclear region of the infected cells, but is also found in the nucleus in a lesser extent (25-30%) (Ferenczy et al., 2012). They all result from alternatively spliced late pre-mRNA (Saribas et al., 2010).

The viral DNA is packaged with histones H2A, H2B, H3, and H4 and creates a mini-chromosome structure that is almost indistinguishable from the host's chromatin.

1.3 Regulatory Proteins

All polyomavirus T-antigens are characterized by four conserved domains: the J domain, origin-binding domain (OBD), zinc (Zn)-binding domain, and ATPase domain (Figure 2).
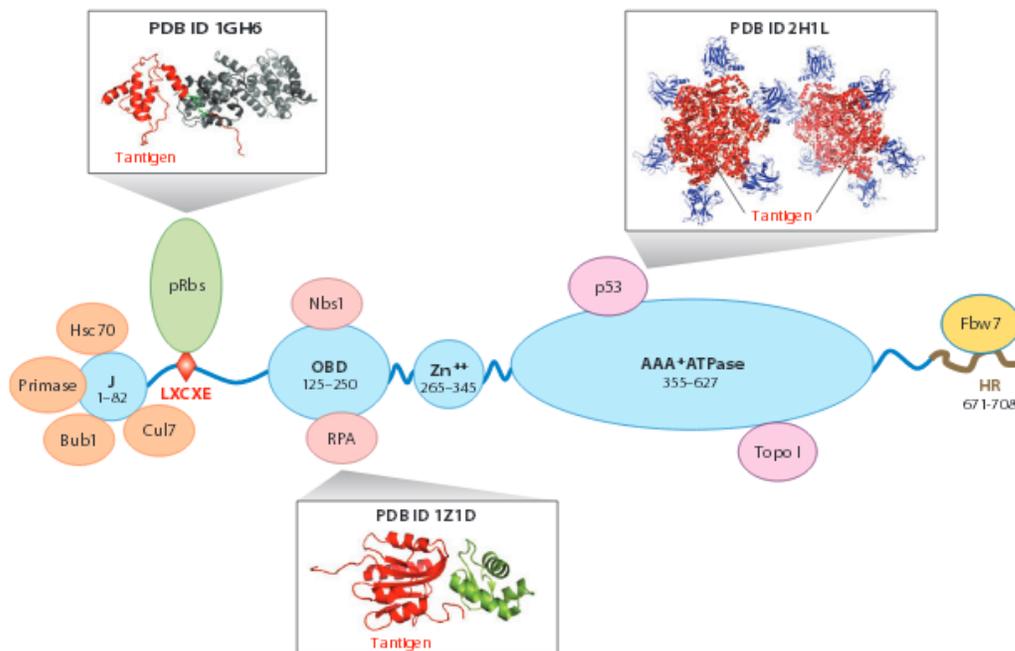
Figure 2: Domain structure and biological activities of SV40 large T antigen and cellular binding partners*. (a) SV40 large T antigen consists of four well-folded domains [J domain, origin-binding domain (OBD), zinc (Zn)-binding domain, and AAA+ ATPase domain; represented by blue ovals] and two large variable disordered regions (shown by curves). Boundaries of each domain are indicated by the amino acid residue numbers. The J domain binds to Hsc70 and functions as its co-chaperone. The J domain also interacts with DNA polymerase (Pol) α primase. The N-terminal disordered region immediately downstream of the J domain harbors the LXCXE motif (diamond). This motif is critical for the interaction between the T antigen and the pRb proteins. Additional cellular targets of this region include Bub1 and Cul7. The OBD binds to the SV40 replication origin, as well as to two host proteins, replication protein A (RPA) and Nijmegen breakage syndrome 1 (Nbs1). The Zn-binding domain mediates oligomerization of T antigen. The AAA+ ATPase domain binds to and hydrolyzes ATP, which is essential for SV40 T antigen helicase to unwind its template DNA during viral DNA replication. This domain also interacts with two cellular proteins, p53 and topoisomerase I (Topo I). The C-terminal disordered region contains the host range (HR) activity and the adenovirus-helper function. The thick brown curve highlights the region critical for the HR activity. SV40 T antigen also binds the Fbw7 ubiquitin ligase through a phosphodegron motif within the HR region. The boxes next to pRb, RPA, and p53 show the crystal structures of T antigen in complex with the corresponding cellular targets. Protein Data Bank identifiers (PDB ID) are indicated.. Reprinted with permission. (Ping An et al., 2012)*

The N' terminal region of all T antigens is also known as a J domain, due to its homology to bacterial DnaJ chaperone (Srinivasan et al. 1997). Mutations in the J domain are defective for viral DNA replication in cell culture (Peden and Pipas 1992). However, the J domain is dispensable for DNA replication in vitro (Collins BS, Pipas JM. 1995).

This could be explained by considering that the chaperone activity of T antigen is required to remove an inhibitor of replication which is already present in cells but that has been eliminated from cell-free replication systems.

The J domain of SV40 also binds and stimulates Hsc70, assisting in the release of bound cell cycle regulators. Hsc70 only interacts with cell cycle regulators when it is bound by SV40 Large-T. It is known that the binding of Hsc70 to Large-T is a critical step for the viral lifecycle. In fact, when this interaction does not occur, Large-T is unable to enhance replication of viral DNA (Borowiec et al., 1990).

Moreover, the J domain allows E2F transcription factors to dissociate from retinoblastoma (Rb) proteins. The released E2F is capable of binding to DNA, stimulating the transcription of its products, and causing cell cycle progression (Wu et al. 2004). Large-T (LT) is able to interact with retinoblastoma (Rb) family members through its LXCXE motif and with p53 in its C' terminal ATPase domain (Wessel et al., 1992).

Interactions of LT with p53 and Rb allow for cell cycle progression. This is a key step in the lifecycle of polyomaviruses without this step the genome is not replicated and the capsid proteins are not produced. The dissociation of E2F requires the binding of Rb and the LT J domain activity to work in cis (Srinivasan et al., 1997).

The OBD is a sequence-specific DNA-binding domain that recognizes the sequence GAGGC. The viral ORI is centered by four of these elements and this interaction is essential for

the initiation of viral DNA replication. Another essential mechanism for replication is the association of OBD with replication protein A (RPA), as shown in Figure 2 and Figure 3.
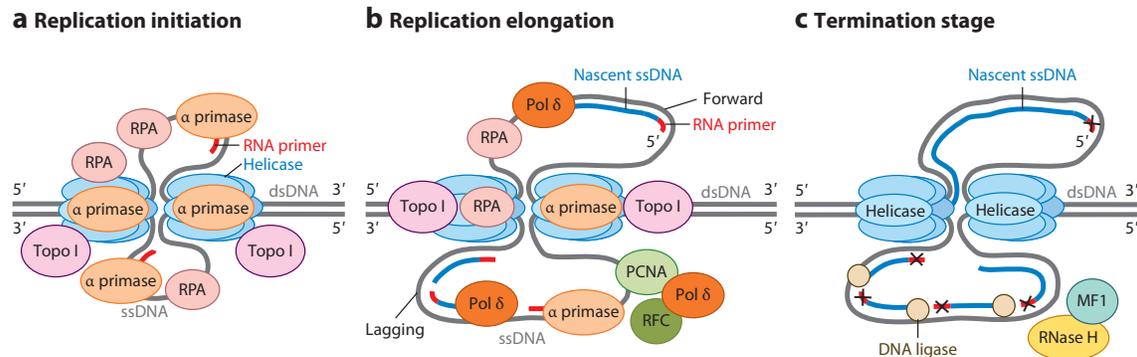

Figure 3: Simian virus 40 (SV40) large T antigen is the master molecule directing viral DNA replication. (a) Simplified schematic of the initiation process of SV40 viral DNA replication. T antigen double hexamer helicase (two sets of six ovals) initiates the distortion and melting of SV40 viral origin and subsequently unwinds the double-stranded DNA (dsDNA) template bidirectionally (represented by two parallel gray lines). The unwound single-stranded DNA (ssDNA) is shown by disordered gray curves. In addition to T antigen, nine cellular factors are required to reconstitute SV40 DNA replication in vitro. The cellular proteins that interact with T antigen at this stage are RPA, DNA polymerase α primase, and topoisomerase I (Topo I). Replication protein A (RPA) is a ssDNA-binding protein necessary for unwinding the double-stranded template DNA, whose C-terminal domain is required for interaction with T antigen. DNA polymerase α primase synthesizes RNA primers (short red curves) about 11 nucleotides in size, which serve as a starting point of DNA synthesis. Topo I and II function to resolve topological problems caused by unwinding and to establish and maintain the double helical configuration of daughter dsDNA. (b) Replication elongation of SV40 DNA. SV40 T antigen helicases continue to unwind template DNA and recruit RPA, α primase, and Topo I through specific interactions. More cellular replicative factors are involved in the elongation process. Replication factor C (RFC) and proliferating cell nuclear antigen (PCNA) facilitate the switch from α primase to DNA polymerase (Pol) δ, which then extends the nascent ssDNA (blue curves) from the primer. For synthesis of the lagging strand, the α primase has to produce primers repeatedly. (c) During the termination stage of viral DNA replication, RNase H and maturation factor 1 (MF1), a 5' to 3' nuclease, are required to remove the primer. Finally, DNA ligase covalently closes the gaps of the newly synthesized strands and completes the replication. Reprinted with permission. (Ping An et al., 2012)

The last two domains are characterized by Zn-binding and ATPase domains which together constitute the enzymatic core for Large-T's DNA helicase activity. The former domain is responsible for T antigen hexamer formation, which represents the active helicase form, whereas the latter domain is responsible for providing the energy needed for this enzyme. In order to

achieve proper helicase functionality, the synergic interaction of OBD, Zn-binding and ATPase is required.

As a consequence of these domains' interaction with ORI, nucleotide-binding and hydrolysis conformational changes occur. Understanding such conformational changes is fundamental to shed light on the mechanisms that regulate the functions of multi protein machines. Computational studies suggest that all polyomaviruses have a partially unstructured region between J domain and OBD, where several binding motifs for cellular proteins and nuclear localization signals can be found. This region is also the target for Rb proteins.

The specific capacity of J domain alone to adopt different conformations due to its flexible nature, predicts that the J domain–Hsc70 chaperone function can be positioned to act on different T antigen–cell protein complexes. The role played by small t antigen in JCV infections has not been studied in detail yet.

It has been shown that SV40 small t is able to interact with the protein phosphatase 2A (PP2A), which is a cellular phosphatase which plays important roles in both cell growth and transformation (Valle et al., 2006).

SV40 small t is therefore capable of interacting with PP2A and inhibiting its activity. Such inhibition has a stimulating effect on extracellular signal-regulated kinase (ERK) and mitogen-activated protein kinases (MAPK) pathways, leading to up-regulation of AP-1 transcriptional activity (Frost et al., 1994).

Protein–protein interaction studies have demonstrated that PP2A associates with agnoprotein, a JCV late viral protein highly involved in proper capsid maturation process. PP2A association to Agno causes its dephosphorylation at PKC-specific sites. Therefore Sm t-Ag by interacting with PP2A, inhibits the dephosphorylation of agnoprotein (Sariyer et al., 2008).

Finally, T' proteins were discovered in 1995 and originally thought to be degradation products of large T (Trowbridge and Frisque 1995).

All of these have the N' terminal J domain, but their sequences are different at the C' terminus region. Such difference is thought to change the phosphorylation status and therefore influence their interactions with the Rb family members p107 and p130 (Bollag et al., 2000).

It is hypothesized that T'135, T'136, and T'165 all play a very important role controlling changes in the cell cycle needed for viral replication and transcription (Bollag et al., 2006;.Prins, and Frisque 2001).

The T-antigens result from alternative splicing of a common pre-cursor pre-mRNA. They are classified as large T, small t, and sliced variants such as T'_{135}, T'_{136}, and T'_{165}. Large-T antigen is the major key regulatory protein, and plays a key role in deregulation of cell cycle and also in viral DNA replication. To promote all of these actions, Large-T protein is structurally composed of a variety of domains capable of interacting with cellular factors. Besides Large-T antigen, Small t and the T' proteins also have a regulatory function, but their roles have not been as fully characterized. The N' terminal region of all T antigens has been described as a J domain, due to its homology to bacterial DnaJ chaperone. This domain has been shown to stimulate the ATPase activity of Hsp70 (DnaK) and is able to functionally substitute for the bacterial DnaJ.

Additionally, human DnaJ homologues can substitute for the SV40 J domain. The J domain of SV40 binds and stimulates Hsc70, assisting in the release of bound cell cycle regulators. Hsc70 only interacts with cell cycle regulators when it is bound by SV40 Large-T. The binding of Hsc70 to Large-T is critical step for the viral lifecycle. In fact, when this interaction does not occur, Large-T is unable to enhance replication of viral DNA.

Large-T (LT) is able to interact with retinoblastoma (Rb) family members through its LXCXE motif and with p53 in its C' terminal ATPase domain (Figure 3). These physical interactions have been demonstrated by immunoprecipitation assays of virally infected cells. In addition, these contacts are required for the transforming ability of large T, demonstrated by soft agar assays. Interactions of LT with p53 and Rb allow for cell cycle progression. Rb negatively regulates the E2F transcription factor; large T breaks this association. The released E2F is competent to bind to DNA, stimulate the transcription of its products, and cause cell cycle progression. This is a key step in the lifecycle of polyomaviruses: without cell cycle progression, the genome is not replicated and the capsid proteins are not produced. The dissociation of E2F requires the binding of Rb and the LT J domain activity to work in *cis*.

Recently, microRNAs (miRNAs) were found during the late phase of the JCV lifecycle. PolyomavirusmiRNAs were first discovered in SV40, using an algorithm aimed at recognizing pre-miRNA in small genomes. A pre-miRNA was identified in the SV40 genome that produced a hairpin capable of being processed by RNA-induced silencing complex (RISC). This hairpin was able to produce two miRNAs targeting the early mRNA of SV40. Similar analysis has since been performed for JCV. This analysis showed JCV also contains a homologous miRNA that targets the early mRNAs. This miRNA is unique in that both cleavage products target the same early transcript. The miRNA for JCV down-regulates Large-T antigen late during the viral lifecycle. SV40 was still infectious in the absence of the pre-miRNA in vitro. However, it is hypothesized the viral miRNAs are important for downregulating Large-T to evade immune response in vivo.

The role small t plays in JCV infection has not been extensively studied. We can gain insights into its role through its known functions in SV40 infection. SV40 small t has been

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shown to interact with the protein phosphatase 2A (PP2A). PP2A is a cellular phosphatase with roles in both cell growth and transformation. SV40 small t interacts with PP2A and blocks its inhibition of protein kinase C. This release of inhibition stimulates extracellular signal-regulated kinase (ERK) and mitogen-activated protein kinases (MAPK) pathways, leading to increases in NF1B gene expression.

Recently, small t in JCV has also been shown to interact with PP2A. However these studies showed this interaction blocked the effect of PP2A on the late viral protein Agno. The authors suggest this regulation of Agno is critical for proper capsid maturation. The T' proteins were discovered in 1995 and originally thought to be degradation products of Large-T. They all share the N' terminal J domain, but their sequences diverge at their C' terminus. This difference is thought to change their phosphorylation status and influence their interactions with the Rb family members p107 and p130.

All three are hypothesized to be important to tightly control the changes in the cell cycle needed for viral replication and transcription.

1.4 Late Proteins

The late region contains agno, Vp1, 2, and 3. The JC virus capsid is composed of V antigens (VAg). Specifically, the capsid consists of 360 molecules of the major coating protein Vp1 arranged in 72 pentameters, which create an icosahedral shape (Yan et al., 1996). Either one of the two minor coating protein, Vp2 or Vp3, lies in the center of each pentameter (Figure 3) (Chen et al.,1998). The pentameters are linked together through N' terminal regions of Vp1 that invade the next protein, while the C' terminal Vp1 tails bind to the adjacent pentameters, tying the viron together.

Vp1 presents a barrel structure with three large exterior loops and it constitutes the outer region of the capsid. Vp1 is capable of interacting with cellular receptors (see Figure 4).

Figure 4: Vp1 pentamer and virus capsid. (A) JCV Vp1 pentamer created using SwissModel software. The structure was created using known SV40 Vp1 structure as a model. The molecule has a #-barrel structure with the outer, receptor interacting loops shown in yellow, purple, and red. (B) Vp1 molecules form a pentamer, each color indicates a unique monomer. The N' and C' terminal tails from each molecule are used to tie the virion together. The N' terminal tails interact within the pentamer, the C' terminal tails tie adjacent pentamers together. Stehle et al, Structure 1996, used with written permission from Cell Press. (C) Arrangement of the pentamers on the viral capsid surface. The virion has 5- and 6-fold symmetry. Stehle et al, Structure 1996, used with written permission from Cell Press. (D) Cross-section of the virion. Inside each pentamer there is a minor protein, Vp2 or Vp3. The minor proteins also interact with the viral DNA, which is complexed with histones and act as a bridge for the entire structure. Reprinted with permission Kate Manley.

Vp3 and the terminal two-thirds of Vp2 are identical. This shared domain is composed of the Nuclear Localization Signal (NLS), the DNA binding domain and the Vp1 interacting domain (Barouch and Harrison 1994; Clever et al., 1993; Clever and Kasamatsu 1991; Gharakhanian and Kasamatsu 1990).
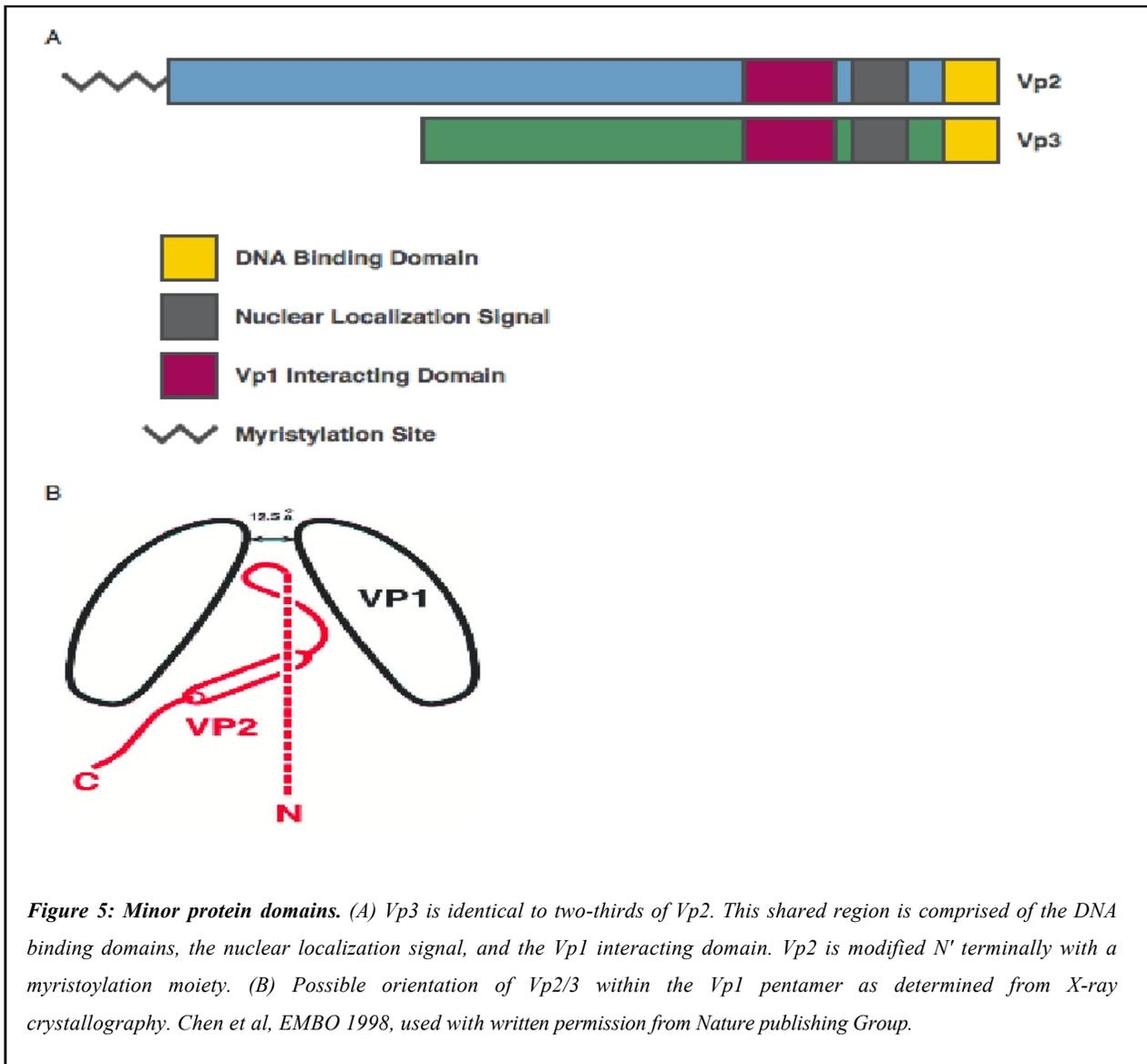
Vp2 N' terminus can also be modified by a myristoylation moiety (Figure5). This is a process by which a fatty acid is co-translationally added to a protein. Myrostoyl proteins can either be cytoplasmatic or membrane-associated. After the methionine is removed and an N' terminal glycine residue is recognized, the myristoyl group is transfected by the enzyme N-myristoyltansferase (NMT). In order to anchor a protein within a membrane, the myristoylation process needs a basic region adjacent to the insertion point or an additional anchor such as a palmitylation. A series of modifications can also modulate the above-mentioned membrane association. For instance, if a membrane-associated protein becomes phosphorylated, the negative charge will repel it out of the membrane. Conformational changes can also influence the myristoylation site exposure, for example, it can be captured in a hydrophobic pocket until a stimulus exposes it. Viral proteins are fundamental for viral uncoating and for viral release from membrane-bound compartments.

In a previous study (Gharakhanian et al., 2003), the role of minor coat proteins in SV40 was assessed and it was found that Vp2 was unessential while Vp3 was necessary for infection. These studies also suggested that the importance of Vp3 lay in its ability to activate poly (ADP-Ribose) polymerase (PARP). It is because of this over-activation of PARP that intracellular ATP seems to be depleted. This, in turn, causes cellular necrosis, releasing the virus (Gordon-Shaag et al., 2003).

In recent work, the importance of both these minor proteins has been demonstrated for SV40 infection. In addition, these proteins' ability to lyse bacteria could represent a further tool that the virus could benefit from in order to release itself from the cell (Daniels et al., 2006).

Recently, a new minor protein Vp4 has been identified. Vp4 is not present in the virion, but it is found in cellular lysates during late time points of infection. Some investigators have

hypothesized that Vp4 is a lytic factor produced to complete the viral lifecycle (Daniels et al., 2007).

Figure 5: Minor protein domains. *(A) Vp3 is identical to two-thirds of Vp2. This shared region is comprised of the DNA binding domains, the nuclear localization signal, and the Vp1 interacting domain. Vp2 is modified N' terminally with a myristoylation moiety. (B) Possible orientation of Vp2/3 within the Vp1 pentamer as determined from X-ray crystallography. Chen et al, EMBO 1998, used with written permission from Nature publishing Group.*

Related work on minor proteins has been carried out on mouse polyoma (mPy), with Vp3 and Vp2 myristoylation mutants being produced. In this study, it was proven that Vp2 and Vp3 were fundamental for both early and late events in the viral lifecycle. Specifically, the myristoylation site was switched with alanine, glutamate, glutamine and histidine using site-

directed mutagenesis. Although the alanine change showed a delay in early kinetics, with Vp1 production occurring later than in normal circumstances, this mutant did not show any virion stability defects. In a single round of infection, both the changes to glutamate and glutamine did not show any significant delayed kinetics. However, in the long term, these changes displayed a reduced re-infection capability, which was likely due to structure interactions with the host cells. Moreover, the glutamate substitution also generated a virion morphology modification. Finally, the histidine change generated an inability to either enter or release from the cells, similar to what happens with Vp2 and Vp3 mutants (Krauzewicz et al., 1990;Mannova et al.,2002;Sahli et al., 1993).

JCV requires the presence of both Vp2 and Vp3 minor proteins for its viral replication. In addition, it needs the myristoylation site on Vp2, as large groups are not able to prevent the loss of the myristoylation site (Gasparovic et al., 2006).

	Large T	Vp1	Vp2	Vp3	Agno
SV40	72%	78%	79%	75%	62%
BKV	82%	75%	72%	66%	79%

Table 2: Sequence similarity to JCV. Comparison of the sequence similarity between SV40 and BKV to JCV early and late proteins.

During the late viral lifecycle phase, Agno-protein is produced, even though it is not packaged within virions. This phenomenon makes understanding Agno-protein's role in JCV infection difficult. Despite recent studies having begun to shed some light on the properties of Agno-protein in JCV infection, much of what is known of this protein derives from studies carried out on Sv40.

All the late transcripts are produced in a polycistronic manner and its reading frame is the leader sequence for all the late gene products (Hay et al., 1982). However, for a long time, the fact that the leader sequence actually produced a protein product remained unknown.

The SV40 Agno-protein was discovered in the early 1980s. It is a basic small protein (~ 61 a.a.), with very short half-time life (~ 2 hours), which suggests that it is a regulatory protein (Jay et al., 1981). SV40's basic properties give it a special affinity for DNA binding. Agno has often been associated with both replicating DNA and partially assembled virions (Jackson and Chalkley, 1981).

Interestingly, JCV exhibits growth defects if Agno-protein is removed. On the other hand, the removal of this protein does not affect the early gene production, DNA replication, or late gene transcription or translation. Virionsare still produced, but at a lower rate and they are releasedin smaller amounts (Resnick and Shenk. 1986).

Another interesting property of the Agno-protein is that it has been often characterized as localized to the cytoplasm and perinuclear space by indirect immunofluorescence (Nomura et al.,1983;Safakand Khalili 2001).

Furthermore, Agno-protein has multiple potential phosphorylation sites that induce a reduction in viral growth. It has been found to be a substrate for PKC and it is thought that variations in Agno phosphorylation also change its cellular localization (Sariyer et al., 2006).

To this point, recent studies have demonstrated the role of Agno as substrate for PP2A and that small-t proteins' interaction with PP2A seems to regulate the Agno dephosphorylation (Sariyer et al., 2008.). Considering the highly basic nature of Agno, it has been hypothesized that changes in the phosphorylation status of Agno-protein control its DNA binding characteristic (Safak et al., 2001)

1.5 Transcription of JCV Genes

Once the viral genome is delivered to the newly infected cells, it acquires the histone H1 and resembles cellular chromatin (Major and Imperiale 2007). Once in the host nucleus, the JC virus genome serves as a template for RNA polymerase II (pol II) transcriptional machinery. The regulation of such machinery depends on the sequence of the NCCR, but also from the availability of host transcription factors (Ferenczy et al., 2012).

The NCCR is thought to be the key of cell type specificity and it is composed of well conserved regions surrounding the transcription start sites of both early and late coding regions. Additionally, it is also composed of a central region which contains many transcription factor binding sites. The NCCR early –proximal side contains pre-origin and origin of replication (ORI).

The original viral sequence isolated from a PML patient is known as Mad-1, since it was isolated at the University of Wisconsin-Madison (Ferenczy et al., 2012).

The Mad-1 NCCR is composed of two 98-bp tandem repeats, each one composed of a TATA box which can position mRNA start site (Ghosh et al., 1981) and multiple transcription factor binding sites (Frisque RJ. 1983). The NCCR Mad-1 tandem repeat is known as the "prototype" sequence and is composed of three sets of sequences, "a" (where the TATA box is), "c" and "e" respectively.

It is acknowledge that TATA boxes contained in the 98-bp tandem repeat are essentials for the transcription of the early and late viral genes (Daniel and Frisque 1993). Although the Mad-1 variant was the first variant isolated, it has been shown that many JCV isolates from PML

patients are actually missing the second TATA box which may not be required for JC replication (Martin et al., 1985).

The JC virus promoter contains multiple binding sites for transcription factors and transcriptional repressors.

The Nuclear factor for activated T-cells (NFAT) is a transcription factor required for JCV transcription. NFAT4 is activated by calcium release, presumably triggered by virus-receptor interactions. Once activated, it moves to the nucleus where it is able to interact with the JCV genome and drive transcription (Manley K, et al. 2006). Additionally, JCV has binding sites for NF1-X (Monaco MC, et al., 2001), NFkB (Ranganathan and Khalili, 1993), SP-1 (Henson et al., 1992) and many others, who bind certain variants of the NCCR activating transcription of early genes.

On the other hand, NF1-A (Ravichandran and Major 2008), c-jun (Ravichandran et al., 2006;Kim J, et al. 2003), c-fos (Kim J, et al. 2003) SF2/ASF (Sariyer and Khalili 2011) and others have been shown to repress early transcription levels.

The NF1 family on cellular DNA binding proteins is critical to JCV transcription and replication. Three NFI binding sites have been identified in the NCCR of JCV (Amemiya et al., 1989).

Dimerization, DNA binding, and DNA replication domains of NF1proteins are found in the N terminus and are separable from the transcriptional activating domain (Gronostajski 2000). All NF1 genes (-A, -B, -C, -X) share homology on the N-terminus portion but differ at the C-terminus, which is responsible for transactivation and the repressive activity (Gronostajski 2000). It is known that all NF1 proteins are able to homo- and heterodimerize and are able to compete for the same binding site influencing transcription levels. This could explain why overexpression

of the NF1-X gene supports increased viral activity whereas NF1-a reduces the ability of permissive cell types to support JC virus infection (Ferenczy et al 2012).

Members of the activating protein 1 (AP-1) family play a key role regulating the activation of JCV transcription (Amemiya et al., 1992).

NF1 binding to and activation of JCV are reduced by the presence of c-jun (Amemiya et al., 1992). This is thought to be due to the overlapping AP-1 and NF1 binding sites in the NCCR of JCV, suggesting that c-jun is able to physically block NF1-induced activation (Ferenczy et al 2012).

Interestingly both NF1 and AP-1 family members interact with Large-T antigen but in an antagonistic manner. NF1 appears to increase Large T –dependent early and late gene expression (Amemiya et al., 1989), and therefore contribute to enhanced viral replication (Ravichandran et al., 2006.)

AP-1 members c-jun and c-fos, instead, have been shown to interact with Large T and suppress its activation and, consequently, viral DNA replication (Kim J, et al. 2003).

It is well established that Large T antigen is able to facilitate binding of YB-1 to the viral lytic control element (LCE), and that YB-1 together with Large T increases the displacement of Pur-α from the viral promoter, and therefore stimulate late gene expression (Chen et al., 1995.; Chen and Khalili K 1995).

T antigen therefore promote late transcription by interacting with components of the basal transcriptional machinery, including TATA binding protein (TBP), TBP-associated factors (TAFs), and transcription factors, including Sp1 (Kim et al., 2000), but they can also function directly as a TAF (Damania et al., 1998).

1.6 JCV Lifecycle

Virus Entry, Trafficking, and Uncoating

In order to infect glial cells, JCV has to bind to specific cell surfacereceptors, penetrate the plasma membrane, then target its double-stranded DNA genome to the nucleus (Pho et al.,2000). JCV binding and entry into the cell requires both an N' linked glycoprotein with an$\forall$ (2-6)- or$\forall$2-3)-linked sialic acid (Liu et al.,1998) and the serotonin receptor 5-HT2A (Elphick et al., 2004; Dugan et al., 2008)

It has still not been established whether the sialic acid is on the serotonin receptor itself. Following binding to the cell surface receptors, the virus is internalized by the ligand inducible clathrin-dependent pathway (Querbes et al., 2004).

The virus is initially trafficked to early endosomes and uses a Rab-5- dependent pathway to access the caveosome, from which it traffics to the endoplasmic reticulum (ER) (Figures 6 and 7) (Querbes et al., 2006).
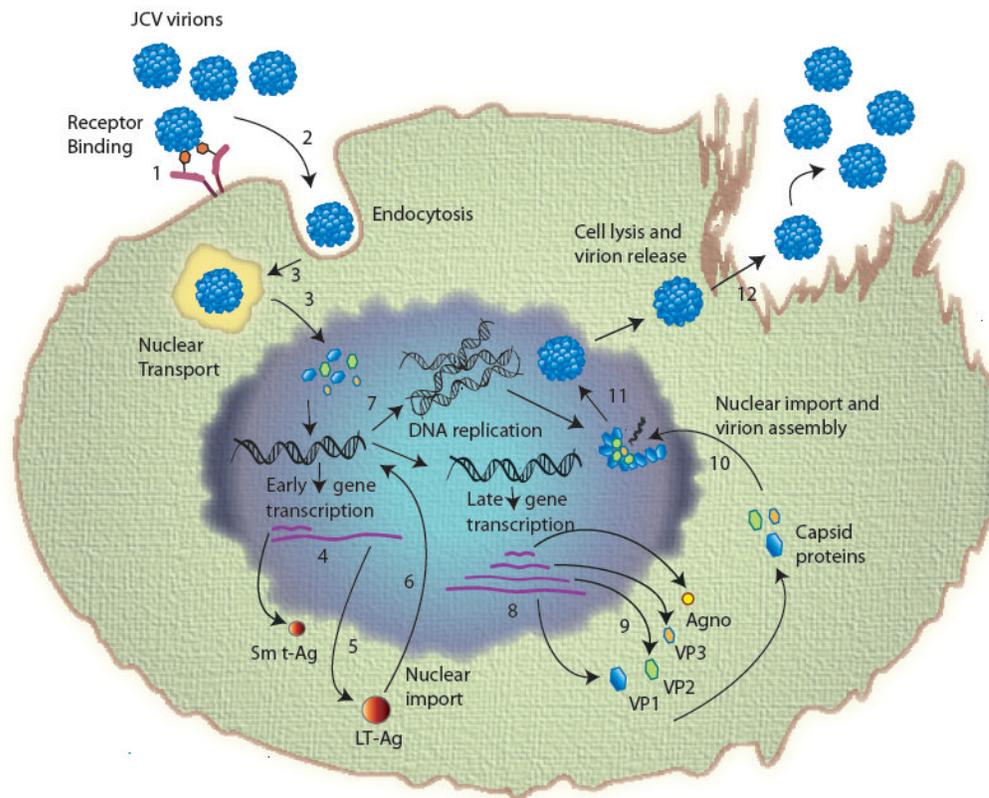
Figure 6: Sequential events during JCV life cycle*(1) Adsorption of virus to the cell surface receptors; (2) entry by clathrin-mediated endocytosis; (3) uncoating of virions and nuclear transport (uncoating takes place either in the either in endoplasmic reticulum or in the nucleus); (4) transcription of early coding region; (5) translation to produce early regulatory proteins, LT-Ag, Sm t-Ag and T' proteins (T'$_{135}$, T'$_{136}$ and T'$_{165}$); (6) import of LT-Ag into nucleus to initiate viral DNA replication and late gene activation; (7) replication of viral genome; (8) transcription of viral late genome; (9) translation of viral late transcript to produce agnoprotein and capsids (VP1, VP2 and VP3); (10) Nuclear import of capsid proteins; (11) assembly of viral progeny in the nucleus; (12) release of virions from infected cells. Agno: Agnoprotein; JCV: JC virus; LT-Ag: Large T antigen; Sm t-Ag: Small t antigen Reprinted with permission. (Saribas et al., 2010)*

Virus trafficking is pH dependent, as demonstrated by an increase in endosomal pH causing a reduction in virus infection (Ashok and Atwood. 2003). Many viruses require low pH for one of three main reasons: viral membrane fusion, protease activation and vesicular trafficking.

Influenza has three proteins in its envelope, hemagglutinin (HA), neuraminidase (NA) and M2**,** the proton channel. Upon entering the endosome, the acidic pH causes HA to undergo conformational rearrangement, exposing a membrane penetrating form of the HA protein

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(Bullough et al., 1994). In the endosome, M2 will pump protons into the viral particle, thereby releasing viral-genome complexes from the envelope (Pinto et al., 1992). Ebola virus also requires acidification of the endosomes, but low pH does not allow for membrane fusion. Low pH activates cathepsins, which are endosomal cysteine proteases.
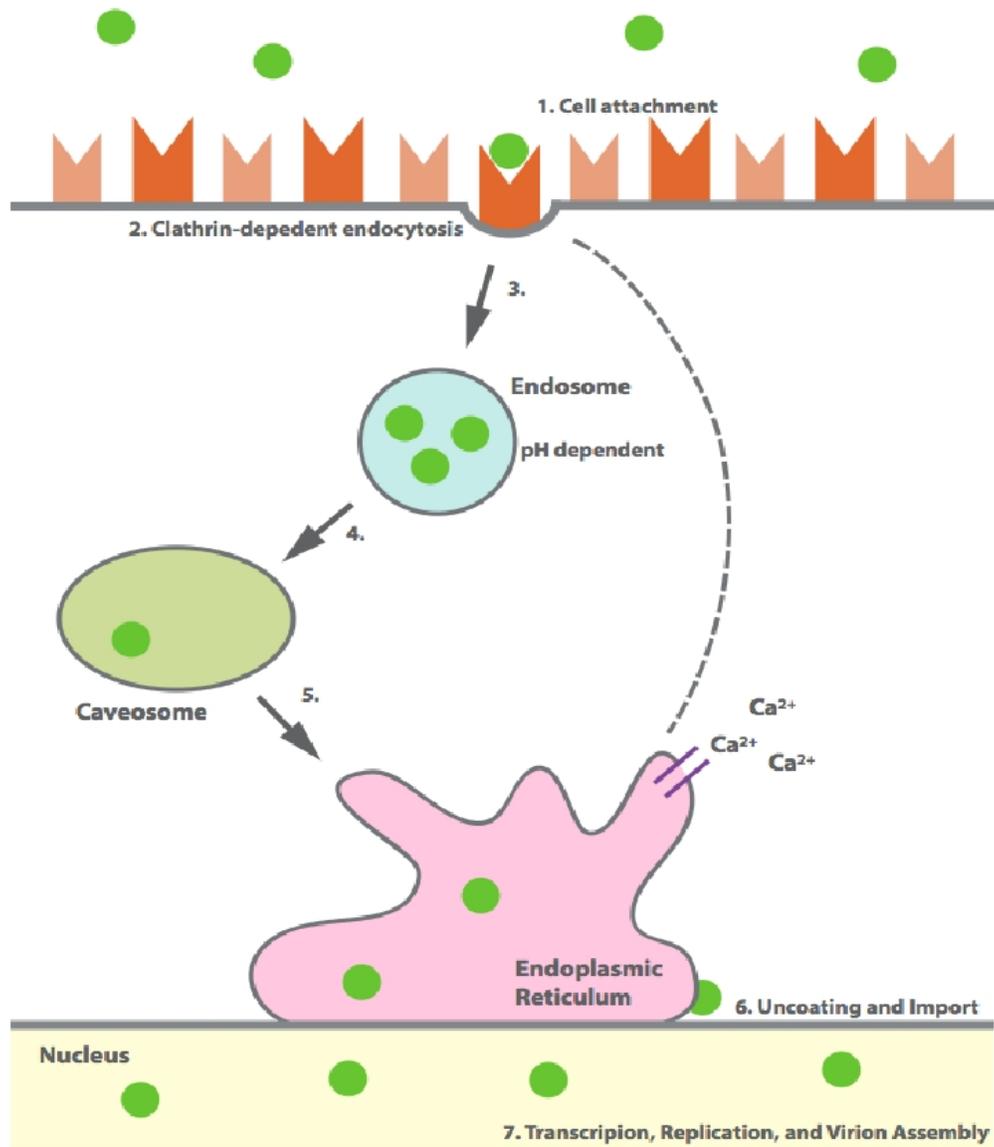
Figure 7: JC virus lifecycle. *JC virus binds to cells using an ∀(2-6)- or ∀(2-3)-linked sialic acid and the serotonin receptor 5-HT$_{2A}$R (1). After binding, JCV is internalized using clathrin-dependent endocytosis where it traffics to early endosomes (2 and 3). JCV requires pH at early times during infection to complete its trafficking to the caveosome and the ER (4 and 5). Uncoating is hypothesized to occur in the ER. The virus is then delivered to the cytoplasm where it can import into the nucleus using nuclear pores (6). Once inside the nucleus, the virus transcribes its early genes, replicates its genomes, and transcribes late genes. Virus assembly also takes place in the nucleus (7).*

Ebola requires both cathepsin B and L to create a viral peptide which is then able to induce endosomal membrane fusion (Chandran et al., 2005; Schornberg et al., 2006). Reoviruses also require low pH and cathepsin B and L for efficient disassembly and membrane penetration. This

is confirmed by observation that when reoviruses are digested prior to infection to generate their infectious subvirion particle (ISVP), they are able to overcome the requirement for low pH and cathepsins (Baer et al., 1999; Ebert et al., 2002). One of the rate-limiting steps in the viral lifecycle is the uncoating of the viral genome and its delivery to the nucleus. Recently, a role for ER chaperones has been discovered for both SV40 and mouse polyoma. For mouse polyoma, interactions with ERp29, a protein disulfide isomerase (PDI) family member, cause conformational changes within the viral capsid (Magnuson et al., 2005).These conformational changes allow the virus to interact with lipid membranes so it can deliver its genome to the cytoplasm.

Furthermore, in vitro studies of mouse polyoma reveal Vp2 is capable of binding to and penetrating into the lipid membrane of the ER (Rainey-Barger et al., 2007). After the genome has reached the cytoplasm, it is able to import into the nucleus using the traditional nuclear pore pathway.

SV40 localizes and exposes its minor proteins in the ER (Norkin et al., 2002). It is hypothesized that upon delivery of the SV40 genome to the ER, chaperones uncoat the virus, where it becomes a candidate for the ER-associated degradation (ERAD) pathway (Schelhaas et al., 2007). The ERAD pathway then pulls the partially assembled virus into the cytoplasm. SV40 is unable to undergo this retrotranslocation when the proteasome and membrane protein Derlin-1 are inhibited, which further support the role of an ERAD pathway in SV40 infection. JCV makes its way through the cell through a series of filamentous networks. Treating cells with nocodazole, cytochalsin D and acrylamide disrupts these networks and renders JCV no longer infectious. This indicates that JCV infection requires microtubules, microfilaments and intermediate filaments during its lifecycle (Ashok and Atwood. 2003). The current model suggests actin is important

during early points of infection, either by directly interacting with virus-containing vesicles or indirectly affecting clazthrin-dependent endocytosis. Microfilaments and microtubules are used for subsequent steps in the lifecycle as the virus continues to be transported in vesicles to the caveosome and then the ER.

1.7 Infection and Latency of the JC virus

Seroepidemiological studies have indicated that more than 70% of the human population have been exposed to JCV during their childhood but exhibit no symptoms of clinical disease (Walker and Padgett, 1983). However, inimmunocompromised patients suffering from lymphoproliferative diseases, in AIDS patients or patients undergoing immunosuppressive therapy, JCV reactivates and leads to development of PML (Chang et al., 1996), a rare disease characterized by a lytic infection of oligodendrocytes in the central nervous system (CNS) that generally affects adults but rarely children (Brew B.J. et al., 2010).

Since its development in patients who were known to be seropositive long before clinical manifestation is not associated with the increase in JCV-specific IgM antibody titer, it's possible that the establishment of PML is consequent to a reactivation of JCV from a latent state (Major E.O. et al., 1992). How the viral infections occur remains unclear, however it is known that primary infection occurs most likely in stromal or immune cells of the upper respiratory system (Berger et al. 2006). The virus then appears to be transported by infected lymphocytes to kidneys and bone marrow where it remains latent (Ferenczy et al., 2012). It is known that viral reactivation occurs outside the CNS, and that, once the reactivation is completed, it crosses the

blood-brain barrier transported by B cells and enters the brain where it replicates vigorously in oligodendrocytes, leading todemyelination (Brew B.J. et al., 2010; Saribas et al., 2010).

Several observations indicate that a vital role in controlling the virus is played by the cellular immune response which requires the collaboration of both innate and adaptive immunity: NK-cells destroy virus infected cells. As for adaptive immunity, B-lymphocytes produce antibodies able to neutralize free virus in fluids, whereas T-lymphocytes can kill infected cells before the viral maturation and therefore its release, preventing cell-to-cell transmission (Koralnik I.J. 2002). Therefore, a reduction of CD4+ T cells can cause a lack of immune control of JCV and thus increase the likelihood of JCV reactivation and PML development (Bayliss J. et al., 2013). It is likely that, in healthy individuals, the immune system retains the virus in a latent state. Therefore, alterations in immune system function could promote reactivation of viral gene expression and start the lytic phase of infection (Chang et al., 1996).
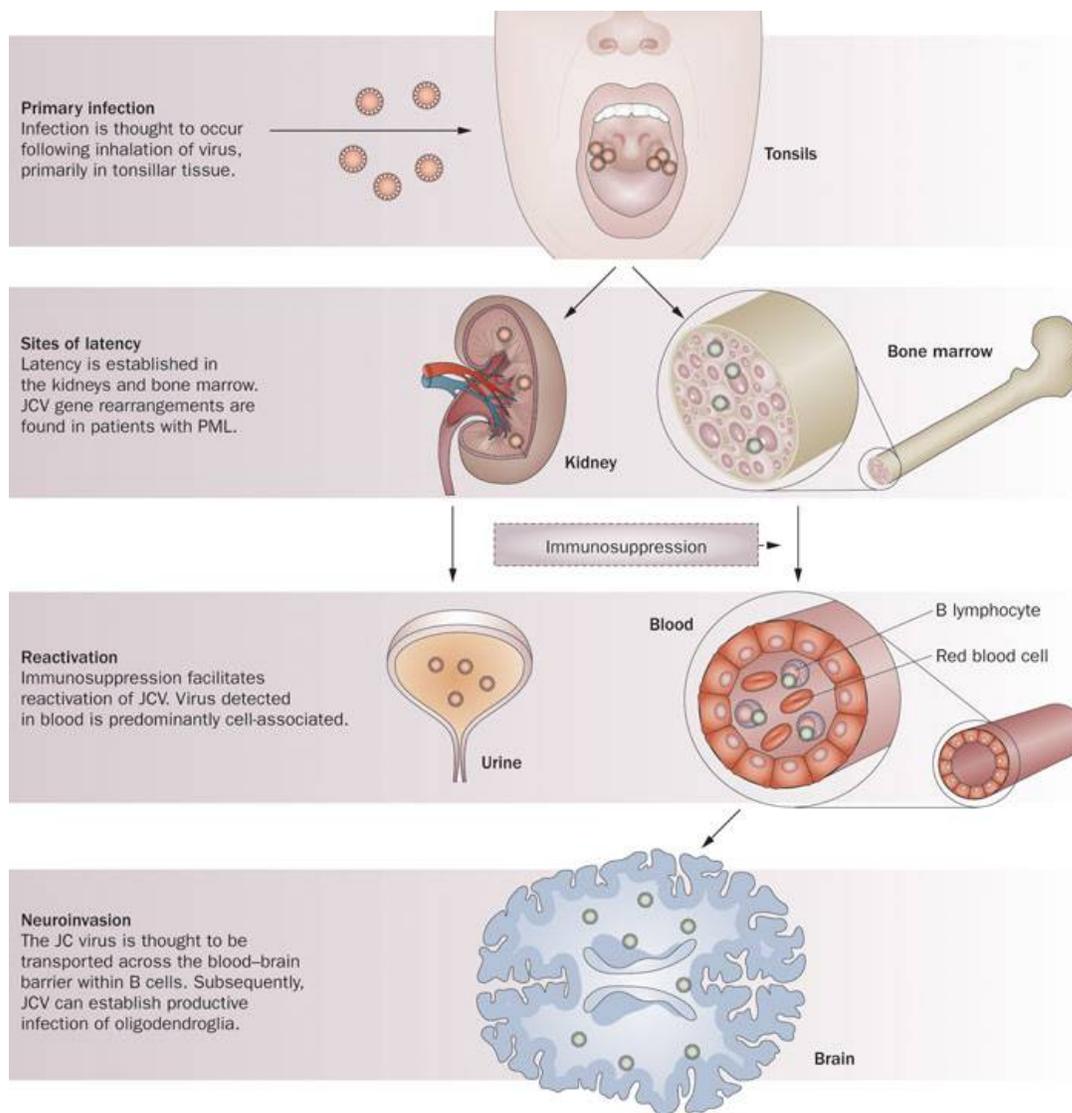
Figure 8: initial JCV infection is thought to occur in tonsillar tissue after inhalation. *Lymphocytes infected with JCV carry virions to the kidney and bone marrow, which are thought to be the primary sites of viral latency. Following reactivation of JCV, the virus is thought to cross the blood–brain barrier within B cells and infect oligodendroglia. The change in JCV color from red to green indicates genetic rearrangement. Abbreviations: JCV, JC virus; PML, progressive multifocal leukoencephalopathy. Reprinted with permission. (Brew et al., 2010)*

CHAPTER 2

JCV ASSOCIATED DEMYELINATING DISEASES

2.1 Progressive Multifocal Leukoencephalopathy

Progressive Multifocal Leukoencelopathy (PML) is a fatal disease that develops from a lytic infection of the myelin-producing oligodendrocytes in the central nervous system (CNS). JC virus (JCV) has been identified to be the causative agent of PML. JCV has been shown to have a limited tropism, in fact its effects are limited to oligodendrocytes, astrocytes, B-lymphocytes, tonsils and kidney epithelial cells. It is very common in humans, studies estimate that about 70% of the human population is seropositive for JCV. It is not completely understood how most humans get infected, but it is hypothesized that the initial infection is subclinical and contracted during childhood. JCV is mostly latent, but can be reactivated in case of immunosuppression and lytically infect oligodendrocytes and cause PMC .

PML was first identified in the 1950s, but it took a decade to discover the viral origin of this disease. Considering that many of the early patients also had lymphatic leukemias or Hodgkin's Disease, PML was thought to be related to complications of lymphoproliferative diseases. Over time, the pathology became more studied and it was found in patients with quite different conditions. It was soon discovered that all the patients had in common the fact that their immune system was impaired, thus researchers started attributing a viral nature to PML (Karl and Astrom 2001). In the 1960s, using electron micrographs of brain tissues collected from PML patients, for the first time viruses resembling papilloma were seen (Zu Rhein, 1965). At the time, human polyomaviruses had not yet been characterized. Then when better staining techniques were available, these brain tissue virions were identified as polyoma and not papilloma (Zu Rhein, 1965). Furthermore, they were found to be present in every brain tissue sample harvested from patients with PML. It was during the same period that cell culture techniques were introduced and these allowed researchers to grow and investigate Simian Virus 40, that is another polyomavirus. Simultaneously, the first primary human fetal glial cell cultures (PHFG) were introduced. In 1970s, for the first time the JC polyoma virus was identified in the brain biopsy of a patient whose name was John Cunningham. In 1971, using Cunningham's brain sections, that were larger and with more virions than usual, these virions were isolated from brain matter and cultured in PHFG cells. This is why the virus was named after John Cunningham's initials (JC).

PML was once thought to be a rare disease, but it has recently become more widespread because of the Acquired Immune Deficiency Syndrome (AIDS). It has been estimated that 4-6% of AIDS patients will develop PML (Major et al., 1992), but this is not the only group at risk of contracting PML. Others at risk are patients undergoing chemotherapy, transplant recipients and patients with Multiple Sclerosis (MS) or Crohn's Disease who are treated with natalizumab.

Although PML can affect all the above-mentioned patient groups, 85% of cases are reported in Human immunodeficiency Virus (HIV)- positive patients.

In more detail, PML is known to cause multiple large demyelination areas, large bizarre astrocytes and nuclear inclusions in oligodendrocytes (See Figure 9). The common PML diagnostic tools are Magnetic Resonance Imaging or Cerebrospinal Fluid (CSF) Polymerase Chain Reaction (PCR). PML is also commonly known to cause lesions that are diffused and subcortical. Interestingly, lesions caused by PML are quite different from those caused by MS. These lesions are characterized by having edges that are not neat, they are not regularly shaped and tend to grow asymmetrically.
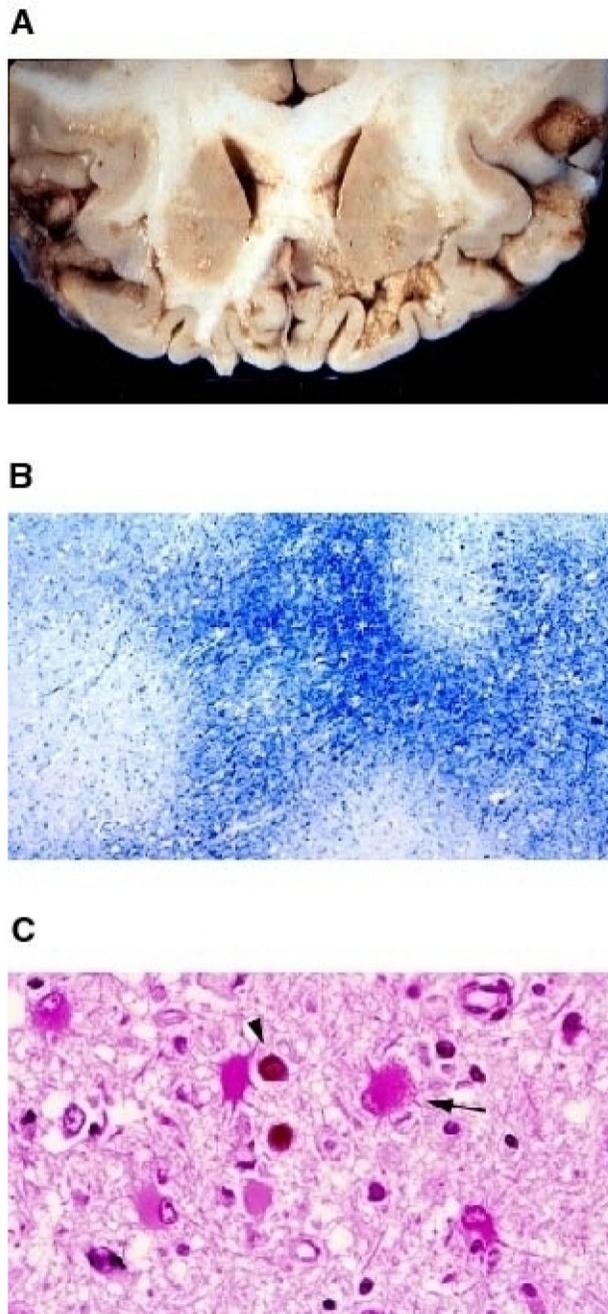
Figure 9: Histological features of PML. *(A) Gross examination of JCV- induced lesions occurring at the subcortical white matter. A coronal section of the frontal lobe of the brain from a PML patient is shown. (B) Apparent myelin loss, as result of JCV infection of oligodendrocytes, is made detectable by Luxol blue staining (40×). Demyelinated areas are visibly distinguishable as white plaque areas. (C) Hematoxilin and Eosin staining of the brain sections from a PML patient. Infected oligodendrocytes are indicated with a round dark staining of the eosinophilic inclusion bodies (arrow head). An arrow points to an infected astrocyte (400×). Reproduced with permission from (Saribas et al., 2010).*

PML might cause different symptoms, such as limb weakness and ataxia at first and as the disease progresses cognitive, speech and visual impairments may occur. Patients rarely survive more than one year since disease onset.

As of today, no cure has been found for Progressive Multifocal Leukoencephalopathy. All the clinical trials that have been implemented, using different drugs such as cytosincearabinoside, topotecam, cidofovir and high dose of azodothymidine (AZT), have proven not effective. They have failed to improve the symptoms and they were even toxic in some cases. The current gold standard treatment is Highly Active Anti-Retroviral Therapy (HAART) in HIV infected patients, whose goal is to alleviate the underlying immunosuppression to slow down the disease progression.

In fact, recent studies have showed how the use of HAART with a high central nervous system penetration effective score might be associated with prolonged survival of patients with HIV-related PML (Yoganathan et al., 2012).

2.2 Immune response against infectious agents: innate and adaptive

A healthy immune response against infectious organisms requires the collaboration of both innate and adaptive immunity.

The first line of defense against pathogens is called the innatenon-antigen specific immunity, and is carried out by macrophages, neutrophils, the competent system and NK (natural killer) cells. Among all of these, only the activated NK cells are actually able to destroy the virus-infected cells.

As for the adaptive, or antigen-specific immunity, is mainly carried out by B and T lymphocytes where B cells produce antibodies to neutralize free virus, and T cells can kill infected cells prior viral maturation, and by doing that, limiting viral transmission (Koralnik 2002).

T cells are subdivided into CD4+ helper cells, and CD8+ cytotoxic T lymphocytes (CTLs). CD4+ cells, whose roll is mainly to stimulate macrophages, and CD8+ cells (TH1 response) or B cells (TH2 response) by producing specific cytokines are able to recognize viral epitopes presented on MHC class II molecules. Stimulated CD4+ cells will proliferate and then produce cytokines such as interferon (IFN), granulocyte-macrophage colony stimulating factor (GM-CSF), and tumor necrosis factor (TNF) in case of TH1 cells, or interleukin (IL)-4, IL-5 and IL-10 in TH2 cells.

CD8+ T cellswho are responsible for the destruction of viral-infested cells, are able to recognize viral epitopes bound to MHC class I molecules. This happens when a newly synthesized viral protein gets tagged for destruction in the cytoplasm of an infected cell and is degraded into peptides by the proteasome system (Koralnik 2002).

2.3 Inflammation

Inflammation is the cellular response to pathogen invasion that results from vascular dilation and an increased movement of immune cells into the affected area resulting in clinical appearance of swelling and reddening. Many diseases originate from or are exacerbated by the inflammatory response such as chronic inflammation resulting in asthma, infections in the limbs of patients with diabetes and advancement of certain cancers. Thymoquinone may have a therapeutic role in inflammation, cancer and diabetes. The immune response is often manifested by the release of pro-inflammatory cytokines and chemokines from cells in the innate immune system. Cytokines and chemokines can work to activate immune cells or elicit a chemoattractive response in other cells in the vicinity. Cytokines such as IL-1 and TNF-α are markers of inflammation. Chemokines and adhesion molecules including MIP-1 and sICAM-1 are also primary indicators of inflammation. Th1- and Th2-dependent immune responses result in the production of various cytokines. When Th1 cells are activated, they produce pro-inflammatory cytokines such as IL-1, IL-2, IL-12, IFN-γ, and TNF-α that stimulate macrophages, Natural Killer cells, cytotoxic T cells and movement of other cells into the affected area. Whereas when the Th2 cells are activated, they stimulate B cell proliferation and antibody production. In response to Th-2 mediated or humoral immunity, anti-inflammatory cytokines IL-4, IL-5, IL-10 and IL-13 are released. The ability of a substance to control the balance between Th1- and 3 Th2-associated releases of cytokines has been associated with both pro- and anti-inflammatory properties.

2.4 Immune system within the CNS

A range of mechanisms exists to limit immune responses in the CNS; in fact, the CNS is considered to be an immune-privileged site. The idea of an "immune privilege" comes from the presence of the blood-brain barrier (BBB) and the blood–cerebrospinal fluid barrier (BCSFB), that play a role in delaying the immune response to non-tumor foreign tissue in the CNS (Galea et al., 2007).

This delay is related to several factors. The CNS lacks conventional lymphoid drainage (Weller et al., 2010) and CNS-derived antigen may be transported to cervical lymphnodes in the fluid phase (Weller et al., 1996) or associated with dendritic cells (DC) (Karman et al., 2004). Since it contains few APCs, and neurons only express MHC under exceptional conditions, the parenchyma of the normal brain and spinal cord has poor capacity for antigen processing and presentation (Neumann et al., 1995). Moreover, lymphocytes have to be activated before they can cross the BBB (Wekerle et al., 1986; Prendergast et al. 1998), and once they arrive in the CNS the environment remains hostile to activated lymphocytes expressing FAS.

In fact, FAS ligands (FASLor CD95L) are a type-II transmembrane protein belonging to the tumor necrosis factor (TNF) family. Its binding with its receptor results in death by apoptosis (Bechmann et al., 1999; Flugel et al., 2000). Moreover, Fas ligand/receptor interactions play an important role in the regulation of the immune system and the progression of cancer.

Microglia, the innate immune cells of the CNS, further respond to inflammation by up-regulation of immune-regulatory molecules including B7-H1 (Magnus et al., 2005) and IDO (Kwidzinski et al., 2005), while neurons protect themselves by secreting TGF- B upon contact with activated lymphocytes (Liu et al., 2006).

The Lymphocytic migration within the CNS is regulated by the interaction of chemokines with their receptors (Wraith DC. and Nicholson LB. 2012). Without any inflammation occurring, CD4+ migration outside of blood vessels is constrained to pathways that run along their axes (Siffrin et al., 2009) and it highly differs from the randommotility of CD8+ cells (Siffrin et al., 2009; McCandless et al., 2006; McCandless et al., 2008).

This confinement is regulated by the interaction of the chemo-attractant CXCL12 with the receptor CXCR4, expressed on the surface of lymphocytes.

The migration of leukocytes into the CNS may be modulated by sequestration of CXCL12 by other receptors (Cruz-Orengo et al., 2011), or by the physical redistribution of CXCL12 (McCandless et al., 2006; McCandless et al., 2008).

In addition, antagonistic effect on CXCR4 allows CD4 T cells to escape and therefore penetrate deeper into brain parenchyma (Siffrin et al., 2009; McCandless et al., 2006).

Treatment with natalizumab, an anti–α 4-integrin, increases the risk of progressive multifocal leukoencephalopathy (PML) is caused by the reactivation of the JC Virus Polyomavirus in the CNS of immune-compromised individuals (Koralnik 2006; Kappos et al., 2011). This is virtually never seen in immune-competent individuals (Weber 2008), attesting to the effectiveness of the immune surveillance of CNS tissue.

CHAPTER 3

MATERIAL AND METHODS

3.1 Cell lines and culture

Human derived T98G glioblastoma cell lines and SVG-A (SV40 T-antigen transformed human glial cells) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics (penicillin/streptomycin, 100 μg/ml). They were maintained at 37 °C in a humidified atmosphere with 7% CO2.

PBMCs were isolated from whole blood by density gradient centrifugation on Ficoll-Paque solution (AMERSHAM Biosciences). PBMCs were then cultured in RPMI medium with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics (penicillin/streptomycin, 100 μg/ml).

3.2 Plasmid constructs and viral strains

JCV -LT-Ag was cloned into aeukaryotic expression vector pcDNA3.1 (+), at EcoRI site and designated as pcDNA3.1(+)–JCV LT-Ag, which expresses both LT-Ag and Sm t-Ag, as previously described (Saribas et al., 2014). Reporter constructs, JCV- Early-LUC and JCV-Late-LUC contained the JCVpromoter from the Mad-1 strain linked to the luciferase gene in the early and late orientations as previously described (Wollebo et al., 2011). Autophagic flux was measured by transfecting a mRFP-GFP tandem fluorescent ptfLC3 plasmid (Addgene) (Kimura et al., 2007).

3.3 Western blots

Briefly, 50 μg of protein was resolved by SDS-PAGE,transferred to a nitrocellulose membrane, and immunoblotted with primary antibody in 10% PBST/non fat dry Milk and secondary antibody (2/5000 dilution) in 5% PBST/non fat dry Milk. Bound antibody was detected with Licor Biosciences

The following antibodies were used for Western blot at a dilution of 1:1000: mousemonoclonal anti-T-antigen, Oncogene Science pAb416; rabbit polyclonal B-tubulin Santa Cruz Z-5; α-VP1 Mouse monoclonal antibody (Ab587) against JCV capsid protein VP1.

3.4 PBMC cultures

Blood samples were obtained from healthy donors. PBMCs were isolated from whole blood by density gradient centrifugation on Ficoll-Paque solution (AMERSHAM Biosciences). Briefly, the freshly drawn blood was diluted at a ratio of 2:1 (vol/vol) with PBS at room temperature and 20 ml of the mixture was layered above 20 ml Ficoll-Paque solution (ratio 1:1) in 50 ml tubes. After centrifugation at 1200 g for 30 min at 25°C without break, PBMCs were then collected and resuspended in RPMI media and centrifuged again at 2000 RPM for 10 Min at room temperature. Pellets were then washed twice with PBS and centrifuged again as described. Finally PBMCs were then washed with RPMI media supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics (penicillin/streptomycin, 100 μg/ml), and resuspendend in culture media at a concentration of 3x 10^{6}/ml.

48 h after isolation, PBMCs were then divided into two groups, as Uninduced and Induced. PBMCs were induced by PMA and Ionomycin for 2 hours and media was changed with fresh media. Cells were incubated for 48 hours and conditioned media was collected and used in the experiments.

3.5 Luciferase Reporter Assay

Luciferase reporter constructs, pGL3.7-JCV-Early and pGL3.7-JCV-Late contained the JCV Mad-1 strain promoter linked to the luciferase gene respectively in the early and late orientations as described previously (Wollebo et al., 2011).

T98G cells (2.5 x 104per well) were transfected with 0.5 μg of reporter plasmid using Fugene6 reagent according to the manufacturer's instructions (Roche). Luciferase activities were detected at 48 using the Dual-Luciferase Reporter Assay system (Promega).

3.6 JCV infection

Transfection/infection of PHFA cells with the full-length JCV Mad-1 genome as previously described (Sariyer et al., 2010).

Briefly cells were co-transfected/infected at a confluence of 1 x 10^6cells per T 75-cm^2 tissue culture flask, with the JCV-Mad1–WT DNA (10μg/flask) using Fugene6 transfection reagent as indicated by the manufacturer (Roche). After 8 and 15 days post-infection, cells were trypsinized and whole cell protein lysates were processed for VP1. Browth media of infected cells were also collected, and processed for Q-PCR analysis of viral copy numbers.

3.7 Quantitative-PCR (Q-PCR) analyses of JCV copy numbers in growth media

Transfection/infection of cells with the full-length JCV-Mad1 genome was performed as described before.

Culture media containing the viral particles was collected 8 and 15 days post infection, in parallel to whole cell extracts, and centrifuged for 10 minutes at 13 000 RPM in order to remove cell debris. Supernatants were collected and incubated at 95^0 C for 10 minutes for inactivation of the virus. Ten microliters of the medium was used as a template in the Q-PCR reactions. JCV Q-PCR-forward: 5'-AGTTGATGGGCAGCCTATGTA-3' and JCV Q-PCR-reverse: 5'-

TCATGTCTGGGTCCCCTGGA-3'. The probe for the Q-PCR was 5'-/5HEX/CATGGA TGCTCAAGTAGAGGAGGTTAGAGTTT/3BHQ_1/-3'.

A serial dilution of a plasmid containing the whole genome of JCV Mad-1 strain (pJCV) served to create the standard curve, that was then used to extrapolate the viral load from each sample.

Each reaction was run with both positive and negative controls and each sample was tested in triplicate

3.8 DpnI assay and detection of replicated-viral DNA by Southern blotting

Replication assay was performed using T98G cells. These cells were plated at a confluence of 1 x 106cells per T 75-cm2 tissue culture flask and 24 hours later transfected for 72 hours with Cat3-Mad1-Early plasmid and pcDNA3.1-LT.

Low molecular weight DNA purified from JCV-infected cells was digested with Dpn I and BamH1 enzymes. Low molecular weight DNA extraction was performed by using QiagenMiniprep Kit.

 Digested-DNAsamples were separated on 1% agarose gel and were transferred to a nylon membrane. Replicated viral DNA was visualized upon incubation of the membrane with Cat3-Mad1-Early plasmid

3.9 Cytokine array

PBMCs were isolated from whole blood as described above. 48 h after isolation, PBMCs were then divided into two groups, as Uninduced and Induced. PBMCs were induced by PMA and Ionomycin for 2 hours and media was changed with fresh media. Cells were incubated for 48 hours at 37°C, 5% CO2. Conditioned media was then collected and centrifuged at $1,500 \times g$ to remove cell debris before being applied to a cytokine array kit (RayBiotech, Inc.,). The array membranes were processed according to the manufacturer's instructions. Briefly, membranes were blocked with a blocking buffer, and then 1 ml of both conditioned media was incubated at room temperature for 2 h.

3.10 Autophagic imaging and flux assays

PHFA cells were seeded on coverslips at 50% confluency and were transiently transfected with either the ptfLC3 encoding LC3fused to MRFP and EGFP. Twenty-four hours after transfection, cellculture media were replaced with fresh and cells were subjected to a 36 hour treatment with either CM-Uninduced, Cm-Induced or IL-2 [1ng/ml]. Cells were then fixed in 4% Formaldehyde for 15 minutes, then rinsed with PBS and mounted with Vectashield DAPI, used to identify nuclei.

After recovering from transfection, cells were subjected to a 32-hour treatment with either CM-Uninduced, CM-Induced and IL2 [1ng/ml] that was directly implemented to cell's growing media.Cells were then fixed in a 4% Formaldheide solution and analyzed by fluorescent microscopy.

Total Lc3 dots obtained by the addition of the number of yellow Lc3 dots with red Lc3 dots was counted in more than 10 in each condition

3.11 RT-PCR

RT-PCR reactions of the JCV-early region splicing were performed by using following primers: PF (Mad-1 4801-4780): 5'- CCTGATTTTGGTACATGGAA -3' and PR (Mad-1 4291-4313): 5'-GTGGGGTAGAGTGTTGGGATCCT -3'. Amplified gene products were resolved on a 3% DNA-agarose gel

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CHAPTER 4

RESULTS

4.1 Conditioned-media from induced PBMCs inhibits JCV infection on glial cells.

To investigate the possible impact of soluble immune mediators on JCV gene expression and replication, we utilized PBMCs as the source of immune mediators in infection studies. PBMCs were isolated from a healthy patient's whole blood and either induced or left un-induced in culture using PMA and Ionomycin for 2 hours as can be seen in the cartoon illustration in Figure 10 panel A, that describes the experimental approach implemented in this work. More in detail, 48 hours after induction with PMA and Ionomycin, conditioned media were collected and supplemented, in a 1:1 ratio (%50), into Dulbecco's minimal essential medium (DMEM) (Mediatech Inc., Herndon, VA), supplemented with 10% heat-inactivated fetal bovine serum (Mediatech Inc.), in which SVGA cells infected by the virus are grown. SVG-A cells are a subclone of the original SVG human glial cell line established by transformation of human fetal glial cells by an origin-defective SV40 mutant (Major et al.,1985). SVG-A cells were maintained in a humidified 37°C CO2 incubator.

Whole cell extracts and growth media of the cells were collected at 8 and 15 dpi, and processed for Western Blot analysis and Q-PCR as shown respectively in panel B and panel C.

As hypothesized, Western Blot analysis of viral protein VP1 levels, show a progressive JC virus infection occurring in SVGA cells as early as 8 days post infections (8 dpi) with a peak at 15 dpi (results are shown in the top graph of Figure 10B lanes 3 and 4).

Our results suggest that CM-Uninduced treatment slightly modulate VP1 levels already after 8 dpi, as shown in lanes 5 and 6, but it is after CM-induced treatment that Vp1 levels are strongly down-regulated (lanes 7 and 8) compared to control. The bottom section of Figure 10B is a bar graph that represents the quantification of VP1 band intensities normalized to Tubulin.

As shown in panel C, Q-PCR analyses of the growth media, also shows the presence of viral particles in SVGA cells infected with JCV, as visible in 8 dpi and 15 dpi controls. Our results show how after CM-induced treatment, viral copy numbers are drastically reduced, especially after 15 dpi, indicating a strong inhibiting effect of CM-induced on viral infection.
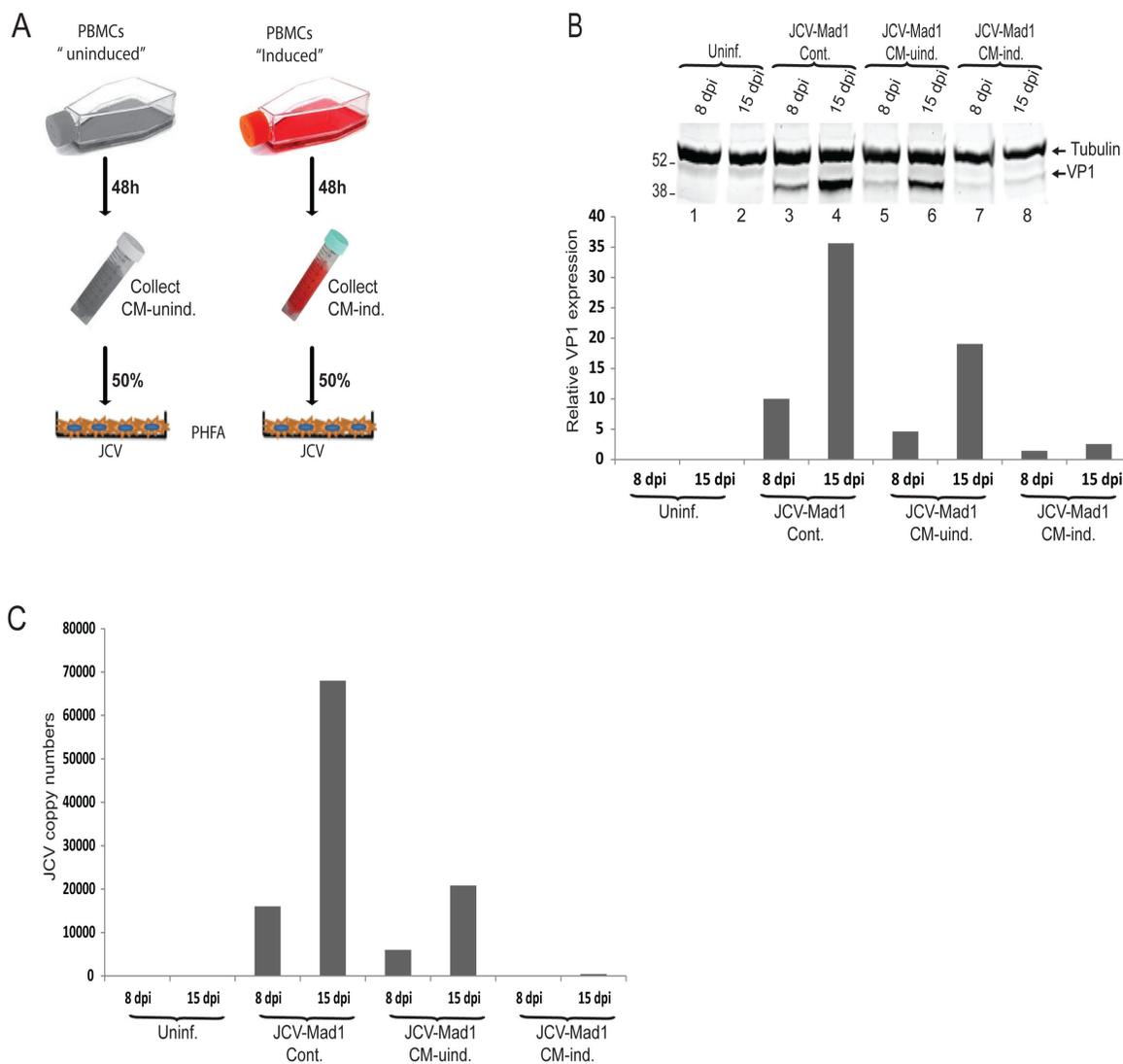
Figure 10: Immune mediated suppression of JCV propagation. *A. Graphic representation of experimental approach. Conditioned media were collected after 48h induction with PMA and Ionomycin, and supplemented into the media of infected SVGA cells. **B.** Western blot analysis of whole extracts from JCV infected SVGA cells. Upper part represents autoradiograph of VP1. Bar graph represents the quantification of VP1 band intensities normalized to tubulin. **C.** Q-PCR analysis of viral copy numbers in growth media of SVGA cells infected with JCV. Growth media was collected in parallel to whole cell extracts in the same infection studies presented in panel B.*

4.2 Soluble immune mediators secreted by activated PBMCs inhibit JCV early and late gene transcription in glial cells.

As a follow up of the results we obtained from the previous experiment, we were interested to investigate the impact of Conditioned Media obtained from PBMCs on JCV transcription. In this experiment we decided to use T98G cell line, derived from a human glioblastoma multiform tumor. It is known that T98G cells are a polyploid variant of T98, and that they express a unique combination of normal and transformed aspects of the control of cellular proliferation. T98G cells are like normal cells in that they become arrested in G1 phase under stationary phase conditions, yet they also exhibit the transformed characteristics of anchorage independence and immortality.

T98G cells were transfected with luciferase reporter plasmids pGL3.7 which consists of either JCV- Mad1 Early (Figure 11A) or Late (Figure 11B) promoter. The Luciferase Assay System was developed for reporter quantitation in mammalian cells. Firefly luciferase, a monomeric 61kDa protein, catalyzes luciferin oxidation using ATP-Mg^{2+} as a co-substrate, generating light as oxyluciferin returns to the ground state. After recovering from transfection, cells were then treated for 32 hours with CM obtained once again from induced or uninduced PBMCs in a 1:1 ratio with 10%FBS/DMEM. After 48 hours from the beginning of the experiment, cells were lysed and Luciferase activity (LUC) was determined with the dual-luciferase assay kit according to Manufacturer's instructions (Promega). As shown in Figure 11A, a 32hour treatment with CM induced from PBMCs greatly decreases Early gene transcripts levels indicating a direct effect of soluble immune mediators on Early gene transcription.

JCV Mad-1 Late promoter activity was also down-regulated after treatment, but in a less significant manner, as indicated by the p-values in Figure 11A and Figure 11B.

The asterisks indicate that test results were statistically significant, as measured by the obtained T-test p-values. T-tests were performed on three independent sets of experiments.
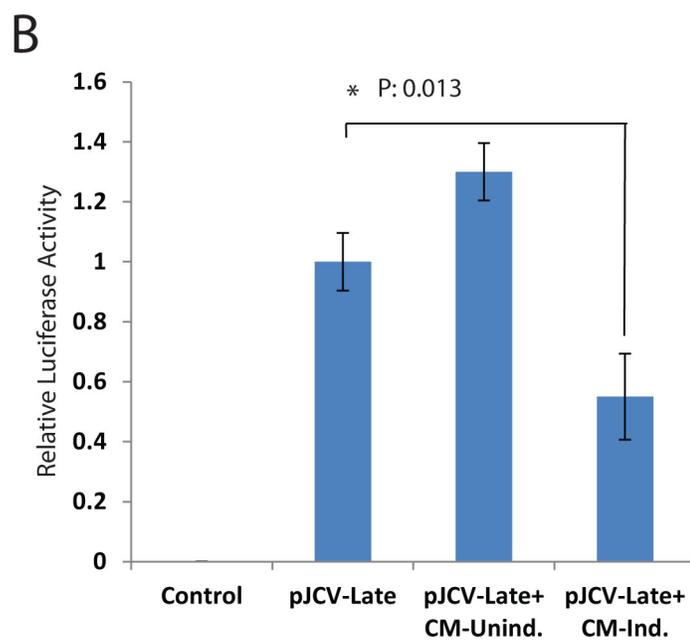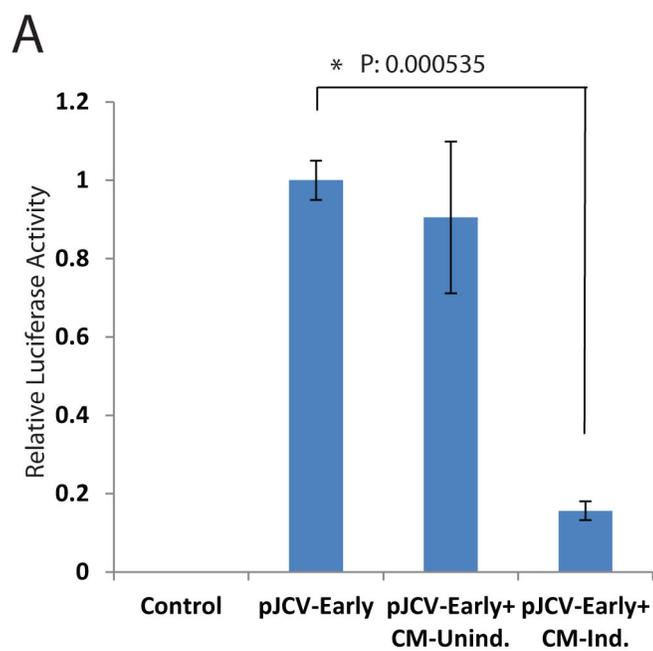
A

B

Fig.11 Induced CM from PBMCs negatively modulates JCV early and late gene transcription.T98G human glioblastoma cells were transfected with luciferase reporter plasmids pGL3.7 which consists of either JCV-Mad1 Early (Figure 2A) or Late (Figure 2B) promoter and then treated for 32 hours with conditioned media obtained from either induced or uninduced PBMCs. Cells were then harvested for luciferase assay. LUC activity was normalized to untreated cells (second bar in both panels), the results were presented as a histogram. The asterisks indicate that experiments were conducted in three independent experiments and are therefore statistically significant.

4.3 Conditioned media from PBMCs inhibits viral replication induced by T-Ag.

The next experimental step consisted in investigating whether immune mediators also played a modulating effect on JCV viral DNA replication. We therefore performed a viral replication assay utilizing pBLCAT3-JCV-early plasmid, which contains whole viral NCCR including origin of replication.

T98G cells were harvested from human fetal brain tissue obtained from elective abortions in full compliance with National Institutes of Health guidelines. T98G cells were then transfected with this pBLCAT3-JCV-early construct. Since it is well known that large T-antigen is able to activate its own promoter and that it also enhances the activity of both early and late promoters, we chose to also transfect T98G cells with an expression plasmid encoding large T-antigen.

Cells were harvested for low molecular weight DNA at day 4 post-transfections. DNA was then digested with Dpn I and BamH1 enzymes. Digested-DNA samples were separated on 1% agarose gel and were transferred to a nylon membrane. Replicated viral DNA was visualized upon incubation of the membrane Cat3-Mad1-Early plasmid.

As expected, our results showed that the band corresponding to replicated-DNA was only detectable in the presence of large T-antigen (Fig.12A, compare lanes 4 and 5). Interestingly, treatment of cells with conditioned media from induced PBMCs but not from uninduced PBMCs

showed a significant reduction in the levels of replicated DNA (compare lanes 6 to 7). These data suggested that immune mediators secreted by active PBMCs had a negative impact on JCV replication mediated by large T-antigen. In parallel to the DNA samples, we also prepared whole cell protein extracts from the same experiments and analyzed by western blot for detection of large T-ag expression (Fig.12B). Surprisingly, conditioned media from induced PBMCs showed a significant decrease in large T-ag levels (compare lane 5 with lane 3 and 4).
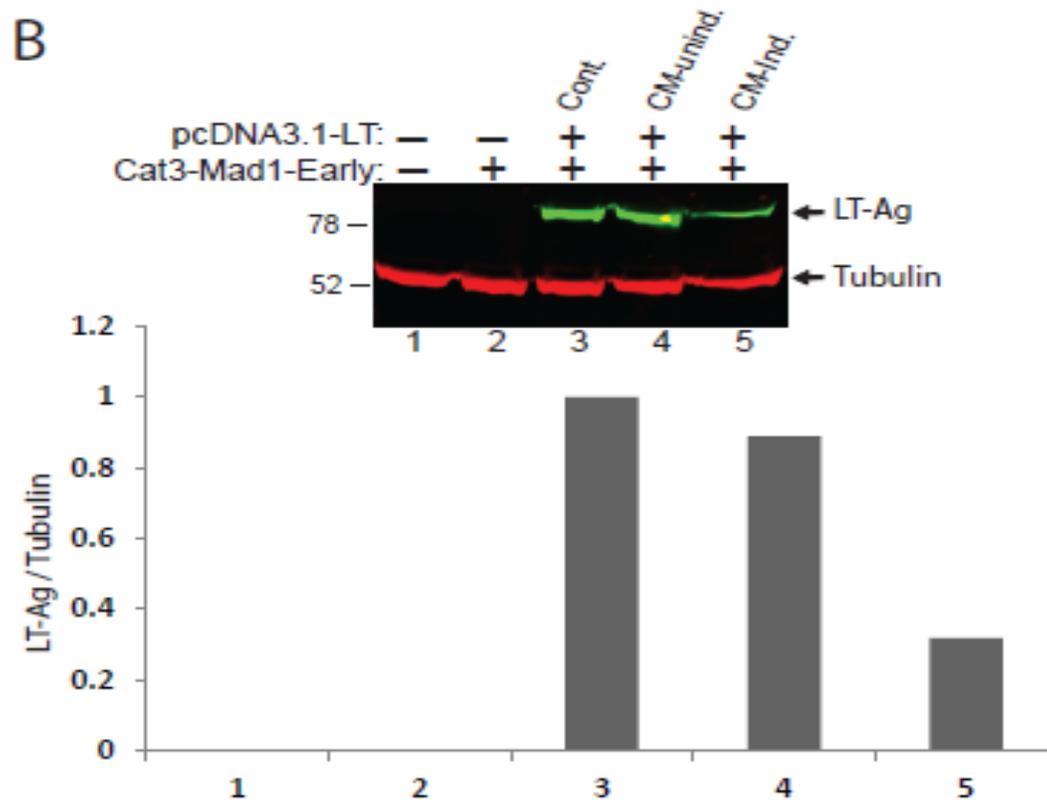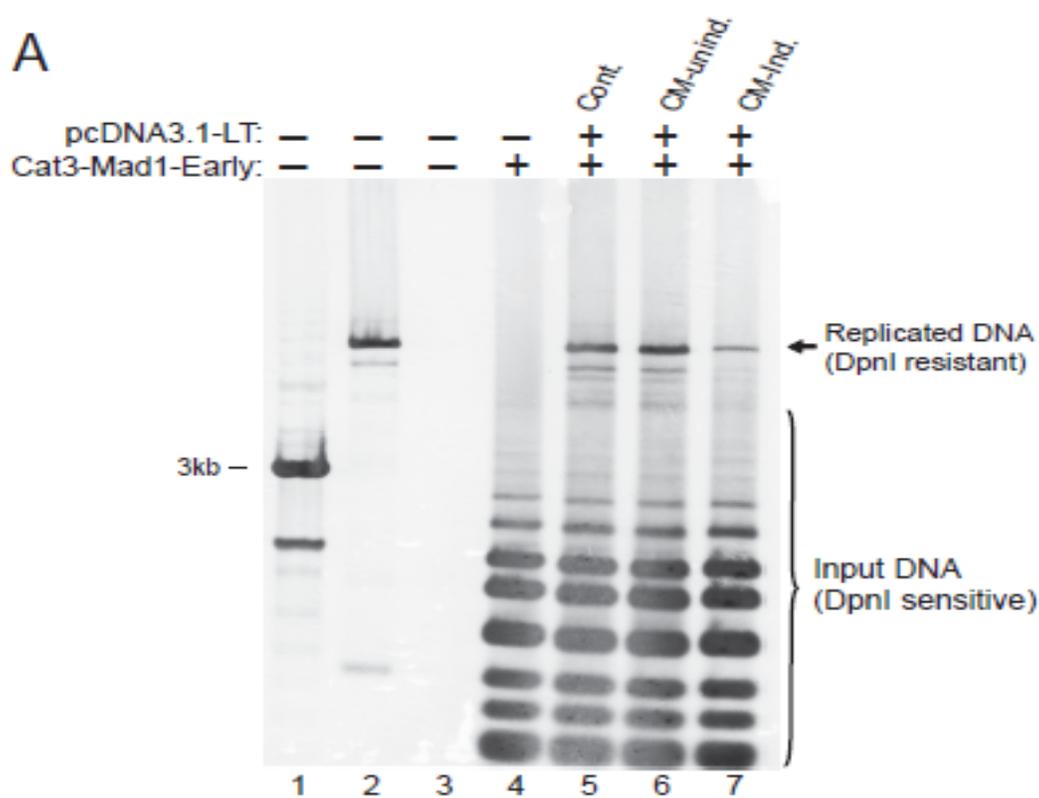
Fig.12 Conditioned media from induced PBMCs inhibits viral replication induced by LT-Antigen Replication assay was performed as previously described.T98G cells were transfected at a confluence of 1 x 106 cells per T 75-cm2 tissue culture flask for 24 hours. Low molecular weight DNA purified from cells was digested with Dpn I and BamH1 enzymes. Low molecular weight DNA extraction was performed by using QiagenMiniprep Kit. Digested – DNA samples were separated on 1% Agarose gel and then transferred to a nylon membrane (Fig. 3 A). Replicated viral DNA was visualized upon incubation of the membrane with a [32P]- labeled JCV DNA probe. In parallel, same extracts were also analyzed by western blot for the expression of large T-ag (Fig.5B). Surprisingly, after treatment with conditioned media from induced PBMCs, Large T-ag stability appeared altered and this led us to investigate on Large T stability after treatments.

Conditioned media from 3 different sets of PBMCs inhibits Large-T-Ag expression.

Another experimental question that was addressed in this work was to identify the mechanism involved in the negative modulation of Large-t levels induced by conditioned media as found in the above-mentioned experiments. T98G cells werethentransfected with a plasmid able to overexpress Large T antigen (pcDNA 3.1-LT-ag) for 24 hour. After recovery, cells were then treated with 50% of either uninduced or induced CMs collected from 3 different sets of PBMCs for 32 hours. Whole cell extracts were then analyzed by western blotting to determine Large T-ag expression. As seen in Figure 13A, western blot analyses of samples show that LT-Ag levels do appear highly down-regulated in all three cases after induced CM treatment compared to the uninduced CM treatment. In Figure 13 panel B is drawn a graphic representation of Large T expression level modulation normalized to tubulin after treatments. The results shown in Figure 13A and Figure 13B indicate that there is a direct effect on Large-T expression level modulation induced by soluble immune mediators secreted by induced PBMCs.
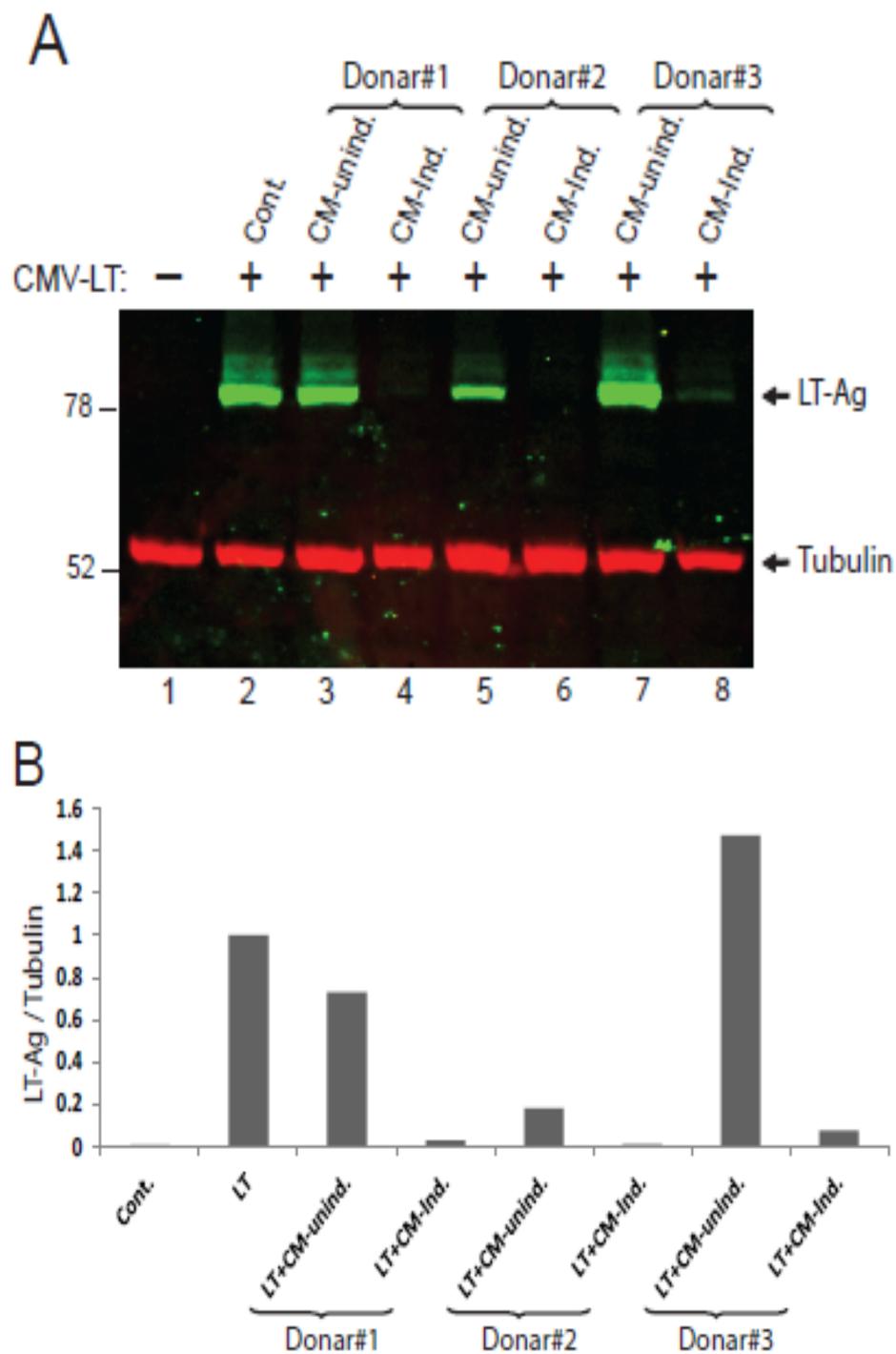
Figure 13. CM induced collected from 3 different donors all affect Large-T expression levels. *T98G cells transfected for 24 hours with an expression plasmid encoding large T- antigen, and then treated for 32 hours with either uninduced or induced CM. Cells were then harvested and Western blot analysis of whole cell extracts was performed in order to determine large T-ag expression levels. Large-t antigen does appear down-regulated after CM-induced treatment in all 3 cases. Bar graph represents the quantification of LT-Ag band intensities normalized to tubulin.*

4.4 Conditioned-media from PBMCs induced by PMA and Ionomycin down-regulates LT-Ag in a glioblastoma cell line.

To determine the effect of immune mediators secreted by PBMCs on JCV gene expression, we decided to use again T98G cells, a human GBM cell line, and transfect them with pcDNA3.1 LT+ smt-WT plasmid, which expresses the entire JCV early region, and therefore, encodes the major early regulatory protein of JCV, Large T-ag and small-t antigen. After a 4-hour recovery from transfection, T98G cells were treated with CMs from either induced or uninduced PBMCs for a 48h time period. Whole cell extracts were analyzed by western blotting to determine Large T-ag expression levels. As seen in Figure 14 A, CM-induced treatment suppressed the expression levels of Large T-ag, but the same effect was not present for the CM-uninduced treated samples. This indicated that immune mediators secreted by PBMCs had a regulatory function on the early viral protein expression levels. In parallel to western blot analysis of protein products, we also analyzed viral RNA products from early gene by RT-PCR. As shown in Figure 14 panel D, our data revealed no alteration on either LT-Ag nor small-t mRNA, whose schematic structures are displayed in Figure 14 panel C. These data suggested that the observed reduction in protein levels was most likely occurring at a protein quality control level rather than transcription or splicing modification.

Figure 14: Immune mediated suppression of early viral protein level. *A. and B. Western Blot analysis demonstrate how 32 hours treatment with CM-Induced, causes down-regulation of LT-Ag in transfected glioblastoma cell lines. C. Schematic structure of LT mRNA and Sm-t mRNA. D. RT-PCR analysis of JCV early proteins Lt-Ag and Sm-t gene products T98G cells non transfected and transfected with JCV early genome. In lane 1, Kb ladder was loaded as molecular weight marker. In lane 2, JCV Mad-1 genome was used as a positive control.*

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4.5 Cytokine array analysis of conditioned media.

Our preliminary data from infection studies showed that immune mediators secreted from induced PBMCs suppress JCV gene expression and replication in glial cells, suggesting a possible role of cytokines in the control of JCV propagation. Our next goal was then to identify which cytokines appeared to be highly activated after 48-hour stimulation with PMA and Ionomycin, in order to assess and identify which cytokines could be the ones responsible for such regulation. In order to determine the cytokine profiles of conditioned media upon stimulation with Ionomycin/PHA treatment, we utilized a commercially available cytokine array kit (RayBiotech, Inc.). Results are shown in Figure 6. The key cytokines, that appeared highly increased in PBMCs after stimulation with PMA and Ionomycin for 48 hours, are marked up in red, whereas marked up in blue, are the cytokines which result less active in induced conditioned media compared to the uninduced CM. A representative graph displayed in Figure 15 panel B helps quantify the fold change in the cytokine profiles of conditioned media from induced PBMCs compared to uninduced PBMCs.

Our preliminary cytokine arrays data revealed, among all, a robust increase in the expression of IL2, INFγ, RANTES, IL3, IL1β, and IL13, and an important decrease in the expression of MCP1, MIG, and MDC.

A

PBMCs-uninduced

PBMCs-induced
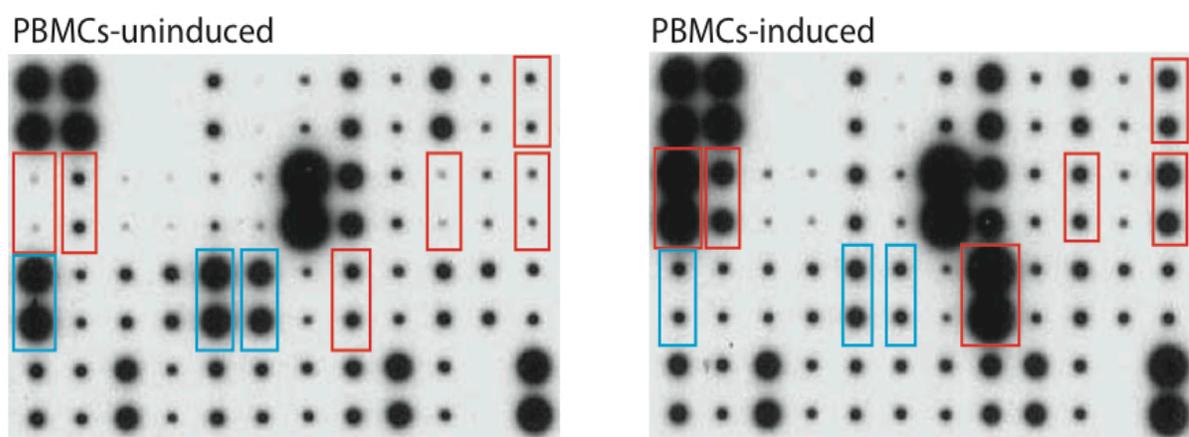
| Pos | Pos | Neg | Neg | ENA-78 | GCSF | GM-CSF | GRO | GRO-α | I-309 | IL-1α | IL-1β |
Pos	Pos	Neg	Neg	ENA-78	GCSF	GM-CSF	GRO	GRO-α	I-309	IL-1α	IL-1β
IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10	IL-12 p40p70	IL-13	IL-15	IFN-γ
IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10	IL-12	IL-13	IL-15	IFN-γ
MCP-1	MCP-2	MCP-3	MCSF	MDC	MIG	MIP-1δ	RANTES	SCF	SDF-1TA	RC	TGF-β1
MCP-1	MCP-2	MCP-3	MCSF	MDC	MIG	MIP-1δ	RANTES	SCF	SDF-1TA	RC	TGF-β1
TNF-α	TNF-β	EGF	IGF-I	Angiogenin	Oncostatin M	Thrombopoetin	VEGF	PDGF BB	Leptin	Neg	Pos
TNF-α	TNF-β	EGF	IGF-I	Angiogenin	Oncostatin M	Thrombopoetin	VEGF	PDGF BB	Leptin	Neg	Pos

B
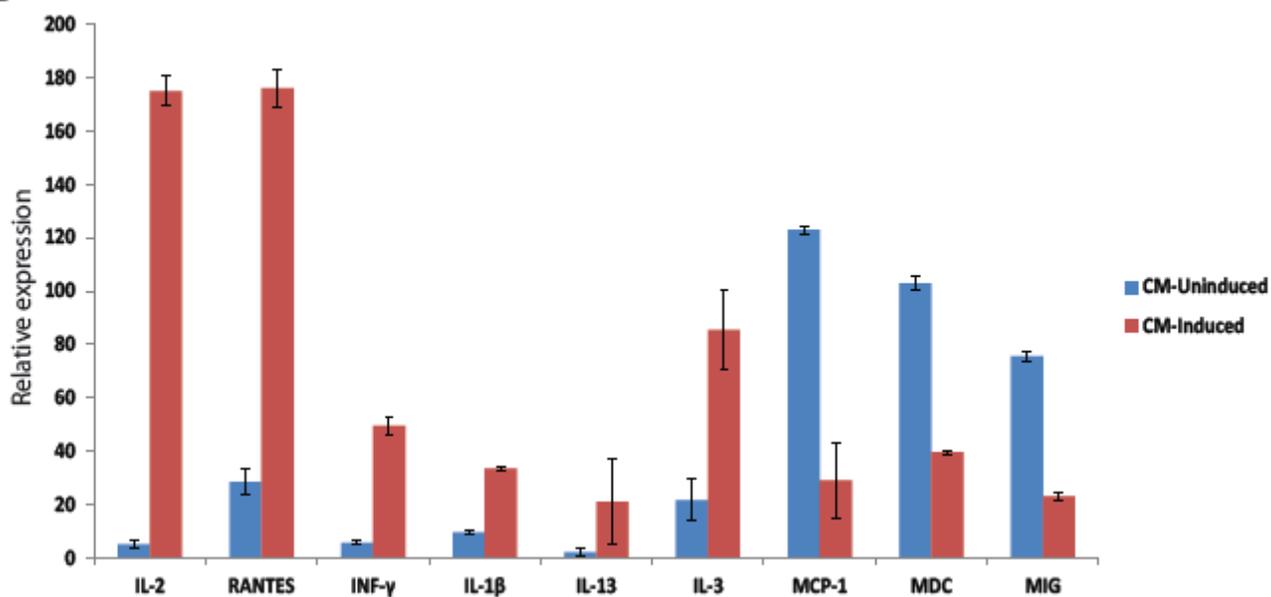
*Figure 15.Cytokine array with conditioned media from uninduced and induced PBMCs. A. Schematic representation of the cytokine array (**RayBiotech, Inc.**).Uninduced PBMCs (upper left panel) and induced PBMCs (upper right panel). Red boxes show the cytokines with increased production. Blue boxes show the cytokines with decreased production. B. Quantification of the fold change in the cytokine profiles of conditioned media from induced PBMCs compared to uninduced PBMCs.*

4.6 Effect of IL-2, RANTES and IL-13 alone and in combination on LT down regulation

The data presented in this work, demonstrate how immune mediators secreted by induced PBMCs were able not only to inhibit viral replication in glial cells, but could also alter Large T-ag expression levels at a post-transcriptional level. We therefore decided to investigate the direct effect of what we thought could be the key cytokines affecting Large T stability. Based on our previous findings, we decided to investigate the possible direct effect of three cytokines which in the cytokine array, appeared to be highly secreted after stimulation for 48 hours with PHA.

 In order to investigate the effect of the soluble immune mediators identified in the previous experiment, T98G cells were transfected with pCDNA 3.1-LT-ag, a construct characterized by the presence of CMV promoter, for 24 hours. They were then treated with uninduced CMs implemented with IL2 [1 ng\ml], RANTES [40 ng\ml] and IL-13 [5 ng\ml] separately or in combination.

Cells were then harvested, and whole cell extracts were analyzed by western blotting to determine Large T-ag expression. As seen in Figure 16, Large T-Antigen levels appeared to be drastically down-regulated after cytokine treatment compared to uninduced CM alone (compare lane 2 with lanes 3, 4, 5 and 6). The two cytokines which appeared to affect in a greater extent

Large T antigen expression levels, were IL-2 and RANTES. The bottom bar graph represents the quantification of LT-Ag band intensities normalized to tubulin. These findings strongly suggest a direct involvement of Interleukin-2 and RANTES in the post-transcriptional suppression of T-ag expression induced by conditioned media from PBMC.
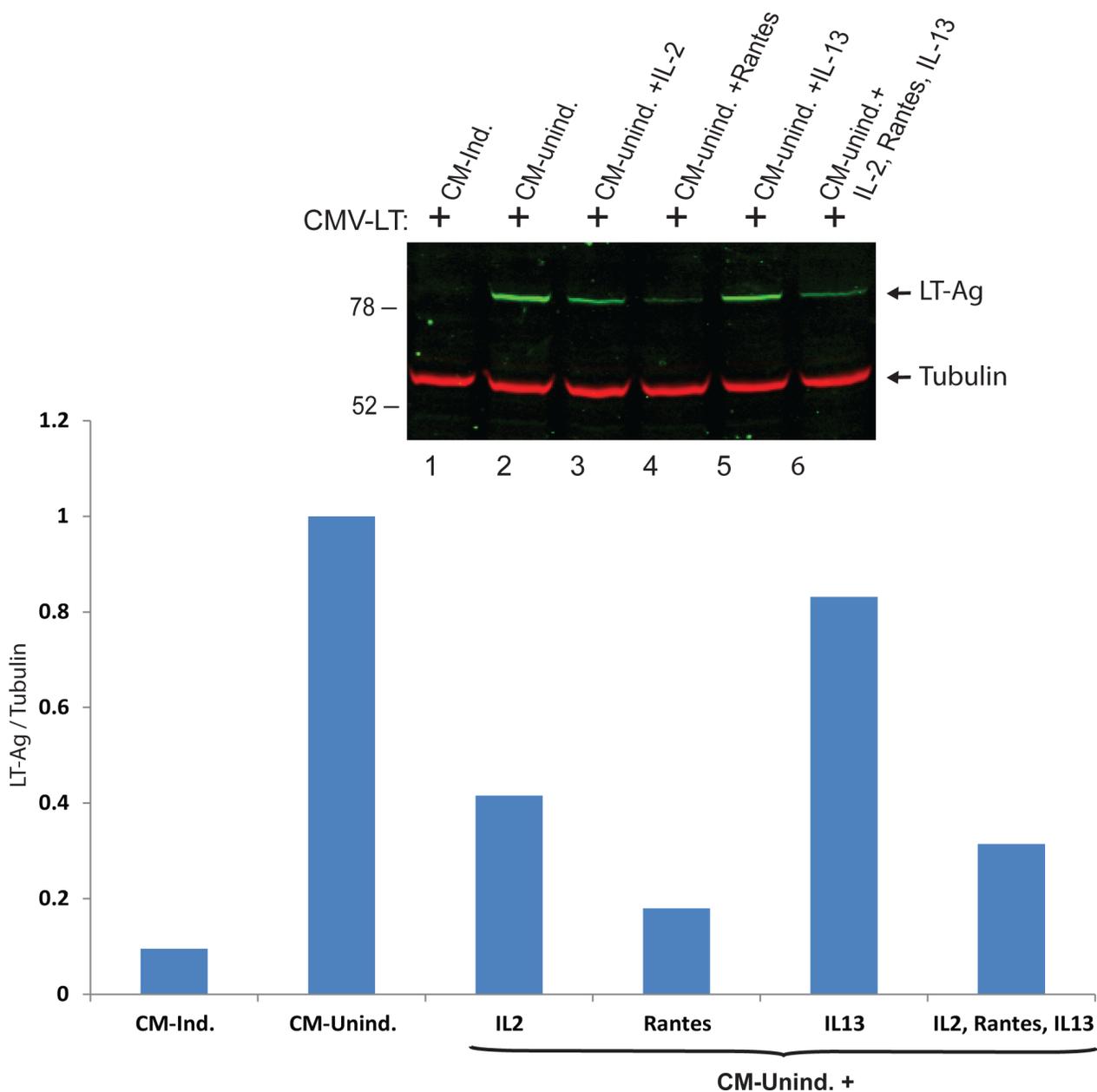
Figure 16.Direct effect of key cytokines, on LT expression. T98G cells transfected for 24 hours with an expression plasmid encoding large T- antigen, and then treated with IL2 [1 ng/ml], RANTES [ng/ml], IL-13 [ng/ml] alone or in combination, in the presence of conditioned media obtained from uninduced PBMCs. Western blot analysis from whole cell extracts was performed to determine large T-ag expression levels. The results obtained show a strong downregulation in large-T antigen expression levels when IL-2 and RANTES were implemented to uninduced conditioned media compared to uninduced-CM alone. This proves the direct effect of these cytokines on large-T expression level modulation. Bar graph represents the quantification of LT-Ag band intensities normalized to tubulin.

4.7 IL-2 and RANTES down regulate JCV Early and Late transcripts in a dose-dependent manner in glial cells.

After having identified these key cytokines as modulators of Large-T ag expression levels, we decided to investigate in more detail at what stage this downregulation effect actually occurs.

In order to determine at what level these cytokines regulate JCV, we transfected T98G cells, a human GBM cell line, with Luciferase reporter plasmids for the JCV Mad-1 early (Figure 9A) or late (Figure 17B) promoter. These constructs are integrated with JCV Mad-1 promoter in either the early or late orientation integrated with Luciferase enzyme which allows to quantify the promoter activity calculating the emission of light obtained from oxyluciferin when it returns to the ground state.

After recovery, cells were treated with increasing concentrations of IL-2 and RANTES for 32 hours. We observed a dramatic decrease in pJCV-Early gene levels after treatment indicating a strong dose-dependent effect of these cytokines on Early gene transcription. JCV Mad-1 Late promoter clone also appeared down-regulated after treatment.

We finally tested the effect of these cytokines implemented in conditioned media obtained from uninduced PBMCs, in T98G glioblastoma cells that had been transfected once again with Luciferase reporter plasmids for the JCV Mad-1 early promoter. Such an approach helped exclude any possible interference from other soluble immune mediators secreted from PBMCs,

and prove that such an inhibitory effect is actually due to a direct effect of IL-2 and RANTES on JCV- Early transcription machinery (Figure 17C).

Once again, the results we obtained demonstrated how the effect of these cytokines implemented to conditioned media obtained from uninduced PBMCs also leads to a dramatic reduction in JCV- Early transctipts levels.

We hypothesize therefore that IL-2 and RANTES not only play a key role in the down-regulation of Large-T expression levels, but that they are also capable of reducing viral transcription of both early and late genes.
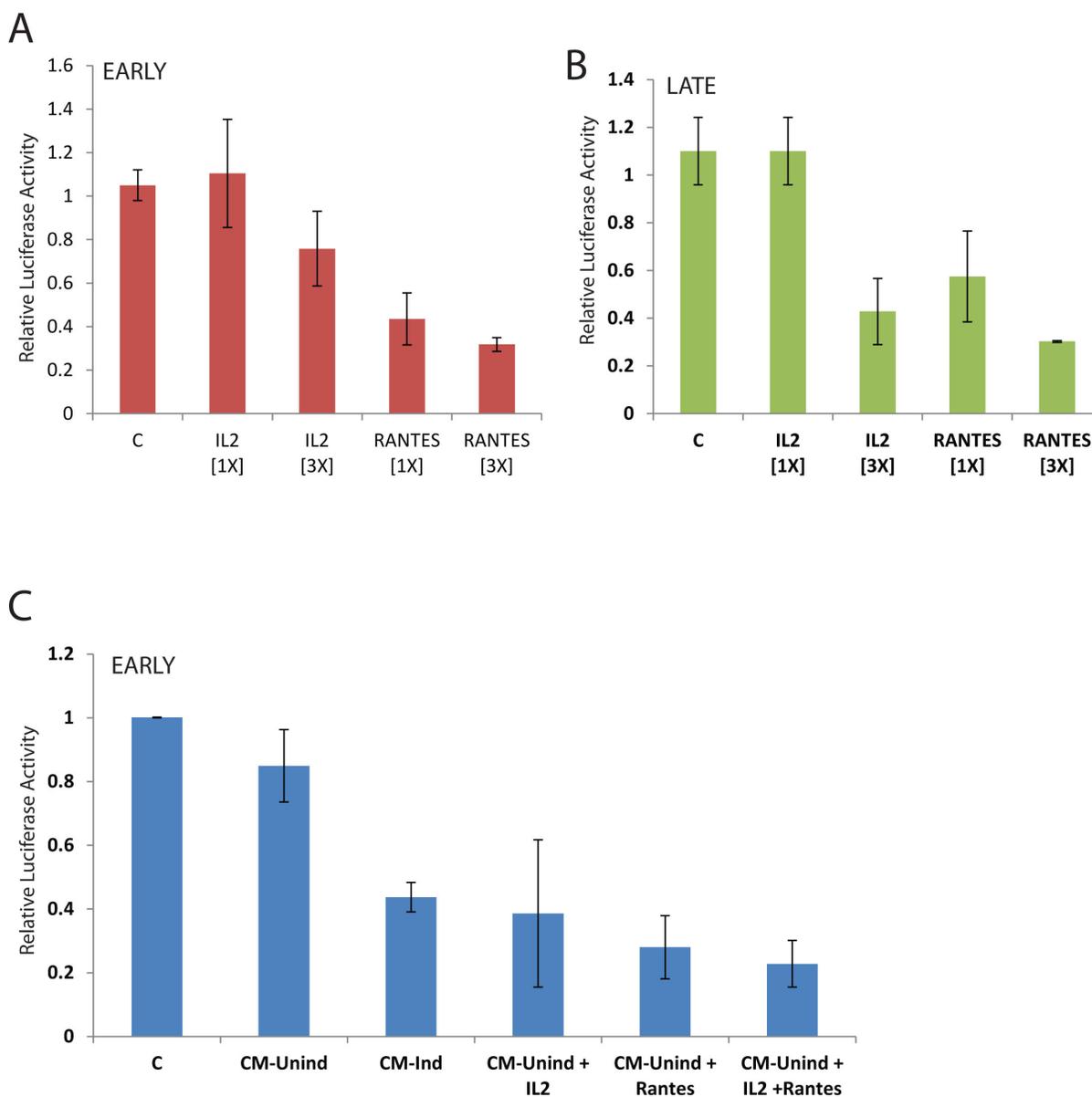
Figure 17. Dose dependent effect of cytokines on JCV Early and Late gene transcription. *T98G cells were transfected with Luciferase reporter plasmids for the JCV Mad-1 early (Panel A) or late (Panel B) promoter. We then treated them with increasing concentration of IL-2 and RANTES for 32 hours. Cells were then harvested for luciferase assay. LUC activity was presented as a histogram. A. and B. panels demonstrate how transcriptional levels of both JCV-Early and Late gene result significantly decreased after dose-dependent treatment with IL-2 and RANTES compared to control. Panel C. shows how the effect of these cytokines implemented to conditioned media obtained from uninduced PBMCs also leads to a dramatic reduction in JCV- Early transctipts levels.*

4.8 Time dependent effect of IL2 on LT-Ag regulation

After having identified the key cytokines involved in LT-Ag regulation, we focused on the kinetics of one the major responsible cytokines: IL2. We decided therefore to investigate the possible effect of this cytokine on Large-T expression levels in a time-dependent manner.

T98G glioblastoma cells were transfected with an expression plasmid encoding large T-antigen (pcDNA 3.1-LT-ag), for 24 hours. After recovery from transfection, cells were then starved [1% FBS\DMEM] overnight, in order to prevent any possible interference between the FBS implemented to the cell medium and the cytokine's effect. The following day, cells were either treated or not with IL2 [1 ng/ml] which was added directly to the culture media. Whole cell extractions were performed every hour and each time, new cytokine was implemented to the media. Cells were then harvested and western blot analysis of protein extracts was performed in order to investigate the time-dependent effect of IL2 on Large-T expression levels.

As shown in Figure 18, a massive down-regulation in LT-Ag levels already occurred 1 hour after IL-2 treatment, whereas the strongest down-regulation appears 4 hours after treatment (Figure 8 lane 10) compared to untreated cells. The bottom bar graph represents the quantification of LT-Ag band intensities normalized to tubulin.

Once again these findings indicated that IL-2 is capable of modulating one of JCV major regulatory proteins, Large-T antigen, even after a very short period of treatment.
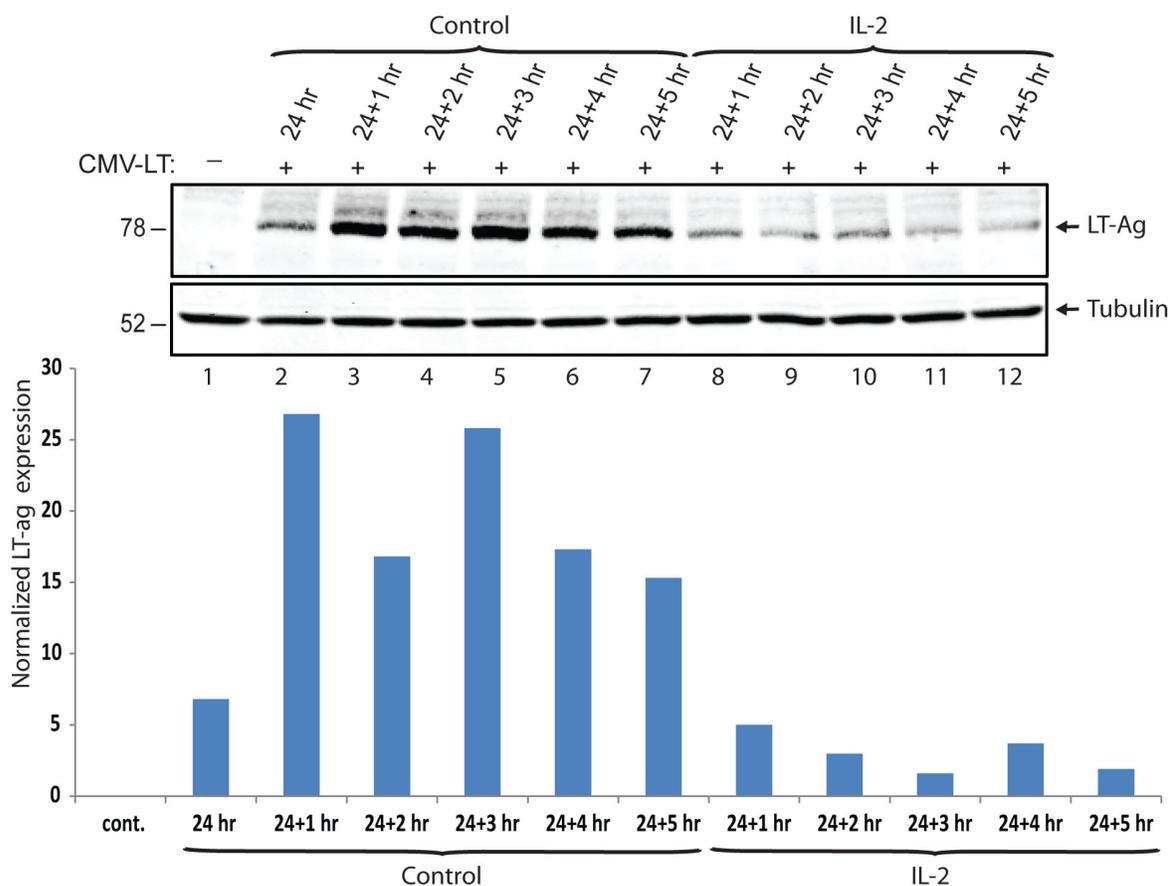
Figure 18.Time dependent effect of IL2 on LT-Ag regulation. *T98G cells were transfected for 24 hours with an expression plasmid encoding large T-antigen, and underwent starvation with a 1%FBS\DMEM overnight. The following day they were either treated or not hourly with IL2 [1 ng/ml] which was directly implemented to the media. Western blot analysis of whole cell extracts from T98G was performed in order to determine large T-ag expression levels. Large-t antigen does appear extensively down-regulated after only one-hour treatment, with a pick of downregulation registered after 4 hours treatment, compared to untreated samples. Bar graph represents the quantification of LT-Ag band intensities normalized to tubulin.*

4.9 IL2 down-regulates Large-T expression levels through autophagy

The above results demonstrated how IL-2 is capable of down-regulating, even after a very short period of treatment, Large T antigen expression levels. We therefore decided to investigate which key mechanism had a negative impact on Large T stability. Sariyer et al. (2012) have previously demonstrated how by overexpressing Bag3, a key protein deeply involved in the activation of the autophagic machinery, Large T antigen expression levels appeared to be drastically decreased. Thus we sought to investigate the possible role of CM-Induced and Il-2 in Large-T antigen modulation by autophagy. Consequently, we transfected PHFA cells with a construct named Plasmid 21074: ptfLC3 (Kimura et al., 2007) mRFP-GFP tandem fluorescent-tagged L3 (tfLC3). Such a construct is commonly used for assessing the fusion step of autophagosomes with lysosomes. Autophagosomes marked by this marker protein show both mRFP and GFP signals. After fusion with lysosomes, GFP signals results attenuated, and only mRFP signals are observed. After recovering from transfection, cells were subjected to a 32-hour treatment with either CM-Uninduced, CM-Induced and IL2 [1ng/ml] that was directly implemented to cell's growing media. Cells were then fixed in a 4% Formaldheide solution and analyzed by fluorescent microscopy. As shown in figure 19 upper panel A, we found that not only CM-Induced but also IL-2 treatment increased both yellow (i.e., mRFP and GFP) and red (i.e., mRFP only) punctae, indicating that it led to the degradation of the GFP moiety due to the increased autophagic flux. These effects were not observed in the control samples. In panel B a schematic representation of total Lc3 dots obtained by the addition of the number of yellow Lc3 dots with red Lc3 dots. Percentage of the red Lc3 dots from the total Lc3 dots from (B). More

than 10 cells were counted in each condition. These results demonstrate that Large T down-regulation induced by IL-2 treatment is due to the activation of the autophagic machinery.
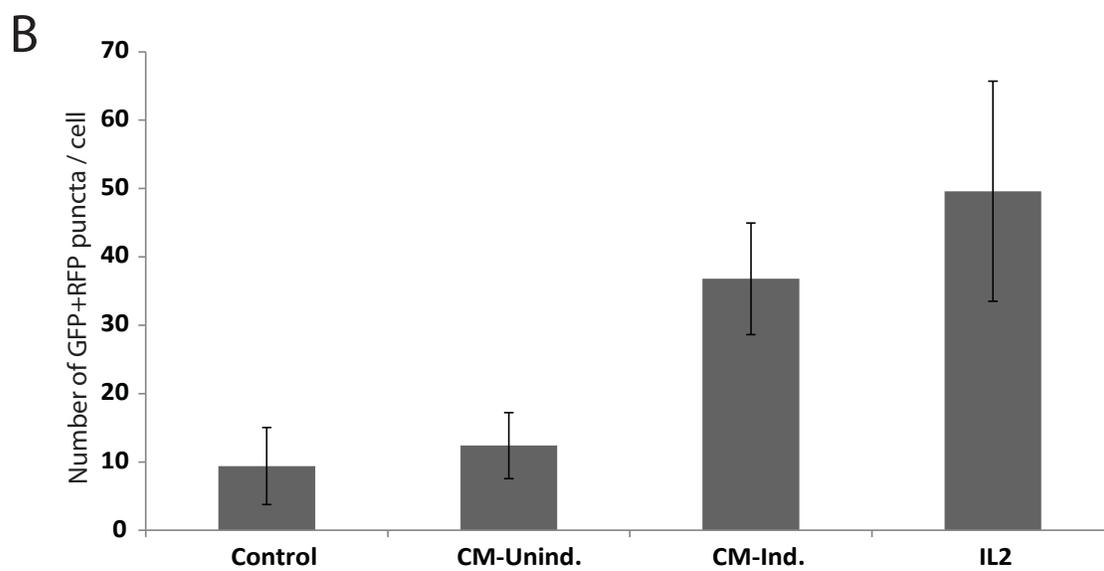
A

	GFP	RFP	MERGE
CONTROL			
CM-UNIND			
CM-IND			
IL2			

B
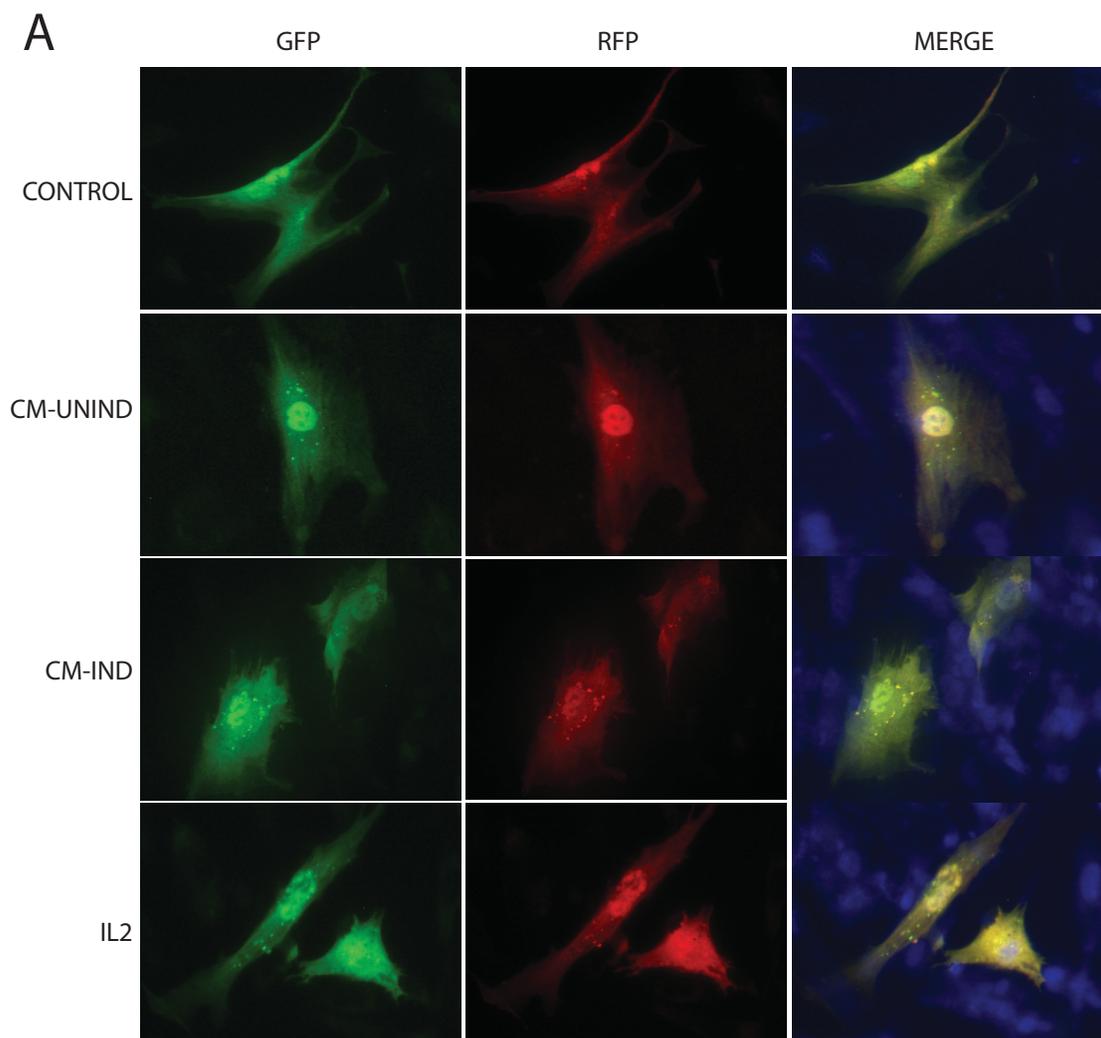
Figure 19. IL-2 induces Large T down-regulation through autophagy. A) T98G cells were transfected with the tandem mRFP-GFP-LC3 plasmids and then treated for 36 hours with either CM-Uninduced, Cm-Induced and IL2 [1ng/ml]. cells were then fixed with 4% Formaldheide followed by fluorescent microscopy.Total Lc3 dots are the addition of the number of yellow Lc3 dots with red Lc3 dots. Percentage of the red Lc3 dots from the total Lc3 dots from (B). More than 10 cells were counted in each condition.

CHAPTER 5

CONCLUSION

JC virus (JCV) is a member of Polyiomaviridae family, characterized by icosahedral capsids, circular and double-stranded DNA. JC viral genome is characterized by a specific bipartite organization that is composed of two regions, called early and late transcription units, which despite being similar in size, are transcribed in opposite directions. Early region encodes for early regulatory proteins, such as small-t, Large-T, T'135 and t'165.

The late side of the viral genome is transcribed concomitant with DNA replication and it encodes for all structural capsidic proteins such as VP1, VP2 and VP3, and the regulatory protein Agno, which function still remains unclear. JC virus reactivation in the host is the causative agent of the fatal demyelinating disease Progressive Multifocal Leukoencephalopathy (PML). PML is characterized by a lytic infection of oligodendrocytes, that are the myelin-producing cells in the Central Nervous System (CNS). Studies have estimated that about 70% of human population has been infected during childhood, but not everyone develops PML disease since JCV reactivation only occurs in those cases in which there are immune-compromised conditions. This is one of the main reasons why PML is often closely associated with conditions such as

Acquired Immune Deficiency Syndrome (AIDS), Lymphoproliferative diseases and patients undergoing immunosuppressive therapies.

Since it is known that JCV only reactivates in immunocompromised conditions, we aimed to recreate an experimental model in which an activated immune system could modulate JCV infections in glial cells. Specifically, in order to investigate the possible impact of soluble immune mediators on JCV gene expression and replication, we utilized PBMCs as the source of immune mediators in infection studies. The model was composed of two systems, an active immune system model in which conditioned-media samples created by stimulating PBMCs for 48 hours with PHA. A second system that we called CM-uninduced, in which PBMCs were not stimulated. In both systems, PBMCs were obtained from a single healthy patient's peripheral blood. Then SVGA cells were infected with JC virus and treated with either CM-uninduced or CM-induced. Cells were then harvested after 8 and 15 days post infection. Viral capsid protein VP1 levels were then analyzed by Western blot analysis in order to assess whether infection took place and to monitor VP1 level changes after CM treatment.

Our results suggested that CM-Uninduced treatment slightly modulate VP1 levels already after 8 dpi, but it is after CM-induced treatment that Vp1 levels are strongly down-regulated compared to control. In this view, our analysis showed that the presented approach is a feasible model to investigate the effect of CM on JCV.

Furthermore, Q-PCR analysis of the growth media was performed to assess if after CM-induced treatment, viral copy numbers in SVGA cells infected with JCV, were altered. We found that viral copy numbers were indeed drastically reduced, already after 8 dpi but especially after 15 dpi. This indicates a strong inhibiting effect of CM-induced on viral infection.

We were then interested in investigating whether CM-Induced was also able to affect JC virus transcriptional levels. In order to evaluate at what level this inhibition occurs, we chose to test if CM-Induced was able to modulate JCV Early and Late transcriptional levels using Luciferase activity (LUC) assay.

Our luciferase activity (LUC) assay results demonstrated how CM induced treatment greatly decreases Early gene transcripts levels indicating a direct effect of soluble immune mediators on Early gene transcription. JCV Late promoter activity resulted down-regulated after treatment as well, although in a less significant manner.

Another important factor we believed important to study was whether immune mediators also played a modulating effect on JCV viral DNA replication. In order to test such an effect we performed Southern blot analysis on PHFA cells.

Interestingly our data suggested that treatment of cells with conditioned media from only induced PBMCs showed a significant reduction in the levels of replicated DNA. Surprisingly, Large-T antigen expression levels also appeared to be strongly down-regulated after CM-induced treatments. This suggested that immune mediators secreted by active PBMCs had a negative impact on JCV replication mediated by large T-antigen.

In order to prove that these findings were due to a direct effect of CM-induced on Large-T antigen expression levels we chose to perform Western blot analysis on T98G cells transfected with a plasmid overexpressing Large-T and then treated with CM-uninduced and CM-induced obtained from three different donors.

Our results show that the down-regulating effect on Large-T antigen expression levels was consistently present in all the analyzed samples treated with CM-induced. This indicates that

soluble immune mediators secreted by induced PBMCs do have a direct effect on Large-T mRNA expression levels.

After evaluating this direct effect of induced CMs on the early viral protein expression levels, we planned to investigate whether CM-induced was also able to affect viral gene expression levels.

We therefore analyzed viral RNA products from early gene by RT-PCR and we did not come across any alteration on either LT-Ag or small-t mRNA. These data suggested that the observed reduction in protein levels was most likely occurring at a protein quality control level rather than transcription or splicing modification levels.

Our preliminary data from infection studies showed that immune mediators secreted from induced PBMCs suppress JCV gene expression and replication in glial cells, suggesting a possible role of cytokines in the control of JCV propagation. Our next goal was then to assess and identify which cytokines could be the ones responsible for such regulation. Thus we performed cytokine arrays of both CM-uninduced and CM-induced. Our preliminary data revealed, among all cytokines, a robust increase in the expression of IL2, INFγ, RANTES, IL3, IL1β, and IL13, and a substantial decrease in the expression of MCP1, MIG, and MDC.

The results presented in this work demonstrate how immune mediators secreted by induced PBMCs were able not only to inhibit viral replication in glial cells, but could also alter Large T-ag expression levels at a post-transcriptional level. We therefore decided to investigate the direct effect of what we thought could be the key cytokines affecting Large T stability. We decided to test the direct effect of three cytokines which appeared strongly increased in the CM-induced population, IL-2, RANTES and IL-13, alone or in combination. Based on our results, the two

cytokines that appeared to affect to a larger extent Large T antigen expression levels, were IL-2 and RANTES.

After having identified these key cytokines as modulators of Large-T ag expression levels, we then investigated in greater detail at what stage this down-regulation effect actually occurs. We therefore tested whether these key cytokines were able to modulate, in a dose-dependent manner, JCV Early and Late transcriptional levels using Luciferase activity (LUC) assay. Our results showed a dramatic decrease in Early gene levels after treatment indicating a strong dose-dependent effect of these cytokines on Early gene transcription. Late promoter also appeared down-regulated after treatment.

Next we decided to test the effect of these cytokines implemented in conditioned media obtained from uninduced PBMCs. Once again, we found demonstrated how the effect of these cytokines implemented to conditioned media obtained from uninduced PBMCs also led to a dramatic reduction in JCV- Early transcripts levels.

We hypothesized therefore that IL-2 and RANTES not only play a key role in the down-regulation of Large-T expression levels, but that they are also capable of reducing viral transcription of both early and late genes.

We then focused on the kinetics of one of the major responsible cytokines: IL2. We decided therefore to investigate the possible effect of this cytokine on Large-T expression levels in a time-dependent manner. In order to do so, we transfected T98G cells with a plasmid overexpressing Large-T antigen, and then treated them with IL2 that was added directly to the culture media. Whole cell extractions were performed every hour and each time, new cytokine was implemented to the media. Cells were then harvested and western blot analysis of protein extracts was performed.

IL-2 treatment appeared to strongly down-regulate Large T protein levels 4 hours after treatment. Once again these findings indicated that IL-2 is capable of modulating one of JCV major regulatory proteins, even after a very short period of treatment.

Finally, our last experiment was designed to test which mechanism was affecting Large-T antigen stability.

Previous studies (Sariyer et al., 2012) demonstrated how Large T antigen expression levels appeared to be significantly reduced after the overexpression of Bag3, one of the most important proteins deeply involved in the autophagic machinery,.

BAG3, in fact, cooperates with the molecular chaperones Hsc70 and HspB8 to induce the degradation of mechanically damaged cytoskeleton components in lysosomes. This process is called chaperone-assisted selective autophagy (CASA).

We therefore decided to investigate whether CM-induced, and especially IL-2, were capable of triggering the autophagicmachinery .

In order to test such activation, we used a mRFP-GFP tandem fluorescent-tagged L3 (tfLC3) construct (Kimura et al., 2007).

This novel model allows us to assess the fusion step of autophagosomes with lysosomes. GFP-LC3 punctate signals do not colocalize with lysosomes (Bampton et al., 2005; Kabeya et al., 2000). In contrast, RFP (and other red fluorescent proteins, such as mCherry) exhibits more stable fluorescence in acidic compartments (Katayama et al., 2008), and mRFP-LC3 can readily be detected in autolysosomes. By exploiting the difference in the nature of these two fluorescent proteins, autophagic flux can be morphologically traced (Kimura et al., 2007). With this novel construct, autophagosomes and autolysosomes are labeled with yellow (i.e., mRFP and GFP) and red (i.e., mRFP only) signals, respectively. If autophagic flux is increased, both yellow and red

punctae are increased; however, if autophagosome maturation into autolysosomes is blocked, only yellow punctae are increased without a concomitant increase in red punctae.

PHFA cells were transfected with the above mentioned tfLC3 construct and subjected to a 32-hour treatment with either CM-Uninduced, CM-Induced and IL2 [1ng/ml].

Cells were then fixed in a 4%Formaldheide solution and analyzed by fluorescent microscopy.We found that were subjected to either CM-Induced but especially IL-2 treatment, resulted in an increased number of both yellow (mRFP-GFP merged) and red (i.e., mRFP only) punctae, indicating that in IL-2 treated samples, the increased autophagic flux led to the degradation of the GFP moiety. These effects were not observed in the control samples.

These findings indicate that autophagosomes in CM-induced and IL-2 treated cells are able to fuse with the lysosomes and that autophagic flux or turnover is increased.

These observations present a novel role of immune mediators in regulation of JCV gene expression, and provide a new avenue of research to understand in depth molecular mechanism of viral reactivation in patients who are at risk of developing PML, and identifying which immune subpopulation may be the main responsible of the downregulation of JC virus Large T antigen.

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