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Viral trafficking pathways of JC virus to the brain in Progressive Multifocal Leukoencephalopathy

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Abstract

Progressive multifocal leukoencephalopathy (PML) is a highly fatal, demyelinating disease of the brain caused by lytic infection of oligodendrocytes with the JC polyomavirus (JCPyV). Emergence of PML is considered rare and is always associated with an underlying deficit in immune surveillance. In accordance, the majority of PML cases are detected in patients with HIV-induced severe immunodeficiencies. Fortunately, the introduction of cART significantly reduced the incidence of PML in HIV-infected patients. In 2005, however, a surprising increase in PML cases was observed with the use of monoclonal antibodies, including natalizumab and rituximab. As these therapies modulate the immune system rather than suppressing it, questions were raised regarding the conventional route of JCPyV pathogenesis. Owing to the lack of both animal models, and sufficient *in vitro* models to sustain effective replication of JCPyV, the mechanisms of JC virus infection remain largely uncertain and current understanding in pathogenesis is obtained from human autopsy material. In particular, transport of JCPyV through the blood brain barrier is a challenging concept and requires critical analysis. This review summarizes the current understanding of JCPyV pathogenesis, and revisits commonly accepted mechanisms of JCPyV trafficking to the brain.

Introduction

In 1958 two patients were described with a syndrome characterized by progressive dementia, motor dysfunction and vision loss, rapidly followed by death [1]. Subsequent histopathological studies displayed severe demyelination, abnormal oligodendroglial nuclei and giant astrocytes leading to the concept of Progressive Multifocal Leukoencephalopathy (PML). Remarkably, both patients were diagnosed with underlying lymphoproliferative disorders [1].

Despite the clinical and pathologic description of PML, demonstration of the causative agent remained indecisive until in 1965 papova-like virions in nuclei of injured oligodendrocytes were displayed by electron microscopy [2]. Though, isolation of the virus was not until 1971 [3], when it was named JC, after the initials of the patient. This newly acquired virus is a double-stranded DNA polyomavirus, abbreviated as JCPyV [4]. Subsequently, seroprevalence against JCPyV was detected in a large percentage of the population [5].

Prior to the onset of the HIV-1 pandemic in the mid-80s, PML was considered a very rare viral disease in patients suffering from hematologic malignancies [6]. However, a sharp increase in the occurrence of PML was observed in HIV-1 infected patients, with an incidence of approximately 3 to 5% in patients diagnosed with AIDS [7]. Moreover, a considerably higher frequency of PML has been observed in AIDS patients compared to individuals with other underlying causes of immunosuppression [7], as approximately 85% of the PML cases is associated with AIDS as underlying immunodeficiency [6]. Fortunately,the incidence of PML in HIV-infected patients was sharply reduced after the introduction of cART [8]. More recently, it was unexpectedly observed that patients treated with immunomodulatory therapies, in particular with monoclonal antibodies such as natalizumab, are predisposed to the onset of PML [9, 10]. In contrast to immunodeficient individuals, immunity in these patients is not directly suppressed, though their normal immune functions are dramatically altered. Considering the comprehensive distribution of JCPyV within populations, the emergence of PML is relatively rare and only occurs in patients with a suppressed or altered immune system. As a result, the pathogenesis of JCPyV is highly complex and current understanding in the mechanisms of infection are inconsistent. In particular, little is known of how the virus acquires entry to the brain in order to successfully induce lytic infection of oligodendrocytes. To elucidate the puzzling emergence of PML in immunosuppressed individuals, critical analysis of JCPyV pathogenesis must be conducted. This review therefore aims to summarize the extensive literature on JCPyV pathogenesis, though, more importantly, to shed light on the complex route of JCPyV trafficking through the blood-brain barrier.

Clinical manifestations of PML

Clinical manifestations in PML are quite diverse, as practically any area in the brain may be affected by the JC virus. However, the majority of affected patients show behavioral and cognitive abnormalities. Most commonly, patients show symptoms as visual fields impairments, motor dysfunctions or dementia [7] and may also show speech and language disturbances, personality changes, emotional lability and headaches [11, 12]. Seizures have been unexpectedly observed in 18% of PML cases when lesions were present directly adjacent to the hemispheric cortex [13]. Despite the seemingly random distribution of plaque lesions, foci are mainly detected in the posterior brain regions, including the brain stem, cerebellum and occipital lobe as is demonstrated by the expression of clinical symptoms [4]. Emergence of PML is progressive and nearly always fatal, and so far, no uniform treatment of PML has been demonstrated despite experimental and clinical trials with antiviral drugs [14]. Therefore, current treatments aim on regaining immune control over JC viral replication, as represented by the reduced incidence of PML after the introduction of cART [8].

However, even though recovery of the cellular immune response may lead to remission of the disease, rapid restoration can potentially trigger Immune Reconstitution Inflammatory Syndrome (IRIS), as has been observed in HIV-related PML patients [15].

JCPyV genome organization

The JC virus consists of a non-enveloped, T=7 icosahedral virion, is approximately 42nm in diameter and is known as a member of the *polyomaviridae* family. The JCPyV genome is a circular double-stranded DNA of roughly 5100 base pairs and, once it has entered the host nucleus, composes a nucleosomal structure with host cell histones, as has been determined for the polyomavirus-member SV40 [16]. The genome of the polyomavirus family can be divided into early coding regions, expressing genes prior to the onset of DNA replication and the late coding regions, encoding genes after initiation of DNA replication. These domains are physically separated by the non-coding control region (NCCR), harboring the origin of DNA replication, TATA sequences and multiple promoter/enhancer elements [4, 17]. Moreover, both the early and late regions are transcribed in opposite directions and from opposite strands of DNA (**Fig. 1***A*).

The early region harbors the large T-antigen, small T-antigen genes and various splice variants that are derived by alternative splicing of one major pre-mRNA transcript, known as T'135, T'136 and T'165. These genes are involved in viral replication and transcription of the late region genes. Accordingly, the late region genes are composed of the capsid proteins VP1, VP2 and VP3 and an accessory protein known as agnoprotein. Of note, as the viral genome does not harbor any DNA or RNA polymerases, it must take advantage of the host replication machinery in order to initiate viral replication or transcription. Once in the nucleus, the viral genome serves as a template for transcription by the host RNA polymerase II and solely host-derived proteins will be used for the transcription of the viral early genes. Consequently, both the availability of the host transcription factors, as the sequence of the NCCR are of high importance in order to regulate JC viral transcription. The NCCR sequence has a size of roughly 200 bp and is considered the primary modulator of viral activity, regulating early and late viral gene expression. The most conserved strain, the archetype NCCR strain, is a sequence of six blocks, denoted A,B,C,D,E and F [17, 18] (**Fig. 1***B*). Each block harbors binding sites for host transcriptional factors, essential for the regulation of viral transcription. The archetype strain is rarely associated with PML tissue, though is frequently found in the kidney and urine and does not support infection in *in vitro* cultures [19]. Contrary, NCCR sequence variants have been found in brain isolates, cerebrospinal fluids and blood of PML-patients [20] and show effective replication in glial cells, resulting in lytic infection [19]. These variants are presumably derived from rearrangements, deletions or insertions acquired during viral reactivation of the archetype virus [11], and hence are referred to as prototype. The originally isolated prototype is the Mad-1 strain, characterized by a 98-bp tandem repeat, resulting in duplication of the TATA-box and occurrence of multiple transcription factor binding sites [14] (**Fig. 1***C*). However, subsequent isolated JC viruses from pathologic affected tissues have shown to contain strains that are highly diverse in comparison to the Mad-1 strain [20], highlighting the extreme variability between PML-cases. In addition, individual PML-patients have shown to harbor a predominant NCCR genotype, coexisting with multiple minor subtypes expressing small variations [20]. Regardless of the detailed rearrangement of the NCCR sequences, the neurotropic prototype strain generally displays repeat structures that express significantly more binding sites for host transcription factors [20], and consequently, has striking effects on viral replication rate and multiplication. Accordingly, it has been observed that the host transcription factors Spi-B, NF-1X, C/EBPβ, NFκB, Oct-6 and YB-1/Purα support viral activity by binding to the repeat element on the neurotropic NCCR [11, 14, 21] (**Fig. 1***B*/*C*).

Rather than the 98-bp insertion, multiple independent studies have reported different deletions of segment D as the most commonly affected part in prototype JCPyV isolated from PML-patients [20, 22]. *In vitro* deletion of block D in archetype NCCR displayed strongly elevated expression of the viral early genes [20], indicating a positive prerequisite for viral activity in infected cells. Moreover, point mutations in the VP1-capsid protein, situated near the sialic acid binding pocket, have been demonstrated in the neurotropic strains isolated from PML-patients [22, 23] and these mutations were not present in the archetype NCCR harvested from the urine of these patients [22]. Hence, these modifications have demonstrated to induce a loss or substantial change in the binding specificity of the virus to sialic acid,

thus significantly increasing the pathogenicity of this strain [22, 23]. These results highlight the relevance of rearranged NCCR sequences in order to increase replication and cytypathology in JCPyV susceptible cells.

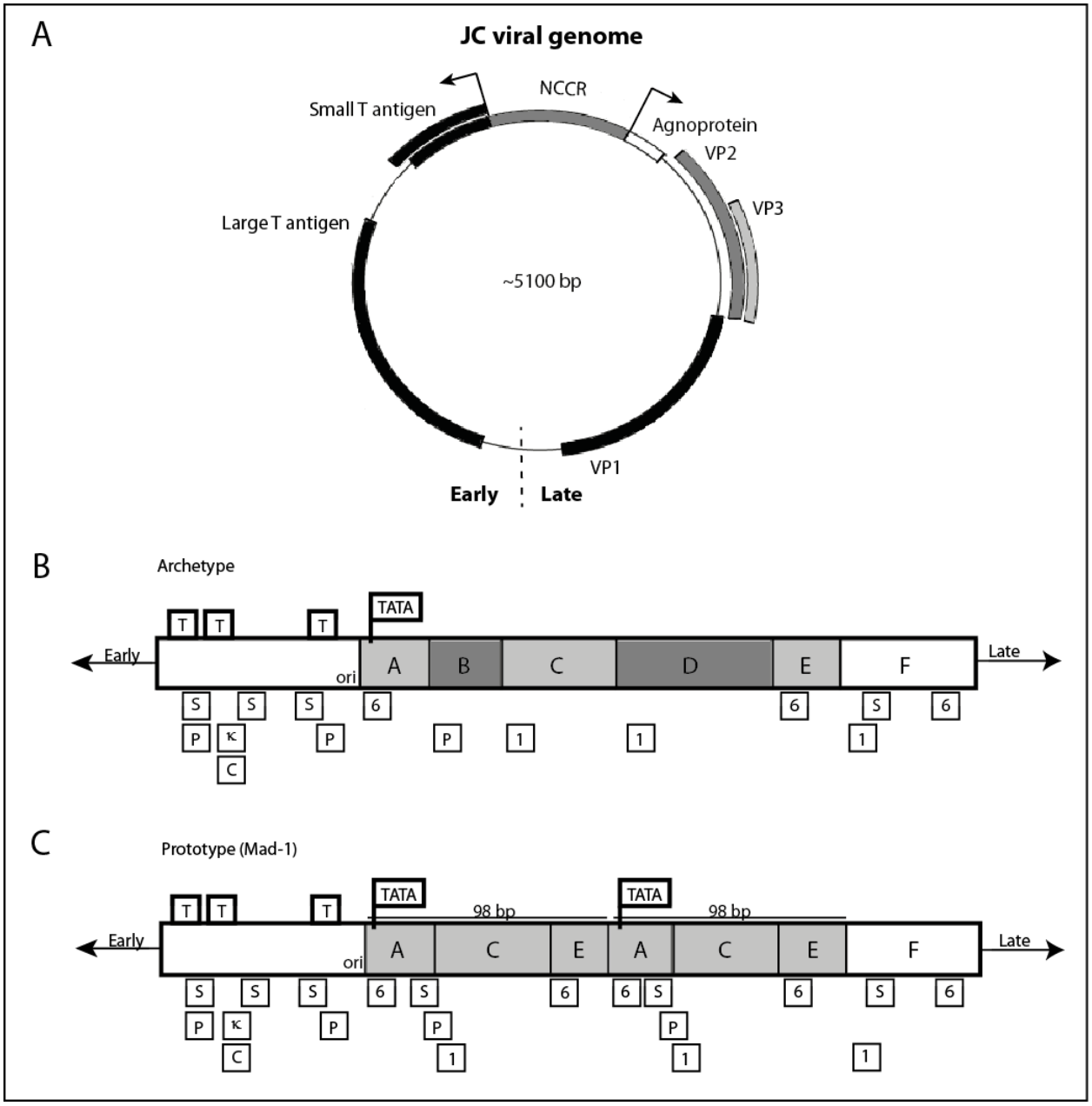

Figure 1. The JC virus genome.

A. Schematic organization of the JC virus genome. Showing the viral early genes, including large T-antigen and small T-antigen, and the viral late genes, as indicated by the VP1, VP2, VP3 and agnoprotein. The early and late gene segments are separated by the NCCR-control region.

Archetype (**B**) and prototype (**C**) regulatory region sequences, representing DNA sequence block arrangements. The origin of replication (ori) contains three binding sites for large T-antigen, as illustrated by the boxes containing the letter 'T'. Transcription factor bindings sites are represented by boxes under the regulatory control region and letters indicate abbreviations for their corresponding transcription factors: 1, NF1; 6, Oct-6; κ, NFκB; C, C/EBPβ; P, YB-1/purα; S, Spi-B.

B. Archetype NCCR-sequences consists of six sequence blocks, denoted A,B,D,C,D,E and F.

C. The mad-1 prototype NCCR sequence, the original isolate of JCPyV from a PML-patient, is typically recognized by a 98-bp tandem repeat, containing sequence blocks A,C and E. Rearrangement of the NCCR results in duplication of the TATA-box and a significant increase in transcription factor binding sites.

Epidemiology of JC virus infection and PML

Soon after the discovery of the JC polyomavirus in 1971, it was shown that JCPyV could induce hemagglutination of human type O erythrocytes [24]. By taking advantage of this property, antibodies against JCPyV could be measured by hemagglutination inhibition assays. Early seroepidemiological studies indicated that prevalence of antibodies against JCPyV can be detected in 50% to 92% of healthy individual [5, 6]. Though, these rates are divergent among various studies, owing to different techniques that are employed for the detection of JCPyV-specific antibodies [5, 25, 26]. As there is a tolerably high concordance between seroprevalence rates within these studies, it is believed that seroconversion is acquired slowly during childhood and continues into early adolescence [25, 26]. By adulthood, a large percentage of the population shows positive for antibodies against JCPyV [25-27]. These data indicate that exposure to the virus in a population occurs in two phases, with the highest rates of initial infection occurring prior to adolescence, and naïve individuals are subsequently exposed to the virus in a slow, but persistent manner [28]. Remarkably, despite an increase in seroprevalence with age, there appears no association between acquisition of the virus and development of PML [27]. Moreover, primary infection induces no recognizable clinical disease, and once an individual becomes infected either a persistent or latent infection is established, as indicated by the lifelong presence of JC virus in the kidney [29].

It has been estimated that the incidence of PML in the general population is approximately 0.3 per 100.000 person-years [17]. This rate is significantly higher in immunocompromised patients with HIV-AIDS, allogeneic hematopoietic stem cell transplantations (HSCT), hematological malignancies or solid organ transplantations [17, 30]. Interestingly, rare but occasional emergence of PML has been described in individuals with minimal or occult immunosuppression [31].

The highest rates of PML incidence has been found in patients suffering from HIV-AIDS, with respectively 2.4-3.3 cases per 1000 person-years in AIDS patients before the use of cART [8, 17, 32]. With the introduction of cART, this incidence decreased to 1.3 cases per 1000 person-years [8]. The median age of HIV-patients diagnosed with PML ranged between 32-33 years old [8, 32]. Moreover, at PML diagnosis median $CD4^+$ T-cell counts were lower than 200 cells/µl [32], reaching numbers of 60 cells/µl in a Swiss cohort study of HIV-infected patients [8], clearly indicating a state of immunodeficiency. Of note, JCPyV-infected HIV-positive patients with low $CD4^+$ T-cell numbers do not automatically develop PML, as the decisive parameter is believed to be the JCPyV-specific $CD4^+$ T-cell count, rather than the overall $CD4^+$ T-cell counts [17].

A high incidence of PML has been observed in patients treated with immunomodulatory therapies [7]. The estimated incidence of Multiple Sclerosis (MS) patients treated with natalizumab for minimally 1 month is 2.13 cases per 1000 patients [33]. However, incidence is increased to 11.1 cases per 1000 patients [17, 33] when patients are treated with natalizumab >24 months, received immunosuppressive drugs as prior therapy for MS and showed positive for anti-JCPyV antibodies [33].

Finally, emergence of PML has been observed in patients receiving a transplantation, presumably due to the administration of immunosuppressive treatment. Although data collection was limited, incidence has been estimated by 1.24 cases per 1000 post-transplant patient years [34] and lesions occur approximately within 17 months of transplantation [34, 35]. Renal transplant recipients, however, tend to have a later onset of plaque lesions, though merely nine cases have been reviewed [35].

Pathogenesis

It has been hypothesized that development of PML only occurs after a number of barriers have been breached [28] (**Fig. 2**). However, the exact pathogenesis of JCPyV remains challenging, as *in vitro* studies have been poorly available to represent JCPyV-routes of infection or transmission, and to date there have been no reports of an animal model with PML [17]. As a result, controversy regarding the mechanism of pathogenesis remains. Available evidence suggests that once primary infection with JCPyV has occurred, the virus disseminates through the body and establishes persistent or latent infection with episodic emergence of JCPyV reactivation [17]. Importantly, reactivation might induce

rearrangements of the JC virus NCCRs, potentially resulting in the formation of a neurotropic strain. Upon immune suppression, the rearranged virus might establish productive infection in oligodendrocytes and subsequently, virions will spread to adjacent cells, leading to progressively enlarged lesions and severe neurologic deficits.

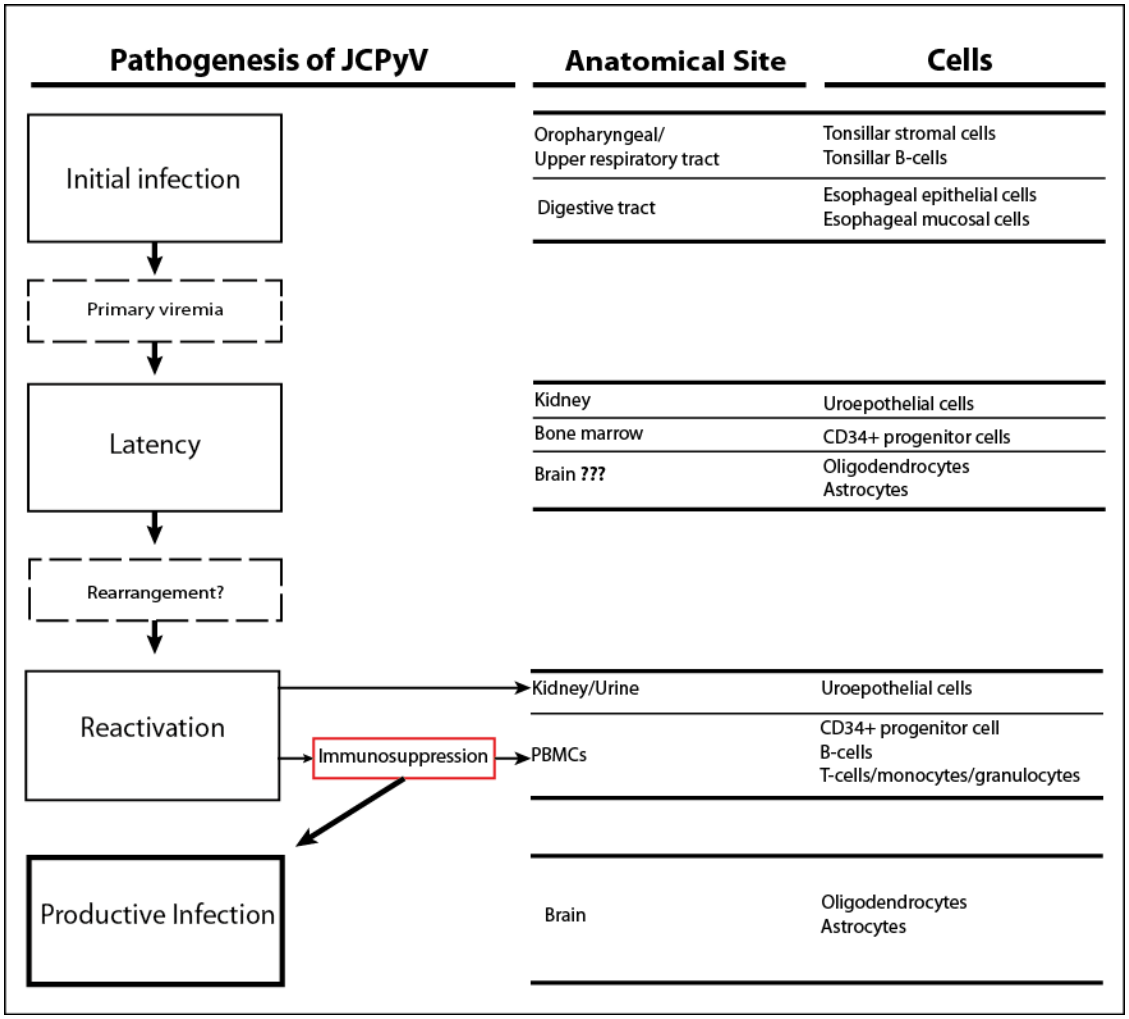

Figure 2. Schematic overview of JCPyV pathogenesis.
The proposed pathway of JC virus-induced disease from initial infection to productive replication in the brain, leading to severe demyelination. Anatomical sites in the body, and the cells associated, are represented. Dashed boxes indicate presumed occurrences that have not yet been demonstrated *in vivo*.

JCPyV transmission

Even though it has been over 40 years since JCPyV was isolated, the mode of viral transmission is not yet well defined. Consensus regarding transmission has been complicated, as it is difficult to distinguish the initial site of infection, since the virus has shown to maintain a lifelong persistent infection in a variety of tissues [18]. However, presence of JCPyV DNA has been detected in stromal cells and B-lymphocytes of the tonsils in children and adults [36, 37]. Furthermore, JCPyV is able to productively infect stromal cells isolated from tonsillar tissue in culture [38]. It was therefore proposed that tonsils may serve as an initial site for viral infection, and the virus might acquire entrance to the upper respiratory tract by close interpersonal contact or through fomites. However, viral DNA could not be detected in the saliva of HIV-

positive or immunocompetent individuals [39]. Despite this observation, evidence for an oropharyngeal or respiratory route was subsequently found in the detection of archetype JCPyV in both human tonsils [37] as in 98% of the sewage samples collected from diverse geographic regions [40]. Therefore, individuals might be infected through ingestion of virus-contaminated materials. This hypothesis shed light on the possibility of infection through the digestive tract, and was evidenced by the presence of JCPyV DNA in epithelial cells of the esophagus [41] and in mucosal cells of the upper- and lower gastrointestinal tract [42]. In addition, it has been observed that JCPyV particles remain intact after treatment at pH 5 for 90 minutes, or pH 3 for 30 minutes [43], and thus the virus is feasible to survive in an acidic environment, such as the stomach and duodenum.

Although the routes of JCPyV dissemination in an individual remain unclear, intact JC virus has been detected in urine of 20-40% of healthy individuals [29, 44], and can even reach 70% within select populations [39], indicating that the kidneys are a preferred site for viral latency. Moreover, the JCPyV found in urine is almost exclusively of the archetype strain [27, 45], and therefore might illustrate the comprehensive distribution of archetype JC virus found in sewage. However, next to urinary excretion, shedding of JC virus has also been detected in stool specimens of healthy adults [46], providing evidence that JCPyV could persist in the gastrointestinal tract. As a consequence, fecal-oral transmission could be an important route of JCPyV dissemination in the human population.

Importantly, as the archetype JCPyV strain has been found globally in urine and sewage, it is believed that this form of JC virus is the transmittable form [6].

JCPyV cellular tropism

In order to induce primary or lytic infection, the JCPyV has to acquire entrance to cells. Polyomaviruses are known to display restriction towards specific species and establish infection in confined cellular specificities [47]. Hence, JC viral infection has only be detected in humans, and presence of JCPyV DNA has been restricted to kidney epithelial cells, tonsillar stromal cells, $CD20^+$ B-cells ,$CD34^+$ hematopoietic progenitor cells, oligodendrocytes and astrocytes [4, 38, 48]. Of these, glial cells are known as the preferred site for viral replication and lytic infection. JCPyV has not been detected in cerebral neurons or in human neuronal cultures, although there have been reports of JCPyV infection in cerebellar granule cell neurons [49].

It is not completely understood why JCPyV has such a major tropism for human glial cells, however multiple factors are likely responsible to determine susceptibility to JC viral infection. To acquire entry to a host cell, it is established that JCPyV should initially interact with negatively charged α2,6-linked sialic acid-bearing structures [50]. This receptor type is abundantly expressed on oligodendrocytes, astrocytes, human B-cells in tonsils and spleen, and on kidney and lung tissue, but to a lesser extent on human T-lymphocytes and not on cortical neurons [51]. More recently, the proteinaceous moiety has been identified, known as Lactoseries tetrasaccharide C (LSTc), and is a pentasaccharide with a terminal sialic acid linked by a α2,6-bond [52]. However, JCPyV has also been shown to require the serotonin receptor $5HT_{2a}R$ to infect cells [53], as was evidenced by the uptake of JC virus in cells expressing $5HT_{2a}R$, that are usually non-infectable [53]. Because JCPyV uptake has also been demonstrated in cells lacking $5HT_{2a}R$ expression, this receptor is considered as an additional or alternative route for JC viral entrance. More importantly, cell-type specific factors, such as transcription factors, are highly critical in defining cellular susceptibility to JCPyV infection, as they regulate viral DNA replication and transcriptional activity [11] (**Fig. 1***B*/*C*). Specifically, these transcription factors have shown to increase viral activity by binding to their specific binding sites on the viral NCCR [14]. In particular, the transcription factors Spi-B and NF-1X showed increased levels in cells susceptible to JCPyV infection [14, 54]. Cells non-permissive to JCPyV became susceptible after induced expression of NF-1X [55], whereas induced downregulation of this protein blocked cellular permissibility [56]. Therefore, cells non-permissive to JCPyV infection might not contain the appropriate protein factors to promote viral transcription and, consequently, could even restrict viral replication.

Latency and viral reactivation

Polyomaviruses have the biological characteristic to maintain a lifelong viral infection in their host, once initial infection has been eliminated by the immune system. However, the latent state of JCPyV is not yet well defined, and is considered as an asymptomatic, chronic and persistent infection, wherein JCPyV DNA can be detected by highly sensitive PCR techniques, in contrast to JCPyV protein expression [6]. It is currently indistinct if JCPyV achieves a truly latent state or whether JCPyV remains as a persistent infection. Nevertheless, latency, as indicated by the presence of JCPyV DNA but without the appearance of viral proteins, has been detected in the kidney/urinary system [45], bone marrow/lymphoid tissues [57] and the brain of immunocompetent non-PML individuals [58-60]. Importantly, detection of JCPyV DNA in the brain [58, 60] illustrates the possibility that JC virus might enter the brain during primary viremia, and subsequently retains as a latent infection [6].

It is thought that reactivation of the JC virus occurs episodically, as indicated by the frequent shedding of JCPyV in the urine of healthy individuals [17]. In a comprehensive study of 400 immunocompetent individuals, JC virus was detected in 33% of the urine from JCPyV-infected healthy individuals [27]. Moreover, the frequency of JCPyV shedding in urine was shown to increase with age [27, 61], though no increase in shedding has been observed in immunodeficient HIV-infected patients [62]. In addition, urinal shedding of JCPyV has been observed in MS patients, but showed no correlation with duration or number of natalizumab administrations [63]. These studies indicate that a reduced immune control does not show to increase replication of JCPyV. Taken together, detection of JCPyV in the urine represents ongoing replication in immunocompetent individuals, and is not readily affected by an altered state of immune control. Interestingly, JCPyV replication and urinal shedding show no pathologic significance, and there is no data revealing what might trigger viral persistence, and sudden reactivation [47].

Regarding PML, it has been hypothesized that emergence of disease is more likely the consequence of reactivation of a latent infection, rather than the result of a primary infection. This concept is evidenced by the observation that PML-patients are almost invariably positive for JCPyV-specific IgG antibodies [28]. Additionally, natalizumab treated PML-patients did all show antibodies directed against JCPyV 16 to 187 months prior to disease [17, 28]. Moreover, JCPyV obtained 0.5-4.1 years before diagnosis of PML from spleen, bone marrow tissue and lymphoid cells showed the same genetic rearrangement as the one isolated from the brain [28]. Finally, although PML has been reported in HIV-infected children [64], this observation is extremely rare in the pediatric population [28].

These data indicate that reactivation of latent infection in JCPyV-infected individuals is independent of the variant of JC virus present, as reactivation occurs with both archetype as prototype strains. However, reactivation of the prototype strain is always associated with development of PML, and typically arises in immunocompromised patients [4].

Immune response

The healthy immune system conducts a tight surveillance on JC viral infection, as has been illustrated by the presence of JCPyV-specific $CD8^+$ T-cells in healthy individuals that are thought to prevent emergence of PML [65]. In addition, various studies have indicated that presence of viral-specific $CD8^+$ T-cells in immunocompromised JCPyV-infected patients correlated with prevention of PML onset, and even improved diagnosis of PML [66, 67].

Progression of disease will become inactive in 85% of PML-patients when JCPyV-specific $CD8^+$ T-cells are present [67]. In contrast, in the absence of viral- specific $CD8^+$ T-cells, 82% of the PML-diagnosed patients will have an active progress of disease [67]. The incidence of PML rose sharply during the HIV-AIDS pandemic [8], indicating that a HIV-induced reduction in $CD4^+$ T-cells might alter JCPyV control. In addition, onset of PML was observed in non HIV-infected patients with idiopathic $CD4^+$ T-cell lymphocytopenia, resulting in decreased $CD4^+$ T-cell counts [68]. However, in a study comparing HIV-infected PML non-survivors with HIV-infected patients, both groups having equal numbers of $CD4^+$ T-cells, no JCPyV-specific $CD4^+$ T-cells were observed [69]. This observation might imply that $CD4^+$ T-cells

do not exercise direct control on JCPyV infection.
In a similar cohort study, JCPyV-specific $CD8^+$ T-cells were detected with a common specificity for the $VP1_{p100}$ and the $VP1_{P36}$-epitope expressed by JCPyV, and these cytotoxic lymphocytes were present in 71% of the PML-survivors, but not in the PML-non survivors [70]. Considering that $CD4^+$ T-cells are a critical component to sustain a $CD8^+$ T-cell response in chronic infections [18], these results illustrate a narrow interrelationship between $CD8^+$ T-cells and $CD4^+$ T-cells in controlling JCPyV infection. With a dominant role for JCPyV-specific $CD8^+$ T-cells to control JC virus infection, supported by $CD4^+$ T-cells to sustain a proper function of the cytotoxic lymphocytes. Indeed, PML-progressors showed a remarkably lower amount of $CD8^+$ T-cells in comparison to PML-survivors, and detectable $CD4^+$ T-cells did not prevent the progression of disease [66]. However, when increased numbers of $CD4^+$ T-cells were detected, there was an improved response in controlling disease [66].
Remarkably, despite the comprehensive presence of JC virus-specific antibodies, the humoral immune response shows no effect on the surveillance of JCPyV infection [70]. However, this might not seem surprising, as it has been suggested that the virus escapes immune recognition by assembling in the nucleus [4].

Histopathological features of JCPyV infection in brain tissue
Upon immune suppression, emergence of clinical disease may occur. PML is exclusively found in the brain tissue, and does not infect the spinal cord or optic nerves [15, 71]. In general, lytic infection is predominantly established in oligodendrocytes, and to a lesser extent in astrocytes, allowing spreading of the virus from the lytic cell to neighboring cells. Consequently, multifocal lesions with irregular borders arise [71]. As disease progresses, these necrotic plaques can enlarge into asymmetrical macroscopic plaque lesions that may become confluent [15]. These asymmetrical lesions might potentially disregard the gray/white-matter border, and affect the deep gray matter structures [71]. Of note, even though JCPyV mainly infects the white matter, initial infection of the gray matter has been reported in a HIV-negative woman [72].
On a microscopic level, PML can be characterized by hyperchromatic and substantially enlarged nuclei in infected oligodendrocytes, owing to viral inclusion bodies [7, 11, 71]. In addition, bizarre enlarged astrocytes can be detected, featured by irregular and lobulated nuclei [11, 71].
Plaque lesions are commonly invaded by macrophages, but not lymphocytes, and serve as scavengers to eliminate debris from damaged myelin or oligodendrocytes [4, 11].

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JC virus trafficking routes to the brain
PML manifests as multiple foci heterogeneously spread throughout the white matter of the brain. However, these lesions are not affiliated to specific vascular areas [15], nor do these lesions correlate to neuronal spread [73]. As a consequence, the random distribution of these foci indicate that JCPyV might enter the brain via the hematogenous route either as a cell-free virus, or by virus-infected lymphocytes [17, 74].
Trafficking of JCPyV towards the brain via JC virus-infected cells has been evidenced by the presence of JCPyV in mononuclear cells of bone marrow and spleen [75], and in circulating lymphocytes [57]. Consistent with these observations was the detection of JCPyV in $CD34^+$ hematopoietic progenitor cells and primary B-lymphocytes [38]. In addition, *in vitro* studies using the $CD34^+$ hematopoietic progenitor cell lines KG-1 and KG-1a, and primary B-cells showed low, though persistent levels of JCPyV infection [38]. Subsequent experiments further differentiated the KG-1 cell line into either a macrophage lineage or a lymphocytic lineage and these cells were thereafter exposed to JCPyV. Susceptibility to JCPyV infection was only maintained in the lymphocytic cell lineage, but was blocked in the macrophage-like lineage [38]. Based on these observations, many researchers have focused on lymphocytes as primary site of JCPyV dissemination, with particular interest in B-cells [4, 77-79]. This hypothesis is bolstered by the observation that both archetype as prototype NCCRs have been detected in circulating lymphocytes [80] and $CD138^+$

plasma cells [81]. Since B-cells maintain the enzymes critical for immunoglobulin gene rearrangements, it has been put forward that the prototype arrangement arises from archetype JCPyV as B-cells mature [18]. This concept is supported by the fact that infection of JCPyV has shown to induce chromosomal damage in culture cells, including lymphocytes [82]. Therefore, it has been suggested that upon JCPyV infection, the infected cells will activate their DNA repair response and consequently, these mechanisms might contribute to viral recombination, resulting in the acquisition of multiple binding sites for host transcription factors important in pathogenesis [18]. This theory is in accordance with the assumption that cellular susceptibility to JCPyV is regulated on a molecular level, in particular by the presence of specific host transcription factors. As B-cells develop and mature, inducible transcription factors guide the cell how to differentiate [83], resulting in upregulation of specific transcription factors during different phases of B-cell development. Accordingly, Spi-B and members of the NF-1 family have shown to increase viral replication in JCPyV susceptible cells [11, 54] and are present in both glial cells as B-cells [54, 84]. Taken together, these results indicate that B-cells provide attractive mechanisms to regulate JCPyV replication and rearrangement, and could play an important role in JCPyV trafficking towards the brain.

However, *in vitro* only low numbers of JCPyV-infected cells have been detected in the total population of B-cells studied [38][78, 85]. Moreover, EBV-transformed B-cells showed no replication of JCPyV after incubation with the virus for 2 hours [78], despite the detection of low numbers of replication in the BJAB and Namalwa B-cell lines [86]. If JCPyV is not able to productively replicate within their host, the virus needs to survive within the B-cell until it has crossed the blood brain barrier (BBB). Copy numbers of JCPyV Tag- and VP1- DNA in infected B-cells reduces severely two weeks after infection [78, 85], indicating that JCPyV can only stay intact in B-cells for two weeks. Likewise, even though JCPyV DNA copy numbers can be detected in PBMCs obtained from healthy volunteers, natalizumab-treated patients [87, 88] or HIV-infected patients with and without PML [89, 90], no or only a small population of B-cells is infected with the virus in these individuals, and viral copy numbers in these cells are low [38][81, 91]. These data indicate that B-cells are not necessarily the preferred site for JCPyV infection and latency.

The emergence of PML in patients treated with the immunomodulatory therapy rituximab [92] further questioned the role of B-lymphocytes as primary site of JCPyV trafficking. The monoclonal antibody rituximab binds to the CD20 complement expressed on B-cells, inducing downregulation of the receptor and subsequent apoptosis of $CD20^+$ B-lymphocytes [18]. As a consequence, circulating B-cells are depleted from the population. Treatment with rituximab has shown to deplete B-cells in the CSF of MS patients, and is subsequently associated with the removal of T-cells [93]. The resulting abrogated immune surveillance might therefore potentially allow active replication of JCPyV present in the brain. However, as B-cells are depleted from the population, these observations imply that JCPyV is either latently present in the brain or that transport of JCPyV is not necessarily dependent on B-lymphocytes, but might be regulated by other hematopoietic subpopulations.

Indeed, analysis of peripheral blood mononuclear cells (PBMCs) obtained from HIV-1 infected PML patients, HIV-1 infected non-PML patients and healthy individuals indicated that the association of JCPyV DNA in hematopoietic subpopulations is highly heterogeneous [90, 94].

Depending on the study, presence of JCPyV DNA has been detected in $CD34^+$ hematopoietic progenitor cells [38][87], T-cells, monocytes and granulocytes [90, 91, 94] (**Table 1**). Importantly, the infected hematopoietic compartment differs considerably between individuals and JCPyV DNA is not always detectable in hematopoietic cells of PML patients with underlying HIV-infection [90, 94].

In addition, trafficking of JCPyV via a cell-bound route is significantly hampered by the BBB, which is known as an almost impermeable barrier that separates blood from brain parenchyma [95]. Entry of leukocytes is only possible via three distinctive routes [95] and after migration through the endothelial cells and glial limitans [18]. It has been estimated that approximately 80% of the leukocytes present in the brain consists of T-cells, and only a small percentage of B-cells [96]. Granulocytes infiltration is limited and only detectable in sites of BBB inflammation [97]. Thus, the BBB considerably impedes influx of many of the cells susceptible for JCPyV infection.

Study	Population	# Participants	Cell type					Technique
			CD34$^+$	T	B	Mono	Granulo	
Koralnik, '99[90]	Healthy	3	ND	-	33%	-	-	PCR
	HIV$^+$	6	ND	17%	-	17%	17%	
	HIV$^+$/PML$^+$	4	ND	25%	25%	25%	-	
Frohman '14[87]	Healthy	18	6%	ND	11%	ND	ND	qPCR
	MS	49	22%	ND	16%	ND	ND	
Chalkias '14[94]	MS	43	19%	14%	16%	16%	14%	qPCR

Abbreviations: ND, Not Determined; minus sign, no detection of JCPyV DNA; PML, Progressive Multifocal Leukoencephalopathy; MS, Multiple Sclerosis; PCR, polymerase chain reaction.
Cell types: CD34$^+$ progenitor cells, CD3$^+$ T-lymphocytes, CD19$^+$CD20$^+$ B-lymphocytes, CD14$^+$ monocytes, CD66b$^+$ Granulocytes

Table 1. Summary of reported JCPyV DNA detected in hematopoietic subpopulations.
Studies that have focused on the presence of JCPyV DNA in the subpopulations of hematopoietic cells have been summarized. Percentages indicate number of individuals that show positive for JCPyV in a particular cellular compartment. Variety in percentages between studies might be assigned to differences in techniques used, and the variable numbers of participants available in a population. Since focus mainly has been on B-cells and CD34$^+$ progenitor cells as primary site of JC viral trafficking route, Frohman, et al.[87] did not take other hematopoietic subpopulations into consideration.
The MS patients studied by Frohman, et al.[87] and Chalkias, et al.[94] were all treated with natalizumab, with the exception of five patients that were on treatment with IFN-β1a[94].

Furthermore, emergence of PML is associated with treatment of the monoclonal antibody natalizumab, which exerts an antagonistic effect by binding to the α4-chain of α4β1- and α4β7-integrins expressed on hematopoietic cells [18]. As a result, adhesion of integrins to the vascular cell adhesion molecule 1 (VCAM-1) is blocked, preventing leukocytes from passing through the BBB.
Taking into account the relatively low infection efficiency of JCPyV in hematopoietic subpopulations present in immunocompetent and immunocompromised individuals, together with the impermeable barrier for leukocytes to enter the brain, it might be expected that transport of JCPyV to the brain occurs via a viremic route. Accordingly, although this has not yet been demonstrated *in vivo*, it has been observed *in vitro* that JCPyV productively infects and replicates in HBMV cells, the main component of the BBB, and these produced viruses could induce efficient replication in PHFG cells [74]. In addition, JCPyV DNA, but not protein, has been observed in the brain of healthy immunocompetent individuals [58-60], indicating that the virus is transported to the brain in an early stage of infection, presumably during primary viremia. Assuming that initial infection occurs via an oropharyngeal or respiratory route, primary viremia must be pursued in order to establish infection of the reno-urinary tract. Concomitantly, while viremia is present, the virus might infect JCPyV-susceptible cells other than renal cells, including hematopoietic cells, stromal cells of the tonsils, lung and spleen and eventually the brain [17], where it can persist as latent infection. However, various studies focusing on viremia as a predictive marker for viral spread in non-PML patients treated with natalizumab [87, 88, 91] concluded that presence of viremia is extremely rare in these patients. In addition, viremia was not detected in a cohort study including 400 adult healthy adults [29]. This observation might not seem surprising as it has been estimated that exposure to JCPyV occurs early in life [47], suggesting that latency has already been established once the patient is treated with natalizumab. However, the lack of detectable viremia implies that treatment with natalizumab is not associated with an increased JC viral load in plasma, and therefore it seems unlikely that the virus establishes a second viremia in order to infect the brain.
Of note, recent advances in natalizumab-treated patients significantly challenge the current perspectives in JCPyV dissemination and subsequent transport to the brain. Emergence of PML has solely been detected in MS patients that were treated with immunosuppressants prior to the use of natalizumab, though onset of PML only occurs after the use of natalizumab for more than 24 months [33]. Therefore, if the brain would surely be a site of latency, it should be expected that PML develops directly after the start of natalizumab treatment. Moreover, even though JCPyV could not be detected in plasma of natalizumab-treated patients, two recent studies observed JCPyV in a variety of hematopoietic subpopulations,

including $CD34^+$ progenitor cells, lymphocytes and monocytes [87, 91] (**Table 1**). As an additional effect, by preventing homing to the bone marrow, natalizumab has shown to increase the circulation of $CD34^+$ progenitor cells [98], $CD19^+$ mature B-cells and $CD19^+CD10^+$ pre-B cells [99]. These results imply that trafficking of the virus is cell-associated rather than cell-free. However, by blocking binding of the α4β1-integrin, highly expressed on lymphocytes, monocytes [100] and $CD34^+$ progenitor cells [98], natalizumab hampers transport of infected cells through the BBB. These observations are puzzling and counter the current understanding in JCPyV pathogenesis. As an alternative route, JCPyV might remain associated to the cell surface, rather than entering the cell [78]. As these cells migrate through the body, the cells might reach the CNS and the virus could detach from the surface and enter the brain. However, in order to establish effective reactivation, it is critical that the virus enters the cell to take advantage of the host replication mechanisms. *In vitro* studies indicate that JCPyV infects EBV-transformed B-cells, rather than attaching to the cell membrane [78] and therefore, this route of transmission remains debatable. Regarding to PML in HIV-infected patients, it has been observed that HIV upregulates expression of the macrophage chemoattractant protein-1 (MCP-1) in the CSF and this is associated with increased incidence of PML [101]. Furthermore, it has been put forward that HIV-associated Tat-protein increases the BBB permeability by upregulating CCR5-expression on monocytes, and thus facilitating migration of these cells to the CNS [102]. The increased permeability might contribute to the influx of hematopoietic cells, other than monocytes, potentially infected with JCPyV. As the highest rates of PML incidence are associated with HIV-infection, these results indicate a synergistic role of both HIV and JCPyV. Alternatively, HIV-infection of the brain induces increased levels of pro-inflammatory cytokines [103], including TNFα [104]. TNFα induces phosphorylation of the inhibitor protein IκB, allowing the transcription factor NFκB, normally present in the cytoplasm, to enter the nucleus and initiate transcription of both cellular and viral proteins [105]. The HIV-induced upregulation of TNFα also increases expression of the transcription factor C/EBPβ [106], and both C/EBPβ as NFκB have shown to regulate JCPyV transcription [107]. Therefore, it was postulated that the TNFα-induced upregulation of NFκB and C/EBPβ control reactivation of latent JCPyV present in astrocytes and oligodendrocytes [107]. Upon immune suppression, the reactivated virus might spread and infect neighboring cells, resulting in development of progressive disease.

Taking into account the different data found regarding emergence of PML between HIV-infected patients and patients treated with immunomodulatory therapies, it appears that trafficking of JCPyV differs depending on the clinical setting (**Table 2**). However, considering the seemingly random distribution of foci within the brain, and the fact that JCPyV is not able to productively infect neurons [51], it is feasible to conclude that transport of JCPyV occurs via a hematogenous route. Nevertheless, future studies are of high importance to determine the preferential sites of JC viral latency, rearrangement and reactivation.

Discussion

Development of PML is a rare, though severe disease caused by lytic infection of the JC virus in oligodendrocytes, and is only present in patients suffering from an underlying immunodeficiency. Regarding JCPyV pathogenesis, many gray areas remain present. For instance, viral latency and persistence are thought to serve as a source for JC viral infection, though their corresponding sites within the body remains unclear. In addition, there is no accordance on when and how the neurotropic NCCR rearrangement or capsid VP1-capsid proteins are required, and neither is the moment of when JCPyV enters the brain. Most importantly, even though multiple routes have been proposed, consensus on the pathway of JC virus to the brain is lacking. Available evidence suggests that the virus might transport to the brain via various routes, depending on the underlying clinical disease. Future studies should therefore focus on factors that distinguish shared mechanisms in pathogenesis from unique mechanisms. However, it appears that dissemination generally occurs via a hematogenous route, either as a cell-bound or a cell-

HIV-infected patients		
	Pro	**Con**
B-cell hypothesis	Detection of JCPyV in hematopoietic cells, including B-cells[38,75,77]	Only very low JCPyV DNA copy numbers detectable in infected cells[38,78,85]
	HIV increases BBB permeability[102]	Low percentage of cells is infected compared to total population of B-cells[87-90]
		JCPyV DNA not always present in hematopoietic cells of PML-patients[90,93]
		High heterogeneity between patients in cellular compartment infected[87-90]
Hematopoietic cell transport	Detection of JCPyV in hematopoietic subpopulations, including $CD34^+$ progenitor cells, T-cells, monocytes and granulocytes[87-90]	Only very low JCPyV DNA copy numbers detectable in infected cells[38,78,85]
	HIV increases BBB permeability[102]	JCPyV DNA not always present in hematopoietic cells of PML-patients[90,93]
Reactivation latent virus in brain	HIV-induced upregulation of TNFα in brain increases expression of NFκB and C/EBPβ, regulating transcription of JCPyV = reactivation of JCPyV[103-107]	
Cell-free	HIV increases BBB permeability[103]	
	Detection of JCPyV in plasma of HIV-infected patients[90,93]	

Patients on Immunomodulatory therapies*		
	Pro	**Con**
B-cell hypothesis	Detection of JCPyV in hematopoietic cells, including B-cells[38,75,77]	Natalizumab prevents entry of hematopoietic cells into brain[18]
	Natalizumab increases circulating levels of pre-B and B-cells[99]	Rituximab depletes circulating B-cells from the population[92]
Hematopoietic cell transport	Detection of JCPyV in hematopoietic subpopulations, including $CD34^+$ progenitor cells, T-cells, NK cells and PMN[87-90]	Natalizumab prevents entry of hematopoietic cells into brain[18]
	Natalizumab increases circulating levels of CD34 progenitor cells[98]	
Reactivation latent virus in brain		No development of PML during prior treatment with immunosuppressants[33]
		Development of PML occurs after >24 months of natalizumab treatment[33]
Cell-free		No presence of viremia in plasma = no cell-free transport[86,93]

* Immunomodulatory therapies include natalizumab (Tysabri®) and rituximab (Rituxan®)

Table 2. Overview of proposed JC viral trafficking routes compared to the underlying clinical disease, including HIV-infected patients and patients treated with immunomodulatory therapies.
Likelihood of the occurrence of proposed viral trafficking routes is displayed in "Pro's" and "Con's", as is shown for HIV-infected patients and patients treated with immunomodulatory therapies. Clearly, the JC virus transport route to the brain differs between clinical settings and the lack of convincing data prevents the ascertainment of a primary trafficking route for the JC virus.

free virus. In particular, the brain as a site of viral latency remains debatable. If JCPyV arrives in the brain early after initial infection, rearrangement into the neurotropic strain has to occur directly after entry into the body. Therefore, both neurotropic as archetype strains should be present in tonsillar tissues of healthy individuals, as this is the initial site of infection. Quantitative PCR has shown highly sensitive in detecting JCPyV DNA in CSF [108], and might therefore be a good technique to detect viral DNA present in these

tissues. In addition, as the T-antigen is highly conserved within JCPyV [30], this region should be targeted in PCR analysis, rather than the variable VP1 sequence.

Occurrence of reactivation in specific areas of the brain would result in JCPyV strains with highly similar NCCR rearrangements. Contrary, if rearrangement and reactivation arises from anatomic compartments outside the brain, it is expected that a high variety in NCCR genome sequences can be observed, as variants are obtained from different replication sites. As a consequence, distinct JCPyV strains must be found in different sites of a PML-affected brain. Whole genome sequencing of JCPyV strains isolated from various compartments of healthy- and PML-brain biopsies should therefore be conducted in future studies.

In natalizumab treated patients, JCPyV has solely been detected in circulating hematopoietic cells, but not in plasma [87]. This remarkable observation might indicate that natalizumab induces a favorable environment for JCPyV to enter the cell. Hence, future studies must determine if binding of natalizumab induces molecular changes on the surface of hematopoietic cells, perhaps increasing the JCPyV-susceptibility of these cells. In addition, despite blocking the entry of hematopoietic cells to the brain, leakage of potentially infected cells through the BBB might occur. Therefore, studies should focus on the exact mechanisms of natalizumab in preventing leukocyte entry to the brain.

Taken together, there is a clear need to increase our current understanding in JCPyV pathogenesis. Elucidating the specific routes of JC virus dissemination could shed light on potential therapeutic targets that might block JCPyV from inducing lytic infection, and consequently, might decrease the high morbidity and mortality risk in patients with PML.

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