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# Fate of Microglia during HIV-1 Infection: From Activation to Senescence?

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## Abstract

Microglia support productive human immunodeficiency virus type 1 (HIV-1) infection and disturbed microglial function could contribute to the development of HIV-associated neurocognitive disorders (HAND). Better understanding of how HIV-1 infection and viral protein exposure modulate microglial function during the course of infection could lead to the identification of novel therapeutic targets for both the eradication of HIV-1 reservoir and treatment of neurocognitive deficits. This review first describes microglial origins and function in the normal central nervous system (CNS), and the changes that occur during aging. We then critically discuss how HIV-1 infection and exposure to viral proteins such as Tat and gp120 affect various aspects of microglial homeostasis including activation, cellular metabolism and cell cycle regulation, through pathways implicated in cellular stress responses including p38 mitogen-activated protein kinase (MAPK) and nuclear factor κB (NF-κB). We thus propose that the functions of human microglia evolve during both healthy and pathological aging. Aging-associated dysfunction of microglia comprises phenotypes resembling cellular senescence, which could contribute to cognitive impairments observed in various neurodegenerative diseases. In addition, microglia seem to develop characteristics that could be related to cellular senescence post-HIV-1 infection and after exposure to HIV-1 viral proteins. However, despite its potential role as a component of HAND and

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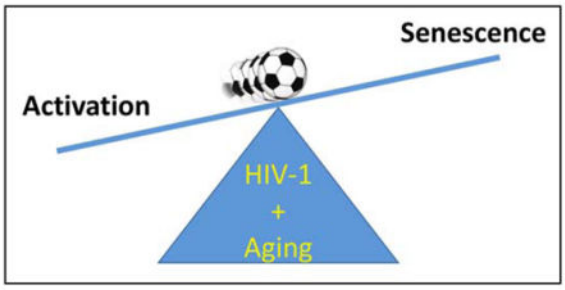
likely other neurocognitive disorders, microglia senescence has not been well characterized and should be the focus of future studies, which could have high translational relevance.

## Graphical Abstract




### Keywords

Chronic infection; Microglia dysfunction; Cellular senescence; HAND

## Introduction

With the advent of combination anti-retroviral therapy (cART), which for most treated patients effectively controls HIV-1 viral replication, the proportion of patients developing HIV-1-associated dementia (HAD) has declined dramatically. However, recent population-based studies suggest that around 50% of all infected patients continue to develop HIV-1-associated neurocognitive disorders (HAND) to varying degrees (Chan and Brew 2014; Heaton et al. 2011; Nightingale et al. 2014; Sacktor et al. 2016). Remarkably, patients with even the mildest form of HAND -- asymptomatic neurocognitive impairments (ANI) -- have much higher chances of displaying impairment with daily activities due to cognitive decline as they age, compared to non-infected age-matched controls. This suggests that ANI potentiates age-associated cognitive impairment and having ANI is predictive of more serious impairment later on (Grant et al. 2014). Currently there are no effective treatments designed specifically for HAND and patients are managed with regimens commonly prescribed for patients with aging-related dementia such as antioxidants, N-methyl-D-aspartate (NMDA) antagonists and life style adjustments, such as exercising. More efforts are required to elucidate the molecular mechanisms underlying the development of HAND in order to design targeted therapies that could more effectively ameliorate the neurocognitive impairments.

Together with macrophages, microglia are the major cell type productively infected by HIV-1 in the CNS, and therefore it is also likely a major contributor to neurotoxicity observed during chronic HIV-1 infection (Gonzalez-Scarano and Martin-Garcia 2005). Various groups have proposed that the pro-inflammatory sequelae of microglia activation during HIV-1 infection, including altered cytokine secretion, comprise a major tenant of HAND development (Gonzalez-Scarano and Martin-Garcia 2005; Lull and Block 2010). Advanced technology and dedicated efforts heralded significant advancement in our understanding of microglia physiology including unique molecular programs during

microglial activation and aging, which could add new meanings to the implication of HIV-1 infected microglia during HAND.

The objective of the present review is to critically assess functional changes of microglia during chronic HIV-1 infection taking into consideration the aging demographics of the HIV-1-infected population, in order to identify molecular pathways that could serve as potential therapeutic targets.

## Microglia Origin and Function

Introduced as the non-astroglial, non-neuronal “third element” of the CNS, microglia were first characterized in the 1930’s by del Rio Hortega using silver staining technique (Rio-Hortega 1939). It was noted that microglial cells have the capacity to migrate, proliferate and phagocytose. Since then, advanced staining techniques and genetics-based studies elucidated that microglia belong to the myeloid phagocytic/monocytic lineage (Murabe and Sano 1982; Perry et al. 1985; Smith et al. 2013). Unlike other tissue resident macrophages, which are hematopoietically derived, the majority of microglia at steady state originate from a self-renewing cohort of yolk sac-derived erythro-myeloid progenitors (Ginhoux et al. 2013; Gomez Perdiguero et al. 2015; Sheng et al. 2015). Although there are significant variations in microglia density (0.5%–16.6%) and morphology depending on brain regions, whether region-dependent heterogeneity in microglia function exists remains largely unknown (Mittelbronn et al. 2001; Olah et al. 2011). Recent murine studies demonstrate differential genetic profiles depending on the brain regions, suggesting region-dependent functional differences (Grabert et al. 2016). At the basal level, microglia act as sentinels to survey the environment of the CNS through their motile processes and dynamic contacts with neural cells, including astrocytes and neurons (Kettenmann et al. 2011). Recent evidence suggest that microglia prune pre-synaptic axons and post-synaptic dendritic spines to fine-tune synapses (Kettenmann et al. 2013; Tremblay et al. 2010). During neuronal development and maturation, a period of significant cell turnover, microglia also contribute to the clearance of dead cell debris through phagocytosis (Michell-Robinson et al. 2015). In the context of neurodegenerative diseases, microglial cells play essential roles in the clearance of beta amyloid (Aβ) proteins (Liu et al. 2010), the accumulation of which correlates significantly with neurocognitive impairments. Microglia express receptors for various neurotransmitters, cytokines as well as sensors for innate immunity including pattern recognition receptors such as Toll-like receptors (TLR) (Neumann and Wekerle 2013). Upon exposure to activating stimuli such as invading pathogens, microglial cells quickly mobilize to the site of insult and mount an innate immune response, and may help establish adaptive immunes responses, through phagocytosis, secretion of cytokines and chemokines, and antigen presentation (Aloisi 2001; Pascual et al. 2012; Shrikant and Benveniste 1996; Smith et al. 2010; Wraith and Nicholson 2012). Whether microglia activation is neurotoxic or neurotrophic is highly context-dependent. For instance, “acutely activated” (24 h treatment with the TLR4 ligand lipopolysaccharide [LPS]) microglia release pro-inflammatory cytokines and contribute to reduced neuronal survival rate, whereas “chronic activation” (72 h LPS exposure) of microglia induces secretion of anti-inflammatory cytokines and development of a neuroprotective phenotype (Cacci et al. 2008). As a result, the nature, duration and strength of microglial response to foreign insults are tightly regulated by inputs

from both neural cells and components of the immune system. For example, depending on the type of stimuli, microglia could assume a pro-inflammatory/antigen-presenting activation state or anti-inflammatory/tissue-repairing activation state, similar to the M1 and M2 phenotypes traditionally used to describe macrophages, as demonstrated predominantly with murine models (Ponomarev 2011) (Table 1). Evidence obtained with primary human cells suggests, however, that microglia initiate slightly different activation programs compared to that of monocyte-derived macrophages (MDMs). When stimulated with M1-polarizing agents such as interferon (IFN)γ, human adult and fetal microglia and MDMs all express elevated levels of CD80 and CCR7 with fetal microglia exhibiting the least elevation in CD80 and CCR7. On the other hand, treatment with M2 polarizing agents such as interleukin (IL)-4 leads to significantly increased CD23, CD163, CD206 and CD209 in MDMs, CD209 in adult microglia and CD209 and CD206 in fetal microglia (Durafourt et al. 2012). The different activation patterns between microglia and MDMs is just one of many functional characteristics that differentiate microglia from macrophages. For instance, adult microglia express qualitatively similar but quantitatively lower levels of antigenic markers including CD45, CD11b, HLA-DR, CD14, CD200R, CD16, CD32, CD64, CD86, CD163, CD68, CD4 and Iba-1 compared to macrophages (Kettenmann et al. 2011; Melief et al. 2012). Gene profiling and quantitative mass spectrometry analysis of murine cells demonstrate that microglia uniquely and highly express a group of transforming growth factor β (TGF-β)-dependent molecular signatures compared to other neuronal and immune cells, including resident macrophages in peripheral tissues (Butovsky et al. 2014). To conclude, microglia are an important and unique class of CNS-resident cells that perform various immunemodulatory functions to insure neuronal integrity. Disturbed microglial function has been attributed to the development of various neurological disorders including Alzheimer's, Huntington's, and Parkinson's, as well as HAND (Block et al. 2007; Nakajima et al. 2007).

## Age-Dependent Changes in Microglial Function – Activation and Senescence

Microglial function evolves during the course of an individual's lifespan (Figure 1). Immunohistochemical staining of brain sections of pre-term fetuses detected the presence of microglial cells in human CNS as early as 9 gestational weeks, which precedes birth and onset of hematopoiesis in the human bone marrow and liver (Verney et al. 2010). This observation is consistent with the discovery that the majority of adult microglia derive from embryonic progenitors, rather than from circulating monocytes generated during post-natal hematopoiesis. Fetal microglia strikingly differ from their mature, adult counterparts. In early gestational weeks, microglia appear amoeboid resembling activated microglia morphology and go through gradual ramification with increasing gestational age. Phenotypic analysis demonstrate that fetal microglia at pre-myelinating 16–20 weeks of gestational age express CD4, CCR5, CD11b, CD11c, CD14, CD45, CD68, CD86 and human leukocyte antigen-D-related (HLA-DR), and the staining intensity of antigenic markers weakens with increasing gestational age (Esiri et al. 1991; Rezaie and Male 1999; Wang et al. 2002). These observations suggest that fetal microglia have a partially activated state, which diminishes with maturation.

At the other end of the age spectrum, microglia also exhibit elevated activation status. Positron emission tomography (PET) demonstrated increased R-[$^{11}$C] PK11195 ligand binding, suggesting elevated microglial activation, in healthy aged humans (Schuitemaker et al. 2012). Additionally, molecular markers associated with microglia activation including major histocompatibility complex II (MHC II), CD11b and Iba1 have been shown to be elevated in microglia in tissues of aged individuals without overt neurocognitive deficits (Frank et al. 2006; Rogers et al. 1988; Ziv et al. 2006). The potential consequences of such chronically elevated activation of microglia during aging have garnered significant scientific interest in recent years, since the “microglial dysfunction hypothesis” suggests that age-related alteration of microglial function contributes to the onset of various neurodegenerative diseases (Wong 2013).

Another very important aspect of the age-associated alterations in microglia function is the development of the microglial senescence program. The concept of cellular senescence was first introduced by Hayflick and Moorhead to describe the limited lifespan of primary human cells in vitro, and has since been observed to play important roles in regulating human tissue aging and aging-related pathology (Hayflick and Moorhead 1961). It has been proposed that senescence is beneficial to reproductive fitness early in life through fine-tuning organ development during embryogenesis and as an anti-tumor mechanism by inducing permanent cell cycle arrest of neoplastic cells (Childs et al. 2014). As an organism ages, however, senescence appears to have detrimental health effects and contributes to the progressive loss of tissue and organ function during aging. More specifically, senescent phenotypes have been observed in various cell types residing in different tissues including bronchial cells, adipocytes and pancreatic β cells, as well as in astrocytes, and development of senescence in those cells has been shown to contribute to and/or to be associated with the onset of pulmonary fibrosis, obesity, type 2 diabetes and Alzheimer’s disease, respectively (Bhat et al. 2012; Chesnokova et al. 2009; Chinta et al. 2013; Minagawa et al. 2011; Minamino et al. 2009; Sone and Kagawa 2005). Although it still remains to be clarified whether cellular senescence directly promotes aging, eliminating senescent cells appears to prolong the health span of both BubR1-hypomorphic progeroid and normal mice (Baker et al. 2011; Baker et al. 2016), and ‘senolytic’ drugs that can selectively kill senescent cells may potentially have remarkable beneficial effects in terms of health span and/or life span (Chang et al. 2016; Zhu et al. 2015a; Zhu et al. 2015b). It has been proposed that neutralization of detrimental effects of senescent cells or elimination of senescent cells could prevent the onset of various aging-related diseases, including neurodegenerative diseases (Salminen et al. 2011). As a result, much effort has been spent on characterizing senescence in various cell types in vivo and in vitro. Senescent cells in vitro have many distinguishing characteristics including elevated expression of p53-p21 axis and/or p16$^{Ink4a}$ pathway that ultimately prevent entry into the S phase of the cell cycle; permanent DNA damage foci with accumulation of the p53-binding protein 53BP1 and/or the topologically-changed histone γH2Ax; increased enzymatic activity of the lysosomal hydrolase senescence-associated β-galactosidase (SA-β-Gal); and the development of the so-called senescence-associated secretory phenotype (SASP), which is a distinct secretory profile consisting of various pro-inflammatory cytokines, chemokines and metalloproteinases (Munoz-Espin and Serrano 2014) (Table 1). It was also shown that the onset of SASP is dependent on the initiation of

DNA damage response and not present in all senescent cells (Rodier et al. 2009). The aforementioned traits need to be used in combination to characterize the senescent phenotype since any individual characteristic can be found independent of the initiation of the cellular senescence program. Cell culture experiments demonstrate that microglia undergo telomere shortening, which is a classic inducer as well as characteristic of senescence (Flanary and Streit 2004). Additionally, emerging evidence suggest that microglia could potentially develop a senescence-like phenotype with in vitro passaging. Compared to 2 days in vitro (DIV) microglia, 16 DIV murine microglia demonstrate elevated SA-β-Gal positivity, which might suggest the onset of cellular senescence (Caldeira et al. 2014). Importantly, transition to a potentially senescent phenotype correlated with reduced phagocytic and migratory capacities, indicating that what the authors describe as an "age-like phenotype" in microglia could result in impairment of its normal functions (Caldeira et al. 2014). However, it seems striking that the establishment of senescence may occur in microglia after a relatively short time of in vitro culture. In addition, the observation of reduced phagocytosis and mobility in these cells is not in agreement with very recent evidence obtained from aging mice using high spatial-resolution electron microscopy, which describes more active microglia in 14-month old than in 3-month old mice. Imaging captured increased instances of microglia extending their processes to encircle neuronal synapses including pre-synaptic axon terminals and post-synaptic dendritic branches, which, together with the shrunken appearances of and empty spaces surrounding these neuronal processes, suggests active digestion of neuronal synapses by microglia in the aging mice (Bisht et al. 2016). These discrepancies could be due to the fact that microglia exhibit differential rates of phagocytosis depending on the objects being ingested – the in vitro study used beads for phagocytosis assay while the in vivo experiment observed microglial synaptic pruning, which is a more physiologically-relevant model. In addition, it is possible that sub-populations of microglia may exist in the aged brain, and that some may exhibit a phenotype more similar to that described in vitro. Although microglial senescence has not been examined systematically in human tissues, analysis of aged human brain tissues revealed the presence of dystrophic microglia, characterized as having altered cytoplasmic structures including de-ramified, tortuous processes with spheroidal bulbous swellings, which often progresses to fragmented cytoplasm (Streit et al. 2004). This dystrophic microglia phenotype is observed to increase with aging and has been detected in neuropathological conditions including Alzheimer's disease (AD) (Yang et al. 1998). Thus, the cumulative evidence suggests that aging is a significant modifier of microglial function. Although the progression and exact nature of microglial "aging" remains to be clarified, activation and senescence appear to be integral processes of the microglial "aging program".

## Microglia During HIV-1 Infection: Persistent Viral Presence During cART Era

Despite stable courses of cART that may result in undetectable levels of virus in blood, HIV-1 persists in most infected patients and it is possible that the CNS may be one anatomical reservoir where the virus resides. Microglia constitute the only resident cells in the human brain parenchyma that can support productive HIV-1 infection and could thus serve as one of the potential cellular reservoirs within the CNS during chronic HIV-1 infection. This is supported by evidence demonstrating significant cerebrospinal fluid (CSF)

viral escape in some patients on effective cART that have low or undetectable plasma HIV-1 RNA levels. In a retrospective analysis that examined viral load in patients' CSF and plasma, Eden et al. detected a median viral load of 121 HIV-1 RNA copies/ml in the CSF of 7 patients out of a total of 69 patients analyzed, all of whom had undetectable plasma viral load by standard assays (below 50 copies/ml) and were asymptomatic for neurocognitive diseases (Eden et al. 2010). In two additional retrospective studies that selected for patients who had developed neurocognitive deficits and brain magnetic resonance imaging (MRI) abnormalities, CSF viral escape (median viral loads of 880 and 3900 copies/ml, respectively) was detected in all patients with undetectable plasma viral load (Canestri et al. 2010; Peluso et al. 2012). Furthermore, phylogenetic analyses have revealed compartmentalization of HIV-1 in the CSF compared to peripheral blood early post-infection, which suggests that HIV-1 evolves very quickly to adapt to the CNS microenvironment early on (Schnell et al. 2010; Sturdevant et al. 2012). This is in addition to the well-established compartmentalization of viral strains between brain and blood or lymphoid tissues determined with autopsy samples derived from patients with neurocognitive manifestations associated with HIV-1 infection (Gonzalez-Scarano and Martin-Garcia 2005). Furthermore, genetic and functional studies of the envelope gene of viral strains isolated from brain, and the comparison with those from blood or lymphoid tissues of the same infected patients, have demonstrated genetic changes that contribute to a reduced dependence on the primary receptor for infection, namely CD4; this reduced CD4 dependence confers these strains an increased ability to infect cells with low CD4 expression, compared to that of CD4+ T cells, such as microglia and macrophages (Gonzalez-Scarano and Martin-Garcia 2005; Martin-Garcia et al. 2005; Martin-Garcia et al. 2006; Rossi et al. 2008). The importance of macrophages and microglia during HIV-1 infection is further emphasized in a model of simian immunodeficiency virus (SIV)-infected rhesus macaques with depletion of CD4+ T cells, in which infection is sustained by macrophages and microglial cells (Micci et al. 2014). The above evidence suggests that HIV-1 has evolved to reside in long-lived cells such as microglia within the CNS, to achieve certain levels of viral replication refractory to immune pressure and antiretroviral therapies that, in most patients, effectively restrict viral load in the peripheral blood.

The presence of HIV-1 viral reservoir in the CNS is bolstered by the detection of stable SIV viral DNA in infected macaques even when viral RNA in circulation decreases to an undetectable level, suggesting a state of viral latency (Clements et al. 2005; Clements et al. 2011; Zink et al. 2010). Isolated microglia from SIV-infected animals and HIV-1-infected human brains start to produce infectious virus upon in vitro culture, which further validate the observation that microglia harbor infectious HIV-1 virions (Brinkmann et al. 1993; Ghorpade et al. 2005). Overall, ex vivo and in vitro evidence suggest that the CNS, and microglial cells in particular, can potentially serve as one of the anatomical and cellular reservoirs, respectively, where HIV-1 may persist during chronic infection despite successful cART. As a result, it is important to better understand the interaction between HIV-1 and microglia during chronic HIV-1 infection.

## The Effect of HIV-1 Infection on Microglial Function

### Microglia activation during HIV-1 infection

Abundant clinical evidence suggests a state of immune activation in the CNS of patients infected with HIV-1. Brain imaging of HIV-1-infected patients on cART using positron emission tomography imaging and 11C-PK11195 as an in vivo marker of microglia activation, reveals activation of microglia even in the absence of neurological symptoms (Garvey et al. 2013). In vivo and ex vivo studies of HIV+ patients demonstrate that increased immune activation in the CNS of infected patients compared to uninfected controls could be attributed to infected microglia (Andersson et al. 1998). Immunocytochemistry staining of brain sections from HIV-1-infected patients indicates that, compared to tissues from uninfected controls, there are significant increases in MHC class II, CD163, IL-1 and tumor necrosis factor (TNF)α levels, indicative of immune activation. The CSF from HIV+ patients also contains increased levels of TNFα, β2-microglobulin and neopterin, which are also markers of macrophage activation (Tyor et al. 1992). IL-1α was elevated in microglial cells derived from brains obtained from HIV-1-infected patients, and S100β, a cytokine that induces intraneuronal calcium levels, was also found to be elevated in the CNS of HIV-1-infected patients, which could contribute to neuronal degeneration (Stanley et al. 1994). Combined reverse transcriptase/polymerase chain reaction, in situ hybridization and immunohistochemistry in brain and spinal cord sections of patients infected with HIV-1 demonstrate that cells of the macrophage lineage, including microglia, are positive for TNFα mRNA and therefore represent possible sources of TNFα in the brain during HIV-1 infection (Wesselingh et al. 1997). This was in contrast with the results of an ex vivo study utilizing microglia isolated from brain tissues of patients who were HIV seropositive. The cells were confirmed to be CD68+ and to express p24 antigen by immunofluorescence and were subsequently stimulated with LPS for 24 h. Surprisingly, HIV-1-infected microglia secreted lower levels of TNFα both in basal conditions and post-LPS challenge than control microglia, suggesting that microglia chronically infected by HIV-1 may exhibit subdued pro-inflammatory profile compared to uninfected cells, despite the fact that most of the microglia stain positive for HLA-DR, indicative of activation (Ghorpade et al. 2005). Unfortunately, a more extensive secretory profile was not investigated to fully examine the activation status of infected microglia in this particular study.

Immunohistochemistry staining of brain sections from chronically-infected patients who have developed HIV-1 encephalitis (HIVE) revealed elevated levels of IL-1 and caspase-1 in microglia, which suggests that inflammasome activation, as a part of the microglia activation program, could contribute to the development of HAND (Zhao et al. 2001). This was confirmed in an in vitro study in which HIV-1 infection resulted in inflammasome activation, as indicated by the release of IL-1β and caspase-1 activation (Walsh et al. 2014). Using an in vitro primary human microglia model, HIV-1 infection results in secretion of IL-8, IL-6, CCL2/monocyte chemotactic protein 1 (MCP-1), TNFα and CCL5/RANTES (or “regulated on activation, normal T cell expressed and secreted”), in a Beclin-1-dependent manner (El-Hage et al. 2015). Microglial activation is also observed in animal models of HIV-1 infection of the CNS. In brain tissues of rhesus macaque monkeys infected with SIV, in situ hybridization showed that a majority of infected microglia expressed MHC class II,

indicating glial activation (Brinkmann et al. 1993). Astroglial activation, as indicated by microglial nodule formation and increased IL-1β and IL-6 expression, was also observed in severe combined immunodeficiency (SCID) mice that developed encephalitis upon receiving xenografts of HIV-1-infected cells (Persidsky et al. 1996). Although most of the evidence suggest that microglial cells predominantly exhibit pro-inflammatory status in the context of HIV-1 infection, surface marker analysis on brain sections from patients with HIVE detected elevated levels of CD163 in association with ramified microglia, suggesting a de-activated or alternatively-activated phenotype (Roberts et al. 2004).

All evidence considered, it is likely that microglia exist in a spectrum of activation status in the context HIV-1 infection, however the mechanisms regulating differential microglial activation during the course of HIV-1 infection remain largely unknown. Pro-inflammatory profiles seen in microglia during HIV-1 infection could be due to multiple factors including infection itself, viral protein-mediated or caused by cytokine secretion, possibilities which can be further explored in an in vivo setting. Using a primary human fetal microglia model, it was shown that HIV-1 integrase inhibitor Raltegravir significantly reduced the levels of multiple cytokines including IL-8, IL-10, TNFα and IL-6, which suggest that the infection definitely plays an important role in either the initiation or propagation of the pro-inflammatory phenotype (Tatro et al. 2014).

## HIV-1 infection affects the apoptosis vs. survival pathway of microglia

HIV -1 infection also affects the balance of microglial survival and death. Recent evidence suggests that terminally differentiated tissue macrophages have the potential to proliferate in order to self-maintain under favorable conditions (Hashimoto et al. 2013). The intricate balance of this process is perturbed during HIV-1 infection, since it was shown to inhibit granulocyte-macrophage colony-stimulating factor-mediated CD68+ macrophage/microglia proliferation observed in vitro as well as in brain tissues using Ki67 and BrdU (5-bromo-2′-deoxyuridine) staining (Cosenza-Nashat et al. 2007). In vivo and in vitro evidence also suggest that productive HIV-1 infection promotes a pro-apoptotic environment by up-regulating Bax (pro-apoptotic) and inactivating Bcl-2 and Bcl-x (anti-apoptotic) (Krajewski et al. 1997). However, it is unclear whether microglial apoptosis occurs, and how widespread it is, during HIV-1 infection. On the other hand, there have been also studies demonstrating that microglial cells avoid HIV-1 infection-mediated cell death through up-regulation of pro-survival pathways. For instance, primary human microglia promote cell survival by up-regulating BAG3, whose down-modulation seems to be required for caspase-3-mediated apoptosis following infection (Rosati et al. 2009). Other studies suggest that HIV-1 infection could modulate cell survival at the level of cell cycle regulation. For example, an aberrant profile of cell cycle regulators was observed using the SIV model. In brain tissues with SIV encephalitis, levels of E2F1 and retinoblastoma susceptibility gene product (pRB) were shown to be elevated in neurons and glial cells in both frontal cortex and basal ganglia using immunofluorescence staining (Jordan-Sciutto et al. 2000). E2F and pRB are required for the initiation of S phase during cell cycle progression; however, in terminally differentiated cells such as neurons, E2F could cooperate with p53 to initiate other p53-mediated cellular processes including apoptosis (Pan et al. 1998). Unfortunately, expression of proteins mediating apoptosis such as caspase-8 and -9 was not examined to determine the potential

link between E2F1 up-regulation and apoptosis during infection. In subsequent studies, no difference was detected in E2F target genes including proliferating cell nuclear antigen (PCNA) and cyclin A, which are important for cell cycle progression, or $p19^{ARF}$, which mediates p53-dependent apoptosis in neurons (Wang et al. 2010). Thus, the consequences of altered cell cycle proteins including E2F and pRB in neurons during HIV-1 infection remain to be fully clarified.

Additionally, it has been shown that the level of MDM4, a homologue of E3 ligase murine double minute 2 (MDM2) that negatively regulates p53 activity, is reduced in the brain tissues of HIV-1-infected patients and patients with HAD (Colacurcio et al. 2013). This was consistent with up-regulated protein levels of p53 in both HIV-1-infected human and SIV-infected macaque brain tissues (Garden et al. 2004; Jayadev et al. 2007; Jordan-Sciutto et al. 2000; Jordan-Sciutto et al. 2002). In addition to an elevated p53 signaling pathway, target genes of p53, including p21 and Bax, were both up-regulated in neurons and glial cells in HAD tissues (Jayadev et al. 2007). Although p53 was up-regulated in both astrocytes and microglia identified with cell type-specific markers, no distinction between the two glial cell types was made regarding the expression of its target genes. To conclude, the levels of various cell cycle regulators are modulated in HIV-1-infected tissues and appear to be associated with the onset of HAND. Specifically, the p53-p21 pathway, which typically inhibits cell cycle progression, is elevated in various CNS cell types, including microglia, during infection. Although the p53-p21 axis is well known for its role in cell cycle regulation, evidence suggests its relevance in other cellular pathways in a cell type-dependent manner. In addition to regulating the expression of cell cycle inhibitors, p53 was also shown to control macrophage activation (Su et al. 2013). In murine models, p53 knock-out abrogates IFN$\gamma$-induced microglia activation, which suggests that the p53 pathway also promotes activation of microglia (Jayadev et al. 2011). Therefore, it is important to consider the effect of elevated p53 on microglia activation, in addition to cell cycle progression, during HIV-1 infection. Although, the consequential impact of the altered cell cycle machinery remains to be clarified, HIV-1 infection or bystander effects of HIV-1 infection seem to disrupt the delicate balance of cell survival, cell cycle progression and apoptosis, which could contribute to the development of HAND.

## HIV-1 infection affects cellular metabolism in microglia

Individuals with chronic HIV-1 infection often display severe metabolic disorders including disturbance in protein turnover, insulin resistance and altered amino acid metabolism (Gostner et al. 2015; Hommes et al. 1990; Vigouroux et al. 1999). Metabolomic screening of CSF from HIV-1-infected patients detected elevated levels of metabolites typically associated with mitochondrial dysfunction (succinate) and microglial activation (glutamate and arachidonate) in patients with HAND compared to those without any neurocognitive impairment (Cassol et al. 2014). Elevated kynurenine pathway downstream of tryptophan metabolism has also been shown to be associated with neurocognitive impairments during HIV-1 infection (Kandanearatchi and Brew 2012). Increased concentrations of kynurenine pathway metabolites such as quinolinic acid and heightened activities of the kynurenine-catabolizing enzymes have been detected in brain tissues as well as CSF of HIV-1-infected patients and SIV-infected macaques (Heyes et al. 1998; Sardar et al. 1995; Valle et al. 2004).

Microglia is a major source of CNS quinolinic acid, which can induce neurotoxicity as an NMDA receptor agonist. Importantly, increased production of quinolinic acid is correlated with microglial activation and contributes to neurotoxicity during HAND (Drewes et al. 2015; Guillemin et al. 2005; Heyes et al. 2001). Infection of primary human microglia using neurotropic HIV-1 also results in the release of quinolinic acid and, in return, quinolinic acid treatment seems to promote HIV-1 viral replication and elevated CCR5 expression in microglia (Chao et al. 2000; Kandanearatchi and Brew 2012). Thus, altered kynurenine metabolism in microglia during HIV-1 infection could contribute to HAND development.

In addition to increased amino acid metabolism, activation of autophagy, which is a catabolic process that recycles cellular components in the event of nutrient starvation, is also observed in microglia during HIV-1 infection. In primary human microglia cells, HIV-1 infection induces activation of autophagy in a time-dependent manner, and knock-down of beclin 1, which is required for multiple stages during autophagy, results in reduced p24 release as well as reduced secretion of pro-inflammatory cytokines (El-Hage et al. 2015). This study suggests that autophagy activation is essential for viral replication as well as infection-mediated immune activation of microglia cells (El-Hage et al. 2015). In a large-scale small interfering RNA screen, various proteins essential for regulating autophagy, including autophagy-related factors Atg7, Atg8 and Atg16L2, had been found to be required for HIV-1 infection (Brass et al. 2008). This had been also confirmed in T cell lines with stable knock-down of various Atgs (Eekels et al. 2012). The dependency of HIV-1 replication on autophagy was highly unexpected since autophagy has always been considered as an effective immune defense mechanism against intracellular pathogens, including viruses, by promoting the degradation of viral materials (Talloczy et al. 2006). It is important to note, however, that HIV-1 infection activates the initiation but inhibits the maturation of autophagy, to simultaneously promote autophagy-dependent processing of viral materials and inhibit degradation of viral proteins by autophagy in macrophage lineage cells (Kyei et al. 2009). Interrupted autophagic processes could compromise the turnover of various cellular organelles including the mitochondrion. As a result, abnormal autophagic activation is detrimental to mitochondrial health, which is also observed during HIV-1 infection (Lee et al. 2012; Miro et al. 2003; Miro et al. 2004). In fact, abnormal mitochondrial fusion and fission, which are critical processes for maintaining overall mitochondrial dynamics, have been observed in the brains of patients with HAD compared to HIV-1-infected but neurocognitively normal subjects (Fields et al. 2016b). Specifically, the protein levels of dynamin 1-like protein, which is a GTPase that promotes mitochondrial fission, are reduced in the frontal cortex tissue of patients with HAD (Fields et al. 2016b). Accelerated mitochondria turnover by activating autophagy was sufficient to reverse microglial activation in a preclinical, transgenic murine model of HAND (Fields et al. 2013; Fields et al. 2016a). Taken together, these results suggest a role for altered mitochondrial homeostasis in microglial dysfunction during HAND.

Interestingly, the altered metabolomic profile, inefficient autophagy activation and reduced mitochondrial turnover observed in the CNS during HIV-1 infection are comparable to what may occur during the aging process, which suggests that altered cellular metabolism in microglia may underlie accelerated aging in HIV-1 infected patients with neurocognitive impairment (Cassol et al. 2014; Torok et al. 2016).

## Microglia processing of Aβ protein during HAND

Clearance of Aβ protein constitutes an essential function of microglia cells. Amyloid protein can undergo either the non-amyloidogenic or amyloidogenic pathways. In the amyloidogenic pathway, amyloid precursor protein (APP) is enzymatically processed to produce $A\beta_{42}$ or $A\beta_{40}$, which can then form oligomers and insoluble fibrils. Elevated Aβ fibril deposition in brain tissues and correlatively reduced CSF soluble $A\beta_{42}$ levels have been described as hallmarks of some neurodegenerative diseases including AD (Aguzzi and O'Connor 2010). Intracellular Aβ accumulation has also been linked to neuronal damage during aging and development of AD (Baker-Nigh et al. 2015). However, clinical studies on amyloid homeostasis during HAND development are not as clear-cut. While some groups have described reduced CSF level of $A\beta_{42}$ comparable to that of patients with AD (Brew et al. 2005; Krut et al. 2013), others have found no significant differences (Ances and Ellis 2007; Ances et al. 2010). Studies that report significantly reduced CSF $A\beta_{42}$ levels examined subjects with HAD, whereas those that do not report reduction enrolled patients with HAND encompassing all stages, including the mildest form. It is possible that CSF $A\beta_{42}$ reduction is only detectable in the latest stages of HAND. All studies on CSF $A\beta_{42}$ examined patients with mean ages of less than 50 yrs old, and it is unclear whether an older demographic would yield different results. Furthermore, positron emission tomography (PET) studies do not detect elevated $A\beta_{42}$ fibrils in the brains of patients with HAND compared to cognitively normal HIV+ or HIV− controls, and no difference was detected between cognitively normal HIV+ and HIV− subjects (Ances et al. 2012; Ortega and Ances 2014). It is important to note, however, that the brain imaging studies to date are limited by the small numbers of patients enrolled. Furthermore, even though patients between the age of 35 and 65 were recruited in the above-mentioned studies, results were not stratified by age within each group, which could have revealed different patterns, considering that $A\beta_{42}$ fibrils deposition correlates positively with age (Ortega and Ances 2014). Therefore, due to the limitations of current PET studies, the issue of whether $A\beta_{42}$ fibrils brain deposition is altered during HIV-1 infection as the infected population ages remains unresolved.

Our understanding of amyloid metabolism during HIV-1 infection is further clouded by conflicting reports on the association between HAND development and Apolipoprotein E (ApoE) expression, which is a significant risk factor for abnormal amyloid deposition resulting in sporadic AD development (Kim et al. 2009; Morris et al. 2010). While some early reports demonstrate increased frequencies of HAD development in ApoE carriers, more recent studies do not find a correlation between ApoE phenotype and HAND development characterized by either neurocognitive impairments or detrimental neuroimaging outcomes (Burt et al. 2008; Cooley et al. 2016; Corder et al. 1998; Morgan et al. 2013; Valcour et al. 2004b). These differential results could be due to disparities in stages of neurocognitive impairments examined (HAD vs. milder forms of HAND) or in ages of study subjects since ApoE was shown to be an independent risk factor for HAD in HIV-infected patients who are 50 year of age and older, but not in younger subjects (Valcour et al. 2004a). ApoE status did not modify amyloid fibril brain deposition during HAND in a cohort largely composed of patients younger than 50 years old (Ances et al. 2012). Unfortunately, similar studies conducted with patient cohorts older than 50 years old have not been performed. Recently, utilizing a HIV+ cohort in which 30% of the patients are 60+

years old, it has been demonstrated that ApoE ε4 phenotype correlates with reduced CSF Aβ levels during prolonged HIV-1 infection (Cysique et al. 2015), suggesting that the ApoE ε4 status could have also contributed to the inherent heterogeneities in the CSF Aβ studies discussed above. ApoE ε4 levels have been shown to be correlated with cognitive impairments in HIV-1+ ApoE ε4+ individuals with mean age less than 50 years old, and it would be extremely valuable to determine whether ApoE ε4 status modifies CSF Aβ levels during HIV-1 infection and HAND development (Andres et al. 2011).

The distribution of amyloid protein in the CNS of HAND patients has also been examined through immunofluorescence staining of human brain tissues. Using antibodies targeting APP/Aβ, both intraneuronal and extracellular diffuse APP/Aβ accumulation has been detected in the brains of HIV-1-infected patients (Green et al. 2005; Nebuloni et al. 2001). HAND patients do not display the typical plaques of Aβ fibrils observed in AD patients. However, the increased intracellular as well as extracellular diffuse Aβ accumulation detected in HAND patients suggests an increased pool of soluble Aβs, which could be associated with the presence of dysfunctional microglia.

## The Effect of Viral Proteins on Microglial Function

In addition to their susceptibility to infection by HIV-1, microglia in vivo are also exposed to several viral proteins such as Tat, gp120, Vpr and Nef, which are released by infected cells and can be detected in the CNS (Chang et al. 2011; Mukerjee et al. 2011). Various studies suggest that exposure to those viral proteins may also result in altered microglial function and that these effects could underlie the microglial dysfunction observed both in vivo and in in vitro models of infection.

Tat is the trans-activating protein of HIV-1 that plays a vital role in HIV-1 viral transcription and has been shown to affect multiple cellular functions, thereby contributing to the pathogenesis of HIV-1 infection (Romani et al. 2010). An in vitro study exposing microglial cells to increasing concentrations of Tat demonstrated a dose-dependent stimulation of the secretion of cytokines and chemokines (Sheng et al. 2000). When exposing microglia to Tat in the presence of specific inhibitors of various signaling pathways, it was demonstrated that Tat stimulates the secretion of CCL2/MCP-1 and CCL4/macrophage inflammatory protein (MIP)-1β by activating extracellular-signal-regulated kinases (ERK)1/2 MAPK; CXCL10/IFNγ-induced protein (IP)-10 through phosphatidylinositide 3-kinase (PI3K) activation; and CXCL8/IL-8 through p38 MAPK activation (D'Aversa et al. 2004). Tat also stimulates the production of CCL2/MCP-1, IL-1β, TNFα and inducible nitric oxide synthase (iNOS) in a cyclooxygenase (Cox)-2-dependent manner, downstream of NF-κB activation (Flora et al. 2006). Finally, activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidases also promotes the secretion of various pro-inflammatory cytokines and associated neurotoxicity following Tat exposure (Bokhari et al. 2009; Jadhav et al. 2014; Turchan-Cholewo et al. 2009).

In addition to pro-inflammatory cytokines, Tat activation of p38, p42/44 MAPK and NADPH oxidase pathways leads to increased glutamate release, which could potentially induce excitotoxicity and further contribute to neurotoxicity (Gupta et al. 2010). Tat

exposure also promotes microglial migration towards, and phagocytosis of, axonal arbor, resulting in loss of neuronal synapses (Eugenin et al. 2005; Marker et al. 2012).

The surface gp120 sub-unit of the viral envelope glycoprotein is non-covalently bound to the transmembrane sub-unit gp41, and is known to shed in CD4-dependent and –independent manners from viral envelope glycoprotein trimers present both in the surface of virions and in the plasma membrane of infected cells (Ruprecht et al. 2011; Selhorst et al. 2013). This soluble gp120 can engage receptors in multiple cells and could thus result in activation of microglia through diverse mechanisms. Exposure to gp120 results in up-regulation of intercellular adhesion molecule (ICAM)-1 expression in a human astroglioma cell line and primary rat microglia, mediated by protein kinase C and tyrosine kinase (Shrikant et al. 1996). Exposure of microglia to gp120 also induces an outward K+ current and a concomitant increase in voltage-gated K(v) channel proteins, which could lead to elevated cytokine secretion, and subsequently contribute to neuronal damage that can be attenuated with K(v) channel blockers (Xu et al. 2011). In rat microglia, it was further shown that gp120 enhancement of voltage-gated K(v) channels depends on the activation of p38 MAPK pathway (Liu et al. 2012). Exposure of microglial cells to gp120 has also been recently shown to induce inflammasome activation and associated IL-1β secretion (Walsh et al. 2014).

Although these in vitro studies reveal important information on the effects of viral proteins on cellular functions, and specifically in microglia, their physiological relevance in vivo needs to be critically assessed, especially with regard to the concentrations of viral proteins used in vitro. In in vitro studies, concentrations of Tat in the range of 100 to 1000 ng/ml are routinely used; this is much higher than the concentrations of Tat detected in sera of HIV-1-infected patients, which usually ranges from 1 to 40 ng/ml depending on the study (Westendorp et al. 1995; Xiao et al. 2000). It is also possible that Tat release from infected cells may lead to locally higher concentrations, but these have not been demonstrated to date. Similar criticisms can be made for the studies utilizing gp120 proteins. While earlier studies may have detected higher serum concentrations of the envelope glycoprotein gp120 (Gilbert et al. 1991; Oh et al. 1992), more recent studies reported lower levels in the plasma of HIV-1-infected patients in the range of 0.5–15.6 ng/ml (Rychert et al. 2010), and even undetectable amounts in serum samples from patients with low-to-undetectable viremia (Santosuosso et al. 2009). It has also been shown in high viral load, rhesus macaques acutely-infected with a simian-HIV (or SHIV, encoding the HIV-1 envelope glycoproteins in an SIV genome) that HIV-1 gp120 was present in a much higher concentration in lymph node lysates than in plasma (218 ng/ml vs. 1.5 ng/ml, respectively) (Stevceva et al. 2008). However, in another study, cell-free tissue extracts from lymph nodes and spleens from patients with low-to-undetectable viremia showed that gp120 was undetectable or below 9 ng/ml (Santosuosso et al. 2009). To our knowledge, no study has quantified gp120 levels in brains of HIV-1-infected patients, but because one would expect the number and proportion of infected cells in the brain to be lower than in lymph nodes or spleens, it seems likely that a lower concentration of gp120 would be found in the brain than in those other tissues. However, concentrations of 200–500 ng/ml of gp120 are regularly used in in vitro studies, and these seem to be well above what might be expected in the brain. In addition, the vast majority of in vitro studies have used the soluble gp120 of a CXCR4-using strain, HIV-$1_{IIIB}$,

while the viruses isolated from brain tissues are almost exclusively using the alternative co-receptor for infection, namely CCR5. This is because infection of macrophages and microglia is only efficient for a subset of CCR5-using viruses, and for a few exceptions among the CXCR4-using strains. Thus, rather than focusing on the effects of IIIB gp120 in microglia, more studies should be directed to address the effects of macrophage/microglia-tropic, CCR5-using gp120 proteins from brain- or CSF-derived isolates.

Any effects of viral proteins on HIV-1 disease progression in patients likely occur through chronic exposure at physiologically-relevant concentrations, which are conditions that cannot be appropriately modeled with in vitro cell culture systems that most often use higher concentrations over acute time courses. To complement results obtained using in vitro models, in vivo animal models treated with or expressing viral proteins for longer periods of time at lower concentrations have been used to study the effects of viral protein exposure during chronic HIV-1 infection. In a non-infectious HIV-1 transgenic rat model that expresses 7 of 9 HIV-1 viral proteins including gp120, Tat and Nef, microglia activation characterized by elevated Iba-1 expression is detected in younger groups and declines with age (Reid et al. 2016). Elevated Iba-1 expression post-gp120 exposure is also demonstrated using a well-characterized transgenic mice model expressing soluble CXCR4-using HIV-1 LAV gp120, and could be reversed by modulating pathways that facilitate mitochondria regeneration (Fields et al. 2013; Fields et al. 2016a; Maung et al. 2014; Toggas et al. 1994). In a rat model that has been transduced to express HIV-1 NL4-3 gp120, also a CXCR4-utilizing envelope, microglia activation peaks at week two and then transitions into the onset of apoptosis as indicated by significant TUNEL staining in CD68+ cells at one and three months, which can be partially protected with antioxidant treatment (Louboutin et al. 2009; Louboutin et al. 2010), suggesting that activated microglia might eventually undergo apoptosis during protracted gp120 exposure, possibly due to prolonged oxidative stress. Results generated using these models have limited impact, however, considering that, as discussed above, gp120s from CCR5-utilizing viral strains are more relevant for studying effects on microglia function during HIV-1 infection, and the concentrations of gp120 obtained in both models have not been examined.

Microglia activation is also observed using various in vivo models of Tat exposure. For example, microglia activation was detected in mice that were injected with Tat in the striatum for 7 days (El-Hage et al. 2006; Puccini et al. 2015), and in the brain tissues of a rat model two weeks post-stereotaxical Tat injection in the striatum as well (Agrawal et al. 2012). Microglia activation upon Tat exposure has been reversed with over-expression of antioxidant enzymes including Cu/Zn superoxide dismutase (SOD1) and glutathione peroxidase (GPx1), suggesting a role of oxidative stress in Tat-induced microglia activation (Louboutin et al. 2014; Louboutin and Strayer 2014). An important caveat of the Tat injection models in mice and rats is that the initial injected concentrations of Tat are usually much higher than the physiological levels, although the actual final concentrations of Tat in the animal brains could be significantly lower due to distribution to wider areas as well as Tat degradation in cells and tissues over time (Passiatore et al. 2009). Another limitation of the stereotaxical injection models is the localized effects around the injection site, which has been the striatum in all studies. While the striatum is a brain area with high rate of productive HIV-1 viral replication, other areas such as the frontal gyrus are also affected

during HAND development and should be examined during Tat exposure (Glass et al. 1995). Compared to direct stereotaxical injection of Tat in animal brains, a more physiologically-relevant model appears to be the conditional Tat transgenic mouse model that could be induced to express Tat in astroglial cells at 0.01–0.85 ng/ml. Three months of Tat expression in this model also results in elevated microglia activation evidenced by nitrosative cellular stress (Hahn et al. 2015; Kim et al. 2003).

In addition to gp120 and Tat, other viral proteins including Nef and Vpr have also been shown to stimulate production of pro-inflammatory cytokines in microglia as a result of oxidative stress (Si et al. 2002; Vilhardt et al. 2002). Overall, HIV-1 viral proteins affect multiple functions of microglia including activation, migration, phagocytosis and glutamate secretion, all of which are relevant aspects of microglia dysfunction during HIV-1 infection and could contribute to the development of HAND. While directly exposing microglia in vitro to viral proteins does not necessarily recapitulate all biological processes during HIV-1 infection, this approach does afford us the opportunity to dissect mechanisms underlying functional changes of both infected and uninfected microglia. This type of studies has revealed that many of the phenotypes observed during HIV-1 infection could be due to activation of various cellular processes that mitigate oxidative stress responses, including inflammasome, p38, p42/44 MAPK, NADPH oxidase and NF-κB signaling, many of which are known to facilitate cellular senescence (Freund et al. 2010) and could be the pathways upon which HIV-1 infection and activation of cellular senescence might converge.

## Concluding Remarks

With the aging of the HIV-1-infected population in the cART era, health care professionals working with HIV-1-infected patients are faced with new challenges of treating both aging-associated loss of functions and health risks, as well as the chronic viral infection and inflammation associated with it. In addition, it seems well-established now that the latter seem to have an effect on the former. Although a large recent study suggested that HIV-1 infection may cause accentuated but not accelerated aging (Althoff et al. 2015), many others have presented evidence of accelerated aging in chronically HIV-1-infected populations (Angelovich et al. 2015; Cassol et al. 2014; Chou et al. 2013; Gianesin et al. 2016; Horvath and Levine 2015; Levine et al. 2015; Martin et al. 2013; Pathai et al. 2013; Pathai et al. 2014; Pfefferbaum et al. 2014; Schrack et al. 2015), involving multiple immune system- and CNS-related functional deficits. This review highlighted the many commonalities between functional changes of microglia during HIV-1 infection and during aging, including cellular activation, arrested cell cycle progression and altered cellular metabolism. Although microglia exhibit various signs of activation including secretion of elevated levels of pro-inflammatory cytokines, we do not think that activation captures the complexity of altered microglia function during chronic HIV-1 infection, especially given the interplay between HIV-1 infection and aging. It is also known that some pro-inflammatory cytokines are part of the unique secretory phenotype of cellular senescence, a cellular process not only known to occur during aging, but that can be at the origin of tissue/organismal aging. Moreover, microglia also express higher protein levels of cell cycle inhibitors and exhibit altered autophagy, both of which are integral parts of cellular senescence (Table 1), but not of activation. Thus, we propose that, although microglia senescence has not been fully

characterized, it is likely an important part of the processes triggered in the CNS during chronic HIV-1 infection. Examining pathways implicated in the induction and consequences of cellular senescence during HIV-1 infection could potentially lead to novel therapeutics for the treatment of HIV-1-associated end organ diseases, including HAND.

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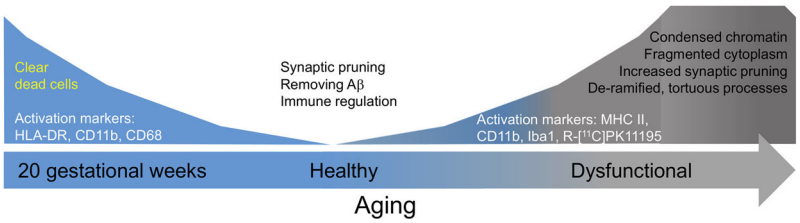


**Figure 1. Age-dependent changes in microglia**

Microglia exhibit phenotypic changes over an individual's lifetime. During fetal development, microglia express activation markers including HLA-DR, CD11b and CD68, indicating an activation state (denoted by blue) that seems to be needed to accomplish proper differentiation and development, and clearing of dead cells. This activation state decreases with maturation and usually leads to a healthy, homeostatic status that predominates until later in life, when a build-up of chronic, persistent activation may occur. In this context, chronic activation may result in deleterious changes that associate with dysfunctional microglia (transition from blue to grey), characterized by morphological changes such as condensed chromatin and fragmented cytoplasm, and functional changes such as increased synaptic pruning that contributes to loss of neuronal synapses. This dysfunctional state may be reflective of a senescence phenotype and could contribute to the development of neurodegenerative diseases.

**Table 1**

Comparison between the main changes in microglia with HIV-1 infection and other processes.

| | HIV-1 infection/Viral proteins | M1 polarized | M2 polarized | Aging | Senescence |
|---|---|---|---|---|---|
| **Immunological profile** | Pro-inflammatory cytokines<br>Chemokines<br>Metalloproteinases<br>Reduced phagocytosis<br>R-[$^{11}$C]PK11195 | IL-6, IL-8 and TNFα | IL-10 and TGF-β | Immune amplification<br>Reduced endocytosis<br>R-[$^{11}$C]PK11195 | Pro-inflammatory cytokines<br>Chemokines<br>Metalloproteinases |
| **Cellular metabolism** | Induction of autophagy<br>Elevated glutamate | Elevated glycolysis | Elevated mitochondrial respiration (β-oxidation) | Elevated lipid synthesis | Altered autophagy<br>Impaired mitochondrial function |
| **Cell cycle regulation** | Elevated p53, p21, and E2F<br>Reduced MDM4 | | | | Elevated p53, p21, and p16 |