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Investigators in Training I

I.1

Activation of the cell cycle protein CDC2 may cause calcium dysregulation in postmitotic neurons contributing to HIV-induced excitotoxicity and neuronal cell death

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The chemokine receptor CXCR4 is involved in HIV infection and contributes to HIV neuropathology in a direct and indirect manner. It has been suggested that abnormal activation of CXCR4 alters neuronal/glial signaling causing neuronal damage, whereas proper CXCR4 signaling promotes neuronal survival. Previous work from our group showed that dysregulation of cell cycle proteins, namely the CDK/Rb/E2F pathway, is involved in the neurotoxicity induced by X4-using HIVgp120s; we also reported that E2F1 and its transcriptional target cdc2 (also known as cdk1), are up-regulated in the brain of HIV/HAD patients. This study aims to establish the role of cdc2 in gp120-induced neuronal injury and determine the molecular mechanisms involved in neurotoxicity. We report that expression of a Cdc2 dominant negative mutant in rat cortical neurons rescues them from HIVgp120-induced apoptosis. Also, down-regulation of cdc2 expression by shRNA in human cell lines reduced phosphorylation of apoptotic proteins downstream cdc2. Furthermore, as recent evidence suggests that Cdc2 is implicated in the regulation of intracellular Ca2+ homeostasis via phosphorylation of IP3 receptors, we are currently testing the hypothesis that cdc2 may also lead to aberrant Ca2+ signaling in neurons, by using calcium imaging and IP3-uncaging. Alterations of Ca2+ homeostasis by gp120 and other HIV-1 proteins are well-documented, and know to affect neurotransmission and neuronal survival, which lead to HIV-associated neuronal deficits. These studies show the importance of cdc2 in gp120-induced neurotoxicity and suggest that therapeutic approaches aimed at preventing stimulation of cdc2 or its targets in neurons may turn useful in HIV neuropathology. (Supported by NIH grants DA 19808 and 15014 to OM).

I.2

T-cell infiltrates in HSV-1 latently infected human geniculate ganglia

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In humans, a primary infection with Herpes simplex virus type-1 (HSV-1) is followed by a lifelong viral persistence in the sensory neurons of the cranial nerve ganglia, especially in the trigeminal ganglia (TG), and less frequently in the geniculate (GG) and vestibular ganglia (VG). In some individuals HSV-1 reactivates repeatedly from the TG and causes 'herpes labialis'. In contrast, reactivation from the GG and VG is rather a once-in-a-lifetime event.

Immunohistochemical and quantitative RT-PCR studies in the TG have revealed that HSV-1 latency is accompanied by an active chronic immune response composed of CD8+ T-cells showing an effector memory phenotype. Although the infiltrating T-cells reflect an active immune response, it is not clear whether the T-cells control viral latency and reactivation or whether they represent an inflammatory residue after the frequent silent or overt reactivations in the TG.

In the present study, using immunohistochemistry, we found that T-cells are also present in the GG where the latency-associated transcript (LAT) was detectable by in situ hybridization. In the VG no T-cells could be found and LAT was detectable only after amplification by RT-PCR. Hence we suppose that the T-cell infiltration in the cranial nerve ganglia monitors HSV-1 latency and does not only represent an inflammatory sequel of a previous reactivation.

I.3

HIV-1 and cytokine (IFN-γ/TNF-α) synergy in CXCL10 release from astrocytes: implications in HIV-associated dementia

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There are 40 million people world wide infected with Human Immunodeficiency Virus-1 (HIV-1). Shortly after infection, HIV-1 penetrates the brain and can

eventually cause HIV-1 associated CNS disease, of which HIV-associated dementia (HAD) is the most severe manifestation. HIV encephalitis (HIVE), the pathologic correlate of HAD is characterized by astrogliosis, cytokine/chemokine dysregulation, and neuronal degeneration. The severity of HAD/HIVE correlates better with the presence of activated astroglial and microglial cells rather than with the presence and amount of HIV-1 infected cells in the brain. Also, correlated with the pathogenesis of HAD/HIVE are the increased levels of the proinflammatory cytokines, IFN-γ and TNF-α. These two cytokines are released by activated immune cells and have the ability to synergize with other cellular factors and HIV-1 viral proteins resulting in increased inflammation and a more severe disease state. Astrocytes, the most populous cell type within the brain, are activated not only by IFN-γ and TNF-α, but also by HIV-1. Once activated, astrocytes provide an important reservoir for the generation of inflammatory mediators including CXCL10, a neurotoxin and chemoattractant. CXCL10 is implicated in the pathophysiology of HAD since high levels of CXCL10 are also correlated with disease severity. The underlying central hypothesis of these studies is that the interplay of viral and cellular factors in astrocytes can result in the synergistic induction of CXCL10 expression resulting in neuronal degeneration. Our preliminary studies suggest a synergistic induction of CXCL10 protien in astrocytes exposed to HIV-1, IFN-γ and TNF-α. We observed that the HIV-1 proteins nef and tat were the key determinants involved in synergy with IFN-γ and TNF-α. Transcriptional and translational regulatory mechanisms involved in the synergistic enhancement of CXCL10 will be discussed.

This could have possible implications for enhanced CNS disease because of the neurotoxic functions of CXCL10 and its ability to enhance viral replication. Thus understanding the cellular and molecular mechanisms involved in the induction of proinflammatory responses by astrocytes is critical to the development of interventional therapies for the treatment of HAD.

I.4

The role of JC virus minor capsid proteins in the viral lifecycle

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JC virus (JCV) is a human polyomavirus for which 70% of the human population is seropositive. It is responsible for the fatal demylinating disease Progressive Multifocal Leukoencephalopathy (PML). JCV contains an icosahedral capsid that consists of 72 pentamers of the major capsid protein Vp1 with a minor coat protein Vp2 or Vp3 in the center of the pentamer. It is known that Vp1 contributes to virus tropism through receptor interactions. However, little is known about the role the minor coat proteins in pathogenesis. Using site-directed mutagenesis we show that both Vp2, the myristylation site on Vp2, and Vp3 are necessary for the correct packaging of viral DNA. We suspect that these proteins also play critical roles early in infection and have engineered tags in Vp2 and Vp3 to facilitate their detection in infected cells. The tagged viruses are viable and Vp2 and Vp3 localize to subcompartments within the nucleus. Co-localization studies with Vp1 show that Vp1 is uniformly distributed in the nucleus and Vp2 and Vp3 are excluded from the peripheral margins of the nucleus. We are currently using the tagged virions to study the trafficking of virus from the plasma membrane to the nucleus with the goal of understanding when and where the minor capsid proteins become exposed in the cell.

I.5

Transmigration of HIV infected monocytes/macrophages and lymphocytes into the central nervous system

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Human immunodeficiency virus 1 (HIV-1) invades the brain and results in neurologic dysfunction in a large percentage of individuals. Even with adequate suppression of systemic virus due to therapy, central nervous system (CNS) disease continues to evolve. Both the penetration of HIV into the brain and the subsequent damage are thought to be mediated by transmigration of infected monocytes across the blood brain barrier (BBB). The number of activated macrophages in the CNS appears to be a more reliable predictor of HIV associated cognitive impairment than viral load, suggesting that monocyte infiltration and CNS damage are tightly correlated. Monocyte/macrophage trafficking is of interest in studies of the pathogenesis of HIV infection because of their ability to cross the BBB, to elaborate factors that are harmful to the CNS, and to establish viral reservoirs. Monocytes may circulate in the peripheral blood for 1-3 days before entering and differentiating into macrophages in the brain. It is important to determine the phenotypic markers and the stage of maturation of monocytes as they enter the CNS, with the ultimate goal of therapeutic intervention to limit CNS infection and inflammation associated with NeuroAIDS. We first designed experiments to establish the optimal culture system for human peripheral blood mononuclear cells (PBMC) that promote monocyte/macrophage survival and examined the expression of specific phenotypic markers as cells mature in culture. Flow cytometry was used to determine monocyte/macrophage maturation using antibodies to CD14, CD71, CD68 and CD16. We found Macrophage Colony Stimulating Factor (MCSF) supports the survival of CD14+ isolated monocytes and their expression of maturation markers under non-adherent

conditions. However, when cultured as PBMC, the expression of CD14 and CD68 decreases over time when cultured with MCSF. In addition, HIV infection of PBMC does not affect the expression of monocyte markers. This knowledge was then used to characterize the transmigration of HIV infected monocytes and T cells across our in vitro model of the BBB using FACS analysis and confocal microscopy. Lastly, we examined the route, transcellular or paracellular, by which HIV infected leukocytes transmigrate, using various microscopic techniques. Preliminary data suggest that HIV infected cells transmigrate by both mechanisms. Data from these studies contribute to our understanding of how HIV infected monocytes/macrophages and lymphocytes transmigrate across and disrupt the BBB, rendering the CNS vulnerable to virus mediated inflammatory damage that leads to cognitive impairment.

I.6

Signal transduction mechanisms of HIV-1 gp120-induced proinflammatory cytokine interleukin-1 beta (IL-1b) production by primary human macrophages

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HIV-associated dementia (HAD) is a common neurological complication of AIDS that involves activation of macrophage/microglial cells in the brain, with subsequent release of toxins including proinflammatory cytokines that lead to neuronal death. In addition to direct infection, uninfected bystander macrophages can be activated by viral proteins such as the Env glycoprotein gp120 to induce cytokine production. Interleukin-1 beta (IL-1b) is one of the proinflammatory cytokines upregulated in brain, CSF and blood of HAD patients. Although gp120 has been reported to induce IL-1b production by macrophages in vitro, the signal transduction pathways responsible have not been defined. In this study, we set out to elucidate the mechanisms mediating gp120-induced IL-1b release by primary human monocyte-derived macrophage (MDM). Recombinant gp120 from CCR5-using (R5) HIV-1 stimulated IL-1b production by MDM as measured by ELISA in both time- and concentration-dependent manner. The gp120-induced IL-1b secretion was blocked by a CCR5 antagonist (M657) and inhibited by pertussis toxin, suggesting it is mediated by binding to CCR5 and coupling to Gi/o protein. Env-mediated IL-1b production was abrogated by upstream inhibitors of Pyk2 (AG17 & dantrolene) and direct inhibitors of PI3K (wortmannin & LY294002), as well as the src kinase inhibitor PP2 and a pseudosubstrate peptide inhibitor KRX123.302 specific for the src family kinase Lyn. We further demonstrated that exposure of MDM to gp120 activated Pyk2, PI3K & Lyn in a time-dependent fashion with direct (Pyk2 or Lyn phospho-specific immunoblot) or indirect (PI3K-dependent Akt activation) approaches. IL-1b release triggered by gp120 was also impeded by inhibitors of the MAP kinase ERK (U0126 & PD98059) and gp120 induced phosphorylation of ERK in a time-dependent manner, implicating MAP kinases in the HIV-1 envelope-mediated cytokine production. We then demonstrated via both coimmunoprecipitation and immunocytochemistry that gp120 triggered an activation-induced physical association between Pyk2, PI3K & Lyn, involving translocation of cytoplasmic Pyk2 and PI3K to membrane-bound Lyn. Finally, we demonstrated by immunofluorescent labeling and confocal microscopy that native gp120 on the surface of HIV-1 virions also induced colocalization of Pyk2, PI3K & Lyn in a CCR5-dependent fashion. Our results indicate that HIV-1 gp120 induces IL-1b release by macrophages through CCR5 coupled to Gi/o protein, subsequently activates multiple protein kinases including Pyk2, PI3K, Lyn and induces formation of a multimeric signaling complex and downstream MAP kinase activation. Defining the signaling pathways responsible for gp120-mediated proinflammatory cytokine production by macrophages could help understand the neuropathogenesis of HAD and contribute to the development of pharmacological agent that attenuate the progression of HAD by specifically blocking these signaling pathways.

Investigators in Training II

I.7

Clade specific variation in HIV-1 transactivating protein (Tat) induced neurotoxicity in human neurons

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Human immunodeficiency virus-1 (HIV-1) infection of central nervous system often results in mild to severe cognitive impairment and dementia due to significant neuronal death. The transactivator HIV-1 protein Tat, has been implicated in HIV associated neurological deficits. HIV-1 is classified into A-K clades that are unequally distributed in world of which HIV-1 clade B and C together account for almost all infections around the globe. Recently, clade B and C specific differences in Tat mediated cytokine and chemokine function have been demonstrated in human peripheral blood monocytes, however it is unknown if these differences affect neurons in human brain. The fact that HIV-1 clade C accounts for over 50% of HIV-1 cases in world today but its effect on neuropathogenesis is unknown, warrants an immediate need to investigate effect of Tat C on human neurons. We used human fetal brain derived neural precursor cell culture system to understand the pathological changes in terms of neuronal apoptosis, glial activation as well as elevation of certain inflammatory parameters. Our experimental data suggest that there are clade specific differences in Tat induced oxidative stress, degree of neuronal death which may be mediated due to disturbances in mitochondria membrane potential and release of cytochrome-c. We also observed that expression of Tat modulated cell growth and proliferation of neural precursor cells. We are currently studying the role of MAP kinase pathway as it plays an important role in neuronal cell function and differentiation. Our observations provide a better understanding of the neuropathogenesis and complications that may arise in human population infected with HIV-1C.

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I.8

JC virus latency in the normal human brain

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JC Virus, a member of the Polyomaviridi family is a neurotropic virus with wide distribution among the human population of the world, according to seroepidemiological studies. After a sub-clinical primary infection in childhood, the virus remains in latent state, presumably in the kidney. Reactivation of JCV under immunosuppressive conditions results in Progressive Multifocal Leukoencephalopathy, a fatal demyelinating disease of the brain, result of the cytolytic destruction of oigodendrocytes. The histopathological features of PML are demyelinated plaques in the subcortical white matter, enlarged oligodendrocytes harboring intranuclear inclusion bodies, and bizarre astrocytes. These two cells represent the sites of active viral infection. Although JCV has been shown to infect lymphocytes, which can act as carriers into other organs, the time at which JCV enters the brain, and the mechanism of such entry are still unknown. Differences in the strain of JCV that is isolated from the kidney (CY) and the one that causes PML (Mad-1) suggest rearrangements in the control region during the time of latent and active infection. Another subject of controversy is the establishment of latency in the brain. To elucidate this question we have collected samples from 5 regions of 7 normal brains from individuals who died of non-neurological conditions, extracted DNA and PCR amplified viral sequenced from the early and late regions of JCV with specific primers. We found JCV DNA sequences in at least one region of all cases studied.

Furthermore, in order to demonstrate the cell type in which JCV is present, we have performed PCR amplification in laser-captured cells of different phenotypes labeled with specific markers for neurons, astrocytes and oligodendrocytes. Results from these experiments suggest that JCV establishes a latent infection in astrocytes and oligodendrocytes, but not in neurons. Finally, immunohistochemical studies showed no expression of viral proteins, T-antigen, capsid proteins (VP-1), and the accessory product Agnoprotein, ruling out productive infection.

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I.9

Non SIV-infected MAC387+ macrophages accumulate within encephalitic lesions of SIV-infected rhesus monkeys: MAC387 as a potential marker of disease

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HIV in humans and SIV in non-human primates cause immunodeficiency and can induce encephalitis. Monocytes/macrophages are targets of infection and play a key role in AIDS-associated neuropathogenesis. HIV/SIV enter the central nervous system (CNS) at primary infection (peak viremia), which corresponds to an increase of the number of CD14+CD16+ monocytes in peripheral blood and macrophages in the brain. We investigated the characteristics of macrophages present in brain lesions of SIV-infected CD8+ cell-depleted rhesus macaques treated or not with anti-retroviral drugs (PMPA and RCV). SIV-infected untreated animals were sacrificed with AIDS, whereas the treated animals had histopathological evidence of SIV infection, but not AIDS. We focused on MAC387 expression, a marker of newly infiltrating monocytes/macrophages. PMPA/RCV-treated SIV-infected animals showed no encephalitis and no MAC387+ cells (n = 4). By contrast, we detected encephalitis in all non-treated SIV-infected animals that showed SIV RNA and an accumulation of MAC387+ cells within brain lesions around blood vessels (n = 4). Interestingly SIV-infected cells were CD68-MAC387-cells. Moreover preliminary phenotypic analysis showed that peripheral blood CD14+CD16+ monocytes from uninfected macaques expressed MAC387 and CCR2. We also detected accumulation of MAC387+ cells in brain lesions of HIV-infected patients with dementia. These data suggest that MAC387+ cells detected in brain lesions of SIV-infected CD8+ cell-depleted macaques are newly infiltrating monocytes/macrophages that are not cellular reservoirs of SIV-productive replication possibly because of their differentiation stage. However they could serve as potential markers of CNS disease. Whether brain MAC387+ macrophages of SIV-infected CD8+ cell-depleted rhesus macaques are derived from blood CD14/CD16 monocyte subpopulations expanded after HIV/SIV infection remains to be determined. This should contribute to decipher the ontogeny of such populations in AIDS-associated neuropathogenesis.

I.10

Effects of minocycline and PMPA on dopamine and dopamine metabolite levels in the caudate nucleus of SIV-infected pigtailed macaques

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Acquired immunodeficiency syndrome (AIDS) and virus-related neurological disease are significant consequences of human immunodeficiency virus (HIV) infection. Even with the advent of highly active antiretroviral therapy (HAART), the prevalence of HIV-associated neurological disease has increased, as many HAART drugs do not cross the blood-brain barrier. Minocycline is an off-patent, tetracycline derivative that effectively crosses the blood-brain barrier and has been shown to be neuroprotective in animal models of several neurodegenerative diseases such as multiple sclerosis, Parkinson's disease, and amyotrophic lateral sclerosis. Using our accelerated, consistent model of simian immunodeficiency virus (SIV) infection we showed that minocycline reduces the severity of neurodegenerative indicators such as macrophage activation and infiltration, astrocytosis, levels of beta-amyloid precursor protein, and macrophage chemoattractant protein-1 in addition to brain viral loads.

Previous studies on post-mortem AIDS patients showed decreased levels of dopamine (DA) in the caudate nucleus (Sardar AM et al 1996), which was also shown to occur in the putamen early in infection in a SIV model (Scheller C et al 2005). Our hypothesis was that minocycline would be able to abrogate the decreases in dopamine levels seen in the brains of SIV-infected macaques. We used high performance liquid chromatography (HPLC) with electrochemical (EC) detection to examine levels of dopamine and its metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in the caudate nucleus of terminally infected pigtailed macaques that were treated with varying regimens of minocycline with or without PMPA (tanofavir), a nucleotide reverse transcriptase inhibitor. Our data showed that uninfected animals had significantly higher levels of DA in the caudate than terminally infected animals and that this decrease was alleviated with early minocycline treatment or later treatment with minocycline in combination with PMPA. Surprisingly the level of DA in the caudate nucleus of terminally infected macaques did not correspond to severity of CNS lesions or several other markers of neurodegeneration.

I.11

Regulation of CXCR4 expression and impact on HIV-1 infection by the mu-opioid agonist DAMGO in a bone marrow progenitor cell line model

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Several studies now suggest that opioids act as a co-factor in enhancing susceptibility to HIV-1 infection in immune cell populations as well as modulating innate, humoral, and cell-mediated immunity. Studies have

also shown that under chronic exposure to mu opioid receptor ligands like morphine there is an increased detection in the amount of HIV-1 long terminal repeat (LTR) DNA in cells of the monocyte-macrophage lineage. CD34+/CD38 − progenitor cells within the bone marrow are refractile to HIV-1 infection, probably due to their low level expression of HIV-1 co-receptors, CXCR4 and CCR5. We have previously shown that the human CD34+/CD38+ TF-1 erythromyeloid progenitor cell line can be utilized as a model to study the differentiation process of hematopoietic progenitor cells and how this differentiation process effects the cell surface expression of the HIV-1 receptor and co-receptors and HIV-1 susceptibility. Given these observations, studies have been initiated to identify the presence of the mu opioid receptor on the TF-1 bone marrow progenitor cell line. Studies have also been initiated to determine the functional relevance of this receptor in altering HIV-1 co-receptor expression, susceptibility to HIV-1 infection, impact on HIV-1 LTR activity, and impact on HIV-1 replication during chronic opioid exposure.

I.12

Astrocytic activation causes impaired glutamate clearance and altered CD38/cADPR signaling: Mechanistic links to HIV-1-associated dementia

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Reactive astrogliosis is a key pathological feature of HIV-1-associated dementia (HAD) and is associated with neural injury. However, to date, molecular mechanisms through which astrocyte functions are altered in HAD are incompletely understood. Glutamate is the primary excitatory neurotransmitter in the central nervous system. The regulation of synaptic glutamate concentration by high affinity transporter such as EAAT2 is crucial to avoid excitotoxicity. Astrocytes are known to express EAAT2 that is responsible for over 90% of synaptic glutamate clearance. CD38 is a 45 kD ectoenzyme involved in synthesis and translocation of the potent calcium (Ca2+) mobilizing agents, cyclic adenosine diphosphate-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP+). Recently, the expression of CD38 in astrocytes has been proposed to play a key role in glutamate-mediated bi-directional astrocyte-to-neuron communication. Our data suggest that the glutamate clearance capability of astrocytes is impaired in neuroinflammatory disorders such as HAD and the dysregulation of CD38 expression in astrocyte contributes to this impairment. Primary human astrocytes were cultured and treated with pro-inflammatory cytokine IL-1σ or HIV-1gp120, or infected by HIV-1ADA. The glutamate uptake ability of the cells was determined using the Amplex Red Glutamic Acid/Glutamate Oxidase Assay. The astrocyte CD38 and EAAT2 levels were tested by real-time PCR, immunocytochemical and immunohistochemical analyses and confocal microscopy. Our results show that either IL-1σ or HIV-1gp120, or HIV-1ADA induced astrocytic activation, and both IL-1σ and HIV-1gp120 significantly upregulated astrocyte CD38 levels. Importantly, our data confirmed that IL-1σ time-dependently downregulated astrocyte glutamate uptake, which was completely reversed by co-incubation with 8-Br-cADPR, a specific cADPR-antagonist. In contrast, 3-Deaza-cADPR, a non-hydrolysable analog of cADPR downregulated astrocyte glutamate uptake in a time-dependent manner. Downregulation of astrocytic EAAT2 expression induced by IL-1σ was also reversed by co-incubation with 8-Br-cADPR. Our data provide new evidence that astrocyte activation is accompanied by impaired glutamate uptake in the context of CD38 dysregulation, thereby contributing to the pathogenesis of neuroinflammatory disorders such as HAD. Our data implicate that the dysregulation of astrocyte CD38/cADPR signaling pathway plays an important role in the maintenance of synaptic glutamate homeostasis.