Monocyte / Macrophage Traffic Plays an Essential Role in HIV and SIV Pathogenesis

Author: Jennifer Helen Campbell

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Boston College

The Graduate School of Arts and Sciences

Department of Biology

MONOCYTE / MACROPHAGE TRAFFIC
PLAYS AN ESSENTIAL ROLE IN
HIV AND SIV PATHOGENESIS

a dissertation

by

JENNIFER HELEN CAMPBELL

submitted in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

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CHAPTER II

Minocycline Inhibition of Monocyte Activation

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACM</td>
<td>Astrocyte Conditioned Media</td>
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<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency Syndrome</td>
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<tr>
<td>ART</td>
<td>Antiretroviral therapy</td>
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<tr>
<td>BBB</td>
<td>Blood Brain Barrier</td>
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<tr>
<td>BrdU</td>
<td>5'-bromo-2'deoxuryridine</td>
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<tr>
<td>cART</td>
<td>Combination Antiretroviral Therapy</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>CPE</td>
<td>CNS penetration-effectiveness</td>
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<tr>
<td>Cr</td>
<td>Creatine</td>
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<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
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<tr>
<td>CCL</td>
<td>C-C chemokine ligand</td>
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<td>CCR</td>
<td>C-C chemokine receptor</td>
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<td>CXCL</td>
<td>C-X-C chemokine ligand</td>
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<tr>
<td>CXCR</td>
<td>C-X-C chemokine receptor</td>
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<tr>
<td>CTL</td>
<td>Cytotoxic T Lymphocyte</td>
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<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
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<td>DPI</td>
<td>Days post infection</td>
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<td>GALT</td>
<td>Gut associated lymphoid tissue</td>
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<td>GI</td>
<td>Gastrointestinal</td>
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<td>GP</td>
<td>Envelope glycoprotein</td>
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<tr>
<td>1H MRS</td>
<td>Proton Magnetic Resonance Spectroscopy</td>
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<tr>
<td>HAART</td>
<td>Highly Active Antiretroviral Therapy</td>
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<td>HAD</td>
<td>HIV-Associated Dementia</td>
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<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
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<td>HIVE</td>
<td>HIV Encephalitis</td>
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<tr>
<td>HPSC</td>
<td>Hematopoietic stem cells</td>
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<td>HTLV</td>
<td>Human T-cell leukemia virus</td>
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<tr>
<td>ICAM</td>
<td>Intercellular Adhesion Molecule</td>
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<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IV</td>
<td>Intravenous</td>
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<tr>
<td>LFA</td>
<td>Lymphocyte Function Antigen</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>MAdCAM</td>
<td>Mucosal Vascular Addressin Molecule</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-Activated Protein Kinase</td>
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<tr>
<td>MCP</td>
<td>Monocyte Chemotactic Protein</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>M-CSF</td>
<td>Macrophage-Colony Stimulating Factor</td>
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<td>MFI</td>
<td>Median Fluorescence Intensity</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>NAA</td>
<td>n-acetylaspartate + n-acetylaspartylglutamate</td>
</tr>
<tr>
<td>NCI</td>
<td>Normalized Cell Index</td>
</tr>
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<td>NF</td>
<td>Nuclear Factor</td>
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<tr>
<td>NNRTI</td>
<td>Non-Nucleoside Reverse Transcriptase Inhibitor</td>
</tr>
<tr>
<td>NRTI</td>
<td>Nucleoside Reverse Transcriptase Inhibitor</td>
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<tr>
<td>OI</td>
<td>Opportunistic Infection</td>
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<tr>
<td>PAMP</td>
<td>Pathogen Associated Molecular Pattern</td>
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<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cell</td>
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<tr>
<td>PMA</td>
<td>Phorbol 12- myristate 13- acetate</td>
</tr>
<tr>
<td>sCD14</td>
<td>soluble CD14</td>
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<tr>
<td>sCD163</td>
<td>soluble CD163</td>
</tr>
<tr>
<td>SDF</td>
<td>Stromal Cell-Derived Factor</td>
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<tr>
<td>SIV</td>
<td>Simian Immunodeficiency Virus</td>
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<tr>
<td>SIVE</td>
<td>SIV Encephalitis</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-Like Receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular Cell Adhesion Molecule</td>
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<tr>
<td>VLA</td>
<td>Very Late Antigen</td>
</tr>
<tr>
<td>WPI</td>
<td>Weeks post infection</td>
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CHAPTER I

Introduction
ABSTRACT

MONOCYTE/MACROPHAGE TRAFFIC PLAYS AN ESSENTIAL ROLE IN HIV AND SIV PATHOGENESIS

Author: Jennifer H. Campbell

Advisor: Kenneth C. Williams, Ph.D.

Elucidating the mechanisms through which viral infection and persistence in CNS occurs is critical to understanding the development and progression of neurological disease. To date, no study has demonstrated that monocyte traffic in HIV and SIV infection directly results in neuronal injury. The central hypothesis in this thesis is that continuous trafficking of monocytes into tissues is essential for pathogenesis with viral infection. In the dissertation work presented here, two studies addressed this hypothesis. In Chapter 2, experiments examining the role of peripheral monocyte activation in the development of neuroAIDS using the tetracycline antibiotic minocycline will be described. We hypothesized that decreased monocyte activation with minocycline treatment would play a neuroprotective role in the context of rapid SIV infection with a high incidence of SIV encephalitis (SIVE). We observed a reversal of neuronal injury within days of minocycline treatment that correlated with loss of monocyte activation. From these findings we concluded that decreased activation of
monocytes results in lower CNS traffic. However this effect may have occurred due to lower plasma virus, decreased SIV infection of monocytes, or the ability of minocycline to cross the BBB and modulate changes within the CNS directly.

In Chapter 3 of this thesis, we hypothesized that continuous traffic of activated monocytes from the periphery into the CNS is required for neuronal injury with AIDS, and that by effectively stopping monocyte accumulation, CNS pathology can be blocked or reversed. We also hypothesized that monocyte trafficking is necessary for the seeding of brain and small intestine with cell-associated virus. In order to test these hypotheses, we utilized the anti-α4 blocking antibody natalizumab (Tysabri; Biogen Idec), which selectively binds to the α4 subunit of α4β1 (VLA-4) and α4β7 integrins, preventing the interaction between α4 and its various ligands. To address the first hypothesis, natalizumab was administered after four weeks of infection once significant neuronal damage had already occurred. We found that preventing cell traffic with natalizumab is sufficient to stabilize neuronal injury and loss, demonstrating conclusively that stopping monocyte traffic stabilizes CNS disease. To address the second hypothesis, rhesus macaques were treated with natalizumab on the day of SIV infection. Natalizumab treatment completely blocked SIV infection in the brain, and virus traffic to the small intestine was significantly suppressed. Overall, these studies demonstrate that continuous traffic of monocytes is required for neuronal injury and the formation of CNS lesions, and that early trafficking of leukocytes is critical for seeding of the CNS and contributes to seeding of the small intestine with virus.
I. Human Immunodeficiency Virus

A. An annotated history of HIV and AIDS

The Human Immunodeficiency Virus (HIV) pandemic continues to be a monumental challenge for research, public health, and medicine. Since HIV was first identified more than thirty years ago, it is estimated that ~ 59 million people have become infected, with over 25 million deaths resulting from complications of the disease [1]. Retrospective sequence analyses have demonstrated that an individual in Africa was infected with HIV as early as 1959 [2], however the virus quietly spread around the world from the 1960’s until 1981, when several cases of *Pneumocystis carinii* pneumonia and Kaposi’s Sarcoma were reported across the United States in a short period of time [3,4]. The severe lymphopenia and immune suppression observed in these individuals was described as acquired immune deficiency syndrome (AIDS) by the Centers for Disease Control before the end of 1981 [5]. A few years later, the first human retrovirus was isolated from patients with AIDS [6], prompting intense research into the etiology and role of viral infection in immune deterioration. The virus was first termed human T-cell leukemia virus (HTLV) – I, and HTLV II and III were also described before researchers demonstrated that this retrovirus caused AIDS [7,8], and appropriately named the virus HIV [9]. Around the same time, a systematic description of central nervous system (CNS) complications with AIDS were initially described as subacute encephalitis [10], which was later termed HIV-
associated dementia (HAD) [11]. In 1987, based on promising changes in viral load and CD4+ T cell count, azidothymidine (AZT) was rapidly approved as the first anti-retroviral drug for the treatment of AIDS [12], however it was soon evident that there was little improvement in the time to death with this medication. Nearly a decade later, highly active antiretroviral therapy (HAART) was introduced and immediately resulted in improved clinical outcomes relative to patients on monotherapies [13-15]. Since then, great strides have been made in the treatment of HIV. There are currently 27 antiretroviral drugs on the market in the United States [16], that when used in combinations of 3 or more (combination antiretroviral therapy, cART), significantly improve the longevity of HIV-infected individuals [17,18].

B. HIV Transmission and clinical features of HIV infection

Mucosal exposure remains the primary route of HIV transmission, accounting for approximately 80% of new infections worldwide [19]. The other 20% result from percutaneous/intravenous infection or mother to infant transmission [20]. There are several well-characterized periods in the course of HIV infection: (1) an early asymptomatic phase of uncontrolled viral spread around the body until ~2 weeks post infection (wpi), (2) acute infection and viremia within 2-4 wpi, (3) a period of virologic latency lasting an average of 10 years, and (4) progression to AIDS, which occurs in the absence of cART, or upon treatment failure [21] (Figure 1.1).

Viral infection begins with attachment of the envelope glycoprotein gp120 portion of the gp120/gp41 heterotrimeric protein to the CD4 receptor on the host
cell [22,23]. Once the virus has bound to the host cell, conformational changes in gp120 allow it to form additional interactions with co-receptors C-C chemokine receptor type 5 (CCR5) and/or C-X-C chemokine receptor type 4 (CXCR4). During acute disease, virions initially target leukocytes expressing CD4 and CCR5, including monocytes, macrophages, dendritic cells, and T cells [19]. Over time, HIV evolves to also utilize CXCR4 for infection of target cells [24].

**Figure 1.1 Pathogenesis of HIV infection.** A schematic diagram of the pathogenic effects that occur from initial infection with HIV through the development of clinical disease. (Modified from Pantaleo and Fauci, Current Opinion in Immunology 1994)
Binding of gp120 to its co-receptor results in insertion of the gp41 fusion peptide into the host cell membrane [25]. This allows the viral core, including the genome, reverse transcriptase, and integrase, to be released into the cytoplasm [26]. Uncoating of the viral core during transport from the cytoplasm to the nucleus facilitates the formation of the pre-integration complex [27,28]. Once in the nucleus, the integrase protein incorporates the viral genome into the host cellular DNA, where it can now be used as a template for the production of new virions [29]. Integration occurs within hours of infection, and once integrated, the provirus persists throughout the life of the host cell [30]. Initiation of replication requires interaction between the transcriptional activator protein tat and the transactivation response (TAR) RNA element [31], which together promote recruitment of the positive transcription elongation factor P-TEFb [32]. Direct binding of tat to P-TEFb results in phosphorylation of RNA polymerase [33], marking the transition from initiation of HIV replication to transcript elongation [34]. With the help of other viral and cellular proteins, HIV RNA is spliced, transported, translated, and assembled into virions [35]. Finally, particles bud from the cell surface and HIV protease activity renders newly formed virions infectious, completing a full cycle of HIV replication in 1-2 days [36]. During reverse transcription HIV replicates and mutates frequently, which, over time leads to high levels of variation in the viral sequence and ultimately diminishes the potential of current antiretroviral interventions [37]. Based on mathematical models, activated CD4+ T lymphocytes produce the majority of viral particles, however these cells are short-lived, surviving for approximately a day after
becoming infected [38]. In contrast, infection with HIV is not lytic to monocytes and macrophages [39], and allows these cells to produce virions and harbor provirus long-term [40,41].

Regardless of the route of transmission, productive viral replication typically occurs first in the draining lymph nodes, and from there infected leukocytes travel through the bloodstream to the gut, brain, spleen, liver, lungs, and bone marrow, which promotes an exponential expansion of infected cells throughout the body [42] (Figure 1.2).

**Figure 1.2** HIV-1 transmission and acute infection. Preferential CCR5 HIV transmission is illustrated along with potential roles for macrophages and dendritic cells. Virus-host cell interactions in the initial days of infection have been elucidated primarily in SIV-infected rhesus macaques. (Adapted from Shaw and Hunter, Cold Spring Harbor Perspectives in Medicine 2012)
The gut-associated lymphoid tissue (GALT) harbors <90% of the body’s lymphocytes, macrophages, and dendritic cells [43,44], and the majority of these cells express the viral co-receptor CCR5 [45,46]. During the early asymptomatic phase of disease, this pool of activated leukocytes is preferentially infected and provides HIV with an ideal environment for viral replication [47,48], leading to severe CD4+ T lymphocyte depletion in the gastrointestinal (GI) tract [49,50]. Changes in the intestinal epithelium are also evident soon after exposure to HIV, including breakdown of the mucosa that prompts translocation of lipopolysaccharide (LPS) and other bacterial products into the blood [51,52]. Increased production of inflammatory cytokines and chemokines including interferon alpha (IFN-α), interferon gamma (IFN-γ), interleukin 15 (IL-15), tumor necrosis factor (TNF)-α, monocyte chemotactic protein (MCP)-1, IL-6, IL-8, and CXCL10, as well as the expansion of CD4+ T lymphocytes in the blood occur within the first days to weeks after infection, and aid in further dissemination of virus throughout the body [53,54]. This results in high plasma viremia (≥ 10^7 copies of HIV RNA per mL of blood) [55,56], which is frequently accompanied by the earliest clinical signs of HIV infection, such as fever, fatigue, rash, sore throat, swollen lymph nodes, and flu-like symptoms [57-59]. Intriguingly, GI symptoms are not typically reported during this time [60]. With peak viremia, HIV-specific cytotoxic CD8+ T lymphocytes (CTLs) start to develop and contribute to the initial decline in plasma viral load [61,62]. About 3 months after exposure, the CTL response and neutralizing antibodies contribute to the control of viral replication [63]. At this point, an individuals' set-point for viremia is
established, beginning the asymptomatic phase of disease [64]. Over time, HIV progressively destroys the immune system, leading to the transient depletion of the CD4+ T cells necessary to control the types of opportunistic infections (OIs) that are typically non-pathogenic to healthy individuals. Once numbers of CD4+ T lymphocytes fall below ~200 cells per microliter of blood, the immune system can no longer fight adventitious infections or the virus itself, signaling the onset of AIDS. In the absence of effective memory cells, there is recrudescence of plasma viremia and development of OIs, resulting in progression to AIDS and ultimately death of the infected individual (Figure 1.3).

![Figure 1.3 Time course of typical HIV. Patterns of CD4+ T cell decline and viremia vary greatly from one patient to another. (Modified from Coffin and Swanstrom, Cold Spring Harbor Perspectives in Medicine 2013)]
C. HIV infection in the cART era

Fortunately, the advent of cART has greatly improved the health of HIV infected individuals. When an appropriate drug regimen is found and if adherence is good, patients can exhibit improved immune function and suppress plasma virus to levels below the threshold of clinical detection (50 copies of HIV RNA/mL of plasma) for several years [65]. Yet, despite living longer with HIV infection, individuals are at a much higher risk of developing non-AIDS related cancers, kidney, liver, cardiovascular, gastrointestinal, and neurologic diseases than their age-matched HIV-negative counterparts [66]. The reasons for this increased susceptibility are not fully understood, however it appears that chronic inflammation and immune dysfunction with HIV infection are likely at the root of these pathologies. As more than 33 million people around the world are currently living with HIV, it is evident that cART alone is not sufficient to rid the body of virus [67]. Therefore, it is imperative that predictive biological markers, underlying causes, and ultimately improved therapies for these increasingly prevalent co-morbidities be more closely explored.

Gastrointestinal manifestations of HIV infection with cART

Even when cART is initiated very early in infection there is irreversible damage to the gut, as lymphocytes in the small intestine get depleted by 2 wpi [68,69] before HIV is detected in the blood, and because it is difficult for most drugs to cross into the GI tract [70]. The GALT remains CD4+ T lymphocyte depleted throughout the course of HIV infection [49], and residual translocation of LPS across the gut mucosa is positively correlated with ongoing levels of
peripheral immune activation and inefficient CD4+ T cell recovery in cART treated individuals [56,71-73]. In addition, after almost a decade of cART therapy, concentrations of HIV DNA in the gut remain unchanged despite complete suppression of virus in plasma [74,75]. As a result, GI symptoms and complications such as dysbiosis, diarrhea, nutrient malabsorption, weight loss, abdominal pain, and intestinal bleeding persist in the majority of cART treated patients [76-78]. Persistent inflammation and immune dysfunction may be the root of these pathologies, and events occurring during the acute phase of HIV infection are likely critical for determining the overall course and severity of disease. Notably, chronic immune activation has also been repeatedly associated with monocyte expansion, disease progression, and the severity of neurological complications in HIV+ patients [79-83].

**Neurological manifestations of HIV infection with cART**

Prior to widespread antiretroviral use, many HIV-infected individuals were developing HAD and corresponding neuropathology, or HIV encephalitis (HIVE). In the cART era the incidence of HAD has declined, however the prevalence of milder forms of HIV-associated neurocognitive disorders (HANDs) continues to increase [84]. Data published within the last year indicates that almost a third of HIV+ individuals with long-term viral suppression report significant deficits in cognitive and motor domains, and that more than 50% of patients meet formal criteria for HAND [85,86]. HAND diagnoses are based on deficiencies in motor skills, attention, and memory performance. These disorders fall along a spectrum of severity from asymptomatic neurocognitive impairment (ANI),
through mild neurocognitive disorder (MND), to the most severe CNS involvement with HAD [87]. Importantly, development of HANDs are repeatedly associated with higher rates of AIDS-defining illnesses [88] as well as increased mortality [89] (Figure 1.4). Some reports indicate that cognitive impairment is improved if individuals begin treatment early in the course of HIV infection [90-92].

![Figure 1.4](image_url)

**Figure 1.4 Changes in the prevalence of HIV-associated brain disorders and survival in the eras pre-cART and post-cART.** The predominant transition since the introduction of potent combination antiretroviral therapies (cART) has been a replacement of HAD and MND by a milder, chronic asymptomatic neurocognitive impairment (ANI), with which individuals can live for many years. The impact of a neurocognitive disorder that is severe in a patient who lives for a relatively short period of time might actually be less than the impact of a milder cognitive disorder in an individual who lives for decades. Question marks denote uncertainty as to the longevity of cART-treated individuals. (Ellis et al., Nature Reviews Neuroscience 2007)

On the other hand, conflicting studies have demonstrated that even in patients initiating therapy during acute infection, immune activation is still evident in the CNS after more than four years of effective cART [93], with brain tissue
expression of more than 100 genes with roles in adaptive immunity, interferon signaling, cell cycle and myelin pathways remaining dysregulated [94]. Moreover, despite effective cART regimens, undetectable viral loads, and having no neurological symptoms at the time of death, 80-90% of HIV+ individuals have evidence of neuronal injury and loss, myelin pallor, and elevated numbers of activated and productively infected macrophages in the CNS upon examination at autopsy [95,96]. Thus, while there remain no established cART guidelines for HAND, it is evident that future treatment will require adjunctive therapies that target macrophage infection and impede the cascade of inflammatory events that result from and perpetuate HIV infection in the CNS.

II. Simian Immunodeficiency Virus

A. SIV and emergence of AIDS in SIV infected macaques

Soon after the HIV epidemic began, simian immunodeficiency virus (SIV) was fortuitously transmitted from sooty mangabys (Cercocebus atys; SIVsm) to rhesus macaques (Macaca mulatta; SIVmac) at multiple primate research centers in the United States, resulting in AIDS-like disease [97,98]. One feature that initially distinguished the HIV and SIV lentiviruses from other retroviruses was the number of genes in their viral genomes [99]. Retroviruses are typically capable of replication with just the \textit{env}, \textit{gag}, and \textit{pol} genes, however HIV and SIV also contain regulatory (\textit{tat}, \textit{rev}, and \textit{nef}) and auxiliary (\textit{vif}, \textit{vpr}, and \textit{vpu} or \textit{vpx}) genes, rendering them much more virulent and pathogenic than most retroviruses [100,101]. HIV type 1 (HIV-1) was the first lentivirus identified in
humans, and is responsible for the vast majority of infections worldwide [102].
HIV type 2 (HIV-2) is less easily transmitted and less pathogenic than HIV-1, and is predominantly found in western Africa [103]. HIV-2 is most closely related to SIVsm and SIVmac, while HIV-1 likely evolved from chimpanzee SIV (SIVcpz) [104]. Sooty mangabeys and African green monkeys (Chlorocebus sabaeus) are natural hosts to SIV, and thus have low levels of immune activation with viral infection and do not develop AIDS [105,106]. On the other hand, Asian primates such as the rhesus, pigtailed (Macaca nemestrina), and cynomolgus (Macaca fascicularis) macaques are unnatural hosts of SIV, with exposure to the virus in these species resulting in a disease course very similar to that seen in humans with HIV [107]. Because HIV and SIV are closely related in their genetic structure and pathogenicity both viruses induce persistent infection, resulting in immune deficiency, progressive wasting, neurodegeneration, opportunistic infections, and ultimately death [108,109]. Since the discovery of HIV, experiments studying infection in Asian primates have consistently corroborated what is seen with HIV-1 infection, and have greatly expanded our understanding of HIV pathogenesis.

B. Utility of SIV infected macaques in understanding acute HIV infection

Because there is significant viral replication and depletion of immune cells early in HIV infection, it is critical that the effects of HIV pathogenesis on the GALT, as well as the role of aberrant GI tract activation in subsequent neuropathology, be more closely examined. Yet, it is difficult to monitor changes
occurring during acute disease as seroconversion can happen without patients even knowing that they are infected. It is also important to consider that if recently identified as HIV-positive, someone will be dealing not only with the physical symptoms of acute infection, but with the emotional stresses that come along with this type of diagnosis, making it challenging to obtain essential gut biopsies or to study CNS effects. For this reason, SIV infected macaque models have been indispensable to research progress, providing important insights into the early stages of HIV infection and how these events affect overall outcomes [110]. Unlike human patients, tissues can be obtained at any point SIV infection, allowing researchers to characterize the cellular components and their roles in various tissues at different periods throughout infection, thus providing highly useful model of early HIV infection in the CNS, the formation of viral reservoirs, and the pathogenesis of disease.

High numbers of SIV-infected monocytes and macrophages populate the bone marrow by 3 days post infection (dpi) and persist throughout acute infection, whereas low-to-no infected CD3+ T lymphocytes are evident in the bone marrow during this time [111]. In general, there are higher numbers of monocytes in the blood of SIV infected macaques than in HIV infected humans, and as a primary target of SIV, monocytes and macrophages are an important contributor to the dissemination of virus in tissues during early infection [112]. Viral RNA is found in T lymphocytes and macrophages resident to the small intestine by 2-3 dpi [113-115], with significantly increased production of proinflammatory cytokines in the gut evident by 6-10 dpi [116]. Similar to HIV-
infected individuals, the majority of CD4+ T cells are massively infected and depleted from the intestinal compartment within weeks of exposure to SIV [50]. Early damage to the gut mucosa results in increased translocation of microbial products into the circulation, driving immune cell activation in the periphery. From 2-3 wpi, mucosal tissues are responsible for the generation of SIV-specific CTL responses [117]. Comparable to what is seen in humans, SIV infected macrophages can be detected in the GI tract throughout the course of disease [118], while numbers of macrophages in gut tissues predominate during acute infection and with progression to AIDS [119].

C. A SIVmac251 infected, CD8 lymphocyte depleted rhesus macaque model of rapid neuroAIDS

Intravenous (iv) inoculation of rhesus macaques with SIVmac251 typically results in progression to AIDS within 1-3 years, with 30-40% of animals developing SIV encephalitis (SIVE) [120]. Treating SIV infected macaques with a chimeric humanized mouse anti-CD8 monoclonal antibody on 6, 8, and 12 days post infection (dpi) results in depletion of lymphocytes and natural killer cells and leads to a significant elevation in plasma viremia by 12 dpi, providing an excellent model of rapid CNS disease [121,122]. Animals that are depleted long-term (> 28 days of CD8 depletion) develop AIDS within 3-4 months post infection, and SIVE is evident in more than 75% of macaques [120,123]. SIV infected rhesus macaques develop cognitive, motor, and behavioral deficits with CNS infection that resemble neuropsychological changes observed in HIV+ individuals [124,125]. Moreover, accompanying neuropathology is analogous to what was
observed pre-ART in HIV infected patients with HIVE, including cerebral atrophy, neuronal damage, microglial nodules, multinucleated giant cells, productive infection in macrophages, and white matter disease, though more acute and severe in macaques [120,126]. The rapid progression and comparable nature of CNS disease in SIVmac251 infected, CD8 depleted rhesus macaques provides an ideal model for the identification and examination of therapeutics that could be beneficial for the treatment of HIV infection and the targeting of viral reservoirs in the brain.

III. Immune regulation in the Central Nervous System

A. The Central Nervous System as an immune privileged site

The CNS has long been considered an immune privileged site [127]. It is not thought to have a lymphatic system, and is protected by both an endothelial blood-brain barrier (BBB) and an epithelial blood-cerebrospinal fluid (CSF) barrier. Interactions between tight junctional proteins expressed by endothelial cells and astrocytic foot processes cover the entire surface of brain capillaries, regulating and restricting movement of cells and macromolecules across the BBB. Under normal conditions, the number of cells traversing the BBB and entering the CNS is very low [128,129], however there are four types of mononuclear phagocyte lineage cells in the CNS that are capable of antigen presentation, namely parenchymal microglia, meningeal macrophages, choroid plexus macrophages, and perivascular macrophages [130]. Microglia, the resident CNS macrophages, are yolk sac derived and arrive in the brain during
embryonic development and for a brief time postnatally [131]. Microglia are rarely turned over by cells from the bone marrow [132], though some research suggests they may stimulate B cell and T cell populations under certain conditions [133]. In response to activation, microglia undergo morphological changes, as well as produce proinflammatory chemokines, cytokines, and other soluble mediators that negatively affect neuronal function [134]. Perivascular macrophages are distinct from microglia and other CNS mononuclear lineage cells in that they are phagocytic in the absence of inflammation [135]. Due to the way perivascular macrophages are strategically situated between glial and endothelial basement membranes, they are capable of responding to changes within the brain and in the periphery [136]. These cells constitutively express receptors for antigen recognition (DC-SIGN, mannose receptor) [137], antigen presentation (major histocompatibility complex (MHC) II) [138], and several receptors for pathogen-associated molecular patterns (PAMPs). Importantly, perivascular macrophages and very highly activated microglia [139] express the haptoglobin-hemoglobin complex receptor CD163, allowing these cells to scavenge iron complexes in the blood [135].

B. CNS Perivascular Macrophages are turned over by bone marrow derived cells

Early studies in rats approximated that every three months, 30% of perivascular macrophages are repopulated by bone marrow derived monocytes migrating into the CNS from the circulation [140]. Moreover, in response to inflammation, there is increased release of monocytes out of the bone marrow
In the bone marrow, CD34+ myeloid progenitor cells undergo three stages of differentiation (monoblast, promonocyte, monocyte) to become mature CD14+ monocytes [143]. As part of normal immune surveillance, CD14+ monocytes continuously leave the bone marrow, circulate in the blood for 3-5 days, and enter tissues where they differentiate into macrophages and myeloid dendritic cells [144]. By transplanting autologous EGFP-expressing bone marrow progenitor cells into healthy rhesus macaques, our lab conclusively established that CD163+ perivascular macrophages are slowly turned over by bone marrow derived monocytes [145]. Four years after transplantation, a significant number of blood monocytes remain EGFP+, and all EGFP+ cells in the CNS are CD163+ perivascular macrophages found in the cerebrum, cerebellum, and choroid plexus. Furthermore, there was a direct relationship between the percent of EGFP+ monocytes in the blood and the percent of EGFP+ perivascular macrophages in the CNS [145], indicating that the rate of perivascular macrophage turnover is dictated events including viral infection and heightened immune activation in the periphery [146].

C. Relationship between myeloid cell turnover and CNS disease progression

Similar to what was achieved through the transplantation EGFP-transduced CD34+ cells in rhesus macaques, the thymidine analog 5’-bromo-2’-deoxyuridine (BrdU) has been used to precisely quantify the kinetics of monocyte traffic out of the bone marrow, through the blood, and into tissues [147].
Monocytes leave the bone marrow immediately after completing S-phase of the cell cycle, and do not divide again in the blood. BrdU is incorporated into DNA during S-phase, and is thus a specific marker for myeloid cells that have emigrated from the bone marrow. Recent studies utilizing BrdU during SIV infection have recapitulated what was previously seen in response to inflammation in rodents. In response to SIV infection in rhesus macaques, there is increased egress of monocytes from the bone marrow, and these monocytes remain in the circulation for a shorter amount of time before entering the CNS and other tissues [147-150]. Importantly, the magnitude of BrdU+ monocyte turnover is a more accurate indicator of the rate of progression to AIDS than plasma viral load or CD4+ T cell count [148]. Furthermore, the degree of BrdU+ monocyte expansion directly predicts the severity of SIVE as early as 8, and consistently by 27 dpi [150], underscoring the importance of monocyte dynamics during acute infection to overall disease outcomes.

IV. Dynamics of monocyte infection, activation, and traffic during HIV and SIV infection

A. Monocyte subsets under normal and inflammatory conditions

Phenotypically independent subpopulations of monocytes exist in the blood, and can be delineated based on their size, granularity, expression of surface markers, and the types of chemokines and cytokines that they produce [151]. The LPS receptor CD14 is expressed to some degree by all circulating monocytes. With the help of the toll-like receptor 4 (TLR4), CD14 binds to LPS,
resulting in cleavage of CD14 from the cell and release of both LPS and CD14 (soluble CD14, sCD14) into the circulation. In healthy people, CD14+CD16- cells make up 90-95% of blood monocytes. These monocytes express FcγRI (CD64) and the MCP-1 receptor C-C chemokine receptor type 2 (CCR2), and release cytokines such as IFN-β and IL-10 [152] (Figure 1.5). The percentage of peripheral blood monocytes expressing the CD16 (Fc receptor for immunoglobulin G; FcγRIII) receptor is low under normal conditions, but expands significantly in several inflammatory states such as atherosclerosis, sepsis, and rheumatoid arthritis [153-155]. In response to peripheral immune activation with HIV and SIV infection, there is a bi-phasic expansion in the number of CD16+ monocytes in the blood, occurring first with high plasma viral load during acute infection, and again prior to and with the development of AIDS, when these cells can represent up to 40% of the total circulating monocyte population [156-158]. The CD16 receptor mediates functions such as removal of immune complexes and antibody-dependent cell-mediated cytotoxicity (ADCC) [159]. As a result, CD14lo/+CD16+ monocytes have a more mature, macrophage-like morphology, and are more phagocytic and efficient at antigen presentation than the CD14+CD16- subpopulation [151,160]. CD14lo/+CD16+ monocytes strongly express CD49d, CX3CR1, and HLA-DR, and produce high levels of inflammatory chemokines and cytokines such as IFNγ, IL-1β, IL-6, and TNFα [161-164]. In addition, CD14lo/+CD16+ monocytes are immunophenotypically similar to perivascular macrophages in the CNS, with the majority CD16+ monocytes expressing the haptoglobin-hemoglobin scavenger receptor CD163, and
perivascular macrophages expressing CD14 and CD16 [165,166]. Upon activation, monocytes and perivascular macrophages can actively shed CD163 from the cell surface as soluble CD163 (sCD163). Levels of sCD163 are elevated with increased expansion of activated CD14+CD16+ monocytes [167]. There is also evidence for LPS induction of CD163 shedding [168]. While classical CD14+CD16- monocytes are relatively resistant to infection with HIV and SIV, CD14+CD16+ monocytes up-regulate expression of the viral co-receptor CCR5, rendering these activated cells more susceptible to viral infection [169,170]. CD16+ cells may also be infected through phagocytosis of IgG coated virion immune complexes [171]. Monocytes restrict viral replication, and the viral life cycle only goes to completion after monocytes differentiate into macrophages [172].

![Figure 1.5 Monocyte populations in normal and SIV infection states.](image)

Subpopulations of circulating monocytes display distinct phenotypic characteristics and immune functions.
B. Transmigration of monocytes across the blood brain barrier (BBB)

Interestingly, CD163 expression improves the adherence of activated monocytes endothelial cells prior to migration into the CNS [173]. When CD16+ monocytes migrate across the BBB, they can acquire a macrophage phenotype, which stimulates productive viral replication. HIV and SIV infection and increased release of chemokines and cytokines (IL-1β, TNFα, IFNα) on both sides of the activated brain microvascular endothelium recruit circulating CD16+ monocytes to the BBB and induce their transmigration into the CNS [174]. The process of monocyte extravasation from the circulation into the brain involves several players including selectins, integrins, and cell adhesion molecules [175]. In both physiological and pathological settings, chemokines activate blood vessel endothelial cells to up-regulate expression of E-selectin, L-selectin, and P-selectin molecules [176]. CD16+ monocytes engage these selectin molecules to initiate a rolling movement along the surface of the endothelium [177,178]. Firm arrest to the blood vessel wall is triggered by the interaction of integrins including lymphocyte function-associated antigen 1 (LFA-1), very late antigen 4 (VLA-4 / α4β1), and α4β7 on monocytes with cognate receptors intercellular adhesion molecule 1 (ICAM1), intercellular adhesion molecule 2 (ICAM2), mucosal vascular addressin cell-adhesion molecule 1 (MAdCAM1), and vascular cell-adhesion molecule 1 (VCAM1) expressed by the endothelium [179-181]. Lastly, intraluminal crawling and diapedesis through transcellular and paracellular routes are mediated by the sequential interaction of junctional proteins activated
leukocyte cell adhesion molecule (ALCAM), junctional adhesion molecule A (JAM-A), platelet endothelial cell adhesion molecule-1 (PECAM-1), and CD99, that are expressed on the endothelium and the cell surface, allowing monocytes to complete extravasation into the brain [182,183] (Figure 1.6).

Figure 1.6  Monocyte transmigration across the blood-brain barrier. Monocyte transmigration is a multistep process, (A) consisting of capture and rolling, chemokine-mediated activation of the monocyte and subsequent firm arrest, intravascular crawling with the formation of focal adhesions to the vasculature, and ultimately, diapedesis. (B) An enlarged image illustrating some of the homotypic monocyte-endothelial cell interactions, which mediate diapedesis. (Williams et al., Journal of Leukocyte Biology 2012)
Several proinflammatory chemokines and cytokines have been implicated in the traffic of monocytes across the BBB, including MCP-1, CX3C-chemokine ligand 1 (CX3CL1, also known as fractalkine), CXCL10, and CCL3 (also known as macrophage inflammatory protein 1α, MIP-1α). Production of these inflammatory mediators stimulates up-regulation of adhesion molecules by endothelial cells [184], allowing for increased binding and transmigration of activated monocytes into the CNS [185]. Levels of CCL2, CX3CL1, and CXCL10 in CSF are used as biomarkers of HIV and SIV infection in the CNS, as these chemokines correlate with the severity of neuropathology. By the same token, increased recruitment of CD16+ monocytes across the BBB is associated with more rapid progression to SIVE [150], and high numbers of CD14+CD16+ cells are observed in the brains of HIV-positive individuals with HIVE [186].

V. HIV and SIV infection of the CNS

A. Resident myeloid lineage cells are susceptible to infection

While parenchymal microglia, meningeal macrophages, choroid plexus macrophages, and perivascular macrophages all express viral co-receptors and can be infected [187,188], perivascular macrophages are a consistent target for the HIV and SIV in the CNS [135,189,190]. There are increased numbers of perivascular macrophages in the brain with HIV and SIV infection [191-193], and productively infected CD163+ cells are present throughout the CNS during acute disease and with progression to AIDS [135,194,195]. In humans and rhesus macaques, expression of CD16+ and CD68+ on activated resident macrophages
and microglia is also up-regulated during CNS viral infection [196-198]. In addition, HIV and SIV infection of CNS perivascular macrophages and microglia stimulates the production of macrophage colony stimulating factor (M-CSF), a pro-survival cytokine that protects these cells from apoptosis [199]. Release of pro-inflammatory chemokines and cytokines by macrophages and microglia serves to increase M-CSF production even further, which contributes to up-regulation of CD4 and CCR5 expression, viral infection, and viral persistence that is observed in the brain throughout infection [200,201].

B. Neuronal damage with HIV and SIV infection

HIV and SIV do not directly infect neurons, however increased production of proinflammatory chemokines, cytokines and other excitotoxictoxic factors by infected macrophages and microglia all contribute to neuronal injury and death [202-205]. Release of inflammatory mediators such as stromal cell-derived factor (SDF)-1, TNF-α, MCP-1, IL-1β, and IL-6 in response to inflammation in the CNS results in the activation of macrophages and microglia in the surrounding environment [206,207]. Several other viral and cellular factors are also released from macrophages and microglia in the presence of brain inflammation, including viral proteins gp120, gp41, nef, tat, and vpr, platelet activating factor, arachidonic acid, quinolinic acid, and nitric oxide, which accelerate damage to neurons and result in neuronal apoptosis [202,203,208-213]. These events lead to reduced neuronal dendrites and axonal damage, as well as astrocytosis, myelin pallor, and increased BBB permeability, which are linked to the severity of neuropathology with HIV and SIV infection [214-216].
C. HIV and SIV enter the CNS very early in disease

It is generally assumed that virus is carried into the brain within infected monocytes, but this has not been directly demonstrated. Free virus may cross the BBB through endothelial cells [217] or through the choroid plexus [218,219]. The sole evidence that virus enters the brain soon after iatrogenic exposure in humans came from an unfortunate case of inadvertent inoculation with HIV [195]. A seronegative man was admitted to the hospital with complaints of left hip pain and fever. To determine whether he had a bone infection, an indium scan was ordered. However, rather than receiving an infusion of his own radiolabeled white blood cells (WBCs) prior to the scan, the individual was accidentally infused with WBCs from an HIV-infected patient. The man was treated with a host of antiretrovirals within 45 minutes of the infusion, and virus could not be detected in the blood of the individual on day 0, 1, or 8, but was present by 14 dpi. The patient died from hepatic encephalopathy and hepatorenal syndrome on 15 dpi, when examination of CNS tissue revealed perivascular cuffing and HIV infected perivascular macrophages in several brain regions [195]. Early infection of the CNS was confirmed in SIV-infected rhesus macaques, where after only 3-7 days of infection, gliosis, glial nodules, and productively infected cells were found in frontal lobes, temporal lobes, and white matter [194,220]. Later experiments revealed that SIV RNA is only evident during acute infection (7 and 14 dpi), and in animals that developed SIVE, whereas viral DNA could be isolated from brain throughout disease [197] (Table 1.1).
Table 1.1  Assessment of Viral DNA, RNA, and Protein in the CNS as a Function of Time After SIVmac251 Infection.  (Adapted from Williams et al., Journal of Experimental Medicine 2001)

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Data presented here are the results of \( n = 2 \) animals per data point. Viral DNA was assessed using PCR for gag. Viral RNA was assessed using in situ hybridization with SIV-specific riboprobes. Viral protein was assessed by immunohistochemistry for SIV-gp120 and SIV-p28. --, not detected; +/-, detected in scattered cells or samples; +, detected in all tissues or samples examined.

This was also observed in pigtailed macaques, with the additional observation that levels of SIV DNA remain consistent from acute infection through the asymptomatic phase of disease. Only viral DNA can be isolated from brains of asymptomatic HIV-infected individuals [221], and concentrations of HIV DNA during this period are generally very low. With progression to AIDS, however, there are high numbers of productively infected cells, and significantly increased concentrations of HIV RNA in the brain [222].

D.  Conflicting hypotheses regarding the etiology of neurological manifestations

While HIV and SIV enter the brain soon after exposure to the virus, clinical presentation with neurologic syndromes traditionally occurs during the chronic stage of disease in individuals that have been HIV-positive for a long time [86,88,89,223]. This suggests that viral infection of the CNS is necessary, but not sufficient for the induction of neurological sequelae [85]. There are two conflicting viewpoints regarding the etiology and progression of CNS disease.
The first is the ‘Trojan Horse’ model, which proposes that virus enters the brain very early and establishes persistent infection. During asymptomatic disease, latent infection is maintained, and is the source of recrudescing productive infection that manifests with high levels of immune activation and progression to AIDS. HIV sequencing studies supporting this view have demonstrated that there is compartmentalized and independent evolution of quasispecies in the CNS throughout disease, and that these viruses are phylogenetically distinct from sequences found in the plasma, spleen, and lymph nodes [224-227]. In other sequence evolution studies, lesions in the CNS of HIV-infected patients with HAD contain viral RNA that originated in the brain very early after infection [228]. These studies reinforce the notion that the virus that infects the CNS early is contributing to the progression of HAND seen in late-term infection with AIDS.

The second is termed the ‘Late Invasion’ model, suggesting that the development neuropathology is late in the course of disease and is independent of virus that is already in the brain, instead dependent on events occurring in the periphery [229]. Support for the ‘late invasion’ hypothesis comes from the repeated observation that the presence of viral RNA in the CNS coincides with the bi-phasic expansion of circulating CD14+CD16+ monocytes with acute infection and progression to AIDS [113]. It is possible that during the asymptomatic period of disease, reduced inflammation in the periphery results in diminished traffic of cells into the brain, leading to suppression of CNS viral replication. Further evidence for the ‘late invasion’ model comes from the identification of a population of recently infiltrating MAC387+ macrophages in the CNS that are
believed to play an active role in lesion formation and expansion. Most MAC387+ cells are BrdU+, indicating that they have recently emigrated from the bone marrow [150,230]. These MAC387+ macrophages express CCR1 and migrate into the CNS in response to CCL3 (macrophage inflammatory protein 1-α, MIP-1α) [231,232]. The majority of MAC387+ macrophages also express CD16+, however these cells are not productively infected. A heterogeneous population of activated resident CD68+ macrophages and microglia, CD163+ perivascular macrophages, and recently infiltrating MAC387+ macrophages composes multi-nucleated giant cells in macaques with SIVE [230]. Furthermore, the age and severity of a particular lesion can be determined by the relative frequency of MAC387+ and CD68+ macrophages composing the lesion. MAC387+ cells predominate in animals with mild encephalitis, whereas higher numbers of CD68+ macrophages are found in lesions from macaques with severe SIVE [230]. This indicates that different types of monocytes and macrophages influence the development and severity of CNS lesions and are required for the progression of neuropathology. Recent studies examining viral sequence evolution within the CNS compartment have revealed that distinct viral variants arising from the periphery seed the CNS throughout the course of disease [233,234], implying that there is continuous transmigration of monocytes into the brain with HIV and SIV infection. Phylogenetic analyses have demonstrated that viral sequences isolated from deep white matter are most closely related to the viral sequences recovered from circulating monocytes just five months earlier [235]. This data provides additional evidence for rapid influx
of HIV-infected monocytes into the CNS during late-stage disease. Thus, it is possible that an expansion of activated monocytes with AIDS results and increased recruitment of monocytes into the brain and is likely playing a significant role in the appearance of neurological symptoms late in disease. Finally, with antiretroviral treatment, control of viral replication in the blood results in the reduction of macrophage activation and neurotoxin production in the CNS [236] which has led to a significant decline in the incidence of HAD and HIVE in the cART era [237,238]. Overall, these studies indicate that suppression of inflammation in the periphery may dictate the severity of neuropathology with HIV infection.

VI. Barriers to HIV eradication

A. Latently infected cellular and anatomic reservoirs of viral infection

After proviral DNA is incorporated into the host genome, the production of provirus and expression of viral antigens are dictated by the activation status of the cell. If transcription is silent or levels of transcription are very low, the cell can maintain infection while subverting immune surveillance [239,240]. There are a number of cellular and anatomic reservoirs of HIV and SIV infection that are capable of avoiding detection by the innate and adaptive immune systems [241,242] (Figure 1.7). Resting CD4+ T cells and CD16+ monocytes in the blood are latently infected [243-247], and these two cell populations harbor genetically distinct viral variants [248,249]. In patients on successful cART,
replication competent HIV DNA can be isolated from both T cells and monocytes in the periphery throughout the course of disease, indicating that despite therapy, these cells are capable of maintaining a reservoir of infectious virus [224,239,250]. In addition, there is significant evidence to suggest that HIV and SIV seed the CNS and the small intestine during acute infection when viral reservoirs are first established. HIV and SIV DNA is found throughout infection in perivascular macrophages and microglia in the brain, and in macrophages resident to the GI tract [118,198,221].

**Figure 1.7 HIV’s many reservoirs.**
Beyond lying in wait in dormant memory T cells, HIV may reproduce at a low rate in macrophages and dendritic cells. Further, HIV infected cells in a few parts of the body may be physically shielded from the immune system and certain drugs. HIV made in cellular and anatomical reservoirs does not reach the blood readily in aggressively treated patients but might generate a vigorous infection if treatment stops. (Stevenson, M., Scientific American 2008)
Macrophages and microglial cells are unique in that they are capable of harboring virus long-term without cytopathic effects, thus contributing greatly to the viral persistence throughout disease in these tissues [198,228]. The brain and gut are considered anatomic ‘sanctuaries’, because even when virions are released from productively infected cells, they typically remain within tissues [251,252]. As a result, replication can persist for a long time before changes in viral load can ever be detected in the blood [253]. Complications arising throughout infection such as treatment interruption, drug toxicity, or when a drug is ineffective against newly-emergent strains, can all induce latently infected cells to produce infectious virions, ultimately leading to a rebound in viral load [254].

Even with effective cART, these cells often become infected before therapy is initiated. What is more, by the time an individual becomes symptomatic and converts to an HIV-positive status, latently infected cells have already taken up residence in the lymph nodes [42], eliminating the possibility of HIV eradication using anti-retroviral drugs alone [255]. It is particularly difficult to treat latent infection as mechanisms underlying persistence of HIV in the brain and GI tract, as well as the cell types responsible for the formation of viral reservoirs early in the course of disease have not been elucidated. However, because monocytes and macrophages represent the primary cell type harboring HIV, adjunctive therapies targeting these cells may be effective in reducing latent infection in the blood and in these tissues.

**B. Ongoing peripheral immune activation during HIV and SIV infection**
Despite viral suppression in cART treated patients, innate immune activation persists throughout disease. The numbers of CD14+CD16+ monocytes and inflammatory mediators such as MCP-1, sCD14, sCD163, LPS, CXCL10, and IL-6 in the blood are significantly higher in HIV+ patients on therapy than in seronegative individuals, and are associated with disease progression, morbidity, and mortality [71,256-262]. In addition, even in the unique cohort of “elite controllers”, or HIV-positive patients who naturally control the virus without antiviral drug treatment, have persistently elevated levels of proinflammatory monocytes, soluble mediators (sCD14, sCD163), chemokines and cytokines [263-265]. As a result, it is evident that identification of and treatment with novel therapies that prevent or reverse peripheral immune inflammation will be necessary to improve the overall health and longevity of HIV-infected individuals.
REFERENCES


54. Stacey AR, Norris PJ, Qin L, Haygreen EA, Taylor E, et al. (2009) Induction of a striking systemic cytokine cascade prior to peak viremia in acute human immunodeficiency virus type 1 infection, in contrast to more


96. Kumar AM, Borodowsky I, Fernandez B, Gonzalez L, Kumar M (2007) Human immunodeficiency virus type 1 RNA Levels in different regions of human brain: quantification using real-time reverse transcriptase-


188. Lavi E, Strizki JM, Ulrich AM, Zhang W, Fu L, et al. (1997) CXCR-4 (Fusin), a co-receptor for the type 1 human immunodeficiency virus (HIV-1), is expressed in the human brain in a variety of cell types, including microglia and neurons. Am J Pathol 151: 1035–1042.


197. Williams KC, Corey S, Westmoreland SV, Pauley D, Knight H, et al. (2001) Perivascular macrophages are the primary cell type productively


CHAPTER II

Minocycline Inhibition of Monocyte Activation Correlates with Neuronal Protection in SIV NeuroAIDS

Jennifer H Campbell, Tricia H Burdo, Patrick Autissier, Jeffrey P Bombardier, Susan V Westmoreland, Caroline Soulas, R Gilberto Gonzalez, Eva-Maria Ratai, and Kenneth C Williams
Abstract

Background: Minocycline is a tetracycline antibiotic that has been proposed as a potential conjunctive therapy for HIV-1 associated cognitive disorders. Precise mechanism(s) of minocycline’s functions are not well defined. Fourteen rhesus macaques were SIV infected and neuronal metabolites measured by proton magnetic resonance spectroscopy (\(^1\text{H MRS}\)).

Methods: Seven received minocycline (4 mg/kg) daily starting at day 28 post-infection (pi). Monocyte expansion and activation were assessed by flow cytometry, cell traffic to lymph nodes, CD16 regulation, viral replication, and cytokine production were studied.

Results: Minocycline treatment decreased plasma virus and pro-inflammatory CD14+CD16+ and CD14\(^{lo}\)CD16+ monocytes, and reduced their expression of CD11b, CD163, CD64, CCR2 and HLA-DR. There was reduced recruitment of monocyte/ macrophages and productively infected cells in axillary lymph nodes. There was an inverse correlation between brain NAA/ Cr (neuronal injury) and circulating CD14+CD16+ and CD14\(^{lo}\)CD16+ monocytes. Minocycline treatment in vitro reduced SIV replication CD16 expression on activated CD14+CD16+ monocytes, and IL-6 production by monocytes following LPS stimulation.

Conclusion: Neuroprotective effects of minocycline are due in part to reduction of activated monocytes and monocyte traffic. Mechanisms for these effects include CD16 regulation, reduced viral replication, and inhibited immune activation.
Introduction

Human immunodeficiency virus (HIV) infection of the central nervous system (CNS) can result in cognitive impairment, behavioral deficits, and motor dysfunction. With the use of anti-retroviral therapy (ART) the incidence of HIV-associated neurological disease has declined [1]. While ART prolongs health and longevity of HIV-infected individuals, the majority of anti-retroviral drugs have poor CNS penetration. As a result, the prevalence of neurologic complications in HIV-infected patients continues to rise [2]. Factors mediating inflammatory responses outside the CNS likely play critical roles in CNS dysfunction. Monocyte/macrophage traffic likely plays a significant role in driving CNS neuropathogenesis [3-6].

Monocyte traffic across the blood-brain barrier (BBB) occurs at a basal level that increases with immune activation [7]. Such traffic likely serves as a primary route of viral entry into the CNS [8] and regulates the accumulation of macrophages in encephalitic lesions, which are the histopathological correlate of HIV-associated neurocognitive disorders (HAND). The majority of monocytes express the lipopolysaccharide (LPS) receptor CD14, while only approximately ten percent also express the FcyIII receptor CD16 under normal conditions [9,10]. Following viral infection, with inflammation, the number of monocytes as well as the percentages of activated monocyte subsets increase, resulting in increased traffic to and accumulation within tissues including the brain [11,12]. Once activated, CD14+CD16+ and CD14loCD16+ monocytes express high levels of pro-inflammatory cytokines that are linked to the development of HAND and
simian immunodeficiency virus encephalitis (SIVE) [12,13]. With HIV and SIV infection, the number of CD14+CD16+ monocytes increases [14,15]. HIV and SIV DNA and RNA are found in both CD14+CD16- and CD14+CD16+ monocyte subsets in acute infection and AIDS. Viral DNA is consistently found in CD14+CD16+ monocytes throughout disease [16,17]. We have shown that perivascular macrophages are repopulated from bone marrow in normal rhesus macaques [18] and are a primary cell productively HIV and SIV infected in the CNS [19,20]. Populations of monocytes are immunophenotypically similar to CNS perivascular macrophages; both express CD14, CD16, and CD163. Thus, it is likely that subsets of CD14+CD16+CD163+ monocytes, some of which are infected, repopulate CNS perivascular macrophages [6,21]. Thus, therapies targeting monocyte/macrophages outside the CNS can potentially affect neuronal injury.

Minocycline, a lipid soluble tetracycline antibiotic that has putative effects on immune system cells, fortuitously can also effectively cross the blood brain barrier (BBB) into the CNS parenchyma [22]. Several studies established that minocycline possesses anti-inflammatory and possibly direct neuroprotective properties independent of its antimicrobial effects [23,24]. Animal studies indicate minocycline inhibits the production of immune activators by macrophages, microglia [25-28], and neurons [25,27,28]. Minocycline inhibits activation, proliferation, and viral replication of microglia, macrophages, and lymphocytes in vitro [25,26,29,30]. In SIV-infected pigtailed macaques, minocycline reduced plasma virus, the pro-inflammatory monocyte
chemoattractant protein 1 (MCP-1)/CCL2, and viral DNA in the CNS [25].

Whether decreased monocyte/macrophage activation by minocycline also plays a neuroprotective role via such mechanisms is not well-defined. To date studies correlating neuronal injury simultaneously with viral infection and monocyte/macrophage activation have not been done.

Here, we report the effects of minocycline on monocyte/ macrophage numbers and activation, and neuronal injury in a pathogenesis study. We used a CD8+ T lymphocyte depletion model of SIV infection in rhesus macaques, which results in rapid progression to AIDS (3–4 months) with a high incidence of SIVE [31]. Using this model and magnetic resonance (MR) spectroscopy we found that minocycline treatment resulted in stable N-acetylaspartate to Creatine (NAA/Cr) levels in the brain (representing neuronal protection) compared to non-treated animals, which continued to decline (consistent with neuronal injury) [32]. In the current study, using the same cohort and three additional control non-minocycline treated animals, we report minocycline treatment reduced activation of monocytes that inversely correlated with neuronal injury, reduced the accumulation of monocyte/macrophages in lymph nodes of treated animals, and inhibited the expression of CCR2, CD163, CD11b, and CD64 on monocytes. These results suggest that minocycline, by down-regulating CD16 and viral replication, inhibiting monocyte activation and immune cell traffic, is neuroprotective.
Results

Fourteen animals were SIV-infected and treated with an anti-CD8+ T lymphocyte antibody (cM-T807), administered at 6, 8, and 12 days post infection (dpi). Three were transiently CD8+ lymphocyte depleted (≤21 dpi), while the remaining eleven were persistently CD8+ lymphocyte depleted (>28 dpi) (Table 2.1). Over the course of the study, there were no significant differences in the plasma viral load or numbers of monocyte subsets between the transiently and persistently CD8 lymphocyte depleted animals. We have previously shown that persistent CD8+ lymphocyte depletion results in rapid AIDS (3–4 months) with a high incidence of SIVE (85%) [32,33]. Minocycline (4 mg/kg/day) was initiated 28 dpi given daily as we previously reported [34]. Animals were sacrificed with the development of AIDS or at a previously determined timed sacrifice. Plasma SIV RNA peaked at 10^8 copy eq. / mL by 12 dpi. Plasma virus decreased by approximately one log after 7 days of minocycline treatment and remained at that level until sacrifice (Table 2.1).

Flow cytometric analyses were completed by employing a gating strategy where peripheral blood monocytes were initially identified according to forward scatter and side scatter properties (Figure 2.1 A) [10,33]. A small population of CD14-negative, HLA-DR-negative cells, likely representing lymphocytes or dendritic cells, was excluded by gating on all CD14+ HLA-DR+ cells. Within this gate, monocyte subsets were defined by expression of CD14 versus CD16. The absolute numbers of classical CD14+CD162 monocytes were comparable between groups prior to and after minocycline treatment (Figure 2.1 B).
absolute number of activated CD14+CD16+ (Figure 2.1 C) and CD14\textsuperscript{lo}CD16+ (Figure 2.1 D) monocytes increased in the untreated group but were significantly reduced in minocycline treated animals at all time points. The median fluorescence intensity (MFI) of CD11b, CD163, CCR2, CD64 and HLA-DR on monocyte subsets prior to minocycline treatment (day 27) and terminally was examined (Table 2.2). Day 27 was selected because it was a time point immediately preceding minocycline treatment and is a point of peak monocyte activation. All markers studied on monocyte subsets from SIV-infected macaques without minocycline treatment were increased one to four-fold at sacrifice compared to day 27 pi. In contrast, there was a two to seven-fold decrease in MFI expression of CD11b, CD163, CCR2, CD64, and HLA-DR between minocycline treated and untreated animals terminally. Interestingly, the MFI of these markers on monocytes from minocycline treated animals terminally was very similar to the values found the prior to treatment indicating that minocycline treatment reduces monocyte/macrophage activation in vivo. Additionally, the MFI for HLA-DR decreased on all monocyte subsets two-fold less than the pretreatment values (Table 2.2). Thus, minocycline treatment reduces the expression of several markers critical for monocyte traffic and function at late stage of infection, and in the case of HLA-DR is reduced below that found prior to minocycline treatment.

Next, we examined possible correlations between longitudinal changes in monocyte numbers and the ratio of N-acetylaspartate to Creatine (NAA/Cr) in different brain regions of the same animals with and without minocycline
treatment. The NAA/Cr ratio in the frontal cortex, parietal cortex, white matter, and basal ganglia was previously determined and reported [34] where decreases were found in all SIV-infected animals prior to minocycline treatment (dpi 27) [34]. At four weeks pi, following minocycline treatment, NAA/Cr in treated animals was stabilized, whereas untreated animals had a continued decline [34]. Linear regression analyses revealed a highly significant relationship between the absolute number of pro-inflammatory CD14+CD16+ and CD14^loCD16+ monocytes and NAA/Cr (in all brain regions). The relationship between monocyte subsets and NAA/Cr in the frontal cortex (CD14+CD16+: r^2 = 0.59, p = 0.0004; CD14^loCD16+: r^2 = 0.45, p = 0.04) representative of the other brain regions is illustrated in Figure 2.2. In the parietal cortex we found: CD14+CD16+: r^2 = 0.67, p = 0.0003; CD14^loCD16+: r^2 = 0.55, p = 0.0007. In the basal ganglia we found: CD14+CD16+: r^2 = 0.53, p = 0.039; CD14^loCD16+: r^2 = 0.26, p = 0.36. In the white matter: CD14+CD16+: r^2 = 0.52, p = 0.0012; CD14^loCD16+: r^2 = 0.50, p = 0.0006. We only found a significant correlation between CD14+CD162 monocytes and NAA/Cr in the parietal cortex (CD14+CD162: r^2 = 0.36, p = 0.02). Correlations between CD14+CD162 monocytes in other brain regions were not significant (data not shown). The inverse relationship between activated CD14+CD16+ and CD14^loCD16+ monocytes with NAA/Cr coupled with the observation that minocycline treatment reduces the number of activated monocytes, supports the notion that there is a link between alterations of NAA/Cr and the number of activated monocytes.

We previously reported that none of the minocycline treated animals
developed SIVE (defined as the accumulation of monocyte/macrophages, virally infected cells, and multi-nucleated giant cells) [35]. Examining axillary lymph nodes we found a statistically significant reduction in the relative numbers of resident mature CD68+ macrophages (Figure 2.3 A–B, Figure 2.4 A; p = 0.0023), recently recruited MAC387+ monocytes/macrophages (Figure 2.3 C–D, Figure 2.4 B; p = 0.0033), and productively infected SIV p28+ cells (Figure 2.3 E–F, Figure 2.4 C; p = 0.0070) with minocycline. This finding is consistent with reduced traffic and activation of monocyte/macrophages, as well as productive infection in lymph nodes (Figure 2.4 A–C) similar to the decreased infection we reported in the CNS [34].

In vitro experiments were used to determine the effect of minocycline on monocyte CD16+ with viral infection. CD16 expression on monocyte/macrophages was reduced following 10 mM minocycline for 24 hours, and was significantly reduced using 20 mM minocycline for 24 hours (Figure 2.5). By 72 hours of treatment, CD16 expression was significantly decreased on minocycline treated cells at both concentrations (Figure 2.5 A–B) while CD14 expression was unchanged (data not shown). These data suggest that by down-regulating CD16, minocycline treatment may prevent differentiation, activation, or both on monocyte/macrophages. Such inhibition of monocyte/macrophage activation or differentiation in vivo may result in decreased replication or abundance of CD14+CD16+ target cells for HIV and SIV. In addition, 20 mM minocycline in vitro significantly reduced SIV replication by monocyte/macrophages 96 hours post-infection (Figure 2.5 C). Whether the
inhibition of viral replication in monocytes in vitro is due to a block of viral entry or post entry event requires further study. Minocycline did not result in monocyte cell death as measured by LIVE/DEAD cell staining (data not shown). We note that in vitro doses of minocycline used here (10 mM and 20 mM) are similar to those found in serum of minocycline treated humans [36]. Collectively, these data indicate that both CD16 expression and viral replication are reduced with minocycline treatment, consistent with the effects of minocycline observed in vivo.

Further in vitro experiments were completed to determine the effect of minocycline on pro-inflammatory cytokine production in response to LPS stimulation (Figure 2.6). The percentage of CD14+ monocytes producing IL-6 after 3 hours of culture without stimulation was significantly lower with 10 mM (p = 0.04) and 20 mM (p = 0.009) minocycline treatment. Following 3 hours of stimulation with 10 ng/mL LPS, the percent of IL-6 producing monocytes was significantly lower with 20 mM than with 10 mM minocycline (p = 0.016). With a higher concentration of 100 ng/mL LPS, minocycline treatment at both 10 mM (p = 0.058) and 20 mM (p = 0.03) doses significantly inhibited IL-6 cytokine secretion in CD14+ monocytes (Figure 2.6). TNF production in response to LPS stimulation was also examined, however there was no significant difference between untreated and minocycline treated monocytes following 10 ng/mL or 100 ng/mL LPS (data not shown).
Discussion

Here, we demonstrate a correlation between expansion of activated monocytes and neuronal protection with minocycline in a rapid model of SIV-neuropathogenesis. We found decreased traffic of monocyte/macrophages to lymph nodes in minocycline treated animals, and in vitro evidence of a down-regulation of CD16 expression, a marked decrease in viral replication, as well as inhibition of IL-6 production following LPS stimulation. In minocycline treated animals we did not observe an expansion of CD14+CD16+ and CD14^loCD16+ monocytes that was observed in untreated animals with AIDS. These data parallel our previous results showing a direct relationship between the expansion of activated monocyte populations and decreased NAA/Cr [34].

Although it is difficult to determine the exact timing of CNS pathological changes, neuronal injury as measured by decreased NAA/Cr was detected by two weeks pi coincident with an elevation in activated CD14+CD16+ monocytes. Virus enters the CNS consistently by two weeks pi likely through trafficking of monocytes into the brain [33,37]. This appears to be sufficient for the induction of neuronal damage. We observed a decrease in the absolute number of monocytes with minocycline treatment with no further CNS damage, supporting the notion that monocyte expansion is required to drive disease.

In previous work, we observed a bi-phasic increase in the number and relative percentage of activated monocytes with the second peak occurring with peripheral immune system dysfunction and a steep decline in NAA/Cr ratios [32]. The levels of circulating monocytes in untreated animals followed this well-
described pattern of biphasic monocyte expansion with the development of AIDS, but this second peak was not seen in the minocycline treated animals. In fact there was a decrease in the number of such cells as well as the level of immune activation and accessory molecules on total monocytes.

The neuroprotective effects of minocycline confirm previous observations by Zink et al. [25] who found decreased activation of tissue macrophages, CNS viral load, and severity of CNS disease with minocycline treatment in SIV-infected pigtail macaques. Our results extend these findings by demonstrating that the effects of minocycline are directly correlated with reduced number of activated monocyte/macrophage and decreased activation markers on these pro-inflammatory cells. Zink and colleagues also examined alterations in the potent monocyte chemoattractant protein (MCP-1) throughout infection and with minocycline treatment. MCP-1 concentration in CSF followed a biphasic pattern with elevations during acute infection that declined after 10–14 dpi and again increased after four weeks of infection. In macaques treated with minocycline during chronic infection, the second peak in MCP-1 levels in CSF was not observed [38]. This is very similar to our observations that minocycline treated macaques did not have a second wave of activated CD14+CD16+ and CD14loCD16+ monocytes. In addition, although expression of the MCP-1 receptor CCR2 was also significantly increased on the pro-inflammatory monocytes of untreated animals in our study, we found CCR2 levels remained unchanged on activated monocytes from minocycline treated animals, suggesting that minocycline may disrupt the recruitment and trafficking of highly
activated monocytes into the CNS. This was further supported by our findings of reduced recruitment, viral replication, and activation of macrophages in the lymph nodes of minocycline treated animals.

Classically activated CD14+CD16- monocytes express CD64 (FcγRI), CCR2, low levels of HLA-DR, and release cytokines such as IFN-γ and IL-10 [39][38]. In response to inflammation and viral infection, there is an expansion of more mature CD14+CD16+ and CD14loCD16+ monocyte populations first with acute infection and again with the development of AIDS, when these cells can represent up to 40% of the total circulating monocyte population [10,32]. These monocytes express high levels of CD11b, CD163, and HLA-DR and release high levels of pro-inflammatory cytokines including TNF-α, IFN-γ, and IL-6 [19]. Interestingly we found minocycline significantly reduced the number of pro-inflammatory monocytes, but did not affect the number of classical CD14+CD16- monocytes. This may have resulted from suppressed activation and maturation of monocytes, down-regulation of CD16 in vivo, and/or decreased turnover and trafficking of this subset of myeloid cells from the bone marrow.

In addition to its anti-inflammatory effects, minocycline inhibits the growth of a wide variety of Gram-negative and Gram-positive bacteria. Following oral administration, concentrations of minocycline are highest in the bile and small intestine [40]. It is therefore conceivable that in our SIV infection model of rapid neuroAIDS, minocycline binds to and eliminates microbial products in the gut, resulting in decreased TLR4 signaling and inhibited expansion of highly activated CD16+ monocytes. In this study, we assayed for LPS in plasma, but did not find
significant differences in levels of LPS between the treatment groups. This does not rule out that minocycline might directly effect the response of monocytes in vivo to translocated bacterial products. Decreased expression and therefore potentially crosslinking of Fcγ receptors (CD16 and CD64) by antibody opsonized microbes in minocycline treated monocytes could have resulted in decreased transcription of inflammatory genes [41]. Minocycline treatment has also been shown to strongly chelate iron, which is an essential nutrient required by bacteria to survive and multiply [42].

Recent studies by Szeto et al. [43] indicate that by suppressing lymphocyte activation, minocycline treatment reduces HIV replication in CD4+ T lymphocytes. We observed a similar effect in CD14+ monocytes in vitro, where reduction of viral replication was directly related to the extent of CD16+ expression. It is important to note that the concentrations of minocycline that we used in vitro are physiologically similar to those found in humans with minocycline treatment [36,44]. These results indicate that the antiviral effects of minocycline are linked to its ability to reduce activation of monocytes and their permissiveness to viral infection. CD14+CD16+ and CD14loCD16+ monocytes are considered to be at an advanced stage of maturation, and it has been proposed that these cells are preferentially infected and harbor viral particles long-term [16,17,45]. Current evidence suggests that restriction of viral replication in less mature CD14+CD16- monocytes is mediated by differentiation-dependent cofactors such as apolipoprotein B mRNA-editing enzyme 3G (APOBEC3G) and APOBEC3A [46]. Upon CD16-mediated activation, the
transcriptional activators NF-κβ and C/EBPβ, which are essential factors for viral replication in monocytes, are also induced [47]. Based on the results from our study, it is conceivable that minocycline treated monocytes display a restriction to SIV replication similar to that of classically activated CD14+CD16- monocytes.

Despite the reported beneficial effects of minocycline in several animal models of CNS disease, including ALS [48-50], a recent clinical trial with ALS patients found patients deteriorated significantly faster than the placebo control group [51]. These results underscore that caution and more studies are required before additional clinical work with minocycline. In addition, this study underscores the importance of understanding differences between animal models of disease and disease. Our data support the notion that inhibition of monocyte/macrophage activation, and possibly viral infection, correlates with neuronal protection assessed by MRS. Our results suggest that minocycline may be beneficial as an adjunctive therapy, to antiretroviral therapies, that are less effective in crossing the BBB. This data was found using an SIV model of CNS neuroAIDS, which might more accurately mirror CNS pathology, than mouse models of ALS mirror the human disease.

We report here that suppression of chronic immune activation with minocycline treatment results in the reduced expansion of highly activated and potentially infected pro-inflammatory monocytes. Decreased expression of receptors such as CD11b, CD16, and CCR2 critical for trafficking of monocytes into the brain demonstrates that minocycline prevented the recruitment of these highly invasive cells into the CNS.
Experimental Procedures

Ethical Treatment of Animals

These studies were performed with the approval of the Massachusetts General Hospital Subcommittee on Research and Animal Care and the Institutional Animal Care and Use Committee of Harvard University. Animals were housed according to the standards of the American Association for Accreditation of Laboratory Animal Care. Treatment of animals was in accordance with the Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources.

Animals, SIV infection, CD8+ T lymphocyte depletion, and Minocycline treatment

The cohort of animals used in this study was reported in a recent publication of the effects of minocycline on CNS neural metabolites using MR spectroscopy [34]. Three additional non-minocycline treated animals were also included in the experiments presented here for a total of n=14 animals. In the current manuscript, we report the effects of minocycline on monocytes from animals in this cohort, and perform correlations of monocyte numbers vs. n-acetylaspartate/creatine (NAA/Cr), a marker of neuronal injury. Fourteen rhesus macaques (Macaca mulatta) were intravenously inoculated with SIVmac251 (20 ng SIV p27; a generous gift from Dr. Ronald Desrosiers, NERPC) as previously described [34]. CD8+ T lymphocyte depletion was achieved using cM-T807, an anti-CD8+ antibody that was administered subcutaneously (10 mg/kg) on day 6 post infection (pi) and intravenously (5 mg/kg) on days 8 and 12 pi [31,52,53].
Minocycline was orally administered twice daily (2 mg/kg) to seven animals beginning four weeks pi and continuing throughout the study [34]. Macaques were sacrificed upon development of AIDS or at a predetermined timed sacrifice following four weeks of minocycline treatment.

**Viral load, MRI and MRS**

Plasma SIV RNA was quantified using real-time PCR as previously described [53]. NAA/Cr measured values using MRI and $^1$H magnetic resonance spectroscopy (MRS) were recently published [34]. Here we correlate the NAA/Cr ratios in different brain regions with monocyte activation and the expansion of subpopulations.

**Flow cytometry studies of monocytes**

Peripheral blood was drawn on days 27, 6, 8, and 12 pi, and weekly thereafter. Complete blood counts were obtained using a CBC Hematology Analyzer (Hema-True, HESKA). Flow cytometric analyses were performed with 100 ml samples of blood as previously described [10]. Fluorochrome-conjugated primary antibodies including anti-CD3-FITC (SP34-2), anti-CD4-FITC (L200), anti-CD14-FITC (M5E2), anti-CD16-PE (3G8), anti- HLA-DR-PerCP-Cy5.5 (G46-6), and isotype control anti-IgG1, k-FITC (MOPC-21) all from BD Pharmingen, anti-CD64-FITC (22) and anti-CD163-FITC (Mac2-48) from Trillium Diagnostics, anti-CD8-PE (DK25; Dako), and anti-CD11b-APC (M1/ 70.15.11.5; Miltenyi Biotec) were used. Samples were fixed in PBS containing 2% formaldehyde, acquired on a FACSARia cell sorter (Becton-Dickinson) and analyzed with Tree Star Flow Jo version 8.7. Monocytes are first selected based on size and
granularity (FSC vs. SSC). From this gate, HLA-DR+ CD14+ monocytes were selected. We note all monocytes analyzed by FSC vs. SSC are HLA-DR+. The absolute number of peripheral blood monocytes was calculated by multiplying the total white blood cell count by the total percentage of each monocyte subset population as determined by flow cytometric analysis.

**Immunohistochemistry**

Axillary lymph nodes were collected in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 5 µm. Tissues were deparafinized, rehydrated and incubated with blocking reagents. Newly infiltrating monocyte/macrophages were identified by the expression of myeloid/histiocyte antigen MAC387 (MAC387; Dako) [54]. Mature resident monocyte/macrophage and microglia were assessed using anti-CD68 (KP1; Dako) [55]. Cells that were productively SIV infected were studied using anti- SIV-p28 (MX-0322; Microbix Biosystems) [56]. For quantification, at least 3 non-serial axillary lymph node sections from each of the fourteen macaques were stained for each marker. The number of MAC387+, CD68+, and p28+ cells was counted from 4 arbitrary fields, and the data are expressed as the number of chromogen-positive cells per unit area (mm$^2$). Sections were visualized with a Zeiss Axio Imager M1 microscope (Carl Zeiss MicroImaging, Inc.) using a Plan-Apochromat 620/0.8 Korr objective and analyzed using Adobe Photoshop v4 software.

**In vitro infection and minocycline treatment**

Peripheral blood mononuclear cells (PBMC) were prepared from EDTA-coagulated blood obtained from healthy animals by Ficoll density gradient
separation. CD14+ monocytes were isolated using CD14 MACS microbeads (Miltenyi Biotec). Isolated CD14+ monocytes (.95% purity) were adjusted to a final concentration of 5x10^5 cells/mL in RPMI 1640 supplemented with 11 g/L sodium pyruvate, 10% fetal bovine serum (Atlas Biologicals), and 10 ng/mL M-CSF (Peprotech Inc). Using M-CSF all monocytes in vitro were CD14+CD16+ prior to minocycline treatment. Monocyte/macrophages were infected with a highly macrophage tropic clone SIV316STOP virus (30 ng of SIV p27; a generous gift from Dr. Ronald Desrosiers, NERPC) at 37°C for 24 hours, then washed with PBS containing 2% FBS to remove excess virus. Cells were cultured for 24 or 72 hours with 10 mM and 20 mM minocycline (Sigma Aldrich). Myeloid markers were assessed by flow cytometry using anti-CD14-Pacific Blue (BD Pharmingen; M5E2), anti-CD16-PE, anti-HLA-DR-PerCpCy5.5, and anti-CD163-FITC antibodies. Viability of cells was determined using a LIVE/DEAD Fixable Dead Stain Kit (Invitrogen). Viral replication in conditioned media was quantified by SIV p27 ELISA (Advanced BioScience Laboratories, Inc).

**IL-6 and TNF induction by monocytes in vitro**

CD14+ monocytes were isolated and cultured as described in the previous section at a concentration of 1x10^6 cells/mL for 16 hours in non-adherent conditions at 37°C. Monocyte/macrophages were then incubated for 3 hours at 37°C with or without 10 ng/mL or 100 ng/mL LPS (Sigma Aldrich), and 10 mg/mL brefeldin A (Sigma Aldrich) for intracellular detection of cytokines. After stimulation, cells were fixed and permeabilized with BD Cytofix/Cytoperm™ buffer (BD Biosciences) for 20 minutes at 4°C. Cells were washed and incubated
with anti-CD16-PeCy7 (3G8) anti-IL-6-PE (MP5-20F3), anti-TNF-APC (MAb11), anti-IgG1, k-APC (MOPC-21), and anti-IgG2a, k -PE (R35–95) all from BD Pharmingen, anti-HLA-DR ECD (Immu-357; Beckman Coulter), and anti-CD14-Pacific Blue antibodies for 30 minutes at room temperature. Viability of cells was determined using a LIVE/DEAD Fixable Dead Stain Kit and dead cells were excluded. Data are expressed as the percent of total monocytes producing IL-6 or TNF.

**Statistical methods**

We have previously described kinetics of NAA/Cr over time in different brain regions of minocycline treated versus non-treated animals [34]. Here we used a least-squares means model to identify correlations between our previously determined NAA/Cr in different brain regions and the absolute number of different monocyte subsets. This method allows for the correlation of data points that are not independent of one another, such as repeated measurements of NAA/Cr or monocytes from the same animal. Cross terms between animals and monocytes were performed where a significant cross term indicated that at least one animal’s slope ((NAA/Cr) / absolute monocytes) was contrary to a randomly chosen reference animal. If such significance existed, the statistic was considered invalid. Statistical analysis was performed using JMP 7.0 (SAS, Cary, NC). Mann-Whitney U tests were used for all other statistical analyses and performed using Prism version 5.0b (GraphPad Software, Inc., San Diego, CA) software.
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Table 2.1

<table>
<thead>
<tr>
<th>Animal no.</th>
<th>Minocycline</th>
<th>Length of infection (days)</th>
<th>CD8+ lymphocyte depletion&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Terminal plasma viral load (copy eq. /mL)&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td>74 – 05</td>
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<td>5.6 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
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<tr>
<td>156 - 04</td>
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<td>Persistently depleted</td>
<td>3.6 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
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<tr>
<td>346 - 04</td>
<td>None</td>
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<td>6.2 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
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<td>Persistently depleted</td>
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<tr>
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<td>Persistently depleted</td>
<td>9.0 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
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<td>62</td>
<td>Transiently depleted</td>
<td>2.7 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
</tr>
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Note- dpi = days post infection.

* Untreated animals time-sacrificed at 6 weeks pi; all other animals sacrificed at 8 weeks pi.

<sup>a</sup> Transiently CD8+ lymphocyte depleted (≤ 21 dpi), persistently CD8+ lymphocyte depleted (> 28 dpi).

<sup>b</sup> Viral RNA quantitated using RT PCR and are results of duplicate measurements.
Table 2.1 SIV-infected, CD8+ T Lymphocyte depleted animals used in this study.
Figure 2.1

A

B

C

D

CD_{14} + CD_{16} monocytes / L

Days post infection

CD_{14} + CD_{16} monocytes / L

Days post infection

CD_{14} + CD_{16} monocytes / L

Days post infection
Figure 2.1 Minocycline reduces expansion of activated monocytes.

(A) Flow cytometric analysis of CD14+CD16−, CD14+CD16+, and CD14loCD16+ monocyte populations. Using flow cytometric analyses, monocytes were first selected based on size and granularity (FSC vs. SSC). From this gate, HLA-DR+ CD14+ monocytes were selected. A small population of CD14-negative, HLA-DR-negative cells, likely representing lymphocytes or dendritic cells, were excluded. We note that all monocytes are HLA-DR+ based on FSC vs. SSC and that the number of HLA-DR+ and absolute number of monocytes are equivalent. From the CD14+ HLA-DR+ gate monocyte subsets were fractionated based on CD14 and CD16 expression. (B) Comparisons were made between SIV-infected, CD8+ T lymphocyte depleted animals with (filled symbols) and without (open symbols) minocycline treatment. Minocycline treatment was initiated at day 28 post-infection (start date is marked by a solid vertical line). The absolute number of CD14+CD16− monocytes was comparable between groups throughout the course of the study. In contrast, numbers of (C) CD14+CD16+ and (D) CD14loCD16+ monocytes were significantly higher in untreated animals at all points compared to minocycline treated animals. Data points represent the mean ± standard error of the mean (n = 7 animals per group until 6 weeks pi when two untreated animals were time-sacrificed). P values were calculated using a Mann-Whitney U test. p < 0.05 *, p < 0.01 **.
### Table 2.2

**Activation Markers on Monocyte Subsets**

<table>
<thead>
<tr>
<th>Monocyte Subset</th>
<th>Day 27 (MFI)</th>
<th>Terminal (MFI)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All Animals</td>
<td>Untreated</td>
<td>MN Treated</td>
</tr>
<tr>
<td>CD14+CD16-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD11b</td>
<td>11085 (5382)</td>
<td>37149 (17100)</td>
<td>10425 (1603)</td>
</tr>
<tr>
<td>CD163</td>
<td>3178 (823)</td>
<td>8791 (2600)</td>
<td>3651 (1911)</td>
</tr>
<tr>
<td>CCR2</td>
<td>478 (25)</td>
<td>1971 (649)</td>
<td>463 (170)</td>
</tr>
<tr>
<td>CD64</td>
<td>3443 (201)</td>
<td>5746 (842)</td>
<td>3793 (357)</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>3964 (950)</td>
<td>5480 (1747)</td>
<td>1754 (972)</td>
</tr>
<tr>
<td>CD14+CD16+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD11b</td>
<td>11099 (5261)</td>
<td>37560 (16295)</td>
<td>10171 (1184)</td>
</tr>
<tr>
<td>CD163</td>
<td>4156 (1047)</td>
<td>9140 (2381)</td>
<td>3728 (1754)</td>
</tr>
<tr>
<td>CCR2</td>
<td>182 (58)</td>
<td>1172 (306)</td>
<td>303 (109)</td>
</tr>
<tr>
<td>CD64</td>
<td>1209 (162)</td>
<td>2175 (419)</td>
<td>1435 (96)</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>8060 (1875)</td>
<td>13160 (3689)</td>
<td>4145 (2456)</td>
</tr>
<tr>
<td>CD14&lt;sup&gt;+&lt;/sup&gt;CD16+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD11b</td>
<td>2715 (1131)</td>
<td>6807 (2751)</td>
<td>1822 (483)</td>
</tr>
<tr>
<td>CD163</td>
<td>1344 (340)</td>
<td>3328 (572)</td>
<td>1214 (642)</td>
</tr>
<tr>
<td>CCR2</td>
<td>27 (92)</td>
<td>360 (115)</td>
<td>113 (25)</td>
</tr>
<tr>
<td>CD64</td>
<td>390 (20)</td>
<td>763 (206)</td>
<td>434 (179)</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>6010 (1408)</td>
<td>9247 (3465)</td>
<td>1227 (186)</td>
</tr>
</tbody>
</table>

Note: Means are the median fluorescence intensity, MFI, and the SEM is in brackets. P values were calculated by comparing terminal MFI values for untreated and minocycline treated animals. MN = minocycline. *p < .05. ***p < .001.
Table 2.2 Activation Markers on Monocyte Subsets.
Figure 2.2

A

B

C

Untreated

74-05
79-05
156-04
346-04
307-05
118-07
121-07

Minocycline
Treated

94-04
35-07
150-04
150-05
48-07
227-04
258-04

Untreated

74-05
79-05
156-04
346-04
307-05
118-07
121-07

Minocycline
Treated

94-04
35-07
150-04
150-05
48-07
227-04
258-04

Untreated

74-05
79-05
156-04
346-04
307-05
118-07
121-07

Minocycline
Treated

94-04
35-07
150-04
150-05
48-07
227-04
258-04

\[ r^2 = 0.30 \]
\[ p = 0.3776 \]

\[ r^2 = 0.59 \]
\[ p = 0.0004 \]

\[ r^2 = 0.45 \]
\[ p = 0.04 \]
Figure 2.2 Linear regression analyses reveal significant relationships between circulating pro-inflammatory monocytes and NAA/Cr.

Regression analyses were performed between the absolute numbers of each monocyte subset and the percent change in neuronal metabolite values (NAA/Cr) in the frontal cortex relative to pre-infection levels for all animals from 28 days pi until necropsy. Minocycline treated and non-treated animals were examined at 4 time-points except for the two animals that were time-sacrificed at 6 weeks pi, have three time-points. We found a significant inverse relationship between both CD14+CD16+ (B; \( r^2 = 0.59, \ p = 0.0004 \)) and CD14loCD16+ (C; \( r^2 = 0.45, \ p = 0.04 \)) monocytes and decreased NAA/Cr, while no relationship between CD14+CD16− monocytes and NAA/Cr levels were observed (A; \( r^2 = 0.30, \ p = 0.3776 \)).
Figure 2.3
Figure 2.3 Reduced CD68+, MAC387+, and SIV p28+ productively infected cells in lymph nodes with minocycline treatment.

Immunohistochemistry was performed to compare CD68+ resident macrophages, newly infiltrating MAC387+ monocytes/macrophages, and SIV-infected cells in the axillary lymph node from untreated and minocycline treated animals. A reduced number of resident CD68+ macrophages (A–B) and newly infiltrating MAC387+ monocytes/macrophages (C–D) in an axillary lymph node of minocycline treated animals (B, D) and an untreated controls (A, C). In addition, there was a significantly higher number of productively infected SIV p28+ cells in the lymph node of untreated animals (E) compared to minocycline treated animals (F). All scale bars are 50 µm.
Figure 2.4

A

CD68+ cells / mm²

- Untreated
- Minocycline treated

p = 0.0023

B

MAC387+ cells / mm²

- Untreated
- Minocycline treated

p = 0.0033

C

p28+ cells / mm²

- Untreated
- Minocycline treated

p = 0.0070
Figure 2.4 Minocycline reduces the number of CD68+, MAC387+, and p28+ cells in axillary lymph node.

(A) Quantitative analysis of CD68+ revealed significantly fewer CD68+ cells in the axillary lymph node of animals that received minocycline as compared to untreated controls (p = 0.0023); (B) fewer numbers of newly infiltrating MAC387+ monocytes/macrophages (p = 0.0033); and (C) a decreased number of productively SIV infected p28+ cells (p = 0.0070). Numbers are representative of the means from a minimum of twelve fields calculated to represent a single data point for each animal. Horizontal bars indicate group mean values and error bars indicate the standard error of the mean. P values were determined using a Mann-Whitney U test.
Figure 2.5

A

B

C

48 hours post infection

96 hours post infection

Untreated
10 μM minocycline
20 μM minocycline

CD16

SIV p27 (pg/mL)

48 hours post infection
96 hours post infection
**Figure 2.5 Reduction of CD16 expression and viral replication in CD14+ monocytes during *in vitro* minocycline treatment.**

(A) CD14+ monocytes were infected with SIVmac316STOP virus and cultured with M-CSF in the presence or absence of minocycline for 24 and 72 hours. With M-CSF treatment, all monocytes expressed CD14 and CD16 prior to minocycline treatment. By flow cytometry, monocytes were first gated based on size (FSC) and granularity (SSC). From this gate HLA-DR+ CD14+ monocytes were selected and CD16 expression on these cells between treatment groups was compared. (B) Histograms represent the median fluorescence intensity (MFI) of CD16 from one representative experiment out of three. Averages of MFI ± standard error of the mean in a given treatment group are indicated in the upper left hand corner of the graphs. CD16 expression was significantly higher on untreated than 20 μM minocycline treated monocytes at 48 hours pi ($p = 0.021$). Untreated monocytes had significantly higher CD16 expression than both 10 μM and 20 μM treated cells at 96 hours pi ($p = 0.001$). (C) After 96 hours of infection, SIV-p27 was reduced with minocycline treatment, with significant differences between control and 20 μM minocycline ($p = 0.039$). Studies presented here are the results of $n = 3$ three independent experiments with $n = 3$ animals per experiment performed in triplicate wells. P values were determined using a Mann-Whitney U test.
Figure 2.6
Figure 2.6 Minocycline treatment inhibits induction of IL-6 by CD14+ monocytes in vitro.

CD14+ monocytes were cultured in non-adherent conditions with M-CSF and in the presence or absence of minocycline for 16 hours. IL-6 was induced by LPS in the presence of brefeldin A for 3 hours. The percentage of monocytes producing IL-6 was significantly lower with 10 µM (p = 0.04) and 20 µM (p = 0.009) minocycline than in untreated cells. With 10 ng/mL LPS, the percent of IL-6 producing monocytes was significantly reduced with 20 µM as compared to 10 µM minocycline treatment (p = 0.016). With 100 ng/mL LPS stimulation, minocycline treatment at both 10 µM (p = 0.058) and 20 µM (p = 0.03) doses significantly inhibited IL-6 cytokine secretion in CD14+ monocytes. Data presented represent the results of two independent experiments with n = 3 animals per experiment performed in triplicate wells. P values were determined using a Mann-Whitney U test.
REFERENCES


CHAPTER III

Anti-α4 Antibody Treatment Blocks Virus Traffic to the Brain and Gut Early, and Stabilizes CNS Injury Late in Infection

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R Gilberto Gonzalez, Macro Salemi,
Tricia H Burdo, and Kenneth C Williams
Abstract

SIV-infected monkeys with high plasma virus and CNS injury were treated with an anti-α4 blocking antibody 28 days post-infection, and brain and gut infection were assessed. Treatment resulted in stabilization of neuronal injury (NAA/Cr), significantly fewer recruited monocyte/macrophages in brain, and decreased SIV infection (SIV p28+, RNA+) in brain and gut. Treatment initiated at the time of infection blocked monocyte/macrophage traffic and infection in the CNS, and significantly reduced infection in the intestine. SIV DNA was undetectable in brains of five of six animals treated at the time of infection, but proviral DNA in guts of treated and control animals was equivalent. Animals treated at the time of infection had low-to-no plasma LPS or sCD163. Our results indicate that monocyte/macrophage traffic late in infection drives neuronal injury and maintains CNS viral reservoirs. Furthermore, early leukocyte traffic seeds the CNS with virus and contributes to productive infection of the gut.
Introduction

The importance of monocyte/macrophages as a critical cell type bringing human immunodeficiency virus (HIV) to the central nervous system (CNS) is often assumed [1,2], but has not been directly tested. Similarly, the function of lymphocytes seeding the gut early during infection has not been directly assessed. HIV infection of the CNS is associated with compromised motor, behavioral, and cognitive functioning, collectively referred to as HIV-associated neurocognitive disorders (HAND) [3]. Neuropathologic correlates of these clinical conditions include accumulation of perivascular macrophages, microglial activation, decreased synaptic/dendritic densities, neuronal damage and loss [4]. Combination antiretroviral therapies (cART) restore peripheral immune function and control viral replication, however effective cART does not prevent the formation of a CNS viral reservoir early in infection [5]. Consequently, neuroinflammation remains and neurologic impairment affects the majority of HIV-infected individuals [6,7]. Gut-associated lymphoid tissues (GALT) are another important reservoir of HIV RNA and DNA that is established during acute infection and persists despite long-term effective therapy [8,9].

Early after exposure to HIV and SIV, virions and infected cells enter the gut and infect resident CD4+ T lymphocytes. These cells harbor virus and propagate infection, resulting in CD4+ T cell loss within days [10,11]. With CD4+ T cell depletion, there is expansion of activated immune cells and virus in blood that can infect draining lymph nodes, brain, and other tissues [12]. CD4+ T cell apoptosis during acute HIV and SIV infection is thought to contribute to aberrant
immune activation and translocation of microbial products, which can cause
increased trafficking of monocytes into the CNS. It is postulated that this is
closely linked to the development of HAND and SIV encephalitis (SIVE) [13,14].

Similar to the gut, SIV and HIV are found in the CNS as early as 3
[15,16] and consistently by 14 [5,17] days post infection (dpi), and occurs
concurrently with accumulation of perivascular macrophages, some of which are
infected [18,19]. Although neurons are not infected, neuronal damage is evident
even during the acute phase of infection [20-22]. 1H MRS is a sensitive method
of non-invasively measuring neuronal injury by decreased levels of neuronal
metabolites N-acetylaspartate+N-acetylaspartylglutamate (collectively NAA).
Neuronal injury (NAA/Cr) correlates with the expansion of activated monocytes in
the periphery, indicating that neuroinvasion, likely through entry of activated or
infected monocytes into the brain, is required for CNS pathogenesis [23]. Using
BrdU, we have shown that the magnitude of blood monocyte expansion by 8 dpi
is highly predictive of the rate of disease progression and severity of CNS
neuropathology [24]. It is widely accepted that monocyte/macrophage traffic and
accumulation in CNS drives neuronal injury, though no study has tested whether
directly blocking monocyte/macrophage traffic affects neuronal injury, or blocks
infection of the CNS by HIV and SIV.

In this study, we used the anti-α4 antibody natalizumab (Biogen Idec),
which selectively binds the α4 subunit of α4β1 and α4β7 integrins, blocking the
interaction between α4 and its’ ligands [25]. Natalizumab prevents accumulation
of leukocytes (B cells, T lymphocytes, and monocyte/macrophages) in the CNS
of patients with relapsing-remitting Multiple Sclerosis [26] and small intestine of patients with Crohn's disease [27]. We used natalizumab primarily to assess the requirement of monocyte/macrophage traffic on SIV neuropathogenesis, and secondarily to assess the impact of SIV pathogenesis in the gut. To examine the requirement of monocyte/macrophages for neuronal injury, macaques were treated later in infection (28, 34, and 41 dpi, “late”; n=4) and compared to SIV infected non-treated controls (n=4), all sacrificed when they developed AIDS (50 to 62 dpi). To determine if leukocyte traffic seeds the brain and gut with virus, animals received natalizumab at the time of infection (0, 7, and 14 dpi, “early”; n=6) and were compared to untreated controls (n=3), all sacrificed on 22 dpi. In late treated animals, we found decreased accumulation of SIV-infected monocyte/macrophages in the CNS and stabilization of neuronal injury. Early natalizumab treatment prevented macrophage traffic and infection in the CNS, and decreased the number of productively infected cells in the gut. These data underscore the requirement of monocyte/macrophage traffic for neuronal injury and maintenance of the CNS lesions, and indicate that early leukocyte traffic is critical for seeding the CNS and contributes to seeding of gut with virus.
Results

**Natalizumab treatment with ongoing infection stabilizes neuronal injury**

The eight SIV-infected macaques in the later cohort (n=4 natalizumab treated, n=4 non-treated) developed AIDS. One of the four natalizumab treated animals and two of four untreated macaques developed SIVE, defined by productive virus, multinucleated giant cells, and macrophage accumulation in the CNS. Plasma viral loads in all animals remained high regardless of treatment (data not shown). We assessed the requirement of continuing monocyte/macrophage traffic for neuronal injury and maintenance of CNS reservoirs with three weekly natalizumab treatments (30 mg/kg) beginning on 28 dpi, when significant neuronal damage had already occurred [20,21]. Neuronal injury (decreased NAA/Cr) was measured in frontal cortex (FC), parietal cortex (PC), basal ganglia (BG), and white matter semiovale (WM) of the four natalizumab treated and four untreated macaques by MR spectroscopy bi-weekly (Figure 3.1). The mean NAA/Cr ratio declined from pre-infection to 4 weeks post infection (wpi) in FC (-13%, p=0.0028), PC (-8.3%, p=0.0016), BG (-9.7%, p=0.008), and WM (-8%, p=0.036) of all animals (Figure 3.1 A-D), consistent with neuronal damage as previously reported [21,28,29]. Following natalizumab treatment, NAA/Cr decreases stabilized in the FC (+0.5%, p=0.89), PC (-3.3%, p=0.60), BG (-2.1%, p=0.76) and WM (-5.7%, p=0.046). In contrast, SIV infected, non-treated animals had continued reductions of NAA/Cr in the FC (-13.2%, p=0.016), PC (-12.5%, p=0.0008), and WM (-11.9%, p=0.0001), and a trend towards decline in the BG (-6.8%, p=0.13) (Figure 1 E-H).
Late natalizumab treatment suppresses the traffic and accumulation of SIV infected monocyte/macrophages in the brain

In all brain regions examined (frontal cortex, parietal cortex, occipital cortex, brainstem), numbers of SIV p28+ and RNA+ cells were markedly lower in late natalizumab treated versus untreated animals (p28+ p=0.0202, RNA+ p=0.0005; Figure 3.2 A). There were significantly fewer activated CD68+ resident macrophages (p=0.0017; Figure 3.2 B) and recently infiltrating MAC387+ monocytes (p=0.0003; Figure 3.2 C) in late treated macaques. We have previously observed significant numbers of BrdU+ macrophages in the CNS of animals receiving BrdU even 24-hours prior to sacrifice [24], yet no BrdU+ macrophages were found in brains of animals that received BrdU after natalizumab treatment began (33 dpi, 24-hours prior to necropsy) (Figure 3.2 D). In animals that received BrdU throughout infection (-9 dpi, 26 dpi, and 24-hours prior to necropsy), we found lower numbers of BrdU+ cells in natalizumab treated animals versus controls (Figure 3.2 D). These data demonstrate that natalizumab treatment with ongoing infection blocks monocyte/macrophage traffic, reduces the CNS reservoir of productively infected monocyte/macrophages, and stabilizes neuronal injury.

Reduced accumulation of T lymphocytes, monocyte/macrophages, and productively SIV infected cells in the gut with late natalizumab treatment

In the gut (duodenum, jejunum, colon), there were fewer SIV p28+ cells in treated animals (p=0.0187), but no difference in the number of RNA+ cells
observed between late natalizumab treated animals and untreated controls (Figure 3.3 A). There were lower numbers of CD68+ macrophages (p=0.0460; Figure 3.3 B), MAC387+ monocytes (p=0.0182; Figure 3.3 C), and CD3+ T lymphocytes (p=0.0001; Figure 3.3 D) in the guts of natalizumab treated macaques, indicating that late treatment was not sufficient to stop viral infection that has already occurred, but did reduce subsequent traffic of lymphocytes and monocyte/macrophages.

**Early natalizumab treatment blocks traffic and accumulation of SIV infected monocyte/macrophages in the brain and gut, and bacterial translocation**

Next we sought to determine whether weekly natalizumab treatment at the time of infection (“early”) blocks viral seeding of the CNS and gut. Relative to untreated controls (n=3) that were also sacrificed at 22 dpi, there were fewer SIV p28+ (p=0.0004) and RNA+ (p=0.0024) cells (Figure 3.2 A), and CD68+ macrophages (p=0.0016; Figure 3.2 B) in the CNS of early natalizumab treated animals (n=6). When present, SIV p28+ and RNA+ cells were primarily found in vessels outside the parenchyma. Numbers of MAC387+ cells were lower in brains of early treated macaques (p=0.0179; Figure 3.2 C), and recently trafficking BrdU+ cells were absent (Figure 3.2 D). In the guts of animals receiving early natalizumab, there was a significant reduction in SIV p28+ (p=0.0012) and RNA+ cells (p=0.0013) (Figure 3.3 A). There were similar numbers of CD68+ macrophages in early treated and control groups (Figure 3.3 B), but lower numbers of MAC387+ monocytes (p<0.0001; Figure 3.3 C) and
CD3+ T lymphocytes (p=0.0001; Figure 3.3 D) with natalizumab treatment. Interestingly, early treated macaques had significantly lower plasma LPS at 8 (p<0.0001) and 12 dpi (p=0.0019) than untreated controls (Figure 4 A). In contrast, equivalent LPS levels were seen in late treated and non-treated animals (data not shown). Early natalizumab also resulted in reduced soluble CD163 in plasma, with treated macaques exhibiting significantly lower concentrations than untreated macaques at 12 (p=0.0488) and 21 dpi (p<0.0001) (Figure 4 B).

**Early natalizumab blocks viral seeding in brain, but not in gut and lymph nodes**

To determine whether natalizumab treatment on the day of SIV infection blocked latent viral infection in brain and gut, we analyzed tissues for SIV gag DNA using qPCR. Proviral DNA was undetectable in brains of five of six early natalizumab treated macaques (Figure 5 A). One animal had a low level of SIV DNA that was detected only in brainstem, which may be explained by brainstem trauma resulting from a CSF tap. Although natalizumab significantly reduced the number of productively infected cells in the gut, similar numbers of viral DNA copies were found in the duodenum and jejunum of natalizumab and untreated animals. SIV gag DNA levels were lower in colon with treatment, however this difference did not reach significance (Figure 5 B).

**Natalizumab treatment does not affect monocyte/macrophage traffic or the accumulation of productively infected macrophages in lymph nodes**
There was elevated SIV provirus in axillary lymph nodes and similar levels of SIV DNA in mesenteric lymph nodes of early natalizumab treated relative to untreated controls (Figure 5 C), probably reflecting differing degrees of α4β1 and α4β7 utilization in these different compartments. This was not surprising, as comparable numbers of SIV p28+ and RNA+ infected cells were detected in lymph nodes from treated and untreated animals in both late and early cohorts (Figure 6 A). Natalizumab treated animals had fewer CD3+ T lymphocytes in lymph nodes than matched controls (Late p=0.0011, Early p=0.0006; Figure 6 D), yet similar numbers of CD68+ (Figure 6 B) and MAC387+ monocytes (Figure 6 C) were observed in all animals, suggesting that natalizumab did not affect immune recirculation in lymph nodes.
Discussion

While it has been suggested that monocyte/macrophage traffic drives CNS infection and neuron damage, this has not been demonstrated experimentally. Here, we examined whether continuous neuronal injury with HIV and SIV infection depends on monocyte/macrophage traffic, and if cell trafficking to CNS and gut is required for viral seeding. NAA/Cr was monitored throughout infection in four SIV-infected rhesus macaques treated with natalizumab beginning after 28 days of infection, when significant neuronal injury had already occurred. We have previously shown these decreases to correlate with increased monocyte/macrophage activation, accumulation, viral infection, and neuronal injury by immunohistochemical and neuropathologic examination [10,20,23,30]. Despite significant reductions in NAA/Cr, blocking monocyte/macrophage traffic with natalizumab stabilized NAA/Cr declines, consistent with limiting further neuronal injury. Because natalizumab also blocks lymphocyte traffic, it is possible that lymphocytes might also play a role in neuronal injury, however it has been repeatedly demonstrated that there are low-to-no CD4+ T cells in the CNS with HIV and SIV infection [22,31,32], and our animals were CD8 lymphocyte depleted.

To determine whether leukocyte traffic is required for initial seeding of brain and gut, animals were treated with natalizumab beginning on the day of infection. At sacrifice 22 days later, no SIV p28+ or RNA+ cells were found in the CNS, indicating that traffic of monocyte/macrophages from the periphery is necessary for initial viral dissemination in the brain. This is further supported by
the absence of SIV \textit{gag} DNA in brain tissues of five of six natalizumab treated animals. Provirus in the brainstem of the sixth macaque may be a result of a CSF tap trauma, and a lower concentration of SIV \textit{gag} DNA was found in this animal than in brains of non-treated controls.

There were no BrdU+ cells in early or late natalizumab treated animals, indicating that \(\alpha 4\) blockade was sufficient to prevent BrdU+ monocyte/macrophages from entering the brain. We have previously reported that the majority of BrdU+ cells in the CNS of SIV-infected animals are MAC387+ [24,33], underscoring the role of recently recruited MAC387+ monocytes in active CNS inflammation [22]. The few scattered MAC387+ monocytes and CD68+ macrophages seen in brains of treated animals suggests that despite SIV infection and CD8 lymphocyte depletion, very little inflammation occurred in the CNS following natalizumab treatment. Blocking leukocyte traffic later in animals with ongoing inflammation and lesions reduced inflammation to almost undetectable levels. These observations with low numbers of SIV p28+ and RNA+ cells and rapid stabilization of NAA/Cr in the brains of late natalizumab treated animals suggest that ongoing traffic maintains not only neuronal injury, but also productive infection of the CNS.

The small intestine is a primary site for SIV infection, with interaction between the \(\alpha 4\beta 7\) integrin and MAdCAM-1 facilitating traffic of leukocytes [34,35]. Natalizumab reduced numbers of CD3+ T lymphocytes, MAC387+ monocytes, and SIV p28+ cells relative to controls, suggesting that treatment suppressed traffic of cells responsible for early viral replication. It has previously been shown
that loss of α4β7HIGH CD4+ T cells in blood is an indication of decreased numbers of CD4+ T cells in gut [36]. Whether we directly blocked trafficking of α4β7HIGH CD4+ T cells was not assessed. SIV DNA was detected in gut tissues of early natalizumab treated animals, however it is plausible this is non-integrated DNA, as very low numbers of SIV p28+ and RNA+ cells were observed in these tissues. Others have demonstrated that with early infection of GALT with ART given four hours after infection, there is protection against rapid depletion of CD4+ T cells, yet SIV RNA and DNA were detected [37]. Despite high levels of SIV provirus in the gastrointestinal tract with natalizumab treatment, productive viral infection appeared to be controlled. This is in contrast to what was seen in the CNS, which might be accounted for in part by the BBB. It is established that in the CNS, the BBB controls traffic of cells, which can be blocked by natalizumab. Therefore, blocking α4β1 and α4β7 likely has a more limited impact in gut than in the CNS. It is important to note that the majority of T cells trafficking to GALT utilize α4β7, but this is a small population in the blood [35,38]. The viral envelope protein gp120 can bind to the α4β7 receptor expressed by leukocytes homing to the small intestine, which may not affect cell infection, but can result in activation and apoptosis of T lymphocytes by HIV and SIV [39]. This could be why we observed low numbers of CD3+ T cells despite similar levels of SIV DNA in the guts of natalizumab treated animals.

We found low levels of plasma LPS with early natalizumab treatment, but no difference with late treatment, suggesting that inflammation in the intestine early during infection contributes to mucosal damage and endotoxin
translocation. In addition to inhibiting release of microbial products such as LPS from gut, low levels of sCD163 in plasma were also observed. Because significant reductions of chemokine/cytokine production in blood and CSF have previously been shown in natalizumab treated patients [40], this may also have contributed to an overall reduction in peripheral immune activation, as suggested by the reduced sCD163 and chemokine expression on monocyte and T lymphocyte populations in blood (data not shown). We found decreases in ex vivo transmigration and adhesion of PBMCs from natalizumab treated animals, further supporting the diminished ability of cells to traffic to the brain and gut (data not shown).

Similar numbers of MAC387+ and CD68+ monocyte/macrophages in lymph tissues of untreated and treated macaques suggest that natalizumab did not significantly affect traffic of these cells to lymph nodes, a finding made by others using natalizumab in monkeys [41,42]. These same studies also demonstrated normal regulatory immune function in natalizumab treated macaques, but increased numbers of lymphocyte precursors, monocyte/macrophages, and T cells in blood. We observed a similar expansion of CD14+ monocytes, CD4+ T lymphocytes, CD34+ hematopoietic progenitors, and CD20+ B lymphocytes, as well as a decline of CD49d (α4 integrin) expression in the periphery of all treated animals (data not shown). It was surprising to observe fewer CD3+ T lymphocytes in lymph nodes of both groups of natalizumab treated macaques, however this may be explained by higher numbers of SIV p28+ and RNA+ cells and elevated SIV DNA copies in lymph
nodes, and therefore high numbers of infected leukocytes that are susceptible to apoptosis. Several papers have shown normal lymphoid follicle function and no major differences in immune function with natalizumab treatment, as both monocytes and T cells use the interaction between leukocyte function antigen (LFA)-1 with ICAM-1 or ICAM-2 in order to traffic into high endothelial venules [41, 43, 44].

Early initiation of effective cART reduces CNS disease [45], suppresses virus to non-detectable levels, and reduces HIV transmission, however current therapies are not sufficient to eradicate viral reservoirs [46]. Furthermore, many cART therapies have low CNS penetration and do not target monocyte/macrophages that drive cardiac and CNS pathology. While we do not suggest using natalizumab long-term in HIV-infected patients, one might consider whether natalizumab treatment early, in combination with antiretroviral therapy, could stop productive infection of the brain and gut, preventing the establishment of these tissue reservoirs. While PML is a concern in patients receiving natalizumab for extended periods, all reported incidents have occurred after more than a year of antibody treatment. Additionally, patients with JC viral antibodies have received effective natalizumab treatment for more than 245 days without the development of PML [47]. Regardless, the experiments described here underscore the critical role of monocyte/macrophage traffic in ongoing neuronal injury, and establishment and maintenance of viral reservoirs in the CNS and intestinal tissues.
Supplemental Data

A number of publications have demonstrated normal regulatory immune function with natalizumab treatment, but increased numbers of circulating white blood cells, monocytes, B cells, and T cells in humans and rhesus macaques given the same dose of the antibody that was used in the current study [41,48]. Therefore, we monitored the frequency and activation status of leukocyte subsets in the blood of all antibody treated macaques throughout infection. In addition, for analysis of unbound α4 integrin (CD49d), staining was performed with saturating amounts of antibody against CD49d. Plasma viral loads were also measured throughout infection in untreated and natalizumab treated animals. Finally, ex vivo experiments were used to elucidate differences in migratory, attachment, and adhesion capabilities of cells isolated from natalizumab treated and uninfected macaques.

We found that plasma viral loads in all animals remained high regardless of treatment (Figure 3.7). Flow cytometric analyses comparing cell populations at a preinfection timepoint and following the last natalizumab treatment revealed several changes in the blood (Figure 3.8). There was significant expansion in the number of CD20+ B lymphocytes (Late $p=0.034$, Early $p=0.004$) and CD34+ HPSCs (Late $p=0.019$, Early $p=0.008$) in both cohorts of natalizumab treated animals (Figure 3.8 A-B). In late treated animals, there were also significantly higher numbers of CD4+ T lymphocytes in the blood ($p=0.010$), while in early treated animals there were significantly more circulating CD14+ monocytes ($p=0.028$). This indicated to us that antibody treatment rendered cells incapable
of trafficking from the blood into various tissue compartments. Of all cell subsets, only the percent of CD34+ HPSCs circulating in the blood increased in late natalizumab treated animals (p=0.034), whereas there were significant increases in the percentage of CD4+ T cells (p=0.019), CD34+ HPSCs (p=0.003), and CD14+ monocytes (p=0.008) in the early treated cohort (Figure 3.8 C-D). It is also important to keep in mind that the early natalizumab treated macaques were also being CD8 T lymphocyte depleted during the same time, which may have affected the percentages of various cell subsets present in the blood. Following treatment initiation in both groups of animals receiving natalizumab we observed much lower levels of CD49d surface expression on CD14+ monocytes (Late p=0.043, Early p=0.041), CD4+ T lymphocytes (Late p=0.037, Early p=0.008), and CD34+ HPSCs (Late p=0.002, Early p=0.0003), as well as on CD20+ B lymphocytes from late treated macaques (p=0.009) (Figure 3.8 E-F). Overall, all cells expressed less unbound α4 following treatment, implying successful α4 integrin antagonism with natalizumab.

The ability of natalizumab to prevent PBMC adhesion to the extracellular matrix protein fibronectin was measured ex vivo in real time under constitutive and inflammatory (phorbol 12-myristate 13-acetate (PMA) induced) conditions. PBMCs isolated from the late treated animals prior to natalizumab (27 dpi) displayed rapid attachment (initial increase in normalized cell index (NCI) values) and adhesion to fibronectin within two hours of adding PMA (Figure 3.8 A). Following natalizumab, cells from treated animals exhibited a markedly reduced binding capacity. In the early natalizumab treated group, there were also fewer
numbers of cells attaching to the fibronectin matrix after the addition of PMA compared to cells from uninfected and non-treated macaques (Figure 3.8 C). While attachment (normalized cell index) increased significantly during the first two hours after PMA in PBMCs from uninfected controls, there was a lower stable NCI in PBMCs from natalizumab treated animals as well as a decline in the impedance slope, representing inhibited adherence of cells. The number of attaching and adhering PBMCs following antibody administration in all treated macaques was similar (Late, Figure 3.9 B) or even lower (Early, Figure 3.9 D) than that of uninfected animals, indicating that natalizumab treatment resulted in an impaired interaction between cells and their ligand fibronectin.

Lastly, to elucidate potential differences in the ability of cells isolated from natalizumab treated macaques to traffic in response to chemokine stimulation, we compared the capacity of PBMCs to migrate in response to CCL2 (MCP-1), CX3CL1 (fractalkine), SDF-1 (CXCL12), astrocyte conditioned media (ACM), and CCL7 (MCP-3). In late treated animals, the response of CD14+ monocytes to CCL2 (p=0.0061), CX3CL1 (p=0.0135), ACM (p=0.0169), and CCL7 (p=0.0373) was significantly lower after treatment compared to pre-treatment (27 dpi) values (Figure 3.10 A), and CD3+ T lymphocyte responsiveness to CCL2 (p=0.0274) and CX3CL1 (p=0.0220) was also suppressed (Figure 3.10 B). In the early natalizumab treated group, CD14+ monocyte chemotaxis to CCL2 (p=0.0216), CX3CL1 (p=0.0138), ACM (p=0.0320), and CCL7 (p=0.0005), as well as the degree of CD3+ T lymphocyte movement towards CCL2 (p=0.0347), CX3CL1 (p=0.0111), ACM (p=0.0027), and CCL7 (p=0.0003) was significantly inhibited.
relative to pre-treatment (pre-infection) migration levels (Figure 3.10 C-D).

There was no difference in monocyte or T lymphocyte migration towards SDF-1 with natalizumab treatment, as this chemokine regulates leukocyte function antigen (LFA) -1 rather than VLA-4 integrin mediated cell migration [49].

Typically, increased production of chemokines/cytokines CCL2, CX3CL1, CCL7, and fractalkine by endothelial cells, macrophages, and astrocytes during HIV and SIV infection results in increased recruitment and infiltration of monocytes into the brain [50-52], and expression of receptors CX3CL1 (CX3CR1), CCL2 and CCL7 (CCR2) is elevated on monocytes and T cells homing to the small intestine [53]. However, in natalizumab treated patients, significant reductions of chemokine/cytokine production in blood and CSF have previously been reported [40]. In our study, reduced levels of chemokines and cytokines in the blood of the natalizumab treated animals was also observed, and may have contributed to an overall reduction in chemokine expression on monocyte and T lymphocyte populations in blood. Decreased activation of immune cells with natalizumab treatment likely contributed to the blockade of cell traffic into tissues. This was also observed on functional level in ex vivo transmigration and adhesion assays with the continued decline in the capacity of PBMCs to employ α4-mediated adhesion and migration under inflammatory conditions, supporting the notion that there was also diminished trafficking of leukocytes into the brain and small intestine with natalizumab treatment.
**Experimental Procedures**

**Ethical Treatment of Animals**

Animals were housed according to the standards of the American Association for Accreditation of Laboratory Animal Care. The studies were performed with the approval of the Massachusetts General Hospital Subcommittee on Research and Animal Care, the Institutional Animal Care and Use Committees of Harvard University and BIOQUAL, Inc. The treatment of animals was in accordance with the Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources (8th edition).

**Animals, SIV infection, CD8 lymphocyte depletion, and viral load determination**

A total of seventeen rhesus macaques (*Macaca mulatta*) were intravenously inoculated with SIVmac251 (20ng SIV p27; a generous gift from Dr. Ronald Desrosiers, NERPC). CD8 lymphocyte depletion was achieved using cM-T807, an α-CD8 antibody that was administered subcutaneously (10 mg/kg) on day 6 post infection (pi) and intravenously (5 mg/kg) on days 8 and 12 pi [54-56]. Eight macaques (n=4 late natalizumab treated, n=4 untreated) were sacrificed at similar time points with progression to AIDS (50 to 62 dpi). Nine animals (n=6 early natalizumab treated, n=3 untreated) were sacrificed at 22 dpi. Plasma SIV RNA was quantified in all animals at various time points throughout infection using real-time PCR as previously described [56].

**Anti-α4 integrin (natalizumab) administration**
The recombinant humanized IgG4 monoclonal anti-α4 integrin mAb (natalizumab) was kindly provided by Biogen Idec (Cambridge, MA) in a sterile concentrated solution. This antibody has specificity for the α4 subunit of α4β1 (very late activation antigen 4, VLA-4) and α4β7 integrins expressed on the surface of all leukocytes except neutrophils [51]. The rhesus macaque α4 sequence exhibits 96% homology with the human sequence (NCBI), and the anti-α4 antibody binds to the α4 subunit with affinity comparable to that in humans \(K_d = 0.04 \text{ – } 0.07 \mu g/ml\) [41]. The pharmacokinetic half-life of natalizumab in humans is 11 ± 4 days, however more than 70% of α4 integrin sites remain saturated 4 weeks after infusion and cell counts in the CSF are significantly reduced for up to 6 months [57]. The antibody was administered once weekly for three weeks beginning on the day of infection (0 dpi, n=6) or 28 days after infection (28 dpi, n=4). On the day of infusion 30 mg/kg of α-VLA-4 was injected into a 250 mL bag of 0.9% NaCl and administered intravenously (iv) over 30-60 minutes. We chose a high dose of natalizumab and only treated three times with one-week intervals between each treatment to avoid hypersensitivity responses by the monkeys to the humanized antibody. This regimen has previously been shown to maintain high serum levels of natalizumab throughout treatment in rhesus macaques [41]. Chemistry panels including alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were examined at various time points throughout infection and remained below 100 IU/L, indicating that natalizumab treatment did not induce hepatotoxicity.
**BrdU administration**

5-bromo-2'-deoxyuridine (BrdU) (Sigma) was prepared as a 30 mg/mL stock solution in 1X PBS (Ca$^{2+}$/Mg$^{2+}$ free; Mediatech Inc.) and given intravenously at 60 mg/kg as described previously[24]. To monitor levels of monocyte/macrophage trafficking out of the bone marrow, in blood, and into the CNS and gut, BrdU was administered prior to infection (-9 dpi), at peak infection (26 dpi), and 24 hours prior to necropsy in two macaques given natalizumab beginning on 28 dpi and two untreated control animals. In the other thirteen animals, BrdU was administered once natalizumab treatment was initiated, on days 33 and 47 post infection (n=2 late treated, n=2 untreated) or days 6 and 20 post infection (n=6 early treated, n=3 untreated).

**MRI/MRS**

To determine if blocking monocyte/macrophage traffic impacted neuronal injury, n=4 rhesus macaques were treated with natalizumab beginning on 28 dpi. These animals and non-treated controls (n=4) were scanned prior to infection (2x) and biweekly thereafter until sacrifice. For imaging, each animal was tranquilized, intubated, and monitored continuously throughout the scanning procedure as previously described [29,58]. Briefly, MR imaging and spectroscopy were performed on a 3 Tesla whole-body imager (Magnetom TIM Trio, Siemens) with a circularly polarized transmit-receive extremity coil. First a three-plane localizer scan, used for positioning and to ensure 1H voxel reproducibility, was acquired. The 1H MRS volumes of interest (VOI) were then chosen as previously described [29,58]. Single-voxel proton spectra were
acquired from the parietal cortex (PC), frontal cortex (FC), basal ganglia (BG) and white matter semiovale (WM) using the point resolved spectroscopy sequence (PRESS) with WET [59] water suppression. Spectroscopic data were processed using LCModel software and concentrations of NAA (N-acetylaspartate + N-acetylaspartylglutamate) and creatine-containing compounds (Cr) were quantified using the unsuppressed water signal as an internal intensity reference.

**Flow cytometry**

Flow cytometric analyses were performed as published [60] using 100 µl samples of blood stained with the following fluorochrome-conjugated primary antibodies: anti-CD3-Alexa Fluor 700 (SP34-2), anti-CD4-PerCp-Cy5.5 (L200), anti-CD8-APC (RPA-T8), anti-CD11b-Alexa Fluor 700 (1CRF44), anti-CD14-Pacific Blue (M5E2), anti-CD16-PE-Cy7 (3G8), anti-CD20-APC (2H7), anti-CD20-APC-Cy7 (L27), anti-CD25-PE (M-A251), anti-CD34-PE (563), anti-CD49d-PE-Cy5 (9F10), anti-CD95-FITC (DX2), anti-CD195-APC (3A9), and isotype control anti-IgG1, κ-FITC (MOPC-21) from BD Biosciences, HLA-DR-Texas Red PE (Immu-357; Beckman Coulter), anti-CD163-PerCp-Cy5.5 (GHI/61; Biolegend), anti-CD8-PE (DK25; Dako), anti-CD28-PE-Cy7 (CD28.2; eBioscience), anti-CD8-Qdot-655 (3B5; Invitrogen), anti-CD44v6-Biotin (VFF-7; Invitrogen), anti-CD4-Qdot-605 (19Thy-5D7; NIH Nonhuman Primate Reagent Resource), anti-CCR2-PE (48607; R&D Systems), and anti-CD64-FITC (22; Trillium Diagnostics). Samples were fixed in PBS containing 2% formaldehyde, acquired on a FACSARia cell sorter (Becton-Dickinson) and analyzed with Tree
Star Flow Jo version 8.7. Monocytes and lymphocytes were first selected based on size and granularity using forward scatter (FSC) area vs. side scatter (SSC) area. From this gate, doublets were excluded (FSC area vs. FSC height). Populations were further identified using negative selection and positive expression of various cell markers using 12-color flow cytometry panels. Complete blood counts were obtained using a CBC Hematology Analyzer (Hema-True, HESKA) and the absolute number of peripheral blood cell subsets was calculated by multiplying the total white blood cell count by the total percentage of each population as determined by flow cytometric analysis. Median Fluorescence Intensity (MFI) values were calculated by subtracting the MFI of the appropriate isotype control.

**Soluble CD163 ELISA and LAL assay for LPS in plasma**

Levels of sCD163 in plasma were determined using an ELISA kit, according to the manufacturer’s protocol (Trillium Diagnostics) as previously described[24]. Endotoxin lipopolysaccharide (LPS) levels in heat-inactivated plasma were measured using the Limulus Amebocyte Lysate (LAL) test (Associates of Cape Cod Inc.) as previously described [24]. Samples were diluted fivefold with endotoxin-free water and heated (30 min at 65°C) to inactivate plasma components. Following incubation with LAL (30 min at 37°C) and chromogen, duplicate samples were read at 570 nm in a photometric plate reader. LPS concentrations were expressed in endotoxin units (EU), with an assay sensitivity range of 0.005 EU/mL - 50 EU/mL.

**Cell migration induced by chemokines or astrocyte supernatant**
Blood from Tysabri treated and control animals was collected into EDTA coated vacutainers and peripheral blood mononuclear cells (PBMCs) were prepared by Ficoll density gradient separation. Chemokines and astrocyte supernatants were prepared from frozen stocks each day. Astrocyte supernatants were cultured and harvested as described in detail [61,62] and warmed to 37°C prior to use. MCP-3/CCL7 was used at a concentration of 100ng/mL, SDF-1/CXCL12 at 10ng/mL, CCL2/MCP-1 at 35ng/mL, and CX3CL1/fractalkine was used at 90ng/mL (all from R&D systems). Control ± chemokine containing medium or Astrocyte Conditioned medium was added to the lower compartment of 3µm pore multiwell insert filters (Becton Dickinson). 100µL of 10⁶/mL PBMCs were seeded into the upper compartment of the transwell and allowed to migrate at 37°C with 5% CO₂. After 2 hours the filters were removed and the number and phenotype of cells in the lower compartment was assessed by flow cytometry. Samples of input cells were also stained in all studies to determine the ratios of CD14+ monocytes: CD3+ T lymphocytes seeded on the transwells. In the MCP-3, SDF-1, and Astrocyte Conditioned media experiments anti-CD3 (FN18, Biosource), anti-CD11b (Bear1, Immunotech), and anti-CD14 (M5E2, BD Biosciences) and previously described flow cytometric analyses [60,63] were utilized. For the CCL2 and CX3CL1 transmigration assays, PBMCs were stained with anti-CD3-Alexa Fluor 700, anti-CD4-FITC (L200; BD Biosciences), anti-CD14-Pacific Blue, anti-CD16-PE-Cy7, anti-CD20-APC (2H7; BD Biosciences), and HLA-DR-Texas Red PE using a published protocol [64].
Results are expressed as fold change in chemotaxis, which represents the difference in PBMC transmigration between basal and stimulated conditions.

### Automated measure of cell adhesion

The xCELLigence system (ACEA Biosciences) monitors changes in electrical impedance across a network of microelectrodes covering the bottom of a tissue culture well. With these changes, the system calculates the Cell Index (CI), a dimensionless parameter that is directly proportional to the area of a well that is covered by adhering cells. xCELLigence E-plates were coated with 10µg/mL of fibronectin (Sigma) for 30 minutes. Purified PBMCs from Tysabri and uninfected macaques were then seeded into wells of the E-plate at a concentration of 2×10⁴ cells/well. After adding cell suspensions to the E-plate, adhesion and proliferation of PBMCs was monitored in 15-minute intervals for fourteen hours by the xCELLigence system. PMA (Sigma) was added at a concentration of 10ng/mL seven hours after cell seeding in order to induce cell adhesion to fibronectin [64]. Using the RTCA Software 1.2 (ACEA Biosciences) the Normalized Cell Index was calculated by dividing the CI value at each time point by the CI value when PMA was added.

### Immunohistochemistry and In situ hybridization

On the day of sacrifice, animals were anesthetized with ketamine-HCl and euthanized by intravenous pentobarbital overdose. Axillary lymph node, intestinal (duodenum, jejunum, and colon), and cerebral (brainstem, frontal cortex, parietal cortex, and occipital cortex) tissues were collected in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 5µm. For
immunohistochemistry, tissue sections were deparaffinized, rehydrated and incubated with blocking reagents. Mature resident monocyte/macrophage and activated microglia were assessed using anti-CD68 (KP1; Dako) and newly infiltrating monocytes were identified by the expression of myeloid/histiocyte antigen MAC387 (MAC387; Dako) as previously described[33]. T-lymphocytes were stained with anti-CD3 (A 0452; Dako) and BrdU+ cells were examined using anti-BrdU (Bu20A; Dako) as previously described [24]. Productive SIV infection was determined with anti-SIV-p28 (3F7; Fitzgerald Industries International) and by in situ hybridization for SIV RNA using anti-digoxigenin labeled SIVmac239 antisense riboprobes that span the entire SIVmac genome (Lofstrand Labs) as previously described [65]. Hybridization specificity was confirmed in each experiment using the SIVmac239 sense probe and matched tissue from uninfected rhesus macaques. For quantification, at least 3 non-serial blind-coded sections from all tissues were stained for each marker. Tissue sections were examined with a Zeiss Axio Imager M1 microscope (Carl Zeiss MicroImaging, Inc.) using a Plan-Apochromat x20/0.8 Korr objective and analyzed by one unblinded and one blinded observer using Adobe Photoshop v11.0.2 software. The minimum number of arbitrary visual fields analyzed in each tissue was 24. From this number a median number of cells per tissue region was calculated. Data are represented as the number of positive cells per unit area (cells / mm²).

_Nucleic acid isolation and qPCR for SIV DNA loads in tissues_
For each tissue examined, ten 15 µm frozen sections were homogenized and washed in 1X PBS (Ca\(^{2+}/\)Mg\(^{2+}\) free; Mediatech Inc.) prior to genomic DNA isolation using the AllPrep DNA/RNA Mini Kit (Qiagen) according to manufacturers instructions. For each sample, 100ng of gDNA was loaded in triplicate wells. The concentration of the gDNA was calculated using the Qubit® 2.0 Fluorometer (Invitrogen). A standard curve was added to each PCR plate, consisting of a plasmid containing 1 copy of the SIV gag gene that was serially diluted from 1e9 copies down to 1 copy per microliter. Each quantitative PCR reaction contained 5µl of a standard serial dilution or sample (diluted to 20ng/µl) and 20µl of reaction master mix containing 12.5µl Invitrogen 2x TaqMan® Universal Mastermix 2, 2.25µl each of 10uM forward and reverse primers, 0.625µl of 10µM TaqMan® probe, and 2.375µl of water. The forward and reverse primers ShehuF 5'-AATTAGATAGATTTGAGATTAGCAGAAAGC and ShehuR 5'-CACCAGATGACGCAGACAGTATTAT and the MGB TaqMan probe ShehuP 6FAM-CAACAGGCTCAGAAAA-MGBNFQ were used as described previously [66]. The PCR was performed using Applied Biosystems 7500 Fast Real-Time PCR System under the following conditions: 95°C 10 min followed by 45 cycles of 94°C 15 s and 60°C 60 s. The lowest limit of detection of the assay was 50 copies per reaction. The number of viral gag gene DNA copies per 100ng of total tissue gDNA was calculated using Applied Biosystems 750 Software v2.0.5.

Statistical methods
Statistical analyses were conducted using Prism version 6.0 (GraphPad Software, Inc.). To detect significant changes in NAA/Cr metabolite ratios during disease progression, analysis of variance with repeated measures (RM-ANOVA) was used. If significant by RM-ANOVA (P < 0.05), Holm-Šídák post-tests were used to isolate significant differences between time points and treatment groups. All other P values were calculated using Student’s two-tailed, unpaired t tests. Statistical significance was defined as P < 0.05. Data are presented as the mean ± the standard error of the mean (SEM).

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Figure 3.1 Stabilization of neuronal injury in SIV-infected animals following natalizumab treatment.

(A) A decreased NAA/Cr ratio in frontal cortex (FC), (B) parietal cortex (PC), (C) basal ganglia (BG), and (D) white matter (WM), was observed in untreated and natalizumab treated animals by four weeks post infection (WPI). Decreased NAA/Cr stabilized with natalizumab treatment (indicated by arrows at 28, 35, and 42 dpi) in the FC (E), PC (F), BG (G), and WM (H). Each point represents the mean ± SEM. P < 0.05* using Holm-Šídák post-tests following significant repeated measures ANOVA.
Figure 3.2

A

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Figure 3.2 Natalizumab treatment blocks the traffic and accumulation of SIV infected monocyte/macrophages in the brain.

(A) Natalizumab treatment on 28 dpi (“late”), resulted in scattered SIV p28+ and RNA+ cells in the CNS (frontal cortex, parietal cortex, occipital cortex, brainstem) relative to controls, all sacrificed with progression to AIDS. Natalizumab at the time of infection (“early”) prevented the traffic of SIV p28+ and RNA+ cells into the parenchyma, while several SIV p28+ and RNA+ cells were evident in tissues of untreated controls that were all sacrificed on 22 dpi. (B) Late natalizumab treatment resulted in decreased numbers of CD68+ macrophages in the brain relative to controls. Numbers of CD68+ macrophages were significantly reduced in the brains of early natalizumab treated animals compared to matched controls. (C) Fewer MAC387+ cells were observed in late natalizumab treated macaques compared to non-treated animals. Significantly less MAC387+ monocytes were detected in early treated macaques than in untreated controls. (D) To determine the timing of monocyte/macrophage egress into the CNS, animals were administered BrdU at various time points. In animals given BrdU after late natalizumab treatment had begun (33 dpi, 24-hours prior to necropsy), there were no BrdU+ monocyte/macrophages any brain region examined. Lower numbers of BrdU+ cells were detected in natalizumab treated than non-treated animals that all received BrdU throughout infection (-9 dpi, 26 dpi, and 24-hours prior to necropsy). No recently recruited BrdU+ monocyte/macrophages were found in the parenchyma of animals treated with early natalizumab. Scale bars:
50 microns. P values calculated using unpaired t tests. \( p < 0.05^* \), \( p < 0.01^{**} \), \( p < 0.001^{***} \).
Figure 3.3

A

B

C

D

E

F

G

H

I

J

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**Figure 3.3** Natalizumab reduces the accumulation of SIV p28+ and RNA+ cells, CD3+ T lymphocytes, and MAC387+ monocytes to the gut.

(A) There are significantly fewer SIV p28+ infected cells in the duodenum, jejunum, and colon tissues from late natalizumab treated animals compared to untreated animals. The numbers of SIV RNA+ cells were similar between both groups. Less SIV p28+ and RNA+ cells were detected in guts of early natalizumab treated macaques compared to controls. (B) Untreated animals had significantly higher numbers of CD68+ macrophages than late natalizumab treated animals. There were comparable numbers of CD68+ macrophages in gut tissues from early natalizumab treated and non-treated animals. (C) Untreated controls had significantly higher numbers of MAC387+ cells in the intestine than macaques starting natalizumab late in infection. Natalizumab treatment early reduced the number of infiltrating MAC387+ monocytes compared to controls. (D) There were fewer CD3+ T lymphocytes in the gut of late and early natalizumab treated animals compared to controls. Lines and error bars indicate the mean ± SEM for each treatment group. Scale bars: 50 microns. P values calculated using unpaired t tests. p < 0.05*, p < 0.01**, p < 0.001 ***, p < 0.0001 ****.
Figure 3.4
Figure 3.4 Reduced LPS and sCD163 levels in plasma with early natalizumab treatment.

(A) There were high levels of LPS in plasma on 8, 12, and 21 dpi in control macaques, with significant differences between natalizumab treated (represented by arrows at 0, 7, and 14 dpi) and untreated groups on days 8 and 12 pi. (B) With natalizumab treatment, soluble CD163 (sCD163) remained stable throughout the study, whereas sCD163 increased significantly in untreated animals at both 12 and 21 days post infection. Endotoxin (LPS) and sCD163 levels were determined in duplicate. Each point represents the mean ± SEM and p values were calculated using unpaired t tests. $P < 0.05^*, p < 0.01^{**}, p < 0.0001^{****}$. 
Figure 3.5 Natalizumab treatment at the time of infection blocks viral DNA
expression in brain, but not in gut and lymph nodes.

(A) SIV gag DNA was undetectable in all but one of the 24 brain tissue samples analyzed from animals treated with natalizumab on the day of SIV infection.  (B) Similar viral DNA copy numbers were found in the duodenum and jejunum tissues of all macaques sacrificed at 22 dpi, regardless of treatment. The lowest concentrations of SIV DNA were present in the colon of natalizumab treated animals, in contrast to untreated macaques, which had much higher levels of SIV DNA in this region.  (C) The number of proviral DNA copies was higher in the axillary lymph nodes of treated than in non-treated animals whereas SIV DNA was lower with natalizumab treatment in mesenteric lymph node tissue. Viral DNA copies were measured in duplicate. Each bar represents the mean ± SEM for each animal group. P values calculated using unpaired t tests.  p < 0.01 **.

Figure 3.6
Figure 3.6 There are fewer CD3+ T cells, but not monocyte/macrophages in lymph node of natalizumab treated animals.

(A) There were similar numbers of p28+ and SIV RNA+ cells in the axillary lymph nodes of all of animals, regardless of treatment or time of sacrifice. (B) Comparable levels of activated resident CD68+ macrophages were detected in all macaques. (C) The numbers of MAC387+ cells were similar in late and early treated versus non-treated macaques. (D) There was a reduction in CD3+ T lymphocytes from tissues of late and early natalizumab treated macaques relative to untreated controls. Each point represents the mean number of positive cells in the three tissue regions examined from a single animal. Lines and error bars indicate the mean ± SEM for each treatment group. Scale bars: 50 microns. P values calculated using unpaired t tests. p < 0.05*, p < 0.01 **.
Figure 3.7
**Figure 3.7 Natalizumab treatment does not affect plasma viral load.**

Comparable levels of SIV RNA were seen in early untreated (n=3, open circles) and natalizumab treated animals (n=6, filled circles), with no effect of antibody administration on days 0, 7, and 14 post infection (dark grey arrows) on the high viral loads visible by 8 dpi. Natalizumab treatment on days 28, 34, and 41 post infection (light grey arrows) also did not affect plasma SIV RNA. Moreover, sustained concentrations of virus in the plasma of 28 DPI natalizumab treated macaques (n=4, filled squares) were even higher than that of untreated animals sacrificed with AIDS (n=4, open squares).
Figure 3.8

A

B

C

D

E

F

Figure 3.8

A

B

C

D

E

F

Figure 3.8
Figure 3.8 *Increased leukocyte numbers and decreased unbound CD49d expression in the circulation of natalizumab treated animals.*

(A) Examination of immune cell subsets in the blood of macaques prior to and following the last natalizumab treatment revealed an approximate doubling of CD4+ T cells, CD20+ B cells, CD14+ monocytes, and CD34+ HPSCs, with numbers T cells, B cells, and HPSCs reaching significance. (B) In early animals, significantly higher numbers of T cells, monocytes, and HPSCs were retained in the periphery following treatment initiation, with the concentration of B cells in the blood also trending towards significance. (C) Increases in the percentage of leukocyte subsets in the blood of late macaques were generally comparable, with only the percent of CD20+ B cells increasing after the last treatment. (D) There was an increased percentage of CD4+ T cells, CD14+ monocytes, and CD34+ HPSCs with early natalizumab treatment. (E) For analysis of unbound α4 integrin (CD49d) expression, staining was performed with saturating amounts of antibody against CD49d. In late treated animals, pre-infusion (27 dpi) α4 expression levels differed between leukocyte subsets, with CD14+ monocytes and CD34+ HPSCs expressing higher levels of CD49d than CD4+ T cells and CD20+ B cells. Late natalizumab treatment significantly reduced the CD49d MFI on all circulating immune cells, indicating successful α4 integrin antagonism. (F) Basal α4 integrin expression levels were higher on monocytes and HPSCs than B cells and T cells isolated from macaques on the day of SIV infection. Following treatment initiation, we found significantly lower CD49d MFI on monocytes, T lymphocytes, and HPSCs. P values calculated using unpaired t tests.
Figure 3.9

A

B

C

D

Uninfected Control
Pre-infection
Prior to natalizumab (27 DPI)
Following last natalizumab

Uninfected Control
Prior to natalizumab (pre-infection)
Following last natalizumab
Figure 3.9 PBMCs have impaired adhesion and attachment capabilities in inflammatory conditions following natalizumab treatment.

Automated analysis of PBMC binding to the ECM substrate fibronectin in real time under constitutive and inflammatory (PMA induced) using the xCELLigence system. (A) A representative attachment curve produced by PBMCs isolated from SIV infected rhesus macaques on 27 DPI where adhesion and attachment to the activated fibronectin matrix on the bottom of the wells is reflected by an increase in the Normalized Cell Index (NCI). There was minimal interaction of PBMCs with fibronectin in unstimulated conditions (light purple and peach), however there was a quick and drastic increase in cell impedance (pink and green) following addition of PMA after approximately 7 hours (black line). (B) Maximal NCI values were calculated in uninfected (n=3, white bar), as well as for pre-infection (n=2, light grey bar), 27 DPI (n=2, black bar), and following natalizumab (n=2, dark grey bar) PBMCs. (C) An attachment curve demonstrating the difference in NCI between PBMCs from uninfected animals (red and blue) and following the last natalizumab treatment (pink and green) in early treated animals. Purple, peach, and light blue lines illustrate very little PBMC adherence and attachment in unstimulated (- PMA) conditions. (D) The maximal NCI was calculated for PBMCs from uninfected controls (n=5, white bar), pre-infection (n=6, light grey bar) and following the last natalizumab treatment (n=6, dark grey bar).
Figure 3.10
Figure 3.10 Migration of CD14+ monocytes and CD3+ T lymphocytes towards chemokines/cytokines is inhibited with natalizumab treatment.

Differences in migratory capabilities of PBMCs isolated from natalizumab and uninfected macaques were assessed by their the ability to migrate through a 3 µm collagen transwell in response to CCL2, CX3CL1, Astrocyte Conditioned Media (ACM), SDF-1, and MCP-3 stimulation. (A) The ability of CD14+ monocytes to migrate in response to all chemokines except SDF-1 was higher after four weeks of infection (n=4, black) than for uninfected (n=4, white bars) and pre-infection (n=4, light grey) cells. However, there was a significant reduction in the ability of monocytes from late natalizumab macaques to migrate towards CCL2, CX3CL1, ACM, and CCL7 following the last treatment. (B) The capacity of CD3+ lymphocytes isolated from late macaques to migrate towards CCL2 and CX3CL1 was markedly decreased following the last natalizumab treatment, however there were no significant changes in T cell migration towards the other three inflammatory stimuli examined. (C) There was a highly significant inhibition of monocyte chemotaxis towards CCL2, CX3CL1, ACM, and CCL7 with natalizumab beginning on the day of infection (uninfected: n=3, white bars; pre-infection: n=6, light grey bars; following last treatment: n=6, dark grey bars). (D) T lymphocyte responsiveness under proinflammatory conditions was also impacted by early treatment, as cells were much less likely to migrate towards CCL2, CX3CL1, ACM, and CCL7 following the last dose of natalizumab. P values calculated using unpaired t tests. p < 0.05*, p < 0.01 **, p < 0.001***.
REFERENCES


51. Stüve O, Bennett JL (2007) Pharmacological properties, toxicology and scientific rationale for the use of natalizumab (Tysabri) in inflammatory


CHAPTER IV

Summary and Discussion
In the cART era, HIV infection of the CNS can result in the development of HIV-associated neurological disorders (HAND) in more than half of infected individuals, and the prevalence of HAND continues to rise [1]. Events leading to neuronal injury are not well understood, but the presence of activated and productively infected macrophages in the CNS are the best correlates of neurocognitive decline [2,3]. Additionally, many studies have emphasized the relationship between expansion of activated monocytes and the development of neurological disease [4-13]. It appears that persistently elevated numbers of CD16+ monocytes are more tightly linked to the development of neurological symptoms than the number of virally infected cells in the CNS or CSF viral load [12,14,15]. Levels of HIV DNA in CD16+ monocytes are also closely associated with the severity of neuropathology [16,17]. Yet, while activation of immune cells in the periphery is clearly important to the timing of HIV disease progression, the precise role of trafficking monocytes in neuronal damage and the development of HAND are not well defined. The central hypothesis addressed in this thesis is that ongoing traffic of accumulation of activated monocytes/macrophages is essential for CNS pathogenesis with viral infection. To test this hypothesis, we directly examined the effects of both reducing monocyte activation and viral infection, and blocking leukocyte traffic, on neuronal injury and the accumulation of infected macrophages in tissues.

**Is monocyte activation required for CNS damage?**

In the second chapter of this dissertation, we hypothesized that decreased monocyte activation with minocycline treatment plays a neuroprotective role in
the context of rapid SIV infection. In these experiments, we found that suppression of monocyte activation was sufficient to reverse neuronal injury. Treatment with minocycline prevented the development of SIVE, reduced damage to neuronal synapses and dendrites, and stopped further neuronal death [18]. Importantly, the inhibition of monocyte activation that was observed within days of beginning minocycline treatment directly correlated with reversal of neuronal damage. From these findings we concluded that diminished monocyte activation results in decreased numbers of cells trafficking into the brain and reduced neuronal injury.

In addition to suppressing monocyte activation, both plasma viral loads and the number of virally infected macrophages in lymph nodes were significantly reduced in minocycline treated macaques. We found a similar effect in vitro, where minocycline down-regulated CD16 expression, reduced chemokine and cytokine production, and suppressed SIV replication in monocytes/macrophages. While minocycline was not engineered to have antiviral functions, data suggest that minocycline might also control viral replication by binding to the active site of HIV integrase, thus inhibiting enzyme function [19]. Additionally, minocycline has been shown to directly interfere with the release of infectious virions through repression of nuclear factor (NF)-κB activation [20] and to reduce HIV and SIV infection through indirect mechanisms. By preventing the phosphorylation of IκB kinase (IKKα/β) and inhibitor of nuclear factor-κB alpha (IκBα), minocycline modifies chemokine (CXCL10, IL-8, MCP-1,MIP-1α) and cytokine (IFN-γ, IL-6, and TNFα) production [21]. Minocycline can suppress SIV infection in
Box 1. Conclusions from studies in Chapter 2.

- Ongoing monocyte activation is required for the development of neuroAIDS
- Decreased activation of monocytes results in lower CNS traffic
- Lower plasma viral loads, decreased infection of monocytes, and the ability of minocycline to cross the BBB and modulate changes within the CNS directly may also contributed to the neuroprotective effects observed with minocycline treatment

Following the publication of our results describing anti-inflammatory and neuroprotective effects of minocycline in the SIV model of rapid neuroAIDS [10], another study in SIV infected macaques provided additional support for the beneficial effects of minocycline in the CNS with viral infection. These experiments demonstrated that three months of minocycline treatment (4mg/kg/day) starting on 12 dpi (acute infection), but not on 21 dpi (asymptomatic infection) prevented damage to dopaminergic neurons, dopamine loss, and nigrostriatal dysfunction in the CNS [24]. Similar to our findings, this work
emphasizes that significant CNS injury is occurs during acute infection. Our data indicated that minocycline treatment during asymptomatic infection stabilizes neuronal damage that is already present, while these results imply that neuronal deterioration during acute infection can be prevented completely with minocycline treatment. Taken together, these studies suggest that the earlier treatment is initiated, the more effective it is at exerting neuroprotective effects. Overall, our findings in Chapter 2 indicate that early infection of the CNS is not sufficient for the development of neurological disease by demonstrating that suppression of monocyte activation during asymptomatic infection reverses neuronal injury that has already occurred, and prevents the development of SIVE. Based on these results, it is reasonable to suggest that reducing monocyte recruitment into the brain in HIV-infected individuals with minocycline or similar adjunctive therapies could quell macrophage activation and infection, suppress production of chemokines and cytokines, and thus inhibit the induction of CNS pathology.

**Is ongoing traffic of leukocytes necessary for HIV-associated pathogenesis in the CNS and small intestine?**

In the third chapter of the thesis, in order to determine whether continuous recruitment of cells from the periphery into brain and gut tissues is required for SIV-associated damage to these organs, we directly blocked leukocyte trafficking with the anti-α4 antibody natalizumab. Natalizumab has been utilized to prevent the accumulation of leukocytes in the CNS of patients with Relapsing-Remitting Multiple Sclerosis [25] and the small intestine of patients with Crohn’s disease [26], yet no one has used natalizumab in HIV infection or in an animal model to
directly assess the requirement of ongoing monocyte traffic on viral pathogenesis. Natalizumab treatment was initiated at 28 dpi (“late”) when CNS injury was already evident in SIV-infected animals. Despite significant damage, stopping monocyte migration into the brain stabilized neuronal injury, reduced the number of productively infected macrophages, and disrupted CNS lesion formation. These data provide strong evidence that continuous recruitment of cells into the brain is critical for the maintenance and progression of neuropathology, and that blocking leukocyte migration during chronic viral infection is sufficient to prevent further CNS injury. We were also intrigued to find significantly fewer productively infected SIV p28+ and RNA+ cells in the small intestines of “late” natalizumab treated relative to untreated macaques, suggesting to us that leukocyte trafficking is vital to the propagation of SIV replication in the small intestine during infection. To our knowledge, this is the first time that blocking the continuous trafficking of leukocytes has been shown to be necessary for the perpetuation of viral infection in both the brain and the gut.

*Is leukocyte traffic required for the initial seeding of brain and gut tissues with cell-associated virus?*

In the last set of experiments, we addressed the long-standing question of whether leukocyte traffic is required to establish viral sanctuaries in tissues. We found no SIV p28+, RNA+, or DNA+ cells in the brains of natalizumab-treated macaques, indicating that “early” treatment was sufficient to prevent CNS infection. Through these experiments, we demonstrated that migration of infected leukocytes across the BBB is required for viral infection of the CNS and
that during acute disease, monocytes/macrophages play a critical role in the formation of a latent reservoir in the brain. By inhibiting cell traffic on the day of infection, we also gained a greater understanding of how the viral sanctuaries in the small intestine are initially established. Comparable concentrations of proviral DNA were isolated from the duodenum, jejunum, and colon of all animals, suggesting that leukocyte trafficking is not required for the seeding of gut tissues with virus. In general, the mechanisms regulating initial infection in GALT are relatively unclear, but seem to depend on the route and site of transmission [27]. More than a decade ago, two studies demonstrated that when primary transmission occurs across mucosal surfaces, cell-free virus enters the gut through transcytosis across intestinal M cells [28,29]. It may be the case that cell-free virus was also capable of establishing infection in the GALT following intravenous transmission in SIV-infected macaques. Our results indicate that cell migration into the GI tract during acute infection does contribute to significantly increased levels of SIV replication, microbial translocation (LPS), and peripheral immune activation (sCD163), however. Importantly, HIV can directly bind to the α4β7 on CD4+ T cells [30], and while this binding is not sufficient for viral entry [31], it does result in T cell activation and LFA-1 expression, which in turn promotes cell-to-cell transfer of HIV and migration of cells across the gastrointestinal mucosa [32]. We postulate that natalizumab binding likely inhibited the ability of virus to interact with α4β7 expressing CD4+ T cells, reducing mucosal activation and LFA-1 mediated cell-to-cell spreading of infectious virions, and resulting in significantly reduced numbers of productively
SIV p28+ and RNA+ cells present in the gut.

**Box 2. Conclusions from studies in Chapter 3.**

- Continuous traffic of monocytes/macrophages is required for neuronal injury and maintenance of CNS lesions
- Early trafficking of leukocytes is critical for seeding the CNS and contributes to seeding of the small intestine with cell-associated virus
- Natalizumab did not prevent viral invasion in the gut, but significantly inhibited SIV replication, microbial translocation, and immune activation

In summary, our experiments with natalizumab demonstrate that interaction between α4 ligands on leukocytes and integrin molecules on endothelial cells is necessary for transmigration of virally infected cells into tissues. These data are critical to better understanding how to modulate leukocyte transmigration, and highlight the notion that targeting specific interactions (i.e. α4β1 and VCAM1 vs. α4β7 and MaDCAM1) may be useful for designing novel adjunctive therapies that selectively target infection in protected viral sanctuaries. We found that blocking leukocyte traffic with natalizumab during early infection prevents the seeding of brain with cell-associated virus and significantly reduces the number of productively infected cells in the gut, suggesting that along with cART, natalizumab treatment in HIV+ individuals soon after exposure might prevent the development of HIV-associated pathology in these tissues. In addition to preventing the recruitment of cell-associated virus into latent tissue reservoirs, our data in “late” natalizumab-treated macaques indicate that even if natalizumab treatment is initiated during the asymptomatic period of infection, inflammation
and infection in the brain and gut are still suppressed. Thus, it seems that even if HIV is given the opportunity to establish latent infection in the brain and gastrointestinal tract of individuals, preventing further traffic of leukocytes with natalizumab treatment would still be beneficial for reducing inflammation and productive viral replication in these tissues. Moreover, our ex vivo experiments revealed that natalizumab treatment restricted chemotaxis of T cells and monocytes towards potent inflammatory cytokines/chemokines, implying that natalizumab therapy could also serve to diminish the migration of cells into tissues that are typically replenished by leukocytes. It is possible that keeping infected leukocytes in the periphery might also allow antiretrovirals to more effectively target these cells. Because there are no current data to suggest that reactivation strategies can rid latently infected monocytes and macrophages of virus, preventing activated cells from leaving the blood may be an efficient way to combat persistent HIV infection.

**Why are novel therapies needed to target latently infected monocytes/macrophages in the CNS and GALT?**

Despite complete suppression of virus in the plasma, there are several factors that are likely contributing to persistent inflammation and infection in the CNS with effective therapy [33]. Multiple publications have reported that the majority of ART compounds have limited neuropenetrance [34-36], and that CNS levels of antiretrovirals below the therapeutic threshold can result in the development of drug resistant viral strains [37]. There are some data to suggest that treatment with compounds that have the highest CNS penetration (CPE)
scores improve cognitive functioning, medication adherence, employment outcomes, and overall quality of life [38-41]. However, other studies assessing the relationship between CPE and neurocognitive performance have yielded inconsistent results [35,42-44]. This suggests that the ability of drugs to enter the CNS may not be sufficient to determine efficacy in treating the symptoms of cognitive deficits. In addition, most of the currently available antiretroviral drugs that are capable of penetrating the CNS have also been shown to be neurotoxic [45,46]. Three recent studies indicate that the ability of an antiretroviral to target CNS macrophages is the most accurate indication of how useful it will be in treating clinical symptoms of HIV infection in the brain [12,15,47]. These data, along with the fact that viral sequences isolated from the brains of HIV infected individuals have macrophage-trophic motifs [48,49], clearly demonstrate that in order to be effective in the CNS, a therapy will need to target monocytes and macrophages.

Nevertheless, it appears that no existing ART compound sufficiently treats HIV infection in these cells [50-54]. Protease inhibitors are frequently prescribed, and are effective in stopping the release of infectious virions from productively infected macrophages, however this class of drugs is ineffective against latent infection, and is generally more useful for targeting infected lymphocytes [55,56]. Similarly, because nucleoside reverse transcriptase inhibitor (NRTI) activity relies on competition with dNTPs for DNA binding, the ability of these drugs to target macrophages depends on the activation status of the cell [57]. As a result, these drugs are not useful against resting macrophages that contain integrated provirus
The non-nucleoside reverse transcriptase inhibitors (NNRTIs) are another commonly used class of antiviral drugs that function by inhibiting viral reverse transcriptase activity [51], and are effective in preventing new infection and productive viral replication. Yet, like NRTIs, because NNRTI activity affects events occurring upstream of viral integration into the host cell genome, so these compounds have also been rendered ineffective against latent infection in macrophages [58]. A newer and highly promising drug called raltegravir prevents integration of the HIV genome into host cell DNA, and thus is just as potent in targeting macrophage infection as it is lymphocyte infection [59]. Additionally, raltegravir has been shown to reach therapeutic concentrations in the CSF [60]. Yet a single mutation in the integrase gene is sufficient to render macrophages resistant to drug activity [61]. Overall, of the 27 currently available antiretroviral therapies, only a handful can reduce inflammation and prevent viral replication in the CNS. As result, latent infection and activation of CNS macrophages and microglial cells persist despite therapy, and contribute significantly to neuronal injury and the perpetually increasing prevalence of HANDs [62-64].

Similar to the CNS, all currently available antiretroviral drugs are ineffective against latently infected macrophages in the GI tract [65]. While initiating cART within three weeks of exposure to HIV suppresses productive viral replication [66] and limits the extent of damage to the gut mucosa [67], starting therapy very early does not prevent HIV infection or CD4+ T cell depletion in the gut [68]. Even if cART begins during acute infection and is infective long-term, concentrations of proviral DNA do not decay [69,70], viral replication persists
numbers CD4+ T cells in the lamina propria are never completely restored [72-75]. As a result, novel antiretroviral or adjunctive therapies will be necessary to combat perpetual infection and periodic viral replication in the small intestine of HIV-infected individuals.

**How is persistent infection in tissue reservoirs preventing eradication of HIV from the body?**

Regardless of when cART is started, and despite strict adherence to therapy, currently available treatments are not sufficient to completely rid the body of HIV, as all compounds are futile against persistent and periodic viral replication in T cells, monocytes, and macrophages in the blood, brain, and GI tract. These cellular and anatomic reservoirs of infection remain a source of residual viremia throughout the course of infection [76]. Reactivation of these perpetuates peripheral immune activation [16,77,78], and supports the continual transmigration of cells into the blood and other tissues, providing the body with a constant source of new targets for viral infection [79].

Activation of latently infected leukocytes in the GI tract perpetuates mucosal damage, microbial translocation, and contribute to generalized immune activation in the periphery [80-82]. What is more, this reservoir of latently infected cells in the GALT contain viral sequences with distinct drug resistance mutations from the sequences carried by latently infected cells in the blood [83]. As a result, it is common for infectious particles that are released from the gut into the periphery to be impervious to antiretroviral treatment [84]. When administered during acute infection, novel therapies such as natalizumab have
the potential to decrease gut inflammation and prevent disruptions to the gastrointestinal lumen with HIV infection. Consequently, this treatment could prove useful in suppressing chronic peripheral immune activation and the spread of infectious virions from the gut into the circulation.

Although still the source of some speculation, early studies in rodents demonstrated that not only can monocytes emigrate into the CNS, but that perivascular macrophages can also traffic back out of the brain [85]. In addition, phylogenetic analyses of viral sequences in the brain and periphery have also recently confirmed that HIV can transit out of the CNS and reseed the peripheral circulation with infection [86]. Analogous to the GALT, viral sequences in the brain carry different antiviral resistance mutations than sequences in the periphery [87]. It is likely that these drug-resistant strains proliferate and evolve in the CNS [88], then proceed to migrate out of the brain and contribute to infection in the periphery [89].

It is apparent that high levels of inflammation persist in the CNS and gut throughout disease and despite cART, implying that infected cells may be leaving these compartments and reseeding the periphery with drug resistant virions at any point in infection [90,91]. To this end, latent infection in brain and gut tissues is a critical barrier to the eradication of HIV [64,92]. The body will not be free of infection until the virus can be cleared from infected monocytes/macrophages [93], or if novel adjunctive therapies prove to be effective in preventing the establishment of tissue sanctuaries in the first place.
Summary

In conclusion, the results described in this dissertation provide novel clarity around the role of monocyte traffic in the progression of HIV and SIV infection. Our studies emphasize that events during the acute period of disease are critical for determining the overall course and severity of pathology. What is more, these data indicate that continuous migration of monocytes into the brain and gut throughout infection is necessary to perpetuate damage in these tissues. These results provide a deeper understanding of how reservoirs are initially established with viral infection, and about the factors underlying viral persistence in the small intestine and brain. While there remain no antiretroviral or adjuvant therapies that are designed to treat latently infected monocytes/macrophages, the data presented in this thesis underscores the need to specifically target these cells. We demonstrated that minocycline and natalizumab inhibit monocyte/macrophage infection and impede the cascade of peripheral inflammatory events resulting from and perpetuating disease, indicating that these therapies have the potential to effectively limit the establishment and size of viral sanctuaries.
REFERENCES


53. Gavegnano C, Schinazi RF (2009) Antiretroviral therapy in macrophages:


63. Graham DR, Gama L, Queen SE, Li M, Brice AK, et al. (2011) Initiation of HAART during acute simian immunodeficiency virus infection rapidly


