CHRONIC INFLAMMATION IN HIV INFECTION: EFFECTS ON MECHANISMS OF T CELL LYMPH NODE EGRESS, HOMEOSTASIS, AND TURNOVER

by

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Chronic Inflammation in HIV Infection: Effects on Mechanisms of T Cell Lymph Node Egress, Homeostasis, and Turnover

Abstract

by

JOSEPH CHRISTOPHER MUDD

Lymphoid tissues are major sites of inflammation in HIV infection and this is manifested clinically by a generalized lymphadenopathy. We hypothesized that lymphocytes could be sequestered in HIV-1+ lymph nodes (LN) through impairments in the Sphingosine-1-phosphate (S1P)/Sphingosine-1-phosphate-receptor-1(S1P1) axis. To test this hypothesis, we developed novel assays for S1P-induced Akt phosphorylation and actin polymerization. In HIV-1+ LNs, CD4 and CD8 T cells had impaired responses to S1P in all maturation subsets. We find that LN T cells expressing the S1P1-antagonizing factor CD69 are unable to respond to S1P in either assay. Chronic inflammation in HIV-1+ LNs may contribute to impairment in T cell egress through decreased S1P responsiveness and to immune dysregulation in a key organ of immune homeostasis.

Some treated HIV+ individuals display residual inflammation despite undetectable viremia. This has been associated with suboptimal CD4 T cell reconstitution. We explored underlying mechanisms that may link residual inflammation to suboptimal CD4 T cell reconstitution. We find that the pro-inflammatory cytokines IL-6 and IL-1β induce memory T cell turnover \textit{in vitro}. IL-6 and IL-1β also impaired T
cell responsiveness to IL-7, a major determinant of T cell homeostasis. In lymphoid tissues, we find elevated expression of IL-1β in HIV-infected patients that does not normalize with ART. Induction of CD4 T cell turnover and diminished T cell responsiveness to IL-7 by IL-1β and IL-6 exposure may contribute to the lack of CD4 T cell reconstitution in treated HIV infected subjects.

Chronic inflammation that persists in treated HIV-infection has been linked to non-AIDS serious events such as cardiovascular disease. CD8 T cells can express the fractalkine receptor, CX3CR1, that allows rolling and tethering along vascular endothelium. We find that treated HIV-infected patients display increased frequencies of CX3CR1+ CD8 T cells when compared to proportions of CX3CR1 CD8 T cells of healthy controls. The thrombin-binding receptor, PAR-1, is highly expressed on CX3CR1+ CD8 T cells and is increased on CD8 T cells of treated HIV+ patients. Taken together, these findings highlight a central role of inflammation in driving immune dysfunction as well as contributing to the risk of adverse clinical events such as cardiovascular disease.

Introduction

The function of the lymph node

The lymph node (LN) provides a structural background to support the complex interactions among multiple cell types. It is at this anatomical site where both humoral and cell mediated immune responses are generated in response to invading pathogens. The importance of the LN is highlighted by the fact that lymphotoxin-α deficient mice that lack organized lymphoid structures, exhibit profound impairments in immune
responses to foreign antigens (1). Thus, the lymph node is essential in maintaining proper immune function.

The predominant immune cell types present within lymph nodes comprise naïve and central memory T cells as well as B cells that take up residence within lymphoid follicles. T cells home to LNs in a receptor-specific fashion and are selectively retained within these sites where they receive signals mediated by Interlukin-7 (IL-7), promoting their survival and homeostasis. Antigen presenting cells (APC) also home to and accumulate within LNs, and upon uptake of foreign microbes, will present exogenous antigen to naïve and central memory T cells in a peptide-MHC dependent manner. Cognate peptide-MHC interactions between APCs and T cells are extremely rare events. To increase the probability that this event will occur, T cells migrate along specialized conduit networks within the LN where APCs localize, thus enhancing the chances of antigen-specific interactions (2). Upon antigen encounter, carefully orchestrated cognate interactions occur between the APC and T cell, forming the molecular basis of a cell-mediated immune response. Co-stimulatory signals provided in trans by the APC promote clonal expansion and differentiation of the T cell. In the context of CD8+ T cell antigenic responses, cytokines elaborated by the APC such as IL-2 and IL-15 will promote cytotoxic effector function that aids in the killing of target cells (3), whereas cytokine-driven signals in CD4+ T cell differentiation will skew the effector progeny towards a Th1 (Interferon-γ secreting), Th2 (IL-4 secreting), Th17 (IL-17 secreting), T follicular helper (IL-21 secreting), or T regulatory (IL-2/TGFβ secreting) phenotype. Effector T cell maturation is also associated with an up regulation of inflammatory chemokine receptors that direct them to extralymphoid sites. Egress of effector cells
from lymphoid tissues is very important, as their potent cytotoxic function could potentially induce the cell death of critical antigen presenting cells as well as interfere with naïve and central memory T cell homeostasis. Thus, effector T cells are encouraged to not stay very long in lymphoid tissues, and are programmed to efficiently egress from the LN and home to peripheral tissue sites.

**Lymph node inflammation in HIV infection and its effect on T cell egress**

In HIV infection, the function of the lymph node is disrupted. While exact descriptions on how orderly interactions may be impaired within LNs is lacking, profound dysregulation at these sites is evidenced by impaired immune responses to both neo and recall antigens unrelated to HIV (4).

Why are there global immune impairments to a variety of pathogens other than the virus itself? It is important to note that unlike other viral infections, lymphoid tissues are the predominant anatomical site of HIV replication. Viral burden in lymphoid tissues is several fold higher than in peripheral blood (5). In fact, lymphoid tissues contain 5 to 10 times greater frequencies of infected CD4 T cells when compared to frequencies of infected CD4 T cells in peripheral blood (6). Through direct CD4 T cell killing as well as ligation of viral products to pathogen recognition receptors, sustained viral replication at these sites promotes a pro-inflammatory immune environment that persists throughout the chronic phase of HIV infection. This has been shown both at the single cell level (7), as well as in lymph node histocultures of HIV infected patients, where there was constitutive expression of a number of proinflammatory cytokines such as IL-1β, IL-15, IL-2, and IFN-γ (8). Chronic lymphoid inflammation results in an accumulation of TGFβ-producing CD4 T
regulatory cells that promote the deposition of collagen within LNs. Collagen deposition at these sites directly impedes T cell access to IL-7 through destruction of the Fibroblastic Reticular Cell (FRC) network, contributing to loss of the naïve T cell pool (9).

Lymphoid inflammation is manifested clinically in HIV-infected patients by a generalized lymphadenopathy (10), a pathologic characteristic that has been noted since the earliest days of the epidemic. Abnormal T cell accumulation within lymphoid tissues is a characteristic feature of HIV-associated lymphadenopathy. This is evidenced by the entrapment of recent thymic emigrant (RTE) naïve T-cells in HIV(+) LNs, as well as an enrichment of effector memory T-cells that normally take up residence in extralymphoid peripheral tissues (11, 12). Abnormal lymphoid accumulation of T-cells is closely associated with viral replication at these sites. This is demonstrable through the restoration of circulating CD4 T cells that occurs in two distinct phases following inhibition of viral replication with ART. The initial phase of CD4 T cell replenishment occurs very rapidly within the first several weeks of virological suppression, followed by a delayed, more gradual rise in circulating CD4 T cells that occurs within the subsequent months of ART (13). The first phase of rapid CD4 T cell replenishment is reflective of T cell re-distribution rather than de novo T-cell production, as decreases in T cell numbers within lymphoid tissues are concomitant with rises in T-cell numbers found in circulation (14). This implies that something related to viral replication inhibits T-cells from exiting lymphoid tissues, resulting in the abnormal accumulation of T-cells within sites of high viral replication. Taken together, these studies indicate that immune reconstitution induced by
pharmacological control of viral replication is associated with an improvement in T cell exit from lymphoid tissues.

The sphingosine-1-phosphate-receptor axis controls lymph node egress and is negatively regulated by inflammation

Continual circulation of lymphocytes is necessary for immune homeostasis. From the blood, a lymphocyte will home to secondary lymphoid tissues in a CCR7-dependent manner via CCL19 and CCL21 chemokine gradients produced by high endothelial venules (HEVs) which are sites of LN entry. Glycam-1 present on lymphatic HEVs will interact with the L-selectin CD62L that is expressed on T cells. These interactions will mediate T cell adhesion to HEVs and facilitate extravasation into the lymphoid interstitial space. Upon LN entry, T-cells may interact with antigen presenting cells (APC) found within the paracortical zone. APCs presenting self-peptides will promote low affinity TCR interactions that promote T cell survival and homeostasis, whereas much stronger interactions will occur if a T cell binds its cognate peptide presented by the APC. If a T-cell does not encounter its cognate peptide, it will egress through efferent lymphatic vessels to subsequently recirculate into peripheral blood. While mechanisms of T-cell entry into lymphatic tissues have been well characterized, the requirements for T-cell egress from LNs have only recently been elucidated. Mechanistic insights into T cell egress were first discovered through studying the effects of the immunosuppressive drug, FTY720, on lymphocyte trafficking. It was found that in mice, experimental autoimmune encephalitis (EAE) could be attenuated through the administration of this drug (15), and this was due to a functional block on a T-cell’s ability to exit lymphoid tissues. FTY720 is a potent
agonist of four of five G-protein coupled receptors specific for the lysophospholipid S1P: S1P$_1$, S1P$_3$, S1P$_4$, and S1P$_5$ (16). T-cells predominantly express the S1P$_1$ and S1P$_4$ receptor subtypes (17), and it was found that FTY720 exerts an inhibition of T-cell egress by binding the S1P$_1$ receptor to induce its internalization and subsequent degradation (18). A non-redundant role of S1P1 in controlling T cell egress was further reinforced by the observation that conditional knockout mice lacking T cell S1P1 displayed identical T cell migratory phenotypes as mice treated with FTY720 (19).

Structurally, FTY720 is very similar to S1P, the only known physiological ligand of S1P1. S1P is formed by the phosphorylation of sphingosine, a reaction that is catalyzed by two isoforms of sphingosine kinase, SPHK1 and SPHK2 (20). Erythrocytes and platelets are the main producers of S1P in circulation (21). The constitutive production of this molecule by these cell types results in concentrations of S1P in the bloodstream in the low micromolar range (22). S1P is also found in lymph fluid where it is present at nanomolar concentrations. The source of lymph S1P has been shown to be distinct from the source of S1P in circulation, with lymphatic endothelial cells shown to be the main producer (22). Concentrations of S1P are regulated by S1P degrading enzymes. The S1P degrading enzyme S1P lyase is not only critical in tightly regulating plasma and lymph S1P concentrations, but is also essential for maintaining the S1P-free environment of the lymph node, as S1P in lymphoid organs increases considerably after lyase inhibition by treatment with the compound 2-acetyl-4- tetrahydroxybutylimdazole (23). Differential concentrations of S1P in distinct anatomical compartments serve to establish a gradient that chemotactically
guides T-cells from the S1P-free environment of the lymph node to high concentrations of S1P in the bloodstream. This chemotactic migration is dependent upon S1P1 receptor ligation. Upon binding to S1P, the activated S1P1 receptor will immediately transduce a signal to its $G_{\alpha_i}$ G-protein subunit, in turn promoting the activation of phosphatidylinositol-3-kinase (PIP3K) (24). PIP3K lies directly upstream of Akt and the small GTPase Rac, and upon activation, promotes both Akt phosphorylation and Rac activation. These signaling events trigger a polymerization of the actin cytoskeleton (25), resulting in a polarized migration towards the S1P gradient. An additional consequence of S1P1 signaling is the recruitment of proteins such as β-arrestins and G-protein-coupled-receptor kinases (GRKs) that promote S1P1 internalization and receptor desensitization, a process that is induced when a T-cell is exposed to the S1P-high environment of the bloodstream. T-cells will recycle S1P1 back to the cell surface upon transit through the S1P-free environment of the lymph node. From here, a multistep model of T-cell egress has been proposed by Cyster and colleagues in which T-cells migrate into cortical sinuses in an S1P1-dependent manner, at which time these T-cells are carried by lymphatic flow into medullary sinuses and efferent lymph, where higher S1P concentrations produced by lymphatic endothelial cells are present (26).

T-cell egress is ultimately dependent upon the mechanisms that regulate S1P1 expression. These mechanisms were first described through studying the effect of T-cell activation on S1P1 expression. It has been known for many years that within the first hours of an inflammatory stimulus, T-cell exit from draining lymph nodes is blocked, leading to an accumulation of T-cells within inflamed lymph nodes. One of the earliest markers that is expressed on the cell surface following T-cell
activation is the C-type lectin CD69. In murine systems, upregulation of CD69 through \textit{in vivo} exposure to type I interferons was associated with a loss of S1P1 expression, an inability of T-cells to migrate towards S1P, and a disappearance of T-cells from lymph (27). This led to the finding that CD69 associates with S1P1 through hydrophobic interactions to antagonize S1P1 at the cell surface (28), functionally inducing a block to S1P1-mediated T-cell egress. Despite active mechanisms such as CD69 antagonism that inhibit S1P1 surface activity in the early stages of T-cell activation, message levels of S1P1 remain high (29). However, within a day following TCR-driven activation on murine T cells, S1P1 mRNA levels diminish dramatically, implying a loss of S1P1 transcriptional induction. In mice, PI3K activation induced by TCR signaling induces cytosolic translocation of the Forkhead transcription factor Foxo1 that controls KLF2 expression (30), resulting in reductions in levels of the transcription factor KLF2. S1P1 mRNA levels were found to be concomitant with sharp decreases in KLF2 (31), and not surprisingly, it was shown that KLF2 can directly bind the S1P1 promoter in murine lymphocytes to induce S1P1 expression (32). Thus, T-cell dwell time within lymphoid tissues is sustained during adaptive immune priming through negative regulation of S1P1. This is accomplished by the inhibition of S1P1 surface activity through CD69 upregulation, and diminished S1P1 production through decreases in KLF2. It is important to note that egress inhibition of T-cells is merely transient in the setting of an immune response.

Sustained crosstalk with dendritic cells will promote T-cell division and subsequent maturation, and it was found that CD69 surface expression progressively decreases with subsequent rounds of T-cell division (33). Furthermore, KLF2 is re-expressed in
murine T-cells with transition towards a memory phenotype, and this was shown to be dependent upon low doses of cytokines that promote T-cell maturation, such as IL-2, IL-12 and IL-7 (29). Thus, once T-cells mature to effector T cells, they are able to exit lymph nodes through mechanisms that rescue both S1P1 activity and production.

While most studies of T cell egress have been performed in mice, it is likely that the same mechanisms are operative in humans. The S1P1 functional antagonist FTY720 has proven effective in treating patients with relapsing multiple sclerosis (MS), and its primary mechanism of action involves an inhibition of autoreactive T cell trafficking to the CNS through blockade of lymph node egress. In MS patients, FTY720 selectively reduced the numbers of circulating CCR7-expressing naïve and central memory T cells. In contrast, CCR7-negative effector and effector memory T cells remained in blood, presumably because these cells do not migrate in and out of lymphoid tissues. While lymphocyte circulation patterns were dramatically altered with FTY720 treatment, T cell proliferation and cytokine production remained unaffected upon stimulation \textit{ex vivo}. Thus, the immunomodulatory effects of FTY720 are thought to be primarily attributed to alterations in lymphocyte recirculation patterns. It is important to note that the therapeutic efficacy of FTY720 may not solely be attributed to its modulation of S1P1 on T cells, as this receptor is also expressed on neuronal and endothelial cells. The role of S1P1 modulation by FTY720 on these cell types and how it pertains to mechanism of action is less well characterized however.

Inflammation has profound effects on T cell circulation patterns. In addition to HIV, other viral infections can induce a transient shutdown of T cell egress that is associated with lymph node enlargement. Additionally, a well-known side effect of
Interferon-alpha/Ribavirin therapy that is used to treat HCV-infected patients involves a transient lymphopenia. While the mechanisms of egress shutdown in these settings are not characterized, prior work done by our group has shown that interferon, as well as a number of other pro-inflammatory mediators known to be increased in HIV infection can upregulate CD69 expression on CD8 T cells \textit{in vitro}. In the first part of our work, we characterize the S1P1 receptor for the first time in human T cells and show that activity of this receptor is blunted in lymph node T cells from untreated HIV+ patients, providing a link between and chronic lymphoid inflammation and dysregulation of the S1P1/S1P axis that controls LN egress.

\textbf{Residual inflammation in treated HIV infection: Effects on T cell homeostasis, turnover, and CD4 T cell reconstitution}

The natural course of HIV infection can be altered with antiretroviral therapies (ART) that inhibit viral replication. Pharmacological inhibition of viral replication is associated with immune reconstitution that reflects a gradual rise in the circulating CD4 T cell pool. As noted earlier, increases in circulating CD4 T cells in response to ART are biphasic in nature, comprising an initial rapid and dramatic increase in circulating CD4 T cells that is reflective of lymphocyte redistribution, followed by a delayed, more gradual rise of circulating CD4 T cells (13, 14). ART initiation also results in dramatic changes in indices of inflammation, both locally and systemically. Many genes associated with innate host defenses are downregulated in lymphoid tissues in the first several weeks of ART administration (34), and circulating levels of a number proinflammatory cytokines such as TNF and IL-6 also decrease during this period (35).
Importantly, indices of immune activation such as CD38 and HLA-DR expression and cellular turnover that are strong predictors of disease outcome in untreated HIV infection are also decreased on CD4+ and CD8+ T cells after ART treatment (35).

Despite ART-induced suppression of viral replication, a number of treated HIV-infected patients continue to display immune dysfunction. This is particularly apparent in those treated HIV-infected individuals who fail to recover normal CD4+ T cell counts, known as Immune Failures (a population that we define with CD4 T cell counts <350 cells/mm³) (36, 37). In these patients, plasma levels of soluble inflammatory mediators are elevated, and both CD4+ and CD8+ T cells have elevated coexpression of the activation markers CD38 and HLA-DR (36). Increased T cell turnover is also demonstrable in Immune Failures, as intracellular expression of the cell cycle marker Ki67 is elevated in the memory CD4+ T cells when compared to expression of Ki67 in treated HIV+ patients who reconstitute their CD4 T cells to levels comparable to those of healthy control subjects.

Residual immune dysfunction in treated HIV infection also involves impairments in the homeostatic division and survival of CD4+ T cells. T cell homeostasis is mediated both by low-affinity TCR/MHC interactions and the IL-7/IL-7R(CD127) axis. The continual exposure of T cells to IL-7 is vital in maintaining both the overall size and survival of the naïve and central memory T cell pool (38). T cell access to IL-7 is thought to occur within lymphoid tissues, where IL-7 is posted along specialized structures known as Fibroblastic Reticular Cell (FRC) networks (39) (40). T cell access to IL-7 through the FRC network is crucial for the survival of naïve T cells, as blocking T cell contact with IL-7, either through adoptive transfer of T cells into IL-
7-deficient mice, or restricting access to the FRC network through blockade of T cell chemokine receptors needed for LN entry significantly reduces both the size and survival of the naïve T cell pool (39, 41). The IL-7R is a heterodimer composed of a common signaling gamma chain, CD132, that is shared by other cytokine receptors, and a unique alpha chain (CD127). Binding of IL-7 to CD127 induces the rapid activation of Jak proteins associated with the receptor which in turn activate the transcription factor Stat5, allowing for its nuclear translocation (42). CD127 receptor signaling also induces phosphorylation of Akt through activation of the PI3K pathway, although the kinetics of IL-7-induced PI3K activation are significantly more delayed than that of Stat5 activation (43). The result of IL-7 signaling promotes the expression of factors essential for T cell survival and homeostasis. IL-7 maintains T cell survival by regulating the expression of B-cell lymphoma 2 (Bcl-2), which inhibits the mitochondrial apoptotic pathway (42). IL-7 receptor signaling also modulates T cell trafficking to the gut through upregulating the α4β7 integrin on naïve T cells that increases T cell binding activity to MadCam-1 ligands selectively expressed on endothelial cells of the gut mucosa (44).

In HIV infection, the IL-7/CD127 axis is impaired and naïve T cell homeostasis is compromised. To start, while CD4 T cell count diminishes upon HIV disease progression, IL-7 levels typically rise in circulation. Mechanistically, the inverse relationship between plasma levels of IL-7 and the size of the naïve CD4 T cell pool is thought to be attributed to decreased receptor clearance. As CD4 T cell levels fall, the concentration of CD127 also falls, resulting in decreased clearance of IL-7 (45). Within lymphoid tissues naïve T cell numbers also decrease in HIV infection, and this is
directly correlated with the loss of IL-7 at these sites (46). As a counter to the immunopathological events that occur within lymphoid tissues in chronic HIV infection, cellular remodeling processes are triggered. Genes that mediate tissue repair such as matrix metalloproteases are upregulated in HIV+ nodes Transforming growth factor β (TGF-β) expression is also elevated in HIV+ nodes (47), the presence of which has correlated with the deposition of collagen along the FRC network (46). Importantly, collagen deposition was shown to be significantly correlated with the loss of the FRC network in the T cell zone, diminishing both the access and source of IL-7 that is critical for naïve T cell survival (46).

Impairments in CD127 receptor density and cellular responsiveness to IL-7 also exist in treated and untreated HIV infection. Many groups have observed decreased surface expression of the CD127 receptor on both CD4 and CD8 T cells (48, 49). Reduced T cell responsiveness to IL-7 is also apparent in HIV-infected patients and consequently, these subjects show reduced levels of intracellular Bcl-2, increased T cell apoptosis, and attenuated T cell proliferation in response to IL-7 in vitro (50-52).

In the setting of treated HIV infection, reconstitution of circulating CD4 T cell numbers that occurs with pharmacological control of viremia is critically dependent upon the degree to which subjects are able to normalize both CD127 and functional responses to IL-7 itself. For example, treated HIV-infected subjects who respond successfully to ART also show upregulation of CD127, whereas those individuals who do not show immune reconstitution display a reduced recovery of CD127 surface expression, particularly among CD4 T cells (49, 53). An intriguing aspect of these findings is that control of viremia does not necessarily normalize CD127 surface
expression in treated HIV infected subjects, suggesting that residual abnormalities in the IL-7/IL-7 receptor axis may not be driven directly by the virus or by viral replication. Rather, a number of groups have shown that immune activation is a more accurate predictor of CD127 receptor loss. The loss of CD127 on T cells correlates with levels of immune activation, as defined by co-expression of CD38 and HLA-DR (53, 54). Despite the known correlation between viral load and immune activation, a relationship cannot be found between viral load and T cell expression of CD127 (52). Thus, it is likely that immune activation is an important driver of CD127 receptor dysfunction, however the specific mediators of CD127 loss and immune activation in HIV infection have yet to be determined.

As stated earlier, CD127 surface expression on both CD4 and CD8 T cells is reduced in those treated HIV-infected patients with suboptimal CD4 T cell reconstitution. These patients, known as Immune Failures are characterized by a number of other immunological abnormalities, such as heightened markers of inflammation (IL-6, C-reactive protein, and TNF), coagulation (D-dimer), and immune activation (36). What is unclear is whether there is a causative link between inflammation and immune restoration failure. In the second part of our work, we assessed whether inflammatory cytokines could contribute to immune restoration failure. By treating PBMCs from healthy subjects with the inflammatory cytokines IL-6 or IL-1β, we found that we could recapitulate much of the immunophenotype of patients with immune restoration failure- decreased expression of CD127 and increased memory T cell cycling (36, 51, 53, 55).

The role of CD8 persistence in HIV-associated cardiovascular disease risk
While the defining characteristic of HIV disease is a progressive loss of CD4 T cells, the circulating CD8 T cell compartment is expanded in HIV infection. During primary HIV-1 infection, the immune system responds appropriately by clonally expanding a population of highly activated HIV-specific CD8 T cells (56). Yet, even among non-HIV specific CD8 T cells, the bulk CD8 T cell compartment is activated as a whole and in particular subsets such as central and effector memory CD8 T cells, as many as 40% can be shown to be in cell cycle (56). As disease progresses, the CD8 T cell compartment becomes enriched for cells of a highly differentiated phenotype that lack CD27 and CD28 (56). These highly differentiated CD8 T cells were also shown to express high levels of the senescent marker CD57 (57). Upon further examination, it was shown that CD57-expressing CD8 T cells were unable to divide in response to cognate antigen, were highly susceptible to apoptosis \textit{in vitro}, and had a history of more cellular divisions than CD57-negative CD8 T cells as evidenced by short telomere length (57, 58). Thus, uncontrolled HIV infection is characterized by an accumulation of highly differentiated, senescent CD8 T cells that are not entirely all HIV-specific.

Administration of anti-retroviral therapy results in reductions of both the proliferation and overall size of the CD8 T cell pool (59, 60). Yet, a proportion of treated HIV-infected subjects maintain high numbers of circulating CD8 T cells and consequently, an abnormally low CD4/CD8 ratio (61). Abnormally low CD4/CD8 ratios in treated HIV-infected subjects were found to be associated with heightened markers of immune activation and senescence (62, 63). In a large-cohort study designed to determine the relationship of abnormally low CD4/CD8 ratios to immune phenotype in treated HIV infection, low CD4/CD8 ratios were associated with expansion of more
mature effector and effector memory CD8 T cells that lacked CD28 surface expression (61). A number of groups have found that absolute numbers of CD28 negative cells expressing CD57 are increased in HIV infection. Yet proportionally, percentages of CD28- cells expressing CD57 are significantly lower in both treated and untreated HIV-infected subjects when compared to proportions of CD28- CD8 T cells that express CD57 in healthy control subjects (61) (64). The dichotomy between increased absolute numbers of CD28-CD57+ CD8 T cells and decreased proportions of CD28- CD8 T cells that are CD57+ can be attributed to an overall expansion of the CD28- CD8 T cells in HIV infection. Thus, while the numbers of antigen-experienced CD28 negative CD8 T cells are increased in HIV infected subjects, there appears to be a maturation block as these cells less often express CD57 on the cell surface.

In a nested case-controlled cohort of 33 treated HIV-infected patients, both the CD4/CD8 ratio as well as the absolute CD8 T cell count independently predicted the risk of serious non-AIDS events (Serrano-Villar, Plos One ’14). Additionally, in a separate study of 63 HIV-infected patients with more advanced disease (SOCA cohort), the CD4/CD8 ratio was found to be an independent predictor of mortality (61). While there is evidence linking persistently low CD4/CD8 ratios to non-AIDS morbidities and mortalities, mechanistic insights into this association are lacking. A large proportion of serious non-AIDS events are cardiovascular related, and breakdown products of coagulation such as D-dimers are associated with higher mortality (65, 66). In the final section of our work, we assessed whether expanded CD8 T cells may contribute to cardiovascular disease (CVD) associated with HIV infection. In mice, CD8 T cells contributed to the development of atherosclerotic plaques in a perforin and granzyme B
dependent manner (67). While the role of CD8 T cells in CVD is less clear in humans, as many as 50% of lymphocytes found within human plaques can be CD8 T cells and these are preferentially activated (68). Additionally, increased CMV-specific CD8 T cell responses are associated with increased carotid intima media thickness (IMT) in HIV-infected subjects (69). In our study, we sought to explore the potential role of CD8 T cell expansion and maturation in cardiovascular risk.
Chapter 2:

Impaired T cell responses to sphingosine-1-phosphate in HIV-1 infected lymph nodes*

Abstract

The determinants of HIV-1-associated lymphadenopathy are poorly understood. We hypothesized that lymphocytes could be sequestered in the HIV-1+ lymph node (LN) through impairments in sphingosine-1-phosphate (S1P)-responsiveness. To test this hypothesis, we developed novel assays for S1P-induced Akt phosphorylation and actin polymerization. In the HIV-1+ LN, naïve CD4 T cells and central memory CD4 and CD8 T cells had impaired Akt phosphorylation in response to S1P while actin polymerization responses to S1P were impaired dramatically in all LN maturation subsets. These defects were improved with antiretroviral therapy. LN T cells expressing CD69 were unable to respond to S1P in either assay yet impaired S1P responses were also seen in HIV-1+ LN T cells lacking CD69 expression. Microbial elements, HIV-1, and interferon alpha – putative drivers of HIV-1-associated immune activation all tended to increase CD69 expression and reduced T cell responses to S1P in vitro. Impairment in T cell egress from lymph nodes through decreased S1P responsiveness may contribute to HIV-1-associated LN enlargement and to immune dysregulation in a key organ of immune homeostasis.

Introduction

In HIV-1 infection, lymphoid tissues are important sites of disease pathogenesis (3, 4), and in the early years, generalized lymphadenopathy was a sentinel manifestation of infection (5, 6). In the HIV-1-infected lymph node (LN), effector T cells are typically over-represented and there is concurrent over-expression of a variety of inflammatory cytokines (12, 70, 71). This contrasts with the LN environment in health where highly
ordered processes are necessary to facilitate T cell homeostasis and antigen-driven T cell maturation and expansion (72). In untreated HIV-1 infection, these processes are compromised but can be improved with antiretroviral therapy (73) (74-76). In untreated HIV-1 infection, normal lymphocyte trafficking is impaired, with abnormal T cell sequestration in lymphoid tissues (77, 78). Very early after ART initiation, there are both dramatic reductions in lymph node size and rapid increases in numbers of circulating lymphocytes. Replenishment of circulating lymphocytes during this period is suggestive of cellular redistribution rather than *de novo* production (13), as there is no evidence of increased cell cycling (79) and increases in circulating lymphocytes are related to reductions in T cell densities within lymphoid tissues (14). Thus, inappropriate retention of lymphocytes within inflammatory LNs is thought to be a characteristic of untreated HIV-1 infection, but the determinants of this retention are poorly understood.

The principal route of T cell entry into lymphoid tissue involves CD62L selectin-dependent tethering along high endothelial venules (80) facilitating T cell entry into the lymphoid interstitial space through CCL21-CCR7 interaction (81). Egress of T cells from the lymph node is also receptor mediated through activation of the G protein-coupled (82) sphingosine-1-phosphate receptor 1 (S1P1) by tightly regulated gradients of the extra-cellular lipid mediator sphingosine-1-phosphate (S1P) (83), resulting in lymphocyte chemotaxis through efferent lymphatic vessels (19, 26). S1P1 is transcriptionally regulated by Kruppel-like factor 2 (KLF2) (32). Upon T cell activation, KLF2 is rapidly downregulated and subsequently re-expressed with further T cell maturation (84, 85). Furthermore, S1P1 expression can be post-translationally antagonized by the C-type lectin, CD69 (27). CD69 is expressed very early upon T cell
activation and directly binds S1P1, inducing its intracytoplasmic retention and subsequent degradation (28, 33).

This pathway of S1P1-dependent cellular egress from lymphoid tissues has been illuminated and characterized extensively in mice. There is reason to suspect that similar control of lymph node trafficking is operative in humans as an S1P analogue – FTY720 that has been approved for the treatment of multiple sclerosis - promotes receptor internalization and durable unresponsiveness to S1P resulting in profound lymphopenia that is attributed to blockade of cellular egress from lymphoid tissues (86) (87). This stated, there has been only limited study of the role of SIP and its receptors in the human system.

We hypothesized that impaired S1P1-mediated signaling and chemotactic egress from lymph nodes might underlie the excessive lymphocyte sequestration in lymphoid tissues seen in HIV-1 infection. To test this hypothesis, we developed novel methods to evaluate this system in human cells. Herein, we characterize S1P1 bioactivity in human T cells and show that this activity is impaired in lymph node T cells in HIV-1 infection.

Results

*Human T cell responses to S1P can be measured in vitro*

A major limitation to the study of S1P1 in humans has been its low level surface expression on human T cells. Using a murine monoclonal antibody reactive with the extracellular amino terminus of S1P1, we were unable to detect S1P1 on primary human lymphocyte surfaces by flow cytometry although we could detect cell surface S1P1 on a transfected cell line (*Figure 2.1a*). Additionally, an antibody specific to the intracellular
C-terminus of S1P1 was reactive with endothelial cells but not T cells on immunofluorescence staining of fixed human LN tissue sections (Figure 2.1b). We therefore developed assays to characterize S1P1 bioactivity in peripheral blood (PB) T cells by taking advantage of distinct signaling events induced following S1P1 ligation. S1P1 is thought to be exclusively coupled to G alpha i G-protein subunits, and in murine lymphocytes, S1P1 ligation drives polymerization of the actin cytoskeleton and phosphorylation of Akt (88). We assessed S1P-induced Akt phosphorylation at the Ser473 residue using a fluorochrome-labeled monoclonal antibody and monitored actin polymerization by staining with fluorochrome-tagged phalloidin, a molecule that binds to polymerized actin but not to actin monomers (89). In order to assess S1P1 responses relative to the activity of a chemotactic receptor expressed at detectable levels on T cells, CXCR4-dependent signaling was assessed after stimulation with SDF-1α. We found that freshly prepared peripheral blood T cells are unresponsive to S1P (not shown), however after an 18 hour incubation period in serum free medium that contains no S1P, all circulating T cell maturation subsets contained cells responsive to S1P, with both S1P-induced Akt phosphorylation and actin polymerization particularly robust among memory T cells (Figure 2.2a). As Akt phosphorylation and actin polymerization can be induced by signaling through multiple receptors, we next sought to determine if these readouts were specific for S1P1 after S1P exposure. Pre-treatment of PB T cells with the S1P-receptor functional antagonist, FTY720 (16), completely abrogated both S1P-induced Akt phosphorylation and actin polymerization (Figure 2.2b). Similar results were obtained using the S1P1-specific competitive antagonist, W146 (90) (Figure 2.2c). Thus in this system, S1P responses reflect activity mediated solely by S1P1.
Lymph node T cells from HIV-1+ donors are less sensitive to S1P-mediated lymph node egress signals.

We next prepared single cell suspensions from surgically removed lymph nodes of HIV-1-infected patients on or off therapy and uninfected controls. The cell suspensions were incubated for 18 hrs in serum-free medium and then examined for their responsiveness to S1P. In untreated, viremic HIV-1+ patients’ lymph nodes both CD4+ naïve and central memory T cells as well as CD8+ central memory T cells had significantly diminished Akt phosphorylation responses after S1P exposure. (Fig. 2.3a). Diminished Akt phosphorylation was also seen in untreated viremic HIV-1+ patients’ central memory CD4+ T cells in response to SDF-1α (Figure 2.3b). On the other hand, the actin polymerization response to S1P was significantly impaired in all T cell subpopulations in the untreated viremic HIV-1+ LNs (Figure 2.3c) while more subtle impairments in the SDF-1α response were demonstrable only in CD4 and CD8 EM T cells. This dissociation between Akt phosphorylation and actin polymerization responses to S1P indicates that a relatively preserved Akt phosphorylation response (such as seen for example among EM cells) may not assure “normal” actin polymerization in response to S1P. Thus LN T cells in uncontrolled HIV-1 infection demonstrate impairments in response to chemotactic signals provided by the interaction between S1P and its type 1 (S1P1) receptor and this may especially compromise the efficiency with which these cells can exit inflammatory LNs. As can be seen in this figure, these impairments largely resolve or improve after control of HIV-1 replication with suppressive antiretroviral therapies.

*The C-type lectin CD69 antagonizes S1P responsiveness in human LN T cells*
In mice, CD69 can bind to S1P1, inducing its intracytoplasmic retention and degradation (28). To determine if this interaction might take place also in human T cells, we assessed S1P responses in CD69-negative and CD69-positive LN T cells. We find that despite strong responses of both CD69+ and CD69- cell populations to SDF-1α, CD69-expressing T cells fail to phosphorylate Akt and also fail to polymerize the actin cytoskeleton in response to S1P (Fig. 2.4a). Thus we find that human T cells expressing CD69 fail to respond to S1P mimicking findings in mice (27). We next asked whether S1P responsiveness was also impaired in HIV-1+ LN T cells that lacked surface expression of CD69. In both HIV-1+ and uninfected control LNs, both CD4 and CD8+ T cells expressing CD69 failed to polymerize actin or phosphorylate Akt in response to S1P (Fig 2.4b). Yet even among the CD69 negative T cell populations, both CD4+ and CD8+ T cells from the LNs of HIV-1+ viremic subjects had impaired Akt phosphorylation and actin polymerization in response to S1P (Fig. 2.4b). In 3 treated patients who had controlled viremia, the responses to S1P among CD69 negative LN T cells were largely improved. Thus, although CD69 can antagonize S1P responsiveness in human LN T cells, these data suggest that mechanisms independent of CD69 expression also may contribute to reduced T cell sensitivity to S1P in the LN in the setting of HIV-1 viremia.

*In vitro immune activation attenuates T cell responses to S1P*

Because immune activation in HIV-1 infection has been linked to systemic exposure to both viral and bacterial elements (91, 92), we next asked whether in vitro exposure of PB cells to bacterial toll-like receptor (TLR) ligands, to HIV-1 or to the type 1 interferons that these microbial products induce could affect T cell sensitivity to S1P.
As a positive control, cells were also activated with agonistic antibodies to CD3 and CD28. After exposure of peripheral blood cells to these agents, CD4 and CD8 T cell Akt phosphorylation and actin polymerization in response to S1P were inhibited and CD69 surface expression tended to increase (Fig. 2.5a), suggesting that in vivo exposure to these factors might perturb the ability of T cells to exit lymphoid tissues efficiently. Notably, these same stimuli, all failed to significantly affect responsiveness to SDF-1α (not shown).

*Decreased expression of S1P1 in HIV-1+ LN tissues*

As our data indicated that T cells from the LNs of untreated, viremic subjects that lacked CD69 expression also showed impaired responses to S1P, we sought additional explanations for the defects we observed. We measured levels of S1P1 mRNA and found that S1P1 transcript levels were significantly decreased in HIV-1+ LN tissue sections from patients with uncontrolled viral replication when compared to levels in controls’ samples (Fig. 2.6a). And since S1P1 transcription is controlled by KLF2 we also asked whether reduced KLF2 gene expression might underlie the diminished S1P1 mRNA levels we observed. This could be the case as KLF2 mRNA levels also were diminished in LNs from untreated, viremic HIV-1+ patients (Fig. 2.6b). LN Tissue is comprised of a heterogenous mixture of cell types. To determine whether our findings were representative of gene expression in LN T cells, we assessed S1P1 and KLF2 mRNA levels in purified LN T cells. Despite reductions in S1P1 and KLF2 mRNA in the LN tissue, S1P1 and KLF2 mRNA levels in purified LN T cells of untreated, viremic HIV-1+ patients and uninfected controls were not different (Fig 2.6c, d). Our data suggest that there is a reduction of S1P1 and KLF2 mRNA levels in lymph nodes of HIV-1+ patients.
with uncontrolled viral replication, but these reductions may not necessarily reflect mRNA levels of S1P1 and KLF2 in T cells and may reflect reductions in other cell types.

**Discussion:**

The lymph node is an important site of immunopathology in HIV-1 infection. Generalized lymph node enlargement is common and this typically diminishes with disease progression as the inflammatory environment results in the deposition of collagen, disruption of lymph node architecture and fibrosis (71, 93). Even in the absence of clinically detectable lymph node enlargement, with application of antiretroviral therapies, there is rapid redistribution of lymphocytes from inflammatory LNs to the systemic circulation indicating that HIV-1 replication is associated with lymphocyte sequestration within these inflammatory sites (14). Yet the precise mechanisms of lymphocyte sequestration in the HIV-1+ nodes are not well characterized. Other viral infections can involve a transient shutdown of T cell egress from lymphoid tissues (27) that in humans is associated with lymph node enlargement, yet in these settings the determinants of lymphadenopathy are also not well characterized. Temporary egress inhibition is thought to facilitate immune priming, increasing the likelihood of antigen encounter (94). Yet sustained dysregulated lymphocyte sequestration in lymph nodes could be detrimental in chronic HIV-1 infection, where lymphoid tissues are a major site of viral replication, and the inflammatory environment may disrupt immune homeostasis. Thus, we set out to explore possible determinants of lymphocyte sequestration within HIV-1+ LNs.

T cell egress from lymphoid tissues is thought to be governed principally by S1P1 ligation. Yet there are not robust methods to study S1P1 activity in humans.
Immunologic methods to identify cell surface expression are limited by low expression levels and chemotaxis assays have been reported (73) but migratory responses to S1P have been low and difficult to quantify, perhaps because the key T lymphocyte S1P receptor, S1P1 is desensitized immediately following ligation (95). We report here the application of novel assays that allow robust, rapid and reliable detection of S1P1-dependent responses to S1P in human T cells. We show here that we can measure bioactivity of S1P1 through the phosphorylation of Akt and polymerization of the actin cytoskeleton.

In these studies, responses to S1P can be demonstrated in all T cell maturation subsets but demonstrating this response typically requires ex vivo incubation of these cells in S1P-free medium. In blood samples this is likely a consequence of sustained exposure to plasma levels of S1P prior to preparation; it is less clear why human lymph node cells also characteristically fail to respond to S1P immediately after preparation. In mice, freshly-isolated LN T cells are also typically unresponsive to S1P stimulation but regain responsiveness following a period of incubation in S1P-deprived medium (96). It has been suggested that subnanomolar concentrations of S1P are found within lymphoid interstitial spaces (97), and this may suffice to desensitize lymph node T cells to S1P during the isolation process. In our culture conditions, we did not measure S1P in culture supernatants, therefore we cannot rule out the possibility that S1P still may be present within the medium during this incubation period. We can be confident however that our culture conditions are sufficient to allow T cells to become resensitized to S1P following this culture period.
Here we show that T cell responses to S1P are diminished in the HIV-1+ lymph node in uncontrolled infection. While both CD4+ and CD8+ central memory T cells and naïve CD4+ T cells have diminished Akt phosphorylation responses to S1P, modest impairments are also seen in response to SDF-1 among naïve and central memory CD4+ T cells. In untreated infection, actin polymerization responses to S1P are impaired substantially in all LN T cell maturation subsets while SDF-1-induced actin polymerization is decreased only modestly and only within the effector memory compartment. It isn’t clear why these two readouts of S1P1 activity were not completely concordant. They might be differentially regulated after S1P1 ligation or additional signals may be necessary to optimally polymerize actin. Further studies will be necessary to define this defect in more detail. Nonetheless, our data demonstrate that in the HIV-1 infected lymph node, T cells have demonstrable impairment in the activity of a chemotactic receptor that in murine systems is necessary for return of lymphocytes to the systemic circulation. We propose that this defect contributes to the recognized sequestration of T cells within the inflammatory nodes in HIV-1 infection.

In these findings, it is important to point out that this data set does not demonstrate a causative relationship between blunted S1P responses and inappropriate T cell retention in HIV infection. In order to address better this relationship, correlative data between T cell S1P responses and overall T cell density in LNs would be helpful as would implementation of a targeted intervention that selectively increases S1P1 expression and attenuates lymphocyte retention in HIV disease.

In the data set presented, we did not observe a relationship between impaired S1P responses and lymphocyte density in lymphoid tissues. On the other hand, T cell
enrichment within the HIV+ LN may be offset by the increased frequencies of T cells that are known to undergo apoptosis at this site (98-100). Thus, the interpretation of this relationship may be difficult. As an alternative, assessment of T cell S1P responses during the first several weeks of ART administration may prove valuable in assessing the link between S1P responses and inappropriate LN T cell retention. As noted earlier, rapid CD4 T cell replenishment is observed in circulation during the first several weeks of therapy that has been attributed to redistribution of T cells from lymph nodes to peripheral blood (13, 14). If T cell responsiveness to S1P does indeed determine LN retention in HIV infection, one might expect that during this period, CD4 T cell replenishment in circulation would be associated with significant increases in T cell responsiveness to S1P. In a study such as this, a much larger data set could be examined since peripheral blood samples are easier to obtain, and subjects could be followed longitudinally with multiple timepoints tested after ART administration. Additionally, levels of a number of circulating inflammatory mediators such as IL-6, TNF, sCD14, and LPS, diminish during this period (35), and the relationship between changes in these inflammatory mediators and S1P responses could be assessed as well.

Trafficking of T cells in and out of lymphoid tissues is kept at a delicate balance between signals that mediate T cell ingress and signals that mediate T cell egress. It is conceivable that impairments in lymphocyte ingress could also contribute to the lymphadenopathy seen in HIV-1 infection. The CCL19 and CCL21 chemokines promote T cell entry into lymphoid tissues in a CCR7 dependent manner, and these chemokines have been reported to be increased in untreated HIV-1+ patients and treated patients with immune failure (101). Additionally, in untreated HIV-1 infection, lymphoid tissues are
enriched for CXCR3+ effector CD8 T cells (102), and the infiltration of these inflammatory CD8 T cells into sites from which they are normally excluded may also contribute to HIV-1 associated lymphadenopathy.

Lymph node inflammation in uncontrolled HIV infection is associated with inappropriate T cell LN retention, yet which one is responsible for the other? Is T cell LN retention a cause, or merely a consequence of lymphoid inflammation? Determining cause or effect in this setting would be difficult. Yet, in non-HIV infected subjects receiving FTY720, blocking T cell egress from LNs does not lead to any observable lymphadenopathy (103). This suggests the possibility that T cell LN sequestration may be a consequence of inflammation, rather than a cause of lymphadenopathy. Nevertheless, pharmacological LN egress inhibition by FTY720 in non-HIV infected subjects is likely very different from egress inhibition induced by HIV replication. In FTY720-treated patients, relatively quiescent naïve and central memory T cells, but not effector memory T cells are retained in the node (104). In contrast, LN T cell retention in HIV infection reflects entrapment of not just naïve and central memory T cells but also effector memory T cells; the latter secrete pro-inflammatory cytokines and cytolytic molecules that could potentially contribute to inflammation at this site (71, 104, 105). Thus, it is plausible that effector T cell LN sequestration could contribute directly to lymphoid inflammation in untreated HIV infection.

S1P1 activity can be regulated transcriptionally and post-translationally. We confirm here in human lymphoid tissues the observation made in murine systems that CD69 expression identifies cells that cannot respond to S1P. Yet we also find that even CD69 negative T cells from HIV-1 infected lymph nodes demonstrate an impaired
response to S1P. Although we observe decreased RNA levels of S1P1 and its transcription factor KLF2 in whole LN tissues from HIV-1 infected subjects, we could not confirm these findings in purified T cells from these nodes. Thus we do not have a clear understanding as to why there is an impairment in S1P1 function in CD69- cells in the HIV-1 infected LN. We also don’t know yet which cell types within the HIV-1+ LN tissue are responsible for the reductions of S1P1 and KLF2 gene expression that we observed. IHC assays demonstrate S1P1 expression by endothelial cells within lymphatic tissues (figure 2.1b), and in mice endothelial cell S1P1 dampened cytokine storm and innate immune cell recruitment during infection with a pathogenic human influenza virus (106). Thus, the role of endothelial S1P1 within chronically inflamed HIV-1+ lymph nodes merits further investigation.

How might reductions in S1P responsiveness take place? We show here that a number of immune activating signals (T cell receptor engagement, exposure to type 1 interferon, to bacterial TLR ligands and to HIV-1) that are each known or proposed to drive immune activation in the setting of chronic HIV-1 infection tend to increase T cell expression of CD69 and block S1P-induced Akt phosphorylation and actin polymerization. In mice, the double stranded “viral RNA” TLR-3 ligand poly I:C inhibits LN egress of T cells regardless of their antigen reactivity (27). Thus, ongoing viral replication within these sites may induce a non-specific inhibition of T cell egress from the HIV-1 infected lymph node. When viral replication is controlled by antiretroviral therapies there are dramatic reductions in a number of inflammatory mediators within lymphatic tissues (34). We show here that antiretroviral treatment is associated also with improvements in S1P responses in LN T cells.
There are limitations to this study. All control nodes were obtained during pelvic surgeries among women while nodes from all the HIV-1+ subjects were peripheral and these subjects included men and women who tended to be younger than the uninfected controls. We could not see an effect of age or gender on S1P1 responses (not shown). Because our uninfected control group was comprised of only lymph nodes from female donors, we were unable to assess an effect of gender on S1P responses. Nonetheless, the improvement in S1P responses after suppression of HIV-1 replication provides reasonable assurance that the defects we observed in untreated infection is largely related directly or indirectly to viral replication and not so much to other differences in the populations studied.

The controlled movement of T cells into and out from lymphoid tissues is essential for maintaining proper immune homeostasis. Manipulation of this cellular process has profound immunomodulatory effects that have recently been applied in a clinical setting, as the S1P receptor pharmacological agonist, FTY720, is approved for the treatment of patients with multiple sclerosis and is associated with profound circulating lymphopenia confirming that in humans, S1P receptor blockade interferes with systemic lymphocyte trafficking (87). Our identification of cellular assays that reflect T lymphocyte S1P1 bioactivity provides novel methods that now permit an exploration of this important system in human health and disease.

In HIV-1 infection, disruption of T cell egress from lymph nodes could have profound implications for disease progression as well as for the generation of immune responses to new and recall antigens (4, 107). In mice chronically infected with LCMV, prolonged lymph node egress shutdown through FTY720 treatment attenuated CD8 T
cell antiviral activity and led to higher levels of virus (108). Thus, improving T cell responses to signals that control lymphocyte trafficking from the HIV-1 infected lymph node might improve both vaccine responses and disease outcome through ameliorating immune dysregulation in a key immunological organ.

Methods

Study Subjects

All procedures were approved by the Institutional Review Boards at the relevant institutions, and all participants gave written informed consent before all procedures. Whole pelvic lymph nodes were obtained from adult women not known to be HIV-1 infected who were undergoing medically indicated surgery at University Hospitals of Cleveland. Their median age was 63 (range, 43-82 years). ART-naïve HIV-1-infected patients were recruited at the Drexel University College of Medicine (Philadelphia, PA). This group consisted of 5 males, 2 females, and 1 transgender subject. Median age of these subjects was 35 yrs (range 27-48). All were naïve to ART and had HIV-1 levels in plasma ≥ 2000 copies/ml and a median CD4 count of 558. Lymph nodes were also obtained from 5 HIV-1 infected subjects from University Hospitals (Cleveland, OH) who had been receiving suppressive combination antiretroviral therapy. The median age of the treated HIV-1 infected group was 50 (range 42-54). This group consisted of 5 males, and their median CD4 cell count was 665. Lymph nodes were excised from the inguinal region under local anesthesia as described previously (78). Surgeries were performed in the morning and the excised nodes were then placed in RPMI medium and shipped to Case Western Reserve University at 4°C via same day air along with matching peripheral
blood. The time between biopsy and subsequent specimen processing was no more than 7 hours.

**Blood and Lymph Node Processing**

Upon receipt of tissue, LNs were washed once in ice cold PBS. After careful removal of surrounding fatty tissue, LNs were cut into 1 mm X 1 mm tissue blocks and digested with collagenase IV (5 mg/ml in RPMI) (GIBCO) supplemented with 0.5% fatty acid-free BSA (Sigma-Aldrich, St. Louis, MO) and 200 µg/ml DNAse I (Roche. Basel, Switzerland). Collagenase-digested tissues were then mechanically digested with a motorized pestle, and the liberated cell suspensions were passed through a 40-micron nylon mesh and washed once in RPMI 1640.

**Flow cytometry**

Viable cells were gated using LIVE/DEAD-Aqua viability dye (Invitrogen. Grand Island, NY). Lymphocytes were identified by light-scatter properties and then were analyzed for surface marker expression using the following fluorochrome-labeled monoclonal antibodies: anti-CD3–peridinin chlorophyll protein, anti-CD8–alexa fluor 700, anti-CD27–allophycocyanin, anti-CD45RA– phycoerythrin, anti-CD69–phycoerythrin cy7 (all from Becton Dickinson. San Jose, CA), and anti-CD4–Pacific Blue (Biolegend. San Diego, CA). Cells were then washed, fixed in PBS containing 4% formaldehyde, and acquired on an LSRII flow cytometer (Becton Dickinson. San Jose, CA) equipped with lasers emitting at wavelengths 355, 488, 532, 407, and 638 nm. Data were analyzed using DIVA version 6.2 software. Additional analysis was performed using Flowjo software (Tree Star. San Carlos, CA). T cell maturation phenotypes were
defined by CD45RA and CD27 expression. For detection of S1P1 surface expression on HTC-4 cell lines, cells were stained with anti-Edg1—phycoerythrin (R & D systems, Minneapolis, MN).

*Real-Time quantitative RT-PCR*

Fresh LN tissue sections were placed in 500 µl RNAlater (Ambion, Grand Island, NY) and stored at 4°C for 24 hours. Specimens were then transferred to a -80°C freezer. Thawed samples were immediately disrupted in lysis buffer (Qiagen, Valencia, CA) using a homogenizer (Powergen, Pittsburgh, PA) and subsequently passed through a Qiashredder microfuge spin column (Qiagen, City, State). RNA was then isolated from tissue lysates via silica-membrane RNeasy spin columns (Qiagen, Valencia, CA). In concurrent experiments, T cells were purified from the LN cell suspensions by negative selection through incubation with a cocktail of antibodies targeting CD14, CD16, CD19, CD36, CD56, CD123, and Glycophorin A (Miltenyi, Auburn, CA) followed by magnetic bead separation (Miltenyi). Purified LN T cells were then lysed in Trizol and RNA was extracted with isopropanol. Following purification, one µg of total RNA was reverse-transcribed using M-MuLV reverse transcriptase in the presence of oligo(109) primers (Invitrogen, Grand Island, NY). cDNA was amplified by SteponePlus (Applied Biosystems, Carlsbad, CA) real-time quantitative PCR using pre-designed TaqMan Gene Expression assays for S1P1 (Assay ID: Hs01922614_s1) and KLF2 (Assay ID: Hs00360439_g1) (Applied Biosystems, Carlsbad, CA). Target gene expression was normalized to primers amplifying 18S rRNA (Part number 4308329) (Applied Biosystems).
Measurement of S1P-induced bioactivity in human T cells

Peripheral blood T cells were negatively selected by incubation with a cocktail of antibodies targeting CD14, CD16, CD19, CD36, CD56, CD123, and Glycophorin A, (Miltenyi, Auburn, CA) followed by magnetic bead separation (Miltenyi). Separated PB T cells or unpurified LN cell suspensions were cultured in X-VIVO chemically defined serum-free medium (Lonza, Allendale, NJ) at a density of 0.5 x10^6 cells/ml for approximately 18 hours at 37°C in 5% CO2.

To assess ligand-induced Akt phosphorylation and actin polymerization, cells were then stimulated with 50 nM S1P (Sigma-Aldrich) or 0.1 ng/ml SDF-1α (Sigma-Aldrich) dissolved in PBS containing 0.5% fatty acid-free BSA (Sigma-Aldrich) for 30 seconds and immediately fixed in 4% paraformaldehyde. Preliminary experiments identified these conditions as optimal for induction of Akt phosphorylation and actin polymerization. Cells were then permeabilized on ice with a saponin-based permeabilization buffer (Becton Dickinson) for actin polymerization or a methanol-based buffer for Akt phosphorylation (Becton Dickinson) and subsequently incubated with either anti-phospho Akt–PE (anti-ser473)(Becton Dickinson) to assess ligand induced Akt activation, or FITC-labeled phalloidin (Invitrogen) to measure actin polymerization. Cells were additionally stained with anti-CD3–PercP, anti-CD4–Pacific Blue, anti-CD8–PEcy5, anti-CD45RA–PEcy7, and anti-CD27–APC after permeabilization. In some experiments, T cells were pre-treated with sphingosine-1 phosphate receptor inhibitors FTY720 (Cayman, Ann Arbor, MI) or W146 (Avanti Polar Lipids, Alabaster, AL) for 18 hrs or for 1 hr respectively prior to stimulation. To assess CD69 surface expression on
S1P-stimulated T cells, cells were pre-stained with anti-CD69–Alexa Fluor 700 (Becton Dickenson) prior to ligand exposure.

Measurement of S1P responses in T cells pre-activated in vitro

Platelets were depleted by incubation of human PBMCs with anti-CD61 microbeads (Miltenyi) and passage of cells through an Automacs Pro separator (Miltenyi). The negative fraction was collected and incubated in X-VIVO chemically defined serum-free medium for 48 hours or medium supplemented with plate-bound anti-CD3 (Becton Dickenson), 1000 U/ml IFNα subtype 2a (Pestka Biomedical Laboratories. Piscataway, NJ), 100ng/ml LPS (Invivogen. San Diego, CA), 1 µg/ml B. Subtillis Flagellin (Invivogen), 3ug/ml synthetic CpG oligonucleotides (CpG 7909, Coley Pharmaceuticals. Wellesley, MA), 25 ng/ml poly I:C, 1ug/ml R848 (Invivogen), 150 ng/ml aldrithiol-2 (AT-2) inactivated CL.4/SUPT1 CXCR4-tropic and ADA-M/SUPT1 CCR5-tropic HIV-1 strains kindly provided by Dr. Jeffrey Lifson (National Cancer Institute, Frederick, MD). S1P and SDF-1α induced Akt phosphorylation and actin polymerization was then assessed by flow cytometry as above.

Triple Immunofluorescence assay (IFA) in LN tissue sections

Slides containing 5 micron LN tissue sections were incubated for 1 hour at 60 C. Slides were then placed in Xylene for two 5 minute intervals, followed by dehydration in decreasing concentrations of ethanol at 5 minute intervals. After washing slides in dionized water, antigen retrieval was performed on slides in citrate buffer(10mM) pH 5.0 in a pressure cooker for 30 seconds at 125 degrees C. Tissue sections were encircled with a PAP pen and subsequently immersed in Sudan Black B (0.1% in 70% EtOH)
(Sigma) for 30 minutes. Slides were then washed in TBS-T and blocked with Sniper Blocking reagent (Biocare Medical, Concord CA) for 60 minutes. Tissue sections were then incubated with mouse anti-CD3 (Dako, 1:50) and rabbit anti-Edg1 clone H-60 (1:20, Santa Cruz Biotechnology) in TBST + 2% Sniper at 4°C overnight. Tissue sections were then washed in TBS-T three times and subsequently incubated with anti-mouse-Alexa 488, anti-rabbit-Alexa 555 (Invitrogen, 1:400), and DAPI (Invitrogen) in the dark at room temperature for 60 minutes. Tissue sections were washed in TBS-T 3 times and mounted with Aqua Poly/Mount (Polysciences Inc.) Tissue sections were captured on an Aperio ScanScope (Vista, CA) slide scanner at 20x.

**Statistics**

Comparisons were made using the Mann-Whitney Test

**Ethics Statement**

All investigation was carried out according to Declaration of Helsinki principles.
Figure 2.1*

**Supplementary Fig. 1. Failure to detect S1P1 protein in human T-cells.** Staining with a) an N-terminal S1P1 ab from R&D systems on S1P1-null and huS1P1-transfected rat hepatoma HTC-4 cell lines and primary human T-cells. b) a C-terminal S1P1 antibody from Santa Cruz Biotechnology applied to fixed human LN tissue sections recognizes S1P1 on endothelial cells but not on lymphocytes.

Figure 2.2*

Figure 2.2. Monitoring S1P1 activity in human T-cells. S1P responses were measured by flow cytometric analysis in separated PB T-cells after 18 hours’ incubation in serum-free medium. a.) PB T-cell subpopulations: naïve (CD45RA+,CD27+), CM = central memory (CD45RA-,CD27+), EM = effector memory (CD45RA-CD27-) cells identified after permeabilization with a PE-labeled phospho-Akt monoclonal antibody or FITC-labeled phalloidin to detect actin polymerization. Responses to SDF-1a were assessed as a positive control. b.) PB T-cells were pre-treated with 250 nM FTY720 prior to assessment of S1P-induced actin polymerization and S1P-induced Akt phosphorylation. This experiment is representative of three. c.) PB T-cells were pre-treated with 10μM W146 for 1 hour prior to measurement of S1P-induced Akt phosphorylation and actin polymerization. This experiment is representative of two.

Figure 2.3


Figure 2.3. Blunted responses to S1P in the HIV-1+ lymph node. S1P responses were assessed in cell suspensions prepared from lymph nodes of HIV-1+ patients and uninfected controls. a.) S1P-induced Akt phosphorylation. b.) SDF-induced Akt phosphorylation. c.) S1P-induced actin polymerization. d.) SDF-induced actin polymerization.
**Figure 2.4**

*a.* Impaired S1P induction of Akt phosphorylation and actin polymerization in CD69⁺ LN T-cells. This is representative of 13 samples from both HIV- and HIV+ donors.  

*b.* S1P-induced Akt phosphorylation and actin polymerization in CD69 positive and CD69 negative LN cells.

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Figure 2.5.

Induction of CD69 and inhibition of S1P responses in human T cells in vitro. Platelet-depleted PBMCs were treated for 48 hours as noted and S1P-induced bioactivity and CD69 surface expression were assessed (data shown represent means and SD of 3 separate experiments). a.) S1P responses are reported as percentage change relative to non-stimulated conditions.

Fig 2.6. Diminished levels of S1P1 and KLF2 RNAs in HIV-1 + LNs. a.) S1P1 and b.) KLF2 gene expression in LN tissues was assessed by real-time PCR and normalized to levels of 18S rRNA. c.) S1P1 and d.) KLF2 gene expression in purified T cells was assessed by real-time PCR and normalized to levels of 18S rRNA.

Chapter 3:
Inflammatory Cytokines Drive CD4 T cell Cycling and Impaired Responsiveness to Interleukin-7; Implications for Immune Failure in HIV Disease*

*This work was performed in collaboration with Carey Shive, and resulted in a co-first authored manuscript

Summary

Systemic inflammation has been linked to a failure to normalize CD4 T cell numbers in treated HIV infection. Although inflammatory cytokines such as IL-6 are predictors of disease progression in treated HIV infection, it is not clear how, or if, inflammatory mediators contribute to immune restoration failure. We examined the in vitro effects of IL-6 and IL-1β on peripheral blood T cell cycling and CD127 surface expression. The pro-inflammatory cytokine IL-1β induces cell cycling and turnover of memory CD4 T cells and IL-6 can induce low level cycling of naïve T cells. Both IL-1β and IL-6 can decrease T cell surface expression and RNA levels of CD127, the IL-7 receptor alpha chain. Pre-exposure of healthy PBMCs to IL-6 or IL-1β attenuates IL-7 induced Stat5 phosphorylation, induction of the pro-survival factor Bcl-2 and the gut homing integrin α4β7. We find elevated expression of IL-1β in the lymphoid tissues of patients with HIV infection that does not normalize with ART. Induction of CD4 T cell turnover and diminished T cell responsiveness to IL-7 by IL-1β and IL-6 exposure may contribute to the lack of CD4 T cell reconstitution in treated HIV infected subjects.
Introduction

In successfully treated HIV infection, chronic inflammation often persists and has been identified as a major predictor of morbidity and mortality (110, 111). Moreover, in treated patients with controlled viremia, immune activation and elevated plasma markers of inflammation have been associated with poor CD4 T cell restoration (36, 112); yet the mechanistic link between persistent immune activation, inflammation, and CD4 T cell restoration failure is unknown.

Earlier studies of patients with immune restoration failure found a link between higher indices of inflammation and the occurrence of morbidities and mortalities despite antiretroviral therapy (ART)-induced control of HIV replication (110, 113, 114). It is not clear however whether the relationship between inflammation and immune failure is causal and if so, the mechanisms whereby inflammation might promote persistent CD4 T cell restoration failure (or vice versa) are not defined.

An increase in T cell turnover is characteristic of untreated HIV infection (113-115) and recent data demonstrate elevated frequencies of cycling CD4 (but not CD8) T cells in treated HIV-infected patients with immune restoration failure even in the setting of virologic control (36, 116). Diminished expression of CD127, the interleukin-7 receptor alpha chain (IL-7Rα), and an imputed failure of homeostatic CD4 T cell expansion in response to IL-7, have also been identified in treated HIV infection (48, 117) and may contribute to CD4 T cell restoration failure.

During the acute-phase response to infection, IL-1β, IL-6 and TNFα act systemically to induce fever and induction of acute-phase proteins from the liver (118). This work was designed to ascertain if inflammatory cytokines might contribute to CD4 T cell
restoration failure in treated HIV infection. We found that by treating PBMCs from healthy subjects with the inflammatory cytokines IL-6 or IL-1β, we could recapitulate much of the immunophenotype seen in patients with immune restoration failure—decreased expression of CD127(53, 117) and increased T cell cycling especially of memory CD4 T cells(36, 114, 116, 119). Importantly, IL-1β is expressed throughout all lymph node compartments in HIV-infected viremic patients and, while reduced with ART, still remains elevated compared to levels in uninfected controls, providing in vivo support for our in vitro findings.

Results

Both IL-1β and IL-6 exposure can drive CD4 T cells into cell cycle

Microbial products such as LPS have been strongly linked to immune activation in HIV-infected subjects (91, 120). Ligation of microbial products by toll-like receptors expressed on antigen-presenting cells will induce pro-inflammatory cytokines such as IL-6, IL-1β, and TNFα. To begin to understand the impact of pro-inflammatory cytokines on T cell activation and dysfunction in HIV infected patients, PBMC from healthy subjects were stimulated with IL-1β (10ng/mL), IL-6 (10ng/mL) or, as a positive control, IL-7 (2ng/mL) for 7 days then examined for intranuclear Ki67 using flow cytometry. As optimal induction of Ki67 by IL-7 was demonstrable after 7 days, we used this duration of culture to test the effects of inflammatory cytokines on cell cycling. Dose response experiments indicated that both IL-6 and IL-1β induced Ki67 optimally at 10ng/mL and that the optimal dose of IL-1β needed for Ki67 induction varied substantially between subjects.
While IL-7 induced dramatic increases in cycling of both CD4 and CD8 T cells, IL-1β induced cycling preferentially among CD4 T cells and while the increase in cycling induced by IL-1β was substantial, the effect was more subtle after IL-6 exposure (Figure 3.1a). Summary data are shown on Figure 3.11b. Although there was some subject variability, the aggregate induction of cycling by IL-1β was significant (p=0.0002) while the effect of IL-6 was not (p=0.1399). As expected, the cells induced to express Ki67 after exposure to IL-7 included naïve, central memory and effector memory CD4 and CD8 T cells (Figure 3.1c). In contrast, cells expressing Ki67 after IL-1β exposure were primarily central memory and effector memory CD4 T cells (Figure 3.1c). Low level cycling was induced by IL-6 in naïve CD4 and CD8 T cells (Figure 3.1c).

Cell cycle entry induced by IL-7 or IL-1β results in cellular proliferation as detected by dilution of CFSE dye

Induction of Ki67 expression is considered evidence of cell cycle entry(121). Earlier work by others(114) and by our group(122) indicates that in vivo cell cycle entry in HIV infection is associated with an accelerated cellular turnover. To evaluate the effects of inflammatory cytokines on T cell proliferation, CFSE-labeled PBMCs from healthy controls were stimulated with IL-1β, IL-6 or IL-7. With each cell division CFSE is distributed equally between daughter cells resulting in cell populations halving fluorescence intensity with each division. As expected, IL-7 induced substantial dye dilution reflective of cellular division in both CD4 and CD8 T cells (Fig 3.2a,b). Interleukin-1β induced proliferation of CD4 T cells and to a lesser degree, proliferation of CD8 T cells while IL-6 induced negligible cell division (Fig 3.2a,b). Interestingly, dye
dilution induced by IL-1β resulted in most dividing cells undergoing more than four rounds of division, while proliferation induced by IL-7 appeared more controlled with distinct populations undergoing one to more than four rounds of division. This suggests that cells induced to expand after IL-1β exposure may more readily become “exhausted” as a result of many rounds of division. This apparent differential regulation is even more demonstrable when we examined the maturation phenotypes of T cells that had divided in response to these cytokines (Fig 3c,d). Naïve, central memory and effector memory CD4 (Fig 3.2c) and CD8 T cells (Fig 3.2d) underwent one, two, three, or four rounds of proliferation after stimulation with IL-7. In contrast, proliferation induced by IL-1β was restricted predominantly to central and effector memory CD4 T cells (Fig 3.2c,d).

*LPS-induced down-regulation of CD127 on T cells is partially attributable to IL-6*

Loss of CD127 is a characteristic of HIV disease progression and has been linked with immune activation (123). Because microbial products are demonstrable in the circulation of HIV infected patients (124), we asked whether LPS or the cytokines it induces could downregulate CD127 on T cells in vitro. Treatment of human PBMCs with LPS induced loss of CD127 on CD4 T cells (Fig 3.3a). Upon TLR4 engagement, LPS induces the production and secretion of the pro-inflammatory cytokines IL-6, IL-1β, and TNFα by antigen presenting cells. The addition of an anti-IL-6 neutralizing antibody to PBMCs treated with LPS partially rescued the loss of CD127 on T cells. The neutralizing effect was specific for the paratope of the anti-IL-6 antibody, as treatment with the IgA2 constant region had no effect on LPS-induced CD127 downregulation. Thus LPS can drive the reduction of CD127 through IL-6 dependent mechanisms.

*IL-1β and IL-6 down-regulate CD127 on CD4 T cells in vitro*
In murine systems, exposure to IL-6 in vitro can down-regulate T cell CD127 gene expression (125). This appears to be the case in humans as well, as 2 days’ exposure of human PBMCs to IL-6 significantly decreased CD127 surface expression on CD4 T cells, but the effect was not significant on CD8 T cells (Fig 3.4a). Similar findings were seen when purified T cells were exposed to IL-6 suggesting a direct effect (data not shown). Within PBMCs, CD4 T cells, but not CD8 T cells, were also induced to down-regulate CD127 upon exposure to IL-1β (Fig 3.4b). Further characterization revealed that the IL-6 effect on CD127 down-regulation was restricted to naïve CD4 T cells (Fig 3.4c), whereas IL-1β tended to affect naïve and central memory CD4 T cells but this was not significant when the populations were analyzed separately (Fig 3.4d). Dose response and time course experiments showed IL-6 could down-regulate T cell CD127 surface expression at concentrations as low as 10 pg/mL, and the maximal effect was seen at 2 days’ exposure. Maximal down-regulation of CD127 expression by IL-1β was reached at a concentration of 10 ng/ml and after 2 days’ exposure.

CD127 can be regulated both transcriptionally and post-translationally (126); we next asked whether reductions in CD127 surface expression were associated with reductions at the mRNA level. CD127 gene expression was reduced in naïve CD4 T cells in PBMCs treated with IL-1β or IL-6 (Fig 3.4e). Thus IL-1β and IL-6 each can down-regulate CD127 surface expression in CD4 T cells and this is due, at least in part, to decreased mRNA levels.

*Pre-exposure to either IL-1β or IL-6 impairs T cell responses to IL-7*

To ascertain if the down-modulation of CD127 was associated with impairment in responsiveness to IL-7, we assessed IL-7-induced phosphorylation of Stat5 and Akt in
PBMCs pretreated with IL-6 or IL-1β. Although IL-7-induced Stat5 phosphorylation was unaffected in CD4 and CD8 T cells at 15 minutes or 2 days (not shown), maintenance of the p-Stat5 signal was diminished at 5 days post-IL-7 stimulation in both CD4 and CD8 T cells pre-treated with IL-1β or IL-6 for 2 days (Fig 3.5a). Neither IL-1β nor IL-6 affected the IL-7 induced p-Akt signal in either CD4 or CD8 T cells (not shown).

In ART-treated HIV+ patients, gut repopulation of CD4+ T cells is impaired and this may underlie a trafficking defect (Mavigner, *J Clin Invest* '12). Since IL-7 signaling induces a gut homing phenotype through up-regulation of α4β7 integrin on T cells, we tested whether IL-6 or IL-1β can interfere with this process. Signaling via the IL-7 receptor promotes a T cell gut homing phenotype through up-regulation of α4β7 surface expression. Pre-exposure to either IL-1β or IL-6 attenuated the up-regulation of α4β7 on both CD4 and CD8 T cells 5 days after exposure to IL-7 (but not after 2 days) (Fig 3.5b). Attenuation appeared to be specific for CD127 signaling, as pre-treatment with IL-1β or IL-6 had no effect on expression of α4β7 on T cells induced by retinoic acid (data not shown).

Bcl2 is a pro-survival protein that is up-regulated by IL-7 and can inhibit cytochrome c release and subsequent apoptosis (127). Pre-exposure of PBMCs to IL-6 or IL-1β for 2 days attenuated the up-regulation of Bcl-2 seen after 7 days exposure to IL-7 (Figure 3.5c). Thus, in vitro exposure to IL-1β or IL-6 impairs T cell responsiveness to IL-7.

*Elevated inflammatory cytokine expression in HIV infection*

While numerous groups have found elevated levels of IL-6 in the plasma of HIV infected subjects both before and after ART(36, 110, 111, 128-131), information
regarding systemic levels of IL-1β is scant (132-134). Plasma levels of IL-1β were barely detectible, <1pg/mL, in both HIV infected, treated patients and uninfected controls (not shown). We therefore examined tissue sites of HIV replication for evidence of increased IL-1β exposure. Expression of mature IL-1β protein was increased in each of 5 lymph node samples from untreated, HIV-infected, viremic subjects when compared to levels in 6 healthy control lymph nodes (Fig 3.6b). Mature IL-1β was expressed within all anatomical sites of the lymph node in HIV-infected patients, but most prominently within medullary cords, sinuses, and the T cell zone (Fig 3.6a). While IL-1β expression was reduced in patients receiving combination ART, IL-1β expression remained significantly elevated compared to levels in healthy controls’ nodes (Figure 6), and all but one of 8 treated patients had IL-1β levels exceeding the median of controls.

Discussion

Despite control of viremia, immune activation and inflammation persist in a significant proportion of otherwise effectively treated HIV-infected persons (36). Among those who fail to increase circulating CD4 T cells to “normal” levels, activation and inflammation indices are especially elevated, and these indices are strongly linked to morbidity and mortality even in patients on ART (36, 129, 135-137). Here, we attempt to explore the relationship between heightened inflammation and CD4 T cell restoration failure and demonstrate the in vitro effects of two inflammatory cytokines on T cell turnover and responsiveness to homeostatic signals.

Untreated HIV infection is characterized by increased T cell cycling and turnover (113, 114, 119, 122). With control of viremia, CD4 and CD8 T cell cycling is typically diminished; however, cycling and turnover remains elevated in memory CD4 T cells
among subjects with CD4 T cell restoration failure (36, 114). The drivers of cycling in this setting are not well characterized. Here, we show that the inflammatory cytokines IL-1β and, to a lesser degree, IL-6 can drive CD4 T cells into cell cycle and, in the case of IL-1β, to proliferate. Interestingly, the cycling and proliferation induced by these inflammatory cytokines can be distinguished from the proliferation induced by the homeostatic cytokine IL-7. Interleukin-7 typically induces cells to undergo distinct rounds of cell division and this is seen in all CD4 and CD8 T cell maturation subsets, whereas cycling and proliferation driven by the inflammatory cytokine IL-1β occurs predominantly among memory CD4 T cells and typically results in at least 5 rounds of proliferation in almost all cells that divide.

This is the first work that demonstrates an effect of inflammatory cytokines in driving human T cell turnover. An in vivo study in mice demonstrated enhanced memory T cell expansion in response to antigenic stimulation if IL-1β was coadministered (138) and partially purified IL-1β could enhance the antigen induced proliferation of human T cells (139). It is currently unclear whether the phenotype and function of cells induced to divide by IL-6 or IL-1β are different from those of cells induced to divide by homeostatic cytokines such as IL-7. In future work, comparing the transcriptional profiles of CD4 T cells induced to proliferate by pro-inflammatory cytokines to the profile of CD4 T cells induced to proliferate in response to homeostatic cytokines will be important. If a transcriptional signature of pro-inflammatory cytokine-induced proliferation can be identified in vitro, it would be important to assess whether components of this same transcriptional signature are evident in cycling memory CD4 T cells of patients with immune restoration failure, and whether this is distinct from a transcriptional signature of
homeostatic proliferation. These comparative studies may give valuable insights into the drivers of increased CD4 T cell turnover in treated HIV infection.

Interleukin-7 is essential in the maintenance of T cell homeostasis (126). In HIV infection, systemic levels of IL-7 are increased especially as circulating CD4 T cell numbers fall (45, 48, 140, 141). Yet despite elevated levels of IL-7, responsiveness to IL-7 is likely impaired in HIV infection. Decreased expression of CD127 is demonstrable on both CD4 and CD8 T cells in both viremic HIV infection and in subjects with treatment-controlled viremia (48, 53, 117, 141-144) and in some studies is linked to CD4 T cell restoration failure (48, 53, 117). In lymphoid tissues, access to IL-7 also may be impaired as the fibroblastic reticular cell network - conduit for the trafficking of cytokines - is often disrupted by the deposition of collagen and resultant fibrosis (46, 78, 145). We show here that exposure of PBMCs to IL-1β or IL-6 results in down-regulation of CD127 on CD4 T cells but not on CD8 T cells and this effect is likely mediated, at least in part, at the level of RNA expression. The mechanism of these effects is not clear. The effect of IL-6 on CD127 expression may be mediated through Stat3 signaling as a Stat3 inhibitor (ethyl-1-(4-cyano-2,3,5,6-tetrafluorophenyl)-6,7,8-trifluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate) could block IL-6 mediated CD127 down-modulation (not shown). CD127 gene expression can be promoted by guanine and adenine binding protein (GAPBa) and repressed by growth factor independence 1 (GFI-1) (126). The roles of these factors in IL-6 and IL-1β-mediated CD127 down-regulation remain to be determined.

Stat5 phosphorylation in response to IL-7 stimulation is impaired ex vivo in both viremic and ART-treated HIV infected patients (50, 146). As this might be related to decreased expression of CD127 or a downstream impairment of IL-7 responsiveness, we
asked if exposure of T cells to IL-1β or IL-6 decreases functional responses to IL-7 in vitro. We found that pre-exposure to IL-1β or IL-6 did not impair the initial induction of IL-7 mediated Stat5 phosphorylation. Yet the ability of T cells to sustain this signal was significantly diminished 5 days after IL-7 addition. IL-7 signaling promotes the expression of the pro-survival factor Bcl2 and the gut homing integrin α4β7 on T cells, and we demonstrate that the impairment of IL-7 signaling is also associated with diminished induction of Bcl2 and α4β7 in response to IL-7 when cells are pre-exposed to IL-6 or IL-1β in vitro. These effects may mirror what has been seen in HIV infection, as levels of Bcl2 were lower in CD4 and CD8 T cells from viremic HIV positive patients (117), as was the IL-7 induced up-regulation of Bcl2 (48, 117). Although these inflammatory cytokines decreased CD127 expression, the attenuated IL-7 responsiveness was not solely attributable to a loss of CD127 expression, as functional responses to IL-7 were blunted in both CD4 and CD8 T cells despite a relative preservation of CD127 surface expression on CD8 T cells after exposure to these inflammatory cytokines. The mechanism for this effect is currently unclear, but it is plausible that exposure to these inflammatory cytokines may impair signaling events downstream of receptor ligation that are important for optimal responses to IL-7.

In our studies, it is interesting to note the differences in the response to IL-6 and IL-1β between CD4 and CD8 T cells. When compared to IL-6 and IL-1β responses in CD4 T cells, these pro-inflammatory cytokines induced turnover in CD8 T cells to a much lesser extent. Additionally, IL-6 and IL-1β downregulate surface expression of CD127 on CD4 T cells, but not on CD8 T cells. These observations raise an interesting possibility that the pro-inflammatory environment in HIV infection may exert differential
effects on CD4 and CD8 T cell homeostasis. While CD4 T cells are progressively lost in HIV infection, CD8 T cells are typically expanded and remain so even after therapy in many individuals (61) and the mechanism for this sustained expansion is not known. In patients with immune restoration failure, increased cycling is observed in memory CD4 T cells but not in CD8 T cells (36). Therefore our findings of differential susceptibility of CD4 and CD8 T cell turnover by inflammatory mediators may have relevance in vivo.

Viral replication is thought to be a major contributor to CD8 T cell expansion, as a number of groups have observed a direct relationship between circulating CD8 T cell numbers and plasma HIV RNA levels (147, 148). In addition to viral replication, other factors may also contribute to CD8 T cell expansion in HIV infection. In other conditions that result in lymphopenia such as sepsis or chemotherapy, homeostatic proliferation stimulates the reconstitution of CD8 T cells much faster than that of CD4 T cells (149, 150). In addition, deuterated glucose labeling in HIV infected subjects revealed that CD8+ T cells are much less susceptible to cell death than CD4 T cells in vivo (151). Thus, CD8 T cells exhibit higher rates of proliferation and lower susceptibility to cell death when compared to proliferation and cell death rates of CD4 T cells, and this may also contribute to persistent CD8 T cell expansion in HIV infection.

These in vitro effects of IL-1β and IL-6 recapitulate many of the T cell phenotypes of immune failure in treated HIV infection, i.e. an increased cycling of memory CD4 (but not CD8) T cells (36), diminished IL-7 receptor expression on CD4 T cells (48, 54, 117, 142) and a failure of IL-7 responsiveness even among CD127+ T cells (50, 54, 117). While evidence of reduced α4β7 integrin surface expression in treated HIV-infected subjects is lacking, it is known that in treated HIV infection there is a lack
of CD4+ T cell recruitment to the gut that is attributed to defective T cell homing to this site (Mavigner, *J Clin Invest* ’12). CD4+ T cell recruitment to the gut is likely very important, as α4β7 integrin induction in HIV+ patients given therapeutic doses of IL-7 is associated with gut T cell repopulation and decreases in soluble CD14 in plasma (Sereti, *CROI* ’12). These new findings support a model wherein the inflammatory environment that characterizes immune failure in treated HIV infection also may contribute to immune pathogenesis via accelerating CD4 T cell turnover, impairing homeostatic responses and impairing restoration of gut T cell numbers. While it is well documented that systemic levels of IL-6 are elevated in both treated and untreated HIV infection, we have failed to demonstrate an increase in plasma levels of IL-1β in HIV-infected subjects (not shown). Yet, our earlier studies showed increased spontaneous expression of IL-1β in lymph node histocultures from HIV-infected subjects (71) and Doitsh et. al. have found that abortive infection in the HIV positive lymphoid tissues is linked to increases in IL-1β expression(152). In the current study we have confirmed elevated lymph node IL-1β expression by immunohistochemistry in untreated HIV infection and now show that this typically persists even after therapy. The biologic relevance of this finding is supported by the frequent demonstration of a peripheral blood transcriptional signature of inflammasome activation in patients with CD4 T cell restoration failure(153). These new findings suggest that strategies targeting the expression or function of the pro-inflammatory cytokines IL-1β and IL-6 may have utility in treated HIV infection; not only in preventing the clinical complications linked to inflammation, but perhaps also may enhance CD4 T cell restoration. Agents targeting these elements have been approved for treatment of rheumatologic conditions and in those settings, their toxicities, including
risks for infectious complications, are recognized. Whether these targeted immunosuppressive interventions are safer or more effective than more broadly immunosuppressive approaches in HIV infection will require attention to the design and monitoring of clinical intervention studies.

Materials and Methods

Ethics Statement

All subjects provided written informed consent in accordance with the Declaration of Helsinki. Patient studies were approved by the University Hospitals/Case Medical Center Institutional Review Board.

Cell Culture

Peripheral blood mononuclear cells (PBMCs) were purified by centrifugation over ficoll-hypaque (GE Healthcare, Sweden) and cultured in complete RPMI with 10% fetal bovine serum, 1% penicillin/streptomycin, 1% L-glutamine, and 1% sodium pyruvate at 37°C and 5% CO₂. Where indicated, PBMCs were stimulated in the presence of recombinant human IL-6 (R&D Systems, Minneapolis, MN), recombinant human IL-1β (R&D Systems), or recombinant human IL-7 (Cytheris, Issy-les-Moulineaux, France).

Flow Cytometry

Viable cells were gated using LIVE/DEAD-Aqua or yellow viability dye (Invitrogen, Grand Island, NY). Lymphocytes were identified by forward and side scatter and T cell phenotype was assessed using the following fluorochrome conjugated monoclonal antibodies: anti-CD3 peridinin chlorophyll protein (Percp), anti-CD8 allophycocyanin-
cy7 (APC-Cy7), anti-CD127 fluorescein isothiocyanate (FITC), anti-CD45RA phycoerythrin cy7 (PE-Cy7), anti-CD27 allophycocyanin (APC), anti-CD197 AlexaFluor 700 (all from BD Biosciences, San Jose, CA), and anti-CD4 Pacific Blue (Biolegend, San Diego, CA). Anti-α4β7 antibody was obtained from the NIH AIDS Research and Reference Reagent Program and conjugated using an R-phycoerythrin antibody conjugation kit (Abd Serotec, Oxford, UK). Cells were stained for 20 minutes in the dark at room temperature, washed, fixed in PBS containing 2% formaldehyde, and acquired on an LSRII flow cytometer (Becton Dickinson, San Jose, CA). For detection of intracellular Ki67 and Bcl2, cells were surface stained, fixed, and permeabilized with a saponin-based buffer (BD biosciences) followed by incubation with anti-Ki67-PE and anti-Bcl2-FITC (BD Biosciences, San Jose, CA) for 40 minutes on ice. For detection of phospho-epitopes, fixed cells were permeabilized with a methanol-based buffer (BD biosciences) and stained with anti-phospho Akt-PE (S473) and anti-phospho-Stat5 Alexa Fluor 647 (Y694) (BD Biosciences). Data were analyzed using FACSDIVA, (Version 6.2 BD Bioscience, San Diego CA) or FLo-Jo software. Maturation subsets were determined based on CD45RA, CD27 and CCR7 expression.

**CD127 RNA measurement by RT PCR**

Using a cocktail of antibodies targeting CD8, CD14, CD15, CD16, CD19, CD25, CD34, CD36, CD45RO, CD56, CD123, TCRγ/δ, HLA-DR, and Glycophorin A followed by magnetic bead separation (Miltenyi, Auburn, CA), naïve CD4 T cells were separated by negative selection from PBMCs treated for two days with IL-6 or IL-1β. Naïve CD4 T cell purity was consistently >90%. Naive CD4 T cells were lysed in RLT buffer
(Qiagen, Valencia, CA) and stored at -80°C. RNA was isolated with an RNeasy mini kit (Qiagen) and was reverse transcribed using the High Capacity RNA-to-cDNA kit (Applied Biosystems, Grand Island, NY). cDNA was amplified by StepOnePlus (Applied Biosystems, Carlsbad, CA) real-time quantitative PCR in the presence of SYBR Green (Applied Biosystems). Primers for IL-7 receptor transcripts were 5’-AAAGTTTTAATGCACGAT-3’, 5’-TGTGCTGGATAAATTCACATGC-3’. Gene expression was normalized to 18S rRNA using primers obtained from Applied Biosystems (part No. 4308329).

**CFSE dye dilution**

Cell division was assessed by labeling PBMCs with 5(6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE) (Molecular Probes Invitrogen, Grand Island, NY) for 10 minutes at 37°C. Staining was quenched by the addition of FBS for 5 minutes on ice. Cells were then washed and cultured as described.

**Immunohistochemistry and quantitative image analysis**

Immunohistochemical staining using rabbit anti-human mature IL-1β (ab2105; Abcam, Inc.) was performed using a biotin-free polymer approach (Rabbit Polink-1, Golden Bridge International, Inc.) on 5-µm tissue sections mounted on glass slides, dewaxed and rehydrated with double-distilled water. Antigen retrieval was performed by heating sections in 0.01% citraconic anhydride at 122°C for 30 sec. Slides were stained with optimal conditions determined empirically on an IntelliPATH autostainer (Biocare Medical) that consisted of a blocking step (TBS with 0.05% Tween-20 and 0.5% casein)
for 10 min and an endogenous peroxidase block using 1.5% (v/v) H\textsubscript{2}O\textsubscript{2} in TBS (pH 7.4) for 10 min. Rabbit anti-human mature IL-1\(\beta\) was diluted in blocking buffer and incubated for 1h at room temperature. Tissue sections were washed, and the Rabbit Polink-1 staining system (Golden Bridge International, Inc) was applied for 30 min at room temperature. Sections were developed with Impact\textsuperscript{TM} 3,3'-diaminobenzidine (Vector Laboratories), counterstained with Hematoxylin and mounted in Permount (Fisher Scientific). Stained slides were scanned at high magnification (200x) with ScanScope CS System (Aperio Technologies, Inc.) yielding high-resolution data for the entire tissue section. Representative regions of interest (ROIs; 500 \(\mu\text{m}^2\)) were identified and high-resolution images extracted from whole-tissue scans. The percent area of the LN that stained for IL-1\(\beta\) was quantified using Photoshop CS5 and Fovea tools.

**Statistics**

Continuous variables were compared between groups using the Mann-Whitney U test (GraphPad Prism software, Version 5.04). \(P\) values of less than 0.05 were considered nominally significant.
Figure 3.1*

Figure 3.1) IL-6 and L-1β induce cell cycle initiation in T cells.
a) PBMCs from a healthy subject were stimulated with IL-6 (10ng/mL), IL-1β (10ng/mL) or IL-7 (2ng/mL) for 7 days. After 7 days, cells were washed, stained for intranuclear Ki67 and examined by flow cytometry. b) Summary data of Ki67 induction by IL-6 (n=10) and IL-1β (n=11). Group data were compared using the Mann-Whitney U test. c) IL-1β-induced cycling is highest in memory T cells; IL-6 induces cycling in naïve T cells. Naïve= CD45RA+ CCR7+ CD27+; central memory (CM)= CD45RA- CCR7+ CD27+; effector memory (EM)= CD45RA- CCR7- CD27-.

Figure 3.2*

**Inflammatory cytokines drive cell proliferation.**

a) PBMCs from a healthy control were stained with CFSE dye then cultured for 7 days with IL-6 (10ng/mL), IL-1β (10ng/mL) or IL-7 (2ng/mL). After 7 days cells were washed and stained for surface markers and acquired by flow cytometry. b) Summary data of 3 experiments

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Figure 3.2

Inflammatory cytokines drive cell proliferation.

- IL-7 induced proliferation is seen in all CD4 T cell maturation subsets, whereas IL-1β induced proliferation is seen almost exclusively in memory T cells. Data shown are representative of six experiments using 4 different subjects.

Figure 3.2*  

Inflammatory cytokines drive cell proliferation. d) Proliferation of CD8 T cell maturation subsets. Data shown are representative of six experiments using 4 different subjects. Maturation subsets were determined as outlined in Figure 1.

Figure 3.3* a.)

CD127 surface expression on CD4 T cells

Figure 3.3 LPS induces loss of CD127 on CD4 T cells that is partially mediated through IL-6. PBMCs from a healthy donor were treated in the presence or absence of 100ng/ml LPS for 2 days and CD127 surface expression on CD4 T cells was assessed by flow cytometry. In separate conditions, 10ug/ml of anti-IL-6 or IgA2 isotype were added to LPS treated PBMCs.

Figure 3.4

IL-6 and IL-1β down-regulate CD127 surface expression on CD4 T cells.

Representative and summaries of CD127 surface expression assessed on CD4 and CD8 T cells in PBMCs treated for two days in the presence or absence of a) 1 ng/mL IL-6 or b) 10 ng/mL IL-1β. Representative and summaries of CD127 surface expression in CD4 T cell subsets assessed in PBMCs treated in the presence or absence of c) 1 ng/mL IL-6 or d) 10 ng/mL of IL-1β for 2 days. e) CD127 mRNA expression normalized to 18s rRNA as assessed by RT-PCR in naïve CD4 T cells separated from PBMCs treated for two days with or without IL-6 or IL-1β.

Figure 3.5* IL-6 and IL-1β impair T cell responses to IL-7. PBMCs from a healthy donor were treated in medium alone or supplemented with 1 ng/mL IL-6 or 10 ng/mL IL-1β for two days followed by addition of 5 ng/mL IL-7. a) Stat-5 phosphorylation was measured 5 days after IL-7 addition. Representative and summary of 7 independent experiments shown as means and standard errors b) Surface staining for the α4β7 heterodimer 5 days after IL-7 addition. (c) PBMCs from healthy donors were treated in medium alone or supplemented with 10 ng/mL IL-6 or 10 ng/mL IL-1β for two days then intracellular Bcl2 expression was measured in CD4 and CD8 T cells 7 days after addition of IL-7 (2ng/mL).

Figure 3.6*

**Figure 3.6** IL-1β is expressed in lymph nodes from HIV infected patients and is decreased but not normalized in patients treated with ART. a) Mature IL-1β stained lymph node sections from 2 healthy controls, 2 viremic patients, and 2 HIV+ patients on therapy (200x magnification). White bars in lower left represent 200uM. b) Summary data of lymph node IL-1β staining from 6 healthy controls, 5 untreated HIV infected viremic patients and 8 HIV+ treated patients.

Chapter 4:

Expression of CX3CR1 and PAR-1 on mature CD8 T cells may localize these cells to sites of tissue injury and coagulation
Summary:

In spite of virological suppression, many treated HIV-infected patients are at high risk for morbidities such as cardiovascular disease. Mature effector CD8 T cells oftentimes persist in these patients, yet the contributions of CD8 T cell expansion to adverse clinical events are incompletely understood. We show here that treated HIV-infected patients display a skewing of CX3CR1 expressing CD8 T cells that localizes them to blood vessels. CX3CR1 CD8 T cells are found exclusively in circulation and not in lymphoid tissues. The thrombin-binding receptor, PAR-1, is expressed at high levels on CX3CR1+ CD8 T cells and is increased on CD8 T cells of treated HIV-infected patients. Expression of CX3CR1 and PAR-1 on mature CD8+ T cells may localize these cells to areas of tissue injury and clot formation contributing to non-AIDS morbidity and mortality during treated HIV disease.

Introduction

Antiretroviral therapy (ART) has significantly altered the natural course of HIV disease progression. Despite improvements in CD4 T cell counts and immunological function, many treated HIV-infected patients continue to experience increased morbidities compared to the general population (154, 155). Some of these morbidities are cardiovascular, and there is now evidence that treated HIV-infected patients are at a significantly greater risk for venous and arterial thrombotic events (156-158). Although it is currently unclear what drives cardiovascular risk in treated HIV infection, the inflammatory mediators IL-6, C-reactive protein, and the monocyte activation marker...
soluble CD14 independently predict cardiovascular events (159, 160). Increased monocyte activation is also demonstrable in treated HIV-infected subjects, and heightened surface expression of the procoagulant tissue factor on monocytes correlates with plasma levels of the coagulation breakdown product D-dimer, providing a link between innate immune activation and coagulation (161, 162).

While innate immune mechanisms have been shown to play a role in thrombosis, less is known regarding the contribution of adaptive immunity to thrombotic events. CD8+T cells are known to target vascular endothelial and smooth muscle cells (163, 164), and up to 50% percent of lymphocytes in advanced human carotid artery plaques are activated CD8+ T cells (68). Although the role of CD8+ T cells in atherosclerotic risk remains unknown, in mice CD8+ T cells were shown to promote atherosclerotic plaque development in a granzyme, perforin, or tumor necrosis factor-dependent manner (67), indicating an atherogenic role of CD8+ T cells in plaque development.

HIV infection is characterized by a profound expansion of circulating the CD8+ T cell numbers that in some individuals does not normalize even after many years of antiretroviral therapy (61). In treated HIV-infected subjects, these expanded CD8+ T cells comprise mature effector T cells that lack CD28 expression, yet among these cells, they are typically less differentiated than those of healthy control CD28- CD8 T cells, indicated by reduced expression of the cellular senescence marker CD57 (165) (64). CD8+ T cell persistence in treated HIV-infected patients is reflected in an abnormally low CD4/CD8 ratio. In recent studies Serrano-Villar et al, found that treated HIV-infected subjects with abnormally low CD4/CD8 ratios were at a higher risk for morbid events including cardiovascular disease (61). Inversion of the CD4/CD8 ratio was
independently associated with higher intima-media thickness and arterial stiffness, both important predictors of cardiovascular events (61).

While CD8 T cell persistence in HIV infection has been associated with risk factors for cardiovascular disease, the underlying mechanisms behind this association remain undetermined. Certain receptor polymorphisms on CD8 T cells such as the CX3CR1 receptor that mediates rolling along endothelial vessels have been shown to confer reduced risk for atherosclerosis. Additionally, activation of Protease-Activated Receptor-1 (PAR-1) by thrombin on CD8 T cells can enhance CD8+ T cell effector activity, suggesting a link between coagulation and CD8 T cell activation (165). Here, we provide evidence that the expanded CD8 compartment in treated HIV-infected patients is enriched for cells expressing CX3CR1 and PAR-1, a cell phenotype that localizes along vascular endothelium and may be activated in the setting of clot formation.

Results

Distinct homing characteristics of the CD8+ T cell compartment

T cell maturation subsets are distributed differentially throughout tissue sites that can be attributed to their distinct expression patterns of chemokine receptors. Naïve and central memory CD8+ T cells express the lymphoid homing receptor CCR7, whereas mature effector CD8+ T cells preferentially express CX3CR1 that allows rolling along endothelial vessels and subsequent extravasation to peripheral tissues (166, 167). We first assessed the homing characteristics of the circulating CD8 T cell compartment based
on CCR7 and CX3CR1 expression. We found that in healthy controls and treated HIV+ subjects, CD8 T cells expressed either CX3CR1 or the lymph node homing receptor CCR7 exclusively, and virtually never expressed both (Figure 4.1a). CX3CR1-expressing CD8+ T cells in healthy control and treated HIV+ subjects were absent from lymphoid tissues but found abundantly in circulation (Figure 4.1b). We next assessed the maturation phenotype of CCR7 and CX3CR1 expressing CD8 T cells. As expected, CD8 T cells that were more mature were less likely to express CCR7 and more likely to express CX3CR1 (Figure 4.1c), indicating that effector T cells express homing markers that localize them to endothelium.

Skewed migratory phenotypes of the CD8 compartment in treated HIV-infected subjects

Those treated HIV-infected patients that maintain abnormally low CD4/CD8 ratios display a preferential expansion of memory CD8 T cells that are CD28- (61). We asked whether skewed maturation of the CD8 T cell compartment was also associated with changes in the migratory phenotype of this population. CX3CR1 expressing CD8 T cells were proportionally increased in treated HIV-infected subjects when compared to proportions of CX3CR1-expressing CD8 T cells in healthy controls, while proportions of CD8 T cells expressing CCR7 were significantly reduced in treated HIV infected patients (Figure 4.2). Thus, the CD8 T cell compartment in treated HIV-infected patients is skewed towards a CX3CR1-expressing phenotype that localizes to blood vessels rather than to lymphoid tissues.

CX3CR1+ CD8 T cells express PAR-1
The thrombin-binding receptor PAR-1 has been shown to be expressed by CD8 T cells and PAR-1 transcripts have been shown to be increased in T cells from HIV-infected patients (165). We found that PAR-1 was expressed at higher levels on CX3CR1-expressing CD8 T cells than on CCR7-expressing CD8 T cells and that PAR-1 surface expression was increased in both CCR7 and CX3CR1 CD8 T cell populations of treated HIV infected patients when compared to expression on these cells of healthy controls (Figure 4.3), indicating that CD8 T cells localizing to blood vessels may interact with components of the clotting cascade.

**Discussion**

Primary HIV infection induces a rapid expansion of the CD8 T cell compartment that is not fully normalized in many subjects after control of HIV replication (56, 61). Persistently high CD8 T cells are oftentimes reflected by an inverted CD4/CD8 in treated HIV-infected subjects. Recently, it was found that inverted CD4/CD8 ratios were associated with higher incidences of non-AIDS morbidities, and in untreated HIV infection, the CD4/CD8 ratio predicted disease progression better than absolute CD4 T cell count or CD4 T cell percentage alone (168). In this study, we investigated the potential mechanisms linking CD8 T cell expansion to adverse outcomes in treated HIV infection.

Specific patterns of chemokine receptors dictate the tissue tropism of T cells. The CCR7 chemokine receptor specifies T cells that home to lymphoid tissues whereas the CX3CR1 chemokine receptor is important for tissue-bound effector T cell rolling and
adhesion along vascular endothelium. We find here that in healthy controls and treated HIV+ subjects, CD8 T cells expressed either CX3CR1 or the lymph node homing receptor CCR7 exclusively, and virtually never expressed both. We also find that CX3CR1-expressing CD8 T-cells are exclusively found within circulation and absent from lymphoid tissues. CX3CR1+ CD8 T cells show characteristic functions of mature effector cells, expressing high levels of perforin, granzyme B, and Interferon-gamma (166, 169). Thus, the exclusion of these cells from lymphoid tissues is likely important, as their presence could potentially interfere with many of the highly ordered immunological events that take place within these sites.

HIV-disease progression results in a relative loss of naïve and central memory T cells and concomitant gains in more mature effector T cells, skewing their tissue distribution from lymphoid tissues to peripheral extralymphoid sites (170). Here, we show that treated HIV-infected patients display higher proportions of CD8 T cells that express CX3CR1 rather than CCR7, indicating that the CD8 T cell compartment is skewed towards a phenotype that localizes them to blood endothelial vessels rather than to lymphoid tissues. Expression of CX3CR1 is closely associated with cardiovascular disease risk. CX3CR1 and its ligand CX3CL1 are highly expressed in atherosclerotic lesions, and patients with coronary artery disease have significantly increased CX3CR1 expression on peripheral blood mononuclear cells (171, 172). Furthermore, large population studies have identified polymorphisms in CX3CR1 that confer reduced risk for coronary artery disease (173). Thus, the skewing of the CD8 T cell compartment in treated HIV infected patients towards CX3CR1 expressing cells that roll along endothelial vessels may contribute directly to atherosclerotic risk. In future studies, functional analysis of CX3CR1+ CD8 T cells, such as the cytokines they secrete and their capacity to kill target cells, may give insights into the role of these cells in cardiovascular risk.

Although less studied, activation of the vascular endothelium is likely an important determinant of HIV-associated cardiovascular disease as well (174, 175). The
CX3CR1 ligand CX3CL1 (fractalkine) can be expressed on endothelial cells (ECs), and in resting states, ECs do not express fractalkine but can significantly upregulate the ligand upon exposure to inflammatory mediators (176). These elements, as well as the breakdown product of coagulation, D-dimer, are known to be increased in plasma of treated HIV-infected patients (36). Because inflammation is closely linked to progression of atherosclerosis (174), we propose that circulating inflammatory mediators known to be increased in treated HIV-infected subjects could promote CD8 T cell rolling and adhesion along the endothelium through inflammatory cytokine-induced upregulation of CX3CL1 on endothelial cells.

Other factors have been shown to contribute to the development of atherosclerosis in HIV infection as well. Cytomegalovirus (CMV) has been implicated in atherosclerosis in part due to its ability to activate endothelial cells, an important step in development of atherosclerosis (177). In both the general population and HIV-infected subjects, higher frequencies of CMV-specific CD8 T cells are associated with increased risk of atherosclerotic cardiovascular disease (69, 177, 178). While CMV replication alone can promote atherosclerosis through endothelial damage (179), it is likely that the host-directed immune response against CMV is important in driving CMV-associated cardiovascular pathology. CMV-specific CD8+ T cells persist at very high levels in treated HIV-infected subjects, and the frequencies of CMV-specific CD8+ T cells correlate directly with intima-media thickness in these subjects (69, 180). Interestingly, endothelial damage from the CMV-specific host immune response can be prevented by targeted disruption of the CX3CR1/CX3CL1 axis in an in vitro model of CMV
replication in endothelial monolayers (181). In future experiments, it will be important to determine whether the heightened frequencies of CX3CR1-expressing CD8 T cells we observe in treated HIV infected subjects can be attributed in part to a selective expansion of those CD8 T cells that are CMV-specific.

Coagulation results from a series of complex events that can induce cleavage of the prothrombin complex to thrombin. This can be initiated intrinsically through endothelial damage, or extrinsically through cleavage of prothrombin by tissue factor expressed on the surface of circulating monocytes (182). This ultimately results in formation of a fibrin clot and inflammation has been implicated as a driver of clot formation (Funderburg, cardio ’14). In treated HIV-infected patients, inflammatory indeces and the breakdown product of clot formation D-dimer are elevated (36). Here, we show that surface expression of PAR-1, a receptor activated by thrombin, is increased on CD8+ T cells of treated HIV-infected patients and is particularly elevated in CD8+ T cells that express CX3CR1. Pro-inflammatory effects of PAR-1 activation on a number of cell types have been well described (183). On endothelial cells, PAR-1 activation can initiate the production of a number of inflammatory mediators such as IL-6, IL-8, TGF-β, and L-selectin (154 cite). Thrombin can also increase production of IL-1β, TNF, and IL-10 in monocytes (154 cite). The role of PAR-1 activation on CD8+ T cells however is less studied. A recent report has shown that in combination with T-cell receptor signaling, PAR-1 activation can enhance IFNγ production, and can induce CD8 T cell chemokinesis (165). Our data suggest that CD8+ T cells that localize to blood vessels might be influenced by coagulation through activation of PAR-1. The specific effects of PAR-1 activation on CD8 T cell function are under current investigation and will be discussed in further detail in the following chapter.

In-depth phenotyping of leukocyte populations within human arterial plaques have shown that the plaque represents an immunologic compartment that is distinct from peripheral blood
with high frequencies of CD8+ T cells that are preferentially activated (68). While further studies are needed to assess the direct role of CD8+ T cells in atherosclerotic plaque development, it is likely that expression of CX3CR1 on CD8 T cells may play a role in localization to these sites. Our findings of skewed proportions of CD8 T cells that express CX3CR1 and their propensity to interact with thrombin, a mediator of thrombosis, may implicate a role for these cells in cardiovascular disease risk in HIV infection.

**Materials and methods**

*Ethics Statement*

All subjects provided written informed consent in accordance with the Declaration of Helsinki. Patient studies were approved by the University Hospitals/Case Medical Center Institutional Review Board.

*Flow Cytometry*

Viable cells were gated using LIVE/DEAD-Aqua or yellow viability dye (Invitrogen, Grand Island, NY). Lymphocytes were identified by forward and side scatter and T cell phenotype was assessed using the following fluorochrome conjugated monoclonal antibodies: anti-CD3 peridinin chlorophyll protein (PerCP), anti-CD8 AlexaFluor 700 (AF-700), anti-CD45RA Brilliant Violet 510 (BV-510), anti-CD27 Brilliant Violet 785 (BV-785), CX3CR1-Allophycocyanin (APC) (all from Biolegend, San Diego, CA), anti-CD197 Phycoerythrin-CF594 (PE-CD594), CD38 Phycoerythrin-cy7 (PE-cy7), HLA-DR Flourescin (FITC) (all from BD Biosciences, San Jose, CA), and anti-PAR1 Phycoerythrin (PE) (Beckman Coulter, Brea, CA). Cells were stained for 20 minutes in the dark at room temperature, washed, fixed in PBS containing 2% formaldehyde, and
acquired on an LSRII flow cytometer (Becton Dickinson, San Jose, CA). Data were analyzed using FLo-Jo software. Maturation subsets were determined based on CD45RA, CD27 and CCR7 expression.
Figure 4.1

a.) The expression patterns of CX3CR1 and CCR7 were compared in the CD8 compartment.
b.) Matching blood and lymph node samples were assessed for CX3CR1 surface expression.
c.) CX3CR1 and CCR7 expression were compared in CD8 maturation subsets by CD45RA and CD27 expression.

Figure 4.1: Distinct homing phenotypes of the CD8 compartment. a.) The expression patterns of CX3CR1 and CCR7 were compared in the CD8 compartment. b.) Matching blood and lymph node samples were assessed for CX3CR1 surface expression. c.) CX3CR1 and CCR7 expression were compared in CD8 maturation subsets by CD45RA and CD27 expression.
Figure 4.2. CXC3CR1 and CCR7 define mutually exclusive CD8 T cell populations. Proportions of CX3CR1 and CCR7 expressing CD8 T cells were assessed by flow cytometry in healthy controls and treated HIV-infected patients.
Figure 4.3

Figure 4.3. The thrombin-binding receptor, PAR-1 is highly expressed on CX3CR1+ CD8 T cells. PAR-1 expression on CCR7 and CX3CR1 expressing CD8 T cells was assessed by flow cytometry in PBMCs from healthy controls and treated HIV-infected patients.
Chapter 5:

Future directions: Targeting cytolytic cells to lymphoid sites of HIV persistence and studying the role of CD8 T cell lymphocytosis in cardiovascular risk in treated HIV infection.
Summary

HIV infection is characterized by a broad immunological dysfunction that cannot be solely attributed to viral replication alone. Inflammation and chronic immune activation that persist throughout disease course are hallmarks of HIV infection, and it is now clear that these indices are central driving forces in disease pathogenesis. Here, we provide mechanistic insights into some of the known immunological abnormalities that characterize HIV infection. HIV-associated lymphadenopathy is a sentinel manifestation of HIV infection. Through the development of novel assays that detect activity of the receptor that mediates T cell egress from LNs, S1P1, we show that responsiveness to S1P is blunted in T cells from LNs of untreated HIV+ viremic subjects. Interestingly, our findings of blunted T responsiveness to S1P in HIV infection can be recapitulated by treatment of healthy PBMCs with microbial products in vitro. Our data suggest that diminished T cell responsiveness to S1P may contribute to lymphadenopathy in HIV infection and may be driven by inflammation.

We also show that the inflammatory mediators IL-6 and IL-1β can induce CD4 T cell turnover and blunted responsiveness to the homeostatic cytokine IL-7. These findings resemble some aspects of the immunophenotype observed in treated HIV-infected patients with Immune Restoration Failure. Taken together, these studies highlight the central importance of chronic inflammation in HIV infection, and they suggest that therapies designed to target inflammatory mediators in HIV infection may be clinically useful.

Chronic inflammation in treated HIV infection is associated with non-AIDS
related clinical events such as cardiovascular disease. In this setting, persistently high circulating CD8 T cell counts have also been associated with morbid events in treated patients. We provide evidence here that CD8 T cells express high levels of CX3CR1 and PAR-1 on their cell surface. This may localize these cells to areas of tissue injury and clot formation, contributing to non-AIDS morbidity and mortality during treated HIV disease.

We plan to further examine the role of both S1P1 manipulation as a means to reduce the latent viral reservoir, as well as examine the role of PAR-1 and CX3CR1 in contributing to HIV-associated cardiovascular disease risk. In one of our future directions, we propose to concentrate virus-specific lymphocytes within the LN through FTY720 treatment of virologically suppressed Rhesus Macaques. Another future direction will focus on functional studies of PAR-1 and CX3CR1 on CD8 T cells.

**Future Direction**

**Hypothesis:** Treatment of SIV-infected virologically suppressed Rhesus Macaques with FTY720 will reduce the latent viral resevoir through entrapment of virus-specific CD8 T cells within lymphoid tissues.

**Rationale:**

Although antiretroviral therapy suppresses viral replication and alters disease course, the establishment of a reservoir of resting CD4 T cells harboring proviral HIV DNA represents the single most significant hurdle in providing a lifetime cure (184, 185). Thus, therapeutic strategies aimed at erradicating the latent resevoir are of central importance in current HIV research. Multiple lines of evidence suggest that HIV-specific
cytotoxic CD8 T cells (CTL) are important in controlling both the size and diversity of the viral reservoir (186-188). Although comprehensive analysis of tissue sites harboring proviral DNA have not been performed in humans, in a rhesus macaque model of antiretroviral therapy, lymphoid tissues as well as the GI tract were found to contain the high frequencies of proviral DNA when compared to other anatomical sites (189). Thus, concentrating virus-specific CTLs in lymphoid tissues by blockade of T cell egress through FTY720 administration may promote the killing of CD4 T cells harboring HIV-proviral DNA and reduce the size of the viral reservoir. Here, we propose to administer ART treated rhesus macaques (RMs) FTY720 and measure proviral DNA and viral RNA by latent reactivation \textit{in vitro} to measure changes that might occur in the size of the viral reservoir. Here, we propose to administer FTY720 to ART-treated virologically suppressed SIV-infected Rhesus macaques to assess whether concentrating virus-specific effector T cells in lymphoid tissues will affect the size of the latent SIV reservoir.

**Preliminary data:**

To assess whether the proposed study is plausible, we first must determine whether FTY720 abrogates S1P responses in RM T cells, as it does in human T cells. We find that after an overnight incubation period in serum-free medium, S1P-induced actin polymerization is detectable in RM CD4 and CD8 T cells and that pre-treatment with FTY720 abrogated these responses (Figure 5.1). It is likely that FTY720-induced antagonistism of the S1P1 receptor results in blockade of lymphocyte egress in RMs \textit{in vivo} as it does in humans and mice, as a small study of RMs given FTY720 resulted in a mean 30% reduction in circulating lymphocyte numbers (190). Thus, in our study we will measure both S1P responses as well as absolute numbers of circulating lymphocytes in
FTY720-treated RMs as a way to monitor drug efficacy. We will also perform lymph node biopsies on these monkeys to assess the proportions, maturation, and activation state of lymphocytes induced to be retained at these sites.

We propose that treatment of FTY720 to SIV-infected RMs will reduce levels of proviral DNA by sequestering viral-reactive CTLs within the LN where they may lyse SIV infected targets as they express viral peptides during spontaneous blips of viral expression (191). To measure changes in the viral reservoir, we have developed a PCR-based approach that can detect latently infected cells that can produce replication-competent virus upon reactivation. A schematic of this experimental approach is illustrated (Figure 5.2). In brief, PBMCs or LNMCs from RMs would be stimulated \textit{in vitro} with anti-CD3 in the presence of an antiretroviral drug cocktail to prevent further rounds of de novo infection. Viral RNA in the supernatant would be quantified by a nested RT-PCR approach amplifying a portion of the HIV long terminal repeat U5 region against a standard curve of known HIV DNA concentration. The first step of this approach involves reverse transcription of a conserved region of the Gag gene which lies downstream of the 5'-long terminal repeat (5'-LTR) section of the HIV genome. The reverse transcribed cDNA product is composed of the 5'-LTR region followed by a portion of the Gag gene. The 5'-LTR contains terminal sequences known as the U5 region, and in the initial PCR reaction, primers spanning a portion of the U5 region are used to amplify the cDNA product. In the second PCR reaction, primers binding to conserved sequences of the U5 region contained within the PCR product generated in the first step are used to further amplify the product. The absolute number of copies is quantified through utilizing a fluorescent labeled probe complementary to sequences
within a portion of the U5 region generated in the second PCR step. This approach will allow us to quantify the amount of virus that has been processed, packaged, and secreted in the culture supernatant. Quantification of integrated proviral DNA will be measured by Alu-PCR, with primers complementary to the SIV LTR and chromosomal Alu repeats (192). While we have optimized these assays with primers specific for sequences of the HIV genome, using this same approach to detect SIV vRNA and vDNA will require different primer sets, however multiple groups have successfully quantified SIV DNA and RNA in a number of different non-human primate species, therefore translating this approach to amplify regions of the SIV genome will be feasible (193-195).

**Implications:**

Pharmacological blockade of T cell egress has profound immunomodulatory effects, as administration of FTY720 dramatically reduces the rate of relapses in patients with multiple sclerosis (196). What is less clear is how blockade of lymph node egress may influence the course of chronic viral infections. In non-human primates, short-term administration of FTY720 to SHIV-infected RMs had no effect on altering any aspects of disease course (190). In this study however it is unclear of whether the dosing of FTY720 given was sufficient enough to induce lymphocyte retention within lymphoid tissues. Other studies have reported as much as 90% reductions in circulating lymphocytes that was apparent in both CD4 and CD8 populations, whereas the RM study reported mean reductions of circulating lymphocytes by only 30% that did not affect the frequencies of circulating CD8 T cells (108). Reasons for this lack of FTY720 efficacy are unknown, however it is important to highlight the pre-existing blockade of LN egress that is already apparent in uncontrolled chronic viral infections such as SIV or HIV.
Thus in this setting, the effects of FTY720 may have been redundant in that egress blockade may have already been induced by pathogenic effects of the virus itself as would be suggested by our prior publication (197). Our proposed study is novel because FTY720 administration would significantly alter the trafficking patterns of T cells that have returned to a steady state by antiretroviral treatment, and may affect the size of the viral reservoir through sequestering virus-specific CD8 T cells within the lymph node. FTY720 may also induce sequestration of certain NK cell subsets that can be found in LNs, which when primed with IL-2 or IL-15, can acquire antiviral functions (198).

There are some caveats to our proposed study. We hypothesize that FTY720 will trap virus-specific effector CD8 T cells in the LN. It is important to point out however that effector and effector memory CD8 T cells do not express CCR7 or CD62L that control homing to LNs. Thus FTY720 may not mediate effector CD8 T cell LN entrapment because their homing patterns do not direct them to migrate to lymphoid tissues in the first place. In fact, FTY720 treatment in multiple sclerosis patients resulted in significant reductions in circulating naïve and central memory T cells but did not affect proportions of circulating effector T cells (199), indicating a preferential LN entrapment of only those T cells exhibiting tropism for lymphoid tissues. Nevertheless, FTY720 could mediate lymphoid retention of CCR7-expressing SIV-specific central memory CD8 T cells (especially during antiretroviral treatment), and upon antigen-specific activation, could mature to mediate antiviral activity. Additionally, it is plausible that naïve T cells reactive to SIV may be present that have not already matured in chronic infection, and lymphoid entrapment of these cells could also promote an antiviral response.
Additionally, T cell exhaustion that affects the quality of antiviral CD8 T cell responses is apparent in both treated and untreated HIV infection (200). Thus, while FTY720 may increase the frequency at which virus-specific CTLs come into contact with latently infected CD4 T cells, these cells may not be able to efficiently mediate viral clearance due to inhibitory receptors such as PD-1 and CD160 on the CD8 T cell that dampen effector function. To preserve the quality of CD8 CTL responses in our study, it is plausible that FTY720 could be administered in combination with a PD-1 blocking antibody that would block T cell inhibitory signals (201), or with administration of IL-15, a homeostatic cytokine that has been shown to augment CD8 T cell effector function (202). Nevertheless, the profound immunomodulatory effects of FTY720 alone may provide some interesting avenues to explore the effects of LN egress blockade in a model of treated HIV infection.

**Hypothesis:** PAR-1 activation will induce functional responses in CD8 T cell populations that may include CMV-specific CD8 T cells.

**Rationale:**

In mice, CD8 T cells have been shown to contribute to the development of atherosclerotic plaques (67). While less is known about the role of CD8 T cells in plaque development in humans, CD8 T cells can comprise up to 50% of lymphocytes within human plaques (68). A proportion that exceeds their representation in peripheral blood of most HIV uninfected persons (203). Furthermore, we have demonstrated that CX3CR1+ CD8 T cells highly express the thrombin-binding receptor, PAR-1, indicating that CD8 T cells may play a direct role in cardiovascular events such as thrombosis. Here, we plan to
explore the signaling molecules induced by PAR-1 activation on CD8 T cells, and determine how PAR-1 activation may influence CD8 T cell migration and effector function, specifically focusing on CMV-specific CD8 T cells as these may be localized to sites of endothelial injury. Furthermore, we plan to assess the role of CX3CR1 and PAR-1 on CD8 T cell recruitment to areas of endothelial stress through the use of an in vitro flow chamber that facilitates leukocyte rolling along an endothelial monolayer.

Preliminary findings:

While the functional effects of PAR-1 activation on CD8 T cells are not well characterized, more is known about the activation of this receptor on other cell types. On platelets, PAR-1 activation leads to stimulation of RhoA signaling that mediates actin cytoskeletal rearrangement, contributing to platelet aggregation and initiation of clot formation (204). PAR-1 activation on endothelial cells leads to cytoskeletal arrangements also, causing endothelial cell contraction and an increase in vascular permeability (205). In future experiments, we will determine whether PAR-1 activation on CD8 T cells leads to polymerization of the actin cytoskeleton in vitro.

Because CX3CR1+ CD8 T cells are likely localized to the vascular endothelium, they may come into contact with pathogens that replicate within endothelial cells such as CMV. There is evidence that CMV infection can induce a procoagulant response, both in vitro and in vivo (206, 207). Thus, it is plausible that CMV-specific CD8 T cells localized to sites of active CMV replication could be exposed to procoagulant factors such as thrombin, influencing their function. Here, we show that we can measure CMV-
specific CD8 T cells are reactive to peptides derived from pp65, a structural protein of CMV, by fluorochrome-labeled tetramers in HLA-A2 MHC haplotype individuals (Figure 5.3). In future studies, we will determine whether CMV-specific CD8 T cells express PAR-1, whether PAR-1 activation on these cells induces changes in the actin cytoskeleton, and whether PAR-1 activation modulates the effector function of CMV-specific CD8 T cells.

**Future experiments:**

It will first be important to characterize the functional responses induced by PAR-1 activation. Evidence of the functional consequences of PAR-1 activation on CD8 T cells is scant, thus we will assess the impact of PAR-1 activation on CD8 T cells as broadly as possible. We will employ a combination approach through cell signaling assays, gene transcription profiles, and functional measurements of cytokine secretion and proliferation to assess the effects of PAR-1 activation on CD8 T cells. To determine specificity in these experiments, the PAR-1 antagonist Vorapaxar, will be used as a control in our experiments.

PAR-1 stimulation by thrombin may activate intracellular signalling cascades in CD8 T cells. In T cells, PAR-1 can be coupled to $G_{\alpha_{12/13}}$ or $G_{\alpha_4}$ G-proteins that have been shown to mediate calcium influx upon PAR-1 activation and G-protein subunit dissociation (165). Because intracellular calcium mobilization is a known modulator of the MAPK signaling cascades, we will also assess whether phosphorylation of the ERK1/2 or p38 MAP kinases occur with thrombin treatment. To confirm receptor specificity, we will assess thrombin-induced ERK1/2 or p38 MAPK activation in the
presence of a PAR-1. Additionally, we will co-stimulate PBMCs with anti-CD3 and anti-CD28 in the presence or absence of thrombin to determine whether thrombin affects signaling cascades induced by T cell activation. T cell receptor signaling will activate a number of downstream responses that are not limited to MAPK activation. These include phosphorylation of lymphocyte specific protein-tyrosine kinase (Lck), activation of the PI3K cascade, phosphorylation of protein kinase C (PKC), and nuclear translocation of transcription factors NF-kB and NFAT (208). Thus, we will assess whether these TCR signaling cascades in CD8 T cells are affected by co-treatment with thrombin.

Commercially available antibodies that are compatible with the phosflow method exist for many of these signaling molecules. Our group has experience with the phosflow method (197), thus these experiments will be feasible.

The activation of signaling molecules induced by PAR-1 stimulation on CD8 T cells may affect gene transcription. In subsequent experiments, we will assess a wide range of genes that may be affected by PAR-1 activation. To do this, we will stimulate separated CD8 T cells with thrombin and compare the transcriptional profile of these cells to the transcriptional profile of CD8 T cells left unstimulated. PAR-1 activation is known to induce pro-inflammatory functional responses in a number of different cell types (204), thus we will focus our studies on genes related to inflammatory responses. Using this method, we will also assess changes in gene expression profiles in CD8 T cells co-stimulated with thrombin and anti-CD3/anti-CD28 and compare these profiles to gene expression in CD8 T cells stimulated with anti-CD3/anti-CD28 alone. This method may provide an exploratory approach to broadly assess the impact of PAR-1 activation on CD8 T cells.
PAR-1 activation can affect CD8 T cell effector function as well. Recent reports indicate that dual stimulation of PBMCs with anti-CD3 and thrombin can augment CD8 T cell interferon-gamma production when compared to anti-CD3 stimulation alone (165). In future experiments, we will determine whether PAR-1 activation leads to enhancement of other aspects of CD8 T cell effector function. Importantly, the progression of atherosclerotic plaque development in mice was shown to be dependent upon granzyme B and perforin expression in CD8 T cells (67). In future experiments, we will treat PBMCs for 6 hours in the presence or absence of thrombin with anti-CD3/CD28 antibodies and expression of granzyme B and perforin in CD8 T cells will be assessed. We will also look at expression of the T-box transcription factor, T-bet, as this has been shown to control granzyme B expression and CD8 cytotoxicity in both humans and mice (209, 210).

CMV-infected individuals are at higher risk for cardiovascular complications, and in HIV-infected individuals, the frequencies of CMV-specific CD8 T cells correlate directly with increased Intima Medial Thickness (IMT), a measure of cardiovascular disease (69, 211). Thus, in future experiments it will be important to determine whether functional effects of PAR-1 activation on CD8 T cells extend to those that are specific for CMV. We will first determine whether CMV-specific CD8 T cells express PAR-1, by assessing its surface expression on CMV-tetramer+ CD8 T cells. We will additionally measure CMV-specific effector CD8 T cell responses by stimulating PBMCs with the CMV peptides pp65 or IE-1 in vitro. Here, we will assess whether the addition of thrombin influences cytokine production, or granzyme and perforin expression in those CD8 T cells that respond to pp65 or IE-1 CMV peptides. This will allow us to determine
whether PAR-1 mediates effector function of CMV-specific CD8 T cells that may localize to areas of tissue injury and clot formation.

While our proposed experiments may assess how coagulation mediators such as thrombin may influence CD8 T cell function, they cannot assess how PAR-1 may influence CD8 T cell interaction with endothelial cells, a likely important process in atherosclerotic events. While studying T cell/endothelial cell interactions would be difficult in vivo, we can recapitulate some of these interactions that take place through the use of an in vitro flow chamber system that models leukocyte rolling and transmigration through endothelial cells (Figure 5.4) (in collaboration with Dr. R. Ransohoff, Cleveland Clinic). In brief, endothelial cells can be cultured and grown to confluence along a collagen-coated polycarbonate filters. These filters are then placed between top and bottom plates. A gasket is then placed over the top plate to create an area that mimics shear forces of blood flow. PBMCs can then be perfused into the apparatus through a pump. At the end of the defined period of flow, those PBMCs that have transmigrated through the endothelial layer can be collected from the lower chamber to assess their phenotype by flow cytometry. This experimental system has been used by Ransohoff and colleagues to model leukocyte transmigration across a modeled the blood brain barrier (212). In this system, CXCL12 induced monocytes, CD4 and CD8 T cells, as well as B cells to transmigrate across the endothelial cell layer. Additionally, monocytes preferentially adhered to the endothelial cell layer and this was dependent upon CXCL12 (212).

Inflammation is a key mediator in multiple steps of atherosclerosis. Thus, we will first determine whether inflammatory mediators known to be increased in HIV infection
such as TNFα, IL-1β, or thrombin can influence transmigration of CD8 T cells across the endothelial layer (176). Because these cytokines are known to induce CX3CL1 on endothelial cells, we suspect that higher numbers of CD8 T cells will transmigrate across the endothelium treated with inflammatory cytokines when compared to endothelium left untreated, and that these CD8 T cells would express CX3CR1. To determine specificity, we can perform these assays in the presence of CX3CR1 neutralizing antibodies to determine if transmigration is CX3CR1/CX3CL1 dependent. Furthermore, the use of PAR-1 antagonists in this system can allow us to determine whether activation of PAR-1 contributes to CD8 T cell endothelial transmigration as well.

Through the use of flow cytometry, we could further phenotype CD8 T cells that have transmigrated to the bottom chamber. In mice, CD8 T cells have been shown to exacerbate plaque formation in a granzyme B and perforin-dependent manner (67). Thus, it would be interesting to assess whether transmigrated CD8 T cells found in the bottom chamber are cytotoxic and express granzyme B and perforin in response to SEB or anti-CD3/anti-CD28 stimulation, as these cells may play a role in plaque development.

Endothelial damage mediated by CMV is likely an important driver of atherosclerosis in HIV infection (69). To assess the impact of CD8 T cell recruitment to areas of active CMV replication, we could infect endothelium-coated filters with CMV and determine whether endothelial damage mediated by CMV induces arrest, or transmigration of CD8 T cells across the endothelial layer. Frequencies of CD8 T cells specific to CMV that are induced to transmigrate may be low, and would also be restricted by MHC molecules not shared by the endothelial monolayers. This system
however could allow us to determine whether CMV replication damages the epithelium and also whether this affects CD8 T cell transmigration.

In summary, these experiments will allow us to determine whether CD8 T cell function is affected by mediators of coagulation such as thrombin, and whether CX3CR1 and PAR-1 play a role in CD8 T cell recruitment to endothelial cells activated by inflammatory mediators.

Conclusions:

HIV infection impairs many aspects of immunity that are not completely normalized even in patients who restore circulating CD4 T cell numbers to numbers within the range of “normal”. There are still many aspects of HIV disease pathogenesis that are incompletely understood. Here, we give mechanistic insights into some of the known features of HIV pathogenesis such as HIV-associated lymphadenopathy, residual immune failure in treated HIV infection, and cardiovascular risk in treated HIV infection. We provide evidence that inflammatory mediators known to be increased in HIV infection can recapitulate in vitro some aspects of the immune phenotype that is observed in HIV-infected subjects. Thus, targeted blockade of inflammation in chronic HIV infection may improve many aspects of T cell function and promote immune reconstitution. Currently, a number of clinical trials are underway, aimed at blocking type I Interferon or the IL-6 receptor. Our work provides a rationale for the implementation of these therapies in future clinical settings, as we show mechanistically
that some of these pro-inflammatory mediators may play a role in impairing aspects of T cell function that are essential for immune homeostasis.
Figure 5.1

S1P responses can be detected in NHP primary human T cells and are S1P-receptor specific. PBMCs from a rhesus macaque were shipped from Emory University. T cells were separated and T cell actin polymerization responses were assessed following an O/N incubation in serum-free medium.
Figure 5.2

Schematic of high-sensitivity PCR assay that can detect viral RNA in vitro.
**Figure 5.3**

**Staining for CMV-specific CD8 T cells:** PBMCs from a donor of an HLA-A2 haplotype were stained with pp65 tetramers and combined with antibodies to CD28 and CX3CR1. Gating shows CX3CR1 expression in CMV-reactive CD8 T cells that are CD28-negative.
Figure 7.3 An *in vitro* model for leukocyte rolling and transmigration through endothelium (Schumei, *Sci Trans Med* ’12). Endothelial cells are cultured and grown to confluence along a collagen-coated polycarbonate filters. These filters are then placed between top and bottom plates. A gasket is then placed over the top plate to create an area that mimics shear forces of blood flow. PBMCs can then be perfused into the apparatus through a pump. At the end of the defined period of flow, those PBMCs that have transmigrated through the endothelial layer can be collected from the lower chamber to assess their phenotype by flow cytometry.
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