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Cell-to-cell transmission of HIV-1 is required to trigger pyroptotic death of CD4 T cells in lymphoid tissue

Author
Galloway, Nicole Louise Katherine

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CELL-TO-CELL TRANSMISSION OF HIV-1 IS REQUIRED TO TRIGGER PYROPTOTIC DEATH OF CD4 T CELLS IN LYMPHOID TISSUE

by

NICOLE LOUISE KATHERINE GALLOWAY

DISSERTATION

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Dedications

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Cell-to-cell transmission of HIV-1 is required to trigger pyroptotic death of CD4 T cells in lymphoid tissue

Nicole Louise Katherine Galloway

Abstract

The pathway causing CD4 T-cell death in HIV-infected hosts remains poorly understood although apoptosis has been proposed as a key player. Our studies now show that caspase-3-mediated apoptosis only mediates the death of only a small fraction (<5%) of the total CD4 T cells that correspond to activated cells. The remaining >95% of quiescent lymphoid CD4 T-cells die by caspase-1-mediated pyroptosis triggered as a result of abortive viral infection occurring in nonpermissive resting CD4 T cells. Pyroptosis is an intensely inflammatory form of programmed cell death where cytoplasmic contents and pro-inflammatory cytokines including IL-1β and IL-18, are released. This death pathway thus links the two signature events in HIV infection—CD4 T-cell depletion and chronic inflammation—in a single process and creates a vicious pathogenic cycle where dying CD4 T-cells release inflammatory signals that attract more cells to die. This cycle can be broken with caspase-1 inhibitors, including a compound already shown to be safe and well-tolerated in humans. These inhibitors could form a new class of "anti-AIDS" therapeutics that target the host rather than the virus. We further show that cell-to-cell transmission of HIV is obligately required for the induction of pyroptosis. Cell-free HIV-1 virions, even when added in large quantities, due not suffice. These findings underscore the infected CD4 T cell as the major killing unit promoting progression to AIDS and highlight a previously unappreciated biological role for the virological synapse.

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Chapter One:

Introduction

1.1. The HIV-1 Pandemic

Human immunodeficiency virus (HIV) has infected 75 million people and killed more than 39 million, making it one of the most catastrophic infectious diseases in human history (UNAIDS, World Health Organization). Currently, there is no vaccine or curative therapy for HIV-1. With more than 2.1 million people newly infected in 2013 alone (amfAR), we need new strategies to combat HIV-1. Ultimately, to end the HIV pandemic, we need a better understanding of HIV biology that includes a detailed description of the fundamental manner in which HIV interacts with the human host and causes disease.

1.2. HIV-1: a retrovirus

HIV is a member of the *Retroviridae* family and sub-segregated into the Lentivirus genus (Freed 2007). HIV is an enveloped virus with a diploid, RNA genome that is approximately 9.8 kb in size. Members of the *Retroviridae* family reverse transcribe their RNA genomes into double-stranded DNA, which integrates into the genome of the host target cell. Each viral RNA contains nine genes that encode 15 proteins. These genes include the products of the *gag* gene (Matrix, Capsid, Nucleocapsid, and p6), the *pol* gene (Reverse Transcriptase, Protease, and Integrase) the *env* gene (gp120 and gp41), the regulatory genes *tat and rev*, and the accessory genes *vpu*, *nef*, *vif* and *vpr* (Hope 1999; Greene and Peterlin 2002).

1.3. HIV depletes CD4 T cells—the fundamental cause of AIDS

HIV infects cells of the immune system that express the receptor CD4 and either of the chemokine co-receptors CCR5 or CXCR4 (the viral tropism determines which co-receptor is used; R5 viruses use CCR5 while X4 viruses use CXCR4). These cells include macrophages,

dendritic cells, and monocytes; however HIV primarily targets CD4 T cells (Freed 2007). CD4 T cells exist in a resting and naïve state until they encounter their cognate antigen and become activated, after which they undergo proliferation and differentiation (Murphy 2008). Activated CD4 T cells are permissive to HIV infection, meaning that HIV can infect, integrate, and produce new virions in activated CD4 T cells, resulting in a productive infection. However, naïve CD4 T cells are non-permissive to productive HIV-1 infection. Although the virus enters these cells normally by fusion at the plasma membrane, the viral life cycle is halted during the elongation stage of reverse transcription, causing incomplete DNA reverse transcripts to accumulate in the cytosol (Zack, Arrigo et al. 1990; Swiggard, O'Doherty et al. 2004; Zhou, Zhang et al. 2005; Kamata, Nagaoka et al. 2009). The progressive loss of CD4 T cells in HIV-infected subjects is the fundamental cause of acquired immune deficiency syndrome (AIDS), which leaves infected patients unable to fight off opportunistic infections that ultimately cause their death (Moir, Chun et al. 2011). Despite more than three decades of study, the precise mechanisms underlying the demise of CD4 T cells during HIV infection remain poorly understood.

1.4. Direct versus indirect death of CD4 T cells induced by HIV

Two mechanisms of cell death have been implicated in depleting of CD4 T cells during HIV infection. The first mechanism is direct death, in which an infected CD4 T cell dies due to the cytopathic effects of virus production (Laurent-Crawford, Krust et al. 1991; Terai, Kornbluth et al. 1991). While direct death can cause the loss of productively infected CD4 T cells, the number of productively infected cells cannot account for the vast depletion of CD4 T cells observed in patients. The second mechanism is indirect death, in which uninfected “bystander”

cells are the majority of CD4 T cells that die. Often these uninfected bystander cells are clustered around a productively infected cell (Finkel, Tudor-Williams et al. 1995). Many factors have been implicated in indirect death, such as secreted viral proteins, HIV-1 envelope binding. or fusion between either infected cells or free virions and host death signaling proteins (Westendorp, Frank et al. 1995; Gandhi, Chen et al. 1998; Herbeuval, Grivel et al. 2005; Schindler, Munch et al. 2006). However, which method of cell death accounts for the majority of CD4 T-cell loss remains controversial.

1.5. A physiological experimental system using lymphoid tissue

Lymphoid organs are highly organized structures that include lymph nodes, spleen, tonsil and mucosal lymphoid tissues. Over 98% of CD4 T cells reside in lymphoid organs, which are the primary locations where CD4 T cells encounter antigens and interact with other immune cells to mediate successful immune responses (Finkel, Tudor-Williams et al. 1995; Murphy 2008). In addition, lymphoid organs are the primary sites for HIV replication (Pantaleo, Graziosi et al. 1993). Human lymphoid aggregated cultures (HLAC) created from fresh lymphoid tissue are a unique and physiological system for studying HIV infection and CD4 T-cell depletion. HLAC maintains the cellular composition and cytokine milieu of lymphoid organs and supports HIV infection without the adding external mitogens (Eckstein, Penn et al. 2001; (Glushakova, Baibakov et al. 1995), which makes it an ideal system for determining the molecular mechanisms underlying HIV-mediated depletion of CD4 T cells.

1.6. Abortive HIV infection depletes non-permissive CD4 T cells in lymphoid tissue

Using HLAC as an experimental system to examine CD4 T cell killing by HIV-1, Doitsh *et.al.* discovered X4 tropic HIV vastly depletes lymphoid CD4 T cells kills non-productively infected "bystander" cells. Surprisingly, this indirect killing involves abortive infection of resting CD4 T cells. HIV binds, fuses, and starts to undergo reverse transcription in resting CD4 T cells. However, because of the naturally non-permissive nature of these resting cells, reverse transcription cannot be completed, resulting in incomplete viral DNA products to accumulate in the cytosol. The host cell senses these DNA intermediates and triggers a death pathway that involves the secretion of type-1 interferon, activation of caspase-1, and release of bio-active interleukin (IL)-1β (Doitsh, Cavrois et al. 2010). Therefore, the cells do not die from of the toxic action of a viral product, but rather as a result of a host defense response to HIV infection. This cellular "suicide" likely evolved to protect the host by stopping viral spread: however, it massively depletes CD4 T cells and drives HIV pathogenesis.

1.7. Programmed Cell Death

Programmed cell death is critical for development, homeostasis and protection against pathogen infections. There are three main programmed cell death pathways: apoptosis, programmed necrosis (necroptosis), and pyroptosis. These pathways involve cysteine aspartly proteases called caspases, which are inactive zymogens whose activities are controlled through proteolytic cleavage either by autoactivation or by another caspase (Upton and Chan 2014).

1.7.1. Apoptosis

For decades after the discovery of programmed cell death, apoptosis was considered the most known cell-death pathway (Vanden Berghe, Linkermann et al. 2014). Apoptosis is

triggered by external and internal stimuli that activate initiator caspases (e.g., caspase-2, caspase-8, caspase-9, caspase-10) that, in turn, activate the effector caspases (e.g., caspase-3, caspase-6 , caspase-7), which cleave cellular substrates that kill the cell. Apoptosis is characterized by cell shrinkage, mitrochondrial permeabilization, nuclear and chromatin condensation, DNA fragmentation, membrane blebbing, and breakdown of the cell into apoptotic bodies (Kerr, Wyllie et al. 1972; Fink and Cookson 2005; Upton and Chan 2014). During this apoptosis, cellular contents are contained in the dying cell, which are quickly engulfed by phagocytic cells, making apoptosis a non-inflammatory or "silent" form of programmed cell death.

1.7.2. Necroptosis

Previously necrosis was considered as an accidental form of cell death caused by physical and chemical insults. However, recent genetic studies and the discovery of chemical inhibitors of necrosis have firmly demonstrated the existence of multiple tightly regulated pathways of programmed necrosis, or necroptosis (Holler, Zaru et al. 2000; Teng, Degterev et al. 2005; Degterev, Hitomi et al. 2008; Zhang, Shao et al. 2009). Necroptosis is triggered by diverse stimuli and depends on the activity of receptor-interacting protein kinase 1 (RIPK1 or RIP1) and/or receptor-interacting protein kinase 3 (RIPK3 or RIP3), as well as inactivation of caspase-8. If caspase-8 becomes active, it inhibits necroptosis and triggers apoptosis, in part by cleaving RIPK1 and RIPK3 (Holler, Zaru et al. 2000; Zhang, Shao et al. 2009; (Vercammen, Beyaert et al. 1998; He, Wang et al. 2009). Necroptosis is morphologically characterized by cytoplasmic granulation and, organelle and cellular swelling that causes a loss of membrane integrity and release of cytoplasmic contents (Upton and Chan 2014; Vanden Berghe, Linkermann et al. 2014).

1.7.3. Pyroptosis

Pyroptosis was first described as atypical, caspase-1-dependent apoptosis of macrophages (Cookson and Brennan 2001). However, it is now evident that it is a distinct form of programmed cell death that shares properties of both apoptosis and necroptosis. Pyroptosis is characterized by DNA cleavage, nuclear condensation, water influx, cellular swelling, plasma membrane rupture, and osmotic lysis, which causes the cell to release its proinflammatory cytosolic contents (Fink and Cookson 2006; Schroder and Tschopp 2010).

Pyroptosis is triggered by pathogen-associated molecular patterns (PAMPS) and danger-associated molecular patterns (DAMPS). Host proteins called inflammasome proteins sense a PAMP or DAMP and recruit pro-caspase-1, either directly or indirectly, through the adaptor apoptosis-associated speck-like protein containing CARD (ASC) to form a high-molecular weight, multiprotein complex known as the inflammasome. inflammasome formation leads to autocleavage and activation of caspase-1, which is responsible for the processing and secretion of the proinflammatory cytokines IL1-β and IL18 (Schroder and Tschopp 2010; Franchi, Munoz-Planillo et al. 2012; Strowig, Henao-Mejia et al. 2012).

The inflammasome proteins responsible for sensing and inflammasome formation are seperated into two families: NOD-like receptor (NLR) and the PYHIN (pyrin and HIN200). The former family includes NLRP1, NLRP2, NLRP3, NLRP6 and NLRC. These proteins all contain leucine rich repeats (LRRs) involved in sensing PAMPS and DAMPs; a nucleotide binding and oligomerization domain (Nod) responsible for self-oligomerization; and either a PYD or a caspase activation and recruitment domain (CARD), or both, which mediate(s) interactions with ASC or caspase-1, respectively. The PYHIN family includes absent in melanoma 2 (AIM2) and interferon-γ-inducible protein 16 (IFI16), which contain a PYD and a HIN200 domain

responsible for ligand binding (Schroder and Tschopp 2010; Franchi, Munoz-Planillo et al. 2012; Strowig, Henao-Mejia et al. 2012).

The dual release of the proinflammatory cellular contents and cytokines during pyroptosis results in a cell death that is highly inflammatory, which serves to recruit immune cells to the site of infection or injury. However, this potent form of cell death must be tightly regulated by inflammasome sensors to prevent excess inflammation. These sensors are restricted to specific cell types and often their expression is low until the cell receives a priming signal (Hornung and Latz 2010) (also known as signal I), such as bacterial components (e.g. such as LPS, cytokines, or reactive oxygen species (ROS)). After exposure to a priming signal, the components of the inflammasome and cytokines are upregulated; however, pyroptosis will not be triggered untl the cell senses a PAMP or DAMP (Hornung and Latz 2010; Strowig, Henao-Mejia et al. 2012).

1.8. A novel technique to introduce lentiviral vectors into resting CD4 T cells

A major hurdle in studies involving resting CD4 T cells was the inability to knockdown endogenous genes and express exogenous ones. Many delivery techniques had been attempted, but their success was limited by low transfection efficiencies, general cellular toxicity, laborious methods, or altered cell activation (Suzuki, Zelphati et al. 1991; Tahvanainen, Pykalainen et al. 2006; Wheeler, Trifonova et al. 2011). Infection with lentiviral vectors was an attractive means to knock down and express genes in resting CD4 T cells; however, this approach was limited by the non-permissiveness of these cells, in part due to the phosphohydrolase and RNase activity of the cellular protein SAM domain and HD domain-containing protein 1 (SAMHD1) (Goldstone, Ennis-Adeniran et al. 2011; Laguette, Sobhian et al. 2011; Powell, Holland et al. 2011; Baldauf,

Pan et al. 2012; Lahouassa, Daddacha et al. 2012; Ryoo, Choi et al. 2014). HIV-2 and related simian immunodeficiency viruses (SIVs) encode the accessory protein Vpx, which polyubiquitinates SAMHD1, leading to its proteasomal degradation (Laguette, Sobhian et al. 2011; Baldauf, Pan et al. 2012). Geng and Doitsh et. al. took advantage of this function of Vpx and utilized it to remove the restriction imposed by SAMHD1 in resting CD4 T cells. They created a two-step procedure that involves psuedotyping virus like particles (VLPs) with an X4 tropic HIV envelope that promotes efficient and targeted entry of the VLPs. In the first step, they spinoculated resting CD4 T cells from blood or tonsils with VLPs containing Vpx. In the second step, they spinoculated the cells with VLPs containing lentiviral vectors of interest. The first step leads to the degradation of SAMHD1 and creates a permissive window that promotes expression of the lentiviral vector in the second step (Geng, Doitsh et al. 2014). This process efficiently delivers lentiviral vectors into resting CD4 T cells without altering their activation status.

1.9. IFI16 is the DNA sensor that detects HIV in abortively infected lymphoid CD4 T cells

While abortive HIV infection induces a loss of lymphoid CD4 T cells, the host protein that detects incomplete HIV reverse transcripts in the cytosol and triggers death was still unknown. Using an unbiased proteomic approach and gene knockdown methodologies, Monroe et. al. identified interferon-γ-inducible protein 16 (IFI16) as the DNA sensor that detects HIV in abortively infected cells. They mixed biotinylated HIV DNA with tonsillar cytosolic extracts and then identified potential DNA sensor candidates by mass spectrometry. One of the top hits was IFI16, a known antiviral DNA sensor (Unterholzner, Keating et al. 2010; Kerur, Veettil et al. 2011). With two strategies to knock down genes—an "activated-rest" approach and the VLP-

Vpx method created by Geng et. al.—Monroe et al confirmed that knocking down IFI16 rescues the death of lymphoid CD4 T cells infected with HIV-1, while knocking down other candidate genes did not (Monroe, Yang et al. 2014).

IFI16 had been previously shown to assemble into inflammasomes by interacting with ASC and procaspase-1 after infection with Kaposi's sarcoma-associated herpesvirus (KSHV) (Kerur, Veettil et al. 2011). Its unique ability to not only act as a DNA sensor, but also as a component of the inflammasome made it an attractive protein to bridge viral DNA sensing with the triggering of caspase 1 dependent cell death. However, the death pathway activated in lymphoid CD4 T cells abortively infected with HIV was still unknown.

1.10. HIV-1 infection by cell free virus versus cell-to-cell transmission

HIV-1 can spread as cell-free virions and by transmission from an infected cell to a non-infected cell (cell-to-cell transfer). While, the relative contribution of cell-free virus versus cell-to-cell transmission of HIV-1 *in vivo* is unknown (Jolly, Kashefi et al. 2004), cell-to-cell transfer is the predominate method of viral infection *in vitro* (Sourisseau, Sol-Foulon et al. 2007) and is likely important *in vivo* when there are large amounts of cellular contacts, such as in lymphoid organs (Haase 1999). Outside the cell, the infectivity of HIV-1 decreases rapidly, because its envelope trimer spike disassembles over time. Thus, it is essential that HIV-1 quickly engage its target cell (Sattentau 2010). Cell-to-cell transmission of viruses is a faster and more efficient method for viral propagation than infection by cell-free viruses. It has been estimated to be 100-1,000-fold more efficient form compared to cell free virions. Cell-free virions must freely diffuse to find their target and, thus, are less efficient than cell-to-cell transmission that requires less time for HIV to find its target cell and directly pass between cells. Cell-to-cell spread also

protects the virus from immune responses, such as neutralizing antibodies. Lastly, cell-to-cell transmission polarizes viral egress on the infected cell and viral entry receptors on the target cell by forming a macromolecular structure known as the virological synapse (VS) (Jolly, Kashefi et al. 2004; Jolly and Sattentau 2004; Jolly 2011).

The VS was named for its resemblance to the immunological synapse (jolly 2002). In VS formation, the initial event involves gp120 (Env) on the infected cell interacting with CD4 on the target cell (Jolly, Kashefi et al. 2004; Jolly, Mitar et al. 2007; Hornung and Latz 2010). The VS is then further stabilized by binding between integrin proteins intracellular adhesion molecule-1 (ICAM) and lymphocyte function-associated adhesion molecule (LFA-1) (Fais, Capobianchi et al. 1995; Jolly, Mitar et al. 2007; Hornung and Latz 2010). Then Env binds to CD4 to rearrange actin in the target cell and recruit more CD4, CXCR4 and/or CCR5, and adhesion proteins to the sight of interaction (Jolly, Kashefi et al. 2004; Jolly, Mitar et al. 2007; Vasiliver-Shamis, Cho et al. 2009). In addition, the secretory pathway becomes polarized towards sites of cell-to-cell contact, increasing viral assembly at the VS (Jolly and Sattentau 2007). Therefore, the VS forms a high-efficiency channel to transfer large quantities of virus from an infected cell to a target cell.

Chapter Two:

Cell Death by Pyroptosis Drives CD4 T-cell Depletion in HIV-1 Infection

2.1. Introduction

The progressive loss of CD4 T cells in HIV-infected individuals lies at the root of AIDS. Despite more than three decades of study, the precise mechanism(s) underlying the demise of CD4 T cells during HIV infection remains poorly understood and has been highlighted as one of the key questions in HIV research (Thomas 2009). In almost all cases, loss of CD4 T cells has been linked to apoptosis, both in *in vivo* (Finkel, Tudor-Williams et al. 1995; Muro-Cacho, Pantaleo et al. 1995; Gougeon, Lecoeur et al. 1996; Rosok, Brinchmann et al. 1998; Huang, James et al. 2008) and *ex vivo* (Rosok, Brinchmann et al. 1998; Grivel, Malkevitch et al. 2000; Jekle, Keppler et al. 2003) studies. However, various features of apoptotic cell death including maturation of executioner caspase-3, DNA fragmentation, and plasma membrane permeabilization are commonly shared with other programmed cell death pathways (Lamkanfi and Dixit 2010). Importantly, most studies have focused on the death of productively infected cells circulating in peripheral blood (Cooper, Garcia et al. 2013). Very little is known about the death of "bystander" CD4 T cells in tissues that are refractory to productive HIV infection. These resting CD4 T lymphocytes in fact represent the major cellular targets encountered by HIV in lymphoid tissues (Glushakova, Baibakov et al. 1995; Eckstein, Penn et al. 2001; Doitsh, Cavrois et al. 2010).

To investigate how CD4 T cells die during HIV infection, we took advantage of an *ex vivo* human lymphoid aggregate culture (HLAC) system formed with fresh human tonsil or spleen tissues (Glushakova, Baibakov et al. 1995). HLACs can be infected with a small number of viral particles in the absence of artificial mitogens, allowing analysis of HIV cytopathicity in a natural and preserved lymphoid microenvironment (Eckstein, Penn et al. 2001). Infection of these cultures with HIV-1 produces extensive loss of CD4 T cells, but >95% of the dying cells are abortively infected with HIV reflecting their nonpermissive, quiescent state. The HIV life cycle

is attenuated during the chain elongation phase of reverse transcription, giving rise to incomplete cytosolic viral DNA transcripts. Cell death is ultimately caused by a cellular innate immune response elicited by these cytosolic DNA intermediates (Doitsh, Cavrois et al. 2010). This response is associated with production of type I interferon and activation of both caspase-3 and caspase-1. While caspase-3 activation leads to apoptosis without inflammation (Ren and Savill 1998), caspase-1 activation can trigger pyroptosis, a highly inflammatory form of programmed cell death where dying cells release their cytoplasmic contents, including inflammatory cytokines, into the extracellular space (Fink and Cookson 2005; Lamkanfi and Dixit 2010). The consequences of apoptosis-versus-pyroptosis may affect HIV pathogenesis by influencing the state of inflammation and immune activation, but their relative contribution to CD4 T-cell death in lymphoid tissues had remained unexplored.

2.2. RESULTS

2.2.1. Host permissivity determines the form of cell death

Previous reports have implicated caspase-3 activation and apoptosis in most instances of cell death caused by HIV-1 (Finkel, Tudor-Williams et al. 1995; Grivel, Malkevitch et al. 2000; Jekle, Keppler et al. 2003). To explore the role of caspase-1 in dying HIV-infected CD4 T cells, HLACs formed with freshly dissected human tonsillar tissues were infected with a GFP reporter virus (NLENG1), prepared from the X4-tropic NL4-3 strain of HIV-1. This reporter produces fully replication-competent viruses. An IRES upstream of the *nef* gene preserves Nef expression and supports LTR-driven GFP expression (Levy, Aldrovandi et al. 2004), allowing simultaneous quantification of HIV-1 infection and caspase activation in CD4 T cells. NL4-3 was selected because tonsillar tissue contains a high percentage of CD4 T cells that express CXCR4 (90–

100%). Consistent with our previous report (Doitsh, Cavrois et al. 2010), infection with HIV-1 produced extensive depletion of "bystander" non-productively infected CD4 T cells. No more than 4% of the CD4 T cells were productively infected with HIV-1, but most of the remaining CD4 T cells underwent abortive infection and ultimately died after four days in culture (Figure 2.1.a).

To determine the distribution of active caspase-1 and caspase-3 in the dying CD4 T cells, we used fluorescently labeled inhibitor of caspases (FLICA) probes with sequences targeted by specific activated caspases (Bedner, Smolewski et al. 2000). Interestingly, the majority of non-productively infected CD4 T cells exhibited activation of caspase-1. Conversely, essentially no caspase-1 activity was detected in the productively infected cells (Figure 2.1.b). Caspase-3 activity was markedly less abundant, and mainly confined to the productively infected subset of cells (Figure 2.1.c). Treatment, with efavirenz (a non-nucleoside reverse transcriptase inhibitor, NNRTI) or AMD3100 (an inhibitor of CXCR4-dependent HIV entry) prevented activation of both caspases. Infection with the primary, dual-tropic 89.6 HIV isolate (Collman, Balliet et al. 1992) produced similar results (Figure 2.2.). The two FLICA probes appeared to bind their respective caspases with reasonable specificity based on exclusive caspase-3 staining in cells treated with staurosporine, a protein kinase inhibitor known to induce apoptosis versus robust caspase-1 staining in cells treated with the cationic ionophore nigericin that promotes NLRP3 inflammasome assembly, caspase-1 activation, and pyroptosis (Mariathasan, Weiss et al. 2006).

2.2.2. Healthy lymphoid CD4 T cells express pro-IL-1b

IL-1b activity is controlled at multiple levels including pro-IL-1β expression, processing, and secretion. Proinflammatory stimuli induce expression of pro-IL-1b while processing and release

are regulated by caspase-1 activation in inflammasomes (Schroder and Tschopp 2010). The signals required for caspase-1 activation and release of IL-1b differ between immune cells. In circulating human blood monocytes, caspase-1 is constitutively active (Netea, Nold-Petry et al. 2009). Stimulation of these cells with LPS promotes pro-IL-1b expression leading to the rapid release of bioactive IL-1β. In contrast, macrophages and dendritic cells require a second signal to activate caspase-1(Laliberte, Eggler et al. 1999). Nigericin can function as this second signal activating caspase-1 in LPS-primed macrophages (Perregaux and Gabel 1994; Mariathasan, Weiss et al. 2006). Surprisingly, nigericin proved sufficient alone to activate caspase-1 in uninfected lymphoid CD4 T cells (Figure 2.1.b) and to promote the release of the 17-kDa bioactive form of IL-1b (Figure 2.3.a). Treatment with monensin, a different monovalent cationic ionophore, or A23187, a calcium ionophore, did not promote mature IL-1b release (Perregaux, Barberia et al. 1992; Perregaux and Gabel 1994). Maturation and secretion of the bioactive form of IL-1b was inhibited by Z-VAD-FMK (a pan-caspase inhibitor), Z-WEHD-FMK, or Z-YVAD-FMK (two independent caspase-1 inhibitors, which also block other inflammatory caspases i.e. caspase-4 and -5), but not by Z-FA-FMK (negative control for caspase inhibitors) suggesting that caspase-1 activation was required.

Pro-IL-1b expression in human tonsil and spleen HLACs was next examined. Western blotting analysis surprisingly revealed large amounts of intracellular pro-IL-1b in both untreated tonsil and spleen HLACs (Figure 2.3.b). Removal of dead cells by ficoll-hypaque density centrifugation resulted in an even higher intracellular pro-IL-1β signal, suggesting that these normal lymphoid tissues constitutively express high levels of pro-IL-1b. The presence of pro-IL-1b in spleen argued that expression in tonsil is not solely caused by infection (tonsillitis).

Fractionation of the lymphocytes present in these HLACs revealed high levels of intracellular pro-IL-1b in isolated CD4 T cells, but not in CD8 T or B-cell populations.

Most tonsillar CD4 T cells express CXCR4, but only ~5% of these cells also express CCR5 (Eckstein, Penn et al. 2001; Moore, Kitchen et al. 2004). Interestingly, when CCR5-positive and -negative lymphoid CD4 T-cell subsets were isolated and studied, the CCR5-expressing cells displayed much higher levels of intracellular pro-IL-1b (Figure 2.3.b). The CCR5-expressing CD4 T cells also released significantly more 17 kDa IL-1b into the supernatant after infection with HIV-1 (Figure 2.3.c). These results suggest that most of the mature form of IL-1b is released by the small population of CCR5-expressing CD4 T cells. The resident CCR5-expressing cells in lymphoid tissues are primarily memory CD4 T cells, which might be more permissive for productive HIV infection (Schweighardt, Roy et al. 2004). However, the activation status of these cells varied (Figure 2.3.d). Two thirds exhibited a memory phenotype as determined by surface expression of CD45RO but only a small fraction of these cells were permissive to productive infection with either X4-tropic or R5-tropic HIV-1 strains (Figure 2.4.). Notably, lymphoid CCR5-expressing CD4 T cells also express CXCR4 and thus can be targeted by either X4 or R5-tropic HIV-1 strains (Grivel and Margolis 1999; Eckstein, Penn et al. 2001; Zhou, Shen et al. 2008). Memory T cells continually recirculate within lymphoid tissues scanning for presentation of their cognate antigen (Mackay 1993; Sallusto, Lenig et al. 1999; Lanzavecchia and Sallusto 2000). It seems likely that many of these cells have returned to a sufficient state of quiescence that they are susceptible to abortive HIV infection and thus could contribute importantly to chronic inflammation through the release of bioactive IL-1b .

2.2.3. CD4 T-cell death by HIV-1 is mediated by pyroptosis

Caspase-1 is a proinflammatory caspase whose catalytic activity is tightly regulated by signal-dependent autoactivation within inflammasomes (Schroder and Tschopp 2010). Inflammasome-dependent caspase-1 activity results in a highly inflammatory form of cell death known as pyroptosis, primarily described in myeloid cells infected with intracellular bacterial pathogens (Fink and Cookson 2005; Bergsbaken, Fink et al. 2009; Lamkanfi and Dixit 2010). Pyroptosis is caspase-1-dependent by definition and occurs independently of other proapoptotic caspases (Bergsbaken, Fink et al. 2009; Lamkanfi and Dixit 2010). Based on our finding that caspase-1 is activated in lymphoid CD4 T cells following abortive HIV infection, we investigated whether pyroptosis is triggered within these cells.

Fresh HLACs were infected with HIV-1 and cultured for 12 hours to initiate viral spread and then treated with various caspase inhibitors or controls. Extensive and selective depletion of CD4 T cells occurred in untreated, HIV-infected cultures after 3 days. However, treatment with either pan-caspase or caspase-1 inhibitors prevented the depletion of CD4 T cells as efficiently as the viral inhibitors efavirenz and AMD3100 (Figure 2.5.a). Inhibitors of caspase-3 or caspase-6 and the control compound did not prevent CD4 T-cell depletion. Necrostatin-1, a RIP1 inhibitor, did not inhibit CD4 T-cell depletion (Figure 2.6.a,b), suggesting that cell death does not reflect necroptosis. Analysis of spleen cells yielded similar results (Figure 2.6.c). Inhibiting type-I interferon signaling with neutralizing antibodies directed against IFNa/b receptor did not prevent CD4 T-cell death (Figure 2.7.), indicating that this antiviral response is not critical for the innate immune-mediated onset of programmed cell death. Distinct from apoptosis, pyroptosis features cellular swelling, plasma membrane rupture, and release of intracellular content into the extracellular milieu (Fink and Cookson 2005), including cytosolic enzymes like

lactate dehydrogenase (LDH) (Decker and Lohmann-Matthes 1988). LDH release was readily detected after HIV infection (Figure 2.5.b), but completely blocked by an inhibitor of caspase-1, efavirenz and AMD3100, not by a caspase-3 inhibitor. Thus, the form of cell death associated with abortive HIV infection appears to involve caspase-1 activation and the release of cytoplasmic contents. Caspase-1 inhibitors also prevented death of CCR5-expressing CD4 T cells in HLACs infected with a CCR5-dependent strain of HIV-1 (Figure 2.5.c). Inhibition of cell death by the caspase-1 inhibitor was as effective as the CCR5 receptor antagonist TAK779, suggesting that most CCR5-expressing CD4 T cells die by caspase-1-mediated pyroptosis. These findings are consistent with the large amounts of bioactive IL-1b released by these cells after HIV-1 infection.

Because caspase inhibitors are not exquisitely specific. we designed shRNA vectors to silence the expression of caspase-1, the ASC (PYCARD) adaptor, which recruits pro-caspase-1 to inflammasome complexes (Schroder and Tschopp 2010), caspase-3, and NLRP3 (Figure 2.8.). For these experiments, a third generation shRNA-encoding lentiviral vector (shRNA LV) pSico (Ventura, Meissner et al. 2004), bearing an EF1a:mCherry reporter expression cassette was used. To relieve the resistance of lymphoid CD4 T cells to shRNA LV infection, target cells were initially challenged with Vpx-harboring lentiviral particles (Vpx-VLPs), which induce proteasomal degradation of SAMHD1 in non-permissive human resting CD4 T cells (Baldauf, Pan et al. 2012). Infections with Vpx-VLPs did not lead to activation of resting CD4 T cells, as measured by surface expression of the CD69 and CD25 activation markers (not shown). The shRNA LV particles and Vpx-VLPs were pseudotyped with a CXCR4-tropic Env of HIV-1, which supports efficient fusion to quiescent CD4 T lymphocytes (Agosto, Yu et al. 2009). Under these conditions, infection with shRNA LVs markedly suppressed expression of a variety of

targeted genes while the scrambled shRNA LV control did not (Figure 2.5.d). We next investigated whether any of these shRNA LVs inhibited pyroptosis induced by nigericin. Nigericin induced massive pyroptosis in mCherry positive CD4 T cells infected with scramble and caspase-3 shRNA LV particles, but this response was blocked by the caspase-1, ASC, or NLRP3 shRNA LV particles (Figure 2.5.e). Next, the effect of these shRNAs on CD4 T-cell death elicited by HIV-1 was examined. HIV-1 infection caused extensive death of mCherry-positive CD4 T cells expressing shRNAs against scramble, caspase-3 and NLRP3, but not caspase-1 and ASC. Thus, cell death occurring during abortive HIV infection appears to be mediated through caspase-1 dependent pyroptosis involving an inflammasome that contains ASC but lacks NLRP3.

2.2.4. HIV-1 stimulates caspase-1 to secrete IL-1b

To independently confirm that abortive HIV-1 infection leads to the activation of caspase-1, we investigated appearance of the active p10 subunit of caspase-1. As controls for pyroptosis and apoptosis, uninfected cells were treated with nigericin or staurosporine, respectively. An active 10kDa subunit of caspase-1 (p10) was detected in the lysates of HIV-infected cultures as well as in nigericin-treated cells, and in blood monocytes where caspase-1 is constitutively active (Netea, Nold-Petry et al. 2009). Treatments with viral or caspase-1, but not caspase-3, inhibitors prevented caspase-1 cleavage (Figure 2.5.f). These findings confirm the induction of caspase-1 in quiescent CD4 T following abortive infection with HIV-1. Caspase-3 activation in these infected cultures was markedly less abundant (Figure 2.9.). To test whether caspase-1 activation leads to proteolytic maturation of pro-IL-1b, we used various caspase inhibitors and analyzed the culture media for the mature 17-kDa form of IL-1b. Interestingly, release of mature IL-1b

was completely inhibited by a pan-caspase inhibitor, and by two different caspase-1 inhibitors (Figure 2.5.g). Inhibitors of apoptotic caspases, caspase-3, -6, or -8, or necrostatin did not interrupt this release. Similar findings were observed using a quantitative IL-1b enzyme-linked immunosorbent assay (ELISA) (Figure 2.10.a). Thus, caspase-1 activation is specifically required for the release of bioactive IL-1b in lymphoid CD4 T cells infected with HIV-1. In accord with shRNA analyses, treatment with four separate NLRP3 inhibitors did not prevent release of bioactive IL-1b by HIV-1 (Figure 2.5.g), nor CD4 T-cell death by HIV-1 (Figure 2.10.b,c).

2.2.5. In vivo evidence for HIV-mediated pyroptosis

To extend our *ex vivo* HLAC findings, we next examined fresh lymph node tissue obtained from a consenting untreated subject infected with R5-tropic HIV and displaying a high viral load and a low CD4 T cell count. *In-situ* immunostaining revealed a distinct zone of HIV $p24^{gag}$ expression between the mantle zone and germinal centers, where activated CD4 T and B cells proliferate (Ki67) and interact in the follicles (Figure 2.11.). Conversely, staining for caspase-1 revealed abundant activity in the surrounding paracortical zone (CD3) comprised primarily of resting CD4 T cells. Staining of uninfected tonsil or spleen (not shown) tissues revealed no such positive signals (Figure 2.12.). Because this antibody reacts with both the active p20 component of caspase-1 and pro-caspase-1, we cannot completely exclude the possibility that abortive HIV-1 infection produced localized increase in pro-caspase-1 expression. However, large amounts of IL-1b were also detected in the paracortical zone, particularly in the extracellular space between the T cells, as well as the cell death marker annexin V. In sharp contrast, active caspase-3 staining was limited to the areas in the germinal center where HIV-1 $p24^{gag}$ expression was

detected. These findings strongly agree with the HLAC results (Figure 2.1.b) suggesting that caspase-3 activity occurs in a set of productively infected cells, anatomically separated from the majority of resting CD4 T cells undergoing abortive infection, caspase-1 activation, IL-1b processing, and pyroptosis.

2.2.6. Clinically safe drug blocks pyroptosis by HIV-1

Identifying pyroptosis as the predominant mechanism mediating CD4 T-cell depletion during HIV infection provides novel targets, such as caspase-1, for potential therapeutic intervention. The role of caspase-1 in the chronic inflammatory response has attracted therapeutic interest (Boxer, Shen et al. 2010). VX-765 is caspase-1 inhibitor that has been tested in chronic epilepsy and psoriasis (Figure 2.13.a) (Randle, Harding et al. 2001; Stack, Beaumont et al. 2005; Boxer, Quinn et al. 2010; Maroso, Balosso et al. 2011), and found in a phase IIa trial to be safe and well tolerated (Vezzani, Balosso et al. 2010). In our studies, VX-765 inhibited IL-1b secretion by nigericin-induced lymphoid CD4 T cells (Figure 2.10.b), indicating it efficiently blocks caspase-1 activity in these cells. VX-765 also blocked caspase-1 cleavage (Figure 2.5.f), IL-1b secretion (Figure 2.5.g), and CD4 T-cell death in HIV-infected tonsillar and splenic HLACs (Figure 2.5.c, 2.14.a, Figure 2.14.b, Figure 2.13.b). Cell death was not markedly inhibited by VRT-043198 (the active form of the VX765 prodrug), likely because of reduced cellular permeability (Boxer, Quinn et al. 2010). HIV-1 infection was not restored to productive infection when caspase-1 was blocked (Figure 2.15.). These findings demonstrate that a small-molecule inhibitor of caspase-1 shown to be safe in humans suppresses CD4 T-cell death and inflammation elicited in lymphoid tissues by HIV-1.

2.3. DISCUSSION

HIV's lethal attack on its principal cellular target, the CD4 T cell, has been generally attributed to apoptosis (Fevrier, Dorgham et al. 2011). We now demonstrate that the permissivity status of the host cell dictates the pathway through which lymphoid CD4 T cells die following HIV infection. Specifically, when HIV infects permissive, activated CD4 T cells, cell death occurs silently through caspase-3-dependent apoptosis. Conversely, when either R5- or X4-tropic HIV abortively infects nonpermissive, quiescent CD4 T cells from lymphoid tissue, death occurs through caspase-1 dependent pyroptosis, an intensely inflammatory form of programmed cell death. In most human lymphoid tissues including tonsil, lymph node, and spleen, the activated and permissive subset of cells represents 5% or less of the total CD4 T cells, while non-permissive quiescent cells represent 95% or more of the targets encountered by HIV (Eckstein, Penn et al. 2001; Moore, Kitchen et al. 2004). Thus, in sharp contrast to previous studies (Finkel, Tudor-Williams et al. 1995; Muro-Cacho, Pantaleo et al. 1995; Grivel, Malkevitch et al. 2000; Jekle, Keppler et al. 2003; Cooper, Garcia et al. 2013), caspase-1-mediated pyroptosis, not caspase-3-mediated apoptosis, appears predominantly responsible for driving CD4 T-cell death following HIV infection of these lymphoid tissues. These findings are further supported by analysis of fresh lymph nodes from subjects infected with R5-tropic HIV, where caspase-1 and IL-1b are detected in the paracortical zone rich in resting CD4 T cells while caspase-3 activity is detected in the anatomically distinct germinal centers where productively infected cells are found.

Our studies also highlight how lymphoid CD4 T cells are selectively primed to mount inflammatory responses as evidenced by constitutive expression of cytoplasmic pro-IL-1b. This is particularly prominent within the CCR5-expressing subset of lymphoid CD4 T cells. The

pyroptotic death of these cells would lead to high level release of IL-1b potentially further fueling chronic inflammation.

Pyroptosis likely promotes the rapid clearance of various bacterial infections by removing intracellular replication niches and enhancing the host's defensive responses through the release of proinflammatory cytokines and endogenous danger signals. However, in pathogenic chronic inflammation, such as in HIV infection, pyroptosis is not a protective response and does not lead to clearance of the primary infection. In fact, pyroptosis appears to create a vicious pathogenic cycle, where dying CD4 T cells release inflammatory signals that attract more cells into the infected lymphoid tissue to die and to produce more inflammation (Biancotto, Iglehart et al. 2008) (Figure 2.13.c). These events establish a chronic state of inflammation that likely fuels disease progression and tissue injury (Zeng, Smith et al. 2011). Chronic inflammation might also promote maintenance of the latent HIV reservoir through the dysregulated action of the IL-7 or IL-15 cytokines stimulating homeostatic proliferation of memory CD4 T cells. In this regard, it will be interesting to assess to what extent pyroptosis persists in lymphoid tissues of HIV-infected subjects on effective anti-retroviral therapy.

The depletion of CD4 T cells and the development of chronic inflammation are signature processes in HIV pathogenesis that propel disease progression (Deeks 2011). Our studies now reveal how pyroptosis provides an unexpected link between these two disease-promoting processes. In non-pathogenic infections where simian immunodeficiency virus (SIV) infects its natural nonhuman primate hosts, caspase-3-apoptosis in productively infected cells may signal for most of the cell death rather than caspase-1, thus avoiding local inflammation. The pathogenic cycle of cell death and inflammation created by pyroptosis critically depends on the activation of caspase-1. As such, it may be possible to break this pathogenic cycle with safe and

effective caspase-1 inhibitors. These agents could form a new and exciting “anti-AIDS” therapy for HIV-infected subjects where the treatment targets the host instead of the virus.

Figures for Chapter Two:

Figure 2.1: Host permissivity determines the CD4 T-cell death pathway employed following HIV infection. a. Kinetics of spreading viral infection versus depletion of CD4 T cells after infection of HLACs with a replication-competent HIV reporter virus encoding GFP. The relative proportion of CD8 T cells was not altered (not shown). Consistent with our previous report, HIV-infected HLACs contain a small number of productively infected cells, while almost all of the dying cells are abortively infected (Doitsh, Cavrois et al. 2010). **b**. Abortively infected CD4 T cells exclusively activate caspase-1. Nigericin induces abundant caspase-1 activation in uninfected cells. **c**. Productively-infected CD4 T cells activate caspase-3, but not caspase-1. (b) and (c) represent cells from the same infected tonsil culture. Efavirenz and AMD3100 were added to the indicated cultures prior to HIV infection. These data are representative of four independent experiments performed with tonsil cells isolated from four different donors.

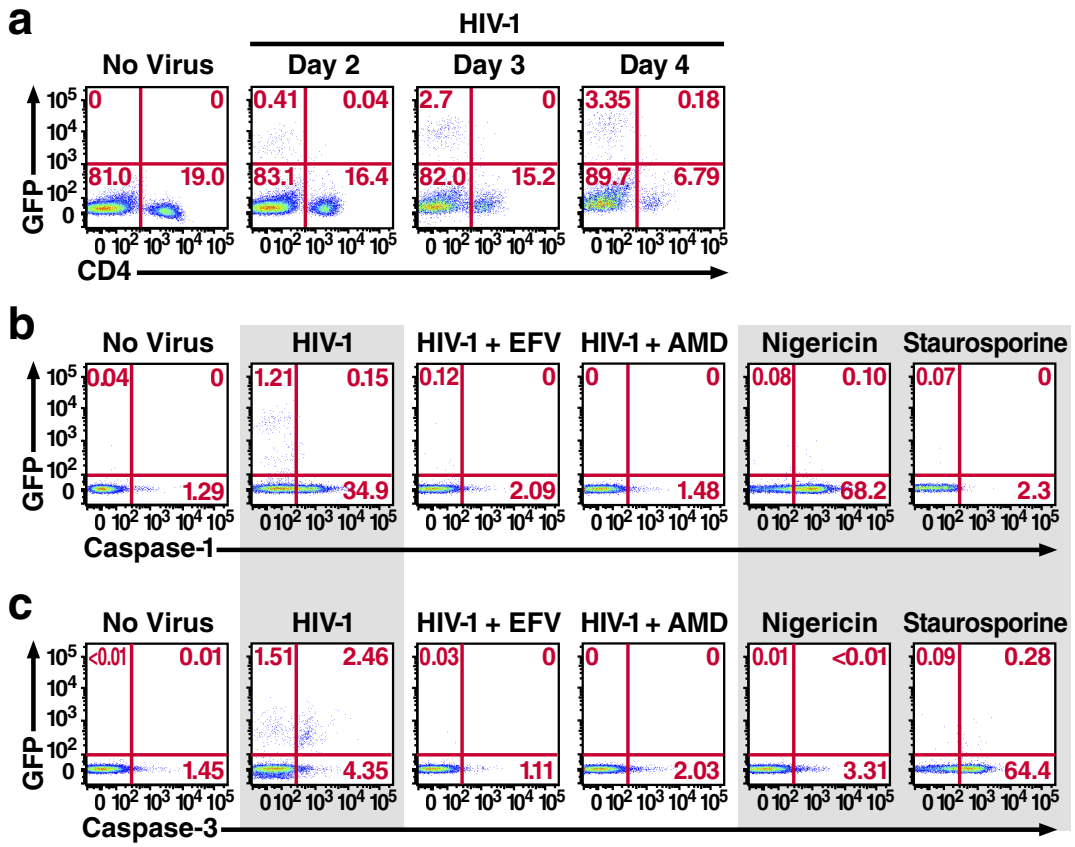


Figure 2.2. Extensive caspase-1 activation in dying lymphoid CD4 T cells infected with either NL4-3 or a primary HIV-1 isolate. a. Dying CD4 T cells activate caspase-1. HLACs were infected with NL4-3 or with a primary HIV-1 isolate 89.6 obtained from a mixed PBMC culture from an AIDS patient. The 89.6 viral isolate replicates to high titers in primary human cells such as macrophages and lymphocytes. It is highly cytopathic and utilizes both CCR5 and CXCR4 as co-receptors (dual-tropic) (Collman, Balliet et al. 1992; Doranz, Rucker et al. 1996). Infected cells were treated either with no drugs or with AMD3100 (250 nM) as indicated. Caspase-1 activity was determined by flow cytometry using FLICA 12 hours after treatment with nigericin (10 mM) or 3 days after infection with HIV. Notably, equivalent levels of caspase-1 activation were observed in CD4 T cells infected with NL4-3 or 89.6 HIV-1 isolate. AMD3100 prevented caspase-1 activity in both viruses, indicating the abundant presence of CXCR4-expressing target CD4 T cells in these cultures. **b.** Low levels of caspase-3 activity in dying CD4 T cells. The same cultures as in (a) were tested for caspase-3 activity using FLICA. Interestingly, compared to caspase-1, infections with NL4-3 and 89.6 HIV-1 isolate induced low levels of caspase-3 activation in dying CD4 T cells. No caspase-3 activation was observed in cells treated with nigericin, which signals the NLRP3 inflammasome to activate caspase-1 (Mariathasan, Weiss et al. 2006), indicating a specific recognition of caspase-1 and caspase-3 activity by the FLICA probes. These data are the representative results of four independent experiments performed in tonsil cells isolated from four different donors.

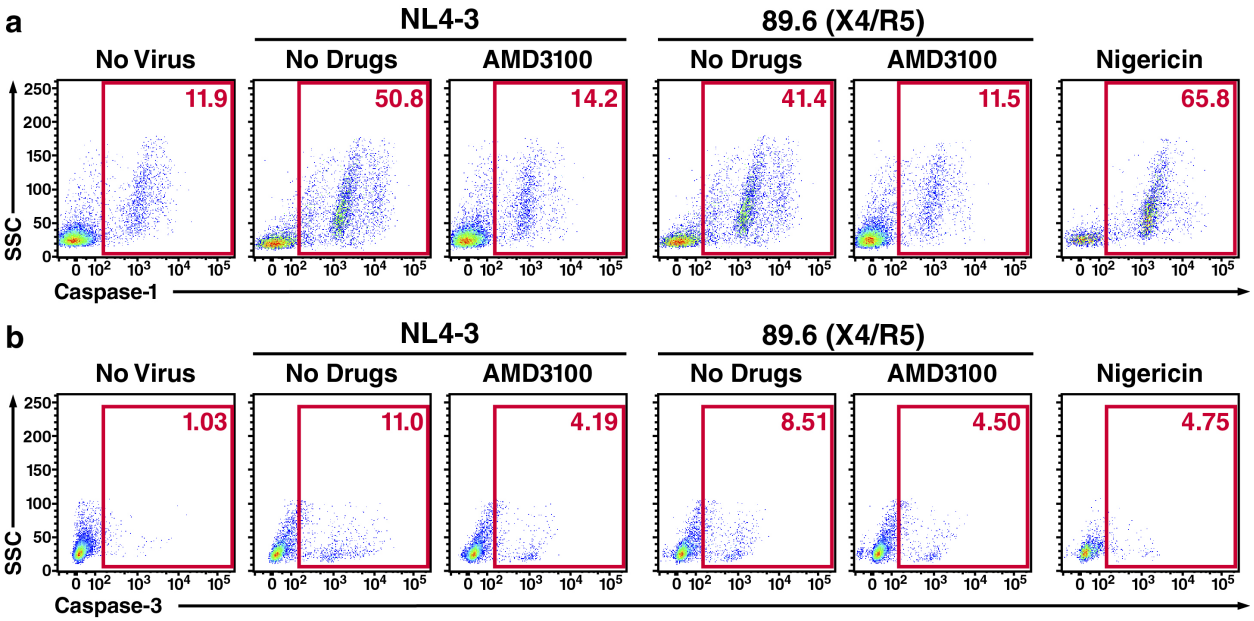

a
NL4-3
89.6 (X4/R5)
No Virus
No Drugs
AMD3100
No Drugs
AMD3100
Nigericin
11.9
50.8
14.2
41.4
11.5
65.8
SSC
Caspase-1
b
NL4-3
89.6 (X4/R5)
No Virus
No Drugs
AMD3100
No Drugs
AMD3100
Nigericin
1.03
11.0
4.19
8.51
4.50
4.75
SSC
Caspase-3

Figure 2.3. Lymphoid CD4 T cells are primed to mount an inflammatory response and constitutively express high-levels of pro-IL-1β. a. A secondary inflammatory stimulus by nigericin induces lymphoid CD4 T cells to process and release bioactive IL-1b. Supernatants from cell cultures were filtered to remove all remaining cells and subjected to SDS-PAGE immunoblotting analyses for bioactive 17-kDa IL-1β. **b**. High levels of constitutive pro-IL-1b are selectively expressed in lymphoid CD4 T cells. Levels of intracellular pro-IL-1β were assessed in HLACs from fresh tonsils or spleen tissue from different donors. Asterisks indicate samples in which dead cells were removed. CD4 T cells were positively isolated from HLAC. Cells were lysed and analyzed for pro-IL-1β expression. **c**. Nearly all bioactive IL-1b produced by HIV-infected lymphoid CD4 T cells is released from CCR5-expressing cells. Indicated CD4 T-cell populations were isolated from HLAC and infected with HIV-1. Supernatants of cultures were filtered and analyzed for bioactive 17-kDa IL-1β. **d**. HLACs were characterized for expression of memory and activation markers by flow cytometry. The majority of CCR5-expressing CD4 T lymphocytes exhibit a memory phenotype. All CCR5-expressing CD4 T cells co-express the CXCR4 receptor.

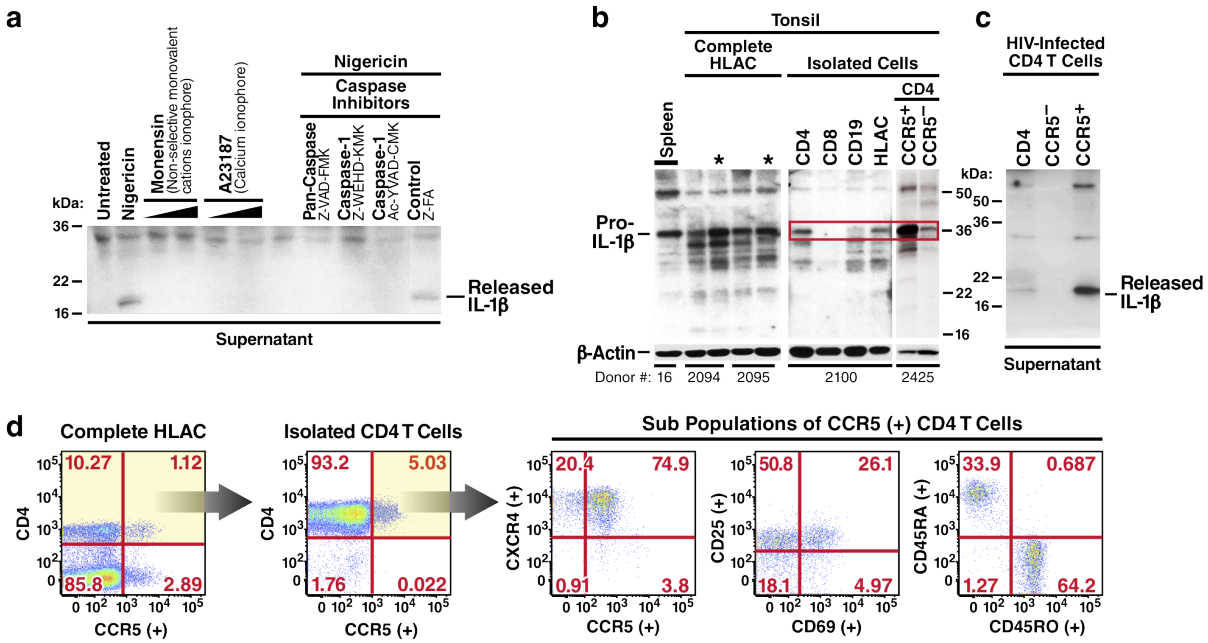

a
Untreated
Nigericin
Monensin
(Non-selective monovalent cations ionophore)
A23187
(Calcium ionophore)
Nigericin
Caspase Inhibitors
Pan-Caspase
Z-VAD-FMK
Caspase-1
Z-WEHD-KMK
Caspase-1
Ac-YVAD-CMK
Control
Z-FA
kDa:
36
22
16
Released IL-1β
Supernatant
b
Tonsil
Complete HLAC
Isolated Cells
CD4
Spleen
CD4
CD8
CD19
HLAC
CCR5+
CCR5−
Pro-IL-1β
β-Actin
Donor #: 16
2094
2095
2100
2425
c
HIV-Infected CD4 T Cells
CD4
CCR5−
CCR5+
kDa:
50
36
22
16
Released IL-1β
Supernatant
d
Complete HLAC
Isolated CD4 T Cells
Sub Populations of CCR5 (+) CD4 T Cells
CD4
CCR5 (+)
10.27
1.12
85.8
2.89
93.2
5.03
1.76
0.022
CXCR4 (+)
20.4
74.9
0.91
3.8
CD25 (+)
CD69 (+)
50.8
26.1
18.1
4.97
CD45RA (+)
CD45RO (+)
33.9
0.687
1.27
64.2

Figure 2.4. CD4 T lymphocytes in lymphoid tissues contain large population of central memory cells. (**a**) To identify the subpopulations of CD4 T cells in human tonsil histocultures we examined the expression pattern of CCR5, CD45RA, CD45RO, CD62L and CD27. Central memory CD4 T cells (T_{CM}) are characterized by expression of $CD45RO^+/CD62L^+$ or $CD45RO^+/CD27^+$ (Sallusto, Lenig et al. 1999; Lanzavecchia and Sallusto 2000; De Rosa, Herzenberg et al. 2001; Brenchley, Schacker et al. 2004). T_{CM} lack effector function and constantly travel through the lymph nodes in large quantities for antigen sampling, while effector memory cell (T_{EM}) mainly migrate to peripheral tissues (Mackay 1993; Sallusto, Lenig et al. 1999; Lanzavecchia and Sallusto 2000). Analysis of these surface markers revealed at least three distinct maturation phenotypes. The majority of CD4 T lymphocytes exhibit a memory phenotype as determined by surface expression of CD45RO, among them more than two thirds were found to be central memory cells ($CD45RO^+$ / $CD62L^+$ and $CD45RO^+$ / $CD27^+$). Similarly, a large population of CCR5-expressing CD4 T cells was found to have central memory phenotype ($CCR5^+$ / $CD62L^+$ and $CCR5^+$ / $CD27^+$). These findings are in accord with previous studies in primary human lymphoid cultures (Bleul, Wu et al. 1997; Eckstein, Penn et al. 2001; Gondois-Rey, Grivel et al. 2002). (**b**) Memory lymphoid CD4 T cells represent preferential target for productive infection by both the R5- and X4-tropic strains of HIV-1. To determine whether cell maturation influence susceptibility for productive infection, we measured the levels of productive infection using GFP reporter viruses harboring either an X4-tropic or R5-tropic of Env of HIV-1. Except for their select V3 loop envelope determinants, both reporters were derived from the same bicistronic Nef-IRES-GFP clone, which produces fully replication-competent viruses (Levy, Aldrovandi et al. 2004). Interestingly, productive infection of both X4-tropic or R5-tropic viral strains was detected in CXCR4-expressing cells, indicating that CXCR4

co-receptor is equally present on CCR5-expressing cells, as was previously shown (Bleul, Wu et al. 1997; Penn, Grivel et al. 1999; Eckstein, Penn et al. 2001; Gondois-Rey, Grivel et al. 2002). Interestingly, memory CD4 T cells ($CD45RO^{+}$) were selectively productively infected in cultures infected with either X4-tropic or R5-tropic reporter virus. Similar findings were found in infected cultures activated with CD3/CD38 beads to achieve higher rates of infection. Among the memory CD4 T cells, T_{EM} cells became productively infected in higher quantities than T_{CM} (not shown). These data are the representative results of eleven independent analyses performed in tonsil cells isolated from four different donors.

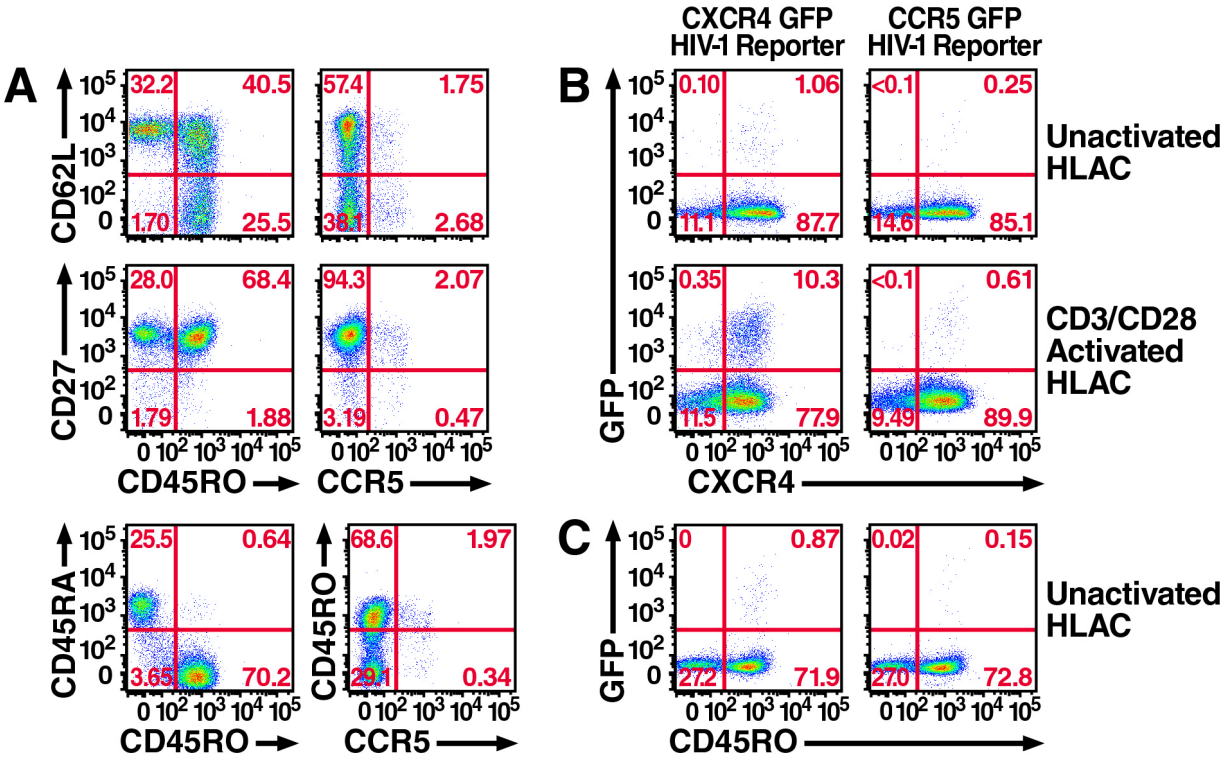


Figure 2.5. Death of HIV-infected lymphoid CD4 T cells and release of bioactive IL-1b are controlled by caspase-1. a, b. Caspase-1 inhibitors are sufficient to prevent CD4 T-cell death in HIV-infected HLACs. Viable CD4 T cells were counted by flow cytometry, and supernatants were analyzed for levels of cytoplasmic LDH enzyme release(Decker and Lohmann-Matthes 1988). **c,** Infection with CCR5-dependent HIV-1 induces pyroptosis of lymphoid CD4 T cells. Death of CCR5-expressing CD4 T cells is prevented by caspase-1 inhibitors and TAK779, but not buy the CXCR4 antagonist, AMD3100. Due to the small number of target CCR5-expressing cells, this experiment was performed by overlaying tonsil cells on a monolayer of 293T cells that had been transfected with an R5-tropic proviral HIV-1 clone, as previously described (Doitsh, Cavrois et al. 2010). The co-culture conditions for the R5 virus experiment induced no activation of the overlaid cells. **d**. Efficient repression of target genes by shRNA-coding lentiviral vectors. **e**. shRNA LV designed to silence either caspase-1 or ASC, key components of the pyroptotic pathway, protect lymphoid CD4 T cells from death by nigericin or HIV-1 infection. To specifically assess non-productively infected cells, cultures were treated with AZT before infections with HIV-1. **f**. Caspase-1 cleavage in HIV-infected CD4 T cells is blocked by specific caspase-1 inhibitors. **g**. Inhibitors of caspase-1, but not NLRP3, prevent release of bioactive IL-1b from HIV-infected lymphoid CD4 T cells. Error bars represent SD/√n of at least three independent experiments utilizing tonsil cells from at least three different donors. Protein analyses represent results from three independent experiments utilizing tonsillar CD4 T cells from three different donors.

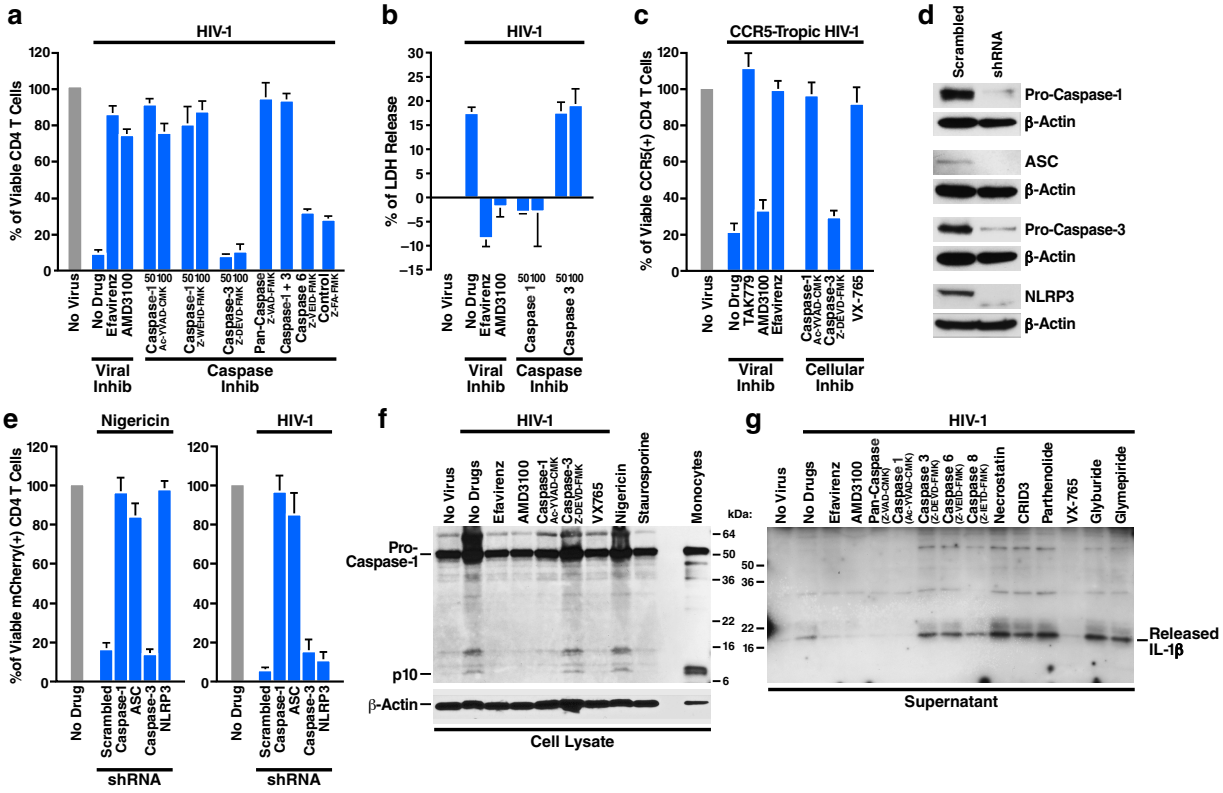

a
HIV-1
% of Viable CD4 T Cells
No Virus
No Drug
Efavirenz
AMD3100
Caspase-1 Ac-YVAD-CMK
Caspase-1 Z-WEHD-FMK
Caspase-3 Z-DEVD-FMK
Pan-Caspase Z-VAD-FMK
Caspase-1 + 3
Caspase 6 Z-VEID-FMK
Control Z-FA-FMK
Viral Inhib
Caspase Inhib
b
HIV-1
% of LDH Release
No Virus
No Drug
Efavirenz
AMD3100
Caspase 1
Caspase 3
Viral Inhib
Caspase Inhib
c
CCR5-Tropic HIV-1
% of Viable CCR5(+) CD4 T Cells
No Virus
No Drug
TAK779
AMD3100
Efavirenz
Caspase-1 Ac-YVAD-CMK
Caspase-3 Z-DEVD-FMK
VX-765
Viral Inhib
Cellular Inhib
d
Scrambled
shRNA
Pro-Caspase-1
β-Actin
ASC
β-Actin
Pro-Caspase-3
β-Actin
NLRP3
β-Actin
e
Nigericin
HIV-1
%of Viable mCherry(+) CD4 T Cells
No Drug
Scrambled
Caspase-1
ASC
Caspase-3
NLRP3
shRNA
f
HIV-1
No Virus
No Drugs
Efavirenz
AMD3100
Caspase-1 Ac-YVAD-CMK
Caspase-3 Z-DEVD-FMK
VX765
Nigericin
Staurosporine
Monocytes
kDa:
Pro-Caspase-1
p10
β-Actin
Cell Lysate
g
HIV-1
No Virus
No Drugs
Efavirenz
AMD3100
Pan-Caspase Z-VAD-CMK
Caspase-1 (Ac-YVAD-CMK)
Caspase 3 (Z-DEVD-FMK)
Caspase 6 (Z-VEID-FMK)
Caspase 8 (Z-IETD-FMK)
Necrostatin
CRID3
Parthenolide
VX-765
Glyburide
Glymepiride
Released IL-1β
Supernatant

Figure 2.6. Necrostatin-1 does not prevent lymphoid CD4 T-cell death and cell lysis in HIV-infected cultures. Necrostatin was tested at 5 mM, a concentration that yields maximal inhibition without inducing toxicity (not shown). Pyroptosis shares cell death features with necrosis, which similarly leads to the release of intracellular contents into the extracellular space (Lamkanfi and Dixit 2010). To test whether cell death involves necrotic signaling we treated HIV-infected CD4 T cells with necrostatin, a specific inhibitor of RIP1, whose kinase activity is essential for programmed necrosis to occur (Cho, Challa et al. 2009). Interestingly, concentrations of necrostatin that block necrotic signaling (not shown) did not inhibit CD4 T-cell depletion in HIV-infected cultures (**a**), and did not prevent the release of intracellular contents into the culture medium, as indicated by LDH activity in the supernatants (**b**). Thus, although pyroptosis shares features with necrosis, these data demonstrate that the signaling pathways linking caspase-1 activation to CD4 T-cell death are specific. Together, these findings indicate that the CD4 T-cell depletion and release of cytoplasmic contents in HIV-infected lymphoid cultures reflects pyroptosis rather than apoptosis or necrosis. Error bars represent standard deviations of at least three independent experiments utilizing tonsil cells from at least three different donors. **(c)** Caspase-1 inhibitors prevent CD4 T-cell death in HIV-infected splenic tissues. Splenic HLACs were cultured with no virus or were infected with HIV-1. The HIV-infected cultures were treated as indicated, either with no drugs, efavirenz (100 nM), AMD3100 (250 nM), the caspase-1 inhibitor Ac-YVAD-CMK (50 mM), or the caspases-3 inhibitor Z-DEVD-FMK (50 mM). After 4 days, viable CD4 T cells were counted by flow cytometry. Viable CD4 T are presented as the percentage remaining live CD4 using CD8 T cells to normalize each HIV-infected or uninfected culture. Error bars represent standard deviations from four independent experiments utilizing tonsil cells isolated from three different donors.

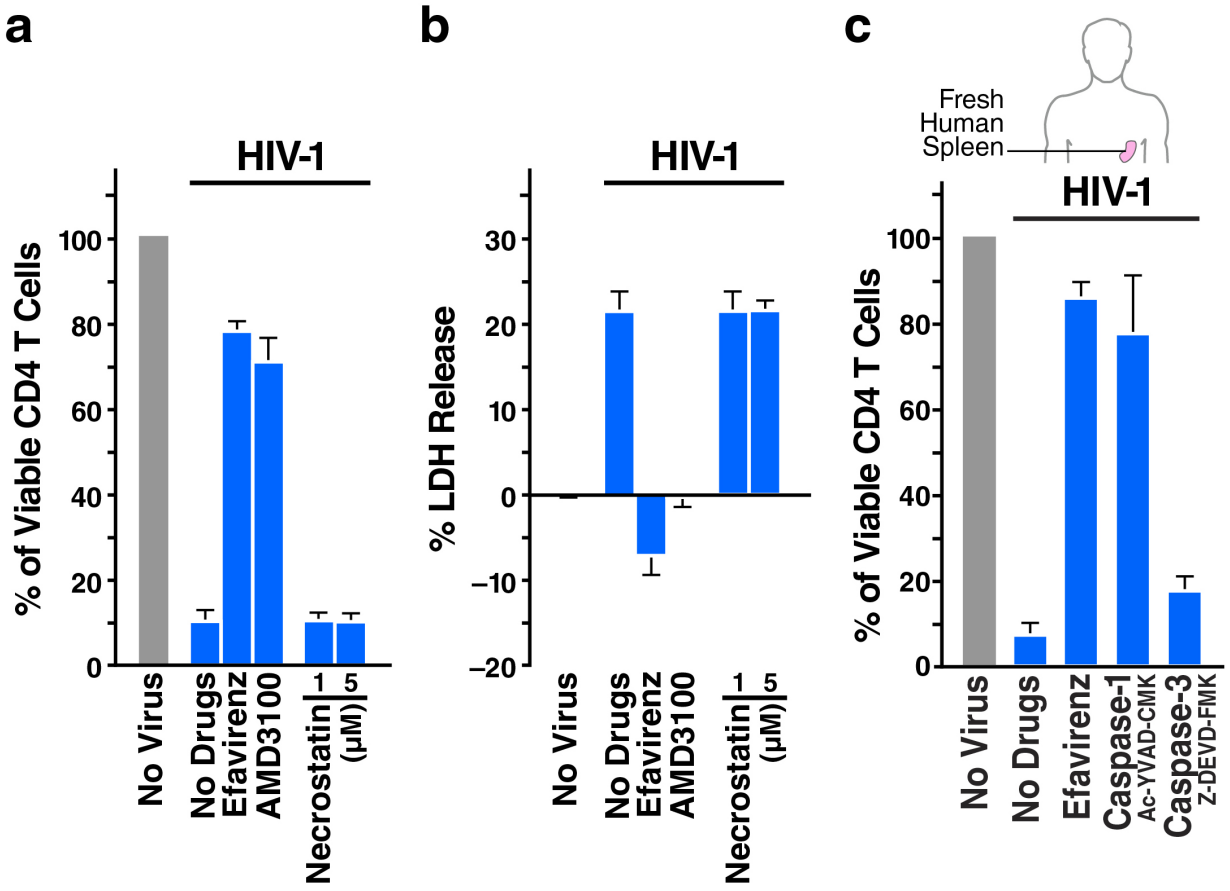

a
HIV-1
% of Viable CD4 T Cells
100
80
60
40
20
0
No Virus
No Drugs
Efavirenz
AMD3100
Necrostatin (μM)
1
5
b
HIV-1
% LDH Release
30
20
10
0
−10
−20
No Virus
No Drugs
Efavirenz
AMD3100
Necrostatin (μM)
1
5
c
Fresh
Human
Spleen
HIV-1
% of Viable CD4 T Cells
100
80
60
40
20
0
No Virus
No Drugs
Efavirenz
Caspase-1
Ac-YVAD-CMK
Caspase-3
Z-DEVD-FMK

Figure 2.7. Induction of type-I interferon is not required to trigger a death response in HIV-infected lymphoid CD4 T cells. HIV-1 infections induce induction of type-I interferon *in vitro* and *in vivo* (Pitha 2011). To test the involvement of this antiviral response in modulating CD4 T-cell death, isolated CD4 T cells were infected with HIV-1 in the presence of neutralizing antibodies against the human interferon alpha receptor (IFNAR-2), which blocks biological action of type I interferons. To determine the state of interferon signaling, cells were analyzed in parallel for the presence of phosphorylated STAT-1, which plays a central role in mediating type-I IFN-dependent biological responses, including induction of antiviral state (Samuel 2001). Phosphorylated STAT readily appeared in HIV-infected CD4 T cells, but not in HIV-infected cells treated with Efavirenz (100 nM), AMD3100 (250 nM), or anti-IFNAR-2 neutralizing antibodies (1–5 mg/ml). Notably, blocking interferon signaling with anti-IFNAR-2 neutralizing antibodies did not prevent the death of CD4 T cells by HIV-1 although tyrosine phosphorylation of STAT-1 was inhibited indicating effectiveness of the antibody blockade. The data suggest that this antiviral IFN induction is not critical to the onset of the innate immune death response leading to caspase-1 activation and pyroptosis. Error bars represent standard deviations from three independent experiments using tonsil cells from three different donors.

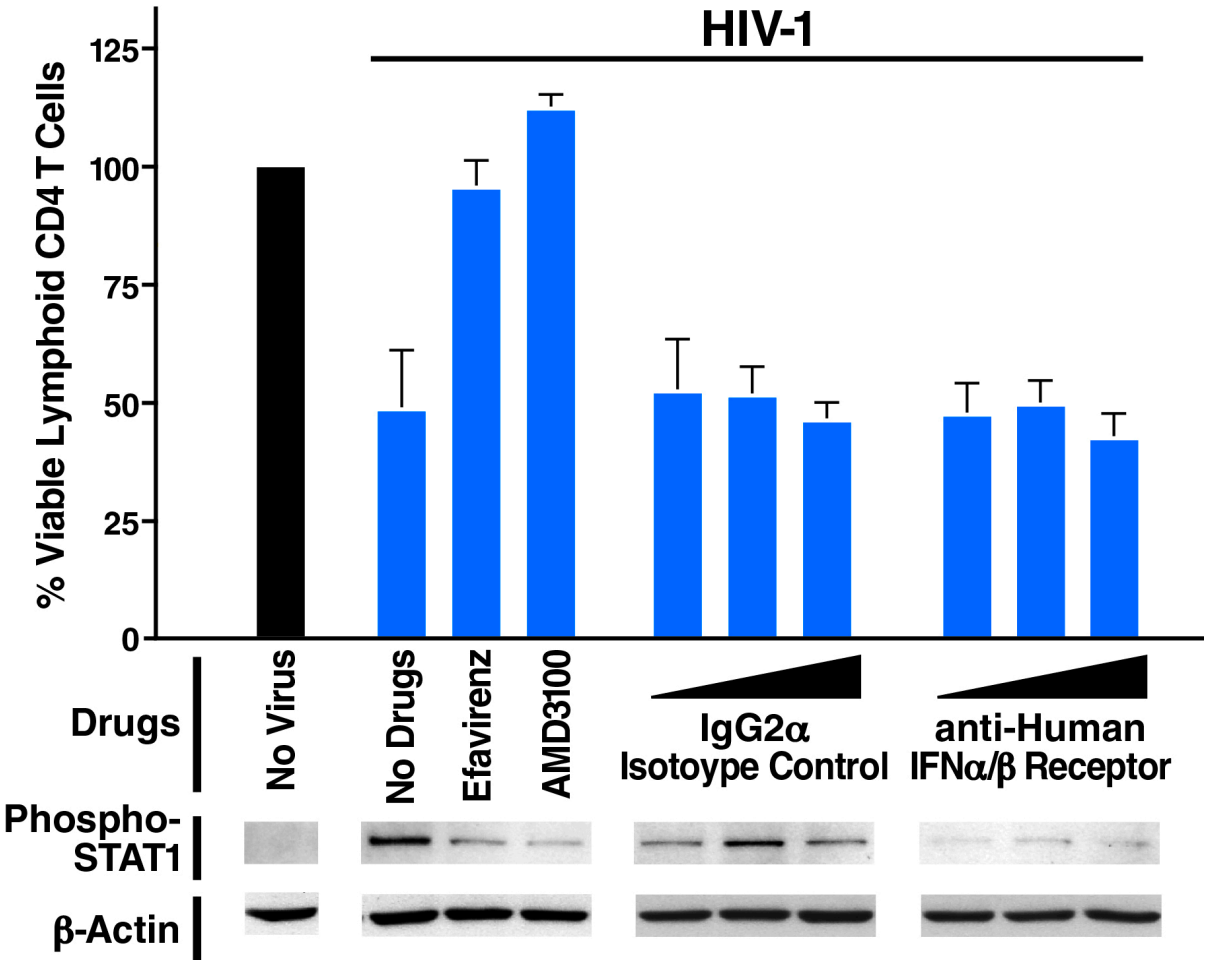

HIV-1
% Viable Lymphoid CD4 T Cells
125
100
75
50
25
0
Drugs
No Virus
No Drugs
Efavirenz
AMD3100
IgG2α
Isotoype Control
anti-Human
IFNα/β Receptor
Phospho-
STAT1
β-Actin

Figure 2.8. Lymphoid CD4 T cells express detectable levels of ASC and NLRP3 relative to blood-derive monocytes. The bipartite adaptor protein ASC (PYCARD) plays a central role in the interaction between (NOD)-like receptor and caspase-1 in inflammasome complexes (Lamkanfi and Dixit 2009). Interestingly, lymphoid CD4 T cells are primed to mount such inflammatory responses, and constitutively express high levels of cytoplasmic pro-IL-1b, but also ASC and NLRP3, compared to blood-derived monocytes. Interestingly, CD4 T lymphocytes express constitutive levels NLRP3. In contrast to lymphocytes, monocytes require stimulation with TLR ligands such as LPS to induce NLRP3 expression (Netea, Nold-Petry et al. 2009). Thus, the release of intracellular 5′-ATP by pyroptotic CD4 T cells may provide a second inflammatory stimulus to induce activation of caspase-1 by the NLRP3 inflammasome in nearby CD4 T cells that are already primed as reflected by their high levels of ASC, NLRP3, and pro-IL-1b expression. Thus, pyroptosis activated initially by HIV may result in an avalanche of new rounds of pyroptosis in primed CD4 T cells by the repeated release of intracellular ATP in a virus-independent manner. Such an "auto-inflammation" scenario could result in persistent rounds of pyroptosis, chronic inflammation, and loss of CD4 T cells even when viral loads are reduced by antiretroviral therapy (ART).

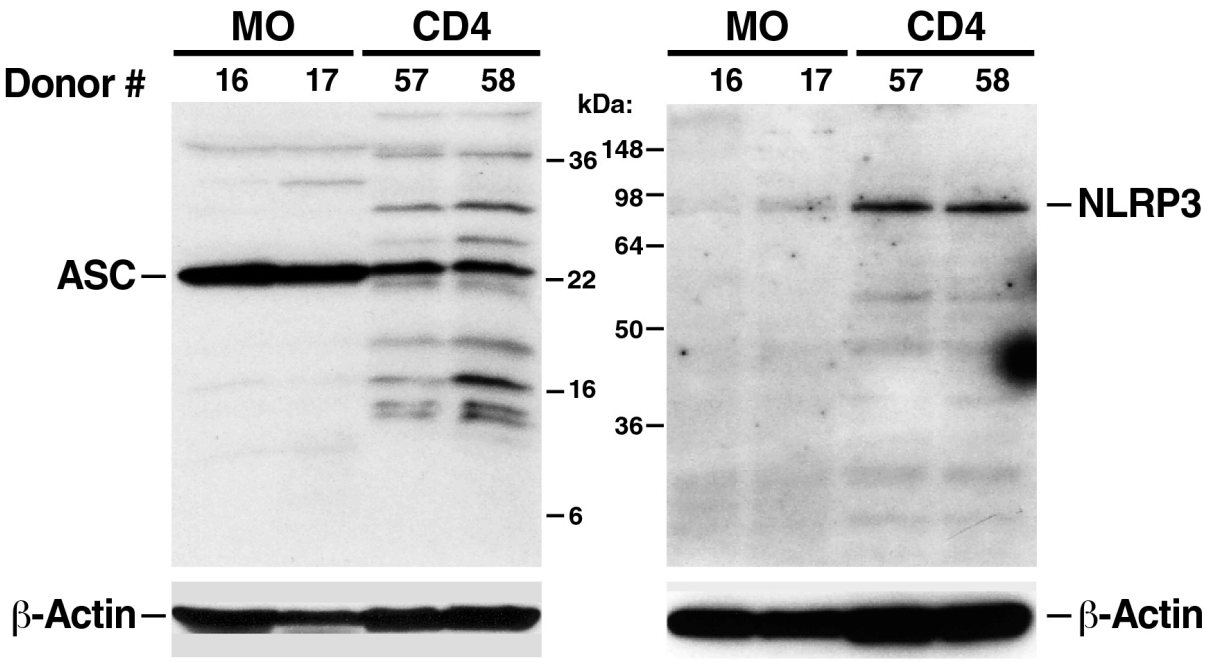

MO
CD4
MO
CD4
Donor #
16
17
57
58
16
17
57
58
kDa:
148
98
64
50
36
36
22
16
6
ASC
NLRP3
β-Actin
β-Actin

Figure 2.9. Low levels of caspase-3 activation in HIV-infected lymphoid CD4 T-cell cultures. Although the endogenous levels of pro-caspase-3 and pro-caspase-1 expression are similar in lymphoid CD4 T cells, caspase-3 activation in these cells was markedly less abundant after infection with HIV-1, compared to caspase-1. These data are in accord with our findings using fluorescently labeled inhibitor of caspases (FLICA) probes in cultures infected with a GFP reporter HIV-1. In these cultures, the majority of CD4 T cells were abortively infected and showed activation of intracellular caspase-1. No caspase-1 activity was observed in productively infected cells (Figure 2.1.c). In sharp contrast, capspase-3 activity in these cultures was markedly less abundant, and specifically occurred in productively infected, but not in non-productively infected cells (Figure 2.1.c). These data are the representative results of three independent experiments performed in tonsillar CD4 T cells isolated from four different donors.

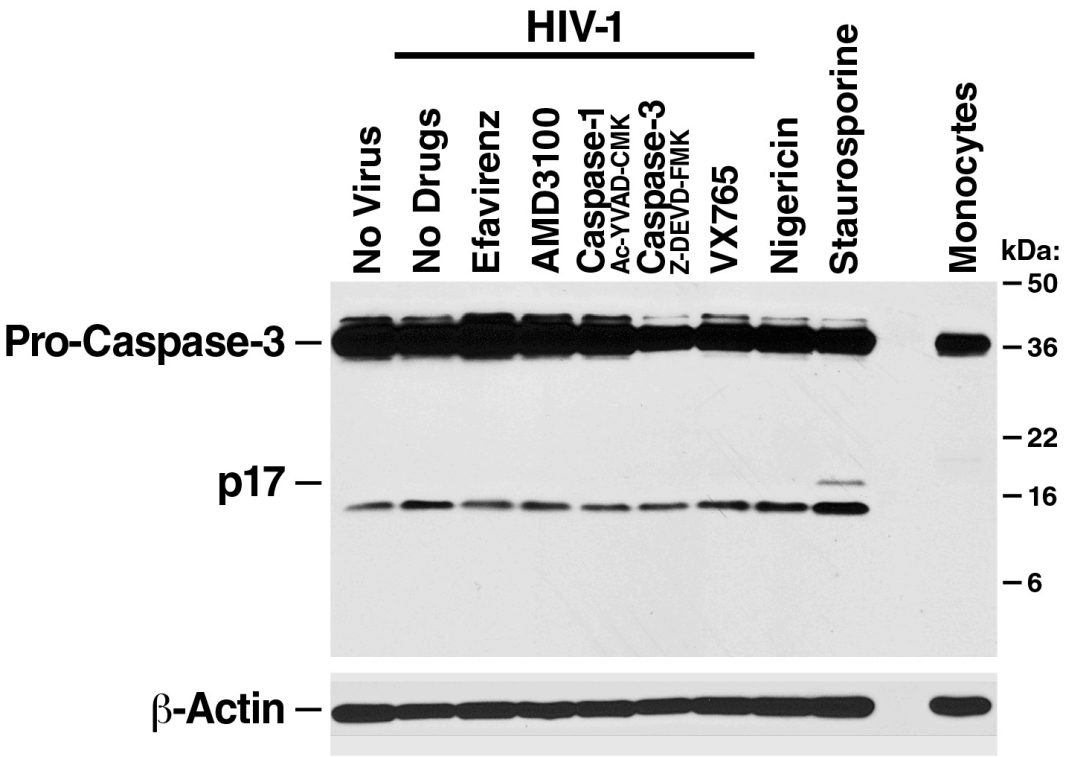


Figure 2.10. Inhibitors of caspase 1, but not of NLRP3, prevent CD4 T-cell death by HIV-1. **(a)** Quantitative evaluation of bioactive IL-1b secreted in HIV-infected CD4 T-cell cultures using ELISA. Isolated tonsillar CD4 T cells were left uninfected or infected with HIV in the presence of the indicated drugs. Four days after infection, supernatants were filtered through 0.22 mm filter plates and subjected to IL-1β ELISA analysis. 200 ml of supernatant from 2 million isolated CD4 T cells was used for each condition. The assay was performed as described by the manufactures description (R&D systems). Interestingly, bioactive IL-1b was detected in supernatants of HIV-infected cultures, in comparable levels to uninfected cells treated with nigericin. Treatments of HIV-infected cultures with viral or caspase-1 inhibitors, but not caspase-3 inhibitor, prevented accumulation of IL-1b in the supernatants comparable to the levels detected in uninfected cultures. These finding demonstrate that caspase-1 activation is specifically required for the release of bioactive IL-1b in lymphoid CD4 T cells infected with HIV-1. Error bars represent standard deviations of three independent experiments utilizing tonsil cells from at least three different donors. Inhibitors of caspase-1 and the NLRP3 inflammasome prevent release of mature IL-1b induced by nigericin, but not CD4 T-cell death by HIV-1. Because nigericin engages the NLRP3 inflammasome to activate caspase-1 in lymphoid CD4 T cells, we sought to determine if NLRP3 also similarly controls caspase-1 activity in response to HIV-1 infection. Cell cultures were treated with four separate NLRP3 inhibitors including CRID3 (Coll and O'Neill 2011), parthenolide (Juliana, Fernandes-Alnemri et al. 2010), and the sulfonylureas glyburide (Lamkanfi, Mueller et al. 2009) and glimepiride. Treatments with CRID3, parthenolide, or sulfonylureas (not shown) completely inhibited NLRP3-dependent release of mature IL-1b by nigericin (**b**), but had no effect on IL-1b release triggered by HIV infection of lymphoid CD4 T-cell cultures (Figure 2.5.f). Additionally, treatments with CRID3,

parthenolide, or sulfonylureas did not prevent HIV-1-mediated CD4 T-cell death (**c**), suggesting that the NLRP3 inflammasome does not control the caspase-1-mediated cytopathic responses in HIV-infected lymphoid CD4 T cells. Cell death results represent ratios of viable CD4 versus CD8 T cells in each HIV-infected or uninfected culture. Error bars represent standard deviations of at four independent experiments utilizing tonsil cells from three different donors.

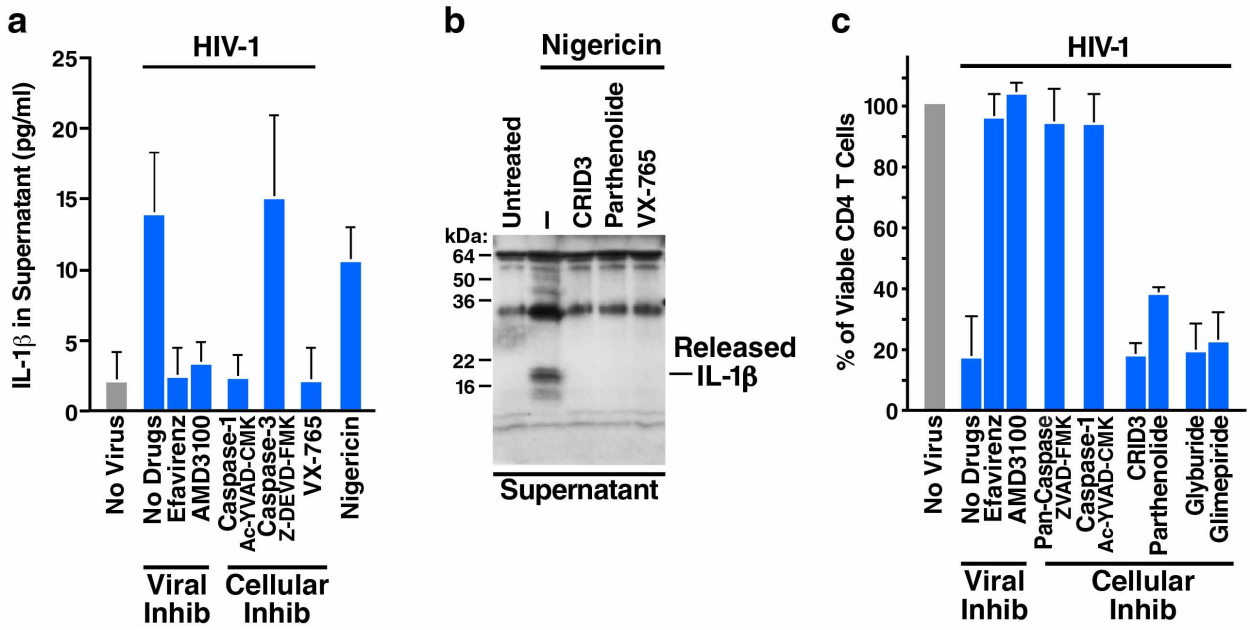


Figure 2.11. Distinct regions of caspase-1 and caspase-3 activity in lymph node of a patient chronically infected with R5-tropic HIV. Inguinal lymph node was collected from a 50-year-old immunosuppressed HIV-1 infected subject during the chronic phase of disease. The patient was first identified with HIV in 1985, has not been on anti-retroviral therapy and displayed CD4 count of 156 cells / ml and viral load of 85,756 copies/ml at the time of lymph node resection. (See also Figure 2.12.). GC, germinal center; MZ, mantle zone; PC, paracortical zone; E, epithelium.

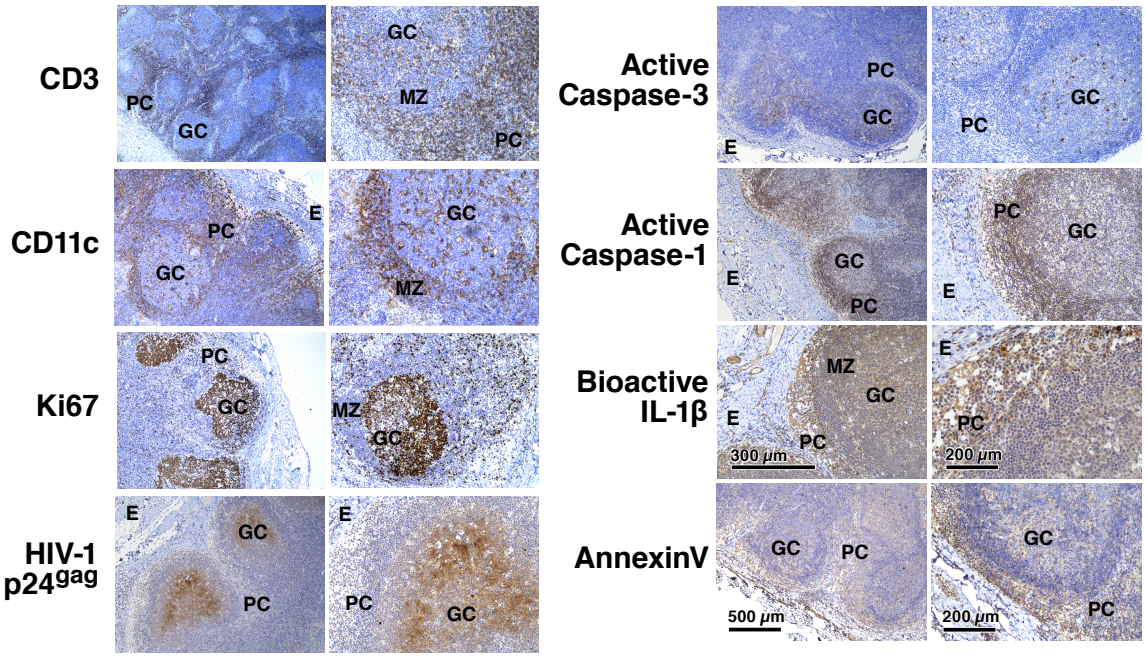


Figure 2.12. Distinct regions of caspase-1 and caspase-3 activity in lymph node of a chronically infected HIV patient. Inguinal lymph node was collected from a 41-year-old African American male, infected with an R5-tropic strain of HIV-1. The patient has been on intermittent anti-retroviral therapy between 2004-2009, and stopped anti-retroviral therapy on 2009. This individual exhibited a viral load of 30,173/ ml, and CD4 T-cell count of 259/ ml. The fresh specimen was immediately subjected to immunostaining in parallel with fresh uninfected human tonsil. Note the immunostain against CD3 highlights the paracortical region, which is almost entirely composed of resting T cells. Note also the sparse presence of CD3-positive T-cells in the mantle zones and germinal centers, where lymphocytes become activated (Ki67) and differentiate into memory and plasma cells. These CD4 T cells are responsible for antigen-dependent activation of B-cells in the follicle. Staining for CD11c reveals scattered dendritic cells (MacDonald, Munster et al. 2002; Merad, Sathe et al. 2013) in the germinal center and largely in the mantle zone. Interestingly, HIV $p24^{gag}$ expression is located between the mantle zone and germinal centers, where activated CD4 T cells reside. Remarkably, caspase-3 activity also occurs in this anatomical region, which is separated from the majority of non-productively T cells in the paracortical zone and exhibit caspase-1 activation, IL-1β processing and pyroptosis. The anti caspase-1 antibody was raised against a peptide mapping at the C-terminus of caspase-1 p20 of human origin and detects both the cleaved p20 subunit and the precursor of caspase-1. Therefore, in the absence of an equivalent uninfected lymph node control it is hard to absolutely determine whether HIV-1 infection affects pro-caspase expression. However, staining of uninfected tonsil or spleen (not shown) tissue revealed no positive HIV $p24^{gag}$, active or pro-caspase-1, bioactive IL-1b, and annexin V signals. These data closely correlate with the findings in HIV-infected HLACs where the 95% of the CD4 T cells are non-productively infected CD4 T

cells and show activation of intracellular caspase-1, while capspase-3 activity is markedly less abundant and specifically occurs in productively infected CD4 T cells. GC, germinal center; MZ, mantle zone; PC, paracortical zone; E, epithelium.

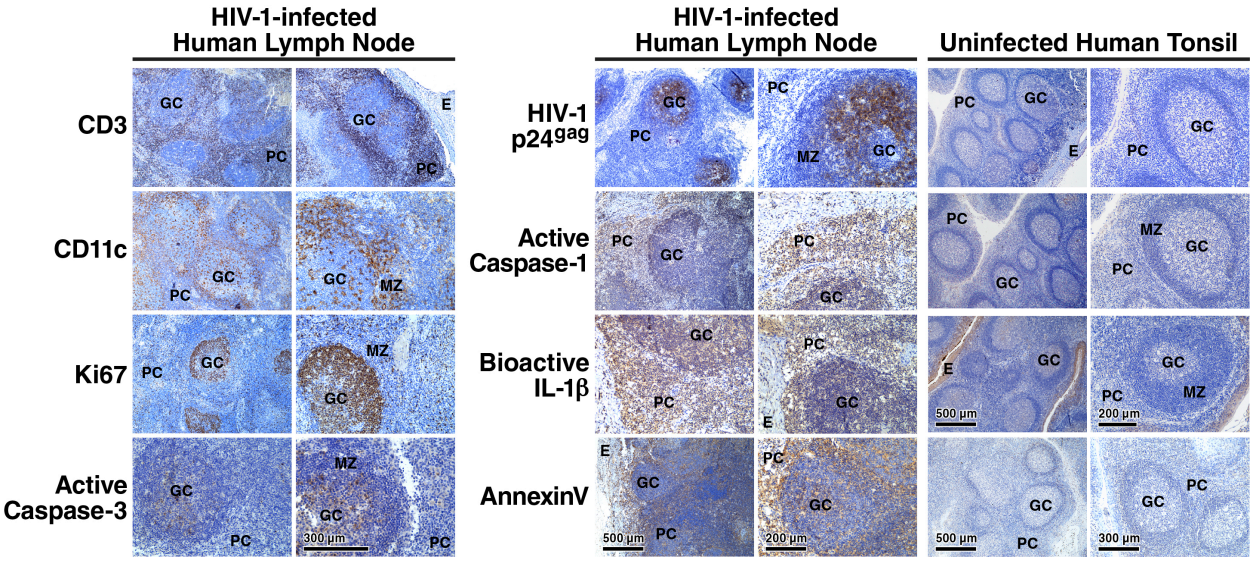


Figure 2.13. Targeting caspase 1 via an orally bioavailable small molecule inhibitor prevents lymphoid CD4 T-cell death by HIV-1. (a) VX-765 is a cell permeable pro-drug (1) that requires intracellular esterase cleavage in the cell to yield the aldehyde functionality (green) of the drug VRT- 043298 (2b), which acts as a potent caspase 1 inhibitor. Adapted from ref. 38 with permission. (**b**) VX-765 prevents CD4 T-cell death in a dose-dependent manner in HIV-infected lymphoid tissues. HLACs were either not infected or infected with HIV-1 in the absence of drugs or in the presence of efavirenz (100 nM), AMD3100 (250 nM) or VX-765 (0.05, 0.5, or 5 mM) as indicated. Flow cytometry plots depict gating on live cells based on the forward-scatter versus side-scatter profile of the complete culture. These results are representative of four independent experiments performed utilizing tonsil cells from four different donors.

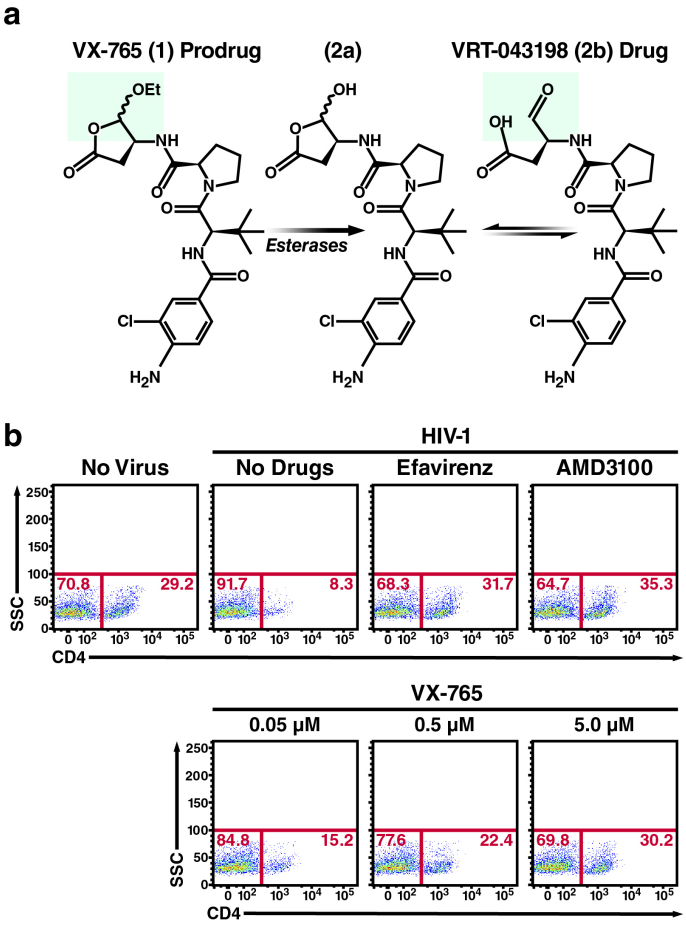


Figure 2.14. **Targeting caspase-1 via an orally bioavailable and safe drug prevents lymphoid CD4 T-cell death by HIV-1. (a, b).** VX-765 efficiently blocks CD4 T-cell death in HIV-infected tonsillar and splenic lymphoid tissues. No toxicity was observed at any of these drug concentrations. Error bars represent SD/√n of three independent experiments utilizing tonsil or spleen cells from three different donors. **c**. Pyroptosis in HIV-infected lymphoid tissues may ensue a chronic cycle of CD4 T-cell death and inflammation that ultimately contributes to disease progression and tissue damage. Inhibitors of caspase-1 such as VX-765 may inhibit pyroptosis in a manner that both preserves CD4 T cells and reduces inflammation.

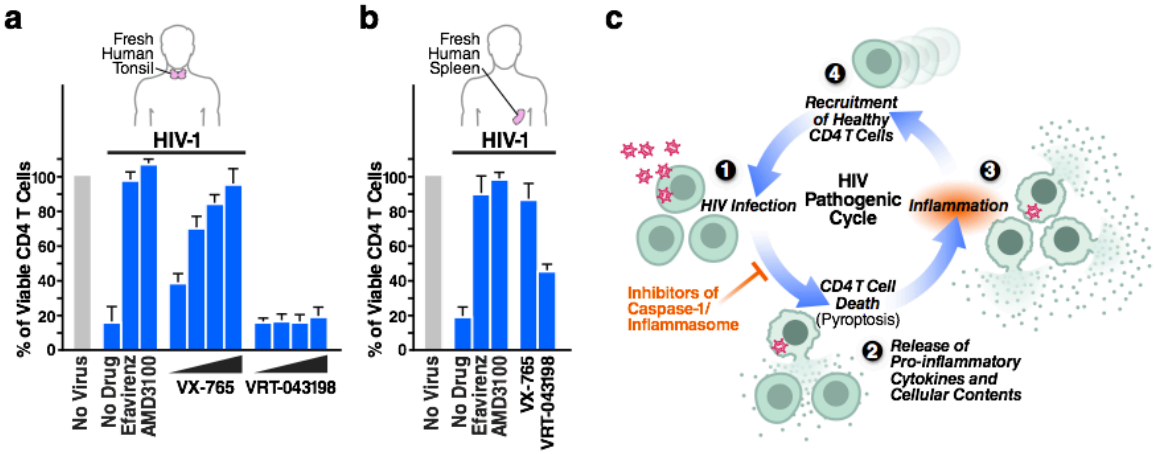
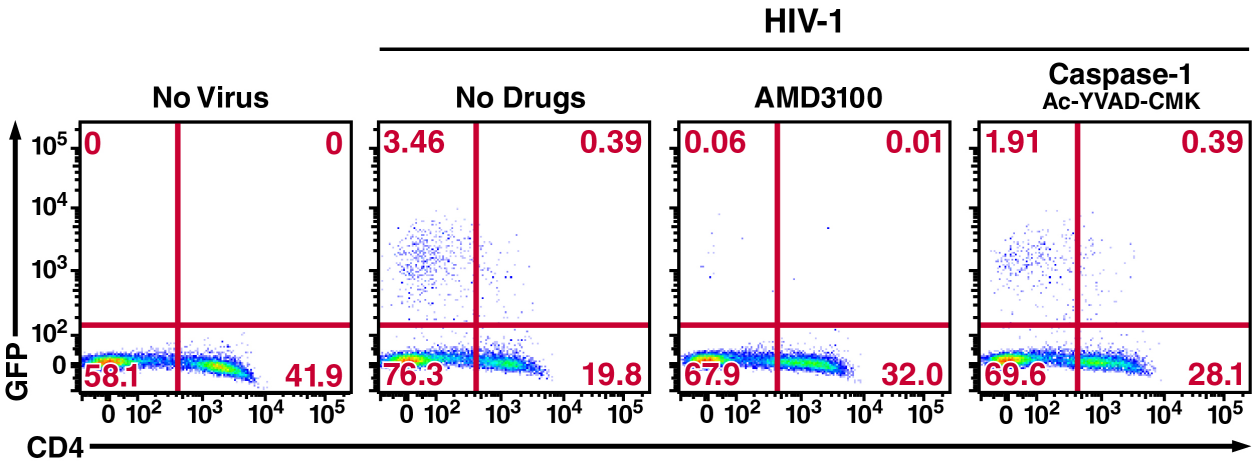


Figure 2.15. Treatment with a caspase-1 inhibitor does not increase productive HIV-1 infection. To determine whether inhibition of caspase-1-mediated pyroptosis would result in higher levels of productive HIV-1 infection, tonsillar HLACs were treated with AMD3100 or with caspase-1 inhibitor Ac-YVAD-CMK before infection with a GFP reporter virus (NLENG1). After 5 days, flow cytometry analysis of the infected cultures revealed no increase in GFP-positive cells in the infected cultures treated with the caspase-1 inhibitor Ac-YVAD-CMK. This result likely reflects the continued function of the host restriction factor SAMHD1(Baldauf, Pan et al. 2012; Descours, Cribier et al. 2012). These findings argue against the possibility that pyroptosis functions as a defense against productive infection. Instead, pyroptosis appears to represent an overall harmful response that centrally contributes to HIV pathogenesis. These results also argue that interdiction of the pyroptosis pathway with caspase-1 inhibitors would produce beneficial rather than harmful therapeutic effects.



Chapter Three:

Cell-to-cell transmission of HIV-1 is required to trigger pyroptotic death of CD4 T cells in lymphoid tissue

3.1. Introduction

The primary cause of AIDS in subjects is the progressive loss of CD4 T cells due to HIV infection (Thomas 2009). The depletion of these cells has often been studied using cell-free virions to infect activated blood-derived CD4 T cells (Cooper, Garcia et al. 2013), because of their accessibility and ability to support productive viral infection. However, the most relevant experimental approach to study HIV pathogenesis involves more than productively infected, activated blood cells. Indeed, most CD4 T cell death occurs in lymphoid tissues and involves resting CD4 T cells abortively infected with HIV (Doitsh, Cavrois et al. 2010). We have used an *ex vivo* human lymphoid aggregate culture (HLAC) system formed with fresh human tonsil or spleen tissues to model these conditions encountered in tissue (Glushakova, Baibakov et al. 1995). HLACs can be infected with a small number of viral particles in the absence of artificial mitogens, allowing analysis of HIV-1 cytopathicity in a natural and preserved lymphoid microenvironment (Eckstein, Penn et al. 2001). Infection of HLACs with HIV-1 produces extensive loss of CD4 T cells — less than 5% of the cells die as a result of productive viral infection while >95% of them die as a consequence of abortive infection (Doitsh, Cavrois et al. 2010). Due to viral nonpermissivity of these quiescent cells, the viral lifecycle attenuates during chain elongation in reverse transcription, giving rise to incomplete transcripts of cytosolic viral DNA. These intermediates are sensed by interferon gamma inducible protein 16 (IFI16) (Monroe, Yang et al. 2014), which activates caspase-1 in inflammasome and pyroptosis to cause a highly inflammatory form of programmed cell death (Doitsh, Galloway et al. 2014).

Retroviruses can disseminate between susceptible cells either by cell-free infection or by direct cell-to-cell spread (Sattentau 2010). The advantage of cell-to-cell spread on viral infectivity has been known for two decades (Sato, Orenstein et al. 1992; Phillips 1994; Jolly and

Sattentau 2004; Sourisseau, Sol-Foulon et al. 2007; Lehmann, Nikolic et al. 2011). For HIV-1, the infectivity of virus-producing cells, as measured in co-culture systems, is approximately 10^2 to 10^3 times higher than the infectivity of cell-free particles from the same infected cells (Jolly 2011). However, in the context of pathogenesis, it remained unclear whether transfer of HIV-1 through cell-to-cell contact triggers the same innate immune responses as cell-free particles in resting CD4 T cells, the predominant target cells depleted by HIV in lymphoid tissues.

3.2. Results

3.2.1. The mode of HIV-1 transfer affects the death response in target lymphoid CD4 T cells

Most studies examining innate immune recognition of HIV-1 have utilized cell-free particles and characterized responses occurring in dendritic cells or macrophages (Hayashi, Nishitsuji et al. 2010; Manel, Hogstad et al. 2010; Yan, Regalado-Magdos et al. 2010; Gao, Wu et al. 2013; Jakobsen, Bak et al. 2013; Lahaye, Satoh et al. 2013; Sun, Wu et al. 2013). More recently, attention has focused on resting CD4 T cells in lymphoid tissue, which are mostly non-permissive for productive HIV infection. We previously have shown that the massive death of lymphoid CD4 T cells that are abortively infected with HIV-1 requires close interaction between uninfected target and HIV-producing cells (Doitsh, Cavrois et al. 2010). These findings were consistent with *in vitro* (Holm and Gabuzda 2005; Garg, Joshi et al. 2007) and *in vivo (Finkel, Tudor-Williams et al. 1995)* studies showing that dying non-productively infected cells in human lymph nodes often cluster near productively infected cells (Finkel, Tudor-Williams et al. 1995). In contrast, we found that cell-free virions accumulating in the supernatants of HIV-infected HLACs, even at high concentrations, were much less efficient at inducing killing of resting target cells by abortive infection. These differences were entirely unexpected. One explanation

was that transfer of cell-free particles may not generate sufficient incomplete reverse DNA transcripts to induce a cytopathic response in target CD4 T cells. Cell-to-cell spread increases infection kinetics by one or more orders of magnitude by directing virus assembly and obviating the rate-limiting step of extracellular diffusion needed for cell-free virus to find a susceptible target cell (Sato, Orenstein et al. 1992; Sourisseau, Sol-Foulon et al. 2007; Martin and Sattentau 2009; Jolly 2011).

To test this hypothesis, we recapitulated the efficient cell-to-cell spread of virus using spinoculation (Geng, Doitsh et al. 2014), which accelerates the binding of cell-free virions to target cells, facilitates synchronized delivery of a large number of particles into the cells (O'Doherty, Swiggard et al. 2000; Saphire, Bobardt et al. 2002), and enhances accumulation of cytoplasmic reverse DNA transcripts (O'Doherty, Swiggard et al. 2000; Pace, Graf et al. 2012). As expected, spinoculation of HLACs with free HIV-1 particles (Figure 3.1a) caused extensive and selective depletion of target CD4 T cells. The relative proportion of CD8 T cells was unaltered. CD3+/CD8– T cells were similarly depleted, indicating that cell loss was not an artifact of downregulated surface expression of CD4 following direct infection (not shown). Consistent with our previous reports (Doitsh, Cavrois et al. 2010; Doitsh, Galloway et al. 2014; Monroe, Yang et al. 2014), loss of CD4 T cells was prevented by the NNRTI efavirenz, which allosterically inhibits HIV-1 reverse transcriptase, and by AMD3100, which prevents viral entry by blocking gp120 engagement with CXCR4. However, unexpectedly and not in keeping with our previous reports, addition of the integrase inhibitor, raltegravir, also blocked CD4 T cell death (Figure 3.1b). Because cell death involves viral life cycle events occurring prior to viral integration, raltegravir acts too late to affect the abortive infection process that activates

pyroptosis. These results were also surprising because spinoculation promoted high levels of HIV-1 fusion into target lymphoid CD4 T cells (Figure 3.2).

To further investigate this surprising result, co-culture studies of CFSE-labeled target CD4 T cells with productively infected HLACs were performed, where raltegravir was added at the time of mixing of productively infected and target CD4 T cells (Figure 3.1c). Under these conditions, marked death of target CD4 T-cell occurred, which was blocked by efavirenz and AMD3100, but not by raltegravir (Figure 3.1d). Thus the timing of raltegravir addition appeared important.

3.2.2. Free HIV-1 particles do not induce cell death of target lymphoid CD4 T cells

One possible explanation for these disparate results was that initial establishment of productive infection in permissive CD4 T cells is required in order to spread the virus to the non-permissive subset of CD4 T-cells and generate cell death. To test this hypothesis, we spinoculated HLACs with either single-round or multiple-round viruses containing a GFP reporter (NLENG1) (Levy, Aldrovandi et al. 2004). These viruses permit the dynamics of HIV-1 infection and T-cell depletion to be simultaneously monitored in the spinoculated cultures. Four after spinoculation, we observed a similar number of GFP-positive, productively infected cells with both the single-round and multiple round viruses, indicating that viral spread was not required to establish an initial population of productively infected cells. However, we observed a massive loss of CD4 T cells only in cultures spinoculated with the multiple-round virus. Notably, spinoculation with an integrase-deficient GFP HIV-1 (NLENG1 D116N) (Gelderblom, Vatakis et al. 2008) resulted in no productive infection and no CD4 T-cell death (Figure 3.3a). These results suggested that viral spread from productively infected cells, but not infection

mediated by cell-free virions, is required to promote the death of non-permissive lymphoid CD4 T cells. In agreement with this conclusion, addition of the AMD3100 entry inhibitor four hours after spinoculation efficiently blocked the ensuing death response while not affecting the number of GFP-positive productively infected cells (Figure 3.3b). These findings indicate that CD4 T-cell death occurs after establishment of productive infection, but not during infection with cell-free viruses.

Single-round and integrase-deficient HIV-1 clones are not competent for cell-to-cell dissemination following spinoculation with HLACs. To confirm that the mode of viral transfer, but not a lack of action or viral product, influenced the death response of target CD4 T cells, we modified the infection system by overlaying HLACs on a monolayer of 293T cells that had been transfected with these single-round proviral clones (Figure 3.3c). Interestingly, when these single-round viruses were transferred to HLACs by direct interaction with virus-producing 293T cells, a massive killing of target lymphoid CD4 T cells was observed (Figure 3.3d). These results demonstrate that recapitulating the cell-to-cell mode of viral transfer is sufficient to restore the killing capacity of these single-round clones.

3.2.3. Death response requires formation of a virological synapse

To further explore whether cell-cell contact was needed to induce death of CD4 T cells, we repeated the co-culture assay using productively infected and target CFSE-labeled HLACs. However, in this experiment the cells were co-cultured under conditions of increasing surface area thereby reducing the likelihood of cell-cell interactions. Using flow cytometry, we analyzed the levels of viable target CD4 T cells in the plates every 24 hours during four days of co-culture. The death of target CD4 T cells decreased as the surface area of the culture increased (Figure

3.4a), even in samples where the volume of culture medium remained constant (Figure 3.4b). These data suggest that the physical distance between HIV-producing and target cells directly affects the kinetics of CD4 T-cell depletion, and argue further against a role for free virions released into the medium in the death response.

Cell-to-cell spread of HIV-1 predominantly takes place across specialized contact-induced structures known as virological synapses (Jolly, Kashefi et al. 2004; Jolly and Sattentau 2004; Jolly, Mitar et al. 2007). These synapses facilitate efficient transmission of virus toward the uninfected and engaged target cell. The synapse gains stability through a rapid actin-mediated recruitment of adhesion molecules, such as the integrin leukocyte function-association antigen 1 (LFA-1) and its cognate ligand ICAM-1 to the junction point of cellular interaction (Jolly, Mitar et al. 2007). To examine whether virological synapse formation between HIV-infected and target cells is required to promote CD4 T-cell death, productively infected and target CFSE-labeled HLACs were co-cultured in the presence of blocking antibodies against ICAM-1 or CD11a, the a-subunit of the LFA-1 heterodimer. Addition of either the anti-ICAM-1 (Figure 3.4c) or anti-CD11a (Figure 3.4d) antibodies, but not isotype matched control antibodies, effectively blocked depletion of target CD4 T cells in the mixed cultures as efficiently as the antiviral drug efavirenz. These findings suggest that the interaction between ICAM-1 and LFA-1 in virological synapses is required to induce CD4 T-cell death in lymphoid cultures infected with HIV.

Western blotting analysis of HLAC revealed high expression levels of ICAM-1 in B cells, but not in CD4 or CD8 T lymphocytes. However, activated CD4 T cells, which correspond to those that become productively infected with HIV-1, express high levels of this adhesion molecule (Figure 3.4e). In contrast to ICAM-1, CD11a expression levels were high in both resting and activated CD4 T cells (Figure 3.4f). Thus, synapse formation between activated

effectors expressing ICAM-1 and uninfected CD4 T cells (either activated or resting targets) expressing LFA-1 likely occur regularly in paracortical zones of lymphoid tissues, which primarily comprises of CD4 T cells. In infected tissues, such interactions may further promote virological synapse formation and cycles of cell-to-cell viral spread leading to productive infection of new permissive targets, and cell death via abortive infection in non-permissive target CD4 T cells. Similarly, elevated levels of ICAM-1 and LFA-1 were observed in HIV-producing 293T cells overlaid with target HLACs (Figure 3.5).

3.2.4 Caspase-1 activation in abortively infected cells requires cell-to-cell spread of HIV-1

Most CD4 T cells in lymphoid tissues infected with HIV die by caspase-1-mediated pyroptosis triggered by abortive viral infection (Doitsh, Galloway et al. 2014). To test whether caspase-1 is induced by cell-free HIV-1 particles or by cell-to-cell spread of HIV-1, we spinoculated HLACs with single-round or multiple-round clones of the GFP reporter NLENG1, and analyzed intracellular caspase-1 activity using a specific fluorescently labeled inhibitor of caspases (FLICA) probe (Bedner, Smolewski et al. 2000). Consistent with our previous reports (Doitsh, Galloway et al. 2014; Monroe, Yang et al. 2014), spinoculating multiple-round HIV-1 particles triggered caspase-1 activity in non-productively, but not productively, infected CD4 T cells (Figure 3.6a). Treatment with AMD3100 four hours after spinoculation efficiently inhibited caspase-1 activation but did not affect the number of GFP-positive cells productively infected by this multiple-round clone of HIV-1. Thus, caspase-1 activity was not induced by the initial spinoculation of cell-free viruses, but rather by the spread of virus from productively infected cells established by spinoculation. Consistent with these results, spinoculation of single-round HIV-1 particles produced a similar level of productive infection, but only background levels of

caspase 1 were observed in the bystander cells. Spinoculation with integrase-deficient HIV-1 particles produced neither productive infection nor caspase-1 activation.

Consistent with pyroptosis as the pathway of programmed cell death (Fink and Cookson 2005), spinoculation with multiple-round HIV particles resulted in the release of the intracellular enzyme lactate dehydrogenase (LDH) (Figure 3.6b). Further, the release of LDH was completely blocked when AMD3100 was added four hours after spinoculation or when single-round or integrase-deficient HIV-1 particles were used for initial infection. Together, these findings indicate that infection with cell-free HIV-1 particles does not lead to caspase-1 activation despite apparent abortive infection of lymphoid CD4 T cells. Rather, capsase-1 activation and the induction of pyroptosis requires the generation of productively infected cells and successful cell-to-cell spread of HIV-1 to quiescent bystander lymphoid CD4 T cells.

3.3. Discussion

The life cycle of HIV-1 involves the release of particles into the extracellular space, where it can leave producer cells and spread to distant susceptible cells and hosts. HIV-1 can also spread directly between target cells via the architecture of lymphoid tissues where zones of direct cell-cell contacts naturally form. Although the superior efficiency of cell-to-cell spread compared to cell-free infection has been known for many years, in the context of HIV-1 pathogenesis the research has focused almost exclusively on transmission of cell-free viruses (Cummins and Badley 2010; Fevrier, Dorgham et al. 2011). One possible reason is that most studies examine viral replication in highly permissive cells, such as activated peripheral blood lymphocytes. These cells are the most common source of primary CD4 T cells and they promote production infection and cell death via caspase-3-mediated apoptosis (Gougeon, Lecoeur et al. 1996; Cooper, Garcia et al. 2013). However, in human lymphoid tissues such as tonsil and spleen,

activated and permissive cells represent only 5% of the total CD4 T cells, while non-permissive quiescent cells represent >95% of the targets encountered by HIV (Eckstein, Penn et al. 2001; Moore, Kitchen et al. 2004; Doitsh, Cavrois et al. 2010). Non-permissive cells die by abortive infection, which elicits caspase-1-dependent pyroptosis, a highly inflammatory form of programmed cell death (Doitsh, Galloway et al. 2014; Monroe, Yang et al. 2014).

Here, we explored the death of lymphoid CD4 T cells in HLACs using experimental strategies that unambiguously distinguish between cell-free and cell-cell modes of HIV-1 transmission. Using this system we now demonstrate that the mode of HIV-1 spread determines the outcome form of cell death. Specifically, cell-to-cell spread of HIV-1 is required to deplete non-permissive lymphoid CD4 T cells via caspase-1-dependent pyroptosis. Free HIV-1 particles, even at high quantities, are unable to trigger innate immune recognition and produce this form of cell death. Conversely, infection with free HIV-1 particles causes the small fraction of permissive cells in HLACs to die via caspase-3-dependent apoptosis. These findings suggest a radical change in the prevailing view of HIV pathogenesis where most of the pathogenic effects of HIV-1 are attributed to killing of CD4 T cells by circulating free virions. We propose that the fundamental "killing units" of CD4 T cells leading to CD4 T-cell depletion and ultimately progression to AIDS are infected cells residing in lymphoid tissues and mediate cell-to-cell spread of the virus. Productive ("direct") and abortive ("bystander") infections are often viewed as independent pathways underlying the progressive depletion of CD4 T cells (Doitsh, Cavrois et al. 2010; Cooper, Garcia et al. 2013; Doitsh, Galloway et al. 2014). Our findings now show that productive and abortive infections are not independent cytopathic events, but rather linked in a single pathogenic cascade (Figure 3.6c). Productively infected cells are obligatorily required to transmit the virus across the virological synapse formed with resting CD4 T cells. The

productively infected cell ultimately dies by apoptosis, while the resting bystander cell dies by pyroptosis.

The interaction of the cognate adhesion molecules ICAM-1 and LFA-1 at the virological synapse is critically important for efficient HIV-1 spread between permissive effector and target CD4 T cells. Our findings in HLACs demonstrate the role of the virological synapse in viral infection and depletion of *non-permissive* CD4 T-cell targets. Human lymphoid tissues predominantly consist of non-permissive CD4 T cells (Glushakova, Baibakov et al. 1995; Eckstein, Penn et al. 2001; Doitsh, Cavrois et al. 2010; Doitsh, Galloway et al. 2014). Therefore, the interaction and formation of virological synapses between productively infected cells expressing ICAM-1 and non-permissive targets expressing LFA-1 likely occur at high frequency in regions of T cells in HIV-infected lymphoid tissues and centrally contribute to the immunopathogenic effects of HIV-1. The interaction of LFA-1 on T cells with ICAM-1 also mediates the arrest and migration of T cells on surfaces of postcapillary venules at sites of infection or injury, as well as the ability of these cells to crawl out of the blood stream between high endothelial venules and into lymph nodes (Girard, Moussion et al. 2012). Importantly, interleukin (IL)-1β increases the expression of adhesion molecules such as ICAM-1 on endothelial cells (Hubbard and Rothlein 2000; Dinarello 2009; Dustin, Rothlein et al. 2011). The release of IL-1b by dying pyroptotic CD4 T cells in HIV-infected lymphoid tissues likely attracts more cells from the blood into the infected lymph nodes to die and produce more inflammation. Thus, the interaction of LFA-1 with ICAM-1 contributes to HIV pathogenesis by both promoting the depletion of CD4 T cells and facilitating a state of chronic inflammation, two key processes that propel disease progression to AIDS (Deeks 2011).

The molecular mechanisms that limit pyroptosis to virus transmission occurring via the cell-to-cell route are unknown. One possibility relates to TREX1, a cellular 3′ DNA exonuclease, and SLX4-associated MUS81-EME1 endonucleases that function as "cytoplasmic cleaners" that degrade single- and double-stranded DNA, respectively (Stetson, Ko et al. 2008; Laguette, Bregnard et al. 2014). Indeed, the intrinsic action of the TREX1 and SLX4-associated endonucleases in the cytoplasm may set a threshold level for reverse-transcribed DNA products needed for either productive infection in permissive cells, or, alternatively, pyroptosis in abortively infected non-permissive cells (Yan, Regalado-Magdos et al. 2010; Laguette, Bregnard et al. 2014). Cell-to-cell spread across the virological synapse may overcome TREX1/SLX4-mediated restriction by rapidly transferring large quantities of viral nucleic acid to the opposing target cell. Ironically, while this mechanism likely evolved for efficient viral spread between permissive cells, it acts against HIV-1 in non-permissive targets where it triggers viral detection, promotes cell death, terminates viral propagation, and drives inflammation and disease in the host.

3.4. Figures for Chapter Three

Figure 3.1: The mode of HIV-1 transfer generates disparate death responses of target lymphoid CD4 T cells. The death of lymphoid CD4 T cells was examined by spinoculation of target cells with large amounts of cell-free virions to target cells **(a, b)**, as previously described (O'Doherty, Swiggard et al. 2000; Doitsh, Galloway et al. 2014; Geng, Doitsh et al. 2014), or in co-cultures of CFSE-labeled target CD4 T with productively infected HLACs (Jekle, Keppler et al. 2003; Doitsh, Cavrois et al. 2010) **(c, d).** All samples were infected with a multiple-round X4-tropic NL4-3 strain of HIV-1. NL4-3 was selected because tonsillar tissue contains a high percentage of resting CD4 T cells that express CXCR4 (90–100%). Target cells were treated with the same concentrations of drugs prior to co-culture with productively infected HLACs or spinoculation with free virions. Inhibitors blocking HIV entry (AMD3100) or early steps of reverse transcription (efavirenz) prevented death of target CD4 T cells. In sharp contrast, inhibiting later events in the viral life cycle (raltegravir) did not prevent cell death in co-cultures with productively infected cells **(b)**, but abrogated the death response of target cells spinoculated with cell-free HIV-particles **(d)**. Error bars represent SD/√n of three independent donors. FACS plots represent results from three independent tonsils.

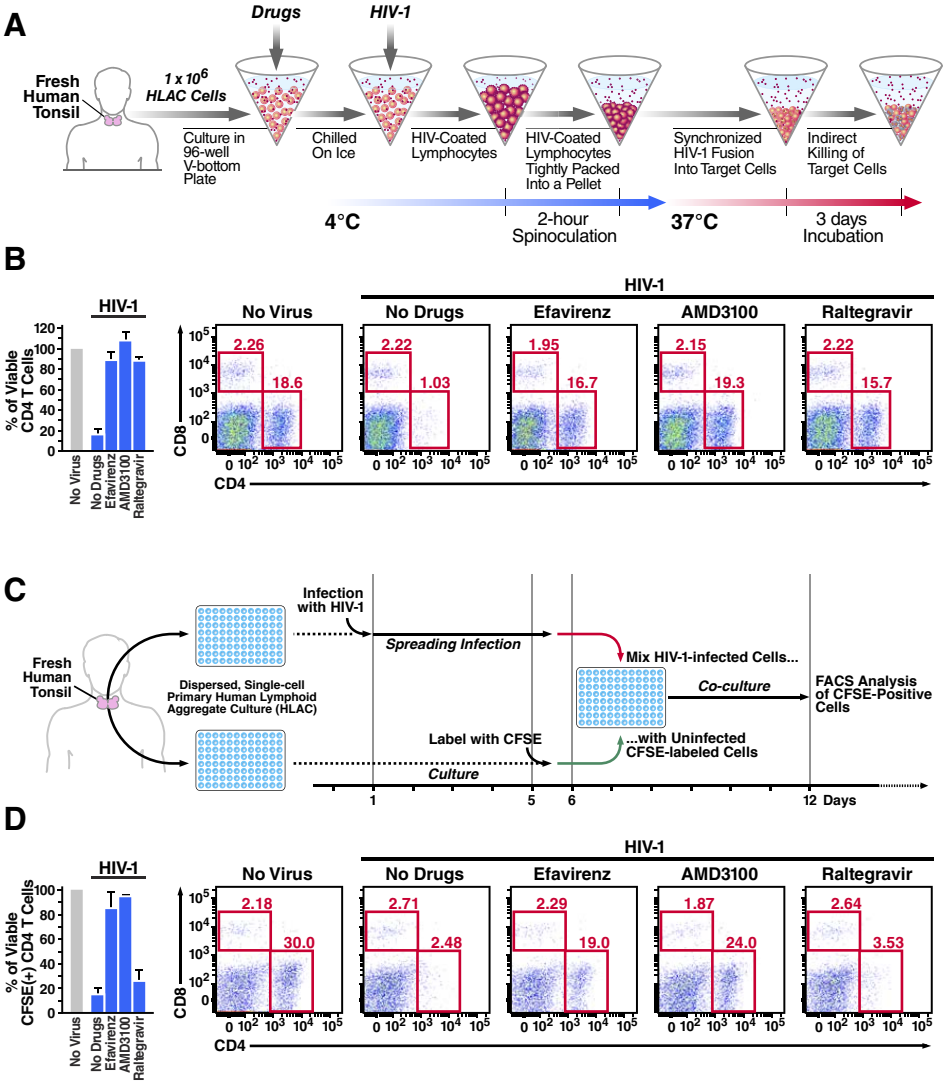

A
Drugs
HIV-1
Fresh Human Tonsil
1 x 10^6 HLAC Cells
Culture in 96-well V-bottom Plate
Chilled On Ice
HIV-Coated Lymphocytes
HIV-Coated Lymphocytes Tightly Packed Into a Pellet
Synchronized HIV-1 Fusion Into Target Cells
Indirect Killing of Target Cells
4°C
2-hour Spinoculation
37°C
3 days Incubation
B
HIV-1
% of Viable CD4 T Cells
No Virus
No Drugs
Efavirenz
AMD3100
Raltegravir
No Virus
No Drugs
Efavirenz
AMD3100
Raltegravir
2.26
18.6
2.22
1.03
1.95
16.7
2.15
19.3
2.22
15.7
CD8
CD4
C
Fresh Human Tonsil
Dispersed, Single-cell Primary Human Lymphoid Aggregate Culture (HLAC)
Infection with HIV-1
Spreading Infection
Mix HIV-1-infected Cells...
Co-culture
FACS Analysis of CFSE-Positive Cells
Label with CFSE
...with Uninfected CFSE-labeled Cells
Culture
1
5
6
12 Days
D
HIV-1
% of Viable CFSE(+) CD4 T Cells
No Virus
No Drugs
Efavirenz
AMD3100
Raltegravir
No Virus
No Drugs
Efavirenz
AMD3100
Raltegravir
2.18
30.0
2.71
2.48
2.29
19.0
1.87
24.0
2.64
3.53
CD8
CD4

Figure 3.2: Free HIV-1 particles do not induce cell death of abortively infected lymphoid CD4 T cells. (a) HLACs were spinoculated with a multiple-round, a single-round (D-Env lacking the gp160 gene, pseudotyped with HIV-1 Env), or D116N (integrase deficient) viral clones containing a GFP reporter (NLENG1). An IRES upstream of the *nef* gene preserves Nef expression and supports LTR-driven GFP expression in productively infected target cells (Levy, Aldrovandi et al. 2004). No drugs were added to the spinoculated cultures. The levels of productive infection and CD4 T-cell depletion in the cultures were analyzed by flow cytometry. Spinoculations with either multiple-round or single-round NLENG1 clones produced similar frequencies of productive HIV-1 infection. However, extensive depletion of CD4 T cells occurred only by the multiple-round, but not the single-round HIV-1 clone. No productive infection or CD4 T-cell depletion occurred in spinoculations with integrase-deficient HIV-1 particles. **(b)** Treatments with the entry inhibitor AMD3100 four hours after spinoculation with multiple-round NLENG1 HIV-1 particles does not prevent productive infection, but efficiently block killing of target resting CD4 T cells. Thus, death of CD4 T cells occurs after establishment of productive infection, but not during initial spinoculation of cell-free viruses. **(c)** A method to assess death of CD4 T cells with non-infectious HIV-1 clones. The single-round and integrase-deficient HIV-1 clones are not competent for multiple rounds of viral replication. Instead, we modified the experimental system by overlaying HLAC cells on a monolayer of 293T cells transfected with these proviral clones, as previously described (Doitsh, Galloway et al. 2014). As illustrated, fresh human tonsil is processed into HLAC and cells are cultured in suspension. After 12 hours, transfected 293T cells in a 24-well plate are washed and overlayed with $4x10^6$ HLAC cells in RPMI. Virus-producing 293T cells directly interact with target overlaying HLAC cells.

After 24–72 hours, HLAC suspensions were collected from wells and analyzed by flow cytometry. **(d)** Single-round and integrase-deficient HIV-1 clones kill target CD4 T cells as efficiently as multiple-round HIV-1 clones when transmitted via virus-producing cells. Error bars represent SD/√n of three independent donors. FACS plots represent results from three independent tonsils.

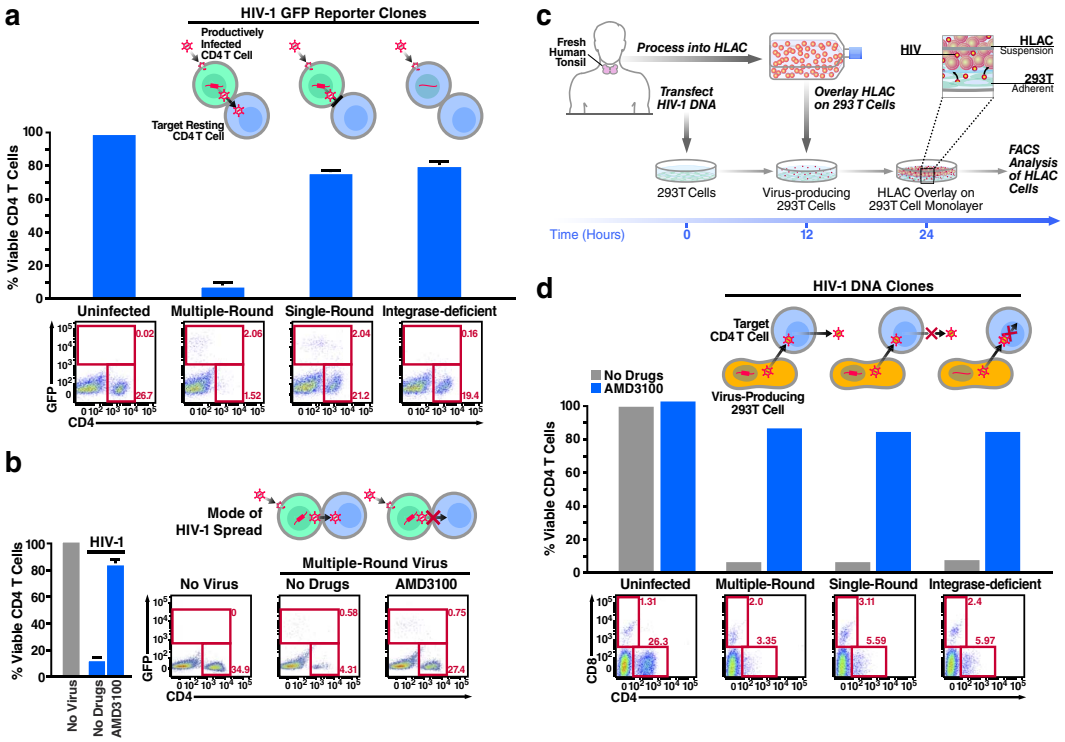


Figure 3.3: HIV-1 fuses into target lymphoid CD4 T cells at high levels. To assess the ability of these viruses to fuse with target cells, we used an HIV virion-based fusion assay that measures β-lactamase (BlaM) activity delivered to target cells upon the fusion of virions containing BlaM fused to the Vpr protein (BlaM-Vpr) (Cavrois, De Noronha et al. 2002). HLAC or CD4 T cells isolated from HLAC were infected with 100 ng of X4-tropic HIV-1 that has the b-lactamse (Blam) enzyme fused with Vpr (Blam-Vpr), which allows Blam incorporation into the HIV-1 virions (HIV-1 Blam-Vpr). Target cells were the loaded with the Blam substrate CCF2, which fluoresces in the green channel. Upon HIV-1 Blam-Vpr fusion into a CCF2 loaded target cell, Blam cleaves CCF2, changing its fluorescence from green to blue and allowing for measurement of the amount of viral fusion into target cells. HIV-1 fused to 62.7% of isolated CD4 T cells and 46.3% of CD4 T cells in HLAC, which was blocked upon the addition of the CXCR4 antagonist AMD3100.

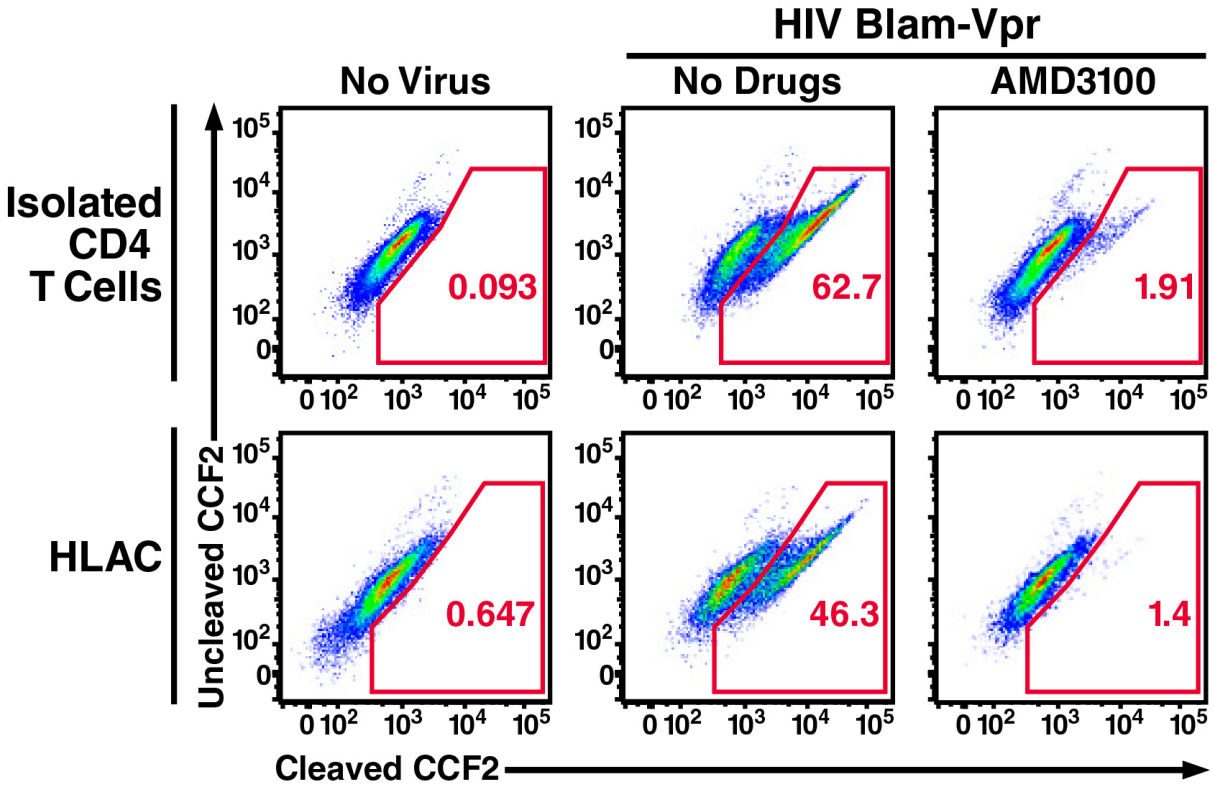


Figure 3.4: Death of lymphoid CD4 T cells requires close interaction with productively infected cells. (a) Increasing the cell culture surface area in HIV-infected cultures decreases cell-to-cell interactions, and reduces the kinetics of CD4 T-cell depletion. **(b)** Inverse correlation between culture surface area and CD4 T-cell death. Death of target CD4 T cells in each vessel was examined after four days of co-culturing with HIV-infected cells. Note that cell death decreases even in vessels where the volume of culture medium remained constant. **(c, d)** Blocking antibodies against either ICAM-1 or CD11a prevent death of target CD4 T cells by productively infected cells. **(e)** Exclusive expression of ICAM-1 on activated (permissive) lymphoid CD4 T cells. High ICAM-1 expression is also high in antigen presenting B cells, but not CD8 T cells. **(f)** High expression of LFA-1 on lymphoid CD4 and CD8 T cells, but not on B cells. In contrast to ICAM-1, LFA-1 expression is not increased on activated CD4 T cells. Error bars represent SD/√n of three independent donors. FACS plots represent results from three independent tonsils. EFV, Efavirenz.

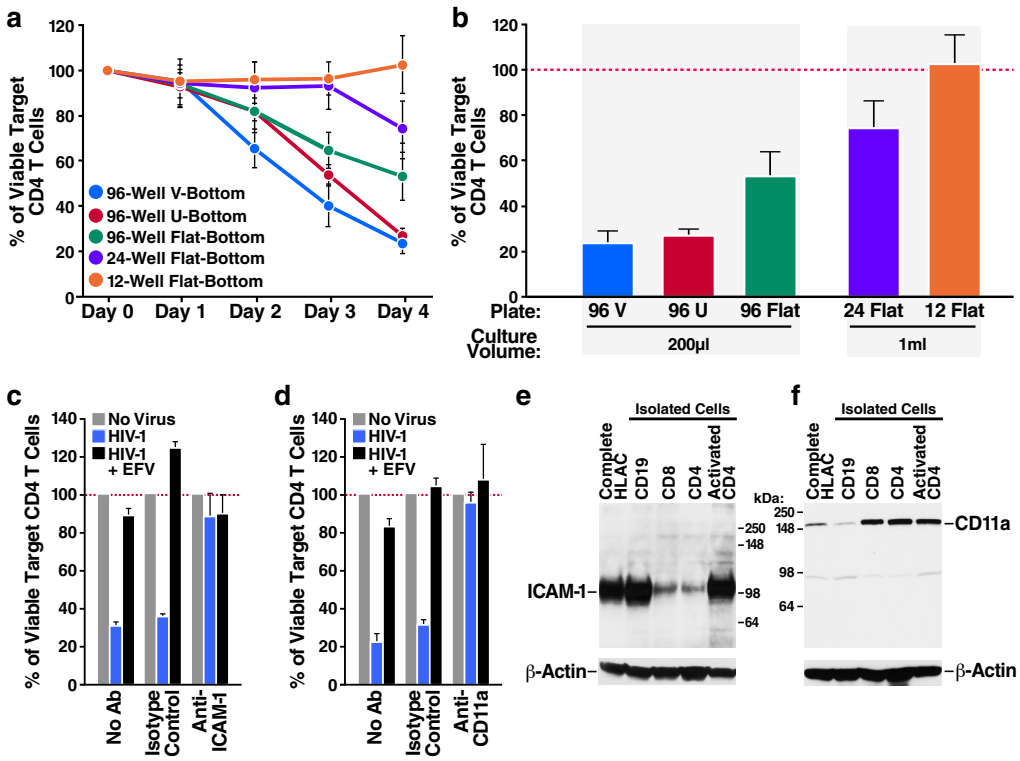

a
% of Viable Target CD4 T Cells
120
100
80
60
40
20
0
96-Well V-Bottom
96-Well U-Bottom
96-Well Flat-Bottom
24-Well Flat-Bottom
12-Well Flat-Bottom
Day 0
Day 1
Day 2
Day 3
Day 4
b
% of Viable Target CD4 T Cells
Plate:
96 V
96 U
96 Flat
24 Flat
12 Flat
Culture Volume:
200µl
1ml
c
% of Viable Target CD4 T Cells
No Virus
HIV-1
HIV-1 + EFV
No Ab
Isotype Control
Anti-ICAM-1
d
% of Viable Target CD4 T Cells
No Virus
HIV-1
HIV-1 + EFV
No Ab
Isotype Control
Anti-CD11a
e
Isolated Cells
Complete HLAC
CD19
CD8
CD4
Activated CD4
kDa:
250
148
98
64
ICAM-1
β-Actin
f
Isolated Cells
Complete HLAC
CD19
CD8
CD4
Activated CD4
250
148
98
64
CD11a
β-Actin

Figure 3.5: Elevated levels of ICAM-1 and LFA-1 in HIV-producing 293T cells overlaid with target HLACs HIV-1. HLACs were labeled with CFSE and overlaid over 293T cells transfected with the HIV-1 clove NL4-3, or untransfected. After three days the surface levels of ICAM-1 and CD18, the b-subunit of the LFA-1 heterodimer, were analyzed by flow cytometry. Note that overlaying target HLAC induces ICAM-1 and CD18 expression on 293T cells. In contrast, the levels of ICAM-1 and CD18 on HLACs remain unaffected upon overlaying 293T cells.

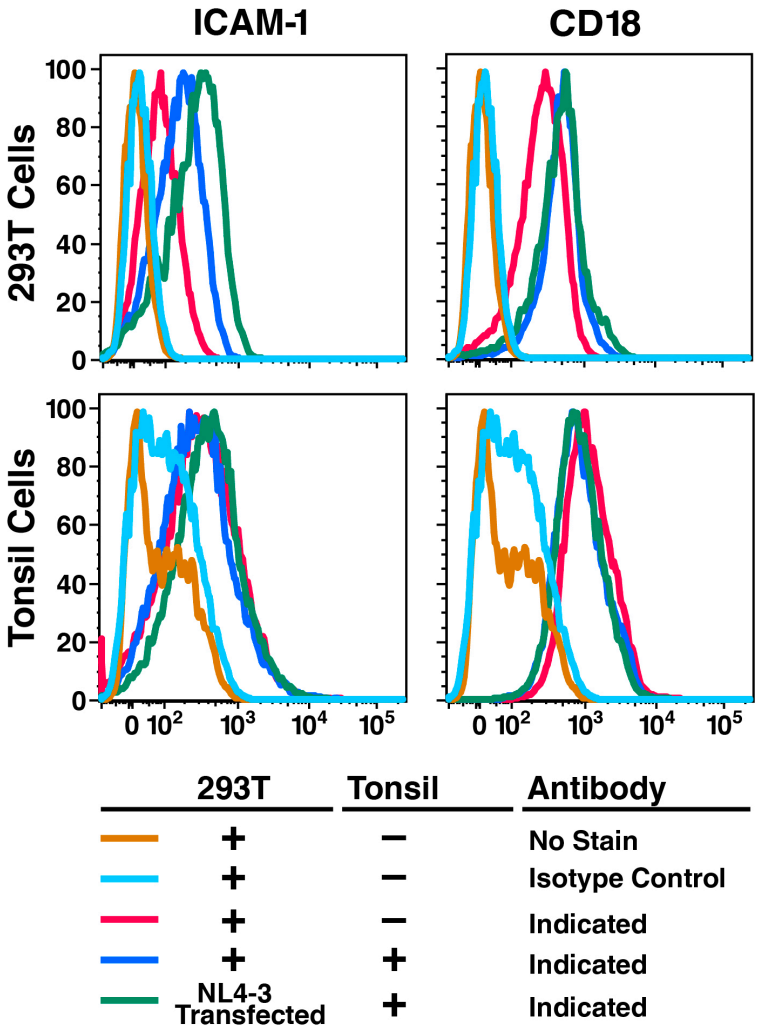
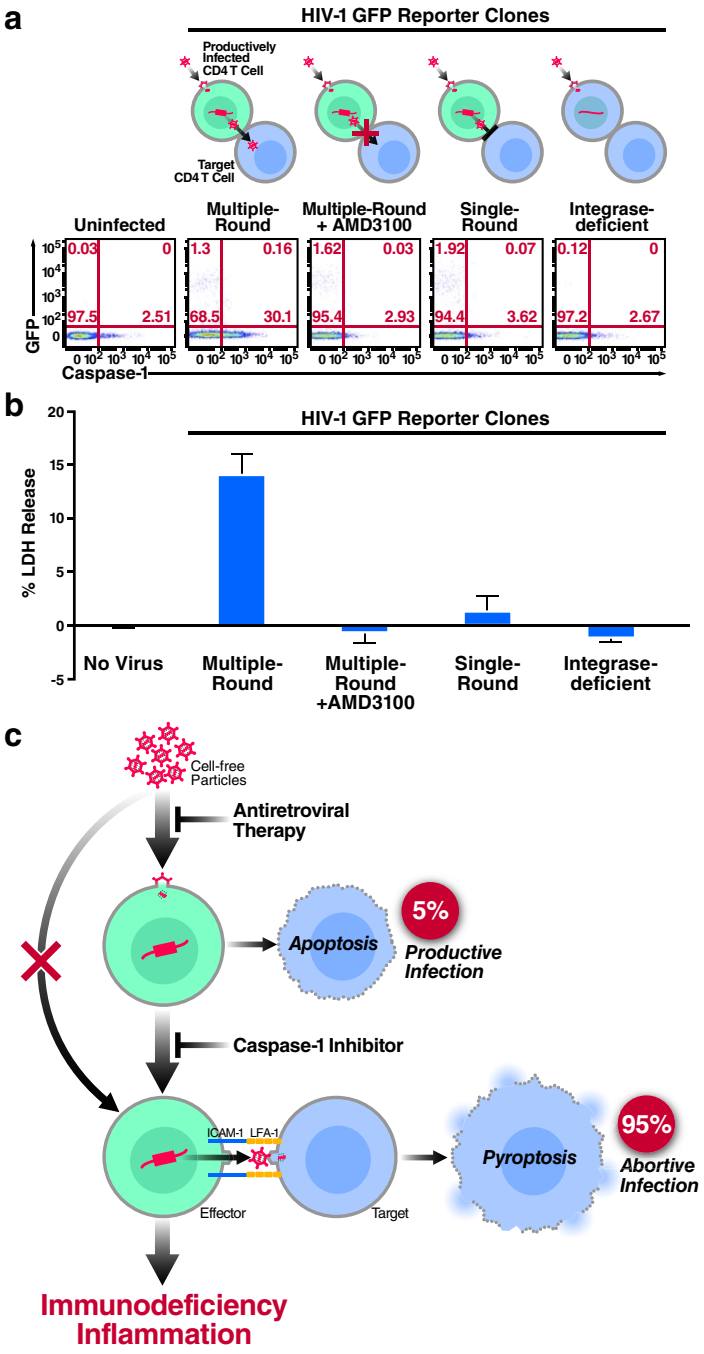


Figure 3.6. Cell-to-Cell transmission of HIV-1 is required to trigger innate recognition and caspase-1-dependent pyroptosis of lymphoid CD4 T Cells. (a) Isolated lymphoid CD4 T cells were spinoculated with multiple- or single-round NLENG1 reporter clones as indicated. AMD3100 was added to the indicated culture four hours after spinoculation. Cells were analyzed by flow cytometry using FLICA probe that contain amino acids sequences specifically targeted by active caspase-1. Abundant caspase-1 activity is exclusively observed in cultures spinoculated with multiple- round HIV-1 clones. Essentially no caspase-1 activity is observed in cultures where cell-to-cell spread of HIV-1 is blocked or does not occur. Note that caspase-1 is exclusively activated in abortively infected cells and that, in all cases, caspase-1 activity is limited to the GFP-negative subset of cells (abortively infected). **(b)** Supernatants from spinoculated cultures were analyzed for levels of released cytoplasmic LDH enzyme, an indicator of pyroptosis (Decker and Lohmann-Matthes 1988) **(c)** The mode of HIV-1 spread determines the outcome form of cell death. Infection of free HIV-1 particles produces productive infection and caspase-3-apoptosis in a small fraction of permissive lymphoid CD4 T cells. Next, cell-to-cell spread of HIV-1 is required to deplete the non-permissive lymphoid CD4 T cells, which represent 95% or more of the target cells in lymphoid tissues, via caspase-1-dependent pyroptosis. Free HIV-1 particles, even at high quantities, cannot trigger innate immune recognition and produce this form of cell death. Thus, in contrast to the previous view of productive and abortive infections by HIV-1 being independent events for cell death (Cummins and Badley 2010; Doitsh, Cavrois et al. 2010; Fevrier, Dorgham et al. 2011; Garg, Mohl et al. 2012), these events are linked in a single pathogenic cascade. Accordingly, antiretroviral therapy such as AZT or raltergravir, which does not prevent death of abortively infected CD4 T cells (Doitsh, Cavrois et al. 2010), effectively inhibits HIV pathogenesis, as it blocks upstream

productive HIV-1 infection. Caspase-1 inhibitors do not inhibit productive HIV-1 infection but block pyroptosis of abortively infected CD4 T cells (Doitsh, Galloway et al. 2014).



Chapter Four:

General Discussion and Future Directions

4.1. Caspase-1 inhibitors as potential therapeutic drugs

Our studies revealed that HIV depletes most CD4 T cells through an innate immune response against the virus. This response causes a highly inflammatory form of cell death that likely creates a vicious cycle of cell death, release of inflammatory signals, leading to the recruitment of new cells to the site where they become infected and die, which, in turn exacerbates the inflammation. This cycle could contribute to a chronic state of inflammation that likely fuels disease progression. This form of cell death requires the activation of caspase-1 and can be blocked by caspase-1 inhibitors, such as VX-765—a safe and well-tolerated compound in humans. These findings create an exciting new area of potential "anti-AIDS" drugs.

Patients on antiretroviral therapy (ART) exhibit low levels of chronic inflammation, despite suppression of viral replication. This inflammation is thought to contribute to premature age-related diseases (such as heart disease, liver failure, and dementia) now being seen in HIV-1 patients on ART. Whether the low level chronic inflammation is due to residual pyroptotic cell death is unknown. Patients on ART could exhibit small amounts of pyroptosis due to lingering viremia (such as viral bursts from latently infected cells) and the use of antiretroviral drugs that block too late in the viral life-cycle to inhibit abortive infection and pyroptosis, such as NRTIs and integrase inhibitors. In addition, because lymphoid CD4 T cells are already primed for pyroptosis and only require a second signal for cell death, the release of ATP from dying cells could trigger new rounds of pyroptosis independent of HIV-1. Casapse-1 inhibitors could potentially inhibit this low level inflammation, preventing the development of age-related diseases and thus expanding and improving the quality of life of HIV-1 patients on ART.

Inflammation driven by pyroptosis may contribute to the survival of the latent reservoir by dysregulating cytokines and increasing homeostatic proliferation of latently infected cells. In this

case, caspase-1 inhibitors may be key components to functional HIV cures. In addition, they could be given to patients with broad-range anti-viral resistances in combination with ART to possibly reduce viral loads and increase CD4 T-cell survival. Thus, we need to determine whether caspase-1 inhibitors rescue CD4 T cells from pyroptosis induced by HIV-1 abortive infection and whether these cells remain fully functional. We speculate that these rescued cells will clear the incomplete HIV-1 DNA transcripts over time through the action of cytoplasmic cleaners, such as TREX1 and the SLX4 complex, and will display normal T-cell functions. Ultimately, drug trials in humans perhaps preceded by studies in humanized mice and rhesus macaques will be required to establish the *in vivo* efficacy of this class of inhibitors.

4.2. Vpx as a modulator of cell death and inflammation

We have shown that non-permissive CD4 T cells abortively infected with HIV-1 die by caspase-1-mediated pyroptosis, while permissive CD4 T cells productively infected with the virus die by caspase-3-dependent apoptosis. Compared to HIV-1, patients infected with HIV-2 display slower CD4 T-cell declines, longer periods of asymptomatic infection, and lower mortality rates (Gottlieb, Sow et al. 2002; de Silva, Cotten et al. 2008). Interestingly, HIV-2 contains a unique accessory protein, Vpx, which HIV-1 lacks. Vpx was recently shown to degrade the host restriction factor SAMHD1, which prevents retroviral infection through its phosophohydrolase and RNase activity (Goldstone, Ennis-Adeniran et al. 2011; Laguette, Sobhian et al. 2011; Powell, Holland et al. 2011; Baldauf, Pan et al. 2012; Lahouassa, Daddacha et al. 2012; Ryoo, Choi et al. 2014). Thus, Vpx may degrade of SAMHD1 to relieve the restriction of HIV infection in target cells, thereby shifting the cell death pathway from the highly inflammatory caspase-1-mediated pyroptosis, to the more silent caspase-3-mediated

apoptosis. Although Vpx may increase productive infection of target cells and viral propagation, it may reduce the vast depletion of CD4 T cells caused by abortive infection and pyroptosis, and therefore reduce inflammation. The reduction in inflammation may result in the milder clinical course seen with HIV-2 infections.

4.3. Determining why cell-to-cell transmission is required for HIV-1 induced pyroptosis

We have demonstrated that pyroptosis induced by HIV-1 abortive infection occurs only via cell-to-cell transmission of HIV virions across a virological synapse. Cell-free virions are ineffective. However, cell-free virions can infect permissive CD4 T cells and promote apoptosis. Therefore, the manner of HIV-1 transmission controls the form of cell death and the resulting level of inflammation. Thus, we need to delineate why cell-to-cell spread of HIV-1 is required to trigger pyroptosis to understanding the immunopathogenic effects of HIV-1.

Cell-to-cell transmission of HIV-1 is approximately 10^2 to 10^3 times more infectious than cell-free particles. We speculate that cell-to-cell spread of HIV-1 is required for sufficient incomplete DNA reverse transcripts to accumulate in the target cell to trigger innate immune sensing and pyroptosis. In addition, the restriction imposed by SAMHD1 as well as the activities of the TREX1 exonuclease and SLX4-associated MUS81-EME1 endonucleases likely limit the amount of incomplete reverse-transcribed DNA in the cytoplasm by blocking its synthesis or promoting its degradation. Cell-to-cell transmission of HIV-1 may overwhelm the ability of these host factors to clear all viral nucleic acid quickly and efficiently transferring large quantities of virus. However, the GTPase activity of SAMHD1 would still limit intracellular dNTP concentrations, thereby stopping reverse transcription and causing an accumulation of reverse transcripts that induce pyroptosis. Interestingly, knocking down

SAMHD1 with Vpx, such as with HIV-2, might now promote the completion of reverse transcription and also protect the cell from undergoing pyroptosis and the host from inflammation.

Cell-to-cell transmission of HIV likely evolved to promote rapid and efficient productive infection of target cells, while SAMHD1 restriction likely evolved to protect the host from infection by cell-free retroviruses. However, in the context of HIV-1 infection of non-permissive CD4 T cells by the cell-to-cell route, SAMHD1 action may not be protective, but instead it may function as a pathogenic factor creating abortive transcripts. The subsequent pyroptotic cell death is a major driver of HIV pathogenesis. Interestingly, monkeys infected with species-specific SIVs do not limit viral replication, yet the animals do not develop inflammation of AIDS-like disease. The virus and the host have evolved to coexist. Understanding the key differences in nonpathogenic versus pathogenic infections could provide new insights enabling a functional cure.

Chapter Five:

Material and Methods

5.1. Preparation of HIV-1 virions

To generate viruses: pNL4-3, pNLENG1, 89.6, NL4-3 Blam-Vpr, NLENG1 ΔEnv + gp160 (an NLENG1 clone lacking the gp160 gene, pseudotyped with HIV-1 gp160 envelope, to create single-round HIV-1 GFP reporter particles), NLENG1 D116N (Integrase-deficient mutant of the NLENG1) and R5-tropic GFP-reporter virus (pBRNL43_005pf135(R5)nef+_IRES_eGFP) proviral expression DNA was transfected into 293T cells by the calcium phosphate method. The medium was replaced after 16 hours. After 48 hours, the supernatants were collected and clarified by sedimentation, and virions were concentrated by ultracentrifugation, and stored at –80°C in 100% fetal bovine serum. All viral stocks were quantitated by measuring $p24^{gag}$ levels by ELISA (1 ng $p24^{gag}$ equals approximately 2 x 10^6 viral particles). The R5-tropic GFP-reporter virus (pBRNL43_005pf135(R5)nef+_IRES_eGFP) was derived from the pNLENG1 clone replaced with gp120 V3 loop sequence of R5-tropic HIV primary isolates as previously described (Papkalla, Munch et al. 2002). For single-round NLENG1, 293T cells were co-transfected with pNLENG1 ΔEnv DNA and HIV-1 gp160 DNA at a 9:1 ratio. For NL4-3 Blam-vpr, 293T cells were transfected with NL4-3, Blam-vpr and pAdvantage at a ratio of 3:1:0.1.

5.2. Processing of HLAC, Isolation of CD4 T Cells and Cell Culture

Human tonsil tissue from routine tonsillectomies were obtained from Cooperative Human Tissue Network, processed into HLAC and cultured as described (Doitsh, Cavrois et al. 2010). CD4, CD8 T cells and B cells were isolated from HLAC by positive selection (Miltenyi), or negative selection (Stem Cell Technologies).

5.3. Infection of HLAC, Co-Culture with CFSE-Labeled Target Cells, Killing Assay with 293T Overlaid HLACs

Infections of HLAC or isolated CD4 T cells from HLAC were performed by spinoculation as described (Doitsh, Cavrois et al. 2010). In brief, cells were plated in a 96 V-bottom plate at $1x10^6$ cells/well/100 ml. Drugs were then added as indicated and allowed to incubate with the cells at 37°C for 15 minutes. Cells were then cooled on ice for 15 minutes before they were mixed with 100 ng of $p24^{gag}$. The virus-cell mixture was incubated on ice for 30 minutes to allow the virus to attach to cells, and then spun for 1 hour at 1200g at 4°C and subsequently shifted to 37°C to allow synchronized viral fusion of the attached virions.

In co-culture experiments, uninfected HLAC were stained with 1 mM CFSE (Molecular Probes) and plated at $1x10^6$ cells/well/200 ml. At 2 days post-spinoculation, infected or uninfected HLAC were co-cultured with CFSE-labeled target cells in a 96 V-bottom plate in 200 ml of tonsil medium. The indicated drugs were added to the CFSE-labeled cells and incubated at 37°C for 15 minutes before co-culture with infected HLAC, unless otherwise noted. Uninfected, infected, and CFSE-labeled cultures were manipulated as described for individual experiments.

Because splenic cells are extremely refractory to HIV infection we modified the infection system by overlaying splenic HLAC cells on a monolayer of 293T cells that had been transfected with HIV-1 proviral clones. Analysis of CCR5-expressing CD4 T-cell death was similarly performed using 293T transfected with the R5-tropic 81A strain of HIV-1. We also used this method for assays using shRNA-infected HLACs. Lastly this method was used to assess the killing of single-round or non-infectious HIV-1 clones. 293T cells were transfected with 50 ng HIV-1 DNA in a 24-well plate. After 12 hours, 293T cells were overlayed with $4x10^6$ HLACs in

tonsil media (i.e. RPMI 1640 supplemented with 15% heat-inactivated fetal bovine serum, 100 μg/ml gentamicin, 200 μg/ml ampicillin, 1 mM sodium pyruvate, 1% nonessential amino acids (Mediatech), 2 mM L-glutamine, and 1% fungizone), in the presence of the indicated drugs. Virus-producing 293T cells directly interact with targets present in the overlaying HLACs. After 24-72 hours, the HLAC suspensions were collected from wells and analyzed by flow cytometry.

Unless otherwise stated, drugs were used at the following concentrations: 250 nM AMD3100; 100 nM Efavirenz; Nigericin 8-10 μM; Staurosporine 50nM; Ac-YVAD-CMK, Z-WEHD-FMK, Z-DEVD-FMK, Z-VAD FMK, Z-VEID-FMK, Z-VAD FMK, Z-IETD-FMK, or Z-FA, all 50 mM (100 mM represents the maximal concentration of these caspase inhibitors that is not associated with toxicity); 10 mM VX-765; 10 mM VRT-043198; 5 mM necrostatin; 50 mM CRID3; 10 mM parthenolide; 20 mM Glyburide; 20 mM Glimepiride (20 mM of Glyburide and Glimepiride represent the maximal drug concentration that does not induce toxicity) and 5 μM Raltegravir. In some experiments, AMD3100 was added 4 hours post-spinoculation.

5.4. FACS Analysis and Gating Strategy

For analysis of cell death by flow cytometry, cells were stained with CD4-PE (BD Biosciences) and CD8-APC (BD Biosciences) at a concentration of 1:200 in FACS buffer (PBS supplemented with 2 mM EDTA and 2% fetal bovine serum) and fixed with a final concentration of 2% paraformaldehyde. For analysis of CCR5-expressing CD4 T cells, HLACs were stained with 1:3 dilutions of mouse anti human CCR5 (BD Pharmingen, clone 2D7/CCR5) on ice for 3 hours. In isolated CD4 T-cell cultures a standard number of fluorescent beads (Flow-Count Fluorospheres, Beckman Coulter) were added to each cell-suspension sample before data acquisition. Data was collected on a FACS Calibur (BD Biosciences) and analyzed with Flowjo software (Treestar).

Cell death was analyzed in spinoculation cultures by gating live cells with forward and side scatter, followed by gating and counting subsets of CD4 and CD8 T cells. Survival of CD4 T cells was calculated by dividing the number of CD4 T cells by the number CD8 T cells in each condition. In isolated CD4 T-cell cultures, survival of CD4 T cells was calculated by dividing the number of CD4 T cells by the number of collected fluorescent beads. All samples were normalized to the number of uninfected cultures.

Cell death was determined in co-culture systems by gating on live cells with forward and side scatter, followed by gating and counting of CFSE-positive CD4 and CD8 T cells. The survival percentage of CD4 T cells was calculated by dividing the number of CD4 T cells by that of CD8 T cells. All conditions were normalized to uninfected conditions.

5.5. Protein Analysis and IFN Inhibition

For stimulating the processing and secretion of IL-1b, CD4 T cells were isolated from HLACs by positive selection and treated with 8-10 μM nigericin (Sigma, Cat) for 12 h at 37ºC. The potassium ionophore nigericin mediates an electroneutral exchange of intracellular K^+ ions for extracellular protons, providing a second inflammatory stimulus, which results in the NLRP3-mediated activation of caspase-1 (Perregaux, Barberia et al. 1992; Perregaux and Gabel 1994). For assessing the processing and secretion of IL-1b in infected CD4 T cells, CD4 T cells were isolated from HLACs as described above, spinoculated with or without NL4-3 (80 ng $p24^{gag}$/1 x 10^6 cells) and the indicated drugs as indicated in figures. For cytoplasmic pro-IL-1b (Figures 2a, 2b) and other intracellular protein analysis, cells were washed in PBS and immediately lysed in cell extraction buffer (Life Technologies) with the addition of a protease inhibitor cocktail (Roche). For NLRP3 detection cells were lysed using Digitonin lysis buffer

(Digitonin 0.5%, Tris-Hcl pH 7.4 20 mM, NaCl 150 mM) with the addition of a protease inhibitor cocktail (Roche). Lysates were subjected to SDS-PAGE protein analysis using mouse anti human IL-1b antibody (R&D systems clone 8516, Cat#MAB201), which recognizes the pro- as well as the cleaved form of IL-1b (Figures 2a, 2b). For analysis of secreted IL-1b (Figures 2c, 3g, 4), cells were cultured in RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum. Supernatants were collected 3-5 days after infection with HIV-1 or 12 h after treatment with nigericin, filtered through 0.22 mm filter plates (Millipore) and subjected to SDS-PAGE protein analysis using rabbit polyclonal anti-human IL-1β (Abcam, Cat # ab2105, which primarily recognizes the cleaved form of IL-1b. For SDS-PAGE immunoblotting analysis, Bio-Rad Criterion 15% pre-cast Tris-HCL gels were used. Gels were wet transferred onto PVDF membranes (Bio-rad) at max current for 3 hours at 4°C and then blocked in 5% non-fat milk for 1 hour at room temperature. Primary antibodies were incubated overnight at 4°C and secondary antibodies for 1 hour at room temperature. Additional primary antibodies used for SDS-PAGE analysis were 1/1000 rabbit anti caspase-1 p10 (clone c-20, Santa Cruz, cat# SC-515), 1/1000 rabbit anti caspase-3 (clone 8G10, Cell signaling, cat# 9665S), 1:1000 mouse anti NLRP3 (Abcam, cat# ab17267), 1/1000 rabbit polyclonal anti-human ASC (Imgenex, Cat # IMG-5662), 1/100 Phospho-Stat1 (ser727, Cell Signaling, Cat #9177), 1:1000 rabbit anti human CD54/ICAM-1 antibody (Cell Signaling Technology, Cat# 4915S), 1:1000 mouse anti Human Integrin alpha L/CD11a antibody (R&D systems, Cat# MAB3595), and 1/10000 of the mouse monoclonal anti-β-Actin (Sigma, Cat. # A5316). The secondary antibody used was 1/5000 anti-rabbit secondary (Thermo Scientific, Cat #32460) or 1/5000 anti-mouse secondary (Thermo Scientific, Cat #32430) developed using 1/4 dilution of SuperSignal West Femto substrate (Thermo Scientific, Cat #34095). To neutralize interferon alpha receptor in HLACs, cultures

were added with 1-5 mg of Anti-Interferon-α/β Receptor Chain 2 Antibody, clone MMHAR-2 (Millipore).

5.6. LDH Assay

HLAC cultures were spinoculated as described above with, NL4-3, NLENG1, NLENG1 ΔENV + gp160, or NLENG1 D116N. At 3–4 days post spinoculation, culture supernatant was collected and assessed for release of cytoplasmic lactate dehydrogenase (LDH), as described (Decker and Lohmann-Matthes 1988). When applicable, cells in the same cultures were also collected and analyzed for productive infection (GFP+ cells) and death as described above.

5.7. FLICA Staining for Active Caspase-1

To determine intracellular activation of caspases-1, fluorescently labeled inhibitors of caspases (FLICA) probe assays (ImmunoChemistry Technologies) were performed. Each FLICA probe contains a 3 or 4 amino acid sequence targeted by a specific activated caspase. There is no interference from pro-caspases or the inactive form of the enzymes (Bedner, Smolewski et al. 2000). FLICA probes are cell-permeable and covalently bind to the active forms of specific caspases. After washing, FLICA fluorescent signal is specifically retained within cells containing the appropriate active form of the caspase while the reagent is washed away in cells lacking the appropriate active caspase.

CD4 T cells were isolated from HLACs as described above and spinoculated with NLENG1, NLENG1 ΔENV + gp160, or NLENG1 D116N. At 2 days post-spinoculation FLICA probes were added directly to the cell cultures, incubated for 15 min at 37°C, and washed five

times with FACS buffer. Cells were then fixed with paraformaldehyde and analyzed by flow cytometry.

5.8. Production and infection of Vpx-VLPs and shRNA-coding HIV LV particles.

SIVmac 251 virus-like particles for Vpx delivery (Vpx-VLPs) were produced using the pSIV3^{+} plasmid, kindly provided by Dr. A. Cimarelli (Goujon, Jarrosson-Wuilleme et al. 2006). These Vpx-VLP particles are non-infectious as they do not contain any viral genetic material, but they are used to transiently deliver Vpx into target cells where it promotes degradation of SAMHD1 thereby rendering the cells permissive to HIV LV infection (Laguette, Sobhian et al. 2011). In contrast to the commonly used VSV-G glycoprotein, we pseudotyped the Vpx-VLPs with the CXCR4-tropic Env of HIV-1, which supports efficient fusion of viral particles to quiescent CD4 T lymphocytes (Agosto, Yu et al. 2009). For production of Vpx-VLPs 293T cells were co-transfected with 8 µg pSIV3^{+} and 2 µg CXCR4-tropic Env (gp160)-encoding plasmid. The amount of lentiviral particles was determined by SIV p27^{gag} ELISA assay. shRNA-coding vectors were cloned using a modified version of the pSicoR (plasmid for Stable RNA interference, conditional) lentiviral vector(Ventura, Meissner et al. 2004), which encodes an mCherry reporter driven by an EF-1α promoter (pSicoR-MS1) (Wissing, Montano et al. 2011). To generate shRNA lentiviral particles, 293T cells were co-transfected with 10µg pSicoR-mCherry shRNA constructs, 9µg HIV-based packaging construct NL4-3 8.91(Grivel, Elliott et al. 2007), and 2µg CXCR4-tropic Env (gp160)-encoding plasmids. Cells were transfected using the standard phosphate calcium transfection protocol (Wigler, Pellicer et al. 1978). The lentiviral particle stocks were quantitated by HIV p24^{gag} ELISA assay (1 ng p24^{gag} equals approximately 2 x 10^{6} viral particles).

To achieve productive infection of shRNA-encoding LV particles, complete HLACs or isolated lymphoid CD4 T cells were initially challenged with Vpx-VLPs, followed by a second infection with an shRNA-coding LV of interest after 24 hours. This sequential infection strategy allowed Vpx to establish an optimal permissive state within the target cells at the time when the shRNA LV infection was performed. To facilitate a synchronized delivery of Vpx and fusion of shRNA LV particles, cells and particles were subjected to high-speed spinoculation at each step. To assess the efficiency of gene silencing by the shRNA-coding vectors, highly infectious SupT1 were infected with shRNA LV (without prior Vpx-VLP infection), and were subjected to protein analysis after 48 hours.

For cloning of caspase-1-coding shRNA vector the following oligos were used:

Sense:

TACACGTCTTGCTCTCATTATTCAAGAGATAATGAGAGCAAGACGTGTTTTTTTC;

Antisense:

TCGAGAAAAAAACACGTCTTGCTCTCATTATCTCTTGAATAATGAGAGCAAGACGTG

TA

For cloning of caspase-3-coding shRNA vector the following oligos were used:

Sense:

TAAAGGTGGCAACAGAATTTTTCAAGAGAAAATTCTGTTGCCACCTTTTTTTTTC;

Antisense:

TCGAGAAAAAAAAAGGTGGCAACAGAATTTTCTCTTGAAAAATTCTGTTGCCACCTT

TA

For cloning of ASC-coding shRNA vector the following oligos were used:

Sense:

TGAAGCTCTTCAGTTTCACATTCAAGAGATGTGAAACTGAAGAGCTTCTTTTTTC;

Antisense:

TCGAGAAAAAAGAAGCTCTTCAGTTTCACATCTCTTGAATGTGAAACTGAAGAGCTT

CA

For cloning of NLRP3-coding shRNA vector the following oligos were used: Sense:

TGAAATGGATTGAAGTGAAATTCAAGAGATTTCACTTCAATCCATTTCTTTTTTC;

Antisense:

TCGAGAAAAAAGAAATGGATTGAAGTGAAATCTCTTGAATTTCACTTCAATCCATTT

CA.

5.9. Immunohistochemistry

5.9.1 Tissue Samples.

HIV-infected lymph node tissue was obtained from a patient participating in the SCOPE cohort at HIV/AIDS clinic of the San Francisco General Hospital (SFGH) Positive Health Program. All tissues were obtained with full consent from the patient and under a protocol fully approved by the Committee on Human Research at UCSF. For the results presented, an inguinal lymph node was harvested from two different HIV-infected patients: A 50-year-old immunosuppressed, untreated R5-tropic HIV-1-infected subject during the chronic phase of disease. This individual exhibited a viral load of 87,756/ ml, and CD4 T-cell count of 227/ ml. A 41-year-old African American male, infected with an R5-tropic strain of HIV-1, has been on intermittent anti-retroviral therapy between 2004-2009, and stopped anti-retroviral therapy on 2009. This individual exhibited a viral load of 30,173/ ml, and CD4 T-cell count of 259/ ml. The

fresh specimens were immediately fixed with 4% PFA and subjected for immunostaining analysis. Sections of the HIV-infected lymph node and of a fresh human tonsil were processed in parallel and analyzed for the indicated markers.

5.9.2. Tissue Preparation and Immunohistochemistry.

Five-micron sections were cut from formalin- fixed paraffin embedded tissue blocks and mounted on X-tra microscope slides (Leica Microsystems). Specimens were stepwise deparaffinized in xylene and rehydrated in descending alcohols to water. Endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide (Sigma Chemicals Cat #H1009) in PBS for 15 min. Antigen retrieval was performed by microwaving the sections in 10mM citrate buffer, pH 6.0. Sections were then blocked in the secondary antibody host's normal serum (Vector labs; horse S-2000, goat S-1000, rabbit Cat #S5000). The following primary antibodies were diluted in PBS with 0.1% bovine serum albumin (BSA) and applied to the slides overnight at 4°C: Monoclonal Mouse Anti-Human CD3 (1/100, Clone F7.2.38 Dako, Cat#M725429-2), monoclonal rabbit anti-human CD11c (1/100, clone EP1347Y, Abcam Cat#ab52632), monoclonal mouse anti-human Ki-67 (1/100, clone MIB1, Dako Cat #M724029-2), monoclonal mouse anti-HIV $p24^{gag}$ (1/50, clone KaI-1, Dako Cytomation Cat #M0857), rabbit anti-human cleaved caspase-3 (1/300, Cell Signalling Technology Cat#9661), goat anti-p20 subunit of active human Caspase-1 (1/200, clone c15, Santa Cruz Biotechnology Cat #sc-1780), rabbit anti-human against bioactive 17 kDa IL-1b (1/100, Abcam Cat #ab2105), and Annexin V (1/50, Abcam Cat #EPR3979). The following day sections were washed in 0.05% Tween-20 in PBS followed by incubation with Vector laboratories biotinylated secondary IgG antibodies diluted 1:200 in PBS for 30 minutes at room temperature (donkey anti-mouse BA-2000, goat anti-rabbit BA-1000, rabbit anti-goat BA-5000). Slides were then rinsed in 0.05%

Tween-PBS, and incubated in streptavidin horseradish peroxidase complex at a 1:200 dilution in PBS for 30 min at room temperature (Vector Laboratories Cat #SA-5004). Specimens were rinsed in 0.05% Tween-20 in PBS then incubated with 3,3-diaminobenzadine (DAB) chromogenic substrate (Sigma Chemical, D-5905, St. Louis, MO) using hydrogen peroxide as a substrate (Sigma Chemicals, Cat #H1009) for 10 min. Sections were counterstained in hematoxylin dehydrated through graded alcohols, cleared in xylene and mounted in depex.

5.10. Virion-Based Fusion Assay

The virion-based fusion assay was performed as described (Cavrois, Neidleman et al. 2004; Doitsh, Cavrois et al. 2010). Briefly, HLAC or isolated CD4 T cells were incubated with 100 ng of $p24^{gag}$ BlaM-Vpr containing virions at 37°C for 2 hours and washed in CO_2-independent medium (Gibco BRL). Cells were then loaded with CCF2/AM dye as described by the manufacturer (Invitrogen) and incubated for 16 hours at room temperature with 2.5 mM probenecid (Sigma Pharmaceuticals). Cells were then washed, fixed with 2% paraformaldehyde, and analyzed by flow cytometry with LSR2 (Becton Dickinson). Data were collected with DiVa software and analyzed with FlowJo software (Treestar).

5.11. Plate Surface Experiments

HLACs were processed and infected, and target cells were stained with CFSE, as described above, with the exception that 5x10 cells/well were spinoculated with 40 ng $p24^{gag}$ NL4-3 and CFSE target cells were plated at 5x10 cells/well. At 2 days post-spinoculation, uninfected or infected HLAC were co-cultured with CFSE target cells in either a 96 V-bottom, 96 U-bottom, 96 flat-bottom, 24-well, or 12-well plate. Co-cultures in 96 V-bottom, 96 U-bottom, and 96 flat-bottom plates were plated in 200 ml of tonsil medium and co-cultures in 24-

or 12-well plates were plated in 1 ml of tonsil medium. Cell death was analyzed 1, 2, 3, and 4 days post co-culture, as described above.

5.12. Virological Synapse Blocking Antibody Experiments

HLACs were processed, infected, and co-cultured as described above. Antibodies against ICAM-1 (Calbiochem, Cat # CP53) and LFA-1 (eBioscience, Cat # BMS102), as well as an isotype mouse IgG1 negative control (clone 1E2.2 EMD Millipore) and Efavirenz, were added to CFES target cells 4 hours before co-culture. At 2.5 days post co-culture, cells were stained and analyzed by flow cytometry as described above.

5.13. Analysis of surface ICAM-1 and LFA-1 expression by Flow Cytometry

To determine the surface expression levels of ICAM-1 and LFA-1 on 293T and HLAC cells, the 293T overlay assay was performed as described above. Prior to overlay, HLACs were labeled with CFSE. 48 hours after overlay, HLACs were either left unstained, stained with an isotype control antibody conjugated with PE (BD Biosciences), ICAM-PE (eBioscience) or CD18-PE (the beta chain of LFA) (eBioscience). The cultures were then fixed and analyzed using a FACS Calibur. To assess the expression levels of ICAM or CD18 on 293T cells and HLAC, non-CFSE labeled cells and CFSE labeled cells were gated upon respectively.

Chapter Six:

Abbreviations and References

6.1. Abbreviations

HIV: Human Immunodeficiency Virus

AIDS: Acquired Immunodeficiency Syndrome

HLAC: Human Lymphoid Aggregate Culture

IRES: Internal Ribosomal Entry Site

LTR: Long Terminal Repeat

GFP: Green Fluorescent Protein

FLICA: Fluorescently Labeled Inhibitor of Caspases

EFV: Efavirenz

NNRTI: Non-Nucleoside Reverse Transcriptase Inhibitor

NLRP3: NOD-like receptor family, pyrin domain containing 3

IL-1b: Interleukin-1 beta

LPS: Lipopolysaccharide

CCR5: Chemokine Receptor Type 5

CXCR4: C-X-C chemokine receptor type 4

RIP1: Receptor-interacting serine/threonine-protein kinase 1

IFN: Interferon

LDH: Lactate Dehydrogenase

ASC: Apoptosis-associated speck-like protein containing a CARD

shRNA LV: shRNA-encoding Lentiviral Vector

Vpx-VLPs: Vpx-harboring Lentiviral Particles

SAMHD1: SAM domain and HD domain-containing protein 1

ELISA: Enzyme-Linked Immunosorbent Assay

SIV: Simian Immunodeficiency Virus

PBMC: peripheral blood mononucleated cell

ENV: Envelope

Tem: Effector Memory T cell

Tcm: Central Memory T cell

AZT: Azidothymidine

IFNAR: Human Interferon Alpha Receptor

STAT: Signal Transducers and Activators of Transcription

TLR: Toll Like Receptor

ATP: Adenosine triphosphate

ART: Antiretroviral Therapy

CRID3: Cytokine Release Inhibitory Drug

CFSE: Carboxyfluorescein Succinimidyl Ester

LFA-1: leukocyte function-association antigen 1

ICAM-1: Intercellular Adhesion Molecule 1

TREX1: Three prime repair exonuclease 1

FACS: Fluorescence-activated cell sorting

BlaM: β-lactamase

RIPK3 or RIP3: Receptor-interacting protein kinase 3

PAMPS: Pathogen-associated molecular patterns

DAMPS: Danger-associated molecular patterns

NLR: NOD-like receptor

PYHIN: Pyrin and HIN200

LRR: Leucine rich repeats

NOD: Nucleotide binding and oligomerization domain

CARD: Caspase activation and recruitment domian

ROS: Reactive oxygen species

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