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Blood CD4 T cells are resistant to cell death by

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By

Isa Arias

A dissertation submitted in partial satisfaction of the requirements

for the degree of Doctor of Philosophy in

Infectious Diseases and Immunology

in the

Graduate Division

of the University of California, Berkeley

Committee in charge:
Prof. Eva Harris, Chair
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Abstract
Blood CD4 T cells are resistant to cell death by pyroptosis during HIV infection
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Progression to AIDS is driven by HIV depletion of CD4 T cells. Most CD4 T cells residing in lymphoid tissues die from abortive HIV infection. During abortive infection, HIV fuses normally to quiescent cells but the elongation step of reverse transcription is very inefficient leading to cytoplasmic accumulation of short DNA transcripts. The host DNA sensor IFI16 detects these incomplete viral DNAs triggering both interferon-β expression and the assembly of inflammasomes containing caspase 1. Caspase 1 activation induces pyroptosis, a highly inflammatory form of programmed cell death. We studied whether this mechanism also promotes the death of quiescent, non-permissive CD4 T cells circulating in the blood. Here we report that blood CD4 T cells are highly resistant to pyroptosis at least in part because they lack expression of the DNA sensor, IFI16. However, blood CD4 T cells undergo HIV induced pyroptosis when co-cultured with lymphoid tissue–derived CD4 T, CD8 T, or B cells. The interactions between these co-cultured cells induce IFI16 expression likely by mimicking events occurring within lymphoid organs. These results highlight a fundamental biological difference between blood and lymphoid tissue–derived CD4 T cells and underscore AIDS as a disease of lymphatic tissue.

Dedication
This work is especially dedicated to my father, Wilfredo Arias, who died too soon in my life. Daddy I would give my life to be able to spend one more day with you.

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When I first applied to a PhD program there were two things I knew for certain: 1) this is something I had to do and 2) I didn't know if I could finish it. These two things have been constant all throughout my career as a graduate student. Unlike many graduates, I am going to tell you the truth, graduate school for me was a struggle and it required so many colleagues, friends, and family member for me to triumph in it at the end.
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Sometimes when you win, you lose, and sometimes when you lose, you win.

Dissertation Outline

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Chapter 1
Section 1: Discovery of a new human pandemic
Many vaccines and antibiotics have been developed to combat infectious diseases. For many years, the majority of people held the belief that infectious disease was an area of medicine that was a worry of the past. On June 5, 1981, the Center for Disease Control (CDC) released a report describing five medical cases with two common characteristics: all cases involved previously healthy, young homosexual men who were afflicted with multiple opportunistic diseases[1]. Opportunistic diseases involve pathogens that inhabit the body of the majority of healthy people, but cause disease only when they are able to abundantly replicate as a result of a compromised immune system. A compromised immune system is common among patients receiving transplants, cancer therapy, also patients with genetic defects, or are advanced in age. None of these conditions accounted for why these men had extremely compromised immune systems. These patients suffered from multiple opportunistic infections, such as Kaposi Sarcoma (cancer caused by a herpes virus), *Mycobacterium pneumoniae*, and *Pneumocystis pneumonia* (respiratory infections caused by bacteria or a fungus, respectively). These were three very diverse infections caused by very different pathogens, which made the cause of their condition more difficult to identify. Moreover, by the date of the report's publication, two out the five patients had already died[2]. Similar cases occurred for many years prior to this, but these cases were so rare that they went unnoticed. Metropolitan epicenters such as San Francisco, New York, London, and Paris were flooded with these mysterious medical cases of multiple opportunistic diseases that lead every single patient to their death, months after this report was released in 1981. Within a month of the report, the New York Times reported 41 men showing similar symptoms. By the end of the year, a total of 270 cases were described in the United States and 120 of those patients died [2,3].

Originally, this condition was named Gay Related Infectious Disease (GRIDS), since it was only seen in homosexual males in the first year[2]. Many alarmed U.S citizens suggested ousting the infected individuals from society, reasoning that not only were their behaviors "repugnant," but also dangerous to others. The first case to disprove that GRIDS was endemic only to the homosexual community involved an infant that received a contaminated transfusion in 1982. The name of the condition was then changed to Acquired Immunodeficiency Syndrome (AIDS). Despite the slow discovery of AIDS in other communities, the disease was used by many to further marginalize homosexuals and later injected drug users[2]. Therefore AIDS was complicated not only in terms of its biology but also from a social point of view.

In 1983, only two years after the first report, 3,000 people were diagnosed with AIDS[2]. The rise of new cases was so rapid and overwhelming that some hospitals overflowed with patients. It was clear to the medical and scientific community that the global human population was stumbling upon a new complicated and deadly epidemic. That same year, the pathogen responsible for so much turmoil, Human Immunodeficiency Virus (HIV), was isolated from patient tissue and proven to infect cells from the immune system[4-7]. The scientific community then gained a false sense of optimism that identifying HIV would easily lead to the development of a vaccine. In 1985, scientists developed a diagnostic test that probed for the virus' core protein. This test allowed scientists to diagnose asymptomatic patients who had unknowingly carried the virus for up to 10 years[2]. Surprisingly, this timeline meant that many patients who were diagnosed in 1981 might have been infected as early as 1971.

Section 2. The HIV Pandemic Today

Over the last 32 years, an estimated 70 million people have contracted HIV and 32 million have died from AIDS[8]. 2011 alone, 2 million new infections were reported and 1.7 million people died of AIDS, including 230,000 children. Scientists estimate that 0.8% of the global human population (average age 15–49) is infected with HIV[2,8]. Sub-Saharan Africa represents 69% of the world infections[8], where poverty, limited education, and very little access to anti-retroviral drugs or HIV testing contribute to the spread of this disease. In fact, only 8 million people from low-income countries have access to anti-retroviral drugs [9] (Figure 1).

Despite these morbid numbers, HIV is not the biggest plague mankind is currently confronting. The World Health Organization (WHO) recently set goals to combat "the big three": HIV, Malaria, [1]and Tuberculosis (TB). Of these diseases, HIV does not compare to the morbidity and mortality of the other two[8]. In 2012, an estimated 207 million cases of malaria and 627,000 malaria related deaths, 482,000 of which were children under the age of 5 and living in Sub-Saharan Africa [10]. Scientists estimate that Malaria kills 1 child every minute [10]. Tuberculosis, on the other hand, is closely related to HIV because HIV infected patients are a lot more susceptible to TB. It has been estimated that one-third of the world population is infected with tuberculosis. In 2012, there were 8.6 million new cases of TB infections, 1.1 million of which were HIV positive patients [11]. That year, there were also 1.3 million TB related deaths, 430,000 of which were also related to HIV [11].

A number of challenges contribute to the persistence of the HIV pandemic: a great percentage of people are unaware they are infected, limited access to therapy in areas with the highest number of cases insures continued spread of infection, and adherence to therapy. In America alone, 60% of young infected people (age 13–24 who make up 7% of people living with HIV in the United States) are unaware that they are infected[12]. This is an astounding statistic, since United States citizens have great access to medical care and HIV/AIDS education and testing in comparison with many third world countries. Globally, only 50% of the 34 million infected people are not aware of their HIV status [9]. If infected people unknowingly continue to spread HIV by having unprotected sex then the end to this pandemic will be impossible, at least not without an aggressive program to vaccinate every uninfected person, such as the controversial implementation of the small pox vaccine, never mind that the scientific community is no where near an HIV vaccine.

According to the 2012 UNAIDS global report, only 8 out of 14.8 million eligible patients (determined by low CD4 counts) receive therapy[9]. This represents a global challenge because access to therapy decreases viral loads to not only prolong a patient's life but also to decrease the likelihood of transmission. The main reason for limited access to therapy is the cost, as generation, transportation, implementation, analysis, and maintenance of adherence to treatment is expensive for infected patients, low-income countries, and medical facilities. Also, pharmaceutical companies are challenged to find a balance between their need for profit and their costs of producing and testing these antiretroviral drugs with the moral imperative to reduce this pandemic.

Poverty not only reduces access to therapy, but also contributes to new infections. In the general female adult population, approximately 15% of HIV is attributable to (unsafe) female sex work. While the highest fraction of this subpopulation is Sub-Saharan Africa, the burden is also substantial for the Caribbean, Latin America, and South and Southeast Asia. Other challenges include increased resistance to antiretroviral drugs and patient adherence to therapy. At the cellular/viral level, scientists question how we can develop a cure for a pathogen that is integrated into our genome. Originally, researchers estimated that 1 in 1 million infected CD4 T cells are latent, unrecognized by the immune system, and are not targeted by any HIV drugs [13]. More recently, a study suggested that this reservoir of latent cells is 50 times larger than previously thought [13]. These questions underscore that the HIV/AIDS pandemic is a combination of social, economic, and scientific challenges that still requires many efforts to find the best ways to combat the disease.
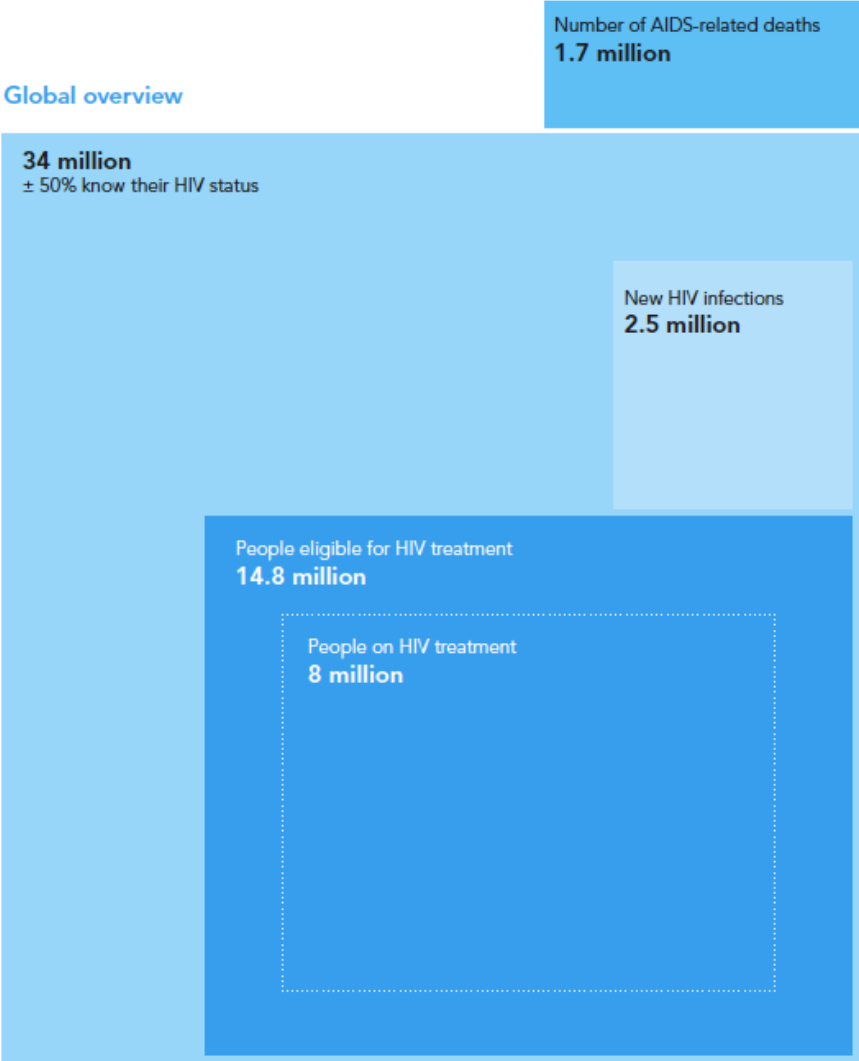
Figure 1. UNAIDS 2011 estimates on the HIV pandemic
In 2011, there were an estimated 34 million global HIV infected cases. Out of 34 million only 50% are aware of that they are infected. In that year alone there were 2.5 million new infection and 1.7 million AIDS related deaths. Based on their CD4 T cell counts, 14.8 million infected people are eligible for HIV treatment but only 8 million are receiving treatment.

Section 3. Stages of infection

Primary or Acute stage of infection is the period between transmission and production of HIV targeting antibodies (Figure 2). The time required for patients to produce antibodies against the virus varies widely. In fact, the variety in the length of time needed to produce antibodies presented a profound problem for the first HIV diagnostic test, an enzyme linked immunoassay (ELISA) which detected patients' anti-HIV core protein antibodies. Initiation of anti-HIV antibody production can take three months. The primary stage of infection is also characterized by viral load (measured by viral RNA in plasma) and CD4 T cell count. Viral RNA can be detected in the plasma at all stages of infection. When plasma viral RNA peaks at $1x10^6$ copies/ml, flu-like symptoms occur, sometimes accompanied by opportunistic infections. This correlation is a direct consequence of the virus killing the same cells that orchestrate the body's immune response to the opportunistic pathogens, the CD4 T cells.

A CD4 T cell count of ≤500 cells/ml, and at least flu-like symptoms. Healthy individuals tend to carry an average of 1000 CD4 T cells/ml, implying that HIV patients in the acute phase only have half of their CD4 T cells left to protect them. At the end of the acute phase and beginning of the chronic stage of infection, a sharp drop in viral load as well as in antibody production, and an increase in the CD4 T cell count occurs. The extent of the increase in CD4 T cells varies between patients and has become a strong predictor of the length of time until the patient will reach the AIDS stage of infection. Very few patients' CD4 counts recover up to 1000 CD4 T cells/ml levels after the acute phase.

The chronic stage of infection was originally thought to be a long period in which the virus that had managed to integrate into CD4 T cells without killing them, would actively signal them to stop producing virus, a latent infection. This type of signal is common among many viruses that chronically infect, including the Herpes virus family. With infections such as with herpes viruses the virus actively stops the infected cells from producing new virions, but such is not the case with HIV. Instead, latency in HIV is a direct result of the activation status of the cell, of how much protein the cell is producing at the time of infection and if it is infected during or close to cell division. When described as such then some cells, including neuronal tissue resident macrophages, can be latently infected by HIV.

The lower the activation state of the cell, the less transcription and protein production that will occur, which presents an obstacle for producing viral proteins and assembling new virions. At the same time, latency has become the primary barrier to the discovery of an HIV cure. Currently, there is no way to distinguish an uninfected cell from a latently infected cell. If there is no mode of differentiation, then how can latently infected cells be targeted? Therefore, current therapy is only able to target productively infected cells. As long as there are latently infected cells in patients' bodies, a recurrent peak in viral load only requires the activation of the latently infected cell by a pathogen, or a tissue injury. Some investigators attempted to make latent cells susceptible to antiretroviral drug therapy by activating as many cells as possible, but these attempts were either too toxic or were unable to sufficiently activate all latently infected cells to make them therapeutic targets. Ironically, activating CD4 T cells is essential for combating most infectious diseases, but in the case of HIV, the very activity that could lead to annihilation of the virus actually leads to the demise of the cavalry.

The hypothesis that originally labeled the chronic stage as latency was dismissed when researchers observed that virus was produced during all stages of infection, even when patients were put on antiretroviral therapy. In fact, the viral load increases while the CD4 T cell count decreases at compatible speeds. This stage can continue for as long as 10–12 years, and with antiretroviral therapy, as long as 30 years. The chronic stage is now more accurately described as "clinical latency," since patients do not display symptoms of infection.

The AIDS stage of infection is defined as the period when patients' CD4 T-cell counts decrease to less than 200 cells/mL. Correspondingly, the viral load can return to as high as 1×10^6 copies/ml.
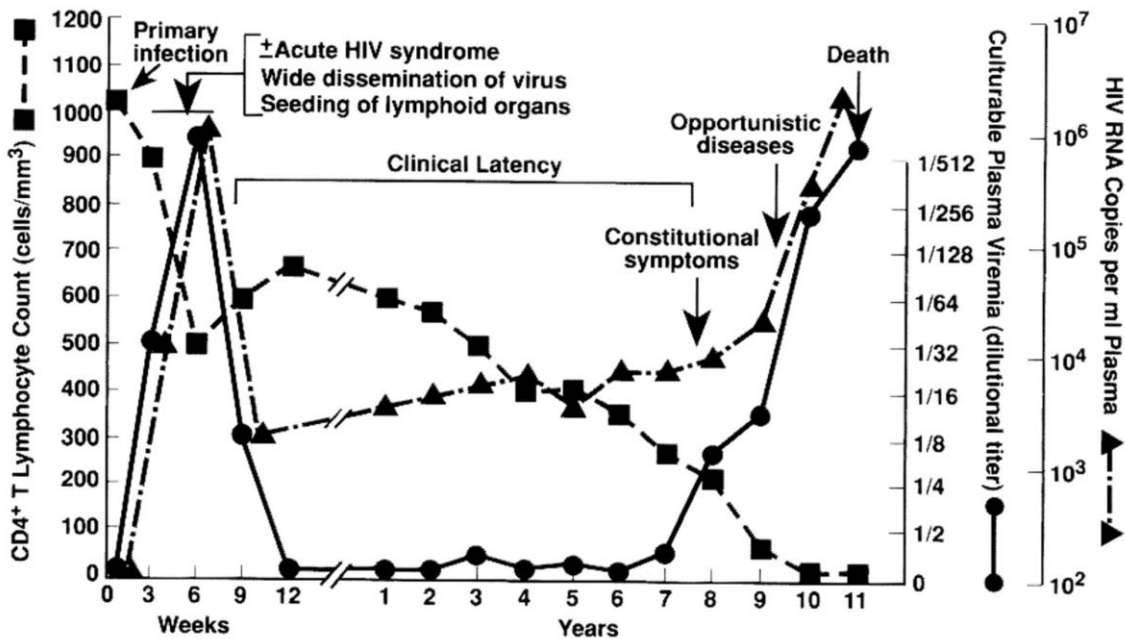
Figure 2. Stages of HIV Infection
The acute phase of infection is marked by an RNA viral load of 1×10^6 copies/mL, and a CD4 T cell count of ≤500 CD4 T cells/ml. The chronic phase of infection is characterized by a slowly increasing RNA viral load and a slowly decreasing CD4 T cell count. At the AIDS stage patients carry ≤200 CD4 T cells/ml, viral load peaks again at 1x10^6 copies/ml or more.

Section 4. The Origin of HIV: Cross Species Transmission

Two of the most important questions that arose with the 1981 HIV cases were: Why did this disease appear now? And where did HIV come from? The first question was easier for researchers to answer. Researchers speculate that HIV has lurked in the human population for a long time, as early as the beginning of the 20th century, but that the disease spread as international travel increased and the sexual revolution of the 1970s began in the United States.

The second question, however, was more difficult for researchers to answer. Up to forty different African non-human primate species are natural hosts to simian immunodeficiency viruses (SIVs) [14]. Most non-human primate species are only infected by a single SIV strain, implicating limited interaction between different simian species (except when they hunt each other), and a long lasting co-evolution between the natural host species and the viruses. In the case of non-natural hosts, these infections lead to the same consequences (CD4 T cell depletion, opportunistic infections, AIDS, death, etc.) as HIV does in humans.

Despite the rarity, recombinations and mosaic SIVs do exist. One of these mosaic SIVs was eventually transmitted to chimpanzees of the subspecies *Pan troglodyte troglodyte* (PTT), as a result, the name of the virus is commonly abbreviated $SIVcpzPTT$[14] (Figure 3B and C). This virus consisted of an envelope protein from the SIV that naturally infected red-capped-monkeys (SIV_{rcm}), a vpu protein from the SIV that naturally infected the greater spot-nosed monkey (SIV_{gsn}), and several viral proteins from other SIVs[14]. Chimpanzees are known to prey some of the primates they coexist with in Africa and may contract these viruses during hunting. It is theorized that SIV_{cpzPTT} acquired its proteins from many different SIVs by being repeatedly transmitted from one species to the next during these hunting activities.

The first suggestion as to the origin of HIV-1 was the discovery of HIV-2 and its genetic similarity to an SIV that naturally infected sooty mangabey primates (SIV_{sm})[14] (Figure 3C). These findings lead researchers to speculate that HIV-1 was also a descendant of another SIV. The second suggestion was when an Asian macaque primate was accidentally infected with an SIV (SIV_{mac}) via blood transfusions from sooty mangabey in a laboratory[14]. Because the Asian macaque was not the natural host to SIV_{mac}, it displayed symptoms of AIDS upon infection. These observations lead researchers to speculate that perhaps HIV-1 was a descendant of an SIV and because humans did not serve as the natural host, they would succumb to AIDS. Both of these suggestions were confirmed with the findings that SIVptt was genetically related to HIV-1. Therefore while non-human primates acquired SIVs by hunting each other, in turn, at the beginning of the 20th century, from hunting chimpanzees, humans have contracted two (M and N) out of the four (O and P) groups of HIV-1. SIV_{cpzPTT} is not only similar to HIV-1 groups M and N in genetics, but also in the mode of transmission (e.g., sexually, mucosal, perinatal), where the probability of sexual transmission ranges from 0.0008 to 0.0015%, the mechanism of pathogenesis and degree of lethality [14].

More recently, chimpanzees of the *P.T. troglodyte* subspecies also cross-transmitted SIV to gorillas (SIV_{gor}), and HIV-1 group P was cross-transmitted from gorillas to humans [15][14]. Another chimpanzee subspecies *Pan troglodyte schwefurthii* (PTS) has been infected with SIV, but no cross-transmissions to other primates, including humans, have been identified.

There have been a total of twelve (HIV1 groups M, N, O, P, and HIV-2 groups A-H)

independent cross-transmissions of HIV from other primates to humans. From those cross species transmissions, HIV-1 M was the only one to become a pandemic. HIV-1 N, O, P and HIV-2 are endemic to West Africa. HIV-1 M diversified into nine subtypes (A, B, C, D, F, G, H, J, and K) which can differ in envelope sequence by as much as 35% and in Gag sequence by 13%[16][17][16][14]. Of these subtypes, C accounts for 50% of the pandemic, and together subtypes F, H, J, and K, accounts for 1%.

Figure 3A

Greater spotted nose monkey

Red capped monkey

Chimpanzee Subspecies: *Pan troglodyte troglodyte*

Figure 3B

Greater spotted nose monkey
SIV_{gsn}
HIV Vpu

Mosaic virus formed
in chimpanzee:
SIVgsn+SIVrcm

Red capped monkey
SIV_{rcm}
HIV Env

(SIVcpz)

Chimpanzee

SIV_{GAB1} → HUMAN
SIV_{US} → HUMAN
SIV_{CAM3} → HUMAN

Figure 3C.

Sooty Mangabey

SIV$_{sm}$ A → Humans HIV-2 A

SIV$_{sm}$ B → Humans HIV-2 B
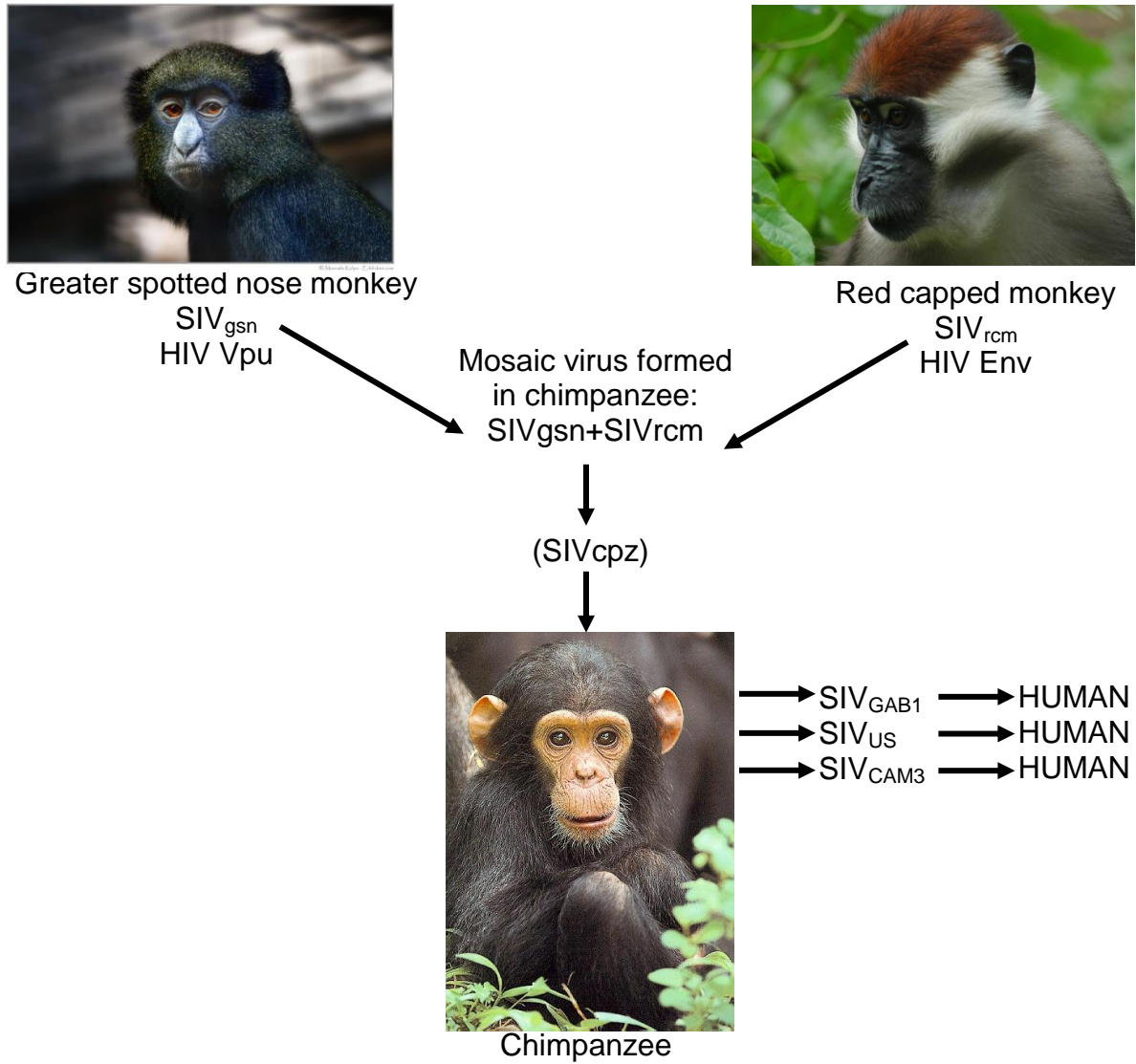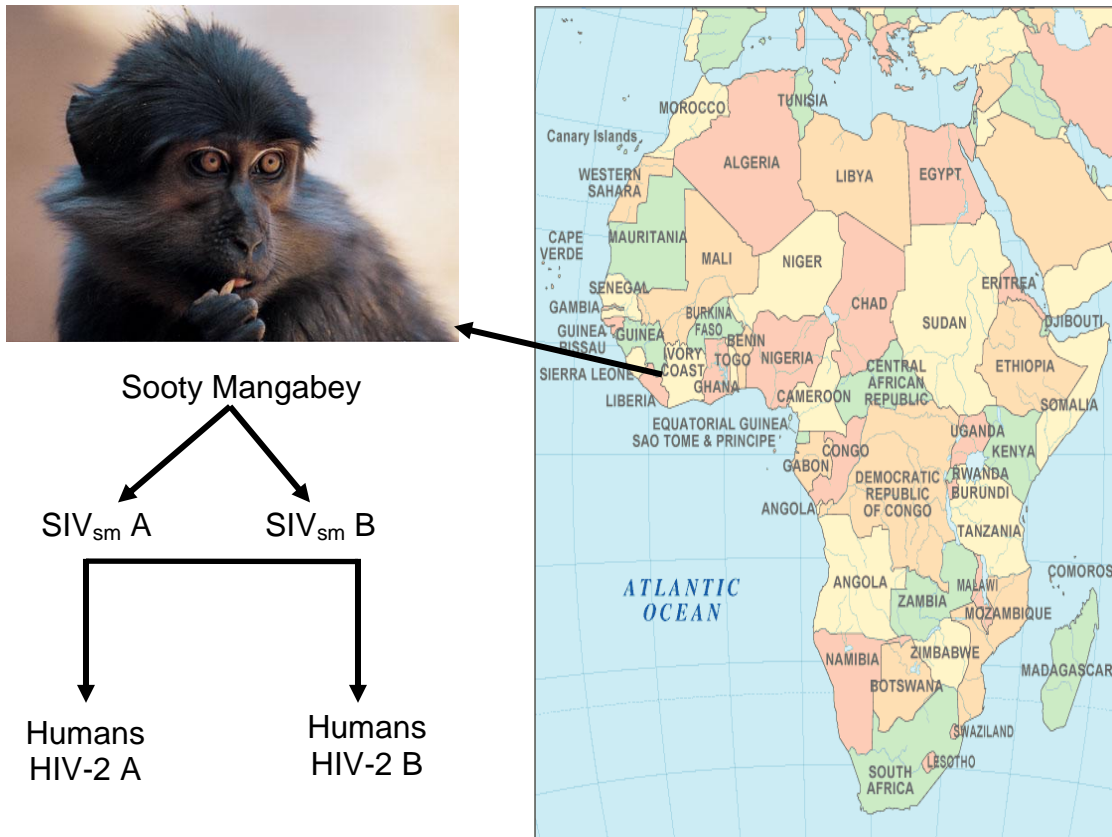
Figure 3. Origin of HIV-1 and HIV-2 A. Chimpanzees share territory in West Africa specifically the country of Gabon with the red capped monkey and the greater spotted monkey. These monkeys are known to interact/hunt each other. It is hypothesized that several hunting events resulted in a co-infection of a chimpanzee. **B.** A mosaic virus (SIV$_{cpz}$) arose from a co-infection of a chimpanzee with the SIV from a red capped monkey (SIV$_{rcm}$) and another SIV strain (SIV$_{gsm}$) from the greater spotted monkey. The SIVrcm provided the gene for Vpu that HIV-1 carries, and the SIVrcm provided the HIV-1 Env. At least three separate cross-species transmissions from chimpanzees to humans have occurred, resulting in the three different HIV-1 groups: M, N, and O. HIV-1 M is pandemic, while cases involving HIV-1 N and O infections can be found in West Africa. **C.** Eight separate cross transmissions occurred from Sooty mangabey monkeys to humans: HIV-2 groups A-H. Only A and B are epidemically spread. Group A can be found in West Africa, Brazil, India and Angola. Group B is limited to West Africa.

Section 5. Transmission and risk factors

All HIV strains are capable of being transmitted amongst humans by sexual contact, injectable drug use through ruptured mucosal surfaces, and from mother to child (vertical transmission) during birth or via breastmilk[18]. Heterosexual transmission accounts for 70% of global infections, while injectible drug use and vertical transmission account for the remaining[18] 30%. The range of susceptibility to each type of transmission differs between strains. For example, the recombinant Group M subtype AE in Thailand is highly transmissible by injectable drug use[18]. Despite the variability in their preferred mechanism of transmission, Group M viruses are consistently transmitted by sexual contact, indicating that this capability was strongly selected for. Risk factors that contribute to sexual transmission include viral load in an infected partner, the stage of disease, genital inflammation/ulcer disease, pregnancy, male circumcision, and route of transmission. Most of these risk factors are self-explanatory, such that a greater viral load leads to higher viral transmission, giving more virions the chance of overcoming barriers to set off the infection. Out of the three stages of infection, the acute/primary stage holds the highest risk of transmission. During this stage, the infection in the transmitting partner is new, so the surviving virus has been recently re-selected for its ability to overcome the barriers to transmission. Additionally, patients are withstanding the highest viral load they will ever have[18,19]. Genital inflammation leads to the recruitment and possible activation of more CD4 T cells, which are the most susceptible cells to productive HIV infection. Under healthy circumstances, CD4 T cells can be found in the layer under the mucosa of the genital tract but not in the lumen. Genital ulcers cause breaks in the mucosa creating a direct gateway for the virus to reach CD4 T cells[18,20]. In the majority of cases, HIV infection is established by one genetic variant, one virion[18,20], but when the at-risk partner has genital inflammation, this bottleneck is overwhelmed and instead, multiple variants establish infection[20]. These results infer that the mucosa represents a formidable barrier to transmission. The establishment of a multivariate infection could possibly lead to a quicker advancement in disease or quicker development of resistance to anti-retroviral drugs. Although certain strains of HIV vary in their capability to infect via certain routes, there is a higher chance of infection with anal-penile exposure with all strains (one in three exposures leads to infection) versus vaginal-penile (one in ten exposures)[18]. This difference is a result of the vaginal mucosa consisting of multiple layers that act as barriers between the virus and the layer containing the CD4 T cells. The anal mucosa only consists of one layer of columnar cells therefore is a lot more susceptible to trauma[18]. Pregnancy dampens inflammatory responses because they can induce termination. Inflammatory responses are the most formidable immune responses to viruses therefore, pregnant women are even more susceptible to HIV infection.

Multiple studies have indicated that in 80–90% of sexual transmission cases, where the at-risk patient does not have an inflammatory genital infection, a bottle neck occurs in which only one virus is responsible for setting off the infection[18,20]. The selection of this one virion is not random, viral characteristics favored by transmission include: the use of CCR5 as a entry co-receptor, shorter and less glycosylated V1-V4 envelope regions (subtypes A, B, C, and D), virions with higher envelope incorporation, and resistance to antibody neutralization from the previously infected partner.

HIVs use the protein CD4 as an entry receptor and either the protein CCR5 or CXCR4 as a co-

receptor. Viruses that use CCR5, and CXCR4 as co-receptors are referred to as R5 and X4 tropic, respectably. There is almost a 100% protection against the establishment of a new infection by a CXCR4 tropic virion, but the factors that contribute to this protection remain poorly understood. Up to 50% of patients infected with HIV-1 subtype C convert to CXCR4 tropic infection during the chronic stage, therefore at least in these patients there is sufficient CXCR4 tropic virions to transmit but even these patients sexually transmit CCR5 tropic viruses only. The purpose of such a strong selection for the use of CCR5 as a co-receptor has not been clarified. Only activated or memory CD4 T cells, express CCR5 while CXCR4 is expressed by all CD4 T cells, therefore, it would make sense that the virus would prefer CXCR4 so that it could infect more targets.

One possible contributor is that the X4 tropic virions have envelope protein (gp120) with the third variable (V3) loop more exposed and therefore more susceptible to neutralization. There is also evidence that post mucosal barrier factors contribute to the selection of CCR5 tropic virus, since this tropism is selected even in infections that circumvent those barriers, such as intravenous infection[19]. Originally, researchers hypothesized that virions would attach to or infect Langerhans dendritic cells, or monocytes/macrophages in the mucosa and that these cells would transfer and/or trans-infect CD4 T cells in the submucosa. The dendrites of langerhans can reach to the surface of the mucosa and bind virus. Thus, researchers hypothesized that these dendrites are used to transport virus to CD4 T cells that reside in the submucosa tissue. More recent studies with subtype A, B, or C indicate that the founder viruses are selected for their capability to fuse CD4 T cells and not monocytes/macrophages. Because CD4 T cells are rare on the mucosal layer, these studies imply that a rupture in this layer may be required for the founder virus to establish an infection[18,20].

Glycosylated V1-V4 loops in the viral envelope lead to more efficient interactions with the entry receptors[18]. Higher envelope incorporations means that the virions will carry more envelope (gp120) protein on them thereby increasing the chances of the virus finding a target cell[18].

Section 6. Characteristics of Retroviruses
Not long after the discovery of HIV as the pathogen causing AIDS, the virus was categorized as a newly discovered member of the retrovirus family from the genus lentivirus. What distinguishes retroviruses from other viral families is that they integrate a DNA copy of their RNA genome into their host genome[16]. This DNA copy is synthesized in the host cell cytoplasm by a viral RNA dependent DNA polymerase reverse transcriptase. These viruses also exit the host cell by budding from the plasma membrane as non-infectious viral particles that require enzyme processing (or maturation) in order to become infectious. Viruses outside this family often carry one or several of those characteristics but not all. For example, Hepatitis B virus carries a functional reverse transcriptase, but it does not integrate its genome into the host, and it codes a DNA genome instead of an RNA genome. All retroviruses carry at least three genes coding for the group specific antigen gene (gag), envelope (env), and polymerase (pol). The simple retroviruses (e.g., genera: alpha, beta, gamma) carry the minimum three genes while the complex retroviruses (e.g., genera: delta, episilon, lenti, spuma) carry those genes along with some accessory genes that regulate certain steps of the life cycle and/or help the virus evade immune detection[16,21]. The genera of the retrovirus family are distinguished by several characteristics including the shape of the core. All of the members of the lentivirus genus are

capable of inserting their DNA copy of the RNA genome into the cell nucleus without cell division or dissolution of the nuclear membrane. Also all members of this genus tend to have a slow life cycle, with the exception of HIV, hence the name lenti.

Chapter 2. VIRAL ARCHITECTURE

The virus consists of an RNA genome, five structural and eight nonstructural proteins, six of which are accessory proteins, and a lipid by-layer high-jacked from the cell producing the virus. These viral building blocks have been studied for the past 30 years, and with every technological innovation, new methods arise to further characterize them. Unfortunately, this means that the extent of our knowledge about the virus is limited by how much technology advances. This makes studying HIV challenging, as researchers are constantly revisiting their findings in an effort to uncover new milestone characteristics about the virus.

Section 1. RNA Genome

The viral genes are coded by two copies of single positive-stranded RNA (7–13 kb) [16]. The copies form a dimer through complementary sequences at their 5' ends, called the dimer linkage structure. Each copy is generated by the host cell via transcription and resembles a host mRNA: it has a cap at the 5' end (7G5'ppp5'Gup) and a poly A tail (200 nucleotides long) at the 3' end. Besides coding the three main open reading frames (genes): gag, pol, and env, the genome also contains regulatory sequences for different steps of the viral life cycle. For example, the genome has a repeated region (R region) at the 5' end just after the cap and also at the 3' end just before the poly A tail. The unique 5 (U5) region contains the ATT sites that are essential for the virus' ability to integrate into the host genome. The primer binding site (PBS) contains 18 nucleotides that bind the lysine tRNA which serves as a primer for (-) strand synthesis. The Psi sequence is required for encapsidation of the genome into the newly produced virion. The polypyrimidine tract site (PPT), coded by a series of nine adenine and guanine residues, serves as the sequence where (+) strand initiation occurs. The untranslated 3' region (U3) is a cis-acting element rich region for viral expression.

Table 2.1.1 Regulatory elements of the viral genome

Regulatory elements coded by the HIV RNA genome	Function
5' Cap	Transcription
R region	
U5 region	Integration
Primer Binding site (PBS)	Binds the lysine tRNA
Psi sequence	Encapsidation
PPT	Reverse transcription (+) strand synthesis
U3	Viral expression
Poly A tail	Stability of mRNA

Section 2. Nucleocapsid Protein

The most closely associated protein to the viral genome is the nucleocapsid protein (NC, 7kDA) [16]. The NC is a product of the viral Gag polyprotein that interacts directly with and stabilized[16] the genome. Approximately, 5000 copies of nuclear capsid are packaged in the virion and bound to viral RNA. Each nucleocapsid molecule binds to six RNA nucleotides. The only specificity that has been characterized has been the affinity for alternating guanine and thymidines and also for the *Psi* sequence found on the non-coding region of the viral RNA. NC has a number of functions, including: stimulating dimerization of viral genome in the virion, duplex formation between the tRNA the viral RNA during viral assembly, facilitating reverse transcription by easing the movement of the polymerase on the RNA genome, stabilizing the viral DNA product

of reverse transcription, protecting viral DNA from degradation, and evading APOBEC 3G and 3F induced hypermutation of the reverse transcribed DNA.

Section 3. The Viral Capsid Protein
The inner most protein layer swaddling the RNA genome is the capsid (CA), also known as the viral core, or p24 (24 kDA). The capsid is also made from the viral poly-protein Gag. The virion carries approximately 5000 copies of capsid. The core is made up of 1300 monomers of p24 arranged into 12 pentagons and 216 hexagons[22]. After cleavage by the viral protease (a process known as maturation), the capsid's shape changes from spherical to conical. The conical shape is one of the viral characteristics used in earlier studies to determine the genus to which the virus belongs to[16]. Recent innovations in crystallography and simulation revealed how these identical monomers of capsid protein fit together to form the stable shape of the conical capsid[22]. Specifically, the simulation showed that although these monomers are made of the exact same proteins, they attach to each other at different angles to form the proper shape of the core. The 12 pentagons attach to the 216 hexagons at more acute angles than the hexagons attach to each other[22].

Section 4. Matrix Protein
The next layer encompassing the core is matrix protein (MA, 17 kDA), which is also made from the Gag poly-protein. MA not only associates with the core, but also with the inner face of the virion's lipid membrane. It is transported to the cell membrane because of its myrostilation. It forms trimer sheets with openings through which the cytoplasmic tail of the viral envelope protein fits. During assembly, the matrix domain interacts with the cytoplasmic tail of gp41 so that envelope protein can polarize[23]. The polarization of the envelope protein ensures sufficient levels of envelope incorporation into the virion so that the virus can fuse[24].

Section 5. Envelope Protein
The viral envelope protein gp160 is translated in the ribosomes in the endoplasmic reticulum (ER) [23]. Once translated, gp160 is glycosylated and oligemerized into trimers in the ER[23] and then transported to the golgi network. In the trans-golgi network, gp160 is partially cleaved by the cellular protein furin, into gp120 and gp41 which remain associated with each other[23]. The two envelope proteins then are transported to the cell membrane. Gp41 becomes anchor to the cell membrane for gp120 in the form of a type one membrane protein, with only one transmembrane domain, where the N-terminus faces the extra-cellular space, and the C-terminus (more than 130 amino acids) faces the cytoplasm[23].
The N-terminus of gp41, immediately proximal to the membrane spanning domain, codes a highly conserved peptide motif (LWYIK) essential for binding cholesterol on the target cells [25]. Depletion of cholesterol from target cells by β-cyclodextrin treatment results in impaired infection, implying that the binding of cholesterol by gp41 is essential for viral entry[25]. This activity is hypothesized promote gp41 aggregation and thereby enhance viral-cell membrane fusion[25]. The cytoplasmic tail of gp41 is multifunctional, and essential for viral replication[23]. An additional not yet characterized function (not involving fusion, assembly or structural stability) is essential for replication in macrophages. The cytoplasmic tail of gp41 interacts with MA to stabilize the envelope protein's location on the plasma membrane so that it can incorporate into the virus. This interaction between MA and the gp41 cytoplasmic tail also ensures that the envelope protein can polarize and assemble with the other viral proteins that form a virion thereby increasing envelope incorporation into the virion[24]. Gp120 is completely extra-cellular

only anchored down to the plasma membrane by its interaction with gp41. Its only responsibility is to bind the cellular entry receptor CD4 and co-receptors CCR5 or CXCR4 (discussed in more detail in viral entry section).

Section 6. Reverse Transcriptase (65 and 55 kDA)
HIV carries their genome in the form of single stranded RNA (ssRNA). Two copies of ssRNA are stabilized together by dimerization initiation sequence in the 5' end long terminal repeat (LTR). To make its genome suitable for integration into the host cell, the virus had to acquire an enzyme that could convert the ssRNA into double stranded DNA (dsDNA). The enzyme acquired was reverse transcriptase, a heterodimer (p51 and p65) polymerase capable of producing DNA from RNA. The p65 domain consists of regions known as palm, fingers, thumb, and the RNAse H. The palm has an activation site that consists of three aspartic residues (Asp-110, Asp-185, and Asp-186) and two Mg^{2+} ions, which are essential for reversing transcription. RNAse H degrades polymerized RNA[16]. Although the p51 domain consist of exactly the same properties as the p65 domain its aspartic residues are not exposed enough for the domain to be able to polymerize[16]. Instead, the p51 domain serves a structural role[16].

Reverse transcriptase is a very dynamic enzyme that can produce a DNA strand with either an RNA or DNA template, degrade unnecessary RNA, and, if it encounters a break in the RNA template, can jump from that template to the other ssRNA copy carried by the virion. Abbondanzieri et. al. showed that the polymerase determines what activity will proceed depending on the primer-template that is bound. If binding a RNA-DNA primer-template, then the polymerase orients the RNAse-H domain to the 3' end of the primer and its polymerase domain to the 5'end of the primer. If binding a DNA-DNA primer-template set, then it reverses the positions of the domains. In this way, the enzyme ensures that the RNAse H domain is always closest to the RNA strand that has already been polymerized and is not useful to the reaction anymore. Studies have modeled this interaction to show that the fingers tighten to trap the template and nucleotides so that the reaction to add the nucleotide to the change can occur. Once the nucleotide is added, the fingers relax, giving the chain enough to move forward and bring in the next nucleotide to add and polymerize[16].

One of the biggest challenges for HIV eradication/therapy is the immense variability of the viruses. This variability is a partial product of the high error rate of the polymerase. Until 1988, HIV reverse transcriptase were the most error prone polymerase found [26]. These observations were obtained by *in vitro* studies and therefore host factors may still enhance the efficiency of the enzyme. More errors were observed with HIV reverse transcriptase than with other retroviruses, such as Mouse Leukemia Virus (MLV), implying that there may be host factors necessary for efficient HIV's but not other viruses' reverse transcription[26]. Certain areas of the template also tend to be more susceptible to errors than others[26]. Another contributor to viral variability is recombination. During reverse transcription, the polymerase can only catalyze the production of DNA from one template at a time; however, if the template has a break or unfavored mutation, the polymerase can jump to the other RNA template and continue polymerizing the same DNA strand thereby producing one dsDNA template with sequences from both strands. Recombination has become a significant problem for the eradication of the pandemic as certain parts of the world frequently infected with recombinants.

Section 7. Integrase
The second most unique retrovirus trait is their ability to integrate into the genome of the host cell. Some DNA viruses, such as Kaposi Sarcoma Herpes virus (KSHV), were shown to integrate fragments of their DNA, but not their entire genone, into the host. The partial integration of herpes viruses are a consequence of host, not viral, enzymes[16,27,28]. Also, these integrated fragments are not essential for viral replication. HIV mutants lacking the ability to integrate were reported to productively infect cells, but only at very low levels that are not sufficient to spread infection[16]. Although the virus may lack the proper machinery to integrate the genome under these circumstances, some cells can still independently integrate the provirus by the same mechanism used to integrate KSHV DNA fragments. The only other known family of viruses known to integrate their whole genome into the host are endogenous retroviruses (ERV), the family of viruses from which retroviruses are theorized to have originated. ERV, like retroviruses, require the integration of their genome in the host to replicate. ERVs are not pathogenic to humans, which may be a result of them integrating into human genomes for millions of years, co-evolving, and sweeping away events that would harm the host to extend the life of the virus.

The integration of the viral genome into the host only requires one viral enzyme, integrase (32 kDA). Approximately 250 copies of integrase are carried in the capsid of the virion. Integrase contains three domains: an N-terminal region, a catalytic central core, and a C-terminal region. Because these three domains are flexible, scientists do not know the exact conformation of the domains. All crystallography studies of the core have generated different conformations, so we do not know which positions are essential for the enzyme's activity. The N-terminal domain contains a conserved pair of His and Cys residues (HHCC) that create a zinc finger binding motif[16,27,28]. Zinc binding to integrase induces multimerization of the integrase, a step that's essential for integration. Both of the terminal domains are involved in DNA binding[28], although the function of the C-terminal domain, other than non-specific DNA binding, remains elusive. The most critical domain for integration is the central catalytic core, specifically the acidic residues in the D,D-35E motif. This motif is a conserved set of acidic residues (Asp64, Asp116, and Glu152) in other retroviral integrases, retrotransposases, and DNA transposons of both prokaryotes and eukaryotes. A point mutation of any of these three residues is sufficient to stop the virus from integrating. Researchers speculate that after binding DNA, conformational changes bring these three residues close together to bind divalent metal ions and catalyze the integration of the substrate DNA. The catalytic core domain is essential for both preparing the DNA for integration and for recognizing the site onto which the viral DNA will be integrated into.

Integrase, like many of the viral proteins, has pleitropic effects, such that it is involved in orchestrating viral assembly, morphology, the initiation of reverse transcription, nuclear import of cDNA, and integration. These effects are presumably influenced by the protein's ability to interact with NC, reverse transcriptase, the reverse transcription primer tRNALys, and/or the viral RNA. Mutation of the HHCC motif at the N-terminus renders the virus 20–10-fold less efficient at producing strong stop DNA. More recent studies have also implicated the C-terminal domain (specifically residues 271–273) in initiating reverse transcription. Integrase contains a total of six Cys residues, two in the N-terminal HHCC motif, three in the core domain (56, 65, and 130), and one (280) in the C-terminus. Mutating the N-terminal Cys residues inhibited both reverse

transcription and integration while mutating the C-terminal Cys residue, as well as Cys 56 and 65 in the core, did not affect viral replication[29]. Mutating Cys130 prevented interactions between integrase and reverse transcriptase and, therefore, blocked reverse transcription[29].

Section 8. Protease
The main function of the viral protease (11 kDA) is to cleave viral polyproteins so that virions exiting productively infected cells can mature. Maturation is a step of the viral life cycle in which proteins carried by the virion transition from being optimal for budding to being optimal for cell entry. Protease is activated only after the virion has budded away from the cell and homodimerized[30]. Because of its antiretroviral drug target potential, protease is one of the most studied enzymes in HIV biology; however we have much more to learn about this enzyme[30,31]. For example, while protease auto-cleaves from the Gag poly-protein, we do not yet know what triggers auto-proteolysis[31].

Once activated, protease recognizes five sites within Gag and another five within the rest of the gag (Gag-Pol-Pro) polyprotein (Figure 4). Protease recognized a variety of sequences, suggesting that protease activity depends on structure and not sequence[30,31]. This implication was confirmed in experiments that introduced protease to substrates with different sequences[31]. Researchers now hypothesize that each structure has a different affinity for the enzyme's binding pocket that ensures the domains are cleaved in a specific order: NC is cleaved from CA, MA is cleaved from CA, p6 is cleaved from NC, and then the two spacer peptides surrounding NC are cleaved away[31].

Cleavage of the Gag polyprotein results in condensation and stabilizes of the viral genome, conformational changes in the capsid needed for proper packaging of the genome, and prepares the virion for cell entry, uncoating, and reverse transcription[24,30]. During assembly, the matrix domain interacts with the cytoplasmic tail of gp41 so that envelope protein can polarize and assemble with the other viral proteins that form a virion. Cleavage of the gp41 tail decreases envelope incorporation[24]. In immature virions, the C-terminus of gp41 that resides inside the virion stays bound to the matrix domain of the Gag polyprotein. Introduction of mutations that allowed the cleavage of all the other sites (sites number 2, 3, and 4) but not between CA and NC (#1 in Figure 4) did not allow proper maturation or viral fusion. Further research is needed to characterize if fusion is limited specifically because of a lack of conformational changes in the Env and/or NC protein, or if the viral genome's interaction with NC inhibits fusion.
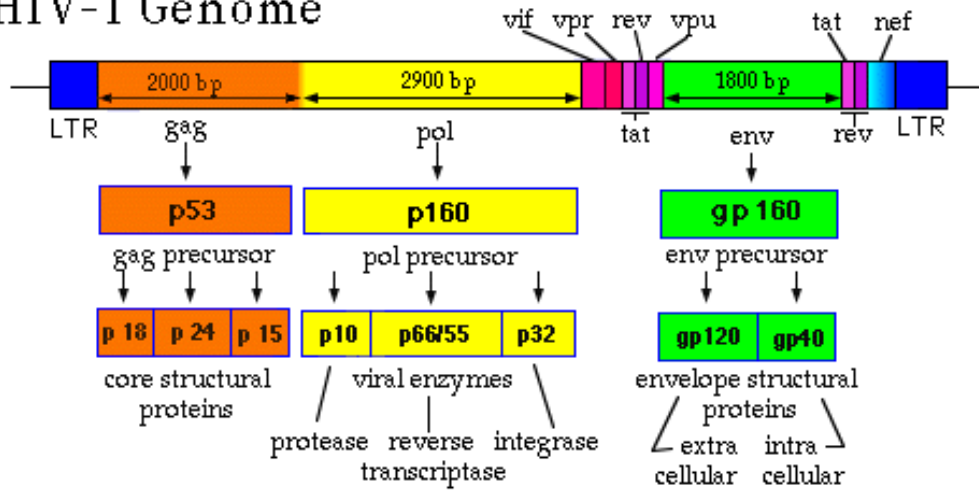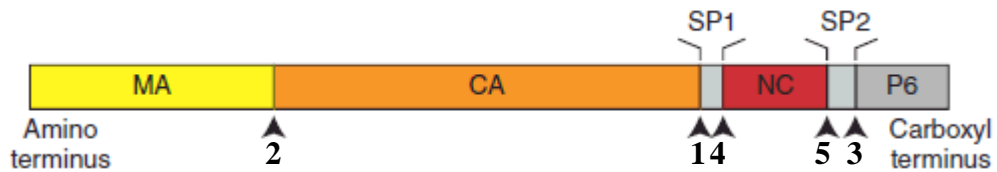
Figure 4 The three poly-proteins and the cleavage sites of Gag
A. The HIV genome is first translated as three poly-proteins: Gag, Pol, and Env. The cellular protease Furin, cleaves Env into gp41 and gp120 inside the cell. Then during viral maturation, the viral protease cleaves Gag and Pol into smaller proteins. **B.** Depiction of the sites and the order in which viral protease cleaves the gag polyprotein. (1) First, NC is cleaved from CA, (2) MA is cleaved from CA, (3) p6 is cleaved from NC, and then (4) the two spacer peptides surrounding NC are cleaved away. Cleavage sites are determined by structures not sequence.

Regulatory Proteins: Tat and Rev
Section 9.1 Tran activator of transcription or Tat (16 kDA)
When the provirus (reverse transcribed viral DNA) integrates into the genome of a resting cell, the level of transcription in the cell is low. Under these conditions, cellular transcription of HIV is abortive, producing RNA transcripts that are only 60 nucleotides long[32]. Some of these abortive mRNA transcripts code for Tat by two exons. The cell splicing machinery cleaves unnecessary sequences from the two mRNA and allows them to anneal, which varies the size of Tat from 14 to 16 kDA (86–104 aa). As a protein, Tat consists of two functional domains: the activation domain, which mediates its interactions with the cell, and the DNA binding domain.
In eukaryotic cells, 146 nucleotides of the genome are wrapped around nucleosomes containing four histones[33]. The transcriptional activity of the genes is regulated by how tightly the chromatin is wound around nucleosomes: the more tightly wound, the less access the transcriptional machinery has to the gene and the more silent the gene becomes. Several modification repress or activate the gene. For example, methylation deep silences while acetylation activates.

To relieve repression of the HIV chromatin, Tat binds to the Tat-responsive element (TAR) motif formed by the HIV LTR mRNA (Figure 5A). The TAR motif is an mRNA stem-loop structure located immediately downstream of the HIV transcription start site. When bound by Tat, the TAR motif becomes the docking site for protein complexes recruited by Tat, including the pTEFb complex (Figure B), many of which have histone-acetyl activity. Tat regulates provirus transcription through a complex regulatory interplay between histone acetyltransferases (HATs) and kinases. Specifically, Tat possesses three lysines (Lys 28, 50, and 51) that are acetylated by at least five HATS (p300/CBP, PCAF, GCN5, Tip60, and TAF$_{II}$250). Thus, the site that is acetylated determines Tat interactions and activities. For example, when Tat interacts with TAR, the protein complex p300/Creb is recruited[33] .Then, once p300/CBP acetylates Lys 50 in Tat, PCAF binds the residues flanking this site[32,34] and acetylates Lys 28 on Tat, which recruits PTEF-b[33], a protein complex between cyclin T1 and Cdk9[32,34]. Cyclin T1 binds to both Tat and Tar to form a ternary complex between Tat, PCAF, Tar, and PTEF-b[32,34]. Cdk9 then phosphorylates the serine residues on the C-terminal domain (CTD) tail of RNA polymerase II[33] and promotes continuous, rather than abortive, transcription of the provirus. Acetylating lysines in nucleosome histones causes chromatin to relax, therefore increasing exposure of the gene promoter to the transcriptional machinery. p300/CBP also acetylates Lys 14 and 8 on histone 3, and Lys 5 and 8 on histone 4. PCAF acetylates Lys 14 of histone 3 and Lys 8 of histone 4. An acetylated histone allows the transcriptional machinery access to the DNA. The presence of Tat, pCAF, and CBP/p300 also allows Tat to interact with other host proteins such as Tip60 and Taf$_{II}$250. Tip60 binding limits its HAT activity to inhibit transcription of Tip60-dependent host genes, while Taf$_{II}$250 binding limits transcription of other host genes. Thus, Tat interacting with host proteins not only enhances provirus transcription, but also limits host gene transcription, allowing the virus to hijack the transcriptional machinery for its own replication.
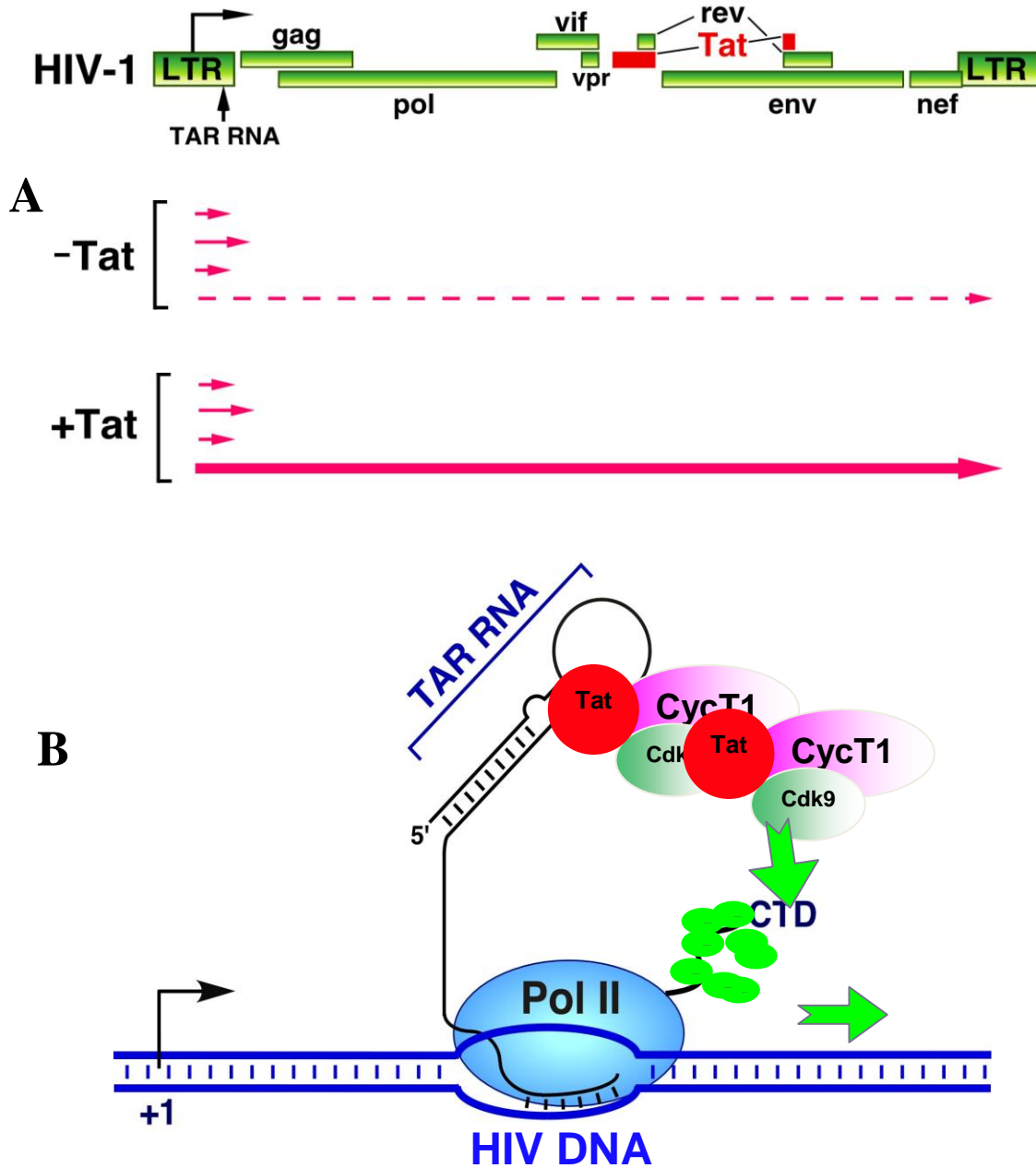
Figure 5. The role of Tat in the HIV Life cycle
A. Abortive transcription of the HIV genome in the absence of Tat **B.** Tat recruits the pTEFb complex (Cyclin T1 and Cdk9)**.** These proteins assemble into a complex using the TAR (HIV RNA stem-loop) as a platform. The pTEFb complex phosphorylates the CTD tail of polymerase II so that transcription elongation can proceed.

Section 9.2 Regulator of expression viral protein (Rev)

In eukaryotic cells, all introns are spliced from the mRNA before nuclear exit. This function is inconvenient for replicating retrovirus, as the virion genome consists of introns itself. Also, unspliced pre-mRNA is the cytoplasmic precursor to the expression of two viral proteins, Gag and Pol. HIV overcame this problem by coding the Rev (18kDA) protein and Rev-responsive element (RRE) on its unspliced or partially spliced RNA[35].

Rev is a phosphoprotein that contains two functional domains. The arginine-rich, N-terminal region is the RNA binding domain, which binds stem IIB and IID nucleotides on the RRE through, residues 35, 39, 40, and 44[35]. Other arginine residues (38, 31–43, 46, and 48) are not essential, but do bind the RNA backbone and sugar. Rev binds RRE as a monomer and then multimerizes via protein-protein or protein-RNA interactions[35]. More than eight Rev molecules have been observed to multimerize on RNA. The leucine-rich, C-terminal domain is the activation domain is needed for nuclear export. In addition to ensuring the transport of RNA that contains introns out of the nucleus, Rev protein also extends the stability of these RNAs in the nucleus from 10 minutes to 6 hours and enhances their translation in the cytoplasm[35].

During HIV infection, the provirus integrates into the genome. The first RNA transcripts to accumulate in the nucleus are those containing multiple splice sites, such as Tat, Nef, and Rev, which can exit the nucleus without Rev. Tat then enhances continuous transcription of the viral genome, allowing unspliced (Gag, Gag-Pol) and singly-spliced (Env, Vif, Vpr, Vpu) transcripts to accumulate in the nucleus[35]. After the N-terminal domain of Rev binds to the RRE, Rev attracts more Rev molecules to multimerize on the RNA. Then, the C-terminal nuclear exit signal binds exportin-1. Exportin-1 then binds related nuclear protein (RAN), which hydrolyzes guanine triphosphate (GTP) to transport across the Nuclear Porin Complex (NPC)[35]. Once in the cytoplasm, exportin-1, guanine diphosphate (GDP), and Rev disassemble from the RNA. Both Rev and RAN are then shuttled back into the nucleus by binding importin-β via its N terminus domain or a complex of GDP and importin-β, respectively[35].

Section 10. The Accessory Proteins: Nef, Vif, Vpr, and Vpu

Of most lentiviruses, HIV and SIV appear to have the most accessory proteins[16]. These proteins are not essential for viral infection or spread in permissive cell lines, but help overcome host restriction factors during infection in non-permissive cell lines and/or primary cells. As more studies investigated the mechanisms by which the virus was restricted, researchers found that these accessory proteins ensure that the virus remains undetected by the intrinsic immune system.

Section 10.1 Nef

Negative factor protein (Nef, 27 kDA) is packaged by the virion, where it is cleaved by viral protease protein and then released into the cytoplasm of the target cell, indicating that Nef is crucial for the early phase of the life cycle. Nef molecules carried by the virion facilitate CD4/CXCR4/CCR5-dependent entry of the virus into the host cell[36,37]. Although researchers speculate that Nef may enhance fusion by altering the viral core or Env molecules on the viral surface, the mechanisms of these functions remain unclear.

Nef is coded by a sequence that overlaps both the envelope and 3' late terminal repeat (LTR). As a consequence of being the first gene after the 3' LTR, Nef is expressed very early after integration. Nef is a multifunctional accessory protein that plays many roles during cell infection. For example, Nef helps the virus evade the immune system and optimize virion production by interacting with at least 16 protein complexes[36]. Nef also participates in signal transduction, host-cell activation, blocking super infection, and enhancing virion assembly, infectivity, and replication[38]. Furthermore, Nef downregulates CD4, CD3, HLA-A, and HLA-B by interacting with different protein complexes.

CD4 downregulation prevents cell activation and more virions from entering an already infected cell (i.e., superinfection exclusion). Besides decreasing cell activation, and preventing superinfection, removal of CD4 from the cell surface also increases the infectivity of newly produced virions. During viral assembly CD4 stabilizes the expression of gp120 at the cell surface so that the proteins can polarize into the area of the cell membrane from where the virions will protrude from. If not enough CD4 proteins are removed from the surface then gp120 protein will likely stay tethered to the cell membrane instead of being carried on the virions. Myrostilated Nef is transported to the inner leaflet of the plasma membrane, where the cytoplasmic tail of CD4 binds to Nef. The Nef bound CD4 complex then recruits clarithin adaptor protein complex 2 (AP2), which transports the complex to the endosome via clarithin coated pits. The endosome then fuses to a lysosome and after acidification the endolysosome then degrades the CD4/Nef complex. The importance of CD4 downregulation from the cell surface is accentuated by the fact that the virus has two other proteins besides nef, Vpu and Env, capable of the same activity[39]. Briefly, Env binds newly synthesized CD4 molecules in the ER, and Vpu then sends these Env bound CD4 complexes to the ubiquitin degradation pathway[39].

Downregulating HLA-A and -B prevents HIV-1 peptide presentation to CD8 T cells. HIV peptide presetation to CD8 T cells can lead to their activation and thereby lysis of productively infectected cells. Coincidently, the peptide that Nef targets is expressed on the cytoplasmic tails of both HLA-A and -B, but not -C. Thus, HLA-C allows infected cells to go undetected by natural killer (NK) cells, which are known to target cells with low expression of HLA-C for lysis[40][41].

Nef is made of 3 domains: N-terminus, central core, and C terminus. Both the N- and C-termini carry a flexible loop[40][40]. The N-terminus is essential for Nef interactions with the membrane and lipid raft. The sequences necessary for interaction with AP2, β-COP, ATPase-VIH can all be mapped to the central core [42]. While all these central core sequences are necessary for the removal and degradation of CD4 from the cell surface, none of them are involved in the downregulation of HLA-A or -B [40].

Nef residues 17-26, referred to as the N terminus alpha helix, contains Met20 which has been shown to anchor Nef to the plasma membrane but also, Arg17 and 19 form a motif that binds β-COP[40]. β-COP is a peripheral protein on the membrane of the Golgi complex and in non-clarithin vesicles[42]. Therefore, Nef anchors itself to both the plasma membrane and golgi complex by binding proteins at those locations, inferring that Nef might interact with HLA-A and B in both compartments. Further research is needed to decipher if Nef removes HLA-A and B from the plasma membrane, from the trans-golgi network, or both. Residues 62–65 bind

PACS-1 and -2, which increase endocytosis and the localization of Nef to the trans-golgi network, where it may also interact with HLA-A and -B[40]. Residues 72–85 form a poly-proline rich region that acts as an SH3 binding motif to interact with Lyn and Hck, two Src kinases expressed in the trans-golgi network that are involved in endocytosis signaling. All three of these motifs (residues 17-26, 62-65, and 72-85) are well conserved in patients infected with HIV-1, unlike the rest of Nef, which is highly variable. The conservation of these motifs in such a variable protein accentuates the importance of HLA-A and -B downregulation.

To capture HLA-A and -B, Nef must first bind AP-1, which changes the conformation of the μ1 subunit of AP-1 to form a pocket that binds the Y320SQA323 sequence in the cytoplasmic tail of HLA-A and -B[40]. The μ1 subunit often binds Yxxϕ motifs (ϕ represents a bulky amino acid). The binding of AP1 and Nef is stabilized by electrostatic interactions with the acidic residues (E62EEE65) and the poly-proline rich area (72–85).

Table 10.1.1 The Functions of Nef Motifs

Nef Functions	Nef Domain/Sequence required	High jacked Cell Pathway	Purpose
CD4 down regulation	Central Core Domain/	AP2, Clathrin-coated vesicle trafficking, β-COP	Efficient assembly Block superinfection Block Immune activation
HLA-A and HLA-B down regulation	Residues: 62–65, 17–26, 72–85	AP1, β-COP, endocytosis	Escape Immune recognition by CD8 T cells

Section 10.2 The counteraction of APOBEC by Vif

The main function of viral infectivity factor (Vif) (23 kDA) is to counteract a family of proteins known as apoliprotein B mRNA editing enzyme, catalytic polypeptide-like proteins (APOBEC). The APOBEC protein family includes activation induced cytidine deaminase (AID), APOBEC 1, 2, 4, and 7, and also A3 (A3A–D and A3F–H) [43]. A3 proteins target HIV at multiple steps of the infection cycle. For example, A3 binds the virion's nucleocapsid protein and blocks Lys-tRNA from binding reverse transcriptase, thereby inhibiting reverse transcription (RT)[43]. A3 also prevent strand transfer thereby blocking the elongation step of RT. Both A3G and A3F are capable of binding integrase to reduce integration of the provirus by 5–50-fold.

APOBEC proteins also restrict HIV by targeting up to 10% of cytoplasmic viral ssDNA for cytidine deamination. In productively infected cells, interferon alpha/beta is activated to induce APOBEC expression. The newly expressed A3 proteins bind the nucleocapsid during viral assembly in the cytoplasm. The RNAse H domain of reverse transcriptase is then encapsulated into the budding virion, where it degrades the viral RNA that keeps A3 inactive. As reverse transcription polymerizes the viral RNA genome into a (-)ssDNA product, the A3 protein starts to mutate cytidine into uridine, The A3 enzyme converts cytidine to uridine by cutting out the amine group from the cytosines. Since uridines are not supposed to be incorporated into DNA transcripts, cellular DNA glycosylases such as UNG2 and SMUG1, remove the uridines[43]. Apurinic/apyrimidimic endonucleases add a 5'-deoxyribose phosphate group to the site from where the uridine is removed, ensuring that the transcript gets targeted by DNA repair enzymes[43]. The viral DNA trasncript is often degraded. The DNA repair machinery upregulates the ULBP2 protein, which is recognized by the NKG2D receptor on NK cells. Upon recognition the ULBP2 expressing cell is lysed by the NKcells[43]. This sequence of events has been characterized in non-permissive cell lines when infected with ΔVif HIV.

All of the APOBEC proteins that target the HIV (-)ssDNA (A3D, A3F, A3G, and A3H) are counteracted by Vif, which implies that the most essential function of Vif is counteracting A3 restriction. The ancestral virus of HIV (SIV_{cpzPTT}) also counteracted APOBEC with another accessory protein Vif. The ancestor virus of SIV_{cpzPTT} carried Vpx instead of Vif, which targets the restriction factor, SAMHD1 [44] [45]. This observation shows that the pressure to counteract APOBEC was higher for than that for SAMHD1 for SIV_{cpzPTT}.

Binding between A3 and Vif is both essential and sufficient to prevent encapsidation of the A3G/F proteins (21). In the cytoplasm of productively infected cells, Vif binds newly expressed A3 proteins and induces conformational changes that render A3 proteins inadequate for packaging. Vif also recruits the E3 ubiquitin ligase complex to mark the proteins for proteosomal degradation[43,46,47]. This complex consists of cullin 5 scaffold protein, elongins B and CD, and Rbx2. Recently, researchers identified the core binding factor beta (CBFβ) as essential for Vif stability and degradation of A3. CBFβ only associated with the ubiquitin ligase complex in the presence of Vif, implying that CBFβ directly associates with and stabilizes Vif [47].

Section 10.3 Vpr (14 kDA)

Viral protein R (Vpr) (14kDA) plays many roles in the viral life cycle that enhance infectivity, including nuclear import of the pre-integration complex, inducting G2-cycle arrest, activating transcription of the viral LTR, and regulating nuclear factor NF-κB. Despite these activities, whether Vpr is essential for the viruses' survival is unknown. Researchers speculate that, like Nef, Vpr may be a multifunctional viral protein that enhances infection at many different steps instead of rescuing the virus from its demise at a specific one. Some of these activities may also be important for a certain part of the life cycle in a specific type of cell, not necessarily all the target cells. For example G2 cycle arrest is irrelevant in macrophages since these cells are already terminally differentiated and therefore do not divide. One activity that has been studied extensively is the induction of a G2-cell cycle arrest. The only characterized advantage of the G2 cycle arrest is the maximizing of viral protein production [41] [47] [45] [48]. The scientific field hypothesizes that G2 cycle arrest is the unintended consequences of the degradation of an unknown host factor. G2 cycle arrest is induced when the N-terminus of DCAF-1 binds to the C-terminus of VPR. DCAF-1 scaffolds the interaction between VPR and Cul4A. Cul4A binds the E2 ligase via DDB1 [48]. DDB1 then recruits the E2 ligase which ubiquitinates the complex. The ubiquitination and degradation of the complex leads to ATR activation. ATR then activates the DNA-damage response which is very well known to lead to cell cycle arrest.

Section 10.4 Viral protein Unique or Vpu

Vpu (16kDA) is a small transmembrane protein found in the trans-golgi network and endosomal pathway. Until 2008, Vpu only had one thoroughly characterized function: targeting CD4 for ubiquitination and proteosome degradation. This mechanism blocks CD4 from reaching the cell membrane in productively infected cells by preventing CD4 protein from binding viral Env during assembly, particularly in the ER. Researchers found it unlikely that Vpu would be evolutionarily selected for infection in four different species of primates (found in HIV-1, SIV_{cpz}, SIV_{gsn}, and SIV_{mon}) for the sole purpose of CD4 down-regulation when both Nef and Env already had that same activity [49].

A new VPU function was then characterized by Neil et. al in which ΔVpu virions are trapped at the plasma membrane by a small (29–33 kDA) host protein named tetherin[50-52] (Figure 6A and B). Tetherin had previously been identified as a tumor antigen (HM1.24), a bone marrow stromal cell antigen 2 (BST-2), and CD317, with constitutive expression in plasmacytoid dendritic cells, mature B cells, and plasma cells [49]. More recent studies indicate that tetherin may play a role in myeloid cell adhesion, B cell development, and/or cancer. Additionally, activating the type I interferon (IFN) pathway induces the expression of tetherin in other myeloid cells and lymphocytes, such as CD4 T cells, which are the primary targets of productive HIV infection.

Prion protein (PrP) is the only other protein that has been characterized to have a similar structure to tetherin. Like PrP, tetherin is a type 2 membrane protein with a short (21 aa) N-terminal cytoplasmic tail, followed by a (25 aa) transmembrane domain, a coiled-coil domain, and C-terminal glycosyl-phosphatidylinositol (GPI) anchor. The cytoplasmic tail contains a tyrosine residue motif (YxYxx) that signals trafficking between the trans-golgi network and the plasma membrane [49]. As a result of this signal, tetherin can be detected at the plasma membrane, the trans-golgi network, and in endosomes. Glycosylation sites between the transmembrane and the coiled-coil domain contribute to efficient transporting, folding, and dimerization of PrP. The coiled-coil domain gives the protein flexibility, and the GPI anchors the C-terminus in

cholesterol-rich microdomains from which many viruses bud [49].

Tetherin orthologs have been found in all tested mammalian cells, which inhibit retroviral infection [49]. All tested retroviruses code proteins that combat tetherin (Env in HIV-2 and Nef in SIVcpz or SIVsmm). Interestingly, tetherin may regulate budding of enveloped viruses from cholesterol-rich microdomains in the plasma membrane, including filoviruses (Ebola and Marburg), arenaviruses (Lassa and Machupo), paramyxoviruses (Nipah), and γ-herpesviruses (Kaposi Sarcoma) [49].

It has been shown that in the absence of Vpu, tetherin homodimerizes at the plasma membrane by 1–2 disulfide bonds between the coiled-coil domains, which is essential for antiviral activity [52]. Normally, when viruses bud from the cell, it takes the cell membrane with it. However, if the virus does not code for Vpu, then the virion enclosed in the cell membrane stays chained by the tetherin molecule on the productively infected cell [51]. When tethered virions cannot break away, they are endocytosed and degraded by the cell [52] [49] (Figure 6B).

Vpu counteracts tetherin in the trans-golgi network[52]. Here, the trans-membrane domain of tetherin, interacts with Vpu's transmembrane domain. Then, a phosphorylated Ser in the cytoplasmic tail of Vpu binds the β-TrCP 1 and 2 (Figure for how the TrCP usually works) and TrCPSCFskp ubiquitinates the Lys, Thr, and/or Ser residues in the cytoplasmic tail of tetherin. Mutating lysine residues in the cytoplasmic tail of tetherin blocks Vpu-dependent degradation. Because tetherin is a type I IFN-inducible protein, then, presumably, Vpu would most often be present in the trans-golgi network before tetherin. This means that Vpu would target tetherin for degradation as soon as tetherin arrives at the trans-golgi network, thereby evading any transport of tetherin to the plasma membrane.

An increasing amount of research indicates that there might be multiple mechanisms by which Vpu antagonizes tetherin. This hypothesis comes from a set of studies showing that even when tetherin cannot interact with the 1-Cull-F-box and target tetherin for ubiquitination, it still enhances viral budding[49]. Also, in certain CD4 T-cell lines, tetherin is only mildly downregulated or degraded by Vpu. Thus, it is speculated that binding of the tetherin and Vpu transmembrane domains will inhibit tetherin dimerization and tetherin antiviral activity.
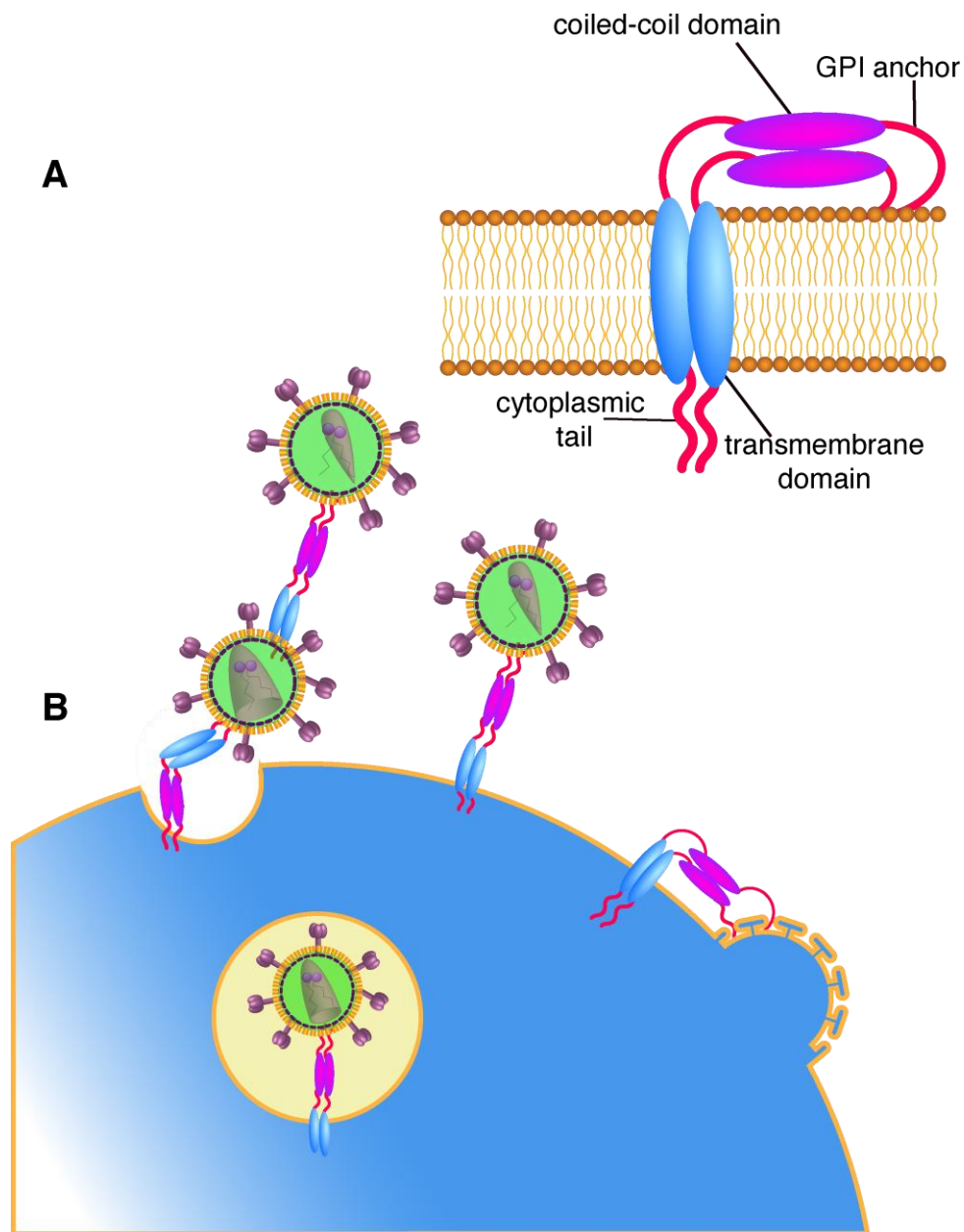
Figure 6. Tetherin restriction[52]
A. Tetherin is a GPI anchored transmembrane protein consisting of cytoplasmic tails at the N and C terminius, a transmembrane domain, and a coiled-coil domain that dimerizes. **B.** ΔVpu-HIV virions accumulate at the surface of interferon treated cells due to the expression of tetherin The N-terminus trasnsmembrane domain of tetherin embeds into the cell membrane, dimerizes with another tetherin molecule via the coiled coil domain, the GPI anchor at the C-terminus is also embedded on the membrane. When the virion attempts to bud, it does it uses the cell membrane as its coat but then tetherin's transmembrane domain and GPI anchor keep the virion tethered to the cell. Eventually, the virion is taken up in the endocytic pathway and degraded.

Section 11. THE HIV LIFE CYCLE
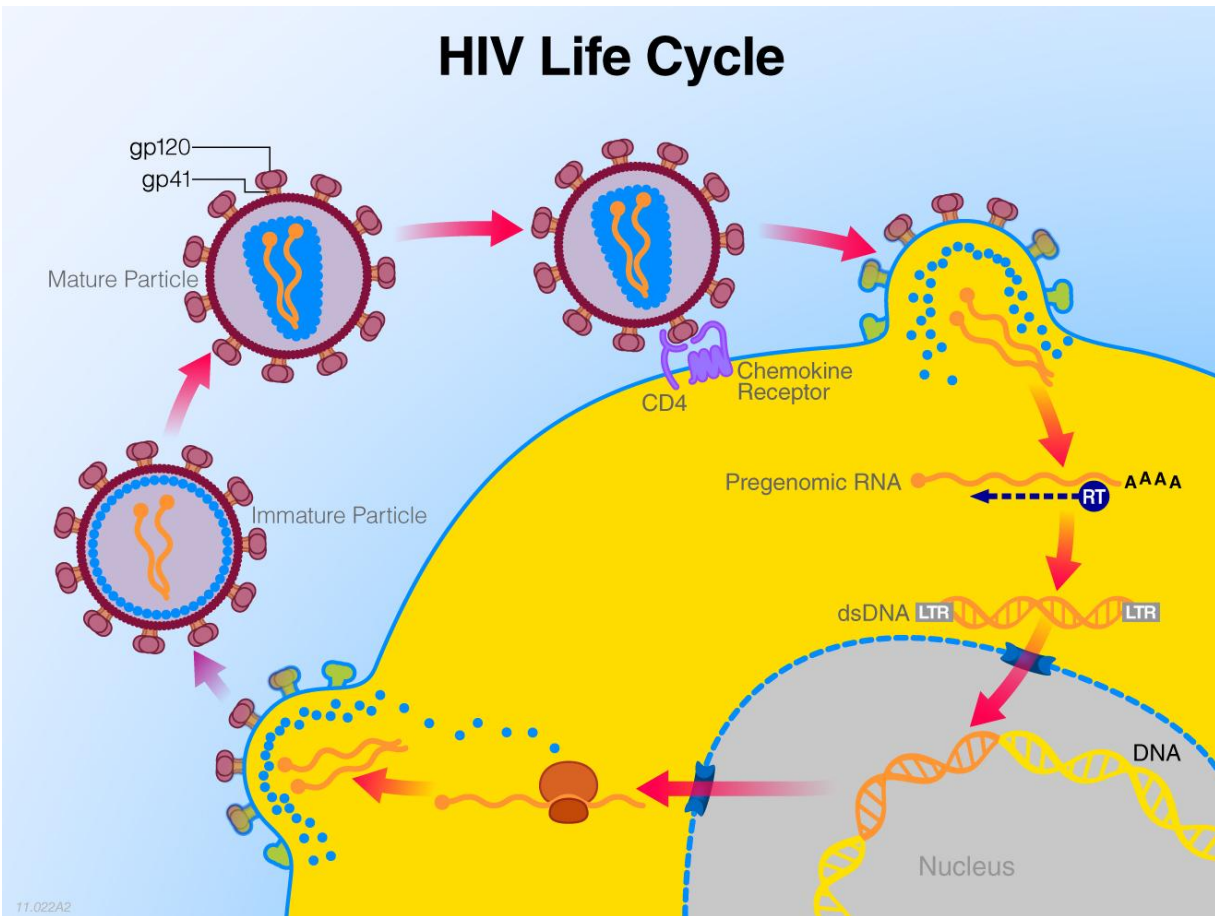
Figure 8. The HIV Life cycle
HIV enters cells that express the glycoprotein CD4, then as reverse transcription polymerizes dsDNA from its genomic dsRNA, the capsid gets filled up until it disintegrates. The dsDNA enters the nucleus via nuclear pores and integrates into the host genome. Activation of the cell leads to transcription, nuclear exit of RNA transcripts, and translation. The viral proteins assemble with the genome at the plasma membrane. Maturation of the virus occurs post budding in preparation for cell entry.

11.1 Entry

For HIVs, fusion to the viral receptors (CD4, and CCR5 or CXCR4) can occur without a target cell, indicating that other cytoskeletal components or molecules are not essential for entry and demonstrating the lack of a strict orientation dependence of the virus-cell fusion machinery. Many of the virions that fuse to the target cells are trapped in the endolysosomal pathway. Trapping by the endolysosomal pathway can occur with or without CD4 binding indicating that HIV binds attachment receptors[16,25]. Characterized attachment receptors include: galactosylceramide, gangliosides monosialotetrahexosylganglioside 1 and 2 (GM1 and GM2) (lipid raft markers), and glycoshpingolipids[25]. Besides acting as possible attachment sites for gp120, glycoshingolipids may promote CD4 and co-receptor clustering by regulating their lateral mobility on the plasma membrane[25]. SIV and HIV can only enter cells that express CD4 and CCR5 or CXCR4. CD4 is the first receptor to which gp120 binds, but then binds to either CCR5 or CXCR4 (Figure 9). CCR5 is expressed by activated or memory (previously activated) CD4 T cells, while CXCR4 is expressed by all CD4 T cells. Once gp120 binds the co-receptor, both gp120 and gp41 change conformation so that gp41 can insert its lipophilic peptide into the cell membrane. After inserting the lipophilic peptide, the viral membrane fuses with the cell membrane and the viral core is released into the cytoplasm of the cell.
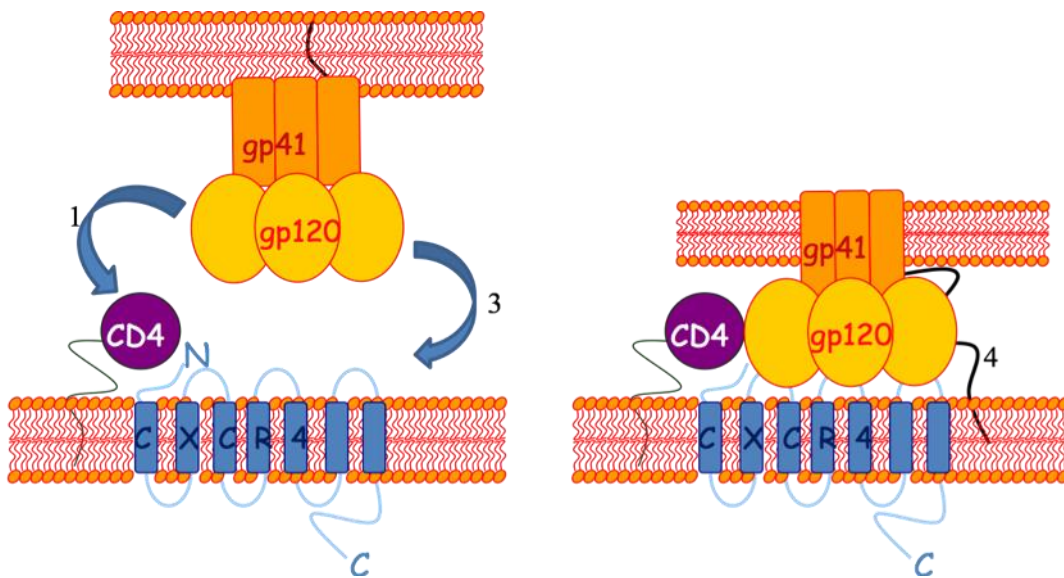
Figure 9. The steps of HIV entry
(1)The envelope protein, gp120 binds the cellular glycoprotein receptor CD4. (2) gp120 changes conformation so that the proper peptides are exposed in order to enable chemokine co-receptor binding (3), either CXCR4 or CCR5. (4) After co-receptor binding gp41 changes conformation to expose a lipophilic peptide that can be inserted into the membrane. Insertion of the lipophilic peptide leads to fusion of the viral and cell membrane.

The importance of CCR5 in HIV infection was first noted when researchers discovered that people who do not express a fully functional CCR5 were resistant to HIV transmission. In these cases, CCR5 was mutated to exclude 32 base pairs from the gene. Up to 4% of people with Northern European descent carry a heterozygous gene for this mutation. Researchers discovered that CCR5 is the preferred co-receptor during transmission, while CXCR4 is only used by virions after or during the chronic stage of infection. The percentage of HIV+ patients whose viral population switch to CXCR4 depends on the subtype with which they are infected. Subtype C accounts for 50% of global infection. Studies have indicated that up to 50% of patients infected with subtype C switch to CXCR4 [53]. While up to 50% of patients infected with subtype B switch to CXCR4 usage[53]. More recently a study involving 495 patients over the span of 10 years found that 60-77% of patients infected with all subtypes, except those infected with subtype C, switch to CXCR4 use as a co-receptor [53]. The cause of the switch is presumably due to the pressure of losing all the CCR5+ CD4 T cells at the beginning of the AIDS stage of infection. Further research is needed to decipher whether the change in CXCR4 is induced by the annihilation of all CCR5+ CD4 T cells throughout the chronic stage of infection or if the change to CXCR4 brings on AIDS. In other words, does change to CXCR4 precede or follow the initiation of the AIDS stage of infection? Interestingly, even in cases where there is no change to CXCR4, patients still reach the AIDS stage of infection.

11.2 Reverse Transcription

Retroviruses carry two copies of its genome in the form of ssRNA in its core, along with hijacked $tRNA_{lys}$ from the productively infected cell. These ssRNA templates are not suitable for permanent integration into the host cell. They can also be recognized as a danger signal by several host cell sensors, suggesting that the ssRNA only serves as a template for RT. The goal of RT is to convert viral ssRNA into a suitable template, dsDNA, for permanent integration into the host genome while keeping them hidden from detection in the viral core. Indeed, evading the intrinsic immune system requires synchronization between RT and uncoating of the viral core. Originally investigators thought that RT began after the virion uncoated in the cytoplasm, which would expose the templates to detection. More recent data indicate that RT most often begins before the virus fuses to the target cell. RT mainly involves three viral proteins: reverse transcriptase, RNAse H, nucleocapsid, and integrase. Reverse transcriptase is the polymerase that produces DNA from the RNA templates provided by the virion (Figure 10). As reverse transcriptase produces DNA, RNAse H degrades the RNA that is unnecessarily bound to DNA. The nucleocapsid then eases the movement of the polymerase on the RNA genome, stabilizes the viral DNA product, and protects it from degradation. Although the primary role of integrase is to optimize integration of the proviral DNA, it also has been shown to play several roles in RT by speeding up initiation, stabilizing the $tRNA_{lys}$-polymerase complex, and enhancing elongation [54].
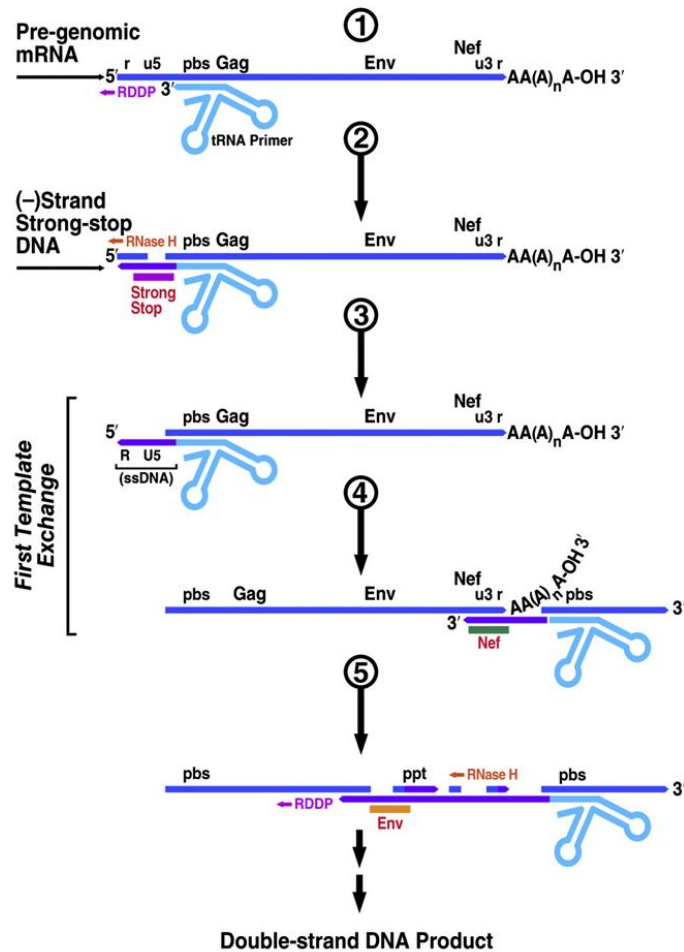
Figure 10. The steps to reverse transcription

Initiation

(**1**) Reverse transcriptase uses $tRNA_{lys}$ as a primer to polymerize at the 3'end of the $tRNA_{lys}$ and transfer over to the viral ssRNA 5' LTR. Polymerization produces a (-) ssDNA bound to viral RNA (i.e., DNA-RNA hybrid). (**2**) RNAse H digests RNA in the hybrid to expose a short ssDNA known as the minus-strand strong-stop DNA.

Elongation

(**3**) First strand transfer step: minus-strand strong-stop DNA is transferred to the opposite end of the viral ssRNA genome (3' LTR). The 3' LTR codes exactly the same sequence as the 5'LTR, so the (-) strong stop binds to the 3' LTR by homology. (**4**) ssRNA template continues to be polymerized using the (-) strong stop as a primer. RNAse H disintegrates any RNA bound to newly generated DNA. Occasionally, RNAse H is inefficient at disintegrating all of the RNA; these short bound sequences become primers for (+) strand synthesis.

(**5**) The central PPT is consistently resistant to RNAse H and serves as the RNA primer for (+) strand synthesis. Once the (+) strand is synthesized, RNAse H degrades the PBS and $tRNA_{lys}$ sequences on the (-) strand, leaving the (+) strand exposed and inducing the second strand

transfer. Here, the (+) strand is delivered to the 3'end of the (-) strand. The two templates bind at the PBS site in the LTR and then polymerization proceeds.

11.3 Uncoating

The HIV provirus enters the nucleus through the nuclear pore, where it can integrate into the genome of both circulating and dividing cells without being limited to proliferating cells like other retroviruses (e.g., MLV) [55] [56]. The nuclear pore is 30nm wide while the protein that cocoons the provirus, the core or capsid, is 60nm. Due to the difference in size, the capsid must disintegrate before nuclear import can occur [55]. Despite many years of rigorous research, this step of the life cycle, as well as the site (near the plasma membrane or the nuclear pore) and time in which uncoating occurs, remains elusive[55] [56]. Also unclear is whether RT occurs before or after uncoating [55] [56] [57] and if some monomers of the core actually stay bound to the proviral complex [56]. What has limited our progress in these areas is the fragility of the HIV core and that results depend on the type of cells tested.

Originally, evidence suggested that the only purpose of the viral core was to cocoon the viral genome to keep it close to the reverse transcriptase complex of proteins (including reverse transcriptase, matrix, nucleocapsid, integrase, and Vpr) until successfully delivered into the target cell. This model insinuated that RT only occurs in the cytoplasm after the shedding of the core. More recent studies, however, implicate that uncoating and RT occur in parallel—the farther RT progresses, the more capsid monomers are shed [55] [56]. This model suggests that accumulating dsDNA within the core pushes the core to its maximum capacity and finally leads to its disintegration. This hypothesis is supported by experiments using mutations that make the core less stable so that the capsid monomers are shed too quickly and the results have indicated that such mutations prevent RT progression. Conversely, treating target cells with antiretroviral drugs that block RT, such as efavirenz or azidothymidine (AZT), lead to accumulation of cores that remain stable for an extended time. This observation is further supported by evidence on a host restriction factor in rhesus macaques known as $TRIM5\alpha_{rh}$. $TRIM5\alpha_{rh}$ specifically restricts HIV infection by binding to the intact core and targeting its monomers for proteasome-dependent degradation [55] [56] [57]. In the presence of $TRIM5\alpha_{rh}$, degradation of the core occurs prematurely (<1 hour post-infection), and results in blocking RT [55] [56] [57]. The current model implies that RT occurs either during uncoating [56] [57]. Multiple studies indicate that not all capsid monomers are discarded from the provirus, and that capsid is essential for nuclear entry via the nuclear pores and integration.

11.4 Integration

Every step of the life cycle challenges the virus with either limited substrates or restriction factors. For example, in the case of viral entry, both CCR5 and CXCR4 expression can be limited. CCR5 is limited to CD4 T cells in a memory state (previously activated), which would present a challenge to CCR5 tropic virions. CXCR4 is expressed on all CD4 T cells including cells that are not permissive to infection post entry. RT is targeted by restriction factors such as APOBEC 3G and 3F, and SAM and HD containing protein-1 SAMHD-1[58-60].

In the case of integration, the challenge to the virus comes in the form of finding a site of integration into a gene that is transcription ally activated and/or avoiding introns. To remedy

these problems, integrase binds to the host protein cellular lens epithelium-derived growth factor (LEDGF/p75) [16] [61] [62]. The N-terminus (Pro-Trp-Trp-Pro) of LEDGF binds to the host genome while its C-terminus binds to integrase to tether the viral enzyme to the target gene where the viral genome will be integrated. Knockdown studies revealed that LEDGF is sufficient and essential for integrating the viral genome into active genes [61] [62]. LEDGF also protects integrase from proteosomal degradation resulting in the accumulation of integrase at the chromosome.

The steps of integration:
  (1) 3' end processing
      Integrase cleaves up to three nucleotides from the 3' end of the viral genome to create an overhang of the blunt end, thereby preparing the DNA for integration into the host genome.
  (2) DNA strand transfer
      Integrase creates a staggered cleavage in the host genome and joins the 3' end of the viral DNA to the 5' end of the host genome and vice versa.
  (3) Gap repair
      When a gap occurs at the integration site, such that the integrase created an overhang on both the ends of the virus and the host, the host DNA repair machinery fills the sites left empty with new nucleotides.

11.5 Assembly
HIV can assemble at different locations, depending on the cell type infected. For example, HIV assembles in the late endosome compartment of monocyte-derived macrophages (MDM) and at the cell membrane in CD4 T cells[52]. Early studies indicated that the viral protein responsible for orchestrating viral assembly and the site at which it occurs was the matrix protein in the Gag poly-polyprotein [52]. Deleting any of the residues between 84 and 88 of matrix, and also in the basic domain, results in viral assembly at the late endosome compartment, recapitulating the phenotype observed in MDM[52] [25]. Although HIV assembly occurs at the cell membrane of most cells, the cell factors involved in selecting the assembly site remain unknown.
Retroviral Gag is transcribed as a poly-protein (Pr55) and transported from the nucleus to the cytoplasm by Rev (see REV chapter 2 section 9.2). After Gag exits the nucleus, the matrix domain immediately directs the poly-protein to lipid rafts in the cell membrane.

Lipid rafts are cholesterol and glycosphingolipid-rich microdomains in the plasma membrane that serve as platforms for several cell functions (e.g., signal transduction, membrane protein sorting and trafficking, cell polarization, immune synapse formation, cell movement) and viral activities (e.g., assembly, budding, viral synapse mediated entry) (Figure 11). Protein markers of lipid rafts include GPI proteins, CD4, DRM, and GM1. The first evidence showing that viruses can assemble and/or bud from specialized compartments of the cell membrane showed that viral lipid membrane composition was enriched for specific lipids. For example, cholesterol is at least 2.5 times more common on the lipid membrane of HIV-1 and other enveloped viruses than on the host cell membrane[25]. Removing cholesterol from the cell membrane reduces viral production specifically by lowering the number of Gag and Env molecules that anchor onto the cell membrane. This process does not allow the virus to fuse, indicating that cholesterol enrichment is necessary at both the site of assembly and in the viral membrane [25].

A proposed mechanism for enriching cholesterol in these sites is the transport of Nef to the cell

membrane by its myristic motif. To move the gag poly-protein to the lipid rafts, the N-terminal glycine residue covalently binds myristic acid the basic residues (17–31) interact with phosphoinositide phosphatidylinositol 4,5 bisphosphate (PI[4,5]P$_2$) on the inner leaflet of the membrane. This binding serves as an anchor for the assembly complex, which results in Gag multimerization via CA-CA interactions[63] and polarization of lipid rafts. After Nef reaches the plasma membrane, it interacts with the ATP-binding cassette transporter A1 in macrophages, changing its distribution and reducing efflux of cholesterol. This interaction enriches cholesterol in the membrane for when assembly occurs[25], although further experiments are needed to determine if this activity occurs in all HIV target cells. The matrix domain of Gag also binds the cytoplasmic tail of gp41 in the Env poly-protein, and the viral nucleocapsid protein then binds to the RNA genome [63].
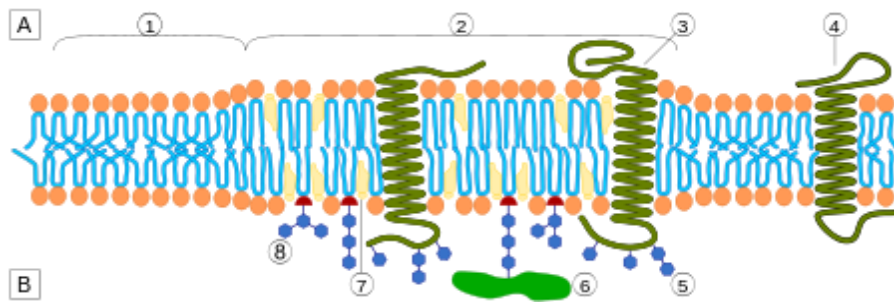
Figure 11. Lipid raft architecture and components
Increasing evidence indicates the virus has to assemble at a lipid raft for efficient infection. **A.** Intracellular space or cytosol, **B.** Extra-cellular space or vesicle/Golgi apparatus lumen. 1) Non-raft membrane (2) Lipid raft (3) Lipid raft associated transmembrane protein (4) Non-raft membrane protein (5) glycosylation modifications on glycoproteins and glycolipids (6)Cholesterol (7)Glycolipid

11.7 Budding

After the viral proteins and genome accumulate at the cytoplasmic side of the plasma membrane, the virion must separate the part of the cell membrane that it is hijacking. Early studies indicated that when deleting or mutating the p6 domain (part of the Gag polyprotein precursor), virions assemble and accumulate at the cell membrane but do not separate or pinch off from the cell [63]. The HIV p6 Gag domain codes two peptide sequences essential for the virus' high-jacking of this pathway and thereby HIV budding. The first sequence PTAP, binds to Tsg101 in ESCRT I and the second sequence LYPXnL binds to Alix in ESCRT III[64]. These short amino acid sequences are also expressed by host cellular proteins that require endosomal sorting complexes required for transport (ESCRT) pathway move to and from the plasma membrane or lysosomes. These sequences are also common to many envelope viruses including Ebola, rouse sarcoma virus (RSV), mouse leukemia virus (MLV), rabies virus, vesicular stomatitis virus, and human T lymphocyte leukemia virus (HTLV-1), among others [65].

The ESCRT pathway consists of 4 multi-protein complexes, ESCRT 0, ESCRT I, ESCRT II, and ESCRT III. ESCRT 0 is responsible for first recognizing any ubiquitinated cargo[66]. All four of these complex, except for ESCRT I, contain at least one protein domain that anchors them down to membranes by interacting with different lipids. VPS28 in ESCRT I recruits and binds EAP45 in ESCRT II[66]. Tsg101 recognition of PTAP leads to activation of ESCRT II[66]. ESCRT II associates with endosomes and recruits ESCRT III(3). EAP20 in ESCRTII then recruits and binds CHMP6. ESCRT III has the scission activity to detach the endosome compartment from the ubiquitinated cargo or HIV p6[64]. CHMP proteins in ESCRT III bind ATPase proteins such as Vps4 and Vta1 so that the ESCRT complexes can be recycled. CMP4A, 4B, and 4C interact with Alix[64].Vps4 binds and hydrolyses ATP and then Vps4 binds Vta1 and then oligermerizes[67]. Vps4 then binds Alix which binds the p6 domain[64]. By a yet to be characterized mechanism Alix cuts away the stem of the loop so that the virion can be released from the membrane.

These viruses have proteins that mimic cellular proteins to hijack the ESCRT pathway for budding from the cell. Steps in viral hijacking of the ESCRT machinery for budding [64,66,67]:
(1) p6 PTAP binds TSG101 (ESCRT I domain)
(2) ALix is activated by changing conformation and dimerization
(3) ALix activation leads to its membrane binding and recruitment of ESCRT III
(4) ALix is bound by the LYPLTSLRSL residues on the C-terminus of p6
(5) ALix binds the carboxy tails of CHMP4 (ESCRT III domain)
(6) CHMP4 polymerizes into filaments at the most narrow contact (neck) between the cell and the virion
(7) CHMP4 filaments recruit CHMP2 (ESCRT III domain)
(8) CHMP2 binds VPS4 (ESCRT III domain)
(9) VPS4 hydrolyzes ATP and uses the energy to remove ESCRT III and divert it back into the cytoplasm
(10) ESCRT III removal results in contraction and fission of the membrane

Importantly, ALix binding to the C-terminus of p6 can bypass any mutations that prevent the PTAP motif from binding TSG101[63]. Therefore, HIV-1 has at least two possible mechanisms, provided by two late domain sequences, by which to hijack this pathway [63]. The steps and/or

additional host proteins involved in the fission of the virion from the cell have yet to be fully characterized.
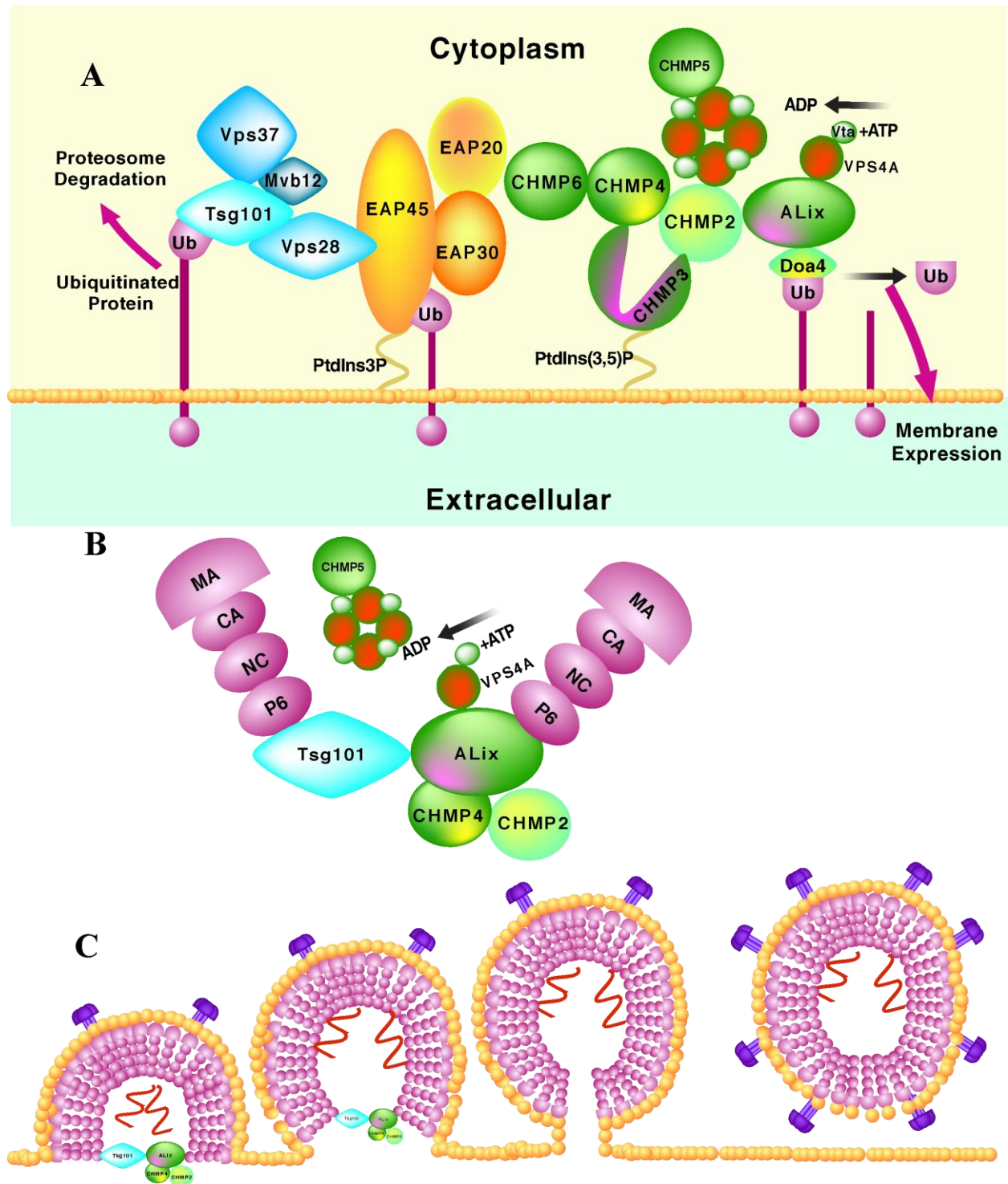
Figure 12. Components of the ESCRT pathway and their highjack by HIV [67] [68]
ESCRTI-Light blue components, ESCRT II Orange components, ESCRTIII Green components, Ub is Ubiquitin. **A** Protein EAP45 anchors ESCRT I and ESCRT II components to the membrane via its interaction with the PtdINs3P lipid. ESCRT III is anchored to the membrane via CHMP3 binding to PtdIns(3,5)P2. Doa4 removes the ubiquitin from the protein so that it doesnot get degraded in the proteasome and stays at the cell membrane surface. **B** The gag polyprotein (MA, CA, NC, and P6) is anchored to the cell membrane by MA interacting with the lipids. P6 consists of two peptide sequences recognized by two different components of the ESCRT system, TSG101 recognizes PTAP while ALix recognizes LYPLTSLRSL. **C** As the neck of the virus forms CHMP4 polymerizes forming filaments, eventually the whole complex is removed. Removal of the ESCRT complex leads to constriction and scission of the virion from the membrane.

CHAPTER 3. Cell Death Pathways and HIV Pathogenesis
Eukaryotic cells initiate programmed suicide via three distinct pathways: apoptosis, necroptosis, and pyroptosis. These pathways involve signaling cascades dictated by distinct proteases that are cysteine-dependent and aspartate-specific (i.e., caspases). They can be distinguished based on many cellular morphological properties and also differ in the degree of inflammation induce. For example, both necroptosis and pyroptosis produce inflammation as a result of the cell exploding and releasing all of its contents to the extra-cellular space. Many of the cytoplasmic and nuclear contents are recognized by neighboring cells as "danger signals" that activate inflammatory pathways. Unlike necroptosis, pyroptosis also programs the cell to secrete inflammatory cytokines such as IL18 and IL1β before its demise.

HIV researchers have debated for many years on whether CD4 T cells die by apoptosis or necrosis. Pyroptosis was not recognized as an independent cell death pathway until 1992 because it shared so many morphological phenotypes (described below) with both apoptosis and necrosis. Additionally, necroptosis was not recognized as a programmed pathway until 2000 because any mutation of the gene, RIP1, which is at the center of the necroptosis pathway, produced a lethal result[69]. The lack of distinction between these pathways challenged scientists to determine which cell death pathway was responsible for driving pathogenesis during HIV infection.

Chapter 3. Section 1.1 Apoptosis
Apoptosis was first described in 1972 by a study investigating the rapid turnover of embryonic cells during development[70][71][69]. The morphology of this process involves condensing nuclei, chromatin, and cytoplasmic molecules/organelles, fragmenting DNA and the nuclear membrane, maintaining an intact cell membrane [70][71], and membrane blebs (pieces of cytoplasm embodied by cell membrane) pinch away from the cell. Biochemically, scientists found a number of proteins called caspases (14 so far) with similar amino acid sequences but distinct functions [71]. The caspases involved in apoptosis include caspase-2, 3, 6, 7, 8, 9, and 10, with caspase-2, 8, 9, and 10 being initiator caspases involved in the early steps of apoptosis[71]. Activating initiator caspases involves dimerizing their domains and cleaving their aspartic residues[71]. One example of apoptotic caspase activation involves the nod-like-receptor protein APAF , which binds cytochrome C at its WD40 domain. The WD40 domain allows APAF to oligomerize with six other APAF molecules to create the apoptosome. This also stops the CARD domain of APAF from inhibiting the WD40 domain[70]. The CARD domain then recruits caspase-9 oligemerizes and cleaves to activate neighboring caspase-9 molecules[70]. These dimerized initiator caspases activate the effector caspases at the late stage of apoptosis (caspase 3, 6, and 7)[71], which leads to assembly of two initiators oligomerized with two effectors, forming a heterotetramer[71].The effector caspases (3, 6, and 7) directly cleave cell-structure molecules. DNA fragmentation is the result of the nuclease caspase-activated-DNase (CAD) being released from the inhibitor of CAD (ICAD) by cleavage courtesy of caspase-3 and 7. Activated CAD then cuts genomic DNA into 180 bp length transcripts[70],[71]. To generate membrane blebs, effector caspases activate the actin-depolymerizing enzyme, gelsolin. Additional structural molecules such as fodrin (cytoskeletal) and lamins (nuclear membrane) dismantle and shrink the cell[71]. Phosphatidylserine exposure at the cell membrane recruits phagocytic macrophages, which consume the shrunken dying cells (apoptotic bodies) and digest them in their endolysosomes [70][71]. Inhibition of consumption of apoptotic bodies can lead to necrosis [70][71].

Section 1.2 Necrosis, Oncosis, or Necroptosis

Originally, necrosis was considered a non-programmed inflammatory cell death accidentally caused by cell trauma/injury and pathogens with no other options for cellular exit. Injury would cause the cell to swell and explode, releasing its contents onto the extra-cellular space [70] [71] [69] (1,7,8). Immunce cells such as macrophages would detect cell debris as danger signals, secrete inflammatory cytokines and recruit more immune cells. These events are still said to occur during necrosis but this form of cell death was rediscovered as a programmed and tightly controlled process similar to pyroptosis, which led to is renaming of necroptosis. Recently specific genes were identified to induce necrosis in a regulated manner: receptor-interacting protein kinases 1 (RIP-1) and 3 (RIP-3), as well as caspase-8[69]. Suppressing caspase-8 activity is essential for necroptosis induction. Capase-8 activation leads to cleavage of RIP-1, RIP3, and CYLD, causing the cell to die via apoptosis instead of necroptosis. After caspase-8 inhibition, RIP-1 is deubiquitinated by CYLD [69]. The RHIM domain of RIP-3 then binds the intermediate domain of RIP-1. The N-terminus kinase domain of RIP-1 autophosphorylates but might also be phosphorylated by RIP-3. RIP-1 is deacetylated by sirtuin-2 and then phosphorylates RIP-3 [69]. The assembly of RIP-1 and RIP-3 and activation by phosphorylation, and deacetylation is termed the necrosome. Phosphorylated RIP-3 acts as a platform that attracts and binds MLKL. RIP-3 then phosphorylates MLKL, which then attracts PGAM5L. Phosphorylated PGAM5L recruits and dephosphorylates DRP-1, which causes mitochondrial fission and respiratory oxygen species (ROS) production. ROS is not essential for inducing necroptosis, but further research is needed to clarify alternative routes. Mitochondrial fission causes cell swelling and cell membrane rupture, although the mechanism remains unclear. Similar to apoptosis and pyroptosis many stimuli can initiate necroptosis, including Toll-like receptor (TLR) 3 and 4 signaling, T-cell receptor (TCR) signaling, tumor necrosis factor (TNF) α, FAS, and TRAIL [69].

Section 1.3 Pyroptosis

Pyroptosis is a programmed cell death that exclusively depends on the activation of caspase-1 and results in cell lysis that releases all cell contents into the tissue environment. Two signaling pathways are essential to caspase-1 activation [72]. The first essential signaling pathway is NF-κB activation, which can be activated by cytokine signaling, TLR signaling, cell-cell interactions, among other mechanisms [72] [73] [74]. Caspase-1 can only be activated to induce pyroptosis by the assembly of an inflammasome. Inflammasomes are complexes of proteins that serve as platforms for interaction, oligermerization, detection of "danger signals" by sensors, and activation of caspase-1. The assembly of these proteins into an inflammasome upon recognition of an antigen is the second essential signaling patway for pyroptosis induction. Examples of sensors that form inflammasomes include absent in melanoma-2 protein (AIM-2) (dsDNA), and Interferon inducible-16 protein IFI16 (ds or ssDNA)(1).

These two essential signaling pathways are not always mutually exclusive, since macrophages were shown to require NF-κB activation for upregulation of the sensor NALP-3 (detects many different antigens) [70] [74] [73]. After recognizing their cognate antigen, these sensors attract apoptosis-associated speck-like protein containing a CARD (ASC), which oligomerizes and forms a platform where pro-caspase-1 docks. Up to seven ASCs have been shown to bind seven molecules of pro-caspase-1[70]. After docking on ASC foci, pro-caspase-1 may change conformation and cleave itself at aspartic residues[70]. ASC is essential for recruiting inactive forms of the inflammatory cytokines IL1β and IL18 to the inflammasome. Pro-caspase-1

cleavage produces two smaller sized proteins (10kDA and 20kDA) that are essential for cleaving cytokines such as pro-IL1β and pro-IL18, forming cell membrane pores, and cell death[70]. Both of these cytokines can then expand the number of cells that are NF-κB activated to prime them for the second essential signaling pathway. ASC is not required for all inflammasomes to activate caspase-1, since certain sensors (e.g., NLRC4) can bind pro-caspase-1 directly. In the inflammasomes not containing ASC, cell death occurs more rapidly[70][72][75][76]. Although pyroptosis does not require pro-IL1β and IL18, this pathway indicates caspase-1 activation with their (IL1 and IL18) secretion.   Researchers speculate that even in the absence of ASC, other factors associated with the inflammasome recruit pro-IL1β and IL18 to be prepared for secretion by caspase-1 dependent cleavage.

Table. 3.1 Apoptosis, Pyroptosis, Necroptosis: Distinctions and Similarities

	Apoptosis	Pyroptosis	Necroptosis
Caspases involved:	2, 3, 6, 7, 8, 9, 10	1, sometimes 4 & 5	Inhibition of 8
Platform of assembly:	Apotosome with APAF	Inflammasome with Sensor	Necrosome with RIP-1 and RIP-3
DNA fragmention	Yes	Yes	Yes
Chromatin condensation	Yes	Yes	No
Membrane blebbing	Yes	No	No
Cell shrinking	Yes	No	No
Phosphotidylcholine exposure to outer cell membrane	Yes (Annexin V+ because of lipid transferase)	No (Annexin v+ because of membrane pores)	No
Nuclear Membrane break	Yes	No	Yes
Cell swelling	No	Yes	Yes
Cell bursting	No	Yes	Yes
Cell membrane pores	No	Yes	Yes
Inflammation	No	Yes	Yes
Respiratory Oxygen Species	No	No	Yes
Inhibitors	ZVAD and ZDEVD	ZVAD and ZWEDH	Necrostatin

Table. 3.2 Pyroptosis Inducing Human Sensors

Pyroptosis inducing Sensors	Family of receptors	Ligands	ASC dependent
AIM-2	PYHIN/HIN-200	DNA	Yes
IFI16	PYHIN/HIN-200	DNA	Yes
NALP-3	NLR	Uric acid, asbestos silica, LPS, fungal zyomogen, external ATP, malaria hemozoin crystals, bacterial RNA, aerolysin toxin, *C. albicans*, aluminum, nigericin	Yes
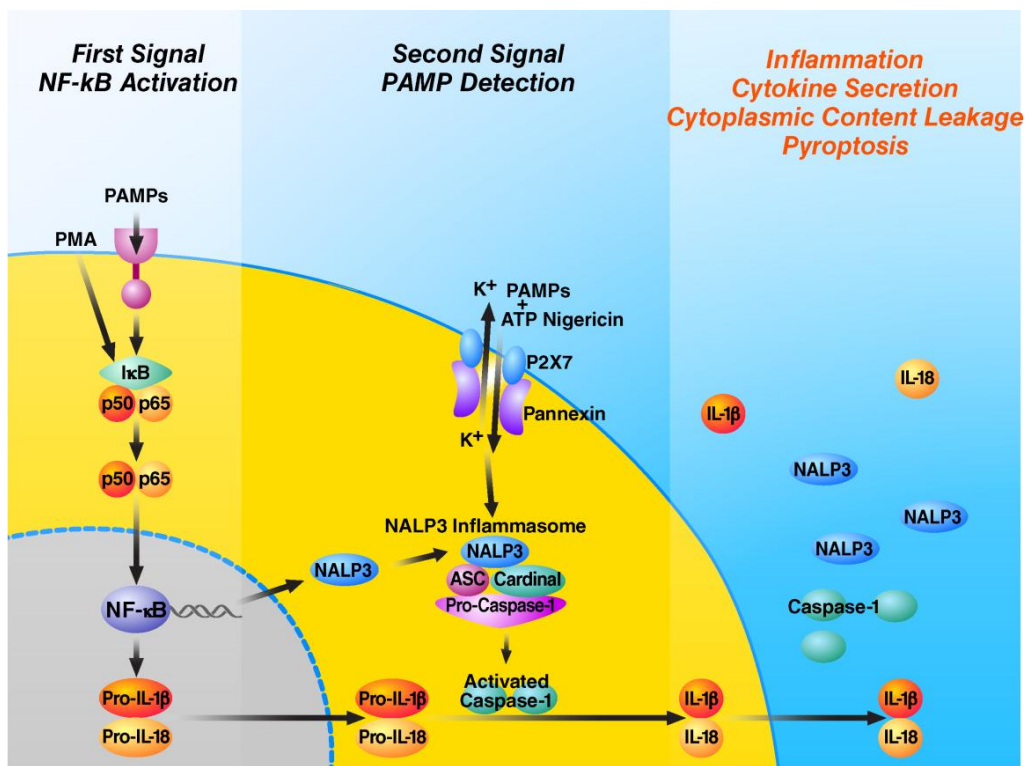
Figure 13. Pyroptosis Induction via the NALP-3 inflammasome
Expression of the sensor NALP3 requires NF-kB activation. The cell surface receptor P2X7 binds nigericin. The binding of nigericin offsets the ions so that there is increased potassium efflux. The NALP-3 inflammasome assembles with ASC, which leads to the recruitment of pro-caspase-1. Pro-caspase-1 is activated by auto-cleavage. Caspase-1 then cleaves the NF-kB dependent pro-IL1 and IL18 cytokines. Cleavage of these cytokines prepares them for their secretion. By unknown mechanisms, caspase-1 induces the breaking of the cell membrane. Cytoplasmic content, inflammatory cytokines are released into the extra-cellular space insuring spread of inflammation.

Chapter 3. Section 2.0 Mechanisms of HIV induced CD4 T cell depletion

After the first described cases of HIV infection, the scientific community rapidly realized that the manifestation of multiple opportunistic diseases must be driven by a profound impairment to the immune system[77], specifically to CD4 T cells. These cells orchestrate the host cells' adaptive immune response to infection by instructing other cells how to best combat pathogens, implicating that loss of CD4 T cells was causing such profound effects on the immune system of patients. In the absence of these cells, the immune system cannot remember past exposure to pathogens and how they were combated, rendering past vaccines insignificant in HIV patients. Thus, one of the five most important unanswered questions in HIV research is: how are CD4 T cells depleted during HIV infection?

Chapter 3. Section 2.1 Direct Killing

Several mechanisms have been proposed to explain the massive loss of CD4 T cells during infection. In immortalized CD4 T cell lines, superficially activated primary CD4 T cells, and CD4 T cells isolated from gut associated lymphoid tissues (GALT), direct killing is the predominant mechanism of cell death[78-81]. Direct killing is defined as death of the cell during productive infection. Productive infection requires a high activation state because most restriction factors are rendered inactive or degraded before cell division[82]. The post integration step at which the cells are rendered dead during direct killing has been difficult to decipher due to the lack of anti-retroviral drugs targeting those phases of the life cycle. The limitation on such drugs is a consequence of those steps being orchestrated mainly by the cell. One possible direct killing mechanism involves generating viral proteins that highjack transcription and translation and therefore starve the cell of essential proteins. Alternatively, budding of an overwhelming amount of virions from the cell surface can lead to damages in the cell membrane's architecture and stability. More recently Cooper, et al. proposed that activated CD4 T cells integrate virus and produce virions but then the viral integration is "sensed" by DNA dependent protein kinase DNA-PK [83]. Further research is needed to decipher why DNA-PK only detects integrated viral DNA after the cell produces viral proteins, as opposed to immediately after integration.

DNA-PK is a Ser/Thr protein kinase involved in DNA damage. Specifically, the genome is susceptible to environmental threats such as radiation, chemicals, and viruses, which can cause double-stranded DNA (dsDNA) breaks. DNA breaks commonly occur during the DNA synthesis (S) phase of the cell cycle, and can result in the loss of an entire chromosome. Several mechanisms exist to remedy these common, but dangerous accidents in the cell cycle, including homologous and non-homologous recombination. Homologous recombination requires an intact chromatin as a template, unlike non-homologous recombination. Non-homologous recombination requires DNA-PK [84].

Steps of DNA-PK mediated non-homologous recombination [84]:
1. Broken ends of the chromosome bind the Ku70/80 heterodimer
2. Ku directs DNA-PK to the ends
3. DNA-PK autophosphorylates
4. When the ends are staggered:
    a. Arteminus is activated via phosphorylation by DNA-PK
    b. Arteminus cleaves over-hanging bases
    c. Polymerase II synthesizes new bases (these bases have little chance of being harmful to genes since there are so many repeats throughout the genome)

5. When the ends are blunt:
    a. Blunt ends recruit DNA ligase IV
    b. RCC4 binds to DNA ligase IV
    c. DNA ligase IV ligates the ends of the chromosomes

Chapter 3. Section 2.2 Bystander killing

Because the majority of CD4 T cells are at such a low activation state that results in resistance to productive infection, researchers have explored many possible mechanisms for depleting uninfected, "bystander" cells [85] [86] [81]. Indirect mechanisms generally describe the loss of "bystander" CD4 T cells that never fused virions. For example, a productively infected cell expresses all viral proteins, including gp120 at cell surface. Surface gp120 can bind to CD4 protein and the co-receptors on the surface of uninfected bystander cells and fuse the two cells together into a syncitia. This event was observed in co-cultures of CD4 T cell lines that are permissive to infection and therefore express high levels of gp120. Bystander killing may also be caused by excessive signaling via CD4, CXCR4, or CCR5 by soluble gp120. Gp120 can become soluble after detaching from virions or productively infected cells.

Chapter 4. Abortive HIV infection mediated CD4 T cell depletion

Indirect killing mechanisms have been mostly characterized in immortalized cell lines, thus, their contribution to HIV pathogenesis is unknown. Direct killing fundamentally contributes to HIV pathogenesis, but it does not explain all mechanisms CD4 T cell depletion [80]. The majority of CD4 T cells in the lamina propria of gut associated lymphoid tissue (GALT) are at the highest activation state (presenting late activation markers, such as CD25 and HLADR, at the cell surface) and are completely permissive to productive infection and therefore the most susceptible to direct killing [78]. In the rest of the lymphoid system, as close to the GALT as the mesenteric lymph node and tonsil, the majority of CD4 T cells appear to be in an intermediate activation state, with early activation markers, such as CD69, upregulated, but not permissive to productive infection[87](Figure 14). These changes in activation and permissivity could determine the mechanisms by which HIV depletes CD4 T cells.

There are multiple restriction factors, including APOBEC 3G and 3F[43,46] (chapter 1 section 10.2), and SAMHD-1[58,60,76], that block the early steps of the viral life cycle in resting CD4 T cells. Most recently, SAMHD1 was characterized as being responsible for blocking the elongation stage of RT[58-60,82]. Resting CD4 T cells have very low amounts of dNTPs, which are essential for the virus to polymerize a dsDNA transcript from its RNA genome. Researchers showed that adding dNTPs alleviates the restriction of these cells to HIV infection by RT, which is supported by early studies in which depleting dNTPs caused SAMHD1 to inhibit HIV RT[59,88-90]. This activity may be regulated by phosphorylation, since activated, but not resting, cells express phosphorylated (Thr 593) SAMHD-1[91]. Later studies, however, indicated that although phosphorylation rendered resting cells permissive, it did not abrogate all dNTPs depletion[92]. Further research is needed to decipher the mechanism by which SAMHD-1 restricts HIV. SAMHD1 may also act as an exonuclease (RNAse) that destroys the viral RNA, which prevents its polymerization. However, we do not yet know how SAMHD-1 degrades viral RNA that is encompassed by the viral core in the cytoplasm, which would require SAMHD-1 to shuttle out of the nucleus, enter into a closed viral core, and degrade RNA.

Even in rare resting CD4 T cells where the virus completes RT[88], the virus still faces the challenge of entering the nucleus, integrating, efficient transcription and translation to produce infectious virions. Many non-essential genes are either deacetylated or methylated in resting CD4 T cells, reducing the opportunities for the viral genome to integrate into genes to transcribe viral proteins. Several studies used tonsil and spleen tissues to investigate the mechanism by which HIV depletes lymphoid CD4 T cells outside the lamina propria of the GALT[87,93] (Glushakova 1995).

We first analyzed the lymphocyte composition in tonsils by processing the tissue into dispersed cell suspension, staining with fluorescent antibodies targeting CD4, CD8, CD19, and CD14, and analyzing the cells with a flow cytometer. The composition of lymphocytes was as follows: 30% CD4 T cells, 5% CD8 T cells, and 60% B cells (Figure 14). The percentage of CD14+ cells was minimal if not obsolete.

B cells   62.5

CD4 T cells   31.3
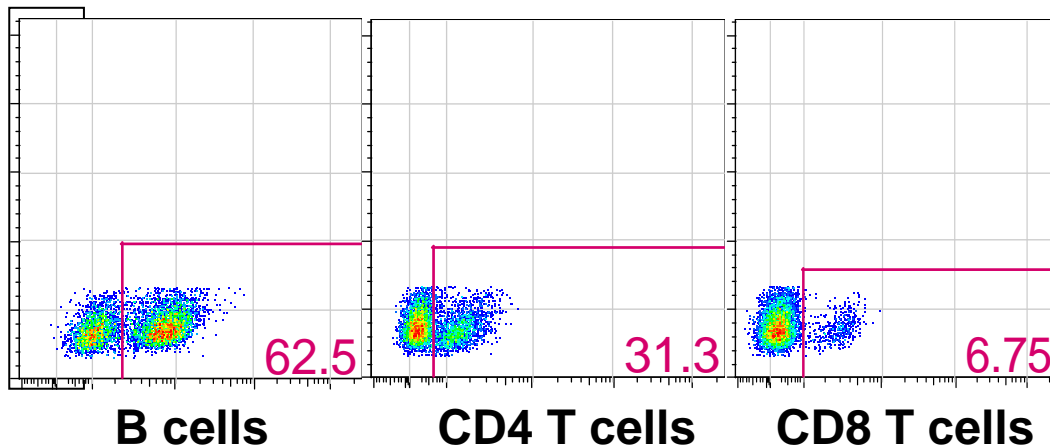
CD8 T cells   6.75

Figure 14. Lymphocyte composition of tonsil tissue
Tonsil tissue consists of 60% B cells (CD19+ lymphocytes), 20% CD4 T cells, and 5% CD8 T cells, at most 0.5% myeloid (dendritic or macrophages) cells but usually undetectable.

The lab-adapted green fluorescent protein (GFP) reporter virus, NLENG1, was used to analyze the percentage of CD4 T cells that become productively infected and are susceptible to direct killing in lymphoid tissues such as tonsil and spleen (Figure 15). Despite our observations that up to 80% of the CD4 T cells were fusing virus (Figure 16), only 5% of them were permissive to productive infection (HIV-GFP+). These results indicated that 95% of lymnphoid tissue derived CD4 T cells are resistant to productive infection and thereby also direct killing, possibly as a consequence of host restriction factors at early steps of the viral life cycle, presumably between viral fusion and integration.
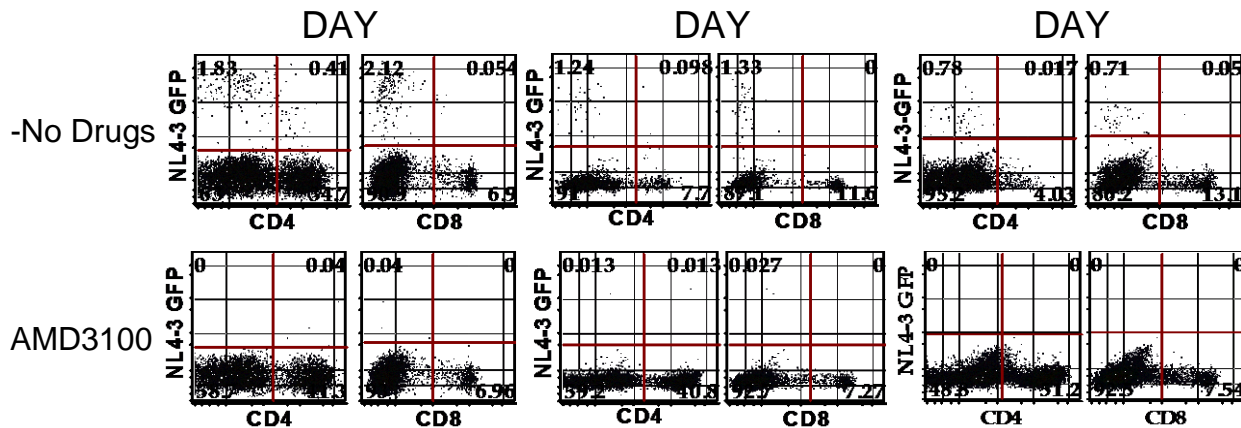
Figure 15. NLENG-IRES-GFP infection of tonsil tissue
The CXCR4 tropic NLENG-IRES-GFP virus was used to infect tonsil tissue. Samples were collected on day 3, day 6 and day 9. Up to 5% of CD4 T cells were productively infected (CD4-GFP+). The other 95% of CD4 T cells are not permissive to productive infection. The non-permissive cells are depleted by day 6 while the productively infected cells are depleted by day 9.

Up to 80% of tonsil CD4 T cells fused virus (Figure 16) but only 5% of CD4 T cells in the tonsil ever become productively infected, indicating that at least 75% of CD4 T cells are susceptible to an abortive infection in which the viruses fuses but does not establish productive infection. Despite their resistance to productive infection, based on the lack of GFP expression (Figure 2), abortively infected CD4 T cells die earlier (Day 6) than productively infected cells (Day 9) (Figure 14).
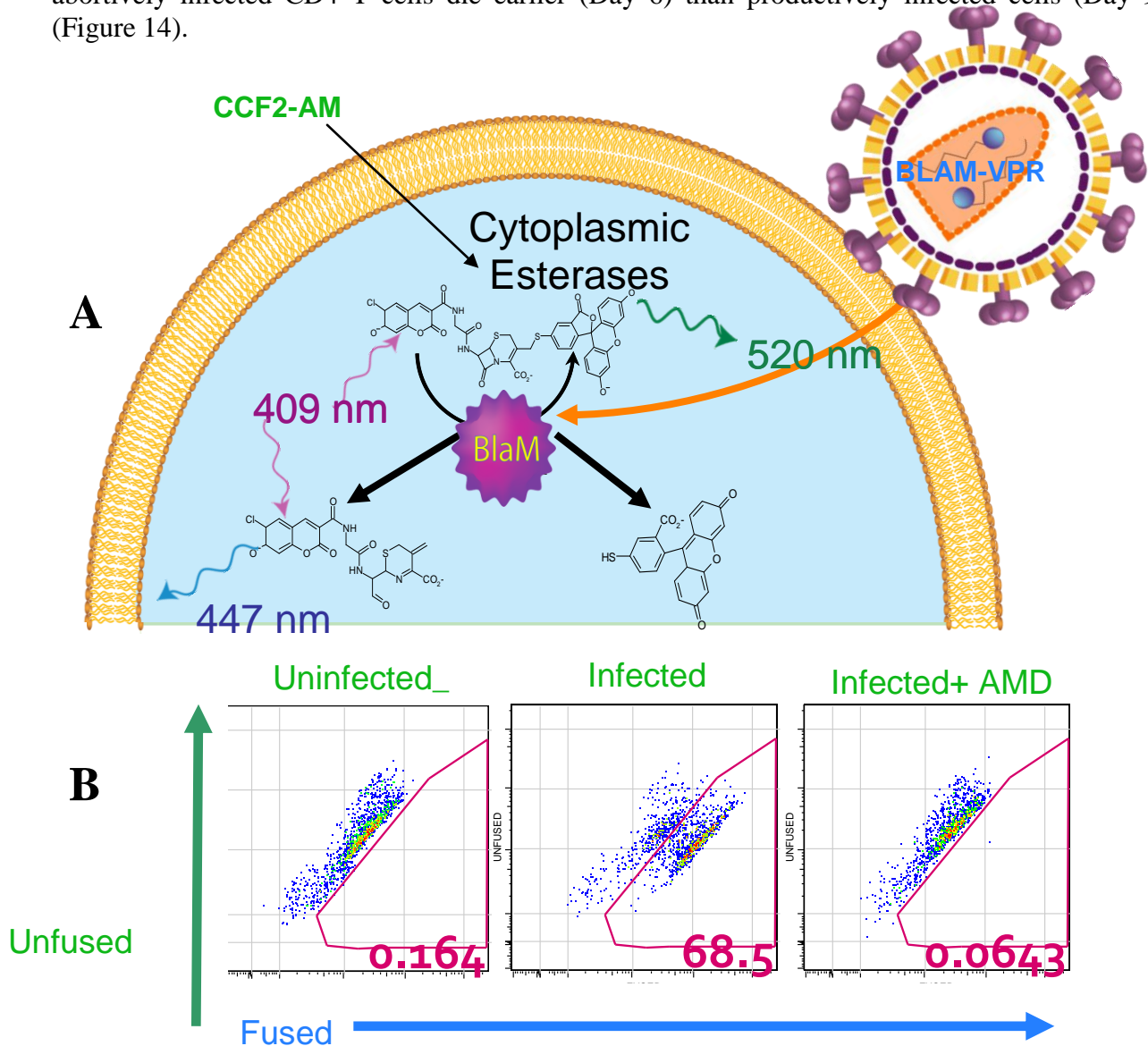
Figure 16. The majority of Lymphoid CD4 T cells are susceptible to viral fusion
**A.** Cells were infected with a BLAM-VPR/HIV-CXCR4 tropic ENV/NL4-3 core mosaic virus for one hour before staining with CCF2. Cells that fuse BLAM-VPR containing virus will transform from green to blue fluorescence due to a FRET. **B.** In infected cultures, up to 70% of CD4 T cells were fused (blue) by virus. This fusion was dependent on CXCR4 expression as the anti-retroviral drug AMD3100 blocked all fusion.

To investigate whether these cells were becoming productively infected at an unobservable pace, we used the human lymphocyte aggregate cultures (HLAC) co-culture system (Figure 16). Briefly, tonsil tissue was processed into dispersed cell suspension, and a portion of these cells was infected immediately after isolation to generate a population of productively infected cells termed effector cells. Infection was allowed to spread for four days, after which uninfected lymphocytes were stained with carboxy-fluoro succinyl ester (CFSE) and either left untreated or pre-treated with antiviral drugs targeting different steps of the life cycle. The anti-retroviral drug AMD2100, was used to block binding of the viral envelope protein gp120 to the entry co-receptor CXCR4. Alternatively, efavirenz (EFV), a non-nucleoside RT inhibitor (NNRTI) that binds to reverse transcriptase to block initiation of RT was used. Also tested was azidothymidine (AZT) is a nucleoside reverse transcriptase inhibitor that integrates into the growing DNA chain during the elongation step of RT[94]. Since the thymidine in AZT has an extra phosphate at its 3' end, the polymerase cannot add another nucleotide, which terminates RT [94]. Finally also tested was raltegravir which prevents the viral enzyme integrase from integrating the pro-virus (reverse transcribed DNA viral genome. The distinct mechanisms of these drugs allowed us to determine which of those steps of the viral life cycle made the cells susceptible to depletion. After treating target cells with different combinations of anti-retroviral drugs and staining them with CFSE, we co-cultured them with effector cells that had been spreading virus for 4 days. Co-cultured cells were allowed to interact with each other and the virus for 5 days before harvesting. Cells were stained with fluorescent antibodies targeting CD4, and CD8. To calculate the percentage of target (CFSE+) CD4 survival, absolute counts of target CD4 T cells were divided by CFSE+ CD8 T cell and then normalized to the corresponding uninfected co-culture ratios.

EFV treated infected co-cultures displayed no depletion of target CD4 T cells. This result implies that RT initiation is essential for the depletion of tonsil derived CD4 T cells. Raltegravir treatment did not protect the cells from depletion, implying that productive infection of target cells is not required for their depletion. Untreated infected co-cultures displayed comparable CD4 T cell depletion to AZT treated co-cultures. This result implies that there is an intrinsic impairment to RT in these cells, since AZT is not necessary to observe their depletion. Since viral entry and the initiation of reverse transcription are required for this mechanism of CD4 T cell depletion we termed it abortive HIV infection mediated depletion. This mechanism was also observed in tissues from lymph nodes (e.g., mesenteric lymph-node and adenoids) and spleen (Figure 26).

Following the first strand transfer, short reverse DNA transcripts accumulate in HIV infected tonsil CD4 T cells[95][87][96]. This accumulation was paralleled by IFNβ production, caspase-1 activation, and IL1β secretion, implicating that pyroptosis was induced in these cells, possibly via sensing of the accumulated DNA transcripts [87]. Tonsil CD4 T cells displayed early markers of activation, such as CD69, and also NF-κB activation as measured by pro-IL1β and NALP3 expression [87][96]. Thus, lymphoid CD4 T cells are primed for pyroptosis by NF-κB activation and inflammasome assembly after infection [95]. Inhibiting and knocking down caspase-1, and ASC protected CD4 T cells from depletion [95].
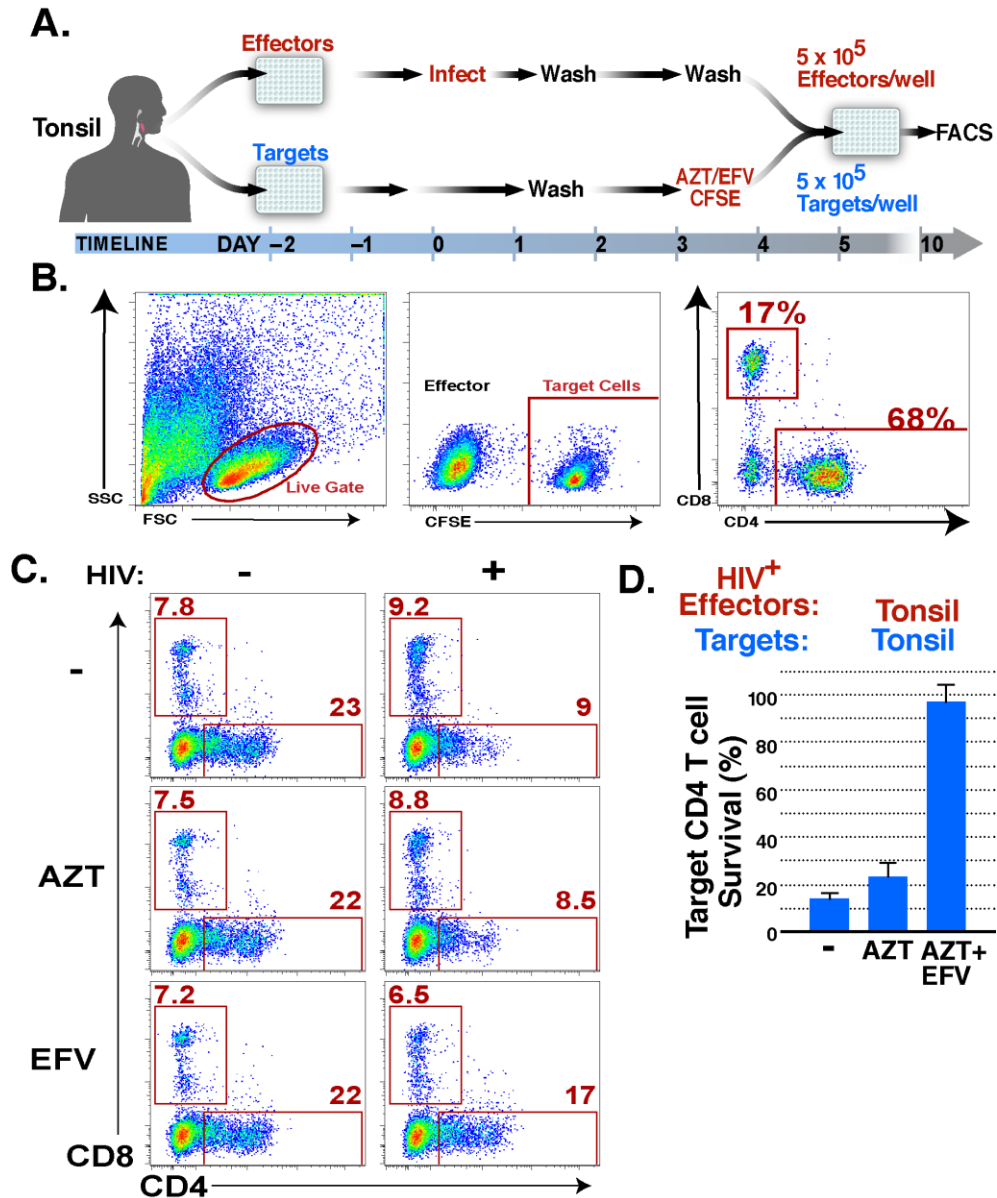
Figure 17. HLAC Co-culture system: Method and Results
A. Tonsil tissue is processed so that lymphocytes are dispersed from each other. Cells (5x10^5/well) are infected with NL4-3 virus (10-50ng measured by p24 content). Infection is allowed to spread for 5 day. Uninfected tonsil cells (5x10^5/well) are stained with CFSE and treated with different combinations of anti-retroviral drugs. CFSE+ (target) cells are co-cultured with productively infected (effector) cells for 5 days. Co-cultures are harvested, stained for CD4 and CD8. Percentages of surviving CD4 T cells are calculated by normalizing the absolute count of CD4 to CD8 ratio to the corresponding uninfected samples. **B.** A lymphocyte gate is contructed upon which the target (CFSE+) cells are gated on and then CFSE+ CD4 and CD8 T cells are gated separately. **C.** FACS Plots of tonsil co-cultures uninfected vs infected, untreated vs AZT or EFV treated. **D.** Target tonsil CD4 T cell depletion was seen in untreated or AZT treated cultures but not in EFV treated cultures.

Chapter 5. The DNA Sensor that Induces CD4 T cell depletion during HIV infection
The Pyrin and Hin-domain Containing Protein (PYHIN) Family
Given the detection of accumulated RT DNA transcripts in CD4 T cells abortively infected with HIV, the PYHIN family DNA sensors became top candidates for the pyroptosis-inducing sensor. The human PYHIN protein family consists of three members: AIM2, IFI16, and IFIX. These proteins share the characteristic of having two domains (HINa and HINb) capable of binding DNA in an electrostatic-dependent manner [97] [98]. These proteins lack an oligemerization domain, so their DNA ligand is used as a platform for them to bind to [98] (Figure 18). The optimal length of DNA for these sensors to bind is 60 base pairs (bp), but they can also bind up to 70 bp[98], which may allow up to 20 AIM-2 molecules bind. Both AIM-2 and IFI16 express the same HINb sequence, the domain that most strongly binds DNA[98]. The first of these proteins that was described as a DNA sensor was AIM2, which induced inflammasome assembly leading to caspase-1 activation, and pyroptosis in a DNA- and ASC-dependent manner [97] [98].

IFI16 was originally identified as a DNA damage protein[98]. The first studies to propose IFI16 as a DNA sensor postulated that it was not capable of binding to ASC for inflammasome assembly[97]. Instead, IFI16 was thought to bind DNA and stimulator of interferon genes (STING), a molecule essential for TBK1-dependent IFNβ expression. Briefly, recruitment and binding to STING first requires IFI16 to bind ss or dsDNA[97]. IFI16 binding to STING activates TBK1, leading to IRF3 phosphorylation by DDX3 then phosphorylated-IRF3 mobilizes and binds the IFNβ promoter and finally stimulates IFNβ expression[97]. Therefore, scientists thought that AIM-2 worked as a sensor for inflammasome assembly via the binding of ASC, while IFI16 bound STING only to induce IFNβ expression but not inflammasome assembly[97]. Then it was shown that IFI16 was constitutively expressed in human dendritic cells, bone marrow–derived macrophages, peripheral blood–derived monocytes, THP cells, and HEFs, but not HEK293T cells [97]. A year later, IFI16 was shown to bind the episomal genomic latent DNA of Kaposi sarcoma virus in the nucleus of endothelial cells to induce pyroptotic cell death[99]. These cells expressed pro-IL1β, a marker of NF-κB activation, even in uninfected conditions[99]. IFI16 formed a complex with episomal DNA and ASC in the nucleus. At early time points after infection, this complex was only seen in the nucleus but, at later time points, was mostly found in the cytoplasm[99]. The investigators postulated that an assembled inflammasome in a complex with IFI16 could be transported out of the nucleus in endothelial cells, although further research is needed to elucidate this pathway. More recently, the same assembled complex and IFNβ secretion were observed after HIV infection of human macrophages[100]. Also, during Human Cytomegalovirus (HCV) infection, IFI16 induced IFNβ, which inhibited expression of early and late viral genes to limit replication [101].
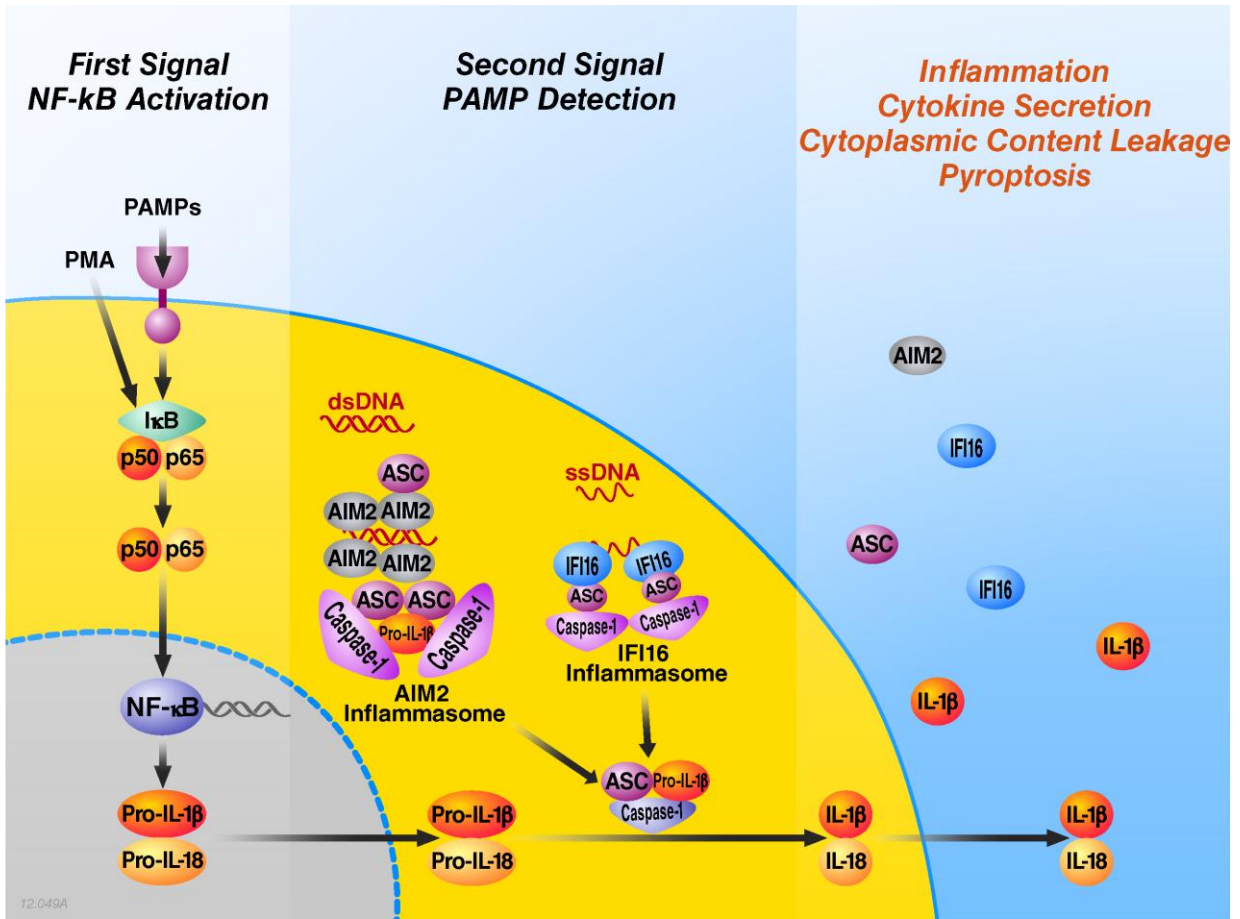
Figure 18. Model of PYHIN sensors
AIM-2 detects dsDNA in the cytoplasm and then forms an inflammasome with ASC which induces caspase-1 activation, IL1β and IL18 secretion and pyroptosis. Up to 7 molecules of both sensors (AIM-2 and IFI16) can bind DNA. Each molecule bound to the DNA can bind ASC molecules. IFI16 can bind stem-loops in ss or dsDNA, form its own inflammasome and then induce cytokine secretion, cytoplasmic leakage, and pyroptosis.

To investigate if PYHIN or other proteins are responsible for detecting the accumulated DNA in abortively infected lymphoid CD4 T cells, and induction of pyroptosis, cytoplasmic enriched lysates of tonsil CD4 T cells were incubated with streptavidin-bound DNA and probed with HRP bound beads that underwent mass spectrometry for DNA binding proteins. We found a number of proteins, including IFIX and IFI16, bound accumulated RT-DNA [102]. Knocking down IFI16 by short hairpin RNA (shRNA) protected CD4 T cells from cell death [102]. Knocking down DNAPK, AIM2, IFIX, or STING did not rescue cell death, implicating that IFI16 was the DNA sensor responsible for pyroptotic cell death of CD4 T cells during HIV infection. Monroe and Yang et al. then showed that knocking down IFI16 in spleen and tonsil CD4 T cells protected them from depletion [102]. Collectively, these results imply that the majority (>95%) of lymphoid CD4 T cells are abortively infected via impaired RT elongation, which causes reverse transcripts to accumulate in the cell. These accumulated RT transcripts are then detected by the DNA sensor IFI16, which forms an inflammasome by binding ASC, and ASC then bridging IFI16 to Caspase-1 so that it can be activated thereby inducing pyroptotic cell death (Figure 19).
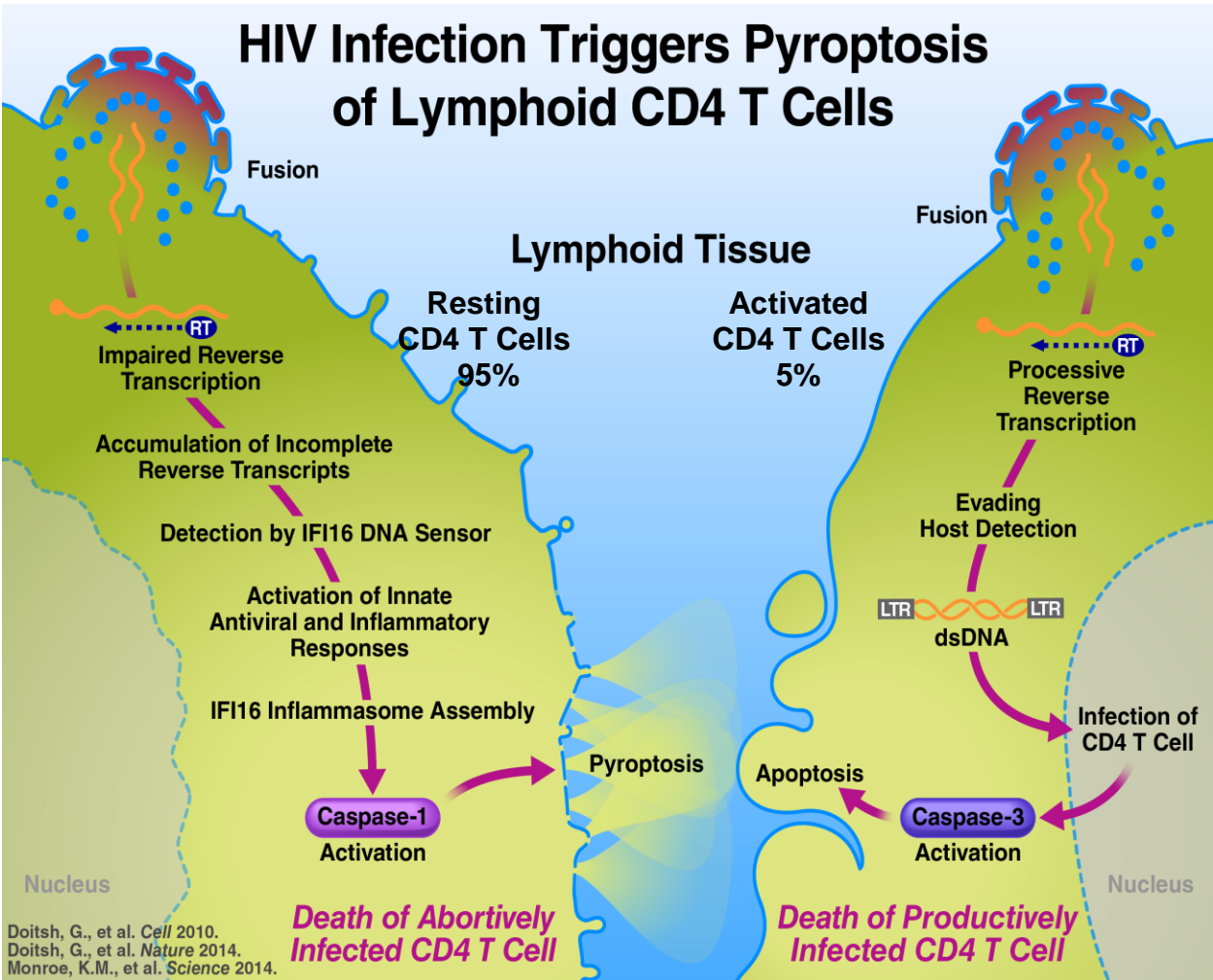
Figure 19. Model of HIV mediated Pyroptosis
Up to 5% of CD4 T cells in the lymphoid tissue (excluding GALT {tonsils, lymph nodes, spleen}) are susceptible to productive infection leading to direct depletion of these cells. Up to 95% of CD4 T cells in those same tissues are abortively infected. This abortive infection ends during reverse transcription when a restriction factor only allows reverse transcriptase to produce short DNA transcripts. Short DNA transcripts accumulate in the cytoplasm and are detected by a sensor. Detection of cytoplasmic DNA leads to Caspase-1 activation, IL1β and IFNβ secretion, and finally cell death by pyroptosis.

Chapter 6. HIV delivery via the virological synapse is required for abortive infection mediated CD4 T cell depletion

Retroviruses, like many other pathogens, take an advantage of close cell-to-cell interactions for efficient spread of infection. The cell-to-cell spread of HIV[103] and HTLV[104] often takes place at the virological synapse. This specialized structure on the cell surface received its name because of its common proteins (CD4, LFA, ICAM-1), structure, and signaling pathways (ZAP70, LCK,) to the immunological synapse. The virological and immunological synapse also share the characteristic of polarization of both surface and secreted proteins to a specific "hot spot" on the plasma membrane of target and effector cells[104]. In the case of the immunological synapse, cytokines such as IFNgamma and IL2 have been characterized in their polarized secretion [105]. In terms of the virological synapse, it has been shown that HIV virions can highjack this same regulated secretory pathway to achieve polarization[106] [105]. This hot spot recruits membrane-bound and secreted proteins, as well as lipid rafts. The recruitment of the proteins is initiated with gp120 binding CD4 and intercellular adhesion molecule-1 (ICAM-1) binding LFA-1 (on target cell's surface). Then, LFA-1 recruits the kinase LCK but not Fyn, although more LCK is recruited and phosphorylated upon CD4 binding to gp120 [107]. LCK phosphorylates CD3ς, which then phosphorylates ZAP70, thereby depleting filamentous (F)-actin. In the cytoplasm of productively infected cells, gp120 binds to CD4, activates ZAP-70 [108], which reorients the microtubule organizing center (MOC) to guide host and viral proteins, including Gag, to the site of contact. Gag (p17) specifically binds the gp41 cytoplasmic tail where the virus assembles.

Cell-to-cell spread is the most common and efficient mechanism of HIV infection *in vitro* systems [103]. In fact, viruses that are just secreted (cell-free virus) by productively infected cells tend to be a thousand fold less infectious than virus spread through the virological synapse. Cell-cell spread of virus may also be the most favored *in vivo* because it offers many advantages over cell free viral infection. The virological synapse increases the kinetics of infection and depletes F-actin [107] from target cell cytoplasm, allowing for more efficient entry and uncoating. This process may also decrease exposure to neutralizing antibodies and/or immunological surveying cells, such as phagocytes [104]. These advantages may also decrease sensitivity to therapy, since cell-to-cell infection is less sensitive to antiretroviral drugs compared to cell-free viral infection [109]. Therefore, prolonged therapy may not only lead to mutations in the virus that resist antiretroviral drugs, but also select virions that are more easily spread via the virological synapse. Under uninfected conditions, cell-to-cell interactions between CD4 T cells usually last up to 10 minutes [104]. HIV infection extends the time of interaction between these cells to as much as 18 hours [104] [110].

The possibility of cell-cell viral infection being the preferred mechanism of infection in vivo was highlighted by the research demonstrating that gp120 binding to activated α4 in the heterodimer α4β7 signals activation of LFA-1 [111]. Incidentally, all CD4 T cells express α4β7, but only GALT derived CD4 T cells exposed to Ca^{2+}, Mn^{2+}, or retinoic acid (produced by GALT resident dendritic cells) express the activated form of α4β7. Activated LFA-1 then clusters with CD4 and α4β7 at the "hot spot" and binds more ICAM-1 than the inactivated conformation of LFA-1. The peptide sequence in the V2 loop of gp120, Leu-Asp-Val (aa182-4), that binds α4 is conserved in 97% of gp120 sequences in the 2006 Los Alamos HIV database of aligned gp120 sequences. Viral envelopes from HIV subtypes A, B, C, and D, as well as in SIVsmm, interact with α4β7 [111]. These results imply that a sequence in the HIV envelope was conserved specifically for activating and recruiting an essential virological synapse receptor, LFA-1.

Most studies on innate detection of HIV have been done with either cell-free virions or viral components. Some examples of innate detection of HIV include: when gp120 attaches to DCSIGN on the surface of dendritic cells, TLR7/8 detection of HIV RNA in endosomes, and when newly synthesized capsid interacts with cyclophillin A leading to IRF3 dependent IFNβ [103]. Less is known about the effects of delivering virus by cell-to-cell transfer on innate HIV detection.

Doitsh et al. showed that cell-free virus, even when concentrated by centrifugation, could not deplete abortively infected cells [87]. On the other hand, infection by spinoculation was able to induce abortive HIV mediated depletion of CD4 T cells (Figure 20). Originally it was hypothesized that spinoculation was allowing the attachment of more virions to cells than when cells were infected with concentrated cell-free virus and that this increase in virion attachment was overwhelming a barrier. This hypothesis was proven wrong when we pretreated tonsil lymphocytes with raltegravir, and then infected these cells by spinoculation (Figure 20). Of note, blocking of integration and therefore productive infection with Raltegravir had previously been shown to not prevent depletion of CD4 T cells in the HLAC system[87]. Instead, in the spinoculation infection system all CD4 T cells pretreated with Raltegravir were safe from depletion.

We developed another system in which HEK293T cells are transfected with viral DNA and then co-cultured with lymphocytes for analysis of CD4 T cell survival (Figure 21). In these cultures HEK293T cells act as the main virus producing cells and can directly transfer virus via the virological synapse to the lymphocytes. Using this system, we compared the depletion in cultures transfected with multi-round virus NL4-3 left untreated, to cultures treated with raltegravir. Under these conditions both the untreated and raltegravir treated cultures were capable of inducing depletion in an EFV dependent manner (Figure 22). This confirmed once again that productive infection of lymphoid tissue derived CD4 T cells was not essential for depletion.

Up to 5% of lymphoid tissue derived CD4 T cells are permissive to productive infection[87] (Figure 15). We next hypothesized that abortive infection induced depletion, in spinoculated cultures, required the infection of the permissive (5%) subpopulation. We speculated that pretreatment with raltegravir was not allowing the 5% to be productively infected and so there was no viral source except for the cell free virus. To test this possibility, Dr. Nicole Galloway, infected tonsil cells with a HIV-GFP reporter virus and then AMD3100 was added to the cultures four hours after spinoculation (Figure 23). Up to 5% of CD4 T cells were once again observed to be productively infected (GFP+). Despite the presence of productively infected cells, the AMD3100 protected the rest of the cells from death. These results confirmed our hypothesis that infection of the permissive subpopulation of lymphoid derived CD4 T cells was required for depletion.

Next, we hypothesized that productive infection of the permissive subpopulation was required because HIV induced depletion required the transfer of virus via the virological synapse. To test this hypothesis, we used blocking antibodies against ICAM-1 and LFA (Figure 24). Under these conditions cell-free virions would be allowed to infect but the virological synapse would not form properly thereby blocking cell-cell spread of infection. When ICAM-1 or LFA were blocked, depletion of lymphoid derived CD4 T cells was prevented. These results show that the depletion of lymphoid derived CD4 T cells requires transfer of virus via the virological synapse.

We speculate that transfer via the virological synapse allows the virus to overcome a barrier. Further research is needed to decipher the mechanism by which the virological synapse increases susceptibility to abortive infection mediated depletion.
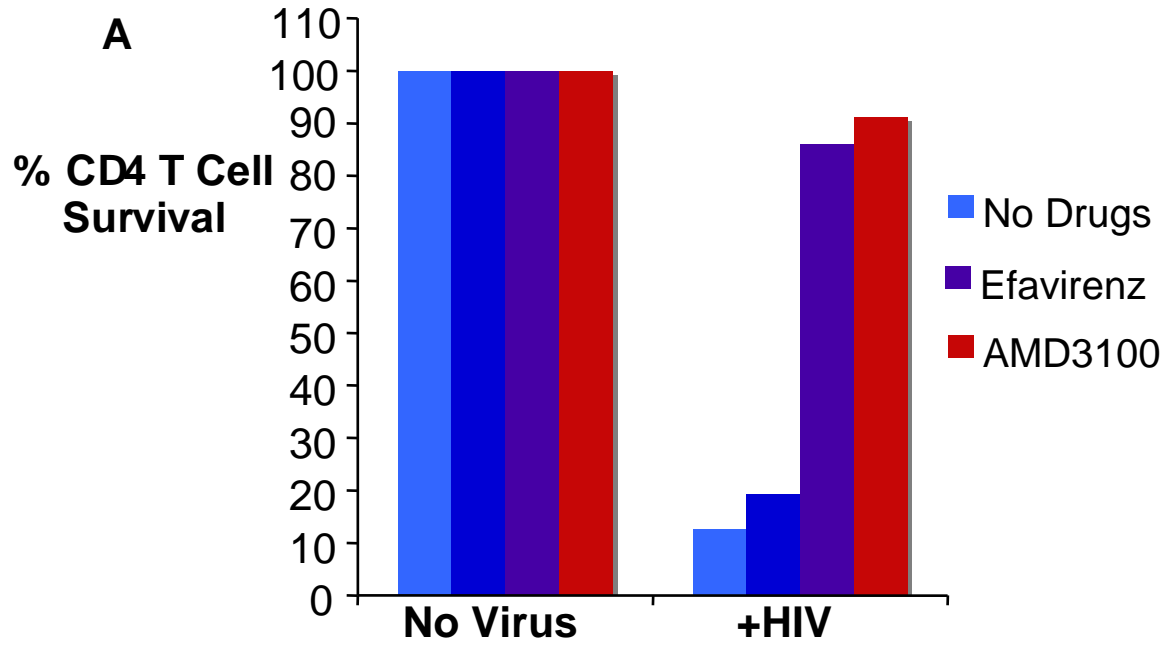
B
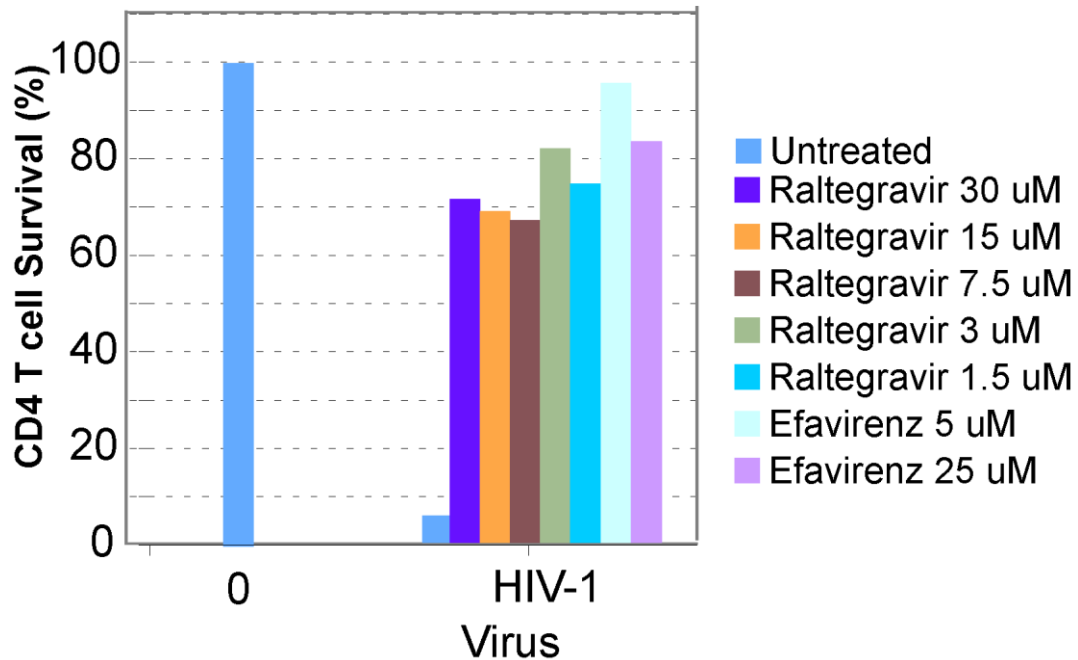
Figure 20. Infection by spinoculation results in lymphoid CD4 T cell depletion but it requires productive infection
A. Tonsil cells were cultured for 2 days post spinoculation with virus. Up to 95% of CD4 T cells were depleted post spinoculation, implying that a enhanced viral attachment to CD4 T cells overcomes the cell-free virus barrier to abortive HIV infection mediated depletion. **B.** Tonsil lymphocytes were left untreated or treated with raltegravir for 1 hour before spinoculation. Raltegravir protected CD4 T cells from depletion implying that either productive infection was necessary.
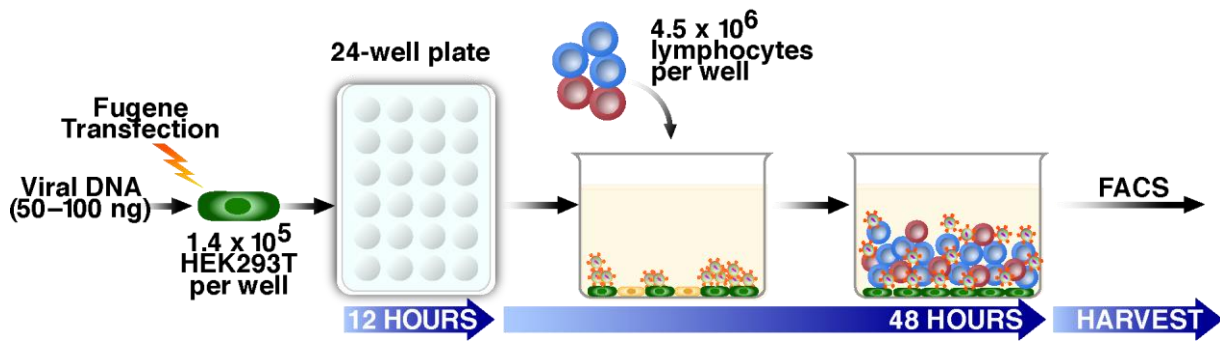
Figure 21. The HEK293T co-culture system
HEK293T cells are plated at 1.6-1.4x10^5 cells/well then transfected with viral DNA. Untreated or raltegravir treated Lymphocytes (4.5 x10^6) are added 12 hours later and then harvested two days after co-culture
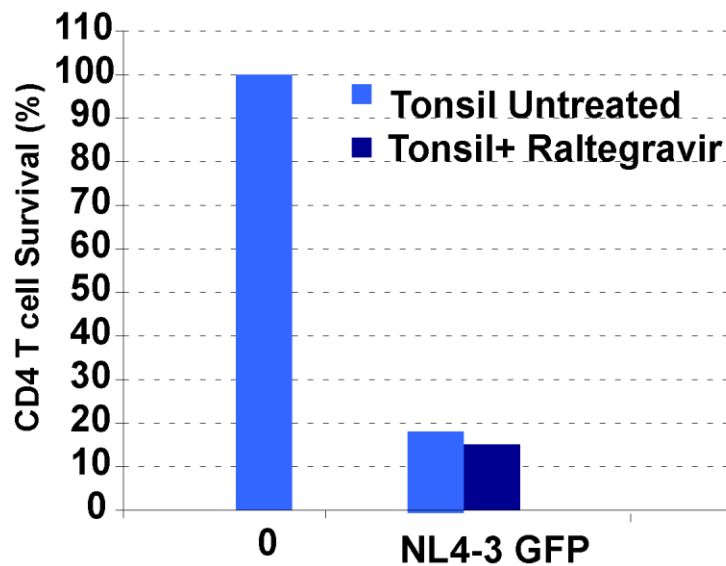
Figure 22. Depletion of lymphoid CD4 T cells does not require productive infection in the HEK293T co-culture system.
When HEK293T cells produce and transfer virus (irrespective of its ability to integrate) via the virological synapse, lymphoid CD4 T cells are depleted.
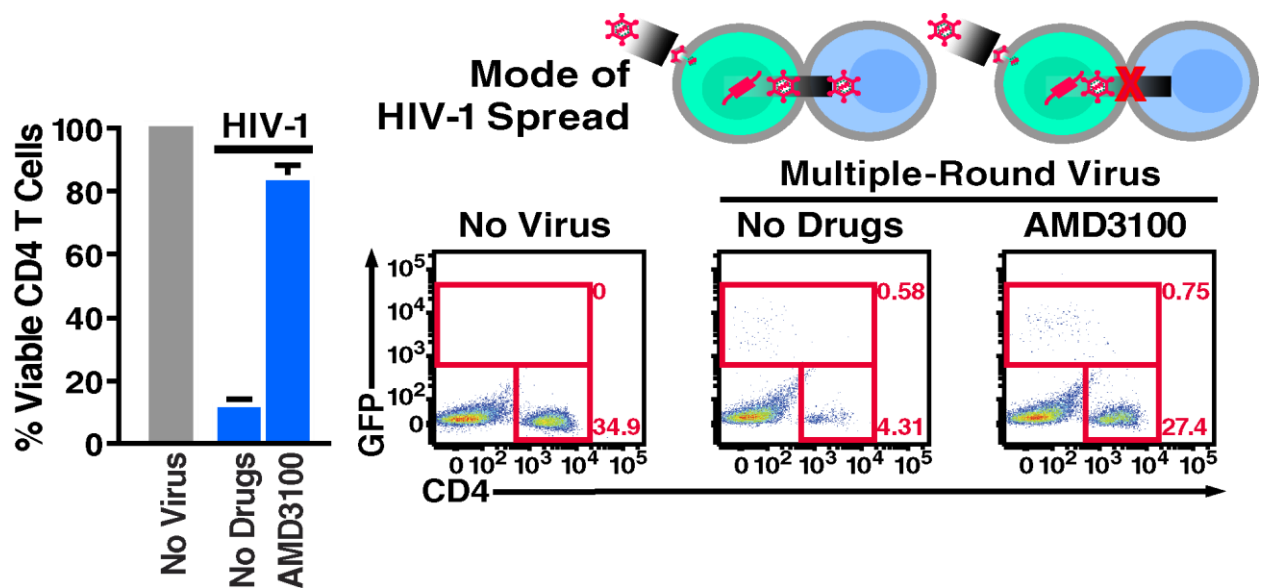
Figure 23. AMD3100 treatment four hours post spinoculation results in productive infection of permissive lymphoid CD4 T cells but no abortive infection mediated depletion Productive infection of permissive CD4 T cells was allowed by only treating spinoculated cells with AMD3100 4 hours post infection. AMD3100 protected the non-permissive CD4 T cells from depletion.
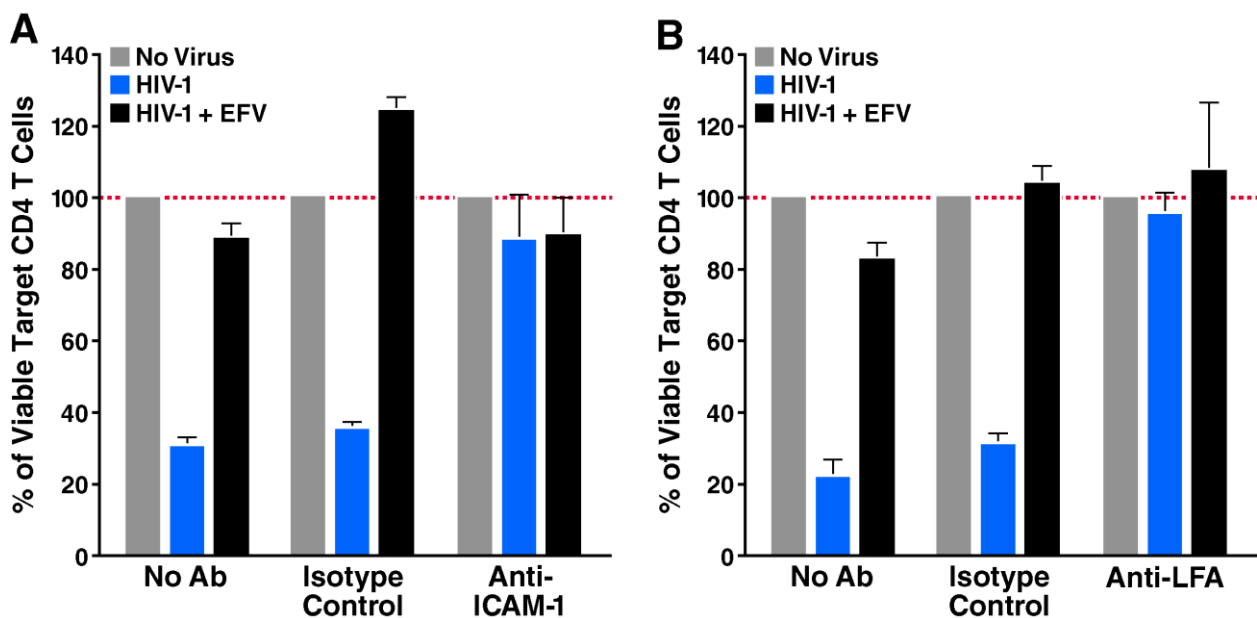
Figure 24. Transfer of virus via the virological synapse is required for CD4 T cell depletion A blocking antibody against the essential virological synapse receptors (A) ICAM-1 and (B) LFA but not an isotype control was able to protect the lymphoid CD4 T cells from abortive infection mediated depletion. These results show that transfer of virus via the virological synapse is essential for the depletion of lymphoid CD4 T cells.

Chapter 7. Characterization of GALT CD4 T cells
The majority of HIV infections begin with virus that uses CCR5 as a co-receptor for cellular entry. All patients progress to AIDS but 50-70% of patients (depending on the viral subtype they are infected with) switch from CCR5 to CXCR4 tropic infection right before reaching the AIDS stage of infection. These observations imply that CXCR4 switching is not required to reach the AIDS stage of infection.

Previous studies concentrating on the mechanism of HIV induced depletion during the acute phase of infection, observed that the highest rate of depletion occurred in the GALT. The massive loss of GALT resident CD4 T cells occurred regardless of the route of infection (vaginal or anal sex vs. injectible drug use) and continued throughout all phases of infection[79]. Characterization of this tissue showed that it consisted 95% of the body's memory CD4 T cells [81]. Concomitantly, CD4 T cells that express CCR5 are more highly activated, which permits productive infection, implying that most of these cells die by direct killing mechanisms. Indeed, Mattapallil et. al examined the mechanism of GALT CD4 T cell depletion and revealed that up to 80% died via direct killing [79]. Li et. al showed that SIV infection killed most GALT CD4 T cells via direct effects, but that indirect mechanisms may occur as well [80]. The difference between these conclusions may be explained by the use of higher viral dose in the studies conducted by Mattapallil et. al. Therefore, we did not know if CCR5 tropic virus could also abortively infect CD4 T cells and induce pyroptosis.

CXCR4 tropic virus better represents the chronic phase of infection. Most of our studies were limited to the use of CXCR4 tropic virus because tonsil, spleen, and blood derived CD4 T cells do not consist of a large population of CCR5 expressing CD4 T cells. We hypothesized that since the mesenteric lymph node is the closest lymph node to the GALT, that a larger subpopulation of CD4 T cells would be susceptible to depletion there. To analyze if a CCR5 virus can induce abortive infection mediated CD4 T cell depletion mesenteric lymph node derived lymphocytes were stained for CCR5 and then infected with CXCR4 (NL4-3) and compared to CCR5 (81A) tropic infected cultures. While CXCR4 tropic virus depleted up to 80% of mesenteric lymph node derived CD4 T cells, CCR5 tropic virus only depleted 20% (Figure 25). Also, TAK779, a CCR5 entry inhibitor, did not rescue killing by the CXCR4 virus. These results suggest that most CD4 T cells in mesenteric lymphoid tissue are susceptible to fusion of CXCR4 tropic virus but not CCR5. Since abortive infection requires fusion of virus, the mesenteric lymph node is not a suitable model for CCR5 tropic analysis.

We compared two protocols of CCR5+CD4 T cell extraction from GALT, scraping cells from the surface of the tissue versus collagenase treatment (Figure 26). While memory (CD45RO) CD4 T cells were obtained by both protocols, collagenase treatment extracted more CCR5+HLADR+ (a late activation marker) CD4 T cells. We also used the collagenase digestion protocol to compare the activation status of CD4 T cells in two different sections of the intestines, the jejunum, ileum, to tonsil (colon tissue was processed but had low viability) (Figure 27).

No fluorescent reporter viruses have been constructed to study R5 tropic infection, therefore we set out to close a BFP or a GFP sequence into the 81A plasmid. To construct the 81A-GFP, we disgested the ENV-GFP-IRES NEF sequence out of the NL4-4-GFP reporter virus and then

ligated the sequence into the 81A plasmid. To construct the 81A-BFP reporter virus we performed overlapping PCR to generate an ENV-BFP-IRES-NEF sequence and then used the same restriction enzymes to ligated into the 81A plasmid. Plasmids were sequenced and then virus was generated by HEK293T transfection. A HeLa cell line (TZMBL) which expresses both CXCR4 and CCR5 was infected with 81A-BFP and 81A-GFP. Infection was either blocked with TAK779 (blocks R5 infection) or AMD3100 (blocks CXCR4) infection. Up to 80% of TZMBL were susceptible to infection by either virus (Figure 28). All 81A infections were blocked by TAK779 (data not shown).
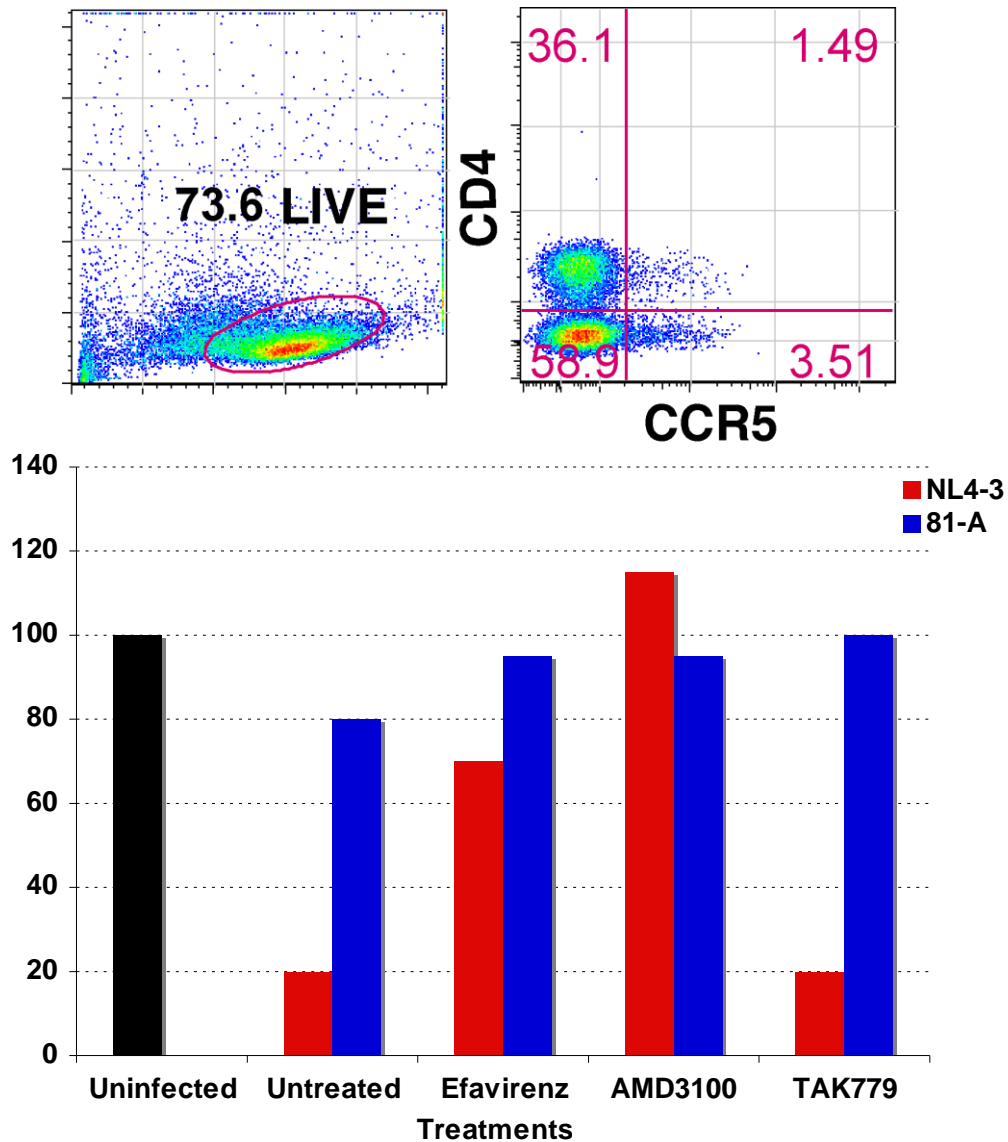
Figure 25. Abortive HIV infection mediated depletion of mesenteric lymph node
A. The majority of CD4 T cells in the mesenteric lymph node do not express enough CCR5 to be detectable by FACS analysis. **B.** The majority of mesenteric lymph node derived CD4 T cells are susceptible to abortive HIV infection mediated depletion when infected with CXCR4 tropic (NL4-3) but not CCR5 tropic (81-A) HIV virus. These results indicate that most CD4 T cells do not express enough CCR5 protein to be susceptible to fusion by an CCR5 tropic virus.
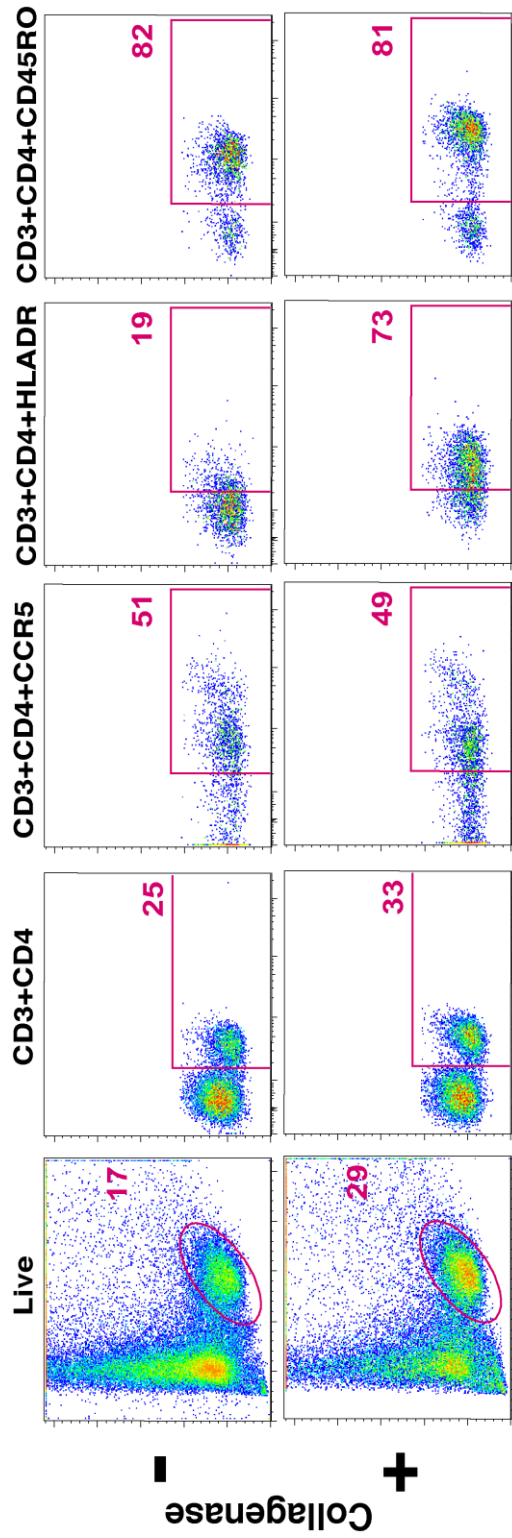
Figure 26. Comparison of two protocols for CCR5+CD4 memory T cell extraction
We attempted to either just scrape the surface of intestinal tissue or to treat the tissue with collagenase in order to extract CD4 T cells. Collagenase treatment resulted in being the most efficient method to extract memory CD4 T cells since more HLADR+ (late activation marker) CD4 T cells were extracted than by the scraping method.
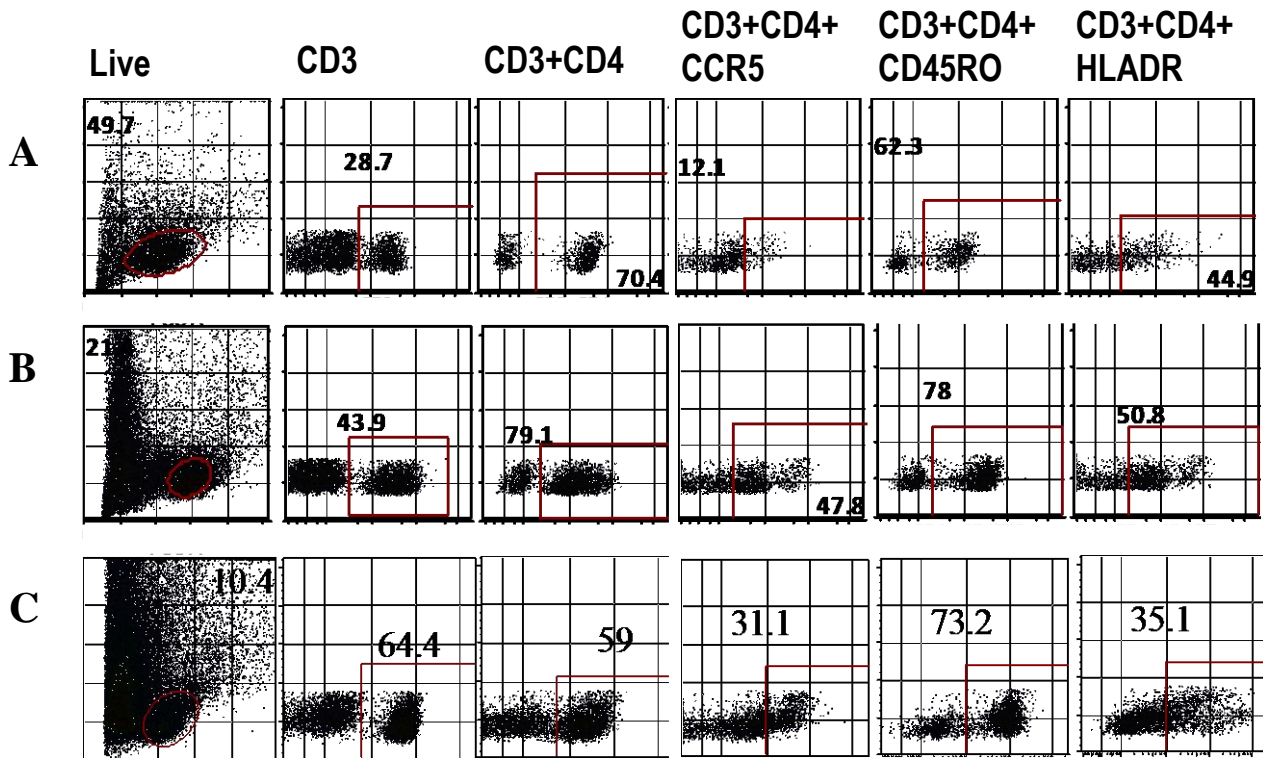
Figure 27. Comparison of the activation status of GALT vs tonsil derived CD4 T cells A. Analysis of tonsil tissue. **B.** Analysis of ileum GALT. **C.** Analysis of jejunal GALT. A much larger percentage of GALT (intestine) derived CD4 T cells express CCR5 when compared to tonsil tissue indicating that the GALT is more suitable for studying depletion of CD4 T cells infected with CCR5 tropic virus than tonsil.

81A-IRES-BFP
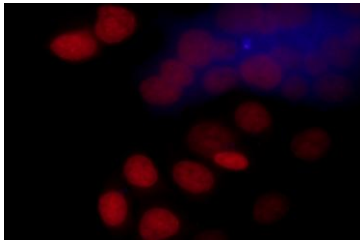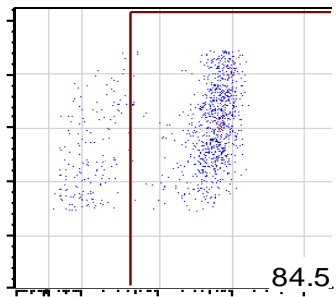
81A-IRES-GFP
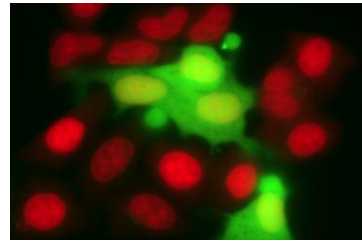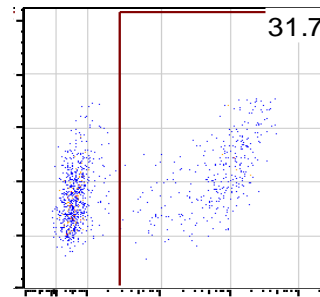
Figure 28. Testing of a CCR5 BFP and GFP reporter HIV virus
A BFP-IRES-NEF sequence was cloned into the lab adapted viral plasmid 81-A to detect productively infected cells in tonsil versus GALT. C. The infection of the permissive monocytic cell line TZMBL with 81A-BFP and 81-A-GFP resulted in productive HIV infection.

CHAPTER 8: Resting Blood CD4 T cells are Resistant to cell death by Pyroptosis during HIV infection

Peripheral blood tissue is used to analyze progress in HIV patients, monitor the phases of infection by counting CD4 T cells and determining viral load, and to study the mechanisms of pathogenesis. Despite our previous results showing that lymphoid derived CD4 T cells die via pyroptotic cell death mediated by abortive infection, we still did not know if blood derived CD4 T cells died by the same mechanism. To compare the depletion of peripheral blood to tonsil derived CD4 T cells, we performed experiments with the HLAC system (Figure 29A).

Similar to previous results, target tonsil CD4 T cells were depleted in the presence or absence of AZT but rescued from death when the cultures were pre-treated with EFV [87] (Figure 29B). These results confirm that tonsil CD4 T cells are intrinsically susceptible to depletion by abortive infection. These data also suggest that the viral replication cycle must progress beyond the initiation phase of RT for cell death to occur and does not require integration.

Most blood CD4 T cells are not permissive to productive infection. Thus, to generate an efficient population of productively infected effector cells, we needed to activate peripheral blood lymphocytes (PBL) with the mitogen phytohemagluttin A (PHA) and IL2 (Figure 29A). Co-cultures were then used to analyze depletion of resting target blood CD4 T cells (Figure 29 C). Strikingly, CD4 T-cell depletion was not observed in PBL co-cultures (Figure 29C). These results raised the possibility that interaction with the lymphoid tissue derived cells may impact the blood cell susceptibility to abortive infection mediated pyroptosis.

Given the striking difference in pathogenesis between these two tissues, we wanted to further explore the cause of the target PBL's resistance to depletion. To analyze the efficacy of viral production and viral dissemination from productively infected (effector) PBL, effector PBL were then co-cultured with target tonsil cells (Figure 29D). Under these conditions, target tonsil CD4 T cells once again died with or without AZT treatment, but were protected from depletion by EFV pretreatment. These results indicate that PHA/IL2 pretreatment of PBL generates a large and efficient enough population of virus producers to induce HIV depletion of tonsil but not blood derived CD4 T cells.

CD4 T cells continuously migrate between the peripheral blood and lymphoid tissue. Unless they encounter cognate antigen, naive lymphocytes reside in lymphoid tissue for 12–18 hours before re-entering the peripheral circulation [112] [113]. Since the lymphoid tissue environment can change the activation status of naive lymphocytes [93,114], we assessed whether target PBLs were susceptible to depletion induced by abortive infection following co-culture with productively infected tonsil cells (Figure 29E). Remarkably, blood CD4 T cells were readily depleted when co-cultured with tonsil effectors. The depletion of blood CD4 T cells in these co-cultures displayed the same drug sensitivity as tonsil CD4 T cells: EFV but not AZT blocked the depletion. Spinoculated co-cultures replicated the same phenotypes as the HLAC system (data not shown). These results show that co-culture with infected tonsil cells enhances pyroptosis of blood CD4 T cells abortively infected with HIV. These results also imply that lymphoid tissues

but not blood, contain one or more factors that enhance this mechanism of HIV induced CD4 T cell depletion.

The tonsil and blood lymphocytes in these experiments, originated from different donors, thus we excluded the effects of a mixed lymphocyte reaction on blood CD4 T cell depletion by co-culturing blood derived lymphocytes from different donors (Figure 30). Upon co-culture with PBL from a different donors, no target blood CD4 T cell depletion was observed. These results show that heterologous co-culture is not sufficient to increase susceptibility to HIV induced depletion.
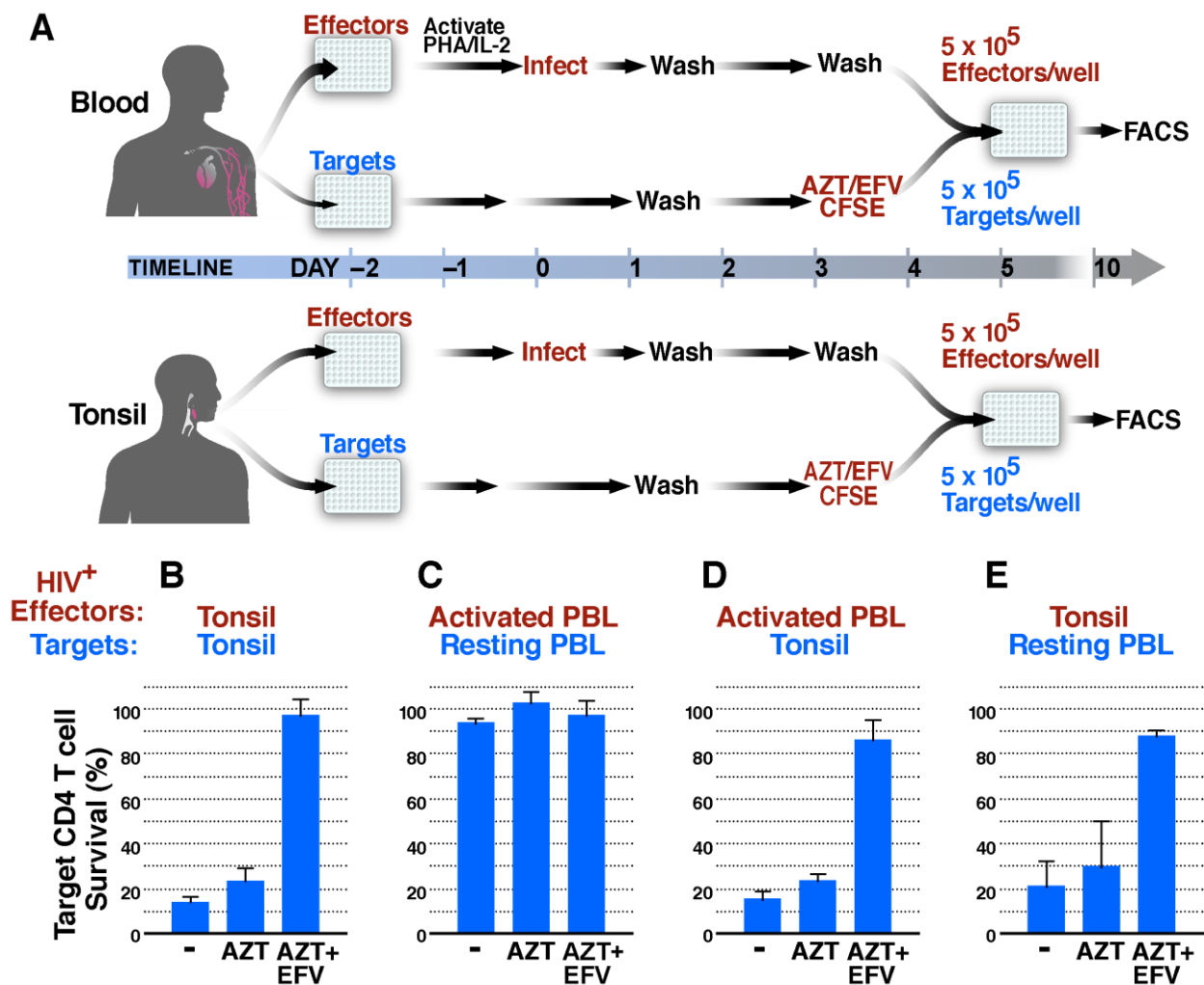
Figure 29. Blood CD4 T cells are resistant to HIV mediated depletion unless co-cultured with productively infected tonsil cells
A. Scheme of the HLAC system when applied to blood CD4 T cells co-cultures. Unlike the tonsil derived CD4 T cells, where up to 5% are naturally permissive to infection, blood derived CD4 T cells need to be activated for efficient productive infection to occur. **B.** At least 80% of target tonsil CD4 T cells are depleted when co-cultured with productively infected tonsil cells. Depletion requires reverse transcription initiation. **C.** No depletion of target resting blood-derived CD4 T cells is observed when co-cultured with productively infected blood CD4 T cells. **D.** Co-culture of target tonsils with productively infected blood CD4 T cells is sufficient to induce their depletion, therefore blood CD4 T cells produce and transfer sufficient virus to kill tonsil CD4 T cells. **E.** Co-culture with productively infected tonsil cells enhances depletion of target blood CD4 T cells in a reverse transcription initiation dependent manner (EFV blocks killing).

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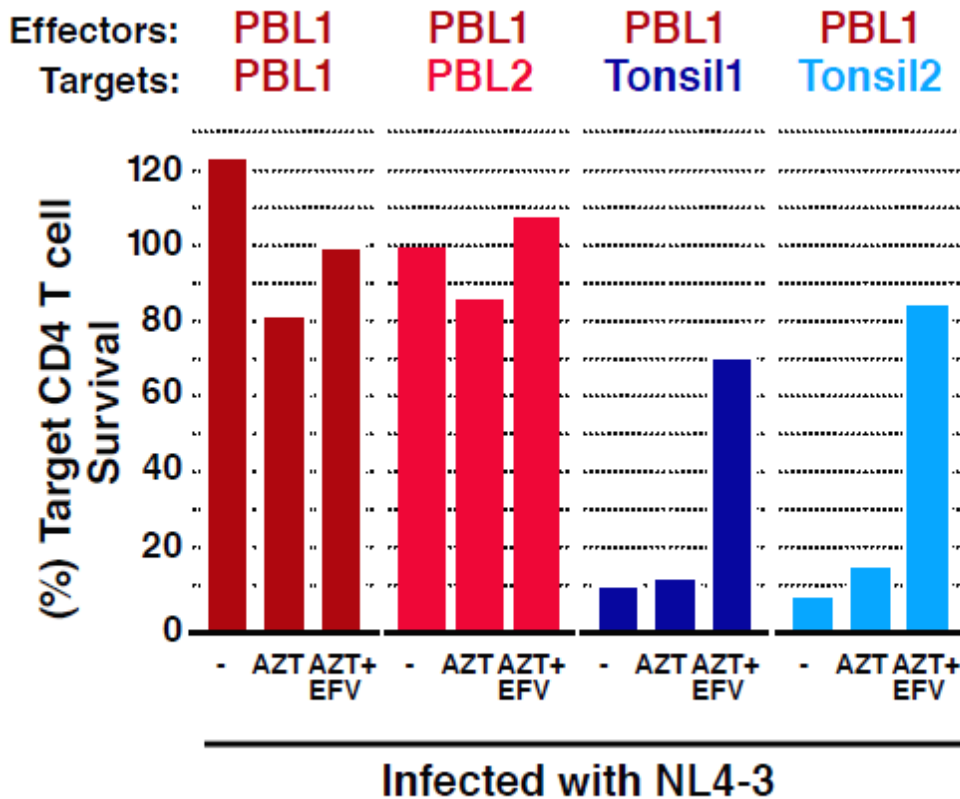
Figure 30. Heterologous co-culture is not sufficient to induced HIV mediated blood CD4 T cells depletion. Co-culture with productively infected blood lymphocytes from one (donor 1) does not result in depletion of target resting blood CD4 T cells from a different donor (donor 2) showing that a mixed lymphocyte reaction between tonsil and blood CD4 T cells from different donors would not be sufficient to enhance depletion of blood CD4 T cells in our system.

Both the HLAC and spinoculation co-culture systems present various limitations. The HLAC system requires up to 10 days of culture. One of the limitations is the requirement of an efficient viral producing population of lymphoid derived-lymphocytes to see death of target cells (Chapter 6). This limitation in particular requires that at least 50% of the cells be present just for viral production, making it difficult to characterize the role of lymphoid derived cells in enhancing blood CD4 T cell depletion. For example, we cannot characterize if blood CD4 T cells remain susceptible to depletion after purification from tonsil co-culture because then there would be no virus producing cells. We cannot analyze whether transfer of the virus from tonsil cells are the only requirement for enhancement of blood CD4 T cell depletion. In light of these limitations we set up a system that would not require the tonsil cells as effectors, the 293T co-culture system (Figure 31).

Specifically, both PBL and tonsil lymphocytes were co-cultured with HEK293T cells transfected with an HIV-GFP reporter plasmid, p-NLENG-1 (Figure 32). This plasmid expresses GFP under

the control of the HIV promoter, making GFP a direct reporter of viral gene expression or productive infection.

To determine if the HEK293T co-culture system could show enhancement of blood CD4 T cell depletion upon co-culture with tonsil cells, tonsil derived lymphocytes were first labeled with 7-amino-4-chloromethylcoumarin (CMAC) to differentiate them from PBL before adding them to the transfected HEK293T cultures. This culture system replicated the results previously observed with the HLAC system, e.g blood derived CD4 T cells were only susceptible to depletion when in the presence of tonsil lymphocytes (Figure 32A). Greater depletion of blood CD4 T cells occurred in cultures that contained a higher ratio of tonsil cells (Figure 32B). This ratio could be adjusted depending on the number of amount of viral DNA transfected. In some co-cultures depletion of blood CD4 T cells was observed when tonsil cells made 10% of the lymphocytes (90% PBL) in the co-cultures (Figure 32B), but in order to insure rescue by EFV we kept the amount of viral DNA between 50-100ng and therefore required at least 50% of the cells to be lymphoid tissue derived cells. Spleen cells (splenocytes), also enhanced depletion of blood CD4 T cells, implying that this phenotype is not limited to tonsil tissue (Figure 33) . These results demonstrate that enhancing HIV-induced depletion is not limited to tonsil tissue, but results from fundamental differences in the peripheral blood versus lymphoid tissue environments.
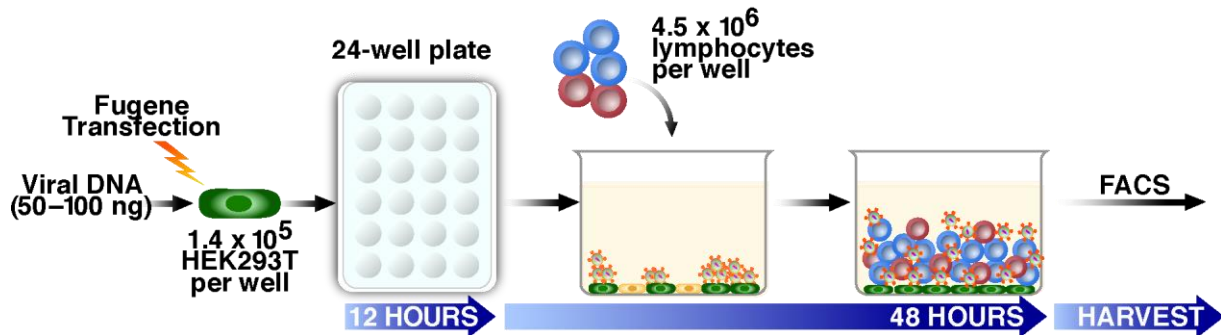
Figure 31. The HEK293T co-culture system
HEK293T cells are plated at 1.6-1.4x10^5 cells/well then transfected with viral DNA. Lymphocytes (4.5 x10^6) are added 12 hours later and then harvested two days later.
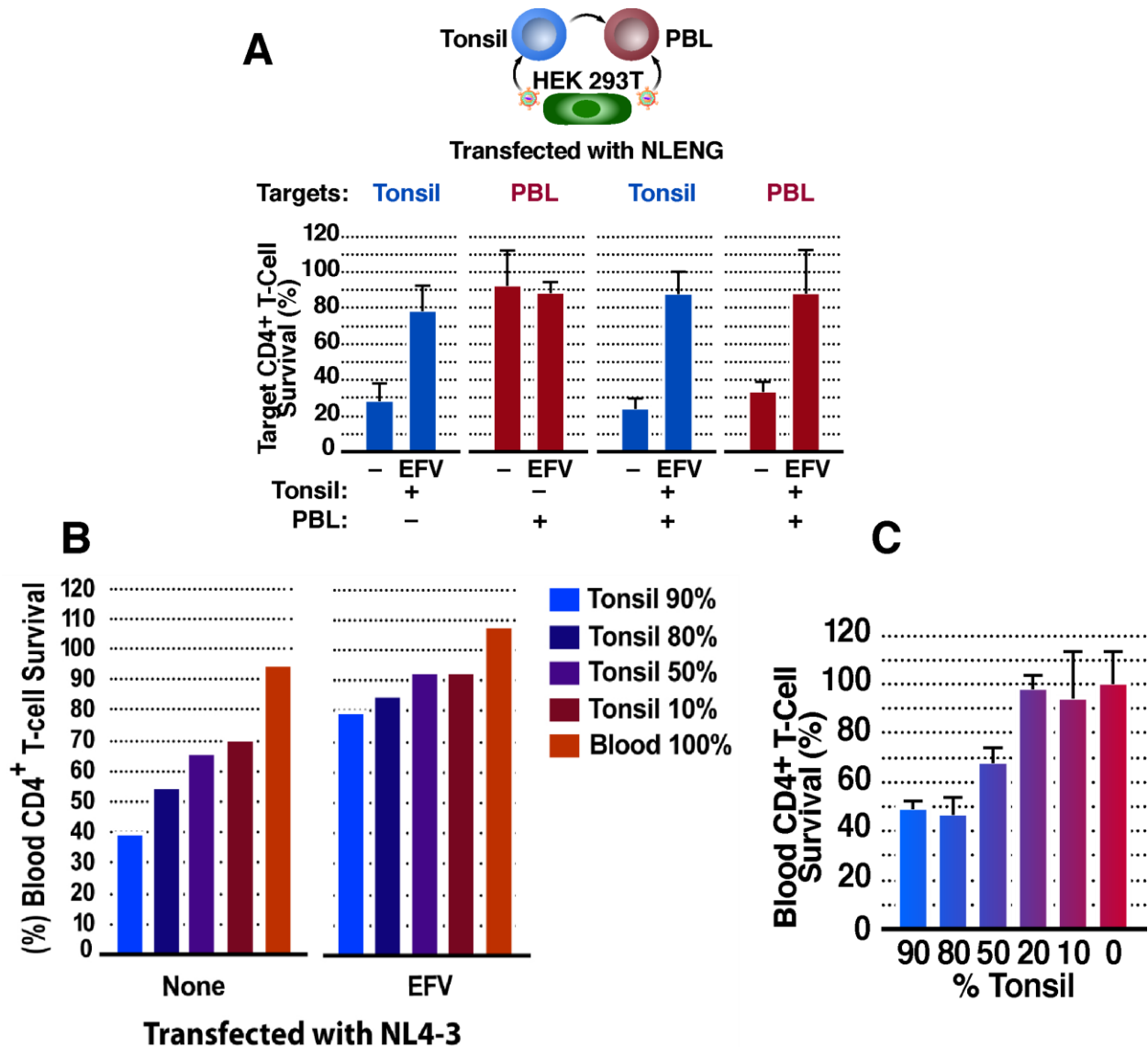
Figure 32. Co-culture with tonsil cells via the HEK293T co-culture system results in blood derived CD4 T cell depletion

A. Blood derived lymphocytes were co-cultured with CMAC stained tonsil cells and either left untreated or treated with EFV before adding to NLENG1-IRES-GFP transfected HEKHEK293T. Blood CD4 T cells were only susceptible to depletion in the presence of tonsil cells. **B.** Depletion of blood CD4 T cells can be observed in cultures containing as low a ratio of tonsil cells as 10% tonsil cells/90% blood lymphocytes. The depletion at all ratios of tonsil is efavirenz dependent. **C.** Although depletion can be observed with as low a ratio of tonsil cells as 10%, A significant amount of depletion can only be see when co-cultures contain 50% or more tonsil cells.
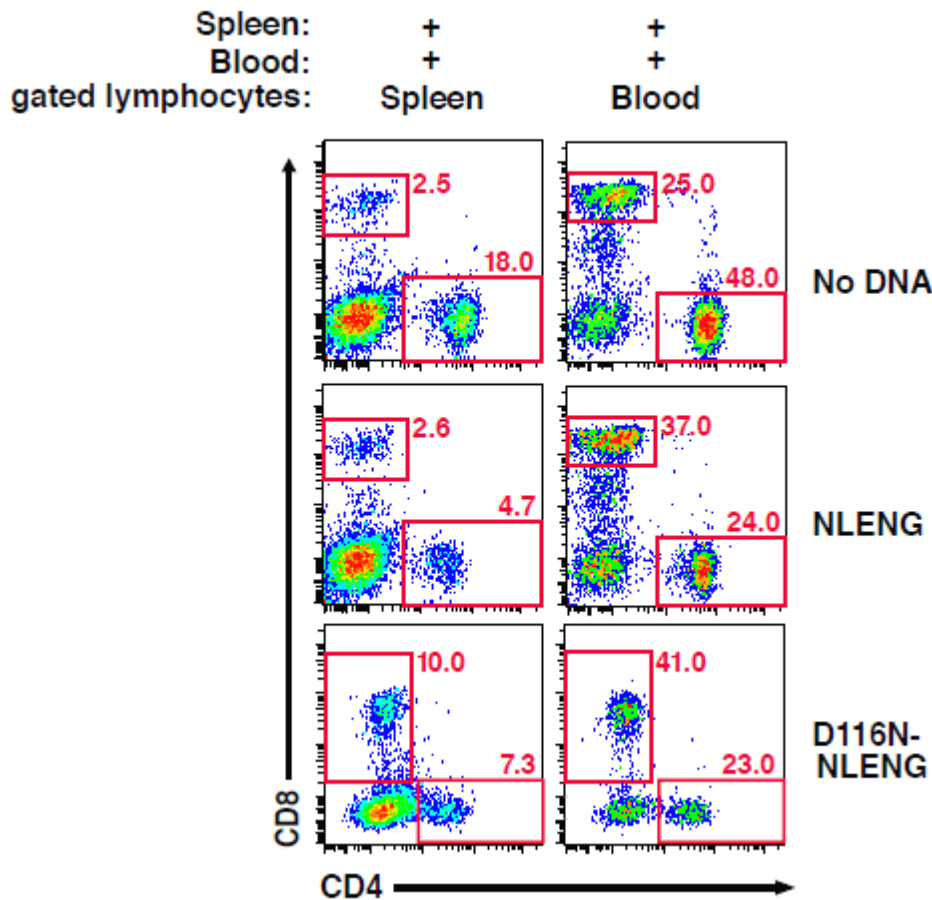
Figure 33: Co-culture with splenocytes results in blood CD4 T cell depletion
Blood derived lymphocytes were co-cultured with CMAC stained splenocytes and NLENG1-IRES-GFP transfected HEK293T. Blood CD4 T cells were only depleted in co-cultures containing splenocytes implying that the ability to enhance depletion of CD4 T cells during HIV infection is not limited to tonsil tissue.

We next investigated whether close cell-cell interactions between tonsil lymphocytes and blood CD4 T cells were required for death of blood CD4 T cells. PBLs were added to NLEG-IRES-GFP transfected HEK293T cells and separated from tonsil cells by a 0.4-μm transwell. Under these conditions, HEK293T cells directly delivered the virus to blood CD4 T cells, but the transwell interrupted any surface interactions between blood and lymphoid cells. Under these conditions, blood CD4 T cells were not depleted (Figure 34), indicating that close interactions between lymphoid and blood CD4 T cells were required. This finding is consistent with the inability of tonsil supernatants to render blood CD4 T cells sensitive to pyroptosis induced by HIV abortive infection (data not shown).
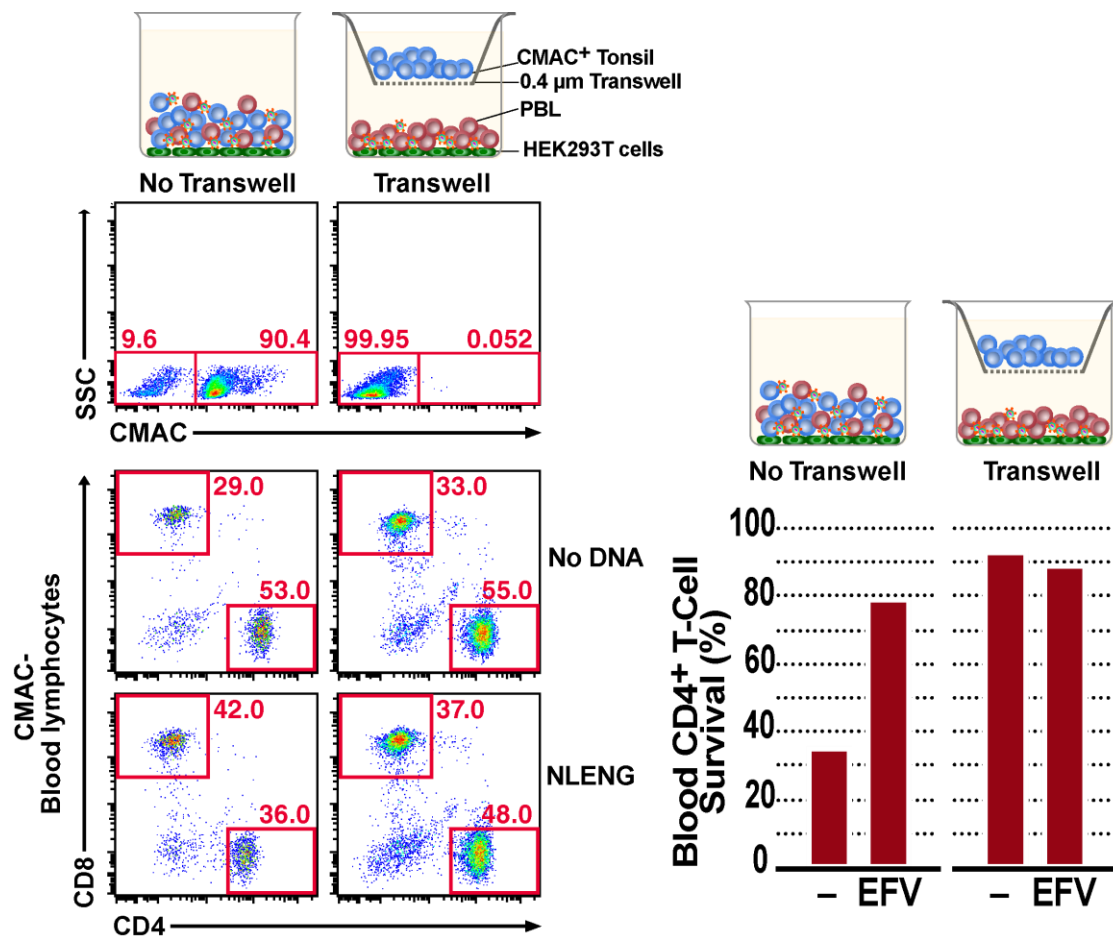
Figure 34: Blood derived CD4 T cell depletion requires close interactions with lymphoid tissue lymphocytes

Blood derived cells were allowed to interact directly with NLENG1-IRES-GFP transfected HEK293T while tonsil cells were separated by a 0.4um transwell. Blood CD4 T cell depletion was only observed in cultures not separating blood CD4 T cells from tonsil cells.

Blood CD4 T cell depletion may require direct delivery of virions from productively infected tonsil CD4 T cells, which would have been disrupted by the transwell system. To investigate this possibility, HEK293T cells were transfected with an integrase defective viral plasmid, D116N-NL4-3-GFP[115]. Here, HEK293T cells served as the only source of virus, since the virus cannot integrate into the genome of tonsil CD4 T cells. Despite the inability of the virus to integrate within tonsil CD4 T cells, they were effectively depleted in these cultures, (Figure 35). Confirming once again that productive infection of tonsil CD4 T cells is not required for their depletion. Blood CD4 T cells placed in co-culture with tonsil cells and D116N-transfected HEK293T were also depleted. These results indicate that tonsil CD4 T cells need not be productively infected to render the blood CD4 T cells sensitive to abortive infection and pyroptosis.
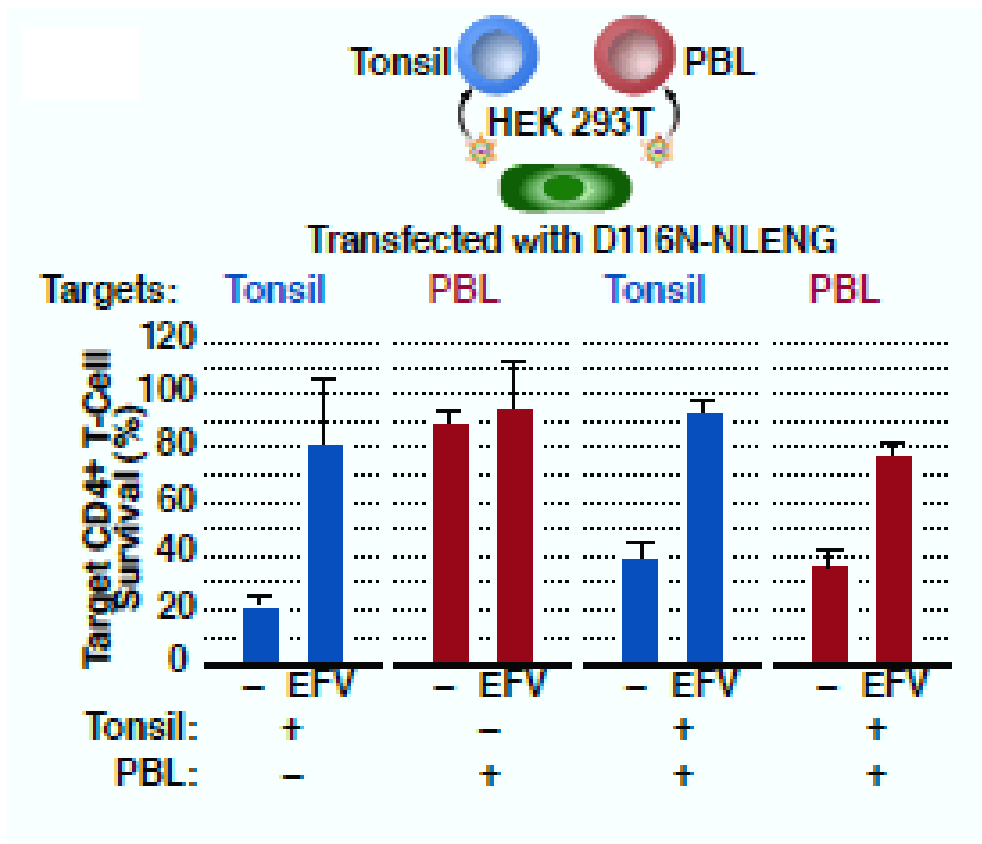
Figure 35: Productive infection of lymphoid CD4 T cells is not essential for blood CD4 T cell depletion
CMAC stained tonsil, and blood derived CD4 T cells were co-cultured with the integrase deficient viral plasmid D116N-NLENG1-IRES-GFP transfected HEK293T. Depletion of blood derived CD4 T cells was only observed when in the presence of tonsil cells.

Next, we performed studies to determine whether tonsil CD4 T cells uniquely contained the factor(s) needed to make blood cells sensitive to death. CMAC-labeled CD4 T, CD8 T, and B cells were purified from tonsil tissue using MACS Miltenyi beads. Purity of each sample was checked by staining for CD4 and CD8 (Figure 36A). Purified populations with less than 90% purity were not used. These purified lymphocytes were then individually co-cultured with PBL and HEK293T cells. All three populations of lymphocytes rendered blood CD4 T cells susceptible to depletion (Figure 36B). Thus, many different types of lymphoid tissue-derived cells express lymphoid factor(s) that enhance HIV induced CD4 T cell depletion. These results also refute a mixed lymphocyte reaction being involved in the depletion of the blood CD4 T cells since samples lacking tonsil-CD8 T cells (purified B and CD8 T cell samples) were still capable of sensitizing blood CD4 T cells to death. These results also refute the need for productive infection or pyroptosis of lymphoid CD4 T cells to enhance depletion of blood CD4 T cells, since samples lacking tonsil CD4 T cells (purified B and CD8 T cell) samples still enhanced depletion of blood CD4 T cells.
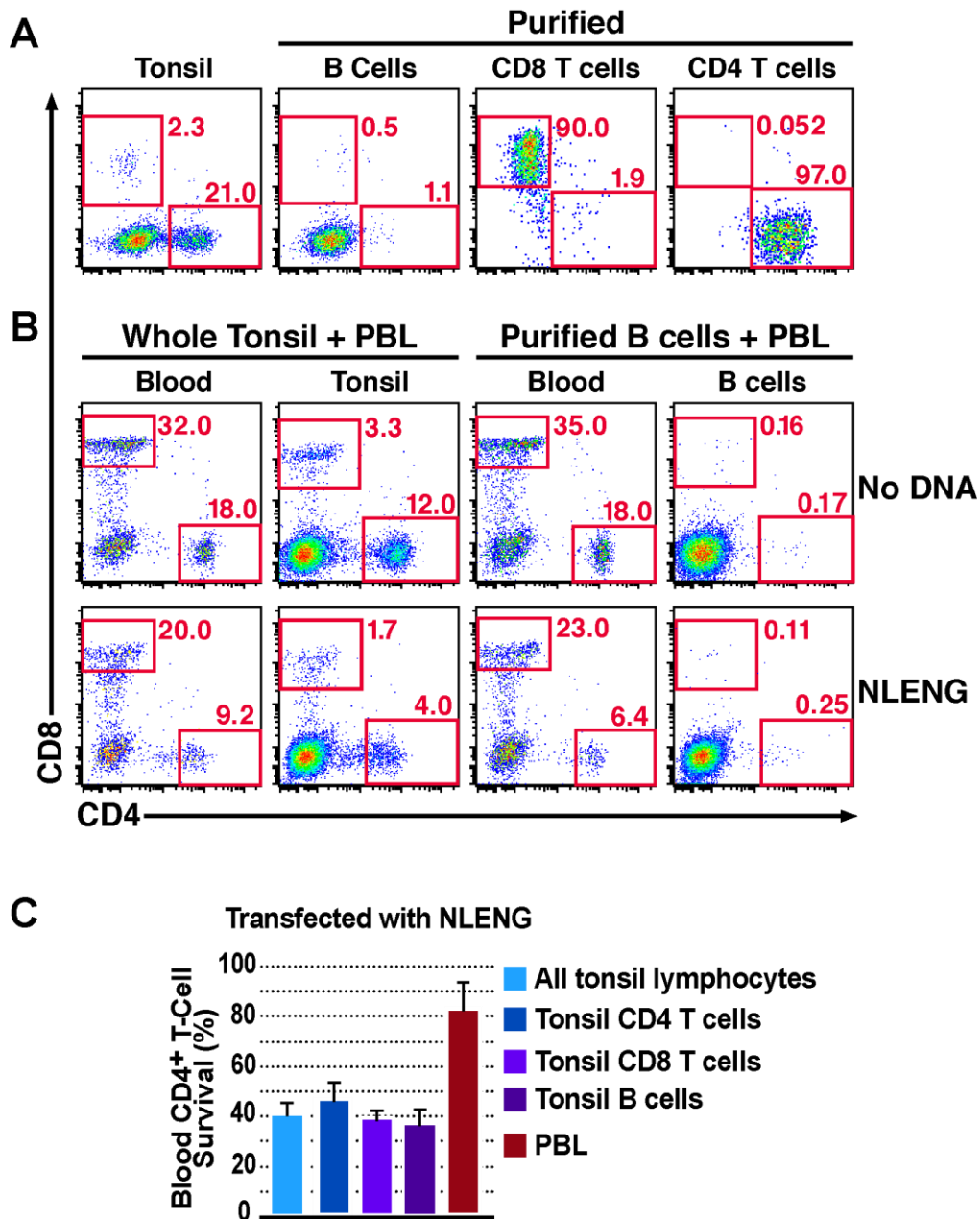
Figure 36: All subsets of tonsil lymphocytes enhance blood CD4 T cell depletion
A. CMAC stained B, CD8 and CD4 T cells were purified from CMAC stained tonsil tissue. Purity was confirmed to be greater than 90% (for an efficient depletion of blood CD4 T cells at least a 50% ratio of tonsil is required) with each purification before the purified cells were co-cultured with blood derived lymphocytes. **B.** Shown are the percentages of (tonsil and blood derived) CD4 and CD8 T cells in wells containing all the tonsil lymphocytes or just the B cells. **C.** Blood CD4 T cell depletion was seen when these cells were co-cultured with any of the purified tonsil lymphocytes subpopulations (monocytes, dendritic cells, and macrophages are rare (>0.5%) in tonsil tissue), implying that death or productive infection of tonsil CD4 T cells is not essential for blood CD4 T cell death.

Abortively infected tonsil CD4 T cells die via pyroptosis induced by IFI16 binding to HIV RT products [87,95 Monroe, #94]. We next determined whether co-culture with tonsil lymphocytes depletes blood CD4 T cells in a caspase-1 dependent manner. Of note, pyroptosis requires Caspase-1 activation while apoptosis requires Caspase-3. Lymphocytes were pretreated with the peptide inhibitors targeting caspase-1, ZWEDH-FMK, caspase-3, ZDEVD-FMK, or all caspases, ZVAD-FMK and then overlaid on HIV-transfected HEK293T cells. Both tonsil and blood CD4 T cells died despite Z-DEVD-FMK (Caspase-3 inhibition) pre-treatment (Figure 37). However, Z-WEDH-FMK (Caspase-1 inhibition) and Z-VAD-FMK (Pan-caspase inhibition) pre-treatment effectively blocked depletion of blood CD4 T cells. These results support the conclusion that lymphoid cells sensitize blood CD4 T cells to pyroptosis following abortive HIV infection.
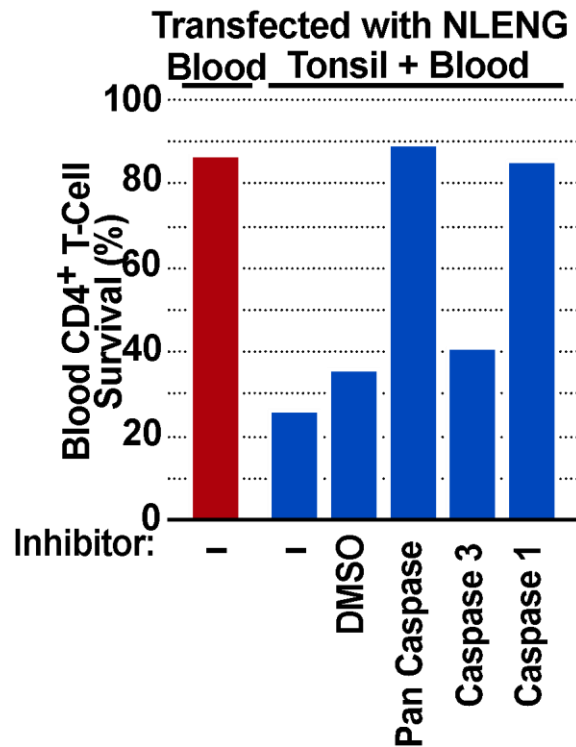
Figure 37: Co-culture with lymphoid cells results in a caspase-1 dependent cell death of blood CD4 T cells.
Co-cultures of blood and CMAC stained tonsil lymphocytes were treated with caspase peptide inhibitors targeting all caspases, caspase-3 (representative of apoptosis), or caspase-1 (representative of pyroptosis). Blood CD4 T cells were protected from depletion when treated with the pan-caspase and caspase-1, but not with the caspase-3 inhibitor implying that blood CD4 T cells are dying via pyroptosis not apoptosis when co-cultured with tonsil cells.

Under certain circumstances, inducing pyroptosis can require two signals. The first signal involves activating NF-κB, while the second involves activating caspase-1 via inflammasome assembly [73,116]. These two pathways are not always mutually exclusive since the activation of caspase-1 itself often requires NF-κB induced sensors such as NALP3 (Chapter 3). To determine the characteristics that make blood CD4 T cells resistant to HIV-induced pyroptosis, we purified tonsil CD4 T cells, and resting (CD25-) and activated (CD25+) blood CD4 T cells and compared the expression of inflammasome components, such as IFI16, and apoptosis-speck-like-protein (ASC). Both IFI16 and ASC were expressed in tonsil CD4 T cells. ASC was expressed in both resting (CD25-) and activated (CD25+ and PHA/IL2-treated) cells (Figure 38), but IFI16 was only expressed in activated (CD25+ and PHA/IL2 treated) CD4 T cells.
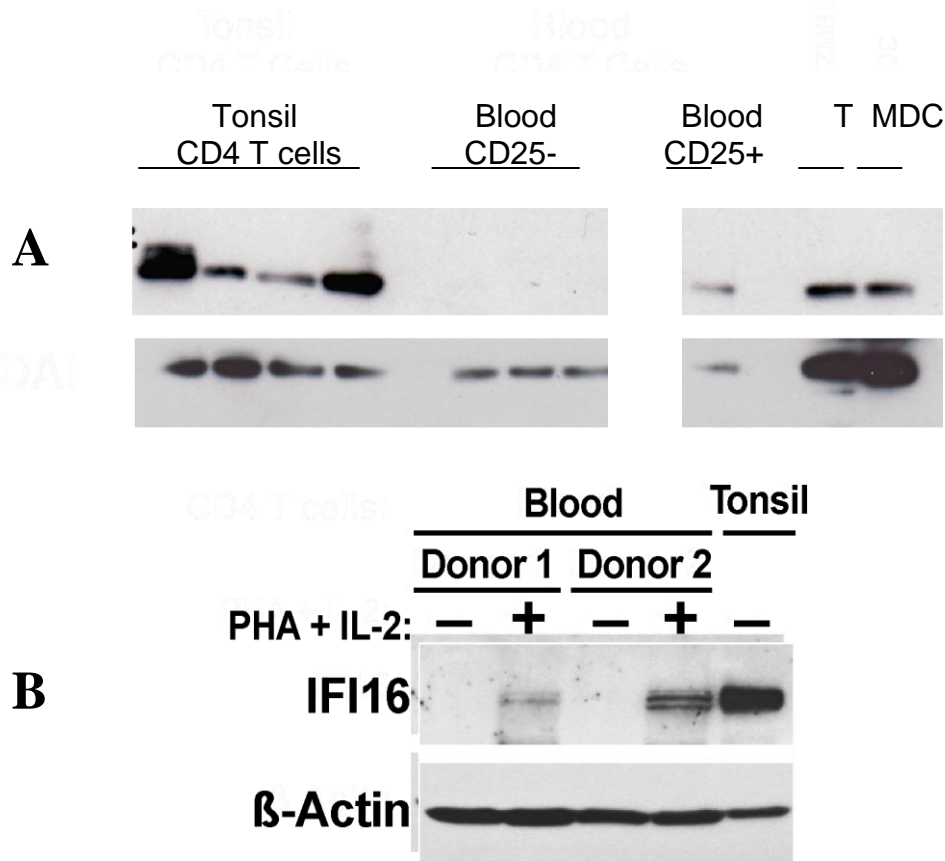
Figure 38: Blood derived CD4 T cells constitutively express ASC but only express IFI16 when activated
A. IFI16 expression was compared in CD4 T cell derived from tonsil and blood. Resting (CD25-) were purified and compared to activated (CD25+) blood CD4 T cells. T indicates the monocytic cell line TZMBL, and MDC indicated monocytes derived dendritic cells. **B.** Cell lysates from tonsil CD4 T cells, or CD25- or PHA/IL2 treated blood CD4 T cells were blotted for ASC, IFI16, and β-Actin. ASC and β-Actin were expressed in all cells. IFI16 was only expressed by tonsil CD4 T cells, or CD25+ and PHA/IL2 activated blood CD4 T cells.

To compare the level of IFI16 mRNA in blood CD4 T cells, we performed qPCR on cells either resting (CD25-) or activated with PHA and IL2 for 48 hours. Activated blood CD4 T cells contained up to 3.5 fold more IFI16 mRNA than resting blood CD4 T cells (Fig 39). These results indicate that the majority (>95% of blood derived CD4 T cells are CD69-CD25-) of blood CD4 T cells do not express IFI16, which is the sensor responsible for inducing pyroptotic cell death in up to 95% of tonsil, spleen, and lymph node CD4 T cells during HIV infection (Chapter 4).
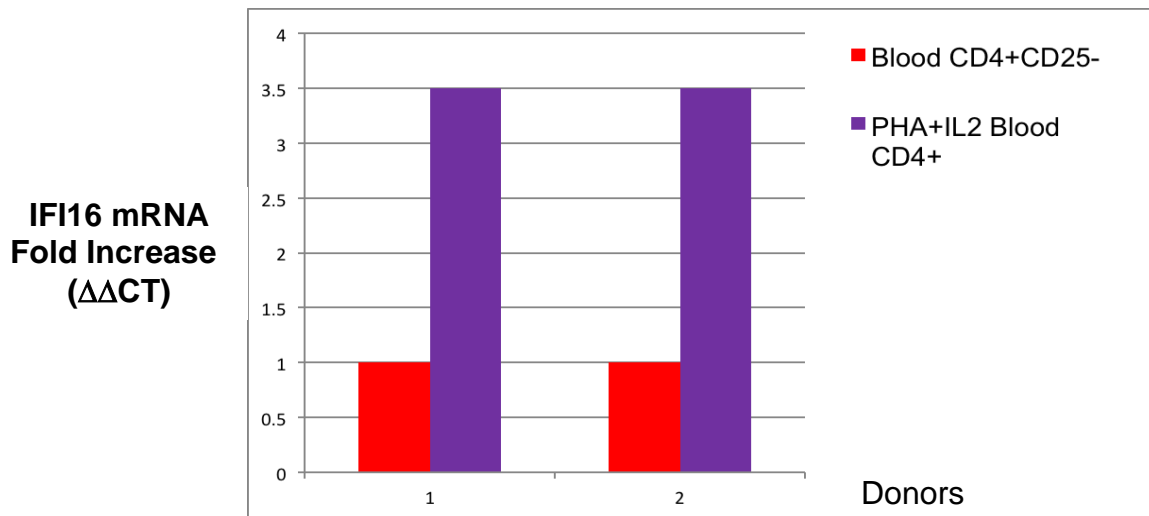
Figure 39: Blood CD4 T cells sorted from tonsil co-culture contain IFI16 mRNA
A. FACS plots of cells before and after purification by sorting. CMAC stained tonsil were co-cultured with blood lymphocytes for two days. Blood CD4+CD25- cells were sorted. **B.** Fold increase in IFI16 mRNA when normalized to resting (not co-cultured) blood CD4 T cells.

Monocytes express a constitutively activated caspase-1[72,117] that impairs the susceptibility of these cells to pyroptosis. In monocytes, caspase-1 activity is maintained through the constant secretion of ATP, which is sensed by the NALP3 sensor[72,117]. Surprisingly, constitutively activated caspase-1 can only cleave inactive cytokines such as pro-IL1b and pro-IL18, but cannot induce pyroptosis. The mechanism that prevents constitutively activated caspase-1 from inducing pyroptosis in these cells remains unknown[117]. Physiologically, monocytes require sensing of a PAMP to differentiate into macrophages, which are then recruited to infected or damaged tissue. Once monocytes differentiate into macrophages, constitutively activated caspase-1 is degraded. Thus, we hypothesized that blood CD4 T cells express constitutively activated caspase-1, which might serve as an added layer of protection for the host by restricting pyroptosis to damaged or infected tissue and thereby avoid systemic inflammation. We hypothesized that besides lack of IFI16 expression, an impaired caspase-1 in blood CD4 T cells, could serve as an additional barrier to pyroptosis in blood CD4 T cells. We compared the expression of pro-caspase-1 (50 kDA) and activated caspase-1 (p20, 20 kDA) in tonsil and blood CD4 T cells to blood monocytes and macrophages (Figure 40). Macrophages served as a negative control for constitutively activated caspase-1. Pro-caspase-1 expression was similar in all cell lysates but constitutively activated caspase-1 was only observed in monocytes.
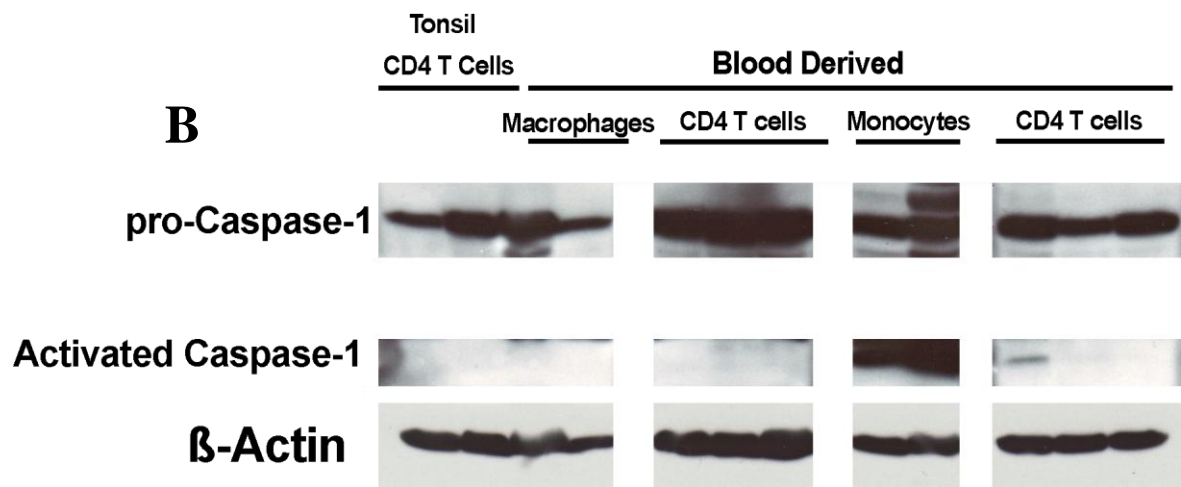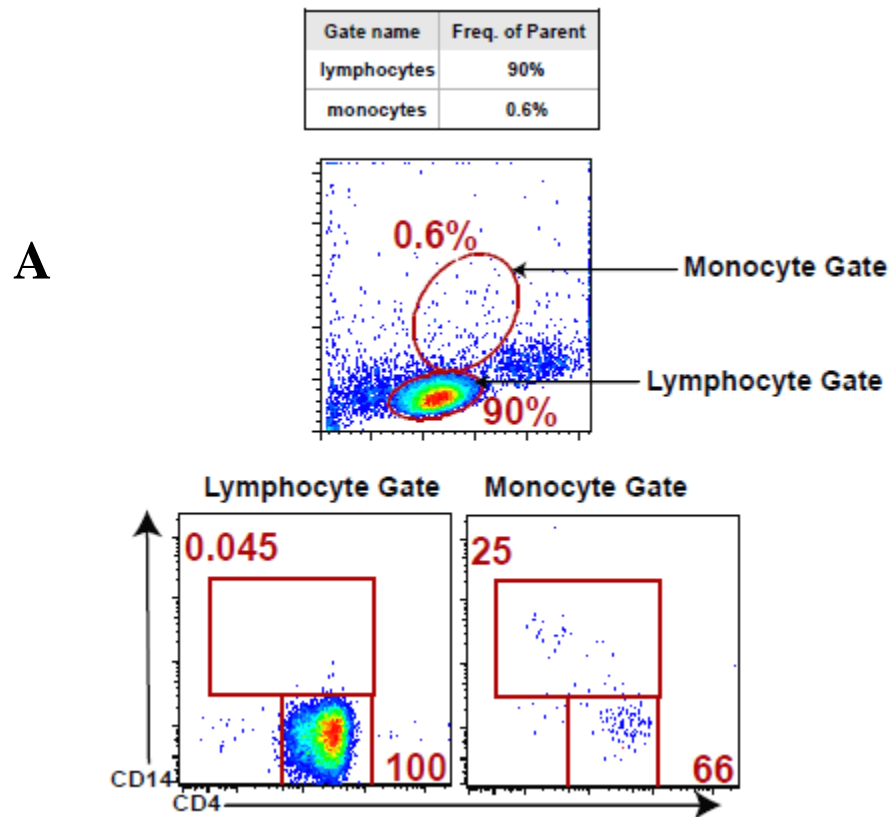
Figure 40: Blood derived CD4 T cells do not express a constitutively activated Caspase-1 Blood derived monocytes, macrophages, and CD4 T cells and tonsil CD4 T cells were blotted for pro-caspase-1 (50kDA) and activated caspase-1 (20kDA). **A.** Cultures had to have a high purity (99.9%) of CD4 T cells so that the constitutively activated signal from monocytes would not show on the blot for CD4 T cells. **B.** Only cultures with blood derived monocytes expressed a cleaved form of caspase-1.

To test if NF-κB activation is induced in blood CD4 T cells co-cultured with lymphoid cells, blood and CMAC-stained tonsil lymphocytes were co-cultured for 2 days, similar to the 293T co-culture system, and then stained with fluorescently tagged antibodies targeting the phosphorylated Ser-536[118] Rel-A subunit of NF-κB. Examination of this activity is convenient for testing NF-κB activation because the phosphorylation is executed by the IKK complex only after stimulus-coupled degradation of IκBα[118,119]. PHA/IL2-treated blood CD4 T cells were used as a positive control. To calculate the fold increase in MFI in cells, CD4+ T cells were gated, then the average MFI of these cells then divided by the average MFI of the resting (isolated) blood CD4 T cells. The average fold increase of the median fluorescence intensity (MFI) for phosphorylated Rel-A/p65 in activated CD4 T cell compared to unstimulated (CD25- blood CD4 T cells) cultures was 3.06. In blood CD4 T cells co-cultured with tonsil cells, there was a average 2.0 fold increase in MFI of Rel-A Ser536 phosphorylation when compared to unstimulated blood CD4 T cells. To test whether this low level of activation could upregulate key proteins required for pyroptosis, we also stained the cells for IFI16 (Figure 41). There was up to a two-fold increase in the MFI for IFI16 in blood CD4 T cells co-cultured with tonsil cells when compared to unstimulated blood CD4 T cells.

To test if co-culture with lymphoid-derived cells induced transcription of IFI16, blood CD4+CD25- T cells were first sorted (Figure 42A) from 48 hours of co-culture with tonsil cells. Then mRNA was purified from the CD4+CD25- cell lysates, and analyzed by qRT-PCR. IFI16 mRNA was increased up to two-fold in blood CD4 T cells sorted from co-culture as compared to blood CD4 T cells cultured alone (blood CD4 T cells cultured alone contained 3.3 copies of IFI16 mRNA) (Figure 43B).

To analyze if this low level of NF-κB activation was sufficient to induce target protein expression we compared expression of surface NF-κB activation markers such as CD69, CD25, and HLADR. The expression of these proteins on the surface of CD4 T cells serve as markers of early, intermediate, and late stages of activation, respectively[120]. The majority of blood CD4 T cells did not upregulate these proteins upon co-culture with tonsil cells, although there was an increase in the percentage of CD4 T cells expressing them. Collectively, these results imply that upon close interaction with lymphoid cells, blood-derived CD4 T cells are sensitized to pyroptosis by inducing a very mild activation. This activation is strong enough to induce NF-κB phosphorylation via the IKK complex, up-regulate IFI16, an essential component for HIV mediated pyroptosis, but not all targets (CD25, CD69, HLADR)(Figure 43). Also, this activation is sufficient to sensitize the cells to pyroptosis but not to productive HIV infection or direct killing, as AZT pretreatment does not rescue the cells from death (Figure 29, 32, and 35).

To investigate if NF-κB could directly induce IFI16 expression, we blasted the consensus NF-κB sequence in the IFI16 promoter sequence and found one target non-canonical sequence (TGGGCTTTCC) in the minus strand at -568 to -558 implying that NF-κB may directly induce IFI16 expression in lymphoid CD4 T cells. NF-κB can induce genes equally from both DNA strands. As previously reported, the consensus sequence for AP-1 (TGACTC) is located at 73–78 on the (+) strand[121].

To test if NF-κB directly induced IFI16 up-regulation, the promoter sequence of IFI16 was ligated to a luciferase reporter and transfected with or without Rel-A into HEK293T cells and

then the fold increase in luciferase was calculated (Figure 44). Under these conditions we found no luciferase-IFI16 induction, implying that NF-κB does not directly induce IFI16 expression. Further experiments are necessary to test if NF-κB induces IFI-16 via activation of c-Jun, a component of the AP-1 complex which has been shown to induce IFI16 expression post type I interferon signaling.
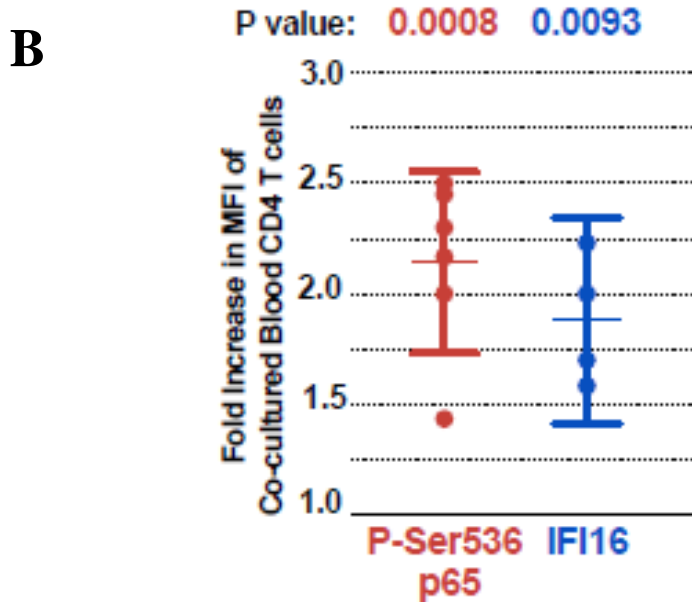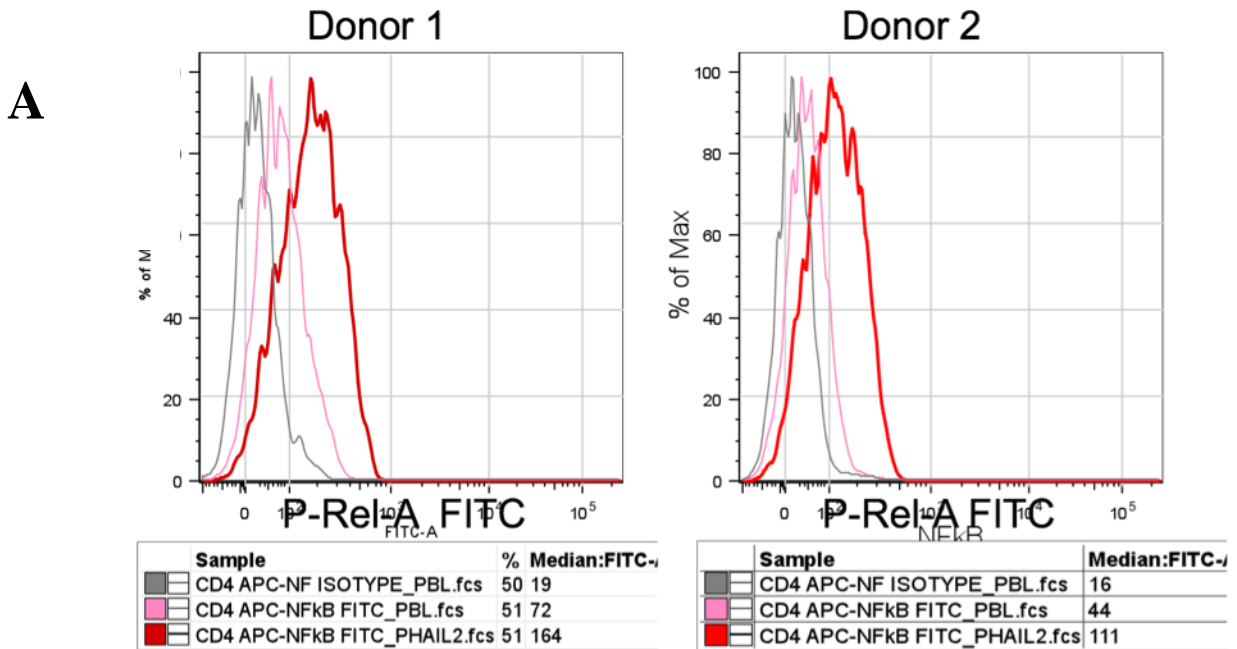
Figure 41: Co-culture with lymphoid cells induces NFkB activation and IFI16 up-regulation

Uninfected tonsil lymphocytes, blood lymphocytes or co-cultures of tonsil and blood lymphocytes were cultured for two days as in the HEK293T co-culture system and then intracellular staining was performed for IFI16 and P-Ser536 RelA/p65. The median fluorescence intensity (MFI) in co-cultured gated blood (CMAC-) CD4 T cells for both antibodies were normalized to isotype controls and then normalized to resting blood CD4 T cells. **A.** Shown are the FACS plots of the resting blood CD4 T cells when stained with an Isotype control vs IFI-16 before and after activation via PHA/IL2. **B.** Shown is the fold increase in MFI for co-cultured (with tonsil cells) blood CD4 T cells. There was a 2 fold increase in MFI for RelA phosphorylation indicating NFkB activation, and up to a 2 fold increase in the MFI for IFI16 in blood CD4 T cells co-cultured with tonsil cells.

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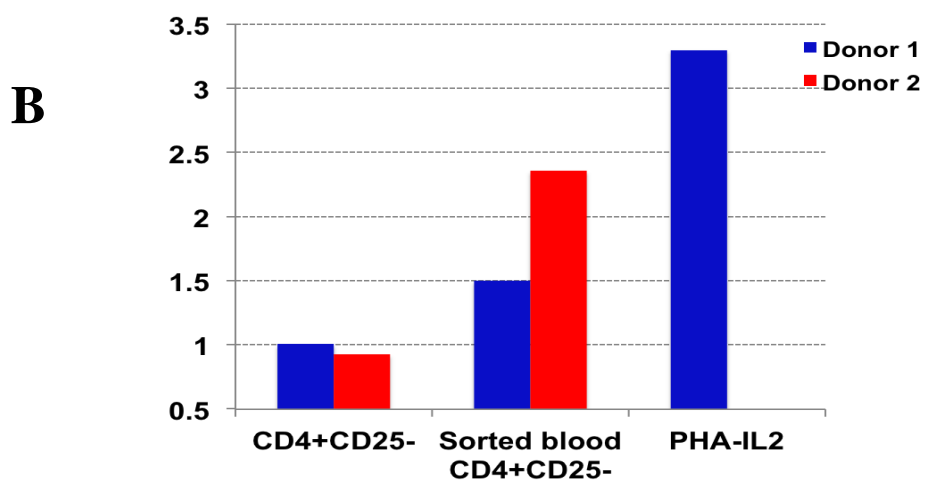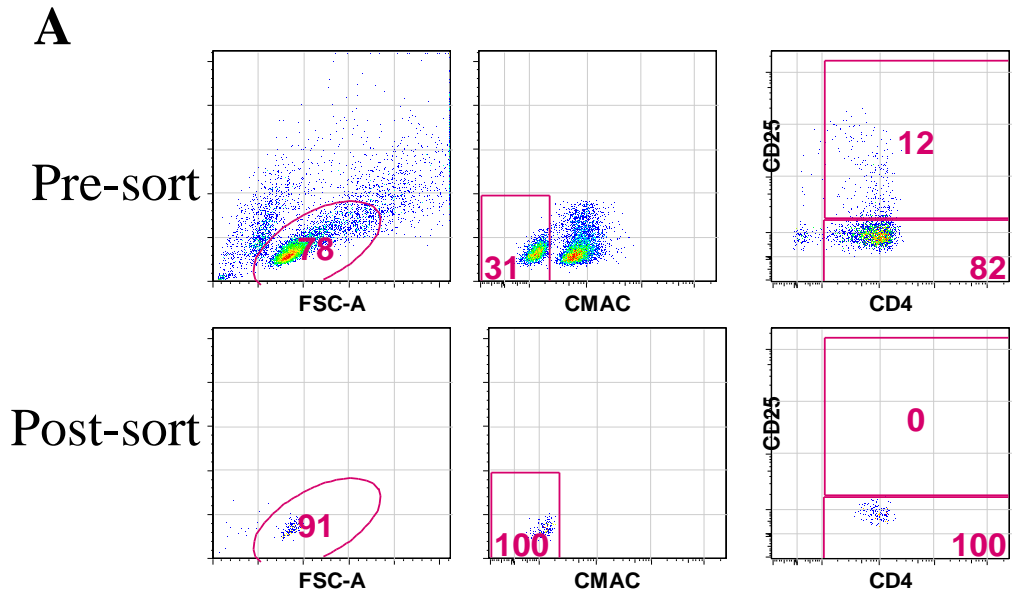
Figure 42: Blood CD4 T cells sorted from tonsil co-culture contain IFI16 mRNA
A. FACS plots of cells before and after purification by sorting. CMAC stained tonsil were co-cultured with blood lymphocytes for two days. Blood CD4+CD25- cells were sorted. **B.** Fold increase in IFI16 mRNA when normalized to resting (not co-cultured) blood CD4 T cells.
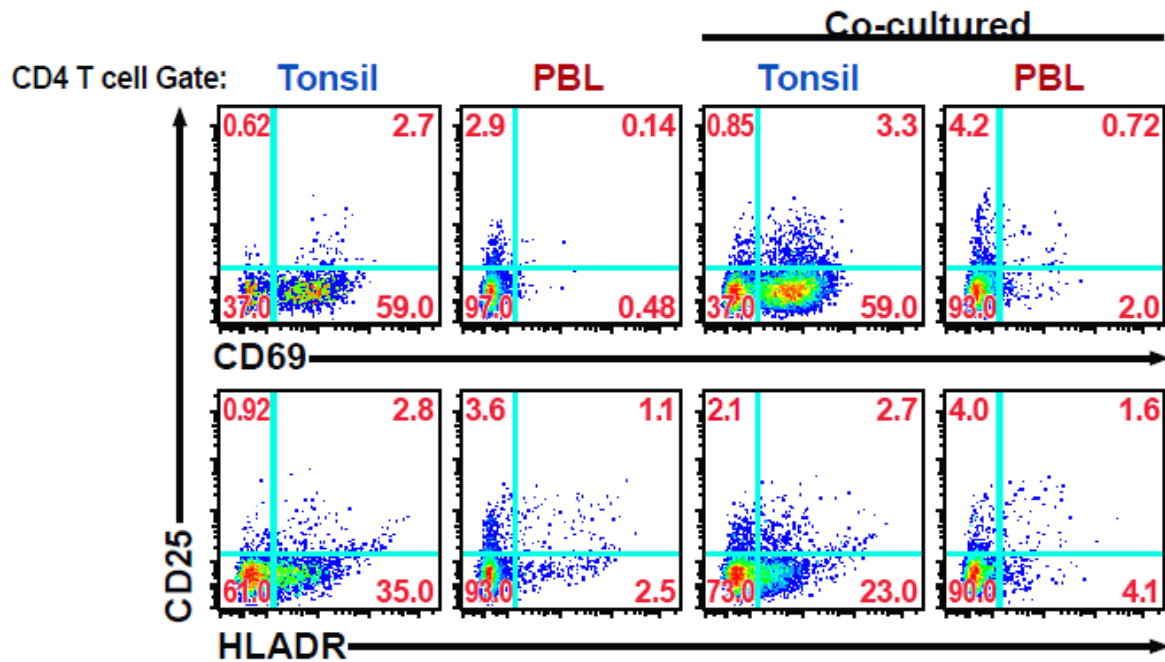
Figure 43: Activation status of blood CD4 T cells before and after co-culture with tonsil cell Blood and tonsil lymphocytes were either cultured alone or co-cultured for two days. Cultures were then stained for CD69, CD25, and HLADR. The majority of tonsil CD4 T cells express the early lymphoid tissue homing receptor and activation marker CD69, a portion also express the late marker for activation HLADR. These activation markers are not expressed in the majority of blood CD4 T cells even after co-culture.
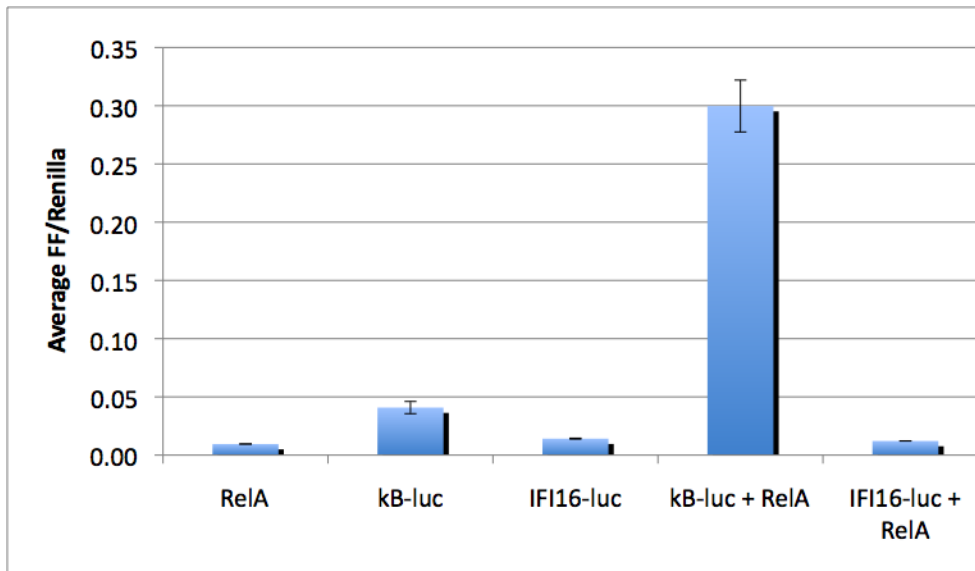
Figure 44: NF-kB does not directly induce IFI16 expression
Shown is the fold increase in luciferase-IFI-16 expression upon transfection of Rel-A/p65 into 293T cells.
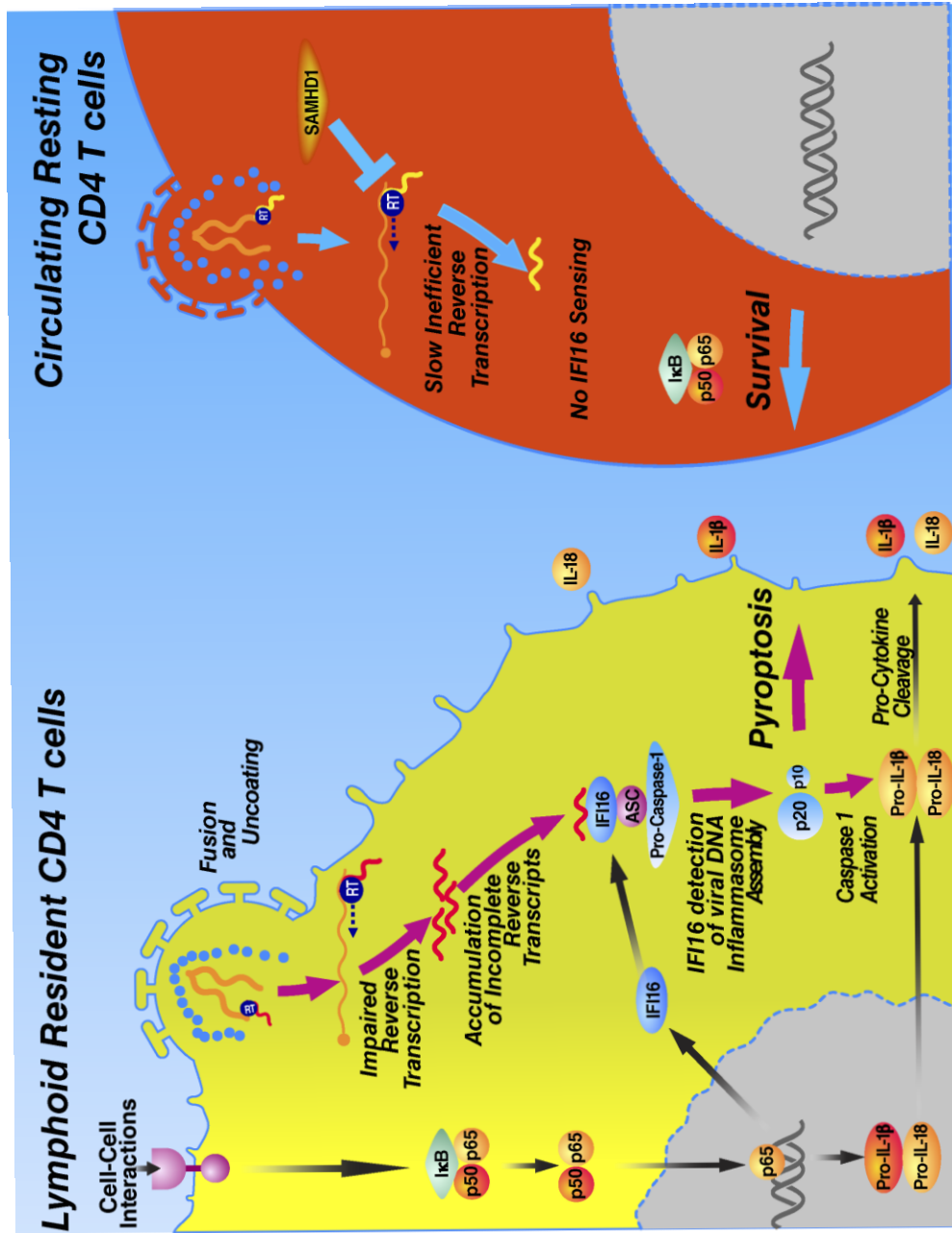
Figure 45. *Model of tissue dependent abortive HIV mediated CD4 T cell depletion Via cell-cell interactions the majority of CD4 T cells in the lymphoid tissues die via an abortive infection mediated depletion dependent on the detection of short viral DNA RT products by IFI16. The majority of CD4 T cells circulating in the peripheral blood lack expression o fIFI16. Lack of IFI16 expression renders blood derived CD4 T cells resistance to abortive HIV infection mediated pyroptosis.*

CHAPTER 9: Activated Blood CD4 T Cells Resist Pyroptosis Induced by Abortive HIV Infection

The majority of lymphoid derived CD4 T cells die during HIV infection as a consequence of abortive infection [87] [95]. Depletion of these cells by abortive infection differs from direct and bystander killing in that it requires viral entry and RT, but not integration [87]. Accumulated reverse transcripts are sensed by IFI16 [102] in the cytoplasm, which assembles into an inflammasome with ASC and pro-Caspase-1 [102]. Caspase-1 auto-cleaves into activation and then induces pyroptotic cell death. Resting blood CD4 T cells are resistant to this mechanism of depletion (chapter 7).

To explore if activating blood CD4 T cells was sufficient to sensitize them to HIV-induced pyroptosis, target PBL were activated with PHA and IL2 for 48 hours and pre-treated with AZT before co-culture with activated effector PBL. Activating target PBL allows RT to progress faster and more efficiently[90] and also IFI16 up-regulation. AZT pre-treatment promotes accumulating reverse transcripts and averts productive infection [94]. Surprisingly, target blood CD4 T cells remained resistant to depletion, even under these conditions (Figure 46).
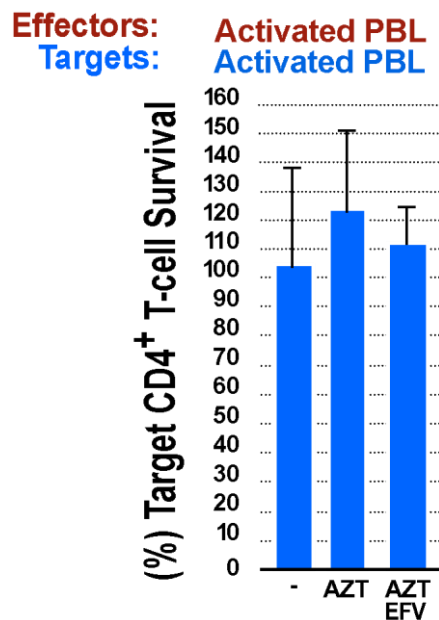
Figure 46: Activated blood CD4 T cells are resistant to abortive infection mediated depletion in the HLAC system
Target blood derived lymphocytes were activated with PHA/IL2 for 48 hours before co-culture with activated productively infected blood cells. No depletion of activated target blood CD4 T cells was observed.

We attempted the same conditions by using the HEK293T co-culture system. Using this system, we compared cell depletion induced after activation of PBLs treated with PHA and IL2 treatment or CD3 and CD28 activation beads. PMA/ionomycin treatment was not attempted since PMA induces CD4 downregulation (which is essential for infection). All cultures were treated with AZT to ensure accumulating reverse transcripts and blocking productive infection [94]. Blood CD4 T cells activated by either method were still resistant to HIV-induced pyroptosis (Figure 47). These results suggest that enhancing accumulation of reverse transcripts by PHA/IL2 or CD3/CD28 and up-regulating IFI16 are not sufficient to sensitize blood CD4 T cells to abortive infection mediated depletion. These results confirm those of Cooper [83] et. al in which activating blood CD4 T cells makes them resistant to pyroptosis induced by abortive infection.
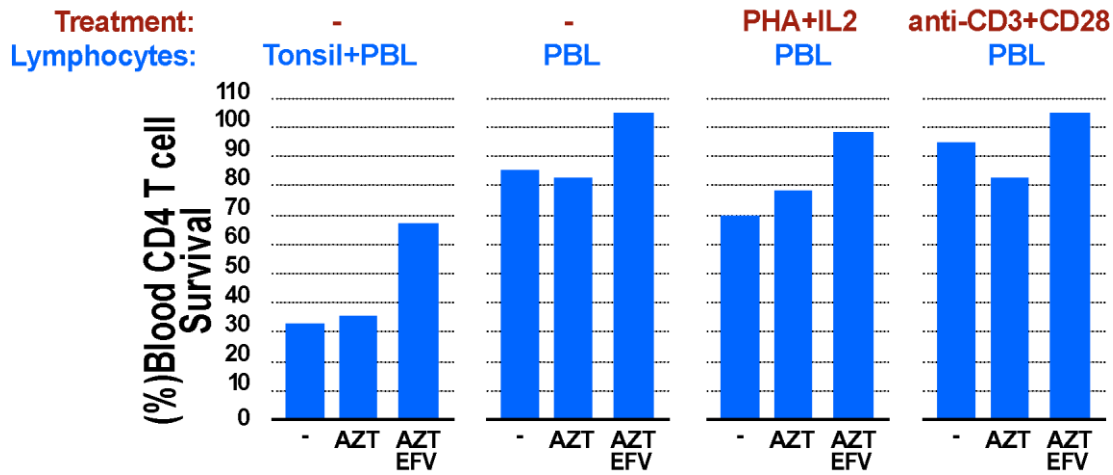
Figure 47: Activated blood CD4 T cells are resistant to abortive infection mediated depletion in the HEK293T system
Blood lymphocytes were either activated by PHA/IL2 or CD3/CD28 treatment for two days and then cultured with NLENG-IRES-GFP transfected HEK293T. No depletion was observed under either conditioned.

Despite the presence of all the known IFI16 inflammasome components (IFI16, ASC, and pro-Caspase-1), one or several of them may be rendered non-functional. To determine which step of pyroptosis is impaired in activated blood CD4 T cells, we compared the death of tonsil, resting, and activated blood CD4 T cells after treating them with Nigericin. Nigericin is a potent inducer of pyroptosis via the NALP3 inflammasome [117][116][74][73] (Figure 48). Since NALP3 is induced by NF-κB [73], resting blood CD4 T cells served as a negative control. After nigericin treatment, tonsil and activated blood CD4 T cells were depleted but not resting blood derived CD4 T cells (Figure 49). These results imply that all the known components involved in forming the NALP3 and IFI16 inflammasomes (e.g., NALP3, IFI16, ASC, pro-caspase-1, activated caspase-1) are not compromised in these CD4 T cells. We hypothesize that the resistance to pyroptosis in these cells was not a consequence of a compromised inflammasome component, but instead a host factor affecting the accumulation of reverse transcripts.
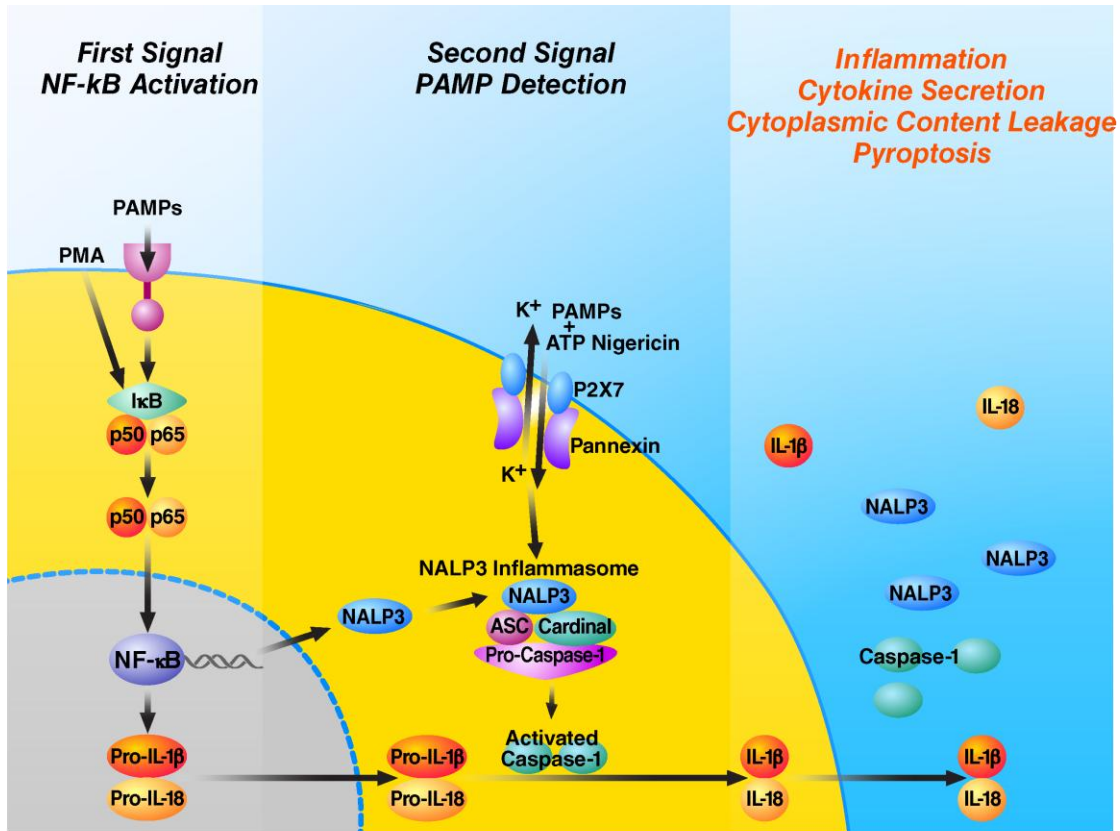
Figure 48. Pyroptosis Induction via the NALP-3 inflammasome by Nigericin treatment
Expression of the sensor NALP3 requires NF-kB activation. The cell surface receptor P2X7
binds nigericin. The binding of nigericin offsets the ions so that there is increased potassium
efflux. The NALP-3 inflammasome assembles with ASC, which leads to the recruitment of pro-
caspase-1. Pro-caspase-1 is activated by auto-cleavage. Caspase-1 then cleaves the NF-kB
dependent pro-IL1 and IL18 cytokines. Cleavage of these cytokines prepares them for their
secretion. By unknown mechanisms, caspase-1 induces the breaking of the cell membrane.
Cytoplasmic content, inflammatory cytokines are released into the extra-cellular space insuring
spread of inflammation.
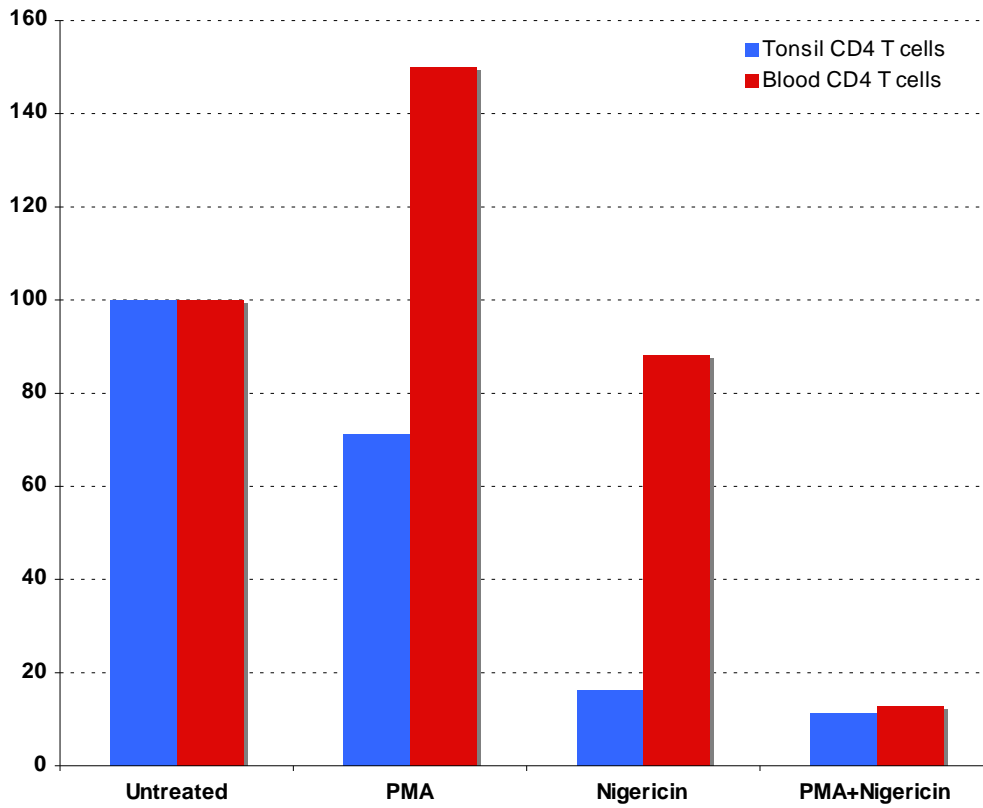
Figure 49: Activated but not resting blood CD4 T cells are susceptible to nigericin mediated pyroptosis Blood CD4 T cells were either left untreated or treated with PMA (4 hrs) before Nigericin treatment (16hrs). Activated blood CD4 T cells but not resting cells (which lack the NALP3 sensor) were depleted, indicating that activated blood CD4 T cells contain all the known and essential components of the NALP3 and the IFI16 inflammasome to induce pyroptosis. Perhaps the resistance to HIV induced pyroptosis is due to the dynamics of HIV infection rather than a defect in inflammasome assembly or pyropotosis induction.

One host factor known to affect the accumulation of late reverse transcripts in activated HIV-infected cells is the ssDNA exonuclease, TREX-1[122]. TREX-1 is essential for the host to degrade cytoplasmic DNA generated by endogenous retroviruses, therefore avoid detection and induction of the inflammatory host response via IRF3 and INFβ. Mutations that render TREX-1 non-functional cause cytoplasmic DNA accumulation and INFβ secretion and have been linked to inflammatory autoimmune diseases such as Aicardi-Goutières syndrome (AGS)[122].

Few HIV proviral integration events occur in permissive cells[122]. Yan et. al showed that HIV infected cells lacking TREX-1 secrete INFβ and IL6[122]. Secretion of these cytokines was inhibited by NNRTIs, such as nevirapine, but not by the integrase inhibitor raltegravir[122]. These results imply that the nuclease activity of TREX-1 on HIV DNA prevents its detection during

HIV RT. Despite low TREX-1 expression, tonsil CD4 T cells accumulate reverse transcripts and secrete INFβ [87] after HIV infection when pretreated with raltegravir, but not in the presence of an NNRTI. TREX-1 is essential for evading the INFβ pathway in macrophages derived from blood monocytes (MDM)[122]. It remains unknown if TREX-1 helps activated/permissive blood CD4 T cells evade HIV-induced.

Resting and activated blood CD4 T cells peak in the generation of strong stop DNA at the same time post infection, 12hrs, but strong stop DNA is degraded much faster in activated cells (by 4.5 days) than in resting CD4 T cells [88]. In fact a subset of resting CD4 T cells have been shown to harbor reverse transcribed DNA for long enough so that when activated the provirus integrates and the cells become productively infected. In another words in some resting CD4 T cells these RT products are never degraded [88]. For example, when detecting late gag DNA after HIV infection, where activated CD4 T cells start degrading late gag DNA by 2 days post-infection, while resting CD4 T cells do not peak until 4 days post-infection [88]. Therefore while resting blood CD4 T cells progress through RT at a very slow pace and accumulate late reverse transcripts, activated CD4 T cells progress rapidly and degrade late gag DNA. We hypothesize that the degradation of the late gag DNA in activated CD4 T cells is executed by TREX-1 or a similar exonuclease and that this degradation protects activated CD4 T cells from IFI16 sensing and pyroptosis. We are currently pursuing experiments that will answer these questions.

Chapter 10. DISSERTATION SUMMARY

After thirty years of research, we have characterized the mechanism by which non-permissive CD4 T cells are depleted during HIV infection. We show that non-permissive CD4 T cells die as a consequence of an abortive infection that leads to the accumulation of reverse DNA transcripts in lymphoid tissue derived CD4 T cells. These short transcripts are detected by the sensor IFI16. IFI16, then forms its own inflammasome with ASC and pro-caspase-1. Pro-caspase-1 is activated upon its association with ASC and DNA-bound-IFI16. Activated caspase-1 induces the secretion of the pro-inflammatory cytokines, IL1-β and IL18 and also induces cell death via pyroptosis. This death pathway requires the direct transfer of virus via the virological synapse but not specifically from lymphoid derived CD4 T cells.

This mechanism of HIV mediated depletion remained uncharacterized for so long because most studies analyzing CD4 T cell depletion use GALT or peripheral blood tissue [78] [79] [80]. In this regard, the majority of CD4 T cells in lamina-propria of GALT display a state of cellular activation that allows for productive viral infection and death as a result of apoptosis. Most CD4 T cells outside the GALT are not permissive to productive [123] [124] [82] infection therefore the question still remained how non-permissive cells were killed during HIV infection. It was possible that CD4 T cells need to be in GALT in order to become susceptible to depletion but this would not explain the continuous loss of CD4 T cells through all stages of infection. It would also not explain the massive loss of both permissive and non-permissive cells during the acute and AIDS stage of infection.

In contrast, spleen, tonsil, and mesenteric lymph node derived CD4 T cells (>95%) are much less activated (as measured by CCR5, CD25, and HLADR) when compared to GAT derived CD4 T cells and do not support productive HIV infection [87]. Therefore researchers studying GALT would likely miss the abortive infection mediated depletion pathway. Here we demonstrate that blood CD4 T cells represent the most highly resting population of cells and are naturally resistant to both productive and abortive viral infection. This resistance to abortive infection mediated depletion is at least in part because of a lack of IFI16 expression. Of note, IFI16 is constitutively expressed in most cells but is directly induced by the AP1 complex, interferon signaling. Co-culture with lymphoid derived lymphocytes leads to blood CD4 T cells increased susceptibility to abortive infection mediated depletion. This sensitization requires close cell-cell interactions with any (B-cells, CD8 or CD4 T cells) of the lymphocytes subpopulations found in the lymphoid tissue. Of note, tonsil tissue does not contain a detectable amount of myeloid cells. Sensitization does not require productive infection or the death of lymphoid derived CD4 T cells. Also not required is the induction of a mixed-lymphocyte reaction. Instead, close interactions seem to be inducing signaling that leads to a low level of NF-κB activation as well as IFI16 upregulation but not CD69, CD25, or HLADR expression.

Currently, we are working to identify the lymphoid factor(s) that sensitize blood CD4 T cells to HIV-induced pyroptosis through low-level activation. With a better understanding of this signaling event, we may be able to confer the same resistance to pyroptosis displayed by circulating blood CD4 T cells onto lymphoid cells. We are also trying to decipher why activated blood CD4 T cells do not dies by abortive infection mediated pyroptosis. We are investigating why this mechanism of CD4 T cell depletion requires viral-synapse delivery of HIV. We are also confirming that blood CD4 T cells purified from HIV infected patients do not express IFI16 and

therefore remain resistant to abortive infection mediated pyroptosis. Our work underscores how AIDS is principally a disease of lymphoid tissues. Briefly, when CD4 T cells are circulating in the peripheral blood not only are they resistant to direct killing but they are also resistant to abortive infection induced pyroptosis, in high contrast to when they are residing in lymphoid tissue.

Appendix A: Materials and Methods

Preparation of viral stocks. To generate viral stocks, HEK293T cells were transfected with pNL4-3 (David N. Levy, University of Alabama, Birmingham) (80 µg/T175 flask) by the calcium-phosphate method (See detail protocol). The medium was replaced 12–14 h after transfection. Forty-eight hours after transfection, viral supernatant was concentrated by ultracentrifugation for 1.5–2 h at 53,000 *g*. The pellet was resuspended in 30 ml of medium consisting of 90% fetal bovine serum (FBS) and 10% RPMI and frozen at -80°C until needed for determination of p24^{gag} or infection. p24 ^{gag} levels were determined with the p24 ELISA kit (Perkin Elmer).

Isolation of primary tissue lymphocytes. Human lymphoid tissues were obtained from the Cooperative Human Tissue Network and processed as described[1,19]. Briefly, tissue was passed through a 40-µm cell strainer, and red blood cells and dead cells were removed by Ficoll density-gradient centrifugation (See detailed Protocol). B cells, CD4 T cells, or CD8 T cells were purified with anti-CD19, anti-CD4, or anti-CD8 microbeads (Miltenyi Biotec) respectively using a Miltenyi Automacs separator. PBL were purified from buffy coats (Stanford University Blood Bank) or concentrated leukocyte preparations (Blood Centers of the Pacific) by Ficoll density-gradient centrifugation. Monocytes were removed by adherence or by magnetic CD14 microbead depletion (Miltenyi Biotec). Purity was confirmed by FACS staining for surface markers on flow cytometric analysis.

HLAC system. PBL were activated with phytohemagglutinin (10 µg/ml) and IL-2 (100 units/ml) for 48 h before infection to generate an effector population. Activation of tonsil effector cells was unnecessary, as 5% of tonsil CD4 T cells are naturally permissive to productive infection[1]. Dispersed lymphocyte cultures (of activated PBL or tonsil cells) were infected with NL4-3 (10–50 ng p24 ^{gag} /200 µl) or left uninfected. Four days later, uninfected target cells were labeled with CFSE (1 µM) (Molecular Probes) and left untreated, treated with AZT (5 µM) for 12–16 h, or with AZT and efavirenz (5 µM) for 1 h before co-culture. Effector and target cells were co-cultured at a 1:1 ratio. Drug-treated target cells were mixed with uninfected or infected effector cells. Co-cultures were harvested on day 5 or 6 and stained for FACS analysis. Absolute counts of target (CFSE+) CD4 T cells were normalized to absolute counts of target CD8 T cells in the same well; CD8 T cells served as an internal control because they are not depleted by HIV-1 infection[1,19]. The percentages of target CD4 T cells were normalized to those in corresponding uninfected samples.

HEK293T co-culture system. HEK293T cells (1.4×10^5/well) were cultured in 24-well plates and transfected with 50–100 ng of viral DNA via Fugene. To distinguish lymphoid from the blood cells, these cells were stained with 7-amino-4-chloromethylcoumarin (CMAC) (Molecular Probes) for 1 h at 37°C before co-culture with PBL, HEK293T cells, or both. Treated (efavirenz, 25 µM) and untreated lymphocytes (4.5×10^6) were added to each well 12–16 h after transfection. After 48 h of co-culture, the lymphocytes were harvested and FACS stained prior to flow analysis. Before co-culture, cells were pretreated for 1 h at 37°C with the peptide inhibitor ZWED-FMK (inhibits caspase 1) (R and D Systems), ZVAD-FMK (pan-caspase inhibitor) (R and D Systems) or caspase 3-inhibitor VII (EMD Millipore). All caspase inhibitors were added at a final concentration of 100 µM.

FACS staining and analysis. Antibodies were diluted 1:100 with 2% FBS/PBS. Cells were stained with CD4 PE-CY7 or CD4 PE, CD69, CD25, HLA-DR, and/or CD8 APC (BD Biosciences) for 15–60 min at 4°C in the dark. Stained cells were washed with 2% FBS/PBS and fixed with 4% paraformaldehyde for 1 hr. Fixed cells (10–20,000 events on the live gate) were acquired on a Becton-Dickinson LSRII flow cytometer and the data were analyzed using FlowJo software.

Intracellular staining. Cells were stained for CD4 as described above for 1 h on ice, washed with 2% FBS/PBS, and fixed with 4% paraformaldehyde for 30 min to 1 h at 37°C. The cells were then washed and chilled on ice for 5 min, permeabilized on ice with ice-cold 90% methanol for 30 min to 1 hr, washed with 2% FBS/PBS, and stained with the following antibodies for 1 h on ice: Phospho-Ser536 RelA-Alexa Fluor 488 (4886S, Cell Signaling; 1:50), or IFI-16 (sc-8023, Santa Cruz Biotechnology; 1:200). Finally, the cells were washed, stained with secondary antibodies (APC goat anti-mouse 550826, Becton Dickinson; 1:200) and analyzed with a Becton-Dickinson LSRII flow cytometer. Flow cytometer was set to acquire 2500-5000 CMAC^{-} CD4^{+} events in cultures containing blood cells to ensure adequate comparisons. For cultures only containing tonsil cells, 2500-5000 CMAC^{+}CD4^{+} events were acquired. The histograms are shown with logged x-axis. Data were analyzed using FlowJo software. The median fluorescence intensity (MFI) of all samples was normalized to that of the corresponding isotype control. The normalized MFI of co-cultured PBL was divided by the MFI of isolated PBL to calculate the fold increase in median fluorescence intensity measuring NF-κB phosphorylation or IFI16 expression in co-cultured cells.

Cell Activation. PHA/IL2: Cells were seeded at 2x10^6 cell/200uL of 10%FBS/RPMI media. PHA was added at 10μg/mL Final. IL2 is added at 100 u/ml. Cells are incubated at this concentration for 48-72hrs. Cells are then washed and seeded at 2x10^6 cell/200uL in 10units/ml of IL2 alone.

Appendix B. Detailed Protocols

I. Ficoll Purification from Buffy Coat
Prepare: 500mL of FBS for 2% FBS/PBS
Ficoll Addition:
1. Make sure centrifuge is ready because blood exposure to Ficoll should be minimized. The centrifuge must be RT.
2. Spray scissors and tip of blood down with ethanol. Cut tip and pour blood into a 50mL Falcon tube. Fill the falcon tube containing the blood up to 35 mL FINAL with 2% FBS/PBS.
3. Slowly underlay 10mL Ficoll (Paque-plus Amersham Biosciences; at RT) on the bottom of tube.
4. Spin 2000RPM (400xg) at RT for 25 minutes, making sure that the BRAKE IS OFF.
Wash Steps:
5. Add 10mL RT PBS to a 50mL Falcon. Before transferring mononuclear cell layer, remove all but ~5mL of top plasma layer. Deposit cloudy mononuclear cell layer into 2% FBS/PBS using 2-5mL pipette. Add 2%FBS/PBS to bring final volume of mononuclear cells/PBS to 45mL.
6. Centrifuge at 1400RPM for 5 minutes at RT.
7. While cells are spinning, add bleach to inactivate residual blood in discarded Falcon tubes.
8. After spinning is complete, remove supernatant from first wash making sure not to aspirate accidentally the cell pellet.
9. Repeat wash step 2 times until supernatant is more clear. Centrifuge at 1800RPM for 4 minutes at RT. Resuspend in small volume initially in order to dissolve pellet and then bring total volume to 45mL.
10. Incubate cells in 10% FBS/RPMI at maximum 2x10^6 cells/ml.

II. Fusion Assay
Cell Pretreatment
Make a 10x solution of cells….Ex: if you need a final concentration of 2x10^5 cells/mL then make 20x10^5!
Final concentrations for each cell-line: 2x10^5 for DC, 2 x 10^5 for CEM, i2 x 10^6 PBL,
Controls:
You are going to be adding AMD to the CXCR4 tropic virus and Tak to the CCR5 tropic virus. These work as inhibitors of those viruses. These are negative controls for the fusion assay. If you are working with viruses that you haven't before then testing these inhibitors on them wouldn't be a bad idea. Calculate how many cells you can add to have the following controls for each type of cells.

      1- CCF2-No virus-No other stains
      2-No CCF2- No virus- No other stains
      3- CCF2- No virus - Cell Marker stain (ex: CD1a, CD4)
      4- NLA4- AMD
      5- NLA4- No AMD
      6- 81A-Tak
      7-81A-No Tak

Treat cells with AMD or TAK.
AMD is added at 5 uM in 10 uL/well
Stock 250 uM => 5 uM
      50 <= 1
So for 20 samples: 200 => 4

Add 10 uL of that to the well.

TAK is added also at 5 uM in 10 uL to a 100 uL cell solution.

> Stock 1 mM => 5 uM
>
>> 200 <= 1 (dilution)
>
> Add 10 uL of that to well

1. Place the cells in incubator for 30 min (37^0) It can be more than 30 mins….
2. In the meantime calculate what volume of virus you need (from your p24 ELISA/Flaq assay/Flow p24 stain). Ideally you'll use 500 ng of virus per well.
3. Before adding the virus, spin (3 min, 12 RPM) your cells and take out 50 uL. Each well will have 50 uL of (10 ug) virus and 50 uL of cells (so the virus will be diluted 2x's). This gives you a final concentration of 5 ug = 500 ng!
4. Add the virus. Pipete up and down.
5. Incubate for 1 hr, every 15 min (3x's) pipette up and down at 37 degrees.
6. <u>CO_2 independent Master mix:</u>

There are 2 kinds of CO_2 Independent Master mixes, there is one for incubation and another for loading CCf2

> -Incubation CO_2 Independent Master mix
>
>> CO_2 independent media (big aluminum bottle)
>>
>> 10% FBS
>>
>> Probenecid (1:10 diluted 200 uL of probenecid in 2 ml of FBS)
>
> -Loading CO2 Independent Master mix
>
>> Dilute 10 uL of CCF_2 in 90 uL of Solution b (10x's dilution) vortex
>>
>> Then dilute that solution in 20 mL of CO_2 independent media

1. Spin the cells after incubation.
2. Wash them 1 time with CO_2 independent media (no CCF2)

o Place 100 uL of the CO_2 independent Master mix, to the cell.
o Incubate them at <u>ROOM TEMPERATURE</u> for 1 hour.
o Wash the cells
o Incubate overnight in CO2 independent media at room temperature.
o The next MORNING, stain the cells

III. **HIV production via HEK293T transfection**

Plate HEK293T at 3.5x10^6 cells/T175 flask day before or 7x10^6 cells/T175 flask day of transfection. Make sure each T175 flask has 40 mL of 10% FBS/RPMI

2XHBSS:

- 280 mM   Liquid NaCl
- 50 mM   Liquid HEPES buffer
- 10 mM   Powder KCl
- 1.5 mM   Powder $Na_2HPO_4*7H_2O$
- 12 mM   Powder Dextrose
- Adjust to pH:7.05 by adding water

1. Place 80ug/ T175 flask of Viral DNA in 50ml Falcon tube
2. Add 2ml of HBSS per T175 flask (12 flasks = 24 mL of HBSS)
3. Add water up to 4mL/T175 flask
4. Slowly add 250 uL of $CaCl_2$ (2M)/flask (12flask = 3mL)
5. Incubate at room temperature for 10 minutes
6. Drop by drop add mix of DNA/CaCl/HBSS/Water to T175 flask
7. Incubate for 12-14 hours at in 37^oC incubator
8. Change media in flask, final volume of flask does not need to be more than 20ml
9. Harvest virus 48hrs post transfection

Harvesting Concentrated HIV
Cool down the ultra-centrifuge (the Beckman XL90 in back of TC)

Remember you must activate the vacuum for the centrifuge to cool down.
Grease the red tubes (Red SW28 rotor) and rubber.
Set the program to: SW28, 20,000, 2 hrs, 4°

Collect the supernatant from the flasks with transfected virus.
Place in blue top 50 mL conical falcon tubes.
Spin at 2,000 RPM for 5 min at 4^0 in the TC centrifuge.

Collect all 40 mL of supernatant. Make sure that the volume ends up being exactly 40 mL. Filter the supernatant into 150 mL flasks
Place the 40 mL of filtered supernatant into Round bottom clear tubes. Place the clear tubes into the red metal rotor tubes.
Close the red tubes so that the numbers are aligned and it is closed securely. Hook the tubes onto the rotor hinge.

Press Vac so you can open the centrifuge
Place the rotor in the centrifuge, close it, press vac, and wait until the vac is @ 200, then press enter 2x's and then press Start!
Wait until the speed reaches 20,000 RPM.

Aspirate the supernatant down to 200 uL.
Make sure the pellet is diluted in exactly 400 uL.
Place the pellet in an eppindorf.
Collect 10 uL of virus and place in 96 well plate.
Add 90 uL of media (1:10 dilution).

Prepare the virus for a p24 ELISA (Perkin Elmer kit) to monitor how much virus was produced. You'll need to make several dilutions of the virus in order to make sure you fall within the standard curve's range and to use as duplicates.
10^1, 10^2, 10^3, 10^4

Blood derived monocyte differentiation to macrophages

Isolate CD14+ cells with CD14 Miltenyl beads (protocol in Miltenyl kit).

Plate cells on a 24 well plate at maximum 5x10^6 monocytes/well in 10% Human AB serum RPMI media. Leave cells on plate for 5 days.

At 5 days, change media.

Leave cells on plate for 5 more days.

Once the cells are adherent to the plate and they have grown dendrites they have differentiated.

To detach the macrophages wash the cells with PBS.

Then add trypsin.

Allow to sit for 5 minutes at 37^oC and then spin down the cells.

GALT collagenase protocol [125]

Digestion Media	Final
Collagenase (Type II)	2mg/mL 1276.6units/ml
Penicillin/Streptomycin	1%
Hepes Buffer	1%
L-Glutamine	1%
Ampicillin	100uL of 100mg/ml
Fungizone	500 uL
DNAse I	40ug/ml
FBS	5%
RPMI	Fill to final volume

Cut the tissue into very small pieces with a scapel.

Plate small pieces of tissue with collagenase media at 37^oC for 5 minutes.

Pipette tissue in collagenase media up and down and return to 37^oC.

Every 5-10 minutes pipette up and down and then return to 37^oC for a total of 1-2hrs until the tissue is degraded and the cells are dispersed.

Filter the cells through a 40um filter.

After the cells have been dispersed, isolate live lymphocytes in a Ficol.

GALT does not have high viability.

The extent of viability depends on the freshness of the cells.

Tissue cannot last more than overnight.

Do not be surprised if your viability is 10% even after the Ficol.

GALT scraping protocol

Scrape the surface (gelatin like) of the GALT tissue.

Collect cells and extract live lymphocytes with Ficol.

Overlap PCR: Creating BFP-IRES-Nef
Primers ENVBAMH1: GTTTCTGGATCCTTAGCACTTATCTGGGAC
Primers NEF-FW: CGG CAT GGA CGA GCT GTA CAA GTA A
Primers NEF-REV: GTT TCT CTC GAG ATA CTG CTC CCA CCC C

<u>PCR product 1: IRES</u>
Primer-1 EnvBAMH1
Primer-2 NEF-REV
Template-1 NLENG1

<u>PCR product 2: BFP</u>
Primer-1 BFP Fw
Primer-2 BFP Rv
Template-1 pBFP

<u>PCR product 2: Nef</u>
Primer-1 Nef Fw
Primer-2 Nef Rv
Template-1 NLENG1
<u>PCR product 3: IRES-BFP</u>
Primer-1 EnvBAMH1
Primer-2 BFP2
Template-1-IRES
Template-2-BFP
<u>PCR product 4: IRES-BFP-Nef</u>
Primer-1 EnvBAMH1
Primer-2 NefRV
Template-1-PCR product 1
Template-2-Nef

Cut the viral plasmid NLENG1 with BAMH1 and Xho I.
Cut PCR product 4 (Nef-IRES-BFP) with BAMH1 and Xho I.
Treat with shrimp phosphatase or Calf (CIP 2uL 1 hr at 37°C).
Ligate Nef-IRES-BFP and plasmid NLENG1.

Appendix C: Dictionary

ATR-ATM and RAD3-related protein or FRAP-related protein 1. A Ser/Thr protein kinase that is involved in the sensing of DNA damage and activating the DNA damage checkpoint that leads to cell cycle arrest. It senses single stranded DNA strands.

Apurinic /Apyrimidinic endonucleases- involved in DNA damaged repair. Creates a nick in the phosphodiester backbone on the sites where DNA glycosylases remove damaged bases.

activation induced cytidine deaminase (AID)- a deaminase that induces point mutations in antibodies and B-cell receptors to increase their affinity to their cognate antigen.

AIM2-Absent from melanoma cells-a dsDNA sensor.

ATP-binding cassette transporter A1- An ABC transporter protein family member. ABC transporter proteins hydrolyze ATP in order to transport substrates across membranes of organelles in the cell.

AP-1 complex-the heterodimer of c-Jun and c-Fos which induces interferon type I transcription.

APAF-1-apoptotic protease activating factor- Binds cytochrome C and dATP which leads to its oligermerization. The oligermerized version is called the apoptosome. Apotosome binds, dimerizes caspase-9. Caspase-9 autocleaves resulting in its activation. Caspase-9 then induces activation of one of the effector caspases of apoptoisis such as caspase-3.

AP2-heterodimer made of c-FOS and c-JUN, works as a transcription factor inducing expression of many proteins including type I interferon alpha.

ALix- binds ubiquitin or the LYPLTSLRSL peptide on the C-terminus of the p6 domain of HIV Gag. Binding to the ubiquitin or peptide leads to its activation via conformational changes and dimerization. Activation leads to ALix membrane binding/anchoring. Activation also leads to recruitment of the rest of the ESCRT III complex. Upon recruitment of ESCRT III, ALix binds the carboxy tails of CHMP4, and Vps4 in the ESCRT III complex. Alix is responsible for cutting away the virion from the cell membrane or cutting away ubiquitinated cargo.

Arteminus- a DNAse activated by DNA-PK that cleaves overhanging bases during non-homologous recombination

α4β7-the dimer of two integrins that homes lymphocytes to the gut associated lymphoid tissue. Upregulated by memory CD4 T cells. Binds MADCAM-1 which expressed by endothelials in the gut.

AMD3100-a peptide that binds to CXCR4 therefore blocks binding of gp120 to the co-receptor. AMD3100 is not used in anti-HIV therapy because it would dis-regulate the homing of cells to the bone marrow.

AZT-azido-thymidine-is a nucleoside reverse transcriptase inhibitor (NRTI). Inhibits reverse transcription by integrating into the growing DNA chain. Since AZT does not have the last phosphate the next nucleotide cannot be added by the polymerase and so the chain terminates. It was the first anti-retroviral drug developed.

ASC-apoptosis associated speck-like protein-a bridging protein between "sensors" that do not have a CARD to directly bind to CASPASE-1.

B cells-a bone marrow generated and maturing lymphocyte that matures and expresses membrane bound antibody and also secretes them upon interacting with antigen. Presents antigens via the MHC II complex to CD4 T cells.

β-COP-a golgi transmembrane protein.

βTrCP- a ubiquitin ligase (E2) a group of proteins involved in the ubiquitination of proteins involved in the cell cycle that recognize their target proteins in a phosphate dependent manner.

CFSE-carboxy-fluoro-succinyl ester-a small green fluorescent sugar used to stain cells. The sugar binds to proteins on the surface or inside the cell.

CMAC- 7-Amino-4-Chloromethylcoumarin-similar to CFSE, CMAC is a sugar that binds proteins on or in cells used to stain them. Except that it fluoresces blue instead of green.   **CD3ς-** a transmembrane chain of the CD3 receptor. Only has 9 amino acids in the extracellular space, 113 of its amino acids are in the cytoplasmic region. It forms a homo-dimer. The cytoplasmic tail has 3 ITAM sites which interact with protein kinases which are critical for signal transduction that leads to the activation of T cells upon antigen presentation. The ITAMS consist of tyrosines that are phosphorylated by protein kinases.

CD4- a glycoprotein found on the surface of one class of T cells (Not CD8 T cells), dendritic cells, and macrophages. Essential for proper activation of T cells during antigen presentation.
CD8-A protein expressed by the second main class of T cells (Not CD4 T cells) that serves as a co-receptor for antigen presentation via the MHC-I complex.
DNA-sensor-a protein that binds DNA and induces the intrinsic immune sytem, including type I interferon secretion leading to restriction of bacterial/viral infections, activation of cells, and induction of inflammation
Cul1-1-is a scaffold subunit of a multi-protein E3 complex. Cul-1 is activated by Nedd8 binding. Upon Nedd8 binding, Cul-1 can bind to Rbx and Skp1 to form a whole E3 ligase. Rbx works as the E2 ligase. Skp1 then binds F-box which directly recognizes the substrate protein that's to be ubiquitinated in a phosphorylation dependent manner. Therefore in the case tetherin, the F box would directly bind after phosphorylation of tetherin and mark tetherin with a ubiquitin for degradation.
Cul4A- an E2 ubiquitin ligase (see E2) protein involved in the degradation of proteins that participate in the DNA damage response.
CBP-CREB-binding protein, co-activating protein that interacts with many transcription factors to induce gene expression.
CD-cluster of differentiation, or Cell Surface Markers. The CD system is commonly used as cell markers, allowing cells to be defined based on what molecules are present on their surface. These markers are often used to associate cells with certain immune functions.
CCR5-a chemokine receptor. Binds the chemokines, RANTES, MIP1α, and MIP1β. Binding of the chemokines recruits lymphocytes to specific tissues. Expressed on activated or memory T-cells.
Δ32CCR5-A mutated version of the CCR5 chemokine receptor which is missing 32bp in the gene, causing a frame-shift mutation. As a result of the frame shift mutation, less transcripts of CCR5 are expressed. This mutation is carried is homozygous in 1% and heterozygous in 10% of the Caucasian population. This mutation was not found in Japanese or African descendants. It was believed that this mutation was selected by the pressure of the black plague but studies have not indicated any effects on disease in mouse models. People carrying this mutation tend to be more susceptible to West Nile virus infection.
CHMP- Charged multivesicular body proteins- proteins in ESCRT III complex. Bind ATPase proteins such as Vps4 and Vta1 so that the ESCRT complexes can be recycled.
CHMP6 or Vps20-binds CHMP4
CMP4- polymerizes into filaments at the most narrow contact (neck) between the cell and the virion, filaments then recruit CHMP2 (ESCRT III domain) which recruits Vps4

CHMP2-recruits and binds Vps4

CXCR4-R4, fusin or CD184. A chemokine receptor that specifically binds stromal derived factor-1 (SDF). SDF is expressed in the bone marrow its binding to CXCR4-expressing cells directs them to to the bone marrow. CXCR4 is also a co-recpetor for HIV. The recognition of CXCR4 is done by the V3 loop of the envelope protein gp120.

Cyclin T1–a subunit of p-TEFB complex. Necessary and sufficient for HIV transcription. Involved in phosphorylation of the CTD tail, thereby enhancing transcription elongation.

Cdk9-cyclin dependent kinase 9. Associates with pTEFB. Involved in cell cycle regulation. Also works as an elongation factor for RNA polymerase II-directed transcription and functions by phosphorylating the C-terminal domain.

cytochrome C–a protein that associates with the inner leaflet of mitochondria's. Essential for the electron transport. Upon release into the cytoplasm it binds APAF-1 to initate apoptosis.

CARD-Caspase recruitment domain. The domain that directly binds caspases.

CAD-caspase activated DNase-cleaves genomic DNA during the late stages of apoptosis into 180bp fragments. This activity is used as a marker of late stage apoptosis or pyroptosis by staining cells with ethidium bromide.

Caspase 8- Induces apoptosis by activating effector apoptotic caspases and inhibits necrosis by cleaving of RIP-1, RIP-3 and CYLD.

CYLD-cylindromatosis- de-ubiquitinates Lys63 of RIP-1 so that RIP-1 can be recruited to complex I and thereby initiate necroptosis.

c-Jun-transcription factor that dimerises with c-Fos to induce interferon type I transcription.

DCSIGN- an integrin expressed by some dendritic cells, acts as a attachment receptor for HIV.

DNA-PK- is a Ser/Thr protein kinase involved in DNA damage

DNA ligase IV-ligates the ends of chromosomes.

DRP-1- Dynamin family of large GTPases which causes mitochondrial fission and respiratory oxygen species (ROS) production

dNTPs-nucleotide tri-phosphates

DCAF-1- Vpr binding protein. Binds Cul4A.

E1-ubiquitin activating enzyme. It is the first enzyme in the protein degradation process to bind the ubiquitin. Upon binding the ubiquitin on its cysteine residue in the active site, the ubiquitin is activated and then transferred to an E2 enzyme. See ubiquitin for more details.

E2-ubiquitin ligase enzyme. Transfers the ubiquitin to an E3 enzyme. See ubiquitin for more details.

E3-Transfers the ubiquitin to a lysine residue on the protein that's being marked for degradation. See ubiquitin for more details.

ESCRT pathway or Multivesicular (MVB) body biogenesis- endosomal sorting cargo required for transport- Without ESCRT machinery, these proteins can build up and lead to neurodegenerative disease. This pathway decides which proteins will go to the surface of the cell, which proteins will be removed from the cell membrane, or be degraded via the lysosome or proteosomes. It has 4 complexes (ESCRT 0, ESCRT I, ESCRT II, and ESCRT III) all complexes contain at least one protein that anchors it to membrane by binding lipids, and all have at least one protein with the capability of binding ubiquitin on a ubiquitinated protein. This process is essential for cells to destroy misfolded or damaged proteins, and also at excess proteins. A dysfunctional ESCRT pathway can lead to degenerative diseases.

EAP20 or Vps25- in ESCRTII then recruits and binds CHMP6 ({Vps20}part of the ESCRTIII complex).

EFV-efavirenz- a non-nucleoside reverse transcriptase inhibitor (NNRTI). Inhibits reverse transcription by binding reverse transcriptase in a site outside the activation site.

FASL-CD95L-a signaling molecule that binds its receptor Fas receptor to induce death of cells.

F-Actin-a linear microfilament form of actin, important for contraction and movement of the cell. Can act as a barrier to viral entry because it needs to be activated in order to induce movent of cargo in the cytoplasm. Therefore the virus needs to circumvent the actin so that the virion can move inside to the essential compartments.

GTP-guanine tri-prosphate

GM1-a ganglioside marker for lipid rafts.

HAT-histone acetyl transferase.

Hck-a tyrosine kinase involved in the coupling of the Fc receptor to the activation of the respiratory burst. May play a role in neutrophil migration and in the degranulation of neutrophils.

HEK293T cells –human embryonic kidney cells 293 containing the SV40 T antigen- HEK239 cells were generated by transformation with adenovirus DNA. The transforming activity of the SV40 T antigen is due in part to its blocking the retinoblastoma (pRB) and p53 tumor suppressor proteins. The T antigen enhances transfections by binding several other cellular factors, such as transcriptional co-activators.

HLA-human leukocyte antigen. The human version of the MHC (mouse histocompability comples). These proteins present antigens (produced by the disgestion of antigens by proteosomes and lysosomes).

HLA-human leukocyte antigen. The human version of the MHC (mouse histocompability comples). These proteins present antigens (produced by the disgestion of antigens by proteosomes and lysosomes).

HLADR- The human version of the MHC (mouse histocompability complex) class II. These proteins present antigens (produced by the disgestion of antigens by proteosomes and lysosomes). This particular HLA is upregulated on the surface of CD4 T cells upon activation after CD69 and CD25.

Importin-β-a protein that helps other proteins enter the nucleus by binding the nuclear localization signal on the protein.

ICAD-inhibitor of caspase activated DNase-inhibits CAD's DNase activity to keep genomic DNA intact.

intercellular adhesion molecule-1 (ICAM-1)- an adhesion molecule that stabilizes the synapse of cells during antigen presentation or HIV infection via the viral synapse. Expressed by antigen presenting cells such as B-cells, macrophages, dendritic cells. Binds LFA-1.

IFI16-Interferon inducible protein 16-a DNA sensor. Has been characterize to sense DNA from KSH and HCMV.

IL2- interleukin 2-is an essential cytokine for T cell activation/proliferation. Secreted by T cells.

Ionomycin- Produced by the bacteria *Streptomyces conglobatus*. Used in research to stimulate Ca production and thereby stimulate cell activation/proliferation

Ku70/80 heterodimer-two proteins that complex and are essential for the DNA-PK repair machinery.

lamina propria-the layer of the gut associate lymphoid tissue particularly the intestines where the largest population of memory/activated CD4 T cells reside.

Lyn- a tyrosine kinase involved in the activation of cells.

LFA-1-lymphocytes function associated antigen- an integrin that binds ICAM-1. Expressed by

T cells, B cells, macrophages, and ncutrophils. Works as an adhesion molecule to stabilize the interaction between antigen presenting cells and their targets.

LCK-a lymphocyte specific tyrosine kinase protein essential for the activation of T cells via the immunological synapse. LCK is usually sequestered away from the T cell receptor in lipid rafts. Upon antigen presentation the TCR complex is recruited to lipid rafts closer to LCK. It phosphorylates the ITAMs on the cytoplasmic tail of the CD3 chains.

MLV-murine leukemia virus

microtubule organizing center (MOC)

MLKL-Mixed lineage kinase domain like-recruited to the necroptosome complex II by phosphorylation of Ser227 on RIP-3. An essential substrate of RIP-3 for necroiss induction.

NF-κB-transcription factor that homo or hetero dimerizes upon activation by many signal transduction pathways and leads to the transcription of many genes upon activation. Essential for immune reactions as it stimulates the expression of many cytokines and cell proliferation.

NLENG1-a GFP reporter CXCR4 tropic lab adapted HIV virus.

NALP3-cryopyrin-a sensor that recognizes many different antigens including Uric Acid (which leads to Gouts diseases via NALP3 recognizing it and causing pyroptosis), nigericin (an antibiotic that disregulates the ion transportation in/out of the cell), flagella of different bacteria.

PMA-phorbol myristate acetate-a mitogen that induces activation of cells

PHA-phyto-hemagluttin A-a mitogen extracted from red kidney beans. Activates T cells to proliferate.

PTEF-b-positive transcription elongation factor. A multi-subunit protein kinase. Subunits include Cdk9, and either cyclin T1, T2, and K. Enhances HIV transcription in a Tat and Tar dependent manner.`

PACS-1- plays a role in directing trans golgi network localization of furin by binding to furin's phosphorylated cytosolic domain. In addition, the human protein plays a role in HIV-1 Nef mediated downregulation of cell surface MHC-1 molecules to the TGN, thereby enabling HIV-1 to escape immune surveillance.

p300-(EP300), E1A bind protein, co-activating protein that binds transcription factors to induce expression from genes.

PCAF-p300/CBP-associated factor, has an acetyltransferase, bromodomain, and an E3 ubiquitin ligase domain. Contains several sites of acetylation and ubiquitination. Competes with E1A for the p300/CBP binding site. Its acetyltransferase activity and localization is regulated by acetylation by HDAC3.

phosphoinositide phosphatidylinositol 4,5 bisphosphate (PI[4,5]P$_2$)-membrane phosphor lipid

PYHIN–pyrin and HIN domain containing proteins -is a family of DNA binding proteins

RIP-1 kinase-Receptor interacting Ser/Thr protein-a kinase recruited by TNF alpha receptor signaling to form complex I (with RIP3, caspse-8, and FADD) and phosphorylate the protein RIP-1 which leads to necroptosis. When RIP-1 is ubiquitinated by cIAP and LUBAC,it leads to NFkB activation, which saves the cell from both apoptosis and necroptosis, when it is de-ubiquitinated at Lys63 it leads to necroptosis.

RIP3-cleaved at ASP328 to avert necroptosis. Binds and phosphorylates RIP-1 to induce necroptosis. Phosphorylation RIP-3 by RIP-1 kinase on Ser227 is necessary for necroptosis induction.

RHIM domain-RIP homotypic interaction motif – the domain of RIP3 that binds RIP-1

Sirtuin-2 acetylates RIP-1 to activate it.

SMUG1-A uracil DNA glycosylase. Targets single-stranded DNA as substrate to remove

uracils from it but is also able to remove uracils from double-stranded DNA. SMUG1 can also remove 5-hydroxyuracil, 5-hydroxymethyluracil, and 5-formyluracil bearing an oxidized group.

SAMHD1-SAM domain and HD domain containing protein-A phosphohydrolase enzyme which converts nucleotides (dNTPs) to nucleosides (dNDPs) in cells that are not proliferating. The depletion of dNTPs does not allow retroviruses to efficiently polymerize during reverse transcription. It is suspected that this restriction factor has multiple mechanisms of restriction but alternative mechanisms have yet to be characterized. Mutations that render SAMHD1 non-functional or not expressed have been found in a small percentage of people who as a result have Aicardi- Goutières syndrome (AGS) an auto-immune disease with chronic inflammation due to continuous type I interferon secretion.

SIVcpz, a strain of simian immunodeficiency virus (SIV) that infects chimpanzees.

STING-stimulator of interferon- a protein that binds to sensors and stimulates type I Interferons via IRF3 activation.

TrCPSCFskp-a multi-protein E3 complex that ubiquitinates proteins including the cytoplasmic tail of tetherin. See Cull-1.

tRNA$_{lys}$-lysine tRNA produced by the cell, which reverse transcriptase uses as a primer for DNA synthesis.

TRIM5α$_{rh}$-Tri-partite motif 5 alpha coded by rhesus macaques-a restriction factor that restricts HIV but not SIV infection of macaques by binding the viral core and inducing its degradation via the proteosome.

Tsg101-Tumor susceptibility gene 101orVps23. Part of the ESCRT I complex. Binds protein bound ubiquitin and the PTAP sequence of the HIV p6 domain of the Gag polyprotein. Recognition of PTAP leads to recruitment and activation of ESCRT II.

TNFα-Tumor necrosis Factor alpha-an inflammatory cytokine that induces NFkB activation or Necrosis upon binding its cognate receptor TNFR

TRAIL-TNF related apoptosis inducing ligand-binds its cognate receptor to induce cell death.

Tip60-Tat interacting protein 60. Histone acetylation transferase protein.

TAF$_{II}$250-Tata binding protein associated factor **250-**

TAR-HIV Trans-activation response element. An RNA stem-loop structure in the HIV-LTR which serves as a platform for Tat and the pTEFB co-activating subunits.

T cell- a lymphocytes that is generated and matures in the thymus and expresses the T cell receptor (CD3). There are two main classes of T cells, CD4 (CD4 T cells have many subclasses including TH1, TH2, TH17, Tregs etc) and CD8 expressing T cells.

TBK1-TANK binding kinase 1-phosphorylates IRF-3 the transcription factor that induces transcription of the interferon alpha gene.

THP cells- a human monocytic cell line derived from an acute monocytic leukemia patient.

TLR7 or 8 toll like receptor 7 and 8-Are proteins expressed to bind and detect common antigens in pathogens- pathogen associated microbial patterns (PAMP). Toll 7 detects ssRNA.

TAK779-(N,N-dimethyl-N-(4-[[[2-(4-methylphenyl)-6,7-dihydro-5H-   Benzocyclohepten-8-yl]carbon-yl]amino]benzyl)-tetrahydro-2H- Pyran-4-aminium chloride. A non-peptide HIV entry inhibitor that binds to CCR5 therefore blocking all CCR5 dependent infection.

UNG2-uracil DNA glycosylase 2-DNA glycosylases

ULBP2 protein- Binds to the human NKG2D receptor, an activating receptor expressed on NK cells, NKT cells, gamma delta T cells, and CD8+ alpha beta T cells

Ubiquitin-a 76aa length protein which convalently binds to other proteins to tag them as ready

to be degraded via the proteosome. Ubiquitin is recognized by the 19s proteosome. Proteins usually need to bind ubiquitin at several lysine residues before they can be degraded. Upon recognition of the ubiquitin by the 19s proteosome an ATP dependent unfolding of the protein occurs so that it can pass through the proteosome core where many proteases participate in cleaving it into short peptides.

VPS28- vacuolar protein sorting 28- in ESCRT I recruits and binds EAP45 in ESCRT II.

Vps4-CHMP3- Vacuolar protein sorting-associated protein 4-recruited to the complex by both Alix and CHMP2. Binds and hydrolyses ATP. Vps4 binding to Vta1 enhances and accelerates VPs4 ATP hydrolysis. ATP hydrolysis allows dimerization. Upon dimerization Vps4 Alix. Eventually Vps4 oligermizes even more with Vta1. ATP hydrolysis allows removal of the ESCRT III complex back into the cytoplasm. Removal of the ESCRT III allows more contraction at the neck and fission to occur between the membrane of the virion and that of the late endosome.

ZAP70- a protein kinase docks on the ITAMS of the CD3and is responsible for phosphoryalation of a few essential proteins (such as Vav-1, Shc, LAT, and SLP-76) for T cell activation. Normally expressed near the surface membrane of T cells and natural killer cells. It is part of the T cell receptor, and plays a critical role in T-cell signaling.

Z-WED-FMK (flouro-methyl ketone)- a peptide (WED) inhibitor of Caspase-1. The fluoromethyl ketone insures that the inhibition is not reversible.

Z-DEVD-FMK-a peptide (DEVD) inhibitor of Caspase-3. The fluoromethyl ketone insures that the inhibition is not reversible.

Z-VAD-FMK –a peptide (VAD) inhibitor of all caspases. The fluoromethyl ketone insures that the inhibition is not reversible.

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