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Dissecting How CD4 T Cells Are Lost During HIV Infection

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Abstract

Although the replicative life cycle of HIV within CD4 T cells is understood in molecular detail, less is known about how this human retrovirus promotes the loss of CD4 T lymphocytes. It is this cell death process that drives clinical progression to acquired immune deficiency syndrome (AIDS). Recent studies have highlighted how abortive infection of resting and thus nonpermissive CD4 T cells in lymphoid tissues triggers a lethal innate immune response against the incomplete DNA products generated by inefficient viral reverse transcription in these cells. Sensing of these DNA fragments results in pyroptosis, a highly inflammatory form of programmed cell death, that potentially further perpetuates chronic inflammation and immune activation. As discussed here, these studies cast CD4 T cell death during HIV infection in a different light. Further, they identify drug targets that may be exploited to both block CD4 T cell demise and the chronic inflammatory response generated during pyroptosis.

During the last three decades, HIV virologists have sought to understand how HIV attacks and destroys its principal cellular target, the CD4 T lymphocyte. It is the death of this subset of lymphocytes that lies at the root of AIDS. Initially, depletion was thought to reflect a viral cytopathic effect occurring in productively infected CD4 T cell (Alimonti et al., 2003). This notion found support in studies involving immortalized T cell lines or activated cultures of peripheral blood cells. Infection of these cells with laboratory-adapted strains of HIV resulted in productive infection and ultimately apoptotic death of the virus-producing cells. However, the frequency of these activated CD4 T cells appeared too limited to explain the massive loss of CD4 T cells observed in vivo (Jekle et al., 2003; Meyaard et al., 1992; Muro-Cacho et al., 1995). Other studies suggested that most of the dying cells in lymph nodes of infected patients were bystander CD4 T cells that themselves were not actively infected (Finkel et al., 1995). Various mechanisms have been proposed to contribute to the death of these bystander cells, including the action of host factors (e.g., tumor necrosis factor-α, Fas ligand, and TRAIL [Gandhi et al., 1998; Herbeuval et al., 2005]), and various viral factors (e.g., HIV-1 Tat, Vpr, and Nef) released from infected cells (Schindler et al., 2006; Westendorp et al., 1995). For example, the release of exosomes containing the viral

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accessory protein Nef from HIV-infected cells was shown to cause death of bystander CD4 T cells in vitro (Lenassi et al., 2010). Considerable interest has also focused on the role of gp120 Env protein in bystander killing, suggesting death signals involve gp120 binding to its chemokine receptor or occur through subsequent gp41-mediated fusion events (Perfettini et al., 2005).

It should be noted that not all CD4-expressing cells are rapidly depleted by HIV. For example, monocyte-derived macrophages do not die quickly; instead they produce virus over a period of weeks (Cassol et al., 2006). Infected microglia appear to survive for months if not years (Jones and Power, 2006). These findings suggest that viral infection and replication are not inherently linked with cell death. Indeed, it is well known that many retroviruses infect cells without killing their hosts (Swanstrom and Coffin, 2012). Unlike non-enveloped viruses that usually exit infected cells by inducing their lysis, enveloped viruses leave their cellular hosts by budding leaving the plasma membrane intact. Therefore, other features of HIV and its interaction with the host must be responsible for the massive CD4 T cell loss in AIDS.

Key Role for Human Lymphoid Cultures in Discovering HIV Death Pathway

In contrast to the productive infection and direct killing observed with activated blood CD4 T cells and CD4 T cell lines, studies of HIV infection employing primary lymphoid tissue highlighted a key role of death occurring within the bystander cell population (Finkel et al., 1995; Jekle et al., 2003; Rosok et al., 1997). We have explored HIV-associated CD4 T cell death using an ex vivo human lymphoid aggregate culture (HLAC) system formed with fresh human tonsil or spleen tissues (Glushakova et al., 1995). This system recapitulates many of the conditions encountered by HIV in vivo and, thus, offers a biologically relevant approach for modeling the molecular and cellular events that occur during HIV infection in vivo. Importantly, HLACs can be infected with a low number of viral particles in the absence of artificial mitogens or cytokine activation, allowing analysis of CD4 T cell death in a natural and preserved lymphoid environment. Infection of HLACs with HIV-1 resulted in the near complete depletion of CD4 T cell population without changes in the CD8 T cell and B cells compartments. However, only approximately 5% of these CD4 T cells became productively infected with the virus. Conversely, 95% of the dying CD4 T cells were resting, nonpermissive CD4 T cells. A key question was how HIV promotes the death of these frequently bystander cells.

Identifying a “Death Window”

To better understand how these bystander cells were dying, anti-viral drugs affecting distinct sequential events in the retroviral life cycle were employed. Early experiments revealed that bystander CD4 T cell death is prevented by entry inhibitors (for example, AMD3100 that blocks the engagement of gp120 with CXCR4) and fusion inhibitors (for example, T20 that blocks six-helix bundle formation by gp41, a prerequisite for virion fusion and core insertion). Conversely, cell death was not prevented by a number of nucleoside reverse transcriptase inhibitors (NRTIs) including AZT. These key findings raised the possibility that CD4 T cell death associated with HIV-1 involves a non-productive form of infection of

the resting CD4 T cells (Swiggard et al., 2004; Zack et al., 1990; Zhou et al., 2005). Subsequent studies focused on events that occur after HIV-1 entry. Specifically, our laboratory examined whether blocking early DNA synthesis with non-nucleoside reverse transcriptase inhibitors (NNRTIs) produced the same negative effect as AZT. Strikingly, the NNRTIs efavirenz and nevirapine blocked CD4 T cell death in HIV-infected HLACs as efficiently as entry or fusion inhibitors, suggesting that a certain degree of DNA chain elongation during reverse transcription is required to elicit the cell-death response (Doitsh et al., 2010). Using independent in vitro assays, viral ssDNA and dsDNA oligonucleotides exceeding 500 bases in length were shown to be sufficient to induce this cell death response.

These findings revealing a “death window” associated with the chain elongation phase of reverse transcription suggested a different mechanism of bystander cell death than previously described. Rather than being driven by membrane signaling events through CD4 or chemokine co-receptors or involving the elaboration of cytotoxic viral proteins or host factors, the cell death observed in these HLAC involved abortive viral infection of the resting CD4 T cells (Doitsh et al., 2010). Specifically, HIV binds and effectively fuses to these bystander CD4 T cells; however, because of their resting state, the viral life cycle attenuates during the chain elongation phase of reverse transcription, giving rise to incomplete cytosolic viral DNA transcripts.

Death by Cell Suicide

The accumulation of incomplete reverse transcripts within the abortively infected cells raised the possibility that their death involved innate sensing of these DNAs. Accordingly, affinity chromatography and mass spectroscopy studies were used to identify this cellular sensor. These studies revealed that sensing of the viral DNA fragments was mediated through interferon-gamma-inducible protein 16 (IFI16) (Monroe et al., 2014). shRNA-mediated knockdown of IFI16 rescued bystander CD4 T cells from death in HIV-infected HLACs. As discussed below, at present, IFI16 is the only mammalian DNA sensor identified that recognizes both single- and double-stranded (ds) DNA.

By examining how these CD4 T cells were dying, our laboratory found that CD4 T cell depletion was blocked in the presence of caspase-1 inhibitors. Similarly, shRNA-mediated knockdown of caspase-1, but not caspase-3, blocked CD4 T cell death in HIV-infected HLAC cultures. These findings indicated that death of abortively infected CD4 T cells in HLACs is elicited by caspase-1-mediated pyroptosis, a highly inflammatory form of programmed cell death (Doitsh et al., 2014). Consistent with this notion, shRNA knockdown of the inflammasome adaptor protein, ASC (apoptosis-associated speck-like protein containing a CARD) effectively blocked CD4 T cell death. Interestingly, shRNA-mediated knockdown of NLRP3 did not alter cell death, suggesting that cell death is mediated through caspase-1-dependent pyroptosis involving an inflammasome that contains ASC but lacks NLRP3. This finding was consistent with the fact that IFI16 can form its own ASC-containing inflammasome where caspase-1 is activated.

Together, these findings revealed a fundamentally different mechanism of CD4 T cell death by HIV, emphasizing that most cells are not dying because of a toxic action of products

encoded by HIV. Rather, death occurs as a consequence of a powerful defensive innate immune response launched by the host against the virus leading to a cellular form of suicide rather than virological murder (Figure 1).

This paradox—that pathogenesis is not produced when HIV successfully replicates but instead where it encounters resistance by the host and fails to replicate—raises new questions. What immune cells and tissues are involved in HIV pathogenesis? Why are some lentiviral infections pathogenic and others nonpathogenic? What role does pyroptosis play in the chronic inflammation observed during HIV infection? Does pyroptosis play any role in the residual inflammation observed in HIV patients treated with antiretroviral therapy (ART)?

Are All Resting CD4 T Cells Created Equal?

Because the pyroptotic death pathway was readily detected in HLAC formed with human tonsil or spleen, subsequent studies investigated whether resting CD4 T cells circulating in the blood are similarly sensitive to pyroptosis in the presence of HIV. Remarkably, although quiescent lymphoid and blood CD4 T cells supported HIV entry and fusion with equivalent efficiency (Cavrois et al., 2011), blood cells were highly resistant to the pyroptotic death pathway (Muñoz-Arias et al., 2015). At least in part, this difference reflects their deeper state of cellular rest. This deeper resting state is associated with the formation of fewer incomplete reverse transcripts following abortive infection and lower expression of innate immune sensors, specifically IFI16 (Figure 2A) (Muñoz-Arias et al., 2015). Pyroptosis involves multiple events and an array of endogenous DNA sensors and innate immune mediators, such as ASC, NLRP3, and cytoplasmic pro-IL-1β, that are constitutively expressed in lymphoid tissue CD4 T cells (Doitsh et al., 2014). Resting blood-derived CD4 T cells lack the expression of these key components, likely further contributing to their resistance for pyroptosis. Interestingly, when these blood cells are activated, higher levels of IFI16 are detected, reverse transcription improves, yet the cells still display resistance to pyroptosis, arguing for yet additional blocks. The pyroptotic response is not the only arm of IFI16 signaling that is defective. Within these activated blood CD4 T cells, IFI16 effectively recruits STING and TBK1 following sensing of DNA. However, this response does not lead to the expression of IFNβ- and IFN-stimulated genes (Berg et al., 2014). Thus, IFI16 may be involved in a yet higher level of regulation in CD4 T cells. Such regulation may allow IFI16-mediated function in select tissue compartments but not in others, perhaps serving to prevent inappropriate cell-autonomous responses. These findings highlight a striking difference in the biology of resting CD4 T cells residing in lymphoid tissue versus blood. Moreover, the primary experimental use of mitogen-activated blood CD4 T cells for the study of HIV pathogenesis may have inadvertently created observational bias favoring the direct killing model, in which activated CD4 T cells are productively infected with HIV-1 and die via caspase-3-mediated apoptosis (Cooper et al., 2013; Gandhi et al., 1998; Laurent-Crawford et al., 1991; Terai et al., 1991; Zhang et al., 1997).

Not a Good Time to Be in the Neighborhood

Although viral load is commonly measured in blood, HIV principally replicates within lymphoid tissue (Haase et al., 1996, 1999; Hufert et al., 1997; Pantaleo et al., 1993; Racz et al., 1990; Tenner-Racz et al., 1998; Zeng et al., 2012b). Many CD4 T cells continually traffic between the blood stream and lymphoid tissue compartments. In the absence of engagement of their cognate antigens, naive lymphocytes may reside in lymphoid tissue for only 12–18 hr before migrating back into the bloodstream (Cyster, 2005; Ho et al., 1995; Wei et al., 1995). While in the lymph nodes, CD4 T cells are in intimate contact with other cells and exposed to various cytokines. One consequence of this interaction is that a small subpopulation of these cells achieve a sufficient state of cellular activation to support productive HIV infection (Kreisberg et al., 2006). In contrast, CD4 T cells in the blood are not exposed to these cytokines, and most of these cells remain in a deep resting state that is unable to support productive HIV infection (Gao et al., 1993).

Innate immune mediators and the microenvironment within lymphoid tissues are evidently crucial in the massive bystander killing of CD4 T cells that occurs during HIV infection (Figure 2B). Remarkably, when cocultured with either CD4 or CD8 T cells or B cells from lymphoid tissues, normally resistant peripheral blood CD4 T cells readily died from caspase-1-induced pyroptosis (Figure 2C). Currently, the nature of sensitization is poorly defined. These effects are quickly lost when the cocultures are disassembled, which resembles CD4 T cells trafficking in and out of lymphoid tissues where they rapidly gain and lose sensitivity to pyroptosis (Muñoz-Arias et al., 2015). Conditioned medium from lymphoid tissues fails to consistently render peripheral blood cells sensitive to cell death by pyroptosis, suggesting that key signals are generated through cell-to-cell interactions. It is still unclear whether one or multiple signals participate in conferring this response on peripheral blood cells. By understanding the nature of the signaling between lymphoid tissue and blood cells, it might be possible to use antibodies or small molecules to block sensitization in lymphoid tissues, thereby rendering all CD4 T cells resistant to pyroptosis, like blood CD4 T cells. However, it would be key to assess the effects of such an intervention on the normal human immune response to ensure that one form of immunodeficiency is not replaced by another.

A Starring Role for Cell-to-Cell Transmission in HIV Pathogenesis

Retroviruses fuse and enter their target cells either as cell-free virions or through cell-to-cell spread (Sattentau, 2010). The pathway utilized greatly affects the infectivity yield. Cell-to-cell spread is 10^2 to 10^3 times more efficient than cell-free particles emanating from the same cells (Jolly, 2011). Although the increased effectiveness of cell-to-cell spread has been known for 20 years (Dimitrov et al., 1993), its contribution to HIV pathogenesis—and particularly to abortive infection, innate immune detection, and pyroptosis—has not been directly explored.

Remarkably, our studies in HLACs show that the mode of viral spread sharply affects the outcome of HIV infection. Infection with free HIV-1 particles, even at high quantities, is unable to trigger innate immune recognition and pyroptotic cell death. However, these free

virions are able to establish productive infection in a small subset of activated CD4 T cells that die via caspase-3-dependent apoptosis after producing new virions. Conversely, cell-to-cell spread of HIV-1 across virological synapse leads to massive abortive infection of the far more prevalent resting non-permissive CD4 T cells. These cells ultimately die as a consequence of caspase-1-dependent pyroptosis (Galloway et al., 2015). These findings differ from the prevailing view of HIV pathogenesis—where most of the pathogenic effects of HIV arise from the killing of CD4 T cells by circulating free virions. Instead, we propose that the fundamental “killing units” that lead to CD4 T cell depletion and progression to AIDS are infected cells that reside in lymphoid tissues and disseminate virus from cell to cell. Importantly, these findings provide a key mechanistic link between (previously considered independent) bystander and direct killing models. Death of abortively infected bystander cells critically depends on the presence of productively infected cells (Figure 3).

The role of cell-to-cell spread in CD4 T cell death further underscores the contribution of lymphoid tissues to HIV pathogenesis, where such intimate cellular interactions occur. Dying bystander CD4 T cells in human lymph nodes often cluster near productively infected cells, both ex vivo (Doitsh et al., 2010) and in vivo (Finkel et al., 1995). Cell-to-cell spread of HIV predominantly takes place across specialized contact-induced structures known as virological synapses (Agosto et al., 2015; Jolly et al., 2004, 2007; Jolly and Sattentau, 2004). These synapses facilitate efficient transmission of virus toward the uninfected and engaged target cell. The synapse gains stability through actin-mediated recruitment of adhesion molecules, such as the integrin leukocyte function-association antigen 1 (LFA-1) and its cognate ligand, intracellular adhesion molecule 1(ICAM-1), to the junction point of cellular interaction (Jolly et al., 2007). Ironically, while close cell-cell interaction is exploited by HIV for efficient viral spread between permissive cells, it acts against HIV when it spreads to nonpermissive targets, causing termination of viral propagation, abortive infection, and cell death by pyroptosis driving inflammation and disease.

Why would cell-to-cell transmission of HIV trigger cell death yet free virions do not? One possibility relates to the low infectivity of retroviral particles. Because of this property, cell-free virions may fail to generate sufficient quantities of cytoplasmic DNA intermediates to elicit an innate immune response in the abortively infected cells. Notably, the DNA-binding domain within IFI16 displays surprisingly low affinity for DNA perhaps explaining why IFI16 has evolved to contain two DNA-binding HIN domains, whereas other PYHIN proteins, such as AIM2 (another inflammasome-forming DNA sensor), exhibit only one (Jakobsen and Paludan, 2014). To enhance recruitment to the sensed DNA, IFI16 molecules (and other inflammasome-forming sensors) undergo cooperative DNA binding through homotypic interactions between non-DNA-binding pyrin domains to form filamentous clusters (Lu et al., 2014; Morrone et al., 2014). Thus, a minimum concentration of foreign DNA products (with an optimal length >500 bp) may be required to engage IFI16 into a functional inflammasome complex and activate caspase-1. The physical polarization and the targeted delivery of virus particles by cell-associated virus transmission may result in particularly high local concentrations of DNA products prone to detection and induction of pyroptosis (Jolly, 2010). TREX1, a cellular 3′DNA exonuclease, and other endonucleases, such as SLX4-associated MUS81-EME1, may further antagonize this process by degrading reverse-transcribed DNA products in the cytoplasm (Crow et al., 2006; Laguette et al., 2014;

Perrino et al., 2009; Stetson et al., 2008). Indeed, intrinsic TREX1 activity in the cytoplasm may create a threshold of DNA products necessary for productive infection in permissive CD4 T cells or, alternatively, for innate sensing and activation of the pyroptotic pathway in abortively infected CD4 T cells (Yan et al., 2010). Cell-to-cell spread across the virological synapse may overcome TREX1 enzymatic restriction by rapid transfer of large quantities of viral DNA, facilitated by the concomitant clustering of adhesion synapse molecules on the target cell. Cell signaling events may be involved, as well. Cell-free virion infection may not exceed this threshold and thus fails to trigger pyroptosis. It will be interesting to determine whether silencing endogenous TREX1 and SLX4 will allow cytoplasmic DNA intermediates from cell-free HIV particles to accumulate in sufficient quantity to elicit pyroptosis.

Making Sense of HIV DNA Sensing

The immunostimulatory activity of exogenous DNA has been known for 50 years (Isaacs et al., 1963; Rotem et al., 1963). However, a decade ago, almost nothing was known about the cellular mechanisms for detecting viruses and producing IFNs and proinflammatory cytokines. It is now clear that viruses, like bacteria and fungi, are initially recognized by host pattern recognition receptors (PRRs), which are widely expressed on the membranes, and within the cytoplasm and endosomes of the host cells. These PRRs form a first line of defense initiating cell-intrinsic anti-viral innate immune responses. The most recent addition to the family of viral PRRs includes the cytoplasmic DNA sensors. The newly identified sensors for HIV reverse-transcribed DNA are cyclic GMP-AMP synthase (cGAS) active in monocyte-derived macrophages and monocyte-derived dendritic cells (Sun et al., 2013; Wu et al., 2013) and IFI16 active in lymphoid tissue CD4 T cells (Monroe et al., 2014). Both cGAS and IFI16 directly bind cytosolic DNA and activate the adaptor protein STING on the endoplasmic reticulum (IFI16 by direct binding and cGAS through a dinucleotide product, cyclic GMP-AMP), leading in turn to the binding of TBK1 and TBK1-dependent phosphorylation of IRF3 followed by induction of a type-I IFN response (Paludan and Bowie, 2013). This antiviral response to intracellular DNA is commonly referred to as the “interferon-stimulatory DNA” (ISD) pathway.

Initially, cGAS and IFI16 appeared to mediate parallel ISD sensing pathways that operated during HIV infection. However, in fact, we now understand that these sensors recognize distinct forms of ISD, are expressed and operate in specific cell types, and produce different biological outcomes. cGAS binds most efficiently to dsDNA (Civril et al., 2013; Kranzusch et al., 2013; Zhang et al., 2014), while IFI16 recognizes both single-stranded (ss) DNA and stem-rich forms of dsDNA (Brázda et al., 2012; Jakobsen et al., 2013; Monroe et al., 2014; Unterholzner et al., 2010). In macrophages, IFI16 is expressed and recognizes HIV and herpes simplex virus-1 (HSV-1) DNA (Horan et al., 2013; Jakobsen et al., 2013; Kerur et al., 2011), but knockdown of cGAS is sufficient to inhibit the ISD pathway induced by retroviruses, such as HIV, simian immunodeficiency virus (SIV), murine leukemia virus, and DNA viruses, such as HSV-1 and vaccinia virus (Gao et al., 2013; Sun et al., 2013). In lymphoid-derived CD4 T cells, knockdown of IFI16 prevents both ISD and pyroptotic death pathways in cells abortively infected with HIV-1 (Monroe et al., 2014). In contrast, cGAS protein could not be detected in resting lymphoid tissue-derived CD4 T cells, nor bound to any cytoplasmic form of DNA in affinity chromatography-mass spectrometry experiments

(Monroe et al., 2014). Thus, while cGAS appears to be a primary sensor of cytoplasmic DNA in myeloid cells, IFI16 may preferentially function as the primary DNA sensor in lymphoid tissue CD4 T cells. Of note, knockdown of IFI16 does not render abortively infected CD4 T cells permissive to HIV-1, suggesting that post-entry restriction is mediated independently of these innate antiviral sensing pathways. In addition, inhibition of the downstream ISD pathway by knockdown of STING (as well as antibody-mediated blockade of surface IFNα/β receptors) does not prevent pyroptosis, suggesting that inflammasome assembly and induction of IFNβ by IFI16 occur independently in abortively infected CD4 T cells. Much remains to be learned about the protein interaction network engaged by the IFI16 and the circumstances surrounding its seemingly redundant function with cGAS.

The functions of IFI16 and other PRR are likely cell type-specific and are regulated at a level beyond expression. The surveillance activity of IFI16 in lymphocytes and macrophages is modulated by numerous posttranslational modifications involving acetylation and phosphorylation. For example, when the nuclear localization signal of IFI16 is acetylated on two lysine sites by the acetyltransferase p300, IFI16 nuclear import is inhibited, and the sensor accumulates in the cytoplasm (Li et al., 2012). These modifications allow for dynamic changes in the intracellular localization of IFI16 and extend its range of DNA surveillance. Indeed, nuclear IFI16 localization is essential for sensing Kaposi sarcoma-associated herpesvirus (KSHV) and HSV-1 DNA in the nucleus, and it requires a functional nuclear localization signal (Ansari et al., 2015; Kerur et al., 2011). Both lysine sites are highly conserved among IFI16 homologs and HIN200 family members such as AIM2, suggesting a common regulation of subcellular localization by acetylation. These findings contradict the canonical paradigm that the nucleus is “immune privileged” for sensing of foreign DNA, suggesting that both cytoplasmic and nuclear IFI16 participate in viral DNA surveillance. Predicted acetylation site have also been identified on and between the C-terminal HIN domains of IFI16 that bind viral DNA. Modification at these sites might influence the target DNA specificities of the protein (Li et al., 2012). We have detected three distinct isoforms of endogenous IFI16 in the cytoplasm of lymphoid-derived CD4 T cells, each exhibiting effective binding activity for either dsDNA or ssDNA forms (Monroe et al., 2014). These isoforms, which likely correspond to IFI16 splicing variants (Johnstone et al., 1998), or perhaps different posttranslational modifications, may respond to distinct types of HIV DNA intermediates in the cytoplasm (Figure 4A).

Interestingly, our unbiased analysis of cytoplasmic DNA-binding proteins revealed selective binding activity among the array of DNA sensors expressed in tonsillar CD4 T cells (Monroe et al., 2014). For example, the known DNA sensors AIM2 and DAI did not appear to bind HIV DNA, even though their cytoplasmic expression levels were as high as that of IFI16 (Figure 4B). Particularly intriguing was that, of the known inflammasome DNA sensors, IFI16, but not AIM2, recognized HIV DNA. AIM2 efficiently binds dsDNA and was expected to be a top candidate for HIV sensing, but it was completely absent from the mass-spectrometry list (Figure 4C). These findings suggest that the surveillance activity of host innate sensors may be regulated at multiple levels. More insight is needed into how the recognition of pathogenic nucleic acids by these sensors is regulated (Figure 4D).

Sorting Out Pathogenic and Nonpathogenic Lentiviral Infections

Infection with HIV is characterized not only by development of profound immunodeficiency but also by sustained inflammation and immune activation (Deeks, 2011; Klatt et al., 2013; Salazar-Gonzalez et al., 1998). Chronic inflammation likely plays a role as a critical driver of immune dysfunction, the premature appearance of aging-related diseases, and the emergence of immune deficiency (Deeks, 2011; Ipp and Zemlin, 2013). Many now regard HIV infection not only as an evolving virus-induced immunodeficiency but also as chronic inflammatory disease (Nasi et al., 2014; Wang et al., 2015). Even after the introduction of effective antiretroviral therapy, chronic inflammation persists. Animal studies also support the relationship between immune activation and progressive cellular immune deficiency: SIVsm infection of its natural nonhuman primate hosts, the sooty mangabey, causes high-level viral replication (Brenchley et al., 2004; Mattapallil et al., 2005) but limited evidence of disease (Brenchley et al., 2010; Milush et al., 2011; Rey-Cuillé et al., 1998). This lack of pathogenicity is accompanied by a lack of inflammation, immune activation, and cellular proliferation. In sharp contrast, experimental SIVsm infection of rhesus macaque produces immune activation and AIDS-like disease with many parallels to human HIV infection (Brenchley et al., 2010; Chakrabarti et al., 2000). In fact, in many cases, upon cross-species jumps, viruses can result in severe or fatal disease in the novel, nonnatural hosts, while these same viruses replicate at high levels in their natural hosts but cause little or no pathology. In these infections, the balance between protective and pathogenic host immune responses seems to be critical (Rouse and Sehrawat, 2010). Innate immune effectors that detect the presence of viral products by PRRs likely play a pivotal role in this balance (Iwasaki, 2012). The immunological processes during zoonotic viral infections leading to host pathology or tolerance have been the subject of an excellent recent review (Mandl et al., 2015).

Pyroptosis Can Breed More Pyroptosis

What causes chronic inflammation in HIV-infected subjects? The precise stimulus that triggers chronic inflammation is not clear and remains a key open and widely debated question in the field. Among the potential causes is microbial translocation caused by breakdown of the gastrointestinal epithelia barrier that follows extensive depletion of CD4 T cells in mucosal tissues (Ancuta et al., 2008; Brenchley et al., 2006; Marchetti et al., 2008; Nazli et al., 2010; Nowroozalizadeh et al., 2010). However, CD4 T cell depletion in the gut mucosa also occurs during non-pathogenic SIV infection but gut barrier function is not compromised (Marchetti et al., 2013). Another area of uncertainty pertains to the natural history of HIV-infected patients, who continue to exhibit low levels chronic inflammation despite effective long-term combination antiretroviral therapy and full suppression of plasma HIV RNA levels (Hunt et al., 2003; Kuller et al., 2008; Lichtfuss et al., 2011). As these subjects grow older, they appear to experience a higher-than-expected risk for a number of diseases typically associated with aging, including cardiovascular disease, cancer, osteoporosis, and other end-organ diseases (Deeks, 2011). Importantly, a subset of these patients is classified as “immunological nonresponders.” Despite the introduction of antiretroviral therapy, CD4 T cell counts remain low in these individuals. They also are distinguished by higher-than-expected levels of inflammation and immune activation (Gazzola et al., 2009; Massanella et al., 2015; Robbins et al., 2009). Thus, paradoxically,

systemic inflammation in HIV-infected patients appears to persist in these individuals even when viral replication is effectively suppressed.

We found that tissue-derived CD4 T cells are naturally primed to mount inflammatory responses as evidenced by their production of high levels of cytoplasmic pro-IL-1β, as well as the caspase-1 adaptor ASC, and NLRP3 inflammasome (Doitsh et al., 2014). The release of proinflammatory cellular contents, including ATP, by pyroptotic CD4 T cells may provide a second inflammatory stimulus, leading to activation of caspase-1 by the NLRP3 inflammasome in surrounding CD4 T cells. Thus, pyroptosis initiated by HIV may trigger an avalanche of new rounds of pyroptosis in primed CD4 T cells by the repeated release of intracellular ATP in a virus-independent manner. Such an “auto-inflammation” scenario could generate persistent rounds of pyroptosis, chronic inflammation, and loss of CD4 T cells even when viral replication is reduced by antiretroviral therapy (Figure 5). In this scenario, pyroptosis and inflammation would be initially triggered by abortive infection of resting CD4 T cells leading to IFI16 sensing, caspase-1 activation within IFI16 inflammasomes, and death by pyroptosis. The subsequent release of propyroptotic mediators like ATP from these dying cells could then trigger new rounds of pyroptosis involving a switch to NLRP3 inflammasomes.

Inflammatory signals induce upregulation of endothelial selectins and immunoglobulin superfamily members, particularly ICAM-1 and/or VCAM-1, which mediate the transendothelial migration of various types of circulating leukocytes to inflamed lymphoid organs (Luster et al., 2005) (Figure 3). The most abundant blood-borne leukocytes in healthy humans are neutrophils. These short-lived cells express a large number of adhesion molecules for rapid binding to inflammation-induced counter-receptors on activated endothelial cells and accumulate within hours at sites of acute inflammation. Neutrophils are essential for combating bacterial and fungal infection; however, upon activation, they release cytototoxic mediators that promote necroptosis and tissue damage (Linkermann et al., 2014). Furthermore, neutrophil enzymes, such as elastase, proteinase-3, chymases, granzyme A, and cathepsin G process extracellular deposits of pro-IL-1β precursor (Fantuzzi et al., 1997; Guma et al., 2009; Joosten et al., 2009). Monocytes are long lived and also express a broad range of adhesion molecules and chemoattractant receptors. After recruitment into lymph nodes, monocytes (unlike neutrophils) differentiate into tissue-resident macrophages or dendritic cells. These professional antigen-presenting cells are specifically attracted to extracellular “danger signals,” including ATP and uridine triphosphate released by dying cells (Ravichandran, 2011), and are primed to activate NLRP3-mediated pyroptosis upon binding of ATP to their surface P2X7 purinergic receptors (Mariathasan et al., 2006). Differentiated myeloid cells constitutively express pro-IL-18 and release high levels of bioactive and proinflammatory IL-18 during pyroptosis (Puren et al., 1999). Sustained high levels of IL-18 are observed in the plasma of HIV-infected patients, especially in later stages of the disease (Torre and Pugliese, 2006). Thus, while the main source of IL-1β is HIV-mediated pyroptosis of lymphoid tissue CD4 T cells, the source of IL-18 (and associated pro-inflammatory cytokines such as IL-6, IL-15, and TNF-α) may result from an auto-feed form of pyroptosis involving tissue-resident macrophages and dendritic cells during the chronic phase of infection.

Conclusions and Implications for Therapeutic Strategies

Pyroptosis contributes to the host's ability to rapidly limit and clear infection by removing intracellular replication niches and enhancing defensive responses through the release of proinflammatory cytokines and endogenous danger signals. However, in pathogenic inflammation, such as that elicited by HIV-1, this beneficial response does not eradicate the primary stimulus. In fact, it goes into overdrive and creates a vicious cycle, where dying CD4 T cells release inflammatory signals that trigger “bystander pyroptosis” and attract more cells into the infected lymph nodes to die and produce more inflammation.

Identifying pyroptosis as the predominant mechanism that causes the two signature pathogenic events in HIV infection—CD4 T cell depletion and chronic inflammation—provides therapeutic opportunities, namely caspase-1, which controls the pyroptotic pathway. In this regard, pyroptosis of CD4 T cells and secretion of IL-1β can be blocked in HIV-infected HLACs by addition of the caspase-1 inhibitor VX-765 (Doitsh et al., 2014), which has already proven to be safe and well tolerated in phase II human clinical trials (http://clinicaltrials.gov/ct2/show/NCT01048255). These findings could open the door to an entirely new class of “anti-AIDS” therapies that act by targeting the host rather than the virus. By altering the host response to tolerate the virus rather than suppressing its replication, VX-765 or related drugs may mimic the evolutionary strategy observed in natural hosts, where a stable equilibrium has been established between viral growth and host survival. This new class of AIDS therapies could be particularly beneficial (1) for patients with broad-spectrum resistance or limited access to antiretroviral therapy, (2) for blocking chronic inflammation that likely drives the earlier onset of age-related diseases in patients treated for HIV, and (3) as a potential component of an HIV cure, if the inflammation associated with persistent pyroptosis maintains the latent reservoir through cytokine dysregulation and increased homeostatic renewal.

The question of how CCR5-expressing CD4 T cells die during HIV-1 infection is of central importance. For many years, this question has been difficult to address in cultures of human lymphoid tissues due to the small number of CCR5-expressing CD4 T cells in the cultures (~5%), and the availability of only weakly reactive antibodies for detecting CCR5 expression by flow cytometry. In fact, previous studies reported no cytopathic effect in tonsillar tissues following infections with R5-tropic strains of HIV-1 (Jekle et al., 2003; Penn et al., 1999). Two important questions regarding the death of CCR5-expressing CD4 T cells are as follows: (1) If only a few CD4 T cells in lymphoid tissues express the CCR5 coreceptor, how are CD4 T cells massively depleted in HIV-infected patients? (2) Do CCR5-expressing CD4 T cells die via abortive HIV infection and pyroptosis?

Collectively, the findings discussed here indicate that it is the activation state, not the pattern of coreceptor expression or the mode of viral entry, that determines nonpermissivity. Indeed, pseudotyping HIV-1 with an amphotropic MLV envelope does not lead to productive infection of nonpermissive lymphoid-derived CD4 T cells (Doitsh et al., 2010). Although CCR5-expressing CD4 T cells in tonsillar tissues are more permissive to HIV infection (possibly effector memory T cells) (Roy et al., 2005; Schweighardt et al., 2004), a significant fraction of this cell population (possibly central memory T cells) becomes

abortively infected by HIV-1 and dies via caspase-1-mediated pyroptosis (Doitsh et al., 2014). Of note, in situ immunostaining of fresh lymph nodes obtained from untreated subjects infected with R5-tropic HIV-1 revealed abundant caspase-1 activity in the paracortical zone comprised primarily of resting CD4 T cells (Doitsh et al., 2014). As discussed above, such large area of caspase-1 activity may occur by the release of intracellular content including DNA and ATP from dying cells that trigger an avalanche of new rounds of pyroptosis in primed nearby CD4 T cells.

Studies by Steele and colleagues, who used a lamina propria aggregate culture system demonstrated that R5-tropic HIV infection of these cultures, drive caspase-1-dependent pyroptosis (Steele et al., 2014). In these cultures, exposure to gut microbes promoted a shift to caspase-3-dependent apoptosis, likely reflecting an increased state of cellular activation and permissivity. Additional studies are now underway using humanized mice infected with R5-tropic HIV and in vivo analysis of lymphoid tissue from longitudinal HIV patients.

Together, these findings are shifting the paradigm for how HIV exerts its pathogenic effects. Rather than the virus playing a major role, it is the host response to viral DNA produced during abortive infection that triggers CD4 T cell death. These findings also link the two pathogenic signatures of HIV infection—CD4 T cell depletion and chronic inflammation—through a common mechanistic pathway. These findings reveal a prominent role of the host as a major driver of HIV pathogenesis. It may be possible to exploit these insights for the therapeutic benefit of HIV-infected subjects. We can imagine that HIV-infected individuals will benefit from combined treatment with antiviral drugs and agents like caspase-1 inhibitors that interrupt the pyroptotic pathway. These agents could be particularly useful in subjects exhibiting persistently high levels of inflammation after antiviral drug initiation or in the immunological nonresponders subset of HIV-infected individuals, who may be experiencing unexpectedly high levels of ongoing pyroptosis despite excellent control of viremia.

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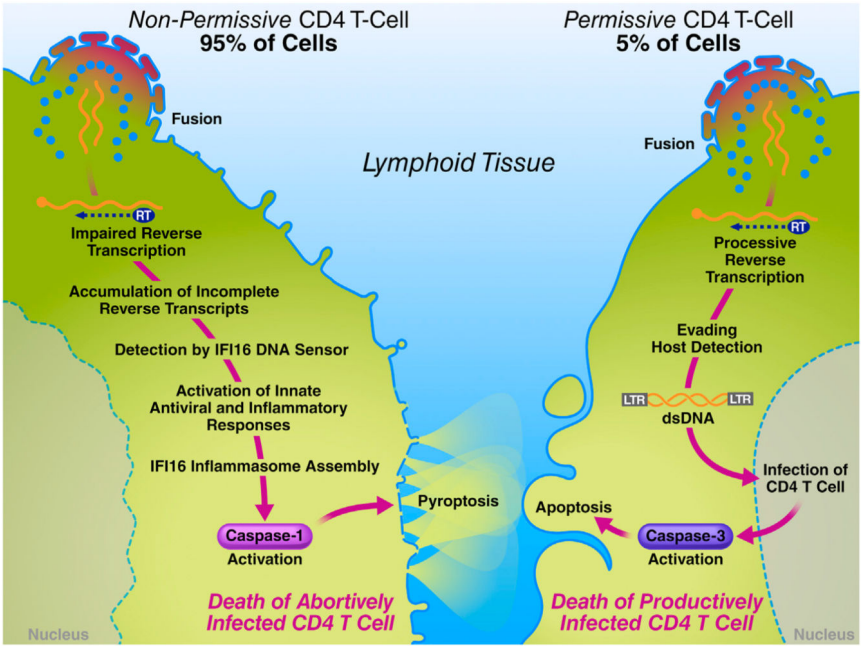

Figure 1. Roles of Caspase-3-Dependent Apoptosis and Caspase-1-Dependent Pyroptosis in CD4 T Death during HIV Infection

HIV-1 infection in a biologically relevant human lymphoid aggregate culture (HLAC) system reveals that the permissivity status of target CD4 T cells dictates how they die. If the virus infects an activated and thus permissive cell (i.e., 5% of the total CD4 T cells), productive infection ensues, and the cell dies from a silent caspase-3-mediated apoptosis. Conversely, if the virus infects a resting, nonpermissive cell (i.e., >95% of target CD4 T cells), abortive infection occurs, leading to the accumulation incomplete cytosolic viral DNA transcripts that are detected by IFI16. This sensor assembles into an inflammasome where caspase-1 becomes activated, which in turn triggers pyroptosis, a highly inflammatory form of programmed cell death. Elicitation of pyroptosis absolutely requires cell-to-cell spread of the virus; cell-free virions are not able to activate this response (see Figure 3). During chronic infection, most HIV-1 replication and loss of CD4 T cells occurs in such secondary lymphoid tissues (Zeng et al., 2012a).

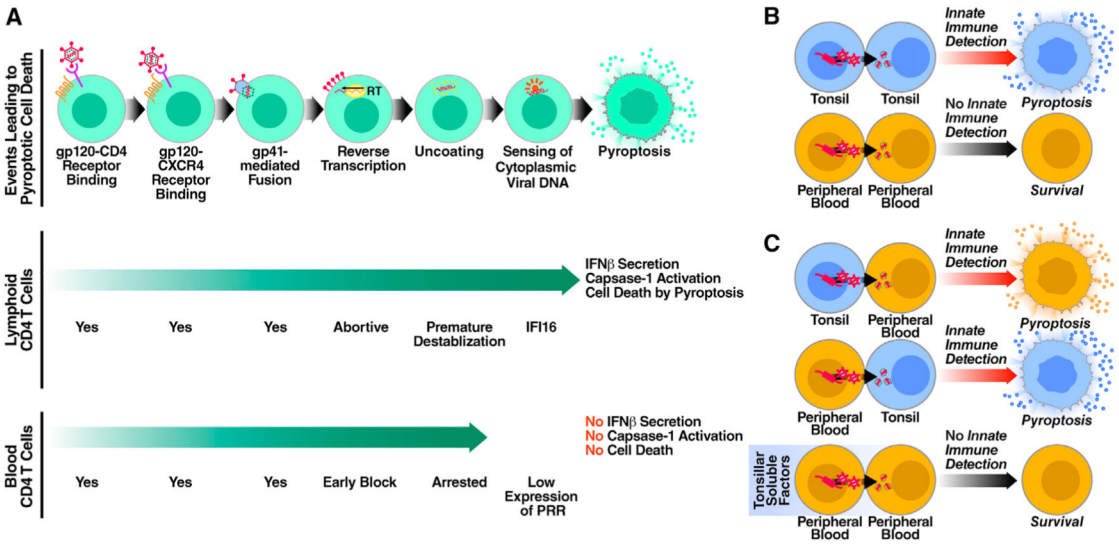

Figure 2. Biological Differences between Tissue-Derived and Blood CD4 T Cells Have Important Implications for Viral Pathogenesis

(A) Target CD4 T cells derived from lymphoid tissue undergo abortive infection and pyroptotic cell death. Resting peripheral blood CD4 T cells are susceptible to HIV-1 fusion (with X4-tropic virus) and become abortively infected but are highly resistant to pyroptosis. This intrinsic resistance likely involves factors affecting both viral replication (i.e., early block of reverse transcription, arrested or delayed uncoating as a result of unsuccessful reverse transcription, reverse-transcribed DNA intermediates below the optimal length required for recognition), and the ability of peripheral blood CD4 T cells to mount an efficient antiviral response (i.e., low expression of IFI16 and other PRRs, a general defect in innate antiviral pathways in response to cytosolic DNA [Berg et al., 2014]).

(B) Viral spread from productively infected cells extensively depletes target CD4 T cells in lymphoid cultures but not in cultures of peripheral blood cells.

(C) Resting blood-derived CD4 T cells are rendered sensitive to cell death and massively depleted when cocultured with lymphoid cells (CD4 T, CD8 T or B cells), suggesting that the lymphoid microenvironment sensitizes CD4 T cells to depletion by abortive HIV infection and caspase-1-mediated pyroptosis. Thus, the resistance of target blood cells to pyroptosis may not be due to inefficient viral production or transfer from blood-derived CD4 T cells. Supernatants from tonsil cultures do not render blood CD4 T cells susceptible to pyroptosis, indicating that close interactions between the lymphoid-derived and blood cells are required for sensitization, as occurs when CD4 T cells in bloodstream traffic back to lymphoid tissue.

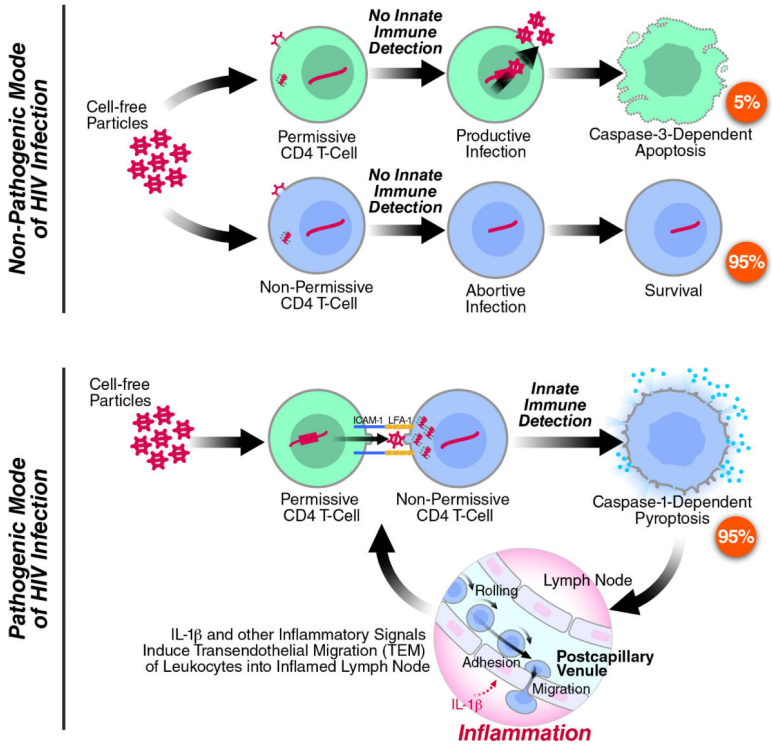

Figure 3. The Mode of HIV-1 Spread Determines the Outcome Form of Programmed Cell Death and Has a Key Role in HIV Pathogenesis

Free HIV particles kill only CD4 T cells that are permissive, undergo productive infection, and die from caspase-3-mediated apoptosis. However, in human lymphoid tissues such as tonsil and spleen, activated and permissive cells constitute <5% of all CD4 T cells. Free HIV-1 particles, even in large quantities, cannot directly trigger innate immune recognition and pyroptosis of nonpermissive target CD4 T cells, which constitute >95% of CD4 T cells in lymphoid tissues. Similarly, infection with lentiviral vectors does not kill nonpermissive target CD4 T cells (Doitsh et al., 2014). Thus, HIV particles themselves do not directly cause pathogenesis and AIDS. Conversely, it is the small fraction of permissive cells that become productively infected and mediate cell-to-cell spread across viral synapses culminating in the pyroptotic death on nonpermissive CD4 T cells. Thus, productively infected cells, not free HIV particles, are the fundamental “killing units” of CD4 T cells in lymphoid tissues. Productive (“direct”) and abortive (“bystander”) infections are therefore not independent pathways of CD4 T cell depletion; they are linked in a single pathogenic cascade. Along with playing a critical role in the virological synapse the interaction of LFA-1 on T cells with ICAM-1 also mediates the arrest and migration of leukocytes on surfaces of postcapillary venules at sites of infection or injury, as well as the ability of these cells to crawl out of the blood stream between high endothelial venules and into lymph nodes (Girard et al., 2012). Importantly, IL-1β and other inflammatory signals increase the expression of adhesion molecules such as ICAM-1 on endothelial cells (Barreiro et al., 2002; Carman and Springer, 2004; Dinarello, 2009; Dustin et al., 2011; Hubbard and Rothlein, 2000). The release of IL-1β by dying pyroptotic CD4 T cells in HIV-infected lymphoid tissues likely attracts more cells from the blood into the infected lymph nodes to die and produce more inflammation. Thus, the interaction of LFA-1 with ICAM-1

contributes to HIV pathogenesis by both promoting the depletion of CD4 T cells and facilitating a state of chronic inflammation, two key processes that propel disease progression to AIDS.

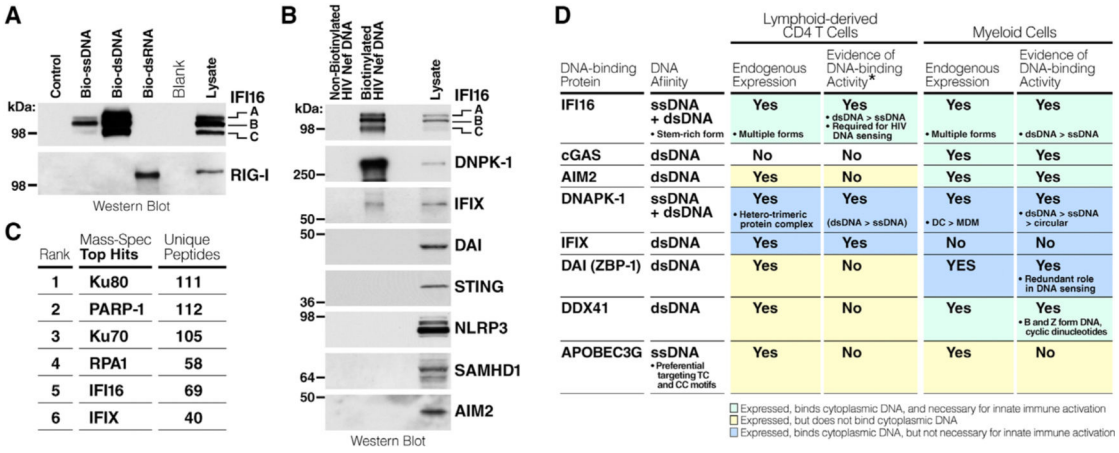

Figure 4. The Set of DNA Sensors Expressed in Cells Does Not Necessarily Define the Innate Response Pathway Against Intracellular DNA

(A) IFI16 displays two HIN domains (designated HIN-A and HIN-B) separated by a spacer region containing several serine, threonine, and prolines. The length of this region is regulated by alternative mRNA splicing, giving rise to three IFI16 isoforms (designated A–C). The predominant B isoform of IFI16 is detectable in various cell types, including human fibroblasts, epithelial cells, macrophages, and T cells (Dell'Oste et al., 2015). Interestingly, while all A–C IFI16 isoforms are equally expressed in tonsillar CD4 T cells and bind dsDNA, the B form specifically possess high affinity to ssDNA.

(B) Biochemical analysis of cytosolic DNA-binding proteins in tonsillar CD4 T cells to identify potential viral DNA sensors (Monroe et al., 2014). Despite their significant endogenous expression, the known DNA binding proteins DAI, STING, and AIM2 were not recovered by immunoprecipitation of biotinylated HIV DNA. APOBEC3G, another endogenously expressed DNA-binding proteins, which has high affinity for ssDNA, was not identified in these analyses. The DNA sensor cGAS was detected neither at the protein level in tonsillar CD4 T cells nor in the affinity chromatography-mass spectrometry experiments. Conversely, the proteins DNA-PK and IFIX were expressed and associated with cytoplasmic HIV-1 DNA but were not involved in IFN-β induction and pyroptosis of CD4 T cells abortively infected with HIV-1. Thus, the surveillance activity of host innate sensors comprises a diverse set of PRRs that act nonredundantly against cytoplasmic DNA ligands.

(C) Top-ranked hits of cytoplasmic DNA-binding protein in tonsillar CD4 T cells based on DNA affinity chromatography and mass spectrometry protein discriminant scores. Among the broad array of DNA-binding proteins identified, only IFI16 was required and sufficient to induce IFN-β and pyroptosis of lymphoid-derived CD4 T cells abortively infected by HIV-1. Some of the identified proteins might not be true sensors but instead have other regulatory roles involving innate sensing pathways, DNA damage repair, or the cell cycle.

(D) A comparison of evidence supporting the function of the various receptors in lymphoid CD4 T cells and myeloid cells in detecting intracellular DNA. Despite some redundancy at the level of expression, most sensors act in a cell-type-specific manner. More detailed studies are needed to identify the dynamic role of individual sensors in the context of disparate viral infections and to assess crosstalk between the different sensing pathways. (A)–(C) were adapted from (Monroe et al., 2014). (D) is based on experimental data sets from Civril et al. [2013]; DeYoung et al. [1997]; Ding et al. [2004]; Ferguson et al. [2012];

Fernandes-Alnemri et al. [2009]; Gao et al. [2013]; Hornung et al. [2009]; Jakobsen et al. [2013]; Jin et al. [2012]; Lee [2013]; Li et al. [2007]; Lu et al. [2015]; Shindo et al. [2012]; Sun et al. [2013]; Takaoka et al. [2007]; Unterholzner et al. [2010]; Wang et al. [2008]; Wu et al. [2013]; Yan et al. [2008]; Zhang et al. [2011a, 2011b].

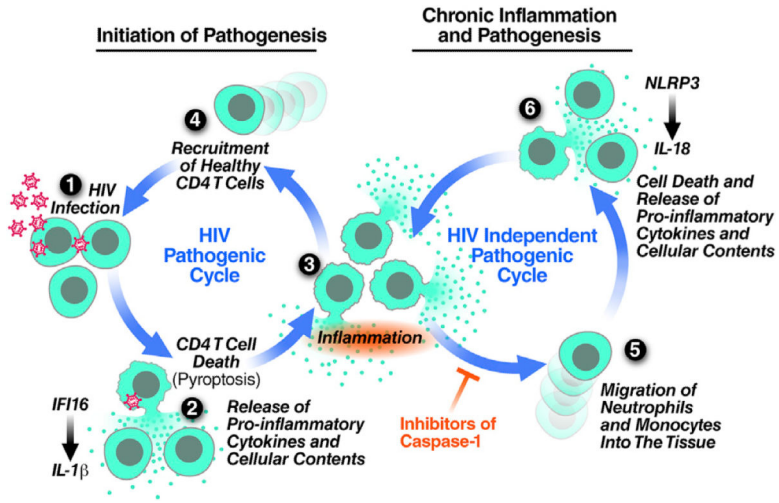

Figure 5. Caspase-1 Activity in Lymphoid Tissue May Persist Independently of Viral Replication and Promote a Chronic State of Inflammation and Immune Activation

Pyroptosis provides a nexus between CD4 T cell death and inflammation—the two key drivers of HIV pathogenesis. Abortive HIV infection of CD4 T cells in lymphoid tissues results in sensing of the viral cytosolic DNA products by IFI16 leading to caspase-1 activation in inflammasomes and pyroptosis (steps 1 and 2). Dying cells release large amounts of proinflammatory cytokines including IL-1β and cellular contents such as 5′-ATP into the extracellular milieu (step 2). These events promote local inflammation (step 3), which mediates the migration of new circulating CD4 T cells (predominantly central memory CD4 T cells, containing large amounts of pro-IL-1β) into the lymph node (step 4) and establishes a vicious cycle of HIV spread, CD4 T cell death, and inflammation. Inflammatory signals induce transendothelial migration of other types of circulating leukocytes into the inflamed lymphoid organs, particularly neutrophils and monocytes; the latter cells differentiate into tissue-resident macrophages or dendritic cells (step 5). These tissue-resident cells are primed to mount inflammatory responses and constitutively express high levels of cytoplasmic pro-IL-18, as well as the caspase-1 adaptor ASC and NLRP3 inflammasome. The release of proinflammatory cellular contents and ATP by nearby pyroptotic cells may activate the NLRP3 inflammasome in nearby primed cells (including primed naive CD4 T cells), leading to new rounds of pyroptosis (step 6). Such an “autoinflammation” scenario could generate persistent rounds of pyroptosis, chronic inflammation, and loss of CD4 T cells even when viral replication is suppressed by antiretroviral therapy.