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Role of the NLRP3 Inflammasome in HIV Pathogenesis and Inflammation

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The Thesis of Brian Faung is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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DEDICATION

I would like to dedicate my thesis to my parents, Richard and Angela Faung, and to my sister Evelyn. I would also like to thank my close friends for all their support. Finally, thank you to everyone in Dr. Spector's lab who has helped me in my learning and growth.

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ABSTRACT OF THE THESIS

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Antiretroviral therapy (ART) has ameliorated substantially human immunodeficiency virus type 1 (HIV-1) associated disease. Even so, persons living with HIV-1 (PLWH) on ART still exhibit chronic inflammation and various forms of HIV-1-associated disorders. The continued existence of HIV-1-associated disorders is thought to result from persistent inflammation present in PLWH despite sustained viral suppression and normalization of the

CD4+ T cell count. Recent literature suggests that inflammasome activation is central to persistent inflammation with the NLRP3 inflammasome being the most studied. Upon formation of the NLRP3 inflammasome complex, the cytokines IL-1β and IL-18 are produced. PLWH have increased activation of the complex that results in excess cytokine production, causing excessive inflammation. This activation is thought to contribute to a broad range of associated diseases including the early onset of aging, cardiovascular disease, and cognitive disorders. In this review, the mechanisms associated with the activation of the NLRP3 inflammasome in HIV-1 infected cells as well as the role of NLRP3 in persistent inflammation and HIV-associated pathogenesis are discussed. A summary of the NLRP3 inflammasome in HIV pathogenesis and inflammation as well as its role in other diseases will be given. Consequently, possible therapeutics using NLRP3 inflammasome inhibitors will also be discussed. By understanding the NLRP3 inflammasome and its connection to HIV-1, effective methods to treat HIV-1-associated diseases caused by excessive inflammation can be identified.

Introduction

HIV-1 Infection and Viral Production

Human immunodeficiency virus type 1 (HIV-1) is an obligate intracellular pathogen. The virus is dependent on host cell machinery for replication and growth. During primary infection, HIV-1 binds to CD4 and most often uses CCR5 as the co-receptor for entry into CD4+ helper T cells (Siliciano et al., 2000). During later stages of infection, the virus undergoes mutation and X4 tropic variants emerge as immune activation increases. The virus then uses CXCR4 as a co-receptor, which is associated with increased viral replication and a rapid decline in the CD4+ T cells. After infection, the virion enters the host cell and uses reverse transcriptase to convert its viral RNA into double stranded DNA. The viral DNA then enters the nucleus and integrates into the host genome to create a provirus. Synthesis of mature, infectious particles are then made and the virion buds off and infects new cells (Abbas et al., 2018).

HIV-1 Pathogenesis and AIDS

HIV-1 infection occurs first through acute infection where memory CD4+ T cells are infected in mucosal lymphoid tissue, resulting in the death of many of these cells (Appay et al., 2008). Dendritic cells then transport the virus to lymph nodes, resulting in viremia and spreading of the virus throughout the body (Abbas et al., 2018). The adaptive immune system mounts a humoral and cell-mediated immune response to control viral replication. During the chronic phase, also known as the latent period, most of the infection is controlled. However, the number of CD4+ T cells decreases over time (Douek et al., 2002). Once the number of CD4+ T cells falls below 200 cells/mm^3, the risk of opportunistic infections increases (Abbas et al., 2018). This can be brought about by microbial infections and cytokines, leading to AIDS progression. To remove

infectious microbes, the immune system upregulates cytokine production to activate the immune response. However, this leads to increased viral HIV-1 production.

Low CD4+ T cell Count and Chronic Inflammation

The development of antiretroviral therapy (ART) has decreased mortality and allows individuals living with HIV-1 to enjoy a greater quality of life. ART typically involves three drugs from at least two different classes, such as two reverse transcriptase inhibitors and an integrase inhibitor, that helps to control the production of viral particles (Abbas et al., 2018). However, ART-treated patients are still subject to chronic inflammation due to elevated levels of cytokines (Decrion et al., 2005). Inflammation can be induced through the mechanism of pyroptosis, a caspase-1 mediated programmed cell death, causing the loss of bystander cells. This occurs in naïve T cell lymphocytes and CD4+ T cell lymphocytes, which are lost or become dysfunctional (Doitsh et al., 2014). Loss of these T cells result in the chronic inflammatory issues associated with HIV-1.

HIV-1 and Autophagy

The mechanism of survival of HIV-1 is through the avoidance of host cell defenses. With its high mutation rate, HIV-1 is able to avoid detection by antibodies and T cells (Abbas et al., 2018). A natural response to pathogens is autophagy, which HIV-1 modulates in order to increase its viral production (Campbell et al., 2013). Autophagy destroys pathogens using autophagosomes to capture and subsequently target them for eradication by lysosomes. HIV-1 downregulates autophagy, preventing the mechanism from removing the pathogen (Zhou et al., 2008). Interestingly, autophagy is not completely eradicated as the virus uses the mechanism's

early stages for viral replication in CD4+ T cells and macrophages. Later stages of autophagy result in the increase of autophagosomes, which is prevented by HIV-1 (Kyei et al., 2009). Thus, this mechanism is down modulated in primary cells during HIV permissive infection but not removed since it is necessary for cell survival (Campbell et al., 2013). Since autophagy is not completely removed by HIV-1, further studies can be conducted to discover ways to bypass the block of autophagosome production.

The NLRP3 Inflammasome

The NLR family pyrin domain containing 3 (NLRP3) inflammasome is used in the immune response against pathogens, such as parasites, bacteria, fungi, and viruses (Zhao et al., 2020). Inflammasomes function to mediate the activation of inflammatory mediators and are important in the innate immune response. They work to fight off invading pathogens and become activated during cellular infections or stressors, resulting in a multitude of inflammatory responses (Wang et al., 2020). The NLRP3 inflammasome is integral in maintaining proper function in various inflammatory diseases, including metabolic and central nervous system (CNS) disorders. Inflammatory responses and pyroptotic cell death can result from the formation of the NLRP3 inflammasome in response to pathogens (Wang et al., 2020).

Although activation of the NLRP3 inflammasome is important for proper immune response, excessive activation has been shown to be involved in various diseases caused by overexpression, resulting in excessive inflammation (Masters et al., 2009). In PLWH, this results in HIV-related conditions that may include early aging, kidney disease, and minor cognitive disorders (Shao et al., 2015).

NLRP3 Inflammasome Activation

Inflammasomes are multiprotein complexes that can be found in dendritic cells, macrophages, and other immune cells. One main type is the nucleotide-binding oligomerization-(NOD-) like receptors (NLRs). This is a form of pattern recognition receptors (PRRs) and is used in the control of pathogens in immune responses (Broz et al., 2016). PRRs are highly conserved sensors that include toll-like receptors (TLRs) and sense the pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) from viral infections to cause an immune response (Próchnicki et al., 2017). Proteins involved with NLRs are termed NOD-like receptor proteins (NLRPs) and a major type is seen in NLRP3, which is used in inflammation and antiviral responses (Zhao et al., 2020). The NLRP3 inflammasome is a complex that includes the NLRP3 protein, the apoptosis speck-like protein (ASC), and procaspase-1 (Inoue et al., 2013). ASC serves as the connecting point between the NLRP3 protein and procaspase-1. The main role of the NLRP3 inflammasome is regulating the activation of procaspase-1 to caspase-1, a proteolytic enzyme, which subsequently causes the release of IL-1β and IL-18 cytokines from their immature pro-IL-1β and pro-IL-18 forms. The increased levels of IL-1β and IL-18 in infected cells result in pyroptosis, a highly inflammatory form of cell death as a result of intracellular infection (Y. Yang et al., 2015). The three forms of NLRP3 inflammasome activation are the Canonical (Classical), Non-canonical, and Alternative pathways.

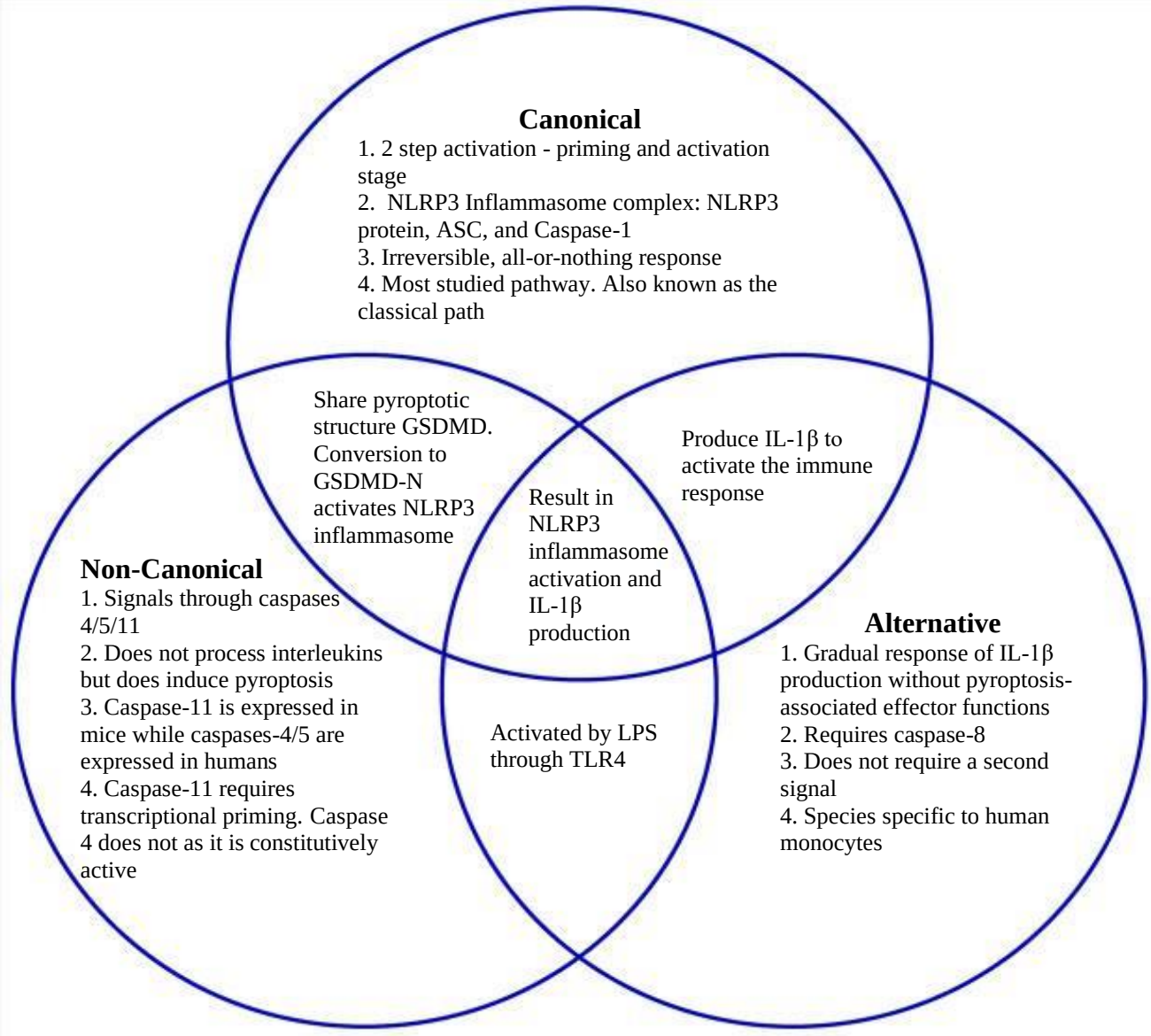

Figure 1: Canonical, Non-canonical, and Alternative Pathways of NLRP3. Summary of the similarities and differences of NLRP3 inflammasome activation using the different pathways of the canonical, non-canonical, and alternative paths

Canonical (Classical) Activation

In the canonical NRLP3 pathway, an immune activator is required for assembly of the NLRP3 inflammasome complex. Other processes that may trigger the activation of the NLRP3 inflammasome complex are calcium signaling, mitochondrial dysfunction, and protein aggregates (Wang et al., 2020). Without immune activators, the internal interaction between the

NOD domain and the leucine-rich repeats (LRRs) suppress the interaction between NLRP3 and ASC (Inoue et al., 2013). The complex is activated as a result of pathogen-associated molecular patterns (PAMPs), which can come from viruses, and/or damage-associated molecular patterns (DAMPs), also known as danger signals (Shao et al., 2015). PAMPs and DAMPs are both sensed by PRRs. Activation takes place in a two-step process. In the first step, the sense or priming stage, PAMPs and DAMPs are recognized by TLRs. Interferon-alpha/beta receptor (IFNAR), which binds endogenous type I interferon cytokines, can also activate the first step. As a result, NF-κB signaling is activated, causing increased release of the NLRP3 protein, pro-caspase-1, pro-IL-1β, and pro-IL-18 (Bauernfeind et al., 2009). In the second step, known as the assembly or activation stage, the NLRP3 inflammasome is constructed. This results from various stimuli due to infection, tissue damage, and metabolic imbalances, and can result from ionic flux (potassium efflux) and reactive oxygen species (ROS) production. Associated stimuli may be ATP, invading pathogens, nucleic acids, pore-forming toxins, and crystalline substances (Hari et al., 2015; Lamkanfi et al., 2014). The components of the NLRP3 protein, ASC, and procaspase-1 join to form the mature NLRP3 inflammasome complex (Wang et al., 2020). Once created, procaspase-1 is cleaved to form mature caspase-1, which is used to transform pro-IL-1β and pro-IL-18 into IL-1β and IL-18 (Lu et al., 2014). Maturation of caspase-1 also regulates the production of gasdermin D (GSDMD), which makes pores in infected and uninfected cell membranes, causing increased IL-1β and IL-18 production and the triggering of pyroptosis (Shi et al., 2017; He et al., 2015).

Non-canonical Activation

In contrast to the canonical activation of the NLRP3 inflammasome, the non-canonical pathway occurs through caspase 11/4/5 activation instead of caspase-1 (Ding et al., 2017). Caspase-11 is seen in mouse macrophages (Kayagaki et al., 2011; Viganò et al., 2013) while caspases-4 and -5 are seen in humans. Caspase-11 requires transcriptional priming by lipopolysaccharide (LPS), IFN-αβ, or IFN-γ in the presence of ISRE/GAS or NF-kB binding elements in the promoter region. Cytosolic LPS causes caspase-11 dependent pyroptosis and enters the cell through TLR4/MD2/CD14 receptor complex-mediated endocytosis or bacterial OMV-mediated entry (Vanaja et al., 2016). Host GBPs or genetic mutation in bacteria increase cytosolic access to LPS. Host-derived lipids oxPAPC (oxidized phospholipid) can enhance dendritic cell activation in mouse cells via caspase-11-dependent NLRP3 inflammasome-mediated IL-1β release. Caspase-4, on the other hand, does not require transcriptional priming. This caspase is constitutively expressed in monocytes and some non-monocytic cells, and is also activated by cytosolic LPS (J. Yang et al., 2015). Lipid A, a lipid component of LPS, is an agonist of the TLR4/MD2 membrane receptor and is responsible for caspase-11-dependent pyroptosis. LPS or lipid A directly bind to caspase-4/5 (Viganò et al., 2015) and to caspase-11. CARD, a caspase recruiting domain, in caspase 11/4/5 recognizes lipid A and undergoes oligomerization for caspase activation. Binding induces caspase oligomerization and activates cysteine protease activity to cleave downstream targets. oxPAPC also activates cysteine protease activity specifically in caspase-11 to activate downstream NLRP3 inflammasome independently of caspase-11 catalytic activity. Unlike caspase-1, caspase 11/4/5 do not process interleukins but do induce pyroptosis (Ding et al., 2017). The pyroptotic structure Gasdermin D (GSDMD) is shared by caspase-1 and caspase 11/4/5 (Shi et al., 2015). LPS-activated caspase 11/4/5 cleaves GSDMD to GSDMD-N, which binds the GSDMD pore and causes pyroptosis (Pellegrini et al.,

2017). GSDMD-N binding to the GSDMD pore can activate the NLRP3 inflammasome to produce IL-1β (Ding et al., 2017). The non-canonical pathway is associated with the activation of TLR4–MyD88 and toll/IL-1 receptor homology-domain-containing adapter-inducing interferon-β (TRIF) pathways, with a consequent nuclear translocation of NF-κB, which in turn promotes the transcription of IL-1β, IL-18, NLRP3, interferon regulatory factor (IRF)-3 and IRF7 genes (Ding et al., 2017).

Alternative Pathway

A third pathway for NLRP3 inflammasome activation has been identified and has been called the alternative pathway (Gaidt et al., 2016). LPS and TLR4 trigger the alternative pathway in human monocytes (Netea et al., 2009), which appears to be species specific as it has only been identified in humans to date (Gaidt et al., 2016). The alternative pathway occurs independently of potassium efflux, pyroptosome (inflammasome complex) formation, and pyroptosis. The TLR4-TRIF-RIPK1-FADD-CASP8 complex upstream of NLRP3 is activated and produces mature IL-1β. Caspase-8 is believed to cleave an intermediate protein needed for NLRP3 inflammasome activation. In contrast to the canonical pathway as an irreversible, all-or-nothing response (Liu et al., 2014), the alternative pathway has a gradual response. Inflammasome activation and pyroptosis uncoupling allows for IL-1β production without pyroptosis-associated effector functions. Caspase-8 appears to be required as experiments with primary monocytes show the caspase-8 inhibitor Z-IETD-FMK blocks activation of the alternative pathway (Gaidt et al., 2016).

Summary of NLRP3 Inflammasome Activation Pathways

To summarize, there are three known forms of NLRP3 inflammasome activation. The canonical pathway has a two-step activation. In the first step, a signal from pathogens is sensed and upregulates pro-IL-1β (Wang et al., 2020). The second step is caused by a second signal, most commonly with cytosolic potassium efflux, and is where the inflammasome complex is formed. Upon activation of the complex, caspase-1 is released resulting in the subsequent production of IL-1β (Zhao et al., 2020). In the non-canonical pathway, LPS is sensed by caspase-11/4/5 to trigger pyroptosis and activate the NLRP3 inflammasome. This pathway does not process interleukins itself but does induce pyroptosis resulting in cytokine production (Ding et al., 2017). In contrast, the alternative pathway is specific to human monocytes, has a gradual response, and requires only one signal for activation; IL-1β production is separate from pyroptosis (Gaidt et al., 2016). Although there are differences between these pathways, they all ultimately result in NLRP3 inflammasome activation and IL-1β production. A concise summary comparing and contrasting these pathways can be found in Figure 1.

The NLRP3 Inflammasome in HIV-1 Pathogenesis and Inflammation

Polymorphisms in NLRP3 Contribute to HIV Chronic Inflammation

Single-nucleotide polymorphisms (SNPs) in inflammasome related genes are associated with HIV chronic inflammation (Pontillo et al., 2010). These SNPs have been studied to see the effects on IL-1β production and subsequent activation of the NLRP3 inflammatory response (Verma et al., 2008). The genetic variants identified are involved in innate and acquired immunity, and play a role in HIV-1 infection susceptibility and in AIDS progression (Kaslow et al., 2005). Pontillo et al. (2010) tested *NLRP1* and *NLRP3* SNPs in HIV-1 infected children born to infected mothers and in HIV-1 infected adults. The study used two SNPs each for the *NLRP1* gene and the *NLRP3* gene. Their results showed a 3'UTR SNP in the *NLRP3* gene (rs10754558) exhibited susceptibility to HIV-1 infection. Previous studies found the G allele of this polymorphism enhanced NLRP3 mRNA stability (Hitomi et al., 2009). NLRP3 was hypothesized to be used by HIV-1 as an intracellular receptor leading to inflammasome activation with increased production of IL-1β, and excessive inflammation (Pontillo et al., 2010). These results suggest that the NLRP3 inflammasome is implicated in HIV-1 infection susceptibility. Further studies are needed to confirm these findings since there may be confounding variables where the genotypes of the mothers affected the results in the children.

HIV-1 Induces NLRP3 Inflammasome Activation

Hernandez et al. (2014) demonstrated that HIV-1 acts as a first signal inducer in the activation of the NLRP3 inflammasome, leading to the production of IL-1β, as seen in monocyte-derived macrophages (Xing et al., 2009). This production of IL-1β can lead to AIDS progression by causing increased inflammation and by increasing viral production. NLRP3

activation results in the induction of pyroptosis (Fink et al., 2006), which also causes inflammation as well as a subsequent decrease in CD4+ cells. HIV-1 activates the NLRP3 inflammasome by inducing the priming signal through the NF-κB signaling pathway, suggesting its role as an activator of the inflammasome through the canonical pathway. Of note, HIV-1 alone fails to lead to IL-1β release as it does not induce the second signal for NLRP3 inflammasome activation but rather requires a separate second signal (Hernandez et al., 2014).

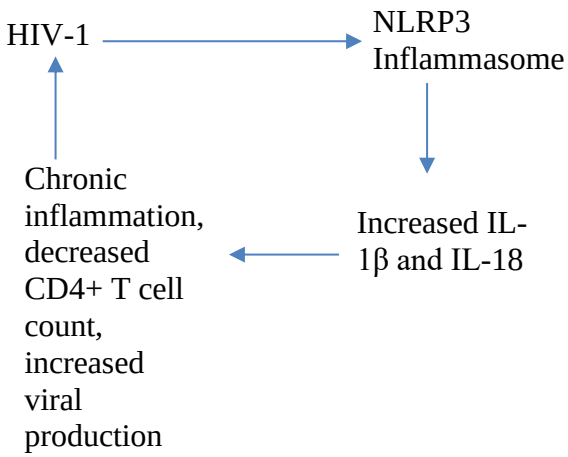

Figure 2: HIV-1 and the NLRP3 inflammasome. HIV-1 activates the NLRP3 inflammasome to produce IL-1β and IL-18. Although production of these interleukin cytokines is needed for natural and proper immunity, overproduction leads to excessive inflammation and increased HIV production. This presents the double-edged sword dilemma of the NLRP3 inflammasome where overactivation causes associated diseases implicated with chronic inflammation

As noted earlier, although ART can lead to viral suppression, chronic inflammation persists in persons living with HIV-1 and is thought to be a major factor in many chronic conditions associated with HIV-1 infection. Bandera et al. (2018) showed that an upregulation of NLRP3 and caspase-1 in immunological non-responder (INR) patients results in increased inflammation and persistent immune activation. This may also result in increased caspase-1 activation, causing CD4+ T-cell loss due to pyroptosis and decreased CD4+ T-cell recovery (Doitsh et al., 2014). The study compared NLRP3 activation between patients with HIV-

stimulated peripheral blood mononuclear cells (PBMCs) of immunological non-responders (INRs) and those of patients with complete immune recovery (IRs) on ART. The results showed a significant increase in NLRP3 activation in INR patients and a decrease in CD4+ T-cell recovery under ART (Bandera et al., 2018). Using LPS, a potent activator of the inflammasome pathway (J. Yang et al., 2015), INR PBMCs were found to be more responsive to LPS stimulation, showing that greater immune activation results in increased NLRP3 expression and caspase-1 activation. This resulted in increased production of IL-1β and IL-18. These effects were also seen with HIV-1, which had less NLRP3 activation than with LPS but still showed an upregulation of NLRP3, IL-1β, and IL-18 when compared to IRs. Caspase-1 levels in INRs were increased as well in comparison with IRs, suggesting its role in CD4+ T-cell death and decreased immune recovery as a result of pyroptosis. Thus, HIV-1 appears to activate the NLRP3 inflammasome and caspase-1, leading to the production of inflammatory cytokines in HIV-infected ART treated patients with defective immune recovery (Bandera et al., 2018). Although these findings are intriguing, the *in vitro* comparisons between LPS and HIV may not be completely valid as different TLRs are used for signaling. LPS uses TLR4, which is on the surface of the cell, while HIV has been found to use TLR7/8, which are intracellular. Thus, further studies must be conducted to confirm these results, perhaps incorporating *in vivo* studies.

Further studies show how the effects of excessive inflammation due to increased cytotoxic cytokines leads to disease progression. During HIV pathogenesis, gut-associated lymphoid tissue (GALT) is the main organ affected and has vast degradation of CD4+ T-cells, causing GALT structural damage and increased microbial translocation that results in increased immune activation (Brenchley et al., 2006). This begins during acute infection of HIV-1 and

persists during chronic infection to differing degrees between HIV-1-infected persons. Feria et al. (2018) studied these effects by comparing expression of IL-1β, IL-18 and caspase-1 genes in HIV-1-progressors (patients with progressing viral replication) and -controllers (patients with controlled viral replication) in their GALT and PBMCs. Consistent with the hypothesis that increased inflammation is associated with disease progression, HIV-1-progressors had increased IL-18 and IL-1β production in comparison to controllers associated with increased caspase-1 activation in both GALT and PBMCs. Additionally, increased ASC in PBMCs was observed in HIV-1-progressors (Feria et al., 2018).

Immune activation occurs early in the acute phase of infection, which causes GALT changes, ultimately resulting in viral replication and immune exhaustion. With increased production of these cytotoxic cytokines, induction of inflammation leads to pyroptosis. The activation of caspase-1 in GALT is associated with a decrease in CD4+ T-cell count and an increase in viral load. Furthermore, IL-18 activates naïve T cell differentiation into Th1 and Th17 cells (Tominaga et al., 2000), both of which are very susceptible to HIV infection. Thus, the increased production of IL-18 in HIV-progressors results in immune activation and increased viral replication (Feria et al., 2018). The inflammasomes tested in this study were the protein absent in melanoma 2 (AIM2) inflammasome, NLRC4 inflammasome, and NLRP1 inflammasome. All of these inflammasomes studied showed increased IL-1β production in HIV-1-progressors due to caspase-1 activation, leading to subsequent pyroptosis resulting in GALT degradation. Although this was not specifically seen in the NLRP3 inflammasome, the same cytokines produced as a result of NLRP3 inflammasome complex formation and subsequent inflammation shows the similarities between the inflammasomes and the processes that lead to HIV pathogenesis and progressive disease.

-Kidney Function and NLRP3 Inflammasome

The induction of the NLRP3 inflammasome by HIV-1 also has an effect on renal function. Podocytes, cells in the Bowman's capsule in kidneys, are affected and play a major role in HIV-1-associated nephropathy (HIVAN). Lack of regeneration of these cells after injury prevents recovery from proteinuric kidney diseases. The dysregulated growth and loss of podocytes leads to HIVAN (Husain et al., 2009). The NLRP3 inflammasome complex is implicated as contributing to this pathology. Familiar activators of the inflammasome in PAMPs and DAMPs result in complex formation and subsequent effects that lead to HIVAN. Additionally, transforming growth factor (TGF-β) also appears to be important in the development of HIVAN. IL-1β increases TGF-β in kidney cells, leading to HIV-induced activation of NLRP3, causing increased TGF-β. Haque et al. (2016) examined the effects of the NLRP3 inflammasome complex on pyroptosis of podocytes and subsequent HIVAN in HIV-transgenic mice. In the transgenic model, mice were observed to have increased NLRP3, ASC, caspase-1, and IL-1β indicating the formation of the NLRP3 inflammasome complex in podocytes. This was observed both *in vivo* and *in vitro* in these HIV-transgenic mice (Tg26) when compared to the control (FVBN) mice. Other activators, such as ROS and potassium efflux, contributed to HIV-induced pyroptosis in both a dose and time dependent manner.

Inhibition of caspase-1 decreased podocyte expression of IL-1β production and provided protection against pyroptosis. Tempol, a superoxide dismutase mimetic agent, and glyburide, a potassium efflux inhibitor (Lamkanfi et al., 2009), decreased HIV-induced podocyte pyroptosis. Tempol also provided partial protection against HIV-induced podocyte pyroptosis (Haque et al., 2016). Furthermore, HIVAN is a proteinuric disease and is considered a podocytopathy. This can

be caused by lysosomal cysteine protease cathepsin L (CTSL) leakage, causing the loss of podocytes in podocytopathies (Rüster et al., 2006) and can be caused by ROS (Chen et al., 2012). Lastly, the renin angiotensin system has been seen to cause HIVAN development and progression. Studies showed Ang II infusion led to increased renal lesion progression in HIVAN mouse models, leading to HIVAN podocyte injury, while inhibiting Ang II production led to reduced HIVAN progression in humans and mice. Ang II also contributed to podocyte CTSL expression, leading to podocytopathies, which are kidney diseases resulting from damaged podocytes in the glomerulus (Salhan et al., 2012). This may lead to proteinuria or nephrotic syndrome where there is an increased level of protein in the urine (Haque et al., 2016). Future therapeutics for HIVAN can be created targeting the relationship between HIV-1 and the NLRP3 inflammasome and can further expand on the inhibitors used in this study for HIV-associated diseases.

-The Role of NLRX1 in HIV-1 Progression and NLRP3 Comparison

How HIV infection results in the upregulation of inflammatory cytokines in HIV-infected ART treated patients continues to be debated. A study conducted by Nasi et al. (2015) analyzed the major inflammasome components and studied the innate mitochondrial sensing (IMS) pathways in HIV-1 positive patients undergoing ART (Nasi et al. 2015). Results showed no differences in the expression of inflammasome components (sensors, adaptors, regulators, and downstream signaling) but did identify a significant decrease in mRNA expression of *NLRX1*, a mitochondrial protein used in the regulation of apoptosis (Soares et al., 2014; Jaworska et al., 2014). This protein is a part of the same NLR family as NLRP3 and serves as a regulator of immune system function. Normally, the overexpression of *NLRX1* increases ROS, regulates

apoptosis in transformed cells, and regulates type I interferon (IFN-I) and autophagy through activation of Tu translation elongation factor (TUFM), another mitochondrial protein (Tattoli et al., 2008; Lei et al., 2012). *NLRX1* and TUFM together may play a role in controlling host antiviral responses since their reduced levels are associated with increased IFN-I activation. With the decreased mRNA expression of *NLRX1*, it is seen that HIV-1 can downregulate pathways that result in cell death in various immune cell types, allowing the virus to avoid host restriction factors. This suggests that decreased expression of *NLRX1* may play a key role in HIV-1 infection (Nasi et al. 2015).

Contrasting Views

-Inhibition of HIV-1 Infection by NLRP3

The role of NLRP3 has been mentioned throughout this review to cause chronic inflammation upon activation by releasing cytotoxic cytokines that lead to excessive inflammation. This activation occurs during HIV-1 infection. However, NLRP3 may also play a dual role in the prevention of HIV-1 entry. This is caused by the interaction between NLRP3, HIV-1, and the purinergic receptor P2Y2 (Paoletti et al., 2019). NLRP3 prevents viral entry by blocking F-actin reorganization while HIV-1 avoids this by activating a P2Y2 dependent pathway, causing NLRP3 degradation (Paoletti et al., 2019). The HIV-1 envelope (Env) binds to host cell receptors (CD4, CXCR4, CCR5), which activate P2Y2. This causes recruitment of E3 ubiquitin ligase CBL to NLRP3, resulting in its degradation and preventing the NLRP3 block of HIV-1 (Stolp and Fackler, 2011). To test this, Paoletti et al. (2019) observed the interaction between NLRP3 and P2Y2, which are both expressed in CD4+ T-cells, during HIV-1 infection. Their results show that expression of the NLRP3 protein is regulated by post-translational

modifications in the early stages of HIV-1 infection. When HIV-1 Env binds to its host cell receptors, the NLRP3 and P2Y2 interaction increases, resulting in the recruitment of E3 ubiquitin ligase CBL by P2Y2 and degrading NLRP3. As a result, NLRP3 can act as an inhibitor of HIV-1 infection since its degradation allows for viral entry while its presence blocks entry. However, during post-entry stages, NLRP3 contributes to the known chronic inflammation and excessive immune activation. This finding can be used in the creation of treatments that target the NLRP3-P2Y2 interaction in order to prevent viral entry. This is further seen with NLRP3 activators, which reduce the susceptibility of HIV-1 target cells by regulating P2Y2-dependent F-actin remodeling in the initial stages of HIV-1 infection (Paoletti et al., 2019).

-NLRP3 and its Role in Vaccine Production in HIV-Infected Patients

Another contrasting view of the NLRP3 inflammasome is its role in the production of vaccines. Outside of the discussed effects of overproduction of cytokines leading to excess inflammation, this inflammasome is needed for the proper activation of dendritic cells (DC) to enhance vaccine response. This is seen in healthy individuals DCs (HD-DC) but not in DCs of HIV-infected patients (HIV-DC), even those undergoing ART, due to a defect in the NLRP3 of HIV-DC patients (Reis et al., 2019). DC are specialized antigen presenting cells (APC) that creates and maintains the primary immune response against antigens. DC maturation into APC occurs after recognizing PAMPs and DAMPs, resulting in increased CD4+ T lymphocyte activation and inducing the immune response (Steinman, 2007). Vaccines must properly activate DC to result in memory protective immunity through the use of adjuvants (Reis et al., 2019). NLRP3 is needed for adjuvant responsivity, thus HIV-DC patients with a defect in NLRP3 have decreased DC activation and an impaired immune response to vaccines (Li et al., 2008).

Reis et al. (2019) hypothesized that bacterial flagellin (FLG) could activate the NLRP3 inflammasome in HIV-DC. FLG, a ligand of the NAIP/NLRC4 inflammasome, activates this inflammasome and subsequently activates dendritic cells. This occurs where NAIP binds FLG and this complex is then recognized by NLRC4 to create the NAIP/NLRC4 inflammasome, which then produces IL-1β and IL-18 that leads to the immune response (Diebolder et al., 2015). The study tested FLG as an adjuvant to activate monocyte-derived dendritic cells (MDDC) of HIV-DC and tested its efficacy against lipopolysaccharide (LPS). This experiment was compared to regular donors (HD-DC). Their results showed FLG activates DC in both the HIV-DC and HD-DC conditions and did so to greater degrees than LPS in HIV-DC. These findings show dendritic cells of HIV-infected patients undergoing ART can still be activated and result in normal immune responses and bypass the NLRP3 defect, allowing for proper responses to vaccines for these individuals (Reis et al., 2019). Activation of the NLRP3 inflammasome to produce IL-1β and IL-18 leads to chronic inflammation in many HIV associated diseases but is also needed in the immune response and in vaccine therapy. This presents the double-edged sword of the NLRP3 inflammasome as both needed in proper immunity but also where overactivation can result in further exacerbation of inflammatory diseases. A greater understanding of this inflammasome is needed to find the best methods to elicit robust immunity against pathogens while at the same time preventing the overproduction of cytotoxic cytokines leading to excessive inflammation.

NLRP3 and HIV-1-associated Cognitive Impairment

ART treatment in HIV-1 infected patients has allowed for a great decrease in HIV-associated dementia due to successful viral suppression. However, many patients still have

inflammation and minor neurocognitive issues. This may be the result of viral RNA still being made at low levels by infected cells even in the absence of infectious virus or by low level HIV replication causing the persistent inflammatory response (Edén et al., 2016). These symptoms have been attributed to the activation of the NLRP3 inflammasome, causing increased production of inflammatory cytokines and activation of caspase-1. Rawat et al. (2019) tested this using GU-rich ssRNA within the HIV LTR (ssRNA40), which signals through TLR7/8 located within the cell, to see the effects on microglia in producing inflammatory markers as a result of NLRP3 inflammasome activation. Results showed an increase in NLRP3 inflammasome activation increases the production of IL-1β and IL-18. Inflammasome activation also caused an upregulation of ROS, causing damage to mitochondria. The use of ssRNA40 also resulted in inhibition of mitophagy, a form of autophagy associated with mitochondria, from negatively regulating the NLRP3 inflammasome from producing subsequent cytokines and activating caspase-1, resulting in increased microglial cell death by pyroptosis. Mitophagy is used to maintain healthy cells by removing damaged mitochondria that can lead to cellular degeneration (Lazarou et al., 2015). Results from this study showed that inhibition of mitophagy increased NLRP3 inflammasome activation and associated inflammation. The pathway found from this study with increased NLRP3 activation due to blocking of mitophagy and resulting subsequent cytokine production leading to inflammation may be the cause of cognitive issues and chronic inflammation in HIV-infected patients with ART treatment (Rawat et al., 2019). Future studies should explore how blocking or silencing TLRs reduce NLRP3 inflammasome activation and the production of inflammatory cytokines, possibly serving as a treatment for HIV-infected patients with chronic inflammation.

The regulatory genes of HIV are needed for viral production and may also play a role in NLRP3 activation. One particular protein is Transactivator of Transcription (Tat), which is elevated in HIV-1 infected patients, even those undergoing ART, and can activate NLRP3 through activation of the NF-kB pathway (Fiume et al., 2012). Chivero et al. (2017) hypothesized that Tat primes and activates the NLRP3 inflammasome resulting in an excessive inflammatory response. Issues associated with increased inflammation can be seen in HIV-1-associated neurocognitive disorders (HAND). HIV-1 infected patients undergoing ART still exhibit mild forms of HAND in asymptomatic neurocognitive impairment (ANI) and mild neurocognitive disorder (MND) whereas HIV-1-associated dementia (HAD) has decreased significantly with ART. Patients who develop ANI and MND exhibit increased NLRP3 activators in comparison to HIV-1 infected patients without deficits (Chivero et al., 2017). These activators can cause NLRP3 activation and IL-1β production (Chivero et al., 2017). Previous studies have shown that Tat stimulates IL-1β release in monocytes. To further this research, Tat was tested to see if it would also result in the same production of IL-1β by the activation of the NLRP3 inflammasome in microglial cells. Results show Tat primes the NLRP3 inflammasome by activating caspase-1 resulting in IL-1β release. Inhibition of NLRP3 activation by glyburide and NLRP3 silencing (*NLRP3* siRNA) resulted in a decrease in caspase-1 maturation and IL-1β production. Tat primes the NLRP3 inflammasome by reducing the effects of miR-223, a regulator of NLRP3. Upon exposure of microglia to Tat, the expression levels of miR-223 are reduced. After priming of the inflammasome, endogenous agonists, such as ATP and calcium, then activate NLRP3. Tat plays a role in the activation by increasing calcium levels (Mayne et al., 2000), which is an activator of NLRP3 (Lee et al., 2012). These findings show that Tat plays a novel role in the priming and activation of the NLRP3 inflammasome in microglial cells

(Chivero et al., 2017). Thus, targeting the interaction between Tat and NLRP3 can result in the inhibition of the inflammasome and be a novel therapeutic to decrease and even prevent neuroinflammation and diseases associated with HAND in HIV-1 infected patients. Furthermore, studies targeting the other HIV regulatory proteins should be conducted to observe their effects with NLRP3 and prevention of neuroinflammation.

TLR4 Signaling and NLRP3 in Liver Function

Another TLR associated with HIV-1 and NLRP3 inflammasome activation is TLR4, which is located on the cell surface. Zhang et al. (2019) observed the effects through the TLR4-NLRP3 pathway in both HIV-infected and healthy macrophages. This study showed HIV-1 infection in CD68+ liver macrophages activated the TLR4-NLRP3-caspase-1 axis and leads to increased IL-1β response to LPS, resulting in dysregulation of hepatic immune responses and progression of liver injury. HIV-1 infected liver macrophages exhibited increased NLRP3 expression, resulting in increased IL-1β production. Results show increased TLR4 expression in HIV-1 infected CD68+ macrophages, suggesting TLR4 is a major activator of NLRP3 expression. Findings show HIV-1 infection increases IL-1β expression through the activation of the NLRP3 inflammasome and caspase-1 in CD68+ hepatic macrophages (Zhang et al., 2019). Finding a way to block TLR4 activation can be used to prevent NLRP3 inflammasome activation and the production of inflammatory cytokines, a possible treatment to reduce chronic inflammation and associated liver issues in HIV-infected patients.

Antiretrovirals and NLRP3 Activation

Antiretrovirals have served as the cornerstone of treatment approaches for PLWH. However, in some cases certain antiretrovirals have been associated with severe adverse reactions in a subpopulation of recipients. An example of this is abacavir (ABC), a nucleoside analog reverse transcriptase inhibitor (NRTI) (White et al., 2015). For some patients ABC is associated with severe inflammation and an allergic response through the activation of an innate immune response. In these cases, ABC is a specific activator of the NLRP3 inflammasome (Toksoy et al., 2017). An allergic response known as abacavir hypersensitivity syndrome (ABC-HS) is seen, a response similar to organ graft rejection, in some patients. This response occurs approximately five days after treatment, showing a *de novo* generation of drug-reactive T-cells (Lucas et al., 2015). Activation is caused by danger signals of DAMPs or external cues, such as infections, as noted in the "danger model", resulting in drug-induced innate immune activation in TLRs and inflammasomes. TLR activation has immediate release of cytokines TNF and IL-8 through IKK2/NFκB signaling pathway while inflammasome activation occurs through the canonical two step priming and activation process. Inflammasome activation appears to be caused by lysosomal damage with cytoplasmic cathepsin B release or mitochondrial damage causing increased mitochondrial ROS and mitochondrial DNA and mitochondrial-localized lipid cardiolipin, both of which need further studies. Additionally, previous activators of inflammasome activation are seen in potassium efflux and necrosis. Patients with the major histocompatibility complex (MHC) class I allele human leukocyte antigen (HLA) 5701 are more likely to develop ABC-HS, which may be life-threatening (Mallal et al., 2002).

Toksoy et al. (2017) hypothesized that NLRP3 inflammasome activation leads to developing drug hypersensitivity of ABC-HS. Results showed that ABC stimulation did not cause a direct innate immune signal in THP1 cells and could not activate the inflammasome by

itself, suggesting other factors are needed for priming *in vivo*, such as the HIV-1 virus. In contrast, TLR8 stimulation was enough to prime the ABC-induced activation of the inflammasome. Upon NLRP3 inflammasome activation, the release of cytotoxic cytokines leads to cellular injury and allergic responses (Toksoy et al., 2017). With this knowledge of ABC as an inflammasome activator and drug allergen that induces delayed allergy depending on innate immune activation, screening patients for the presence of HLA 5701 has led to the elimination of ABC hypersensitivity in patients treated for HIV-1 infection.

NLRP3 Inflammasome Inhibitors

Excessive inflammation caused by the activation of the NLRP3 inflammasome is implicated in many different diseases. In order to ameliorate these issues, inhibitors of the NLRP3 inflammasome components, cytokines, and the activators of the complex have been tested to reduce chronic inflammation.

One such issue associated with the overproduction of inflammatory cytokines due to NLRP3 inflammasome activation is seen in aging and obesity. Patients who are older or obese tend to have increased cytotoxic cytokine levels as a result of chronic inflammation (Forsey et al., 2003; Vandanmagsar et al., 2011). The effects in obesity can be due to over nutrition, which leads to insulin resistance, causing problems with glucose homeostasis (He et al., 2020). An activator of the NLRP3 inflammasome is acetylation (He et al., 2020). Ways to deacetylate the inflammasome have been proposed as methods to reduce the effects of chronic inflammation. He et al. (2020) studied one such type in sirtuin 2 (SIRT2), a metabolic sensor and a cytosolic deacetylase dependent on NAD+ (Jing et al., 2016). SIRT2 was hypothesized to target NLRP3 and reverse aging-associated inflammation as well as insulin resistance. Using macrophages in mice, this study observed how acetylation activated the NLRP3 inflammasome, resulting in an acute response and the effects in aging and insulin resistance, which leads to glucose dysregulation. In contrast, the use of SIRT2 deacetylated the inflammasome and reversed these effects. SIRT2 regulates two downstream targets in the NLRP3 inflammasome activation pathway by targeting the NLRP3 protein and tubulin, which leads to the suppression of NLRP3 inflammasome assembly transport (Misawa et al., 2013). Mice with SIRT2 knocked out were found to have aging-associated chronic inflammation and compromised glucose homeostasis while those with SIRT2 showed decreased inflammation and improved glucose homeostasis (He

et al., 2020). These findings are exciting as further deacetylases can be implicated and studied to see their effects as therapeutics against diseases associated with excessive inflammation. Deacetylases can be further tested as a treatment for excessive inflammatory HIV-associated diseases.

Another NLRP3 inflammasome inhibitor that can be used to decrease the effects of overproduction of cytokines in HIV-1 is glyburide, a potassium efflux inhibitor (Lamkanfi et al., 2009). Commonly used to treat type-2 diabetes, glyburide is a medication used to maintain glucose homeostasis and has also been found to be anti-inflammatory. HIV-infected microglia that have NLRP3-associated neuroinflammation can use glyburide to directly prevent the formation of the NLRP3 inflammasome complex or prevent the production of IL-1β (Wang et al., 2020). Glyburide inhibits the inflammasome by acting downstream of the purinergic receptor, P2X7R, and inhibits ATP-sensitive potassium channels, inhibiting ASC aggregation. This prevents the NLRP3 inflammasome complex from forming and thus prevents the production of IL-1β and IL-18 (Wang et al., 2020). Glyburide reduces the excessive inflammation found in HIV-1 associated microglia neuroinflammation and further research can test its effects in other HIV-associated diseases caused by excessive inflammation.

More novel NLRP3 inflammasome inhibitors are the small molecule inhibitors MCC950 and β-Hydroxybutyrate (BHB) (Shao et al., 2015). MCC950 is a direct inhibitor that prevents caspase-1-dependent-processing of IL-1β and blocks NLRP3 inflammasome activation by inhibiting NLRP3 and ASC oligomerization in both the canonical and non-canonical pathways (Coll et al., 2015). This prevents IL-1β and IL-18 secretion through directly acting on the inflammasome. Similarly, BHB blocks ASC oligomerization, preventing NLRP3 inflammasome complex formation and the production of IL-1β and IL-18 by the inflammasome in human

monocytes. This inhibitor blocks potassium efflux from macrophages and only inhibits the canonical pathway (Youm et al., 2015). Both of these inhibitors can be tested to reduce cytokine levels in HIV-infected patients to decrease chronic inflammation and may prove useful as a treatment for these individuals.

More established inhibitors can be seen in Type I interferon (IFN) and IFN-β, both of which have been used in autoimmune and autoinflammatory diseases, most notably in multiple sclerosis (Shao et al., 2015). Type I IFN is produced by macrophages as a result of extracellular bacteria, viruses, and environmental irritants (Meylan et al., 2006). IFN-β is used to block IL-1β production through two separate paths. The first is where the STAT1 transcription factor phosphorylation represses NLRP1 and the NLRP3 inflammasome, blocking caspase-1 from activating IL-1β from pro-IL-1β. The other path is where Type I IFN increases IL-10 production through a STAT-dependent mechanism resulting in autocrine signaling and reducing pro-IL-1α and pro-IL-1β (Guarda et al., 2011). Future treatments can observe the effects of these inhibitors on HIV-associated inflammation.

Inhibitors in need of further research and testing are seen in autophagy inducers (Shao et al., 2015). One type is resveratrol, which activates autophagy by suppressing macrophage mitochondrial damage, preventing NLRP3 inflammasome activation and subsequent mediated IL-1β production (Chang et al., 2015). Another inhibitor is Arglabin, which activates autophagy in macrophages, reducing cholesterol levels and inflammation. Arglabin blocks NLRP3 inflammasome mediated IL-1β and IL-18 production and secretion (Abderrazak et al., 2015). Lastly, cannabinoid receptor-2 (CB2R) agonist blocks the priming step of NLRP3 inflammasome activation, preventing NF-kB signaling and the production of pro-IL-1β, pro-IL-18, and pro-caspase-1 (Shao et al., 2014). A better understanding of the pathways activating the NLRP3

inflammasome are needed in order to find the correct inhibitor to treat the associated illness. Further work on these inhibitors can be studied to see their effects in decreasing excessive inflammation in HIV-associated diseases.

Discussion/Conclusion/Future Works

This review has discussed how the NLRP3 inflammasome complex is activated and produces cytotoxic cytokines leading to excessive inflammation in various diseases, especially in HIV-1-infected patients. HIV-1 infection also leads to NLRP3 inflammasome activation, resulting in a continuous cycle of chronic inflammation. With the ability to inhibit components of the NLRP3 inflammasome complex, the production of cytokines, and the activators of the inflammasome, a reduction in chronic inflammation and associated diseases can be seen. This can help to treat the associated pathologies implicated in HIV-infected patients, suggesting the need for increased studies in NLRP3 inflammasome inhibitors. In addition, future studies need to have more primary cell work and research on humans, rather than merely studies on monkeys and mice. This will allow for more direct results to be seen in HIV-infected patients.

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