Title: The sooner the better: innate immunity as a path towards the HIV cure

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Abstract

To combat the diverse pathogens that infect humans, the immune system has evolved complex and diverse transcriptional signatures, which drive differential cellular and humoral responses. These signatures are induced by immune receptor sensing of pathogens and by cytokines produced at the earliest onset of infection. The specific nature of immune activation is as critical to pathogen clearance as the induction of an adaptive immune response. This is particularly true for HIV, which has developed numerous immune evasion mechanisms. In this review, we will highlight recent findings that show the differential role for early innate immune responses in promoting infection versus clearance and demonstrate the need for continued research on these pathways for development of effective HIV treatments.

Highlights

- Virus dissemination occurs rapidly after mucosal SIV infection
- Early innate host immune response after HIV/SIV infection dictates viral load at acute phase
- Specific Interferon signatures are associated to viral dissemination
- Inflammasome activation at early time-points inhibits intrinsic innate anti-viral responses and leads to virus dissemination and seeding

Introduction

Despite the development and implementation of new therapies, HIV infection still leads to high incidence of morbidity and mortality worldwide (WHO 2014). Currently available drugs have had a positive impact on the quality of life but cause many undesirable side effects that result in reduced adherence to treatment regimens [1]. When used continuously antiretroviral therapy (ART) controls the virus to undetectable levels, at least in plasma. Interruption of ART culminates in rebound of viral titers, confirming the presence of long-lived viral reservoirs [2]. HIV is capable of subverting many normal immune mechanisms; hence an effective cure for HIV will need to engage key antiviral pathways normally perturbed during infection. This is confirmed in animal models, which show that the early innate immune responses activated by HIV are not sufficient to interrupt infection and dissemination. Understanding the mechanisms that allow the virus to escape immune clearance will lead to the development of more effective treatments.

HIV infection and early immune activation

Following sexual exposure to HIV, the virus replicates in the vaginal or rectal mucosa [3]. Viral RNA is detectable in the circulation about 10 days after infection with HIV, and even earlier for SIV in non-human primates (NHP) (eclipse phase) (reviewed in [4]). The first line of immune defense in the mucosa is the innate immune response. This response is governed by the activation of conserved receptors termed Pattern Recognition Receptors (PRRs). One of the key mechanisms by which PRRs recognize viruses is through nucleotide sensing of viral RNA/DNA in the cytoplasm of infected host cells [5]. Activation of these receptors leads to signal transduction through multiple pathways including nuclear factor kappaB (NFκB), interferon regulatory factors (IRFs) and mitogen-associated protein (MAP) kinases of which the IRFs may be the most critical for antiviral defense [6, 7].

Integration of these complex signaling cascades leads to a mosaic of innate immune responses needed to fight off the diverse array of pathogens humans encounter and which are usually successful in controlling viral dissemination and provide the required inflammatory signals needed to develop a protective adaptive immunity. The activation of transcription factors, such as IRFs and NFκB, leads to altered gene expression and production of a wide range of pro-inflammatory cytokines. Cytokines

trigger immune cell recruitment and migration to infected sites; they also direct cellular activation. A bad outcome of this cytokine storm also leads to the recruitment of adaptive cells to lymphoid organs, increasing the number of HIV targets, helping virus dissemination to secondary lymphoid tissue including gut-associated lymphoid tissue (GALT) [8]. The consequence of this influx of immune cells is rapid viral seeding and dissemination. After the eclipse phase, virus levels increase exponentially to peak around 21–28 days. By this time, substantial CD4⁺ T cell depletion has occurred particularly in the GALT [9], possibly due to the higher expression of CCR5 [10]. This rapid loss of CD4⁺ T cells, in conjunction with impaired innate responses, creates an immune environment that is incapable of controlling infection and dissemination.

Role of the gut and the microbiome in HIV infection

The infected cells that form the reservoirs, such as resting memory CD4⁺ T cells [11–13], are refractory to ART-induced viral control and invisible to HIV-specific immune responses [14, 15]. Moreover, the anatomic sites of the reservoirs are diffuse with HIV detected in brain [16, 17], adipose tissue [18], spleen, lung, and other organs [11, 19]. Studies testing rapid initiation of ART in *Rhesus* macaques (RMs) on days 3–14 after intra-rectal SIVmac251 infection showed that only treatment with ART on day 3 blocked the emergence of viral RNA and pro-viral DNA in peripheral blood, lymph nodes and gastrointestinal mucosa. Paradoxically, treatment on day 3 also abrogated the induction of SIV-specific humoral and cellular immune responses. Yet, when ART was interrupted after 24 weeks of fully suppressive therapy, virus rebounded in all animals, including those that were treated on day 3. These results highlight the rapid seeding of the viral reservoir prior to detectable viremia in RMs and the need for an early, potent immune response to prevent reservoir establishment ([20], reviewed by [21]).

The human gut microbiota plays an essential role in maintaining immune homeostasis. Previous studies have demonstrated that disruption of the gut epithelial barrier during chronic HIV and SIV infections contributes to microbial translocation, increased T cell turnover and systemic immune activation [22][23–25]. Components of the gut microbiota aid in digestion of nutrients and produce factors that support epithelial growth, barrier function and immune system development (reviewed by [26]). Changes in the composition and diversity of gut microbiota have been linked to inflammation and

disease [27]. Increases in microbial diversity and pathogenic species is reported in HIV-infected patients and in SIV-infected NHPs[28-33]. These findings highlight the importance of normal gut function and microbiome composition in shaping the course of HIV infection.

Inflammation: a double-edged sword in HIV

Researchers at UC Davis •[34]reported that specialized gut cells called Paneth cells are initial viral sensors to SIV and the source of gut inflammation by producing IL-1 β . IL-1 β causes the breakdown of the gut epithelium that provides a barrier to protect the body against pathogens, suggesting another route for viral invasion and dissemination. This group found that addition of the probiotic *Lactobacillus plantarum* to the gut rapidly reduced IL-1 β and accelerated repair. IL-1 β is a highly inflammatory and pleiotropic cytokine capable of activating immune cells as well as inducing symptoms of infection (fever, lethargy, cachexia). Due to the potent nature of this molecule, its production is a tightly regulated process requiring two signals. It was recently discovered that members of the NOD-like receptor family termed inflammasomes mediate the proteolytic cleavage of pro-IL-1 β by inflammatory caspases into its biologically active form. This provides the second signal to initiate a full-fledged immune response, with the first coming from PRR activation. Inflammasomes also induce an inflammatory form of cell death called pyroptosis, which is implicated as a major driver of CD4⁺ T cell depletion and inflammation. This has been highlighted in recent findings from the Greene Lab at UCSF detailing the induction of pyroptosis in HIV ••[35][36-38]. These proinflammatory NLRs are counterbalanced by other NLRs known to antagonize signaling and inflammasome activity. These include anti-inflammatory NLRC3[39, 40], NLRP6[41], NLRP12[42], and NLRX1 ••[43]. The complex interplay of the NLR/inflammasome family highlights not only the critical role for these pathways in governing immune responses but also the importance of negative feedback loops as a means to check aberrant inflammation. These negative feedback loops also provide pathogens with an opportunity to subvert immunity.

While inflammation contributes critically to innate control of infection, it also recruits HIV target cells during acute infection, impairs CD4⁺ T cell recovery, and promotes disease progression. The ability for inflammation to confer beneficial and

detrimental effects, especially in HIV infection, creates a paradox for the immune system. In addition to leading to the production of proinflammatory cytokines, viral sensing by the immune system also triggers the production of type I interferons, mainly by pDCs, causing upregulation of Interferon stimulated genes (ISGs) and activation of innate immune cells triggering inflammation and antiviral defenses[44, 45]. In the African Green Monkey (AGM) and sooty mangabeys (SM) models of non-pathogenic SIV infection, IFN- α and its targets are rapidly induced during acute infection but downregulated during chronic infection[46, 47]. In contrast, they remain elevated in RMs with chronic SIV infection and correlate with progression to simian AIDS (S-AIDS)[47]. Sandler et al., [48] have shown that blockade of the IFN-I receptor caused reduced antiviral gene expression, increased SIV reservoir size and accelerated CD4⁺ T cell depletion with progression to AIDS. In contrast, IFN- α 2a administration initially upregulated expression of antiviral genes preventing systemic infection. However continued treatment induced IFN-I desensitization and decreased antiviral gene expression, enabling infection with increased reservoir size and accelerated T cell loss. These clearly highlight the yin-yang nature of inflammation and immune activation in which a balanced must be struck to promote clearance but limit deleterious effects of prolonged activation.

SIV dissemination and early immune responses

The events described so far occur prior to detectable viremia, making study of these cascades in humans difficult since infection is not detected until after onset of viremia and reservoir establishment. Studies of the initial events after infection need to use SIV in NHPs where the route, timing, and size of inoculum can be controlled. Comprehensive assessment of viral dissemination and immune responses is made possible in the NHP model through necropsy studies.

Utilizing detailed necropsy studies of intravaginally SIVmac251 infected RMs, Barouch et al., (2016) [49] show that SIV rapidly disseminates following infection and triggers components of the inflammasome, both at the site of inoculation and distal sites of viral spread [49]. A pro-inflammatory signature lacking antiviral restriction factors was observed in viral RNA-positive tissues 24 hours post-infection. The early innate response included expression of NLRX1 and activation of

theFOXO3a/TransformingGrowth Factor β (TGF β) pathway. NLRX1 expression was correlated with lack of induction of antiviral ISGs (at day 1 and 3) in virus +tissues. Western blots confirmed the negative correlation between levels of NLRX1 and IRF7, the master switch genes of innate antiviral immune responses. NLRX1 positively correlated with the magnitude of viral load and was reflective of dissemination (Figure 1). NLRX1 also exerts its anti-inflammatory effects by inhibiting the expression/activation of NF κ B. NLRX1 positively correlatedwith NF κ BIA expression, an inhibitor ofNF κ B, and negatively correlated with the expression of NF κ B1, a critical subunit involved inNF κ Bmediated signaling. A gene expression signature downstream of the TGF β pathway, a transcriptional target of the repressor FOXO3A, was negatively associated with Gag-specific CD4⁺/CD8⁺ T cell responses. These data suggest a model in which rapid triggering of NLRX1 and FOXO3A/TGF β by PRRs sensing HIV/SIV will suppress the generation of protective anti-HIV innate and adaptive immune responses and promote infection.

Impact of viral replication on immune activation

In these experiments the impact of viral dissemination was also noted in virus negative tissues. Upregulation of pathways reflective of widespread T cell activation was evident at the site of inoculation and in distal tissues (LN). We also usedAT-2 (aldrithiol-2) inactivated SIV virus to assess the role of viral replication in driving immune activation and the anti-inflammatory state that drives immune senescence.This strain infects CD4⁺ cells, however, the viral life cycle is arrested before initiation of reverse transcription [50].Serial necropsies of RMs after intravaginal inoculation with AT2-SIVmac239 (unpublished data from DrsSekaly and Barouch groups) revealed that SIVmac251 inoculation induced higher levels of IL-15, TLR activation, TNFR2, apoptosis and T cell activation than AT2-SIVmac239. In addition, the number of differentially expressed genesincreased in RMs from day 1 to day 3 following SIVmac251 inoculation, whereas they decreased following AT2-SIVmac239 inoculation.These results demonstrate the role of viral replication in earlyimmune responsesand highlights how HIV manipulates early immune activation to promote infection and persistence, as many of the identified pathways were associated with increased viral loads.

Validation of our findings in in vitro systems paves the way for screens of antagonists of NLRX-1

Similar transcriptional signatures were found in anNHP rectal explant study with activation of inflammatory and innate immune pathways that positively correlate with infection (unpublished observations from the DrsSekaly and Hope groups) (Figure 2). NLRX1 and TGF β may serve as central regulators of immune activation signatures in both SIV and HIV infection as these cascades correlate with increased viral dissemination, immune dysfunction, and disease progression. Corroboration of these findings in both SIV and HIV infection substantially increases the likelihood that targeting these pathways could lead to an effective treatment for HIV. While data from our lab and others shows that NLRX1 and TGF β are critical for immune senescence, the complex interplay of the immune responses dictates that other pathways will also allow the virus to escape early antiviral responses (Figure 3). Identifying the upstream and downstream signals that perturb these pathways during SIV/HIV is crucial for understanding viral seeding/dissemination and will guide future therapy development.

Conclusions

Recent work from our lab and others on models of early SIV pathogenesis have important implications for the development of vaccines and other strategies to block infection. These findings further our understanding of HIV-1 pathogenesis and illuminate the importance of early innate immune responses in mediating viral clearance. Rapid immune subversion and manipulation by HIV can inhibit the generation of antiviral immunity promoting the establishment and maintenance of the HIV/SIV reservoir. Our data also provide a research platform in RMs to assess the impact of vaccines, antibodies, drugs, and other preventative interventions on these early events.

The complex interplay of early immune signaling cascades and the exquisite ability for HIV to manipulate these cascades makes the development of effective therapies and vaccines aimed at HIV eradication difficult. Identifying and detailing pathways that promote and antagonize protective anti-HIV immune responses will allow the generation of novel vaccine adjuncts designed to properly skew immune responses.

This provides the opportunity to get one step ahead of a pathogen that we have, so far, been two steps behind.

Figures/Legend

Figure 1. The balance between FOXO3A and IRF7 controls the anti-viral immune response and viral dissemination. Chart depicting negative feedback loop between FOXO3A and IRF7. FOXO3A is a transcriptional repressor that regulates critical pathways including oxidative stress, metabolism and apoptosis. Elevated levels of FOXO3 and NLRX1 are associated with anti-viral suppression and enhanced virus dissemination•[51].

Figure 2. Pathway Module analysis reveals differences between Virus + and Virus – samples after SIV challenge in the FRT and LN tissue. Jaccard Index cutoffs of at least 25% were used to group enriched pathways ($P < 0.05$) into modules. Method: Module analyses[52] were constructed to group similar pathways and represent an overall biological functions associated with a tissue type at a specific time point. Significantly enriched pathways were organized in sets of related pathways using the enrichment map strategy. This was accomplished by linking gene sets by the number of genes differentially expressed overlapping between gene sets. Overlap between significant gene sets was computed according to the Jaccard Index. A Jaccard index cutoff of at least 0.25 was used to generate the results.

Figure 3. Model of NLR induced immune paralysis in HIV. A schematic model summarizing our findings on the key roles of NLRs, in particular NLRX1 in inducing improper anti-HIV immune responses during early infection. NLRX1 functions to blunt NFκB and IRF responses normally induced by viral signaling, leading to an immune state that cannot properly recognize and combat infection. Targeting of this pathway, and other related pathways, will restore normal immune function and promote viral clearance.

Conflict of interest

The authors report no conflict of interest.

Funding: This work was supported by Bill and Melinda Gates Foundation (SEKA15VIMC2, 10/1/2014 - 6/30/2016) and FNIH(U19 AI095985, 7/15/2011 - 6/30/2016).

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●This work demonstrates how HIV infection leads to loss of mucosal epithelial integrity and bacterial translocation. They further link this to an inflammatory response that can promote viral replication.
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 ●Clearly identify early IL-1 β production by specialized gut cells that leads to loss of epithelial barrier function and inflammation. Reversal of inflammation resulted in restored barrier function.
 [35]Doitsch et al
 ●●Highlights the critical role for inflammasomes and pyroptosis in driving CD4 T cell depletion during HIV infection. Identifies inflammasomes as key targets to prevent cell death and immune dysfunction in HIV.
 [43]Guo et al Cell Host and Microbe 2016
 ●●Show NLRX1 suppresses anti-HIV immune responses through inhibiting STING-TBK1 signaling downstream of HIV cDNA. NLRX1 deficiency enhances antiviral immune responses to viruses
 [49]Barouch et al Cell 2016
 ●●Rapid upregulation of NLRX1 in SIV infection leads to impaired innate and adaptive immune responses through inflammation, suppression of ISGs, and activation of TGF β . Identify NLRX1 as a key target for restoration of immune function in HIV and development of vaccines.
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 ●Identify FOXO3 as a key inhibitor of protective anti-viral immune responses by suppressing *Irf7* expression. Illustrates the complex balance and interplay of innate immune cascades.