REGULATION OF INNATE VIRAL SENSING AND CHRONIC INFLAMMATION

by
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ABSTRACT

Human immunodeficiency virus (HIV) and hepatitis C virus (HCV) are chronic viral infections that affect hundreds of millions of individuals worldwide. Despite significant advancements in treatment, increasing evidence suggests that chronic inflammatory medical illnesses occur more frequently and at earlier ages in HIV and HCV infected individuals. Defining the causes of chronic inflammation is key to understanding the pathogenesis of these infections and to developing novel therapeutic treatments of chronic disease.

Innate sensing of viral infections is key to early control of infection and occurs through multiple pathways. Inflammasomes are large multiprotein complexes that play a central role in inflammation associated with viral infection. Inflammasome assembly and activation of caspase-1 results in the cleavage of the pro-forms of IL-1β and IL-18 into active, mature cytokines that are known to mediate inflammation. Elevated IL-18 has been identified in a number of inflammatory conditions. We have shown that IL-18 is significantly elevated in HIV/HCV coinfection compared to HIV or HCV monoinfection. This elevation is most likely due to innate sensing of both viruses, correlates directly with detectable HIV viral load and inversely with CD4 counts. Given the association with increased IL-18 and inflammatory conditions seen with these infections, this finding may explain the enhanced disease progression observed in coinfected individuals.

Type I interferons (IFN) play a dichotomous role in chronic viral infections. These inflammatory cytokines are essential in controlling acute infection and slowing disease progression, yet contribute to chronic immune activation and pathogenesis of disease. We have shown a novel regulation mechanism for IFN produced by pDCs in response to

HIV. HIV specific antibodies can both suppress and enhance the IFN response by pDCs and may explain the persistent IFN response seen in HIV infection that has been linked to immune activation and pathogenesis.

Inflammatory responses are critical for host response to pathogens and the development of the adaptive immune response while being associated with pathogenesis. Therefore, a greater understanding of the balance between beneficial and detrimental inflammatory responses is needed. Not all inflammation can be considered harmful, therefore therapeutic inhibition of these pathways must be balanced against their beneficial contributions.

Ph.D. DISSERTATION REFEREES

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CHAPTER I: Introduction

Chronic Viral Infections and Inflammation

There are approximately 37 million people worldwide infected with Human Immunodeficiency Virus (HIV). HIV is the causative agent of acquired immune deficiency syndrome (AIDS) that results in an estimated 1.6 million deaths worldwide annually[1]. Hepatitis C virus (HCV) is one of the major causes of viral hepatitis and hepatocellular carcinoma. HCV infects 170 million people worldwide and persists in approximately 75% of infected individuals[2]. In the past decade, treatments for these infections have evolved substantially. Highly active antiretroviral therapy (HAART) has significantly reduced the incidence of HIV-related morbidity and mortality. At present, HIV infected patients have an expected life span that is only slightly shorter than HIV-uninfected individuals[3]. The development of novel direct-acting antivirals (DAAs) for treatment of HCV has rapidly pushed the field to aim for cure rates approaching 100% in some genotypes[4-7]. Despite advancements in the treatment field, increasing evidence suggests that chronic inflammatory medical illnesses including cardiovascular disease, diabetes mellitus, liver disease, osteoporosis, and cancer occur more frequently and/or at earlier ages in HIV infected individuals[8-14]. Slow disease progression seen in HCV infection has left many people undiagnosed and untreated. Chronic HCV infection is similarly associated with liver inflammation and increased risk of diabetes mellitus, kidney disease, and liver cancer [15-17]. It has been reported that once substantial fibrosis or cirrhosis has been established, the risk for liver disease remains even after the viral infection has been cured[18-20]. Additionally, more than 2 million people worldwide are coinfected with HIV and HCV. Coinfection with these viruses is strongly

associated with enhanced progression of hepatic inflammation, HIV-related kidney disease, cardiovascular disease and other proinflammatory illnesses[10, 21].

Chronic viral infections are a significant global health burden and represent a unique challenge to the infected host. Persistently replicating viruses subvert the initial antiviral response, allowing for the establishment of chronic infections and continuous stimulation of both the innate and adaptive immune systems[22]. This chronic immune activation causes profound damage through continuous exposure to proinflammatory molecules such as type I IFNs, interleukin (IL)-18, IL-1β, and tumor necrosis factor alpha (TNF-α). These proteins damage cells and tissue around them by inducing danger signals, recruiting more immune cells, leading to the release of more proinflammatory proteins and causing cell death by apoptosis[23]. The chronic inflammation seen with these viral infections begs the question of if we should not only be treating chronic infections but also the inflammation associated with chronic infections. The pathogenesis of chronic viral infections such as HIV and HCV is complex and not fully understood. Further study could lead to a greater understanding of chronic inflammation and the development of therapies to help prevent systemic damage from mediators of that inflammation.

Understanding Inflammation and the Innate Immune System

Inflammation is an essential aspect of the immune system. It is an evolutionarily conserved response mounted by the innate immune system in response to pathogens and it is tightly regulated by the host. Insufficient inflammation can lead to persistent infection while excessive inflammation can cause chronic and systemic inflammatory disease. Therefore, a delicate balance must exist.

The innate immune system is rapid, powerful and non-specific. Its function depends on the recognition of evolutionarily conserved pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs). PAMPs are molecular structures that are broadly shared by microorganisms and are essential to the survival or infectivity of the pathogen. DAMPs are nuclear or cytosolic proteins that are released by cells as a result of injury or stress. PAMPs and DAMPs are recognized by germ line-encoded pattern recognition receptors (PRRs) that are distinguished by their ligand specificity, cellular localization and activation of unique, but often converging, downstream signaling pathways[22-24]. Sensing by PRRs upregulates genes involved inflammatory responses such as proinflammatory cytokines and chemokines, type I interferon (IFN), and other viral sensing proteins such as inflammasomes. Although PRRs provide some specificity in activation of the innate system, the target of the produced proinflammatory molecules is often the surrounding environment. This leads to widespread immune activation and inflammation that typically dissipates with the resolution of infection[22-24]. However, in the case of chronic infections, there is no resolution of infection, and the systemic activation of the immune system is rarely tempered. Two of the essential inflammation-inducing pathways within the innate system are the activation of inflammasomes and type I IFNs.

Activation of Inflammasomes

One of the most important pathways of inflammation associated with viral infection involves activation of inflammasomes. Inflammasomes are sensors and receptors that induce inflammation through the secretion of proinflammatory cytokines in response to infectious pathogens. They are considered to be a key function of the innate system[24].

In general, inflammasomes are multimeric protein complexes that assemble in the cytosol after sensing PAMPs or DAMPs. They serve as a scaffold to recruit and activate pro-caspase-1 into active caspase-1. Activation of caspase-1 results in the maturation and secretion of the proinflammatory cytokines IL-18 and IL-1β and can induce pyroptosis, an inflammatory form of cell death[24, 25]. Several families of PRRs are important components of inflammasome complexes, including the nucleotide-binding domain, leucine-rich repeat containing proteins (also known as NOD-like receptors, NLRs). The inflammasome known to be the primary responder to viral pathogens is the NLR, pyrin domain-containing 3 (NLRP3) inflammasome. NLRP3 is expressed in myeloid cells and requires two independent signals for activation. The first signal is the engagement and activation of a Toll-like Receptor (TLR) [24]. TLRs are PRRs that recognize PAMPs and segregate between the cell surface and cytosol based on their respective ligand. The intercellular TLRs (3,7,8 and 9) recognized viral nucleic acid motifs from both RNA and DNA viruses. Specifically, TLR3 recognizes double stranded RNA, TLR7 and 8 single stranded RNA and TLR9 double stranded DNA[23]. In innate immune cells such as monocytes and macrophages, HCV has been shown to activate TLR7 and HIV to activate TLR8 and 9, though recognition can differ in other innate cells[26, 27]. Activation of signal 1 results in transcription of pro-IL1β, an increase in pro-IL18 over its constitutive expression, and the production of the pro-forms of these proinflammatory cytokines[23, 24]. The second signal involved in NLRP3 activation is not entirely understood. There is no evidence of direct ligand binding, which has led to the general hypothesis that NLRP3 senses changes in the cellular milieu [28]. It has been suggested that reactive oxygen species (ROS), the release of beta cathepsin, potassium efflux or combination of the

above factors may provide the second signal needed for activation. Activation of NLRP3 results in the oligomerization of NLRP3 and the apoptosis-associated speck-like protein containing a CARD (ASC) adaptor followed by the recruitment of pro-caspase1. This forms an inflammasome complex that then activates caspase-1, resulting in the cleavage of the pro-forms of IL-1β and IL-18 into the mature cytokines, their secretion into the environment and mediation of inflammation[24, 25, 28].

During the past decade, it has become clear that chronic inflammation is a key predictor of metabolic disorders, cancer and liver injury. Increased levels of the proinflammatory cytokines IL-18 and IL-1β have been associated with obesity, atherosclerosis, diabetes mellitus, cancer, kidney and liver disease[28]. Elevated levels of IL-18 in serum is associated with a significantly increased risk of type 2 diabetes. Systemic administration of IL-18 promotes diabetes development in young nonobese diabetic mice [29, 30]. IL-1β has also been implicated as an effector molecule involved in inflammation-promoted β-cell destruction leading to type 1 diabetes mellitus[31]. IL-18 promotes atherosclerotic plaque growth and vulnerability. High levels of circulating IL-18 precede the onset of coronary events in healthy men and in SIV infected and uninfected macaques [32, 33]. IL-18 plays an integral role in the development of renal dysfunction during a variety of inflammatory disease processes and in endotoxin-induced liver inflammation and sepsis [34, 35]. Serum levels of IL-18 have been shown to be high in HIV infection and acute HCV infection and it is hypothesized the IL-1β may follow a similar pattern in tissues[26, 27, 36]. The levels of proinflammatory cytokines in serum can be reduced with HIV treatment or HCV clearance, but prolonged presence of these inflammatory cytokines is thought to be responsible for the development of comorbidities.

Production of Type I Interferons

The type I interferon (IFN) family is a multi-gene cytokine family that encodes 13 partially homologous IFNα subtypes, a single IFNβ and several poorly defined single gene products (ε, τ, κ, ω, δ, and ζ) [37, 38]. IFNα and IFNβ are the best-defined and most widely expressed type I IFNs. These cytokines are best known for their ability to induce an antiviral state in both virus infected cells and uninfected bystander cells. Plasmacytoid dendritic cells (pDCs) are the major producers of type I IFNs. However, almost all cells in the body can produce these cytokines in response to stimulation through PRRs[38]. The main cytosolic receptors associated with type I IFN production in response to viral infections are RNA helicases retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5(MDA5) and the recently described cytosolic GAMP synthase (cGAS) [38, 39]. In addition to cytosolic sensors, the activation of the endosomal TLRs (3,7,8 and 9) can lead to type I IFN production in response to viral infections [23, 38]. In general, these signaling molecules have diverse downstream pathways, but the majority of them converge on a few key players in type I IFN signaling. The major group of transcription factors is the IFN regulatory factor (IRF) family. This family activates the transcription of the genes encoding type I IFNs. In most cases, IRF3 and IRF7 are the fundamental IRFs that are required to induce type I IFN gene transcription. The hallmark of type I IFN production is that the *IFNB* and *IFNA4* genes are induced in the initial stage of transcription and this stage relies on the transcription factor IRF3. The initial IFN burst triggers the transcription of *IRF7*, which then mediates a positive feedback loop, leading to the second wave of IFNα gene transcription[38, 40]. Nuclear factor-κB (NFκB) is also required as a cofactor in type I

IFN signaling, although there is some disagreement about the importance of this pathway[40]. Type I IFNs bind to and signal through a heterodimeric transmembrane receptor called IFNAR. Engagement of IFNAR activates the downstream JAK-STAT pathway and induces interferon stimulated genes (ISGs) [38]. IFNs were named for their ability to "interfere" with viral replication; this ability to restrict replication is largely due to the induction of ISGs. Most commonly, these genes are expressed in response to type I IFNs produced during acute infection. The IFN produced in this setting promotes an antiviral state in bystander cells and restrict the viral replication cycle in cells that have already been infected. [38] The fact that most viruses devote a portion of their genome to the evasion or disruption of type I IFN production and/or signaling, thereby preventing ISGs from being induced, illustrates the importance of this cytokine family in controlling viral infection.

There is a growing understanding that type I IFNs can also be harmful during viral infections. These cytokines have been shown to trigger widespread inflammation and tissue damage that can exacerbate disease, particularly during chronic infections[38]. Prolonged IFN responses have been linked to the pathogenesis of disease. SIV infected monkeys that quickly suppress their IFN response during acute infection are less susceptible to severe disease than those who were unable to mute the IFN response[41, 42]. Similarly in HIV infection, rapid progressors have stronger IFN signatures than viremic non-progressors and have been shown to have increased circulating IFN during the chronic stage of infection[43, 44]. In HCV infection, ISG expression in the liver of both chimpanzees and humans is a predictor of response to therapy. In chimpanzees infected with HCV, highly elevated hepatic ISG expression was associated with no

further induction of ISGs and no antiviral efficacy following IFNα treatment. In humans, high ISG expression patterns within liver infiltrating monocytes (LIM) and hepatocytes is key to predicting outcome. High ISG expression in LIM predicted treatment response, whereas high ISG expression in hepatocytes predicted treatment failure[2]. These studies suggest a link between sustained type I IFN levels, disease progression and treatment response, though the exact mechanisms involved are unclear.

Therapies for Chronic Inflammation

A greater understanding of the balance between beneficial and detrimental inflammasome activation or type I IFN responses is needed. Indeed, these inflammatory responses are critical for host response to microbial pathogens and possibly for optimal response to vaccine adjuvants because cytokine production by the innate immune system shapes the adaptive immune response. Thus, not all inflammation can be considered harmful, and the therapeutic inhibition of these pathways has to be balanced against their beneficial contributions. As insight into the mechanism of inflammasomes and type I IFNs increases, opportunities to create new therapies for patients with inflammatory diseases will enhance proportionately.

Dissertation overview

Chapter two describes the systemic elevation of the proinflammatory cytokine IL-18 in HIV/HCV coinfected individuals. The data demonstrate an additive effect of HIV/HCV coinfection on the induction of IL-18 and suggest that the elevated IL-18 levels may explain the increased incidence and progression of inflammatory illnesses seen in coinfected individuals.

Chapter three describes a new form of regulation of type I IFN responses by plasmacytoid dendritic cells and HIV. The data demonstrate that HIV specific antibodies can both suppress or enhance the IFN response and possibly explain persistent high-level IFN responses in HIV infection.

CHAPTER II: Systemic Elevation of Proinflammatory Interleukin 18 in HIV/HCV Coinfection versus HIV or HCV Monoinfection

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ABSTRACT

BACKGROUND: HIV/HCV coinfection and elevated interleukin (IL)-18 levels are both associated with enhanced progression of hepatic inflammation and increased risk of diabetes, kidney disease, and cardiovascular disease. IL-18 is a proinflammatory cytokine made upon activation of the inflammasome, an innate sensing system. We assessed whether increased IL-18 could explain the increased incidence and progression of inflammatory conditions seen with HIV/HCV coinfection.

METHODS: Serum from 559 subjects with HIV monoinfection, HCV monoinfection, HIV/HCV coinfection, or neither infection was tested for IL-18 by ELISA and for 16 other analytes by electrochemiluminescence immunoassay. IL-18 levels were measured in 14 additional chronically HCV infected subjects who developed incident HIV infection to determine if IL-18 increases with coinfection.

RESULTS: IL-18 was significantly elevated in HIV/HCV coinfected individuals versus both monoinfections ($p<0.0001$) independent of age, sex, and race. IL-18 levels were significantly higher in HIV monoinfection than in HCV monoinfection. High IL-18 levels were correlated with detectable HIV viremia and inversely with CD4 count ($p<0.0001$), consistent with HIV activation of the inflammasome resulting in CD4 T cell depletion. Incident HIV infection of chronically HCV infected subjects resulted in an increase in IL-18 ($p<0.001$), while HIV suppression was associated with normal IL-18 levels. Four additional analytes (IP-10, IL-12/23p40, IFNy, IL-15) were found to be elevated in HIV/HCV coinfection when compared to both monoinfections.

CONCLUSIONS: These data demonstrate that HIV/HCV coinfection results in increased serum IL-18. The elevated levels of this proinflammatory cytokine may explain

the increased incidence and progression of inflammatory illnesses seen in coinfected individuals.

INTRODUCTION

Highly active antiretroviral therapy (HAART) has reduced the incidence of HIV-related morbidity and mortality. However, increasing evidence suggests that chronic inflammatory medical illnesses including cardiovascular disease, diabetes mellitus, chronic kidney and liver disease, osteoporosis, and cancer occur more frequently and/or at earlier ages in HIV infected individuals [8-14]. HCV persists in approximately 75% of infected individuals and chronic infection is similarly associated with liver inflammation and increased risk of diabetes mellitus, kidney disease, and liver cancer [15-17]. Coinfection with HIV and HCV is associated with enhanced progression of hepatic inflammation-linked pathology, increased HIV-related kidney disease and renal failure, cardiovascular disease and higher risk of diabetes relative to monoinfections [10, 21]. The pathogenesis of HIV and HCV- related chronic inflammation is complex and not completely understood.

IL-1β and IL-18 are proinflammatory cytokines known to mediate inflammation. Elevated IL-18 is associated with a variety of inflammatory conditions, including diabetes, atherosclerosis, and kidney and liver disease. Elevated levels of IL-18 in human serum are associated with a significantly increased risk of type 2 diabetes and systemic administration of IL-18 promotes diabetes development in young nonobese diabetic mice[29, 30]. IL-18 promotes atherosclerotic plaque growth and vulnerability and high levels of circulating IL-18 precede the onset of coronary events in healthy men and in SIV infected and uninfected macaques[32, 33]. IL-18 plays an integral role in the development of renal dysfunction during a variety of inflammatory disease processes and in endotoxin-induced liver inflammation and sepsis[34, 35].

Existing studies suggest a role for inflammasome activation in HIV and HCV infection. Previous studies have shown that HIV infected subjects have higher plasma levels of IL-18 compared to uninfected and HAART treated individuals [45, 46]. Our group showed previously that IL-18 plasma levels are low prior to HCV infection, increase significantly with acute HCV infection, then decrease to intermediate levels with progression to chronic infection [47]. Polymorphisms in the IL-18 promoter lead to increased IL-18 and IL-18 binding protein production and increase the odds of spontaneous HCV control [48]. We demonstrated previously that endocytosis of HIV or HCV virions activates the inflammasome in monocytes and macrophages and induces production of IL-18 and other proinflammatory cytokines[26]. Finally, a recent study demonstrated that HIV induced CD4 T cell depletion is driven primarily by inflammasome mediated cell death (pyroptosis) through abortive HIV infection of CD4 T cells in lymphoid tissues[49]. Despite substantial data about the importance of inflammasome activation in HIV and HCV infection, only one previously published study has assessed plasma IL-18 levels in HIV/HCV coinfected individuals[50]. This study suggested that IL-18 levels in coinfection were higher than in HIV monoinfection, but there was no assessment of IL-18 levels in HCV monoinfection and the total number of subjects in all categories combined was 29. The limited sample size did not allow for control of other variables that could affect IL-18 levels, including race, gender, or age.

In this study, we assessed IL-18 levels in in HCV monoinfection, HIV monoinfection, and HIV/HCV coinfection as a potential explanation for the enhanced pathogenesis seen in HIV/HCV coinfection. We found that IL-18 is significantly elevated in coinfected individuals compared to both monoinfections independent of age, sex, and race. High IL-

18 levels correlated with having detectable HIV viremia and inversely with CD4 count. Incident HIV infection of chronically HCV infected subjects resulted in a significant increase in IL-18 ($p<0.001$), while HIV suppression with HAART was associated with low IL-18 production. These data support enhanced induction of IL-18 production *in vivo* with coinfection, that inflammasome activation results in CD4 T cell depletion, and that IL-18 elevation could play a role in the enhanced pathogenesis seen in HIV/HCV coinfection.

METHODS

Study Participants

Serum from HCV monoinfected, HIV monoinfected, and HIV/HCV coinfected subjects was obtained from the AIDS Linked to the IntraVenous Experience (ALIVE) study, an ongoing, prospective, community-recruited, observational cohort study of people who inject drugs (PWID) in Baltimore, Maryland [51]. Participants in this cohort provided written informed consent and received counseling to reduce drug use. A detailed study protocol has been previously described [51]. In brief, The ALIVE cohort enrolled HIV-negative and HIV-positive PWID, 89% of whom were HCV seropositive at baseline, and collected semiannual blood samples during follow-up. Subjects consented to long-term storage of serum and plasma samples collected from them [51]. This study conformed to all relevant ethical guidelines; the institutional review board of the Johns Hopkins University approved this study protocol. Samples were selected from 440 individuals chronically infected with one or both infections and divided into three categories: HIV monoinfected (HIV Ab positive, HCV RNA negative), HCV monoinfected (HIV Ab negative, HCV Ab positive, HCV RNA positive at ≥ 1 visits in the chronic infection phase) and HIV/HCV coinfected (HCV and HIV Ab positive, and HCV RNA positive at one or more visits in the chronic infection phase). An additional 32 samples were included from the John Hopkins Adult HIV clinic to increase the number of Caucasians studied because the ALIVE cohort is primarily African American[52]. Both of these samples derived from the same underlying Baltimore population and approximately 40% of HIV-infected ALIVE participants receive their HIV care from the Johns Hopkins HIV

clinic. None of the 472 subjects tested self-reported being on HAART, but 25 subjects had HIV RNA levels below 500 copies/ml.

HIV/HCV antibody seronegative subjects were defined as uninfected and were obtained from the Baltimore Before and After Acute Study of Hepatitis (BBAASH), an ongoing prospective, community-recruited, observational cohort study of PWID in Baltimore, Maryland [53]. Participants in this cohort provided written informed consent and received counseling to reduce drug use. A detailed study protocol has been previously described [53]. Uninfected donors remained HCV-RNA negative and HCV-Ab negative for twenty-four consecutive monthly visits at the time of release from the study.

To assess the effect of incident HIV, an additional 14 subjects with chronic HCV infection who acquired incident HIV infection were also selected from the ALIVE cohort. In addition, we obtained 55 HIV/HCV coinfected and 21 HIV monoinfected subjects from ALIVE on HAART with undetectable HIV RNA to assess the effects of HAART on IL-18 levels.

IL-18 Measurement

Serum IL-18 was measured using the human IL-18 ELISA kit (MBL). The assay was performed per the manufacturer's recommendations. In brief, all samples were diluted 1:5 in assay buffer and run in duplicate. Average values were then calculated and reported as pg/ml. Data were acquired using a SpectaMax M5 (Molecular Devices). The LLOQ of IL-18 in serum samples is 25 pg/mL.

Cytokine Measurement

The Meso Scale Discovery (MSD) multiplex cytokine, proinflammatory, and chemokine electrochemiluminescence immunoassays were used to assess 16 additional analytes:

IL12/23p40, IL-15, IL-16, IL-7, IFN-g, IL-10, IL-1b, IL-2, IL-6, IL-8, TNF-a, Eotaxin, IP-10, MCP-1, MIP-1a, MIP-1b. The assays were performed per the manufacturer's recommendations. Data were acquired on a SECTOR Imager SI2400. Results were analyzed using MSD Workbench software. The LLOQ is for each analyte is indicated where data is shown.

Statistical Analysis

Before analysis, IL-18 values were normalized by log10 transformation. To compare whether IL-18 levels differed across groups, we used one-way analysis of variance (ANOVA) and subsequent t-tests for pairwise comparisons. Univariable and multivariable linear regression models were run to identify independent associations between HIV/HCV status, demographic characteristics and clinical factors independently associated with IL-18 and other cytokine and chemokine levels. Finally, paired t-tests and were used for intraindividual comparisons of IL-18 levels prior to and after HIV infection.

RESULTS

Subjects

IL-18 and MSD multiplex testing was completed on 559 subjects. Of the 559 subjects, 87 were HIV/HCV uninfected, 86 were HIV monoinfected, 198 were HCV monoinfected, and 188 were HIV/HCV coinfected. Demographic characteristics of the subjects are shown in Table 2.1. The demographic characteristics of the HIV/HCV negative subjects was unavailable. Of the remaining subjects, 71.8% of subjects were male and 81.4% African American, and the median age was 46 years (range, 21-70).

Elevated levels of the proinflammatory cytokine IL-18 are observed in HIV/HCV coinfection

The median values of IL-18 seen in each group were: HIV/HCV uninfected 278 pg/ml (interquartile range [IQR], 156-406), HCV monoinfected 466pg/ml (IQR, 316-669), HIV monoinfected 544 pg/ml (IQR, 347-1095), and HIV/HCV coinfected 1009 pg/ml (IQR, 637-1722). Serum IL-18 levels were significantly higher in HIV/HCV coinfected and HCV and HIV monoinfected subjects than in uninfected subjects ($p<0.0001$, Figure 2.1). Additionally, HIV/HCV coinfected subjects had significantly higher IL-18 levels than either HCV or HIV monoinfected subjects ($p<0.0001$, Table 2.1 and 2.2). Lastly, HIV monoinfected subjects had significantly higher IL-18 levels than HCV monoinfected subjects ($p<0.01$, Figure 2.1). The inflammasome-associated cytokine IL-1β was not significantly elevated with HIV/HCV coinfection or monoinfection when compared to uninfected subjects. However, levels were very low in serum in all groups. Of the 559 subjects tested for IL-1β, 13% had undetectable levels and 86% of those detectable were too low to quantify (Figure 2.7).

IL-18 levels are associated with detectable HIV viral load, CD4 count, and race.

Univariable linear regression was performed to evaluate what factors other than infection status were associated with serum IL-18 levels (Table 2.3). In these models, detectable HIV viral load was strongly associated with higher IL-18 levels, as were lower CD4 counts. We could not assess the relationship between HCV RNA levels and IL-18 levels because multiple methods were used to detect HCV RNA in the ALIVE cohort. However, we have previously shown no correlation between the level of HCV RNA in viremia and IL-18. [47]. In the univariable analysis, no association was found between IL-18 and sex. However, there was an association between older age and lower IL-18. African American subjects had higher IL-18, a finding of borderline statistical significance in univariable analysis. In the multivariable analysis, race became more significantly associated with IL-18 levels (Table 2.3). However, controlling for sex, race and age did not affect the associations of HIV/HCV coinfection versus either monoinfection (Table 2.2 and 2.3).

Detectable HIV viral load remained strongly associated with IL-18 in multivariable analysis (Table 2.3), as did CD4 count. Serum IL-18 levels in subjects with <200 and with 200-500 CD4 cells/ml were significantly higher than levels in HIV uninfected subjects (Figure 2.2 A, $p<0.0001$ and $p=0.0002$, respectively). In contrast, serum IL-18 levels in subjects with CD4 counts >500 CD4 cells/ml were not statistically different from levels in HIV uninfected subjects (Figure 2.2 A). CD4 count was inversely correlated with IL-18 levels (Figure 2.2 B, R^2 0.236, $p<0.0001$).

Infection with HIV and HAART suppression of HIV alter IL-18 production

To test whether IL-18 induction is additive in HIV/HCV coinfection, we obtained an additional 14 subjects from the ALIVE cohort who were chronically HCV infected and subsequently acquired incident HIV infection. Two time points during chronic HCV/pre HIV infection and two time points after HIV acquisition were tested. The first HIV/HCV coinfected time point tested for all subjects was the first visit at which HIV Ab became detectable. IL-18 level within person comparisons did not significantly differ between the two pre-HIV infection specimens or between the two post HIV infection specimens (Figure 2.3A). In contrast, comparison of IL-18 levels pre- and post- HIV infection revealed a significant increase in IL-18 levels upon incident HIV infection in the setting of chronic HCV infection (Figure 2.3A). To further assess IL-18 variability over time in the absence of incident HIV infection, 10 chronically HCV monoinfected subjects from the ALIVE cohort were examined at two time points approximately 10 years apart. We saw no significant difference in IL-18 levels in chronic HCV infection over time (Figure 2.4).

To test whether the HIV treatment is associated with normalization of IL-18 levels, we obtained an additional 55 HIV/HCV coinfected and 21 HIV monoinfected subjects from ALIVE on HAART with undetectable HIV RNA. In HIV monoinfected subjects, we saw that HIV suppression by HAART was associated with significantly lower IL-18 levels versus HIV monoinfected subjects not on HAART ($p<0.001$). In fact, the IL-18 levels in HAART treated HIV monoinfected subjects were not significantly different from that of uninfected subjects (Figure 2.3 B). In coinfected subjects, we saw that HIV suppression by HAART was associated with lower IL-18 levels versus HIV/HCV coinfected subjects not on HAART ($p<0.0001$, Figure 2.3 C). However, IL-18 levels remained significantly

higher than in uninfected subjects and were comparable to IL-18 levels measured in HCV monoinfection. This suggests that the elevated IL-18 levels observed in HIV/HCV coinfection can be reduced by HAART but that HCV infection remains a cause of IL-18 elevation in coinfected patients treated with HAART.

Other serum cytokines and chemokines are also associated with HIV/HCV coinfection

In addition to increased IL-18, HIV/HCV coinfection was also associated with increased levels of IP-10, IL12/23p40, IL-15, and IFN-γ versus either monoinfection (Figure 2.5). Additionally, HCV or HIV monoinfected subjects had significantly higher levels of these analytes than HIV/HCV uninfected subjects (Figure 2.5). Controlling for age, sex, and race in a multivariable analysis showed that IP-10, IL12/23p40, IL-15, and IFN-γ were independently associated with HIV/HCV coinfection when compared to either monoinfection (Table 2.4). IL-18 was formerly known as IFN-γ-inducing factor because it stimulates T cells to make IFN-γ, particularly in the presence of IL-12[54, 55]. Therefore, it is not surprising that a pattern of IL-12 and IFN-γ production similar to that of IL-18 was observed.

Serum levels of ten additional analytes (TNFα, MIP-1β, IL-7, IL-16, IL-6, IL-10, IL-8, Eotaxin, MIP-1α, and IL-2) were assessed in the four groups. No significant differences in the levels of those analytes were found between HIV/HCV coinfection and either monoinfection (Figure 2.6 and 2.7). Median values, IQR and multivariate estimates for all 16 analytes are listed in Tables 2.4 and 2.5.

DISSCUSSION

This study provides a detailed assessment of serum IL-18 levels in HIV and HCV monoinfections, and HIV/HCV coinfection, controlling for the first time for demographic factors. We have shown that IL-18 is higher in coinfected than in monoinfected or uninfected subjects (Figure 2.1 A, Table 2.3). High IL-18 in HIV and HCV monoinfection compared to uninfected individuals has been shown previously. Thus, we chose to focus on comparing IL-18 levels among infections, using HCV monoinfection as our reference. The increased IL-18 observed in HIV/HCV coinfection is novel and may explain the advanced disease progression seen in coinfected individuals[21]. Interestingly, when comparing HIV monoinfection to HCV monoinfection, we saw that IL-18 levels were significantly higher in HIV monoinfection, suggesting that HIV more robustly induces inflammasome activation, possibly leading to greater inflammation (Figure 2.1 A, Table 2.3).

To further investigate the role HIV and HCV each play in coinfection pathogenesis, we assessed the relationship between IL-18 and characteristics of viral infection. We found an association between IL-18 levels and detectable HIV viral load (Table 2.3). Our group has previously shown that there is no correlation between the degree of IL-18 elevation and HCV RNA levels [47]. We also found a strong association between IL-18 level and CD4 count (Figure 2.2 A). Lower CD4 counts strongly negatively correlated with higher IL-18 levels (Figure 2.2 B). The association between CD4 depletion and serum IL-18 *in vivo* supports a prior study. That study demonstrated in explanted lymphoid tissue that CD4 depletion results from abortive HIV infection and activation of the inflammasome, resulting in pyroptosis and release of IL-18 and IL-1β [49]. Unfortunately, we were

unable to accurately assess production of IL-1β because the cytokine is virtually undetectable in serum (Figure 2.7). However, we cannot exclude the possibility that IL-1β follows a similar trend within tissue.

We found that incident HIV infection in subjects chronically infected with HCV resulted in a significant increase in IL-18 (Figure 2.3 B). Conversely, HAART was associated with significantly lower IL-18 levels in both HIV/HCV coinfected and HIV monoinfected subjects than levels seen in comparable subjects not on HAART (Figure 2.3 B, C). These findings support the hypothesis that the presence of virus is what drives IL-18 production. Our group and others have shown that the virion is responsible for activating innate cells to secrete proinflammatory cytokines [26, 36, 56]. We were unable to assess how acquisition or resolution of HCV infection affected IL-18 levels in HIV infected subjects since HCV is almost universally acquired first in PWID and spontaneous HCV resolution in the setting of HIV infection is rare. However, we have previously shown that spontaneous control of HCV monoinfection results in return of IL-18 levels to baseline levels measured prior to infection [47].

Finally, we found three additional cytokines (IL-12/23p40, IL-15, and IFN-γ) and one chemokine (IP-10) to be significantly elevated in HIV/HCV coinfection compared to both monoinfections (Figure 2.5). IP-10 is thought to be a key cross-talk cytokine in HIV/HCV coinfection and has been previous reported as increased in this setting [57, 58]. IL-15 elevation, which also has been previously reported, has been suggested to play a role in inflammation, liver damage and advanced fibrosis seen in coinfected subjects[59, 60]. Increased IL12/23p40 subunit and IFN-γ have not been previously reported in coinfection. However, IL-12 and IL-23 can regulate both IFN-γ and IL-18

responses to viral infections. Additionally, IL-18 is known to induce T cells to make IFN-γ, particularly in the presence of IL-12[54, 55, 61]. Detection of elevated levels of these cytokines that are coordinately regulated with or by IL-18 supports the accuracy our finding of elevated IL-18 in HIV/HCV coinfection.

In summary, we have shown that IL-18 is increased in HIV/HCV coinfection when compared with monoinfection and that this increase is most likely due to additive innate sensing of both viruses. Given the association with increased IL-18 and inflammatory conditions seen more commonly in HIV and HCV infection and coinfection, this may explain the enhanced disease progression observed in coinfected individuals. There is a range of IL-18 levels in coinfection, potentially permitting an assessment of the relationship between elevated serum IL-18 and increased incidence and progression of inflammatory conditions in coinfection. If IL-18 is implicated, this will enhance our understanding of coinfection pathogenesis and direct potential therapeutic interventions.

TABLES

Table 2.1 Demographic and Clinical Characteristics of the Study Participants

Characteristic	**n = 559 (%)**
Infection Status	
HIV/HCV negative	87 (15.6)
HIV monoinfection	86 (15.6)
HCV monoinfection	198(35.4)
HIV/HCV coinfection	188 (33.4)
Sex (infected subjects*)	**n = 472 (%)**
Male	339 (71.8)
Female	133 (28.2)
Race (infected subjects*)	
African American	384 (81.4)
Not African American	88 (18.6)
Age, y, median (range)	46 (21-70)

* Sex and race information was not available for all HIV/HCV negative subjects and is therefore shown only for the remainder of the subjects.. IL-18 levels were very low in nearly all uninfected subjects and not expected to differ by race, gender, or sex.
Abbreviations: HIV, human immunodeficiency virus, HCV, hepatitis C virus

Table 2.2 Multivariate analysis of IL-18 with HIV monoinfection as reference

Characteristic	Univariate Estimate (SE)	p-value	Multivariate Estimate (SE)	p-value
Infection status				
HIV neg/HCV pos	-0.145 (0.04)	0.0006	-0.128 (0.04)	0.0033
HIV pos/HCV neg	Reference		Reference	
HIV pos/HCV pos	0.202 (0.04)	<0.0001	0.205 (0.04)	<0.0001

Abbreviations: HIV, human immunodeficiency virus, HCV, hepatitis C virus, pos, infected, neg, uninfected

Age, sex and race estimates and p values were the same as when HCV monoinfection was used as the reference and therefore not listed here

Table 2.3 Variables associated with serum IL-18 levels

Characteristic	Univariate Estimate (SE)	p-value	Multivariate Estimate (SE)	p-value
Sex				
Female	-0.026 (0.039)	0.478	-0.005 (0.03)	0.879
Age				
5 year increments	-0.005 (0.002)	0.0003	-0.002 (0.001)	0.119
Race				
African American	0.074 (0.04)	0.075	0.077 (0.04)	0.049
Infection status				
HIV neg/HCV pos	Reference		Reference	
HIV pos/HCV neg	0.145 (0.042)	0.0006	0.128 (0.044)	0.0033
HIV pos/HCV pos	0.347 (0.033)	<0.0001	0.332 (0.035)	<0.0001
HIV VL *				
HIV uninfected	Reference		Reference	
VL not detectable	0.179 (0.07)	0.01	0.172 (0.07)	0.015
VL detectable	0.230 (0.03)	<0.0001	0.232 (0.03)	<0.0001
CD4 (cells/mL)				
HIV uninfected	Reference		Reference	
>500	0.103 (0.05)	0.048	0.087 (0.05)	0.103
200-499	0.175 (0.043)	<0.0001	0.171 (0.045)	0.0002
<200	0.407 (0.035)	<0.0001	0.392 (0.037)	<0.0001

*lower limit of detection is 500 copies/mL
Abbreviations: VL: viral load, HIV: human immunodeficiency virus, HCV: hepatitis C virus,
neg: uninfected, pos: infected

Table 2.4 Multivariate analysis of 16 additional cytokines and chemokines
Abbreviations: IL, interleukin, IP, inducible protein, IFN, interferon, TNF, tumor necrosis factor, MCP, monocyte chemoattractant protein, MIP, macrophage inflammatory protein, HIV, human immunodeficiency virus, HCV, hepatitis C virus, neg: uninfected, pos: infected
Reference indicates the group used for comparison, age, sex and race estimates and p values were the same regardless of which group was used as reference
The first 4 analytes listed are statistically significant compared to both reference groups, to be considered significant $p<0.002$

	Age: 5yr increments		Sex: Female		Race: African American		HIV neg/HCV pos		HIV pos/HCV neg		HIVpos/HCVpos	
	Multivariate Estimate (SE)	p-value	Multivariate Estimate (SE)	p-value	Multivariate Estimate (SE)	p-value	Multivariate Estimate (SE)	p-value	Multivariate Estimate (SE)	p-value	Multivariate Estimate (SE)	p-value
IL-12/23p40	0.001 (0.001)	0.33	0.109 (0.083)	0.001	-0.064 (0.033)	0.1	Reference		0.137 (0.043)	0.001	0.337 (0.034)	<.0001
							-0.138 (0.043)	0.001	Reference		0.2 (0.042)	<.0001
IL-15	0.001 (0.001)	0.25	0.007 (0.216)	0.73	0.123 (0.025)	<.0001	Reference		0.015 (0.028)	0.58	0.156 (0.022)	<.0001
							-0.15 (0.028)	0.58	Reference		0.14 (0.027)	<.0001
IP-10	0.006 (0.002)	0.004	0.103 (0.04)	0.01	-0.045 (0.05)	0.36	Reference		0.063 (0.054)	0.24	0.294 (0.043)	<.0001
							-0.063 (0.054)	0.24	Reference		0.231 (0.53)	<.0001
IFNγ	-0.003 (0.002)	0.24	0.013 (0.045)	0.77	0.078 (0.052)	0.14	Reference		0.121 (0.058)	0.04	0.31 (0.046)	<.0001
							-0.121 (0.058)	0.04	Reference		0.2 (0.057)	0.001
Eotaxin	0.007 (0.002)	<.0001	0.065 (0.031)	0.04	-0.204 (0.037)	<.0001	Reference		0.001 (0.04)	0.98	0.033 (0.032)	0.3
							-0.001 (0.04)	0.98	Reference		0.032 (0.04)	0.42
IL-10	-0.007 (0.002)	0.0001	-0.031 (0.038)	0.41	0.066 (0.044)	0.14	Reference		-0.077 (0.049)	0.12	0.001 (0.038)	0.98
							0.077 (0.049)	0.12	Reference		0.078 (0.048)	0.12
IL-1α	0.0006 (0.001)	0.63	0.021 (0.024)	0.39	-0.031 (0.029)	0.27	Reference		0.038 (0.032)	0.23	0.1 (0.025)	<.0001
							-0.04 (0.032)	0.23	Reference		0.062 (0.031)	0.04
IL-1β	-0.0002 (0.0003)	0.55	-0.008 (0.007)	0.24	0.008 (0.008)	0.36	Reference		0.018 (0.009)	0.05	0.002 (0.007)	0.76
							-0.018 (0.009)	0.05	Reference		-0.016 (0.009)	0.07
IL-2	-0.0001 (0.0008)	0.89	0.005 (0.016)	0.74	0.006 (0.02)	0.76	Reference		-0.023 (0.022)	0.28	-0.005 (0.017)	0.78
							0.023 (0.022)	0.28	Reference		0.02 (0.021)	0.37
IL-6	0.006 (0.002)	0.003	0.052 (0.04)	0.2	0.064 (0.05)	0.18	Reference		0.088 (0.053)	0.1	0.127 (0.04)	0.002
							0.064 (0.05)	0.1	Reference		0.039 (0.052)	0.45
IL-7	-0.003 (0.001)	0.02	0.019 (0.026)	0.46	-0.044 (0.03)	0.14	Reference		0.195 (0.034)	<.0001	0.229 (0.027)	<.0001
							-0.195 (0.034)	<.0001	Reference		0.035 (0.033)	0.29
IL-8	0.002 (0.002)	0.23	-0.05 (004)	0.23	0.01 (0.05)	0.83	Reference		-0.008 (0.054)	0.88	-0.045 (0.04)	0.28
							0.008 (0.054)	0.88	Reference		-0.037 (0.052)	0.48
MCP1	0.003 (0.001)	0.008	0.007 (0.024)	0.78	-0.091 (0.028)	0.001	Reference		0.076 (0.031)	0.01	0.142 (0.024)	<.0001
							-0.076 (0.031)	0.01	Reference		0.066 (0.03)	0.03
MIP1α	0.011 (0.002)	0.61	-0.034 (0.048)	0.47	0.113 (0.056)	0.05	Reference		0.041 (0.062)	0.51	-0.118 (0.05)	0.02
							-0.041 (0.062)	0.51	Reference		-0.159 (0.061)	0.01
MIP1β	0.001 (0.001)	0.36	-0.012 (0.029)	0.68	-0.0003 (0.034)	0.99	Reference		-0.169 (0.037)	<.0001	-0.21 (0.029)	<.0001
							0.169 (0.04)	<.0001	Reference		-0.011 (0.036)	0.26
TNFα	0.007 (0.002)	0.001	0.048 (0.04)	0.25	-0.09 (0.05)	0.07	Reference		0.161 (0.055)	0.004	0.159 (0.043)	0.0002
							-0.161 (0.055)	0.004	Reference		-0.002 (0.054)	0.97

Table 2.5 Median and rages for all cytokines and chemokines assessed

Cytokine or Chemokine	HIV/HCV uninfected		HIV mono-infected		HCV mono-infected		HIV/HCV co-infected	
IL-18[a]	278.5	(25-898.2)	465.7	(25-3118)	543.8	(130.9-5020)	1009	(25-12134)
IP-10[a]	104.2	(14.6-728.3)	584.5	(0.7-3707)	629.3	(8.8-9080)	1175	(26.4-9080)
IL-15[a]	1.8	(0.7-3.38)	2.7	(0.7-9.63)	2.6	(0.7-12.4)	3.4	(0.7-27)
IL-12/23p40[a]	78.9	(10.9-06.6)	133.2	(28.7-762.2)	171.5	(27.2-1457)	261.4	(19.5-4550)
IFN-γ[a]	3.9	(3.9-140.6)	3.9	(3.9-1171)	8.4	(3.9-304.2)	15.4	(3.9-265.4)
IL-1β	1.1	(1.1-28)	1.1	(1.1-1.1)	1.1	(1.1-6.7)	1.1	(1.1-4.5)
IL-2	0.5	(0.5-1.6)	0.5	(0.5-24.6)	0.5	(0.5-2.5)	0.5	(0.5-4.1)
IL-6	0.8	(0.8-84.7)	0.8	(0.8-82.1)	0.8	(0.8-42)	1.6	(0.8-46.8)
IL-7	18.3	(7.2-47.1)	13.6	(2.3-88)	22.8	(2.6-75.4)	23.7	(3-160.8)
IL-8	39.5	(4.2-990)	25.7	(5.9-990)	24.7	(3.8-990)	24.6	(6.8-657.2)
IL-10	0.3	(0.3-48.3)	0.7	(0.3-28.6)	0.3	(0.3-31.8)	0.8	(0.3-9.6)
IL-16	221	(64.7-559.1)	195.9	(64.2-935.2)	216.8	(68.6-1980)	223.1	(86.5-1887)
TNF-α	1.9	(0.4-69.7)	2.8	(0.4-78)	3.6	(0.4-110.5)	4.5	(0.4-61.8)
Eotaxin	106.6	(6.2-356.9)	169.3	(6.2-1355)	155.3	(6.2-994.7)	179.4	(6.2-856.4)
MCP-1	197.2	(21.7-780)	256.6	(83.7-1614)	286.9	(82.2-1330)	330.9	(35.9-1248)
MIP-1α	25	(6.9-2795)	37.8	(6.9-3148)	39.9	(6.9-1415)	34.8	(6.9-1191)
MIP-1β	92.76	(30.6-3276)	142.3	(29.9-2506)	80.2	(21.2-1216)	85.5	(23.5-1513)

Data are median (range) values and given in picograms per milliliter

[a] Statistically significant compared to HIV/HCV coinfection

Abbreviations: IL, interleukin, IP, inducible protein, IFN, interferon, TNF, tumor necrosis factor, MCP, monocyte chemoattractant protein, MIP, macrophage inflammatory protein, HIV, human immunodeficiency virus, HCV, hepatitis C virus, neg: uninfected, pos: infected

FIGURES

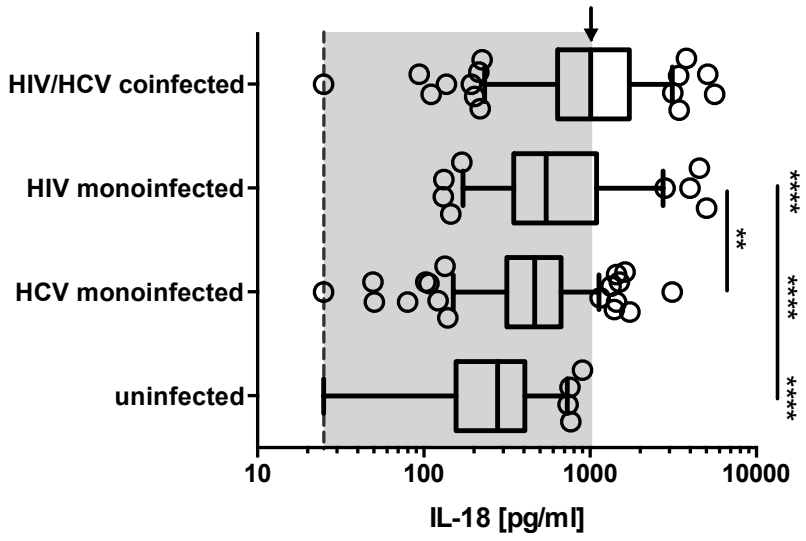


Figure 2.1 Serum IL-18 concentrations are significantly higher in HIV/HCV coinfection

Serum levels of IL-18 in HIV/HCV uninfected, HCV monoinfected, HIV monoinfected, and HIV/HCV coinfected subjects. Dashed line: lower limit of quantitation (LLOQ), Arrow: median of HIV/HCV coinfection group. IL-18 levels in uninfected, HCV monoinfected and HIV monoinfected subjects are statistically different from those in HIV/HCV coinfected subjects (**** $p<0.0001$). IL-18 levels were significantly higher in HIV monoinfected subjects than in HCV monoinfected subjects (** $p<0.001$). Groups were compared using a one way ANOVA and adjusted for multiple comparisons.

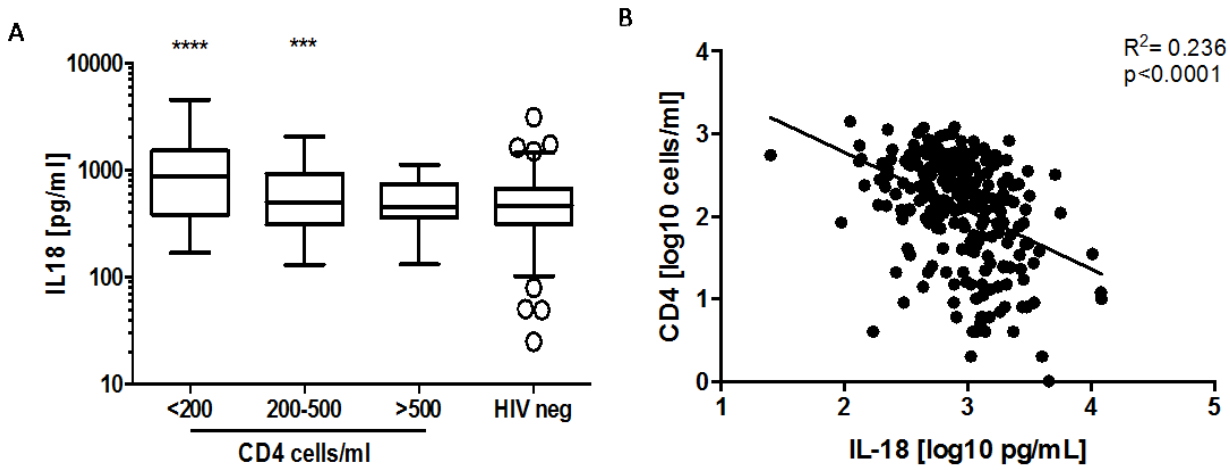


Figure 2.2 Absolute CD4 counts are inversely correlated with serum IL-18 concentration in HIV mono- and HIV/HCV coinfected subjects

A. Serum IL-18 levels grouped by absolute CD4 counts <200, 200-500, >500/ml and HIV uninfected. Serum IL-18 levels in subjects with <200 and 200-500 CD4 cells/ml were significantly higher than in HIV uninfected subjects (**** $p<0.0001$ and *** $p=0.0002$, respectively). Serum IL-18 levels in subjects with >500 CD4 cells/ml were not statistically different from levels in HIV uninfected subjects. B. Linear regression of $\log_{10}$ IL-18 vs. $\log_{10}$ CD4 counts, negative correlation R^2 0.236. Statistical analysis was performed using univariable and multivariable linear regression models.

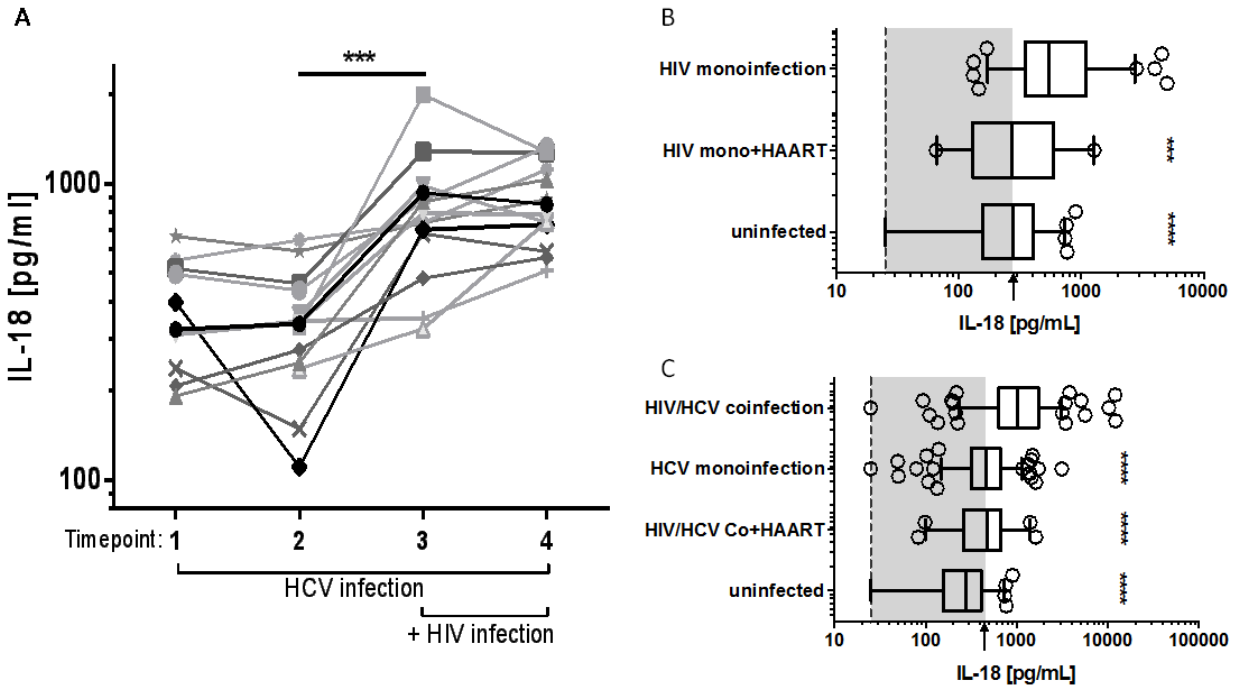


Figure 2.3 Infection with HIV increases serum IL-18 while HAART suppression of HIV is associated with lower IL-18 production.

A. Serum IL-18 levels were measured in 14 subjects with chronic HCV infection at 2 time points prior to and 2 time points after incident HIV infection. IL-18 levels were significantly different upon incident HIV infection (between the last preinfection and first post infection timepoint, *** $p<0.001$, paired student T-test), but not between the two baseline preinfection visits or between the paired post HIV infection visits. Serum IL-18 levels were compared between: B. HIV monoinfected, HIV monoinfected on HAART, and HIV/HCV uninfected subjects. Dashed line: LLOQ, arrow: HIV/HCV uninfected subject median. HIV monoinfected on HAART and the HIV/HCV uninfected groups were both statistically different from the HIV monoinfected group (*** $p<0.001$ and **** $p<0.0001$, respectively), but not from each other. C. HIV/HCV coinfected, HCV monoinfected, HIV/HCV coinfected on HAART and HIV/HCV uninfected subjects.

Dashed line: LLOQ, arrow: HCV monoinfection median. All groups were statistically different from HIV/HCV coinfection, $p<0.0001$. Both B and C were compared using a one way ANOVA and adjusted for multiple comparisons.

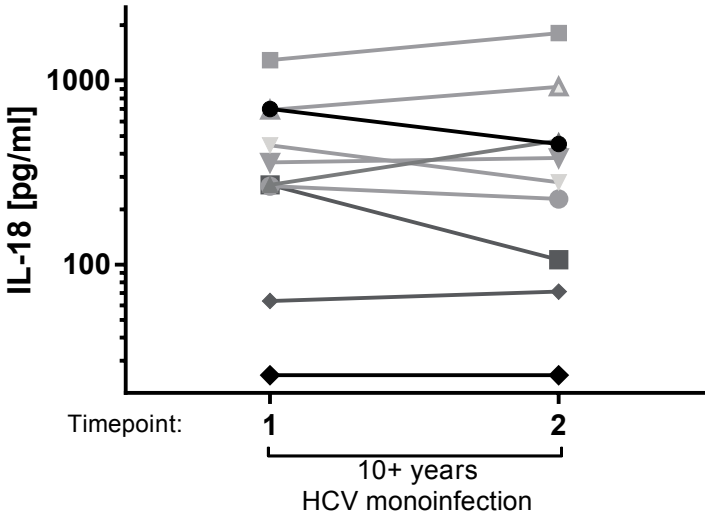


Figure 2.4 IL-18 levels are stable in chronic HCV infection over time

Serum IL-18 levels in 10 chronic HCV monoinfected subjects who failed to acquire HIV were measured at 2 time points approximately 10 years apart. Paired T-tests were used for within person comparisons.

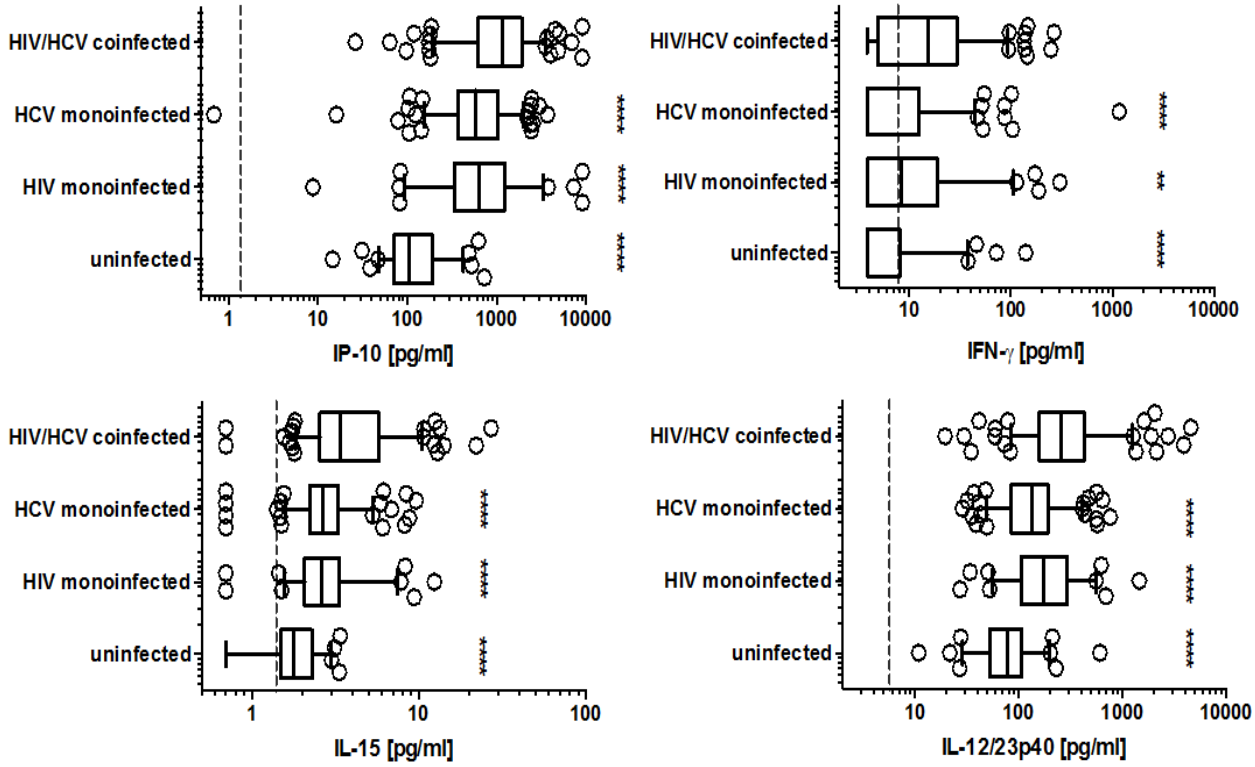


Figure 2.5 Serum IP-10, IFN-y, IL15 and IL12/23p40 are increased in HIV/HCV coinfection

Serum IP-10, IFNy, IL-15 and IL12/23p40 levels were significantly increased in coinfection when compared to either monoinfection or uninfected groups. Dashed line: LLOQ for each analyte, **** $p<0.0001$, ** $p<0.01$. Groups were compared using a one way ANOVA and adjusted for multiple comparisons.

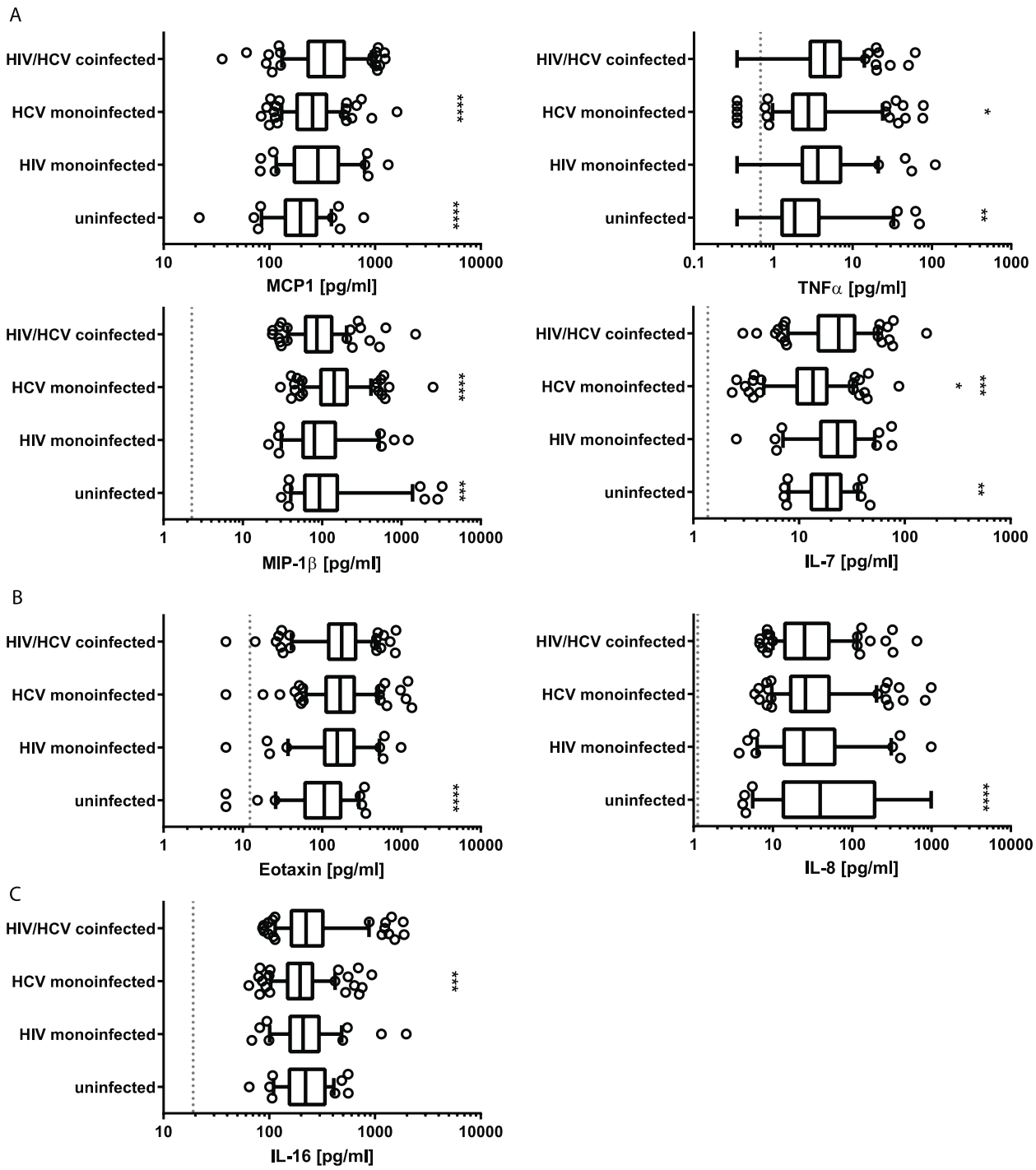


Figure 2.6 Cytokines and chemokines that were not statistically different in HIV/HCV coinfection when compared to monoinfection but were different between other groups.

All cytokine and chemokine levels were measured by MSD. A. Serum IL-7, MCP-1, MIP1b and TNFa levels were statistically different in the HCV monoinfection and HIV/HCV negative groups when compared to the HIV/HCV coinfection group. B. Serum Eotaxin and IL-8 levels were significantly different in the HIV/HCV negative group when compared to HIV/HCV coinfection. C. Serum IL-16 levels were different in the HCV monoinfection group when compared to HIV/HCV coinfection. * $p<0.5$, ** $p<0.1$, *** $p<0.001$, ****$p<0.0001$. Groups were compared using a one way ANOVA and adjusted for multiple comparisons.

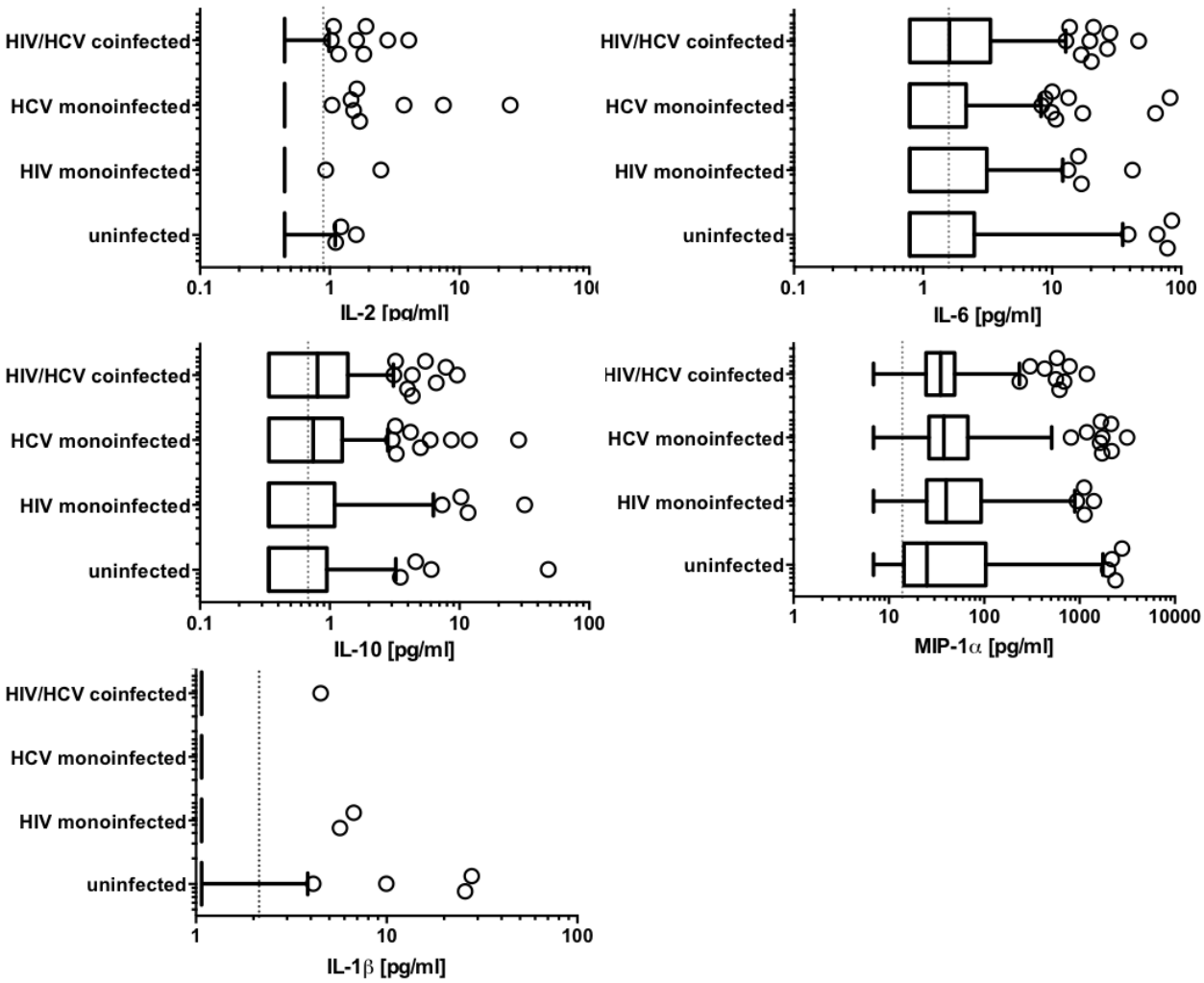


Figure 2.7 Cytokines and chemokine that were not statistically different between any group. Serum IL-2, IL-6, IL-10, MIP-1a and IL-1b levels were measured by MSD. Groups were compared using a one way ANOVA and adjusted for multiple comparisons.

CHAPTER III: HIV-specific antibodies induce greater immune activation by enhancing type I interferon production from plasmacytoid dendritic cells

This chapter is in submission for publication as:

Veenhuis RT, *et al*. 2016 "HIV-specific antibodies induce greater immune activation by enhancing type I interferon production from plasmacytoid dendritic cells."

ABSTRACT

INTRODUCTION: Chronic activation of the immune system is a well-established hallmark of HIV infection, but its etiology remains incompletely understood. Type I inteferon (IFN) production is essential in innate control of acute viral infection, but prolonged IFN production is associated with chronic immune activation in HIV. Despite its potential importance in pathogenesis, the mechanisms that maintain high-level IFN production following the acute phase are unknown. We hypothesized that induction of an HIV-specific antibody (Ab) response might regulate IFN production.

METHODS: We analyzed the mechanism through which HIV activates pDCs, the primary producer of IFN, by testing entry, TLR utilization, and downstream signaling in normal human pDCs. We next analyzed how both monoclonal HIV-specific antibodies and Abs induced in natural HIV infection modulated pDC sensing of HIV.

RESULTS: We found that HIV-driven activation of pDCs to produce IFN requires TLR-7, receptor-mediated entry, fusion, and viral uncoating, but not endocytosis or infection. Downstream signaling in pDCs requires NFκB and CREB followed by IRF7 signaling to produce IFN. Abs directed against the HIV envelope, which do not interfere with CD4 binding, significantly enhanced the IFN response irrespective of Ab capacity to neutralize CD4 T cell infection. Ab-mediated enhancement of IFN production required pDC Fc gamma receptor engagement, bypassed fusion, and initiated signaling through both TLR7 and 9. Polyclonal Ab isolated from HIV infected subjects enhanced pDC production of IFN in response to HIV.

CONCLUSIONS: We demonstrate that Abs generated in persistent HIV infection enhance IFN production by pDCs, potentially driving persistent IFN production and providing a novel explanation for immune activation seen in chronic HIV infection.

INTRODUCTION

Type I IFN is essential in innate control of acute viral infection, but can enhance pathogenesis during chronic infections like HIV and lymphocytic choriomeningitis virus infection (LCMV) [62-64]. In HIV and SIV infection, prolonged Type I IFN production is associated with chronic immune activation [65, 66]. SIV-infected monkeys have been shown to upregulate ISGs following SIV infection. Monkeys whose ISG levels returned to baseline rapidly did not develop chronic immune activation and AIDS, while those with sustained ISG elevation did [41, 42]. Similarly, HIV rapid progressors showed stronger IFN signatures than viremic non-progressors [44]. Increased circulating IFN during the chronic stage of HIV infection has been associated with rapid progression to AIDS [43].

In contrast to chronic infection, the antiviral function of IFN in acute SIV infection prevents disease. Blockade of IFN responses prior to SIV infection led to rapid disease progression, accelerated CD4 T-cell depletion, increased reservoir size and death in macaques [65]. While early exogenous IFN administration upregulated expression of anti-viral genes and prevented systemic infection, prolonged administration induced IFN desensitization [65]. Thus, the role of IFN in HIV/SIV infection is dichotomous, with IFN responses controlling infection in the acute phase but prolonged production enhancing pathogenesis and the establishment of chronic infection.

The mechanism that regulates the level of IFN production outside of the acute phase is unknown despite its potential importance in pathogenesis. The principal source of Type I IFN, specifically IFN-α/β, is pDCs. HIV is sensed by pDCs through endosomal TLR7 recognition of RNA and has been reported to require gp120-CD4 interaction and

HIV entry via endocytosis [67, 68]. We hypothesized that induction of an HIV-specific antibody (Ab) response might modulate pDC production of Type I IFN and HIV disease progression.

In this study, we further define the mechanism through which HIV activates pDC to produce IFN. We demonstrate a new role for Abs in regulation of HIV-induced IFN production. Abs generated in persistent HIV infection enhance Type I IFN production by pDCs, potentially driving persistent IFN production and providing a novel explanation for the immune activation seen in chronic HIV infection.

METHODS

Expansion of HIV cell culture strains and deactivation by AT-2

HIV_{BaL} was obtained from Dr. Joel Blankson (Johns Hopkins) and HIV_{IIIB} from Dr. Suzanne Gartner (Univ. Maryland) and expanded as previously described[26]. In brief, 10-20 x 10^6 PHA activated CD4+ T-cells were spinoculated (1200g, RT, 2 h) with 0.5-1 μg of HIV p24. Spinoculated cells were cultured for 10 days prior to harvest of infectious culture supernatant. HIV p24 Ag was measured using the Alliance p24 ELISA kit (Perkins Elmer, Waltham MA) per manufacture's recommendations. AT-2 deactivated virus was produced as previously described[69]. Briefly, HIV was treated with 300 μM AT-2 (Sigma) for 1 h at 37 ^oC. After incubation, the virus was filtered using a 100 kDa Amicon Ultra 0.5 mL filter (Millipore).

HIV monoclonal antibodies

The following antibodies were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: anti-HIV-1 gp120 monoclonals, IgG1 b12[70-73] from Dr. Dennis Burton and Carlos Barbas; PG16[74]; PG9[74]; and 2G12[75-79] from Dr. Hermann Katinger; anti-HIV-1 gp41 monoclonals 4E10[80], 2F5[75, 81, 82] and 5F3[75] from Dr. Hermann Katinger; 246-D[83-86] from Dr. Susan Zolla-Pazner. 4E10 Ab was also purchased from Polymun Scientific (Klosterneuburg, Austria). Gp120 mAb VRC01 and VRC01 FcγR binding variants were produced as previously described[87].

Rapid and Slow progressor sample selection

Serum samples from rapid and slow progressors were obtained from the Baltimore-Washington DC site of the Multicenter AIDS Cohort Study (MACS;

http://aidscohortstudy.org/). MACS is an ongoing prospective cohort study of the natural and treated history of HIV infection in men who have sex with men at 4 sites (Baltimore, Maryland/Washington, DC; Chicago, Illinois; Los Angeles, California; and Pittsburgh, Pennsylvania) [88, 89]. MACS participants are followed with semi-annual study visits that include questionnaires, a brief physical exam, and phlebotomy for laboratory tests and storage of serum, plasma, and peripheral blood mononuclear cells. HIV-uninfected participants are tested at each visit for HIV seroconversion. Rapid progressors were defined as men who developed an AIDS-defining illness within five years of seroconversion, and slow progressors as men who did not develop an AIDS-defining illness within ten years of seroconversion with no ART. Two frozen plasma samples were obtained from all men 1 and 2 years after the estimated time of seroconversion, which was taken as the midpoint between the last seronegative visit and the first seropositive visit.

Plasmacytoid dendritic cell (pDC) purification and activation

Freshly collected de-identified human blood Leuko Paks were obtained from the Johns Hopkins Blood Donor (Baltimore, MD) center and the Anne Arundel Medical Blood Donor Center (Anne Arundel, MD). PBMCs were isolated by Ficoll-Hypaque gradient centrifugation. pDCs were magnetically sorted by negative isolation per the manufacturer's protocol (Miltenyi Biotec) and cultured in RPMI 1640 media (Invitrogen), 10% heat inactivated pooled human AB serum (Atlanta biological), and 1% MEM non-essential amino acids, sodium pyruvate, HEPES and L-glutamine (Life technologies). Post isolation pDCs were plated at 0.5-1 x 10^6 cells/ml and cultured with 20-40 ng/ml of HIV p24, 0.02-0.1 μM Resiquimod a TLR7 agonist or ODN2216 a TLR9

agonist (InvivoGen). For Ab experiments, HIV was pre-incubated for 1-2 h at 37^{o}C with 5 μg/ml of designated mAb to form immune complexes. The mixture was then added to pDC cultures and IFNα production was measured after 15 h.

Quantitative real-time PCR

RNA was isolated with the RNeasy Kit (Qiagen) and used to synthesize cDNA with Superscript III Reverse Transcriptase system (Invitrogen). Quantitative PCR was performed with IDT pre-designed primers/probe mixes using Light Cycler 480 Probes Master mix (Roche). GAPDH and RPL13A were used as housekeeping controls. The ratio of target gene mRNA copies relative to the average mRNA copies from housekeeping controls was defined as Delta. Ratio of normalized mRNA copies among experimental conditions is defined as Delta/Delta and used to calculate the relative effect of HIV activation in pDCs.

IFNα measurements

Human IFNα was measured with the Human IFNα Tissue Culture kit from Meso Scale Discovery (MSD). Samples were tested per manufacture's protocol using 25μl of undiluted culture supernatant. Data were acquired on a SECTOR Imager SI2400.

Modulation of IFNα by inhibition of HIV entry or infection

Freshly isolated pDCs were pre-incubated with one of the following Abs or inhibitors for 1 h at 37 ^{o}C: 1.25 μg/ml anti-human CD4 mouse mAb (clone SK3, Biolegend), 1.25 μg/ml of isotype control (clone MOPC-21, Biolegend), 10 μM of the CCR5 receptor agonist Maraviroc (MVC, Selleckchem), 1 μM of the fusion inhibitor Enfavirtide (T20, Sigma), 1.5 nM Cyclophillin A inhibitor (CypA, Millipore), 1 μM for the reverse transcriptase inhibitor Emtricitabine (FTC, Selleckchem), 1 μM of the integrase inhibitor

Raltegravir (RAL, Selleckchem). Virus +/- HIV Ab was then added to the pDCs and IFNα production measured after 15 h.

Modulation of IFN by endocytosis, signaling and TLR inhibitors

Freshly isolated pDCs were incubated with Methyl-β-cyclodextrin (MβCD, Sigma) 20 μM, 1 h at 37 ^{o}C, an inhibitor of clathrin-mediated endocytosis inhibitor, the dynamin inhibitor Dynasore 80 μM (Dyna, Abcam); 1 μM of IKKβ inhibitor, which inhibits NFκB signaling, PF184 (Torcis); or 1 μM of MAP Kinase p38 inhibitor SB203580 (Cell Signaling Technology). TLR 7 and 9 inhibitors have been previously described and were synthesized by IDT[90]. Virus +/- HIV Ab was then added to the pDCs and IFNα production measured after 15 h.

Determining Fcγ Receptor usage

Fcɣ Receptor (FcɣR) usage was determined after altering Fc binding in three ways. First, using previously described VRC01 Fc variants [87]. In brief, the Fc domain of VRC01 Ab was point mutated to alter the Ab's affinity for different Fcɣ Receptors. These mutations have no effect on the affinity or avidity of the Fab domain of the VRC01 Ab. The second method was Ab deglycosylation. Abs were digested using the non-denaturing protocol and the PNGase-F Remove-it enzyme (New England Biolabs). In brief, 20 μg of 4E10 was added to GlycoBuffer2 and H_2O to make a 1x 20 μl reaction; the mixture was incubated at 37 ^{o}C overnight. The PNGase-F enzyme was then removed using Chitin Magnetic beads (NEB) per manufacture's recommendations. The third method used a polyclonal Ab against FcR2 to sterically block interactions as previously described[91]. In brief, after isolation pDCs were cultured with 10 μg/ml of goat anti-human FcγR2 pAb (R&D systems) and incubated at 37 ^{o}C for 1 h. Virus +/- HIV Ab was then added to the

pDCs and IFNα production measured after 15 h. Cells were stained with a PE Mouse anti-human CD32 (BD Biosciences) to confirm that FcR2 was blocked during the course of the experiment.

Cell purity and signaling protein phosphorylation by flow cytometry

After sorting, pDCs purity was determined by flow cytometry using known pDC markers PE-Cy™7 Mouse anti-CD123 (BD Biosciences), BV421 Mouse Anti-CD303 and PE Mouse anti-CD4 (Biolegend). The antibodies used to detect phosphorylation were AF647 mouse anti-IRF7 (pS477/pS479), PE-Cy™7 Mouse anti-NFκB p65 (pS529), PE-CF594 Mouse Anti-p38 MAPK (pT180/pY182), PE Mouse anti-JNK (pT183/pY185) and BV421 Mouse Anti-STAT1 (pY701) (BD Biosciences). To assess if the above signaling molecules were phosphorylated in response to HIV activation we used the Phosflow kit and protocol developed by BD. In brief, post stimulation supernatants were removed and cell pellets were incubated with 100 µl of warmed BD Cytofix™ Buffer for 10 min at 37 ^{o}C. Cells were then washed 2 times with 1% BSA in 1x PBS and permeabilized by slowly adding 100 µl of cold BD Phosflow™ Perm Buffer III and incubated on ice at -20 ^{o}C for 30 min. Cells were washed 2 times with 1% BSA in 1x PBS and stained with the panel of phosflow antibodies above per manufacturer's instructions. All data was acquired on a BD LSRII flow cytometer.

Isolation of polyclonal IgG from plasma samples

Polyclonal IgG (pAb) was isolated from plasma samples using protein A columns (Thermo) per manufacturer's instructions. Post isolation IgG was the 5 mL elution was neutralized using 500uL of binding buffer and concentrated on 50 kDa Amicon Ultra

5mL filter (Millipore) and quantified using the Human IgG total ELISA Ready-SET-Go kit (eBioscience) per manufacturer's instructions.

gp120 binding ELISA

Polyclonal Ab binding to HIV_{BaL}-gp120 was quantified by ELISA as previously described [92]. In brief, HEK293T cells were transfected with HIV_{BaL}-gp120 expressing plasmid and lysed 48 h post-transfection. Immulon 2HB flat bottom strips (Thermo) were coated with 500ng/mL *Galanthus nivalis* lectin (Sigma-Aldrich) and blocked with PBS containing 0.5% Tween 20, 1% nonfat dry milk, and 1% goat serum for 1 h at 37 ^{o}C. HIV_{BaL} gp120 cell lysates were added overnight at 4 ^{o}C. Binding of pAb were measured in 5-fold serial dilutions, starting at 125 μg/ml. Binding was detected using horseradish peroxidase (HRP)-conjugated anti-human IgG secondary antibody (BD Pharmingen).

Statistical analysis

One-way ANOVA and paired t-tests were used to evaluate statistical significance of differences between groups. Differences were considered significant when $p<0.05$.

RESULTS

pDCs require receptor-mediated entry to produce Type I IFNs in response to HIV

Purified pDCs produced Type I IFN mRNA (Figure 3.1A) and protein (Figure 3.1B) when incubated with the culture strain HIV_{BaL}. Previous studies have shown that production of Type I IFN by pDCs requires CD4-mediated endocytosis of HIV[67, 68]. However, these studies used inhibitors of endocytosis that also inhibit endosomal acidification, which is necessary for TLR sensing; therefore it was not clear if endocytosis were required. To test whether endocytosis of virus is required, we used MβCD, an inhibitor of clathrin-mediated endocytosis, and Dynasore, a dynamin inhibitor, which inhibits both clathrin and caveolar-mediated endocytosis. Contrary to previous findings, we saw that endocytosis was not required to activate a Type I IFN response in pDCs when using these more specific inhibitors (Figure 3.1C). The lack of IFN suppression by both endocytosis inhibitors lead us to hypothesize that HIV was entering pDCs though another method, possibly receptor-mediated entry. To test if HIV enters pDC by receptor-mediated entry, we used a monoclonal antibody (mAb) against CD4 and a panel of HIV inhibitors that blocked HIV co-receptor binding, fusion, and uncoating. We observed that blockade of the gp120-CD4 interaction (anti-CD4 mAb), co-receptor binding (Maraviroc, MVC, a CCR5 inhibitor), fusion (T20, enfuvirtide), or uncoating (cyclophilin A inhibitor, CpAinh) significantly diminished the IFNα produced by pDCs in response to HIV (Figure 3.1D). Blocking cyclophillin A, is thought to only partially inhibit uncoating, which may explain its partial effect[93]. These data suggest that HIV enters pDCs in the same way it enters CD4 T cells in infection. Previous data have suggested that pDCs may become infected when exposed to HIV[94, 95]. To determine if

infection is necessary for the production of Type I IFN, we used a reverse transcription inhibitor (emtricitabine**,** FTC), an integrase inhibitor (Raltegravir, RAL), and deactivated HIV (2,2-dithiodipyridine aldrithiol-2; AT-2 treated). AT-2 covalently modifies essential zinc fingers in the nucleocapsid protein of HIV virions, thereby inactivating the virus. However, AT-2 treatment maintains viral and host cell-derived proteins on virion surfaces, retaining conformational and functional integrity. Our data demonstrate that infection is not required for the IFN response because the reverse transcription inhibitor, integrase inhibitor, and deactivation of HIV via AT-2 treatment had no effect on IFNα production (Figure 3.1D). To confirm that the inhibitors used affected HIV sensing specifically and did not globally impair pDC function, we treated pDCs with a TLR7 agonist (Resiquimod) with and without our panel of inhibitors and saw that the inhibitors had no effect on the overall IFN production by pDCs in response to Resiquimod (Figure 3.2A). These data show that receptor-mediated entry and uncoating are necessary, but endocytosis or infection are not required, in order for pDCs to release Type I IFN in response to HIV.

HIV signals via TLR7 and IRF7 and requires the NFkB and CREB pathways to induce Type 1 IFN

Next, we sought to determine how HIV triggers TLR signaling once it has entered pDCs. It has previously been reported that HIV and other viruses engage TLR7 to induce IFN production in pDCs[67, 90]. To confirm TLR7 usage, we used antagonists of TLR7 and TLR9, the only TLRs present on human pDCs, [96] in the presence of HIV and observed a significant decrease in IFNα produced in the presence of the TLR7 antagonist with no significant effect on IFNα induction in the presence of a TLR9 antagonist (3.3A).

Specificity of the TLR antagonists was confirmed using agonists for each TLR. The TLR7 inhibitor specifically inhibited induction of the signaling cascade by the TLR7 agonist Resiquimond with no effect on IFN induction with the TLR9 agonist ODN2216 (Figure 3.4A). Conversely, the TLR9 inhibitor specifically inhibited ODN2216 signaling with no effect on Resiquimond signaling (Figure 3.4B). Subsequently, we sought to verify that IRF7 is the adaptor protein required downstream in the TLR7 signaling cascade in response to HIV. IRF7 has been reported to be essential in TLR7 signaling and some data suggest it is involved in HIV signaling in pDCs[97, 98]. To assess IRF7 usage, we looked for a change in phosphorylation of IRF7 and five additional signaling molecules (NFκB p65, MAP kinase p38, JNK, TBK1, and STAT1) after HIV-induced activation. There was a significant increase in phosphorylated IRF7 (p-IRF7) with HIV activation (Figure 3.3B). Cells that had increased p-IRF7 also had increased phosphorylated p65, p38 and JNK (Figure 3.5A). Additionally, all HIV-activated pDCs had increased phosphorylated STAT1 and no phosphorylated TBK1 (Figure 3.5B). STAT1 and TBK1 were used as positive and negative controls, respectively. All cells within the HIV-activated culture should have increased phosphorylated STAT1 because of the positive feedback loop that exists in IFN signaling with IFN engagement of the IFN receptor inducing phosphorylation of STAT1[99, 100]. Conversely, no cells should have phosphorylated TBK1, because TBK1 is involved in the IRF3, not the IRF7, signaling pathway[101]. To assess whether the increases in p65 and MAPK p38 phosphorylation were directly involved in HIV-induced IFN production, we used an IKKβ and a p38 inhibitor to block downstream NFκB and CREB signaling, respectively. We saw that inhibition of either NFκB or CREB signaling completely suppressed IFN

production and prevented phosphorylation of IRF7, p65, and p38 (Figure 3.3C and 3.5C). The pDCs were still alive in the presence of these inhibitors as assessed by flow cytometry. We confirmed they were still capable of producing IFN using a TLR7 agonist. Interestingly, inhibition of CREB signaling did not affect IFN production in response to Resiquimod, but inhibition of NFκB completely suppressed IFN (Figure 3.2B), suggesting that NFκB signaling is central for IFN production in pDCs, but multiple downstream paths from TLR7 can lead to IFN. In sum, these data demonstrate that HIV activates the IFN response by first engaging TLR7, which then signals through NFκB and CREB, resulting in IRF7 phosphorylation and finally, IFNα production.

HIV-specific antibodies regulate Type I IFN production by pDCs

Because the IFN response is crucial in the resolution of acute infection, but potentially detrimental with prolonged exposure in chronic viral infection, we hypothesized that factors present only after the acute phase of infection might alter IFN production in later infection stages. There is prior evidence that the CD4-gp120 interaction is absolutely critical to IFN production in pDCs and that neutralizing Abs that block that interaction inhibit IFN production by pDCs in an FcR-independent way[67, 91, 97]. We would predict this requirement of the CD4-gp120 interaction given that we too demonstrated dependence on CD4 binding for HIV to activate pDCs. We confirmed that Abs known to interfere with CD4 binding (B12, PG9, PG16, and VRC01) suppress IFN production by pDCs (Figure 3.6A, Table 3.1). To assess dependence on FcγR engagement, we tested variants of the VRC01 mAb with constant antigen binding (Fab) portions but mutated or deglycosylated Fc regions resulting in variable binding of FcγR that ranged from no binding to preferential binding of each FcγR[87]. Alteration of Fc did not impair the

capacity of VRC01 to suppress IFN production, confirming that FcγR engagement is not required for Ab-mediated suppression of IFN production by pDC (Figure 3.6B). To test whether the capacity to disrupt CD4 binding determines suppression, we tested an additional neutralizing mAb that binds gp120, but does not disrupt CD4 binding, 2G12. This Ab did not suppress IFN production by pDCs (Figure 3.6A). To further assess the effect of mAbs that bind HIV envelope but do not disrupt CD4 binding, we used mAbs specific for the gp41 portion of the HIV envelope (4E10, 246-D, 5F3, and 2F5). Interestingly, we saw that all gp41-specific mAbs tested enhanced IFN production (Figure 3.6C). Two of the anti-gp41 mAbs tested (246-D and 5F3) do not neutralize HIV infection in CD4 cells. Therefore, enhancement of pDC production of IFN was independent of the mAbs' ability to neutralize infection. Additionally, the epitope specificity of the gp41-specific mAbs does not drive the level of enhancement since mAbs 4E10 and 2F5 bind very similar epitopes on the membrane proximal external region (MPER) of gp41, but have very different capacity to enhance (Figure 3.6C and Table 3.1). Overall, these data demonstrate that HIV-specific antibodies that don't prevent HIV from engaging CD4 on the surface of pDCs can enhance the Type I IFN response to the virus.

Enhancement by HIV-Ab complexes requires receptor mediated binding and IRF7/NFκB signaling pathways

To further understand how HIV envelope-specific mAbs enhance the pDC IFN response, we reexamined the role of pathways defined as critical for HIV sensing without Ab present. Culturing pDCs in the presence of CD4 blocking mAb or CCR5 inhibitor not only negated Ab-mediated enhancement by mAb 4E10, but nearly completely blocked all

IFN production, demonstrating that Ab enhancement of IFN production does not bypass the requirement for CD4 and co-receptor engagement (Figure 3.7A). Additionally, we saw that infection was not required for mAb 4E10 enhancement since inhibition of reverse transcription (FTC) or integration (RAL) did not diminish enhancement (Figure 3.7B). We hypothesized that mAb complexed to HIV might enhance the downstream signaling cascade and leading to enhanced IFN production. To determine if the downstream signaling cascade was altered, we assessed IRF7 phosphorylation and asked whether NFκB and CREB were both required for the enhanced IFN response. We saw an equivalent amount of p-IRF7, p65, p38 and JNK in response to HIV with and without enhancing mAb (Figure 3.8). Additionally, the inhibition of NFκB or CREB signaling led to complete suppression of IFN production whether or not enhancing mAb was present (Figure 3.7C). Our data show that the gp120-CD4 interaction remains critical for HIV-induced activation of pDC IFN production in the presence of enhancing mAbs and that enhancing mAbs do not alter the downstream signaling cascade.

HIV-Ab complexes bypass fusion and require Fcγ Receptor engagement to signal through TLR7 and TLR9 and enhance IFN production

Given no change in the requirement for HIV-CD4 interaction or downstream signaling, we measured how enhancing Abs altered HIV entry into pDCs and TLR engagement. We hypothesized that the addition of Abs may allow CD4-mediated endocytosis as well as receptor-mediated entry, increasing overall viral entry. To assess this, we used MβCD and Dynasore to prevent clathrin and caveolar mediated endocytosis. However, inhibiting endocytosis did not suppress the enhancing effect of HIV specific Ab 4E10 (Figure 3.7D), suggesting that enhancement isn't mediated by endocytosis of virions. Next we

assessed if the HIV-Ab complexes were still entering pDCs via membrane fusion, as seen with HIV alone. While adding the fusion inhibitor T20 significantly suppressed IFN production with HIV alone (Figure 3.1C), the level of IFN produced in response to HIV with 4E10 remained unchanged in the presence of T20 (Figure 3.9A). These data demonstrate that 4E10 mediates HIV entry into pDCs through a mechanism distinct from endocytosis or from that used by HIV in the absence of Abs since enhanced IFN production doesn't require fusion with the cell membrane. This alternate method of entry led us to hypothesize that the HIV-Ab complexes were entering via FcγR engagement. To test dependence on FcγR engagement, we utilized two methods to prevent the HIV-Ab complexes from binding to FcγRs. The first method (PNGase treatment) removed all N-linked glycans from our enhancing mAb 4E10 via digestion (Figure 3.9B, deg 4E10). The removal of glycans prevents the Fc from binding to any FcγR, but has no effect on the Fab portion of the antibody[102-104]. To confirm that deglycosylation didn't affect mAb binding, we compared the neutralization pattern of deglycosylated 4E10 (deg4E10) with intact 4E10 and saw no difference (Figure 3.10). The second method sterically blocks FcγR engagement by using a mAb that binds to, but does not induce signaling through FcγR2[91]. Blockade of FcγRs by engagement with the commercially available Fc Block was not attempted because it non-specifically suppresses the IFN response in pDCs[105]. Deglycosylating our enhancing mAb or sterically blocking FcγR2 engagement prevented enhancement of IFN production by HIV-Ab complexes, but not IFN production by HIV alone (Figure 3.9B). Additionally, we tested whether this alternative method of entry into pDCs altered TLR specificity for the virus. Inhibition of TLR7 signaling by a TLR7 antagonist led to complete suppression of IFN production. However, inhibition of TLR9

signaling led to partial reduction of IFN production, returning IFN production to levels seen in the absence of mAb, indicating that enhanced IFN production mediated by mAb occurs preferentially through TLR9 engagement of HIV (Figure 3.9C).

Abs isolated from subjects with chronic HIV infection enhance IFN production

To assess the role that HIV-specific Abs have in enhancing IFN *in vivo*, we isolated polyclonal IgG from serum collected at years one and two of infection from five rapid and eight slow HIV progressors in the Baltimore-Washington DC Center of the Multicenter AIDS Cohort Study. Rapid progression was defined as progression to an AIDS-defining illness, without HAART treatment, within five years of infection. Slow progression was defined as no progression to an AIDS-defining illness, without HAART treatment, within 10 years of infection. Given previous data suggesting that prolonged high-level IFN production is associated with chronic infection and immune activation, we hypothesized first that IgG from HIV-infected subjects would enhance pDC IFN production in response to HIV and secondly, that IgG from rapid progressors would enhance IFN production more than IgG from slow progressors. Given our previous *in vitro* data suggesting that mAb against gp120 suppress Type I IFN production by pDC, we tested the isolated IgG for gp120 binding in an ELISA. All 26 IgG samples tested (13 subjects at both time points) showed binding to HIV_{BaL} gp120 lysate, although with varying affinity (Figure 3.11). The IgG from the 13 HIV infected subjects was then tested for the ability to suppress or enhance pDC production of IFN in response to HIV_{BaL} in the same assay used to assess the effects of 4E10. Despite showing gp120 binding activity, IgG from 12 of 13 subjects significantly enhanced pDC production of Type I IFN in response to HIV_{BaL} alone and the degree of enhancement varied (Figure 3.12A). When

comparing IgG isolated from one year post infection versus two years post infection, we saw no difference in the degree of IFN enhancement (Figure 3.12B). To test our hypothesis that global binding to gp120, rather than exclusively at the CD4 binding site, does not cause suppression of IFN, we correlated IFN enhancement with gp120 ELISA binding and saw no correlation. This indicates that the amount of gp120 binding does not determine level of IFN suppression or enhancement (Figure 3.12C). When the subjects were stratified by rate of progression, we saw that IgG from rapid progessors had a trend toward increased IFN enhancement when compared to that of slow progressors, but the difference was not significant ($p=0.3$, Figure 3.12D).

Similar results were seen when the IgG specimens were added to pDCs exposed to another HIV laboratory stain, HIV_{IIIB}. All IgG specimens enhanced IFN production and there was no difference between IgG isolated from year one and from year two (Figure 3.13A and B). The degree and rank order of enhancement were similar, but not identical, between the two HIV strains and due to variability in pDC activation seen with the HIV_{IIIB} strain the enhancements were not statistically significant. Additionally, when the subjects were stratified by rate of progression, we saw a similar trend in that IgG from rapid progressors seemed to enhance IFN produced in response to HIV_{IIIB} slightly more than slow progressors, but once again it was not significant ($p=0.18$, Figure 3.13C).

DISSCUSSION

Although type I IFN production is essential in the resolution of acute viral infections, it has been associated with enhanced immune activation seen in chronic infections such as HIV and LCMV. Identifying the cause of persistent, high-level IFN responses during chronic HIV infection is important in understanding the pathogenesis of chronic viral infections. This study demonstrates a possible mechanism for continued production of type I IFN after the acute phase of HIV infection. We have shown that HIV_{BaL} stimulates pDCs to produce type I IFN and that this activity requires receptor-mediated entry, envelope fusion, viral uncoating, and engagement of TLR7 with defined downstream signaling. In contrast to prior reports, our data demonstrate that receptor-mediated entry, not endocytosis, is required for pDC activation. Previous studies that attributed pDC activation to endocytosis observed loss of IFNα production when acidification inhibitors were present in the culture[67]. However, endosomal TLRs require acidification to signal so acidification inhibitors block TLR signaling regardless of the mechanism of virus entry[106]. By targeting the endocytic uptake process independent of endosome function, we have shown that alternative methods of viral uptake activate the type I IFN pathway in pDCs.

Once HIV enters the pDC, there are multiple methods through which viral RNA could access TLR7 in the endosome. Previous studies have shown that autophagy is required for IFN production in viral sensing by pDC, suggesting that the autophagy pathway connects viral RNA in the cytosol and TLR7 within the lysosome[107, 108]. This proposed mechanism of entry and intersection with TLR7 is well supported by our data showing that IFNα production after TLR7 engagement requires NFκB and CREB

signaling followed by IRF7 activation. It has been shown previously that TLR7 signaling within the lysosome leads to NFkB and IRF7 signaling, while TLR7 signaling within the endosome leads to primarily IRF7 signaling[98]. We attempted to test the autophagy pathway, but were unable to find inhibitors that were specific and had no affect on the TLR7 signaling pathway.

In an effort to understand the influence of the adaptive immune responses on type I IFN with persistent infection, we assessed the effect HIV-specific Abs have on IFNα production by pDCs. We demonstrated that mAbs that interfere with HIV gp120-CD4 binding suppress the IFN response independent of their ability to bind FcγR. These data agree with previous pDC HIV studies showing that the gp120-CD4 interaction is critical for IFN production[67, 68, 91, 97]. However, we have also demonstrated for the first time that a subset of HIV specific Abs that do not interfere with CD4 binding significantly enhance the type I IFN response. Using a panel of gp41 mAbs, we determined that neither the gp41 epitope recognized nor the mAb's ability to neutralize HIV infection of CD4 T cells played a role in enhancement of pDC production. Antibody-mediated enhancement required FcγR engagement, bypassed receptor-mediated fusion, and occurred through both TLR7 and 9, with TLR9 signaling unique to HIV sensing in the presence of enhancing mAb. These findings demonstrate dichotomous activity of CD4 binding-site targeting and non-targeting antibodies. Enhanced pDC type I IFN production with HIV mAbs has not been previously shown and represents a novel mechanism for IFN regulation in chronic infection that may in part explain the increased interferon response and immune activation observed in chronic HIV.

We extend these findings by demonstrating that polyclonal IgG isolated from HIV infected patients maintain the activity observed in CD4 binding-site non-targeting antibodies and enhance pDC IFNα response to two different laboratory strains of HIV. This enhancement was not due to a lack of gp120 specific Abs because all polyclonal IgG samples had gp120 binding activity. Thus, in contrast to previous studies with mAbs showing that the role of anti-HIV envelope antibodies is to reduce pDC sensing of virus, Abs generated *in vivo* enhanced IFN production from pDCs in response to HIV. This data suggests that HIV-specific Ab-mediated enhancement of pDC production of type I IFN may be more relevant *in vivo* than the previously described mAb suppression of type I IFN production by pDC *in vitro* through blocking HIV engagement of CD4. In comparing the capacity of IgG isolated from rapid progressors to enhance pDC production of IFN to that of IgG isolated from slow progressors, there was a trend toward enhanced IFN responses with IgG isolated from rapid progressors that was not significantly different. The lack of significance may be due to the fact that small numbers of HIV infected patients were tested, that culture strains rather than autologous HIV were used to stimulate pDCs or that the time point from which we isolated IgG was not during AIDS in the rapid progressors, where one might expect the effect to be most pronounced based on data that serum levels of interferon were highest at the time of AIDS in a previous study[43]. In addition, rapid progression has been shown previously to be associated with multiple factors for which we could not control in our small cohort, including gut microbial translocation and enhanced presentation of antigens by specific HLA types[109, 110]. Therefore, we would not anticipate that any single factor explains disease progression entirely in infected humans. However, HIV-specific Ab isolated

from infected patients enhanced type I IFN production in response to two different culture strains of HIV, suggesting that a humoral response to HIV that fails to control infection may lead to enhanced immune activation.

Although not shown previously in chronic infection, pDC-Ab regulation of type I IFN is supported by data showing that the interaction between pDCs and large self-DNA-Ab complexes is responsible for the IFNα signature seen in autoimmune diseases such as systemic lupus erythematosus [111, 112]. It has been shown that IFNα produced by pDCs causes apoptosis of the pDC secreting the IFN and of pDCs in the surrounding environment[113]. Thus, in an infection that can be controlled by the adaptive immune system, high-level IFN may silence the pDC response as the adaptive immune system clears that infection. In contrast, in HIV and other chronic infections where the immune system is not capable of removing all antigen, continued immune complex formation may persistently stimulate new pDC IFN production. Our data suggest that HIV-specific Ab produced *in vivo* permit persistent high-level IFN responses during chronic HIV infection, representing a novel mechanism of immune activation.

TABLES

Table 3.1 Epitope details for monoclonal antibodies

Monoclonal Antibody	Envelope Protein	Epitope specificity	Interferes with CD4 binding	Effect on Type I IFN
b12	gp120	CD4 binding site	Yes	Suppress
VRC01 (and variants)	gp120	CD4 binding site	Yes	Suppress
PG16	gp120	V1/V2	Yes	Suppress
PG9	gp120	V1/V2	Yes	Suppress
2G12	gp120	Outer glycans	No	Minor enhancement
4E10	gp41	MPER	No	Major enhancement
2F5	gp41	MPER	No	Minor enhancement
5F3	gp41	CC Loop	No	Major enhancement
246-D	gp41	FP/PR	No	Major enhancement

Abbreviations: V(1-3), Variable regions (1-3), MPER, membrane proximal external region, FP, fusion peptide, PR, proximal region

FIGURES

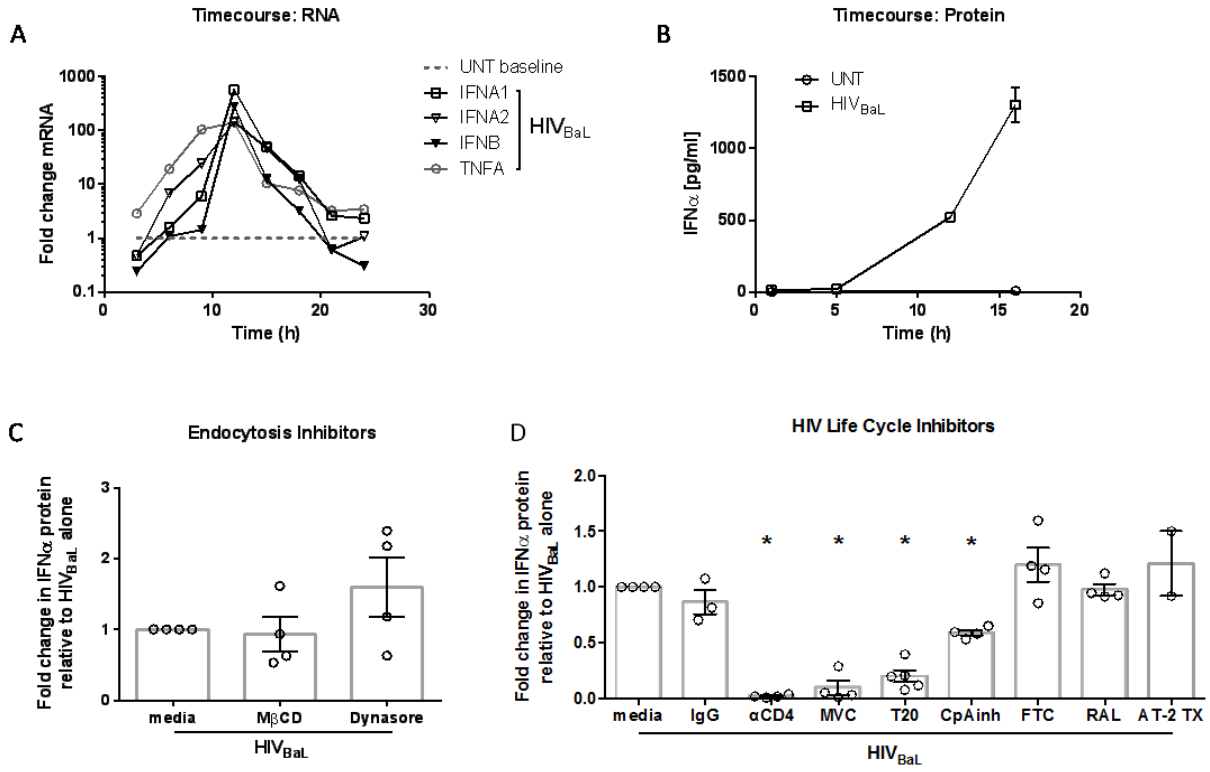


Figure 3.1 Type I IFN induction by HIV requires receptor mediated entry; not endocytosis or infection

Human pDCs were cultured with HIV_{BaL} and cells were harvested and assessed for type I IFN and TNFα mRNA expression (A) or supernatants were harvests and assessed for IFNα protein production (B). P\Human pDCs were cultured with endocytosis (C) or HIV life cycle inhibitors (D) for 1h followed by the addition of HIV_{BaL} for 15h. Supernatants were harvested and assessed for IFNα protein production. Each data point indicates the average IFNα production from one donor's pDCs tested in at least duplicate and normalized to the media condition. Error bars and gray box represent the standard error of the mean and mean, respectively. Conditions were compared using a one way ANOVA with correction for multiple comparisons *$p<0.01$.

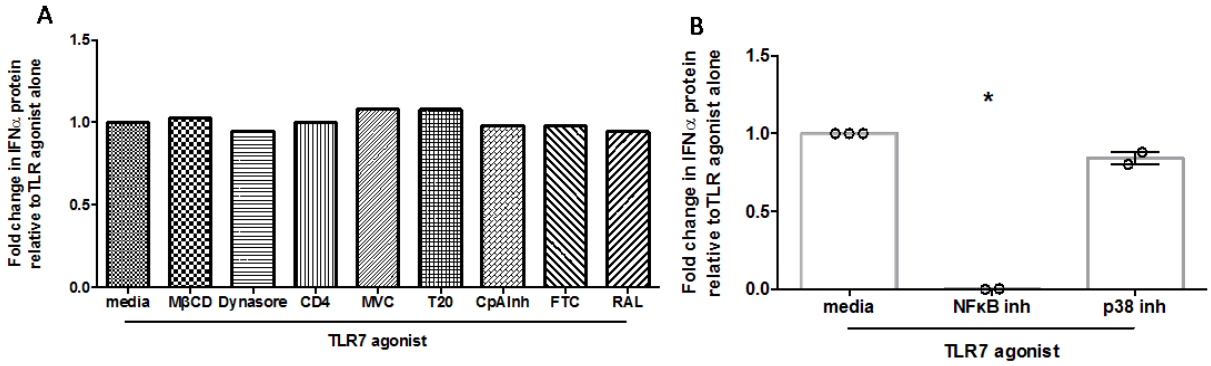


Figure 3.2 pDCs are capable of producing IFNα in the presence of inhibitors

Human pDCs were cultured with endocytosis and HIV life cycle inhibitors (A) or signaling inhibitors (B) for 1h followed by the addition of TLR7 agonist, Resiquimond for 15h. Supernatants were harvested and assessed for IFNα protein production. (A) Is one representative experiment. (B) Each data point indicates the average IFNα production from one donor's pDCs tested in at least duplicate and normalized to the media condition. Error bars and gray box represent the standard error of the mean and mean, respectively. *$p<0.001$

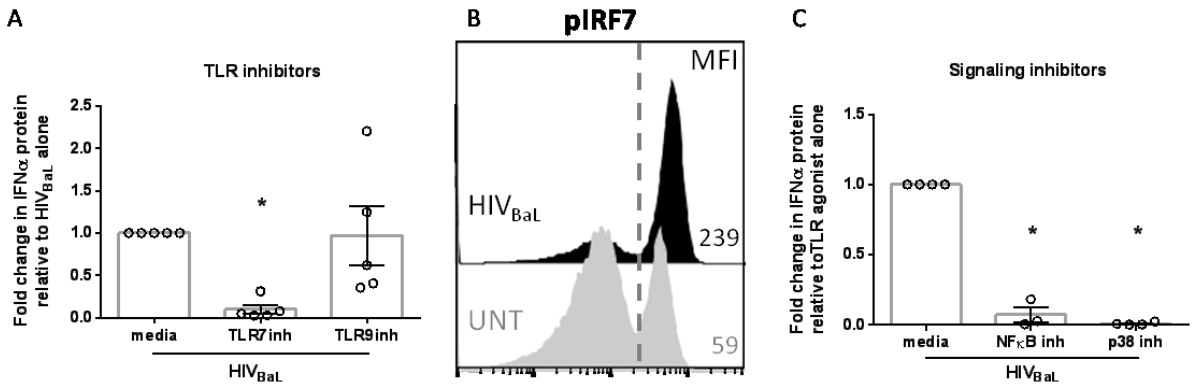


Figure 3.3 pDCs utilizes TLR7, IRF7, NFκB and CREB signaling to produce IFN in response to HIV

Human pDCs were cultured with TLR inhibitors (A) for 1h followed by the addition of HIV_{BaL} for 15h. Supernatants were harvested and assessed for IFNα protein production. (B) Human pDCs were cultured with HIV_{BaL} (black histogram) or without HIV_{BaL} (gray histogram) for 15h cell were then permeabilized and stained for phosphorylated IRF7. The dashed gray line indicates the gate cut off for the positive pIRF7 population. Human pDCs were cultured with signaling inhibitors (C) for 1h followed by the addition of HIV_{BaL} for 15h. Supernatants were harvested and assessed for IFNα protein production. Each data point indicates the average IFNα production from one donor's pDCs tested in at least duplicate and normalized to the media condition. Error bars and gray box represent the standard error of the mean and mean, respectively. *$p<0.05$.

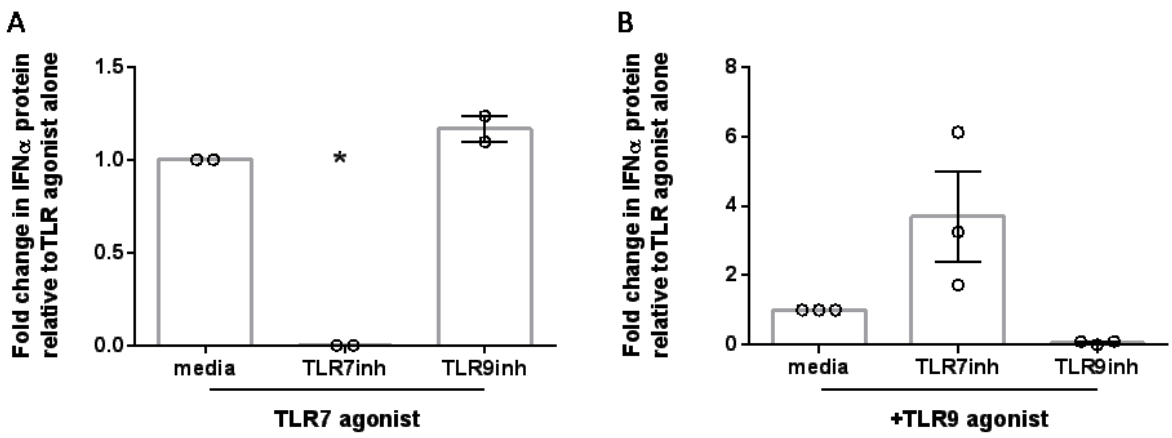
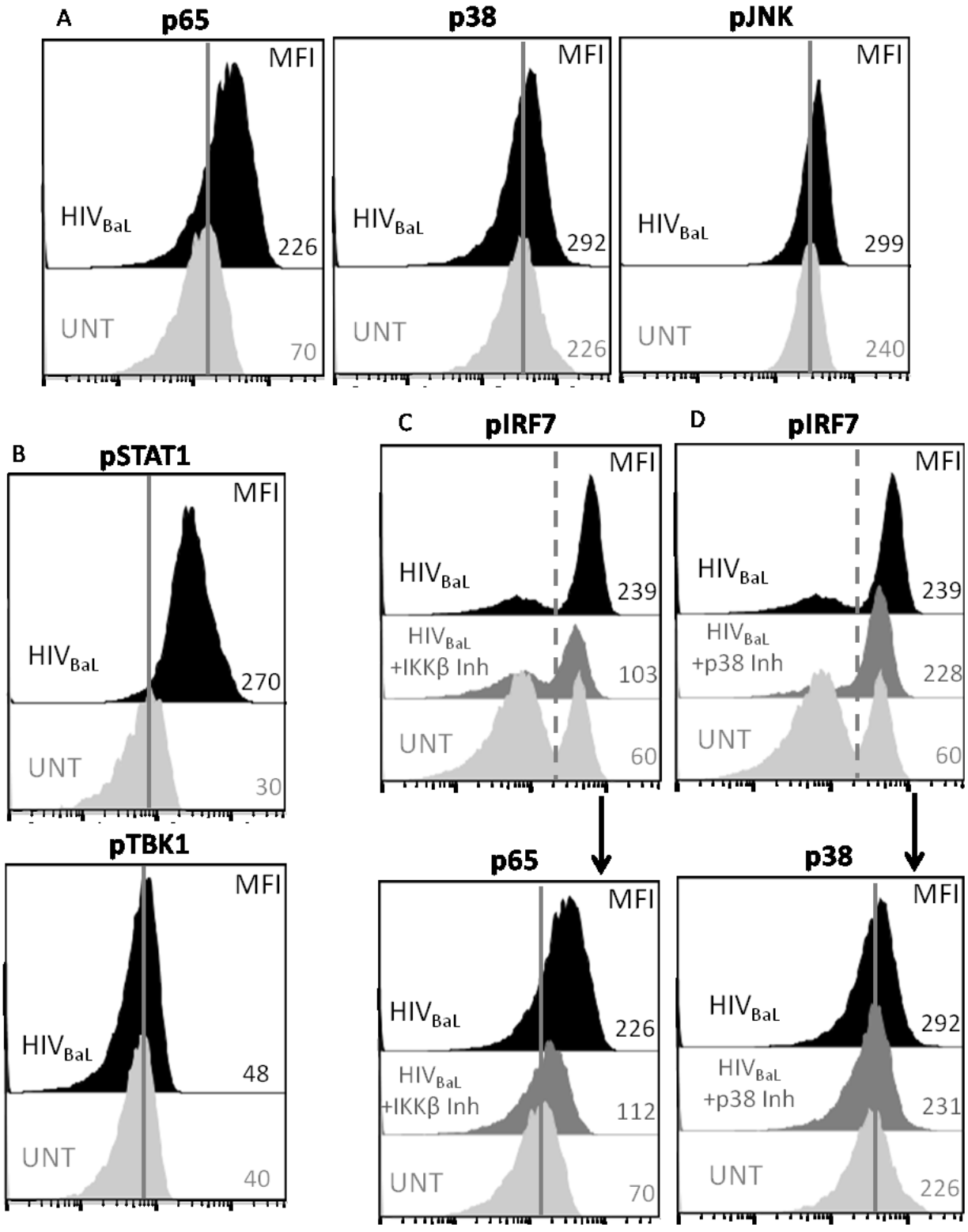


Figure 3.4 Selective Activity of TLR 7 and 9 antagonists

Human pDCs were cultured with TLR inhibitors (A and B) 1h followed by the addition of either TLR7 agonist, Resiquimond or TLR9 agonist ODN2216 for 15h. Supernatants were harvested and assessed for IFNα protein production. Each data point indicates the average IFNα production from one donor's pDCs tested in at least duplicate and normalized to the media condition. Error bars and gray box represent the standard error of the mean and mean, respectively. Conditions were compared using a one way ANOVA with correction for multiple comparisons *$p<0.01$.


A
p65
MFI
HIV$_{BaL}$
226
UNT
70
p38
MFI
HIV$_{BaL}$
292
UNT
226
pJNK
MFI
HIV$_{BaL}$
299
UNT
240
B
pSTAT1
MFI
HIV$_{BaL}$
270
UNT
30
pTBK1
MFI
HIV$_{BaL}$
48
UNT
40
C
pIRF7
MFI
HIV$_{BaL}$
239
HIV$_{BaL}$ +IKKβ Inh
103
UNT
60
p65
MFI
HIV$_{BaL}$
226
HIV$_{BaL}$ +IKKβ Inh
112
UNT
70
D
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239
HIV$_{BaL}$ +p38 Inh
228
UNT
60
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292
HIV$_{BaL}$ +p38 Inh
231
UNT
226

Figure 3.5 Inhibitors block HIV induced phosphorylation of NFκB, MAP kinase, JNK and STAT1

(A and B) Human pDCs were cultured with HIV_{BaL} only (black histogram) or without HIV_{BaL} (light gray histogram) for 15h. Cells were then permeabilized, fixed and stained for phosphorylated NFκB, p38, JNK, STAT1 and TBK1. The solid gray line indicates the peak of the untreated histogram. Human pDCs were cultured with a IKKβ (C) or a p38(D) inhibitor (dark gray histogram) for 1h followed by the addition of HIV_{BaL} (black histogram) or media (light gray histogram) for 15h. Cells were then permeabilized and stained for phosphorylated IRF7, NFκB p65 and p38. The dashed gray line indicates the gate cut off for the positive pIRF7 population and the population shown in both of the p65 and p38 panels, the solid gray line indicates the peak of the untreated histogram.

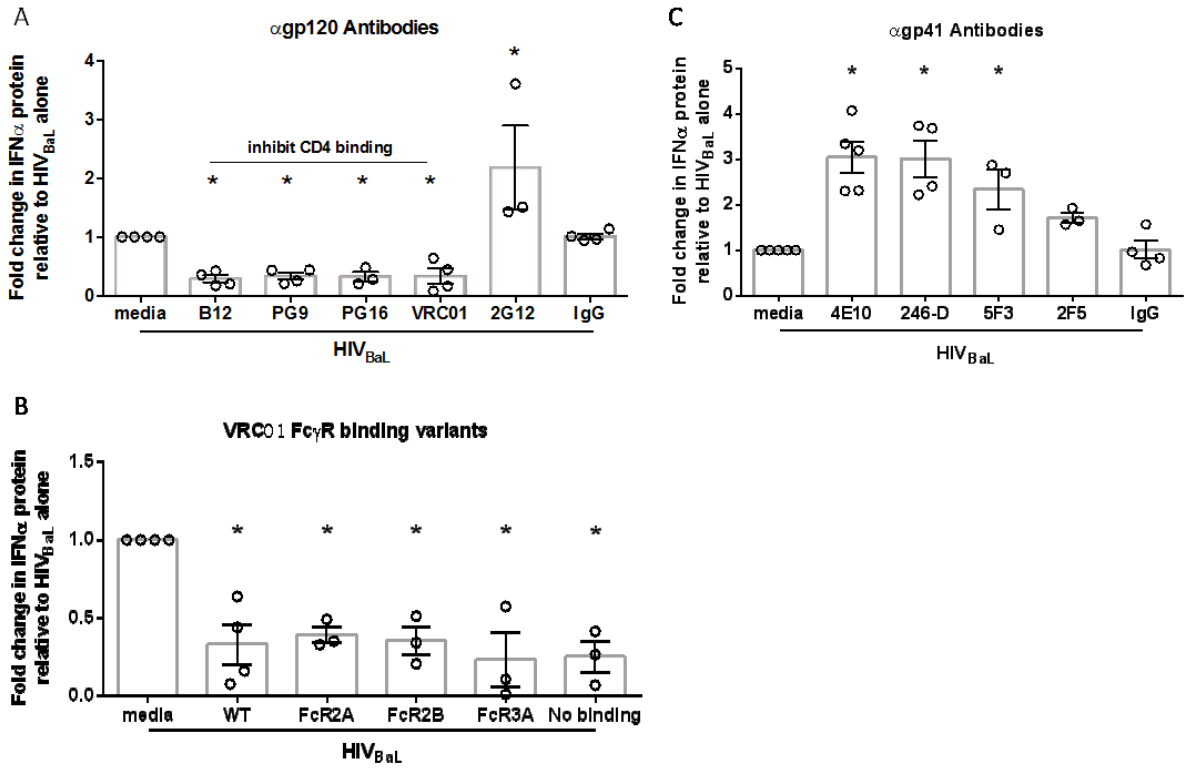

Figure 3.6 HIV antibody specificity regulates Type I Interferon production by pDCs

HIV_{BaL} was cultured with (A) anti-gp120 Abs that interfere with CD4 binding (B12, PG9, PG16 and VRC01) or do not interfere with CD4 binding (2G12) or (B) VRC01 FcγR binding variants or (C) anti-gp41 Abs, for 1-2h and then added pDCs. Supernatants were harvested after 15h and assessed for IFNα protein production. Anti-gp120 Abs that interfere with CD4 binding suppress IFN production independent of binding to FcγR. HIV-specific Abs that do not interfere with CD4 binding enhance IFN production. Each data point indicates the average IFNα production from one donor's pDCs tested in at least duplicate and normalized to the media condition. Error bars and gray box represent the standard error of the mean and mean, respectively. *$p<0.05$.

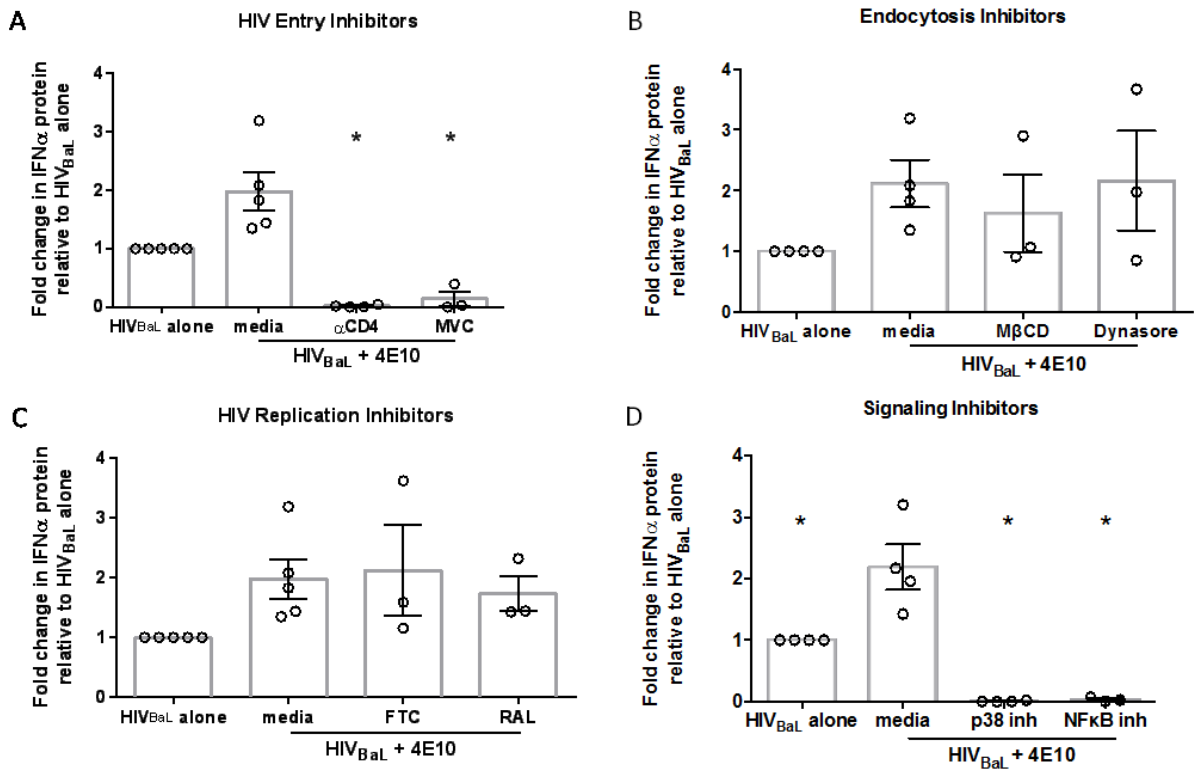

Figure 3.7 IFN enhancement by HIV specific Abs requires gp120 binding and IRF7/NFκB signaling; not endocytosis or infection

Human pDCs were cultured with HIV entry inhibitors (A) or HIV replication inhibitors (B) or signaling inhibitors (C) or endocytosis inhibitors (D) for 1h followed by the addition of HIV$_{BaL}$+/- 4E10 for 15h. Supernatants were harvested and assessed for IFNα protein production. HIV-Specific Ab enhancement of IFN does not require endocytosis or productive infection. However, it does require receptor mediated entry, NFκB and CREB signaling. Each data point indicates the average IFNα production from one donor's pDCs tested in at least duplicate and normalized to the media condition. Error bars and gray box represent the standard error of the mean and mean, respectively. *p<0.01.

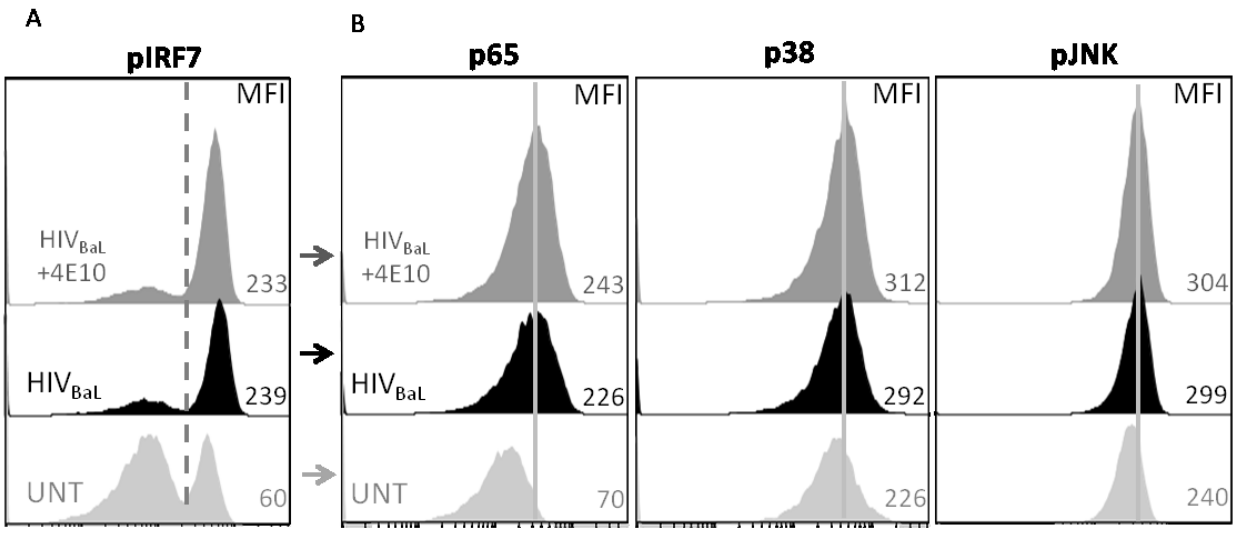

Figure 3.8 The addition of 4E10 does not change the level of phosphorylation of IRF7, NFκB, MAP kinase and JNK

Human pDCs were cultured with HIV$_{BaL}$+4E10 (dark gray histogram) or HIV$_{BaL}$ only (black histogram) or without HIV$_{BaL}$ and 4E10 (light gray histogram) for 15h cells were then permeabilized and stained for phosphorylated IRF7, NFκB p65, p38 or JNK. The dashed gray line indicates the gate cut off for the positive pIRF7 population and the population shown in both of the p65 and p38 panels, the solid gray line indicates the peak of the untreated histogram.

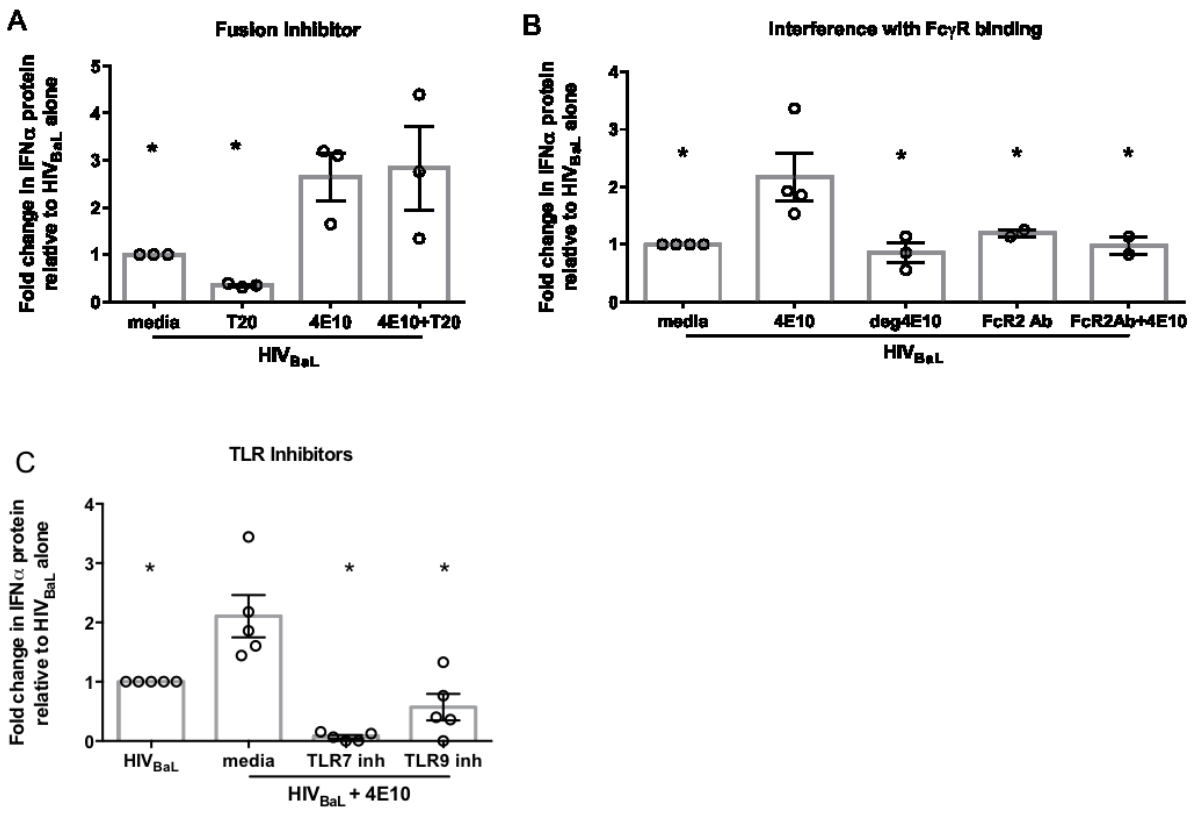

Figure 3.9 HIV-Ab complexes bypass fusion, require FcγR engagement and signal through both TLR7 and 9 to enhance IFNα production

Human pDCs were cultured with an HIV fusion inhibitor, T20 (A) or FcγR2 blocking Ab (B) or TLR inhibitors (C) 1h followed by the addition of HIV_{BaL}+/- 4E10 and +/- deglycosylated 4E10, deg (B only) for 15h. Supernatants were harvested and assessed for IFNα protein production. Each data point indicates the average IFNα production from one donor's pDCs tested in at least duplicate and normalized to the media condition. Each data point indicates the average IFNα production from one donor's pDCs tested in at least duplicate and normalized to the media condition. Error bars and gray box represent the standard error of the mean and mean, respectively. *$p<0.05$.

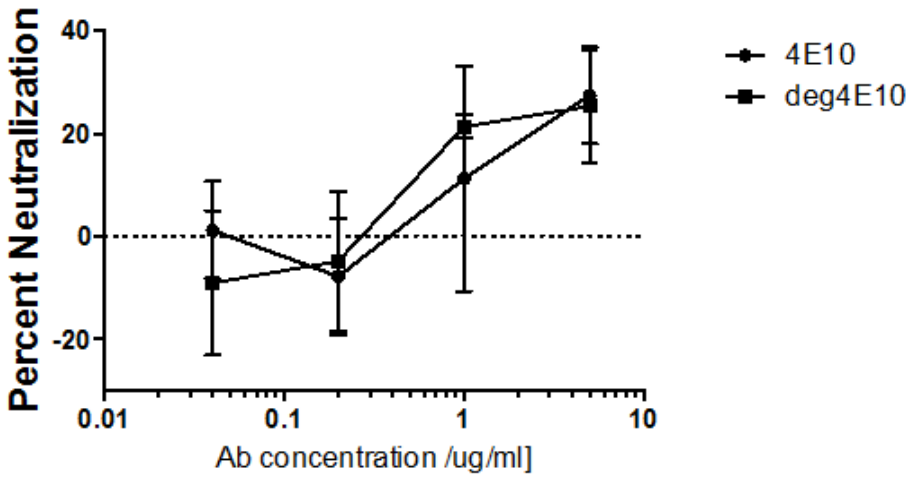

Figure 3.10 Deglycosylated 4E10 maintains the same neutralization capacity as intact 4E10

HIV_{BaL} was cultured with 4E10 and deglycosylated 4E10 for 1-2h following addition to TZM-Bl cells. The cells are incubated for 72hr and then assessed for infection by luciferase. Removing the glycans from 4E10 had no effect on its ability to neutralize.

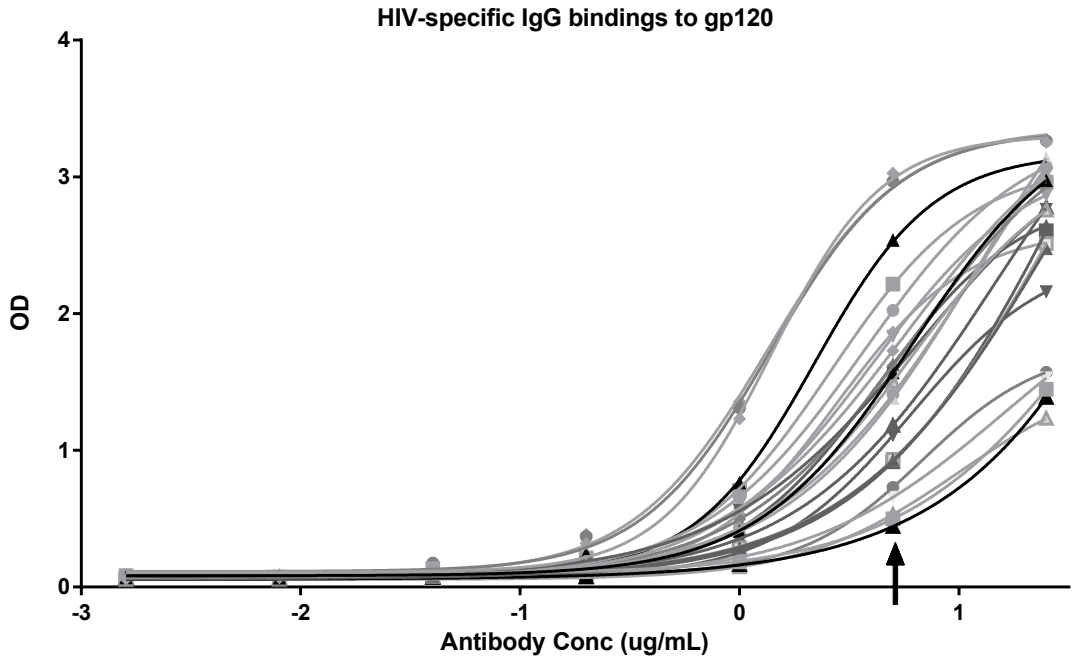

Figure 3.11 IgG isolated from HIV infected subjects bind to gp120 lysates with varying affinities

Polyclonal IgG isolated from 13 HIV infected subjects at two timepoints were assessed for their binding capacity to gp120. Arrow indicates the concentration used in the pDC assay [5ug/ml].

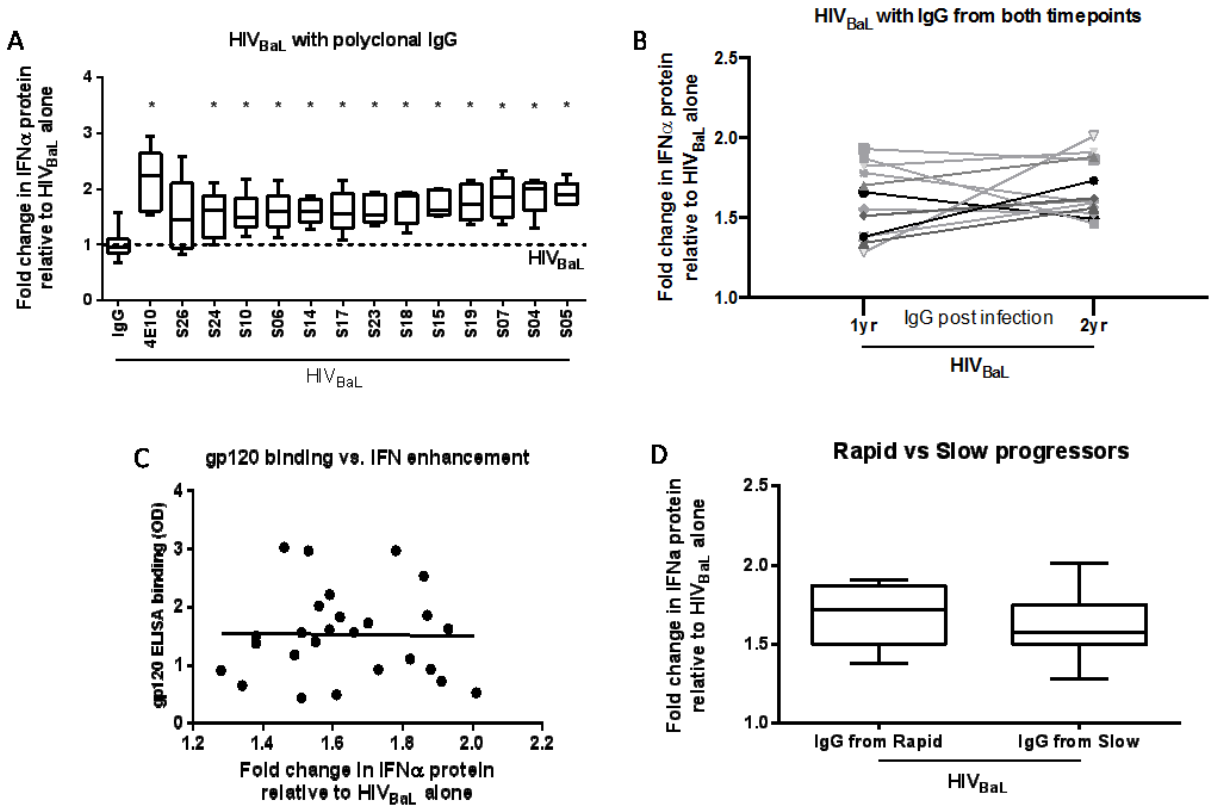

Figure 3.12 IgG isolated from HIV infected subjects enhanced IFN production by pDCs

HIV$_{BaL}$ was cultured with polyclonal IgG isolated from 13 HIV infected subjects (5 rapid progressors=R, 8 slow progressors=S) at two timepoints (1 and 2 years post infection) for 1h and then added to pDCs. Supernatants were harvested after 15h and assessed for IFNα. (A) IgG isolated from 2 years post infection for 13 subjects ordered by increasing IFNα enhancement compared to HIV$_{BaL}$ alone. (B) Comparison of IFNα enhancement by IgG isolated from both 1 and 2 years post infection. (C) Correlation of gp120 binding versus enhancement of IFNα for all IgG samples. (D) IFNα enhancement by all IgG samples stratified by rate of progression. Box plots indicate median, 75 and 25 percent quartiles and 95 and 5 percent outliers. N=5, all samples were normalized to the HIV alone condition indicated by the dashed line (A). *$p<0.05$

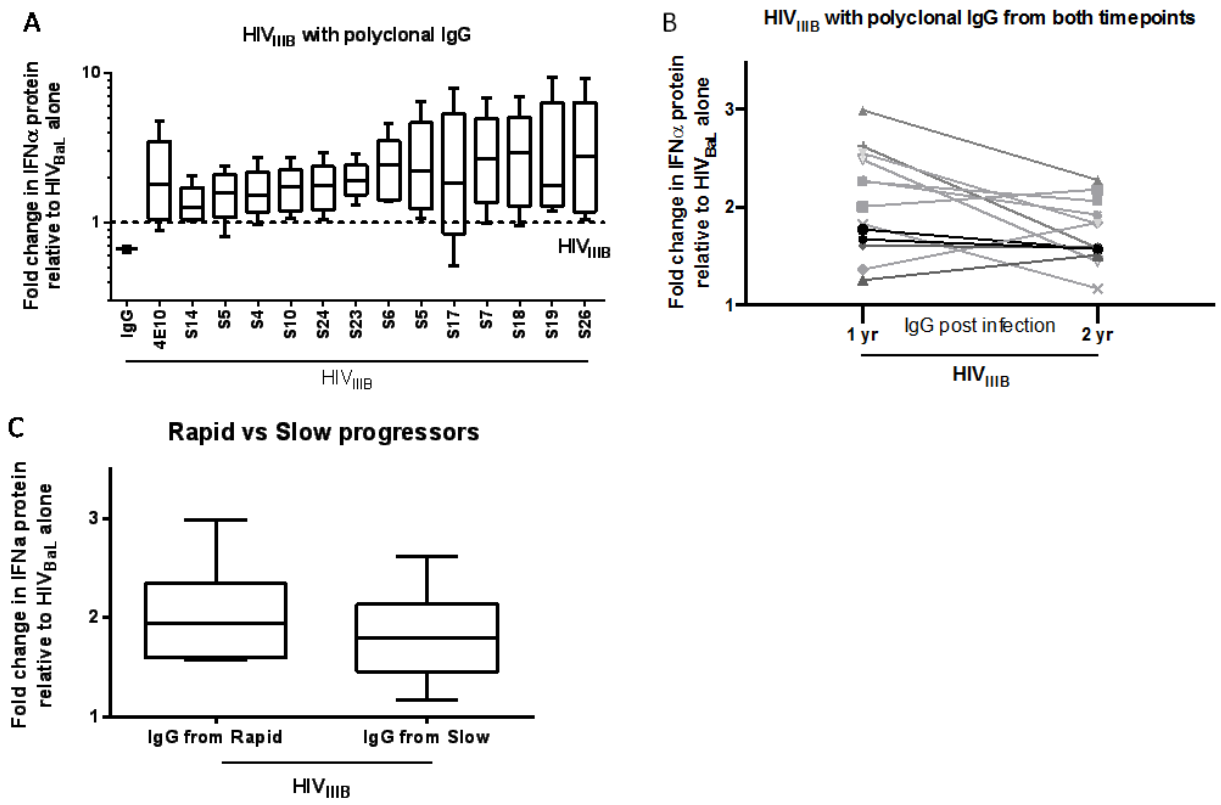

Figure 3.13 IgG isolated from HIV infected subjects enhanced IFN production when pDCs are cultured with HIV$_{IIIB}$

HIV$_{IIIB}$ was cultured with polyclonal IgG isolated from 13 HIV infected subjects (5 rapid progressors, 8 slow progressors) at two timepoints for 1-2h and then added pDCs. Supernatants were harvested after 15h and assessed for IFNα protein production. (A) IgG isolated from 2 years post infection for 13 subjects ordered by increasing IFNα enhancement. (B) Comparison of IFNα enhancement by IgG isolated from both 1 and 2 years post infection. (C) IFNα enhancement by all IgG samples stratified by rate of progression. Box plots indicate median, 75 and 25 percent quartiles and 95 and 5 percent outliers. N=4, all samples were normalized to the HIV alone condition indicated by the dashed line (A).

CHAPTER IV: Discussion

This thesis presents a study of chronic inflammation and avenues of regulation in the chronic viral infections. Chronic viral infections are a significant global health burden and represent a unique challenge to the infected host. Persistently replicating viruses outcompete or subvert the initial antiviral response, allowing the establishment of chronic infections that result in continuous stimulation of both the innate and adaptive immune compartments. This causes a profound reprogramming of the host immune system and creates systemic inflammation. This systemic inflammation leads to enhanced pathogenesis of disease. We sought to understand how persistent elevation of proinflammatory cytokines and their regulation could contribute to disease progression.

In chapter two, we assessed IL-18 levels in HIV/HCV coinfection and monoinfection and identified a potential explanation for enhanced disease pathogenesis in coinfected individuals. We showed that IL-18 was increased in HIV/HCV coinfection when compared with monoinfection and that this increase is most likely due to additive innate sensing of both viruses. The increase in IL-18 could be attributed to several characteristics associated with viral infection. We saw a strong association with both HIV viral load and CD4 counts and IL-18 levels. Viral load set points and CD4 counts that are often determined in part by genetic characteristics, implicating genetics as a possible factor in an IL-18 set points as well. Additionally, we showed that inflammasome activation is an additive process. We found that the presence of virus, shown by acquisition of HIV infection on HCV chronic infection or removal of HIV by HAART suppression, drove IL-18 production. Given the association with increased IL-18 and inflammatory conditions seen more commonly in HIV and HCV infection and

coinfection, this may explain the enhanced disease progression observed in coinfected individuals. We demonstrate that there is a range of IL-18 levels in coinfection, potentially permitting an assessment of the relationship between elevated serum IL-18 and increased incidence and progression of inflammatory conditions in coinfection. If IL-18 is implicated, this will enhance our understanding of coinfection pathogenesis and direct potential therapeutic interventions.

In chapter three, we identified a novel mechanism of IFN regulation in HIV infection. The discovery of a mechanism for persistent IFN production at high levels during chronic viral infections is critical to understanding and treating disease progression. We showed that pDCs produced copious amount of type I IFN in response to activation by HIV. This production of IFN required receptor-mediated entry and engagement of the TLR7, NFκB and IRF7 signaling pathways. We hypothesized that the adaptive immune system must regulate IFN production by pDCs and investigated the role HIV-specific Abs play in IFN production. We show that HIV-specific Abs can both suppress and enhance the IFN response during infection. Suppression of IFN was primarily due to inference with the HIV-pDC interaction and the enhancement of IFN due to Fc Receptor mediated entry and engagement of both TLR7 and 9. This newly identified cross-talk between the innate and adaptive immune systems is not limited to HIV infection. In fact, the pDC-Ab interaction and regulation of IFN has been previously identified in the autoimmunity literature. We would hypothesis that increasing IFN when Abs are present may be a way to down regulate the pDC response because it has been shown that pDCs are self-regulated by the IFN they produce. Perhaps in an infection that can be resolved by the immune system, the final push of IFN shuts down the remaining pDC response, allowing for the adaptive

immune system to clear the infection. Unfortunately, this is not the case in HIV or with other chronic viral infections where the immune system is not capable of removing the antigen, which creates persistent IFN production at high levels.

Overall, this work sheds light on the mechanisms involved in chronic inflammation and its regulation. These inflammatory responses are critical to developing appropriate adaptive immune responses. Therefore, therapeutic intervention will have to be approached very carefully so as to not disrupt beneficial inflammation. However, if specific and targeted therapies can be designed, they could potentially reduce the overall burden of chronic disease.

APPENDIX

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CURRICULUM VITAE FOR Ph.D. CANDIDATES

The Johns Hopkins University School of Medicine

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Educational History

Ph.D. expected	2016	Immunology Johns Hopkins School of Medicine Mentor: Andrea Cox, M.D., Ph.D.
B.S.	2008	Biological Sciences Florida State University

Other Professional Experience

Oak Ridge Institute for Science Education (ORISE) Fellow Aug. 2009 - Aug 2010
Biological Mass Spectrometry Laboratory, Centers for Disease Control and Prevention

Emerging Infectious Disease (EID) Fellow Aug. 2008 - Aug 2009
Biological Mass Spectrometry Laboratory, Centers for Disease Control and Prevention

Summer ORISE Fellow June 2007 - Aug. 2007
NCEH/DLS/OAT, Pesticides Laboratory, Centers for Disease Control and Prevention

Directed Individual Study Student Sept. 2006 - April 2008
Laboratory of Dr. Hengli Tang, Florida State University, Tallahassee, FL

Academic Honors

Magna cum Laude, Phi Beta Kappa Honors Society, Florida State University 2008

Publications

Veenhuis RT, Freeman ZT, Koreleski J, Cohen LK, Tomasi A, Boesch AW, Ackerman ME, Margolick JB, Blankson JN, Chattegoon MA, Cox AL. 2016. : HIV-specific antibodies induce greater immune activation by enhancing type I interferon production from plasmacytoid dendritic cells. *Manuscript in submission.*

Veenhuis RT, Astemborski J, Chattergoon MA, Greenwood P, Jarosinski M, Moore RD, Mehta SH and Cox AL. 2016. Systemic Elevation of Proinflammatory Interleukin 18 in HIV/HCV Coinfection versus HIV or HCV Monoinfection *Manuscript in submission.*

Terilli RR, Cox AL. Immunity and hepatitis C: a review. Curr HIV/AIDS Rep. 2013

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Presentations

Veenhuis RT, Chattegoon MA, Boesch AW, Ackerman ME, and Cox AL. Virus-Specific Antibodies Inhibit Inflammasome Activation in Chronic HCV and HIV Infection, Viral Immunity, Keystone Symosia, 2015, Breckenridge, CO, oral and poster

Veenhuis RT, Chattegoon MA, Boesch AW, Ackerman ME, and Cox AL Virus-Specific Antibodies Inhibit Inflammasome Activation in Chronic HCV and HIV Infection, 21st International Symposium on HCV and Related Viruses, 2014, Banff, AB, Canada, oral

Terilli RR, Moura H, Woolfitt AR, Rees J, Schieltz DM, Barr JR Analysis of Botulinum Neurotoxin G using Endopep-MS and Toxin Proteomics, American Society for Mass Spectrometry, General Meeting 2009, Philadelphia, PA, poster

Terilli RR, Moura H, Woolfitt AR, Rees J, Schieltz DM, Barr JR Analysis of Botulinum Neurotoxin G using Endopep-MS and Toxin Proteomics, American Society for Microbiology, General Meeting 2009, Philadelphia, PA, poster

Terilli RR, Robotham JM, Tang H, Characterization of Hepatitis C Virus Subgenomic Replicon Resistance to Cyclosporine A In Vitro, Third Annual Undergraduate Research Poster Board Competition 2008, Tri Beta Honors Society, Tallahassee FL, poster

Teaching Experience

Spring 2014 & 2015	Graduate Immunology Teaching Assistant,
Aug. 2007 - April 2008	Undergraduate Biology Teaching Assistant,