HUMAN IMMUNODEFICIENCY VIRUS-1 INFECTION AND TYPE 1 INTERFERON RESPONSES

by

Sho Sugawara

A Dissertation submitted to Johns Hopkins University in conformity with the requirements for the degree of Doctor of Philosophy

Baltimore, Maryland

September 2019

© 2019 Sho Sugawara

All rights Reserved
Abstract

HIV-1 infects 37 million people worldwide. Despite the success of antiretroviral therapy (ART), there are still several adverse health outcomes in people living with HIV-1 (PLWH). In addition, most PLWH face lifelong therapy as there is no cure. Type 1 interferons (IFN) are broadly-acting cytokines that promote endogenous antiviral activities. Exogenous type 1 IFN-based therapy can control several viral infections, but its efficacy on HIV-1 is limited. Thus, we hypothesized that antiviral responses to exogenous type 1 IFN are dampened during HIV-1 infection. We confirmed the dampened response to exogenous type 1 IFN in total CD4+ T cells from PLWH with uncontrolled viremia (HIV-UC) compared to healthy controls (HC). Surprisingly, type 1 IFN responses in PLWH with ART (HIV-ART) were not fully restored even after a year of virologic suppression. The dampened type 1 IFN response was associated with upregulation of type 1 IFN regulatory genes, particularly USP18. We confirmed the finding in a separate cohort of HIV-1/HCV co-infected persons who were administered pegylated type 1 IFN (PEG-IFN): baseline expression of USP18 was strongly and inversely associated with the induction of antiviral interferon-stimulated genes (ISGs). Moreover, baseline USP18 levels were inversely associated with the subsequent plasma HIV-1 RNA decline. We also demonstrated that the diminished type 1 IFN response can be transferred to HIV-1 uninfected target cells by soluble mediators, particularly by type 1 IFN from HIV-1 infected cells, and knockdown of USP18 can restore the diminished response. Targeting USP18 may represent an alternative approach to improve type 1 IFN responses in PLWH.
Thesis Advisor:     Ashwin Balagopal, M.D. (Thesis reader)
                    Andrea Cox, M.D., Ph.D.

Thesis Committee:

Janice Clements, M.D. Ph.D.

Robert Siliciano, M.D. Ph.D.

Edward Harhaj, Ph.D. (Thesis reader)
Acknowledgement

First of all, I would like to thank my thesis advisors Dr. Andrea Cox and Dr. Ashwin Balagopal for helping me finishing this challenging project. They have always supported me to troubleshoot any problems I had with the project and given me many insightful ideas to make my projects clinically relevant. I would also like to appreciate the support from my thesis committee members Dr. Janice Clements, Dr. Robert Siliciano, and Dr. Edward Harhaj. Their comments on my projects always helped me to make progress with this project, and it was such a great opportunity for us to hear from both HIV-1 virologists and innate signaling immunologist given that our expertise is in innate signaling in HCV infection. I would also like to thank faculties in Johns Hopkins Viral Hepatitis group, particularly Dr. David Thomas, Dr. Eileen Scully, Dr. Stuart Ray, and Dr. Michael Chattergoon for helping me to learn new techniques and new ideas in order to make progress in my thesis projects. I would also thank Dr. Joel Blankson who gave me some ideas about how to design the experiments with HIV-1 infected persons. In addition, I would like to appreciate Johns Hopkins Graduate Program of Immunology including Dr. Joel Pomerantz, Ms. Angela James and Ms. Lori Fountain who gave me an opportunity to do research in Dr. Cox and Dr. Balagopal’s lab. Moreover, I would like to thank people from my undergrad Soka University, particularly Dr. Sayaka-Yoden Takase, Dr. Hideki Kawai, and Dr. Hirotomo Teranishi who encouraged me to challenge myself to apply for Johns Hopkins Graduate Program of Immunology. I would also like to appreciate Japanese Student Services Organization, who offered me scholarship for 3 years to sustain my life during the program. Finally, I would like to thank my parents who supported me mentally for 5 years in order to get Ph.D.
# Table of Contents

Abstract ................................................................................................................................. ii

Acknowledgement ................................................................................................................ iv

List of Tables .......................................................................................................................... vii

List of Figures ......................................................................................................................... viii

Chapter 1: Background ........................................................................................................... 1

Chapter 2: Upregulated USP18 levels dampen type 1 Interferon responses in HIV-1 infection ........................................................................................................................... 15

1. Abstract .............................................................................................................................. 15

2. Introduction ......................................................................................................................... 15

3. Materials & Methods ......................................................................................................... 17

4. Results ............................................................................................................................... 20

   4.1 Induction of pSTAT1 and ISGs are dampened in T cells from viremic PLWHs
       ....................................................................................................................................... 20

   4.2 Dampening of type 1 IFN responses in CD4+ T cells is associated with
       USP18 upregulation ......................................................................................................... 22

   4.3 The dampened type 1 IFN response phenotype can be transferred to target cells.  ................................................................................................................................. 24
4.4 Type 1 IFN from HIV-1 infected cells triggers USP18 upregulation and downregulation of type 1 IFN response in target cells ........................................25

4.5 USP18 is necessary to dampen type 1 IFN response in target CD4+ T cells .................................................................................................................................................................................................26

4.6 HIV-1 virions contribute to dampened responses to exogenous type 1 IFN ...........................................................................................................................................................................................................................................27

5. Discussion ..................................................................................................................................................................................................................................................................................28

Chapter 3: No Recovery of replication competent HIV-1 from liver Kupffer cells ........................................................................................................................................................................................................................................32

1. Abstract ..................................................................................................................................................................................................................................................................................32

2. Introduction ........................................................................................................................................................................................................................................................................33

3. Materials & Methods ........................................................................................................................................................................................................................................................................33

4. Results ........................................................................................................................................................................................................................................................................37

   3.1 CEMx174 cells are suited for Viral Outgrowth Assay on Liver Macrophages ........................................................................................................................................................................................................................................37

   3.2 Liver Macrophages can harbor HIV-1 but in an inert form ........................................................................................................................................................................................................................................................................37

5. Discussion ........................................................................................................................................................................................................................................................................38

Chapter 4. Figures and Tables ..................................................................................................................................................................................................................................................................................42

Chapter 5: References ..................................................................................................................................................................................................................................................................71

Chapter 6: Curriculum Vitae ........................................................................................................................................................................................................................................................................83
List of Tables

Table 1. Liver macrophage viral outgrowth assay results.

Supplemental Table 1. The size of resting memory CD4+ T cell reservoir measured by quantitative viral outgrowth assay.

Supplemental Table 2. General demographics of participants.
List of Figures

Figure 1. PLWH exhibited reduced pSTAT1 induction by exogenous type 1 IFN.

Figure 2. Diminished expression of ISGs by type 1 IFN in CD4+ T cells from PLWH.

Figure 3. Baseline USP18 level is strongly associated with the response to exogenous type 1 IFN.

Figure 4. Basal USP18 level predicts the ISG induction by Pegylated-IFN based therapy and subsequent plasma HIV-1 RNA decline.

Figure 5. Conditioned media from HIV-1 infected cells can transfer the dampened type 1 IFN responses.

Figure 6. Type 1 IFN from HIV-1 infected cells contributes to the diminished type 1 IFN responses.

Figure 7. USP18 contributes to the diminished type 1 IFN responses.

Figure 8. The diminished type 1 IFN responses are also mediated by HIV-1 virions.

Figure 9. HIV-1 infection can be propagated in CEMx174 cells infected with HIV-1 BaL.

Figure 10. Experimental Procedure for LM separation, and LM VOA.

Figure 11. LMs from HIV-1-infected individuals can transmit HIV-1 infection.

Supplemental Figure 1. Rapid phosphorylation of STAT1.

Supplemental Figure 2. ISG expression in HIV-GFP-infected CD4+ T cells.

Supplemental Figure 3. B18R can neutralize the activity of type 1 IFN.

Supplemental Figure 4. USP18 knockdown in primary CD4+ T cells.

Supplemental Figure 5. Plastic adherence yields better purity of liver macrophages.
Chapter 1: Background

Overview of HIV-1 infection: HIV-1 latent reservoir as a hurdle for cure

HIV-1 infects 37 million people worldwide. Although antiretroviral therapy (ART) has improved the lives of people living with HIV-1 (PLWH) (1), there are still adverse health outcomes that they endure. Moreover, because of the persistence of a long-lived HIV-1 latent reservoir (2), lifelong therapy is required to prevent virologic rebound (2). In addition, ART does not reverse the immune activation in PLWH characterized by many signatures including T cell activation. Immunotherapies have been considered as a strategy to eradicate the latent HIV-1 reservoir. In that context, therapeutically manipulating the endogenous antiviral type 1 interferon (IFN) system may contribute to HIV-1 eradication.

Type 1 IFN-based therapy as an improvement for HIV-1 cure

Type 1 IFNs are potent cytokines that are transcriptionally induced, synthesized and released upon sensing of foreign (often viral) stimuli. In response to type 1 IFN, cells produce hundreds of interferon-stimulated genes (ISGs) that have diverse antiviral actions. HIV-1 replication can be inhibited by exogenously administered type 1 IFN: several lines of evidence have shown that HIV-1 adaptation is partly directed by ISGs (3, 4). However, many off-target effects and toxicity have been observed by exogenous type 1 IFN, which limit its use in humans. In addition, several investigators have suggested that the type 1 IFN system may be involved in the chronic immune activation that continues to result in morbidity in PLWH on ART. The type 1 IFN system can be
divided into: a) the sensing of pathogen-associated molecular patterns (PAMPs) and b) antiviral responses (Fig. 2). Classically, cell surface and intracellular pattern recognition receptors (PRRs) (5, 6) sense PAMPs, which triggers a signaling cascade that results in the phosphorylation and activation of IRF3 and NF-κB transcription factors.

Transcription of cytokines, including type 1 IFN, is driven by these transcription factors (5, 6). PRRs that have been shown to sense HIV-1 are TLR7, cGAS, and IFI16. It was reported that upon cellular entry, TLR7 and TLR9 sense HIV-1 in plasmacytoid dendritic cells (pDCs), activating IRF5 and IRF7 to produce type 1 IFN (7). In human monocyte-derived macrophages (hMDMs) and THP-1 cells, IFI16 appears to sense single-stranded HIV-1 DNA that forms a stem loop structure, triggering the STING complex to activate IRF3, thereby secreting type 1 IFN (8). Separately, another report of HIV-1 infection in THP-1 cells has demonstrated cGAS sensing of products of HIV-1 reverse transcription, also involving STING and IRF3, which then trigger type 1 IFN production (9).

Despite the in vitro findings, whether HIV-1 triggers type 1 IFN secretion in infected primary cells is still controversial: three separate groups (10-14) demonstrated in cell lines that IRF3 is degraded by the HIV-1 accessory proteins Vif, Vpu, and Vpr, while Nasr et al. reported that nuclear translocation of IRF3 is prevented by HIV-1 infection in macrophages (15). In contrast, Hotter et al. have shown that in PBMCs infected with a GFP-tagged HIV-1 virus, HIV-1 infection does not degrade IRF3 (16). Rather, NF-κB activation is inhibited by Vpu (16). Similar results were obtained by Manganaro et al. using the cells that HIV-1 primarily infects (primary CD4+ T cells and macrophages) (17). The report from Harman et al. suggests that IRF3 is not largely affected by HIV-1
infection, but that TBK1 phosphorylation was blocked by Vif and Vpr (18). In a comprehensive investigation into HIV-1 signaling, Rasaiyaah et al. found adaptation in the HIV-1 capsid that allows infection of macrophages without activation of type 1 IFN responses (19). HIV-1 replication may thus be enhanced by evasion of innate sensing: Tsang et al., reported that the degree of evasion from sensing of HIV-1 nucleic acids by macrophages dictates the infectivity of HIV-1 in macrophages (20).

Whether or not HIV-1 infection triggers type 1 IFN signaling in cells, it is quite clear that type 1 IFN is abundantly released in PLWH, and that it contributes to the immune activation state (21-24). Although type 1 IFN can potentially be produced by various types of cells during HIV-1 chronic infection (25-27), specialized IFN producing cells such as plasmacytoid dendritic cells (pDCs) are likely to produce type 1 IFN in these individuals. Consistent with this idea, circulating type 1 IFN in SIV infected macaques was found to be completely abrogated by in vitro depletion of pDCs (28).

The conflicting data on HIV-1 and type 1 IFN signaling have partly been reconciled by recent data showing that pDCs are potently stimulated by HIV-1 infected lymphocytes, and not cell-free virus, to produce type 1 IFNs in a TLR7-dependent manner (29, 30). Herein, we conjecture that during the acute stages of infection, HIV-1-mediated suppression of type 1 IFN signaling may be crucial to establish HIV-1 persistence, but in the context of chronic infection it may be less important because there is abundant secretion of type 1 IFN by cells that include pDCs, which are not susceptible to HIV-1 infection.

After production of type 1 IFN, the cytokines signal in an autocrine or paracrine mode through the interferon alpha/beta receptor (IFNAR1/2) complex. Upon ligation of
type 1 IFN with IFNAR1/2, STAT1 and STAT2 proteins are phosphorylated by receptor-associated Janus kinases (JAKs), which triggers their dimerization and activation. The activated STAT1/2 dimer translocates to the nucleus and binds to IRF9 to form the ISGF3 transcription factor complex. ISGF3 binds to hundreds of interferon-sensitive response elements (ISREs) in the genome, initiating transcription of a distinct set of genes that are defined collectively as interferon-stimulated genes (ISGs) (5, 6).

Many of the upregulated ISGs exhibit antiviral activity that have been well characterized (5). Importantly, several ISGs, including MX2, ISG15, APOBEC3G, and BST2, have been shown to restrict HIV-1 replication. MX2 is known to inhibit nuclear translocation of HIV-1 cDNA following reverse transcription, which prevents proviral DNA integration (31). ISG15 inhibits the ubiquitination of HIV-1 Gag, thereby impeding virion release (32). During reverse transcription, APOBEC3G induces hypermutation within HIV-1 proviral DNA, which results in frequent nonsense mutations thus yielding replication incompetent provirus (33). Also, it has been reported that APOBEC3G inhibits HIV-1 reverse transcription (34). BST2 (also known as tetherin) blocks the budding of new virions (35). Upon the inhibition of virion budding, NF-κB signaling is also activated by BST2 (36).

The importance of type 1 IFN-induced HIV-1 restriction factors is underscored by examining HIV-1 adaptation. For example, HIV-1 isolates taken from PLWH demonstrate successive capsid mutations that are progressively resistant to MX2 restriction (3). Similarly, the HIV-1 Vif protein ubiquititates and degrades APOBEC3G (37), while BST2 is sequestered by Vpu to prevent its binding to new virions (38).
Ongoing type 1 IFN responses, therefore, have partly driven HIV-1 evolution, thereby highlighting their importance.

Research on the inhibition of the type 1 IFN system (i.e., antiviral responses) by HIV-1 is relatively sparse: Ranganath et al. demonstrated that U937 and HK60 myeloid cell lines that harbor integrated HIV-1 proviruses exhibit less ISG induction by type 1 IFN than cell lines without HIV-1 integration, although these findings were not confirmed in primary cells (39).

While type 1 IFN can restrict HIV-1 replication through the upregulation of ISGs in susceptible or infected cells, type 1 IFN is also known to modulate adaptive immune responses, which may control HIV-1 indirectly. For instance, CD8+ T cells exhibit greater expansion, proliferation, and killing of target cells in the presence of type 1 IFN (40, 41). Although there have not been extensive studies with HIV-1, the experience with other chronic viral infections may be a roadmap for the diverse effects of type 1 IFN on adaptive responses (42). In LCMV infection, a well-established model system of chronic viral infection, type 1 IFN was shown to be necessary for early LCMV-specific CD8+ T cell responses (43). Moreover, mice that were administered type 1 IFN formed germinal centers and produced greater amounts of antibodies, while IFNAR knockout reversed this phenotype (40, 44). These results suggest that type 1 IFN also modulates B cell responses. Conversely, type 1 IFN has been also shown to limit host adaptive responses, as LCMV-specific B cell responses were inhibited by type 1 IFN (43). Indeed, several investigators have reported that viral clearance was improved by IFNAR blockade in a model of persistent LCMV infection (45, 46), and later investigations have demonstrated that improved anti-LCMV T cell responses seem to contribute to this
phenomenon (47). It is possible, therefore, that type 1 IFN promotes anti-viral adaptive immune responses early during infection, but may inhibit adaptive responses during chronic viral infection. Nonetheless, further studies are needed to examine the role of type 1 IFN on adaptive responses in HIV-1 infection.

**HIV-1 infection and type 1 IFN responses *in vivo***

Data from animal models and HIV-1 infected subjects have refined our understanding how endogenous type 1 IFN responses are affected by SIV or HIV-1. Notably, the kinetics of ISG expression is different between sooty mangabeys (SMs) and African Green Monkeys (AGMs), natural hosts of SIV that don’t experience pathogenic infection nor develop AIDS, and Rhesus macaques (Rhs), in which pathogenic SIV infection occurs and AIDS develops (48). Whereas all macaques induce MxA (the simian ortholog of human Mx1) expression 7 days after infection, high MxA expression is maintained for up to 29 days after infection only in Rhesus macaques, while MxA expression returned to baseline in SMs and AGMs (48). It has also been confirmed in follow-up studies in non-human primates that during acute SIV infection, AGMs, Rhs, and SMs strongly upregulate ISGs (49); however, ISG expression is attenuated to levels before infection only in AGMs. This could be due to altered sensing in several cell types (28), which does not seem to include pDCs (50). Because AIDS develops in SIV-infected Rhs, but not in AGMs, the authors concluded that abnormally persistent ISG upregulation could lead to pathogenic SIV infection. In fact, Sandler et al. demonstrated that CD4+ T cells and the CD4+/CD8+ T cell ratio significantly decreased in macaques administered type 1 IFN prior to infection (51). In a separate study,
activation of CD4+ and CD8+ T cells is inhibited by type 1 IFN blockade in SIV-infected rhesus macaques (52), further supporting that pathologic infection and AIDS may be partly associated with type 1 IFN responses.

Persistent upregulation of type 1 IFN responses after the acute HIV-1 infection has been confirmed by data from humans. Hardy et al. reported that plasma type 1 IFN levels are correlated with plasma HIV-1 RNA levels (21). Catalfamo et al. showed that higher levels of phosphorylated STAT1 induced by type 1 IFN stimulation are observed in CD4+ T cells from PLWH with uncontrolled viremia than cells from PLWH with suppressed infection (22). A well-characterized marker of HIV-1 related immune activation is CD4+ T cell activation, defined by upregulated expression of a variety of surface markers including CD38, CD25, and HLA-DR. Activated CD4+ T cells in HIV-1-infected individuals express higher ISGs compared to cells in HIV-1 uninfected persons (23). Our group recently reported ART at least partially limits ISG elevation in activated CD4+ T cells in chronic HIV-1 infection (24).

Continued PRR signaling by HIV-1 is likely to contribute to constitutive type 1 IFN responses. Hardy et al. also investigated type 1 IFN signaling in PLWH and reported reduced surface IFNAR expression on monocytes and some classes of dendritic cells, but not on CD4+ or CD8+ T cells (53). Although elevated type 1 IFN signaling is broadly observed in HIV-1 infection, this signaling is possibly dysregulated, and not properly targeted: for example, Kamga et al. found that HSV-1 (herpes simplex virus type 1) in vitro infection of PBMCs from HIV-1-infected individuals produce less type 1 IFN compared with cells from healthy donors (54), and this could be due to the decline in pDC numbers in HIV-1 infection (54).
The resistance of HIV-1 to type 1 IFN may indicate the differences in the antiviral efficacy of type 1 IFN during acute vs. chronic infection. Several studies have revealed that type 1 IFN resistant viruses are likely to be transmitted to new hosts (55-58). In contrast, more type 1 IFN-sensitive HIV-1 viruses have been observed later during infection, indicating perhaps that other selective forces drive HIV-1 adaptation during chronic infection. In contrast, however, Deymier et al., demonstrated that between epidemiologically linked couples, type 1 IFN sensitivity does not change between non-transmitted and transmitted virions during heterosexual transmission (59). These conflicting data, nevertheless, offer intriguing insights on the role of type 1 IFN during different phases of HIV-1 infection.

**Type 1 IFN-based treatment and SIV/HIV-1 infection in vivo**

Therapeutic exogenous type 1 IFN has been investigated in SIV infection. Veazey et al. demonstrated that vaginal transmission of SHIV was prevented by IFN-beta treatment prior to SHIV infection in Rhs (60). Importantly, SIV disease progression and mortality was enhanced by type 1 IFN antagonism by a reversible IFNAR inhibitor before or during acute infection (51). Vanderfold et al. developed a recombinant type 1 IFN agonist that transiently induces ISGs and reduced by approximately ten-fold the plasma SIV RNA level in treated sooty mangabeys (61). These findings support the canonical anti-SIV activity of type 1 IFN in the host (62).

Humanized mice have become a powerful tool for understanding HIV-1 infection *in vivo*. A humanized mouse model revealed that elevated plasma type 1 IFN is mainly derived from plasmacytoid dendritic cells (pDCs) during acute HIV-1 infection (63). In addition, the investigators found that the loss of type 1 IFN production by pDC depletion
enhanced HIV-1 replication (63). Interestingly, IFN-alpha 14 administration exhibited more potent HIV-1 suppression in HIV-1-infected humanized mice than other IFN-alpha subtypes (64).

The antiviral effects of type 1 IFN on HIV-1 were recently demonstrated by two separate groups: persistent type 1 IFN signaling was detected in a model of chronic HIV-1 infection in humanized mice in which CD8+ T cell exhaustion was also observed (65, 66). After infection, the mice were treated with ART until they were virologically controlled and aviremic. Both teams demonstrated improved T cell activation and exhaustion with the administration of a blocking antibody against the IFNAR complex in the mice. More impressively, IFNAR blockade reduced plasma HIV-1 RNA levels in humanized mice. In contrast, in chronic HIV-1 infection of humanized mice without ART, plasma HIV-1 RNA levels increased by IFNAR blockade, although paradoxically the plasma IFN levels were also increased (67). Taken together, these studies indicate that persistent type 1 IFN signaling may contribute to suppressed immune responses in humanized mice with chronic HIV-1 infection. Furthermore, Long et al. reported that humanized mice treated with recombinant IFN alpha-2b had higher activated CD4+ and CD8+ T cell proportions, suggesting that the T cell activation phenotype may be exacerbated by type 1 IFN signaling (68). Notably, T cell activation has been strongly associated with disease progression in PLWH (68). Thus, the data from animal models are interesting but also conflicting. Type 1 IFN antagonism during chronic HIV-1 or SIV infection, after virologic suppression with ART, might reveal similar phenomena in human or macaque studies.

Partial, albeit transient, control of viral replication has been exhibited by PLWH administered with type 1 IFN in numerous clinical trials during the HIV-1 epidemic (69-
Correspondingly, increased T cell activation was also observed by type 1 IFN treatment in many of those trials. It should be noted that subjects who were co-infected with HCV participated in several of these trials. Importantly, the decline in plasma HIV-1 RNA levels was closely associated with the induction of known HIV-1 restrictive ISGs, which strongly suggests that the control of HIV-1 replication is dependent on the induction of intracellular antiviral molecules by exogenously administered type 1 IFN (74). However, it should be noted that the exogenous type 1 IFN administration only resulted in a ten-fold decline in plasma HIV-1 RNA levels (74), although the participants received weeks of administration. Therefore, type 1 IFN treatment exhibits only modest effects in controlling HIV-1 replication in vivo.

The effect of type 1 IFN on the SIV/HIV-1 latent reservoir

Two groups administered IFNAR blockade in HIV-1-infected but ART-suppressed humanized mice and demonstrated intriguing and consistent results (65, 66): both groups reported that the size of the latent HIV-1 reservoir significantly decreased with IFNAR blockade, strongly indicating that maintenance of the size of the latent reservoir is at least partly controlled by type 1 IFN (65, 66). Although these groups did not explore potential mechanisms explaining their findings, it is tempting to hypothesize that the size of the latent reservoir can be manipulated by modulating type 1 IFN signaling. In that light, a trial of type 1 IFN administration in SIV-infected macaques with virologic control by ART failed to demonstrate a difference in the size of the latent reservoir as determined by quantitative viral outgrowth, although a small decline in cell-associated SIV DNA in CD4+ T cells from PBMCs was detected (76).
In humans, several clinical trials have attempted to test whether the size of the HIV-1 reservoir can be diminished by exogenous type 1 IFN treatment: in one trial, a small but significant reduction in integrated HIV-1 DNA in total CD4+ T cells was observed (70). However, an effect on the latent HIV-1 reservoir of replication competent proviruses in resting CD4+ T cells was not investigated in the study. Moron-Lopez et al. showed that short-term type 1 IFN administration did not affect the size of the HIV-1 reservoir in HIV-1/HCV co-infected patients quantified by cell-associated HIV-1 RNA and infectious virions in the supernatant (77). More recently, 20-68 weeks of PEG-IFN treatment in ART-treated and untreated HIV-1/HCV co-infected persons did not show appreciable changes in HIV-1 DNA levels in any of the participants (78). In contrast, significant declines in cell-associated and cell-free HIV-1 DNA were observed in a separate study of ART-suppressed HIV-1/HCV co-infected persons with PEG-IFN treatment; interestingly, a change in NK cell numbers was associated with the decline in HIV-1 measures (79).

Regardless of the significant change in the size of the latent reservoir, hypermutation induced by APOBEC3G provides definitive evidence of the impact of type 1 IFN on the HIV-1 latent reservoir. In a series of papers, major defects have been observed in the majority of HIV-1 proviruses resulting from APOBEC3G-regulated hypermutation. The proviruses became replication incompetent by these defects (4, 80). However, as reviewed earlier, it is likely that APOBEC3G induced hypermutations in proviruses accumulated during active HIV-1 replication, rather than during latency. As HIV-1 cure strategies incorporate immunomodulatory treatments, it will be crucial to determine their effects on the replication competent latent reservoir.
**Resting CD4+ T cells: a major latent reservoir for HIV-1**

Viral latency is defined as a reversible nonproductive state of infection in individual cells (81). Reservoirs are cells that harbor replicative forms of HIV-1 following long periods of ART-suppressed viremia (82, 83). Many cells are targets for HIV-1 infection *in vitro*, but resting memory CD4+ T cells have been the best characterized HIV-1 reservoir (83-85). There may be other long-lived HIV-1 reservoirs, but they have not been examined *in vivo* with the same degree of rigor. The estimated half-life of resting memory CD4+ T cell reservoirs is 44 months, which translates to a duration as long as 73 years to clear the CD4+ T cell reservoir with ART alone (85-87).

**Macrophages: potential candidates as an HIV-1 reservoir.**

Macrophages are mononuclear leukocytes that mostly reside in tissues and play an important role in innate immunity. Historically, the origin of these tissue resident macrophages (TRMs) was ascribed to continual replenishment by the mononuclear-phagocyte system: monocytes produced by the bone marrow were thought to repopulate TRMs after their short life in tissues (88, 89). However, a recent study in murine models indicate that hematopoietic precursors differentiate into TRMs during embryonic development, and that these embryonically-derived TRMs are long-lived. Monocytes only contribute to TRM replenishment during inflammation and injury (90). Indeed, donor alveolar macrophages were found in patients months after they had undergone lung transplantation (91). Thus, TRMs are long-lived cells in tissues (92). In parallel, macrophages do not seem to contribute to the rapid 2nd phase decline of HIV-1 that is observed early during ART (93). In summary, our understanding of the maintenance and longevity of TRMs could be revised by these findings.
It is well established that lab strains of HIV-1 can productively infect macrophages in animal models and *in vitro* (94, 95), although the susceptibility of TRMs can depend on the tissue. For example, many investigations have demonstrated HIV-1 and SIV infection in brain macrophages such as microglia (96, 97). HIV-1 replicates better in vaginal macrophages than intestinal macrophages, which may be attributed to expression levels of entry co-receptors (98). Higher HIV-1 susceptibility of rectal macrophages compared to colonic macrophages has also been demonstrated by comparative *in situ* fluorescence (99).

Several reports in the pre-ART era demonstrated that HIV-1 can infect TRMs in PLWH (84, 100-103). More recently HIV-1 nucleic acids (both proviral DNA and RNA) were detected in alveolar macrophages from individuals on ART (104). Our lab has demonstrated that liver macrophages (Kupffer cells), the largest population of TRMs in the body, can harbor infectious virus from PLWH on ART for as long as 11 years (105). Other tissue macrophages could also harbor HIV-1 including those in the seminal vesicle, duodenum, urethra, adipose tissue, and liver (100, 105-109).

Despite these studies, demonstrating HIV-1 infection in macrophages is still challenging because macrophages phagocytose cells, including CD4+ T cells. Recently, both proviral DNA and T cell receptors (TCR) were detected in myeloid cells *in vivo* during SIV infection: the authors concluded that phagocytosis of infected dying cells rather than *de novo* infection led to the presence of viral DNA in macrophages (110). On the other hand, a subsequent report by Baxter *et al.* showed that HIV-1-infected CD4+ T cells could be selectively captured by primary monocyte-derived macrophages, leading to efficient cell-to-cell macrophage infection (111). Indeed, we
and others have confirmed that T cells and TCRs are successfully excluded in *ex vivo* studies of TRM reservoirs (105, 112).

**Thesis Overview**

In my thesis, I will describe how HIV-1 diminishes type 1 IFN responses in cells from PLWH in Chapter 2. I will also elucidate a mechanism by which HIV-1 modulates the type 1 IFN response without productive infection. In Chapter 3, I will address whether HIV-1 found in liver macrophages (Kupffer cells) can contribute to virological rebound after interruption of ART.
Chapter 2: HIV-1 infection diminishes type 1 IFN responses by upregulating USP18

1. Abstract

Despite the success in antiretroviral therapy (ART), there are still several adverse health outcomes in people living with HIV-1 (PLWH). Type 1 IFN-based therapy has demonstrated a substantial control of several viral infections, but its efficacy on PLWH is limited. Thus, we hypothesized that HIV-1 infection dampens the response to exogenous type 1 IFN. We confirmed the diminished response to exogenous type 1 IFN in total CD4+ T cells from PLWH with uncontrolled viremia (HIV-UC) compared to healthy controls (HC). Surprisingly, PLWH with ART (HIV-ART) do not fully restore responses to exogenous type 1 IFN. The dampened type 1 IFN response is associated with the upregulation of type 1 IFN regulatory genes, particularly USP18, which we confirmed in a cohort of HIV/HCV co-infected persons who were administered pegylated type 1 IFN (PEG-IFN). We demonstrated that the diminished type 1 IFN response can be transferred to HIV-1 uninfected target cells by soluble mediators, particularly by type 1 IFN from HIV-1 infected cells, and knockdown of USP18 can restore responses to type 1 IFN. Therefore, targeting USP18 might be an alternative approach to improve HIV-1 control in PLWH.

2. Introduction

Antiretroviral therapy (ART) has enabled people living with HIV-1 (PLWH) to have a longer and better quality of life (1), although an HIV-1 cure is still not widely
available (2). In addition, PLWH still have worse outcomes than people without HIV-1. For example, PLWH who are co-infected with hepatitis C virus (HCV) or hepatitis B virus (HBV) historically have worse virological responses, especially to exogenous type 1 interferon (IFN) (113-116). It is notable that although type 1 IFN treatment has been considered for HIV-1, PLWH who are treated with exogenous type 1 IFN only achieve a ten-fold decrease in plasma HIV-1 RNA levels (74). Taken together, these results suggest that HIV-1 infection may modulate type 1 IFN responses, resulting in worsened antiviral control.

Type 1 IFN signaling can be conceptually divided into a sensing arm and an effector arm. In the sensing arm, viral nucleic acids are sensed by several pattern recognition receptors (PRRs) including Toll-like receptors (TLRs), RIG-I, and cGAS, which trigger the activation of transcription factors such as NF-κB and IRF3. The activation of transcription factors results in the upregulation of proinflammatory cytokines, and type 1 IFN that functions in an autocrine or paracrine fashion. The effector arm of signaling is initiated by ligation of type 1 IFN with IFN alpha receptor (IFNAR) in recipient cells, activating JAK-STAT signaling. STAT1 and STAT2 are phosphorylated and then hetero-dimerize. The STAT1/2 heterodimer then translocates to the nucleus and activates the expression of interferon-stimulated genes (ISGs). Many ISGs are known to have antiviral functions (5, 6, 117). The effector arm of type 1 IFN signaling is counter-regulated by several type 1 IFN regulatory factors, including SOCS proteins and USP18 (118). It has been demonstrated that HIV-1 infection can regulate the sensing arm of type 1 IFN signaling in several cell types (10-14). However, PLWH experience elevated type 1 IFN levels, implying that the sensing arm of type 1 IFN
signaling is intact in some cell types in PLWH (23). Therefore, we hypothesized that HIV-1 infection modulates the effector arm of type 1 IFN signaling in PLWH, dampening responses to promote viral replication.

3. Materials & Methods

*Patient samples.* Peripheral blood mononuclear cells (PBMCs) from 9 PLWH without antiretrovirals (HIV-UC) and 7 PLWH without antiretrovirals (HIV-ART) were obtained from cohorts from Johns Hopkins AIDS Linked IntraVenous Experience Study (ALIVE) and Johns Hopkins Clinical Cohort. In addition, PBMCs from 9 people who inject drugs (PWIDs) from Johns Hopkins Baltimore Before and After Study of Hepatitis (BBAASH) cohorts, 1 healthy donor, and 2 leukopaks from the Anne Arundel Medical Blood Donor Center (Anne Arundel, Maryland) were tested as a negative control. PWIDs were included to control for exposures from injection drug use.

*Quantification of pSTAT1.* PBMCs were thawed and rested overnight in RPMI (ThermoFisher) with 10% Fetal Bovine Serum (Sigma-Aldrich) (R10). PBMCs from HIV-ART cohorts were rested overnight with 10 μM tenofovir disoproxil fumarate (TDF) (Selleckchem) and 1 μM emtricitabtin (FTC) (Selleckchem). PBMCs were then stimulated with 300 U/ml of universal type 1 IFN (PBL-Assay Science). Five minutes after stimulation, PBMCs were stained with CD3-AlexaFluor700 (eBioscience), CD8-APC, HLA-DR-FITC, pSTAT1-BV421 (BD Bioscience), and CD38-BV711 (Biolegend) along with live-dead staining dye eFluor780 (eBioscience). The level of pSTAT1 in CD3+CD8- cells, CD3+CD8-HLADR+CD38+ cells, CD3+CD8-HLADR-CD38- cells, and
CD3+CD8+ cells, with and without type 1 IFN stimulation, was measured by flow cytometry.

**qPCR for ISGs and type 1 IFN regulatory factors.** PBMCs were thawed and rested overnight. Total CD4+ T cells were isolated by using human CD4+ T cell isolation kits (Miltenyi Biotec, catalog 130-096-533) and stimulated with 10 U/ml of universal type 1 IFN. Six hours after, cells were collected and total cellular RNA was extracted using Zymo DNA/RNA Duet kits (Zymo Research). Total cellular RNA from CD4+ T cells from HC1, 2, HIV-UC1, 2, and HIV-ART1, and 2 was extracted using RNeasy plus mini kits (Qiagen). Total RNA was then reverse transcribed into cDNA using Multiscribe reverse transcriptase kits (ThermoFisher). The levels of MX2, ISG15, PKR, BST2, SOCS1, SOCS3, and USP18 cDNA were quantified by quantitative PCR (qPCR). The expression levels were normalized to 3 housekeeping genes (YWHAZ, SDHA, and RPLP0) and induction of ISGs were calculated by using delta-delta Ct values normalized to unstimulated CD4+ T cells. In addition, the relative expression unit of each gene was calculated. Primers, probes and qPCR mastermix were obtained from Integrated DNA Technologies.

**Collection of supernatant from in vitro HIV-1 infected leukopaks.** Seven healthy leukopaks were thawed, rested for 5 hours and then spinoculated with 1 ng p24 of HIV-1 BaL per 1 million cells from a leukopak at 1,200 g for 2 hours. BaL-infected or -uninfected leukopaks were then cultured in R10. Three days after infection, supernatant from BaL-uninfected or -infected leukopaks were collected and passed through a 0.2 µm filter.
Cytokine screening for USP18 upregulation. Total CD4+ T cells were isolated from healthy leukopaks and stimulated with 20,000 U/ml universal type 1 IFN, 2.5 μg/ml recombinant IL-12/23p40, 2.5 μg/ml IL-15, 2.5 μg/ml IFNγ, 2.5 μg/ml MCP-1, 2.5 μg/ml IP-10 (Peprotech), and 2.5 μg/ml IL-18 (R&D Systems). Six hours after stimulation, cells were harvested and lysed. cDNA was reverse transcribed and the level of USP18 was quantified by qPCR.

Supernatant experiment. Uninfected target CD4+ T cells were isolated from healthy leukopaks using human CD4+ T cell isolation kits (Miltenyi Biotec), and cultured with 10 μM tenofovir disoproxil fumarate (TDF) and 1 μM emtricitabine (FTC) (Selleckchem) in addition to 1 μM T20 (ThermoFisher), or 1.5 nM Cyclophilin A inhibitor (CypA, Millipore) (7). After 24 hours, target CD4+ T cells were incubated with supernatant from BaL-uninfected or -infected leukopaks with the addition of TDF and FTC, T20, or CypA to inhibit various stages of HIV-1 replication. After 24 hours of incubation, target CD4+ T cells were then stimulated with 10 U/ml of universal type 1 IFN and 6 hours later, cells were harvested. Total RNA was extracted using Zymo DNA/RNA Duet kits (Zymo Research). Total RNA was reverse transcribed and qPCR was performed for MX2, ISG15, IFIT3, USP18, and SOCS1.

For neutralization of cytokines, supernatants from BaL-infected or -uninfected leukopaks were pre-incubated with recombinant B18R (eBioscience) or recombinant IFNγ receptor (Peprotech) for 1 hour prior to incubation with target cells.

USP18 knockdown. Healthy leukopaks were thawed and rested overnight. After resting, total CD4+ T cells were separated by magnetic separation and nucleofected with 5 μM
Accel Green non-targeting control RNA or 5 µM SMARTpool human USP18 siRNA (Dharmacon) using the human T cell nucleofector kit (Lonza) and an Amaxa nucleofector (Lonza, Program U-014). Nucleofected cells were resuspended in T cell OpTmizer™ (ThermoFisher) with 10 mM HEPES (Corning) (119). After 24 or 48 hours, cells were conditioned with supernatant from HIV-1 BaL-infected or uninfected leukopaks for 24 hours followed by stimulation with 10 U/ml of universal type 1 IFN for 6 hours. The expression levels of 3 ISGs (MX2, ISG15, and IFIT3) and USP18 were measured by qPCR. We also determined the efficiency of nucleofection 24 hours after nucleofection by fluorescent microscopy.

Correlation between ISG induction, plasma HIV-1 RNA level decline, and baseline USP18 expression. We used archived data from 19 HIV/HCV co-infected participants who were treated with pegylated-IFNα2b (PEG-IFN) who also had plasma HIV-1 RNA measurements at baseline, and 12, 24, 72, and 168 hours after treatment (120). In that study, PBMCs were sampled 0 and 24 hours after IFN treatment and CD38+HLADR+CD3+CD4+ cells were sorted by FACS sorting. Total RNA was extracted and RNA sequencing (RNAseq) was performed. The levels of ISG induction were calculated by dividing the fragments per kilobase million (FPKM) values of ISGs after IFN treatment by their baseline FPKM values. We correlated ISG induction for a defined set of ISGs (change in FPKM values after PEG-IFN), baseline USP18 expression (FPKM value), and plasma HIV-1 RNA decline 72 hours after PEG-IFN treatment.

4. Results
4.1 Induction of pSTAT1 and ISGs are dampened in T cells from viremic PLWHs

We first tested the hypothesis that HIV-1 infection is associated with dampened type 1 IFN responses by investigating the activation of JAK/STAT signaling. Specifically, we quantified the phosphorylation of STAT1 before and after stimulation with type 1 IFN. We focused on CD3+/CD8- T cells as a surrogate for CD4+ T cells because of the well-described down-regulation of surface CD4 levels in HIV-1 infection (121). We observed the maximal level of STAT1 phosphorylation was between 1 to 5 minutes after type 1 IFN treatment (Supplemental Figure 1). We first examined the change in STAT1 phosphorylation in PBMCs from people with uncontrolled HIV-1 (HIV-UC), people who were virologically suppressed with ART for ≥ 1 year (HIV-ART), and from people without HIV (healthy controls; HC). We confirmed that there was no significant difference in baseline pSTAT1 levels among the cohorts. However, when we stimulated PBMCs with exogenous type 1 IFN, the increase in pSTAT1 levels in total CD4+ T cells from HIV-UC was diminished compared to HC (Fig. 1a), thus confirming the phenotype of dampened type 1 IFN responses. We also observed that total CD4+ T cells from HIV-ART exhibited intermediate changes in pSTAT1 levels upon type 1 IFN stimulation, suggesting that antiretrovirals do not fully reverse the dampening of type 1 IFN responses. We then measured the change in pSTAT1 levels in activated (HLAD+CD38+CD3+CD38- cells) and unactivated (HLA-DR-CD38-CD3+CD8- cells) CD4+ T cells, and total CD8+ T cells to test whether the dampening of type 1 IFN responses is generalized in cells that are not susceptible to infection. We demonstrated that HIV-1 non-permissive cells (unactivated CD4+ T cells and total CD8+ T cells) also exhibited changes in pSTAT1 levels in HIV-UC (Fig. 1b).
To confirm further our hypothesis, we measured the mRNA induction of several ISGs (MX2, ISG15, PKR, and BST2) in total CD4+ T cells from participants upon type 1 IFN stimulation. Consistent with the dampened increases in pSTAT1 levels, the induction of the 4 ISG mRNA levels was limited in cells from HIV-UC compared to those from HC (Fig. 2a). Similar to what has been previously reported by other groups (23), there was a statistically significant difference in baseline expression in some of the ISGs that we tested (Fig. 2b). Furthermore, with stimulation of exogenous type 1 IFN, the total expression of 3 ISGs was reduced in cells from HIV-UC compared to HC (Fig. 2c).

Similar to our findings with pSTAT1, we observed an intermediate level of induction of the 4 ISGs in CD4+ T cells from HIV-ART. To confirm that the dampened induction was not the result of direct infection, we infected PHA-activated primary human CD4+ T cells in vitro with a GFP-tagged HIV-1 virus strain; GFP+ cells did not show changes in ISG induction compared to GFP- cells (Supplemental Figure 2). Taken together, we found that HIV-1 infection is associated with the dampening of responses to exogenous type 1 IFN, and that this phenomenon occurs in numerous cell types, irrespective of direct infection.

4.2 Dampening of type 1 IFN responses in CD4+ T cells is associated with USP18 upregulation

We speculated that the dampening of type 1 IFN responses may be due to intracellular counter-regulatory molecular signals. Thus, we measured the baseline mRNA levels of 3 type 1 IFN counter-regulatory genes, SOCS1, SOCS3, and USP18, in total CD4+ T cells. The baseline expression levels of SOCS1 and USP18 were upregulated in CD4+ T cells from HIV-UC compared to HC (Fig. 3a). Moreover, CD4+ T
cells from HIV-ART exhibited intermediate upregulation of baseline SOCS1 and USP18 expression. We then correlated the induction of MX2, a representative ISG, with baseline expression of SOCS1, SOCS3 and USP18. Both SOCS1 and USP18 appeared to predict MX2 induction, but baseline USP18 levels showed a stronger inverse correlation with MX2 induction than baseline SOCS1 expression ($r=-0.731$, $p=2e^{-7}$; Fig. 3b). Indeed, the relationship between baseline USP18 levels and MX2 induction appeared to be conserved across HIV-UC, HIV-ART, and HC, although results from HIV-ART samples clustered bimodally with HIV-UC or HC.

We confirmed in vivo the inverse association between baseline USP18 expression and ISG induction upon stimulation with type 1 IFN. We previously demonstrated heterogenous induction of ISGs in activated CD4+ T cells taken from 19 HIV-1/HCV co-infected people who were treated with pegylated-type 1 IFNα2b (PEG-IFN) (122). In that study, we performed RNAseq on activated CD4+ T cells taken before, and 24 hours after a single administration of PEG-IFN. We identified 99 genes that were robustly upregulated in activated CD4+ T cells that we described as meeting the criteria of ISGs. MX2 was among these ISGs. We then interrogated the RNAseq data set from cells prior to PEG-IFN treatment for reads that mapped to USP18 or SOCS1. We found a strong correlation between the baseline read frequency of USP18 and the induction of MX2 after PEG-IFN ($r=-0.775$, $p=2e^{-7}$), but no correlation for SOCS1 ($r=0.065$, $p=0.787$) (Fig. 4a). We then reasoned that dampened type 1 IFN responses in activated CD4+ T cells might result in worse control of HIV-1 by PEG-IFN. Accordingly, we correlated the baseline read frequency of USP18 with the subsequent plasma HIV-1 RNA decline after PEG-IFN treatment. As expected, we found that
baseline USP18 expression in activated CD4+ T cells was tightly and inversely associated with subsequent decline in plasma HIV-1 RNA levels: persons with high levels of USP18 expression in activated CD4+ T cells had smaller decreases in HIV-1 viremia than persons with low levels of USP18 expression (Fig. 4b). These results strongly indicate that USP18 expression may govern the antiviral control of HIV-1 in response to type 1 IFN.

4.3 The dampened type 1 IFN response phenotype can be transferred to target cells.

We sought to develop a model of type 1 IFN dampening in vitro to uncover its underlying mechanisms. Since we found that dampening of type 1 IFN responses is observed irrespective of bona fide infection, we hypothesized that soluble mediators might transfer the dampening phenotype to other cell types. We tested whether conditioned media from in vitro HIV-1-infected PBMCs would be sufficient to recapitulate the dampening phenotype in uninfected target cells. We infected healthy leukopaks with HIV-1 BaL for 3 days, then filtered and collected supernatant. We incubated uninfected target CD4+ T cells with these conditioned supernatants along with antiretrovirals (TDF and FTC; see Methods) to inhibit de novo HIV-1 infection of target cells. Twenty-four hours after incubation, we stimulated target cells with type 1 IFN and quantified the induction of 3 ISGs (MX2, ISG15, and IFIT3) to test the hypothesis that conditioned supernatant from HIV-1 infected cells is sufficient to dampen type 1 IFN responses in target cells (Fig. 5a). Indeed, we found that ISG induction was dampened in target cells incubated with conditioned supernatants from HIV-1 infected cells (sup-BaL+LP) compared to target cells conditioned with supernatant from HIV-1 uninfected leukopaks (sup-BaL-LP) (Fig. 5b). As observed in
CD4+ T cells from HIV-UC, baseline ISG expression also increased in target cells conditioned with sup-BaL+LP compared to those conditioned with sup-BaL-LP (Fig. 5c). In addition, the total ISG expression upon type 1 IFN stimulation was diminished in cells conditioned with sup-BaL+LP (Fig. 5d). We also found that baseline USP18 mRNA levels in conditioned target cells prior to type 1 IFN treatment were higher in those conditioned with sup-BaL+LP than with sup-BaL-LP (Fig. 5e). We concluded that soluble mediators from HIV-1 infected cells can transfer the dampening phenotype to uninfected target cells and is associated with elevated USP18 expression in target cells.

4.4 Type 1 IFN from HIV-1-infected cells triggers USP18 upregulation and downregulation of the type 1 IFN response in target cells

We investigated which soluble mediators might potentially upregulate USP18 and transfer the dampening phenotype to uninfected target cells. Veenhuis et al. observed elevated levels of IL-12/23p40, IL-18, IFNγ, IL-15, Eotaxin, MCP-1, and IP-10 in plasma from PLWH without controlled HIV-1 infection compared to HIV-1 uninfected individuals (123) (Fig. 6a). In addition to these, we examined type 1 IFN itself since it is known to induce USP18 expression (124), and is not well detected in plasma. We confirmed that USP18 is upregulated in primary CD4+ T cells by type 1 IFN and IL-15, but not by IL-12/23p40, IL-18, IFNγ, Eotaxin, MCP-1, or IP-10 (Fig. 6b). We then measured the amounts of IFNα2a and IL-15 in the conditioned supernatants from sup-BaL+LP and sup-BaL-LP. We found no correlation between IL-15 levels in conditioned supernatants and the dampening of ISG induction (r=0.157, p=0.662), consistent with a limited role of IL-15 in promoting the dampening of type 1 IFN responses. In contrast, levels of IFNα2a in conditioned supernatants were strongly associated with MX2 induction (r=-0.982,
p=2e-7), and with baseline USP18 levels (r=0.802, p=2e-7) in target CD4+ T cells, suggesting that IFNα2a production in cell populations that include HIV-1 infected cells contributed to the dampening of type 1 IFN responses in target cells by way of negative feedback (Fig. 6c, d). We confirmed this hypothesis by neutralizing type 1 IFN in conditioned supernatants with recombinant B18R, a soluble Vaccinia Virus type 1 IFN decoy receptor, prior to incubating with target cells. We demonstrated that pre-incubation of exogenous type 1 IFN with B18R neutralized the effect of type 1 IFN in a dose-dependent manner by measuring the expression of ISGs (MX2 and USP18) (Supplemental Figure 3). Type 1 IFN neutralization in conditioned supernatants led to partial recovery of type 1 IFN responses in target cells (Fig. 6e).

4.5 USP18 is necessary to dampen type 1 IFN response in target CD4+ T cells

To explore the mechanism underlying the dampening of type 1 IFN responses, we examined whether knockdown of USP18 in target cells could recover type 1 IFN responses. We first developed a primary CD4+ T cell USP18 knockdown model using siRNA nucleofection: USP18 mRNA levels were maximally knocked down 12 hours after siRNA nucleofection and USP18 protein expression was knocked down between 24 and 48 hours after nucleofection in primary CD4+ T cells (Supplemental Figure 4). By knocking down USP18 in target CD4+ T cells, we observed changes in baseline and induced ISG expression levels (Fig. 7a). To address the specific role(s) of USP18 in dampening type 1 IFN responses, we calculated several different measures of target cell type 1 IFN modulation: 1) the change in baseline ISG expression (Δbaseline) between target cells conditioned with sup-BaL-LP compared to those conditioned with sup-BaL+LP, prior to type 1 IFN treatment, 2) the change in induction of ISGs with IFN
(Δinduced), calculated by subtracting the difference in ISG expression between target cells conditioned with sup-BaL+LP treated with type 1 IFN to cells conditioned with sup-BaL+LP that were not treated with type 1 IFN, and 3) the total change in ISG expression (Δtotal), calculated by comparing ISG expression in target cells conditioned with sup-BaL+LP and stimulated with type 1 IFN to those conditioned with sup-BaL-LP that did not have type 1 IFN stimulation. We reasoned that the Δtotal calculation of ISG expression modeled how type 1 IFN responses would be enhanced during HIV-1 infection if USP18 were continually inhibited, since type 1 IFN was likely responsible for the initial conditioning and upregulation of USP18.

We demonstrated that target cells with USP18 knockdown had enhanced Δbaseline and Δtotal ISG expression compared to target cells that were not transfected or that were transfected with a scrambled control siRNA (Fig. 7b, c). Paradoxically, we did not find a significant change in Δinduced ISG expression, although this appeared to be explained by the commensurate increase in both Δbaseline and Δtotal ISG expression (Fig. 7d). We concluded that USP18 is partially responsible for dampening type 1 IFN responses in uninfected target cells.

4.6 HIV-1 virions contribute to dampened responses to exogenous type 1 IFN

In a conceptual model of potential soluble mediators during HIV-1 infection that might dampen type 1 IFN responses in uninfected bystander cells (Fig. 8a), we reasoned that non-infectious virions might also contribute to dampening: because TDF and FTC inhibit reverse transcription of uncoated viral RNAs after entry, it is feasible that early events during entry might trigger signaling cascades that resulted in upregulation of USP18. Specifically, sensing of HIV-1 nucleic acids and capsid proteins
that would be found in entering virions have been described (125, 126). Thus, we tested whether addition of HIV-1 fusion inhibitor T20 or cyclophilin A inhibitor (CypA), which blocks uncoating of HIV-1 capsid, might further modulate type 1 IFN responses. We incubated target cells with T20 or CypA in addition to TDF+FTC, conditioned cells with sup-BaL+LP or sup-BaL-LP, and treated with type 1 IFN, measuring ISG induction. We found that addition of CypA partially restored ISG induction in target cells that were conditioned with sup-BaL+LP (Fig. 8), implying that sensing of viral nucleic acids might contribute to dampening in target cells, even in the absence of bona fide infection. Similarly, T20 partially restored type 1 IFN responses. Taken together, HIV-1 virions seem to contribute to the reduced type 1 IFN responses in target cells independent of infection.

5. Discussion

We show here that primary T cells (CD4+ and CD8+) from PLWH demonstrated a dampened response to exogenous type 1 IFN that depends in part on upregulation of USP18 in the same cells. We found that HIV-1 infection led to release of soluble mediators that raised USP18 levels in uninfected target cells and consequently dampened their responses to a second stimulus of type 1 IFN. These mediators include type 1 IFN itself and HIV-1 virions, even in the absence of de novo infection of the target cells. We confirmed that, in vivo, USP18 levels in CD4+ T cells are strongly associated with ISG induction in response to PEG-IFN administered to PLWH. Intriguingly, USP18 levels in CD4+ T cells were closely linked to the subsequent decline in plasma HIV-1 RNA levels after PEG-IFN administration, suggesting a strong link between USP18 levels and HIV-1 control by type 1 IFN in vivo. Surprisingly, we found that a subgroup of
PLWH who have been virologically suppressed with ART for >1 year demonstrated continued dampening of type 1 IFN. Taken together, we describe a persistent defect in innate immune antiviral defenses among PLWH that perpetuates type 1 IFN resistance, thereby limiting control of HIV-1 during chronic infection (7, 74, 127).

Similar to previous findings in PLWH or animal models (23, 49, 65), upregulated baseline ISG expression was observed in CD4+ T cells from PLWH in our study. However, with the stimulation of exogenous type 1 IFN, both STAT1 phosphorylation and ISG induction were diminished in cells from PLWH. Several clinical trials have demonstrated that type 1 IFN-based therapy exhibited modest control of HIV-1 replication (69-75). Our findings could explain the modest effect of type 1 IFN-based treatment on HIV control. In contrast to what we found, Catalfamo et al. reported that pSTAT1 activation was greater in naïve and memory CD4+ T cells, and CD8+ T cells from PLWH without controlled HIV-1 infection compared to those with controlled infection. However, whereas we examined pSTAT1 activation early, within 1-5 minutes of stimulation (based on time course experiments), Catalfamo et al. measured pSTAT1 levels 30 minutes after stimulation. There may be differences in the kinetics of pSTAT1 activation that explain our discrepant findings. It is also possible that naïve and memory CD4+ are resistant to the dampening of type 1 IFN responses, probably due to the differential expression of proteins involved in type 1 IFN signaling.

We suspect that plasmacytoid dendritic cells (pDCs), potent type 1 IFN secretors (7, 127), are the main contributor of the diminished responses (Fig. 9). pDCs sense HIV-1 nucleic acid by TLR7, and produce type 1 IFN (7). In addition, in chronic HIV-1 infection, pDCs are likely to migrate to lymph nodes, and they are capable of secreting
type 1 IFN (128), highlighting the possibility that pDCs can produce type 1 IFN in acute and chronic HIV-1 infection. In future studies, it would be important to test if pDC depletion prior to HIV-1 infection of leukopaks leads to restoration of the type 1 IFN response in target CD4+ T cells.

Interestingly, cells from HIV-ART exhibited an intermediate phenotype between HC and HIV-UC. Because PLWH who are virologically suppressed still release low-levels of HIV-1 virions, and because cell associated HIV-1 DNA and RNA levels decay slowly during ART (129), we speculate that pDC sensing of residual HIV-1 nucleic acids may contribute to constitutive type 1 IFN signaling, either of defective or intact proviruses released from the HIV-1 reservoir (130). We demonstrated that adding fusion or uncoating inhibitors to supernatants partially restored the type 1 IFN responses, indicating that HIV-1 virions may also contribute to diminished type 1 IFN responses independent of infection. Therefore, it is plausible that defective viruses are also responsible for reduced type 1 IFN responses. Recently, Bruner et al. demonstrated that defective proviruses can clonally expand in response to CD3/28 stimulation, while the intact proviruses only showed modest proliferation (131). Therefore, defective proviruses could contribute to constitutive type 1 IFN signaling, which in turn leads to diminished responses to type 1 IFN-based treatment with PLWH.

This study has several limitations that are worth noting. Firstly, our sample size was relatively small; this was mostly because there are only limited numbers of PLWH that are not treated with antiretrovirals. In addition to the small number of participants in this study, we were able to collect only a limited number of cells from PBMCs from PLWH. With the small number of cells, we were not able to interrogate them for the
presence of USP18 protein. Furthermore, USP18 knockdown did not completely restore the reduced type 1 IFN response. The USP18 knockdown experiment was complicated because USP18 is also induced by type 1 IFN in supernatant. Therefore, it was challenging to demonstrate the relative contribution of USP18 to diminished type 1 IFN responses in the absence of a commercially available USP18 specific inhibitor. Therefore, the contribution of USP18 to the dampened type 1 IFN responses was still unclear although USP18 is at least partly involved in the diminished responses.

Our data has shown that elevated type 1 IFN and virions in PLWH triggers USP18 upregulation, surprisingly not Socs genes, resulting in the diminished type 1 IFN responses and, ultimately, in impaired endogenous control of HIV-1. It might be worthwhile to investigate therapies targeting type 1 IFN or USP18 as a novel way to control HIV-1. In a humanized mouse model, IFNAR blockade in HIV-1 infected humanized mice on ART restored T cell exhaustion and decreased plasma HIV-1 RNA levels and the size of the HIV-1 reservoir (65, 66), therefore suggesting a promising alternative strategy for controlling HIV-1. Taken together, targeting USP18 or constitutive type 1 IFN signaling in PLWH might represent a key step in the search for an HIV-1 cure. Furthermore, addition of fusion or uncoating inhibitors to current ART regimens may also have an impact on improving immune responses in PLWH. T20 has been used for HIV-1 treatment regimens. Although T20-based treatment alone has some success in PLWH, many participants with T20 resistant HIV-1 failed to control HIV-1 replication (132). On the other hand, current ART regimens are known to have a lower rate of acquiring drug resistant mutations (133). However, ART alone does not seem to fully restore immune responses, including chronic immune activation (134).
Thus, we propose that combination therapies that include ART in addition to immunomodulatory agents that are aimed at reversing type 1 IFN resistance may be worth considering in the future.
Chapter 3: No recovery of replication competent HIV-1 from liver macrophages

1. Abstract

Long-lived HIV-1 reservoirs that resist eradication by antiretroviral therapy (ART) are a major challenge in attaining an HIV-1 cure. We investigated whether human liver macrophages (LMs), which are the largest tissue macrophage population, could harbor an HIV-1 reservoir. We purified LMs from liver explants and treated them with a T cell immunotoxin to reduce T cell contamination by <1%. Purified LMs were from 9 HIV-1–infected persons, 8 of whom were taking ART (range 8–140 months). We stimulated purified LMs *ex vivo* from 6 of 8 LMs from persons on ART to test whether this would propagate infection in target cells. However, HIV-1 from LMs did not propagate in target cells except from 1 person who was treated with ART for <1 year. Taken together, we found evidence of HIV-1 infection in LMs from PLWH on ART. However, despite developing an assay that could detect HIV-1 outgrowth from infected MDMs *in vitro*, we were unable to find adequate evidence of viral outgrowth from LMs taken from PLWH on ART. These findings suggest that while ART does not eradicate HIV-1 that persists in LMs, the detectable proviruses are inert, suggesting that they are either defective or only exhibit limited propagation.
2. Introduction

Although an HIV-1 cure seems possible (135, 136), there are multiple challenges to developing a durable cure. All HIV-1 infected subjects harbor long-lived HIV-1 reservoirs, hindering HIV-1 eradication. The definition of a long-lived cellular reservoir is one that persists despite combination antiretroviral therapy (ART) based suppression of HIV-1 replication, and from which infectious virus can grow *ex vivo* to establish new infection upon treatment interruption (81). The resting memory CD4+ T cell latent reservoir is the best-characterized HIV-1 cellular reservoir, but there may be other cell types that could serve as a reservoir (137). One plausible candidate for an HIV-1 reservoir are tissue resident macrophages, which are abundant, long-lived, and susceptible to HIV-1 infection; however, their contribution as a long-lived HIV-1 cellular reservoir has been challenging to demonstrate in humans. Recently, the role of tissue resident macrophages in HIV-1 latency has gained more interest (138-140). We performed a proof-of-principle study to investigate whether infectious HIV-1 is harbored by human liver macrophages (LMs), approximately 90% of all tissue macrophages (141), during ART.

3. Methods

*Liver tissue.* Whole liver tissues from persons with HIV-1 infection (*n* = 9) were collected from 2 sources (Supplemental Table 2). Fresh tissues (N7 and N9) from HIV-1–infected demised individuals were provided from The National Disease Research Interchange (NDRI). Other fresh liver tissues from liver transplantation were also obtained from HIV-1–infected patients (LT01, LT02, LT06, LT07, LT08, LT09, and LT10) at Johns Hopkins
Hospital (JHH). Tissues were obtained as soon as liver was explanted and purification of LM was undertaken immediately after liver collection.

**Primary human LMs.** To study their role *in vivo*, we purified LMs from fresh liver tissues collected from both NDRI and from the liver transplantation unit at JHH as previously mentioned. The isolation of LMs from fresh liver tissues was performed following a previously described protocol (142). Briefly, approximately 2 mm³ pieces of liver were sectioned from 50g pieces of liver isolated immediately after explantation, and before pronase digestion. The digest was then filtered and separated by a 16% histodenz (Sigma Aldrich) gradient. The interface was collected followed by the addition of an FcR blocking reagent (Miltenyi Biotec, catalog 130-059-901), after which CD3+ T cells were depleted using CD3 microbeads (Miltenyi Biotec, catalog 130-050-101). The flow-through was plated to allow adherence (e.g. LMs) and subsequently washed after 2 hours to remove non-adherent cells. In a separate experiment, LMs were enriched by staining with CD14 microbeads (Miltenyi Biotec, catalog 130-050-201); however, CD14+ selection demonstrated lower purity than plastic adherence (Supplemental Figure 5). Therefore, LMs from all liver tissues were purified by the plate adherence method in this manuscript. We cultured these cells in collagen-1–coated plates (Life Technologies, catalog A11428-03) to improve adherence.

**LM viral outgrowth assays.** In order to capture infectious viruses from LMs maximally, we developed a modified viral outgrowth assay (VOA) for LMs (143). LMs were stimulated with IFNγ, IL-4 (Miltenyi Biotec, catalog 130-093-920), IL-13 (EMD Millipore, IL012), zolendronic acid (Sigma-Aldrich, catalog SML0223), recombinant HIV-1 tat clade-B (ProSpec, catalog hiv-129), lipopolysaccharide (Sigma-Aldrich, catalog L5293),
and poly I:C (Invivogen, catalog tlrl-picl-v) in order to identify which stimulation induces viral release most effectively (144-149). Two successive doses of IFNγ (200 U/ml) every 48 hours along with 200 ng HIV-1 rTat on 3 consecutive days after IFNγ stimulation gave the optimal and most consistent outcome. LMs were stimulated with rTat 48 hours after the second IFNγ stimulation. The VOA for LMs from only LT01 include stimulation with TLR agonists after IFNγ treatment compared to VOA for the other LMs. All cultured LMs were incubated with antiretrovirals to inhibit infection of cells ex vivo upon virus release from dying or phagocytosed T cells. Following activation, LM supernatants were filtered and transferred to target cells; in addition, target cells and LMs were co-cultured. To enhance viral replication, different batches of uninfected target cells were cultured with LM supernatants collected every 10–11 days, whereas uninfected new target cells were repeatedly added to the co-culture. LMs and target cells were co-cultured for 1 month after IFNγ stimulation. We used either activated CD4+ T (for LT01) or CEMx174 cells (ATCC, catalog CRL-1992) as target cells for VOA. CEMx174 cells were donated by Lucio Gama (Johns Hopkins University, Baltimore, Maryland). We demonstrated in vitro that supernatants from LMs that we infected with HIV-1 could propagate infection in CEMx174 cells, in contrast to prior reports that CEMx174 cells lack CCR5, the co-receptor that is necessary for HIV-1 entry into macrophages (Figure 10). It is worth noting that our findings are consistent with another group that has demonstrated that HIV-1 can be propagated in CEMx174 cells (112).

HIV-1 infections. To study viral kinetics in cell lines, CEMx174, MT4, MOLT4-CCR5+, Sup T1, PM1, Jurkat, and activated CD4+ cells were used for HIV-1 infection. For CD4+ T cell activation, leukopaks were stimulated with 10 μg/ml of phytohaemagglutinin
Three days after stimulation, CD4+ T cells were isolated using the human total CD4+ T cell isolation kit (Miltenyi Biotec, catalog 133-096-533). We quantified the concentration of the viral stock by previously described PCR (150) or HIV-1 p24 ELISA (Perkin Elmer, catalog NEK050001KT). p24 1ng of HIV-1 BaL was spinoculated with 1 x 10^5 cells at 1,200g for 2 hours, and supernatants were harvested and filtered 3 and 7 days after infection. The amount of HIV-1 in the supernatant was measured by an HIV-1 p24 ELISA kit (Perkin Elmer, catalog NEK050001KT).

**Nucleic acid purification and quantification.** Cell-associated DNA was extracted with the DNeasy Blood and Tissue kit. (Qiagen, catalog 69504). For the detection of HIV-1 proviral DNA, a modified PCR protocol for a previously published HIV-1 RNA assay was performed (151). HIV-1 RNA from supernatants was extracted by the QIAmp viral RNA mini kit (Qiagen, catalog 52906). For LT02, we pooled and ultracentrifuged supernatants from CEMx174 cells before RNA extraction so that we were able to maximize the detection of HIV-1 RNA after VOA. Supernatant HIV-1 RNA levels were calculated as described (150). HIV-1 Acrometrix standards (Life Technologies, catalog 942013) were used for setting the lower limit of detection of HIV-1 RNA PCR.

**Antiretroviral therapy.** After purification of LMs from HIV-1–infected subjects taking ART, we prevented *ex vivo* LM infection; therefore, our results, if positive, would only be consistent with HIV-1 infection *in vivo*. Bulk liver tissue and isolated LMs were cultured in media containing tenofovir disoproxil fumarate (10 μM), emtricitabine (1 μM), and raltegravir (1 μM) until we performed VOA. We also confirmed that antiretrovirals can effectively inhibit the *in vitro* infection of LMs with the concentrations previously
published for different cell types (152-155). We confirmed that no toxicity was observed in uninfected LMs treated with antiretrovirals.

**Study approval.** This study protocol was approved by the Johns Hopkins School of Medicine institutional review board, and it was not considered as human subject research because all live samples were collected strictly from liver transplant or demised individuals that were otherwise intended to be discarded.

### 4. Results

**4.1 CEMx174 cells are suitable for VOA using LMs.**

We used a variety of cell lines to identify which would robustly replicate HIV-1 from LMs (Fig. 9). We infected cell lines with the BaL strain of HIV-1, which is macrophage-tropic, and investigated which cell lines could efficiently propagate the infection, and thus which would be best for a VOA. Every cell line tested was capable of propagating the BaL infection including the CEMx174 cell line, despite previous reports of their lack of CCR5 surface expression (156). On the contrary, we observed comparable replication of HIV-1 BaL in CEMx174 cells to CCR5-expressing MOLT-4 cells (157). Also, it was reported that CEMx174 cells was able to propagate SIV from macaque macrophages (112). Thus, we decided to use CEMx174 cells as targets for a VOA to study LM reservoirs.

**4.2 LMs from HIV-1–infected people can harbor HIV-1 in an inert form**

LMs were isolated from liver tissue of HIV-1–infected persons in order to understand the degree of contributions LMs make to HIV-1 infection *in vivo*. Fresh liver tissue was collected from an HIV-1–infected patient (N7) at demise who had not been on ART for
more than 6 months and whose plasma HIV-1 RNA level was greater than 500,000 cp/ml. Eighteen days after purification and culture of LMs, when T cell contamination could not be detected, supernatants from LMs were found to contain detectable polyadenylated HIV-1 RNA that is only present in spliced and genomic HIV-1 RNA (150), demonstrating that in vivo infection of HIV-1 with LMs is possible. We next tested whether we could recover infectious viruses from LMs in HIV-1–infected subjects whose viremia was suppressed by ART. Fresh liver explant tissues were collected from liver transplantation of 7 HIV-1–infected people and from 1 deceased individual (N9). These individuals were treated with ART at the time of liver recovery and their plasma HIV-1 RNA levels were undetectable. We detected low levels of total HIV-1 proviral DNA in bulk liver tissue from 7 of 8 individuals (data not shown). A median of $5.5 \times 10^5$ LMs (range $4.8 \times 10^5 – 1.4 \times 10^6$) were isolated from all people and no T cell contamination was confirmed in all except 1 person (LT02) who contained approximately 1 T cell in 100 LMs (99% pure). LMs from HIV-1–infected individuals were stimulated with IFNγ+rTat more than 30 days after LM isolation (Fig. 10). In one subject (N9), there was bacterial contamination early after LM isolation; therefore, we stimulated LMs only 6 days after purification. Supernatants from stimulated LMs were filtered and CEMx174 cells were cultured with supernatant twice over a 15-day period. We detected proviral DNA in target cells with LM supernatants from 6 of 8 individuals and none in negative control target cells that were maintained with media only (Fig. 11A and Table 1). However, with the exception of LT02, HIV-1 propagation, measured by HIV-1 RNA levels, was not demonstrable in target cells (Table 1). Unlike resting CD4+ T cells (157), high-level replication was not observed. On the other hand, replication-competent HIV-1
was detected in resting CD4+ T cells in this cohort (Supplemental Table 1). It is also important to note that LT02 was on ART for the shortest period before liver explantation (Fig. 11B and Table 1). Taken together, these data demonstrate that HIV-1 can be harbored by LMs for long durations, but the viruses are not able to propagate in target cells unless LMs are collected shortly after viremic suppression by ART.

5. Discussion

In this study we demonstrate using a modified VOA and a novel method to deplete T cells that HIV-1 can be harbored by freshly purified LMs from HIV-1–infected subjects for prolonged periods, despite suppressed viremia with long-term ART. However, despite these findings, replication-competent virus does not seem to be found in LMs from people with long-term virologic suppression, nor could we grow these viruses to propagate effectively.

HIV-1 reservoirs persist for extended durations in HIV-1-infected persons taking ART and lead to virologic rebound after the interruption of ART. Previously, tissue macrophages were thought to be less likely to be a HIV-1 reservoir due to their short half-lives. However, recent reports have demonstrated that macrophages are seeded in tissues during embryonic development and replenish themselves locally, suggesting their longevity (158-163). The estimated longevity of LMs is approximately 14 months in an animal model (164). Also, it was reported that LMs harbor HIV-1 in situ before administration of ART, and HIV-1 can replicate in LMs in vitro (165-169). Therefore, there is sufficient reason to hypothesize that LMs could be HIV-1 reservoirs. Intriguingly,
in our study HIV-1 was detectable in LMs from an individual who was virologically suppressed for 11.7 years with ART (Fig. 11B).

Despite our findings, exponential HIV-1 propagation from LMs does not seem to occur, in contrast to reservoirs in resting CD4+ T cells. Purified LMs from only one person propagated infection to target cells with detectable HIV-1 RNA release (LT02), but this person underwent liver explantation after only 8 months of ART and also had low-level T cell contamination in his LM cultures (Fig. 11B). We hypothesize several reasons for why replication-competent HIV-1 was not detectable in LMs. First, SAMHD1, an HIV-1 restriction factor that has been well-described and is most active in macrophages, may increase the error rate of reverse transcription, producing defective proviral DNAs in macrophages (170). Second, additional restriction factors can contribute to the production of defective HIV-1 in LMs, such as the newly described MARCH8 (171), which reduces the infectivity of released virions from macrophages. Finally, adaptive immune responses may preferentially target LMs harboring replication-competent HIV-1 rather than macrophages with defective HIV-1, similar to the responses to HIV-1 in resting CD4+ T cells (130).

Several limitations can be identified in our approach. Although larger numbers of purified, viable LMs from multiple subjects were tested in our study compared to previous studies (165), the total numbers of LMs were still limited in any one experiment (≤1.4 × 10^6 cells). In addition, because our study was dependent largely on infrequent liver explants from liver transplants in HIV-1 infected individuals (0.4% of all liver transplantations in the United States), the overall size of our research was small. Indeed, we were able to collect only the limited number of liver tissue from almost all
liver transplantations for HIV-1–infected organ recipients at our institution from July, 2013, until July, 2016 (n = 7; Table 1). Because liver disease causes inflammatory responses, which many of our subjects also experienced, distinguishing between inflammatory monocyte-derived LMs, or longer-term liver resident LMs might be an important step that we did not perform (161). Accordingly, our definition of LMs was generalized to adherent mononuclear cells purified from liver explant, which does subset cells based on their origin.

In conclusion, HIV-1 can be recovered from LMs that were isolated from HIV-1–infected subjects taking suppressive ART. However, we were not able to provide sufficient evidence demonstrating that replication competent HIV-1 was harbored by LMs, and HIV-1 did not propagate from LMs in a VOA.
Chapter 4. Figures and Tables

Table 1. Liver macrophage viral outgrowth assay results

Duration of uninterrupted ART is shown if available (no treatment for N7, no clear data for the duration but clear documentation for ART exposure for N9). We couldn’t detect plasma HIV-1 RNA for all subjects when liver was explanted, except for N7. Results of the LM VOA for each person are tabulated. A LT02 was an elite suppressor whose plasma HIV-1 RNA level before ART was 74 cp/ml. B LT09 experienced retransplant, indicating no exposure of active plasma HIV-1 with the explant in vivo.
### Supplemental Table 1

The size of resting memory CD4+ T cell reservoir measured by quantitative viral out growth assay.

<table>
<thead>
<tr>
<th>ID</th>
<th>Time post transplantation</th>
<th>Infectious Units per Million cells (IUPM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LT01</td>
<td>2 weeks</td>
<td>1.65</td>
</tr>
<tr>
<td></td>
<td>34 months</td>
<td>1.69</td>
</tr>
<tr>
<td>LT02</td>
<td>26 months</td>
<td>16.25</td>
</tr>
</tbody>
</table>
Supplemental Table 2. General demographics of participants.

<table>
<thead>
<tr>
<th>ID</th>
<th>Date</th>
<th>Clinical setting prior to obtaining liver tissue</th>
<th>Reason for transplant/ Cause of Death</th>
<th>Liver fibrosis</th>
<th>CD4+ T cell count</th>
<th>Interval from removal to processing</th>
<th>Coinfection</th>
<th>ART duration (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LT01</td>
<td>July, 2013</td>
<td>Explant during transplantation</td>
<td>Decompensated liver disease due to HCV</td>
<td>cirrhosis</td>
<td>607 cells/μL (47%)</td>
<td>&lt; 1 hour</td>
<td>+</td>
<td>140</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(4 days preTx)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LT02</td>
<td>March, 2014</td>
<td>Explant during transplantation</td>
<td>HCC</td>
<td>cirrhosis</td>
<td>116 cells/μL (11.4%)</td>
<td>&lt; 1 hour</td>
<td>+</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5 months preTx</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N7</td>
<td>May, 2014</td>
<td>Post-mortem</td>
<td>HIV (listed on death certificate)</td>
<td>Cirrhosis, portal hypertension</td>
<td>53 cells/μL</td>
<td>3.5 hours</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>20 days prior to tissue</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N9</td>
<td>April, 2015</td>
<td>Post-mortem</td>
<td>HIV/Cancer (listed on death certificate)</td>
<td>NA</td>
<td>NA</td>
<td>17 hours</td>
<td>-</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LT06</td>
<td>March, 2016</td>
<td>Explant during transplantation</td>
<td>Decompensated liver disease due to HCV</td>
<td>cirrhosis</td>
<td>1183 cells/μL (35.4%)</td>
<td>&lt; 1 hour</td>
<td>+</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3 months preTx</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LT07</td>
<td>April, 2016</td>
<td>Explant during transplantation</td>
<td>Decompensated liver disease due to HBV</td>
<td>Cirrhosis</td>
<td>426 cells/μL (39.2%)</td>
<td>&lt; 1 hour</td>
<td>-</td>
<td>&gt; 113</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3 months preTx</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 1. PLWH exhibited reduced pSTAT1 induction by exogenous type 1 IFN. (a) PBMCs from PLWH with uncontrolled viremia (HIV-UC; n=9), ART-controlled viremia (HIV-ART; n=7), or Healthy controls (HC; n=12) were stimulated with type 1 IFN for 5 minutes, and the levels of pSTAT1 with and without IFN were quantified by flow cytometry. Induction of pSTAT1 by type 1 IFN in total (b), unactivated and activated CD4+ T cells, and total CD8+ T cells (c) was calculated for each group (*: p<0.05, **: p<0.01, ***; p<0.005)
Figure 2. Diminished expression of ISGs by type 1 IFN in CD4+ T cells from PLWH. (a) Total CD4+ T cells were isolated from PBMCs from HIV-UC, HIV-ART, and HC and were stimulated with exogenous type 1 IFN for 6 hours. Total RNA was extracted after stimulation and the expression level of 4 ISGs (MX2, ISG15, PKR, and BST2) without (b) and with (c) type 1 IFN was measured by qPCR. The induction of ISGs were calculated for each group of participants (d) (*: p<0.05, **: p<0.01, ***: p<0.005).
Figure 3. Baseline USP18 level is strongly associated with the response to exogenous type 1 IFN. (a) Total CD4+ T cells were isolated from PBMCs from HIV-UC, HIV-ART, and HC and the levels of SOCS1, SOCS3, and USP18 were quantified by qPCR. (b, c, d) The induction of MX2 in total CD4+ T cells by exogenous type 1 IFN is correlated with the baseline expression of SOCS1 (b), SOCS3 (c), and USP18 (d).
Figure 4. Basal USP18 level predicts the ISG induction by Pegylated-IFN based therapy and subsequent plasma HIV-1 RNA decline. 19 HIV/HCV co-infected persons were treated with Pegylated-IFN (PEG-IFN) for 1 week, and the plasma HIV-1 RNA level before and 72 hours after PEG-IFN treatment were quantified. Activated CD4+ T cells from PBMCs before and 24 hours after PEG-IFN were isolated by FACS sorting, total RNA was extracted, and RNA-seq was performed. The induction of MX2 (a, b), and plasma HIV-1 RNA decline (b) was correlated with baseline expression of SOCS1 (a) and USP18 (a, b).
(a) Infect LPs with BaL 3 days

(b) Baseline USP18 level

(c) Incubate CD4+ T cells with media/sup from BaL+LP + ART to prevent target cell infection (24h)

(d) MX2, ISG15, IFIT3

(e) USP18 baseline
Figure 5. Conditioned media from HIV-1-infected cells can transfer the dampened type 1 IFN responses. (a) Schematic of experimental design. Healthy leukopaks (LP) were infected with HIV-1 BaL for 3 days and supernatant from HIV-1 uninfected or infected LPs were harvested. Target CD4+ T cells from healthy LPs were treated with ART for 24 hours and incubated with supernatant from BaL- or BaL+ LP. 24 hours after conditioning, target cells were stimulated with exogenous type 1 IFN and the expression of 3 ISGs (MX2, ISG15 and IFIT3) without (c) and with (d) IFN were measured by qPCR. The induction of 3 ISGs (b) were calculated for each experiment. Baseline expression of USP18 (e) was also measured by qPCR after incubation with BaL- or BaL+ supernatant.
Figure 6. Type 1 IFN from HIV-1-infected cells contributes to the diminished type 1 IFN responses. (a) Upregulated serum cytokines in PLWH. The levels of IL-12/23p40, IL-15, IL-18, IFNγ, MCP-1, TNFα, Eotaxin and IP-10 were measured in serum from 87 HC and PLWH by MSD. (b) Type 1 IFN and IL-15 upregulated USP18 in total CD4+ T cells. Total CD4+ T cells were isolated from healthy LP and stimulated with type 1 IFN, IL-12/23p40, IL-15, IL-18, IFNγ, MCP-1, and IP-10. 6 hours after stimulation, total RNA was extracted and the level of USP18 was quantified by qPCR. (c, d) Supernatant IFNα2a levels, not IL-15 levels are strongly associated with the type 1 IFN response and baseline USP18 levels in target CD4+ T cells. The level of IFNα2a and IL-15 in the supernatant from BaL- and BaL+ LPs were quantified by ELISA and were correlated with the induction of MX2 (c), and basal expression of USP18 (d). (e) Neutralization of type 1 IFN restored the reduced type 1 IFN responses. Type 1 IFN in supernatant from BaL- or BaL+ were neutralized by Vaccinia Virus recombinant B18R, a type 1 IFN decoy receptor for 1 hour. Recombinant IFNγR was added as a negative control for
neutralization. Target CD4+ T cells were then conditioned with the supernatants for 24 hours and stimulated with exogenous type 1 IFN. 6 hours after stimulation the expression of 3 ISGs (MX2, ISG15, and IFIT3) were measured by qPCR and the induction of each gene was calculated for each condition. (*: p<0.05, **; p<0.01, ***; p<0.005).
Figure 7. USP18 contributes to the diminished type 1 IFN responses. (a) Total CD4+ T cells were nucleofected with scramble RNA (si ct), or siUSP18 by using Amaxa
Nucleofector. 24 hours after nucleofection, target CD4+ T cells were incubated with supernatant from BaL- or BaL+ LPs for 24 hours and stimulated with exogenous type 1 IFN. 6 hours later, the level of 3 ISGs (MX2, ISG15, and IFIT3), without and with IFN, were quantified by qPCR and relative expression levels were calculated normalized to delta-Ct of 0. (b) The Δbaseline (the induction of ISGs in cells with BaL+ supernatant and no IFN normalized to cells with BaL- supernatant and no IFN), (c) Δtotal (the induction of ISGs in cells with BaL+ supernatant and IFN normalized to cells with BaL- supernatant and no IFN), and (d) Δinduced (the change in ISG expression from cells with BaL+ supernatant in the absence of type 1 IFN stimulation to those with the stimulation) ISGs were calculated. (*: p<0.05)
Figure 8. The diminished type 1 IFN responses are also mediated by HIV-1 virions.

(a) Model diagram. Upon incubation of target cells with supernatant from BaL-infected leukopaks, soluble mediators, including type 1 IFN and HIV-1 virions are transferred to the target cells. Both type 1 IFN and HIV-1 virions upregulate USP18 expression, thereby dampening the responses to exogenous type 1 IFN. (b) Target CD4+ cells were
treated with T20 or cyclophilin A inhibitor (CypA) in addition to antiretrovirals for 24 hours, and then incubated with supernatant from BaL- or BaL+ LP with T20 or CypA along with antiretrovirals. 24 hours after conditioning, target cells were stimulated with exogenous type 1 IFN, and the level of 2 surrogate ISGs in cells, with or without type 1 IFN, were quantified by qPCR. The induction of 3 ISGs were calculated for each condition.
Figure 9. HIV-1 infection can be propagated in CEMx174 cells infected with HIV-1 BaL. Activated CD4+ T cells and six cell lines were inoculated with 1 pg/mL of HIV-1 BaL and 3 and 7 days after infection, supernatant p24 levels were quantified. All cell types except activated CD4+ T cells and Jurkat cells were able to propagate infection. MOLT-4 CCR5+ cells were maintained in selective media with G148.
Figure 10. Experimental Procedure for LM separation, and LM VOA. (a) Purification of LM. Bulk liver tissues were diced, enzymatically digested, centrifuged on a Histodenz gradient (Sigma-Aldrich), and then T cells were excluded using CD3 microbeads. Mononuclear cell fractions were plated on plastic to allow adherent LMs to attach while non-adherent cells were washed away, and then cultured with Resimmune, a high-affinity T cell toxin that depletes any remaining T cells. LMs were incubated for more than 30 days, without T cell mitogens, and cultured with antiretrovirals (ARVs) to prevent ex vivo infection. (b) Strategy for LM VOA. Primary human LMs from HIV-1–infected individuals were isolated, cultured ex vivo for more than 30 days and VOA conducted on LMs from subjects on ART. Remaining T cells in purified LMs were depleted by 48 hours of Resimmune treatment, and LMs were stimulated with IFNγ and HIV-1 rTat. Supernatants from stimulated LMs were filtered and target cells were cultured with the supernatant twice over 15 days. In addition, target cells were
cocultured with LMs. 11 days after coculture, cell-associated and supernatant HIV-1 DNA and RNA levels were measured in target cells.
Figure 11. LMs from HIV-1–infected individuals can transmit HIV-1 infection. (A) Results from LM VOA for all HIV-1–infected subjects. HIV-1 proviral DNA was measured from lysed target cells that were maintained with LM supernatants from each individual (red diamonds). Error bars denote mean ± SEM for some with multiple positive wells. Negative control target cells incubated with media only are represented by grey triangles. N7 is excluded in this figure because we detected HIV-1 RNA in LM supernatants before LM VOA was initiated. (B) HIV-1 RNA is detectable by LM VOA for only individuals on ART for less than 1 year. HIV-1 RNA was quantified from supernatants from target cells in LM VOA from all subjects, and from N7 LM directly. HIV-1 RNA was detectable only in supernatants from LMs in 2 subjects who had the shortest duration of ART before liver explantation. No data point for N9 is available in this figure because we couldn’t get the duration of ART before liver explantation. LOD, limit of detection; ND, not done.
Supplemental Figure 1. Rapid phosphorylation of STAT1. Jurkat cells were stimulated with type 1 IFN, for 1, 5, 10, 15, 30 and 60 minutes. After stimulation, pSTAT1 was stained and quantified by flow cytometry.
Supplemental Figure 2. ISG expression in HIV-GFP-infected CD4+ T cells. Healthy leukopaks were stimulated with PHA. Three days after stimulations, CD4+ T cells were isolated and infected with GFP-tagged HIV-1 (HIV-GFP). Three days after infection, cells were stained and GFP+ and GFP- cells were sorted by FACS. GFP+ and GFP- cells were rested for 8 hours and cells were collected. The level of 4 ISGs (MX2, ISG15, IP10, and BST2) was quantified by qPCR.
Supplemental Figure 3. B18R can neutralize the activity of type 1 IFN. Healthy leukopaks were thawed and rested overnight. CD4+ T cells were isolated by magnetic separation. 200 U/ml of type 1 IFN was incubated with different concentrations of Vaccinia virus type 1 IFN decoy receptor B18R for 1 hour. After neutralization, the mixture of type 1 IFN and B18R was added to CD4+ T cells. 6 hours later, cells were harvested and the level of MX2 and USP18 was measured by qPCR. The induction of 2 genes normalized to unstimulated CD4+ T cells was calculated.
Supplemental Figure 4. USP18 knockdown in primary CD4+ T cells. (a, b) Total CD4+ T cells were nucleofected with FITC-tagged scrambled RNA (si ct) or siUSP18. 6, 12, 18, and 24 hours after nucleofection, cells were stimulated with type 1 IFN for 6 hours and total RNA was extracted. The level of USP18 was quantified by qPCR. The transfection efficiency of siRNA was assessed by fluorescent microscopy (b). (c) Total CD4+ T cells were nucleofected with FITC-tagged scrambled RNA (si ct) or siUSP18 and cells were stimulated with type 1 IFN 24 and 48 hours after nucleofection. 24 hours after the stimulation, total protein was extracted from the cells, and USP18 and β-Actin were visualized by western blotting.
Supplemental Figure 5. Plastic adherence yields better purity of liver macrophages. CD3+ T cell contamination was detected in liver macrophages purified by CD14+ selection, whereas liver macrophages isolated by plastic adherence did not have CD3+ T cell contamination. 7SL gene was quantified as a readout for housekeeping gene expression.


virus (SIV) infection of natural hosts from pathogenic SIV infection of rhesus macaques. J Virol 84:7886-7891.


dendritic cells suppress HIV-1 replication but contribute to HIV-1 induced immunopathogenesis
64. Lavender KJ, Gibbert K, Peterson KE, Van Dis E, Francois S, Woods T, Messer RJ, Gawanbacht
replication but contribute to T cell depletion and dysfunction during chronic HIV-1 infection. JCI
Insight 2.
67. Long BR, Stoddart CA. 2012. Alpha interferon and HIV infection cause activation of human T
68. Azmy M, Murphy RL, Rosenkranz SL, Lertora JJ, Kottilil S, Cramer Y, Chan ES, Schooley RT,
Rinaldo CR, Thielman N, Li XD, Wahl SM, Shore J, Janik J, Lempicki RA, Simpson Y, Pollard RB,
Team ACTGA. 2010. Safety, tolerability, and mechanisms of antiretroviral activity of pegylated
interferon alfa-2a in HIV-1-monoinfected participants: a phase II clinical trial. J Infect Dis
201:1686-1696.
69. Azzoni L, Foulkes AS, Papasavvas E, Mexas AM, Lynn KM, Mounzer K, Tebas P, Jacobson JM,
Pegylated Interferon alfa-2a monotherapy results in suppression of HIV type 1 replication and
70. Lane HC, Davey V, Kovacs JA, Feinberg J, Metcalf JA, Herpin B, Walker R, Dayton L, Davey RT,
immunodeficiency virus (HIV) infection. A randomized, placebo-controlled trial. Ann Intern Med
112:805-811.
71. Lane HC, Kovacs JA, Feinberg J, Herpin B, Davey V, Walker R, Dayton L, Metcalf JA, Baseler M,
72. Manion M, Rodriguez B, Medvik K, Hardy G, Harding CV, Schooley RT, Pollard R, Asmuth D,
Interferon-alpha administration enhances CD8+ T cell activation in HIV infection. PLoS One
7:e30306.
73. Pillai SK, Abdel-Mohsen M, Guatelli J, Skasko M, Monto A, Fujimoto K, Yukl S, Greene WC,
Gunther HF, Wong JK, Swiss HIVCS. 2012. Role of retroviral restriction factors in the
74. Torriani FJ, Rodriguez-Torres M, Rockstroh JK, Lissen E, Gonzalez-Garcia J, Lazzarin A, Carosi G,
Sasadeusz J, Katlama C, Montaner J, Sette H, Jr., Passe S, De Pamphilis J, Duff F, Schrenk UM,
Dieterich DT, Group AS. 2004. Peginterferon Alfa-2a plus ribavirin for chronic hepatitis C virus
75. Palesch D, Bosingcher SE, Mavigner M, Billingsley JM, Mattingly C, Carnathan D, Paiardini M,
Chahroudi A, Vanderford T, Silvestri G. 2018. Short-term plFb-alpha2a treatment does not


Curriculum Vitae

Sho Sugawara

Address: 3040 Abell Ave, Baltimore, MD, 21218

Email: ssugawa1@jhmi.edu

Phone: 443-600-5435

Education

Johns Hopkins University, Baltimore, MD.

Ph.D., Immunology, expected in June 2019.

Dissertation: HIV-1 infection is associated with increased USP18 levels and dampened type 1 IFN responses

Soka University, Hachio-ji, Tokyo, Japan.

B.S., Bioinformatics,

Thesis: The role of 38-nt region in env gene expression in Murine Leukemia Virus.

Research Interests

viral immunology, HIV-1 infection, type 1 IFN, NK cells, cellular immunology, animal model, T cells, innate immune response.
Laboratory Skills

flow cytometry, cell sorting, cell culture, western blotting, molecular biology, siRNA knockdown, human translational studies

Publications


**Presentations**

**S. Sugawara, R. El-Diwany, D.L. Thomas, A.L. Cox, J.N. Blankson, A. Balagopal.** HIV-1 infection is associated with increased USP18 and dampened type 1 IFN response.

*Accepted for poster by CROI 2019. Abstract# 0211*

**Research Experience**

**Dr. Andrea Cox, Dr. Ashwin Balagopal** Johns Hopkins University, Baltimore, MD.

**PhD. Student.** May 2015 to present

**Professor Edward Harhaj,** Johns Hopkins University, Baltimore, MD.

**Rotation Student.** January 2015 to March 2015

**Dr. Joel Blankson.** Johns Hopkins University, Baltimore MD.

**Rotation Student.** September 2014 to December 2014

**Professor Sayaka Takase.** Soka University, Hachio-ji, Tokyo, Japan.

**Master Student.** April 2014 to August 2014

**Undergraduate Student.** February 2013 to March 2014.
Professor Hideki Kawai. Soka University, Hachio-ji, Tokyo, Japan

**Undergraduate Student.** September 2012 to January 2013.

Professor Izumi Kubo. Soka University, Hachio-ji, Tokyo, Japan.

**Undergraduate Student.** May 2011 to January 2012

- Helped summarizing research papers.

**Fellowship and Awards**