VIRUS-HOST INTERACTIONS IN HIV-1 PATHOGENESIS AND VIRAL IMMUNE EVASION

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ABSTRACT

Li-Chung Tsao: Virus-Host Interactions in HIV-1 Pathogenesis and Viral Immune Evasion
(Under the direction of Lishan Su)

Human viruses such as the Human Immunodeficiency virus (HIV-1) causes chronic diseases and is a major public health concern. My dissertation focuses on the molecular interactions between the virus and the host, tackling two major topics in HIV-1 research. The first topic focuses on HIV-1 pathogenesis and the factors contributing towards AIDS disease progression. Using genetics and pharmacological approaches, we demonstrated the interaction between the viral envelope and the CCR5 receptor contributes to the killing of uninfected bystander CD4 T cells in vitro and in vivo. This study suggests therapeutic strategies targeting CCR5 may reduce HIV-1 pathogenesis and AIDS disease progression.

The second topic focuses on how HIV-1 escapes from restriction factors, a critical part of our innate immune defense against HIV-1. We discovered the TET2 methylcytosine dioxynase is a new HIV-1 restriction factor that binds to HIV-1 cDNA and inhibits viral reverse transcription. The viral accessory protein Vpr hijacks the $CRL4^{VprBP}$ E3 ligase to promote the degradation of TET2 and thereby relieving this restriction. Lastly, we also discovered an alternative role of TET2 in modulating the Type-1 interferon antiviral response in HIV-1 inhibition. We demonstrated TET2 is essential for the interferon-mediated induction of antiviral proteins that targets HIV-1 replication, including MX2 and IFITM3. We report a new restriction factor TET2 that inhibits

HIV-1 through various mechanisms, and we elucidated the mechanism how Vpr promotes HIV-1 escape from this immune defense.

In summary, my dissertation research unraveled the contributions of different virus-host interactions towards viral pathogenesis and viral immune evasion, which may provide new therapeutic targets for HIV-1 treatments.

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LIST OF ABBREVIATIONS

AMD3100	CXCR4 antagonist
AMP	HIV protease inhibitor Amprenavir
APOBEC3G	Apolipoprotein B MRNA Editing Enzyme Catalytic Subunit 3G
CCR5	C-C Chemokine Receptor type 5
CD4	Cluster of differentiation 4
cGAS	Cyclic GMP-AMP synthase
CRL	cullin-ring ubiquitin ligase
CUL4	Cullin 4
CXCR4	C-X-C Chemokine Receptor type 4
DDB1	Damage Specific DNA Binding Protein 1
DENV	Dengue Virus
EBV	Eppstein-Barr virus
ENV	HIV Envelope
G2	Gap 2 cell cycle phase
HAART	highly active anti-retroviral therapy
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HDAC	Histone deacetylases
HIV	Human immunodeficiency virus
HLA-DR	Human Leukocyte Antigen - antigen D Related

HPV	Human Papilloma Virus
HSV-1	Herpes Symplex Virus 1
IAV	Influenza Virus
IFI16	Interferon Gamma Inducible Protein 16
IFITM	Interferon-induced transmembrane protein
IFNAR	Type-1 Interferon receptor
IFN-I	Type-1 Interferon
IFNα	Type-1 Interferon alpha
IFNβ	Type-1 Interferon beta
IL-1β	Interleukin 1 beta
IRF	Interferon Response Factor
ISG	Interferon-Stimulated-Genes
JAK	Janus kinase
KSHV	Kaposi's sarcoma-associated herpesvirus
LTR	Long terminal repeat
MAVS	Mitochondrial antiviral-signaling protein
MG132	Proteasome inhibitor
MIP-1β	Macrophage Inflammatory Proteins Beta
MOI	multiplicity of infections
MUS81	MUS81 Structure-Specific Endonuclease Subunit
MX	myxovirus resistant protein
MYD88	Myeloid differentiation primary response gene 88

Nef	Negative Regulatory Factor
NVP	HIV RT inhibitor Neverapine
OAS	2'-5'-Oligoadenylate Synthetase
p24	HIV capsid protein
PAMPs	pathogen-associated-molecular-patterns
PBMC	peripheral blood mononuclear cell
pDC	plasmacytoid dendritic cell
PKR	Protein Kinase R
Ralt	HIV integrase inhibitor Raltagravir
RLR	RIG-Like-Receptors
RT	Reverse Transcription
RTC	Reverse Transcription Complex
SAMHD1	SAM domain and HD domain-containing protein 1
SERINC	Serine Incorporator
SIV	Simian immunodeficiency virus
SLFN11	Schlafen Family Member 11
SLX4	SLX4 Structure-Specific Endonuclease Subunit
SMC	Structural Maintenance Of Chromosomes
STAT	Signal Transducer and Activators
STING	Stimulator of interferon genes
T20	HIV fusion inhibitor
TAK-779	CCR5 antagonist

Tat	Transactivator protein
TET2	Tet Methylcytosine Dioxygenase 2
TLR	toll-like-receptor
TNFα	Tumor necrosis factor alpha
TRAIL	TNF-related apoptosis-inducing ligand
TRIM5α	Tripartite motif-containing protein 5
UNG2	Uracil DNase Glycosylase
Vif	Viral Infectivity Factor
VLP	Virus-Like particle
Vpr	Viral Protein R
VprBP	Vpr-binding protein
Vpu	Viral Protein U
Vpx	Viral Protein X

CHAPTER 1. INTRODUCTION

1.1. OVERVIEW OF HIV-1 REPLICATION

HIV-1 is a member of the lentivirus genus, part of the family of retroviruses. The virus genome consists of the LTR promoter, which drives the expression of viral proteins including the structural proteins Gag and Env; regulatory and replication proteins Tat, Rev and Pol; and accessory proteins Vif, Vpr, Vpu and Nef (Fig. 1.1A). In my dissertation research, I first focus on HIV-1 Env and its role in HIV-1 pathogenesis (Chapter 2). The next two chapters focus on the viral accessory protein Vpr, and its role in enhancing HIV-1 replication by counteracting host antiviral defenses (Chapter 3-4). HIV-1 infects cells that express the CD4 receptor, including CD4+ T lymphocytes and CD4+ myeloid cells such as monocytes and macrophages. HIV-1 locates the target cells by binding to the CD4 receptor and the co-receptors CCR5 or CXCR4. The co-receptor usage defines the tropisms of the virus. A typical representation of the dynamics of viral tropism, CD4 T cell counts and viral load in infected human subjects is shown in Figure 1.1B. CCR5-tropic viruses dominate in the early stages of infection and are transmitted between human subjects. Infected subjects experience a peak of HIV-1 viral load and a drop of CD4 T cell counts in the first few weeks. Then the disease enters a clinical latency stage, with immune defense being unable to completely clear the virus. In the late AIDS disease stage (~10 years after infection), CXCR4-tropic or dual-tropic HIV-1 emerges in about 50% of HIV-1 patients. A rapid drop of CD4 T cell counts leading to immunodeficiency is observed. The importance of co-

receptor usage by HIV-1 and its role in pathogenesis will be studied and further discussed in Chapter 2 of this dissertation (1).

A schematic overview of the HIV-1 replication is presented in Figure 1.2A. After binding to CD4 receptor and coreceptor, the gp41 domain of HIV-1 Env induces the fusion of viral membrane with the host cell membrane. This allows the viral capsid to enter the cellular cytoplasm, leading to the capsid uncoating and reverse transcription of the viral RNA genome into double-stranded cDNA by the viral polymerase. It is widely believed that capsid uncoating and reverse transcription are orchestrated concomitantly and are dependent on each other (2). The reverse transcribed cDNA forms a pre-integration complex consisting of host and viral proteins, which mediate the import of viral cDNA into the nucleus and subsequent integration into the host genome. HIV-1 transcription is promoted by the viral transactivator protein Tat, and HIV-1 mRNA nuclear export is controlled by HIV-1 Rev. Following RNA translation, newly synthesized viral proteins as well as the viral RNA genome translocate to the plasma membrane to assemble into immature HIV-1 virions. The new viruses bud off from the cell membrane and immature HIV-1 virions are released. Finally, during maturation, the viral protease cleaves the immature Gag-Pol poly protein to form the mature infectious virion.

1.2. VIRAL-HOST INTERACTIONS LEADING TO PATHOGENESIS

The hallmark of HIV-1 pathogenesis and AIDS disease progression is the depletion of CD4+ T lymphocytes (Fig. 1.1B) and the subsequent loss of immune competency. Since the discovery of this phenomenon in 1981, many efforts and research have aimed to explain how HIV-1 infection causes AIDS disease progression. The factors that contribute to HIV-1 pathogenesis are enigmatic and have been widely reported, which include both host factors

and viral factors. The mechanisms of pathogenesis can be generally categorized into two major models that are not mutually exclusive. The first model is based on the strong correlation between chronic immune activation with AIDS disease progression in HIV-infected patients and in pathogenic SIV infected monkeys (3–5). Multiple studies have subsequently shown the aberrant levels of several pro-inflammatory cytokines that may contribute to T cell loss, immune dysfunction and disease progression (6–11). The second model is based on the toxic effects of viral factors acting on the host cells. The suppression of viral replication by highly active anti-retroviral therapy (HAART) substantially halted viral pathogenesis and AIDS disease progression, indicating the levels of viral proteins or viral nucleic acids are playing an important role in HIV-1 pathogenesis. Indeed, studies on many viral factors have elucidated interesting mechanisms and their contributions to viral pathogenesis (1, 12–15).

1.2A. Role of chronic immune activation leading to HIV-1 immunopathogenesis. Chronic immune activation in HIV-1 infected individuals is highly correlated to CD4 T cell depletion and immunodeficiency (3, 11). In several studies, the T cell activation markers (e.g. HLA-DR/CD38 levels) are better predictive markers than viral loads are for AIDS disease progression (6, 16). Compelling evidences supporting this model are found in studies with simian immunodeficiency virus (SIV) infection. SIV infections in Asian monkeys (e.g. Rhesus macaques, pigtail macaques) are pathogenic, and result in persistent immune activation, CD4 T cell depletion and AIDS diseases. In contrast, SIV infections in African Green monkeys and sooty mangabeys are non-pathogenic despite high levels of viral replication, which does not result in CD4 T cell depletion, corresponding with a transient immune activation that is resolved shortly after infection (4, 5, 17, 18). Several mechanisms contributing to chronic immune activation in HIV-1 infection have

been proposed. The loss of gut tissue integrity leading to microbial translocation is proposed to contribute to HIV/SIV induced systemic immune activation in patients and monkeys (11). Recent emerging evidence also indicates type-1 interferons (IFNα and IFNβ) contribute to chronic immune activation in HIV infection, with IFNα produced by plasmacytoid dendritic cells (pDC) likely having the most pronounced effects (5, 6, 18, 19). pDC senses HIV-1 RNA through the toll-like-receptor 7 (TLR7), leading to the production of high levels of IFNα (20). In monocytes and macrophages, HIV-1 reverse transcribed DNA during infection can also be detected by the host DNA sensors cGAS or IFI16 to elicit an IFNβ response (21, 22). The sensing of viral nucleic acids and the induction of the IFN response will be discussed more in Chapter 1.3A. It is now believed that type-1 IFN acts as a double-edged sword in HIV-1 infection. Type-1 IFN efficiently suppresses HIV-1 replication, as demonstrated in cell culture experiments (23). However, HIV-1 replicates effectively in vivo despite high levels of IFNs (6, 7), indicating insufficient virus suppression by the IFN response. This baffling phenomenon is explained by how HIV-1 carries out multiple strategies to evade the innate immune defense (discussed in Chapter 1.3). On the other hand, high levels of persistent type-1 IFN also induce cellular death contributing to HIV-induced CD4 T cell depletion and immunopathogenesis (6, 24). The blockade of type-1 IFN signaling has been shown to reduce persistent LCMV infection (25, 26). Future work on the blockade of IFN signaling in chronic HIV-1 infection will reveal the contribution of IFN to viral replication and immunopathogenesis.

HIV-mediated depletion of CD4 T cells can be broadly categorized into “direct” killing of productively infected CD4 T cells, and killing of uninfected “bystander” CD4 T cells (27). Interestingly, the rate of CD4 T cell decline is discordant with low level of productively infected

cells in HIV-positive individuals. This suggests “direct” death of productively infected cells contributes minimally towards AIDS progression, and bystander cell killing is likely the leading cause for CD4 T cell loss and AIDS progression. Multiple studies have attempted to elucidate the mechanism for direct cell death and bystander cell death. Earlier studies in HIV and SIV infected lymph nodes showed a predominant levels of bystander CD4 T cells undergoing apoptosis (28). Furthermore, the interaction between infected macrophages and pDC with CD4 T cells also contributes to bystander CD4 T cell apoptosis (29–31). Mechanistically, HIV-induced apoptosis can be stimulated by the induction of cell death-inducing ligands such as TRAIL (9, 31), FAS/FASL (32) and TNFα (33), which are upregulated during HIV-induced systemic immune activation. Several HIV-1 proteins have also been reported to directly induce the expression of those death receptors/ligands (discussed in Chapter 1.2B). Recent reports support a new mechanism of CD4 T cell death in HIV-1 infection, involving an alternative form of cell death called pyroptosis. This model is based on the foundation that HIV-1 reverse transcription occurs inefficiently in resting CD4 T cells, leading to the accumulation of incomplete HIV-1 reverse transcript DNA in the cytoplasm (34). These viral DNA induces the activation of DNA sensor IFI16 (22, 35), leading to the activation of the inflammasome and Caspase-1, secretion of the IL1-β cytokine and promoting cellular pyroptosis (36). In addition, HIV-1 infection in monocytes also induces the expression of IL-1β through the activation of TLR8 and NLRP3 inflammasome (37). These reports suggest inflammasome activation and IL-1β is another important factor associated with HIV-1 infection and chronic immune activation, leading to immunopathogenesis.

1.2B. Role of HIV-1 proteins in viral pathogenesis. Many of the HIV-1 proteins have been found to have pro-apoptotic effects on the host cell, probably contributing to viral pathogenesis.

These include viral Env, Tat, Nef, Vpr, Vpu and HIV protease. The viral Env, expressed on surface of virions or of infected cells, can directly interact with CD4 and the co-receptors CCR5/CXCR4 on uninfected neighboring CD4 T cells (38). This suggests HIV-1 Env plays a pivotal role in bystander pathogenesis. Indeed, both membrane-bound and soluble Env induce apoptosis of bystander CD4 T cells, which requires the binding to CD4 and the chemokine co-receptors (1, 39, 40). Membrane-bound Env can also induce the formation of syncytia to kill bystander CD4 T cells, which involves the fusion of an infected cell with neighboring uninfected CD4 cells (38, 41). HIV-1 strains can be broadly divided into three groups based on their Env-coreceptor usage, using CCR5 or CXCR4 or both co-receptors for viral entry. R5-tropic HIV-1 dominates during the early stages of HIV-1 infection. In the late AIDS disease stage, X4-tropic and dual-tropic HIV-1 strains (capable of using both co-receptors) have been reported and are usually more pathogenic (Fig. 1.1B). The role of Env in pathogenesis has been extensively studied using the dual-tropic HIV-1 strain termed R3A. The HIV-R3A strain was isolated from a rapid-progressing AIDS patient, and its encoded viral Env was found to be highly pathogenic (41). R3A depletes CD4 T cells and thymocytes through multiple mechanisms including fusion-induced apoptosis (42) and type-1 IFN mediated cell death (12, 43). In humanized mice, R3A infection causes rapid depletion of both infected and bystander CD4 T cells (1, 44). In the first study of this dissertation (chapter 2), we have extended the mechanism of R3A-induced pathogenesis, demonstrating the interaction of R3A-Env with the co-receptor CCR5 plays a substantial role in bystander CD4 T cell apoptosis (1).

In addition to the HIV-1 Env, other viral proteins have been reported to induce cellular apoptosis. The viral transactivator protein Tat, which promotes HIV-1 gene transcription, has

been shown to promote TRAIL expression in macrophages to induce the apoptosis of bystander CD4 T cells (45). The HIV-1 Vpu protein has also been shown to have pro-apoptotic effects. The expression of Vpu increased the susceptibility of T cells to Fas-mediated apoptosis (14). HIV-1 Nef is one of the earlier proteins produced after HIV-1 infection, and is known to play a multifunctional role including down-regulation of multiple surface proteins, such as CD4, MHC-I and most recently SERINC5/6, to enhance HIV-1 replication (46, 47). Nef is also known to have toxic effects on the host cells, as Nef-expressing T cells have upregulated Fas/FasL levels (48), downregulated levels of anti-apoptotic proteins Bcl-2 (49), and undergo apoptosis by both caspase-dependent and independent mechanisms. HIV-1 Vpr is another accessory protein demonstrated to induce apoptosis. Expression of Vpr induces cell cycle arrest at the G2-phase, which can indirectly contribute to cell death (50, 51). In addition, Vpr has been shown to interact with mitochondrial membrane proteins Bax, ANT and VDAC, causing the release of cytochrome c and activation of Caspases 9 and 3 (50, 52). A more detailed overview on Vpr will be discussed in Chapter 1.3D. Lastly, the viral protease has also been shown to cleave Bcl-2 and the pro-caspase-8 protein (53, 54), which can promote apoptosis in infected cells.

1.2C. Concluding remarks and future directions. In summary, HIV-1 pathogenesis is a complex phenomenon caused by aberrant levels of inflammatory cytokines (IFNα/β, TNFα and IL1β) during chronic immune activation, as well as by the pathogenic attributes of multiple viral proteins including Env, Vpr, Nef, Tat, Vpu and viral protease. The HIV-1 Env is one of the most investigated viral pathogenic factor, which can be easily targeted by drugs and antibodies as it is expressed on virion/cell surfaces. It is therefore important to investigate the interaction

partners of HIV-1 Env in relevant HIV-1 infection models to elucidate how this viral-host interaction results in CD4 T cell depletion.

1.3. VIRAL-HOST INTERACTION LEADING TO HIV-1 EVASION OF THE ANTIVIRAL IMMUNE RESPONSE

1.3A. Overview of type-1 interferons (IFN-I)-mediated antiviral responses. The IFN-I response is the canonical antiviral immune defense, which inhibits a broad range of viral pathogens through the induction of hundreds of interferon-stimulated genes (ISG). Viruses contain conserved molecular features that are not found in the eukaryotic host. These molecular features, also called “pathogen-associated-molecular-patterns, or PAMPs, are recognized by host immune “sensors” to elicit an antiviral response. Viral sensors include Toll-Like-Receptors (TLR7 and TLR9) in the endosome that detect viral RNA and dsDNA, respectively. In addition, there are cytoplasmic viral sensors such as the RIG-Like-Receptors (RLR) that detect virus-specific RNA, and receptors cGAS and IFI16 that detect viral DNA. Upon recognition of viral nucleic acids, the immune sensors initiate a specific signaling cascade that leads to the activation of transcription factors IRF3/IRF7 (and NFkB) and the expression of type-1 interferons (IFNα or IFNβ). IFNs are released outside the cells and signals in a paracrine or autocrine manner. IFN-I engages with the IFN-receptor complex (IFNAR) on cell surfaces, which initiates the IFN signaling pathway. This starts with the activation of Janus Kinase proteins (JAK), which phosphorylate members of the Signal Transducer and Activators (STAT) family of proteins STAT1 and STAT2, leading to STAT dimerization and translocation into the nucleus. This subsequently leads to their binding with IRF9 to drive the transcriptional activation of hundreds of Interferon-Stimulated-Genes (ISG). The large spectrum of ISGs consists of factors that

regulate the IFN-induced response, and many ISGs are antiviral effectors, capable of inhibiting specific steps of virus replication.

1.3B. Viral antagonism of the IFN-induced immune defense. As type-1 IFN induces a hostile cellular environment against viruses, this has also driven evolutionary pressures on viruses to develop a variety of strategies to counteract this host defense. These strategies can be categorized into three groups: inhibition of IFN production, inhibition of IFN signaling, and inhibition of the ISG effectors.

The induction of IFN production can be divided into three parts: recognition of viral RNA/DNA by the viral sensors, downstream signaling by adaptor proteins (such as MYD88, MAVS and STING), and finally the activation of transcription factors IRF3/7 and NFκB. Viruses escape from immune detection by utilizing viral proteins that target a step in the IFN production pathway. To list a few examples, Influenza virus NS1 protein suppresses the ubiquitination of the RIG-I RNA sensor (55). Hepatitis C virus NS5A protein directly binds and suppresses the MYD88 adaptor for TLR signaling (56). The adaptor protein STING for RLR signaling is antagonized by KSHV vIRF1 (57). Finally, the transcription factor IRF3 for IFN expression is commonly antagonized by viruses, by Human Papilloma Virus (HPV) E6 protein and Herpes-Simplex-virus ICP0, both preventing IRF3 translocation to the nucleus (58, 59).

In addition to antagonizing the viral sensing and IFN induction, viruses are also known to counteract the JAK-STAT-IRF9 IFN signaling pathway to prevent ISG induction. The STAT proteins are very critical in this pathway and they are one of the most targeted protein families by viruses through various mechanisms. These include targeting STAT1 and STAT2 for proteasomal degradation by Dengue virus NS5 and parainfluenza virus V protein (60, 61),

preventing STAT phosphorylation by Epstein-Barr virus LMP-1 and Marburg virus VP40 protein (62, 63), and preventing nuclear localization of STAT-IRF9 complex by adenovirus E1A and HPV E7 protein (64, 65).

Upon successful IFN signaling, the cell enters an antiviral state, with multiple antiviral effector proteins being highly expressed in the cell (66). Each effector ISG inhibits a virus-specific feature that is foreign to the host. For example, the myxovirus resistant (MX) proteins MX1 and MX2 are well-known ISG effectors. MX1 blocks endocytic trafficking of incoming viral components and prevents them from reaching their cellular destination (67). MX2 is known to bind with HIV-1 pre-integration complex and prevent viral nuclear import and integration (68–70). Other well-known ISG effector proteins include the 2'-5'-Oligoadenylate Synthetase (OAS) family proteins, Protein Kinase R (PKR) and Interferon-induced-transmembrane (IFITM) proteins. OAS proteins recognize viral RNA and degrade them through RNaseL activation (71). PKR specifically recognizes viral RNA, e.g. RNA lacking the 5'phosphate cap or double-stranded RNA, and inhibits the translation of viral transcripts (72). Lastly, IFITM incorporated in the virions prevent virus fusion with the host cells (66, 73).

1.3C. HIV restriction factors and mechanism of viral evasion. Humans and primates have evolved to express antiviral effector proteins against HIV/SIV, or restriction factors, and in most cases they are induced by type-1 IFN and are ISG effectors. HIV-1 in turn utilizes accessory proteins to directly antagonize those restriction factors. A schematic representation of HIV-1 restriction factors and their antagonism by viral accessory proteins is demonstrated in Figure 1.2A. There are five well-defined examples of this intriguing phenomenon. First, the Serine Incorporator 3/5 (SERINC3/5) are transmembrane proteins incorporated into the nascent HIV-1

virion membrane and inhibits HIV-1 infectivity. This is counteracted by HIV-1 Nef, which induces the downregulation of SERINC from cell surface membrane to prevent its virion incorporation (46, 47). Second, TRIM5α is an ISG that binds to HIV-1 capsids and inhibits capsid uncoating in old world monkeys, which serves as an important barrier for HIV-1 to inhibit primates other than humans. In contrast, SIV has evolved its viral capsid structure to escape the recognition and restriction by TRIM5α (74, 75). Third, APOBEC3G is an ISG that effectively impairs HIV-1 reverse transcription by introducing G to A hyper-mutations in the nascent retroviral DNA, resulting in defective proviral genomes (76). The HIV-1 Vif protein counteracts APOBEC3G by promoting the poly-ubiquitylation of ABOBEC3G by the $CRL5^{ElonB/C-CBF\beta}$ E3 ligase, resulting in proteasomal degradation (77). Fourth, Tetherin is another ISG that broadly inhibits HIV-1 and other viruses by sequestering their release from the cellular plasma membrane (66, 78). HIV-1 Vpu relieves this restriction by hijacking the $CRL1^{\beta\text{-}TrCP}$ E3 ligase and induces poly-ubiquitylation and degradation of tetherin, (79). Fifth, SAMHD1 is a myeloid cell-specific restriction factor that inhibits HIV reverse transcription through the depletion of cellular dNTP pool, which is essential for viral reverse transcription efficiency. The Vpx protein encoded by HIV-2 and SIV can overcome this myeloid restriction by targeting SAMHD1 to polyubiquitylation by $CRL4^{VprBP}$ ligase and proteasomal degradation (80, 81). A summary of HIV-1 accessory proteins, the E3 Ligase complex they hijack, and the restriction factor substrate they target is shown in Figure 1.2B. Other HIV-1 restriction factors such as MX2, IFITM and SLFN11 also inhibit HIV-1 through different mechanisms, but they are not known yet to be specifically antagonized by a viral accessory protein.

1.3D. The enigmatic role of HIV-1 Vpr. The HIV-1 accessory protein Vpr will be a major focus in my dissertation. Like the other HIV accessory proteins, Vpr is believed to enhance HIV-1 replication by helping the virus overcome restriction factors (82). The Vpr gene is conserved among HIV and SIV viruses, and encodes a small 14 kDa Vpr protein. The functional importance of Vpr was initially shown in rhesus macaques infection using Vpr-deficient SIV, which exhibits a decrease in viral replication and delay in AIDS disease progression (83). In the humanized mouse model, researchers have shown Vpr enhances acute HIV-1 infection by promoting their replication in regulatory T cells (84). The role of Vpr has been extensively studied in cell culture models. In addition to inducing cellular apoptosis as discussed earlier (Chapter 1.2B), Vpr is known to increase HIV-1 replication in myeloid cell cultures. Vpr-deficient viruses replicate less well in monocytes and macrophages, but showed no significant differences in CD4 T lymphocytes infection, indicating Vpr may target a myeloid specific restriction factor that inhibits HIV-1 replication (85–87). The Vpr protein was found incorporated inside the HIV virion particle (estimated ~275 Vpr molecules per virion), and therefore Vpr is believed to carry out its function immediately after virion entry into the cytoplasm (88, 89). In 2006, the Vpr-Binding-Protein (VprBP) was discovered as a substrate specificity module for the Cul4-DDB1 ubiquitin ligase (90), and subsequently it was proposed that Vpr carry out its function through its ability to interact with VprBP, thereby targeting host factors to the $CRL4^{VprBP}$ E3 ligase for poly-ubiquitylation and proteasomal degradation (91, 92). One of the most extensively studied attribute of Vpr is its ability to induce G2 cell-cycle arrest, a Vpr feature first reported in 1995 (93). Only recently, the molecular mechanism of G2 arrest induction by Vpr has been elucidated, which involves Vpr-mediated degradation of MUS81 through hijacking $CRL4^{VprBP}$ E3

ligase, resulting in the premature activation of SLX4 complex, a DNA endonuclease. This enhances the cleavage of DNA by SLX4 , resulting in activation of the DNA damage response and cell cycle arrest (94).

How Vpr promotes HIV-1 replication remains unclear. The premature activation of SLX4 by Vpr has been proposed to help the virus escape from immune sensing. This model suggests SLX4 activation result in the cleavage of “excess” viral DNA in the cytoplasm, therefore minimizing viral DNA detection by viral sensors and activation of the IFN response (94). However, how Vpr modulates SLX4 to only cleave “excess” viral DNA without aberrantly inhibiting viral replication, remains a big caveat in this model.

In addition to counteracting the type-1 IFN response, Vpr has also been extensively studied for its impact on specific steps in the virus replication cycle. After cell entry, HIV-1 undergoes capsid uncoating and reverse transcription (RT), with the two processes happening in parallel, while the reverse transcription complex (RTC) is trafficked through the cytoplasm towards the nucleus (2). Several studies have confirmed Vpr co-localizes with HIV-1 RTC, and remains associated with the viral DNA within 4-16h after infection (95, 96). This suggests Vpr is either directly promoting HIV-1 reverse transcription, or it associates with the RTC to antagonize an unknown restriction factor against reverse transcription. Indeed, several studies have suggested Vpr promotes the HIV-1 reverse transcription step. First, Vpr interacts with the primer for viral reverse transcription Lys-tRNA synthetase , suggesting a potential Vpr role in RT initiation (97). However, follow-up studies on this hypothesis has not been conducted. Second, Vpr promotes RT efficiency of virions produced from non-permissive T cell lines (98), suggesting Vpr may counteract a restriction factor affecting RT that are specifically expressed in certain

non-permissive cell lines. Third, in addition to promote RT efficiency, Vpr has been shown to promote RT accuracy and reduce mutation rates introduced in the viral cDNA during reverse transcription in macrophages (99, 100). The mechanism of this phenomenon has been linked to Uracil DNase Glycosylase (UNG2), which is incorporated into HIV-1 virions and is associated with HIV-1 RTC after viral entry (99). However, how UNG2 affects HIV-1 RT accuracy and efficiency remains contradictory. Several reports indicate the interaction of Vpr with UNG2 results in UNG2 incorporation into virions, and that UNG2 enhances viral RT accuracy by repairing dUTP incorporation into viral DNA during reverse transcription (99, 101). Another study has shown virion-associated UNG2 enhances HIV-1 RT efficiency and progression (102). However, multiple reports have challenged this model, showing that UNG2 actually has a detrimental effect on HIV-1 replication (103–106). Indeed, UNG2 is a substrate that is targeted by Vpr to $CRL4^{VprBP}$ poly-ubiquitylation for proteasomal degradation (103, 107). Future work on the activities of UNG2 and SLX4, which are confirmed targets by Vpr to the $CRL4^{VprBP}$ (Fig. 1.2B), may elucidate potential mechanisms of how Vpr promotes virus replication.

It is known that Vpr has the ability to shuttle between the cytoplasm and the nuclear compartments, and that Vpr may play a role in modulating HIV-1 nuclear import (108). Mutational analysis suggests the ability of Vpr to interact with nucleoporins such as hCG1 to localize with the nuclear envelope may be required for Vpr to promote HIV-1 replication in macrophages (109). However, convincing mechanistic evidences are lacking and required to fully elucidate how Vpr can directly or indirectly promote HIV-1 DNA nuclear import.

Lastly, several studies indicate Vpr can increase HIV-1 gene expression by transactivation of the viral LTR promoter (110). One proposed mechanism of this phenomenon

is by arresting cells in G2 phase, the histone acyltransferase CBP as well as transcription factors NF-κB and c-JUN are more likely to be recruited to the LTR promoter leading to increased viral gene expression (111, 112). However, the biological significance of this cell cycle arrest phenomenon by Vpr is not well understood, as it only minimally increased viral gene expression but at a cost of killing the virus-producing cells through this cytostatic effect of Vpr (15). Recently, Vpr has been shown to induce the proteasomal degradation of Histone deacetylases (HDACs) and reducing the epigenetic-mediated silencing of LTR promoter, a new implication of how Vpr modulates LTR activity (110). Interestingly, Vpr has also been reported to promote the gene expression of unintegrated episomal HIV-1 DNA, a virus replication pathway that utilizes RT DNA products that failed to integrate in the host genome (113). Two recent reports discovered that Structural Maintenance Of Chromosomes 5 and 6 (SMC5/6) inhibits episomal DNA activity in hepatitis B virus (HBV) replication. Coincidentally, SMC5/6 is antagonized by the HBV X protein by promoting SMC5/6 polyubiquitylation by CRL4-DDB1 E3 Ligase and proteasomal degradation (114, 115). It will be interesting to investigate whether Vpr also counteracts SMC5/6, a potential mechanism for Vpr in promoting episomal HIV-1 DNA gene expression.

1.3E. Concluding remarks and future directions. The role of Vpr remains enigmatic for several reasons. First, Vpr is a pathogenic factor that contributes to cellular apoptosis and G2 cell cycle arrest. Whether this has evolved as a side effect, or that these attributes are actually beneficial to the virus in natural infection remains debatable in the field. Second, although it is clear that Vpr increases HIV-1 replication in myeloid cell cultures, the mechanism underlying this phenomenon remains poorly understood. Third, the role of Vpr in HIV replication and disease

progression *in vivo* needs further investigations using Vpr mutants that are defective for the different specific Vpr attributes. The most appealing hypothesis remains that Vpr increases HIV replication by targeting a putative restriction factor for proteasomal degradation by the $CRL4^{VprBP}$. Emerging evidences demonstrate Vpr also confers viral resistance against type-1 interferons, leaving this as another appealing mechanism of Vpr functions. In this dissertation, we discovered the TET2 methylcytosine dioxynase is a new HIV-1 restriction factor. Our extensive studies demonstrated three key findings. First, Vpr targets TET2 for proteasomal degradation through hijacking the $CRL4^{VprBP}$ E3 Ligase. Second, TET2 is a direct inhibitor of HIV-1 reverse transcription in myeloid cells by binding with HIV-1 cDNA. Third, TET2 modulates the expression of antiviral genes MX2 and IFITM3 in the type-1 interferon signaling pathway to suppress HIV-1 replication at different steps of the viral life cycle. As other HIV accessory proteins have evolved similar mechanisms to counteract HIV restriction factors, our study adds to the literature on how HIV-1 has evolved to escape from the host immune defense through antagonizing restriction factors and type-1 interferon signaling.

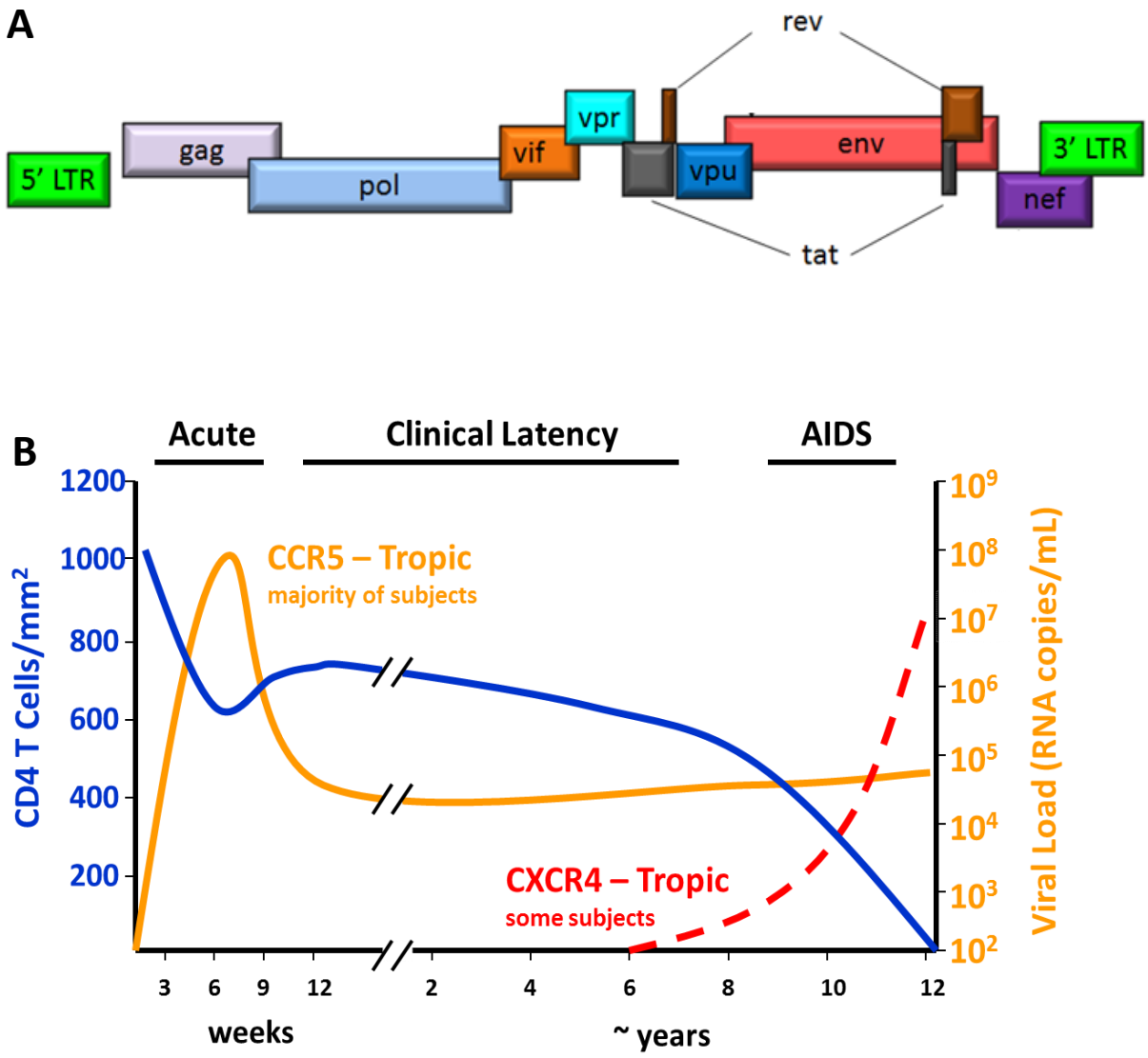

Figure 1.1. Overview of HIV-1 and AIDS disease progression

(A) Schematic representation of the HIV-1 genome, with the LTR promoter and viral gene bodies indicated. Structural genes include the viral capsid Gag and envelope Env. Replication genes includes the viral enzymes reverse transcriptase, intergrase and protease, which are generated from the polyprotein Pol. In addition, the viral transactivation factors Tat and Rev promotes viral gene expression and mRNA export, respectively. The remaining genes encodes viral accessory proteins, which are Vif, Vpr, Vpu and Nef. In HIV-2 and SIV, there is also the Vpx gene (not shown).

(B) A typical representation of HIV-1 tropism, viral load and CD4 T cell levels found in patients over time after contraction of HIV-1. CCR5-tropic HIV-1 are commonly transmitted in human subjects, associated with a peak of viral load at 6-9 weeks of acute infection stage, and a rapid drop of CD4 T cell counts. After a few months, the viral load is suppressed by the immune system, accompanied with a small rebound of CD4 T cell counts. However, HIV-1 remains in the

body, replicating at lower levels, termed clinical latency stage. Over a long period of time (usually >10 years), a rapid CD4 T cell depletion is observed. In 50% of patients, an emergence of CXCR4-tropic or dual-tropic HIV-1 strains emerge during or before the late stage AIDS disease.

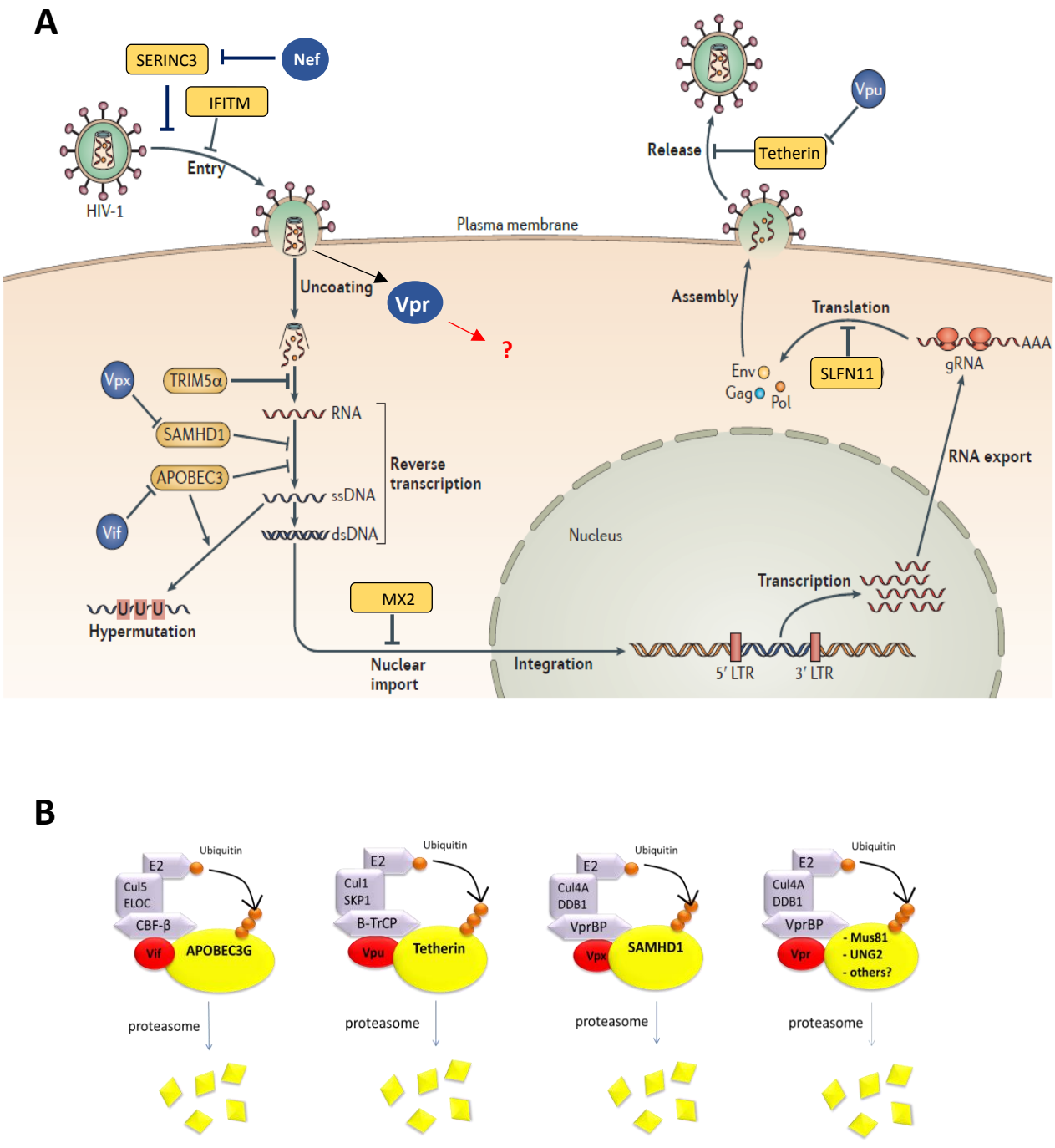

Figure 1.2. Overview of HIV-1 replication steps and evasion from restriction factors
(A) Schematic representation of HIV-1 replication steps. The early steps of replication includes: Virus binding to surface receptors followed by membrane fusion for entry, viral capsid uncoating and reverse transcription of RNA genome into DNA, nuclear import of viral DNA and integration into the human genome. The later steps of replication includes: viral gene expression through the LTR promoter, export of viral mRNA and genomic RNA, translation of viral transcript to make viral proteins, assembly of nascent virion particles near the surface

membrane and budding of virion particles from the surface. Indicated yellow and gray boxes are HIV-1 restriction factors, each known to inhibit a specific step in HIV-1 replication. The tripartite motif-ccontaining protein 5α (TRIM5α) promotes accelerated uncoating of viral capsid, preventing cDNA synthesis in reverse transcription. SAM and HD-containing protein 1 (SAMHD1) depletes cellular dNTPs, which are required for cDNA synthesis. APOBEC3G introduces hypermutations of viral cDNA by cytidine deamination. Tetherin prevent the release of budded virions. Myxovirus resistance 2 (MX2) prevents the nuclear import and integration of viral cDNA. Schlafen 11 (SLFN11) suppressed the translation of viral proteins. Interferon-induced transmembrane proteins inhibit viral entry by interfering with membrane fusion. Viral accessory proteins (shown in blue) are known to antagonize several of these restriction factors. This diagram is modified from Doyle T. et al., Nature Reviews Microbiology 2015.
(B) Schematic representation of viral accessory proteins (red) hijacking a E3 Ligase complexes (gray) to promote poly-ubiquitylation of a host substrate (yellow), which is typically a HIV-1 restriction factor. Vif hijacks the $CRL5^{ElonB/C\text{-}CBF\beta}$ E3 ligase to degrade APOBEC3G. Vpu hijacks the $CRL1^{\beta\text{-}TrCP}$ E3 ligase to degrade Tetherin. Vpx hijacks $CRL4^{VprBP}$ to degrade SAMHD1. Vpr associates with CRL4 through interaction with VprBP, and induces the degradation of MUS81, UNG2 and potentially other substrates, but the biological significance of this in viral replication is poorly understood.

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CHAPTER 2. CCR5 INTERACTION WITH HIV-1 ENV CONTRIBUTES TO ENV-INDUCED DEPLETION OF CD4 T CELLS IN VITRO AND IN VIVO

2.1. SUMMARY

CD4 T cell depletion during HIV-1 infection is associated with AIDS disease progression, and the HIV-1 Env protein plays an important role in the process. Together with CXCR4, CCR5 is one of the two co-receptors that interact with Env during virus entry, but the role of CCR5 in Env-induced pathogenesis is not clearly defined. We have investigated CD4 T cell depletion mechanisms caused by the Env of a highly pathogenic CXCR4/CCR5 dual-tropic HIV-1 isolate R3A. We report here that R3A infection induced depletion of both infected and uninfected "bystander" CD4 T cells, and treatment with CCR5 antagonist TAK-779 inhibited R3A-induced bystander CD4 T cell depletion without affecting virus replication. To further define the role of Env-CCR5 interaction, we utilized an Env-mutant of R3A, termed R3A-5/6AA, which has lost CCR5 binding capability. Importantly, R3A-5/6AA replicated to the same level as wild type R3A by using CXCR4 for viral infection. We found the loss of CCR5 interaction resulted in a significant reduction of bystander CD4 T cells death during R3A-5/6AA infection, whereas stimulation of CCR5 with MIP1-β increased bystander pathogenesis induced by R3A-5/6AA. We confirmed our findings using a humanized mouse model, where we observed similarly reduced pathogenicity of the mutant R3A-5/6AA in various lymphoid organs in vivo. We provide the first evidence that shows CCR5 interaction with a dual-tropic HIV-1 Env played a significant role in Env-induced depletion of CD4 T cells.

2.2. BACKGROUND

The depletion of CD4 T cells is a hallmark of HIV-1 pathogenesis and AIDS progression [1]. Multiple studies have attempted to explain the depletion of CD4 T cells during HIV-1 infection. This can be broadly categorized into killing of uninfected “bystander” CD4 T cells, and “direct” killing of HIV-infected CD4 T cells (2). Interestingly, the rate of CD4 T cell decline is discordant with low level of productively infected cells in HIV-positive individuals. This suggests “direct” death of productively infected cells contributes minimally towards AIDS progression, and bystander cell killing appears to be the leading cause for CD4 T cell loss and AIDS progression (3). The HIV-1 Env, also known as the gp120/gp41 glycoprotein, is expressed on the surface of infected cells or on HIV-1 virions and can interact with bystander cells expressing CD4. This interaction is critical for HIV-1 entry and has been proposed to induce bystander CD4 T cell death (4–6).

HIV-1 strains can be broadly divided into two groups based on their Env tropism, each using CCR5 or CXCR4 chemokine co-receptor for viral entry. The CCR5-tropic HIV-1 Env interacts with CD4 and CCR5, infects CCR5+ CD4 T cells and macrophages, and is sensitive to CCR5 antagonists such as TAK-779. Likewise, the CXCR4-tropic virus interacts with CD4 and CXCR4, infects CXCR4+ CD4 T cells, and is sensitive to CXCR4 antagonists such as AMD-3100 (7, 8). In addition, dual-tropic HIV-1 strains have been reported that are capable to utilize both CCR5 and CXCR4 for entry (9–12). R5-tropic HIV-1 dominates during the early stages of HIV-1 infection. In later stages of infection, X4-tropic viruses emerge and are thought to be responsible for the accelerated decline of CD4 T cells and AIDS progression (13). The highly pathogenic phenotype of late stage X4-viruses has been related to the abundant expression of CXCR4 in virtually all

CD4 T cells, whereas CCR5-expressing CD4 T cells are mostly memory T cells (14). However, in a significant proportion (>50%) of AIDS patients, there is no co-receptor switch detected and their AIDS associated viruses are exclusively R5-tropic (15, 16). Therefore, CCR5-tropic HIV-1 viruses can lead to AIDS progression but the mechanism remains unclear.

Previous reports have studied the pathogenic effect of HIV-1 Env binding to CCR5 by overexpression of R5-tropic Env on cell surface or by using recombinant R5-tropic gp120 proteins (4, 5, 17). However, the pathogenic effect of R5-tropic Env has not been studied in HIV-1 infection models, or directly compared to HIV-1 viral load. In this report, we studied the Env pathogenicity of a highly pathogenic dual-tropic HIV-1 strain (R3A) derived from a rapid progressor (9). The Env gene of R3A is highly pathogenic and has been used for HIV-1 pathogenesis studies (9–11). The interaction of the V3 region of R3A-Env with the co-receptors and its specificity for either CCR5 or CXCR4 has been elucidated in a previous study (8). We took advantage of a mutant R3A strain termed R3A-5/6AA from the study, which has lost the ability to bind and utilize CCR5 but can still use CXCR4 for viral infection, therefore not affecting viral replication capability. Interestingly, the mutant R3A-5/6AA is substantially less pathogenic then the wild type R3A, as evidenced by the reduction of virus-mediated bystander CD4 T cells depletion. Supporting the functional relevance of CCR5 interaction by R3A-Env in CD4 T cells pathogenesis, we found that the inhibition of Env-CCR5 binding by CCR5 antagonistic drug TAK-779 reduced R3A-induced bystander CD4 T cells killing, whereas stimulation of the CCR5 receptor with agonistic drug MIP-1β increased the pathogenesis effect. We confirmed our findings in vivo using a humanized mouse model, and we observed reduced bystander pathogenesis of the mutant R3A-5/6AA compared to the wild type R3A infection in CD4 T cells

in the blood, spleen and bone marrow. We provide the first evidence in two physiologically relevant HIV-1 infection models that shows CCR5 interaction with a dual-tropic HIV-1 Env plays a significant role in Env-induced depletion of bystander CD4 T cells.

2.3. RESULTS

2.3A. A highly pathogenic HIV-1 isolate R3A induces depletion of both productively infected cells and bystander CD4 T cells in activated PBMCs.

We used a primary activated PBMC culture infection model to study the pathogenesis of the highly pathogenic dual-tropic HIV-1 strain (termed R3A) on CD4 T cells. Briefly, freshly isolated PBMCs (peripheral blood mononuclear cells) were infected with an MOI of 0.01 for 3 hours, followed by T cell stimulation with CD3/CD28 activation beads. The kinetics of virus-induced CD4 T cell depletion can be accurately monitored (Fig. 2.1A), as CD4 T cell percentages and numbers in R3A infected PBMCs gradually decreased over time (Fig. 2.1B). R3A pathogenesis was completely prevented by the fusion inhibitor T20, which effectively inhibited viral entry and replication as measured by intracellular HIV-1 p24 staining (Fig. 2.1C). Co-staining of intracellular p24 with a cell viability dye allowed us to analyze the percentage of dying cells in both productively infected and uninfected CD4 T cells (Fig. 2.1C). Compared to mock infection, we observed a significant higher percentage of dying cells in the p24(-) CD4 T cell populations at 6 days post R3A infection, therefore we termed it as bystander cell pathogenesis (Fig. 2.1D).

2.3B. CCR5 antagonist TAK-779 protects CD4 T cells from R3A-induced bystander CD4 T cell depletion.

Expression of R5-tropic HIV-1 Env proteins on cell surface could induce the depletion of neighboring SupT1 cells in a CCR5-dependent manner (5). To investigate how CCR5 interaction during dual-tropic R3A infection contributes to CD4 T cell depletion, we treated R3A infected cells with the CCR5 antagonist TAK-779. TAK-779 is known to block HIV-1 Env interaction with CCR5 and inhibit the infections of CCR5-tropic viruses (18). As the dual-tropic R3A can also utilize CXCR4 for infection, we found R3A replication in CD4 T cells wasn't significantly affected by TAK-779 treatment, as measured by intracellular p24 staining or extracellular HIV-1 RT levels (Fig. 2.2A). Interestingly, CD4 T cell depletion was significantly reduced by TAK-779 treatment (Fig. 2.2B). Furthermore, TAK-779 significantly reduced (p24-) CD4 T cell death to near background levels (Fig. 2.2C and 2.2D). However, cell death of HIV-1 infected (p24+) CD4 T cells was not significantly affected by TAK-779 (Fig 2.2C and 2.2D). This data indicates blocking R3A-Env interaction with CCR5 reduced the pathogenesis on bystander CD4 T cells.

The binding of HIV-1 Env to the other co-receptor, CXCR4, has also been implicated in Env-induced cell death (19). We therefore tested how CXCR4 binding by R3A Env affects viral pathogenesis using AMD3100, a CXCR4 antagonist (20). Unlike TAK-779, the blockage of CXCR4 usage by AMD3100 significantly reduced the entry and replication of R3A in PBMCs (Fig. S2.1A and S2.1B). As expected, the combined treatment of TAK-779 and AMD3100 completely inhibited R3A infection, similar to T20. This suggests the dual-tropic R3A, although capable of using either co-receptor for entry, relies more heavily on CXCR4 for replication in primary CD4 T

cells. Although blocking CXCR4 usage by R3A also reduced CD4 T cell depletion (Fig. S2.1C), the interpretation here is difficult since virus replication was also significantly inhibited.

2.3C. Ablation of CCR5 usage reduces dual-tropic HIV-1 pathogenesis in PBMC infection

Our results with CCR5 antagonist TAK-779 treatment suggest the interaction of R3A-Env with CCR5 receptor contributed to CD4 T cells depletion during R3A infection. To expand this finding, we used a genetic mutant of R3A that is incapable of binding to the CCR5 receptor. As published before (8), the R3A-5/6AA mutant (with two alanine substitutions in the 5th and 6th amino acids of Env V3 region) has lost its capability to infect CCR5-expressing cells but not CXCR4-expressing cells (Fig. S2.2A). R3A-5/6AA could efficiently utilize CXCR4 for virus entry and replication. Accordingly, we observed the virus replication was not significantly affected by its ablation of CCR5 usage, as R3A-5/6AA replicated to wild type R3A viremia levels as measured by both intracellular p24 staining and extracellular HIV-1 genomic RNA levels (Fig. 2.3A and Fig. S2.2B). We then compared the pathogenesis of wild-type R3A with R3A-5/6AA. As shown before, the highly pathogenic R3A can significantly deplete CD4 T cell levels, with 60% depletion at 6dpi and 90% depletion at 10dpi. Remarkably, ablation of CCR5 usage by the mutant R3A-5/6AA significantly reduced CD4 T cell pathogenesis, with only 10% depletion at 6dpi and 35% depletion at 10dpi (Fig. 2.3B and 2.3C). This indicates R3A-Env binding with CCR5 strongly contributed to Env-mediated CD4 T cell depletion. The reduced pathogenicity of R3A-5/6AA observed is not a consequence of reduced virus growth advantage, as R3A-5/6AA replicated to similar levels as R3A (Fig 2.3A). As shown before, R3A infection reduced the viabilities of both p24(-) and p24(+) CD4 T cells (Fig 2.1C). Interestingly, the ablation of CCR5 binding in R3A-5/6AA infection completely rescued death of p24(-) bystander CD4 T cells to background levels (Fig

2.3D). The cell viability of p24(+) cells was not significantly different in R3A and R3A-5/6AA infection (Fig 2.3D). Therefore, our data indicate R3A could efficiently deplete both bystander and infected CD4 T cells, whereas R3A-5/6AA only induced perceivable pathogenesis in productively infected CD4 T cells. The reduced pathogenicity of R3A-5/6AA infection seen in Figure 3c was therefore due to reduced bystander cell death, a consequence of the loss of CCR5 binding capability by the Env mutant. We conclude R3A-induced bystander pathogenesis is dependent on the viral Env interaction with CCR5.

2.3C. CCR5 agonist MIP-1β enhances R3A-5/6AA pathogenesis to promote bystander CD4 T cell depletion

MIP-1β is a CCR5-specific chemokine and agonist (21). We investigated whether activation of CCR5 by MIP-1β could complement for the ablated Env-CCR5 interaction in R3A-5/6AA infection and therefore enhance bystander pathogenesis. We found MIP-1β treatment significantly increased the death of p24(-) CD4 T cells in R3A-5/6AA infected PBMC cultures, without affecting the viability of p24(+) CD4 T cells (Fig. 2.4A and 2.4B). Accordingly, MIP-1β also decreased the CD4 T cell survival (Fig. 2.4C). We conclude MIP-1β binding to CCR5 could partly mimic R3A's Env interaction with CCR5 and increase bystander CD4 T cell depletion.

2.3D. Ablation of CCR5 usage reduces pathogenesis of dual tropic HIV-1 in a humanized mouse model in vivo

To confirm that Env-CCR5 interaction contributes to HIV-1 bystander pathogenesis in a relevant HIV-1 infection model in vivo, we compared the infection and pathogenesis of R3A with R3A-5/6AA in the huHSC mice, a suitable small animal model to study HIV pathogenesis in vivo (22). Similar to infected PBMC cultures, R3A-5/6AA replication was delayed initially at 1

week post infection but reached similar viremia levels as R3A at 2 and 3 weeks post infection as measured by HIV genomic RNA levels in the blood and intracellular p24 staining of CD4 T cells in the spleen (Fig. 2.5A). When analyzed at 3 weeks post infection, we found R3A-5/6AA was significantly less pathogenic than R3A in vivo, with reduced CD4 T cell depletion in the blood, spleen and bone marrow (Fig. 2.5B). Furthermore, R3A infection resulted in higher cell death of p24(-) CD4 T cells than R3A-5/6AA infection in vivo (Fig 2.5C). The viability of infected p24(+) CD4 T cells was similar in R3A and R3A-5/6AA infection (Fig 2.5C). These findings indicate R3A Env interaction with CCR5 induced bystander CD4 T cell pathogenesis in HIV-infected humanized mice.

2.3E. R3A Env induces caspase-3 activation mediated bystander cell death through CCR5 usage

To investigate the cell death pathway induced by R3A Env, we treated the R3A-infected PBMC cultures with inhibitors against several cell death pathway regulators, including pan-caspase inhibitor Z-VAD, caspase-1/pyroptosis inhibitor Y-VAD, and RIP1K/necroptosis inhibitor Necrostatin-1. Only Z-VAD significantly reduced cell death after R3A infection, suggesting the cell death mechanism induced by R3A is not pyroptosis or necroptosis (Fig 2.6A). CD4 T cells in R3A infected PBMC cultures also have increased active caspase-3 levels which can be suppressed by Z-VAD treatment (Fig 2.6B), suggesting apoptosis is the major form of cell death occurring here. Accordingly, we found bystander CD4 T cells in the spleen and bone marrow of R3A infected humanized mice has significantly elevated active caspase-3 levels. In comparison, mice infected with R3A-5/6AA have minimal caspase-3 activation (Fig 2.6C). We conclude that

CCR5 usage by R3A-Env result in caspase-3 activation in bystander CD4 T cells leading to bystander apoptosis.

2.4. DISCUSSION

Multiple viral and host factors determine the variability in HIV-1 disease progression (17, 23–26). Cellular tropism and receptor/co-receptor usage for viral entry are major factors influencing HIV pathogenesis. Despite extensive research, the exact mechanism of how those factors contribute to the gradual loss of CD4 T cells is still enigmatic. Bystander CD4 T cell death plays a major contribution towards AIDS progression. A recent report has revealed a mechanism for HIV-induced CD4 T cell depletion, which involves abortive non-productive HIV infection in resting CD4 T cells, followed by IFI16 activation and caspase-1 dependent pyroptosis (27–29). Besides the abortive RT products in non-productively infected resting cells, several HIV-1 proteins have also been reported to contribute to the depletion of bystander (uninfected and non-productively infected) CD4 T cells, including the Env (4, 5, 17, 19), Vpr (30), Nef (31, 32) and Tat (33). The Env protein is of specific interest in mediating AIDS progression, and has been implicated as an important cytopathic determinant of AIDS-associated CCR5-tropic viruses (34, 35). CCR5 expression levels on cell surface correlate with increased host susceptibility to R5-tropic Env-induced apoptosis (5). Interestingly, humanized mice engrafted with CCR5Δ32+/- donor cells supported HIV-1 replication with reduced CD4 T cell depletion (36). It has been difficult to directly define the role of CCR5 interaction in HIV-induced CD4 T cell depletion because CCR5 interaction is required for the replication of CCR5-tropic HIV-1. Our findings using a dual-tropic HIV-1 confirmed that the interaction between CCR5 and HIV-1 Env is a critical determinant of bystander CD4 T depletion. The dual-tropic R3A virus is known to be

highly pathogenic, depleting thymocytes through multiple mechanisms including fusion-induced apoptosis (10) and fusion-independent interferon-mediated mechanism (11). In humanized mice, R3A infection caused rapid depletion of both infected and uninfected CD4 T cells (22, 37). Using an Env mutant R3A-5/6AA that has lost interaction with CCR5, we were able to show that the ablation of CCR5 usage by the dual-tropic R3A virus effectively decreased viral pathogenicity on bystander CD4 T cells in activated PBMC cultures (Fig. 3) and in humanized mice (Fig. 5). Notably, ablation of CCR5 usage did not significantly affect the killing of productively infected CD4 T cells (Fig 3d and 5c), corresponding with previous reports that the R3A Env fusion activity (10) and other HIV-1 proteins can promote death of directly infected cells (38, 39). It is of note that the replication of mutant R3A-5/6AA was lower compared to R3A at early time points (Fig 5a). The delayed replication peak may have contributed to the decreased pathogenicity of the mutant virus at early time points.

Signal transduction through Env-CCR5 interaction on bystander CD4 T cells may contribute to viral pathogenesis (17, 24). Here we showed that CCR5 antagonist TAK-779 decreased R3A-induced bystander CD4 T cells depletion (Fig 2), whereas CCR5 agonist MIP-1β increased bystander pathogenesis (Fig 4), supporting a role for CCR5 stimulation/signaling in R3A pathogenesis. Mechanistically, HIV-1 Env binding to CCR5 induces a signaling cascade including calcium influx, Pyk2 phosphorylation and downstream activation of p38 MAPK pathway (40–42). Using a p38 inhibitor, Li et al. has shown that CCR5 engagement by HIV-1 Env leads to p38 activation and Fas- and caspase-dependent cell death (43). The signaling through CCR5 by the R3A Env and subsequent p38 activation, together with Env binding to CD4, may be involved in R3A induced bystander T cell killing. MIP-1□ binds to CCR5 and is also known to

similarly activate Pyk2 and p38 MAPK pathways (42, 44), which may mimic the Env-CCR5 interaction and contribute to bystander cell death, as shown in Figure 4. In addition to CCR5, the interaction of CXCR4 with HIV-1 Env is also known to contribute to Env-mediated cell death (19). Although treatment with CXCR4 antagonist AMD3100 decreased R3A pathogenesis, the interpretation in this study is difficult since AMD3100 also significantly inhibited R3A replication (Fig S1). Therefore, like most dual-tropic viruses, R3A relies more heavily on CXCR4 than CCR5 usage for infection in primary PBMC. It remains to be determined whether CXCR4 binding by R3A Env also contributes to CD4 T cell death during infection. Lastly, the cooperative engagement of R3A Env to both CCR5 and CXCR4 may result in stronger binding of the Env to CD4 T cells, which may directly affect Env-mediated cell death. Loss of CCR5 engagement in our studies may have resulted in a weaker binding to CD4/CXCR4 and therefore a reduced Env pathogenicity. Accordingly, MIP-1β may have enhanced this Env-CXCR4 interaction and therefore contributing to the increased cell death.

In summary, we provide the first evidence in relevant infection models with a dual tropic HIV-1 that can efficiently infect primary human PBMC when its binding to CCR5 is blocked genetically or pharmacologically, demonstrating CCR5 interaction with dual-tropic HIV-1 Env played a significant role in Env-induced depletion of bystander CD4 T cells. Our findings suggest that drugs such as Maraviroc or gene therapy targeting CCR5 interaction with HIV gp120 can not only prevent R5-mediated HIV-1 entry, they can also reduce Env-mediated CD4 T cell depletion and AIDS disease progression.

2.5. CONCLUSION

We provide the first evidence in relevant infection models that shows CCR5 interaction with a dual-tropic HIV-1 Env played a significant role in Env-induced depletion of CD4 T cells.

2.6. METHODS

Cell cultures

Total PBMCs were isolated from peripheral blood of healthy donors by Ficoll-PaqueTM Plus (GE Healthcare) density gradient centrifugation and maintained in RPMI 1640 (Gibco) supplemented with 10% FBS, 1X Antibiotic-Antimycotic (Invitrogen) and 1µM L-glutamine. T cell activation in PBMCs were performed with CD3/CD2/CD28 activation beads (Miltenyi Biotech) and cultured in RPMI medium described above containing 20U/mL recombinant human interleukin-2 (IL-2). HEK293T cells were cultured in DMEM (Gibco) containing 10% FBS and 1X Antibiotic-Antimycotic. MAGI cells (NIH AIDS Research and Reference Reagent Program) were maintained in the same medium plus selection antibiotics (45).

HIV-1 virus production

X4/R5 dual tropic HIV-1 (strain pNL4-R3A) was generated by cloning the R3A Env gene in the background of pNL4-3 proviral genome as previously described (9). The mutant R3A proviral strain (pNL4-R3A-5/6AA) was generated and kindly provided by Dr. James Hoxie (8). HIV-1 virions were produced by CaCl2-BES (*N,N*-bis[2-hydroxyethyl]-2-minoethanesulfonic acid) transfection of proviral plasmids in 293T cells. 293T cells cultured on a 10 cm plate were transfected with 30 µg DNA plasmids of pNL4-R3A or pNL4-R3A-5/6AA, and cell supernatants were harvested at 48h post transfection and passed through 45 µm membrane filter. Concentration of virus stocks were measured by p24 ELISA assay (Frederick National Laboratory

for Cancer Research – AIDS and Cancer Virus Program). Infectious titers were determined in CCR5-expressing or CXCR4-expressing MAGI cells as previously described (45).

HIV-1 infection of PBMC cultures

Unless indicated otherwise, infections of PBMCs with R3A or R3A-5/6AA was performed by infecting 1x10^6 freshly isolated PBMCs with MOI of 0.01 (as titered in CXCR4-expressing MAGI cells) for 3 hours at 37 0C in a total volume of 100 µL supplemented RPMI medium containing polybrene (8µg/mL). At 3 hours post infection, viral inoculum were washed with PBS, activated with CD3/CD28 activation beads as described above, and cultured at concentration of 0.5x10^6 cells/mL in the presence of IL-2 (20U/mL). When indicated, PBMCs were treated with 10µg/mL fusion inhibitor T20 (NIH AIDS Reagents Program) before infection and maintained throughout the experiment. TAK-779 and AMD3100 (NIH AIDS Reagents Program) were treated before infection at an IC90 dose of 5µM and 2µM respectively, as determined before in U373-CD4-CCR5/CXCR4 cells. Treatment with 200ng/mL MIP-1β started at 3 hours post infection and maintained.

Flow cytometry analysis

Aliquots of infected PBMC samples (~1E5 cells) were collected for analysis at the indicated time points post HIV-1 infection. Staining for cellular surface CD3, CD4, CD8 (BD Biosciences) and viability dye (LIVE/DEAD fixable yellow dead stain, Invitrogen) were performed in 2% FBS. Intracellular staining was performed after surface antibody staining, with the use of Fixation/Permeabilization Solution Kit (BD Cytofix/CytopermTM), followed by staining with antibodies targeting p24 (Beckman Coulter, #KC-57). Cells were fixed by 1% paraformaldehyde and analyzed with Cyan ADP FACS machine (DAKO). Live total cell numbers were counted by

Guava EasyCytes after staining with viability dye. %CD4 T cells survived at a the time point were analyzed as live CD3(+) CD8(-) populations in PBMCs. Total live CD4 T cell numbers at a given time where calculated using the total live cell numbers multiplied by %CD4 T cells in PBMCs. Total infected CD4 T cell numbers were calculated using total CD4 T cell number multiplied by %p24(+) in live CD3(+) CD8(-) cells.

HIV-1 infection of humanized mice and analysis

DKO-huHSC mice were constructed as previously reported (22). DKO-huHSC mice were infected with HIV-1 at 4000 infectious units/mouse by intravenous injection. At termination, whole animal's blood and lymphoid organs including spleen and bone marrow were harvested as described before (22). Total lymphocytes were isolated from lymphoid organs and red blood cells were lysed with ACK buffer, and remaining cells were stained and fixed before flow cytometry analysis as described above.

RT-qPCR analysis and HIV-1 Reverse transcriptase activity analysis of viral load

Viremia in supernatants from infected PBMC cultures or from blood plasma of infected humanized mice was analyzed by viral RNA extraction (QIAamp viral RNA mini kit, Qiagen), and analyzed by RT-qPCR (Taqman One-Step RT-qPCR Master Mix, ABI) using the following primers and probe targeting HIV-1 Gag region: 765gagF 5'-GGTGCGAGAGCGTCAGTATTAAG-3'; 911gagR 5'-AGCTCCCTGCTTGCCCATA-3'; probe FAM-AAAATTCGGTTAAGGCCAGGGGGAAAGAA-QSY7 (TAMRA). HIV-1 RT activity was analyzed in the infected PBMC supernatant as described before in Lee MH et al., J Clinical Microbiology, 1987

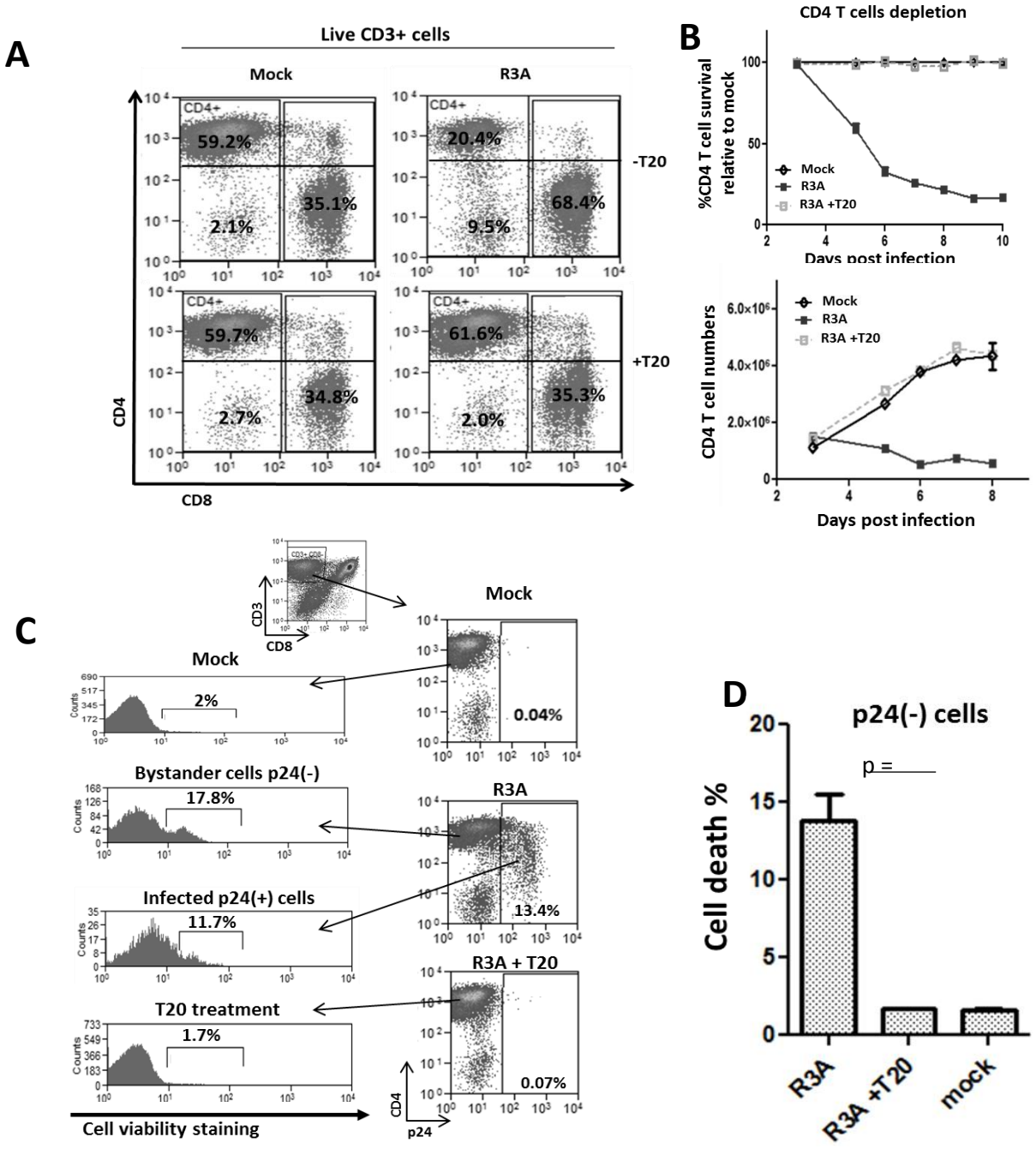

Figure 2.1. A highly pathogenic HIV-1 isolate R3A induces depletion of both productively infected and bystander CD4 T cells

(A) HIV-R3A infection in PBMC leads to efficient depletion of CD4+ T cells. PBMCs were infected with R3A virus, and stimulated with anti-CD3/CD28/CD2 activation beads at 3h post infection and cultured in the presence of IL-2 (20U/mL). FACS Plots show CD4 and CD8 staining of populations gated on live CD3(+) T cells in PBMC at 6 days post infection (dpi). HIV-1 fusion

inhibitor T20 was used as negative control to prevent HIV-1 infection. (B) Graphical summary showing kinetics of CD4 T cells depletion by R3A from 3-10 dpi. CD4 T cells are identified as live CD8 (-) CD3(+) population. Data are presented as %CD4 T cell survival relative to mock infection (*Top*) and total CD4 T cell numbers (*Bottom*) over time. (C) FACS gating strategies are presented. CD4 T cells were identified as CD3(+) CD8(-) population. Uninfected bystander CD4 T cells were identified as p24(-) cells and infected CD4 T cells as p24(+) cells (*Right Graphs*). Cell viability of both bystander and infected populations was quantified by co-staining with a cell viability dye (*Left Graphs*). (D) Graphical summary of bystander p24(-) cell death at 6 days post infection by R3A. *$p<0.05$

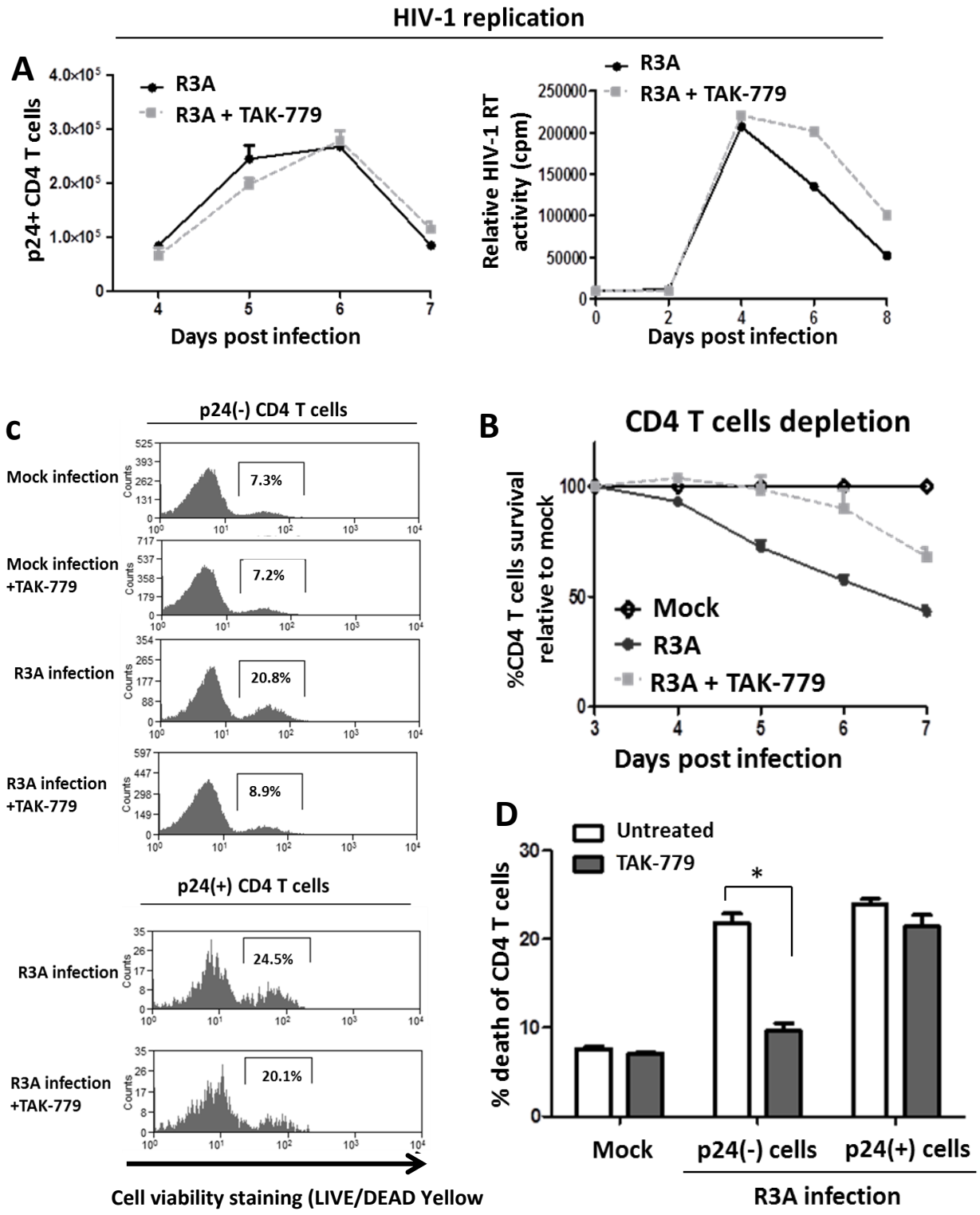

Figure 2.2. CCR5 antagonist TAK-779 protects CD4 T cell from R3A-induced bystander CD4 T cell depletion

(A) PBMCs were treated with CCR5 antagonist TAK-779 (5uM) before infection with R3A and maintained after infection. HIV-1 viral replication in CD4 T cells was measured by intracellular

p24 staining or by extracellular HIV-1 reverse transcriptase levels. (B) CD4 T cell depletion by R3A in the presence of TAK-779 was measured by FACS analysis as described in Figure 1b. %CD4 T cells over time relative to mock infected PBCMCs are presented. (C) Cell viability of bystander and infected CD4 T cells during R3A infection in the presence of TAK-779 was analyzed as described in Figure 2.1C. (D) Graphical summary of viral induced bystander cell death from (C), presented as cell death percentage of p24(-) CD4 T cells at 6 days post infection. Experimental results were repeated with PBMCs from 2 different blood donors. *$p<0.05$

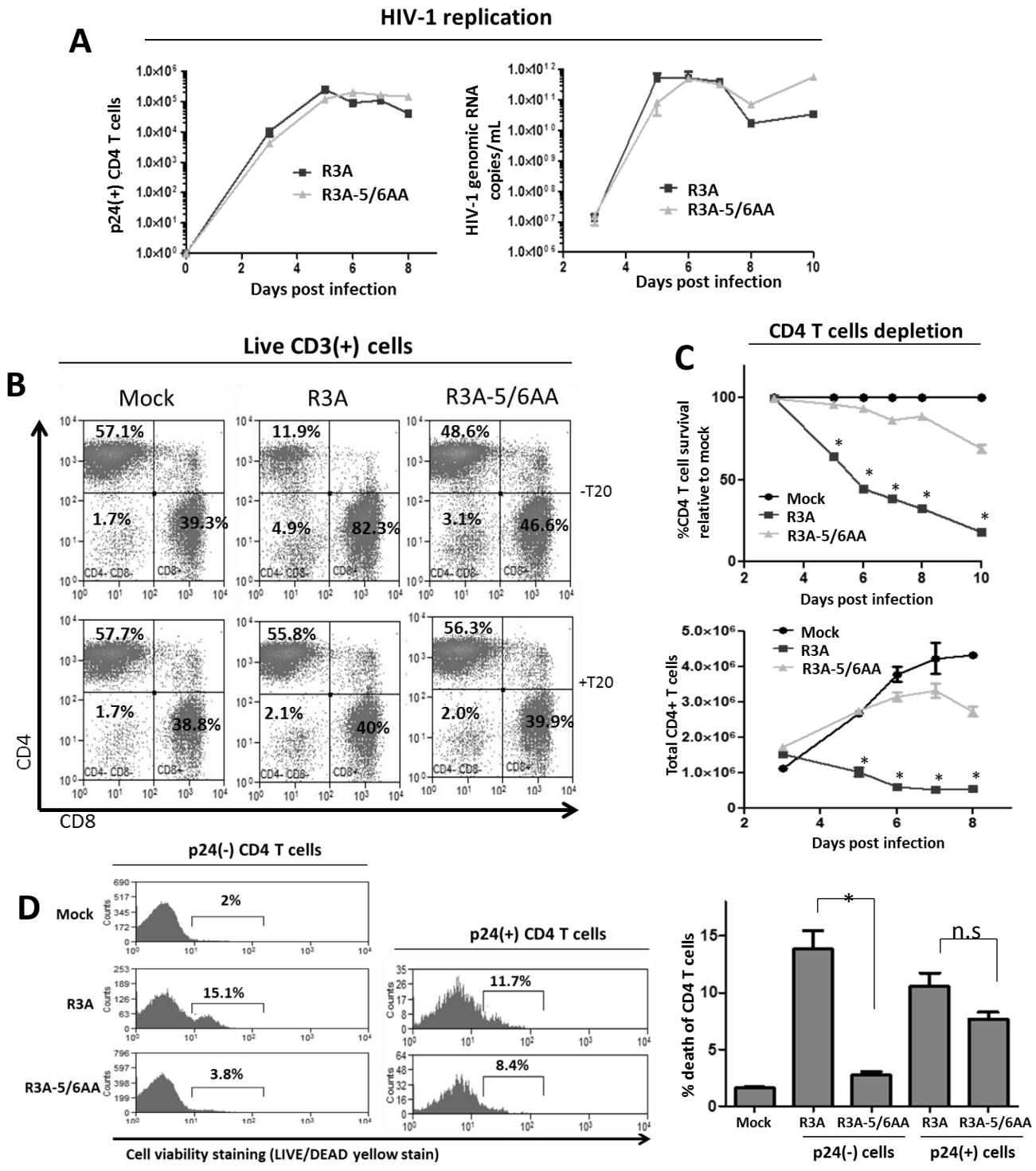

Figure 2.3. Ablation of CCR5 usage reduces dual-tropic HIV-1 pathogenesis in PBMC infection
(A) Intracellular p24 staining and FACS analysis was used to quantify viral replication in infected PBMCs. Infected (p24+) CD4 T cell numbers were calculated by multiplying the percentage of (p24+) cells with total CD4 T cell numbers at each time point (Left). RT-qPCR analysis of HIV-1

genomic RNA levels was used to measure extracellular viral replication of R3A and R3A-5/6AA in infected PBMCs (Right). (B) Representative FACS Plots are presented showing CD4 and CD8 populations gated on live CD3(+) T cells in PBMC at 6 days post infection. HIV-1 fusion inhibitor T20 treatment was used as negative control. (C) Kinetics of CD4 T cell depletion by R3A versus R3A-5/6AA was analyzed as described in Figure 1b. CD4 T cell survival are measured as %CD4 T cells in infected PBMCs relative to mock infection (Top), or live CD4 T cell numbers (Bottom) over time. (D) p24(-) and p24(+) CD4 T cell death in R3A and R3A-5/6AA infected PBMCs are quantified at 6 days post infection, as described in Figure 1C. FACS plots (Left) and graphical summary (Right) from a representative experiment are presented. Experimental results were repeated with PBMCs from 3 different blood donors. *$p<0.05$

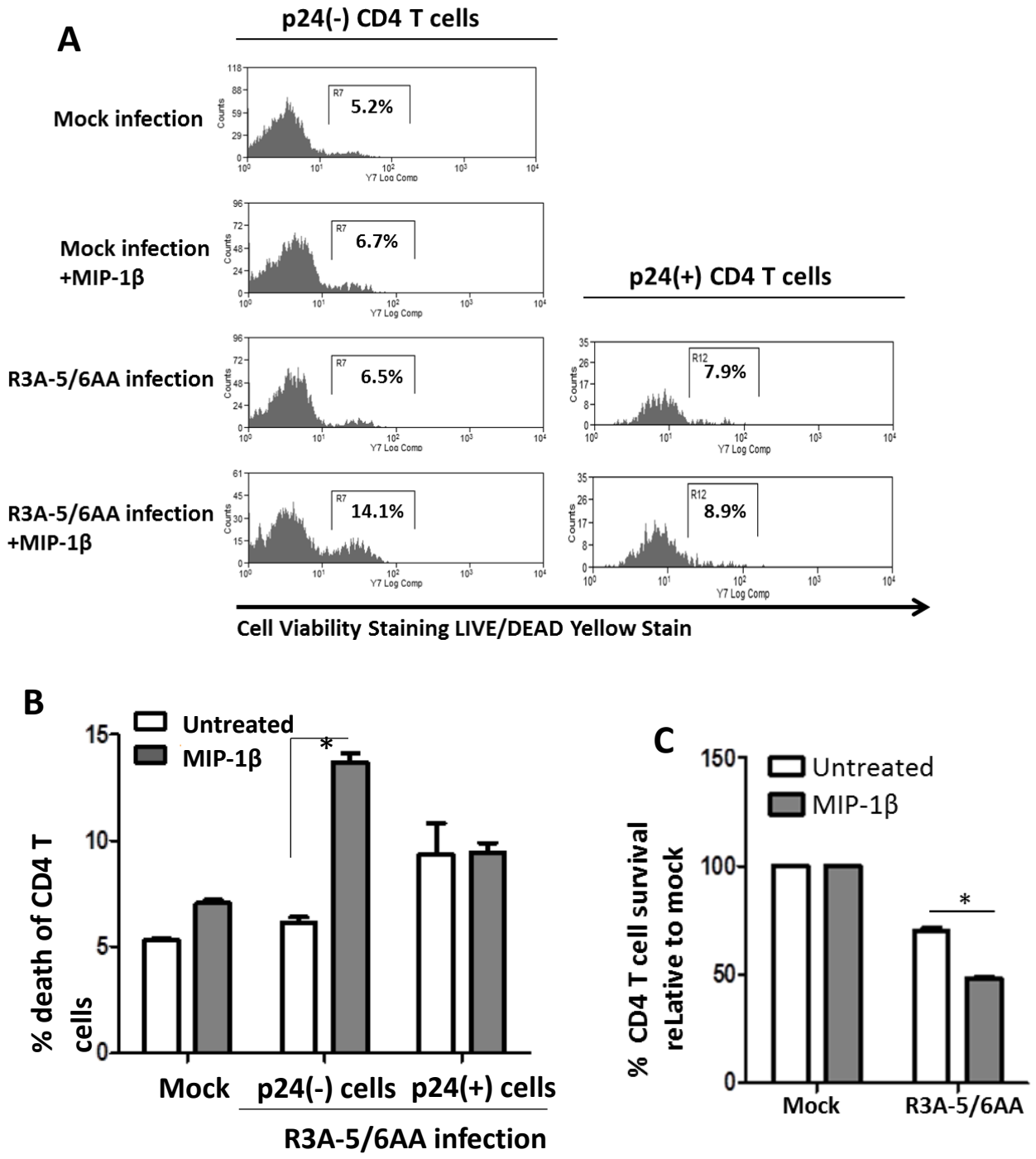

Figure 2.4. CCR5 agonist MIP-1β enhances R3A-5/6AA pathogenesis to promote bystander CD4 T cell depletion

PBMCs were infected with R3A-5/6AA virus and treated with recombinant MIP-1 β (200ng/mL) at 3h post infection. At 6 days post infection, viabilities of p24(-) and p24(+) CD4 T cell were measured as described in Fig 2.1C. (A) Representative FACS plots and (B) graphical summary are presented. (C) %CD4 T cells survival at 6 days post infection was measured by gating on live CD8(-) CD3(+) cells. *p<0.05

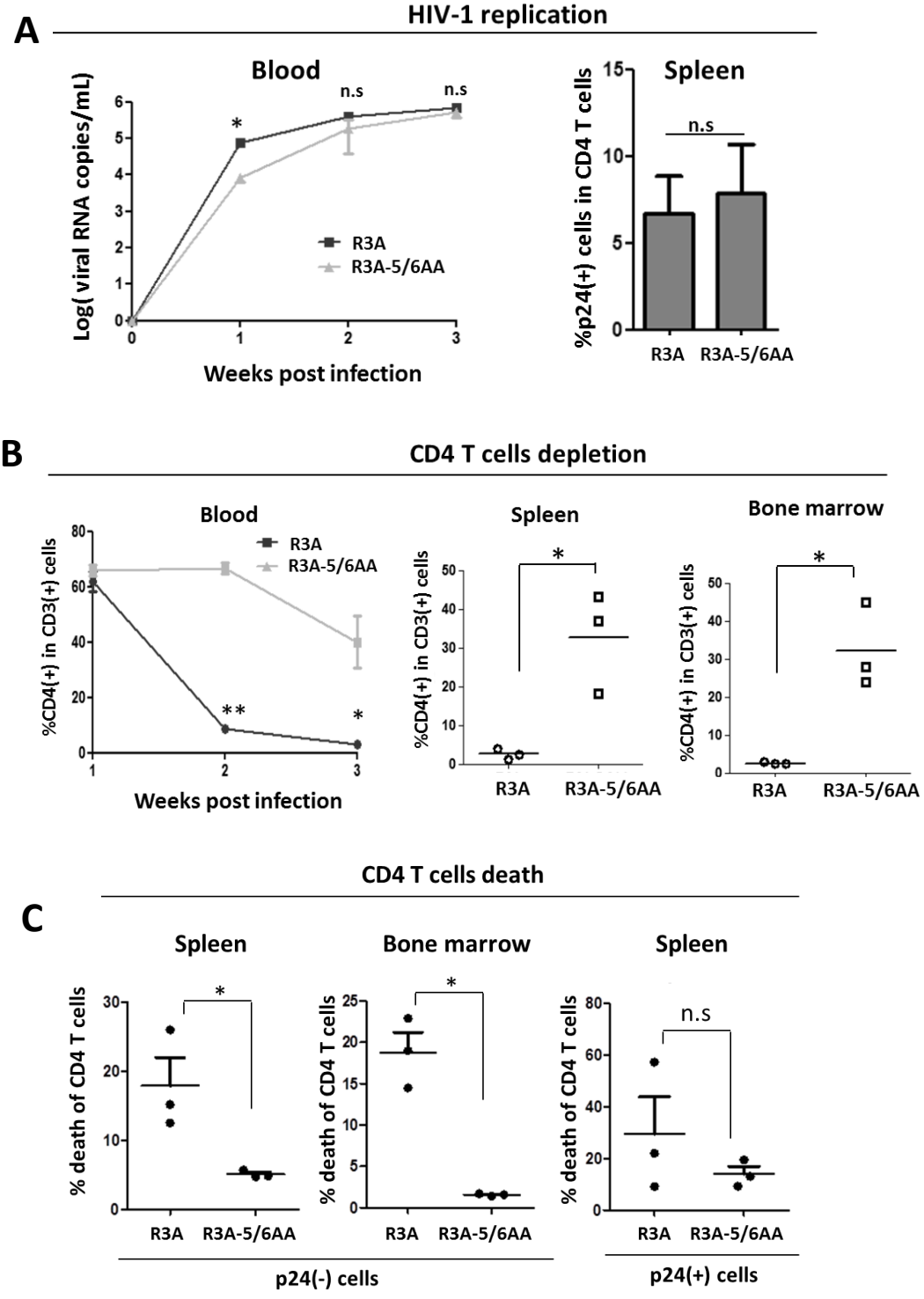

Figure 2.5. Ablation of CCR5 usage reduces pathogenesis of dual tropic HIV-1 in a humanized mouse model

(A) Humanized mice were infected with R3A or R3A-5/6AA as described in Materials and Methods. Viral replication was assessed weekly by RT-qPCR measurement of HIV-1 genomic

RNA in the blood. HIV-1 infection in CD4 T cells was analyzed by intracellular p24 staining of splenocytes isolated from infected mice at 3 weeks post infection (wpi). (B) Total PBMCs in blood, spleen and bone marrow from infected animals were harvested at 3wpi, and CD4 T cell depletion was analyzed by FACS staining as described in Figure 1b. (C) p24(-) and p24(+) CD4 T cell viability in R3A versus R3A-5/6AA infection in the infected animal's spleen and bone marrow were analyzed as described in Figure 1c. *$p<0.05$

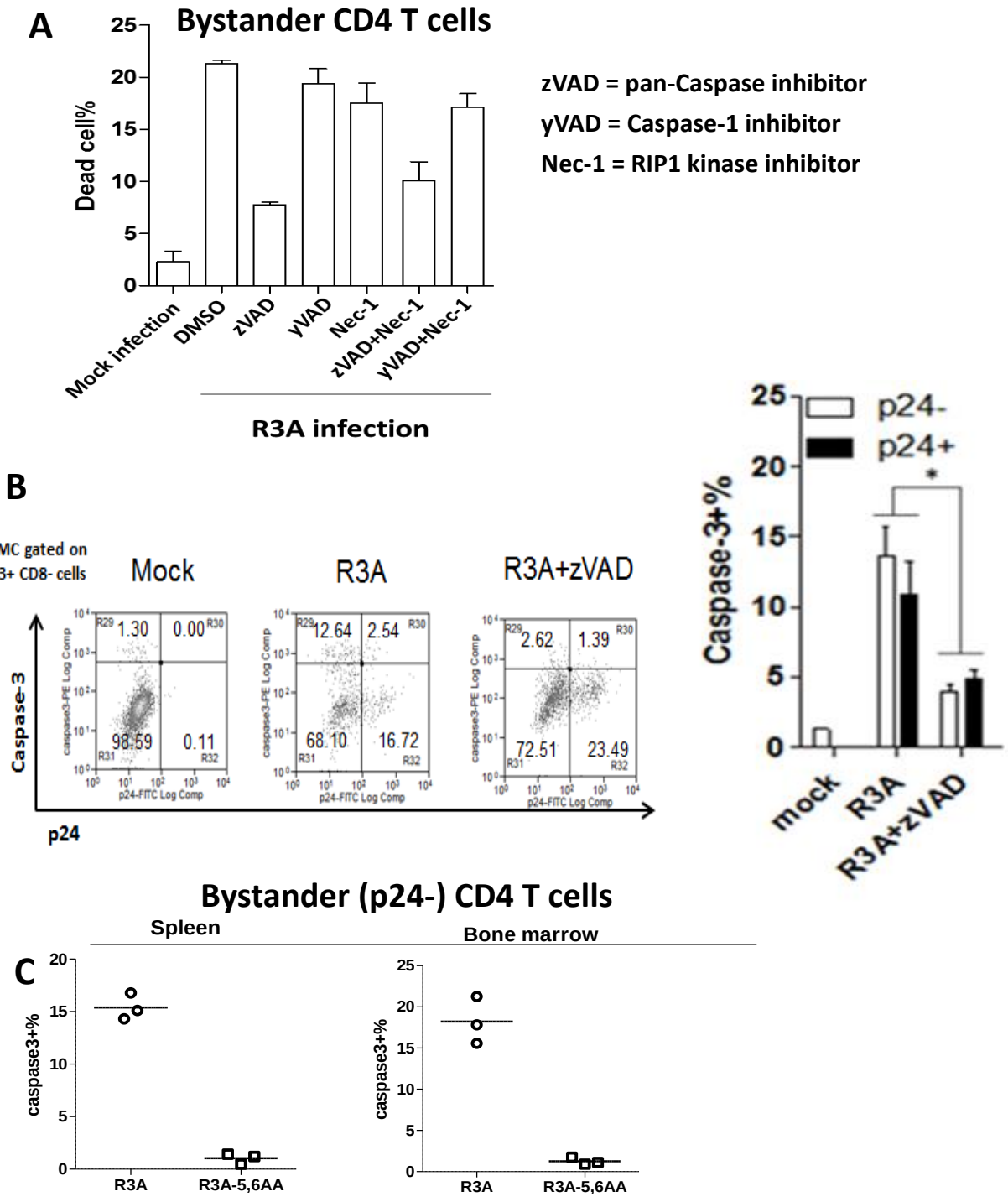

Figure 2.6. R3A Env induces caspase-3 activation mediated bystander cell death through CCR5 usage

(A) PBMCs were pre-treated with 5uM zVAD, 5uM YVAD or 2uM Nec1 and then infected with R3A virus. Drugs were maintained over time course of infection. Virus induced pathogenesis were measured by p24 staining and cell viability staining. (B) R3A infected PBMCs were stained for active caspase-3 levels to measure apoptosis in infected p24+ and bystander p24- CD4 T

cells. (C) Spleen and bone marrow from R3A and R3A-5/6AA infected humanized mice were stained for caspase-3 activation.

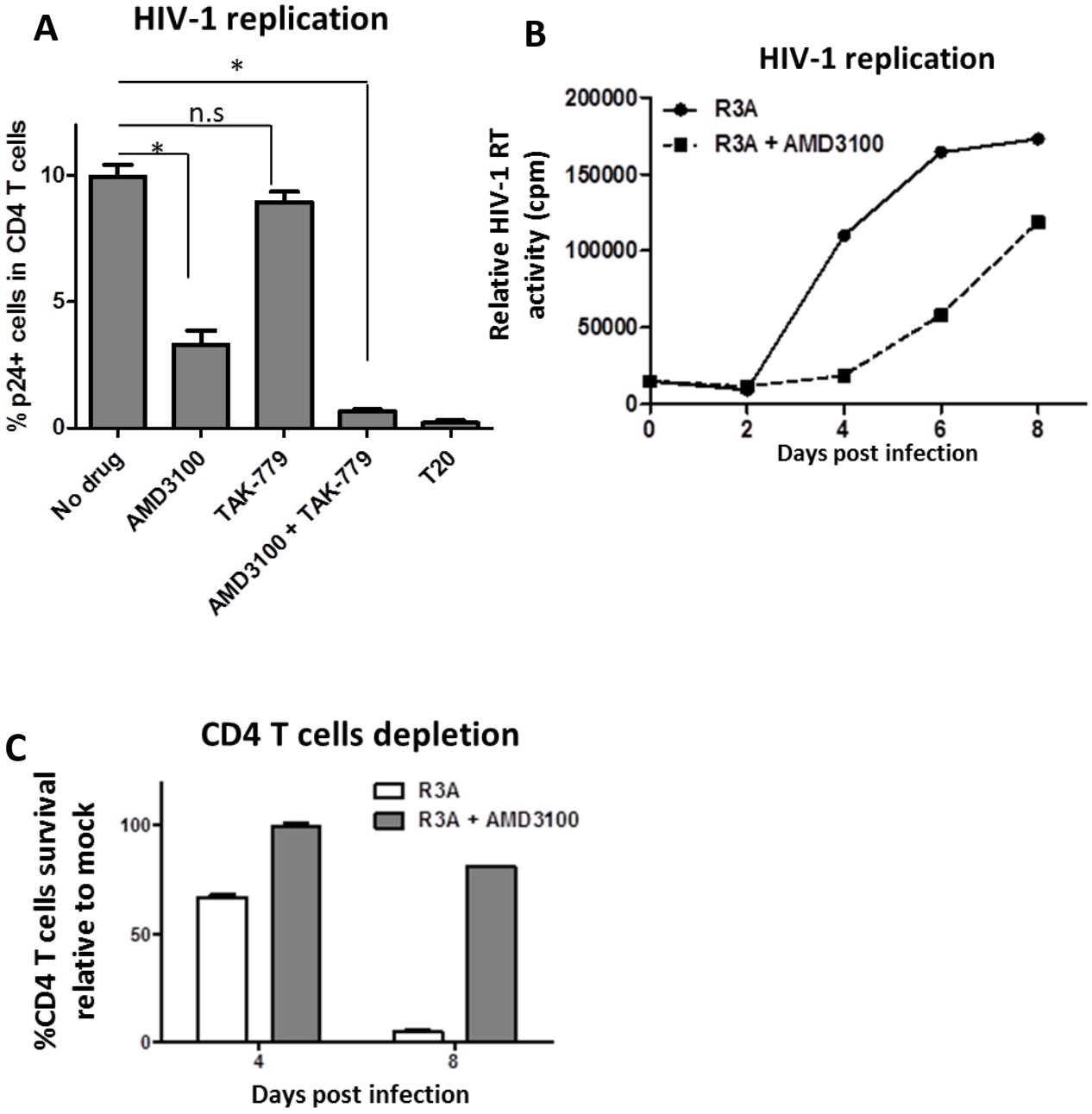

Figure S2.1. CXCR4 antagonist AMD3100 inhibits R3A replication

(A) PBMCs were treated with the indicated drugs before infection with R3A, and viral infection efficiencies were measured by %p24+ cells with FACS analysis at 4 days post infection. Both AMD3100 (2µM and TAK-779 (5µM) were used at >IC90 dose as determined before in U373-CD4-CCR5/CXCR4 cells

(B) PBMCs were treated with AMD3100 before infection with R3A and maintained after infection. HIV-1 replication was measured by extracellular HIV-1 reverse transcriptase activity in the cell supernatant.

(C) CD4 T cell depletion by R3A in the presence of AMD3100 was measured by FACS analysis as described in Figure 1b. %CD4 T cells relative to mock infected PBMCs are presented.

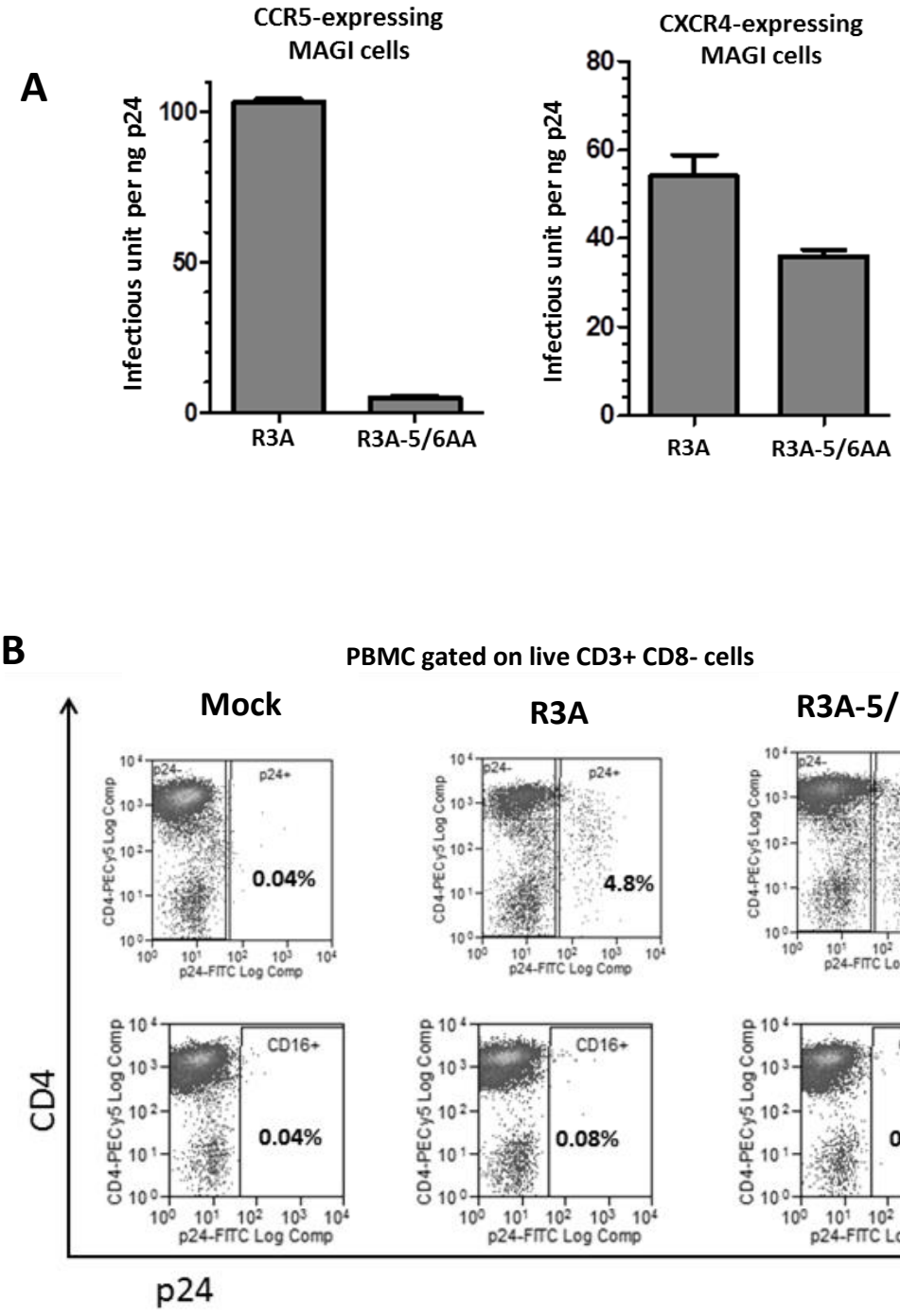

Figure S2.2. Similar infection levels by R3A and R3A-5/6AA in PBMCs

(A) Infectivity of R3A and R3A-5/6AA HIV-1 strain was measured in CCR5-expressing (Top) or CXCR4-expressing (Bottom) U373 MAGI cells. Viral infectivity was presented as infectious units per ng of p24.

(B) PBMCs were infected with 20ng p24 of R3A or R3A-5/6AA virus stock, and viral replication kinetics was analyzed by intracellular p24 staining. Representative FACS plots of p24 staining at 6dpi are presented.

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CHAPTER 3. VPR TARGETS TET2 FOR DEGRADATION BY CRL4^{VPRBP} E3 LIGASE TO ENHANCE HIV REPLICATION

3.1. SUMMARY

Primate lentiviruses, including human immunodeficiency virus 1 (HIV-1), express several accessory proteins to counteract host inhibitors (1, 2). One such accessory protein, Vpr, has been implicated in affecting multiple cellular processes to contribute to HIV-1 infection and disease progression (3–5), but its mechanism remains elusive (6, 7). Here we show that Vpr binds to TET2 methylcytosine dioxygenase and promotes its degradation by the VprBP-DDB1-CUL4-ROC1 (CRL4^{VprBP}) E3 ligase. Virion-associated Vpr can rapidly degrade TET2 in relevant HIV-1 targeted myeloid and T cells. Genetic-mediated inhibition of TET2 expression enhances HIV-1 replication in monocytic cell-lines and macrophages. Vpr promotes TET2 degradation to enhance HIV-1 replication independently of G2 cell-cycle arrest induction by Vpr. The anti-HIV activity of TET2 is dependent on its C-terminal domain, but does not require its enzymatic dioxynase activity. Furthermore, TET2 associates with and inhibits HIV-1 early reverse transcription after viral entry. These results reveal TET2 as a new HIV-1 inhibitor that plays an important role in inhibiting HIV-1 replication and is disrupted by the Vpr protein.

3.2. BACKGROUND

The covalent attachment of ubiquitin to a substrate protein (ubiquitylation) is involved in most cellular processes (8). Ubiquitylation proceeds through sequential reactions promoted by an ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2), and finally a ubiquitin ligase (E3) that binds substrates and determines specificity. Substrate modification

with either a single ubiquitin or various lengths of ubiquitin chain enables recognition by distinct ubiquitin-binding proteins, leading to specific biochemical consequences including degradation, translocation, or recruitment of other proteins. Cullin proteins form a potentially large family of cullin-ring ubiquitin ligase (CRLs) complexes by binding to a small RING protein, ROC1 or ROC2, and various substrate recognition subunits. CUL4 uses damaged DNA binding protein 1 (DDB1) as a linker to interact with multiple DDB1-binding WD40 proteins that serve as substrate recognition subunits(9, 10). One of the strongest and most abundant binding partners of DDB1-CUL4 is VprBP/DCAF1 (11, 12), which was identified as a cellular protein that binds to HIV-1 Vpr protein (13). In a search for the substrate of VprBP, we recently discovered that it binds to the TET family of DNA dioxygenases and targets them for monoubiquitylation by the $CRL4^{VprBP}$ E3 ligase (14). This finding led us to determine the effect of Vpr on $CRL4^{VprBP}$-mediated TET2 ubiquitylation.

3.3. RESULTS

3.3A. HIV-1 Vpr promotes TET2 degradation

TET2 is highly expressed in hematopoietic cells (15), regulates homeostasis and differentiation of hematopoietic cells (16–18), and is frequently mutated in human hematopoietic malignancies of both myeloid and lymphoid lineages (16, 19, 20). To test the effect of Vpr on TET2 protein, we transfected HIV-1 clade B Vpr in THP1 human monocytic cells and found that the level of TET2 protein was significantly decreased (Fig. 3.1A). Moreover, Vpr from HIV-1 clade A Q23, HIV-2 ROD9 and SIV-mac239 were all capable of decreasing TET2 protein (Fig. 3.1B), suggesting that targeting TET2 for degradation represents a conserved function of Vpr. In contrast, Vpx—a homologue of Vpr that is encoded by HIV-2 and most

simian lentiviruses and targets a host restriction factor, SAMHD1, for degradation by $CRL4^{VprBP}$ (21, 22)—had no effect on TET2 protein (Fig. 3.1B and Fig. S3.1A), indicating a different specificity of Vpr from Vpx in promoting host protein degradation. Next, THP1 cells were infected with HIV-1 and the level of TET2 protein was examined at different time points after infection. TET2 protein levels decreased significantly after HIV-1 infection (Fig. 3.1C). Notably, the infection of Vpr-deficient HIV-1 had no effect on TET2 levels in primary human CD4+ T cells or PBMC (Fig. 3.1D), demonstrating that the ability of HIV-1 to decrease TET2 is dependent on Vpr. To determine at which stage of the HIV-1 life cycle Vpr promotes TET2 degradation, human T cell line SupT1 (Fig. 3.1E) and THP1 cells (Fig. S3.1C) were infected with HIV-1 and cells were treated with inhibitors of viral fusion (enfuvirtide, T20), reverse transcription (Nevirapine, NVP), integrase (Raltegravir, RAL) or protease (Amprenavir, AMP). All four drugs effectively inhibited HIV-1 replication, as shown by intracellular p24 flow cytometry analysis (Fig. S3.1B). Only entry inhibitor T20 inhibited TET2 degradation, indicating that the Vpr protein incorporated in HIV-1 viral particles is sufficient to degrade TET2 protein during the early stage of HIV-1 infection.

3.3B. Vpr promotes TET2 degradation by $CRL4^{VprBP}$ E3 Ligase

Quantitative real-time PCR (qRT-PCR) analysis showed that the mRNA levels of TET2 remained unchanged in cells expressing Vpr (Fig. 3.1A), indicating a posttranscriptional regulation of TET2 by Vpr. Two proteolytic pathways have recently been implicated in the regulation of TET proteins: a caspase-mediated cleavage of TET2 that is promoted by a CxxC protein, IDAX28, and the cleavage of all three TET proteins by the calpain family of calcium-dependent proteases29. We treated Vpr-expressing THP1 cells with Z-VAD-FMK, a caspase inhibitor, and calpeptin, an inhibitor of calpain, as well as NH_4Cl, an inhibitor of lysosome

function, and found that these inhibitors did not affect Vpr-promoted TET2 degradation (Fig. 3.2A). Instead, treatment of Vpr-expressing cells with MG132, an inhibitor of the proteasome, effectively blocked TET2 reduction (Fig. 3.2A), indicating that Vpr promotes TET degradation via the proteasomal pathway. Consistent with this notion, MG132 treatment led to significant accumulation of polyubiquitylated TET2 in cells expressing Vpr (Fig. S3.2A).

One obvious candidate E3 ligase for Vpr-promoted TET2 ubiquitylation is the VprBP-DDB1-CUL4-ROC1 ($CRL4^{VprBP}$) E3 complex. To test this hypothesis, individual components of $CRL4^{VprBP}$ E3 ligase were knocked down by siRNA in Vpr-expressing cells and TET2 polyubiquitylation was determined. Knocking down VprBP, DDB1, Cul4A or Cul4B all significantly reduced the ubiquitylation of TET2 (Fig. 3.2B), which also blocked Vpr induced TET2 degradation (Fig. S3.2B). Incubation of immunopurified TET2 with a $CRL4^{VprBP}$ immune complex resulted in robust TET2 poly-ubiquitylation in the presence of Vpr in vitro (Fig. 3.2C), which is dependent on the addition of E1, E2 and ubiquitin (Fig. S3.2C). The Q65R mutation in Vpr, which disrupts its binding with VprBP (23, 24) (Fig. S3.2D), abolished the ability of Vpr to promote TET2 polyubiquitylation in vitro (Fig. 3.2C) and in vivo (Fig. 3.2D) and its degradation (Fig. 3.2E). Vpr did not bind with mouse VprBP and cannot promote TET2 degradation in mouse cells (Fig. S3.2E). Collectively, these results demonstrate that Vpr promotes TET2 degradation by $CRL4^{VprBP}$ E3 ligase via the 26S proteasome, and this function of Vpr requires its binding to VprBP.

We recently discovered that the $CRL4^{VprBP}$ E3 ligase causes monoubiquitylation of TET2 on a conserved lysine residue, K1299 (14) (also see Fig. 3.2C and Fig. S3.2C), thereby promoting TET2 functional activation and binding to chromatin. The finding that Vpr promotes TET2 degradation raises the question of whether Vpr-promoted TET2 polyubiquitylation is distinct

from the monoubiquitylation by the same $CRL4^{VprBP}$ E3 ligase. We found that mutations disrupting the monoubiquitylation site (K1299N or K1299E) or VprBP-TET2 binding (F1300S) did not affect Vpr-promoted TET2 degradation (Fig. S3.2F), indicating that Vpr-promoted TET2 polyubiquitylation is not dependent on either the monoubiquitylation site or the VprBP-TET2 binding in TET2. In the absence of Vpr, TET2 binds to a sequence within the N-terminal 909 residues of VprBP (1 – 909, N909), but not the C-terminal region of VprBP (915 – 1507, C590). The expression of wild-type, but not Q65R mutant Vpr, however, enabled TET2 to bind the VprBP-C590 (Fig. 3.2F and 3.2G). This is similar to Vpx, a homologue of Vpr of HIV-2 and SIV, which also bridges the binding of its substrate SAMHD1 to a sequence (residues 1058 – 1396) within the C-terminal region of VprBP in the proximity of the ROC1 and presumably a favorable position for ubiquitin transfer (25, 26). Like Vpx, which makes direct contact with SAMHD1(26), Vpr also bound to the substrate TET2 (Fig. 3.2H). Taken together, these results indicate that Vpr tethers TET2 to a C-terminal domain in VprBP, thereby promoting its polyubiquitylation by $CRL4^{VprBP}$ E3 ligase.

3.3C. TET2 inhibits HIV-1 replication

HIV-1 Vpr is known to increase viral growth advantage in myeloid cells such as monocytes, DC and macrophages (27–29). As TET2 is known to be highly expressed in myeloid populations and plays an important role in myeloid cell development (18–20), we hypothesize TET2 could be an inhibitor of HIV-1 that is counteracted by Vpr. We therefore generated TET2 knockout THP1 cell lines using the CRISPR-Cas9 approach (Fig. 3.3A) and investigated this effect on Vpr-deficient HIV-1 replication. We found knockout of TET2 drastically increased HIV-1 infection under different multiplicity of infections (MOI), as analyzed by intracellular p24 FACS

(Fig. 3.3B and 3.3C). TET2-KO also increased HIV-1 viremia production in the cellular supernatant as analyzed by p24 ELISA (Fig 3.3D). To confirm this observation, we generated shRNA-mediated knockdown of TET2 in THP1 and in primary human monocytes-derived-macrophages, and found similar enhancement of viral replication in TET2 depleted cells (Fig. 3.3E and 3.3F). Furthermore, rescue experiment by ectopic expression of TET2 in the THP1 knockout cells restored the restriction on HIV-1 infection comparable to the control THP1 cells (Fig. 3.3H). To investigate whether Vpr enhances HIV-1 through TET2 degradation, we took advantage of our finding that virion-associated Vpr is sufficient to rapidly degrade TET2 after viral entry (Fig. 3.1). We generated Virus-Like-Particles that carry Vpr proteins (VLP-Vpr), which can efficiently degrade cellular TET2 after Vpr treatment (Fig. 3.3I). We found VLP-Vpr significantly increased HIV-1 infection in control THP1 cells, but had minimal effect on viral infection in TET2-KO cells (Fig. 3.3J). In comparison, VLP carrying the VprQ65R mutant does not deplete TET2 levels and did not enhance virus infection (Fig. 3.3i and 3.3J). These results indicate Vpr enhances HIV-1 replication in a TET2-dependent manner.

3.3D. Vpr promotes TET2 degradation and enhanced HIV-1 replication independently of G2-arrest

HIV-1 Vpr is known to interact with the $CRL4^{VprBP}$ E3 ligase to induce G2 cell cycle arrest (30), which is reported to contribute to viral replication (31) and pathogenesis (32, 33). We therefore examined the involvement of TET2 in Vpr-induced G2-arrest, a well-known phenotype of Vpr function through the premature activation of the cellular SLX4-Mus81 complex (34). Using the TET2 knockout THP1 cells, we found TET2 was not required for the ability of Vpr to induce G2-arrest (Fig. 3.4A). Similar results were seen in TET2 knockdown cells

(Fig. S3.3). We also found that Vpr degrades TET2 with an indistinguishable efficiency in cells at different phases of the cell cycle (Fig. 3.4B). The VprR80A mutant is known to be defective in causing G2-arrest (23) while retaining other Vpr function such as binding with $CRL4^{VprBP}$ complex and enhancing viral replication (35). We confirmed VprR80A is capable of binding with VprBP and interestingly, it retained its activity in TET2 degradation (Fig 3.4C and 3.4D) while losing its activity in inducing G2-arrest (Fig. 3.4E). This indicates that the two functions of Vpr in TET2 degradation and cell cycle arrest are genetically separable. Importantly, the VprR80A mutant effectively enhanced HIV-1 replication similar as wildtype Vpr when pre-delivered into THP1 or PBMCs by VLP (Fig 3.4C). These results indicate that the functions of Vpr in causing G2 cell cycle arrest and TET2 degradation are independent of each other.

3.3E. TET2 inhibits HIV-1 reverse transcription, which is counteracted by Vpr

Multiple host proteins, called HIV restriction factors, have been reported to inhibit HIV-1 by restricting a specific step in the viral replication cycle (1, 36–38). To investigate how TET2 inhibits HIV-1, we first considered the function of TET2 in gene expression regulation. Recently, TET2 has been reported to shut down IL-6 promoter through the recruitment of HDAC1/2 (39), whereas TET3 has been reported to inhibit IFNβ promoter through similar mechanisms (40). Furthermore, previous reports showed Vpr could transactivate HIV-1 LTR promoter activity (41). To study the effect of TET2 on the LTR promoter, we measured the activity of LTR-driven luciferase reporter in cells ectopically expressed with TET2 or in TET2 knockdown cells. We observed no change in viral LTR promoter activity in 293T cells over expressed with TET2 or in TET2 knockdown cells (Fig. S3.4A). To further confirm the role of TET2 on LTR promoters integrated into the host genome, we utilized the J-LAT Tat-GFP cell-line which contains an

TNFα-inducible LTR promoter expressing GFP (42). Ectopic expression of TET2 did not affect the induction or activity of integrated HIV-1 LTR promoter in J-LAT Tat-GFP cells (Fig. S3.4B).

In addition to LTR transactivation, Vpr is also known to enhance the early HIV-1 post-entry steps, including reverse transcription and nuclear import (28, 43–45). Using a quantitative PCR assay established to measure the HIV-1 post-entry steps (46), we found that knockout of TET2 in THP1 significantly enhanced HIV-1 reverse transcript cDNA production while not affecting the HIV-1 strong-stop cDNA levels (Fig. 3.5A). As the strong-stop cDNA is believed to be completed in the virion particle, our data indicates TET2 inhibits HIV-1 reverse transcription after virion entry into the cells. HIV-1 nuclear import, as measured by 2-LTR circular DNA levels, was also increased in TET2-KO cells (Fig. 3.5A). This may however be a direct consequence of the enhanced HIV-1 reverse transcription in TET2-KO cells. Furthermore, the increase of HIV-1 reverse transcription was also observed in the TET2 knockdown THP1 cells (Fig. S3.5), and ectopic expression of TET2 inhibited HIV-1 reverse transcription (Fig. 3.5B). Pre-treatment of THP1 cells or primary macrophages with VLP-Vpr increased HIV-1 replication at the reverse transcription step (Fig. 3.5C and Fig. S3.5). Importantly, Vpr did not increase HIV-1 reverse transcription in TET2-KO cells, indicating Vpr enhanced HIV-1 RT in a TET2-dependent manner (Fig. 3.5C). To investigate whether TET2 proteins are directly and physically involved in inhibiting HIV-1 reverse transcription, we performed ChIP-qPCR assays using an anti-TET2 antibody to confirm a direct association of TET2 with HIV-1 cDNA shortly (4 hours) after viral infection (Fig. 3.5D). TET2-KO cells were used to assess the background DNA signals that immuno-precipitated with the antibody. We choose to measure the viral strong-stop cDNA, as the total strong-stop cDNA level was not affected by TET2 depletion (Fig 3.5E, left graph).

Interestingly, HIV-1 strong-stop cDNA strongly associated with TET2 by ChIP-qPCR (Fig. 3.5E, right graph). Furthermore, Vpr reduced the association of TET2 with strong-stop cDNA (Fig. 3.5F). We conclude that TET2 directly associates with the viral reverse transcription complex to inhibit HIV-1 reverse transcription, which is prevented by Vpr.

3.3F. The C-terminal domain of TET2 inhibits HIV-1 independently of its enzymatic activity

The C-terminal end of TET2 contains its catalytic domain (TET2-CD), involved in iron-binding and α-ketoglutarate binding, which is required to carry out the oxidation reaction for DNA hydroxyl-methylation (47). To investigate whether the enzymatic activity of TET2 is required for HIV-1 inhibition, we generated constructs that express the TET2-CD and catalytic-dead mutant TET2-CM containing two point mutations in the iron-binding residues (H1382/D1384) (Fig. 3.6A and 3.6B). Expression of TET2-CD inhibited HIV-1 infection as efficiently as the full-length TET2. This suggests the C-terminal domain of TET2 is sufficient for viral inhibition (Fig. 3.6C). Surprisingly, the catalytic-dead mutant of TET2 inhibited HIV-1 comparable to wild type TET2. We conclude that TET2 inhibits HIV-1 directly through its C-terminal domain but independent of its dioxynase activity in DNA hydroxyl-methylation. Accordingly, full-length TET2, TET2-CD and TET2-CM all inhibited HIV-1 reverse transcription in both control and TET2-KO THP1 cells (Fig. 3.6D).

3.3G. The conserved Lysine residue K1229 of TET2 is required for HIV-1 strong-stop DNA binding and inhibition of HIV-1 reverse transcription

Our recent report discovered VprBP regulates the mono-ubiquitylation of TET2 on a conserved lysine residue K1229. This modification is required for TET2 binding with cellular DNA. We therefore hypothesize TET2 may bind to reverse transcribed HIV-1 cDNA through this

domain and prevent reverse transcription progression. Interestingly, TET2 readily bind to HIV-1 strong-stop cDNA in an in vitro DNA binding assay. Mutation of the K1229 residue completely abolished strong-stop cDNA binding capability of TET2. However, mutations that abolish iron-binding (H1881) and α-ketoglutarate-binding (R1896) did not affect strong-stop DNA binding by TET2 (Fig. 3.7A and 3.7B). As we predicted, the wild type TET2 and catalytic mutant R1896S both inhibited HIV-1 infection, whereas the K1229N DNA-binding mutant has lost its anti-HIV activity (Fig. 3.7C). We conclude TET2 directly binds with HIV-1 reverse transcribed cDNA through its K1229 DNA-binding region to inhibit HIV-1 reverse transcription.

3.4. DISCUSSION

HIV-1 encodes several accessory proteins which, while not sharing significant sequence homology, appear to have evolved a common mechanism to promote efficient viral replication by hijacking a host E3 ubiquitin ligase to degrade host restriction factors (1, 36, 48). These include Vif-targeted degradation of APOBEC3 by the $CRL5^{ElonB/C\text{-}CBF\beta}$ E3 ligase (49), Vpu-targeted degradation of BST-2/tetherin/CD317 by the $CRL1^{\beta\text{-}TrCP}$ ligase (50), and SIV Vpx-targeted degradation of SAMHD1 by the $CRL4^{VprBP}$ ligase (21, 22). The function of Vpr in facilitating HIV-1 replication is believed to depend on its ability to interact with VprBP and thereby target a putative restriction factor to the $CRL4^{VprBP}$ E3 ligase for degradation (51). Several cellular proteins have indeed been previously shown to be targeted by Vpr for degradation by the $CRL4^{VprBP}$ E3 ligase, including uracil-DNA glycosylase-2 (UNG2) and single-strand-selective monofunctional uracil-DNA glycosylase 1 (SMUG1) (52, 53), the HLTF DNA translocase (54) and a subunit of the structure-specific endonuclease, MUS81 (34). Whether these proteins play a role in restricting HIV infection remains to be determined. The results presented here

demonstrate that Vpr targets TET2 for degradation by the $CRL4^{VprBP}$ E3 ligase, and that TET2 is an inhibitor of HIV-1 reverse transcription that is antagonized by Vpr.

Vpr incorporated in the virions is sufficient to degrade TET2 proteins and only inhibition of viral fusion, but not subsequent steps, can block TET2 degradation, suggesting that Vpr-promoted TET2 degradation occurs at an early stage during the HIV-1 life cycle after viral entry and represent an important function of Vpr in facilitating the early steps of HIV-1 infection. It is likely that non-productive infection of HIV-1 target cells (e.g., by defective viral particles) may reduce their TET2 levels and condition them for more efficient reinfection. This was demonstrated in our experiments using virus-like-particles that pre-deliver Vpr into monocytic cells, leading to TET2 degradation and enhancement of HIV-1 reverse transcription (Fig. 3.3 and Fig. 3.5). Although Vpr can also promote TET2 degradation in CD4 T cells, it is interesting that Vpr is known to only enhance HIV-1 replication in monocytic cells but not in proliferating T cells (28, 27), indicating the antiviral activity of TET2 is cell type dependent, and may be regulated differentially between myeloid cells and T cells. Indeed, TET2 depletion does not affect HIV-1 replication in CD4 T cells (see Chapter 4).

One of the most extensively studied role of Vpr is its activity to induce G2 cell-cycle arrest, which resulted from Vpr-mediated degradation of Mus81 leading to the premature activation of SLX4 (34). This activity of Vpr has been implicated in viral pathogenesis and also in enhancement of viral growth advantage, with the viral LTR promoter activity reported to be more active in the G2 cell cycle phase (31). Our results here demonstrate that TET2 is not required for G2-arrest induction by Vpr. More importantly, we found that TET2 degradation and

G2-arrest induction are two genetically separable Vpr activities (Fig. 3.4). In addition, TET2 also does not affect the viral LTR promoter activity (Fig. S3.4).

Vpr has been previously reported to enhance post-entry steps of HIV-1 replications, including viral reverse transcription (43, 45, 55, 56) and nuclear import (44, 56, 57). In this report, we found that TET2 specifically inhibits early reverse transcription after viral entry, and that Vpr increases reverse transcript levels in a TET2-dependent manner (Fig. 3.5). Interestingly, although the C-terminal catalytic domain of TET2 was sufficient to inhibit reverse transcription, the enzymatic dioxynase activity of TET2 was not required (Fig. 3.6 and 3.7). This suggests a direct physical interaction between TET2 and the viral reverse transcription complex is involved in TET2-mediated HIV inhibition. Supporting this notion, we found that TET2 is closely associated with HIV-1 reverse transcribed strong-stop cDNA shortly after viral entry. Importantly, mutation on the K1229 residue of TET2 abolished its binding to HIV-1 strong-stop cDNA, and completely impaired the ability of TET2 to inhibit HIV-1 replication (Fig. 3.7). We propose a model where TET2 directly binds to HIV-1 reverse transcribed cDNA to inhibit reverse transcription. The binding to viral cDNA depends on the conserved lysine residue K1299 of TET2. Interestingly, this residue is regulated by the $CRL4^{VprBP}$ complex through mono-ubiquitylation to promote TET2 binding with DNA (14). This suggest Vpr hijacks $CRL4^{VprBP}$ and changes TET2 mono-ubiquitylation into poly-ubiquitylation, which effectively relieves the restriction posed by TET2 on HIV RT.

Recent reports elucidated how TET2 may function independently of its enzymatic activity: TET2 and TET3 function as an adaptor to recruit transcriptional suppressive factors to DNA promoters, such as SIN3 Transcription Regulator Family Member A (SIN3A) and Histone

Deacetylase 1 and 2 (HDAC1/2), leading to the suppression of IL-6 and IFNβ DNA promoter (39, 40). It will be interesting to investigate whether TET2 also recruits suppressive factors to the HIV-1 cDNA to impair reverse transcription. Interestingly, SIN3A and HDACs have indeed been reported to associate with HIV-1 reverse transcription complex, but its biological relevance is not well understood (58, 59).

Lastly, the results presented here identify a new target—Vpr-mediated TET degradation—for therapeutic intervention of HIV-1 infection. Development of therapeutic molecules targeting the Vpr-VprBP interaction or blocking Vpr-mediated TET degradation may merit future exploration.

3.5. METHODS

Plasmids, cell culture, cell transfection and antibodies

Expression constructs for mouse TET proteins and human VprBP were previously described[1, 2]. Human TET2 cDNA was a gift from MCB Lab, Fudan University and was subcloned to the p3XFLAG-CMV destination expression vector (Sigma-Aldrich). Vpx plasmid was a gift from Ronald Swanstrom of University of North Carolina at Chapel Hill and was cloned to a pcDNA3 vector. Point mutations were generated by site-directed mutagenesis and verified by DNA sequencing. MX2-expressing construct was purchased from OriGene #RC206437. Plasmids expressing HIV-1 Q23 Vpr, HIV-2 ROD9 Vpr, SIV-mac 239 Vpr, HIV-2 ROD9 Vpx and SIV-mac 239 Vpx was a gift from Michael Emerman of University of Washington, Seattle.

HEK293T cells and MEFs were cultured in DMEM containing 10% FBS and 1X Antibiotic-Antimycotic (Invitrogen™). THP-1 and SupT1 cells were maintained in RPMI 1640 medium containing 10% FBS with Pen/Strep antibiotics. Human buffy coats were obtained from Gulf

Coast Regional Blood Center. Primary human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats. Primary CD4+ T cells were purified from total PBMCs after Ficoll gradient separation by depletion of non-CD4+ T cells with microbeads (Invitrogen, Cat #: 11346D). CD4 T cells were activated by CD3/CD28 activation beads (GIBCO, Cat #: 11131D) and cultured in 100U/mL IL-2 for HIV-1 infection experiment or lentivirus transduction. CD3/CD28 beads were removed on the third day post stimulation. When indicated, cells were treated with Type 1 interferon (R&D Systems, Minneapolis, MN. Cat #: 11200-1) as indicated for each infection experiment.

Transfection of 293T was performed using lipofectamine 2000 (Invitrogen™). Transfection of THP-1 was performed using Amaxa Cell Line Nucleofector Kit V according to manufacturer's protocol (Lonza Inc. USA). Cells were infected or analyzed at the indicated time points post transfection.

Antibodies to HA (Clone 3F10; Roche Inc.), Myc (Clone 9E10; Roche), Flag (Clone M2; Sigma), TET2 (Cat #: ab94580; Abcam), MX2 (sc-47197), IFITM1 (60074-1-Ig; Proteintech) and IFITM3 (11714-1-AP; Proteintech) were purchased commercially. Antibodies to CUL4, DDB1, ROC1 were described previously[2, 3]. Antibodies to p24 (Cat #: 6458) and Vpr (Cat #: 11836) and BST2 (Cat#: 11721) were obtained from NIH AIDS Reagent Program. Antibodies for WB and ChIP-PCR against TET2 were from Millipore (Cat# MABE462).

siRNA, shRNA, and CRISPR-Cas9-gRNA

All siRNA oligonucleotides were synthesized with 3' dTdT overhangs by Sigma in a purified and annealed duplex form. The sequences targeting each gene were as follows: human CUL4A, 5'-GAACUUCCGAGACAGACCU-3'; Human CUL4B, 5'-AAGCCUAAAUUACCAGAAA-3';

Human DDB1, 5'-CCUGUUGAUUGCCAAAAAC-3'; Human VprBP, 5'-UCACAGAGUAUCUUAGAGA-3'. For transfection of siRNA, OPTI-MEM medium (250 μL) was mixed with Lipofectamine 2000 reagent (Life Technologies, 10 μL) for 5 min and then incubated with another 250 μL OPTI-MEM medium containing 10 μL (20 mM) of siRNA for 20 min at room temperature. The mixtures were added to cells cultured on a 60-mm plate at 30%–40% confluence. The knocking down efficiency was determined 48–72 h after transfection.

pGIPZ-lentiviral vectors expressing control (non-targeting), TET2-targeting shRNA were purchased from Dharmacon, GE Healthcare (Lafayette, CO). shRNA targeting human TET2 has the mature antisense sequence: TAAGTAATACAATGTTCTT (clone ID: V3LHS_363201). Lentiviruses were produced by CaCl2-BES (*N,N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid) transfection in 293T cells using 15ug pGIPZ-shRNA vector, 10 μg packaging plasmid pMDL/pRRE, 2ug pRSV-Rev and 5 μg pCMV-VSV-G envelope-expressing plasmid. Lentiviruses were titered in 293T cells by GFP expression and FACS analysis. Transductions of THP-1 cells were performed with a multiplicity of infection (M.O.I.) of 0.3. Knockdown efficiency of TET2 was assessed by qPCR and western blot seven days after Puromycin selection

CRISPR-Cas lentiviral constructs (Addgene) containing the gRNA targeting the N-terminal region of TET2 (gRNA sequence: GATTCCGCTTGGTGAAAACG) were packaged into lentiviruses as described above, transduced into THP1 cells (estimated M.O.I. of 0.3) and selected with puromycin for 7 days. Single clones of TET2 knockout cells were isolated by limiting dilutions. Control knockout THP1 were similarly obtained using CRISPR-Cas lentiviral construct lacking the gRNA. Monoclonal populations of TET2 knockout THP1 cells were verified by genomic DNA sequencing and by western blot.

In vivo and in vitro ubiquitylation assay

In vitro ubiquitylation assays were performed as described previously (14). Briefly, CUL4-VprBP immune complexes were purified from HEK293T cells transfected with Flag-VprBP. Flag tagged VprBP was immunoprecipitated with anti-FLAG M2 agarose beads for 3 h in a NP-40 lysis buffer (0.3% Nonidet P-40, 50 mM Tris pH 7.5, 150 mM NaCl). Immobilized Flag-VprBP complexes were washed three times in the same lysis buffer and eluted with an excess of Flag peptide (Sigma). Ubiquitylation reactions were performed according to in a 50 µL reaction volume, containing 100 nM E1 (Enzo Life Sci.), 1 µM E2 (Enzo Life Sci.), 1 µM human recombinant ubiquitin (Boston Biochem), 1 unit inorganic pyrophosphatase, 1 mM DTT and 5 mM Mg-ATP, 100 ng of eluted CUL4-VprBP complexes as the source of E3 and 100 ng of human Flag-TET2 as substrate. Reactions were incubated at 37 °C for 30 min, terminated by addition of an equal volume of SDS sample buffer and resolved by SDS-PAGE.

For the in vivo ubiquitylation assay, HEK293T cells were transfected with indicated plasmids and siRNA, and were treated with MG132 (10 µM) for 5hrs before collecting the cells. Cells were lysed under denaturing conditions in a SDS buffer (50 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 1 mM DTT, 1% SDS) by boiling for 10min. Lysate was clarified by centrifugation at 13,000 rpm for 10min and diluted 10-fold with an NP-40 buffer (50mM Tris pH 7.5, 150mM NaCl, 0.3% Nonidet P-40) and then subjected to immunoprecipitation by anti-Flag M2 agarose beads and subsequent SDS-PAGE. Ubiquitylated TET2 was detected with HA antibody.

HIV-1 proviral strains and viral stock production

The NL4 (Vpr+) and (Vpr-) strains containing an HSA reporter in the Nef region were obtained from the NIH AIDS Reagent program. The NL4-LUC Env-deficient Vpr-deficient strain

was also from NIH AIDS Reagents program. X4/R5 dual tropic HIV-1 (strain pNL4-R3A) was generated as previously described. Vpr-mutant R3A was generated by insertion of an HSA reporter into the Vpr open reading frame.

HIV-1 virions were produced by CaCl2-BES transfection of proviral plasmids in 293T cells. 293T cells cultured on a 10 cm plate were transfected with 20 μg DNA of pNL4-3 or pNL4-R3A for production of HIV-1 viruses. For the production of VSV-G pseudotyped HIV-1, 20ug pNL4-LUC-E^- were transfected with 5 μg pVSV-G. For the production of virus-like-particles (VLP) carrying Vpr, 10 μg of pCDNA3 expressing WT, Q65R or R80A mutant Vpr were transfected with 10 μg of pMDL/pRRE, 2ug pRSV-Rev and 3 μg pCMV-VSV-G. Virus stocks were harvested at 48 hr post transfection. Concentration of viral stocks were measured by p24 ELISA kit (Frederick National Laboratory for Cancer Research) and normalized for experiments.

HIV-1 infection

All Infections were performed by spin inoculation for 2 hr at 1500g and 32 ^{0}C, followed by removal of virus inoculum. NL4 or NL4-R3A infections of THP-1, CD4 T cells, PBMC and Monocytes-Derived-Macrophages were performed using an MOI ranging from 0.05 to 1, as indicated in the figure legends for each experiment. Anti-viral drugs (T20 (10 ug/mL), Nevirapine (5 uM), Raltegravir (10 uM), or Amprenavir (10 uM)) were added to the cells as indicated before and after infection. HIV-1 replication were analyzed at 48hpi by HSA or intracellular p24 expression. Infections with Virus-Like-Particles carrying Vpr in THP1 and PBMC were performed using 500 ng p24 of VLP stocks per 10^6 cells. TET2 degradation by VLP-Vpr were measured 12h after VLP treatment. VLP-Vpr treated cells were subsequently infected with NL4 (Vpr-) or NL4-LUC-G (Vpr-) viruses.

Analysis of HIV-1 post-entry replication

Efficiency of HIV-1 entry, reverse transcription and nuclear import DNA were measured by qPCR priming the minus-strand R-U5, U3-U5 and 2-LTR region, respectively. Detailed information of primers and probes are described (46). Briefly, total cellular DNA of infected cells were harvested at 3-24 hours post-infection using DNAeasy blood kit (Qiagen). HIV-1 entry inhibitor T20 and RT inhibitor NVP were used to validate detection assays. Isolated DNA was treated with 0.1 U/µL DpnI for 4h at 37C to remove contaminated proviral plasmid DNA. Real-time qPCR was performed using Taqman universal PCR master mix (ABI Cat #: 4304437). Primer for detection of genomic CCR5 region were used for normalization of cellular DNA input

Flow cytometry analysis

Intracellular p24 staining was performed in BD Cytofix/cytoperm buffers followed by 1% paraformaldehyde fixation. p24-FITC antibody was purchased from Beckman Coulter (Cat #KC-57) and used at concentration according to manufacturer's protocol. Heat-Stable-Antigen (HSA) expression were detected by anti-mCD24 antibody staining (Biolegend #101808). Cell cycle analysis were performed by genomic DNA staining by fixing cells with 100% ethanol at -20 ^{0}C overnight, followed by Propidium Iodide staining of cellular DNA in 1% BSA containing RNase A.

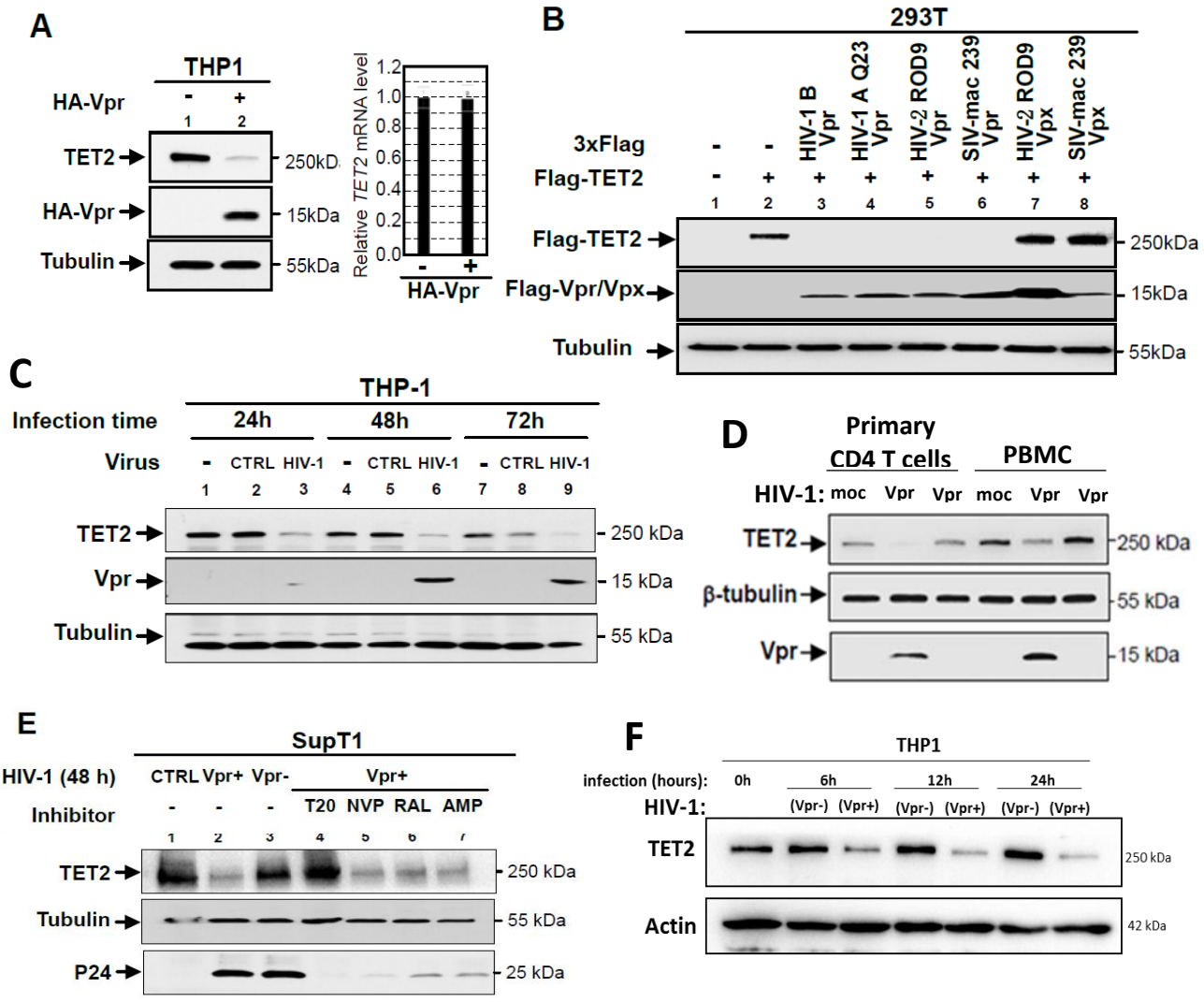

Figure 3.1 HIV-1 Vpr promotes TET2 degradation

(A) HIV-1 viral protein R (Vpr) decreases levels of TET2 protein, not mRNA. THP1 cells were transfected with plasmid expressing HA-Vpr. Western blots were performed with the indicated antibodies, and mRNA level was analyzed by quantitative RT-PCR; error bars represent ±SD for triplicate experiments. (B) Vpr genes from different subtypes of HIV-1, HIV-2 and SIV, but not Vpx, decrease TET2 protein levels. HEK293T cells were transfected with the indicated plasmids and whole-cell extracts were prepared 24hrs after transfection, followed by Western blot (WB) analysis. (C), HIV-1 infection promotes TET2 degradation. THP1 cells were infected with control or HIV-1 (NL4-R3A strain, MOI=1), and cells were harvested at the indicated time points. TET2 levels were detected by WB. (D) Wild-type (WT), but not Vpr-deficient, HIV-1 infection leads to TET2 degradation. Human primary CD4 T cells and human peripheral blood mononuclear cells (PBMCs) were infected with Vpr+ HIV-1 and Vpr- HIV-1 for 24h, 48h or 72h as indicated and TET2 levels were measured. (E) SupT1 cells were infected with HIV-1 in the presence of HIV inhibitors T20 (entry), NVP (RT), Raltegravir (integration) or Amprenavir (maturation). TET2

levels were measured at 48h post-infection. (F) Vpr rapidly depletes TET2 after HIV-1 infection. THP1 cells are infected with Vpr+ or Vpr- HIV-1 for 6h, 12h and 24h to assess kinetics of TET2 degradation

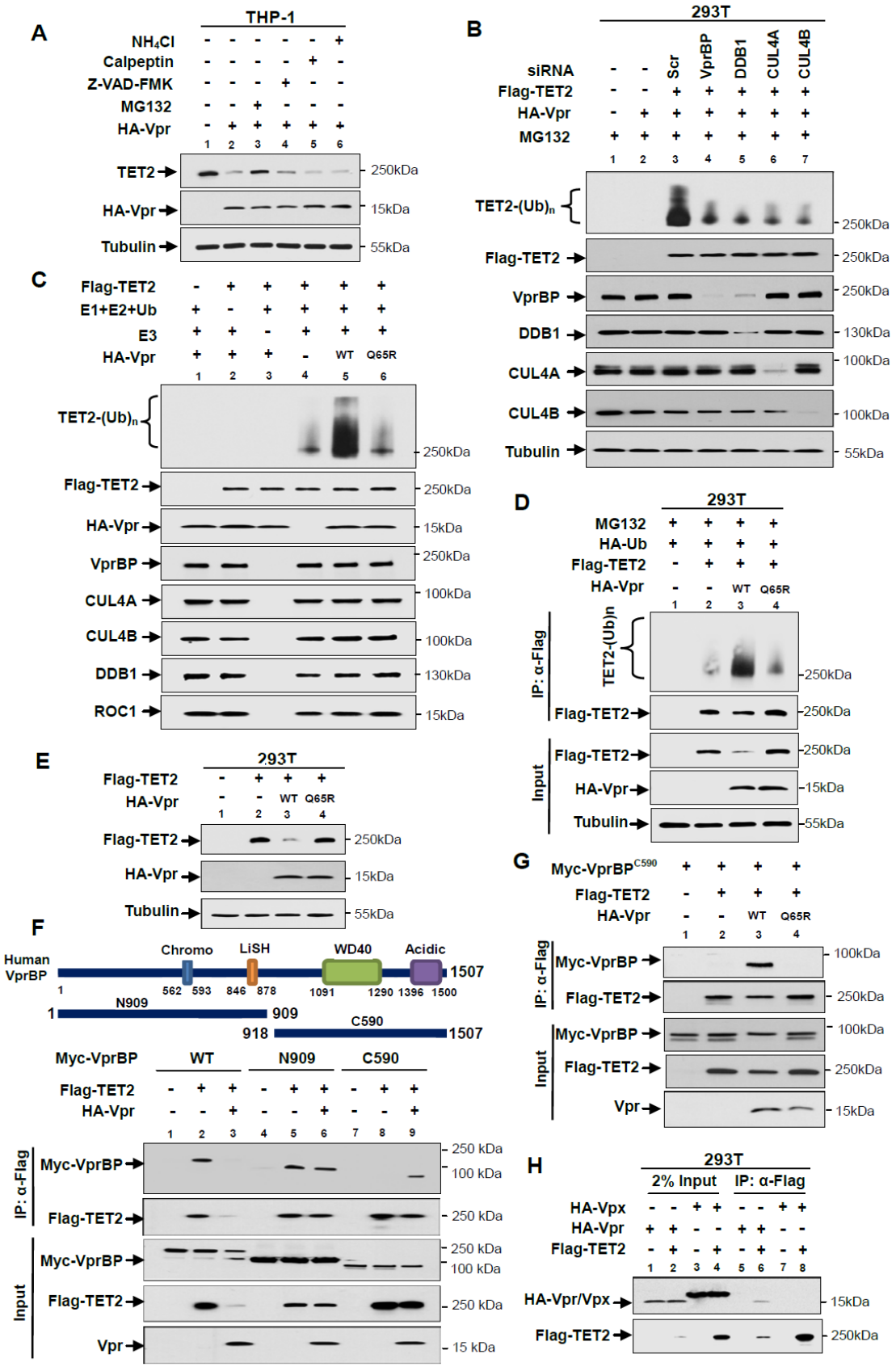
A
THP-1
NH4Cl
Calpeptin
Z-VAD-FMK
MG132
HA-Vpr
TET2
HA-Vpr
Tubulin
250kDa
15kDa
55kDa
B
293T
siRNA
Scr
VprBP
DDB1
CUL4A
CUL4B
Flag-TET2
HA-Vpr
MG132
TET2-(Ub)n
Flag-TET2
VprBP
DDB1
CUL4A
CUL4B
Tubulin
250kDa
130kDa
100kDa
55kDa
C
Flag-TET2
E1+E2+Ub
E3
HA-Vpr
WT
Q65R
TET2-(Ub)n
Flag-TET2
HA-Vpr
VprBP
CUL4A
CUL4B
DDB1
ROC1
250kDa
15kDa
100kDa
130kDa
D
293T
MG132
HA-Ub
Flag-TET2
HA-Vpr
WT
Q65R
IP: α-Flag
TET2-(Ub)n
Flag-TET2
Input
Flag-TET2
HA-Vpr
Tubulin
250kDa
15kDa
55kDa
E
293T
Flag-TET2
HA-Vpr
WT
Q65R
Flag-TET2
HA-Vpr
Tubulin
250kDa
15kDa
55kDa
F
Human VprBP
Chromo
LiSH
WD40
Acidic
1507
562 593 846 878 1091 1290 1396 1500
N909
909
C590
918
1507
Myc-VprBP
WT
N909
C590
Flag-TET2
HA-Vpr
IP: α-Flag
Myc-VprBP
Flag-TET2
Input
Myc-VprBP
Flag-TET2
Vpr
250 kDa
100 kDa
250 kDa
250 kDa
100 kDa
250 kDa
15 kDa
G
Myc-VprBP C590
Flag-TET2
HA-Vpr
WT
Q65R
IP: α-Flag
Myc-VprBP
Flag-TET2
Input
Myc-VprBP
Flag-TET2
Vpr
100kDa
250kDa
100kDa
250kDa
15kDa
H
293T
2% Input
IP: α-Flag
HA-Vpx
HA-Vpr
Flag-TET2
HA-Vpr/Vpx
Flag-TET2
15kDa
250kDa

Figure 3.2. Vpr promotes TET2 degradation by CRL4^{VprBP} E3 Ligase
(A) Vpr-mediated TET2 degradation is blocked by the proteasome inhibitor, MG132. THP1 cells were transfected with HA-Vpr, and then treated with NH4Cl (20mM), Calpeptin (50µM), Z-VAD-FMK (100µM), or MG132 (10µM) for 24hrs. Cell lysates were analyzed by WB with the indicated antibodies. (B) Knockdown of each component of the CRL4^{VprBP} E3 ligase complex inhibits Vpr-promoted TET2 ubiquitylation in vivo. HEK293T cells were transfected with indicated plasmids and siRNA oligonucleotides targeting the indicated genes for 72h. The efficiency of knockdown was verified by immunoblotting. In vivo TET2 ubiquitylation was determined by IP-western blot analysis under denaturing conditions. (C) WT, but not Q65R (VprBP binding deficient mutant Vpr), promotes TET2 polyubiquitylation in vitro. Immunopurified TET2 protein was incubated with VprBP immune-complex and purified wild-type or Q65R mutant Vpr in a buffer containing ubiquitin, recombinant E1, E2 and ATP. Reactions were terminated by adding SDS loading buffer, followed by immunoblotting with the indicated antibodies. (D) WT, but not Q65R mutant Vpr, promotes TET2 polyubiquitylation in vivo. HEK293T cells were transfected with indicated plasmids. Whole cell lysates were prepared under denaturing conditions and TET2 ubiquitylation was examined by immunoblotting with indicated antibodies. (E) Vpr promoted TET2 degradation requires Vpr-VprBP binding. HEK293T cells were transfected with indicated plasmids, TET2 degradation is examined by WB. (F), TET2 binds to different domains of VprBP in the absence and presence of Vpr. HEK293T cells were transfected with indicated plasmids, followed by IP-western analysis with the indicated antibodies. (G) Q65R mutant Vpr cannot bind VprBP to bridge with TET2. HEK293T cells were transfected with indicated plasmids and protein-protein interactions were determined by IP-western analysis. (H) Vpr, but not Vpx, binds to TET2. The indicated plasmids were transfected into HEK293T cells, and protein-protein interactions were determined by IP-western analysis.

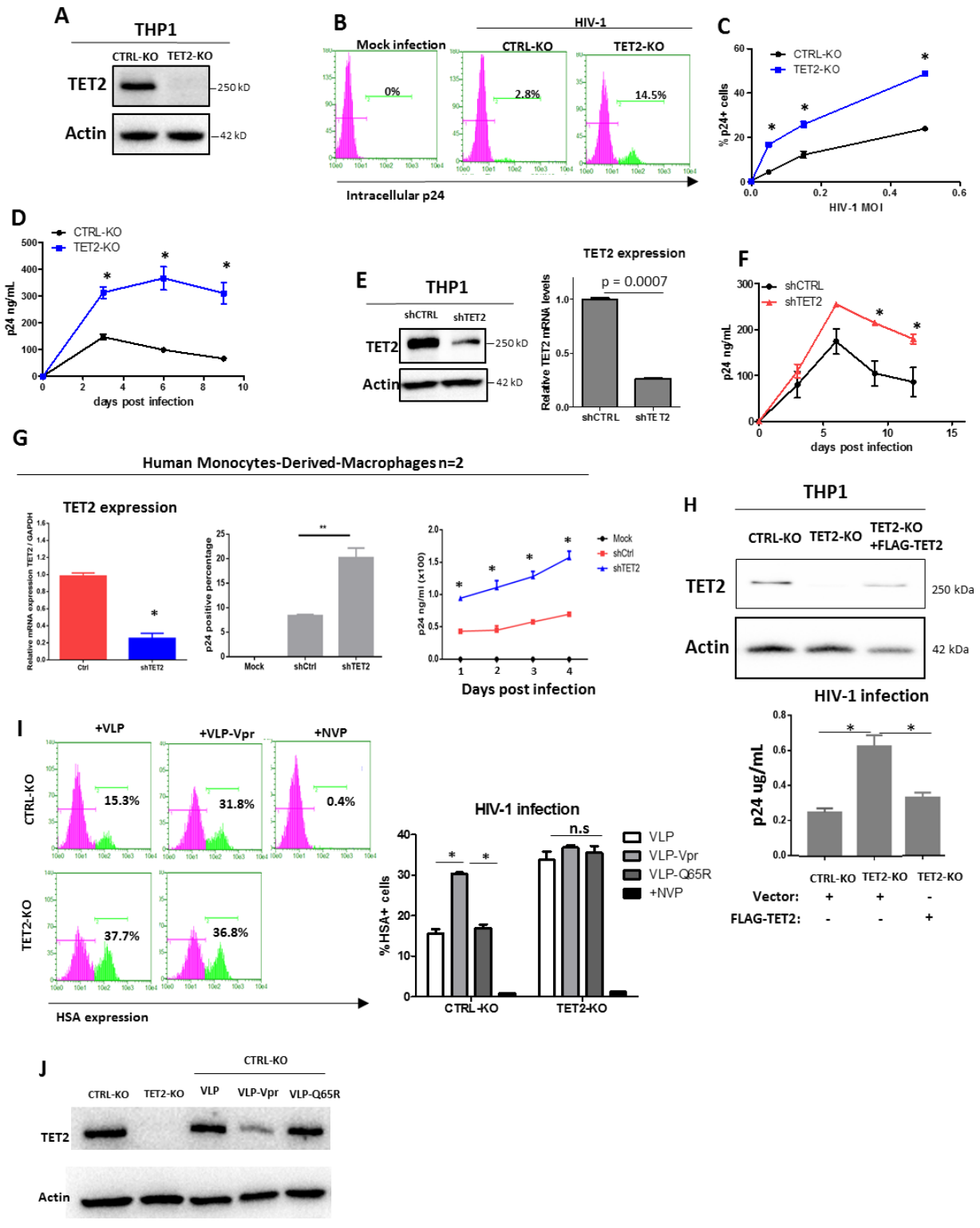

Figure 3.3. TET2 inhibits HIV-1 replication

(A-H), Depletion of TET2 enhances HIV-1 (Vpr-) replication. (A) CRISPR-Cas9 mediated knockout of TET2 were generated in THP1 cells. CTRL-KO cells were transduced by non-targeting gRNA.

Western blot of TET2 protein levels are shown. (B) Control and TET2-KO THP1 cells were infected with HIV-1 (Vpr-) with an MOI of 0.05. Percentage of infected cells were analyzed at 2 days post infection by intracellular p24 FACS. (C) Titrated viral MOIs were used to infect control and TET2-KO THP1 cells and analyzed by intracellular p24 FACS. (D) Viremia of HIV-1 (Vpr-) infected THP1 control and TET2 knockout cell lines were measured over 9 days by p24 ELISA. (E) shRNA-mediated knockdown of TET2 was generated in THP1 cells and confirmed by western blot (left) and RT-qPCR (right) for TET2 expression. Control knockdown cells were similarly transduced with non-targeting shRNA. (F) Control knockdown and TET2 knockdown cells described in (E) were infected with HIV-1 (Vpr-) with an MOI of 0.05. Viral replication was analyzed by p24 ELISA. (G) shRNA-mediated knockdown of TET2 in human Monocytes-Derived-Macrophages (donor n=2) were performed as described in Methods and confirmed by RT-qPCR. MDM were then infected with the R3A(Vpr-) strain and viral replication was analyzed by p24 FACS and p24 ELISA. (H) Rescue of TET2 expression in TET2-KO THP1 cells restored HIV-1 inhibition. TET2-expressing vector were transfected into TET2-KO cells for 24h and verified by Western blot. The transfected cells are infected with HIV-1 (Vpr-), and virus replication was analyzed by p24 ELISA at 3dpi.
(I-J) Vpr enhances HIV-1 replication in a TET2-dependent manner. (I) Virus-Like-Particles (VLP) were produced with Vpr or VprQ65R incorporation, or as empty particles, and treated to CTRL-KO or TET2-KO THP1 cells for 12h. Then cells were infected with HIV-1 (Vpr-). Virus replication was analyzed by HSA reporter expression at 2dpi. (J) TET2 degradation by VLP-Vpr in THP1 were analyzed by western blot and compared to TET2 knockout cells

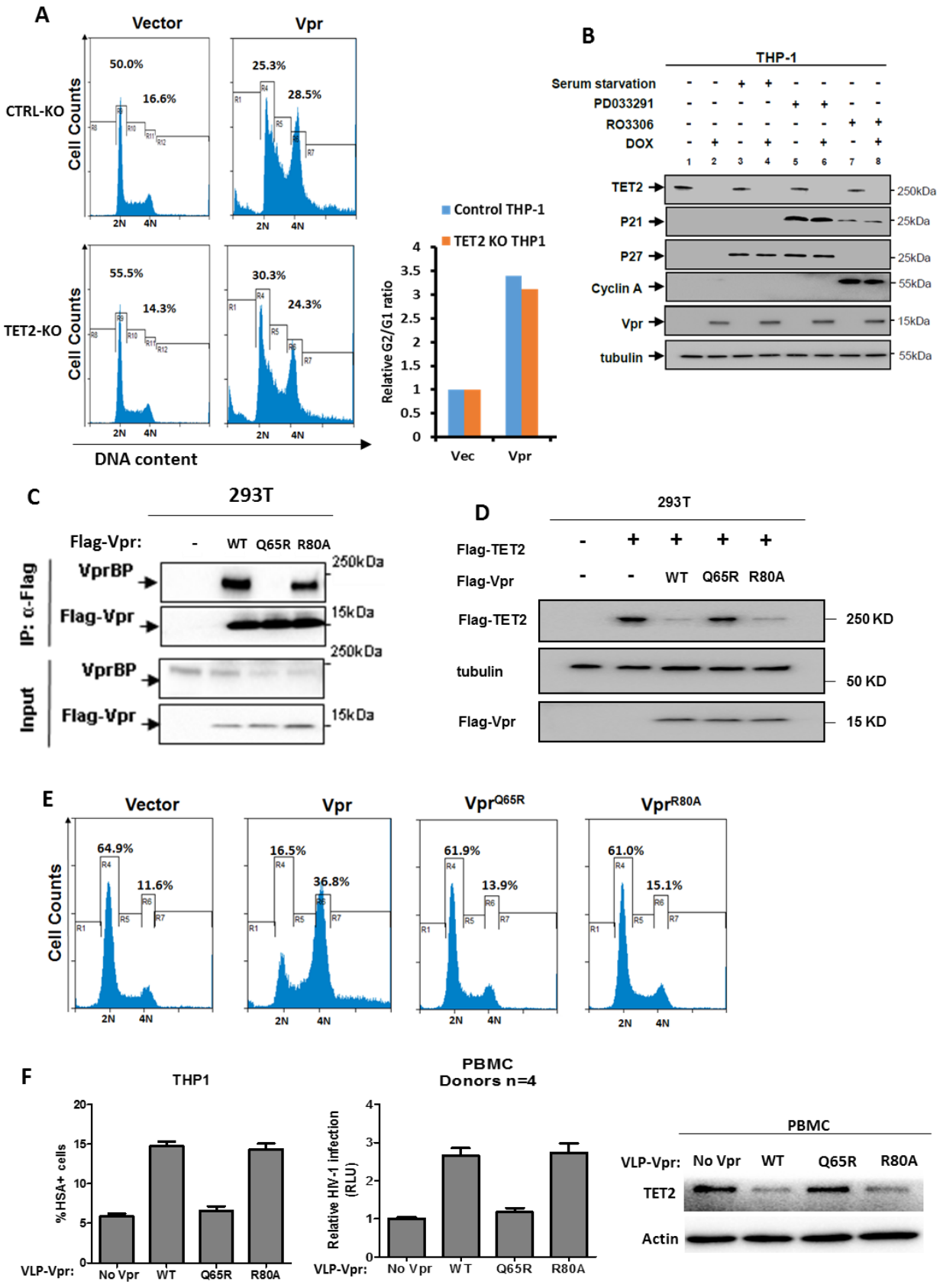
A
Vector
Vpr
CTRL-KO
TET2-KO
Cell Counts
50.0%
16.6%
25.3%
28.5%
55.5%
14.3%
30.3%
24.3%
2N
4N
DNA content
Control THP-1
TET2 KO THP1
Relative G2/G1 ratio
Vec
Vpr
B
THP-1
Serum starvation
PD033291
RO3306
DOX
TET2
P21
P27
Cyclin A
Vpr
tubulin
250kDa
25kDa
55kDa
15kDa
C
293T
Flag-Vpr:
WT Q65R R80A
IP: α-Flag
Input
VprBP
Flag-Vpr
250kDa
15kDa
D
293T
Flag-TET2
Flag-Vpr
250 KD
50 KD
15 KD
E
Vector
Vpr
VprQ65R
VprR80A
64.9%
11.6%
16.5%
36.8%
61.9%
13.9%
61.0%
15.1%
F
THP1
PBMC
Donors n=4
%HSA+ cells
Relative HIV-1 infection (RLU)
VLP-Vpr:
No Vpr
WT
Q65R
R80A
PBMC
TET2
Actin

Figure 3.4. Vpr promotes TET2 degradation and enhanced HIV-1 replication independently of G2-arrest

(A) TET2 is not required for Vpr-induced G2 arrest. TET2 knockout THP1 cells were transfected with control or Vpr-expressing vector, and DNA staining and FACS analysis were performed after 48h for cell-cycle analysis. G2/G1 ratio was used as quantifications of G2 arrest induction by Vpr. (B) Vpr induced TET2 degradation is cell cycle independent. Vpr inducible THP-1 cells were untreated or treated with serum starvation, PD033291, RO3305 respectively for 48h. DOX was added to induce Vpr expression. TET2 protein levels were determined by western blot. (C) 293T cells were transfected with FLAG-tagged Vpr and indicated Vpr mutants. FLAG-immunoprecipitation with VprBP were performed and analyzed by western blot. (D) 293T cells were transfected with TET2 and mutants of Vpr. TET2 protein levels were analyzed at 48h by western blot. (E) Vpr mutants were transfected in THP1 cells, and G2 cell-cycle arrest was analyzed by DNA staining and FACS analysis. (F) VLP packaged with wild type or Vpr mutants Q65R and R80A were generated and quantified by p24 ELISA. Equal amount of VLPs were treated to THP1 or PBMC cultures for 12h. PBMCs were then infected with HIV-1 (Vpr-), and HIV-1 infection were analyzed by HSA or luciferase reporter expression. PBMC data showed relative HIV-1 replication levels from independent experiments of 4 PBMC donors. TET2 depletion by Vpr and VprR80A in PBMC was confirmed by western blot.

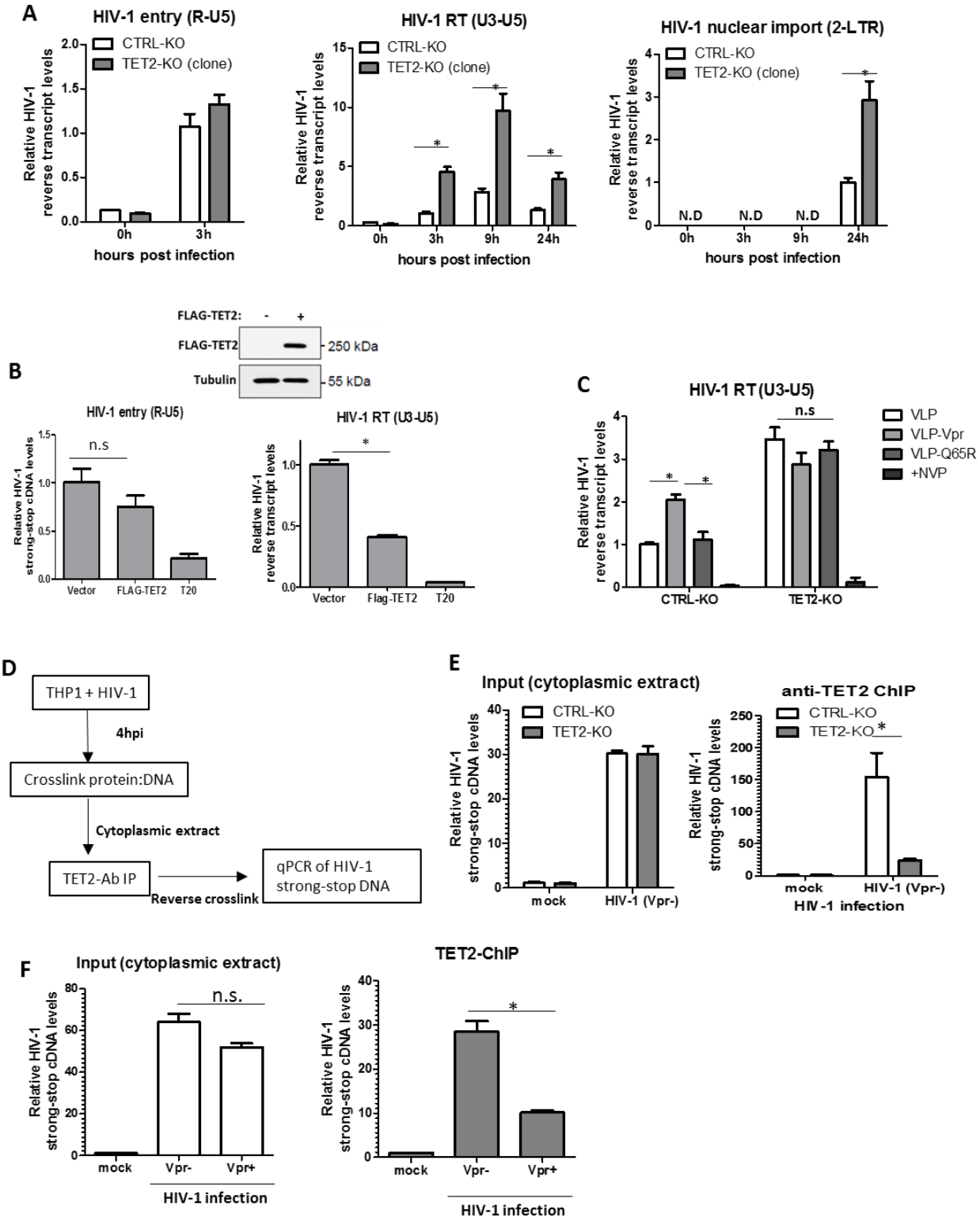

Figure 3.5. TET2 inhibits HIV-1 reverse transcription, which is counteracted by Vpr
(A) Control and TET2-KO THP1 were infected with HIV-1 (Vpr-), and DNA were harvested at the indicated time points for viral post-entry steps analysis by qPCR as described in Methods.

Briefly, strong-stop DNA (R-U5) measured viral entry, (U3-U5) DNA measured early reverse transcription, and 2-LTR DNA indicated nuclear import efficiency. (B) THP1 were transfected with control or TET2-expressing vector, and cells were infected with HIV-1 (Vpr-) virus. DNA were harvested at 6hpi for entry and reverse transcription efficiency analysis. HIV-1 fusion inhibitor T20 and RT inhibitor NVP were used as controls to verify post-entry steps analysis. (C) Vpr enhanced HIV-1 reverse transcription in a TET2-dependent manner. Control or TET2-KO THP1 cells were treated with VLP or VLP-Vpr for 24h, then cells were infected with HIV-1 (Vpr-). HIV-1 reverse transcription were analyzed as described in (B). (D) schematic diagram of ChIP-PCR experiment for TET2 interaction with HIV-1 reverse transcription complex. (E) Control and TET2-KO THP1 cells were infected with HIV-1 (Vpr-) virus for 4h, and TET2 ChIP were performed in the cytoplasmic extract. Total strong-stop DNA levels in the input and TET2-IP fractions were measured by qPCR. (F) Similar TET2-ChIP experiment as in (E) in THP1 cells, using Vpr- and Vpr+ HIV-1 viruses.

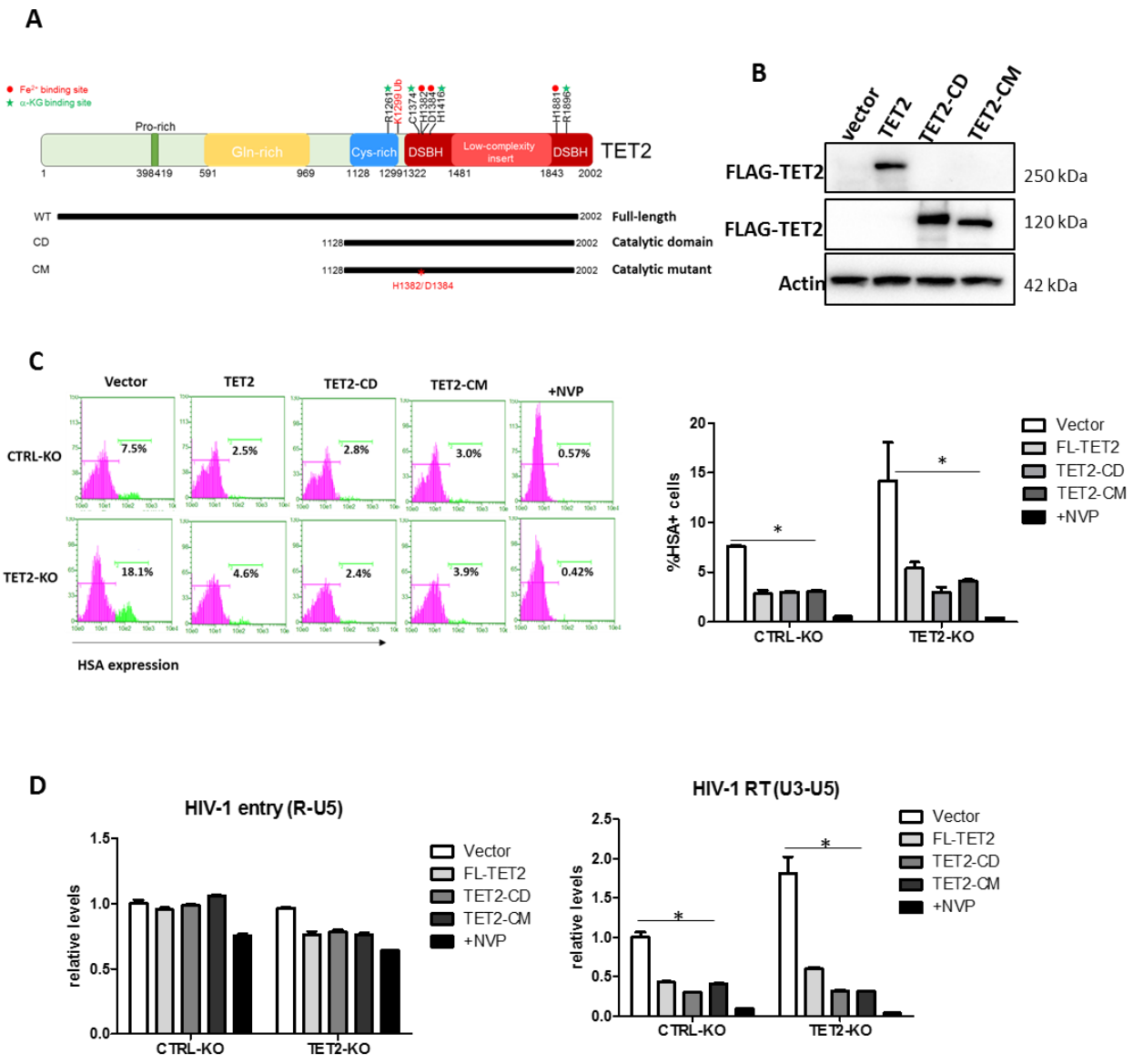

Figure 3.6. The C-terminal domain of TET2 inhibits HIV-1 independently of its enzymatic activity

(A-C) The C-terminal domain of TET2 inhibits HIV-1 infection. (A) schematic representation of TET2 protein domains, with constructs expressing the full-length, C-terminal domain and catalytic iron-binding mutant (H1382/D1384 double mutant) as indicated. (B) Validation of full-length TET2 and and TET2 C-terminal domain (CD) and TET2 catalytic mutant (CM) construct expression in 293T cells by western blot. (C) control and TET2-KO THP1 cells were transfected with the indicated constructs for 24h, and then infected with HIV-1 (Vpr-) virus at MOI=0.1. Viral replication was analyzed by HSA reporter expression after 2 days infection. (D) The C-terminal domain of TET2 inhibits HIV-1 reverse transcription. Similar experiment performed as in (C) with intracellular viral DNA harvested at 6hpi for HIV-1 post-entry analysis by qPCR as described in Figure 3.5.

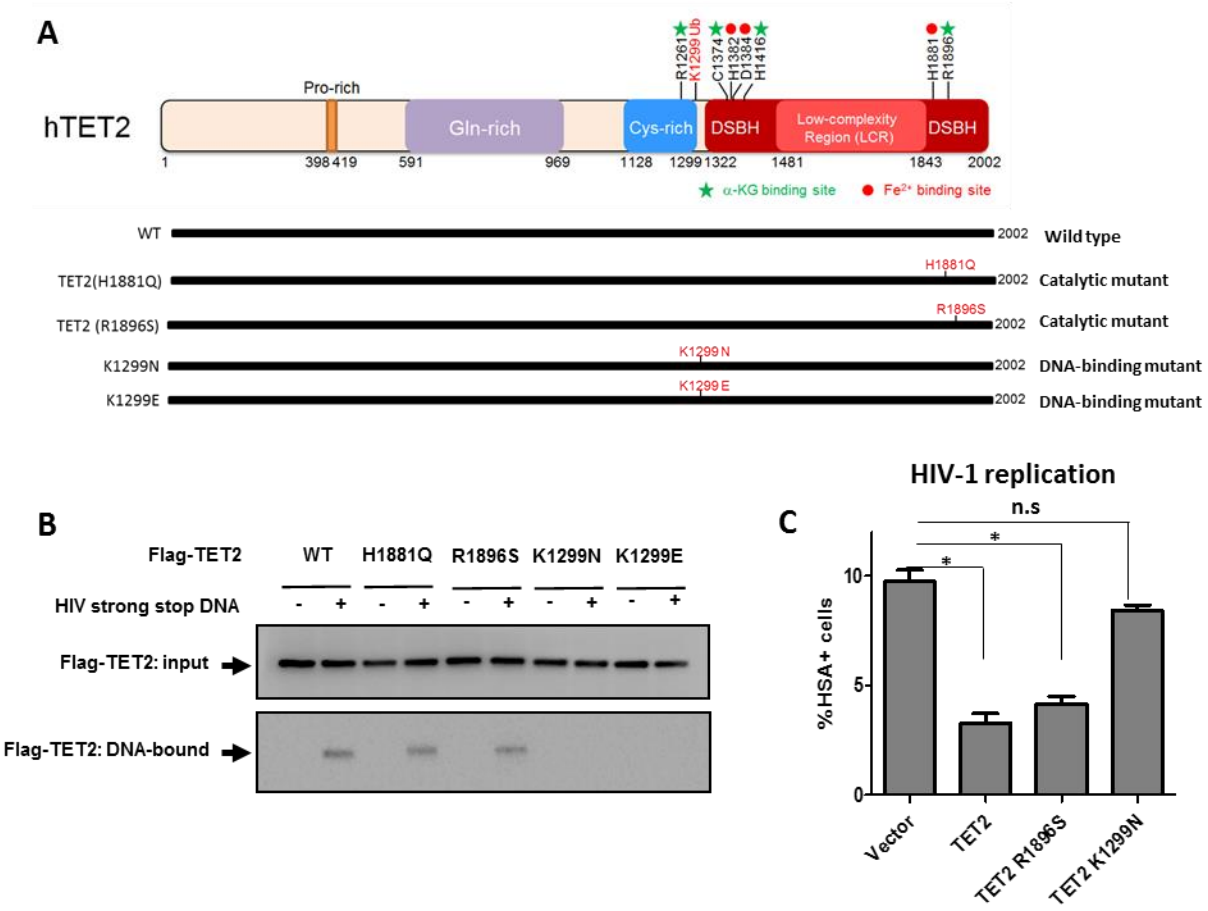

Figure 3.7. The conserved Lysine residue K1229 of TET2 is required for HIV-1 strong-stop DNA binding and inhibition of HIV-1 reverse transcription

(A) schematic representation of TET2 protein domains, with the indicated residues mutants. H1881 is the iron-binding site, R1896 is the α-KG binding site, both required for enzymatic activity. K1229 is the mono-ubiquitylation site, requires for DNA-binding capability. (B) Flag tagged wildtype and mutant TET2 were ectopically expressed in and immunopurified from 293T cells, eluted with Flag peptides. The eluents were incubated with streptavidin beads with or without biotin labeled HIV strong stop DNA. TET2-DNA binding was determined by western blot using Flag antibody. The result shows wildtype and H1881Q and R1896S catalytic mutant TET2 binds to HIV strong stop DNA. In contrast, both K1299N and K1299E DNA-binding mutants does not bind strong stop DNA. (C) TET2-KO THP1 cells were transfected with the indicated constructs for 24h, and the infected with HIV-1 (Vpr-) virus at MOI=0.1. Viral replication was analyzed by HSA reporter expression after 2 days infection. The results show wild type and catalytic mutant R1896S TET2 inhibits HIV infection, the K1299 DNA-binding mutant does not.

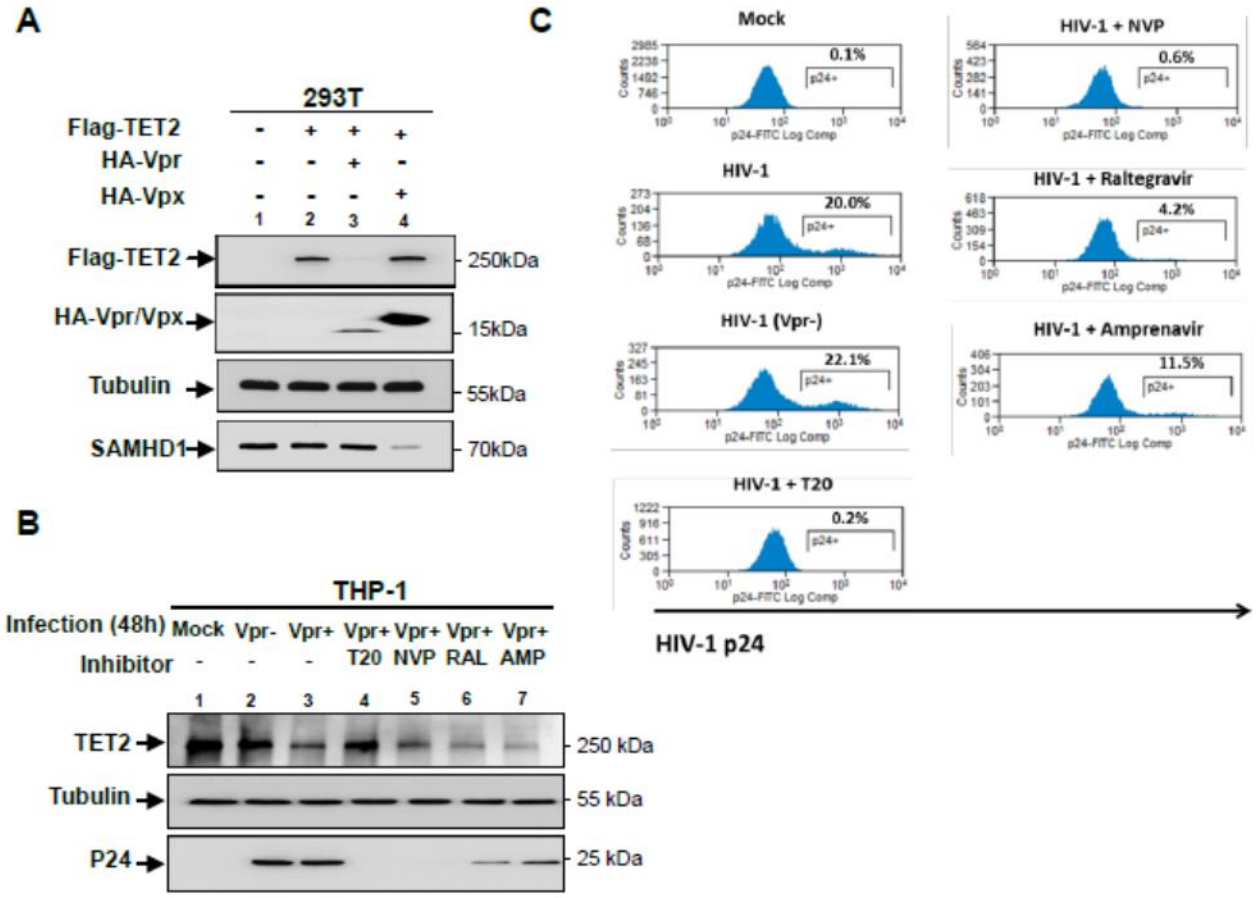

Figure S3.1. HIV-1 viral protein R (Vpr) promotes TET2 degradation.
(A) HEK293T cells were transfected with the indicated plasmids and whole-cell extracts were prepared 30 hrs after transfection, followed by Western blot (WB) analysis.
(B) SupT1 cells were infected with Vpr+ or Vpr- HIV-1 NL4-R3A in the absence or presence of T20, NVP, Raltegravir or Amprenavir. Percentage of infected cell was measured by intracellular p24 staining at 48h post-infection. Related to Figure 1E.
(C) THP-1 cells were infected with NL4-R3A in the absence or presence of T20, NVP, Raltegravir or Amprenavir. TET2 protein levels were measured by western blot at 48h post infection

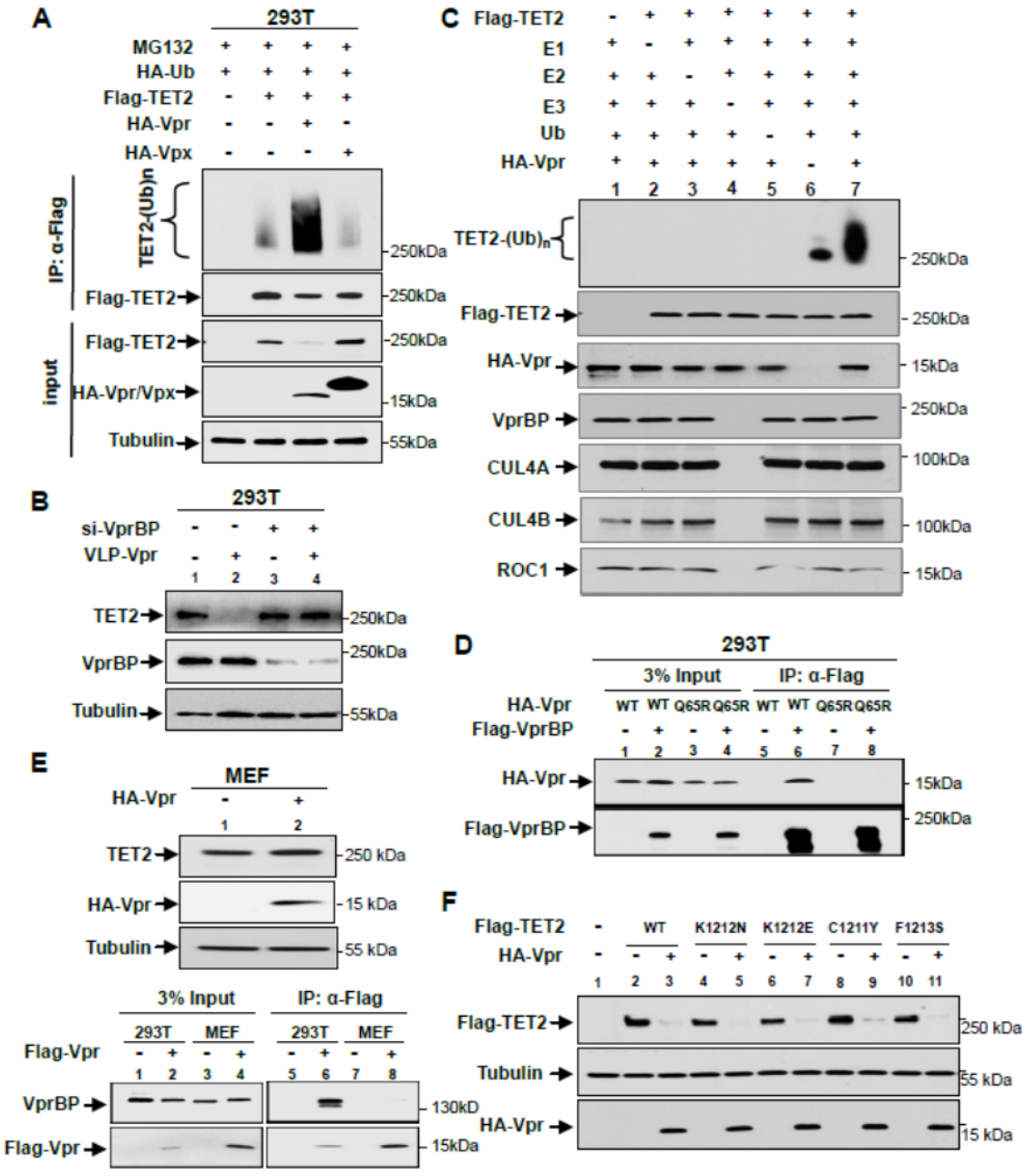

Figure S3.2. Vpr-promoted TET2 ubiquitylation and degradation requires E1, E2 and Vpr-VprBP binding, but is independent of monoubiquitylation site (K1212) and TET2-VprBP binding.

(A) HEK293T cells were transfected with the indicated plasmids. Ubiquitylation of TET2 was examined by coupled IP-western using first Flag antibody to immunoprecipitate TET2 and then HA antibody to detect ubiquitin.

(B) HEK293T cells were transfected with siRNA oligonucleotides targeting VprBP for 48h and then treated with VLP-Vpr for another 24h. The efficiency of knockdown and TET2 degradation was analyzed by immunoblotting.
(C) Vpr promotes polyubiquitylation of TET2 in the presence of $CRL4^{VprBP}$ E3 ligase in vitro. Immunopurified TET2 protein was incubated with VprBP immune-complex with or without immunopurified Vpr in the presence of E1, E2, ATP and ubiquitin. The reaction was terminated by adding a SDS loading buffer and mixtures were resolved by SDS-PAGE, followed by immunoblotting with the indicated antibodies.
(D) Q65R mutation in Vpr disrupts its binding with VprBP. HEK293T cells were transfected with the indicated plasmids. Vpr-VprBP binding was examined by IP and western analysis.
(E) Vpr-promoted TET2 degradation is dependent Vpr binding to VprBP. Mouse embryonic fibroblasts (MEFs) were transfected HA-Vpr. Protein expression and binding was determined by IP-western analysis using the indicated antibodies.
(F) HEK293T cells were transfected with the plasmids indicated and TET2 levels were determined by western blot. K1212N and K1212E in mouse TET2 correspond to K1299 in human TET2 and disrupt the monoubiquitylation site. F1213S in mouse TET2 correspond to F1300S in human TET2 and disrupt TET2-VprBP binding.

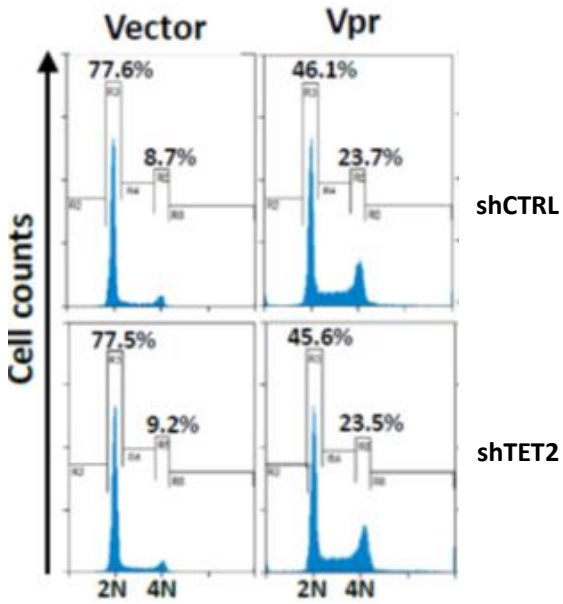

Figure S3.3. TET2 is not required for Vpr-mediated G2-arrest induction
Stable shRNA-mediated knockdown of TET2 were generated in U2OS cells. Control or knockdown cells were transfected with control or Vpr-expressing vector for 48h. Cell-cycle arrest were analyzed by DNA staining and FACS analysis

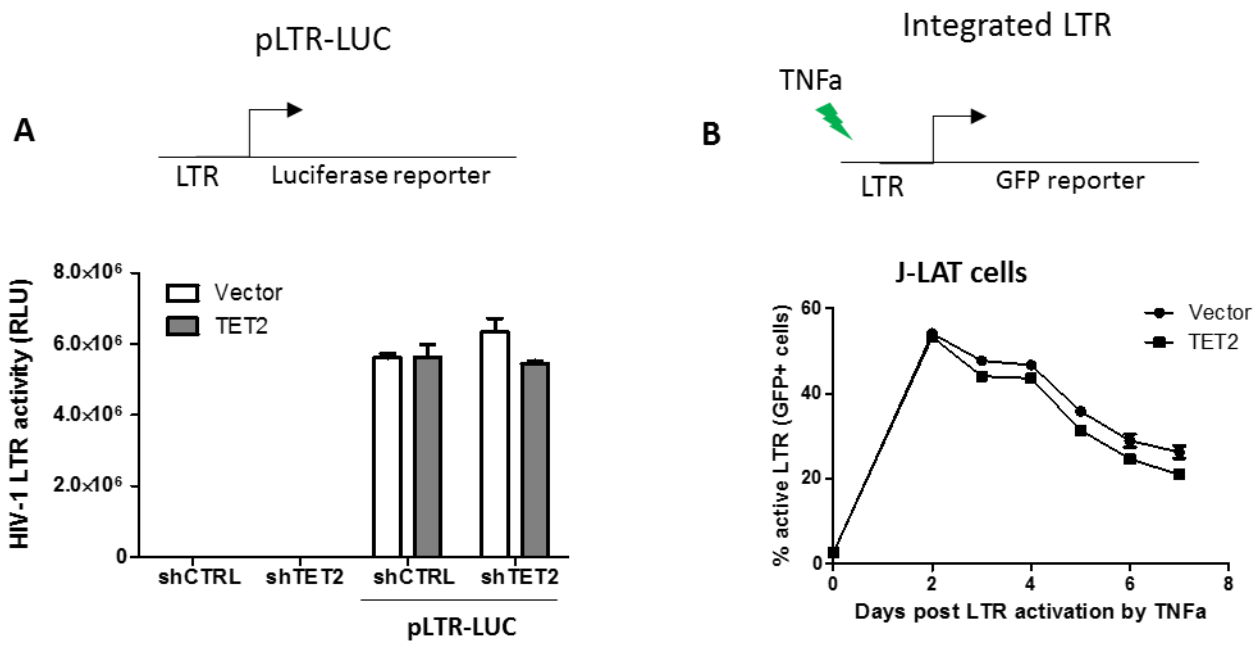

Figure S3.4. TET2 does not control HIV-1 LTR promoter activity
(A) Vectors expressing luciferase under HIV-1 LTR promoters were transfected into control or TET2 knockdown THP1 cells, together with an empty vector control or a TET2-expressing construct at a 1:1 ratio. LTR activity were measured 48h after transfection by luciferase assay. (B) J-LAT2 cells containing a latent integrated LTR promoter controlling a GFP reporter were induced by 100ng/mL TNFa treatment for 6h, then the cells were transfected with TET2-construct or vector control. LTR activity are monitored by GFP expression over 7 days.

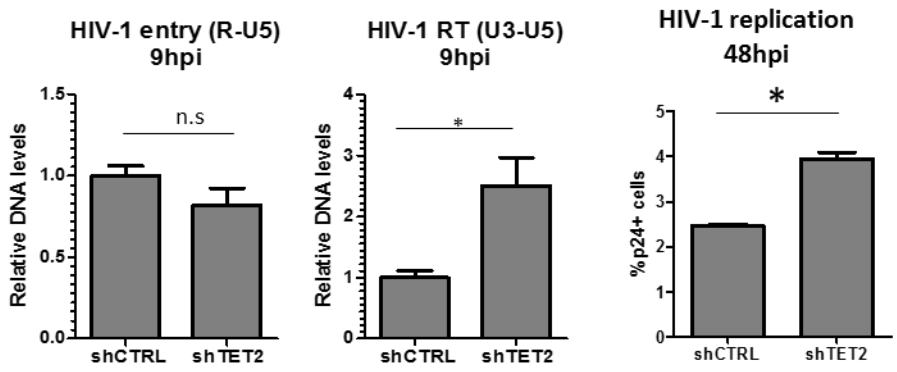

Figure S3.5. TET2 inhibits HIV-1 reverse transcription

Stable shRNA-mediated knockdown of TET2 were generated in THP1 cells. Control or knockdown cells were infected with HIV-1 (Vpr-) with an MOI of 0.05. HIV-1 post-entry steps were analyzed by qPCR as described in Figure 3.5.

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CHAPTER 4. TET2 IS A MODULATOR FOR TYPE-1 INTERFERON-MEDIATED INHIBITION OF HIV-1

4.1. SUMMARY

Viruses have commonly evolved to impair the type-1 interferon-mediated antiviral defense. TET2 is a host restriction factor previously reported to directly inhibit HIV-1 infection and is degraded by HIV-1 Vpr. Here we report that TET2 is also required for efficient inhibition of HIV-1 by the type-1 IFN pathway. Vpr confers IFN resistance to HIV-1 by degrading TET2 in CD4 T cells and THP1 cells. TET2 depletion impairs the induction of MX2 and IFITM3 at a post-transcriptional level. Supporting a functional role of MX2 regulation by TET2, the MX2-resistant Gag^{N57S} HIV-1 is insensitive to TET2 depletion. Furthermore, depletion of TET2 reduced IFITM3 incorporation into nascent HIV-1 virions and enhanced nascent virion infectivity. These results reveal an interferon-TET2-MX2/IFITM pathway that plays a critical role in inhibiting HIV-1 replication.

4.2. BACKGROUND

Viruses including HIV-1, needs a host cell for its replication and survival. The human innate immune defense system primarily limits virus replication through type-1 interferons (IFNα and IFNβ) which are produced after virus detection by the innate immune system (1). Type-1 interferons function in an autocrine or paracrine manner after secreted outside the cells. It binds to the type-1 IFN receptor (IFNAR) to initiate a signal transduction that leads to the activation of TYK2 and JAK1 kinase. These kinases in turn phosphorylate STAT1 and STAT2, causing their dimerization (STAT1/STAT2) and translocation into the nucleus. STAT1/STAT2

dimers binds with IRF9, and recognizes IFN-stimulated-response-elements (ISRE) in the DNA promoter regions of interferon-stimulated-genes (ISGs). The binding of STAT1/STAT2/IRF9 complex, also called the ISGF3 complex, on ISG promoters activates the transcription of hundreds of ISGs (2). ISGs are a diverse range of antiviral effector proteins that directly intervene with a pathogen-specific biological processes that are foreign to the mammalian host (3). The activation of ISGs turns the cell into an antiviral inflammatory state, which becomes a hostile environment for viral replication to occur (4, 5). Viruses in turn has evolved strategies to evade or antagonize the type-1 IFN signaling response (6). Indeed, the IFN signaling cascade is a common process targeted by viruses, such as the EBV LMP-1 protein preventing TYK2-mediated STAT1 phosphorylation (7), DENV NS5 proteins targeting STAT for proteasomal degradation (8), and Adenovirus E1A protein inhibiting the nuclear localization of ISGF3 complex (9).

HIV-1 is known to efficiently elicit the interferon response (10, 11) and yet is still capable of establishing persistent infection (12, 13). Indeed, HIV-1 accessory proteins are well known to counteract host restriction factors that are often induced by interferon (14), which contributes to HIV-1 resistance against IFN. One of the accessory proteins is the viral protein R (Vpr), which has been reported to enhance HIV-1 replication in myeloid cells such as monocytes and macrophages, but not in CD4 T cells (15–17). In Chapter 3, we reported the discovery of a new restriction factor against HIV-1 called TET2, which is targeted by Vpr for proteasomal degradation. TET2 is a direct inhibitor of HIV-1 reverse transcription in monocytes and macrophages. However, how TET2 affect HIV-1 replication in CD4 T cells, the major target cells of HIV-1 infection in vivo, has not been investigated. In this report, we show although TET2

depletion does not directly alter HIV-1 replication in T cells, it is involved in type-1 interferon signaling and required for ISG protein expression and HIV-1 inhibition.

4.3. RESULTS

4.3A. Vpr depletes TET2 to counteract IFN-mediated antiviral effect

In agreement with previous literatures (15, 17), Vpr did not affect HIV-1 replication in activated primary CD4 T cells (Fig. 4.1A, left graph). In contrast to our previous studies in monocytic cells and primary macrophages (Chapter 3), we found the depletion of TET2 by shRNA in CD4 T cells (Fig. 4.1B) has no effect on HIV-1 infection efficiency (Fig. 4.1C, left graph). Type-1 interferon (IFNα and IFNβ) signaling exhibits a broad antiviral effects against HIV-1 in cultured cell models (3, 5, 6). With recent reports suggesting Vpr may confer viral resistance to IFN (16, 18), we hypothesized that TET2 is involved in IFN signaling that is counteracted by Vpr. Interestingly, Vpr significantly enhanced HIV-1 replication in CD4 T cell cultures under treatment with IFNα (Fig. 4.1A, right graph). In addition, when CD4 T cells were treated with IFNα, the knockdown of TET2 by shRNA also significantly enhanced Vpr-deficient HIV-1 infection (Fig. 4.1C). This suggests TET2 is required for the IFNα-mediated inhibition of HIV-1 infection. Indeed, we found that while IFNα inhibits Vpr-deficient HIV-1 by 31-folds, knocking down TET2 reduced the magnitude of IFNα inhibition to 12-folds (Fig. 4.1D). Accordingly, expression of Vpr phenocopied the effect of TET2 knockdown, also reducing the IFN effect to similar extent (Fig. 4.1D). Quantifications of IFN inhibition magnitudes in three independent CD4 T cell donors revealed that both TET2 knockdown and Vpr expression reduced about 70% of the total IFNα activity in inhibiting HIV-1 infection (Fig. 4.1E). These data revealed a potential role of TET2 in

the IFN signaling pathway to inhibit HIV-1 infection. Similar results were obtained in THP1 cells (Fig. 4.1F), suggesting the role of TET2 in IFN pathway is not in T cells exclusively.

4.3B. TET2 is required for the induction of MX2 and IFITM3 proteins

IFN induces many ISGs that are antiviral effector proteins known to inhibit a specific step of HIV-1 replication (see Fig. 1.1A). We therefore investigated the induction of known anti-HIV ISGs in control and TET2 knockdown T cells and THP1 cells. Two of such ISGs – MX2 and IFITM3 – are only expressed after IFN signaling. Indeed, we found MX2 and IFITM3 protein levels were readily induced 24 hours after IFNα treatment. Interestingly, knockdown of TET2 severely impaired the induction of the two antiviral proteins in both T cells (Fig. 4.2A) and THP1 cells (Fig. 4.2C). Surprisingly, when looking at the gene transcriptional level, we found the magnitudes and kinetics of MX2/IFITM3 mRNA induction by IFN were not reduced by TET2 knockdown (Fig. 4.2B and 4.2D). Our results showed an unexpected role of TET2 in regulating ISG expression at a post-transcriptional level. Other anti-HIV proteins, such as APOBEC3G and SAMHD1, were not modulated by TET2 (Fig. 4.2C). We conclude TET2 is important in the IFN response for the induction of a subset of anti-HIV-1 proteins, such as MX2 and IFITM3.

4.3C. MX2-resistant N57S HIV-1 is insensitive to TET2 depletion

We next investigated whether the IFN-TET2 pathway restricts HIV-1 replication through MX2- or IFITM3-mediated mechanisms. MX2 inhibits HIV-1 replication at the viral nuclear import or viral DNA integration step (22, 24, 25), which can be measured by the luciferase reporter for HIV-1 gene expression. Ectopic expression of MX2 in TET2 knockdown cells suppressed HIV-1 infection as measured by luciferase activity, demonstrating the functional antiviral role of MX2 in our viral replication assays (Fig. 4.3A). To prove the involvement of MX2

in the IFN-TET2 pathway on HIV-1 inhibition, we took advantage of a reported MX2-resistant mutant containing the N57S mutation in HIV-1 Gag protein (24). As expected, Gag^{N57S} HIV-1 is resistant to MX2-mediated HIV-1 inhibition (Fig. 4.3B). To prevent the viral Vpr protein from degrading TET2 in our studies, both our WT Gag and Gag^{N57S} HIV-1 were Vpr-deficient. Interestingly, while TET2 knockdown significantly impaired IFN-mediated inhibition of HIV-1, the sensitivity of Gag^{N57S} HIV-1 to IFN was not affected by TET2 depletion (Fig. 4.3C and 4.3D). We conclude the IFN-TET2 pathway inhibits HIV-1 replication in a MX2-dependent manner.

4.3D. TET2 is required for IFN-mediated induction of IFITM3 to inhibit HIV-1 virion infectivity

IFITM3 is incorporated into HIV-1 nascent virions produced during their assembly at the cellular surface membrane. This incorporation of IFITM3 is reported to impair virion fusion capability with the target cells, therefore reducing virion infectivity (23). Transfection of HIV-1 pro-viral DNA constructs into 293T cells allow us to study the step of nascent HIV-1 virions assembly, and the infectivity of the released nascent virions can be measured by titration on target cell lines. HIV-1 pro-viral DNA were transfected in control and TET2 knockdown 293T cells to produce nascent HIV-1 viruses, in the absence or presence of IFNα treatment. As expected, we found the IFN-mediated induction of IFITM3 in 293T cells was impaired in TET2 knockdown cells (Fig. 4.4A). Consequentially, we detected the incorporation of IFITM3 into nascent cell-free virions produced from the control 293T cells (Fig. 4.4B, lane 3) but not in virions produced from the knockdown cells (Fig. 4.4B, lane 4). Viral p24 levels in virion-producing cells (Fig. 4.5A) and in cell-free virions (Fig. 4.4B) were not affected by TET2 knockdown or by IFN, indicating TET2 does not affect HIV-1 protein production, nascent virion assembly or virion release from the cells. In agreement with the reported mechanism of IFITM3

anti-HIV activity, we found the infectivity of HIV-1 virions produced from control cells were significantly impaired by IFN treatment. However, IFN did not affect the infectivity of virions produced from TET2 knockdown cells (Fig. 4.5C). In summary, we conclude TET2 is required for IFN-mediated IFITM3 induction and incorporation into nascent virions that impairs virion infectivity.

4.4. DISCUSSION

The role of HIV-1 Vpr in promoting HIV-1 replication has been enigmatic. Previously, we reported Vpr induced the proteasomal degradation of TET2, and we found TET2 is a direct inhibitor of HIV-1 replication (Chapter 3). This involves the binding of TET2 with HIV-1 reverse transcribed cDNA, leading to suppression of HIV-1 reverse transcription. Interestingly, Vpr has also been reported to help HIV-1 resist type-1 interferon (16, 18). In this report, we found TET2 has an alternative antiviral role, by regulating the type-1 interferon signaling pathway and MX2/IFITM3 induction. The depletion of TET2 and the expression of Vpr strongly impaired the IFNα signaling activity against HIV-1 infection in both T cells and monocytic cells (Fig. 4.1). Importantly, the depletion of TET2 significantly impaired the induction of antiviral effector proteins MX2 and IFITM3 (Fig. 4.2). We showed the MX2-resistant Gag^{N57S} HIV-1 is insensitive to TET2 depletion, demonstrating the IFN-TET2 pathway inhibits HIV-1 in a MX2-dependent manner (Fig. 4.3). In addition, the IFN-TET2 pathway impaired HIV-1 virion infectivity through the induction of IFITM3, which is incorporated into nascent virion particles (Fig. 4.4).

The caveat in this report is TET2 does not control the gene transcription of ISGs (Fig. 4.3B and 4.3C). This is baffling for two reasons: first TET2 is an enzyme for promoter DNA demethylation and gene transcription (20, 28), and second, our preliminary data indicates TET2

is recruited to MX2 and IFITM3 DNA promoters upon IFN treatment through the interaction with STAT1/2 (data not shown). From our results, we conclude that TET2 controls ISG levels at a post-transcriptional step, which may involve ISG protein stability or ISG mRNA translation. Future mechanistic studies on how TET2 proteins may regulate ISGs at a post-transcriptional level is required to explain this puzzling phenomenon. Despite this caveat, we found TET2 plays an important role in IFN signaling to suppress HIV-1. Depletion of TET2 in THP1 and CD4 T cells reduced the magnitude of IFNα antiviral activity by ~70% (Fig. 4.1). By inducing TET2 degradation, Vpr may counteract the direct antiviral effector function of TET2 and enhance HIV-1 reverse transcription (Chapter 3), but concomitantly it also impairs IFN-mediated induction of MX2 and IFITM3 to facilitate HIV-1 replication.

It will be interesting to investigate whether TET2 also controls ISGs to inhibit the replication of other viruses. Future work on the importance of TET2 on the replication different virus classes, type-1 IFN signaling, and potential virus-mediated antagonisms of TET2 may elucidate a broad antiviral role of TET2 in the innate immune response.

4.5. METHODS

shRNA-mediated knockdown

pGIPZ-lentiviral vectors expressing control (non-targeting), TET2-targeting shRNA were purchased from Dharmacon, GE Healthcare (Lafayette, CO). shRNA targeting human TET2 has the mature antisense sequence: TAAGTAATACAATGTTCTT (clone ID: V3LHS_363201). pLKO.1 vectors expressing STAT1-targeting shRNA were purchased from UNC lentivirus core. Lentiviruses were produced by CaCl2-BES (*N,N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid) transfection in 293T cells using 15ug pGIPZ-shRNA vector, 10 μg packaging plasmid ΔNRF and 5

μg pVSV-G envelope-expressing plasmid. Lentiviruses were titered in 293T cells. Transductions of THP-1 cells were performed with a multiplicity of infection (M.O.I.) of 0.3. Transduction of primary CD4 T cells were performed using a M.O.I. of 5. Freshly isolated CD4 T cells were infected with lentivirus and stimulated with CD3/CD28 (Thermo #11132D) and 100U/mL IL-2 for 7 days. Both transduced THP-1 and CD4 T cells were selected with 1ug/mL Puromycin at day 3 post-transduction. Knockdown efficiency of TET2 and STAT1 was assessed by qPCR and western blot seven days after Puromycin selection.

HIV-1 proviral strains and virion stock production

The luciferase reporter Vpr-deficient HIV-1 strain pNL4-LUC-E^-R^- was obtained from the NIH AIDS Reagent Program. The Vpr+ proviral strain pNL4-LUC-E^-R^+ was generated from pNL4-LUC-E^-R^- construct using site-directed mutagenesis (NEB Q5 Site-directed mutagenesis kit) and confirmed by sequencing. N57S mutation in the Gag gene region of pNL4-LUC-E^-R^- construct was also generated using site-directed mutagenesis and confirmed by sequencing. Replication-competent NL4-3-R^+-HSA and NL4-3-R^--HSA strains were obtained from the NIH AIDS Reagent program. HIV-1 virions were produced by CaCl2-BES transfection of proviral plasmids in 293T cells. 293T cells cultured on a 10 cm plate were transfected with 30 μg DNA of NL4-3 for production of replication competent HIV-1 viruses, 25ug pNL4-LUC-E^- with 5 μg pVSV-G for the production of VSV-G pseudotyped luciferase reporter virus. Virus stocks were harvested at 48 hr post transfection. Concentration of viral stocks were measured by p24 ELISA assay (Frederick National Laboratory for Cancer Research – AIDS and Cancer Virus Program).

HIV-1 infection and analysis

Infections of primary CD4 T, THP-1 and 293T cells with HIV-LUC-G were performed by addition of 50 ng p24 of virus per 10^6 cells. When indicated, one day before infection the cells were pretreated with type 1 interferon (R&D Systems, #11200-1) with the indicated amount. Luciferase activity was measured 48 hr post infection using Luciferase Assay System from Promega and normalized to total protein concentration. Replication-competent viruses were analyzed in the culture supernatant by p24 ELISA assays. Nascent HIV-1 virion infectivity were measured by titration of equal amount of p24 virions in Jurkat T cells and analyzed by HSA-reporter expression.

ISG induction analysis

To measure the induction of Interferon-Stimulated-Genes (ISGs), THP1 or Jurkat T cells were treated with IFNα (50U/mL). For ISG mRNA analysis, 100,000 cells were harvested between 2-48h after treatment, and RNA were extracted using RNAeasy Plus mini kit (Qiagen). 9ug total RNA were converted to cDNA (MLV reverse transcriptase, Invitrogen), and ISG gene expression were analyzed by qPCR (2x SYBR Green mastermix, Thermo) normalized to GAPDH levels. Primers used for analysis as follows: TET2:(GATAGAACCAACCATGTTGAGGG, TGGAGCTTTGTAGCCAGAGGT). MX2:(CAGAGGCAGCGGAATCGTAA, TGAAGCTCTAGCTCGGTGTTC). IFITM3:(ATGTCGTCTGGTCCCTGTTC, GTCATGAGGATGCCCAGAAT). For ISG protein analysis, 1 million cells were harvested at 48h after IFNα treatment.

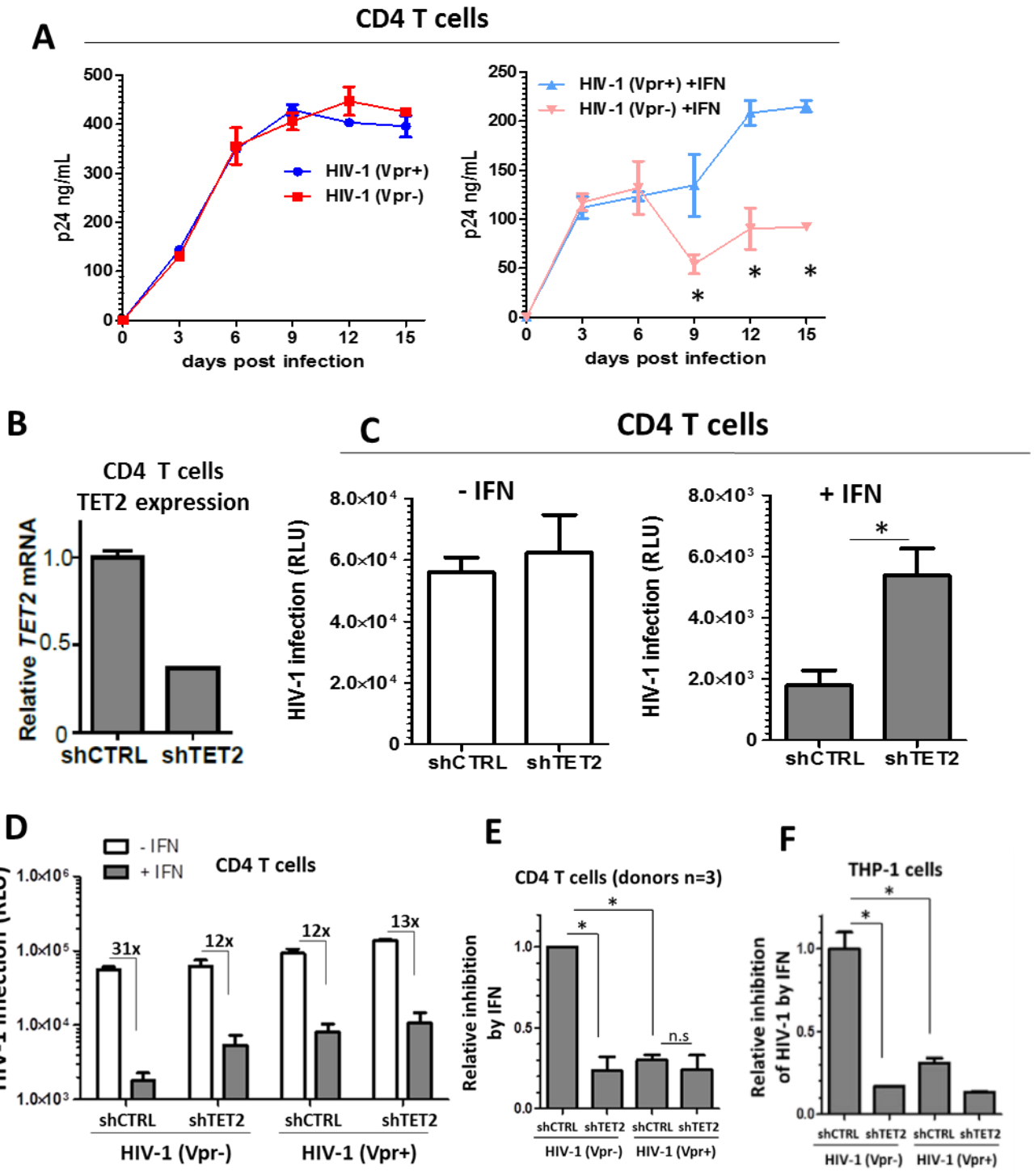

Figure 4.1. Vpr depletes TET2 in CD4 T cells to counteract IFN-mediated antiviral effect
(A) Vpr confers resistance to IFN. Activated CD4 T cells were infected with HIV-1 or Vpr-deficient HIV-1 (NL4 strain) at an M.O.I. of 0.05. Infected samples were treated with 100U/mL of IFNα or left untreated on day 3 after infection. Viral replication was measured by p24 ELISA. (B) confirmation of TET2 knockdown by shRNA in primary CD4 T cells by RT-qPCR. (C) TET2

knockdown confers resistance to IFN. Activated CD4 T cells were stably transduced with control shRNA or shRNA against TET2 expression, and knockdown efficiency were assessed by RT-qPCR (left). Cells were treated with IFN α for 24h, then infected with Vpr-deficient HIV-LUC virus. Viral replication was measured by luciferase expression after 48h. (D-E) Vpr-mediated resistance to IFN is dependent on TET2. Infection experiments with (Vpr+) and (Vpr-) HIV-1 viruses were performed in control or TET2 knockdown CD4 T cells with IFNα treatment. (D) Relative luciferase activity were measured to assess the magnitude of HIV-1 inhibition by IFN. (E) The average fold inhibition by IFN were measured from three independent experiments. (F) Similar experiment as in (E) were performed in THP1 cells.

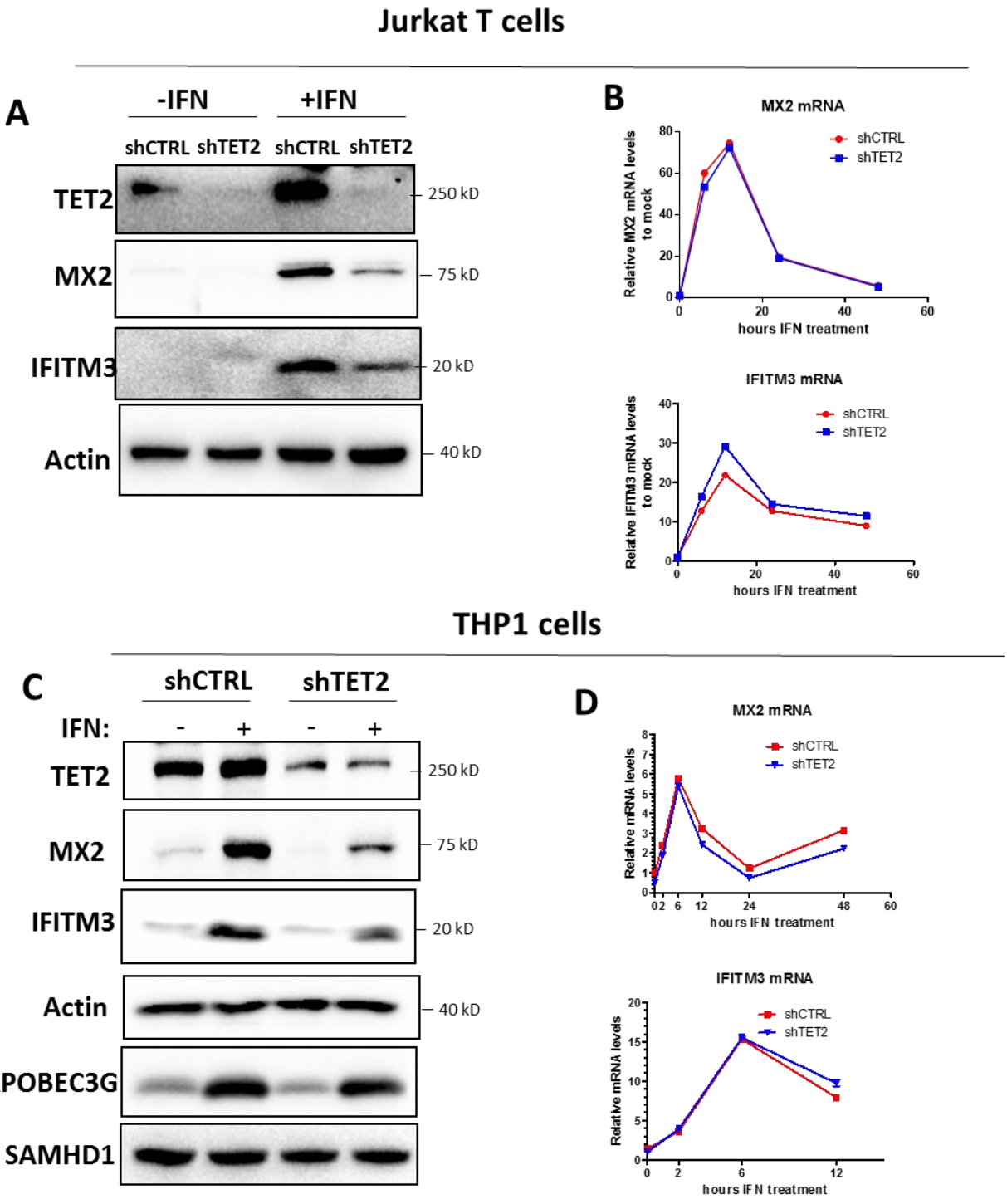

Figure 4.2. TET2 is required for the induction of MX2 and IFITM3 proteins
(A) Stable shRNA-mediated knockdown of TET2 were generated in Jurkat T cells. Cells were treated with IFNα for 24h and the induction of MX2 and IFITM3 proteins were measured by western blot. (B) Aliquots of the IFNα treated cells in (A) were harvested at 6, 12, 24 and 48h

post treatment for MX2/IFITM3 mRNA induction analysis by RT-qPCR. (C) Stable shRNA-mediated knockdown of TET2 were generated in THP1 cells. Induction of MX2, IFITM3, APOBEC3G and SAMHD1 proteins by IFNα were performed at 24h treatment. (D) Aliquots of the IFNα treated cells in (C) were harvested at 2, 6, 12, 24 and 48h post treatment for MX2/IFITM3 mRNA induction analysis by RT-qPCR

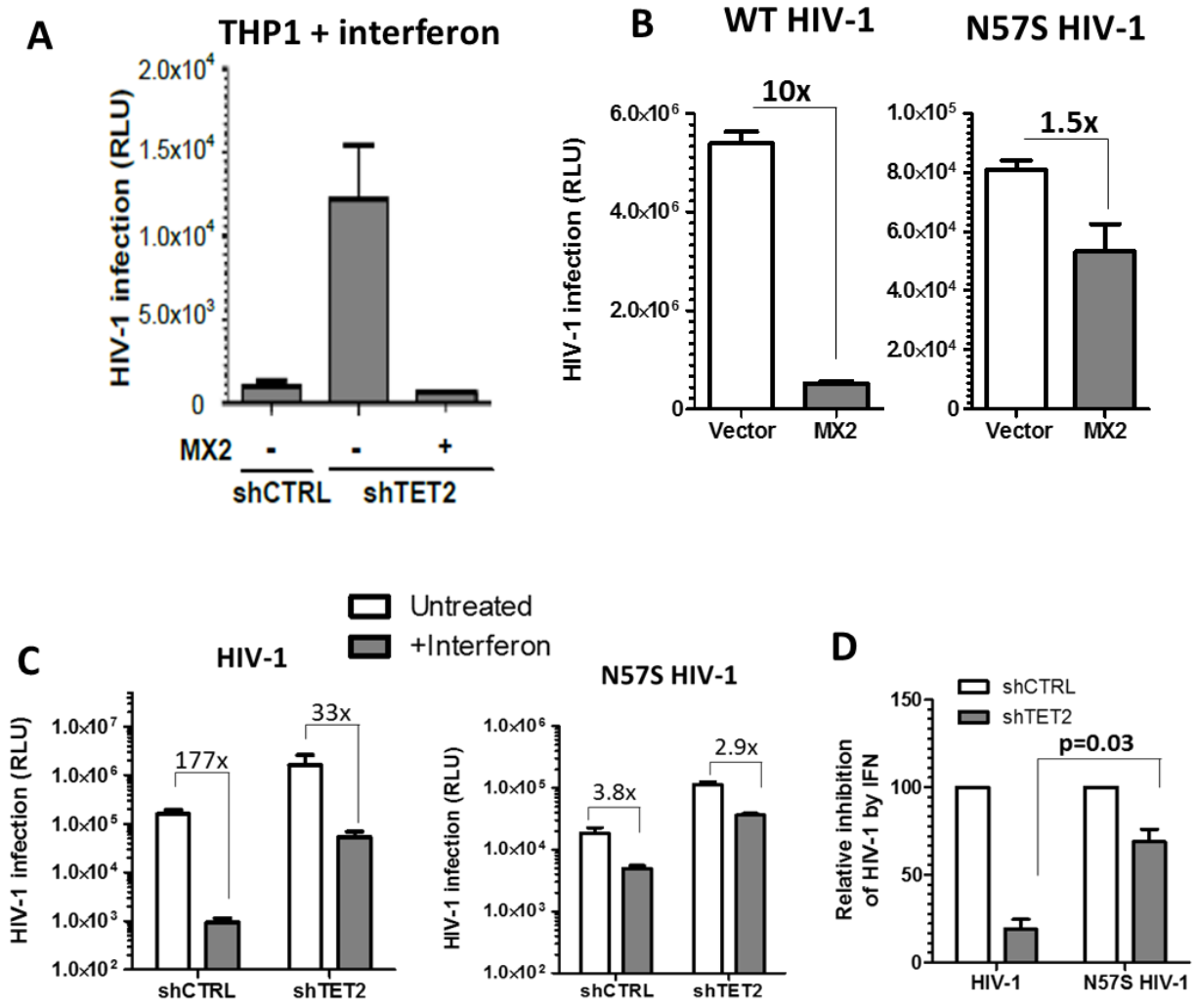

Figure 4.3. MX2-resistant N57S HIV-1 is insensitive to IFN inhibition or TET2 depletion.
(A) Ectopic MX2 expression re-suppress HIV-1 in TET2 knockdown cells. shTET2 THP1 cells were transfected with MX2 and treated with IFN for 24h, and infected with HIV-1 then analyzed after 48h by luciferase assay. N57S HIV-1 is resistant to MX2 antiviral effect. THP1 cells were transfected with MX2 for 24h, then infected with HIV-1 or MX2-resistant GAGN57S HIV-1. (C) N57S HIV-1 is insensitive to TET2 depletion. Control or TET2 knockdown THP1 cells were treated with IFN for 24h, and infected with HIV-1 or N57S HIV-1. Fold inhibition by IFN are indicated on the graphs. (D) Relative HIV-1 inhibition by IFN in control vs TET2 knockdown cells are indicated for the two viruses.

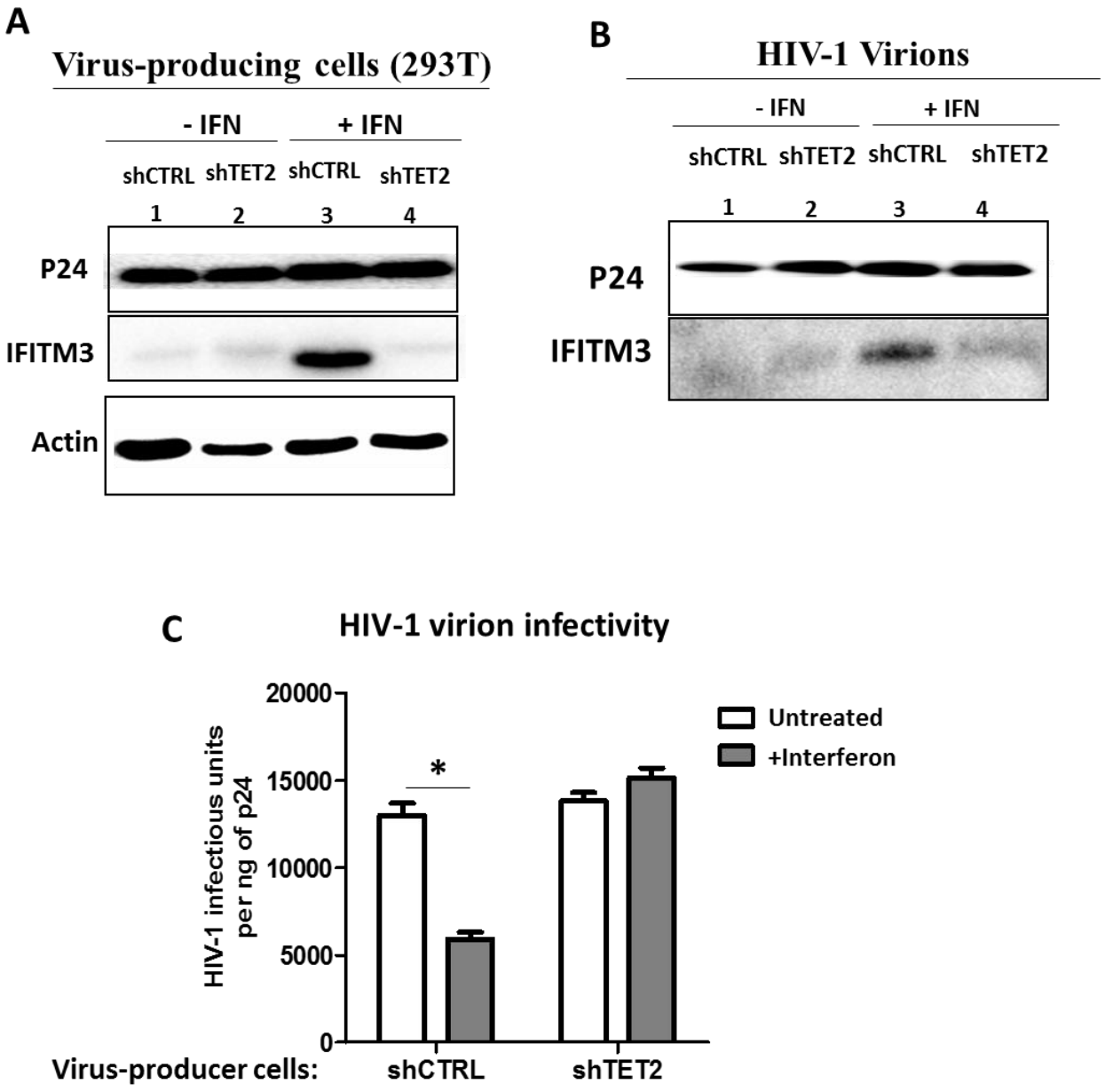

Figure 4.4. TET2 is required for IFN-mediated induction of IFITM3 to inhibit HIV-1 virion infectivity

(A) Stable control-knockdown or TET2-knockdown 293T cells were treated with IFN and transfected with plasmid expressing a Vpr-deficient HIV-1 proviral DNA. Cells were harvested at 48h post transfection for western blot analysis of HIV-1 proteins and IFITM3 expression. (B) HIV-1 virions produced from the transfected cells in (A) were harvested in the culture supernatant, and precipitated by ultra-centrifugation. Western blot was performed to detect IFITM3 incorporation in the virions produced from control or TET2 knockdown 293T cells. (C) Infectivity of HIV-1 virions produced from control or TET2 knockdown cells (A) were titered on T cells and normalized by p24 protein levels.

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CHAPTER 5. DISCUSSION

5.1. INTRODUCTION

This final chapter will summarize and discuss major findings and future directions of the three projects in my dissertation. In the field of HIV research, there are two important areas deserving a lot of attention. First, there is the question on how HIV-1 causes CD4 T cell death and AIDS disease progression. Second, unlike most viruses, HIV-1 persist in our body after infection, and studies on how HIV-1 efficiently evade our immune defense is important. In my dissertation, I focused on these two topics: HIV-1 pathogenesis (Chapter 2, discussed in section 5.2) and HIV-1 evasion from the immune defense (Chapter 3 discussed in section 5.3, and Chapter 4 discussed in section 5.4). Lastly, I will discuss caveats regarding Chapter 4 in section 5.5, and unpublished data with future directions in section 5.6.

5.2. HIV-1 Env induces CD4 T cell pathogenesis – the role of Env and CCR5 interaction

The hallmark of HIV-1 pathogenesis is the depletion of CD4 T cells, with bystander uninfected CD4 T cell death believed to make a major contribution towards AIDS (1). The HIV-1 Env protein is a well-known contributor to the killing of bystander CD4 T cells (2–5). The viral Env is also an ideal therapeutic target, as it is expressed on the virion membrane, making it prone to neutralization by small molecules or antibodies (6–8). Therefore, elucidating the mechanism of Env-induced cell death will have therapeutic values. The viral Env of transmitting HIV-1 binds with CD4 and the co-receptor CCR5. It is known that over the course of HIV disease progression, HIV-1 Env evolves into a more pathogenic Env that emerges during the AIDS

disease stage. Interestingly, the expression of CCR5 levels on cell surface correlate with increased host susceptibility to Env-induced apoptosis (2). However, it has been difficult to directly define the role of CCR5 interaction in HIV-induced CD4 T cell depletion because CCR5 interaction is required for the replication of CCR5-tropic HIV-1. Using the dual-tropic HIV-1 strain R3A, we could prevent its viral Env binding to CCR5 without altering the virus replication fitness. Using genetic and pharmacological approaches, we demonstrated the interaction between CCR5 and HIV-1 Env is a critical determinant of bystander CD4 T cell apoptosis. The *Env* mutant R3A-5/6AA has lost the capability to bind CCR5, which effectively decreased viral pathogenicity on bystander CD4 T cells in activated PBMC cultures and in humanized mice. This indicates binding of R3A Env to CCR5 on the cellular surface, without the targeted cell becoming infected, is sufficient to trigger bystander cell death. A highlight of this work is summarized in Figure 5.1.

Signal transduction through Env-CCR5 interaction may contribute to bystander pathogenesis. Our study showed that CCR5 antagonist TAK-779 decreased R3A-induced bystander CD4 T cell depletion, whereas CCR5 agonist MIP-1β increased bystander pathogenesis, supporting a role for CCR5 stimulation and signaling in R3A pathogenesis. Mechanistically, HIV-1 Env binding to CCR5 induces a signaling cascade including Pyk2 phosphorylation and downstream activation of p38 MAPK pathway, leading to caspase-dependent apoptosis (11, 12). Using a p38 inhibitor, our lab has found that p38 signaling contributes to R3A-induced CD4 T cell depletion in humanized mouse infection (data not shown). Therefore, signal transduction through CCR5 by the R3A Env and subsequent p38 activation may be involved in R3A induced bystander T cell killing. MIP-1□ binding to CCR5 is

known to similarly activate Pyk2 and p38 MAPK pathways (13), which may mimic the Env-CCR5 interaction and contribute to bystander cell death. It is important to note that the R3A Env also binds to CD4 and CXCR4, and the cooperatively binding of Env with all three receptors likely contributes to R3A pathogenicity. Furthermore, R3A Env also can strongly induce IFNα in pDC (14), which may contribute to viral immunopathogenesis in our studies.

In summary, we provide the first evidence in relevant HIV-1 infection models that CCR5 interaction with viral Env played a significant role in Env-induced depletion of bystander CD4 T cells. Our findings suggest that small molecules or gene therapy targeting CCR5 (6, 15), known to prevent CCR5-mediated HIV-1 entry, may also reduce Env-mediated CD4 T cell depletion and AIDS disease progression.

5.3. HIV-1 Vpr induces the degradation of TET2 to enhance HIV-1 replication

HIV-1 encodes several accessory proteins which, while not sharing significant sequence homology, appear to have evolved a common mechanism to promote efficient viral replication by hijacking a host E3 ubiquitin ligase to degrade host restriction factors (16, 17). These include Vif-targeted degradation of APOBEC3 by the $CRL5^{ElonB/C\text{-}CBF\beta}$ E3 ligase (18), Vpu-targeted degradation of tetherin/BST2 by the $CRL1^{\beta\text{-}TrCP}$ ligase (19), and SIV/HIV-2 Vpx-targeted degradation of SAMHD1 by the $CRL4^{VprBP}$ ligase (20, 21). The function of Vpr in facilitating HIV-1 replication is believed to depend on its ability to interact with VprBP and thereby target a putative restriction factor to the $CRL4^{VprBP}$ E3 ligase for degradation (22, 23). Our study in Chapter 3 discovered Vpr targets TET2 for degradation by the $CRL4^{VprBP}$ E3 ligase, and TET2 is a direct inhibitor of HIV-1 reverse transcription that is antagonized by Vpr.

A graphical summary of the highlights in this study is presented in Figure 5.2. Vpr induces the poly-ubiquitylation of TET2 through the $CRL4^{VprBP}$ E3 ligase, leading to the proteasomal degradation of TET2. This function of Vpr is conserved among HIV/SIV species, and happens rapidly after viral entry into cells. Two of the most extensively studied features of Vpr is its activity to induce G2 cell-cycle arrest, and its role in enhancing HIV-1 replication in myeloid cells (24). Using mutational analysis of Vpr, we showed TET2 degradation and G2-arrest are two genetically distinct Vpr functions. Interestingly, the same approach showed TET2 degradation is involved in Vpr-promoted HIV-1 replication. Indeed, genetic deletion of TET2 demonstrated it inhibits HIV-1 replication in monocytic cell lines and primary macrophages. Analysis of HIV-1 at different post-entry steps revealed TET2 inhibits HIV-1 reverse transcription (RT). Accordingly, HIV-1 Vpr promotes viral replication and reverse transcription in a TET2-dependent manner.

Using mutational analysis of TET2, we found while the enzymatic activity of TET2 is not required for inhibition of HIV-1, the DNA-binding mutant $TET2^{K1229N}$ failed to inhibit HIV-1 infection. Accordingly, TET2 binds with HIV-1 reverse transcribed cDNA through this DNA-binding region and is abolished by K1229 mutation. Interestingly, the K1229 residue of TET2 is mono-ubiquitylated by $CRL4^{VprBP}$, which promotes the DNA binding capability of TET2 (25). This suggests Vpr hijacks $CRL4^{VprBP}$ and changes TET2 mono-ubiquitylation into poly-ubiquitylation, which effectively relieves the restriction on HIV reverse transcription posed by TET2.

Of note, TET2 only seems to inhibit HIV-1 replication in myeloid cells but not in T cells (see Chapter 4), suggesting a myeloid-specific co-factor or modification of TET2 may be involved in its anti-HIV activity. This supports previous reports indicating Vpr only enhance HIV-1 replication in myeloid cells but not in T cells (26–28). One example of a myeloid-specific

restriction factor is SAMHD1, which is highly phosphorylated at T592 residue by Cyclin A2/CDK1 in activated T cells (29, 30). This modification shuts off SAMHD1 anti-HIV activity in activated T cells, whereas in myeloid dendritic cells SAMHD1 remains unphosphorylated and inhibits HIV (30). Similar work on the molecular modifications of TET2 in myeloid cells vs T cells is required to elucidate how the antiviral activity of TET2 is regulated. The regulation of TET2 mono-ubiquitylation on its K1229 residue by VprBP is also worth further investigation in different cell types.

It is intriguing to find an enzyme TET2 to function independently of its enzymatic activity in DNA demethylation. However, recent studies have shown examples of this, where the suppression of IL-6 and IFNβ promoters by TET proteins occurs in a catalytic-independent manner (31, 32). These findings shed new light on how TET2 may function as an adaptor protein that serves to recruit transcriptional suppressive factors to DNA promoters, such as the SIN3 Transcription Regulator Family Member A (SIN3A) and Histone Deacetylase 1 and 2 (HDAC1/2) (31, 32). It will be interesting to investigate whether TET2 also recruits suppressive factors to the HIV-1 cDNA that impairs viral reverse transcription. Interestingly, SIN3A and HDACs have indeed been reported to associate with the HIV-1 reverse transcription complex, but its biological relevance is not well understood (33, 34).

In summary, our study in Chapter 3 discovered a new HIV-1 restriction factor TET2, which specifically inhibits HIV-1 reverse transcription. This restriction is antagonized by HIV-1 Vpr, adding to the literature on how HIV-1 has evolved to escape from the host immune defense by hijacking E3 ligases and promoting proteasomal degradation of restriction factor proteins. Lastly, the results presented here identify a new target—Vpr-mediated TET2

degradation—for therapeutic intervention of HIV-1 infection. Development of therapeutic molecules targeting the Vpr-VprBP interaction or blocking Vpr-mediated TET2 degradation may merit future exploration.

5.4. TET2 is required for Type-1 interferon mediated inhibition of HIV-1 and other viruses

Type-1 interferon (IFN) induces the expression of hundreds of interferon-stimulated-genes (ISGs) and is one of the primary defenses against all varieties of viral infections. Viruses have in turn developed a variety of strategies to counteract IFN signaling and ISG induction, using diverse strategies (35). It has been reported that HIV-1 Vpr is known to confer viral resistance against IFN (27, 36, 37). In Chapter 3, we discovered Vpr targets TET2 for degradation, and that TET2 inhibits HIV-1 reverse transcription directly by binding to viral cDNA. However, TET2 is an epigenetic regulator for cellular gene expression through its DNA hydroxyl-methylation activity (38, 39). In Chapter 4, we hypothesized TET2 could play a role in IFN signaling pathway and ISG induction. We found that while Vpr and TET2 depletion did not affect viral replication in CD4 T cells, it significantly enhanced viral replication in the presence of IFNα treatment by impairing IFNα antiviral activity.

The expression of TET2 is required for the induction of ISGs such as MX2 and IFITM3, which are known to inhibit HIV-1. We further provide functional evidences for the IFN-TET2-ISG pathway against HIV-1 infection. Of note, MX2 and IFITM3 inhibits different steps of HIV-1 replication (see Fig. 1.2A). First, MX2 inhibits the viral nuclear import step which can be measured by HIV-1 gene expression (40, 41). We found the MX2-resistant Gag^{N57S} HIV-1 was not affected by TET2 depletion compared to wild type HIV-1, suggesting the IFN-TET2 pathway inhibits HIV-1 through MX2. Second, IFITM3 is induced by IFN and inhibits HIV-1 through its

incorporation into progeny HIV-1 virion's membrane, which will impair virion fusion with the target cell membrane (42). TET2 depletion in virion-producer cells reduced both cellular and virion-associated IFITM3 levels, thereby blocking IFITM3 antiviral effect and rescued HIV-1 infectivity. Our work from Chapter 3 and 4 suggests TET2 has two distinct antiviral roles in HIV-1 infection. First, TET2 may function as a direct antiviral effector protein that binds to HIV-1 reverse transcribed cDNA and inhibit reverse transcription (Chapter 3). Second, TET2 is a modulator for induction of antiviral proteins (MX2 and IFITM3) in the IFN signaling pathway to inhibit HIV-1 at various steps in the viral replication cycle. Vpr may antagonize both antiviral roles of TET2 by inducing its proteasomal degradation. A graphical model is presented in Figure 5.3.

5.5. Discussion of caveat

The major caveat in Chapter 4 is TET2 does not regulate the induction of ISGs at the transcriptional level (Fig. 4.3). This is baffling because TET2 is an enzyme that regulates promoter DNA demethylation and gene transcription (38, 39), therefore we predicted TET2 would regulate ISG transcription. From our results, we conclude that TET2 controls ISG levels at a post-transcriptional step, which may involve ISG protein stability or ISG mRNA translation. Future mechanistic studies on how TET2 proteins may regulate ISGs at a post-transcriptional step is required to explain this puzzling phenomenon. Of note, TET2 directly interacts with the ubiquitin E3 ligase $CRL4^{VprBP}$(25), which may regulate ISG protein poly-ubiquitylation and stability. Another likely possibility is the regulation of ISG mRNA translation by TET2. The ability for the host to promote efficient translation of ISG mRNAs even prior to ISG transcription would provide a mechanism for rapid pathogen suppression. For example, the Protein Kinase R (PKR)

is known to influence ISG mRNA translation upon immune activation (43). it is therefore worth investigating PKR activities in TET2 depleted cells. Several additional mechanisms have been described for controlling the translation of cytokine mRNAs (44), and we can envision that similar regulation may exist for ISG mRNAs that may exist at low basal levels before IFN receptor engagement but are rapidly translated upon IFN signaling.

5.6. Unpublished results and future directions

Given that type-1 IFN has broad antiviral activities, we predict TET2 may be required to inhibit a variety of viruses. Indeed, our unpublished results showed the depletion of TET2 also impairs the IFN-mediated inhibition against Influenza A virus (IAV) and Dengue virus (DENV) (Fig. 5.4A). As viruses are constantly in an evolutionary arm race to escape from the type-1 interferon-mediated antiviral defense (35), we predict the IFN-TET2-ISG pathway is likely frequently disrupted by viruses. Interestingly, we found the infection of IAV, DENV and hepatitis C virus (HCV) resulted in the depletion of cellular TET2 proteins (Fig. 5.4B). These preliminary data revealed TET2 as an important modulator of ISG induction downstream of IFN signaling, which is commonly antagonized by viruses such as HIV, IAV, DENV and HCV. This discovery opens up future directions on how viruses has evolved similar strategies, such as HIV-1 Vpr hijacking the $CRL4^{VprBP}$, to deplete TET2 levels and impair type-1 IFN signaling. Future research on the molecular interactions between TET2 and viral proteins is required to unravel the molecular mechanism of TET2 depletion by different viruses.

5.7. Concluding Remarks

My dissertation focuses on the molecular interactions between HIV-1 and host cell. In my first study, we provide evidence of viral Env interaction with cellular CCR5 contribute to HIV-

induced pathogenesis and CD4 T cell death. The study identifies Env and CCR5 as a potential therapeutic target to prevent AIDS disease progression in HIV patients. In my second study, we discovered a new HIV restriction factor TET2 that directly binds HIV-1 cDNA and inhibits reverse transcription. HIV-1 Vpr protein hijacks the $CRL4^{VprBP}$ E3 ligase to induce the degradation of TET2. This shed light on how Vpr, like other HIV accessory proteins, has evolved to antagonize restriction factors to help HIV escape from host innate immune defenses. In my third study, we identified a second antiviral feature of TET2, providing evidences that TET2 regulates the levels of antiviral proteins induced by the type-1 interferon signaling pathway. We conclude TET2 has broad antiviral roles as a modulator of IFN signaling that inhibits multiple viruses, leading to the evolution of viruses to deplete TET2 and impair IFN signaling.

In summary, my work contributed toward the field of HIV-1 pathogenesis, HIV-1 restriction factors and virus-mediated escape from immune defense. Future research building on these findings would improve our understanding how HIV and other viral pathogens persist after infection and contribute disease progression.

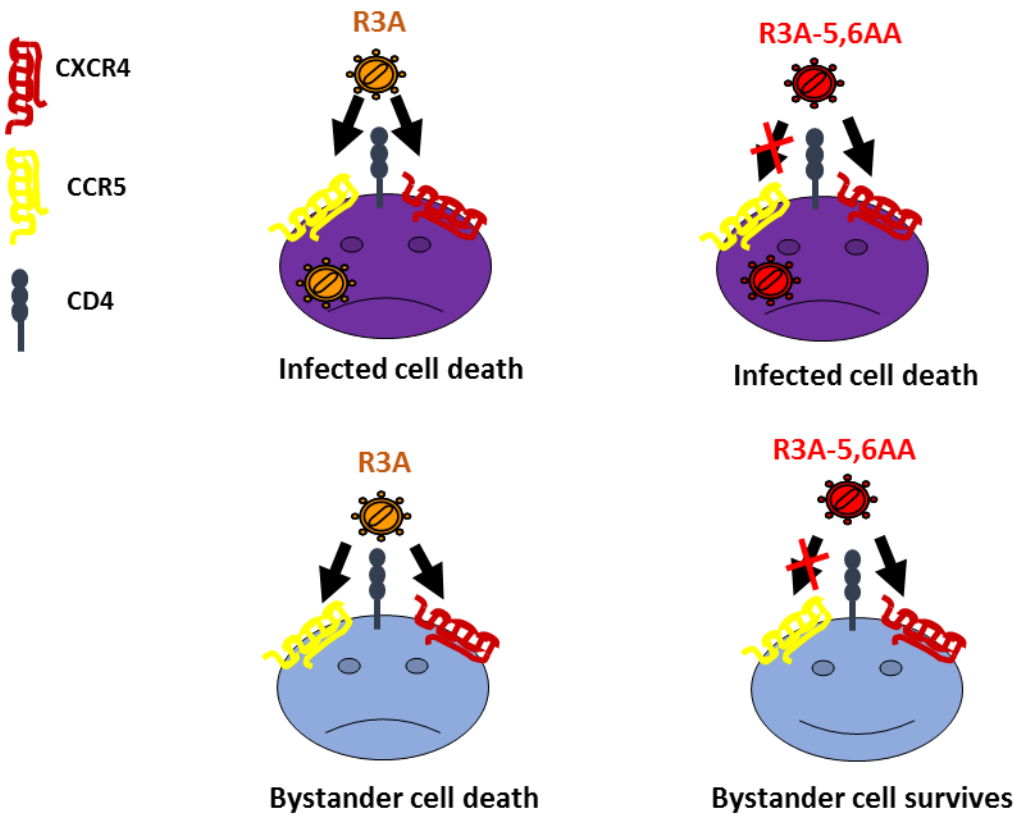

Figure 5.1. HIV-1 Env mediated bystander pathogenesis (Graphical Summary and Highlights)

1. R3A Env rapidly induces CD4 T cell depletion in vitro and in vivo
2. Ablation of Env-CCR5 usage with antagonistic drugs or Env mutation significantly reduced R3A pathogenesis
3. Env-CCR5 binding specifically caused bystander uninfected CD4 T cells death
4. CCR5 agonist MIP-β contributes to Env-mediated bystander cell death

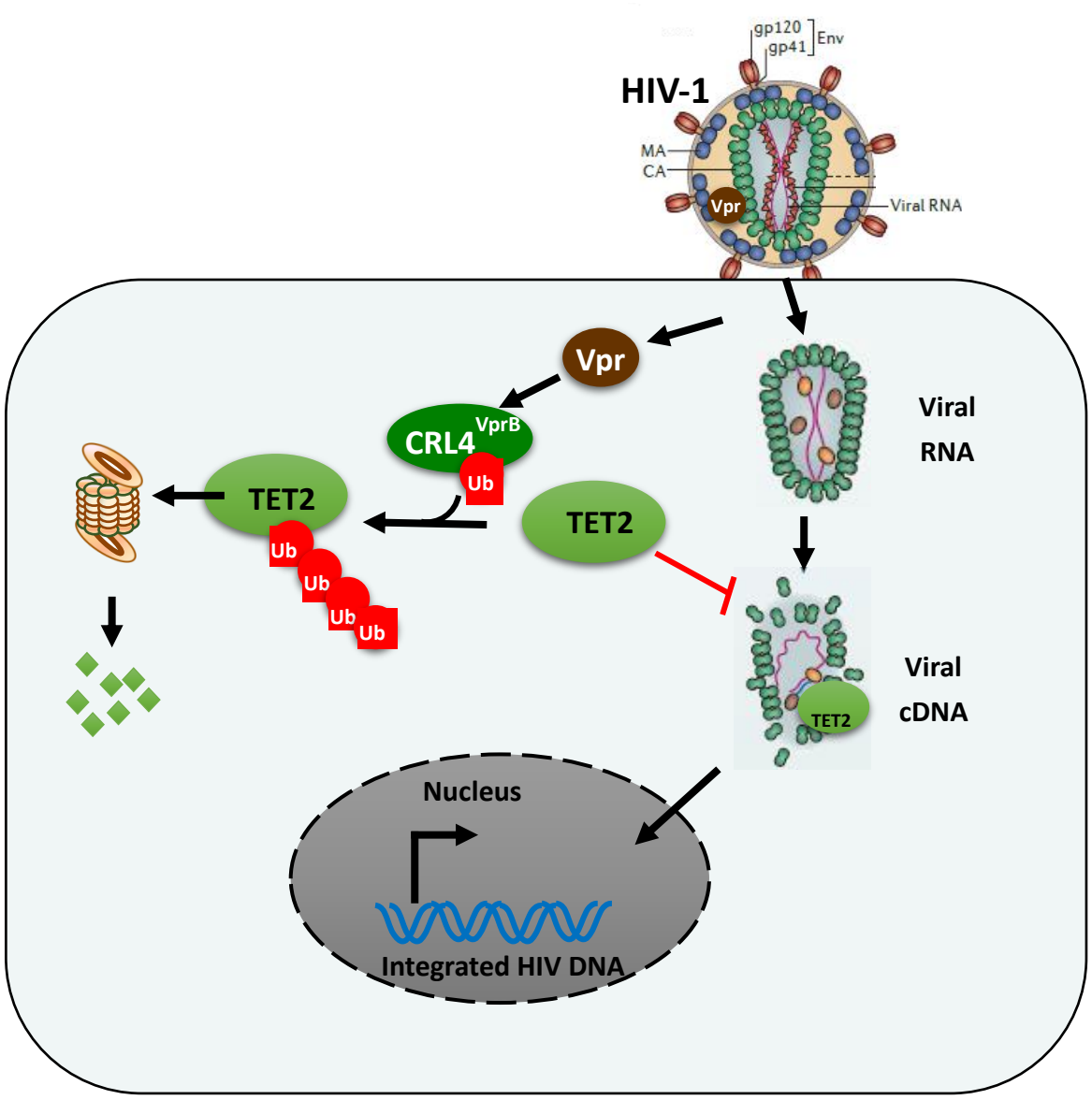

Figure 5.2. TET2 is a restriction factor of HIV-1 reverse transcription that is antagonized by Vpr (Graphical Summary and Highlights)

1. Vpr promotes TET2 poly-ubiquitination by CUL4-DDB1-VprBP E3 Ligase for proteasomal degradation
2. TET2 degradation is not required for Vpr-induced G2-Arrest
3. TET2 inhibits HIV-1 replication and is antagonized by Vpr
4. TET2 binds with HIV-1 reverse transcribed cDNA and inhibit reverse transcription progression

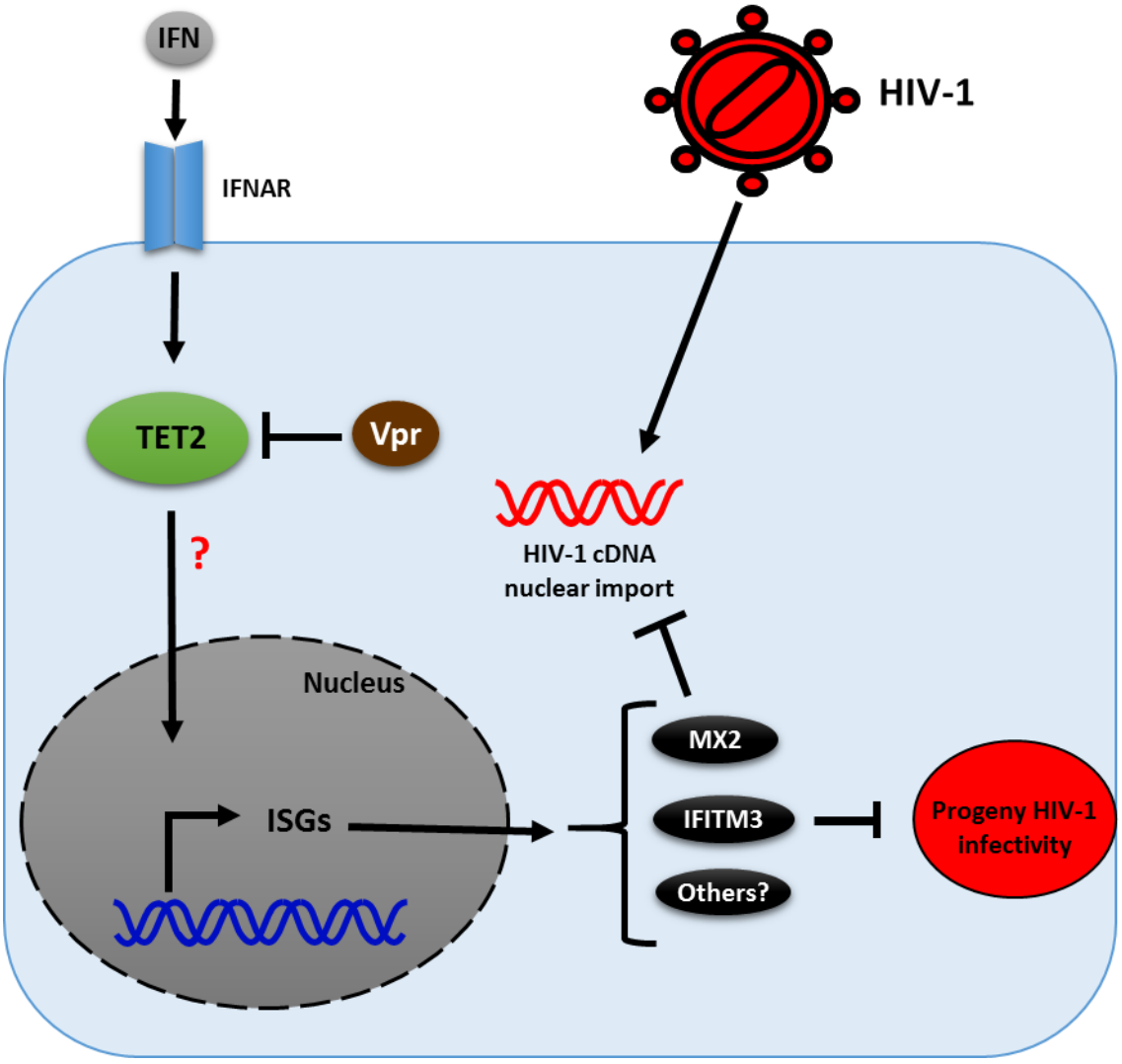

Figure 5.3. The role of TET2 in the IFN signaling pathway against HIV-1 replication (Graphical Summary and Highlights)

1. The IFN-mediated inhibition of HIV-1 requires TET2, which is antagonized by Vpr
2. TET2 modulates the IFN-mediated induction of MX2 and IFITM3
3. IFN-TET2 pathway does not affect MX2-resistant HIV-1
4. IFN-TET2 pathway modulates IFITM3 incorporation into nascent virion to impair virion infectivity

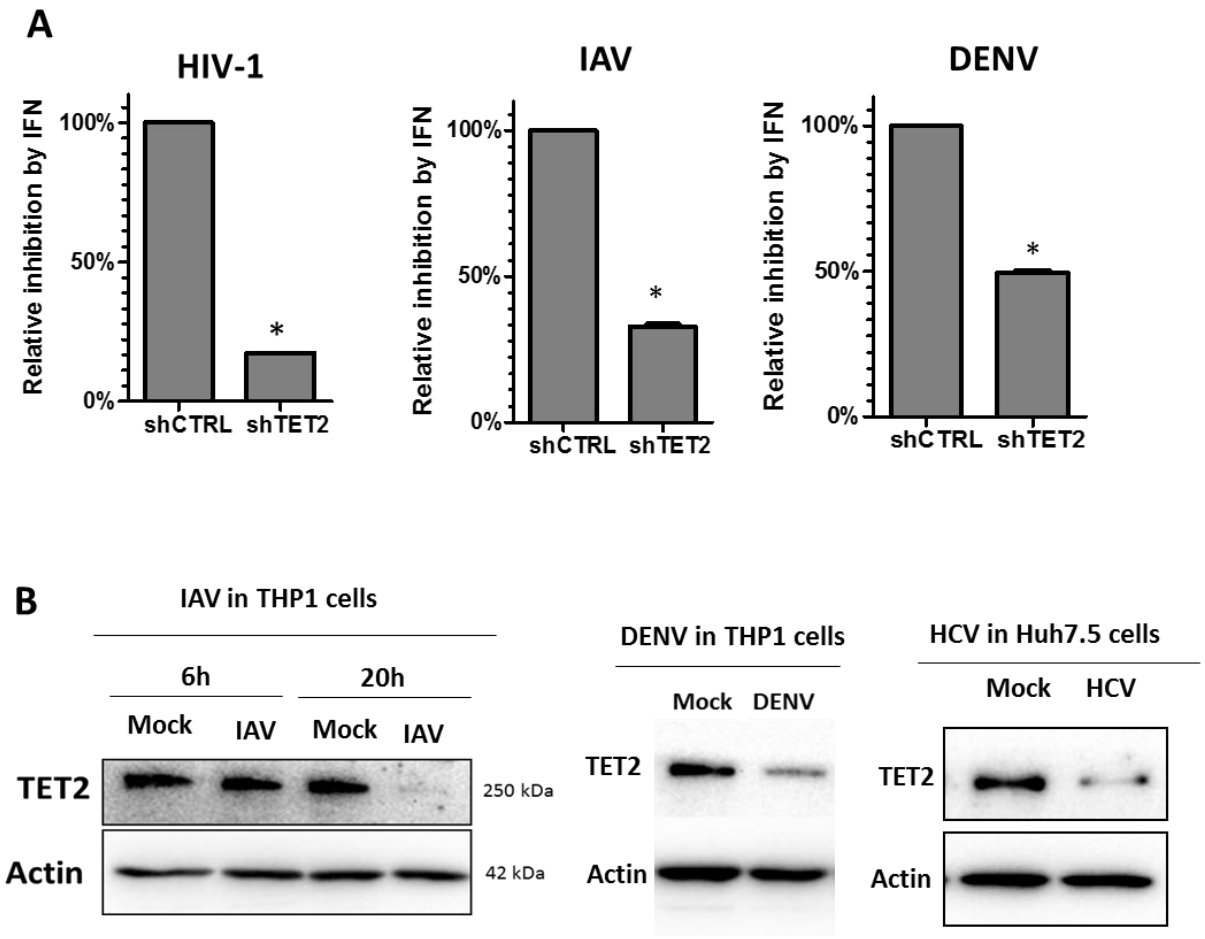

Figure 5.4. Unpublished results – Host pathogen interactions between TET2 vs multiple viruses

(A) TET2 is required for the IFN-mediated inhibition of multiple viruses including HIV-1, influenza A virus (IAV) and Dengue virus (DENV). Relative inhibition of the different viruses by IFNα in THP1 cells are summarized. (B) TET2 protein levels are depleted by multiple viruses after infection. TET2 protein levels are measured after IAV infection in THP1, DENV infection in THP1 and HCV infection in Huh7.5 cells.

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